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ISOLATION AND CHARACTERIZATION OF TWO *Streptomyces* spp. FROM SAW DUST FOR THEIR CELLULASE PRODUCTION AND CELLULOLYTIC ACTIVITY

FAOZIA FALEHA SADIDA[1*] AND M. A. HAKIM[2]

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

ABSTRACT

Two highly cellulolytic actinomycetes, namely SD1 and SD2, were locally isolated from saw dust and provisionally identified as *Streptomyces cirratus* (SD1) and *Streptomyces cellulosae* (SD2), respectively. These isolates were capable of producing cellulolytic enzymes during growth on different cellulosic substrates. They were grown under different conditions and showed that carboxymethyl cellulose (CMC) was the best cellulosic substrates for inducing the synthesis of extracellular cellulolytic enzymes. In addition, both the isolates showed good growth at different pHs, temperature, incubation period, carbon and nitrogen sources when grown in liquid Winstead's media having 1.2% CMC as cellulosic substrate. However, the optimum growth was at pH 6.5-8.5, temperature 30-40°C and 8-14 days of incubation period using CMC as carbon source. The cellulose hydrolysis by both the isolates was also optimum under these conditions with the maximum level of reducing sugar produced due to carboxymethyl cellulase (CMCase) activity of SD1 and SD2 391.76 U/mL and 328.09 U/mL, respectively. The effects of various metal ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Ag^+, Hg^{2+} Mn^{2+}, Li^+ and Fe^{3+}) on the two cellulases were also investigated and some metal ions were found to inhibit cellulase reversibly.

Key words: Saw dust, Cellulases, CMCase, Winstead's medium, Optimization, *Streptomyces cirratus*, and *Streptomyces cellulosae*.

INTRODUCTION

Cellulose is an important structural component of the primary cell wall of green plants and contains a long chain polymeric polysaccharide where glucose units are joined together by 1, 4 β-linkage (Crawford, 1981; Updegraff, 1969). Lignin and cellulose, together termed as lignocellulose, is the most abundant biopolymer on earth. Sawdust, a by-product of wood processing is generally regarded as a waste. It is often heaped near carpenter's shades, burnt or dumped into rivers. Consequently, they block the water ways and if burnt, produce very thick smoke with high environmental consequences. Wastes and their disposal is a

* Corresponding Author : Dr Faozia Faleha Sadida, E-mail : ffsadida69@gmail.com manchur.mbio@cu.ac.bd

subject of environmental concern worldwide especially when they are non-biodegradable (Banjo and Kubuoye, 2000). Sawdust is made up of 3 major components; cellulose, hemicellulose and lignin (Alexander, 1997; Erikson *et al.*, 1990) where lignin is the most recalcitrant and protects the cellulose and hemicellulose from enzymatic attack by some microorganisms (Bonnarme and Jeffries, 1998).

There has been a considerable interest over the past years in the enzymatic degradation of lignocellulosic biomass, a renewable, abundant and inexpensive resource. The reasons for this interest varied and included potential applications in waste treatment, fuel production, oxychemical production, textile industry (Kasana *et al.*, 2008) for biopolishing of fabrics and producing stonewashed look of denims (Noe *et al.*, 1986). Different fungi, bacteria and actinomycetes have been used for the production of cellulases using different substrates. Actinomycetes are important parts of the microbial community in soil environment that are responsible for degradation and recycling of natural biopolymers, such as cellulose, lignin and chitin (Semedo *et al.*, 2001) and also a source of a wide range of other type of bioactive compounds for biotechnological applications (Okami and Hotta, 1988; Bull *et al.*, 1992). Actinomycetes are potential cellulase producers and help considerably in recycling nutrients in the biosphere and are thought to be involved in the primary degradation of organic matter in compost and related materials (Jang and Chen, 2003; Goodfellow and Williams, 1983). Since cellulose is the major component of plant biomass and potentially utilizable source of glucose, the process of microbial degradation of cellulose may be considered as financially viable and seems to be the wise choice. Actinomycetes cellulases are inducible extracellular enzymes (Ibrahim and El-diwany, 2007) that can be produced during their growth on cellulosic materials. Thus, introduction of cellulolytic microorganisms is a beneficial microbiological tool for recovery of bioenergy from degraded cellulose (Balamurugan *et al.*, 2011).

In this pretext, the objectives of the present study were to isolate some actinomycetes, screen them for their cellulolytic potential and optimize the physico-chemical parameters to maximize the yield of cellulase enzyme that can be utilized on commercial scale. In the course of screening for industrially important ones, several aerobic cellulolytic actinomycetes were isolated from saw dust samples collected from different localities of Hathazari, Chittagong, Bangladesh. Two promising cellulose degrading actinomycetes, designated as SD1 and SD2 were selected for the present study to find out the optimum conditions for their growth, cellulase production and enzyme activities.

MATERIALS AND METHODS

Isolation and screening of the cellulase producing microorganisms

Saw dust was collected from local sawmills of Hathazari, Chittagong to isolate cellulolytic Actinomycetes using Czapek's agar medium having 2% CMC as carbon source. The isolates were tested for cellulolysis by Congo red overlay method (Teather and Wood, 1982) using Winstead's medium having 1.2% CMC as carbon source. For the Congo-Red

method, plates were flooded with 0.1% Congo red (Sigma-Aldrich) for 10-15 minutes before destaining with 1M NaCl solution for 15-20 min for several times until the clear zones around the colonies were visualized. The use of Congo-Red as an indicator for cellulose degradation in an agar medium provides the basis for a rapid and sensitive screening test for cellulolytic microbes. Colonies showing discoloration of Congo-Red were taken as positive cellulose-degrading microbial colonies. Then the actinomycetes isolates were further screened (secondary screening) for their cellulolytic potentiality. It was done by using Winstead's medium having 1.2% CMC in separate small conical flasks.

Characterization of microorganisms: Among the numerous isolates SD1 and SD2 were found to be promising cellulose degraders. The isolates were maintained on slants of Nutrient Agar at 4°C with periodic sub culturing. For the characterization of the selected isolate, the basic routine laboratory investigation like morphological, cultural and different biochemical characteristics, which included Indole, methyl red, Voges–Proskauer, citrate utilization, catalase, urease, starch hydrolysis, gelatin hydrolysis, sugar fermentation, caseinase, hydrogen sulfide production and nitrate reduction tests were performed (Cappuccino and Sherman, 2005) and compared with the standard description given in Bergey's Manual of Determinative Bacteriology (8th ed.- Buchanan and Gibbons, 1974; 9th ed. Holt *et al.*, 1984). Forty eight hours old cultures were used for all the tests. Based on morphological, cultural and biochemical characteristics both the isolates SD1 and SD2 were provisionally identified as *Streptomyces cirratus* (Koshiyama *et al.*, 1963) and *S. cellulosae* (Waksman and Henrici, 1948), respectively.

Biomass yield: The filter paper containing biomass residue was dried in an oven at 80°C for a constant weight and amount of biomass was calculated by subtracting the weight of filter paper. Yield was expressed as mg/g cellulose.

Optimization of cultural condition

An attempt was made to determine the optimum culture conditions such as pH, temperature, incubation period, carbon and nitrogen sources requirement for maximum growth and activities. The biomass yields, extracellular protein, reducing sugar level and cellulase production of the isolates were recorded. To be more precise, the effects of pH (3.5, 4.5, 5.5. 6.5, 7.5 and 8.5), temperature (20°C, 25°C, 30°C, 35°C and 40°C) and incubation period (2, 4, 6, 8, 10, 12, and 14 days) on the growth and liquefaction were investigated and recorded (Table 1-3).

Similarly, the production of extracellular cellulase under different carbon and nitrogen sources was studied in the liquid Winstead's culture medium using five carbon sources (Avicel, CMC, Rice bran, Rice straw and Saw dust of 1.2%) and six nitrogen sources (Asparagine, Beef extract, Ammonium sulphate, Yeast extract, Peptone and Urea of 0.2%). Furthermore, effects of these carbon and nitrogen sources on the production of extracellular protein, reducing sugar and biomass yield were recorded (Table 4 & 5). Finally, the substrate concentration was optimized using different concentration of Carboxymethyl cellulose (CMC) (1.0%, 1.5%, 2.0% and 2.5% of CMC). The optimum concentration was

selected based on the maximum production of cellulase, extracellular protein, reducing sugar and biomass (Table 6).

Enzyme assays

The CMCase, FPase, Avicelase, and β-glucosidase, activities were determined by adding 2 ml filtrate 2 ml of 1% CMC (in citrate phosphate buffer, pH 7.0 plus 1 ml phosphate buffer), to 1 ml of phosphate buffer (along with 50 mg Whatman No. 1 filter paper strip, 1×6 cm), to 2 ml of 1% avicel plus 1 ml citrate phosphate buffer, to 2 ml of 1% salicin plus 1 ml citrate phosphate buffer, respectively. In all enzyme assays, the reaction mixtures were incubated at 35^{0}C for 2 hours in water bath followed determinations of of reducing sugars released Nelson's modification of Somogyi method (Nelson, 1944). Enzyme activity was expressed by the amount of glucose released in µg/ml of crude enzyme/hour (U/ml) (Mahadevan and Sridhar, 1982) and soluble protein in culture filtrate was estimated following the Lowry method (Lowry *et al.* 1951). The saccharification percentage was calculated by applying the following equation:

$$\text{Saccharification \%} = \frac{\text{mg of reducing sugar per ml}}{\text{mg of substrate per ml}} \times 100$$

Effect of Metals, Inhibitors and Reductants

Effects of ten different metal chlorides (*viz.* NaCl, KCl, NH_4Cl, $LiCl_2$, $AgCl_2$, $HgCl_2$, $MnCl_2$, $MgCl_2$, $CaCl_2$, and $FeCl_3$) and six different inhibitors and reductants (*viz.* Sodium azide, Sodium dodecyl sulphate (SDS), Urea, Cystein, β-Mercaptoethanol and Ethylene diamine tetraacetic acid (EDTA)) on cellulase activity were determined by adding 2 mL of culture filtrate to 2 ml of 1% CMC prepared in phosphate buffer followed by addition of 1% of metal and inhibitors & reductants solution, respectively. All tubes were incubated for 2 hrs in water bath at 35^{0}C and the enzyme activity was measured according to Nelson's modification of Somogyi method (Nelson, 1944).

RESULTS AND DISCUSSION

Isolation and screening of cellulose degrading actinomycetes

Isolation of the actinomycetes on plates containing Starch Casein Agar resulted in 42 isolates from seven different saw dust samples. They were tested for the production of cellulolytic enzyme on CMC Agar plates. Congo red staining of these plates along with 1N HCl revealed 15 isolates producing clear zones on the plates around the colonies indicating cellulose hydrolysis. Among the 15 isolates, SD1 and SD2 were selected for further investigation on the basis of the area of clear zone (Figure 1).

Cultural morphological and biochemical characteristics of the selected isolates

SD1 forms off white, circular, powdery colonies with radiating margins on the Starch Casein Agar medium (Figure 1A) whereas SD2 forms white colonies with light brown color pigmentation (Figure 1B). Gram's staining and microscopic view revealed gram-positive, filamentous structure of both the actinomycetes (Figure 1C-D). The specified biochemical tests performed on both the isolates and compared with the standard description given in Bergey's Manual of Determinative Bacteriology (8th ed. Buchanan and Gibbons, 1974; 9th ed. Holt *et al.*, 1994). Based on morphological, cultural and biochemical characteristics both the isolates SD1 and SD2 were provisionally identified as *Streptomyces cirratus* (Koshiyama *et al.*, 1963) and *S. cellulosae* (Waksman and Henrici, 1948), respectively.

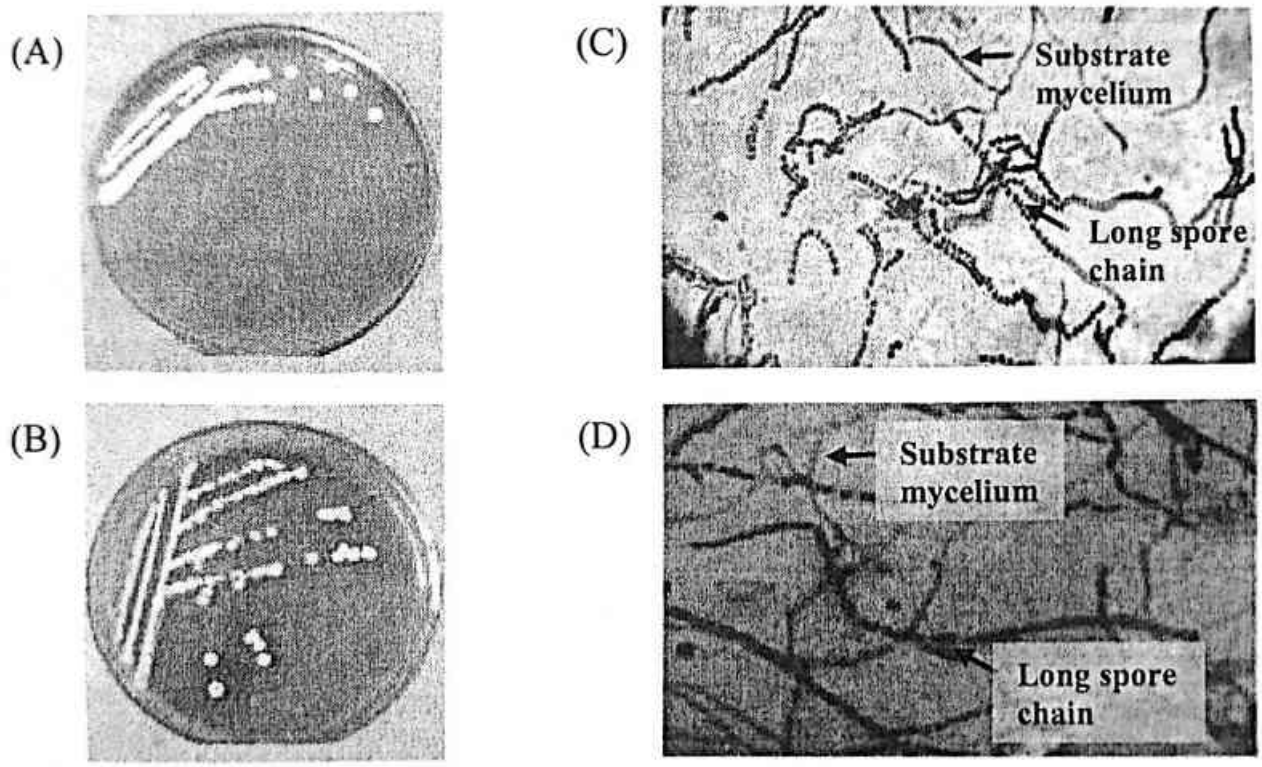

Figure 1: Isolation and characterization of *Streptomyces* spp. Colonies of SD1 (*Streptomyces cirratus*) appeared on Starch Casein Agar (A), SD2 (*Streptomyces cellulosae*) appeared on Starch Casein Agar (B), and microscopic (10x100) view of SD1 (C) and SD2 (D) are shown.

Optimization of culture condition for enzyme production

The effects of medium pH, temperature, incubation period, carbon and nitrogen sources on the production of CMCase, extracellular protein, reducing sugar and biomass yield are shown in Tables 1-6.

Effects of medium pH: Maximum production of CMCase (102.62 U/ml), extracellular protein (368.42 µg/ml), reducing sugar (99.63 µg/ml), saccharification (0.83%) and biomass yield (187.97 mg/g) by SD1 (*Streptomyces cirratus*) was recorded in culture media with pH range 7.5-8.5 whereas maximum production of CMCase (122.47 U/ml), extracellular protein (507.52 µg/ml), reducing sugar (122.47 µg/ml), saccharification (1.02%) and biomass yield (187.97 mg/g) by SD2 (*Streptomyces cellulosae*) was recorded at pH range 6.5-8.5 (**Table 1**). Similar results with different microbes were reported by many workers (Farhana *et al.*, 2000; Malek *et al.*, 1987; Shailendra *et al.*, 1991).

Effects of temperature: Maximum production of CMCase (72.66 U/ml by SD1 and 97.38 U/ml by SD2), extracellular protein (390.23 µg/ml by SD1 and 379.70 µg/ml by SD2), reducing sugar (92.88 µg/ml by SD1 and 67.04 µg/ml by SD2) and saccharification (0.77% by SD1 0.56% by SD2) by both the isolates were recorded in culture media at temperature range 30°C - 35°C, but highest biomass production (300.06 mg/g by SD1 and 450.09 mg/g by SD2) by both the isolates was recorded at 40°C (**Table 2**). Liquefaction of Winstead's medium (with 1.2% CMC) due to enzyme activity at 35°C and 40°C which was recorded herein found similar to the findings of many workers (Malek *et al.*, 1987; Donnelly 1990, Shailendra *et al.*, 1991; Farhana *et al.*, 2000; Hossain *et al.*, 1998; Shibli *et al.*, 2001; Araujo and Ward, 1990; Hachiro and Kazuhiko, 1991; Khanam *et al.*, 2004).

TABLE 1: EFFECT OF pH ON EXTRACELLULAR PROTEIN, REDUCING SUGAR LEVEL, CMCASE ACTIVITY, SACCHARIFICATION (%) AND BIOMASS YIELD OF THE TWO ACTINOMYCETES ISOLATES WHILE GROWN IN WINSTEAD'S MEDIUM HAVING 1.2% CMC AS CARBON SOURCE AND 0.2% ASPARAGINE AS NITROGEN SOURCE

pH	Isolate No.	Change of medium pH after Incubation	Extracellular Protein µg/ml	Reducing Sugar µg/ml	CMCase Activity U/ml	Biomass Yield mg/g	Saccharification (%)
3.5	SD1	4.65	89.47	29.59	27.72	66.68	0.25
	SD2	3.60	20.35	13.11	09.22	22.02	0.02
4.5	SD1	4.54	77.44	35.21	22.72	83.35	0.29
	SD2	4.60	131.80	21.25	18.31	66.68	0.18
5.5	SD1	6.90	114.29	38.58	51.42	83.35	0.32
	SD2	7.10	221.80	41.35	48.31	89.680	0.35
6.5	SD1	7.45	121.80	63.71	55.81	135.02	0.28
	SD2	8.10	282.71	122.47	68.91	105.02	1.02
7.5	SD1	8.45	202.26	99.63	102.62	91.68	0.83
	SD2	8.90	507.52	89.17	65.92	135.02	0.49
8.5	SD1	8.85	368.42	38.20	75.66	187.97	0.318
	SD2	8.90	64.66	80.90	50.56	216.71	0.67

TABLE 2: EFFECT OF TEMPERATURE ON EXTRACELLULAR PROTEIN, REDUCING SUGAR LEVEL, CMCASE ACTIVITY, SACCHARIFICATION (%) AND BIOMASS YIELD OF THE TWO ACTINOMYCETES ISOLATES WHILE GROWN IN WINSTEAD'S MEDIUM HAVING 1.2% CMC AS CARBON SOURCE AND 0.2% ASPARAGINE AS NITROGEN SOURCE

Temperature (°C)	Isolate No.	Change of medium pH after Incubation	Extracellular Protein µg/ml	Reducing Sugar µg/ml	CMCase Activity U/ml	Biomass Yield mg/g	Saccharification (%)
20	SD1	7.97	76.69	63.30	52.06	241.71	0.53
	SD2	8.26	281.95	53.56	54.31	198.37	0.45
25	SD1	8.40	218.05	38.58	24.34	220.04	0.20
	SD2	8.26	302.26	52.81	71.91	196.73	0.44
30	SD1	8.25	390.23	92.88	68.54	150.03	0.77
	SD2	8.26	379.70	67.04	74.16	135.02	0.56
35	SD1	7.08	164.66	27.72	72.66	243.38	0.32
	SD2	7.70	148.87	45.32	97.38	298.96	0.38
40	SD1	6.60	138.35	23.60	43.07	300.06	0.23
	SD2	6.40	57.89	34.08	31.46	450.09	0.28

Effects of incubation period: Maximum production of CMCase (154.31 U/ml), extracellular protein (290.23 µg/ml), reducing sugar (150.94 µg/ml) and saccharification (1.26%) by SD1 was recorded after 14 days of incubation whereas maximum production of CMCase (101.12 U/ml), extracellular protein (198.50 µg/ml), reducing sugar (92.13 µg/ml) and saccharification (0.77%) by SD2 was recorded after 8 days of incubation. But highest biomass yield (300.06 mg/g by SD1 and 333.40 mg/g by SD2) by both the actinomycetes was recorded after 12 days incubation (**Table 3**). Similar results recorded with different microorganisms were also reported by other workers (Das *et al.*, 2010; Nandimath *et al.*, 2016).

TABLE 3: EFFECT OF INCUBATION PERIOD ON EXTRACELLULAR PROTEIN, REDUCING SUGAR LEVEL, CMCASE ACTIVITY, SACCHARIFICATION (%) AND BIOMASS YIELD OF THE TWO ACTINOMYCETES ISOLATES WHILE GROWN IN WINSTEAD'S MEDIUM HAVING 1.2% CMC AS CARBON SOURCE AND 0.2% ASPARAGINE AS NITROGEN SOURCE

Incubation Period (Day)	Isolate No.	Change of medium pH after Incubation	Extracellular Protein µg/ml	Reducing Sugar µg/ml	CMCase Activity U/ml	Biomass Yield mg/g	Saccharification (%)
2	SD1	7.25	147.37	19.10	16.10	35.00	0.16
	SD2	7.15	163.91	13.11	14.98	63.34	0.11
4	SD1	6.43	140.23	38.58	59.92	66.68	0.32
	SD2	6.45	121.05	29.96	75.66	68.34	0.25
6	SD1	6.95	130.08	31.09	40.82	83.35	0.26
	SD2	7.65	57.89	28.84	54.31	75.01	0.24
8	SD1	6.79	135.34	28.46	32.96	100.02	0.24
	SD2	6.62	198.50	92.13	101.12	100.03	0.77
10	SD1	6.80	169.99	17.23	43.56	250.05	0.14
	SD2	6.60	90.98	62.22	24.33	100.03	0.52
12	SD1	7.60	52.63	76.03	50.56	300.06	0.63
	SD2	6.70	75.19	85.77	81.65	333.40	0.71
14	SD1	8.27	290.23	150.94	154.31	233.38	1.26
	SD2	8.19	74.89	70.41	77.15	300.06	0.59

Effects of carbon source: The highest extracellular protein (136.84 µg/ml by SD1 and 144.32 µg/ml by SD2), reducing sugar (189.89 µg/ml by SD1 and 232.96 µg/ml by SD2), CMCase activity (83.90 U/ml by SD1 and 46.07 U/ml by SD2) and saccharification (1.58% by SD1 and 1.94% by SD2) were recorded when CMC was used as carbon source for both the actinomycetes. But highest biomass yield (653.47 mg/g by SD1 and 558.45 mg/g by SD2) was recorded with saw dust **(Table 4).**

TABLE 4: EFFECT OF CARBON SOURCES ON EXTRACELLULAR PROTEIN, REDUCING SUGAR LEVEL, CMCASE ACTIVITY, SACCHARIFICATION (%) AND BIOMASS YIELD OF THE TWO ACTINOMYCETES ISOLATES WHILE GROWN IN WINSTEAD'S MEDIUM HAVING DIFFERENT CARBON SOURCE AND 0.2% ASPARAGINE AS NITROGEN SOURCE

Carbon Source	Isolate No.	Change of medium pH after Incubation	Extracellular Protein µg/ml	Reducing Sugar µg/ml	CMCase Activity U/ml	Biomass Yield mg/g	Saccharification (%)
Avicel	SD1	7.99	52.63	3.00	31.09	213.37	0.02
	SD2	8.00	63.16	4.49	22.47	196.76	0.04
CMC	SD1	7.99	136.84	189.89	83.90	183.37	1.58
	SD2	8.00	144.32	232.96	46.07	116.69	1.94
Saw dust	SD1	8.00	81.20	32.95	28.84	653.47	0.27
	SD2	8.05	99.25	18.73	12.73	558.45	0.16
Rice Bran	SD1	7.69	81.95	90.64	67.04	186.70	0.76
	SD2	7.85	73.68	12.73	22.47	100.06	0.11
Rice straw	SD1	8.17	116.54	23.97	37.83	470.10	0.20
	SD2	8.06	126.36	34.08	11.24	473.43	0.28

Effects of nitrogen source: The maximum extracellular protein (157.89 µg/ml by SD1 and 335.34 µg/ml by SD2), reducing sugar (214.19 µg/ml by SD1 and 252.81 µg/ml by SD2), CMCase activity (244.19 U/ml by SD1 and 269.66 U/ml by SD2) and saccharification (1.78% by SD1 and 2.11% by SD2) were recorded when peptone was used as nitrogen source for both the actinomycetes **(Table 5)**. However, the highest biomass yield (250.05 mg/g by SD1 and 466.76 mg/g by SD2) was recorded with urea.

TABLE 5: EFFECT OF NITROGEN SOURCES ON EXTRACELLULAR PROTEIN, REDUCING SUGAR LEVEL, CMCASE ACTIVITY, SACCHARIFICATION (%) AND BIOMASS YIELD OF THE TWO ACTINOMYCETES ISOLATES WHILE GROWN IN WINSTEAD'S MEDIUM HAVING 1.2% CMC AS CARBON SOURCE WITH DIFFERENT NITROGEN SOURCE

Nitrogen Source	Isolate No.	Change of medium pH after Incubation	Extracellular Protein µg/ml	Reducing Sugar µg/ml	CMCase Activity U/ml	Biomass Yield mg/g	Saccharification (%)
Beef Extract	SD1	7.89	42.04	42.82	49.60	71.68	0.36
	SD2	7.99	151.88	105.24	95.88	171.70	0.88
Asparagine	SD1	7.62	141.35	38.20	36.33	89.95	0.32
	SD2	7.76	114.29	96.63	44.94	100.30	0.81
Urea	SD1	8.43	66.67	34.46	31.46	250.05	0.29
	SD2	8.15	54.89	44.94	37.45	466.76	0.37
Ammonium Sulphate	SD1	6.99	72.18	67.67	56.18	66.68	0.56
	SD2	6.96	75.19	19.48	61.05	50.01	0.16
Peptone	SD1	7.60	157.89	214.19	244.19	216.71	1.78
	SD2	7.91	335.34	252.81	269.66	216.71	2.11
Yeast Extract	SD1	8.35	122.56	95.51	117.60	171.70	0.80
	SD2	8.16	148.87	130.71	97.00	150.03	1.09

Effect of substrate concentration: Maximum production of CMCase (61.32 U/ml by SD1 and 76.89 U/ml by SD2), extracellular protein (306.02 µg/ml by SD1 and 296.24 µg/ml by SD2), reducing sugar (111.24 µg/ml by SD1 and 185.77 µg/ml by SD2) and saccharification (0.93% by SD1 1.55% by SD2) by both the isolates were observed with 2% (w/v) concentration of CMC. However, the highest biomass production by SD1 (365.02 mg/g) and SD2 (470.09 mg/g) was recorded with of 2.0% and 2.5% CMC concentration, respectively (**Table 6**). Similar observations were also made by some other workers (Singh *et al.*, 2004; Kaniz and Manchur, 2015).

TABLE 6: EFFECT ON EXTRACELLULAR PROTEIN, REDUCING SUGAR LEVEL, CMCASE ACTIVITY, SACCHARIFICATION (%) AND BIOMASS YIELD OF THE TWO ACTINOMYCETES ISOLATES WHILE GROWN IN WINSTEAD'S MEDIUM HAVING DIFFERENT CONCENTRATION OF CMC AS CARBON SOURCE AND 0.2% ASPARAGINE AS NITROGEN SOURCE

CMC (%)	Isolate No.	Change of medium pH after Incubation	Extracellular Protein µg/ml	Reducing Sugar µg/ml	CMCase Activity U/ml	Biomass Yield mg/g	Saccharification (%)
1.0	SD1	7.75	262.08	52.07	41.95	210.95	0.43
	SD2	8.25	242.96	48.15	54.31	132.48	0.40
1.5	SD1	7.75	254.14	13.11	43.82	216.71	0.11
	SD2	8.75	263.16	29.59	27.72	116.69	0.25
2.0	SD1	8.20	306.02	111.24	61.32	245.04	0.93
	SD2	8.40	296.24	185.77	76.89	470.09	1.55
2.5	SD1	8.25	279.70	41.57	22.10	365.02	0.35
	SD2	8.75	291.73	27.34	52.06	316.73	0.23

Cellulase (CMCase) production by both the cellulolytic actinomycetes was also tested using optimum condition including all the essential factors, such as pH, temperature, incubation time, carbon and nitrogen sources and substrate (CMC) concentration. Highest value of extracellular protein (728.57 µg/ml by SD1 and 706.77 µg/ml by SD2), reducing sugar (369.29 µg/ml by SD1 and 360.67 µg/ml by SD2), CMCase activity (391.76 U/ml by SD1 and 328.09 U/ml by SD2) and biomass (402.96 mg/g by SD1 and 482.85 mg/g by SD2) production was recorded (**Table not shown**).

Enzyme activity: The quantitative cellulase activity (CMCase activity) of crude enzymes produced by both the actinomycetes grown in liquid Winstead's medium having 1.2% of CMC was investigated using different reaction time, pH, temperature, carbon and nitrogen sources, metals and inhibitors (**Figure 2 & 3**).

Investigation of the effects of enzyme-substrate reaction time on CMCase activity of crude enzyme clearly indicated that SD1 had the highest CMCase activity (72.29 U/ml) at 30 minutes while SD2 had the highest CMCase activity (60.67 U/ml) at 90 minutes, very similar to previous observations (Figure 2A) (Geoffrey, 1989; Nandimath *et al.*, 2016).

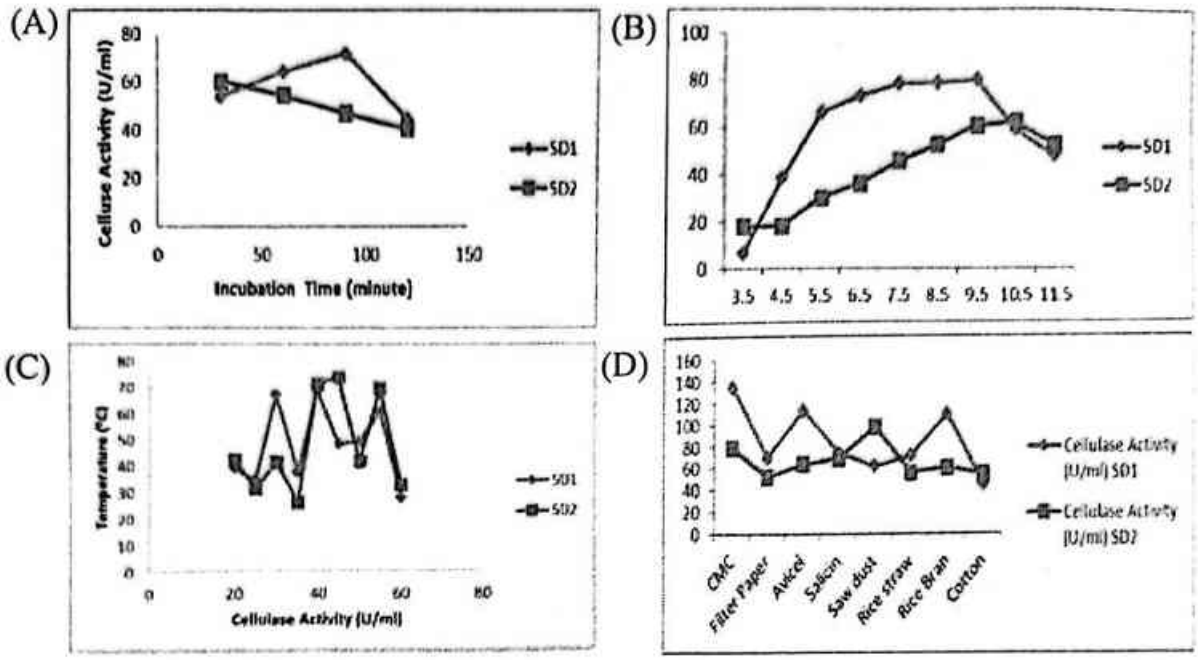

Figure 2: Cellulase activities (reducing sugar released) of crude enzymes of the two actinomycetes isolates in different enzyme-substrate reaction time (A), at different enzyme-substrate pH (B), at different enzyme-substrate incubation temperature (C) and in presence of different carbon sources (D) are shown.

The pH-dependent CMCase activity of crude enzyme produced by SD1 (*Streptomyces cirratus*) and SD2 (*Streptomyces cellulosae*) showed the highest CMCase activity at pH 9.5 (80.90 U/ml by SD1) and 10.5 (62.92 U/ml by SD2), respectively (Figure 2B). Similar findings were also reported by other workers (Immanuel *et al.*, 2006; Khanam and Anwar, 2004; Hossain *et al.*, 1999).

However, Effects of reaction temperature were bit redundant and/or irregular (Figure 2), as reported previously (Goyal and Soni, 2011; Khanam and Anwar, 2004; Shailendra *et al.*, 1991). To be precise, the SD1 and SD2 isolates showed different patterns of temperature-dependent CMCase activities with optimum temperature of 40°C (69.29 U/ml by SD1) and 45°C (75.53 U/ml by SD2). (Figure 2-3).

Effects of Carbon and Nitrogen Sources on Enzyme Activity: The quantitative investigation of the effects of nitrogen sources on CMCase activity of crude enzyme produced by SD1 (*Streptomyces cirratus*) and SD2 (*Streptomyces cellulosae*) clearly

showed that the highest cellulase activity was recorded with SD1 (135.58 U/ml) and SD2 (97.75 U/ml) when CMC and saw dust were used as carbon sources, respectively (Table 8), fully corroborating previous reports (Mandel and Reese, 1957; Mandels *et al.*, 1962; Martin and Eberhart, 1966; Mandel and Weber, 1869; Nishizawa *et al.*, 1972; Breuil and Kushner, 1976; Donald *et al.*, 1995; Kashem, 1998; Hossain *et al.*, 1999; Kaniz and Manchur, 2015). However, both isolates showed The highest cellulase activity (135.58 U/ml by SD1 and 97.75 U/ml by SD2) only when peptone was used as nitrogen source (Table 9), similar to previous observations (Yogita *et al.*, 2015; Ray *et al.*, 2007; Paudel YP and Qin W, 2015).

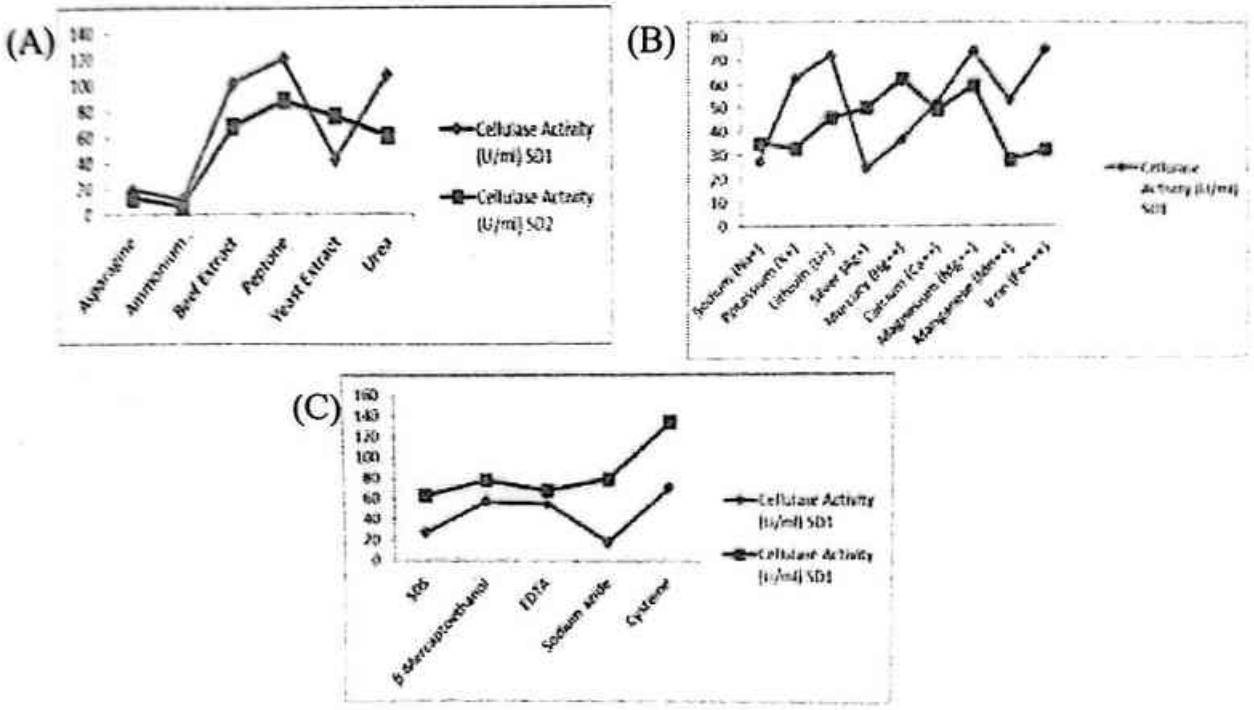

Figure 3: Cellulase activities (reducing sugar released) of crude enzymes of the two actinomycetes isolates in presence of different nitrogen sources (A), different metal ions (B) and different inhibitors and reductants (C) are shown.

Effect of Metals, Inhibitors and Reductants on Enzyme Activity:

The CMCase activity of the crude extracts of the both actinomycetes isolates decreased in presence of any additional metals tested in this study (Figure 3B). However, the maximum inhibition was observed with Ag^{+} and Na^{+} for SD1 (by 24.34 U/ml and 27.34 U/ml, respectively). On the other hand, Mn^{2+} (27.72 U/ml), Fe^{3+} (32.21 U/ml) and K^{+} (32.96 U/ml) resulted significant CMCase inhibition in SD2. In addition, in both cases Mg^{2+} (74.12 U/ml) and Li^{2+} (72.23 U/ml) had minimal inhibition effects on enzyme activity. These observations were quite very similar to other previous reports (Saha, 2004; Lucas *et al.*, 2001; Murashima, 2002).

On the other hand, the presence of inhibitors and reductants (Sodium dodecyl sulphate, β-Mercaptoethanol, Ethylene diamine tetra-acetic acid, Sodium azide and Cysteine) had different effects on enzyme activity (Figure 3C). To be more precise, Cysteine stimulated cellulase activity in SD2 (134.08 U/ml) while Sodium azide inhibited enzyme activity (17.60 U/ml) in SD1 when compared with those observed in the absence of any inhibitors and reductants (for SD1 and SD1 the activity were 135.58 U/ml and 97.75 U.Vml, respectively). Our finding is concurrent with some other workers (Singh *et al.*, 1990; Li-Jung Yin *et al.*, 2010).

Conclusion

Cellulases from two potentially cellulolytic actinomycetes *Streptomyces cirratus* (SD1) and *Streptomyces cellulosae* (SD2) that have been isolated from saw dust collected from local area of Chittagong. They were studied based on optimum cultural condition such as incubation time, pH, temperature, carbon and nitrogen sources maximum production of cellulases. Crude cellulases were analyzed to find out the optimum condition for highest cellulase activity. Besides, both the crude cellulases were tested using different metals, reductant and inhibitors. Cysteine was found to stimulate the enzyme activity of cellulase of *S. cellulosae* (SD2) where as its activity was significantly inhibited by Mn^{2+}.

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