Global Advanced Research Journal of Microbiology (ISSN: 2315-5116) Vol. 6(4) pp. 021-029, June, 2017 Issue.
Available online http://garj.org/garjm

Full Length Research Paper

Optimization of Conditions for Protease Production from *Aspergillus niger* under Solid State Fermentation

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Accepted 04 April, 2017

The main objective of this study was to optimize the conditions for protease production by *Aspergillus niger* under solid state fermentation using locally available agricultural substrates. Fifty one fungal isolates were recovered from different sources. The isolates have shown typical *A. niger* characteristics and they were screened for the production of protease enzyme. Screening was done qualitatively using the Agar Plate Assay and the isolates that gave the widest hydrolysis zone diameters were quantitatively screened in submerged culture using the shake flask method. *A.niger* isolate that showed the highest production value in the shake flask assay was tested for protease production potential in solid state fermentation using five different agro-waste substrates moistened either with water or with a salt solution. The culture conditions that affect production capacity were then investigated. Results showed that 30 of the fungal isolates have shown typical *A.niger* characteristics. Of these, 16 have shown clear zones of casein hydrolysis on skimmed milk agar. The maximum activity of the produced protease (15.42U/ml) was recorded for isolate NAF6ii while the minimum (6.45U/ml) was recorded for isolate NAF7i. Therefore, *A.niger* isolate NAF6ii has been selected for production of protease by solid state fermentation using the five agro-materials as substrates. NAF6ii recorded the maximum protease activity (12.60U/ml) when Mesquite (*Prosopis juliflora*) fruit powder has been moisturized either with salt solution or with water (11.46U/ml). Results also showed that the optimum conditions for protease enzyme production were: 3.0% of spores' inoculum (16.80U/ml), pH 7.0 (18.19U/ml), 5.0% of NH_4NO_3 as a nitrogen source (13.28U/ml), 3.0% of fructose as a carbon source (11.38U/ml) during 96hrs incubation period. These results indicate the potentiality of this fungus as a source for protease production. In addition, the proteases produced from the treatment of local agro-waste could be used in a variety of industrial applications.

Keywords: Alkaline protease, Agro-waste, Fungi, *Prosopis juliflora*

INTRODUCTION

Proteases are enzymes that hydrolyze peptide bonds of protein into peptides and amino acids. It constitute one of the most important groups of industrial enzymes accounting for about 60% of the total worldwide enzyme market because of their various applications in many

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industries such as detergent, food, leather, silk, dairy and pharmaceutical industry (Tunga *et al*., 2003). The use of proteases has been increased remarkably in various industrial processes such as animal feed, cheese and food processing, and X-ray films (Gupta *et al*., 2002; Kumar *et al*., 2002). The proteases market has grown rapidly in the last few years and is estimated to reach a value of $2.21 billion in 2021 at a CAGR of 6% from 2016 to 2021 propelled by the pharmaceuticals segment which is the fastest growing application in the proteases market (Market Reports Hub, 2016). The total market for enzymes used for detergent purposes account for approximately $652.1 million in 2015 and $1.0 billion by 2020 (MicroMarket Monitor, 2016). Proteases have also an important role in baking, brewing and in the production of various oriental foods such as soy sauce, miso, meat tenderization and cheese manufacture (Kirk *et al*., 2002).

The sources of proteases are plants, animal tissues and microorganisms but due to the low production of proteases from the two former sources, they are mainly produced by microorganisms (Mukhtar and Haq, 2009). A variety of microorganisms such as bacteria, fungi, yeast and Actinomycetes are known to produce protease enzymes (Madan *et al.,* 2002). Molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are especially useful for producing proteases, as several species of these genera are generally regarded as safe (Sandhya *et al*., 2005; Devi *et al.,* 2008). Microbial proteases can be produced by both submerged (Sm) and solid-state fermentation (SSF) techniques. Among the various groups of microorganisms used in SSF, filamentous fungi are most widely exploited because of their ability to grow on complex solid substrates and production of wide range of extracellular enzymes (Lekha and Lonsane, 1994). SSF for fungal enzyme production has some advantages over SmF including simplicity, lower production costs, high enzyme yields and low wastewater output (Pandey*,* 2003; Wang *et al.,* 2005).

The use of natural products particularly agricultural residues, offers the advantage of combining the use of a cheap substrate and an interesting way of upgrading the value of these residues. Of the agro-industries by-products that have been commonly used as substrates are sugarcane bagasse, brans and straw from wheat and rice, sugar beet pulp and coffee pulp (Zheng and Shetty, 2000). Commercial wheat and rice brans have been found to be a suitable substrate for producing of mold proteases by SSF in many Asian countries (Sandhya *et al*., 2005). Other agro-industrial wastes that are used in enzymes production constitute spent grain, cocoa husk, chickpea bran, sawdust and other agricultural waste.

The aim of this study was to optimize the conditions for protease production by *Aspergillus niger* in solid state fermentation using locally available agricultural substrates.

MATERIALS AND METHODS

Isolation of *Aspergillus niger*

Fifty one *Aspergillus niger* isolates were recovered from different soil samples and other sources including air and air-contaminated dishes, plants' seeds, fruits, vegetables, and bread.

Isolation from soil was done by suspending 1.0 g of soil in 100 ml sterilized distilled water, shaken thoroughly and left to stand for one hour. A volume of 100µl of soil suspension was transferred to and spread onto the surface of Potato Dextrose Agar (PDA) medium in Petri plates. PDA medium was prepared by suspending 39.1 g of the medium in a liter of distilled water, boiled until completely dissolved and then autoclaved. The cooled medium was aseptically poured in sterile Petri dishes which were stored inverted at room temperature. Clean uncontaminated dishes were used for inoculation by samples. The inoculated Petri-plates were placed in an incubator at 28ºC for 4 – 6 days till culture development. Typical *A.niger* colonies which appeared in the incubated plates were repeatedly sub-cultured for purification, considered as presumptive *A. niger* and preserved onto PDA slants.

For isolation of *A. niger* from air, the sterilized plates were left open in the lab for few hours then covered and incubated as above.

Seeds and sliced bread and surface sterilized fruits and vegetables (1cm² pieces) showing symptoms of diseases were plated onto sterilized PDA in Petri dishes in three replicates. The plates were incubated at 26-30ºC for 4-6 days.

Identification of *Aspergillus niger* isolates

The presumptive *A.niger* isolates were identified by microscopical and cultural characteristics according to Onion *et al*. (1986). Characteristics of the isolates were determined by growing them on three differential media (Pitt, 1973) namely Czapek Yeast Extract Agar (CYA), Czapek Yeast Extract Agar with 20 % sucrose (CYA20S) and Malt extract Agar (MEA). Cultural and microscopical characteristics on each medium were recorded and compared to those given for *A. niger* by Moubasher (1993).

Screening of *A. niger* isolates for protease production

The isolates that showed typical *A.niger* characteristics were screened for protease production qualitatively using the agar plate method (Ellaiah *et al*., 2002) and

quantitatively in submerged culture using the shake flask method (Coral *et al*., 2003).

Agar Plate Assay

A.niger isolates were screened for protease production using skimmed milk agar (Ellaiah *et al*., 2002). Each isolate was inoculated in the center of a Petri plate containing a medium consisting of the following ingredients: KH_2PO_4, 1.0 g; KCl, 0.5 g; Mg $SO_4.7H_2O$; 0.2 g, glucose; 10.0 g, agar; 15.0 g, skimmed milk; 25.0 ml dissolved in a liter of distilled water. The inoculated plates were incubated at 30°C for 96 hrs, and then observed for the zone of clearance (Vermelho *et al*., 1996).

Shake flask method

Ten *A.niger* isolates that had the largest zones in the ager plate assay screening were selected and screened for protease production by submerged fermentation in 250 ml Erlenmeyer flasks containing the production medium, according to Coral *et al*. (2003) with slight lab modification. The medium contained the following ingredients (g/L): glucose; 5.0g, casein; 5.0g, $MgSO_4$.$7H_2O$; 0.2g, yeast extract; 5.0g, KH_2PO_4; 1.0g, NaCl; 0.5g dissolved in distilled water. The medium pH was adjusted to 8 and then each flask containing 50 ml of this medium was inoculated by 1.0 ml of the conidial spores' suspension of the isolate. The inoculated flasks were incubated at 30°C for 96 hrs in an orbital shaker operating at 170 rpm. Later the content of each flask was filtered using Whatman No.1 filter paper and the filtrate was used for enzyme extraction and assay.

Preparation of spore suspension

The conidial suspension was prepared by adding 10 ml of sterilized distilled water to a 3 – 5 days old slants culture of *A. niger* having profuse conidial growth on its surface. A sterile inoculation needle was gently used to break the conidial clumps and then the slant was shaken vigorously to make a homogenous suspension (Sharma, 1989) that has been poured in the flasks containing the production medium.

Extraction of crude enzyme

A volume of 50ml of 5%Tween80 in distilled water was added to each flask and these were shaken on a rotary shaker for one hour at 200 rpm. After that, the contents of the flasks were filtered using Whattman No. 44 filter papers, and the filtrates were used for enzyme assay.

Estimation of protease activity

To each enzyme extract (1ml each) in a test tube, 2.0 ml of 0.5% casein solution was added and the tubes were incubated at 35°C for 10 min. The residual protein was precipitated by adding 3.0ml of 10% ice trichloroacetic acid. The precipitates were allowed to settle for one hour and then centrifuged at 5000 rpm for 5 min. One milliliter of each supernatant was mixed with 5 ml of 1M sodium carbonate. After 20 min, 0.5ml of Folin and Ciocalteu's phenol reagent was added. The concentration of liberated tyrosine in filtrate was measured at 660 nm against a reagent blank using tyrosine standard (Lowry *et al*., 1951). Standard curve was prepared using following concentration range of tyrosine: 27.5, 55, 110, 220 and 275 µM. One protease unit was defined as the amount of enzyme required to releases 1 µM of tyrosine per minute per ml (Mohapatra *et al*., 2003). All the experiments were done in triplicates and mean values are presented. The enzyme activity (U/ml) was calculated by following formula:

$$\text{Enzyme activity (U/ml)} = \frac{\mu\text{mole tyrosine equivalent releases x total volume of assay}}{\text{volume of enzyme taken x incubation time x measured sample volume}}$$

Protease production by solid state fermentation

The *A.niger* isolate that showed the highest production value in the shake flask assay was selected to test its production capacity using the solid state fermentation assay (Lowry *et al*., 1951). Conditions that affect protease production by this technique were determined.

Effect of different substrates on protease production

Five different agro-waste substrates were used for solid state fermentation *viz*., wheat bran, Mesquite plant (*Prosopis juliflora*) fruit powder, sorghum flour, sensitive plant (*Mimosa pudica*) pods powder and groundnut fruit peel. Five grams of each substrate were weighed and hydrated with 10 ml of basal salt solution containing (g): NaCl; 0.5g, $MgSO_4.7H_2O$; 0.2g, KH_2PO_4; 1.0g, NH_4NO_3; 0.5g dissolved in 100ml of distilled water (Coral *et al*., 2003). Flasks containing fermentation medium were autoclaved, cooled and inoculated with 1.0ml of *A.niger* isolate spore suspension prepared as previously described, incubated for 96 hrs at 30°C. Later, 50 ml of distilled water was added to each flask and these were shaken on a rotary shaker for one hour at 150 rpm. After that the content of the flasks were filtered using Whatman No.44 filter paper and the filtrate was used for enzyme assay.

Effect of initial medium pH

The effect of initial pH on protease production was studied by changing the initial growth medium pH from 2-8 with 1N HCl/NaOH before sterilization at 121°C for 15 min.

Effect of incubation period

The culture medium was incubated for varying periods of time (24, 48, 72, 96 and 120hrs) to test the optimum time required for maximum enzyme production.

Effect of the inoculum size

The influence of the inoculum size on protease production was studied by testing the production capacity when each of 0.5, 1.0, 1.5, 2.0, 2.0, 3.0, 4.0, 5.0 and 6.0 ml of the inoculum was added, separately, to 100ml of the fermentation medium. Protease production in cell free supernatant was determined as described previously.

Effect of supplementary nitrogen sources

Whether the addition of supplementary nitrogen sources could enhance the production of protease was tested by supplying an organic nitrogen source (yeast extract, peptone water or urea) or an inorganic nitrogen source ($NaNO_3$ or NH_4NO_3) at a level of 1% w/w in the medium. The flasks were then incubated for 3 days at room temperature. At the end of the incubation period, protease production in cell free supernatant was determined. Further, the best carbon source was optimized with different concentrations [0.5, 1.0, 2.0, 3.0, 4.0 and 5.0% (w/v)].

Effect of carbon sources

The effect of carbon sources on enzyme production was investigated by supplementing the basal salt solution, pH 7, with 0.1% (w/v) of different carbon sources, *viz*., glucose, maltose, fructose, starch and sucrose. The flasks were then incubated for 3 days at room temperature. At the end of the incubation period, protease production in cell free supernatant was determined. Further, the best carbon source was optimized with various concentration range of: 0.5, 1.0, 2.0, 3.0, 4.0 and 5.0% (w/v).

RESULTS AND DISCUSSION

Isolation and identification of *Aspergillus niger*

Fifty one fungal isolates were recovered from soil samples and other sources. These isolates were given code numbers prefixed with NAF. Thirty of these isolates have shown morphological and microscopical characteristics typical to those given for *Aspergillus niger* by Moubasher (1993). Cultural characteristics of the presumptive *A.niger* isolates were studied on three different media according to the guidelines given by Pitt (1973). Growth and development of *A.niger* on these differentiation media was resulted as fellow: On CYA, colonies were floccose with a diameter of 52 – 55mm; stipes vary in length; mycelium white to dull yellow; conidial area dark to black; reverse yellow. On CY20S, colonies diameter were 58 – 75 mm in diameter; obverse black; stipes vary in length; conidia dark to black; mycelium white to dull yellow; reverse yellow to brown. On MEA, colonies were granular to floccose with a diameter of 40 – 48 mm; conidial area black; mycelium white; reverse colorless. The use of these three differential media was a simple and reliable method for identification of *Aspergillus* species. The obtained results are similar to the descriptions given for *Aspergillus niger* by various investigators (Webster, 1980; Moubasher, 1993).

Screening of *Aspergillus niger* **isolates for protease production**

Agar Plate Assay

The thirty *A. niger* isolates were screened for protease production using plate assay; the clear zones around the colonies were measured. Sixteen isolates (53.3%) have shown clear zones of casein hydrolysis, with different zone diameters on skimmed milk agar. Casein or skimmed milk agar plate assays allow principally for qualitative determinations of protease activity, the hydrolysis zone produced on the agar could be related to the amount of protease produced by the fungus (Vermelho *et al*., 1996; Benazir *et al*., 2011; Gnanadoss *et al*., 2011). Therefore, isolates that gave the widest hydrolysis zone diameters (n=8) were selected for further screening using shake flask method.

Shake flask method

Results of protease production screening by submerged fermentation are presented in Figure. 1. The maximum activity of the produced protease (15.42U/ml) was recorded for isolate NAF6ii followed by isolate NAF7m (10.14U/ml), while the minimum activity (6.45U/ml) was recorded for isolate NAF7i. Therefore, *A.niger* isolate NAF6ii had been selected for production of protease by solid state fermentation using five agro-materials as substrates.

Protease p**roduction by solid state fermentation**

The production of proteases by *A.niger* NAF6ii was investigated using five different agro materials that have been moistened either with water or with a salt solution

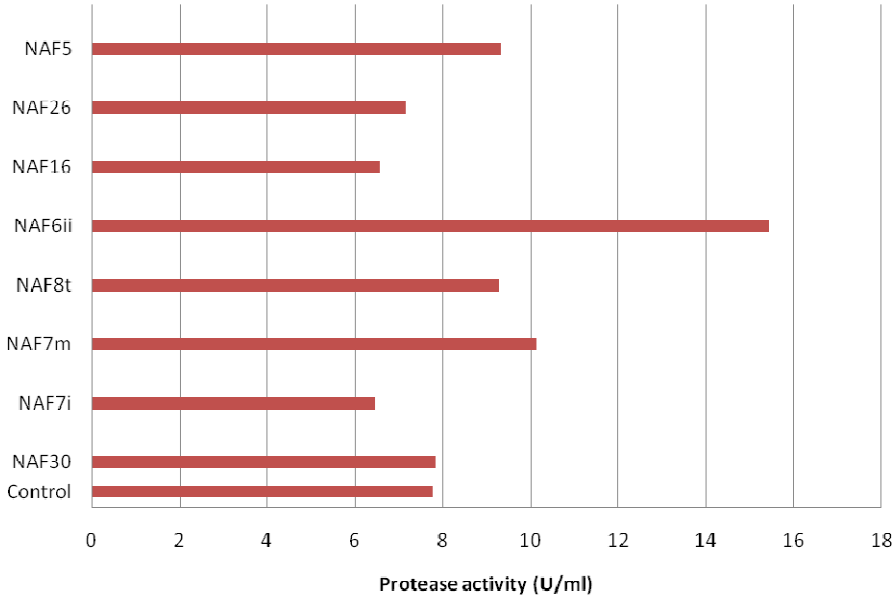

Figure 1: Protease production by *A. niger* isolate using shake flask fermentation

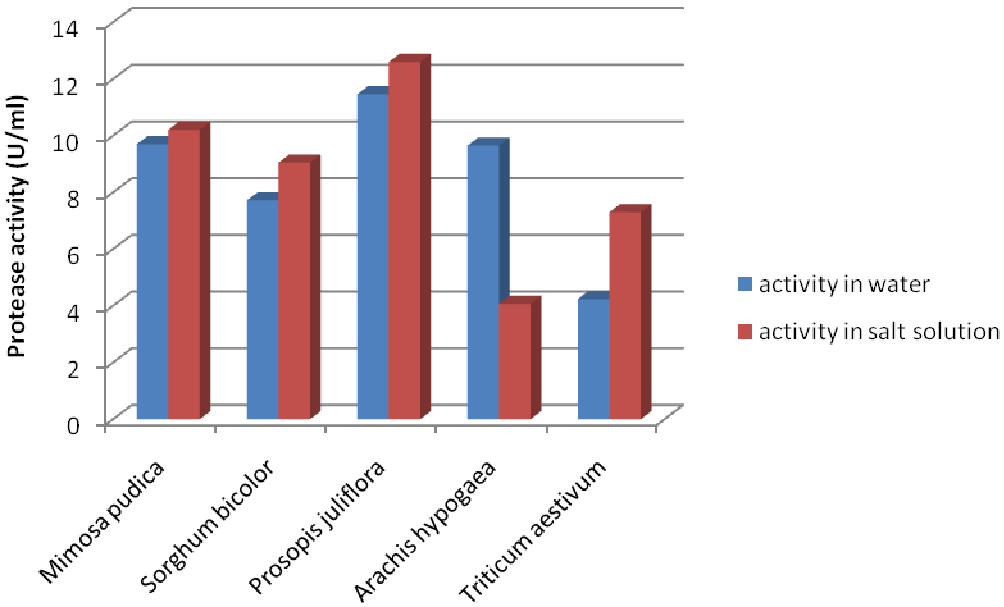

Figure 2: Protease production by *A.niger* isolate using different substrates moistened with water and salt solution

(Figure. 2). With the exception of the case of groundnut fruit peel, the activity of the produced enzymes was higher in salt solution than in water. Results showed that NAF6ii recorded the maximum protease activity when mesquite fruit powder moisturized with water (11.46U/ml) and with salt solution (12.60U/ml). Mulimani and Patil (1999) used different substrates such as Soybean meal, Cotton seed meal, Sunflower meal for the production of protease using *Aspergillus flavus*. Of all these substrates examined, cotton seed meal gave maximum enzyme activity.

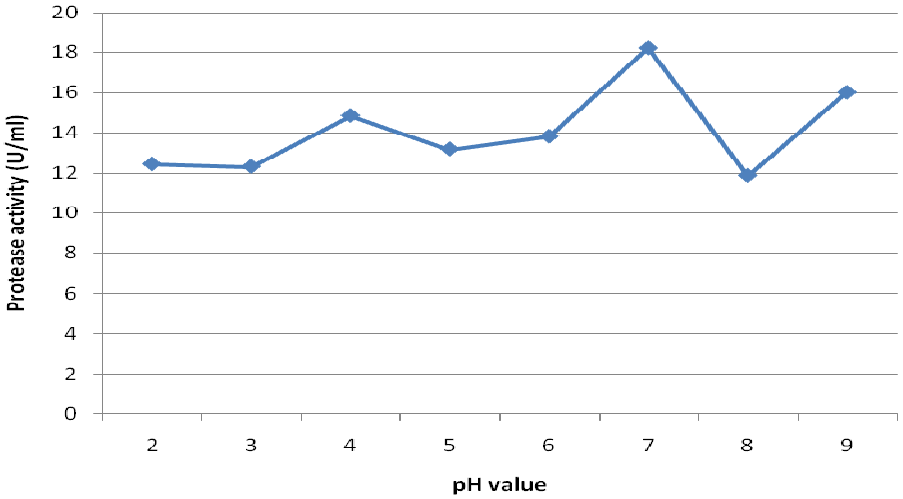

Figure 3: Effect of different pH values on protease production by NAF6ii

Optimization of fermentation conditions for protease production by NAF6ii

Different culture conditions that may affect production of protease from *A.niger* NAF6ii isolate were optimized as follows:

The inoculum size

The effect of spores' inoculum size on the production of protease by *A. niger* NAF6ii was determined using different inoculum volumes. Results indicated that the maximum enzyme activity (16.80U/ml) was produced when 3.0% of the spores' inoculum was added to the fermentation medium. An appropriate inoculum size is essential for optimum growth and enzyme production by the microorganism. Highest yield at certain inoculum size lies in the fact that a sufficient quantity of mycelium was formed, which produced optimum level growth of the mould and subsequently maximum enzyme production. As the amount of mycelium was increased, it rapidly consumed majority of the substrate for growth purposes, hence enzyme synthesis was decreased (Carlile *et al*., 2001).

Optimum pH

Production of the enzymes by mould culture mostly depends on the medium pH. Therefore, the effect of different pH values on the production of protease by *A. niger* NAF6ii was studied. Production of the protease has increased with the increment in pH value, reaching the maximum (18.19U/ml) at pH 7.0 (Figure. 3). This result indicates that *A. niger* NAF6ii was capable of producing alkaline protease. Similar finding was also reported by Dubey *et al*. (2010). Generally, protease production by microorganism depends on the extracellular pH because culture pH strongly influences many enzymatic processes and transport of various components across the cell membranes, which in turn support the cell growth and product production (Paranthaman *et al*., 2009). Changes in the pH may also cause denaturation of enzyme resulting in loss of catalytic activity. It may also cause change in the ionic state of substrate which may result in the formation of charged particles which may not correspond with the ionic active sites of enzyme. Thus enzyme substrate complex will not be formed and the substrate will become unavailable to the microorganism (Karuna and Ayyanna, 1993).

Effect of different nitrogen sources

The effect of different organic and inorganic nitrogen sources on the protease production by NAF6ii isolate was also tested. Results (Figure. 4) showed that ammonium nitrate was the best nitrogen source giving the maximum enzyme activity (13.28U/ml). Among the various organic and inorganic nitrogen sources, the maximum enzyme activity was obtained with ammonium nitrate followed by ammonium, ammonium citrate and potassium nitrate (Nascimento and Martins, 2004). Some other inorganic nitrogen sources that gave better enzyme production were reported by many investigators. Shumi *et al*. (2004) have reported that *Aspergillus funiculosus* produced maximum protease when supplemented with potassium nitrate. The best nitrogen source for protease production recorded by El-Safey and Abdul-Raouf (2004) was $(NH_4)_2SO_4$. In contrast, Mukhtar and Haq (2009) reported that peptone

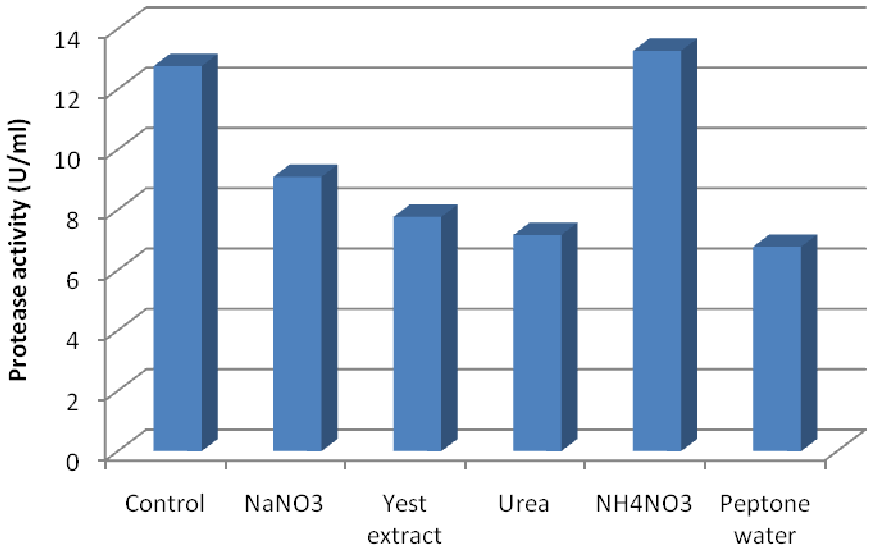

Figure 4: Protease activity in different nitrogen sources

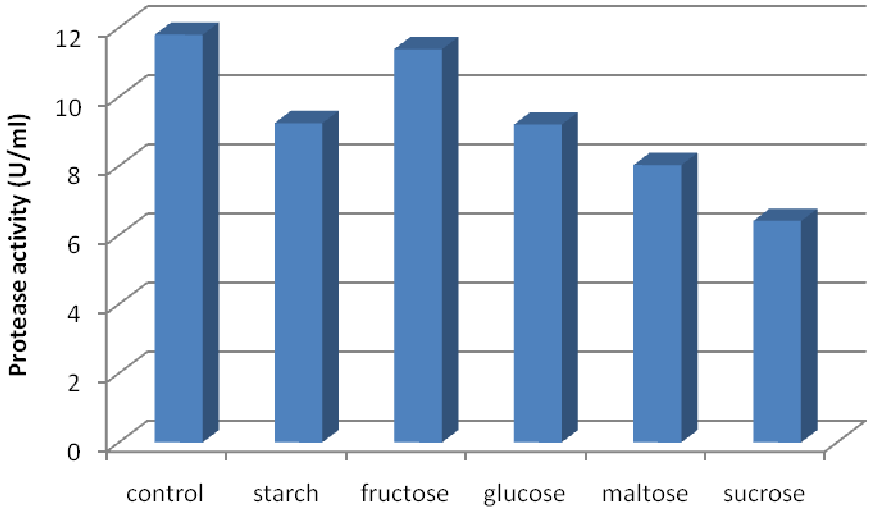

Figure 5: Protease activity in different carbon sources

was the best nitrogen source for protease production by *Rhizopus oligosporous*.

Effect of different concentrations of the nitrogen source

The effect of the concentration of NH_4NO_3 on the protease production was studied using NH_4NO_3 in the range of 0.05-5.0%. A gradual increase in protease activity was observed with the increment of NH_4NO_3 concentration.

Effect of different carbon sources

Results in Figure. 5 indicate that the highest protease activity (11.38U/ml) was obtained when fructose was used as a carbon source while the least protease activity (6.41U/ml) was produced when sucrose was used. These results are in accordance with those reported by Sutar *et al*. (1992) who found that fructose acted as a best carbon source for the production of alkaline protease from

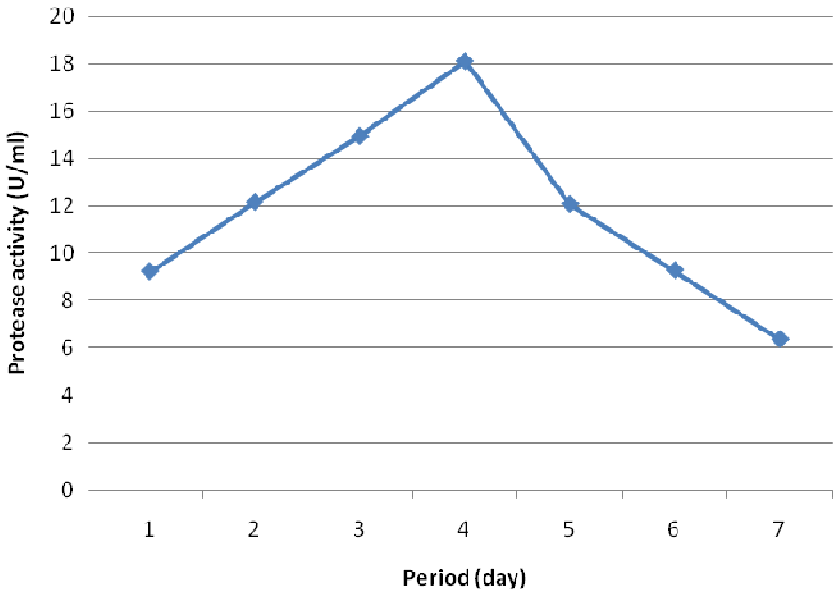

Figure 6: Effect of incubation period on protease production by NAF6ii

Conidiobolus coronoatus. It has also been reported that the absence of a proper carbohydrate (C-source) in the production medium results in a dramatic decrease in enzyme production (Gajju *et al*., 1996). In contrast, Madzak *et al*. (2000) recorded that sucrose was a good substrate for production of extracellular proteases. Glucose was found to be the optimum carbon source for protease activity by four *Bacillus* isolates followed by sucrose, fructose, maltose, starch and cellulose (Boominadhan and Rajakumar, 2009). However, other investigators reported that glucose has drastically inhibited protease production (Fukushima *et al*., 1989; Puri *et al*., 2002) while El-Hadj-Ali *et al*. (2007) has reported an opposite result. In a study by Johnvesly and Naik (2001) starch caused high level of enzyme expression in *Bacillus* species while Shafee *et al*. (2005) reported that maltose, starch and cellobiose caused low protease production.

Effect of the carbon source concentration

The highest protease activity (12.69U/ml) was recorded in 3% fructose and the lowest activity (8.83U/mg) was recorded in 5% fructose.

Effect of the incubation period

The effect of the incubation period on proteases production was tested during 7 days; results are shown in Figure. 6. As clear in this figure, the production activity was increasing gradually during the first four days (96hrs) then a sharp decrease was observed in day 7.

CONCLUSION

In this study, the conditions for protease production by *A.niger* in solid state fermentation medium were optimized. *A.niger* may be considered as a potential source of protease enzyme using Mesquite pods as a promising substrate under solid state fermentation conditions. The optimum conditions for protease production were: 3.0% of spores' inoculum, pH 7.0, 5% of NH_4NO_3 as a nitrogen source, 3% of fructose as a carbon source during 96hrs incubation period. Efforts are needed to isolate more *A.niger* isolates and to screen them for protease production.

REFERENCES

Benazir JF, Suganthi R, Hari A, Kumar V, Aswathi MP, Niraimathi G, Mala MK, Sukanya S, Santhi R (2011). Bio utilization of agro industrial waste in solid state fermentation by *Aspergillus niger* for the production of Protease. Asian J. Biotechnol. Resour. 2, 422-435.

Boominadhan U, Rajakumar R (2009). Optimization of protease enzyme production using *Bacillus* sp. isolated from different wastes. Bot. Res. Int. 2, 83-87.

Carlile MJ, Watkinson SC, Goody GW (2001). The fungi, 2nd Edn. Academic Press, London, Pp.475-476.

Coral G, Arikan B, Ünaldi MN, GÜvenmez H (2003). Thermostable alkaline protease produced by an *Aspergillus niger* strain. Ann. Microbiol. 53(4), 491 – 498.

Devi MK, Banu AR, Gnanaprabhal GR, Pradeep BV, Palaniswamy M (2008). Purification, characterization of alkaline protease enzyme from native isolate *Aspergillus niger* and its compatibility with commercial detergents. Indian J. Sci. Technol. 1(7), 1-6.

Dubey R, Adhikary S, Kumar J, Sinha N (2010). Isolation, production, purification, assay and characterization of alkaline protease enzyme from *Aspergillus niger* and its compatibility with commercial detergents. Dev. Microbiol. Mol. Biol. 1(1), 75-94.

El-Hadj-Ali N, Agrebi R, Ghorbel-Frikha B, Sellami-Kamoun AS, Kanoun Nasri M (2007). Biochemical and molecular characterization of a detergent stable alkaline serine-protease from a newly isolated *Bacillus licheniformis* NH1. Enzyme Microb. Technol. 40, 515-523.

Ellaiah P, Srinivasulu B, Adinarayana K (2002). A review on microbial alkaline proteases. J. Sci. Ind. Res. 61, 690 – 704.

El-Safey EM, Abdul-Raouf UM (2004). Production, Purification and Characterization of Protease enzyme from *Bacillus subtilis*. International Conferences for Development and the Environment in the Arab World, Assiut University, Egypt.

Fukushima Y, Itoh H, Fukasa T, Motai H (1989). Continuous protease production in a carbon-limited chemostat culture by salt tolerant *Aspergillus oryzae*. Appl. Microbiol. Biotechnol. 30, 604-608.

Gajju H, Bhalla TC, Agarwal HO (1996). Thermostable alkaline protease from thermophilic *Bacillus coagulans* PB-77. Indian J. Microbiol. 36, 153-155.

Gnanadoss, J.J., Robert, R. and Jebapriya, R.G., 2011. Production of protease from *Aspergillus niger* and *Mucor mucedo* under submerged and solid state fermentation. Int. J. Cur. Res. 3, 75-78.

Gupta R, Beg QK, Khan S, Chauhan B (2002). An overview on fermentation, downstream processing and properties of microbial alkaline proteases. Appl. Microbiol. Biotechnol. 60, 381-395.

Johnvesly B, Naik GR (2001). Studies on production of novel thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus* sp. JB99 in a chemically defined medium. Process Biochem. 37, 139-144.

Karuna J, Ayyanna C (1993). Production of semi-alkaline protease enzyme from *Aspergillus* spp. Proceedings of the Ninth National Convention of Chemical Engineers and International Symposium on Importance of Biotechnology in Coming Decades, Viskhapatnam India.

Kumar A, Sachdev A, Balasubramanyam SD, Saxena AK, Lata A (2002). Optimization of conditions for production of neutral and alkaline protease from species of *Bacillus* and *Pseudomonas*. Indian J. Microbiol. 42, 233–236.

Lekha PK, Lonsane BK (1994). Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL104 in solid state, liquid surface and submerged fermentation. Proc. Biochem. 29, 497–503.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951). Protein measurement with the folin phenol reagent. J. Biol. Chem. 193, 265-275.

Madan M, Dhillon S, Singh R (2002). Production of alkaline protease by a UV mutant of *Bacillus polymyxa*. Indian J. Microbiol. 42, 155-159.

Madzak C, Treton B, Blanchin-Roland S (2000). Strong hybrid promoters and integrative expression/secretion vectors for quasi-constitutive expression of heterologous proteins in the yeast *Yarrowia lipolytic*a. J. Mol. Microbiol. Biotechnol. 2(2), 207-216.

Market Reports Hub (2016). Proteases Market (Microbial, Animal, Plant) to Hit 6% CAGR to 2021 Led by Pharmaceuticals Sector. Market Reports Hub, PUNE, India, June 17, 2016. Available online from: http://www.kctv5.com/story/32243523/proteases-market-microbial-animal-plant-to-hit-6-cagr-to-2021-led-by-pharmaceuticals-sector#ixzz4Bp7PQMzV.

Micro Market Monitor (2016). Global Protease Market Research Report. available online from: http://www.micromarketmonitor.com/market-report/protease-reports-4007197055.html.

Mohapatra BR, Bapuji M, Sree A (2003). Production of industrial enzymes (amylase, carboxymethylcellulase and protease) by bacteria isolated from marine sedentary organisms. Acta. Biotechnol. 23, 75-84.

Moubasher AH (1993). Soil Fungi in Qatar and other Arab Countries. Doha Modern Printing Press 1st edn., 566 pp. Doha, Qatar.

Mukhtar H, Haq I (2009). Production of acid protease by *Aspergillus niger* using solid state fermentation. Pakistan J. Zool. 41(4), 253 – 260.

Mulimani VH, Patil GN (1999). Production of proteases by *Aspergillus flavus* under solid state fermentation. Indian J. Exp. Biol. 37, 1248-1250.

Nascimento WCA, Martins MLL (2004). Production and properties of an extracellular protease from thermophilic *Bacillus* sp. Brazilian J. Microbiol. 35, 91-96.

Onion A, Allosopp D, Eggins OW (1986). Smith Introduction to Industrial Mycology. 7th ed., Edward Arnold publisher, London, Pp, 187 – 188.

Pandey A (2003). Solid-state fermentation. J. Biochem. Eng. 13, 81–84.

Paranthaman R, Alagusundaram K, Indhumathi J (2009). Production of protease from rice mill wastes by *Aspergillus niger* in solid state fermentation. World J. Agric. Sci. 5, 308-312.

Pitt JI (1973). An appraisal identification method for *Penicillium* species: Novel taxonomic criteria based on temperature and water relation. Mycologyia 65, 1135 – 1157.

Puri S, Beg QK, Gupta R (2002). Optimization of alkaline protease production from *Bacillus* sp. using response surface methodology. Curr. Microbiol. 44, 286-290.

Sandhya C, Sumantha A, Szakacs G, Pandey A (2005). Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid-state fermentation. Biochem. Proc.40, 2689–2694.

Shafee N, Aris SN, Rahman RNZA, Basri M, Salleh AB (2005). Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium *Bacillus cereus* strain 146. J. Sci. Res. 1, 1-8.

Sharma PD (1989). Methods in Microbiology, in: Microbiology and Plant Pathology, 1st Edn., Rastogi Company Meerut, India, Pp. 33 – 35.

Shumi W, Hussain MdT, Anwar MN (2004). Isolation and purification of fungus *Aspergillus funiculosus* and its enzyme protease. Pakistan J. Biol. Sci. 7, 312–317.

Sutar M, Sriniva MC, Vartak HG (1992). Production of an alkaline proteinase from *Conidiobolus coronatus*. World J. Microbiol. Biotech. 8, 254-258.

Tunga R, Shrivasthava B, Banerjee R (2003). Purification and characterization of a protease from solid state cultures of *Aspergillus parasiticus*. Proc. Biochem. 38, 1553-1558.

Vermelho AB, Meirelles MNL, Lopes A, Petinate SDG, Chaia AA, Branquinha MH (1996). Detection of extracellular proteases from microorganisms on agar plates. Mem. Inst. Oswaldo. Cruz. 91, 755 – 760.

Wang R, Law RCS, Webb C (2005). Protease production and conidiation by *Aspergillus oryzae* in flour fermentation. Proc. Biochem. 40, 217–227.

Webster J (1980). Introduction to fungi, 2nd Edn., Cambridge University Press, Cambridge, London, New York, Rochel Melbourne Sydney. Pp: 140-148.

Zheng Z, Shetty K (2000). Solid state production of polygalacturonase by *Lentinus edodes* using fruit processing wastes. Proc. Biochem. 35, 825-830.