

Full Length Research Paper

Optimization of α -amylase production from free and immobilized cells of *Aspergillus niger*

Arti Gupta^{1*}, Neelam Gautam² and Dinesh Raj Modi²

¹G.B. Pant University of Agriculture and Technology, Pantnagar 263145, (Uttarakhand) India.

²Department of Biotechnology, B.B.A. University, Lucknow 226025, (U.P.) India

Accepted 4 October 2010

In the present study, characterization of amylase producing *Aspergillus niger* isolate, optimization of medium composition, cultural conditions for amylase production, extraction and partial purification of extra cellular enzyme from a potential isolate was investigated using both free and immobilized cells. The alpha amylase of *A. niger* had the pH optima ranged at 4-6 and temperature optima ranged at 30-40 °C, however the optimum pH, temperature and incubation period for enzyme production was 5.0, 35 °C and 5th day for Immobilized cells and 5.0, 30 °C and 5th day for free cells respectively. Of the carbon sources, starch was recorded to be the best carbon source for enzyme production. Peptone at 0.03% was ideal nitrogen source. However, surfactants Tween -80, Triton X-100 and Sodium dodecyl sulphate at 0.02%, 0.002% and 0.0002 % concentration were most effective for enhancement of α -amylase production. Finally the enzyme was characterized by chromatography and confirmed as alpha amylase.

Key words: *Aspergillus niger*, α -amylase, Free cells, Immobilized cells.

Introduction

α -amylase enzymes are all α -1,4-glucan 4-glucanohydrolases (E.C. 3.2.1.1) that are important enzymes employed in the starch processing industries for the hydrolysis of polysaccharides such as starch into simple sugar constituents as reported by Akpan *et al.* (1999a, 1999b); Fogarty *et al.* (1980); Haq *et al.* (2002). Starch degrading enzymes like amylase have received a great deal of attention because of their perceived technological significance and economic benefits. This enzyme is also used for the commercial production of glucose. In storage tissues such as seeds, starch a polysaccharide of glucose is hydrolyzed for utilization by the growing seedlings to meet its energy requirement. Nowadays the new potential of using microorganism as biotechnological sources of industrially relevant enzymes has stimulated renewed interest in the exploration of extra cellular enzymatic activity in several microorganisms. Sources of amylases in yeast, bacteria and moulds have been reported and their properties have

also been described by Adebisi *et al.* (1998); Bilinski *et al.* (1995); Buzzini, *et al.* (2002). The purified thermostable alpha amylase from thermophilic fungal origin *Thermomyces lanuginosus* was found to be more stable than the bacterial enzyme (Duochaun *et al.*, 1997). To prepare these extra cellular enzymes on a commercial scale, many attempts have been made to specify cultural conditions and to select superior strains of the fungus (Ichishima, 1971; Windish 1965). Few attempts have been made to elucidate the control mechanism involved in the formation and secretion of the extra cellular enzymes. Among mold species producing high levels of amylase, *Aspergillus niger* is used for commercial production of alpha amylase. This enzyme is an extra-cellular enzyme and therefore can be easily separated from the cell mass. Immobilized enzymes are also used in food technology, biotechnology, biomedicine and analytical chemistry. They have various advantages over free enzymes including easy separation of the reactants, products and reaction media, easy recovery of the enzyme, and repeated or continuous use. Initially, most work was carried out on the immobilization of bacterial cells. Eventually, yeasts and filamentous fungi received increasing attention (Anderson, 1975). An appreciation of

the potential of immobilized fungal cells for the production of enzymes such as glucose oxidase and glucoamylase were demonstrated by Fiedurek and Ilczuk (1991). It is possible to extend the use of amylases under extreme condition of pH and temperature using thermo acidophilic and alkaline amylase confirmed by Kindle (1983). It is worthwhile to isolate a suitable strain of *A. niger* for efficient mechanism. Selection of suitable production media is very essential for the growth of the microorganism as well as production of the enzyme. The production of alpha amylase by moulds has been greatly affected by cultural and nutritional requirements (Pedersen and Nielson, 2000; Prescott and Dunn 1987). Therefore, the objective of this study was the characterization and optimization of cultural conditions for the production of alpha amylase from free and immobilized cells of *A. niger*.

Material and Methods

Isolation and identification of *Aspergillus niger* strain

The *Aspergillus* culture was isolated from the soil by the serial dilution method of Clark et al. (1988), one gram soil sample was dissolved in 100 ml sterilized distilled water. The soil suspension was diluted up to 10^{-3} to 10^{-3} and 0.5ml of diluted suspension was used and the micro-organism producing the starch digesting α -amylase screened according to method the described by Bergmann et al. (1988). *Aspergillus niger* colonies producing large clear zones were picked up and purified by streaking on PDA. Identification was based on cell and colony morphology characteristics as per the method described by Rasper and Fennel (1965). The young colonies of *Aspergillus niger* were aseptically picked up and transferred to PDA slants and incubated at 27 °C for 4-5 days for maximum growth.

Immobilization of *Aspergillus niger* cells

Aspergillus niger cells were immobilized and enzyme was isolated as per the method described by Abraham et al. (1991).

Growth Medium

The medium contained (% w/v): Starch (1%); KH_2PO_4 (0.2%); $(\text{NH}_4)_2\text{SO}_4$ (0.14%); CaCl_2 (0.03%); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.03%), Urea (0.03%), Peptone (0.1%), trace element solution (0.01%), Triton X-100 (0.02%). Trace element solution contained in 500ml (2.5 g FeSO_4 , 1.0 g CoCl_2 , 1.76 g ZnSO_4 , 0.98 g MnSO_4). The pH of the medium was adjusted to 4.8 with concentrated HCl, medium was sterilized by autoclaving at 121 °C and 15-17 psi for thirty minutes.

Inoculum and fermentation

A standardized inoculum size of conidia (each ml of cells suspension contained 2.0×10^6 cells) was transferred from a stock culture in 250ml flask containing 50 ml of growth medium. The flasks were incubated for 72 hrs at $28 \pm 2^\circ\text{C}$ on a rotary shaker at 150 rpm. On the last day of incubation period (72 hrs), the fungal mass was separated by centrifugation at 4500 rpm for 10 min. The clear supernatant (Crude enzyme) was used for estimation of alpha

amylase; the enzyme activity was expressed in number of units. 1 unit of enzyme was defined as the amount of enzyme (protein) in milligram required for hydrolysis of starch to produce a millimolar of reducing sugar (glucose) in one hour under assay conditions. The specific activity was defined as number of units per gram protein.

Preparation of Enzyme

The enzyme was precipitated from clear a supernatant at 4 °C by adding solid ammonium sulphate to achieve 85% saturation. The ammonium sulphate was added slowly, keeping the solution in ice and the protein was allowed to precipitate by keeping it overnight at 4 °C. The protein was separated by centrifugation at 2000 X g for 30 minutes at 4 °C, dissolved in minimum volume of phosphate buffer (50mM, pH 7.2) and used immediately for activity determination.

Enzyme Assay

Enzyme activity was determined by DNS method described by Mandels et al. (1976) using starch as the substrate. The reaction mixture contained the following in a total volume of 2 ml: 10 mg starch, 1 to 1.8 mg protein and 50 mM Sodium – phosphate buffer of pH 7. The reaction mixture was incubated at 37 °C for 30 minutes and the reaction was terminated by addition of 3 ml of DNS solution (3, 5-dinitro Salicylic Acid). After stopping the reaction the tubes were placed in boiling water bath for 5 minutes and then cooled and absorbance was determined at 540 nm. The amount of glucose produced was calculated by referring to the standard plot using glucose as the reducing sugar (The standard plot was prepared simultaneously).

Nutritional Requirement for Extra Cellular α -Amylase Production

Growth and incubation period for maximum alpha amylase production was quantified along with the effect of carbon; nitrogen source on production of the enzyme as follows: percentage of carbon (0.5 to 2.0%); nitrogen source (0.3%); variable temperature range (20 °C to 50 °C) and pH range of (3 to 8) for productive fermentation media. The initial pH of medium was adjusted using concentrated HCl. Effect of surfactants and their concentration (Tween-80, Tween-20, Triton X-100 and SDS at the rate of 0.0002 to 0.2% by w/v) was studied. The characterization of the type of amylase is performed by thin layer chromatography.

Thin Layer Chromatography (TLC)

Sugars produced by the hydrolysis of the raw native starch with crude amylase of *A. niger* were identified by Thin Layer Chromatography (TLC). Commercially prepared TLC plates (Polygram, UK) were used as the stationary phase. Aliquot of free and immobilized cells were spotted on the TLC plates (Figure 1). The reference sugar solution contained 0.1 g each of maltose, sucrose, glucose and raffinose, dissolved in 100 ml of 10% isopropanol. A one dimensional ascending chromatography was done at room temperature using a solvent system of n-butanol: acetic acid: diethyl ether: water (9:6:3:1, (v/v/v/v)). After 2 h, all the chromatograms were treated by dipping in reagents made up of 4-amino-benzoic acid in methanol. The plates were air-dried and placed in the oven at 100 °C for 15 min. The sugar spots appeared as dark brown spots. Identification of the sugars was done by comparing the relative fraction (Rf) values of the samples with that of the standards.

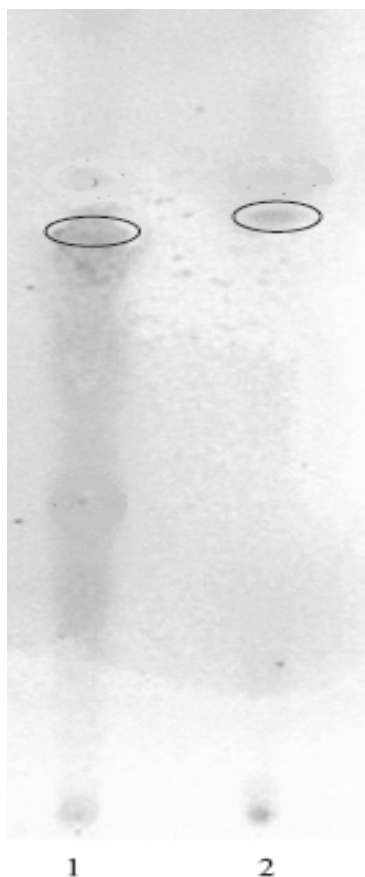


Figure. 1 Thin Layer Chromatography of *Aspergillus niger* (1= Free cells, 2= Immobilized cells)

RESULTS

Aspergillus niger isolated from soil was screened for maximum production of alpha amylase by observing clear zone of starch hydrolysis in Petridishes.

Effect of Temperature and pH on Extra Cellular α -Amylase Production

Assay of enzyme production was carried out at various temperature ranges 20 to 50°C for 24 hrs. It was found that both free and immobilized cells of *Aspergillus niger* showed considerable amount of growth at 20°C but there was less enzyme production. However, the optimum temperature for enzyme production was 30°C for free cells and followed by 35°C for immobilized cells of *A. niger* (Figure 2). The initial pH of medium was adjusted to variable pH range by adding the 0.1N HCl. Enzyme purified was tested in the pH range (pH 3 to 8). The production of alpha amylase was found to be the best at pH 5.0 both free and immobilized cells of *Aspergillus*

niger. Below and above this pH production of alpha amylase was significantly lower (Figure 3).

Effect of Incubation Period on Extra Cellular α -Amylase Production

The alpha amylase activity was determined after every 24 hours of incubation in order to determine the optimum incubation period for maximum production of extra cellular alpha amylase. The enzyme production however, started after 24 hours of inoculation and showed maximum production on 5th day of incubation period for both free cells and immobilized cells of *Aspergillus niger* (Figure 4).

Effect of Carbon Sources on Extra Cellular α -Amylase Production

Flasks containing production were media supplemented with carbon sources (glucose, sucrose, starch, carboxymethyl cellulose, fructose, sorbitol, xylose, galactose and dextrin). The influence of these carbon sources were tested at different concentrations (0.5 to 2.0%). Starch, Sucrose and dextrin and galactose were good carbon sources for amylase production. Xylose, fructose and sorbitol could be considered as moderate source while carboxymethyl cellulose was a poor source of amylase as it was found that 0.5 % concentration is good carbon source for amylase production but carboxymethyl cellulose and glucose at 0.5% concentration produced low amount of alpha amylase. Starch was recorded to be the best carbon source for production of alpha amylase from both free and immobilized cells of *Aspergillus niger* (Table 1).

Effect of Nitrogen Sources on Extra Cellular α -Amylase Production

Effect of different nitrogen sources on the production of alpha amylase was studied, it was observed that casein and gelatin caused poor enzyme production. Peptone supported maximum production of enzyme whereas urea produced considerable amount of alpha amylase from both free and immobilized cells of *Aspergillus niger*. The optimum concentration of peptone was 0.03% (Figure 5).

Effect of Surfactants on Extra Cellular α -Amylase Production

The detergent Tween-80, Triton X-100 and SDS favored more amylase production in culture media. Tween-80 at 0.02% caused maximum enhancement where as Triton X-100 and SDS increased amylase activity at 0.002%

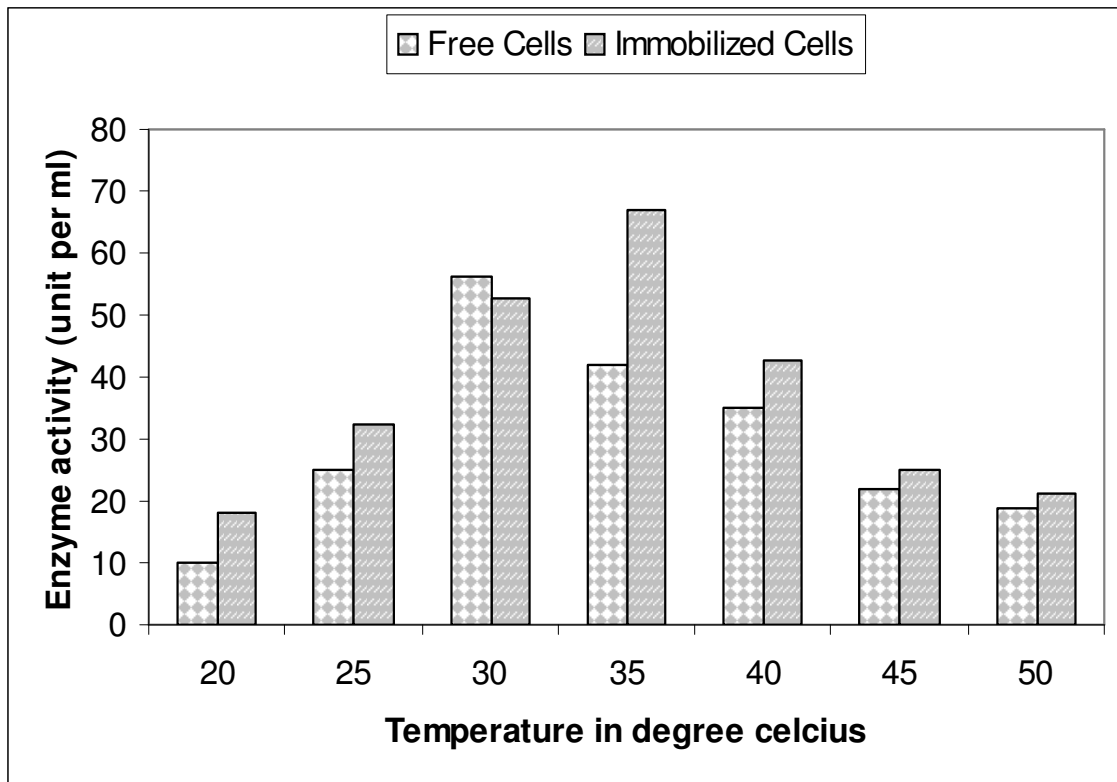


Figure. 2 Effect of temperature on extra cellular α -amylase production.

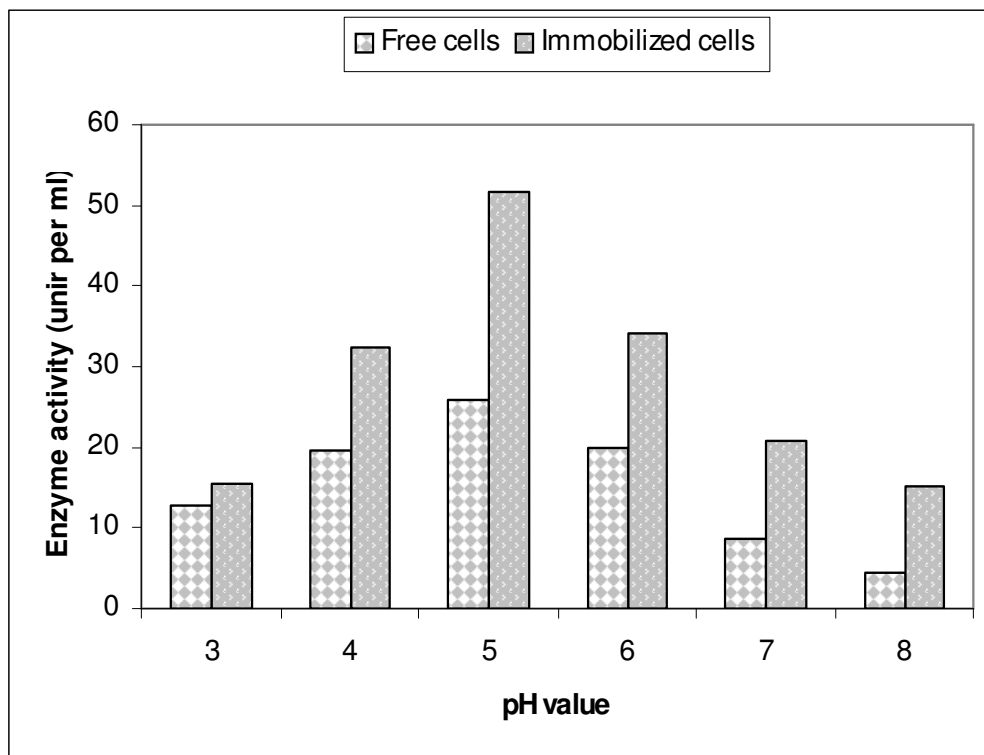


Figure.3 Effect of pH on extra cellular α -amylase production.

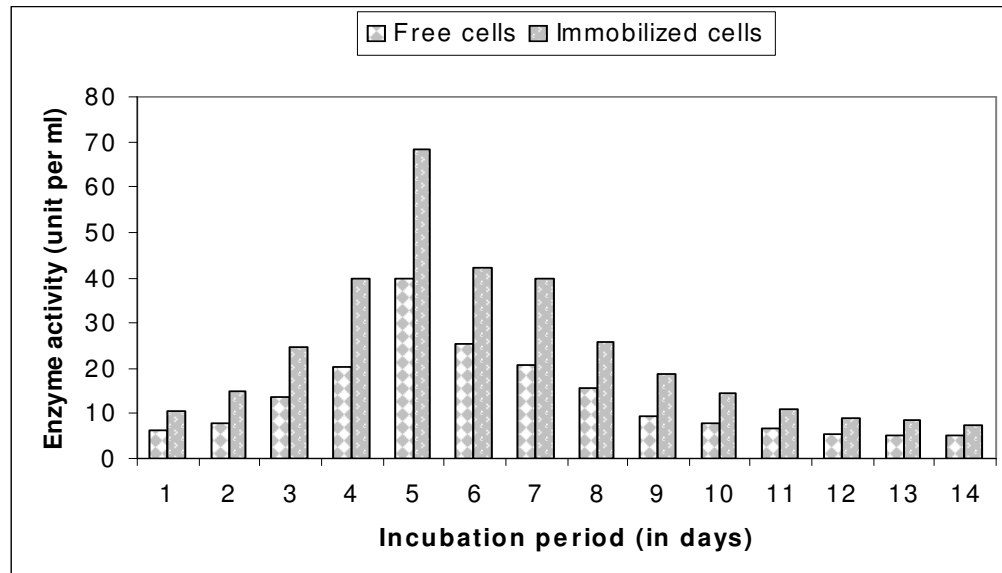


Figure. 4 Effect of incubation period on extra cellular α -amylase production.

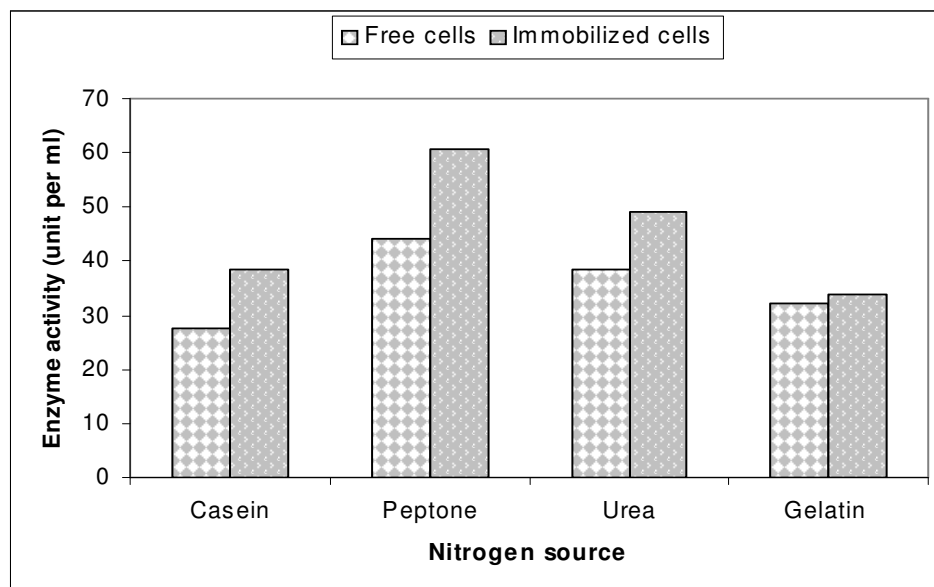


Figure. 5 Effect of different nitrogen sources at 0.03% concentrations on extra cellular α -amylase production.

and 0.0002% respectively for both free and immobilized cells of *A. niger* (Table 2).

Characterization of Amylases

The enzyme separated from *Aspergillus niger* isolates were separated in thin layer chromatography. The thin layer chromatography (TLC) analysis of the starch digest, showed glucose as the predominant product

of hydrolysis with small amount of maltose for all the starches and other sugars tested by free and immobilized cell of *A. niger* (Data not shown)

Discussion

The occurrence of amylolytic organism from the soil agrees with earlier report that the soil is known to be a repository of amylase producer. *Aspergillus niger*

Table 1. Effect of different carbon sources at different concentrations (0.5%, 1.0% and 2.0%) on extra cellular α -amylase production

Carbon sources	Concentration of carbon source (%)	Enzyme Activity (unit per ml)	
		Free cells	Immobilized cells
Sucrose	0.5	44.4	52.25
	1.0	34.6	42.68
	2.0	27.2	35.86
Starch	0.5	48.8	56.85
	1.0	36.8	45.32
	2.0	28.8	36.12
Glucose	0.5	10.44	11.22
	1.0	7.52	10.82
	2.0	1.88	4.22
Dextrin	0.5	42.6	45.26
	1.0	33.5	45.62
	2.0	26.6	32.42
Fructose	0.5	37.5	46.58
	1.0	30.4	40.26
	2.0	24.2	34.88
Carboxymethyl Cellulose	0.5	11.82	16.28
	1.0	8.12	15.66
	2.0	2.42	5.56
Sorbitol	0.5	32.2	42.36
	1.0	28.5	36.86
	2.0	22.8	31.26
Xylose	0.5	35.5	45.22
	1.0	29.6	40.26
	2.0	23.6	32.66
Galactose	0.5	41.4	50.23
	1.0	31.2	44.12
	2.0	25.5	36.55

requires no flooding, no prior replication of colonies on slants; the zones are very sharp and contrast with the blue-black background. Identification and characterization of alpha amylase producing strains were studied by Onion et al. (1987). These thermostable α -amylases differ in their pH optimum, temperature optimum, temperature stability and in several other physiochemical properties depending on the species origin. Hence different enzymes have found specific applicability in different industries. A number of reports exist regarding the influences of various environmental conditions like effect of pH value and temperature optimum, incubation period, carbon sources, nitrogen sources and metal ion on the production of α -amylase by *Aspergillus niger* Gupta et al. (2008). Increase in the incubation period resulted in a decrease in the production of alpha amylase by culture of *Aspergillus niger*. It may be due to the fact that after maximum production of alpha amylase enzyme (maximum incubation time), the production of other by products result in the depletion of nutrients. These

byproducts inhibited the growth of fungi and hence enzyme formation. The enzyme is very sensitive to pH. Therefore, the selection of optimum pH is very essential for the production of alpha amylase. In *A. niger*, production of extracellular proteases and phosphatases are regulated by ambient pH, e.g., acid proteases expressed at an acidic pH were found by Denison (2000). *A. niger* is well known for acidifying the medium as it grows. A pH regulatory system may be especially important. Apart from the regulatory effect on gene expression, cultivation pH can also affect fungal morphology greatly (Whitaker and Long, 1973). Thus, development of an optimal pH control strategy is helpful in obtaining higher protein productivity. Swift et al. (1998) reported that, in the pH auxostat culture of *A. niger* B1, pH values had effects on morphological mutants formation and the recombinant glucoamylase production. It was concluded that acidic medium was required for optimum production of alpha amylase. Enzyme production started at pH 3.0 and cease at pH 8.0

Table 2. Effect of different surfactants at different concentrations (0.0002%, 0.002%, 0.02 and 0.2%) on extra cellular α -amylase production.

Detergents (W/V)	Concentration of surfactants	Enzyme Activity (unit per ml)	
		Free cells	Immobilized cells
Tween-80	0.2	42.8	52.2
	0.02	55.69	66.4
	0.0002	45.66	56.1
	0.0002	44.3	52.4
Triton X-100	0.2	50.2	52.8
	0.02	46.2	53.2
	0.0002	59.88	64.4
	0.0002	50.1	63.2
SDS	0.2	41.85	52.2
	0.02	42.8	54.2
	0.0002	45.66	56.1
	0.0002	55.69	63.4

maximum enzyme production of enzyme occurred at pH 4 to 6, very little growth was observed without enzyme production in medium at initial pH 3 to 4. Agger et al. (2001) suggested the ability of filamentous fungi to secrete large amounts of extra-cellular proteins makes them well suited for protein production. The carbon and nitrogen source are two important factors affecting cell growth and product formation of microorganisms. Carbon and nitrogen sources may have either repressing or inducing effects on enzyme productions. Glucoamylase, amylase, and alpha-glucosidase are all upregulated (induced) by starch and down-regulated (repressed) by glucose as demonstrated by (Archer and Peberdy, 1997). As filamentous fungi especially *A. niger* have long been employed in the fermentation industry and continue to be the principal source of antibiotics and enzymes, advances within the last decade have provided commercially promising recombinant fungal strains, thus ushering in a new era in fermentation biotechnology (Punt et al., 2002; Wang et al., 2003).

Archer and Peberdy (1997) suggested the conventional, mutagenesis-based, strain improvement methods to be applied for enzyme production from filamentous fungi. And the application of recombinant DNA techniques is beginning to reveal important information on the molecular basis of fungal enzyme production and this knowledge is now being applied both in the laboratory and commercially. At the molecular level free and immobilized *A. niger* cells can be encoded for hydrolytic enzyme production, which provides a valuable source for researchers interested in enzyme properties and applications (Semova et al., 2006).

Conclusion

It was concluded in our present study that both nutritional

and cultural condition were required for optimum growth and production of alpha amylase from free and immobilized cells of *A. niger*, for a final breakthrough to achieving commercially relevant quantities of this enzyme and will review the current state of knowledge on the molecular basis of enzyme production by filamentous fungi in coming future prospects.

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