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RESEARCH ARTICLE

PRODUCTION OF MICROBIAL α- AMYLASE BY SOLID STATE FERMENTATION-AN OVERVIEW

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ABSTRACT

α- amylases are most important industrial enzyme and hold maximum market share of enzyme sales having lots of potential application in food processing industries such as sugar, baking, brewing and many starch based industries. The α- amylases hydrolyse α-1,4-glycosidic linkages in starch and related substrates. The conventional Submerged Fermentation method of α- amylase production is being replaced by Solid State Fermentation during recent years due to manifold advantages over Submerged Fermentation such as use cheap substrate, higher yield, avoids substrate inhibition etc. The commercial source of α- amylases are usually bacteria and filamentous fungi. This study reviews the microbial sources, fermentation characteristics and potentiality of α- amylase production through Solid State Fermentation.

INTRODUCTION

α- amylases (1,4-α-D-glucan glucanohydrolase ; E.C. 3.2.1.1.) are endo-acting enzymes which randomly hydrolyse α-1, 4-glycosidic bonds between adjacent glucose units in a starch polymer leading to the formation of linear and branched oligosaccharides such as glucose, maltose and maltotriose. Amylases contribute about 30% to the world's overall enzyme production (van der Maarel *et al*., 2002). 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). 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 (Aiyer, 2005 ; de Souza and e Magalhaes, 2010 ; Gupta *et al*., 2003; Rosell *et al*., 2001). Moreover, due to advances in biotechnology, amylases find application in clinical, medicinal and analytical chemistry as well (Das *et al*., 2011; de Souza and e Magalhaes, 2010; Gupta *et al*., 2003).

α-amylases are ubiquitous enzymes occurring in plant, animal and microbial kingdoms. However, microbial sources, especially thermophilic bacteria and mesophilic molds provide industrial need of α- amylases (de Souza and e Magalhaes, 2010; Sivaramakrishnan *et al*., 2006). Microbial production of α-amylases provides certain advantages over the other sources such as commercial bulk production capacity and easy to manipulate to obtain enzyme of desired characteristics. (de Souza and e Magalhaes, 2010; Gupta *et al*., 2003). Though a numbers of species are reported to produce α- amylase, only a few species of *Bacillus* and *Aspergillus* and their improved strains have dominated application for the production of α-amylases. Although, conventionally α- amylases have been obtained from

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Submerged Fermentation (SmF), Solid State Fermentation (SSF) holds remarkable prospective for the production of α-amylases. SSF system offers numerous advantages over SmF system, including high volumetric productivity, higher concentration of the products, less effluent generation, requirement for simple fermentation equipments and low cost substrates etc. (Pandey *et al*., 1999 ; Pandey *et al*., 2000a, b ; Robinson *et al*, 2001; Sivaramakrishnan *et al*., 2006). Moreover, SSF is like a natural microbiological process such as composting and its controlled utilization can generate desirable industrial products (Couto and Sanroman, 2006; Sivaramakrishnan *et al*., 2006). The increasing demand for amylases in various industries have generated enormous interest for the development of enzymes with better properties suitable for industrial applications such as in raw starch degradation along with the development of cost effective production techniques (Sivaramakrishnan *et al*., 2006). Further, through protein engineering and improvement of strains by mutagenesis, ribosome engineering, genetic recombination etc., it has been trying to get suitable α-amylases for various industrial purposes (Das *et al*., 2011). In this review, it is tried to present various aspects of SSF for the production of microbial α- amylases.

Why SSF is preferred for the production of α- amylases

Submerged Fermentation (SmF) technique is employed to produce most of the industrially important enzymes. However, in the last few decades, there has been a rising tendency towards the exploitation of Solid State Fermentation (SSF) techniques to produce industrially important enzymes including α- amylases (Fadel, 2000 ; Lonsane and Ramesh, 1990; Sodhi *et al*., 2005). 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 (Tengerdy, 1998). 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 operation is cost demanding and poorly understood (Datar, 1986).

These problems can be efficiently overcome by using SSF system as the yield is several times higher, cost effective (Ghildyal *et al*., 1985) and the product is recovered from the fermentation unit in lower volume of solvent (Regulapati *et al*., 2007), thus attaining much simpler and cheaper downstream process. The catabolite repression shown by SmF system in the production of α-amylase can be significantly overcome by SSF (Ramesh and Lonsane, 1991**)** which leads to overall economy of the production process eliminating the need to use low substrate concentrations and costly operation strategies (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; Pandey *et al*., 1999; Sivaramakrishnan *et al*., 2006). Thus, the utilization of the organic wastes not only provides an alternative substrate, but also contributes to solid waste management.

Sources of microbial α- amylases

α- amylases are found universally in plants, animals and microbes, where they play a central role in carbohydrate metabolism. In spite of the wide distribution of α- amylases, microbial sources, namely bacteria and fungi are preferred for the industrial production due to advantages such as cost effectiveness, consistency, less time and space required for production, ease of process modification and optimization as well as easy manipulation and modification of microbial characteristics to get desired quantity and quality of the enzyme (Gupta *et al*., 2003 ; Sivaramakrishnan *et al*., 2006). A large number of microorganisms have been reported to produce α- amylase in both SmF and SSF cultures. Usually, bacteria are the source of thermostable α- amylases. 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 the ability to produce α- amylase in commercial scale (de Souza and e Magalhaes, 2010; Gupta *et al*., 2003; Regulapati *et al*., 2007; Sivaramakrishnan *et al*., 2006). Some other species of bacteria producing α- amylase under SSF 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) etc. Similarly, several mold species along with some yeast have shown 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). Some other species of fungi being exploited for the production of α- amylase through SSF are *A. kawachii* (Sudo *et al*., 1994), *Saccharomyces capsularis* (Soni *et al*., 1996), *Rhizopus* sp. (Soccol *et al*, 1994), *Beauveria feline* (Agrawal *et al*., 2005), *Penicillium janthinellum*(NCIM 4960) (Sindhu *et al*., 2009), *Penicillium decumbens* (Sun *et al*., 2005) etc. The fungal α- amylases are preferred over other microbial sources due to their more accepted GRAS (generally recognized as safe) status (Gupta *et al*., 2003).

Substrates used for α- amylase production in SSF

Studies on selection of suitable substrates for SSF is concentrated on agro-industrial residues due to their promising advantages for filamentous fungi, which can penetrate and colonize into the hardest of the solid substrates, assisted by the presence of turgor pressure at the tip of the mycelium (de Souza and e Magalhaes, 2010; Sivaramakrishnan *et al*., 2006). Thus, SSF is confined to the processes involving fungi in general. However, latest findings have shown successful growth and production of enzymes e.g., α- amylase from bacteria in SSF (Babu and Satyanarayana, 1995; Lonsane and Ramesh, 1990; Sodhi *et al*., 2005). Various organic residues studied as substrate for SSF are - sunflower meal, rice husk, cottonseed meal, soybean meal, pearl millet, rice bran, wheat bran, black gram bran, banana waste, wheat straw, corncob leaf, rye straw, loquat kernel, mustard oil cake, coconut oil cake, cassava flour etc. (Baysal *et al*., 2003, Erdal and Taskin, 2010; Ikram-ul-Haq *et al*., 2003 ; Kalaiarasu and Vivekananthan, 2010 ; Kunamneni *et al*., 2005b). Reports on the use of different substrates for the production of α-amylases in SSF have shown that wheat bran is the most promising one (Regulapati *et al*., 2007; Sivaramakrishnan *et al*., 2006; Sodhi *et al*., 2005). A summary of various agro-industrial residues reported for α-amylase production is given in Table-I.

Factors affecting α- amylase production in SSF systems

The major factors that affect microbial α- amylase synthesis in a SSF system are - a suitable substrate and microorganism, pretreatment of the substrate, particle size (inter particle space and surface area) of the substrate, initial moisture content and relative humidity of the substrate, pH, incubation temperature, additional nutrients, aeration, inoculums size, incubation period and the control of contamination during fermentation (Couto and Sanroman, 2006; Das *et al*., 2011). The results obtained from the optimization of process parameters have clearly revealed their impact on the gross yield of α- amylase as well as their independent nature in influencing the organism's ability to synthesize the enzyme. Determination of particle size corresponding to optimal growth and enzyme production is important because adherence and penetration of microorganisms as well as enzyme action on substrate clearly depend upon the physical properties of the substrate such as the crystalline or amorphous nature, the accessible area, surface area, porosity, etc. (Nandakumar *et al*., 1996 ; Pandey,

1991). Therefore, selection of appropriate particle size is crucial for optimum production of α-amylase. The suitable particle size depends upon the organism and the substrate used in the media. One report states that wheat bran having a particle size between 500-1000µ gave better yields by *Bacillus subtilis* (Baysal *et al.*, 2003). Sindhu *et al.* (2009) observed that the particle size of 450-500µ favoured maximum production of α-amylase when the substrate was wheat bran. Krishna and Chandrasekaran (1996) reported that banana fruit stalk particles of 400µ favoured maximal α-amylase production by *B. subtilis* CBTK106. However, most of the studies have shown that particle size of 400-1000µ is very suitable for the production of α-amylase.

The optimum temperature depends on whether the culture is mesophilic or thermophilic. The fungi being mesophilic have shown a temperature range of 25-37^{0}C (de Souza and e Magalhaes, 2010, Francis *et al.*, 2003). Thermophilic fungi such as *Thermomyces lanuginosus* requires optimum temperature of 50-55 ^{0}C (Kunamneni *et al*., 2005b; Sivaramakrishnan *et al.*, 2006). Bacterial α- amylases are produced at a much 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 (Mielenz, 1983 ; Syu and Chen, 1997). 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 α- amylase production at 4^{0}C (Feller *et al*., 1998).

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). 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). Several comparative studies have demonstrated that in SSF, media supplemented with certain simple carbon, nitrogen and mineral sources give enhanced production of α-amylase. The α-amylase is an inducible enzyme and is generally induced by starch or its hydrolytic product maltose (Sindhu *et al.*, 2009; Kunamneni *et al*., 2005a). Glucose, fructose, sucrose etc. generally have inhibitory effect on the production. Presence of glucose and other sugars act as catabolite repressor (Morkeberg *et al*., 1995 ; Alva *et al*., 2007 ; Nandakumar *et al*., 1999). In one report, Erdal and Taskin (2010) showed that addition of soluble starch in the medium resulted 20% increase in the enzyme production. Balkan *et al*. (2011) reported that wheat bran medium supplemented with 1% lactose increase enzyme production when *Trichothecium roseum* was used as inoculums. There are other reports as well which showed that lactose and maltose induce α-amylase production (Ramachandran *et al*., 2004b; Eratt *et al.*, 1984). Both organic and inorganic nitrogen sources are used to supplement the production media for α-amylase. 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).

Literatures for the production of α-amylase through SSF reveal that the production media consisting of various agro-industrial wastes such as wheat bran, rice husk, sunflower meal, cottonseed meal, soybean meal, rice bran, black gram bran, banana waste, etc. are supplemented with some mineral salts which are necessary for the enhanced secretion of α-amylase. This is because the fermentation medium containing organic wastes may not provide all the essential nutrients for proper growth and production. They may also regulate the buffering activity of the medium. 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). The incubation time is mainly governed by the characteristics of the culture organism and the enzyme production pattern. 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.

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

Table I: Various agro-residual substrates having high potentiality for α- amylase production

Substrate	Organism	Activity(U/g)	Reference
Amaranthus grain	*A. flavus*	1 920	Viswanthan and Surlikar, 2001
Banana waste(fruit stalk & peel)	Aspergillus oryzae	690 (mg/ml)	Kalaiarasu and Vivekananthan, 2010
Coconut oil cake	*A.oryzae*	3 388	Ramachandran *et al*., 2004a
Corn bran	*Bacillus* sp. PS-7	97 600	Sodhi et al., 2005
Gram bran	*B. coagulans*	8 984	Babu and Satyanarayana, 1995
Groundnut oil cake	Aspergillus oryzae	6547	Ramachandran *et al.*, 2004b
Loquat kernels	*P. expansum MT-1*	1012	Erdal & Taskin, 2010
Maize bran	*B. coagulans*	22 956	Babu and Satyanarayana, 1995
Mustard oil cake	*B. coagulans*	5 953	Babu and Satyanarayana, 1995
Mustard Oil seed cake	*Bacillus* sp.	5400	Saxena and Singh, 2011
Rice bran	*Bacillus* sp. PS-7	145 000	Sodhi *et al*., 2005
Rice husk	*B. subtilis*	21 760	Baysal *et al*., 2003
Spent brewing grain	*A. oryzae* NRRL 6270	6 583	Francis *et al*., 2003
Wheat bran	*Penicillium janthinellum*	300	Sindhu *et al*., 2009
Wheat bran	*Bacillus* sp. PS-7	464 000	Sodhi *et al*., 2005

Table II: Applications of α- amylases in various industries

Sector	Uses	References
Food processing industries	Induce softness, taste and shelf life ; diminish staling in baking industry. Pretreatment of animal feed to improve the digestibility of fiber. Decrease in haze development in juices.	Aiyer, 2005; Das *et al*., 2011; de Souza and e Magalhaes, 2010; Gupta *et al*., 2003; Sivaramakrishnan *et al*., 2006 ; Zou *et al*., 2008 ; Craigen *et al*., 2011.
Starch conversion processes	Reduction in viscosity of sugar syrups. Production of high fructose corn syrups. Production of glucose syrups, crystalline glucose. Production of maltose syrups. Solubilization and saccharificarion of starch for alcohol fermentation in brewing industry and ethanol production.	
Detergent industry	Used as an additive to remove starch based dirts.	
Paper industry	Reduction in 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.	

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).

Downstream studies

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 (Gupta *et al*., 2003). 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; Gupta *et al*., 2003). This is followed by chromatography, usually affinity, ion exchange and/or gel filtration. Gel electrophoresis (SDS-PAGE) may constitute the final step in the series (Nouadri *et al*., 2010 ; Patel *et al*., 2005; Regulapati *et al*., 2007; Sodhi *et al*., 2005; Tengerdy, 1998). In a study, 12.7 fold partial purification of α-amylases from Bacillus sp. PS-7 under SSF was obtained by subjecting the cell free supernatant to ammonium sulphate precipitation, gel filtration on Sephadex G-75 followed by Phenyl Agarose Hydrophobic interaction chromatography (Sodhi *et al*., 2005).

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 (van der Maarel *et al*., 2002). The global annual sale of α- amylases is estimated to be **$**11 million (Sivaramakrishnan *et al*., 2006). The present projected value of world market is about US$ 2.7 billion and is estimated to increase by 4% annually through 2012. The key industries which utilize about 75% of industrially produced enzymes are Detergents (37%), textiles (12%), starch (11%), baking (8%) and animal feed (6%). Lee, (1996) reported that the world production of α- amylases from *B. licheniformis* and *Aspergillus* sp. was about 300 tones of enzyme protein per year. Table-II depicts various uses of α- amylases in Industry.

Conclusion

The analysis of the literatures has revealed that the production of α- amylases in SSF is an ideal method which is the most economic and provide manifold advantages in all respects over SmF. Due to increasing demand of α- amylases in

various industries like food processing, textile, detergent, biotechnology etc., there is immense potential to develop suitable production technology for large scale yield through SSF in future.

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