The Chittagong Univ. J. B. Sci.,Vol. 10(1 &2):167-179, 2020.

ISOLATION AND CHARACTERIZATION OF CELLULOLYTIC *Pseudomonas aeruginosa* FROM TEAK FOREST SOIL FOR ITS CELLULASE PRODUCTION AND CELLULOLYTIC ACTIVITY

FATIMA BINTE KAMAL[1], FAOZIA FALEHA SADIDA[2] AND M. A. MANCHUR[1*]

[1]Department of Microbiology, Faculty of Biological Sciences, University of Chittagong, Chittagong 4331, Bangladesh; [2]Institute of Education and Research (IER), Faculty of Arts and Humanities, University of Chittagong, Chittagong 4331, Bangladesh

ABSTRACT

A cellulolytic bacterium was isolated from *Tectona* forest soil and provisionally identified as *Pseudomonas aeruginosa*. The bacterium showed the highest level of reducing sugar and extracellular protein production when incubated for 3 days at 40°C, pH 8.5 in Winstead's broth having 1.5% carboxymethyl cellulose (CMC) and 0.2% Yeast Extract as carbon and nitrogen sources, respectively. The bacterium produced the maximum 462 µg/ml reducing sugar and 450 µg/ml extracellular proteins. Crude cellulase produced by the bacterium showed the highest carboxymethyl cellulase (CMCase) activity rather than filter paperase (FPase), Avicelase and β-Glucosidase activities. The crude cellulase showed highest CMCase activity when incubated for 90 minutes at pH 8.5, 25°C using CMC and Yeast Extract as carbon and nitrogen sources, respectively as well as in presence of Hg^{2+} ion and SDS (inhibitor and reductant). The highest enzyme activity was found to be 315 U/ml. The molecular weight of the crude cellulase was found as 33 kDa through SDS PAGE analysis. According to Worthington Enzyme Manual it may be Endoglucanase IV.

Key Words: *Pseudomonas aeruginosa*, Winstead's broth, CMCase, cellulase enzyme, Endoglucanase IV.

INTRODUCTION

Earth's body of soil is a mixture of organic matter, minerals, gases, liquids and organisms that, together support life. Plants also depend on soil for their life and nutrition. Plants use the mechanism of photosynthesis, which results in the production of plant biomass containing cellulose as the major component. Cellulose is an organic compound consisting of a linear chain of several hundreds

[*] Correspondence to M. A.Manchur; E-mail: manchur.mbio@cu.ac.bd

to many thousands of β - (1, 4) linked D-glucose units (Crawford 1981). It is an important structural component of the primary cell wall of green plants, many forms of algae and the Oomycetes. Some species of bacteria secrete cellulose to form biofilms (Romeo and Tony 2008). Among all naturally occurring organic compounds, which accumulate on earth in the large quantities is cellulose, found most abundantly, and most frequently present in the form of agricultural, industrial, forest and residential wastes (Mandels 1975). Plant litter decomposition is a critical step in the mineralization of organic nutrients, and it influences the carbon (C) balance in terrestrial ecosystems (Berg and McClaugherty 2014).

Cellulose is the structural component of plant materials and the biological degradation of cellulose has a great importance in the activity of living system. Maintenance of the cellulosic waste materials and their disposal system are not satisfactory. One of the major treatment systems for these waste materials is incineration, but huge amount of CO_2 is released in this process. If the biological degradation of cellulosic substances does not occur, the earth would be converted with masses of dead vegetation; therefore, it is obvious that strong cellulolytic activities exist in nature.

The primary cell wall of green plants is made up of primarily cellulose; the secondary wall contains cellulose with variable amount of lignin. Lignin and cellulose, together called lignocellulose, which as wood is one of the most common biopolymers on the earth.

Like several other enzymes cellulase is produced chiefly by fungi, bacteria and protozoans that catalyze cellulolysis, the decomposition of cellulose and of some related polysaccharides. Cellulases break down the cellulose molecules into monosaccharides (simple sugars) such as β-glucose, or shorter polysaccharides or oligosaccharides. The specific reaction involved is the hydrolysis of the 1, 4 β-D-glycosidic linkages in cellulose, hemicellulose, lichenin, and cereal β-D-glucans. As cellulose molecules strongly bind to each other, cellulolysis is relatively difficult compared to the breakdown of other polysaccharides such as starch (Bignell *et al*., 2011). Most mammals only have very limited ability to digest dietary fibers such as cellulose by themselves. In many herbivorous animals such as ruminants like cattle and sheep and hindgut fermenters like horses, cellulases are produced by symbiotic bacteria. Cellulases are produced by a few types of animals, such as some termites, earthworms and snails (Watanabe *et al*., 2001).

In this study, we describe the isolation and characterization of a cellulolytic bacterium from the soil under the *Tectona* forest to find out the optimum conditions for cellulase production and cellulolytic activities as well as to characterize the enzyme.

MATERIALS AND METHODS

Microorganism

A bacterium was isolated from *Tectona* forest soil. After isolation the bacterial culture was purified through repeated plating in Nutrient Agar Medium. On the basis of morphological, cultural and 16S rRNA analysis result, the bacterium was identified as *Pseudomonas aeruginosa*. The amplified sequence of the bacterium was also used to design its phylogenetic tree to find out its genetic relatives.

Observation of clear zone of cellulose hydrolysis and Winstead's broth liquefaction

The isolated bacterium was grown into Enrichment medium (CMC-0.25 g, Agar-15.0 g, Peptone-5.0 g, Beef extract-3.0 g, Distilled water-1000 ml) on Petri plate. After 24 hours of incubation, the plate was flooded with Gram's iodine solution (KI- 2 g, Iodine – 1.0 g, and Distilled water- 300 ml). After 30 seconds, 22 mm zone of cellulose hydrolysis appeared which approved its ability of cellulase production. The bacterium was again incubated into Winstead's broth (CMC-12.0 g, Asparagine-2.0 g, K_2HPO_4 – 3.0 g, $MgSO_4.7H_2O$-2.5 g, and Distilled water-1000 ml) and incubated at (35±2)˚C for 24 hours in shaking incubator. It showed significant amount of cellulose hydrolysis which again revealed its ability to degrade cellulose.

Optimization of cultural conditions

To determine the optimum physico-chemical conditions for the highest cellulase and reducing sugar production by the bacterium, it was incubated in different temperature, pH, for different incubation period using different carbon and nitrogen sources as well as different CMC concentration.

Incubation period

The bacterium was incubated for 1 day, 2 days, 3 days, 4 days and 5 days using Winstead's broth having 1.2% CMC and 0.2% asparagine as carbon and nitrogen source respectively to observe the optimum incubation period for the highest

reducing sugar and cellulase production in shaking incubator. After incubation, the crude enzyme was collected through centrifugation at 10,000 rpm in 4˚C temperature for 20 minutes.

Medium pH

The bacterium was incubated at pH 3.5, 4.5, 5.5, 6.5, 7.5, 8.5 and 9.5 using Winstead's broth having 1.2% CMC and 0.2% asparagine as carbon and nitrogen source respectively to observe the optimum pH for the highest reducing sugar and cellulase production in shaking incubator. After incubation, the crude enzyme was collected through centrifugation at 10,000 rpm in 4˚C temperature for 20 minutes.

Temperature

The bacterium was incubated at 15˚C, 20˚C, 25˚C, 30˚C, 35˚C, 40˚C and 45˚C using Winstead's broth having 1.2% CMC and 0.2% asparagine as carbon and nitrogen source respectively to observe the optimum temperature for the highest reducing sugar and cellulase production in shaking incubator. After incubation, the crude enzyme was collected through centrifugation at 10,000 rpm in 4˚C temperature for 20 minutes.

Carbon and Nitrogen Sources

The bacterium was incubated using Winstead's broth having different carbon sources in 1.2% concentration such as CMC, avicel, salicin and filter paper (Gray *et al.,* 1978) and also using different nitrogen sources in 0.2% concentration such as asparagine, peptone, urea, yeast extract, ammonium sulphate to observe the optimum carbon and nitrogen sources for the highest reducing sugar and cellulase production in shaking incubator. After incubation, the crude enzyme was collected through centrifugation at 10,000 rpm in 4˚C temperature for 20 minutes.

Quantitative Estimation of Reducing Sugars

Reducing sugars of culture filtrate and enzyme-substrate reaction were estimated through Nelson's modification of Somogyi method (Nelson 1944).

Enzyme assay

Carboxymethyl Cellulase (CMC-ase) Assay

2 ml of filtrate was added to 2 ml of 1% CMC prepared in phosphate buffer at pH 6.5 and then added 1 ml of phosphate buffer in a test tube and incubated at 35˚C for 2 hours in a water-bath.

Filter Paper (FP-ase) Assay

2 ml of filtrate was added to 1 ml of phosphate buffer at pH 6.5 along with 50 mg Whatman No-1 filter paper strip [(1×6) cm] in a test tube and incubated at 35˚C for 2 hours in a water-bath.

Avicelase Assay

2 ml of filtrate was added to 2 ml of 1% avicel prepared in phosphate buffer at pH 6.5 and then added 1 ml of phosphate buffer in a test tube and incubated at 35˚C for 2 hours in a water-bath.

β-Glucosidase Assay

2 ml of filtrate was added to 2 ml of 1% salicin prepared in phosphate buffer at pH 6.5 and then added 1 ml of phosphate buffer in a test-tube and incubated at 35˚C for 2 hours in a water-bath.

Measurement

The amount of reducing sugar released in CMC-ase, FP-ase, Avicelase, β-Glucosidase assay after incubation was measured by Nelson's modification of Somogyi method (Nelson, 1944). Enzyme activity was expressed by the amount of glucose released in U/ml of crude enzyme/hour enzyme-substrate reaction in given conditions (Mahadevan and Sridhar 1982).

Enzyme activity

To determine the optimum conditions for cellulase activity, the crude enzyme was incubated for different reaction times (30, 60, 90 and 120 minutes), at different temperatures (15˚C, 20˚C, 25˚C, 30˚C, 35˚C, 40˚C and 45˚C), at different pH (4.5, 5.5, 6.5, 7.5, 8.5 and 9.5), using different carbon sources (CMC, Avicel, Filter paper and Salicin) and nitrogen sources (Urea, Peptone, Ammonium sulphate, Asparagine and Yeast extract). Its enzyme activity was tested against different metals as metal chlorides (NaCl, KCl, $MgCl_2$, and $HgCl_2$) and also assayed using different inhibitors and reductants (SDS, Cysteine, EDTA and Sodium azide) in 1% CMC produced in phosphate buffer using water bath.

SDS PAGE Analysis

The isolated bacterium was inoculated into Winstead's broth containing 1.5% CMC and 0.2% yeast extract as carbon and nitrogen source respectively and incubated for 3 days, at 40˚C temperature, pH 8.5 in shaking incubator for the maximum production of cellulase. After incubation, the culture filtrate or crude enzyme was collected by centrifugation at 10,000 rpm, 4˚C temperature for 20

minutes. Then, it was transferred to sterile test tube for the determination of molecular weight by SDS-PAGE.

SDS-PAGE (Sodium dodecyl-sulphate Polyacrylamide gel electrophoresis) of the crude cellulase was carried out to separate and identify the proteins with molecular masses (Laemmli 1970).

RESULTS AND DISCUSSION

Effect of incubation period

The highest level of extracellular protein and reducing sugar produced by the bacterium were obtained after 3 days of incubation period using Winstead's broth (1.2% CMC). It produced 249.75 µg/ml reducing sugar and 277.5 µg/ml extracellular proteins. Vimal *et al.,* (2016) also reported that, the production of reducing sugar and extracellular protein by *Bacillus subtilis* and *Bacillus cereus* were increased after 3 days of incubation period and were gradually decreased after 4 days of incubation period. The isolate showed highest level of CMCase activity (105 U/ml) after 3 days of incubation period (Figure 1).

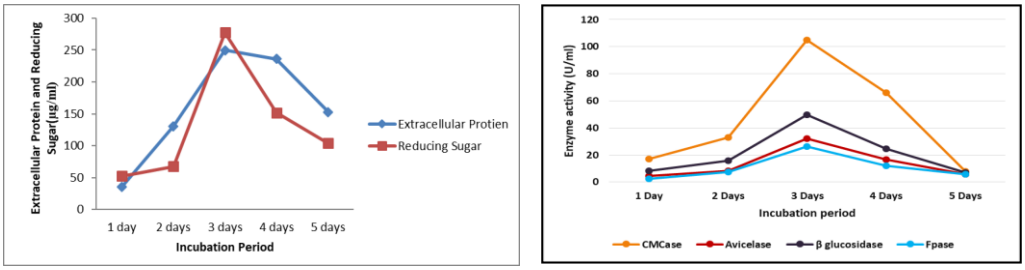

FIGURE 1: EFFECT OF INCUBATION PERIOD ON EXTRACELLULAR PROTEIN AND REDUCING SUGAR PRODUCTION (LEFT) AND ON ENZYME ACTIVITY (RIGHT).

Effect of temperature

The highest level of extracellular protein and reducing sugar produced by the bacterium were obtained at 40˚C temperature using Winstead's broth (1.2% CMC). It produced 462 µg/ml reducing sugar and 313 µg/ml extracellular proteins. El-Naggar *et al.,* (2012) isolated *Streptomyces spp.* strain NEAE-D, and similarly, reported its highest extracellular protein production at 40˚C temperature. The isolate showed highest level of CMCase activity (97.5 U/ml) at 40˚C temperature (Figure 2).

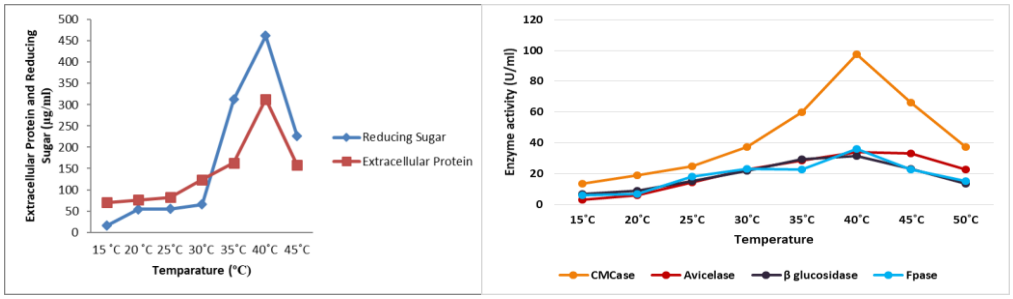

FIGURE 2: EFFECT OF TEMPERATURE ON EXTRACELLULAR PROTEIN AND REDUCING SUGAR PRODUCTION (LEFT) AND ENZYME ACTIVITIES (RIGHT).

Effect of medium pH

To obtain the highest level of extracellular protein and reducing sugar, the isolate was incubated at different pH using Winstead's broth (1.2% CMC). It produced highest 261 µg/ml reducing sugar and 302 µg/ml extracellular proteins at pH 8.5. Yin *et al.*, (2010) isolated *Bacillus subtilis* YJ1 and also reported its optimum pH range from 6.5 to 8.5 for extracellular protein production. The isolate showed highest level of CMCase activity (85.5 U/ml) at pH 8.5 (Figure 3).

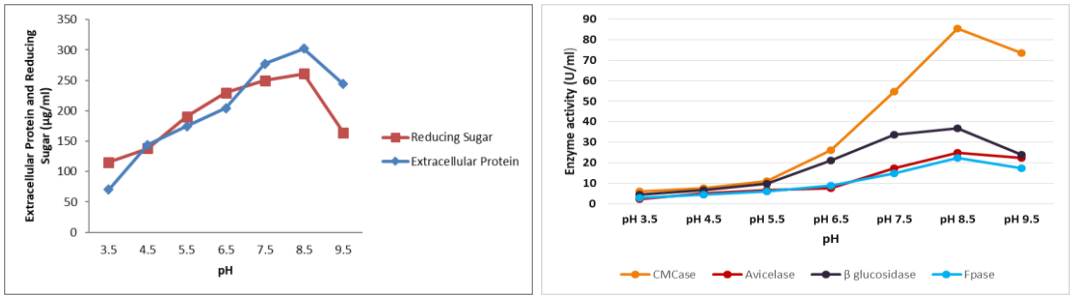

FIGURE 3: EFFECT OF PH ON EXTRACELLULAR PROTEIN AND REDUCING SUGAR PRODUCTION (LEFT) AND ENZYME ACTIVITIES (RIGHT).

Effect of carbon and nitrogen source

For producing the highest level of extracellular protein and reducing sugar, the isolate was incubated using Winstead's broth containing different carbon sources such as glucose, avicel, salicin, CMC, treated rice straw and untreated rice straw in 1.2% concentration. It produced highest 337.5 µg/ml reducing sugar and 435 µg/ml

extracellular protein, using CMC as carbon source. It showed 150 U/ml CMCase activity (Figure 4).

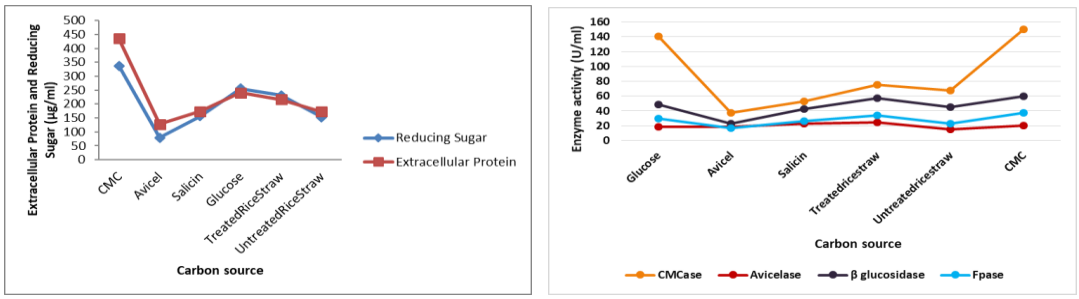

FIGURE 4: EFFECT OF CARBON SOURCES ON EXTRACELLULAR PROTEIN AND REDUCING SUGAR PRODUCTION (LEFT) AND ENZYME ACTIVITIES (RIGHT).

To optimize the nitrogen source for the production of highest level of extracellular protein and reducing sugar by the isolates, the isolate was incubated using Winstead's broth (1.2% CMC) having different nitrogen sources (in 0.2% concentration) such as asparagine, peptone, yeast extract, urea, ammonium sulphate. It produced highest 324.75 µg/ml reducing sugar and 415 µg/ml extracellular protein using yeast extract as nitrogen source. El-Naggar *et al.,* (2012) also reported that, amount of reducing sugar and extracellular protein reached at highest level by *Streptomyces spp.* strain NEAE-D when yeast extract was used. The isolate showed highest level of CMCase activity using yeast extract as nitrogen source (90 U/ml) (Figure 5).

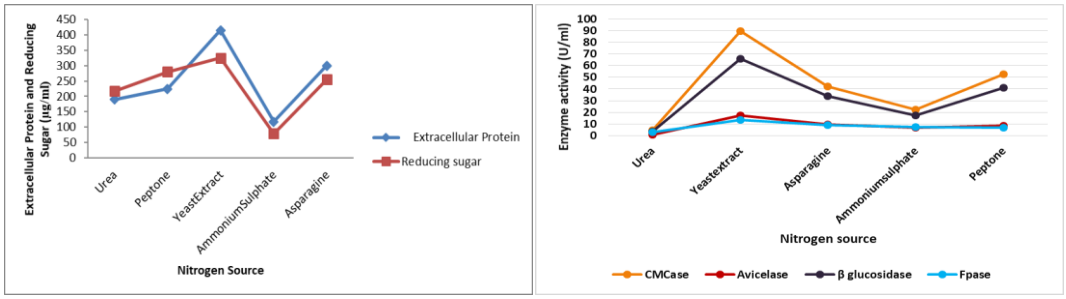

FIGURE 5: EFFECT OF NITROGEN SOURCE ON EXTRACELLULAR PROTEIN AND REDUCING SUGAR PRODUCTION (LEFT) AND ON ENZYME ACTIVITIES (RIGHT).

Concentration of CMC

The highly cellulolytic isolate was grown in Winstead's broth having different concentrations (1%, 1.5%, 2% and 2.5%) of CMC. It showed highest level of reducing sugar (341.25 µg/ml) and extracellular protein production (450 µg/ml) in Winstead's broth having 1.5% CMC (Figure 6left)). Sherief *et al.*, (2010) reported that, highest extracellular protein production occurred when CMC concentration was 1.5% to 2%; because, increase in substrate concentration made binding sites more available for the protein (Dixon and Webb 1971).

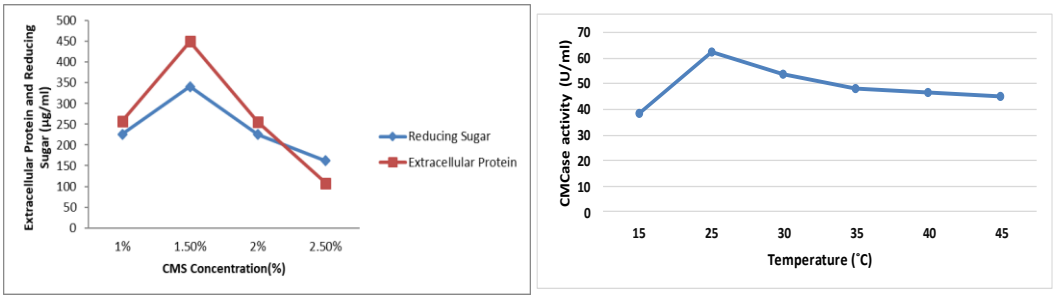

FIGURE 6: EFFECT OF CMC CONCENTRATION ON EXTRACELLULAR PROTEIN AND REDUCING SUGAR PRODUCTION (LEFT) AND EFFECT OF INCUBATION PERIOD ON CMCASE ACTIVITY (RIGHT).

Enzyme activity: Effect of incubation period

Crude enzyme collected from the bacterium (*Pseudomonas aeruginosa*) was incubated using 1% CMC produced in phosphate buffer. Crude enzyme showed highest CMCase activity after 90 minutes (67.5 U/ml) (Figure 6 right). Similar result was reported by Yasmin *et al.*, (2013) for *Trichoderma viridae*.

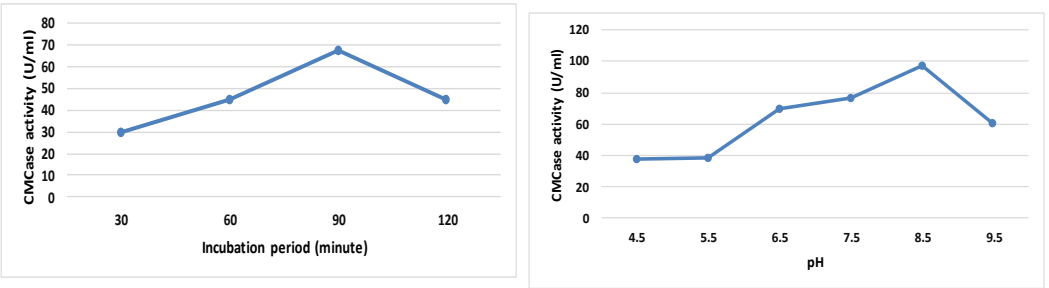

FIGURE 7: EFFECT OF TEMPERATURE ON CMCASE ACTIVITY (LEFT) AND EFFECT OF PH ON CMCASE ACTIVITY (RIGHT).

Effect of temperature

Crude enzymes collected from the bacterium (*Pseudomonas aeruginosa*) was incubated using 1% CMC produced in phosphate buffer. Crude enzyme released by bacterium showed highest CMCase activity at 25˚C (62.25 U/ml) (Figure 7 left). Cheng *et al.,* (2011) also reported the similar result for *Pseudomonas spp.* CL3.

Effect of pH

Crude enzyme of the isolate (*Pseudomonas aeruginosa*) showed highest CMCase activity at pH 8.5 (97.5 U/ml) (Figure 7 right). The result was similar with the work of Gao *et al.,* (2008) who worked on *Aspergillus terreus* M11.

Effect of carbon and nitrogen source

Crude enzyme of the bacterium (*Pseudomonas aeruginosa*) showed highest CMCase activity using CMC as carbon source (76.5 U/ml) (Figure 8 left). Crude enzyme of the bacterium showed highest CMCase activity using yeast extract as nitrogen source (69.75 U/ml) (Figure 8 right). The results agreed with the findings of Cheng *et al.,* (2011) who worked on *Pseudomonas* spp., CL3.

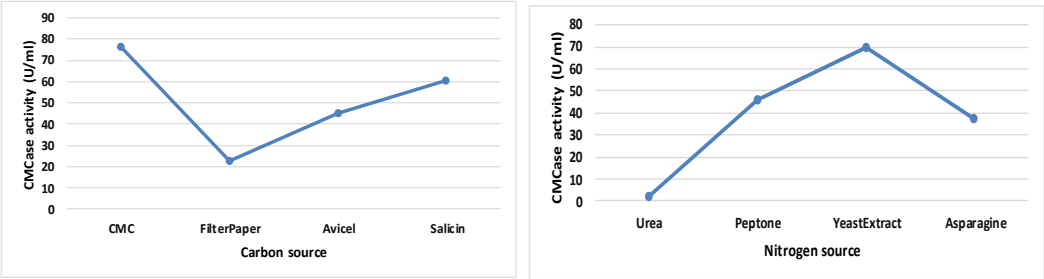

FIGURE 8: EFFECT OF CARBON SOURCE ON CMCASE ACTIVITY (LEFT) AND EFFECT OF NITROGEN SOURCE ON CMCASE ACTIVITY (RIGHT).

Effect of metal ions

Crude enzyme of the bacterium (*Pseudomonas aeruginosa*) showed highest CMCase activity while mercuric chloride ($HgCl_2$, Hg^{2+} ion with Cl^- as counter ion) was used (127.5 U/ml) (Figure 9). Irfan *et al.,* (2012) worked on *Cellulomonas spp.* strain ASN2 and reported the same result on metal ions.

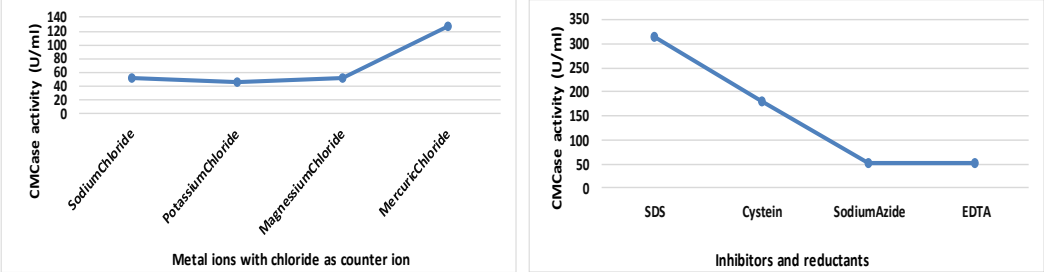

FIGURE 9: EFFECT OF METAL IONS ON CMCASE ACTIVITY (LEFT) AND EFFECT OF INHIBITORS AND REDUCTANTS ON CMCASE ACTIVITY (RIGHT).

Effect of inhibitors and reductants

Crude enzymes of the bacterium (*Pseudomonas aeruginosa*) showed highest CMCase activity while SDS (sodium dodecyl sulphate) was used as inhibitor and reductant (315 U/ml) (Figure 9). Irfan *et al.*, (2012) worked on *Cellulomonas* spp. strain ASN2 and reported the same result on inhibitor and reductant.

SDS PAGE analysis

As the cellulase activity was predominantly present in the isolate (*Pseudomonas aeruginosa*), an attempt was made to characterize cellulase enzyme of the isolate through SDS PAGE analysis. The data of electrophoresis showed that, cellulase enzyme of the bacterium had the molecular weight of 33 kDa (Figure 9). According to Worthington Enzyme Manual (2014), it may be Endoglucanase IV.

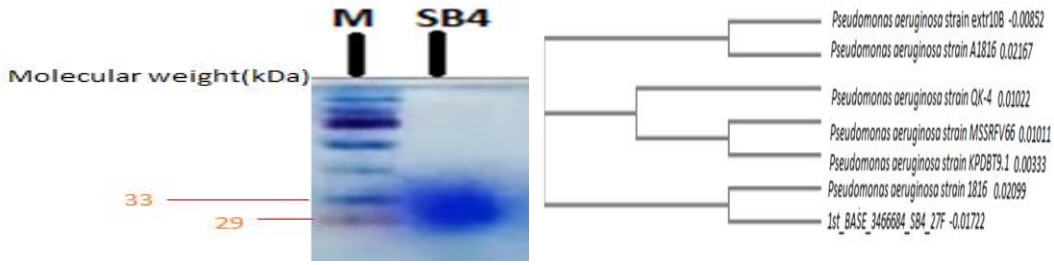

FIGURE 10: SDS PAGE ANALYSIS OF ENZYME PRODUCED BY THE ISOLATE (LEFT) AND THE EVOLUTIONARY RELATIONSHIPS OF TAXA. THE EVOLUTIONARY HISTORY WAS INFERRED USING THE NEIGHBOR-JOINING METHOD (SAITOU AND NEI 1987).

Yin *et al.,* (2010) reported that molecular weight of cellulase of *Bacillus subtilis* YJ1 was 32.5 kDa. Rohini *et al.,* (2017) isolated *Stenotrophomonas maltophilia* identified the molecular weight of cellulase was 38 kDa. The phylogenetic tree of the bacterium has been constructed based on the amplified sequences found after 16S rRNA analysis (Figure 10).

REFERENCES

CRAWFORD, R. L., 1981. Lignin biodegradation and transformation. *New York: John Wiley and Sons.*

ROMEO AND TONY, 2008. Bacterial biofilms. *Berlin: Springer*. pp. 258-263.

MANDELS, M., 1975. Microbial sources of cellulases. *Biotechnol and Bioeng*. Symp. No., **5:** 81-105.

BERG, B., AND C. McCLAUGHERTY. 2014. Plant litter: decomposition, humus formation, carbon sequestration. Third edition. Springer Berlin Heidelberg, New York, USA.

BIGNELL, D. E., ROISIN, Y., LO, N., 2011. Biology of termites: a modern synthesis. *Dordrecht: Springer.*

WATANABE, H., TOKUDA, G., 2001. "Animal cellulases". *Cellular and Molecular Life Sciences*, **58** (9): 1167-1178.

GRAY, P. P., HENDY, N. A., AND UDDIN, N. W., 1978. Digestion by cellulolytic enzymes of alkali pretreated bagasse. *J Aust Inst Agric Sci*, **44:**210-212.

NELSON, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. *J Biol Chem*, **153**(2):375-380.

MAHADEVAN, A., AND SRIDHAR, R., 1982. Methods in physiological plant pathology. Sivakami Publications, Madras.

LAEMMLI, 1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature*, **227**(5259):680-685.

VIMAL, J., VENU, A., JOSEPH, J. 2016. Isolation and Identification of Cellulose Degrading Bacteria and Optimization of the cellulase production. *Int'l J Res Biosci*, **5**(3)**:**58-67.

EL-NAGGAR, N. EL-AHMADY, AND ABDELWAHED, A. M., 2012. Optimization of process parameters for the production of alkali-tolerant carboxymethyl cellulase by newly isolated *Streptomyces sp.* NEAE-D. *Afr J Biotechnol*, **11** (5): 1185-1196.

YIN, L. J., LIN, H. H., XIAO, Z. R., 2010. Purification and Characterization of Cellulase from *Bacillus subtilis* YJ1. *J Marine Sci and Technol*, **18**:466-471.

SHERIEF, A. A., EL-NAGGAR N. E., HAMZA, S. S., 2010. Bioprocessing of Lignocellulosic Biomass for Production of Renewable Bioethanol Using Thermotolerant *Aspergillus fumigatus* Under Solid-state Fermentation Conditions. *Biotechnology*, **9**:513-522.

DIXON AND WEBB. 1971. *Enzymes*, 2nd Edition, Longman group Ltd. London. pp. 67-188.

YASMIN, S., MATTO, R. L., NEHVI, F. A., 2013. Isolation, Characterization and Molecular weight determination of Cellulase from *Trichoderma viride*. *Afr J Biotechnol*, **12**(28): 4512-4518.

CHENG, C. L., AND CHANG, J. S., 2011. Hydrolysis of lignocellulosic feed stock by novel cellulases originating from *Pseudomonas sp.* CL3 for fermentative hydrogen production. *Bioresource Technology*, pp. 8628-8634.

GAO, J., WENG, H., ZHU, D., YUAN, M., GUAN, F., XI, Y., 2008. Production and characterization of cellulolytic enzymes from the thermoacidophilic fungal *Aspergillus terreus* M11 under solid-state cultivation of corn stover. *Bioresource Technology*, pp. 7623-7629.

IRFAN, M., SAFDAR, A., SYED, Q., NADEEM, M., 2012. Isolation and screening of cellulolytic bacteria from soil and optimization of cellulase production and activity. *Turkish J Biochem*, **37**(3):287-293.

WORTHINGTON Biochemical Corporation, 2014. Cellulase. Accessed on 2014-07-03.

ROHINI, T., VELAYUDHAN, S. S., EBENEZER, S., HARSHAVARDHAN, S., GUPTA, P., 2017. Purification and characterization of extracellular cellulase produced by using alkali pretreated rice straw by *Stenotrophomonas maltophilia*. *Int'l J Biol Res*, **2**: 45-54.

Manuscript received on 14.11.2019; Accepted on 18.08.2021
The Chittagong University Journal of Biological Sciences, Vol. 10 (1 & 2). Page No.167-179.