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Production of Acidic protease using Submerged Fermentation by *Rhizopus arrhizus*

[1*]Azeem Haider and [2]Shakil Ahmed
[1]Institute of Agricultural Sciences, University of the Punjab, Lahore-54590
[2]Department of Botany, University of the Punjab, Lahore-54590
*For correspondence: Azeemhaider_iags@yahoo.com

ABSTRACT
The study is concerned with utilize whey of milk factory as a carbon source for the production of protease. The project was planned to produce maximum protease from whey by *Rhizopus arrhizus* at pH 4 and 37 °C temperature. Growth media employed to culture *Rhizopus arrhizus* for the production of enzyme were developed and fermentation conditions was optimized through various trials. Substrate water ratio, different nitrogen sources and concentration of nitrogen source were optimized. The fermented materials were harvested after 72 hours. These were filtered and centrifuged at 10,000 rpm at -10 °C. The filtrates were subjected to enzyme assay. Absorbance of the enzyme sample was determined at 700 nm on spectrophotometer. It was observed that 90% Whey and 2.5% cotton seed meal enhanced the production of protease by *Rhizopus arrhizus*. Maximum enzyme activity was observed (149.26 IU/ml/min) in flask level at pH 4 and 37 °C temperature. These optimized conditions of growth media was again used in Air-Lift fermenter and determined the activity (169.78 IU/ml/min) that is greater than flask level. This is due to proper aeration and proper temperature in Air-lift fermenter.

Keywords: Acidic protease, Air-lift fermentor, submerged fermentation and *Rhizopus arrhizus*

INTRODUCTION

Proteases are a group of enzymes, whose catalytic function is to hydrolyze peptide bonds of proteins and break them down into polypeptides or free amino acids. They constitute 59% of the global market of industrial enzymes, which is expected to exceed $ 2.9 Billion by 2012 (Deng *et al.*,2010). They have got wide range of commercial usage in detergents, leather, food and pharmaceutical industries (Bhaskar *et al.*,2007 and Jellouli *et al.*,2009). They are high temperature resistant with high specific activities and superior physical and chemical characteristics which seem to be good for future biotechnological applications, that is why they have wide applications in a large number of industrial processes (Rao *et al*., 1998; Temiz *et al*., 2008).

The sources of proteases are plants, animal tissues and microorganisms but due to the low production of proteases from above sources, they are mainly produced by microorganisms which involve fungi, bacteria and some other microorganisms. The enzymes of fungi are largely acid proteases however; many fungi have also been reported to produce neutral and alkaline proteases (Tremacoldi *et al.*, 2004). Acid proteases are without doubt the most interesting group of proteases with respect to use in the food industry. They are characterized by maximum activity and stability at pH 2.0 to 5.0. Sources of proteases include all forms of life, that is, plants, animals and microorganisms. Based on their acid-base behavior, proteases are classified in to three groups, that is, acid, neutral and alkaline proteases. Acid proteases performed best at pH range of 2.0-5.0 and are mostly produced by fungi. *Rhizopus* sp. are important major moulds in fermentation. Several reports describe the efficient protease biosynthesis by fungi belonging to the genera *Aspergillus* (Fan-Ching and Lin 1998), *Penicillium* (Chrzanowska *et al*, 1993), *Rhizopus* (Farley and Akasari 1992), and *Humicola* (Aleksieva and Peeva 2000). Although bacterial proteases have long been used in the industry, the main drawback of their use is that they require cost-intensive filtration methodologies to obtain a microbe-free enzyme reparation. On the other hand, proteases of fungal origin offer an advantage that is the mycelium can easily be removed by filtration (Phadatare *et al* 1993).

Pakistan being an agricultural country has a plenty of agricultural by products. So different agricultural by-products such as soybean meal, sunflower meal, rice bran, wheat bran, cottonseed meal and rapeseed meal have been evaluated for the biosynthesis of protease. Soybean meal was reported as a best agricultural by product for the production of protease from *Penicillium griseoroseum* (Haq *et al*., 2004). *Rhizopus oligosporous* was reported to produce protease using rice bran and sunflower meal as best agricultural by products (Ikasari and Mitchell, 1994; Haq *et al*., 2003). Chakraborty *et al.* (2000) reported the production of protease from *A. niger* using wheat bran as a substrate.

The present work was undertaken to produce acidic protease by employing different substrates in submerged fermentation. Various process parameters such as substrate water ratio, nitrogen sources and their different concentrations were optimized to get the maximum yield of enzyme from *Rhizopus arrhizus*.

MATERIALS AND METHODS

Substrate

The whey was used as substrate. It was obtained from Halla Milk Factory, Walton Road Lahore, Cantt. Substrate was boiled and left it for settle down. After one day separate upper and lower layers.

Fermentation Organism

The pure culture of *Rhizopus arrhizus*, isolated from filter paper (dung) was procured form the first fungal culture bank of Pakistan, Institute of Agricultural Sciences, University of the Punjab, Lahore, Pakistan. The culture was revived on Potato Dextrose Agar (PDA) at 37 ℃ and was maintained on PDA (Oxoid) at 4 ℃.

Preparation of Spore Suspension

A spore suspension of 1 $x10^{6-8}$ was prepared by adding 10 mL sterile distilled water in 7 days old slant. The spores were scratched by sterile wire loop to break clumps to form homogenous spore suspension. 5 mL of the spore suspension was used for inoculation.

Optimization of Substrate

Effect of Substrate Water Ratio

Six different substrate water ratio viz 50%, 60%, 70%, 80%, 90% and 100 %, were tested for optimal enzyme production. Screening of substrates was carried out in triplicates in 250 mL Erlenmeyer flasks, each with 50 mL medium. The flasks were agitated at 37 ℃ for 72 hrs on a shaker.

Different Nitrogen Sources

Different agro-industrial residues such as rice soybean meal, cotton seed meals, sunflower meal of optimum concentration (2.5%) were used for the selection of best substrate for acidic protease production. The pH of the medium was adjusted at 5.0 with 1 N HCl/ NaOH before sterilization.

Effect of Nitrogen Source

The production medium supplemented with nitrogen source like cotton seed meal. For the optimization of production medium the experiment was carried out with different concentrations of best source obtained from this experiment, ranging from 1.25 to 5.0 percentages.

Air-Lift Fermenter

The air-lift bioreactor, prepare growth medium (table 1), then attach different equipments for the calibration of Air-lift bioreactor. pH sensor meter introduced in the medium which sense that pH of medium was not change. Water pump for circulation of water from downwards to move upward. DO probe for aeration in the growing medium.

Table 1. Growth medium for Air lift Fermenter

Sr.#	Ingredients	Quantity (ml/g)
1	Whey	950 mL
2	Cotton seed meal	15 gm
3	Inoculum	50 mL
4	Distilled water	Up to 1000 mL mark

*pH = 4 *Temperature = 37 °C

Sample Harvesting

After optimum incubation period, both experimental and control flasks (in each experiment) were harvest. The biomass was filtered through Whatman filter paper No.1 and the filter was centrifuged at 1000 rpm for 15 minutes at 10 °C. The filtrate so obtained was stored in clear, dry and sterilizes glass bottles in refrigerator to prevent the contamination. The filtrate was subjected to enzyme assay.

Enzyme Assay

The method of McDonald & Chen (1965) was used for the assay of proteases. Casein (1%) was incubated with one ml of enzyme sample at 30 degrees C for one hour. The reaction was arrested by the addition of five ml of

trichloroacetic acid (TCA) solution. The mixture was centrifuged and one ml of supernatant was mixed with five ml of alkaline reagent. To this mixture one ml of 1N NaOH was added to make the contents of the tube alkaline. After 10 min., 0.5 ml of Folin and Ciocalteau reagent was added to the test tubes and mixed. The blue colour produced was measured with UV spectrophotometer (CECIL, CE 7200, Cambridge, England) at 700 nm after 30 min. One unit of protease activity is defined as the amount of enzyme required to produce an increase of 0.1 in optical density at 700 nm under defined conditions.

Statistical Analysis

All the data thus obtained were analyzed statistically using ANOVA under CRD and DMR test (Steel and Torrie, 1992).

RESULTS AND DISCUSSION

Effect of Different Concentration of Water

For the optimization of substrate water ratio triplicate flasks i.e. 50%, 60%, 70%, 80%, 90% and 100 % were fermented with *Rhizopus arrhizus* for 72 hours. The samples were harvested and culture filtrate was subjected to protease assay. It was observed that 90% substrate water ratio resulted in maximum protease production 139.48 (IU/mL/min) and 50% substrate level showed the minimum enzyme activity 104.77 (IU/mL/min) of all others ratios tested. The results was also showed that protease activity continuously increased up to 90% substrate level and decrease therefore up to 100% substrate. Analysis of variance of the data showed significant ($P<0.05$) difference between enzyme produced with varying water levels. Comparison of treatment means by Duncan's Multiple Range Test showed that all the treatment means differed significantly ($P<0.05$) as it is clear alphabetically from.

Effect of Nitrogen Sources

Different nitrogen sources i.e., Soybean meal, Cotton seed meal, Sunflower meal, were used in fermentation medium with *Rhizopus arrhizus* for 72 hours. All the treatments were applied in triplicate. Maximum protease activity 95.7 (IU/mL/min) was obtained with cotton seed meal as nitrogen source. It was observed that maximum protease activity was produced with cotton seed meal while it decreases with all other nitrogen sources. Analysis of variance of the data showed significant ($P<0.05$) effect of Cotton seed meal on protease production.

Effect of Cotton Seed Meal Concentration

For the maximum production of protease five different levels of cotton seed meal i.e., 1.25, 1.75, 2.50, 2.75 and 5.0% was used along with 90% whey. After 72 hours, the sample were harvested and subjected to enzyme assay. Results showed that protease production was enhanced by addition of cotton seed meal into the fermentation medium. The maximum activity of protease was observed with 2.5% cotton seed meal whereas growth medium containing 1.25%, 1.75%, 5.0% urea showed lower enzyme activities. Results revealed that 2.5% cotton seed meal was the optimum nitrogen source for protease production 174.50 (IU/mL/min) by *Rhizopus arrhizus* in whey medium. While lowest protease activity showed by medium without nitrogen source 139.21 (IU/mL/min). Results of statistical analysis revealed significant ($P<0.05$) difference in enzyme production with different concentrations of cotton seed meal. Duncan's Multiple Range Test showed that the difference between mean activities of enzyme was significant ($P<0.05$).

DISCUSSION

For thousands of years, man has used naturally occurring microorganisms like bacteria, yeast, fungi and mould used for making different kinds of food. Fungi would normally require suitable condition for their growth. *Rhizopus arrhizus* was cultured on waste potato extract for the production of protease. Different parameters like optimum substrate water ratio, concentration of cotton seed meal, different nitrogen sources were studied for maximum production of protease.

Different isolates of mould culture was evaluated for protease production. Of all the culture tasted maximum production of protease (169.78 IU/mL/min) *Rhizopus arrhizus* was used for the production of protease using submerged fermentation. Different nitrogen sources such as Soybean meal, Cotton seed meal, Sunflower meal for the production of protease. However, Mulimani and Patil (1999) used similarly agriculture by-product for the production of protease using *Aspergillus flavus* as organism of choice. Of all the substrates examined cotton seed meal give maximum enzyme activity (169.78 IU/mL/min). The enzyme production decreased in the following order cotton seed meal (169.78 IU/mL/min)>soybean meal (158.32 IU/mL/min)>sunflower meal (148.48 IU/mL/min).

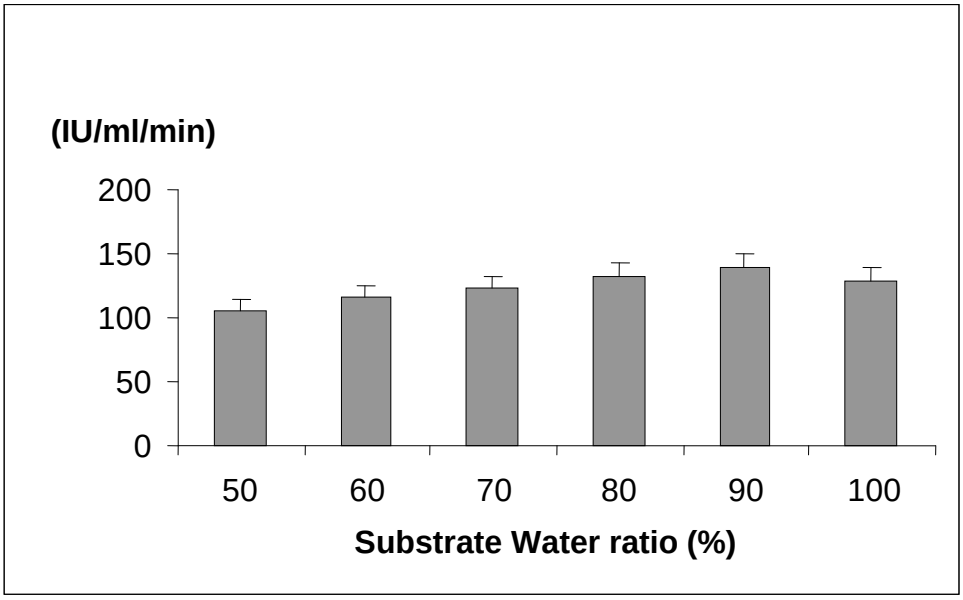

Fig 1 Effect of different substrate water diluents on the production of acid proteases by *R. arrhizus*. (Inc. temperature: 37 ℃; Substrate: whey ; Inc. period: 72 h)

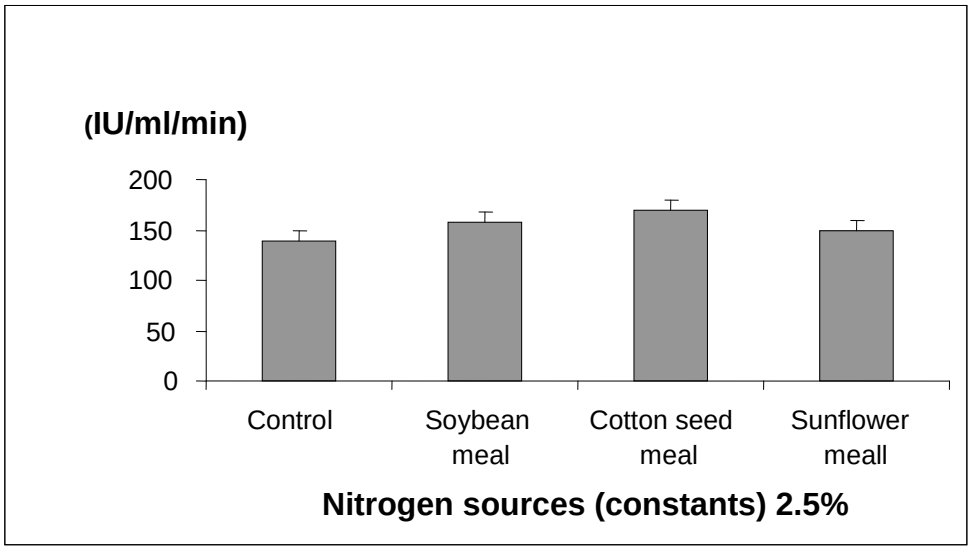

Fig. 2. Selection of substrate for acid proteases production by *R. arrhizus* (Temperature: 37 ℃; Diluent: 90%; Inc. period: 72 h)

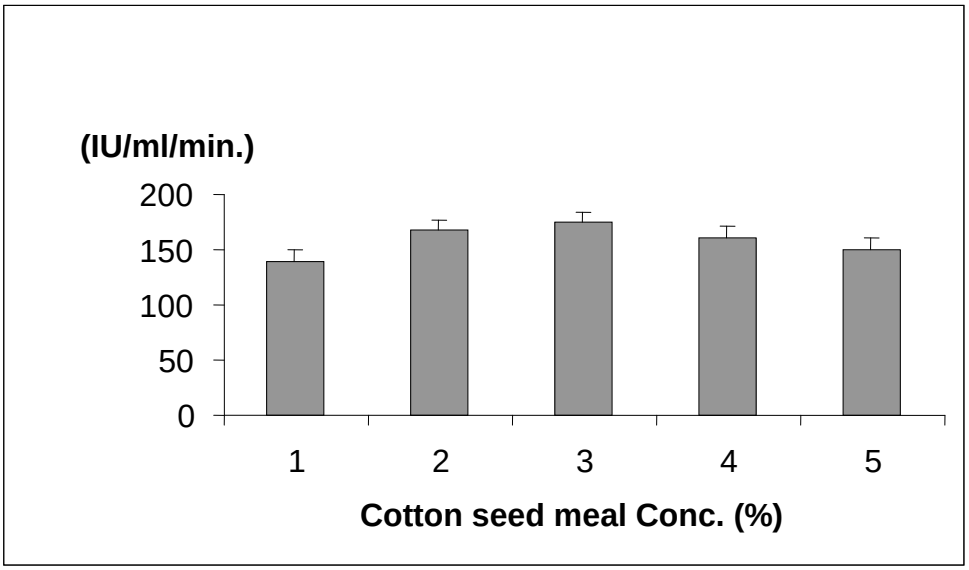

Fig. 3. Effect of different diluents of cotton seed meal for the production of acid proteases by *R. arrhizus* (Inc. temperature: 37 ℃; Substrate: Cotton seed meal; Inc. period: 72 h)

REFERENCES

Aleksieva P and Peeva L. 2000. Investigation of acid proteinases biosynthesis by the fungus *Humicola lutea* 120-5 in an airlift bioreactor. Enzyme Microbiol Technol 26: 402-405.

Bhaskar N, Sudeepa ES, Rashmi HN and Selvi AT: Partial purification and characterization of protease of *Bacillus proteolyticus* CFR3001 isolated from fish processing waste and its antibacterial activities. *Bioresour. Technol*. 2007; 98: 2758-2764.

Chakrabarti, S.K., Matsumura, N. and Ranu, R.S., 2000. Purification and characterization of an extracellular alkaline serine protease from *Aspergillus terreus* (IJIRA 6.2). *Curr. Microbiol.*, 40: 239-243.

Chrzanowska J, Kolaczkowska M and Polanowski A. 1993. Production of exocellular proteolytic enzymes by various species of *Penicillium.* Enzyme Microb Technol. 15:140-143.

Deng A, WU J, Zhang Y, Zhang G and Wen T: Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus* sp. B001. *Bioresour. Technol*. 2010; 101: 7100-7116.

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

Farley PC and Ikasari L. 1992. Regulation of secretion of *Rhizopus oligosporus* extracellular carboxylproteinase. J Gen Microbiol 138:2539-2544.

Haq, I., Mukhtar, H., Butt, Z. and Riaz, N., 2004. Protease biosynthesis by mutant strain of *Penicillium griseoroseum* and cheese formation. *Pak. J. biol. Sci*., 7: 1473-1476.

Haq, I., Mukhtar, H., Daudi, S., Ali, S. and Qadeer, M.A., 2003. Production of proteases by a locally isolated mould culture under lab conditions. *Biotechnology*, 2:30-36.

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

Jellouli K, Bougatef A, Manni L, Agrebi R, Siala R., Younes I and Nasri M: Molecular and biochemical characterization of an extracellular serine-protease from *Vibrio etschnikovii*. *Microbiol.Biotechnol*. 2009; 36: 939-948.

Mcdonald, C.E. and Chen, L.L., 1965. Lowry modification of the Folin reagent for determination of proteinases activity. *Ann. Biochem.*, 10: 175.

Mulimani,V.H. and G.N. Patil, 1999. Production of proteases by *Aspergillus flavus* under solid state fermentation. *Indian J. Exp. Biol*., 37: 1248-1250.
Phadatare SU, Deshpande VV and Srinivasan MC. 1993. High activity of alkaline protease from *Conidiobulus coronatus* (NCL 86.8.20): Enzyme production and compatibility with commercial detergents. Enzyme Microbiol Technol 15:72-76.

Rao, M.B., Aparna, M., Tanksale, Ghatge, M.S. and Deshpande, V.V., 1998. Molecular and biotechnological aspects of microbial proteases. *Microbiol. mol. Biol. Rev.*, 62: 597-635.

state fermentation. *Indian J. Exp. Biol*., 37: 1248-1250.

Steel, R.G.D. and J.H. Torrie. 1984. Principles and Procedures of Statistics, McGrew Hill Book Co. Inc., New York, USA

Temiz, H., Okumus, E., Aykut, U., Dervisoglu, M. and Yazici, F., 2008. Partial purification of pepsin from turkey proventriculus. *Wld. J. Microbiol. Biotech*., 24: 1851–1855.

Tremacoldi, C.R., Watanabe, N.K. and Carmona, E.C., 2004. Production of extracellular acid proteases by *Aspergillus clavatus*. *Wld. J. Microbiol. Biotech*., 20: 639-642.

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