P. J. Bot., 43(4): 2211-2219, 2011.

PROCESS OPTIMIZATION FOR A POTENT WILD AND MUTANT STRAIN OF *ASPERGILLUS NIGER* FOR BIOSYNTHESIS OF AMYLOGLUCOSIDSAE

SHAZIA MALIK[1], TEHREEMA IFTIKHAR[2*] AND IKRAM UL HAQ[1]

[1]*Institute of Industrial Biotechnology, GC University, Lahore*
[2]*Department of Botany, GC University, Faisalabad*
[*]*Corresponding author E-mail: pakaim2001@yahoo.com*

Abstract

The present study is concerned with the selection of a potent strain of *Aspergillus niger* and optimization of the cultural conditions for the biosynthesis of amyloglucosidase. The cultural conditions were optimized for the enzyme production. Twenty percent (50/250ml flask) was found to be optimum volume of the medium. Optimum temperature was 30°C after 72 h of incubation, with the initial pH of the medium 5.0. 2% Starch with 1% glucose as an additional carbon source gave maximum amyloglucosidase production Addition of 0.3% ammonium sulphate in the fermentation medium increased the enzyme production while 2% spore inoculum showed best amyloglucosidase production.

Introduction

Amyloglucosidase is an enzyme of great importance in the starch industries. It has the ability to hydrolyze starch, thus converting it into glucose (Ford, 1999; Reilly, 1999). Glucose is a necessary compound in various food industries (Polakovic and Bryjak, 2004). Amyloglucosidase occurs in microorganisms as an extracellular enzyme and it has characteristic property of hydrolyzing ∝-1, 4 and ∝-1, 6 linkages of the saccharides formed by the action of other amylases on starch. Glucoamylase hydrolyze terminal non-reducing ∝-1, 4-gluco pyranose (Kelly *et al*., 1983). Amyloglucosidase can be produced both by submerged and solid state fermentations (Ramadas *et al.*, 1996, Metwally, 1998; Nandakumar *et al*., 1999). However, submerged fermentation is found to be more pronounced because there is more availability of nutrients and air supply in this fermentation process due to proper agitation (Prescott and Dunn, 1987). *Aspergillus* species are used in the production of industrial enzymes because they possessed a superior ability to secrete proteins (Gwiazdowska *et al.*, 2004). *Aspergillus niger* remained the organism of choice for the commercial production of amyloglucosidase (Pandey and Radhakrishan, 1993; Arassaratnam *et al.*, 1997; Haq *et al*., 2002; Khalaj *et al*., 2001; Omemo *et al.*, 2005; Spier *et al*., 2006; Costa *et al.*, 2007). Pavezzi *et al*. (2008) worked on the effect of addition of various carbon sources for the production of amyloglucosidase by *Aspergillus spp* and its characterization. Haq *et al*. (1997) reported that by addition of yeast extract in a very low concentration in a medium with $(NH_4)_2SO_4$ and starch increased the production of amyloglucosidase by *Aspergillus niger* in submerged fermentation. The pH and temperature values had a decisive effect in the induction of the glucoamylase synthesis (Schmidell *et al*., 1988; Mishra and Debnath, 2002). The growth in the form of small pellets favoured glucoamylase production while larger pellets lowered the amount of glucoamylase produced (Pamboukian *et al*., 1999). The effect of *Aspergillus niger* strain, inoculum type and addition of inducers was studied and it was reported that amyloglucosidase production was greatly influenced by fungal strain and inoculum type but the inducers had no effect (Costa *et al*., 2007). The main objectives of present study was isolation of potent strains of *Aspergillus niger* and optimization of cultural conditions for the enzyme production in shake flask.

Materials and Methods

Isolation and screening of organism: Different strains of *Aspergillus niger* strains were isolated from soils of different habitats. The sterile polythene bags were used to collect soil samples from different places. Serial dilution method was used for making dilutions of the samples. To 100 ml of distilled water (sterilized), 1.0 g of the soil was added and was shaken to dissolve the soil in it. Then dilutions were made from the suspension up to 10^{-4} to 10^{-6} times. 1 ml of diluted soil suspension was poured with the help of sterilized pipettes in the petriplates containing sterilized malt extract- starch agar medium. The petriplates were rotated clockwise and anti clockwise to spread soil suspension on medium (Iftikhar *et al.*, 2003). After pouring 1 ml of soil suspension, the petriplates were incubated at 30°C for 3-4 days. The young colonies of *A. niger* were transferred to the petriplates containing potato dextrose starch agar medium for primary screening. The colonies with large clear zones of starch hydrolysis were picked up. The strain selected after primary screening was maintained on potato dextrose agar medium (Merck, Germany) and was stored in a cold cabinet (Sanyo) at 4°C for culture maintenance. Cultures were maintained on 4% potato dextrose agar slants. The subculturing of the fungus was done regularly after every 15 days (Abbas *et al.*, 2010).

Screening of *Aspergillus niger* strains: Primary screening of the *A. niger* strains was done firstly by starch hydrolysis method. The young colonies showing clear zone of starch hydrolysis in petriplates were selected and tested for amyloglucosidase production in shake flasks for secondary screening (Iftikhar *et al* 2010).

Inoculum

Conidial: Inoculation was done by conidia from the slant culture (3-5 days old). The conidial suspension was prepared by adding 10 ml of 0.005% Monoxal 0.T (Di-octyl ester of sodium sulpho succinic acid) to the slant having profused growth of conidia. The inoculating

needle was used to break the conidial clumps. Then the tube was shaken vigorously to form a homogeneous conidial suspension. The density of the conidial suspension was measured on a haemocytometer (Neubauer Precicdor HBG, Germany). The counting chamber was a ruled glass slide with a cover which holds a definite volume of a fluid. The conidia in a square (0.1 mm depth) were counted under microscope and then the number of conidia in one ml of the conidial suspension was counted. It was found to be $1.2x10^6$ conidia/mL (Iftikhar *et al.*, 2007).

Culture media: The media used for the production of amyloglucosidase by *A. niger* are: **M_1:** (g/L; Starch, 15.0; $NaNO_3$, 3.0; KCl, 2.0; KH_2PO_4,1.0; $MgSO_4.7H_2O$, 0.5; Distilled water, 1000 ml; pH, 5.0). **M_2:** (g/L; Starch, 10.0; Lactose, 10.0; $(NH_4)_2SO_4$, 5.0; $MgSO_4.7H_2O$, 2.0; $CaCl_2.2H_2O$, 2.0; KH_2PO_4, 1.50; K_2HPO_4, 0.1; Distilled water, 1000 ml; pH 5.5). **M_3: (**g/L; Starch, 10.0; yeast extract, 3.0; $MgSO_4.7H_2O$, 0.005; $CaCl_2.2H_2O$, 0.2; $FeSO_4$, 1.0; Peptone, 20.0; Phosphate buffer, 1000 ml pH 6). **M_4:** (g/L; Starch, 10.0; $MgSO_4.7H_2O$, 0.005; $CaCl_2.2H_2O$, 0.2; $FeSO_4$, 0.1; $(NH_4)_2SO_4$, 2.0; Phosphate buffer, 1000 ml; pH 6). **M_5:** (g/L; Starch, 10.0; Glucose monohydrate, 10.0; NH_4Cl, 2.0; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.5; trace metal solution, 0.12 ml; Distilled water, 1000ml; pH 5.0).

Fermentation technique

Shake flask: Twenty five mL of fermentation medium was poured in each 250 mL conical flask and were cotton plugged. The flasks were sterilized in an autoclave at 121^{o}C, (15 lb/inch^2)for 15 min. After cooling the medium at room temperature, one mL of the conidial inoculum was added in each flask under aseptic condition. These flasks were then placed in the rotary incubator shaker (Model: 10*400-xx2c, SANYO, Gallen Kamp PLC, UK) rotating at 200 rpm for 72 hours at 30^{o}C. After 72 hours, the fermented broth was filtered and filtrate was used for the estimation of amyloglucosidase. All the experiments were run parallel in triplicate (Malik *et al.*, 2011).

Analysis

Dry cell mass: Fermented broth was filtered from a pre-weighed filter paper. The residue left on the filter paper (cell mass) was oven dried at 100^{o}C overnight and then placed in a desiccator. It was then reweighed (Malik *et al.*, 2011).

Enzyme assay: The assay of amyloglucasidase was carried out according to the method of Cadwell *et al.*, (1968). One unit of activity is the amount of enzyme, which liberates one mg of glucose per hour from 5% soluble starch. The enzyme activity was then converted into U/ml/min as reported by Malik *et al.*, (2011a).

Results and Discussion

Isolation and selection of a suitable strain is necessary for maximum production of amyloglucosidase. About one hundred and fifty strains of *Aspergillus niger* were isolated from different soil samples and were identified morphologically. The mycelium was yellow in colour and produced dark brown conidial heads which were globose and had an average diameter of 550μm. Conidia were present on metullae. The conidia were globose and had a diameter of 2.5-10.0 μm. The primary screening of the strain was carried out by starch hydrolysis method. Eighty strains were further screened in 250 ml flasks by submerged fermentation method (Table 1a). The amount of amyloglucosidase and dry cell mass produced ranged from 1.21-7.46 U/ml/min and 8.59-11.33 g/L respectively. Table 1b shows the subgrouping of the amyloglucosidase producing isolates. Thirty one isolates produced the enzyme in the range of 1.0-3.0 units. Thirty four stains produced in the range of 3.1-6.0. Only fifteen produced the enzyme more than 6.0 U/mL/min. Isolate No. 52 that produced maximum enzyme i.e., 7.46 ± 0.44 U/ml/min was selected for further studies and it was assigned the name BT.

The composition of the medium affects both the growth and the enzyme production. Figure 1 shows the effect of different media on the enzyme production and dry cell mass for both wild strain (BT) and the mutant strain (M4 120). M2 shows highest production of amyloglucosidase i.e., 11.05 U/mL/min and dry cell mass i.e., 11.77 g/L for the wild strain and 19.49 U/mL/min enzyme activity 12.43 g/l dry cell mass for the mutant strain. M4 also gave good results for the enzyme production (10.40 U/mL/min) and dry cell mass (11.40 g/L) for the wild strain. While for the mutant strain the enzyme activity was low with M4 (13.6 U/ml/min). Similarly, M1 showed better enzyme activity for the mutant strain as compared to the wild strain. However, highest enzyme production was achieved with M2 for the wild and mutant strain. Hence, M2 was selected for optimum production of amyloglucosidase.

The volume of the fermentation medium exert a great influence on the enzyme production. So, the effect of the volume of the medium on amyloglucosidase production and dry cell mass in 250 ml Erlenmeyer flask was investigated as shown in Fig. 2. The production of the enzyme was maximum in 50 mL, of the medium in 250 ml flasks i.e.,11.90 U/mL/min and 20.32 U/mL/min for the wild and mutant strain respectively. When the volume of the medium was decreased below 50 mL the enzyme production also decreased. As the volume of the medium was increased above 50 ml, the amyloglucosidase production again decreased. In 150 ml of the medium the enzyme production was greatly affected (1.39 U/mL/min and 9.46 U/ml/min for the wild and mutant strain respectively). But dry cell mass increased with the increase of the volume of the medium and reached at its maximum value i.e., 14.36 g/l for the wild strain in 75 ml volume after which it also started decreasing. But the maximum dry cell mass for the mutant strain was achieved in 100 ml medium (13.89 g/L). Therefore 50 ml volume was selected for maximum production of amyloglucosidase.

Table 1a. Screening of *Aspergillus niger* isolates for amyloglucosidase production.

Isolate No.	Amyloglucosidase production (U/ml/min)	Dry cell mass (g/l)	Mycelial morphology
1.	1.21 ± 0.54	9.29 ± 0.66	Small pellet
2.	1.57 ± 0.36	8.59 ± 0.84	Small pellet
3.	1.86 ± 0.36	10.58 ± 0.84	Small pellet
4.	1.43 ± 0.49	9.41 ± 0.55	Small pellet
5.	1.34 ± 0.26	10.78 ± 0.41	Small pellet
6.	2.44 ± 0.42	10.19 ± 0.28	Medium pellet
7.	3.77 ± 0.60	9.54 ± 0.49	Small pellet
8.	4.21 ± 0.31	8.61 ± 0.41	Small pellet
9.	5.36 ± 0.48	9.44 ± 0.28	Small pellet
10.	7.1 ± 0.66	8.85 ± 0.36	Small pellet
11.	3.54 ± 0.38	9.45 ± 0.33	Small pellet
12.	4.72 ± 0.64	10.29 ± 0.30	Fine pellet
13.	6.92 ± 0.42	10.43 ± 0.7	Small pellet
14.	5.34 ± 0.61	10.12 ± 0.22	Small pellet
15.	6.37 ± 0.49	8.86 ± 0.43	Small pellet
16.	6.73 ± 0.44	9.44 ± 0.36	Small pellet
17.	5.94 ± 0.48	9.55 ± 0.34	Small pellet
18.	3.28 ± 0.70	10.57 ± 0.42	Small pellet
19.	3.22 ± 0.39	8.46 ± 0.45	Small pellet
20.	3.0 ± 0.14	8.74 ± 0.27	Small pellet
21.	4.39 ± 0.49	9.28 ± 0.38	Very small pellet
22.	4.24 ± 0.36	9.16 ± 0.38	Small pellet
23.	2.6 ± 0.32	10.41 ± 0.86	Small pellet
24.	5.36 ± 0.41	10.61 ± 0.29	Medium pellet
25.	6.18 ± 0.25	10.27 ± 0.36	Small pellet
26.	2.30 ± 0.42	10.10 ± 0.50	Small pellet
27.	2.54 ± 0.61	9.77 ± 0.29	Small pellet
28.	2.0 ± 0.51	9.71 ± 0.48	Small pellet
29.	1.63 ± 0.30	10.44 ± 0.35	Very small pellet
30.	1.38 ± 0.16	10.96 ± 0.53	Small pellet
31.	1.73 ± 0.92	11.33 ± 0.62	Small pellet
32.	2.2 ± 0.33	9.52 ± 0.19	Very small pellet
33.	3.48 ± 0.61	9.70 ± 0.37	Small pellet
34.	3.11 ± 0.44	9.62 ± 0.34	Small pellet
35.	1.85 ± 0.29	9.28 ± 0.27	Small pellet
36.	4.48 ± 0.28	9.72 ± 0.23	Medium pellet
37.	6.41 ± 0.33	10.51 ± 0.37	Small pellet
38.	3.63 ± 0.59	10.64 ± 0.40	Small pellet
39.	3.29 ± 0.26	10.51 ± 0.38	Small pellet
40.	6.33 ± 0.44	8.99 ± 0.18	Small pellet

Table 1a. (Cont'd.).

Isolate No.	Amyloglucosidase production (U/ml/min)	Dry cell mass (g/l)	Mycelial morphology
41.	6.06 ± 0.21	9.51 ± 0.30	Small pellet
42.	3.15 ± 0.87	9.67 ± 0.27	Small pellet
43.	1.62 ± 0.52	10.58 ± 0.39	Small pellet
44.	3.29 ± 0.76	10.37 ± 0.28	Small pellet
45.	5.98 ± 0.14	10.37 ± 0.38	Medium pellet
46.	5.40 ± 0.38	10.57 ± 0.28	Small pellet
47.	1.56 ± 0.36	10.59 ± 0.32	Medium pellet
48.	6.49 ± 2.53	10.77 ± 0.60	Small pellet
49.	2.67 ± 0.45	10.24 ± 0.08	Medium pellet
50.	4.27 ± 0.27	9.77 ± 0.25	Small pellet
51.	1.75 ± 0.26	10.49 ± 0.34	Medium pellet
52.	7.46 ± 0.44	10.67 ± 0.41	Medium pellet
53.	6.34 ± 0.45	9.8 ± 0.43	Small pellet
54.	1.75 ± 0.22	10.14 ± 0.26	Small pellet
55.	3.45 ± 0.33	9.78 ± 0.20	Small pellet
56.	3.50 ± 0.50	9.76 ± 0.05	Small pellet
57.	6.30 ± 0.55	10.32 ± 0.18	Small pellet
58.	1.43 ± 0.34	10.51 ± 0.27	Small pellet
59.	5.47 ± 0.44	10.43 ± 0.22	Medium pellet
60.	1.71 ± 0.36	10.62 ± 0.31	Medium pellet
61.	6.36 ± 0.46	9.77 ± 0.6	Small pellet
62.	4.32 ± 0.56	9.79 ± 0.14	Small pellet
63.	3.33 ± 0.22	9.49 ± 0.34	Small pellet
64.	1.57 ± 0.32	10.53 ± 0.63	Small pellet
65.	2.72 ± 0.34	10.29 ± 0.81	Small pellet
66.	5.08 ± 0.21	9.78 ± 0.43	Small pellet
67.	6.12 ± 0.23	10.31 ± 0.15	Medium pellet
68.	3.36 ± 0.38	10.65 ± 0.52	Medium pellet
69.	4.21 ± 0.34	9.99 ± 0.19	Small pellet
70.	6.58 ± 0.52	9.74 ± 0.13	Small pellet
71.	1.42 ± 0.31	10.45 ± 0.28	Small pellet
72.	2.75 ± 0.27	10.99 ± 0.19	Medium pellet
73.	3.20 ± 0.23	9.43 ± 0.48	Small pellet
74.	1.62 ± 0.33	9.63 ± 0.31	Small pellet
75.	2.33 ± 0.52	10.16 ± 0.33	Medium pellet
76.	3.29 ± 0.42	9.67 ± 0.25	Small pellet
77.	1.59 ± 0.49	9.83 ± 0.08	Small pellet
78.	1.22 ± 0.18	9.77 ± 0.22	Small pellet
79.	1.49 ± 0.32	9.87 ± 0.13	Small pellet
80.	1.26 ± 0.21	9.99 ± 0.22	Small pellet

Table 1b. Subgroping of amyloglucosidase producing isolates of *Aspergillus niger*.

No. of isolates	Range of amyloglucosidase production (U/ml/min)
31	1.0 – 3.0
34	3.1 – 6.0
15	More than 6.0

Each value is an average of trip replicates, ± denotes standard deviation among replicates

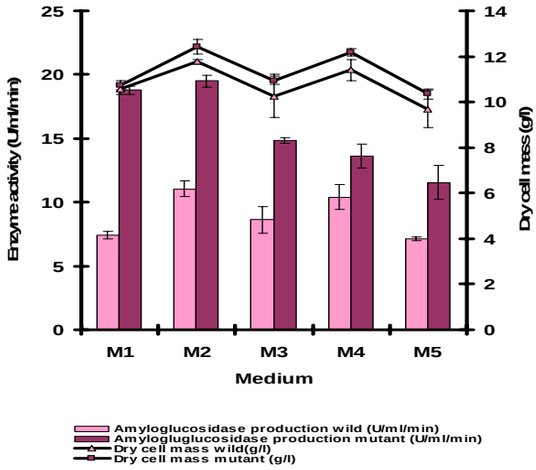

Fig. 1. Selection of medium for the production of amyloglucosidase by *A. niger* BT (wild) and *A. niger* M4 120 (mutant) in shake flask.

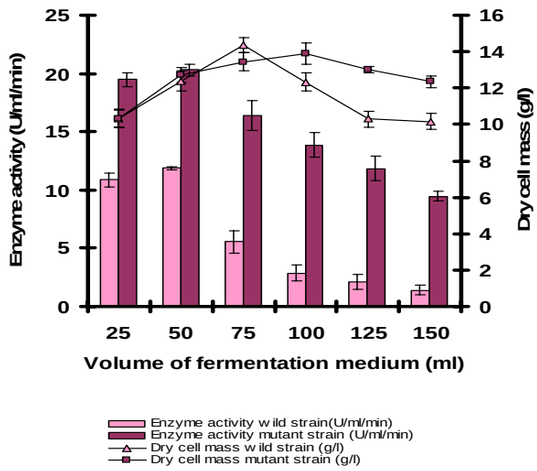

Fig. 2. Effect of volume of fermentation medium on the production of amyloglucosidase by *A. niger* BT (wild) and *A. niger* M4 120 (mutant) in shake flask.

Figure 3 shows the effect of temperature on the production of amyloglucosidase and dry cell mass production. Initially, at 20°C the production of amyloglucosidase was extremely low i.e.,7.93 U/mL/min (wild strain) and 9.99 U/ml/min (mutant strain). When the temperature was increased up to 30°C, the production of amyloglucosidase increased to 12.18 U/mL/min and 20.30 U/ml/min for the wild and mutant strain respectively. After this temperature, amyloglucosidase production decreased as a result of increase in the temperature and at 45°C the production of the enzyme decreased drastically i.e., 2.05 U/mL/min and 10.47 U/ml/min for the wild and mutant strain respectively. Same pattern was observed in case of dry cell mass. It was maximum (11.15 g/L and 12.51 g/L for the wild and mutant strain respectively) at 30°C and minimum at 45°C (4.62 g/L and 7.4 g/l for wild and mutant strain respectively). So, 30°C temperature was selected for further studies.

The incubation period for the production of amyloglucosidase by *A. niger* BT and *A. niger* M4120 was optimized as shown in Fig. 4. The cultures were incubated for different time intervals i.e.,12-96 h at 30°C. The enzyme production increased initially with the increase in the period of incubation and reached maximum value for both the wild and mutant strain i.e., 12.10 U/mL/min and 20.46 U/mL/min respectively at 72 h after inoculation. Further increase in the incubation period resuted in the decrease of amyloglucosidae production. Dry cell mass increased continuously with increase in incubation period and reached its maximum value 14.13 g/l for the wild strain and 15.86 g/l for the mutant strain at 96 h after inoculation. Thus, optimum time of enzyme production was found to be 72 h after inoculation.

The figure 5 depicts the effect of pH on the production of amyloglucosidase by *A. niger* BT and mutant strain of *A,niger* M4 120. The initial pH of the medium was varied from 3.0-8.0. The enzyme production was found maximum (13.28 U/mL/min for wild strain and 21.86 U/ml/min for mutant strain) at pH 5.0. When the pH of the medium was increased or decreased from the pH value 5.0, the production of the enzyme reduced. At pH 3.0, the production of the enzyme was low for wild and mutant strain (9.39 U/ml/min and 11.67 U/mL/min respectively). Similarly at pH 8.0, amyloglucosidase production was very low (6.05 U/ml/min for wild and 6.02 U/mL/min for mutant strain). Dry cell mass increased with increase in pH from 3-4 afterwards it started decreasing in case of the wild strain and it was maximum at pH 4.0 (10.76 g/l). But for the mutant strain maximum dry cell mass was achieved at pH 5.5 (12.45 g/L). At pH 8.0 dry cell mass was 7.10 g/L and 6.50 g/L for the wild and mutant strain respectively. Therefore, pH 5.0 was optimized for the production of enzyme by *A. niger* BT and *A. niger* M4 120.

The effect of replacement of starch by fructose, lactose, sucrose, glucose, maltose and mannitol was investigated for the production of amyloglucosidase by *A. niger* BT (Fig. 6). The sugars were added in the medium at 1% level. Maximum production of enzyme (13.18 U/mL/min) was obtained in the medium containing starch by *A. niger* BT and *A. niger* M4 120 (21.63 U/mL/min). The other sugars were found to be insignificant for the production of amyloglucosidase. When mannitol was added in the medium, the result was low enzyme production i.e., 7.36 U/mL/min and 15.0 U/mL/min for *A. niger* BT and *A. niger* M4 120 respectively. Dry cell mass was maximum 12.20 g/L for the wild and 12.80 g/l for the mutant strain when maltose was added in the medium but amyloglucosidase production with maltose was quite low.

However, the production of amyloglucosidase following growth of the organism was found to be maximum by the addition of starch in the fermentation medium for both the wild and the mutant strain. Thus, starch as carbon source was selected for optimum production of amyloglucosidase.

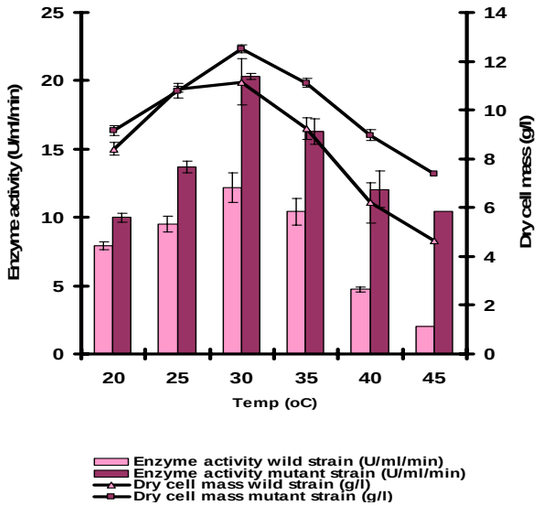

Fig. 3. Effect of temperature on the production of amyloglucosidase by *A. niger* BT (wild) and *A. niger* M4 120 (mutant) in shake flask

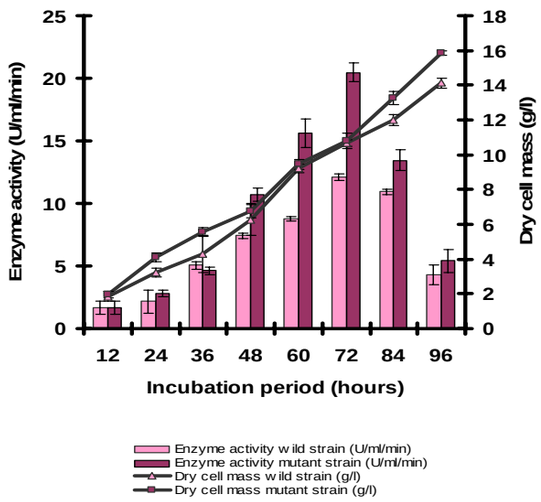

Fig. 4. Rate of amyloglucosidase production by *A. niger* BT (wild) and *A. niger* M4 120 (mutant) in shake flask.

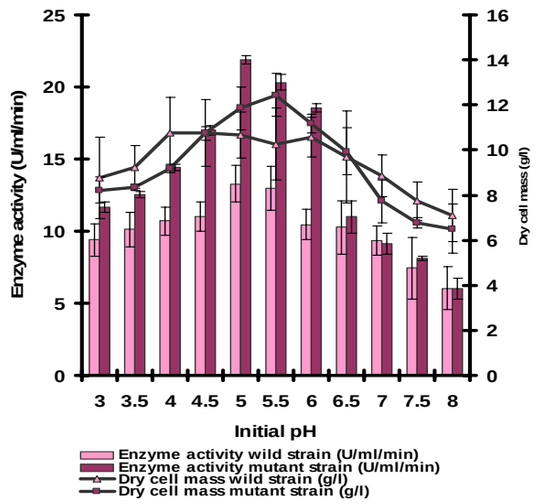

Fig. 5. Effect of initial pH on the production of amyloglucosidase by *A. niger* BT (wild) and *A. niger* M4 120 (mutant) in shake flask.

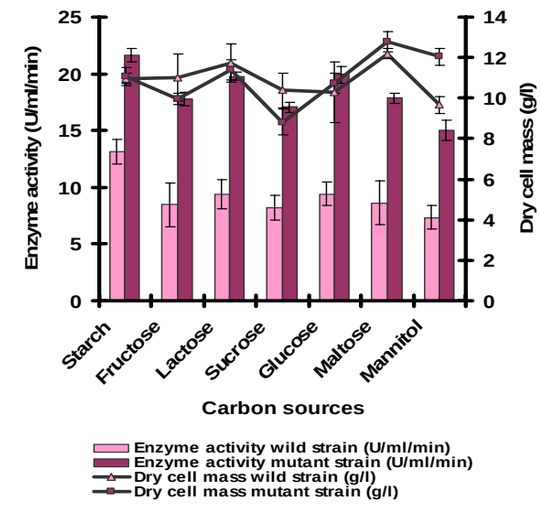

Fig. 6. Effect of replacement of starch by other carbon sources for amyloglucosidase production by *A. niger* BT (wild) and *A. niger* M4 120 (mutant) in shake flask.

Figure 7 shows the effect of different concentrations of raw corn starch on amyloglucosidase production and dry cell mass by *A. niger* BT and *A. niger* M4 120 respectively. Seven different concentrations were checked (0.5%, 1%, 1.5%, 2% 2.5%, 3.0% and 3.5%). Maximum production of amyloglucosidase for the wild and mutant strain (13.73 U/mL/min and 22.84 U/ml/min respectively) was achieved at 2% concentration of starch. At 0.5% concentration of starch the amyloglucosidase production was less (11.46 U/ml/min for wild strain and 18.81 U/ml/min for mutant strain), it increased up to 2% and as the starch concentration was further increased the enzyme production started decreasing. At 3.5% of starch, amyloglucosidase production was very less for both wild and mutant strain (9.35 U/mL/min and 20.35 U/mL/min respectively). Dry cell mass on the other hand increased with increase in starch concentration. At 0.5% starch concentration dry cell mass was less i.e., 10.39 g/L for wild and 10.91 g/L for the mutant strain. It increased with increase in percentage of starch and reached its maximum value at 3% starch (12.68 g/L) for the wild strain while for mutant strain it was maximum at 2% starch concentration (12.71 g/L). Thus 2% concentration of starch was selected for maximum production of amyloglucosidase by the wild and mutant strain.

The effect of addition of glucose, maltose, lactose, sucrose, fructose and mannitol in addition to starch were investigated for the production of amyloglucosidase by *A. niger* BT and *A. niger* M4 120 (Fig. 8). The sugars were added in the medium at 1% level. Maximum production of enzyme by the wild and mutant strain (14.17 U/mL/min and 24.19 U/mL/min respectively) was obtained in the medium containing glucose. The other sugars were found to be insignificant for the production of amyloglucosidase. When mannitol was added in the medium, the result was low amyloglucosidase production i.e., 8.13 U/mL/min for the wild strain and 5.41 U/mL/min for the mutant strain. Dry cell mass was maximum for the wild and mutant strain (12.75 g/L and 14.39 g/L respectively) when maltose was added in the medium but amyloglucosidase production with maltose was quite low.

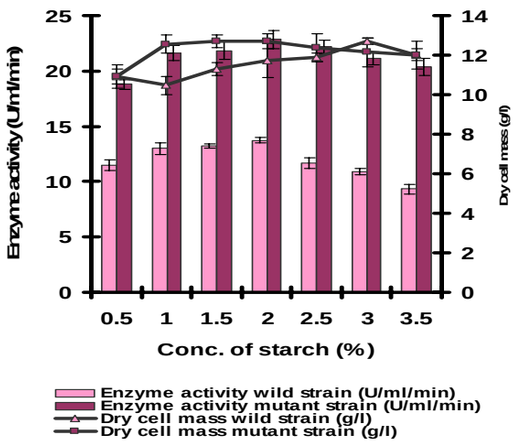

Fig. 7. Effect of different conc. of starch on the production of amyloglucosidase by *A. niger* BT (wild) and *A. niger* M4 120 (mutant) in shake flask.

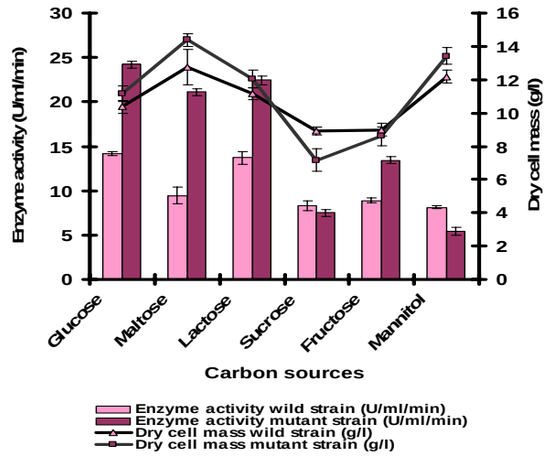

Fig. 8. Effect of different carbon sources on the production of amyloglucosidase by *A. niger* BT (wild) and *A. niger* M4 120 (mutant).

The production of amyloglucosidase following growth of the organism was found to be maximum by the addition of glucose in the fermentation medium. Thus, glucose along with starch as carbon source was selected for maximum production of amyloglucosidase by the wild and mutant strain.

The effect of different concentration of glucose on amyloglucosidase production and dry cell mass by *A. niger* BT and *A. niger* M4 120 was investigated (Fig. 9). Seven different concentrations were checked (0.5%, 1%, 1.5%, 2%, 2.5%, 3% and 3.5%). Maximum production of amyloglucosidase i.e., 14.21 U/ml/min for the wild strain and 24.13 U/ml/min for the mutant strain was achieved at 1% concentration of glucose. At 0.5% concentration of glucose, the amyloglucosidase production was less for the wild and mutant strain (13.39 U/mL/min, 22.34 U/ml/min), it increased up to 1% glucose. As the glucose concentration increased above 1%, the enzyme production started decreasing. At 3.5% of glucose, amyloglucosidase production was less (8.30 U/ml/min and 13.11 U/ml/min) for the wild and mutant strain respectively. Dry cell mass on the other hand increased with increase in glucose concentration. At 0.5% glucose concentration dry cell mass was less (11.41 g/l and 10.99 g/l) for the wild and mutant strain. It increased with increase in percentage of glucose and reached its maximum value at 2% glucose (12.85g/l) for the wild strain after which it also decreased. But for the mutant strain dry cell mass increased upto 3% concentration of glucose and the maximum dry cell mass (13.93 g/l) was achieved when 3% glucose was added in the medium. Thus 1% concentration of glucose was selected for maximum production of amyloglucosidase by *A. niger* BT and *A. niger* M4 120.

The different nitrogen sources such as ammonium sulphate, corn steep liquor, urea, ammonium biphosphate, sodium nitrate, peptone, yeast extract, ammonium nitrate and casein were evaluated for the production of amyloglucosidase by *A. niger* BT and *A. niger* M4 120 (Fig. 10). Nitrogen sources on the basis of 0.5% nitrogen were added in the fermentation medium. The maximum production of enzyme was achieved for the wild and mutant strain (14.19 U/ml/min and 24.16 U/ml/min respectively) when $(NH_4)_2SO_4$ was added in the medium. Dry cell mass was maximum in the presence of casein for the wild and mutant strain (15.67 g/l 14.87 g/l respectively), but the amount of amyloglucosidase production was less than ammonium sulphate. Therefore, ammonium sulphate was selected as nitrogen source and its various concentrations were also tested for the production of amyloglucosidase.

The different concentrations of $(NH_4)_2SO_4$ i.e., 0.1-0.5% were also evaluated for the production of enzyme (Fig. 11). The maximum production of amyloglucosidase (14.68 U/ml/min) was obtained when 0.3% nitrogen was added in the medium for the wild strain. But for mutant strain maximum enzyme production (25.29 U/ml/min) was obtained when 0.4% ammonium sulphate was added in the medium. Further increase in the concentration of nitrogen source resulted in decrease in the production of the enzyme. Hence, 0.3% nitrogen source in the form of $(NH_4)_2SO_4$ was selected for the wild strain while 0.4% ammonium sulphate was selected for the mutant strain for optimum production of amyloglucosidase.

The effect of different sizes of inoculum in the form of spores was investigated for the production of amyloglucosidase for both the wild and the mutant strain. The size of inoculum was varied as 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10% (1.2 x 10^6 conidia/ml). The production of the enzyme was found to be maximum (14.47 U/ml/min) with the addition of 2% spore inoculum (1.2 x 10^6 conidia/ml) for the wild strain. Similar results were obtained by the mutant strain which showed maximum enzyme production (25.25 U/ml/min) at the same concentration (Fig. 12). The production of enzyme following growth of the organism was found to be optimum at 2% (1.0 ml) spore inoculum.

The effect of addition of vegetative inoculum was also investigated for the production of amyloglucosidase by *A. niger* BT and *A. niger* M4 120 (Fig. 13). The size of vegetative inoculum was varied from 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9% and 10%. The production of the enzyme following growth of the wild and mutant strain was found to be optimum when 4% vegetative inoculum (16 hours old) was added. Further increase in size of inoculum resulted in the decrease of amyloglucosidase production. Although, with 4% vegetative inoculum, amyloglucosidase production was high (12.27 U/ml/min and 20.53 U/ml/min) for the wild and mutant strain respectively but it was less than with 2% spore inoculum. Thus 2% spore inoculum (1.0 ml) was selected for the production of amyloglucosidase by *A. niger* BT and *A. niger* M4 120.

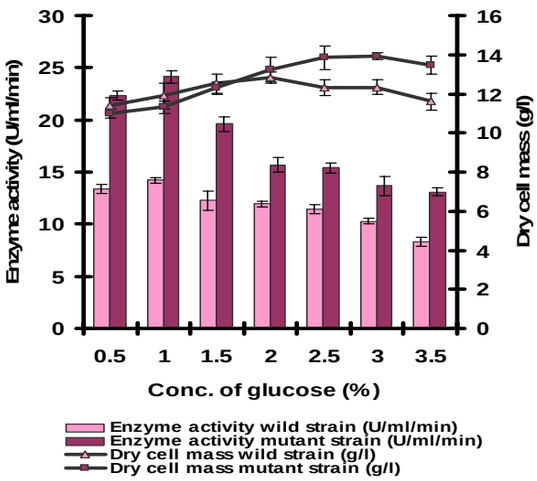

Fig. 9. Effect of different conc. of glucose on the production of amyloglucosidase by *A. niger* BT (wild) and *A. niger* M4 120 (mutant) in shake flask.

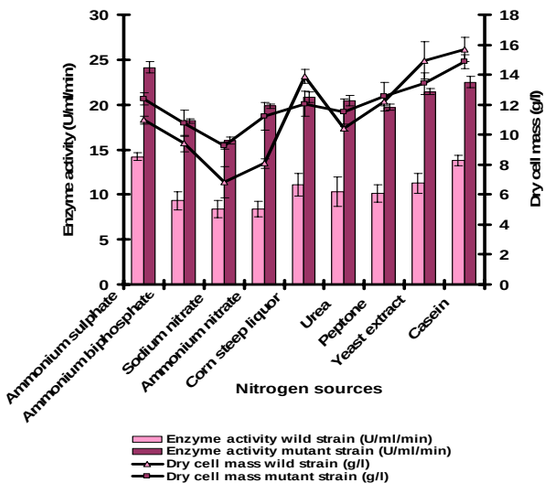

Fig. 10. Effect of different nitrogen sources on the production of amyloglucosidase by *A. niger* BT (wild) and *A. niger* M4 120 (mutant) in shake flask.

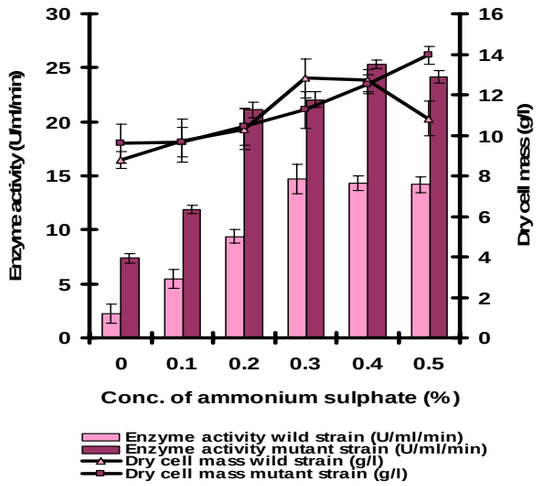

Fig. 11. Effect of different concentration of ammonium sulphate (%) on the production of amyloglucosidase by *A. niger* BT (wild) and *A. niger* M4 120 (mutant) in shake flask.

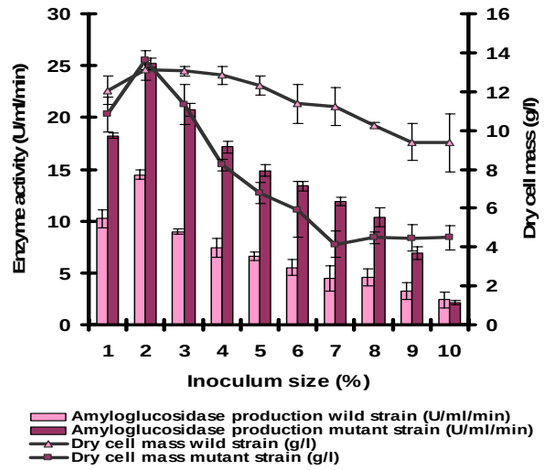

Fig. 12. Effect of inoculum size (spore inoculum) on the production of amyloglucosidase by *A. niger* BT (wild) and *A. niger* M4 120 (mutant) in shake flask.

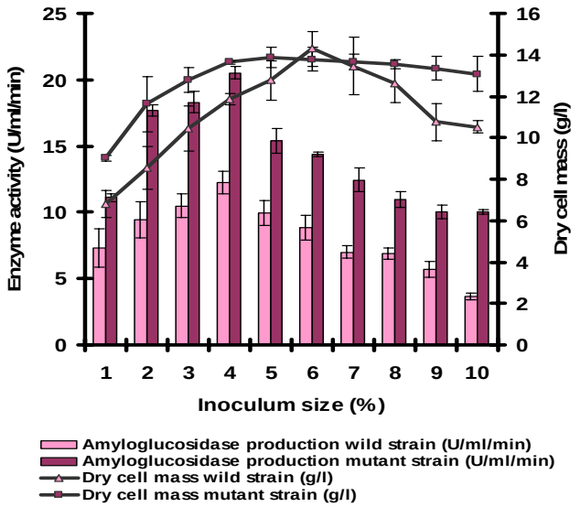

Fig. 13. Effect of inoculum size (vegetative) on the production of amyloglucosidase by *A. niger* BT (wild) and *A. niger* M4 120 (mutant) in shake flask.

References

Abbas, S.Q., R. Ayesha, M. Niaz, I. Ali and T. Iftikhar. 2010. New fungal records on *Morus alba* from Faisalabad Pakistan I. *Pak. J. Bot*., 42(1): 583-592.

Arassaratnam, V., K. Mylvagunam and T. Balasubramanian. 1997. Paddy husk support and rice bran for production of glucoamylase by *Aspergillus niger*. *J. Food Sci. Technol*., 32(4): 299-304.

Costa, J.A.V., Colla, E., Magagnin, G., Santos, L.O., M. Vendruscolo and T.E. Bertolin. 2007. Simultaneous amyloglucosidase and exopolygalacturonase production by *Aspergillus niger* using solid state fermentation. *Braz. Arch. Boil. Technol.*, 50(5): doi:10.1590/S1516-89132007000500003.

Ford, C. 1999. Improving operating performance of glucoamylase by mutagenesis. *Curr Opin Biotechnol.*, 10: 352-357.

Gwiazdowska, A.W., Olejniczak, O.I., G. Kostka and D. Czajkowska. 2004. An *Aspergillus awamori* mutant with enhanced glucoamylase activity. *Acta Biotechnologica*., 11(3): 235-239.

Haq, I., Ashraf, H., S. Omar and M.A. Qadeer. 2002. Biosynthesis of amyloglucosidase by *Aspergillus niger* using wheat bran as substrate. *Pak. J. of Biol. Sci*., 5(9): 962-964.

Haq, I., M. Ashraf, S. Ali and M.A. Qadeer. 1997. Biosynthesis of amyloglucosidase by *Aspergillus niger* in shake flasks. *Biologia*, 43(2): 53-59.

Iftikhar, T., I.U. Haq and M.M. Javed. 2003. Optimization of cultural conditions for the production of lipase by submerged culture of *rhizopus oligosporous* T^{uv} -31. *Pak. J. Bot*., 35(4): 519-525.

Iftikhar, T., M. Niaz, M.A Zia, M.A. Qadeer, S.Q. Abbas, M.I. Rajoka and I.U. Haq. 2007. Screening of media and Kinetic studies for the production of lipase from a mutant strain of *Rhizopus oligosporus*", *Biotechnologies International Symposium "Which biotechnologies for south countries*" *Proceedings of Biotech World 2007*. Oran Algerie, 24-25.

Kelly, C. T., F.O. Reilly and W.M. Fogarty. 1983. Extracellular alpha-glucosidase of an alkalophilic microorganism, *Bacillus* spp., ATCC 21591. *Microb. Lett*., 20: 55-59.

Khalaj, V., J.L. Brookman and G.D. Robson. 2001. A study of the protein secretary pathway of *Aspergillus niger* using a glucoamylase-GFP Fusion protein. *Fungal Genet. Biol*., 32(1): 55-65.

Malik, S., T. Iftikhar and I.U. Haq. 2011. Enhanced amyloglucosidase biosynthesis through mutagenesis using *Aspergillus niger. Pak. J. Bot*., 43(1): 111-119.

Metwally, M. 1998. Glucoamylase production in continuous cultures of *Aspergillus niger* with special emphasis on growth parameters. *World J. Microbiol. Biotechnol*., 14(1): 113-118.

Mishra, A. and M. Debnath. 2002. Effect of pH on simultaneous saccharification and isomerization by Glucoamylase and Glucose isomerase. *Appl. Biochem. and Biotechnol.*, 102(1-3): 193-200.

Nandakumar, M.P., M.S. Thakur., K.S.M.S Raghavarao and N.P. Ghildyal. 1999. Studies on catabolite repression in solid state fermentation for biosynthesis of fungal amylases. *Lett. Appl. Microbiol*., 29(6): 380-384.

Omemu, A.M., I. Akpan, M.O. Bankole and O.D. Teniola. 2005. Hydrolysis of raw tuber starches by amylase of *Aspergillus niger* AM07 isolated from the soil. *Afric. J. of Biotechnol*., 4(1): 19-25.

Pamboukian, C., R. Denser, R. Facciotti and R.C. Maria. 1999. Correlation between pellet size and glucoamylase production in submerged cultures of *Aspergillus awamori. Rev. Microbiol*., 29(1): 23-26.

Pandey and S. Radhakrishan. 1993. The production of glucoamylase by *Aspergillus niger* NCIM 1245. *Process Biochem*., 8: 305-309.

Pavezzi, F.C., E. Gomes and R. Silva. 2008. Production and characterization of glucoamylase from fungus *Aspergillus awamori* expressed in yeast *Saccharomyces cerevisiae* using different carbon sources. *Braz. J. Microbiol*., 39(1): doi: 10.1590/S1517-83822008000100024.

Polakovic, M and J. Bryjak. 2004. Modelling of potato starch saccharification by an *Aspergillus niger* glucoamylase. *J.Biochem. Eng.*, 18: 57-64.

Prescott and Dunn. 1987. *Industrial Microbiology*, 4th ed., CBS Publishers and distributors, India, pp. 225-300.

Ramadas, M., O. Holst and B. Mattiasson. 1996. Production of amyloglucosidase by *Aspergillus niger* under different cultivation regimens. *World J. Microb. Biotechnol*., 12: 267-271.

Reilly, P.J. 1999. Protein engineering of glucoamylase to improve industrial performance – a review. *Starch/Starke.*, 51: 269-274.

Schmidell, W., B.V. Facciotti., M.C.R. Kolikian., H. Aboutboul and Aguero. 1988. Influence of pH oscillations in amyloglucosidase production by *Aspergillus awamori. J. Microbiol*., 19: 71-77.

Spier, M.R., A.L. Woiciechowski., L.P.S. Vandenberghe and C.R. Soccol. 2006. Production and characterization of Amylases by *Aspergillus niger* under solid state fermentation using Agro industrials products. *International Journal of Food Engeenering*., 2(3): 1-20.

(Received for publication 30 December 2009)