
Isolation production and partial purification of protease from an endophytic *Acremonium* sp.

Pranay Jain^{1*}, Vibhor Aggarwal¹, Archit Sharma¹ and Ram Kumar Pundir²

¹Department of Biotechnology, University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra, India, ²Department of Biotechnology, Ambala College of Engineering and Applied Research, Mithapur, Ambala, India

Pranay Jain, Vibhor Aggarwal, Archit Sharma and Ram Kumar Pundir (2012) Isolation production and partial purification of protease from an endophytic *Acremonium* sp. Journal of Agricultural Technology 8(6):1979-1989.

Protease production by bioprocesses is of great value as this enzyme is used commercially for many purposes such as biscuit manufacturers use it to lower the protein level of flour. It also removes cloudiness produced during storage of beers. It is also used in contact lens cleaners to remove proteins on contact lens to prevent infections. It has great value in photographic industries as it dissolve gelatin off scrap film, allowing recovery of its silver content. Considering these industrial important applications a study was carried out for the production of protease from an endophytic fungus using skimmed milk media. Secondary screening of these isolates resulted in a protease producing fungus identified as *Acremonium* sp. The media was first optimized for carbon and nitrogen sources. Role of pH was also considered during growth and production of enzyme. The fructose and ammonium sulphate were found to be optimum carbon and nitrogen sources. The optimum pH was found to be 7 for growth of fungus and for enzyme production. The enzyme activity was found to be 3.34 U/ml. The protein content was found to be 20 µg/ml. The specific activity of enzyme was 0.167 U/µg. Further studies can be carried out to characterize the enzyme for its industrial applications.

Key words: Endophytic fungus, *Acremonium* sp., Enzyme production, Protease

Introduction

Endophytic microorganisms (fungi and bacteria) are those microorganisms that inhabit inside of a plant at least in a period of its vital cycle, and are found in tissues such as leaves, branches and roots. Apparently, they do not cause any damage to the host, which distinguishes them from the phytopathogenic microorganisms (Azevedo, 1998). Mutualism interaction between endophytes and host plants may result in fitness benefits for both

* Corresponding author: Pranay Jain; e-mail: drpranayjain@gmail.com

partners (Kogel *et al.*, 2006). The endophytes may provide protection and survival conditions to their host plant by producing a plethora of substances which, once isolated and characterized, may also have potential for use in industry, agriculture, and medicine (Strobel and Daisy, 2003; Strobel *et al.*, 2004). Approximately 300 000 plant species growing in unexplored area on the earth are host to one or more endophytes (Strobel, 2003) and the presence of biodiverse endophytes in huge number plays an important role on ecosystems with greatest biodiversity (Strobel and Daisy, 2003). The production of bioactive substances by endophytes is directly related to the independent evolution of these microorganisms, which may have incorporated genetic information from higher plants, allowing them to better adapt to plant host and carry out some functions such as protection from pathogens, insects, and grazing animals. Endophytes are chemical synthesizer inside plants, in other words, they play a role as a selection system for microbes to produce bioactive substances with low toxicity toward higher organisms (Strobel, 2003).

A protease (also termed peptidase or proteinase) is any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. They have diverse application in wide variety of industries like biscuit manufacturing, brewing industries. It is also used in contact lens cleaners to remove proteins on contact lens to prevent infections. It also has great value in photographic industries as it dissolve gelatin off scrap film, allowing recovery of its silver content. Although proteases can be derived from several sources such as plants, animals and micro-organisms, enzymes from microbial sources generally meet industrial demands. Microbial enzymes are preferred to those from both plants and animal sources because they are cheaper to produce, and their enzyme contents are more predictable, controllable and reliable (Burhan *et al.*, 2003).

Saraca asoca (Asoka) is a plant belonging to the *Caesalpinaceae* subfamily of the legume family. It is an important tree in the cultural traditions of the Indian Subcontinent and adjacent areas. The bark of the herb is strongly astringent and uterine sedative. It acts directly on the muscular fibers of the uterus. It has a stimulating effect on the endometrium and the ovarian tissue. It is also used for the uterine/ovarian fibroids, menorrhagia, bleeding hemorrhoids, bleeding dysentery (Pradhan *et al.* 2009). Considering the industrial importance of protease the objective of this study was to isolate and identify proteolytic endophytic fungus from *Saraca asoka*, and to perform partial characterization of the enzyme production and its properties with regard to the effect of pH, carbon and nitrogen sources.

Materials and methods

Collection of Sample

Leaves from *Saraca asoca* (Asoka) were taken with the help of sterilized scalpel. The leaves were sterilized for getting the growth of endophytic fungi & avoiding epiphytic fungi. The leaves were washed with tap water. Then leaves were washed with distilled water. After that leaves were optimally cut with the help of scalpel and then were dipped in ethanol for 30 seconds. After that leaves were kept in mercuric chloride for 5 minutes. Then these pieces of leaves were washed 3-4 times with autoclaved water. These pieces were then placed on Potato Dextrose Agar plates containing Streptopenicillin. (Aneja, 2003).

Screening for Proteolytic activity

The fungus plug was cut and kept on the skimmed milk agar plates. The clear zone of hydrolysis around the disc showed the proteolytic activity of fungus. The zone formation around the bacterial colony indicated the protease positive strain which may be due to hydrolysis of casein (Kuberan *et al.*, 2010).

Identification of endophytic fungus

The isolated endophytic fungus was further stained with lactophenol cotton blue for its identification. The prepared slides with observed under trinocular microscope. Detailed morphological study was carried out.

Production of Protease

Growth media development and inoculation

The growth media was selected which contained (g/L) skimmed milk 20, yeast extract 0.7, KH_2PO_4 1.9, KH_2PO_4 0.85, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0015. Then a fungal plug was cut from the master plate & put in the growth media for growth of proteolytic fungi. This growth media was kept undisturbed for 5 days of incubation.

Optimization of fermentation conditions

Effect of Carbon source

The Carbon source was optimized for the fungal growth and enzyme activity. The Carbon sources viz. glucose, fructose and Galactose were taken in the media for checking the fungal growth. The flasks were incubated at 37°C for 3 days. Samples were taken at regular intervals and analyzed for protease activity. The absorbance was taken at 660 nm. The most suitable carbon source was determined (Kuberan *et al.*, 2010).

Effect of Nitrogen source

The Nitrogen source was optimized by using Ammonium Sulphate, Urea, and Ammonium Sulphamate. The flasks were incubated at 37°C for 3 days. Samples were taken at regular intervals and analyzed for protease activity. The suitable nitrogen source was determined (Kuberan *et al.*, 2010).

Effect of pH on enzyme activity

The pH's selected for optimizations were 7, 10, and 13. Adjustments of the pH were done by addition of hydrochloric acid (0.1N) and 0.1N sodium hydroxide to achieve acidity and alkalinity respectively. The flasks were incubated at 37°C for 48 h. Samples were taken at regular intervals and analyzed for protease activity. The optimum growth was seen at pH 7 & was selected for the production media.

Production Media development and inoculation

The production media contained skimmed milk 20, yeast extract 0.7, KH_2PO_4 1.9, KH_2PO_4 0.85, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.0015 and the supplements of optimized carbon and nitrogen source were added. The pH 7 was adjusted with NaOH with the help of pH meter. The growth media was then transferred to production. This was kept for 5-6 days of incubation.

Purification

After incubation the fungi growth occurred. Then it was filtered through Whatman filter paper. The filtrate was used which was then precipitated through Ammonium Sulphate by the process of Ammonium Sulphate fractionation (20-100%) and allowed to stand overnight and then centrifuged

(7500 g, 15 min, -1°C). The precipitate obtained was then stored in a minimal volume of 3 M ammonium sulphate dissolved in 0.05 M K_2HPO_4 . The 20% precipitated crude enzyme was then dialyzed through dialysis tubing 0.02 M Tris-HCl buffer (pH 8.0), The buffer was intermittently changed. Buffer was changed after 2 hours and the buffer was overall changed for 4 times. After that the enzyme was eluted through it and was stored at 4°C (Joshi, 2011).

Assay of enzyme activity

The protease activity was determined as described by Agrawal *et al* (2005). 1 ml of enzyme was mixed with a 5 ml solution of 2% (g per 100mL) casein dissolved in 0.5 mol/l carbonate buffer, pH 10. The resulting solution was incubated at 40°C for 30 min. An aliquot (0.5 mL) of the reaction mixture was withdrawn and the reaction was quenched by adding 1.5 ml pre-chilled trichloroacetic acid (10%). The reaction tube was immersed in an ice bath (5 min) to completely precipitate the protein. The supernatant was recovered by centrifugation (1000 rpm, 10 min). Tyrosine liberated during casein hydrolysis was measured in the supernatant using the method of Lowry *et al*. A unit of protease activity was defined as the amount of enzyme liberating 1 μg tyrosine/min at the incubation temperature of 40°C .

Assay of protein concentration

Protein was quantified by the method of Lowry *et al*. (1951), with bovine serum albumin as standard.

Results and discussions

Endophytic microorganisms (fungi and bacteria) are those that inhabit inside of a plant at least in a period of its vital cycle, and are found in tissues such as leaves, branches and roots. Apparently, they do not cause any damage to the host, which distinguishes them from the phytopatogens microorganisms (Azevedo *et al.*, 1998). A protease (also termed peptidase or proteinase) is any enzyme that conducts proteolysis, that is, begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein. They have diverse application in wide variety of industries like biscuit manufacturing, brewing industries. It is also used in contact lens cleaners to remove proteins on contact lens to prevent infections. It also has great value in photographic industries as it dissolve gelatin off scrap film, allowing recovery of its silver content. Although proteases can be derived from several sources such as plants, animals and

micro-organisms, enzymes from microbial sources generally meet industrial demands. In the present study, the work was carried out on the enzymatic activity of endophytic fungi.

Strain selection

In the present study, two fungal isolates were obtained from different plants used that had cottony colonies and degrade casein. One of the isolate was further proceeded as it showed the larger zone of clearance. The fungus was found to be of *Acremonium sp.* when identified with lacto phenol cotton blue. The fungus was found to be *Acremonium sp.* when stained with lacto phenol cotton blue (Fig.1). *Acremonium* is a genus of Fungi in the *Hypocreaceae* family; it was previously known as "*Cephalosporium*". *Acremonium* species are usually slow growing and are initially compact and moist. *Acremonium* hyphae are fine and hyaline and produce mostly simple phialides. Their conidia are usually one-celled (i.e. ameroconidia), hyaline or pigmented, globose to cylindrical, and mostly aggregated in slimy heads at the apex of each phialide. The colony is shown in Fig. 1.

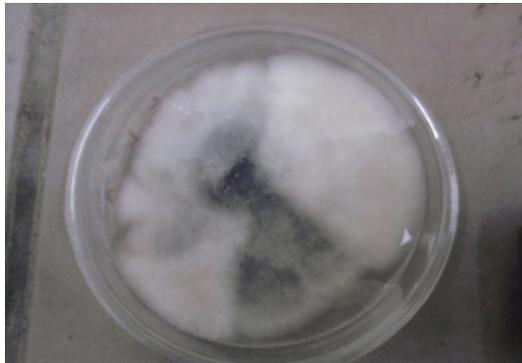


Fig. 1. Isolated fungus *Acremonium sp.* showing proteolytic activity

Optimization of fermentation conditions

The Carbon sources viz. glucose, fructose and galactose were taken in the media for checking the fungal growth. The flasks were incubated at 37°C for 3 days. Samples were taken at regular intervals and analyzed for protease activity. The absorbance was taken at 660 nm. The Optical Density for glucose, fructose and Galactose were 0.22, 0.37, 0.06 respectively. So when fructose as carbon source was used the optimum production was obtained. This effect is shown in Fig. 2.

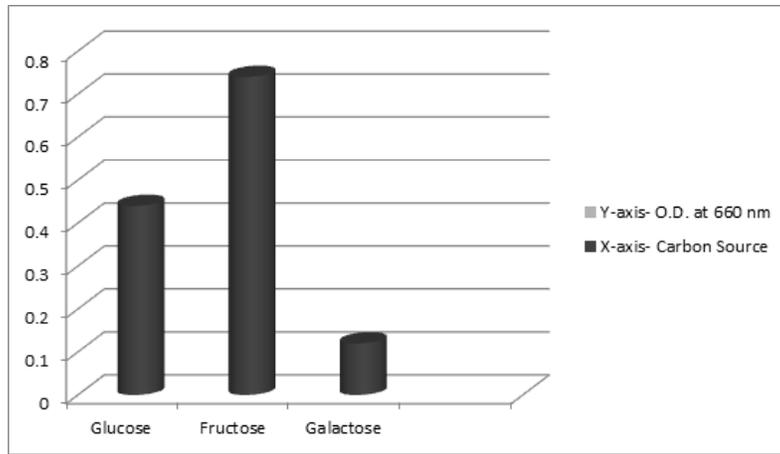


Fig. 2. Optimization of Carbon source for the production of protease

The Nitrogen source was optimized by using Ammonium Sulphate, Urea, and Ammonium Sulphamate. The flasks were incubated at 37°C for 3 days. Samples were taken at regular intervals and analyzed for protease activity. The Optical Density for Ammonium Sulphate, Urea, and Ammonium Sulphamate were 0.09, 0.01, and 0.04 respectively. So when Ammonium Sulphate as nitrogen source was used the optimum production was obtained. This effect is shown in Fig. 3.

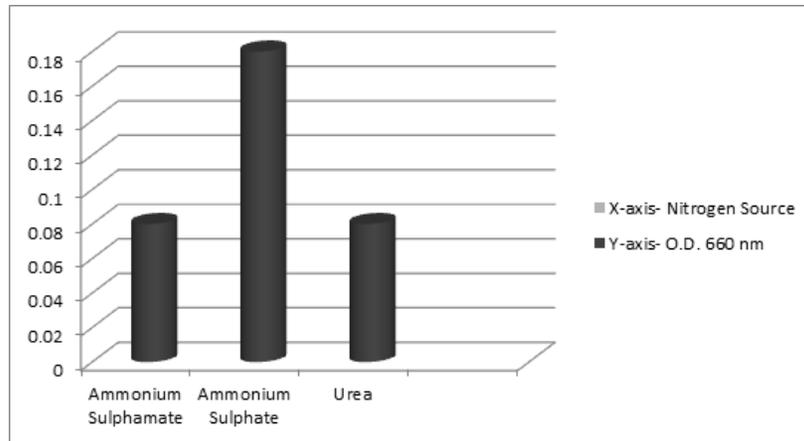


Fig. 3. Optimization of Nitrogen Source for the production of protease

The pH's selected for optimization was 7, 10, and 13. Adjustments of the pH were done by addition of hydrochloric acid (0.1N) and 0.1N sodium hydroxide to achieve acidity and alkalinity respectively. The flasks were

incubated at 37°C for 48 h. Samples were taken at regular intervals and analyzed for protease activity. Optical Density for pH 7, 10, 13 was 0.67, 0.22, 0.18 respectively. The pH 7 showed the optimum growth and production. This effect is shown in Fig. 4.

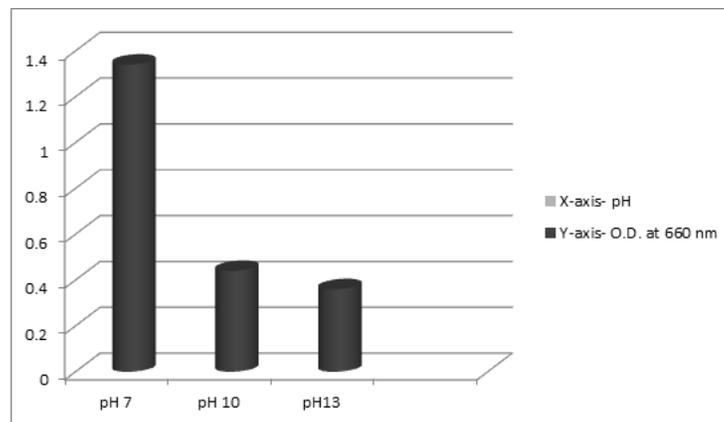


Fig. 4. Optimization of pH for the production of protease

Optimum pH 8.4 has been reported for alkaline protease of *Conidiobolus coranalis*. Likewise pH 7 has been reported to be optimum for *Aspergillus flavus* (Sutar *et al.*, 1992).

Purification

The enzyme was precipitated using Ammonium Sulphate Fractionation Technique. The precipitated crude enzyme was dialyzed through dialysis tubing 0.02 M Tris-HCl buffer (pH 8.0). The dialysis through dialysis tubing is shown in Fig 7.

Specific Activity of enzyme

The enzyme activity was calculated according to the method of Agrawal *et al.* (2005). The amount of tyrosine liberated during casein hydrolysis according to the method of Lowry *et al.* (1951). The standard tyrosine curve was plotted and the amount of tyrosine was calculated from that standard curve. The tyrosine standard curve is shown in Fig 5. The Optical Density obtained for the sample was found to be 0.2. When the corresponding amount of tyrosine was taken, the enzyme activity was found to be 3.34 U/ml.

Similarly standard curve for BSA was plotted and the experiment was performed 5 times within run precision. The mean of the values were taken and

plotted. The plotted curve is shown in Fig 6. The enzyme was assayed for determination of Protein content using Lowry's method and the readings were plotted on the standard BSA curve. The Optical Density of sample was found to be 0.1. When the protein content was calculated from standard curve it was found to be 20 $\mu\text{g/ml}$. The specific activity of enzyme was calculated and found to be 0.167 U/ μg .

Paranthaman *et al.* (2009) carried out the study of maximum enzyme production period. Maximum enzyme production was observed at of 67.7 U/g biomass was observed in PONNI, while minimum protease production 44.7 U/g biomass was noticed with ADT-66 variety.

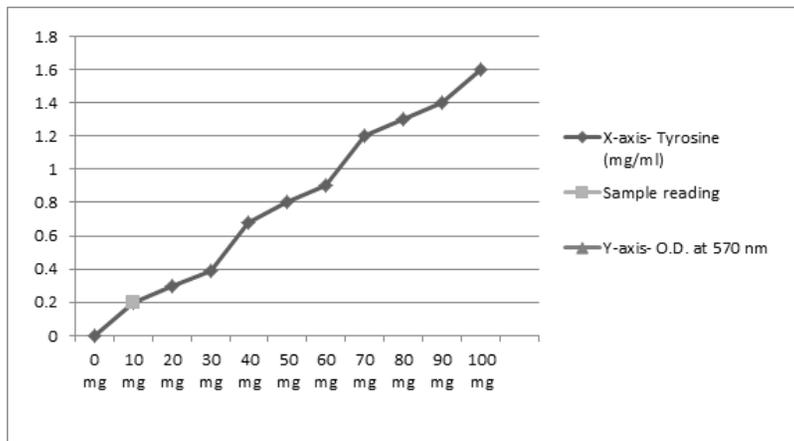


Fig. 5. Tyrosine standard curve plotted for the calculation of enzyme activity

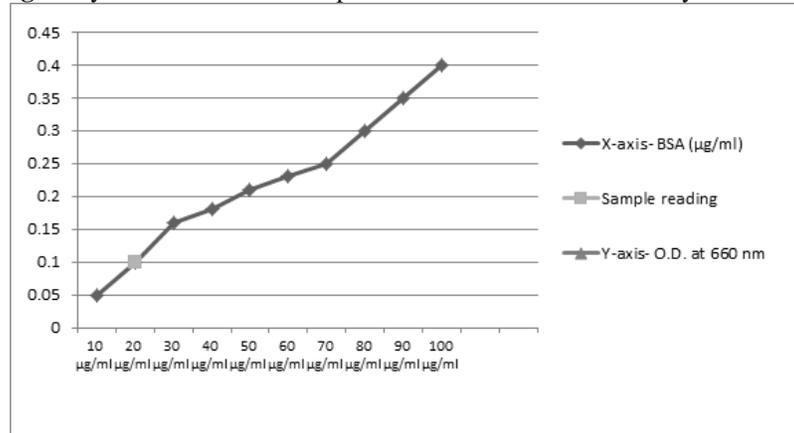


Fig. 6. BSA Standard Curve plotted for calculation of enzyme activity



Fig. 6. Dialysis of Amylase for partial purification

Conclusion

The enzyme is very sensitive to pH. Therefore, the selection of optimum pH is essential for the production of proteases. In this study, the effect of initial pH of substrate was studied (Figure 5). The substrate was maintained at different pH (7 - 13). Production of protease was the best at pH 7. Further the effect of carbon and nitrogen sources was observed. Fructose and Ammonium Sulphate were selected as carbon and nitrogen sources respectively as the optimum growth was observed when they were used. Protease has diverse application in wide variety of industries like biscuit manufacturing, brewing industries. It is also used in contact lens cleaners to remove proteins on contact lens to prevent infections. It also has great value in photographic industries as it dissolve gelatin off scrap film, allowing recovery of its silver content. As protease is found to be an important enzyme so *Acremonium sp.*, can be industrially exploited for the synthesis of protease and strain improvement studies can be carried out to enhance enzyme production.

Acknowledgement

The authors are grateful to the Hon'ble Vice-Chancellor, Kurukshetra University, Kurukshetra and the Director, University Institute of Engineering and Technology, Kurukshetra University, Kurukshetra for providing infrastructure facilities to carry out research work.

References

- Agrawal, D., Partidar, P. Banerjee and Tand Patil, S. (2005). Alkaline protease production by a soil isolate of *Beauveria feline* under SSF condition: parameter optimization and application to soy protein hydrolysis. *Process Biochem* 40:1131–1136.
- Aneja, K.R. (200). *Experiments in Microbiology, plant pathology and biotechnology*: Fourth edition: pp. 472.

- Azevedo, J.L. (1998). Microorganismos endofíticos. In: Melo IS, Azevedo JL (Eds.). *Ecologia Microbiana*. Jaguariúna: Embrapa Meio Ambiente. pp. 117-137.
- Burhan, A., Nisa, U., Gokhan, C., Ashabil, A., Osmair, G. (2003). Enzymatic Properties of a novel thermostable thermophilic alkaline and chelator resistant amylase from an alkaphilic *Bacillus* sp isolate ANT-6. *Process Biochemistry* 38:1397–1403.
- Joshi, B.H. (2011). A novel thermostable alkaline a-amylase from *Bacillus* circulans PN5. *Asian journal of Biotechnology* pp. 58-67.
- Kalpana, D.M., Rasheedha, B.A., Gnanaprabhal, G.R., Pradeep, B.V. and Palaniswamy, M. (2008). Purification, characterization of alkaline protease enzyme from native isolate *Aspergillus niger* and its compatibility with commercial detergents. *Indian Journal of Science and Technology* 1: pp. 7.
- Kogel, K.H., Franken, P. and Huckelhoven, R. (2006). “Endophyte or parasite-what decides?” *Current Opinion in Plant Biology*. 9:358–363
- Kuberan, T., Sangaralingam, S. and Thirumalai Arasu, V. (2010). Isolation and optimization of Protease producing Bacteria from Halophilic soil. *J. Biosci. Res.* 1:163-174.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951). Protein measurement with the Folin-Phenol reagents. *J. Biol. Chem.* 48:17-25.
- Nehra, K. S., Dhillon, S., Chaudhary, K. and Singh, R. (2002). Production of alkaline protease by *Aspergillus* species under submerged and solid state fermentation. *Ind. J. Microbiol.* 42:43-47.
- Nehra, K.S., Singh, A., Sharma, J., Kumar, R. and Dhillon, S. (2004). Production and characterization of alkaline protease from *Aspergillus* species and its compatibility with commercial detergents. *Asian J. Microbiol. Biotech. Env. Sc.* 6:67-72.
- Paranthaman, R., Alagusundaram, K., Indhumathi, J. (2009). Production of protease from rice mill wastes by *Aspergillus niger* in solid state fermentation. *World J of Agri Sci.* 5:308-312.
- Strobel, G.A. (2003). Endophytes as sources of bioactive products. *Microbes and Infection.* 5(6):535–544.
- Strobel, G. and Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiology and Molecular Biology Reviews.* 67 (4): 491–502.
- Strobel, G., Daisy, B., Castillo, U. and Harper, J. (2004). Natural products from endophytic microorganisms. *Journal of Natural Products.* 67:257–268.
- Sutar, I.I., Srinivasan, M.C. and Vartak, H.G. (1992). Production of an alkaline protease from *Conidiobolus coronatus* and its use to resolve DL-phenylalanine and DLP-phenylglycine. *World J. Microbiol. Biotechnol.* 8:254-258.
- Tejesvi, M.V., Nalini, M.S., Mahesh, B., Prakash, H.S., Kinii, K.R., Shetty, H.S. and Subbiah, Ven (2007) New hopes from endophytic fungal secondary metabolites. *Bol. Soc. Quim. Mex.* 1:19-26.
- Wang, Y. and Lee, M. (1996). Influence of culture and nutritional condition on the production of protease from Thermophilic strain *Aspergillus* species NTIJ-FC-671. *J. Chinese Agric. Chem. Soc.* 34:732-742.

(Received 18 March 2012; accepted 30 October 2012)