Aspergillus niger- A potential enzyme producer on cost effective agro industrial wastes

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Abstract

Solid state fermentation (SSF) techniques are gaining foothold in commercial processes to produce a wide variety of enzymes mainly from fungal origin. Production of amylase and protease by solid state fermentation employing two strains of Aspergillus niger isolated from bread was evaluated. Various substrates used were cheap agro industrial wastes such as wheat bran, rice bran, black gram bran, coconut oil cake, gingely oil cake and groundnut oil cake. The process parameters tested were incubation time, incubation temperature and pH, carbon and nitrogen sources. The strain Aspergillus niger BAN3E exhibited the highest amylase production.

The maximum amylase activity was recorded in groundnut oil cake supplemented with starch as carbon source and ammonium sulphate as nitrogen source after an incubation period of 6 days at temperature 37 ºC and pH 7. The best protease producing strain was Aspergillus niger BAN1E which gave the maximum yield of the enzyme in wheat bran supplemented with 0.2 % maltose and ammonium sulphate as carbon and nitrogen sources respectively on the sixth day. The optimum pH and temperature for enzyme production was found to be 7 and 40°C respectively. The optimum parameters were utilized to formulate production media for the two fermentation processes. The proteins were separated on SDS-PAGE and compared with markers. Zymogram analyses were conducted with starch and gelatin as the respective substrates for amylase and protease respectively. This study could be instrumental in the large scale industrial production of amylase and protease for commercial utilization.

Keywords: *Aspergillus niger,* Agro industrial wastes, Solid state fermentation.

Introduction

Solid state fermentation (SSF) holds tremendous potential for production of a large variety of enzymes. Most of the recent research activity on SSF is being done in developing nations as a possible alternative for conventional submerged fermentations which are the main process in pharmaceutical and food industries in industrialized nations. It also has the added advantages of simple media, concentrated product and hence ease of purification as well as reduced risk of microbial contamination due to the low moisture content of the substrate. The *Aspergillus* species produce a large variety of extra cellular enzymes of which amylases and proteases are of significant industrial importance[26]. Amylases have not only been used in fermentation processes but also in processed food industry and in the textile and paper industries. Proteolytic enzymes account for nearly 60% of the industrial enzyme markets in the world. They have found wide application in several industrial processes such as cheese production, meat tenderization, baking industry and in many other fields, including textiles and leather industries and as an additive to detergents.

Aspergillus niger is mainly concentrated for protease and amylase production because of its cosmopolitan and ubiquitous nature and non-fastidious nutritional requirement. The hyphal mode of growth and tolerance to low water activity and high osmotic conditions make *Aspergillus niger* suitable for bioconversion of solid substrates. Comparative evaluation of the production of amylase and protease was carried out by optimizing the various parameters involved in fungal growth and enzyme production.

Material and Methods

Isolation and Screening of Enzyme producing *Aspergillus niger:* Spoiled bread samples and onion were collected from GRD Women's hostel for isolation of *A. niger*. The samples were serially diluted and spread on potato dextrose agar medium plates and incubated for three days at room temperature. Five strains of *A. niger* were isolated and identification was done. The isolated cultures were maintained in PDA (Potato infusion-200 g/L, Dextrose-20 g/L, Agar-15 g/L, pH- 6.4) slants at 4°C.

All strains of *A. niger* were screened on nutrient agar medium containing 1% starch and 1% casein for amylase and protease production respectively. A patch of culture isolated from bread samples was inoculated on the medium and the incubated at 30 °C for 3 days. *A. niger* was further screened for protease production by inoculating a patch of culture on nutrient agar plates containing 1 % gelatin and incubated at 30 °C for 3 days. The plate was then stained with 15% $HgCl_2$ solution in 20% concentrated HCl. Morphological properties of the isolated strains were determined by Lacto phenol Cotton Blue staining (LPB). The scanning electron micrographs of *A. niger* spores were taken in JEOL 6360 JSM model of the Scanning Electron Microscope (SEM) in the metallurgy laboratory of PSG College of Technology**.**

Production of Extracellular Enzyme

Enzyme production was done by two methods- Submerged fermentation and Solid state fermentation**.**

Submerged Fermentation for the Production of Extracellular Amylase and Protease: The respective *A. niger* strains which showed maximum amylase production (BAN3E) and protease production (BAN1E) were inoculated in PD broth and incubated at room temperature for 4 days. The medium was then centrifuged and the supernatant was collected for separating extracellular enzyme**.**

Solid State Fermentation: Six different substrates were used for solid state fermentation viz. rice bran, wheat bran, coconut bran, gingely oil bran, ground bran and black gram bran. 5 g of each bran were weighed and hydrated with 5 ml of basal salt solution (Ammonium sulphate – 2 g/L, Potassium dihydrogen phosphate – 1 g/L, Magnesium sulphate – 0.5 g/L, Zinc sulphate – 0.1 g/L) to adjust the moisture content from 43 to 81 %. 1 % of inoculum of each enzyme producing strain was inoculated after sterilization and incubated at room temperature for 1-7 days.

Enzyme Extraction

22 ml of 0.1M Phosphate buffer saline (Solution A- 2.48 g of monobasic sodium phosphate in 10 ml of distilled water, Solution B- 3.12 g of dibasic sodium phosphate in 10 ml of distilled water. Mix 3.9 ml of solution A + 6.1 ml of solution B and make up to 200 ml to get 0.1 M PBS of pH 7) was added to each of the inoculated and incubated substrate beds and was vigorously shaken in rotary shaker for 15 min at 120 rpm. The mixture was filtered through cheese cloth and centrifuged at 8000 rpm at 4°C for 15 min. The supernatant was used as the crude enzyme preparation.

Optimization: Optimization of growth conditions and enzyme production was done using different parameters such as incubation time, pH, temperature, carbon source and nitrogen source.

Incubation Time: The effect of incubation period on enzyme production (pH 7, 30°C) was investigated by checking the enzyme activity on 4^{th}, 5^{th}, 6^{th}, 7^{th} and 8^{th} days of incubation.

pH: The effect of pH enzyme production was investigated by solid state fermentation in different substrates by adjusting the pH of basal salt solutions to 4, 5.5, 6.5, 7, 7.5, 8 and 9 using concentrated acetic acid for acidic pH and concentrated NaOH for alkaline pH. The substrates were then incubated for 7 days at 30°C as the maximum enzyme production was at 7^{th} day of incubation**.**

Temperature: The effect of temperature on enzyme production was investigated by SSF in different substrates and incubation at 30°C, 37°C, 40°C, 45°C and 50°C for 7 days.

Carbon Sources: The effect of carbon sources on enzyme production was investigated by supplementing the basal salt solution, pH 7 with 0.2% of different carbon sources such as glucose, maltose, lactose, starch and sucrose. The substrates were then incubated for 7 days at room temperature**.**

Nitrogen Sources: The effect of nitrogen sources on enzyme production was studied by replacing the nitrogen source in basal salt solution, pH 7 with 0.2% of nitrogen sources such as sodium nitrate, ammonium nitrate, ammonium sulphate, ammonium chloride and potassium nitrate and incubating at room temperature for 7 days.

Estimation of Protein Content

The concentration of protein in crude/ purified sample was determined by Lowry's method[18] using Bovine Serum Albumin as standard.

Assay for Amylase (Dinitrosalicylic Acid Method): 1 ml of starch solution was added to 1 ml of properly diluted enzyme in a test tube and incubated at 27^{o}C for 15 minutes. The reaction was stopped by the addition of 2 ml of dinitrosalicylic acid reagent followed by incubation in a boiling water bath for 5 minutes. While the tubes were warmed, 1 ml of potassium sodium tartarate solution was added and then the tubes were cooled in running tap water. The volume was made up to 10 ml by addition of water. Absorbance was read at 560 nm. The reaction was terminated at 0 time in control tubes. The standard graph was prepared with glucose. One unit of amylase activity was defined as the amount of enzyme, which released one micromolar glucose per minute per milligram protein (U/mg).

Assay for Protease (Lowry's Method): Enzyme assay was done using tyrosine as standard by the method followed by Lowry et al[18]. The reaction was carried out in a reaction mixture containing 1 ml of 1 % (w/v) casein, 1 ml of phosphate buffered saline (pH 7) and 1 ml of crude enzyme extract. The mixture was incubated at 40°C for 15 min. 3 ml of 10 % ice cold TCA was added to terminate the reaction and was incubated for 10 min. The mixture was centrifuged at 8000 rpm for 10 min and to 100 µl of supernatant, 900 µl of water was added for dilution. From this, 500 µl of the extract was taken for protease assay, using Lowry's method and the absorbance at 670 nm was obtained. One unit of protease activity is defined as the micro molar concentration of tyrosine liberated per minute under controlled assay conditions.

$$\text{Specific Activity} = \frac{\text{Enzyme Activity}}{\text{Total Protein Content}}$$

Partial Purification and Characterization: Purification of the crude extract was done using the following steps: Ammonium sulphate precipitation, dialysis, acetone precipitation, SDS-PAGE and zymogram analysis. To 22 ml of each crude enzyme extract, 9.91 g of ammonium sulphate was added so that the final volume was 27.26 ml at 4°C. This was done to achieve 60% saturation. The mixture was left for precipitation overnight by continuous stirring on a magnetic stirrer at 4°C. The resulting precipitate was collected by centrifugation at 8000 rpm for 15 min at 4°C. The pellet was dissolved in 10 ml of 25 mM Tris – HCl, pH 8.

A pre-treated dialysis bag was used for dialysis of the enzyme collected after ammonium sulphate precipitation. About 15 ml of the partially purified protease was dialyzed against 10 mM Tris- HCl, pH 8 while the same amount of partially purified amylase was dialyzed against 0.8M citrate buffer, pH 5. This setup was kept overnight at 4°C on a magnetic stirrer. The used buffer was checked for the presence of ammonium sulphate by adding 2 drops of Nessler's reagent and observing for reddish brown colour formation. The buffer was changed frequently and the partially purified sample was assayed for enzyme activity and protein content. The dialysate was further concentrated by acetone precipitation. To 1 volume of enzyme solution, 4 volumes of ice cold acetone were added and incubated overnight at -80°C. The pellet was collected by centrifugation at 10,000 rpm for 15 min at 4°C. The air-dried pellet was suspended in sample buffer, incubated in boiling water bath for 5 min and transferred to ice. It was later brought to room temperature and used for SDS-PAGE.

SDS-PAGE: SDS-PAGE was carried out in order to determine the molecular weight of the purified enzyme sample[17]. Varying dilutions of the sample were loaded next to the standard protein marker. The internal surfaces of the gel plates were cleaned and joined to form the cassette and clamped in a vertical position. The separating gel was mixed gently and poured into the gel cassette without any air bubble and left for polymerization for 30 min. Once the separating gel was polymerized, the stacking gel mixture was prepared. Stacking gel mixture was added to the gel cassette until the solution reached the cut away edge of the gel plate. The well forming comb was placed in this solution and allowed to set. After the stacking gel set, the comb was carefully removed. The samples to be run were denatured in sample buffer by heating at 95-100°C for 3-5 min. The gel was placed in the tank and was filled with electrophoresis buffer. The samples were carefully loaded into the wells. The gel was run at 100 V until the bromophenol blue reached the bottom of the gel. The stacking gel was then discarded and the separating gel was transferred to staining solution for 1hr in rocker. Then the staining solution was decanted and the destaining was done overnight using the destaining solution. The destaining solution was changed frequently.

Zymogram Analysis: Amylase activity was localized after electrophoresis by immersing the gel for 1 hour in a solution of 1% soluble starch in 0.1M Citrate buffer (pH 4) at room temperature. The gel was kept in same buffer for 10 minutes without soluble starch and stained with iodine solution. For protease enzyme, gelatin zymography was performed in polyacrylamide slab gels containing SDS and 0.1% gelatin as a co polymerized substrate. After electrophoresis the gels were washed in 0.25 % (w/v) Triton x-100 for 1 hr. at 4°C and then transferred to 0.1 M Tris HCl buffer (pH 9) for removing SDS and incubated for 2 hr at 40°C. The gel was developed by soaking in Coomassie brilliant blue R 250 and rinsed with methanol: acetic acid: water solution (4:1:5).

Results

Isolation and screening of protease producing *A. niger:* Five strains of *A. niger* (BAN1E, BAN2E, BAN3E, BAN4E and BAN5E) were isolated from bread samples and maintained in PDA slants at 4 °C. The organisms were confirmed as *A. niger* by lacto phenol cotton blue staining that showed mycelia with spores and septate hyphae, which is the typical *A. niger* morphology. All the five cultures were screened for amylase production on starch agar medium and on the basis of the area of clearance, *Aspergillus niger* BAN3E was selected for further studies on amylase production. Among the five isolated strains, the *A. niger* (BAN1E) produced more protease when compared to the other four, by forming a zone of hydrolysis with diameter of 3 and 5 cm in both casein and gelatin medium respectively.

Enzyme Production: Initially the amount of enzyme production was checked in submerged fermentation. The specific activity of protease was 16 U/mg while that of amylase was 52 U/mg. A comparative study was made on enzyme production with solid state fermentation. As solid state fermentation gave higher yields than submerged fermentation, further studies were carried on with solid state fermentation only.

Solid State Fermentation: Solid state fermentation was carried out with six different substrates and the protein values of the crude extract were quantified by Folin Lowry method with tyrosine as standard. Wheat bran supported the maximum production of the enzyme protease with the maximum specific activity of 33.64 U/mg at pH 7 with maltose as carbon source, ammonium sulphate as nitrogen source at 40°C after 7 days of incubation. This substrate also supported a good amount of amylase activity by *Aspergillus niger* BAN3E as was demonstrated by the specific activity of 190 U/mg when incubated at 30°C on the 8th day at a pH of 7.5 when supplemented with starch and ammonium sulphate as carbon and nitrogen sources.

The maximum amylase production was observed in groundnut oil cake (specific activity 320 U/mg) supplemented with starch as carbon source and ammonium sulphate as nitrogen source after an incubation period of 6 days at temperature 37 ºC and pH 7. Bioconversion of groundnut by *Aspergillus niger* BAN1E to yield protease gave maximum specific activity (21.0 U/mg) at pH 7 with incubation at 37 °C for 6 days with sucrose as the carbon source and sodium nitrate as the nitrogen source.

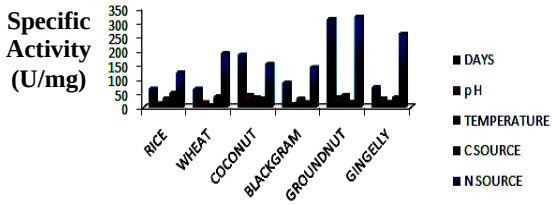
The second best substrate for protease production was rice bran which gave maximum specific activity of 27.44 U/mg on the seventh day at 40°C with sucrose carbon source as and ammonium sulphate as nitrogen source with optimum pH as 4. However rice bran was the least utilized substrate by *Aspergillus niger* BAN3E to produce amylase as the specific activity obtained (122 U/mg) was very less in comparison with the other agro industrial residues.

Gingely oil cake was the second best suited substrate for amylase production as it gave a high enzyme yield of 260 U/mg when supplemented with lactose and ammonium sulphate as the carbon and nitrogen sources and then incubated at pH 7.5 and at a temperature of 30°C. The maximum production was obtained on the sixth day of incubation. The maximum specific activity of protease in gingely oil cake (23.6 U/mg) was obtained at pH 9, 37 °C on 4 days of incubation when supplemented with starch as the carbon source and ammonium sulphate as the nitrogen source.

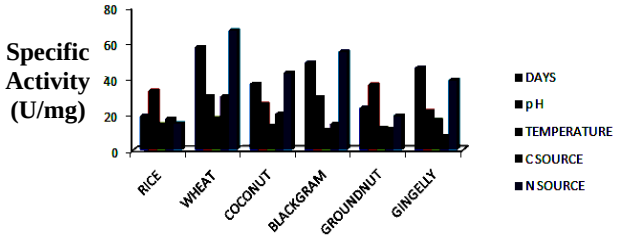
The fourth in terms of protease production was black gram bran with the highest specific activity of 22.4 U/mg on the 6th day of incubation at 50 °C, pH 4 with maltose as the carbon source and ammonium sulphate as the nitrogen source. This substrate was fifth in terms of amylase production. It gave a specific activity of 141 U/mg on the fifth day of incubation at pH of 6.5 and at temperature of 45°C when glucose and ammonium nitrate were incorporated into the substrate.

Coconut oil cake was not as efficiently utilized as the rest of the agro industrial wastes for protease production. The highest specific activity (18.4 U/mg) in its case was obtained at pH 4 on incubation at 40°C for five days with sucrose as carbon source and sodium nitrate as nitrogen source. Coconut oil cake was the fourth best substrate for amylase production yielding a specific activity of 185 U/mg in six days at pH 7 and 40°C utilizing starch and ammonium chloride as substrate.

Partial Purification and Characterization: Amylase was extracted with phosphate buffer and subjected to ammonium sulphate precipitation. The precipitate was dissolved in citrate buffer and dialyzed with same buffer at pH 5. The dialysate was further concentrated with acetone and loaded in SDS PAGE and electrophoresed. The gel was stained with Coomassie Brilliant blue and found very faint band. Zymogram staining of amylase revealed visible enzyme activity as a pale yellow band in the dark colored gel. Extra cellular protease was purified from the culture filtrate of *A. niger*. The enzyme was partially purified by ammonium sulphate precipitation (60%). About 82.47% of enzyme yield (partially purified) was obtained with 1.17 purification fold. The purified enzyme seems to be homogenized because it showed a single band in SDS-PAGE and zymogram analysis. The molecular mass of the purified protease was estimated to be 60 KDa by SDS-PAGE. On zymogram analysis the activity band was observed as a clear colourless area depleted of gelatin in the gel against blue background.



Specific activity of the optimum parameters for amylase production in different substrates



Specific activity of the optimum parameters for protease production in different substrates

Discussion

The production of enzymes by various strains of *A. niger* which can utilize cheap agro-industrial residues have been enjoying a lot of attention in the recent times since there is a growing need to exploit fungal proteases. Enzymes of fungal origin are preferred as comparatively larger quantities of enzyme can be produced by fungal expression systems than bacterial expression systems. The present study focuses on amylase and protease production by two different strains of *A. niger* through bioconversion of cheaper solid substrates such as wheat bran, rice bran, gingelly oil cake, groundnut oil cake, coconut oil cake and black gram bran that showed higher yields than submerged fermentation. In addition the rate of recovery of the enzyme is also higher than in submerged fermentation[34]. In this study, submerged fermentation yielded just 16 U/mg of protease and 52 U/mg of amylase. This could be because the nutrients are released in excess from the medium during fungal growth possibly resulting in nutrient repression and as a result, in low enzyme yield.

However, much higher yield of the enzyme was obtained in solid state fermentation. This is because in SSF, the nutrients liberated from the substrate are directly consumed by the fungi[31]. Microscopic observations can also represent a good way to estimate fungal growth in SSF. Naturally, optic examination is not possible at high magnitude but only at stereo microscope. Scanning Electron Microscope (SEM) is a useful tool to observe the pattern of growth in SSF [29].

Effect of Moisture Content: Presence of water in the substrate makes the nutrients more easily accessible for mold growth. Moreover, water has an impact on physico-chemical properties of the substrate, which in turn affect enzyme production[26]. Too much water adversely affects oxygen diffusion in the substrate[9]. The moisture content of the substrate in the present study is between 43-81%. Previous studies on the effect of moisture content on fungal growth and utilization of the substrate by Ghildyal et al[13] stated the optimum moisture content as between 40-70%. This is also in accordance with Chutmanop et al[10]. However, Negi and Banerjee[23] observed that maximum production of amylase and protease occurred at a relative humidity of 90% using a strain of *Aspergillus awamori* in a concomitant fermentation.

Effect of Incubation Time: The time of incubation has an important role in cheaper commercial production of enzymes. The maximum yield of protease was obtained in 7 days of growth, the production being initiated from the 4th day onwards. Earlier reports suggested maximum yield at 3 days of incubation in other *Aspergillus* sp. However, *A. awamori* showed a peak of protease production at 120 h of incubation and the production of amylase increased with the increase in incubation period up to 96 h and beyond this there was no remarkable change in the yield of amylase[23]. Thus, the optimum incubation time for glucoamylase production was reported as 84 hrs. In a study by Paranthaman et al[27], *A. niger* gave the maximum yield of protease at 72 h of incubation in a SSF process with rice bran as the substrate. Optimizing the time of incubation is an important step in making commercial protease production economically feasible.

Effect of pH: The present study of pH optimization of protease production was completely in accordance with Ikasari and Mitchell[16]. *A. niger* produced proteases at all pH ranges (4.4 - 9), the maximum activity being at a pH range of 7-9 after which the production ceased. These results clearly indicate the alkalinophilic nature of the fungus as reported by Devi et al[12]. For all the culture media, the difference between the optimum pH and the other pH values is very little in absolute terms, which suggests a possible pH insensitivity of enzyme over a pH range of 4 to 9. Fungal proteases are active at neutral and alkaline pH with an optimum between pH 7 to 11 as reported by Coral et al.[11] Similar studies on the influence of pH on protease production have been studied in different *Aspergillus* sp. In case of *A. niger* the optimum pH was reported to be 7.8 by Barthomauf et al[5] in *A. terrus* between 5.5 and 9.5.[7] *A. parasiticus*, *A. fumigatus* and *Aspergillus nidulans* the optimum pH was 8.[36,39] However an optimum pH of 7 for *A. flavus* has been reported by Sutar et al[35] which is similar to our study. This is also supported by Paranthaman et al[27]. In our study the effect of pH on the amylase activity indicates that the amylase is active in the pH range 7 – 8, both neutral and alkaline. This suggests that the enzyme would be useful in processes that require wide range of pH change from neutral to slightly alkaline range and vice versa. Similarly multiple pH optima were observed for amylolytic activities in the crude amylase preparation in literature.[1,3,6,15,41,42]

Effect of Temperature: There was increase in yield in 40°C in coconut oil cake medium and in Rice Bran medium when the temperature was 45°C. Then a gradual decrease in yield was observed. Ueno et al[37] and Kundu et al[17] support great yield of amylase in temperature range of 30°C-37°C. However the optimum temperature for enzyme production was reported as 30°C in literature[3,14]. Previously 30°C and 45°C were reported as optimum temperature for amylase production by *A. flavus* and *Myceliophora thermophilia* respectively.[22,30]

The maximum protease activity was recorded at 40°C and considerable activity was also noted at 30°C and 37°C, however increase in temperature (45°C and 50°C) resulted in considerable loss of activity and poor or no growth. Devi et al[12] have demonstrated the production of proteases at varied temperatures and confirm the protease production increased with increase in temperature from 35 to 45 °C. Maximum production of protease was obtained at 45 °C. Growth and protease production ceased at higher temperatures (50°C). Similar observations are also shown in our study. It was revealed that environmental temperatures not only affect the growth, but also have a marked influence on protease production. *Aspergillus niger* has been reported to produce maximum proteases at 40°C.[11] Temperatures of 30 °C, 35 °C and 37°C have been optimal for protease production in case of *A. saitoi*, *A. tamari, A. nidulans* and *A. awamori* respectively[8,23]. Solid state fermentation is more sensitive to temperature variation than broth culture, not only the temperature regulates the synthesis of the enzyme but possibly the secretion of the enzyme by changing the properties of the cell wall[32].

Effect of Carbon and Nitrogen Source: Various carbon sources such as glucose, maltose, sucrose, lactose and starch were evaluated for their effect on amylase and protease production by *A. niger*. In protease production, the suitable carbon source was maltose for wheat bran and coconut oil cake, starch for gingelly oil cake and sucrose for rice bran, black gram bran and groundnut oil cake respectively. Although all the carbon sources tested supported good growth of *A. niger*, maltose was the best

followed by sucrose. However, glucose was found to be more effective than maltose. Negi and Banerjee[23] reported that the incorporation of lactose as carbon source increased the production of protease by 1.91 fold and that of amylase by 1.62 fold respectively. In their study NH_4NO_3 acted as enhancer for production of amylase and protease by 1.21 and 2.1 fold, respectively. Among other inorganic sources $(NH_4)_2SO_4$ and NH_4Cl increased protease production by 2.05 and 1.48 fold respectively but both led to reduction in amylase production. However in our study, ammonium sulphate and ammonium nitrate were the best utilized nitrogen sources in the production of amylase. Among the various substrates tested, maltose and ammonium sulphate were found to be the most effective for protease production as recently reported[12].The mechanism that shows the formation of extracellular enzymes is influenced by the availability of precursors for protein synthesis.

Effect of Substrates: Oil cakes such as groundnut and gingelly oil cakes promoted high yields of amylase more than the protein rich substrates like wheat and rice brans. They were more efficient in production of amylase than in protease production. Wheat bran was well utilized by the fungus in the production of protease as well as amylase. However the highest amylase production was observed in black gram bran. This is a unique observation compared to most of the available literature.

All the six different substrates described previously were suitable for protease production by *A. niger* (BAN1E) as substrates rich in proteins may act as protease inducers. However, the maximum yield (33.64 U/ml) was obtained in wheat bran. Wheat bran is found to be an excellent substrate for protease production in solid state fermentation for its protein rich composition, texture, which gives an adequate surface area with good porosity, acting as a physical support and allowing the fungi to access the nutrients. Earlier reports on proteases from *Aspergillus* sp. and *Mucor* sp. are produced in higher yields in SSF using wheat bran as a substrate. The second best substrate was rice bran in our study. Rice bran has reduced porosity for satisfactory solid state fermentation and has found to be more effective only when used in combination with wheat bran[10] for protease production by *A. oryzae*. On the other hand, rice bran proved to be an effective substrate for enzyme production by *A. niger* (BAN1E). This may be due to the rapid growth of this strain on the rice bran and also due to its effective utilization of the available nutrients. The next best substrates were oil cakes (21 U/mg, 22.4 U/mg and 23.6 U/mg) in case of groundnut oil cake, coconut oil cake and gingelly oil cake respectively.

The use of oil seed cakes in the fermentation medium for protease production has been reported by several groups[28]. Sumantha et al [34] have worked on the production of proteases by *Aspergillus oryzae* using different oil cakes as substrates. Coconut oil cake proved to be the most efficient in their case with an enzyme activity of 9 U/g however it is much higher in our case. They have also further highlighted on the use of oil cakes in combination with brans that may prove to be more effective.

After the analysis of various parameters involved in protease production, wheat bran was found to be the best substrate and therefore the production medium was designed with wheat bran as the substrate, maltose as the carbon source, ammonium sulphate as the nitrogen source, pH 7, temperature 40°C and incubation for 7 days. For amylase production, media was designed with groundnut oil cake as the substrate, supplemented with starch as carbon source and ammonium sulphate as nitrogen source after an incubation period of 6 days at temperature 37 ºC and pH 7.

Purification of the extra cellular protease from *A. niger* (BAN1E) was carried out by a combination of conventional purification procedures and electro elution from a polyacrylamide gel containing 0.1% SDS. In the present study, approximately 1.17 fold purification was achieved with enzyme yield of 82.47 % and the molecular weight of protease was estimated to around 60 KDa and the characters suggest these enzymes as an alkaline protease. Siala et al[33] succeeded in purifying an acid protease from the culture supernatant of *A. niger* l1 by a three step process involving ultrafiltration, Sephadex G-75 gel filtration and CM-Sephadex C-50 ion exchange chromatography with a 3.55-fold increase in specific activity and 56.29% recovery. Alkaline proteases with a molecular weight above 60 KDa from *A. niger* has also been reported by Coral et al.[11]

Amylase was separated in SDS PAGE and the molecular weight was compared to protein marker. As per the literature, it could be predicted that the molecular weight of fungal amylase was nearly 43KDa.[38]

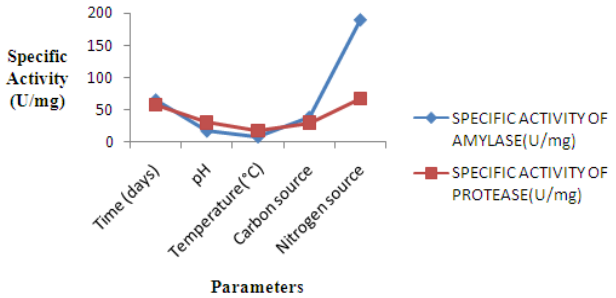


Fig. 1: Specific activity (U/Mg) of optimal parameters for amylase and protease production in wheat bran

Conclusion

The study has demonstrated the potential of a novel isolate of *Aspergillus niger* in the production of amylase and protease in solid state fermentation. It also illustrates the bio-utilization of wastes for commercial applications. The strain isolated can thus be used for further applications.

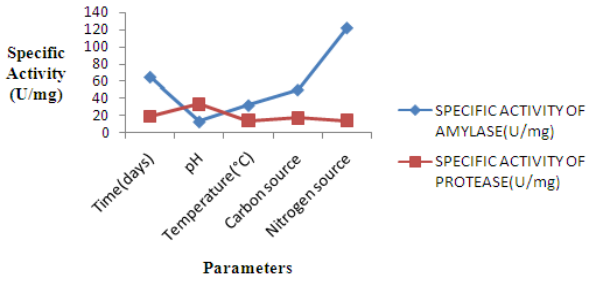


Fig. 2: Specific activity (U/Mg) of optimal parameters for amylase and protease production in rice bran

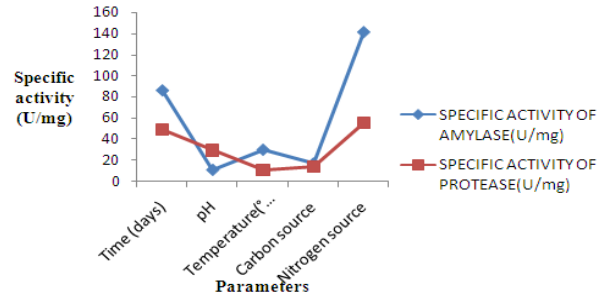


Fig. 3: Specific activity (u/mg) of optimal parameters for amylase and protease production in black gram bran

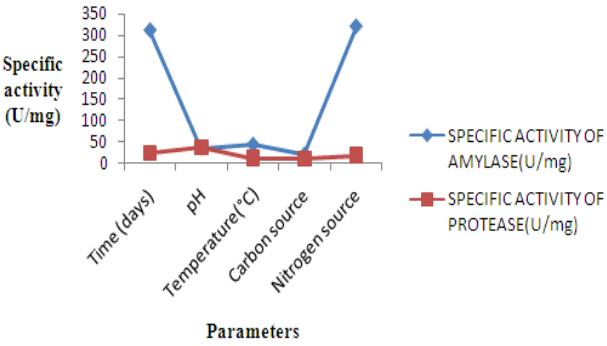


Fig. 4: Specific activity (U/Mg) of optimal parameters for amylase and protease production in groundnut oil cake

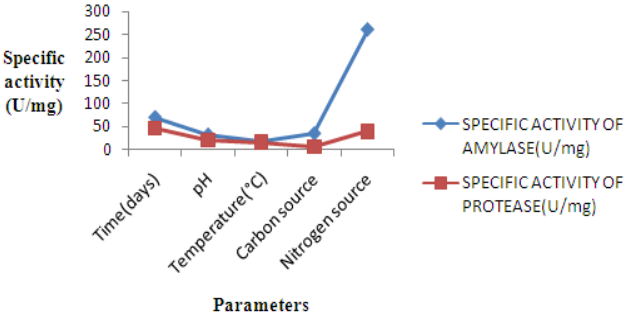


Fig. 5: Specific Activity (U/Mg) of optimal parameters for amylase and protease production in gingelly oil cake

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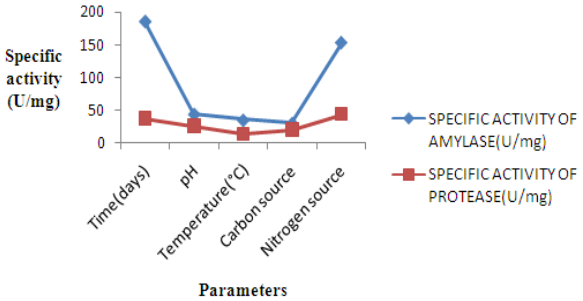

Fig. 6: Specific activity (U/Mg) of optimal parameters for amylase and protease production in coconut oil cake

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