BIOINFOLET 18 (3) : 334 - 341, 2021

STRUCTURAL AND FUNCTIONAL PROPERTIES OF AMYLASES

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ABSTRACT

Amylases have applications in food, textile, detergent, fermentation, paper and pharmaceutical industries. The production of amylases is normally carried out under submerged fermentation. However, solid state fermentation also appears promising. With the advancement in biotechnology, the application of amylases have expanded in medicinal, clinical and analytical chemistry. In view of increased application of amylases in various fields, development of novel sources of amylase production, qualitative improvement in enzyme and quantitative enhancement in its production is essential. Present communication gives information on sources, functional properties, production and application of enzyme amylases.

Key words: Amylase, Strain improvement, Application, Optimization

In recent years, hydrolytic enzymes e.g. proteases, amylases, cellulases, invertases are playing an important role in brewing, detergent, and food industries (Doss and Anand, 2012).

Amylases

Amylases hydrolyze glycosidic linkages in starch molecule. Out of these α Amylase is an endo-enzyme, catalyzing hydrolysis of α -1,4- glycosidic linkages of amylose and amylopectin molecules. The end products of α amylase mediated reaction are oligosaccharides of varying length (Vander Maarel et al., 2002), which constitute a mixture of maltose , maltotriose, and branched oligosaccharides of 6-8 glucose units containing both α -1,4 and α -1,6 glycosidic linkages (Whitcomb and Lowe, 2007). Liquefying α amylases hydrolyze about 30 to 40 % and saccharifying α amylases about 50 to 60 % glycosidic linkages in starch.

The enzyme α Amylase is a calcium containing meatloprotein, eherein Caacts as a cofactor. It is monomeric, with a single polypeptide chain folded into three domains. The calcium (Ca^{+2}) seems to be responsible for stabilization of the three dimensional structure of amylase enzyme (Muralikrishna and Nirmala, 2005).

β-Amylase is, however, an exo-hydrolytic enzyme, which hydrolyses α-1, 4 glucan linkages from non-reducing end of a polysaccharide, and to yields maltose units. β - Amylases are tetrameric proteins made up of four identical subunits (Henrissat and Bairoch, 1993).

On the other hand, γ Amylase is responsible for cleavage of α (1-6) glycosidic linkages, in addition to the α (1-4) linkages, at the non-reducing end of amylose and amylopectin molecules, yielding glucose as an end product (Aleshin et al., 1992)

Sources of amylase

As amylase play crucial role in carbohydrate metabolism, all living systems produce enzyme amylase. The enzyme abundantly occurs in wheat, rye, barley, rice and maize, (Qadeer et al*.,* 1980). Enzyme α-amylase has been reported in the pancreas and saliva of animals. Calcium and chloride ions are necessary for its stability in animals (Granger et al., 1975). Salivary amylases hydrolyze starch releasing dextrin and maltose at optimum pH value of 6.7. addition,

microorganisms like bacteria, fungi and actinomycetes are good producers and sources of amylases. Those are employed for Industrial production of amylase. Production of microbial amylase is cost effective, reproducible and require limited time and space (Burhan et al., 2003). In addition, the microorganisms can easily be subjected to genetic manipulations for greater amylase production. In general, it has been observed that microbial enzymes have higher stability, broader specificity and greater turn over number, making them more useful in variety of applications (Saroja et al., 2000; Teodoro and Martin, 2000; Shiau and Hung, 2003; Sajedi et al., 2005; Sodhi et al., 2005; Ten et al., 2005; Sajitha et al., 2010; Mohammadabadi and Chaji, 2012).

Various fungal species have been proved to be the sources of amylases. Commercial production of amylase has been reported by employing *Aspergillus spp.* e.g. *A. fumigatus, A. niger, A. flavaus, A. oryzae, A. Tamarie,* and *A. kawachii*. (Haq et al., 2002; Shiau and Hung, 2003; Nagamine et al., 2003; Bakri et al., 2009). Efficient amylase-producing species of *Penicillium* (*P. brunneum, P. fellutanum, P. expansum, P. chrysogenum, P. roqueforti, P. janthinellum, P. camemberti, and P. olsonii), Pycnoporus sanguineus, Cryptococcus flavus,* and *Mucor sp.* (Hussain et al., 2013; Sundarramand and KrishnaMurthy, 2014; De Souza and Magalhaes, 2010) have also been reported.

Aquatic actinomycetes can also be considered as a good and important source of amylase. There are various reports on amylase production by actinomycetes, *Streptomyces clavifer*, *Strepttomyces spp*. *Streptomyces rimosus, Thermomyces lanuginosus, Thermomonospora curvata* being prominent among them. *Streptomyces aureofasciculus* **(**Poornima et al., 2008) and *Streptomyces guancidicus*-ASD-KT852565 (Krishnan and Kumar., 2015) isolated from marine soil produce high levels of amylase.

Amylase production from bacteria is cheaper and faster than that from other microorganisms. Among the bacteria, *Bacillus sp.* Is most commonly used for α-amylase production *B. subtilis,* (Rajput et al., 2013), *B. stearothermophilus , B. licheniformis and B. amyloliquefaciens* (Niazi et al., 2010) are also known to be good producers of α-amylase.

Extreme thermophilic and mesophilic bacteria (*Rhodothermus marinus, Bacillus megaterium, Bacillus macerans* and *B. coagulans*) were employed by Saroja et al., (2000) for the production of stable amylases. Most thermo-stable α amylase is produced from *B. licheniformis* (Hmidet et al., 2010) for Industrial use. Highly thermo-stable α amylases are also obtained from hyper-thermophilic and thermophilic species such as *Pyrococcus furiosus*, *Thermococcus hydrothermalis*, *T. profundus*, *Sulfolobus acidocaldarius* and *S. solfataricus*.

Similarly, halophillic amylases have been isolated from bacteria, *Chromohalobacter sp.*, *Halobacillus sp*. *Haloarculahispanica*, *Halomonas meridian*, and *Bacillus dipsosauri* for use in many industrial processes (Prakash et al., 2009).

Many alkaline amylases have been found in the cultures of *Bacillus sp*. These alkaline amylases are saccharifying type, except for the enzymes from *Bacillus sp.* strain 707 and *B. licheniformis*TCRDC-B13 (Bajpai and Bajpai, 1989).

Purification of amylase

In pharmaceutical, clinical sectors highly purified enzymes are required along with its. homogeneity (Gupta et al., 2003). Laboratory scale purification for α-amylase includes various combinations of gel filtration, ion exchange, hydrophobic interactions and reverse phase chromatography. Alternatively, α-amylase extraction protocols using ethanol, acetone and ammonium sulfate precipitation (Khoo et al., 1994), and ultrafiltration have been proposed (De Moraes et al., 1999) for this purpose.

During the studies undertaken on amylase production from *Preussia minim* by Erdal and Taskin, (2010), the enzyme was precipitated using trichloroacetic acid. It was then fractionated using Sephadex G-200 gel filtration and DEAE- Sepharose ion exchange

chromatography

Bolton et al., (1997) purified α-amylase using a combination of ammonium sulphate precipitation, ion exchange chromatography and gel filtration. Similarly, α-amylase from *Clostridium perfringens* Type A using DEAE Sephacel chromatography and Sephacryl S-100 HR column was also purified (Demirkan, 2011).

Amritkar et al. (2004) purified amylase from *Bacillus subtilis* using precipitation with ammonium sulfate, TSK Toyopeal column chromatography, ultrafiltration, dialysis and SP Sepharose column chromatography.

Functional properties of Amylase

Molecular weight of most microbial α-amylases fluctuate within the range of 40 - 70 kDa. Gupta et al., (2003) reported the highest molecular weight of α-amylases (210 kDa) isolated from *Chloroflexus aurantiacus*, whereas, lowest molecular weight (10 kDa) was reported, when it was isloated from *Bacillus caldolyticus*. Among the amylase-producing yeasts, molecular weights of amylases isolated from *Cryptococcus, Lipomyces kononenkoae* CBS 5608, *S. cerevisiae and Schwanneoimyces alluvius* UCD 5483 were 66.0, 76.0, 54.1 and 61.9 kDa (Wanderley et al., 2004).

Temperature

The optimum temperature for maximum activity of α amylase is closely related to the optimum temperature required for the growth of organism, from which it is isolated. The highest optimum temperature has been reported as 100 and 130 °C for amylases isolated from *Pyrococcus furiosus* and *Pyrococcus woesei*, respectively (Fogarty and Kelly, 1980), while lowest optimum temperature was recorded within the range of 25 - 30°C for enzyme amylase isolated from *Fusarium oxysporum* .

Most of the commercial applications of amylases are at higher temperatures, and hence, thermal stability of amylase is an important attribute. It has been reported that amylase from *Bacillus licheniformis* CUMC 305 was quiet stable at 100°C for 4 hr (Krishnan and Chandra, 1983), while that from *Thermococcus profundus* DT5432 was found to be stable at 90°C for 15 min. (Chung et al., 1995).

pH

α Amylases from most of the bacteria, fungi, and terrestrial yeasts have an optimum pH in acidic to neutral range (Pandey et al., 2000). Optimum pH for amylase from *Alicyclobacillus acidocaldarius* is 3.0 (Schwermann et al., 1994), whereas optimum pH for amylase from alkalophilic *Bacillus sp*. is 9.0 to 10.5 (Shinke et al., 1996). Extremely alkalophilic α -amylase from *Bacillus sp.* GM8901 shows optimum pH of 11-12 (Kim et al., 1995). Thus α-Amylases show stability over wide range of pH values from 4.0 to 11 (Khoo et al., 1994).

Effect of Metal Ions

Most amylases depend on the presence of divalent ions like Ca^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Fe^{2+}, etc. (Pandey et al., 2000). There are some examples of Ca-independent amylases, Malhotra et al. (2000) reported thermostable α-amylase from *B. thermooleovorans* NP54, which is independent and does not require calcium ion for its activity.

Inhibitors

Various inhibitors have been investigated for different amylases e.g. heavy metal ions, N-bromosuccinimide, sulphydryl group reagents, p-hydroxyl mercuribenzoic acid, iodoacetate, dimethyl aminobenzaldehyde, p-chloromercuribenzoic acid, guanidine hydrochloride, EDTA and EGTA. (Gupta et al., 2003).

Substrate

Starch molecule is heterogeneous in its structure, in terms length of polymer and extent of branching, as a result of which. amylases from different sources tend to show different activity on different starch substrates.

α-amylase show highest activity with starch (wheat, maize, corn and sago); amylose, amylopectin, cyclodextrin, glycogen and maltotriose as substrates, in the decreasing order of preference (Kunamneni et al., 2005). In addition substrate specificity of α-amylase varies with microorganism.

Stabilization

Operational and storage stability of an enzyme plays an important role in its industrial use (Fagain, 2003). Ca^{+2}, ethylene glycol, propenediols, glycerol, dimethyl formamide and dimethyl sulfoxide enhance stability of amylases (Burhan et al*.,* 2003).

Laboratory scale production

There are two methods for production of α amylase, Submerged fermentation (SmF) and Solid state fermentation (SSF). Submerged fermentation is carried out by using liquid broth cultures, grown in under optimum conditions, either in shaking flask or in fermenters. Most of the industrial enzymes are produced under submerged fermentation, as various parameters, affecting the production of amylase can be easily manipulated in this method. Amylase production under submerged fermentation has been recommended particularly with the cultivation of *Bacillus amyloliquefaciens* (Tanyildizi et al., 2007), *Halobacillus* sp MA-2 (Amoozegar et al., 2003), *Aspergillus niger* ATCC 16404 (Djekrif-Dakhmouche et al., 2006) and *Penicillium fellutanum* (Erdal and Taskin, 2010).

Amylases have also been produced through solid state fermentation by cultivating *Aspergillus niger*, *Aspergillus oryzae* CBS 125-59 (Murado et al., 1997), *Thermomyces lanuginosus* ATCC 58160 (Kunamneni et al., 2005), *Pycnoporus sanguineus* (Siqueira et al., 1997), *Bacillus* sp. AS-1 (Soni et al., 2003). This method is economically feasible, since the production does not involve costly fermenter vessels and associated inputs in terms of power, steams and antifoam agent etc. (Counto and Sanroman, 2006).

Applications

Amylases play important role in food, textile, detergent, fermentation, paper and pharmaceutical industries. The most widespread application of amylases appears in the production of fructose and glucose syrup. It causes liquefaction, and saccharification of starch. Partial hydrolysis of starch is due to amylase activity results in decreased viscosity, followed by production of glucose and fructose. Thermo-stable α-amylase from *B. licheniformis* and *B. amyloliquefaciens* are used for manufacturing of maltose (Sugimoto,1977). Amylases are used in baking industries in order to improve texture of the dough. The enzyme serves as anti-staling agent, thereby improving shelf life of baked products. More than 90 % liquid detergent preparations have amylase as one of the ingredients (Mitidieri et al., 2006). In textile industries amylases are used to remove starch based size, for improved and uniform wet processing. In paper industries amylases are used to reduce the viscosity of starch, for more effective sizing (Bruinenberg et al., 1996). Starch present in the waste water can be treated with amylase, to eliminate it (Kingspohn et al., 1993). Enzyme amylase, in the form of taka-diastase, is used as a digestive enzyme. Now a days amylases are gaining diagnostic significance, as during acute pancreatitis, peptic ulcer, ileum strangulation, torsion of an ovarian cyst, the levels of amylase increases (Das et al., 2011).

Optimization of various parameters and manipulation of media are necessary for increased production of enzymes (Tanyildizi et al., 2005). Various physical and chemical factors, such as time of incubation, temperature, pH, carbon sources, sources of starch, concentration of starch, nitrogen sources, phosphate, different metal ions, agitation are known to affect the production of amylase.

Mutagenesis is one of the approaches to isolate mutants with high yielding capacities of amylase. Chemical mutagens such as nitrous acids, ethyl methyl sulphonate (EMS), N-Methyl-N'-Nitro-N-

Nitrosoguanidine (NTG) are used for mutations in bacteria (Adelberg et al., 1965). Production of α-amylase by the existing microorganisms may get considerably improved through mutagenesis. Haq et al., (2002) used UV radiations as a mutagenic agent to obtain stable mutant of *Bacillus* with enhanced amylase production. Shah et al. (2003), also isolated mutant of *Bacillus subtilis* using NTG and UV radiation. This resulting mutant produced five-fold increased amylase production. Similarly a mutant of *Bacillus subtilis,* with higher yields of amylase was produced by Rani and Rukmini, (2011). Shafique et al.(2009, 2010), used UV radiation and ethyl methane sulphonate (EMS) treatment to produce higher amylase producing Af-UV-5.3 and Af-Ch-5.7 mutants of *Aspergillus flavus* and An-UV-5.6 , An-Ch-4.7 mutants of *Aspergillus niger*.

Genetic engineering and recombinant DNA technology are current molecular techniques, which are used to promote efficient enzyme production. Table 1 gives an account on genetic source, recombinant host and vector for amylase cloning.

Table 1 Cloning of amylase gene from various sources

Gene source	Recombinant host	Vector	Reference
Halothermothrix orenii	*E.coli*	pTrcHis	Mijts and Patel, 2002
Bacillus stearothermophilus	*Bacillus stearothermophilus and Bacillus subtilis.*	pTB90 and pTB53.	Aiba et.al.,1983
Thermococcus hydrothermalis	*Escherichia coli*	pBluescript II KS-	Leveque et al., 2000
Aspergillus kawachii IFO 4308 pYcDE1	*Saccharomyces cerevisiae*	pYcDE1	Kaneko et.al.,1996
Lipomyces starkeyi	*Escherichia coli*	pGEM-T	Kang et al., 2004
Bacillus licheniformis	*Escherichia coli*	pET24d	Shahhoseini et al., 2003
Pyrococcus furiosus	*Bacillus amyloliquefaciens*	pUBC19	Wang et al., 2016
Halothermothrix orenii	*Escherichia coli*	pBluescript SK+	Mijts et.al.,2002
Bacillus amyloliquefaciens	*Escherichia coli*	pETAM (derived from pKK233-2 and pET21d)	Demirkan et al., 2005
Lipomyces kononenkoae	*Saccharomyces cerevisiae*	YIp5	Knox et al., 2004
Alteromonashalo planktis (psychrophile)	*Escherichia coli*	pUC12	Feller et al., 1998

Enzyme engineering is a promising technique for development of enzyme with novel properties. Engineering of enzymes involves integration of desired properties in the gene, which is responsible for enzyme production. These properties include high thermo-stability, wide pH profile, Ca independence, raw starch degrading ability, stability at high concentration of starch, protease resistance, insensibility to catabolic repression and hyper-production.

Acknowledgements:

The author is thankful to the Principal, Tuljaram Chaturchand college of Arts, Commerce and Science for providing necessary research facilities to undertake the study.

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