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Enzymes, proteins and bioinformatics
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Chapter 3

Industrial enzymes and their applications

3.1 Industrial enzymes

Microbial enzyme production is an essential industrial process, owing to the extraordinary performance and multitude of applications of enzymes from various microbes. These microbes are active under a varied range of physical and chemical conditions [1]. Microbial enzymes are a potential source of replacement in the absence or deficiency of human enzymes. In addition, microbial enzymes are the favored source of industrial enzymes as they can be produced in large quantities in a short time. Microbes have short generation times, and genetic manipulation can be performed more easily on bacterial cells to enhance enzyme production. The industry is still seeking novel microbial strains to furnish different enzymes to meet requirements [1]. Of the 3000 enzymes reported to date, only a few are industrially used, such as hydrolytic enzymes which degrade naturally occurring polymers such as starch, proteins, pectins and cellulose. Progress in biochemistry leading to the isolation and characterization of many enzymes made it essential to standardize the enzyme nomenclature. Based on the type of reaction catalyzed, enzymes are classified into six main classes, as listed in table 3.1.

Broad applications of enzymes in different bioprocesses can be employed to deliver a range of products in different industries. Table 3.2 summarizes a number of applications used to deliver a range of products. The industrial production of microbial enzymes is presented in figure 3.1.

3.2 Bacterial α-amylases

Amylases are one of the key enzymes used in industry. These enzymes breakdown starch molecules into polymers composed of glucose units [2, 3]. Amylases have wide applications at the industrial level, e.g. in the food, fermentation and pharmaceutical industries. Based on the source, α-amylases are classified into various categories such as α-amylases from bacteria, fungi, plants, animals and microorganisms, whereas enzymes from fungal and bacterial sources have a

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Table 3.1. Enzyme classification.

S.no.	Enzymes	Class	Reactions
1	Dehydrogenases, oxidases, oxygenases, peroxidases	Oxidoreductases	Transfer of hydrogen or oxygen or electrons between molecules.
2	Fructosyltransferases, transketolases, acyltransferases, transaminases	Transferases	Transfer of groups of atoms from one molecule to another.
3	Isomerases, epimerases, racemases	Isomerases	Transfer of a group from one position to another within one molecule.
4	Pectate lyases, hydratases, dehydratases, decarboxylases, fumarase, argino succinase	Lyases	Non-hydrolytic cleavage by elimination or addition reactions.
5	Proteases, amylases, acylases, lipases, phosphatases, cutinases	Hydrolases	Hydrolytic cleavage of bonds.
6	Synthetases, ligases	Ligases	Covalent joining of two molecules coupled with the hydrolysis of an energy rich bond in ATP or similar triphosphates.

considerable number of applications in industry. α-amylase production is necessary for the conversion of starches into oligosaccharides. Starch, a major storage product of many economically important crops, is a vital constituent of the human diet. Starch-converting enzymes are used in the production of maltodextrin, modified starches, and glucose and fructose syrups. A large number of microbial α-amylases have applications in different industrial sectors such as the food, textiles, paper and detergent industries [2, 3]. α-amylase production is usually performed using submerged fermentation, although solid-substrate fermentation systems also provide reliable technology. The thermostability, pH profile, pH stability, Ca independence and other properties of each α-amylase play an important role in the development of fermentation processes [2, 3].

α-amylases (both bacterial and fungal) are enzymes which have been produced industrially on a large scale. The general procedure for amylase production is depicted in figure 3.2. The bacterial α-amylase endoenzyme breaks down α-1,4 bonds in amylose and amylopectin, resulting in a sudden fall in the viscosity of gelatinized starch solutions (endohydrolysis of starch, also known as starch liquefaction) [2, 3]. After the action of α-amylase, the final product recovered is dextrans, together with small quantities of glucose and maltose. Before treatment with amylases, the native starch is exposed to hydration (gelatinization of starch). According to the literature, α-amylase derived from *Bacillus amyloliquefaciens* is

Table 3.2. Enzymes and their applications.

Enzyme	Microorganism(s)	Function
Acid proteinase	*Aspergillus* spp.	Milk coagulation
Acyltransferase	*Bacillus* sp. APB-6	Synthesis of hydroxamic acids
Alkaline protease	*Alcaligenes faecalis*	Dehairing, bating
Amidase	*Rhodococcus erythropolis*	Degradation of nitriles containing wastes
Aminopeptidase	*Lactobacillus* spp.	Faster cheese ripening
Aminopeptidases	*Lactobacillus brevis*, *Lactobacillus plantarum*	Protein breakdown during mashing
Amylase	*Aspergillus* spp., *Bacillus* spp.	Flour adjustment, bread softness
Amylase	*Bacillus licheniformis*	De-inking, drainage improvement
Amylase	*Aspergillus* spp., *Bacillus subtilis*	Carbohydrate stain removal
Amylase	*Aspergillus* spp., *B. subtilis*	Fiber splitting
Amylase	*B. licheniformis*, *Aspergillus* spp.	Bioremediation of vegetable waste
Amyloglucosidase	*Aspergillus niger*	Starch hydrolysis for bioremediation
Catalase	*A. niger*	Cheese processing
Cellulase	*A. niger*, *Trichoderma atroviride*	Fruit liquefaction
Cellulase	*Bacillus* spp., *A. niger*	De-inking, drainage improvement
Cellulase	*A. niger*, *Bacillus* spp.	Color clarification
Cutinase	*Fusarium solani f. pisi*	Triglyceride removal
Cutinase	*F. solani f. pisi*	Degradation of plastics, polycaprolactone
Endoglycosidase	*Mucor hiemalis*	Teeth and gum tissue care
Glucose isomerase	*Corynebacterium* spp., *Streptomyces murinus*	Production of high-fructose corn syrup
Glucose oxidase	*A. niger*, *Penicillium chrysogenum*	Dough strengthening
Glucose oxidase	*A. niger*	Oxygen removal from beer
Glucose oxidase	*A. niger*, *P. chrysogenum*	Polymerization of anilines
Glycosyl tranferase	*Bacillus* spp.	Synthesis of oligosaccharides
Laccase	*B. subtilis*	Non-chlorine bleaching, delignification
Laccase	*Trametes hirsute*	Polymerization of bisphenol A
Laccase	*B. subtilis*, *Trametes versicolor*	Hair dye
Laccase	*T. versicolor*, *B. subtilis*	Production of textile dyes, cosmetic pigments, flavor agents and pesticides
Laccase	*T. versicolor*	Degradation of waste containing olefin unit, polyurethane and phenolic compounds
Lactase (β-galactosidase)	*Escherichia coli*, *Kluyveromyces* spp.	Lactose reduced milk and whey products

(*Continued*)

Lignin peroxidase	*Phanerochaete chrysosporium*, *Coprinus cinereus*	Degradation of phenolic compounds
Limoninase	*A. niger*, *Aspergillus oryzae*	Debittering
Lipase	*A. niger*, *A. oryzae*	Faster cheese ripening, flavor customized cheese
Lipase	*A. niger*	Dough stability and conditioning
Lipase	*Candida antarctica*	Pitch control
Lipase	*C. antarctica*	Polycondensation, ring-opening polymerization of lactones, carbonates
Lipase	*A. oryzae*, *Aspergillus flavus*	Fat stain elimination
Lipase	*A. oryzae*, *A. flavus*,	Degreasing
Lipase	*A. oryzae*, *A. flavus*	Skin care
Lipase	*A. oryzae*, *A. flavus*	Synthesis of pharmaceuticals, polymers, biodiesels, biosurfactants
Lipase	*A. oryzae*, *Candida tropicalis*	Degradation of crude oil hydrocarbons
Maltogenic α-amylase	*Bacillus stearothermophilus*	Enhancing the shelf life of breads
Manganese peroxidase	*P. chrysosporium*, *C. cinereus*	Degradation of phenolic compounds
Mannanase	*Bacillus* spp.	Mannan spot removal
Naringinase	*A. niger*	Debittering
Neutral protease	*A. niger*, *A. flavus*, *B. subtilis*	Dehairing, soaking
Neutral proteinase	*B. subtilis*, *A. oryzae*	Faster cheese ripening, debittering
Nitrile hydratase	*Rhodococcus rhodochrous* PA-34, *Bacillus* spp. APB-6	Synthesis of acrylamide, butyramide and nicotinamide
Nitrile hydratase	*Rhodococcus* spp.	Degradation of nitriles containing wastes
Oxygenase	*Pseudomonas* spp., *Rhodococcus* spp.	Degradation of halogenated contaminants
Pectinase	*A. oryzae*, *Penicillium funiculosum*	Depectinization
Phytase	*A. niger*	Hydrolyze phytic acid to release phosphorous
Protease	*A. niger*	Restrict haze formation
Protease	*B. subtilis*	Biofilm removal
Protease	*A. oryzae*, *B. subtilis*	Protein stain removal
Protease	*A. niger*, *A. flavus*, *B. subtilis*	Removal of dead skin
Protease	*Chrysosporium keratinophilum*	Bioremediation of keratinic wastes
Pullulanase	*Bacillus* spp., *Klebsiella* spp.	Starch saccharification
Superoxide dismutase	*Corynebacterium glutamicum*, *Lactobacillus plantarum*	Free radical scavenging, skin care

Transglutaminase	*Streptomyces* spp.	Protein crosslinking
Transglutaminase	*Streptoverticillium* spp., *Streptomyces* spp.	Laminated dough strength
Transglutaminase	*Streptomyces mobaraensis*	Protein crosslinking
Tyrosinase	*Trichoderma reesei*	Polymerization of lignin and chitosan
Xylanase	*A. niger*	Dough conditioning
Xylanase	*Aspergillus* spp., *Bacillus* spp.	Enhanced digestibility of starch
Xylanase	*Trichoderma reesei*, *Thermomyces lanuginosus*, *Aureobasidium pullulans*	Bleach boosting
α-amylase	*Bacillus* spp., *Aspergillus* spp.	Starch hydrolysis
β-amylase	*Bacillus* spp., *Streptomyces* spp., *Rhizopus* spp.	Starch hydrolysis
β-glucanase	*B. subtilis*, *Aspergillus* spp.	Restrict haze formation
β-glucanase	*A. niger*	Digestive aid

active up to 90 °C and this high temperature stability (for gelatinization purpose) has been used at the industrial scale. Another strain belonging to the same genus, *Bacillus licheniformis*, has also been used industrially for the production of α-amylases. α-amylases are metallo-proteins, containing at least one mol of calcium ions (they are stabilized by Ca^{++} and inhibited by chelate forming agents) [2, 3]. The activity and stability of α-amylase obtained from *B. licheniformis* is not dependent on the calcium content of the solution, in contrast to α-amylase from *B. amylolequefaciens*. pH 6.5–7 is the optimum range of pH, at which these enzymes show maximum activity. At neutral pH and temperature (30–40 °C), fermentation of bacterial amylases is performed in submerged culture. Cereal meal and starch rich medium are usually used along with an organic source of nitrogen. After an interval of 10–20 h, α-amylase formation starts and lasts for another 100 h [2, 3]. The pH of the medium must be less than 6 during fermentation to avoid the denaturation of α-amylase. The α-amylase is accompanied by other extracellular enzymes. *B. licheniformis* synthesizes a serine protease, whereas *B. amyloliquefaciens* synthesizes a neutral protease along with hemicellulase and β-glucanase. Starch liquefaction using α-amylase is performed in a continuous or batch reactor. The degree of anticipated starch hydrolysis is determined by the intended later use. The manufacture of coating compounds, surface sizing of paper and paints requires a colloidal starch solution with a final viscosity [2, 3]. Incomplete degradation of starch is brought about by α-amylase to attain a specific viscosity. During the production of glucose syrup, α-amylase is used in the first step of enzymatic degradation resulting in a mixture of glucose and fructose with high-fructose content. During the production of dextrans, the degradation of starch required is as high as 10 dextrose

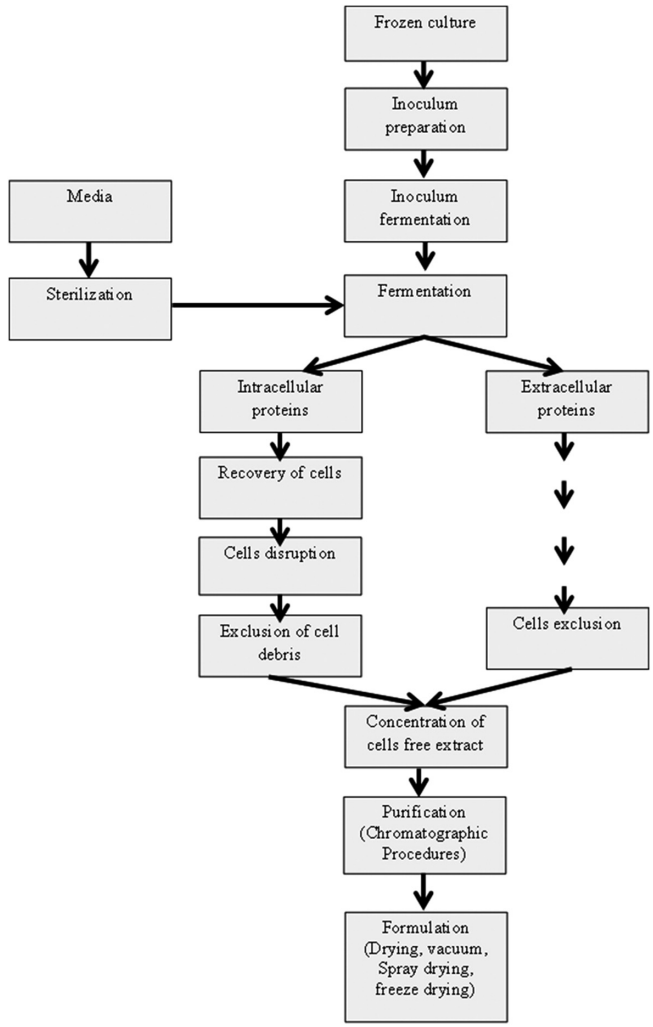

Figure 3.1. Basic outline of industrial production of enzymes.

equivalents. The increasing production of alcohol as a fuel from starch-containing raw material is opening greater prospects for the utilization of α-amylases and glucoamylases at large scales [2, 3].

3.3 Fungal α-amylases

Fungal amylases have been extensively used in large-scale industrial production owing to their many merits, for example, their cost effectiveness, consistency, smaller temporal and spatial requirements, and ease of process modification and optimization [4]. These enzymes account for around 30% of the world's enzyme production [3]. Amylases belong to a class of starch degrading enzymes that catalyze the breakdown of internal glycosidic bonds in polysaccharides with the retention of

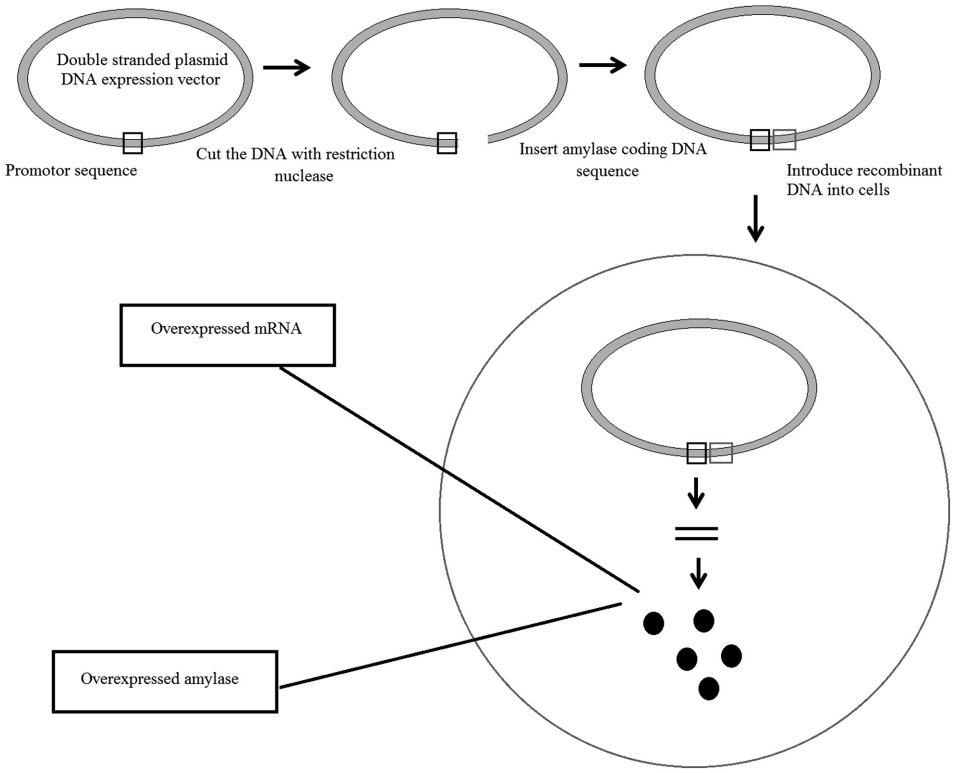

Figure 3.2. Amylase production using rDNA technology.

the anomeric configuration in the products. As mentioned above, most amylases are metalloenzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability [5]. Endo-amylases, exo-amylases, debranching enzymes and glycosyltransferases are further examples of the starch degrading class of enzymes [6]. In the starch processing industry, enzymatic hydrolysis is favored over acid hydrolysis, owing to the specificity of the reaction, stability of the synthesized products, lower energy requirements and exclusion of neutralization steps [7]. Due to the high demand for these enzymes at large scales, there is great interest in developing enzymes with better properties, e.g. raw starch degrading amylases suitable for industrial applications and cost effective production techniques. Most amylases have been synthesized from soil fungi, e.g. *Aspergillus*, *Penicillium* and *Rhizopus* [7]. Only little evidence is available on amylases from endophytic fungi, which are primarily explored for beneficial secondary metabolites with different bioactivity [8]. Fungal α-amylases are mainly derived from the *Aspergillus* genus (*A. niger*, *A. oryzae*). Because of their low deactivation temperature, low optimum pH (pH 4–5) and high saccharifying action, they significantly differ from the bacterial amylases [3–8]. They are less fit for the liquefaction of starch than bacterial α-amylases. The synthesis of fungal α-amylase is accomplished in solid-substrate culture and occasionally in submerged culture for specific strains of *Aspergillus*.

The fermentation medium used remains the same as for bacterial α-amylases, but the concentration of glucose hinders the formation of amylase, thus the concentration of glucose in the fermentation medium must be measured and kept low [3–8]. Other fungal enzymes are produced in solid-substrate culture. The protease content is found to be higher when produced by solid-substrate culture in comparison to α-amylase produced by submerged culture [3–8]. The glucoamylase level is low in both methods. Fungal α-amylases are employed in the manufacturing of baked goods, where this enzyme is added to amylase-poor flour to reduce and standardize the proving time of dough. In addition, they are also used in the production of maltose-rich syrups and various other areas of the food industry [3–8].

3.4 Bacterial proteases

Microbial proteases are among the most important hydrolytic enzymes and have been studied widely. Proteases derived from micro-organisms have gained great attention in the last decade because of their biotechnological applications in various industrial processes, e.g. detergent, textile, leather, dairy and pharmaceutical preparations. Proteolytic enzymes derived from micro-organisms are preferred in industrial applications of enzymes owing to their technical and economic advantages [9]. The category of microbial proteases signifies one of the largest groups of industrial enzymes and accounts for approximately 60% of total enzyme sales in the world [9]. The majority of bacterial proteases are produced from *B. licheniformis*. The bacterium is cultured in a fermentation medium supplemented by protein and protein hydrolysate. The medium's pH is adjusted to neutral and the temperature is kept between 30–40 °C. Supplementation of carbohydrates *in situ* enhances the productivity of enzymes. Production begins when the culture passes into the stationary phase (after 10–20 h) and takes place at an almost constant rate for several hours, providing that the protein is still present in the medium. Starch hydrolysates are degraded and assimilated by *B. licheniformis,* however, at the end of the fermentation serine protease is virtually the only protein in the culture suspension. Proteases can also be synthesized by fermentation *of B. amyloquefaciens*, but the final yield of protease is higher in the case of *B. licheniformis* (at least 10% more protein added to the medium). In the case of *B. amyloliquefaciens,* the culture supernatant contains other enzymes, in particular, a large amount of α-amylase. Alkaline proteases derived from *Bacillus amyloliquefaciens* and *Bacillus licheniformis* represent the lead molecules for subtilisin. Serine proteases have been derived from species of *Bacillus*. These proteases have better pH stability (up to pH 12) compared to subtilisins (pH 8–11). The bulk of alkaline proteases are used as additives to detergents in the form of granulates, prills or marumerizer pellets. Proteases enhance the cleansing power of a detergent and, in addition, this allows the elimination of protein-containing soils, e.g. blood, egg yolk and chocolate, and enhances the detachment of soil from the fibers. The stability of subtilisins is independent of metal ions, hence complexing additives in detergents, e.g. EDTA or tripolyphosphates, do not significantly interfere. Currently, almost 80% of the detergents on the market contain enzymes in concentrations of about

0.015–0.025%. The high alkali resistance of alkaline proteases makes them suitable for the leather industry, for the dehairing (nuding) of skins and to reduce softening times. They exhibit much better stability in detergents when the complexing agent EDTA or tripolyphosphate is substituted by citrate or gluconate.

3.5 Fungal proteases

Fungal proteases have attracted the attention of researchers as fungi can grow on low-cost substrates and secrete large quantities of enzymes into the culture medium, which could ease downstream processing [10]. Fungal proteases can be employed for hydrolyzing protein and other components of soybeans and wheat in soy sauce production. Proteases can be synthesized in large amounts in a short time by established methods of fermentation [10]. Parameters such as the C/N ratio and the presence of some sugars, in addition to several other physical factors, are significant in the development of the fermentation process. Fungus-derived proteases can be produced cost effectively, and also have the advantages of fast production, ease of enzymes modification and the easy removal of mycelium by filtration. Protease production has mainly been achieved by using submerged fermentation, however, the conditions in solid-subtrate fermentation result in several potential merits for the production of fungal enzymes [10].

Fungal proteases are synthesized from the genus *Aspergillus*, in particular the two species *A. oryzae* and *A. niger* [11, 12]. When cultured on a solid substrate, large quantities of proteases are produced by these species. The yield can be further improved by the addition of inorganic nitrogen. Other than proteases, reasonable amounts of α-amylase, glucoamylase, cellulases and pectinases are also produced. As far as applications are concerned, *Aspergillus* proteases are employed for hydrolysis of soybean protein in the production of soya sauce, as a component of enzymatic preparations for the treatment of digestive disturbances and in the baking industry. The supplementation of protease into flour incompletely hydrolyzes the gluten, reduces the viscosity of dough and thus reduces the kneading time. Fungal proteases are deactivated at 50 °C, so a quick denaturation takes place in the beginning of the baking process and circumvents the complete hydrolysis of proteins. Acid proteases can be derived from *Mucor* strains, in particular from the species *M. pusillus* and *M. michei*. During the fermentation of *Mucor* strains, esterases and lipases are also formed, which are mainly used in the manufacture of cheese [13].

3.6 Glucose isomerase (D-xylose ketol-isomerase; EC. 5.3.1.5)

Glucose isomerase is the first intracellular enzyme used on a large scale. It catalyzes the reversible isomerization of D-glucose and D-xylose to D-fructose and D-xylulose, respectively [14]. This enzyme has the largest market in the food industry because of its application in the production of high-fructose corn syrup (HFCS) [14]. This mixture contains 42% fructose, 50% glucose and 8% other mono- and disaccharides used as liquid sugar in the food industry. The entire process is based on starch hydrolysis by α-amylase and glucoamylase, and then isomerization to a mixture of glucose and fructose. Fructose has a better sweetening power than sucrose and

glucose. HFCS, an equilibrium mixture of glucose and fructose, is 1.3 times sweeter than sucrose and serves as a sweetener for use by diabetics [14]. Interconversion of xylose to xylulose by glucose isomerase acts as a nutritional requirement in saprophytic bacteria and has a potential application in the bioconversion of hemicellulose to ethanol. The enzyme is generally found in prokaryotes. The microbial strain development accomplished by utilizing xylan-containing raw materials for the development of or screening for constitutive mutants of glucose isomerase is expected to result in discontinuation of the use of xylose as an inducer for the production of the enzyme [14]. Removal of Co^{2+} from the fermentation medium is necessary to prevent health issues arising from human consumption of HFCS. Glucose isomerase is synthesized by submerged cultivation of the various organisms listed in table 3.3 in the pH range 6.5–8.5 at 30 °C. Enzyme isolation is typically omitted and whole or disintegrated cells are immobilized. Glucose isomerase immobilization requires adsorption or heat treatment together with cross linkage [14]. Glucose isomerase offers an efficient means for its easy recovery and reuse, and lowers the cost. X-ray crystallographic and genetic engineering reports support a hydride shift mechanism for the action of glucose isomerase. Glucose isomerase cloning in homologous as well as heterologous hosts has been performed, with the main aim of overproducing the enzyme and interpreting the genetic makeup of individual genes (xylA, xylB and xylR) in the xyl operon of different micro-organisms [14]. Using a low-cost inducer in the fermentation medium devoid of Co^{2+} and redesigning a tailor-made glucose isomerase with improved thermostability, greater affinity for glucose and lower optimum pH will contribute considerably to the growth of an economically viable industrial process for enzymatic isomerization of glucose to fructose [14]. Various glucose isomerase sources and immobilization techniques are listed in table 3.3.

Different varieties of reactor have been compared and it has been reported that continuous fixed-bed reactors are the most suitable [14]. The isomerization is in a pH range of 7.0–8.5 at 60–65 °C. The substrate solution contains 40–50% glucose and oligosaccharide and has a viscosity of 0.8–3 mPa s. A magnesium salt concentration 0.5–5 mM l^{-1} is supplemented to activate and stabilize the enzyme. Alkali-catalyzed transformation of fructose into mannose and psicose can be averted by decreasing

Table 3.3. Glucose isomerase sources and immobilization techniques.

Name of organism	Immobilization technique	Final form
Streptomyces olivaceus	Whole cell; cross-linked with glutaraldehyde	Granules
Streptomyces olivochromogenes	Aluminum oxide support	Powder
Arthrobacter	Immobilized in the cells with a flocculating agent	Cylindrical particles
Actinoplanes missouriensis	Mycelium encapsulated in gelatin cross-linked with glutaraldehyde	Microspheres

the pH of the product solution. The purification of fructose syrup is achived by treatment with activated charcoal and using ion exchangers. The fructose syrup is concentrated to 71% dry matter. The procedure for the production of fructose syrup is represented in figure 3.3.

3.7 Penicillinase

Penicillinases are produced on the inner surface of the bacterial cell membrane and perhaps pass through the membrane to the external surface while they are being synthesized [15]. Generally, a gram-positive bacterium produces an ample amount of penicillinase and the majority is released into the surrounding medium. This is called exopencillinase. On the other hand, gram-negative bacteria synthesize β-lactamase in smaller amounts. The production and processing of penicillinase by the gram-positive organism *Bacillus licheniformis* has been investigated. When the bacterium is exposed to inducers (e.g. penicillin), there is a sudden rise in penicinillase production [15]. This process is accompanied by the development of many vesicles with high enzymatic activity. There are considerable differences in the way that β-lactamases protect gram-positive and gram-negative bacteria from the β-lactam antibiotic. Generally, β-lactamase production in a gram-positive organism

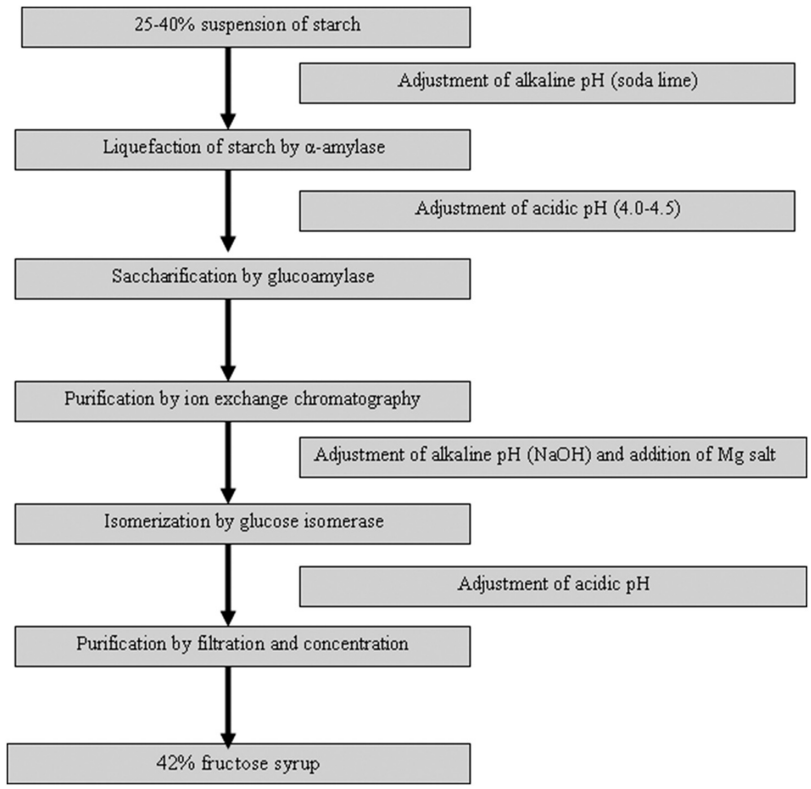

Figure 3.3. Flow diagram for the production of fructose syrup.

is inducible and a large quantity of penicillinase is released into the surrounding medium [15].

Because of its inactivation property against penicillin and certain cephalosporins, penicillinase (a bacterial enzyme) has gained wide attention. Several other hydrolytic enzymes which are also capable of inactivating penicillin have been explored, and a number of penicillinase producing pathogenic bacteria have been explored, e.g. staphylococcus species and some gram-negative bacteria of intestinal origin. This was first reported when some species showed resistance against penicillin. In particular, it was evidenced among patients infected by *Staphylococcus aureus*. Many common genera of fungi, such as *Penicillium*, *Aspergillus* and *Mucor*, synthesize the enzyme penicillin amidase [16–18]. Based on their activity penicillinase can be divided into two major classes:

- p-lactamase or penicillinase, and
- penicillin amidase or penicillin acylase.

Penicillinase reverses the therapeutic potential of penicillins. The activity of the enzyme penicillin amidase is based on a specific attack on the acyl group attached to the basic nucleus, i.e., 6-aminopenicillanic acid [16–18], therefore, it is also called penicillin acylase. Penicillin acylase specifically acts on the enzyme which is more specific to penicillin V and K. p-lactamase acts specifically on the basic nucleus itself, i.e., by cleaving the β-lactam bond and finally generating penicilloic acid. p-lactamase is more specific against penicillin G and X, and to a lesser extent against penicillin V [16–18].

As discussed earlier, certain bacteria have the capability to synthesize penicillinase in constitutents, whereas in others it may be inducible. The final effects can be established with the following case. If the antibiotic concentration is found to be non-lethal in a penicillin-treated patient, at that point, it can encourage the formation of penicillinase after which the bacteria becomes resistant to the antibiotic. A similar example is also reported when the penicillinase synthesizing bacteria are greater in number in comparison to the amount of antibiotic present in a certain time period. Under these conditions, although the bacteria lesser numbers are vulnerable to penicillin, they are resistant when present in greater numbers, as the concentration of penicillinase released by each bacterium adds up to an amount which can inactivate the antibiotic. In several clinically resistant strains of bacterium, the synthesis of β-lactamase is regulated by extra-chromosomal genetic elements. There are a number of notable differences between the enzymes synthesized by gram-positive and gram-negative bacteria. Considering molecular weight as a factor of comparison, gram-negative bacterium enzymes are generally small in molecular weight (22 000 Da), whereas gram-positive bacterium enzymes are large in molecular weight (28 000 Da) [19]. The concentration of β-lactamase synthesized by gram-positive bacteria is greater and, since the antibiotic is more specific against gram-positive bacteria than gram-negative bacteria, the enzyme β-lactamase is more sensitive against penicillin when synthesized by gram-positive bacteria. This is because the concentration of enzyme synthesized by gram-negative bacteria is far less and they do not secrete the enzyme into the external environment.

This can also be linked in an alternative way, i.e., as there is no β-lactamase secreted into the external environment, there is no dilution (loss) of β-lactamase in gram-negative bacteria, and therefore the need for production of β-lactamase by the cells is quenched.

3.8 Chloramphenicol acetyltransferase

Chloramphenicol has been widely used to treat typhoid fever since 1948 and is the drug of choice in the treatment of typhoid fever and certain infections caused by organisms such as *Haemophilus influenza* [20]. A major typhoid epidemic was first countered by chloramphenicol in Mexico [22]. During this period certain strains of the gram-negative bacterium *Salmonella typhi* came to light. It was later reported that the chloramphenicol resistance facilitated by R-factors in gram-negative bacteria and by transducible plasmids in *Staphylococcus aureus* is due to the occurrence of the enzyme chloramphenicol acetyltransferase (CAT), which acetylates the hydroxyl groups of the side chain of the chloramphenicol (see figure 3.4) [21].

The product derived from the two-stage inactivation process involving chloramphenicol acetyltransferase is 1,3-dicetoxychloramphenicol (which is inactive) [23]. Chloramphenicol acetyltransferase production in gram-positive bacteria is stimulated by chloramphenicol. Chloramphenicol acts as a protein synthesis inhibitor, thus producing an initial lag period in which the antibiotic prevents protein synthesis with simultaneous induction of chloramphenicol acetyltransferase. Therefore, an initial low amount of chloramphenicol acetyltransferase does not affect the antibiotic, however, at a later phase, it brings down the inhibitory level, i.e., when the amount of chloramphenicol acetyltransferase is high enough to break the drug moiety. As mentioned earlier for penicillinase, there exists a difference between enzymes released by gram-positive bacteria and gram-negative bacteria. However, in this case there is no such substantial difference. The molecular weight of chloramphenicol acetyltransferase produced by both gram-positive and -negative cells is almost the same (80 000 Da).

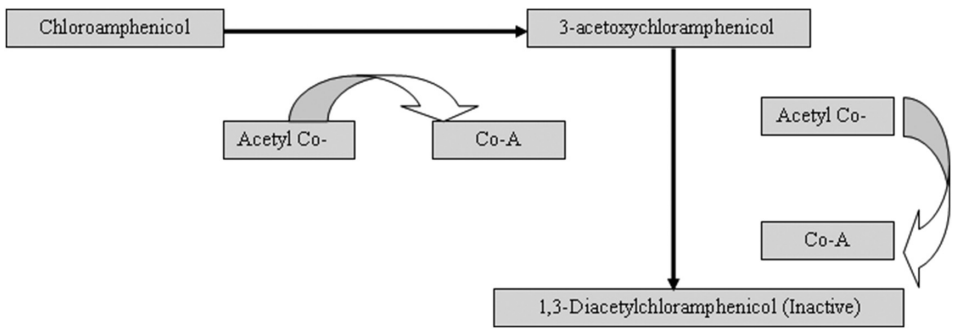

Figure 3.4. The two-stage chloramphenicol inactivation reaction involving chloramphenicol acetyltransferase.

3.9 Aminoglycoside antibiotic inactivating enzymes

Aminoglycoside antibiotics are also susceptible to inactivation. Different strains of *Streptomyces* have aminoglycoside-modifying enzymes. *Streptomyces kanamyceticus* synthesizes an enzyme that acetylates the 6′-amino group of kanamycin A and B, gentamicin C(1a) and neomycin. *Streptomyces spectabilis* synthesizes an enzyme that acetylates the 2′-amino group of the hexose ring of gentamicin C(1a). These enzymes catalyze reactions similar to those catalyzed by enzymes present in gram-negative bacteria having R(antibiotic resistance)-factors. The breakthrough of these enzymes proposes the likelihood of an evolutionary relationship between the aminoglycoside inactivating enzymes (synthesized by resistance determinants) in bacteria containing R-factors and similar enzymes found in the actinomycetes.

Despite the range of enzymes presenting full or partial resistance to antibiotics, there are only three types of inactivation reactions:

- N-acetylation of susceptible amino groups.
- Adenylation of hydroxyl groups. Phosphorylation of hydroxyl groups. Table 3.4 shows different enzymes that are accountable for the inactivation of aminoglycoside antibiotics.

Acetylation of the drug is performed by the co-factor, acetyl-CoA which serves as the source of the acetyl group, while the source of the phosphoryl and adenyl groups is adenosine triphosphate (ATP). Inactivation occurs in the periplasmic space (periplasm) or on the external surface of the cytoplasmic membrane. Intracellular accumulation of active aminoglycosides is determined by the induction of an energy-dependent process. The modified aminoglycoside is unable to stimulate intracellular accumulation after inactivation of the aminoglycosides by the enzymes. If somehow they succeed in entering a cell, they are incapable of restricting ribosomal function.

3.10 Fibrinolytic enzymes

Conversion of fibrinogen to fibrin inside blood vessels leads to thrombosis, resulting in myocardial infarction and other cardiovascular diseases. Generally, there are four therapy options: surgical operation or the intake of antiplatelets, anticoagulants or

Table 3.4. Different aminoglycoside antibiotic inactivating enzymes.

Enzymes	Substrates (aminoglycoside antibiotics)
Streptomycin/spectinomycin adenyltransferases	Streptomycin, spectinomycin
Streptomycin phosphotransferase	Streptomycin
Lividomycin phosphotransferase	Neomycin, paromomycin
Kanamycin/neomycin phosphotransferases I and II	Kanamycins, neomycins, gentamicin A
Kanamycin acetyltransferase	Kanamycin A and B, neomycin, gentamicin
Gentamicin adenyltransferace	Kanamycins, gentamicins
Gentamicin acetyltransferases I and II	Gentamicin

fibrinolytic enzymes [15]. Microbe-derived fibrinolytic enzymes have been gaining much attention compared to traditional thrombolytic agents because the latter are expensive and have side effects. Fibrinolytic enzymes have been successively derived from various micro-organisms, the most significant among which is the genus *Bacillus* [15]. Microbial fibrinolytic enzymes, particularly those from food-grade micro-organisms, have the capability to be established as functional food additives and drugs to avert or cure thrombosis and other associated diseases. There are numerous assay methods for these enzymes; this might be due to the insolubility of the substrate, fibrin. Current assay approaches can be categorized into three main groups:

- The first group involves the assay of fibrinolytic activity with natural proteins as substrates, such as fibrin plate methods.
- The second and third groups of assays are appropriate for kinetic investigations and are based on the determination of hydrolysis of synthetic peptide esters.

The fibrinolytic enzyme dissolves intravascular clots due to the action of plasmin (enzyme that digests fibrin) [15]. Plasminogen (the inactive precursor) is converted to plasmin by breakdown of a single peptide bond. For this reason, infusion of two types of enzymes can be considered, those with direct and general proteolytic activity, such as plasmin or trypsin, and those such as the plasminogen activators which increase the level of native plasmin [15]. In this respect, lyokinase plasminogen activators, such as streptokinase urokinase, and tissue plasminogen activators (t-PA) have gained more attention.

3.10.1 Streptokinase

Streptokinase is a proteolytic enzyme (47-Kda protein) derived from the culture of β-hemolytic streptococci of Lancefield group C. It is a potent activator of the fibrinolytic enzyme system in humans [26]. The protein molecule has mol. wt. 47 600 Da and an isoelectric point of pH 4.7. The thrombolytic capability of this enzyme catalyzes the transformation of human plasminogen to plasmin by hydrolytic cleavage of the Arg–Val bond. The enzymatic action of streptokinase is based upon a 1:1 noncovalent complex with a zymogen from which the active plasmin is released [26]. This protein complex can be dissociated into two parts, one with plasmin activity and the other with plasminogen activity. Figure 3.5 depicts a positive feedback mechanism, in which complexation first occurs between streptokinase and the plasmin contaminant of plasminogen. This complex is later free to convert a second plasminogen molecule [26].

As mentioned above, streptokinase can be utilized in the management of thromboembolic occlusive vascular disease. The major reasons behind acute arterial occlusion are embolism, thrombosis and trauma. The objective of thrombolytic treatment is to restore blood flow and to preserve life and limb. Antithrombotic therapy during chronic disease is considered to avoid the progression and development of thrombotic occlusion, and also to avoid thrombotic problems after vascular

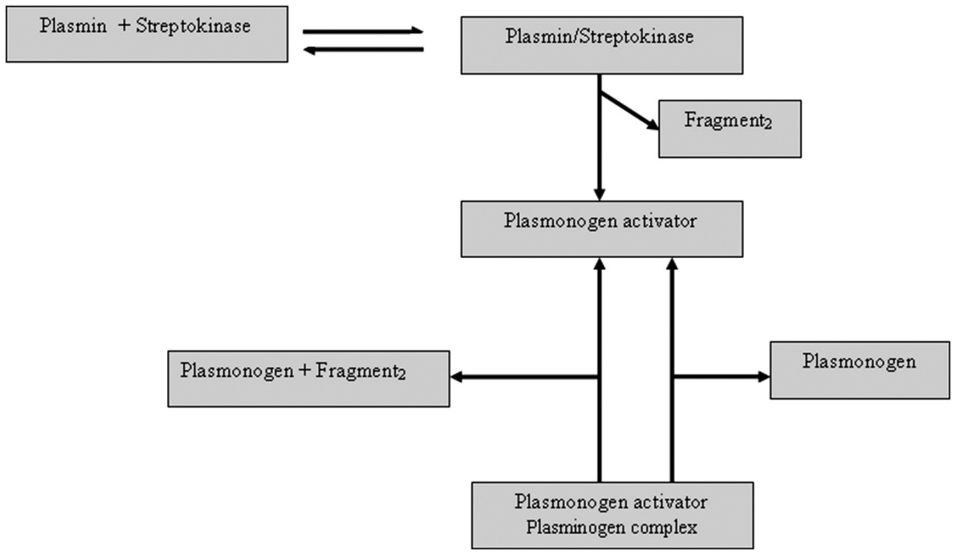

Figure 3.5. Proposed feedback mechanism attributed to streptokinase.

reconstructions. During acute arterial occlusion in the form of embolism or thrombosis, active anticoagulant treatment will avoid the spread of thrombi and also avoid re-occlusion after surgical or interventional procedures to re-establish flow; or, in the case of embolism, may prevent recurrence. In this thrombolytic treatment of occlusive vascular disease, both circulating and intrinsic thrombus plasminogen are activated. This is usually done by forming an activator complex (plasmin, as well as plasminogen, can form an activator complex with streptokinase) with the plasminogen molecule itself. Simultaneously, streptokinase can also encourage coagulation defects (depending on the dosage used) due to the induction of a hyperplasminaemic state [26]. So far the role of streptokinase therapy in the management of occlusive vascular disease is not clear. However, as per a recent report, streptokinase-based treatment can be utilized in acute massive or major pulmonary embolism, arterial occlusion and in deep-vein thrombosis (iliofemoral) and peripheral venous segments [26]. Based on a recent report it was suggested that zinc chelation (Ca-EDTA) can improve the efficacy of streptokinase in thrombolysis. Thus zinc reduced streptokinase-induced thrombolysis and zinc chelation can promote the effects of streptokinase-induced thrombolysis [30]. It was also observed that PEGylation of truncated streptokinase resulted in a formulation with increased *in vivo* half-life, reduced immune-reactivity and clot-specificity [31]. Additionally, streptokinase has also been employed in the management of chronic arterial occlusions and acute myocardial infarction. Streptokinase can also be utilized in the management of several other diseases associated with thromboembolic occurrences, however, more supporting research is essential before these conditions become definite indications for streptokinase treatment. Streptokinase-based thrombolytic treatment differs significantly with certain conditions. It has been observed that newly formed thrombi can be more easily broken down than old ones.

Similarly, venous thrombi can be more easily broken down than arterial thrombi. The action of streptokinase is also dependent on the degree of occlusion and the amount of blood supply in that particular region. Similarly to other thrombolytic agents, streptokinase also faces problems related to bleeding from sites of vascular puncture [26]. Another challenge is the development of antibodies after foreign protein (streptokinase) administration, which limits the course of treatment to 3–5 days. There should be two years between repeated administrations of streptokinase. Reports have also confirmed its cross reaction with anti-streptococcal antibodies. This may result in the development of resistance against therapy. However, this can be overcome by the administration of booster doses.

Streptokinase is superior in artificially dissolving induced coronary thrombi and pulmonary embolisms. The power of streptokinase over heparin in decreasing mortality in myocardial infection has also been well recognized. A streptokinase–plasminogen complex (antistreplase) in which lys-plasminogen is acylated at its catalytic site, serine is also used for coronary thrombolysis. Under *in vivo* conditions, the acyl group is allowing complex to bind to fibrin before activation, and this change confers some specificity towards clots during the fibrinolytic process. Owing to its bacterial origin, streptokinase is antigenic and may bind to antibodies and nonspecific inhibitors. The immune response presented by streptokinase affects the modification of urokinase.

3.10.2 Urokinase

Urokinase (molecular weight 54 500 Da) is a serine protease comprising 411 amino acid residues, synthesized and isolated from cultured human kidney cells [27]. This enzyme is single polypeptide and very stable against temperatures and extremes of pH [27]. Urokinase efficiently digests blood clots. Human urokinases often act as plasminogen activators by specifically binding to cleave peptide bonds adjacent to lysine and arginine side chains. As it is derived from humans, it is non-immunogenic and its action is very similar to that of trypsin, catalyzing the activation of plasminogen in a first order reaction with cleavage of the Arg–Val bond [27]. The current focus on the management of mortality due to thrombovascular disorders is providing openings for various cardiovascular agents, particularly plasminogen activators, to avoid strokes and heart attacks [27]. Therefore, urokinase, one of the most potent plasminogen activators, is gaining a great deal of attention. Progress in cell lines and bioprocessing technique has made it conceivable to synthesize urokinase from *in vitro* cell cultures. Efforts are now underway to increase urokinase synthesis from cell culture through medium optimization, bioreactor cultivation and advanced purification methods [27].

3.10.3 Tissue plasminogen activator (t-PA)

t-PA (527 amino acid residues) is a protein involved in the breakdown of blood clots. This enzyme belongs to the serine protease class and binds to fibrin via lysine binding sites at its amino terminus, and further activates bound plasminogens several hundred fold more quickly than it activates plasminogen in circulation.

In the absence of fibrin it acts as a poor plasminogen activator. Under biological conditions (5–10 ng ml^{-1}), the specificity of t-PA for fibrin restricts the systemic development of plasmin and the introduction of a systemic lytic state. It can successfully break down thrombi during treatment of acute myocardial infarction. t-PA is produced by recombinant DNA technology. Currently it is recommended for coronary thrombolysis. The major side effect is severe hemorrhage. The cost of t-PA is high, several times more than streptokinase per therapeutic dose.

The production of this drug in plants such as cucumber, one of the more common vegetables in the world, could reduce its production costs. In a study it was concluded that the presence of three expressions of regulatory factors (CaMV 35S, Kozak, NOS) and the KDEL signal in the construct caused the increase of t-PA gene expression in cucumber plants [28]. In another study a protease-deficient strain of *Aspergillus niger* was used as a host for the production of human tissue plasminogen activator (t-PA). Production was increased (up to 1.9 mg t-PA (g biomass)(−1)) by the addition of soy peptone to the defined medium [29]. However, the total t-PA (detected by enzyme-linked immunoassay) also eventually disappeared from culture supernatants, confirming significant extracellular proteolytic activity, even though the host strain was protease-deficient [29].

3.11 Biotechnological applications of enzymes

3.11.1 Algae and plant research

As mentioned above, there are various applications of enzymes in different disciplines, however, some of the applications are restricted to plant discipline. Recently, interest related with plant enzymes has augmented considerably. Plants are considered as the main source for secondary metabolites. Plant based enzymes such as peroxidases are extensively used in medicine as diagnostic tools and in the bioremediation and biobleaching industries, among others. Earlier these enzymes were derived from a natural source, a process that is sometimes difficult and influenced by environmental conditions and low yields. To prevent this obstacle, some inputs have been made to develop plant cell cultures *in vitro* to use the system as a continuous source of plant enzymes.

3.11.2 Immobilization

Different types of carriers and procedures have been implemented in the recent past to improve traditional enzyme immobilization targeted to increase enzyme loading, activity and stability to reduce the enzyme biocatalyst cost at large scale [32–42]. These include:

- recently nanoparticle-based immobilization of enzymes;
- microwave-assisted immobilization;
- mesoporous supports;
- cross-linked enzyme aggregates;
- click chemistry technology.

In nanotechnology method, mixture of the specific physical, chemical, optical and electrical properties of nanoparticles, especially catalytic properties of biomolecules, has resulted in the appearance of countless novel biotechnological applications [42–49]. Nanoparticles offer high surface-to-volume ratio resulting in an increase in the concentration of the immobilized entity that is considerably higher than that afforded by experimental protocols based on immobilization on planar 2-D surfaces. Enzymes immobilized on nanoparticles presented a broader working pH and temperature range and higher thermal stability than the native enzymes. In contrast with traditional procedures, nanoparticle based immobilization served three important features:

- nano-enzyme particles are easy to synthesize in high solid content without using surfactants and toxic reagents;
- homogeneous and well-defined core–shell nanoparticles with a thick enzyme shell can be obtained;
- particle size can be conveniently changed within utility limits. Moreover, with the growing attention paid to cascade enzymatic reaction and *in vitro* synthetic biology, it is possible that co-immobilization of multi-enzymes could be achieved on these nanoparticles.

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