OPTIMIZATION OF CULTURAL CONDITIONS FOR THE PRODUCTION OF ALPHA AMYLASE BY *ASPERGILLUS NIGER* (BTM-26) IN SOLID STATE FERMENTATION

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Abstract

The present study deals with the isolation, screening and selection of native fungal strain for the alpha amylase production. Forty fungal strains were isolated from different soil samples. These strains were initially screened qualitatively on starch agar medium and quantitative screening was carried out in solid state fermentation. A strain of *Aspergillus niger* showing maximum production (432 ± 0.9 U/ml/min) of enzyme was selected and assigned the code BTM-26. The yield on various agricultural products, namely, coconut oil cake (COC), rice bran (RB), vegetable wastes or banana peel and wheat bran (WB) was compared. Wheat bran proved to be the best substrate for alpha amylase production. The effect of incubation temperature, initial pH, and inoculum size was investigated for the enzyme production. The maximum enzyme production was obtained at 30°C, pH 5, and inoculum size of 1 ml. The rate of fermentation was also studied and the highest yield of enzyme was obtained after 72 h of inoculation. Addition of 1.5% lactose as carbon source and 0.2% $(NH_4)_2SO_4$ and 0.3% yeast extract as inorganic and organic nitrogen sources respectively gave enzyme production 990 ± 0.81 U/ml/min which reflects about 1.87 fold increase in alpha amylase production as compared to the medium containing wheat bran alone as substrate.

Introduction

Alpha amylase (α-1,4-D- glucan glucanohydrolase) is a starch digesting enzyme that randomly cleaves α - 1,4-D- glucosidic linkages in starch molecules. It releases short chain oligosaccharides and alpha limit dextrin, in addition to maltotriose and maltose. Neither terminal glucose residues nor α -1,6 linkage can be cleaved by α-amylase. Alpha amylase is widely distributed in nature. It is the most important enzyme and is of great significance in the present day biotechnology. The enzyme has wide range of applications in many industries including textile, starch liquefaction, brewing, food, sugar and paper. It has also remarkable applications in sectors such as pharmaceutical, clinical, medicinal and analytical chemistry (Battle *et al*., 2000; Khan & Yadav, 2011). Alpha amylase can be obtained from animals, plants and microorganisms. However, the amount of enzyme synthesized by plants and animals is so low that it cannot meet the demands of the market. In contrast, adequate amount of alpha amylase can be obtained from native microbial sources. This has, therefore, necessitated the screening of microorganisms like bacteria and fungi for α-amylase production (Shibuya *et al*., 1992; Ivanova *et al*., 2001). In nature, micro-organisms are not found in pure culture and we are not likely to find a material, for example, a speck of soil that contains only a single kind of microorganisms without the existence of others. Therefore, this necessitates the isolation, identification and screening of a particular fungal strain that could provide us with a reasonable amount of alpha amylase.

Fungal alpha amylases are of particular significance as compared to those derived from other microbial sources. Filamentous fungi, in general, are most appropriate microorganisms for solid state fermentation (SSF). This owes to their morphological characteristics that allow fungi to colonize and penetrate the solid substrate most efficiently. The fungal hyphae have the ability to grow and spread due to efficient cyclosis of cytoplasm. They have good tolerance to low water availability and high osmotic pressure. These are the properties that make fungi efficient and competitive in natural microflora for bioconversion (Poornima *et al*., 2008). Among the filamentous genera of fungi that serve as a source of alpha amylase, *Aspergillus* and *Penicillium* are most significant. Many species of *Aspergillus* (especially *A. niger*) and other species *A. oryzae, A. tamarii, A. flavus* etc have become a source of a variety of enzymes including alpha amylase, and several other fungi like *Trichoderma* sp. *Thermomyces Ianuginosus, Penicillium griseoreseum, Fusarium moniliformis, Actinomyces* sp. and *Alternaria* sp. have the ability to produce large quantity of extracellular enzymes making them suitable for enzyme production at industrial scale (Poornima *et al*., 2008).

Alpha amylase can be produced both by using solid state and submerged fermentation techniques (Rahardjo *et al.,* 2005). In recent years, however, solid state fermentation (SSF) has been used more and more for α-amylase production because the technique is simple & involves low capital investment with little energy requirements. The volumetric productivity is high with desirable characteristics and better recovery (Edral & Taskin, 2011). The type of substrates used plays a key role of alpha amylase production. Various substrates like wheat bran, mustard oil cake, gram bran, spent brewing grain, rice bran, coconut oil cake, gingerly oil cake, vegetable waste, banana peel etc were evaluated for the alpha amylase production. Wheat bran is proved to be a good substrate for the alpha amylase production by *A. niger* (Iwano *et al*., 1999).

Optimization of cultural conditions is of utmost importance for better production of alpha amylase. The purpose of optimization is to find out conditions under which the fungus can flourish luxuriantly and produce alpha amylase in maximum amounts, which paves the

way to its effective and fruitful commercialization. Inoculum size is an important factor for the alpha amylase production because it determines the extent and quality of enzyme produced.

The determination of optimum time and temperature is important for the better yield of the enzyme. Power of H^+ (pH) is an important factor that determines the growth of the enzyme producing microorganisms as well as the extent to which they can produce alpha amylase (Kasim, 1983; Gupta *et al*., 2008). In view of the importance of pH in enhancing alpha amylase production, the determination of the initial pH of the medium becomes obligatory in the process of optimization. The addition of various carbon sources, namely, sucrose, glucose, maltose, lactose, fructose, galactose, starch etc have greatly affected the rate of carbohydrate metabolism as well as the mode of alpha amylase formation (Dubey *et al*., 2000; Abdullah *et al.*, 2011). A lot of experimental evidence exists concerning the role of soluble starch and maltose as inducer of alpha amylase production by *Aspergillus* species (Varalackhsmi *et al*., 2009). It was found that starch strongly increased enzyme productivity, whereas, glucose resulted in very low productivity of alpha amylase. Starch and lactose have showed better yield in some cases. Since, different fungi respond to the added carbon source differently as to the production of enzymes, it becomes remarkably important to select a suitable carbon source for the enhanced alpha amylase productivity by a fungus (Abdullah *et al.,* 2011).

The growth of a fungus and its ability to synthesize alpha amylase is greatly influenced by the addition of inorganic and organic nitrogen sources (Pandey 2000). The commonly used inorganic sources of nitrogen include sodium nitrate, ammonium nitrate, ammonium sulphate etc. Among the organic nitrogen sources, urea, peptone, yeast extract, tryptophan, corn steep liquor, casein hydrolysates, beef extract are widely used for enzyme production (Pedersen & Nielsen, 2000; Sivaramakrishnan *et al*., 2007).

The general purpose of the present work is to add a drop to the ocean of enzymology and thus contribute to the tides leading to the service of humanity. Moreover, in view of the extensive use of alpha amylase in a number of industries, it seems more economical to produce alpha amylase locally than to import it from abroad. In this way, foreign exchange can be saved. The present work is concerned with the optimization of cultural conditions for the enhanced production of alpha amylase by fungal isolate using solid state fermentation technique.

Materials and Methods

Isolation of microorganism: For the isolation of fungal strains, different soil samples were collected from various habitats. Each of these samples was collected in separate polythene bag. Serial dilution method was used to isolate fungus from each soil sample (Clark *et al*., 1958). One gram of soil sample was dissolved in 100 ml of sterilized distilled water. The soil suspension was diluted up to 10^5-10^7 times and 0.5 ml of the diluted soil suspension was transferred to the petriplates containing

duly sterilized culture medium and uniformly speared. The petriplates were placed in an incubator at 30°C for 3-4 days for culture development. The initial colonies on the incubated petriplates that formed clear zones of starch hydrolysis were picked up and morphological characteristics of fungal isolates were identified according to Onion *et al*., (1986). The conidia of the isolated fungus were aseptically transferred to the slants containing starch agar medium. The slants were then incubated at 30°C for 3-5 days for maximum growth of the fungus and stored in a refrigerator at 4°C for culture maintenance. The screening of all the selected cultures for the production of alpha amylase was done by solid state fermentation.

Conidial inoculum: Conidia from 3-4 days old slant cultures were used for inoculation. The conidial suspension was prepared in sterilized 0.005% dioctyl ester of sodium sulpho succinic acid (Monoxal O.T). Ten ml of sterilized Monoxal O.T was transferred to each slant having profuse conidial growth on its surface. The clumps of conidia were broken with the help of an inoculating needle. The test tube was shaken vigorously to make a homogeneous suspension. The number of conidia was counted with the help of Haemacytometer. Each milliliter of the suspension contained 3.6×10^6 CFU (Haq *et al*., 2002).

Solid state fermentation: Ten grams of solid substrate such as wheat bran moistened with Mineral salt medium was transferred to each of cotton wool plugged (250ml Erlenmeyer) flask. The flasks were sterilized at 121°C for 15 minutes in an autoclave. Thereafter, the medium in the flasks was cooled at room temperature. One ml of the conidial suspension was added to each flask. The flasks were incubated at 30°C for 72 h. All the experiments were carried out in triplicates. After fixed period of incubation, the 100ml of distilled water was transferred to each flask containing fermented bran. The flasks were placed in incubator shaker at 160 rpm for one hour. After one hour contents of the flasks were filtered and filtrate was used for the estimation of enzyme. (Haq *et al*., 2002).

Culture media: Different culture media were tested for the production of alpha amylase by the selected strain (Table 1).

Enzyme assay: The estimation of alpha amylase was carried out according to the method of Rick and Stegbauer (1974). "One unit of enzyme activity is that amount of enzyme, which liberates 1 mg of reducing group from 1% Litner's soluble starch corresponding to 1mg of maltose hydrate in 30 min". One ml of enzyme was incubated with 1.0 ml of 1% soluble Litner soluble starch solution for 30min at 40°C.The reducing sugar was measured by DNS method (Miller 1959).

Statistical analysis: After the completion of research the data was tabulated and results analysis was done statically (standard deviation and standard error) by using Minitab version 15.

Table 1. Medium composition for alpha amylase production.

Medium	Composition	Reference
M_1	Wheat bran, 5 g, 8ml mineral salt medium (MSM) containing mg/l Zn $SO_4.7H_2O$, 6.2; $FeSO_4.7H_2O$, 6.8; Cu $SO_4.7H_2O$, 0.8; Distilled water, 1000 ml	(Varalakshmi *et al*., 2009)
M_2	Coconut oil cake, 5 g; Salt solution 2 ml containing g/l KH_2PO_4,2; NH_4NO_3, 5; NaCl 1.0; $MgSO_4.7H_2O$, 1.0, Distilled water, 1000 ml	(Ramachandran *et al*., 2004)
M_3	Rice bran 5 g, 10 ml Mineral salt solution containing g/l KH_2PO_4, 2; $NH_4 NO_3$, 5; NaCl 1.0; $MgSO_4.7H_2O$, 1.0, Distilled water, 1000 ml	(Anto *et al.,* 2006)
M_4	Potato peel 20 g, MSM containing g/l NaCl, 0.8, KCl, 0.1, $CaCl_2$, 2.0, Na_2HPO_4, 0.2; $FeSO_4$, 8.0, Glucose, 2.0; NH_4Cl 2.0	(Khan & Yadav, 2011)
M_5	Wheat bran 5 g, 5ml distilled water	(Balkan *et al*., 2011)
M_6	Wheat bran 10 g, acetate buffer 10 ml	(Haq *et al.,* 2002)

Results

Forty different fungal strains were isolated and identified according to Onion *et al*., (1986). Quantitative screening of fungal isolates for alpha amylase production was carried out using solid state fermentation technique (Table 2). The range of enzyme activity of the fungal isolates is given in (Table 3). Out of the tested isolates, isolate No. 26 gave the maximum α-amylase production. The desired isolate was identified as *A. niger* and assigned the code BTM-26. The selected hyper producer (BTM-26) was set aside for subsequent optimization of cultural conditions for the production of alpha amylase.

In the present study, six different media were evaluated for α-amylase production by *A. niger* (BTM-26). Out of these media, the M5 medium was found to be best for maximum enzyme production as well as growth of *Aspergillus niger* BTM-26 (Fig. 1). The rest of the fermentation medium did not give significant enzyme production as compared to M5 medium. Figure 2 indicated the influence of using various concentrations of wheat bran (2.5- 12.5%) on the alpha amylase production from *Aspergillus niger* (BTM-26). The alpha amylase production increased progressively up to 10g of concentration and then it declined. The maximum production of enzyme was found at 10g of wheat bran concentration. This concentration was, therefore, used in further studies.

The effect of different incubation time on the production of alpha amylase by *Aspergillus niger* (BTM 26) was investigated (Fig. 3). The time ranged from 0 - 96 hours. The production of alpha amylase increased gradually with time upto 72 hours. Beyond this level decrease in the production of enzyme was observed. So this incubation time is selected for subsequent optimization.

The influence of various temperatures on α-amylase production by *A. niger* (BTM-26) was evaluated in order to find the optimum temperature for enzyme production (Fig. 4). The temperature range was kept 20°C-50°C with a difference of 5°C between successive temperatures. In the present study 30°C proved to be the optimum temperature for the enzyme production. Higher temperature resulted in gradual decrease in enzyme production. At 50°C enzyme production decreased considerably. The effect of various pH (3-9) was investigated in this study. Enzyme production was maximum at pH 5 (Fig. 5).

Figure 6 showed the impact of varying inoculum sizes (0.5 ml-2.5ml) on the α-amylase production by *Aspergillus niger* (BTM-26). The size difference between successive inocula was kept 0.5 ml. In this study, the inoculum size that was most appropriate for maximum enzyme production was 1 ml. Inoculum size greater or less than 1 ml reduced the rate of enzyme production. The effect of the addition of various sources of carbon, *viz*., lactose, maltose, glucose, xylose, and sucrose were investigated for production of alpha amylase (Fig. 7). Out of all these carbon sources, lactose in the concentration of 1.5 % showed maximum enzyme production (Fig. 8).

Various inorganic and organic nitrogen sources, namely, $NaNO_3$, KNO_3, $(NH_4)_2SO_4$, yeast extract, peptone, corn steep liquor and urea were used in this study to test their ability to influence enzyme production (Figs. 9, 11). Out of these nitrogen sources 0.2% $(NH_4)_2SO_4$ and 0.3 % yeast extract proved the best for enzyme production (Figs. 10, 12).

The effect of different moistening agents namely, acetate buffer, HCl, phosphate buffer, citrate phosphate buffer and distilled water on the alpha amylase production by *Aspergillus niger* (BTM-26) was examined (Fig. 13). Of all the moistening agents, distilled water showed maximum enzyme production.

Discussion

The selection of the native fungal strain is a crucial step for the production of alpha amylase. It involves the processes, namely isolation, identification and screening. In the present investigation forty different fungal strains were isolated & assigned the codes accordingly. Further, all the strains were identified and exploited for alpha amylase production using solid state fermentation technique. Of all the identified fungal isolates, the potent producer was found to be *Aspergillus niger* (BTM-26) which gave maximum production of alpha amylase. Similar reports were also found for the production of alpha amylase using *A. niger* (Varalakshmi *et al.,* 2009; Khan & Yadav, 2011). In the present study, six different media were evaluated for α-amylase production by *A. niger* (BTM-26). Out of these media, the M5 medium gave maximum production of enzyme. The reason might be that the suitability of wheat bran for optimum enzyme production lies in its chemical composition because it contain an appropriate proportion of fiber, carbohydrates, proteins, and fats required for growth of microorganism and alpha amylase production (Haq *et al*., 2012) The less enzyme production on other media was either due to the lack of some components in media that were necessary for fungal growth and enhanced enzyme production or due to the presence of repressor substances in media component (Zambare, 2010).

Table 2. Isolation and screening of fungal strains for the production of alpha amylase.

S. No.	Isolate name	Code	Enzyme activity (U/ml/min)
1.	*Aspergillus niger*	BTM- 1	235 ± 0.5
2.	*Rhizopus* sp.	BTM-2	115 ± 0.3
3.	*Aspergillus tamarii*	BTM- 3	357 ± 0.5
4.	*Trichoderma viridi*	BTM- 4	134 ± 1
5.	*Aspergillus niger*	BTM-5	342 ± 1
6.	*Aspergillus flavus*	BTM- 6	212 ± 0.7
7.	*Fusarium* sp.	BTM- 7	161 ± 0.8
8.	*Aspergillus oryzae*	BTM- 8	376 ± 1
9.	*Aspergillus* sp.	BTM- 9	274 ± 0.8
10.	*Rhizopus* sp.	BTM-10	384 ± 0.6
11.	*Aspergillus niger*	BTM- 11	119 ± 0.6
12.	*Aspergillus* sp.	BTM-12	213 ± 0.8
13.	*Fusarium* sp.	BTM- 13	124 ± 0.1
14.	*Aspergillus oryzae*	BTM- 14	332 ± 1
15.	*Rhizopus* sp.	BTM- 15	266 ± 0.8
16.	*Aspergillus niger*	BTM- 16	399 ± 1.5
17.	*Rhizopus* sp.	BTM- 17	258 ± 0.9
18.	*Aspergillus niger*	BTM- 18	365 ± 0.7
19.	*A. oryzae*	BTM- 19	362 ± 0.8
20.	*Rhizopus* sp.	BTM- 20	377 ± 0.6
21.	*Fusarium* sp.	BTM- 21	281 ± 0.5
22.	*Aspergillus niger*	BTM- 22	406 ± 0.7
23.	*Rhizopus oligosporous*	BTM- 23	397 ± 0.9
24.	*Fusarium* sp.	BTM- 24	201 ± 0.3
25.	*Rhizopus* sp.	BTM- 25	309 ± 0.5
26.	*Aspergillus niger*	BTM- 26	432 ± 0.9
27.	*A. oryzae*	BTM- 27	303 ± 0.7
28.	*Rhizopus* sp.	BTM- 28	231 ± 0.5
29.	*Aspergillus oryzae*	BTM- 29	327 ± 0.8
30.	*Fusarium* sp.	BTM- 30	238 ± 0.7
31.	*A.niger*	BTM- 31	238 ± 0.9
32.	*Aspergillus oryzae*	BTM- 32	174 ± 0.9
33.	*A. niger*	BTM- 33	254 ± 0.8
34.	*Aspergillus niger*	BTM- 34	360 ± 0.7
35.	*Trichoderma viridi*	BTM- 35	200 ± 0.8
36.	*A. niger*	BTM- 36	269 ± 0.5
37.	*Aspergillus oryzae*	BTM- 37	183 ± 0.6
38.	*Rhizopus* sp.	BTM- 38	377 ± 0.7
39.	*A. oryzae*	BTM- 39	285 ± 0.9
40.	*Aspergillus niger*	BTM- 40	399 ± 0.9

The mean difference is significant at the level of 0.05; ± indicates the standard deviation (SD) among the three parallel replicates in each column. *Fermentation period 72h, pH 6, temperature of incubation 30°C

Table 3. Sub grouping of alpha amylase producing fungal isolates.

Sr. No.	No of fungal isolates	Range of alpha amylase activity (U/ml/min)
1.	6	101- 200
2.	13	201- 300
3.	16	301- 400
4.	2	401- 500

One isolate gave a maximum production of α-amylase 432 ± 0.9 and it was assigned the name BTM-26* this isolate was selected for further studies

Incubation period plays a vital role in the enzyme production. The α- amylase production increased with the increase in the incubation period and it reached its maximum at 72 h of incubation. The reason might be that the flourishing fungus reached the end of the log phase and entered the stationary growth phase. This was evidenced by Prescott and Dunn (1987) who reported that the accumulation of alpha amylase by a fungal strain reached the optimum level in the stationary growth phase. Incubation beyond 72 h resulted in decreased enzyme yield. The reduced enzyme production might be due to the decline in nutrients and sugar contents. Moreover, inhibitors generated by the fungus could also be the possible cause (Ramesh & Lonsane, 1990; Kirshna & Chandrasekaran, 1996).

Temperature is one of the important factors which strongly affect SSF process (Pandey *et al*., 2000). In the present study, 30°C proved to be the optimum temperature for the enzyme production. Higher temperature resulted in gradual decrease in enzyme production. At 50°C, enzyme production decreased considerably. The decreased enzyme production is related to reduce moisture content. At increased temperature, evaporation in SSF resulted in decreased level of moisture content. Reduction in moisture content, in turn lowered the rate of growth of the fungus leading to decreased enzyme production. The effect of various pH was investigated in this study. Enzyme production was maximum at pH 5. Any increase or decrease in pH directly affected the enzyme production. This is because enzymes function most effectively over a narrow range of pH and are highly sensitive to small changes in pH (Gupta *et al.,* 2008). The growth of the fungus and its ability to produce enzyme is related to the size of inoculum. The decrease in enzyme production at larger inoculum sizes was due to over growth of *A. niger* (BTM-26) which produced anaerobic conditions. This consumed major portion of the substrate affecting metabolic process of enzyme synthesis. Gradual reduction in alpha amylase synthesis was due to production of inadequate mycelia on account of lesser number of conidia.

In the present study lactose in the concentration of 1.5 % showed maximum enzyme production. The decrease in enzyme production at lower lactose concentration might be due to lower amount of carbon which is required for the synthesis of the enzyme. The increase in lactose concentration provided excess carbon that caused catabolic repression (Gupta *et al.,* 2008). The growth of the fungus and enzyme synthesis requires nitrogen. The increase in concentration of $(NH_4)_2SO_4$ beyond 0.2% decreased the synthesis of α-amylase because of the toxic and inhibitory effect of nitrogen on enzyme synthesis as pointed out by Anto *et al*., (2006).

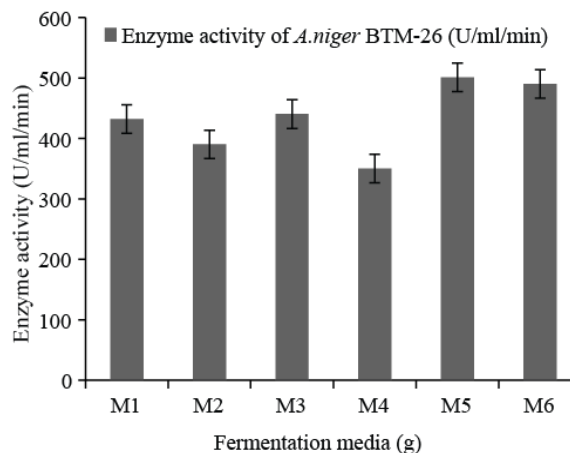

Fig. 1. Effect of different culture media on the production of alpha amylase by *Aspergillus niger* (BTM-26)*
*Fermentation period 72h, pH 6, temperature of incubation 30°C.

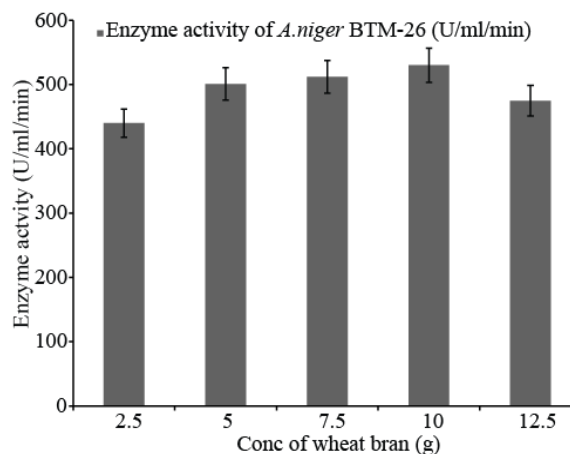Fig. 2. Effect of different concentrations of wheat bran on the production of alpha amylase by *Aspergillus niger* (BTM-26)*
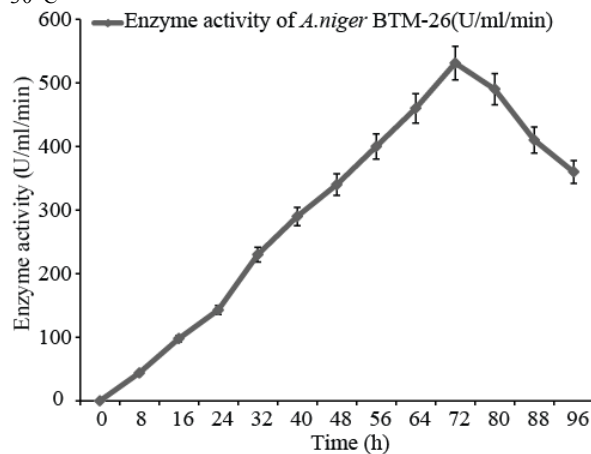*Fermentation period 72h, pH 6, temperature of incubation 30°C

Fig. 3. Effect of different incubation time on the production of alpha amylase by *Aspergillus niger* (BTM-26)*
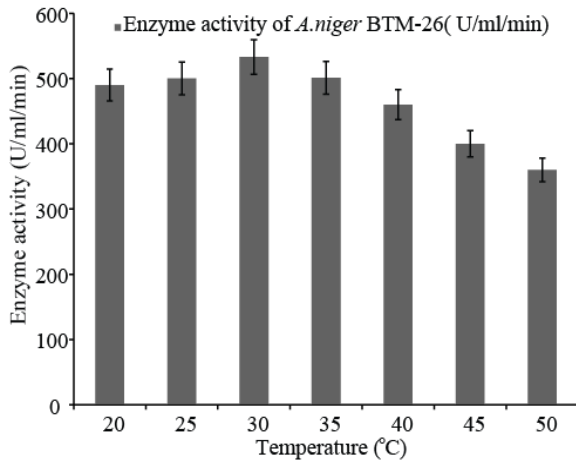*Temperature of incubation 30°C, pH 6.0.

Fig. 4. Effect of varying incubation temperatures on the production of alpha amylase by *Aspergillus niger* (BTM-26)*
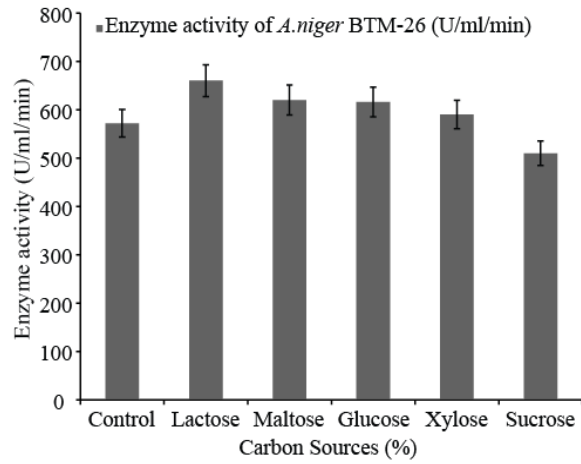* Fermentation period 72h, pH 6.0

Fig. 7. Influence of different carbon sources on the production of alpha amylase by *Aspergillus niger* (BTM-26)*
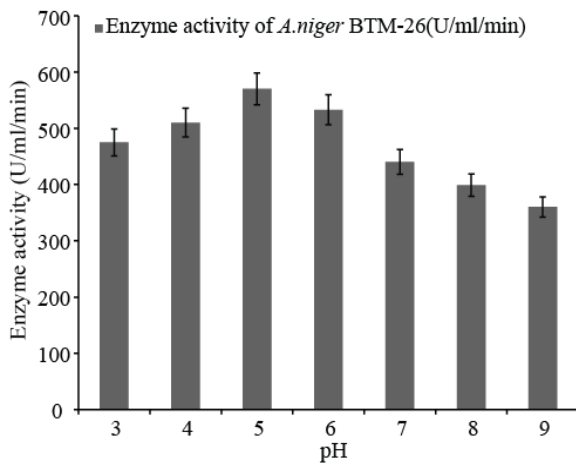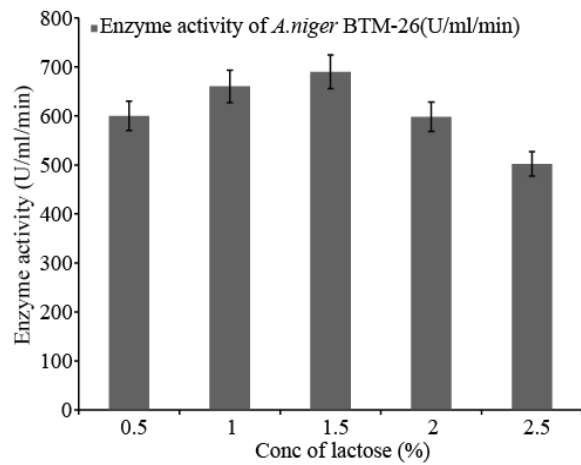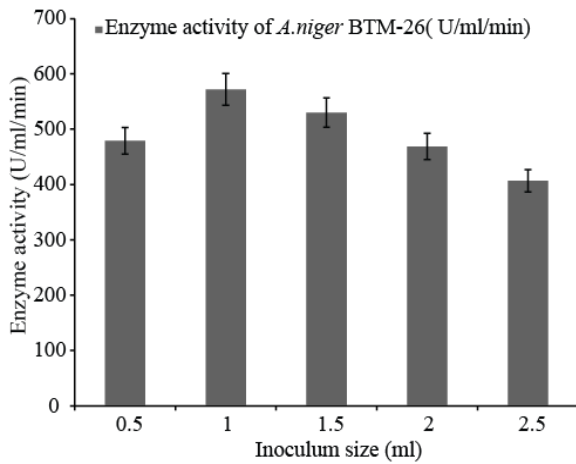*Fermentation period 72h, pH 5, temperature of incubation 30°C.

Fig. 5. Effect of different pH on the production of alpha amylase by *Aspergillus niger* (BTM-26)*
*Temperature of incubation 30°C, Fermentation period 72h.

Fig. 8. Effect of different Concentrations of lactose on the production of alpha amylase by *Aspergillus niger* (BTM-26)*
*Fermentation period 72h, pH 5, temperature of incubation 30°C

Fig. 6. Influence of varying inoculum sizes on the production of alpha amylase by *Aspergillus niger* (BTM-26)*
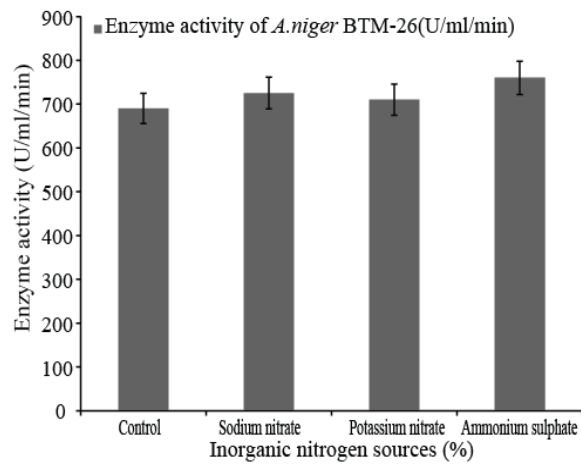*Fermentation period 72h, pH 5, temperature of incubation 30°C

Fig. 9. Effect of different inorganic nitrogen sources on the production of alpha amylase by *Aspergillus niger* (BTM-26)*
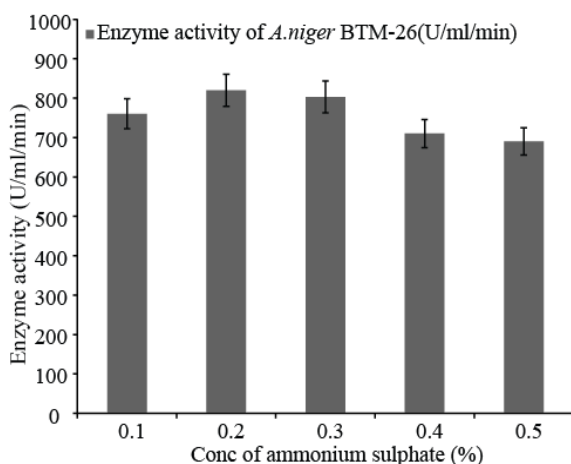*Fermentation period 72h, pH 5, temperature of incubation 30°C

Fig. 10. Effect of different concentrations of ammonium sulphate on the production of alpha amylase by *A.niger* (BTM-26)*
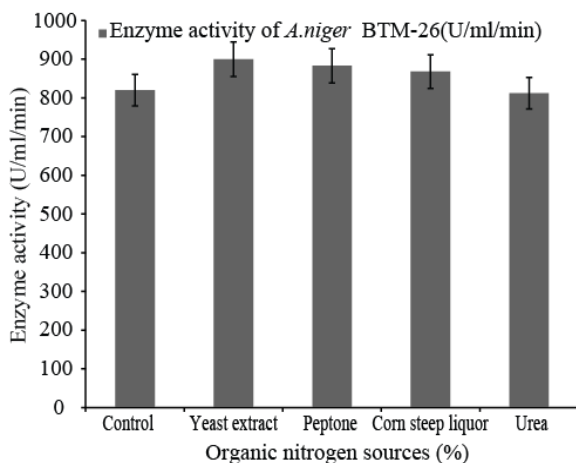*Fermentation period 72h, pH 5, temperature of incubation 30°C

Fig. 11. Effect of different organic nitrogen sources on the production of alpha amylase by *Aspergillus niger* (BTM-26)*
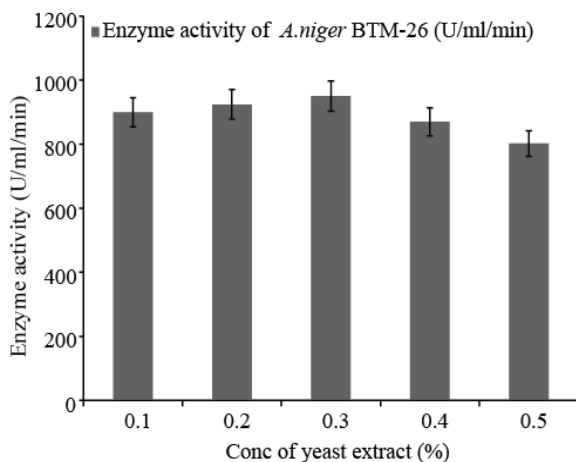*Fermentation period 72h, pH 5, temperature of incubation 30°C

Fig. 12. Effect of different concentrations of yeast extract on the production of alpha amylase by *Aspergillus niger* (BTM-26)*
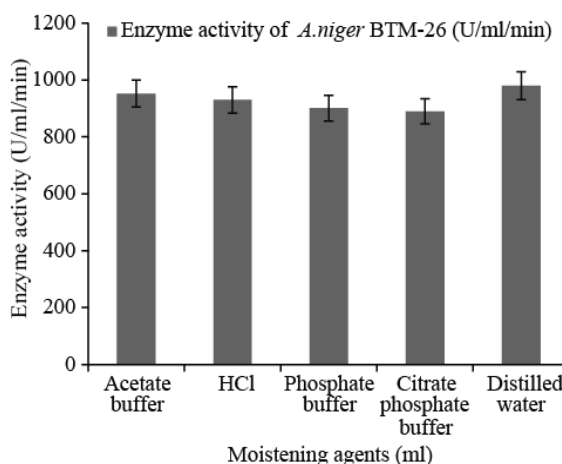*Fermentation period 72h, temperature of incubation 30°C

Fig. 13. Effect of different moistening agents on the production of alpha amylase by *A. niger* (BTM 26)*
*Fermentation period 72h, temperature of incubation 30°C

Acknowledgement

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