Paper No.: 13
Paper Title: Food Additives
Module 29 Developments In Enzyme Technology And Developing Enzyme Modified Food Ingredients

1. Introduction

For many thousands of years, man has used naturally occurring micro-organisms - bacteria, yeasts and moulds - and the enzymes they produce to make foods such as bread, cheese, beer and wine. For example in bread-making the enzyme, amylase, is used to break down flour into soluble sugars, which are transformed by yeast into alcohol and carbon dioxide. This makes the bread rise.

Enzymes catalyze chemical reactions with great specificity and rate enhancements. These reactions are the basis of the metabolism of all living organisms, and provide tremendous opportunities for industry to carry out elegant, efficient and economical biocatalytic conversions.

Today, enzymes are used for an increasing range of applications: bakery, cheese making, starch processing and production of fruit juices and other drinks. Here, they can improve texture, appearance and nutritional value, and may generate desirable flavours and aromas. Currently-used food enzymes sometimes originate in animals and plants (for example, a starch-digesting enzyme, amylase, can be obtained from germinating barley seeds) but most come from a range of beneficial micro-organisms.

Enzyme technology is an interdisciplinary field, recognized by the Organization for Economic Cooperation and Development (OECD) as an important component of sustainable industrial development. Its applications range from straightforward industrial processes to pharmaceutical discovery and development

In food production, enzymes have a number of advantages:

- They are welcomed as alternatives to traditional chemical-based technology, and can replace synthetic chemicals in many processes. This can allow real advances in the environmental performance of production processes, through lower energy consumption and biodegradability.
- They are more specific in their action than synthetic chemicals. Processes which use enzymes therefore have fewer side reactions and waste by-products, giving higher quality products and reducing the likelihood of pollution.
- They allow some processes to be carried out which would otherwise be impossible. An example is the production of clear apple juice concentrate, which relies on the use of the enzyme, pectinase.

Enzyme technology has recently been finding several directions for its development. These directions may be classified as follows; (1) new industrial catalysts, (2) Tools for food production and processing, (3) pharmaceutical uses, (4) analytical and measurement tools, (5) aids for screening new physiologically active substances, and (6) aids for creation of new sources of energy and raw materials.

2. Some uses of enzymes in food production

Market	Enzyme	Purpose / function
Dairy	Rennet (protease)	Coagulant in cheese production
	Lactase	Hydrolysis of lactose to give lactose-free milk products
	Protease	Hydrolysis of whey proteins
	Catalases	Removal of hydrogen peroxide
Brewing	Cellulases, beta-glucanases, alpha amylases, proteases, maltogenic amylases	For liquefaction, clarification and to supplement malt enzymes
Alcohol production	Amyloglucosidase	Conversion of starch to sugar
Baking	Alpha-amylases	Breakdown of starch, maltose production
	Amyloglycosidases	Saccharification
	Maltogen amylase (Novamyl)	Delays process bread staling
	Protease	Breakdown of proteins
	Pentosanase	Breakdown of pentosan, leading to reduced gluten production
	Glucose oxidase	Stability of dough
Wine and fruit juice	Pectinase	Increase of yield and juice clarification
	Glucose oxidase	Oxygen removal
	Beta-glucanases	
Wine and fruit juice	Pectinase	Increase of yield and juice clarification
	Glucose oxidase	Oxygen removal
	Beta-glucanases	
Meat	Protease	Meat tenderising
	Papain	
Protein	Proteases, trypsin, aminopeptidases	Breakdown of various components
Starch	Alpha amylase, glucoamylases, hemicellulases, maltogenic amylases, glucose isomerases	Modification and conversion (eg to dextrose or high fructose syrups)
	dextranases, beta-glucanases	
Inulin	Inulinases	Production of fructose syrups

Source: www.eufic.org

3. Goals for New or Improved Enzyme Processes

Goals	Means to achieve the goals
Cost reduction	Yield increase Biocatalyst reuse and increased productivity by immobilization (e.g. glucose isomerization) Better utilization of the raw material Reduction of process costs for • filtration • energy • desizing of fibers • cheese ripening • malting in beer production Reduction of residence time in starch processing
Improvement of biological properties and quality	Produce only isomers with the desired biological property – e.g. racemate resolution Improved preservation of foods - e.g. Juice concentrates) Improvement of technical properties - e.g. Protein modification, flour for baking, transesterification of vegetable oil, etc. Improved taste (sweetness) - e.g. glucose isomerization to glucose-fructose syrup
Utilization of new regenerable sources of raw materials	Utilization of wastes from food and wood industry (whey, filter cakes with starch and protein from vegetable oil production, cellulose) - e.g. Drinks from whey
Reduction of environmental impact	Reduction of non-recyclable waste Waste recycling (e.g. Utilization of whey)

4. Advances in Enzyme technology

Biotechnology offers an increasing potential for the production of goods to meet various human needs. In enzyme technology – a sub-field of biotechnology – new processes have been and are being developed to manufacture both bulk and high added- value products utilizing enzymes as biocatalysts, in order to meet needs such as food (e.g., bread, cheese, beer, vinegar), fine chemicals (e.g., amino acids, vitamins), and pharmaceuticals. Enzymes are also used to provide services, as in washing and environmental processes, or for analytical and diagnostic purposes. The goal of these biotechnological approaches is to design innovative products and processes that are not only competitive but also meet criteria of sustainability.

Since the early 1980s, companies which produce enzymes have been using genetic engineering techniques to improve production efficiency and quality and to develop new products. There are clear advantages here for both industry and consumers, with major improvements in enzyme production giving better products and processes. However, progress is being slowed down because the debate on some other, more controversial applications of biotechnology - such as genetic engineering in animals - is continuing throughout Europe.

At present, modern biotechnology can be used to give a range of advances in enzymatic production technology:

- Improved productivity and cost-effectiveness in existing processes. By producing enzymes more efficiently, the amount of raw materials, energy and water needed to make a product can be reduced by as much as one-half by changing from a traditional strain of microbe to a genetically modified one.
- Companies can tailor their enzymes more precisely to customer demands for products with specific properties.
- Manufacturers can supply enzymes which otherwise could not be produced in large enough quantities, giving the consumer access to a wider variety of products. An example is the amylase-based product which makes bread stay fresh for longer.

4.1. Biocatalysis in Non-Aqueous Media

A major advantage of biocatalysis is the use of water as a reaction medium owing to the low costs and waste associated with these environmentally friendly processes. Water is non-toxic, nonflammable, odourless and colourless, widely available and inexpensive and is well suited for biphasic catalysis. However, in some instances use of water as solvent is also a chief limitation of biocatalysis as many of the biocatalytic substrates are poorly water soluble and product extraction may prove difficult *e.g.* dehydration reactions such as esterifications do not proceed in an aqueous medium. Side reactions such as hydrolysis, polymerization or racemization can occur leading to product mixtures. In some instances these challenges can be circumvented through use of alternative solvents, organic solvents, supercritical fluids and ionic liquids.

4.1.1. **Organic solvents**: Replacement of an aqueous medium with an organic medium would seem challenging in the light of the conventional view that enzymes (and other proteins) are denatured (lose their native structure and thus catalytic activity) in organic solvents. However, studies over the past 15 years have established firmly that many enzymes can work in organic solvents containing little or no water and the employment of organic solvents as a reaction medium has been reviewed in detail. Biocatalytic solvent systems commonly include monophasic aqueous-organic mixtures, biphasic aqueous-organic mixtures and enzymes suspended in pure organic solvents.

4.1.2. **Supercritical fluids**: In recent years enzyme catalysed processes have been explored in novel media such as supercritical fluids and ionic liquids. A supercritical fluid (SCF) is defined as the physical state of a compound or element above its critical temperature and critical pressure but below the pressure required to condense it to a solid. Apart from most commonly used supercritical carbon dioxide (scCO2), other supercritical fluids including freons (CHF3), hydrocarbons (ethane, ethene and propane) or inorganic compounds (SF6, N2O) have also been reported as media for biocatalysis.

4.1.3. **Ionic liquids**: The employment of ionic liquids (ILs) for biocatalytic reactions has received a lot of attention in the literature in the last decade and has been recently reviewed in great detail. Ionic liquids are organic salts which are liquids at room temperature. Ionic liquids possess unique properties; they are not volatile or flammable and possess excellent chemical and thermal stability, furthermore, they have been described as environmentally benign which make them an attractive alternative to traditional organic solvents. Ionic liquids have been reported to improve activity, selectivity and the stability of enzymes like lipases, oxidoreductases, peroxidases, etc.

4.1.4. **Fluorous solvents in product isolation following biocatalysis:** An interesting application of solvents for biocatalysis was developed in 2002 by Theil and his co-workers.

Initially lipase-mediated kinetic resolution of a range of alcohols with fluorous esters was performed and repeated washing with the fluorous solvent removed the transformed ester in high enantiopurity with the untransformed alcohol remaining in the organic phase also in excellent enantiopurity. This methodology was adapted to lipase-mediated hydrolysis of highly fluorinated esters with similar results. Combination of the kinetic resolution by enzymatic deacylation with fluorous triphasic reaction and subsequent separation yielded the enantiomeric alcohols in excellent enantiopurities and good yields.

4.2. Enzyme Immobilization

An immobilized enzyme is an enzyme that is attached to an inert, insoluble material such as calcium alginate (produced by reacting a mixture of sodium alginate solution and enzyme solution with calcium chloride). This can provide increased resistance to changes in conditions such as pH or temperature. It also allows enzymes to be held in place throughout the reaction, following which they are easily separated from the products and may be used again - a far more efficient process and so is widely used in industry for enzyme catalyzed reactions.

Immobilization typically involves attachment or dispersion of an enzyme or cell to an insoluble support material to create a heterogeneous system. The principal types of immobilisation are outlined in the following Figure 1.

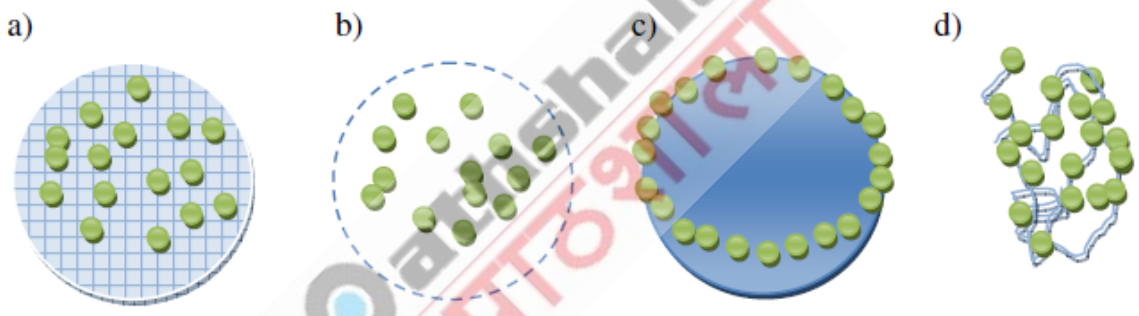


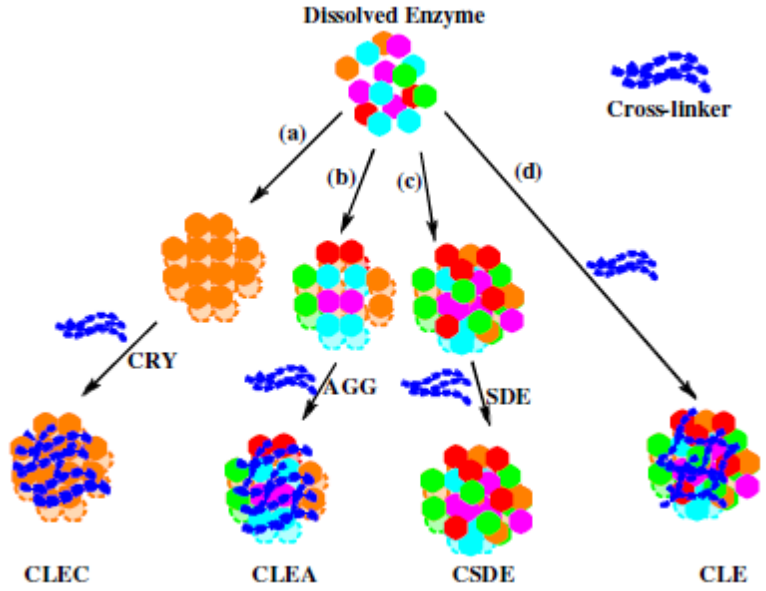
Figure 1: Enzyme immobilization strategies: (a) entrapment, (b) encapsulation, (c) solid support, (d) enzyme crosslinking (*adapted from Maguire and Sinead, 2012*).

The principal advantages of immobilization are that it allows facile recycling and repeated reuse of the biocatalyst in batch operations which significantly improves the commercial viability of enzyme-mediated processes. Immobilization also facilitates the recovery and reuse of costly enzymes, facile handling of the enzyme and easier product recovery. Other advantages associated with enzyme immobilization are improved enzyme performance, and increased pH and temperature stability. Enzyme immobilization has been reported to significantly increase (several hundred folds) enzyme activity in organic solvents. Furthermore, modification of enzyme substrate selectivity has been reported by direct immobilization through attachment of a support to a specific site on the enzyme which can lead to changes in enzyme structure and thus function.

4.2.1. **Cross-linked enzyme aggregates**

In recent years, carrier-bound cross-linked enzyme aggregates (CLEAs) have attracted increasing attention, due to their simplicity, broad applicability, high stability, and high volume activity. Studies in the early 1960s led to the discovery that cross-linking of dissolved enzymes *via* reaction of surface amino groups with a chemical cross-linker such as glutaraldehyde resulted in the formation of insoluble cross-linked enzymes. Carrier-free immobilised enzymes are generally prepared by cross-linking enzyme preparations such as crystalline, spray-dried, dissolved or physically aggregated enzymes, resulting in the

formation of cross-linked enzymes. The different approaches to carrier-free immobilised enzymes are illustrated in Figure 2.



CRY = Crystals

AGG = Aggregates

SDE = Spray Dried Enzymes

(a) Crystallization, (b) Aggregation, (c) Spray drying and (d) Direct cross-linking

Figure 2: Formation of a cross-linked enzyme crystal (CLEC), a cross-linked enzyme aggregate (CLEA), a cross-linked spray-dried enzyme and a cross-linked dissolved enzyme (CLE), (adapted from Maguire and Sinead, 2012).

CLEAs have received increasing attention in recent years due to their facile preparation and as a cheaper alternative to expensive supports; they achieve higher volumetric activities (10-1000 U/g time higher) than carrier bound enzymes. The most efficient of the CLEA methods is the physical aggregation of enzymes followed by chemical crosslinking. Enzymes which have been successfully immobilised using cross-linking enzyme methodology include horseradish peroxidase, lipases, nitrilase and esterases.

4.3. Genetic Engineering of Enzymes

Genetic engineering of the enzyme is required to alter the stereoselectivity, substrate scope and/or improve the enzyme activity. The early 1990s saw the development of new approaches to the enzyme optimisation technologies methods with the emergence of gene library generation *via* DNA shuffling and PCR techniques. Two principal processes routinely used to achieve this are rational design and directed evolution.

In rational protein design, mutants are planned on the basis of their protein structure. They are prepared by site directed mutagenesis. Following transformation into the host expressing organism *e.g. E. coli*, the variant is expressed, purified and analysed for the desired traits. Some specific examples include the employment of rational design to increase the stability of enzymes by the introduction of proline residues, disulfide bonds, or mutation towards the consensus for a given enzyme family. Rational design has also been employed to alter cofactor specificity and modify enzyme specificity by a redesign of the substrate binding site, or changing the position of a charged residue to favour transformation of one substrate over another. Unlike directed evolution, improvements or inversion of enantioselectivity are rarely reported by groups examining protein engineering through rational design.

Directed evolution is employed to improve the stability and enzymatic function of proteins by repeated rounds of mutations and selection and this method has been thoroughly reviewed in the literature. Directed evolution commences with a parent protein and an engineering goal such as enhanced selectivity or protein stability on a particular substrate.

The parent gene is subjected to a number of random point mutations to produce a library of mutants. Proteins encoded by these mutant genes are then produced and screened for the desired function and the proteins are used as the parents for another round. The beneficial mutations are collected until the desired outcome is achieved or no other improvements are practically feasible. This methodology requires careful experimental design, for a protein that is composed of 350 amino acids, 20,400 possible sequences exist. Both Rational protein Design and Directed evaluation techniques are shown in Figure 3 below.

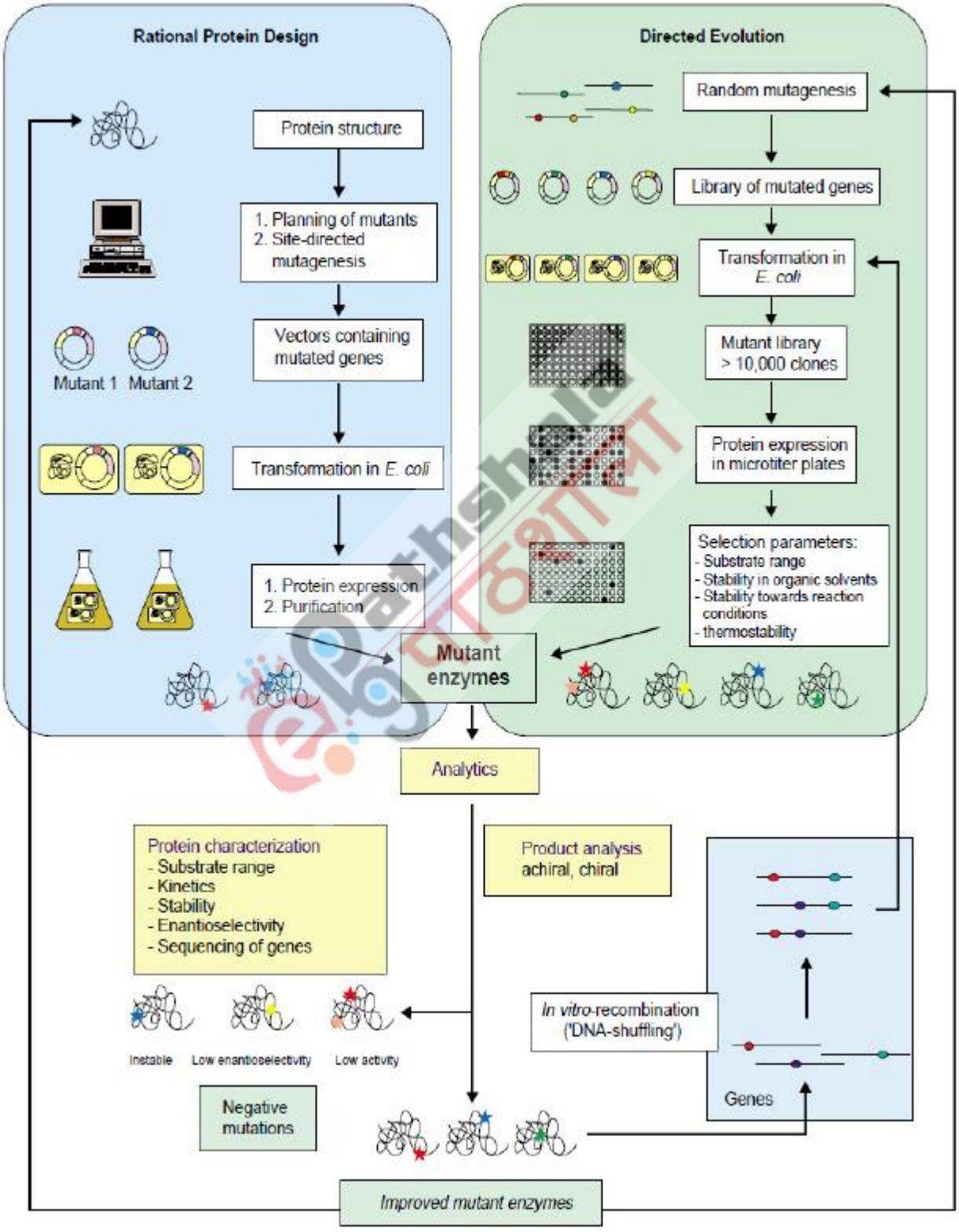


Figure 3: adapted from Maguire and Sinead, 2012

Table 4.3: Marketed enzymes produced using gene technology

Principal enzyme activity	***Application***
Alpha-acetolactate decarboxylase	Brewing
Alpha-amylase	Baking, brewing, distilling, starch
Catalase	Mayonnaise
Chymosin	Cheese
Beta-glucanase	Brewing
Alpha-glucanotransferase	Starch
Glucose isomerase	Starch
Glucose oxidase	Baking, egg mayonnaise
Hemicellulase	Baking
Lipase	Fats, oils
Maltogenic amylase	Baking, starch
Microbial rennet	Dairy
Phytase	Starch
Protease	Baking, brewing, diary, distilling, fish, meat, starch, vegetable
Pullulanase	Brewing, starch
Xylanase	Baking, starch

Source: www.eufic.org

5. Enzyme modified Foods

Enzymes have a wide range of application, not just as ingredients in food but also in the modification of other components. Applications vary from mild food preservation to the development of products with a low glycemic index (GI) and a satiating action. Reformulation of traditional food systems to introduce new ingredients may change their structure and perceived texture, since products result from interactions between different components. The development of protein enriched products has focused the attention of

food technologist when aimed at the design of innovative food with improved nutritional properties. Interactions between proteins and starch during processing can markedly influence starch gel network structure and rheological profile. Enzyme modification of food ingredients, especially proteins and starch, can lead to formation of various new products with improved nutritional and functional properties and texture characteristics, can also be applied to remove odour, flavour, and toxic or antinutritive components.One example is the a-glucose additive of stevia, a-glucosyltransferase-treated stevia (enzymatically modified stevia) gives improved taste quality, and is widely used in Japan.

5.1 Enzyme modified starch

Modified food starch is corn starch that has been processed either chemically, or with enzymes to give it desired properties like withstanding heat and acidity, retaining water, or gelling in cold solutions. Modified food starch is essential to, and exemplifies, the world of processed food. Powdered eggs and powdered cheese must flow when poured without caking and dissolve smoothly, reaching the perfect thickness when mixed with water. Frozen foods must maintain their texture and consistency, and thaw without dripping water. Instant pudding must gel without cooking. Oil based flavorants must be prevented from floating to the top of soft drinks. Nuggets, and breading must hold together without crumbling. And once the perfect texture, viscosity, and uniformity are achieved, the product must stay that way, unaltered, for weeks. All of these things are accomplished with the use of modified food starch.

Modified food starch is also useful to food manufacturers in adding bulk. It is used as a filler to increase the volume and mass of a product while reducing the use of more expensive ingredients like meat. Starch that is modified to hold moisture is used as a fat replacement. Its gluey texture gives the feel of fat and keeps low-fat processed meat from being dry.

When starch is modified, the molecules are chemically engineered into a new structure that gives the desired property. Some starch is modified just to withstand processing by machines.

Enzymatic modification of starch on an industrial scale is mainly based on the use of starch hydrolyzing enzymes such as a-amylase, pullulanase and glucoamylase. E.g. Amylomaltase-treated potato starch showed thermoreversible gelation at concentrations of 3% (w/v) or more, thus making it comparable to gelatin.

5.2 Enzyme modified proteins

Proteins are increasingly being utilized to perform functional roles in food formulations. Common food proteins possess good functional properties including solubility, gelation, emulsification and foaming. The functional properties of proteins are impaired near their pl (isoelectric point), as is the case in most acidic foods. Enzymatic modification of food proteins by controlled proteolysis can enhance their functional properties over a wide pH range, and other processing conditions. Proteins play an important role in nutrition, allergies, taste, texture, structure, and processing and yield performance. Enzyme modification of proteins can be used to produce hypoallergenic product by hydrolyzing milk proteins, modification of viscosity or emulsifying properties of ingredients to create new texture, to suppress the bitter taste like in case of a fish by-product to miximize the use of this scarce marine resource or to clean membranes in membrane processing equipment. One such example is of enzyme hydrolysis, using trypsin, chymotrypsin and pepsin that improved the solubility of the milk protein concentrate in the pH range of 4.6–7.0 inclusive. Hydrolysis also improved emulsification capacity of milk protein concentrate.

Proteases are also used in the baking industry. Where appropriate, dough may be prepared more quickly if its gluten is partially hydrolysed.

5.3 Enzyme modified egg products

Eggs are very good emulsifiers and are used as thickening agents in various recipes. For example, eggs are used to thicken sauces and custards. The drawback is that egg yolk is sensitive to high temperatures and begins to coagulate, leading to loss in emulsification properties. This limits their use in food processing. However, egg yolks can be modified by the addition of enzymes, which makes them retain and improve their emulsifying properties at high temperatures.

Modified egg yolks refer to egg yolks to which enzymes like phospholipase have been added as emulsifiers. Phospholipase (PLA), is harvested from pig pancreas. PLA is available in all mammal tissues and is one of the major irritants found in snake and insect bites. Commercially, enzymes that are obtained from a microbial source, namely *Aspergilus niger*, are also available.

Modified egg yolk has better heat resistant properties than untreated egg yolks, meaning that they can be used in recipes that require higher temperatures. It is also possible to pasteurize products containing modified egg yolks because of their high degree of stability at elevated temperatures. Pasteurizing, which isn't possible for products with untreated egg yolks, improves the microbial quality of the end product and ensures that the product remains fit for consumption for a longer period. Modified egg yolks also have improved viscosity, which is essential for certain food industry applications.

Enzyme modified egg yolks enhance the thickness of products like mayonnaise. They increase the viscosity of dressings and sauces. Since modified egg yolks add considerable thickness to products, the need for adding synthetic or chemical emulsifiers reduces. These are needed in very small quantities if at all.

5.4 Enzyme modified Cheese

Enzyme-modified cheeses (EMCs) are concentrated cheese flavors used to standardize cheese flavor in applications, or to boost flavor in applications where the amount of cheese that can be used may be limited (non-fat dishes, for example).

Enzyme-modified cheeses (EMCs), which provide intense cheese flavor, are made from special blends of natural cheese with added lipases and other natural food-grade enzymes. Flavor concentrations of 10- to 20-fold as high as that of the ripened cheeses develop in one to three days. The first step in production of EMCs generally involves blending freshly made cheese curd, and perhaps other ingredients such as other sources of fat and protein, with water and emulsifying salts to form a paste which is then pasteurized (to inactivate microorganisms and enzymes) and may be homogenized. A blend of enzymes (e.g., proteinases, peptidases and lipases) is then added sometimes together with starter organisms and the paste is incubated for a few days before being heat-treated to inactivate the added enzymes and to stabilize the product. EMCs are available as pastes or dried to form powders. EMCs, which may have approximately 15-30 times the flavour intensity of natural cheese, are used to give a cheese flavour note to products such as processed/analogue cheese, cheese powders, soups, sauces, dips, crackers, salad dressings and in coatings for snack foods. Enzyme-modified cheeses are generally added to foods at levels of 0.1 to 2.0%, although they can be used at 5% of the formulation to add dairy or cheesy notes to foods and to reduce the requirement for aged cheese in food formulations.

6. Conclusion

A large number of new enzyme processes (>100) have been introduced during the past 30 years. The study data show that hydrolases, lyases and oxidoreductases are used in two-thirds of all processes, while only about 1 % of the about 3000 known enzymes are used in larger amounts for enzyme technological and therapeutic purposes. During the past 10 years, the three-dimensional structures and detailed mechanisms of the reactions that they catalyze have been determined for many of the enzymes seen to be important in enzyme technology.

Fields where large amounts of enzymes will be required in order to realize more sustainable new enzyme processes to meet human needs include:

- The production of optically pure therapeutics and fine chemicals.
- The synthesis of antibiotics.
- Paper production or recycling to reduce waste and energy consumption.
- The regio- and stereoselective synthesis of oligosaccharides for food and pharmaceutical purposes.
- The selective glycosylation of peptides, proteins and other drugs.
- Environmental biotechnology.

Anticipating the swelling market demand, number of new enzyme processes is expected to increase further during the next few decades. The rational and sustainable design of these processes – and the improvement of existing processes – requires the interdisciplinary cooperation of (bio)chemists, micro- and molecular biologists and (bio)chemical engineers. The (bio)chemist must determine the mechanism and properties of the catalyzed process, the kinetics of the enzyme-catalyzed process and other relevant properties of substrate, product and free and immobilized enzyme (stability, solubility, pH- and temperature dependence of equilibrium constants, selectivities), and select the suitable support for the immobilization together with the engineer.