

## Isolation, Screening and Characterization of Cellulase Producing Bacterial Isolates from Municipal Solid Wastes and Rice Straw Wastes

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### Abstract

Cellulolytic bacteria were isolated and screened from municipal solid wastes and rice straw wastes using Carboxy Methyl Cellulose (CMC) agar medium as a selective medium. Production of clear zones by the bacterial isolates on CMC agar medium supplemented with 1% CMC was considered as indicative of extracellular cellulase activity. The size of transparent zone diameter was considered as proportional to the level of cellulase production. These bacterial isolates were identified as *Bacillus* sp., *Pseudomonas* sp. and *Serratia* sp. based on morphological, cultural and biochemical characteristics. A basal medium containing CMC,  $\text{KH}_2\text{PO}_4$ ,  $\text{K}_2\text{HPO}_4$ ,  $\text{MgSO}_4$ ,  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{CaCl}_2$  and  $\text{FeSO}_4$  at pH 7.0 was used for cellulase production. The assay of cellulase in term of CMCase was performed by measuring the release of reducing sugar. Different physicochemical parameters were optimized for cellulase production at shake flask fermentation. Time course study revealed that maximum level of cellulase was produced by *Bacillus* and *Serratia* isolates after 24 h of cultivation and by *Pseudomonas* isolates after 42 h of cultivation. Optimum level of cellulase was produced by *Pseudomonas* and *Bacillus* isolates at 37°C, and that by *Serratia* isolate was at 35°C. Optimum pH for cellulase production by these bacterial isolates was 7.0. Optimum temperature and pH for the activity of cellulase from these isolates were 40°C and 7.0, respectively. The cellulase from these isolates was found almost stable up to 55°C and at pH 7.0 for 1 h. The crude cellulase could liberate reducing sugar from filter paper through hydrolysis. Results showed that bacterial isolates produced significant level of cellulase with cellulose degrading capability.

**Keywords:** Cellulase; Bacterial isolates; Rice straw wastes; Sea sands; Biodegradation

**Abbreviations:** CFU: Colony Forming Units; CMC: Carboxymethyl Cellulose; CMCcase: Carboxymethyl Cellulase; DNS: Dinitrosalicylic Acid; MR: Methyl Red; VP: Voges-Proskauer.

### Introduction

Cellulose and hemicelluloses are the most abundant biomasses on the earth and have the greatest potential to resolve both the energetic and environmental demands of bioenergy [1,2]. Cellulose is a linear polysaccharide which consists of 1000-1200 glucose residues with  $\beta$ -1,4-glycosidic linkages [3]. Cellulases can effectively hydrolyze cellulose into glucose via synergistic action of three enzymes, endo- $\beta$ -1,4-glucanase (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and  $\beta$ -D-glucosidase (EC 3.2.1.21) [4,5]. The process is known as cellulolytic system. The endoglucanase randomly hydrolyzes the  $\beta$ -1,4 bonds in the cellulose molecule, and the exo cellobiohydrolases in most cases release a cellobiose unit showing a recurrent reaction from chain extremity. Finally, the cellobiose is converted to glucose by  $\beta$ -glucosidase [6,7]. Cellulases are inducible enzymes synthesized by a large diversity of microorganisms including both fungi and bacteria during their growth on cellulosic materials. These microorganisms can be aerobic, anaerobic, mesophilic or thermophilic [8,9]. Filamentous fungi are the major source of cellulases and hemicellulases [10] but the production costs of these enzymes are very high. Bacteria which have high growth rate and short generation time in compared to fungi have good potential to be used in cellulase production [11]. The furthestmost potential importance is the ease with which bacteria can be genetically engineered. During the last two decades, the use of cellulases, hemicellulases and pectinases has been increased considerably, especially in textile [12,13], food and feed [14], brewery and wine [13] as well as in pulp and paper industries [15-17]. However, the most important use of cellulase is in the bioconversion of plant based cellulosic and lignocellulosic waste,

which opens the possibility of virtually inexhaustible and unique source of renewable biofuel [4,18,19]. Nowadays, these enzymes account for approximately 20% of the world enzyme market used on industrial basis [6].

Currently, cellulases are not produced commercially in Bangladesh and tons of cellulases are imported every year to use in different industries. According to Bangladesh Bank, cellulase of 17.8 million US dollar was imported during FY 2011-2012 to bare only in the textile sector, which imposed tremendous demand of cellulase production in Bangladesh. About 7,690 tons of Municipal Solid Wastes (MSW) are produced daily at the six major cities of Bangladesh namely, Dhaka, Chittagong, Rajshahi, Khulna, Barishal and Sylhet which may be increased up to 47,000 tons per day by 2025 owing to increase in population and urbanization. About 75-85% constituents of the MSW is organic and approximately 80% of this organic content is cellulosic which can be utilized as raw materials for cellulase production. Currently, MSW in Bangladesh are generally collected and dumped in low lands or disposed haphazardly causing environmental pollution and public health hazards. Methane gas is emitted from the rotten MSW. Methane is the second potent greenhouse gas having 20 times more impact on climate change compared to the carbon dioxide. Therefore, cellulosic

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biomass of MSW needs to be bioconverted into bioresources through production of value added products such as enzymes. Cellulase can be produced by bacteria using cellulosic MSW as the raw material. With this view, we herein isolated and screened cellulase producing bacterial isolates from MSW and Rice Straw Wastes (RSW) and optimized some parameters for cellulase production. Cellulases produced from these bacterial isolates were partially characterized.

## Materials and Methods

### Isolation, screening and identification of cellulase producing bacterial isolates

Samples were collected from RSW and MSW from different areas of Sylhet district, Bangladesh. One gram of sample was suspended in 9 ml of sterile distilled water. After serial dilution of this suspension ( $10^{-1}$  to  $10^{-6}$  times), 200  $\mu$ l of each dilution was spread on carboxymethyl cellulose (CMC) agar plates (1% CMC, 0.1%  $\text{KH}_2\text{PO}_4$ , 0.1%  $\text{K}_2\text{HPO}_4$ , 0.04%  $\text{MgSO}_4$ , 0.005% NaCl, 0.000125%  $\text{FeSO}_4$  and 1.8% Agar, pH 7.0) and incubated at 37°C for 24-48 h. The isolated bacterial colonies forming clear-zones after application of Gram's iodine solution [20] were selected as cellulase producers. Bacterial isolates producing significant clear zone on CMC agar were identified based on cultural, morphological and biochemical characteristics as described by Cowan and Steel [21].

### Crude cellulase production

A basal media (1% CMC, 0.1%  $\text{KH}_2\text{PO}_4$ , 0.1%  $\text{K}_2\text{HPO}_4$ , 0.04%  $\text{MgSO}_4$ , 0.005% NaCl, and 0.000125%  $\text{FeSO}_4$ , pH 7.0) was used for production of cellulase. For seed culture, a fresh isolated colony was inoculated in 5 ml basal media and incubated at 37°C and 120 rpm for 24 h. The seed culture (5%) was then inoculated in 50 ml production media in a 250 ml conical flask and incubated at the conditions as indicated. The cell free supernatant obtained by centrifugation at 8,000 rpm for 15 min at 4°C was used for determining the cellulase activity or further investigations.

### Cellulase activity assay

The carboxy methyl cellulase (CMCase) activity was assayed using a method described by Miller [22], with some modifications [6,23]. A 0.5 ml of culture supernatant was added to 0.5 ml of 1% CMC prepared in 50 mM sodium citrate buffer (pH 4.8) in a test tube and incubated at 60°C for 30 min. The reaction was terminated by adding 3.0 ml of dinitrosalicylic acid (DNS) and subsequently placing the reaction tubes in a water bath at 100°C for 15 minutes. One ml of Rochelle salt solution (40 gm Rochelle salt in 100 ml distilled water) was then added to stabilize the color. The absorbance/Optical Density (OD) was recorded at 575 nm wave length against a blank of 50 mM sodium citrate buffer. One unit of CMCase activity was defined as the amount of enzyme that liberated 1  $\mu$ mol of reducing sugar (glucose) in 1 min at 37°C and pH 7.0 [24].

### Time course study for cellulase production

To determine the optimum cultivation period for maximum cellulase production, the seed culture was inoculated into the production media and incubated at the conditions as described above. Culture samples were withdrawn at 6 h interval up to 60 h of cultivation and the CMCase activity was assayed. Simultaneously, bacterial growth was also observed by counting viable cells in Colony Forming Units (CFU).

### Optimization of temperature and pH for cellulase production

To study of effects the temperature on cellulase production, production medium at pH 7 was inoculated with the seed culture and

incubated at different temperatures (25, 30, 35, 37, 40 and 45°C). In order to study the effect of pH on cellulase production, the initial pH of the production medium was adjusted to different pH (pH 4.0 to 9.0). Different suitable buffers such as 50 mM sodium acetate (pH 4.0), 50 mM sodium citrate (pH 5.0), 50 mM potassium phosphate (pH 6.0-7.0) and 50 mM Tris-HCl (pH 8.0-9.0) were used. An inoculum size of 5% was maintained during fermentation at the conditions as indicated [25].

### Effects of temperature and pH on cellulase activity and stability

To investigate the effects of temperature and pH on cellulase activity, 500  $\mu$ l of the crude enzyme was added to 500  $\mu$ l of 1% CMC in 50 mM citrate buffer (pH 4.8). The reaction mixture was incubated for 30 min at various temperatures 30, 35, 40, 45, 50, 55 and 60°C. Cellulase activity was then measured as described above. To study the effects of pH on cellulase activity, different buffers such as 50 mM of sodium citrate (pH 4.0 and 5.0), potassium phosphate (pH 6.0-7.0) and Tris-HCl (pH 8.0-9.0) were used to assay the CMCase activity. To 0.5 ml of 1% CMC prepared in a suitable buffer of a particular pH (pH 4-9), 0.5 ml of crude enzyme was added.

To investigate the temperature stability, 1 ml of crude enzyme was treated for 1 h at various temperatures 30, 35, 40, 45, 50, 55 and 60°C. The residual CMCase activity of crude cellulase was measured according to the standard assay procedure. To study the pH on cellulase stability, the crude cellulase enzyme was treated for 1 h at room temperature with buffers of different pH 4-9 as described above. Standard assay procedure was followed to measure the residual activity of crude cellulase.

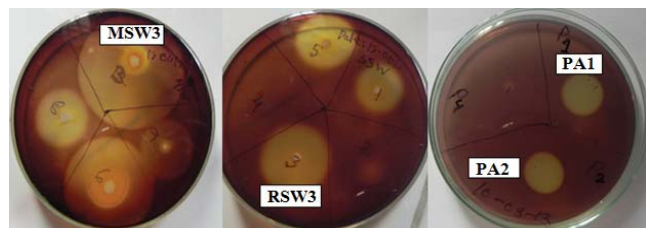
### Determination of cellulose degradation capability of the crude cellulase

A 200 mg filter paper strip was placed in a conical flask containing 50 ml of crude enzyme (total CMCase activity ~2.0 U) from each bacterial isolate, and incubated in a shaker incubator for 7 days. The amount of glucose liberated from the filter paper was estimated according to the procedure described by Miller [22].

## Results and Discussion

### Isolation and screening of cellulase producing bacterial isolates

Cellulolytic bacteria were isolated from samples collected from RSW and MSW. Appropriate dilutions of each sample were inoculated on CMC agar plates. Using of CMC as the sole carbon source plays a pivotal role for achieving the highest level of cellulase production because CMC induces cellulase gene expression. Isolated bacterial colonies with higher cellulase activity were further screened to obtain the pure culture. The pure culture produced clear zone when it was flooded with Gram's iodine solution due to hydrolysis of CMC (Figure 1). Gram's iodine forms a bluish-black complex with cellulose but not with hydrolyzed cellulose, giving a sharp and distinct clear zone around the cellulase-producing bacterial colonies within 3 to 5 minutes. Clear zone producing bacterial isolates were then subjected to various morphological, cultural and biochemical tests (Table 1). Based on the biochemical tests, morphological and cultural characteristics, isolate MSW3 was identified as *Bacillus* sp., isolate PA1 was identified as *Serratia* sp. and isolates RSW3 and PA2 were identified as *Pseudomonas* spp. In our study, isolate MSW3 was obtained from MSW and isolates PA1, PA2 and RSW3 were from RSW.



**Figure 1:** Visualization of cellulase activity with Gram's iodine solution. Cellulolytic bacteria were screened from the master plate to obtain pure culture. Cellulolytic activity was observed on CMC agar media after incubation at 37°C for 24 h. Typical results of three independent experiments for cellulolytic bacteria on CMC agar media are shown.

Morphological and Biochemical tests	Bacterial isolates			
	MSW3	SSW3	PA1	PA2
Bacterial Shape	Rod	Rod	Rod	Irregular
Motility Test	Motile	Motile	Motile	Motile
Gram Staining	+	-	-	-
Catalase Test	+	+	-	-
Oxidase Test	+/-	+	+	+
Spore Test	-	-	-	-
Indole test	-	-	-	-
Glucose Fermentation Test	+	+	+	+
Lactose Fermentation Test	-	+	+/-	-
MR- VP test				
MR	+	+	-	+
VP	-	-	-	-
Results	<i>Bacillus</i> sp.	<i>Pseudomonas</i> sp.	<i>Serratia</i> sp.	<i>Pseudomonas</i> sp.

**Table 1:** Biochemical tests for Genus identification of Bacterial isolates.

### Fermentation conditions for cellulase production

Fermentation period, cultivation temperature, initial pH of the culture media and agitation are the most striking features to influence the production of enzymes [26,27]. Time course study reveals that *Bacillus* and *Serratia* isolates (MSW3 and PA1) show maximum cellulase production at 24 h of cultivation, whereas *Pseudomonas* isolates (RSW3 and PA2) show optimal cellulase production at 42 h of cultivation (Figure 2). Along with cellulase production, bacterial cell growth was also observed by counting the colony forming units, the results indicate that cellulase production was growth associated (Figure 2). Cellulolytic bacterial isolates from the Persian Gulf showed their optimal production of cellulase after 48 h of cultivation [28]. However, optimum cultivation period varies from strain to strain with their characteristics and cultural conditions [29]. Temperature is one of the most important variables controlling bacterial growth and enzyme production.

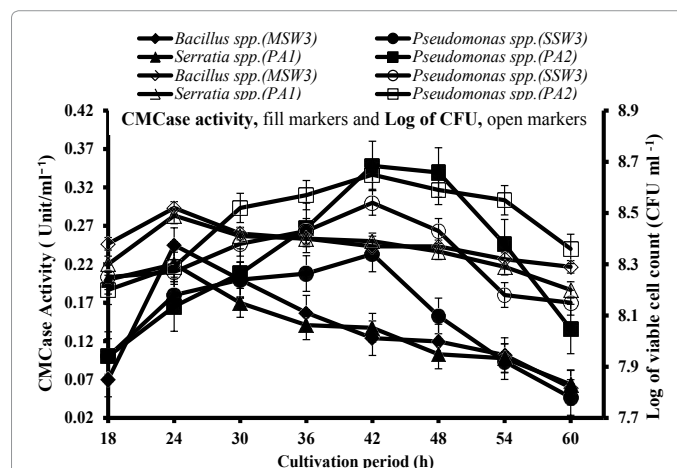
We optimized temperature for cellulase production. *Bacillus* and *Pseudomonas* isolates showed production optima at 37°C, while *Serratia* sp. showed maximum cellulase production at 35°C (Figure 3). However, temperature optima for cellulase production may vary from bacterial species to species [30,31].

The cellulase production by bacterial isolates is controlled by the extracellular pH as the pH of the culture media strongly influences many enzymatic processes. We investigated the effects of initial pH of the media on the production of cellulase by the bacterial isolates. It was observed that the optimum production of cellulase by the all bacterial isolates was observed at initial pH 7 (Figure 4). However, significant

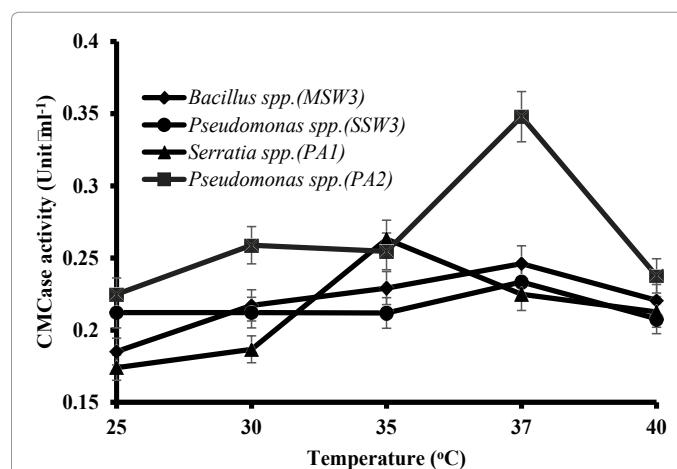
levels (approximately 60-70% of the peak levels) of cellulase production by *Pseudomonas* sp. were supported by a pH range between 6.0 and 8.0. This result indicated that the optimum level of cellulase from *Pseudomonas* isolates was secreted at neutral pH. It was previously reported that cellulolytic bacteria produced highest level of cellulase at a pH range of 6.0-7.5 [32,33].

### Partial characterization of crude cellulase

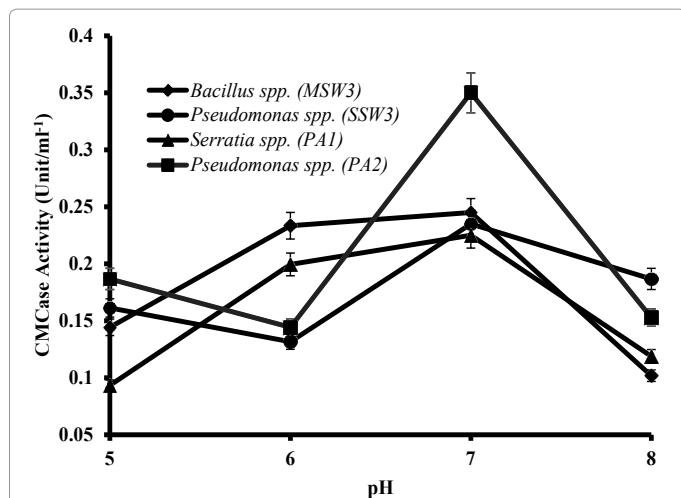
It is necessary to optimize the physicochemical conditions for maximum activity of an enzyme before its application. We determined the optimum temperature and pH for the activity of cellulase produced by the bacterial isolates. Temperature has marked effect on the activities of enzymes [34]. Results showed that the optimum temperature for the cellulolytic activity of cellulase secreted by the bacterial isolates in this study was 40°C (Figure 5). Furthermore, effect of temperature on cellulase stability was also studied. The results indicated that crude cellulase was almost stable up to 40-55°C. However, the stability of



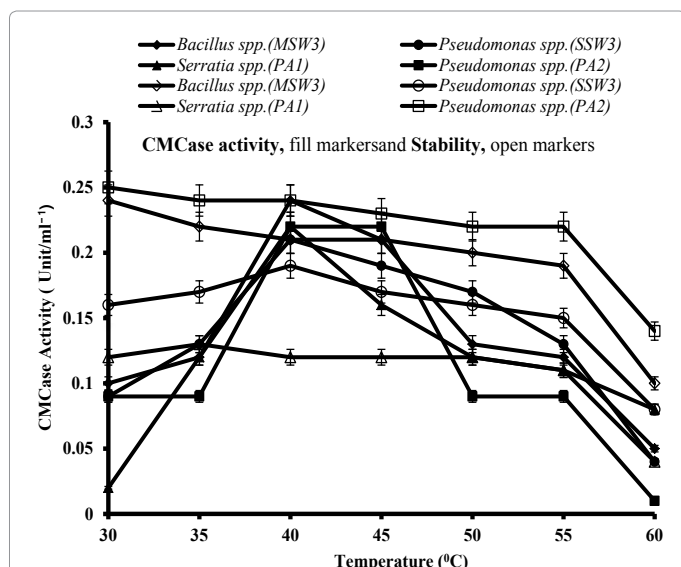
**Figure 2:** Time course for cellulase production. The fermentation at shake flask was carried out at 37°C and 120 rpm with 5% inoculum size. The initial pH of the media was adjusted to 7.0. The CMCCase activity was determined at 6 h interval during fermentation. CMCCase activity and Viable cell count (Log of CFU) are shown as filled and opened marks, respectively.



**Figure 3:** Effects of cultivation temperature on cellulase production. Fermentation by *Pseudomonas* isolates was done at temperature at 25, 30, 35, 37 and 40°C as indicated at 120 rpm for 42 h and *Bacillus* and *Serratia* isolates was done under the conditions for 24 h. The initial pH of the culture media was adjusted to 7.0.



**Figure 4:** Effects of initial pH of the media on cellulase production. The initial pH of the culture media was adjusted to pH 4.0, 5.0, 6.0, 7.0, 8.0 and 9.0. Fermentation by *Bacillus*, *Pseudomonas* and *Serratia* was carried out at 37°C and 120 rpm for 42 h and that by *Serratia* was done at 35°C for 24 h. Cellulase production was optimum at pH 7.



**Figure 5:** Effects of temperature on cellulase activity. The effects of the temperature on the crude cellulase activity of bacterial isolates were examined at various temperatures ranging from 30°C to 60°C. Cellulase from bacterial isolates show optimum activity at 37°C. Enzyme activity decreases significantly above 40°C. CMCase activity at different temperature and temperature stability shown as fill marker and open markers, respectively.

the crude cellulase decreased sharply when the enzyme solution was treated above 55°C (Figure 5).

The effects of pH on cellulase activity were investigated using CMC in 50 mM buffer solution with pH ranging from 4.0-9.0. The maximum cellulase activity was found at pH 7, with activity decreasing significantly both in acidic and neutral pH (Figure 6). We also examined the pH stability of the crude cellulase by treating it with buffer solutions of different pH. The cellulase was almost stable for 1 h at pH 6.0-8.0. However, cellulase activity was significantly lost when it was treated under pH 6.0 or above pH 9.0 (Figure 6). Although bacterial cellulase can be stable at neutral to alkaline conditions, cellulase from fungal sources are unstable at alkaline condition [35].

### Degradation of cellulosic material by crude cellulase

A 200 mg of filter paper was treated in conical flasks containing 50 ml of crude enzyme. After 3 days of incubation at 37°C, it was noted by visible inspection that the crude cellulase degraded the filter paper while the filter paper treated with distilled water remained unchanged [36]. Estimation of reducing sugar released in the solution on the 3<sup>rd</sup> day and 7<sup>th</sup> day revealed that the filter paper was degraded by crude enzyme (Table 2) [37].

### Conclusion

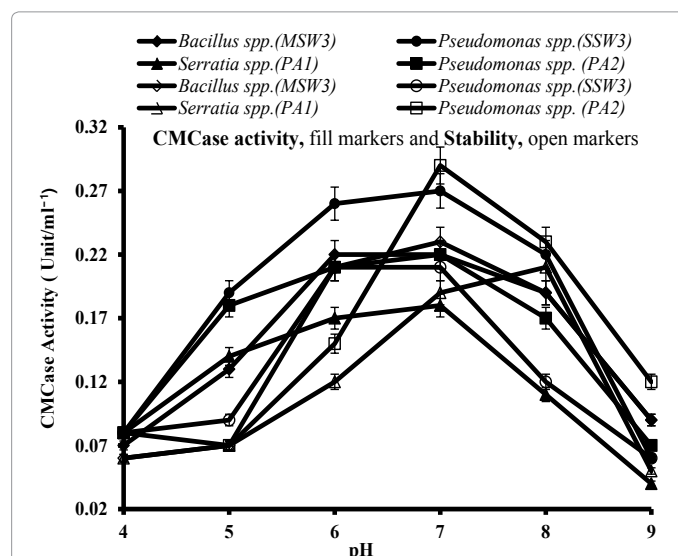
The present study has isolated four cellulolytic bacteria that have been identified as *Bacillus* sp., *Pseudomonas* sp. and *Serratia* sp. based on morphological, cultural and biochemical characteristics. Different parameters such as fermentation period, cultivation temperature and initial pH of the culture media during fermentation have been optimized for the production of cellulase by these four bacterial isolates. Moreover, the crude cellulase of these bacterial isolates has been characterized in terms of effects of temperature and pH on the CMCase activity and stability. In addition, the CMCase activity of the crude enzyme has been characterized based on the capacity of filter paper degradation. Currently, we are performing scale-up of the cellulase production by these bacterial isolates in the bioreactor (Fermac 320, Electrolab, UK) by using cellulosic waste materials of MSW for purification, molecular characterization and application of the cellulase.

### Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Crude cellulase of Isolate	Amount of reducing sugar liberated (mg/ml) at the end	
	3 <sup>rd</sup> day	7 <sup>th</sup> day
MSW3	10.7	18.0
RSW3	7.07	14.6
PA1	5.89	11.6
PA2	9.54	17.5

**Table 2:** Reducing sugar liberated through degradation of filter paper by crude cellulase.



**Figure 6:** Effects of pH on cellulase activity. The effects of the pH on the crude cellulase activity of bacterial isolates were examined at various pH ranging from pH 4.0 to 9.0. The enzyme has a broad range of pH activity (pH 6-8) with optimal at pH 7. CMCase activity at different pH and pH stability shown as fill marker and open markers, respectively.

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