Enzymes for pharmaceutical and therapeutic applications

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Abstract

Enzymes are highly efficient and selective biocatalysts, present in the living beings. They exist in enormous varieties in terms of the types of reactions catalyzed by them for instance oxidation–reduction, group transfers within the molecules or between the molecules, hydrolysis, isomerization, ligation, bond cleavage, and bond formation. Besides, enzyme based catalyses are performed with much higher fidelity, under mild reaction conditions and are highly efficient in terms of number of steps, giving them an edge over their chemical counter parts. The unique characteristics of enzymes makes them highly applicable fora number of chemical transformation reactions in pharmaceutical industries, such as group protection and deprotection, selective acylation and deacylation, selective hydrolysis, deracemization, kinetic resolution of racemic mixtures, esterification, transesterification, and many others. In this review, an overview of the enzymes, their production and their applications in pharmaceutical syntheses and enzyme therapies are presented with diagrams, reaction schemes and table for easy understanding of the readers.
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Abbreviations: *3-ATfBA, (R)-3-amino-4-(2,4,5-triflurophenyl) butanoic acid; 6APA, 6-amino penicillanic acid; 7-ACA, 7-aminocephalosporanic acid; ADA, adenosine aminohydrolase; CF, cystic fibrosis; CSID, congenital sucrose-isomaltase deficiency; DKR, dynamic kinetic resolution; FDH, format dehydrogenase; GDH, glucose dehydrogenase; HIV, human immunodeficiency virus; Kred, keto reductases; LeuDH, leucine dehydrogenase; MRSA, methicillin resistant* Staphylococcus aureus*; NHase, Nitrile Hydratase; PAL, phenylalanine ammonia lyase; PGA, penicillin G amidohydrolase; PKU, phenylketonuria; SCID, Severe Combined Immunodeficiency Disease; SmF, submerged fermentation; SSF, solid-state fermentation.*

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1. Introduction

Enzymes are the functional proteins or nucleic acids (Ribozymes), also known as biocatalysts that facilitate the execution of biochemical reactions at the rates which are suitable for the normal functioning, growth, and proliferation of any living system, including unicellular or multicellular plants as well as animals [1–3]. The ability of enzymes to remain viable and perform catalytic activities even outside their source organism, that is, under *in situ* conditions [4] allows them to be exploited for carrying out a number of industrial processes that rely on chemical transformations of substrates to their corresponding products. The reactions catalyzed by enzymes are highly efficient, that is, they occur under ambient environmental conditions, that is, temperature, pH, and pressure (the conditions depend on the physiological conditions of the source organisms and its environmental conditions). For instance, an enzyme obtained from a mesophilic organism (optimal growth temperature is 37 °C and growth temperature range from 20 to 40 °C), inhabiting neutral environments, shall work efficiently at mild temperatures, neutral pH, and atmospheric pressure. Enzymes may be used as isolated free forms or immobilized forms (as whole cells or isolated enzyme immobilized on a suitable support) [5, 6]. As enzymes are functional outside the cells (or organisms), they are used in a number of industrial applications such as synthesis of pharmaceuticals such as drugs, process grain juices into lager and wine, leaven dough for bread production, production of agrochemicals, artificial flavors, biopolymers, waste remediation, and many others [7].

Majority of the industrial enzymes come from microorganisms as they are the most convenient sources, which gives the propensity of faster production, easy scale up, recovery and purification, strain manipulation for over-expression, enzyme activity, specificity modulations, and so on [8]. At present, around 200 types of microbial enzymes from 4,000 known enzymes are used commercially [9]. Nearly 75% of the total enzymes are produced by three top enzyme companies, that is, Denmark-based Novozymes, US-based DuPont, and Switzerland-based Roche [10].

According to the report "Industrial Enzymes Market: Growth, Trends and Forecast (2019–2024)" from Mordor Intelligence, the Global industrial enzymes market is expected to grow at a compound annual growth rate of 6.8% during the forecast period of 2019–2024 [11]. Some of the leading international players in the Industrial enzyme market are El du Pont de Nemours and Company, Novus International Inc., Associated British Foods (ABF), Koninklijke DSMNV, and AB Enzymes GmbH [12]. Insights related to the market forecast of the most widely used enzymes like proteases, carbohydrases, and lipases and their market for pharmaceutical and healthcare sectors is presented here for the reference of the readers. According to the Industry Report of Global Market Insights [13], the global market for proteases shall be around USD 2 billion by 2024. The main growth in proteases' market shall be in the health industry due to many benefits offered by them, such as, boosting the immune system, preventing inflammatory bowel diseases, and curing skin burns and stomach ulcers. However, other sectors like animal feed segment, where protease are used for improving the nutritional or digestive properties of fodder and upkeep of animal gut health shall hold a great share of protease market. Carbohydrases crossed USD 2.5 billion in 2016 and are expected to show a good increase of more than 1/3 over the existing figure by 2024. The prominent application sectors shall be in food, beverage, and pharmaceutical industry. Lipases are expected to achieve the increase in sale by around 6.8% by 2024. The main area of lipase market shall be in the field of healthcare for the treatment of obesity, which is becoming an emerging issue in developed countries. As lipase break down fats into glycerol and fatty acids under natural conditions, its demand is expected to rise in healthcare industry as an aid for weight control and management in obese people [13].

Highlights

1. Enzymes are highly selective biocatalysts, catalyzing varieties of reactions such oxidation–reduction, group transfers within the molecules or between the molecules, hydrolysis, isomerization, ligation, bond cleavage, and bond formation.
2. Enzymes are applicable in pharmaceutical industries, for group protection and deprotection, selective acylation and deacylation, selective hydrolysis, deracemization, kinetic resolution of racemic mixtures, esterification, transesterification, and many other reactions.
3. In this review, an overview of the enzymes' production and applications in pharmaceutical syntheses and enzyme therapies has been discussed.

Several drugs or pharmaceutical formulations are comprised of active pharmaceutical ingredients (APIs) that are synthesized using enzymes as important components of the manufacturing process [2,14]. The usage of biocatalysts for pharmaceuticals' production as well as rising interest in the production of chiral intermediates and green synthetic processes has substantiated the interest in the applications of biocatalysts in these fields [15]. These have been possible due to improvements in the technologies for biocatalyst selection, screening, improvement, production, and supply. Although the applications of enzymes in pharmaceuticals may be vast, the aim of this review is to focus on the prospects of biocatalysts in the manufacturing of APIs, as aids for the production of health supplements, and as enzyme therapy for treatment of a number of diseases.

2. Production of Pharmaceutical Enzymes

Most of the industrial enzymes including those used in pharmaceutical industries are produced through fermentation of

suitable microbial strains mainly belonging to bacteria and fungi due to their easy handling, fast growth rates, and convenient scale up in large vessels (fermenters) [16–18]. Bacteria like *Escherichia coli*, *Bacillus subtilis*, lactic acid bacteria, and the filamentous fungi such as *Aspergillus oryzae, Aspergillus niger, Trichoderma atroviride*, and Yeasts, for example, *Saccharomyces cerevisiae, Pichia pastoris*, and so on are the most exploited microorganisms for enzyme production by the biotechnology industries over the world [8, 18]. As the strains have been improved through genetic engineering, they produce enzymes in very high yields [8,19]. Selection of suitable microbial strains for the production of various industrial enzymes is a very important aspect for their successful industrial applications. Ideally, the enzymes produced should be secreted out in the fermentation medium by the producing microbial strain as it makes the downstream processing more convenient and economically feasible; however, this may not be the case with most of the industrial strains. Pharmaceutical enzymes are produced by employing the fermentation technology, mainly using the microorganisms (bacteria and fungi), which comes under the Generally Recognized as Safe category [8,19]. This production is mainly carried out employing two processes, that is, submerged fermentation (SmF) and solid-state fermentation (SSF) [20]. Both the processes have their own benefits and limitations. Most industries have adopted SmF process for enzyme production; but there has been a renewed interest in SSF for certain specific industries [18, 21].

3. Enzyme Production Through SmF

Submerged fermentation, as the name suggests, is carried out in a liquid medium, wherein the nutrients and other medium components are either dissolved or remain suspended in an aqueous medium. The microorganisms also grow and proliferate in the liquid fermentation medium in the suspended state. Generally, aerobic microorganisms are grown in submerged cultures in a stirred-tank reactor for industrial production processes for extracellular enzymes and even for the intracellularly overexpressed enzymes as in the case of recombinant *E. coli* and other bacterial and yeast strains [8]. The fermenters for SmF applications have been developed successfully and are being used for the production of enzymes and other biotechnological products [22, 23]. The main reason behind the success of SmF is feasibility of process scale up from pilot scale fermenters (~100 L) to production scale fermenters having capacities of millions of liters (vessel volumes) with well-developed offline and/or online control over several parameters such as, temperature, dissolved oxygen, pH, and foam formation. Further, there is no problem of mass transfer and heat dissipation; however, it is generally achieved at the cost of higher energy consumption [18, 23].

Submerged fermentation for industrial production processes are manly carried out in four ways, namely batch culture, continuous culture, perfusion culture, and fed batch culture. In batch culture, the production strains are inoculated in a fixed volume of medium and thereafter no further addition of medium is carried out during the entire fermentation process (except the addition of acid, alkali, and antifoam). In continuous culture, the fermentation process is initiated in the batch mode. However, after certain time period fresh medium or nutrient concentrate is added into the fermenter at a rate which approximately matches the growth rate of the microorganism used and simultaneously the medium containing the product and biomass is continuously withdrawn from the overflow line of the vessel [24, 25]. A perfusion culture is somehow similar to a continuous culture process, in that it also involves the constant feeding of fresh media and removal of spent media and product. However, it differs from the previous in retention of high numbers of viable cells by using alternating tangential-flow and standard tangential-flow filtration or by binding the cells to a substrate (capillary fibers, membranes, microcarriers in fixed bed, and so on) in the fermenter [26]. In the case of fed batch culture, the concentrated components of the nutrients are added to the batch culture in small lots at regular time points or based on the requirement of the process [24, 25].

4. Enzyme Production Through Solid-State Fermentation

Solid-state fermentation has a great potential for enzyme production [18]. This process offers several advantages over the SmF process, such as high product titer, lesser effluent generation, use of simple fermentation equipment, less trained labor, and so on [27]. However, SSF is in general more suitable for those processes where the crude fermented products themselves are used as the final product rather than the isolated enzymes.

Agroindustrial residues are commonly used substrates for the SSF processes, including those used to produce enzymes. A variety of substrates have been used for the cultivation of enzyme producing microorganisms. Examples of the substrates used are wheat bran, rice bran, sugar cane bagasse, wheat straw, rice straw, saw dust, corncobs, banana waste, cassava waste, palm oil mill waste, oil cakes, and so on [18, 28].

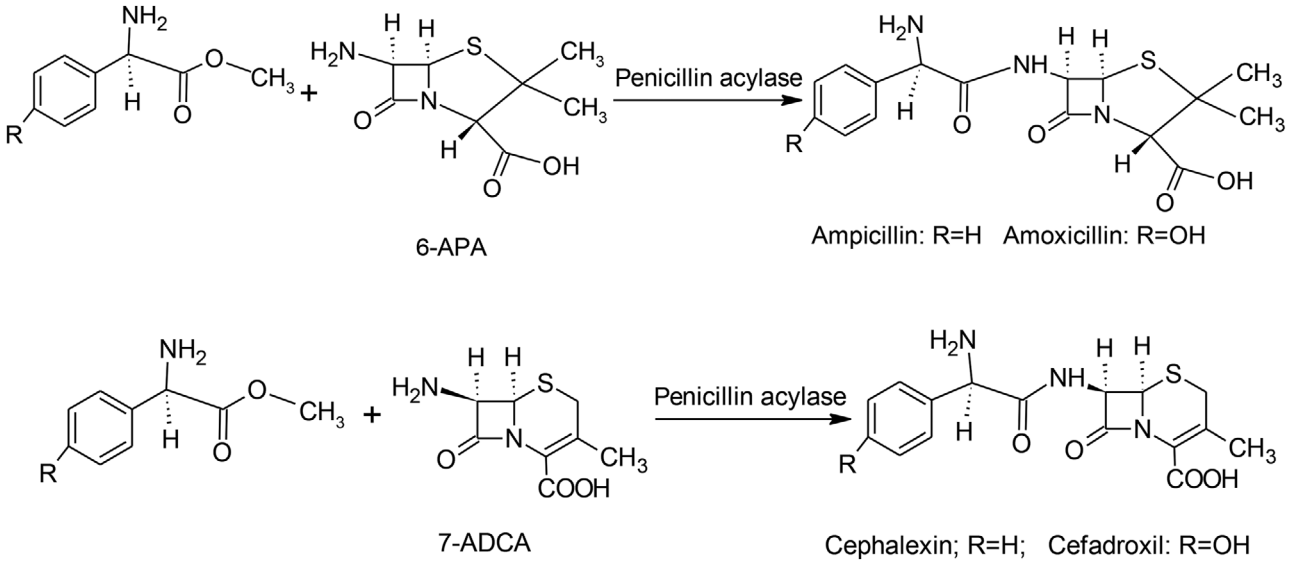
5. Applications of Enzymes in Pharmaceutical Manufacturing

5.1. Enzymes for the synthesis of antimicrobials

5.1.1. Synthesis of 6-amino penicillanic acid by penicillin acylases

Penicillin acylases are a group of enzymes that cleave the acyl chain of penicillins to yield 6-amino penicillanic acid (6-APA) and the corresponding organic acid [29]. Screening of penicillin acylase-producing microorganisms has shown that these enzymes are produced by bacteria, actinomycetes, yeasts, and fungi [30–32].

Based on their substrate specificity, penicillin acylases have been classified into three groups: penicillin G acylases, penicillin



SCHEME 1 *Penicillin acylase catalyzed synthesis of some important semi-synthetic β-lactam antibiotics (adopted from Ref. [43])*

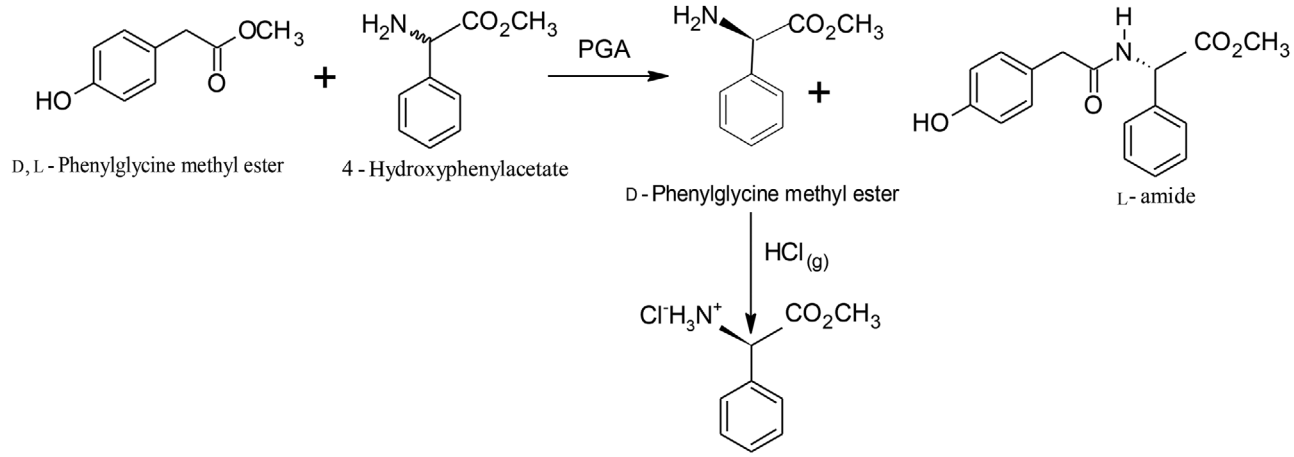
V acylases, and ampicillin acylases. Penicillin acylase-catalyzed synthesis of 6-APA has replaced the traditional chemical processes used for its synthesis. The conventional chemical method of 6-APA synthesis, started in 1970s, was dependent on a one-pot diacylation reaction of penicillin G (produced through fermentation), using hazardous reagents and solvents. This process of production continued for next 15–20 years and thereafter it was replaced substantially by penicillin G acylase-based method, as the enzymatic method allowed the production of 6-APA in good yields. Some researchers proposed an alternative process for the synthesis of 6-APA, based on penicillin V acylase and penicillin V [33, 34]. The advantage of this process relied on the fact that the penicillin V exhibited higher stability at lower pH during the extraction process from the fermented broth, which led to higher yield of 6-APA. In addition, penicillin V acylases-based processes afforded 6-APA production even at higher substrate concentrations compared with penicillin G acylases. Further, their wider optimal pH range alleviated the need of pH regulation during hydrolysis [32, 35]. Enzyme-based production of 6-APA is economically viable with immobilized forms of the enzymes. It results in great cost savings in terms of easy separation of the enzyme from the product, reuse of the enzyme and enhanced stability. Penicillin acylases from *Bacillus megaterium, E. coli*, and *Alcaligenes faecalis* have been immobilized for efficient application [36–41].

5.1.2. Synthesis of semisynthetic penicillins by penicillin acylases

Semisynthetic penicillins have been shown to have better properties than penicillin G and V, such as higher stability, easier absorption and lesser side effects and are also better candidates against the adaptive microbial resistance to antibiotics [42]. Large-scale production of semisynthetic antibiotics that are derived from penicillin are based on the condensation of the β-lactam nucleus with appropriate D-amino acid catalyzed by penicillin acylases (Scheme 1) [43–46]. Another application of penicillin G acylase is kinetic enantioselective acylation of the racemic azetidinone intermediate for the synthesis of the carbacephalosporin antibiotic Loracarbef, an analogue of Cefaclor and anti-platelet agent Xemilofiban [47, 48]. Penicillin G acylase has also been reported for enantioselective acylation of the L-enantiomers of methyl esters of phenylglycine and 4-hydroxyphenylglycine in organic solvents (Scheme 2) [49]. The process resulted in easy isolation of the enantiomerically pure D-enantiomer, which is of practical use in the preparation of β-lactam antibiotics.

5.1.3. Synthesis of β-lactam antibiotic key intermediate, 7-aminocephalosporanic acid

The cephalosporin-derived antibiotics are regarded as one of the most efficient and key drugs in the treatment of bacterial infections/diseases and presently a broad range of drugs have been derived from hetero-cyclic key moiety of the cephalosporins [50, 51]. Several cephalosporin-based semisynthetic β-lactam antibiotics have been commercialized such as cefotaxime, ceftriaxone, cefuroxime, and cefdinir. The lately developed ceftobiprol and ceftaroline fosamil are capable of treating the severe infections caused by MRSA (methicillin resistant *Staphylococcus aureus*). Cephalosporin C is the most feasible starting material for the synthesis of 7-aminocephalosporanic acid (7-ACA), as it is available in the desirable quantities at affordable cost, obtained through microbial fermentation. Additionally, cephalosporin C is a direct precursor leading to 7-ACA by amide bond cleavage [52]. Enzymatic synthesis of 7-ACA involves a combined enzymatic and spontaneous reaction cascade, which consists of a D-amino acid oxidase-catalyzed oxidation of the amino acid side chain in to the corresponding α-keto acid. This is followed by a



SCHEME 2 *Penicillin G acylase catalyzed enantioselective acylation of the L-enantiomers of methyl esters of phenylglycine and 4-hydroxyphenylglycine (adopted from Ref. [49])*

spontaneous decarboxylation of the α-keto acid. Finally, the resulting intermediate is hydrolyzed by glutaryl-7-ACA hydrolase to produce the 7-ACA (Scheme 3).

5.2. Enzymes for the DKR of drugs

Enzymes are exceptional in performing enantioselective reactions because of their high chemoselectivity. Enantioselective enzymes act one of the enantiomers (either *R* or *S* form) and selectively transform it into the corresponding product, whereas the other enantiomer remains untouched. At this point, the product can readily be separated from the other enantiomeric form of the substrate. However, the conventional method for resolution of racemates (i.e., the enzymatic resolution of a racemic mixture of a compound like amino acids or any synthetic or natural biomolecule) results in 50% yield of the required form of the isomers [53]. To overcome this barrier, the dynamic kinetic resolution (DKR) principle has been used recently in a variety of enzymatic reactions. Yasukawa et al. [54] reported the production of optically pure α-amino acids via enzyme catalyzed DKR of α-aminonitriles. This process is performed in three sequential steps: (i) nonselective hydrolysis of racemic α-aminonitrile by a Nitrile Hydratase (NHase), (ii) breakdown of the resulting amide by a stereoselective amino acid amide hydrolase and (iii) simultaneous racemization of the α-amino acid amide by α-amino-ε-caprolactam racemase (Scheme 4). Many compounds were hydrolyzed using the combinations of these enzymes; the results were promising wherein both (R)- and (S)-amino acids were obtained in excellent yields and ee's (>99%). An investigation with *Burkholderiacepacia* lipase to catalyze the DKR of racemic α-aminonitrile, resulted in enantioselective production of acetylated amine with substantial yield of 87%–90% and ee's of 85%–88% [55] (Scheme 5).

In another study DKR of racemic mandelic acid, was catalyzed sequentially using three enzymes, that is, mandelate racemase, D-mandelate dehydrogenase, and L-amino acid dehydrogenase to yield the corresponding enantiopure α-amino acid L-phenylglycine, with high conversion and ee in excess of 97% [56] (Scheme 6).

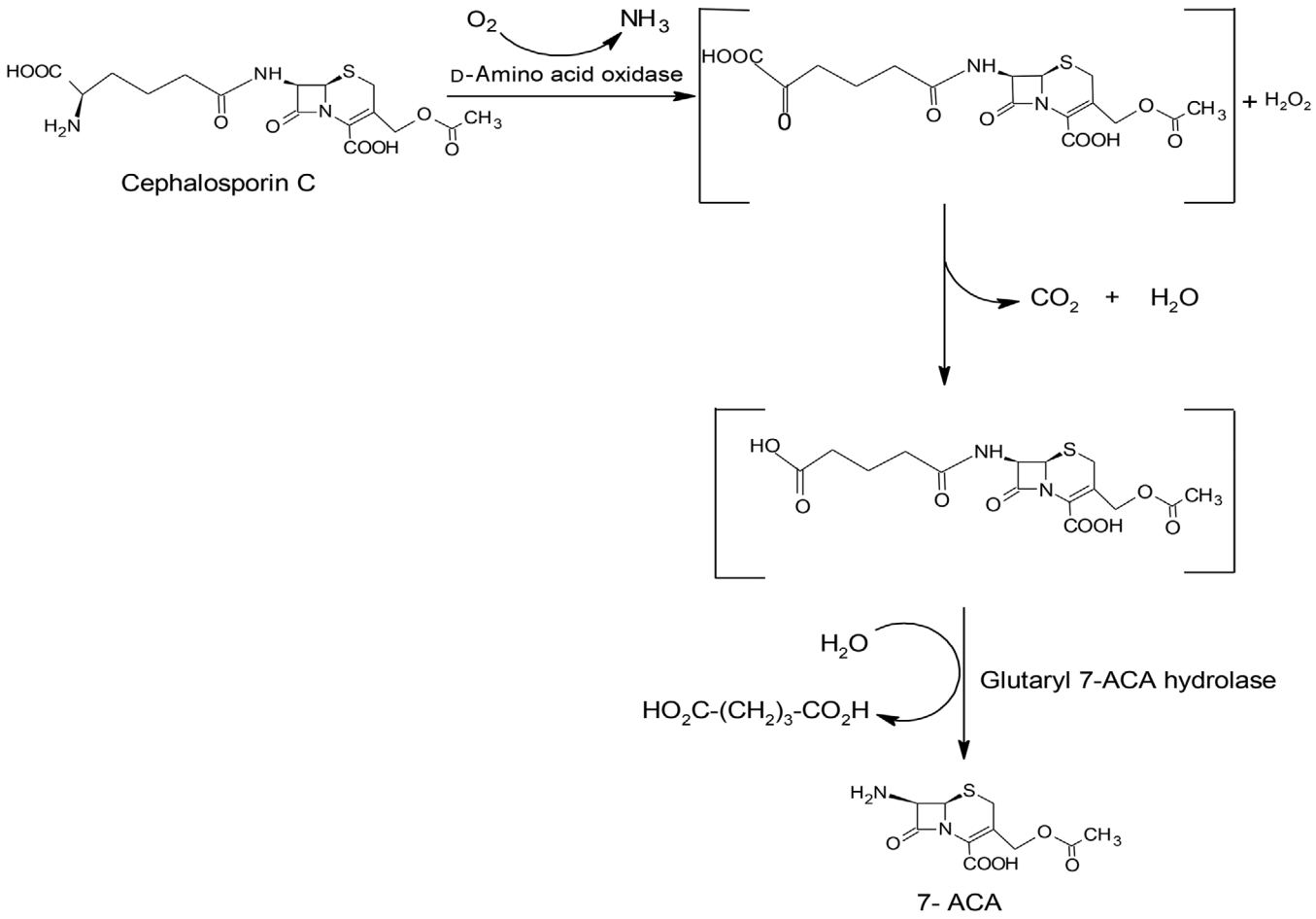
5.3. Enzymes for the synthesis of amino acids

Amino acids are building blocks of life and thus are in dispensable for proper health and nutrition of both humans and animals [57–59]. In addition, due to their chirality, they are biochemically very significant and are very useful in chemical synthesis [60,61]. The nine essential amino acids, that is, L-leucine, L-isoleucine, L-valine, L-lysine, L-histidine, L-methionine, L-phenylalanine, L-threonine, and L-tryptophan are not synthesized in animals and humans and therefore must be ingested with food. Enzymes and whole cell biocatalysts have been important in the production of proteinogenic and nonproteinogenic D- and L-amino acids, and enantiomerically pure amino acid derivatives, which are used as important building blocks for active ingredients that are used in the manufacture of pharmaceuticals, cosmetics, and agrochemicals [62]. The rapid expansion in the amino acid market from 1980s is mainly due to cost effective production and isolation of amino acid products [58]. Out of different methods of amino acids' production the two methods, that is, the fermentation, and enzymatic catalysis are predominant, owing to their cost effective and ecofriendly nature [63].

5.3.1. Enzymatic production of proteinogenic amino acids

Enzymes have been exploited from the last 40 years for the production of L-amino acids in Japan (Scheme 7) [58, 64, 65]. The production of L-methionine, which is used in special diets, has been carried out using enzymatic resolution with acylase of *Aspergillus oryzae* [66]. Several hundred tons of L-methionine and L-valine are now produced each year using enzyme membrane reactor technology to minimize the enzyme loss [66].

Another amino acid that is preferably obtained through enzyme catalysis is L-aspartic acid. The enzyme aspartase catalyzes the addition of amino group from ammonia to fumaric



SCHEME 3 *Enzymatic transformation of cephalosporin C into 7-ACA catalyzed by D-amino acid oxidase and glutaryl-7-ACA acylase (redrawn from Ref. [52])*

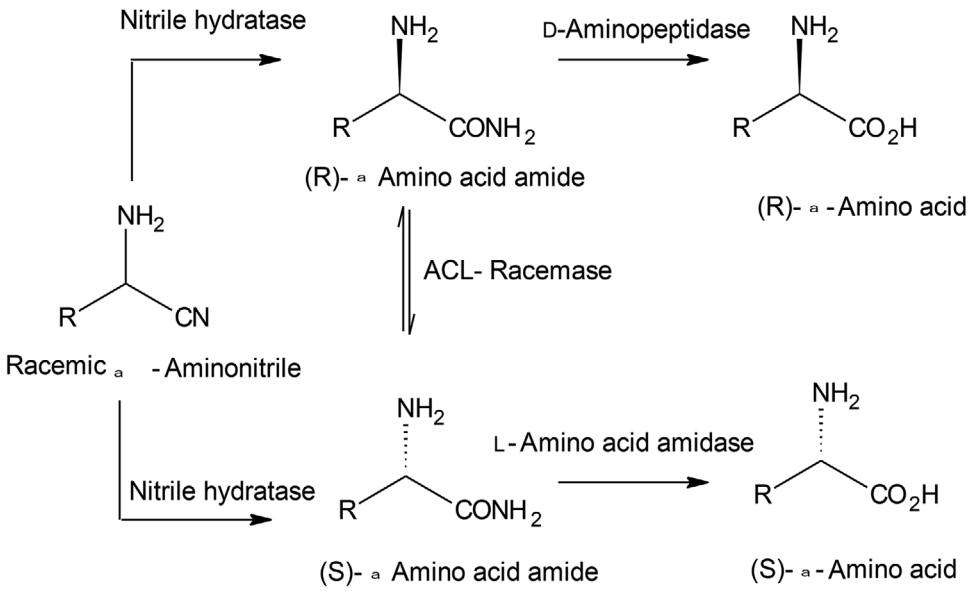
acid to directly produce the L-aspartate, which is used on industrial scale to produce the artificial sweetener L-aspartame. L-Alanine is produced from L-aspartate using aspartate β-decarboxylase enzyme [67, 68]. L-Cysteine has been produced from L-cystine mainly through electrochemical reduction process. However, now its industrial production is feasible through enzymatic hydrolysis and recemization of the substrate DL-2-amino-2-thiazoline-4-carboxylic acid (ATC) carried out by three enzymes, that is, L-ATC hydrolase, S-carbamoyl-L-cysteine hydrolase, and ATC racemase [69]. Recently, substantial production of L-cysteine has been achieved through fermentation of an *E. coli* strain with disrupted L-cysteine regulon, that is, the *yciW* gene [70].

5.3.2. Enzymatic production of nonproteinogenic amino acids

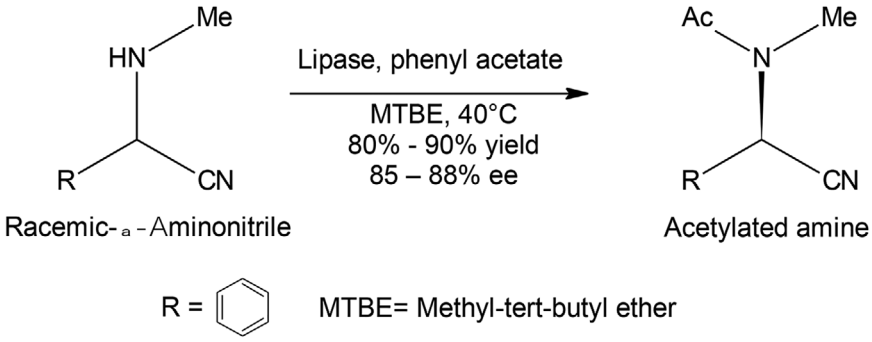
Enzymatic production of D-amino acids and nonproteinogenic L-amino acids are becoming a more acceptable and environmental-friendly method. D-Amino acids can be obtained as the byproducts through enzymatic resolution of racemic mixtures of DL-amino acids used to produce L-amino acids [65]. However, it is also possible to produce D-amino acids directly, for example, using a D-specific acylase, from racemic acetyl amino acids [66]. Still another method of industrial importance is the production of building blocks for the semisynthetic antibiotics ampicillin and amoxycillin, that is, the D-phenylglycine and *p*-hydroxy-D-phenylglycine, which are produced using the enzyme system hydantoinase/carbamoylase. The recent advances in modern molecular biological methods (directed evolution), has made it possible to switch the D-specificity of hydantoinases to L-specificity [71]. Further, it is now possible to coexpress racemases with D- or L-selective hydantoinases and carbamoylases in highly potent recombinant whole-cell systems capable of producing a variety of D- and L-amino acids (Scheme 8) [72]. Recently, a more cost-effective enzymatic method for D-amino acid production has been reported involving dynamic kinetic resolution of *N*-succinyl amino acids carried out by two enzymes namely the D-succinylase and *N*-succinyl amino acid racemase, which enantioselectively hydrolyzed the *N*-succinyl-D-amino acids to their corresponding D-amino acids [73].

5.3.3. Enzymatic synthesis of pragabalin intermediate, (S)-3-cyano-5-methylhexanoic acid

Pregabalin is a powerful anticonvulsant, used to treat seizure disorders, neuropathic pain, and fibromyalgia [74]. The



SCHEME 4 *Dynamic kinetic resolution of α-aminonitriles to produce chiral α-amino acids (adopted from Ref. [54])*



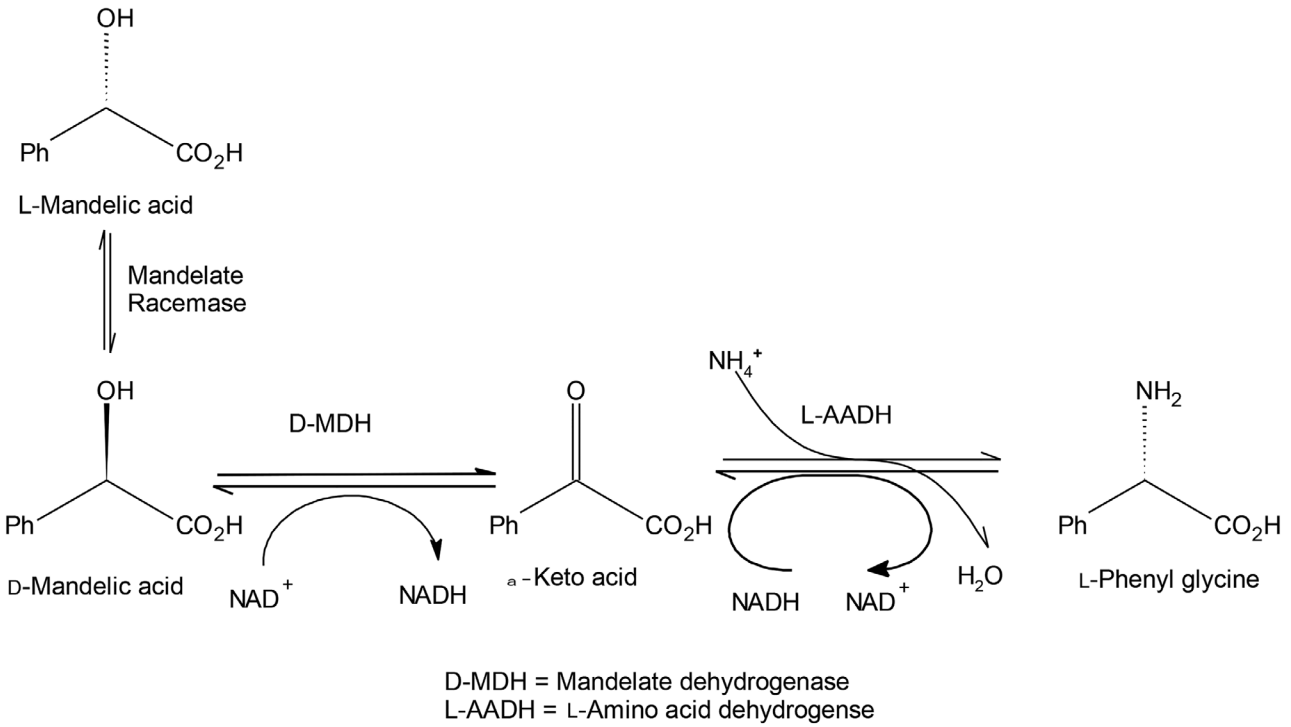
SCHEME 5 *Kinetic resolution of α-aminonitriles catalyzed by lipase (adopted from Ref. [55])*

pharmacological activity of pregabalin is confined to its (S)-enantiomer [75], so its asymmetric synthesis has gained great importance in the pharmaceutical industry [76]. Many chemocatalytic and biocatalytic routes have been used for the asymmetric synthesis of the key intermediate (S)-3-cyano-5-methylhexanoic acid of (S)-pregabalin. For instance, bisphosphine rhodium [77] and nitrilase [78] have been reported for the asymmetric synthesis of (S)-3-cyano-5-methylhexanoate in appreciable yields and good enantiomeric excess viz. 98% and 97% ee, respectively. However, both approaches were unsustainable in terms of environmental and cost effectiveness [79]. In this context, an efficient biocatalytic route for the synthesis of (S)-3-cyano-5-methylhexanoic acid, has been reported wherein an esterase from *Arthrobacter* sp. ZJB-09277 (whole cell catalysis) was used to catalyze the kinetic resolution of racemic 3-cyano-5-methylhexanoic acid esters to yield (S)-3-cyano-5-methylhexanoic acid in 44.6 mM with 95.1% ee [79] (Scheme 9).

5.3.4. Enzymatic synthesis of sitagliptin intermediate, (R)-3-amino-4-(2,4,5-triflurophenyl) butanoic acid

(R)-3-amino-4-(2,4,5-triflurophenyl) butanoic acid (3-ATfBA), a β-amino acid is a crucial intermediate for the synthesis of important antidiabetic drugs such as sitagliptin, retaglptin, and evogliptin [80]. A two-step enzymatic process has been reported for the synthesis of 3-ATfBA, wherein the first step is *Candida rugosa* lipase mediated conversion of β-ketoester substrate to β-keto acid, which was subsequently aminated by the ω-Transaminase (ω-TA) of *Ilumatobacter coccineus* to its corresponding β-amino acid. The process resulted in about 92.3% conversion of 100 mM β-keto ester substrate to 3-ATfBA. Scaleup of the reaction showed excellent conversion (81.9%) and enantioselectivity (99% *ee*) [81] (Scheme 10).

Enzymatic synthesis of another key intermediate of stigaliptin, Boc-(*R*)-3-amino-4-(2,4,5-trifluorophenyl) butyric acid, has been reported by Hou et al. [82]. The reaction was performed at 100 mM scale, resulting in 82% conversion in 24 H. The amino ester product was further transformed to Boc-(*R*)-3-amino-4-(2,4,5-trifluorophenyl) butyric acid, the key intermediate of sitagliptin (Scheme 11).



SCHEME 6 *Dynamic kinetic resolution of mandelic acid (adopted from Ref. [56])*

5.3.5. Enzymatic synthesis of l-*tert-leucine*

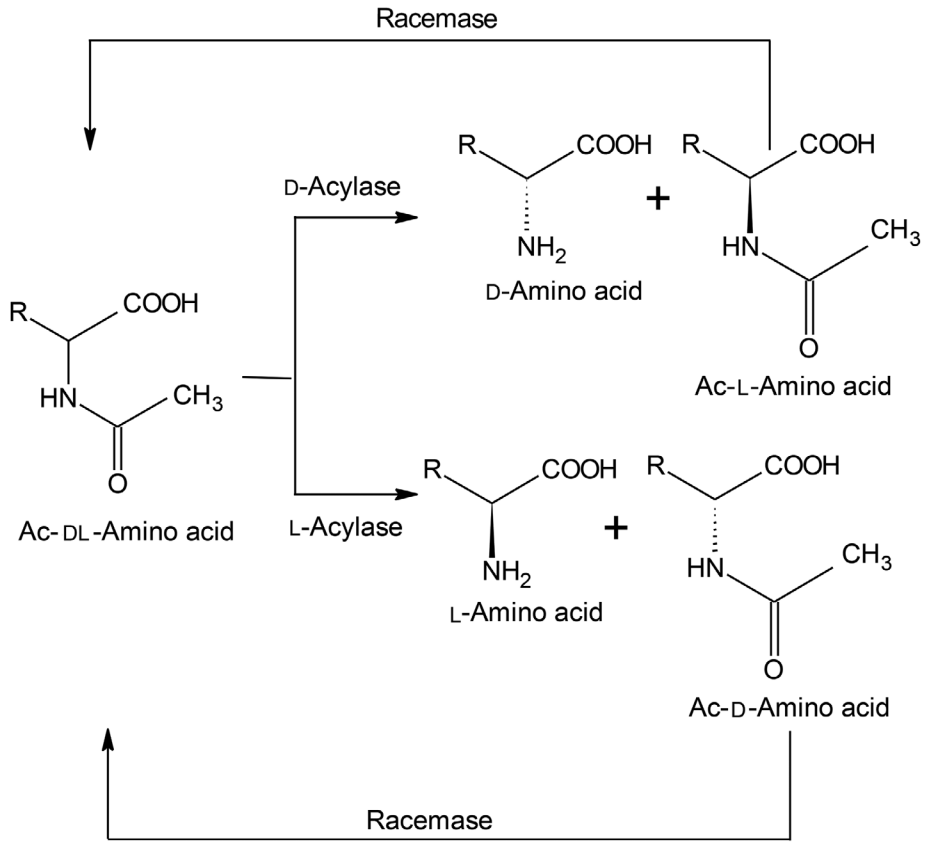
L-*Tert*-leucine is the basic chiral building block for many pharmaceutically active compounds such as atazanavir, boceprevir, and telaprevir, which are potent protease inhibitors for human immunodeficiency virus (HIV) protease, hepatitis C virus genotype 1 protease, and hepatitis C NS3-4A serine protease, respectively [83, 84]. Li et al. [83] has reported the successful coexpression of *Exiguobacterium sibiricum* leucine dehydrogenase (LeuDH) with *Bacillus megaterium* glucose dehydrogenase (GDH) in *E. coli* BL21 for the production of L-*tert*-leucine. Here, LeuDH was used to convert the substrate trimethylpyruvic acid to L-*tert*-leucine and GDH was used to regenerate the cofactor NADH from NAD^+, which was required to continue the synthesis reaction. By using the coexpressed whole cells, they were able to prepare decagram of L-*tert*-leucine at 1 L scale from 0.6 M (78.1 g L^{-1}) of substrate with 99% conversion after 5.5 H, resulting in 80.1% yield and >99% ee (Scheme 12). In another report, recombinant LeuDH from *Thermoactinomyces intermedius* and recombinant format dehydrogenase (FDH) from *Pichia pastoris* were used to catalyze the synthesis of L-*tert*-leucine from trimethylpyruvic acid and NADH regeneration, respectively (Scheme 13). Using this system, a reaction yield of >95% with an ee of >99.5% was obtained at 100 g/L substrate input [84].

5.3.6. Enzymatic synthesis of statin intermediates

Statins such as atorvastatin and rosuvastatin are primarily used as lipid lowering agents and for prevention of cardiovascular diseases [84]. These drugs act as specific inhibitors of the hydroxylmethylglutaryl coenzyme A reductase, which reduces HMG-CoA into mevalonate, a rate-limiting step in the biosynthesis of cholesterol [85, 86, 87, 88]. Two intermediates that are converted to atorvastatin and rosuvastatin have been discussed here. First, is t-butyl 6-chloro-(3*R*,5*S*)-dihydroxyhexanoate and another is (3S,5R)-dihydroxy-6-(benzyloxy) hexanoic acid, ethyl ester. Synthesis of t-butyl 6-chloro-(3*R*,5*S*)-dihydroxyhexanoate has been carried out using recombinant *E. coli* cells harboring the alcohol dehydrogenase (KleADH) of *Klebsiella oxytoca*. KleADH catalyzed the conversion of t-butyl 6-chloro-(5*S*)-hydroxy-3-oxohexanoate to t-butyl 6-chloro-(3*R*,5*S*)-dihydroxyhexanoate with more than 99% diastereomeric excess (de) and 99% conversion in 24 H without adding any expensive cofactors [89] (Scheme 14). In another approach (3S,5R)-dihydroxy-6-(benzyloxy) hexanoic acid, ethyl ester or tert-butyl ester have been synthesized via enantioselective reduction of the corresponding diketoesters, 3,5-dioxo-6-(benzyloxy) hexanoic acid, ethyl or tert-butyl esters, catalyzed by recombinant *E. coli* cells harboring the ketoreductase of *Acinetobacter calcoaceticus* [90, 84] (Scheme 15).

5.3.7. Enzymatic synthesis of antiplatelet agent, Xemilofiban

A racemic mixture of ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate was resolved through an enantioselective acylation catalyzed by penicillin G amidohydrolase (penicillin G acylase) from *E. coli* to yield the S-isomer (Scheme 16), which can be used as a chiral synthon for the synthesis of the antiplatelet agent Xemilofiban [91]. The first step is the synthesis of desilylated phenylacetamide racemic ethyl



SCHEME 7 *L-Amino acid/D-amino acid production using acylases (adopted from Ref. [66])*

3-amino-5-(trimethylsilyl)-4-pentynoate, which is a nonenzymatic step. In the second step, phenylacetamide is enzymatically hydrolyzed to produce (R)-amide in a quantitative yield and (S)-amine with yield of 90%.

6. Enzyme Therapy

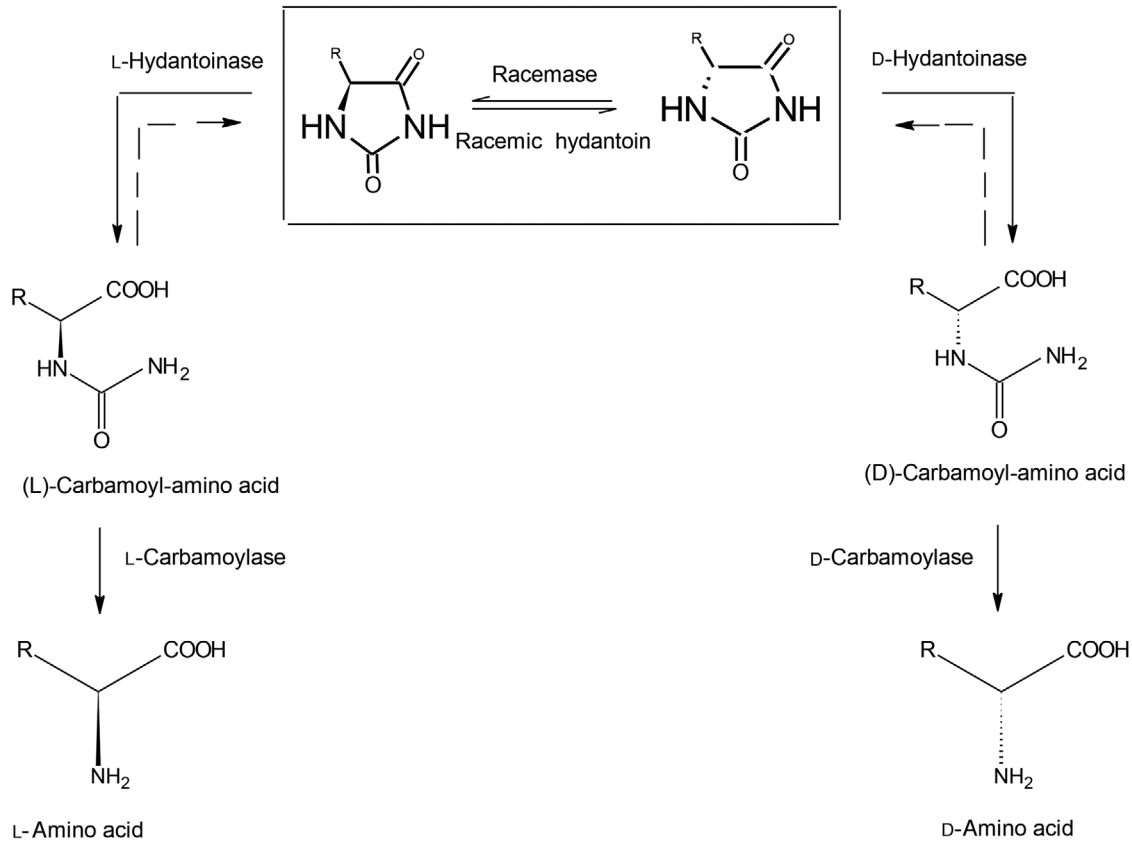
Enzyme therapy refers to the applications of enzymes for treating enzyme deficiencies and other medical conditions in human beings. In humans, enzymes assist in food digestion, body detoxification, strengthening of the immune system, muscle contraction, reduction of stress on the vital organs like pancreas and others. In this regard, enzyme therapy has numerous possible medical applications, for example, treatment of pancreatic insufficiency and cystic fibrosis (CF), metabolic disorders, lactose intolerance, removal of dead tissues, cancers or tumors, and so on. The therapy may be systemic or nonsystemic, and may be administered via multiple routes of administration, most often orally, topically or intravenously [92]. A summary of enzymes used as therapeutic agents is presented in Table 1.

6.1. Adenosine deaminase

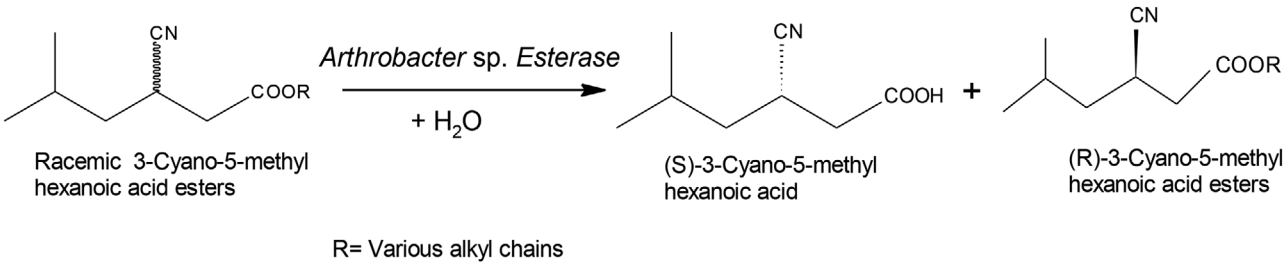
Adenosine deaminase (adenosine aminohydrolase, or ADA) is an enzyme involved in metabolism of purine bases. It performs the breakdown of adenosine obtained through food and from the turnover of nucleic acids in tissues. Its primary function in humans is the development and maintenance of the immune system [101]. This enzyme has emerged as therapeutic agent for the treatment of immunological disorders. Adenosinedeaminase (Adagen: pegadamase bovine) is used for the treatment of Severe Combined Immunodeficiency Disease (SCID) [102]. It is the first successful use of an enzyme for the treatment of an inherited immunological disorder [103]. ADA plays an important role in the metabolism of excess adenosine present in the circulation of these patients and decreases the toxicity to the immune system of the raised adenosine levels. The success of the treatment depends upon the modification of ADA with PEG [104]. PEG enhances the half-life of the enzyme (originally less than 30 Min) and reduces the possibility of immunological reactions due to the bovine origin of the drug [105, 106]. The PEGylated forms of enzyme are commercially available as pegadamase bovine with the brand name Adagen.

6.2. β-Glucocerebrosidase

β-glucocerebrosidase (D-glucosyl-N-acylsphingosinegluco hydrolase, EC 3.2.1.45) is an enzyme with glucosylceramidase activity that hydrolyze the β-glucosidic linkage of the chemical glucocerebroside, an intermediate in glycolipid metabolism. Mutations in the glucocerebrosidase gene cause Gaucher's disease, a lysosomal storage disease characterized by an accumulation of glucocerebrosides [107, 108]. Mutations in the glucocerebrosidase gene are also associated with Parkinson's



SCHEME 8 *D-Amino acid/L-amino acid production using hydantoinases/carbamoylases (adopted from Ref. [67])*



SCHEME 9 *Enzymatic kinetic resolution of racemic 3-cyano-5-methylhexanoic acid esters using* Arthrobacter *sp. esterase (adopted from Ref. [79])*

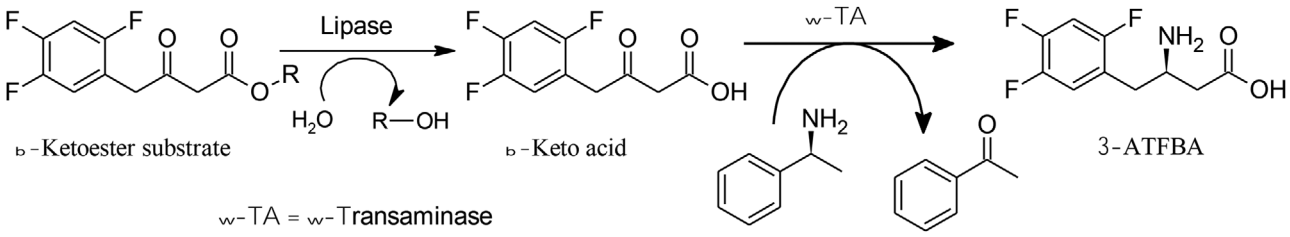
disease [108, 109]. This enzyme was approved for use in enzyme replacement therapy of Gaucher disease in which an exogenous modified placental glucocerebrosidase was targeted to its correct compartment within the body [108]. The commercial formulations of the enzyme were available as alglucerase injection with the brand name Ceredase.

6.3. Sacrosidase

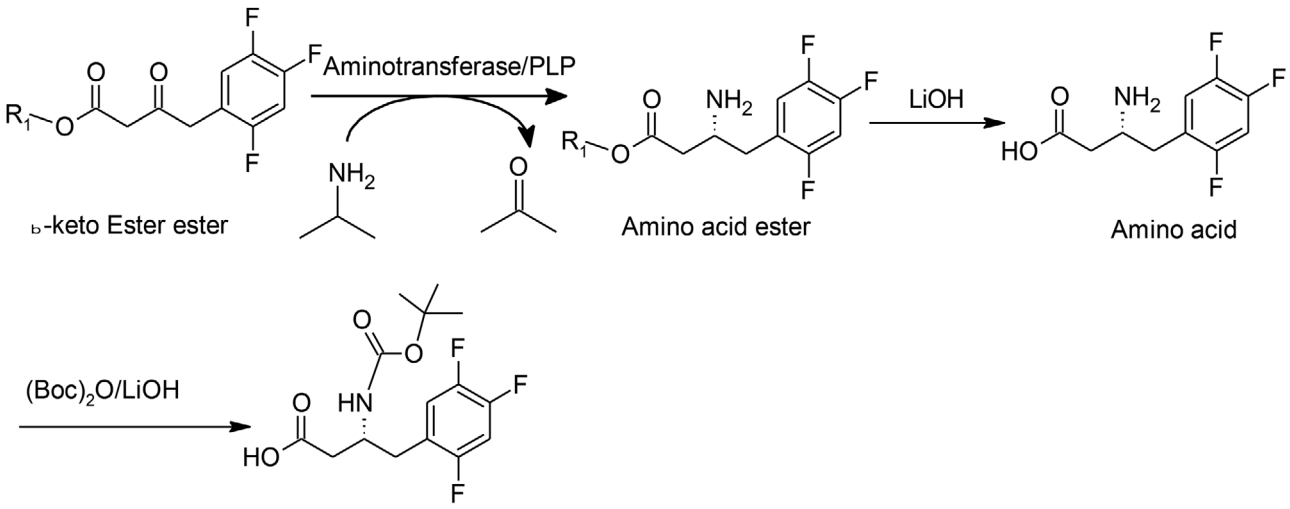
Sacrosidase (β-fructofuranoside fructohydrolase) is another enzyme that is used for the treatment of congenital sucrase-isomaltase deficiency (CSID). CSID patients cannot digest the disaccharide sucrose. The sacrosidase-based drug hydrolyses sucrose, helping in its digestion allowing the consumption of regular food items having added sucrose or otherwise sucrose-containing fruits [110]. A sacrosidase from *S. cerevisiae* that can be taken orally has been used for the treatment [111].

6.4. Enzymes used as digestive aids

A number of enzymes are employed to cure the digestive problems induced by some sugars. The α-galactosidase enzyme is taken as a digestive aid for people who develop the symptoms of bloating, gas, and diarrhea upon ingesting foods like beans and *Brassica* vegetables such as, cabbage, broccoli, *Brussels*, and so on [112]. The hydrolase α-galactosidase assists in breakdown of terminal α-galactosidic residues of sugar substrates



SCHEME 10 *Enzymatic synthesis of 3-ATfBA [(R)-3-amino-4-(2,4,5-triflurophenyl) butanoic acid] from corresponding keto acid (adopted from Ref. [81])*



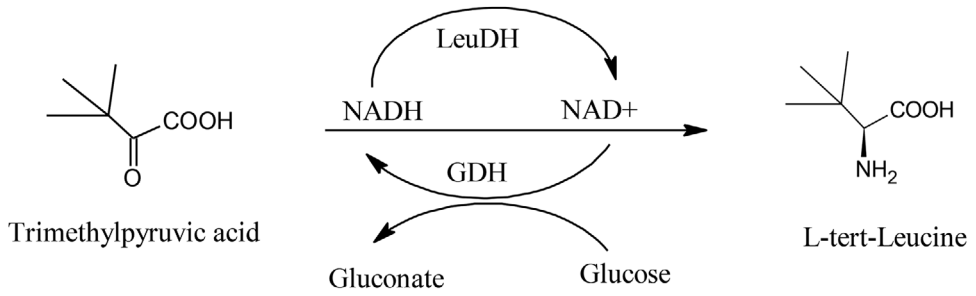
SCHEME 11 *The chemo-enzymatic route for the synthesis of Boc-(*R)-3-amino-4-(2,4,5-trifluorophenyl)-butyric acid *(adopted from Ref. [82])*

present in these foods, which when remain undigested cause the discomfort, probably due to the bacterial fermentation of the undigested sugars [113]. Now-a-days α-galactosidase and lactase are available as a number of ready to use supplements.

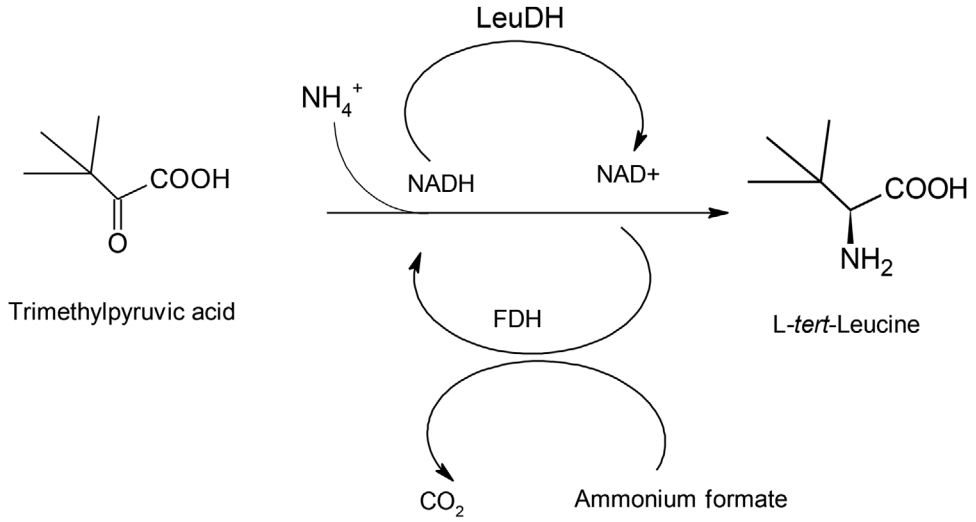
Lactose intolerance is a condition in which the patient is unable to produce sufficient quantities of lactase enzyme that is responsible for the digestion of the milk sugar lactose [114]. The lactose intolerant people cannot digest lactose-containing foods and therefore, suffer from stomach upset when they consume lactose containing food such as milk and milk products containing lactose [115]. For such people, lactase supplements such as milk fortified with lactase, lactase powder, and so on are available. These supplements help to break down the lactose into its monomers, that is, glucose and galactose and alleviate the symptoms of lactose intolerance such as bloating, gas, and diarrhea [116, 117].

Another disease (genetic disorder) is phenylketonuria (PKU), an inheritable disorder, requires strict compliance with a specialized diet [118]. PKU occurs due to little or absence of phenylalanine hydroxylase enzyme. This enzyme helps in converting phenylalanine to tyrosine, thereby maintaining its normal levels in the body [118]. An oral treatment for the disorder has been developed, which is composed of a plant phenylalanine ammonia lyase (PAL) overexpressed and obtained from a recombinant yeast, available in market with the trade name Phenylase™. PAL has been demonstrated to hydrolyze phenylalanine in the gastrointestinal tract [119].

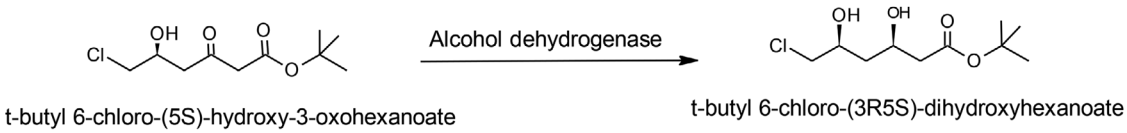
As a general digestive aid for the benefit of people with weak immunity a cocktail of pancreatic enzymes, including amylases proteases, and lipases, has been developed. The cocktail has been shown to be effective in the alleviating the problem of fat malabsorption in patients suffering from HIV [120, 121]. This enzyme cocktail is also beneficial in treating the pancreatic insufficiency, which is common in patients of CF [122]. A cocktail of pancreatic enzymes with the trade name "TheraCLEC Total™" is available commercially.



SCHEME 12 *Reductive amination of trimethylpyruvic acid using recombinant leucine dehydrogenase (LeuDH) and glucose dehydrogenase (GDH) for the production of optically pure* L-tert-*leucine (modified from Ref. [83])*



SCHEME 13 *Reductive amination of trimethyl pyruvic acid to optically pure* L-tert-*leucine using recombinant leucine dehydrogenase (LeuDH) and formate dehydrogenase (FDH) (modified from Ref. [84])*



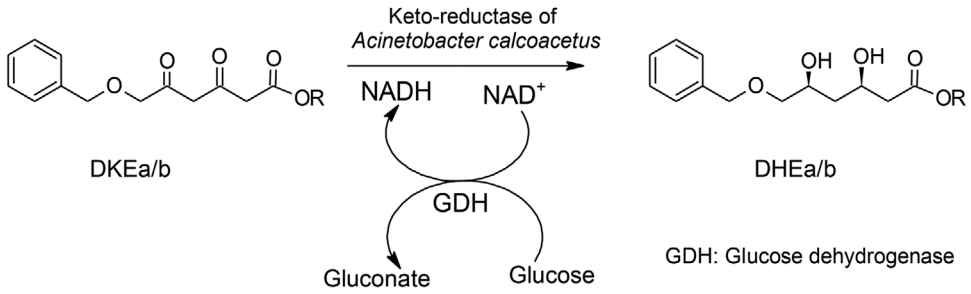
SCHEME 14 *Enzymatic preparation of t-butyl 6-chloro-(3R,5S)-dihydroxyhexanoate by alcohol dehydrogenase of* Klebsiella oxytoca *(adopted from Ref. [90])*

6.5. Proteolytic and glycolytic enzymes for treating damaged tissue

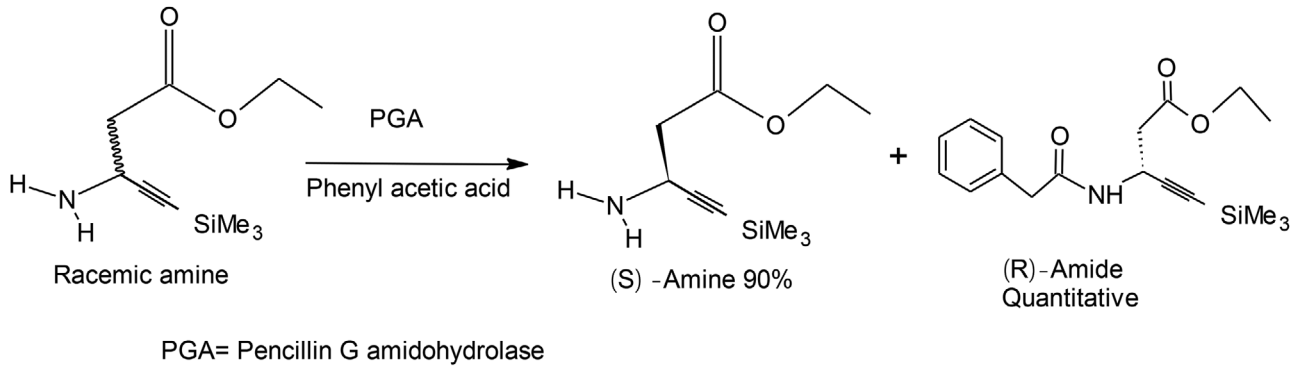
Proteolytic enzymes of bacterial and plant origin have been investigated for debridement (removal of dead skin) of burns [123]. However, due to inconsistent results and lower efficacy of these enzymes of natural origins, commercialization of the technology could not be done. However, with the intervention of recombinant DNA technology, efficient enzyme formulations like Debrase gel dressing, with consistent results is now available. Debrase gel dressing is composed of a mixture of enzymes extracted from pineapple, was approved by US FDA in 2002, and is now in phase IIb clinical trial, in patients with deep partial thickness or full thickness thermal burns, in Europe and the United States [124].

6.6. Enzymes for the treatment of infectious diseases

Lysozyme a bactericidal enzyme produced naturally in human body is added in many food products. It has been discovered that this enzyme has activity against HIV, similar to RNase A and urinary RNase U, it selectively degrades viral RNA [125] and therefore is a promising candidate for the treatment of



SCHEME 15 *Enzymatic preparation of (3S,5R)-dihydroxy-6-(benzyloxy) hexanoic acid, ethyl (DHEa) or tert-butyl (DHEb) ester by keto reductases (Kred) of* Acinetobacter calcoaceticus *from the corresponding diketo ethyl ester (DKEa) or diketo tert-butyl ester (DKEb) (modified from Refs. [84, 90])*



SCHEME 16 *Acylation of racemic amine (ethyl 3-amino-5-(trimethylsilyl)-4-pentynoate) using penicillin G amidohydrolase (PGA) (adopted from Ref. [93])*

HIV infection. Other examples of antimicrobial enzymes are chitinases. Chitin is a good target for antimicrobials because it is a major component of the cell wall of different pathogens like fungi, helminths, and protozoa [126]. On the other hand, lytic bacteriophage-derived enzymes have been known for killing bacteria such as *Streptococcus pneumonia*, *Clostridium perfringens*, and *Bacillus anthracis* [127–128]. Thus, the application of lytic bacteriophages as a treatment for bacterial infections is also under development and is assumed tobe effective against antibiotic resistant bacterial strains.

6.7. Enzymes for the treatment of cancer

Application of therapeutic enzymes in cancer treatment is an emerging field of research. Lately, it has been discovered that PEG immobilized arginine deaminase (an arginine-degrading enzyme) can inhibit human hepatocellular carcinomas and skin cancers which are deficient in arginine due to lack of arginosuccinate synthetase activity [129]. A better enzyme therapy has been developed using PEGylated L-asparaginase with the name Oncaspar (pegaspargase). It has shown better results for the treatment of acute lymphoblastic leukemia, acute myeloid leukemia, and non-Hodgkin's lymphoma [130]. In other words, the normal cells, that is, the noncancerous cells can synthesize asparagine, whereas the cancer cells cannot, and die in the presence of enzymes which degrade asparagine. Although the cost of the PEG–asparaginase formulations is higher than the native enzyme the overall cost of the treatment is very similar in both cases [131]. In fact, asparaginase and PEG-asparaginase are better alternatives to standard chemotherapy.

7. Future Prospects of Enzymes in Pharmaceuticals

As enzymes have successfully replaced many conventional chemical catalysts-based pharmaceutical manufacturing processes, the future of enzymes in this sector is very promising. There are some enzymes that have been shown to have cellular detoxification function such as superoxide dismutase and catalase. Both these enzymes work in conjunction to detoxify the body. The superoxide dismutase transforms the highly toxic superoxide anion to moderately toxic hydrogen peroxide [132]. The catalase then converts hydrogen peroxide to harmless water and oxygen. However, both these enzymes could not be used for these desired activities even in their PEGylated forms [133]. Nevertheless, these enzymes have been reported for

TABLE 1 *Enzymes for therapeutic uses*

Trade Name	*Generic Name*	*Indication*	*References*
ADAGEN	Pegademase bovine	For enzyme replacement therapy for ADA in patients with SCID	[93]
CEREDASE	Alglucerase injection	For replacement therapy in patients with Gaucher's disease type I	[94]
PULMOZYME	Dornase alpha	To reduce mucous viscosity and enable the clearance of airway secretions in patients with CF	[95]
CEREZYME	Imiglucerase	Replacement therapy in patients with types I, II, and III Gaucher's disease	[96]
SUCRAID	Sacrosidase	Treatment of congenital sucrase-isomaltase deficiency	[97]
ELITEK	Rasburicase	Treatment of malignancy-associated or chemotherapy-induced hyperuricemia	[98]
FEBRAZYME	Agalsidase beta	Treatment of Fabry's disease	[99]
NATTOKINASE NSK-SD	Nattokinase	Support healthy blood clotting, circulation, and platelet function	[100]

prolonging the life of the nematode, *Caenorhabditis elegans* and it is assumed that they can be used for prolonging the human life as well [134]. Improvement in the structure and functional properties of these enzymes shall be helpful in avoiding organ damage in hemorrhagic shock [135]. A serum detoxifying enzyme as an example the human butyrylcholinesterasehas been shown to be effective in the treatment of cocaine overdose [136]. Structural engineering of this enzyme has been shown to increase its detoxifying activity toward cocaine [137]. In this respect, directed evolution is proposed to provide more efficient forms of the therapeutic enzymes and other enzymes that will catalyze the pharmaceutical biotransformations with great efficiencies [138].

8. Conclusions

Enzymatic processes have replaced the conventional chemical-based methods for the synthesis of many APIs such as the production of different semi-synthetic antibiotics, active enantiomers of drugs through kinetic resolution, synthesis of enantiomerically pure forms of amino acids (D- and L-amino acids) and others. Enzymes have also been used for therapeutic purposes, for example, as enzyme therapy for the treatment of various metabolic disorders and genetic diseases such as SCID, Gaucher's disease, Parkinson's disease, PKU, and so on. More recent uses of enzymes are in cancer treatment and treatment of infectious diseases, where antibiotics are no longer useful (due to appearance of resistance in bacteria against the antibiotics). They are also used as digestive aids, for example, proteases, lipases, and amylases, and have been used separately or as cocktails for the treatment of digestive problems. However, despite the current progress, there is still ample scope for enzymes in the pharmaceutical sector. In future, the established enzymes will be tried for novel applications and new enzymes, discovered from natural sources or developed through enzyme engineering or directed evolution, shall be used to catalyze still unexploited reactions.

9. References

[1] Nelson, D. L., Lehninger, A. L., and Cox, M. M. (2008) Lehninger Principles of Biochemistry. 5th ed., W.H. Freeman, New York.
[2] Mitchell, J. B. (2017) Curr. Opin. Struct. Biol. 47, 151.
[3] Brasil, B. F., Siqueira, F. G., Salum, T. F. C., Zanette, C. M, and Spier, M. R. (2017) Algal Res. 25, 76.
[4] Copley, S. D. (2017) Curr. Opin. Struct. Biol. 47, 167–175.
[5] Cao, S., Xu, P., Ma, Y., Yao, X., and Lou, W. (2016) Chin. J. Catal. 37, 1814–1823.
[6] Meller, K., Szumski, M., and Buszewski, B. (2017) Sens. Actuat. B: Chem. 244, 84–106.
[7] Newton, M. S., Arcus, V. L., Gerth, M. L., and Patrick, W. M. (2018) Curr. Opin. Struct. Biol. 48, 110–116.
[8] Yang, H., Li, J., Du, G., and Liu, L., Eds. (2017) Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications. pp. 151–165, Academic Press Books, Elsevier.
[9] Liu, X., and Kokare, C., Eds. (2017) Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications. pp. 267–298, Academic Press Books, Elsevier.
[10] Li, S., Yang, X., Yang, S., Zhu, M., and Wang, X. (2012) Comput. Struct. Biotechnol. J. 2, e201209017.
[11] Dublin, (2019) Globe Newswire – The "Industrial Enzymes Market - Growth, Trends, and Forecast (2019 - 2024)". https://www.researchandmarkets.com
[12] Industrial enzymes market to attain revenue of $12.8 bn by 2025, (2019) News-Transparency Market Research.
[13] https://www.gminsights.com/industry-analysis/enzymes-market

[14] Cormode, D. P., Gao, L., and Koo, H. (2018) Trends Biotechnol. 36, 15–29.
[15] Walther, R., Rautio, J., and Zelikin, A. N. (2017) Adv. Drug Deliv. Rev. 118, 65–77.
[16] Saxena, R. K., Malhotra, B., and Batra, A. (2004) Handbook of Fungal Biotechnology. pp. 287–297, Marcel Dekker, Inc. New York.
[17] Saxena, R. K., Agarwal, L., and Meghwanshi, G. K. Eds. (2006) Microbial Diversity: Current Perspectives and Potential Applications. pp. 791–814, I.K. International Pvt. Ltd.
[18] Patel, A. K., Singhania, R. R., Pandey, A., Eds. (2017) Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications. pp. 13–41, Academic Press Books.
[19] https://www.novozymes.com/en/biology
[20] Meghwanshi, GK, and Vashishtha, A. (2018). Biotechnology of Fungal Lipases. In Fungi and their Role in Sustainable Development: Current Perspectives. pp. 383–411, Springer Nature Singapore Pte Ltd. ISBN: 9789811303937
[21] Thomas, L., Arumugam, M., and Pandey, A. (2013) Indian J. Exp. Biol. 51, 875–884.
[22] Charles, M. (1985) Trends Biotechnol. 3, 134–139.
[23] Thiry, M., and Cingolani, D. (2002) Trends Biotechnol. 20, 103–105.
[24] Stanbury, P. F., Whitaker, A., and Hall, S. J. (1995) Principles of Fermentation Technology, 2nd ed, Elsevier.
[25] Shuler, M. L., and Kargi, F. (2001) Bioprocess Engineering: Basic Concepts, Prentice Hall.
[26] Challener, C. (2016) Bioprocess Int. 14, 44.
[27] Thomas, L., Larroche, C., and Pandey, A. (2013) Biochem. Eng. J. 81, 146–161.
[28] Pandey, A., Selvakumar, P., Soccol, C. R., and Nigam, P. (1999) Curr. Sci. 77, 149–162.
[29] Shewale, J. G., and SivaRaman, H. (1989) Process Biochem. 24, 146–154.
[30] Sudhakaran, V. K. and Borkar, P. S., (1985a) Hindustan Antibiot. Bull. 27, 63.
[31] Sudhakaran, V. K. and Borkar, P. S., (1985b) Hindustan Antibiot. Bull. 27, 44.
[32] Illanes, A., and Valencia, P. Eds. (2017) Biotechnology of Microbial Enzymes: Production, Biocatalysis and Industrial Applications. pp. 267–305, Academic Press Books - Elsevier.
[33] Shewale, J. G., and Sudhakaran, V. K. (1997) Enzyme Microb. Technol. 20, 402–410.
[34] Avinash, V. S., Ramasamy, S. R., Suresh, C. G., and Pundle, A. (2015) Int. J. Biol. Macromol. 79, 1–7.
[35] Demain, A. L. (2000) Biotechnol. Adv. 18, 499–514.
[36] Vroom De, E. (1997) An Improved Immobilized Penicillin G Acylase. WO Patent WO 1997004086 A1.
[37] Vroom De, E. (2000) Penicillin G Acylase Immobilized with a Crosslinked Mixture of Gelled Gelatin and Amino Polymer. US Patent 6060268.
[38] Bianchi, D., Bartolo, R., Olini, P., Cesti, P. (1998) Chim. Ind. Milan 80, 879–885.
[39] Wedekind, F., Daser, A. and Tischer, W. (1998) Immobilization of Penicillin G Amidase, Glutaryl 7-ACA Acylase or D-Amino Acid Oxidase on an Amino Functional Organosiloxane Polymer Carrier. US Patent 5780260.
[40] Parmar, A., Kumar, H., Marwaha, S. S., and Kennedy, J. F. (2000) Biotechnol. Adv. 18, 289–301.
[41] Zhang, B., Wang, J., Chen, J., Zhang, H., and Zhang, Q. (2017) Biochem. Eng. J. 127, 43.
[42] Grulich, M., Brezovský, J., Štěpánek, V., and Palyzová, A., Kyslík, P. (2016) J. Mol. Catal. B: Enzym. 133, s53-s59.
[43] Arroyo, M., dela Mata, I., Acebal, C., and Pilar Castillon, M. (2003) Appl. Microbiol. Biotechnol. 60, 507–514.
[44] Hoople, D. W. T. (1998) Ed., Biotransformations I, Vol 8a. Biotechnology. pp. 243–275, 2nd edWiley-VCH, Weinheim, Germany.
[45] Wegman, M. A., Janssen, M. H. A., and Van Rantwijk, F., Sheldon, R. A. (2001) Adv. Synth. Catal. 343, 559–576.
[46] Martens, E., and Demain, A. L., Eds. (2017) Microbial Resources: From Functional Existence in Nature to Applications. pp. 149–168, Academic Press Books – Elsevier.
[47] Zmijewski, M. J., Briggs, B. S., Thompson, A. R., and Wright, I. G. (1991) Tetrahedron Lett. 32, 1621–1622.
[48] Cainelli, F., Giacomini, D., Galletti, P., and DaCol, M. (1997) Tetrahedron Asymmetry 8, 3231–3235.
[49] Basso, A., Braiuca, P., De Martin, L., Ebert, C., Gardossi, L., and Linda, P. (2000) Tetrahedron Asymmetry 11, 1789–1796.
[50] Von Nussbaum, F., Brands, M., Hinzen, B., Weigand, S., and Häbich, D. (2006a) Angew. Chem, 118, 5194–5254.
[51] von Nussbaum, F., Brands, M., Hinzen, B., Weigand, S., and Häbich, D. (2006b) Angew. Chem Int. Ed. 45, 5072–5129.
[52] Gröger, H., Pieper, M., König, B., Bayer, T., and Schleich, H. (2017) Sustain. Chem. Pharm. 5, 72-79.
[53] Rachwalski, M., Vermuea, N., and Rutjes, F. P. J. T. (2013) Chem. Soc. Rev. 42, 9268–9282.
[54] Yasukawa, K., Hasemi, R., and Asanoa, Y. (2011) Adv. Synth. Catal. 353, 2328–2332.
[55] Vongvilai, P., Linder, M., Sakulsombat, M., Svedendahl Humble, M., Berglund, P., Brinck, T., and Ramström, O. (2011) Angew. Chem. Int. Ed. 50, 6592.
[56] Resch, V., Fabian, W. M. F., Kroutil, W. (2010) Adv. Synth. Catal. 352(6), 993–997.
[57] Bercovici, D., and Fuller, F. Eds. (1995) Biotechnology in Animal Feeds and Animal Feeding. pp. 93–113, VCH, Weinheim.
[58] Fan, Y., Evans, C. R., and Ling, J. (2017) Biochim. Biophys. Acta 1861, 3024–3029.
[59] Li, B., Zhang, J., Xu, Y., Yang, X., and Li, L., (2017) Tetrahedron Lett. 58(24), 2374–2377.
[60] Leuchtenberger, W., Eds. (1996) Biotechnology 2nd Ed, Vol 6. Products of Primary Metabolism. pp. 465–502, VCH, Weinheim.
[61] Siebert, A., Wysocka, M., Krawczyk, B., Cholewiński, G., and Rachoń, J. (2018) Eur. J. Med. Chem. 143, 646–655.
[62] Ya-Ping, X., Cheng-Hao, C. and Yu-Guo, Z. (2018) Chem. Soc. Rev. 47, 1516–1561
[63] Cheong, J. E., Pfeiffer, C. T., Northrup, J. D., Parker, M. F. L., and Schafmeister, C. E. (2016) Tetrahedron Lett. 57, 4882–4884.
[64] Chibata, J. (1978) Immobilized enzymes- Research and Development, Kodanska Scientific Limited. p. 284, Tokyo and John Wiley and Sons Ltd, New York and London, Tokyo.
[65] Leuchtenberger, W., Huthmacher, K., and Drauz, K., (2005) Appl. Microbiol. Biotechnol. 69, 1–8.
[66] Woeltinger, J., Karau, A., Leuchtenberger, W., and Drauz, K. Eds. (2005) Advances in Biochemical Engineering/Biotechnology, 92. pp. 289–316, Springer, Berlin Heidelberg New York.
[67] Calton, G. J., Eds. (1992) Biocatalytic Production of Amino Acids and Derivatives. pp. 59–74, Hanser, München.
[68] Calton, G. J. (1992) In J. D.Rozzell and F.Wagner, eds., Biocatalytic Production of Amino Acids and Derivatives. pp 3–21, Hanser, München.
[69] Pae, K. M., Ryo, O. H., Yoon, H. S., and Schin, C. S. (1992) Biotechnol. Lett. 14, 1143–1148.
[70] Ohtsu, Y. K. I., Takumi, K., Tamakoshi, A., Nonaka, G., Funahashi, E., Ihara, M., and Takagi, H. (2015) J. Biosci. Bioeng. 119, 176.
[71] May, O., Nguyen, P., and Arnold, F. (2000) Nat. Biotechnol. 18, 317.
[72] May, O., Verseck, S., Bommarius, A., and Drauz, K. (2002) Org. Process Res. Dev. 6, 452.
[73] Sumida, Y., Iwai, S., Nishiya, Y., Kumagai, S., Yamada, T., and Azuma, M. (2017) J. Biosci. Bioeng 125, 282–286.
[74] https://www.drugs.com/monograph/pregabalin.html#r1
[75] Silverman, R. B. (2008) Angew. Chem. Int. Ed. 47, 3500–3504.
[76] Chen, Y. S., Li, X., Chen, R. N., and Zhao, S. Y. (2011) Chin. J. Org. Chem. 31, 1582–1594.
[77] Hoge, G., Wu, H. P., Kissel, W. S., Pflum, D. A., Greene, D. J., and Bao, J. (2004) J. Am. Chem. Soc. 126, 5966–5967.
[78] Xie, Z., Feng, J., Garcia, E., Bernett, M., Yazbeck, D., and Tao, J. (2006) J. Mol. Catal. B: Enzym. 41, 75–80.
[79] Zheng, R-C, Zhenga, Y-G, Li, A-P, and Li, X-J. (2014) Biochem. Eng. J. 83, 97–103.

[80] Ramisetti, A., Boggu, J. M. R., Shekhar, P., and Subba Reddy, B. V. (2016) Chem. Select 1, 5445–5447.
[81] Kim, H., Jeon, H., Khobragade, T. P., Patil, M. D., Sung, S., Yoon, S., Won, Y., Choi, I. S., and Yun, H. (2019) Enzyme Microb. Technol. 120, 52-60.
[82] Hou, A., Deng, Z., Ma, H., and Liu, T. (2016) Tetrahedron 72(31), 4660–4664.
[83] Li, J., Pan, J., Zhang, J., and Xu, J-H. (2014) J. Mol. Catal. B: Enzym. 105, 11–17.
[84] Patel, R. N. (2018) Bioorg. Med. Chem. 26(7), 1252-1274.
[85] Xingyuan, S., Hanbing, S., Hongxia, B., and Zhimin, O. (2013) Korean J. Chem. Eng. 30(1), 166–171.
[86] Wu, Y., Xiong, F.-J., and Chen, F. E. (2015) Tetrahedron 71(45) 8487–8510.
[87] Huang, Y., Liu, N., Wu, X., and Chen, Y. (2010) Curr. Org. Chem. 14, 1447–1460.
[88] Istvan, E. S., and Deisenhofer, J. (2001) Science 292 (5519), 1160–1164.
[89] Xu, T., Wang, C., Zhu, S., and Zheng, G. (2017) Process Biochem. 57, 72–79.
[90] Patel, J. M. (2009) J. Mol. Catal. B: Enzym. 61, 123–128.
[91] Topgi, R. S., Ng, J. S., Landis, B., Wang, P., and Behling, J. R. (1999) Bioorg. Med. Chem. 7, 2221–2229.
[92] Enzyme therapy, http://www.naturallivingcenter.net/
[93] https://leadiant.com/wp-content/uploads/2019/06/AdagenPI.pdf
[94] Alglucerase injection (Ceredase®) – Gaucher disease, https://www.ninds.nih.gov/
[95] https://www.pulmozyme.com/
[96] https://www.cerezyme.com/
[97] https://www.sucraid.com/
[98] https://www.elitekpro.com/
[99] https://www.fabrazyme.com/
[100] https://www.pureencapsulations.com
[101] Wilson, D. K., Rudolph, F. B., and Quiocho, F. A. (1991) Science 252, 1278–1284.
[102] Aiuti, A. (2002) Curr. Opin. Mol. Ther. 4, 515–522.
[103] Fejerskov, B., Olesen, M. T. J., and Zelikin, A. N. (2017) Adv. Drug Deliv. Rev. 118, 24.
[104] Hershfield, M. (1995) Clin. Immunol. Immunopathol. 76, 228–232.
[105] Greenwald, R. B. (2001) J. Controll. Rel. 74, 159–171.
[106] Roberts, M. J., Bentley, M. D., and Harris, J. M. (2002) Adv. Drug Deliv. Rev. 54, 459–476.
[107] Entrez Gene: Gene-centered information at NCBI, http://www.ncbi.nlm.nih.gov
[108] Erdem, N., Buran, T., Berber, I., and Aydogdu, I. (2018) J. Natl. Med. Assoc. 110, 330–333.
[109] PDGene, http://www.pdgene.org/
[110] Lwin, A., Orvisky, E., Goker-Alpan, O., LaMarca, M. E., and Sidransky, E. (2004) Mol. Genet. Metab. 81, 70–73.
[111] Matta, M. C., Vairo, F., Torres, L. C., and Schwartz, I. (2018) Blood Cells Mol Dis. 68, 200–202.
[112] Lule, V. K., Garg, S., Tomar, S. K., Khedkar, C. D., Nalage, D. N., eds. (2016) Reference Module in Food Science-Encyclopedia of Food and Health. pp. 43–48, Elsevier.
[113] Shang, Q. H., Ma, X. K., Li, M., Zhang, L. H., Piao, X. S. (2018) Feed Sci. Technol. 236, 48-56.
[114] Treem, W. R., McAdams, L., Stanford, L., Kastoff, G., Justinich, C., and Hyams, J. (1999) J. Pediatr. Gastroenterol. Nutr. 28, 137–142.
[115] Parker, A. M., and Watson, R. R., eds. (2017) Nutrients in Dairy and their Implications on Health and Disease. pp. 205–211, Academic Press Books – Elsevier.
[116] Kumar, R., Henrissat, B., and Coutinho, P. M. (2019) Sci. Rep. 9, 10346.
[117] Hertzler, S., Savaiano, D. A., Dilk, A., Jackson, K. A., Fabrizis, S. N. B., and Suarez, L., eds. (2017) Nutrition in the Prevention and Treatment of Disease (4th Edition)–. pp. 875–892, Academic Press Books – Elsevier.
[118] Wallig, M. A., eds. (2018) Fundamentals of Toxicologic Pathology (3rd Edition). pp. 395–442, Academic Press Books – Elsevier.
[119] MacDonald, A., eds. (2013) Brenner's Encyclopedia of Genetics (Second Edition). pp. 300–303, Elsevier.
[120] Sarkissian, C. N., Shao, Z., Blain, F., Peevers, R., Su, H., Heft, R., Chang, T. M., and Scriver, C. R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 2339–2344.
[121] Carroccio, A., Guarino, A., Zuin, G., Verghi, R., Berni-Canani, R., Fontana, M., Bruzzese, E., Montalto, G., and Notarbatolo, A. (2001) Aliment. Pharmacol. Ther. 15, 1619.
[122] Schibli, S., Durie, P. R., and Tullis, E. D. (2002) Current Opin. Pulm. Med. 8, 542–546.
[123] Harish, B. S, and Uppuluri, K. B. (2018) Int. J. Biol. Macromol. 107 (Pt B), 1373–1387.
[124] Biocentury, NexoBrid, https://bciq.biocentury.com/products/nexobrid (debrase gel dressing)
[125] Lee-huang, S., Huang, P. L., Sun, Y., Kung, H. F., Blithe, D. L., and Chen, H. C. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 2678–2681.
[126] Fusetti, F., Moeller, H. V., Houston, D., Rozeboom, H. J., Dijkstra, B. W., Boot, R. G., Aerts, J. M., and Aalten, D. M. (2002) J. Biol. Chem. 227, 2537.
[127] Loeffler, J. M., Nelson, D., and Fischetti, V. A. (2001) Science 294, 2170–2172.
[128] Schuch, R., Nelson, D., and Fischetti, V. A. (2002) Nature 418, 884–889.
[129] Zimmer, M., Vukov, N., Scherer, S., and Loessner, M. (2002) Appl. Environ. Microbiol. 68, 5311–5317.
[130] Ensor, C. M., Bomalaski, J. S., and Clark, M. A. (2002) Cancer Res. 62, 5443–5450.
[131] Avrami, V. I., Sencer, S., Periclou, A. P., Bostrom, B. C., Cohen, L. J., Ettinjer, A. G., Ettinjer, L. J., Franklin, J., and Gaynon, P. S. (2002) Blood 99, 1986–1994.
[132] Kurre, H. A., Ettinger, A. G., Veenstra, D. L., Gaynon, P. S., Franklin, J., Sencer, S. F., Reaman, G. H., Lanje, B. J., and Holcenberg, J. S. (2002) J. Pediatr. Hematol. Oncol. 24, 175.
[133] Veronese, F., Calceti, P., Schiavon, O., and Sergi, M. (2002) Adv. Drug Deliv. Rev. 54, 587–606.
[134] Melov, S., Ravenscroft, J., Malik, S., Gill, M. S., Walker, D. W., Clayton, P. E., Wallace, D. C., Malfroy, B., Doctrow, S. R., and Lithgow, G. J. (2000) Science 289, 1567–1569.
[135] Izumi, M., McDonald, M. C., Sharpe, M. A., Chatterjee, P. K., and Thiermann, C. (2002) Shock 18, 230–235.
[136] Duysen, E. G., Bartels, C. F., and Lockridge, O. (2002) J. Pharmacol. Exp. Therap. 302, 751–758.
[137] Sun, H., Pang, Y. P., Lockridge, O., and Brimijoin, S. (2002) Mol. Pharmacol. 62, 220–224.
[138] Huisman, G. W., and Gray, D. (2002) Curr. Opin. Biotechnol. 13, 352–358