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Production and optimization of acid protease by *Aspergillus spp* under submerged fermentation

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ABSTRACT

Proteases are the most important industrial enzymes and comprise about 25% of commercial enzymes in the world. Two third of the industrially produced proteases are from microbial sources. The proteolytic fungi Aspergillus spp was isolated from soil contaminated with abattoir waste and screened on skim milk agar medium for proteolytic activity. The enzyme was optimized supplementation of cheese whey and other favorable conditions like pH, temperature and incubation time to the medium. The optimized conditions for enzyme production were 5.0, 32 ± 2^0C and 5 days intervals respectively. The fermentation medium was supplemented with different nitrogen and carbon sources to improve the enzyme production. Among the sources tested in the present study, soybean meal (1%) was proved the best nitrogen source whereas glucose and rice flour were shown at 1.5% and 1% for enzyme production by Aspergillus spp respectively.

Key Words: Acid Protease, Submerged fermentation, *Aspergillus spp.*

INTRODUCTION

Proteases are the most important class of industrial enzymes and comprise about 25% of commercial enzymes in the world [1]. Proteases are classified as acid, neutral and alkaline proteases. These enzymes are widely using in dairy industry as milk clotting agent and meat tenderizing agent in food industry. Reduction of tissue inflammation (clinical and medical) application [2]. Proteases are mainly produced by submerged fermentation; the microorganisms and the substrate are present in the submerged state in the liquid medium, where a large quantity in the form of solvent is present. Since the contents are in submerged state in the liquid medium, the transfer of heat and mass is more efficient, and is amenable for modeling the process [3].

A variety of microorganisms such as bacteria, fungi, yeast and Actinomycetes are known to produce these enzymes [4]. Molds of the genera *Aspergillus*, *Penicillium* and *Rhizopus* are potent strains for proteases, as several species of theses genera are generally regarded as safe [5].

Molasses is an interesting raw material, it is rich in nutrients and minerals, cheap in price as well as it is present in plenty hence a by-product of sugar industry [6]. Only few reports are available on the protease production by microorganisms from molasses. In the present study, the potentiality of molasses as a main substrate and its combinations with cheese whey and other optimal conditions for fermentation medium to produce extra cellular protease by *Aspergillus* spp isolated from soil contaminated with abattoir waste.

MATERIALS AND METHODS

Collection of sample

Soil samples were collected from the sites contaminated with abattoir waste from rural areas of Tirupati, A.P, and India.

Isolation and screening of proteolytic fungi

The fungal culture was isolated from soil contaminated with abattoir waste on potato, dextrose and agar medium by serial dilution method. The isolated fungal culture was identified as *Aspergillus spp* based on its morphological and microscopic characteristics and these values matched with values in standard reference book compendium of soil fungi [7]. Further the culture was screened on skimmed milk agar medium for protease production.

Inoculum preparation

The fungal inoculum was prepared with addition of 10ml of sterile distilled water to the 7^{th} day slant and was shaken well to obtain homogeneous spore suspension.

Fermentation

Fermentation was carried out in 250ml Erlenmeyer flask containing 50 ml of sterilized molasses. The medium was cooled to room temperature and was inoculated with 2ml of fungal spore suspension. The flasks after inoculation were placed in the orbital shaker rotating at 150 rpm and at 32^0C for 7 days. Later, the contents of the flasks were filtered using Whatman No.1 filter paper and the filtrate was used for the assay of protease enzyme by modified Anson's method [3].

Mycelia dry weight

For this purpose, the fermented broth was filtered using preweighed Whatman 1 filter paper. It was washed with water thrice and then dried at 105^0C over night in a hot air oven and weighed [8].

To improve the production medium for higher protease activity the experiment was conducted by various combinations of cheese whey and molasses as main cheap sources.

Effect of temperature Enzyme

Enzyme production is mainly influenced by temperature. In the present study, the experiment was performed at various temperatures like 25 ± 2^0C, 32 ± 2^0C and 55 ± 2^0C.

Time of Incubation

The time of incubation was determined by carrying out the fermentation for 3, 4, 5, 6 and 7 days with medium consisting of equal volumes of molasses and cheese whey with initial pH of 6.2 at temperature of 32^0C.

Effect of pH
To determine the impact of pH the medium was prepared by using 1:1 ratio of molasses and whey with different pH ranging from 2 to 9.

Effect of nitrogen source on protease production
The production medium (Basal medium) supplemented with various nitrogen sources like potassium nitrate, sodium nitrate, ammonium nitrate, casein, beef extract, peptone, yeast extract and soya bean meal at 1% were added to molasses and cheese whey medium. For the optimization of production medium the experiment was carried out with different concentrations of best source obtained from this experiment, ranging from 0.5 to 6 percentages.

Effect of carbon source on protease production
The effect of various carbon sources like glucose, mannitol, sucrose, lactose, cellulose and starch on protease production.

Effect of cereals on acid protease production
The medium was amended with 1 % of different flours of cereal like rice, wheat, oat, corn, millet, black gram, green gram for higher acid protease production by *Aspergillus* spp.

RESULTS AND DISCUSSION

The fungal culture *Aspergillus* spp isolated from the soil contaminated with abattoir waste was able to produce protease enzyme with 0.277U ml^{-1} in cane molasses as fermentation medium (Fig 1). The sugar industry molasses can be used as production medium for protease production [6].

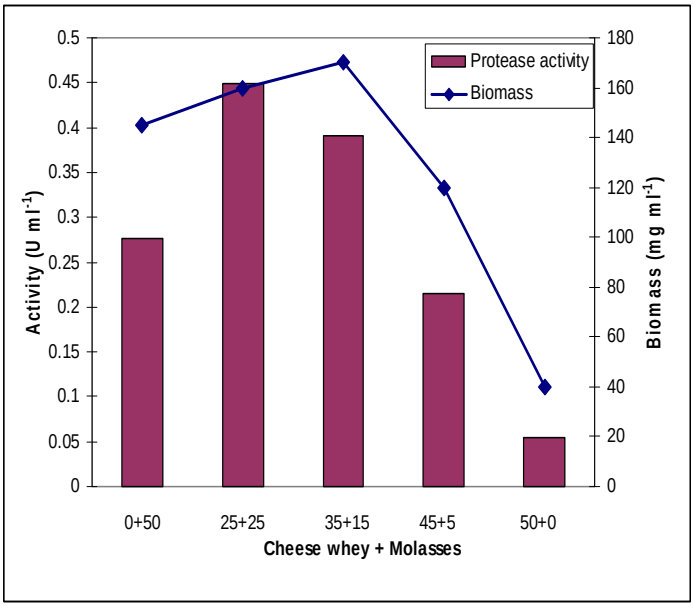

Figure 1: Protease Production from the various combinations of cheese whey and molasses
**Values represented in the figure are mean of two replicates*

Protease production from cheese whey and molasses
To increase the protease production, the fermentation was carried out by using various combinations of molasses and cheese whey and the results were depicted in fig.1. From the observations it was noticed that cheese whey can be used as a nitrogen source for protease

production. Similar observation was made R.C.S.Thys *et al*., 2005 [9] and Francisco *et al*. (2008) [10] used cheese whey for production of protease by *Serratia marcescens*. Usama F.Ali (2008) [11] reported that protease production by *Aspergillus terreus* improved by using whey with some agro-industrial by-products.

Supplementation of each 25 ml of whey and molasses to the medium improved the protease activity by *Aspergillus spp* with 0.4481 U ml^{-1} from 0.2769 U ml^{-1}. A combination of 2.5 parts of whey and 1 part of molasses was better than molasses and produced higher amount of fungal biomass. But least amount of protease enzyme was produced by fungal culture grown in the fermentation medium contains only cheese whey as main source.

Effect of cultural conditions on protease production

The effect of inoculums size on protease production was studied and listed in fig.2. The fermentation medium was inoculated with 2%, 4% and 6% spore inoculum. The optimum inoculums was found to be 4% for protease production for *Aspergillus* sp.

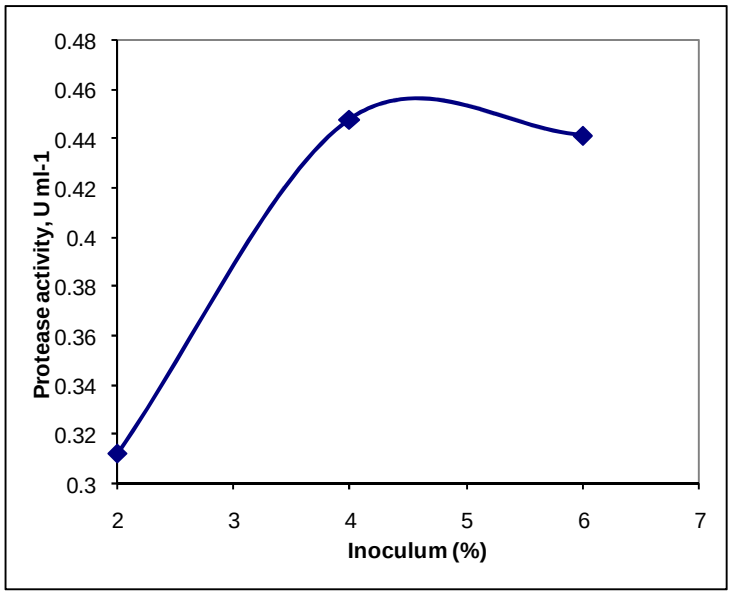

Fig. 2 Effect of inoculum on protease production in submerged fermentation
**Values represented in the figure are mean of two replicates*

Table 1: Protease production at different temperatures by *Aspergillus* spp.

Temperature, ^{0}C	Protease activity	Fungal dry weight
25±2	0.3350	32.25
32±2	0.4481	56.40
55±2	0.2315	25.24

The effect of temperature on protease production by fungal culture studied and shown in table.1. Various temperatures tested in the present study, the optimum temperature is 32±2 ^{0}C for enzyme production. Similarly Ganesh kumar *et al*. (2008) [12] reported the optimum temperature for protease production for by was in mesophilic fungi *Synergistes* species at 35^{0}C.

The productivity of the enzyme from fungal culture is dependent on medium pH [8]. Therefore, the effect of initial pH (3.0 – 9.0) for enzyme production was studied and listed in fig 3.

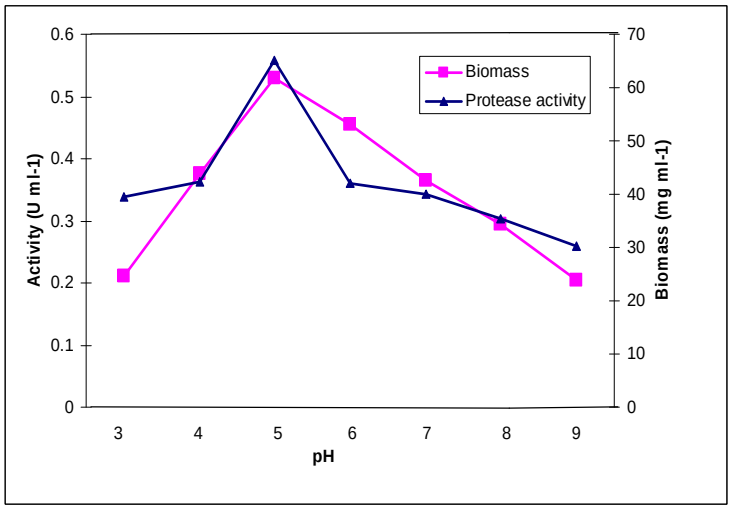

Fig. 3 Effect of different pH on protease production
**Values represented in the figure are mean of two replicates*

In the present study the protease activity was gradually increased from pH 3.0 to 5.0 and was declined at neutral and alkaline pH (fig.2). The pH of fermentation broth after 120h of incubation was measured as 2.25. Fungal acid proteases have an optimal pH range from 4 – 4.5 and they can be stable at pH values from 2.5 – 6.0 [13]. Maximum production of enzyme and fungal dry mass were observed at pH of 5.0. Similarly Ganesh Kumar *et al*. (2008) [12] reported that the optimum pH was in the acidic range of 5.5- 6.5 for acid protease production from solid waste from tannery by *Synergistes* species.

Effect of different nitrogen sources on protease production

The impact of different inorganic and organic nitrogen sources on biomass and protease production were shown in figure 3. The maximum protease activity was 2.307 U ml^{-1} with potassium nitrate followed sodium nitrate used in the present study than the control.

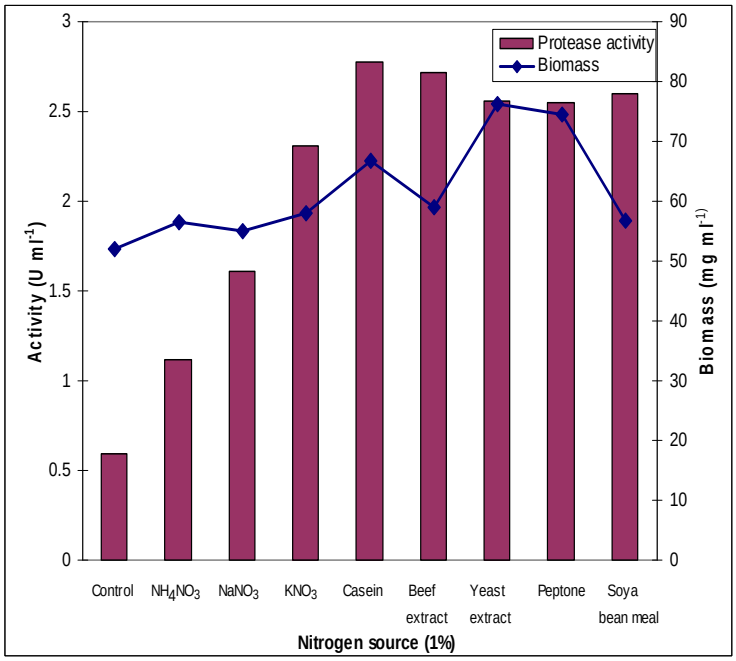

Fig. 4: Effect of different nitrogen sources on protease production
**Values represented in the figure are mean of two replicates*

In this study, the organic nitrogen sources were shown the proteiolytic activity at the ranges of 2.4-2.8 U/ml by *Aspergillus spp*. Medium supplemented with organic nitrogen sources supported higher protease production when compared to inorganic nitrogen sources. Similar reports were made by Narayana *et al*. (2008) [1] the effect of nitrogen sources like peptone, beef extract, casein, yeast extract, tryptone, $NaNO_3$, KNO3 and NH_4Cl on production of protease by *Streptomycin albidoflavus*. According to their studies organic nitrogen sources were better than inorganic nitrogen sources. Mussarat Shaheen *et al*. (2008) [14] also reported that soyabean and casein proved as the best nitrogen sources for protease production. Shirish *et al*. (2009) [3] reported that the enzyme synthesis was maximum when soybean oil seed cakes was used as substrate followed by sesame oilseed cake in solid state fermentation by *Aspergillus* spp. On the economic basis soya bean meal was selected as nitrogen source for further study.

Effect of various concentration of soy bean meal on protease production

It was further desired to find optimum levels of soyabean meal in the production medium for higher protease activity of fungal culture. For this purpose the fermentation was conducted with the soy bean meal concentration range of 0.5 to 6 percentages and the results were depicted in fig.4).

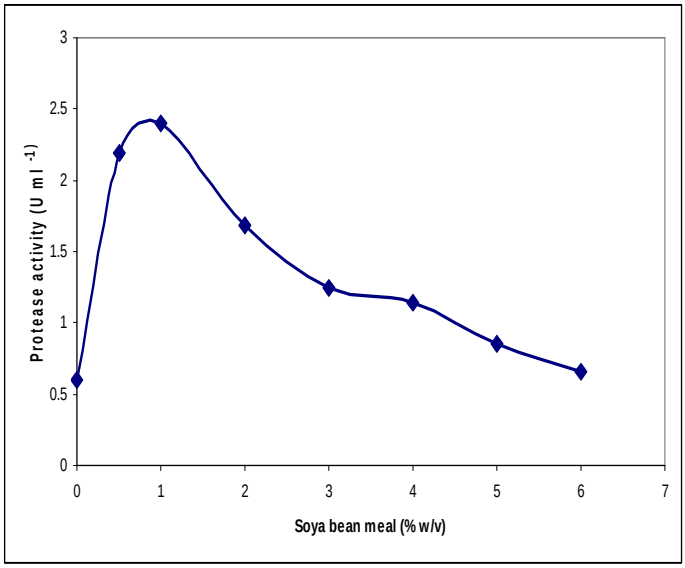

Fig. 5 Effect of concentration of soya bean meal on protease production

**Values represented in the figure are mean of two replicates*

Higher protease activity with 2.599 Uml^{-1} was obtained by fungal culture grown in medium at 1% soyabean meal as a substrate. There was gradual decrease in protease activity from 2% soy bean meal concentration to 6%. From this study it was cleared that the soya bean meal concentration greater than 1% could inhibit the growth of *Aspergillus* spp in submerged medium.

Effect of different carbon sources on protease production

The impact of different carbon sources on the protease production was tested and listed in fig. 6. In this study glucose yielded higher titers of protease activity of 2.818 U ml^{-1} when compared to mannitol. But the impact of sucrose on protease was lower than that of lactose. Among monosaccharides, disaccharides and polysaccharides used in the study, the monosaccharide improved the proteolytic activity. Sinha *et al*. (2009) [3] compared the effect of carbon sources such as glucose for protease production, among these lactose induced the protease production with 0.118 U ml^{-1} on 4^{th} day of fermentation. In the present study It was observed that the medium supplemented with 1% starch favored the biomass growth rather than protease production by the fungal culture. Similarly Narayana *et al*. (2008) [1] was reported that carbon

sources like glucose, maltose, starch were indispensable components for protease production by *S.albidoflavus* in submerged fermentation.

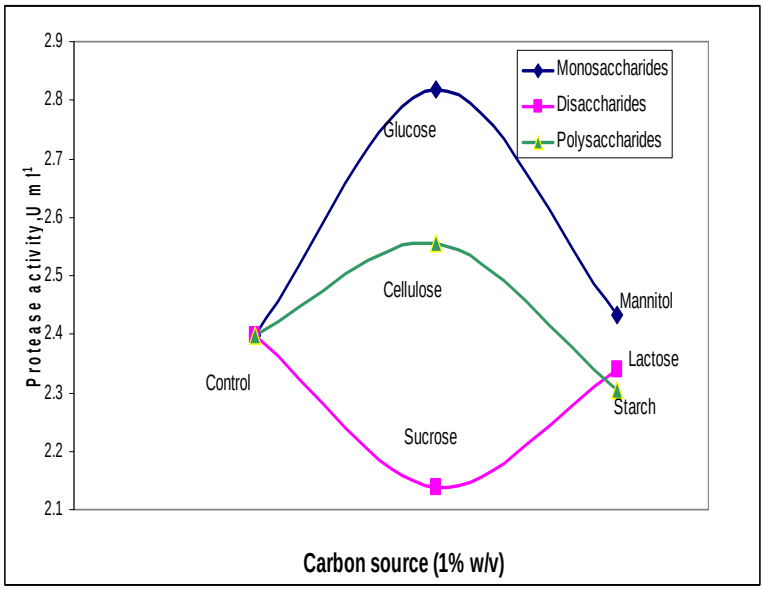

Fig. 6 Effect of carbon source on acid protease production
**Values represented in the figure are mean of two replicates*

The fermentation medium was further optimized with various glucose concentrations in the range of 0.5 to 3.0 % (fig. 7).

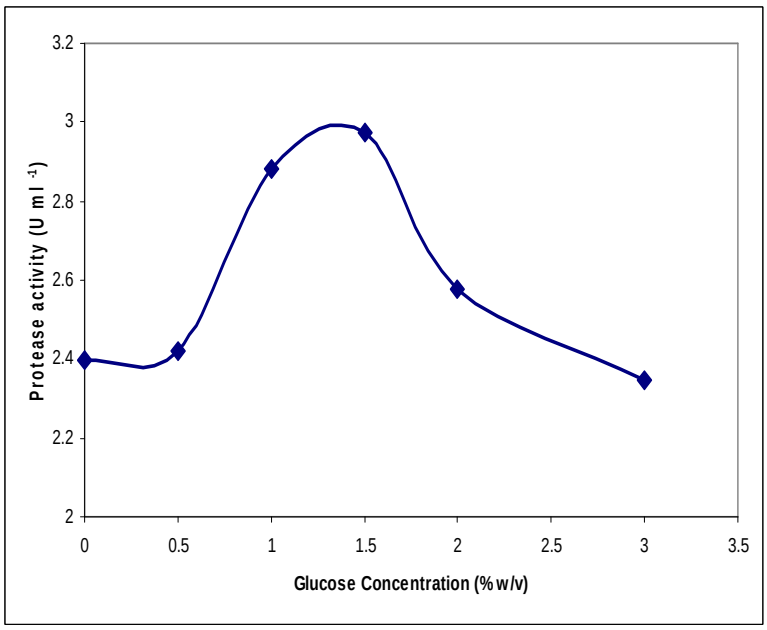

Fig. 7 Effect of different concentrations of glucose on protease production
**Values represented in the figure are mean of two replicates*

The proteolytic activity of *Aspergillus spp* was 2.914 Uml^{-1} when grown in medium consists glucose at 1.5%. Sinha et al. (2009) [3] and Kalpana et al. (2008) [4] also made a similar observation on enhanced protease activity by glucose concentration upto 1-1.5 percentages.

Effect of different flours of cereals on protease production

The effect of cereals flours on protease production by *Aspergillus spp* was studied and listed in fig .8

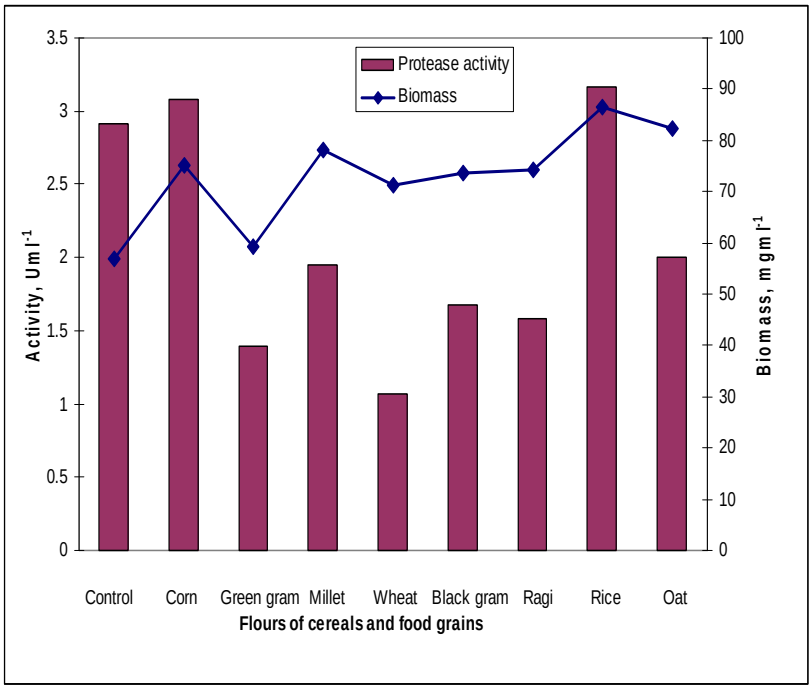

Fig. 8 Effect of different flours of cereals on acid protease production

**Values represented in the figure are mean of two replicates*

The impact of addition of 1% flours of cereals and food grains to the 1:1 volume ratio of molasses and cheese whey were shown in fig. 8. Both the corn flour and rice flour showed the significant increment in protease production by fungal culture when compared to the control. In this study, Rice flour at 1% enhanced the protease production from 2.918 U ml^{-1} to 3.167 U ml^{-1} with maximum biomass growth of 86.4 mg ml^{-1} followed by corn flour with an activity of 3.068 U ml^{-1}. The remaining food grains and cereals were not favored for the protease production.

CONCLUSION

Based on the results from this study, we finally concluded that the molasses and cheese whey were proved to be suitable substrates for protease production in submerged fermentation by *Aspergillus* spp. The cultural conditions were optimized as pH 5.0, temperature 32 ± 2^0C and incubation time 120 h and inoculum 4%. Among the all tested nitrogen sources soya bean meal (1%) was the cheaper substrate and was able to produce protease activity at 13% less than that of casein. Similarly, glucose (1.5%) was found to be suitable carbon source among all the tested carbon sources and Rice flour also enhanced the protease activity.

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REFERENCES

[1] K.J.P.Narayana, M.Vijayalakshmi, *Asian Journal of Biochemistry*, **2008**, 3, 3, 198-202.

[2] C.Djamel, T. Ali, C. Nelly, *European journal of Scientific Research*, **2009**, 25, 3, 469-477.
[3] S. Sinha, S. Sinha, *International Journal of Food Engineering*, **2009**, 5, 1.
[4] M. Kalpana Devi, A. Rasheedha Banu, G.R. Gnanaprabhal, B.V.Pradeep, M.Palaniswamy, *Indian Journal of Science and Technology*, **2008**, 1, 7, 1-6.
[5] C. Sandhya, A. Sumantha, G. Szakacs, A. Pandey, Process Biochem, **2005**, 40, 2689-2694.
[6] Magdi A.M.Younis, Francis F. Hezayen , Moustafa A.Nour Eldein , Mohammed S.A.Sahbeb, *Global Journal of Biotechnology and Biochemistry*, **2009,** 4(2): 132-137.
[7] K. H. Domsch, W. Gams and Trevte-Teidi Anderson (Eds.), Compendium of soil fungi, Academic Press, London, **1980**, 1.
[8] I. Haq, H. Mukhtar, H. Umber, *Journal of Agriculture & Social Sciences*, **2006**, 2, 1, 23-25.
[9] R.C.S. Thys, Samanta O.Guzzon, Florencia Cladera-Olivera and Adriano Brandelli, *Science Direct - Process Biochemistry*, 2005.
[10] Francisco J.U., Adriana L, and Luis A. Garcia y Mario Diaz, **2008,** *Rev.Tec.Ing.Univ.Zulia*, 31,1.
[11] Usama F.Ali, *Research Journal of Agriculture and Biological Sciences*, **2008**, 4(6): 886-891.
[12] A. Ganesh Kumar, N. Nagesh, T.G. Prabhakar, G. Sekharan, *Bioresource Technol*, **2008**, 99, 7,2364-72.
[13] N.A. Cristobal, G.S. Gerardo, A.B. PLilia, R.R.Herrera, L. José, M. Hernandez, C. Juan. Contreras, *American Journal of Biochemistry and Biotechnology*, **2008**, 4, 4, 354-366.
[14] M. Shaheen, A. A. Shah , A. Hameed, F. Hasan, *Pak.J.Bot*, **2008**, 40, 5, 2161-2169.