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Original Article

Identification of Locally Isolated Cellulolytic *Stenotrophomonas maltophilia* from Rice Straw and Optimization of its Cellulase Activity

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A highly cellulolytic bacterium was locally isolated from rice straw and identified as *Stenotrophomonas maltophilia*. Identification of the isolate based on the morphological, cultural and biochemical characteristics was confirmed with 16S rDNA analysis. The bacterium showed the highest level of reducing sugar and extracellular protein production when incubated for 3 days (348.75 µg/ml and 288.5 µg/ml respectively) at 40°C temperature (463.0 µg/ml and 333.0 µg/ml respectively) and pH 6.5 (360.0 µg/ml and 349.0 µg/ml respectively) in Winstead's broth having 1.5% CMC and 0.2% Yeast Extract as carbon and nitrogen source respectively. Crude cellulase enzymes produced by the bacterium showed the highest CMCase activity rather than FPase, Avicelase and ²-Glucosidase activities. Cellulase activity of the crude enzyme was also determined using the same parameters. The crude cellulase enzyme showed the highest CMCase activity when incubated for 60 minutes (232.5 U/ml), at pH 6.5 (105.0 U/ml), 35°C temperature (69.75 U/ml) using CMC and Peptone as carbon and nitrogen source respectively. Crude cellulase showed the highest activity in presence of mercury and SDS as metal and detergent respectively. Substrate specificity and SDS-PAGE analysis reveals that the cellulase may be an endo-1,4-glucanase.

Keywords: Rice straw, cellulase, CMCase, *Stenotrophomonas maltophilia*, extracellular protein, reducing sugar.

Introduction

Rice straw is mainly cellulose and hemicellulose encrusted by lignin, in addition to only small amounts of protein, it is resistant to microbial decomposition compared to straw from other protein-rich grains such as wheat and barley[1]. In many countries, massive amounts of the post-harvest rice residues are eliminated through Weld open-air burning, which represents a threat to public health and poses an environmental pollution problem[2].

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 composed of independently folding, structurally and functionally discrete units called domains or modules, making cellulases module. Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including fungi, bacteria, and actinomycetes.

Cellulose is derived from D-glucose units, which condense through β-(1,4)-glycosidic bonds. This linkage motif contrasts with that for α-(1,4)-glycosidic bonds present in starch and glycogen[3]. It is a straight chain polymer. It is the structural component of plant materials and the biological degradation of cellulose has a great importance in the activity of the living system since cellulosic waste materials play key roles in the environmental pollution. Maintenance of the cellulosic waste materials and their disposal system is not satisfactory. One of the major treatment systems for these waste materials is incineration, but a huge amount of CO_2 is released in this process. The potential cellulolytic microorganisms use a complex enzyme-substrate interaction to degrade and decay celluloses. Cellulase is an important and essential kind of enzyme for carrying out the depolymerization of cellulose into fermentable sugar. Microorganisms produce extracellular cellulases such as Endoglucanases or Endo-1, 4-β-D-Glucan Glucanohydrolases (EC 3.2.1.4), Exoglucanase or 1,4-β-D-Glucan Cellobiohydrolases (EC 3.2.1.91), Exoglucanases or 1,4-β-D-Oligoglucan Cellobiohydrolases or Cellodextrinases (EC 3.2.1.74) that are either free or cell-associated to hydrolyze and metabolize insoluble cellulose[4].

Cellulases have a wide variety of applications because of their ease of extraction and purification process. Cellulase production at the industrial level is economically beneficial and these enzymes are used in biofuel, food and feed, oil extraction, paper and ink, fertilizer production, cotton mills, and textile industries, etc.[5].

The objectives of the present study were to isolate and identify highly cellulolytic bacteria, screen them for their cellulolytic potential and optimize the physicochemical parameters to maximize the yield of cellulase enzyme that can be utilized on a commercial scale. In the course of screening for industrially

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important ones, several aerobic cellulolytic bacteria were isolated from rice straw samples collected from different localities of Hathazari, Chittagong, Bangladesh. One promising cellulose-degrading bacterium, designated as SB23 (*Stenotrophomonas maltophilia*) was selected for the present study to find out the optimum conditions for its growth, cellulase production, and enzyme activities.

Materials and Methods

Isolation and Screening of the Cellulase Producing Microorganisms

Rice straw samples were collected from Hathazari area, Chittagong to isolate cellulolytic bacteria. One gram of sample was suspended in 9 ml of sterile distilled water. After serial dilution of this suspension (10^{-1} to 10^{-5} times), 200¼l of each dilution was spread on carboxymethyl cellulose (CMC) agar plates (1% CMC, 0.1% KH_2PO_4, 0.1% K_2HPO_4, 0.04% $MgSO_4$, 0.005% NaCl, 0.000125% $FeSO_4$ and 1.8% Agar, pH 7.0) and incubated at 37°C for 24-48 hrs. The isolated bacterial colonies forming clear-zones after application of Gram's iodine solution were selected as cellulase producers[6]. Then the bacterial 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 Microorganism

Among the numerous isolates SB23 was found to be a promising cellulose degrader. It was 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[7] and compared with the standard description given in Bergey's Manual of Determinative Bacteriology[8,9]. Forty eight hours old culture was used for all the tests. Based on and biochemical characteristics the isolate SB23 was provisionally identified as *Stenotrophomonas maltophilia.* Later it was confirmed by the construction of phylogenetic tree using 16S rDNA analysis.

16S rDNA sequencing of the Microorganism

Genomic DNA of the bacterium was extracted using Favorgen Cultured Cell Genomic DNA Extraction Mini Kit according to manufacturer instruction (Favorgen® Biotech Corp., Taiwan). The 16S rDNA amplification was carried out in a thermal cycler SimpliAmp™ (Thermo Fisher Scientific Inc., USA) using universal 16S rDNA specific primer pair 5′-AGAGTTTGATCC TGGCTCAG-3′ (forward) and 5′-ACGGCTACCTTGTTACGACTT-3′ (reverse). The PCR products were purified from agarose gel by FavorPrep™ Gel/ PCR Purification Mini Kit (Favorgen® Biotech Corp., Taiwan) and sequenced by a DNA sequencer (Automated Analyzer, Japan). Then the 16S rDNA sequence was subjected to NCBI BLAST (http://www.ncbi.nlm.nih.gov/blast/) to get the most similar sequences. The top matched sequences were then used to construct a phylogenetic tree for genetic alignment using 'Clustal Omega' (www.ebi.ac.uk/Tools/msa/clustalo).

Optimization of cultural conditions for cellulase production

To determine the optimum physicochemical conditions for the highest cellulase and reducing sugar production the bacterial isolate SB23 was incubated for different incubation period at various temperatures and pH using different carbon and nitrogen sources as well as different CMC concentration.

Effect of the incubation period, temperature, and pH

The bacterial isolate SB23 was inoculated into Winstead's broth having 1.2% CMC and 0.2% asparagine as carbon and nitrogen source respectively to observe the optimum incubation period (1-5 days), Temperatures (15ºC- 45ºC) and pH (3.5-9.5) for highest reducing sugar and cellulase production. After incubation, the crude enzyme preparation was collected by centrifugation at 10,000 rpm in 4ÚC temperature for 20 min.

Effect of carbon and nitrogen Sources

The bacterium was grown in Winsted's broth containing different carbon sources at a concentration of in 1.2% including glucose, avicel, salicin, treated rice straw, untreated rice straw and CMC.[10] and different nitrogen sources such as urea, yeast extract, peptone, ammonium sulphate and asparagine at a concentration of 0.2% to observe the optimum carbon and nitrogen sources for the highest reducing sugar and cellulase production. After incubation, the crude enzyme was collected by centrifugation at 10,000 rpm in 4ÚC temperature for 20 min.

Quantitative estimation of reducing sugars and extracellular proteins

Reducing sugars of culture filtrate and the enzyme-substrate reaction was estimated using Nelson's modification of Somogyi method[11]. Extracellular protein concentration was determined by the colorimetric method[12].

Enzyme assay

The crude enzyme (culture filtrate) was used for cellulolytic activities. The reaction mixture contained 2 ml of culture filtrate, 2 ml of substrate solution in phosphate buffer of pH 6.5 (1% CMC for CMCase, 1% avicel for avicelase or 1% salicin for and β-glucosidase) and 1 ml of phosphate buffer. For FPase, the assay mixture contained 2 ml culture filtrate and 50 mg Whatman No 1 filter paper strip [(1×6) cm] in 1 ml phosphate buffer. The enzyme assays were performed in a waterbath at 35ºC for 2 hrs.

The amount of reducing sugar released after enzyme-catalyzed reaction was measured by Nelson's modification of Somogyi method[11]. One unit (U) of the enzyme activity corresponded to the amount of enzyme required to produce 1 µmol of glucose (as

reducing sugar equivalent) in 1 hour under the specified condition[13].

Optimization for Enzyme activity

To determine the optimum conditions for cellulase activity, the crude enzyme was incubated for different reaction periods (30-120 minutes), at various temperatures (15 - 45ºC) and pH values (4.5 - 9.5), using different carbon (CMC, avicel, filter paper and salicin) and nitrogen (urea, peptone, ammonium sulphate, asparagine and yeast extract) sources. The enzyme activity was also tested under standard condition using 1% CMC as substrate in presence of different metal chlorides (NaCl, KCl, $MgCl_2$, and $HgCl_2$), inhibitors and reductants (SDS, Cysteine, EDTA and Sodium azide).

SDS PAGE Analysis

The isolate SB23 was cultivated in Winsted's broth containing 1.5% CMC and 0.2% yeast extract as carbon and nitrogen source respectively and incubated for 3 days, at 40ºC temperature, and pH 6.5 in a shaking incubator for the maximum production of cellulase enzymes. After incubation, the culture filtrate or crude enzyme was collected by centrifugation at 10,000 rpm, 4ºC temperature for 20 minutes. Then, it was transferred to a 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[14].

Results

Characterization and Identification of the isolate

The morphological, cultural and biochemical characteristics of the isolate SB23 was compared with the standard description given in Bergey's Manual of Determinative bacteriology. The identification of the isolate was confirmed by 16S rDNA sequence analysis. Based on the morphological, biochemical and 16S rRNA sequences, the bacterial isolate (SB23) was identified as *Stenotrophomonas maltophilia*, which showed 99% homology with the reference sequence. A phylogenetic tree was constructed using neighbor joining (NJ) distance-based algorithm (Figure 1).

Optimization of culture condition for cellulase production

The isolate SB23 (*Stenotrophomonas maltophilia*) was grown in Winstead's media containing 1.2% CMC as carbon source to optimize the culture conditions such as incubation time, temperature and pH, and carbon and nitrogen sources for maximum production of cellulase. The cultures were grown t for different incubation periods (1-5 days) at various temperatures (15-45ºC) and pH values (3.5-9.5) The highest reducing sugar and extracellular protein was obtained after 3 days of incubation (348.75 µg/ml and 288.5 µg/ml respectively), at 40ºC (463.0 µg/ml and 333.0 µg/ml respectively) with pH value of 6.5 (360.0 µg/ml and 349.0 µg/ml respectively) (Figure 2 to 4).

Effect of carbon and nitrogen source on enzyme production

The isolate SB23 was grown in the media containing different carbon (in 1.2% concentration) sources such as glucose, avicel, salicin, CMC, treated rice straw and untreated rice straw (in 1.2% concentration) and nitrogen sources such as asparagine, peptone, yeast extract, urea, ammonium sulphate (0.2% concentration) in Winstead's broth containing 1.2% CMC. It produced the highest 390.0 µg/ml reducing sugar and 375.0 µg/ml extracellular protein using CMC as a carbon source whereas maximum 385.0 µg/ml reducing sugar and 388.0 µg/ml extracellular protein was recorded when peptone was used as a nitrogen source (Figure 5 and 6).

The highly cellulolytic isolate SB23 was grown in Winstead's broth having different concentrations (1%, 1.5%, 2%, and 2.5%) of CMC. It showed the highest level of reducing sugar (341.25 µg/ml) and extracellular protein (450 µg/ml) production in Winstead's broth having 1.5% CMC.

Factors effect enzyme production

The time-course of enzyme production was carried out at various temperatures and initial pH values. Highest level of CMCase

Figure 1. *Phylogenetic tree based on the 16S rDNA gene sequence of isolate SB23 and related microorganisms*

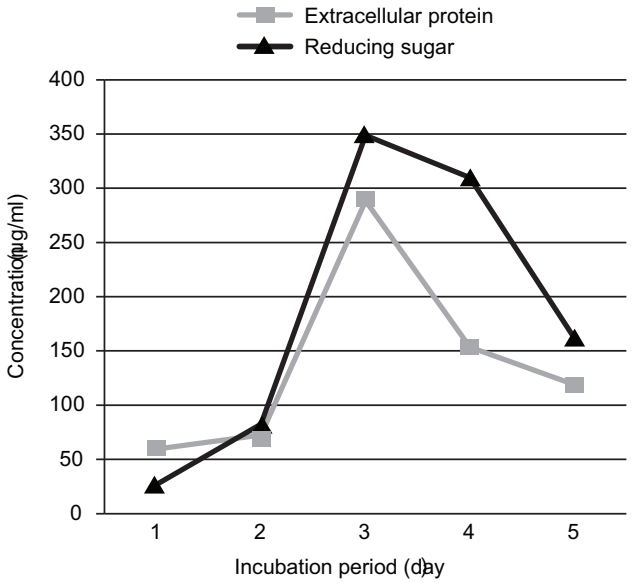

Figure 2. *Effect of incubation period on extracellular protein and reducing sugar production.*

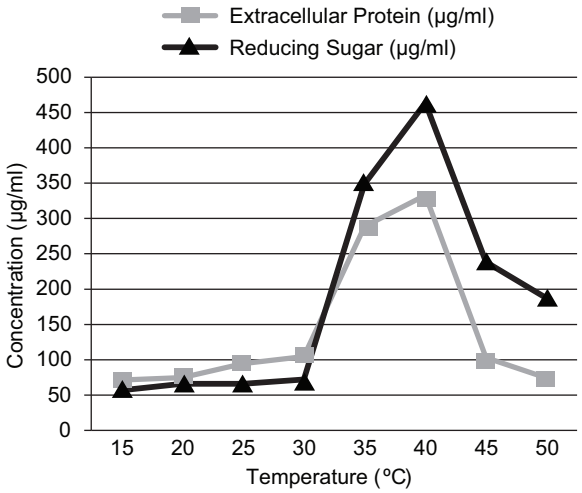

Figure 3. *Effect of temperature on extracellular protein and reducing sugar production.*

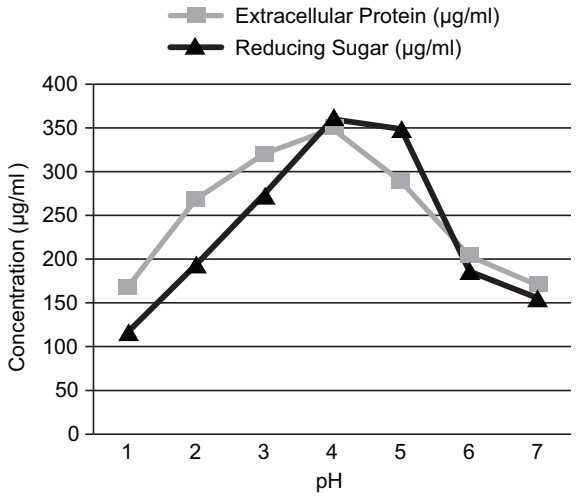

Figure 4. *Effect of pH on extracellular protein and reducing sugar production.*

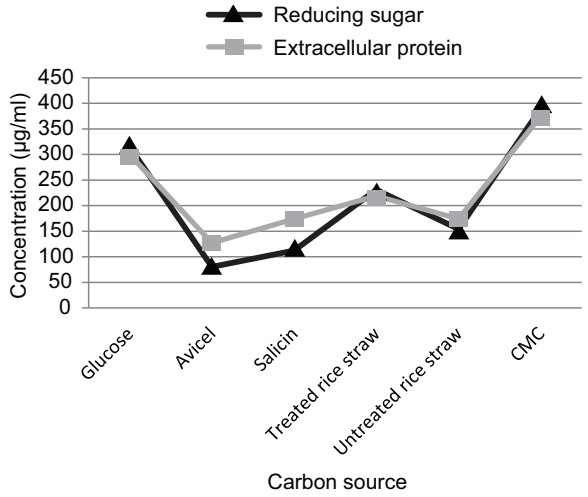

Figure 5. *Effect of carbon sources on extracellular protein and reducing sugar production.*

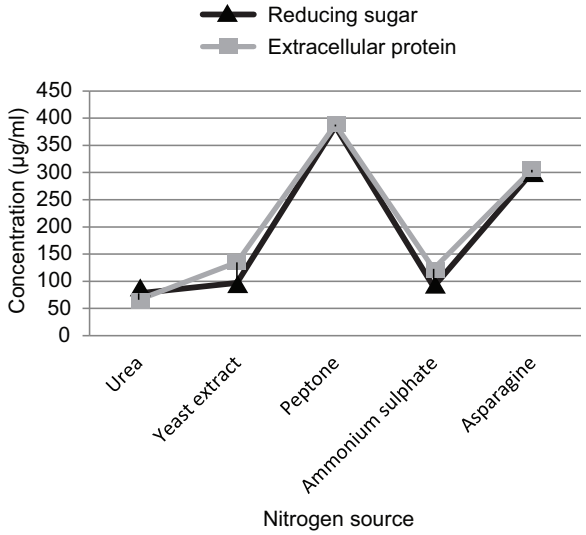

Figure 6. *Effect of nitrogen source on extracellular protein and reducing sugar production.*

activity was contained after 3 days of incubation period (121.5 U/ml) at pH 6.5 (90.0 U/ml) and 40˚C (92.25 U/ml) (Figure 7 to 9). Enzyme production was also carried out using four different carbon (CMC, filter paper, avicel and salicin) and nitrogen (urea, peptone, yeast extract and asparagine) sources. The crude enzyme showed highest level of CMCase activity when CMC and peptone were used as carbon and nitrogen sources respectively (Figure 10 and 11).

Optimization of cellulase activity

Effect of Reaction time, Temperature and pH

CMCase activity was determined for different assay periods (30-120 min) at various reaction temperatures (20-40°C) and pH values (4.5-9.5). The enzyme showed highest activity after 60 min of incubation (232.5 U/ml) at 35ÚC (69.75 U/ml) and at pH 6.5 (105.0 U/ml) (Figure 12[A], 12[B] & 12[C]). The crude

enzyme showed highest its activity when CMC was used as the substrate (90.0 U/ml) (Figure 12[D]).

Effect of Carbon and Nitrogen sources

The crude enzyme of SB23 (Stenotrophomonas maltophilia) was extracted and prepared using 1% CMC produced in phosphate buffer for the optimization of its cellulase activity. The effect of different carbon (CMC, Filter paper, Avicel & Salicin) and nitrogen (Urea, Peptone, Yeast extract & Asparagine) sources on the crude enzyme were also determined. The crude enzyme of the isolate showed the highest CMCase activity when CMC was used as a carbon source (90.0 U/ml) whereas the highest CMCase activity was recorded when peptone was used as a nitrogen source (66.0 U/ml) (Figure 12[D] and 12[E]).

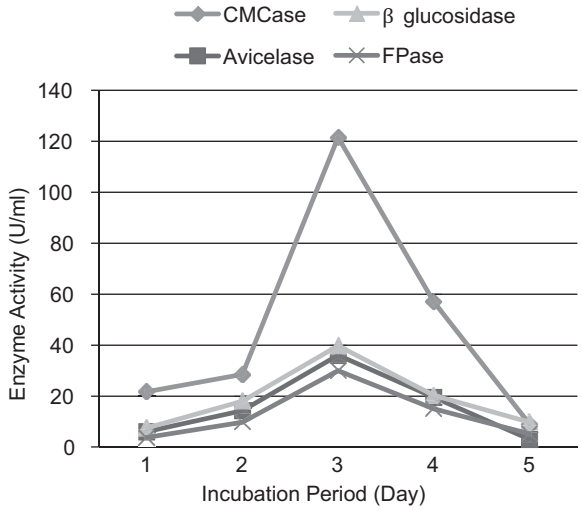

Figure 7. *Effect of incubation period on enzyme activities*

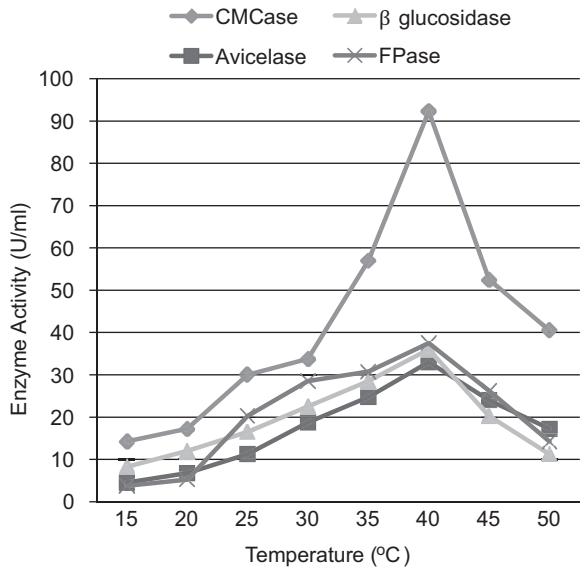

Figure 8. *Effect of temperature on enzyme activities*

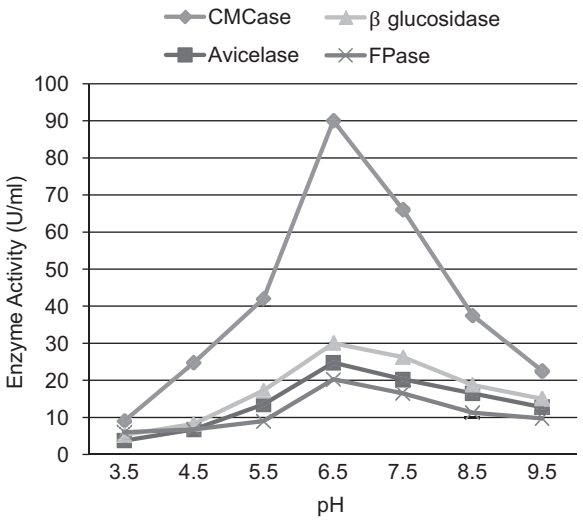

Figure 9. *Effect of pH on enzyme activities*

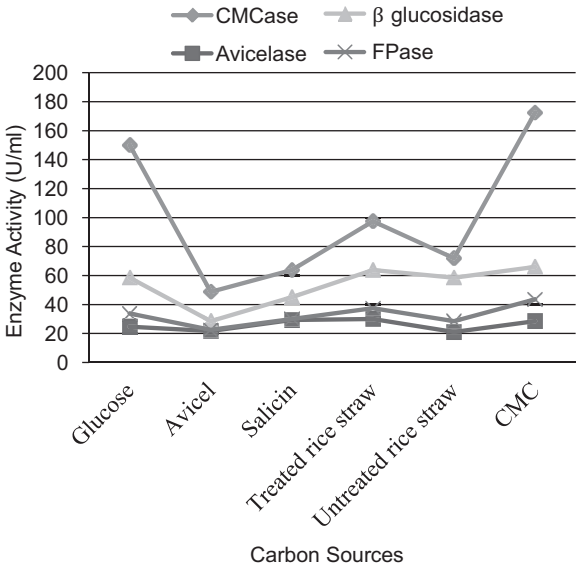

Figure 10. *Effect of carbon sources on enzyme activities*

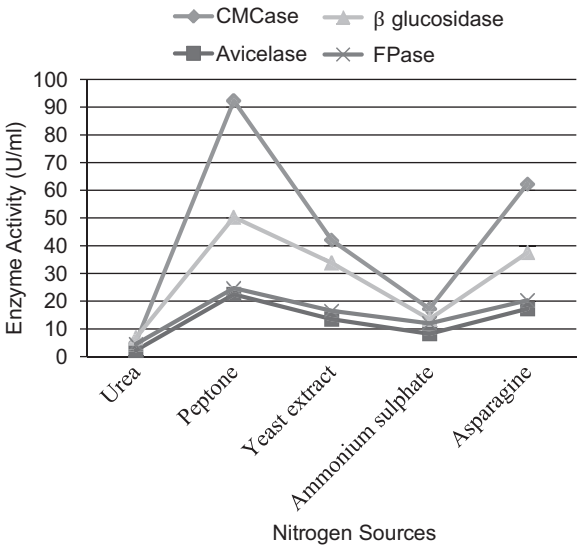

Figure 11. *Effect of nitrogen sources on enzyme activities*

Effect of metals, reductants, and inhibitors on enzyme activity

The crude enzyme of SB23 (*Stenotrophomonas maltophilia*) was also tested using Enzyme assays were also performed under standard conditions in the presence of different metals (NaCl, KCl, $MgCl_2$ and $HgCl_2$), inhibitors and reductants (SDS, sodium azide, EDTA and cystein). Highest CMCase activity (135.0 U/ml) was obtained in the presence of mercuric chloride ($HgCl_2$, Hg^{2+} ion with Cl^- as counter ion). SDS (sodium dodecyl sulphate) also increased the enzyme activity (397.5 U/ml) (Figure 12[F] and 12[G]).

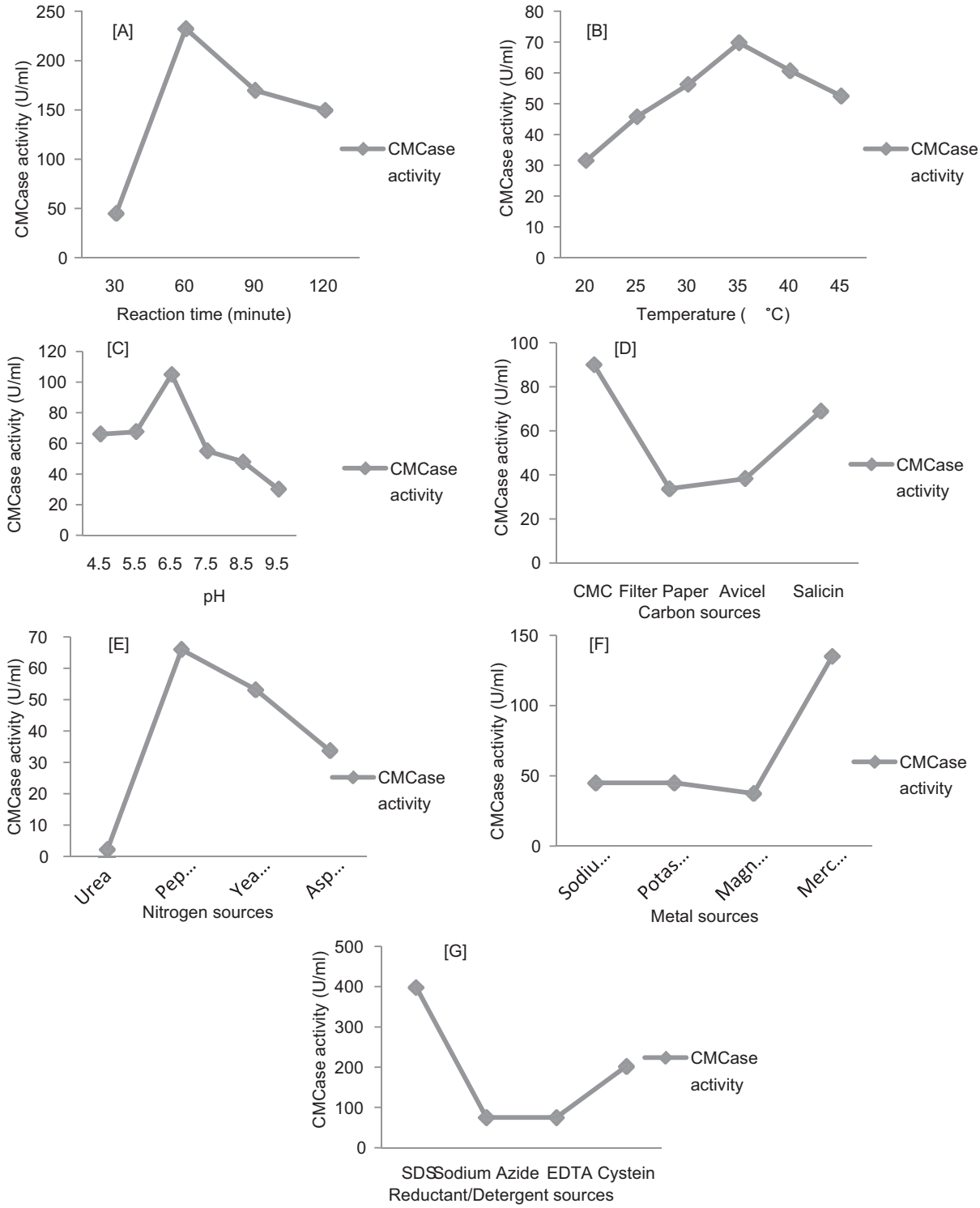

Fig 12: Optimization of cellulase activity. Effect of [A] Enzyme-Substrate reaction time [B] Temperature. [C] pH. D) Carbon source; [E] Nitrogen source [F] Metals, and [G] Reductants/Detergents on CMCase activity.

SDS PAGE analysis

As the cellulase activity was predominantly present in the isolate (*Stenotrophomonas maltophilia*), an attempt was made to characterize the cellulase enzyme of the isolate through SDS PAGE analysis. The data of electrophoresis showed that the cellulase enzyme of the isolate had a molecular weight of 33 kDa.

Discussion

Several cellulolytic microorganisms were isolated from rice straw samples collected locally from Hathazari, Chittagong. They were screened for their cellulase production and cellulolytic activity potential. Among them, only one bacterial isolate (designated as SB23) was found promising and selected for a detailed study. Based on the cultural, morphological, and biochemical studies it (SB23) was identified as *Stenotrophomonas maltophilia* and the identification was confirmed with 16S rDNA analysis.

Effect of different physicochemical parameters such as pH, temperature, and incubation period as well as carbon and nitrogen sources on cellulase production by the isolate (SB23) were studied. It (*Stenotrophomonas maltophilia*) produced maximum reducing sugar and extracellular protein after 3 days of incubation (348.75 µg/ml and 288.5 µg/ml respectively), at 40ÚC temperature (463.0 µg/ml and 333.0 µg/ml respectively) and at pH 6.5 (360.0 µg/ml and 349.0 µg/ml respectively). Similar finding are also in case of the other mesophilic organism like *Streptomyces* spp. strain NEAE-D[15] and *Bacillus* species[16, 17]. Substrate concentration on the production of cellulase was also tested using different concentrations of CMC in Winstead's broth. It showed the highest level of reducing sugar (341.25 µg/ml) and extracellular protein (450.0 µg/ml) production in Winstead's broth having 1.5% CMC. Sherief *et al.,* (2010) reported that the highest extracellular protein production occurred when CMC concentration was 1.5% to 2%; because an increase in substrate concentration made binding sites more available for the protein[18, 19].

Based on some vital factors such as enzyme-substrate reaction time, temperature, pH, carbon, and nitrogen sources, as well as metals and reductants the in vitro cellulolytic activity of the isolate, were tested.

Isolate SB23 produced the highest 390.0 µg/ml reducing sugar and 375.0 µg/ml extracellular protein using CMC as carbon source whereas maximum 385.0 µg/ml extracellular protein and 388.0 µg/ml reducing sugar using peptone as the nitrogen source. El-Naggar *et al.,* (2012) also reported that the amount of reducing sugar and extracellular protein reached at the highest level by *Streptomyces* spp. strain NEAE-D when yeast extract was used[15].

Crude cellulase enzymes produced by the isolate showed the highest CMCase activity rather than FPase, Avicelase, and β-Glucosidase activities. These results are in agreement with those of [20] who found CMC was the best carbon source for cellulase production. This indicates that CMA is more readily utilizable substrate and inducer than the other carbon substrates[21].

Cellulase activity of the crude enzyme was also determined using the parameters-pH, temperature, enzyme-substrate reaction time, carbon and nitrogen sources, metals, and reductants. The crude cellulase enzyme showed highest CMCase activity when incubated for 60 minutes (232.5 U/ml), at pH 6.5 (105.0 U/ml), 40°C temperature (69.75 U/ml) using CMC (90.0 U/ml) and Peptone (66.0 U/ml) as carbon and nitrogen source respectively. Crude cellulase showed the highest activity in presence of mercury (135.0 U/ml) and SDS (397.5 U/ml) as metal and detergent respectively. The increase in cellulase activity with SDS has also been observed by other workers[22, 16]. The detergents can modify the surface property and help to minimize the irreversible reaction of cellulase as reported[23].

The extracellular cellulase extracted from the bacterium *Stenotrophomonas maltophilia* (SB23) has high specificity to the substrate CMC. SDS-PAGE analysis showed that it was has a molecular weight of 33 kDa. Effects of metals ions and reductants and inhibitors were determined to characterize the crude cellulase. Based on the study and according to the substrate specificity and molecular weight detected by SDS PAGE analysis it seems to be an endo-1,4-glucanase. Similar findings were reported by some other workers[24, 25, 26].

Conclusion

The results of the present work indicated that the cellulolytic *Stenotrophomonas maltophilia* plays a vital role in role in the breakdown of rice straw into simple sugars and it was abundantly found in the paddy field. Several microorganisms capable of converting cellulosic matter into simple carbohydrates had been discovered for decades. However, the needs for newly isolated cellulolytic microbes still remain. *Stenotrophomonas maltophilia* showed potential to convert cellulose into reducing sugars which could readily be used in many applications such as composting of agricultural wastes using as organic manures, in animal foods, and feedstock for the production of organic compounds.

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