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Isolation, screening of *Aspergillus flavus* and its production parameters for α-amylase under solid state fermentation

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Abstract: The amylase producing fungi were isolated from spoiled fruits, vegetables and soil, in and around Bangalore, Karnataka, India. The isolates were identified and five fungal species were screened. The best amylase producer among them, *Aspergillus sp* was selected for enzyme production by both sub merged fermentation using mineral salt medium (MSM) and solid state fermentations using wheat bran as a solid substrate. The various parameters influencing solid state fermentation were optimized. The most important factors are such as pH, incubation temperature, incubation period, carbon sources, nitrogen sources and moisture content. The maximum amount of enzyme production was obtained when solid state fermentation was carried out with soluble starch as carbon source and beef extract (1% each) as nitrogen source, optimum conditions of pH 7.0, an incubation temperature of 25 (±2) °C, incubation time 96 h and 62% moisture content.

Keywords: Isolation, Screening, Production, α -amylase, *Aspergillus flavus*, Solid state fermentation

INTRODUCTION

Alpha-Amylases are universally distributed enzymes throughout the animal, plant and microbial kingdoms. Amylolytic microorganisms play a vital role in most of the food industries. In the present day many such microorganisms have been harvested from fruits, vegetables and soil. Enzymes present in them actively participate in breaking down starch substrates into its simple forms. Many microbial enzymes are commercially exploited and successfully used on industrial scale to catalyze several chemical processes. The enzyme proved to be better, cheaper and eco friendly compared to the use of chemicals. Recently, enzymes have also been exploited in bioremediation of complex waste substances (Whiteley and Lee, 2006). Therefore enzyme production now became a multibillion dollar business (Bhat, 2000). Two major classes of starch degrading enzymes are identified in microorganisms-a-amylases and glucoamylases. α-amylases (E.C-3.2.1.1) are extracellular enzymes that randomly cleave α -1,4 glucosidic linkages between adjacent glucose units in the linear amylase chain and glucoamylase (E.C-3.2.1.3) hydrolyses single glucose units from the non-reducing ends of amylase and amylopectin in a stepwise manner and also able to hydrolyse the a-1,6 linkages at the branching points of amylopectin at a slower rate than a-1,4 linkages (Sindhu *et al.*, 2009). Microbial enzymes involved in the degradation and transformation of plant cell-wall polysaccharides have found many biotechnological applications (De Vries and Visser, 2001). Starch is a reserve source of glucose in plants and readily hydrolyzed by amylases produced by almost all living organisms. However, microbial amylases are of great industrial importance (Aiyer, 2005). Beside these carbohydrolases are another important group of industrial enzymes that is widely used in detergent, baking and some other industries. In the glow of current biotechnology, α-amylases are now in advance importance in biopharmaceutical applications. Still, their application in food and starch based industries are the most important market and thus the demand of a-amylases would always be high in these sectors. Alpha-Amylases find application in baking, brewing, detergent, textile, paper and distilling industry (Pandey *et al.*, 2000). The use of agro industrial residues make solid state fermentation more economic (Ellaiah *et al.*, 2002). Baysal *et al.* (2003) reported amylase production using wheat bran as substrate. The effects of the starch, protein and soluble oligosaccharides contents in wheat bran on the production of extracellular amylase were reported in *Penicillium decumbens* by Sun *et al.* (2007). The present investigation deals with the isolation of amylolytic fungi from air, spoiled fruits, vegetables and soil samples collected from in and around Bangalore, India and optimization of process parameters for maximal production of amylase under SSF. In this paper we report a number of factors that influence amylase production by *Aspergillus flavus* under solid state fermentation.

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MATERIALS AND METHODS

Isolation and identification of amylase producers: The fungi were isolated by two methods: Air exposure plate methods in which fungi were isolated by settle plate technique and the spoiled fruits, vegetables and soil by serial dilution method in which isolates of fungi were obtained by serial dilution as per Aneja (2010). The samples collected from local markets and three different places in and around Bangalore, Karnataka, India. These materials were transferred to laboratory and maintained in optimum 28°C. The isolation of fungi was carried out by inoculating the samples on to Czapek Dox agar plates supplemented with streptomycin. After the incubation period, all the plates were observed for macroscopic culture such as colony diameter, colour, texture, conidial colour, mycelia colour and nature of the spores, maintained in Potato dextrose agar slants. For the microscopical observation of fungal species, lacto phenol cotton blue staining method was performed as described by Cappuccino *et al.* (2004).

Screening of amylase producers: All the isolates obtained were screened for actual and efficient starch degraders. For screening purpose starch hydrolysis test was performed. The cultures were inoculated on starch agar medium and plates were incubated for 72 to 96 h at room temperature. After obtained colonies of each plate iodine solution was layered on the agar plates and zone of clearance was observed for screening of the amylolytic fungi (Aneja, 2010) and stored at 4°C.

Substrate: Wheat bran substrate was dried and ground into coarse powder with an electronic blender.

Culture conditions: 10 ml of distilled water containing 0.01% Tween-80 was transfered in to a fully sporulated PDA slant culture. The spores were dislodged using the inoculation needle under aseptic conditions. The spore suspension was appropriately diluted for the required density spores. The total number of viable spores in PDA slant was determined by colony count technique after serial dilution (Patel *et al*., 2005).

Amylase production under sub merged fermentation: Wheat bran (100g) was boiled and filtered, to which a mineral composition of $ZnSO_4 \times 7H_2O$ 6.2mg, $FeSO_4 \times 7H_2O$ 6.8mg and $CuSO_4 \times 7H_2O$ 0.8mg was added and volume was made up to 1litre with distilled water. pH was adjusted to 4.5. This is the Mineral Salt Medium (MSM). One milliliter of spore suspension was used as inoculum. Fermentation was carried out for four days (Varalakshmi *et al*., 2009).

Amylase production under solid state fermentation: Solid state fermentation was carried out in 250 ml Erlenmeyer flask containing 10 g of wheat bran moistened to 16ml with mineral salt medium (MSM) and 2 ml of spore suspension was used as inoculum. Fermentation was carried out for four days.

Optimization of culture conditions for enzyme production: The effect of process parameters on enzyme production was determined by incubating at different pH (3 to 11), temperature (20- 40°C), additional carbon source (1% of glucose, maltose, sucrose and soluble starch), nitrogen source (1% each of beef extract, meet extract, casein and urea), moisture content (43 - 81%) and incubation time (24 - 120 h) were used to determine their effect on amylase production.

Enzyme extraction: After 96 h, from submerged fermentation the fermented broth was centrifuged at 8,000 rpm for 15 min. supernatant was filtered through Whatman no-1 filter paper and filter was used the enzyme source. From the solid state fermentation, 22ml of 0.1M phosphate buffer (pH 6.5) was added to the culture flasks and mixed well for 30 minutes in a rotary shaker (140 rpm) at 19°C. The mixer was filtered through two fold cheese cloth and then filtering through Whatman No: 1 filter paper. The filtrate was used as source of amylase (Alva *et al*., 2007).

Enzyme assay: The amylase activity was assayed by measuring the reducing sugar liberated in the reaction mixture. The reaction mixture (3ml) consisted of 0.5ml of 1% (w/v) soluble starch and 0.5ml appropriately diluted enzyme source in 2 ml of 0.1M phosphate buffer (pH 6.5). After incubation at room temperature for 20 minutes the reaction was stopped by addition of dinitrosalicylic method (DNS). Then boiling tubes in a water bath for 15 min and thus the reducing sugars released by enzymatic hydrolysis of starch were determined (Miller, 1959). One unit of amylase activity was defined as the amount of enzyme that releases one micromole of reducing sugar as glucose per minute under assay conditions and enzyme activity is expressed as the specific activity, which is represented as U/mg of protein. The experiments were carried out in triplicates and standard deviations were calculated.

Protein estimation: Protein content of the enzyme extracts was estimated by the method of Lowry *et al.* (1951), using bovine serum albumin as the standard. Enzyme activity is expressed as the specific activity, which is equivalent to U/mg protein.

Effect of pH of the medium: The effect of pH on enzyme yield by fungus during solid state fermentation was studied by adjusting the pH of the mineral salt medium used to moisten the substrate to various pH levels (pH 3 to11) using 1.0 N NaOH and 1.0 N HCl. The other conditions were kept constant. (Sindhu *et al*., 2009).

Effect of temperature: The effect of temperature on enzyme production by fungi during Solid state fermentation was determined by incubating the flasks at different temperature (20 to 40°C) and keeping other conditions constant (Chimata *et al*., 2010).

Effect of time: The effect of incubation time on enzyme

production by the fungi was studied by incubating the inoculated flask for a total period of 120 h and estimating the enzyme production at regular intervals of 24 h (Ramachandran *et al.*, 2004).

Effect of carbon source: The effect of carbon source on enzyme production by the fungi during solid state fermentation was determined by incorporating at 1% (w/w) level in the medium. The carbon sources tested include glucose, maltose, sucrose and soluble starch (Erdal and Taskin, 2010). The other conditions were kept constant.

Effect of nitrogen source: The effect of nitrogen source on enzyme production by the fungi was studied by incorporating 1% (w/w) level of nitrogen in the SSF medium. The nitrogen sources tested include 1% each of beef extract, peptone, casein and urea (Bhatti *et al.*, 2007). The other conditions were kept constant.

Effect of moisture content: The effect of initial moisture content of the solid medium on enzyme production was determined by preparing the solid substrates with varying moisture contents in the range of 43, 54, 62, 73, 77 and 81**%** (Souza *et al.*, 2001). This was achieved by altering the quantity of sterile distilled water used to moisten the substrates. The other conditions were kept constant.

RESULTS AND DISCUSSION

Isolation and screening: Sixteen fungi were isolated from spoilt fruits, vegetables and soil. The screening was carried out by starch hydrolysis method and totally five out of sixteen possessed were found to be amylolytic activity (Table 1) that is *Rhizopus sp*, *Aspergillus flavus*, *Penicillium sp Trichoderma viride* and *Trichoderma sp*. Among the isolates the fungal isolate *Aspergillus sp* was exhibited higher amylolytic activity in starch agar medium and was selected further studies.

Enzyme production: The *Aspergillus flavus* subjected to sub merged and solid state fermentations and was found to be the best amylase producer with values 17.36 U/mg protein for solid state and 11.08 U/mg proteins for sub merged fermentation. So, this potential species was selected for further studies under solid state fermentation. This value is higher than that reported on *Aspergillus sp* JGI-12 by Alva *et al.* (2007). The observations made with respect to optimization of growth conditions and process parameters that administrate maximal production of a-amylase by this species potential of the organism for industrial use.

Effect of pH of the medium: Among physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium. Most of the earlier studies revealed the optimum pH range between 6.0 and 7.0 for the growth of bacterial strains and enzyme production (Gupta *et al.*, 2003; Kundu

Table 1. Starch hydrolysis test performed on isolated organisms for amylase activity.

S. No	Isolates	Zone of Inhibition
1	*Rhizopus sp*	+
2	*Aspergillus sp*	++
3	*Penicillium sp*	+
4	*Trichoderma viride*	+
5	*Trichoderma sp*	+

Note: ++ = Strongly positive, + = positive

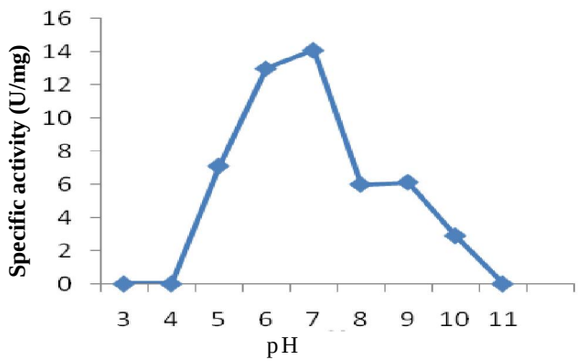

Fig. 1. *Effect of pH on fungal α-amylase production.*

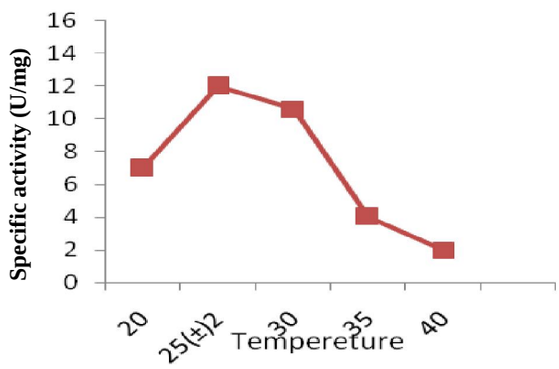

Fig. 2. *Effect of temperature on fungal α-amylase production.*

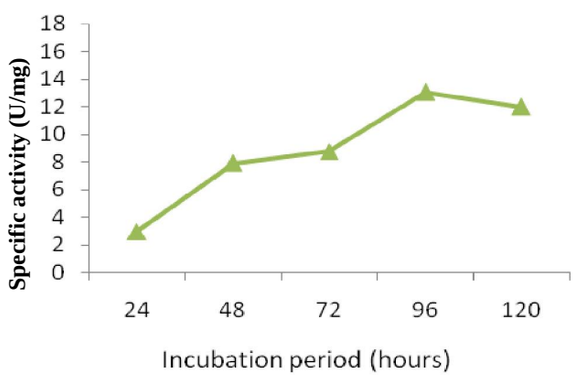

Fig. 3. *Effect of incubation period on fungal α-amylase production.*

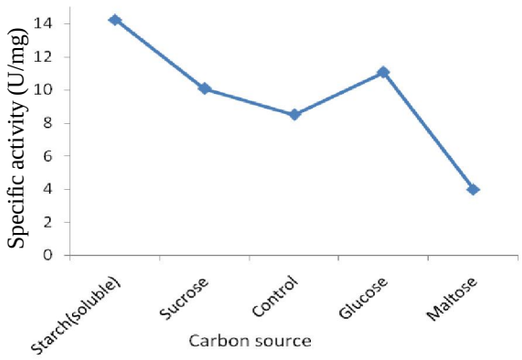

Fig. 4. *Effect of carbon sources on fungal α-amylase production.*

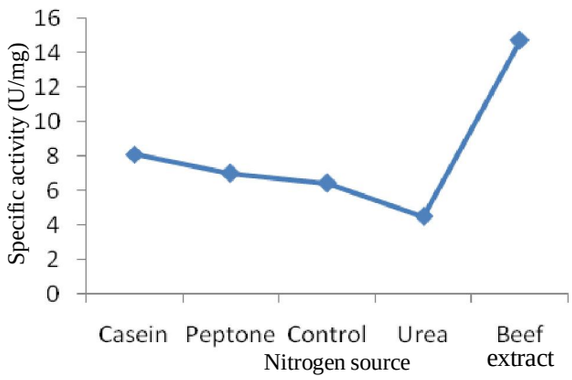

Fig. 5. *Effect of nitrogen sources on fungal α-amylase production.*

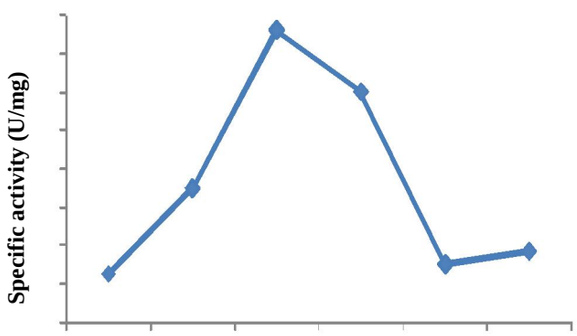

Fig. 6. *Effect of moisture content on fungal α-amylase production.*

et al., 1973; Castro *et al.*, 1992). However, *Aspergillus oryzae* released amylase only in alkaline pH above 7.2 (Yabuki *et al.*, 1977). The selected *Aspergillus flavus* showed best enzyme production at pH 6.0 by solid state fermentation. The results available in Fig. 1 showed that maximum amylase and was produced at pH 7.0 (14.08 U/mg). Baysal *et al.* (2003) were earlier reported the same observations in *B. subtilis*.

Effect of temperature: Solid state fermentation is usually carried out in the temperature range of 25 to 40°C. A significant level of enzyme was produced by the fungi over a range of temperature 20 to 25°C, with an optimum at 25 (± 2) °C (Fig. 2). Temperature optimum for amylase was found to be in a range between 25 and 37°C for the mesophilic fungi (Kundu *et al.*, 1973 and Gupta *et al.*,2003) and the present study recorded 25(± 2) °C as optimal, which agrees with earlier findings. The influence of temperature on amylase production is related to the growth of microbes.

Effect of incubation time: The incubation period varies with enzyme production. Short incubation period offers potential for inexpensive production of enzymes (Silva *et al.*, 1995). In the present study, amylase production was 3.00 U/mg in 24 h and gradually increased up to 13.03 U/mg in 96 h of incubation. After 96 h there was gradual decrease in enzyme production (Fig. 3). Kathiresan *et al.* (2006), reported, that maximum activity was detected in 96h (136 U/ ml) by *Penicillium fellutanum* under sub merged fermentation and as against a short duration of 24 h in the case of bacteria.

Effect of carbon source: α-amylase is an inducible enzyme and is generally induced in the presence of carbon sources such as starch, its hydrolytic product (Yabuki *et al.*, 1977). The results presented in Fig. 4 indicate that Starch (soluble) enhanced amylase production (14.24 U/mg) when compared to other carbon sources. The same observations were earlier reported by Varalakshmi *et al.*, 2009 and Erdal and Taskin (2010).

Effect of nitrogen source: The results presented in Fig.5 indicate that beef extract was a superior maximal amylase production (14.7 U/mg) compare to other nitrogen sources. These same observations were earlier reported by Varalakshmi *et al.*, 2009. Michelena *et al.* (1984) reported that the supplementation of nitrogen salts greatly increased the enzyme yields in *Aspergillus foetidus*.

Effect of moisture content: The results presented in Fig. 6 indicate that moisture content of 62% was optimum for maximum enzyme yield. Optimum yield was observed as 15.21 U/mg at 62% moisture content which decreased at 81% moisture content. Highest content moisture in solid-state systems could increase the processes of diffusion (Souza *et al.*, 2001). Increase in moisture content resulted in clumping of the solid particles and consequent reduction in enzyme yield and microbiological activity on a substrate will progressively decrease at lower water contents (Sindhu *et al.*, 2009).

The present study indicated that *Aspergillus flavus* produced high amount of amylase in wheat bran with minimal salt medium, which has been modified with certain carbon and nitrogen source. So it is concluded that wheat bran with minimal salt medium can be used under solid

state fermentation for the production of amylase under controlled conditions with a pH 7 and at temperature 25 (±2)°C, incubation time 96 h, carbon source soluble starch, nitrogen source beef extract and 62% moisture content.

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