Journal of Applied Sciences Research, 5(10): 1645-1651, 2009

Purification and Characterization of Extracellular Cellulase from *A. oryzae* ITCC-4857.01

[1]Ferdousi. Begum, [2]Nurul Absar and [1]M. Shah Alam

[1]Department of Botany, University of Rajshahi, Rajshahi-6205
[2]Department of Biochemistry, University of Rajshahi, Rajshahi-6205

Abstract: A cellulose splitting enzyme, cellulase (extracellular) extracted from *A. oryzae ITCC*-4857.01 was purified by ion-exchange chromatography using DEAE-cellulose followed by Gel filtration.The purification achieved was 53 fold from the crude extract with a yield of 49 %. The purified enzyme was homogenous as judged by disc gel electrophoresis. The molecular weight as determined by gel filtration and SDS-polyacrylamide gel electrophoresis was 41,500 and 41,000 respectively. The purified extracellular cellulase contained only one subunit. The purified extracellular cellulase in aqueous solution gave absorption maximum at 290 nm and minimum at 260 nm. The enzyme is a glycoprotien as nature and contained 0.72 % neutral sugar. The apparent km value of the enzyme against cellulose was 0.67 % . The affinity of the enzyme with different substrates showed as the highest relative ativities on CMC followed by avicel, salicin and filter paper.

Key words: *Aspergillus*, cellulase, DEAE-cellulose chromatography, SDS-PAGE, km value

INTRODUCTION

Cellulase is an important enzyme required for catabolism of cellulose into smaller sugars. Since last two decades, the use of cellulases, hemicellulases and pectinases has increased considerably, especially in textile, food, brewery and wine as well as in pulp and paper industries[8,19,26,23]. Cellulose, a polymer of glucose, is the primary structural component of most plant cell walls. Although this polysaccharide is the most common carbohydrate on earth, relatively few animals are able to utilize this resource efficiently[9]. Utilization of cellulose as a nutrient source requires enzymes that cleave beta- 1, 4 glycosidic bonds between constituent sugars. The enzymes referred to as cellulases, are require to split beta- 1, 4 glycosidic bonds in the polymer to release glucose units[2]. A large number of microorganisms are capable of degrading cellulose, only a few of these microorganisms produce significant quantities of cell-free enzymes capable that of completely hydrolysing crystalline cellulose *in vitro*. Fungi are the main cellulase-producing microorganisms, though a few bacteria and actinomycetes have also been recently reported to yield cellulase activity. It was also reported that *Trichoderma reesei* is capable to increase the production of cellulase from substrate and shows both the challenging rheology and a cellulase complex product that is shear- sensitive[25]. Today these enzymes account for approximately 20% of the world market[12], mostly from *Trichoderma* and Aspergillus[7, 23].

Commercial production and efficient utilization of these enzymes are infested with many problems. To increase process efficiency, one can search for more active cellulase, or develop cheap methods for pretreatment of the substrates, making them more accessible for enzymic degradation. So, for mitigation of this problem the present investigation describes the purification and characterization of extracellular cellulase from *A. oryzae* ITCC-4857.01.

MATERIALS AND METHODS

Aspergillus oryzae ITCC-4857.01 were isolated from rice bran (poultry feed wastes) which was collected from Plant pathology, Mycology and Microbiology Laboratory, Department of Botany, University of Rajshahi, Rajshahi-6205, Bangladesh. DEAE cellulose was purchased from Sigma Chemical Co., U.S.A. Sephadex G-75 was purchased from Pharmacia Fine Chemicals, Uppsala Sweden. Molecular weight markers were the products of Fluka Biochemica, Switzerland. All other reagents used were of analytical grade.

Preparation of Extracellular Crude Enzyme Solution: *A. oryzae* ITCC-4857.01 was cultivated in 100 ml of Czapek's broth medium (with 1 % CMC as a source of carbon) and pH of the medium was adjusted to 5.6. The medium was taken in 250 ml of conical flask and sterilized. The stock culture was sub cultured and organism was inoculated in the medium and allowed to grow at 28°C with manual shaking for 7 days. When the organism was grown profusely, the

Corresponding Author: Ferdousi Begum, Department of Botany, University of Rajshahi, Rajshahi-6205.
Email: ferdrita@yahoo.com

culture medium was filtered and the filtrate was centrifuged at 10,000 rpm for 15 minutes. The supernatant was used as the crude enzyme solution for the experimental purposes.

Purification of the Enzyme:

DEAE-Cellulose Chromatograghy: The crude enzyme solution after dialysis against distilled water and 0.02 M aceted buffer, pH 5.2 for 24 hour was applied to DEAE-cellulose column which was previously equilibrated with the same buffer at 4 ^{0}C. The separation of protein from the column was performed by stepwise elution with the buffer containing increasing concentrations of NaCl (0.05M, 0.1M, 0.2M, 0.5M and 1M). About 3.0 ml fraction was collected in different test tubes. The absorbency at 280 nm and enzymatic activity of each fraction was estimated. The fraction (F-1) containing enzymic activity, obtained from DEAE cellulose column was purified further by gel filtration.

Gel Filtration: The enzyme containing fraction (F-1) was dialyzed against distil water and 0.02 M sodium acetate buffer pH 5.2 for 24 hours at 4°C. After centrifugation, the clear sample was loaded onto the gel bed. After diffusion the sample, about 1 ml of elute buffer was poured on the top of gel bed and was allowed to diffuse. An additional amount of buffer was then added, so that the space about 3-4 cm above the gel bed was filled with elute. The buffer was allowed to flow continuously through the column and 3 ml fraction of elute was collected by an automatic fraction collector and monitored for enzyme activity as well as for protein concentration at 280 nm.

Determination of Protein Concentration: Concentration of protein was determined following the method of Lowry *et al*.[10] using BSA as standard and the protein in column elute fraction was also monitored spectrophotometricaly at 280 nm.

Assay of Cellulase Activity: Cellulase activities were assayed by the method of Mohadevan and Sridhar[11] using cellulose powder as substrate (0.05%, w/v) in 0.02 M acetate buffer, pH 5.2. The amount of reducing sugar released was determined by dinitrosalicylic acid (DNS) method[13]. The enzyme activity was expressed as the amount of reducing sugar released/ml of the sample / unit time.

Polyacrylamide Disc Gel Electrophoresis: Polyacrylamide disc gel electrophoresis was performed in 7.5% polyacrylamide gel at 28°C, pH 8.5 as described by Ornstein[16].

Molecular Weight Estimation :

Gel Filtration: Molecular weight of purified cellulase was determined by gel filtration following the procedure as described by Andrews[1]. Lysozyme (MW=14 KDa), Trypsin (MW=20 KDa), Egg albumin (MW=45 KDa), Bovine serum albumin (MW=66 KDa) and b-glactosidase (MW=116 KDa) were used as marker proteins.

Sds-polyacrylamide Gel Electrophoresis (SDS-PAGE): SDS-PAGE was conducted in 10% gel at 28°C, pH 7.0 according to the method of Weber and Osborn[24]. Denature protein was prepared by incubating protein in boiling water for 3 minutes in solution containing 0.15 % SDS with and without 0.1% beta-mercaptoethanol and 25% glycerol. The standard protein was same as those used in gel filtration. The gels were stained with 1 % coomassie briliant blue R-250 in 7.5 % acetic acid for an hour at room temperature, and destaining was performed by washing the gels in 7 % acetic acid (v/v) solution.

Ultraviolet Absorption Spectrum of the Purified Enzyme: Ultraviolet absorption spectrum of enzyme was recorded in aqueous solution with double beam spectrophotometer (Shimadzu Model UV-180) at 28°C.

Analysis of Carbohydrate: The total sugar content of purified extracellular cellulase was determined by phenol sulfuric acid method of Dubios *et al*.[5] with D-glucose as the standard sugar.

Determination of Km value: Michaelis constant (km) of purified extracellular cellulase of *A. oryzae* ITCC-4857.01 was determined by Lineweaver-Burk double reciprocal plot. The initial velocity was equal to the amount of product formed per unit time. The initial velocity (Vi) is determined by quantitavely measuring the amount of one of the product at various time intervels[18].

Affinity of Purified Cellulase to Different Substrates: Percentage of relative activities of purified enzyme was determined by DNS method for detection of affinity of purified cellulase to different substrates viz. carboxylmethyl cellulose (CMC), avicel, salicin and filter paper.

RESULTS AND DISCUSSION

Purification of Extracellular Cellulase:

Deae Cellulose Chromatography: The elution profile of extracellular crude enzyme solution of *Aspergillus oryzae* ITCC-4857.01 from DEAE-cellulose column is shown in Fig. 1. As shown in Figure, the protein of

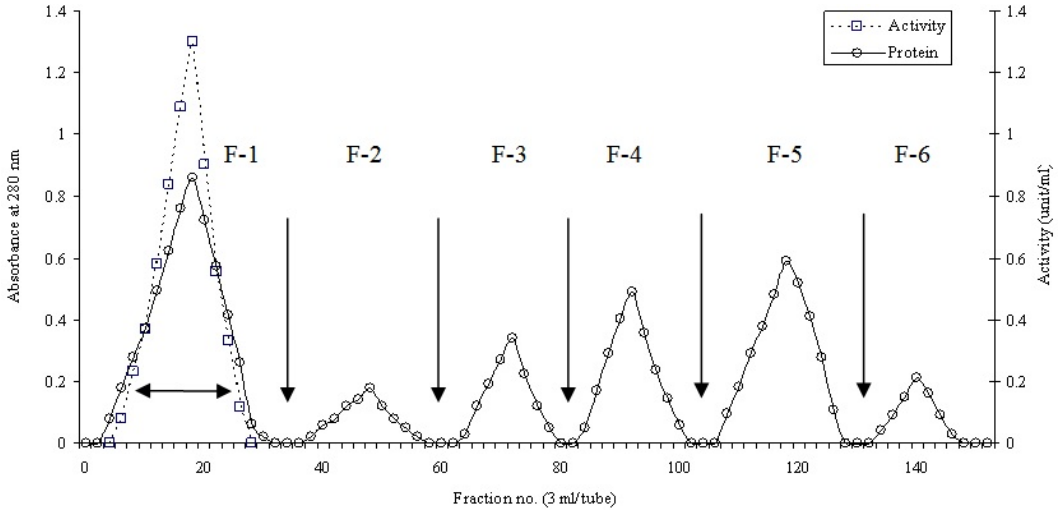

Fig. 1: Ion-exchange chromatography of extracellular crude enzyme solution on DEAE cellulose. The crude enzyme solution (35 mg) was applied to the column (2.1× 25 cm) preequilibrated with 0.2M sodium acetate buffer pH 5.2 at 4°C and eluted by stepwise increases of NaCl concentration in the same buffer. Flow rate 36 ml/hour.

crude enzyme extract was separated in six fractions as F-1, F-2, F-3, F-4, F-5 and F-6. The major fractions i.e. F-1 was eluted from the column by buffer only, while other five fractions were eluted by the buffer containing different concentrations of NaCl. It was found that only the fractions F-1 contained cellulase activity. This fraction as indicated by solid bar was pooled and subjected to gel filtration for further purification.

Gel Filtration: The component of the active fraction F-1, obtained after DEAE cellulose chromatography was separeted by gel filtration and gave only one peak i.e. F-1a (Fig. 2) and the fraction also found to be contained cellulase activity.

The purity of F-1a as indicated by solid bar was checked by polyacrylamide disc gel electrophoresis. The fraction must contain pure cellulase enzyme as it gave single band on polyacrylamide gel (Fig. 3).

A brief summary of the purification procedure was presented in Table 1. As given in the table, the specific activity of cellulase was 64.17 U/mg, which was increased at each purification step. Although the yield was only about 49 % but over 99 % of the extracted protein was removed during the purification steps and the enzyme was purified with an increase in purification fold more than 53. This decrease in yield might be due to denaturation of enzyme during the purification steps or other reasons. Olama *et al.*[15] purified cellulase from *Trichoderma viride* by DEAE-Sephadex A- 50 chromatography method followed by CM- Sephadex C-50 and observed 99.8% loss of protein, and the specific activity was increased to about 22.8 fold. Sultana[21] observed 13.71 U/mg specific activities which were increased to about 32 fold in *Aspergillus* sp. by DEAE- cellulose chromatography. Po-Jui *et al.*[17] reported that the specific activity was 38.22 U/ml increased to about 9.04 fold from *Sinorhizobium fredee* by DEAE- Sepharose anion-exchange column and followed by Phenyl-Sepharose column. The present results showed good similarity with the obsevation of Olama *et al.*[15] and Sultana[21].

Characterization of Cellulase:

Molecular Weight: The molecular weight of extracellular cellulase as determined by gel filtration and SDS polyacrylamide gel electrophoresis was found to be 41.5 and 41 KDa, respectively. This value is very similar to that purified from *T.viride*, M.W. - 42 KDa[3]; *T. viride,* M.W. 38-54 KDa[14]; *T. viride,* MW-58 KDa[15]; *Aspergillus* sp., M.W.- 31.2 KDa[21] and *Volvariella volvaceae,* M.W.- 42 KDa[20]. Further, the purified extracellular cellulase might be contained only one subunit as the molecular weight was found to be unchanged both in presence and absence of beta-marcaptoethanol on SDS-polyacrylamide gel electrophoresis. It was also reported that cellulase purified from *A. saitoi*[22], *T. viride*[14,15] and *Aspergillus* sp.[21] contained only one subunit.

Ultraviolet Absorption Spectrum of Purified Extracellular Cellulase: Purified extracellular cellulase in aqueous solution gave absorption maximum at 290 nm and minimum at 260 nm (Fig. 4).

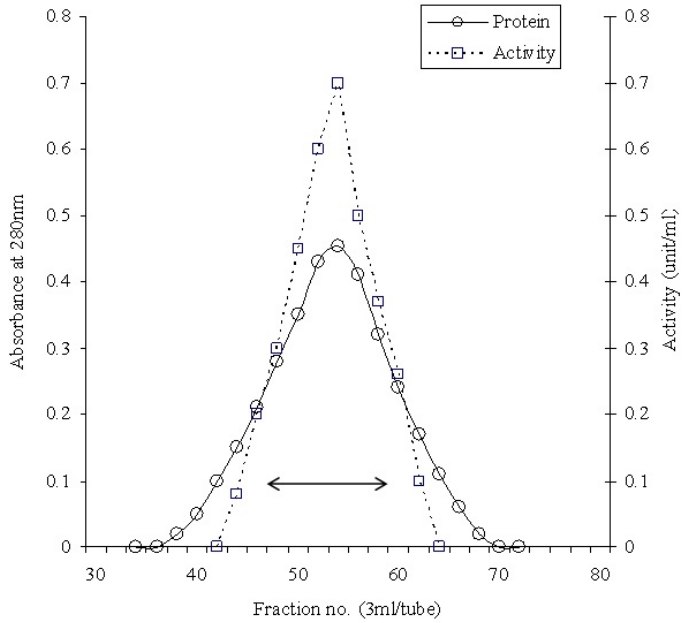

Fig. 2: Gel filtration of F-1 fraction on Sephadex G-75. Fractions F-1 obtained by DEAE-cellulose chromatography was applied to the column (3 × 120 cm) preequilibrated with 0.2M Sodium acetate buffer, pH 5.2 at 4°C and developed with the same buffer.

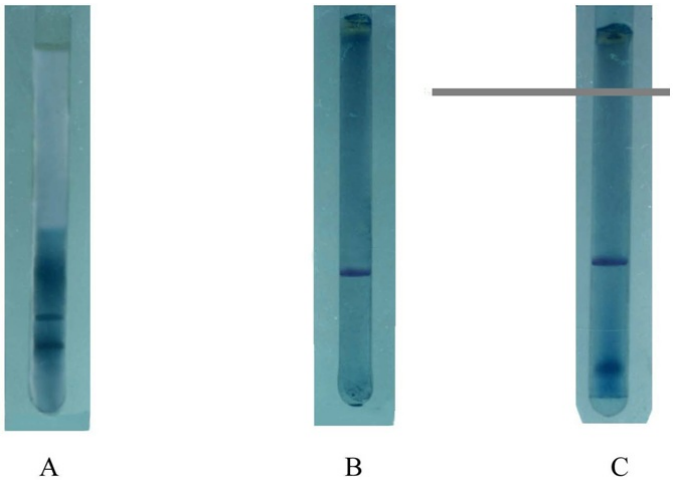

Fig. 3: Disc electrophoretic pattens of different fractions on 7.5 % polyacrylamide gel.

A= Crude enzyme;
B = F-1 (obtained after DEAE-cellulose);
C=F-1a (obtained after gel filtration).

Table 1: Summary of data on the course of purification of extracellular cellulase from *A. oryzae* ITCC-4857.01.

Steps of purification	Total protein (mg)	Total activity (unit)	Specific activity (unit/mg)	Yield (%)	Purification (fold)
Crude cellulose	121	146.6	1.20	100	1
DEAE cellulose	6	111	18.50	75.70	15.40
Gel filtration	0.90	71.3	64.17	48.60	53.40

nit= Release of reducing sugar in mg/min.

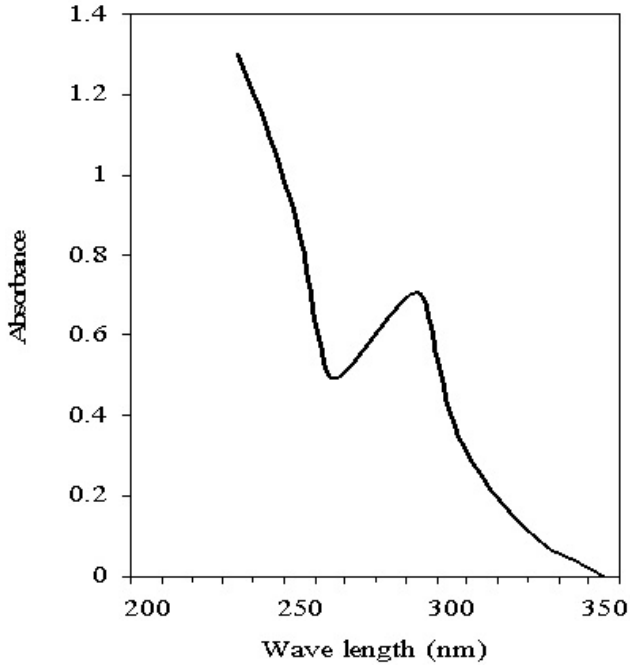

Fig. 4: Ultraviolate absorbtion spectra of extracellular cellulase.

Analysis of Carbohydrate: The purified extra cellular cellulase gave slight chocolate color in the presence of phenol-sulphuric acid, indicating that the enzyme is glycoprotein in nature and the amount of sugar present was calculated to be 0.72%. Neutral sugar from *T. viride* was reported to be 6.3 - 15.1%[14] which indicates that the presently purified extracellular cellulase contained comparatively less amount of neutral sugar.

Determination of Km Value of Extracellular Cellulase: Km value of purified extracellular cellulase was determined by Lineweaver-Burk double reciprocal plot (Fig. 5) and was found to be 0.67 % using CMC as substrate. The Km value of cellulase from *Favouls arcularicas* was 0.28%[6] and 1.32% in *T. reesei*[4]. The difference in km value of the presently purified extracellular cellulase may be due to variation of sources from which it was isolated.

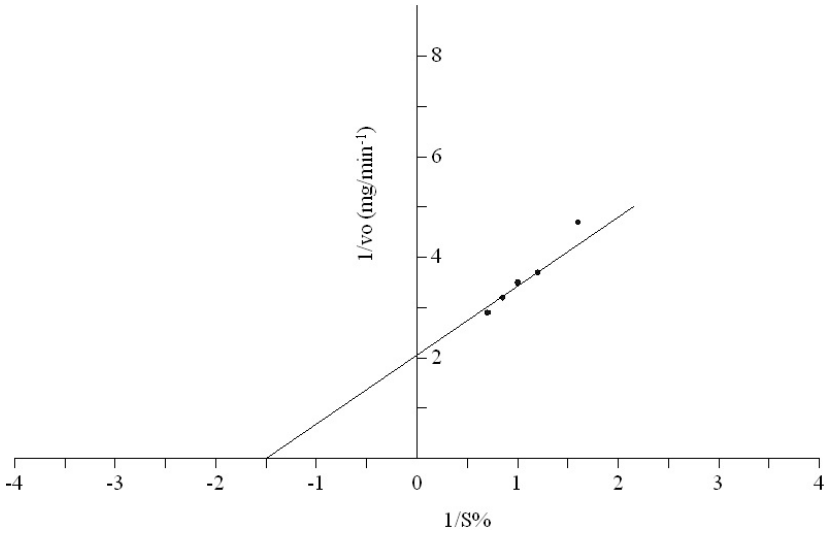

Fig. 5: Lineweaver-Burk double reciprocal plot for the determination of km value of purified extracellular cellulase form *A. oryzae* ITCC-4857.01

Affinity of Purified Enzyme to Different Substrates: The purified extracellular cellulase gave about 100% relative activity in hydrolyzing carboxylmethyl cellulose (CMC) but showed 89% and 60% relative activity when avicel and salicin were used as substrate and filter paper is accounted as very poor substrate (27 %).

REFERENCES

1. Andrews, P., 1965. The gel filtration behaviour of proteins related to their molecular weights over a wide range. Biochem. J., 96: 595-605.
2. Barr, B.K., Y. Hsieh, B. Ganem and D.B. Wilson, 1996. Identification of two functionally different classes of exo-cellulases. Biochem., 35: 586-592.
3. Berghem, L., L. Pettersson and U. Asio-Fredriksson, 1975. The mechanism of enzymatic ecllulose degradation, characterization and enzymatic properties of a b-1-4-glucan cellobiolydrolase from *Trichoderma viride*. Eur. J. Biochem., 53: 55.
4. Busto, M.D., N. Ortega and M.M. Pereg, 1996. Location, kinetics. and stability of cellulases induced in *Trichoderma reesei* cultures. Biores. Tech., 57(2): 187-192.
5. Dubois, M., K.A. Gilles, J.K. Hamilton, P.A. Rebers and G. Smith, 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem., 28(3): 23-25.
6. Enokibara, S., N. Mori and K. Kitamoto, 1991. Purification and characterization of endo-glucanases from *Favouls arcularicus*. J. Fermen. Bioeng, 73(3): 230-232.
7. Godfrey, T., 1996. Textiles. In: Godfrey, T. and West, S. (editors.) Industrial enzymology, 2nd ed. London: Macmillan Press.
8. Godfrey, T. and S. West, 1996. Industrial Enzymology (2nd. ed) London: Macmillan Press.
9. Goodenough, S. and P. Goodenough, 1993. Who needs cellulase?.J. Biol. Educ., 27: 97-102.
10. Lowery, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the Folin-Phenol reagent. J. Biol. Chem., 193: 265-275.
11. Mahadevan, A. and R. Sridhar, 1982. Methods in Physiological plant pathology. Sivakami publications. Madrus.
12. Mantyla, A., M. Paloheimo and P. Suominen, 1998. Industrial mutants and recombinant strains of *Trichoderma reesei*. In: Harman G.F, Kubicek C.P. editors. *Trichoderma* & *Gliocladium*-Enzymes, biological control and commercial applications, (2nd. ed). Taylor & Francis, Ltd. London, 291-309.
13. Miller, G.L., 1959. Use of dinitrosalicylic acid for determination of reducing sugar. Anal. Chem., 31: 426-428.
14. Ogawa, K., 1989. Fractionation and purification of cellulases from *Trichoderma viride*. Bull. Fac. Agr. Myazaki Univ., 36(2): 271-280.
15. Olama, Z.A., M.A. Hamza, M.M. El-Sayed and M. Abdel-Fattah, 1993. Purification, properties and factors affecting the activity of *Trichoderma viride* cellulase. Food Chem., 47(3): 221-226.
16. Ornstein, L., 1964. Disc electrophoreses I-Background and theory. Ann. N.Y. Acad. Sci., 121: 321-349.
17. Po- Jui, C., W. TaoChun, C. YaoTsung, and L. Liangling, 2004. Purification and characterization of carboxylmethyl cellulase from *Sinorhigobium fredii*. Bot. Bull. Acad. Sinica, 45(2): 111-118.
18. Robyt, J.F. and B.J. White, 1990. Biochemical Techniques: Theory and practical. Waveland Press. Inc. Illinois, U.S.A.
19. Saddler. J.N., 1993. Bioconversion of forest and agricultural plant residues Biotechnol. Agricul., 9: 349.
20. Shaojun, D., G. Wei and J.A. Busuell, 2001. Endoglucanase 1 from the edible straw mushroom, *Volvariella volvaeeae*: Purification, Characterization, cloning and expression. European J. Priochem, 268(22): 5687-5695.
21. Sultana, S., 1997. Isolation of cellulolytic microorganism and their activities. M. Phil Thesis. Biochemistry Department, Rajshahi Univ. Bangladesh.
22. Takahashi, F., F. Tokita and T. Nakanishi, 1974. The antigenicities of caseins treated by the various protease preparations from *Aspergillus oryzae* strain B. Japanese J. Dairy Sci., 23(2): 49-56.
23. Uhlig H., 1998. Industrial enzymes and their applications: John Wiley & Sons, New York, pp: 435.
24. Weber, K. and M. Osborn, 1969. The reliability of molecular weight determination by SDS-PAGE. J. Biol. Chem., 244: 4406-4412.
25. Weber, J. and F.A. Agblevor, 2005. Microbubble fermentation of *Trichoderma reesei* for cellulase production. Process Biochemist, 40: 669-676.
26. Wong, K.K.Y. and J.N. Saddler, 1993. Applications of hemicellulases in the food, feed, and pulp and paper industries. In: Coughlan MP, Hazlewood GP, editors. Hemicellulose and hemicellulases. London: Portland Press, 127-143.