Enzymes in Food Technology

ROBERT J. WHITEHURST
BARRY A. LAW,
Editors

Sheffield Academic Press
CRC Press

Enzymes in Food Technology

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Edited by R.J. Whitehurst and B.A. Law

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Edited by

ROBERT J. WHITEHURST
Kerry SPP
Cambridge, UK

and

BARRY A. LAW
Food Science Australia
Victoria
Australia

CRC Press

First published 2002

Published by
Sheffield Academic Press Ltd
Mansion House, 19 Kingfield Road
Sheffield S11 9AS, UK

ISBN 1-84127-223-X

Published in the U.S.A. and Canada (only) by
CRC Press LLC
2000 Corporate Blvd., N.W.
Boca Raton, FL 33431, U.S.A.
Orders from the U.S.A. and Canada (only) to CRC Press LLC

U.S.A. and Canada only:
ISBN 0-8493-9782-0

Printed on acid-free paper in Great Britain by
Antony Rowe Ltd., Chippenham, Wiltshire

British Library Cataloguing-in-Publication Data:
A catalogue record for this book is available from the British Library

Library of Congress Cataloging-in-Publication Data:
A catalog record for this book is available from the Library of Congress

Preface

Enzymes may be described as 'functional catalytic proteins'. I do hope, however, that this volume will re-introduce them as useful and targeted workforces. Like the more familiar human workforces, they have preferred working conditions, may be trained (cultured) to carry out very specific tasks, and they cannot function when their food (substrate) runs out.

The purpose of this volume is to provide both a basic grounding, for those not experienced in the use of enzymes, and a state-of-the-art account of today's enzyme technology as applied to food and drink. Authors have been selected not only for their practical, working knowledge of enzymes but also for their infectious enthusiasm for the subject.

Enzymes are introduced first according to their nomenclature and then by their nature and mode of action. Chapters go on to describe the basic theory and practical applications of exogenous enzymes in food and drink technology, and how enzymes improve raw materials and influence and modify the biochemical and physical events that we describe as 'food processing'. Finally, methods of culturing and manufacturing enzymes in commercial quantities are described, together with the role that genetic engineering has to play in their further development.

Indigenous enzymes in food raw materials have long played a role in food production. Today, however, enzymologists, working together with food development technologists and with a view to market requirements, have helped and improved upon nature to bring us varieties of food and drink that were unheard of a relatively short time ago. Examples of these are wines which mature earlier and have enhanced aroma and colour stability, 'naked' but undamaged citrus fruit (devoid of its pith or peel), bread that resists staling, and enzyme-modified cheeses. Furthermore, production yields and purities of intermediate raw materials have been improved, and by-products, previously thought of as waste, may now be utilised.

My thanks to all the contributors to this book for sharing their practical approach to the subject. I hope that the reader finds the volume as rewarding as I found its preparation.

R. J. Whitehurst
B. A. Law

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Contributors

Ms Rose-Marie Canal-Llaubères	Novozymes, Cité Mondiale, 23 Parvis des Chartrons, F-33074 Bordeaux Cédex, France
Mrs Cornelia Drost-Lustenberger	Novozymes Switzerland AG, Neumatt, 4243 Dittingen, Switzerland
Dr Pierre Fauquembergue	DSM Food Specialties, 20 rue du Ballon, 59041 Lille Cédex, France
Mr Mari J. M. C. van Gestel	PB 28, Quest International, PO Box 2, 1400 CA Buss, The Netherlands
Dr Catherine Grassin	DSM Food Specialties, 20 rue du Ballon, 59041 Lille Cédex, France
Professor Barry A. Law	Food Science Australia, Sneydes Road, Private Bag 16, Werribee, Victoria 3030, Australia
Mr Per Munk Nielsen	Novozymes A/S, Laurentsvej 55, DK-2880 Bagsvaerd, Denmark
Mr Hans Sejr Olsen	Novozymes A/S, Laurentsvej 55, DK-2880 Bagsvaerd, Denmark
Mr Maarten van Oort	Novozymes, Biltseveste 12, 3432 AR Nieuwegein, The Netherlands
Mr Richie Piggott	Quest International, 5115 Sedge Blvd., Hoffman Estates, Illinois 60192, USA
Ms Joan Qi Si	Novozymes SA, 79 Avenue François Arajo, 92017 Nanterre Cédex, France
Mr Diederik J. M. Schmedding	PB 28, Quest International, PO Box 2, 1400 CA Buss, The Netherlands
Mr Reinhold Urlaub	Röhm Enzyme Gmbh, Kirschenallee 45, D-64293 Darmstadt, Germany

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1 The nature of enzymes and their action in foods

Barry A. Law

1.1 Introduction

Enzymes are proteins that are produced by all living organisms. They speed up chemical reactions selectively as part of essential life processes such as digestion, respiration, metabolism and tissue maintenance. In other words, they are highly specific biological catalysts. The enzymes work under more or less mild conditions (they have to, in order to operate in living cells and life-sustaining environments), making them ideal catalysts to use in food technology, in which the manufacturer wants to modify food raw materials selectively without destroying essential nutrients. The historical uses of enzymes to make beer, wine, cheese and bread are elegant examples of the industrial exploitation of the power and selectivity of enzymes. Early food enzyme technologists were clever craftsmen, but they did not realise that this is what they were doing, and that they could have adopted such a splendid job title.

To understand modern food enzyme technology, it is important to realise that these early enzymatic processes were not only fermentations, but also complex and coordinated enzyme-mediated processes. The enzymes were then, and remain, essential for the provision of fermentation substrates (beer and bread), the development of flavour and aroma (wine) or the creation of the very structure of the product (cheese). The following chapters of this volume will describe and discuss how enzymes do all of these things in these and other foods. The picture will emerge of a technology that began as a craft and has become a sophisticated high-technology, high value-added industrial sector. Enzyme production and application in the food manufacturing industry is based on a profound understanding of the role of enzymes in traditional foods, from which technologists have improved the basic processes to supply bigger markets with safer and higher quality products. This understanding, together with improvements in enzyme sourcing and production technology, has also yielded novel enzyme technologies to create new foods and food ingredients, as will also be revealed by the authors. This opening discussion will introduce the reader to the basic properties of enzymes, the methods used to quantify them for technological use, their non-ideal behaviour in food systems, and the range of food technologies in which they are used.

1.2 Enzymes in food

1.2.1 Enzyme nomenclature

Any particular enzyme only catalyses reactions between one type (or a narrow range) of chemical compound (its substrate). This defines its 'specificity' and provides the basis of its classification and name. Often, the trivial name of the enzyme, derived from the truncated substrate name with 'ase' added, identifies the substrate or substrate range better for food technologists than does its systematic name or its International Union of Biochemistry Enzyme Commission (IUB or EC) number [1]. For example, the trivial name for lipid-hydrolysing enzymes is lipase, but the official name and number is triacylglycerol acylhydrolase (IUB/EC 3.1.1.3). The latter is precisely descriptive of what such enzymes do, and the term/number is vital for unambiguous communication between biochemists and product chemists. As far as food technologists are concerned though, lipases act on lipids and break them down to their component fatty acids and glycerol. This is the important information to be used in food product and process terms, and the trivial names of all food enzymes will be used throughout this chapter. Thus, proteinases (usually shortened to protease) chop up proteins by breaking the amide bonds that join their component amino acids into the protein polypeptide chain. Carbohydrates would logically be broken down by carbohydrases, though this is not a commonly used term, largely because carbohydrate building blocks are very diverse, as are the polymeric structures formed from them (the starches, cellulose, pectins). This diversity will become clearer to the reader as this volume unfolds. For now, suffice it to say that more useful general terms for members of this class of enzyme are derived from individual substrates. Thus lactase breaks down lactose, maltase breaks down maltose, pectinase breaks down pectin and cellulase breaks down cellulose. There is no 'starchase' because there are too many structural isomers of starch on which to base the obvious trivial name for starch degrading enzymes. The most ubiquitous 'starchase' is called *alpha*-amylase because it chops up the amylose component of starch at the most common glucose-to-glucose bond in the amylose polymer (*alpha*-1–4).

Although the majority of enzymes (over 90%) used currently in commercial scale food technology are hydrolytic substrate degraders like those mentioned above, some enzymes used to improve and modify food materials catalyse synthetic reactions and substrate interconversions. Most are quite easy to identify with their biochemical function because their names describe the main substrate and the reaction; for example, glucose oxidase oxidises glucose to gluconic acid, using up oxygen. However, this name does not tell us much about the technological functions it performs, namely reducing glucose content of egg whites to reduce Maillard browning, and scavenging oxygen in packaging technology. There is nothing to be done about this, other than for the food technologist to be aware of the technological significance of glucose oxidation

by a nice gentle enzyme. Similarly, the names glucose isomerase or lipoxygenase tell all about the biochemistry involved, but we just have to remember the technology. The isomer of glucose produced by glucose (strictly, 'xylose') isomerase is fructose which, mole for mole, is sweeter than glucose so the enzyme is used to make the sweetener, high fructose corn syrup (section 1.3). The reactions catalysed by lipoxygenases used in bread improvement are quite complex [2] and not altogether understood by research biochemists. Fortunately, food technologists understand empirically that the enzyme catalyses reactions between lipids in flour and oxygen. Directly and indirectly these reactions bleach the flour and make nice white bread, and also modify wheat proteins so that they help in forming good crumb structure and high loaf volumes (chapter 2).

Some enzymes are easier to identify with their food applications than others, but many of these connections between nomenclature and technological function will become familiar to the reader throughout this volume.

1.2.2 Enzyme kinetics

The underlying mechanisms of enzyme action and the interactions of enzymes with their physical and chemical environment can be described mathematically with reasonable precision. However, most of the equations, constants and ground rules (enzyme kinetics) have been worked out for idealised situations in which single enzymes act on simple, single substrates under predictable conditions found within living cells. The reader who wants to understand these parameters in mathematical terms can find an excellent summary and reference list by Fullbrook [3] in '*Industrial Enzymology*' [4]. In the same work, Fullbrook [5] also goes on to explain some of the pitfalls of applying 'classical' enzyme kinetics to non-ideal industrial processes.

Food technologists not only need to know which enzymes degrade, synthesise or interconvert which food material substrates, but also need to have a means of working out how much of the chosen enzyme to use under any particular conditions to achieve an economical rate and efficiency of material conversion. Qualitative and quantitative enzyme kinetics show us that enzymes behave quite predictably in simple ideal systems such as those used to classify and characterise enzyme preparations in research and QA laboratories. They work at peak rates at particular pH values, temperatures and substrate concentrations according to well-established rules (figure 1.1).

At fixed substrate concentrations, enzymic reaction rates depend on enzyme concentration up to a maximum, dependent on the turnover efficiency of the particular enzyme preparation. The 'activity curves' of the type shown diagramatically in figure 1.1 are used to derive the numerical values of these parameters, and they are vital basic inputs into deciding which enzyme preparation(s) should be used in which food modification process [3].

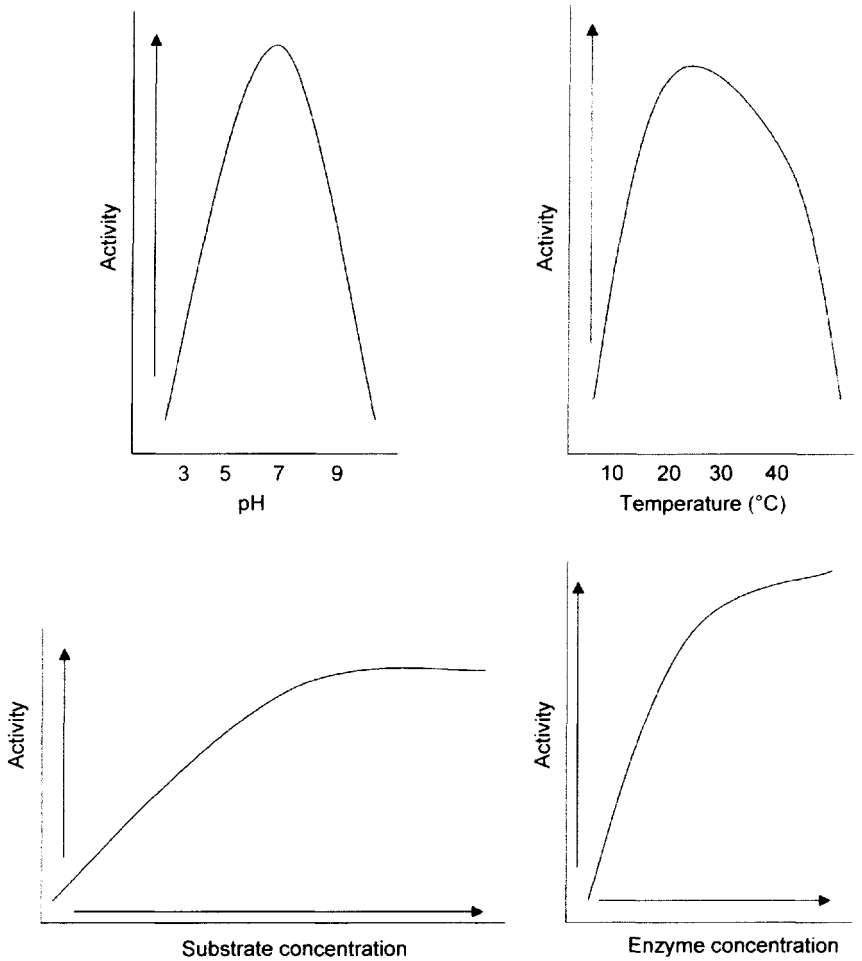

Figure 1.1 Effect of pH, temperature, enzyme concentration and substrate concentration on the initial rate of enzyme catalysed reactions in solution.

The temperature and pH 'profiles' derived from simple test conditions are usually applicable to complex food environments because they are dependent on the molecular properties of the enzyme protein itself, rather than on those of the substrate. Enzyme proteins are precisely folded polypeptide chains, held together by relatively weak molecular forces. The folded structure determines the integrity of the catalytic site (the 'active site', figure 1.2) within the enzyme, and this is easily disrupted by energy changes in the enzyme's environment (temperature being a prime example). This phenomenon is called 'denaturation'

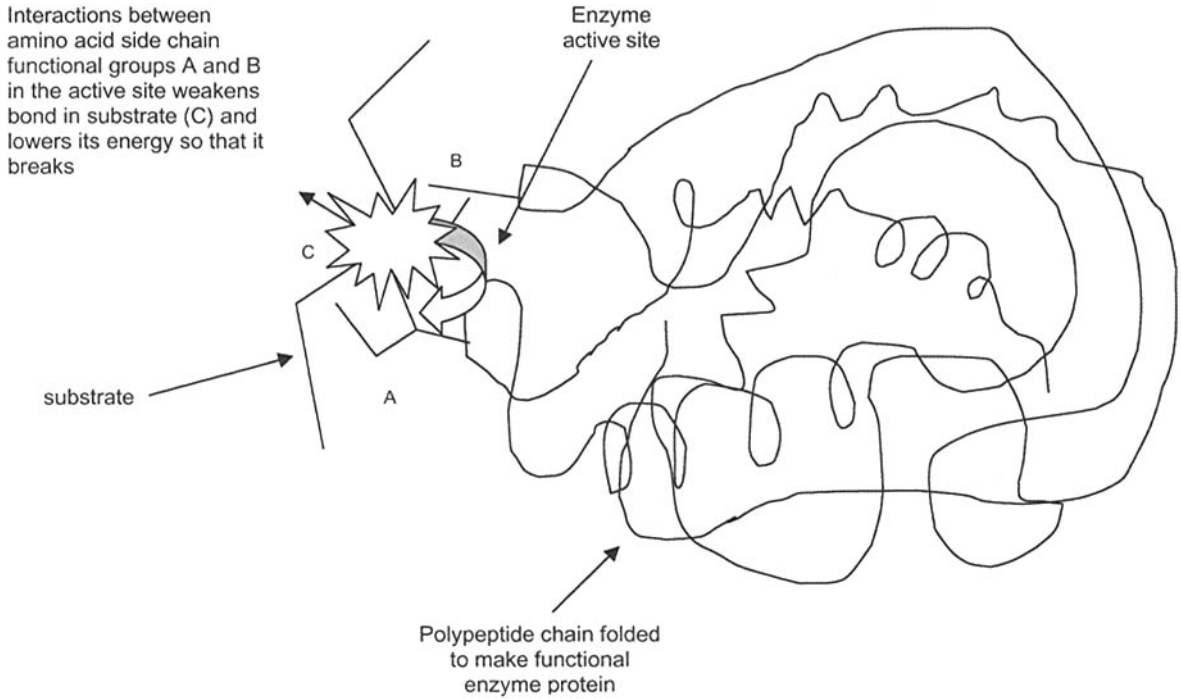

Figure 1.2 The three-dimensional folded polypeptide chain of an enzyme protein, illustrating the interactions between active site functional groups (A and B) and substrate (C) which lower the energy needed to break the chemical bond in the substrate and thus create the catalytic activity of the enzyme.

and it can be reversible or irreversible dependent on the severity of structural deformation and damage.

However, even small changes in intramolecular forces in the enzyme, such as those caused by small temperature changes, or pH-dependent charge differences on the amino acids making up the primary polypeptide chain structures, can also cause conformational changes in the structure that fall short of denaturation. Changes of this magnitude are manifested in the typical temperature and pH/activity curves in figure 1.1 and illustrate how precise the spatial juxtaposition of active site functional groups must be to reach the maximum rate of catalysis. Thus, as reaction temperature is increased, classical chemical kinetics dictate that the reaction will speed up, but beyond a certain temperature (characteristic of any particular enzyme) the disruption to the enzyme protein folded structure reduces its catalytic efficiency and activity tails off again.

In the case of pH, the bell-shaped curve is the manifestation of an optimal spatial structure of the enzyme protein which forms at a certain pH, when the relative ionisations within the structure combine to orientate the active site to give maximum substrate binding, bond modification, and release of product. Either side of the optimum pH for activity, changes in charge and hydrogen bonding efficiency can either distort the active site through changes in the three-dimensional fold of the protein polypeptide chain (figure 1.2), or reduce bond dipoles in active site functional groups, reducing their capacity to lower the activation energy for conversion of the substrate(s).

The understanding of the relationships between the amino acid sequences, three-dimensional structures and catalytic efficiencies of food enzymes is now sufficiently comprehensive to allow molecular enzymologists to change the structures of food enzymes to improve their technological processing properties such as heat resistance, pH optimum, resistance to catalytic poisons/inhibitors and even substrate preference. This is called 'protein engineering', and is reviewed by Goodenough [6] and Law [7] specifically in relation to the advantageous modification of enzymes for food ingredients synthesis. Other examples appear later in section 1.5.

1.2.3 Enzyme (in)stability

Enzymes have a finite working life, or half-life, due to inherent physical instability, the action of antagonists/inhibitors, and 'poisoning' by contaminants in the reaction mixture. In foods and food technology, physical instability can be induced by the pH and temperature effects outlined above, but also by relatively mild forces such as surface tension in foams and emulsions.

Most enzyme inhibitors would not be present in foods because they are generally poisonous (e.g. heavy metals and organometallic compounds), but many enzyme antagonists and catalytic poisons are common in foods and food raw materials (e.g. respectively, proteolytic enzymes and free radicals from oxidised

unsaturated fatty acids). Enzyme instability measured for purified proteins in aqueous buffers can give guidance to technologists as to the comparative stability of rival enzyme preparations under real processing conditions, but absolute stability can only be determined in real food systems. The first decision to make is whether the process will benefit from stable or unstable enzymes.

Highly stable enzymes are normally used in processes that take a long time to complete, such as malting and Koji fermentations, or where the enzyme is part of a diagnostic kit, and has to survive drying and long storage before use, without losing its standardised activity at manufacture. On the other hand, some enzymes used in food manufacture are only required to be active for a short time to scavenge oxygen, to precipitate milk proteins or to assist ripening for example, and their long-term persistence in the food is either irrelevant, or even detrimental. Specific examples of these applied scenarios and the criteria used to pick the right enzyme for the job will emerge in the following chapters. Some of the special factors that influence the stability and half-life of enzymes in food systems and food processing are listed in table 1.1.

There are no hard and fast rules to guide food technologists through this largely unexplored area of applied enzyme science because the research community has not systematically studied the effects of phase structures and interfaces in food on all of the factors used to predict enzyme activity. However, based on table 1.1, it may be possible to avoid some pitfalls, or even exploit some

Table 1.1 Special factors that influence the stability of enzymes in food

Condition	Effect on stability	Underlying cause
Phase concentration of enzyme	Stabilises	Increased energy per unit volume required to denature enzyme
Presence of other non-enzyme proteins	Stabilises	Lowers proportion of denaturing energy or molecular source available to denature enzyme protein
Presence of other enzyme proteins	Destabilises	If accompanying enzymes or proteinases, they may degrade the added enzyme protein
Presence of impurities	Destabilises	Catalytic poisoning • of lipases by free radical-induced chemical damage to the enzyme (especially lipases) • of any enzyme by heavy metal atoms blocking the active site • of metal requiring enzymes by chelating agents such as citric acid or polyphosphates
Phase interfaces	Destabilises	Denaturation by surface tension forces

opportunities. For example, a supplier might recommend an enzyme addition rate of x units per kilogram of food product, based on the activity measured against the substrate in solution. The food manufacturer calculates the cost of the enzymatic conversion based on this figure, adjusts it to compensate for differences in pH and temperature, and decides how much the enzymatic stage will cost. This decision process is based on the assumption that the enzyme will be acting in a homogeneous environment similar to that under which the producer assayed it. However, most foods are heterogeneous, consisting of discontinuous solid composites of fat, protein, carbohydrates and water, or emulsions and foams with distinct phase boundaries. Such structures in food tend to cause any added substance to be concentrated by affinity, osmosis or differential solubility into one particular component or phase of the food. It is therefore worth seeking from the supplier data on the solubility, hydrophobicity and resistance to surface tension denaturation of the enzyme, so that the costing figures can be adjusted to 'x units per kg bulk protein, fat or carbohydrate', or even 'per unit volume of water in an emulsion'. This might produce a cost figure substantially more or less than that based on assumptions of homogeneity.

1.2.4 Composition and activity of commercial enzyme preparations

Most commercial enzyme preparations contain not only the specific enzyme whose activity is printed on the label, but also other enzymes that happen to be produced by the same source material/organism [8]. The enzyme user must always be aware of this factor in the enzyme product specification to avoid side effects in complex foods (e.g. starch breakdown by an enzyme preparation bought for its proteinase function). Even if the application is for an isolated food component such as whey protein, a heterogeneous enzyme preparation can pass unexpected and unwanted enzyme activities on to the customer who buys the enzyme-modified food ingredient unless steps are taken to inactivate it after processing.

Thus, enzyme/substrate interrelationships are more complex and less predictable in food reaction environments because food materials are not pure chemicals, but complex structures and/or poorly defined mixtures of potential (possibly competing) substrates and inhibitors.

The amount of enzyme required to convert a given amount of substrate is measured in 'Enzyme Units'. In simple systems the original International Union of Biochemistry Unit (U) was the 'amount of enzyme that will catalyse the transformation of one micromole of substrate per minute under defined conditions'. The SI unit for enzyme activity is the 'katal', defined as the 'amount of enzyme that will cause the transformation of one millimole of substrate per second under specified conditions'.

Unfortunately for food technologists, even a substrate that is definable in food technology terms (e.g. fungal protein, vegetable protein extracts, animal

muscle, corn starch, milk fat) is heterogeneous as far as an enzyme is concerned, and the above unit definitions are of little use to them in process design. For example, corn starch consists of glucose polymers of infinitely-variable molecular weight with huge batch-to-batch differences, rendering impossible any fixed standard enzyme dose to achieve an agreed specification for a hydrolysed starch ingredient product. Indeed, it would be impossible to define an enzyme unit in relation to such a substrate simply because a millimole or micromole of a mixed molecular weight polymer cannot be defined. These factors mean that there is no simple, consistent way to define or pre-determine how much enzyme to add to how much raw material in food processing.

In practice, enzyme suppliers provide essential information for their customers by defining units in terms of the technological function of the enzyme. This not only allows the user to define and control the enzymatic process on an industrial scale, but sets out the basis for relating the cost of the enzyme to the value of the product produced, and allows comparison of the productivity of enzymes from different suppliers. A good example of such a pragmatic and direct approach to quantifying enzymes for sale and dosing is the use of 'international milk clotting units' (IMCU) for quantifying milk-coagulating enzymes (chymosin, microbial rennets) for sale and use in cheesemaking. The enzymes are all acid proteinases that degrade caseins, but casein breakdown itself is not usefully related to the technological function of destabilising casein micelles and causing milk to clot. The IMCU is a measure of the ability of acid proteinases, sold as cheese milk coagulants, to clot a standard amount of milk under standard conditions in a standard time relative to an international enzyme reference standard [8]. Other function-based units of food processing enzyme activity are based on such parameters as viscosity (reduction or increase), conductivity and colour binding.

1.3 Sources and range of enzymes for food technology

The traditional sources of food technology enzymes have been the tissues of plants and animals (table 1.2). Although these are still widely used in food manufacture, there are many influences driving food enzyme production and use towards microbial alternatives, including genetically-modified derivatives of these organisms (GMOs).

There are many examples of the use of carbohydrate-degrading enzymes in food manufacture, particularly in baking, beer brewing and fruit juice production, and these will be covered in detail in chapters 2, 3 and 7 of this volume. However, their economical production from efficient microorganisms in industrial-scale fermenters means that the utilisation of the enzymes such as amylase and pectinase present in the traditional raw materials (wheat, barley, citrus fruits, flour) is confined now to their action *in situ*, and they are not widely extracted for exogenous use.

Table 1.2 Enzymes widely sourced from animals and plants used in food manufacturing technology

Enzyme	Source	Action in food	Food applications
alpha-Amylase	Cereal seeds e.g. wheat, barley	Starch hydrolysis to oligosaccharides	Breadmaking brewing (malting)
beta-Amylase	Sweet potato	Starch hydrolysis to maltose	Production of high malt syrups
Papain	Latex of unripe papaya fruit	Food and beverage protein hydrolysis	Meat tenderisation chill haze prevention in beer
Bromelain	Pineapple juice and stem	Muscle and connective tissue protein hydrolysis	Meat tenderisation
Ficin	Fig fruit latex	As bromelain	As bromelain and papain but not widely used due to cost
Trypsin	Bovine/porcine pancreas	Food protein hydrolysis	Production of hydrolysates for food flavouring (mostly replaced now by microbial proteinases)
Chymosin (rennet)	Calf abomasum	*kappa*-Casein hydrolysis	Coagulation of milk in cheesemaking
Pepsin	Bovine abomasum	As chymosin + more general casein hydrolysis in cheese	Usually present with chymosin as part of 'rennet'
Lipase/esterase	Gullet of goat and lamb; calf abomasum; pig pancreas	Triglyceride (fat) hydrolysis	Flavour enhancement in cheese products; fat function modification by interesterification
Lipoxygenase	Soy bean	Oxidation of unsaturated fatty acids in flour	Bread dough improvement
Lysozyme	Hen egg white	Hydrolysis of bacterial cell wall polysaccharides	Prevention of late blowing defects in cheese by spore-forming bacteria
Lactoperoxidase	Cheese whey; bovine colostrum	Oxidation of thiocyanate ion to bactericidal hypothiocyanate	Cold sterilisation of milk

Certain plant and animal proteinases, on the other hand, remain in widespread use through their well-established effectiveness in some fundamental processes in cheese production and meat processing (chapters 5 and 6). Of particular note are papain (and the related proteinases bromelain and ficin) in meat

tenderisation, and chymosin (with a little pepsin for good measure) in the milk coagulation stage of cheesemaking. Chymosin extracted from calf offal has been displaced in some countries by the same enzyme produced by fermentation of yeasts and fungi containing cloned chymosin genes (chapter 5), but it is still the preferred choice of many traditional cheesemakers, despite the supply and purity advantages of the fermentation-produced product.

Bovine and porcine trypsin are still used for producing food protein hydrolysates as food flavour ingredients, but there are so many good microbial alternatives to these classical serine proteinases now on the market (table 1.3), especially those with less tendency to make bitter products, that trypsin is no longer as important to food manufacturers.

Like proteinases, the food grade animal lipases that have been the mainstay of the dairy flavours industry in the past (chapter 5) are gradually being replaced by equivalent enzymes of microbial origin. This is especially the case in the newer technologies such as fat function modification by enzymatic interesterification [10] in which complex and interdependent enzyme reactors put new demands on long-term enzyme stability, catalytic efficiency, compatibility with multi-step processes and resistance to poisoning by impurities in commodity food fats. However, some 'traditional' applications remain, particularly in the production of piquant-flavoured Italian cheese varieties (chapter 5).

Soy and wheat lipoxygenase is an important enzyme in bread baking (chapter 2) and has traditionally been used in the form of the endogenous wheat flour enzyme, supplemented with soy flour. The latter not only adds to the lipoxygenase activity in the dough, but also supplies lipid substrates to both enzyme sources to improve the texture, 'workability' and colour of bread dough, through (probably) sulfhydryl group oxidation in wheat proteins, and oxidative bleaching of plant pigments.

Lysozyme and lactoperoxidase are both animal enzymes extracted from natural sources (table 1.2), but they differ in their application from most animal and plant enzymes in that they are both antimicrobial, and can be used to control different types of spoilage in cheese and milk (chapter 5).

More and more enzymes for food technology are now derived from specially selected or genetically modified microorganisms grown in industrial scale fermenters (chapter 10) and table 1.3 lists a range of examples and applications.

The number and range of these examples of microbially sourced alternatives reflect the logistical and commercial advantages of using microbial fermentation rather than animal or plant extraction to produce food enzymes. Logistics are based on both political geography and transportation costs; clearly it is desirable for an enzyme producer and user to have a reliable, predictable source of an enzyme that is pivotal for a manufacturing process. This rule holds good for quantity, quality and price, and the fermentation alternative delivers on all counts. It can be produced anywhere in the world, irrespective of climate and agro-economics, the enzyme yield is predictable from the fermentation

Table 1.3 Enzymes derived from microorganisms and used in food manufacturing technology

Enzyme	Source	Action in food	Application in food technology
alpha-Amylase	*Aspergillus* spp. *Bacillus* spp.* *Microbacterium imperiale*	Wheat starch hydrolysis	Dough softening, increased bread volume, aid production of sugars for yeast fermentation
alpha-Acetolactate decarboxylase	*Bacillus subtilis**	Converts acetolactate to acetoin	Reduction of wine maturation time by circumventing need for secondary fermentation of diacetyl to acetoin
Amyloglucosidase	*Aspergillus niger* *Rhizopus* spp.	Hydrolyses starch dextrins to glucose (saccharification)	One stage of high fructose corn syrup production; production of 'lite' beers
Aminopeptidase	*Lactococcus lactis* *Aspergillus* spp. *Rhizopus oryzae*	Releases free amino acids from N-terminus of proteins and peptides	De-bittering protein hydrolysates accelerating cheese maturation
Catalase	*Aspergillus niger** *Micrococcus luteus*	Breaks down hydrogen peroxide to water and oxygen	Oxygen removal technology, combined with glucose oxidase
Cellulase	*Aspergillus niger* *Trichoderma* spp.	Hydrolyses cellulose	Fruit liquifaction in juice production
Chymosin	*Aspergillus awamori** *Kluyveromyces lactis**	Hydrolyses *kappa*-casein	Coagulation of milk for cheesemaking
Cyclodextrin glucanotransferase	*Bacillus* spp.*	Synthesise cyclodextrins from liquified starch	Cyclodextrins are food-grade micro-encapsulants for colours, flavours and vitamins
beta-Galactosidase (lactase)	*Aspergillus* spp. *Kluyveromyces* spp.	Hydrolyses milk lactose to glucose and galactose	Sweetening milk and whey; products for lactose-intolerant individuals; reduction of crystallisation in ice cream containing whey; improving functionality of whey protein concentrates; manufacture of lactulose
beta-Glucanase	*Aspergillus* spp. *Bacillus subtilis**	Hydrolyses beta-glucans in beer mashes	Filtration aids, haze prevention in beer production
Glucose isomerase	*Actinplanes missouriensis* *Bacillus coagulans* *Streptomyces lividans** *Streptomyces rubiginosus**	Converts glucose to fructose	Production of high fructose corn syrup (beverage sweetener)

Glucose oxidase	*Aspergillus niger** *Penicillium chrysogenum*	Oxidises glucose to gluconic acid	Oxygen removal from food packaging; removal of glucose from egg white to prevent browning
Hemicellulase and xylanase	*Aspergillus* spp.* *Bacillus subtilis** *Trichoderma reesei**	Hydrolyses hemicelluloses (insoluble non-starch polysaccharides in flour)	Bread improvement through improved crumb structure
Lipase and esterase	*Aspergillus* spp.* *Candida* spp. *Rhizomucor miehei* *Penicillium roqueforti* *Rhizopus* spp. *Bacillus subtilis**	Hydrolyses triglycerides to fatty acids and glycerol; hydrolyses alkyl esters to fatty acids and alcohol	Flavour enhancement in cheese products; fat function modification by interesterification; synthesis of flavour esters
Pectinase (polygalacturonase)	*Aspergillus* spp. *Penicillium funiculosum*	Hydrolyses pectin	Clarification of fruit juices by depectinisation
Pectinesterase	*Aspergillus* spp.*	Removes methyl groups from galacose units in pectin	With pectinase in depectinisation technology
Pentosanase	*Humicola insolens* *Trichoderma reesei*	Hydrolyses pentosans (soluble non-starch polysaccharides in wheat flours)	Part of bread dough improvement technology
Pullulanase	*Bacillus* spp.* *Klebsiella* spp.*	Hydrolyses 1-6 bonds that form 'branches' in starch structure	Starch saccharification (improves efficiency)
Protease (proteinase)	*Aspergillus* spp.* *Rhizomucor miehei* *Cryphonectria parasitica* *Penicillium citrinum* *Rhizopus niveus* *Bacillus* spp.*	Hydrolysis of *kappa*-casein; hydrolysis of animal and vegetable food proteins; hydrolysis of wheat glutens	Milk coagulation for cheesemaking; hydrolysate production for soups and savoury foods; bread dough improvement

*These enzymes are commercially available from GMO versions of the source microorganism.

parameters, and the purity is guaranteed by both the fermentation specification and the downstream processing technology. Also, the fermentation source completely avoids problems posed by the threat of the spread of diseases in the plant and animal population.

Nothing illustrates these points better than the use of food-grade microorganisms to make chymosin, the cheese milk coagulant. This topic is dealt with in detail in chapter 5, so a summary will suffice here. Chymosin is an acid proteinase that is traditionally a by-product of the milk and veal industry. It is extracted as 'rennet' from the calf abomasum after slaughter, and ideally has a high ratio of chymosin to pepsin, the adult bovine acid proteinase. Even before the spread of bovine spongiform encephalopathy (BSE) and its human form CJD, the supply of calf offal from the meat industry was unpredictable, and insufficient to match the demands of the global cheese industry. Now the supply situation is even worse, and unlikely to recover in the forseeable future. Fortunately the shortfall can be made up through the supply and use of microbial alternatives produced by the growing food enzyme industry. These alternatives range from fungal acid proteinases (mainly from *Rhizomucor miehei*) to calf chymosin produced by gene cloning technology so that the identical calf enzyme can be produced by fermenting food yeast or mould under strictly controlled conditions.

The following chapters of this volume describe and discuss many more examples of the application of microbial food enzymes in the commodity sectors of the food manufacturing and processing industry. Indeed, the availability and use of microbial enzymes is so widespread that a considerable body of national legislation has emerged and matured to ensure environmental and consumer safety. This is true of enzymes derived from both GM and non-GM microorganisms.

1.4 Food enzyme legislation

The following is a summary only to complete this overview of the special considerations attached to the use of enzymes in food technology. In food enzyme legislation, most enzymes are regarded as processing aids because they are added during processing for technical reasons, and have no function in the food itself. Additives have a definite function in the food product, such as preservation, antioxidation, colouring, flavouring or stabilisation. There are grey areas, of course, most notably in the case of egg white lysozyme in cheese, where it continues to inhibit gas-forming bacteria up to the time of consumption. However, the majority of food enzyme applications are as processing aids, and they are often denatured by heat during processing.

Food enzymes are regulated in the UK and European Union (EU) member states by permitted list. For an enzyme to be on the list it must pass stringent

testing to prove absence of toxins, allergens, heavy metals, pathogenic microorganisms and other hazardous contaminants, as specified by the WHO/FAO Joint Expert Committee for Food Additives (JECFA). Thus, although enzymes are not by definition food additives, they are treated as such for scrutiny before going into the Permitted List. Thereafter they 'become' processing aids and need not be labelled (unless the source organism is a GMO—see below). US legislation and regulation of food enzymes is based on safety in use, whatever the source, and relies heavily on the concept of 'Generally Regarded as Safe' (GRAS) status for additives, ingredients and their sources.

UK and EU Food Law is harmonised for additives by Directives and Regulations which must become Statute Law in Member States, but not for processing aids. This can lead to differences, not in the rigour of safety regulation, but in the need to label foods. US and EU Law is not harmonised at all and enzyme applications in food must be handled on a case-by-case basis.

Although there is a Permitted List of food enzymes that specifies origin, application field and attests to safety, this does not permit an existing enzyme preparation on the list to be used if it has been sourced from a new organism, or by a new production process, or if it is to be used in a novel food process. In the UK, all such intended new sources and processes must be referred to the Advisory Committee on Novel Foods and Processes (ACNFP); the European Commission (EC) operates an equivalent system of approval. ACNFP was formerly an advisory body to the Ministry of Agriculture, Fisheries and Food (MAFF) that was consulted by the industry on a voluntary basis, but its increasing role in regulating GMOs and their products has led to it becoming a statutory body within the new Food Standards Agency (FSA). Clearance from ACNFP is necessary before any new food enzyme/enzymatic process (GM or non-GM) can be brought to market within general Food Law.

ACNFP operates a system of decision trees that lead to sets of information/testing requirements which the would-be producer or user of the enzyme must provide/carry out before ACNFP will consider the case for commercial application to food. This ACNFP system is the basis of new EU legislation placing the same stringent requirements on proposed new uses of enzymes in EU Member States [Regulation (EC) No. 258/97 on Novel Foods and Novel Food Ingredients]. It also incorporates specific information systems, checks and rules to regulate the introduction of enzymes from GMOs into foods.

For the purposes of EU Food Law, a GMO is 'an organism, capable of replicating or transferring genetic material, in which the genetic material has been altered in a way that does not happen naturally by mating and/or natural recombination'. This is a very comprehensive definition, and it is important to note that there are now many methods available for constructing GMOs that either do not use any DNA 'foreign' to the host organism, or use methods that produce a final construct that contains no unnatural mutations. As such, these are inherently as safe as their wild-type equivalents but must, under EU

regulations, be regarded as GMOs in the same way as those constructs that contain 'foreign' genes (e.g. maize and soy resistant to weedkiller and rot-proof tomatoes). Such organisms and enzymes produced from them would have to be referred to ACNFP (or equivalent), but would not necessarily have to appear on the food label. In the US, the type of genetic modifications described above would not result in GMO classification, and would only require normal proof of safety in use.

All GMO-derived food enzymes that pass scrutiny by ACNFP and equivalent must also pass the stringent safety tests and regulations required by JECFA.

1.5 Modification of food enzyme activity by protein engineering

Just as basic and applied research in enzymology has brought about GMO technology to improve the range, reliability and purity of food enzymes, it has also made available the technology of enzyme protein engineering. The principles involved in protein engineering as they specifically relate to food enzymes are comprehensively discussed by Goodenough [6] and need not be repeated here. Suffice it to say that protein engineering is a knowledge-based method of changing the forces and interactions within the three-dimensional structure of an enzyme protein by directed changes to its amino acid sequence through manipulation of the gene controlling its production in the source organism. The aim is to alter the 'technological properties' of the enzyme to make it more or less stable, change its optimum operating range, or change its substrate converting range (specificity).

Because this technology can alter the fundamental properties of naturally occurring enzyme proteins, regulators and enzyme producers alike are not yet considering food applications through which consumers would eat or be exposed to these novel materials. As safety assurance and surveillance methods become more sophisticated, the benefits of the technology may be fully realised, but for the present, engineered enzymes are only use in non-food applications and in one specific food ingredient technology involving tightly-immobilised enzymes in a bioreactor. This is the well-established process for sequentially converting corn (maize) starch to dextrins, glucose, and finally fructose, to produce a sweet syrup (high-fructose corn syrup, HFCS) that is used extensively in carbonated soft drinks [11, 12].

The key enzyme in this process is xylose isomerase (also known as glucose isomerase within the industry). It is immobilised and used in a packed column reactor as the final processing stage in which the glucose released by starch saccharification is isomerised to fructose. The conditions under which the native microbial xylose isomerase is used in the complete process are set by a compromise between the optimum reaction temperature for enzymatic isomerisation, and the rate of enzyme denaturation as a result of destabilisation

of the folded (quarternary) structure of the enzyme protein. The active site of the enzyme is formed through a molecular association of protein monomers held together by interfacial hydrogen bonding and salt bridges (figure 1.3). The process-limiting inactivation of xylose isomerase involves destabilisation of this molecular association through the chemical glycosylation of lysine epsilon-amino groups that mediate the molecular association. The glycosylation happens quite quickly because the enzyme reactor temperature is relatively high, and starch-derived glucose is present at high concentrations to react with the amino groups. Redesign engineering strategies, based on computer graphics-aided protein engineering, identified a particular lysine residue (253) at the interface of three of the glucose isomerase monomers (A, B and D; figure 1.3) which, when substituted with arginine through site-directed mutagenesis, reduced the rate of amino group glycosylation, maintained the spatial orientation of position 253 in the quarternary structure, and created a new stabilising hydrogen bond between the monomers [13]. The practical result of the engineering exercise has been the creation of a new catalytically-active xylose isomerase which is not only more stable in solution at 60–70°C, but less susceptible to glycosylation by glucose at the elevated operating temperature. The 'new' enzyme has a volumetric half-life at 60°C of 1550 h over a 10 week test period, whereas the equivalent value for wild-type xylose isomerase is 607 h.

Figure 1.3 The effects of replacing lysine residue 253 in the amino acid sequence of a monomer of *Actinoplanes missouriensis* xylose isomerase by an arginine residue. Bold lines represent the carbon skeleton of lysine 253 (dashed) in the wild-type enzyme, and the replacement arginine (solid) in the mutated enzyme. The other amino acids and their sequence numbers are merely representations of their approximate positions near the monomer boundaries of protein monomers A, C and D. Hydrogen bond interactions are indicated by dotted lines between the monomer boundary interfaces. Reproduced with permission of Society of Chemical Industry.

1.6 Summary and conclusions

The food manufacturing and ingredients industry makes widespread use of enzymes in both traditional sectors such as baking, brewing and cheesemaking, but also in new areas such as fat modification and sweetener technology. A degree of care and ingenuity is often needed to adapt these fragile biological catalysts to industrial processes, but a combination of basic biochemical knowledge and modern biotechnology is opening up new areas of application, especially for enzymes of microbial origin, and animal enzymes produced in microbes by genetic engineering technology.

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2 Enzymes for bread, pasta and noodle products

Joan Qi Si and Cornelia Drost-Lustenberger

2.1 Introduction

Bread, noodles and pasta are among the most basic, common and low-cost foods and their manufacture is often dependent on the traditions of the countries or regions in which they are consumed. However, ancient as bread, noodle and pasta-making are, they are also closely related to biotechnology.

This chapter reviews the effects of different types of enzymes and the synergistic effects when they are combined with each other during the bread-making process. It will also give examples of enzyme use in Asian noodles and pasta.

2.2 Bread

Consumers have certain quality criteria for bread, including appearance, freshness, taste, flavour, variety, and a consistent quality. It is a great challenge for the baking industry to meet these criteria, for several reasons. Firstly, the main ingredient of the bread—flour—varies due to wheat variety, weather during the growing season, and milling technology. Although millers attempt to blend wheat from different sources to produce flour with a good and consistent baking quality, it often proves difficult to satisfy both high-quality and low-cost standards at the same time. Secondly, because bread preferences differ, the baking industry uses ingredients with different qualities and employs different baking procedures. For instance, English sandwich bread, with its fine crumb structure and very soft texture, is not popular with the French, who want baguettes with crispy crust, large holes, and good crumb chewiness. Thirdly, consumer preferences are shifting towards healthier products. It is sometimes possible to make new bread varieties by simply adjusting the formulation or baking procedure. However, in other cases, bakers may have to develop new techniques. Therefore, both millers and bakers need ingredients or process aids such as chemical oxidants, emulsifiers and enzymes to standardise the quality of the products and diversify the product range.

For decades enzymes such as malt and microbial *alpha*-amylases have been used for bread making. Due to the changes in the baking industry and the demand for more varied and natural products, enzymes have gained more and more importance in bread formulations. Through new and rapid developments in biotechnology, a number of new enzymes have recently been made available to

the baking industry. One example is pure xylanase, with single activity instead of traditional hemicellulase preparations, which improves the dough machinability. A lipase has a gluten strengthening effect that results in more stable dough and improved crumb structure similar to DATEM or SSL/CSL and a maltogenic *alpha*-amylase that has a unique anti-staling effect.

2.2.1 *Fungal* alpha-*amylases*

Wheat and thus wheat flour contains endogenous and indigenous enzymes, mainly amylases. However, the level of amylase activity varies from one type of wheat to another. The amount of *alpha*-amylases in most sound, ungerminated wheat or rye flours is negligible [1]. Therefore, most bread flours must be supplemented with *alpha*-amylases, added in the form of malt flour or fungal enzymes.

Many methods are available for the determination of amylase activities as reviewed by Kruger and Lineback [2]. The baking industry and millers use other methods such as falling number (FN) and Brabender amylograph to determine the amylase content and correlate to bread making quality. Although FN is excellent for measuring the activity of cereal amylases including those in malt flour, it is not suitable for measuring the activity of fungal *alpha*-amylases. Fungal *alpha*-amylases are generally less thermostable; they are inactivated at temperatures near 65°C. Therefore, fungal *alpha*-amylases cannot be detected by the standard FN method, which is conducted at 100°C (AACC method 56-81B, 1972). Perten and co-workers reported a modified FN method for measuring the fungal *alpha*-amylases activity in flour [3].

Fungal *alpha*-amylases act on the damaged starch content, which can vary depending on wheat variety and milling conditions. Generally, flour made from hard wheat contains more damaged starch than the soft wheat. The *alpha*-amylases widely used in the baking industry can hydrolyse amylose and amylopectin to release soluble intermediate-size dextrins of DP2–DP12 [4]. The *alpha*-amylases provide fermentable sugar, which results in an increased volume, better crust colour, and improved flavour. Due to hydrolysis of the damaged starch, a suitable dosage of *alpha*-amylases results in a desirable dough softening. However, extensive degradation of the damaged starch due to an overdose of *alpha*-amylases commonly leads to sticky dough.

Figure 2.1 illustrates the effect of a fungal amylase on bread quality in terms of bread volume, crumb structure and dough characteristics. The volume and crumb structure improve with increasing dosage of fungal *alpha*-amylases. Although a high dosage can provide a larger volume increase, the dough would be too sticky to work with. The optimum dosage is thus defined as the dosage with maximum reachable volume without a sticky dough. For the examples in figure 2.1, the optimum dosage for both flours is around 15 FAU/kg flour (FAU = fungal *alpha*-amylase units).

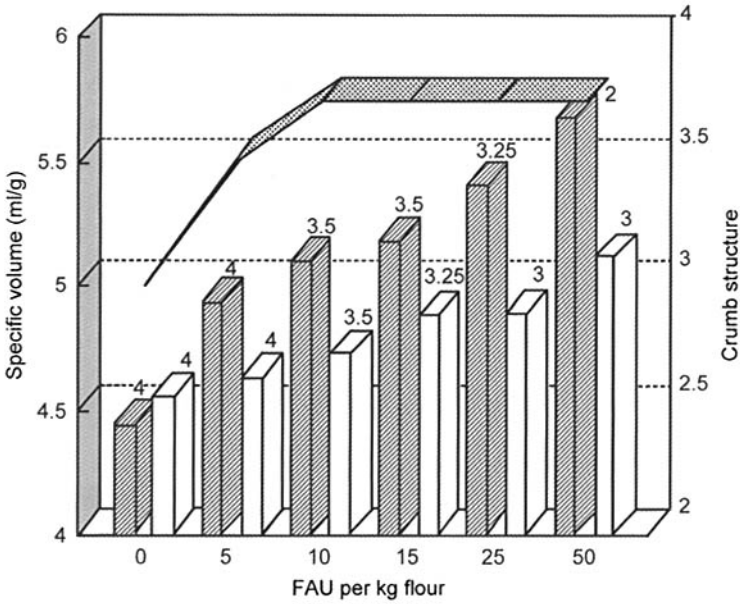


Figure 2.1 Dosage response of fungal amylase in different flours. ▨ = flour type 1; ⊠ = crumb structure; □ = flour type 2. FAU is units of fungal *alpha*-amylase.

2.2.2 *Amylases to extend shelf life*

Bread staling is responsible for significant financial losses to both consumers and bread producers. Staling corresponds to loss of freshness in terms of flavour, texture, perceived moisture level and other product characteristics. It is estimated that 3–5% of all baked goods produced in the US are discarded due to a loss of freshness, which may exceed 1 billion US$ [5].

Crumb softness and crumb elasticity are important characteristics for the description of crumb freshness perceived by consumers. Softness indicates the force needed to compress the crumb, whereas elasticity indicates the resiliency or the resistance given by the crumb while being pressed. The two texture characteristics may not necessarily correspond to each other: that is, the softest bread may not necessarily have the most elastic crumb texture or vice versa. Bread staling results in reduction of crumb elasticity and increase of crumb firmness. Figure 2.2 depicts how crumb softness and elasticity are determined from the graph of a texture analyser.

2.2.2.1 *Factors influencing the crumb softness and elasticity*

It is important to discuss the differences between the true anti-staling effect (i.e. the effect on the starch retrogradation) and other factors which have an effect

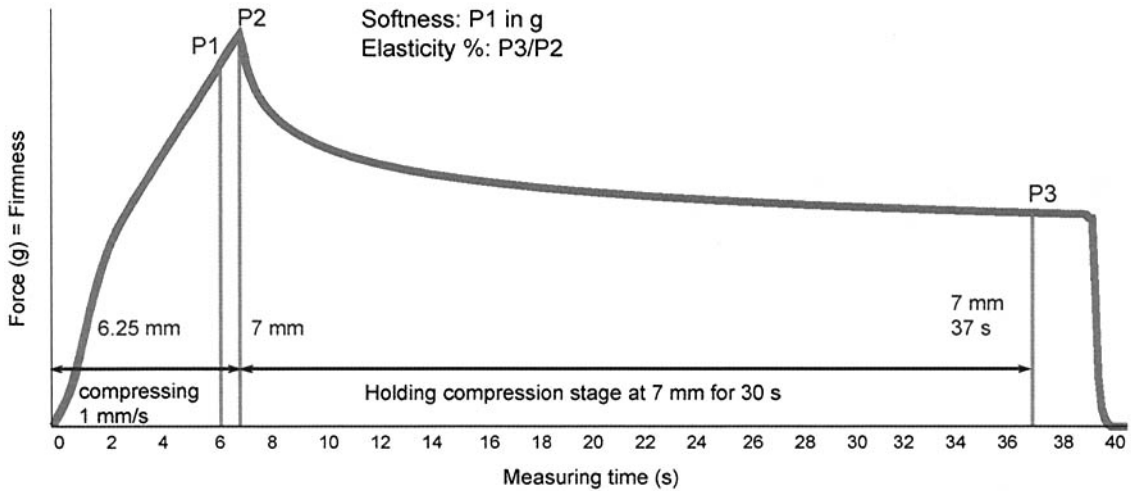


Figure 2.2 Curve of a softness/elasticity measurement using a texture analyser.

on crumb softness without necessarily involving starch retrogradation. A few of the factors influencing the crumb texture are described below.

Quality of wheat flour. First of all, the level of indigenous and endogenous amylases will influence the starch retrogradation as well as the yeast action, thereby affecting properties of the final bread quality such as volume. Secondly, the level of damaged starch in the flour has an influence on the action of cereal and fungal *alpha*-amylase, and therefore on the final quality of the bread.

Bread volume. A larger loaf, that is, one with high specific volume, has a softer crumb than a smaller loaf with a low specific volume and dense crumb.

Crumb structure. A fine crumb structure with thin cell walls and uniform crumb cells gives a softer crumb than a coarse crumb structure with thick cell walls.

Formulation and procedures. Besides flour, most of the ingredients used for bread making have an influence on the crumb texture. For instance, addition of shortening will result in softer crumb because of significantly increased volume and complexes with starch. Any ingredient or procedure that has an influence on loaf volume or crumb structure will influence the crumb texture. Figure 2.3 shows the changes of the crumb softness and elasticity of European sandwich (straight dough) bread and American sponge and dough bread during nine days storage at 24°C. The freshly produced sponge and dough loaves are generally softer and less elastic than the straight dough loaves due to the different procedures and ingredients used. When bread staling occurs during storage, crumb firmness increases and the elasticity decreases regardless of the procedure and ingredients.

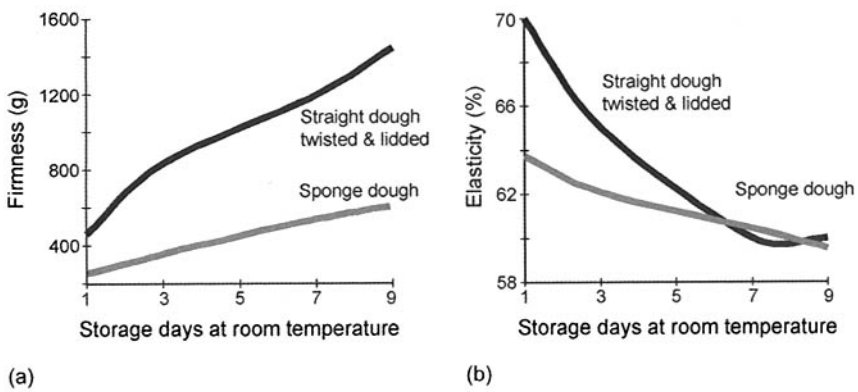


Figure 2.3 The crumb softness (a) and elasticity (b) of sponge and dough compared to straight dough procedure.

A process factor or an ingredient can improve the crumb softness (i.e. reduce crumb firmness), but may not have any influence on starch retrogradation. Figures 2.4 and 2.5 show one example of how the process influences crumb softness and elasticity through improvement of crumb structure. Both loaves were baked using identical ingredients and baking procedures except panning. Both were baked at the same time and stored under the same conditions. As a result of a twisting step before panning, the crumb structure produced from the twisted method is much more uniform than the non-twisted. The improved

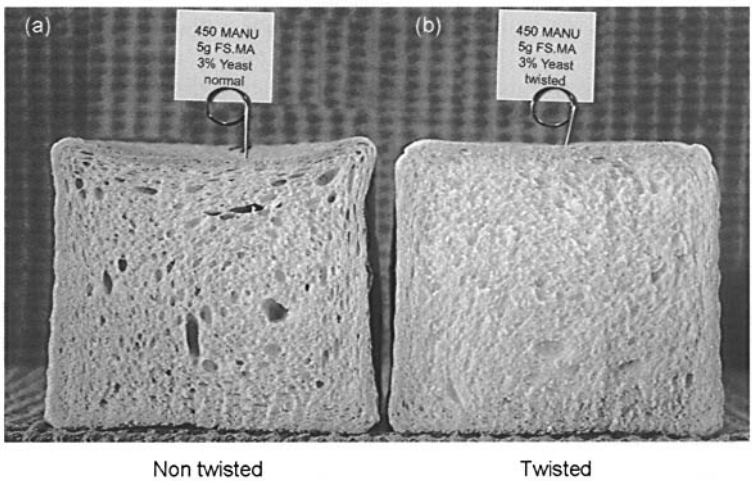


Figure 2.4 Crumb section of European toast bread from (a) one piece dough method; (b) two piece dough twisted method.

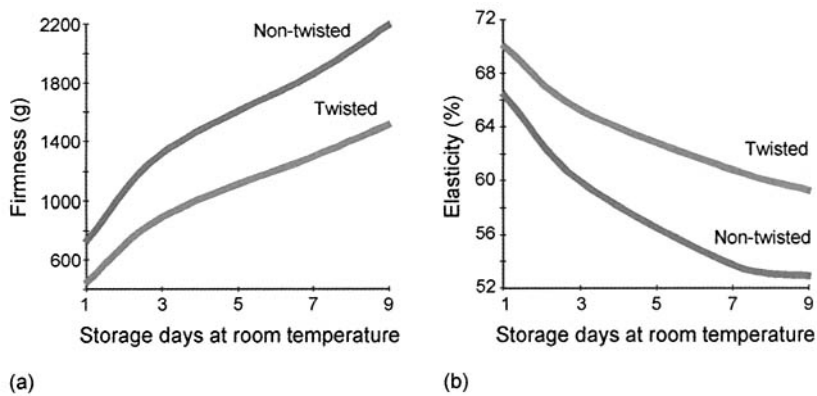


Figure 2.5 The crumb softness (a) and elasticity (b) of European sandwich bread: influence of crumb structure on crumb texture.

crumb structure is the reason for a significantly softer crumb and higher elasticity throughout the whole storage period of nine days as shown by figure 2.5.

Storage condition. Storage temperature and package quality have an impact on the crumb texture. It is well known that cold temperatures induce the retrogradation of starch and thereby increase the staling rate. Moisture loss because of to poor packaging results in a faster staling of bread.

Starch retrogradation. Bread staling has been investigated for nearly 150 years, but the precise mechanism is still far from being understood, and the debate continues as to the general nature of the processes involved [6]. However, most researchers consider starch retrogradation as the primary cause of crumb staling. Recently, several publications presented new results on their studies on the mode of action of amylases and their role in preventing staling [7–9]. To discuss all the studies that have been conducted is beyond the scope of this chapter. The consensus among them appears to be that changes in starch (especially the amylopectin part) modified by the *alpha*-amylase play the major role in the anti-staling effect.

Although the consumer's perception of crumb freshness may be independent of the cause of a given crumb texture, it is critically important for those involved in product development in the baking industry to understand and differentiate between these factors. When comparing or selecting the optimum ingredient for the desired crumb texture, it is important to control or standardise the above factors when setting up the experimental design. For instance, the volume of the loaf samples should be controlled before measuring softness and elasticity.

A longer shelf life means the stored bread has as soft a crumb and as high a resiliency as fresh bread. The optimum anti-staling agent should be the one that can maintain the crumb softness as well as the crumb elasticity throughout the storage of the bread.

2.2.2.2 *Effect of different* alpha-*amylases on bread staling and bread quality*

While fungal amylases are effective in partially hydrolysing damaged starch, and are often added to flour as supplements to develop desirable properties such as ovenspring and a brown colour in the crust, they have limited anti-staling effect due to their limited thermostability. They are, for the most part, inactivated prior to the onset of starch gelatinisation during baking when the bulk of the starch is available for modification.

The bacterial *alpha*-amylase from *Bacillus subtilis* is able to inhibit staling by hydrolysing glycosidic linkages within the amorphous areas of gelatinised starch. However, this thermostable bacterial *alpha*-amylase can easily be overdosed. Its pure endo-action excessively degrades the starch during baking, causing collapsing of the bread immediately after removal from the oven.

Due to the high degree of thermostability, the enzymes can persist throughout baking and cooling and produce an excessive level of soluble dextrins. As a result the final product is often unacceptable, with gummy crumb texture or even sticky crumb causing problems during slicing and retail storage.

Maltogenic *alpha*-amylase has thermostability between that of fungal *alpha*-amylase and thermostable bacterial *alpha*-amylase [10]. Therefore, it is able to hydrolyse the glycosidic linkages of the gelatinised starch during the baking process, but it does not excessively degrade the starch because it is inactivated during the later stage of baking [6]. Figure 2.6 depicts the difference in action pattern between the bacterial and maltogenic *alpha*-amylases.

A major advantage with maltogenic *alpha*-amylase is its tolerance to over-dosing during the bread making process in the bakery. Even fungal *alpha*-amylase can cause sticky dough or over-browning of the crust if overdosed. Maltogenic *alpha*-amylase does not affect dough rheological properties due to its low activity at a temperature under 35°C. It is highly active only at a temperature during starch gelatinisation while it does not excessively degrade the starch, but mainly produces small soluble dextrins. The overdosing risk is, therefore, much lower than with the other two types of amylases. Figure 2.7 shows loaves of bread baked with three different kinds of amylases.

Addition of maltogenic *alpha*-amylase in the sponge and dough bread can prolong shelf life for at least four days longer compared to 0.5% powdered distilled monoglycerides. As seen in figure 2.8, the crumb softness of the loaf with maltogenic *alpha*-amylase at day 7 was as soft as the loaf with 0.5% distilled monoglycerides at day 3; the elasticity of the loaf with maltogenic *alpha*-amylase at day 7 is even higher than the loaf with distilled monoglycerides

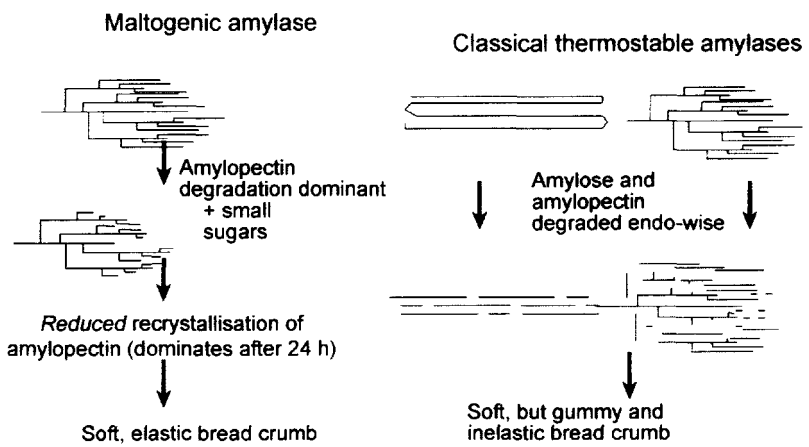


Figure 2.6 A model suggestion of modified amylopectin by maltogenic and thermostabile bacterial *alpha*-amylases.

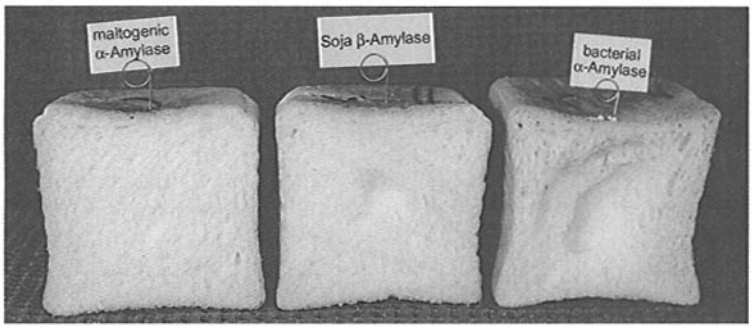


Figure 2.7 Toast bread containing maltogenic *alpha*-amylase, soy *beta*-amylase, bacterial *alpha*-amylase that has been hand squeezed after three days at 24°C. The loaf with the bacterial amylase is unacceptable. Due to the excessive degradation of starch during baking and during storage, the crumb is sticky.

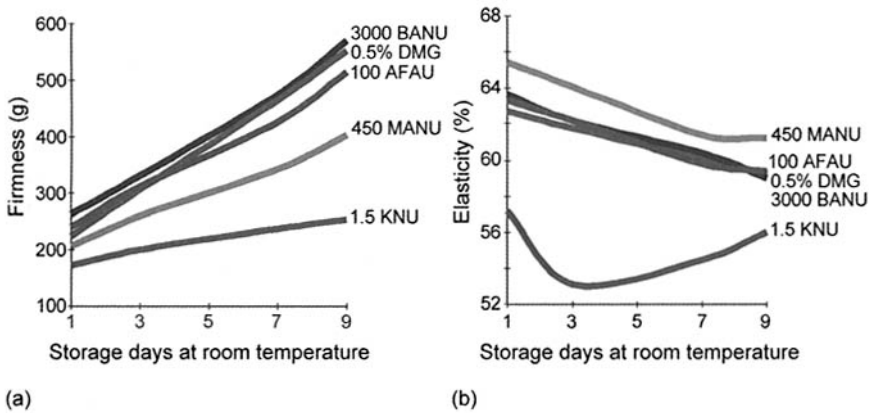


Figure 2.8 Sponge and dough procedure using American flour; (a) crumb softness, (b) crumb elasticity. Volume difference: max. ±3%. DMG = fine powdered distilled monoglycerides; MANU = units of maltogenic *alpha*-amylase/kg flour; KNU = units of bacterial *alpha*-amylase/kg flour; BANU = units of soy *beta*-amylase/kg flour; AFAU = units of acid fungal *alpha*-amylase/kg flour.

at day 3. Although the bacterial *alpha*-amylase gave the softest crumb, it was very gummy with an extremely low elasticity value.

In one study [4] it was shown that maltogenic *alpha*-amylase produces mainly DP2 (i.e. maltose). It also produces small fractions of DP1–DP6 in a starch-water gel that has a similar starch:water ratio as that in normal bread, whereas fungal *alpha*-amylase releases small amounts of DP1–SP12. Later studies [9] showed that maltogenic *alpha*-amylase could release linear maltodextrins up to at least DP7 under actual baking conditions, whereas *beta*-amylase and glucoamylase only form maltose and glucose respectively.

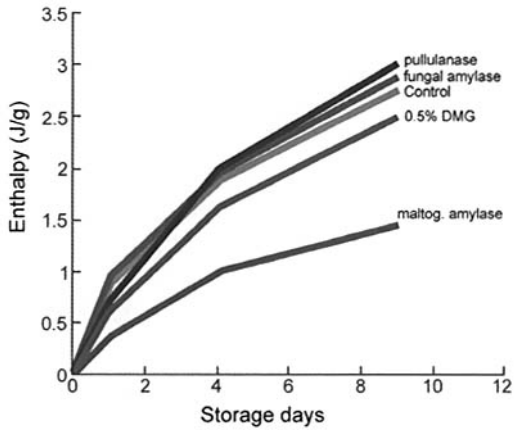


Figure 2.9 Effect of maltogenic *alpha*-amylase on retarding starch retrogradation measured by differential scanning calorimetry (DSC). The maltogenic *alpha*-amylase reduces the endothermic area (enthalpy J/g) significantly after storage of 2–9 days.

The dextrins produced by the amylases in the baked bread give different dextrin distributions to those in the control loaf [6], therefore these can undoubtedly be correlated to the changes in the staling rate. These dextrins produced in the baked bread have a different function than that of added maltodextrins.

Starch retrogradation can be measured using differential scanning colorimetry (DSC). Figure 2.9 shows DSC measurement of the effect of treating a starch-water gel with maltogenic *alpha*-amylase compared to treatment with fungal *alpha*-amylase, pullulanase, distilled monoglycerides and a control.

2.2.3 Xylanases/pentosanases/hemicellulases

There are 3–4% (w/w) pentosans in normal wheat flour, partially soluble and partially insoluble. Xylanase or pentosanases, often commonly called hemicellulases, have long been used as dough-conditioning enzymes, especially in European-type bread, because they have demonstrated desirable effects as dough conditioning enzymes. At the optimum dosage they can improve dough machinability, dough stability, oven-spring, larger loaf volume and improved crumb structure [11, 12]. Because of the beneficial effects they have on loaf volume and crumb structure, the addition of hemicellulases results in a softer crumb. With the presence of fungal *alpha*-amylase in the product, this effect on softness is even more pronounced.

Through recombinant gene technology, some xylanases made from genetically modified organisms (GMO) are now on the market [13]. The benefit of using a xylanase instead of a traditional pentosanase is that there are far fewer side activities in the xylanase product. Consequently, a lower dosage is

needed to achieve the same effect with less risk of possible interference from side activities. The enzyme quality is also more consistent.

Pentosanases and xylanases can be overdosed due to overdegradation of wheat pentosans, thereby destroying the water-binding capability of the wheat pentosans. The result of overdosing is a sticky dough. A suitable dosage of these enzymes results in a desirable softening of dough, thereby improving the machinability. An optimum dosage is therefore defined as the dosage that gives the maximum improvement of the dough and bread properties without causing dough stickiness. The optimum dosage of pentosanases or xylanases varies according to the flour, as illustrated in figure 2.10. The optimum dosage of this particular xylanase for type 1 flour is 120–200 FXU (fungal xylanase units) and for type 2 flour is 80 FXU.

The true mechanism of xylanase in bread making has not been clearly elucidated, although a number of different approaches have been examined. The composition of pentosans varies depending on the flour type [14]. The interaction between pentosans and gluten plays an important role that has not yet been defined. Most commercially available enzyme preparations used for studies consist of several enzyme activities including amylase, protease, and several

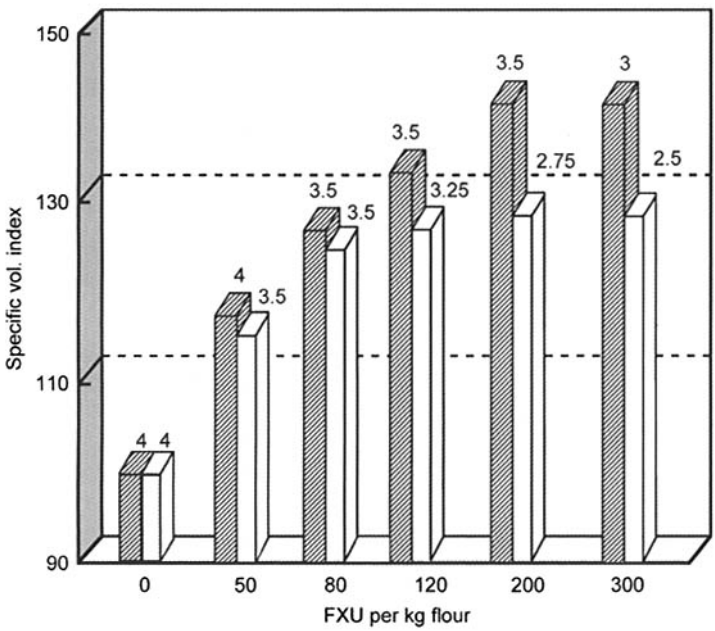


Figure 2.10 Dosage response of xylanase in different flours. Dough stickiness: 4 = normal; 3.5 = acceptable; 3 = sticky; 2.5 = too sticky. ▨ = flour type 1; □ = flour type 2.

hemicellulases. The work of Hamer and co-worker [11, 15] indicates that the use of pentosanases increases gluten coagulation in a diluted dough system. Si and Goddik [17] reported that a good baking xylanase increases the gluten strength measured by dynamic rheological methods on a Bohlin rheometer VOR. By measuring the gluten extracted from a dough system, the good baking xylanase increased storage modulus G′ and G″. At the same time the gluten became more elastic because the phase angle δ decreased, whereas the two other xylanases, which did not show good baking performance, had no significant effect on gluten strengthening.

Figure 2.11 shows the effect of a commercially available baking xylanase on the rheological properties of gluten extracted from a wheat flour dough measured on a Bohlin rheometer VOR [18]. At the dosages that give good baking performance, 50–100 FXU/kg flour, the gluten was strengthened and more elastic. These improved rheological properties explain the positive effect of this xylanase on bread making (i.e. increased oven spring, resulting in larger volume and improved dough stability). At an excessive level of 400 FXU/kg flour, which gave a very sticky dough, G′ is the same as for the control and the phase angle shows that the gluten is more viscous.

Different xylanase or pentosanase preparations have different effects on arabinoxylans (pentosans) in terms of their scission points and reaction products: therefore, they have different effects on bread making [16]. Although many analytical methods are available for assaying pentosanases or xylanases using a wide range of substrates, there is still no analytical method in which the enzyme units can correlate to the baking performance when comparing different types of xylanases or pentosanases. It has been reported that no correlation to the baking performance was found when different xylanase preparations from different sources were submitted to the same standardised assay using purified substrate

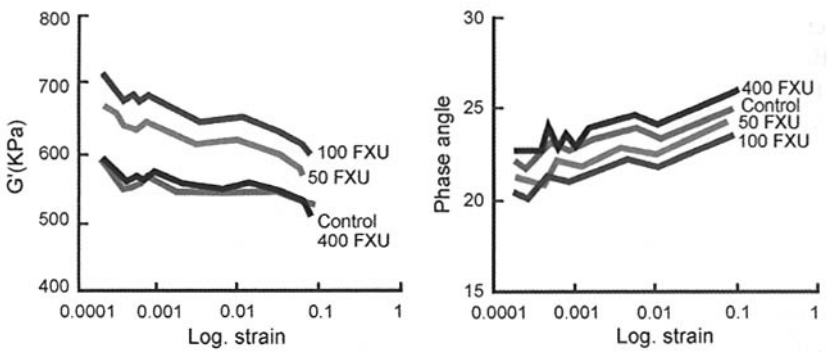


Figure 2.11 The effect of xylanase on the rheological properties of gluten. The diagram on the left shows the storage modulus G′ of gluten for different levels of FXU/kg flour. The diagram on the right shows the phase angle δ.

and baking tests [19]. One possible explanation is that once the wheat pentosans, both soluble or insoluble, were extracted from the flour, the mechanism of the interactions between wheat pentosans and gluten could no longer be assayed. Xylanase indirectly influences the gluten, as discussed above.

A possible mechanism of pentosanases mentioned by Hamer [11] is that the enzyme can offset the negative effects of insoluble pentosans present in the flour, because insoluble pentosans are regarded as having a negative effect on loaf volume and crumb structure. A study conducted by Jacobsen and Si [16] using four different pure xylanases concluded that the best enzyme in terms of baking performance was the xylanase with a certain level of activity towards both soluble and insoluble wheat arabinoxylans (pentosans) in a dough system. The xylanase which had most activity towards the insoluble arabinoxylans gave a dough too sticky to be acceptable.

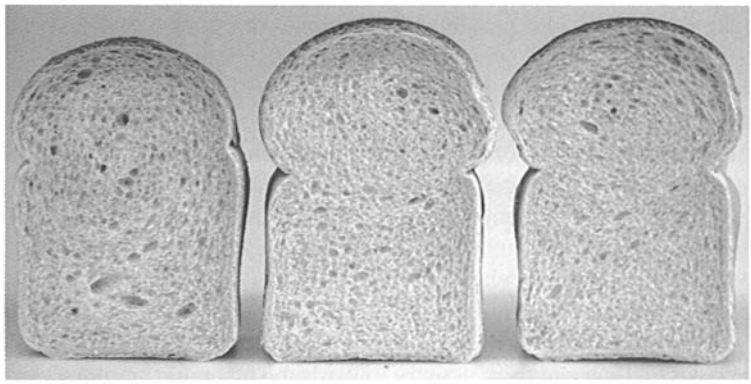
Pentosanases also find application in the production of bread from rye and wheat/rye blends. The baking performance of a rye flour is related to the swelling capacity and solubility of its pentosans and to its starch/pentosan ratio.

Appropriate application of pentosanases balances the water absorbtion capacities of the pentosans with the starch. This initially provides better dough extensibility and handling and prevents further separation of crumb from crust during baking.

2.2.4 Lipase

Lipase has recently been recognised as a strong dough-conditioning enzyme. It shows excellent effects on bread performance. The loaf volumes of various bread types increase significantly and the crumb structure is more silky and uniform with a whiter appearance. As shown in figure 2.12, the lipase can partially or fully replace emulsifiers such as DATEM or SSL/CSL; its overall performance depends on the procedure, formulation and raw material and the presence of other improving ingredients.

Both the influence of wheat lipids in bread making and the mode of action of the lipase [32, 33] are not yet fully understood. The hypothesis that monoglycerides are produced *in situ* cannot explain the effects of the lipase demonstrated above. Monoglycerides are known to have mainly an anti-staling effect; they have little dough conditioning effect compared with lipase. Furthermore, the contents of total lipids in most wheat flours is in the range of 1–1.5%. Regarding the limited hydrolysis degree [20] and limited quantity of triglycerides of saturated fatty acids in a dough system, an insufficient amount of monoglycerides of saturated fatty acids is produced to exhibit the effects described earlier. Besides, the monoglycerides produced by this 1,3-specific lipase would be second-position monoglycerides. It was reported that first and third-position monoglycerides are more able to form complexes with starch, thus having a retarding effect on starch staling [21].



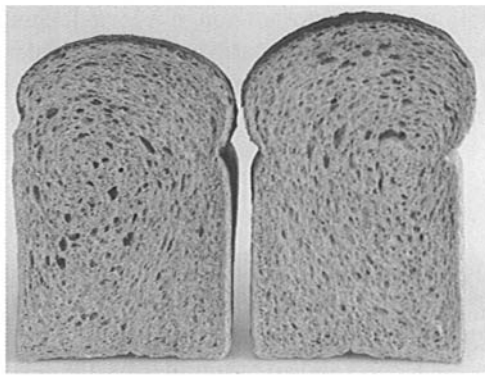


Figure 2.12 A new baking lipase mimics the improving effects of the SSL in white bread (a) or emulsifier blends in multigrain breads (b) to yield loaves with equivalent or even improved volume and crumb structure.

Addition of a fungal lipase to a flour dough does not significantly change the rheological properties of the dough measured by both farinograph and extensograph [18] with optimum water absorption. This is a good characteristic for bread making, because most bakers do not like major changes in the dough system. However, when the rheological property of the gluten treated by the lipase was measured using the dynamic rheological method, it was found that the reason for the good dough conditioning effect of this lipase is the increase of gluten strength, as shown in figure 2.13. The gluten taken from lipase-treated wheat flour dough is significantly stronger, with an increased G′, and more

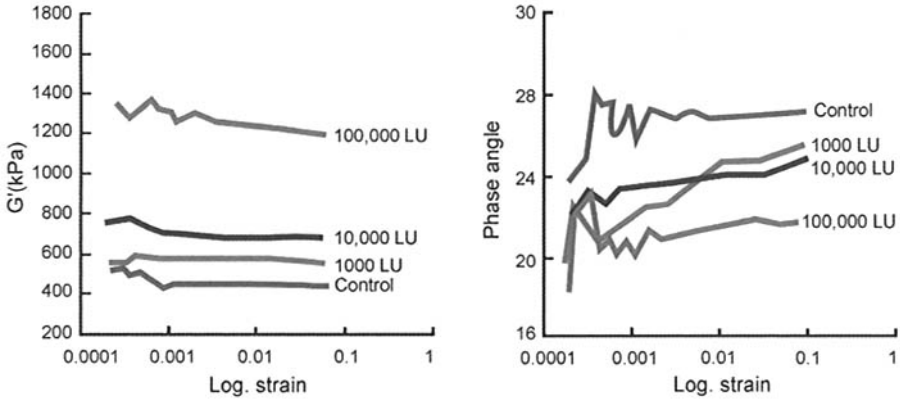


Figure 2.13 The effect of lipase on the rheological properties of gluten. The diagram on the left shows G′, the storage modules of gluten. The diagram on the right shows the phase angle δ. LU is units of lipase/kg flow.

elasticity, shown by a lower δ than the control. Excess lipase results in too strong a gluten complex with a very high G′, which gives a dough that is too stiff and a smaller volume increase, as mentioned earlier. The gluten treated with lipase has a high G′ and increased relaxation time, indicating an increase of cross-links in the gluten networks measured by the dynamic stress relaxation method [19]. The nature of these extra cross-links has yet to be elucidated.

It was assumed that glycolipids form a linkage between gliadin and glutenin through hydrogen bonds and hydrophobic interactions [22, 23]. Work at the University of Lund indicated that the lipase increased the thermostability of the reversed hexagonal phase of the liquid-crystalline phase during heating up to 100°C [24]. This observation is suggested as a possible mechanism for lipase in bread making [25].

Because lipases are gaining more and more attention in the baking industry, studies in the near future should give us a better understanding of the mode of action of lipase for bread making.

2.2.5 *Oxidases*

Oxidants, such as ascorbic acid, bromate and ADA, are widely used for bread making and have been thoroughly studied. However, the true mechanism of the oxidants in bread making has not been established, although a number of hypotheses have been offered. It is generally known that bromate is a slow-acting oxidant that becomes active at high temperatures. In relation to baking, it has the maximum effect in the later stages of proving and in the early stages of baking, whereas ascorbic acid and other oxidants are fast-acting oxidants, having the maximum effect during mixing and proving.

Increasing demands by consumers for more natural products with fewer chemicals, and especially concerns about the possible risks of bromate in food, have created the need for bromate replacements. Therefore, oxidases are gaining increasing attention in the baking industry. Glucose oxidase for bread making has been known since 1957 [26]. Although lipoxygenase, lysyl oxidase, sulfhydryl oxidase [27], peroxidase [28, 29], laccase [30] and transglutaminase have been reported to have good oxidising effects, there is little known about oxidases for bread making.

Glucose oxidase has good oxidising effects that result in a stronger dough. It can be used to replace oxidants such as bromate and ascorbic acid in some baking formulations and procedures. In other formulations it is an excellent dough strengthener along with ascorbic acid. Examples of applications of glucose oxidase combined with other enzymes for bread making are given in the next section.

2.2.6 Synergistic effects of enzymes

2.2.6.1 Enzymes for dough conditioning

Using enzyme combinations for baking bread is not new. It is well known that combining a hemicellulase or xylanase with fungal *alpha*-amylase has a synergistic effect. As shown in figure 2.14 a high dosage of a pure xylanase may give some volume increase, but the dough with this dosage of xylanase would be too sticky to be handled in practice. When the xylanase is combined with

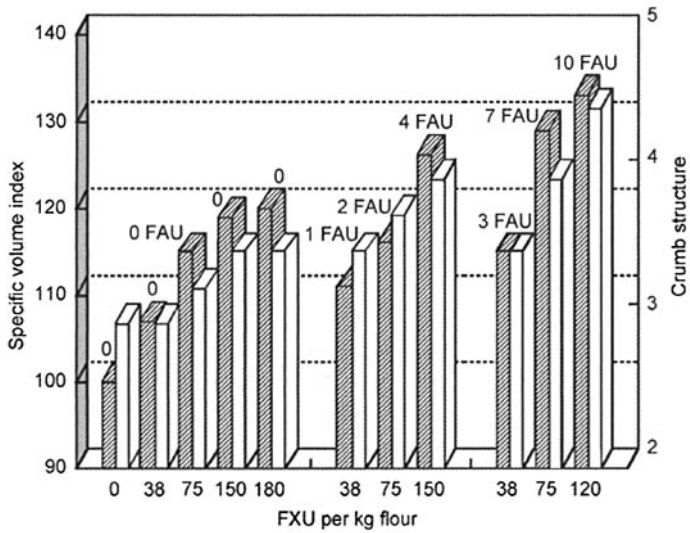


Figure 2.14 Synergistic effect of amylase and xylanase. ▨ = volume index; □ = crumb structure.

even a very small amount of fungal *alpha*-amylase, a lower dosage of xylanase with the *alpha*-amylase provides a larger volume increase and better overall score without the problem of dough stickiness.

Possible new combinations with improved functionality are continuously arising from new enzymes through product development in the enzyme industry [31]. For instance, as lipase does not make the dough sticky and significantly improves dough stability and crumb structure, synergistic effects between xylanase, amylase and lipase could improve bread quality. Due to its effect on gluten strengthening, lipase improves dough stability against over-fermentation. Figure 2.15 shows the combination of the enzymes for a French baguette (trials were conducted at the ENSMIC Baking Laboratory, Paris). Without any enzyme, the baguette had poor stability against over-fermentation. With the addition of *alpha*-amylase alone or in combination with xylanase, the volume and overall scores of the bread improved. When *alpha*-amylase, xylanase, and lipase were used together, the overall scores were further improved. The dough exhibited notably good stability against over-fermentation, resulting in significantly better baguettes.

Figure 2.16 shows that on addition of xylanase, loaf volume is slightly improved. Using a xylanase in combination with fungal *alpha*-amylase, the volume is further increased; it also improves the crumb structure, so the cell walls are thinner and the crumb is silkier. When combining lipase with the other enzymes, not only is the loaf volume further increased, but also the crumb

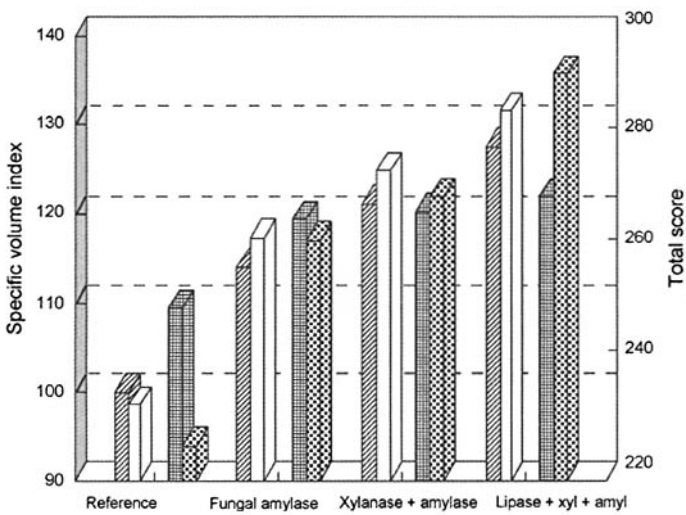


Figure 2.15 The synergistic effect of enzymes for the French baguette. ▨ = volume; □ = volume over proved; ▦ = total scores; ▩ = total score, over proved.

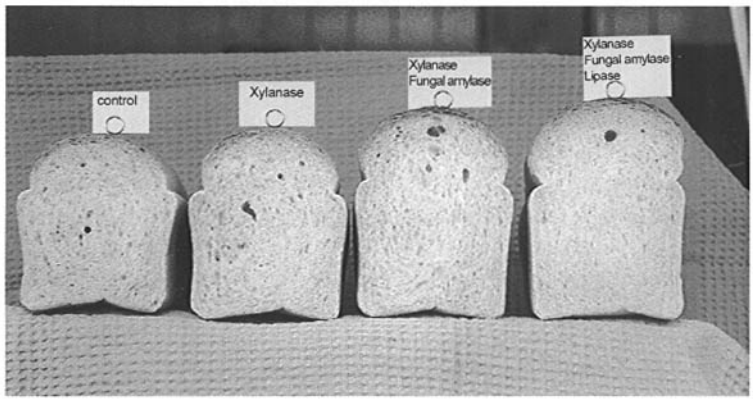


Figure 2.16 Improvement of loaf volume on addition of xylanase.

structure is significantly improved with more uniform crumb cells. Consequently, this loaf would have the softest crumb among these samples. This shows that the combination of lipase with *alpha*-amylase or xylanase can provide a fine, silky and uniform crumb structure to loaves made with a straight dough process.

2.2.6.2 Combining enzymes to maximise shelf life

As mentioned before, maltogenic *alpha*-amylase is a true anti-staling enzyme that affects neither bread volume nor crumb structure. Therefore, it is quite feasible to use this enzyme in combination with enzymes such as fungal *alpha*-amylase, xylanase and lipase to ensure that other bread quality parameters such as volume, dough stability, and crumb structure are improved. As shown in figure 2.17, a combination of the four enzymes gave the softest crumb throughout the entire storage period of 9 days. The softness of the bread with these four enzymes at day 9 was the same as that of the loaf with 0.5% of distilled monoglycerides at day 3.

It is, however, important to note that these enzymes, except the maltogenic *alpha*-amylase, have no effect on the crumb elasticity. The crumb elasticity, which indicates the resilience of the crumb upon storage, is only affected by the increasing dosage of the maltogenic *alpha*-amylase.

2.2.6.3 Enzymes for dough strengthening

Glucose oxidase has good oxidising effects that result in stronger dough. In combination with other enzymes such as xylanase and *alpha*-amylase, it can be used to replace oxidants such as bromate and ADA in some baking formulations and procedures. In other formulations when it is added with ascorbic acid, it is an excellent dough strengthener.

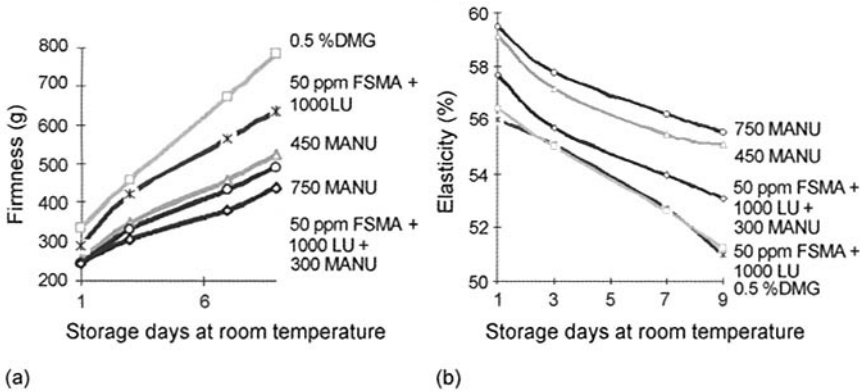


Figure 2.17 The synergistic effect of maltogenic *alpha*-amylase in combination with fungal *alpha*-amylase, xylanase and lipase compared to distilled monoglycerides in American type sponge and dough bread; (a) crumb softness, (b) crumb elasticity. DMU = fine powder distilled monoglycerides. 50 ppm FSMA = 12.5 FAU units of fungal *alpha*-amylase and 100 FXU units of xylanase per kg flour. LU = units of lipase/kg flour, MANU = units of maltogenic *alpha*-amylase/kg flour, DMG = fine powder distilled monoglycerides.

There are benefits in adding a combination of glucose oxidase, xylanase and fungal amylase to ascorbic acid when baking French batârds. Adding the enzymes improves the stability of the dough during processing which results in batârds with greater volume. The enzyme combination also improves the bloom, yielding a more desirable crispy crust.

Figure 2.18 shows a similar benefit obtained by adding a combination of glucose oxidase, xylanase and fungal amylase to ascorbic acid when baking Kaiser rolls. Addition of the enzymes results in a markedly improved volume and a more defined cut pattern.

Figure 2.18 The roll on the right has had a combination of enzymes added to ascorbic acid, while that on the left has untreated ascorbic acid.

Figure 2.19 shows that adding a combination of glucose oxidase and fungal amylase to ascorbic acid makes a bagel with significantly improved volume.

Glucose oxidase combined with fungal *alpha*-amylase can replace the bromate in some bread formulations. Figure 2.20 shows a type of South American

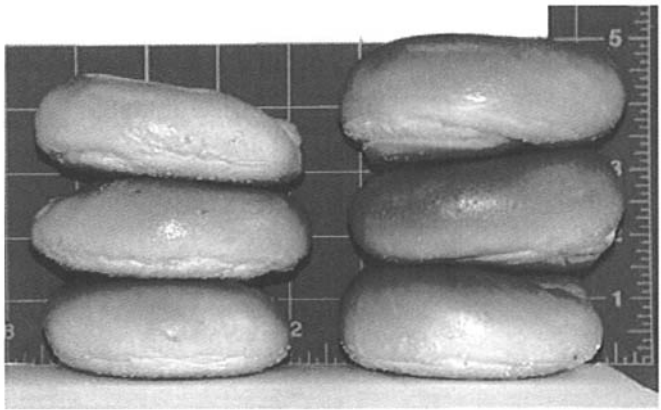


Figure 2.19 The bagels on the right have had a combination of enzymes added to ascorbic acid: those on the left have untreated ascorbic acid.

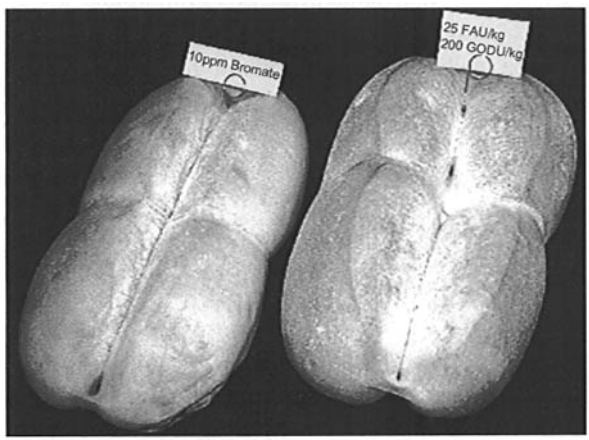


Figure 2.20 The effect of adding enzymes to Marraquetta.

bread, Marraquetta. This procedure requires dough with high stability because after two 50 min proving sessions, the dough is turned around (subjected to mechanical stress) before baking. Dough without strong dough stability will collapse when turned. The basic formulation contains both ascorbic acid and potassium bromate. By adding glucose oxidase and fungal *alpha*-amylase instead of bromate, the final bread has a much improved appearance and a volume increase of approximately 40%.

2.2.7 *Enzymes for frozen dough and part-baked bread*

Frozen dough has gained more and more popularity within the baking industry because it creates the opportunity to save on labour and equipment while providing freshly baked goods for the consumer at in-store bakeries, food services and in restaurants.

The major problem with production of frozen dough is deterioration in volume and overall quality of the products. Many studies have shown that frozen dough stability is related to factors such as flour quality, ingredients, formulation, process, freezing and thawing conditions [34, 35].

The growth of ice crystals during freezing, freeze-thaw-cycles or frozen storage may cause physical disruption of the membrane of yeast cells. The loss of cytoplasm causes the yeast cells to die and production of carbon dioxide is no longer ensured. Reduced volume or prolonged proving time and undesirable appearance of the thawed and baked bread is the result. The autolysis of yeast cells, caused by concentration of organic metabolism products during freezing out the water phase, has been reported [34]. Furthermore, ice recrystallisation contributes towards mechanical disruption and weakening of the three-dimensional protein network which is responsible for gas retention. Weakening of the gluten structure and of the dough leads to less retention of gas, and lower volumes [36, 37].

The effects of different ingredients or chemical improving agents on the quality of bread made from frozen dough have been extensively reported [34, 38, 39]. This section describes the use of some enzymes and enzyme combinations to reduce quality deterioration in frozen systems. It focuses on unproved frozen dough, partially proved frozen dough and part-baked frozen baguettes and buns.

2.2.7.1 *Unproved frozen dough*

Xylanase and amylase. The functions of xylanases and fungal amylases have been previously discussed in this chapter. The indirect effect of xylanase on the strengthening of gluten in fresh dough also applies to frozen dough. Improvements in dough stability, volume and crumb structure are achieved during long frozen storage.

Figure 2.21 shows the volume improvement when a combination of xylanase and fungal *alpha*-amylase is added to the formula. The crumb structure is also

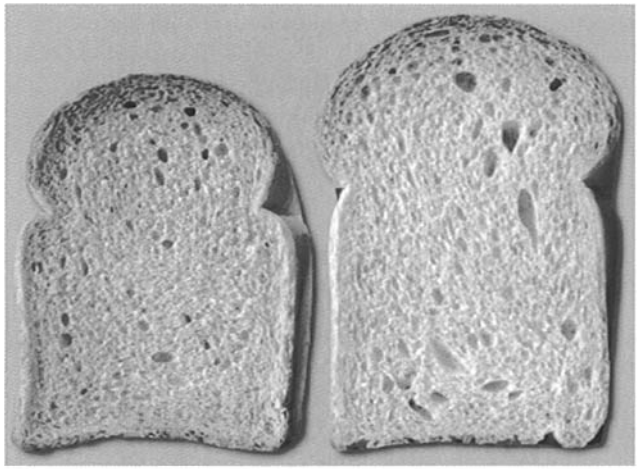


Figure 2.21 Effect of xylanase and fungal *alpha*-amylase in bread baked from unproved frozen dough. In the bread on the right containing 50 ppm of a product containing xylanase and fungal *alpha*-amylase, volume increase and improved crumb structure in terms of less density, elongated cells and thinner cell walls are clearly visible. The dough was stored for 14 weeks at −18°C. Both breads contain ascorbic acid, DATEM and vegetable shortening in the basic recipe.

improved. After several weeks of frozen storage, the control bread has a rather dense crumb with small round cells and thick cell walls. The addition of xylanase and fungal *alpha*-amylase reduces the density of the crumb and results in more elongated cells and thinner cell walls. Because of the larger specific volume and the improvement of crumb structure, softness and elasticity of the crumb are also improved.

In figure 2.22, and in most of the following figures, the volumes of the control or reference during frozen storage were taken to be 100%, in order to eliminate day-to-day variations in the results. That means that the figure shows the relative improvement of bread containing xylanase and fungal *alpha*-amylase, compared to the control. The increasing slopes of the graphs thereby do not necessarily mean that the volume increased as result of storage, but rather that it decreased at a lower rate, compared with the control.

Figure 2.22 shows a dosage response of xylanase and fungal *alpha*-amylase in terms of volume. The improvement in volume may be explained by the improved extensibility and less bulky dough structure. A more extensible dough probably moves more easily against growing ice crystals. The destruction of the gluten network by growth and sharpness of ice crystals is reduced and more of the gluten stays intact.

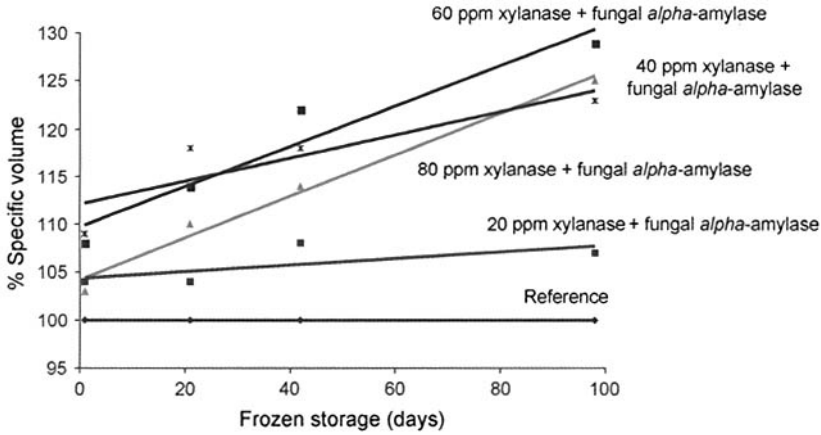


Figure 2.22 Xylanase and fungal amylase.

Glucose oxidase. As described earlier in this chapter, glucose oxidase is an effective gluten-strengthening enzyme, resulting in loaves with increased volume and a better appearance. It is somewhat surprising that in some cases, as illustrated in figure 2.23, the addition of glucose oxidase, even low levels, can have a detrimental effect on the quality of baked goods made from unproved frozen dough.

Of course actual results depend on other ingredients in the formula, but in this case, the gluten (not being fully developed at the time of freezing) becomes

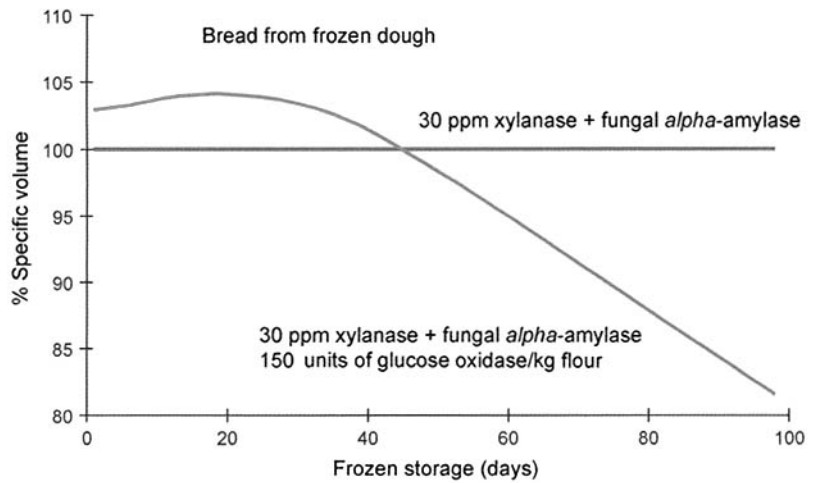


Figure 2.23 Effect of glucose oxidase on bread made from frozen dough.

too rigid with the reaction of glucose oxidase. It becomes brittle and even more susceptible to ice crystal damage during frozen storage or freeze-thaw cycles.

2.2.7.2 Pre-proved frozen dough

Frozen pre-proved dough presents a particular challenge because when the dough is frozen, the dough is at its weakest point. However, even in this case, enzymes can play an important role to ensure a good quality product.

Xylanase and amylase. Figure 2.24 clearly shows that in pre-proved frozen dough significant improvement in volume was achieved by the addition of xylanase and fungal *alpha*-amylase, or fungal *alpha*-amylase alone. For this application, dough pieces were made up to small baguettes and rolls and were proved for half the time of optimal proving before they were shock frozen.

Glucose oxidase. Glucose oxidase improves the quality of pre-proved or partially proved frozen dough, unlike its effect on unproved frozen dough. The gluten network in a pre-proved dough is developed. Through its oxidising effect, glucose oxidase makes this network stronger and less susceptible to damage by growing ice crystals. Figure 2.25 depicts the volume of rolls produced from pre-proved frozen dough with different glucose oxidase levels in combination with

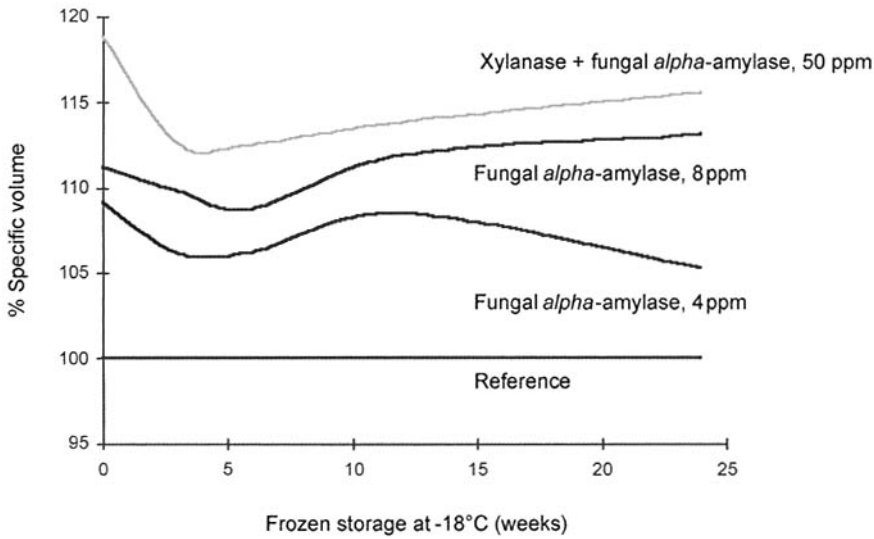


Figure 2.24 The volume of baked baguette from pre-proved frozen dough during frozen storage at −18°C. The graph shows increased volume of baguettes containing fungal *alpha*-amylase or the combination of fungal *alpha*-amylase with xylanase.

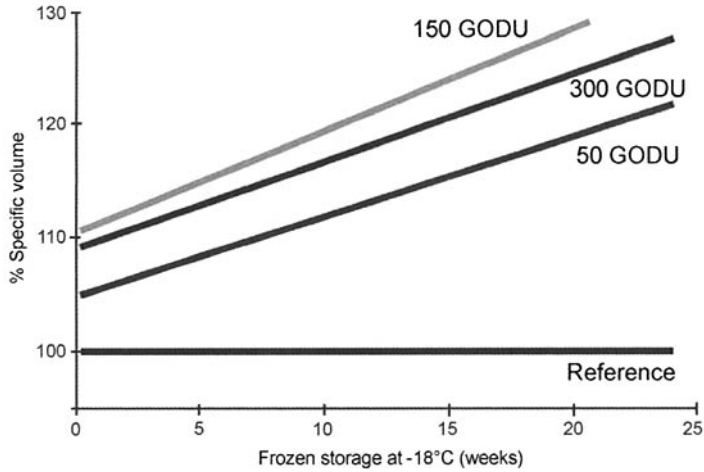


Figure 2.25 Volume of rolls baked from pre-proved frozen dough. Rolls containing glucose oxidase show significantly increased volumes during frozen dough storage and lower rate of deterioration. 30 ppm of a combination of xylanase and fungal *alpha*-amylase is added to the basic recipe. GODU = units of glucose oxidase/kg flour.

xylanase and fungal *alpha*-amylase. Glucose oxidase strengthens the gluten and stabilises the gluten network without causing the rigidity seen in unproved frozen dough. Addition of glucose oxidase resulted in an improved volume and reduced rate of deterioration during frozen storage.

2.2.7.3 Part-baked, frozen bread

Baguettes. In part-baked baguettes, effects of enzymes are similar to their effects in freshly baked baguettes. Volume and bloom are significantly improved by using a combination of xylanase and fungal *alpha*-amylase. The quality of the part-baked baguettes is further improved using the combination of xylanase and fungal *alpha*-amylase and glucose oxidase, as shown in figure 2.26.

Buns. Several enzymes and their combinations were also tested in part-baked Hamburger buns using a straight dough procedure, European flour and a formula with limited chemical additives and low water absorption. As shown in figure 2.27, 120 ppm of a combination of hemicellulase and fungal *alpha*-amylase gives a significant improvement in the volume and symmetry of the buns. Using a combination of xylanase and fungal *alpha*-amylase instead of hemicellulase and fungal *alpha*-amylase resulted in exactly the same effect (data not shown here). Combining glucose oxidase at a dosage of 200 GODU/kg flour with 120 ppm of a combination of hemicellulase and fungal *alpha*-amylase

Figure 2.26 Effect of 30 ppm of a combination of xylanase and fungal *alpha*-amylase and 150 GODU in partially baked, frozen baguettes. Frozen storage conditions were −18°C for 3 months.

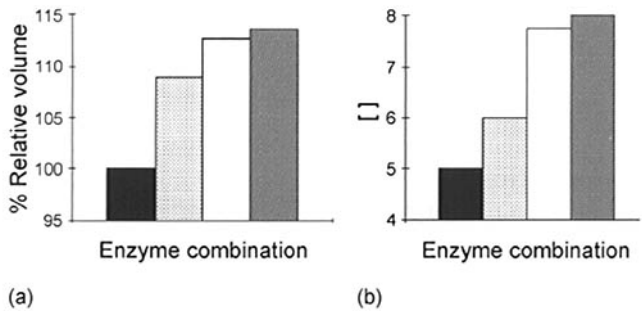
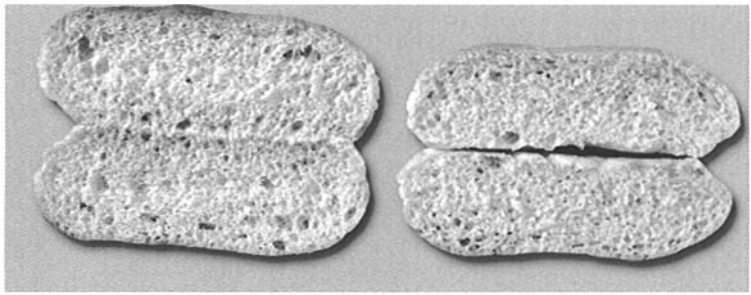


Figure 2.27 Part-baked straight dough Hamburger buns; (a) relative volume, (b) symmetry of bun. Dosage: 120 ppm of a combination of hemicellulase and fungal *alpha*-amylase, 200 GODU, 450 MANU. ■ = reference; ▨ = hemicellulase; □ = hemicellulase + glucose oxidase; ■ = hemicellulase + maltogenic *alpha*-amylase + glucose oxidase.

further increased volume and especially improved the symmetry and overall appearance of the buns.

One important parameter, particularly for hot dog or sandwich buns, is the hinge strength. This term describes the 'holding together' of two halves of a bun when nearly cut apart. The two halves should hold together under mechanical



(a) (b)

Figure 2.28 Hinge strength of buns with (a) and without (b) enzyme.

strain. Using an enzyme combination of amylases, hemicellulase and glucose oxidase can significantly improve this hinge strength. Figure 2.28 shows that the reference bun without enzymes broke into two after having been bent back and forth a few times. The bun with the described enzyme mix shows a strong 'holding effect' between the two halves. This effect is mainly pronounced in formulae with relatively low water absorption.

2.2.7.4 Shelf life extension of bread made from frozen dough

Bread baked from frozen dough is reported to be even more susceptible to firming than bread baked from freshly made dough [41–43]. Bread baked from unproved frozen dough that has been frozen for 7 weeks is firmer than bread made from dough frozen for 5 days. As in bread made from fresh dough using amylases, in particular maltogenic *alpha*-amylase, the staling rate can be reduced significantly. Bread baked from frozen dough containing maltogenic *alpha*-amylase or a combination of maltogenic *alpha*-amylase and xylanase and fungal *alpha*-amylase is significantly softer after baking than the reference containing 0.5% distilled monoglycerides. Again, as in bread made from fresh dough, maltogenic *alpha*-amylase provides much more crumb elasticity after baking compared to bread containing monoglycerides. These results show that maltogenic *alpha*-amylase prolongs the shelf life of bread not only during storage of the baked bread, but that it is also effective after a long freezing process and provides better quality with a softer and more elastic crumb texture.

Figure 2.29 again shows the effect of maltogenic *alpha*-amylase and its combination with fungal *alpha*-amylase and xylanase compared to that of distilled monoglycerides in bread baked from dough that was stored for 7 weeks at −18°C. The results show that the effect of enzymes on crumb texture is not only significantly better than that of distilled monoglycerides during bread storage, but also for fresh bread.

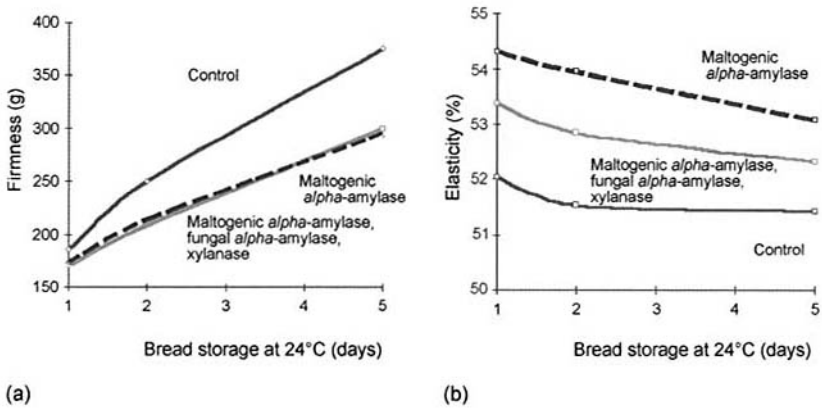


Figure 2.29 Softness (a) and elasticity (b) of bread baked from frozen dough after 7 weeks of frozen storage at −18°C. After baking the bread was stored at 24°C and measured after 1, 3 and 6 days. Volume was controlled. DMG = fine powdered distilled monoglycerides; 900 MANU = units of the maltogenic *alpha*-amylase per kg flour; 30 ppm FSMA = 7.5 FAU units of fungal *alpha*-amylase and 67.5 FXU units of xylanase.

2.3 Enzymes for Asian noodles and non-durum pasta [44]

The application of a fungal lipase in pasta or noodle production could improve product quality. This section reviews some of the positive effects of lipase on the quality characteristics of pasta or noodles, such as colour, colour stability, biting quality, stickiness and cooking tolerance. Results from DSC and light microscopy have confirmed increased formation of amylose–lipid complexes as a result of the lipase treatment. These complexes inhibit the swelling of the starch granules, especially in the outer layer of the noodle or spaghetti strand, resulting in a firmer texture and a smooth surface.

2.3.1 Reducing speckiness

A common problem in noodles and pasta is speckiness (i.e. small dark spots) the quantity of which is mainly dependent on the level of flour purification (extraction) or ash content. An addition of the fungal lipase reduces noodle speckiness. The lipase addition also reduces the increase in speckiness over time (figure 2.30).

2.3.2 Increasing brightness and colour stability

Darkening of raw noodles or noodle dough sheets during production or shelf life can be a serious problem. Figure 2.31 shows colour measurements of raw, white, salted noodle dough sheets. Treatment with lipase increased the brightness of noodles produced from two different flours made with slightly

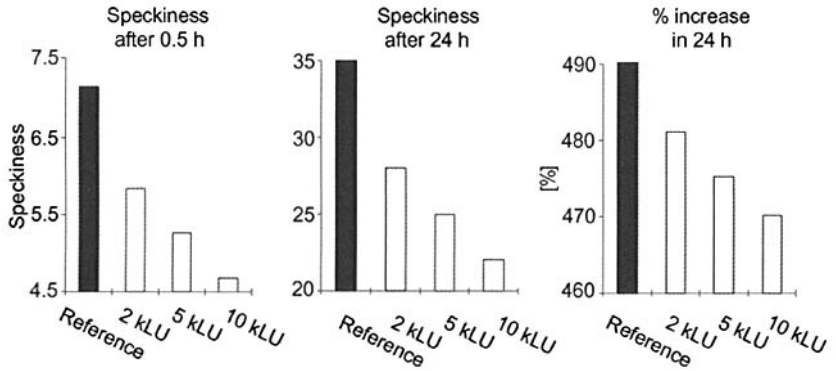


Figure 2.30 Effect of lipase on reducing speckiness in white salted noodles (dough sheets). Australian Eradu wheat, measured by Branscan. Dosage: kLU/kg flour. LU = lipase units.

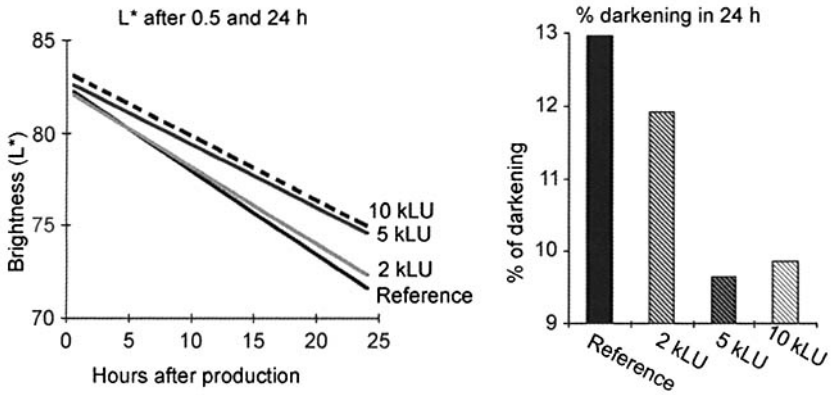


Figure 2.31 Brightness of white salted noodles (raw dough sheets), Australia Eradu wheat, measured by Minolta CR310. Dosage: kLU/kg flour. LU = lipase units.

different production procedures and different colour measuring equipments. Lipase addition also reduced the darkening rate during storage of raw noodles.

During production of instant noodles, the colour of the noodle sheet can change within the time frame of production before the noodles are steamed. Addition of lipase gave substantially higher L* values than the control and also reduced the rate of darkening in the first 30 min (figure 2.32).

Brightness is also a very important parameter for cooked noodles. Noodles with smooth surface characteristics are brighter than noodles that have a mushy and uneven surface. Cooked white salted noodles (figure 2.33) showed increased in brightness when the dough was treated with lipase.

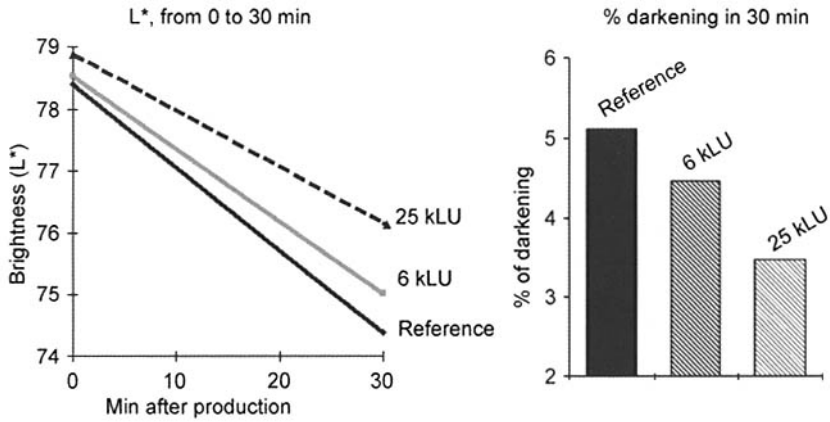


Figure 2.32 Darkening of instant noodle dough within 30 min. Flour: Australian Prime Hard. colour measured by Minolta CR310.

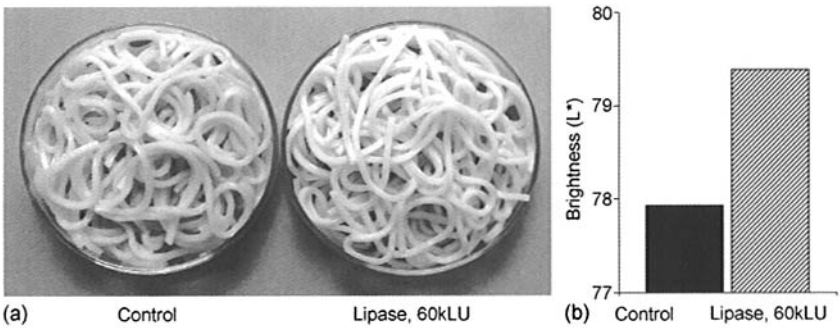


Figure 2.33 Effect of lipase on colour of cooked white salted noodles, shown 2 h after cooking (a). (b) L* of cooked noodle dough sheets. Cooking time: 3 min; flour: Meneba type 550. L* measured by LabScan XE.

2.3.3 *Improving texture*

Firmness, or biting quality, of cooked pasta and noodles is another important characteristic. Firmness can be measured instrumentally either by determining the cutting force of a single noodle strand or by measuring the relative depth of penetration at a specified cutting force (0.1 Newton). The relative depth of penetration at 0.1 N is a better indication of the firmness of the outer layer of a noodle or pasta strand than cutting force. As noodles become firmer, cutting force increases and the relative depth of penetration at a specified cutting force decreases.

Firmness of cooked white salted noodles increased after treatment of the dough with increasing dosage of lipase (figure 2.34). Increasing lipase dosage also improved the over-cooking tolerance of noodles (figure 2.35). The degree of softening over-cooked control noodles was more than 25% compared to around 15% for cooked noodles treated with lipase.

Stickiness of cooked white salted noodles was assessed using sensory evaluations. Triangle tests were used to compare control noodles to noodles treated with lipase at 40 kLU/kg of flour. The sensory panel was able to detect a significant

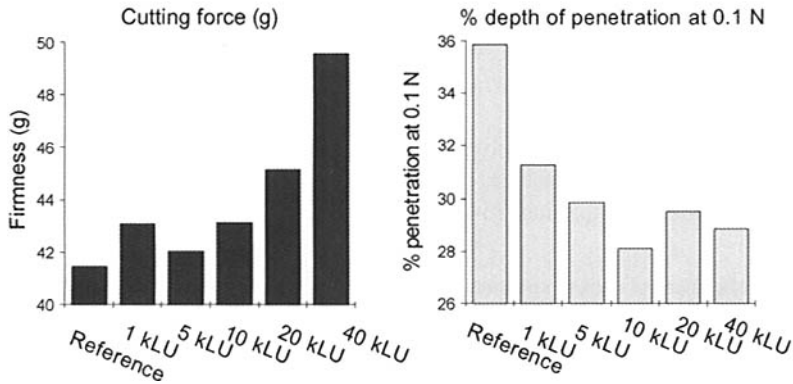


Figure 2.34 Firmness of cooked white salted noodles (European flour) measured by texture analyser. Cutting force increases with firmness; % depth of penetration at 0.1 N decreases as firmness of the outer layer increases. LU = lipase unit. Dosage: kLU/kg flour.

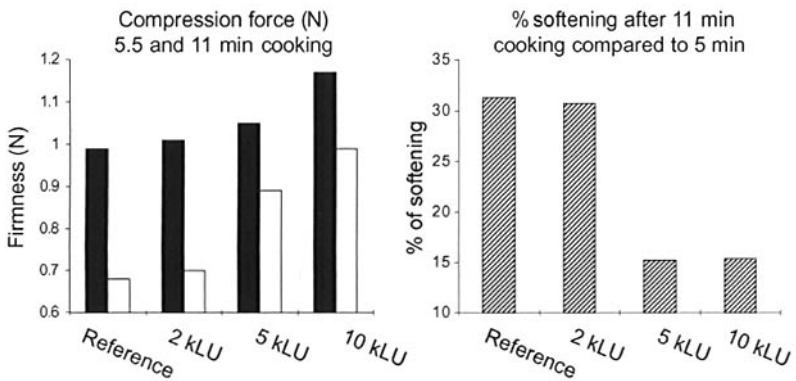


Figure 2.35 Comparison of firmness of normally cooked and over-cooked white salted noodles (European flour) measured by texture analyser. Cutting force increases with firmness; % depth of penetration at 0.1 N decreases as firmness of the outer layer increases. Dosage: kLU/kg flour. ■ = cooked for 5.5 min; □ = cooked for 11 min.

difference ($p < 0.001$) in stickiness between the control and the noodles treated with lipase. All those who could detect a difference between the samples were able to indicate that the control noodles were stickier than the lipase treated noodles.

When pasta is produced from bread-wheat flour, the quality in terms of texture, colour and cooking behaviour differs substantially from the pasta made from durum semolina. Figure 2.36, for example, shows the stickiness of pasta produced from bread-wheat flour. When a spaghetti sample was cooked 1 min longer than the '*al dente*' point and the stickiness was measured by texture analyser using the integrated adhesive force on the sample towards a withdrawing probe, lipase addition reduced the stickiness of pasta. Furthermore, the lipase also increased the surface firmness of pasta. The pasta sample treated with lipase at 60 kLU per kg flour had a surface firmness level close to that of pasta made from durum semolina.

The lipase can improve the physical appearance of the dried pasta products, especially if the raw material had poor gluten quantity and quality, for example, a wet gluten quantity of less than 23–24%. Figures 2.37 and 2.38 show an example

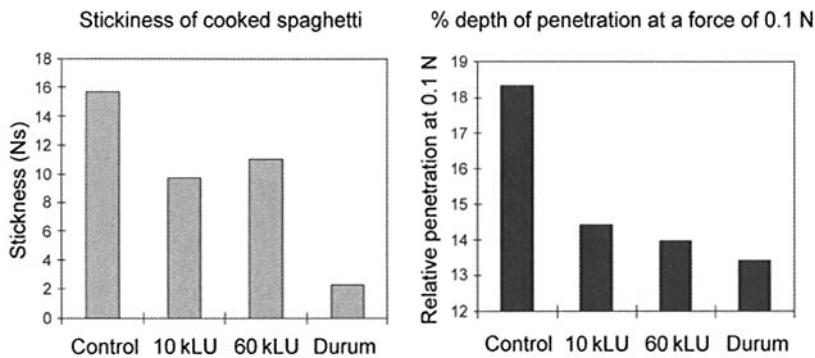


Figure 2.36 Stickiness and firmness of spaghetti produced from European wheat flour (type 550), measured by Zwick. Dosage kLU/kg.

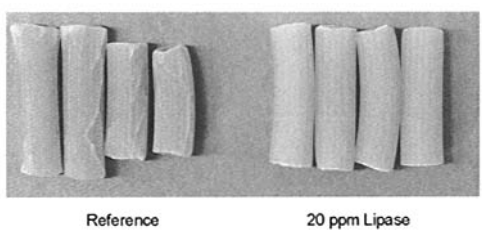


Figure 2.37 Dry macaroni produced with soft wheat flour. Lipase can reduce cracks caused by low gluten quantity and quality of the flour.

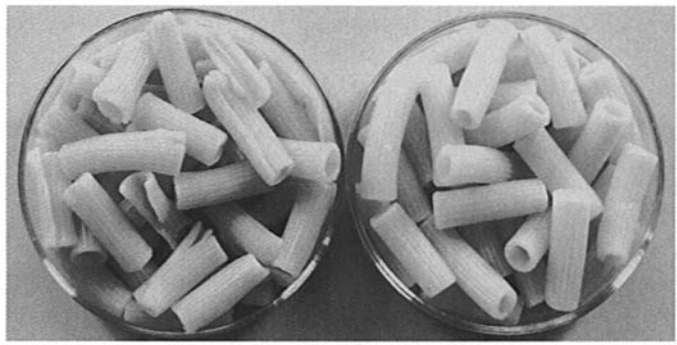


Figure 2.38 Cooked macaroni produced with soft wheat flour. Due to the reduction of cracks by adding lipase, the physical appearance can be improved.

of how the lipase eliminates the cracking problem of macaroni in both dried and cooked product. The cracks occurred when poor quality flour was used. With addition of the lipase at a dosage of 12 kLU/kg flour, and using exactly the same process, the cracks had disappeared.

2.3.4 Mechanisms for the effect of lipase

The mechanism of the beneficial effect of lipase in pasta or noodles is not yet fully elucidated and may be due to modification of both the protein and the starch fractions of the flour. Results from differential scanning calorimetry (DSC) measurements of uncooked and cooked pasta produced from common wheat flour indicated that complexes of hydrolysis products of lipase with starch may take place. The formation of amylose–lipid complexes changes the starch fraction of pasta or noodles because the complexes inhibit the swelling of starch granules during cooking. The results from DSC (table 2.1) show that the melting enthalpy of the amylose–lipid complexes in uncooked pasta increased

Table 2.1 Amylose–lipid melting enthalpy of uncooked and cooked pasta produced from bread wheat flour

Pasta	Amylose–liquid enthalpy (J/g db)
Uncooked	
Control	1.37 (96.7°C)
Lipase	2.29 (95.4°C)
Cooked	
Control	0.68 (101.3°C)
Lipase	1.41 (100.8°C)

Dosage: 60 kLU/kg flour.

by approximately 62% when lipase was added to the dough. The cooked pasta treated with lipase showed an increase of around 75% in its melting enthalpy compared to the control. Increased formation of amylose–lipid complexes may reduce the leaching of amylose during cooking, resulting in a firmer texture and a less sticky and smoother surface.

Figures 2.39a and 2.40a show light micrographs of cross-sections of the outer layer of white salted noodles produced without lipase at magnifications of 50× and 200×, respectively; the light micrographs of figures 2.39b and 2.40b show the cross-sections of the outer layer of the noodles treated with lipase at similar magnifications. The noodles were cooked to optimum (3 min) prior to cutting the cross-sections. The light flecks mark the protein fraction. Starch appears dark grey (amylose) or lighter grey (amylopectin and complexed amylose) due to iodine staining. The lack of colouring in the outer layer of the reference noodles indicates that more amylose has leached from the reference sample into the

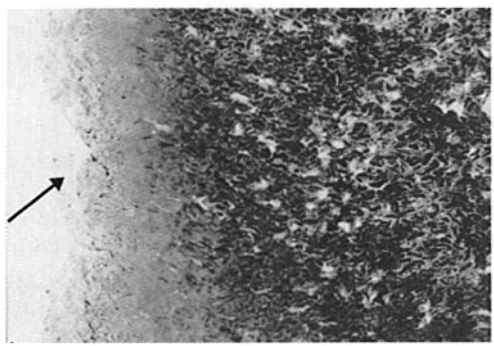


Figure 2.39 (a) Light micrograph of the outer layer and surface of reference noodles. Magnification: 50×.

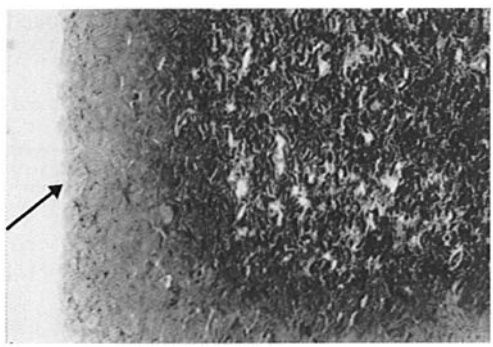
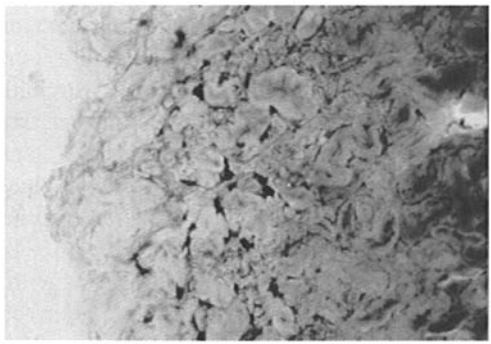
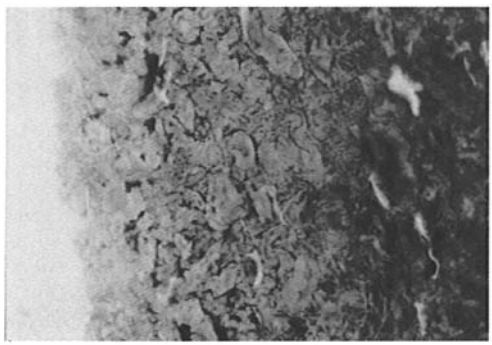


Figure 2.39 (b) Light micrograph of the outer layer and surface of noodles treated with 60 kLU lipase/kg flour. Magnification: 50×.



igure 2.40 (a) Light micrograph of the outer layer and surface of reference noodles. Magnification: 200×.



igure 2.40 (b) Light micrograph of the outer layer and surface of noodles treated with 60 kLU lipase/kg our. Magnification: 200×.

ooking water than from the sample treated with lipase, where amylose is both rapped into the structure or bound in the form of amylose–lipid complexes. his observation confirms the results found by DSC and also gives a direct ndication that amylose leaching is reduced as a result of enhanced amylose–ipid complexing. Comparing the surface characteristics of noodles with and vithout lipase (see arrows) shows, that the surface of the lipase-treated noodles s much smoother compared to the surface of the control noodles.

In the noodles treated with lipase, the starch granules appear less swollen nd the thickness of the outer layer is reduced compared to the reference. This s especially shown very clearly in the micrographs with the large magnification f 200×. Light microscopy of cross-sections from the centre of the noodles (not hown here) also showed less swelling of starch granules in the centre of noodles vhen treated with lipase compared to the reference sample without lipase.

This effect may explain the firming effect of lipase found in texture measurements of the noodles and pasta.

Another potential mode of action of the lipase is its strengthening effect on gluten. This was illustrated for bread earlier in the chapter. This property may also account for part of the increased firmness and cooking tolerance of pasta and noodles after lipase addition. The combined effect on both gluten strengthening and increased amounts of lipid-complexed amylose make lipase a very good aid in improving the quality of noodles and pasta.

Acknowledgement

This work contains contributions from the following persons: Dr. B. Conde and Dr. Ch. Zweifel from ETH, Zurich, and Todd Forman, Jack B. Nielsen, Andy Christen, Fritz Glansmann, Niclaus Neyelin and Silvia Strachan at Novozymes. Special thanks are given to Todd Forman and Antonia Huber, for their tireless efforts in editing this chapter.

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3 Enzymes in brewing

Diederik J.M. Schmedding and Mari J.M.C. van Gestel

3.1 Introduction

This chapter describes the use of enzymes in the brewing process. It will provide a basis for understanding which enzyme activities in a commercial enzyme preparation are acting on specific components of the raw materials. It will not show typical enzymes and the dose rates used, and so the practical matter of the cost–benefit relationship will not be covered, as this depends heavily on the conditions in the specific brewery, on the quality of its raw materials, and on the cost of the enzyme.

Enzymes from the raw materials used in brewing play an extremely important role in the process, as will be shown in this chapter, but due to natural variations, these enzyme levels can vary significantly in the raw materials. Therefore exogenous (i.e. added) enzymes allow more tolerance in the quality of the raw materials and the conditions of the brewing process.

3.1.1 History of brewing

The brewing of 'beer' is an activity that goes back to about 5000 BC in the area of the Middle East (Nile, Tigris Euphrates). The 'beer' being made then probably bore little resemblance to the beer that we consume nowadays. The process was probably not developed initially to make 'beer', but arose rather from the preparation of other grain-based foods, where the stored (mashed and malted) grain was 'infected' with microorganisms, such as yeast, which generate ethanol (alcohol) under anaerobic conditions. These early 'beers' must have been very nutritional and porridge-like, and the 'spirit' in these foods must have stimulated early humans to optimise on the production of this indulgent foodstuff. Development took place in the Middle East, and also in other regions, with the basic idea of fermenting grain. Brewing technology today still makes use of the various types of starch sources, such as maize (from the Incas), sorghum (Africa) and rice (from China).

There was no understanding of the processes taking place, but it must have been quite clearly understood that generating acidity through lactic acid bacteria fermentation, and alcohol through yeast fermentation, played an important role in preventing food spoilage by pathogenic microorganisms. In medieval times, there was an increase in size from small 'private' to 'industrial' scale operations.

Only after the development of the 'microscope' by Anthonie van Leeuwenhoek in 1680, could microscopic particles be seen, and it was not until Louis Pasteur's invention in 1870 that an understanding of the role of living microorganisms could begin. About ten years later (1883), the Dane, Hanson, developed a system for the cultivation of pure yeast on an industrial scale, facilitating more controlled brewing. From 1900 onwards, the understanding of the processes in yeast (biochemistry) grew, leading ultimately to a role for enzyme technology in brewing in general.

3.2 Raw materials

3.2.1 Malt and adjuncts

It was quickly realised that grain needed to be germinated to make more digestible foods and, as is now known, to break down cell wall structures and develop enzymes to modify starch granules to soluble starches. This process is called 'malting'. The remainder of the endosperm cell walls in malt are only present in low quantities, but can have a significant effect on the brewing process. β-Glucan in barley is usually present at levels of 3–4% (w/w) and due to the germination process is reduced to about 0.5–1% in the malt. The most important contribution from malt in the brewing process is to deliver fermentable sugars and the maltodextrins. The fermentable sugars are the largest part of the malt-derived components and will yield ethanol in the final beer. The maltodextrins are not degraded any further by the malt enzymes and are (partly) responsible for creating some 'mouth feel' in the final beer. Apart from these very important ingredients, malt also delivers a number of other important ingredients such as proteins, lipids, polyphenols and complex Maillard reaction products. The latter are responsible for the typical malt flavour, and for the colour. These can be adapted according to the wishes of the brewer, by modifying the last step in the malting process, which will be described later.

Proteins can be divided into two groups. Firstly, the non-catalytically active protein delivers a nutritional element to beer, both for the human consumer, but also, when degraded by enzymes to free amino acids and peptides, for the yeast to serve as a nitrogen source during the growth phase in fermentation. Some particular non-catalytic proteins are responsible for the foaming character of beer and they also contribute to some extent to mouth feel. The second group of proteins are the enzymes, which are catalytically active. They can convert one component into another and are the key topic of this chapter.

Polyphenols play a role in the brewing process, as will be discussed later, but also have an impact on colour, mouth feel (astrigency) and stability of the final beer. Lipids play a role during fermentation, as they provide the yeast with membrane material during growth. Otherwise they may impart off-flavour

formation (e.g. *trans*-2-nonenal), either through auto-oxidation or enzyme catalysed (lipoxygenase) oxidation during the mashing (and malting) process.

From all this it can be seen that the understanding of malt, and thus also the malting process, is of key importance. With current knowledge, it is clear that alternative substrates for malt, such as barley, maize, wheat, rice or sorghum may deliver fermentable sugars (or soluble starch), but will not provide the brewer with all the other ingredients. Therefore, the complete omission of malt is rare, and is only done in cases where major constraints exist: for example in some countries in Africa the import of malt is either banned or, through import duties, made extremely expensive. One way to make up for the missing ingredients is to employ commercial enzymes.

3.2.2 Hops

Hops are the flower of the female '*Lupulus humulus*' plant. In ancient times, they were probably added to the final beer as a type of 'spice', but also served to prolong the shelf life of the brew. Nowadays, hops are usually added early in the brewing process, where they deliver mainly bitterness and flavour. Due to the point of addition, during boiling, the role of enzymes from hops is not significant.

3.2.3 Yeast

Although most brewers consider their yeast as a raw material, since it is declared an ingredient on the label of the bottle, it is in fact a processing aid. Raw materials are being consumed (converted), while yeast is growing and is removed from most commercial beers, or it is at least inactivated (pasteurised). In fact, yeast is a biological 'catalyst', a 'living bag of enzymes', which converts the multiple substrates present in wort to the alcohol-containing, flavoursome beer.

3.2.4 Water

Water is normally a source of minerals, which will affect processing and also taste, but which normally have a negligible effect on the performance of enzymes, with the exception of amylase (see section 3.4.3). A more important parameter is the pH of the mash. The aim of the brewer is to work optimally with a mashing pH of around 5.5 ($\pm$ 0.1).

3.2.5 Exogenous enzymes as processing aids

In the brewing process a number of processing aids, like fining agents and enzymes, have been used for a long time. The main (traditional) reason is that the enzymes in malt are not always present in sufficient quantities to run the process optimally. Exogenous enzymes, in contrast to endogenous enzymes, are added separately to a particular process step.

3.2.5.1 Origin of exogenous enzymes

Most exogenous enzymes are of microbial origin, although there are two exceptions: β-amylase from high enzyme malt, and papain, a proteolytic enzyme from the papaya fruit.

3.2.5.2 Production of commercial brewing enzymes

The plant-derived enzymes are commercially produced through extraction of the plant material, and subsequent purification, concentration and standardisation (see chapter 10).

The microbial enzymes are of bacterial or fungal origin (seldom from yeast) and are produced by means of fermentation. The two methods of fermentation are either submerged, a dispersion of microbial cells in an aqueous medium, or through surface solid state (Koji). This surface culture fermentation is typically performed with fungi (moulds), while submerged fermentation can be undertaken with either bacteria or fungi. The most common bacterial production strain is from the genus *Bacillus*. Typical enzymes produced with this bacterium are glucanases (less relevant for brewing are the alkaline proteases). Fungal production strains are from the genus *Aspergillus* (amylase, amyloglucosidase, glucanase and hemicellulase), *Trichoderma* (glucanase and hemicellulase), *Penicillium* (glucanase), and *Rhizopus* (amylase and amyloglucosidase).

The name of the enzyme preparation relates to one specific enzyme activity, but commercial non-GMO (genetically modified organism) enzyme preparations are the sum of all proteins, active and inactive, excreted by the microbe. Growth conditions, however, can be chosen such that a particular enzyme activity is predominant.

3.2.5.3 Enzyme activity assay

Although most enzyme preparations consist of multiple enzymes, the enzyme assay makes use of the specificity of a typical enzyme. By selecting a purified substrate (e.g. glucan) one can measure only the glucanase activity in the enzyme preparation, as all other enzymes do not act on this substrate. The idea behind these assays is that the correlation between the enzyme assay and the functionality has already been established, so that the often more tedious and laborious functional tests can be avoided. It should be mentioned, however, that not every enzyme assay is standardised. Therefore comparisons between literature data always need to be checked for the assay conditions (e.g. incubation time, pH, temperature) and unit definition.

3.3 The processes of malting and brewing

For such an ancient product as beer, the process of brewing is surprisingly complex, beginning with the malting process and followed by the brewing

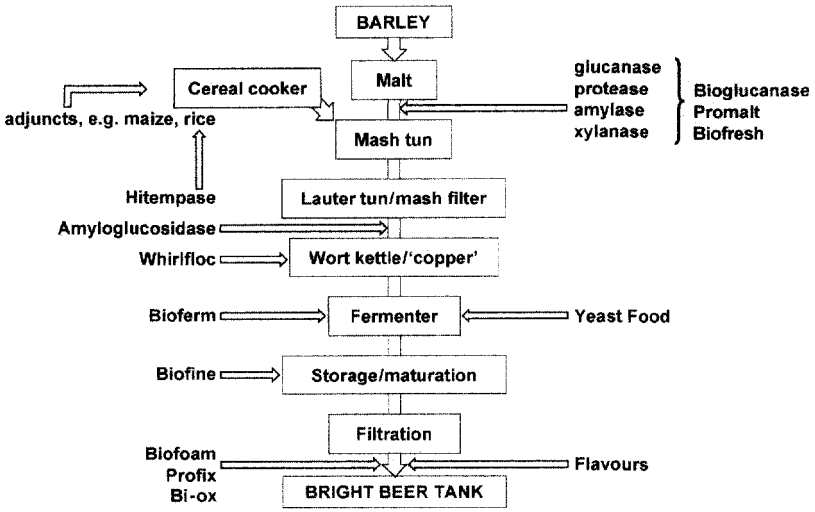

Figure 3.1 The brewing process. Source: Quest International.

process (see figure 3.1). In modern times, these two processes are generally performed by different companies, despite the close interaction between them.

3.3.1 The malting process

In the malting process, kernels of barley grain are wetted (the term for this is 'steeping') which activates the grain to germinate (grow). This activation involves intermittent steps of wetting and aeration, to avoid 'drowning' the grain. It takes about 48 h. The next process step is 'germination', which takes about 4–5 days. The kernels are put on a trough with a perforated floor while continuously humidified air is blown through the bed of grain. Temperatures are kept relatively low (around 15°C), in order to minimise growth of the rootlets and acrospire ('leaf') as this occurs at the expense of energy (starch). The last step (kilning) is the inactivation of the growing kernel, by heating the grains in hot dry air. Depending on the temperature–time regime, more colour and flavour components are formed. It should also be realised that a significant amount of enzymes (among others, β-glucanase) are from microbial origin, as in all stages of the malting process microbial growth also takes place. The last step performed by the maltsters is the removal of rootlets from the grains.

3.3.2 Malt specification

Maltsters and brewers use a large number of malt specifications in order to ensure an optimum brewhouse performance and final beer quality. These quality

specifications are part of delivery contracts used by maltsters and brewers. Usually the quality parameters are estimated with recommended methods, from the Institute of Brewing (IOB), the American Society of Brewing Chemists (ASBC) or the European Brewery Convention (EBC), organisations whose methods show many similarities. Table 3.1 shows the most important parameters used in a typical malt analysis and their significance for the brewing process and final beer quality.

The parameters in table 3.1 give a good indication of the general malt quality but are no guarantee of an ideal brewhouse performance because they are mainly based on an average analysis of a whole population of malt kernels. In particular, undermodification of parts of malted barley grains is not always recognisable in modification parameters, and can cause unexpected problems in brewhouse performance.

3.3.3 The brewing process

3.3.3.1 Mashing

During mashing, milled malt (also called grist) is added to water (brewing liquor) in order to extract all the components from the grain. Usually the liquor to grist

Table 3.1 Malt quality parameters

Malt quality parameter	Significance
Moisture content	Malt storage potential Pricing of malt
Extract recovery (yield)	Total amount of extractable material (finally amount of beer) likely to be obtained
Endosperm cell wall modification friability wort β-glucan level wort viscosity fine/coarse difference	Extract yield Rate of wort separation Rate of final beer filtration
Protein and protein modification total malt protein soluble protein soluble protein in relation to total malt protein (Kolbach) amino acids in wort	Foam stability Beer haze (colloidal stability) Yeast food (fermentation) Beer colour Mouth feel
Wort fermentability, degree of attenuation, limit to which fermentation proceeds	Potential alcohol formation Mouth feel, fullness and sweetness of beer
Malt enzyme activity diastatic power (combined activity of α- and β-amylase) α-amylase activity β-glucanase activity	Speed and degree of solubilisation of malt solids Brewhouse performance
Wort colour	Beer colour

ratio is 3:1, which leads ultimately, after the various sparging steps, to about 17 kg of malt/hectolitre of beer. Extract recovery (yield) is the most important criterion for success of this process step. Extract recovery is usually about 80% of which approximately two-thirds is suitable for fermentation. As well as these fermentable sugars the extract contains, for example, maltodextrins and protein. The amount of fermentable sugar is related to the so-called 'attenuation' level. This is the limit to which fermentation proceeds.

There are a number of ways (temperature regimes) in which this extraction can be undertaken, but it encompasses various enzymatic reactions. These will be discussed later (section 3.4.2).

3.3.3.2 Lautering

After the extraction is completed, the insoluble material, or 'spent grain', is separated from the mash. The resultant extract is called 'sweet wort'. Traditionally this is done in a 'Lauter tun', a vessel with a coarse 'sieve' in the bottom (false bottom). This type of separation makes use of the hulls of the malt, which act as a filter aid. It is important that care is taken during milling to ensure that the hull remains intact. Nowadays more breweries are making use of mash filters, whereby the malt can be milled much more finely, as the filterbed function of the grain is no longer required. The use of mash filters results in shorter process times and higher extract recovery. In either case, the mash, containing the highest levels of β-glucan, results in a retarded extract separation. The remaining extract in the spent grain is washed out with 'sparge water'. The whole process is carried out at about 75°C for 2–5 h.

3.3.3.3 Wort boiling

After all the sweet wort has been collected, it is boiled and hops are added. During boiling, some steam is released, also removing particular volatiles such as aldehydes and sulfur components. The high temperature is also required to convert hop acids into the bitter *iso*-α-acids. The boiling stage takes about 90 min. After boiling the wort, insoluble proteins, polyphenols and (depending on the hop product used) hop solids are removed from the wort in the so-called whirlpool. Subsequently the wort is cooled and oxygenated (aerated).

3.3.3.4 Fermentation

The nutrient-rich wort is pumped in a fermenter, and yeast can now be added in order to let the primary fermentation take place. In the first aerobic phase, yeast starts to grow to about four to five times in biomass, after which conditions become anaerobic. This stops the yeast from growing and changes its metabolism into producing ethanol and other volatile components. The primary fermentation finishes when most of the fermentable sugar has been converted, which takes about a week, at a preferred temperature of around 10°C, in the case of a 'lager' fermentation.

3.3.3.5 Maturation, filtration and clarification

At the end of primary fermentation, a large amount of yeast has settled to the bottom of the tank and is taken out first, as part of the yeast will be reused to initiate the next batch of fermentation. This 're-pitching' is done several times (typically 5 to 12 times) depending on the vitality of the separated yeast. The 'green beer' is pumped to a lagering tank, where it is matured. During the maturation phase, some components, like diacetyl, will be converted by the remaining yeast to more preferred compounds. Traditionally the lagering could take several months, but nowadays it can be reduced to days.

After this process, the remainder of yeast cells are removed ('fined') and the beer is usually (colloidally) stabilised upon filtration by removing proteins and/or polyphenols by respectively silica and/or PVPP. This conditioned beer is collected in so-called 'bright beer tanks', in order to be checked on quality, before it is bottled.

3.4 Enzymes in the brewing process

3.4.1 Enzymes in malting

During the malting process the enzymes native to the barley (amylases, glucanases, proteases and hemicellulases) are activated. As a result the malted barley is made more amenable to milling and starch/carbohydrate extraction. The structure of the barley grain and the enzymes involved in the breakdown of its constituents are shown in figure 3.2.

Apart from the endogenous enzymes present in malt (and barley), no exogenous enzymes are applied in the malting process. The key target of the maltster is to generate a well-modified, homogeneous malt. The modification relates to the enzymatic conversion of the endosperm. As the enzymes are released through the aleurone layer, it will be clear that the modification gradually moves on through the endosperm as germination time progresses. The maltster has to find an optimal limit for the extent the modification (germination) proceeds, as this occurs at the expense of valuable sugars for the germinating kernel and thus of the yield of this process. The homogeneity of the malt relates partly to the modification process in the grain kernel, but is also dictated by the size of the operation. The germination and the kilning stages of the process suffer particularly from the physical differences a kernel encounters at the top of the bed of grain or on the bottom. The main differences in the germination bed are gradual changing levels of oxygen and carbon dioxide and to a lesser extent temperature. During kilning it is mainly a temperature gradient affecting the 'kill rate' of the germinating grain and the concomitant colour formation (Maillard). The consequence of all this, plus different agricultural conditions is that by definition malt is not a homogenous product, despite all efforts of the maltster to make it so.

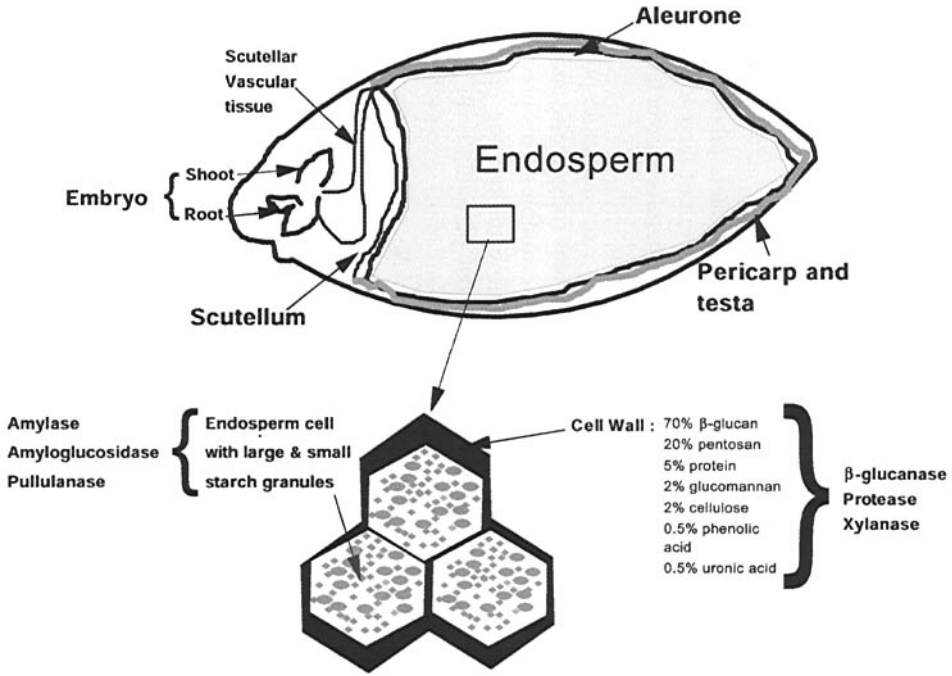

Figure 3.2 Structure of the barley grain and the enzymes involved in the breakdown of its constituents.

3.4.2 Enzymes in mashing

During the malting process some enzymes have already been active in degrading the various polymeric substrates, such as starch, glucan, protein and many more low molecular weight components (e.g. oil). However, particularly in case of undermodified malt or low quality heterogeneous malt, exogenous (added) enzymes such as proteases, amylases and glucanases can help to increase the degradation of these polymeric substrates.

The most important benefit of using exogenous enzymes is that they give the brewer the required tolerance in using his malt, particularly as the mashing process is a more homogenous process than malting (see section 3.4.1).

3.4.2.1 Protein converting enzymes

Mashing usually starts with the degradation of malt protein (about 10% w/w in malt) by means of proteases and peptidases, as a result of their lower thermostability compared to the other enzymes involved. Therefore lower temperatures (e.g. 45–55°C) are usually applied at this stage.

These enzymes are referred to respectively as exo- and endo-protease and produce, respectively, free amino acids and peptides (oligomeric amino acids), and smaller proteins (thereby making them more soluble). This process step is also referred to as 'the proteolytic stand'. During the malting process some protein degradation has already taken place, but particularly in the case of undermodified malt (or low quality heterogeneous malt), exogenous enzymes can help to increase the free amino nitrogen (FAN), which is required during fermentation as the nitrogen source for the yeast. However, some care should be taken in applying excessive protein degradation, as this can cause excessive colour formation (through the Maillard reaction), and may affect the foam potential of the final beer.

3.4.2.2 Cell wall degrading enzymes

Degradation of cell walls (in good malt 1% (w/w), of which about 75% is β-glucan and 25% is hemicellulose, also called xylan or pentosan), is carried out by glucanases. The main problem with incomplete cell wall degradation, as is the case with heterogeneous and/or undermodified malt, is the negative impact these polysaccharides can have on the mash filtration (lautering) and beer filtration process steps, and also on the colloidal stability (haze) of the final beer. The most important polysaccharide to degrade is β-glucan. This polymer contributes most to the viscosity of wort, and in beer it has the tendency to form insoluble complexes. Xylan is usually already sufficiently degraded in the malt. Any residual xylan will also be degraded by the side activities present in commercial glucanase preparations. Barley (malt) glucan consists mainly of β 1-4 glucose units, interspersed with β 1-3 linkages (see figure 3.3). The

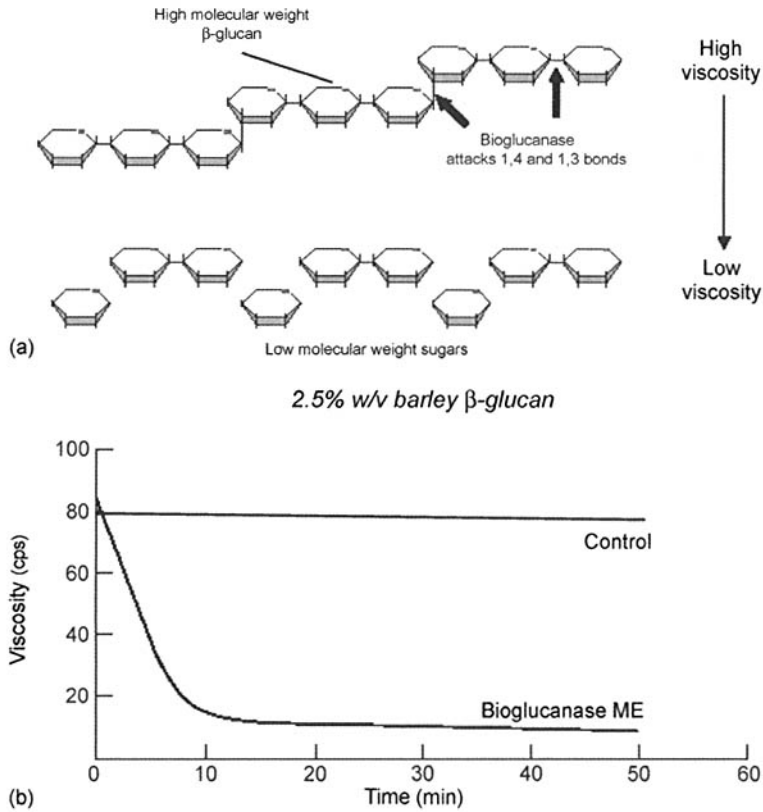

Figure 3.3 Barley glucan degradation. (a) schematic; (b) practical viscosity reduction at 60°C. Source: Quest International.

commercial β-glucanases contain activities to degrade this type of glucan and are therefore called β 1-3,1-4 glucanase.

As can be seen from table 3.2, malt glucanases are less temperature-stable than microbial enzymes. Fungal glucanases are seen to be the most stable in the relevant pH range.

3.4.2.3 Starch-converting enzymes

Complete starch conversion (in malt about 60% (w/w) of which about 25% is amylose and 75% is amylopectin) is carried out by a range of amylolytic enzymes. Amylose consists of glucose units linked through α 1-4 linkages, while amylopectin also has α 1-6 linkages, making it into a branched polymer (see figure 3.4). The amylolytic enzymes in malt are α-amylase, β-amylase and limit dextrinase. From microbial origin, the enzymes pullulanase (the much more

Table 3.2 Characteristics of some typical β-glucanases

	Barley malt	*B. subtilis*	*Asp. orzyes*	*Asp. niger*
pH optimum range	4.5–5.3	4.5–7.0	4.0–6.0	3.0–6.0
Temperature optimum range (°C)	40–45	45–50	45–55	45–65
Degrades:				
barley β-glucan	+	+	+	+
laminarin	+	–	+	+
CMC	+	–	+	+
lichenin	+	+	+	+

Source: Quest International.

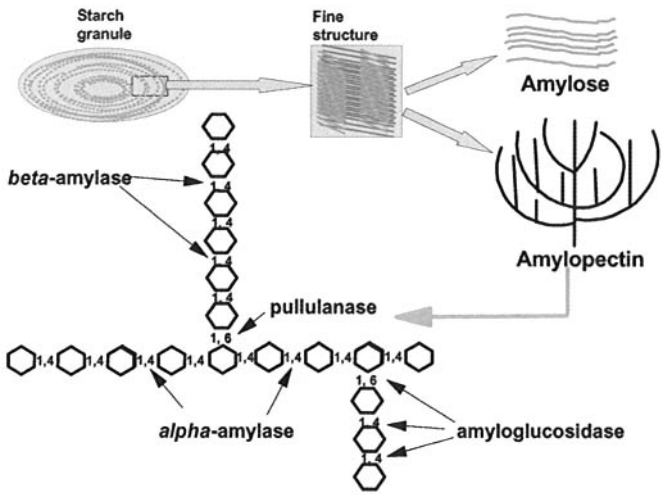

Figure 3.4 Starch hydrolysis.

stable microbial equivalent of limit dextrinase) and amyloglucosidase can be added (see figure 3.4). α-Amylase splits the polymer in an 'endo-' manner, and therefore leaves a considerable amount of branched maltodextrins from amylopectin. β-amylase splits only maltose units from the (non-reducing) end of the polymer (exo-activity). It can act on both amylose and amylopectin, but stops if it encounters an α 1-6 link. While β-amylase is less stable than α-amylase, limit dextrinase is even less stable. Its activity, as the name suggests, relates to the degradation of the partially hydrolysed amylopectin remaining after the α-amylase degradation. It does not act on single α 1-6 linked glucose units, but requires at least an α 1-4 linked glucose on each side of the α 1-6 link. As this enzyme is not able to operate at temperatures of 60°C

and higher, amyloglucidase is often used to increase the fermentability of wort. Amyloglucosidase (or also called glucoamylase) consists of both α 1-4 and an α 1-6 cleaving activity, but only acts in an 'exo-' manner.

This stage of the mashing process is often referred to as the 'saccharification stage'. Some typical differences between the various types of α-amylases are shown in table 3.3.

Apart from the previously mentioned analysis of the enzyme activities, the performance of the enzymes is also measured on a laboratory scale (EBC methods), analysing starch degradation (iodine staining and fermentability), glucan degradation (wort viscosity, mash filtration and staining) and protein degradation [free amino nitrogen (FAN) analysis], as shown in figure 3.5. The complete mashing process can take 2 to 4 hours.

Table 3.3 Properties of amylases for commercial application

	A. oryzae	*B. subtilis*	Barley malt	Porcine pancreas
Amylase type	α-amylase	α-amylase	α-amylase	α-amylase
pH optimum range	4.8–5.8	5.0–7.0	4.0–5.8	6.0–7.0
pH stability range	5.5–8.5	4.8–8.5	4.9–9.1	7.0–8.8
Temperature optimum range (°C)	45–55	60–70	50–65	45–55
Effective temperature range (°C)	Up to 60	Up to 90	Up to 70	Up to 55

Source: Quest International.

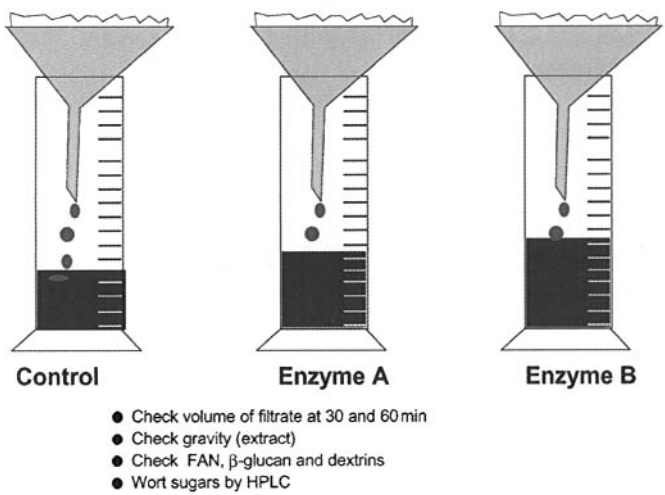

Figure 3.5 Laboratory brewing optimisation trials. Source: Quest International.

3.4.3 *Enzymes in adjunct cooking*

As mentioned before, adjuncts are applied in brewing as they are a relatively cheap alternative of fermentable extract compared to barley malt. Most common sources are maize, rice, barley or wheat. All these cereals require the use of starch degrading enzymes, or else a sugar syrup (an acidic starch hydrolysate) should be used. The drawback of these sugar syrups is that they may have a slowing effect on the fermentation, due to an unbalanced sugar composition. The most optimal enzyme is a bacterial (*Bacillus*) α-amylase which is heat stable and does not generate many free sugars, which would otherwise have an adverse effect on fermentation. The application of this type of enzyme is particularly suitable when the source of starch is other than barley (e.g. maize, rice, sorghum); the gelatinisation temperature of these starches is between 68° and 80°C whereas that of barley is around 63°C. The most stable enzyme is derived from *B. licheniformis* (performance up to 105°C), while the enzyme from *B. subtilis* has 'intermediate' stability (performance up to 80°C). These enzymes require a minimum of 50 ppm of calcium in the mash water.

In the case of barley brewing, the same enzymes are required as in regular malt mashing (i.e. proteolytic, glucanolytic and amylolytic activities). Indeed, one could consider barley brewing as an extreme case of brewing with undermodified malt. Often the high thermostable amylases are used for the starch conversion.

3.4.4 *Enzymes in lautering/mash filtration*

Lautering and other forms of mash filtration are usually performed at so-called 'mashing-off' temperatures of 75–78°C. As can be seen in table 3.4, almost all malt enzymes are already inactivated at these temperatures. Only a small amount of residual α-amylase activity will be present where a relatively low mash filtration temperature is used.

During sparging of the spent grains in the filterbed, water at 75–78°C is used, the higher temperature being preferred because of reduced wort viscosity, increased rate of wort separation and to some extent increased extract recovery. If large quantities of adjunct or undermodified malt are used, starch may be released late in the mashing cycle. The amylolytic enzymes only show a limited amount of activity at this stage during mashing; this could result in starch polymers being carried over into the filtered wort, especially if using sparging

Table 3.4 Inactivation of malt enzymes

Malt enzyme	Inactivation temperature (°C)
β-Glucanase	55
Proteases	70
β-Amylase	68–70
α-Amylase	75–78

water at temperatures above 75°C (where α-amylase is rapidly inactivated). Lower sparging temperatures could minimise these problems but would have a negative effect on such factors as wort viscosity. Addition of thermostable exogenous α-amylase during mashing can reduce this wort-starch problem. At the same time it allows the brewer to use higher sparging temperatures with the accompanying benefits.

3.4.5 Enzymes in fermentation

The wort to which the pitching yeast will be pitched (to start fermentation) has no enzyme activity. The boiling process has inactivated all enzyme activity, either malt derived or from exogenous sources.

Through enzyme activity within yeast cells, sugar molecules (DP < 4) are taken up and in the anaerobic phase transformed into ethanol and carbon dioxide and amino acids are transformed into yeast proteins and some flavour components, e.g. phenyl ethanol. Wort components such as dextrins, β-glucan and soluble proteins are not metabolised by strains of brewer's yeast.

Exogenous enzymes, apart from the application areas described below, are used in fermentation to help prevent difficulties occurring later in the process. In case of beer filtration problems, β-glucanase can be added to the fermenter (or during maturation), degrading residual glucans which would otherwise cause the filters to block. Another area for enzyme application during fermentation is to reduce haze problems in the final beer. After analysing the type of haze material (which can be done using enzymes), fungal α-amylase or β-glucanase (for respectively starch or glucan haze) can be applied.

3.4.5.1 Low calorie beer production

Since the malt-derived limit-dextrinase is not very temperature stable, a large amount of non-fermentable dextrins will be present in finished beer (typically 2.4% in Pilsener beer), as brewer's yeast is not able to convert these branched maltodextrins. Together with other beer components such as proteins, these dextrins are responsible for the mouth feel and fullness of the beer and also contribute heavily to its caloric value. Low calorie beer can be produced with the application of exogenous amyloglucosidase during fermentation, which will degrade dextrins to fermentable sugars. In this way normal alcohol/low dextrin beer can be made from wort containing a reduced amount of extract.

One step further is the production of low alcohol or non-alcohol low calorie beer where the application of exogenous amyloglucosidase is combined with the fermentation of low extract-containing wort or with removal of alcohol, for example by vacuum distillation.

3.4.5.2 Hanging fermentation

Lager fermentation of 5% alcohol beer usually takes 6 to 10 days. After this period the beer will only contain a small amount of residual fermentable

extract which will be fermented during maturation. In the fermentation, the sugars glucose, maltose, maltotriose and sucrose are fermented, while maltodextrins with more than three glucose units cannot be fermented. A typical profile of fermentable sugars in 12 °Plato wort (5% alcohol beer) is given below in table 3.5.

One of the most troublesome problems for a brewer is that of 'hanging' fermentations where complete attenuation is not reached within normal fermentation time (or even not at all). Hanging fermentations can be caused by a number of factors:

- unbalanced wort carbohydrate spectrum; too small an amount of fermentable sugars as a result of incomplete enzymatic saccharification during mashing, usually related to the use of adjuncts or undermodified malt
- failure of the yeast, although still in suspension, to utilise all the fermentable wort sugars, usually due to the yeast's inability to take up and metabolise maltotriose, and believed to be the result of a spontaneous mutation which can occur in most brewing strains
- deficiency of yeast nutrients like amino acids or zinc (especially related to adjunct brewing)
- premature flocculation of the yeast culture in the fermenting wort.

The problem of an unbalanced wort carbohydrate composition can be controlled during fermentation with the help of exogenous α-amylase (acting on α-1-4 bonds) which will correct a possible incomplete saccharification during the mashing process.

Exogenous amyloglucosidase may also be used to save batches of beer if the problem of the inability to metabolise maltotriose (yeast mutation) occurs. Maltotriose will then be degraded to smaller sugars, and these can be fermented subsequently.

A possible shortage of amino acids (yeast food) can be overcome in the brewhouse by ensuring a better protein breakdown in the mashing cycle, either by prolonged protease rests at the start of mashing or the use of exogenous exo-peptidases.

Table 3.5 Carbohydrate composition of 12 °Plato wort

Sugar	% in wort	Fermentable
Fructose	0.1–0.2	+
Glucose	0.9–1.3	+
Maltose	5.8–6.8	+
Sucrose	0.2–0.4	+
Maltotriose	1.4–1.7	+
Dextrins	1.9–2.4	–

3.4.6 Enzymes in maturation

3.4.6.1 Enzymes to correct problems (filtration and beer haze)

Exogenous enzymes can correct incomplete degradation of starches and glucans causing beer filtration problems or haze problems in the final beer. This was described in section 3.4.5 for enzymes in fermentation, but exogenous enzymes can also be used during maturation. The point of addition is best upon transfer to the maturation tank, so that the mixing of the enzyme is controlled.

3.4.6.2 Enhanced maturation with acetolactate decarboxylase (ALDC)

As mentioned before, a number of conversions resulting in flavour changes are taking place during maturation. One of the aims of maturation is to 'reabsorb' diacetyl, which is considered a (buttery) off-flavour in lager beer. This component is produced by the yeast during the main fermentation. The spontaneous conversion of the precursor α-acetolacate into diacetyl is particularly slow. This reaction can be speeded up by raising the temperature at the end of the primary fermentation, but this will also increase the rate of other reactions, some of which are undesirable (related to yeast stress, e.g. autolysis). Therefore a microbial enzyme (*Bacillus*) was developed to convert α-acetolactate into acetoin, before it can be converted into diacetyl. This enzyme is called α-acetolactate decarboxylase (ALDC). The relevant transformations are depicted in figure 3.6.

3.4.7 Chill proofing enzymes

If no preventive actions are taken, beer will loose its clarity quite quickly. Apart from the earlier mentioned glucan hazes (permanent haze), so called 'chill haze'

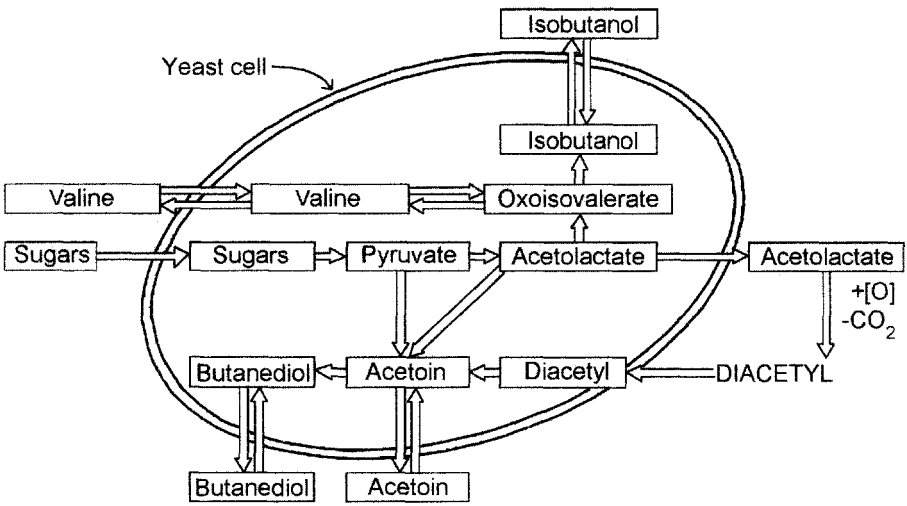

Figure 3.6 Diacetyl formation and reabsorption. Source: Quest International.

can play a major role in beer. The chill haze particles usually develop through the complexing of proteins and polyphenols (also called tannins). This process is reversible upon heating and cooling, hence the name 'chill haze'. Therefore strategies have been developed to increase the colloidal stability (figure 3.7).

The enzymatic approach is to degrade the haze-forming proteins so the protein–polyphenol complexes will not be able to aggregate over time, and the beer thus remains clear. Typically, the enzyme selected for this purpose is papain. Obviously care should be taken not to apply the enzyme at very high dosage levels, as foam stability may be reduced. If the beer is pasteurised at temperatures above 70°C, the papain will be completely destroyed (inactivated). Compared to other stabilisation techniques (e.g. PVPP), this method is very simple to operate and also cost effective. Another enzymatic approach using polyphenol oxidase (Biofresh) is under investigation (see section 3.4.8).

3.4.8 Future developments

One of the major concerns in brewing has been how to control beer flavour stability. Nowadays there are many methods for controlling colloidal stability of beer (see chill proofing) which has led to colloidal stability in excess of a year. However strategies controlling flavour stability are still scarce.

One of the ideas in the past was to apply an oxygen scavenging enzyme, such as glucose oxidase, into the bottle of beer. Although technically possible, the idea of having an active enzyme in the final beer was not very appealing. Alternative ways have been since developed to limit oxygen in the bottle with improved packaging technology.

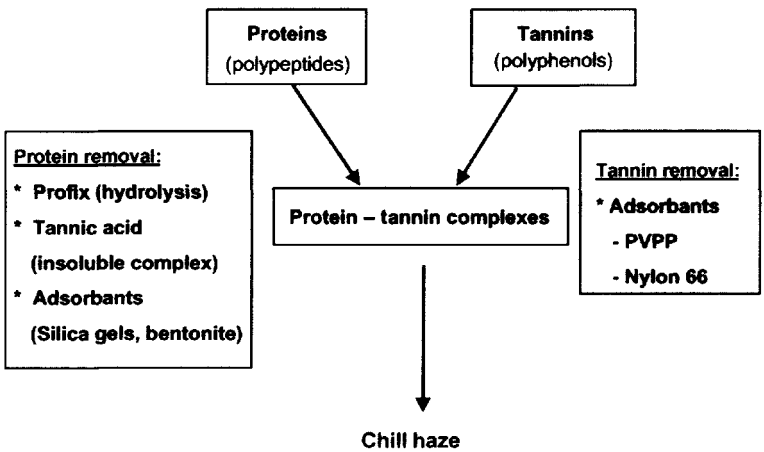

Figure 3.7 Strategies for preventing 'chill haze'. Source: Quest International.

Another general worry for brewers is the oxidative damage that occurs during mashing. It is generally believed that lipoxygenase-catalysed lipid oxidation (and/or auto-oxidation) plays a role in the formation of cardboard off-flavours (*trans*-2-nonenal) upon ageing. Also here a technological solution is being developed, by milling and mashing under oxygen limited conditions. Currently, we are busy pursuing a less capital intensive route, by adding oxygen-scavenging enzyme systems in the mash (Biofresh). Preliminary work has shown that this approach seems feasible. Suitable oxygen scavenging enzymes can be selected from the group consisting of glucose oxidase, hexose oxidase, sulfhydryl oxidase, superoxide dismutase, peroxidase and polyphenyl oxidases such as laccase (and combinations of these enzymes). In particular, the polyphenol oxidase enzyme has the benefit of not only scavenging oxygen, but at the same time converting polyphenols into less soluble complexes, subsequently removed from the wort upon mash filtration (or lautering). Another benefit of this new enzyme system is that the temperature stability is such that it even remains active for some time after mashing off (78°C). Thereby it also protects the wort lipids from oxidation, which occurs particularly quickly at these high temperatures. Ultimately, the enzyme system is destroyed during boiling in the copper. Later on in the colloidal stabilising strategies, the beer needs less severe protection, although considerable amounts of polyphenols are introduced in the copper if standard hopping regimes are used.

Acknowledgements

We wish to thank our brewing colleagues in Quest for gathering information on brewing over the past 20 years. Some of them have contributed specifically to the contents of this chapter. We are grateful to Eoin Lalor and Chris Smith for their technical contributions and to Vicky Fritzsche for her assistance on editorial matters. Finally we wish to thank Mike Woulfe for giving permission to publish this chapter.

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4 Enzymes in wine production

Maarten van Oort and Rose-Marie Canal-Llaubères

4.1 Introduction

The production of wine is a biotechnological process marked with tradition. However, research into winemaking is providing an understanding of the biochemical reactions that occur in grape juice during its transformation into wine.

Enzymes play a definitive role in the process of winemaking. Indeed, wine can be considered the product of enzymatic transformation of the grape juice. Enzyme activity is required as early as possible in the pre-fermentation stage. The enzymes originate not only from the grape itself, but also from yeasts and fungi and other microorganisms. Moreover, the action of such endogenous enzymes is nowadays reinforced and extended by the use of exogenous industrial enzymes. Enzymes coming from adventitious microorganisms infecting the grapes may also play a role. This chapter will review only the application of industrial enzymes.

4.2 Legal aspects of the use of enzymes in winemaking

Industrial enzymes are considered as technological tools, used during processing and not recoverable from the end products. Everything that remains can be considered as technically inevitable residues. The use of enzymes in winemaking is covered by three levels of regulation in the European community [1]. Of these, the first two are community level and national level regulations, which supplement each other and are directly applicable by the end users. At the third level are international rules from the 'Office International de la Vigne et du Vin' (OIV). This organisation takes decisions which are not directly applicable by the member states, but should be seen as recommendations, regarding methods of analysis and applications of enzymes in the process of winemaking.

In 1997 the common legislation on winemaking overruled the procedures of national user licenses. The text of regulation CE 822/87, that applies to enological applications, was revised and rewritten in 1999. The current community legislation is described in regulation CE 1493/1999 from the European council dealing with the common organisation of the agricultural markets. This regulation is based on the use of positive lists. The accepted enological applications and practises are described in Appendix IV of this regulation. The modifications made in 1999 deal with the use of glucanases and lysozyme.

Pectolytic enzymes and β-glucanase preparations are described for clarification purposes. The use of lysozyme is also described. Only the applications in this Appendix are allowed.

4.2.1 Recommendations from international organisations

Enzyme preparations used in winemaking must comply with the specifications recommended by JECFA (joint FAO/WHO Expert Committee on Food Additives) and by FCC (Food Chemical Codex) for food enzymes.

The OIV takes decisions on agricultural lands suited for winemaking, on analysis methods and on enological applications and treatments. In 1996 the work of OIV made it possible to amend the 'Codex Oenologique International' with a monograph on general prescriptions for enzyme preparations ('résolution oeno 6/95, Bulletin de l'OIV, 1996, 779-780). This monograph clearly states that

> 'All enzyme preparations serving a technological purpose that is convincingly demonstrated in practise and that meets the requirements of the Codex, are allowed for treatment of grapes and their derivatives'.

Until 1995 pectinases were the only class of enzymes allowed for winemaking in Europe (regulation CE 822/87, JO of the European Community no. L84/18 dated 27/03/87). Nowadays, glucanases have been added to the list of enological applications (resolution CE 2624/95). The OIV also recommends the use of a new enzyme, urease, for treatment of wines to reduce the risk of ethyl carbamate formation. Also this enzyme is taken up in the International Code of Winemaking.

4.3 Enzymes in winemaking: history and definitions

Enzymes have always been used by mankind, either in the form of enzyme-rich vegetable materials or in the form of microorganisms used for different purposes, such as brewing, baking or alcohol production. Since the 1970s the use of enzymes has been introduced in the technology of winemaking. The first enzyme preparation was a pectolytic preparation sold under the name of Ultrazym G 100. It was used for hydrolysis of pectic substances in the must. At that time enzymes were mainly used for improvement of the pressing and clarification of must and wine. From the 1980s onwards enzymes have been used for colour extraction, filtration and aroma liberation. Purified enzyme preparations were introduced in the 1990s. Very recent investigations focus on new enzymes, such as urease and lysozyme for reduction of ethyl carbamate formation and microbial stability improvement, respectively.

Enzymes are natural proteins with catalytic properties, produced by every living cell (chapter 1). Like other proteins, enzymes are large biological molecules

built from amino acids linked to each other by peptide bonds. Due to their nature, proteins (and thus also enzymes) are easily biologically degradable.

Industrial enzymes are commercial products obtained by purification and formulation of a microbial ferment or of plant or animal tissue, and standardised with regard to enzyme activity or activities. Most industrial enzymes are produced nowadays by fermentation of microorganisms (yeasts, bacteria or fungi).

Industrial enzymes for winemaking are also preparations made by fermentation of microorganisms. Pectinases are mainly produced by *Aspergillus* species, whereas glucanases are mainly derived from *Trichoderma* species. The enzymes must be purified sufficiently to ensure no effect on organoleptic properties of the wine [2].

4.4 Enzyme properties and composition

The endogenous enzymes present in grapes play a fundamental role in ripening [3]. Nevertheless, their role is very limited during winemaking under winemaking conditions due to very low and limited activities. Therefore enzymes from exogenous sources are seen as helpful processing aids because they can reinforce or replace the grape enzyme activities. It has been reported that such enzymes have been used successfully in winemaking since the early 1970s [4–7]. Improved knowledge of the nature and structure of macromolecules in must and

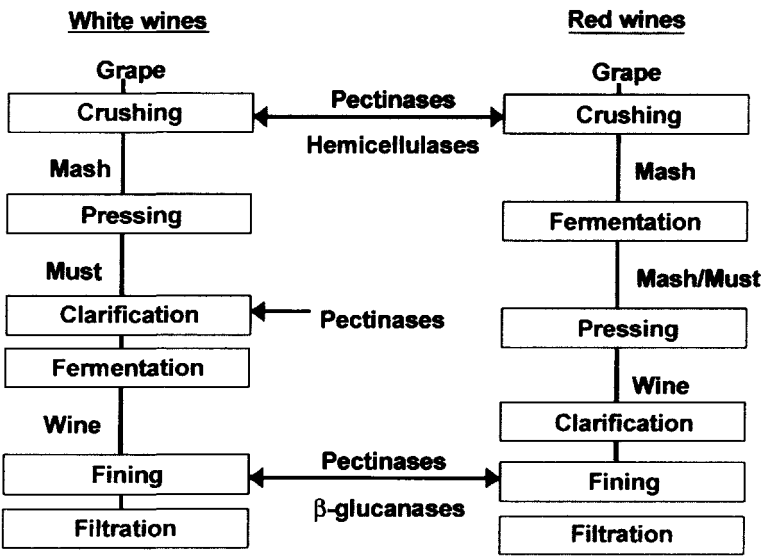

Figure 4.1 Application of industrial enzyme preparations in white and red wine production.

wine offers new possibilities for the application of enzymes in winemaking, in clarification and filtration, extraction and stabilisation processes in both white and red wine production. Today, the main application of industrial enzymes in winemaking concerns the use of pectinase, hemicellulase and glucanase preparations, as schematically shown in figure 4.1 [8].

Enzyme preparations can be supplied in granular, liquid or powder form. The powder form is not recommended for use because of the allergic potential of enzyme dust. In granular form enzyme preparations do not produce dust due to fixation of the enzyme particles. Other advantages of granular enzyme products are the lack of preservatives and the good storage stability. Liquid enzymes normally contain preservatives.

4.5 Applications of enzymes

Enzyme preparations are used throughout the whole winemaking process:

- on the grapes (weakening, maceration)
- in the must and the press wine (clarification and sedimentation)
- in the young wine at the end of the fermentation

Enzymes are added to the process in different ways [6]. In all cases solutions are made of the enzyme (granular enzyme preparations are also readily soluble). Actual addition proceeds via:

- spraying the enzyme solution on the grapes
- dosing the solution via a pump system directly into the inlet of the press
- adding the solution to the tank before clarification.

Due to their specific character and action, enzymes release molecules that play a role in the taste characteristics of wine (colour, aroma, structure) or breakdown molecules that limit the effect and efficiency of technological treatments, like pressing, clarification and filtration [9]. Better control of fermentation processes and enzyme production has lead to the possibility of purifying enzymes, for example, excluding the presence of cinnamyl esterase [2, 10].

Simultaneous use of bentonite and enzymes should be avoided because the enzyme will be inhibited due to specific adsorption to the bentonite and the bentonite will become less effective due to blocking of the active sites by enzyme protein. Bentonite treatment should preferably take place after the enzyme treatment. Addition of bentonite will help in flocculation of enzymatically-hydrolysed pectins.

The activity and efficiency of an enzyme can vary enormously, depending on temperature and pH. Must pectinases can be used in the temperature range of 10–55°C. Below 10°C the enzyme dosage should be increased. Above 55°C the enzyme will be inactivated. β-Glucanase can only be used above 15°C and

requires a longer incubation time. Enzyme dosages should be also increased at low pH values.

Enzymes are not inhibited by sulfur dioxide (SO_2) at levels that are acceptable in wine. Inhibition by polyphenols may occur in red wines. Increasing the enzyme dosage can counteract the effect. Alcohol up to a level of 14% (w/v) has no negative influence on enzyme action. In fact it has been reported that alcohol has an activating effect on β-glucanases used for aroma liberation.

4.5.1 Enzymes for pressing and maceration

Increased understanding of the way enzymes act on the polysaccharides of the grape cell walls, such as arabinans, galactans, arabinogalactans and arabinoxylans, makes it possible to optimise the usage of enzymes in wine-making. The mechanism of action of pectolytic enzyme preparations, usually called pectinases, is shown in figure 4.2.

As far as is currently known, the enzyme activities involved in hydrolysing pectic substances are pectin esterase, polygalacturonase, pectin lyase, rhamnogalacturonase, rhamnogalacturonan acetylesterase, arabinase and galactanase. Other enzyme activities are of hemicellulase and cellulase type and are normally present in varying amounts in pectinase preparations. The combined action of all these enzymes leads to a partial hydrolysis and solubilisation of acid and neutral polysaccharides present in the pectocellulose wall and middle lamella of the grape cells. These structures are responsible for the characteristic stiffness of the berries. The result of this hydrolysis is the release of juice, aromas and colour in a selective and controlled way. The remaining cell wall fragments are solubilised in the colloidal wine solution. This enzyme action is further shown in figure 4.3.

Pectinases partially hydrolyse the pecto-cellulose wall of the pulp cells. Their combined action causes the wall to become more porous. These walls

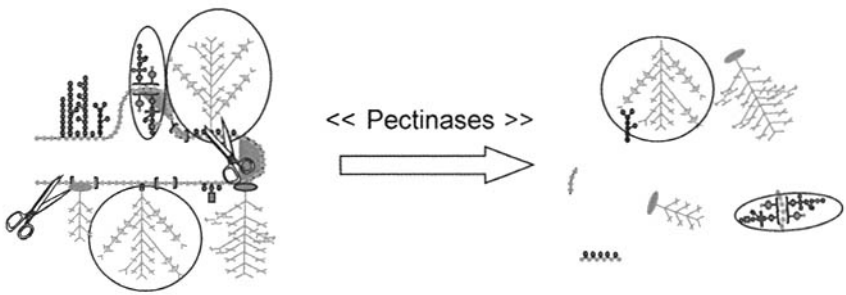

Figure 4.2 Action of pectinases on the polysaccharides of grapes [11].

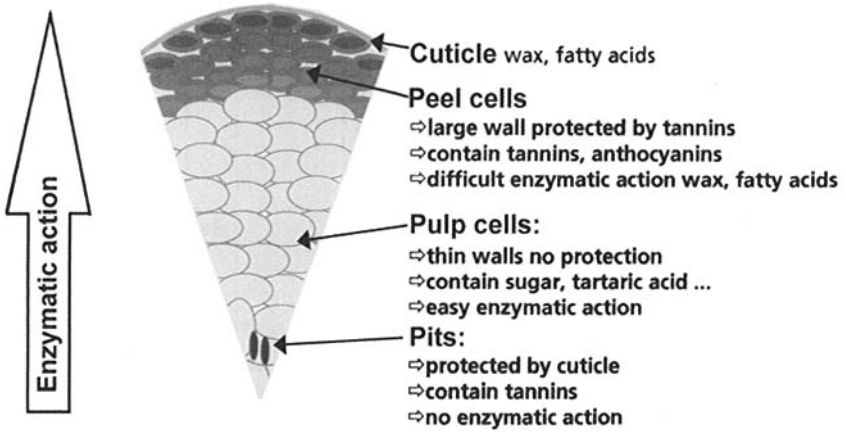

Figure 4.3 Action of pectinases during maceration of grapes [12].

are relatively sensitive to enzyme action due to their limited thickness and low tannin content. Enzymes also act on the peel cells, but with less effect, because peel cells have thicker walls and higher tannin content. These tannins have an inhibitory effect on enzymes. Maceration with enzymes does not lead to extraction of tannins from the pits. The lipid layer surrounding the pits strongly reduces their accessibility.

4.6 Enzyme applications for white and pink wine grape varieties

Extraction of grape juice is enhanced by enzyme action as can be seen in figure 4.4 [13]. The result depends on the amount of pectins present in the grapes and this depends on the ripeness and type of grapes, on the enzyme preparation (type of enzyme activities present in the preparation) and on the conditions applied for the enzyme action (incubation time, pH, temperature and presence of inhibitors). The increase in highest quality first press juice yield can go up to at least 10% and the pressing time can be reduced by 20–50% as a result of the presence of enzymes.

It is possible to vary the effect of the enzyme preparation, depending on the quality of the grape and the desired end result. The incubation time has a particularly strong influence. When enzymes are applied without pre-maceration of the peel, the enzyme action mainly occurs during pressing. In the case of pre-maceration, the enzymes are added immediately upon arrival of the grapes. This improves the pressing efficiency and the level of the enzyme effects. Pre-maceration is usually undertaken for a period of 3–4 h at temperatures around 20°C or 6–10 h at temperatures below 15°C.

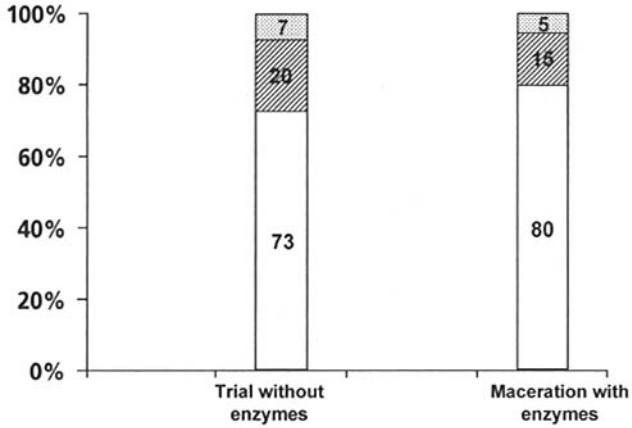

Figure 4.4 Effect of enzymes on the extraction of grape juice. ☐ = 1st press; ▨ = 2nd press; ▩ = 3rd press.

For production of white and pink wines it is recommended that enzyme preparations that have been purified to remove cinnamyl esterase activities are used. Enzyme preparations used for making of white wines should have none of this activity. It is produced in nature by *Aspergillus niger* and *Botrytis cinerea* species, and is responsible for the hydrolysis of coumaric and ferulic acids, which, after decarboxylation, lead to the formation of vinyl-4-phenol and vinyl-4-guaiacol. These compounds give a characteristic unwanted 'pharmaceutical' off-flavour in white wines [10]. Investigations by Chatonnet [14] have shown that it is possible to reduce the level of vinyl-4-phenol in white wine from the

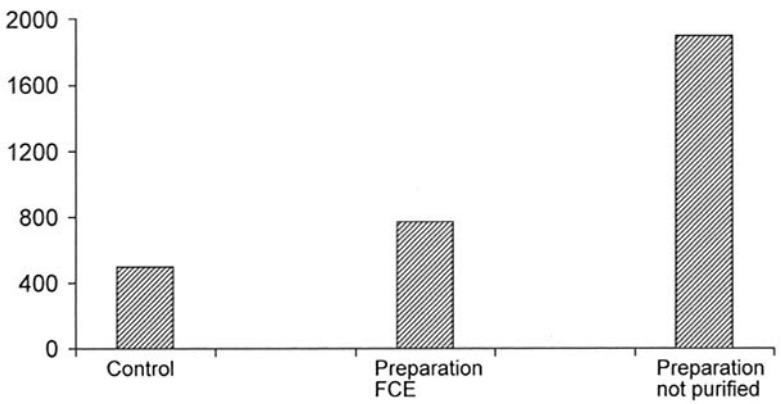

Figure 4.5 Comparison of effects of a purified pectolytic enzyme (Vinozyme FCE®) and a non-purified enzyme on vinyl-4-phenol levels.

Sauvignon grape by more than 50%, just by using purified pectolytic enzymes (figure 4.5).

There are generally two main reasons for using enzymes in the production of white and pink wines. The first is a quality issue. After a short pre-incubation with enzymes, a more controlled pressing process can be applied, leading to less oxidation, better quality of first press wine and better extraction of flavour compounds leading to better organoleptic properties. The second reason is technical: the use of enzymes allows higher loading of press and centrifuge equipment, shorter pressing times and faster rotation of the total equipment. Besides the effect of enzymes on maceration and pressing there is also a positive effect on settlement and clarification of the pressed juice. Overall this leads to higher efficiency and higher capacity.

4.7 Enzyme applications for red wine grape varieties

In red wine production the enzymes act on the pulp and peel cells. The method of enzyme addition is similar to that described for white wines (i.e. at the start of the maceration process). Enzyme dosage depends on harvest quality of the grapes (very ripe, ripe, unripe, affected) and on wine type (short or long fermentation).

Selective action of the enzymes on the peel polysaccharides is especially interesting with regard to the tannins of the red grape. Only free tannins in the vacuoles and tannins present in complex structures together with polysaccharides are released. Protein agglutinated tannin complexes are too big to be released in the cell vacuoles. Release of carbohydrate-tannin complexes is reported to have a positive effect on red wine, which is evaluated as less astringent and less bitter in comparison with untreated wines.

Higher enzyme dosage and longer incubation times result in more intense enzyme action and thus more extraction of peel components [15]. With red wines there is thus more variation possible with regard to enzyme dose rate/incubation time. At higher dose rates there is a greater contribution from the tannins; at longer incubation times there is a greater contribution from the pit-tannins.

4.8 Enzyme applications for must and press wines: clarification enzymes

The clarification of must before alcoholic fermentation is of utmost importance. Proper settlement of floating particles considerably reduces the formation of aromatic C_6 compounds, which lead to spicy or savoury flavours.

Enzyme preparations for clarification purposes have predominantly pectolytic activities. Hydrolysis of pectic substances leads to significant reduction of the viscosity of the must and for reduction of the protecting colloidal effect of macromolecules.

Settlement proceeds in three stages. The first stage is depectinisation, characterised by partial breakdown of pectins and decrease of the viscosity of the must. The end of the depectinisation step can be determined by an alcohol test. The second stage, the flocculation, is characterised by an increase in turbidity and formation of insoluble complexes. The third stage, the sedimentation, is mainly characterised by strong reduction of the turbidity and precipitation of the complex molecules. The enzymes improve the first stage, thereby accelerating the following stages.

After this enzyme step winemakers have several options. The must can be flocculated and decanted, followed by filtration of liquid, or centrifuged directly after the enzyme step.

The quality of the enzyme preparations used determines the degree of separation of the different phases in the fermentation tub, making it easier to work on the side streams of the wine production. Finally, as a result of the increased speed of the whole process there is less risk of spontaneous fermentation of endogenous microflora.

4.9 Enzyme applications for young wines: maturation and filtration enzymes

Maturation and filtration enzymes mainly consist of pectinases and β-glucanases. Pectinases partially hydrolyse polysaccharides coming from the grapes, releasing smaller polysaccharide fragments. These smaller fragments, often with a linear molecular structure, can obstruct the various stages of wine filtration and should be removed prior to filtration. The β-glucanases hydrolyse the glucan type polysaccharides, coming from *Botrytis cinerea* or from yeast cell walls. Such polysaccharides usually have a very high molecular weight and hinder filtration to a very large extent or even make it impossible. The amount of glucans released in the wine by wine yeasts (*Saccharomyces cerevisiae*) depends on the media used for the yeast fermentation [16]. At the same time, however, β-glucans can promote the release of certain macromolecules as mannoproteins. These compounds play an important role in protein stabilisation in the wines [17–19]. Furthermore, a flavour-carrying role has also been described. The use of exogenous mannoproteins for wine stabilisation has resulted in a patent application [20]. A reduction in the size of these components makes them more soluble, keeps the colloidal structures in wine during filtration and lowers the risk of filter blocking.

The effect of enzyme treatment on the filtration of wine is very obvious and in some cases even spectacular. Pectin hydrolysis generally results in 2–5 times greater amounts of wine filtered during one filtration cycle (see table 4.1), leading to a greatly increased capacity of the installation and a reduction of the filtration costs [9]. Qualitatively, the hydrolysis of blocking polysaccharides

Table 4.1 Effect of enzyme (Vinozym® 2.5 g/hl) on treatment of the filtration of Sylvaner wine over Kieselguhr [6]

	Untreated wine		Enzyme treated wine	
Filtration time (min)	Volume filtered (hl)	Pressure (bar)	Volume filtered (hl)	Pressure (bar)
30	35	3	35	3
60	65	6*	70	3
90	–		105	3
120	–		135	3
180	–		155	6*

*filter clogged

makes it possible to obtain improved filtrations without affecting the sensory properties of the wines.

It is strongly recommended that enzymes are added at the end of the alcoholic fermentation in order to be sure that the temperature is higher than 15°C. This ensures that the maturation and filtration of the wines is much easier.

4.10 Enzyme preparations for aroma liberation

The varietal character of (white) wines is especially defined by the presence of aromatic molecules, among which monoterpene alcohols play a well-defined role [21]. These compounds are found in grapes as free, volatile, odorous molecules, and as non-volatile glycosidic precursors, also known as 'bound terpenes'. In many grape varieties the amount of bound terpenes can be higher than the amount of free aromatic terpenols [22]. Consequently, the distinctive character of wines could be increased by releasing the glycosidically-bound terpenes [23–26, 30].

During the late 1980s enzyme preparations containing glycosidase activity (β-glucosidase, α-arabinosidase, α-rhamnosidase, β-aposidases) were developed to improve the aromatic profile of certain wines. In figure 4.6 the effect of an enzyme preparation on the liberation of several terpenes in Gewürztraminer wine is shown [27]. In table 4.2 a similar effect is shown for various grape varieties.

These enzyme preparations are added at the end of the alcoholic fermentation and during 'retapping' of wines (pouring into another vessel), which are not treated with bentonite, in order to prevent enzyme inhibition. The optimal temperature for this enzyme treatment should be above 15°C and an incubation time of several weeks to one month is required. The aroma development has to be controlled by tasting, and the enzyme action is finally inhibited by addition of bentonite. Small amounts of this compound (20 g/hl) are usually sufficient to block all enzyme activity. Application of these enzymes has to be approved

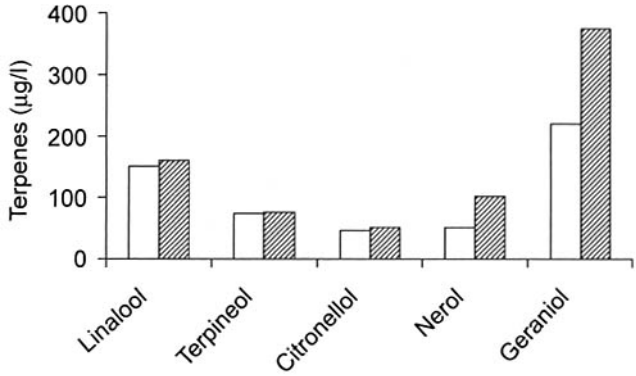

Figure 4.6 Influence of enzyme treatment on liberation of free aromatic terpenes in Gewürztraminer wine. □ = control; ▨ = enzyme treated.

Table 4.2 Influence of enzyme treatment on liberation of free aromatic terpenes in wines from various grape varieties [27]

		Terpenes (µg/l)						
Grape variety	U or E	L	T	C	N	G	Total	Change (%)
France 1989								
Muscadelle	U	71.5	32.3	1.7	4.1	10.9	120.5	
	E	85.4	39.4	2.1	6.3	23.4	156.6	30
Sauvignon	U	16.2	9.0	3.8	0.8	3.3	33.1	
	E	23.1	10.9	4.3	2.5	7.7	48.7	47
Sémillon	U	21.9	–	1.5	2.7	1.6	27.7	
	E	25.7	–	1.9	3.1	5.8	36.5	32
Colombard	U	11.7	10.2	2.6	1.9	3.1	29.5	
	E	16.5	11.9	3.1	2.3	7.5	41.3	40
Grenache	U	22.5	27.7	2.6	2.1	3.4	58.3	
	E	27.6	36.7	3.3	5.3	7.5	80.4	38
Austria 1990								
Spätlase Traminer-	U	454.6	137.8	7.8	19.0	61.8	681.0	
Muskat Ott.	E	476.0	127.2	11.6	41.4	162.3	818.5	20
South Africa 1991								
Chardonnay	U	16.6	22.4	2.1	2.1	3.2	46.4	
	E	23.2	31.5	0.9	3.2	7.2	66.0	42
Chenin	U	7.4	4.7	–	1.4	–	13.5	
	E	9.6	6.0	0.8	2.3	2.0	20.7	53

L: linalool; T: terpineol; C: citronellol; N: nerol; G: geraniol; U: untreated; E: enzyme treated.

by the 'Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes (DGCCRF)'. Even though these enzymes are produced by *Aspergillus niger*, European regulation does not mention the application of β-glucanases in winemaking.

Since the mechanism of hydrolysis of terpenyl glycosides has been elucidated, various different fungal enzyme preparations have been developed capable of enhancing wine aroma [28]. Trials with *Aspergillus* spp. pectinase and glucanase preparations have shown that good results can be obtained under winemaking conditions. These enzymes can be used in fermented juice as soon as glucose has been consumed by the yeast, or in young wines. In this way the variety in aroma and bouquet of certain wines can be improved.

4.11 Enzymes for colour extraction

The extraction of phenolic compounds generally occurs with the maceration of the mash during alcoholic fermentation and depends on the variety and quality of the grapes and on technological parameters such as crushing, maceration time, temperature and pumping. Even though colour extraction trials are difficult to conduct because of the heterogeneity of the mash and the influence of processing factors, good results have been obtained on an industrial scale using enzymatic treatments [29]. Improved anthocyanin extraction was obtained by enzyme addition at the beginning of maceration. However, more fundamental research is required to understand all mechanisms involved in colour extraction fully.

4.12 Other enzymes used in winemaking

4.12.1 Urease

The presence of ethyl carbamate in wine has been regulated since 1985 when the Canadian government set a maximum ethyl carbamate content in alcoholic drinks of 30 μg/l. The source of this molecule, suspected to be carcinogenic and mutagenic at high dose rates, is predominantly urea. It has been demonstrated that of 1800 wines tested only 1% of white wines and 3% of red wines have ethyl carbamate levels above 30 μg/l; the average level was 5.8 μg/l. Predominantly wines from northern regions contained higher levels of this component.

An enzyme preparation containing the enzyme urease was developed in Japan. Urease catalyses the hydrolysis of urea to carbon dioxide and ammonia. This limits the formation of ethyl carbamate. The enzyme is produced by *Lactobacillus fermentum*. Experiments with this enzyme in Elzas and Burgundy have shown positive results. The use of this enzyme has been permitted in France since 1997, although it is not mentioned in the new European regulations.

4.12.2 Lysozyme

Microbial stabilisation of wines is accomplished by the addition of sulfur dioxide (SO_2). Research carried out by ITV (Institute Technique de la Vigne et du Vin) has shown that stabilisation is guaranteed also by lowering the sulfur dioxide level in combination with treatment with lysozyme [31]. Lysozyme is derived from egg white protein and is used in the pharmaceutical and food industries. The enzyme has the ability to break down the cell walls of lactic bacteria and its activity increases with increasing pH, in contrast to SO_2. Lysozyme treatment is recommended for:

- blocking the malolactic fermentation (FML) in white wines
- stabilising red wines after FML
- treatment of problematic end stages of alcoholic fermentation
- preventing premature FML in wines of the latest vintage

Lysozyme has been discussed by OIV for use in must and wine. The enzyme is mentioned in the new European regulations, although the conditions and limitations have not yet been included.

4.13 New developments

Recent investigations have stimulated interest in another group of enzymes, oxidases. In particular, a polyphenol oxidase originating from the fungus *Myceliophtera thermophile* and produced by *Aspergillus oryzae* has been developed for application in the wine industry.

4.13.1 Cork cleaning

This application deals with the corks used for closing wine bottles. The enzyme removes phenolic compounds (which can give an off-flavour to the wines) and is applied during cleaning of the corks. This enzyme treatment significantly reduces the amount of water-extractable total polyphenols in comparison with crude, unwashed corks. It is comparable with chlorine washing, but has the advantage that the level of trichloroanisol (TCA) is reduced instead of increased, as is the case with chlorine treatment [14].

4.13.2 Colour stabilisation

Removal of phenolic compounds to prevent oxidation, haze and flavour changes is usually undertaken with fining agents such as gelatin or polyvinylpyrrolidone (PVP). Treatment with enzymes such as laccases, tannases and peroxidases has been investigated for juice or wine stabilisation. The effect of fungal laccase has been closely examined because of its ability to react with a wide range of

phenolic compounds. Laccase treatment can enhance the effect of conventional fining treatments.

4.13.3 Health issues

Since the early 1990s there has been an increasing interest in health aspects of wine. Enzymes can play a certain role by eliminating antioxidants such as polyphenols [32].

4.14 Conclusions

Selected enzymes for winemaking are increasingly being appreciated for their possibilities during the various stages of winemaking.

Dependent on vintage, the raw materials and the method of processing, enzymes can reduce the intensity of mechanical treatments during soaking, pressing, clarification and filtration. The intrinsic properties of the grapes are maintained and the enzymes have only a very limited influence on the economics and quality of the wines. The production of waste material can be neglected and the use of non-specific clarifying agents or filtration agents can be reduced.

Enzymes are natural compounds having a specific action and are fully biodegradable. Nevertheless, the enzyme composition, the way enzymes are produced and the nature of enzyme activities present in a preparation play a predominant role in the mechanisms described and the quality of the wines. It is therefore very important that enzyme preparations are carefully selected and purified to have no negative influence on the wines.

In the near future biotechnology will allow the development of new enzymes for new applications. Research on genetic engineering of microorganisms has already resulted in highly specific and purified enzymes. This will lead to a better understanding of the reactions catalysed by different enzymes in different enzyme preparations. Enzyme producers face these challenges and authorities must be given all means to evaluate the technology properly and to limit the normally time-consuming approval procedures, which are sometimes difficult for the wine industry to accept, yet are vital to protect consumers.

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5 Enzymes in the manufacture of dairy products

Barry A. Law

5.1 Introduction

The dairy foods sector of the food manufacturing industry is a traditional user of enzymes. The best known dairy enzyme preparation is, of course, rennet, a collective name for commercial preparations containing acid proteases extracted from animal tissues [1]. These products clot milk by removing a highly charged peptide fragment from *kappa*-casein on the surface of micellar casein, the major form of milk protein. Destabilised casein micelles aggregate and form the structure of the milk clot, which is then acidified by lactic cultures to make cheese curd [2]. Although this use of enzymes is the single most important in the dairy sector, modern production methods have made possible other applications to meet changing needs and priorities. For example, in the UK and US, the shortage of calves from which to source traditional rennet has led to the development of enzyme production technology, with yeasts, moulds and fungi as the primary source. Over half of all milk coagulating enzymes used in these regions is microbial in origin, mostly from genetically-modified (GM) food yeast and mould containing copies of the calf gene for the production of chymosin, the main acid proteinase involved in milk clotting. The other main type of microbial rennet (coagulant) is made from non-GM mould, *Rhizomucor miehei*. This topic of coagulant production and use in the dairy industry will be described and discussed in detail in this chapter.

In addition to the use of milk clotting enzymes to make cheese, the dairy industry also makes use of enzymes such as lipases, non-coagulant proteases, aminopeptidases, lactase, lysozyme and lactoperoxidase. Some of these applications are traditional (lipase for flavour enhancement) while others are relatively new (lactose hydrolysis, accelerated cheese ripening, control of microbiological spoilage, modification of protein functionality) and all will be discussed in detail below. Table 5.1 summarises the range of enzymes used in dairy products.

5.2 Milk-clotting enzymes

5.2.1 The nature and identity of rennets and coagulants

The first commercial standardised rennet preparation was made and sold by Chr. Hansen A/S, Denmark in 1874, and was probably the first commercial

Table 5.1 Enzymes used in dairy technology

Enzymes	Application examples
Acid proteinases	Milk coagulation
Neutral proteinases and peptidases	Accelerated cheese ripening; de-bittering; enzyme modified cheese; production of hypoallergenic milk-based foods
Lipases	Accelerated cheese ripening; enzyme-modified cheese; flavour-modified cheese; structurally-modified milk fat products
beta-Galactosidase	Lactose-reduced whey products
Lactoperoxidase	Cold sterilisation of milk; milk replacers for calves
Lysozyme	Nitrate replacer for washed-curd cheeses and cheeses with eyes (e.g. Emmental)

enzyme of any type. It was then and still is by definition an extract of ruminant abomasum, ideally containing mainly chymosin, the enzyme which is specific for *kappa*-casein hydrolysis and casein destabilisation. However, dependent on the age of the calves from which it is extracted, rennet can contain more or less pepsin, another acid protease with a wider casein substrate range. Both chymosin and pepsin, and indeed all of the milk coagulating enzymes used in cheese technology, are classified as aspartic proteinases with the Enzyme Commission (EC) number 3.4.23. Because there are now several different types and sources of milk clotting enzymes on the market, the International Dairy Federation (IDF/FIL) official definitions decree that the name 'rennet' be reserved for enzyme preparations from ruminant stomachs, and other milk clotting enzymes (mainly the microbial ones) should be named 'coagulants' [1].

As far as the cheese technologist is concerned, the rennets and coagulants are most usefully categorised by their source, not only to distinguish animal, vegetable, microbial and GMO-sourced preparations, but also to select the most suitable enzyme for any particular cheese variety. This is a very important aspect of cheese manufacturing technology, affecting the yield of cheese, its storage life and its final quality of flavour/texture after maturation. These interrelationships are discussed in detail in section 5.4 of this chapter. For now, we will consider the range of rennets and coagulants available, in table 5.2.

5.2.2 *Main characteristics of rennets and coagulants from different sources*

Of the animal rennets, calf rennet is widely regarded as the ideal milk-clotting enzyme for cheesemaking. This preference arises partly through traditional familiarity with the product through long use, but also has a sound scientific basis in that calf rennet is typically 80–90% chymosin (EC 3.4.23.4). This means that most of the casein breakdown in the cheese vat is directed very specifically at *kappa*-casein to clot the milk, and not at the other caseins.

Table 5.2 Main types and sources of milk-clotting enzymes (rennets and coagulants) used in cheesemaking

Type	Source	Component enzyme(s)	Technological characteristics
Animal			
Calf rennet; bovine rennet	Bovine stomachs	Chymosin A and B, pepsin and gastricin	High ratio of *kappa*-casein : general casein hydrolysis; maxim yield; traditional texture and flavour in hard and semi-hard cheeses
Rennet paste	Calf, kid or sheep stomachs	As calf rennet + pregastric lipase	Optimum coagulation and yield, with piquant flavour production
Sheep, pig and kid rennet	Corresponding animal stomach	Chymosin, pepsin and gastricin	Not widely used; best used with milk from same animal
Vegetable			
Plant coagulant	*Cynara cardunculus*	Cyprosin, cardosin	Produced locally on small scale for artisanal cheeses
Microbial			
Miehei coagulant type L, TL and XL	*Rhizomucor miehei*	*R. mucor* aspartic proteinase	Native L-type enzyme too heat stable and proteolytic for hard cheese manufacture; also produced as destabilised TL and XL
Pusillus coagulant	*Rhizomucor pusillus*	*R. mucor* aspartic proteinase	Similar properties to L-type miehei enzyme, but more pH-sensitive
Parasitica coagulant	*Cryphonectra parasitica*	*C. parasitica* aspartic proteinase	Very heat-resistant; use confined to high-cook cheeses like Emmental
Fermentation-produced chymosin (Chymax™; Maxiren™)	**Kluyveromyces lactis*; **Aspergillus niger*	Calf chymosin	Identical to calf chymosin in all respects

*Genetically-modified to express copies of the calf prochymosin gene.

Non-specific proteolysis of *alpha-* and *beta*-casein during curd formation can result in the loss of casein nitrogen in the whey, reducing the yield of the cheese process. The second enzyme in calf rennet, pepsin (EC 3.4.23.1), is thought by traditional cheesemakers to add to the ripening qualities of rennet in maturing cheese, but there is no hard evidence for this. Indeed, cheesemakers now make excellent quality long-hold cheeses (especially Cheddar) using pure chymosin from GM yeast and fungi expressing cloned copies of the calf (pro)chymosin gene.

Sheep, goats and pigs can provide rennet preparations that are enzymatically similar to calf rennet, but not ideally suited to clotting cows' milk [3]. Rennet 'paste' is a crude form of rennet made from the macerated stomachs of suckling calf, lamb or kid, and containing pregastric lipase to add piquancy to the flavour of the cheese. It is mainly used in traditional Italian cheeses.

Many plants produce proteinases that clot milk. However, plant coagulants are not produced on a commercial scale, but made locally (mainly in Portugal) for artisanal cheesemaking [1].

The best-known and most widely used microbial coagulant is that produced from *Rhizomucor miehei* (table 5.2). The commercial preparation is a mixture of aspartyl proteinases (EC 3.4.23.23) and is commercially available in three types. The native, unmodified enzyme ('type L') is very heat stable and hydrolyses all of the caseins, not just *kappa*-casein. Although this has been used successfully to make soft, short-hold cheese varieties, its non-specific proteolytic action reduces yields of cheeses whose curd spends a long time in the whey (hard and semi-hard cheese), and caused bitterness in long-hold cheeses. The heat resistance of the enzyme is also a potential drawback in cheese plant from which the whey is processed as a food ingredient. The heat treatment and processing does not eliminate the activity of the coagulant and it can cause protein breakdown in other food products in which whey protein is a supplementary ingredient (e.g. sausages, meat pies, soups).

To overcome these problems, dairy enzyme producers have developed heat-labile versions of the *R. miehei* coagulant ('TL' and 'XL') using chemical oxidation to modify methionine side chains. These enzymes can be denatured by pasteurising the whey, and they are also less generally proteolytic than the native proteinase. These coagulants are a good alternative to fermentation-produced chymosin (see below) in the manufacture of 'vegetarian' cheeses, but the texture of hard cheese made with them becomes crumbly more quickly than that of cheese made with chymosin. Also the flavour profile of hard cheese made with fungal rennet is not the same as that of chymosin-made cheese.

The most widely used alternative to calf rennet in the cheese industry worldwide is fermentation-produced chymosin (FPC). It is produced by large-scale fermentation of GM *Kluyveromyces lactis* or *Aspergillus niger*. In both cases the microorganism has been modified using gene technology by the incorporation of the pro-chymosin gene from the calf into the host organism with a suitable

promoter to ensure its efficient secretion into the growth medium. The enzyme is relatively easy to harvest and purify from the culture, unlike the earlier production system using *E. coli* to produce chymosin in inclusion bodies [1, 4].

The details of cheesemaking with alternative rennets and coagulants are beyond the scope of this chapter and the reader should study '*Technology of Cheesemaking*' [5] for full coverage of the topic.

5.2.3 Production of rennets and coagulants

Animal rennets are secreted from the stomach mucosa as inactive proenzymes that can easily be extracted by maceration with water, weak brine or a buffer solution. A preservative (usually sodium benzoate) is normally added at this stage to prevent microbial growth during the next stages of production, involving filtration and acidification to activate the proenzymes. After neutralisation to pH 5.5 and a second filtration to clarify the extract, the preparation is standardised to the 'advertised' milk clotting activity, sterile-filtered and packaged as a liquid enzyme product to be transported and stored refrigerated. Animal rennets are not purified products, but contain whatever enzymes were secreted by the mucosal tissue at the time of slaughter. However, the enzymes in good quality calf abomasal tissue are mainly chymosin and pepsin and only standardisation is necessary.

Microbial coagulants are produced by submerged fed-batch fermentation of the production organisms *Rhizomucor* or *Cryphonectria*. The fermentation is usually for several days, after which the enzyme is recovered as a crude filtrate, concentrated by ultrafiltration and standardised. No attempt is made by the manufacturer to purify the product by removing other co-produced enzymes such as lipases and starch hydrolases, though the production strains of the mould are selected to minimise these contaminants. Nevertheless, it is important to note that *Rhizomucor* coagulants contain significant amounts of starch-degrading enzymes which pass through to the cheese whey and are not removed by whey processing. Whey protein concentrates (WPCs) from cheese plants using microbial coagulants should not therefore be used in the formulation of sauces and other food products containing starch as a thickener.

Fermentation-produced chymosin (FPC) is mainly sourced from either GM yeast (*Kluyveromyces lactis*) or the GM filamentous fungus (*Aspergillus niger*). Both organisms have a long history of safe use in food fermentations, and in food enzyme production, and the genetic modifications employed are fully approved as safe in Europe and North America. Law and Goodenough [6] summarised the GM techniques used to enable these organisms to secrete calf chymosin, and Harboe and Budtz [1] described the generic production scheme used for the fermentation and isolation of the coagulant product, which is similar to that used in the production of microbial rennets. Special emphasis is put on an acidification step in the FPC production process to ensure that the source

organism is killed and that the DNA is broken down before the enzyme is isolated from the fermentate. The FPC sourced from *Aspergillus* by Chr Hansen A/S is purified by chromatography to minimise the activity of non-coagulant enzymes and to provide added product purity assurance for cheesemakers and consumers [1].

5.2.4 Formulation and standardisation of rennets and coagulants

The most common type of rennet/coagulant product is the liquid form, inexpensive to produce, easy to measure out for addition to the cheese milk, and easy to mix. All products are formulated in a similar way irrespective of their source, and the same types of stabilisers (sodium chloride, buffer, sorbitol, glycerol) are used. The only permitted preservative is sodium benzoate, but some manufacturers also sterilise their products by filtration to prevent microbial growth in the stored liquid products. Some rennets and coagulants are sold as powders or tablets, especially for shipment to hot countries. Whether dry or liquid, the products are all standardised so that a particular volume or weight always has the same milk-clotting activity, or 'strength'. Rennet/coagulant strength is determined by an international standard method, IDF standard 157A [7], developed jointly by IDF, International Standards Organisation (ISO) and the Association of Official Analytical Chemists (AOAC). This standard uses International Milk Clotting Units (IMCU) and measures clotting at the pH of most milk (6.5). Standard 157A uses a calf rennet preparation as the reference standard and is used to standardise rennets and FPC. A similar standard method is also available for microbial coagulants (IDF Standard 176; [8]). The standard method for determining rennet composition (% chymosin and pepsin) is IDF 110B [9]. The application technology and enzymology of rennets and coagulants is too great a subject to cover in this chapter, but is described and discussed in great detail in '*Technology of Cheesemaking*' [5].

5.3 Lactoperoxidase

Lactoperoxidase (LP) (EC 1.11.1.7) occurs naturally in raw milk, colostrum and saliva; it is thought to be part of the protective system for suckling animals against enteric infections. Lactoperoxidase is bactericidal to Gram-negative bacteria, and bacteriostatic to Gram positives. It is a peroxidase that used hydrogen peroxide to oxidise the thiocyanate ion to hypothiocyanate, the active bactericidal molecule. Law and John [10] demonstrated that the LP 'system' (LP + thiocyanate + hydrogen peroxide) irreversibly inhibits the membrane-energising D-lactate dehydrogenase in Gram negative bacteria, leading to cell death. In Gram-positive bacteria, the membrane ATP-ase is reversibly inhibited and may be the basis of bacteriostasis, rather than death.

Although all raw milk contains LP and thiocyanate, there is not sufficient natural hydrogen peroxide to activate the enzyme system (LPS), and several methods have been devised to increase hydrogen peroxide levels in commercial raw milk supplies to provide a 'cold sterilisation' system for countries with insufficient energy resources for heat treatment to preserve raw milk before consumption. Its efficacy in eliminating psychrotrophic Gram-negative spoilage bacteria from raw milk stored at 4°C was demonstrated by Reiter and Marshall [11]. Although hydrogen peroxide alone can be used as a preservative in these countries, it must be used at a dosage of 300–500 mg/l to be effective, and at this concentration it destroys some vitamins and impairs the functionality of the milk proteins. With the LPS, hydrogen peroxide can be generated *in situ* using glucose oxidase, and free hydrogen peroxide levels are too low to damage the milk. Even if chemical peroxide dosage is used instead of glucose oxidase, it need be added at only 10 ppm to activate the LPS. Law and Goodenough [6] have summarised these options.

5.4 Cheese ripening enzymes

5.4.1 Types of enzyme available commercially

The enzymes and enzyme 'packages' used to modify, enhance or accelerate the maturation of cheese are generally composed of more than one class of enzyme, and for the sake of clarity they are discussed here as a technological group, rather than as individual classes. The classes used in commercial ripening technology include many hydrolases represented by proteinases, peptidases and lipases, and if current research is successful, this list may soon extend to metabolic enzymes such as acetyl-CoA synthases and amino acid-catabolising enzymes to generate volatile esters and sulfur compounds.

Considering the very extensive worldwide research effort and literature on the enzymology of cheese ripening [12], few enzyme companies have successfully developed commercial enzyme packages for cheese technology, other than the ageing Enzyme-Modified Cheese (EMC) production methods used to make flavour ingredients for processed cheese and cheese-like foods [13,14].

This state of affairs is partly due to the poor availability of commercial enzyme preparations that are dedicated to cheese ripening, and of proven efficacy. The research literature contains hundreds of reports of small-scale and pilot scale enzyme trials with well-known cheese varieties, but few reach the market. Thus, in contrast with the widespread use of animal (bovine, porcine, caprine) and fungal (*Aspergillus*, *Candida*, *Rhizomucor*) lipases and proteolytic enzymes in the manufacture of EMC, only one commercial system, Accelase®, has been widely tested in the manufacture of the established cheese varieties and their reduced-fat variants.

This product was developed by IBT Ltd., now part of Rhodia UK, from the author's basic and applied research into the role of starter enzymes and cell lysis in flavour development [15,16]. Smith [17] has described the efficacy of the commercial system. It is made up of a food grade microbial endopeptidase (proteinase) active against all of the casein components in cheese, together with general and specific LAB aminopeptidases, and undefined esterases and flavour enzymes present in LAB cell homogenates. Extensive trial data from commercial cheese manufacture suggest that, when the product is added to cheese curd, the cheese reaches the equivalent of nine months' maturity in only five months. In addition, this enzyme treatment is claimed to reduce bitterness due to certain cultures, and to enhance flavour notes such as 'sulfur', acid and Cheddar. The precise mechanism of flavour enhancement is not defined but, like its research prototype, this product increases the amino acid pool in cheese, providing taste enhancement directly, and increasing the supply of flavour and aroma precursors, in the presence of added LAB biomass. The emerging research on amino acid catabolising enzymes in LAB also suggest that Accelase® may not only increase the cheese amino acid pool, but also increase its enzymatic turnover to flavour and aroma compounds.

Enzymes such as 'Rulactine' (Rhone–Poulenc) and 'Flavorage' (Chr Hansen, US, Inc.) have been marketed as cheese ripening enzymes, but little information is available as to their efficacy and market uptake. 'Rulacine' is a proteinase from *Micrococcus* sp. and 'Flavorage' contains a lipase from *Aspergillus* sp. together with proteolytic enzymes.

The research literature suggests that one day cheesemakers will *knowingly* benefit from enzymes that convert amino acids into sulfur volatiles, esters, aldehydes, amines, ammonia and fatty acids. However, many obstacles stand in the way of commercialisation, not least the instability and low production levels of these enzymes in their natural host microorganisms. Also, some of these conversions require enzyme/cofactor complexes that are only sustainable in whole cell environments, and GM variants of the wild-type cheese bacteria with selectively-enhanced activities may be the only route to the technology.

Before leaving the topic of enzyme availability, it is worth mentioning the very interesting approach that uses urokinase added to the cheese milk to activate plasminogen to plasmin in the cheese [18]. The increased plasmin activity accelerates proteolysis during maturation, and would presumably accelerate texture development in hard and semi-hard cheese as a consequence. If this enzyme technology could be economically viable, it would link up well with various forms of peptidase enrichment ('Accelase' and GM peptidase mutants). The activation of plasmin as an indigenous milk proteinase component of a ripening system, after whey separation and curd formation, would also overcome one of the practical difficulties of incorporating enzymes intimately into the cheese matrix.

5.4.2 *Enzyme addition technology*

As is the case with most good scientific ideas, technology transfer to the real world of process innovation and product manufacture presents unique and often unforeseen challenges. Thus it was for those of us who have tried to put proteolytic enzymes into cheese. Figure 5.1 illustrates the stages in the manufacture of hard and semi-hard cheese that could be the addition points for ripening enzymes. This plan applies to any enzyme, but the process implications are more serious in the case of proteinases, hence the emphasis.

Proteinases added to break down casein in cheese are needed only in very small amounts because, like all enzymes, they are catalysts, and a small quantity will convert a large amount of substrate. This is fine from the point of view of cost and conversion efficiency, but it means mixing grams of the active enzyme with tonnes of cheese. Putting enzymes evenly into the complex cheese matrix is difficult enough in itself, but the problem of distributing such *small* amounts is far from trivial. Enzyme addition to the cheesemilk at point **a** (figure 5.1) would be ideal logistically, because the starter and rennet are also added here and thoroughly mixed in. However, unlike these traditional parts of the recipe, ripening proteinases quickly begin removing soluble peptides from caseins. These peptides are lost into the whey when the cheese curd is separated, causing unacceptable losses to cheese yield. Also, the early breakdown of caseins disrupts their orderly structure, prevents proper gel formation, and renders the curds to soft and unworkable in the later stages of curd acidification, prior to salting and pressing into cheese. Add to these problems the loss of added enzymes into the whey (at a rate of about 95%) and it is clear that addition of proteinases directly to the milk is not an option. If peptidase preparations were

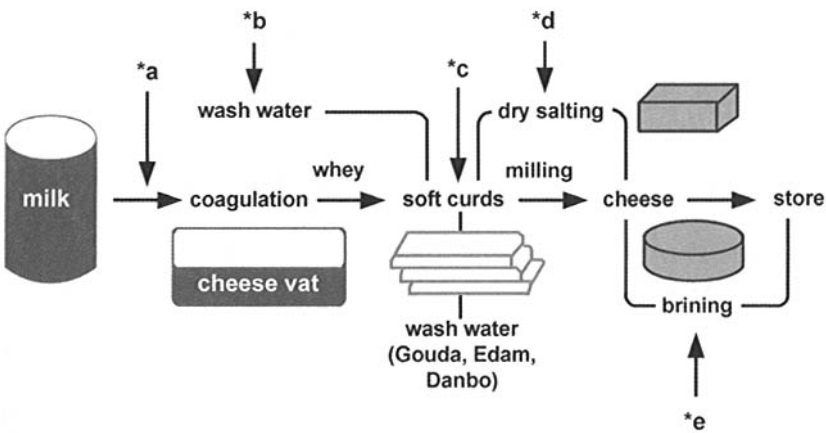

Figure 5.1 Possible enzyme addition points* during the manufacture of hard and semi-hard cheese.

very inexpensive, they could be added by this route, but most large cheese plants sell their whey as concentrates to be added to foods for their functionality. Any carry-over of ripening enzymes would have to be removed or destroyed before the whey was processed and sold.

Enzyme microencapsulation is the obvious solution to the above problem, to protect caseins in the milk and ensure their physical entrapment in the curd gel matrix. Options include fat, starch or gelatine capsules, but none of these has a satisfactory 'release' mechanism in cheese. The author's research group has developed a special type of phospholipid liposomes [19, 20] as an effective technology for overcoming this problem. Proteinases and peptidases were entrapped in the liposomes and added to cheese milk. Most of the ripening enzymes were entrapped in the water spaces between the curd particles and very little was lost in the whey. The liposomes were degraded naturally in the cheese matrix after whey separation, and this allowed full contact between the protein matrix and the ripening enzymes. However, although the technique has since been adopted in numerous experimental cheese trials [21], the high cost of the pure phospholipids necessary to make stable, high-capacity liposomes rules this out as a large-scale commercial technology. More recently, liposomes have been available commercially in the form of pro-liposome mixture (Prolipo-Duo™, Lucas Meyer, France) and used to put enzymes into Cheddar cheese [22], but the author is not aware of any economic assessment of this technology compared with the earlier work with 'home-made' liposomes.

The semi-hard cheeses typified by Gouda and Edam are made by a process which includes a curd 'washing' stage to replace some of the whey with water to reduce acidity. Although this stage (point **b** in figure 5.1), and the soft curd stage (point **c**) offer a further opportunity to introduce enzymes uniformly into the cheese matrix, they both create problems through premature curd softening, yield reduction and loss of 'unincluded' enzyme from the curds. Washed-curd cheeses are brine-salted, rather than dry-salted, and this may appear to offer an enzyme addition point (**c** in figure 5.1), but researchers can predict that enzyme penetration from brine into the closed texture of pressed cheese would be very low, ruling out this route, and making washed-curd cheese very difficult to treat with ripening enzymes.

In dry-salted cheese varieties, such as Cheddar, the addition of enzymes to milled curd with the salt (point **d**, immediately before pressing to form cheese blocks) was originally proposed for laboratory-scale cheesemaking [23], and this was successfully adapted to a 180 l vat scale [24]. However, this technique is difficult to adapt and scale up to automated salting equipment in large-throughput cheese plant, and although enzymes can be granulated with dry salt, this is an expensive process for cheap ingredients and is not widely used. An alternative method of enzyme addition was patented recently, involving mixing curds and enzyme in a vessel to which 'negative pressure' is applied, so that the enzyme is 'sucked in' to the curd matrix [25]. However, it is not clear how

the problems of even distribution and alteration of curd moisture and structure are solved by this invention. Whatever physical method is employed to place enzymes into cheese curd, some kind of vehicle is needed to disperse them and this is either water, or some other natural constituent of cheese such as salt or fat.

Thus, this whole area of enzyme addition technology is in urgent need of radical new ideas from the research base, but researchers also need feedback from cheese technologists and business economists. For example, there is sufficient expertise in molecular and applied enzymology to devise matrices and support materials to create micro-particulate enzyme complexes which could both liberate and metabolise amino acids, fatty acids and sugars to known flavour and aroma compounds. Such expertise has been generated in the fields of low-water enzymology, immobilised enzyme science, cellular enzymology and membranology, but as yet there has been no incentive to apply this to cheese ripening research. This is understandable in current circumstances, because the logical route to the use of complex enzyme systems that are easy to put into cheese is via whole cell technology—Nature has done the work for us, so why undo it? The simple answer is that Nature designed microbial cells for efficient life processes, not for efficient cheese ripening technology, and the natural microbial cell chemistry and architecture needs modification to put the (technologically) right combinations together. Gene technology achieves this within the whole cell technology concept but however safe this technology is made, the tide of media and consumer opinion is firmly against developments along this route. Nevertheless, whole cell options are available, some without GM and some with, and they are currently favoured by companies involved in developing cheese ripening systems [12].

5.4.3 Enzyme-modified cheese (EMC) technology

Enzyme-modified cheese is not really cheese from the consumer food point of view. It is a highly-flavoured ingredient for processed cheese, cheese flavoured snack foods and sauces, made by incubating emulsified cheese homogenates with animal or GRAS (generally recognised as safe) microbial lipases and proteinases. This technology was first approved in the US in 1969 and its products quickly came into large-scale use in processed cheese in 1970. All of the major dairy ingredient suppliers now have extensive and relatively sophisticated EMC-based flavour product lines; their production methods are based on patents and a large body of proprietary knowledge [14] but the general flow of the process is universal (figure 5.2).

The raw material for EMC manufacture is young bland hard or semi-hard cheese, cheese off-cuts and/or fresh salted Cheddar cheese curd. This is blended to homogeneity with emulsifying salts as a semi-liquid slurry (40–45% solids), pasteurised for 10 min at 72°C, then cooled ready for enzyme treatment. The incubation temperature for enzyme treatment depends on the flavour reactions

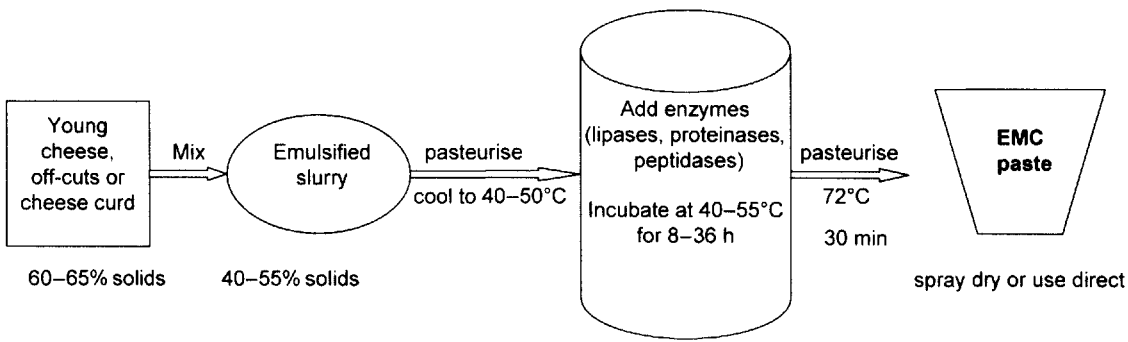

Figure 5.2 Production process for enzyme-modified cheese (EMC).

to be used to make the product. For example, a temperature of 25–27°C is suitable for making a blue cheese flavour product using a *Penicillium roqueforti* mould culture to grow and metabolise milk fatty acids to the characteristic methyl ketones. However, the slurry would need a pre-treatment at 40–45°C with a lipase to generate sufficient short and medium chain free fatty acids for rapid development of blue cheese flavour.

The most common emulsifiers and stabilisers used in EMC production include monoglycerides and diglycerides, phosphates, citric acid and Xanthan gum. Antioxidants are usually added in the form of plant oils and fat-soluble vitamins (e.g. tocopherols). The basic flavours generated enzymatically can be 'topped up' using food-grade nature-identical aroma compounds such as aldehydes, lactones, and alcohols, or refined by fermentation with dairy cultures of lactic acid bacteria and moulds.

Cheddar, Parmesan, Romano, Swiss-type and Gouda flavoured EMCs require a composite enzyme treatment, using lipases, proteinases and peptidases to develop the characteristic savoury, pungent and lipolysed notes in balance. The choice of incubation temperature (or indeed temperatures) is critical for the balance of these flavour notes, but is generally in the range of 40–55°C. This range is also governed by a compromise between the need for a short incubation time at high temperature for process efficiency and reduction of microbiological spoilage on the one hand, and the need to avoid temperature denaturation of the enzymes. EMC producers would like to have more robust microbial enzymes that could produce the required flavour biochemicals from the raw material in a few hours at temperatures up to 70°C. However, this might create new shelf life and users' problems from residual enzyme activity in the product. Currently, EMC products are pasteurised at 72°C for 30 min after enzyme incubation to destroy residual activity and eliminate spoilage microorganisms.

EMCs are spray-dried or packaged as pastes of different water content, dependent on customer preference and intended food use.

5.5 Lysozyme

Lysozyme (EC 3.2.1.17) is a hydrolase widely distributed in Nature; it is bactericidal to many Gram-positive species because it breaks down their cell walls. The enzyme is a mucopeptide *N*-acetyl muramoylhydrolase, available commercially from hen egg white or *Micrococcus lysodiekticus*. The food-grade preparations are from egg albumin.

Lysozyme is sold by the major dairy enzyme suppliers as an alternative control agent for 'late blowing', the textural defect of slits and irregular holes caused by the butyric fermenation in Gouda, Danbo, Grana Padano, Emmental and other important hard and semi-hard cheese varieties. Traditionally the defect, caused by *Clostridium tyrobutyricum* in raw milk, has been controlled by the addition

of potassium nitrate to the cheesemilk. However, this practice will be phased out because it is associated with the production of carcinogens, and lysozyme has become the preferred control agent [26, 6]. *Clostridium tyrobutyricum* is a sporeformer and as such, cannot be killed by pasteurisation, hence the need to treat the milk by alternative methods. Lysozyme kills vegetative cells and also inhibits outgrowth of spores in cheese; it is stable for long periods in the cheese matrix and because it binds to the cheese curd, little of the enzyme is lost on whey separation. Although lysozyme also inhibits the lactic acid bacteria used as starters in cheesemaking, they are less sensitive than the Clostridia, and a typical enzyme dose rate of 500 units/ml is sufficiently selective (commercial lysozyme preparations contain about 20,000 units/mg). Nevertheless, some thermophilic lactobacilli used in Grana cheesemaking are very sensitive, but can be 'conditioned' by unknown mechanisms by repeated growth on lysozyme-containing media [26].

Lysozyme also inhibits the growth of *Listeria monocytogenes* in yoghurt and fresh cheese with high acidity (< pH 5.0), but the effect is not consistent enough to rely on in commercial fermented milk products, and in any case high acidity is usually sufficient in itself to inhibit these pathogens.

5.6 Transglutaminase

With the recent availability of commercial microbially-derived transglutaminase (protein-glutamine *gamma*-glutamyltransferase; EC 2.3.2.13) preparations, there has been considerable interest in their application to the gelation of caseins and whey proteins, though this is not a widespread technology yet. However, transglutaminase is effective in reducing syneresis in acid milk gels and has been investigated as a method of improving the texture and shelf life of yoghurt [27].

5.7 Lipase

Although lipases are used in cheese flavour technology as components of the ripening systems already discussed in this chapter, they are also used to produce modified milk fat products for other food applications [28].

5.7.1 Lipolysed milk fat (LMF)

LMF has a creamy, buttery and cheesy aroma derived from short to medium chain fatty acids and fatty acid chemical derivatives released from milk fat by lipases. The raw material substrate for manufacturing LMF is either condensed milk or butter oil emulsified to maximise the fat surface area to activate the lipase. Lipases are added and left in contact with the substrate at the optimum

temperature for the enzyme(s) used, until the required flavour/aroma is achieved, or until a predetermined acid degree value (ADV) is reached, corresponding to a measurable release of fatty acids by the lipase.

The product is pasteurised, spray dried or otherwise adjusted to a standard solids content, and packaged. LMF products include chocolate coatings and syrups, butter flavours for margarine, artificial creams and sauces, flavourings for coffee whiteners, and cheese flavour additives [29].

The type of lipase used to make LMF products depends on the intended food application. Generally, good LMF products for use in baked products can be made using pancreatic lipase preparations, lamb and kid pregastric esterase and fungal/mould lipases from *Aspergillus niger*, *Geotrichum candidum* and *Penicillium roqueforti*. Some bacterial lipases are also suitable (*Achromobacter lipolyticum* and *Pseudomonas* lipase) but the LMFs for bread baking should not be prepared with *Achromobacter*, *Penicillium* or *Geotrichum* lipase to avoid soapy and musty flavours, and the pregastric esterases also produce too high a proportion of butyric acid for bread making, in which they tend to produce rancid, sweaty flavour notes [28].

This technology and its food applications has been comprehensively reviewed recently, and the interested reader can find detailed information therein [30].

5.7.2 Lipase-catalysed intra- and intermolecular modification of milk fat

Chemical interesterification, acidolysis, alcoholysis and transesterification have been used for many years to modify the physical/functional properties of milk fat, but more recently lipase technology has replaced this chemical technology to give more precise and 'cleaner' processing [30–32]. In particular, milk fat substitutes have been prepared as a partial replacement for the milk fat in baby foods [33]. However, fat fractionation by physical methods is the commercially-preferred option for milk fat modification in dairy product applications, and for coverage of lipase-catalysed molecular modification reference should be made to the reviews cited above.

5.8 Lactase

Lactase (*beta*-galactosidase; EC 3.2.1.23) hydrolyses lactose to its constituent monosaccharide sugars, galactose and glucose. The enzyme is widespread in animals, but it has only become important technologically since microbial sources have become readily available [34]. The principal commercial preparations are sourced from *Aspergillus niger*, *Aspergillus oryzae*. *Candida pseudotropicalis* and *Kluveromyces lactis*. Lactase applications are in batch and immobilised enzyme technology, favouring the *Aspergillus* and *Kluyveromyces* sources, respectively.

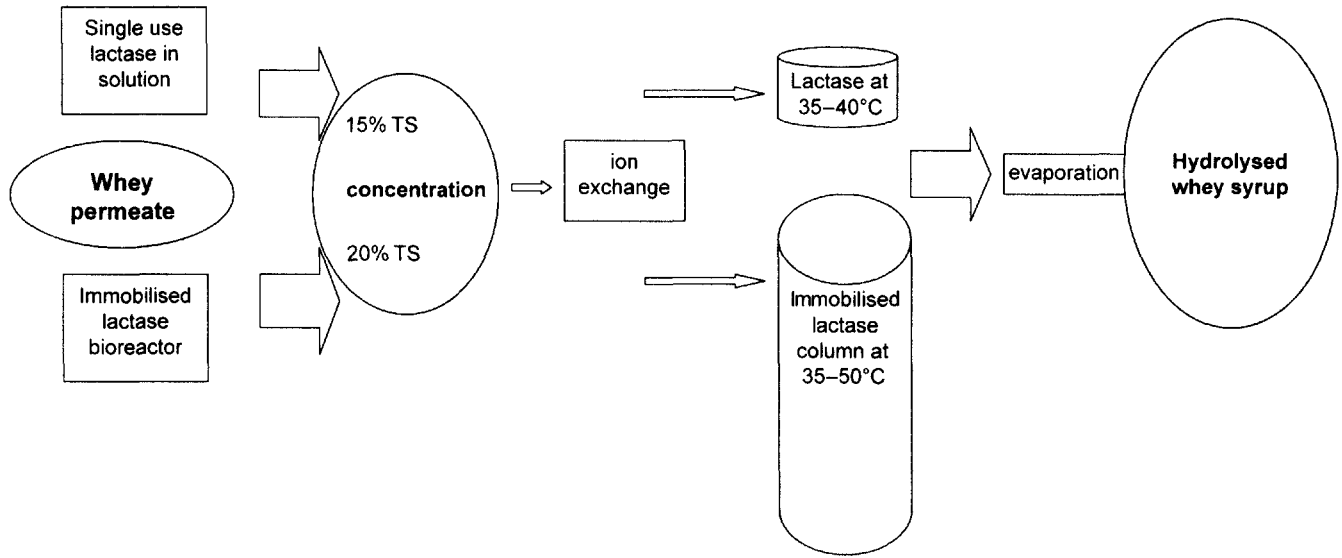

Figure 5.3 Applications for lactase in the process of lactose hydrolysis for whey technology.

5.8.1 Commercial dairy products of lactase technology

Lactase in tablet form (e.g. Lactaid®) is sold as an *in vitro* remedy for lactose intolerance, a widespread condition (affecting 30–50 million people in the US, for example) caused by a deficiency of lactase in the digestive tract. Suffers experience stomach cramps, bloating and diarrhoea due to the accumulation of lactose in the gut lumen. Lactaid® tablets deliver active lactase to the gut to break down the ingested lactose and alleviate the intolerance symptoms.

Hydrolysed whey syrup is produced from whey, the by-product of cheese-making and casein production. The hydrolysis step can be on the whey itself, or on the permeate from UF plant used to make whey protein concentrate. The UF permeate still contains some whey protein but is enriched in lactose. It is concentrated to 15–20% total solids (TS), demineralised, usually by ion exchange, though electrodialysis or nanofiltration can be used for this, then heated according to the type of lactase treatment to be used. The hydrolysis step can be by batch treatment with yeast lactase, though the use of immobilised enzyme reactor columns is more efficient, using *Aspergillus* lactase. Immobilised lactase reactors can achieve up to 90% lactose hydrolysis in whey permeate, though the batch process converts only about 70%. However, both processes produce sufficient free glucose and galactose to make the product sweet, and this property is enhanced by evaporation of the hydrolysate to 60% TS to make the final syrup. A typical flow diagram for the process is shown in figure 5.3.

The whey syrup is made sticky by the high concentration of glucose and galactose, so it is not dried, but sold and used in that form. It is used in food manufacture to replace sweetened condensed milk, sugar and skim milk in many products such as ice cream, milk desserts and sauces. The syrup is also an excellent caramel ingredient and as a sweetener/binder in cereal bars.

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6 Enzymic modification of food protein

Per Munk Nielsen and Hans Sejr Olsen

6.1 Introduction

Modification of the molecular structure of food proteins with enzymes is an attractive way of improving the functional and nutritional properties of these proteins. A limited and specific example of this is the conversion of milk to cheese by the action of protease. Today, various enzymes are applied to food proteins for the manufacture of new and valuable products.

Protein structure is modified to improve solubility, emulsification, gelling and foaming properties. Chemical modification is not desirable for food applications because of the harsh reaction conditions, non-specific chemical reagents and the difficulties of removing residual reagents from the final product. Enzymes, however, provide several advantages, including fast reaction rates, mild conditions and (most importantly) high specificity. In addition, there is a need for gentle methods that modify the food itself, in order to limit the use of additives. The most frequently used enzymes for protein modification are proteases and transglutaminases; carbohydrases and lipases are also used for structural, functional and aromatic modifications of protein food products.

6.2 Industrial proteases

It is difficult to tell which application first spurred the development of new proteases. As well as their application in the production of traditional foods like cheese, beer, and shoyu (soy sauce), proteases have been developed for the tanning industry and also for the detergent industry. Scientific interest in the action of proteases on different food proteins has resulted in the development of new proteases for extraction processes as well as for production of functional ingredients for the food industry.

By far the largest tonnage production of protease is based on microbial sources. Recombinant DNA methods and protein engineering are today's means of developing important industrial proteases for both food and non-food applications.

Proteases are classified according to their source of origin (animal, plant, microbial), their catalytic action (endo-peptidase or exo-peptidase), and the nature of the catalytic site (active site). Common names, trade names, typical pH range, and preferential specificity are also used to characterise them.

Table 6.1 Proteolytic enzymes used in food processing

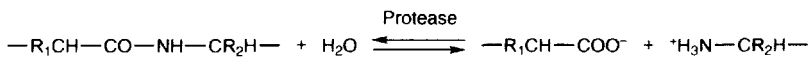
Type of protease	Source	Common names and some trade names	Practical application range for pH	Practical application range for T °C
Serine protease	*Bacillus licheniformis*	Subtilisin Carlsberg, Alcalase®	6–10	10–80
	Bacillus lentus	Subtilisin Esperase	7–12	10–80
	Bacillus GMO	Subtilisin Savinase, Novo-Pro™ D	8–11	15–80
	Ox or pig pancreas	Trypsin	7–9	10–55
Metalloprotease	*Bacillus amyloliquefacus*	Neutrase®	6–8	10–65
Aspartic protease	*Rhizomucor miehei*	Microbiol coagulant Hannilase® or Fromase®	3–6	10–50
	Calf abomasum	Rennet	3–6	10–50
Mixtures of aspartic protease, metalloprotease and carboxypeptidase	*Bacillus*	Protamex™	6–8	10–65
	Aspergillus oryzae	Flavourzyme™	4–8	10–55

Based on a comparison of active sites, catalytic residues, and three-dimensional structures, four major protease families are recognised: serine, thiol, aspartic and metalloproteases. The serine protease family contains two sub-groups; the chymotrypsin-like proteases and the subtilisin-like proteases, which are the most important industrial products. Many commercial proteases are mixtures of the different types. This is especially true for pancreatin, papain (crude), and some proteases from *B. amyloliquefaciens*, *A. oryzae*, *Streptomyces*, and *P. duponti*, mainly used within the food area.

Some proteolytic enzymes used in food processing have the characteristics shown in table 6.1. Examples of thiol (or cysteine) proteases are plant proteases from papain latex (papain), from pineapple stem (bromelain), and from fig latex (ficin).

6.3 Protein hydrolysis in enzyme applications

Proteases catalyse the hydrolytic degradation of the peptide chain, as shown for reactants and products in scheme 6.1. In aqueous solutions and suspensions of protein, the equilibrium lies so far to the right that degradation and not synthesis of larger molecules is thermodynamically favoured.



Scheme 6.1 The hydrolysis reaction: enzymatic cleavage of a peptide bond

When a protease acts on a protein substrate, there is a catalytic reaction of consecutive reactions. At very high degrees of hydrolysis, the water molecule added at each bond cleaved is important. The amount of water 'added' can be as much as 3% of the dry matter [1].

6.3.1 Control of hydrolysis reaction

Over the past 20 years, enzymic hydrolysis of food proteins has become a significant area of modern food processing. Fundamental description of the principles, the methods of controlling the hydrolysis reaction, ways of carrying out industrial processing, and characterisation of functional properties of proteins treated with proteases have been intensively researched and developed at Novozymes A/S. A great deal of this work was introduced by Adler-Nissen [2].

The most important parameters of the hydrolysis reaction are *S* (% protein in the reaction mixture), *E/S* (enzyme/substrate ratio in activity units per kg of protein), pH and temperature. Together with the specificity and properties of the enzyme itself, these parameters are responsible for the course of the reaction on a given protein raw material. The quantitative criterion of a proteolytic reaction is the degree of hydrolysis, calculated from determination of the number of peptide bonds cleaved and the total number of peptide bonds in the intact protein.

As a quantitative measure for the hydrolytic reaction, hydrolysis equivalents (h) are used. The unity of h is equivalent peptide bonds cleaved per kg of protein. h is used for kinetic investigations of the protein hydrolysis; but in other connections, the derivative quantity *DH* (degree of hydrolysis) is preferred. *DH* is calculated from equation 6.1.

$$DH = \frac{h}{h_{tot}} \times 100\% \tag{6.1}$$

h_{tot} is estimated on the basis of the amino acid composition of the protein. For most food proteins, the average molecular weight of amino acids is about 125 g/mol, making h_{tot} about 8 g equivalents per kg of protein (calculated as 6.25×N). Table 6.2 gives some more exact figures for Kjeldahl conversion factors f_N and for h_{tot} for some common proteins [3].

The degree of hydrolysis of enzyme-treated proteins determines the properties of relevance to food applications. It is important that the degree of hydrolysis can be measured while the reaction is going on. Only in this way will it be possible to stop the reactions at a well-defined stage when the desired property of the product has been obtained.

When a sufficient amount of substrate is available, relatively simple analytical tools, which can be applied directly during the reactions, are often used. Examples are the pH-stat technique, pH-drops, osmometry, viscosimetry and chemical determination of free amino groups, using for instance a measurement of liberated α-amino groups by reaction with trinitrobenzenesulfonic acid (TNBS),

Table 6.2 Kjeldahl conversion factors and content of peptide bonds for various food proteins [3]

Protein	Kjeldahl conversion factor, f_N	h_{tot} equiv/kg (N × f_N)
Casein	6.38	8.2
Whey	6.38	8.8
Meat	6.25	7.6
Fish muscle	6.25	8.6
Egg white	6.25	approx. 8
Soy	6.25	7.8
Cotton seed	6.25	7.8
Red blood cells	6.25	8.3
Wheat protein	5.70	8.3
Gelatine	5.55	11.1

whereby a coloured derivative of the peptide is formed. The TNBS method has been adapted to be usable for hydrolysates of food proteins, which are usually partly soluble; that is, the sample should be dispersed in SDS (sodium dodecyl sulfate) [4]. The O-phthaldialdehyde and the ninhydrin reaction with liberated α-amino groups may also be used, with some limitations [1]. The pH-stat technique and the osmometry technique are quicker and less labour demanding than the 'chemical' methods. When, as an effect of the protein hydrolysis, a simultaneous solubilisation of protein occurs, measurements of soluble nitrogen are carried out, for example by Kjeldahl analysis or a simple detection of soluble dry matter by measuring the increase of refraction index (°Brix).

6.3.2 Calculation of the degree of hydrolysis of proteins using the pH-stat technique

Originally, the pH-stat method was developed at the Carlsberg Laboratory [5]. It is based on the principle that pH is kept constant during hydrolysis by means of automatic titration with base when hydrolyses are carried out under neutral to alkaline conditions. When the hydrolysis is carried out under acid conditions, as for instance by use of pepsin at pH 3, the titration must be made with acid. The high buffer capacity of proteins means that at extreme pH values (i.e. pH > 11 or pH < 3), the pH-stat is inoperable [5].

The free carboxyl groups and the free amino groups found after hydrolyses will be more or less ionised, depending on the pH of the hydrolysis reaction. Working in the region pH 6–9.5, the amino groups will be partially protonated. This means that the hydrolysis of protein in this pH region is accompanied by a release of H^+, as previously noted by Sörensen [6]. Consequently, at pH values above 7.5–7.8 (the pK values at 25°C), the amino group will be less than half protonated, but the carboxyl groups will be fully dissociated. This leads to a net release of 0.5–1 mol H^+ for each mol of peptide bonds cleaved. In

the pH range 4–6.5, amino groups and carboxyl groups will be approximately equally dissociated. Therefore, in this pH area the pH-stat is not accurate. *DH* is calculated on the basis of the titration equations in the following way:

$$DH = \frac{h}{h_{tot}} \times 100\% \tag{6.1}$$

$$DH = B \times N_b \times \frac{1}{\alpha} \times \frac{1}{MP} \times \frac{1}{h_{tot}} \times 100\% \tag{6.2}$$

where B is base consumption in ml (or l); N_b is normality of base; α is average degree of dissociation of the α-NH_2 groups (see below); MP is mass of protein ($N \times f_N$) in g (or kg); h is the hydrolysis equivalents in meqv/g protein (or eqv/kg protein); h_{tot} is total number of peptide bonds in the protein substrate (meqv/g protein or eqv/kg).

The degree of dissociation, α, is calculated using equation 6.3.

$$\alpha = \frac{10^{pH-pK}}{1 + 10^{pH-pK}} \tag{6.3}$$

pK is the average pK value of the α-amino groups liberated during the hydrolysis, and it can be determined from a direct assay of these amino groups by use of the TNBS method. pK also varies significantly with temperature because the ionisation enthalpy of the amino group is considerable [2]. Inserting the ionisation enthalpy (+45 kJ/mole) in the Gibbs–Helmholtz equation, it was found that pK changed by about 0.23 pH units for a change of 10°C in the hydrolysis temperature [7]. Table 6.3 gives the results of calculations of the degrees of dissociation, α, from equation 6.3, for various pH values as a function of the temperature using the method described above.

Table 6.3 Degree of dissociation (α values)

	Temperature (°C)					
	40	50	60	70	75	80
pK	7.3	7.1	6.9	6.7	6.6	6.5
pH						
6.5		0.20	0.29	0.39	0.44	0.50
7.0	0.33	0.44	0.55	0.67	0.71	0.76
7.5	0.61	0.71	0.80	0.86	0.89	0.91
8.0	0.83	0.89	0.93	0.95	0.96	0.97
8.5	0.94	0.96	0.97	0.98	0.99	0.99

6.3.3 *Calculation of the degree of hydrolysis of proteins using the osmometry technique*

This method can be used for calculation of the degree of hydrolysis of proteins when no soluble components are added during the reaction; that is, in such ranges of the pH scale where the pH-stat principle does not function [8]. A freezing point osmometer can record the osmolality in a few minutes of a sample taken directly from the hydrolysis mixture. By drawing of samples as a function of time during a hydrolysis reaction, a reaction curve can be constructed. By means of the osmometer, ΔT is measured and converted to osmol through the simple proportionality of: $\Delta T = K_f \times osmolality$.

From the increase in osmolality, ΔC, DH is calculated by use of the following equation:

$$DH = \frac{\Delta C}{S\% \times f_{osm}} \times \frac{1}{\omega} \times \frac{1}{h_{tot}} \times 100\% \tag{6.4}$$

where ΔC is the increase in osmolality measured in milli-osmol/kg H_2O; $S\%$ is the protein substrate concentration (N×6.25), w/w%; ω is the osmotic coefficient ($\omega = 0.96$ for most actual concentrations of protein [2]); f_{osm} is the factor to convert % to g per kg of H_2O. f_{osm} is calculated using equation 6.5.

$$f_{osm} = \frac{1000}{100 - D\%} \tag{6.5}$$

where $D\%$ is the percentage of dry matter present in the reaction mixture; h_{tot} is the total number of peptide bonds in the protein substrate (mequiv/g protein or equiv/kg).

6.3.4 *Some kinetic aspects of protein hydrolysis*

The pH-stat technique has been utilised at Novozymes A/S to study the proteolytic degradation of haemoglobin, and to understand the reaction mechanism. In discussing initial proteolysis, Linderstrøm-Lang [9] mentioned the reaction of a protease on the native haemoglobin molecule 'one-by-one', indicating that a particular protease molecule degraded one substrate molecule at a time. No appreciable amounts of intermediary products will be present. The reaction mixture will consist of native proteins and end products only.

The opposite case mentioned was the one where the native protein molecules were rapidly converted into intermediary forms, which would be more slowly degraded to end products ('Zipper reaction').

As seen from the hydrolysis curves in figure 6.1, made by using Alcalase™ (table 6.1) at 50°C and 55°C, respectively, in the pH-stat, the slight bending at low DH values of the blood cell fraction hydrolysis curves indicates a thorough initial degradation to small peptides (a zero order reaction). This degradation

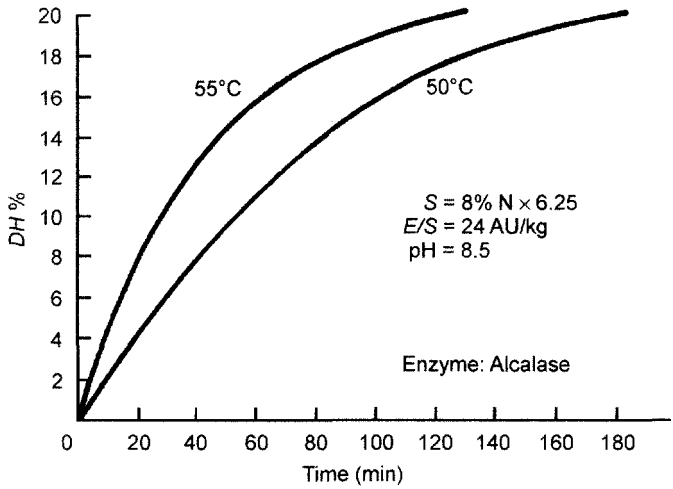


Figure 6.1 Hydrolysis curves for a haemolysed red blood cell fraction. *S* is % substrate protein in reaction mixture; *E/S* is enzyme:substrate protein ratio.

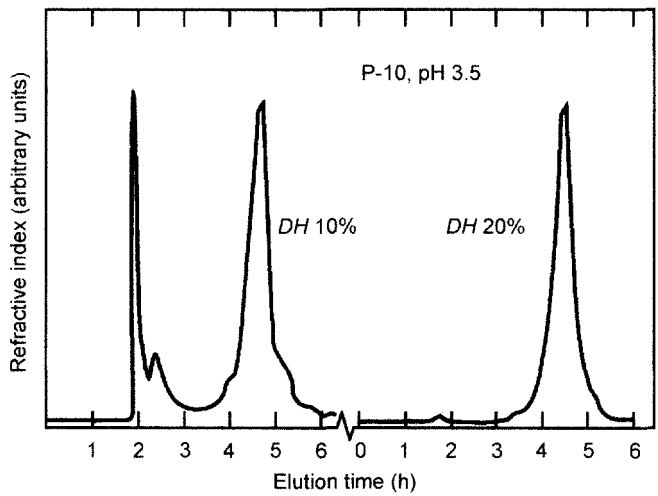


Figure 6.2 Gel chromatography of haemoglobin hydrolysate [4]. *DH* is degree of hydrolysis.

is close to the ideal one-by-one reaction. The hydrolysis proceeds rapidly with little decrease in velocity until the stage where the substrate concentration is so low that first order kinetics take over.

A gel chromatogram of the soluble part of the haemoglobin hydrolysate at *DH* 10% and *DH* 20% (figure 6.2) fully confirms the degradation pattern

of the haemoglobin substrate. At *DH* 10%, the hydrolysate consists of high molecular weight material and small peptides in accordance with the one-by-one mechanism. At *DH* 20%, the high molecular weight material has completely disappeared, as predicted by Linderstrøm-Lang in 1952 [9]. This is an important factor behind the application of ultrafiltration in the industrial process for separation of the hydrolysate from precipitated haemin, called the sludge [10]. If the hydrolysis was not of the one-by-one type, large peptides would also be present and consequently, the yields and fluxes would have been much lower. One should be careful not to draw too simple conclusions about kinetic models on enzymatic hydrolysis on even a fairly simple and well-defined substrate as the haemoglobin. The HPLC spectrum of (blood cell haemoglobin) BCH is shown in figure 6.3. At least 50 different peptide compounds are present under the single peak in the gel chromatogram of *DH* 20%.

6.3.5 The effect of proteolysis

When the protein structure is modified by proteolysis, the various effects thereof are considered positive or negative depending on whether the effect is desired or not. The developments within enzymic hydrolysis have, of course, been within both criteria. Desired effects of importance include increased solubility, reduced surface tension, increased emulsification, increased foaming properties and effective cleaning of fibres and hard surfaces. Undesired effects such as increased bitterness and other undesired taste developments have been key problems to solve within the food area.

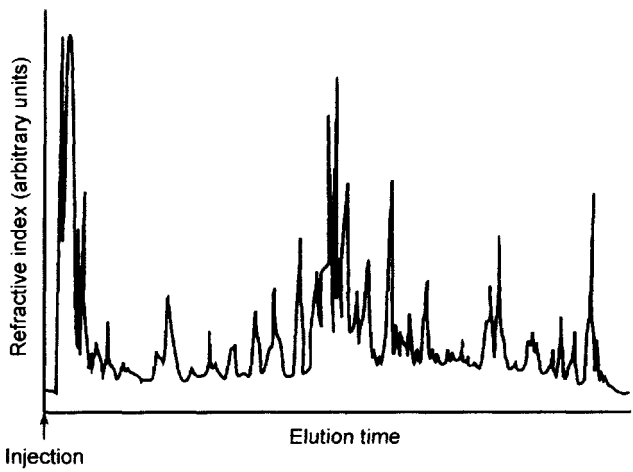


Figure 6.3 HPLC chromatogram of blood cell haemoglobin (BCH). *DH* 20% [4].

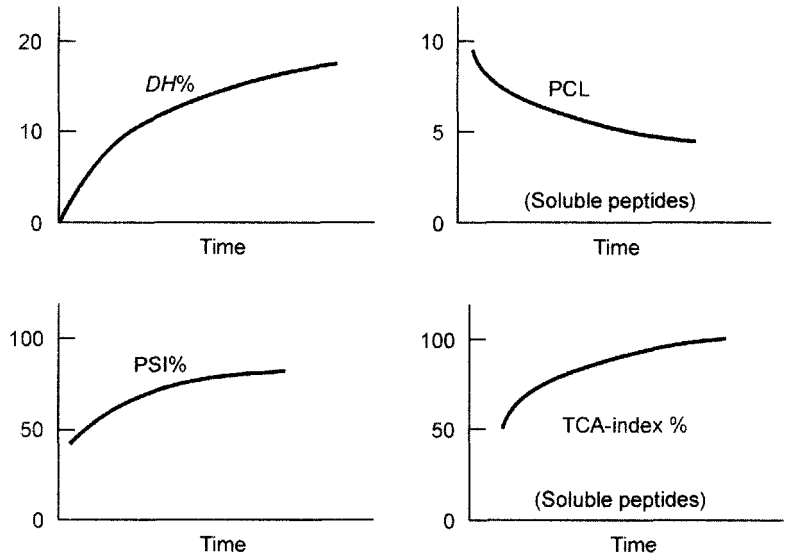


Figure 6.4 Time dependence of key indices during enzymatic hydrolysis of protein [4]. PCL is peptide chain length; PSI% is protein solubility index; TCA is trichloracetic acid.

During an enzymatic hydrolysis of proteins, the size character of the soluble peptides produced is changed. Key indices such as *%DH*, protein solubility index (PSI), and protein solubility in 0.8 M TCA (TCA index) increase, whereas the peptide chain length (PCL) is reduced, as shown in figure 6.4.

6.4 The bitterness problem

Unpleasant bitterness was observed at an early stage for many protein hydrolysates. Today, this problem can be managed by proper selection of the reaction parameters, and by the enzymes used. Bitterness is a complex problem, which can be influenced by at least the following variables [2, 11]:

- Hydrophobicity of the substrate, since hydrolysates become bitter if amino acid side chains containing hydrophobic groups become exposed, due to hydrolysis of the protein [12].
- High *DH*, and therefore the concentration of soluble hydrophobic amino acids, and their peptide chain length. The specificity of the enzyme used plays an enormous role to bitterness of protein hydrolysates. Separation steps applied in the downstream processing; for example centrifuging at isoelectric pH, and carbon treatment.

- Masking effects from other components in the protein hydrolysate. Organic acids like malic acid and citric acid exert a significant masking effect in soy protein hydrolysates [13]. This masking effect can be utilised in the final formulation of the food into which the hydrolysate is incorporated.

Other solutions to the problem of bitterness in production of protein hydrolysates with high *DH* values are available. For example, a debittering of protein hydrolysates can be performed by a selective extraction or removing of hydrophobic peptides to a 2-butanol phase [2, 13]. This may explain why it was 'surprisingly' possible to produce a *DH* 20% soy protein hydrolysate using Alcalase (table 6.1) from full fat soybean meal, which was fully acceptable from an organoleptic point of view [14]. The oil was simultaneously recovered by centrifugation.

Reaction parameters like pH and temperature, by which the specificity of the proteases are changed, also play a very important role in managing bitterness. Combinations of enzymes can change the overall mode of degradation of a protein whereby bitterness is reduced. The application of exo-peptidases, which remove the hydrophobic side chains, is a generally recognised way of removing bitterness from high *DH* hydrolysates.

6.4.1 Other off-flavour problems related to bitterness

Animal raw materials considered useful for protein hydrolysis do not usually contain many *in situ* bitter compounds of any significance. Bitterness can appear from rancid traces of fat or from extended hydrolysis of proteins.

Oil seeds contain a variety of phenolic compounds, in particular bitter tasting phenolic acids [15]. Some volatile compounds are hydrophobic and bind to the protein phase—in particular to denatured protein. By enzymatic hydrolysis of such proteins, these off-flavours may be released. Some can be removed, but usually not all. Reduced acceptability of such hydrolysates can be the result. A solution to such a problem has been described for the industrial production of a soluble enzymatic hydrolysate of soy protein [16]. In this example, an isoelectric, soluble enzymatic hydrolysate of soy protein with no bitterness and a bland taste was produced economically from white soya flakes. The white flakes were washed at pH 4.5 with water in a four-step extraction process to produce a concentrate with a low level of 'beany' off-flavour. The concentrate was immediately hydrolysed with Alcalase™ to a specific degree of hydrolysis. Reducing the pH terminated the hydrolysis reaction and the supernatant was recovered by centrifugation at the isoelectric point. The hydrolysate was carbon treated, concentrated, and used for protein-fortified fruit juices as well as for extension of cured whole meat products.

Some proteins are natural inhibitors of proteases. Trypsin and chymotrypsin inhibitors of legume and cereal grains have thus been reviewed and studied [17]. Protease inhibitors for bacterial proteases, such as subtilisin, have been purified and characterised from legume seeds [18] and from egg albumen.

The probably best-known and most studied protease inhibitors are the trypsin inhibitors of soybeans. The nutritional significance of the anti-tryptic activity of soybeans and soybean foods and feed materials has long been known. The way to inactivate these inhibitors has been an inherent part of most manufacturing processes based on soybean material [19].

In liquid formulations used in the food industry, proteases are stabilised against loss of catalytic activity by denaturation and against microbial growth by a combination of low water activity and reduced pH.

6.5 Protein hydrolysis for food processing

The hydrolysis of proteins with enzymes is an attractive way of giving better functional and nutritional properties to food proteins of vegetable origin or from by-products (e.g. scraps of meat from slaughterhouses). Some important properties of proteins, their application in foods, and enzymes used for modification of their properties are shown in table 6.4.

Industrial baking of biscuits and conversion of milk to cheese are examples of the use of proteases for production of the food itself. For the production of functional ingredients, the structure of protein is often modified when using enzymes. In this way, the solubility, emulsification and foaming properties may be improved.

Over the years, many different protein raw materials have been used with different objectives. Examples of extraction processes giving enhanced yields include the production of soya milk, recovery of scrap meat, cleaning of bones from slaughterhouses, recovery of gelatine, and production of meat extracts (for flavour) and yeast extracts. Furthermore, proteases facilitate the evaporation of

Table 6.4 Functional properties of proteins in foods, enzyme types and their applications

Property	Enzyme types for modification	Application
Allergenicity	Proteases	Baby food formula
Emulsification	Proteases	Meats, coffee whiteners, salad dressings
Hydration	Amylases, proteases	Doughs, meats
Viscosity	Carbohydrases or proteases	Beverages
Gelation	Transglutaminases, rennin	Sausages, gel desserts, cheese
Foaming	Protease	Toppings, meringues, angel food cakes
Textural properties	Transglutaminase, protease	Textured foods
Solubility	Protease	Beverages
Flavour enhancement	Protease, carbohydrases	Condiments, meat extracts, yeast extracts

fish/meat stickwater, the rendering of fat, meat tenderisation, and removal of the membrane from fish roe.

Functional food ingredients in the form of soluble protein hydrolysates are also being produced. The hydrolysates are used for nutritional purposes or for foaming and emulsification. Examples of such products are isoelectric soluble soya protein (ISSPH), egg white substitute from soya protein, emulsifiers from soya protein, soluble wheat gluten, foaming wheat gluten, blood cell hydrolysate, whey protein hydrolysates, casein hydrolysates, soluble meat proteins and gelatine hydrolysates.

Development of new enzyme applications should include the selection and optimisation of the parameters that can be varied according to the desire of obtaining the best processing result. Very often it is not possible to change parameters without affecting the quality of the final product. Figure 6.5 shows some practical aspects of enzyme applications for new processes that should be taken into consideration.

6.5.1 Inactivation of enzyme activity and downstream processing

The degree of hydrolysis of a substrate may be managed by the selection of the reaction parameters as explained earlier. In order to control the functional

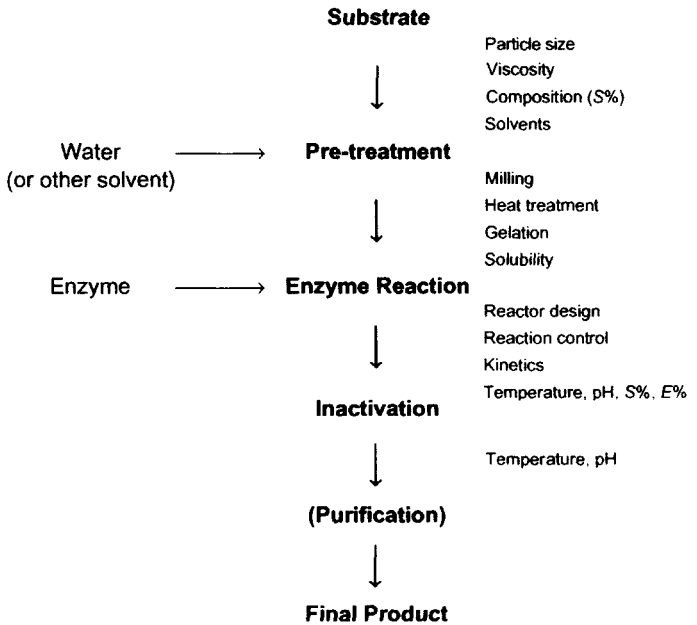


Figure 6.5 Optimisation and parameters of enzymatic processes.

properties of the hydrolysate, it may be important to stop the enzyme reaction at a closely defined *%DH* value.

In most enzymatically treated food products, active enzyme is not wanted in the final product to avoid post production changes that could appear in the product. Therefore, an efficient method for irreversible inactivation of the enzyme must be developed. Most frequently, an efficient heat treatment is used, but neutral and alkaline proteases are also effectively inactivated when pH is adjusted to pH 4 or below. All of the proteases can be irreversibly inactivated by heat treatment. Table 6.5 suggests treatment times for inactivation at a given pH and temperature [3]. However, inactivation by heat treatment is very much dependent on the substrate (substrate concentration, pH and so on). Thus, the documentation for efficient elimination of protease must be based on actual analysis for the detection of residual activity. The proteases Alcalase™, Neutrase™, Protamex™ and PTN™ are all inactive at pH 4 or below. In consequence, the reaction can be stopped instantaneously by addition of a convenient acid, for example, hydrochloric, phosphoric, malic, and lactic or acetic acid. Methods for detection of residual protease activity in protein hydrolysate are available [3].

An instantaneous increase in temperature is difficult to achieve under industrial conditions and hence, it can be difficult to control the *%DH*, as hydrolysis will continue during the inactivation step. To overcome this inactivation problem in cases where a definite (low) *%DH* is wanted, the enzyme reaction can be carried out at an elevated temperature, whereby the enzyme is inactivated during the hydrolysis stage. This possibility is for instance described in figure 6.14 for modification of wheat gluten.

Engineering aspects regarding the scaling up of inactivation have to be taken into account when the enzyme treatments are going to be carried out

Table 6.5 Suggested treatment times for inactivation at a given pH and temperature [3]

		Temperature		
Protease	pH	°C	°F	Time (min)
Alcalase	4	50	122	30
	8	85	185	10
Esperase	4	70	158	10
	6–8	90	194	10
Flavourzyme	6–8	90	194	10
Neutrase	4	50	122	30
	7	80	176	4
Protamex	4	50	122	30
	8	85	185	10
PTN	4	85	185	10
	7–8	85	185	10

at production scale. Examples of inactivation procedures are shown in the following. Purification of the products made enzymatically may in some cases be needed; for example, where the enzymatic method does not produce the product *in situ*. The selection of unit operations and the understanding of the performance of downstream processes are therefore also important parts of the application technology.

6.6 Functional protein hydrolysates

A limited hydrolysis of soy protein isolates was made with *Aspergillus oryzae* protease at varying ratios of enzyme to substrate (*E/S*) [20]. The degree of hydrolysis (*DH*) was not directly measured; but according to the data given in the publication, the maximum *DH* value obtained was 5%. It was shown that the whipping and emulsifying capacities were improved [20].

The first application of the pH-stat technique for a controlled production of enzymatically hydrolysed soy proteins was by use of Alcalase™ (table 6.1) at pH 8, and Neutrase™ (table 6.1) at pH 7 [21]. The isoelectric solubility, the foaming capacity and the emulsifying capacity were all increased. If the reactions were continued to higher *DH* values than 5%, the foaming capacity and the emulsifying capacity decreased again. Also a significant difference of the functional capacities was observed, dependent on the two different proteases applied. Alcalase™ was found to have a generally much better performance. This effect could be due to the fact that foams and oil emulsions are stabilised better by peptides having a higher content of non-terminal hydrophobic amino acids than by peptides having terminal hydrophobic amino acids. Figure 6.6 shows the whipping expansion versus *DH* from the early demonstration mentioned above.

In the further research work on functional ingredients based on enzymatic modification, improved products were developed when other soy raw materials were used, or when physical treatment like ultrafiltration was used during purification of the active components responsible for the specific effect. When a soy protein concentrate was hydrolysed to *DH* 3% and 5% with Alcalase™ a functional protein hydrolysate with a high foam expansion and remarkably good foam stability was obtained [22]. On the other hand, the emulsifying capacity was considerably lower than when modifying soy isolate the same way.

When using a native soy protein isolate produced by ultrafiltration of an aqueous extract of defatted soy bean meal [23], the shape of the hydrolysis curve was remarkably different compared to the one obtained for acid precipitated protein, as can be seen in figure 6.10 [24]. The hydrolysis curves are shown for acid precipitated soy protein isolate (denatured and nearly insoluble in water) and ultrafiltered soy protein isolate (native and soluble in water). It appears from figure 6.7 that the ultrafiltered protein isolate is hydrolysed more slowly than the

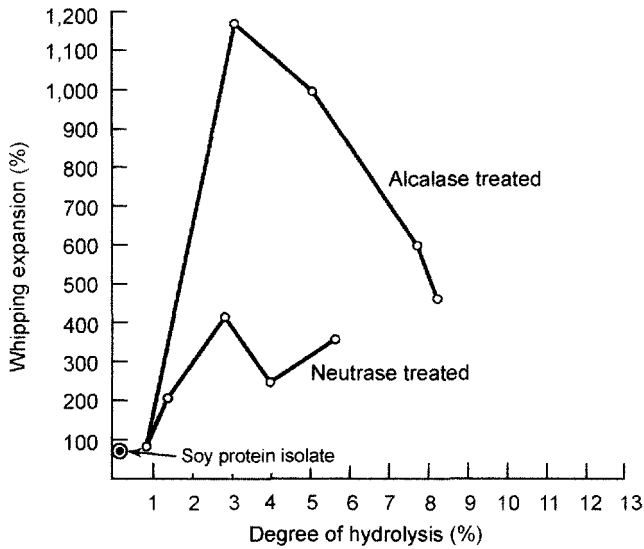


Figure 6.6 Whipping expansion versus *DH* [21].

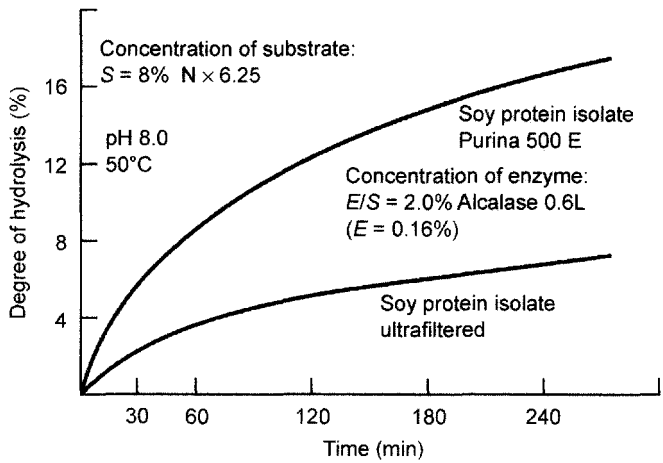


Figure 6.7 Hydrolysis curves for acid precipitated soy protein isolate and ultrafiltered soy protein isolate [24]. *S*=%(w/v) substrate protein; *E*=%(w/v) enzyme present; *E/S*=ratio of enzyme amount:substrate amount.

acid precipitated protein. This is due to the compact molecular structure of the ultrafiltered protein, which is still in its native stage. It has long been known that the degree of denaturation of a protein substrate has a profound influence on the kinetics of proteolysis [25].

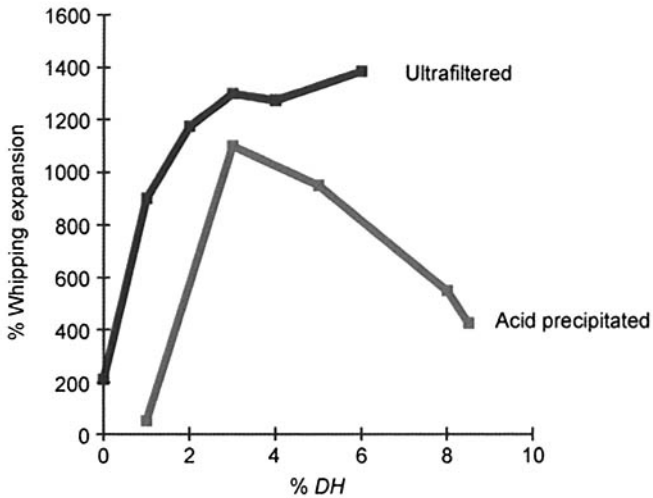


Figure 6.8 Whipping expansion as a function of the degree of hydrolysis (*DH*) for soy protein hydrolysates [24].

Using this soy protein isolate, a functional protein hydrolysate with improved foam expansion and foam stability was obtained compared to those from the acid precipitated protein (figure 6.8). If the small peptides in this hydrolysate were removed by ultrafiltration, the foam stability was improved even further. The use of ultrafiltration before and/or after the enzymatic hydrolysis was investigated in pilot plant scale in a number of process combinations [24, 26]. The high molecular weight hydrolysates had excellent whipping properties and furthermore, they were able to heat-coagulate so that they could substitute egg white completely on a protein basis in a meringue batter.

6.7 Low allergenic peptides for baby food formulae

One of the best established enzyme applications within food proteins is in baby food for reducing the risk of allergenicity in human breast milk substitutes. This application dates back more than 50 years [27]. Some babies react to sequences of amino acids by producing antibodies. This results in allergic reactions on repeated exposure of the same protein. The reaction is caused by specific sequences of amino acids, the epitopes. These sequences differ in size as well as composition of the amino acids. One of the well-known proteins for this application is whey protein, due to its high nutritional value. Several epitopes have been identified in *beta*-lactoglobulin [28]. These epitopes are 20, 11, and 21 amino acids, respectively, in chain length. Results from hydrolysing whey protein with different proteases shows that not only the *DH* but also the type of

Table 6.6 Reduction of antigenicity response in whey protein hydrolysates produced by different proteases

Enzyme	Specificity	*DH* (%)	ARI=RF/*DH*
Trypsin	Lys, arg	13.8	362
Bac. liceniformis	Glu,asp	3.5	143
Chymotrypsin	Phe, tyr, trp	4.7	19
Papain	Arg, lys, phe, gly	11.7	14
Subtilisin A	Broad	8.2	12
Alcalase	Broad	19.1	12
Fusarium protease	Glu, asp	9.1	2

DH = degree of hydrolysis; RF = (allergenicity) reduction factor; ARI = allergenicity reduction index.

protease influences how much the allergenicity is reduced [1]. Measuring the immunological response by the ELISA method follows the allergenicity. In an experiment with different enzymes, hydrolysate from whey protein concentrate was produced. Table 6.6 shows the results from analysing the samples. In order to express the efficiency of the proteases in reduction of the ELISA response, an Antigenicity Reduction Index (ARI)=Antigenicity Reduction Factor (ARF) is defined, measured in ELISA analysis divided by *DH*. The difference in ARI value indicated in the table clearly shows how the efficiency differs from enzyme to enzyme.

In a study of casein antigenicity as a function of the *DH*, a reduction in the immunological active protein from 10^6 µg/g of protein to $10^{2.7}$ µg/g of protein by hydrolysing to *DH*=55% was shown [29]. This also shows that the immunological response can never be completely eliminated by the hydrolysis process itself. There will be residual peptide structures that are able to cause reactions even after an extensive hydrolysis. The risk of immunological reactions can, of course, be further reduced by different processing, such as membrane filtration. In another study [30], a reduction in antigenicity of 10^6 by extensive hydrolysis was found. No peptides were larger than 1200 Daltons. It has been debated which peptide size is the maximum without causing any risk of allergenicity. No precise answer can be found, as peptides smaller than 1000 Daltons caused antibody production when injected into rabbits [31].

6.8 Meat extracts

Hydrolysed protein products with a strong meat flavour are used in soups, sauces and ready meals. Proteinaceous material recovered using proteases can be produced from coarse and fine scrap-bone residues from the mechanical fleshing of beef, pig, turkey or chicken bones. The flavour intensity depends on the content of free amino acids and peptides and their reaction products. Reactions that develop flavour include Maillard reactions between reducing sugars and

amino acids, thermal degradation caused by Maillard reactions, deamination, decarboxylation, or the degradation of cysteine and glutathione. The latter reaction can give rise to a large number of volatile compounds important to aroma and taste.

The first step in the production of protein hydrolysates from meat involves efficient solubilisation of the product by endo-proteases. Figure 6.9 shows a process layout for production of meat extract.

The application of exo-peptidases is a generally recognised way of removing any bitterness of high-*DH* hydrolysates. Protein hydrolysates based on a relatively low degree of hydrolysis possess functional properties that are ideal for use as marinades for meat products such as ham or bacon. These functional extracts can be used to improve meat products with respect to flavour, cooking loss and ease of slicing. Other important applications for meat extracts are as flavour enhancers in soups, sauces, snack food and 'pot noodles' (a type of instant meal).

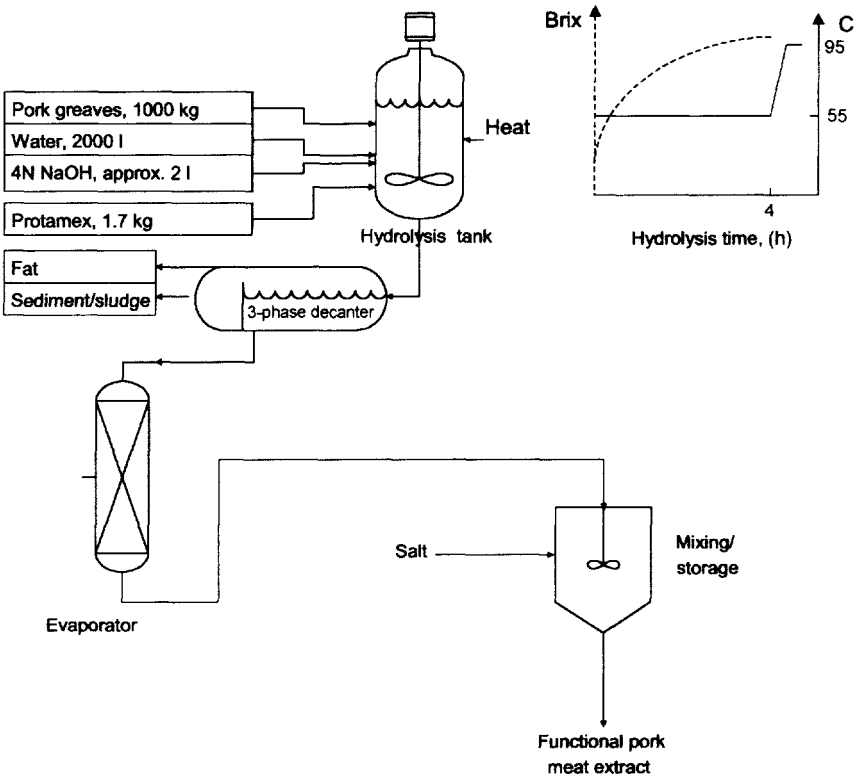


Figure 6.9 Production of functional pork meat extract.

6.9 Bone cleaning

As an alternative to rendering, an enzymatic process can be used to upgrade fresh bones to valuable products; that is, cleaned bone material suitable for gelatine production and meat protein hydrolysate for the food industry. A two-step enzyme process can be performed where functional meat extracts are produced by the first enzymatic extraction and cleaned bones by the second [32].

The fresh bone material (beef or pork) is crushed and mixed with hot water. The enzymes Neutrase™, Alcalase™, Esperase™, and Flavourzyme™ (table 6.1) (singly or in combination) are added and the slurry is agitated and extracted batch-wise or continuously at 60–75°C and at neutral pH (6.5–7). Poultry material may also be used; however, the bone material is not generally used for gelatine.

Bone cleaning trials have been carried out at the Novozyme A/S pilot plant for process development [33]. Using a bone crusher, 250 kg of beef bones were milled to a particle size of approximately 20 mm. This material was poured into 250 kg of 80°C water and the temperature was adjusted to 65–70°C. After that, the enzymatic hydrolysis reaction was carried out under powerful stirring. Samples as a function of time were centrifuged for 3 min before determination of °Brix (dry matter), osmolality and pH. The reaction mixture was sieved and the liquid phase was pasteurised, defatted by centrifugation, concentrated and dried. The bone material was washed and dried. During the enzymatic hydrolysis, data shown in figure 6.14 were recorded using Esperase™ (table 6.1).

Within the 20 minutes of reaction, the °Brix value and the osmolality were still increasing. This indicates that the enzyme was not inactivated at this temperature (70°C). The value mOsm/°Brix is an expression of the molecular size of the soluble phase. In the beginning when the content of soluble protein is low, mOsm/°Brix is a high value because of the high content of low molecular weight compounds. Later on, this value stabilises (figure 6.10). This indicates that when the protein is solubilised from the bone surface, no further degradation occurs. The 'one-by-one' reaction system seems to be confirmed.

Working at a sufficiently high temperature, inactivation of the enzyme may be achieved simultaneously. The reaction should then be carried out at a temperature range, which is adapted to the enzyme product in use. Centrifuging at 90°C during defatting of the hydrolysate pasteurises the product. Such protein solutions are used as ingredients in the meat industry, or possibly as a clear soluble meat extract for soups and seasonings. The cleaned bones make an excellent raw material for production of gelatine [34].

6.9.1 Hydrolysis of meat protein in relation to bone cleaning

Based on the osmolality difference, ΔC in equation 6.4, DH can be calculated and compared with those from hydrolysis of lean beef meat. A number

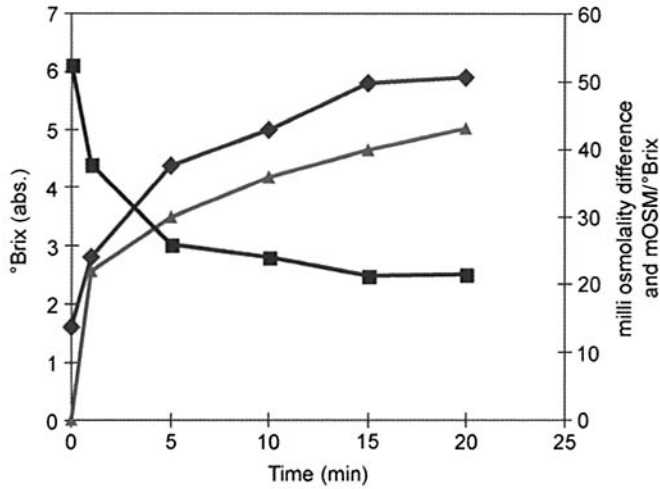


Figure 6.10 Data from a pilot plant bone cleaning trial [33]. —◆— °Brix —▲— mOSM(diff.) —■— mOSM/°Brix. Enzyme: Esperase 7.5 FG. Dosage: 2.0 kg/ton of bone °Brix = dry matter; mOSM = osmolality. T = 70°C, pH = 7.1–6.7.

of hydrolyses using Esperase™ were carried out on raw and cooked minced beef meat, respectively. Both pH-stat trials and hydrolysis followed by osmometer measuring were undertaken at temperatures in the high region. pH was adjusted to 7.5 so no appreciable hydrolysis of triglycerides occurred. Figures 6.11 and 6.12 show hydrolysis curves and corresponding protein solubility measured in osmometer trials.

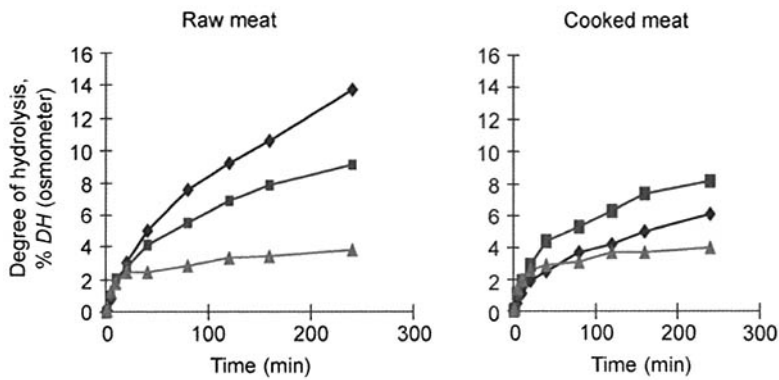


Figure 6.11 Hydrolysis curves (osmometer) at 8% protein of beef [33]. —◆— = 55 C: —■— = 65 C: —▲— = 75°C. Enzyme: Esperase 7.5 FG. Dosage: 0.5% of the protein.

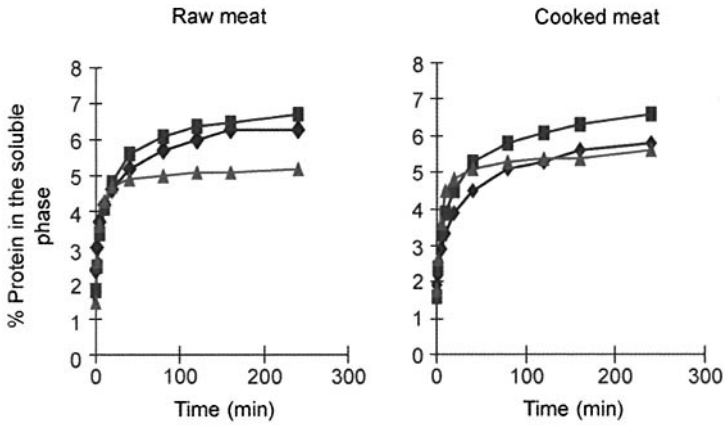


Figure 6.12 Solubilisation of protein by hydrolysis of beef meat [33]. —◆— = 55°C; —■— = 65°C; —▲— = 75°C. Enzyme: Esperase 7.5 FG. Dosage: 0.5% of the protein.

Table 6.7 Bone or meat treated by Esperase at 65–70°C [33]

	Bone			Raw meat		
Time (min)	*% DH*	pH	% Soluble protein	*%DH*	pH	% Soluble protein
0	0	7.1	1.2	0	7.5	1.8
1	5.7	6.8	2.0	0.1	7.3	2.5
5	7.7	6.8	3.2	1.0	7.0	3.4
10	9.3	6.7	3.7	2.1	6.9	4.1
15	10.3	6.7	4.2	2.5	6.8	4.5
20	11.1	6.7	4.3	2.8	6.7	4.8

% DH = degree of hydrolysis as % of unhydrolysed protein.

For both the raw meat and the cooked meat, the optimal performance was at 65°C (figures 6.11 and 6.12). This was also seen for pH-stat trials. In table 6.7, the hydrolysis data are compared for the bone cleaning trial (figure 6.10), and for hydrolysis of raw meat. Even the enzyme dosage based on protein was the double for the bone trial as compared to the meat trial. The excellent protein solubilising properties of Esperase™ are clearly seen in both cases. However, it was surprising that a lower *%DH* secured a higher solubility of the raw meat than for the meat on bones.

6.10 Enzymatic tenderisation of meat

The use of the plant proteases papain, ficin, and bromelain for enzymatic tenderisation of meat has been known for many years. These enzymes have

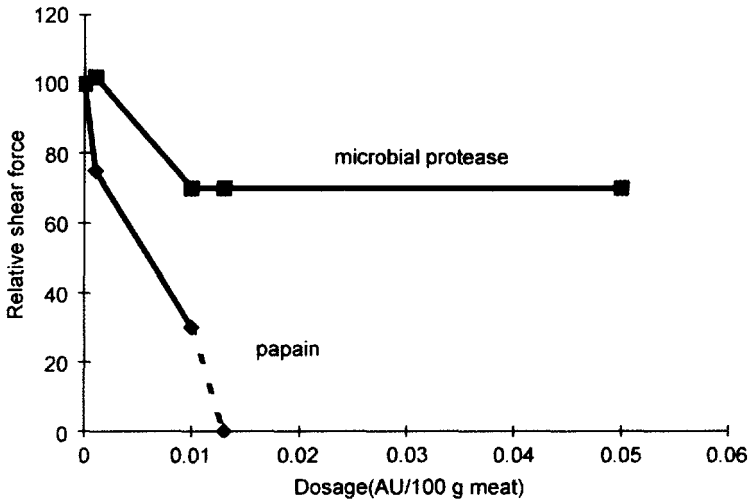


Figure 6.13 Measurement of tenderness (relative shear force) in beef meat treated with protease. AU=Anson unit of protease activity.

relatively good potency on muscle tissue components like collagen and elastin. Microbial proteases have also shown the ability to tenderise meat. In figure 6.13, the results from experiments with an acidic microbial protease are shown.

In the experiments reported above in figure 6.13, the enzymes were applied by injection. The meat was cooked at 70°C for 30 min. Shear force was measured after cooling. The data clearly shows the tendency of 'over-tenderising' the meat when using papain. This risk is not present when using the microbial enzymes.

The enzymes are applied to the meat by sprinkling the enzyme powder onto the thin slice of meat, by dipping the meat in an enzyme solution, or by spraying an enzyme solution into the meat cut by means of an injection system. Whatever method is used, the difficulty in using external enzymes for tenderising meat seems to be obtaining an even distribution of the enzyme throughout the tissues.

6.11 Modification of wheat gluten

The protease is conveniently inactivated simultaneously during the hydrolysis if the reaction is carried out at a temperature above the denaturation temperature of the enzyme [32]. Examples of hydrolysis curves obtained using a pH-stat for the monitoring of the progress of the reaction are shown in figure 6.14.

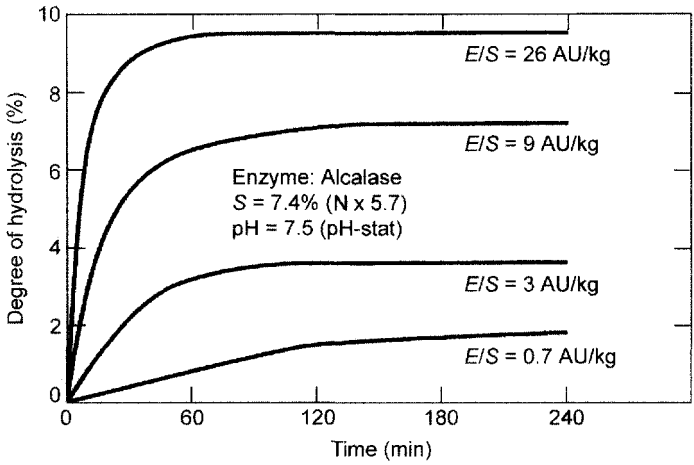


Figure 6.14 Enzymatic hydrolysis of wheat gluten at 72.5°C. One Anson Unit (AU) is the amount of enzyme, which under standard conditions digests haemoglobin at an initial rate, liberating per minute an amount of TCA soluble products, which gives the same colour with phenol reagent as one milli-equivalent of tyrosine [35].

6.12 Use of membranes in protein hydrolysis processes

Membrane processes are used within many areas of industrial enzymatic hydrolysis of proteins. Membrane processes can be applied for pre-treatment of proteins, for the reaction step and for the purification step. Some examples are discussed below.

6.12.1 Functional protein hydrolysates

Examples of the application of ultrafiltration processes in various process combinations for production of highly functional protein hydrolysates of soy protein were mentioned earlier. In principle, the technique of removing small peptides from total hydrolysates may be used on many substrates other than soy. Thereby, excellent foaming and coagulating protein products may be isolated (purified). Normally, the first process step after inactivation is centrifugation, to remove insoluble material that may reduce the whipping expansion and the foam stability. In order to perform the purification of the supernatant as effectively as possible, the ultrafiltration step can be carried out in three steps. In the first step, the concentrate obtains a protein content of about 10%. Hereafter, a diafiltration process is performed by addition of water at a flow similar to the permeate flux. During this step, small low-functional peptides are washed out. The final ultrafiltration can bring the protein content up to approximately 20%. This concentrate can be then spray-dried. The efficiency of these processes and the

overall yields are dependent on the actual ultrafiltration equipment used, the membrane cut-off value, and the character of the hydrolysate (enzyme used, *%DH*, pH, protein type) [24, 26].

6.12.2 Low molecular weight hydrolysates

These are produced from raw materials that are hydrolysed to a high degree. One example of such products is the mild-tasting decolorised blood cell hydrolysate developed from hygienically collected red cell fraction of slaughterhouse blood [10]. Another example is the isoelectric soluble soy protein hydrolysate called ISSPH [16]. Flow charts for these hydrolysates are shown in figure 6.15 (blood cell hydrolysate) and figure 6.16 (ISSPH).

These two examples demonstrate how the membrane processes can be used for different purposes. In the blood cell hydrolysis process, the pH = 4.2 insoluble haem product may be removed by centrifugation or by ultrafiltration. Comparisons of the centrifugation process and the ultrafiltration process showed that the ultrafiltration process was most economical when new plants were

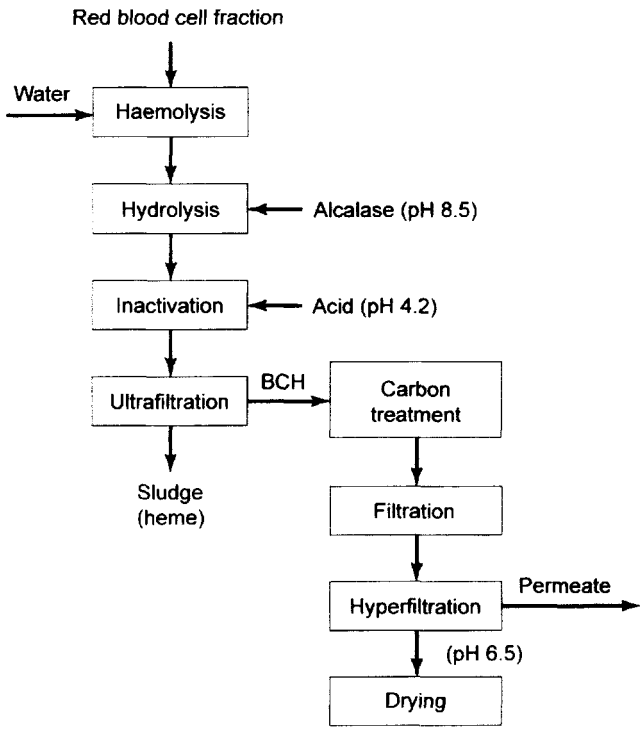


Figure 6.15 Flow chart for production of blood cell hydrolysate.

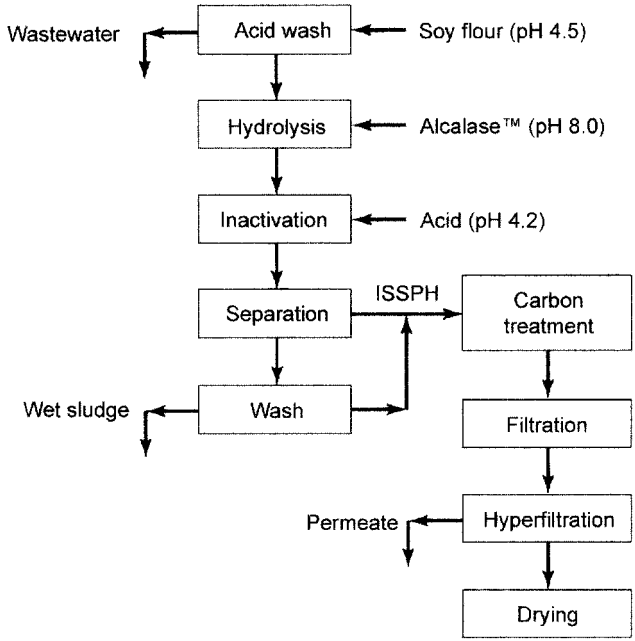


Figure 6.16 Production of isoelectric soluble soy protein hydrolysate (ISSPH) [16].

installed [10, 36]. Ultrafiltration equipment from various suppliers has been tested with technical success.

During production of the soy protein hydrolysate (figure 6.16), the clear filtrate after carbon treatment is concentrated by hyperfiltration as an alternative to evaporation. A falling film evaporator can concentrate the hydrolysate to above 50% dry matter. Hyperfiltration can only be used efficiently up to about 25% dry matter, but allows for a simultaneous removal of salt if the proper membranes are used for concentration. In consequence, about 75% of the salt in ISSPH was removed by direct concentration, and 93% if a diafiltration was included in a pilot plant trial on a selected cellulose acetate membrane [15].

6.12.3 The membrane reactor

This is an ultrafiltration system in which a high concentration of hydrolytic enzyme is confined. High molecular weight substrate is fed continuously to the reactor and the low molecular weight products are removed simultaneously as permeate through the membrane. Ideally, a steady state is reached at which the degradation of substrate is carried out indefinitely with high efficiency and negligible loss of enzyme. A considerable number of publications on the use of membrane reactors for enzymatic hydrolysis of proteins appeared 10–15

years ago. However, use of the system has not been widespread. One of the reasons may be the fact that the economical advantage of the membrane reactor depends critically on the need for purging of the reactor. A small part of the substrate is always non-degradable. For that reason, inert material will rapidly build up in the reactor causing mechanical problems. A considerable purge is therefore necessary if the concentration of this material should be kept at a reasonably low level. This has a drastic, negative influence on the yield of the product. Also the enzyme loss during purging will be considerable. Kinetic investigations of protein hydrolysis in the membrane reactor have been presented and a mathematical model established [24]. Based on these calculations, it has been found that the concomitant loss of enzyme in certain cases may lead to an overall enzyme consumption close to that of the batch hydrolysis process.

6.13 Flavour enhancers

6.13.1 Production of flavour enhancers

In their natural state, proteins do not contribute chemically to the formation of flavour in foods. However, the products of protein hydrolysis (peptides and amino acids) do have a flavour. They are also much more reactive so they react with other components in food such as sugars and fats to create specific flavours.

Many savoury products from different sources are available on the market. Hydrolysed protein, mainly produced using hydrochloric acid, is a common ingredient for products such as soups, stock cubes, and savoury sauces. Hydrolysed protein is one of the largest contributors to flavouring produced from protein. The hydrolysis with hydrochloric acid usually takes place in the presence of triglyceride fat material. It is well known that chloropropanols and dichloropropanols can be formed during acid hydrolysis if the process is not controlled extremely well. Concern for the safety of the products of hydrochloric acid hydrolysis of proteins has led to limits of 10 ppb in the hydrolysed product. Considerations such as these have increased the desire for an alternative process for hydrolysed protein. The obvious option is use of proteases to perform the hydrolysis process.

Glutamic acid in the form of monosodium glutamate (MSG) is by far the most widely used flavour enhancer of proteinogenic origin. Glutamates are known as the fifth basic taste sensation, in addition to sweet, sour, salty, and bitter. This fifth basic taste is called *umami* by the Japanese, and savoury in English. MSG is used at concentrations of 0.2–0.8% in a variety of foods, such as soups, broths, sauces, gravies, flavouring and spice blends, canned and frozen meats, poultry, vegetables and finished dishes. As an alternative way of producing glutamic acid, glutaminases are of interest as a way of producing *in situ* flavour enhancers of the MSG type in protein hydrolysates. Such a hydrolysate allows people to reduce their intake of sodium [34, 37, 38].

Through their reactions at different taste sites on the tongue, peptides may result in flavours that are bitter, sweet, salty or *umami*. Sourness and astringency have also been attributed to peptides isolated from protein hydrolysates or by synthesis. Many studies have been made of the effects of flavour in dairy products, meat and fish products, and yeast extracts. Flavour products can be produced directly using proteases on their own or in combination with a fermentation process, chemical treatment, or heat treatment, for example, Maillard reactions. Soy and wheat hydrolysates are used for flavouring and flavour enhancement of soups and seasonings, meat hydrolysates for addition of flavour to meat products, soups, bouillon, and sauces, and milk/cheese hydrolysates for production of cheese flavours [38].

6.13.2 Enzymatically hydrolysed vegetable proteins (e-HVP)

Enzymatic hydrolysis usually leads to products that are light in colour and have a much less pronounced meaty or savoury flavour than yeast extracts for example [39]. Meat flavour and similar condiments are produced from many sources and their production often involves a natural microbial fermentation. The oriental products that are based on soy protein can be included among these. Wheat and maize gluten are also useful because of their hydrolysis to flavour-intense peptides.

The content of free glutamic acid is important for the *umami* flavour. A way of exhibiting flavour enhancement characteristics using mild acid hydrolysis of the protein, followed by enzymatic hydrolysis, has been described [40]. The preferred protein for producing savoury flavours is wheat gluten. A mild acid hydrolysis is carried out for 1 h to obtain a deamidation, at about 95°C and a hydrogen ion concentration of about 1.0 M. The enzymatic hydrolysis was preferably carried out by use of an *Aspergillus oryzae* protease having both endo- and exo-proteases. The overall degree of hydrolysis was 50–70%. Conditions during the acid treatment were mild enough to avoid the formation of substantial amounts of monochloropropanols.

Flavourzyme™ can be used in combination with Alcalase™ to obtain a degree of hydrolysis of 60–70% for hydrolysis of soy protein isolate [38]. The same degree of hydrolysis could thus be obtained without the acid treatment.

An enzymatically hydrolysed soy protein, a so-called 'e-HVP', was developed by Miwon C. Ltd. [41]. The e-HVP gave no dosage limitations in formulation as a flavour enhancer. Another e-HVP is wheat gluten hydrolysate, used for addition to moromi during fermentation of soy sauce.

6.13.3 Hydrolysed animal proteins (e-HAP)

Products with a high meat extract flavour may be used in soup, sauces and prepared meals. Enzymes can be used to recover proteinaceous material from

coarse and fine scrap-bone residues from mechanical fleshing of beef, pig, and turkey or chicken bones. The flavour intensity is dependent on the content of free amino acids and peptides and related reaction products. The reactions that develop flavour are series of reactions such as those between reducing sugars and amino acids (Maillard), thermal degradations due to the Maillard second stage, deamination, decarboxylation, or degradation of cysteine and glutathione. This last reaction can give rise to a large number of volatile compounds of importance in aroma and taste [42].

The first step in the production of protein hydrolysates from meat is an efficient solubilisation of the product. The bacterial serine proteases and metalloproteases are very efficient agents for this purpose. However, the hydrolysates are usually found to be bitter at degrees of hydrolysis above 10%, which is needed for sufficient solubilisation [43]. A method has been patented that consists of a series of steps, using raw meat, which is hydrolysed with a specified combination of neutral and alkaline proteases [44]. This meat hydrolysate exhibits excellent organoleptic properties and can be used as a meat flavoured additive for soup concentrate. A degree of hydrolysis above 20% showed no bitterness when such specified enzyme combinations were used. The reason for this effect may be the preferential specificity being favourable when metalloprotease and serine protease are used simultaneously.

A method for further enhancement of the flavour of a natural beef juice concentrate is a hydrolysis using endo-proteases (metalloprotease, serine protease or combinations thereof) followed by treatment with an exo-protease like Flavourzyme^{TM}, or by incubation with a culture of a food-grade microorganism, capable of producing exo-proteases, such as aminopeptidase [45].

6.14 Yeast extracts

The demand for yeast extracts has increased as a result of the doubts concerning the safety of acid hydrolysed proteins. Yeast extract is a source of amino acids, peptides, sugars, nucleotides, lipids, and B vitamins, all of which can act as meat flavour precursors. Aroma components of yeast extracts with different aroma properties have been analysed [44]. Several hundreds of components were identified. Most of the aroma components arose from sugar and/or amino acid interactions, or thiamine degradation. Variations between conditions used to manufacture the yeast extracts reflected the aroma.

Mixtures of a primary yeast extract and protein hydrolysate are used both as savoury flavours and as flavour enhancers. In order to avoid the use of strong acids like hydrochloric acid for degradation of proteins and carbohydrates, most yeast extracts are produced by the following steps: plasmolysis, autolysis, pasteurisation, clarification, and concentration [45]. The autolysis step in particular is a protein hydrolysis reaction [38].

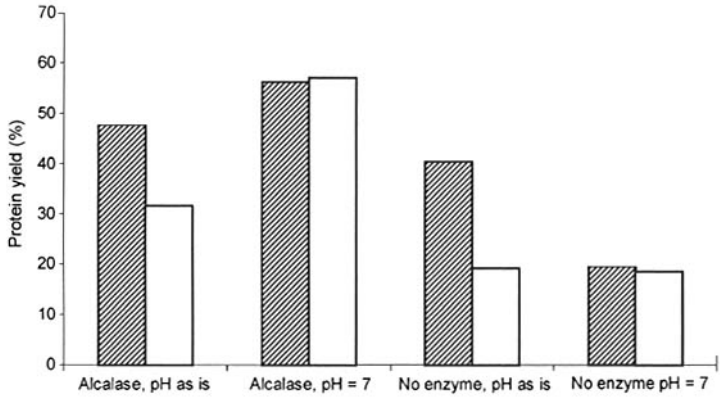


Figure 6.17 Protein yield in the yeast extract process. Alcalase 2.4 l at a dosage of 0.2% (w/w on dry matter) compared to no enzyme addition. ▨ = 50°C; □ = 60°C.

It has been demonstrated that addition of proteolytic enzymes during incubation of yeast cells with lytic enzymes acting on the cell walls of the yeasts caused the concurrent hydrolysis of the yeast proteins [46]. The autolysis and the hydrolysis processes may be carried out simultaneously. The effect of an added enzyme system during the autolysis process is seen in figure 6.17. The positive effect on protein yield is clear. In particular increase of the initial pH to 7 has a major impact on the protein yield when the Alcalase™ protease is added.

6.15 Fish processes

A newly developed process using backbones and trimmings from fish fillet production has been used for improving the end product quality and economy of fillet production [50]. The mixture of off-cuts is used to produce salmon marinade. The raw material is heated to 55°C and held there for 45 min during hydrolysis. The enzyme used is Protamex™ at 1 kg per 1000 kg of raw material. After the hydrolysis, heating to 90°C and holding for 15 min inactivates the enzymes. The mixture then drained through a sieve and separated in a 3-phase decanter centrifuge. A crystal clear product is obtained by filtration. Concentration is by evaporation and addition of salt. The hydrolysate can be concentrated to 45% dry matter, after which 12% salt (12% of dry matter) is added for preservation. Alternatively, the protein solution can be used without concentration directly as a base for marinade production by addition of salt [50].

The use of proteases as processing aids for reduction of the viscosity of stickwater before and during evaporation in a fishmeal plant or in a meat rendering plant could reduce the fouling on heat surfaces. The capacity of the

evaporators could then be increased due to reduction of the overall heat transfer coefficients, and a higher degree of dry solids could be obtained. This process offers direct economical benefits [51, 52].

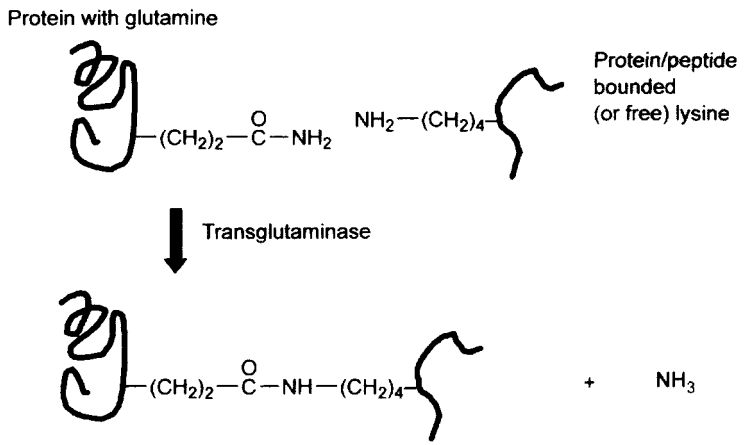
6.16 Protein cross-linking in food processing

In recent years, the cross-linking reaction has made its way into food applications. It is based upon the availability of the transglutaminase enzyme that catalyses the covalent bond between peptide-bond glutamine and an amino group, normally from lysine. The general reaction for the formation of the ε-(γ-glutamyl)lysine cross-link is shown in scheme 6.2.

The covalent bond gives rise to a gel network that significantly changes the rheological properties of the protein. Figure 6.18 shows the effect of a transglutaminase reaction measured by release of ammonia and increase in viscosity. This can, of course, be utilised in food products [53–55]. It is important to note that no negative effect of the released ammonia on flavour is described.

The transglutaminase reacts differently on different substrates. The more non-globular proteins/peptides are better substrates than native globular proteins. For instance, casein and gelatine are very good substrates compared to soy or whey proteins. With extensive cross-linking, gelatine forms gels that are insoluble at high temperatures [53].

The utilisation of transglutaminase in industrial applications depends on the availability of the enzyme. Transglutaminase is available everywhere in the nature, but in amounts so small that it is very difficult to detect. It was in the second half of the 1980s that microbial enzymes were isolated and used in food



Scheme 6.2 Formation of the ε-(γ-glutamyl)lysine cross-link by the transglutaminase reaction

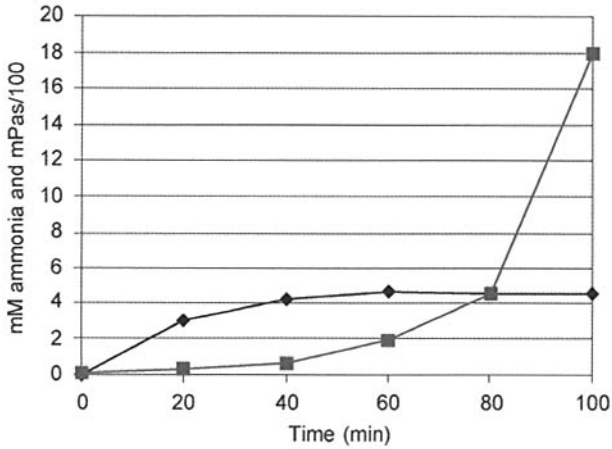


Figure 6.18 Monitoring transglutaminase reaction by release of ammonia. Viscosity increase in a caseinate solution (8% protein concentration) cross-linked by FXIII. ◆ = mM ammonia; ■ = mPas.

applications. Since then, the number of patent applications has been enormous. There is an alternative to the microbial enzyme available, which is isolated from bovine blood plasma. It is used as a plasma isolate including fibrin, which needs to be activated in the application by addition of the specific thrombin protease. In this concept, part of the blood coagulation pathway from the body is transferred to the food system and used for cross-linking of fibrin [56, 57]. Transglutaminase—activated by thrombin—is part of the cascade reaction that heals wounds in mammalians, and is here called Factor XIII.

The iso-peptide bond, which is formed by a cross-linking reaction, is found in food products. Of 127 food products analysed, 96 items contained between 0.2 μmol and 135 μmol. The highest levels were found in fish and meat products [58].

6.16.1 Applications of transglutaminase

From the literature it seems that most applications have been or will be developed in the fish, meat, and dairy industries.

6.16.1.1 Fish applications

The low temperature gelation of fish protein is reported to arise from the endogenous transglutaminase in the fish meat [59]. The importance of the ε-(γ-glutamyl)lysine cross-link for gel strength has been documented in the low-temperature setting of sardine meat [60]. The quality of fish meat differs, which can be ascribed partly to different contents of endogenous transglutaminase present in the fresh meat [61].

It has been found that addition of transglutaminase was most efficient when the raw material was of relatively low quality [62]. In other words, it will be possible to utilise raw materials for gelled fish products also in seasons of the year where the quality is normally too low.

6.16.1.2 Meat applications

One of the most useful recent technologies in the meat industry is binding pieces of meat together to form whole meat servings. This has been suggested both for Factor XIII [63, 64] and the microbial enzyme [53].

The applications have been extensively expanded by utilising cross-linking between meat proteins and other proteins such as soy, casein and gluten [65]. To provide the best possible binding, some substrate protein can be added together with the enzyme. For instance, casein and/or gelatine included in the recipe will improve the binding.

6.16.1.3 Dairy applications

Although the different caseins react differently with transglutaminase [66], it has been shown to be a very good substrate [67]. Micellar κ-casein is cross-linked [66] but the same researchers later showed that whey proteins cross-linked incompletely [68].

Options for making different dairy products have demonstrated [69] that gelled products can be made from casein as well as skim milk powder, and that protein solutions as well as caseinate-stabilised emulsions gel very efficiently by the transglutaminase reaction.

It has been found that transglutaminase assists in gelling yoghurt at a lower dry matter content in the yoghurt compared to the amount normally used [70].

The transglutaminase-catalysed cross-linking reaction has great potential within the food industry [71]. One major issue is still the availability of the enzyme at a sufficiently low cost.

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7 Enzymes in fruit and vegetable juice extraction

Reinhold Urlaub

7.1 Introduction

The manufacture of fruit and vegetable juices is an important economic operation that provides the consumer with valuable fruit and vegetable constituents. Basically it involves extracting the liquid portion of fruit—the juice—and preserving it by pasteurisation or concentration, either in clear (filtered) or cloudy form.

The theoretical foundations for the use of pectolytic enzymes in fruit processing were laid by Kertèsz [1] and Mehlitz [2] around 1930. Mehlitz described the increased ease of filtering of various juices following enzymatic treatment [2]. Shortly afterwards, the first pectinase products were developed, including Pectinol K, launched on the market in 1938 by Röhm & Haas, and used mainly to manufacture clear apple juice. In the early 1950s, enzyme–gelatin clarification found acceptance in fruit juice technology, making it possible to obtain juices of higher quality and stability. To facilitate enzymatic treatment of the mash at high temperatures Koch [3] and Krebs [4] developed the 'hot fermentation' method for berry fruit, enabling the production of high-quality berry juices. Pectinases are able to act within the pH range of the fruit even up to 55°C. This was put to use to improve the press performance of crushed berry fruits, thus providing a higher yield in both colour and extract. Modified production methods in the 1960s led to the introduction of fungal *alpha*-amylase for pome fruit, to avoid the post-clouding of apple juice concentrate due to starch. These studies by Krebs [5] were pursued by Grampp [6], leading to the so-called hot clarification of apple juice, which was linked with the utilisation of glucoamylase for starch degradation.

Enzymatic mash treatment of pome fruit was also performed more frequently in the early 1980s, in order to obtain higher juice yields from the stored fruit that was increasingly being processed. The new maceration and extraction processes made it necessary to employ arabanases during depectinisation in order to manufacture stable apple and pear juice concentrates [7].

Enzymatic maceration then led to the development of enzymatic liquefaction processes using cellulases and hemicellulases. Enzymatic 'total liquefaction' was marked by the work of Pilnik and Voragen [8, 9].

At the same time, cross-flow membrane systems, in particular ultrafiltration, were introduced to replace conventional fining methods in the fruit juice industry [10]. This in turn led to the utilisation of enzymes to help solve problems with cleaning these membranes.

The historical development of fruit processing demonstrates its close connection with the enzymes industry. The enzymes either had to meet the rising demands of industry, or newly developed products prompted modifications of fruit juice technology. Many processes were modified, improved, optimised and rationalised by means of enzymes, the focal point always being the quality of the end products.

Today, all types of fruit juices have become commodities. Consumers have grown accustomed to the manifold possibilities of buying orange, apple, mango and other juices at relatively low prices, independently of the season or geographic location. Consumers react sensitively and demand constant quality with regard to appearance, nutritional value, taste and stability of the product; hence the need for optimised production methods.

Enzymes are an integral component of modern fruit juice manufacture and are highly suitable for optimising processes. With their characteristic properties, they meet the criteria for sustainable protection of the environment. One can therefore safely presume that they will find an increasing number of applications.

7.2 The legal situation

Enzymes are employed in very small quantities during fruit processing and are completely inactivated during the process. Generally speaking, the finished foodstuff only contains a technically unavoidable and inactive enzymatic residue, in proportions that are inoffensive from the point of view of health, odour and taste.

In EU Member States, enzymes are generally used and considered as processing aids, since most are used during food processing. In most Member States, there is no general statutory notification or approval system for enzymes used as processing aids. Instead, the use of processing aids is controlled by general regulations requiring the safety of all food ingredients. Only France and Denmark have in place a statutory procedure for notification of the use of enzyme products which is based on specific legislation for food enzymes.

The enzymes employed for food processing are completely inoffensive. Most of the microorganisms that serve to produce enzymes have been in use for decades and are considered as absolutely safe. New production strains are subjected to extensive toxicological tests to ensure their safety. International organisations like the JECFA or the FAO/WHO have issued generally accepted guidelines for the toxicological assessment and purity criteria of enzymes.

Current legal regulations on fruit and vegetable processing specify which enzymes may be used for a defined application. According to the EU Directive, only pectolytic, proteolytic and amylolytic enzymes may be used to manufacture fruit juices, fruit juice concentrates and dried fruit products. The Directive further specifies that cellulolytic enzymes may additionally be used for vegetable juices and beverages based on vegetable juice. Although, strictly speaking, these legal regulations only allow the use of the pure enzymes pectinase, amylase and protease mentioned above, the products employed for decades by the fruit juice industry contain further secondary or foreign activities. That is why various international organisations use the word 'enzyme preparation' in manufacturing and treatment regulations, and do not refer to one specific enzyme.

In 1989, the Joint FAO/WHO Expert Committee on Food Additives discussed 'technologically suitable enzyme preparations' (Specifications for Identity of Certain Food Additives, Joint FAO/WHO Expert Committee on Food Additives 35th Session, Rome, 29 May to 7 June 1989). The Codex Alimentarius Commission (FAO and WHO committee based in Rome) also spoke only of 'enzyme preparations' in the last version of their fruit-juice standards on 28 June to 3 July 1999, point 4.2.: permissible processing aids. The OIV (Office International de la Vigne et du vin) has adopted the same position as the FAO/WHO experts.

EC Directive 90/219/EC amended by Directive 98/81/EC applies to the production of enzymes derived from genetically modified organisms (GMO), and has already been implemented into national law in some countries, such as Germany and Finland. The Directive regulates the contained use of the genetically modified production microorganism but not the enzyme itself. Depending on the kind of modification of the used production strain, the Directive classifies them as GMO or non-GMO. This means that homologous transformations do not result in a GMO strain.

7.3 Definition and characteristics

By definition, enzymes are biocatalysts. As is typical for catalysts, they speed up biochemical reactions without being consumed in the process. The accelerated reaction is due to the reduction of the energy barrier between the initial substance and the product. This conversion takes place under very mild reaction conditions, safeguarding the nutritional value and palatability of the foods concerned. Enzymes are high molecular weight proteins and thus display the characteristic behaviour of proteins with regard to temperature and pH sensitivity. All enzymes show their own characteristic pH and temperature optimum (figure 7.1).

That is particularly important for the targeted use of enzymes, since each enzyme can only unfold its desired activity under the right ambient conditions. Unfavourable pH and temperature conditions may reduce the effectiveness of the enzyme or even damage its structure in such a way that it becomes

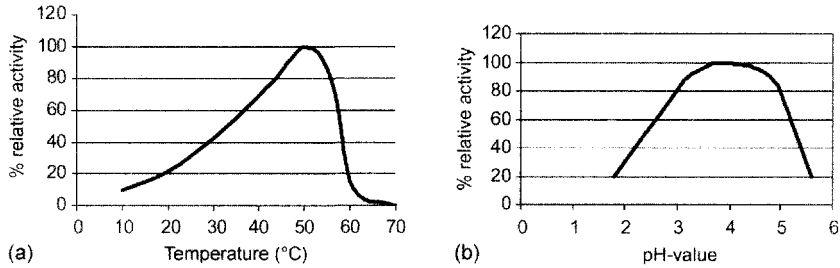

Figure 7.1 Pectinase activity as a function of (a) temperature and (b) pH.

inactive. This characteristic can be put to good use in the food industry. After targeted application, the enzyme is usually inactivated by subsequent work steps (cooking, baking or pasteurisation) and remains in the end product as a denatured, inactive protein. Enzymes react on specific substrates and display specific activity making it very easy to control their effect and eliminate secondary reactions. Among the many constituents of a given fruit, a particular type of enzyme recognises only one substance as a substrate and only catalyses one specific type of reaction. For a wider discussion of these aspects of enzyme use, the reader should consult chapter 1 of this volume.

7.4 Pectins

Fruits contain a high proportion of pectic substances that have the greatest influence on processing of all polymeric constituents. They belong to the group of acid polysaccharides which are found in the middle lamella of the primary cell wall of plants.

The texture of fruit and vegetables is determined by the quantity and properties of pectic substances. In unripe fruit, it is mainly present in the form of insoluble protopectin which is converted to soluble pectin during ripening [11]. This conversion is brought about by the pectinases within the fruit and makes the fruit become soft. The same breakdown mechanism can be effected by a microbial polygalacturonase with macerating properties, also termed protopectinase. This type of hydrolysis is also called maceration.

Chemically speaking, pectins are not a homogenous group of substances (figure 7.2). On the contrary, they are very heterogenous mixtures of polysaccharides with different molecular weights and degrees of esterification. A relatively large proportion, some 60–90%, consists of the so-called smooth-region pectins. Their main components are non-esterified galacturonic acid units or such units esterified with methanol. These are smooth regions or blocks of *alpha*-1,4-galacturonic acid with polymer linkages [13].

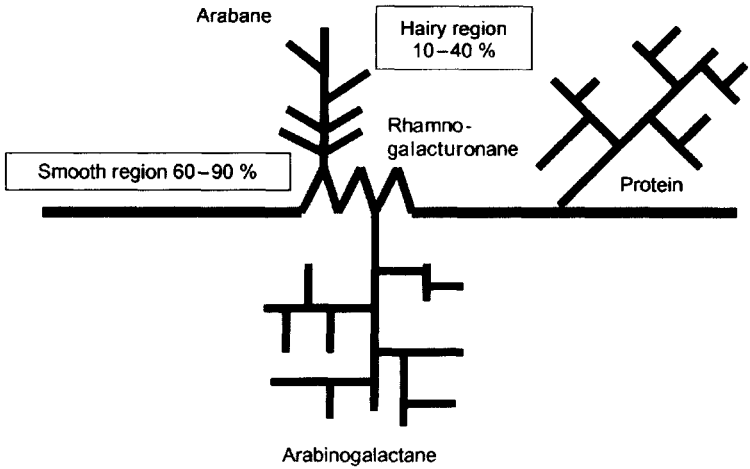

Figure 7.2 Pectin structure [12].

The water-insoluble protopectin consists of methoxylated poly-D-galacturonic acid chains cross-linked via metal ions (Ca^{2+}, Mg^{2+}), polyhydroxy compounds (arabinose, xylose, rhamnose, galactose), phosphoric acid, carboxyl ester compounds and hydrogen bridges [14]. The water-soluble pectins formed by macerating enzymes present in the fruit as it ripens are usually highly esterified. Their degree of esterification is 65–98%, with a degree of polymerisation ranging from a few dozen to several hundred [13]. Most of them are smooth-region pectic substances whose solubility rises with an increasing degree of esterification and decreasing molecular weight. The hairy-region pectins which account for some 10–40% mainly consist of a mixture of araban, rhamnogalacturonan and arabinogalactans, as well as proteins.

7.4.1 Smooth-region pectinases

The term 'smooth-region' pectinases covers a mixture of enzymes which degrade high molecular weight pectin within the polygalacturonic acid chain (depolymerases) and on the other hand de-esterify pectins (esterases). Pectinesterase provokes enzymatic de-esterification of the pectin by splitting off methanol. This process converts the pectin to a partially demethylated version (pectinic acid) or pectic acid. Depolymerisation can occur in two ways, by hydrolytic reaction or highly specific *beta*-transelimination. The enzymes involved in the first case are polygalacturonases; in the second case lyases.

Among the polygalacturonases (PGs) we distinguish between exo- and endo-PGs. The exo-enzymes degrade the pectin polymers from the terminal end, whereas the endo-enzymes split the polymers at sites along the molecular chain.

The endo-PGs are of particular importance, and can be divided into macerating and liquefying PGs. Although both PG types have similar properties and cause a rapid drop in the viscosity of pectin solutions, for example, they show clear differences with regard to substrate preference.

Macerating PGs prefer to react with protopectin, dissolving the cell tissue and converting it to soluble pectin. In combination with sufficient quantities of pectinesterase, they provide complete pectin breakdown. The ratio of PG to PE is of crucial importance in this context, because pectic acid is the substrate of preference for PG.

Liquefying PGs show reduced reaction with protopectin, but are capable together with pectinesterase of hydrolysing water-soluble pectin. Since this type of pectin is usually highly esterified, a relatively large quantity of pectinesterase is required. The breakdown of water-soluble, highly esterified smooth-region pectins is of importance when fruit processing includes pressing processes and the primary concern is to improve juice separation. This enzyme combination is ideal for the mash treatment of pome fruit and white grapes, for instance, because the degradation of the soluble pectin reduces its viscosity and therefore effects a more rapid run-off of juice. On the other hand, the mash structure is retained because the matrix-building protopectin is less severely attacked and the juice can escape freely via drainage channels.

The lyases are divided into pectin and pectate lyases. Endopectin lyases are an important constituent of technical enzyme products. Owing to their ability to statically depolymerise highly esterified pectin, they give rise relatively quickly to pectin fragments of medium chain length, leading to a rapid drop in the viscosity of pectin solutions. The endopectin lyase (PL) is excellently suited for rapid viscosity reduction and is used particularly for manufacturing cloudy juices and purées. It predominantly degrades highly esterified, insoluble and water-soluble pectin. The cleavage occurs between two esterified galacturonic acids. The degradation products are pectin fragments with sufficient cloud stability. The enzyme activity decreases together with the chain length, and no methanol is released as a reaction product.

Mixtures of macerating polygalacturonases with pectinesterase and pectin lyase are suitable for breaking down all the smooth-region pectins. Pectin lyase accelerates complete degradation of the pectin by more rapidly breaking down the medium-sized pectin fragments of the PG/PE combination to mono-, di- and tri-galacturonic acid (synergy effect). This type of pectinase is used both as a mash enzyme for releasing valuable fruit constituents such as colour and aroma, and for complete depectinisation to manufacture clear juices and concentrates.

7.4.2 Hairy-region pectinases

Hairy-region pectinases can also be grouped under the term 'arabanases'. One of the most important of these is endoarabanase. It splits the *alpha*-1,4-bonds

within the araban chain. Arabinofuranosidase, an exoenzyme, mainly splits the *alpha*-1,3-bonds of the side chains. Further important enzymes are rhamnogalacturonase, acetylesterase and the arabinogalactanases, which exist in *beta*-1,3 and *beta*-1,6 variants. In keeping with the pectin model, the hairy-region pectins are divided into araban, rhamnogalacturonan and arabinogalactan. The quantity and quality of these hairy-region pectic substances depends on the raw material and process. Of the pome fruits, pears in particular present greater problems with araban. The two enzymes endoarabanase and arabinofuranosidase hydrolyse arabans and are especially important for preventing post-clouding due to araban in pome fruit concentrates.

With increasing maceration and the breakdown of all the pectic substances, hairy-region pectins are also increasingly dissolved in apples and other fruits, and need to be partially hydrolysed during further processing. Maceration or liquefaction processes dissolve far greater quantities of hairy-region pectins, particularly rhamnogalacturonan and arabinogalactan, than more traditional pressing processes. That is why greater concentrations of special arabanases are required in such processes, principally to avoid filtration problems. Three enzymes are mainly responsible for breaking down rhamnogalacturonan: arabinofuranosidase, rhamnogalacturonase and acetylesterase. Arabinogalactan is degraded by the two enzymes arabinofuranosidase and arabinogalactanase.

The much higher colloid values of such juices and concentrates also show that it has not been possible so far to effect complete breakdown. As practical experience shows, the enzymes responsible for such breakdown are often not present in sufficient quantities. If corresponding processes, such as pome fruit liquefaction, dissolve more hairy-region pectins, the colloid concentration of the juice increases and its ease of filtering decreases. This particularly affects cross-flow membrane processes which react very sensitively to colloids [10].

7.5 Cellulose and hemicellulose

Cellulose is one of the cell-wall constituents of fruits and is linked by xyloglucans with the smooth and hairy-region pectins. According to present knowledge, the breakdown of native cellulose calls for the interaction of three different hydrolases. The first step is the splitting of the amorphous regions by an endoglucanase, also called Cx-cellulase. This causes the formation of new terminal groups as a substrate for the next step, the splitting of cellobiose by the so-called cellobiohydrolase or C1-cellulase.

The synergistic effect of the Cx- and C1-cellulases gives rise to cellobiose molecules which are broken down into glucose by the third enzyme, cellobiase, also known as *beta*-glucosidase [13].

Hemicelluloses are a group of polymer carbohydrates which can be classed in the wider sense as hairy-region pectins. The main representatives of this group

are galactomannans, xylans and *beta*-glucans. Vegetable gums also belong to this family of substances. Depending on the fruit and processing technology, these substances dissolve, and impede processing or filtration. Suitable enzymes in this case come under the category of hemicellulases and occur regularly as secondary activities in pectolytic enzyme products. The hemicellulases obtained from *Aspergillus niger* are particularly rich in *beta*-glucanases, galactomannanases, xylanases, and so on.

The use of cellulases and hemicellulases in fruit processing is not allowed in the EU for legal reasons. This concerns enzyme products with the relevant main activities. They are, however, allowed without any legal restrictions for vegetable processing. As secondary activities, though, cellulases and hemicellulases can in fact be detected in commercially available pectinase, amylase and protease products. Their proportion depends on the strain of enzyme used and may vary widely. It also takes considerable effort to differentiate between cellulases and hemicellulases.

Cellulase products derived from *Trichoderma* species are particularly suitable for fruit and vegetable processing, and are mainly used in combination with pectolytic enzymes. These enable further viscosity reduction, especially in maceration and liquefaction processes, and facilitate solid/liquid separation. The main objective, as well as an increased yield, is to optimise the process.

Higher cellobiose values in fruit juice concentrates have led to the misunderstanding that there is a connection between cellulase application and the formation of cellobiose. This has led to restrictions on the use of cellulases in the fruit juice industry. Investigations by Will, Bauckhage and Dietrich [17] showed that no rise in cellobiose values was ascertained when using cellulases with the usual cellobiase activity. Higher cellobiose values in juices and concentrates cannot therefore be taken to indicate the use of cellulase.

7.6 Starch

Starch as a polymeric carbohydrate plays a rather minor role in fruit processing. Whereas pectin and cellulose are the essential building blocks of the cell walls, starch mainly serves as an energy store. Its presence is of technical importance for processing apples, pears and bananas.

Starch consists of an unbranched amylose fraction and a branched fraction, amylopectin. Amylose mainly consists of glucose molecules, linked by *alpha*-1,4-glycosidic bonds. Several thousand glucose molecules may enter into the constitution of amylose; they are arranged in spiral form and divided into so-called helical parts. These helical parts are capable of forming inclusion compounds with iodine, with one mole iodine per eight glucose residues.

In contrast with amylose, amylopectin contains not only *alpha*-1,4- but also *alpha*-1,6-glycosidic bonds. Amylopectin has a strong swelling capacity and

usually turns purple when coloured with iodine. Amylose, which is only slightly soluble in cold water, dissolves in pure form as soon as it is even slightly heated. This dissolution process is reversible; at low temperatures, amylose precipitates in the course of time—it retrogrades. Amylopectin requires pronounced heating at temperatures in excess of 60°C before it dissolves. Since the solution becomes very viscous during the dissolution process, it is also termed gelatinisation. Unlike amylose, pure amylopectin forms a stable solution even at low temperatures and shows no retrogradation even after prolonged storage.

Amylose and amylopectin are not found separately side by side: they are linked by hydrogen bridges. The ratio of amylose to amylopectin varies, depending on the type of starch, and is about 27% amylose and 73% amylopectin in apple starch. Owing to the pH requirements of fruit juices, the only enzymes which can be used are those derived from mould and fungi, since bacterial enzymes only show an effect from pH 5 onwards, and are also strongly temperature-dependent.

Depending on their specific action, one distinguishes between four different amylase activities: *alpha*-amylase, *beta*-amylase, glucoamylase and pullulanase. Of course, these may occur individually or in combination, and can also be employed in both forms.

The first of these types of amylase, *alpha*-amylase, is an endohydrolase that splits the *alpha*-1,4-bonds of starch in a non-systematic way inside the molecule, with release of water. It brings about a rapid drop in viscosity of the gelatinised starch, and because of this, *alpha*-amylase is also called a 'liquefaction enzyme'.

Beta-amylase is an exoamylase that successively splits the *alpha*-1,4-bonds from the non-reducing end of the glucose chain. This mainly leads to the formation of maltose, which is why the enzyme is also called maltase. *Beta*-amylase cannot split the *alpha*-1,6-bond, which means that enzymatic degradation of the amylopectin stops at the *alpha*-1,6-glycosidic branch points. Therefore, when *alpha*- and *beta*-amylase are used in combination, oligomeric sugar compounds (maltotriose, maltotetraose, etc.) are always left over.

Glucoamylase is also an exohydrolase that splits the *alpha*-1,4-bonds of the starch molecule successively from the non-reducing end of the glucose chain. It splits off individual glucose units, which is why it is called glucoamylase. Cleavage of the *alpha*-1,4-glycosidic bond occurs at a high conversion rate. Glucoamylase can also split the *alpha*-1,6-bond, albeit at a relatively low conversion rate. That makes glucoamylase the only amylolytic enzyme that can completely hydrolyse starch (amylose and amylopectin) all on its own. The final and practically sole product of complete starch degradation is glucose.

Pullulanases are endohydrolases which exclusively split the *alpha*-1,6-bond of amylopectin, which is why they are called 'debranching enzymes'. Hydrolysis of the branched bonds destroys the spatial structure of the amylopectin. The reaction products are linear amylose fragments.

All amylases mentioned here are manufactured on an industrial scale by means of specially selected bacterial or mould/fungal cultures. There are bacterial *alpha*-amylases, *beta*-amylases and pullulanases, but no bacterial glucoamylase exists. Depending on the production organism utilised, there are different enzyme profiles with activity characteristics specific to a certain strain, and therefore special fields and conditions of application. This naturally applies equally to fungal amylases, which are available as *alpha*-, *beta*- and glucoamylases. Whereas bacterial cultures are mainly used to produce ultrapure *alpha*-amylases, fungal *alpha*-amylases are usually combined products with high *beta*-amylase secondary activity. Owing to the predominantly acid pH reaction conditions encountered with fruit, the fruit juice industry almost exclusively employs fungal amylases.

When enzymes were first employed for fruit processing, only fungal *alpha*-amylases were used, because there were no other fungal amylases available at the time and starch problems were more or less a rarity. Following the introduction of horizontal presses (Bucher), the repressing of the pomace greatly increased the quantity of starch in the juice.

Only with the introduction of hot clarification, developed and patented by Röhm GmbH, Darmstadt, were glucoamylases used for the first time. The fungal *alpha*-amylases employed until then were not suitable for hot clarification, owing to their low temperature stability in the pH range of fruit juices. Since these glucoamylases also had a better price/performance ratio than fungal *alpha*-amylases, they initially ousted these almost completely from the market, being used for cold clarification as well. Fungal *alpha*-amylases are, however, once again increasingly being used for cold clarification.

7.7 Protein

Fruits contain only small quantities of protein, but these are becoming more important in the light of new processing technologies. Currently, proteases are not widely used in fruit processing, although European legislation explicitly allows this application. As membrane technology becomes more and more popular, this neglected group of enzymes is meeting with increasing interest. Bentonites are employed to precipitate proteins in conventional fining and clarification processes, but this type of protein separation does not take place in cross-flow membrane processes. That is why proteins may accumulate on the membrane, impairing filtration properties such as flux and capacity. The quality of the fruit concentrates may also be affected negatively because protein fragments pass through the membrane and cause post-clouding.

Basically, the only suitable proteases are those whose pH optimum lies in the acid range. That is why only corresponding fungal proteases are worth

considering. They show very good activity in the pH range of 3–5, leading to the desired effect. Proteinases can be divided into the following categories:

- Peptidases produce high quantities of amino acids, but also di- and tripeptides.
- Endoproteinases reduce the chain length of peptides, transforming high molecular weight proteins into large proportions of low molecular weight protein or protein fragments [13]. This type of protease is particularly interesting for fruit processing.

Special fungal proteases derived from *Aspergillus niger*, with an acid pH optimum between pH 3 and 5, work very efficiently in grape juices [15]. By hydrolysis of high molecular weight protein into protein fragments, these juices lose their ability to bind tannins and to form protein–tannin complexes. Such protein–tannin complexes are liable to clog the membrane. This is particularly the case in citrus processing, where one is dealing with relatively large amounts of protein on the one hand, and new membrane technologies are gaining ground on the other. Experience with stabilising pome fruit concentrates by means of suitable proteases has also been positive. A further problem that can be solved with the aid of suitable proteases is pronounced foam formation during processing.

7.8 Application of technical enzyme products

Technical enzyme products are basically mixtures of main and secondary activities. The main activities of pectolytic enzyme products are polygalacturonases, pectinesterase, pectin lyase and various arabanases. In modern enzyme technology, it is now normal for the various pectinase products to contain different main activities, depending on the application. The major secondary activities are cellulases, hemicellulases, proteases and *beta*-glucanase. These secondary or foreign activities are contained in commercially available pectinase products, depending on the production strain, but are usually not standardised. To ensure optimum technical efficiency and economy, the selected enzymes must be suitable for the specific process. There are several preconditions for their application, particularly with regard to enzyme characteristics:

- The pH value must be adjusted to the enzyme characteristics, or, if the natural pH value cannot be changed, the corresponding enzyme must be selected.
- The speed of the reaction can be influenced by the choice of reaction temperature. Reactions can be considerably accelerated by working at

just below inactivation temperature, but enzyme reactions still proceed even at low temperatures.

- If the reaction time cannot be altered for technical reasons, the course of the reaction can be controlled by selecting the temperature or the enzyme dose.

Stabilisation of the enzyme products also follows the requirements of the industry. Although the current preference is given to liquid products manufactured without preservatives, stabilisers are frequently used in fruit juice enzymes (glycerine, potassium chloride, sorbitol), so that they keep for one to two years in a cool place (4–6°C). The activity loss under ideal storage conditions is usually less than 10% in one year. For solid enzyme products, granulated products are preferred to powder enzyme products, since the latter have high allergic potential due to their dust formation, and call for special working regulations to be observed during use.

7.9 Pome fruit processing

About 60 million tonnes (t) of pome fruit are produced on average around the world. Some 21% or 10–12 million t of which are processed to juice. The most important product is apple juice concentrate, with an annual production capacity of 800,000–1,000,000 t. The US is foremost among producing countries, with a production of about 200,000 t per annum. At the same time, the US imports roughly the same quantity, so that annual consumption exceeds 400,000 t per year, or approximately 40% of annual world production. China probably comes second among producing countries, with a capacity of 120,000–140,000 t apple juice concentrate and rapidly rising processing quantities, almost exclusively for export. The most important producers also include the countries of the former Soviet Union (CIS: 100,000–150,000 t). The major European producer is Poland (90,000–130,000 t), followed by Germany and Italy (80,000–100,000 t each). The largest producers in South America are Argentina (100,000–120,000 t) and Chile (50,000–60,000 t).

Various commercial methods are employed for processing pome fruit to obtain clear apple or pear juice concentrate. The most important processes are summarised in figure 7.3. These are first divided into pressing and maceration processes. Whereas classical enzyme treatment of the mash is performed in pressing processes, further maceration usually takes place in the decanting processes. The pomace produced during pressing can be mixed with condensate from the concentrate unit and subjected to further enzyme treatment. This step also involves either extraction or maceration. The cloudy press juices and extracts obtained in this way are subsequently depectinised and freed from starch, before clarification/filtration and concentration steps are performed.

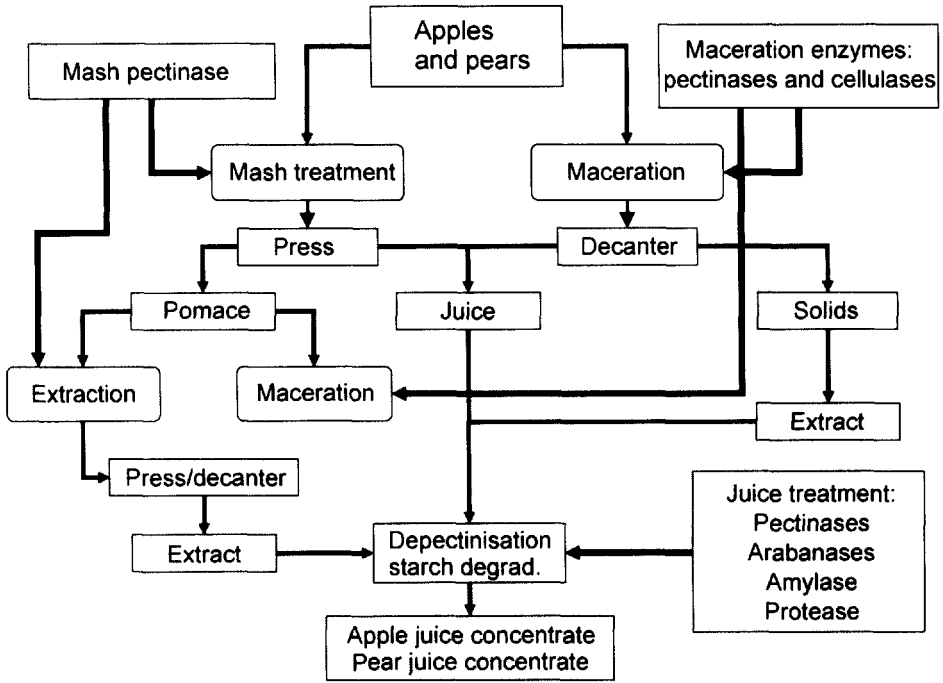

Figure 7.3 Processing of pome fruit.

7.9.1 Enzymatic treatment of the mash

Enzymatic treatment of the mash renders it more suitable for pressing, as expressed by increased throughput and greater yield (figure 7.4). Pectinases with a high proportion of pectinesterase and liquefying polygalacturonases are suitable both for mash treatment and pomace extraction.

The hydrolysis of the protopectin that binds the cells weakens the fruit tissue, causing the protopectin to dissolve and increasing the juice viscosity. This leads to a reduction in press performance and juice yield. Mash treatment is performed under mild conditions at temperatures of 20–30°C and in a reaction time of 30–120 min. The breakdown of the dissolved pectin in the juice phase immediately reduces the viscosity of the juice and makes it run off the press more quickly. This provides a greater yield and press capacity.

Pectinase products suitable for mash treatment therefore contain mainly liquefying PGs and a high proportion of pectinesterase. The reaction leads to short-chain pectin fragments with low water-binding capacity which prevent the pomace from sticking. If the protopectin matrix is largely preserved, drainage channels in the pomace ensure optimum press behaviour. In some pressing processes, the addition of rice hulls (1–2% w/w) improves press performance.

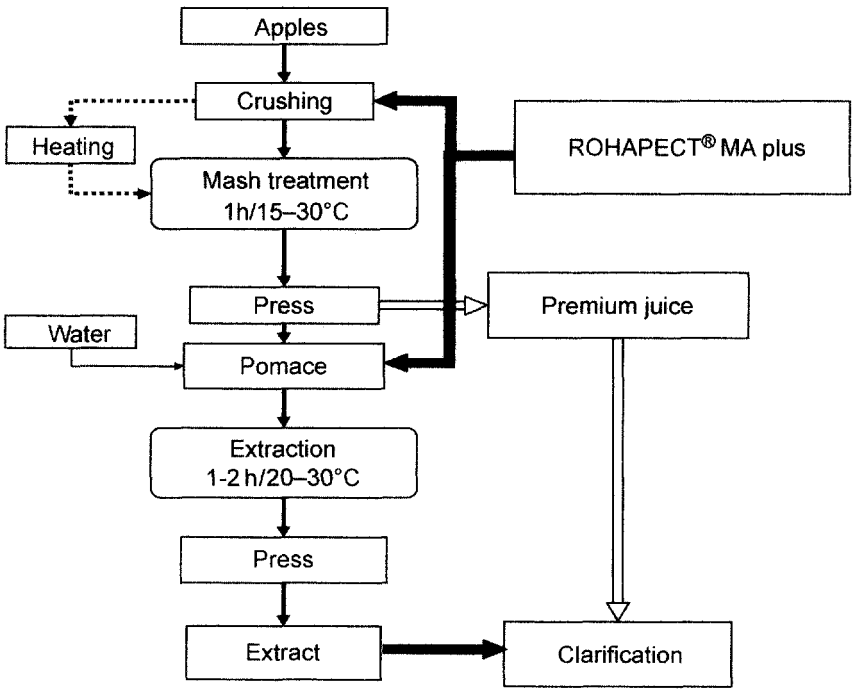

Figure 7.4 Enzymatic treatment of the mash and pomace extraction.

At temperatures over 40°C, thermal weakening of the fruit tissue occurs, supported by macerating protopectinases present in the fruit, which greatly impedes pressing. Unsuitable pectinase products with too great a proportion of macerating polygalacturonases show a similar effect.

The special pectinase ROHAPECT® MA plus from AB Enzymes GmbH has proved particularly suitable for mash treatment. Comparisons with competitive products, based on operating results on PC-controlled Bucher presses of type HPX 5005 i, demonstrate the excellent effect of this product. ROHAPECT® MA plus contains high quantities of pectinesterase and a liquefying polygalacturonase as its main constituents. These enzymes preferentially degrade highly esterified, soluble pectin. Enzymes with lower proportions of pectinesterase and macerating polygalacturonase, such as those in conventional mash enzymes, are less suitable. These enzymes weaken the protopectin and thus the mash structure. The resulting pomace is stickier and wetter, and lower juice yields are obtained. The reduced flow rate of the juice also results in lower press capacity.

Plocharski [16] from the Polish Institute of Pomology established in his tests that an increased dosage of ROHAPECT® MA plus leads to greater yields and lower cloud content in the press juice. A high degree of clouding or high nephelometric turbidity unit (NTU) values in the juice also result in large quantities of soluble pectin. It is noteworthy that the optimum enzyme composition during mash treatment provides press juices with lower amounts of residual pectin.

The colloid contents, another indication for undegraded hairy-region pectins, are also much lower. This shows that the selection of the right mash enzyme triggers a chain reaction that extends from juice yield to filterability.

Measurements of the pectin quantity in the pomace revealed that much higher quantities of pectin remain in the pomace when the mash is treated with ROHAPECT® MA plus than with conventional mash enzymes.

7.9.2 Pomace extraction

Extracting pomace by means of vapour condensate is now a very widespread method, and is usually performed before the second pressing, in order to achieve a greater yield (figure 7.4). There is a distinction made between continuous processes, such as those on belt filter presses, and cascaded presses. In these, the pomace from the previous pressing step is mixed with vapour condensate and pressed again in a second press. The technical benefits of this process are offset by disadvantages such as lower extraction efficiency due to the short retention time between the addition of water and pressing.

Much higher yields are obtained with discontinuous processes if the extraction step is carried out with the help of suitable enzymes. The pomace is mixed with about 30–50% (v/v) vapour condensate. After continuous enzyme dosage, extraction is performed in a container for 1–3 h without stirring before the second pressing is effected. Reaction temperatures of 20–30°C are preferred

for reasons of quality. As in enzymatic treatment of the mash, suitable products for this purpose are pectinases with liquefying polygalacturonase and a high proportion of pectinesterase. Pectinases with a low proportion of macerating polygalacturonases are most suitable for retaining the mash structure.

Very good extraction results can be obtained by combining suitable pectinases and longer extraction periods. Enzymatic action makes the cell walls more permeable, improving the extraction of soluble dry matter. Since protopectin and hairy-region pectins are only slightly soluble, the subsequent processing steps pose no major problems. This is also demonstrated by the relatively moderate increase in the galacturonic acid and colloid contents of the concentrates.

7.9.3 Maceration

In contrast to enzymatic treatment of the mash or pomace extraction, maceration involves complete disintegration and liquefaction of the fruit tissue. This involves the extensive hydrolysis of protopectin, soluble pectin and other hydrocolloids.

This technology is often used in decanter separation processes and can basically be divided into two variants:

(a) treatment and processing of the entire pome fruit mash
(b) treatment and processing of the pome fruit pulp (purée) after separation of the cores, peel and stems in a finisher.

Whereas variant (a) involves total liquefaction or whole-fruit liquefaction, this does not apply to variant (b), since major constituents of the fruit (cores, peel and stems) are separated before processing actually begins, by heating the mash to about 60°C and then using a finisher after heating (figure 7.5). Enzymatic maceration of pome fruit is performed with highly concentrated pectinase activities with specific liquefaction properties and a very high proportion of arabanase. The aim is complete hydrolysis of the soluble and insoluble pectin under optimum reaction conditions (about 50°C, 1–2 h). The criteria for particularly suitable products are the liquefying effect and the ease of filtering and stability of the juices and concentrates. This can be ascertained by the alcohol-insoluble material (AIM) test. Cellulases can also be employed to support maceration, where legally permissible. They improve liquefaction and thus help to optimise the process. Initial decanting is followed by leaching, the solids obtained being mixed with vapour condensate and extracted.

Variant (b) usually makes it possible to manufacture juices and concentrates of better quality. Frequent disadvantages of variant (a) are poorer quality as established in sensorial tests, the higher polyphenolene and tannin content and the pronounced browning and oxidation of the juices and concentrates obtained.

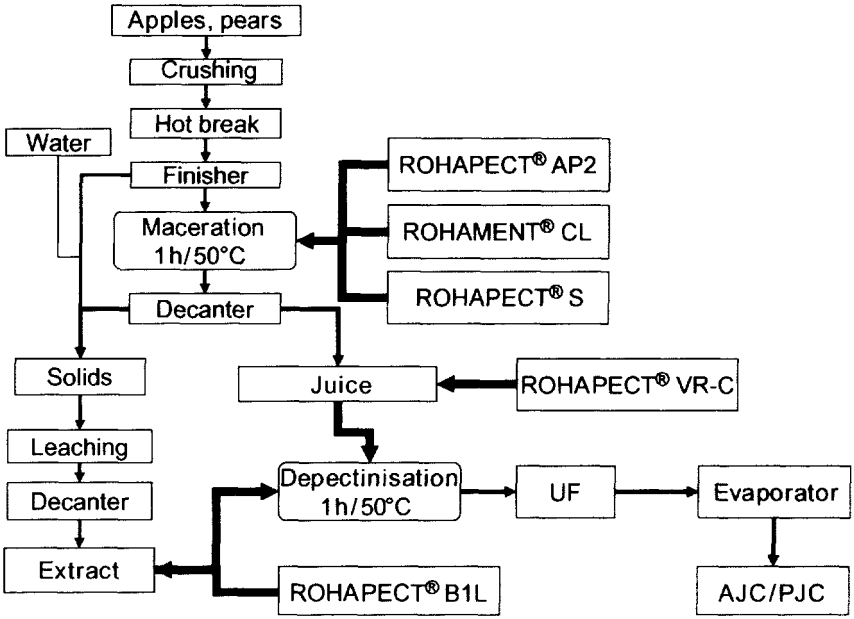

Figure 7.5 Enzymatic maceration of pome fruit.

7.9.4 Pomace maceration

The standard two-step process involves normal enzymatic treatment of the mash and pressing to obtain a premium juice (figure 7.6). The pomace produced in the process is mixed with water (1:1) and macerated by means of enzymes, leading to almost total liquefaction. A second press or decanter provides an extract, which can either be mixed with premium juice or processed separately.

Enzymatic maceration is performed under optimum reaction conditions at about 50°C for roughly 1–2 h with constant stirring, aiming at the greatest possible degree of liquefaction. This is done using highly concentrated pectinases with optimum macerating properties to ensure degradation of all the smooth-region pectin. Special highly concentrated arabanases are required to break down the hairy-region pectins. Suitable cellulases optimise the process.

The benefit of this process (i.e. obtaining the maximum yield) is offset by technical disadvantages. In particular, the up to 100 times higher colloid content of the extract makes filtration more difficult. The output of ultrafiltration units drops to values that are 30–50% lower than those achieved in conventional juice manufacture.

The extracts contain large quantities of poly-, oligo- or monosaccharides. Their analytical composition is quite different to that of conventionally manufactured juices and concentrates, which is why they are not so readily accepted.

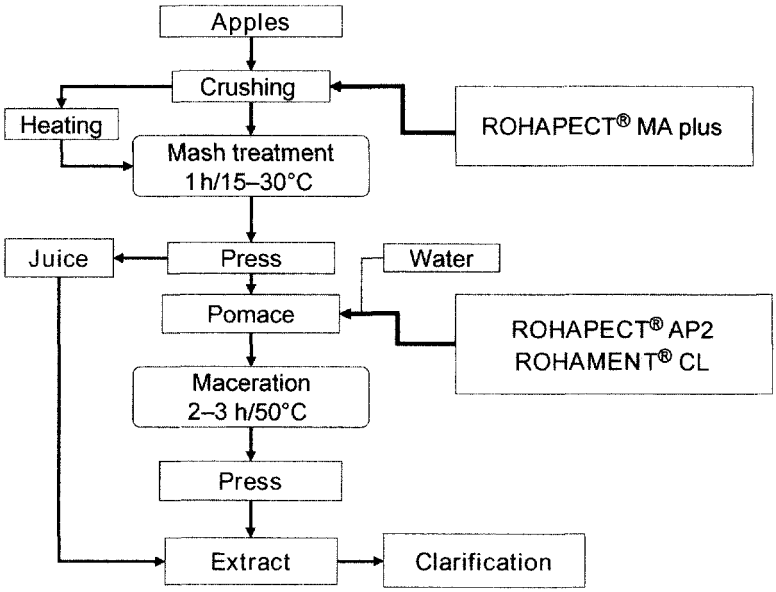

Figure 7.6 Pomace maceration.

Recent examinations by Will, Bauckhage and Dietrich [17] have shown that these extracts contain highly interesting constituents of nutritional importance. Compared to juices obtained by conventional methods, they have a higher content in soluble, partially depolymerised fibre and bioactive secondary metabolites (flavanoids). Moreover, they contain three to seven times as much total phenol, which increases the antioxidative capacity (TEAC). *In vivo* and *in vitro* tests have also showed positive nutritional results.

7.9.5 Juice treatment

Depending on the technology used for juice extraction and on the subsequent clarifying and filtration process, enzymatic juice treatment must meet different requirements, which can generally be divided into the individual steps of depectinisation and starch degradation (figure 7.7).

7.9.5.1 Depectinisation

Depectinisation of apple juice is nowadays successfully performed with pectinases and arabanases, which effect the most rapid possible pectin hydrolysis, enabling the subsequent clarification and filtration steps to proceed smoothly. The adequate effect of the arabanases increases the stability of the juice and prevents undesirable post-clouding by araban. Since pear juices have much higher araban contents than apple juices, the enzyme products used for their

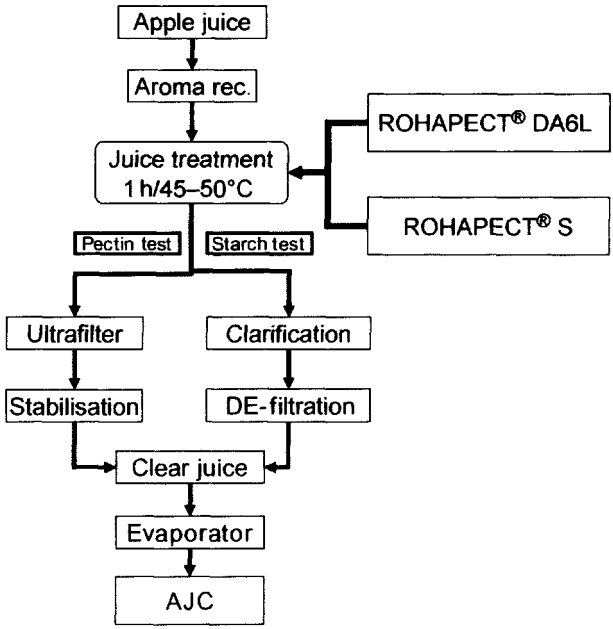

Figure 7.7 Juice treatment.

depectinisation must contain much more arabanase. Depending on the composition of the juice, suitable proteases and hemicellulases can also be used for juice treatment, which contribute equally to improved ultrafiltration (i.e. increased flux and output) or stability of the concentrates.

Pectin test. The pectin test makes it possible to determine more or less large quantities of residual pectin in fruit juices, and is based on a mixture of fruit juice and alcohol (96% ethyl alcohol). Above a certain size, pectic substances become insoluble and either precipitate as gels with inclusion of water, or form flocs. This test, also known as the alcohol test, can be used both for process control and to determine the optimum enzyme dosage. In the 1:1 pectin test (one volume fruit juice to one volume ethyl alcohol), pectins with more than 8–10 galacturonic acid units become insoluble in 50% ethyl alcohol and precipitate as gels (figure 7.8). The tested fruit juice is pectin-free when no more separation is detectable and the juice/alcohol mixture remains uniformly cloudy.

The 1:2 pectin test (one volume fruit juice to two volumes ethyl alcohol) detects even shorter-chain pectic substances which precipitate in the higher alcohol concentration of approximately 66% and form flocs (figure 7.9). In order to detect predominantly acid polysaccharides such as pectins, the ethyl alcohol can be acidified with 1% conc. HCl.

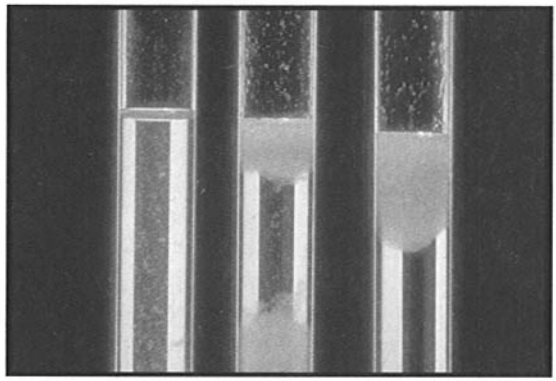

Figure 7.8 Pectin test (1:1).

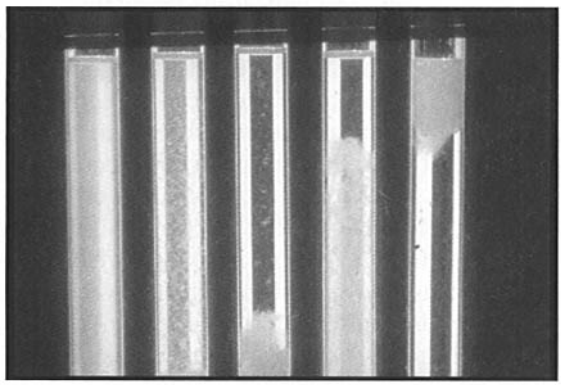

Figure 7.9 Pectin test (1:2).

AIM test. The AIM test mainly detects hairy-region substances and is therefore used in particular when processing pears and in liquefaction processes. Centrifuged, cloud-free juice is mixed with isopropyl alcohol (acidified with 1% HCl) in a volume ratio of 1:2, and centrifuged after standing for 5 min. The quantity of centrifuged AIM should be as small as possible, for example, $< 1\%$ (w/v).

Stability test. The test allows the juice to be examined and assessed before concentration. It gives information on how the juice behaves after concentration and its expected stability. Filtered juice is heated to approximately 100°C, then cooled and frozen. After defrosting, the turbidity is measured and the value compared with the one obtained before heating. The difference measured in

NTU should be as small as possible. Sufficient stability of the concentrate can be expected if the initial value is < 1 and the increase is < 0.5 NTU.

7.9.5.2 Starch degradation

Owing to technical progress and the increased use of early-ripening raw material, the starch content of raw apple juices has increased sharply in recent years, leading to a considerable rise in demand for amylase.

Heating the juice, particularly during aroma recovery, causes the native starch to dissolve. That is an important precondition for rapid and complete starch breakdown, and means that the temperature and retention period when heating the juice must be selected so as to ensure the complete dissolution of the starch granules.

Enzymatic breakdown of dissolved starch is essential in order to avoid problems in subsequent process steps, or post-clouding of the concentrate. Starch-containing juices pose problems during ultrafiltration (UF), where they may reduce throughput and capacity. Starch dextrins (partially hydrolysed starch) may also diffuse through UF membranes and get into the juice and concentrate. The same thing happens during conventional fining and filtration, and in microfiltration processes. The starch molecules can retrograde, which leads to post-clouding after prolonged storage, particularly in concentrates.

Only fungal *alpha*-amylases used to be employed for cold clarification (up to 30°C). These endoenzymes initially split the starch into large dextrin fragments and are subsequently hydrolysed to maltose and maltotriose.

When hot clarification (45–55°C) was introduced, these *alpha*-amylases could no longer be used because of their instability towards heat. Nowadays, glucoamylases are mainly used (also known as amyloglucosidases).

Glucoamylase splits glucose from the non-reducing chain end of amylopectin, amylose or glycogen by the exo mechanism. The major glucoamylase derived from *Aspergillus niger* for industrial manufacture and application splits not only *alpha*-1,4 bonds, but also *alpha*-1,6 and *alpha*-1,3-bonds.

The enzyme is a glycoprotein which at higher dosages leads to turbidity caused by thread-like particles that resemble glass fibres. This is also termed post-bottling haze and occurs shortly after hot apple juices have cooled down in the bottle. This precipitation is a protein–tannin reaction caused by denaturation and coagulation of the glycoprotein present in the enzyme due to the high filling temperatures. The enzyme protein cannot be completely removed by means of cross-flow filtration. Apparently, it is a linear molecule which is capable of passing through pores as small as 20 kilodaltons. To avoid this reaction, process control needs to be optimised, firstly by complete dissolution of the starch, so that relatively small amounts of glucoamylase are required for the subsequent enzyme reaction. At a maximum dosage of about 30 ppm of a commercially available glucoamylase, one can avoid the formation of these threads which occur at high dosages. Owing to the reduced reaction speed, the higher dosages

of glucoamylase required for cold clarification are better replaced by fungal *alpha*-amylases. In contrast to glucoamylase, the enzyme protein of the fungal *alpha*-amylase can be completely removed from the juice during the usual three-component (bentonite, gelatin, silica sol) fining process.

Starch test. High molecular weight starch is dissolved by heating the pome fruit juice (e.g. during aroma extraction). It can be detected as a blue colour by adding iodine solution. Partially degraded starch (dextrin) shows a purple to maroon colouring. At high degrees of degradation (sugars), starch cannot be coloured with iodine, hence the usefulness of this test to determine the presence of residual starch in the juice (figure 7.10).

7.9.6 Traditional clarification and filtration

Traditional clarification processes are nowadays mainly performed in small and medium-sized processing plants. Frequently, the process also serves to prepare for subsequent bottling. Three-component fining with bentonite, gelatin and silica sol can be followed either by filtration with diatomaceous earth and a layer-type filter, or by microfiltration. The membranes used for cross-flow microfiltration have pore diameters between 0.1 and 0.3 µm, and higher flux rates are achieved than with ultrafiltration. The advantage of using clarifying agents is that, if properly employed, they provide very stable juices and concentrates. Since most of the tannins are precipitated by the gelatin, such juices and concentrates also show less tendency to become dark. Some processing plants also employ this technique to process rediluted, cloudy semi- or full concentrates outside the season.

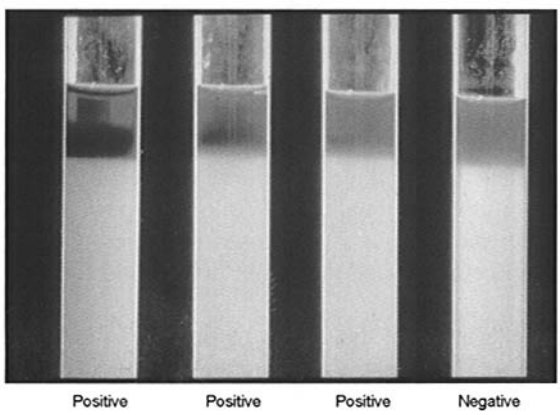

Figure 7.10 Starch test with 0.1 n iodine solution.

7.9.7 Ultrafiltration of apple juice

Ultrafiltration is without doubt a more modern filtering system than conventional fining and filtration. Moreover, ultrafiltration offers the possibility of filtering clear, sterile fruit juices in one step, without the addition of clarifiers. Ultrafilters are now used predominantly for manufacturing apple and pear juice concentrate. Ultrafiltration is a cross-flow process; that is, the juice flows through the membranes horizontally.

With ultrafiltration membranes, the separation capacity is characterised not by pore size, but by the so-called separation limit. This states whether a molecule of defined size can be held back by a membrane. Common ultrafiltration membranes have a separation limit of 10 to 50 kilodaltons. Modern plants contain polymer membranes of asymmetric composition (i.e. the pores are conical, with the wider end pointing downwards). This means that all particles which enter the narrow pore can emerge at the bottom and do not clog the membrane. A module is formed from the membrane, through which the juice to be filtered is pumped under pressure, at high speed and high turbulence. The low molecular weight constituents of the juice pass through the membrane, whereas the high molecular weight substances are held back and concentrated. The liquid phase that passes through the membrane is usually termed the permeate, and the concentrated product stream, the retentate.

The stream of matter through the membrane is termed the flux and is expressed in litres per m^2 and hour. It is a measure of the capacity of a unit and depends on various influencing factors such as the temperature, viscosity, pressure and mode of operation. Membrane fouling is a particularly important factor to take into account from an enzyme technology perspective. This problem is caused by colloidal constituents of the juices, which build up and subsequently clog the membranes. When ultrafiltration was first introduced, there were claims that it could reduce the enzyme dosage for depectinisation and starch degradation to one fifth. Today, it is known that ultrafiltration technology in fact requires *larger* quantities of enzymes to guarantee the complete hydrolysis of pectic substances and starch, and to minimise the risk of irreversible blockage of the costly membranes.

The flux or output of a cross-flow filtration unit is, however, largely influenced by the colloid content of the juice being filtered, because the colloids may cause membrane fouling. The quantity of colloids depends on the processing method. The further the pomace is processed (see sections 7.9.2 and 7.9.4), the higher the colloid content, and the lower the flux and capacity of the ultrafiltration unit.

Despite these relationships, the aim is to optimise treatment of the juice prior to ultrafiltration. That means using pectinases with a very high content in hairy-region pectinase activity for colloid-producing processes. In addition, the AIM test makes it possible to examine and control the reaction. In individual

cases, very good results have been obtained through combined treatment with pectinase, hemicellulase and protease.

7.9.7.1 Fining agents

General statements cannot be made on the use of fining agents. Three-component fining with bentonite, gelatin and silica sol is also employed in individual cases for ultrafiltration, to prevent post-clouding and to conform to the high quality standards to be met by the concentrate. Alternatives for reducing post-clouding are offered by caseinates, activated carbon or PVPP. A further alternative is treatment of the ultrafiltered juice with adsorber resins to prevent tannin clouding and colour changes. The resins are selected so as to bind selectively undesired substances such as pigments, and polyphenols in the acid range and remove them from the juice.

7.9.8 Cloudy apple juice or apple juice concentrate

The stability of suspended matter in naturally cloudy apple juices and apple juice concentrates is determined by the content in water-soluble pectin, the size of the suspended particles and the charge intensity of the hydrated pectin in the suspended matter.

As a general rule, the content in water-soluble pectin must not fall below a certain value in order to prevent the finely suspended matter from settling. Moreover, the suspended particles should be as small as possible, since their weight and ability to form a sediment increases together with their size. The proportion of pectin in the suspended matter is surrounded by a hydrate shell, which results in a negative electric charge of the suspended particles. The more pectin that is present, the higher the negative charge. Particles with the same charge repel each other and therefore keep each other in suspension. The higher the charge intensity, the better the particles remain in suspension.

Pectin is extremely important for the stability of naturally cloudy juices, which means that pectinases are not normally used for the production of such juices. Under certain circumstances, the starch contained in the juice may have a negative effect on the cloud stability. Native starch dissolved by heating processes initially increases the viscosity of the juice and thus also the conditions for better cloud stability. However, during storage the dissolved starch retrogrades in the juice and particularly in the concentrate, and precipitates. The initially increased viscosity then drops again. The suspended particles stabilised by the previous rise in viscosity can then no longer be kept in suspension, and settle. Part of the retrograded starch settles immediately. Another part reacts with polyphenols to form specifically 'heavy' starch/phenol complexes, which also settle. Overall, therefore, the starch in naturally cloudy juices and concentrates is more likely to lead to cloud instability than to increased cloud

stability. The latter can be ensured by targeted treatment of the juice with suitable glucoamylases.

Prompt processing is essential for manufacturing cloudy apple juices of high quality. In the most favourable case, it takes only a few minutes from crushing of the apples to pasteurisation, which minimises the effect of the enzymes contained in the fruit, such as pectinases and oxidases. This is where continuous decanter separation processes have proved most useful. The cloudy apple juices thus obtained are very cloud-stable and show less tendency to browning. The addition of ascorbic acid affords further protection against oxidation, enabling the manufacture of cloud-stable juices and concentrates of particularly light colour.

7.10 Grapes

The manufacture of grape juice and grape juice concentrate for the alcohol-free fruit juice industry has a long tradition, particularly in the US. The highly popular hybrid grape Concord provides purple-coloured juice with a pronounced 'foxy' flavour, which is usually undesirable in Europe but is a standard feature of grape juice in the US. The Concord grape is mainly cultivated in the cooler regions of the US and Canada. In Europe and California, the main varieties processed into grape juices and concentrates are white *Vitis vinifera* grapes such as Gorda, Thompson Seedless and Muscat. Red *Vitis vinifera* grapes are also used in smaller quantities.

7.10.1 Processing of Concord grapes

The particularly high demands made on the colour yield and colour stability of Concord grape juices have influenced the processing technology employed. The best colour values are obtained when the mash is first flash-heated to 88–90°C and then exposed to enzyme treatment at 58–60°C for about one hour (figure 7.11). The high mash temperature destroys the oxidases present in the fruit, which have a negative influence on the colour stability. Very high demands are therefore also made on the pectinases used for mash treatment, with regard to their oxidase secondary activities and temperature stability. For this reason, only pectinases with very good temperature tolerance are suitable. In the course of further processing, relatively low temperatures around 0°C are used for cold settling and tartrate stabilisation, which again makes extreme demands on the chosen pectinase product. Obtaining a juice of optimum clarity depends less on the pectinase and more on the secondary activities. The best suited pectinases are therefore characterised not by a particularly high pectinase activity, but rather by the composition and quantity of secondary activities, such as hemicellulases and proteases.

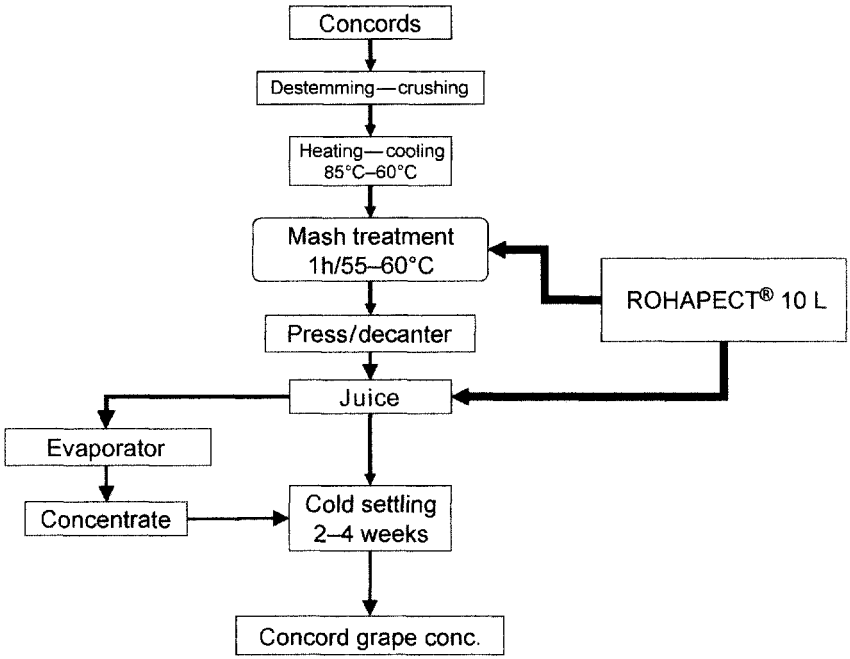

Figure 7.11 Processing of Concord grapes.

7.10.2 Manufacturing white grape juice and grape juice concentrate

The lowest possible temperatures are employed for processing white grapes, because of quality considerations. This is particularly necessary to retain the temperature-sensitive grape aroma, in Muscat varieties, for example.

Pectinases with a large proportion of pectinesterase, liquefying polygalacturonase and pectin lyase are suitable for the mash treatment of white grapes. Treatment is best performed at temperatures of about 20°C (figure 7.12). The hydrolysis of soluble pectin promotes juice separation in the drainer, making it possible to obtain larger quantities of free-run juice. Owing to the selective breakdown of soluble pectin, the matrix-forming protopectin remains largely intact, leading to a drainage effect that promotes the juice flow in the subsequent pressing step. A further advantage is that free-run and press juice with lower cloud content is obtained, which has a favourable effect on further processing. During subsequent aroma extraction, the enzymes are inactivated by heating. The best-suited pectinases for subsequent depectinisation are those which still contain arabanases and thus optimise pectin degradation, clarification and filtration. Some grape varieties contain hairy-region pectins, and in these cases the use of suitable arabanases leads to better clarification. Depending on the

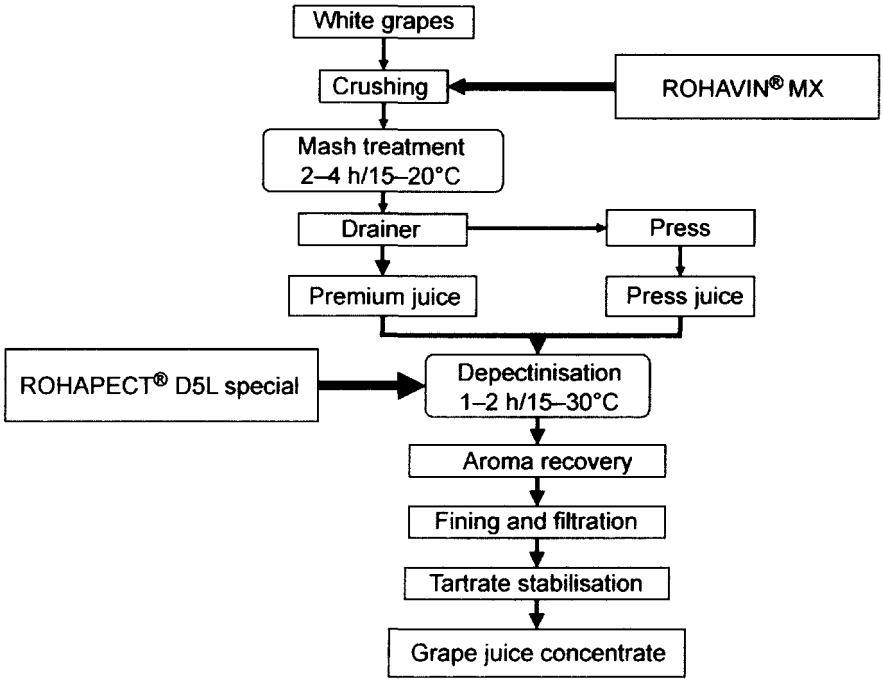

Figure 7.12 Manufacture of white grape juice and grape juice concentrate.

type of grape, pectinases with high hemicellulase and protease activity can also be employed, especially when filtration is performed with membrane systems.

7.11 Berries

The primary aim when processing berries is to achieve an optimum yield in colouring matter and valuable constituents. Since the major berry varieties (cranberry, blackcurrant, raspberry and strawberry) contain large quantities of pectic substances, enzyme treatment also calls for much larger quantities of enzymes in order to produce a clear and stable berry juice concentrate (figure 7.13). When manufacturing non-concentrated juices, depectinisation can be dispensed with, leading to higher viscosity and a better mouth feeling of the juice. Pectinases with good acid stability are suitable for mash treatment, especially of blackcurrants and cranberries. The pectinases suitable for total pectin hydrolysis are those with a high proportion of pectinesterase and macerating polygalacturonase, as well as a rather low content in pectin lyase. Treatment is performed at optimum temperature conditions of 50°C, and has a pronounced

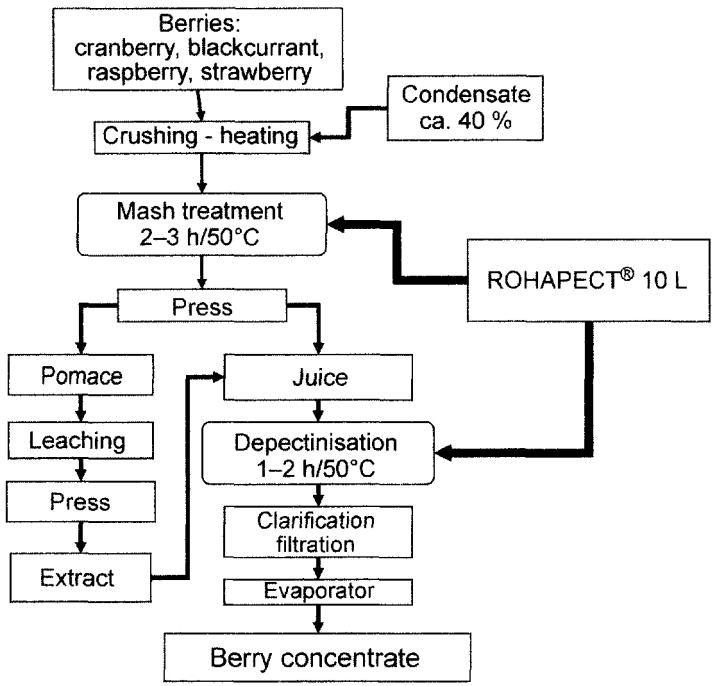

Figure 7.13 Processing of berries.

liquefying effect. This improves colour extraction—the main consideration—as well as optimising press performance and yield. Excessive maceration and liquefaction due to cellulolytic and hemicellulolytic secondary activities should, however, be avoided, since this is accompanied by the release of a greater quantity of undesired acid polysaccharides that may have a negative effect on the filtration and stability of clear berry juice concentrates.

Juice depectinisation is usually performed with the same kind of pectinases as mash treatment, normally after aroma extraction of the juice. Some fruits, such as raspberries, naturally contain large amounts of colloids. With these, the use of hairy-region pectinases or hemicellulases leads to improved filtration. Raspberries infected with botrytis present particular difficulties, because the raspberry glucan formed by this fungus is extremely difficult to degrade.

Acid stability is naturally an important criterion in suitable pectinases, because many berry fruits have high acid values and low pH values. For most pectinases, that means a reduced reaction speed which is no longer within their optimum range. Moreover, treatment is often performed at temperatures above 50°C in order to optimise colour extraction. These conditions impair the stability of the pectinase, which means that more and more of the enzyme is inactivated at

higher reaction speeds. It is therefore important to select pectinase grades that provide optimum results under the given circumstances.

Fruits with pigments and aromas that are sensitive to oxidation (e.g. strawberries) are best subjected to enzyme treatment at low temperatures of less than 25°C. Tests at low temperatures with a combination of pectinases and cellulases have shown the best results. This is of course subject to the restriction on the use of cellulases in the EU.

Pectinases with highly macerating polygalacturonase and pectin lyase are suitable for manufacturing purée concentrates. Their application aims at viscosity reduction and ease of concentration.

The process for manufacturing clear berry juice concentrates consists of complete depectinisation, controlled by the pectin test, followed by clarification, filtration and concentration. Cross-flow ultrafiltration cannot be recommended for berry juice manufacture owing to the pronounced colour loss. Cross-flow microfiltration, however, is suitable, because of the much larger membranes, usually combined with fining agents such as bentonite, gelatin and silica sol.

7.12 Stone fruit

Stone fruits such as peaches and apricots are mainly processed into purées or purée concentrates. Enzymes are being increasingly employed for the latter application, suitable products being pectinases with highly macerating polygalacturonase and pectin lyase. This combination provides good results with regard to maceration, viscosity reduction and cloud stability. High pectinesterase values, on the other hand, have a negative influence on cloud stability.

These macerating polygalacturonases can also be employed for ordinary purée manufacture and provide more homogenous products with greater cloud stability. Pectinases with hemicellulase secondary activities are particularly suitable for manufacturing clear juices and concentrates from peaches, sour cherries and other stone fruit varieties. The main target in this context is ease of filtering, particularly during ultrafiltration.

7.13 Citrus fruit

Enzymes have become increasingly significant in citrus processing where they are more and more involved in the manufacturing of specialities produced from the various by-products. New processing techniques, such as the debittering process, can also be optimised by using specific enzymes. Tailor-made enzyme products offer advantages both in terms of quality improvement and cost saving. Citrus processors can increase their flexibility by producing various kinds of products and by-products. Furthermore, their manufacturing

processes can be optimised to reduce operational problems. Pectinases with high endopectin lyase are particularly suitable for the most important citrus applications: peel extraction, pulp wash or 'WESOS' (water-extracted soluble solids).

7.13.1 WESOS

The main focus with oranges is to process the internal portions of the citrus (pulp, rags, cores) and peel into water-extracted soluble solids (WESOS) (figure 7.14). This term covers a variety of by-products, the most important being pulp wash, although it is becoming less significant since the in-line addition of this extract during orange-juice concentrate manufacture has become generally accepted. Instead, other by-products are establishing themselves in the market, known as core wash or cloudy peel (figure 7.15). These products also incorporate sections of peel, which often require debittering. Pectinase treatment is necessary with all WESOS products in order to thicken the extracts to the desired concentration. Pectinases with a high proportion of pectin lyase are especially suited for this purpose, because they effect rapid viscosity reduction while retaining the cloud stability. Highly purified pectin lyases are also advantageous with regard

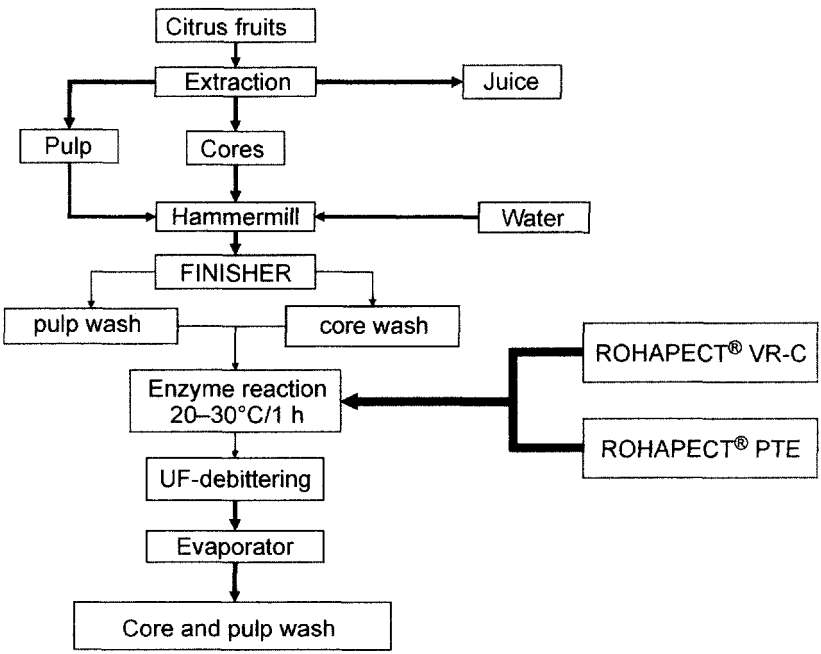

Figure 7.14 Processing of pulp and core wash.

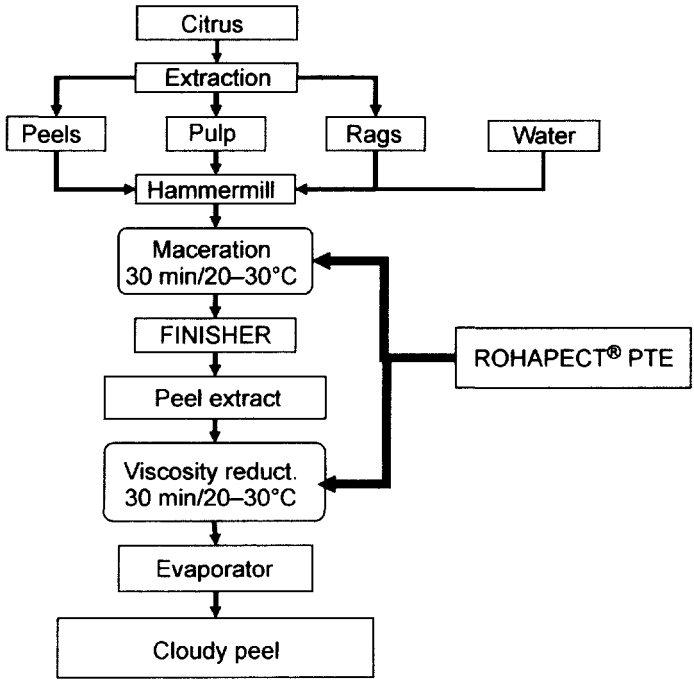

Figure 7.15 Processing of peel.

to dosage tolerance, because their effectiveness towards average-sized pectin fragments is often greatly restricted and relatively high dosages are required in order to hydrolyse all of the cloud-stabilising pectin.

The debittering processes that have been popular for quite some time place special demands on enzymatic treatment. During this process, the juices and extracts containing bitter substances are separated into retentate and permeate on an ultrafiltration unit. The clear permeate is then fed through an ion exchanger. The resins in the exchanger bind the bitter substances limonin and naringin, so that the product obtained by recombining the permeate with the cloudy retentate contains a much lower amount of bitter substances. This is particularly interesting in connection with grapefruit products and improves their flavour acceptability.

Pectinases combined with proteases are suitable for the required enzyme treatment, which considerably improves the flux and capacity of the UF unit.

Citrus juices contain relatively large amounts of protein which is largely responsible for membrane fouling. Suitable proteases can be used to hydrolyse the macromolecule to lower molecular weight peptides that are less likely to clog the membrane.

7.13.2 Citrus juice concentrates

So far, enzyme treatment has only been used in exceptional cases for manufacturing cloudy citrus juice concentrates (figure 7.16). This means that the majority of concentrated orange juice available on the market has not been enzymatically treated. Such treatment is necessary for manufacturing orange or grapefruit juice concentrate when the corresponding debittering processes involve ultrafiltration. A further application for pectinases is for targeted viscosity reduction to improve the concentratability of the juice. Early-season orange varieties or the in-line addition of pulp wash may make such treatment necessary in highly viscous juices, to avoid problems with concentrate manufacture.

Pectinases with high pectin lyase activity rapidly split the high molecular weight, soluble pectin into medium-sized pectin fragments, thus reducing viscosity within a very short time by 25–50%. The selective pectin lyase activity prevents total degradation of the pectin, which would cause cloud destabilisation.

The enzyme product is best added continuously after the finisher. The enzyme reaction takes place in the buffer tank and takes 10–30 min. depending on the

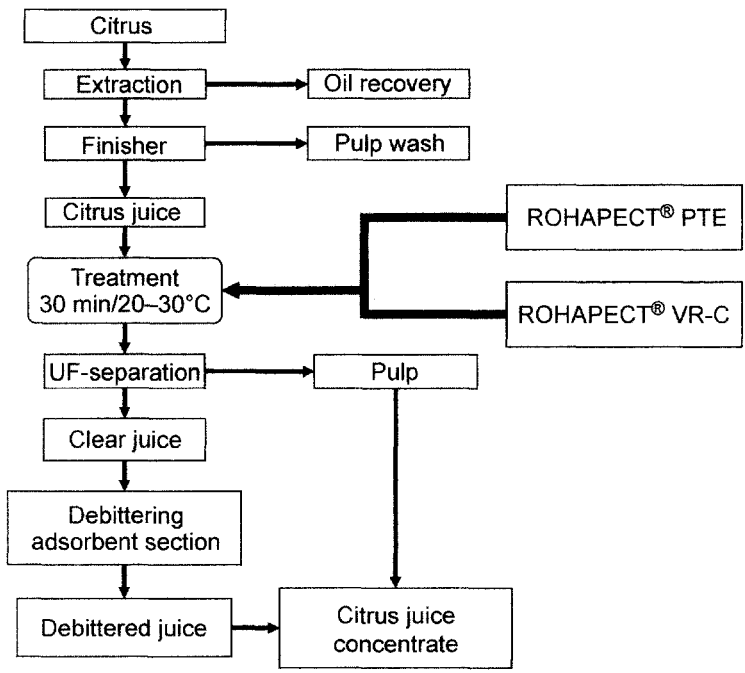

Figure 7.16 Processing of citrus juice concentrates.

process parameters. The enzymes are inactivated during subsequent heating, and the reaction stopped.

7.13.3 Clear and semi-cloudy citrus concentrates

The demand for clear and semi-cloudy citrus concentrates has grown continually in recent years. Oranges and mandarins as well as lemons are increasingly being processed into citrus concentrates (figure 7.17). These generally require particularly acid-stable pectinases because the main product, lemon juice, may exhibit pH values of less than 2.5. For successful depectinisation, it is important to have a balanced ratio of PE, PGs and PL with a high proportion of hemicellulolytic secondary activities.

The reaction temperature is selected as a function of the pH value of the juice, particularly in the case of lemons. This is because, under unfavourable conditions, the pectinases are inactivated before all of the pectin can be degraded. Depending on the chosen pectinase product, lower reaction temperatures therefore need to be used at lower pH values; for example, about 25–30°C at pH 2–2.5 and about 30–40°C at pH 2.5–3.

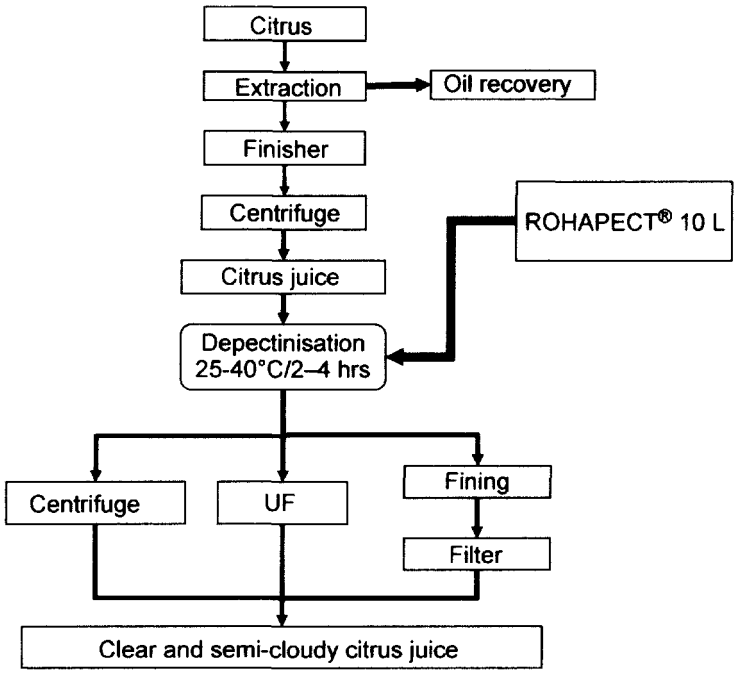

Figure 7.17 Processing of clear and semi-cloudy citrus concentrates.

After depectinisation of the juice, there are various options for further processing, depending on the situation:

(a) centrifugation to obtain a semi-cloudy concentrate
(b) ultrafiltration to manufacture a clear concentrate
(c) traditional fining with bentonite and silica sol, followed by filtration with diatomaceous earth to provide a clear juice or concentrate.

7.13.4 Extraction of citrus oil

Citrus oils are very much in demand as essences and flavouring substances for the food and perfume industry. Various methods exist for their extraction (figure 7.18).

In the FMC process, dejuicing and oil separation are performed in a single step. Owing to mechanical treatment of the peel during the pressing step, the oil emerges from the flavedo, is washed off with water and is collected separately. With the Brown method, the peel of the citrus fruit is pricked before dejuicing and the emerging oil is rinsed off. With the Indelicato method, the flavedo is

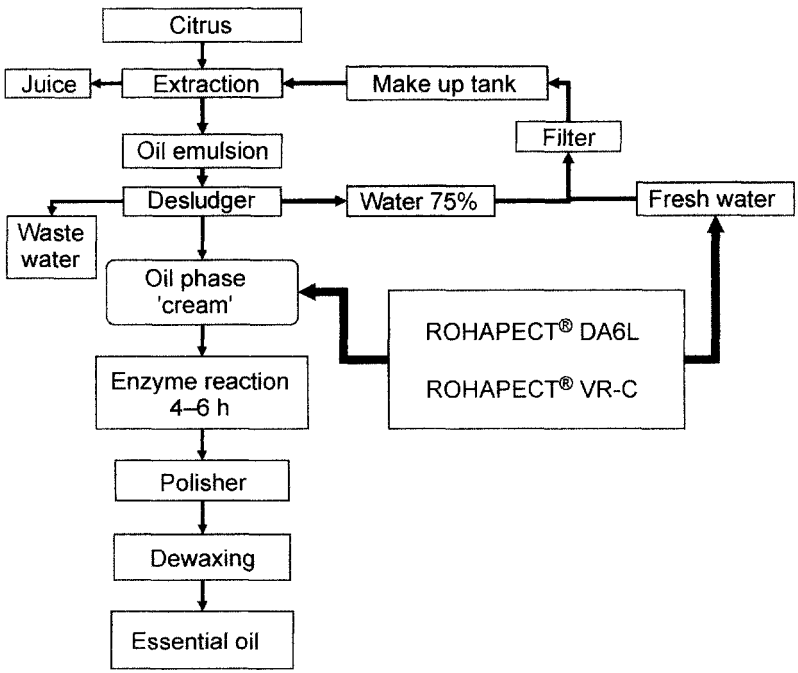

Figure 7.18 Extraction of citrus oil.

grated off, mixed with water and subjected to further processing. However, an oil emulsion with 1–3% oil is obtained, which is concentrated to 50–80% in the next step by means of centrifuging.

A polishing separator is employed to remove the residual water and fine solid particles from the essential oil, which is also referred to as 'cream' or 'rich emulsion'. Depending on the extraction system employed, unwanted waxes enter the oil phase. Cold storage results in the precipitation and separation of the cold-sensitive wax portion.

Pectinase prepations containing hemicellulases and proteases as side activities are best suited to treat the 'cream'. The use of this enzyme cocktail effects the breakdown of pectins and proteins as well as of various polysaccharides which may contribute to the separation of the oil/water emulsion. Furthermore, it improves the dewaxing process by producing a faster and more complete precipitation of these substances. Cost and ecological considerations have induced an increasing number of citrus processors to recycle the water separated through centrifuging. The advantages of this water-recycling process are lower water consumption, less waste water and less pollution in the waste water, because citrus oil has a bacteriostatic effect, even in very small concentrations. The separated water, however, has to be appropriately treated to ensure a smooth process. During recycling, pectins also accumulate in the water and may impede filtration. The breakdown of these pectins simplifies the process, ensuring trouble-free system operation.

7.13.5 Cloudifier

Natural cloudifier products are special concentrates, initially produced with high amounts of citrus peel. High-quality cloudifiers (figure 7.19) which satisfy certain special requirements necessary for their use in lemonades, for example, can be obtained by the so-called 'disintegration process', providing:

- excellent cloud stability (Imhoff test: 0)
- high turbidity and optical density
- good colour and flavour
- sufficient 'concentratability'

Enzymatic treatment can be divided into two steps:

- Maceration with suitable pectinases containing a high proportion of macerating polygalacturonase and cellulases. The raw material consisting of peel, pulp and rags is very finely crushed, mixed with water and finally pasteurised. All of the enzymes present in the fruit are thereby inactivated. Optimum reaction conditions, particularly the correct temperature, are chosen for enzymatic maceration. The fruit tissue thus dissolves and is liquefied.

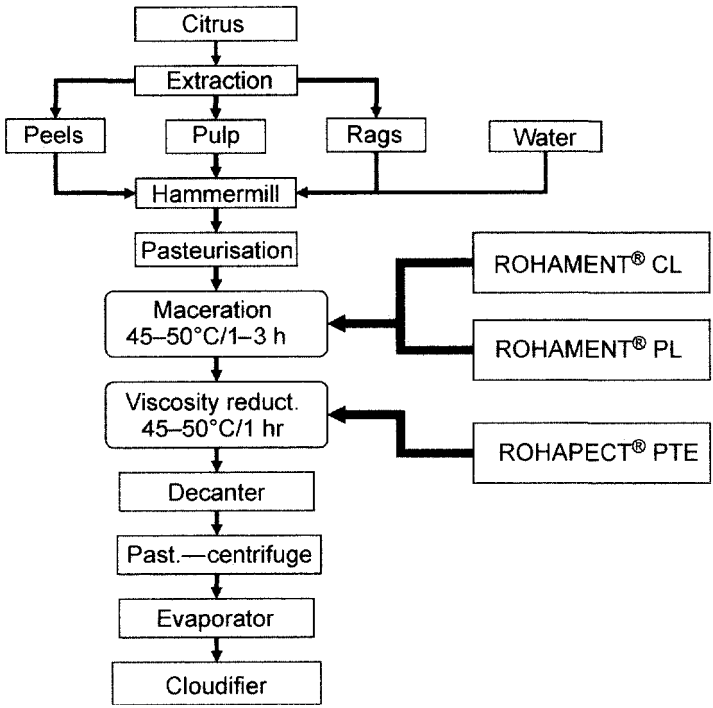

Figure 7.19 Enzymatic treatment of cloudifier products.

- Pectinases with a high proportion of pectin lyase are suitable for subsequent viscosity reduction of the soluble pectin. These pectinases split high molecular weight pectins into short-chain pectin fragments. The related viscosity reduction makes the product more suitable for concentration. Pectinases with high degrees of pectinesterase activity are not suitable for enzymatic treatment because of their negative influence on cloud stability.

The subsequent processing steps effect the complete separation of particles which can be precipitated (e.g. by using decanters and centrifuges). Homogenisation, which helps to emulsify the citrus oil and improves cloud stability, is often conducted prior to concentrating. The concentrates obtained by this disintegration process are usually of a higher viscosity and can therefore only be concentrated to 40–50°Brix.

7.14 Tropical fruit

Clear concentrates can be manufactured from pineapples, guavas and bananas, for example, using pectinases and hemicellulases. Pineapples contain large

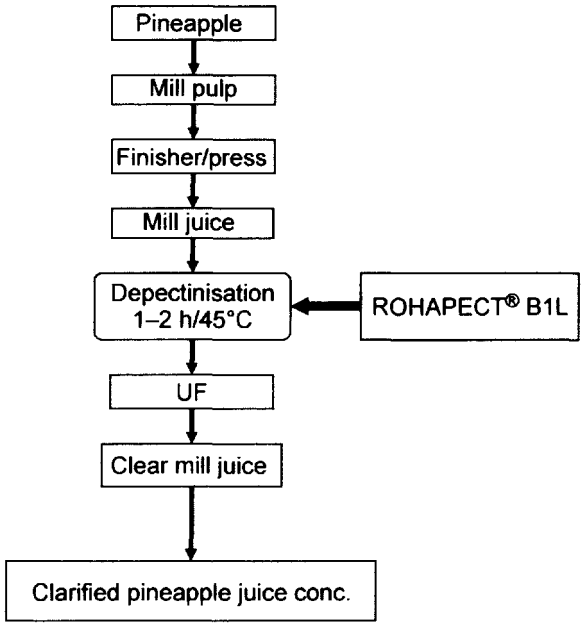

Figure 7.20 Processing of pineapple.

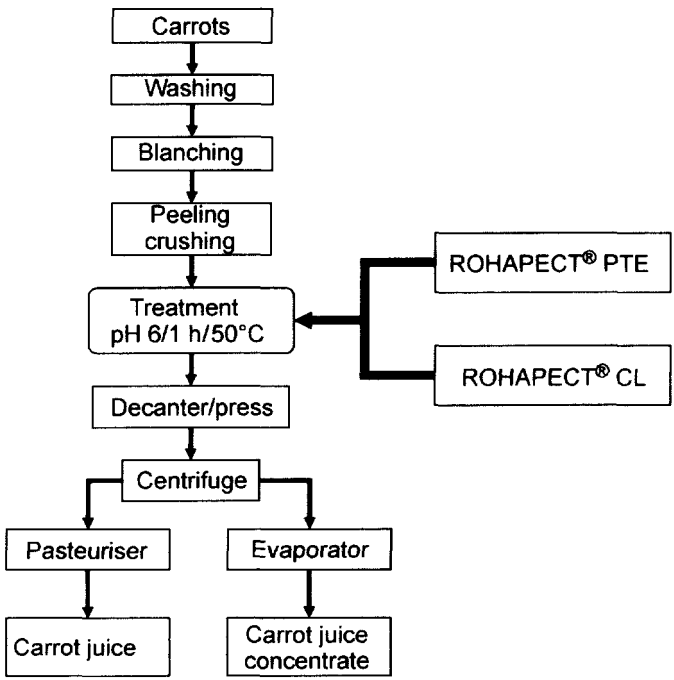

Figure 7.21 Processing of carrots to make carrot juice and carrot juice concentrate.

quantities of galactomannan, which means that the use of galactomannanase improves the ease of filtration, especially during cross-flow ultrafiltration (figure 7.20). Pectinases are also occasionally employed for manufacturing purées and purée concentrates. Pectin lyase reduces the viscosity and prevents concentrated banana purée, for example, from gelling. Macerating polygalacturonases are suitable for processing mangoes and passion fruit. They liquefy the fruit tissue and facilitate processing. Moreover, they improve cloud stability and homogeneity.

7.15 Vegetables

Carrots are the most commonly processed vegetables, and are either used to manufacture carrot juice and concentrate (figure 7.21), or carrot purée (figure 7.22). Pectinases with high pectin lyase activity, combined with cellulase, are suitable for juice or concentrate manufacture. In this case, the legislator allows the use of cellulase, which leads to much better results as far as juice yield and *beta*-carotene yield are concerned.

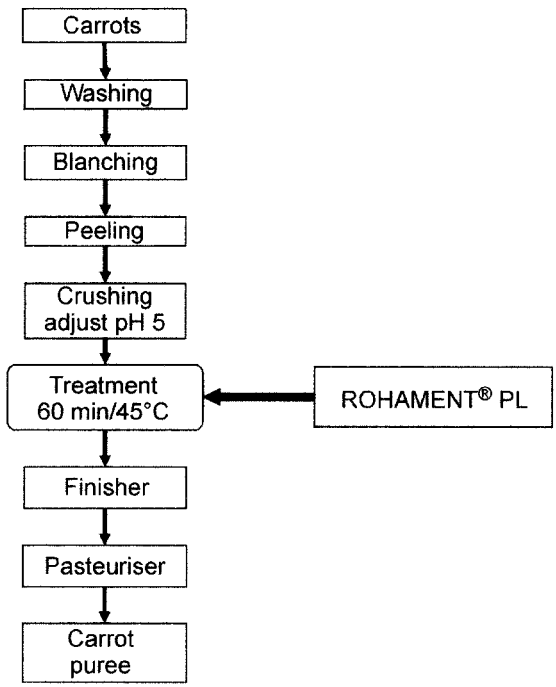

Figure 7.22 Processing of carrots to make carrot purée.

It is important that the enzymes employed still show sufficient activity at pH values of more than 6. There is then no need to acidify the mash, which would lead to an undesired change in taste of the carrot juice or concentrate.

Pectinases with predominantly macerating polygalacturonase are used for manufacturing carrot purée. These are able to dissolve the high molecular weight protopectin. This treatment results in a cell suspension made up of dissolved pectin. The purée thus obtained is fine, homogenous and highly viscous. Clear vegetable concentrates are also of interest, such as those made from beetroot, used as a natural pigment in the food industry. These can be obtained by means of pectinases with hemicellulase, to optimise pectin degradation and colour extraction.

7.16 Membrane cleaning

A relatively new field of application for enzymes is the cleaning of the UF membranes that are ever more widely used in the fruit juice industry. This treatment has nothing to do with the final product, the fruit juice, which is why the legal provisions naturally do not apply in this case. However, it does belong to fruit juice technology and therefore deserves mention here.

Various concepts are being discussed. Highly concentrated arabanases and cellulases can be used for this purpose. Good results can also be obtained with a combination of suitable cellulases and proteases. Basically, two different processes suggest themselves: alkaline cleaning at pH 7–9 or acid cleaning at pH 4–6. The optimum pH range is selected for the respective enzyme. Experience so far has shown a preference for alkaline cleaning, particularly among pome fruit processors.

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8 Enzymes in fruit processing

Catherine Grassin and Pierre Fauquembergue

8.1 Citrus peeling

Consumption of fresh and processed citrus products has risen to an output of 65 million tons. Average US consumption is about 60 kg, while in Western Europe it is around 40 kg per person. Frozen concentrated orange juice was initially developed in the 1940s and it is now the most widely consumed fruit juice in the world. Although juice is the most important product, the fruit is also processed to obtain by-products, the most significant of which are pectin and essential oil. Although in certain countries the use of enzymes is not permitted in the production of premium orange juice, they can be used for increasing the value of by-products [1]. Enzymes can increase the yield of solid recovery during pulp washing, facilitate the production of highly concentrated citrus bases, improve essential oil recovery from peel, debitter juice, clarify lemon juice or increase the worth of waste products [2].

An example of enzyme use in the citrus industry is the pre-peeling of citrus fruit. The only industrial enzyme infusion process with a commercial application is that of applying vacuum or pressure infusion of pectinases to soften citrus fruit peel for removal. Treating orange or grapefruit by pectinase infusion results in the digestion of the white layer albedo of the fruit peel, below the coloured flavedo, but without enzyme penetration into the fruit juice segments [3]. Citrus peel composition is described in the table 8.1 [4]. The successful infusion of an enzyme solution is dependent on the porosity of fruit tissue. Citrus fruit are ideally suited to peeling by vacuum infusion of pectinases, because the edible portion of the fruit is relatively solid and free of voids, whereas the albedo and core of the fruit are extremely porous. Pectinase solution is preferentially drawn into these porous tissues, maximising its effectiveness. Although gases within citrus white albedo can be removed under vacuum through pores, aqueous enzyme solution cannot enter easily.

The flavedo must therefore be scored to permit the entry of the enzyme solution. When properly infused with pectinase solution, citrus peel separates cleanly at the outer surface of the segments, providing an attractive peeled fruit. Thus fresh citrus fruit can be converted to an easily consumed convenience food. Several food plants in the US, Japan, South Africa and the UK are currently using this technology to peel citrus fruit and market the peeled fruit as a fresh refrigerated item. The process for peeling of fresh citrus fruit using enzyme

Table 8.1 Citrus peel composition

In % of fresh weight	**50% of whole fruit weight**
Water	76–80%
Total soluble solids	16%
Ash	0.7%
Citric acid	0.7%
Volatile oil	0.4%
Crude fibre	1.7%
Protein	1.1%
Pectin	4%
Total sugars	6.35%
Pentosans	0.8%
Calcium pectate	3.2%
Naringin	0.4%
pH of peel	5.9–6.4
pH of pulp juice	3.0–3.3
In % of dry weight	**20% of whole fruit weight**
Total sugars	25–37%
Hydrolysable polysaccharides	7–11%
Insoluble solids	30–40%
Ratio of flavedo to albedo weight	0.75
In % of alcohol insoluble solids (AIS)	**50–68% of dry weight**
Pectic substances	42–52%
Hemicellulose	9–12%
Cellulose	30–40%
Polygalacturonic acids	23%
Araban	7–10%
Galactan	5–6%
Xylan	2%

infusion is extensively described in different patents from Sunkist Growers Company [5]. In the first stage of the process, the peel of the fresh citrus fruit is scored or broken so as to penetrate the peel's thick albedo layer but not to penetrate any of the underlying fruit segment or segment membranes. The scored fruit is then dropped into a 2% pectinase solution, before the infusion stage. Rapidase Citrus Peeling (DSM) and Peelzym (Novozymes) are enzymes proposed for this technology. They contain pectinesterase and polygalacturonase which degrade albedo pectin around segments. The pectinase solution is infused into the scored fruit using either a vacuum process or a pressure process. In vacuum infusion, the fruit and enzyme solution are placed together in a vacuum chamber, and a vacuum of 25–30 inches of mercury is applied to draw off air from the fruit peel. Releasing the vacuum infuses the enzyme solution into the spaces in the peel previously occupied by air. The pectinase-infused fruit is then removed from the vacuum chamber and placed into storage tubs at room

temperature for 45 min to 2 h for albedo digestion inside the fruit. The fruit peel can thereafter be readily removed with only minimal amounts of albedo binding to the fruit sections. Segments can be separated without damaging the membrane of the segment. Peeled fruits are then rinsed, cooled and packed. In this process there is no need to heat the fruit.

Successive batches of fruit can be processed effectively using the same enzyme solution by replenishing the solution each time to replace the amount infused into the previous batch of fruit. This process results in an easy fruit hand peeling, providing individual fruit segments having fresh fruit flavour and appearance.

8.2 Citrus peel processing

After sampling and testing of incoming fruits, for which the maturity standard is the ratio brix/acid, oranges enter the extractor, the first stage of the process. The extraction equipment and finishing operations affect yield, quality and characteristics of the citrus juice produced. There are two main types of extractors and finishers manufactured by the companies FMC and Brown in the US and Indelicato in Europe. The expressed juice from the extractors contains too much pulp and cell membrane residue, thus processing in a finisher (rotating screw principle) is required to reduce the pulp content. Standard finishing reduces the pulp content of juice to about 12%. The excess pulp and tissue by-products of extracted juice leaving the primary finishers are processed through a pulp wash system [6]. The processing of citrus fruit into citrus juice generates enormous amounts of by-products. Approximately 40–60% of the weight of fruit ends in waste peel, segment membranes, rags, seeds and other by-products. Pectin and essential oils are the main valuable products. However, the commercial producer of pectin can utilise only a limited amount of the available peel residue for conversion to by-products. Citrus waste is ultimately dried and marketed as a cattle feed. However, due to the high cost of water evaporation, poor nutritional value and low selling price, the trend is to transform citrus by-products into more valuable products such as natural sugars, or aromatic sugar fibre bases for soft drinks. The process is summarised in figure 8.1.

To avoid the addition of artificial cloudifier to citrus beverages, there is a demand for a natural cloudifier, obtained from the citrus fruit itself. Cloudy peel products can be obtained using the enzymatic process described in figure 8.2. Communited products, peels and rags are ground to a particle size of 3–5 mm and mixed with water, heated at 90°C to destroy the citrus pectinmethylesterase to avoid clarification, cooled down at 50°C, then dosed with pectinases like Rapidase Peel Pressing (DSM), Citrozym (Novozymes) or Rohament PC (Röhm). Commercial pectinase contains a high pectin lyase activity that is stable under these process conditions. It increases the yield of sugar and fibres, improves

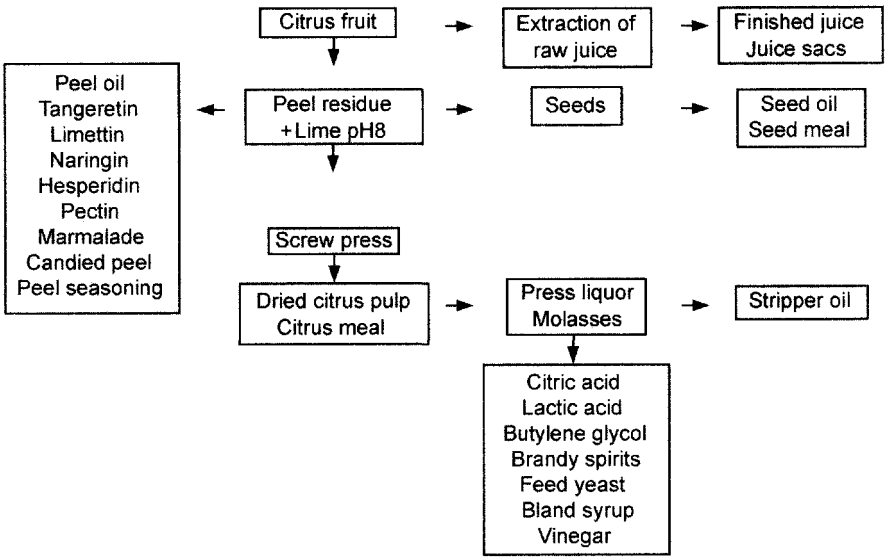

Figure 8.1 Processing of citrus peel.

the cloud stability, decreases the viscosity and makes the concentration of the extract easier. The final concentrate at 65° brix has a high content of pectin, polysaccharides, glycoproteins, waxes and essential oils. It can be used as a cloudy aromatic sugar and fibre source for soft drinks and other citrus taste beverages or desserts. Citrus peel extract can be sold as a clear colourless sugar after de-aromatisation, de-ionisation and decolourisation with resins prior concentration.

8.3 Fruit firming

The manufacture of food, comprising entire fruit or fruit pieces, is conventionally performed by mixing fruit, sucrose and other ingredients, such as starch or pectin, then heating the mixture in order to form a gel (and also to prevent microbial contamination). However, the use of fruit preparations is limited by the weak texture of fruit or fruit pieces, especially when they are mixed with dairy products (i.e. ice cream, fruit yoghurt). Most fruits such as strawberries are damaged by thermal treatment, freezing or pasteurisation, or as a result of osmotic transfer of water and sugar in fruit preparation or in syrup. The heating stage has a negative effect on the fruit texture and firmness, giving a fluffy appearance and mouth feel.

The texture of fruit is attributed to the structural integrity of the primary cell wall and the middle lamella. The primary cell wall consists of cellulose

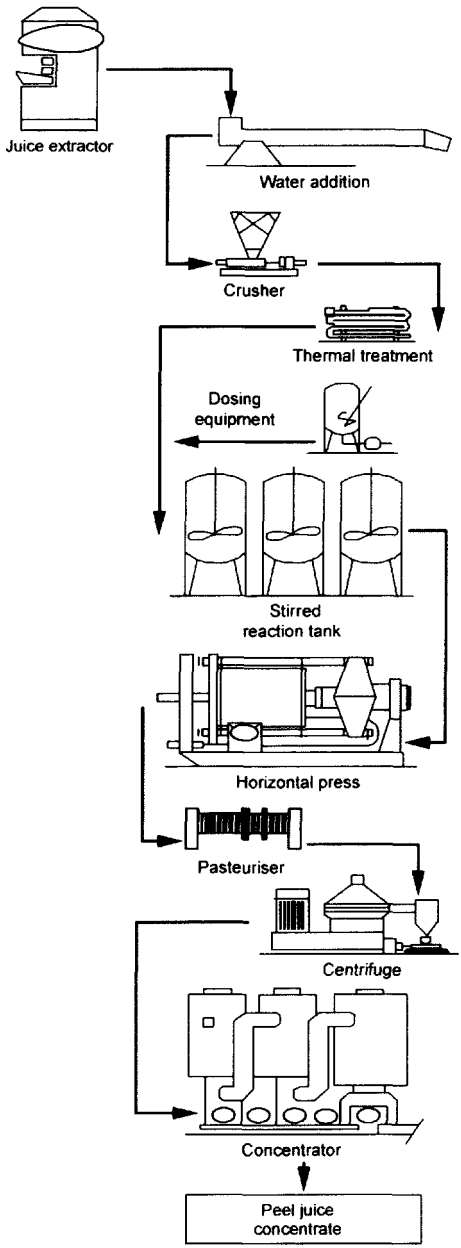

Figure 8.2 Enzymatic processing of citrus peel. Peels are added 1:1 to hot water, and the mix is milled to fine particles. The mash is heated to 90°C for 3 min and cooled to 50°C. Enzyme is added. Maceration takes place in the stirred reaction tank, at 50°C for 1.5–3 h. Horizontal presses or decanters are used to extract peel juice, which is then pasteurised, centrifuged and concentrated.

micro-fibrils aligned in parallel, cross-linked with hemicellulosic xyloglucan polymers [7]. Pectin is the major structural polysaccharide of fruit lamella and cell walls. Pectin contains smooth regions (60–90%) as homogalacturonan and hairy regions (10–40%) as highly branched rhamnogalacturonan type I [2] (see figure 8.3). Homogalacturonan is a homopolymer of (1,4)-α-D-galacturonic acid residues, partly esterified with methanol and sometimes with acetyl groups. Hairy regions are composed of rhamnogalacturonan (RGI) [1,2-α-L-rhamnosyl-(1,4)-α-D-galacturonic acid] with α-1,5-linked L-arabinans and β-1,3-1,6-D-linked arabinogalactan type II as side chains covalently attached to RGI. Pectins are divided into two groups on the basis of their different gelling properties. High methoxyl pectins (HM pectins) have usually more than 50% methoxylated polygalacturonic acid units, and thus practically no reaction with calcium ions. The gel strength depends on acid content and soluble solids, among other factors. The degree of esterification correlates with the gel setting rate and gel texture. Very high methoxylated pectins gel more quickly at higher temperatures than lower methoxylated pectins. They form elastic and brittle gel textures. This accurate correlation requires a very homogeneous intermolecular and intramolecular carboxyl group distribution, as in apple pectin. Low methoxyl pectins (LM pectins) have less than 50% methoxylated polygalacturonic acid units that can gel with calcium ions. LM pectins do not only form gels with sugar and acid but also with less soluble solids and with calcium ions compared to HM pectin. Factors that affect gel strength are: pectin volume, type of pectin, dry soluble solids, pH range and concentration of buffer salts and calcium ions contained in the environment. A balance between pectin and calcium concentration will lead to an optimal texture.

The association of pectin chains leads to the formation of three-dimensional networks: gel formation. Two or more chain segments of methoxyl homogalacturonan bond together and start to interact. Fruit pectin with a low methoxyl content or after methanol group removal with pectinesterase (endogenous or exogenous enzymes), may form bonding zones (egg-box conformation) with bivalent cations and form gels relatively independently of soluble solids content and pH range. The calcium concentration required for gelling depends on pH of the product, soluble solid content and buffer system. With addition of a small amount of calcium, pectin chains will start to cluster over calcium bonds. With increased calcium concentration, a gel will form. Exceeding the calcium optimal concentration will produce a brittle gel with tendency towards syneresis or ultimately to calcium pectate formation. Over-dosage of calcium ions will lead to calcium pectate formation and precipitation of this insoluble form of pectin. Precipitation of calcium is only reversible to a limited extent, even when the gel is once more heated above its setting temperature and cooled down without destruction [8]. Fruit pectin composition and concentration are

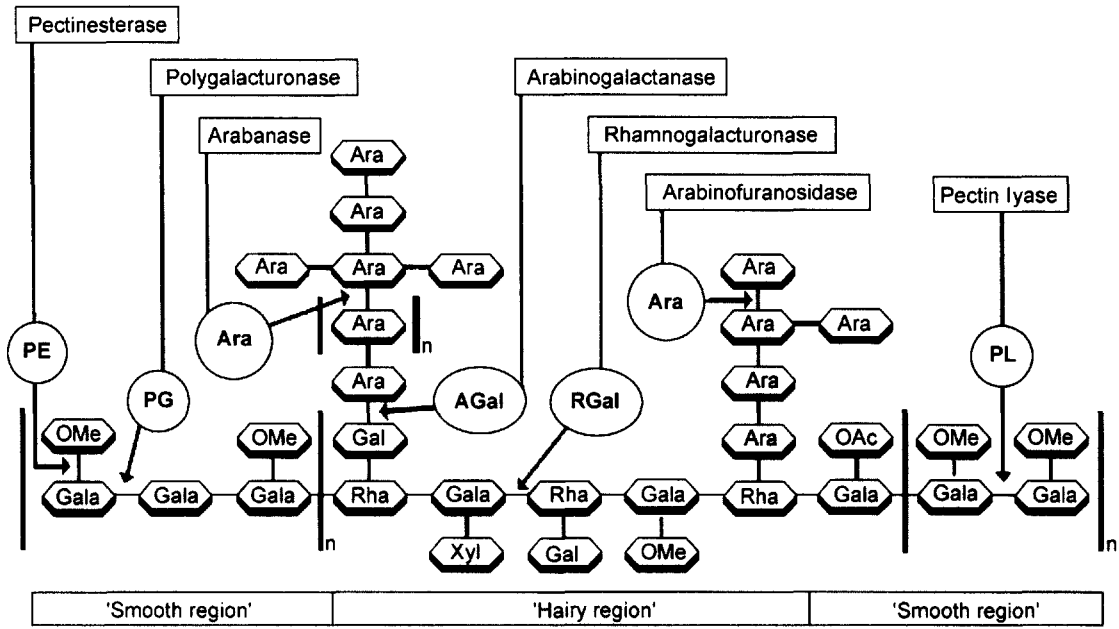

Figure 8.3 Fruit pectin composition. Gala: galacturonic acid; Ara: arabinose; OMe: methyl ester; OAc: ethyl ester; Xyl: xylose; Gal: galactose; Rha: rhamnose; n: number of units.

very variable. Pectin content and esterification percentage (methyl and acetyl esters) are specific to the fruit species (see table 8.2). However, during fruit ripening and depending on climatic conditions, pectin molecular weight and methoxyl percentage tend to decrease due to fruit pectinases (endogenous pectinmethylesterase and polygalacturonase).

Consumer demand increasingly requires that fruit in food products should have well defined characteristics of fruit identity, firmness and stability (e.g. shape, colour). In order to improve the resistance of fruit, increase firmness and prevent fruit damage due to severe conditions of heating and pumping during preparation, a fruit treatment with pectinmethylesterase has been proposed [9]. The FirmFruit® concept is the innovative application of a pure fungal pectinmethylesterase (PME) with calcium to any whole fruit, fruit pieces or fruit puree being processed in fruit preparations or in fruit sauce. When PME removes the methyl ester group from the fruit pectin *in situ*, pectic acid binds to calcium ions forming insoluble calcium pectate (see figure 8.4). The major advantage of the process is that fruit damage and disintegration after heating is minimised. This effect is markedly strong where a combination of PME and calcium ions (from calcium chloride or lactate) is used. Neither the PME nor calcium ions give by themselves the same positive effect. The process improves integrity, firmness and texture of the fruit. The release of fruit aromas is enhanced, the red colour is better maintained and the resulting fruit preparation is preferred by the consumer for taste and flavour compared to an untreated fruit preparation [9]. The commercial pectinmethylesterase Rapidase® FP Super is produced by *Aspergillus niger* and is free of other pectin depolymerase activities such as pectin lyase, polygalacturonase or rhamnogalacturonase. PME from *Aspergillus niger* has a molecular weight around 45,000 Daltons. If fungal PME is compared with plant PME, the enzyme from *Aspergillus niger* has an optimal pH at 4.5 (not too far from crushed fruit pH), while it is between pH 7.0 and pH

Table 8.2 Fruit pectin and calcium content

Fruit	Pectin (% wet weight)	Pectin methylation (%)	Total calcium in fruit (ppm)
Apple	0.7–1.6	65–85	90
Blackcurrant	1.0–1.2	50–80	180
Grape	0.1–0.4	50–65	250
Orange	0.6–0.9	65	350–1500
Orange peel	3.5–5.5	65	Nd*
Peach	0.3–0.4	60–80	160
Pear	0.7–0.9	50–70	150
Pineapple	0.04–0.1	22–40	Nd*
Raspberry	0.4–0.5	20–40	250
Strawberry	0.5–0.7	20–60	350
Tomato	0.3–0.5	55–60	100

*Nd: non determined.

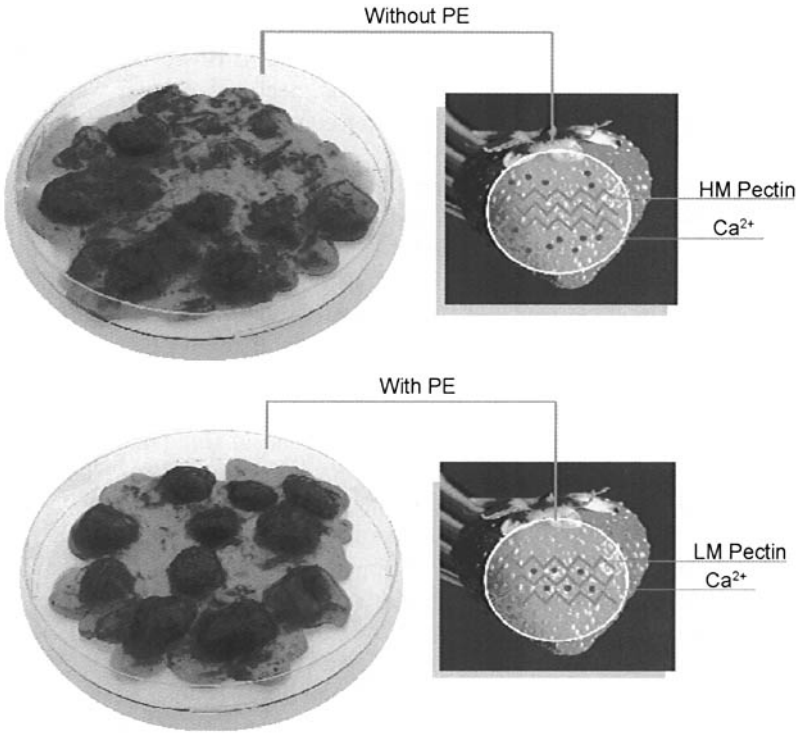

Figure 8.4 Comparison of appearance of strawberry fruit preparation both untreated and treated with Rapidase® FP SUPER (PE).

8.0 for plant PME. *Aspergillus niger* PME demethylates pectin randomly when plant PME would demethylate blockwise. However, both modes of action have recently been described for the sole apple PME, its properties depending on the pH and ion environment [10]. PME removes methoxyl groups from pectin with a nucleophilic attack forming an acyl-enzyme, then releasing methanol. Pectic acid formed after the reaction can be assayed by titration. This assay can be used to quantify PME activity.

The presence of pectin depolymerases which decrease pectin molecular weight (such as polygalacturonase or pectin lyase contained in fruit or in commercial enzyme products together with the PME) can make the gel formation impossible. For this reason PME must be as pure as possible. Another reason is that fruit PME activity is very weak in fruit (no activity can be detected after 20 min at pH 4.5). Gist-brocades has developed a purified concentrated PME for fruit firming from a non-genetically modified *Aspergillus niger* strain with the trade name Rapidase FP Super. For the same application, Novozymes has recently proposed Rheozyme as a trade name for a pectin esterase preparation

produced by *Aspergillus oryzae*, expressing the gene encoding a pectin esterase from *Aspergillus aculeatus*. The pectinmethylesterase and calcium ions must be present in sufficient amounts for efficiency. Optimisation of enzyme and calcium dosage must be defined depending on raw material and on process temperature and duration [9]. Many types of fruit may be processed with the Firmfruit® concept, including red berries such as strawberries or raspberries, apples, pears, tomatoes and any other pulpy fruit, whether fresh, frozen or thawed. The process can be applied to whole fruit, fruit pieces, or sliced, diced or puréed fruit. General conditions are detailed in the Firmfruit® process diagram (figure 8.5). It can be applied to fruit before any kind of heat treatment such as blanching, cooking, baking or pasteurisation. The method is particularly suitable for the preparation of dairy, bakery food compositions, canned fruits in syrup or candy fruit.

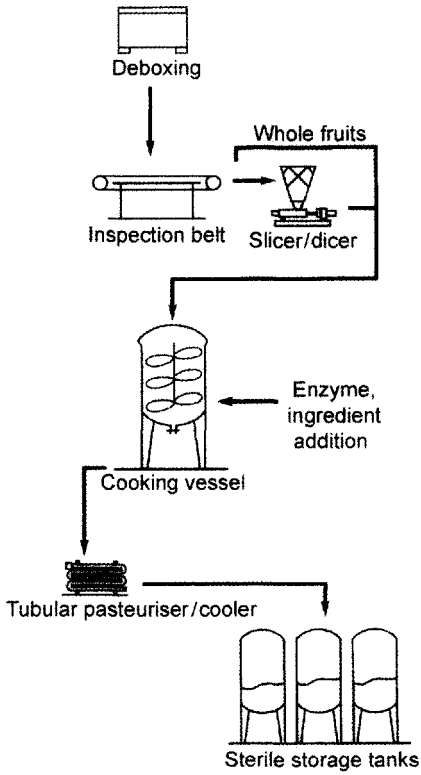

Figure 8.5 The Firmfruit® process. Steps in the process: a) defrosting; b) sorting, dicing or slicing, introduction to the cooking vessel; c) Rapidase® FP Super is added, preferably at 40–45°C for 10–20 min, dosage about 1 kg/ton; d) addition of other ingredients; e) heating at 90°C; f) storage of fruit preparation.

8.3.1 Strawberries

Strawberries are soft fruit with a high pectin content of around 0.5 to 0.7% of fruit weight. Strawberry pectin composition is basically similar to that shown in figure 8.3. Arabinose and galactose are the major neutral sugars, linked in arabino-galactan type II as pectin hairy-region side chains [11]. Strawberry pectin methoxylation ranges from 20% to 60% depending on raw material, justifying the use of PME for LM pectin formation *in situ* (see table 8.2). Calcium content is relatively high (on average 300 ppm) but can also vary and can be in the fruit as bound or free form, depending on the ionic environment. Free calcium is only available for pectate formation. For this reason calcium is added in the Firmfruit® process to complement the natural calcium, resulting in a more extensive pectate formation.

The traditional process of fruit preparation involves the use of stabiliser systems such as pectin, starch or gums, sugar and citric acid, for increasing the viscosity of the dispersing phase to maintain fruit shape, colour and taste after cooking. Fruit texture, especially of strawberries or raspberries, is usually destroyed in the final preparation. A strawberry fruit preparation process can be similar to the one described in figure 8.5. The treatment with Rapidase® FP Super improves the texture, mouth feel and taste of fruit greatly (see figure 8.4). Fruit identity and texture are improved in the PME treated fruit preparation, as shown by the change of pectate and soluble pectin proportions, compared to the control (see table 8.3). In the example described in the table, pectin methylation decreased after PME action from 46% to 25%. At the same time, pectin content decreased when pectate content increased in parallel from 23% to 48%. With PME treatment and calcium addition, fruit firmness was multiplied by a factor of almost five. The treated fruit is more easily processed and can be incorporated in numerous products with minimal deterioration. For example, a strawberry

Table 8.3 Strawberry pectin analysis after treatment with Rapidase® FP SUPER

Strawberry composition	No treatment	Calcium only 5 mmol	Treatment with Rapidase® FP SUPER 1 kg/ton of fruit – $CaCl_2$ 5 mmol 20 min 45°C
Alcohol insoluble solids (AIS)	5.8%	5.6%	5.8%
Galacturonic acid (GalA) % of AIS	7.5	9.0	10.4
Pectin methylation	46%	43%	25%
Soluble pectin % GalA	46.7%	33%	20.2%
Pectate % GalA	22.7%	28.9%	48.1%
Protopectin % GalA	30.7%	37.8%	31.7%
Fruit firmness kgF*	3.6	9.2	14.8

*Fruit firmness measured with Texture Analyser extrusion force expressed in kg Force (Stable Micro System - Ottawa cell).

preparation processed with the Firmfruit® process, then added to yoghurt, gives firmer fruit or fruit pieces compared to the untreated fruit. Moreover, after three weeks of cold storage, the fruit firmness in yoghurt seems even firmer compared to the same sample after only one week. The slight increase could be due to calcium rearrangement within the fruit–milk system.

Increasing certain fruit firmness after treatment with pectinmethylesterase is sometimes slow due to fruit texture or difficult transfer of the enzyme to the fruit pectin. For this reason the technique of vacuum infusion has been investigated.

Impregnation is defined as penetration of a fluid in a solid substance. Impregnating a fruit or vegetable involves replacing air in the intercellular space or space surrounding fruit akenes of strawberries or raspberries, with a liquid containing one or several soluble substances. The vacuum can be applied via two different processes. In the first process, the fruit product is put in a vacuum chamber before addition of the impregnation solution. The solution is then introduced and, after fruit immersion, the vacuum is stopped and the solution impregnates the fruit as the pressure increases until atmospheric conditions are attained. In the second process, the fruit product is immersed in the solution before the vacuum is applied. Fruit impregnation occurs after the vacuum is stopped. Penetration of particles in a porous fruit is an exponential function of pore size, until pores aggregate or are full. This is why it is necessary to impregnate fruit species with a high number of pores as completely as possible. Citrus fruit, strawberries or raspberries are very good candidates (see section 8.1). Impregnation is less easy with apple, cherry or carrot. The contact between fruit or fruit pieces with the impregnation solution is sometimes difficult because fruit tends to float. Total immersion is required for taking advantage of the maximum contact surface of fruit with the solution. Mass transfer is then easier for fruit impregnation. There are four different stages in the vacuum impregnation process:

1. Porous fruit is immersed in a solution containing ingredients (e.g. sugar, enzymes, hydrocolloids) at atmospheric pressure P1.
2. Fruit in solution is subjected to a pressure P2 that is slowly decreased below atmospheric pressure P1.
3. The vacuum is maintained at P2 value during a defined time.
4. P2 is quickly increased up to P1 and treated fruit is sieved, then processed further.

At atmospheric pressure, pores in fruit contain air but no liquid. Capillarity can occur when fruit is immersed in solution. When pressure decreases, air tends to expand and leave the intercellular space. Capillarity also occurs at this stage. Finally, when the vacuum is stopped and when the pressure increases to atmospheric pressure, external pressure is the active mechanism. Optimisation of vacuum degree, time of vacuum application, and concentration of the liquid solution is necessary for each process. The efficiency of fruit impregnation by

compounds in the solution depends on the concentration and viscosity of the solution. The latter is a function of the temperature. For example, apple cubes can gain + 6.5% mass if temperature is increased from 25°C up to 50°C for a constant sugar concentration in the solution, which is directly related to the viscosity of the solution. Vacuum impregnation has already been successfully tried with different fruit and vegetables. For example, vacuum impregnation of *Agaricus bisporus* with cellulose pectin or alginate improves the yield by 13% according to Singh and coworkers [12]. Vacuum impregnation or vacuum infusion of an enzyme like pectinase for pectin gelling of fruit or fruit pieces requires a sound knowledge of fruit pectin composition (see table 8.2). In 1951 Barton was the first to show that impregnation of strawberry slices with sugar and texture agents could be facilitated by the vacuum process [13]. The initial texture of strawberries vacuum impregnated with alginate or pectin was maintained after processing. In addition, the anthocyanin pigment that gives strawberries their red colour was less altered in vacuum processing compared to processing at atmospheric pressure.

Vacuum impregnation has also been applied to prevent peach softening before pasteurisation [14]. Peaches were vacuum impregnated with a water solution containing pectinmethylesterase and sodium chloride and then heated at 104°C for 12 min. A four fold increase in peach firmness was reported after fruit impregnation with the enzyme PME.

The vacuum infusion used for citrus fruit peeling (see section 8.1) has also been tried with the Firmfruit® process, to help PME solution entering the fruit tissue or strawberry akenes. Fruit firmness has been compared after industrial scale trials with frozen strawberries via three processes: (i) the traditional process; (ii) with enzymatic maceration (described in figure 8.5); (iii) with the vacuum infusion technique. Results are illustrated in figure 8.6. Strawberry cubes or diced strawberries were treated for 20 min at 40°C with 2.5% $CaCl_2$ and 0.6% Rapidase® FP Super. Improvement of strawberry firmness in fruit preparation was significant after treatment with PME but even more so if the enzyme was added via vacuum infusion (at least double in the example given in figure 8.6). Mass increase or yield was also increased from 9 to 14%.

8.3.2 Tomato

Considerable quantities of tomato are industrially processed for the preparation of preserved peeled tomato. Processing consists of cutting tomatoes in different ways with partial removal of seeds and skin, and packaging as such or with the addition of partially concentrated juice at 8° brix or puree at about 12° brix. The most important requirement in the preparation of these products is a high consistency of raw material. In the case of tomato, there must be a high polysaccharide content (cellulose and pectic substances), a considerable thickness of pulp, no discoloured parts and easy detachment of the skin and few seeds [15]. Because

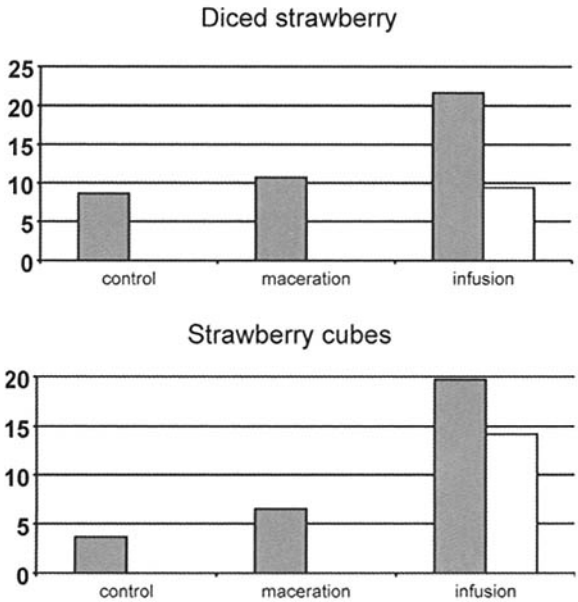

Figure 8.6 Effect of pectinesterase maceration or vacuum infusion process on strawberry firmness. ▨ = texture kg force; □ = mass gain %.

the improvement of the mechanical characteristics of peeled tomato and derived products is relevant to the food industry, numerous studies have been performed to improve tomato texture by mechanical, thermal or enzymatic processes.

Tomato consistency is related to fruit composition (variety and ripening) and processing technology. Tomato softening during ripening and post-harvest handling is due to the degradation of cell wall polysaccharides, primarily of pectic substances, in which several fruit endogenous hydrolases are involved. Cellulase, polygalacturonase, pectinmethylesterase and recently xylogalacturonase activities have been described in tomato during ripening [16]. These hydrolytic activities increase during fruit ripening, leading to a slow pectin solubilisation together with molecular weight decrease and loss of neutral sugars. Endo-polygalacturonase activity is the main activity responsible of tomato softening. It is even more active after fruit crushing. This is why the first stage of tomato processing consists of thermal stabilisation of texture by inactivating any endogenous enzyme activity. However, it has been noted that the thermal treatments employed for endogenous enzyme denaturation and for the microbial stabilisation of the product have also an adverse effect on the fruit consistency and on the end product quality. From the technological point of view, numerous factors may adversely affect the final quality of semi-processed product, in particular the various handling operations (cutting, draining, extrusion, pumping

and mixing). To counterbalance this loss of consistency due to mechanical and thermal processes, it is common to add calcium chloride to the product to increase its firmness. Calcium ions react with the free carboxyl groups of tomato pectin to produce a polymeric network of calcium pectate, strengthening the cell wall, and thus increasing the product firmness [17]. This technology has also the advantage of increasing the drained weight and the fruit integrity. However, the calcium concentration necessary to obtain a significant increase in firmness is sometimes around 500–600 ppm, close to the limit above which effects on the product taste (bitterness) can be observed.

It was observed that the addition of the enzyme pectinmethylesterase in the immersion bath containing calcium ions increased the product consistency much more and permitted a decrease in the calcium dosage. Batch or continuous processes have been developed to improve the consistency of peeled or diced tomato based on product immersion in PME calcium solution.

Before canning, the commercial pectinmethylesterase Rapidase® FP Super is added with calcium chloride to whole peeled tomato, cubes or sauce for a few minutes at 35–40°C. Tomato firmness is improved greatly (up 40%) and yield after draining is also increased (up 10% to 20% tomato pieces). Treated tomato products have a better mouth feel, more pleasing appearance and stronger consistency (figure 8.7). In the case of tomato sauce processing, viscosity can be increased by 35% after treatment with Rapidase® FP Super. These results demonstrate that it is possible to increase the consistency of tomato products considerably by taking advantage of the synergy provided by pectinmethylesterase and calcium ions for tomato firming.

In conclusion, commercial enzymes specifically chosen for the function of the fruit composition and the desired degradation or modification can increase industrial productivity with higher yield together with higher quality (taste, appearance) and longer shelf life of finished fruit products.

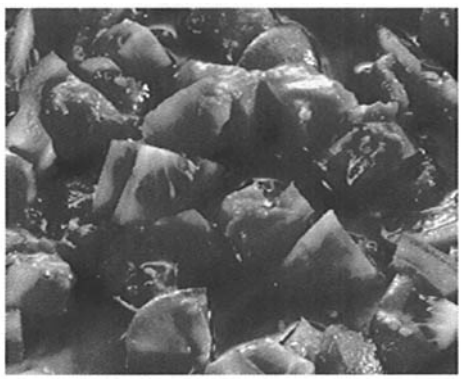

Figure 8.7 Tomato pieces treated with the Firmfruit® process.

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9 Enzymes in starch modification

Hans Sejr Olsen

9.1 Introduction

The first major application of industrial microbial enzyme preparations in the food industry began in the early 1960s with the introduction of a glucoamylase. Because of clear product benefits—such as greater yields, a higher degree of purity and easier crystallisation—most glucose production plants changed from acid hydrolysis to enzymatic hydrolysis. For example, compared to the old acid process, the enzymatic liquefaction process cut steam costs by 30%, ash by 50% and by-products by 90%.

From 1973 onwards, with the development of immobilised glucose isomerase, the industrial production of high fructose syrup became feasible. Special types of syrup that could not be produced using conventional chemical hydrolysis were the first compounds made entirely by enzymatic processes.

Because many different products are derived from starch, there has been heavy investment in research in this field. The starch industry has rapidly adopted new enzymes produced using the latest techniques of molecular biology and genetic engineering. To support these enzyme breakthroughs, there has been intensive development work on application processes.

Enzymes are ideal catalysts for the starch industry. Simple equipment can be used, and the moderate temperatures and pH values used for the reactions mean that few by-products affecting flavour and colour are formed. Furthermore, enzyme reactions are easily controlled and can be stopped when the desired degree of starch conversion is attained.

Depending on the enzymes used and by controlling the enzyme reactions, various valuable products may be produced to suit almost any requirement of the food industry. Syrups and modified starches with different compositions and physical properties can be obtained. The syrups are used in a wide variety of foodstuffs: examples are soft drinks, confectionery, meat products, baked products, ice cream, sauces, baby food, canned fruit and preserves.

The major steps in conversion of starch are liquefaction, saccharification and isomerisation. In simple terms, the further a starch processor goes, the sweeter the syrup obtained.

A slurry of the starch is cooked in the presence of a thermostable bacterial endo-*alpha*-amylase. The enzyme hydrolyses the *alpha*-1,4-glycosidic bonds in pre-gelatinised starch, whereby the viscosity of the gel rapidly decreases

and the so-called maltodextrins are produced. The process may be terminated at this point, the solution may be purified and dried, and the maltodextrins utilised as bland tasting functional ingredients in dry soup mixes, infant foods, sauces and gravy mixes, for example. Further hydrolysis by means of amyloglucosidase leads to the formation of sweet-tasting, fermentable sugars. Sweet starch hydrolysates with special functional properties may be obtained by using fungal *alpha*-amylase either alone or in combination with amyloglucosidase. Alternatively a vegetable *beta*-amylase may be used to increase the yield of maltose.

Also many non-food products made by fermentation are based on the use of enzymic modified starch products. Enzyme hydrolysed starches are used for instance in production of alcohol, polyols, cyclodextrins, ascorbic acid, enzymes, monosodium glutaminate (MSG), lysine and penicillin.

9.2 Processing and enzymology

The corn wet milling industry is utilising modern enzyme technology to a very large extent. Current developments focus on refinements of the basic enzyme conversion processes in order to improve process yields and efficiency. An overview of the major steps in conversion of starch is given in figure 9.1.

9.2.1 Starch liquefaction

As native starch is only slowly degraded by *alpha*-amylases, gelatinisation and liquefaction of a 30–40% dry matter suspension is needed to make the starch susceptible to enzymatic breakdown. The gelatinisation temperature differs for different starches [1]. Maize starch is the most widespread source, followed by wheat, rice, tapioca and potato. A good liquefaction system is necessary for successful saccharification and isomerisation. For liquefaction an efficient temperature stable *alpha*-amylase is added to the starch milk. The mechanical method of performing the liquefaction may be by use of stirred tank reactors, continuous stirred tank reactors (CSTR) or a jet cooker. The jet cooker secures homogenisation as well as liquefaction, due to injection of direct steam into the starch milk.

In most plants for sweetener production the starch liquefaction is made in a single dose jet-cooking process as shown in figure 9.2.

The *alpha*-amylase Termamyl® is metered into the starch slurry after pH adjustment, and the slurry is pumped through a jet cooker. Steam is injected to raise the temperature to 105°C, and subsequent passage through a series of holding tubes provides a 5 min residence time, which is sufficient to gelatinise the starch fully. By flash cooling the partially liquefied starch the temperature is reduced to 95–100°C, and the enzyme is allowed to react further at this temperature for 1–2 h until the required dextrose equivalent (DE) is obtained.

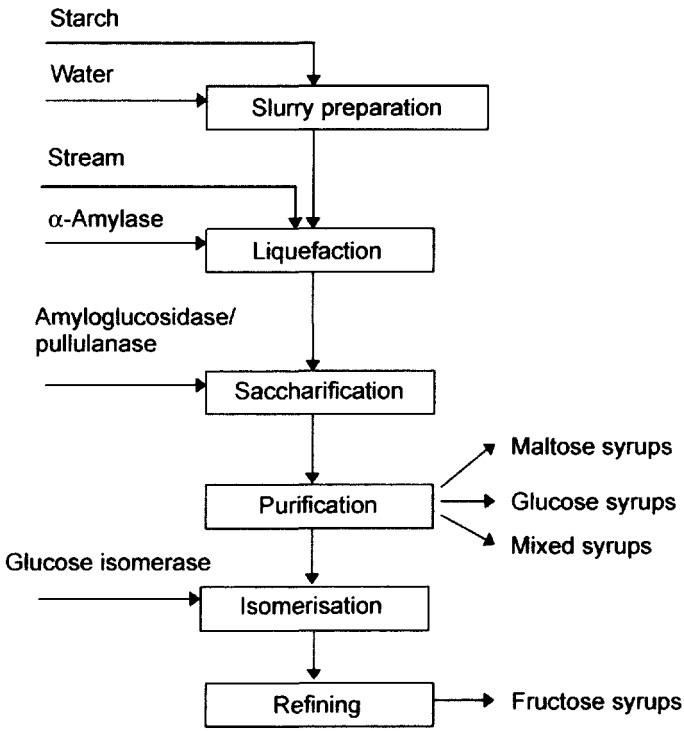

Figure 9.1 Major steps in the conversion of starch.

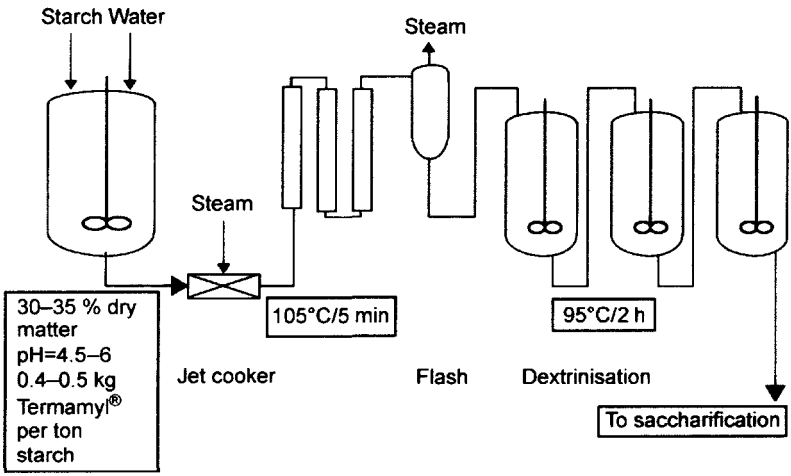

Figure 9.2 Starch liquefaction process. Termamyl is a bacterial thermophillic α-amylase produced by Novozymes A/S [22].

The jet-cooking process is carried out under the following general reaction conditions: dry substance (DS) 30–40% (w/w), pH 5.3–6.5, temperature: T=105–110°C, and a Ca^{2+} content of 5–80 ppm (w/v).

The dose range is between 0.3 and 0.6 kg Termamyl per ton of starch (DS). The stability depends on the following parameters: temperature, Ca^{2+} concentration, ion content, pH and DS. Rosendal and coworkers [2] described stability data from pilot plant trials using the first generation Termamyl. From such data it was possible to estimate optimum design parameters for the use of Termamyl in individual processes, thus minimising the dosage of enzymes. The relationship between half-life and temperature was derived from an Arrhenius-type of diagram (plot of half-life versus the reciprocal absolute temperature). It was assumed that the stability at other conditions could be expressed as the standard half-life multiplied by correction factors for each parameter:

$$T_{1/2} = T^*_{1/2} \times K_1(\text{pH}) \times K_2(\text{DS}) \times K_3(\text{Ca}) \times K_4(\mu\Omega)$$

where $T^*_{1/2}$ is the any half-life value for the temperature effect taken from table 9.1 at the reference set of parameters; K_1(pH) is the proportion of the half-life from the reference parameter and the actual value of pH (table 9.1); K_2(DS) is the proportion of the half-life from the reference parameter and the actual value of dry substance (DS) (table 9.1); K_3(Ca) is the proportion of the half-life from the reference parameter and the actual value of [Ca] (table 9.1); and $K_4(\mu\Omega)$ is the proportion of the half-life from the reference parameter and the actual value of conductivity (non calcium). This factor is set to 1.0 in most cases.

In an example, the reference set of parameters could be 105°C, DS = 35%, pH = 6.5, $[Ca^{2+}]$ = 40 ppm. An actual case considered could be at the following set of parameters: 110°C, pH = 5.5, DS = 30%, and 70 ppm Ca. Using the values from table 9.1, $T^*_{1/2}$ = 15 min K_1(pH) = 110/200 = 0.55, K_2(DS) = 30/50 =

Table 9.1 Stabilities of first generation Termamyl (expressed in half-lives)

Reaction parameters		Half-life (minutes)
Temperature°C:	110	15
at DS = 35%, pH = 6.5, $[Ca^{2+}]$ = 40 ppm	105	50
	100	200
	95	700
Dry substance % w/w:	35	50
at 105°C, pH = 6.5, $[Ca^{2+}]$ = 40 ppm	30	30
	25	20
pH:	6.5	200
at 100°C, DS = 35%, $[Ca^{2+}]$ = 40 ppm	6.0	110
	5.5	40
Calcium concentration, ppm Ca^{2+}:	70	100
at 100°C, DS = 35%, pH = 6.5	40	50
	10	15

0.60, $K_3(\text{Ca}) = 100/50 = 2.00$, $K_4(\mu\Omega) = 1.0$ (see above) the half-life under the new conditions is $T_{1/2} = 15 \times 0.55 \times 0.60 \times 2.00 = 9.9$ min.

From a practical point of view stabilities of Termamyl (expressed in half-lives) are a function of temperature, dry substance (DS), pH and calcium concentration (see table 9.1).

Using data such as those shown in table 9.1 it will be possible to deduce the enzyme stability at almost any set of conditions. The enzyme activity decay follows a first order reaction scheme according to the formula:

$$A_t = A_0 \times \exp -\left(\frac{\ln 2}{T_{1/2}} \times t\right)$$

where A_t is the residual activity at t; A_0 is the activity at time 0; $T_{1/2}$ is the enzyme stability (half-life) in min; and t is the reaction time in min.

The characteristics for new optimal performing liquefying *alpha*-amylases are calcium independent stability, high heat stability, high specificity and high activity at pH 4.5. Novozymes A/S has introduced calcium independent and acidic *alpha*-amylases. When using corn the sweetener industry want to perform the entire process, from wet milling through liquefaction to saccharification to glucose, at pH 4.5. A lot of process benefits (e.g. less formation of unwanted side products, less colour formation, fewer unit operations) would be the result of this.

For wheat starch, potato starch and others, liquefaction is in general carried out at pH 5.0–6.5. Higher pH values should be avoided due to increased by-product formation (e.g. maltulose precursors). Maltulose formation is enhanced at high temperatures, high pH and long residence times, and is more pronounced at increasing DEs. One advantage of Termamyl is that the temperature is increased only to 105°C at a very low DE. In processes with hydrochloric acid (at pH 1.8–2) a temperature of 140°C is often used to obtain DE 7–8. A *B. subtilis alpha*-amylase treatment at 85°C is some times used for thinning of the starch before the pressure-cooking with acid.

The above-mentioned process (figure 9.2) can be used for the production of maltodextrins (DE 15–25). In that case, a higher enzyme dose and/or a longer residence time are needed.

Without great success, cooking extruders have been studied for starch liquefaction but due to the high temperature in the extruder, inactivation of the enzyme activities demands 5–10 times higher dosages than in a jet cooker [3].

9.2.2 alpha-*Amylases*

alpha-Amylases are used for liquefaction of starch in sweetener production, in the detergent, textile and brewing industries and for the fuel and distilling industries. Enzymatic starch liquefaction is an old market segment developed by Novozymes A/S early in the 1970s.

An *alpha*-amylase hydrolyses the *alpha*-1,4 glycosidic bonds characteristic f the starch backbone to water-soluble oligosaccharides of 2 to 10 sugar units. he reaction occurs by attachment of the active site in the enzyme to an *alpha*-1,4 ond in the polymer molecule where hydrolysis can occur (enzyme–substrate mplex), followed by 'clipping' of the bond. This reaction continues, causing ie degradation of the polymer chain. Figure 9.3 is a molecular model of n *alpha*-amylase, showing the enzyme's active site associating with five of ie glucose units in the polysaccharide chain. These enzymes typically have iolecular weights on the order of 25–75 kDaltons and diameters of 5–10 nm. lence, amylases are smaller than the polysaccharides they destroy and have a ery different shape. The complex shape of the protein is shown on the model.

Molecular modelling is the key to understanding enzyme reaction mechaisms and designing better enzymes for a specific purpose. Figures 9.4 and 9.5 lustrate the chemical reaction mechanism for hydrolysis.

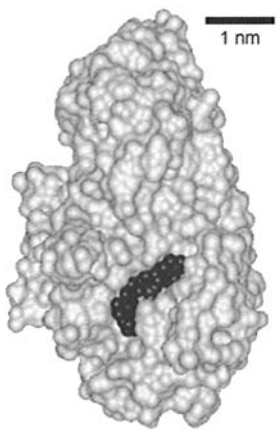

Figure 9.3 Structure of enzyme–substrate complex for α-amylase.

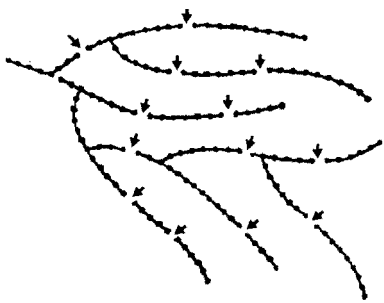

Figure 9.4 α-Amylase activity on amylopectin.

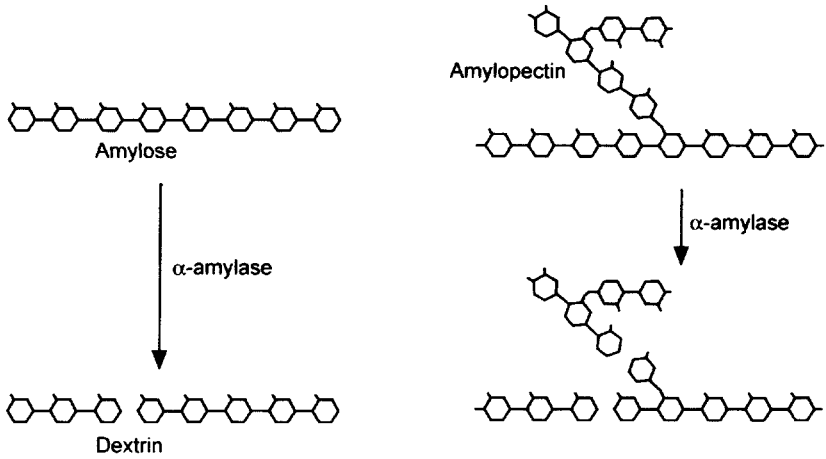

Figure 9.5 Reaction mechanism for the degradation of amylose and amylopectin to oligosaccharides by α-amylase.

During the liquefaction the *alpha*-1,4-linkages are hydrolysed at random as illustrated in figure 9.4. This reduces the viscosity of the gelatinised starch and increases the DE, a measure of the degree of hydrolysis of the starch. The liquefaction is carried out in such a way as to give the required DE for the subsequent process. For saccharification to dextrose, a DE of 8–12 is commonly used. Higher DE values are often necessary for maltodextrin production. The maximum DE that can be obtained is about 40.

Industrial important *alpha*-amylases are made from *Bacillus licheniformis*, *Bacillus stearothermophilus*, *Bacillus subtilis and Aspergillus oryzae*. In table 9.2 some application conditions and a few comparative characteristics are shown for various enzymes available for starch processing. Examples of

Table 9.2 Industrial important starch degrading enzymes

Enzyme	Microorganism	Application temperature range (°C)	Application pH range	Minimum Ca^{2+} dosage (ppm)
Bacterial mesophilic α-amylase	*Bacillus subtilis*	80–85	6–7	150
Bacterial thermophilic α-amylase	*Bacillus licheniformis* or *Bacillus stearothermophilus*	95–110	5–7	5
Fungal α-amylase	*Aspergillus oryzae*	55–70	4–5	50
Amyloglucosidase	*Aspergillus niger*	55–65	3.5–5	0
Pullulanase	*Bacillus acidopollulyticus*	55–65	3.5–5	0

Table 9.3 Some important commercial starch processing enzymes

Producer	*alpha*-Amylases	Saccharifying enzymes	Isomerising enzymes	Maltogenic enzymes
Enzyme Bio-Systems Ltd	G-zyme G 995	G-zyme G 990	–	–
Genencor International Inc	Spezyme AA	Optidex	Gensweet SGI	Spezyme BBA
	Spezyme	Distillase		Optimalt
	Delta	Optimax	Gensweet IGI	Glarase
	Spezyme Fred	Fermenzyme®		Transglucosidase L
Gist-brocades NV	Maxamyl	Amigase	–	–
Novozymes A/S	Termamyl Classic	AMG	Sweetzyme IT	Fungamyl 800 L
		AMG E		Maltogenase 4000 L
	Termamyl varieties (see table 9.4)	Promozyme 400 L		
		Promozyme D		
	BAN 240 L	Dextrozyme E		

the current most important commercial market amylases are given in table 9.3.

9.2.2.1 Bacterial thermophilic alpha-*amylase*

Termamyl® (Novozymes A/S) is an endo-amylase which hydrolyses the *alpha*-1,4-linkages in starch (amylose and amylopectin) almost at random. The breakdown products formed are mainly soluble dextrins and oligosaccharides (figures 9.4 and 9.5). In a concentrated solution of starch, the hydrolysis results in a rapid viscosity reduction. In consequence Termamyl is often referred to as a 'liquefying amylase'.

In table 9.4 a survey is shown of the reaction requirements and effects for different varieties of Termamyl developed by protein engineering and produced by Novozymes A/S over the years. The trend has been to reduce pH-optimum, to reduce the need for calcium, to increase the initial reaction rates, and to improve thermostability. Significant saving of costs is found in the industrial starch conversion processes of these effects [4].

The wild type amylase SPEZYME® AA from Genencor International Inc. can perform adequately down to pH 6.1. The engineered amylase SPEZYME Delta AA performed down to pH 5.8 and the variant SPEZYME FRED showed acceptable performance as low as pH 5.3 [5].

9.2.2.2 Fungal alpha-*amylase*

Fungamyl® is a fungal exo-amylase, which hydrolyses the *alpha*-1,4-linkages in liquefied starch (amylose and amylopectin); a prolonged reaction results in

Table 9.4 Reaction requirements and effects for varieties of Termamyl

Enzyme (Trade name)	Microorganism	Dosage (kg/MT)	Application temperature range (°C)	Application pH range	Minimum free Ca^{2+} dosage (ppm)
Termamyl® 120L Type S	Origin: *Bacillus stearothermophilus* Production organism: *Bacillus licheniformis*	0.2–0.4 (For alcohol processes)	85–110	5.7–6	10–20
Termamyl® Type SC	Origin: *Bacillus stearothermophilus* Production organism: *Bacillus licheniformis*	0.2–0.4 (For alcohol processes)	85–110+	5.0–6.0	5
Termamyl® 120L Type L	*Bacillus licheniformis*	0.60	80–105	6.0–6.5	40–60
Termamyl® 120 L Type LS	*Bacillus licheniformis*	0.45	80–105	5.7–6.5	40
Termamyl® 120 L Type LC	*Bacillus licheniformis*	0.45	85–110	5.4–5.6	5
Termamyl® 120 L Type Supra	Origin: *Bacillus stearothermophilus* Production organism: *Bacillus licheniformis*	0.30–0.45	85–110	5.3–5.7	5

the formation of large amounts of maltose. Fungamyl is used for production of high maltose syrups or high conversion syrups (see table 9.5).

9.2.2.3 Glucoamylase (amyloglucosidase)

AMG®E is a glucoamylase (exo-amylase), which hydrolyses *alpha*-1,4-linkages as well as *alpha*-1,6-linkages in liquefied starch (amylose and amylopectin). The hydrolysis proceeds in a stepwise manner. The breakdown product formed is glucose, which has been split off from the non-reducing end of the substrate molecule. Maltotriose and in particular maltose are hydrolysed at a lower rate than higher saccharides, and 1,6-linkages are broken down more slowly than 1.4 linkages. Eventually, almost complete conversion of starch into glucose is obtained. In consequence AMG is referred to as a 'saccharifying amylase'. AMG E is produced by a genetically modified strain of *Aspergillus niger* and is available in liquid form with a standardised activity. An often used activity measurement for amyloglucosidase is the AGU (amyloglucosidase unit), which is the amount of enzyme that hydrolyses one micromole of maltose per minute at 25°C under standard conditions.

Table 9.5 Various specifications of saccharification products and some applications

Saccharification product	Maltose content (%)	Glucose content (%)	Application	Products and effects
High maltose syrup–by Fungamyl®	50–55	2–5	Confectionery	Moisture and texture control in soft confectionery
High maltose syrup–produced at 65°C	50–55	8–10	Baking	Moisture retention and colour control in final products
			Frozen desserts	Control of softness and freezing characteristics
High maltose syrup special–produced at 60°C to 65°C	55–65	8–12	Brewing	Control of fermentation via balanced fermentable sugar spectrum High percentage of fermentable sugars (97+)
Very high maltose syrup	70–75	0–3	Ice cream	Control of softness and freezing characteristics
Ultra high maltose syrup	82–88	5–9	Candy fruit	No crystallisation Less hygroscopic product
High conversion syrup[a]	30–37	35–43	Jam and jellies	Viscosity and osmotic profiles Sweetness control
			Brewing	Fermentation control
			Canning	'Bodying' and sweetness in fruit canning
			Confectionery	Candies for high sucrose replacement. Less hygroscopic products.
			Baking	Moisture retention and colour control in final products
			Soft drinks	Stabilisation of flavour profile during shelf-life.
Glucose syrup[b]	1–2	94–97	Baby food, dietetic foods	Instant energy source.
			Baking	Dough properties and caramelisation.

[a]High conversion syrups should be stable enough to resist crystallisation at temperatures above 4°C and 80–83% DS. The content of glucose in these syrups should therefore not exceed 43% on DS basis.
[b]Apart from the food industry, syrups and crystalline glucose (dextrose) have applications also in the pharmaceutical and chemical industry. Furthermore glucose is used as a substrate for fermentation processes, for example, in the production of monosodium glutamate (MSG).

9.2.2.4 beta-*Amylases*

beta-Amylases are exo-enzymes, which attack amylose chains resulting in efficient successive removal of maltose units from the non-reducing end. In the case of amylopectin the cleavage stops two to three glucose units from the 1,6-α-branching points. *beta*-Amylase is used for the production of maltose syrups and in breweries for adjunct processing. The most important commercial

products are made from barley or soybeans. SPEZYME®BBA, from Genencor International is a barley *alpha*-1,4-D-glucan maltohydrolase (E.C. 3.2.1.2) and is commonly referred to as *beta*-amylase. It is extracted from barley grain. *beta*-Amylase is an exo-amylase that catalyses the release of successive maltose units by hydrolysing *alpha*-1,4-D glycosidic linkages from the non-reducing end of the dextrin chain. The enzyme is free of *alpha*-amylase activity; therefore virtually no glucose is formed.

9.2.2.5 Maltogenic amylase

A maltogenic amylase (exo) like Maltogenase™ (from Novozymes A/S), hydrolyses *alpha*-1,4-glycosidic linkages from the non-reducing end of the maltodextrin chain. Maltodextrins, oligosaccharides and maltotriose are hydrolysed mainly to maltose.

9.2.2.6 Isoamylase and pullulanase

Isoamylase (glycogen-6-glucanohydrolase) and pullulanase (pullulan-6-glucanohydrolase) hydrolyse *alpha*-1,6-glycosidic bonds of starch, which has been partly hydrolysed by *alpha*-amylase, provided that there are at least two glucose units in the side chain. When amylopectin is treated with pullulanase, linear amylose fragments are obtained. Using heat-stable and acid-stable pullulanase in combination with saccharification enzymes makes the starch conversion reactions more efficient. Norman described the pullulanase Promozyme® which is well suited for debranching starch after liquefaction [6].

9.2.3 Saccharification of liquefied starch

Maltodextrin (DE 15–25) produced from the liquefied starch is commercially valuable for its rheological properties. Maltodextrins are used in the food industry as fillers, stabilisers, thickeners, pastes and glues. When saccharified by further hydrolysis using glucoamylase or fungal *alpha*-amylase a variety of sweeteners can be produced, having DEs in the ranges 40–45 (maltose), 50–55 (high maltose), 55–70 (high conversion syrup) [7]. Applying a series of enzymes including *beta*-amylase, glucoamylase and pullulanase as debranching enzymes, intermediate-level conversion syrups having maltose contents close to 80% can be produced [6].

A high content of 95–97% glucose may be produced from most starch raw materials (corn, wheat, potatoes, tapioca, barley and rice). The effect of action of amylases and debranching enzymes is shown in figures 9.6 and 9.7a and b.

9.2.4 Tailor-made glucose syrups

Glucose syrups, in commercial practice called glucose, are obtained by hydrolysing starch mainly from wheat, maize (corn) and potato. The method and the extent of hydrolysis (conversion) affects the final carbohydrate composition,

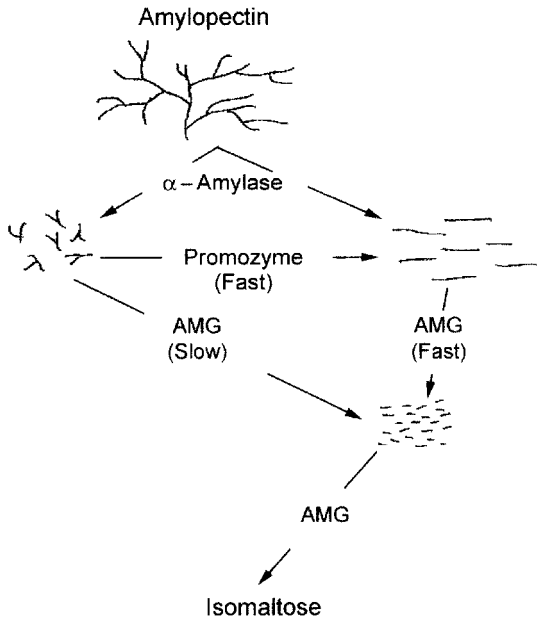

Figure 9.6 The effect of the action of starch degrading enzymes.

and hence many functional properties. The degree of hydrolysis is commonly defined as the dextrose equivalent (DE), expressing the reducing power as a percentage of pure dextrose, calculated to dry weight basis.

Originally, acid conversion was used to produce glucose syrups. Today, because of their specificity, enzymes are frequently used to predetermine exactly how hydrolysis will take place. In this way, tailor-made glucose syrups with specific sugar spectra can be manufactured.

The sugar spectra can be analysed by different techniques, such as high performance liquid chromatography (HPLC) and gel permeation chromatography (GPC). They give information about the molecular weight distribution and overall carbohydrate composition of the glucose syrups, which, at the same time, define and characterise the product type (e.g. high maltose syrup). Although these techniques help to optimise glucose syrup profiles for specific applications, indirect methods, such as viscosity measurements, are also important in obtaining tailor-made glucose syrups.

9.2.5 Use of syrups

Maltose syrups have a low glucose content and a high maltose content. Because of the low glucose content, high maltose syrups show a low tendency to crystallise, and they are relatively non-hygroscopic. Various specifications (maltose

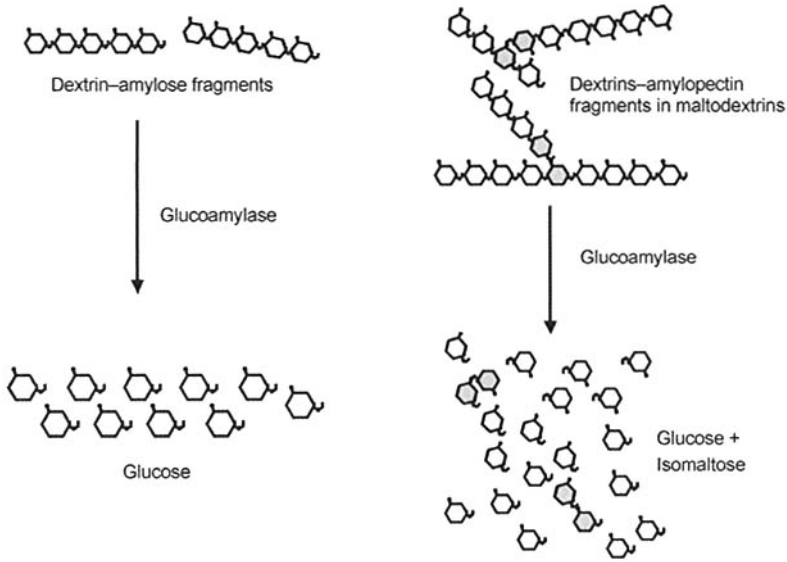

Figure. 9.7a Hydrolysis of maltodextrin using glucoamylase.

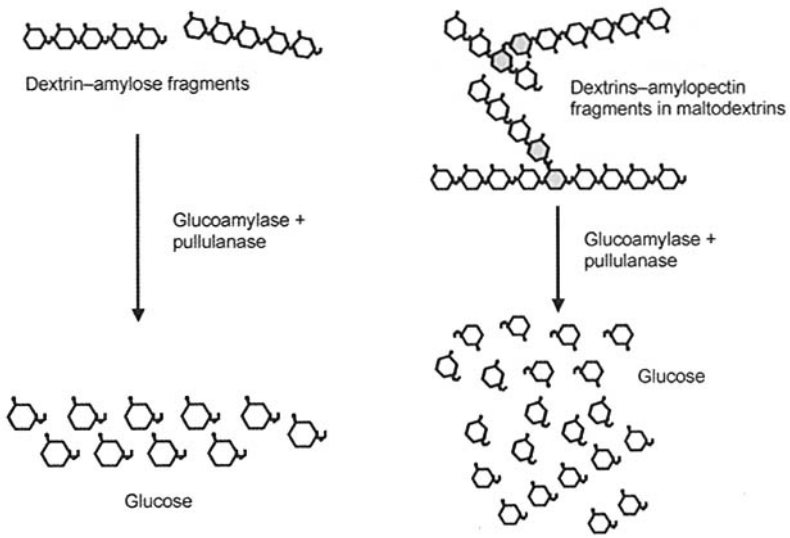

Figure. 9.7b Hydrolysis of maltodextrin using glucoamylase and pullulanase.

Table 9.6 Compositions of maltose syrups obtained by usage of different enzyme systems

Enzymes	Dextrose	Maltose	Maltriose	Dextrins
'Traditional maltose syrups' *	5–10	50–55	10–15	25–30
Fungamyl (0.3 l/t DS)	5	55	20	20
Fungamyl (1.0 l/t DS)	8	60	12	20
Maltogenase	8	65	2	25
Malt extract+Promozyme	2	70	17	11
Maltogenase+Promozyme	10	75	1	14
Malt extract+Maltogenase+ Promozyme	7	80	5	8

Sugars are in % of dry substance – approximate figures.
*Approximate sugar spectrum in a malting adjunct.

and glucose content) of saccharification products and some applications of maltose syrups as well as glucose syrups are shown in table 9.5. Syrups are generally called glucose syrups when the glucose content is higher than the maltose content.

9.2.6 Production of maltose syrups

Maltose syrups are produced by saccharifying liquefied starch with maltogenic exo-enzymes. Enzymes for this purpose are fungal *alpha*-amylase (e.g. Fungamyl®), Maltogenase™, Promozyme® or barley *beta*-amylase, also known as malt extracts. Saccharification is conducted at 55–65°C, pH 4.8–5.5, and at solids level DS 30–40%. Enzyme dosage around in the range of 0.3–3 kg enzyme products per ton of starch (DS) and the reaction time may be in the range of 20–40 h. The sugar spectrum obtained depends on the enzyme dosages and on the reaction time used.

As shown in table 9.6 various sugar spectra may be obtained dependent on the enzyme systems being used.

9.2.7 DX, DE and reducing value

Syrups and breakdown products of starches are usually characterised by the following terms:

DE = dextrose equivalent (degree of hydrolysis)
DX = the true dextrose content
DP = degree of polymerisation
Note: dextrose = glucose

The number of glucose molecules is also characterised by the reducing value. Thus glucose (DP1) has a reducing value of 1.00, maltose (DP2) has 0.5 and maltotriose (DP3) has 0.33.

The calculation of DE and DX is illustrated by the following example:

A sugar composition has been found to be the following:

93% dextrose (DP1)
4% maltose (DP2)
3% maltotriose (DP3)
100% total DS

$$DE = 93 \times 1.00 + 4 \times 0.50 + 3 \times 0.33 = 96\%$$
$$DX = 93\%$$

9.2.8 *High conversion syrup*

Figure 9.8 illustrates the ranges of sugar spectra that can be obtained using combinations of only two enzymes. The example shows that so-called 'high conversion syrups' can be produced from acid-liquefied starch with a combination of Fungamyl and AMG E. Higher maltose and lower glucose levels are obtained with enzyme-liquefied starch compared to acid liquefied starch.

The following conditions can be used for the production of high conversion syrups. The process may be carried out at 55°C and pH 5.0. The concentration of dry substance should be in the range 30–40% and no adjustment of dry substance concentration after liquefaction is therefore necessary. The saccharification time is normally about 40 h, but shorter or longer reaction times may be used with proportionately higher or lower enzyme dosages.

It is very important to inactivate the AMG, when the required DE and sugar composition have been reached. If the reaction is allowed to proceed, more

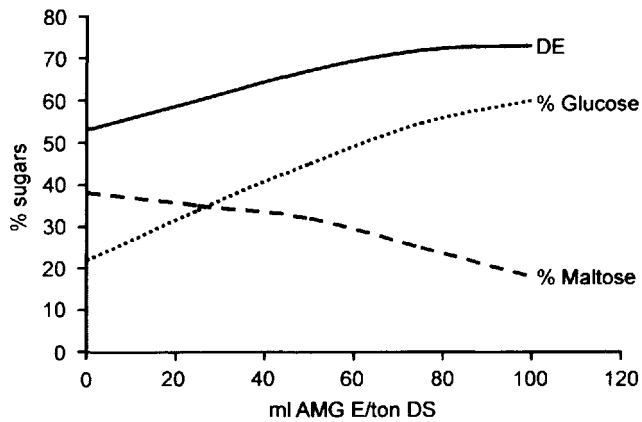

Figure 9.8 Production of high conversion syrups. Substrate: 44 DE acid liquefied starch, 45% DS; enzymes: Fungamyl 800 l: 150 g/t DS, AMG E: (dose varied); reaction conditions: 55°C, pH = 5.0, reaction time 42 h.

glucose will be produced, which crystallises when the syrup is concentrated and stored. Heating the syrup to 80°C for about 40 min or to 85°C for about 5 min may stop the reaction. The syrup is then filtered, carbon-treated and, if desired, ion-exchanged. High conversion syrups are used, for example, in ice cream and in candy fruits.

9.2.9 *Production of high dextrose syrups*

Amyloglucosidase hydrolyses the *alpha*-1,4-linkages rapidly, but during the saccharification process the *alpha*-1,6-linkages of the highly branched amylopectin are hydrolysed much more slowly. Normally the dosage of AMG E is adjusted to obtain the desired final degree of saccharification, DX, within 48 h to 70 h as shown in figure 9.9.

At the start of the saccharification, the rate of dextrose formation is high, but it gradually decreases towards the end of the process. This is partly due to the accumulation of branched dextrins, and partly because the increasing concentration of dextrose accelerates reversion (re-polymerisation of dextrose into isomaltose and other saccharides). At a certain point, the rate of reversion outbalances that of dextrose formation, and if the saccharification is not stopped at this point (maximum DX), the dextrose level will gradually decrease towards chemical equilibrium (around 85% dextrose at 30% DS, 60°C).

Using a pullulanase like Promozyme together with the amyloglucosidase like AMG E at the start of the saccharification the *alpha*-1,6-linkages of the branched dextrins are rapidly hydrolysed. In consequence fewer branched oligosaccharides accumulate towards the end of the saccharification.

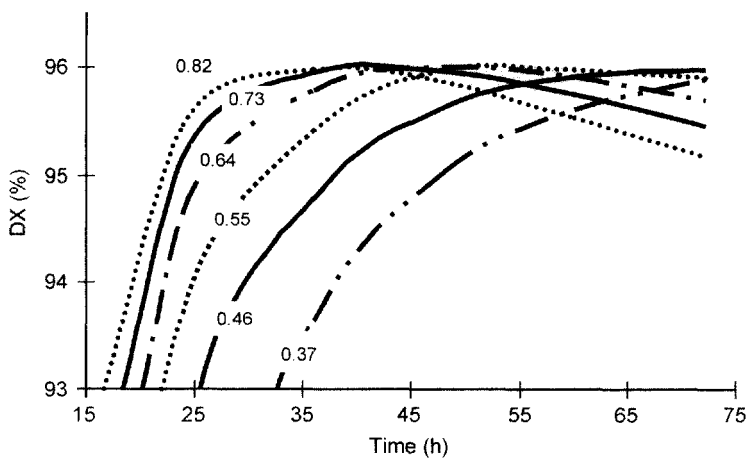

Figure 9.9 DX as function of time and dosage (litre AMG E/1000 kg DS). Conditions: dry solids 30% (initial; pH 4.3; temperature 60°C).

The maximum DX obtainable with AMG and Promozyme mainly depends on the enzyme dosage ratio (PUN/AGU) and dry substance level. Further details about the favourable practical effects of combining AMG and Promozyme are described by Olsen [8]. To summarise, higher glucose yield, higher dry substance and decreased saccharification time at lower enzyme dosage may be some of the effects that can be obtained.

9.2.10 Amyloglucosidase/pullulanase combination in the production of high dextrose syrup

Dextrozyme® E is one example of a balanced mixture of amyloglucosidase and pullulanase. Figure 9.10 shows that, when dosed equally, this combination gives higher glucose yield (about 2 DX) than AMG (glucoamylase) or a shorter time (about 40%) is used to reach the same glucose yield as with AMG.

Figure 9.11 illustrates the influence of the dry substance content and the reaction time on the maximum obtainable DX value, the yield of glucose.

The time necessary to reach the maximum obtainable DX depends on the enzyme dosage. The maximum obtainable DX is a function of mainly the dry substance level and the nature of the enzyme used. Figure 9.12 illustrates the influence of enzyme dosage and reaction time on the amount of glucose formed during saccharification. The glucose level begins to fall if the reaction is continued beyond the maximum. This is due to the reverse reaction, which is a condensation of glucose molecules to mainly isomaltose. Figure 9.12 illustrates the importance of optimising dosage and time to get the highest DX.

The purification processes of saccharified starch may be adapted to each raw material and may be different from plant to plant. When the starch milk is liquefied in the jet cooker (see figure 9.2) the saccharification process is carried

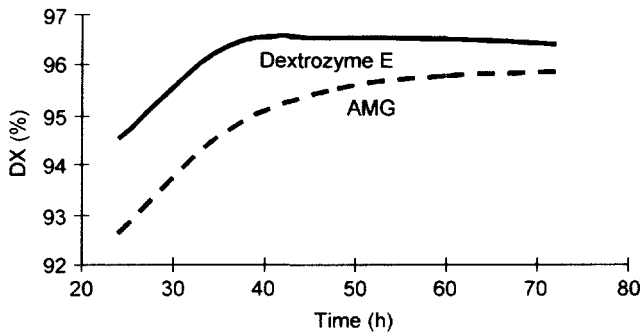

Figure 9.10 Influence of Dextrozyme E on saccharification. Conditions: enzyme dosage 0.73 l/1000 kg DS; pH 4.4; temperature 60°C; dry solids 30% (initial).

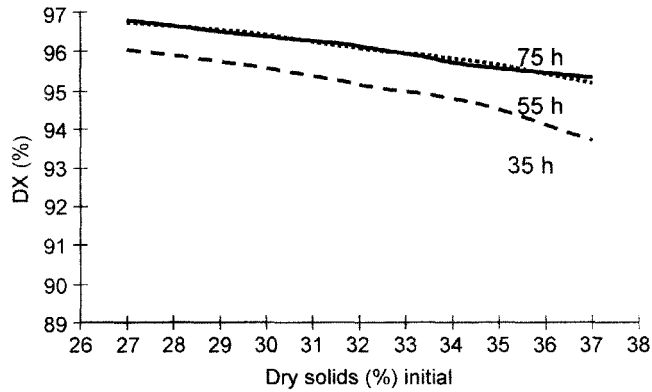

Figure 9.11 Effect of dry substance on maximal obtainable % DX. Conditions: Dextrozyme E dosage 0.60 l/1000 kg DS; pH 4.3; temperature 61°C.

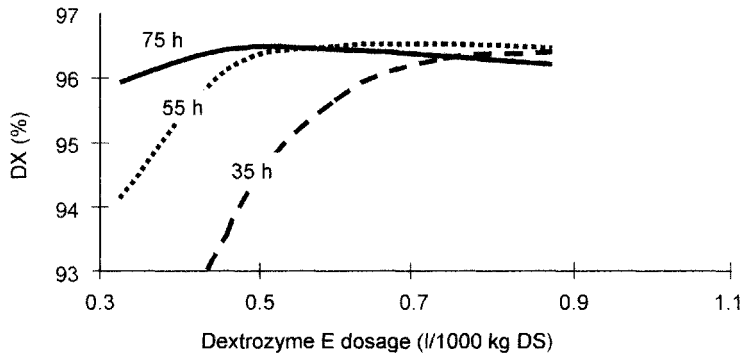

Figure 9.12 Optimisation of the dosage and time to get the highest DX. Conditions: dry solids 29.5% (initial); pH 4.3; temperature 61°C.

out at 55–65°C, pH 4–4.5 for 24–72 h. The saccharified liquid is filtered to remove insoluble impurities such as denatured protein and fat (a mixture). In most cases the filtration is followed by carbon treatment, for removal of soluble organic impurities, and ion exchange. Finally evaporation is performed to make a storage-stable product.

9.2.11 Continuous saccharification

It is theoretically possible to obtain exactly the same final DX by continuous saccharification as in a batch process under the same reaction conditions.

It would be necessary, however, to use either a plug-flow (tube) reactor or an infinite series of infinitely small continuous-flow stirred tank reactors (CSTR). A limited number of saccharification tanks results in a final DX lower than a theoretical use of an infinite number of infinitely small continuous-flow stirred tank reactors [8]. A series of a minimum number of eight tanks is recommended.

9.2.12 Continuous saccharification in a membrane reactor

Membrane processing is used in the starch industry for removal of 'mud' (mixture of fat and protein released after the saccharification of pure starch) by microfiltration, substituting rotary vacuum filters, centrifugation or pressure filtration. Ultrafiltration is used for production of clear juices by removing suspended solids, large molecules like proteins, enzymes and to some degree limit dextrins. Nanofiltration is used for improving hydrolysate quality (e.g. increasing the DX of glucose syrup or increasing the maltose content in maltose syrups).

A continuous hydrolysis process for the saccharification of liquefied corn starch has been developed using a membrane reactor [9]. A residence time distribution confirmed that the membrane could be modelled as a simple CSTR. Kinetic studies indicated that the continuous reactor operated in the first-order region with respect to substrate concentration at substrate concentrations greater than 200 g/l. The productivities were claimed to be 10 to 20 times higher than those obtained in a batch reactor [10]. All saccharifications were carried out using amyloglucosidase from *Aspergillus niger* (AMG from Novozymes A/S) at 55°C and pH 4.5 in a dosage which were about 15 times higher than normally recommended in batch processes using a saccharification time of 40–70 h. At a residence time of 1 h in the membrane reactor the apparent Michaelis constant (K'_m) was 562 g/l, which is 2–7 times greater than that obtained in a batch reactor [10]. It seems evident that more work is needed to all technical and economical aspects and possibilities of the use of membrane technology for the saccharification of starch.

A continuous membrane reactor was also used successfully for the production of maltose from soluble starch using a combination of *beta*-amylase and pullulanase [11].

9.3 Enzymes as processing aids in the purification of saccharified wheat starch

A particular problem with wheat starch is that a minor amount of impurities in the starch milk adheres to the starch granules. To facilitate the refining (e.g. filtration operations) cellulases, pentosanases, glucanases, proteases and pectinases can be used. Wheat starch is known to form precipitates or hazes that are difficult to filter. Konieczny-Janda and Richter [12] claimed arabinoxylan, pentosanes, and lysophospholipids to be responsible for this problem. Frandsen and Olsen [13] described how filtration problems might be solved by addition of an *Aspergillus*

niger lysophospholipase. At the end of the saccharification, filtration rates were enhanced significantly and clear oligosaccharide solutions were obtained. Poor filtration properties are due to lysophospholipids that increase the viscosity of the sugar solution. The content of phospholipids in wheat starch might be higher than 0.8%, in particular in starch samples with a high proportion of starch granules having a small particle size. In wheat, the main phospholipids are lysophospholipids. Lysophospholipases selectively remove fatty acids from lysophospholipids, by cleavage of an ester linkage. Enzymatic hydrolysis of the lysophospholipids therefore destroys the inherent emulsifying ability of these molecules and increases filtration rates.

9.4 Glucose isomerisation

Enzymatic isomerisation of glucose to fructose was the process that provided a real alternative to white sugar (sucrose) from cane or beets. The commercial product obtained was high-fructose corn syrup (HFCS). Two grades of the syrup have established themselves in the world market, HFCS-42 and HFCS-55. They contain 42% or 55% fructose based on dry substance. These products account for over a third of the caloric sweetener market in the US. Annually more than 8 million tons of HFCS are produced using glucose isomerase. This represents the largest commercial application of immobilised biocatalysts in industry.

9.4.1 The isomerisation reaction

Glucose can reversibly be isomerised to fructose. The equilibrium conversion for glucose to fructose is 50% under industrial conditions, and the reaction is slightly endothermic [14]. The reaction is usually carried out at 60°C, pH 7–8. To avoid excessive reaction time, the conversion is normally limited to about 45%.

The isomerisation reaction can only be economic by using immobilised enzyme. The reaction parameters in this system have to be as optimal as possible in order to obtain reasonable yield of fructose. The pH must be about 7.5 or higher in order to secure high activity and stability of the enzyme. Under these conditions glucose and fructose are rather unstable and decompose easily to organic acid and coloured by-products. To overcome these problems the reaction time must be limited. This is done by using an immobilised isomerase in a fixed-bed reactor process in a column through which glucose flows continuously. The enzyme granules must be rigid enough to prevent compaction during the operation. Sweetzyme IT from Novozymes A/S is produced by a mutant of a selected *Streptomyces murinus* strain. The immobilisation procedure for Sweetzyme IT consists of a disruption of a cell concentrate through a homogeniser with a single stage-homogenising valve. The cells are then cross-linked with glutaraldehyde, diluted and flocculated. The concentrated aggregate is extruded and finally fluid-bed dried and sieved [15].

9.4.2 Isomerisation conditions

The activity of the enzyme is determined by the rate of glucose to fructose conversion obtainable at any time. The stability is reflected in the ability of the enzyme to retain its activity during operation. Productivity is the combined effect of activity and stability, and is defined as the amount in kg of 42% fructose syrup (dry substance) produced per kg of enzyme throughout its lifetime. Sweetzyme IT is a robust version with improved flow characteristics, which results in easier handling during column loading as well as during its lifetime. During its lifetime, one kg of this isomerase can produce at least 18,000 kg syrup dry substance. The activity of Sweetzyme IT decreases with time at a constant rate (linear activity decay). When discarding the enzyme at approximately 10% residual activity, lifetime average activity is 55%, a considerable improvement compared to a first order decay isomerase. Depending on parameters such as temperature, pH feed purity, and so on, the operating lifetime of this isomerase will typically be 200–360 days.

9.4.3 Isomerisation temperature

The isomerisation temperature strongly affects the enzyme activity and stability as well as the by-product formation. The standard operating temperature is 60°C. Higher temperatures will result in higher enzyme activity (increased glucose conversion rate), but the stability and productivity will be lower. This means that an increasing activity decay and therefore enzyme consumption will be the result. Furthermore, by-product formation due to glucose and fructose decomposition will accelerate with increasing temperature.

Lowering the operating temperatures will lead to increased risk of microbial infection as well as to reduced activity (lower glucose conversion rate), but enzyme stability and productivity will be improved. Syrup production plants operating at a high level of hygiene and feed purity may prefer to lower the isomerisation temperature towards 55°C or even lower during periods with ample production capacity.

In figure 9.13, the effect of the isomerisation temperature on the relative performance (syrup flow rate) and productivity of glucose isomerase is illustrated.

9.4.4 Isomerisation pH

The isomerisation pH affects the enzyme activity, stability and by-product formation. Maximum activity is obtained at a pH above 8, whereas maximum stability is found between pH 7.2 and 7.5. By-product formation due to glucose and fructose decomposition increases at higher pH levels.

With the aim of obtaining optimal enzyme economy as well as high syrup quality, the inlet pH should be selected within the range 7.5–8.0 (measured at

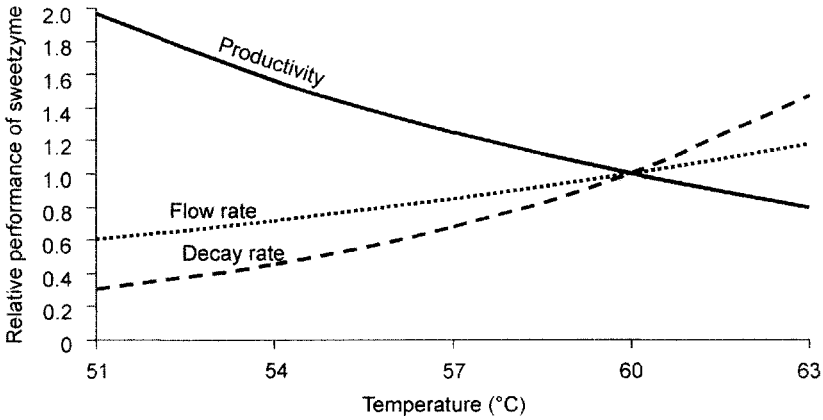

Figure 9.13 Effect of isomerisation temperature (Sweetzyme is a glucose isomerase). Substrate: 45% w/w glucose; pH 7.5.

25°C) depending on feed syrup purity. Feed impurities will catalyse glucose and fructose decomposition into acid by-products, thereby leading to lower outlet pH. Outlet pH readings lower than 7.0 (measured at 25°C) should be avoided. A mild buffering of the feed syrup with Na_2CO_3 or $NaHCO_3$ will facilitate pH control. (The use of Na_2CO_3 for feed pH adjustment will result in sufficient buffering in most cases).

9.4.5 Isomerisation glucose (dextrose-DX) and fructose concentration

The feed syrup DX should be as high as possible in order to obtain the maximum isomerisation rate. With low feed DX, the isomerisation reaction must come closer to chemical equilibrium in order to attain a given fructose concentration; for example, the standard 42%.

The rate of isomerisation decreases with increasing fructose concentration as chemical equilibrium is approached. In consequence, any fructose content in the feed syrup (e.g. from fructose enrichment recycle streams) should be limited to a minimum.

The dry substance content of the feed syrup should generally be 40–50%. Higher syrup concentration and higher viscosity will result in a reduced isomerisation rate due to diffusion resistance in the pores of the immobilised enzyme.

9.4.6 The immobilised enzyme system

The glucose isomerases used are immobilised and granulated to a particle size between 0.4 and 1.0 mm. Mg^{2+} acts as activator and stabiliser of the enzyme, and

is therefore added to the feed syrups in the form of $MgSO_4 \cdot 7\ H_2O$. The amount necessary depends on the presence of calcium ion, which acts as an inhibitor in the system by displacing the magnesium ion activator from the isomerase molecule. Therefore the Ca ion content should be kept as low as possible. At a Ca ion content in the feed syrup of 1 ppm or lower, the addition of 45 ppm Mg (e.g. approximately 0.6 g of $MgSO_4 \cdot 7\ H_2O$ per litre) will be sufficient. At higher concentrations of Ca ion, a proportionate weight ratio between Mg and Ca ion should be provided for.

Each reactor load of Sweetzyme is used for a long period of time, thereby being exposed to a very large quantity of syrup. In consequence, the accumulated effect of even low levels of feed syrup impurities may lead to significant reduction of the enzyme lifetime. Some impurities may chemically inactivate the isomerase, whereas others may adsorb to the isomerase particles, gradually blocking their active surface. In order to attain maximum enzyme lifetime, it is therefore very important to purify the feed syrup (including possible recycle streams) thoroughly before isomerisation.

The raw high-DX liquor from saccharification is filtered to remove particulate material, which might clog the isomerase particles. Soluble impurities (peptides, amino acids, ash and so on) are potential inhibitors of the isomerase, and must therefore be removed by carbon treatment and ion exchange. The efficiency of the carbon and ion exchange treatments should be checked by measuring the UV absorbance and conductivity of the purified feed syrup.

Dissolved oxygen in the feed syrup increases by-product formation, and some by-products may inhibit the enzyme. Therefore, the oxygen content of the syrup should be minimised by vacuum de-aeration after heating to isomerisation temperature. Sulfite can be used as an oxygen scavenger, but should not be used in amounts exceeding approximately 100 ppm SO_2 in the feed syrup.

Before entering the isomerisation reactor the syrup should be passed through a 5–10 micron check filter.

Some impurities (e.g. maltulose), which are difficult to remove by the procedures above, may have been formed during the starch liquefaction process. This is minimised by avoiding excessive exposure of the gelatinised starch to high temperature and pH.

The main criteria for selecting the feed syrup specifications are optimisation of enzyme productivity and limitation of by-product formation. Typical feed syrup specifications are shown in table 9.7.

9.4.7 Process lay-out

The glucose syrup must be purified by filtration to obtain clear syrup and to remove insoluble impurities (mainly protein and fat). A carbon treatment to obtain colourless syrup by removal of soluble coloured impurities (mainly yellow/brown 'caramel' compounds) should be performed. Ion exchange should

Table 9.7 Typical feed syrup specifications

Parameter	Value
Temperature	55–60°C
pH	7.5–8.0
Dry substance content	40–50 wt%
Glucose content	≥95%
SO_2	0–100 ppm
Calcium ion	≤1 ppm
$MgSO_4 \cdot 7H_2O$ (activator)	0.15–0.75 g/l
Conductivity	≤ 100 μS/cm
UV absorbance (280 nm)	≤0.5

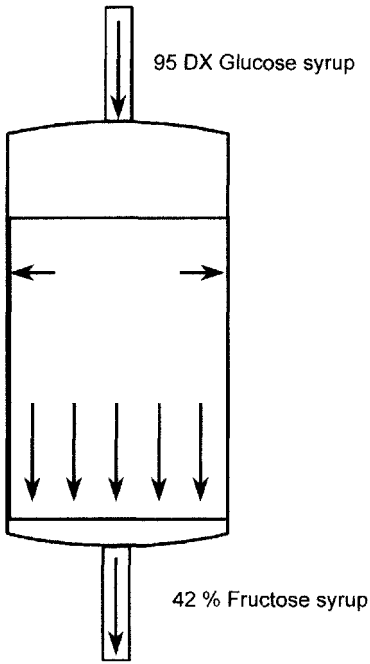

Figure 9.14 Enzyme column layout for glucose converting to fructose syrup with immobilised glucose isomerase.

be carried out to reduce the ash content so that the conductivity after adjustment of pH to approximately 7.5 is < 100 μS/cm. The result is the removal of mainly mineral salts and adjustment of the magnesium content can now take place. Before reaction on the immobilised column, evaporation is carried out to obtain a storage-stable syrup during the isomerisation reaction. Figure 9.14 shows a column layout for the isomerisation. After the reaction a final carbon treatment,

ion exchange and evaporation to the dry matter content (>70% dry matter) at which the final syrup is stored are carried out.

9.4.8 Enzyme decay

Typically, a reactor load of glucose isomerase is replaced after three half-lives; that is, when the activity has dropped to around 12.5% of the initial value. The most stable commercial glucose isomerases have half-lives of 200–360 days in industrial practice.

To maintain a constant fructose concentration in the product syrup, the feed flow rate is adjusted according to the actual activity of the enzyme. With only one isomerisation reactor in operation, there would be excessive variations in syrup production rate. To avoid this, several reactors containing enzyme of different age are operated in combination.

Reactor design for glucose isomerisation in the US has been described [16]. Reactor diameters are normally between 0.6 and 1.5 m. Typical bed heights are 2–5 m. Minimum bed height–diameter ratio for one reactor is 3:1 to ensure good flow distribution. Plants producing more than 1000 t of HFCS (based on dry matter) per day use at least 20 individual reactors.

9.4.9 Controlling isomerisation costs

Most fructose refineries have high control over the cost of isomerisation. While the costs of equipment, labour, and energy continue to rise, the cost of producing fructose has fallen significantly over the past decade. Some guidelines for maximising isomerisation productivity are given below:

- Over-conversion should be avoided. The cost of averaging any given fructose level is always higher than the cost of running each column at the average. The tighter the distribution of fructose levels from the columns, the lower the costs will be.
- The total monosaccharide level should be watched. Isomerisation is an equilibrium reaction. The quantity of enzyme required will rise rapidly as the conversion approaches equilibrium. Therefore, the lower the content of monosaccharides in the syrup, the lower the equilibrium, and the more costly the product will be as indicated in figure 9.15.
- Low temperature is not always good. As temperature is decreased, flow rate drops slightly, but enzyme productivity increases markedly. Problems occur when the temperature is so low that bacteria and moulds are able to propagate in the packed bed of isomerases. Columns can usually run infection-free at 40% DS and 60°C.
- Calcium is an inhibitor. Calcium, which is normally found in saccharified syrup and the dextrose-rich raffinate stream from the fructose enriching adsorptive chromatography units, displaces magnesium that activates the isomerase as mentioned above. Calcium displaces the magnesium during

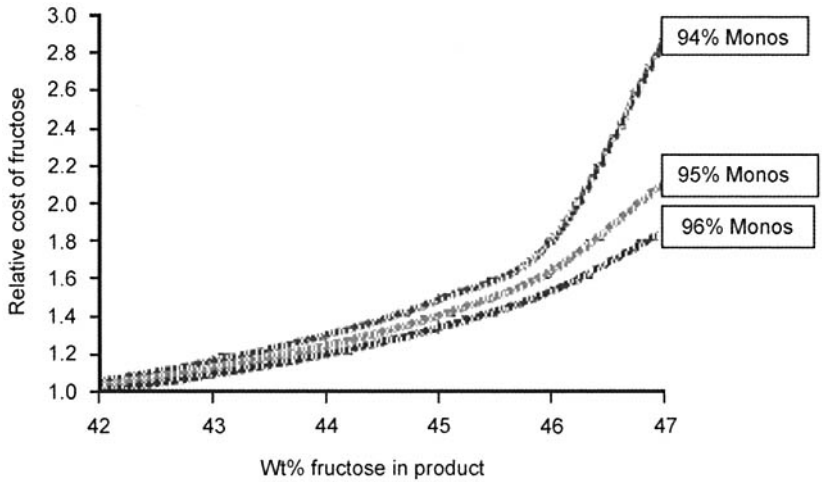

Figure 9.15 Costs of fructose as a function of % fructose and the monosaccharide content in the feed syrup.

peaks of calcium concentration. The calcium levels should be below 1 ppm in the isomerisation feed syrup. Occasional peaks of 2–3 ppm can be accommodated by the recommended 40 ppm magnesium level.

- Recycling of fructose should be avoided. Recycling fructose is a waste of the high fructose syrup which have already been paid for to produce. Furthermore fructose is very thermo-labile. It readily denatures into colour precursors and organic acids.

9.5 Use of high-fructose corn syrups (HFCS)

Guideline figures for sweetness of various sweeteners depend on dry substance, pH, temperature and viscosity. In table 9.8 the relative sweetness of HFCS-products is compared with that of other sugars. Table 9.9 indicates the areas in which high-fructose syrup is used, the relative replacement of sucrose and some specific properties.

Table 9.8 Relative sweetness of sugars

Sugar type	Relative sweetness (%)
Fructose	150–170
Sucrose	100
Dextrose	70
Maltose	30
HFCS 42%	90
HFCS 55%	100
HFCS 90%	120–160

Table 9.9 Uses of high-fructose syrup

	Replacement of sucrose (%)	Specific food properties
Baby foods	50–100	sw
Bakery products	100	sw, f.e., h, fer, b.r.
Breakfast foods	50–100	sw
Ketchup, tomato sauce	100	sw, f.e.
Cereals; prepared	50–100	sw, h
Chilli sauce	100	sw, f.e.
Chocolate products	25–50	sw
Confections	5–25	sw, h, c.c.
Fruit juices	100	sw, f.e.
Fruits, canned	50–100	sw, f.e., o.p.
Fruits, frozen	50–100	sw, f.e.
Ice cream, frozen desserts	50–100	sw, f.e.
Jams, jellies, marmalades	75–100	sw, f.e., c.c.
Liquors	100	sw
Pickles	100	sw, o.p.
Pie fillings, cream and fruit	50–100	sw, f.e., c.c.
Port and beans, canned	50–100	sw, f.e.
Soft drinks	100	sw, f.e.
Sweet potatoes, canned	100	sw, f.e., o.p.
Wine	50–100	sw, fer

sw = sweetness; f.e. = flavour enhancement; h = humectancy; o.p. = osmotic pressure; fer = fermentability; b.r. = browning reaction; c.c. = crystallisation control.

9.6 Cyclodextrins

Cyclodextrins are crystalline, water soluble, cyclic, non-reducing oligosaccharides, of six, seven, or eight glucopyranose units linked together by *alpha*-1,4-bonds. Depending on the number of glucose moieties in the ring they are named α-, β-, and γ-cyclodextrin. Cyclodextrins have long been known as products that can form inclusion complexes. Successful applications have been made in the areas of agriculture, analytical chemistry, biotechnology, cosmetics, diagnostics, electronics, foodstuffs, pharmaceuticals and toxic waste treatment. When cyclodextrins are added, medicines insoluble in water become more soluble, and/or more stable because cyclodextrins form inclusion complexes with medicines. They can bind other compounds in their cavity and thereby stabilise, solubilise or precipitate compounds [17]. Almost all applications of cyclodextrins involve complexation. In liquid and solid phases, organic and inorganic molecules of appropriate size can be incorporated into the cyclodextrin cavity to form inclusion complexes. The incorporated compounds and cyclodextrins are referred to as guests and hosts, respectively. The main driving force for this binding is that of hydrophobic interactions. Molecules, or functional groups of molecules, that are hydrophobic can be included in the cyclodextrin cavity in the presence of water, if their molecular dimensions correspond to those of the

cyclodextrin cavity. CDs are doughnut shaped and can bind a variety of organic 'guest' compounds inside their apolar cavities in aqueous solution.

Novel developments in enzymic modification of starch are concerned with production of cyclodextrins, among other things. Cyclodextrins are produced during liquefaction of starch by use of the class of enzymes called cyclomaltodextrin glycosyltransferase (CGT-ase) [18]. Such enzyme may be produced by a genetically modified strain of Bacillus (host), which has received the gene for CGTase from a strain of Thermoanaerobacter (donor). Novozyme A/S produces such an enzyme (Toruzyme™). The enzyme catalyses the formation of α-, β-, and γ-cyclodextrin from starch. Initially, equal levels of α-, β-cyclodextrin are formed, but if the reaction is prolonged, β-CD is the major component. The CGT-ase will also catalyse the formation of 'coupling sugars' by transferring glucosyl units from starch to a suitable acceptor such as sucrose.

For the cyclisation reaction, the pH should be in the range of 5.0–5.5, and the temperature should not exceed 80–90°C. Due to the stabilising effect of the starch, this CGT-ase can also be used for starch liquefaction at 105°C for short periods (5–15 min). For liquefaction in a jet-cooking process the pH can be reduced to 4.5–4.7.

The CGT-ase reaction can be terminated by lowering the pH. The enzyme will be completely inactivated by maintaining 80°C at pH 3.0 for 5 min [19].

In the enzymatic production of cyclodextrins from milled corn starch, membranes can be incorporated into the reactions and post-treatment steps to enhance productivity and lower downstream costs [20]. A four-fold reduction in enzyme usage and halved the reaction time could be obtained in such processes.

9.7 The future

Refined starch sweeteners have proved immensely important to the food industry. For example, since 1985 the Americans have used more corn products than sucrose for their nutritive sweetener needs [21]. The industry maintains its emphasis on science and the creation of new products. During recent years a wide range of value-added food ingredients have been introduced. Examples are fat replacers from starch, cyclodextrins for encapsulation, crystalline fructose, and new modified starches for microwave applications.

9.8 Conclusion

Glucose syrups as starch-derived bulk sweeteners have been shown to be much more than just sweeteners. Many food products and chemicals are derived from the utilisation of starch as a raw material for enzymatic conversions. Thus it has been demonstrated that the major steps in conversion of starch, liquefaction, saccharification and isomerisation may lead to a lot of valuable products, when processing and enzymology go hand in hand.

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10 Commercial enzyme production and genetic modification of source organisms

Richie Piggott

10.1 Brief history

Commercial enzyme production dates back to the early 1890s, with Takamine's pioneering study on fungi as a source of enzymes [1]. In 1894, he patented the production of Takadiastase, a preparation of diastatic enzymes obtained by cultivating strains of *Aspergillus oryzae* using surface-culture fermentation of wheat bran. The first patent on the use of bacteria as a source of commercial enzymes appeared in 1917, when Boidin and Effront [2] described the production of amylases using different strains of Bacilli.

Production of enzymes steadily increased through the 1940s and 1950s, and a 1958 report by Underkofler, Barton and Rennert [3] recorded the commercial production of the following enzymes: amylase, catalase, cellulase, glucose oxidase, invertase, lactase, lipase, pectinase and protease.

Commercial enzyme production continued to thrive over the following 30–40 years, with improvements in manufacturing techniques, chemical engineering and increasing application of enzymes to the food processing industry. The impact of recombinant DNA technology on commercial manufacture since the 1980s has been phenomenal, with the potential for improving yield, purity and variety of enzymes far greater now than ever dreamed possible.

10.2 Sources of commercial enzymes

There are three principal sources of commercial enzymes currently used in food processing, namely:

- animal
- plant
- microbes

By far the largest group of commercial enzymes currently used in the food industry are produced by microbial fermentation but several animal-derived enzymes and a smaller group of enzymes derived from plants are still being applied.

10.2.1 Animal-derived enzymes

The enzymes used in food processing today derived from animal sources are typically extracted from the organs of different species (table 10.1). An example of such an enzyme is pepsin, which is isolated from the stomach mucosa of pig in the process as outlined below. It has preferential cleavage of peptide bonds where the carbonyl group is from phenylalanine, leucine or glutamic acid (pepsin A) and has limited applications in cheese manufacture, protein hydrolysis and as a digestive aid.

10.2.1.1 Pepsin production process

Frozen stomach linings are collected from meat packaging plants and coarsely ground into a stainless steel, steam-jacketed stirred vessel. This ground material is digested with hydrochloric acid for 4–6 h at a temperature of 50°C. Fat separation is usually facilitated by the further addition of small quantities of hexane. The pepsin preparation is precipitated from the aqueous solution with the use of isopropyl alcohol. The precipitate is collected and dissolved in water. This solution is then filtered and dried (usually at temperatures of 45–55°C under vacuum for up to 48 h). Residual hexane and isopropyl alcohol are vaporized and removed under these drying conditions. The dry pepsin is milled and can be blended using standard powder blending techniques to the desired enzyme activity ready for market. Typical diluents or carriers used for pepsin powder blending include maltodextrin, lactose or sucrose.

10.2.2 Plant-derived enzymes

The smallest group of enzymes used in food processing today are derived from plants (table 10.2).The origin of the use of plant enzymes goes back centuries to

Table 10.1 Animal-derived enzymes

Enzyme	Source	Typical application
Lipase	Pig pancreas	Cheese manufacture
Trypsin	Pig pancreas	Protein hydrolysis
Pepsin	Pig stomach	Protein hydrolysis
Chymosin	Calf stomach	Cheese manufacture

Table 10.2 Plant-derived enzymes

Enzyme	Source	Typical application
Papain	Papaya (*Carica papaya*)	Brewing, meat tenderizing
Bromelain	Pineapple (*Ananas comosus*)	Meat tenderizing
Ficin	Fig (*Ficus carica*)	Meat tenderizing

the time when the natives of tropical countries wrapped meat in the leaves of the papaya plant before cooking and found this had a tenderizing effect. Those early users were not aware of the biochemical reactions taking place; however, with increased knowledge of biochemistry it was found that the tenderizing property was due to the presence of proteolytic enzymes in the papaya plant acting on the meat proteins.

Currently these properties have been commercially exploited in the food industry and the three main plant-derived enzymes used in the food industry are papain from the papaya fruit, bromelain from the pineapple and ficin from figs. Of the three, papain is by far the most important and is used extensively today primarily in brewing and in meat tenderizing.

10.2.2.1 Commercial papain production

The initial stage of papain production involves the collection of the crude latex from the papaya fruit. This is done by tapping the fully grown, unripened fruit of the papaya tree early in the morning when latex flow is maximal, making a series of incisions on each fruit with a sharp stainless steel blade. The latex which flows for only one or two minutes is then collected and dried. This crude latex is further processed to produce eventually a spray-dried powder product or concentrated, stabilized liquid preparation ready to formulate for product to market. A typical process-flow sheet on the commercial production of papain is presented in figure 10.1.

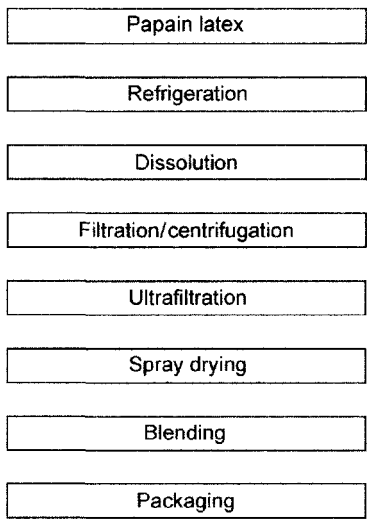

Figure 10.1 Commercial papain production.

Papain is formulated to market by blending the final concentrate to a specified activity which will vary depending on the specific application. Spray-dried papain is blended on a carrier such as a maltodextrin of similar particle size which will avoid layering during transport or storage. Liquid concentrate is blended to the desired enzyme activity using water-containing stabilizers and preservatives to ensure the integrity of the final product for a given period of time at a specified storage temperature.

10.3 Microbial enzyme fermentation

The largest group of industrial enzymes used in the food industry today is derived from microbial fermentation. Fermentation is the process whereby a microbe is grown under very controlled environmental conditions of pH, temperature, dissolved oxygen and so on, which allows for the maximal production and expression of the desired enzyme product. All three classes of microorganisms (bacteria, fungi and yeast) are sources of commercial enzymes and typical examples of strains and their products are listed in table 10.3.

An overview of industrial enzyme fermentation can be seen in figure 10.2. The process begins with revitalization of the preserved culture and the gradual build up of cell number through a multi-series inoculum development which culminates in the seed fermenter. At this stage the culture is ready for full fermentation (submerged fermentation in deep tank liquid medium or surface-culture [Koji] fermentation on a solid substrate) where maximum growth of the microorganism and enzyme production takes place. The final stages of manufacture consist of downstream processing steps aimed at recovery of maximum amounts of the enzyme produced during fermentation.

Table 10.3 Examples of microbial sources of commercial enzymes

Microbial source	Enzyme	Typical application
Bacteria		
Bacillus subtilis	Neutral protease	Brewing
Bacillus subtilis	α-Amylase	Starch modification
Fungi		
Aspergillus niger	Pectinase	Fruit juice processing
Trichoderma longibrachiatum	Cellulase	Brewing
Aspergillus niger	Amyloglucosidase	Starch processing
Rhizopus oryzae	Lipase	Lipid modification
Rhizomucor spp.	Acid protease	Cheese production
Yeast		
Sacharomyces cerevisiae	Invertase	Confectionery
Kluyveromyces lactis	Lactase	Dairy

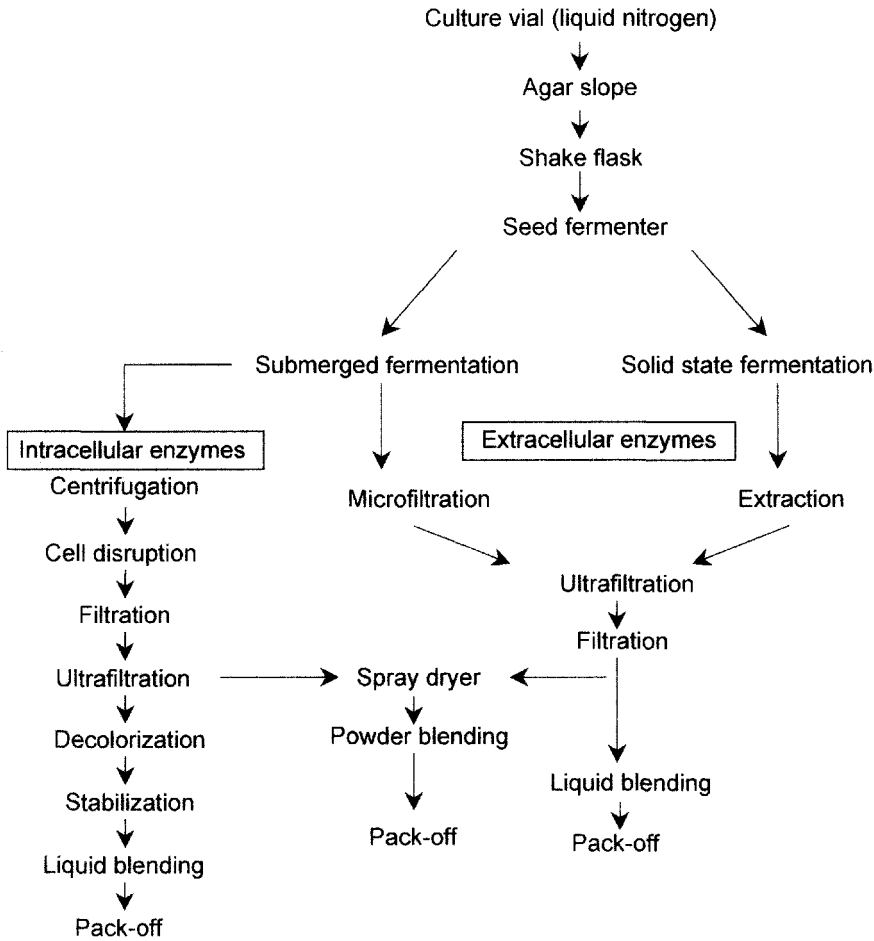

Figure 10.2 Microbial enzyme fermentation.

10.4 Preservation of industrial microorganisms

One of the most important assets of any commercial enzyme manufacturer is its culture collection and therefore the preservation and long-term storage of these microorganisms is of prime importance. Ideally, microbial preservation should provide conditions in which the highly productive strains are preserved for long periods of time free from phenotypic change. There are a number of different ways to preserve cultures, the most common of which are listed below.

10.4.1 Freezing

Broth cultures or cells harvested from slants are dispensed into vials and stored frozen. This can be done by storing under standard freezing conditions of −5 to −20°C which will maintain viability for up to two years. For longer-term preservation, temperatures ranging from −50 to −80°C are used with mechanical freezers. Alternatively, cultures can be stored in liquid nitrogen (−196°C) or the vapor phase of liquid nitrogen (−156°C) for long-term preservation. Liquid nitrogen is regarded as one of the most successful methods of culture preservation and stability in industry today because of its ability to preserve microorganisms reliably over long periods of time.

10.4.2 Freeze-drying

Freeze-drying or lyophilization of cultures involves the removal of water from frozen cell suspensions by sublimation under reduced pressure. There is no requirement to refrigerate freeze-dried cultures and most strains can be preserved in this state for periods of up to ten years.

10.4.3 Subculturing

Subculturing is a method of maintaining the viability of cultures by periodic transfer onto fresh agar, followed by incubation at a suitable growth temperature. The cultures should then be stored in a refrigerator at 5°C. This method is convenient and inexpensive but is not recommended for long-term preservation.

10.5 Inoculum development

The first stage of commercial enzyme production involves the revitalization of the preserved culture and a multi-staged accumulation of biomass, and is called inoculum development. A small quantity of the stored culture is added to a liquid medium in test tubes or flasks to begin the process. After incubation at a suitable temperature for a defined period of time (one to two days for bacteria, several days for fungi) the culture is ready for multi-staged development. This typically begins with the transfer of inoculum into increasing volumes of a liquid medium in shake flasks ranging in size from 50 mls up to 10 l. The inoculum is subcultured into each flask in a controlled environment to prevent contamination. The flasks are placed on a reciprocating shaker and shaken vigorously to ensure oxygen does not become limiting for the defined period of time. After the desired level of growth has been achieved at the flask stage, the contents of the final flask are transferred into the first stirred tank stage, known as the seed fermenter. These fermenters can vary in size from a capacity of a few hundred litres up to 20,000 l. Depending on the scale of

the operation, one or more stirred tank seed fermenters may be required to achieve the desired inoculum size to seed the production fermenter. For example, 10,000 l is required if a 10% inoculum is to be used for a 100,000 l production fermenter. The inoculum is now ready to be delivered into the production fermenter, if the preferred process is submerged fermentation (deep, stirred tank vessels) or to be transferred onto solid media in the case of surface-culture or Koji fermentation.

10.6 Submerged fermentation

In submerged fermentation the enzyme is produced by the microorganism during growth in liquid medium contained in a large, deep production fermenter. The fermenter size in commercial enzyme manufacture today can vary from 20,000 l to 200,000 l. The fermenter is prepared for commercial production by first adding water, followed by addition of the nutrient ingredients which constitute the prescribed production media for that specific fermentation. The production media provides all carbon, nitrogen, minerals and vitamins required by the specific microorganism for maximum growth and enzyme yield. The fermenter contents are sterilized typically by steam injection at 121°C for 30–60 min.

Fermentation media may be divided into two categories, namely chemically defined and undefined ingredients. Defined media generally produce a consistent fermentation with less foaming and do not cause much interference in the recovery stage of commercial enzyme manufacture. This type of media is used most often in laboratory scale fermentations but because of cost considerations, chemically defined media are not typically employed in commercial enzyme production. The carbon and nitrogen sources in commercial enzyme fermentation media are derived from by-products of the food processing industry. Typical sources of carbon and mineral are cellulose, whey, molasses, soybean and corn while nitrogen sources include fish meal, corn steep liquor, yeast extract and flour. Apart from the requirement for consistent growth and enzyme yield in the fermenter, factors such as reliability of supply, consistent quality, food grade, non-GM, kosher and halal certification and cost are all extremely important considerations to be taken into account during the initial stages of fermentation media development. Depending on the substrate feeding strategy, submerged fermentation can be divided into three groups: batch, fed-batch and continuous fermentations. In batch fermentations, the full media composition is added to the fermenter and sterilized prior to the fermentation process. When less defined complex nutrients are used with varying concentrations of inhibitors, cell growth may be inconsistent. Typical batch fermentations may not be ideal for optimum culture growth and enzyme production where there is catabolite repression as a result of the presence of certain substrates. This problem

can be overcome by adding certain substrates in small quantities at different intervals of time during the fermentation process to meet the physiological requirements of the growing culture. In such a system, known as fed-batch fermentation, the primary metabolite concentration will build up together with the cell mass. Substrate concentration is kept at a certain minimum level by the intermittent feeding which avoids substrate inhibition. Several researchers have shown major improvements in yield with the use of fed-batch fermentations [4–6]. In continuous fermentations, the sterile liquid nutrient medium is fed continuously to the growing culture in the fermenter, while an equivalent volume of the fermentation broth is simultaneously drawn off. Although continuous fermentation may result in higher yields, this method is not used widely for commercial enzyme production because of the complexity of the system in large scale.

The duration of any given submerged fermentation process will depend on the type of organism being fermented also whether the enzymes are constitutively synthesized during the growth phase or synthesized during the late exponential and/or stationary phase. In general, bacterial fermentation can take as little as 24 h for full completion of enzyme yield to be achieved in the fermenter, while fungal fermentations can take as long as 6–10 days to achieve the same goal. The fermentation is constantly monitored (usually in a fully automated manner) to ensure constant growth of the culture and yield of enzyme. Factors such as agitation, dissolved oxygen, pH profile, temperature and off-gas analysis will all be fully checked and controlled to ensure optimum production of the enzyme by the culture in the fermentation broth. The next step in commercial enzyme manufacture is the recovery of the product in a series of post fermentation steps known as downstream processing.

10.7 Separation of broth

Several techniques are used to separate the liquid phase containing the enzyme from the solid phase post fermentation. Typically the techniques used are filtration with an appropriate filter aid, cross-flow microfiltration or centrifugation.

10.7.1 Filtration with filter aid

Two methods are predominantly used, namely plate and frame filtration and precoat rotary vacuum filtration.

Plate and frame filtration involves the precoating of a suitable support medium with a layer of filter aid. Fermentation broth also containing filter aid is then pumped through the filter, retaining the filter aid and solids on the support material. This procedure will result in a clear filtrate that will require some further processing.

Rotary vacuum filtration is a suitable alternative to plate and frame filtration. In this process a precoat filter is used where a deep layer of filter aid is built up on a rotating drum with a vacuum applied to keep the filter aid in place. The filter aid is kept moist by applying a water spray which also serves to wash the filtrate through the bed. The drum is rotated through a trough containing the fermentation broth and some of the broth is drawn onto the bed by the effect of the vacuum. The broth is washed and then a thin layer cut away allowing clean filter aid to come into contact with the fermentation broth again. The spent cake is discarded.

10.7.2 Microfiltration

It is possible to microfilter fermentation broth to recover a clear filtrate. The membranes used for this process can either be polymer or ceramic, with the pore size being determined by application trial work. The pore size used for recovery of fermentation broth ranges from 0.1 to 0.5 micron. The process involves circulating the fermentation broth around a system that keeps all the broth ingredients in suspension. This requires a high flow rate on the membrane surface to reduce fouling; usually 4 m/s with a low trans membrane pressure of 1.5 bar can be used. Microfiltration systems tend to be tubular in makeup with a minimum diameter for enzyme recovery of approximately 6 mm. Permeate flow rates are variable depending on the material in contact with the membrane surface; however, flow rates of up to 100 l/m^2/h are achievable. The broth is flushed with water to ensure full recovery of metabolites, however a balance is required between target yield, the volume of water added and length of process time. It is possible to sterilize the spent material and dispose through wastewater treatment.

10.7.3 Centrifugation

Fermentation broth may be centrifuged, the level of solids present will determine the type of separator required. A desludging bowl centrifuge can be used up to 25% wet solids and consideration must be given to the separator specification which takes into account the makeup of the broth ingredients. The particle size and viscosity of these ingredients will be a significant factor in the overall design and efficiency of the system chosen. Separation is also unlikely to be achieved in one pass, so a system is required to resuspend and reseparate the solids discharged. This may require two or more passes through the centrifuge depending on the efficiency of extraction required.

10.8 Concentration

Following separation from the fermentation broth solids, the filtrate which now consists of a large volume of dilute enzyme is ready for the next step in the

downstream process, which is concentration. This is the process whereby the protein (enzyme) content of the filtrate is concentrated, by eliminating large volumes of water containing minerals, vitamins and so on from the filtrate.

There are many methods of concentration possible and the method chosen will be dependent on the physical characteristics of the enzyme being recovered, such as molecular size, pH stability, thermal properties and cost of the selected process. The most common concentration technique used for enzyme recovery is ultrafiltration, where a selectively permeable membrane retains the required material and the waste is allowed to permeate through the membrane and is discarded. Ultrafiltration membranes have a typical molecular size ranging from 4,000 to 200,000 Daltons. These membranes may only be selected based on experimental trials, as enzyme protein molecular size and shape are variable. Different polymers showing the same molecular size characteristics can show different results when applied to ultrafiltration membranes. This process utilizes a higher trans membrane pressure than microfiltration, typically 5 bar and a lower flow rate on the membrane surface. Using this technique it is possible to achieve concentrates at approximately 30% soluble solids.

The enzyme concentrate may now be stored and is ready for blending into a liquid product for market or it may be further processed by drying, if a powder finished product is required.

10.9 Drying

There are many drying methods available to process the concentrate further to a powder. The most common method used is spray drying. Other techniques such as freeze-drying are considerably more expensive to operate from both a capital and running cost perspective.

Spray drying will result in a fine dusty powder, unless steps are taken to agglomerate this powder within the process or separately using a fluidized bed dryer. The suitability of the drying technique will have to be established by trial work to determine the optimal process conditions. Typically an inlet temperature of 200°C, with an outlet temperature of 80°C, may be used to dry most enzymes successfully. The powder produced in this way can be granulated using a fluid bed dryer at a temperature of 45°C. This technique will produce a micro-granular product of uniform particle size that is both free flowing and readily dispersible in liquid.

Enzymes, like many proteins when inhaled as dust or aerosols, may lead to respiratory allergy in susceptible individuals. If enzyme dust is inhaled excessively, the mucous membranes of the respiratory tract will be irritated and may cause coughing and/or congestion. The symptoms can be compared with those of pollen allergy and like hay fever they disappear when the exposure is eliminated. Respiratory allergies are caused by the inhalation of respirable particles below

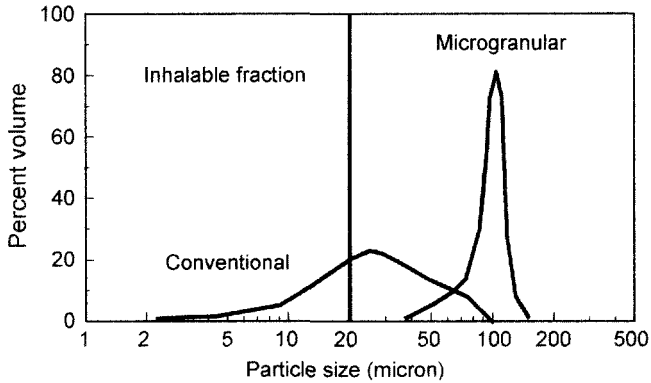

Figure 10.3 Comparison of the particle size profile of microgranulated and conventional spray dried powder.

20 micron. The presence of these particles can be dramatically reduced by the microgranulation process as described above. The data in figure 10.3 shows the profile of a traditional spray dried powder compared with a microgranulated powder. The data shows how respirable particles have been reduced to negligible levels in microgranulated enzymes.

10.10 Enzyme formulation

The final stage in preparation of an enzyme product for market is the formulation of that enzyme to a specific activity by blending of powder concentrate or dilution of liquid concentrate.

10.10.1 Powder blending

Formulation of a powder product for market involves the blending of powder concentrate from the spray dryer, which will typically have a very high enzyme titre. A suitable carrier is used to dilute the dry enzyme concentrate to the target activity of the product. Typical carriers or diluents used in commercial enzyme blending include starch, maltodextrins, lactose and flour fractions. The final selection of the carrier will depend on many factors, including particle size (matching that of the enzyme concentrate powder) and colour. There is a wide variety of different blenders available, ranging from Ribbon type, double and single cone, through to systems that involve tumbling individual box containers. The selection of a blender depends on factors such as the material, quantity, fragility and bulk density, along with many other physical characteristics of the ingredients to be blended.

Table 10.4 FCC* and JECFA** food grade enzyme specifications

Analysis	JECFA	FCC
Enzyme activity	–	85–115%
Arsenic	Not more than 3 ppm	–
Lead	Not more than 10 ppm	Not more than 5 ppm
Heavy metals	Not more than 40 ppm	Not more than 30 ppm
Salmonella	Not detected in 25g	Not detected in 25 g
Coliforms	Not more than 30/g	Not more than 30/g
E. coli	Not detected in 25g	–
Total viable count	Not more than 50,000/g	–
Antibiotic activity	Not detected (microbial sources)	–
Mycotoxins [4]	Not detected (fungal sources)	–

*Food Chemical Codex; ** Joint Expert Committee on Food Additives.

10.10.2 Liquid blending

Commercial enzyme products sold in liquid form are formulated by dilution of the high activity liquid enzyme concentrate with water, to the specific activity required of the final product. This process is carried out in diluting tanks which can range from a few hundred to several thousand litres in capacity. The final liquid enzyme product will also contain certain stabilizers, such as sorbitol or glycerol, to ensure stability of the enzyme protein in storage. It will also contain preservatives such as sodium benzoate and potassium sorbate to prevent microbial contamination of the final product in prolonged storage.

Prior to sale, powder and liquid enzyme products are finally checked to ensure they meet the required specifications and an example of such specifications may be seen in table 10.4.

10.11 Surface culture (Koji) fermentation

Surface culture fermentation is the second process type currently used to produce enzymes in commercial quantities. This method is primarily used in the East and has its origins in the production of fermented foods such as soy sauce in Japan, hence the commonly used term Koji (meaning 'bloom' in Japanese) fermentation. This fermentation process also goes under the name of solid state fermentation (SSF).

Surface culture fermentation can be briefly defined as fermentation in which microbial growth takes place on the surface of a solid substrate and in the absence or near absence of free water. The initial stage of the fermentation process, inoculum development, follows the exact process steps as outlined above for submerged fermentation. Once ready, this inoculum is seeded onto a solid medium which has been previously sterilized and the fermentation and enzyme production takes place as the culture grows on the solid substrate.

Substrates traditionally used in surface culture fermentation include a variety of agricultural products, such as wheat bran, rice bran or soy grits. The substrate is sterilized by chemical or heat treatment and once cooled is seeded with the inoculum. The fermentation is allowed to proceed under controlled conditions of temperature and humidity in static reactor trays, stored in sterile rooms. During this time, microbial growth and product formation occur at or near the solid surfaces of the specific substrate and the duration of fermentation can range from five to twelve days. At the end of this process, the static reactor trays containing the substrate are completely covered with microbial biomass and the contents of each tray are emptied into a large vessel known as the extraction vessel. The contents of this vessel (biomass, residual substrate and enzyme) are then washed with volumes of water to remove maximum amounts of the enzyme product formed during the fermentation process. The remainder of the downstream processing steps, beginning with ultrafiltration of the dilute enzyme solution post extraction, is exactly as described earlier for the submerged fermentation process, again yielding either a liquid or spray dried powder product, ready for market.

10.12 Intracellular enzyme production

All of the enzyme manufacturing described thus far, in both submerged and solid state fermentation, concentrates on the commercial production of extracellular enzymes. These are enzymes which are produced and excreted into the surrounding media by the growing microbial culture. There are, however, a number of commercially important intracellular enzymes produced by submerged fermentation, which remain within the cell wall of the growing cells and require separate downstream processing to prepare for market (figure 10.2).

An example of such an enzyme product is invertase (*beta*-fructofuranosidase). This enzyme is produced by the submerged fermentation of *Saccharomyces cerevisiae*. The first post fermentation step requires harvesting the microbial biomass which is usually carried out by centrifugation. This process is followed by disruption of the cells which can be carried out using enzymatic or chemical methods. The purpose of this procedure is to release the contents of the cells, which will include the intracellular target enzyme, in a mild manner to ensure maximum yield of enzyme. This is followed by a series of filtration steps to remove all cellular debris, then ultrafiltration and decolorization resulting in the desired liquid concentrate. This concentrate, now containing a high titre of invertase enzyme, is then stabilized with glycerol (30–50%) and finally blended, using standard liquid blending techniques, to the desired enzyme activity specification prior to pack-off. Alternatively, the liquid concentrate is spray dried and the resulting powder concentrate is blended to the desired enzyme activity specification.

10.13 Genetics of producer organisms

The economics of enzyme manufacture are of extreme importance to the companies involved in commercial production. A major part of the research and development within these companies is concentrated on the continuous improvement of the producer organism. Development areas have focused on increasing the yield of enzyme expression during fermentation, decreasing or removal of undesirable co-metabolites and improving the ability of the strain to utilize inexpensive nutrients during the fermentation process. Therefore strain improvement has primarily concentrated on modification of the genome to produce hyperproducing strains which will overproduce specific enzymes during fermentation. The traditional method used is the manipulation of the genome of the producer organism by exposure to a powerful mutagen which will destroy the majority of the microbial population. The surviving cells are then screened for genomic variants which have a positive affect on expression of the particular target enzyme. This method of gene manipulation, known as classical mutation, dates back to the 1930s with the publication of the research of Thom and Steinberg [7]. The process was further developed in the pioneering work on increasing the production of penicillin during the early to mid 1940s. The most common mutagens used in this process include UV light, X-rays, gamma rays and chemical agents, the principal one being *N*-methyl-*N*-nitro-*N*-nitrosoguanidine. For each mutagen and organism, there is a combination of mutagen concentration, time exposure and conditions of treatment to produce the highest proportion of mutants among the surviving population. These mutants are then screened for an increase in enzyme production and after stabilization of that particular mutant the procedure is repeated in a continuous effort to increase enzyme production. In this way, enzyme companies have increased the production capability of their strains to make specific enzymes and improvements of three to six-fold in enzyme production have occurred from the 1950s to the 1970s using these procedures.

The ability to increase expression of enzyme from an organism took a major step forward in the early 1970s with the development of what we now call genetic engineering or recombinant DNA (rDNA) technology. Using this technique, the specific gene coding for a given enzyme may now be isolated, purified and over-expressed in either the donor or a separate organism thus increasing the yield and purity of enzyme many times. The first cloning experiments reported in the early 1970s [8, 9] were carried out in *E. coli* which was the initial host organism of choice for the pioneering work in genetic modification using rDNA technology. As techniques developed, interest in the application of this technology using important industrial strains for commercial enzyme production grew, and the 1980s saw a major proliferation in cloning of commercially important enzymes in strains such as *Aspergillus niger*, *Trichoderma longibrachiatum* and *Bacillus subtilis*. The first petition for a food-grade enzyme produced by a genetically

Table 10.5 Commercial enzymes derived from rDNA technology

Enzyme name	Donor organism	Producer organism
α-Amylase	*B. stearothermophilus*	*B. subtilis*
Alphagalactosidase	Guar seed	*S. cerevisiae*
β-Glucanase	*B. subtilis*	*B. subtilis*
Cellulase	*T. reesei*	*T. reesei*
Chymosin	Calf	*A. niger, K. lactis*
Glucoamylase	*A. niger*	*A. niger*
Lactase	*K. marxianus* var *lactis*	*K. marxianus* var *lactis*
Phytase	*A. niger*	*A. niger*
Xylanase	*A. niger*	*A. niger* var *awamori*

B = Bacillus; S = Saccharomyces; T = Trichoderma; K = Kluyveromyces.

engineered microorganism was submitted by CPC International, Inc. to the US Food and Drug Administration (FDA) for GRAS status for *alpha*-amylase. The donor strain for the *alpha*-amylase gene was *Bacillus amyloliquefaciens* and was expressed in the producer organism or genetically modified organism (GMO) *Bacillus subtilis* ATCC 39,701. Since that time most enzyme companies have adopted this technology into their development programmes. Several genetically engineered enzyme products are now available commercially and this list will certainly continue to grow (table 10.5).

There are many reasons for the application of rDNA technology to commercial enzyme production.

- Commercial enzymes produced from GMOs are in general purer than their wild-type counterparts. This has major implications in food processing where the use of specific enzymes in a purer form can greatly enhance performance. Examples of this may be found in the bakery industry, where the application of amylases and pentosanases, now substantially free of protease activity, have a far greater effect on dough handling during processing without the danger of breakdown of the gluten network.
- Production of enzymes from GMOs results in higher yields compared to wild-type organisms, which means lower production costs. A recent paper by van den Hondel and coworkers [10] describes the overproduction of glucoamylase and endoxylanase in strains of *Aspergillus* with yields of between 10 and 30-fold increase over the wild-type strains.
- It is now possible to produce commercially certain enzymes in far greater amounts and more easily than ever before. The best example of such an enzyme is chymosin, which was originally derived from calf stomach and is now commercially produced by microbial fermentation as the bovine gene has been cloned into both *Aspergillus niger* var *awamori* (Chymax™) and *Kluyveromyces marxianus* var *lactis* (Maxiren™).

- Expression systems are now developed called 'plug-in' systems, which allow for the insertion of the specific purified gene of choice into an expression vector leading to the overproduction of that enzyme. This means that the same fermentation system, using the same producer organism, may now be used to produce many different types of enzymes, which is a major advantage to the commercial producer. The fermentation expertise can now be limited to a few or even a single organism into which many different genes may be inserted, thus simplifying the commercial production process.

As already indicated, most of the commercial enzyme producers have embraced this technology and are producing enzymes using rDNA techniques. There is widespread acceptance of enzymes derived from GMOs in non-food applications such as the textile and paper and pulp industries, but concerns still exist in the application of such enzymes to food processing. It remains a major task of the commercial enzyme industry to keep their customers aware of the origins of each enzyme on the market, to offer a choice of GMO or non-GMO derived product where possible and to continue to highlight the benefits to the consumer, the environment and the manufacturer as this new technology becomes an integral part of commercial enzyme production into the 21st century.

Acknowledgements

I wish to thank many of my colleagues, especially Richard Dring and Lynda Dubovi, for their kind assistance.

To my mother, Margaret Piggott, for her constant encouragement to follow my curiosity in science and nature, I dedicate my chapter.

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