Research & Reviews: Journal of Microbiology and Biotechnology

e-ISSN:2320-3528
p-ISSN:2347-2286

Partial Purification and Characterization of Cellulolytic Enzyme from *Bacillus pantothenticus* Isolated from a Dumpsite

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Research Article

Received date: 15/12/2016
Accepted date: 14/04/2017
Published date: 27/04/2017

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Keywords: Cellulase; *Bacillus pantothenticus*; Purification characterization

ABSTRACT

Cellulose hydrolyzing organism, *Bacillus pantothenticus*, was isolated from a dumpsite soil. Partial purification of the enzyme with precipitation with 60% chilled acetone and on Biogel P-100 gel filtration yielded an enzyme with specific activity of 253.50, 10.40% yield and purification fold of 2.41. The apparent K_m and V_{max} for Carboxymethylcellulose (CMC) hydrolysis was 1.167 mg/ml and 0.833 µg of glucose/ml/min respectively. The native molecular weight of the enzyme from *B. pantothenticus* was estimated as 51.48 kDa. The cellulase produced showed activity over broad range of temperature (30-70°C) with maximum activity at 60°C. The enzyme also showed activity in a wide pH range of 4-11 with optimum activity at pH 4.5. At concentration of 10 mM, KCl, $MgCl_2$, NaCl and $NiCl_2$ inhibited the enzyme while $CoCl_2$ activated the enzyme. The enzyme showed highest activity with microcrystalline cellulose as substrate followed by CMC. Significant activity was also observed with organic substrates such as sugarcane bagasse, orange bagasse and corn cub. The study concluded that the cellulose enzyme obtained had suitable catalytic properties for use in biodegradation of waste and other industrial applications.

INTRODUCTION

Municipal Solid Waste Management (MSWM) is a globally challenging issue especially in developing countries, due to its adverse environmental effects [1-3]. Solid waste which is composed of 50% cellulosic materials which include paper waste, yard trimmings, wood, textiles and food scraps is one of the three major environmental problems (other major environmental issue include flooding and desertification) in Nigeria, many other developing countries and even the developed countries. The standards of waste management is still poor and outdated in many developing countries, with poor documentation of waste generation rates and its composition, inefficient storage and collection systems.

Beside hemicellulose and lignin, cellulose is a major component of agricultural wastes and municipal residues [4-6]. The cellulose and hemicellulose comprise the major part of all green plants and this is the main reason of using such terms as 'cellulosic wastes' or simply 'cellulosics' for those materials which are produce especially as agricultural crop residues, fruit and vegetable wastes from industrial processing, and other solid wastes from canned food and drinks industries [6,7]. For the complete hydrolysis of cellulose to glucose, the cellulase systems must contain: endoglucanase (1,4-β-glucan glucanohydrolase, EC 3.2.1.4), exoglucanase (1,4-β-glucan cellobiohydrolase, EC 3.2.1.91) and β-glucosidase (β-D-glucoside glucohydrolase or cellobiase, EC 3.2.1.21) [8-10]. Only the synergy of these enzymes makes possible the cellulose hydrolysis to glucose [10]. In addition, crystalline native cellulose (CI) hydrolysis activity is necessary for splitting off the elementary fibrils from the crystalline cellulose [11,12].

Cellulase is used in many industries like textile [13], paper [14], laundry and detergent [15], animal feed industry [16] and biofuel production [17]. A good bacteria candidate could serve as a good source of the enzyme. In this study, cellulase producing organism was isolated from a dung hill and the enzyme was partially purified and characterized.

e-ISSN:2320-3528
p-ISSN:2347-2286

MATERIALS AND METHODS

Carboxymethylcellulose, Trizma hydrochloride, Trizma base and dipotassium hydrogen orthophosphate were obtained from Sigma-Aldrich Chemical Company, Limited, St. Louis, MO., USA. Glucose, lactose, sucrose, maltose, zinc sulphate, calcium chloride, acetone, crystal violet, safranin and peptone were obtained from BDH Chemicals Limited, Poole, England. Sodium hydroxide was purchased from Merck Millipore International, Darmstadht, Germany. Biogel P-100 was purchased from Bio-Rad, Hercules, CA., USA. Dinitrosalicylic acid was purchased from Avishkar Scientific and Surgicals, Hyderabad, India. Disodium hydrogen phosphate and sodium metabisulphite were purchased from Tianjin Kermel Reagent Company Limited, Tianjin, China. Isolation and Screening for Cellulolytic Bacteria.

Soil sample was obtained from a dumpsite in Onibueja, Osogbo, Nigeria. Isolation was carried out by weighing 1 g of the soil sample aseptically into 10 ml of pre-sterilized de-ionized water in a conical flask. The suspension was mechanically shaken for 10 min at room temperature. One milliliter (1 ml) of the suspension was diluted with 9 ml distilled water. One milliliter (1 ml) was also measured from the second dilution and was diluted with 9 ml distilled water. This was repeated four times resulting in 10-4 serial dilution. Aliquot (100 µl) of the diluted sample was spread on correspondingly labeled sterile nutrient agar (NA) plates in duplicates. The plates were inverted and sealed in plastic bags to reduce evaporation from the plates and incubated at 37°C for 24 h. After incubation, plates with 30-300 colony forming units (c.f.u.) were viewed and representative colonies were isolated from each plate for further identification. The representative colonies were purified by repeated streaking on NA and screened for cellulolytic activities. Isolates which showed positive cellulolytic activities were preserved on NA slopes in the refrigerator.

Bacterial strains isolated from the soil sample were screened for cellulase activity by spotting on cellulose-agar plates containing: peptone (2% w/v), KH_2PO_4 (0.1% w/v), $MgSO_4.7H_2O$ (0.5% w/v), NaCl (0.075% w/v) and high viscousity carboxymethylcellulose (0.5% w/v). Plates were incubated at 37°C for 48 h. Thereafter, plates were flooded with 0.1% congo red and destained with 1 M NaCl solution. Cellulase-producing colonies were detected by the appearance of a clear halozone around them [18]. Characterization of the bacterial isolates was carried out by observing their macroscopic and microscopic morphology. The bacterial isolates obtained were classified using Bergey's Manual of Determinative Bacteriology [19].

Cellulase Assay

Cellulase activity was assayed by measuring the amount of reducing sugar produced in the reaction mixture containing carboxymethylcellulose and the enzyme using the modified dinitrosalicyclic acid (DNSA) method of Miller (1959) [20].

The reaction mixture consisted of 0.75 ml of 1.0% w/v carboxymethylcellulose in Tris-HCl buffer (pH 8.0) and 0.25 ml of crude enzyme solution. Control experiment tubes (Enzyme blank) contained the same amount of substrate and 0.25 ml of the crude enzyme solution boiled for 20 min. Both the experimental and control tubes were incubated at 50°C for 20 min. The reaction was terminated by the addition of 1.5 ml of DNSA (10 g sodium hydroxide (NaOH); 40 g sodium potassium tartarate; 10 g 3, 5-dinitrosalicyclic acid and distilled water up to 1000 ml) reagent. The reaction mixture was heated at 100°C for 15 min in boiling water. The tubes were then cooled under running water. The optical density (OD) was read at 540 nm. The amount of reducing sugars produced was interpolated from a glucose standard curve.

One unit of cellulase activity was expressed as the amount of enzyme that liberated reducing sugar equivalent of 1 µg of glucose per minute under the assay condition.

Protein Determination

The protein concentration was determined by the method of Bradford (1976) using bovine serum albumin (BSA) as standard.

Production and Partial Purification of Cellulase

The organism was cultured on modified liquid basal medium containing peptone (0.5 g), yeast extract (0.5 g), K_2HPO_4 (0.1 g), KH_2PO_4 (1.15 g), $MgSO_4.7H_2O$ (0.004 g), $FeSO_4.7H_2O$ (0.00125 g), $CaCl_2.H_2O$ (0.05 g), glucose (0.5 g), capuric acid (0.001 g) and carboxymethylcellulose (1 g), pH 7.0 after the method of Sakthivel et al. [21] Aqueous suspension of pure bacterium isolate was made in sterile distilled water and the medium containing 0.2% (w/v) CMC was inoculated with a suspension containing the cells. Cultures were grown in 250 ml Erlenmeyers flasks with 100 ml of medium in a rotary shaker (140 rpm) at room temperature. After 72 h the biomass was separated by centrifugation at 10,000 rpm at 4°C for 30 min and the supernatant was used as the crude enzyme [22].

The enzyme solution was further precipitated by 60% (v/v) by the gradual addition of chilled acetone with continuous stirring and kept in a refrigerator at 4°C for 1 h. The resulting precipitate was collected by centrifugation at 12,000 rpm at 4°C for 30 min and dissolved in minimum amount of Tris-HCl buffer (pH 8.0) after which the enzyme was dialyzed against the same buffer using acetylated dialysis bag [23].

The dialysate obtained from acetone precipitation was layered on a 2.5 cm X 80 cm column of Biogel P-100 which had previously been equilibrated with 0.05 M Tris-HCl buffer, pH 8.0. Fractions (5 ml) were collected at a flow rate of 8 ml/h. Cellulase activity and the protein profile of the fractions was determined. The active fractions were pooled and stored in the freezer.

e-ISSN:2320-3528
p-ISSN:2347-2286

Properties of the Enzyme

Determination of kinetic parameters: The K_m and V_{max} of cellulase was determined by varying the concentration of carboxymethylcellulose and measuring the initial reaction velocities at 50°C for 20 min. The concentration of CMC was varied between 0.8 and 4 mg/ml. The assay mixture contained 0.1 ml of enzyme. The data was plotted according to Lineweaver-Burk.

Effect of temperature on enzyme activity: An aliquot of enzyme (0.1 ml) and 0.75 ml of the substrate was incubated at varying temperatures of 10°C to 80°C.

The thermal stability experiment was carried out by assaying the enzyme at various temperatures ranging from 30-80°C for 8 h. Five milliliters (5 ml) of the enzyme was incubated for 1 h and 0.1 ml was withdrawn at intervals and the cellulase activity was assayed. The residual activity was plotted against time intervals at various temperatures.

Effect of pH on *B. pantothenticus* cellulase activity: The substrate (0.75 ml) was solubilized in the appropriate buffers; 50 mM citrate buffer (pH 4.0-6.0), 50 mM sodium phosphate (pH 7.0), 50 mM Tris-HCl (pH 8.0-9.0) and 50 mM glycine-NaOH (pH 10.0-11.0). A 0.75 ml of the substrate was mixed with 0.1 ml of the enzyme and assayed for cellulase activity for 30 min at 50°C. The residual activity of the enzyme was then quantified according to the standard assay procedure.

Enzyme (0.5 ml) was incubated with 0.5 ml of the above buffers for 1 h at 50°C. The residual activity of each sample for hydrolysis of CMC was then assayed and was plotted against the various pH.

Effect of salts on *B. pantothenticus cellulase* activity: The enzyme (0.1 ml) was pre-incubated with 0.1 ml of 10 mM of NaCl, KCl, $MgCl_2$, $NiCl_2$ and $CoCl_2$ for 30 min at 50°C before the addition of substrate (0.75 ml) (1 g CMC was dissolved in 100 ml of 0.05 M Tris-HCl buffer, pH 8) The residual cellulase activity was assayed. A sample without any salt was taken as the control with 100% activity.

Substrate specificity of *B. pantothenticus* cellulase: The hydrolytic ability of the cellulase against 1% CMC and micro-crystalline cellulose in 0.05 M Tris-HCl buffer (pH 8.0). One gram (1 g) of dried and powdered sugarcane bagasse, orange bagasse and corn cob was solubilized in 10 ml of 0.05 M Tris-HCl buffer pH 8.0. The substrate (0.75 ml) and the enzyme (0.25 ml) were mixed together for the reaction.

RESULTS

Isolation of Cellulolytic Bacteria

Five different bacterial strains were isolated from the dumpsite soil sample. Three of the isolates showed clear halozone around the line of streak on Carboxymethylcellulose agar (CMCA) showing cellulase production and were labeled A, B and C. Two of the three isolates were identified as *Bacillus pantothenticus* and the third as *Lactobacillus casei*. Isolate B which was *Bacillus pantothenticus* with the highest activity was picked for further studies. Enzyme produced from *Bacillus pantothenticus* was partially purified and characterised.

Partial Purification of Enzyme

Precipitation using 60% v/v acetone yielded an enzyme of increased activity with 2% purification fold **(Table 1)**. The gel filtration on Biogel P-100 yielded an activity peak.

Table 1. Summary of partial purification of cellulase produced by *Bacillus pantothenticus*.

Fraction	Vol (ml)	Activity (units/ml)	Total Activity (units)	Protein (mg/ml)	Total Protein(mg)	Specific Activity (units/mg protein)	Yield (%)	Purification Fold
Crude	200	307.69	61538.40	5.567	1113.40	55.28	100	1
Acetone precipitation	65	1320.00	85800	12.60	818.76	104.80	139.42	1.90
Biogel P- 100	40	223.08	8923.08	0.88	35.20	253.50	10.40	2.41

e-ISSN:2320-3528
p-ISSN:2347-2286

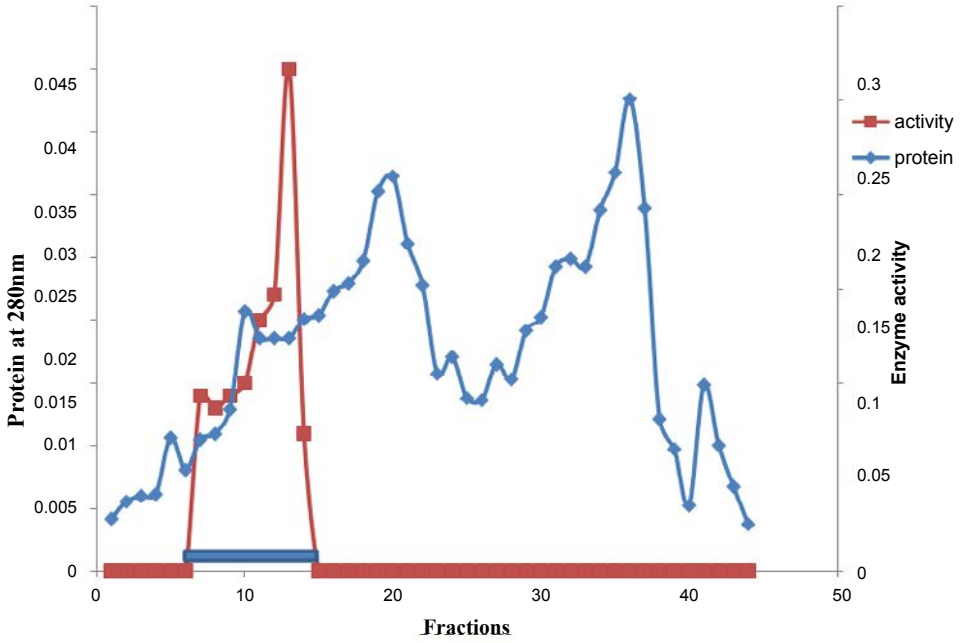

Figure 1. Gel filtration of cellulase from *Bacillus pantothenticus* on Biogel P-100. (column 2.5 X 80 cm) equilibrated with 0.05 M Tris-HCl-HCl buffer (pH 8.0). 5 ml fractions were collected at flow rate of 10 ml/h.

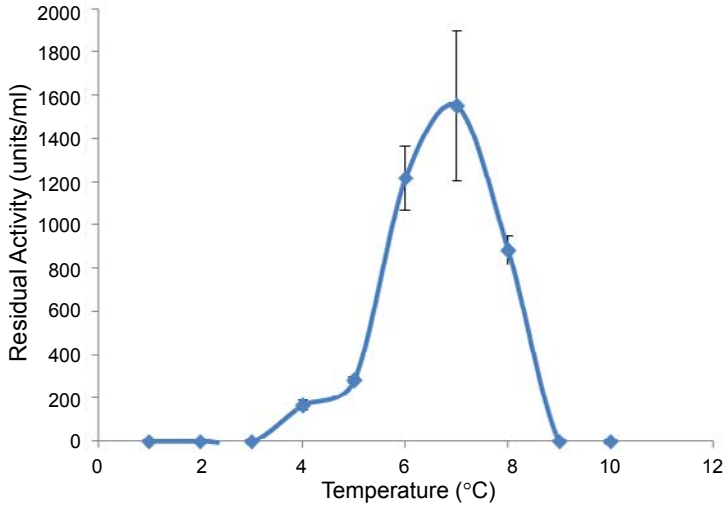

Figure 2a. Effect of temperature on cellulase activity from *Bacillus pantothenticus.*

The activity-temperature profile of cellulase from *Bacillus pantothenticus* was obtained by varying the temperature between 10 °C and 80 °C. The values shown represent averages from triplicate experiments. Error bars represent the standard deviation.

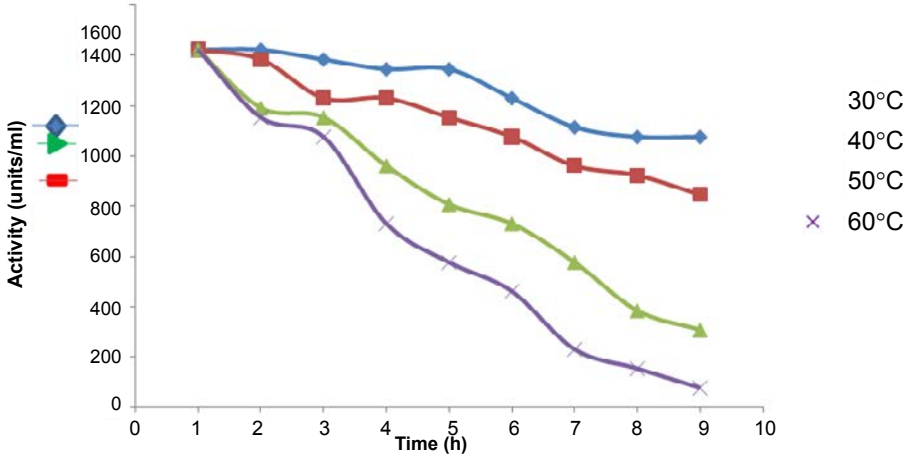

Figure 2b. Thermostability of Cellulase from *Bacillus pantothenticus.*

e-ISSN:2320-3528
p-ISSN:2347-2286

Thermostability was determined by incubating the enzyme for 8 h and at 1 h intervals up to the 8 h, samples were withdrawn and the residual cellulase activity was assayed. The values shown represent averages from triplicate experiments.

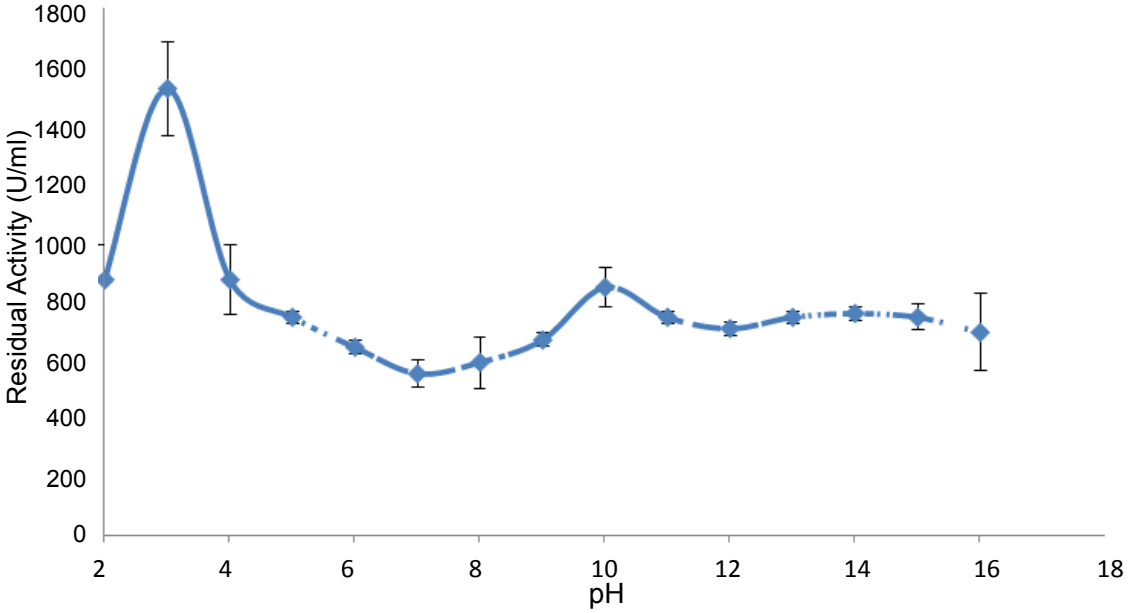

Figure 3a. Effect of pH on the activity of *Bacillus pantothenticus* cellulase. Optimum pH was determined using 50 mM citrate buffer (pH 4.0-6.0), 50 mM sodium phosphate (pH 7.0), 50 mM Tris- HCl (pH 8.0-9.0), 50 mM glycine-NaOH (pH 10.0-11.0).

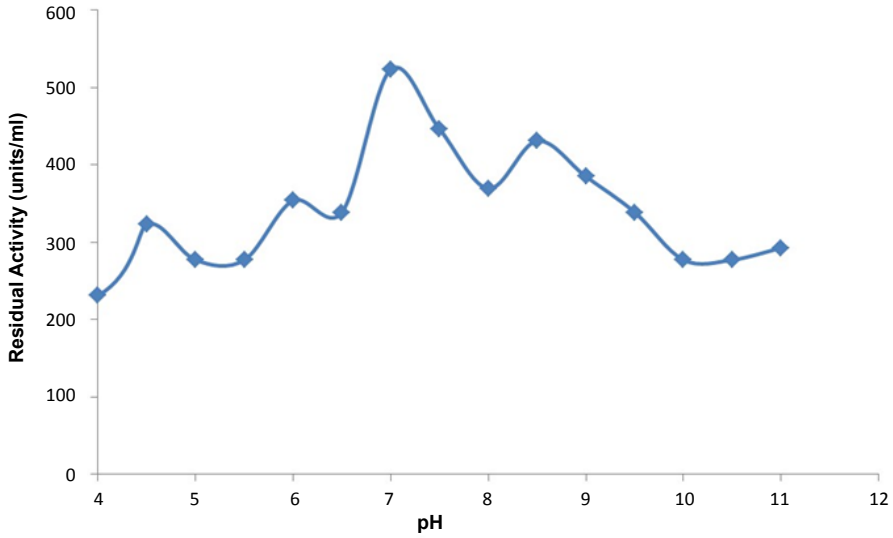

Figure 3b. Effect of pH on the stability of *Bacillus pantothenticus* cellulase. pH stability was determined using 50 mM citrate buffer (pH 4.0-6.0), 50 mM sodium phosphate (pH 7.0), 50 mM Tris- HCl (pH 8.0-9.0), 50 mM glycine-NaOH (pH 10.0-11.0). 0.5 ml of buffer (4-11) was incubated with 0.5 ml of enzyme for 1 h at 50°C **(Table 2)**.

Table 2. Substrate specificity of cellulase from *Bacillus* pantothenticus.

SUBSTRATES	RELATIVE ACTIVITY (%)
Carboxylmethyl Cellulose (CMC)	100
Microcrystalline Cellulose (MCC)	107.44
Sugarcane Bagasse	41.93
Orange Bagasse	22.93
Corn Cub	38.00

1% Sugarcane bagasse, orange bagasse, microcrystallinecellulose and corn cob. The substrates were prepared by suspending 0.1 g of each subatrates in 0.05 M Tris-HCl buffer, pH 8.0. The cellulase activity of the substrates was expressed as a percentage activity of carboxymethylcellulose.

RESULT AND DISCUSSION

In this study three cellulolytic bacteria were isolated from dumpsite soil sample obtained from Onibueja, Osogbo, Osun

e-ISSN:2320-3528
p-ISSN:2347-2286

State. The isolate with widest halozone which was identified tentatively as *Bacillus pantothenticus* was used for further studies. Acetone precipitation had better purification fold (1.90) and percentage yield (6.2%) **(Table 1)** compared to ammonium sulphate precipitation (data not shown). The gel filtration profile indicated a single peak of enzyme activity **(Figure 1)**. This suggests that this enzyme never existed as isoforms.

Kinetic parameters, K_m and V_{max} were estimated as 1.167 mg/ml and 0.833 units/ml respectively. Karnchanatat et al. [24] reported similar Km value of 1.74 mg/ml for endoglucanase from *Daldinia eschscholzii* and also V_{max} of 0.63 U/ml. The kinetic parameters reported in this study is lower than that reported by Wang et al. [25] from *Sporocytophaga* sp with K_m and V_{max} of 9 mg/ml and 27.3 units/ml.

The enzyme showed an optimum temperature 60°C is similar to that of cellulase produced by *Bacillus subtilis* with optimum temperature at 60˚C reported by Yin et al. [26] but higher than optimum temperature of 35°C from *Pseudomonas fluorescens* reported by Bakare et al. [27]. Arrifin et al. [28] also reported cellulase from *Bacillus* pumilus with optimum temperature of 60˚C while an optimum temperature of 50°C was reported by Wang et al. [25] for the enzyme from *Sporocytophaga sp*. The optimum pH obtained in this study was 4.5 and a shoulder at pH 8 **(Figure 3)**.

Cellulase from *Bacillus pantothenticus* was stable at 30°C-60°C and retaining half of its activity after 4 h of incubation **(Figures 2a and 2b)**. The enzyme thermostability was similar to wide temperature range of 30-100°C and optimum of 60°C reported by Bajaj et al. [29] and Ariffin et al. [28] with temperature range of 30-70°C and 60°C optimum. The enzyme showed activity at both acidic and alkali range. The enzyme was stable at all the pH range with optimum stability at pH 7.5 as shown in **Figures 3a and 3b**. Bajaj et al. [29] also reported a pH stable endoglucanase from *Bacillus* strain M-9 with maximum activity at pH 5 and significantly high activity at pH 4, 6, 7, 8, 9 and 10. Other acidophilic cellulases have been reported; optimum pH of 5.5 from cellulomonas sp ASN2 [30] also reported cellulase from Aspergillus oryzae with optimum pH of 5.5. Alkalophilic cellulase from *cellulomonas* sp ASN2 with optimum pH of 7 was reported by Irfan et al. [30] and *Paenibacillus elgii* with optimum pH of 8 [31].

Similar pH range was reported by Abo-State et al. [32] from *Bacillus* species with optimum activity at pH 3 and 9. The pH stability of the cellulase produced by *B. pantothenticus* over a broad pH range seems to be a common characteristic of many *Bacillus cellulases* [33,34].

The enzyme activity was activated by $CoCl_2$ by 175.33% while KCl, $MgCl_2$, $NaCl_2$, and $NiCl_2$ reduced the activity of the enzyme by 36.22%, 53.74%, 68.92% and 61.45% respectively. Vijayaraghavan and Vincent [33] had earlier reported inhibition of cellulase by KCl, $MgCl_2$ and NaCl. Activation by $CoCl_2$ is similar to results of Saha [34] and Yin et al. [25] from Mucor circineloides with 44% increase and 58% from *Bacillus subtilis* respectively.

The cellulase degraded microcrystalline cellulose, CMC, sugarcane bagasse, orange bagasse and corn cub. Microcrystalline cellulose showed higher activity compared to CMC while other substrates had low activity as shown in **Table 2**.

The higher activity of the enzyme for microcrystalline cellulose (107.44%) than CMC (100%) infers presence of exoglucanase and endoglucanase [35]. Significant activity was also observed with the microcrystalline forms of cellulose by Vijarayagahvan and Vincent [33] and similar results were obtained with two other bacterial strains, *Bacillus* CH43 and HR68 [36-39].

CONCLUSION

In conclusion, the *Bacillus pantothenticus* cellulase was active at both acidic and alkali pH, thermostable and had the ability to degrade various substrates. The characteristics exhibited by the enzyme makes it suitable for use in biodegradation of waste and other industrial applications.

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e-ISSN:2320-3528
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