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Isolation, production and characterization of amylase enzyme using the isolate *Aspergillus niger* FAB-211

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Amylase enzymes are industrially important enzymes used in food, sugar, textile, pharmaceutical, paper and detergent industries. The main objective of this study was to isolate, produce and optimize α-amylase enzyme using a fungal strain isolated from fruit peel soil wastes. Media optimization was done by one-factor at a time method. Average values of duplicate experiments were taken. Microsoft office Excel worksheet 2010 was used for data analysis. Soil samples were collected from three places and a total of 89 fungal isolates were isolated. All isolates were screened for their potential to produce amylase based on the clear zone formation on starch agar media, of which isolate FAB-211 showed the maximum potential to produce amylase and considered for further study. The isolate was further characterized based on colony morphology and microscopic mount and the isolate FAB-211 was *Aspergillus niger*. Important process parameters affecting amylase activity with the fungal isolate were optimized. The maximum activity (0.483 U/ml) was observed at pH of 6.0 and temperature at 45°C was found to be the best for amylase activity (1.241 U/ml). The highest and least alpha-amylase production was found when 6 and 2 discs spore of *A. niger* FAB-211 were used, respectively. Maximum yield of alpha amylase (0.281 U/ml) was observed on the 3rd day of incubation period followed by 4, 6 and 5th days. Maltose and yeast extract were found to be the best carbon and nitrogen sources, respectively. Therefore, further optimization of parameters and characterization of *A. niger* FAB-211 amylase is important for their application in industries.

Key words: Fungi, amylase enzyme, *Aspergillus niger*, FAB-211.

INTRODUCTION

Amylase enzyme has received a great deal of attention because of their economic and technological significance. Because of the importance of amylases, isolation of new microorganisms suitable for amylase production could provide potential new sources of the enzyme (Aullybux and Puchooa, 2013). In present day, biotechnology amylase accounts approximately for 25% of the enzyme market (Dabai et al., 2001). This enzyme has diverse

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applications in a wide variety of industries such as food, fermentation, textile, paper, detergent, pharmaceutical, and sugar industries. The hydrolysis of α-D-(1,4) glycosidic linkage in starch components and related polysaccharides to release maltose and a disaccharide is possibly due to this enzyme (Avwioroko and Tonukari, 2015). Major advantage of using fungi for the amylase production is the economical bulk production capacity (Shah et al., 2014).

The production of amylase is dependent on the strains, methods of cultivation, cell growth, nutrient requirement, metal ions, pH, temperature, time of incubation and thermotability. In addition, selection of a suitable substrate is critical for fermentation processes and investigating the potential of agriwastes for producing amylase could lead to the availability of new alternative substrates for this purpose (Aullybux and Puchooa, 2013). Many different species of fungi inhabit the soil, especially near the soil surface where aerobic conditions prevail. Such fungi are active in degrading a wide variety of biological materials present in the soil (Saranraj and Stella, 2013). The production of this enzyme is vital by investigating the potential fungal isolates and optimizing different parameters that will enhance the amylase activity with desirable properties intended to be used in different industries. Exploitation of the potential fungal isolate for the production of α-amylase enzyme is vital intended to be used in many industrial application like starch degradation and liquification. Therefore, the main objective of this research was the isolation, cultivation and characterization of the potential fungal isolate from fruit peel soil waste for the production of α-amylase enzyme.

MATERIALS AND METHODS

Soil sample was collected from fruit peel wastes and transferred to the laboratory using sterile polythene bags. Potato dextrose agar media was used for the isolation and maintenance of pure cultures of fungi (Mukunda et al., 2012). Potato dextrose agar (39 g) was dissolved in 1000 ml of sterile distilled water. The starch agar media was used for the primary and subsequent screening of amylolytic fungi isolates. Starch agar was prepared following the method by Ugoh and Ijigbade (2013).

Isolation of amylolytic fungi isolates

Serial dilution was used to isolate the fungus followed the method Clark et al. (1958). The inoculated Petri plates were incubated at 28°C for 3 to 4 days (Khan and Yadav, 2011). The initial fungal isolates were identified according to Sharma and Rajak (2003) and morphological characteristics. The isolates were picked up and further inoculated on sterile potato dextrose agar plates by point inoculation and incubated at 28°C for 48 h in order to obtain pure fungal plates.

Screening and selection of potential isolates

The amylolytic fungal isolates were screened following the method of Morya and Yadav (2008) for their best enzymatic starch hydrolysis. The isolate with maximum clear zone was further studied and selected as the potential single strain.

Culture maintenance and preparation of pure isolates

The cultures were subsequently sub cultured and used regularly following the method of Ugoh and Ijigbade (2013). The spore of the isolated fungus was aseptically transferred to the slants containing potato dextrose agar medium. The slants were then incubated at 28°C for 3 to 4 days for maximum growth of the fungus and stored in a refrigerator at 4°C for culture maintenance.

Staining of the pure isolate

The microscopic morphology of the pure isolate mount was done following the method by Shamly et al. (2014) and colonial characteristics such as size, surface appearance, texture, reverse and pigmentation of the colonies were used for microscopic view of the isolate.

Production of α-amylase from isolate

Submerged fermentation was carried out using the isolate *Aspergillus niger* FAB-21 for α-amylase production. Mineral media as described by Singh et al. (2009) was used for the production of enzymes. The pH was adjusted to 6.5 before sterilization. Mineral media (50 mL) were prepared in 250 mL Erlenmeyer. The fungal isolate spore disc was inoculated with sterilized 8 mm size cork borer into 250 ml Erlenmeyer flasks containing 50 ml production medium followed by incubation at 28°C for 72 h in rotary shaker at 150 rpm. The supernatant was collected by agitating the flask in shaker at 180 rpm for 1 h, the mixture was filtered through Whatman No. 1 filter paper and centrifuged at 8000 rpm at 4°C for 5 min and treated as crude enzyme.

Amylase assay

Amylase activity was determined as described by Miller (1959). The absorbance was measured at 540 nm by spectrophotometer. The concentration of the enzyme produced and kinetics were evaluated against the standard amylase enzyme. One unit (U) of alpha-amylase activity was described as the amount of enzyme that released µmol of reducing sugar per minute, under the assay conditions.

Optimization of condition for amylase production

Important process parameters affecting amylase activity with the fungal isolate were optimized. The methods described by Shah et al. (2014) were used with some modification to determine the optimum pH and effect of inoculum size on α-amylase production. The effects of various temperatures and incubation period on α-amylase production were determined using the method described by Puri et al. (2013) with some modification. The effect of nitrogen sources were optimized following the method by Liu et al. (2016) with some modification. The method by Abdullah and Ikram-ul-Haq (2014) was used to optimize carbon sources.

Experimental design and statistical analysis

Media optimization was done by one-factor-at-a-time method.

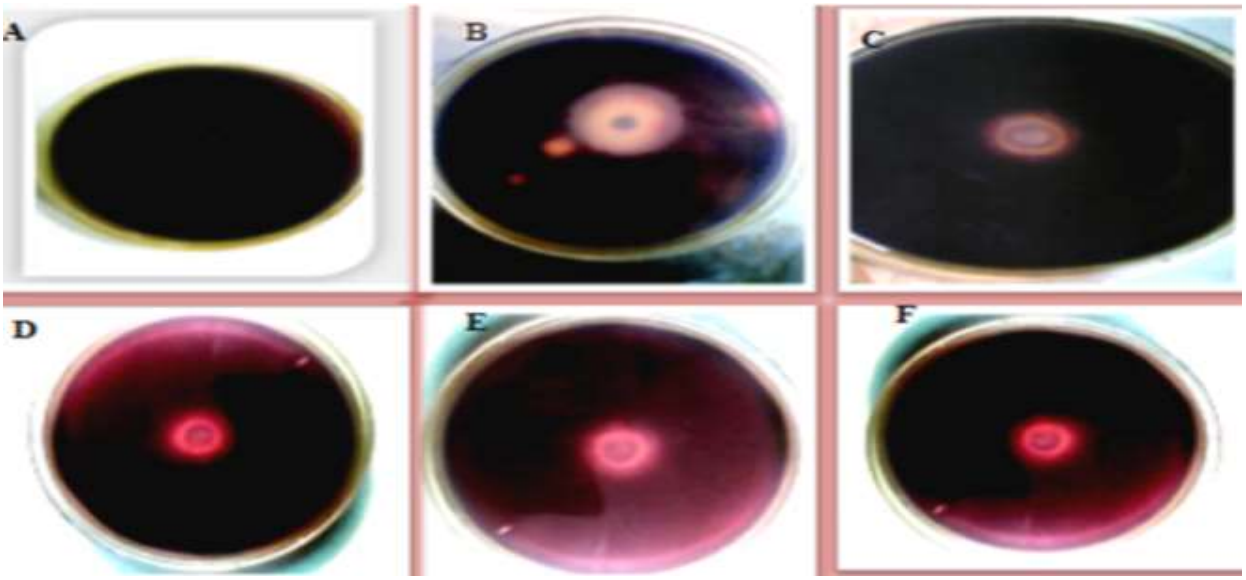

Figure 1. Zone of hydrolysis at 28°C, pH 6.5 after 4 days of incubation by promising isolates (B-F). A=control, PDA without the fungal isolate flooded with iodine solution; B=FAB-211; C=FAN-211; D=FAF-213; E=FAM-222; F=FIM-111.

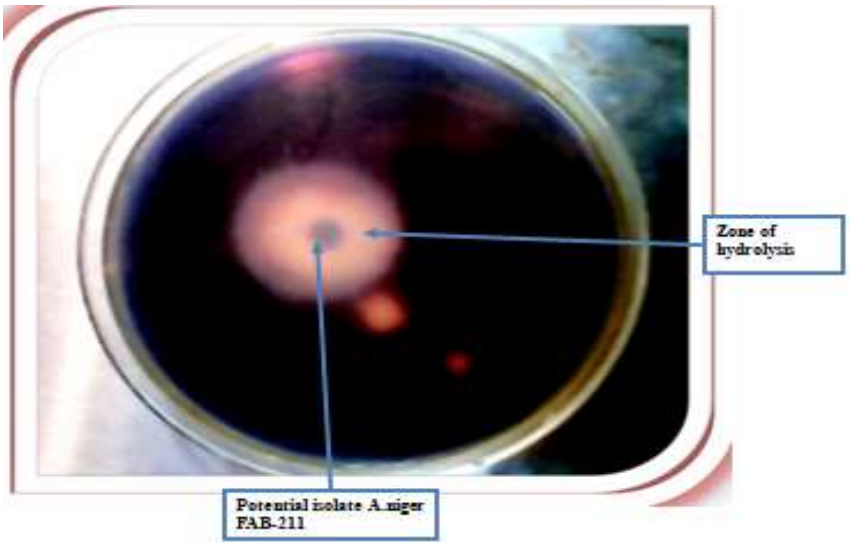

Figure 2. Plate indicating zone of hydrolysis (clear zone) by FAB-211 isolate.

Average values of duplicate experiments were taken. Microsoft office Excel worksheet 2010 was used for data analysis.

RESULTS AND DISCUSSION

Isolation and screening of amylolytic fungal isolates

The totals of 89 fungal isolates were isolated in the first phase of screening based on colony morphology and microscopic mount of the isolates. From the total of 89 fungal isolates, 29 isolates with relatively higher clear zones formation by starch hydrolysis were selected and further studied (Figure 1). In the second phase of screening, 5 potential isolates (Figure 2) were selected for further characterization and the isolates belonged to the genera *Aspergillus* (*A. niger, Aspergillus flavus, Aspergillus fumegatus, Aspergillus nidulans*, and *Aspergillus oryzae*) and the isolate with maximum α-amylase production was found to be FAB-211. This isolate was further characterized and *A. niger* was found (Figure 3). Therefore, the maximum clear zone formation

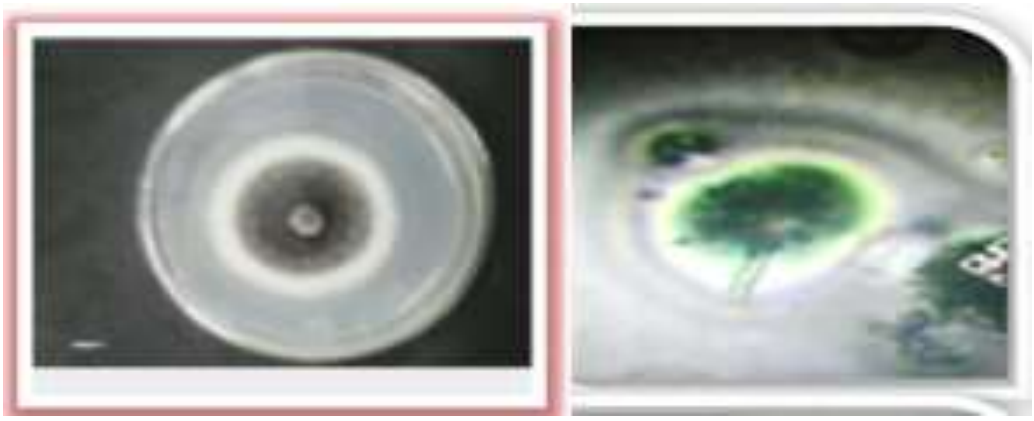

Figure 3. Colony morphology on PDA and microscopic mount of the potential isolate.

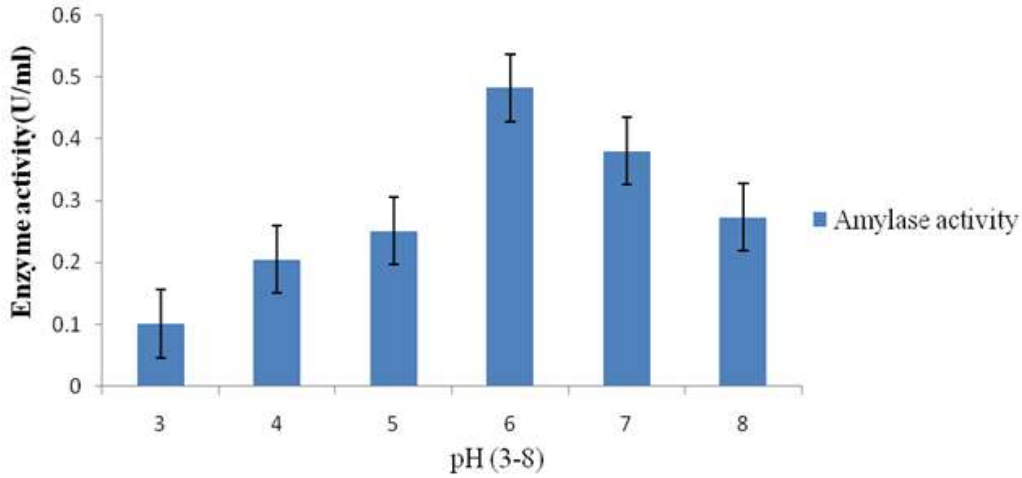

Figure 4. Effect of pH on enzyme activity at 28°C for 96 h period of incubation.

shown in Figure 2 on starch agar media by the fungal isolate confirmed that the isolate is a candidate for α-amylase producer.

Effects of optimization parameters on enzyme production

Effect of initial pH

The enzyme activity is markedly affected by pH. This is because substrate binding and catalysis are often dependent on ion distribution on both substrate and enzyme molecules (Shah et al., 2014). As shown in Figure 4, maximum activity (0.483 U/ml) was observed at pH of 6.0, since this was chosen as media pH for further optimization studies. With increase in pH value from 3.0 to 6.0, the activities of amylase attained the maximum followed by a gradual decrease thereafter.

Similar findings were reported by Shinde et al. (2014) who found maximum enzyme activity by *A. niger* and *Bacillus licheniformis* at pH 6 and Ellaiah et al. (2002) reported that *A. niger UO-01* had a preference to pH around 6.0 for amylase production but its production capacity decreased for pH levels higher and lower, probably as a consequence of a reduction in the metabolic activity of the amylase producing strain.

Effect of incubation temperature

Incubation temperature is an important parameter that affects the growth and metabolic activities of the isolate. The optimum incubation temperature is required for maximum production of the enzyme. The result as shown in Figure 5 revealed that temperature at 45°C was found to be the best for amylase activity (1.241 U/ml). At the beginning of 20°C, the activities of alpha-amylase was

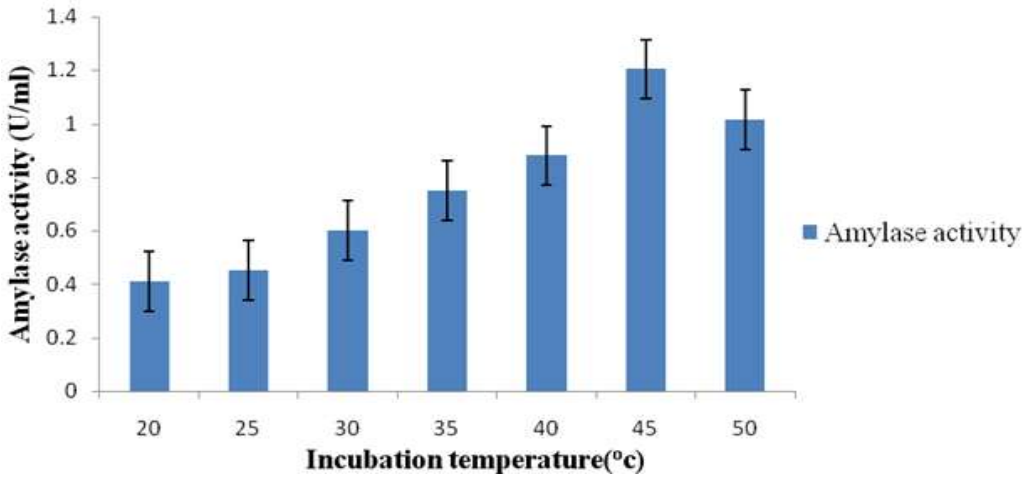

Figure 5. The effect of incubation temperature on enzyme production.

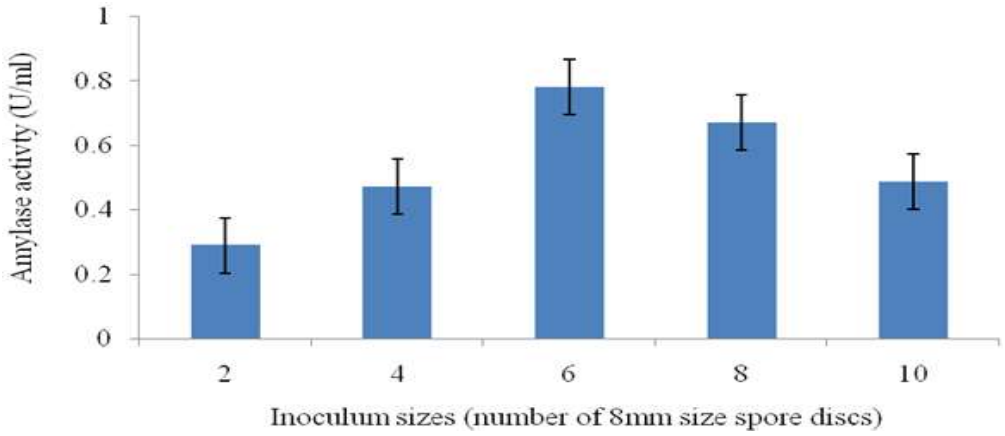

Figure 6. The effect of inoculum size on enzyme production at 28°C, pH 6 and 7 days of incubation.

low and showed gradual increases with the increase in temperature till it reaches its maximum at 45°C.

Similarly, Spier et al., (2006) reported that 45°C was optimum for amylase activity by *Aspergillus* species*;* however, Suganthi et al. (2011) and Ugoh and Ijigbade (2013) reported that temperatures at 30, 37 and 40°C were optimum for amylase activity by *Aspergillus* spp., respectively. The isolate *A. niger* FAB-211 exhibited maximum activity at 45°C. Variation in temperature was probably due to the preference of the strains to their optima growth. In this study, it was observed that further increase in temperature resulted in decrease in production of alpha-amylase. This is probably because the cell activity of the isolate increases gradually with increase in temperature until it reaches the maximum growth of mycelium to capture nutrients and growth retarded beyond optimum temperature (45°C). At higher temperature, the moisture content becomes lower than the optima for growth of the isolate and thereby greatly influences enzyme production.

Effect of inoculum size

The size of inoculum was important during the production of alpha-amylase thereby affecting the utilization rate of the production media by the fungal isolate. Maximum and minimum alpha-amylase production was found in 6 and 2 discs spore of *A. niger* FAB-211, respectively (Figure 6). After 6 discs spore of *A. niger* FAB-211, production declined gradually. The minimum activity at lower inoculum size probably because the number of active cells in the production medium was lower and therefore long time was needed to grow to an optimum number to utilize the nutrients in substrate and for enzyme production. Less enzyme production at higher inoculum level may be due to decreased nutrient availability for the large number of viable cells, or rapid accumulation of toxic metabolites (Haq et al., 2012). The result was partly in agreement with those of Shah et al. (2014), who reported that maximum amylase production was found when inoculum size was 5 discs for *A. oryzae*. Further

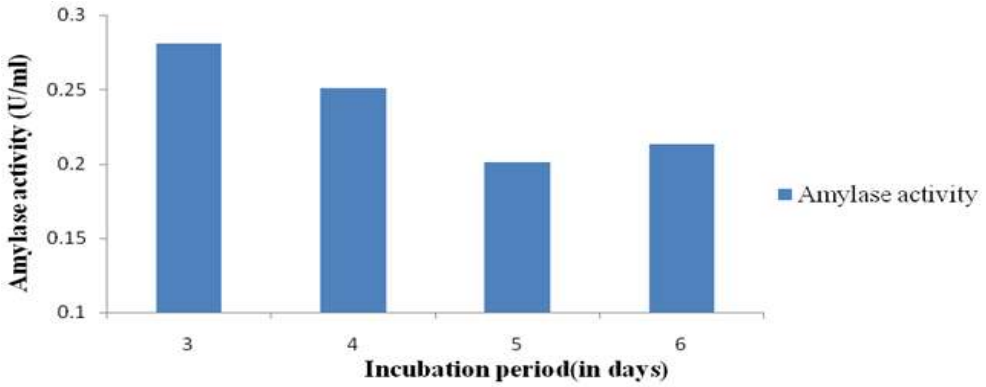

Figure 7. Effect of incubation period by *Aspergillus niger* FAB-211.

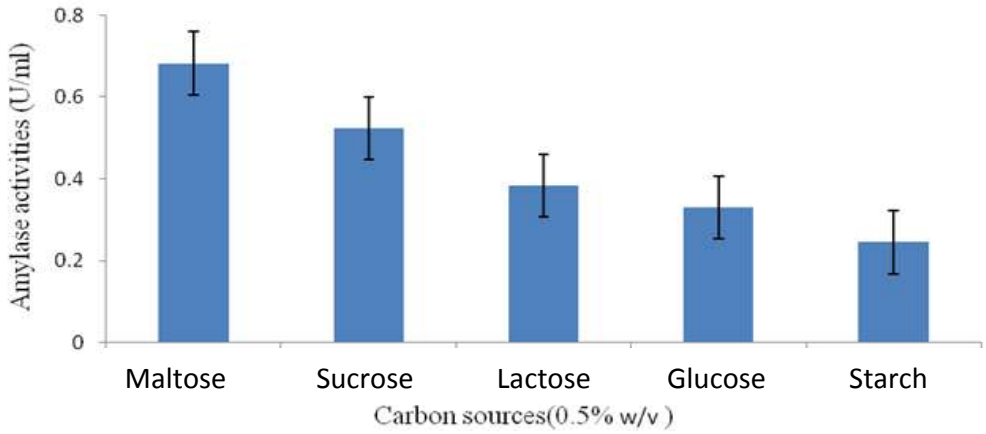

Figure 8. Effect of carbon sources on enzyme production at 28°C, pH 6 and 96 h of incubation.

increase or decrease in inoculum size affects alpha-amylase production; this was probably related with the limitation of nutrients and the growth activity of the isolate.

Effect of incubation period

Optimization of incubation period is an important parameter for maximum growth of the fungal isolates and thereby greatly affects enzyme production. Maximum yield of alpha amylase (0.281 U/ml) was observed on the 3rd day of incubation followed by 4, 6 and 5th days. The study revealed that as shown in Figure 7 the activity of the enzymes decreased as the incubation period increased but it decreased gradually from 3rd day with the increase in incubation period. This is probably due to the availability of desired nutrient and moisture in the substrate that contribute to the growth of the isolate. The result is similar to those reported by Shah et al. (2014) for *Aspergillus* spp.

Effect of carbon sources

The composition of media plays an important role in the production of enzymes. Growth and enzyme production of any organism are greatly influenced by both environmental conditions as well as the nutrients available in the growth medium (Singh et al., 2011). Carbon was one of the major elements in the medium composition for the metabolic activities of the isolate. As shown in Figure 8, maximum (0.684 U/ml) and least α-amylase production were observed during incorporation of maltose and starch, respectively as carbon sources. Similarly, Varalakshmi et al. (2009) reported maltose and on the contrary to this findings starch significantly increased the production of α-enzyme from *A. niger* JGI 24. Esfahanibolandbalaie et al. (2008) reported on the contrary to this findings that starch has substantial effect on the production α-amylase from *A. oryzae.* Varalakshmi et al. (2007) reported that maximum production of amylase was achieved from *Aspergillus* spp. JGI 12 when glucose was the carbon supplement.

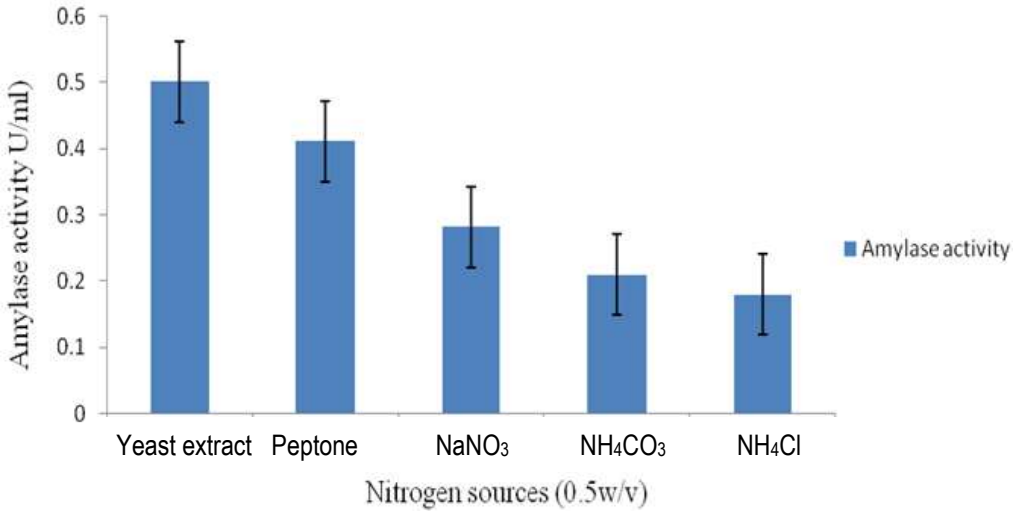

Figure 9. Effect of nitrogen sources on enzyme production at 28°C, pH 6 and 96 h of incubation.

Effect of nitrogen source

The production of α-amylase is enhanced using nitrogen source like peptone and yeast extract. As shown in Figure 9, the highest α-amylase production (0.501 U/ml) was attained with yeast extract but the least α-amylase production was observed with ammonium chloride. Similarly, Sharanappa et al. (2011) reported that optimum activities were realized using yeast extract and peptone as a nitrogen sources. However, Suganyadevi et al. (2012) reported in their investigation that *A. niger* under submerged fermentation showed the highest α-amylase production using ammonium nitrate and media supplemented with peptone showed maximum amylase activity. The maximum α-amylase production is shown in Figure 9 by the augmentation of yeast extract which is in line with those of Esfahanibolandbalaie et al. (2008) who reported that sound effects of α-amylase production by yeast extract from *A. oryzae* might be due to the presence of vitamin B group (promoting growth), amino acids and carbohydrate.

Conclusion and recommendations

The results suggest that the fungal isolate *A. niger* FAB-211 is a potential strain that can easily degrades starch. The effect of various process parameters on the enzyme activity was found to be significantly influenced by pH, temperature, inoculum size, incubation period, carbon, and nitrogen sources. Maximum amylase production during optimization processes was achieved at pH 6.0, 45°C and 4 days of incubation with 6 disc spore of *A. niger* FAB-211. Therefore, in the future, further optimization and characterization of *A. niger* FAB-211 amylase should be studied. This study showed that agro-soil wastes would be useful for the exploitation and screening of amylolytic potential of fungal isolates.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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