Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 3, Issue, 09, pp.075-078, September, 2011

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

ISSN: 0975-833X

RESEARCH ARTICLE

PRODUCTION OF PROTEASE FROM *Aspergillus niger* AND *Mucor mucedo* UNDER SUBMERGED AND SOLID STATE FERMENTATION

***Joel Gnanadoss, J., Rebecca Robert and Roseline Jebapriya, G**

Department of Plant Biology and Biotechnology, Loyola College (Autonomous), Chennai – 600 034, Tamil Nadu, India

ARTICLE INFO

Article History:
Received 14th June, 2011
Received in revised form
29th July, 2011
Accepted 19th August, 2011
Published online 17th September, 2011

Key words:
Protease,
Aspergillus niger,
Mucor mucedo,
solid state fermentation,
Agro wastes.

ABSTRACT

Fungal strains were isolated from oil cakes and were screened for protease production on casein agar plate. Organisms capable of casein hydrolysis were selected and enzyme assay was carried out. Organisms with maximum enzyme activity were selected for further studies. *Aspergillus niger* and *Mucor mucedo* were used for the present investigation. Optimization of parameters such as pH and substrate concentration was carried out for production of protease. A pH of 5 was found optimal for maximal enzyme production. Casein at 10% concentration showed significant increase in protease activity. Effect of UV mutagenesis on protease production was also studied. For experiments on solid state fermentation several agro wastes (cotton seed oil cake, wheat bran and soya bean) were used as substrates. Among the substrates tested, cotton seed oil cake was the best for production of protease in *Aspergillus niger*. However, in *Mucor mucedo* more enzymes were produced when soya bean meal was used as substrate.

INTRODUCTION

Proteases catalyze hydrolytic reactions in which protein molecules are degraded to peptides and amino acids. Proteases which are one among the three largest groups of industrial enzymes, account for about 60% of the total worldwide sale of enzymes. They are widely used in several industries that include detergent, leather processing, meat processing, and diary, preparation of organic fertilizer, as digestive aid, silk industry (Mala *et al*., 1998) and also for the recovery of silver from used X-ray films (Singh *et al*., 1999). Microbial proteases account for about 60% of the total enzyme market sale (Rao *et al*., 1998). Although the majority commercial proteases originated from bacteria belonging to the genus *Bacillus*, fungi exhibit a wider variety of proteases than bacteria. In addition, fungi are normally GRAS (generally regarded as safe) strains and they produce extracellular enzymes, which can be recovered easily from the fermentation broth (Sandhya *et al*., 2005). Numerous molds, especially those belonging to the genera *Aspergillus* (Fan-Ching & Lin, 1998), *Penicillium* (Chrzanowska *et al*., 1993) and *Rhizopus* (Farley and Ikasari, 1992) have been known to produce variety of proteases. Proteases are classified into acid, neutral and alkaline proteases based on the pH in which their activity is maximum. Neutral proteases are important for food industry, because they possess specific function in hydrolyzing hydrophobic amino acid bonds at neutral pH, thereby reducing the bitterness of food protein hydrolysates. Neutral proteases also find application in rice starch isolation (Wang and Wang, 2001). Certain genera such as *Aspergillus, Penicillium and Fusarium* have been reported to produce neutral proteases. Few examples for organism producing acidic and alkaline proteases having commercial values are *Aspergillus* sp., *Penicillium* sp. and *Mucor* sp. Proteases have been produced through submerged (SmF) and solid-state fermentations (SSF) (Sandhya *et al*., 2005). Each organism has its own individual growth condition as well as enzymes production. 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 *et al*., 1994). SSF has been reported to be an economical alternative to SmF for the production of high titres of proteases. SSF for fungal enzyme production has advantages include simplicity, lower production costs, high enzyme yields and low wastewater output (Pandey *et al*., 2003). 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. By-products generated by agro-industries have been used as substrates including sugarcane bagasse, brans and straw from wheat and rice, sugar beet pulp and coffee pulp (Zheng and Shetty, 2000). The present investigation aimed to maximize the production of protease from *Aspergillus niger* and *Mucor*

Corresponding author: joelgna@gmail.com

mucedo under submerged and solid state fermentation. Effect of UV induced mutagenesis on protease production has also been carried out.

MATERIALS AND METHODS

Isolation of organism

Different varieties of oil cakes were procured from the local market. The oil cakes (Cotton seed and Sesame oil cake) were soaked in water for 48 hrs for the growth of fungal spores. After 48 hrs, the soaked oil cakes were suspended in 100 mL of sterile water and mixed thoroughly for even distribution of the sample and evenly mixed sample were serially diluted in the range of 10^{-3} – 10^{-6} dilutions. 0.1ml from dilutions 10^{-4}, 10^{-5}, 10^{-6} was inoculated into potato dextrose agar (PDA) plates. The plates were incubated at room temperature for 48 hrs.

Inoculum preparation

The isolated fungal strains were inoculated into basal medium containing 5g starch, 10g skimmed milk powder, 2g potassium nitrate, 2g sodium chloride, 2g dipotassium hydrogen phosphate, 0.05g magnesium sulphate, 0.02g calcium carbonate, 0.01g ferrous sulphate in 1L distilled water. Isolated fungal strains were inoculated separately into 100 mL of the basal medium. The inoculated flasks were kept on a shaker (90 rpm) at 35˚C for 7 days.

Screening of protease producer by plate assay

After incubation, the basal medium containing the active enzyme was filtered using muslin cloth. Using cork-borer, a well was punched in the center of the casein agar plate containing 20g casein, 15g agar in 1L distilled water. 4 to 5 drops of the culture filtrate was placed into the wells on the casein agar plates. The plates were incubated overnight and then observed for the zone of clearance.

Enzyme assay

0.5 ml of culture filtrate was taken and 0.5 ml of 2% casein was added and incubated for 1 hour. After incubation, 0.1ml of 10% trichloroacetic acid (TCA) was added and centrifuged. To the 0.5 mL of supernatant, 2.5 mL of reagent A (2.4 g sodium hydroxide, 5.8 g sodium carbonate and 200 mL distilled water) and 0.75 mL of folin phenol reagent was added to neutralize TCA and incubated for 20 minutes. The readings were taken at 650 nm and the enzyme activity was expressed per mL of filtrate. One unit of protease is that amount of enzyme which produced an absorbance increase of 0.01 per hour under the assay conditions. The specific activity of the enzyme is calculated as:

$$\text{Unit/mL enzyme} = \frac{\text{r A 650nm/min test} - \text{r A 650nm/min blank}}{(0.001)\,(0.5)}$$

rA	-	Relative absorbance
0.001	-	The change in A650 nm / minute per unit of protease
0.5	-	Volume (in mL) of enzyme used

The enzyme activity for all the isolated fungal strains was calculated from the standard graph. Based on maximum enzyme activity, the fungal strain was selected and culture was maintained on PDA slants at 4˚C and subcultured once in every 3 weeks.

Effect of pH on protease production

Basal medium was prepared and pH was adjusted to a range of 5.0 – 8.0 by adjusting with 1N HCL or 1N NaOH. The fungal strains were inoculated separately into the basal medium which was set at various pH concentrations. All the flasks were incubated at 35˚C in a shaker for 7 days. After incubation, the enzyme assay was carried out and the absorbance was read at 650nm.

Effect of substrate concentration on protease production

The fungal strains were inoculated separately into the basal medium containing various concentration of casein. The concentration of casein ranging from 1, 4, 7 and 10% were used. All the flasks were incubated at 35˚C in a shaker for 7 days. After incubation, the enzyme assay was carried out and the absorbance was read at 650nm.

Effect of UV mutation on protease production

The spores of fungal stains were exposed to UV radiation for time duration such as 5, 10 and 15 minutes. The spores from the exposed plates were again inoculated into PDA plates. The plates were incubated at room temperature for 48 hrs. After incubation, the fungal strains were inoculated into basal medium. All the flasks were incubated at 35˚C in a shaker for 7 days. After incubation, the enzyme assay was carried out and the absorbance was read at 650nm.

Solid-state fermentation

Various agro-industrial residues such as cotton seed oil cake, wheat bran and soya bean meal were used for evaluation their potential as substrate in SSF for protease production. The substrates were cut into small pieces, washed and soaked overnight and water was drained off. SSF was carried out by taking 200g of the substrates in saline bottles and the bottles were plugged, wrapped and autoclaved at 121˚C for 15 minutes. After sterilization, the bottles were cooled and inoculated with 5 discs of the fungal culture. The inoculated bottles were incubated for 2 weeks at room temperature. After incubation, 50 mL of 0.2 M phosphate buffer (pH 7.0) was poured into the bottles. The bottles were kept in shaker for 30 minutes for allowing the buffer to extract the enzyme which was followed by overnight incubation. The enzymes was filtered and stored. The enzyme assay was carried out by reading the absorbance at 650nm.

RESULTS AND DISCUSSION

Different types of oil cakes which were used for the isolation of protease producing organism. The isolated fungal strains were labeled as LCJ 21- 29. After incubation, the inoculated fungal strains (LCJ 21- 29) present in the basal medium showed different size and colour of fungal colonies. Generally, protease produced from organisms is constitutive or partially inducible in nature and strongly influenced by medium components such as carbon / nitrogen ratio and

presence of easily metabolizable sugars, such as starch etc. (Beg *et al*., 2002).

Screening of protease producer by plate assay

Protease producing organisms showed clear zone of casein hydrolysis on milk agar (Ellaiah *et al*., 2002), which was taken as basis in the present study to screen the strains. In present work the fungal strains LCJ 21- 28 showed clear zone of casein hydrolysis on milk agar. Zone formation was not seen on plates inoculated with LCJ 29 strain which indicated that there is no protease production by the organism (Table 1.). Therefore, strains LCJ 21 – 28 were selected for further studies. There are earlier reports also on protease production by strains of *Aspergillus* sp. (Malathi and Chakraborty, 1991). Selected fungal strains LCJ 21- 28 was carried out for protease assay based upon the protease assay, the fungal strains LCJ 26, LCJ 28 were selected and the strains were identified as *Aspergillus niger* (LCJ 26) and *Mucor mucedo* (LCJ 28).

Table 1. Screening for protease production

Strain No.	Zone formation (zone of hydrolysis)	Protease production
LCJ 21	+	++
LCJ 22	+	++
LCJ 23	+	++
LCJ 24	+	++
LCJ 25	+	++
LCJ 26	+	++
LCJ 27	+	++
LCJ 28	+	++
LCJ 29	-	--

(+ Zone of hydrolysis, - no zone of hydrolysis); (++ protease production, -- no protease production)

Effect of pH on protease production

Production of the enzymes by mould culture mostly dependant on pH of the medium. Therefore, the effect of pH was studied for the production of protease by *Aspergillus niger* and *Mucor mucedo* The results clearly indicate that for *Aspergillus niger* and *Mucor mucedo,* the optimum pH for production of protease was at pH 5 and there was less enzyme production at pH 6 (Fig.1). For *Aspergillus niger* and *Mucor mucedo* the optimum pH for protease production was pH 5, and both the strains were capable of producing acid protease. Similar result was also observed in *Aspergillus fumigatus* (Oyeleke *et al*., 2010). Generally microbial strains depend on the extra-cellular 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 formation (Paranthaman *et al*., 2009).

Effect of substrate concentration on protease production

The maximum enzyme production was observed when medium contained 10% casein in both *Aspergillus niger* and *Mucor mucedo* (Fig.2) Abdul Rauf *et al.,* (2010) reported maximum enzyme production was found at 20 g/L of sunflower meal. Similar results were also reported in *Rhizopus oligosporus* (Sumantha *et al*., 2006). Warin Samarntarn *et al.,* (1999) reported the influence of casein concentration in the production of protease by *A.oryzae.* They suggested that aminoacids or peptides in casein could serve as specific inducers for increasing protease production.

Effect of UV mutation on protease production

The mutation is important for the successful improvement of strains for enzyme production. *Aspergillus niger* and *Mucor mucedo* were treated with UV irradiation for different duration to improve protease production. Results showed that

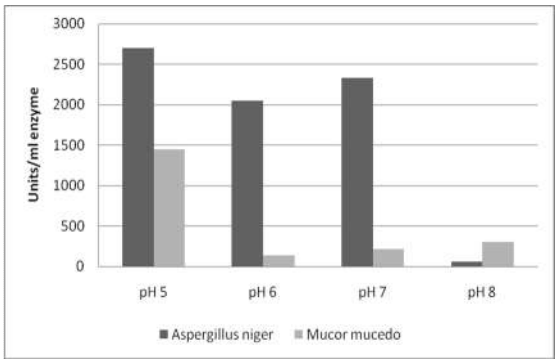

Fig.1. Effect of initial pH of the medium on protease production by *Aspergillus niger* and *Mucor mucedo*

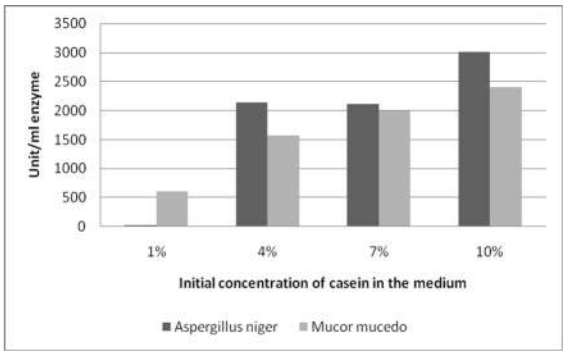

Fig.2. Effect of initial substrate concentration in the medium on protease production by *Aspergillus niger* and *Mucor mucedo* under submerged conditions

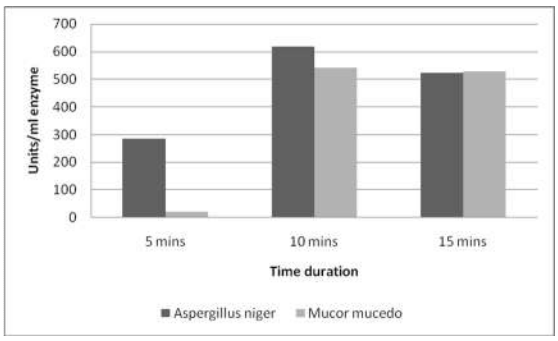

Fig.3. Effect of UV mutation on protease production by *Aspergillus niger* and *Mucor mucedo*

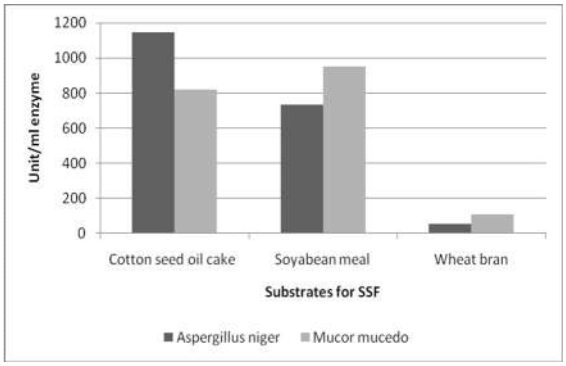

Fig.4. Effect of different solid substrates on protease production by *Aspergillus niger* and *Mucor mucedo*

Aspergillus niger and *Mucor mucedo* produced more amount of protease when it was exposed to 10mins in UV light (Fig.3). Thus, mutation by UV irradiations played a vital role for protease production with reference to *Aspergillus niger* and *Mucor mucedo*. Dutta and Banerjee (2006) observed increased alkaline protease production by UV mutant of *Psuedomonas* sp. Similar results were also observed in *Mucor mucedo* (Bhargavi Moturi and Singara Charya, 2010). Rao *et al* (1998) reported that mutagenesis either by conventional methods or by recombinant DNA technology played an important role in improving the yield of protease.

Solid-state fermentation

The study on the potential of agro-industrial residues as substrates for protease production by *Aspergillus niger* and *Mucor mucedo* varies from different substrates (Fig.4). In SSF, the selection of a suitable solid substrate for fermentation process is a critical factor. In the present study cotton seed oil cake, wheat bran and soya bean meal were selected as substrates. All the substrates used supported the growth and enzyme production, while cotton seed oil cake was found superior to the other substrates. *Aspergillus niger* produced maximum amount of protease utilizing cotton seed oil cake as substrate whereas *Mucor mucedo* showed more enzyme activity when soya bean meal was used as a substrate. There are few reports suggest that wheat bran also more effective in protease production (Malathi & Chakraborty, 1991). In another study, a mixture of rice bran, rice husk and gram hull in the ratio 5:3:2 was found to be suitable for protease production (Nehra *et al*., 2002). The finding reported by Ikasari and Mitchell (1994) on *Rhizopus oligosporus* showed that higher levels of protease production were inducted by rice bran than other substrates. The production of protease in solid state fermentation might be due to natural growth conditions available to the cells in solid cultures as well as due to nutrients availability in the solid state medium.

Acknowledgement

The authors would like to acknowledge the encouragement and support provided by the management, Loyola College, Chennai - 600 034.

REFERENCES

Abdul Rauf, Muhammad Irfan, Muhammad Nadeem, Ishtiaq Ahmed, and Hafiz Muhammad Nasir Iqbal. 2010. Optimization of Growth Conditions for Acidic Protease Production from *Rhizopus oligosporus* through Solid State Fermentation of Sunflower Meal. *International Journal of Agricultural and Biological Sciences.,* 1:40-43.

Beg, Q.K., Saxena, R.K. and Gupta, R. 2002. De-repression and subsequent induction of protease synthesis by *Bacillus mojavensis* under fed-batch operations. *Process Biochem.,* 37: 1103-1109.

Bhargavi Moturi and Singara Charya, M. A. 2010. Influence of physical and chemical mutagens on dye decolourising *Mucor mucedo*. *African Journal of Microbiology Research*, 4(17), pp. 1808-1813.

Chrzanowska, J., Kolaczkowska, M., Polanowski, A. 1993. Production of exocellular proteolytic enzymes by various species of *Penicillium*. *Enzyme Microb Technol.,* 15:140–3.

Dutta, J.R., and Banerjee, R. 2006. Isolation and characterization of a newly isolated *Pseudomonas* mutant for protease production. *Braz. Arch. Biol. Technol.,* 49, 37-47.

Ellaiah.P., Adinarayana, K., Rajyalakshmi, P and Srinivasulu, B. 2003 Optimization of process parameters for alkaline protease production under solid state fermentation by alkalophilic *Bacillus* sp. *Asian J Microbiol Biotechnol Environ Sci*., 5: 49-54.

Fan-Ching, Y. and Lin, I H. 1998. Production of acid protease using thin stillage from rice spirit distillery by *Aspergillus niger*. *Enzyme Microb Technol.,* 23:397–402.

Farley, PC. and Ikasari, L. 1992. Regulation of secretion of *Rhizopus oligosporus* extra cellular carboxyl proteinases. *J Gen Microbiol.,* 138: 2539–44.

Ikasari, L. and Mitchell, D.A. 1994. Protease production by *Rhizopus oligosporus* in solid-state fermentation. *World J Microbiol Biotechnol.,* 10:320–324.

Lekha, P.K., Lonsane, B.K. 1994. Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL104 in solid state, liquid surface and submerged fermentation. *Process Biochem.,* 29:497–503.

Mala, B.R., Aparna, M.T., Mohini. SG., Vasanti, VD. 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiol Mol Biol Rev.,* 62(3):597–635.

Malathi, S., Chakraborty, R. 1991. Production of alkaline protease by a new *Aspergillus flavus* isolate under solid-state fermentation conditions for use as a depilation agent. *Appl Environ Microbiol.,* 57:712–6.

Nehra, K.S., Dhillon, S., Kamala, C., Randir, S. 2002. Production of alkaline protease by *Aspergillus sp*. under submerged and solid substrate fermentation. *Ind J Microbiol.,* 42:43–7.

Oyeleke, S.B., Egwim, E.C. and Auta, S.H. 2010. Screening of *Aspergillus flavus* and *Aspergillus fumigatus* strains for extracellular protease enzyme production. *Journal of Microbiology and Antimicrobials*, Vol. 2(7), pp. 83-87.

Pandey, A.2003. Solid-state fermentation. *J Biochem Eng.*, 13:81–4.

Paranthaman, R., Alagusundaram, K. and Indhumathi, J. 2009. Production of Protease from Rice Mill Wastes by Aspergillus *niger* in Solid State Fermentation. *World Journal of Agricultural Sciences,* 5 (3): 308-312.

Rao, M.B., Tanksale A.M., Ghatge M.S. and Deshpande, V.V. 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiol. Molecul. Biology,* 62, 597-634.

Sandhya, C., Sumantha, A., Szakacs, G., Pandey, A. 2005. Comparative evaluation of neutral protease production by *Aspergillus oryzae* in submerged and solid state fermentation. *Process Biochem.,* 40:2689–94.

Singh, J., Voohra, R.M., Sahoo, D.K. 1999. Alkaline protease from a new obligate alkalophilic isolate of *Bacillus sphaericus*. *Biotechnol Lett.,* 21:921 4.

Sumantha, A., Deepa, P., Sandhya, C., Szakacs, G., Soccol, C. R, and Pandey, A. 2006. Rice Bran as a Substrate for Proteolytic Enzyme Production. *Braz Arch Biol Technol.,* vol. 49, no. 5, pp. 843-851.

Wang, L., Wang, Y. J. 2001. Comparison of protease digestion at neutral pH with alkaline steeping method for rice starch isolation. *Cereal Chem.,* 78:690–2.

Warin Samarntarn, Supaporn Cheevadhanarak and Morakot Tanticharoen. 1999. Production of alkaline protease by a genetically engineered *Aspergillus oryzae* U1521. *J. Gen. Appl. Microbiol.,* 45, 99–103

Zheng, Z., Shetty, K. 2000. Solid state production of polygalacturonase by *Lentinus edodes* using fruit processing wastes. *Process Biochem.,* 35:825-30.