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Production of Milk Clotting Protease by Rhizopus Stolonifer through Optimization of Culture Conditions

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***Abstract*—**The present study describes the biosynthesis of a milk-clotting protease by solid state fermentation (SSF) of a locally isolated mould, *Rhizopus stolonifer*. The production medium was prepared using wheat bran at 50% (w/v). The production conditions are optimized by varying 7 parameters: carbon and nitrogen sources, medium moisture, temperature, pH, fermentation time and inoculum's size. The maximum enzyme synthesis was measured after 96 h of incubation time at temperature of 28°C. The optimum pH determined was 6 and the inoculum size was 3.10^6spores/ml. The optimum initial moisture content is comprised between 50 to 70%. The formation of milk clotting protease is enhanced when galactose and peptone are used at 10% (w/v) and 1% (w/v) concentrations respectively. The maximum production of milk clotting protease is 120 US/ml.

***Keywords*—**Milk clotting activity, protease production, *Rhizopus stolonifer*, Solid state fermentation.

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I. Introduction

ACID proteases are a well known group of proteolytic enzymes; it plays an important role in the food processing industry. They have isolated and characterized from plants [10], [12], [24], fungi, yeasts, and bacteria [2], [5], [31]. However, attention has focused on the production of milk-clotting enzymes (MCE) from microbial sources for use as rennin substitutes. Although there are many microorganisms that can produce MCE; actually, only some strains are used for industrial production: *Rhizomucor miehei* [33], [36], *Rhizomucor pusillus* [4], [18], *Rhizopus sp* [15], [23],[34], [35] and *Enthothia parasitica* [28].

Industrial enzyme production by molds is usually carried out by solid state fermentation (SSF) because it has a better productivity [1], [20], [21], [26], good yields of enzymes, the products in fermentation are relatively dilute and therefore the downstream process results in high vessel [1], [11].

However, the metabolic processes of the microorganisms are influenced by the temperature, pH variations, water content, inoculums concentration, and carbon, nitrogen sources. These conditions vary considerably from species to species [1], [9], [30], [38]. So, it becomes very important to know the environmental conditions of the microorganism for maximum production.

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In this paper we have studied a number of factors which influence the protease production by *Rhizopus stolonifer* through SSF.

II. Materials And Methods

A. Microorganisms and conservation

The mould is isolated from Boumerdes forest (Algeria); it is maintained on potato-dextrose-agar (PDA) plates at 4°C.

B. Inoculum preparation

The cultures of 7 days old are wetted by adding 10 ml of sterile water containing solution of tween 80. The spores were scratched by sterile wire loop to break clumps and obtain homogeneous spore suspension. One ml of spore suspension is used for inoculation.

C. Fermentation procedure

Erlenmeyer flasks of 250 ml containing 5 g of wheat bran moistened with 10 ml of salt solution [24], the flasks are autoclaved at 121°C and after cooling; each flask is inoculated with 1 ml of spore suspension ($3x\ 10^6$ spore/ ml) then incubated for 5 days. The enzyme of each flask is extracted with 50 ml of distilled water, which are taken on rotator shaker for 1h at 200 rpm and then filtered through Whatman filter paper. The extract is centrifuged at 5000 rpm for 15 min at 4°C, the clear supernatant constitute the crude extract.

D. Enzyme assay

Milk-clotting activity is determined according to the method of Arima [4]. The amount of enzyme that clotted 1 ml of 10% skim milk containing 10 mM $CaCl_2$, pH 6.0, in 40 min at 35˚C is defined as one milk-clotting unit.

E. Protein Assay

The protein concentrations of the samples are done by the method of Bradford [7]. Bovine serum albumin is used as the standard.

Optimization of process parameters for the production of protease

Various process variables are studied to monitor their effects on acid protease production in SSF. These are incubation temperature (20, 25, 30, 35, 40, 45°C); pH of medium (4, 4.5, 5, 5.5, 6, 6.5, 7); incubation period (24, 48,

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72, 96, 120,144, 168, 192, 116, 240 h); initial water content of the substrate (1, 2, 3, 4, 5, 6, 10 ml), inoculum size (1, 2, 3, 4, 5 ml) of $3x10^6$ spore suspension, carbon sources (galactose, glucose, fructose, mannose, sucrose, starch, lactose) at 1% concentration. To investigate the effect of nitrogen sources on the MCE production, experiments are carried out with different organic nitrogen sources namely: peptone, yeast extract, malt extract, casein at 1% concentration and different inorganic nitrogen sources, namely ammonium sulfate (NH_3SO_4), ammonium nitrate (NH_4NO_3), ammonium chloride (NH_4Cl), calcium chloride ($CaCl_2$) and dihydrogene phosphate potassium (KH_2PO_4) at 1% concentration.

IV. Results And Discussion

Effect of incubation temperature on protease synthesis in SSF

The enzyme production by *Rhizopus stolonifer* at 25-45°C temperature range revealed that there is an increase in protease production when the incubation temperature is increased from 25°C (11.3US/ml) to 30°C (10.5 US/mL), the optimum incubation temperature for the production of protease is found as 28°C (13.05 US/ml). The enzyme production is sudden decrease when the incubation temperature is increased from 30°C to 45°C (Fig. 1).

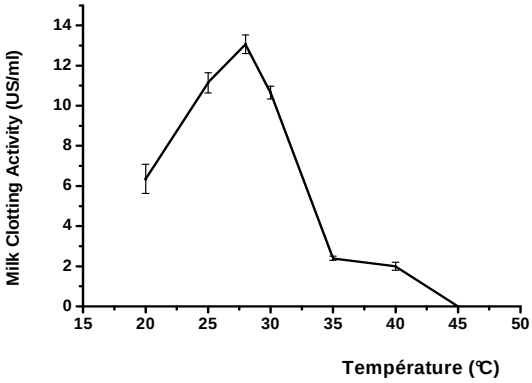


Fig. 1. Effect of incubation temperature on milk-clotting activity of *Rhizopus stolonifer*.

This result is reported by UI-Haq [34, 35], so fungal proteases are usually thermolabile and show reduced activities at high temperatures which have some adverse effects on metabolic activities of microorganism and cause inhibition of the growth of the fungus [38]. However, Sathya [29] reported that the optimal temperature for the production of *Mucor circinelloides* protease is 30°C.

The enzyme is denatured by losing its catalytic properties due to breaking of weak hydrogen bonds within enzyme structure [37].

Effect of initial pH on protease synthesis in SSF

Productivity of the enzyme by mould culture is very much dependant on pH of the fermentation medium [6]. Therefore, the effect of initial pH (4-8) on the production of protease by *Rhizopus stolonifer* is studied. The data on Fig. 2 shows that the organism is an acidophilus and produced maximum amount of protease (6.033 US/ml) at pH 6.0.

Changes in external pH optima alter the ionization of nutrient molecules and reduce their availability to the organism.

Our results are similar to Tunga [38] and Cavalcanti [8], who report that *Rhizopus oryzae* and *Mucor pusillus* protease act optimally at pH 6.00. However, Ul-Haq [35], report an optimum activity at pH 5.0 for *Rhizopus oligosporus*.

Effect of water content and inoculums size for protease production

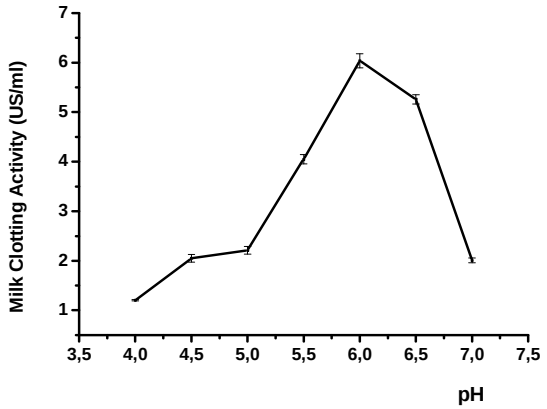


Fig. 2 Effect of medium pH on milk-clotting activity of *Rhizopus stolonifer* (28°C).

Moisture content and inoculums size are the key factors that strongly influence microbial growth and activity in SSF. Filamentous fungi, cultivated on agro-industrial residues during SSF, grow best when the substrate moisture content is generally between 50 and 75% [30].The fermentation was carried out under various initial relative moisture content (22.5-70%).

The results represented in figure 3, show that the optimum activity production (62.41 US/ml) is obtained when the water content is 50, 6 %.

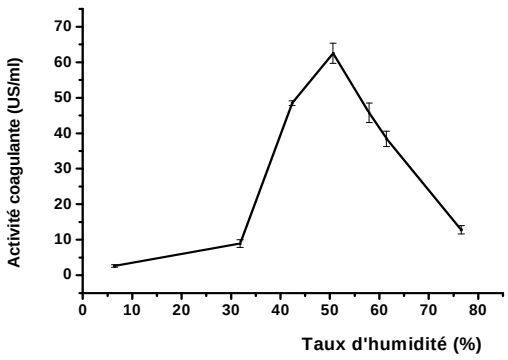


Fig 3. Effect of water content on milk-clotting activity of *Rhizopus stolonifer* (28°C, pH 6).

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This result is in agreement with those of authors, who described the requirement of 55 and 63% initial moisture content for maximum protease production by *Penicillium* LPB-9 [19] and *A. flavus* [3, 13]. Higher moisture levels are not suitable for enzyme production because it caused the decrease of enzyme activity, due to scum formation and decreased aeration [34, 35].

Importance of inoculums size on microbial fermentation processes is widely studied. An inoculums size of 1 ml containing $3x10^6$ spores is found to be optimal for protease production by *Rhizopus stolonifer* with 102.12 US/ml (Fig 4).

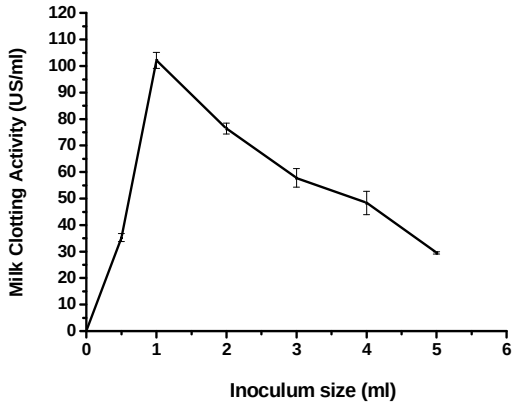


Fig. 4. Effect of inoculums size on milk-clotting activity of *Rhizopus stolonifer* (28°C, pH 6, 50.6%).

A decrease in enzyme production is noted when the inoculums size increased, which is due to the shortage of nutrients available for the larger biomass and faster growth of the culture [34].

The results are similar to that found by UI-Haq [35], which reports that the milk clotting activity of *Rhizopus oligosporus* is optimal when the inoculums volume is between 0.5-2 ml containing 10^6 spores/ml.

However, the studies of El-Safey [16] and Sathya [29] show that *Bacillus subtilis* and *Mucor circinelloides* protease production are optimal for inoculums size of $7x10^3$ spores/ml and $3x\ 10^9$ spores/ml respectively.

- *Effect of supplementation of carbon and nitrogen sources*.

The impact of supplementation of external carbon and nitrogen sources on protease production are studied and the results are shown in Fig 5 and Fig 6.

All the carbon sources are found to enhance the protease production, but *Rhizopus stolonifer* had a preferential choice toward galactose, lactose and glucose wich produced high titers of enzyme (58.53 US/ml., 52.74 US/ml and 48.97 US/ml) respectively.

Addition of starch resulted in decrease on protease activity (362.59 UP/ml) against control (418.44 UP/ml).

The result is similar to that report by Odeniyi [25], which found that the best carbon sources for the production of *Rhizopus stolonifer* protease are galactose, lactose and tréhalose. However, for *Bacillus subtilis* and *Mucor circinelloides* proteases, lactose and sucrose are the best carbon sources [16, 29].

Addition of nitrogen sources to the medium showed maximum production with peptone (125 US/ml) followed by casein (109.31 US/ml) as organic nitrogen source (Fig 6). Malt and beef extract have no effect on protease production (76.49 and 61.63 US/ml) respectively compared to control (77.42 US/ml). However, the addition of inorganic nitrogen sources such as ammonium chloride and potassium nitrate (41.81 US/ml and 26.26 US/ml) respectively cause a decrease in protease production compared to control (77. 42 US/ml).

Our results are similar to those reported by Dutt [14]; Sathya [29] and Phadatere [27] that showed that casein and pepton are inducers of the protease production of *Bacillus subtilis*, *Mucor circinelloides* and *Conidiobolus coronatus* respectively.

Effect of incubation time of fermentation

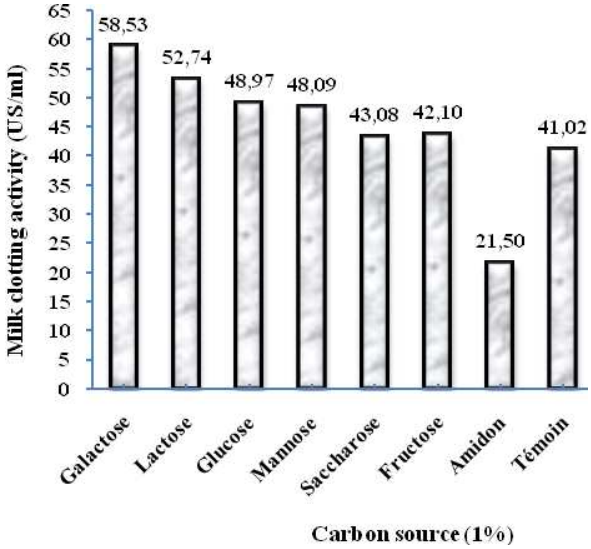


Fig. 5. Effect of carbon sources on milk-clotting activity of *Rhizopus stolonifer* (28°C, pH 6, 50.6%, 3×10^6 spores/ml).

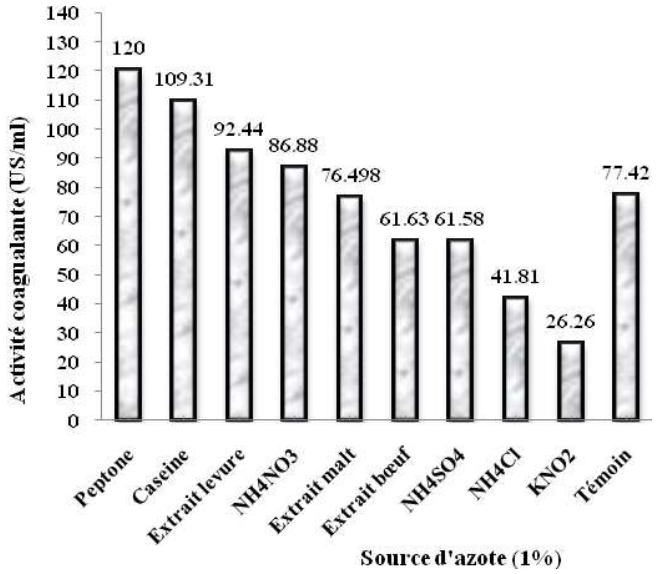


Fig. 6. Effect of nitrogen sources on milk-clotting activity of Rhizopus stolonifer (28°C, pH 6, 50.6%, 3 × 106 spores/ml, 10% galactose).

Time course for the production of protease by *Rhizopus stolonifer* was studied in solid state flasks. The enzyme production was gradually increased with the passage of time and highest enzyme activity (124.67 US/ml) was obtained after 96 h of incubation, it's obviously prolonged incubation time decreases after this time, the enzyme activity reaches 53.69 US/ml (see figure 7).

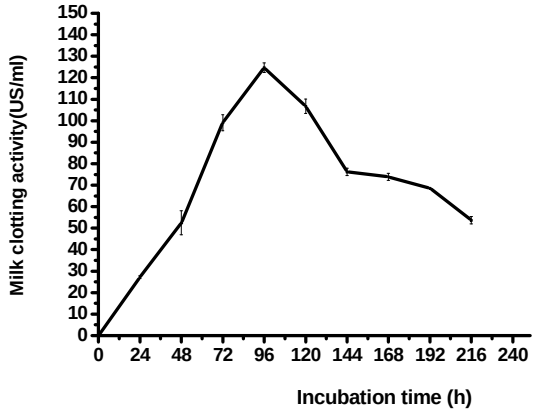


Fig. 7. Effect of incubation time on milk-clotting activity of *Rhizopus stolonifer* (28°C, pH 6, 50.6%, 3 × 10^6 spores/ml**,** 10% galactose, 1% peptone).

Our results are consistent with work reported by Fernandez-Lahore [18] and Barnett & Escobar [17], for *Mucor sp* protease that optimal activity is achieved after 4 days of incubation.

However, optimal production of *Nocardiopsis sp* and *Mucor circinelloides* is observed after 56 hours and 5 days respectively [8, 29].

The incubation period is directly related with the production of enzymes and other metabolites up to a certain extent, after that the enzyme production and growth of the microorganism decreases, which can be attributed to the reduced availability of nutrients and the production of toxic metabolite [6].

IV. Conclusion

Rhizopus stolonifer proved potent producer of protease by solid state fermentation. The enzyme production was considerably enhanced under the set of conditions optimized in this study and remarkable milk-clotting activity is obtained at 124.67 US/ml.

References

[1] A.L. Abdullah, and M.V.G. Tengerdy, "Optimization of solid substrate fermentation of wheat bran", Biotech. Bioengin. vol. 27, pp. 20-27, 1985.
[2] C.N. Aguilar, G.G. Sanchez, P.L.A.R. Barragan, R.R. Herrera, J.L.M. Hernandez, and J.C. Esquivel, "Perspectives of solid state fermentation for production of food enzymes", Americ. J. Biochem .Biotechno., vol 4, no 4, pp. 354-366, 2008.
[3] D. Anandan, W.N. Marmer, and R. L. Dudley,"Isolation characterization and optimization of culture parameters for production of an alkaline protease isolated from Aspergillus tamari ", J . Ind. Microbiol. Biotechnol., vol. 34, pp. 339-347, 2007.
[4] K. Arima, J. Yu, and S. Iwasaki, "Milk-clotting enzyme from Mucor pusillus var.Lindt", Metho. Enzymo., vol. 19, pp. 446-459, 1970.
[5] M.J. Bailey, M. Siika-aho, "Production of microbial rennin", Biotech. Letters., vol. 10, no. 3, pp. 161-166, 1988.
[6] B. Botton, A. Breton, M. Fevre, S. Gauthier, P. Guy, J.P. Larpent, P. Reymond, J.J. Sanglier, Y. Vayssier, and P. Veau, « Moisissures utiles et nuisibles importance industrielle », Collec Biotech., pp. 512, 1990.
[7] M.M Bradford, "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding", Anal. Biochem. Vol. 72, no. 1-27, pp. 248-254, 1976.
[8] M. T. H. Cavalcanti, C. R. Martinez, V. C. Furtado, B. B. Neto, M. F. Teixeira, F. J. L. Lima, and A. L. F. Porto, "Milk-clotting protease production by Nocardiopsis sp. in an inexpensive medium", World. J. Microb. Biotech., vol. 21, pp. 151-154, 2005.
[9] P.S. Channe, and J.G. Shewale, "Influence of culture conditions on the formation of milk-clotting protease by Aspergillus niger MC4", World. J. Microbio. Biotech., vol. 14, pp. 11-15, 1998.
[10] S. Chazarra, L. Sidrach, D. Lopez-Molina, and J. N. Rodrıguez-Lopez, "Characterization of the milk-clotting properties of extracts from flowers artichoke (Cynara scolymus, L.)", Intern. Dairy. J., vol. 17, pp. 1393–1400, 2007.
[11] S.R. Couto, and M. A. Sanroma, "Application of solid-state fermentation to food industry", J. Food. Engineer., vol. 76, pp. 291-302, 2006.
[12] M.U. Dahot, M.Y. Khan, and A. N. Memon, "Screening of some Pakistani plants for milk clotting activity", J. Islamic. Acad. Scienc., vol. 3, no. 4, pp. 284-286, 1990.
[13] R. Davidson, A. Gertler, and T. Hofmann, "Aspergillus oryzae acid proteinase purification and properties and formation of 7r-Chymotrypsin", J. Biochem., vol. 147, pp. 45-53, 1975.
[14] K. Dutt, P. Gupta, S. Saran, M. Swati, and R. Kumar Saxena, "Production of milk-clotting protease from Bacillus subtilis". Appl. Biochem. Biotech., vol. 158, no.3, pp. 761-772, 2009.
[15] A.M. Jurus, and W.J. Sundberg, "Penetration of Rhizopus oligosporus into soybeans in tempeh", Appl. Environ. Microbio. Vol. 32, no. 2, pp. 284-287, 1976.
[16] E.M. El-Safey, and U.M. Abdul-Raouf, "Production, purification and characterization of protease enzyme from Bacillus subtilis". Interna. Confer. Develop. Environ. In The Arab World., pp. 14, 2004.
[17] J. Escobar, and S. M. Barnett, "Effect of agitation speed on the synthesis of Mucor miehei acid protease", Enzym. Microbio. Techno. ,vol. 15, pp. 1009-1013, 1993.
[18] H. M. Fernandez-Lahore, R. M. Auday, E. R. Fraile, O, Cascone, M. Biscoglio de jimenez.Bonino, L. Pirpignani and C. Machalinski, « Purification and characterization of an acid proteinase from mesophilic Mucor sp on solid state cultures", J. Pept. Res., vol. 53, pp. 1-7, 1997.

Open Science Index, Nutrition and Food Engineering Vol:3, No:6, 2009 publications.waset.org/15170/pdf

[19] H. Hashimoto, Y. Kaneko, T. Iwaasa, and T. Yokotsuka,. "Production and Purification of Acid Protease from the Thermophilic Fungus Penicillium duponti K1014", Appl. Microbio., vol. 25, no. 4, pp. 584-588, 1973
[20] C.W. Hasseltine, "Solid state fermentation". Biotech. Bioeng., vol. 14, pp. 517-532, 1972
[21] G. V. González, E.F. Torres, C.N. Aguilar, S.J.R. Gomez, G. D. Godınez, and C. Augur, "Advantages of fungal enzyme production in solid state over liquid fermentation systems", Biochem. Engin. Journal., vol. 13, pp. 15-167, 2003.
[22] A. Kazemi-Vaysari, A. Kheirolomoom, M. Arjmand and M. Habibollahi,. Optimization of Mucor miehei rennin production and recovery. Scientia. Iranica. 9 (1): 99-104. 2002.
[23] S. Kumar, N.S. Sharma, M.R. Saharan, and R. Singh, "Extracellular acid protease from Rhizopus oryzae purification and characterization", Process. Biochem., vol. 40, pp. 1701-1705, 2005.
[24] S. Kumar, J. Sharma, A. Kumar Mohanty, S. Grover, V. Kumar Batish, "Purification and characterization of milk clotting enzyme from goat (Capra hircus)", Comparative. Biochem. Physio., vol. 145, pp. 108-113, 2006.
[25] O. A. Odeniyi, A. A. Onilude, and M. A. Ayodele, "Growth and substrate utilization patterns of a Rhizopus stolonifer strain isolated from depolymerising rice husk", World. Appl. Scien. J., vol. 6, no. 5, pp. 595-599, 2009.
[26] A. Pandey, "Solid-state fermentation", Biochem. Engin., vol. 13, pp. 127–135, 2003.
[27] S. U. Phadatare, M. C. Srinivasan, and V. V. Deshpande, "High activity alkaline protease from Conidiobolus coronatus (NCL 86.8.20):enzyme production and compatibility with commercial detergents", Enzym. Microbio. Techno., vol. 15, pp. 72-76, 1993
[28] J. L. Sardinas, "Rennin enzyme of Endothia parasitica". Appl. Microbiol., vol. 16, pp. 248–53, 1968.
[29] R. Sathya, B.V. Pradeep, J. Angayarkanni, and M. Palaniswamy. Production of milk clotting protease by a local isolate of Mucor circinelloides under SSF using agro-industrial wastes. Biotechn. Bioprocess. Engin., vol. 14, pp. 788-794, 2009
[30] R. A. Sparringa, M. Kendall, A. Westby, and J.D. Owens, "Effects of temperature, pH, water activity and CO2 concentration on growth of Rhizopus oligosporus NRRL 2710", J. Appl. Microbio., vol. 92, pp. 329-337, 2002.
[31] A. Sumantha, C. Larroche, and A. Pandey, "Micorbiology and Industrial Biotechnology of food-grade proteases: A perspective". Food. Technol.Biotechnol., vol. 44, no. 2, pp. 211-220, 2006.
[32] A. Sumantha, P. Deepa, C. Sandhya, C. Szakacs, R. C. Soccol, and A. Pandey, "Rice bran as a substrate for proteolytic enzyme production", Brazilian . Arch . Biol.Technol., vol. 49, no.5, pp. 843-851, 2006.
[33] M. Z. Sternberg, "Crystalline Milk-Clotting Protease from Mucor miehei and some of its Properties". J. Dairy. Scien., vol. 53, no. 2, pp. 159-167, 1970.
[34] I. Ul-Haq, H. Mukhtar, S. Daudi, S. Ali, and M.A. Qadeer, "Production of protease by locally isolated mould culture under lab conditions". Biotechno., vol. 2, no. 1, pp. 30-36, 2003.
[35] I. Ul-Haq, and H. Mukhtar, "Biosynthesis of protease by Rhizopus oligosporus IHS13 in low-cost medium by solid state fermentation", J. Basic.Microbiol., vol. 44, pp. 280-287, 2004.
[36] Z. A. Tubesha, and K. S. AL-Delaimy, "Renin-like milk coagulant enzyme produced by local isolated of Mucor", Intern. J. Dairy. Techno., vol. 56, no. 4, pp. 237-241, 2003.
[37] R. Tunga, "Influence of Temperature on Enzyme Production", Tech.M. Thesis, II. T. Kharagpur, India, 1995.
[38] R. Tunga, R. Banerjee, and B. C. Bhattacharyya, "Optimizing some factors affecting protease production under solid state fermentation", Bioprocess . Engin., vol. 19, pp. 187-190, 1998.

Open Science Index, Nutrition and Food Engineering Vol:3, No:6, 2009 publications.waset.org/15170/pdf