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REVIEW

α-Amylases from Microbial Sources and Its Potential Applications in Various Industries

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Abstract Amylases are widely distributed and are one of the most studied enzymes. Such enzymes hydrolyze the starch molecules into polymers composed of glucose units. Amylases have potential application in a wide number of industrial processes such as food, fermentation and pharmaceutical industries. Amylases can be obtained from plants, animals and microorganisms. However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. The microbial source of amylase is preferred to other sources because of its plasticity and vast availability. The production of α-amylase is essential for conversion of starches into oligosaccharides. Starch is an important constituent of the human diet and is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato. The properties of each α-amylase such as thermostability, pH profile, pH stability, and Ca-independency are important in the development of fermentation process. This review focuses on the isolation, substrates of α-amylases, production of bacterial and fungal α-amylases, properties of α-amylases, and the use of these enzymes in industrial applications.

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Introduction

α-Amylases (E.C.3.2.1.1) are starch degrading enzymes that catalyses the hydrolysis of internal α-1,4 and α-1,6-glycosidic linkages in starch in low molecular weight products, such as glucose, maltose and maltotriose units. Amylases are among the most important enzymes and are of great significance for biotechnology, constituting a class of industrial enzymes. Amylases can be obtained from several sources such as plants, animals and microbes [1]. The microbial source of amylase is preferred to other sources because of its plasticity and vast availability. Today a large number of microbial amylases are available commercially and they have almost completely replaced chemical hydrolysis of starch in starch processing industry. The amylases of microorganisms have a broad spectrum of industrial applications as they are more stable as compared to plant and animal α-amylases.

Starch degrading amylolytic enzymes is of great importance in biotechnological application ranging from food, fermentation, and textile to paper industries etc. Most amylases used in industry are from microbial source due to several factors, for example, the great microbial genetic diversity present in the environment, high enzymatic activity in a wide range of conditions (extreme pH, temperature, osmolarity, pressure, etc.), and simple and cost effective production. α-Amylase is a key enzyme in metabolism of spacious diversity of living organisms which utilize starch as carbon and energy sources. Microbial amylase has almost surpassed the synthetic sources in

different industries [2]. These enzymes account for about 30 % of the world's enzyme production market [3]. Based on the mode of actions enzymes that hydrolyze starch can be divided into endoamylases (α-amylase), exoamylases (β-amylase, glucoamylase, and α-glucosidase) and debranching enzymes [4, 5]. Amylases are important in starch-processing industries; endoamylases initiate starch degradation (liquefaction process) to produce maltodextrins, and exoamylases are usually used to further degrade maltodextrins into glucose and maltose (saccharification process) [6].

The α-amylase family can roughly be divided into two groups: the starch hydrolyzing enzymes and the starch modifying, or transglycosylating enzymes. The enzymatic hydrolysis is preferred to acid hydrolysis in starch processing industry due to a number of advantages such as specificity of the reaction, stability of the generated products, lower energy requirements and elimination of neutralization steps. This review illustrates an overview of microbial α-amylases.

Endoamylases

Endoamylase randomly cleave α-1,4-D-glycosidic linkage between adjoining glucose units in the product chain retaining the anomeric carbon configuration in the product [7]. α-Amylases are well known as endoamylases, cleavage of internal α-1,4 bonds result in α-anomeric products [8]. Majority of α-amylases are extracellular, however a few others were found to be intracellular.

Exoamylases

Exaoamylases act at the non-reducing ends of polysaccharides and produce low molecular weight products, e.g., glucose and maltose. These enzymes exclusively either cleave α-1,4-glycosidic bonds as β-amylase or cleave both α-1,4 and α-1,6-glycosidic bonds like glucoamylase and α-glycosidase [8]. The starch hydrolysates are also different: glucoamylase and α-glucosidase produce only glucose, whereas β-amylase results in maltose and β-limit dextrin. β-Amylase and glucoamylase also convert anomeric configuration of the liberated product from α to β [2].

Debranching Enzymes

The branch points containing α-1,6-glycosidic linkages present in starch and glycogen are resistant to attack by α- and β-amylase resulting in a α/β limit dextrins respectively. Pullulanase, first discovered in 1961 attracted interest because of its specific action on pullulan, a linear D-glucose polymer with maltotriosyl units joined by α-1,6 bonds. Pullulanase is produced by mesophillic organisms such as *Klebsiella aerogenes* and *Aureobasidium pullulans* and are capable of specifically attacking α-1,6 linkages present in starch and glycogen [5]. Glucoamylase can also attack α-1,6 linkages but the reaction proceeds at relatively slow rate compared to pullulanase action.

Isolation of α-Amylases

Various α-amylases are produced by plants, animals, and microorganisms. Microorganisms have become increasingly important as producer of industrial enzymes. Due to their biochemical diversity and the ease with which enzyme concentrations may be increased by environmental and genetic manipulation, attempts are now being made to replace enzymes, which traditionally have been isolated from complex eukaryotes [9]. Starch degrading amylolytic enzymes are most important in the biotechnology industries with huge application in food, fermentation, textile and paper [2]. Among the microbial origin, amylases of fungal origin were found to be more stable than the bacterial enzymes on a commercial scale. Thus the attempts have been made to optimize the culture conditions for suitable strains of fungi [10]. On the other hand, as bacterial α-amylase has generally been produced from the strains belonging to genus *Bacillus* [11, 12], several attempts have been made at their purification and characterization from both mesophilic and thermophilic strains [1, 2].

Attempts have been made to isolate amylase from different sources [11–14]. Amylases have been isolated from mangrove associated fungi *Pestalotiopsis microspora* and *Aspergillus oryzae* [13]. Haloalkalophillic amylases have been isolated from bacterium *Bacillus agaradhaerens* which was quiet tolerant to a range of organic solvents such as alkanes and alcohol [14]. Production of haloalkalophillic amylases has a great importance in starch liquefaction, pulp processing and detergent making industries [15–17].

Irrespective of the wide range of applications one major limitation of the amylases is their ineffectiveness in the detergent industry [18]. Earlier research have shown that alkaliphilic amylases can be good source for detergent industry. Alkaliphiles are the extremophiles that occupy extreme pH environments [19]. The first alkaline amylase of an alkaliphilic *Bacillus* strain was reported by Horikoshi [20]. The main reasons for selecting enzymes from alkaliphiles are their long term stability in detergent products, energy cost saving by lowering the washing temperature, quicker and more reliable product, reduced effluent problem during the process and stability in the presence of

detergent additives such as bleach activators, softeners and perfumes. Attempts have been made to isolate alkaline amylases from various *Bacillus* species [21]. Similarly Yang et al. [22] reported that the recombinant alkaline α-amylase from *Bacillus alcalophilus* and *Bacillus subtilis* was stable at pH from 7.0 to 11.0 with an optimum pH of 9.5. For the optimization of production protocol to reduce cost of production at industrial level, efforts have been made to isolate amylase from *Penicillium camemberti* using orange waste as substrate [23]. Vasant [24] isolated a thermostable amylase form *Acremonium sporosulcatum* which was able to withstand high temperature variations dissipated during production processes in industries.

Substrates for Amylase Production

To meet the growing demands in the industry it is necessary to improve the performance of the system and thus increase the yield without increasing the cost of production [23]. Natural sources could serve as economical and readily available raw material for the production of valuable enzymes. Wheat bran and rice flakes were used as cheap and efficient carbon source for the production of amylases [21]. Similarly waste potato starch in liquid medium is used for the enhanced production of α-amylases [11]. Use of soybean meal as a substrate for amylase production has been optimized for the production of amylases from *B. subtilis* [22]. Use of different carbon and nitrogen sources as substrate has been studied [25]. They induced the α-amylase production with soybean meal, wheat bran, corn protein, hazelnut cake and whey as natural substrates by *B. amyloliquefaciens*, after the studies it has been observed that medium containing soybean meal is best for the amylase production.

Production of α-Amylases

Both solid state fermentation (SSF) and submerged fermentation (SmF) could be used for the production of amylases, although traditionally these have been obtained from submerged cultures because of ease of handling and greater control of environmental factors such as temperature and pH. Mostly synthetic media have been used for the production of bacterial amylase through SmF [26, 27]. However, SSF is generally defined as the growth of microorganisms on moist solid substrates with negligible free water [28]. The solid substrate may provide only support or both support and nutrition. SSF constitutes an interesting alternative since the metabolites so produced are concentrated and purification procedures are less costly [2, 8, 29–32]. The expenditure of enzyme production in SmF is high, thus creating the need to develop a system which lessens the cost by substitute methods. To reduce the cost of production and enhance efficient commercialization technique, α-amylase production in solid-state fermentation with wheat bran and rice husk as substrates have been reported [33]. Ikram-ul-Haq et al. [34] has illustrated the selection of an appropriate low cost fermentation medium for the production of α-amylase by using agricultural by-products. The optimization of fermentation parameters for α-amylase production has been carried out to study the purification and characterization of amylase from *Ganoderma tsugae* through the process of solid-state fermentation [35, 36].

SSF technique is generally confined to the processes involving fungi. However, successful bacterial growth in SSF is known in many natural fermentations [9, 37]. The production of α-amylase by SSF is limited to the genus *Bacillus. B. subtilis*, *B. polymyxa*, *B. mesentericus*, *B. vulgaris*, *B. coagulans*, *B. megaterium* and *B. licheniformis* have been used for α-amylase production in SSF [38]. Production of α-amylase in fungi is known to depend on both morphological and metabolic state of the culture. Growth of mycelium is crucial for extracellular enzymes like α-amylase [39]. Various physical and chemical factors have been known to affect the production of α-amylase such as temperature, pH, period of incubation, carbon sources acting as inducers, surfactants, nitrogen sources, phosphate, different metal ions, moisture and agitation with regards to SSF and SmF, respectively. Interactions of these parameters are reported to have a significant influence on the production of the enzyme.

Properties of α-Amylase Affecting Its Production and Application

α-Amylase probably has the most widespread use in enzyme industry. Besides their use in the saccharification or liquefaction of starch, these enzymes are also used for the preparation of viscous stable starch solutions used for the warp sizing of textile fibers, the clarification of haze formed in beer or fruit juices, or for the pretreatment of animal feed to improve the digestibility [3]. An important area of application of α-amylases is in the fields of laundry and dish-washing detergents. A modern trend among consumers is to use colder temperatures for doing the laundry or dishwashing. At these lower temperatures, the removal of starch from cloth and porcelain becomes more problematic. Detergents with α-amylases optimally working at moderate temperatures and alkaline pH can help solve this problem. In addition to this several patents exist describing the potential use of branching enzyme in bread as an anti-staling agent [40], or for the production of low-viscosity,

high molecular weight starch for, e.g. the coating of paper [41] or warp sizing of textile fibers, thus making the fibers stronger [42]. However the applications of branching enzymes are limited by the lack of commercially available enzymes that are sufficiently thermostable or alkalistable. Continuously, growing area of industrial application is pressurizing the demand to discover the enzymes with a broad spectrum of activity. The various factors which affect the enzyme production and its application are as follows.

pH Optima and pH Stability of α-Amylases

Starch gelatinization is the process carried out at low pH of about 4.5. The extreme conditions required for such pretreatment necessitate the use of an enzyme that is resistant to high temperatures and low pH. Acid hydrolysis of peptide bonds at low pH has been reported to occur most often at the C-terminal side of Asp residues, with the Asp–Pro bond being the most susceptible. This may be due to the facts that the nitrogen of proline is more basic than that of other residues, and Asp has an increased propensity for α-β isomerization when linked on the N side of a proline [43]. Peptide bond hydrolysis never occurs in the helical and beta structures. Thus, it appears that Asp residues, or Asp–Pro bonds, occurring in regions except helical and beta structures are susceptible to hydrolysis at low pH.

However, amylases have been isolated with a pH optima varying from 2 to 12 [44]. α-Amylases from most bacteria and fungi have pH optima in the acidic to neutral range [2]. α-Amylase from *Alicyclobacillus acidocaldarius* showed an acidic pH optima of 3 [45, 46] in contrast to the alkaline amylase with optima of pH 9–10.5 reported from an alkalophilic *Bacillus* sp. [4, 22]. Extremely alkalophilic α-amylase with pH optima of 11–12 has been reported from *Bacillus* sp. GM8901 [47]. Studies have also shown an influence of polyols indicated that stability towards high temperature and proteolytic digestion of enzymes was markedly enhanced in the presence of additives such as sorbitol, glycerol and trehalose. Moreover, enzymes were resistant to acidic digestion [48].

Temperature Stability and Temperature Optima of Amylases

Temperature is one of the most important parameter that affect the rate of enzyme hydrolysis. It is desirable that α-amylases should be active at high temperatures of gelatinization (100–110 °C) and liquefaction (80–90 °C) to economize the process; therefore, there has been a need and continual search for more thermophilic and thermostable α-amylase [49]. The Ca^{2+} is found to be necessary for enzyme folding and enzyme stability. The temperature optimum for the activity of α-amylase is related to the growth of the microorganism [44]. The lowest temperature optimum is reported to be 25–30 °C for *Fusarium oxysporum* amylase [50] and the highest of 100 and 130 °C from archaebacteria, *Pyrococcus furiosus* and *Pyrococcus woesei*, respectively [51]. Thermophilic amylases has also been isolated from *B. subtilis* and *B. firmus* [11, 52]. Isolation of amylases with temperature optima of 55 °C has been reported from actinomycetes [53]. Temperature optima of enzymes from *Micrococcus varians* are calcium dependent [54] and that from *H. meridiana* is sodium chloride dependent [55].

Effect of Inhibitors on Amylase Activity

Many metal cations, especially heavy metal ions, sulphydryl group reagents, *N*-bromosuccinimide, phydroxyl mercuribenzoic acid, iodoacetate, BSA, EDTA and EGTA inhibit α-amylases. In amylases, Ca^{2+} is loosely bound to enzyme and can be removed by treating with metal chelators such as EDTA, EGTA, etc. [56].

Calcium and Stability of α-Amylases

α-Amylase is a metalloenzyme, which contains at least one Ca^{2+} ion [57, 58]. The affinity of Ca^{2+} ions to α-amylase is much stronger than that of other ions. The amount of bound calcium varies from one to ten. Ca^{2+} ions add to the stability of the enzyme, however, they can be removed from amylases by dialysis against EDTA or by electrodialysis. The stabilizing effect of Ca^{2+} on thermostability of the enzyme can be explained due to the salting out of hydrophobic residues by Ca^{2+} in the protein, thus, causing the adoption of a compact structure [59]. Calcium free enzymes can be reactivated by adding Ca^{2+} ions. Some studies have been carried out on the ability of other ions to replace Ca^{2+} as Sr^{2+} in *B. caldolyticus* amylase [60]. In the presence of Ca^{2+}, α-amylases are much more thermostable than without it [61]. There are also reports where Ca^{2+} did not have any effect on the enzyme [62]. Calcium independent amylases have also been reported the presence of a thermostable α-amylase from *B. thermooleovorans* NP54, which did not require calcium ions for its activity or production [46, 63].

Effect of Different Substrates on Amylase Activity

α-Amylase is an inducible enzyme and is generally induced in the presence of starch or its hydrolytic product, maltose.

Amylases show substrate specificity. The substrate specificity of the α-amylase was evaluated on soluble starch, amylose, amylopectin, glycogen, maltodextrins, and α- and β-cyclodextrins. Natural starches such as maize starch [29], raw sago starch [64], corn starch [65, 66] and wheat starch [33] increased α-amylase activities.

The carbon sources as glucose and maltose have been utilised for the production of α-amylase. However, the use of starch remains promising and ubiquitous. A number of other non-conventional substrates as lactose, casitone, fructose, oilseed cakes, sugarcane bagasse [30, 31], dairy effluent [67], industrial waste [68], date waste [69], fermented cassava waste water [70], starch processing waste water and bread waste [36] have also been used for the production of α-amylase while the agro-processing byproduct, wheat bran has been used for the economic production of α-amylase by SSF [29]. The use of wheat bran in liquid state fermentation (LSF) for the production of α-amylase from *Aspergillus fumigatus* and from *Calvatia gigantea*, respectively, has also been reported [71]. Use of low molecular weight dextran in combination with either Tween 80 or Triton X-100 for α-amylase production in the thermophilic fungus *Thermomyces lanuginosus* (ATCC 200065) has also been reported [72]. Triton X-100 had no effect, whereas Tween 80 increases the α-amylase activity by 27-fold.

Industrial Applications of Amylases

Production of Bioethanol

Bioethanol production from starch has increased rapidly as the demand for renewable sources of fuel escalates. In industry, bioethanol is generated from sugars through the fermentation process, which is carried out by microorganisms, mainly *Saccharomyces cerevisiae*. *S. cerevisiae* is unable to directly utilize starch for cell growth and fermentation but rather requires starch to be pre-treated to release the glucose residues. Traditionally, the industrial process involves the cooking of starch granules at high temperatures in order to solubilize the starch molecules, which is followed by the addition of starch-degrading enzymes. However, high temperature cooking greatly contributes to the energy consumption of the fermentation process, thus reducing the total energy output of a bioethanol producing plant [73, 74]. Thus, a genetically engineered yeast strain that can express a raw starch-hydrolyzing enzyme would greatly reduce the cost of bioethanol production using a cold starch hydrolysis process. Liao et al. [75] engineered barley α-amylase in *S. cerevisiae* for the conversion of starch to bioethanol which lead to a considerable lowering in the cost of production of bioethanol at industrial level.

Starch Conversion

One of the most widespread applications of α-amylases is in the starch industry, which is used for starch hydrolysis. Starch liquefaction process converts starch into fructose and glucose syrups [76]. The enzymatic conversion of all starch includes: gelatinization, liquefaction and saccharification processes. Gelatinization involves the dissolution of starch granules, thereby forming a viscous suspension. Liquefaction, involves with the partial hydrolysis and loss in viscosity of syrup and saccharification, involving the production of glucose and maltose via further hydrolysis [6, 77]. Initially, α-amylase of *B. amyloliquefaciens* was used but it has been replaced by the α-amylase of *B. stearothermophilus* or *B. licheniformis* [3]. The enzymes from the fungal origin and *Bacillus* species are of special interest for large scale biotechnological processes due to their remarkable thermostability and because efficient expression systems are available for these enzymes [77].

Detergent Industry

The use of enzymes in detergents formulations enhances the detergents ability to remove tough stains and making the detergent environmentally safe. Amylases are used in detergents for laundry and automatic dishwashing to degrade the residues of starchy foods such as potatoes, gravies, custard, chocolate etc. to dextrins and other smaller oligosaccharides [78, 79]. Detergent industries are the primary consumers of enzymes, in terms of both volume and value. Amylases are the second type of enzymes used in the formulation of enzymatic detergent, and 90 % of all liquid detergents contain these enzymes [6, 80, 81]. Amylases have activity at lower temperatures and alkaline pH, maintaining the necessary stability under detergent conditions and the oxidative stability of amylases is one of the most important criteria for their use in detergents where the washing environment is very oxidizing [82]. Removal of starch from surfaces is also important in providing a whiteness benefit, since starch can be an attractant for many types of particulate soils. Examples of amylases used in the detergent industry are derived from *Bacillus* or *Aspergillus* [81].

Textile Industry

Starch is a very attractive size in textile industries, because it is cheap, easily available in most regions of the world, and it can be removed quite easily. Amylases are used in textile industry for desizing process. Desizing involves the removal of starch from the fabric which serves as the strengthening agent to prevent breaking of the warp thread during the weaving process. Sizing agents like starch are applied to yarn before fabric production to ensure a fast and secure

weaving process. Starch is later removed from the woven fabric in a wet-process in the textile finishing industry. The α-amylases remove selectively the size and do not attack the fibers [6, 83, 84]. Amylase from *Bacillus* strain was employed in textile industries for quite a long time.

Paper Industry

The use of α-amylases in the pulp and paper industry is for the modification of starch of coated paper, i.e. for the production of low-viscosity, high molecular weight starch [3, 6]. The coating treatment serves to make the surface of paper sufficiently smooth and strong, to improve the writing quality of the paper. In this application, the viscosity of the natural starch is too high for paper sizing and this can be altered by partially degrading the polymer with α-amylases in a batch or continuous processes. Starch is a good sizing agent for the finishing of paper, improving the quality and erasability, besides being a good coating for the paper. The size enhances the stiffness and strength in paper [6, 41]. Examples of amylases obtained from microorganisms used in paper industry includes Amizyme® (PMP Fermentation Products, Peoria, USA), Termamyl®, Fungamyl, BAN® (Novozymes, Denmark) and α-amylase G9995® (Enzyme Biosystems, USA) [85].

Baking Industry

α-Amylases have been widely used in the baking industry. These are extensively employed in processed food industry such as baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups [86]. The addition of α-amylase to the dough results in enhancing the rate of fermentation and the reduction of the viscosity of dough, resulting in improvements in the volume and texture of the product. These enzymes can be added to the dough of bread to degrade the starch in the flour into smaller dextrins, which are subsequently fermented by the yeast. Moreover, it generates additional sugar in the dough, which improves the taste, crust colour and toasting qualities of the bread. Besides generating fermentable compounds, α-amylases also have an anti-staling effect in bread baking, and they improve the softness retention of baked goods, increasing the shelf life of these products [3, 6]. Currently, a thermostable maltogenic amylase of *B. stearothermophilus* is used commercially in the bakery industry [3]. Amylases are also used for the clarification of beer or fruit juices, or for the pretreatment of animal feed to improve the digestibility of fiber [3, 87, 88].

Conclusion

The α-amylase family comprises a group of enzymes with a variety of different specificities that all act on one type of substrate, being glucose residues linked through an α-1,1; α-1,4; or α-1,6-glycosidic bond. Members of this family share a number of common characteristics but at least 21 different enzyme specificities are found within the family. These differences in specificities are based not only on subtle differences within the active site of the enzyme but also on the differences within the overall architecture of the enzymes. The α-amylase family can roughly be divided into two subgroups: the starch-hydrolysing enzymes and the starch-modifying or transglycosylating enzymes. As evident from the foregoing review, amylases are among the most important enzymes used in industrial processes. With increase in its application spectrum, the demand is for the enzyme with specificity. Research is focused on developing thermotolerant and pH tolerant α-amylase from microbes, modifying them genetically or applying site-directed mutagenesis to acquire desired properties in the enzyme. Commercially most of the production of α-amylase is carried out in SmF, but solid-state fermentation is being looked at as a potential tool for its production, especially applying agroindustrial residues as substrate.

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