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review

α-Amylases from Microbial Sources – An Overview on Recent Developments

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Summary

This review covers the progress made in research on microbial α-amylase, a highly demanded industrial enzyme in various sectors such as food, pharmaceuticals, textiles, detergents, *etc*. Amylases are of ubiquitous occurrence and hold the maximum market share of enzyme sales. The article surveys the α-amylase family and the major characteristics, microbial sources, production aspects, downstream processing, salient biochemical properties, industrial applications, enzyme engineering and some recent research developments.

Key words: α-amylase, α-1,4-*O*-glycosidic bonds, fermentation, process optimization, enzyme characteristics, enzyme engineering

Introduction

α-Amylases (E.C. 3.2.1.1.) are starch-degrading enzymes that catalyze the hydrolysis of internal α-1,4-*O*--glycosidic bonds in polysaccharides with the retention of α-anomeric configuration in the products. Most of the α-amylases are metalloenzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability. They belong to family 13 (GH-13) of the glycoside hydrolase group of enzymes (*1*). Amylases are one of the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to sugar syrups, to the production of cyclodextrins for the pharmaceutical industry. These enzymes account for about 30 % of the world's enzyme production (*2*). 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 (*3*). Due to the increasing demand for these enzymes in various industries, there is enormous interest in developing enzymes with better properties such as raw starch degrading amylases suitable for industrial applications and their cost effective production techniques (*4*). This review illustrates an overview of microbial α-amylases.

Alpha Amylase Family

The α-amylase family, *i.e.* the clan GH-H of glycoside hydrolyses, is the largest family of glycoside hydrolases, transferases and isomerases comprising nearly 30 different enzyme specificities (*5*). A large variety of enzymes are able to act on starch. These enzymes can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases (*2*):

1. endoamylases: cleave internal α-1,4 bonds resulting in α-anomeric products,

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2. exoamylases: cleave α-1,4 or α-1,6 bonds of the external glucose residues resulting in α- or β-anomeric products,
3. debranching enzymes: hydrolyze α-1,6 bonds exclusively leaving long linear polysaccharides, and
4. transferases: cleave α-1,4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor forming a new glycosidic bond.

Glycoside hydrolases are able to metabolize a large variety of saccharides. They have been divided into classes based on their mode of reaction and families based on their well-defined amino acid sequence similarities. Most of the starch converting enzymes belong to GH-13 family. GH-13 family can be further classified based on a larger unit called clan, which is the three dimensional structure of catalytic module. A clan may consist of two or more families with the same three-dimensional structure of catalytic domain but with limited sequence similarities, indicating that protein structure is better preserved by evolution than amino acid sequence. Among the fourteen clans (A–N) defined for glycosidases and transglycosidases, α-amylase family (GH-13) belongs to the eighth clan, clan GH-H (*6*).

The concept of this group of enzymes as the α-amylase was proposed in 1992 (*7*). According to that definition the members of this family must satisfy the following requirements: (*i*) they must act on α-glucosidic linkages and hydrolyze them to produce α-anomeric monosaccharides and oligosaccharides or form α-glucosidic linkages by transglycosylations; (*ii*) have four highly conserved regions in their primary structures consisting of catalytic and important substrate-binding sites; (*iii*) have Asp, Glu, and Asp residues as catalytic sites corresponding to the Asp206, Glu230 and Asp 297 of Taka amylase A; and (*iv*) possess a $(\beta/\alpha)_8$ or TIM barrel catalytic domain.

Characteristics of Catalytic Domain of α-Amylases

Four conserved sequence regions covering the strands β_3, β_4, β_5 and β_7 of the catalytic $(\beta/\alpha)_8$-barreled domain were identified and used for defining the α-amylase family**.** The two dimensional structure of α-amylase prototype consists of three domains, namely A, B and C. Domain A is the N-terminal TIM barrel structure, domain B consists of a long loop that protrudes between β-strand 3 and α-helix 3 and C domain with a β sheet structure linked to A domain. The $(\beta/\alpha)_8$ barrel consists of eight alternate β-strands and α-helices. The β-strands are placed parallel to one another as if on a cylinder and α-helices lie outside the cylinder. This TIM barrel contains four highly conserved regions closely related to the active site present in all α-amylases (*8*). They are: (*i*) first region: C-terminal end of β-strand 3 and histidine residue which interacts with the glucose residue of the substrate, (*ii*) second region: β-strand 4 with Asp residue, which acts as the nucleophile during catalysis, (*iii*) third region: β-strand 5 with glutamic acid residue acting as proton donor/acceptor, and (*iv*) fourth region: β-strand 7 with a histidine residue and an Asp residue that may form hydrogen bonds with glucose residue of the substrate. While the four original conserved sequence regions contain the catalytic and substrate binding residues of the individual members of the family, three additional conserved sequences were later identified and they are shown to contain amino acid residues connected to a given enzyme specificity. Two of these three regions roughly cover the strands of α_2 and α_8 of the catalytic $(\beta/\alpha)_8$ barrel and one is located near the C-terminus of domain B (*9*).

Sources of α-Amylases

α-Amylases are ubiquitous enzymes produced by plants, animals and microbes, where they play a dominant role in carbohydrate metabolism. Amylases from plant and microbial sources have been employed for centuries as food additives. Barley amylases have been used in the brewing industry. Fungal amylases have been widely used for the preparation of oriental foods. In spite of the wide distribution of amylases, microbial sources, namely fungal and bacterial amylases, are used for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production and ease of process modification and optimization (*4*).

Among bacteria, *Bacillus* sp. is widely used for thermostable α-amylase production to meet industrial needs. *B. subtilis, B. stearothermophilus, B. licheniformis* and *B. amyloliquefaciens* are known to be good producers of α-amylase and these have been widely used for commercial production of the enzyme for various applications. Similarly, filamentous fungi have been widely used for the production of amylases for centuries. As these moulds are known to be prolific producers of extracellular proteins, they are widely exploited for the production of different enzymes including α-amylase. Fungi belonging to the genus *Aspergillus* have been most commonly employed for the production of α-amylase. Production of enzymes by solid-state fermentation (SSF) using these moulds turned a cost-effective production technique. Detailed literature is available on various microbial sources for the production of amylases (*10,11*).

Fermentative Production

To meet the demand of industries, low-cost medium is required for the production of α-amylase. Both 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 (*12–15*). The contents of synthetic media such as nutrient broth, soluble starch, as well as other components are very expensive and these could be replaced with cheaper agricultural by-products for the reduction of the cost of the medium. SSF resembles natural microbiological processes such as composting and ensiling, which can be utilized in a controlled way to produce a desired product. SSF has been used for long to convert moist agricultural polymeric substrates such

as wheat, rice, soy, cassava, *etc*. into fermented food products including industrial enzymes (*16*). SSF is generally defined as the growth of microorganisms on moist solid substrates with negligible free water (*17*). 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 (*11,18–20*). SSF is preferred to SmF because of simple technique, low capital investment, lower levels of catabolite repression and end-product inhibition, low waste water output, better product recovery, and high quality production (*21*). Among the different substrates used for SSF, wheat bran has been reported to produce promising results (*22–25*). Other substrates such as sunflower meal, rice husk, cottonseed meal, soybean meal, and pearl millet and rice bran have been tried for SSF (*25,26*).

SSF technique is generally confined to the processes involving fungi. However, successful bacterial growth in SSF is known in many natural fermentations (*27,28*). The production of α-amylase by SSF is limited to the genus *Bacillus*. *B. subtilis*, *B. polymyxa*, *B. mesentericus*, *B. vulgarus*, *B. coagulans*, *B. megaterium* and *B. licheniformis* have been used for α-amylase production in SSF (*29*). The production of bacterial α-amylase using the SSF technique requires less fermentation time, 24–48 h (*30*), which leads to considerable reduction in the capital and recurring expenditure. Research on the selection of suitable substrates for SSF has mainly been centered around agroindustrial residues due to their potential advantages for filamentous fungi, which are capable of penetrating into the hardest of these solid substrates, aided by the presence of turgor pressure at the tip of the mycelium (*31*). In addition, the utilization of these agroindustrial wastes, on one hand, provides alternative substrates and, on the other, helps in solving pollution problems, which otherwise may cause their disposal (*32*). Table 1 summarizes various agroresidues reported for microbial α-amylase production.

Table 1. Various agrosubstrates used for α-amylase production

Substrate	Organism	Activity U/g	Reference
Wheat bran	*Bacillus* sp. PS-7	464 000	(*33*)
Spent brewing grain	*A. oryzae* NRRL 6270	6 583	(*34*)
Maize bran	*B. coagulans*	22 956	(*29*)
Rice bran	*Bacillus* sp. PS-7	145 000	(*33*)
Rice husk	*B. subtilis*	21 760	(*26*)
Coconut oil cake	*A. oryzae*	3 388	(*31*)
Mustard oil cake	*B. coagulans*	5 953	(*29*)
Corn bran	*Bacillus* sp. PS-7	97 600	(*33*)
Amaranthus grains	*Aspergillus flavus*	1 920	(*35*)
Gram bran	*B. coagulans*	8 984	(*29*)

Process optimization

Optimization of various parameters and manipulation of media are one of the most important techniques used for the overproduction of enzymes in large quantities to meet industrial demands (*36*). 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 (*37*). 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.

Temperature

The influence of temperature on amylase production is related to the growth of the organism. Hence, the optimum temperature depends on whether the culture is mesophilic or thermophilic. Among the fungi, most amylase production studies have been done with mesophilic fungi within the temperature range of 25–37 °C (*31,34*). A raw starch degrading α-amylase was produced by *Aspergillus ficuum* at 30 °C (*38*). Yeasts such as *Saccharomyces kluyveri* and *S. cerevisiae* were reported to produce α-amylase at 30 °C (*39*). Amylase production at optimum level has been reported between 50–55 °C for the thermophilic fungal cultures such as *Talaromyces emersonii*, *Thermomonospora fusca* and *Thermomyces lanuginosus* (*40,41*). Bacterial amylases are produced at a much wider range of temperature. *Bacillus amyloliquefaciens*, *B. subtilis*, *B. licheniformis* and *B. stearothermophilus* are among the most commonly used *Bacillus* sp. reported to produce α-amylase at temperatures 37–60 °C (*42–45*). Certain hyperthermophiles such as *Thermococcus profundus* and *Thermatoga maritima* have been reported to produce α-amylase at 80 °C (*46*). *Rhodothermus marinus*, a marine thermophilic bacterium was reported to give maximum yields of thermostable α-amylase at 61 °C (*47*). A cold active α-amylase from Antarctic psychrophile *Alteromonas haloplanktis* was reported to exhibit maximum α-amylase production at 4 °C (*48*).

pH

pH is one of the important factors that determine the growth and morphology of microorganisms as they are sensitive to the concentration of hydrogen ions present in the medium. Earlier studies have revealed that fungi required slightly acidic pH and bacteria required neutral pH for optimum growth. pH is known to affect the synthesis and secretion of α-amylase just like its stability (*49*). Fungi of *Aspergillus* sp. such as *A. oryzae*, *A. ficuum* and *A. niger* were found to give significant yields of α-amylase at pH=5.0–6.0 in SmF (*38,50,51*). α-Amylase producing yeast strains such as *Saccharomyces cerevisiae* and *S. kluyveri* exhibited maximum enzyme production at pH=5.0 (*39,52*). Bacterial cultures such as *B. subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* required an initial pH of 7.0 (*36,42,53*). *Rhodothermus marinus* was reported to yield good enzyme levels at initial pH range 7.5 to 8 (*47*). Hyperthermophilic archae such as *Pyrococcus furiosus*, *P. woesei* and *Thermococcus profundus* yielded optimum α-amylase at pH=5.0 (*46*). Thermophilic anaerobic bacteria *Clostridium thermosulfurogenes* gave maximum titres of α-amylase at pH=7.0 (*54*).

Carbon sources

Carbon sources such as galactose, glycogen and inulin have been reported as suitable substrates for the production of amylases by *B. licheniformis* and *Bacillus* sp. I-3 (*55*). Starch and glycerol were known to increase enzyme production in *B. subtilis* IMG22, *Bacillus* sp. PS-7 and *Bacillus* sp. I-3 (*33,36,56*). Soluble starch has been found as the best substrate for the production of α-amylase by *B. stearothermophilus* (*57*). *Bacillus* sp. was noted to give a maximum raw starch digesting amylase in a medium containing lactose (1 %) and yeast extract (*15*). *Thermomyces lanuginosus* was reported to give maximum α-amylase yield when maltodextrin was supplemented to the medium (*58*). A synthetic analogue of maltose, α-methyl-D-glucoside, resulted in 3-fold higher α-amylase production than inducers such as starch and maltose when supplemented to the medium as the only carbon source (*59*). Agricultural wastes are being used for both liquid and solid fermentation to reduce the cost of fermentation media. These wastes consist of carbon and nitrogen sources necessary for the growth and metabolism of organisms. These nutrient sources include orange waste, pearl millet starch, potato, corn, tapioca, wheat and rice as flours (*51,53,54*).

Nitrogen sources

Soya bean meal was found as the best nitrogen source for α-amylase production by *Bacillus* sp. I-3 (*33, 34,56*). Tanyildizi *et al.* (*36*) reported that peptone increased enzyme activity, while yeast extract exhibited no effect on α-amylase production. Strains of *B. stearothermophilus* and *B. amylolyticus* secreted maximum α-amylase in a medium supplemented with 1 % peptone, 0.5 % yeast extract and 0.5 % maltose under vigorous shaking conditions (*60*). Aiyer (*61*) compared the influence of organic and inorganic nitrogen sources and reported peptone to be a better nitrogen source for enzyme production by *B. licheniformis* SPT 278 than ammonium hydrogen phosphate, the best among inorganic nitrogen sources. L-asparagine was reported to be one of the most promising nitrogen sources for α-amylase production by *Thermomyces lanuginosus*. Yeast extract also resulted in significant α-amylase yield (*58*). Supplementation of casein hydrolysate to the medium resulted in 143 % increase in α-amylase productivity by *A. oryzae* A1560 compared to ammonia. Yeast extract along with ammonium sulphate also gave significant enzyme productivity (110 %) by *A. oryzae* (*62*). Peptone (1 %) and ammonium nitrate (1 %), when supplemented individually, gave an increase in enzyme yield in SSF using coconut oil cake as substrate, while sodium nitrate exhibited a negative influence (*31*). Addition of corn steep liquor and ammonium hydrogen phosphate to SSF involving *Amaranthus* grains as substrate by *A. flavus* gave high enzyme yield (*35*).

Surfactants

Surfactants in the fermentation medium are known to increase the secretion of proteins by increasing cell membrane permeability. Therefore, addition of these surfactants is used for the production of extracellular enzymes. Addition of Tween 80 (1.3 %) to the fermentation medium increased α-amylase production by 2-fold in *Thermomyces lanuginosus* (*63*). A study on the effect of supplementation of polyethylene glycols (PEG) (molecular mass of 600, 3000, 4000, 8000 and 20 000) in fermentation medium for α-amylase production by two *Bacillus* sp. indicated that 5 % PEG 600 and PEG 3000 yielded 31 % increase in enzyme production by *B. amyloliquefaciens* and 21 % increase by *B. subtilis* (*64*). Rao and Satyanarayana (*65*) compared the effect of various surfactants in the production media of Ca^{2+} independent α-amylase by *Geobacillus thermoleovorans*. Cholic acid, an anionic surfactant, and Tween 80, neutral surfactant, gave 2-fold enzyme yields at a concentration of 0.03 % mass per volume ratio. A mixture of PEG 8000 and SDS showed an inhibitory effect on enzyme production. Surfactants such as SDS, cholic acid, Tweens, *etc.* were reported to increase cell permeability, thereby enhancing enzyme yield (*65*).

Metal ions

Supplementation of salts of certain metal ions provided good growth of microorganisms and thereby better enzyme production (as most α-amylases are known to be metalloenzymes). Ca^{2+} ions are reported to be present in majority of these enzymes. Addition of $CaCl_2$ to the fermentation media increased the enzyme production (*34,66*). Positive results of the influence of $CaCl_2$ (0.1 %) and NaCl (0.1 %) on α-amylase production in SSF using *Amaranthus* grains as substrate were recorded (*35*). $LiSO_4$ (20 mM) and $MgSO_4$ (1 mM) increased α-amylase production by *Bacillus* sp. I-3 (*33,56*), but $FeCl_3$ and $MgSO_4$ exhibited negative influence on α-amylase production (*35*).

Moisture

Moisture is one of the most important parameters in SSF that influences the growth of the organism and thereby enzyme production. Low and high moisture levels of the substrate affect the growth of the microorganism resulting in lower enzyme production. High moisture content leads to reduction in substrate porosity, changes in the structure of substrate particles and reduction of gas volume. Bacteria are generally known to require initial moisture of 70–80 %. α-Amylase production by *Bacillus licheniformis* M27 was highest with 65 % initial moisture content in an SSF system. Significant decrease in enzyme production was observed with high increase in moisture content, which was due to the decrease in the rate of oxygen transfer. Studies indicated that enzyme titres could be increased significantly by agitation of the medium with high moisture content (*67*). A thermotolerant *B. subtilis* required initial moisture of 30 % for its growth and maximum enzyme production (*26*). Moisture content of 65 % was required by *B. coagulans* for optimum α-amylase production on wheat bran (*24*). *A. oryzae* (on wheat bran) and *Streptomyces rimosus* (on a mixture of sweet potato and peanut meal residue) required an initial moisture content of 65 % for maximum enzyme yield in SSF (*31,68*). Maximum α-amylase production by *Thermomyces lanuginosus* ATCC 58157 by SSF on wheat bran was at 90 % moisture content (*22*).

Particle size of substrate

In SSF, particle size of the substrate affects growth of the organism and thereby influences the enzyme pro-

duction. The adherence and penetration of microorganisms as well as the enzyme action on the substrate clearly depend upon the physical properties of the substrate such as the crystalline or amorphous nature, the accessible area, surface area, porosity, particle size, *etc*. In all the above parameters, particle size plays a major role because all these factors depend on it (*24*,*69*). Smaller substrate particles have greater surface area for growth but inter-particle porosity is lower. For larger particle sizes, the porosity is greater but the saturated surface area is smaller. Hence, determination of particle size corresponding to optimum growth and enzyme production is necessary (*69*). A mathematical model has been described, which enables the quantification and prediction of the extent of degradation of the substrate particles with fermentation time for any given initial size of substrate particles. Studies on release of α-amylase have been recorded and a gradual increase was noticed up to 72 h for *A. niger* and 36 h for *B. coagulans*. The reduction of particle size was up to 60 % at the end of the fermentation time, 72 and 36 h for *A. niger* and *B. coagulans*, respectively. Wheat bran having a particle size between 500–1000 μm gave better enzyme yields by *B. subtilis* when compared with the larger particles (*26*).

Oxidative stress

Increase in enzyme production must be attained without altering the economics of enzyme production. Induced oxidative stress is used for enhanced enzyme production. Oxidative stress results when the production rate of reactive oxygen species (ROS) exceeds the capacity of the cell for disposal. *B. subtilis* treated with ROS inducing agents such as hydrogen peroxide and hypochlorous acid (2.2-fold) exhibited increased specific levels of extracellular enzymes as well as growth rate. Microarray data analyzed indicated that the increase in enzyme productivity was due to the adaptive responses induced under oxidative stress (*43*).

Purification

Downstream processing for the production of pure enzymes can generally constitute a major percentage of overall production cost, especially if end purity requirements are stringent. Purification processes in downstream processing after fermentation strongly depend on the market, processing cost, final quality, and available technology. Most enzymes are purified by chromatographic techniques after crude isolation by precipitation and membrane separations. The need for large-scale cost effective purification of proteins has resulted in evolution of techniques that provide fast, efficient and economical protocols in fewer processing steps (*70*). Purification techniques that produce homogeneous preparation of amylases in a single step are given in Table 2.

Enzyme Characteristics

The properties of α-amylases such as thermostability and pH profile should match its application. Hence, the diversity of the applications creates the need to search for novel α-amylases with novel and improved properties. The rate of hydrolysis of starch by α-amylase depends on many process conditions such as temperature, pH, nature of substrate, substrate concentration, enzyme concentration, presence of Ca^{2+} ions and other stabilizing agents. α-Amylases with characteristics suitable for the industrially relevant process conditions and applications have to be appropriately selected as per the demand.

Effect of temperature

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 (*4*). Most of the α-amylases are reported to have one, or two intrinsic Ca^{2+} ions, present near the active centre formed by the two domains, A and B. The Ca^{2+} is necessary for enzyme folding and enzyme stability. Secondary calcium binding sites have also been reported, which enhanced the thermostability. Saboury (*75*) reported the presence of 17 different secondary binding sites for calcium in α-amylase of *B. amyloliquefaciens*, which were responsible for stabilization of the enzyme against thermal and surfactant denaturation. Weemaes *et al*. (*76*) studied the stability of α-amylases produced by *B. amyloliquefaciens*, *B. licheniformis* and *B. stearothermophilus* under combined high temperature and pressure and the results indicated that α-amylase produced by *B. licheniformis* was the most stable enzyme among the three. Different sources of α-amylases exhibiting considerable temperature stability are given in Table 3.

Effect of pH

Natural pH of starch slurry is generally around 4.5. It is known that gelatinized starch is more susceptible to degradation than nongelatinized starch. 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

Table 2. Methods of one-step purification of α-amylases

Method	Adsorbent	Yield/%	Purification fold	Reference
Affinity adsorption chromatography	β-cyclodextrin-iminodiacetic acid-Cu^{2+}	95	–	(*71*)
Expanded bed chromatography	Alginic acid-cellulose cell beads	69	51	(*70*)
High speed counter current chromatography	PEG4000-aqueous two-phase system	73.1	–	(*72*)
Magnetic affinity adsorption	Magnetic alginate microparticles	88	9	(*73*)
Substitute affinity method	Insoluble corn starch at 4 °C	78	163	(*74*)

Table 3. α-Amylases exhibiting considerable temperature stability

Organism	Temperature range/°C	Residual activity/%	Temperature optimum/°C	Reference
Lactobacillus manihotivorans	50–60	70 (50 °C for 1.0 h)	55	(*77*)
Bacillus sp. I-3	65–100	50 (80 °C for 2.5 h)	70	(*56*)
Pyrococcus furiosus	80–100	50 (98 °C for 13 h)	100	(*46*)
Thermobifida fusca NTU22	50–60	70 (60 °C for 3 h)	60	(*78*)
Cryptococcus flavus	50–60	60 (60 °C for 60 min)	50	(*79*)
Aspergillus tamarii	50–60	90 (65 °C for 3 h)	55	(*80*)
Scytalidium thermophilum	55–65	50 (55 °C for 25 min)	60	(*81*)

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 (*46*). Peptide bond hydrolysis never occurs in the helical and beta structures (*82*). 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. Studies on 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 (*83*). α-Amylases having pH optima and stability in acidic, neutral and alkaline range are given in Table 4.

Effect of metal ions

Most of amylases are known to be metal ion-dependent enzymes, namely divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, *etc.* (*11*). Ca^{2+} was reported to increase α-amylase activity of an alkaliphilic *Bacillus* sp. ANT-6 (*4*). 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 (*56*). Ca-independent enzymes have also been reported. Malhotra *et al.* (*87*) reported the presence of a thermostable α-amylase from *B. thermooleovorans* NP54, which did not require calcium ions for its activity or production. Zn^{2+} has varied effect on different amylases. Zn^{2+} was reported to inhibit thermostable α-amylases from a thermophilic *Bacillus* sp. suggesting that the inhibition with the ion determined the thermostability of enzyme. The presence of Zn^{2+} decreased the activity of ANT-6 enzyme indicating its high thermostability (*4*). Detailed lists of metal ions showing either positive or negative effects on amylase activity are shown in Table 5.

Effect of inhibitors

The effect of metal chelators and chemical compounds helps to study the characteristics of the enzyme. The effect of inhibition of metal chelators indicates the requirement of certain metal ions for enzyme activity. Najafi and Kembhavi (*74*) studied the effects of chemical modifiers on enzyme activity of α-amylase from marine *Vibrio* sp. The results suggested the involvement of amino acids such as Lys, Trp, Asp/Glu and His in enzyme activity. Table 6 shows effect of some inhibitors on amylase activity.

Effect of substrate

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. Amylose was found to be the best substrate for *L. manihotivorans* LMG 18010T α-amylase. The relative activity observed on amylose was 1.6 times higher than that obtained on starch. The hydrolysis of amylopectin was 90 % in relation to starch. Glycogen and dextrins with 10 and 20 DP were hydrolyzed at low rate reaching 6.7, 7.3, and 8.9 %, respectively, and no activity was found on α- and β-cyclodextrins (*77*). Natural starches such as maize starch (*91*), raw sago starch (*78*), corn starch (*74,92*) and wheat starch (*85*) increased α-amylase activities.

Table 4. α-Amylases having different optimal pH and stability

Organism	pH range	Residual activity/%	pH optimum	Reference
Bacillus sp. I-3	5.0–5.5	80 stable	7.0	(*56*)
Lactobacillus manihotivorans	4.0–6.0	90	5.5	(*77*)
Bacillus sp. ANT-6	9.0–13	55 (pH=10 for 15 h)	10.5	(*4*)
Bacillus KSM-K38	6.0–11.0	80 (pH=11 for 30 min)	8.0–9.5	(*84*)
Bacillus sp. PS-7	5.0– 8.0	96 (pH=5.0 for 90 min)	6.5	(*33*)
Cryptococcus sp. S-2	5–7		6.0	(*85*)
Aspergillus kawachii IFO 4308	2.0–6.5	90 (pH=2.0 for 30 min)	5.0	(*86*)

Table 5. Concentration effect of metal ions on the activity of α-amylases

Metal ion	Concentration effect/mM	Organism	Reference
Ca^{2+}	5 promoting	*Lipomyces starkeyi*	(*88*)
	5 promoting	*Bacillus* sp. ANT-6	(*4*)
K^+	10 promoting	*L. manihotivorans* LMG 18010	(*77*)
Na^+	5 promoting	*Bacillus* sp. L1711	(*89*)
	5 inhibitory	*Bacillus* sp. ANT-6	(*4*)
Co^{2+}	5 promoting	*Bacillus* sp. L1711	(*89*)
Mg^{2+}	5 promoting	*Vibrio* sp.	(*74*)
Ba^{2+}	10 promoting	*L. manihotivorans* LMG 18010	(*77*)
Mn^{2+}	1 promoting	*Bacillus* sp. I-3	(*56*)
	1 inhibitory	*Bacillus* KSM-K38	(*84*)
Zn^{2+}	10 promoting	*L. manihotivorans* LMG 18010	(*77*)
	5 inhibitory	*Bacillus* sp. ANT-6	(*4*)
Fe^{2+}	1 promoting	*Bacillus* sp. I-3	(*56*)
	20 inhibitory	*Cryptococcus flavus*	(*79*)
Cu^{2+}	1 promoting	*Bacillus* sp. I-3	(*56*)
	10 inhibitory	*L. manihotivorans* LMG 18010	(*77*)
Ni^{2+}	5 promoting	*B. halodurans* LBK 34	(*90*)
Al^{3+}	10 inhibitory	*L. manihotivorans* LMG 18010	(*77*)
Fe^{3+}	5 inhibitory	*B. halodurans* LBK 34	(*90*)
Hg^+	1 inhibitory	*Bacillus* sp. I-3	(*56*)
	1 inhibitory	*Thermobifida fusca* NTU22	(*78*)

Table 6. Concentration effect of denaturing agents on α-amylase activity

Inhibitor	Concentration effect	Organism	Reference
EDTA	(10 mM) resistant	*Bacillus* sp. L1711	(*89*)
	Inhibitory	*Bacillus* sp. I-3	(*56*)
	100 mM resistant	*Bacillus* KSM-K38	(*84*)
SDS	(1 %) slight inhibition	*B. halodurans* LBK 34	(*90*)
Urea	8 M inhibitory	*Bacillus* sp. ANT-6	(*4*)
H_2O_2	1.8 M resistant	*Bacillus* KSM-K38	(*84*)
EGTA	1 mM inhibitory	*L. starkeyi*	(*88*)
	10 mM resistant	*Bacillus* sp. L1711	(*89*)

Raw starch digesting property

Gelatinization of starch requires a high-energy input resulting in increased production cost of starch-based products. Hydrolysis of raw starch below gelatinization temperatures has gained importance in view of energy costs, effective utilization of natural resources and viscosity problems (*56*). It turns out to be an economically superior alternative to the conventional process, which uses pregelatinised starch as substrate (*93*).

Among microorganisms, fungi such as *Aspergillus* sp., *Rhizopus* sp. and *Corticium rolfsi* were reported to be good producers of raw starch digesting amylases. *Bacillus* sp. (*e.g. B. stearothermophilus*) was also reported to produce amylases capable of digesting raw starch granules. A thermostable α-amylase from *Bacillus* sp. I-3 hydrolyzed raw potato starch at a concentration of 12.5 % within 12 h (*56*). The digestibilities of α-amylase reported from *A. niger* from rotting cassava bagasse were of the order maize starch>cassava starch>sorghum starch>soluble potato starch (*93*). Raw starch degrading α-amylase from a protease-negative *A. ficuum* mutant dissolved raw corn starch granules (*38*). Raw starch digesting α-amylases was reported to lose raw starch binding domain by the action of protease present in the culture filtrate. A liquefying α-amylase (RBLA) from *Bacillus* sp. was reported to act on raw starches over a wide range of pH=5.0–9.0 (*38,94*). Iefuji *et al.* (*85*) compared raw starch digesting abilities of a thermostable α-amylase from the yeast *Cryptococcus* sp. S-2 and Taka amylase. α-Amylase from *Cryptococcus* sp. S-2 exhibited a stronger digestibility towards raw potato starch and weaker activity for wheat, corn, rice and sweet potato starches while Taka amylase was reported to exhibit negligible raw starch digesting activity.

Stabilization

Enzymes are mostly proteins with a labile nature. Inactivating agents such as temperature, pH, chemicals, *etc.* impair the native conformation of an enzyme, thus affecting its catalytic activity. The utility of an enzyme depends mainly on its operational and storage stability (*95*). Therefore, enzyme stability and its stabilization are crucial factors in the application of enzymes. Stability of enzymes can be achieved by screening intrinsically stable enzymes, adding stabilizing agents, chemical modification, immobilization, protein engineering, *etc.* Among the stabilizing additives, as mentioned previously, Ca^{2+} has been widely used to attain thermostability (*4,96,97*). It has also been documented that Na^+, K^+, NH_4^+ and bovine serum albumin have protective effect on α-amylase (*98*). Polyols (ethylene glycol, propenediols and glycerol), dimethylformamide and dimethyl sulfoxide increased the half-life of *B. stearothermophilus* α-amylase twofold. These compounds stabilized the enzyme against thermal denaturation through ionic interactions (*99*). Effect of the addition of polyethylene glycols of different molecular mass ranging from 400–8000 Da has been studied and was found to increase the activity and stability of α-amylases from different sources (*100*). Comparative study of various stabilizing agents was done and sorbitol was found to provide the highest degree of protection against denaturation by increasing T_m and preventing deamidation of amino acid residues (*101*). Heat inactivation kinetics of α-amylase of *A. oryzae* was studied and results indicated that the thermal stability could be maintained better in maltodextrin systems at reduced moisture content than in aqueous solutions (*102*). Enhancement of thermal stability of α-amylase from *B. amyloliquefaciens* was achieved by the modification of Lys residues using citraconic anhydride (*103*).

Immobilized Amylases

The catalytic activity of an enzyme depends on its three dimensional structure and conformation. Any permanent or temporary change in its natural conformation

caused by any physical or chemical agent affects its catalytic function. Thus, it becomes necessary to protect enzymes from its destructive elements in order to sustain its catalytic activity. Immobilization of enzymes is one of the methods for protecting and stabilizing the enzymes, thereby enhancing their properties and their repetitive utilization either in batch, or continuous mode (*104*). Immobilization of enzymes prevents their deactivation by various physical and chemical denaturing agents and thereby enhancing their operational stability. Certain immobilization techniques developed for α-amylases are given in Table 7.

Cloning of α-Amylase Genes

Cloning of genes has been done extensively for the molecular study of proteins, their hyperproduction and protein engineering. Cloning of α-amylases has been done for studying their sequence, characteristics, hyperproduction and expression and for enzyme engineering (*11*). α-Amylase genes from different fungal and bacterial sources have been cloned in appropriate host organisms using suitable vectors (Table 8).

Enzyme Engineering

Demands for α-amylases with different physiological and biochemical properties by different industries necessitate the search and development of enzymes with novel properties. Enzyme engineering is known to be a promising technique to achieve this goal. Engineering of enzymes consists of integrating desired properties in the appropriate gene and these properties may include high thermostability, wide pH profile, Ca independence, raw starch degrading ability, activity at high concentration of starch, protease resistance, insensibility to catabolite repression and hyperproduction. Introduction of three mutations Asn172→Arg, His156→Tyr and Ala181→Thr was reported to increase the thermostability of α-amylase of *B. licheniformis* by 5-fold (*116*). Thermostability at low pH was achieved by substitutions Met15→Thr/Asn 188→Ser (*117*). Negative regulatory mechanism in the presence of glucose, observed in *Streptomyces* sp., was relieved by cloning an α-amylase gene (amt TO1) and a mutated dyatic symmetric element present in promoter region (3-bp change) of α-amylase gene in high copy number replicating cloning vector pIJ702 and was expressed in both the original host *Streptomyces* sp. TO1 and *S. lividans* Tk24 (*118*). Stabilizations of the proteins have also been done by insertion of prolines in loop regions (*119*). Error prone PCR in combination with gene shuffling of α-amylase gene of *B. amyloliquefaciens* and two of its mutants resulted in another mutant, which was active over a broader pH range than the wild type, also exhibiting a 5-fold higher activity at pH=10 (*120*). It has also been shown that incorporation of hydrophobic residues at the surface of the enzyme of *B. licheniformis* has resulted in increased resistance to high temperature (*121*).

Table 7. Prominent immobilization methods for α-amylases

Source	Immobilizing agent	Retained activity after immobilization/%	Reference
Bacillus sp.	Copolymers of butyl acrylate and ethylene glycol dimethacrylate activated with glutaraldehyde	60.2 (79.2 % activity after 1 month's storage at 4 °C)	(*105*)
A. oryzae	Cu^{2+} chelated poly(ethylene glycol dimethacrylate-*n*-vinyl imidazole) matrix *via* adsorption	– (70 % retained for 20 cycles)	(*106*)
B. subtilis	Bis(2-ethylhexyl) sulfosuccinate sodium salt (AOT) coated α-amylase entrapped into butylacrylate-acrylic acid copolymer (BuA/AAc)	90 (60 % activity retained for 15 cycles)	(*107*)
B. licheniformis	Poly(hydroxyethyl methacrylate-co-glycidyl methacrylate)	76	(*108*)
B. subtilis	Ca-alginate gel capsules	70 (90 % activity retained for 20 cycles)	(*109*)
α-amylase: glucoamylase coenzymes (1:3)	(1) Hydrophilic silica gel (2) DEAE-cellulose entrapped in alginate beads	92.3 % activity retained for 10 cycles 88.9 % activity retained for 10 cycles	(*110*)

Table 8. Cloning of α-amylase genes from different sources

Gene source	Recombinant host	Vector	Reference
Aspergillus kawachii IFO 4308	*Saccharomyces cerevisiae*	pYcDE1	(*111*)
Bacillus amyloliquefaciens	*Escherichia coli*	pETAM (derived from pKK233-2 and pET21d)	(*112*)
Halothermothrix orenii	*Escherichia coli*	pBluescript SK^+	(*113*)
Alteromonas haloplanktis (psychrophile)	*Escherichia coli*	pUC12	(*114*)
Lipomyces starkeyi	*Escherichia coli*	pGEM-T	(*88*)
Lipomyces kononenkoae	*Saccharomyces cerevisiae*	YIp5	(*52*)
Thermococcus hydrothermalis	*Escherichia coli*	pBluescript II KS^-	(*115*)

Table 9. Uses of amylases in various sectors of industry

Sector	Uses	Reference
Food industry	Production of glucose syrups, crystalline glucose	(*2*,*122*,*125*)
	Production of high fructose corn syrups	
	Production of maltose syrups	
	Reduction of viscosity of sugar syrups	
	Reduction of haze formation in juices	
	Solubilization and saccharification of starch for alcohol fermentation in brewing industries	
	Retardation of staling in baking industry	
Detergent industry	Used as an additive to remove starch based dirts	
Paper industry	Reduction of viscosity of starch for appropriate coating of paper	
Textile industry	Warp sizing of textile fibers	
Pharmaceutical industry	Used as a digestive aid	

Applications

α-Amylases are the enzymes first to be commercially produced and marketed. Dr. J. Takamine established the first industrial production of α-amylase from *A. oryzae* known as »Taka diastase«, which was used as a digestive aid. The global market for enzymes was about $2 billion in 2004. It is expected to have an average annual growth rate of 3.3 %. The share of carbohydrases comprising amylases, isomerases, pectinases and cellulases is about 40 % (*122*). The food and beverage sectors utilize 90 % of the carbohydrases produced. The annual sale of α-amylases in global market is estimated to be $11 million (*123*). The world production of α-amylases from *B. licheniformis* and *Aspergillus* sp. was about 300 tonnes of pure enzyme protein per year (*124*). Table 9 shows vivid applications of amylases.

Conclusions

α-Amylases are one of the most widely used enzymes required for the preparation of fermented foods. Apart from food and starch industries, in which demand for them is increasing continuously, they are also used in various other industries such as paper and pulp, textile, *etc.* (*125*). 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 submerged fermentation, 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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