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3	Texto de la Comunicación	Respuesta al Auto dictado por la Dirección de Nuevas Creaciones mediante notificación por fijación en lista de Marzo 22, 2016, correspondiente al Oficio No. 2708 (Ver Anexos).

4	Anexos

☒ Anexos

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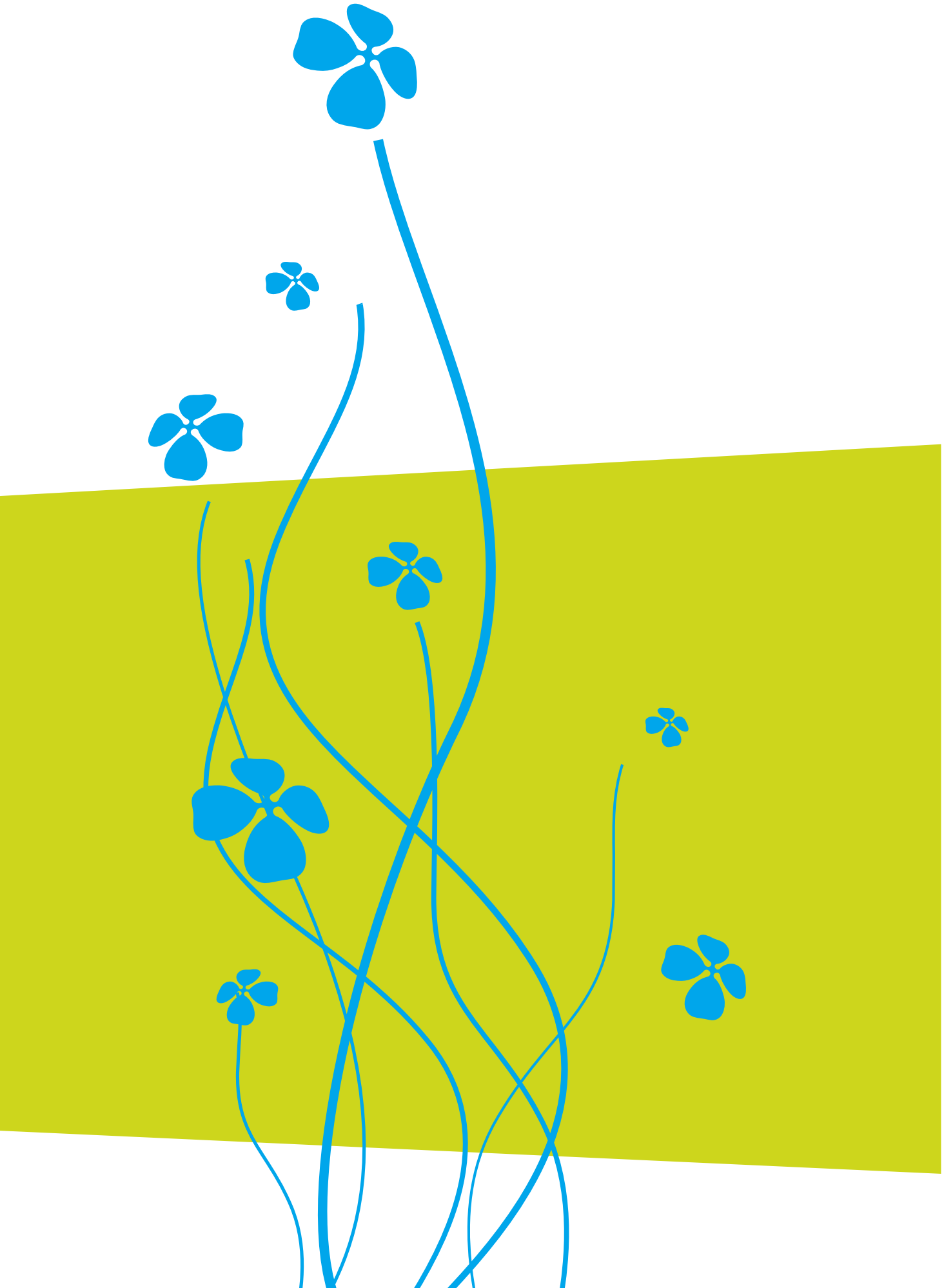
Rethink Tomorrow

Enzymes at work

Rethink Tomorrow...



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1. Why use enzymes for industrial processes?

Many chemical transformation processes used in various industries have inherent drawbacks from a commercial and environmental point of view. Nonspecific reactions may result in poor product yields. High temperatures and/or high pressures needed to drive reactions lead to high energy costs and may require large volumes of cooling water downstream. Harsh and hazardous processes involving high temperatures, pressures, acidity, or alkalinity need high capital investment, and specially designed equipment and control systems. Unwanted by-products may prove difficult or costly to dispose of. High chemicals and energy consumption as well as harmful by-products have a negative impact on the environment.

In a number of cases, some or all of these drawbacks can be virtually eliminated by using enzymes. As we explain in the next section, enzyme reactions may often be carried out under mild conditions, they are highly specific, and involve high reaction rates. Industrial enzymes originate from biological systems; they contribute to sustainable development through being isolated from microorganisms which are fermented using primarily renewable resources.

In addition, as only small amounts of enzymes are needed in order to carry out chemical reactions even on an industrial scale, both solid and liquid enzyme preparations take up very little storage space. Mild operating conditions enable uncomplicated and widely available equipment to be used, and enzyme reactions are generally easily controlled. Enzymes also reduce the impact of manufacturing on the environment by reducing the consumption of chemicals, water and energy, and the subsequent generation of waste.

Developments in genetic and protein engineering have led to improvements in the stability, economy, specificity, and overall application potential of industrial enzymes.

When all the benefits of using enzymes are taken into consideration, it's not surprising that the number of commercial applications of enzymes is increasing every year.

Table 1 presents a small selection of enzyme types currently used in industrial processes, listed according to class, for example:

1. A laccase is used in a chlorine-free denim bleaching process which also enables a new fashion look.

2. A glucosyltransferase is used in the food industry for the production of functional sweeteners.

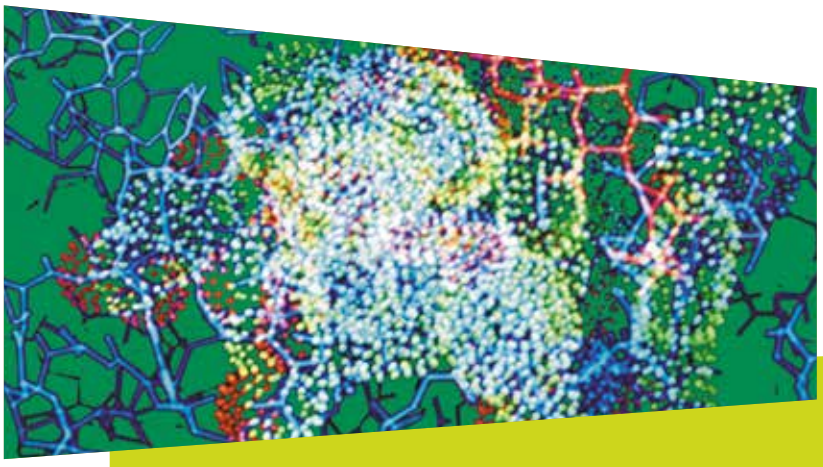
3. Hydrolases are by far the most widely used class of enzymes in industry. Numerous applications are decribed in later sections.

4. An alpha-acetolactate decarboxylase is used to shorten the maturation period after the fermentation process in beer brewing.

5. In starch processing, after hydrolyzing it to glucose, a glucose isomerase is used to convert glucose into fructose, which increases the sweetness of the syrup.

ENZYME CLASS	INDUSTRIAL ENZYMES
EC 1: Oxidoreductases	catalase
	glucose oxidase
	laccase
EC 2: Transferases	glucosyltransferase
EC 3: Hydrolases	amylase
	cellulase
	lipase
	mannanase
	pectinase
	phytase
	protease
	pullulanase
	xylanase
EC 4: Lyases	pectate lyase
	alpha-acetolactate decarboxylase
EC 5: Isomerases	glucose isomerase
EC 6: Ligases	not used at present

Table 1. A selection of enzyme types used in industrial processes.
(The classes are defined in Table 2.)



CLASS IN THE EC (Enzyme Commission) CLASSIFICATION	REACTION PROFILE
EC 1. Oxidoreductases	These enzymes catalyze *redox reactions*, i.e., reactions involving the transfer of electrons from one molecule to another. In biological systems we often see the removal of hydrogen atoms from a substrate. Typical enzymes catalyzing such reactions are called *dehydrogenases*. For example, alcohol dehydrogenase catalyzes reactions of the type $R\text{-}CH_2OH + A \rightarrow R\text{-}CHO + AH_2$, where A is a hydrogen acceptor molecule. Other examples of oxidoreductases are *oxidases* and *laccases*, both catalyzing the oxidation of various substrates by dioxygen, and *peroxidases*, catalyzing oxidations by hydrogen peroxide. *Catalases* are a special type, catalyzing the disproportionation reaction $2\ H_2O_2 \rightarrow O_2 + 2\ H_2O$, whereby hydrogen peroxide is both oxidized and reduced at the same time.
EC 2. Transferases	Enzymes in this class catalyze *the transfer* of groups of atoms from one molecule to another or from one position in a molecule to other positions in the same molecule. Common types are *acyltransferases* and *glycosyltransferases*. *CGTase* (cyclodextrin glycosyltransferase) is one such enzyme type, which moves glucose residues within polysaccharide chains in a reaction that forms cyclic glucose oligomers (cyclodextrins).
EC 3. Hydrolases	Hydrolases catalyze *hydrolysis*, the cleavage of substrates by water. The reactions include the cleavage of peptide bonds in proteins by *proteases*, glycosidic bonds in carbohydrates by a variety of *carbohydrases*, and ester bonds in lipids by *lipases*. In general, larger molecules are broken down to smaller fragments by hydrolases.
EC 4. Lyases	Lyases catalyze the addition of groups to double bonds or the formation of double bonds though the removal of groups. Thus bonds are cleaved by a mechanism different from hydrolysis. *Pectate lyases*, for example, split the glycosidic linkages in pectin in an elimination reaction leaving a glucuronic acid residue with a double bond.
EC 5. Isomerases	Isomerases catalyze rearrangements of atoms within the same molecule; e.g., *glucose isomerase* will convert glucose to fructose.
EC 6. Ligases	Ligases join molecules together with covalent bonds in biosynthetic reactions. Such reactions require the input of energy by the concurrent hydrolysis of a diphosphate bond in ATP, a fact which makes this kind of enzyme difficult to apply commercially.

Table 2. Enzyme classes and types of reactions.

2. The nature of enzymes

Enzymes are biological catalysts in the form of proteins that catalyze chemical reactions in the cells of living organisms. As such, they have evolved – along with cells – under the conditions found on planet Earth to satisfy the metabolic requirements of an extensive range of cell types. In general, these metabolic requirements can be defined as:

1) Chemical reactions must take place under the conditions of the habitat of the organism
2) Specific action by each enzyme
3) Very high reaction rates

2.1 Chemical reactions under mild conditions

Requirement 1) above means in particular that there will be enzymes functioning under mild conditions of temperature, pH, etc., as well as enzymes adapted to harsh conditions such as extreme cold (in arctic or high-altitude organisms), extreme heat (e.g., in organisms living in hot springs), or extreme pH values (e.g., in organisms in soda lakes). As an illustration of enzymes working under mild conditions, consider a chemical reaction observed in many organisms, the hydrolysis of maltose to glucose, which takes place at pH 7.0:

$$\text{maltose} + H_2O \rightarrow 2\ \text{glucose}$$

In order for this reaction to proceed nonenzymatically, heat has to be added to the maltose solution to increase the internal energy of the maltose and water molecules, thereby increasing their collision rates and the likelihood of their reacting together. The heat is supplied to overcome a barrier called "activation energy" so that the chemical reaction can be initiated (see Section 9.2).

As an alternative, an enzyme, maltase, may enable the same reaction at 25 °C (77 °F) by lowering the activation energy barrier. It does this by capturing the chemical reactants – called substrates – and bringing them into intimate contact at "active sites" where they interact to form one or more products. As the enzyme itself remains unchanged by the reaction, it continues to catalyze further reactions until an appropriate constraint is placed upon it.

2.2 Highly specific action

To avoid metabolic chaos and create harmony in a cell teeming with innumerable different chemical reactions, the activity of a particular enzyme must be highly specific, both in the reaction catalyzed and the substrates it binds. Some enzymes may bind substrates that differ only slightly, whereas others are completely specific to just one particular substrate. An enzyme usually catalyzes only one specific chemical reaction or a number of closely related reactions.

2.3 Very high reaction rates

The cells and tissues of living organisms have to respond quickly to the demands put on them. Such activities as growth, maintenance and repair, and extracting energy from food have to be carried out efficiently and continuously. Again, enzymes rise to the challenge.

Enzymes may accelerate reactions by factors of a million or even more. Carbonic anhydrase, which catalyzes the hydration of carbon dioxide to speed up its transfer in aqueous environments like the blood, is one of the fastest enzymes known. Each molecule of the enzyme can hydrate 100,000 molecules of carbon dioxide per second. This is ten million times faster than the nonenzyme-catalyzed reaction.

2.4 Numerous enzymes for different tasks

Because enzymes are highly specific in the reactions they catalyze, a diverse supply of enzymes must be present in cells to carry out all the different chemical transformations required. Most enzymes help break down large molecules into smaller ones and release energy from their substrates. To date, scientists have deposited around 35,000 known structures of enzyme molecules in the Enzyme Structures Database (http://www.ebi.ac.uk/thornton-srv/databases/enzymes/). Because there are so many, a logical method of nomenclature has been developed to ensure that each one can be clearly defined and identified.

Although enzymes are usually identified using short trivial names, they also have longer systematic names. Furthermore, each type of enzyme has a four-part classification number (EC number) based on the standard enzyme nomenclature system maintained by the International Union of Biochemistry and Molecular Biology (IUBMB) and the International Union of Pure and Applied Chemistry (IUPAC).

Depending on the types of reactions catalyzed, enzymes are divided into six main classes, which in turn are split into groups and subclasses. For example, the enzyme that catalyzes the conversion of milk sugar (lactose) to galactose and glucose has the trivial name lactase, the systematic name β-D-galactoside galactohydrolase, and the classification number EC 3.2.1.23.

Table 2 lists the six main classes of enzymes and the types of reactions they catalyze.

3. Industrial enzyme production

At Novozymes, industrial enzymes are produced using a process called submerged fermentation. This involves growing carefully selected microorganisms (bacteria and fungi) in closed vessels containing a rich broth of nutrients (the fermentation medium) and a high concentration of oxygen (aerobic conditions). As the microorganisms break down the nutrients, they produce the desired enzymes. Most often the enzymes are secreted into the fermentation medium.

Thanks to the development of large-scale fermentation technologies, today the production of microbial enzymes accounts for a significant proportion of the biotechnology industry's total output. Fermentation takes place in large vessels called fermentors with volumes of up to 1,000 cubic meters.

The fermentation media comprise nutrients based on renewable raw materials like corn starch, sugars, and soy grits. Various inorganic salts are also added depending on the microorganism being grown.

Both fed-batch and continuous fermentation processes are common. In the fed-batch process, sterilized nutrients are added to the fermentor during the growth of the biomass. In the continuous process, sterilised liquid nutrients are fed into the fermentor at the same flow rate as the fermentation broth leaving the system, thereby achieving steady-state production. Operational parameters like temperature, pH, feed rate, oxygen consumption, and carbon dioxide formation are usually measured and carefully controlled to optimize the fermentation process (see Fig. 1).

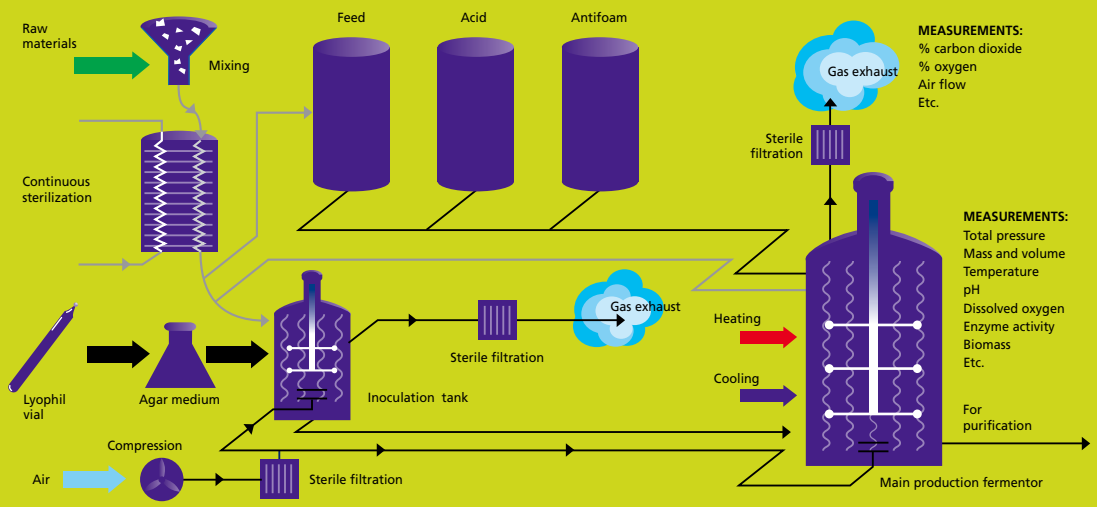


Fig. 1. A conventional fermentation process for enzyme production.

The first step in harvesting enzymes from the fermentation medium is to remove insoluble products, primarily microbial cells. This is normally done by centrifugation or microfiltration steps. As most industrial enzymes are extracellular – secreted by cells into the external environment – they remain in the fermented broth after the biomass has been removed. The biomass can be recycled as a fertilizer on local farms, as is done at Novozymes' major production sites. But first it must be treated with lime to inactivate the microorganisms and stabilize it during storage.

The enzymes in the remaining broth are then concentrated by evaporation, membrane filtration or crystallization depending on their intended application. If pure enzyme preparations are required, for example for R&D purposes, they are usually isolated by chromatography and/or by electrophoresis.

Certain applications require solid enzyme products, so the crude enzyme is processed into a granulate for convenient dust-free use. Other customers prefer liquid formulations because they are easier to handle and dose along with other liquid ingredients. Glucose isomerase used in the starch industry to convert glucose into fructose is immobilized, typically on the surfaces of particles of an inert carrier material held in reaction columns or towers. This is done to prolong their working life; such immobilized enzymes may go on working for over a year.

4. Enzymes for detergency and personal care

Enzymes have contributed greatly to the development and improvement of modern household and industrial detergents, the largest application area for enzymes today. They are effective at the moderate temperature and pH values that characterize modern laundering conditions, and in laundering, dishwashing, and industrial & institutional cleaning, they contribute to:

- A better cleaning performance in general
- Rejuvenation of cotton fabric through the action of cellulases on fibers
- Reduced energy consumption by enabling lower washing temperatures
- Reduced water consumption through more effective soil release
- Minimal environmental impact since they are readily biodegradable
- Environmentally friendlier wash water effluents (in particular, phosphate-free and less alkaline)

Furthermore, the fact that enzymes are renewable resources also makes them attractive to use from an environmental point of view.

4.1 The role of detergent enzymes in general

Enzyme applications in detergents began in the early 1930s with the use of pancreatic enzymes in presoak solutions. It was the German scientist Otto Röhm who first patented the use of pancreatic enzymes in 1913. The enzymes were extracted from the pancreases of slaughtered animals and included proteases (trypsin and chymotrypsin), carboxypeptidases, alpha-amylases, lactases, sucrases, maltases, and lipases. Thus, the foundation was laid already in 1913 for the commercial use of enzymes in detergents. Today, enzymes are continuously growing in importance for detergent formulators whether the cleaning task is for laundry, automatic dishwashing or cleaning of industrial equipment in the food industry.

Although the detailed ingredient lists for detergents vary considerably across geographies, the main detergency mechanisms are similar. Soils and stains are removed by mechanical action assisted by enzymes, surfactants, polymers and builders.

Surfactants of various kinds help the wash liquor wet fabrics by lowering the surface tension at the interface and assist in removing various kinds of soilings. Anionic surfactants and polymers furthermore increase the repulsive force between the original

soil, enzymatically degraded soil and the fabric and thereby help to prevent soil redeposition.

Builders act to chelate, precipitate, or ion-exchange calcium and magnesium ions, to provide alkalinity and buffering capacity, and to inhibit corrosion.

Enzymes in heavy-duty detergents degrade, and thereby help solubilize, substrate soils attached to fabrics or hard surfaces (e.g., dishes). Cellulases also clean indirectly by gently hydrolyzing certain glycosidic bonds in cotton fibers. In this way, particulate soils attached to microfibrils are removed. A further desirable effect of cellulases is to achieve greater softness and improved color brightness of worn cotton surfaces. Many detergent brands are based on a blend of two or more, even up to eight different enzyme products.

One of the driving forces behind the development of new enzymes or the modification of existing ones for detergents is to make enzymes more tolerant to other ingredients, for example builders, surfactants, and bleaching chemicals, and to alkaline solutions. The trend towards lower wash temperatures, at least in Europe, has also increased the need for additional and more efficient enzymes. Starch and fat stains are relatively easy to remove in hot water, but the additional cleaning power provided by enzymes is required in cooler water.

4.2 Enzymes for laundry detergents

The most widely used detergent enzymes are hydrolases, which remove soils consisting of proteins, lipids, and polysaccharides. Research is currently being carried out with a view to extending the types of enzymes used in detergents.

Many problem stains come from a range of modern food products such as chocolate ice cream, baby food, desserts, dressings and sauces. To help remove these stains, and classical soilings like blood, grass, egg and animal and vegetable fat, a number of different hydrolases are added to detergents. The major classes are proteases, lipases, amylases, mannanases, cellulases and pectinases. Historically, proteases were the first of these to be used extensively in laundering for increasing the effectiveness of detergents.

Cellulases contribute to cleaning and overall fabric care by maintaining, or even rejuvenating, the appearance of washed cotton-based garments through selective reactions not previously available when washing clothes.

Some lipases can act as alternatives to current surfactant technology targeting greasy lipid-based stains, and lipases are thus an essential part of enzyme solutions used to replace surfactants.

Recent investigations show that often multienzyme systems may replace up to 25 % of a laundry detergent's surfactant system without compromising the cleaning effect. This leads to a more sustainable detergent that allows cleaning at low wash temperature.

Mannanases and pectinases are used for hard-to-remove stains of salad dressing, ketchup, mayonnaise, ice cream, frozen desserts, milkshakes, body lotions, and toothpaste as well as banana, tangerines, tomatoes, and fruit-containing products like marmalades, juices, drinking yogurts and low-fat dairy products.

The obvious advantages of enzymes make them universally acceptable for meeting consumer demands. Due to their catalytic nature, they are ingredients requiring only a small space in the formulation of the overall product. This is of particular value at a time where detergent manufacturers are compactifying their products.

4.2.1 Easyzyme®: making it easy to wash with laundry bars

In many parts of the world, strongly colored and stubborn stains from blood, sebum, food soils, cocoa, and grass are removed with the help of laundry detergent bars. After decades of very little performance enhancement for laundry bars, a new solution that allows the incorporation of enzymes has been developed. A specially formulated protease empowers the producer to create products that stand out from non-enzymatic laundry detergent bars, offering effective and effortless washing. Stain removal and washing by hand is one of the more time-consuming and physically demanding domestic duties. With the protease product Easyzyme® in laundry bars, washing is shortened by at least one rinse and requires much less scrubbing. In addition to obtaining a superior result, laundry bars containing the enzyme may be formulated to be milder to the hands than old type bars without enzymes.

4.2.2 Washing cold

Most of the energy spent during a household machine wash is used for heating the water. Thus, for saving energy and thereby helping to reduce carbon dioxide emissions, the most efficient measure is to lower washing temperatures. Increased use of enzymes from a spectrum of types available today, combined with a choice of appropriate other ingredients, including surfactants and bleaching systems specifically selected to work at low temperatures, has enabled manufacturers to produce 'cold water detergents'.

4.3 Enzymes in dishwashing

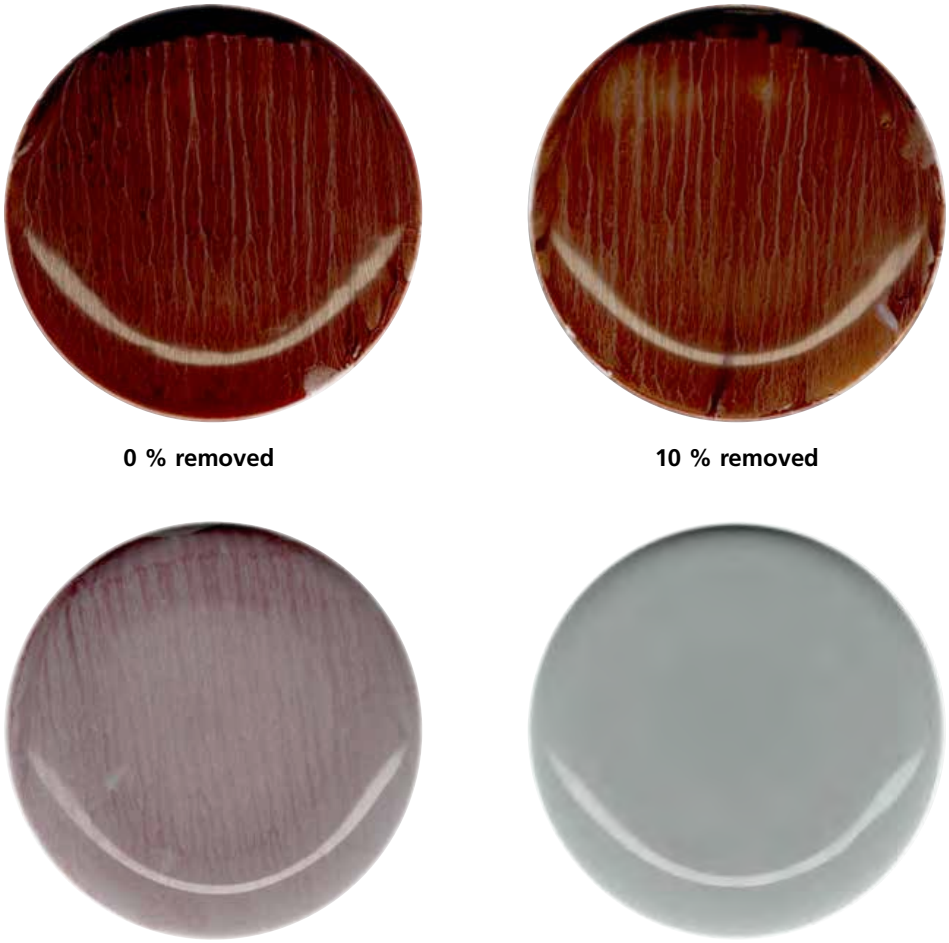
Modern dishwashing detergents face increasing consumer demands for efficient cleaning of tableware. Enzymes are key ingredients for effectively removing difficult and dried-on soils from dishes and leaving glassware shiny. Enzymes clean well under mild conditions and thereby assist to reduce clouding of glassware. In addition, enzymes also enable environmentally friendly detergents. Phosphates have been used in dishwashing detergents to get dishes clean, but they harm the aquatic environment and are increasingly being banned in detergents around the world. The combination of modifying detergent compositions and using multienzyme solutions enables the detergent manufacturers to replace phosphates without compromising the cleaning performance.

For removal of starch soils, amylases are used; and proteases are used for removal of protein soil.

4.3.1 Amylases for cleaning dishes from starch-containing soils

In automatic dishwashing, most of the soil is washed off by the water jets. However, foods usually leave behind a thin film of starch/protein-containing soils. If they are not removed, these films will build up over time. Larger lumps of burnt-on soils may also remain. These soils are the main target for enzymes.

The performance of automatic dishwashing detergents is determined by washing artificially soiled items with a range of enzyme dosages as shown on the pictures below. The residual starch films on the dishes were here dyed with an iodine solution to make them more visible.

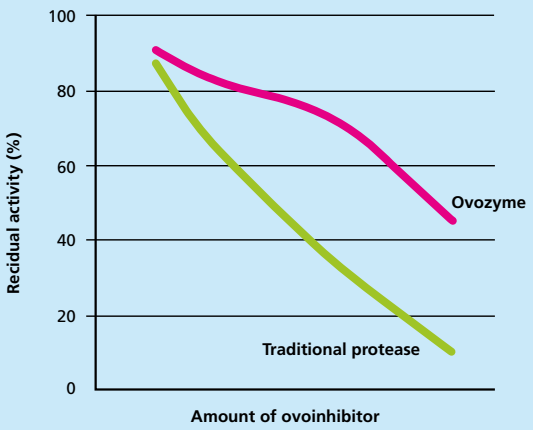


Dyed starch on porcelain plates.

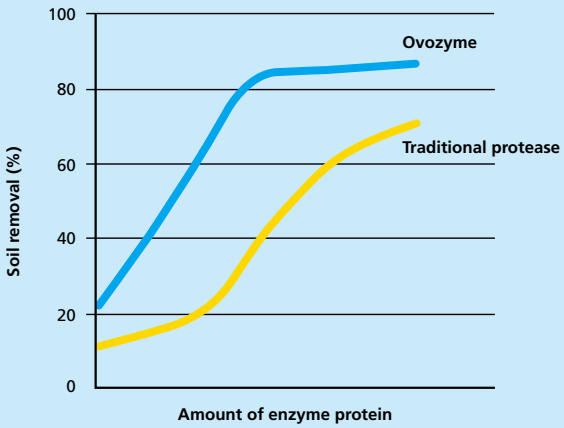
4.3.2 Proteases for cleaning dishes and cutlery

Some of the more difficult soilings on dishes and cutlery were blends of egg yolk/milk, egg yolk, whole egg and egg white as well as minced meat and oatmeal. The reason for this was the content of protease inhibitors in these foods. The protease Ovozyme® has been specifically engineered to overcome high levels of protease inhibitors from eggs. These inhibitors effectively inactivate detergent proteases, resulting in reduced cleaning performance not just on the egg stain itself, but on all protein-containing soils in the same dishwasher load. The effects of the ovoinhibitors on protease activity are illustrated below in Graph 1 and 2.

Graph 1. Inhibition of detergent proteases by ovoinhibitor.



Graph 2. Soil removal effect of Ovozyme® compared to a traditional protease in a wash with ovoinhibitor present.



4.4 Enzymes for cleaning-in-place (CIP) and membrane cleaning in the food industry

For many years, proteases have been used as minor functional ingredients in formulated detergent systems for cleaning reverse osmosis membranes. Now various enzymes are also used in the dairy and brewing industries for cleaning microfiltration and ultrafiltration membranes, as well as for cleaning membranes used in fruit juice processing. As most proteinaceous stains or soils are complexes of proteins, fats, and carbohydrates, beneficial synergistic effects can be obtained in some cases by combining different hydrolytic enzymes.

4.5 Personal care

The following examples illustrate the large potential of enzymes in the personal care sector:

Some brands of toothpaste and mouthwash already incorporate a multiple-enzyme system that helps supplement the natural antibacterial system in the mouth. An amylase and a glucoamylase hydrolyze starch (included in the toothpaste, or present as a food residue in the mouth) to produce glucose; a glucose oxidase oxidizes glucose, producing hydrogen peroxide; a lactoperoxidase helps hydrogen peroxide oxidize thiocyanate (added as the potassium salt KSCN to the toothpaste, but also naturally present in the mouth) to hypothiocyanite, $OSCN^-$, which is the true antibacterial agent. This has a positive effect in preventing plaque formation even though people normally brush their teeth for only 2–5 minutes. Dentures can be efficiently cleaned with products containing a protease.

Enzyme applications are also established in the field of contact lens cleaning. Contact lenses are cleaned using solutions containing proteases or lipases or both. After disinfection, the residual hydrogen peroxide is decomposed using a catalase.

5. Enzyme applications in nonfood industries

The textile industry has been quick to adopt new enzymes. So when Novo developed enzymes for stonewashing jeans in 1987, it was only a matter of a few years before almost everybody in the denim finishing industry had heard of them, tried them, and started to use them.

The leather industry is more traditional, and new enzyme applications are slowly catching on, though bating with enzymes is a long-established application. One of the prime roles of enzymes is to improve the quality of leather, but they also help to reduce waste. This industry, like many others, is facing tougher and tougher environmental regulations in many parts of the world. The consumption of chemicals and the impact on the environment can be minimized with the use of enzymes. Even chrome shavings can be treated with enzymes and recycled.

As regards pulp and paper, enzymes can minimize the use of bleaching chemicals. Sticky resins on equipment that cause holes in paper can also be broken down.

A growing area for enzymes is the animal feed industry. In this sector, enzymes are used to make more nutrients in feedstuffs accessible to animals, which in turn reduces the production of manure. The unwanted effect of phosphorus compounds on the environment can therefore be reduced.

The use of enzymes in oil and gas drilling, and in the production of biopolymers and fuel ethanol are also briefly discussed in this section.

The term "biofuels" normally refers to either bioethanol, biodiesel or biogas. Bioethanol is based on crops or other biomass and is produced by the help of enzymes and subsequent fermentation by yeast. Biodiesel is manufactured from vegetable oils.

The transformation of nonnatural compounds by enzymes, generally referred to as biocatalysis, has grown rapidly in recent years. The accelerated reaction rates, together with the unique chemo-, regio-, and stereoselectivity (highly specific action), and mild reaction conditions offered by enzymes, makes them highly attractive as catalysts for organic synthesis.

5.1 Textiles

Enzymes have found wide application in the textile industry for improving production methods and fabric finishing. One of the oldest applications in this industry is the use of amylases to remove starch size. The warp (longitudinal) threads of fabrics are often coated with starch in order to prevent them from breaking during weaving.

Scouring is the process of cleaning fabrics by removing impurities such as waxes, pectins, hemicelluloses, and mineral salts from the native cellulosic fibers. Research has shown that pectin acts like glue between the fiber core and the waxes, but can be destroyed by an alkaline pectinase. An increase in wettability can thus be obtained.

Cellulases have become the tool for fabric finishing and bioblasting. Their success started in denim finishing when it was discovered that cellulases could achieve the fashionable stonewashed look traditionally achieved through the abrasive action of pumice stones. With bioblasting the textile finishing process uses cellulases to modify the surface of cotton fabrics. Novozymes' bioblasting solutions permanently prevent pilling and maintain smoothness and softness of fabrics over time.

Catalases are used for degrading residual hydrogen peroxide after the bleaching of cotton. Hydrogen peroxide has to be removed before dyeing.

Proteases are used for wool treatment and the degumming of raw silk.

5.1.1 Enzymatic desizing of cotton fabric

Although many different compounds have been used to size fabrics over the years, starch has been the most common sizing agent for more than a century and this is still the case today.

After weaving, the size must be removed to prepare the fabric for the finishing steps of bleaching or dyeing. Starch-splitting enzymes are used for desizing woven fabrics because of their highly efficient and specific way of desizing without harming the yarn. As an example, desizing on a jigger is a simple method where the fabric from one roll is processed in a bath and re-wound on another roll. First, the sized fabric is washed in hot water (80–95 °C/176–203 °F) to gelatinize the starch. The desizing liquor is then adjusted to pH 5.5–7.5 and a temperature of 60–80 °C (140–176 °F) depending on the enzyme. The fabric then goes through an impregnation stage before the amylase is added. Degraded starch in the form of dextrins is then removed by washing at (90–95 °C/194–203 °F) for two minutes.

The jigger process is a batch process. By contrast, in continuous high-speed processes, the reaction time for the enzyme may be as short as 15 seconds. Desizing on pad rolls is continuous in terms of the passage of the fabric. However, a holding time of 2–16 hours at 20–60 °C (68–140 °F) is required using low-temperature alpha-amylases before the size is removed in washing chambers. With high-temperature amylases, desizing reactions can be performed in steam chambers at 95–100 °C (203–212 °F) or even higher temperatures to allow a fully continuous process. This is illustrated in Fig. 2.

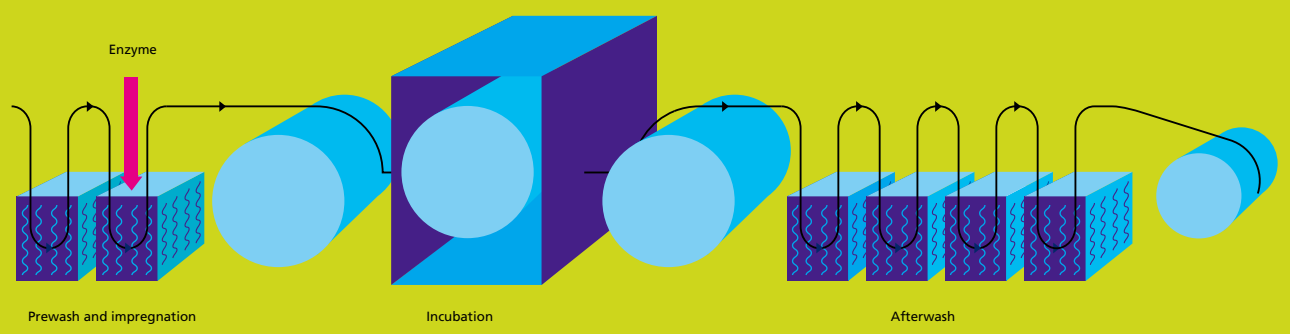


Fig. 2. A pad roll process.

5.1.2. Enzymes for denim finishing

Most denim jeans or other denim garments are subjected to a wash treatment to give them a slightly worn look. In the traditional stonewashing process, the blue denim is faded by the abrasive action of lightweight pumice stones on the garment surface, which removes some of the dye. However, too much abrasion can damage the fabric, particularly hems and waistbands. This is why denim finishers today use cellulases to accelerate the abrasion by loosening the indigo dye on the denim. Since a small dose of enzyme can replace several kilograms of stones, it results in less damage to garments, less wear on machines, and less pumice dust in the working environment. The need for the removal of dust and small stones from the finished garment is also reduced. Productivity can furthermore be increased through laundry machines containing fewer stones and more garments. There is also no sediment in the wastewater, which can otherwise block drains.

The mode of action of cellulases is shown in Fig. 3. Denim garments are dyed with indigo, a dye that penetrates only the surface of the yarn, leaving the center light in color. The cellulase molecule binds to an exposed fibril (bundles of fibrils make up a fiber) on the surface of the yarn and hydrolyzes it, but leaving the interior part of the cotton fiber intact. When the cellulases partly hydrolyze the surface of the fiber, the blue indigo is released, aided by mechanical action, from the surface and light areas become visible, as desired.

Both neutral cellulases acting at pH 6–8 and acid cellulases acting at pH 4–6 are used for the abrasion of denim. There are a number of cellulases available, each with its own special properties. These can be used either alone or in combination in order to obtain a specific look. Practical, ready-to-use formulations containing enzymes are available.

Application research in this area is focused on preventing or enhancing backstaining depending on the style required. Backstaining is defined as the redeposition of released indigo onto the garments. This effect is very important in denim finishing. Backstaining at low pH values (pH 4–6) is relatively high, whereas it is significantly lower in the neutral pH range. Neutral cellulases are therefore often used when the objective is minimal backstaining.

The denim industry is driven by fashion trends. The various cellulases available (as the DeniMax® product range) for modifying the surface of denim give fashion designers a pallet of possibilities for creating new shades and finishes. Bleaching or fading of the blue indigo color can also be obtained by use of another enzyme product (DeniLite®) based on a laccase and a mediator compound. This system together with dioxygen from the air oxidizes and thereby bleaches indigo, creating a faded look. This bleaching effect was previously only obtainable using harsh chlorine-based bleach. The combination of new looks, lower costs, shorter treatment times, and less solid waste has made abrasion and bleaching with enzymes the most widely used fading processes today. Incidentally, since the denim fabric is always sized, the complete process also includes desizing of the denim garments, by the use of amylases.

5.1.3 Cellulases for bioblasting of cotton fabric

Cotton and other natural and man-made cellulosic fibers can be improved by bioblasting. The main advantage of bioblasting is the prevention of pilling. A ball of fuzz is called a "pill" in the textile trade. These pills can present a serious quality problem since they result in an unattractive, knotty fabric appearance. Cellulases hydrolyze the microfibrils (hairs or fuzz) protruding from the surface of yarn because they are most susceptible to enzymatic attack. This weakens the microfibrils, which tend to break off from the main body of the fiber and leave a smoother yarn surface.

After bioblasting, the fabric shows a much lower pilling tendency. Other benefits of removing fuzz are a softer, smoother feel, and superior color brightness. Unlike conventional softeners, which tend to be washed out and often result in a greasy feel, the softness-enhancing effects of bioblasting are washproof and non-greasy.

5.1.4 Cellulases for the bioblasting of lyocell

For cotton fabrics, the use of bioblasting is optional for upgrading the fabric. However, bioblasting is almost essential for the new type of regenerated cellulosic fiber *lyocell* (the leading make is known by the trade name Tencel®). Lyocell is made from wood pulp and is characterized by a high tendency to fibrillate when wet. In simple terms, fibrils on the surface of the fiber peel up. If they are not removed, finished garments made of lyocell will end up with an unacceptable pilled look. This is the reason why lyocell fabric is treated with cellulases during finishing. Cellulases also enhance the attractive, silky appearance of lyocell. Lyocell was invented in 1991 by Courtaulds Fibers (now Acordis, part of Akzo Nobel) and at the time was the first new man-made fiber in 30 years.

5.1.5 Enzymes for wool and silk finishing

The bioblasting of cotton and other fibers based on cellulose came first, but in 1995 enzymes were also introduced for the bioblasting of wool. Wool is made of protein, so this treatment features a protease that modifies the wool fibers. "Facing up" is the trade term for the ruffling up of the surface of wool garments by abrasive action during dyeing. Enzymatic treatment reduces facing up, which significantly improves the pilling performance of garments and increases softness.

Proteases are also used to treat silk. Threads of raw silk must be degummed to remove sericin, a proteinaceous substance that covers the silk fiber. Traditionally, degumming is performed in an alkaline solution containing soap. This is a harsh treatment because the fiber itself, the fibrin, is also attacked. However, the use of specific proteolytic enzymes is a better method because they remove the sericin without attacking the fibrin. Tests with high concentrations of enzymes show that there is no fiber damage and the silk threads are stronger than with traditional treatments.

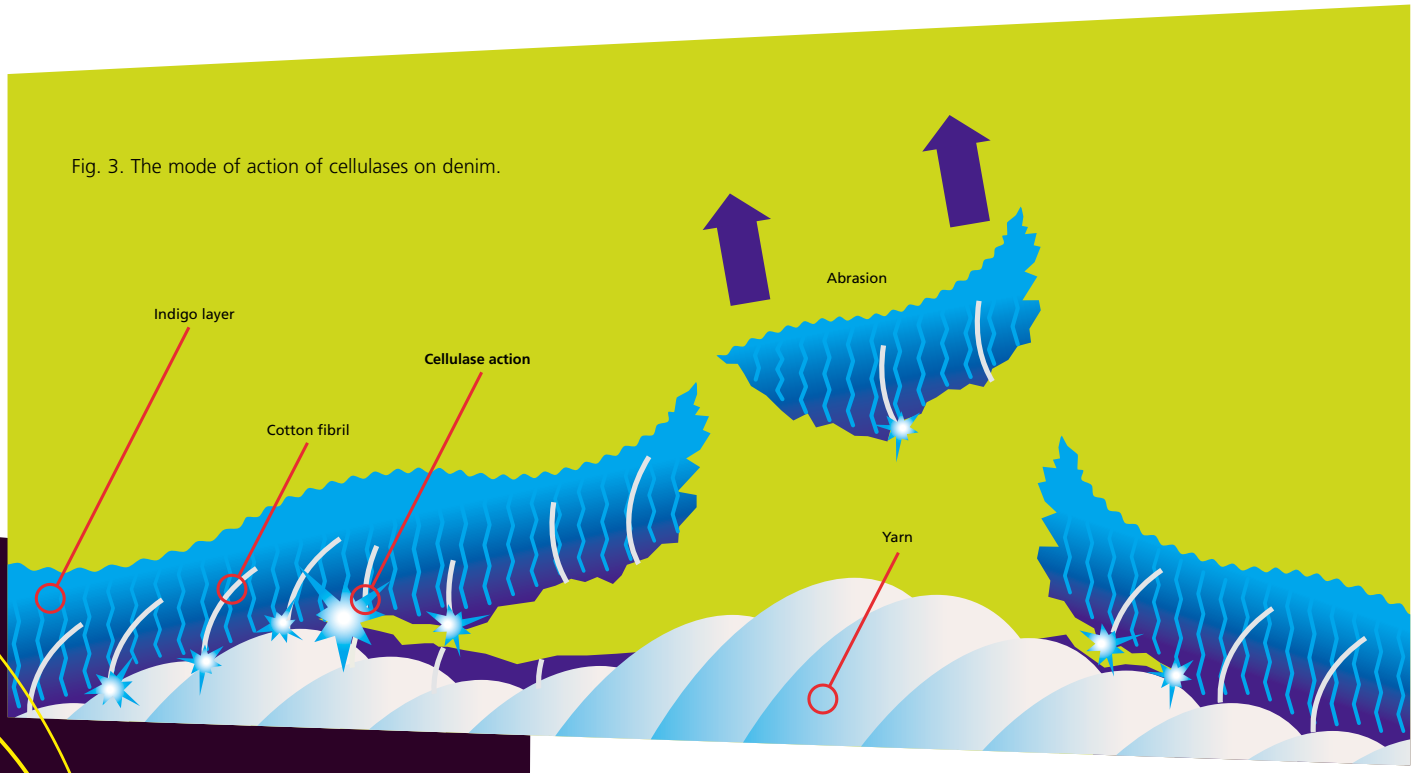


Fig. 3. The mode of action of cellulases on denim.

5.1.6 Scouring with enzymes

Before cotton yarn or fabric can be dyed, it goes through a number of processes in a textile mill. One important step is scouring – the complete or partial removal of the noncellulosic components of native cotton such as waxes, pectins, hemicelluloses, and mineral salts, as well as impurities such as size and lubricants. Scouring gives a fabric with a high and even wettability that can be bleached and dyed successfully. Today, highly alkaline chemicals such as sodium hydroxide are used for scouring. These chemicals not only remove the impurities but also attack the cellulose, leading to a reduction in strength and loss of weight of the fabric. Furthermore, the resulting wastewater has a high COD (chemical oxygen demand), BOD (biological oxygen demand), and salt content.

Alternative and mutually related processes introduced within the last decade, called Bio-Scouring and Bio-Preparation, are based on enzymatic hydrolysis of pectin substrates in cotton. They have a number of potential advantages over the traditional processes. Total water consumption is reduced by 25 % or more, the treated yarn/fabrics retain their strength properties, and the weight loss is much less than for processing in traditional ways. Bio-Scouring also gives softer cotton textiles.

Scourzyme® L is an alkaline pectinase used for Bio-Scouring natural cellulosic fibers such as cotton, flax, hemp, and blends. It removes pectin from the primary cell wall of cotton fibers without any degradation of the cellulose, and thus has no negative effect on the strength properties of cotton textiles or yarn.

5.2 Leather

Enzymes have always been a part of leather-making, even if this has not always been recognized. Since the beginning of the last century, when Röhm introduced modern biotechnology by extracting pancreatin for the bating process, the use of enzymes in this industry has increased considerably.

Nowadays, enzymes are used in all the beamhouse processes and have even entered the tanhouse. The following outlines the purposes and advantages of using enzymes for each leather-making process.

5.2.1 Soaking

Restoration of the water of salted stock is a process that traditionally applied surfactants of varying biodegradability. Proteases, with a pH optimum around 9–10, are now widely used to clean the stock and facilitate the water uptake of the hide or skin.

The enzyme breaks down soluble proteins inside the matrix, thus facilitating the removal of salt and hyaluronic acid. This makes room for the water. Lipases provide synergy. A cross section of a piece of skin is shown in the picture on page 21.

5.2.2 Liming

The main objective of liming is to remove hair. The conventional process used lime and sodium sulfide. The mechanism of the process is different if proteases and lipases are used in the liming process. Protein that surrounds the basement membrane of the hair root is hydrolyzed. Thereby the "cementing substances" such as proteoglycans, glycosaminoglycans and dermatan sulfate are removed, and the hair is loosened. Degradation of hair is achieved more quickly and completely as a result of the joint action of the chemicals which convert hair into a kind of "hair pulp" in conventional unhairing.

5.2.3 Bating

In this final beamhouse process, residues of noncollagen protein and other interfibrillary material are removed. This leaves the pelt clean and relaxed, ready for the tanning operation.

Traditionally, pancreatic bates have been used, but bacterial products are gaining more and more acceptance.

By combining the two types of proteases, the tanner gets an excellent bate with synergistic effects which can be applied to all kinds of skins and hides.

The desired result of a clean grain with both softness and tightness is achieved in a short time.

5.2.4 Acid bating

Pickled skins and wetblue stock have become important commodities. A secondary bating is necessary due to non-homogeneity.

For skins as well as double face and fur that have not been limed and bated, a combination of an acid protease and lipase ensures increased evenness, softness, and uniformity in the dyeing process.

Wetblue intended for shoe uppers is treated with an acid to neutral protease combined with a lipase, resulting in improved consistency of the stock.

5.2.5 Degreasing/fat dispersion

Lipases offer the tanner two advantages over solvents or surfactants: improved fat dispersion and production of waterproof and low-fogging leathers.

Alkaline lipases are applied during soaking and/or liming, preferably in combination with the relevant protease. Among other things, the protease opens up the membranes surrounding the fat cell, making the fat accessible to the lipase. The fat becomes more mobile, and the breakdown products emulsify the intact

Figure showing a cross section of a piece of skin.
The two upper layers - grain and the junction of grain and corium - is what is left after the processes called liming, bating and degreasing.

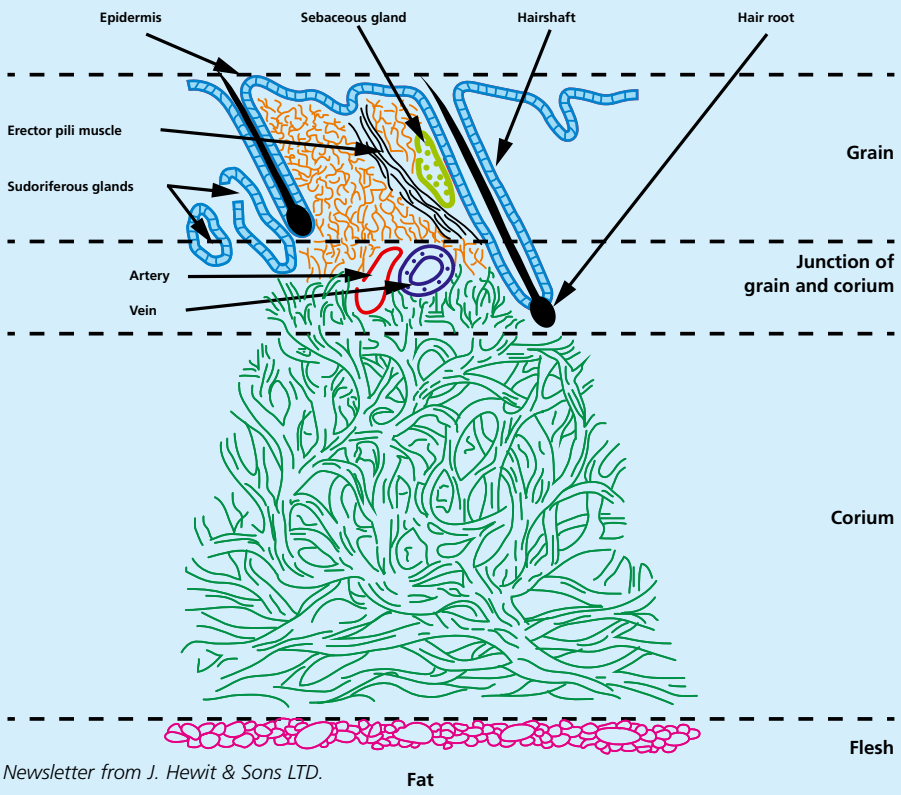


Figure from *The biannual Newsletter from J. Hewit & Sons LTD.* No.2 - Autumn 1996

fat, which will then distribute itself throughout the pelt so that in many cases a proper degreasing with surfactants will not be necessary. This facilitates the production of waterproof and low-fogging stock.

Lipases can also be applied in an acid process, for example for pickled skin or wool-on and fur, or a semi-acid process for wet-blue.

5.2.6 Area expansion

Elastin is a retractile protein situated especially in the grain layer of hides and skins, see picture c. Intact elastin tends to prevent the relaxation of the grain layer. Due to its amino acid composition, elastin is not tanned during chrome tanning and can therefore be partly degraded by applying an elastase-active enzyme on the tanned wetblue.

The results are increased area and improved softness, without impairing strength.

As well as the above-mentioned increase in area of the wetblue, application of NovoCor® AX can often increase the cuttable area into the normally loose belly area, resulting in an even larger improvement in area.

5.3 Forest products

Over the last two decades the application of enzymes in the pulp & paper industry has increased dramatically, and still new applications are developed. Some years ago the use of amylases for modification of starch coating and xylanases to reduce the consumption of bleach chemicals were the most well known applications, but today lipases for pitch control, esterases for stickies removal, amylases and cellulases for improved deinking and cellulases for fiber modification have become an integral part of the chemical solutions used in the pulp and paper mills. Table 3 lists some of the applications for enzymes in the pulp & paper industry.

5.3.1 Traditional pulp and paper processing

Most paper is made from wood. Wood consists mainly of three polymers: cellulose, hemicellulose, and lignin. The first step in converting wood into paper is the formation of a pulp containing free fibers. Pulping is either a mechanical attrition process or a chemical process. A mechanical pulp still contains all the wood components, including the lignin. This mechanical pulp can be chemically brightened, but paper prepared from the pulp will become darker when exposed to sunlight. This type of paper is used for newsprint and magazines. A chemical pulp is prepared

Amylases	Starch modification Deinking Drainage improvement Cleaning
Xylanases	Bleach boosting Energy saving
Cellulases	Deinking Drainage improvement Energy saving Tissue and fiber modification
Lipases and esterases	Pitch control Stickies control Deinking Cleaning

Table 3. Examples of enzyme applications in the pulp and paper industry.

by cooking wood chips in chemicals, hereby dissolving most of the lignin and releasing the cellulosic fibers. The chemical pulp is dark and must be bleached before making paper. This type of bleached chemical pulp is used for fine paper grades like printing paper. The chemical pulp is more expensive to produce than the mechanical pulp. Enzymes applied in the pulp and paper processes typically reduce production costs by saving chemicals or in some cases energy or water. The enzyme solutions also provide more environmentally friendly solutions than the traditional processes.

5.3.2 Amylases for starch modification for paper coatings

In the manufacture of coated papers, a starch-based coating formulation is used to coat the surface of the paper. Compared with uncoated paper, the coating provides improved gloss, smoothness, and printing properties. Chemically modified starch with a low viscosity in solution is used. As an economical alternative to modifying the starch with aggressive oxidizing agents, alpha-amylases can be used to obtain the same reduction in viscosity. Enzyme-modified starch is available from starch producers or can be produced on site at the paper mill using a batch or continuous process.

5.3.3 Xylanases for bleach boosting

The dominant chemical pulping process is the Kraft process, which gives a dark brown pulp caused by lignin residues. Before the pulp can be used for the manufacture of fine paper grades, this dark pulp must undergo a bleaching process. Traditionally, chlorine or chlorine dioxide has been used as the bleaching agent, resulting in an effluent containing chlorinated organic compounds that are harmful to the environment. Treatment of Kraft pulp with xylanases opens up the hemicellulose structure containing bound lignin and facilitates the removal of precipitated lignin–carbohydrate complexes prior to bleaching. By using xylanases, it is possible to wash out more lignin from the pulp and make the pulp more susceptible to bleaching chemicals. This technique is called "bleach boosting" and significantly reduces the need for chemicals in the subsequent bleaching stages. Xylanases thus help to achieve the desired level of brightness of the finished pulp using less chlorine or chlorine dioxide.

5.3.4 Lipases for pitch control

In mechanical pulp processes the resinous material called pitch is still present in the pulp. Pitch can cause serious problems in the paper production in the form of sticky deposits on rollers, wires, and the paper sheet. The result is frequent shutdowns and inferior paper quality. For mechanical pulps triglycerides have been identified as a major cause of pitch deposit. A lipase can degrade the triglycerides into glycerol and free fatty acids. The free fatty acids can be washed away from the pulp or fixed onto the fibers by use of alum or other fixatives.

Lipase treatment can significantly reduce the level of pitch deposition on the paper machine and reduce the number of defects on the paper web, and the machine speed can often be increased as well. Lipase treatments of mechanical pulps intended for newsprint manufacture can also lead to significant improvements in tensile strength, resulting in reduced inclusion of expensive chemical pulp fibers.

5.3.5 Esterases for stickies control

Stickies are common problems for most of the mills using recycled paper and paperboard. Stickies, which originate from, for example, pressure-sensitive adhesives, coatings, and binders, can cause deposit problems on the process equipment. Often stickies are found to contain a significant amount of polyvinyl acetate or acrylate, esters that are potential enzyme substrates. Esterases can modify the surface of the very sticky particles preventing a potential agglomeration. Hereby the mill can prevent microstickies, which can be handled in the process, from forming problematic macrostickies.

5.3.6 Enzymes for deinking

Recycled fibers are one of the most important fiber sources for tissue, newsprint, and printing paper. Enzymatic deinking represents a very attractive alternative to chemical deinking. The most widely used enzyme classes for deinking are cellulases, amylases, and lipases. A significant part of mixed office waste (MOW) contains starch as a sizing material. Amylase can effectively degrade starch size and release ink particles from the fiber surface. Different from amylases, cellulases function as surface-cleaning agents during deinking. They defibrillate the microfibrils attached to the ink and increase deinking efficiency. For deinking of old newsprint (ONP) cellulases and lipases have shown the most promising results. The increase in environmental awareness has resulted in the development of printing inks based on vegetable oils. It has been demonstrated that use of lipases for deinking of vegetable oil-based newsprint could achieve remarkable ink removal and brightness improvement.

5.4 Animal feed

Many feed ingredients are not fully digested by livestock. However, by adding enzymes to feed, the digestibility of the components can be enhanced. Enzymes are now a well-proven and successful tool that allows feed producers to extend the range of raw materials used in feed, and also to improve the efficiency of existing formulations.

Enzymes are added to the feed either directly or as a premix together with vitamins, minerals, and other feed additives. In premixes, the coating of the enzyme granulate protects the enzyme from deactivation by other feed additives such as choline chloride. The coating has another function in the feed mill – to protect the enzyme from the heat treatments sometimes used to destroy *Salmonella* and other unwanted microorganisms in feed.

Enzyme products in a liquid formulation are developed for those cases where the degree of heat treatment (conditioning) for the feed is high enough to cause an unacceptable loss of activity. Thereby addition can be performed accurately after the conditioning with insignificant loss of activity.

A wide range of enzyme products for animal feed are now available to degrade substances such as phytate, beta-glucan, starch, protein, pectin-like polysaccharides, xylan, raffinose, and stachyose. Hemicellulose and cellulose can also be degraded.

As revealed by the many feed trials carried out to date, the main benefits of supplementing feed with enzymes are faster growth of the animal, better feed utilization (feed conversion ratio), more uniform production, better health status, and an improved environment for chickens due to reductions in "sticky droppings".

5.4.1 The use of phytases

Around 50–80 % of the total phosphorus in pig and poultry diets is present in the form of phytate or phytic acid. The phytate-bound phosphorus is largely unavailable to monogastric animals as they do not naturally have the enzyme needed to break it down – phytase. There are two good reasons for supplementing feeds with phytase.

One is to reduce the harmful environmental impact of phosphorus from animal manure in areas with intensive livestock production. Phytate or phytic acid in manure is degraded by soil microorganisms, leading to high levels of free phosphate in the soil and, eventually in surface water. Several studies have found that optimizing phosphorus intake and digestion with phytase reduces the release of phosphorus by around 30 %. Novozymes estimates that the amount of phosphorus released into the environment would be reduced by 2.5 million tons a year worldwide if phytases were used in all feed for monogastric animals.

The second reason is based on the fact that phytate is capable of forming complexes with proteins and inorganic cations such as calcium, magnesium, iron, and zinc. The use of phytase not only releases the bound phosphorus but also these other essential nutrients to give the feed a higher nutritional value.

5.4.2 NSP-degrading enzymes

Cereals such as wheat, barley and rye are incorporated into animal feeds to provide a major source of energy. However, much of the energy remains unavailable to monogastrics due to the presence of nonstarch polysaccharides (NSP) which interfere with digestion. As well as preventing access of the animal's own digestive enzymes to the nutrients contained in the cereals, NSP can become solubilized in the gut and cause problems of high gut viscosity, which further interferes with digestion. The addition of selected carbohydrases will break down NSP, releasing nutrients (energy and protein), as well as reducing the viscosity of the gut contents. The overall effect is improved feed utilization and a more "healthy" digestive system for monogastric animals.

5.5 Oil and gas drilling

In underground oil and gas drilling, different types of drilling muds are used for cooling the drilling head, transporting stone and grit up to the surface, and controlling the pressure underground. The drilling mud builds up on the wall of the borehole a filter cake which ensures low fluid loss. Polymers added to the mud "glue" particles together during the drilling process to make a plastic-like coating which acts as a filter. These polymers may be starch, starch derivatives, (carboxymethyl)cellulose, or polyacrylates.

After drilling, a clean-up process is carried out to create a porous filter cake or to completely remove it. Conventional ways of degrading the filter cake glue involve treatment with strong acids or highly oxidative compounds. As such harsh treatments harm both the environment and drilling equipment in the long term, alternative enzymatic methods of degrading the filter cake have been developed.

Many wells operate within the range 65–80 °C (149–176 °F), which may be tolerated by some enzymes under certain conditions. In particular, certain alpha-amylases can bring about a significant degradation of starch at even higher temperatures.

A technique called fracturing is used to increase the oil/gas production surface area by creating channels through which the oil can easily flow to the oil well. Aqueous gels containing crosslinked polymers like guar gum, guar derivatives, or cellulose derivatives are pumped into the underground at extremely high pressures in order to create fractures. An enzymatic "gel breaker" (e.g., based on a mannanase) is used to liquefy the gel after the desired fractures have been created.

5.6 Biopolymers

The biopolymer field covers both current and next-generation materials for use in products such as biodegradable plastics, paints, and fiberboard. Typical polymers include proteins, starch, cellulose, nonstarch polysaccharides (e.g., pectin and xylan), and lignin, and biodegradable plastic produced by bacteria (e.g., polyhydroxybutyrate). Enzymes are used to modify these polymers for the production of derivatives suitable for incorporation in synthetic polymers for paints, plastics, and films.

Laccases, peroxidases, lipases, and transglutaminases are all enzymes capable of forming cross-links in biopolymers to produce materials *in situ*. Enzymes that can catalyze a polymerization process directly from monomers for plastic production are under investigation.

5.7 Fuel ethanol

In countries with surplus agricultural capacity, ethanol produced from biomass may be used as an acceptable substitute, extender, or octane booster for traditional motor fuel. Sugar-based raw materials such as cane juice or molasses can be fermented directly to ethanol. However, this is not possible for starch-based raw materials which have to be catalytically converted to fermentable sugars by use of alpha-amylases, glucoamylases and related enzymes. This conversion can be like the mashing process used in beer brewing or can be a cold mashing including simultaneous saccharification and fermentation processes.

The American Energy Information Administration (DOE/EIA) estimated that the global production of fuel ethanol reached 85 x 10^6 L in 2012, to compare with the 2011 world production of gasoline of 1230 x10^6 L.

Worldwide, approximately 900,000 t grain per day (2011) were processed into whole-grain mashes for whisky, vodka, neutral spirits, and fuel ethanol. Although the equipment is different, the principle of using enzymes to produce fuel ethanol from starch is often the same as that for producing alcohol for beverages (see Section 6.5 for more details). The main stages in a dry-milling production of alcohol using grains such as corn, wheat, rye, or barley are shown in Fig. 4.

Improvements in dry-milling processes on the one hand, and achievements within modern biotechnology on the other, have highlighted the importance of thorough starch liquefaction for the efficiency of the grain alcohol process. Novozymes has developed alpha-amylases (Termamyl® SC and Liquozyme® SC) that are able to work without addition of calcium and at lower pH levels than traditionally used in the starch industry (Section 6.1.3). This allows them to work efficiently under the conditions found in dry milling, whereas previous generations of enzymes often resulted in inconsistent starch conversion.
Producing fuel ethanol from cereals such as wheat, barley, and rye presents quite a challenge. Non-starch polysaccharides such as beta-glucans and arabinoxylans create high viscosity, which has a negative impact on downstream processes. High viscosity limits the dry substance level in the process, increasing energy and water consumption and lowering ethanol yield. Non-starch polysaccharides reduce the efficiency of separation, evaporation, and heat exchange. The Viscozyme® product family includes enzymes that degrade non-starch carbohydrates and give higher ethanol production capacity and lower operating costs. Greater flexibility in the choice of cereal and raw material quality together with the ability to process at higher dry substance levels are facilitated using these enzymes.

To minimize the consumption of steam for mash cooking, a pre-liquefaction process featuring a warm or a hot slurry may be used (see Fig. 5). Alpha-amylase may be added during the pre-liquefaction at 70–90 °C (158–194 °F) and again after liquefaction at approximately 85 °C (185 °F). Traditionally, part of the saccharification is carried out simultaneously with the fermentation process. Proteases can be used to release nutrients from the grain, and this supports the growth of the yeast.

Thus over the years novel technologies and the creation of new microbial strains for enzyme production have improved each major stage of the process flow – liquefaction, saccharification, fermentation, and downstream processing. The fuel ethanol industry has demanded enzymes for the so-called "cold-cook" liquefaction for the development of entirely new ethanol production processes based on raw-starch hydrolysis (RSH) technology. The enzyme industry has fulfilled this demand so that the results today are high-efficiency processes using fractionated corn, lower total energy costs, increased throughput and yield, and improved value of the co-products.

Collaborations between ethanol producers and Novozymes have initiated a beneficial change of this industry due to tailor-making of a new enzyme technology, optimization of processes and utilization of the fractions like the distiller´s dry grain (DDGS). (See Fig. 4).

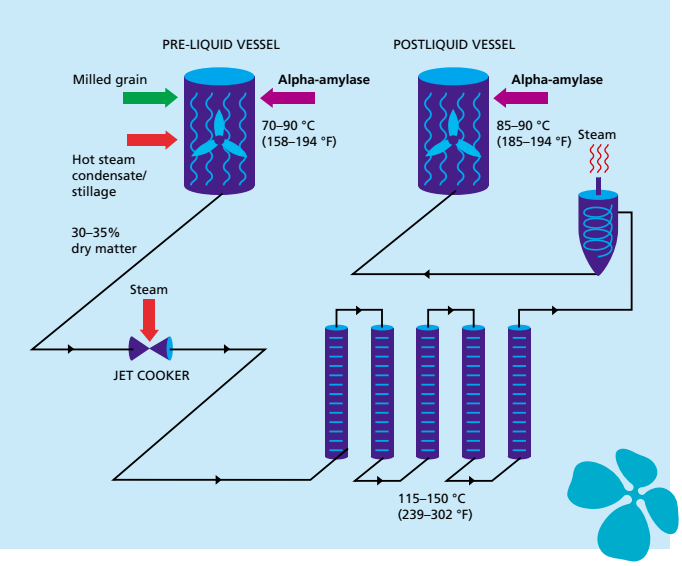


Fig. 5. Warm or hot slurry liquefaction processes.

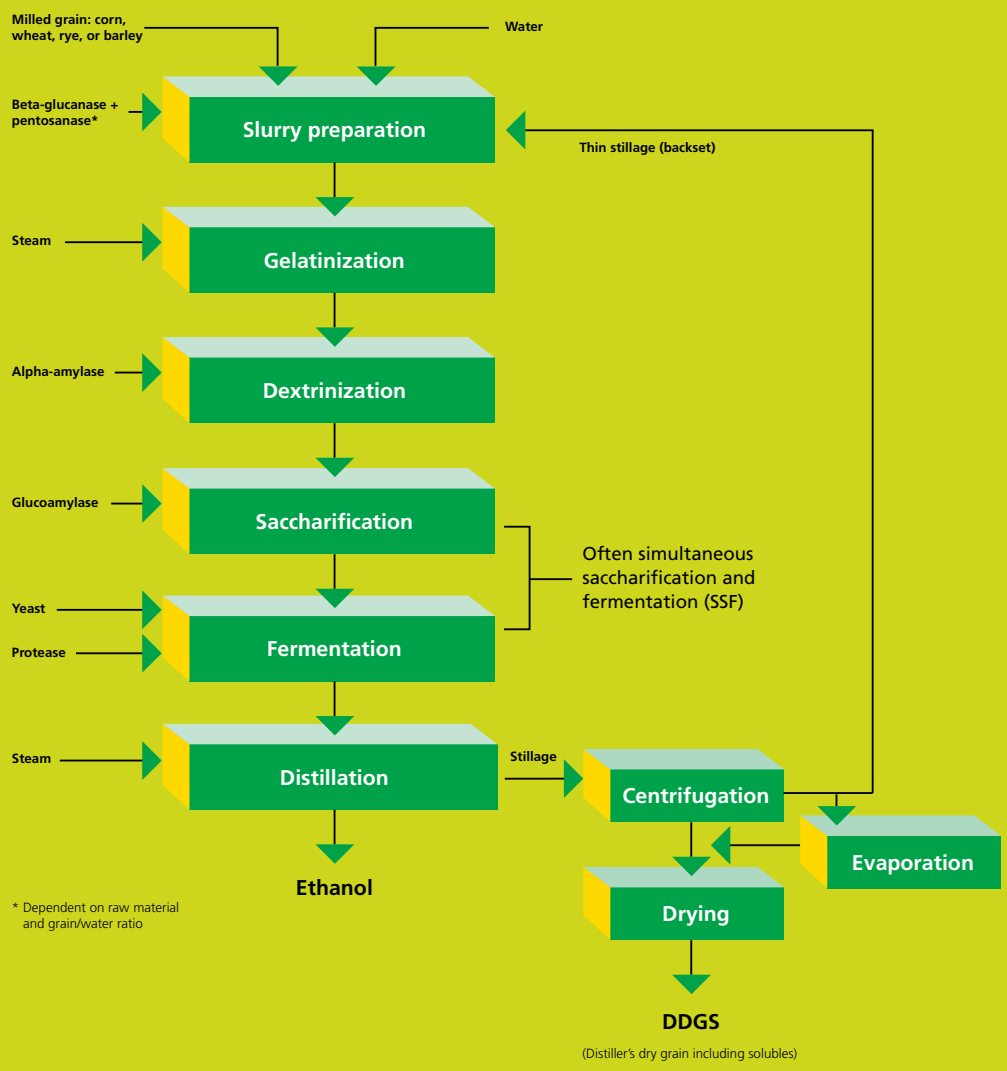


Fig. 4. Main process stages in dry-milling alcohol production.

5.7.1 Cellulosic ethanol

Looking ahead, an increased need for sustainable products acting as fuel extenders or gasoline additives, like anti-knocking agents, will demand that different feedstocks are evaluated for economical conversion of biomass. The ingredients can be combined to environmentally safe motor fuels that also result in reduced amounts of toxic exhaust. An increasing airborne pollution with toxic combustion products is the challenge for many big cities around the world.

The endeavor to solve the problems mentioned goes hand in hand with an increasing production of waste from households (municipal solid wastes) and food and agricultural production. A good and highly demanded solution can be to utilize enzymatic conversion of these wastes for ethanol production, for biodiesel or for biogas (see the following sections).

Compared to the processes for ethanol production from starch-containing feedstock, the greatest breakthrough in enzyme technology for ethanol is deemed to come in the area of cellulosic ethanol. Because of the widespread availability and low feedstock costs for e.g., corn stover, woody biomass and municipal waste, cellulosic ethanol can be a major source of sustainable energy and gasoline boosters.

The complex structure of biomass is however more difficult to convert into ethanol than traditional starch substrates. This has until today presented unique technical and economic challenges in bringing cellulosic ethanol to the market. Enzymes are vital in the conversion of biomass to ethanol, and it has been a key focus area to make this technology available at a commercially viable cost.

Research efforts

The R&D efforts in this area began in 2000 as part of a U.S. Department of Energy (DOE) grant with the purpose of developing a cellulosic ethanol production process based on lignocellulose. This biomass is composed of 35–50 % cellulose, 20–35 % hemicelluloses, 10–25 % lignin and 5–10 % minerals. A result of pre-treatments is a release or separation of three major fractions: cellulose, hemicellulose, and lignin. Fig. 6 illustrates the substrate complexity.

Before hydrolysis to fermentable sugars, the fibrous carbohydrates must be released from a recalcitrant structure. Processes originating from methods used for production of paper are basically adapted in many of today's proposed pre-treatments, usually carried out at high temperature and pressure.

The glucose-based cellulose chains are bundled into strong fibers in an organized crystalline structure.

Lignin is a complex three-dimensional polymer composed of phenol rings linked to various carbon chains and other chemical functionalities. Lignin is non-crystalline, and its structure has been described as analogous to a gel or foam. Lignin forms covalent cross-links to polysaccharides in cell walls.

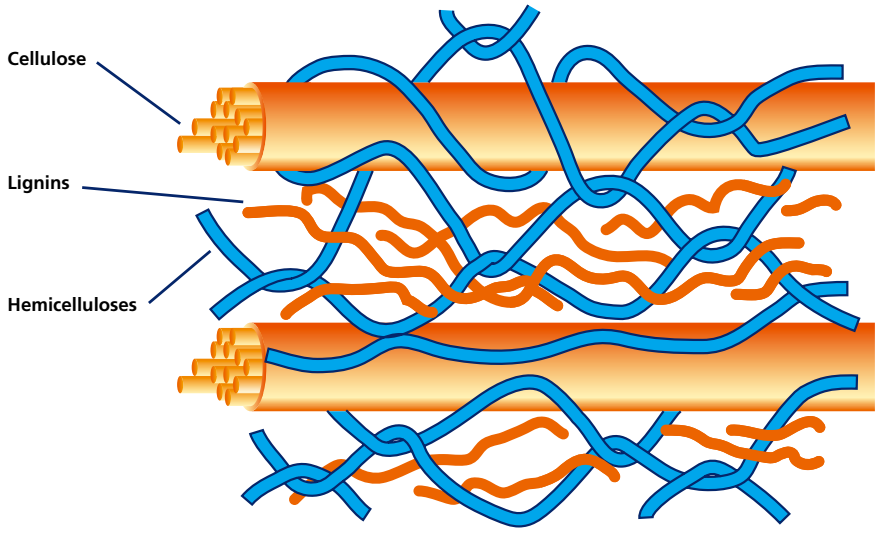


Fig. 6. Substrate complexity of lignocellulose.

Hemicellulose is composed of various five-and six-carbon sugars in a highly branched structure. Hemicellulose attaches weakly to cellulose and fills out in the lignin structure.

The complex structure of hemicellulose requires the use of different enzyme classes of which endoxylanases, beta-xylosidases and arabinofuranosidases contribute to the production of C5-sugars like xylose and arabinose.

Owing to intensive research and process development efforts the production is approaching commercial viability. Demonstration plants are under construction at several places.

5.7.2 Enzymatic hydrolysis of cellulose

Enzymatic cellulose hydrolysis uses the cellulase types shown for the reactions in Fig. 7.

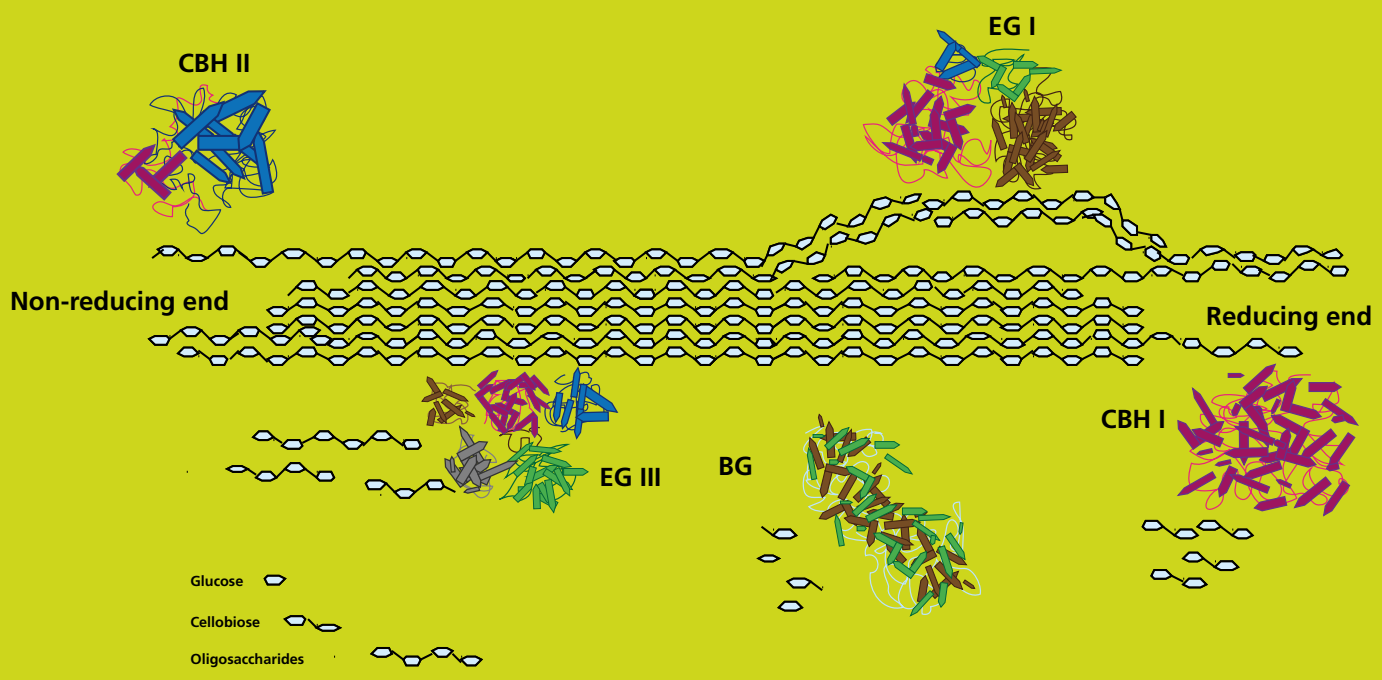


Fig. 7. Types of cellulases for hydrolysis of cellulose.

Cellobiohydrolase CBH I and CBH II cut two glucose units (cellobiose) at a time from the end of the cellulose chain (from the reducing (R) and the non-reducing (NR) ends, respectively). Endoglucanases (EG I and EG III) work from the inside of the cellulose chains and preferably in the amorphous regions, where the substrate is more accessible, and release oligosaccharides of intermediate lengths. The beta-glucosidase (BG) hydrolyses cellobiose to glucose.

The figure is modified from: Rosgaard, L., Pedersen, S., and Meyer, A. Fra halm til alkohol, *Aktuel Naturvidenskab* 2005, 3: 11–14.

The classification of cellulases into glycosyl hydrolase (GH) families is based on degree of sequence identity. Based on mechanism, cellulases can be grouped into exo-1,4-β-D-glucanases or cellobiohydrolases (CBHs, EC 3.2.1.91), endo-1,4-β-D-glucanases (EGs, EC 3.2.1.4) and beta-glucosidases (BGs, EC 3.2.1.21). A model for the reaction mechanism could be the following:

- Cellulases, in principle, act to catalyze hydrolysis at a multitude of sites by moving from site to site. Cellulases are often modular, containing a catalytic core, a linker and a carbohydrate-binding module.
- Endocellulases (EGs) cleave glycosidic bonds within cellulose microfibrils, acting preferentially at amorphous cellulose regions. Thereafter the crystalline structures of cellulose disrupt and expose cellulose polysaccharide chains individually.
- EGs fragment cellulose chains to generate reactive ends for CBHs to attack.
- The two main types of exocellulases (CBH), working from either the reducing (CBH I) or the non-reducing (CBH II) ends of cellulose chains, generate mainly cellobiose.
- At high concentrations, cellobiose inhibits the CBH activity.
- Cellobiase or beta-glucosidase hydrolyses cellobiose into glucose and is required for optimum performance of cellulase for glucose production.

In this mode, small amounts of the enzymes are able to catalyze the degradation of a large amount of cellulose. However when lignin is present, the enzymes are blocked from reaching some sites and the enzymes become irreversibly bound to the lignin and are no longer able to serve as mobile catalysts. In this situation, the enzymes act more as consumable reagents than as catalysts, and the dosage must be increased. Net enzyme usage in this reagent mode may be as much as ten times what is required in the catalyst mode.

Over the past 25 years the mode of action of cellulases has been intensively investigated; and a combination of breakthroughs has been achieved within recombinant DNA technology for enhancing cellulolytic activities.

5.7.3 Industrial demonstration plants

Different pre-treatment principles mentioned briefly in Table 4 are under testing using Novozymes-developed enzyme families, e.g., Cellic® CTec/Cellic® HTec.

Dilute acid (+ steam explosion)
Hydrothermal treatment and washing (acidic, alkaline or neutral)
Ammonia explosion / percolation
Autohydrolysis (neutral thermo-chemical conversion)
Organosolv (various types)
Oxidative processes
Size reduction
Swelling

Table 4. Physical/chemical pre-treatment methods of lignocellulosic materials.

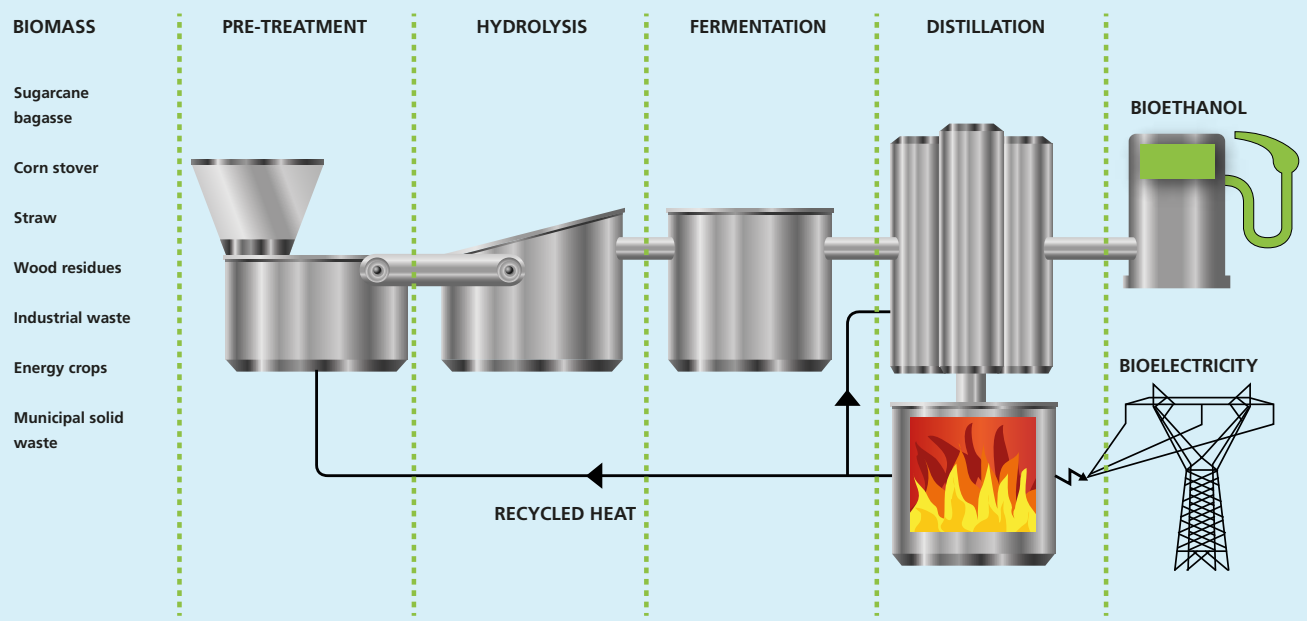


Fig. 8. General process flow for second-generation ethanol production.

The various pre-treatment methods differ significantly from one another in reaction conditions, process efficiency, complexity, and impact on the rest of the process. But all pre-treatments can increase the accessibility of the biomass for enzymes. The optimal enzyme recipes can be different for different pre-treatments. The process flow from pre-treatment to ethanol and energy production is briefly shown in Fig. 8.

5.7.4 Economic considerations for cellulosic bioethanol

Total cost reductions have come from a focus on a holistic approach to the production of cellulosic bioethanol. Over the last years a significant reduction in enzyme use cost per liter of ethanol for a range of feedstocks has been published. The cost of enzymes is just one factor in the total cost of ethanol production, and it is no longer the highest variable cost.

The ethanol fermentation processes utilize new developments of yeast strains that are able to convert both C5- and C6-sugars to ethanol in simultaneous saccharification and fermentation processes.

5.8 Other biofuels made by assistance from enzymes

Biofuels include products made via sustainable processing; substantiated by reducing the need for energy from fossil fuel, obtaining better production efficiencies and reducing environmental impact. Biodiesel is an example of such a product having combustion properties like petro-diesel.

Biogas is a renewable energy source resulting from biomass – mainly waste products from industrial or agricultural production.

A biorefinery is a facility that integrates biomass conversion processes and equipment to produce fuels, power, and value-added chemicals from biomass. Enzymatic catalysis is needed as the way to a sustainable, selective and mild production technique.

5.8.1 Biodiesel

Biodiesel is methyl or ethyl esters of fatty acids made from renewable biological resources: vegetable oils or animal fat. The esters are typically made by catalytic reactions of free fatty acids (FFA) or triglycerides (triacylglycerols) with alcohols, preferably methanol or ethanol.

The overall reaction is a sequence of consecutive and reversible reactions, in which diglycerides and monoglycerides are formed as intermediate compounds. The complete stoichiometric reaction requires 1 mol of triglycerides and 3 mol of alcohol as indicated in Fig. 9. The reaction is reversible and therefore excess alcohol is used to shift the equilibrium to the products' side.

Methanol and ethanol are frequently used in the process. Transesterification as an industrial process is usually carried out by heating an excess of the alcohol under different reaction conditions in the presence of an acid or a base, or by heterogeneous catalysts such as metal oxides or carbonates, or by a lipase. The yield of biodiesel in the process of transesterification is affected by process parameters like moisture, content of free fatty acids (FFAs), reaction time, reaction temperature, catalyst type and molar ratio of alcohol to oil.

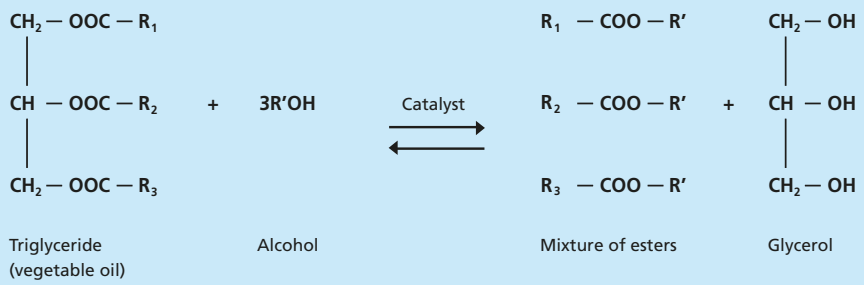


Fig. 9. Transesterification reaction of triglycerides.

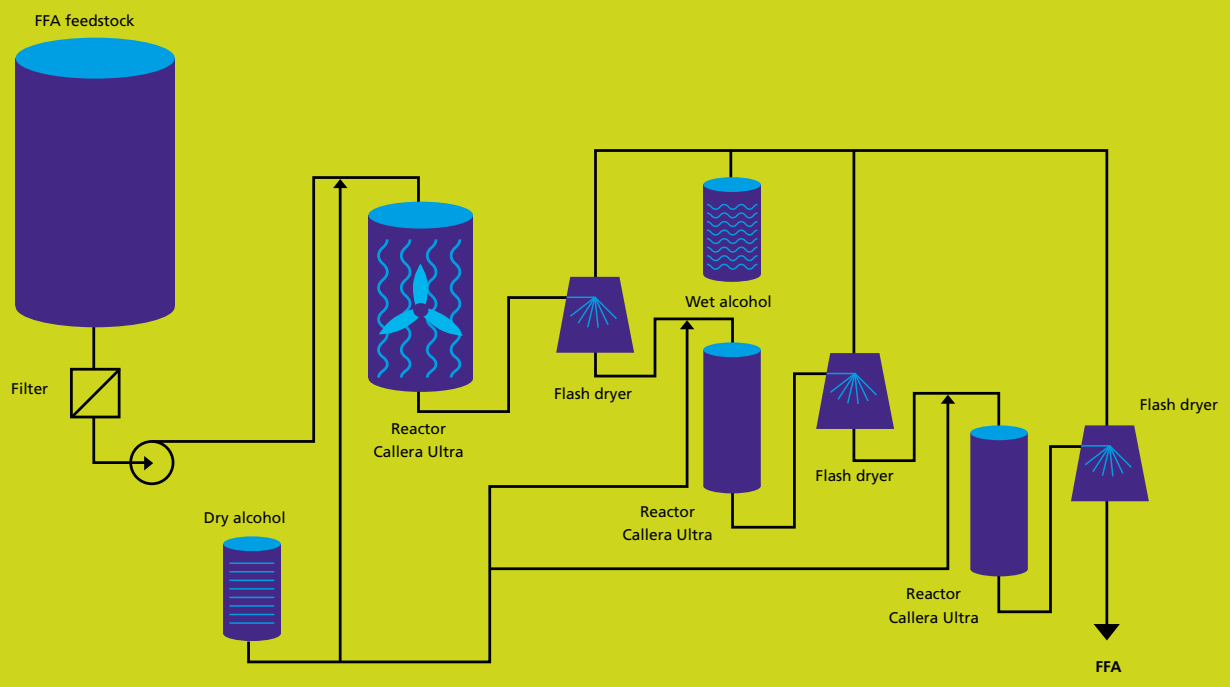


Fig. 10. Continuous biodiesel production from FFA using packed-bed reactors loaded with the immobilized lipase Callera™ Ultra IM produced by Novozymes.

Enzymatically catalyzed FFA esterification offers another simple, high-yield process. The FFA is converted to biodiesel with low alcohol surplus. The esterification of 100 % FFA material will produce approximately 6 % of water. The FFA level can be reduced to approximately 10 % FFA without water removal, but the water needs to be removed to drive the equilibrium of the reaction toward full FFA esterification.

A process involving continuous water removal is illustrated in Fig. 10, where the enzyme reactor is operated as a packed-bed column.

Biodiesel is a preferred motor product because the flash point is lowered after transesterification compared to liquid oil. This is an important property for use in cold climate. Furthermore the biodiesel can be used alone, or blended with petro-diesel.

5.8.2 Biogas

Biogas primarily consists of methane and carbon dioxide made by the biological breakdown of organic matter in the absence of oxygen. Cleaned biogas can substitute fossil natural gas and be used for cooking, heating and production of electricity.

Methane can be concentrated and upgraded to the same standards as fossil natural gas, which itself has had to go through a cleaning process. If compressed, it can replace compressed natural gas for use in vehicles that are configured for it. Biogas is produced by the anaerobic digestion or fermentation of biodegradable materials such as biomass, manure, sewage, municipal waste, green waste, plant material, and crops. Biogas is generated during a 4-step biological process. The first step of the process is the hydrolysis where carbohydrates, fats, and proteins are converted to sugars, fatty acids, and amino acids.

The second step is the acidogenesis where short chain acids, alcohols, CO_2 and H_2 are produced. The third step, acetogenesis, produces acetic acid, carbon dioxide, and hydrogen. In the final step, methanogenesis, methane is formed by specific microorganisms called methanogens. Fig. 11 shows the steps in the anaerobic digestion.

Using fibrous substrates, the methane formation process is supported by enzymes. In the hydrolysis phase, enzyme systems from the biogas-producing bacteria can to some degree convert the substrate components (cellulose, hemicellulose, pectin, starch, protein, lipid) into cell membrane-permeating substances (sugars, amino acids, fatty acids).

To enhance the biogas production support from external fiber-splitting enzyme systems are used for separate pretreatments and hydrolysis reactions of the biomass. Such enzyme systems introduce a drastic improvement of the yield of biogas based on convertible organic material. In biogas processes using external enzymes the complete process time including the steps shown in Fig. 11 can be reduced to only a few days. Thereby the overall production of biogas from plant cell wall material, lignocellulosic material and municipal solid wastes can be carried out at a higher rate compared to traditional processes.

5.8.3 Biorefineries

The biorefinery concept is analogous to today's refineries, which produce multiple fuels and products from petroleum. Both cellulosic and oleaginous biomass are suitable raw materials for a bio-based economy where fuel, plastics and chemicals are made. The International Energy Agency Bioenergy Task 42 on biorefineries has defined biorefining as the sustainable processing of biomass into a spectrum of bio-based products like food, feed, chemicals, materials, biofuels, power and heat.

By producing multiple products, a biorefinery takes advantage of the various components in biomass and their intermediates and maximize the value derived from the feedstocks. A biorefinery could, for example produce one or several low-volume but high-value chemicals or nutraceutical products. Low-value, but high-volume products such as biodiesel, biogas or bioethanol can at the same time generate electricity and process heat, through combined heat and power (CHP) technology. This energy can be for the refinery's own use or it can be sold to the local utility. The high-value products increase profitability, the high-volume fuel and the power production helps to form a sustainable overall production. Examples of enzyme-based biorefinery concepts are mentioned in the following:

5.8.3.1 Bioraf: A whole-crop biorefinery project

Traditional rapeseed processing (pressing and hexane extraction) often results in significant degradation of glucosinolates - also called mustard oil glycosides. Glucosinolates are at high doses known for their goitrogenic effects in both humans and animals whereby an enlargement of the thyroid gland can cause a malfunction.

The biorefinery process uses cell wall degrading enzymes to facilitate the extraction of pure vegetable oil in an aqueous process. In the process, all the components of the cell, i.e., protein,

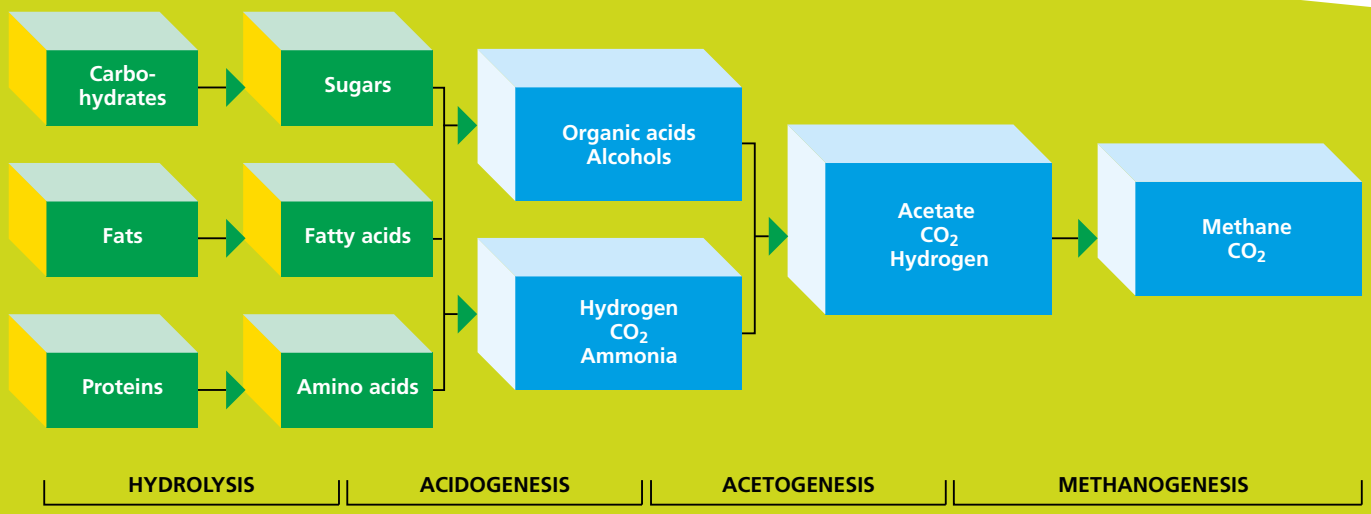


Fig. 11. Steps in anaerobic digestion.

oil and polysaccharides are transferred to the aqueous phase. Separation of the pure components is performed by centrifuge processes like in a dairy.

The traditional expeller process gives rise to only two products, oil and press cake.

The enzymatic process produces oil, protein, sugars, and a hull fraction. This is illustrated in Fig. 12. The oil is of high quality for food use, but it may also be applied for production of biodiesel (Section 5.8.1). The protein might be used for feeding of piglets as a milk substitute. The "sugars" and the hull fraction might be used for cattle feed, for biogas or for direct fuel in a power plant.

5.8.3.2 Biorefinery for processing potatoes and oil seed

This is a multi-purpose production in an enzyme-based biorefinery which for one third of the year processes potatoes and for the rest of the year processes an oil seed, e.g., rapeseed. The basic idea is to link the two types of production, thereby achieving production synergies with regard to process equipment and opportunities to produce diversified products from the two separate raw materials.

The products are enzymatically modified starches, concentrated feed products from potatoes, and enzymatically extracted vegetable oil and rapeseed protein flour. High degree of refining is obtained due to the enzymatic processes. Virtually no waste products are produced. Modified starches can be used in the paper industry and in the food industry. The oil product can be used for food or for biodiesel. It is assumed that the protein products and the by-products from the rapeseed process may be sold as animal feed.

In view of an annual production season of 300,000 tons of potatoes, and 100,000 tons of rapeseed, a technical layout of each process type is based on the optimum utilization of each operating unit. A financial study showed a direct financial synergy of such biorefining processes.

5.8.4 Enzymes commonly used for organic synthesis

Biocatalysis has been the focus of intense scientific research and is now a well-established technology within the chemical industry. Biocatalysis is the general term for the transformation of non-natural compounds by enzymes. The accelerated reaction rates, together with the unique stereo-, regio-, and chemoselectivity (highly specific action) and mild reaction conditions offered by enzymes, makes them highly attractive as catalysts for organic synthesis. Additionally, improved production techniques are making enzymes cheaper and more widely available. Enzymes work across a broad pH and temperature range, and often also in organic solvents. Many enzymes have been found to catalyze a variety of reactions that can be dramatically different from the reaction and substrate with which the enzyme is associated in nature.

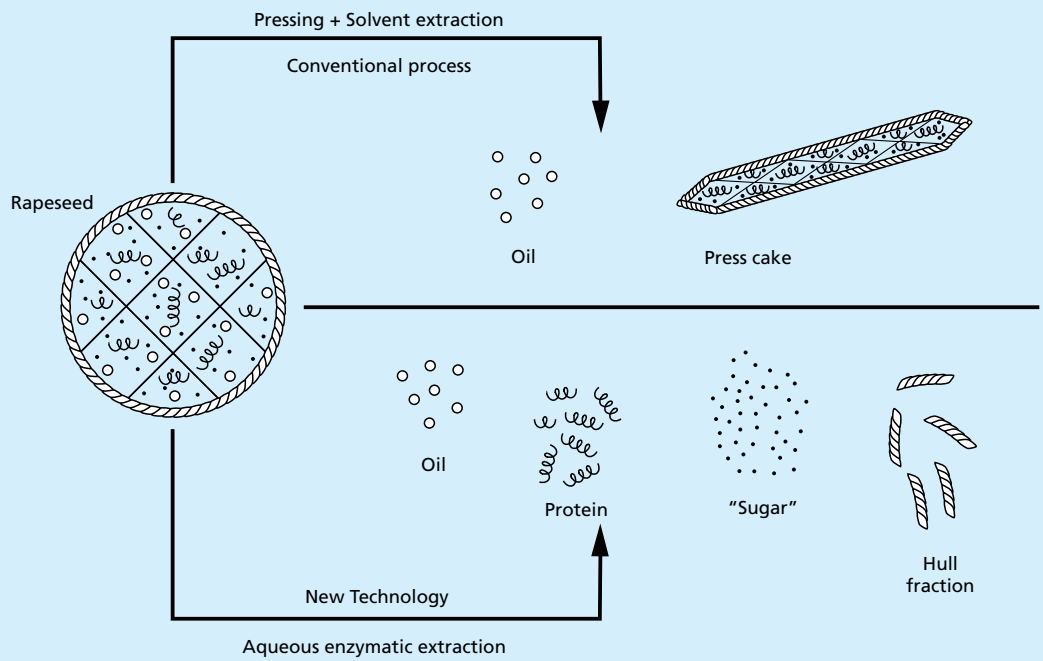


Fig. 12. Conventional process versus aqueous enzymatic extraction.

Enzymes commonly used for organic synthesis are listed in Table 5.

Lipases and other esterases (ester formation including transesterification; aminolysis and hydrolysis of esters)
Proteases (ester and amide hydrolysis, peptide synthesis)
Nitrilases and nitrile hydratases
Other hydrolases (hydrolysis of epoxides, halogenated compounds, and phosphates; glycosylation)
Oxidoreductases (e.g., enantioselective reduction of ketones)

Table 5. Enzymes most commonly used for organic synthesis.

Lipases are among the most versatile and flexible biocatalysts for organic synthesis (they are highly compatible with organic solvents), and therefore the most frequently used enzyme family. Oxidoreductases (e.g., alcohol dehydrogenases) have been used in the preparation of a range of enantiomerically enriched and pure compounds.

Due to the chiral nature of enzymes and their unique stereochemical properties, they have received most attention in the preparation of enantiomerically pure compounds. For many stereospecific and regioselective reactions enzymes are necessary for synthesis of carbohydrates, amino acids and peptides. Such reactions are also applied the introduction and/or removal of protecting groups in complex polyfunctional molecules. Even though the unique properties of enzymes are accordingly well documented, their potential is still far from being fully explored.

Biocatalysis is used in the preparation of a number of pharmacologically active compounds on both laboratory and commercial scale. More and more large-scale processes involving biocatalysis are being used today by fine-chemicals companies and bulk-chemicals manufacturers to produce commercial quantities of intermediates and chemicals. Table 6 gives examples of enzyme catalysts for producing commercial quantities of intermediates and chemicals.

The recent developments in the discovery or engineering of enzymes with unique specificities and selectivity that are stable and robust for synthetic applications will provide new tools for the organic chemist. The increasing demand for enantiomerically pure drugs and fine chemicals, together with the need for environmentally more benign chemistry, will lead to a rapid expansion of biocatalysis in organic synthesis.

ENZYME	SUBSTRATE	PRODUCT	APPLICATION
nitrile hydratase	pyridine-3-carbonitrile	nicotinamide	Pharmaceutical intermediate
nitrile hydratase	acrylonitrile	acrylamide	Intermediate for water-soluble polymers
D-amino acid oxidase & glutaryl acylase	cephalosporin C	7-aminocephalosporanic acid	Intermediate for semisynthetic antibiotics
penicillin acylase	7-aminodeacetoxy-cephalosporanic acid	cephalexin	Antibiotics
penicillin G acylase	penicillin G	6-aminopenicillanic acid	Intermediate for semisynthetic antibiotics
ammonia-lyase	fumaric acid + ammonia	L-aspartic acid	Intermediate for aspartame
thermolysin	L-aspartic acid + D,L-phenylalanine	aspartame (after methylation)	Artificial sweetener
dehalogenase	(*R*,*S*)-2-chloropropionic acid	(*S*)-2-chloropropionic acid	Intermediate for herbicides
lipase	(*R*,*S*)-glycidyl butyrate	(*S*)-glycidyl butyrate	Chemical intermediate
lipase	isosorbide diacetate	isosorbide 2-acetate	Pharmaceutical intermediate
lipase	(*R*,*S*)-naproxen ethyl ester	(*S*)-naproxen	Drug
lipase	racemic 2,3-epoxy-3-(4-methoxyphenyl)propionic acid methyl ester	(2*R*,3*S*)-2,3-epoxy-3-(4-methoxyphenyl)propionic acid methyl ester	Pharmaceutical intermediate
acylase	D,L-valine + acetic acid	L-valine	Pharmaceutical intermediate
acylase	acetyl-D,L-methionine	L-methionine	Pharmaceutical intermediate

Table 6. Examples of the use of biocatalysts in organic synthesis.

6. Enzyme applications in the food industry

The first major breakthrough for microbial enzymes in the food industry came in the early 1960s with the launch of a glucoamylase that allowed starch to be completely broken down into glucose. Since then, almost all glucose production has changed to enzymatic hydrolysis from traditional acid 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 %.

Since 1973, the starch-processing industry has grown to be one of the largest markets for enzymes. Enzymatic hydrolysis is used to form syrups through liquefaction, saccharification, and isomerization.

Another big market for enzymes is the baking industry. Supplementary enzymes are added to the dough to ensure high bread quality in terms of volume and a uniform crumb structure. Special enzymes can also increase the shelf life of bread by preserving its freshness longer.

A major application in the dairy industry is to bring about the coagulation of milk as the first step in cheesemaking. Here, enzymes from both microbial and animal sources are used.

In many large breweries, industrial enzymes are added to control the brewing process and produce consistent, high-quality beer.

In food processing, animal or vegetable food proteins with better functional and nutritional properties are obtained by the enzymatic hydrolysis of proteins.

In the juice and wine industries, the extraction of plant material using enzymes to break down cell walls gives higher juice yields, improved color and aroma of extracts, and clearer juice.

A detailed description of these processes is given in this section.

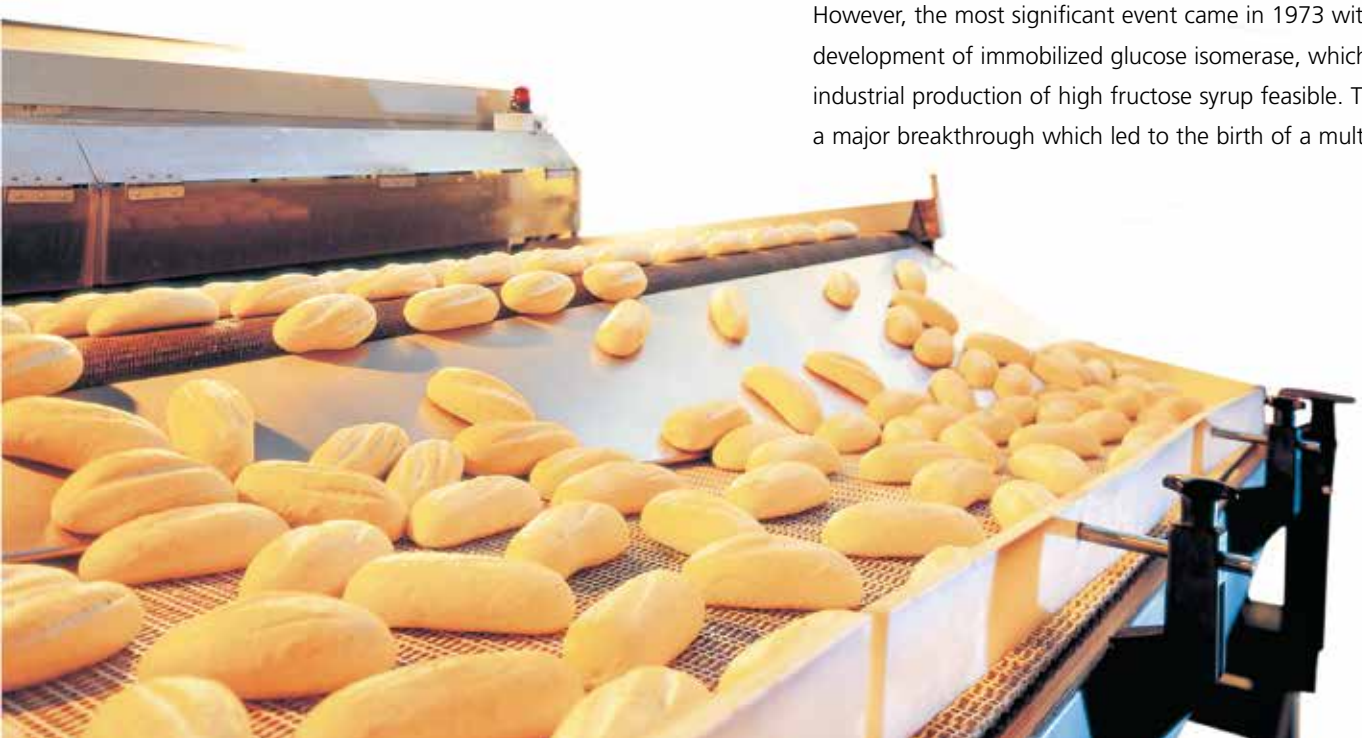
6.1 Sweetener production

The starch industry began using industrial enzymes at an early date. Special types of syrups that could not be produced using conventional chemical hydrolysis were the first products made entirely by enzymatic processes.

Many valuable products are derived from starch. There has been heavy investment in enzyme research in this field, as well as intensive development work on application processes. Reaction efficiency, specific action, the ability to work under mild conditions, and a high degree of purification and standardization all make enzymes ideal catalysts for the starch industry. The moderate temperatures and pH values used for the reactions mean that few by-products affecting flavor and color are formed. Furthermore, enzyme reactions are easily controlled and can be stopped when the desired degree of starch conversion is reached.

The first saccharification enzyme (glucoamylase) for the food industry in the early 1960s was the real turning point. After liquefaction this enzyme completely breaks down starch to glucose. Soon afterwards, almost all glucose production switched from acid hydrolysis to enzymatic hydrolysis because of the clear product benefits of greater yields, a higher degree of purity and easier crystallization.

However, the most significant event came in 1973 with the development of immobilized glucose isomerase, which made the industrial production of high fructose syrup feasible. This was a major breakthrough which led to the birth of a multi-billion-



dollar industry in the US for the production of high fructose syrups.

6.1.1 Enzymes for starch modification

By choosing the right enzymes and the right reaction conditions, valuable enzyme products can be produced to meet virtually any specific need in the food industry. Syrups and modified starches of different compositions and physical properties are obtained and used in a wide variety of foodstuffs, including soft drinks, confectionery, meat products, baked products, ice cream, sauces, baby food, canned fruit, preserves, and more.

Many nonfood products obtained by fermentation are derived from enzymatically modified starch products. For instance, enzymatically hydrolyzed starches are used in the production of alcohol, polyols, ascorbic acid, enzymes, lysine, and penicillin.

The major steps in the conversion of starch are liquefaction, saccharification, and isomerization. In simple terms, the further the starch processor goes, the sweeter the syrup obtained.

6.1.2 Tailor-made glucose syrups

Glucose syrups are obtained by fully hydrolyzing starch (mainly from wheat, corn, tapioca/cassava, and potato). This process cleaves the bonds linking the dextrose units in the starch chain. The method and extent of hydrolysis (conversion) affect the final carbohydrate composition and, hence, many of the functional properties of starch syrups. The degree of hydrolysis is commonly defined as the dextrose equivalent (see box).

Dextrose equivalent (DE)
Glucose (also called dextrose) is a reducing sugar. Whenever an amylase hydrolyzes a glucose–glucose bond in starch, two new glucose end groups are exposed. One of these acts as a reducing sugar. The degree of hydrolysis can therefore be measured as an increase in reducing sugars. The value obtained is compared to a standard curve based on pure glucose – hence the term "dextrose equivalent".

Originally, acid conversion was used to produce glucose syrups. Today, because of their specificity, enzymes are frequently used to control how the hydrolysis takes place. In this way, tailor-made glucose syrups with well-defined sugar spectra are manufactured.

The sugar spectra are analyzed using different techniques, two of which are high-performance liquid chromatography (HPLC) and gel permeation chromatography (GPC). HPLC and GPC data provide information on the molecular weight distribution and overall carbohydrate composition of the glucose syrups. This is used to define and characterize the type of product, for example high maltose syrup. Although these techniques help to optimize the production of glucose syrups with the required sugar spectra for specific applications, indirect methods such as viscosity measurements are also used in the production of tailor-made products.

6.1.3 Processing and enzymology

Modern enzyme technology is used extensively in the corn wet-milling sector. Current research focuses on refining the basic enzymatic conversion processes in order to improve process yields and efficiency.

An overview of the major steps in the conversion of starch is shown in Fig. 13. The enzymatic steps are briefly explained below.

Liquefaction

Corn starch is the most widespread raw material used, followed by wheat, tapioca, and potato. As native starch is only slowly degraded using alpha-amylases, a suspension containing 30–40 % dry matter needs first to be gelatinized and liquefied to make the starch susceptible to further enzymatic breakdown. This is achieved by adding a temperature-stable alpha-amylase to the starch suspension. The mechanical part of the liquefaction process involves the use of stirred tank reactors, continuous stirred tank reactors, or jet cookers.

In most plants for sweetener production, starch liquefaction takes place in a single-dose, jet-cooking process as shown in Fig. 14. Thermostable alpha-amylase is added to the starch slurry before it is pumped through a jet cooker. Here, live steam is injected to raise the temperature to 105 °C (221 °F), and the slurry's subsequent passage through a series of holding tubes provides the 5-minute residence time necessary to fully gelatinize the starch. The temperature of the partially liquefied starch is then reduced to 90–100 °C (194–212 °F) by flashing, and the enzyme is allowed to further react at this temperature for one to two hours until the required DE is obtained.

The enzyme hydrolyzes the alpha-1,4-glycosidic bonds in the gelatinized starch, whereby the viscosity of the gel rapidly decreases and maltodextrins are produced. The process may be terminated at this point, and the solution purified and dried. Maltodextrins (DE 15–25) are commercially valuable for their rheological properties. They are used as bland-tasting functional ingredients in the food industry as fillers, stabilizers, thickeners, pastes, and glues in dry soup mixes, infant foods, sauces, gravy mixes, etc.

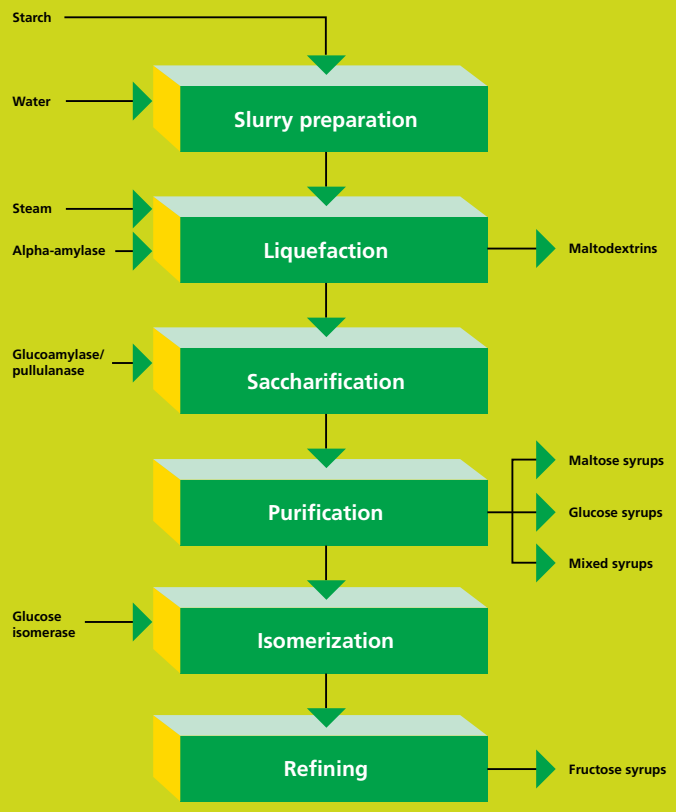


Fig. 13. Major steps in enzymatic starch conversion.

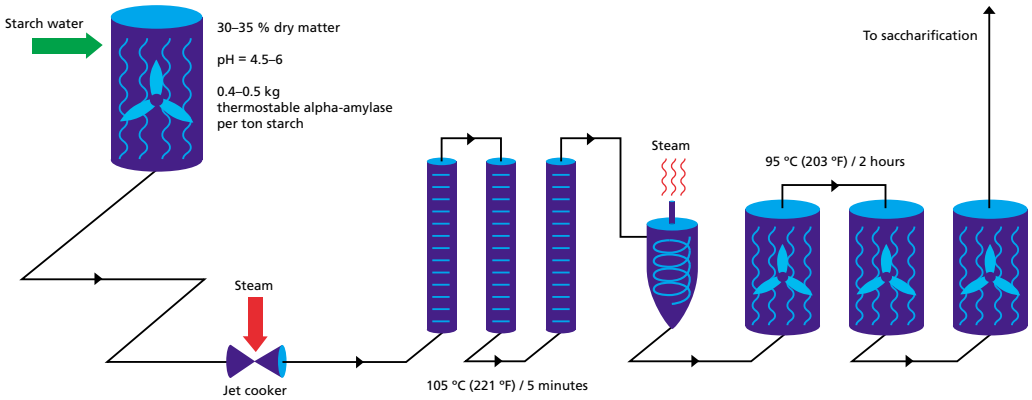


Fig. 14. Starch liquefaction process using a heat-stable bacterial alpha-amylase.

Saccharification

When maltodextrins are saccharified by further hydrolysis using glucoamylase or fungal alpha-amylase, a variety of sweeteners can be produced. These have dextrose equivalents in the ranges 40–45 (maltose), 50–55 (high maltose), and 55–70 (high conversion syrup). By applying a series of enzymes, including beta-amylase, glucoamylase, and debranching enzymes, intermediate-level conversion syrups with maltose contents of nearly 80 % can be produced.

A high yield of 95–97 % glucose may be produced from most starch raw materials (corn, wheat, potatoes, tapioca, barley, and rice). The action of amylases and debranching enzymes like pullulanase is shown in Fig. 15. The debranching enzyme most often used is alpha-dextrin endo-1,6-alpha-glucosidase (pullulanase).

Isomerization

Glucose can be isomerized to fructose in a reversible reaction (see Fig. 16).

Under industrial conditions, the equilibrium point is reached when the level of fructose is 50 %. The reaction also produces small amounts of heat that must be removed continuously. To avoid a lengthy reaction time, the conversion is normally stopped at a yield of about 45 % fructose.

The isomerization reaction in the reactor column is rapid, efficient, and economical if an immobilized enzyme system is used. The optimal reaction parameters are a pH of about 7.5 or higher and a temperature of 55–60 °C (131–140 °F). These parameters ensure high enzyme activity, high fructose yields, and high enzyme stability. However, under these conditions glucose and fructose are rather unstable and decompose easily to organic acids and colored by-products. This problem is countered by minimizing the reaction time by using an immobilized isomerase in a column through which the glucose flows continuously. The enzyme granulates are packed into the column but are rigid enough to prevent compaction.

The immobilized enzyme loses activity over time. Typically, one reactor load of glucose isomerase at a time is replaced when the enzyme activity has dropped to 10–15 % of the initial value. The most stable commercial glucose isomerases have column lifetime up to 200–400 days when used on an industrial scale.

To maintain a constant fructose concentration in the syrup produced, the flow rate of the glucose syrup fed into the column is adjusted according to the actual activity of the enzyme. Thus, towards the end of the lifetime of the enzyme, the flow rate is much slower. With only one isomerization reactor in operation, there would be great variation in the rate of syrup production over a period of several months. To avoid this, a series of reactors are operated together, and some or all of the enzymes in the columns are renewed at different times.

Reactor designs used in the US for glucose isomerization are described in the technical literature. Reactor diameters are normally between 0.6 and 1.5 m, and typical bed heights are 2–5 m. Plants producing more than 1,000 tons of high fructose corn syrup (HFCS) per day (based on dry matter) use at least 20 individual reactors.

6.1.4 Sugar processing

Starch is a natural component of sugar cane. When the cane is crushed, some of the starch is transferred to the cane juice, where it remains throughout subsequent processing steps. Part of the starch is degraded by natural enzymes already present in the cane juice, but if the concentration of starch is too high, starch may be present in the crystallized sugar (raw sugar). If this is to be further processed to refined sugar, starch concentrations beyond a certain level are unacceptable because filtration of the sugar solution will be too difficult.

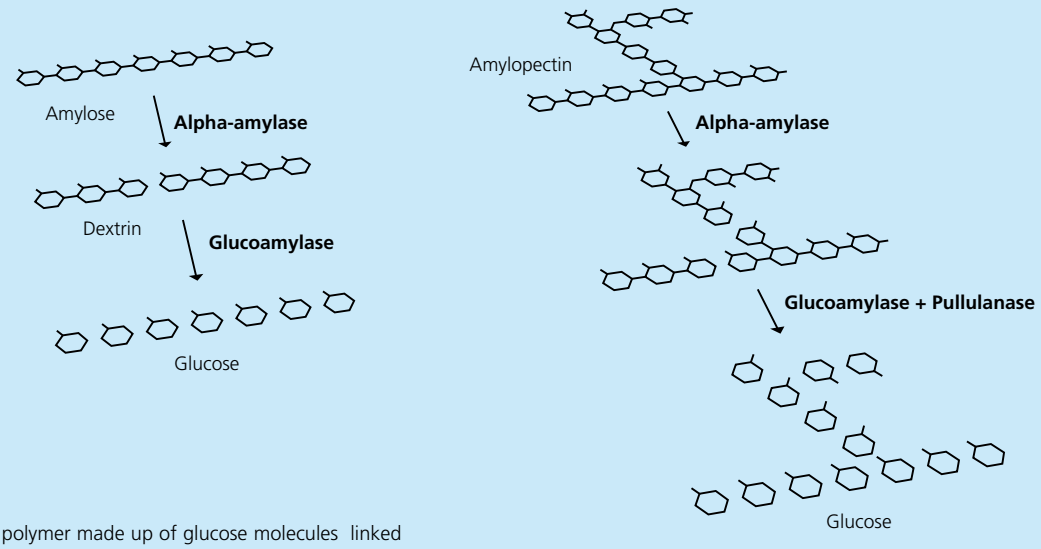
In order to speed up the degradation of starch, it is general practice to add concentrated enzymes during the evaporation of the cane juice.

A thermostable alpha-amylase may be added at an early stage of the multistep evaporation of the cane juice. Thereby the crystallization process will be facilitated because a complete degradation of starch is obtained.

Another polysaccharide, dextran, is not a natural component of sugar cane, but it is sometimes formed in the sugar cane by bacterial growth, in particular when the cane is stored under adverse conditions (high temperatures and high humidity). Dextran has several effects on sugar processing: Clarification of the raw juice becomes less efficient; filtration becomes difficult; heating surfaces become "gummed up", which affects heat transfer; and finally, crystallization is impeded, resulting in lower sugar yields.

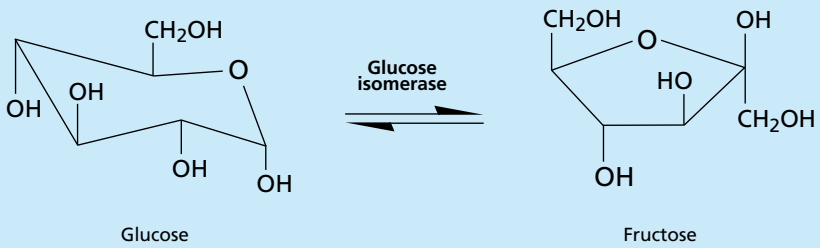
These problems may be overcome by adding a dextran-splitting enzyme (a dextranase) at a suitable stage of the process. It should be added that dextran problems may also be encountered in the processing of sugar beets, although the cause of the dextran is different. In this case, dextran is usually a problem when the beets have been damaged by frost. The cure, however, is the same – treatment with dextranase.

Fig. 15. Effect of the action of starch-degrading enzymes.



Native starch is a polymer made up of glucose molecules linked together to form either a linear polymer called amylose or a branched polymer called amylopectin. Glucose units are linked in a linear way with alpha-1,4-glycosidic bonds. The branching in amylopectin takes place with alpha-1,6-glycosidic bonds occurring at every 24-30 glucose molecules.

Fig. 16. Isomerization of glucose.



6.2 Baking

For decades, fungal alpha-amylases and enzymes from malt have been used in bread-making. Rapid advances in biotechnology have made a number of exciting new enzymes available for the baking industry. The importance of enzymes is likely to increase as consumers demand more natural products free of chemical additives. For example, enzymes can be used to replace potassium bromate, a chemical additive that has been banned in a number of countries.

The dough for bread, rolls, buns, and similar products consists of flour, water, yeast, salt, and possibly other ingredients such as sugar and fat. Flour consists of gluten, starch, nonstarch polysaccharides, lipids, and trace amounts of minerals. As soon as the dough is made, the yeast starts to work on the fermentable sugars, transforming them into alcohol and carbon dioxide, which makes the dough rise.

The main component of wheat flour is starch. Amylases can degrade starch and produce small dextrins for the yeast to act upon. There is also a special type of amylase that modifies starch during baking to give a significant antistaling effect.

Gluten is a combination of proteins that forms a large network during dough formation. This network holds the gas in during dough proofing and baking. The strength of this network is therefore extremely important for the quality of all bread raised using yeast. Enzymes such as hemicellulases, xylanases, lipases, and oxidases can directly or indirectly improve the strength of the gluten network and so improve the quality of the finished bread.

Table 7 lists some of the bread properties that can be improved using industrial enzymes.

6.2.1 Flour supplementation

Malt flour and malt extract can be used as enzyme supplements because malt is rich in alpha-amylases. Commercial malt preparations can differ widely in their enzyme activity, whereas an industrial enzyme is supplied with a standardized activity.

ENZYME	EFFECT
Amylase	Maximizes the fermentation process to obtain an even crumb structure and a high loaf volume
Maltogenic alpha-amylase	Improves shelf life of bread and cakes through antistaling
Glucose oxidase	Cross-links gluten to make weak doughs stronger, drier and more elastic
Lipase	Modifies the natural lipids in flour to strengthen the dough
Lipoxygenase	Bleaches and strengthens dough
Asparaginase	Reduces the amount of acrylamide formed during baking

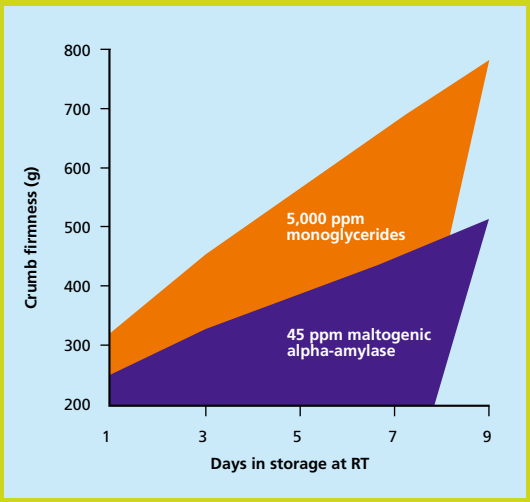
Table 7. Typical benefits of using enzymes in baking.

The alpha-amylases degrade the damaged starch granules in wheat flour into small dextrins, which allows yeast to work continuously during dough fermentation, proofing, and the early stage of baking. The result is improved bread volume and crumb texture. In addition, the small oligosaccharides and sugars such as glucose and maltose produced by these enzymes enhance the Maillard reactions responsible for the browning of the crust and the development of an attractive "baked" flavor.

Bread and cake staling is responsible for significant financial loss for both consumers and producers. For instance, every year in the US, bread worth more than USD 1 billion is discarded. However, the main saving on prolonging the shelf life is actually savings in transportation and fuel costs due to a more efficient distribution. Staling is associated with a loss of freshness in terms of increased crumb firmness, decreased crumb elasticity, and loss of moistness. Staling is believed to be due to changes in starch structure during storage. When the starch granules revert from a soluble to an insoluble form, they lose their flexibility; the crumb becomes hard and brittle. For decades, emulsifiers have been used as antistaling agents. However, they actually have a limited antistaling effect and are subject to special labeling rules.

By contrast, Novozymes' bacterial maltogenic alpha-amylase has been found to have a significant antistaling effect. It modifies the starch during baking at the temperature when most of the starch starts to gelatinize. The resulting modified starch granules remain more flexible during storage. Bread produced with maltogenic alpha-amylase has a far softer and more elastic crumb than bread produced with distilled monoglycerides as emulsifiers.

As the graphs in Fig. 17 show, the addition of maltogenic alpha-amylase at 45 ppm results in a much softer and more elastic crumb than the addition of high-quality distilled monoglycerides (DMG) at 5,000 ppm.



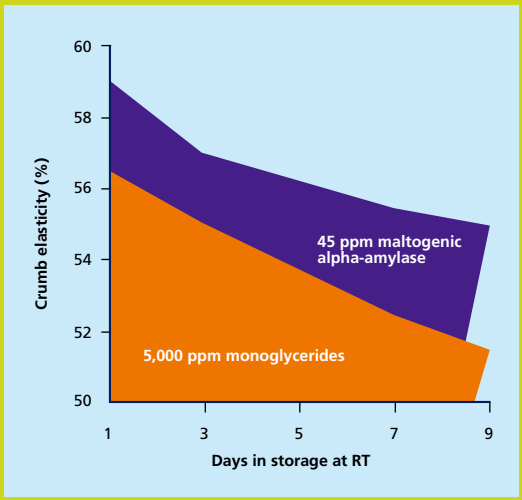


Fig. 17. Softness and elasticity of American (sponge & dough) pan bread using maltogenic alpha-amylase compared with distilled monoglycerides (DMG).

6.2.2 Dough conditioning

Flour contains 2.5–3.5 % nonstarch polysaccharides, which are large polymers (mainly pentosans) that play an important role in bread quality due to their water absorption capability and interactions with gluten. Although the true mechanism of hemicellulase, pentosanase, or xylanase in bread-making has not been clearly demonstrated, it is well known that the addition of certain types of pentosanases or xylanases at the correct dosage can improve dough machinability, yielding a more flexible, easier-to-handle dough. Consequently, the dough is more stable and gives better ovenspring during baking, resulting in a larger volume and improved crumb texture.

Normal wheat flour contains 1–1.5 % lipids, both polar and nonpolar. Some of these lipids, especially the polar lipids such as phospholipids and galactolipids, are able to stabilize the air bubbles in the gluten matrix. The addition of functional lipases modifies the natural flour lipids so they become better at stabilizing the dough. This ensures a more stable dough in case of overfermentation, a larger loaf volume, and significantly improved crumb structure. Because of the more uniform and smaller crumb cells, the crumb texture is silkier, and the crumb color appears to be whiter. It also reduces the need for addition of emulsifiers that otherwise are commonly added to dough in order to stabilize it. This in turn means that emulsifiers can be removed from the label.

Chemical oxidants such as bromates, azodicarbonamide, and ascorbic acid have been widely used to strengthen the gluten when making bread. As an alternative, oxidases such as glucose oxidase can partially replace the use of these chemical oxidants and achieve better bread quality.

As shown in Fig. 18, glucose oxidase and fungal alpha-amylase can be used not only to replace bromate but also to give a larger bread volume.

Fig. 18. Glucose oxidase and fungal amylase (right-hand loaf) were used to replace bromate in maraquetta (South American bread).

6.2.3 The synergistic effects of enzymes

Each of the enzymes mentioned above has its own specific substrate in wheat flour dough. For example, lipases work on the lipids, xylanase works on the pentosans, and amylases work on the starch. Because the interaction of these substrates in dough and bread is rather complex, the use of enzyme combinations can have synergistic effects that are not seen if only one enzyme is used – not even at high dosages. Quite often an overdose of enzymes will have a detrimental effect on either the dough or the bread. For instance, an overdose of fungal alpha-amylase or hemicellulase/xylanase may result in a dough that is too sticky to be handled by the baker or baking equipment. It is therefore beneficial for some types of bread formulations to use a combination of lower dosages of alpha-amylase and xylanase with low dosages of lipase or glucose oxidase to achieve optimal dough consistency, stability and bread quality. Another example is to use maltogenic alpha-amylase in combination with fungal alpha-amylases and xylanase or lipase to secure optimal crumb softness as well as optimal bread quality in terms of crumb structure, bread volume, etc.

6.2.4 Reduction of acrylamide content in food products

During recent years it has been shown that the amount of the potentially carcinogenic substance acrylamide is relatively high in a number of cereal- and potato-based products like biscuits, crackers, crisp bread, french fries, and potato crisps. Contents have been found in the interval of about 400 to 1000 ppb in some of these products.

Acrylamide is a substance that is formed at high temperatures

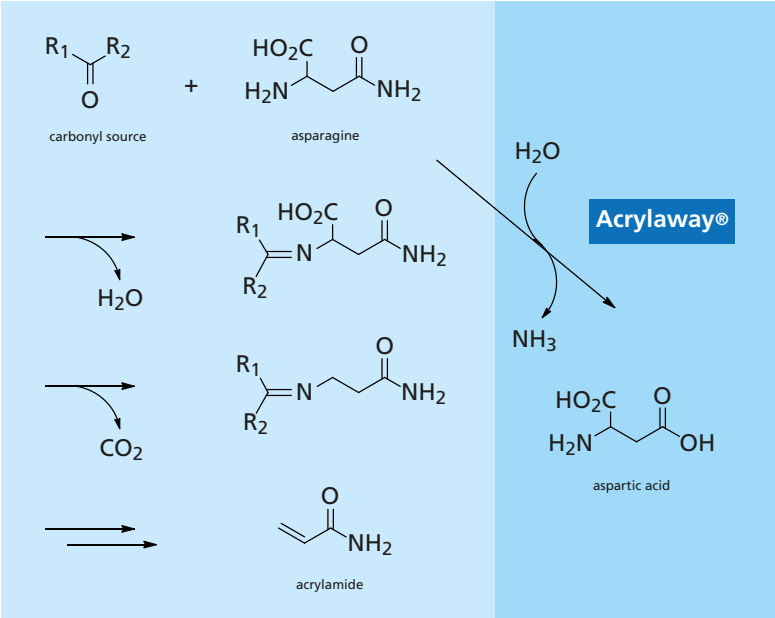


Fig. 19. A carbonyl source and asparagine react when heated, and the side chain of asparagine is converted to acrylamide. Acrylaway reduces the formation of acrylamide by instead converting asparagine to aspartic acid.

when the amino acid asparagine reacts with a reducing sugar like glucose as shown in Fig. 19, a Maillard reaction.

Maillard reactions, also known as non-enzymatic browning, typically occur at temperatures above 100 °C and are responsible for important color and flavor development in fried and baked starchy products. Thus the same reactions that make fried and baked foods tasty give rise to a potential carcinogen.

To address this issue, the enzyme asparaginase has been developed to reduce the formation of acrylamide. By adding asparaginase before baking or frying the food, asparagine is converted to aspartic acid and ammonium. With the use of an asparaginase, the formation of acrylamide can be reduced by up to 90 % in a range of food products.

6.3 Dairy products

The application of enzymes in the processing of milk has a long tradition. In ancient times, calf rennet was used for coagulation during cheese production. The rennet contains the enzyme chymosin, and nowadays there are many industrially produced chymosin products or similar proteases available as substitutes.

Proteases are also used to accelerate cheese ripening, to modify the functional properties of cheese, and to modify milk proteins to reduce allergenic properties of some dairy products.

Protein is not the only possible allergen in milk. We begin life drinking our mother's milk but many adults are unable to drink milk later in life. Cow's milk contains 5 % lactose, and in order to break it down we need the enzyme lactase. Lactase levels in humans are high at birth, but only low levels are found in

certain sections of the world's population during adulthood. Lactase (beta-galactosidase) is used to hydrolyze lactose in order to increase digestibility or to improve the solubility or sweetness of various dairy products.

Finally, lipases are used mainly in cheese ripening.

6.3.1 Cheesemaking

For industrial cheesemaking, the protein and fat content of milk must be standardized. This is normally achieved by blending different milk batches that have undergone centrifugation to alter the fat content, and membrane filtration to alter the protein content. The standardized milk is normally pasteurized at 72 °C (162 °F) for 15 seconds and then cooled to 30 °C (86 °F) before being transferred to the cheese tank. Starter culture is added to the pasteurized milk to initiate fermentation. Chymosin (the milk-clotting enzyme) and calcium chloride are added to promote the milk protein clotting reaction that forms a gel.

After about 25 minutes at 30 °C, the clotted milk (coagulum) is cut or stirred to promote syneresis – the exudation of whey. Syneresis is further promoted by heating the curd–whey mixture. Specialized equipment is used to drain the whey. After drainage, the cheese is pressed, and the lactic acid fermentation continues during pressing and subsequent storage. Afterwards, the cheese is steeped in brine. The fermentation of the lactose continues until virtually all of it has been fermented. So when the cheese is removed from the brine, it is practically free of lactose. The cheese is then ripened in storage for an appropriate period.

6.3.2 Rennet and rennet substitutes

Rennet, also called rennin, is a mixture of chymosin and pepsin. It is extracted from the gastric mucosa of the abomasum (stomach) of young mammals, for example calves and lambs. Rennets are also made by fermentation. It is far cheaper to use microbial enzymes than standard animal rennet.
Microbial rennets are produced by submerged fermentation of selected strains of fungi such as *Rhizomucor miehei* and have properties similar to those of chymosin. The enzyme product is marketed under the name HANNILASE® Liquid by CHR. HANSEN A/S. In practice, only slight modifications need to be made to the cheesemaking process when using microbial rennet.

Chymosin is nature's own enzyme for clotting milk. CHY-MAX® is a 100 % pure chymosin coagulant produced by fermentation of *Aspergillus niger var. awamori* and is identical in structure to that produced by the calf stomach and provides the same performance as pure calf rennet in terms of enzymatic activity, usage and storage.

6.3.3. Cheese ripening and yield increase

Fresh curd obtained by milk clotting and drainage is composed of casein, fats, carbohydrates, and minerals. In their natural state, these compounds have a very mild taste. Cheese flavor is developed during the ripening period as a controlled hydrolysis of these compounds by enzymes. Cheese ripening is defined as the enzymatic modification of these substrates to give the texture and flavor of mature cheese. Enzymes synthesized by curd microorganisms play a major role in these biochemical modifications. The huge variety of cheeses available is due to variations

in different physicochemical properties and the use of different microfloras.

Cheese ripening requires storage space and controlled temperatures and is relatively expensive. Accelerating the ripening process can therefore save costs, especially with low-moisture, slow-ripening cheese varieties, provided that the right conditions can be maintained throughout the entire process.

Research into accelerating cheese ripening has concentrated on proteolysis in cheddar. This process produces peptides and amino acids; just enough for the microflora to accelerate the conversion to minor aroma products.

Although there is potential for industrial enzymes to accelerate ripening, controlling the process is difficult, and enzymes are therefore not widely used.

However, one related application in which enzymes are becoming established is as a substitute for rennet paste. Lipases are used in blue and Italian cheeses to develop their piquancy, which is due primarily to short-chain fatty acids. Originally, this flavor was produced by the action of lipases in rennet pastes, traditionally added during the preparation of these cheeses. Using a special technique, rennet pastes were prepared from the stomachs of calves, lambs, or kids slaughtered after suckling. Because of possible health risks to the public, these rennet pastes are prohibited in some countries. One alternative is to use pregastric lipases extracted from animals. Several attempts have been made to develop safe processes for producing this type of lipase, but the most obvious and safest technique is to use lipases derived from microorganisms instead. Lipases such as Novozymes' Palatase®, which is derived from *Mucor miehei,* have shown satisfactory results in Italian cheesemaking.

YieldMAX is an advanced enzyme solution increasing the yield in production of mozzarella and pizza cheese. YieldMAX is a standardized solution of a phospholipase produced by submerged fermentation of an *Aspergillus oryzae* strain.

In the process of making mozzarella and pizza cheese, the role of YieldMAX is to optimize the clotting process, thus improving the cheese yield, by improving the emulsification properties of phospholipids in cheese milk. YieldMAX is marketed by CHR. HANSEN A/S.

6.3.4 Infant milk formulas

Proteases have been used for more than 50 years to produce infant milk formulas from cow's milk. The proteases are used to convert the milk proteins into peptides and free amino acids. The main reason is that nondegraded cow's milk protein can induce sensitization in infants when they are fed the milk. By degrading a high percentage of the milk protein, the risk of inducing sensitization or an allergic reaction can be minimized. This is very important for infants who belong to the high-risk group for developing allergies or who are already allergic to cow's milk.

Only some parts of intact milk proteins – the epitopes – present a potential risk for infants. The epitopes are eliminated by cutting one or more of their internal peptide bonds. In this way, proteases provide the means to make an important nutritional product that can be used if a mother cannot breast-feed her child. In addition, the nutritional value of the infant milk is increased when the proteins are broken down into smaller peptides.

When producing low-allergenic infant formulas, the type of enzyme used is very important, especially its specificity. Endoproteases with a preference for degrading peptide bonds between amino acids in the highly hydrophilic regions of a protein molecule are used for this application.

6.4 Brewing

Traditionally, beer is produced by mixing crushed barley malt and hot water in a large circular vessel called a mash copper. This process is called mashing. Besides malt, other starchy cereals such as corn, sorghum, rice, and barley, or pure starch itself, are added to the mash. These are known

as adjuncts. After mashing, the mash is filtered in a lauter tun. The resulting liquid, known as sweet wort, is then run off to the copper, where it is boiled with hops. The hopped wort is cooled and transferred to the fermentation vessels, where yeast is added. After fermentation, the so-called green beer is matured before final filtration and bottling. This is a much-simplified account of how beer is made. A closer look reveals the importance of enzymes in the brewing process.

The traditional source of enzymes used for the conversion of cereals into beer is barley malt, one of the key ingredients in brewing. If too little enzyme activity is present in the mash, there will be several undesirable consequences: The extract yield will be too low; wort separation will take too long; the fermentation process will be too slow; too little alcohol will be produced; the beer filtration rate will be reduced; and the flavor and stability of the beer will be inferior.

Industrial enzymes are used to supplement the malt's own enzymes in order to prevent these problems. Furthermore, industrial enzymes can be used to ensure better adjunct liquefaction, to produce low-carbohydrate beer ("light beer"), to shorten the beer maturation time, and to produce beer from cheaper raw materials.

A diagram of the brewing process is shown in Fig. 20.

6.4.1 Mashing

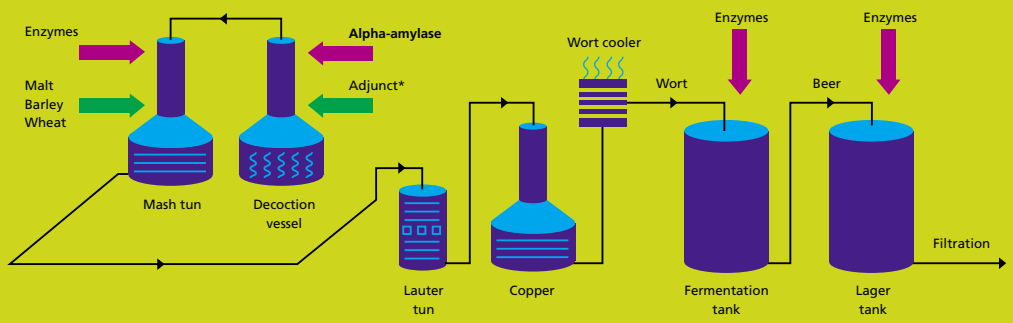
Malt is the traditional source of alpha-amylase for the liquefaction of adjuncts. The action of alpha-amylase ensures simpler liquefaction and shorter process times. Heat-stable alpha-amylase preparations (e.g., Novozymes' Termamyl®) are becoming more popular for three main reasons:

- They enable a more predictable and simpler production process. As heat-stable amylases are much more stable than malt amylases, simpler liquefaction, shorter process times, and an overall increase in productivity can be achieved.
- The malt enzymes are preserved for the saccharification process, where they can be used to better effect. This safeguards the brewhouse operation and results in a better wort and, ultimately, a better beer.
- Eliminating the malt from the adjunct cooker means less adjunct mash and thus more freedom in balancing volumes and temperatures in the mashing program – a problem for many brewers who use a high adjunct ratio.

6.4.2 Brewing with barley – the Ondea® Pro concept

Traditionally, the use of barley was limited to 10–20 % of the grist when using high-quality malts. To convert barley grain into fermentable wort, enzymes are needed to break down cell walls and release the amino acids and the desired sugars needed for fermentation.

The Ondea Pro brewing concept allows the strict malting barley specifications to be broadened without detriment to the final beer quality and opens the possibility of directly using local crops for sustainable beer production. This benefit mainly arises



* Adjuncts are starchy cereals such as corn, rice, wheat, sorghum, barley, or pure starch materials added to the mash.

Fig.20. The processing steps in brewing.

because the added enzyme combination has superior activities and better heat stability than the malt enzymes, and parameters that are especially necessary for a successful malting process. The composition of Ondea Pro ensures attenuation control and an efficient reduction of viscosity, turbidity and protein degradation. This new enzyme system works in synergy with the barley's endogenous enzymes (proteases, beta-amylase) for the production of fermentable worts.

As any raw material for beer production, the barley must comply with national and regional regulations for food use. The extract yield from barley is the same as the extract yield from malt on dry basis. Ondea Pro ensures the enzymatic breakdown of the barley grain components, resulting in high-quality standard wort made by a simple infusion mashing for a real degree of fermentation (RDF) of 70 % within 2 h using a dosage of 2 kg Ondea Pro/t barley. The mashing temperature profile like the one shown in Fig. 21 worked for successful mashing in a brewery.

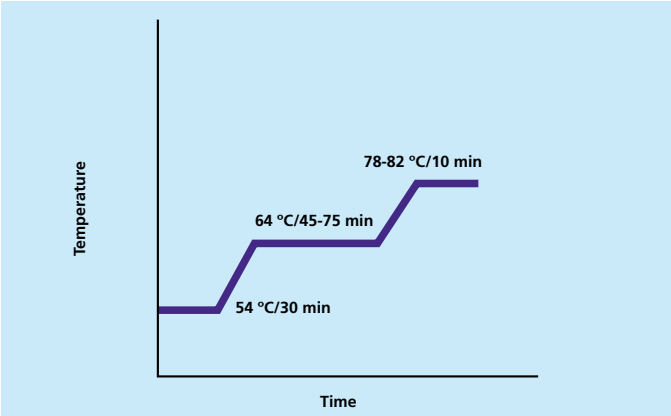


Fig. 21. Infusion mashing for a 70 % RDF within 2 h using a dosage of 2 kg Ondea Pro/t barley.

For measuring the success of a mashing, the level of digestible free amino nitrogen (FAN) and the sugar profile must be measured. The barley wort has about 9–12 mg FAN/L/°P and a sugar profile of 5-10 % DP1 (glucose), 45-60 % DP2 (maltose), < 15 % DP3 (maltotriose) and < 22 % DP4+ (maltotetraose and higher oligosaccharides).

6.4.3 General filtration problems

Wort separation and beer filtration are two common bottlenecks in brewing.

Poor lautering not only reduces production capacity but can also lead to lower extract yields. Furthermore, slow lautering negatively affects the quality of the wort, which may lead to problems with filtering the beer, and with the flavor and stability of the beer. A thorough breakdown of beta-glucans and pentosans during mashing is essential for fast wort separation.

Nondegraded beta-glucans and pentosans carried over into the

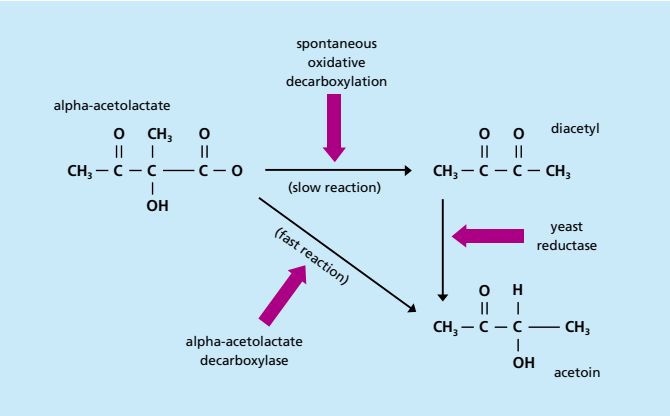


Fig. 22. The removal of alpha-acetolactate during fermentation.

fermentor reduce the beer filtration capacity and increase the consumption of diatomaceous earth (kieselguhr).

A wide range of beta-glucanase/pentosanase preparations for use in mashing or fermentation/maturation are available to solve these problems.

6.4.4 Enzymes for improving fermentation

Small adjustments in fermentability can be achieved by adding a fungal alpha-amylase at the start of fermentation or by adding a debranching enzyme (e.g., Novozymes' Promozyme®) together with a glucoamylase at mashing-in.

Beer types with very high attenuation (light beer) can be produced using saccharifying enzymes. Fungal alpha-amylases are used to produce mainly maltose and dextrins, whereas glucoamylase produces glucose from both linear and branched dextrins.

The alcohol content is another parameter that brewers are interested in controlling. The amount of alcohol in a beer is limited by the amount of solids (extract) transferred from the raw mate-

rials to the wort, and by the level of fermentable sugar in the extract. In turn, the sugar content is controlled by the amount of starch degradation catalyzed by the amylases in the mash, and by the saccharifying enzymes used during fermentation.

Yeast is a living organism and needs proteins in order to grow and multiply. If the yeast is not supplied with enough free amino nitrogen, the fermentation will be poor, and the beer quality will be inferior. A neutral bacterial protease added at mashing-in can be used to raise the level of free amino nitrogen. This is beneficial when working with poorly modified malt or with high adjunct ratios.

6.4.5 Diacetyl control

When exactly is a beer mature? This is an important question for brewers because it determines when they can "rack" the beer to make room for the next batch. The simple answer to the above question is when the diacetyl level drops below a certain limit (about 0.07 ppm). Diacetyl gives beer an off-flavor like buttermilk, and one of the main reasons for maturing a beer is to allow the diacetyl to drop to a level at which it cannot be tasted.

Diacetyl is formed by the nonenzymatic oxidative decarboxylation of alpha-acetolactate, which is produced by the yeast during primary fermentation. The diacetyl is removed again by the yeast during the beer maturation stage by conversion to acetoin, which has a much higher flavor threshold value. In fact, acetoin is almost tasteless compared with diacetyl. By adding the enzyme alpha-acetolactate decarboxylase (e.g., Novozymes' Maturex®) at the beginning of the primary fermentation process, it is possible to bypass the diacetyl step (Fig. 22) and convert alpha-acetolactate directly into acetoin. Most of the alpha-acetolactate is degraded before it has a chance to oxidize and less diacetyl is therefore formed. This makes it possible to shorten or completely eliminate the maturation period. The brewery thus enjoys greater fermentation and maturation capacity without investing in new equipment.

6.5 Distilling – Potable alcohol

The production of fermented alcoholic drinks from crops rich in starch has been practised for centuries. Before the 1960s, the enzymatic degradation of starch to fermentable sugars was achieved by adding malt or *koji*, which is fermented rice containing active microorganisms. *Koji* is used as an enzyme source for alcohol production in Japan and China.

Today, in many countries malt has been completely replaced in distilling operations by industrial enzymes. This offers many advantages. A few liters of enzyme preparation can be used to replace 100 kg of malt, making enzymes much easier to handle and store. When switching to commercial enzymes, savings of 20–30 % can be expected on raw material costs. Furthermore, since industrial enzymes have a uniform standardized activity, distilling becomes more predictable with a better chance of obtaining a good yield from each fermentation. The quality of malt, on the other hand, can vary from year to year and from batch to batch, as can *koji*.

Microbial amylases are available with activities covering a broad pH and temperature range, and therefore suitable for the low pH values found in the mash. Given these advantages, it is hardly surprising that commercial enzymes have replaced malt in all but the most conservative parts of the distilling industry.

The choice of raw material differs around the world. In North America, corn and rye are the ingredients for whisky, whereas in the UK barley is used for malt whisky and other cereals for grain spirits. In Scandinavia, potatoes and/or grain are used to produce *Akvavit*. In Germany, wheat is used for *Kornbranntwein*, whereas potatoes and grain are used for other types of spirits. And in the Far East, rice is used to make *sake*.

In the alcohol industry, starch is usually hydrolyzed by enzymes in two stages – liquefaction and saccharification. The yeast can then transform the smaller molecules – mainly glucose – into alcohol.

6.5.1 Starch liquefaction

Novozymes' enzymes bring more savings and increased efficiency to modern alcohol production. Enzymes enable the benefits of the nonpressure cooking (NPC) process, which has maximum operating temperatures between 60 and 95 °C (140–203 °F).

In the past high temperatures (150 °C/302 °F) and high energy consumption made standard alcohol production expensive. Novozymes' bioinnovation makes this expense a thing of the past. The NPC process cost-effectively breaks down and gelatinizes starch in grain, potatoes, or other raw materials, preparing them for subsequent enzymatic breakdown into fermentable sugars.

6.5.2 Starch saccharification

The second step is saccharification. A glucoamylase is used to break down starch molecules and dextrins. This enzyme is able to completely degrade starch into fermentable sugars (glucose). During fermentation these sugars are converted into alcohol by yeast cell metabolism – or in a simultaneous saccharification and fermentation process. This saves tank capacity.

Cereals, in particular corn, tend to be low in soluble nitrogen compounds. This results in poor yeast growth and increased fermentation time, which can be overcome by adding a small amount of protein-degrading enzyme to the mash. To facilitate

the distillation process it may be necessary to reduce the viscosity of the fermented broth using beta-glucanase/pentosanase preparations.

6.5.3 Viscosity reduction – High gravity fermentation

Correct gelatinization, liquefaction, and saccharification of starch-based raw materials can still result in highly viscous mashes. The extraction and solubilization of highly viscous polysaccharides such as starch, dextrins, cellulose, pentosans, xylans, and beta-glucans during the process is highly dependent on the type of raw material used. In order to save energy and reduce water consumption and effluent by running the process at a high dry-solids level, viscosity-reducing enzymes are needed for many raw materials.

Balanced combinations of xylanase, beta-glucanase, alpha-amylase and cellulase have been developed for the beverage alcohol industry.

6.6 Protein hydrolysis for food processing

The hydrolysis of proteins with enzymes is an attractive means of giving better functional and nutritional properties to food proteins of vegetable origin or from by-products such as scraps of meat from slaughterhouses. Some important properties of proteins and their application in foods are shown in Table 8.

PROPERTY	APPLICATION
Emulsification	Meats, coffee whiteners, salad dressings
Hydration	Doughs, meats
Viscosity	Beverages, doughs
Gelation	Sausages, gel desserts, cheese
Foaming	Toppings, meringues, angel food cakes
Cohesion binding	Textured products, doughs
Textural properties	Textured foods
Solubility	Beverages

Table 8. Functional properties of proteins in food and their applications.

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

The food industry is demanding milder methods of modifying food in order to limit the use of additives. Chemical modification is not desirable for food applications because of the harsh reaction conditions, nonspecific chemical reactions, and difficulties when removing residual reagents from end products. Enzymes, on the other hand, have several advantages, including fast reaction rates, mild conditions, and – most importantly – high specificity.

Over the years, many different protein raw materials have been used with different objectives. Examples of extraction processes giving enhanced yields include production of soy milk, recovery of scrap meat, cleaning of bones from slaughterhouses, recovery of gelatine, and production of meat extracts (for flavor) and yeast extracts. Furthermore, proteases facilitate the evaporation of fish/meat stickwater, the rendering of fat, meat tenderization, and the removal of the membrane from fish roe.

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

6.6.1 Flavor enhancers

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

A wide variety of savory products from different sources are available on the market. Hydrolyzed protein, mainly produced using hydrochloric acid, is a common ingredient for products such as soups, stock cubes, and savory sauces. Concern over the safety of products resulting from the hydrolysis of proteins using hydrochloric acid has led to the development of proteins that have been enzymatically hydrolyzed.

Glutamic acid in the form of monosodium glutamate (MSG) is

by far the most widely used flavor enhancer originating from protein. 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. MSG is used at concentrations of 0.2–0.8 % in a variety of foods such as soups, broths, sauces, gravies, flavor 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 means of producing *in situ* flavor enhancers of the MSG-type in protein hydrolysates. Such a hydrolysate allows people to reduce their intake of sodium.

Through their reactions at different taste sites on the tongue, peptides may result in flavors which 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 flavor in dairy products, meat and fish products, and yeast extracts. Flavor products can be produced directly using proteases on their own or in combination with a fermentation process.

6.6.2 Meat extracts

Products with a strong meat extract flavor 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 flavor intensity depends on the content of free amino acids and peptides and their reaction products. Reactions that develop flavor include Maillard reactions between reducing sugars and amino acids, thermal degradation caused by Maillard reactions, deamination, decarboxylation, and the degradation of cysteine and glutathione. The latter reaction can give rise to a large number of volatile compounds important to aroma and taste.

In the production of protein hydrolysates from meat, the first step involves efficient solubilization of the product by endoproteases. It is well known that meat hydrolysates usually taste bitter when the degree of hydrolysis (DH) is above the 10 % required for satisfactory solubilization. However, the application of exopeptidases is a generally recognized way of removing the bitterness of high-DH hydrolysates. For example, with Novozymes' Flavourzyme®, it is possible to degrade the bitter peptide groups and obtain a degree of hydrolysis of 20 % without bitterness.

Protein hydrolysates based on a relatively low degree of hydrolysis have functional properties that are ideal for use as a marinade for meat products such as ham or bacon. These functional extracts can be used to improve meat products with respect to flavor, cooking loss and sliceability. Other important applications for meat extracts are as flavor enhancers in soups, sauces, snack food and pot noodles (a type of instant meal).

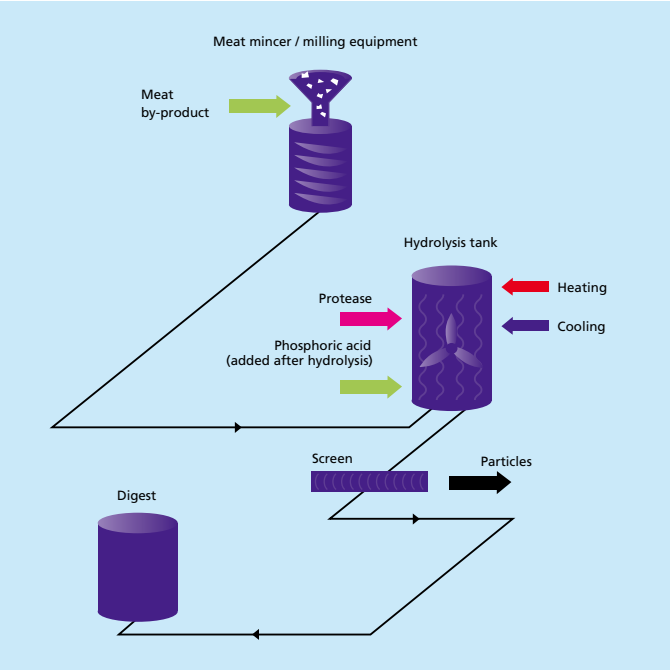


Fig. 23. Meat digest production.

6.6.3 Pet food

The most important application of enzymes in the pet food industry is in the production of digest, which is coated onto or mixed into dry pet food to improve its palatability. Digest is produced using proteases that hydrolyze meat or meat by-products, thus liquefying the raw material and creating a good flavor.

Fig. 23 shows an example of meat digest production. The raw materials originate from poultry, pigs, sheep, lambs, etc. and consist of by-products such as intestines, livers, and lungs. Before the material is filled into the hydrolysis tank, it is put through a mincer or a milling system. This allows the enzyme to gain better access to the meat protein. The pH of the minced meat substrate should be as close as possible to the pH optimum for the enzymes used. If necessary, the pH can be adjusted by adding a base. A protease is added to the tank, and enough reaction time should be allowed to completely liquefy the raw material. The exact time depends on the raw material. The reaction is then terminated by adding phosphoric acid (or other food-grade acids) to adjust the pH to 2.8 – 3.0. To ensure total inactivation of the enzyme, the digest should be heat-treated at 95 °C (203 °F) for 10 minutes. Apart from inactivation, heat treatment usually serves to improve the flavor.

6.7 Extraction of plant material

Plant material is widely used for the production of valuable food and feed products. Many ingredients used for beverages, food, and feed are produced by the extraction of plant raw materials.

Examples are proteins, starch, and other polysaccharides (e.g., pectins, gums, alginate, carrageenan, agar, celluloses, hemicelluloses), sugars, juices (from fruit and vegetables), oils, flavors, color components, phenolic and astringent components, and fibers.

All these components are found intracellularly in plant material such as seeds, fruit, and vegetables.

6.7.1 Plant cell walls and specific enzyme activities

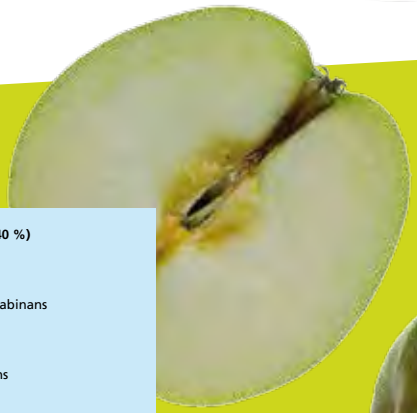
An important development has occurred in the enzymatic degradation of highly complex polysaccharides found in the cell walls of unlignified nonwoody plant material. These cell walls are composed of cellulose fibers to which strands of hemicellulose are attached. The fibers are embedded in a matrix of pectic substances linked to structural proteins. The content of cell wall polysaccharides in different plant material varies in composition and quantity, and so the composition of enzyme complexes used in industrial applications must be optimized according to the kind of material to be treated. One of the first efficient multienzyme complexes for this purpose was launched by Novozymes, who developed a strain of *Aspergillus aculeatus* that was able to express 10–15 different enzyme activities.

Conventional enzyme preparations capable of breaking down plant cell walls usually contain different activities: pectinases, hemicellulases, and cellulases. However, these products are unable to completely degrade the heteropolysaccharide nature of pectic substances. The latest-generation pectinases have a wider range of activities, enabling them to degrade the so-called hairy parts of pectin's molecular structure much better than conventional pectinases.

6.7.2 Fruit juice processing

Pectolytic enzyme preparations have been used for more than 60 years in fruit juice production. Today, they play a key role in modern fruit juice technologies. They are a prerequisite for obtaining clear and stable juices, good yields, and high-quality concentrates. They make it possible to achieve good process economy.

Pectins are composed of galacturonic acid units glycosidically linked to form polygalacturonic acid that is partially esterified (see Fig. 24). In the past, it was believed that the application of pectinesterase, polygalacturonase, and pectin lyase was suf-



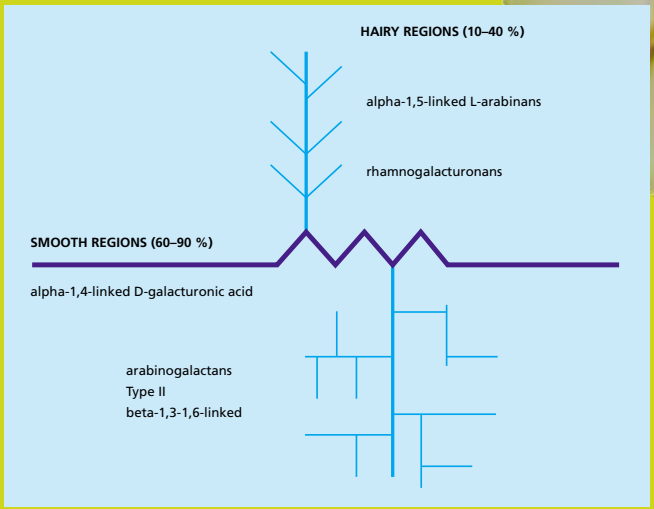


Fig. 24. The new model of the structure of pectin. The old models of pectin only included the smooth regions. The latest models include the hairy regions (shown as branches), which are more difficult to break down.

ficient to decompose pectic substances. However, more has now been learnt about the heteropolysaccharide nature of pectins. We now know that other sugars (e.g., rhamnose, xylose, galactose, arabinose) are incorporated into the pectin molecule, and we distinguish between "smooth" and hairy" regions.

As a result of this greater understanding of the substrate, there has been a logical change in the composition of the enzymes used in juice processing. For example, Novozymes' Pectinex® SMASH contains not only the three activities mentioned above, but also various other pectinolytic enzymes and hemicellulases (e.g., rhamnogalacturonases, xylanases, galactanases, and arabinanases). These additional enzymes achieve a higher degree of decomposition of pectins. Trials with Pectinex SMASH show that the time taken to press a batch of apples in a Bucher HPX-5005i press can be reduced by a further 20–30 % compared to a traditional mash enzyme. Press capacity increases from about 10 tons of apples per hour to 12–16 tons per hour.

Apart from mash treatment, there are several other enzyme applications in fruit juice processing.

6.7.3 Citrus fruit

Special pectolytic enzyme preparations are also used in the citrus juice industry. In the pulp wash process, enzymes are used to reduce viscosity and increase extraction yields. Viscosity reduction is important for avoiding jellification of the pectin during concentration. Pectolytic enzymes are also used in the clarification of citrus juices (particularly lemon juice), the extraction of essential oils, and the production of highly turbid extracts from the peels of citrus fruit. These cloudy concentrates are used in the manufacture of soft drinks.

Enzymatic peeling of citrus fruit can be used in the production of fresh peeled fruit, fruit salads, and segments. Enzymatic treatment results in citrus segments with improved freshness as well as better texture and appearance compared with the traditional process using caustic soda.

6.7.4 Fruit preparations

In the manufacture of fruit preparations, industrial processes such as mixing, bulk pulping, and sterilization are hard on the fruit pieces. Up to 50 % of the fruit pieces are converted to "pulp" during processing. However, a pure pectinesterase from Novozymes called NovoShape® is able to increase the number of intact fruit pieces in fruit preparations, which are incorporated into yoghurts, ice cream, and pastries. Intact fruit pieces clearly improve the visual appearance and mouthfeel of these products.

NovoShape utilizes the fruit's own pectin. It demethylates the endogenous fruit pectin, which thus becomes capable of forming a gel in the presence of calcium. This gel maintains fruit integrity during processing.

6.7.5 Winemaking

The grape's own enzymes consist mainly of pectin esterase and polygalacturonase, but these are often insufficient to break down pectic substances and have no effect on complex polysaccharides found in the cell wall. Since the introduction of pectinases into the wine industry in the 1970s, the development of specific cell wall-degrading enzymes offers winemakers the opportunity to improve wine quality and increase production flexibility. Enzyme preparations are used in a number of applications:

- For maceration (mash treatment) to release colors and aroma compounds, as well as increasing juice yield
- For clarification (must treatment) to speed up settling
- For wine maturation – aroma liberation, wine stabilization and filtration

Novozymes produces tailor-made preparations with pectinase, hemicellulase, and cellulase activities. Vinozym® Process gives efficient maceration of black grape skins and helps to optimize the extraction of valuable tannins, anthocyanins and aroma compounds. Furthermore, it has been observed that red wines produced with Vinozym clarify easily and have a pleasant fruity aroma with an enhanced mouthfeel. Maceration time can be reduced by 20 %. Vinozym is dosed directly into the crusher or into the mash tank. A specially purified preparation called Vinozym FCE is used for the maceration of white grapes.

Concentrated pectinases such as Ultrazym® 100 and NovoClair® Speed are used for clarification purposes to reduce grape must viscosity and speed up settling. These enzyme preparations are added immediately after the press. The major benefits are better juice quality and quicker processing.

To enhance the wine aroma of Muscat or similar grape varieties containing bound terpenes, Novozymes has developed Novarom® Blanc. By adding Novarom, it is possible to liberate more aromatic compounds into the wine and thus to increase its aroma intensity. The best time to add Novarom is after the alcoholic fermentation. The enzyme is completely inactivated by the addition of bentonite.

Late harvest grapes often become affected by "noble rot", the grey fungus *Botrytis cinerea* (Fig. 25).

Fig. 25. "Noble rot" on Riesling grapes.

This fungus produces beta-1,3-D-glucan in the wine. This type of glucan can also be released by the wine yeast. For the treatment of wines infected with *B. cinerea*, Novozymes has developed the enzyme system Vinoflow® Max A containing beta-glucanases.

Vinoflow Max is added towards the end of alcoholic fermentation or before malolactic fermentation, whichever is preferred. It allows the removal of colloids, which have a tendency to clog filters and slow down sedimentation. When these colloids are degraded, clarification, filtration, and wine stabilization improve. Furthermore, the use of Vinoflow Max on wine lees helps to speed up the aging process, giving a reduced contact time and faster clarification of the wine.

6.7.6 Oil extraction

Oil from rapeseed, coconut, corn germ, sunflower seed, palm kernels, and olives is traditionally produced by expeller pressing followed by extraction with organic solvents. The solvent most commonly used in this process is hexane, which has been identified as a hazardous air pollutant by recent environmental regulations.

Cell wall-degrading enzymes offer a safe and environmentally responsible alternative. They can be used to extract vegetable oil in an aqueous process by degrading the structural cell wall components. This concept has already been commercialized in olive oil processing, and it has been thoroughly investigated for rapeseed oil, coconut oil, and corn germ oil. In olive oil processing, the efficacy of cell wall-degrading enzymes has been proved by numerous independent studies in most olive oil-producing countries. Both yield and plant capacity can be improved, while no negative effects on the oil have been found. On the contrary, the quality of the oil is enhanced in many cases.

6.8 Enzymatic modification of lipids

A number of specific lipases are used for ester synthesis, interesterification, and hydrolysis reactions. These reactions are carried out in oils (triglycerides), glycerol, free fatty acids, esters, and alcohols. Lipases enable the oils & fats industry to produce new types of triglycerides, esters, and fatty acids, or to improve the quality of existing products. Examples of novel products include: edible oils that are nutritionally balanced in terms of saturated and unsaturated fatty acids; cocoa butter extenders; esters for lubricants and cosmetics; monoglycerides as emulsifiers; and carbohydrate-based surfactants.

6.8.1. Enzymatic degumming

Enzymatic degumming is a physical refining process in which one group of phospholipases converts nonhydratable phosphatides into fully hydratable lysolecithin. In industrial degumming this facilitates gum removal as shown on Fig. 26.
In most physical refining methods, a fundamental criterion should be that the crude oil is degummed as effectively as possible.

Using different phospolipases a variety of products, for example lyso-phospholipids, free fatty acids, diacylglycerols, choline phosphate, and phosphatidates are produced. Traditionally, chemical refining uses large amounts of caustic soda (NaOH) as a main refining component. The enzymatic degumming process has many benefits. An overall higher yield is obtained because the gums contain up to 25 % less residual oil, and because no soapstock is produced, no oil is lost. Furthermore enzymatic degumming works with crude oil as well as water-degummed oil.

6.8.2. Enzymes in simple fat production

Enzymatic interesterification is an efficient way of controlling the melting characteristics of edible oils and fats. No chemicals are used in the process and no trans fatty acids are formed. Until recently, the technology was not widely used due to the high cost of the enzyme, but now enzymatic interesterification is a cost-effective alternative to both chemical interesterification and hydrogenation since neither washing nor bleaching of the inter-

esterified fat is required, and the low-temperature enzymatic process produces no side products.

The capital investment costs are low because the enzymatic process requires only one simple column/tank as special equipment. A specific melting profile of the fat is achieved by passing the oil once through the enzyme column. Unlike both hydrogenation and chemical interesterification, the enzymatic process requires no chemicals. The enzyme is fixed in the column throughout the production, so the only handling of the enzyme is when it is changed after the production of many hundreds of tons of fat.

6.9 Reduction of viscosity in general

Enzymes are ideal for breaking down soluble compounds responsible for high viscosity in upstream and downstream processes. They are highly specific and work under mild conditions.

One example of this application is in sweetener production (see Section 6.1) where alpha-amylases reduce the viscosity of gelatinized starch. The thick gel can be transformed into a liquid that flows like water.

During evaporation processes, proteases are used to lower the viscosity of fish and meat stickwater. This reduces fouling of the evaporator's heat surfaces, thus minimizing downtime for cleaning.

Another application is in improving the separation of starch and gluten when processing wheat. The wheat contains gum-like polysaccharides known as pentosans or arabinoxylans. They can have a major influence on the process by downgrading the quality of wheat starch and reducing yields. A xylanase can be added to the wheat flour slurry right at the beginning to reduce the viscosity. Apart from increasing the yields of starch and protein, a higher production capacity from the separation equipment is obtained. A reduction in both water and energy consumption is also seen. This is the reason for an overall improvement in the process economy when using these types of enzymes.

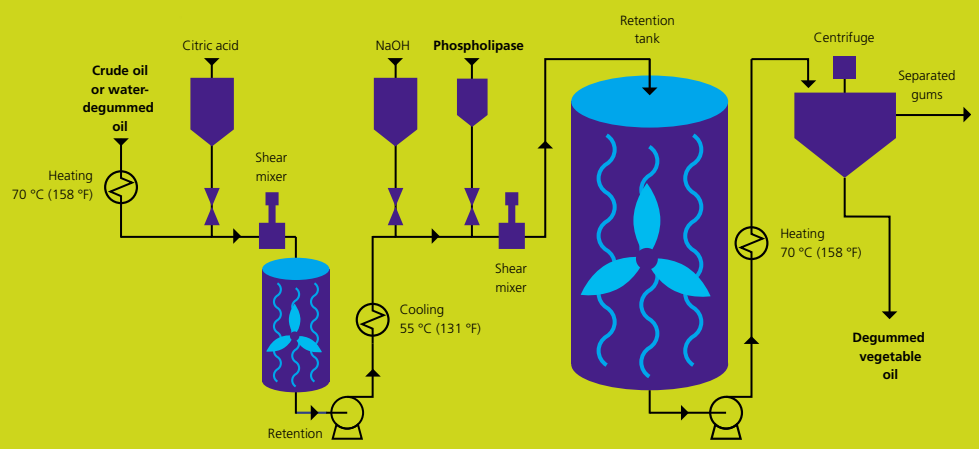
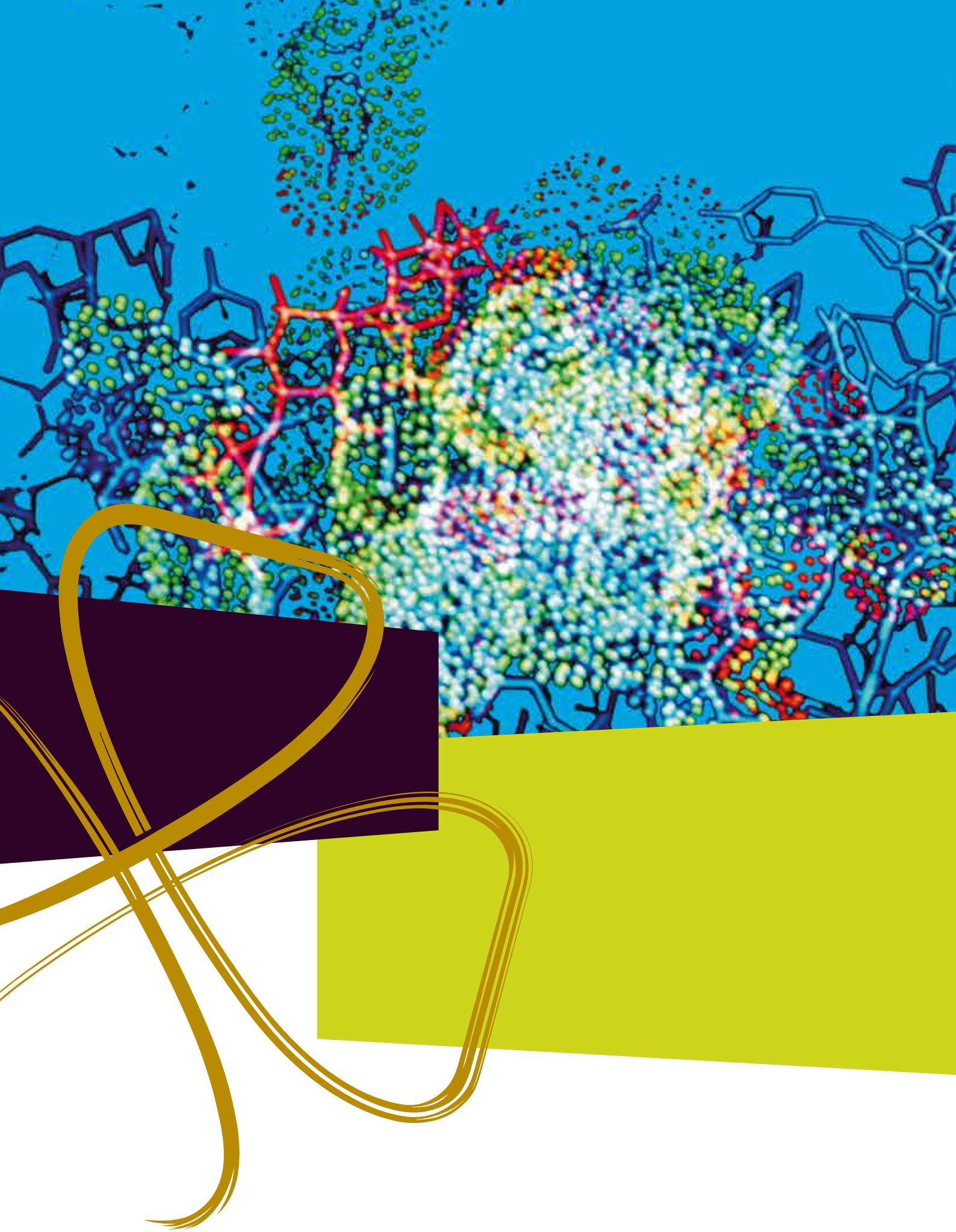


Fig. 26. Degumming of lipid oil with phospholipase.



7. Safety

Proteins are abundant in nature. Proteins from pollen, house dust mites, animal dander, and baking flour contain proteins which can cause allergies. Like many other proteins foreign to the human body, enzymes are potential inhalation allergens. The inhalation of even small amounts of foreign protein in the form of dust or aerosols can stimulate the body's immune system to produce specific antibodies. In some individuals, the presence of these specific antibodies can trigger the release of histamine when re-exposed to the allergen. This compound can cause symptoms well known to hay fever sufferers such as watery eyes, a runny nose, and a sore throat. When exposure ceases, these symptoms also cease.

Enzymes must be inhaled for there to be a risk of causing sensitization that may lead to an allergic reaction. It may be necessary to monitor the working environment in facilities where enzymes are used, especially if large quantities are handled on a daily basis. Monitoring is used to confirm that threshold limit values (TLVs) for airborne enzymes are not being exceeded. In many countries, the TLVs for enzymes are based on the proteolytic enzyme subtilisin and are stated as 60 ng (nanogram)/m^3 of pure crystalline subtilisin in air[1].

One industry that has come a long way in the safe handling of enzymes is the detergent industry. The use of encapsulated enzymes, combined with improved industrial hygiene and operating practices, has brought levels of airborne enzyme dust down dramatically in developed countries since the occupational problem of enzyme allergies first came to light in the late 1960s. The trade association AISE has generated a guide to safe handling of enzymes in the detergent industry[2].

It should be emphasized that allergy to enzymes is solely an occupational hazard, and no effects on end consumers using products containing enzymes have ever been reported during more than 40 years of use. In one of the most important reports on the subjects, the National Research Council (NRC) concluded that consumers using enzymatic laundry products did not develop respiratory allergies[3]. Further studies of enzyme allergy over the years have confirmed that enzymatic laundry and dishwashing detergents are safe for consumers to use. The HERA Risk Assessment document[4] gives a comprehensive overview of consumer safety in regard to enzyme application within the household cleaning sector.

The safe use of enzymes in food processing has been documented in a recent study by Novozymes and the University Hospital of Odense (Denmark)[5].

1. American Conference of Governmental Industrial Hygienists. Documentation of the Threshold Limit Values, 5th edition, 1986; 540–541.

2. AISE, Association Internationale de la Savonnerie, de la Détergence et des Produits d'Entretien. Guidelines for the Safe Handling of Enzymes in Detergent Manufacturing, 2002.

3. PB 204 118. Report of the *ad hoc* Committee on Enzyme Detergents. Division of Medical Science. National Academy of Science – National Research Council. Enzyme Containing Laundering Compounds and Consumer Health. Supported by the Food and Drug Administration, November 1971; 1–31.

4. www.heraproject.com, HERA, Risk Assessment. (Visited June 2013)

5. Bindslev-Jensen, C., Skov, P.S., Roggen, E.L., Hvass, P., and Brinch, D.S. Investigation on possible allergenicity of 19 different commercial enzymes used in the food industry. *Food and Chemical Toxicology*, 2006; 1909–1915.

8. Enzyme regulation and quality assurance

8.1 Detergent enzymes

In most countries, the regulatory status, classification, and labeling of enzymes are determined according to existing product control procedures for chemicals. Many enzyme types are listed in chemical inventories, for example EINECS in the EU and TSCA in the US. In some cases, enzymes are considered natural substances exempt from listing. In other cases, they are regulated by specific legislation covering biotechnology products.

The Association of Manufacturers of Fermentation Enzyme Products (AMFEP) has defined a Good Manufacturing Practice (GMP) for microbial food enzymes. This practice is generally also followed for detergent enzymes, the most important element being to ensure a pure culture of the production organism.

When an enzyme is used for a nonfood and nonfeed industrial technical application, its regulatory status is determined by its properties as a naturally occurring substance. These properties determine the classification and consequent labeling in accordance with existing regulations for chemicals.

8.2 Food enzymes

The application of enzymes in food processing is governed by food laws. Within the EU, large parts of the food laws of individual member states have been harmonized by directives and regulations. For general purposes, the FAO/WHO Joint Expert Committee on Food Additives (JECFA) and the Food Chemicals Codex (FCC) have made guidelines available for the application of enzymes as food additives. AMFEP in Europe and the Enzyme Technical Association (ETA) in the US work nationally and internationally to harmonize enzyme regulations.

AMFEP members ensure that the enzymes used in food processing are obtained from nonpathogenic and nontoxicogenic microorganisms, that is, microorganisms that have clean safety records without reported cases of pathogenicity or toxicosis attributed to the species in question. When the production strain contains recombinant DNA, the characteristics and safety record of each of the donor organisms contributing genetic information to the production strain are assessed.

The majority of food enzymes are used as processing aids and have no function in the final food. In this case, they do not need to be declared on the label because they are not present in the final food in any significant quantity. A few enzymes are used both as processing aids and as food additives. When used as additives, they must be declared on the food label.

Good Manufacturing Practice is used for industrial enzymes for the food industry. The key issues in GMP are microbial control of the microorganism selected for enzyme production, the control and monitoring systems ensuring pure cultures and optimum conditions for enzyme yield during fermentation, and the maintenance of hygienic conditions throughout the recovery and finishing stages.

Commercial enzyme products are usually formulated in aqueous solutions and sold as liquids or processed into nondusting, dry products known as granulates or microgranulates. Both liquid and dry preparations must be formulated with the final application in mind. It is important for both the producer and customer to take into account storage stability requirements such as stability of enzyme activity, microbial stability, physical stability, and the formulation of the enzyme product itself.

9. Enzyme origin and function

9.1 Biochemical synthesis of enzymes

Like other proteins, enzymes are produced inside cells by ribosomes, which link up amino acids into chains. Although the majority of industrial enzymes are produced by microorganisms, the enzymes are formed in exactly the same way as in human cells.

The structure and properties of the enzymes produced by a particular cell are determined by the genetic instructions encoded in the deoxyribonucleic acid (DNA) found in chromosomes of the cell.

DNA enables the production of specific enzymes through a code consisting of four bases: adenine (A), guanine (G), cytosine (C), and thymine (T). DNA's characteristic double helix consists of two complementary strands of these bases held together by hydrogen bonds. A always pairs with T, while C always pairs with G. The order in which these bases are assembled in the DNA double helix determines the sequence of amino acids in the enzyme protein molecule. Each fully functional segment of DNA – or gene – determines the structure of a particular protein, with each of the 20 different amino acids being specified by a particular set of three bases.

The information encoded in the DNA is converted into a protein – perhaps an enzyme – molecule by ribonucleic acid (RNA). An enzyme called RNA polymerase binds to one of the DNA strands and, moving along one base at a time, matches each base with a new RNA building block. This results in a growing chain of messenger RNA (mRNA) – a copy of the DNA code. Pieces of this mRNA then move to the ribosomes, which translate the code into a protein. As a ribosome moves along the mRNA molecule, successive amino acids are brought into position and linked together until the entire protein has been assembled. This is illustrated in Fig. 28.

9.2 How enzymes function

During and after their formation by ribosomes, the chains

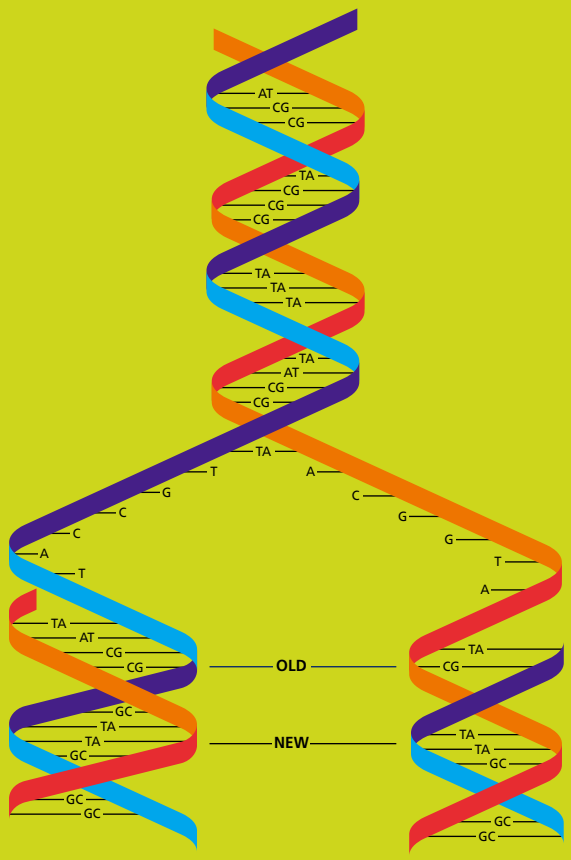


Fig. 27. A DNA molecule undergoing replication.

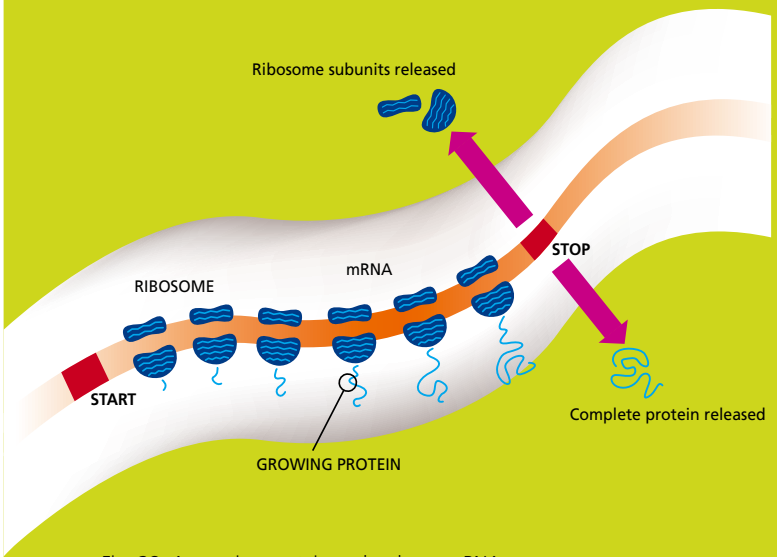


Fig. 28. A growing protein molecule on mRNA.

of amino acid residues (polypeptides – the primary structure) undergo a controlled folding (to give the secondary structure) and end up having a three-dimensional (tertiary) structure that has a major bearing on the finished enzyme's catalytic specificity and activity. Some enzymes are active only in the presence of a cofactor, which may be inorganic like a metal ion (e.g., Zn^{2+}, Ca^{2+}) or one of a series of complex organic molecules called coenzymes, which have their origins in vitamins like thiamine and riboflavin.

Enzymes have molecular weights ranging from about 12,000 to over 1 million dalton and demand physical space for movement and to be able to act on the much smaller functional groups in substrates.

Enzymes are true catalysts. They greatly enhance the rate of specific chemical reactions that would otherwise occur only very slowly. They cannot change the equilibrium point of the reactions they promote. A reaction such as S ("substrate") → P ("product") takes place because at a given temperature, there is at any instant a certain fraction of substrate molecules possessing sufficient internal energy to bring them to the top of the energy "hill" (see Fig. 29) to a reactive form called the transition state. The activation energy of a reaction is the amount of energy required to bring all the molecules in one mole of a substance at a given temperature to the transition state at the top of the energy barrier. At this point there is an equal probability of them undergoing reaction to form the products or falling back into the pool of unreacted S molecules (see Fig. 29). The

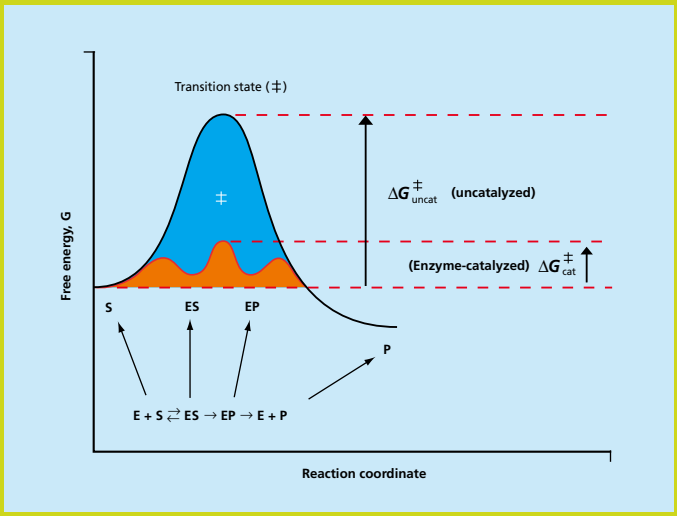


Fig. 29. A comparison of the enzyme-catalyzed and the uncatalyzed reaction S → P. The $\Delta G^{\ddagger}$'s are the free energies of activation of the uncatalyzed and catalyzed reactions, respectively.

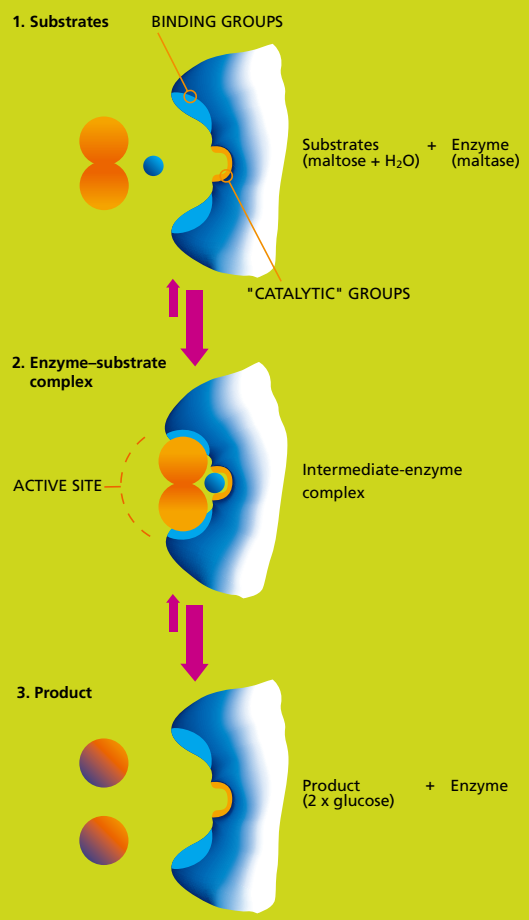


Fig. 30. The course of an enzyme reaction.

rate of any chemical reaction is proportional to the concentration of the transition state species.

There are two general ways of increasing the rate of a chemical reaction. One is to increase the reaction temperature in order to increase the thermal motion of the molecules and, thus, increase the fraction having sufficient internal energy to enter the transition state.

The second way of accelerating a chemical reaction is to add a catalyst, e.g., an enzyme. Catalysts enhance reaction rates by lowering activation energies.

In enzymatic reactions, binding groups and catalytic centers ("active sites") in enzyme molecules bind substrate molecules to form intermediate complexes with lower energy contents than those of the transition states of the uncatalyzed reactions. These complexes undergo certain atomic and electronic rearrangements, after which the products are released, see Fig. 30. Thus, the enzymes work by providing alternative reaction pathways with lower activation energies than those of the uncatalyzed reaction, see Fig. 29.

Mere recognition of a substrate is far from enough to guarantee that catalysis will take place – and if a bound compound is recognized, but no reaction takes place, it becomes an inhibitor rather than a substrate.

9.3 Basic enzyme kinetics

Many enzyme reactions may be modeled by the reaction scheme

$$E + S \rightleftarrows ES \rightarrow E + P$$

where E, S, and P represent the enzyme, substrate and product, respectively, and ES represents an *enzyme–substrate complex*. Usually it is assumed that the equilibrium between S and ES is established rapidly, so that the second reaction is the one mainly determining the rate d[P]/dt of appearance of the product P. This reaction will follow a first-order rate law, i.e.:

$$d[P]/dt = -k_{cat}\,[ES]$$

with a rate constant k_{cat} called the *catalytic constant* or the *turnover number*.

Under given conditions and at given initial concentrations [E] and [S] of enzyme and substrate, respectively, the rate of appearance of P will typically decrease over time. The rate observed during conversion of the first few percent of the substrate is called the *initial rate V*. In 1913, Leonor Michaelis and Maud Menten showed that the above model leads to the following relation between the initial rate *V* and the initial substrate concentration [S] at any given enzyme concentration:

$$V = \frac{V_{max}\,[S]}{K_M + [S]}$$

where K_M is a constant called now the *Michaelis constant* and V_{max} is a constant dependent on the enzyme concentration. This dependence of *V* on [S] leads to the characteristic curve shape shown in Fig. 31. At low substrate concentrations the initial rate is, with good approximation, proportional to [S], and at high values of [S] (substrate saturation) it approaches the limit value V_{max}, aptly called the maximum rate.

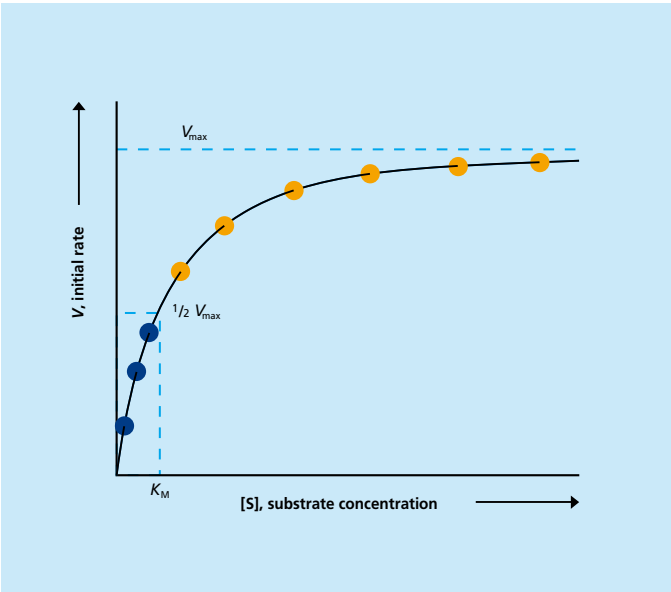


Fig. 31. Initial rate *V* as a function of substrate concentration at a given enzyme concentration.

The calculations further show that $V_{max} = k_{cat}$ [E].

The Michaelis constant is independent of the enzyme concentration, and it can be seen from the formula above that K_M can be found as the substrate concentration for which $V = V_{max}/2$.

In general, for a given enzyme, different substrates and different sets of conditions (temperature, pH) will give different values of k_{cat} and K_M and thus different initial rates will be measured under otherwise identical conditions. This means in practice that each enzyme has an optimum range of pH and temperature for its activity with a given substrate. The presence or absence of *cofactors* and *inhibitors* may also influence the observed kinetics.

Enzyme activity is usually determined using a rate assay and expressed in activity units. The substrate concentration, pH, and temperature are kept constant during these assay procedures. Standardized assay methods are used for commercial enzyme preparations.

10. A short history of industrial enzymes

Fermentation processes for brewing, baking, and the production of alcohol have been used since prehistoric times. One of the earliest written references to enzymes is found in Homer's Greek epic poems dating from about 800 BC, in which the use of enzymes for the production of cheese is mentioned.

The modern history of enzymes dates back to 1833, when Payen and Persoz isolated an amylase complex from germinating barley and called it *diastase*. Like malt itself, this product converted gelatinized starch into sugars, primarily maltose. In 1835, Berzelius demonstrated that starch can be broken down more efficiently with malt extract than with sulfuric acid and coined the term *catalysis*. In 1878, Kühne introduced the term *enzyme* for the substances in yeast responsible for fermentation (from the Greek *en* for in and *zyme* for yeast). In 1897, the Buchner brothers demonstrated that cell-free extracts from yeast could break down glucose into ethanol and carbon dioxide. In 1894, Emil Fischer developed the lock-and-key theory based on the properties of glycolytic enzymes. Fundamental enzyme kinetics date back to 1903, when Victor Henri concluded that an enzyme combines with its substrate to form an enzyme–substrate complex as an essential step in enzyme catalysis. Based on this idea, Michaelis and Menten developed the kinetic model described in Section 9.3.

The fact that enzymes are a type of protein was discovered in 1926 by James Sumner, who identified urease as a protein after purification and crystallization. Other important contributors to the development of enzyme chemistry include K. Linderstrøm-Lang and M. Ottesen, who were the first to isolate and characterize a subtilisin, a type of alkaline protease produced by bacteria.

Enzymatic desizing is one of the oldest nonfood applications of bacterial amylases. In 1950, Novo launched the first fermented enzyme, a bacterial alpha-amylase. The use of enzymes in detergents – their largest industrial application – began slowly in the early 1930s based on Röhm's 1913 patent on the use of pancreatic enzymes in presoak solutions. 1963 saw the arrival of a protease with a low alkaline pH optimum (Alcalase®), which heralded the real breakthrough for detergent enzymes. 1974 saw the launch of an immobilized glucose isomerase, which became a breakthrough in the starch industry.

The discovery by Avery in 1944 that genetic information is stored in the chromosome as deoxyribonucleic acid (DNA) was perhaps the first major step towards the now widespread use of genetic engineering and the related technique of protein engineering. Another important breakthrough came in 1953 when Watson, Crick and Franklin proposed the double-helical structure for DNA. In this molecule, genetic information is stored as a linear sequence written in a four-letter chemical alphabet. Today, scientists understand most of the significance of the information contained in DNA. For instance, the linear message laid down in an individual gene of, say, 1,200 letters can be translated into the chain of 400 amino acids making up a particular enzyme; the genetic code has been broken.

The first commercialized enzyme expressed in a genetically modified organism was a lipase for detergents called Lipolase®. It was developed by Novo and introduced in 1988 for immediate incorporation into the Japanese detergent Hi-Top made by the Lion Corporation.

Recombinant DNA technology has brought about a revolution in the development of new enzymes, as you can read in Section 11.

11. Production microorganisms

As mentioned earlier, most industrial enzymes are produced using microorganisms. Most production organisms belong either to the genus *Bacillus* (which are gram-positive bacteria) or to the genus *Aspergillus* (filamentous fungi).

The diversity of microorganisms in nature is staggering. More than 400,000 species are known, and this is just a fraction of the likely number; it is estimated that there are between four and five million different species of microorganisms. As a result, microorganisms can be found in virtually every biotope around the world. The enzyme industry is keen to exploit this diversity by gathering soil and water samples from the four corners of the Earth – often at places with extreme physical and chemical conditions – and testing these samples for the presence of microorganisms that produce enzymes of particular interest. Here it should be mentioned that the samples are collected in compliance with the Convention on Biological Diversity. This ensures, among others, that no microbial strain or natural material will be obtained without prior informed consent from the country of origin.

Enzyme molecules are far too complex to synthesize by purely chemical means, and so the only way of making them is to use living organisms. The problem is that the useful enzymes produced by microorganisms in the wild are often expressed in tiny amounts and mixed up with many other enzymes. These microorganisms can also be very difficult to cultivate under industrial conditions, and they may create undesirable by-products. Traditionally, the solution has been to breed more efficient production organisms by altering their genetic material through mutation induced by chemicals or radiation. However, these techniques are highly inefficient because the mutations are random.

Genetic engineering is a far more efficient option because the changes are completely controlled. This process basically involves taking the relevant gene from the microorganism that naturally produces a particular enzyme (donor) and inserting it into another microorganism that will produce the enzyme more efficiently (host). The first step is to cleave the DNA of the donor cell into fragments using restriction enzymes. The DNA fragments with the code for the desired enzyme are then placed, with the help of ligases, in a natural vector called a plasmid that can be transferred to the host bacterium or fungus. The DNA added to the host in this way will then divide as the cell divides, leading to a growing colony of cloned cells each containing exact replicas of the gene coding for the enzyme in question.

Since the catalytic properties of any enzyme are determined by its three-dimensional structure, which in turn is determined by the linear combination of the constituent amino acids, we can also alter an enzyme's properties by replacing individual amino acids. Detergent enzymes can be made more bleach-stable using this type of protein engineering (known as site-directed mutagenesis). Bleach-stable protein-engineered enzymes have been on the market for a number of years, for example Novozymes' Everlase®. Furthermore, enzymes can be given other useful properties using this technique, for example improved heat stability, higher activity at low temperatures, and reduced dependency on cofactors such as calcium.

12. Future prospects – in conclusion

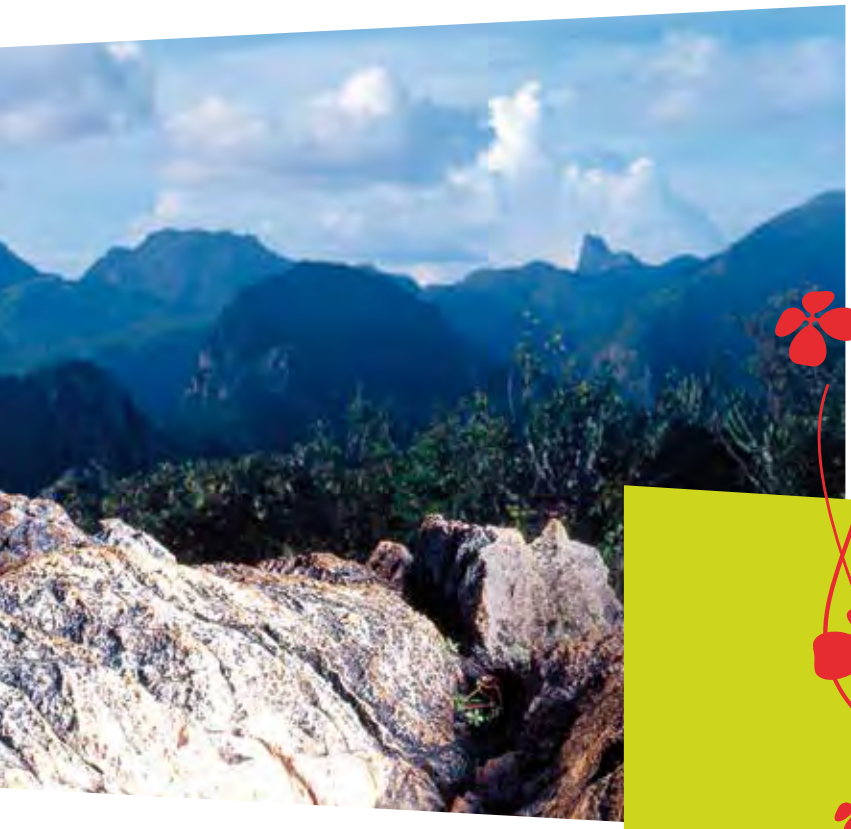
Detergents currently represent one of the largest single markets for industrial enzymes, and so we begin our look into the future here.

Enzymes have been responsible for numerous improvements in wash performance since the early 1960s. Enzymes have also contributed to more environmentally adapted washing and cleaning because they are biodegradable, they can replace harsh chemicals, and they enable reducing the wash temperature in certain cases. Nevertheless, the process of washing laundry or dishes in a machine still requires large quantities of chemicals, energy, and water. In future, this trend could lead to the development of effective detergent systems that use much smaller quantities of chemicals, less water, and less energy to attain maximum washing or cleaning performance. One possibility is the development of special dosing techniques that add active ingredients as and when they are needed at a particular stage in the washing or cleaning cycle and so enhance their performance.

The continued development of new enzymes through modern biotechnology may, for example, lead to enzyme products with improved cleaning effects at low temperatures. This could allow wash temperatures to be reduced, saving energy in countries where hot washes are still used.

New and exciting enzyme applications are likely to bring benefits in other areas: less harm to the environment; greater efficiency; lower costs; lower energy consumption; and the enhancement of a product's properties. New enzyme molecules capable of achieving this will no doubt be developed through protein engineering and recombinant DNA techniques.

Industrial biotechnology has an important role to play in the way modern foods are processed. New ingredients and alternative solutions to current chemical processes will be the challenge for the enzyme industry. Compared with traditional chemical reactions, the more specific and cleaner technologies made possible by enzyme-catalyzed processes will promote the continued trend towards natural processes in the production of food.



13. Glossary

Alpha-amylase: Amylase that catalyzes the hydrolysis of internal alpha-1,4-bonds in starch molecules and starch breakdown products.

Amino acid: An organic compound containing an amino group (–NH_2) and a carboxy group (–COOH). In particular, any of 20 basic building blocks of proteins.

Amylase: An enzyme that catalyzes the breakdown (hydrolysis) of starch. Names such as alpha-amylase or endoamylase, beta-amylase, amyloglucosidase (glucoamylase), etc., refer to enzymes that attack starch or starch breakdown products in slightly different ways.

Antibiotic: A substance, such as penicillin or streptomycin, produced by – or derived from – certain fungi, bacteria, or other organisms, that can destroy or inhibit the growth of other microorganisms.

Arabinoxylans: Carbohydrates that are major components of plant cell walls and act as storage nutrients.

ATP: Adenosine triphosphate, an energy-rich molecule that is important as a source of energy in cells.

Bating: The treatment of delimed animal skins and hides with enzymes in order to produce a clean, relaxed, and open structure ready for tanning.

Beta-amylase: An enzyme that hydrolyzes starch from the reducing end and releases maltose.

Beta-glucan: Carbohydrate found in e.g., barley.

Bleaching chemical: Any chemical providing a bleaching (decolorization) effect such as required in the textile industry and in laundry detergents. Bleaching chemicals include hydrogen peroxide and sources of hydrogen peroxide such as sodium perborates and percarbonate, hypochlorite (household "bleach"), dichlorine, dioxygen chloride (chlorine dioxide) and ozone. Only the hydrogen peroxide-based chemicals are used in laundry detergents.

Builder: A substance added to detergents to increase their cleansing action, primarily by removing the water hardness cations (magnesium and calcium) that would otherwise interfere with the action of some types of surfactants. Common builders are sodium triphosphate (which removes the hardness ions by complexation), various types of zeolites (insoluble materials that remove the hardness ions by an ion-exchange effect), and sodium carbonate (soda ash), which precipitates the hardness ions as carbonates.

Carboxypeptidase: An enzyme that removes the C-terminal amino acid from a peptide. There are carboxypeptidases that are produced in the pancreas and function as digestion enzymes.

Cellulase: An enzyme that degrades cellulose – the basic structural building block in plants and the main constituent of cotton. Endocellulases attack cellulose chains at positions away from the ends, whereas exocellulases degrade the chains from one end. Areas for cellulase applications include laundry detergents, the textile industry, and biofuel.

Chromosomes: The self-replicating genetic structure containing the cellular DNA that carries the genes. Chromosomes consist of single long molecules of DNA packed into a very compact structure. Different kinds of organisms have different numbers of chromosomes. Humans have 23 pairs of chromosomes, 46 in total.

Chymotrypsin: Specific protease from the pancreas that cleaves peptides and proteins.

Cofactors: Nonprotein substances that help an enzyme to carry out its catalytic action. Cofactors may be cations or organic molecules known as coenzymes. Unlike enzymes themselves, cofactors are often heat stable.

Deamination: Elimination of amino groups from a molecule.

Decarboxylation: The removal of a carboxy group (–COOH) from a molecule.

Desizing: Removal from a woven textile fabric of a protective coating (size) originally laid on warp threads to reduce mechanical wear during the weaving process.

Dextrin: Any of a range of soluble polysaccharides produced by partial hydrolysis (degradation) of starch such as achieved by exposing the suspension of starch to high temperature for a short time or by the action of suitable enzymes.

Diatomaceous earth: Sediments consisting of dead photosynthetic marine algae (diatoms) fossilized into a soft, chalky substance. Diatomaceous earth is used as a filtration aid. Also called kieselguhr.

DNA : DeoxyriboNucleic Acid.

DOE/EIA : US. Department of Energy/Energy Information Administration

Endoprotease: Protease that catalyzes the hydrolysis of internal peptide bonds in protein molecules.

Exoprotease: Protease that catalyzes hydrolysis from the N-terminal end or C-terminal end of a protein molecule.

FAN: In brewing, free amino nitrogen (FAN) is a measure of the amount of individual wort amino acids and small peptides that can be utilized by yeast for cell growth and proliferation.

FAN/L/°P: FAN/L of wort/degree Plato. X °Plato means the wort contains X g of solids per 100 g of wort.

Feed conversion ratio (FCR): Is the amount of feed (kg) required to produce a unit (kg) of animal. When a feed has a low FCR, it takes less feed to produce one kilogram of animal than it would if the FCR were higher. A low FCR is a good indication of a high quality feed.

Flashing: Sudden release of pressure.

Glucoamylase: Also called amyloglucosidase. An enzyme that catalyzes the hydrolysis of dextrins to glucose.

Glucosinolates: A class of organic compounds that contain sulfur and nitrogen and are derived from glucose and an amino acid. They occur as secondary metabolites of almost all plants of the order *Brassicales* (including the families *Brassicaceae, Capparidaceae* and *Caricaceae*). Glucosinolates are also called mustard oil glycosides and known for their goitrogenic effects.

Glycolytic enzyme: An enzyme involved in the conversion of glucose in living cells.

Grist: Brewers' term for milled grains, or the combination of milled grains to be used in a particular brew. Derives from the verb to grind.

Indigo: An important and valuable vat dyestuff obtained up to about 1900 entirely from plants of the genera *Indigofera* and *Isatis*. It is used for dyeing cotton for work clothes (blue denim); for a long time it was used to produce heavy (navy blue) shades on wool.

Inhibitor: An agent that partially or fully destroys the normal activity of an enzyme. A reversible inhibitor may be removed again, giving back the enzyme its full activity.

Isoelectric soluble soy protein: Enzymatically hydrolyzed soy protein that is soluble at pH 4.5.

Isomerization: A rearrangement of atoms and bonds within a molecule with no change of the molecular formula.

Jet cooker: A continuous injector system in which live steam is mixed with a slurry of starch held under pressure to raise the temperature above the boiling point.

Lautering: Separation of wort in a lauter tun.

Lignocellulose: The essential part of woody cell walls; consists of cellulose intimately associated with lignin.

Lipase: An enzyme that catalyzes the hydrolysis of fats into fatty acids and glycerol. Lipases are present in the pancreatic and intestinal juice of vertebrates.

Maillard reaction: Condensation reaction between amino acids and carbohydrates.

Maltase: An enzyme that catalyzes the hydrolysis of the disaccharide maltose into two molecules of glucose.

Maltodextrin: Maltodextrin is produced from starch by partial hydrolysis. It consists of D-glucose units connected in chains of variable length.

Molasses: The brownish liquid byproduct left after heat crystallization of sucrose (commercial sugar) in the refining process.

Octane rating or octane number: The higher octane rating or number a fuel has, the more compression it can withstand before selfignition or detonation results in knocking. Gasoline with too low octane numbers may lead to engine knocking, resulting in reduction of power and efficiency. Fuels with a high octane number are used in high-compression engines that generally have higher performance. In contrast, fuels with low octane numbers are ideal for diesel engines. (The term originally related to the content of a particular branched isomer of octane.)

Octane booster: An anti-knocking agent. When ethanol is added the octane ranking of the final gasoline blend increases. The motor runs more smoothly and at increased horsepower.

Particulate soil: Very small soil particles that have attached to the surface of a textile, etc., such as dust, clay, soot, or rust.

Pectinase: Any enzyme capable of degrading pectin.

Pentosan: A polysaccharide consisting (mainly) of pentoses.

Peroxidase: Any of a class of enzymes that oxidize substrates using hydrogen peroxide as the primary oxidant, turning it into water.

pH: A measure of the hydrogen ion (H^+) concentration (more correctly activity) on a logarithmic scale, with low pH values (pH < 7) corresponding to acidic solutions and high pH values (pH > 7) corresponding to basic (alkaline) solutions.

Phospholipase: An enzyme that catalyzes the hydrolysis of a phospholipid.

Pitch control: Avoiding processing problems with paper sheet caused by the presence of spots of sap (pitch) on the rollers.

Polypeptide: A peptide consisting of a large number of amino acids.

Pregelatinized starch: Starch that is cooked and then dried. Thereby a starch product with viscosity without the need for cooking is provided.

Premix: A mixture of ingredients such as micronutrients added to animal feed products.

Protease: An enzyme that catalyzes the hydrolysis of proteins.

Ribosomes: The site of protein synthesis in the cell. Small organelles made of rRNA and protein in the cytoplasm of cells.

RNA : RiboNucleic Acid. The nucleic acid that carries the DNA message into parts of the cell where it is interpreted and used.

Stickwater: Protein-containing water pressed out of cooked meat or fish when producing meat or fish meal.

Sticky droppings: Particularly humid or wet manure from poultry which can lead to unhealthy living conditions for the birds.

Substrate: A molecule that reacts in a reaction catalyzed by an enzyme.

Sucrase: An enzyme that catalyzes the hydrolysis of the sugar saccharose (sucrose, cane sugar).

Sugar spectra: Result of the analysis of the molecular weight distribution and composition of syrups.

Sulfhydryl groups: Thiol (-SH) groups from cysteine in proteins.

Surfactant: A substance that accumulates at surfaces, changing the behavior of the phases meeting at the surface. For example, soap spreads over a water surface and lowers its surface tension, thus enhancing its ability to wet a fabric being washed.

Syneresis: Separation of liquid from a gel.

Tanning: Chemical treatment of raw animal hide or skin to convert it into leather. A tanning agent displaces water from the interstices between the protein fibers and cements these fibers together. The three most widely used tanning agents are vegetable tannin, mineral salts such as chromium sulfate, and fish or animal oil.

Transesterification: A reversible reaction in which one ester is converted into another by interchange of the alcohol groups. These reactions are catalyzed by acid or base or by lipases.

Trypsin: A digestive protease.

Wine lees: Refers to deposits of dead yeast or residual yeast and other particles that precipitate, or are carried by the action of "fining", to the bottom of a vat of wine after fermentation and aging.

Wort: The aqueous solution produced by mashing grist (malt) after separating spent grains is called the sweet wort. It is boiled with hops. The subsequent cooling down of the wort is typically carried out in a plate heat exchanger in order to reach yeast pitching temperature.

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Notes

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YOU WILL ALREADY FIND OUR INNOVATIONS HERE
JUST IMAGINE WHERE WE CAN GO TOMORROW WITH OUR PARTNERS

AGRICULTURAL WASTE ANIMAL FEED ANIMAL WASTE A
JUICE APRICOTS AQUARIUMS BATHROOM CLEANING BE
STOCK BEER BERRY MASH BIOPOLISHING BIOFUEL BISCU
BLEACH CLEAN-UP BREAD CAR FUEL CARROT JUICE CEL
CULTURE CEREAL FOOD CHEESE CLEANING FLUIDS COLO
CARE CONTACT LENSES COOKIES COSMETICS COTTON
CRACKERS DEINKING DETERGENTS DOG FOOD DOUGH
DRAINLINE TREATMENT DRESSINGS DRYING FLUIDS EGG
EYE CARE FABRIC CARE FABRIC CLEANING FAT SUBSTITU
FERTILIZERS FISH FARMS FLOOR CLEANING FLOUR GRAP
GRASS HAIR CARE ICE CREAM JEANS JUICE TREATMENT
LEATHER LOW-ALCOHOL BEER MANGO JUICE MARGARI
MAYONNAISE MEAT MILK NOODLES ODOR CONTROLLE
ORANGE JUICE ORANGES PAINTS PAPAYA PAPER PASTA
PET FOOD PINEAPPLE JUICE PIP FRUIT PLASTICS POLYME
POND WATER PORK POULTRY RAW SILK RED WINE SHRI
PONDS SEPTIC SYSTEMS SKIN MOISTURIZER SOFT DRINK
SOFTER COTTON TEXTILES STAIN REMOVAL STARCH STA
MODIFICATION STONE FRUIT SWEETS TEXTILE LAUNDRY
TEXTILES TOOTHPASTE VARNISHES VEGETABLE OIL VEGE
WASTE DEGRADATION WASTE ELIMINATORS WASTEWA
TREATMENT WHITE WINE WOUND CARE YOGHURT

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COLORANTE-POLÍMERO

RESUMEN-OBJETO Y FINALIDAD DE LA INVENCIÓN

La presente invención provee colorantes-polímeros que comprenden polietilenimina covalentemente unida a un colorante reactivo, en donde los protones de los átomos del nitrógeno de amina primarios y secundarios de la polietilenimina no sustituida están sustituidos por grupos de alcohol isopropílico. Los colorantes-polímeros de la presente invención se presentan en composición útiles para el lavado de ropa.

SECTOR: Química. Colorantes poliméricos.

REIVINDICACIONES

1. Un colorante-polímero azul o violeta que comprende una polietilenimina covalentemente unida a un colorante reactivo, donde la polietilenimina tiene desde 6 hasta 1.000.000 átomos de nitrógeno de amina, caracterizado porque desde un porcentaje que abarca desde el 20 hasta el 95 % mol de la totalidad de los protones de los átomos de nitrógeno de amina primarios y secundarios de la polietilenimina no sustituida son protones sustituidos por grupos de alcohol isopropílico.

2. Un colorante-polímero de acuerdo con la reivindicación 1, caracterizado porque la polietilenimina contiene desde 15 hasta 45 átomos de nitrógeno de amina.

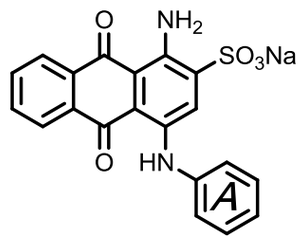
3. Un colorante-polímero de acuerdo con una cualquiera de las reivindicaciones 1 ó 2, caracterizado porque un porcentaje que abarca desde el 57 hasta el 80 % mol de los protones de los átomos de nitrógeno de amina primarios y secundarios son protones sustituidos por grupos de alcohol isopropílico.

4. Un colorante-polímero de acuerdo con una cualquiera de las reivindicaciones anteriores, caracterizado porque el grupo reactivo del colorante reactivo se selecciona entre grupos reactivos heterocíclicos, 2-bromoprop-2-enamido, 2,3-dibromopropanamido, y un grupo reactivo sulfooxietilsulfonilo (-$SO_2CH_2CH_2OSO_3Na$).

5. Un colorante-polímero de acuerdo con una cualquiera de las reivindicaciones anteriores, caracterizado porque el cromóforo del colorante reactivo se selecciona entre mono-azo, bis-azo y antraquinona.

6. Un colorante-polímero de acuerdo con la reivindicación 5, caracterizado porque el cromóforo del colorante reactivo es antraquinona.

7. Un colorante-polímero de acuerdo con la reivindicación 6, caracterizado porque el colorante reactivo es un colorante antraquinona de la forma:



,

en donde el anillo A está sustituido por un grupo reactivo seleccionado entre diclorotriazinilo, difluorocloropirimidina, monofluorotrazinilo, monofluoroclorotrazinilo, dicloroquinoxalina; difluorotriazina, monoclorotriazinilo, tricloropirimidina 2-bromoprop-2-enamido, 2,3-dibromopropanamido, y un grupo reactivo sulfooxietilsulfonilo ($-SO_2CH_2CH_2OSO_3Na$).

8. Un colorante-polímero de acuerdo con la reivindicación 7, caracterizado porque el anillo A está sustituido por uno o más grupos orgánicos seleccionados entre alquilo C1-C8 y SO_3Na.

9. Un colorante-polímero de acuerdo con la reivindicación 1, caracterizado porque la antraquinona colorante se selecciona entre Reactivo azul 1; Reactivo azul 2; Reactivo azul 4; Reactivo azul 5; Reactivo azul 6; Reactivo azul 12; Reactivo azul 16; Reactivo azul 19; Reactivo azul 24 ; Reactivo azul 27; Reactivo azul 29; Reactivo azul 36; Reactivo azul 44; Reactivo azul 46 ; Reactivo azul 47; Reactivo azul 49; Reactivo azul 50; Reactivo azul 53; Reactivo azul 55; Reactivo azul 61; Reactivo azul 66; Reactivo azul 68; Reactivo azul 69; Reactivo azul 74; Reactivo azul 86; Reactivo azul 93; Reactivo azul 94; Reactivo azul 101; Reactivo azul 103; Reactivo azul 114; Reactivo azul 117; Reactivo azul 125; Reactivo azul 141; Reactivo azul 142; Reactivo azul 145; Reactivo azul 149; Reactivo azul 155; Reactivo azul 164; Reactivo azul 166; Reactivo azul 177; Reactivo azul 181; Reactivo azul 185; Reactivo azul 188; Reactivo azul 189; Reactivo azul 206; Reactivo azul 208; Reactivo azul 246; Reactivo azul 247; Reactivo azul 258; Reactivo azul 261; Reactivo azul 262; Reactivo azul 263; Reactivo azul 172; Reactivo violeta 22; Reactivo violeta 31; y Reactivo violeta 34.

10. Una composición para el lavado de ropa que comprende desde 0,001 hasta 2,0 % en peso del colorante-polímero tal como se define en una cualquiera de las reivindicaciones anteriores, y desde 2 hasta 70 % en peso de agente tensoactivo.

Señor
SUPERINTENDENTE DE INDUSTRIA Y COMERCIO
DIVISIÓN DE NUEVAS CREACIONES
E. S. D.

Trámite: 11
Evento: 379
Actuación: 444

Re: PI.-**UNILEVER N.V.**-Solicitud de Patente de Invención en Fase Nacional PCT para **"COLORANTE-POLÍMERO"**.-Clase **C09B 69/10**.-Expediente número **16-048.983**.-

1. Me refiero al auto dictado por ese Despacho mediante notificación por fijación en lista de Marzo 22 de 2016.

2. Con el fin de dar respuesta al Oficio **N° 2708** emitido por la Dirección de Nuevas Creaciones, a continuación me permito presentar los siguientes comentarios en relación con los requerimientos y objeciones formulados por ese Despacho:

3. **RESUMEN.**

En primer lugar, atendiendo lo sugerido por el Señor Examinador en relación con el resumen de la presente solicitud, me permito adjuntar al presente memorial un resumen modificado, en el cual se ha incluido información de la invención que permite precisar el objeto de la invención, sus elementos característicos y el campo de aplicación. Así pues, atentamente me permito solicitar a ese Despacho que la objeción a este respecto sea retirada.

4. **DESCRIPCIÓN.**

Atendiendo a las objeciones del señor Examinador frente a la claridad del capítulo descriptivo de la presente solicitud, me permito señalar que la especificación de enzimas particulares correspondiente a dichas marcas no es una tarea fácil, teniendo en cuenta que las enzimas se definen a partir de su clase, por ejemplo: *lipasas, celulasas, esterasas, proteasas, amilasas, etc.*

Ahora, es preciso resaltar que los productos comerciales suministrados por, por ejemplo, Novozymes y otros productores son bastante consistentes en que se mantienen fiel a los respectivos nombres comerciales. Estas enzimas comerciales (*representadas a través de sus marcas*) son secretos comerciales y son extremadamente difíciles de identificar, excepto que la clase del tipo de enzima sí se da a conocer. Las enzimas mencionadas en la presente invención son parte de la descripción y no de las reivindicaciones, por lo que, dentro del contexto de patentes (*y teniendo en cuenta que se trata de una descripción*) se acostumbra a identificar las enzimas comerciales de esta manera (con sus nombres comerciales).

En vista de lo anterior, me permito facilitar un documento de la empresa Novozymes que da información sobre las clases de enzimas que produce comercialmente y su uso. Es importante mencionar que las enzimas comerciales descritas se suministran bajo los términos de un acuerdo de no-análisis. Por todo lo anterior, me permito señalar que la definición particular de enzimas de la descripción no es posible (*en vista que son secretos comerciales y sólo se puede definir la clase de enzima*). Consecuentemente, solicito amablemente a ese Despacho que retire la objeción al respecto.

5. REINVINDICACIONES.

5.1. Modificaciones al Capítulo Reivindicatorio.

Inicialmente, me permito manifestar que es deseo del solicitante presentar un **capítulo reivindicatorio modificado**, que reemplaza al actualmente presentado, con el propósito de superar las objeciones de claridad presentadas por ese Despacho.

En conexión con el mencionado **capítulo reivindicatorio modificado**, me permito resaltar que éste no constituye de ninguna manera una ampliación al alcance de la solicitud tal como fue originalmente presentada, toda vez que define claramente el novedoso colorante-polímero azul o violeta que comprende polietilenimina covalentemente unida a un colorante reactivo, el cuál es el objeto de la presente invención de acuerdo con las enseñanzas del capítulo descriptivo. Dichas modificaciones se exponen a continuación:

- **Nuevas Reivindicaciones 1 a 10:** corresponden a las respectivas reivindicaciones 1 a 10 del capítulo reivindicatorio actualmente presentado.

De acuerdo a lo anterior, me permito señalar que las reivindicaciones **11 a 13** del Capítulo Reivindicatorio actualmente presentado fueron **eliminadas** del documento modificado. Asimismo, me permito aclarar que el orden de enumeración y dependencia de las nuevas reivindicaciones se ha reajustado de conformidad con las modificaciones previamente descritas.

De esta forma, **el capítulo reivindicatorio modificado queda constituido por 10 reivindicaciones,** las cuales se refieren de manera más clara y concisa a las características técnicas esenciales de los colorantes-polímeros azul o violeta que comprenden polietilenimina covalentemente unida a un colorante reactivo que son objeto de la presente solicitud, y que cuentan con pleno sustento en las enseñanzas de la descripción.

5.2. **Claridad y concisión del Capítulo Reivindicatorio.**

Ahora bien, frente a las objeciones de claridad y concisión planteadas por ese Despacho, el señor Examinador aduce lo siguiente:

- *"Las reivindicaciones 1 a 9 no son claras, ni concisas cuando emplean las expresiones (…) toda vez que da lugar a un número indefinido de compuestos que no tienen soporte en la descripción (…) Se solicita restringir el capítulo reivindicatorio de acuerdo a lo soportado en la descripción".*

Respecto al anterior señalamiento, me permito muy amablemente resaltar que, contrario a lo que señala el señor Examinador, las reivindicaciones 1 a 9 son totalmente claras, puesto que el objeto a proteger se encuentra debidamente definido y soportado por la descripción de la invención.

Particularmente *–y frente a las objeciones relacionadas con la polietilenimina-*, me permito señalar que el hecho de que la imina de polietileno de la invención tenga protones de amina significa que puede reaccionar con un colorante reactivo de manera que el colorante reactivo queda unido, resultando así, en un polímero azul o violeta.

Es preciso entonces resaltar que el solicitante ha realizado una invención que se ejemplifica en las enseñanzas de la descripción y que tiene el derecho a hacer una postulación generalizada de su comprensión de la tecnología relevante para la invención.

En vista de lo anterior, solicito muy amablemente a ese Despacho retirar la objeción al respecto.

- *"La reivindicación 10 no es clara cuando señala que el componente de la composición es un "agente tensioactivo", toda vez que define el componente por su función o actividad, lo que da lugar a un número indeterminado de composiciones, algunas sin soporte en la descripción. Se solicita definir los componentes de la composición mediante compuestos específicos de acuerdo a lo soportado en el capítulo descriptivo".*

Frente a tal objeción, me permito señalar el término *"agente tensioactivo"* en ningún momento pretende definir la composición a través de su función o actividad. Todo lo contrario, tal término corresponde a una expresión genérica de un componente clave en las composiciones de la invención, que se utiliza de forma ubicua en las patentes y en las descripciones de productos para el hogar.

Como una persona versada en la materia entendería, tal expresión en ningún momento pretende caracterizar la composición a partir de una supuesta funcionalidad o actividad. En realidad, dicha expresión corresponde a la descripción de un posible componente o elemento de la composición, cuya definición se detalla en las páginas 9 a 11 de la memoria descriptiva:

> *"La composición para el lavado de ropa comprende desde 2 hasta 70 % en peso de un agente tensoactivo; con mayor preferencia, desde 10 hasta 30 % en peso. En general, los agentes tensoactivos no iónicos y aniónicos del sistema de agentes tensoactivos se puede elegir entre los agentes tensoactivos descritos en Surface Active Agents, Vol. 1, de Schwartz & Perry, Interscience 1949, Vol. 2 de Schwartz, Perry & Berch, Interscience 1958, en la edición actual de McCutcheon's Emulsifiers and Detergents, publicada por Manufacturing Confectioners Company o en Tenside-Taschenbuch, H. Stache, 2da Edición, Carl Hauser Verlag, 1981. Preferentemente, los agentes tensoactivos usados están saturados"*

De lo anterior es claro que existe una gran cantidad de agentes tensioactivos que son adecuados para un lavado de lavandería. El tensioactivo está en la composición para proporcionar una función de limpieza que es el propósito general de agentes tensioactivos.

En este sentido, es evidente que su <u>significado</u> es comprendido por la persona versada en la materia, quien contextualiza dicha expresión y complementa su interpretación con las claras enseñanzas de la memoria descriptiva. Así las cosas, la persona versada en la materia, utilizando el capítulo descriptivo, las nuevas reivindicaciones y su conocimiento en el campo técnico correspondiente, es capaz de comprender el alcance del término y de apreciar que el mismo sirve para limitar el alcance del objeto reclamado.

En este punto, considero importante resaltar que dichos términos/expresiones no pueden sacarse de contexto y considerarse como "aislados", sino que deben interpretarse en el contexto de la reivindicación en el que se encuentran y deben ser entendidos por el experto en la materia a quien se dirigen.

En conclusión, me permito manifestar que todos los términos incluidos en el nuevo capítulo reivindicatorio presentado junto al presente memorial son claros y permiten la fácil identificación y directa comparación del objeto de la materia reclamada con lo divulgado en el estado de la técnica anterior. Por lo tanto, se considera que se superan completamente las objeciones del señor Examinador a este respecto.

5.3. **Reivindicaciones relativas a usos o segundos usos.**

Por otra parte, el Señor Examinador establece que las reivindicaciones 11 a 13 están definidas en términos de la forma de usar la composición reclamada, por lo que se considera que dichas reivindicaciones no son patentables de acuerdo al Artículo 14 de la Decisión 486/2000.

Tomando en consideración la anterior objeción, me permito aclarar que, de acuerdo con las modificaciones expuestas en el numeral 5.1 del presente memorial, las reivindicaciones 11 a 13 han sido eliminadas del capítulo reivindicatorio, con lo cual se supera cualquier objeción de ese Despacho al respecto.

En vista de todo lo discutido anteriormente, me permito resaltar que el nuevo capítulo reivindicatorio es totalmente claro y conciso al reclamar los colorantes-polímeros azul o violeta que comprenden polietilenimina covalentemente unida a un colorante reactivo, dado que se define la invención a través de sus características técnicas esenciales. Asimismo, dicho capítulo modificado supera completamente las objeciones de claridad presentadas por el Señor Examinador y, por ende, cumple a cabalidad con lo estipulado por el Artículo 30 de la Decisión Andina 486 de 2000.

6. Anexo al presente memorial, me permito presentar:

i) Copia del resumen modificado;

ii) Copia de las páginas **23** a **25** que corresponden al nuevo capítulo reivindicatorio, el cual está conformado por **10 reivindicaciones** y que reemplaza al documento presentado originalmente ante ese Despacho; y

iii) Copia del Artículo de la empresa Novozymes.

7. Ruego a usted tomar atenta nota de lo anterior y proceder de conformidad.

8. Mi dirección es Calle 72 No. 5-83, Piso 6º, Bogotá D.C., Colombia.

Señor Superintendente,

ALICIA LLOREDA RICAURTE

T.P. 53.215