Review paper

MICROBIAL ENZYMES: THERAPEUTIC APPLICATIONS

Abstract

Enzymes are biomolecules with highly specialized catalytic functions produced by all living organisms and are responsible for biochemical reactions in plants, animals , microorganisms and human beings. Nowadays enzymes are considered as core of biotechnology because they are the main tools for the application of basic biotechnological techniques , they act as the target of the therapeutic drugs and are indispensable intermediates in all biotechnological processes. The concept of the therapeutic enzymes has been around for at least 40 years . Microbial enzymes are preferred over other sources and in this review different types of microbial enzymes are discussed for their therapeutic applications.

MICROBIAL ENZYMES: THERAPEUTIC APPLICATIONS

Enzymes are biomolecules with highly specialized catalytic functions produced by all living organisms and are responsible for biochemical reactions in plants, animals, microorganisms and human beings. The use of enzyme in processing raw materials from plants and animals have been practiced for a long time. The first observation of the enzymatic degradation was in 1783 by Spallanzani. In 1814 Kirchhoff found that the barley contain a substance that convert starch in to sugars. The term enzyme was coined by Kuhne in 1878. Enzyme preparations were used in ancient times without much knowledge about the nature and properties of enzymes. Today industrial application of enzymes began with Jokichi Takamine, who developed an enzyme preparation takadiastase a mixture of carbohydrases and proteases(Uhlig, 1998).

Enzymes have been used as catalysts in various industries like brewing, tanning, bakery, diary etc along centuries. Nowadays the enzymes are

considered as core of biotechnology because they are the main tools for the application of basic biotechnological techniques, the targets of the therapeutic drugs and the indispensable intermediates in all biotechnological processes. Apart from the function as targets in therapy, enzymes are novel in that they find application as therapeutic molecule itself (Vitolo, 2009). This review focuses on the application of various microbial enzymes as therapeutic agents.

Microbial therapeutic enzymes

Enzymes were largely ignored as drugs until Emmerich and his associates observed in 1902 that an extracellular secretion of *Bacillus pyocyaneus* was capable of killing anthrax bacilli. He deduced that the secretions contain nucleases which is the responsible element for the bacterial lysis. This milestone study gradually opened the way for the use of enzymes in the treatment first of infections, then of cancer and finally of a diverse spectrum of diseases (Gonzalez and Issacs, 1999).

The concept of the therapeutic enzyme has been around for at least 40 years. For example, a therapeutic enzyme was described as part of replacement therapies for genetic deficiencies in the 1960s by de Duve (1966). Enzymes as drugs have two important features i) they often bind and act on their targets with great affinity and specificity and ii) they are catalytic and convert multiple target molecules to the desired products. These two features make enzymes specific and potent drugs for a wide range of disorders (Vellard, 2003).

Sources of therapeutic enzymes include animals, plants and microorganisms (bacteria and fungi). Microbial enzymes are preferred because they are generally cheaper to produce, their enzyme content is more predictable and controllable and

the availability of reliable supplies of raw materials of constant composition. As they are foreign in nature, some of them are unadapted with the human body. Plant and animal sources contain more harmful materials than microbes which include phenolic compounds (from plants), endogenous enzyme inhibitors and proteases (Kaur and Sekhon, 2012).

Microbial enzymes have found wide application in medicine and pharmacology and their use in this field is recognized recently. Therapeutic enzymes have a wide variety of specific uses such as oncolytics, thrombolytics, or anticoagulants and as replacements for metabolic deficiencies, anti-inflammatory agents etc (Gurung etal., 2013). Various microbial therapeutic enzymes are described below.

L- asparginase

L-Asparaginase(EC 3.5.1.1) is broadly distributed among the plants, animals and microorganisms. Microbes are preferred source of L-asparaginase, because they can be cultured easily and the extraction and purification of L- asparaginase is also comfortable with the large-scale production. A wide range of bacteria, fungi, yeast, actinomycetes and algae are very potent producers of L-asparaginase (Savitri et al ,2003; Verma et al ,2011).

L- asparginase from *Erwinia carotovora* or *Escherichia coli* is used in the treatment of acute lymphocytic leukemia (Eden etal., 1990). Its activity depends upon the fact that tumour cells lack aspartate-ammonia ligase activity, which stops the synthesis of nonessential amino acid L-asparagine(Gurung etal., 2013). Hence, they are extracted from body fluids. Thus leukemic cells require L –aspargine, unlike normal cells, for their survival (Kidd and Sobin, 1966). By injecting L-

asparginase the availability of the aminoacid is reduced, so the leukemic cells fail to survive(Mashburn and Wriston, 1964).

Collagenase(Ec 3.4.24.3)

True bacterial collagenases are consensually described as enzymes that cleave helical regions of fibrillar collagen molecules under physiological conditions (Harrington, 1996). Microbial collagenases belong to the MEROPS peptidase family M9 (INTERPRO: IPR002169; PFAM: PF01752), which comprises bacterial metalloproteinases (predicted to be zinc-dependent) from Vibrio and Clostridium with presumable collagenolytic activity (Rawlings et al., 2012). Collagenases are applied in the pharamaceutical world for the treatment of various disorders listed below.

Treatment of damaged tissues

For treating damaged tissues, several studies, comparing the use of enzymatic methods with surgical/ mechanical procedures as well as comparing the effect between several enzymes were made. Data are controversial, while a few studies state that wound debridement is more efficient by using enzymatic procedures, reducing hospital staying and the demand for surgical debridement (Karagol et al., 2011).

Enzymatic debriding agents are effective alternative for removing necrotic material from pressure ulcers, leg ulcers, and partial-thickness wounds. They may be used to debride both adherent slough and eschar. Enzymatic agents may be used as the primary technique for debridement in certain cases, especially when different approach such as surgical or conservative sharp wound debridement (CSWD) are not feasible due to bleeding disorders or other

106 considerations(Ramundo and Gray, 2008).

107 For the removal of dead skin of burns, the use of a large number of bacterial
108 and plant enzymes have been studied. Among the microbial enzymes, a proteolytic
109 enzyme from *Vibrio proteolyticus* was found to be effective, and it successfully
110 finished phase1b clinical trials in 2004. Now it is used under the trade name
111 Vibrilase TM, especially for the serious secondary burn treatments(Gurung, 2013).

112 **Dupuytren's disease (DD)**

113 Clostridium collagenase(Ec 3.4.24.3) is also applied in the treatment of
114 Dupuytren's disease. .Dupuytren's disease is a fibroproliferative disorder of the
115 palmar fascia that limits hand functions, ultimately disabling the hand, and lowering
116 life's quality (Hurst et al., 2009). This progressive disorder results in the permanent
117 and symptomatic flexion contracture of the digits.

118 Although some side reactions have been noted for injectable *C. histolyticum*
119 collagenase like skin lacerations, edema, hemorrhage, injection site pain and
120 bruising and less frequently tendon and pulley rupture (Hallock, 2012; Kaplan,
121 2011), its effectiveness has been proved by several in vivo studies (Foissac et al.,
122 2013; Martin-Ferrero et al., 2013) with contracture reduction in more than 60% of
123 the patients injected with injectable clostridial collagenase (Hurst et al., 2009;).
124 Collagenase injection is more worthwhile than surgical fasciotomy (Martin-Ferrero
125 et al., 2013), has less and milder side-effects, and demonstrated a better total
126 reduction of Dupuytren's contracture leading to higher patient satisfaction (Vollbach
127 et al., 2013).

128 **Chronic total occlusions (CTO)**

129 Microbial collagenases (more precisely clostridial collagenases) have also

been applied to the treatment of chronic total occlusions (CTO) in animal models (Segev et al., 2005; Strauss et al., 2003). CTO is defined as a 3-month-old total obstruction of a coronary artery, and is one of the more difficult challenges for coronary interventionists (Aziz & Ramsdale, 2005). It consists of various degrees of fibroatheromatous plaque and thrombus, depending on the occlusion mechanism and its duration, and occurs in approximately 30% of the patients with coronary artery disease. Presently, clinical trials have showed that in human subjects, local delivery of collagenase into coronary chronic total occlusion is feasible and safe (Strauss et al., 2012).

PROTEASES

Proteases constitute the single most important group of industrially important microbial enzymes which are capable of hydrolysing peptide bonds in to aminoacids based on the size of the molecules they can attack or preferably attack. These may be proteinases or petidases. Since the later years of the nineteenth century, crude proteases are used for the treatment of gastrointestinal disorders. Microbial proteases are used either directly or indirectly in the field of medicine for diagnostic or therapeutic purposes (Morihara etal., 1950).

Streptokinase (EC 3.4.24.29)

Pathologies involving a failure of hemostasis and the clot formation require clinical intervention consisting of intravenous administration of thrombolytic agents (Collen et al., 1988; Collen, 1990; Francis and Marder, 1991). Streptokinase is one such agent.

Streptokinase is an extrcellular enzyme produced by ß hemolytic streptococci. Streptokinase, produced by certain strains of streptococcus, is used

as a therapeutic agent in the treatment of cardiovascular diseases. It is a single chain polypeptide that exhibits its fibrinolytic action by indirectly activating the circulating plasminogen. Streptokinase is used in the treatment acute myocardial infarction , it is certainly more cost effective, however its use is not risk free.

When Streptokinase binds with circulatory plasminogen or plasmin, the resulting 1:1 stoichiometric complex is a high specificity protease that proteolytically activate other plasminogen molecules to plasmin (Bajaj and Castellino, 1977). Comparative clinical trials and cost effective considerations suggest that streptokinase is the drug of choice for thrombolytic therapy(Mucklow, 1995). Streptokinase is a non human protein and its introduction in to the circulatory system can elicit severe anaphylatic response including death (Lee, 1995). This immunogenicity restricts multiple applications of the streptokinase.

Staphylokinase (EC 3.4.99.22)

Staphylokinase is a protein produced by certain strains of staphylococcus and possesses fibrinolytic activities. Staphylokinase is a single polypeptide chain with a molecular weight of approximaltely 15.5 KDa and length of 163 amino acids. Natural staphylokinase has been purified from S. aureus strains that were transformed with bacteriophages containing the staphylokinase gene, or that had undergone lysogenic conversion to staphylokinase production (Lijinen et al., 1992).

Staphylokinase converts inactive proteolytic enzyme plasminogen to its active form, plasmin. Staphylokinase is used for the treatment of myocardial infarction. It can stimulate the lysis of both erythrocyte rich and platelet rich clots(Szarka etal., 1999).

Serrazime(EC 3.4.24.40)

Serrazyme, a proteolytic enzyme from *Aspergillus oryzae* and *Aspergillus meeus,* is used as an alternative of serratiopeptidase and is used as a dietary supplement for cardiovascular, antiinflammatory or immune support.

Serrapeptase (EC 3.4.24.40)

Serrapeptase is available for clinical use more than a decade. Serratiopeptidase binds to alpha -2-macroglobulin in the blood in the ratio of 1:1, which helps to mask its antigenicity but retains its enzymatic activity and is slowly transferred to site of inflammation. Serratiopeptidase hydrolyses bradykinin, histamine and serotonin responsible for the oedematic status. Serratiopeptidase reduces swelling, improves microcirculation and expectoration of sputum, etc(Mohankumar, 2009).

Serrapeptase or serratiopeptidase from *Serratia marcescens* is used as a therapeutic enzyme and possesses applications, as antiinflammatory agent, for treating carpel tunnel syndrome, for fibrocystic treatment and as agent to enhance the activity of antibiotics against biofilm formation(Preethi,2012).

Glutaminase

L-glutaminase (EC.3.5.1.2) is an amidohydrolase which catalyses the hydrolytical deamination of L-glutamine resulting in the production of L-glutamic acid and ammonia. L-Glutaminases are ubiquitous in the biological world (Ohshima et al., 1976; Iyer and Singhal, 2010) and organisms ranging from bacteria to human beings have the enzyme.

Acinetobacter glutaminisificans , Bacillus licheniformis, Bacillus subtilis ,Erwinia cartowora ,Micrococcus luteus etc are some of the representatives of the microbial world with potential glutaminase production capacities(Holchenberg, 1976

,Cook et al., 1981 , Shimizu et al., 1991).

L-Glutaminase, in combination with or as an alternative to asparaginase, could be of significance in enzyme therapy for cancer especially acute lymphocytic leukemia(Roberts etal., 1970). Glutaminase from microbes exhibit antitumour activity and recombinant glutaminase from Pseudomonas is patented for its activity against HIV and cancer therapy.

Lysostaphin(EC 3.4.24.75)

Lysostaphin is a 27 KDa zinc metalloenzyme secreted by certain strains of Staphylococcus simulans which has a specific lytic action against *Staphylococcus aureus*. It posess two functional domains an N terminal catalytic peptidase domain and a C terminal targeting domain which bind to the peptidoglycan substarte. Lysostaphin has activities of three enzymes namely, glycylglycine endopeptidase, endo-β-N-acetyl glucosamidase and N-acteyl muramyl-L-alanine amidase. Glycylglycine endopeptidase specifically cleaves the glycine–glycine bonds, unique to the interpeptide cross-bridge of the S. aureus cell wall(Wu et al., 2003).

Due to its unique specificity, lysostaphin could have high potential in the treatment of antibiotic-resistant staphylococcal infections(Kumar, 2008). Lysostaphin is found to reduce surface colonization by S.aureus and S. epidermidis. Thus the drug is more effective in preventing the nasal colonization of S. aureus. Lysostaphin acts synergistically with some membrane active agents plymixin and ranalexin against MRSA. Recombinant lysostaphin was found effective in the treatment of aortic endocarditis(Preethi etal., 2011).

Laccases

Laccase (EC 1.10.3.2) or p-diphenol oxidase is one of a few enzymes that have

been studied since the 19th century. Yoshida first reported laccase in 1883 from the exudates of the Japanese lacquer tree, Rhus vernicifera (Thurston 1994; Levine 1965). However in 1896, for the first time, both Bertrand and Laborde demonstrated laccase to be a fungal enzyme (Thurston 1994; Levine 1965).

Laccases are copper-containing enzymes that catalyze the oxidation of a wide variety of organic and inorganic substrates, including mono-, di-, and polyphenols, amino phenols, methoxy phenols, aromatic amines and ascorbate with the concomitant four electron reduction of oxygen to water (Galhaup et al. 2002). Laccase is a member of the large blue copper proteins or blue copper oxidases (Thurston 1994). The ability of laccases to oxidize phenolic compounds as well as their ability to reduce molecular oxygen to water has led to intensive studies of these enzymes (Thurston 1994). Laccase activity has been reported only in few bacteria, including *Azospirillum lipoferum, Marinomonas mediterranea, Streptomyces griseus, and Bacillus subtilis* (Octavio et al. 2006).

The first bacterial laccase was detected in the plant root-associated bacterium *Azospirillum lipoferum*, where laccase was associated with the melanin production for cell pigmentation. Recently some bacterial laccases have also been characterized from *Azospirillum lipoferum*, *Bacillus subtilis, Streptomyces lavendulae, S.cyaneus* and *Marinomonas mediterranea*. Many products generated by laccases are antimicrobial, detoxifying or active personal-care agents. Laccase can be used in the synthesis of complex medical compounds as anesthetics, anti-inflammatory agents, antibiotics, sedatives, etc, including triazolo(benzo)cycloalkyl thiadiazines, vinblastine, mitomycin, penicillin X dimer, cephalosporins, and dimerized vindoline (Pazarloglu , 2005, Shi, C., Clemmons, 2003).

Lipases

Lipases(tri acyl glycerol acyl hydrolases E.C 3.1.1.3) are hydrolases that catalyse the hydrolysis of triacylglcerol to glycerol and free fatty acids over an oil water interface. Bacterial lipases are glycoproteins but some extracellular lipases are lipoproteins. In addition to this, the enzyme catalyzes the transesterification and hydrolysis of other esters and also synthesis of some others. Such transformations enable them to be used in food, cosmetic and especially in pharmaceutic industry.

Among bacteria, *Achromobacter* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Pseudomonas* sp.,*Staphylococcus sp*., and *Chromobacterium* sp. have been exploited for the production of lipases.

Microbial lipases are used to enrich PUFAs from animal and plant lipids, and their mono and diacylglycerides are used to produce a variety of pharmaceuticals. Many PUFAs are essential for normal synthesis of lipid membranes and prostaglandins. Free PUFAs and their mono and diacylglycerides are subsequently used to produce a variety of pharmaceuticals. Considerable effort is being made to obtain optically pure compounds, which are pharmacologically more active than its antipode. Profens, a class of nonsteroidal anti-inflammatory drugs, are active in the (s)-enantiomer form.

Lee et al(1995) and Xie et al (1988) synthesized pure (s)-ibuprofen using lipase-catalyzed kinetic resolution via hydrolysis and esterification, respectively. Optically active homochiral intermediates for the synthesis of nikkomycin-B, non steroid anti-inflammatory drugs (naproxen, ibuprofen, suprofen and ketoprox), the

potential antiviral agent lamivudine, and for the enantiospecific synthesis of alkaloids, antibiotics, vitamins, and anti- arteriosclerotic, anti tumour and antiallergic compounds(Pandey etal., 1999). Lipase from *Candida rugosa* is used to synthesize lovastatin, a drug that lowers serum cholesterol level. The asymmetric hydrolysis of 3-phenylglycidic acid ester which is a key intermediate in the synthesis of diltiazem hydrochloride is a widely used coronary vasodilator and is synthesized using *S. marcescens* lipase(Matsumae etal., 1993).

Alginate lyase (EC 4.2.2.3)

Alginate lyase can digest alginate through the beta elimination of the glycosidic bond(Wong et al., 2000). They yield various oligosaccharides with unsaturated uronic acid at the non reducing terminus and unsaturated duronic acid monomers. The oligosaccharides released by the enzyme seems to possess biological activities like enhancing the growth of endothelial cells and stimulate secretion of cytokines from human macrophages. The enzyme possesses pharmaceutical activty(Courtois, 2009., Iwamoto etal., 2005, Kawada etal, 1999).

One of the leading causes of illness and death in cystic fibrosis (CF) patients is *Pseudomonas aeruginosa* infection of the respiratory tract. Patients colonised by mucoid, alginate-producing strains have a particularly poor prognosis (Govan and Deretic,1996), and the infection is rarely eliminated by antibiotic treatment. Co-administration of alginate lyase with gentamicin increased the killing of biofilms of mucoid *P. aeruginosa* growing in conditions similar to those found in the CF respiratory tract (Cotton et al., 2009).

Microbial enzymes also find their application in various lysosome storage diseases. The lysosomal storage diseases are due to the deficiency of a particular

enzyme such as β- glucuronidase or sphingomyelinase which lead to incomplete digestion of particles and results in clinical symptoms. Recombiant enzymes from *E.coli* is used in the treatment of such disorders.

CONCLUSION

Enzymes are known to mankind since the ancient times. Even in the period when there was no much knowledge on enzymes, people used them in various forms in fields like brewing etc,. Later on, in 18^{th} century, the entity was identified as enzymes. Now the global use of enzymes is estimated to be worth $4.2 billion in 2014, and it is estimated to to develop at a compound annual growth rate (CAGR) of approximately 7 % over the period from 2015 to 2020 to reach nearly $6.2 billion. Microbial enzymes are considered as the highly effective therapeutic agents of this century. To discover more and more new enzymes and also to explore their novel applications, research is going on world wide. To achieve this goal, intense research in the field is necessary.

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