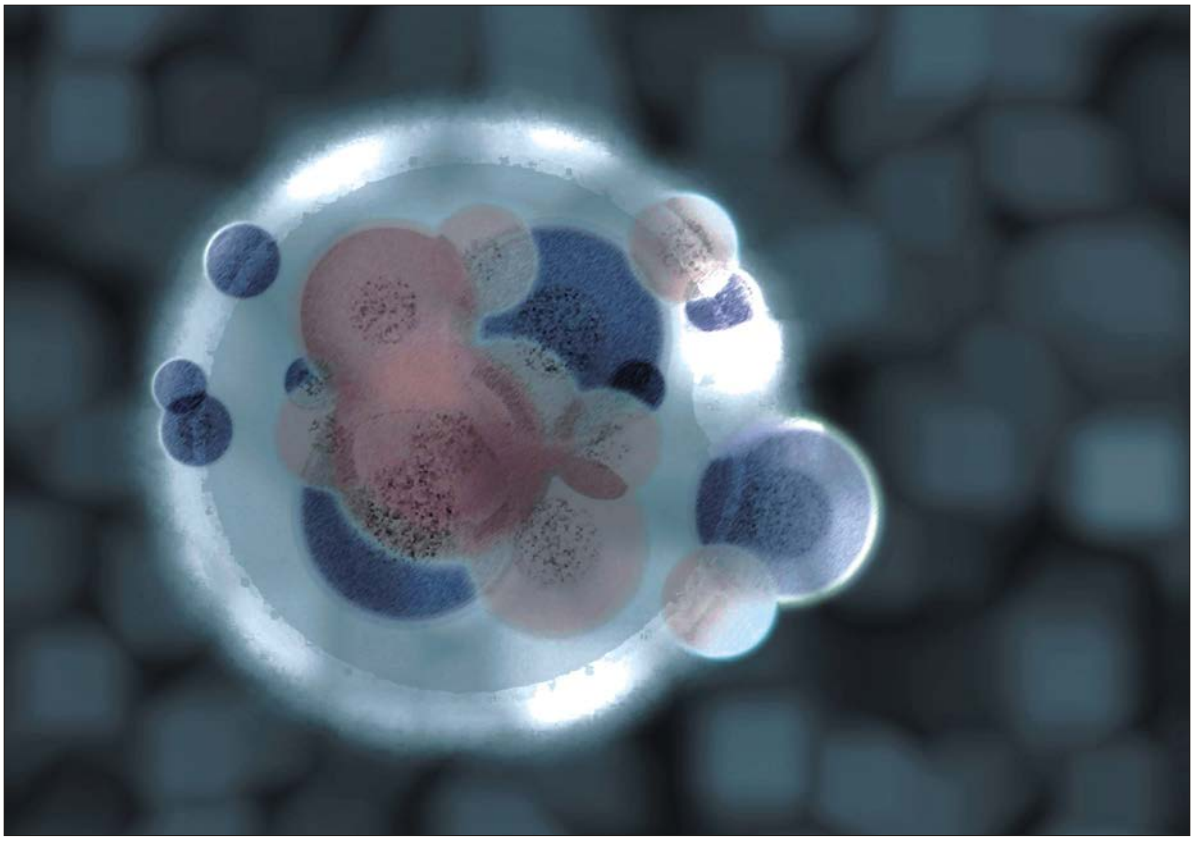

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Proteolytic Activity of a Bacterial Isolate *Bacillus fastidiosus* den Dooren de Jong

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Abstract: Following the enrichment technique in total 25 microbial colonies were isolated, purified, preserved and tested for their proteolytic ability. Among the bacterial isolate D_1 showed the maximum ability to degrade egg albumin, milk casein and gelatin. On the basis of cultural, morphological, physiological and biochemical characteristics the isolate D_1 was identified as *Bacillus fastidiosus* den Dooren de Jong. The isolate (D_1) produced maximum protease at 37°C temperature and pH 7.0. Cellobiose (carbon source) and $(NH_4)_2HPO_4$ (nitrogen source) were found to induce the protease production of the isolate and showed highest proteolytic activity at temperature 35°C and pH 6.5.

Key words: *Bacillus fastidiosus*, proteolytic activity

INTRODUCTION

The core of traditional biotechnology is the use of enzymes. Enzymes are widely used in several industries, notably in detergent, food processing, brewing and pharmaceutical industries. They are also used for diagnostic, scientific and analytical purposes. Enzymes are potentially of great use because of both their enormous catalytic activity and high degree of specificity. The use of microorganisms to produce enzymes has a number of technical and economic advantages and in recent years has become the predominant mode of enzyme production. During production of enzymes, the nature of the whole process must be known before attempt can be made to optimize any one step because a particular step may influence the stability of other methods to be used. For this emergence, in recent years novel techniques of enzyme extraction and purification promise markedly to improve such processes. Scientist now searching for such methods of optimum conditions for maximum enzyme production, extraction and purification[1-5]. At present, the economically most important industrial enzymes are extracted from bacteria (*Bacillus* sp, *Staphylococcus* sp., *Pseudomonas* sp.), fungi (*Aspergillus* sp., *Candida* sp., *Saccharomyces* sp.) and actinomycetes (*Streptomyces* sp.). Over 300 tons of enzymes are being annually produced from *Bacillus* sp. and most of them are proteases[6]. Microbial proteases play an important role in biotechnological processes accounting for approximately 59% of the total enzymes used[7].

MATERIALS AND METHODS

Isolation, purification and screening of protease producing microorganisms: Enrichment and nutrient agar media were used during isolation period. The isolates were purified through repeated plating method in nutrient agar medium. The purified bacterial isolates then transferred to the nutrient agar slants and then preserved as stock culture. The primary screening was done by the hydrolysis of Egg Albumin, Skimmed Milk Casein and Gelatin. The isolate was finally selected by observing its activity in liquid medium.

Protease production in liquid culture medium: Tryptone-dextrose-yeast extract broth medium (Tryptone 1%, dextrose 0.1%, yeast extract 0.5%)[8] was used for protease production by the isolate. Media was inoculated with 2 days old culture of the isolate and incubated at 37°C for 1 to 2 days. After incubation, the culture medium was filtered and centrifuged at 12,000 rpm for 15 min. Thus the cell free supernatant was used as crude enzyme and stored at 4°C with few drops of toluene.

Protease activity measurement: Protease activity was measured by the modified method of Hayashi *et al.*[9] as followed by Meyers and Ahearn[10] and the absorbance of the solution was measured at 650 nm in a spectrophotometer and calculated the amounts of tyrosine released in the reaction from a standard curve plotted from known concentration of tyrosine. The enzyme activity was expressed in unit. One unit of

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enzyme was defined as the amount of enzyme that released 1 μg of tyrosine ml^{-1} of crude extract h^{-1}.

Identification of selected isolate: For the identification of selected isolate different morphological characteristics and physiological behaviors were observed.

Biomass yield: Bacterial biomass can be measured by measuring the absorbance at 600 nm[11].

Culture conditions determination for maximum production of protease: To observe the effects of cultural condition for maximum protease production, conical flask containing 50 ml of selected suitable medium with selected pH (such as 5.0, 6.0, 7.0, 8.0 and 9.0) were incubated at different incubation periods (1, 2, 3 and 4 days) at different temperatures (30±2, 37±2 and 45±2°C). The effects of temperature, pH and incubation periods on biomass characteristic, biomass yield and protease production was recorded. The production of proteases under different carbon and nitrogen availability were also studied in the liquid culture medium[8] containing four carbon (glucose, lactose, cellulose, cellubiose) and nitrogen [peptone, tryptone, KNO_3, $(NH_4)_2HPO_4$] sources and the effects of these carbon and nitrogen sources were recorded.

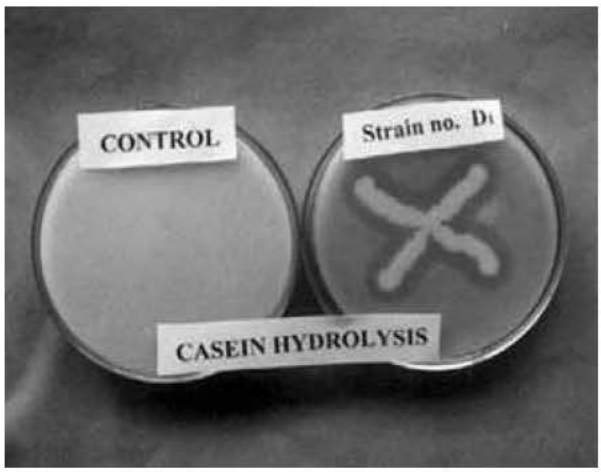
Optimum conditions determination for the crude enzyme activity: The isolate D_1 (*Bacillus fastidiosus*) was allowed to grow in optimized medium with its optimum medium pH 7.0 and incubation temperature 37°C. After incubation, the crude enzymes were collected and studied at various temperature (30, 35, 40 and 45°C) and pH (5.5 to 8.0).

RESULTS AND DISCUSSION

Twenty five isolates were collected from various proteinaceous sources and then purified, preserved and tested for their proteolytic ability (Fig. 1 and 2). The isolates, which showed proteolytic ability in liquid culture media were finally selected for further studies. Of these the bacterial isolate D_1, showed higher proteolytic activity and was selected for detail studies.

Identification of the selected isolates: The isolate (D_1) was characterized on the basis of their morphological, cultural and biochemical properties (Table 1). All these properties were then compared with the standard characteristics described in Bergey's Manual of Determinative Bacteriology (8th Edn.). The bacterial isolate D_1 was found to belong to the genus *Bacillus* and was provisionally identified as *Bacillus fastidiosus* den Dooren de Jong.

A



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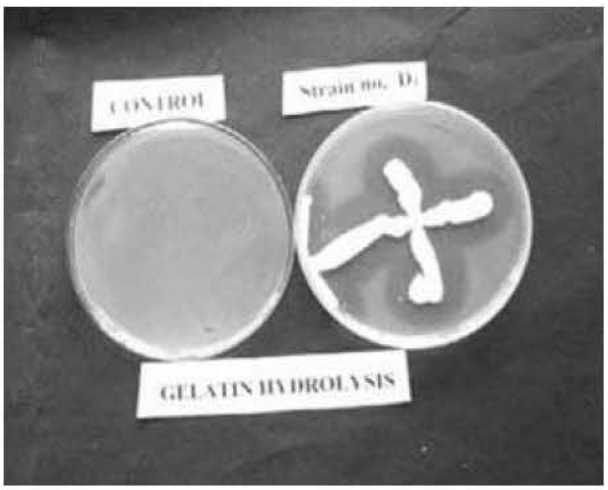

Fig. 1: Primary screening by casein hydrolysis (A) and gelatin hydrolysis (B) method by the isolate D_1 (*Bacillus fastidiosus*)

Effects of culture conditions on protease production

Incubation period: Table 2 showed the effect of incubation period on the biomass yield of the isolate D_1. The biomass increased with the increase of the incubation period. The highest biomass was recorded after 4 days of incubation period. The maximum enzyme activity was recorded after 1 day of incubation period (Fig. 3). The pH (6.2 to 7.8) of different media was changed with incubation period.

Temperature and pH: Table 3 showed the change of pH at different incubation temperature and the change varies from 6.0-6.4, 6.2-7.5 and 6.2-7.9 at 30, 37 and 45°C, respectively. The maximum enzyme production was recorded at pH 7.0 and at 37°C temperature (Fig. 4).

Table 1: Morphological, cultural and biochemical characteristics of the bacterial isolate D_1

Vegetative cells	:	Short rod, single to short chain. Size 2.6-0.3 μm							
Spore staining	:	Spore formed, central							
Gram staining	:	Positive							
Acid fast staining	:	Not acid fast							
Motility test	:	Positive							
Catalase test	:	Positive							
Oxygen relationship	:	Aerobic							
Indole test	:	Not formed							
Nitrate reduction test	:	Positive							
H_2S production	:	Positive							
Casein hydrolysis	:	Hydrolysed the skimmed milk casein							
Methyl red test	:	Positive							
Voges- Proskaur test	:	Negative							
Starch agar plate	:	Hydrolysed							
Growth at different temperature	:	10°C	27°C	30°C	37°C	45°C			
		-	+++	+++	++++	+			
Growth at different NaCl concentration (%)	:	0	1	2	3	4	5	6	7
		+++	++++	++++	++++	+++	++	+	-
Gelatin hydrolysis	:	Positive							
Urease test	:	Positive							
Oxidese test	:	Negative							
Fermentation of different carbohydrates	:	No change in Glucose Fructose, Xylose, Arabinose, Raffinose, Inulin, Rhamnose, Mannitol, Lactose, Galactose, Starch, Sucrose, Glycerol, Cellulose.							
Identification	:	Above characteristics indicates that the isolate D_1 belongs to the genus *Bacillus* and closely related to the species *Bacillus fastidiosus* den Dooren de Jong, 1929.							

Table 2: Effect of incubation period on the biomass yield by the isolate D_1

	Colour and pH of the medium after incubation period			
Incubation period (in days)	Colour	pH	Biomass characteristics	Biomass yield (absorbance at 600 um)
1	Sand gold	6.2	Membranous growth	0.96
2	Primrose	6.6	,,	1.08
3	Pale lemon	7.5	Sedimentation occur	1.43
4	,,	7.8	Profuse sedimentation	1.95*

Note: Initial colour of the medium was Golden brown, pH 6.5 and incubation temperature 37°C, during enzyme substrate reaction phase temperature 35°C, pH 5.5, *Maximum biomass yield

Table 3: Effects of incubation temperature and pH on the biomass yield by the isolate D_1

	pH of the medium before/ after inoculation			
Incubation temperature	Before	After	Biomass characteristics	Biomass yield (mg g^{-1} of protein)
30°C	5.0	6.0	Membranous growth	7.32
	6.0	6.3	"	7.69
	7.0	6.2	Sedimentary growth	8.09
	8.0	6.3	"	8.13
	9.0	6.4	"	8.20*
37°C	5.0	6.2	Membranous growth	7.73
	6.0	6.3	"	7.89
	7.0	7.2	"	8.10
	8.0	7.5	Sedimentation occur	8.23
	9.0	7.4	"	8.67*
45°C	5.0	6.9	Scanty growth	0.10
	6.0	6.2	"	0.01
	7.0	6.2	Moderate growth	0.10*
	8.0	7.9	"	0.09
	9.0	7.8	Scanty sedimentary growth	0.08

Note: During enzyme substrate reaction temperature 35°C, pH 5.5, *Maximum biomass yield

Table 4: Effects of the carbon and nitrogen sources on the biomass yields by the isolate D_1 (*Bacillus fastidiosus*)

	Biomass yields (mg g^{-1} of protein) Carbon sources			
Nitrogen sources	Glucose	Lactose	Cellulose	Cellobiose
Peptone	8.1	7.7	7.9	8.1
Tryptone	8.9	8.6	8.7	9.2*
KNO_3	4.6	5.0	4.3	5.1
$(NH_4)_2HPO_4$	5.8	5.9	6.1	7.4

Note: Incubation period = 1 day, Incubation temperature=37°C, Initial medium pH= 7.0

Carbon and nitrogen sources: The maximum biomass yield was recorded with cellubiose and tryptone as carbon and nitrogen sources (Table 4), whereas maximum protease production was recorded with cellubiose and $(NH_4)_2HPO_4$ as carbon and nitrogen sources of the medium, respectively (Fig. 5). Present results suggest that use of $(NH_4)_2HPO_4$ and cellubiose induced the protease production by the isolate D_1 (*Bacillus fastidiosus*).

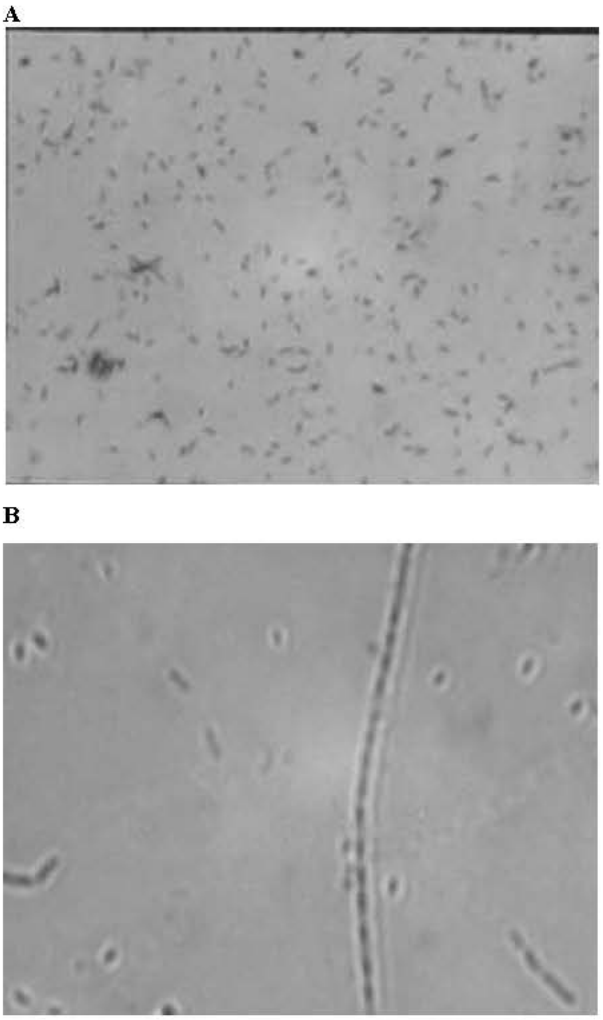

Fig. 2: Microscopic feature of the isolate showing vegetative cells (A) and endospore and sporangium (B) under 12xX40x D_1 (*Bacillus fastidiosus*)

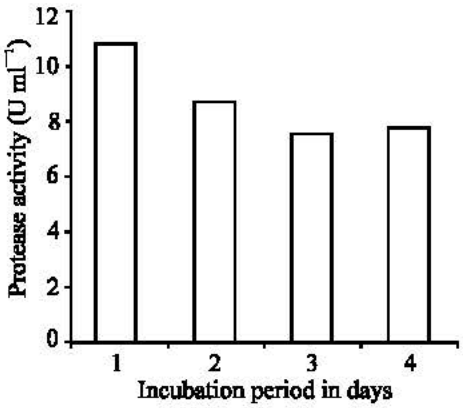

Fig. 3: Effect of incubation periods on protease production by isolate D_1

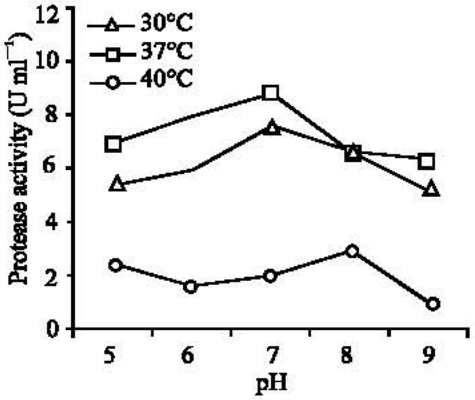

Fig. 4: Effect of pH and temperature on production of protease by the isolate D_1, grown in liquid culture with $(NH_4)_2$ HPO_4 1%, cellubiose 0.1%, yeast extract 0.5%

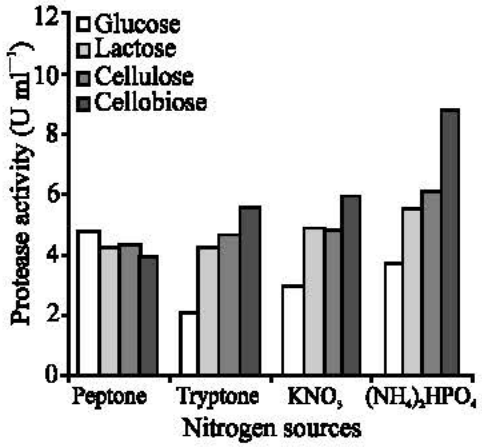

Fig. 5: Effects of carbon and nitrogen sources on protease production by isolate D_1

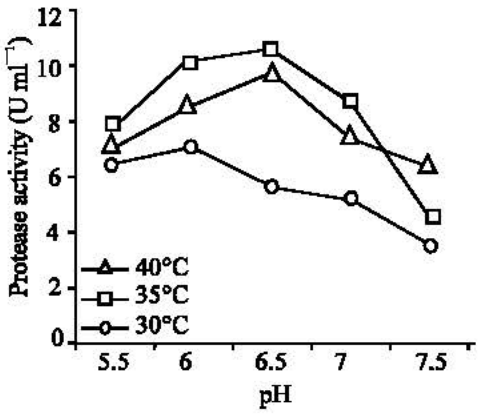

Fig. 6: Effects of pH and temperature on protease activity of isolate D_1

Similarly, induction of protease production with carbohydrate was also reported by many workers[12-15]. Besides these, effects of protease production by different carbon and nitrogen sources were reported by Ogrydziak and Yamada[16], Kalisz *et al.*[17] and Michael[18].

Determination of optimum conditions for the maximum crude protease activity: Figure 6 showed the effect of temperature and pH on the crude enzymes activity of the selected isolate D_1. From the Fig. 6 it was found that the enzyme activity was highest at 35°C with the reaction pH 6.5. But the activity was also found better at temperature 40°C and pH 6.5. Similar protease activity at acid to neutral pH was reported by many workers[4,19-25].

REFERENCES

1. Rao, M.B., A.P. Tanksale, M.S. Ghatge and V.V. Deshpande, 1998. Molecular and biotechnological aspects of microbial proteases, Microbiol. Mol. Biol. Rev., 62: 597-635.
2. Alvarez-sanchez, M.E.A., L. Avila-Gonzalez, C. Becerril-Garcia, L F. Fattel-Facenda, J. Ortega-Lopes and R. Arroyo, 2000. A novel cysteine proteinase (CP65) of *Trichomonas vaginalis* involved in citotoxicity. Microbial. Pathogenesis, 28: 193-202.
3. Gianna, P., B. Carmen, C. Giovanna, G. Paola, M. Gennaro, M. Maria and S. Giovanni, 2001. Purification, Characterization and Functional Role of a Novel Extracellular Protease from *Pleurotus ostreatus*. Applied Environ. Microbiol., 67: 2754-2757.
4. Thangam, B.E. and R.G. Suseela, 2002. Purification and characterization of alkaline protease from Alcaligenes faecalis. Biotechnol. Appl. Biochem., 35: 149-154.
5. Shumi, W., Md. Towhid Hossain and M.N. Anwar, 2004. Isolation and purification of *Aspergillus funiculosus* G. Smith and its enzyme protease. Pak. J. Biol. Sci., 7: 312-317.
6. Kumar, H.D., 1998. A text book of biotechnology. Affiliated East-West press private limited. New Delhi, India, pp: 173.
7. Maugh, T., 1984. Need a catalyst? Design an enzyme, Sci., pp: 269-271.
8. Matta, H., V. Punj and S.S. Kanwar, 1997. An immuno-dot blot assay for detection of thermostable protease from *Pseudomonas* sp. AFT-36 of dairy origin. Appl. Microbial., 25: 300-302.
9. Hayashi, L.D., D. Fukushima and K. Mogi, 1967. G.P. Agarwal and K. S. Bilgrami (Ed.). Today and tomorrow's printers and Publishers, New Delhi, India, pp: 194.
10. Meyers, S.P. and D.G. Ahearn, 1977. Mycologia, 69: 646-651. cited from Physiology and Parasitism. G.P. Agarwal and K.S. Bilgrami (Ed.). Today and tomorrow's printers and Publishers, New Delhi, India, pp: 195.
11. Henriette, C., S. Zinebi, M.F. Aumaitre, E. Petitdemange and H. Petitdemange, 1993. Protease and lipase production by a strain of *Serratia marcescens*. J. Industrial Microbiol., 12: 129-135.
12. Sabita, R.R., M.M. Rahman and N. Choudhury, 1995. Extracellular proteolytic activity of *Saccharomyces cerevisiae* strain DSM 1848. Bangladesh J. Microbiol., 12: 45-49.
13. Suseela, R., 1998. Hydrolysis of chrome- tanned leather wastes and production of alkaline protease BBY A *Penicillium* species. International Symposium on Ecology of Fungi, Goa University, Goa-403 206, India.
14. Hossain, M.T., S. Akter, M.S. Rahman, M.A. Manchur and M.N. Anwar, 1999. Isolation of protease producing fungi and their protease activity. The Chittagong Univ. J. Sci., 23: 101-106.
15. Marzan, L.W., Md. Abul Manchur, Md. Towhid Hossain and M.N. Anwar, 2002. Protease production by a strain *Aspergillus funiculosus*. Bangladesh J. Life Sci., (In press).
16. Ogrydziak, D.M. and T. Yamada, 1983. Extracelluar acid proteases produced by *Saccharomycopsis lipolytica*. J. Bacteriol., 154:23-31.
17. Kaliz, H.M., D. Moore and D.A. Wood, 1986. Protein utilization by basidiomycete fungi. Trans. Brit. Mycol. Soc., 86: 519-525.
18. Micales, J.A., 1992. Proteinases of the brown rot fungus *Postia placenta*. Mycologia, 84: 815-822.
19. Fermor, T.R. and D.A. Wood, 1981. Degradation of bacteria by *Agaricus bisporus* and other fungi. Gen. Microbial, 126: 377-387.
20. Matsushima, K., M. Hayakawa, M. Ito and K. Shimada, 1981. Features of the proteolytic enzyme system of hyper-acid-productive and non-acid-productive fungi. J. Gen. Appl. Microbial., 27: 423-426.
21. Kawai, M., 1973. Hydrolytic enazymes of basidiomycetes. Productivity of prolytic enzymes and distrubution of its milk clotting activity among the Basidiomycetes. J. Agric. Chem. Soc. Japan, 47: 467-472.
22. Groninger, H.S. and M. W. Ekland, 1966. Characteristics of a proteinase of a *Trichosporon* species isolated from Dungeness crab meat. Appl. Microbiol., 14: 110-114.
23. Vorbeck, M.L. and J.F. Cone, 1963. Characteristics of an intracellular proteinase system of a *Trichosporon* species isolated from Trappist-type cheese. Appl. Microbiol., 11: 23-27.
24. Jawahar, L.D, A.K. Dutta and A.K. Bishwas, 1991. pH dependence of protease and amylase activity and amylase isozymes in control and mutant lines of *Nigella sativa* L. Bangladesh J. Bot., 20: 117-123.
25. Michael, J. N., 1982. Comparative biochemistry of the proteinases of eucaryotic microorganisms. Microbiol. Rev., 46: 308-340.