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Amylase Production from Solid State Fermentation and Submerged Liquid Fermentation by *Aspergillus niger*

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Abstract

The purpose of this work is to study the optimized cultural conditions for the production of amylase by *Aspergillus niger* in solid state and submerged liquid fermentation. Four solid substrates banana peel, corn, potato and tapioca with different moisture conditions were taken for solid state fermentation (SSF). Basal medium was used for submerged liquid fermentation (SLF) with different pH (3 to 8), temperature (25, 30, 35 and 40ºC), carbon concentration (1, 2 and 3 g) and nitrogen source (0.1, 0.2 and 0.3 g). In SSF, tapioca yielded highest amylase activity and specific activity (4.43U/ml and 4.58U/mg) at 50% moisture content. In SLF, 2 g starch and 0.3 g peptone concentration showed 0.78 and 1.23 U/ml amylase activities under the optimum pH (5) and temperature (30ºC) the amylase activities reached to 0.86 U/ml and 0.76 U/ml respectively. In SSF using tapioca as substrate the enzyme yield is about five times higher than that achieved with submerged liquid culture.

Key words: Tapioca, Solid state fermentation, Submerged liquid fermentation, pH, Temperature, Amylase activity

Introduction

Microorganisms made significant contribution for the production of foods and beverages in the past three centuries. Various industries, such as food, brewing, textile, pharmaceutical and confectionaries depends mainly on various products especially extracellular enzymes. In recent years, using of microorganisms as biotechnological sources as industrially relevant enzymes, have stimulated renewed interest in the exploration of extracellular enzymatic activity in several microorganisms (Ibukun and Adindumila, 1998).

Enzymes are the most important products, obtained through microbial sources for human needs. Among these biomolecules, amylase an exo-acting enzyme that yields maltose on hydrolysis of various starches. The α-amylase is being isolated from a number of microbial sources and every enzyme moiety isolated from various microbial sources is unique in their characteristics. Various attempts have been made so far on studying the yield and potential of α-amylase from different microbial strains. Fungal and bacterial amylases are widely used for commercial application in food processing industries (Burhan *et al.,* 2003). The *Aspergillus* species produce a variety of extracellular enzymes of which amylases are of the world-wide interest in fermentation, food, pharmaceutical, textile and paper industries (Rao and Satyanarayana, 2007; Bhargav *et al.,* 2008).

Production of amylases by *Aspergillus* strains in both submerged liquid fermentation (SLF) and solid-state fermentation (SSF) by using different food wastes or agricultural residues has been thoroughly studied (Ellaiah *et al.,* 2002; Francis *et al.,* 2002, 2003; Salas *et al.,* 2006). However, comparative studies between SLF and SSF claim higher yields and other advantages for products obtained by SSF, such as low energy requirements, lower availability of water that reduces the possibilities of contamination by bacteria and yeast, small volumes of polluting effluents and low downstream processing cost (Guerra *et al.,* 2003). The production of amylases in SSF is affected by a variety of physicochemical factors, including the type, nutrient composition of the substrate, incubation temperature, pH, aeration, concentration and the type of carbon, phosphate and nitrogen sources, concentration and age of the inoculum, particle size and moisture level of the substrate (Balkan and Ertan, 2007; Rodriguez and Sanroman, 2006). Therefore, after selecting a culture medium for amylase production, the fermentation

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conditions must be optimized to improve enzyme production at a low production cost (Balkan and Ertan, 2007; Spier *et al.,* 2006). The purpose of this work was to study the production of the enzyme, amylase by *Aspergillus niger* in solid state and submerged liquid cultures and optimized the cultural conditions such like appropriate substrate, pH, temperature, moisture level, total sugars concentrations, concentrations of nitrogen sources.

Materials and Methods

Microorganism

Aspergillus niger was isolated from infected potato and maintained in potato dextrose agar (PDA) (Hi-media, Mumbai). Isolated colonies were identified based on the morphological characters by fungal staining procedure and it was maintained on potato dextrose agar slants at 4°C (Ellaiah *et al.,* 2002; Salas *et al.,* 2006).

Inoculum

The inoculum was prepared by the addition of sterile distilled water into the freshly grown potato dextrose agar slants, from this 0.5 ml of cell suspension was inoculated in to 50 ml (250 ml Erlenmeyer flasks) of sterilized fermentation medium and incubated at 35°C and 220 rpm/min for 48 hrs (Salas *et al.,* 2006). The composition of the fermentation medium (gl^{-1}): glucose, 20; $(NH_4)_2SO_4$, 6.6; KH_2PO_4, 3.5; $FeSO_4.7H_2O$, 0.15; $MgSO_4.7H_2O$, 0.10; $MnCl_2.2H_2O$, 0.45 and mycological peptone, 3.0; at pH 6.8.

Fermentation Mediums

Solid substrates

Banana peel, corn, potato and tapioca were cut into small pieces and air dried at 40°C. The substrates were moistured with water to adjusted water content of 40%, 50%, and 60% in conical flasks

Liquid mediums

The fungus was grown in basal medium. Liquid state fermentation was carried at varying pH (3 to 8), temperature (25, 30, 35 and 40ºC) concentration of the carbon source (1 g, 2 g and 3 g), and concentration of the nitrogen source (0.1 g, 0.2 g, and 0.3g).

Inoculation of samples

Solid state fermentation

The solid media were sterilized in 250 mL conical flask along with sterile water. To the *Aspergillus niger* slant tube, sterile water was added and scratched with inoculation loop to obtain a spore suspension. Each conical flask was inoculated with 1 ml of fungal suspensions and was incubated in rotatory shaker at 200 rpm for 48 h.

Liquid state fermentation

Liquid media were sterilized in and then each conical flask, inoculated with 1 mL of liquid suspension of *Aspergillus niger* and then incubated in rotatory shaker at 200 rpm for 48 h.

Extraction of the enzyme

After fermentation, the fermented slurry was mixed with water to 100ml. Contents were mixed thoroughly (150 rpm/min, at room temperature for 1 h) in a rotary shaker and the suspension was then centrifuged at 7000 rpm at 4°C for 10 min and the supernatants were used for enzyme assay.

Analytical method

Amylase assay

Amylase activity was determined as described by Okolo *et al*. (1995). The reaction mixture consisted of 1.25 mL of 1% soluble starch, 0.25 ml 0.1 M acetate buffer (pH 5.0), 0.25 ml of distilled water, and 0.25 mL of crude enzyme extract. After 10 min of incubation at 50°C, the liberated reducing sugars (glucose equivalents) were estimated by the dinitrosalicylic acid (DNS) method of Miller (1959). The colour developed was read at 575 nm using a Hitachi 220s UV spectrophotometer. Glucose was used as the standard. The blank contained 0.5mL of 0.1 M acetate buffer (pH 5.0), 1.25 ml 1% starch solution and 0.25 mL distilled water. One unit (IU) of α-amylase was defined as the amount of enzyme, releasing one µmol glucose equivalent per minute under the assay rendition.

Estimation of soluble protein

Soluble protein concentrations were determined in the aqueous extract of fermented matters were determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard.

Results and Discussion

In recent years, application of the agro industrial residues (*i.e*., sugar cane bagasse, sugar beet pulp, apple pomace, wheat bran) provided an alternate way to replace the pure and costly raw materials. In industrial process the use of such materials would help to solve many environmental hazards (John *et al.,* 2006). Extensive works are going on all over the world to select the suitable organisms and efficient inducers for the production of concentrated α-amylases using biomass wastes in solid-state fermentation. The selection of a suitable solid substrate in solid state fermentation (SSF) is a critical factor. In literature different solid substrates were found to influence the production of enzymes (Beckord *et al.*, 1945; Satyanarayana,1994). Hamilton *et al.* (1999) reported that the growth and enzyme production by microorganisms were greatly influenced by both environmental conditions and nutrients available within the growth medium. The critical importance of moisture level in SSF media, its influence on the biosynthesis and selection of enzymes can be attributed to the interference of moisture in the physical properties of the solid particles. An increase in moisture level is believed to reduce the porosity of the wheat bran, resulting in limited oxygen transfer (Sadhukhan *et al.,* 1990). Low moisture content causes reduction in the solubility of nutrients of the substrates and low degree of swelling (Feniksova *et al.,*1960).

The first optimization experiment was performed to check the influence of different moisture content, which was varied by adding water to different concentrations (40, 50 and 60%). In solid state fermentation banana peel was used as a substrate for amylase production. In this experiment amylase activity and specific activity on banana peel were shown in Fig. 1. The maximum amylase activity (0.39942 U/mL) was observed at 40% moisture and maximum specific activity (1.8 U/mL) was observed with 60% moisture content. Amylase activity and specific activity on corn are shown in Fig. 2. The maximum amylase activity and specific activity were respectively 0.70224 U/mL and 1.5 U/mL at 50% moisture content. Fig .3 shows the enzyme activity and specific activity on potato. The maximum amylase activity (0.46788 U/mL) and specific activity (1.1 U/mL) was observed at 50% moisture. On tapioca, the maximum amylase activity (0.43512 U/mL) and specific activity of (4.58 U/mL) was observed at 60% moisture (Fig. 4). Among these solid substrates tapioca yielded highest amylase activity (4.43U/mL) at 60% moisture content.

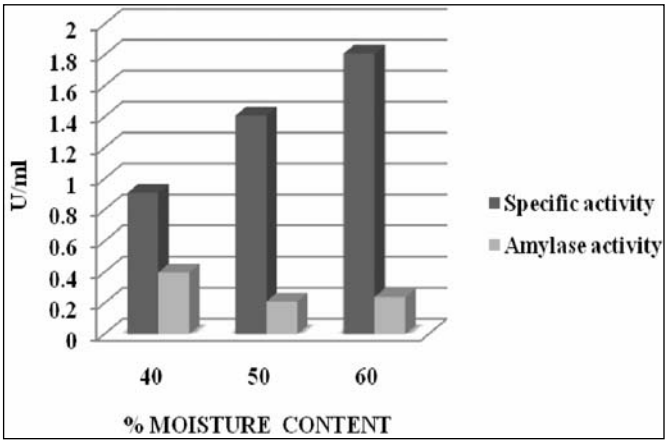

Fig. 1: Effect of banana peel on amylase activity and specific activity in different moisture contents

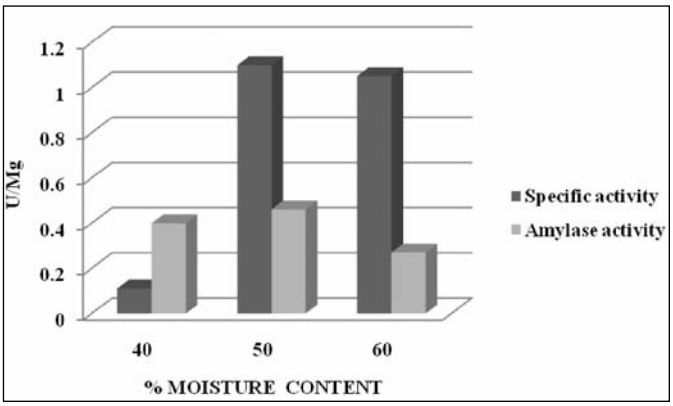

Fig. 2: Effect of corn on amylase activity and specific activity in different moisture contents

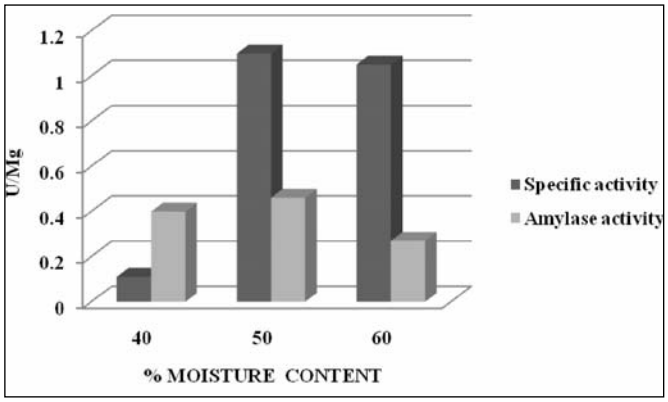

Fig. 3: Effect of potato on amylase activity and specific activity in different moisture contents

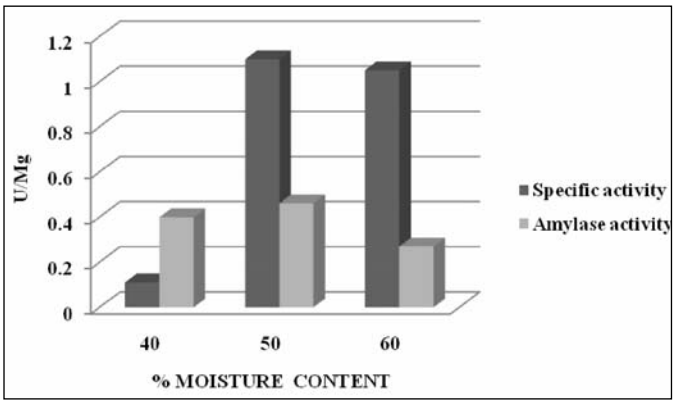

Fig. 4: Effect of tapioca on amylase activity and specific activity in different moisture contents

Irrespective of the substrates used, 60% moisture content was found to be optimal for amylase production. Tapioca, among the substrates has appreciable fibrous content in addition to starch, which avoided the compaction of the solid mass in due course of fermentation, there by promoting good aeration reaching the whole core of the substrate, in turn yielding more amylase. Whereas the other substrates like potato and corn could not promote better yield because of their compressible nature of solid packing, which reduced aeration. So from the above study, amylase production from tapioca was found to be the suitable solid substrate and the system can be scaled up for commercial production of amylase from solid substrate. Tapioca is traditionally used as a dessert or breakfast meal. As the development of technology become more sophisticated, tapioca starch is useful in textile industry, paper industry and for miscellaneous uses (Van damme *et al.*, 2002). Tapioca contains almost 70-75% of starch. Therefore it is suitable for use as a substrate. Ramesh *et al.* (2001) reported that the biomass production by *Clostridium thermosulfurogenes* using barley and tapioca flour did not differ significantly when soluble starch was used as a carbon source.

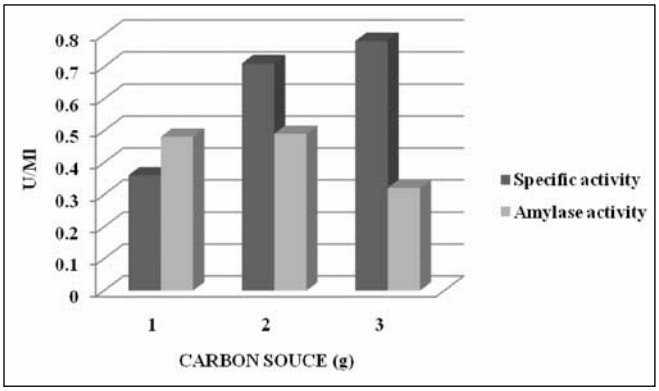

Fig. 5: Effect of carbon source on amylase activity and specific activity using *Aspergillus niger*

In submerged liquid fermentation, amylase activity was influenced by varied carbon (starch) and nitrogen concentrations (Fig. 5 and Fig. 6). The maximum amylase activity (0.49 U/ml) and maximum specific activity (0.78 U/mL) was detected using 2 g and 3g of starch concentration respectively. Highest enzyme activity (1.23 U/mL) and specific activity (0.89 U/mL) was observed in the highest concentration of nitrogen source used. From the above study it was seen that solid state fermentation yielded nearly 5 times more amylase activity as compared to submerged fermentation. Temperature and pH plays vital role in enzyme production. The fungal production highest amount of α-amylase at temperature between 30and 35 °C (Fig. 7), and at pH between 5 and 6 (Fig. 8). These results are in agreement to the other studies. *Aspergillus niger* UO-01 exhibited its best performance for enzyme production in the mesophilic range (T = 30.2ºC), as

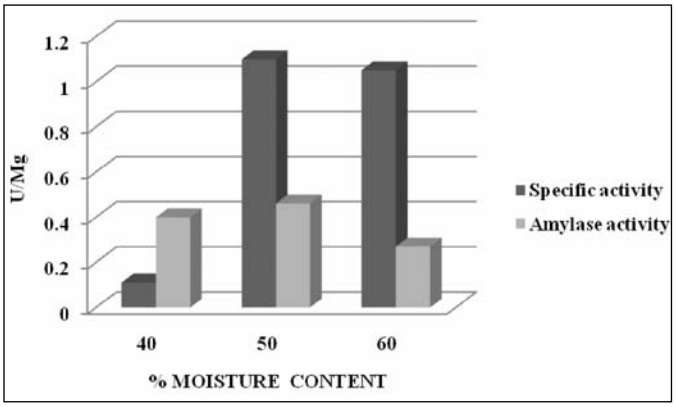

Fig. 6: Effect of nitrogen source on amylase activity and specific activity using *Aspergillus niger*

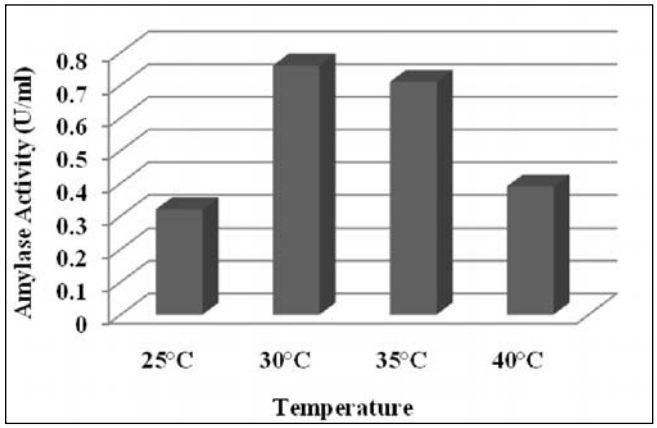

Fig. 7: Effect of temperature on amylase production

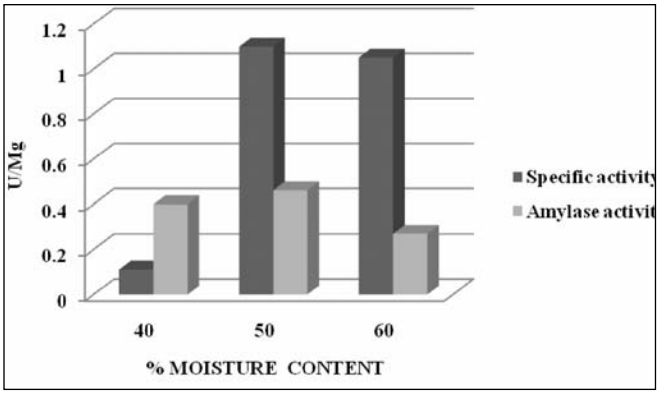

Fig. 8: Effect of pH on amylase production

it was reported before for *A. niger* LPB 28, which produce the highest amylase level at 30ºC (Spier *et al.*, 2006). Temperature values higher than 30ºC may lead to enzymatic inactivation (Mazutti *et al.,* 2006) or suppression of cell viability (Francis *et al.,* 2002). In contrast, low temperature values may reduce the metabolism of the microorganism (Mazutti *et al.,* 2006) and consequently, the enzyme synthesis. In this study, these results of present study suggest that Tapioca yielded highest amylase activity at 50% moisture and it was also verified that *A. niger* has a preference for acidic (pH 5) and moderate temperature (30°C) probably as a for growth and extracellular amylase production.

Conclusion

The purpose of this work is to study the optimized cultural conditions for the production of amylase by *Aspergillus niger* in solid state and submerged liquid fermentation. Four solid substrates Banana peel, Corn, Potato and tapioca with different moisture conditions were taken for solid state fermentation (SSF). From these substrates tapioca yielded 5 times higher than submerged liquid fermentation. So the commercial production of Amylase enzyme will be made feasible by successful scale up of these optimized parameters to industrial production in large scale fomenters.

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