Production of microbial α-amylases – an overview

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Abstract:
α-amylases are key industrial enzymes having highest market share of enzyme sales (about 30%) with lots of potential application in food processing industries such as sugar, baking, brewing and many other starch based industries. This group of enzyme catalyses α-1,4-glycosidic linkages of starch and related substrates. In recent years, the traditional Submerged Fermentation method of α-amylase production has gradually been substituted by Solid State Fermentation for its multiple advantages as compared to Submerged Fermentation. The advantages include use of inexpensive substrates, higher yield, avoids substrate inhibition etc. α-amylases are commercially produced by different species of bacteria and filamentous fungi. This study reviews about the microbial sources, aspects of fermentative production and potential fields of α-amylase application.
Keywords: α-amylase, fermentation, enzyme, submerged fermentation, solid state fermentation.

1. Introduction:

α-amylases (1,4-α-D-glucan glucanohydrolase ; E.C. 3.2.1.1.) are the endo-enzymes which hydrolyse α-1,4-glycosidic bonds of adjacent glucose units randomly of starch forming linear and branched oligosaccharides such as glucose, maltose and maltotriose. Amylases contribute about 30% to the world's overall enzyme production (Mostafa et al., 2024). α-amylases have been extensively used in sugar, textile, alcohol, detergent and paper industries (Bruins et al., 2001; Gupta et al., 2003; Sivaramakrishnan et al., 2006; Far et al., 2020). They have also been used in various processed food industries like baking, brewing, preparation of digestive aids, production of cakes, fruit juices and starch syrups (Rosell et al., 2001; Gupta et al., 2003; Aiyer, 2005; de Souza and e Magalhaes, 2010). Moreover, due to advances in biotechnology, amylases find application in clinical, medicinal and analytical chemistry as well (de Souza and e Magalhaes, 2010; Das et al., 2011; Saini et al., 2017).

α-amylases are ubiquitous enzymes occurring in plant, animal and microbial kingdoms. However, microbial sources fulfill the industrial need of α-amylases (de Souza and e Magalhaes, 2010; Sivaramakrishnan et al., 2006). Microbial production provides several advantages over the other sources such as large scale commercial production ability and easy manipulation to get enzymes of desired character (Gupta et al., 2003; de Souza and e Magalhaes, 2010). Although, numbers of α-amylase producing microbes are described, only a few species of *Bacillus* and *Aspergillus* and their improved strains have been employed for industrial production of α-amylases.

Traditionally, α-amylases have been obtained from Submerged Fermentation (SmF), Solid state Fermentation (SSF) is a promising technology for the production of α-amylases. SSF technology present multiple advantages over SmF such as higher productivity, higher concentration of the products, less effluent formation, simple fermentation equipments and inexpensive substrates etc. (Pandey et al., 2000a, b ; Robinson et al., 2001; Sivaramakrishnan et al., 2006; Sahu et al., 2024).

The increasing demand for amylases in various industries necessitate the development of enzymes with unique properties suitable for industrial applications for instance raw starch degradation associated with cost effective production techniques (Sivaramakrishnan et al., 2006). Moreover, through modern techniques of protein engineering, ribosome engineering, improvement of strains by mutagenesis, genetic recombination etc., there has been efforts to get suitable α-amylases for diverse industrial requirements (Das et al., 2011).

2. Sources of microbial α-amylases:

α-amylases universally occur in plants, animals and microbes. However, for industrial production microbial sources particularly bacteria and fungi are favored because of manifold advantages such as cost effectiveness, consistency, less time and space required for production, simple process optimization as well as easy modification of microbial characteristics to get desired quantity and quality of the enzyme (Gupta et al., 2003 ; Sivaramakrishnan et al., 2006). Various workers have reported numbers of α-amylase producing microorganisms in both SmF and SSF cultures. Although, a good number of bacterial species are being exploited for α-amylase production in SSF, only a few species of *Bacillus* and their improved strains such as *B. licheniformis, B. subtilis, B. amyloliquefaciens and B. stearothermophilus* have been recognized as commercial producer of α-amylase (Gupta et al., 2003; Sivaramakrishnan et al., 2006; Regulapati et al., 2007; de Souza and e Magalhaes, 2010). Some other reported bacterial species are *Aeromonas caviae* (Pandey et al., 1999), *B. coagulans* (Babu and Satyanarayana, 1995), *B. megaterium* (Ramesh and Lonsane, 1987), *Streptomyces megasporus* (Dey and Agrawal., 1999), *Thermomyces lanuginosus* (Kunamneni et al., 2005b), *B. cereus* (Abo-Kamer et al., 2023) etc.

In addition, several filamentous fungi and some yeast have revealed to produce α-amylase. However, only some species of *Aspergillus* (e.g., *A. oryzae and A. niger*) and *Penicillum* (e.g., *P. expansum*) have gained the status as commercial producer (de Souza and e Magalhaes, 2010; Erdal and Taskin, 2010 ; Kalaiarasu and Vivekananthan, 2010). Various other species of fungi being explored for α-amylase production of are - *Rhizopus* sp. (Soccol *et al.*, 1994), *A. kawachii*

(Sudo *et al.*, 1994), *Saccharomyces capsularis* (Soni *et al.*, 1996), *Beauveria feline* (Agrawal *et al.*, 2005), *P. janthinellum* (NCIM 4960) (Sindhu *et al.,* 2009), *P. decumbens* (Sun et al., 2005), *P. citrinum* (Metin et al., 2010), *A. terreus* (Sethi et al., 2016) etc. The fungal α-amylases are favored over bacterial sources in food processing industries due to their GRAS (generally recognized as safe) status (Far et al., 2020).

3. Fermentation process:

To fulfill the industrial demand, low cost medium is required for the production of α-amylase (Aliyu et al., 2011, Jujjavarapu et al., 2018). Both SSF and submerged fermentation (SmF) have been used for the production of amylase, although traditionally these have been obtained from submerged cultures because of easy handling and better control of cultural conditions such as temperature and pH (Xusheng et al., 2011; Singh et al., 2022). Generally, synthetic media have been used for the production of microbial amylase through SmF (Ajay et al., 2010). The constituents of synthetic media such as peptone, yeast extract, starch, as well as other components are very expensive and these can be substituted by cheaper agricultural by-products to reduce the cost (Solange et al., 2010). The solid substrate provides both support and nutrition (Hashemi et al., 2011; Singh et al., 2022). SSF is considered as suitable alternative of SmF. The transformed attention for α-amylase production by SSF is due to several economic and engineering advantages over SmF (Carrizales and Jaffe, 1986; Pandey et al., 2000a, b and c ; Regulapati et al., 2007). The special importance of SSF lies in that the crude fermented product can be directly used as enzyme source (Gupta et al., 2003). The chief advantages of SSF over the conventional SmF are - higher yields in a shorter time period, better oxygen circulation, resemblance to the natural habitat for filamentous fungi, high volumetric productivity, relatively higher concentration of the products, less effluent generation, requirement for simple fermentation equipment, much simpler and cheaper downstream process and use of low cost substrates (Nigam and Singh,1995; Pandey et al., 2000a,b ; Regulapati et al., 2007; Sivaramakrishnan et al., 2006). On the contrary, production of amylase using SmF system is known to cause potential problems (Regulapati et al., 2007) such as presence of product in low concentration, handling and disposal of large volumes of water during downstream processing. Moreover, SmF is a costly operation and sometimes inadequately understood (Pandey et al., 2000a). These problems can be efficiently overcome by using SSF system as the yield is several times higher, cost effective and the product recovery can be achieved in less amount of solvent (Regulapati et al., 2007) thus offering simpler and cheaper downstream operation.

The catabolite repression shown by SmF in α-amylase production can be significantly overcome by SSF which leads to overall economy of the production process eliminating the need to use low substrate concentrations and costly operation strategies (Gupta et al., 2003; Sivaramakrishnan et al., 2006; Regulapati et al., 2007). In addition, in SSF system for α-amylase production uses very low cost substrates such as solid organic wastes, agro-industrial residues etc. (Couto and Sanroman, 2006 ; Gupta et al., 2003; Sivaramakrishnan et al., 2006; Paul et al., 2021; Singh et al., 2022). Thus, the utilization of the organic wastes not only provides an alternative substrate, but also contributes to solid waste management.

4. Process optimization:

Optimization of cultural conditions and manipulation of media constituents are one of the most important parameters used for the production of enzymes in enormous quantity (Balasubramaniem et al., 2011; Paul et al., 2021). Production of α-amylase using fungi depends on both morphological and metabolic state of the culture. Mycelial growth is crucial for extracellular enzyme like α-amylase (Sangeeta et al., 2009). Fermentation conditions including both physical and chemical that have an effect on the optimum production of α-amylase are temperature, pH, Incubation period, carbon, nitrogen sources, surfactants, phosphate, different metal ions, moisture and agitation relating to SSF and SmF (Ellaiah et al., 2002; Far et al., 2020).

4.1. Temperature:

Temperature affects the growth of the organism which in turn controls the amylase secretion. Hence, the optimum temperature is dependent on whether the culture is mesophilic or thermophilic. Among fungi mainly amylase production studies have been carried out with mesophilic fungi within the temperature range of 25-37^{0}C (Takahiro et al., 2011; Abdullah *et al*., 2017; Balakrishnan *et al*., 2021). Thermophilic fungi such as Thermomyces lanuginosus requires optimum temperature of 50-55 ^{0}C (Kunamneni et al., 2005b; Sivaramakrishnan et al., 2006). Bacterial α-amylase production shows a wider range of temperature. Most common bacterial species such as *B. amyloliquefaciens*, *B. subtilis, B. licheniformis and B. stearothermophilus* are reported to produce α-amylase at temperatures 37-60^{0}C (Gupta et al., 2003). Certain hyperthermophiles such as *Thermococcus profundus* and *Thermotoga maritime* have been reported to produce α- amylase at 80^{0}C (Vieille and Zeikus, 2001). A cold active α-amylase from Antarctic psychrophile *Alteromonas haloplanktis* was reported to exhibit maximum production at 4^{0}C (Feller *et al.*, 1998).

4.2. pH:

pH is an important factor which affects the growth and enzyme production during SSF (Kunamneni et al., 2005a). The growth and production of α-amylases occur at slightly acidic pH for fungi and bacterial species require nearly neutral pH for α-amylase production both in case of SSF and SmF. The optimum pH range for fungi in SSF have been reported to be 5-7(Sindhu et al., 2009; Kalaiarasu and Vivekananthan, 2010; Vidya *et al*., (2012). Bacterial species generally require an optimum pH range of 6-8 (Sivaramakrishnan et al., 2006; Regulapati et al., 2007). Normally, pH of the media changes during fermentation due to the production of organic acids. However, it has been reported that agro-industrial substrates posses exceptional buffering action and consequently have advantages for enzyme production (Erdal and Taskin, 2010). Only 0.1 decrease in medium pH was observed after 5 days during α-amylase production by *Penicillium expansum* at initial pH 6.0 (Erdal and Taskin, 2010).

4.3. Carbon sources:

Carbon sources like galactose, glycogen and Inulin have been found suitable for the production of amylases by *B. licheniformis* and *Bacillus.sp.1-3* (Xusheng et al., 2011). Starch and glycerol were reported to increase enzyme production in *B. subtilis* IMG22, *Bacillus* sp, PS-7 and *Bacillus* sp.1-3 (Sundarram, and Krishna Murthy, 2014). However, in most of the studies soluble starch was reported to be desirable substrate for the production of α-amylase by both bacterial and fungal species (Far et al., 2020). Agricultural wastes are being used for both SmF and SSF to reduce the cost of fermentation media. The wastes contain carbon and nitrogen sources necessary for the growth and metabolism of organism. These nutrients sources include orange waste, peer millet starch, potato, corn, tapioca, wheat bran and rice husk as flours (Hui et al., 2011).

4.4. Nitrogen sources:

Both organic and inorganic nitrogen sources are used to supplement the production media for α-amylase (Far et al., 2020). Balkan et al. (2011) reported that among the nitrogen sources, urea increased α-amylase production to 870 U/g. Presence of organic nitrogen sources, urea and peptone have been reported to enhance α-amylase production by *A. niger* in Wheat Bran medium (Anto et al., 2006). Niaz et al. (2010) reported that corn steep liquor in wheat bran accelerated α-amylase production (1338 U/ml) by *B. licheniformis*. Ammonium nitrate (1%) showed highest enzyme production as compared to other inorganic and organic nitrogen sources by *A. oryzae* IFO-30103 in a medium containing oil cakes (Ramachandran *et al*., 2004a). 1% peptone and starch in the medium gave enhanced production of α-amylase by *A. niger* JGI24 (Kunamneni et al., 2005a). Peptone is considered as ideal source for amylase synthesis and favours the growth and product formation (Tiwari *et al*., 2007; Premalatha *et al*., 2022)

4.5. Surfactants:

Surfactants in fermentation medium are reported to increase the secretion of proteins by increasing cell membrane permeability. Therefore, surfactants are added for the production of extracellular enzymes (Samrat et al., 2011). A surfactant, of tween 80 (1.3%) when added to the fermentation medium increased α-amylase production by 2 fold in *Thermomyces lanuginosus* (Sivaramakrishnan et al., 2006). In a study on the effect of addition of Poly ethylene glycols (PEG) (molecular mass of 600, 3000, 4000, 8000 and 20,000) in fermentation medium for α-amylase production by two *Bacillus* spp., it was found that 5% PEG's 600 and PEG 3000 yielded 31% increase in enzyme production by *B. amyloliquefaciens* and 21% increase by *B. subtilis* (Goesaert et al., 1999).

4.6. Metal ions:

Supplementation of mineral salts of certain metal ions good growth of microorganisms and enhanced secretion of α-amylase (Zoe et al., 2006). Among the minerals used in the preparation of fermentation media are KCl, NaCl, KH_2PO_4, $MgSO_4$, $FeSO_4$, Na_2HPO_4, NaH_2PO_4, $CaCl_2$, $ZnSO_4$, etc. (Sindhu et al., 2009; Erdal and Taskin, 2010; Balkan et al. (2011); Ramachandran et al., 2004b; Varalakshmi et al., 2009 ; Ahmed, 2011 ; Saxena and Singh, 2011). It is apparent from different research works that varied inorganic salts are important for desired production enzymes like α-amylases (Abdullah *et al*., 2017; Balakrishnan *et al*., 2021; Premalatha *et al*., 2022). In majority of the cases α-amylase production media require Ca^{2+} ion to enhance the productivity as α-amylase is depends on Ca^{2+} for its activity (Far et al., 2020).

4.7. Moisture content:

Moisture is one of the most important parameters in SSF that influence the growth of the microorganism and thereby enzyme production. For the fungi the optimum moisture content of the substrate varies from 55-70% (Balkan and Etran, 2006 ; Erdal and Taskin, 2010; Kalaiarasu and Vivekananthan, 2010 ; Sindhu et al., 2009). Normally bacteria are reported to require initial moisture content of 70-80% (Sivaramakrishnan et al., 2006). 65% of moisture content was required by *B. coagulans* for optimum α- amylase production on wheat bran (Nandakumar et al., 1996). A thermotolerant *B. subtilis* required initial moisture content of 30% for its growth and maximum enzyme production (Baysal et al., 2003). Moreover, the nature of the moistening agent also determines the enzyme production. *Bacillus* sp. PS-7 gave optimum α- amylase production when moistening agent was tap water (Sodhi et al., 2005). Various other moistening agents used are distilled water (Soni et al., 2003), salt solution (Babu and Satyanarayana, 1995; Sindhu et al., 2009), phosphate buffer (Ramesh and Lonsane, 1991), acetate buffer (Balkan and Etran, 2006), etc. Surfactants such as Sodium Dodecyl Sulphate (SDS), Cholic acid, Tweens, etc. in fermentable medium were reported to increase cell permeability, thereby, enhancing enzyme yield (Rao and Satyanarayana, 2003; Sindhu et al., 2009). Addition of Tween-80 (1.3%) to the fermentation medium increased α- amylase production by 2-fold in *Thermomyces lanuginosus* (Arnesen et al., 1998).

4.8. Incubation time:

The optimum incubation time is dependent on the characteristics of the culture organism and related to the growth curve and the enzyme production pattern (Jujjavarapu et al., 2018). Prolonged incubation is not favourable because it may cause loss of moisture especially when the microorganism is thermophilic. Most reports reveal that the optimum period of incubation ranges between 48-96 hrs for α- amylase production (Patel et al., 2005; Regulapati et al., 2007; Sindhu et al., 2009; Sivaramakrishnan et al., 2006). However, some fungi such as Penicillium expansum MT-1 (Erdal and Taskin, 2010), *Aspergillus* sp. JGI12 require 6-7 days of incubation for optimum production.

5. Downstream processes:

It has been found that in most applications, α-amylases do not require them to be of high purity and the crude or partially purified enzyme preparations are quite effective and popular (Regulapati et al., 2007; Gupta et al., 2003). However, α-amylases applied in pharmaceutical and clinical sectors require high purity. Furthermore, high purity of the enzyme is also required in the studies of structural, functional and biochemical properties (Singh et al., 2022).

In majority of the cases, the application of α-amylase from microbial sources involves classical purification methods for both SmF and SSF. These methods involve several steps including separation of the culture from the media, selective precipitation using ammonium sulphate or organic solvents such as chilled acetone followed by membrane separation (Sivaramakrishnan et al., 2006; Paul et al., 2021). This is followed by chromatography, usually affinity, ion exchange and/or gel filtration (Singh et al., 2022). Purification can also be done by HPLC (high-performance liquid chromatography) method (Bano et al., 2011). Gel electrophoresis (SDS-PAGE) may constitute the final step in the series for testing purity and molecular weight of the enzyme (Paul et al., 2021).

6. Applications of α- amylases:

α-amylases are the most significant hydrolytic enzyme for all starch based industries. About 30% of the world's enzyme production is shared by these enzymes (Paul et al., 2021). α-amylase market was estimated as USD 10.53 Billion in 2023 and was expected to drive at a rate of 7.5% to turn into USD 17.54 billion till 2030 (Verified Market Reports, 2024). About 400 small suppliers and 12 large firms provide the global enzyme need. Denmark-based Novozymes Switzerland-based Roche and US-based DuPont are the top manufacturers of α-amylases. Table-I depicts various applications of α- amylases in Industry.

Table-I: Utilization of α-amylases in various industries

Area	Application	References
Food processing industries	Induce softness, taste and shelf life; diminish staling in baking industry. Primary treatment of animal feed to improve the digestibility of fiber. Decrease in haze development in juices.	de Souza and e Magalhaes, 2010; Das et al., 2011;; Craigen et al., 2011; Paul et al., 2021, Singh et al., 2022; Sahu et al., 2024
Starch conversion processes	Reduction in viscosity of sugar syrups. Production of high fructose corn syrups and glucose syrups, crystalline glucose. Production of maltose syrups.	
Detergent industry	Used as an additive to remove starch based dirts.	
Paper industry	Decrease of viscosity of starch for appropriate coating of paper.	
Textile industry	Drape sizing of textile fibers.	
Pharmaceutical/clinical & analytical industry	Used as a digestive aid. Used to make α-amylase biosensors. Removing *Staphylococcus aureus* biofilms.	
Fuel alcohol production	Solubilization and saccharificarion of starch for alcohol fermentation in brewing industry and ethanol production.	

7. Conclusion:

α-amylases are amongst the extensively used industrial enzymes. Due to advancement in biotechnological fields, there is a continuous rise in the enzyme market. With the extension of new spectrum of applications of α-amylases in medical, clinical chemistry, molecular biology, bioremediation etc., the demand for α-amylase with high specificity, stability and efficiency is increasing. Therefore, production of α-amylase with novel characteristic features is in demand in recent times.

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9. References :

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