POTENTIAL OF CHEAP CELLULOSIC RESIDUE AS CARBON SOURCE IN AMYLASE PRODUCTION BY *ASPERGILLUS NIGER* SH-2 FOR APPLICATION IN ENZYMATIC DESIZING AT HIGH TEMPERATURES

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The present study explores the potential of indigenous fungal flora for cost-effective production of amylases for efficient use in enzymatic textile desizing. *Aspergillus niger* SH-2, found to produce the highest amylase production among the fungal isolates, was subjected to conditions of solid state fermentation (SSF), using wheat bran as solid substrate. It produced 37.6 IU/mL of amylase and 8.94 mg/mL of supernatant protein concentration after 6 days of incubation. The incubation temperature of 37 ^{o}C (38.99 IU/mL of amylase activity), pH 6.0 (40.30 IU/mL of amylase activity) and beef extract as nitrogen source (40.19 IU/mL of amylase activity) were found optimum for amylase production by the test fungi. The test fungi produced 49.00 IU/mL of amylase activity and 11.08 mg/mL of supernatant protein concentration under the above-mentioned optimized conditions of SSF. The crude amylase of the test fungal strain was found to be stable at high temperature and alkaline pH, as even at 80 ^{o}C and pH 6.0, 23.31 and 50.25% of its maximum activity was retained, respectively. The crude amylase preparation obtained from the test strain, under optimum conditions of SSF, exhibited significant desizing of grey cotton fabric where 73.5% size (starch) was removed at a temperature of 70 ^{o}C and incubation time of 60 minutes. The findings promise successful desizing by the said enzyme preparation at high temperatures during textile processing.

Keywords: amylase, desizing, solid state fermentation (SSF), fungi

INTRODUCTION

A continuous search of environment friendly technologies has led to increased attention towards microbial sources of enzymes and thus, a number of industrial applications are being tested upon for the incorporation of microbial enzymes to decrease the environment pollution and the overall cost of the industrial processes.[1-5] In addition, these enzymes provide highly specific reactions, thereby making them all the more important. α-Amylase (endo-1,4-α-D-glucanglucohydrolase, EC 3.2.1.1) is one such industrially important enzyme and is extensively explored for industrial applications. α-Amylases are extracellular enzymes that randomly cleave the 1,4-α-D-glycosidic bonds between adjacent glucose units in the linear amylase chain and are classified according to their action and properties.[6-13] Amylases can be derived from various sources, such as plants, animals and micro-organisms, but microbial sources generally meet industrial demands.[14,15] As far as microbial sources of amylase are concerned, fungal amylases have been found to fare better, as compared to bacterial sources. The hyphal mode of growth and, good tolerance to low water activity (a_w) and high osmotic pressure conditions seem to make fungi the most attractive source of amylase.[16] The use of solid state fermentation using cheap, lignocellulosic substrates for amylase production is gaining great importance due to their distinct advantages.[16-20] Amylases have been shown to play a significant role in a number of industrial applications,[21-22] including textile desizing.[12] Sizing agents like starch are applied to yarn before fabric production to ensure a fast and secure weaving process. Desizing involves the removal of starch from the fabric on which it serves as a strengthening agent to prevent breaking of the warp thread during the weaving process. After weaving the cloth, the starch has to

be removed, and it is at this point of the process that α-amylase is introduced to help with the removal of the starch.[8,11] The amylases remove selectively the size and do not attack the fiber, which adds to its significance in textile industry.

The current study explores the indigenous fungal microflora of Jalandhar region, Punjab, India, for its amylase producing ability. The fungal strain *Aspergillus fumigatus* NTCC1222, showing the highest amylase activity among the isolates, is further explored for desizing grey cotton fabric to explore its use in textile desizing.

EXPERIMENTAL

Materials and methods

All the chemicals were of analytical grade and purchased from Himedia Pvt. Ltd., India, and Lobachemie Pvt. Ltd., India. Wheat bran (WB) was procured from a local market and used as a solid substrate. The solid substrate was first washed with cold water and then with warm water to remove dirt and impurities. The washed substrate was then dried in sunlight. The dried substrate was ground in a laboratory grinder and a particle size of 0.5 mm was selected for further studies. The grey cotton fabric used for desizing experiments was purchased from a local market of Ludhiana, Punjab, India.

Isolation and primary screening of fungal amylase producers

Soil samples were collected from different regions of Jalandhar, Punjab, India, by sweeping off the debris from the top of the soil, and scooping the sample from the top soil (about 100 g, into a "Ziploc" bag. The soil samples were serially diluted and spread plated on starch agar medium (SAM). The plates were incubated at a temperature of 35 ^{o}C for 3 days and were subsequently stained with iodine solution (1% iodine w/v, 2% potassium iodide w/v) and observed for amylase production.

Maintenance of stock cultures

Stock cultures of purified isolates were maintained on potato dextrose agar (PDA) slants, incubated at 25 ^{o}C for 24 hours and subsequently stored at 4 ^{o}C. The cultures were maintained as a suspension of spores and hyphal fragments in 15% (v/v) sterile glycerol at -20 ºC for long term preservation.

Secondary screening of amylase producers

Amylase positive fungal strains were subjected to solid state fermentation (SSF).[23] 5 g of wheat bran was taken into Erlenmeyer flasks, to which 15 mL of nutrient salt solution (NSS) (potassium dihydrogen orthophosphate 5 g/L, ammonium nitrate 5 g/L, sodium chloride 1 g/L, magnesium sulphate 1 g/L)[24] was added, thereby maintaining a substrate to moistening agent ratio of 1:3.[25] The flasks were autoclaved, cooled, inoculated with two discs of fungal isolates (each of a diameter of 5 mm) and incubated at 25^{o} C for 3 days.

Enzyme harvesting

At the end of fermentation, the contents of flasks were crushed with a glass rod and 15 ml of distilled water was added. The contents were then mixed by shaking for 10 minutes at 55 ^{o}C on a rotary shaker at 200 rpm. The slurry obtained was squeezed through four layers of cheese cloth. The extract was filtered with a Whatman filter paper 1 and then centrifuged at 5000 rpm for 10 minutes.[18] The filtrate obtained was treated as crude enzyme.

Estimation of amylase activity and supernatant protein concentration

The amylase activity was determined by the dinitrosalicylic acid (DNS) method[26] at 540 nm and reported as IU/mL using glucose as a standard. One unit of amylase is defined as the amount of enzyme that releases 1 µ mole of reducing sugar per minute (IU/mL) with glucose as a standard, under the assay conditions described above. The supernatant protein concentration was determined by the Lowry method[27] at 660 nm, using bovine serum albumin (BSA) as a standard and reported as mg/mL.

Identification of the test fungal strain

The test fungus was identified based on morphological and microscopic features. A drop of lacto-phenol cotton blue stain was placed on a clean glass slide to which fungal mycelia and spores of the test strain were added. The stain was gently mixed with fungal structures and a cover slip was placed over the preparation. The prepared slide was examined microscopically at 40x under compound microscope.[28]

Effect of incubation period, incubation temperature and initial pH on amylase production by the test fungal strain under SSF

The effect of incubation period on amylase activity was determined by incubating the inoculated flasks for variable incubation period (1 to 8 days) at pH 6.0, using wheat bran as carbon source, beef extract as nitrogen source, at a temperature of 37 ^{o}C. The wheat bran was moistened with NSS in the ratio of 1:3, as previously described. The enzyme was harvested after every 24 hours and amylase activity[26] and supernatant protein concentration[27] were determined. Similarly, the inoculated flasks were incubated at variable temperatures (27 ^{o}C to 52 ^{o}C), using wheat bran as carbon source at pH of 6.0 for optimized incubation period and variable initial pH (3.0 to 8.0), using wheat bran as carbon source for optimized incubation time and temperature. The pH of the fermentation medium was not monitored after autoclaving and only the

initial pH was maintained. The enzyme was subsequently harvested and amylase activity[26] and supernatant protein concentration[27] were determined.

Effect of nitrogen sources on amylase production by the test fungal strain under SSF

Erlenmeyer flasks containing 5 g of wheat bran as solid substrate and 15 ml of NSS, containing different nitrogen sources (peptone, beef extract, tryptone, yeast extract, ammonium nitrate), were inoculated with the test strain under optimized conditions of incubation period, temperature and pH.[29] The amylase activity and supernatant protein concentration were determined.

Effect of simple sugars on amylase production by the test fungal strain under SSF

The effect of additional sugars on the amylase production by the test fungi was determined by supplementing the basal fermentation medium with 1% of different simple sugars (dextrose, lactose, maltose, sucrose) and comparing it with the amylase activity for non-supplemented basal fermentation medium under optimized conditions of temperature, pH and time.

Large scale production of crude amylase by test fungi under optimized conditions of SSF

The test fungal strain was subjected to SSF, as optimized above, in a 1000 mL Erlenmeyer flask, using 20 g of wheat bran and 60 mL of NSS. The amylase activity and supernatant protein concentration were subsequently determined.

Effect of pH and temperature on amylase activity and stability

The effect of incubation pH, incubation temperature and incubation time on the crude amylase activity of the test fungi was studied by incubating the same at variable pH (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0), incubation temperature (40 ^{o}C, 50 ^{o}C, 60 ^{o}C, 75 ^{o}C and 80 ^{o}C) and determining the amylase activity.[26]

Application of crude enzyme of test strain for desizing of grey fabric

A stiff piece of cotton fabric (grey cotton fabric procured from a local market of Ludhiana, Punjab, India, which had been starched (1% w/v) was used in the study. Equal size (5 x 5 inch) fabric pieces were weighed on electric balance before and after starching. The fabric strips were then dipped in 50 mL of enzymatic solution and then incubated at variable temperatures of 50 ^{o}C, 55 ^{o}C, 60 ^{o}C, 65 ^{o}C 70 ^{o}C for 60 min. The fabric strips were washed with tap water and then oven dried. After drying, the cloth strips were weighed again.[30] The percent (%) removal of starch was calculated by applying the following formula:

Desizing (%) = {Weight of starch removed by enzyme (g)/Total starch present on the fabric strips (g)} x 100

Statistical analysis

All the cultures were replicated in triplicates and the results presented are the mean standard deviation (±) of the value.

RESULTS AND DISCUSSION

A total of 8 fungal strains (S1-S8) were found to be positive for amylase production, as indicated by the area of clearance (zone of hydrolysis) against a bluish-black background, formed around the fungal colonies. As shown in Table 1, S4 showed the best amylase activity.

Table 1
Primary screening to isolate amylase positive fungal strains

S. No.	Fungal strains	Appearance of clear zones on starch agar medium
1.	S1	++
2.	S2	+
3.	S3	++
4.	S4	+++
5.	S5	+
6.	S6	++
7.	S7	+
8.	S8	++

*Growth conditions: incubation period: 3 days; incubation temperature: 35 ^{o}C
+ = average, ++ = good, +++ = very good

Fungal strains S1, S3, S6, S8 showed comparable amylase activity, while S2, S5, S7 showed comparable, but the least amylase activity. The secondary screening (Table 2) confirmed that S4 (11.90 IU/mL) was the best amylase producer among the isolates.

The rest of the fungal isolates can be arranged in decreasing order of amylase activity: S6 > S1 >

S3 > S8 > S5 > S2 > S7. The best amylase producer, S4, was identified as *Aspergillus niger* on the basis of morphological and microscopic features. The strain was designated as *Aspergillus niger* SH-2 for future reference.

Table 2
Secondary screening of fungal isolates for amylase activity

S. No.	Fungal strains	Amylase activity (IU/mL)
1	S1	8.03±0.21
2	S2	3.64±0.14
3	S3	7.45±0.17
4	S4	11.90±0.10
5	S5	4.90±0.10
6	S6	8.20±1.02
7	S7	2.21±0.10
8	S8	6.90±2.11

Fermentation conditions: incubation period: 3 days; temperature: 25 °C; pH = 6.0
Assay conditions: incubation time: 30 minutes; temperature: 40 °C; pH = 6.0

As graphically represented in Figure 1, the maximum amylase activity (37.6 IU/mL) and corresponding supernatant protein concentration (8.94 mg/mL) were reported on the 7th day of incubation. On the 6th day of incubation, the amylase activity was 28.1 IU/mL and on the 8th day of incubation the amylase activity decreased from 37.6 IU/mL to 31.1 IU/mL. The amylase production (38.99 IU/mL) and supernatant protein concentration (8.99 mg/mL) were maximum at 37 °C (Figure 1). Even at 50 °C, the enzyme activity was found to be 9.70 IU/mL, indicating slight thermophillic nature of the test fungal strain. The amylase production (40.3 IU/mL) and supernatant protein concentration (9.12 mg/mL) were maximum at an initial pH 6.0 (Figure 1).

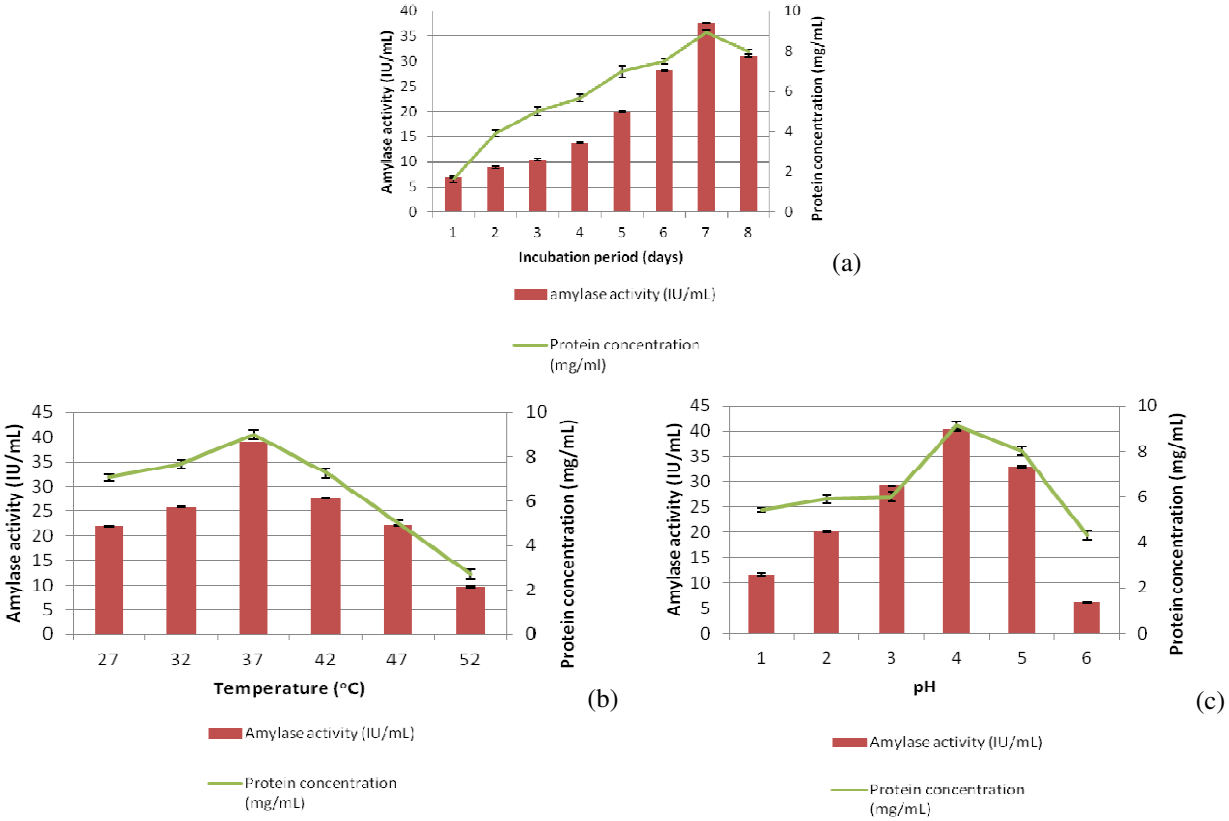

Figure 1: Effect of (a) incubation period, (b) temperature, and (c) pH on amylase production

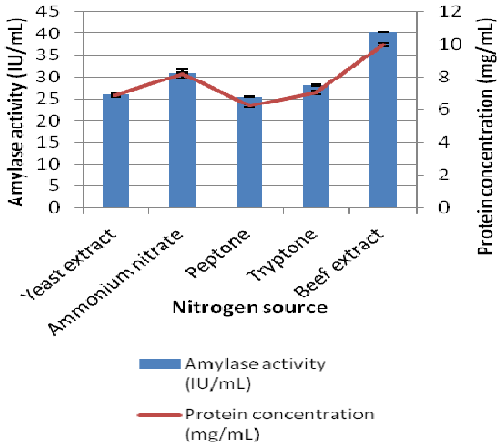

Figure 2: Effect of nitrogen sources on amylase production

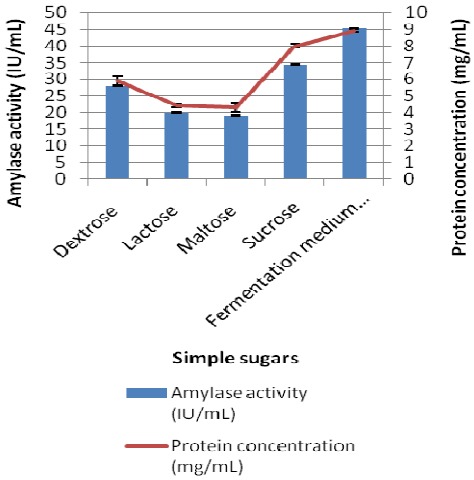

Figure 3: Effect of simple sugars on amylase production

At pH 8, the amylase retained 15.17% of its highest activity, while there was a gradual decrease in amylase activity at a pH lower than 6.0, retaining 28.79% of its highest activity at pH 3.0. The results indicated that slightly acidic pH was required for the amylase to be produced by the test strain under SSF.

Figure 2 indicates that beef extract served as the best nitrogen source for amylase production (40.19 IU/mL) and supernatant protein concentration (9.98 mg/ml) followed by the other nitrogen sources in a decreasing order (related to the amylase production and supernatant protein concentration, respectively) as: ammonium nitrate (31.00 IU/mL, 8.19 mg/mL) > tryptone (28.1 IU/mL, 7.05 mg/mL) > yeast extract (25.98 IU/mL, 6.89 mg/mL) > peptone (25.5 IU/mL, 6.21 mg/mL). The higher enzyme production observed for the beef extract might be attributed to a better absorption of amino acids of the yeast extract directly through the mycelia of the test strain.[31,32] As Figure 3 indicates, all the additional sugars (dextrose, sucrose, lactose and maltose) added to the basal fermentation medium, were found to decrease amylase activity, which might be due to catabolite repression.[33,34] Sucrose, maltose, lactose and dextrose repressed the amylase activity by 23.71%, 57.81%, 55.66% and 37.90%, respectively.

As shown in Table 3, under optimized conditions of SSF (wheat bran as solid substrate, NSS as moistening agent, beef extract as nitrogen source, incubation period of 6 days, at 35 °C and pH 6.0), amylase activity and supernatant protein concentration were found to be of 49.00 IU/mL and 11.08 mg/ml, respectively.

The maximum amylase activity (48.00 IU/mL) was obtained at pH 6.0 (Figure 4), when crude amylase preparation was incubated at different pH values for 30 minutes at 40 °C. At pH 7.0 and 8.0, the enzyme retained 64.8 and 50.25%, respectively, of its maximum activity. At pH 5.0, 4.0 and 3.0, the amylase activity was 21.12, 20.10 and 16.90 IU/mL, respectively. The maximum amylase activity (46.90 IU/mL) was obtained at 60 °C (Figure 4) when crude amylase preparation was incubated at different temperature values for 30 minutes at optimized pH. The enzyme retained 23.31% of its maximum activity even at very high temperature – of 80 °C. The findings are significant as they indicate good stability of the crude amylase of the test fungi at high pH and temperatures, thereby improving its applicability in conventional industrial textile processing.

Table 3
Production of amylase under optimized conditions of SSF

Amylase activity (IU/mL)	Protein concentration (mg/mL)
49.00±0.10	11.08±0.12

Fermentation conditions: incubation period: 6 days; temperature: 35 °C; pH = 6.0; solid substrate:moistening agent ratio = 1:3. Assay conditions: incubation time: 30 minutes; temperature: 40 °C; pH = 6.0

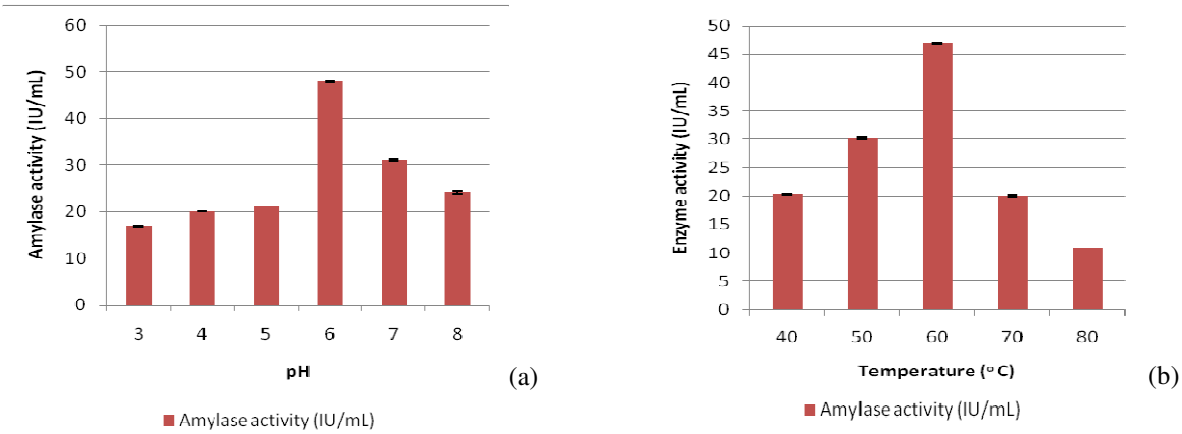

Figure 4: Effect of (a) and pH (b) temperature on crude amylase preparation

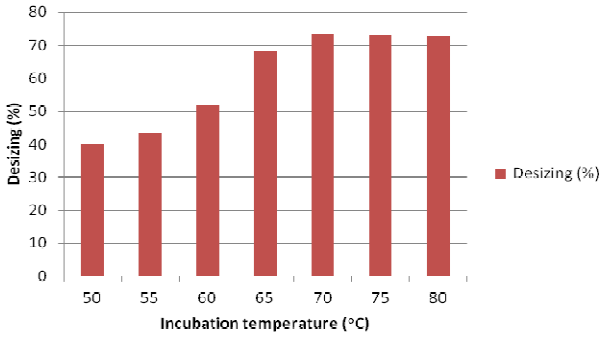

Figure 5: Application of crude enzyme preparation in desizing of grey fabric

The crude amylase obtained from *Aspergillus niger* SH-2 under optimized conditions of SSF, successfully desized (Figure 5) grey fabric to a significant extent. As can be seen, the extent of desizing gradually increased as the temperature of incubation increased from 50 °C (40.0%) to 70 °C (73.5%) and then became more or less constant. Thus, maximum desizing can be obtained with the said crude amylase by incubating the grey fabric in the presence of crude amylase of the test fungi at 70 °C. The results prove that *Aspergillus niger* SH-2 can be successfully used as a source of amylase for application in textile desizing.

CONCLUSION

The study has led to promising results, showing that *Aspergillus niger* SH-2 can be an important source of α-amylase, which can be successfully applied in environment friendly textile desizing. The potential of wheat bran as a cheap, but efficient lignocellulosic substrate was explored for the production of amylase by indigenously isolated fungal strains. Eight fungal isolates from soil samples collected from different locations of Jalandhar city, Punjab, India, were found to be amylase positive. Fungal strain S4, later identified as a strain of *Aspergillus niger* and designated as *Aspergillus niger* SH-2, showed the maximum amylase activity. An incubation temperature of 37 °C, incubation time of 6 days, pH of 6.0 and beef extract as nitrogen source were found to be the best culture conditions for production of amylase by *Aspergillus niger* SH-2 under solid state fermentation (49 IU/mL amylase activity). The maximum desizing (73.5%) can be obtained by incubating grey fabric in the presence of crude amylase of the test fungi at 70 °C. Though the amylase activity was not too high, and many microbial strains have already been found to report better amylase activities, the study is yet significant as the crude enzyme is found to be a good textile desizer and the desizing efficiency is maintained even at very high temperatures (75 °C and 80 °C), thereby adding to the search of thermostable α-amylases for use as textile desizer.

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