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PRODUCTION AND OPTIMIZATION OF CELLULASE ACTIVITY OF *THERMOMONOSPORA VIRIDIS* ISOLATED FROM RICE STRAW

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Abstract

A highly cellulolytic actinomycete SR1 was locally isolated from rice straw and provisionally identified as *Thermomonospora viridis*. Optimum pH, temperature, carbon and nitrogen sources for its cellulase production were 6.5, 35°C, Carboxymethyl cellulase (CMC) and yeast extract, respectively whereas those of cellulase activity were 7.5, 40°C, CMC and peptone respectively. The effects of various metal ions and different reductant and inhibitors on its cellulase activity were investigated. Univalent Ag^{+} was found to decrease the enzyme activity whereas increased by bivalent Mg^{2+}. Ethylene diamine tetraacetic acid (EDTA) caused remarkable decrease of cellulase activity but β-Mercaptoethanol stimulated its cellulase activity.

Introduction

Rice cultivation produces large quantities of straw waste in Bangladesh as well as most part of the world. As the components of rice straw are mainly cellulose and hemicellulose encrusted by lignin, it is resistant to microbial decomposition in compared to straw from other protein-rich grains such as wheat and barely (Parr *et al*. 1992). Generally, on-field burning and incorporation of rice straw was practiced by farmers to manage this waste. Incomplete combustion of rice straw due to burning causes disturbance to air quality in the environment, losses of soil nutrient which negatively impact the soil fertility and release of greenhouse gases (Rosmiza *et al*. 2014).

Biotechnological conversion of cellulosic biomass is potentially sustainable approach to develop novel bioprocesses and products. Microbial cellulases have become the focal biocatalysts due to their complex nature and wide spread industrial applications. Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including fungi, bacteria and actinomycetes. Among the cellulose degraders actinomycetes are well known for their ability to decompose complex molecules, particularly lignocellulose components, which make them important agents in decomposition processes (Lacey 1997). Additionally, the apparent widespread ability of actinomycetes to generate soluble lingo-carbohydrate from straw has been confirmed (Ball *et al*. 1990, Mason *et al*. 2001). Another approach of dealing with rice straw was mixing it into the soil. It is comparatively easy to mix the rice straw to soil as the soil inhabiting microorganisms transform the components of lignocellulose into compost. However, it usually takes a long time to decompose and impoverish the soil easily. Like bacteria and fungi, actinomycetes also produce and release multiple enzymes involved in the degradation of lignocellulose (Ball and McCarthy 1989). *Streptomyces* had been in the main focus point as the lignocellulose degrading ability of some *Streptomyces* spp. was reported (Spear *et al.* 1993). Therefore, it was necessary to search for more efficient actinomycetes for the biodegradation of rice straw.

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Different fungi, bacteria and actinomycetes have been used for the production of cellulases using different substrates. Actinomycetes are important part of the microbial community in soil environment because they are responsible for degradation and recycling of natural biopolymers, such as cellulose, lignin and chitin (Semedo *et al*. 2001). 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 (Goodfellow and Williams 1983, Jang and Chen 2003). Cellulose is the major component of plant biomass and potentially utilizable source of glucose. Therefore, microbial degradation process of cellulose can be considered as financially viable and seems to be the wise choice.

In this pretext, the present study was aimed to isolate and identify potential cellulolytic actinomycetes, optimize the physicochemical parameters to maximize the yield of cellulase enzyme that can be utilized on commercial scale. Based on the screening for cellulolytic potential one promising cellulose degrading actinomycete, SR1 that has been identified as *Thermomonospora viridis* was selected for the present study to find out the optimum conditions for its growth, cellulase production and enzyme activities.

Materials and Methods

Rice straw was collected from local paddy field of Hathazari, Chittagong to isolate actinomycetes using Starch Casein Agar medium. 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 min 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.

Among the numerous isolates SR1 was found to be promising cellulose degrader. For the characterization of the 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 (Buchanan and Gibbons 1974. Holt *et al*. 1994). Based on morphological, cultural and biochemical characteristics the isolate SR1 was provisionally identified as *Thermomonospora viridis*.

An attempt was made to determine the optimum culture conditions such as pH, temperature, carbon and nitrogen sources requirement for maximum cellulase production. The biomass yields, extracellular protein, reducing sugar level and cellulase production of the isolates were recorded. To be more precise, the effects of pH (4.5, 5.5, 6.5. 7.5, 8.5, 9.5 and 10.5) and temperature (20, 25, 30, 35, 40 and 45°C) on the growth and liquefaction were investigated and recorded (Tables 1 and 2). 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 (Tables 3 and 4). Finally, the substrate concentration was optimized using

different concentrations 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 5).

Physicochemical parameters such as different pH (3.5, 4.5, 5.5, 6.5, 7.5, 8.5, 9.5 and 10.5), temperature (20, 25, 30, 35, 40, 45, 50, 55 and 60°C), carbon sources (avicel, CMC, rice straw, rice bran, saw dust, filter paper, cotton and salicin) and nitrogen sources (asparagine, ammonium sulphate, beef extract, peptone, yeast extract and urea) on the activities of crude cellulase were determined. To determine cellulase activity, the reaction mixtures (2 ml filtrate + 2 ml of 1% substrate in citrate phosphate buffer + 1 ml phosphate buffer) were incubated at 35°C for 2 hrs in water bath followed by the determinations of reducing sugars released by 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:

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

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.

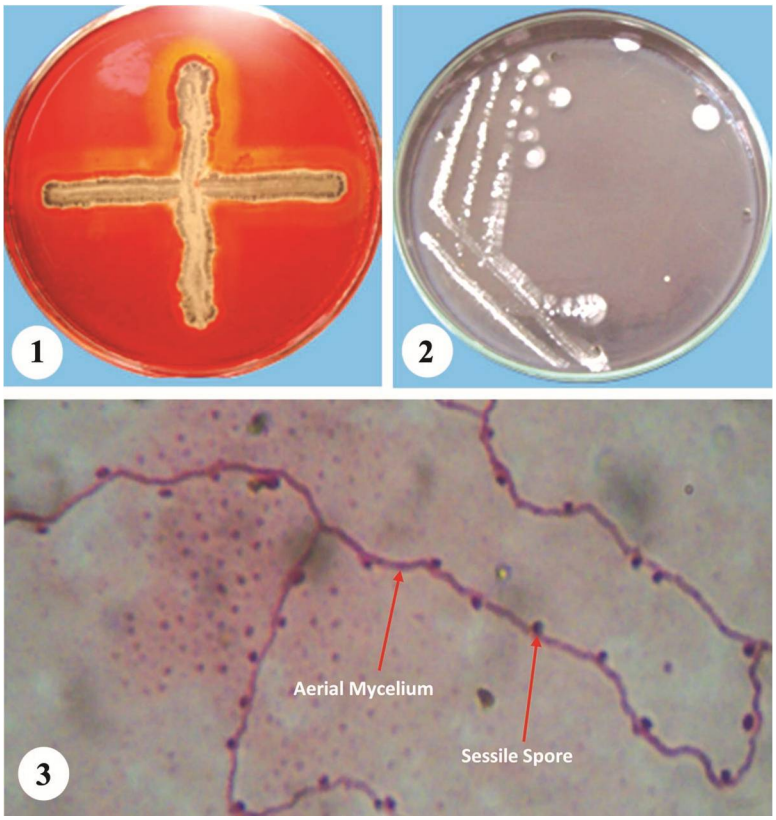
Effect of different metal ions as well as reductant and inhibitors on the activities of crude cellulase was investigated. Effects of ten different metal chlorides [*viz.* NaCl, KCl, NH_4Cl, $LiCl_2$, $AgCl_2$, $HgCl_2$, $MnCl_2$, $MgCl_2$, $CaCl_2$, and $FeCl_3$] and six different inhibitors and reductants [*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 and reductants solution, respectively. All tubes were incubated for 2 hrs in water bath at 35^0C and the enzyme activity was measured according to Nelson's modification of Somogyi method (Nelson 1944).

Results and Discussion

Isolation of the actinomycetes on plates containing Starch Casein Agar resulted in 20 isolates from seven different rice straw samples. They were tested for the production of cellulolytic enzyme on CMC Agar plates. Congo red staining of these plates along with 1M NaCl revealed 6 isolates producing clear zones on the plates around the colonies indicating cellulose hydrolysis. Among the 6 isolates, SR1 was selected for further investigation on the basis of the area of clear zone (Fig. 1).

The isolate SR1 forms light ash colour, circular, powdery colonies with filamentous margins on the Starch Casein Agar medium (Fig. 2). Gram's staining and microscopic view revealed gram-positive, filamentous structure with sessile spore (Fig. 3). The specified biochemical tests performed on the isolate and compared with the standard description given in Bergey's Manual of Determinative Bacteriology (Buchanan and Gibbons 1974, Holt *et al*. 1994). Based on morphological, cultural and biochemical characteristics SR1 was provisionally identified as *Thermomonospora viridis*.

Culture condition for Cellulase production by the microbes is greatly influenced by different physicochemical parameters. Therefore effects of medium pH, temperature, carbon and nitrogen sources and substrate concentration on the production of CMCase, extracellular protein, reducing sugar and biomass yield were determined.



Figs 1-3: 1. Primary screening for cellulolytic activity of Isolate SR1 (*Thermomonospora viridis*). 2 Single colonies of SR1 (*Thermomonospora viridis*) appeared on Starch Casein Agar. 3. Microscopic view (10×100) of SR1 (*Thermomonospora viridis*).

Maximum production of CMCase (136.329 U/ml), extracellular protein (293.233 µg/ml), reducing sugar (149.438 µg/ml), saccharification (1.245%) and biomass yield (151.11 mg/g) by the isolate SR1 (*Thermomonospora viridis*) was recorded in culture media with pH range 6.5-7.5 (Table 1). *Streptomyces* sp. NEAE-D isolated from rice straw showed highest cellulase production at pH 6.5 (El-Naggar and Abdelwahed 2012) and similar report was made by other workers with four *Streptomyces* spp. isolated from different cellulosic substrates (Alam *et al*. 2011).

Maximum production of CMCase (169.06 U/ml), extracellular protein (248.87 µg/ml), reducing sugar (122.27 µg/ml) and saccharification (1.02%) by the isolate was recorded in culture media at temperature 35°C, but highest biomass production (72.89 mg/g) was recorded at 20°C (Table 2). Liquefaction of Winstead's medium (with 1.2% CMC) due to enzyme activity at 35°C which was recorded herein was found to be similar to the findings of many workers such as *Serratia* sp. isolated from Municipal solid wastes and Rice straw (Khatiwada *et al*. 2016) and

Bacillus pumilus from soil under the Jarul forest (Kaniz *et al.* 2016) produced maximum cellulase at 35°C.

Table 1. Effect of pH on extracellular protein and reducing sugar production and CMCase activity by *Thermomonospora viridis* (SR1).

pH	Extracellular protein (µg/ml)	Reducing sugar (µg/ml)	CMCase activity (U/ml)	Biomass yield (mg/ml)	Saccharification (%)
4.5	221.052	13.483	21.722	0.60	0.112
5.5	254.112	85.767	96.629	1.20.	0.714
6.5	293.233	149.438	136.329	1.22	1.245
7.5	287.969	73.408	70.411	151.11	0.611
8.5	162.406	44.449	47.191	2.32	0.370
9.5	22.90	60.10	30.11	1.40	0.50
10.5	12.80	36.10	14.11	32.11	0.27

Temperature 35ºC.

Table 2. Effect of Temperature on extracellular protein and reducing sugar production and CMCase activity by *Thermomonospora viridis* (SR1).

Temp. (^{0}C)	Extracellular protein (µg/ml)	Reducing sugar (µg/ml)	CMCase activity (U/ml)	Biomass yield (mg/ml)	Saccharification (%)
20	221.804	80.149	79.026	72.89	0.67
25	196.10	68.164	102.621	1.80	0.57
30	108.270	113.483	149.063	1.62	0.95
35	248.87	122.27	169.06	2.86	1.02
40	178.195	118.726	18.992	2.20	0.99
45	60.15	90.23	77.81	44.50	0.75

Medium pH 7.5.

The highest extracellular protein (146.12 µg/ml), reducing sugar (144.19 µg/ml), CMCase activity (50.56 U/ml) and saccharification (1.21%) were recorded when CMC was used as carbon source for the actinomycete. But highest biomass yield (395.07 mg/g) was recorded with saw dust (Table 3). Previously maximum production of cellulase by *Bacillus* sp. using CMC as carbon source was reported by other workers (Das *et al*. 2010, Kaniz *et al.* 2016) and similar findings with *Aspergillus niger* was reported by Gautam *et al.* (2011).

Maximum extracellular protein (192.48 µg/ml), reducing sugar (359.93 µg/ml), CMCase activity (283.90 U/ml) and saccharification (3.0%) were recorded when yeast extract was used as nitrogen source for the actinomycete (Table 4). However, the highest biomass yield (243.39 mg/g) was recorded with urea. El-Naggar and Abdelwahed (2012) reported maximum cellulase production in presence of yeast extract by *Streptomyces* sp. strain NEAE-D.

Maximum production of CMCase (154.31 U/ml), extracellular protein (309.78 µg/ml), reducing sugar (122.85 µg/ml) and saccharification (1.02%) by the isolates was observed with 2% (w/v) concentration of CMC. However, the highest biomass production (300.06 mg/g) was recorded with 2.5% CMC concentration (Table 5). Similar observations were also made by

El-Naggar and Abdelwahed (2012) and Das *et al.* (2010) whereas Sadhu *et al.* (2014) reported best cellulase production with 8% CMC by *Bacillu*s sp.

Table 3. Effect of carbon sources on extracellular protein and reducing sugar production and CMCase activity by *Thermomonospora viridis* (SR1).

Carbon source	Extracellular protein (µg/ml)	Reducing sugar (µg/ml)	CMCase activity (U/ml)	Biomass yield (mg/ml)	Saccharification (%)
Avicel	69.17	1.87	22.85	183.37	0.01
CMC	146.12	144.19	50.56	228.37	1.21
Saw dust	112.78	24.72	37.83	395.07	0.21
Rice bran	75.19	11.61	11.99	140.03	0.10
Rice straw	134.59	39.70	10.86	150.03	0.33

Legend: Temperature 35ºC; Medium pH 7.5.

Table 4. Effect of nitrogen sources on extracellular protein and reducing sugar production and CMCase activity by *Thermomonospora viridis* (SR1).

Nitrogen source	Extracellular protein (µg/ml)	Reducing sugar (µg/ml)	CMCase activity (U/ml)	Biomass yield (mg/ml)	Saccharification (%)
Beef extract	73.68	317.60	108.99	190.03	2.65
Asparagine	106.77	187.27	177.53	100.20	1.56
Urea	58.65	132.96	62.17	243.39	1.11
$(NH_4)_2SO_4$	69.92	205.24	143.07	16.67	1.71
Peptone	151.88	154.31	178.65	233.38	1.29
Yeast extract	192.48	359.93	283.90	126.69	3.00

Temperature 35ºC, Medium pH 7.5.

Table 5. Effect of substrate concentration on extracellular protein and reducing sugar production and CMCase activity by *Thermomonospora viridis* (SR1).

CMC (%)	Extracellular protein (µg/ml)	Reducing sugar (µg/ml)	CMCase activity (U/ml)	Biomass yield (mg/ml)	Saccharification (%)
1.0	228.43	38.21	47.20	102.00*	0.32
1.5	250.38	35.21	114.23	180.03	0.29
2.0	309.78	122.85	154.31	253.38	1.02
2.5	240.60	102.62	102.62	300.06	0.86

Temperature 35ºC, Medium pH 7.5.

Cellulase (CMCase) activity is also greatly influenced by some physicochemical parameters such as pH, temperature, carbon and nitrogen sources etc. Therefore, the quantitative cellulase (CMC-ase) activity of crude enzymes produced by the isolate *Thermomonospora viridis* (SR1) was investigated using different pH, temperature, carbon and nitrogen sources.

The quantitative CMCase activity of crude enzyme produced by *Thermomonospora viridis* (SR1) was investigated at 35°C using their suitable carbon and nitrogen sources at different pH is shown in Fig. 4a. The highest CMCase activity by the actinomycete was recorded at pH 7.5 (172.92 U/ml). A pH range of 5.0 - 9.0 was reported as optimum for maximum xylanase activity by *Thermomonospora fusca* (George *et al.* 2001) whereas maximum CMCase activity by *Bacillus pumilus* at pH 7.5 was reported (Kaniz *et al.* 2016).

The results presented in Fig, 4b on the quantitative CMCase activity of crude enzyme produced by *Thermomonospora viridis* (SR1) using suitable pH, carbon and nitrogen sources at different temperature showed that the optimum temperature during enzyme substrate reaction of crude enzyme of the actinomycete was the best at 40°C (217.85 U/ml). Similar observation with enzyme-substrate reaction temperature was reported by other workers (Kaniz *et al*. 2016, Khatiwada *et al.* 2016;). The cellulases of *Thermomonospora curvata* and *T. fusca* active at wide range of temperature have been reported by other workers (Stutzenberger 1995, Tuncer *et al.* 1999).

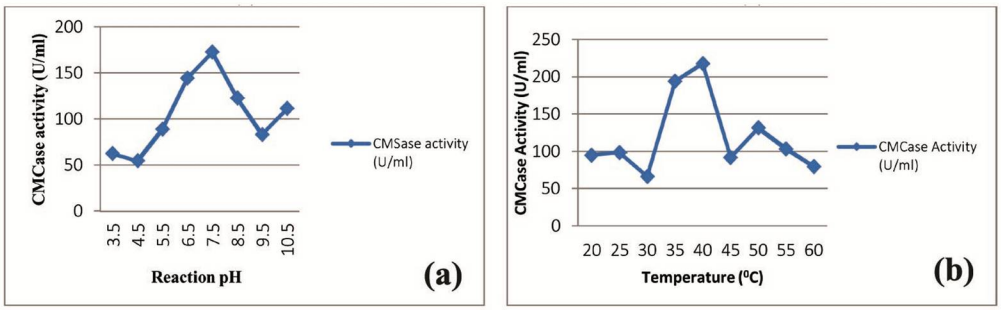


Fig. 4. Cellulase activity of crude enzyme in presence of different pH and temperature. (a) Effect of pH on cellulase activity, (b) effect of temperature on cellulase activity.

The quantitative CMCase activity of crude enzyme produced by *Thermomonospora viridis* (SR1) was investigated with suitable pH, temperature and nitrogen source in presence of different carbon sources (Fig. 5a). The highest cellulase activity (139.70 U/ml) was recorded when CMC was used as carbon source. This result is more or less similar to the findings reported by Das *et al*. (2010) who found that media containing CMC enhance the cellulase activity of a thermophilic *Bacillus* sp. Li-Jung Yin *et al.* (2001) also reported the highest CMCase activity of cellulase of *Bacillus subtilis* when CMC was used as carbon source (substrate).

The quantitative CMCase activity of crude enzyme produced by *Thermomonospora viridis* (SR1) was investigated with suitable pH, temperature and carbon sources in presence of different nitrogen sources (Fig. 5b). The highest cellulase activity was recorded with SR1 (200.0 U/ml) when peptone was used as nitrogen source. Similarly Das *et al* (2010), Yogita *et al* (2015) reported highest CMCase activity by the cellulase of *Bacillus* sp. when peptone was used as nitrogen sources with CMC as carbon source during the optimization for highest CMCase activity.

In Fig. 6a the quantitative CMCase activity of crude enzyme produced by *Thermomonospora viridis* (SR1) using nine metals is presented. Enzyme activity of cellulase extracted from SR1 was significantly inhibited in presence of Ag^{+} (14.61 U/ml) ions whereas stimulated activity of cellulase was recorded in presence of Mg^{2+} (148.69 U/ml). These results are almost similar to the findings of Saha (2004) who obtained stimulated cellulase activity by *Mucor circinelloides* in

presence of divalent metal Mg^{2+} whereas Lucas *et al*. (2001) reported univalent metal Ag^{+} as significant inhibitor of cellulase activity of *Chalara paradoxa*.

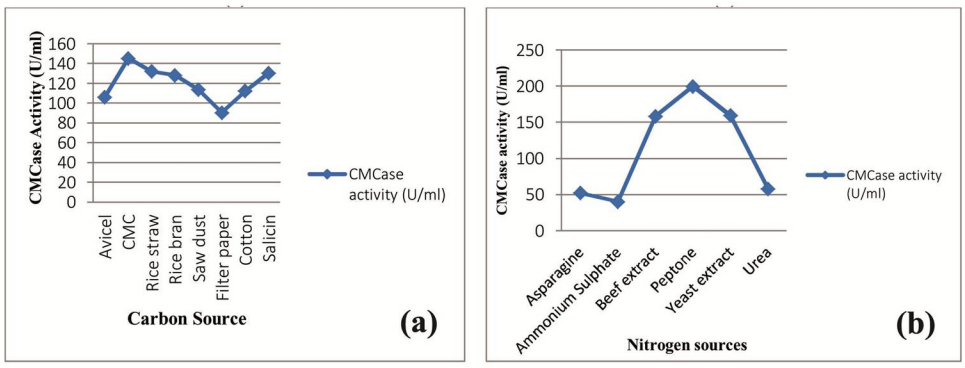


Fig. 5. Cellulase activity of crude enzyme in presence of different carbon and nitrogen sources. (a) Effect of carbon source on cellulase activity, (b) effect of nitrogen source on cellulase activity.

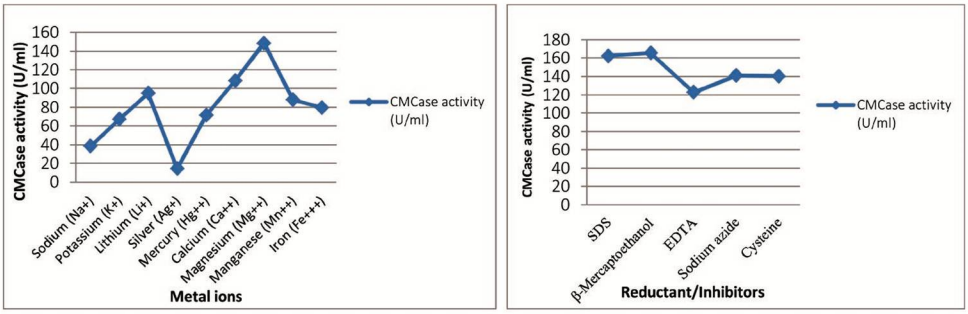


Fig. 6. Cellulase activity of crude enzyme in presence of different metal ions and reductant and inhibitors. (a) Effect of metal ions on cellulase activity, (b) effect of reductant and inhibitors on cellulase activity.

The quantitative CMCase activity of crude enzyme produced by *T. viridis* (SR1) using five inhibitors and reductants is shown in Fig. 6b. Cellulase activity of the isolate was inhibited by EDTA (122.85 U/ml) whereas that was stimulated by β-Mercaptoethanol (165.54 U/ml). The present findings are in agreements with the results reported by Lucas *et al*. (2001) who reported the inhibition of cellulase activity of *Chalara paradoxa* by EDTA whereas Li-Jung Yin *et al*. (2010) found maximum cellulase activity of *Bacillus subtilis* YJ1 by β-Mercaptoethanol.

Finally data obtained from the cultural, morphological and biochemical studies were compared with the standard description given in the Bergey's Manual of Determinative Bacteriology and the cellulolytic actinomycete isolate was identified as *T. viridis*.

Cellulose degrading actinomycete SR1 (*Thermomonospora viridis*) isolated from rice straw was found to produce highest amount of cellulase in an optimized condition such as, pH 6.5, temperature 35^0C, CMC as carbon source and yeast extract as nitrogen source. Optimum reaction condition for highest cellulase activity of crude enzyme revealed at pH 7.5, temperature 40^0C, 2.5% CMC as carbon source and 0.2% peptone as nitrogen source. CMCase production and its activity in pH 6.5 - 7.5 and temperature range of 35 - 40^0C are commonly evident. Carboxymethyl Cellulose (CMC) is widely used for endoglucanase production and its activity. Its highest biomass

yield was recorded at pH 7.5, temperature 20^0C whereas highest biomass yield was found when saw dust and urea were used as carbon and nitrogen sources, respectively.

Besides, the crude cellulase of the isolate was tested using different metals, reductant and inhibitors. It was evident that univalent cation (Ag^+) have less influence whereas divalent cation (Mg^{2+}) have greater influence on the cellulase activity. EDTA was found to inhibit the CMCase activity whereas that was stimulated by β-Mercaptoethanol. The cellulase could be an endoglucanase as it showed highest cellulolytic activity on CMC which has been reported by many workers (Li-Jung Yin *et al.* 2001, Lucas *et al.* 2001, Saha 2004).

Present study suggested that the actinomycete SR1 (*Thermomonospora viridis*) potentially degrades rice straw *in vitro* optimized condition. Partial characterization of its cellulase also revealed that it can be used as a potent cellulolytic strain for the degradation of biowastes such as rice straw in paddy field. Therefore, the actinomycete can be an important tool for the bioremediation of rice straw in paddy field; thereby environmental pollution through open air burning of rice straw can avoided.

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