Türk Biyokimya Dergisi [Turkish Journal of Biochemistry–Turk J Biochem] 2012; 37 (2) ; 222–230.
doi: 10.5505/tjb.2012.47955

Research Article [Araştırma Makalesi]

Yayın tarihi 30 Eylül, 2012 © TurkJBiochem.com

[Published online 30 September, 2012]

Purification and characterisation of a cellulase obtained from cocoa (*Theobroma cacao*) pod-degrading *Bacillus coagulans Co4*

[*Bacillus coagulans Co4*'dan selülaz enziminin saflaştırılması ve karakterizasyonu*]

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Registered: 15 December 2011; Accepted: 19 March 2012

[Kayıt Tarihi : 15 Aralık 2011; Kabul Tarihi : 19 Mart 2012]

ABSTRACT

Objective: Some properties of cellulase purified from the culture supernatant of *Bacillus coagulans Co4,* isolated from cocoa pod dumpsite were investigated for possible biotechnological applications.

Methods: The crude cellulase was purified to apparent homogeneity using a combination of acetone precipitation, CM Sepharose CL-6B ion exchange chromatography and gel filtration on Sephadex G-100. The molecular and thermodynamic properties of the purified enzyme were studied following standard procedures.

Results: The specific activity of the purified cellulase rose from 0.10 to 47 units/mg of protein, at the end of purification. The molecular weight was found to be 14.5 kDa; and an apparent K_m value of 0.18±0.06 mg/ml of carboxylmethylcellulose. The optimum pH and temperature were 7.5 and 60°C respectively. The activation energy for carboxylmethylcellulose hydrolysis (E_a) was 16.5 kJ/mol. Na^+ and K^+ had no effects on its activity at concentrations up to 200 mM, whereas Ca^{2+} and Mg^{2+} served as inhibitors at concentrations above 25 and 40 mM respectively. The cellulase retained 40% residual activity when heated at 60°C for 40 minutes.

Conclusison: On the basis of these properties, it is concluded that the purified cellulase is moderately thermostable and may have applications in the bioconversion of agricultural wastes into economically useful products.

Key Words: *Bacillus coagulans Co4*, cellulase, cocoa pod, bioconversion, agricultural wastes

Conflict of interest: The authors declare that there was no conflict of interest in this work.

ÖZET

Amaç: Çalışmada, çöp atıklarındaki kakao kozasından izole edilen *Bacillus coagulans Co4* kültür süpernatanından saflaştırılmış selülaz enziminin bazı özellikleri saptanarak olası biyoteknolojik uygulamalar için uygunluğu araştırılmıştır.

Yöntem: Kaba selülaz aseton çöktürmesi, CM Sefaroz CL-6B iyon değişim kromatografisi ve Sefadeks G-100 jel filtrasyonu yöntemleri kullanılarak saflaştırılmıştır. Saflaştırılan enzimin moleküler ve termodinamik özellikleri standart yöntemler kullanılarak saptanmıştır.

Bulgular: Selülazın spesifik aktivitesi saflaştırma basamakları sırasında 0.10'dan 47 ünite/mg protein'e yükselmiştir. Molekül ağırlığı 14.5 kDa olarak bulunmuştur. Karboksimetilselüloz için K_{mapp} 0.18±0.06 mg/ml olarak hesaplanmıştır. Optimum pH ve sıcaklık sırasıyla 7.5 ve 60°C olarak ölçülmüştür. Karboksimetilselülozun hidrolizi için aktivasyon enerjisi (E_a) 16.5 kJ/mol'dür. Na^+ ve K^+'un 200 mM'a kadar olan derişimleri enzim aktivitesi üzerine etki etmemektedir. Bununla birlikte Ca^{2+} ve Mg^{2+} sırasıyla 25, 40 mM ve üzeri derişimlerde kullanıldığında inhibe edici etkiye sahiptir. Selülazın, 60°C'de 40 dakika ısıtıldığında aktivitesinin %40'ını koruduğu gözlenmiştir.

Sonuç: Bu özelliklerden yola çıkarak, saflaştırılmış olan selülazın kısmen ısıya dayanıklı olduğu ve tarımsal atıkların ekonomik olarak yararlı ürünlere biyodönüşümünde kullanılabileceği düşünülebilir.

Anahtar Kelimeler: *Bacillus coagulans Co4*, selülaz, kakao kozası, biyodönüşüm, tarımsal atıklar

Çıkar çatışması: Yazarlar arasında çıkar çatışması bulunmamaktadır.

Introductizon

Cellulases (EC 3.2.1.21) refer to a class of enzymes produced chiefly by fungi, bacteria and protozoan that catalyze the hydrolysis of cellulose. Unlike chemical methods, enzymatic hydrolysis of lignocelluloses offers an attractive method and relatively pure products can be obtained from the hydrolytic process. Such products can serve as raw material for the production of bio-ethanol, glucose and a few other compounds [1,2]. Cellulose is the major component of plant biomass and the major biopolymer found in abundance on earth, and much of the cellulose exists as wastes. Such wastes include straw, corn cobs, wood wastes, peat, bagasse and waste paper [3].

In principle, all lignocellulosics can be converted into simple sugars which can serve as useful raw materials in the production of fuel, animal feedstock and feedstock for chemical synthesis [4]. Against this backdrop, there has been several studies on the use of different agricultural wastes which include bagasse [1,3], corn cob [3,5], sawdust [6,7], wheat bran [5] and wheat straw [8] as lignocellulosic substrate for cellulase hydrolysis.

Relatively high temperatures are often necessary in some industrial processes involving hydrolytic enzymes. Therefore, there has been thrust to source such enzymes from thermophiles because enzymes from thermophiles are usually stable at moderate to high temperatures.

Little research has been done on ways to put to use, the bulk of waste from cocoa processing industries especially the pod that is usually discarded after the fruit has been removed. A cocoa fruit on the average contains about 20 to 60 seeds (usually called cacao beans) which are embedded in the white pulp. The cocoa pod makes up about 75% of the total weight of the fruit [9] and becomes an agricultural waste, and a health hazard for the healthy immature cocoa pods, as it harbors cocoa stem borers. Hence, this research was conceived to look into the possibility of isolating cellulase from cellulase producing thermophilic bacteria from dumpsites of discarded cocoa pods and characterize it for possible biotechnological applications.

Materials and Methods

Preparation of crude extract

The organism previously isolated from dumpsite of discarded cocoa pod was cultured on modified liquid basal medium (BM) containing high viscosity carboxylmethylcellulose (CMC) 2g, yeast extract 2g, KH_2PO_4 1g, $MgSO_4.7H_20$ 5g, NaCl 0.75g and Peptone 20g dissolved in 1000 ml of 100 mM sodium citrate buffer pH 6.5 [10,11]. Aqueous suspension of pure bacterial isolates was made in sterile distilled water and standardized to 0.5 McFarland standards. The basal medium containing 0.2% (w/v) CMC was inoculated with an aqueous suspension of the organism from a 24 hour old culture. The mixture was incubated at 55°C for 48 hours on a rotary shaker at 100 rpm. The culture was then centrifuged at 6,000g for 15 minutes. The clear supernant was collected aseptically as the crude extract.

Cellulase assay

Cellulase activity towards carboxymethylcellulose (CMC) was measured by the appearance of reducing end groups released by the action of the enzyme on the substrate using modified method of Nelson [12] and Somogyi [13]. One unit of cellulase activity was expressed as the amount of enzyme that liberated reducing sugar equivalent to 1 µg of glucose per minute under assay condition. The specific enzyme activity was expressed as the unit of enzyme activity per mg of protein.

Determination of protein concentration

Protein concentration of the samples was determined using Lowry's method [14] with bovine serum albumin (BSA) as standard protein.

Enzyme purification

Acetone precipitation

The proteins in the crude preparation were precipitated by the addition of cold acetone at a ratio of 1:4 (supernatant:acetone). The precipitate was allowed to form at -20°C overnight. The precipitate was redissolved in a minimal amount of 100 mM sodium citrate buffer pH 6.5.

Purification by ion exchange chromatography on CM Sepharose CL-6B

Preswollen CM Sepharose CL-6B was packed into a column (2.5 x 40 cm) at a flow rate of 36 ml per hour. The column was equilibrated with 300 ml of 100 mM sodium citrate buffer, pH 6.5. The acetone precipitated sample (30 ml) containing 411 mg per ml of protein was layered onto the column and was eluted with the same buffer at the same flow rate. Three ml fractions were collected. A gradient of 1 M NaCl in 100 mM sodium citrate buffer, pH 6.5 was applied to elute bound proteins. The protein concentration of the fractions was monitored by measuring absorbance at 280 nm. The fractions were also assayed for cellulase activity. The fractions with high enzyme activity were pooled together. The pooled eluates were precipitated with cold acetone.

Gel filtration on Sephadex G-100

Sephadex G-100 slurry was packed into a column (1.0 x 50 cm) followed by equilibration with 100 mM sodium citrate buffer, pH 6.5. An aliquot (0.75 ml) of acetone precipitated enzyme was layered and eluted with 100 mM sodium citrate buffer, pH 6.5. Fractions of 0.5 ml were collected at a flow rate of 18 ml/hr. Fractions containing cellulase activity were pooled for further analysis.

Native and subunit molecular weight determination

For the subunit molecular weight measurement, SDS-PAGE was performed on 4% stacking gel and 12% separating gel together with a mixture of a set of marker proteins. Native molecular weight of the purified cellulase was estimated on a Sephadex G-100 column that had been calibrated with a set of molecular weight standard proteins.

Determination of kinetic parameters

The effect of varying concentrations of substrate on the purified cellulase was determined using carboxymethyl cellulose as substrate.

The apparent kinetic parameters (V_{max} and K_m) of the cellulase were determined by varying the concentration of carboxymethylcellulose from 0.018 mg/ml to 3.2 mg/ml in 100mM sodium citrate buffer, pH 6.5. The assays were performed with the enzyme which had been diluted appropriately with 100 mM sodium citrate, pH 6.5. The apparent kinetic parameters were estimated from Lineweaver-Burk plots [15].

Effect of pH on cellulase activity

For the measurement of the effect of pH, aliquots of the purified cellulase were incubated with carboxylmethylcellulose in buffers at pH values ranging from 4.0 to 9.0. The buffers were sodium citrate (pH 4.0-6.5) and potassium phosphate (pH 7.0-9.0). The activity assay was then carried out as previously described.

Effect of temperature on cellulase activity

Aliquot (0.05 ml) of the purified cellulase and the substrate were incubated at temperatures ranging from 35°C to 75°C. The residual activity was assayed by measuring the amount of reducing ends produced as earlier described. The effect of heat on the stability of the enzyme was examined by placing a stock solution of cellulase at 60°C in water bath. At 0, 30, 60, 90, 120 and 150 minutes, aliquot (0.05 ml) of the enzyme was taken at intervals and assayed for residual activity.

Effect of cations and EDTA on cellulase activity

The effect of cations on cellulase activity was determined with K^+ Na^+, Mg^{2+} and Ca^{2+} at different concentrations ranging from 0 to 200 mM. The residual activity was measured in the presence of the salt of each of the ions. For the effect of EDTA on the enzyme activity, the following concentrations of EDTA (0, 4, 12, 15, 20, 30, 50, 80, 100, 120 mM) were used

Results

Enzyme purification

Acetone precipitation

Acetone precipitation was selected over ammonium sulphate precipitation because of better recovery of activity. In the acetone precipitation, the crude enzyme gave a yield of about 70% and a 3-fold purification. The specific activity of the precipitated enzyme was 0.29 units/mg of protein.

Purification of enzyme using ion exchange chromatography on CM Sepharose CL-6B

Ion exchange chromatography of the partially purified enzyme preparation on CM Sepharose CL-6B gave a single peak. The peak was pooled and precipitated using cold acetone. Rerun of the redissolved enzyme on CM Sepharose CL-6B gave rise to two peaks. The elution profile of the rerun on CM Sepharose CL-6B is shown in Figure 1. The peaks (X and Y) were separately pooled.

Rerun of partially purified cellulase on CM Sepharose CL-6B

Purification by gel filtration chromatography on Sephadex G-100

Gel filtration of the acetone precipitated fractions of each of the peaks (X and Y) on Sephadex G-100 produced a single peak. The summary is shown in Table 1.

Molecular weight determination

The purified enzyme has a native molecular weight of 14,700 ±2900 Da from estimation on the calibration curve of the gel filtration on Sephadex G-100. Using SDS-PAGE, the purified cellulase showed a single band (Figure 2). The molecular weight was estimated to be 14,500 Da.

Effect of substrate concentration and determination of kinetic parameters (K_m and V_{max})

The activity of the purified cellulase increased with increase in substrate concentration until a maximum was reached at 1.25 mg/ml of CMC above which there was no further increase in enzyme activities. The activity of the enzyme was inhibited at substrate concentrations above 1.8 mg/ml. The plot of cellulase activity against varying substrate concentrations is shown in Figure 3.

The Michaelis-Menten constants (K_m and V_{max}) of the purified cellulase for carboxylmethylcellulose were estimated from the Lineweaver-Burk plot. The K_m and V_{max} values obtained for the purified cellulase was 0.18 ±0.06 mg/ml of CMC and 37.94 ±2.98 units/mg of protein.

Effect of pH on cellulase activity

A plot showing the effect of pH on the activity of the enzyme is shown in Figure 4. The optimum pH for the purified cellulase was 7.5. At pH 4 to 5 there was a steady increase, in enzyme activity. At pH 7.5 the enzyme activity was 42 units/min and then dropped to 34 units/min at pH 8.5 and pH 9.

Effect of temperature on cellulase activity

Cellulase activity gradually increased as the temperature increases up to the optimum temperature of 60°C (Figure 5). Thereafter, there was a steady decline in activity.

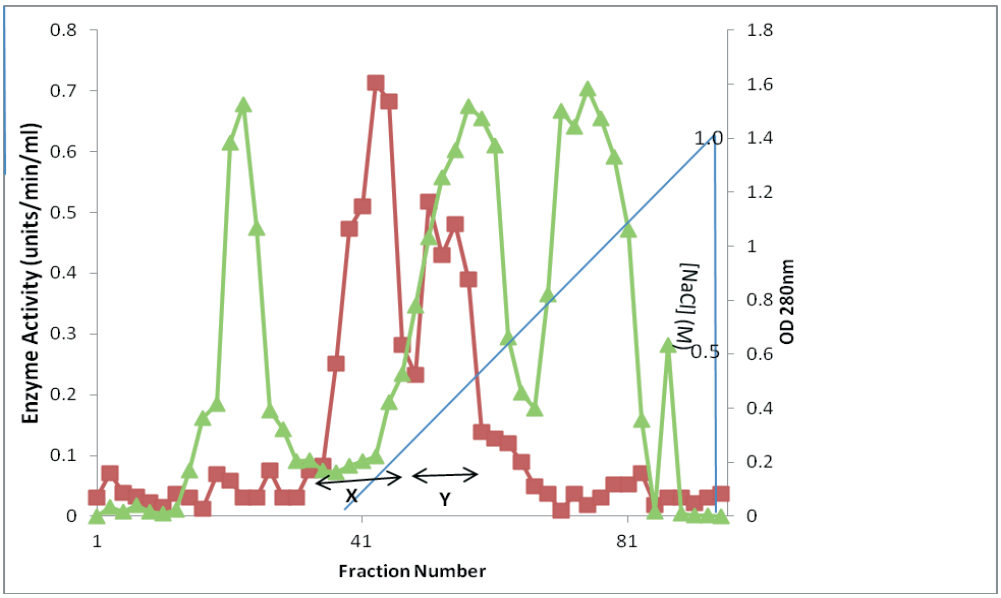
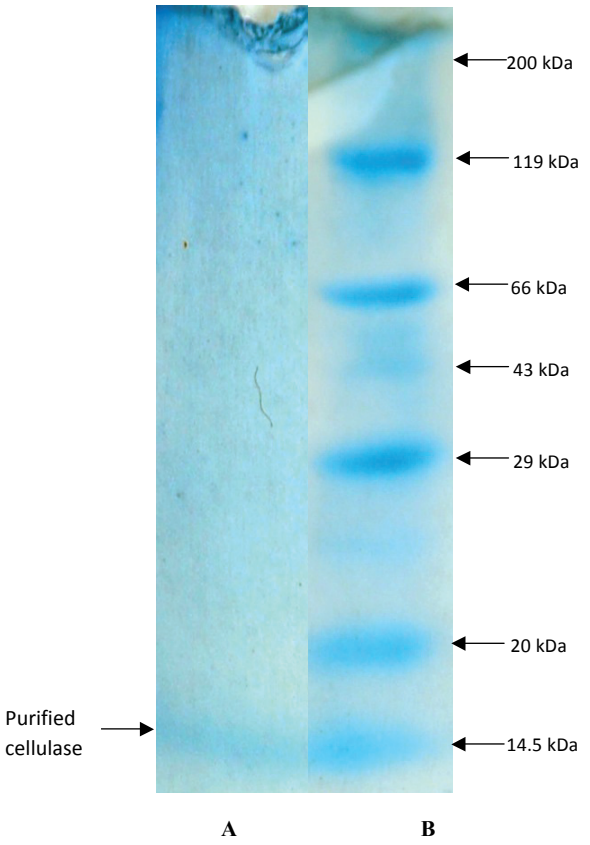


Figure 1. Elution profile of Rerun of Cellulase obtained from *Bacillus coagulans Co4* on CM Sepharose CL-6B. Active fractions from a previous CM-Sepharose CL-6B ion-exchange chromatography were concentrated by acetone precipitation, re-dissolved in small volume of elution buffer (100 mM sodium citrate buffer pH 6.5) and re-layered on a freshly prepared column. Fractions of 3 ml each were collected at a flow rate of 30 ml/hr. A gradient of 0-1 M NaCl was used to elute bound proteins. The fractions were monitored for enzymatic activity(-■-■- by assaying individual fractions as stated in the text. The protein profile (-▲-▲-) was determined by measuring the absorbances of each fraction at 280 nm.

Table 1. Summary of purification of cellulase obtained from *Bacillus coagulans Co4*

Purification Step	Total Volume (ml)	Protein concentration (mg/ml)	Enzyme Activity (units/ml/min)	Total Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Yield (%)	Purification fold
Crude enzyme	1000	46.3	9.88	46,300	4830	0.1	100	1
Acetone precipitation	30	411	110	12,300	3300	0.29	70	2.64
Acetone ppt.	20	19.4	105	387	2100	5.4	43	52.2
Rerun on CM Sepharose CL-6B	X: 90	0.21	12.4	19	1120	59	23	567
	Y: 48	6.04	11.5	290	550	1.9	11	18.3
Acetone ppt	X: 6	1.92	117	11.5	700	61	15	61
	Y: 7.5	17.6	53.3	132	400	3.03	8.3	30.3
Sephadex G-100	X: 40	0.033	7.3	1.32	382	290	8	2790
	Y: 50	0.08	3.76	3.98	188	47	4	454



Lane A: Aliquot of purified cellulase
Lane B: Standard marker proteins (Roti-Mark STANDARD)

Figure 2. Photograph of SDS-PAGE of the purified cellulase from Bacillus coagulans Co4. Aliquots (5 µg) of both the purified cellulase and standard marker proteins were layered on wells A and B respectively, following established protocols stated in the text. The mixture of the standard proteins contains lyzozyme from chicken (14.5 kDa), soya bean trypsin inhibitor (20 kDa), carbonic anhydrase (29 kDa), chicken ovalbumin (43 kDa), bovine serum albumin (66 kDa), *E. coli* β-galactosidase (119 KDa), and myosin from beef (200 kDa).

A plot of log V against the inverse of temperature (K^{-1}) was used to determine the activation energy of the reaction. The activation energy was determined from the negative slope of the graph (Figure 5b). The activation energy (E_a) of the reaction was estimated to be 16.5 kJ/mol of purified cellulase.

Effect of cations and EDTA on cellulase activity

The range of concentrations of cations (K^+ and Na^+) employed in this study had no inhibitory effect on the purified cellulase. There was no significant change in the residual activity of the purified cellulase up to 200 mM concentration.

For Ca^{2+}, the highest cellulase activity was observed at 30 mM concentration and thereafter there was a gradual decrease in activity (Figure 6). Concentrations of Mg^{2+} above 50 mM were inhibitory to the activity of the purified cellulase. At 160 mM Mg^{2+} concentration, more than 50% residual activity was lost. At concentrations above 40 mM, EDTA was inhibitory to the activity of the purified cellulase (Figure 7).

Effect of heat on stability of cellulase activity

A plot of residual activity of purified cellulase against temperature of incubation of the enzyme is shown in Figure 8.

Heating of the purified cellulase at 60°C over a period of 90 minutes reduced the residual activity to 20%. About 50% of the cellulase activity remained after heating for 30 minutes. At about 60 minutes, more than 50% of the residual activity was lost.

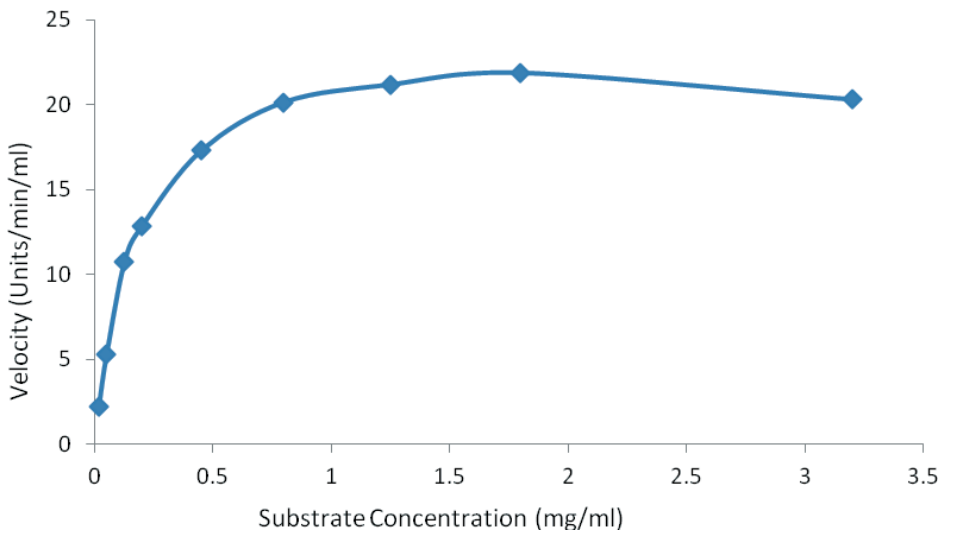


Figure 3. Effect of substrate concentration on purified cellulase obtained from *Bacillus coagulans Co4*. Effect of substrate concentration on cellulase activity was measured by incubating an aliquot (5 µg) of the enzyme solution with the substrate at each of the indicated concentrations for 1 hr. The amount of reducing sugar liberated by the enzyme was then quantified.

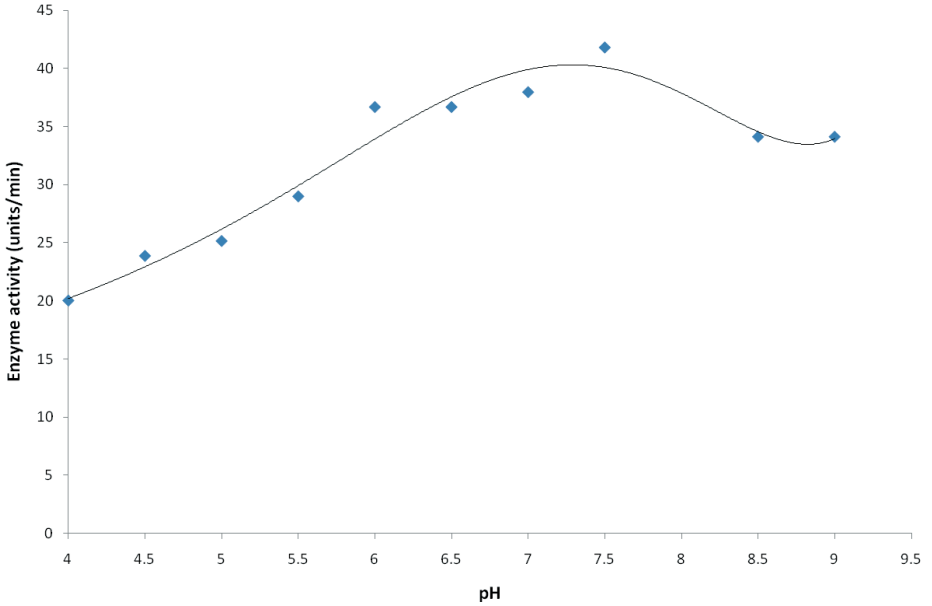


Figure 4. Effect of pH on purified cellulase of *Bacillus coagulans Co4* isolated from heap of cocoa pods. 5 µg of the purified enzyme was incubated with the substrate (CMC) dissolved in the buffers at the respective pHs indicated. The buffers were sodium citrate (pH 4-6.5) and potassium phosphate (pH 7-9.0).

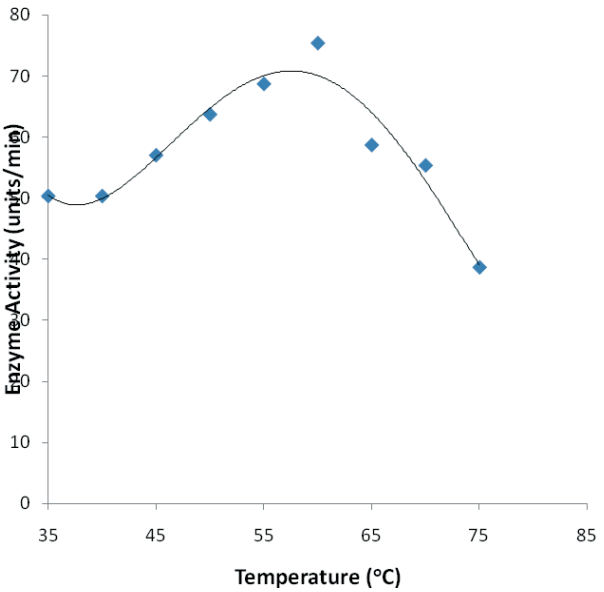


Figure 5a. Effect of temperature of purified cellulase obtained from *Bacillus coagulans Co4* isolated from a heap of discarded cocoa pods

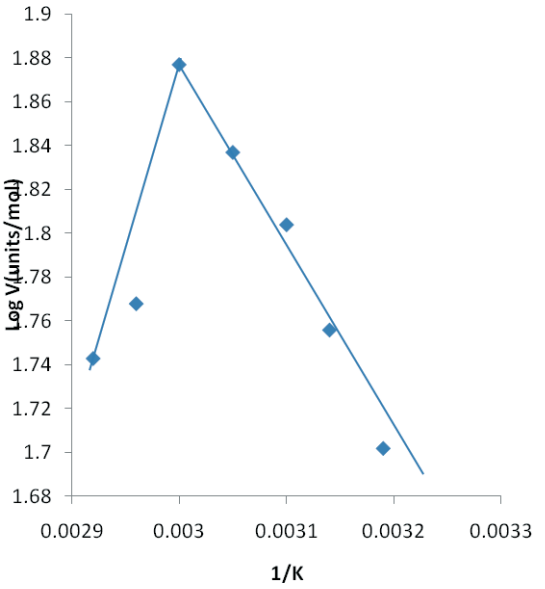


Figure 5b. A plot of log (Activity) against 1/K for the determination of activation energy (E_a) of the reaction catalyzed by purified cellulase obtained from Bacillus

Discussion

Growth of the isolated bacteria species-*Bacillus coagulans Co4* in basal medium (BM) containing carboxymethylcellulose led to the expression of large quantity of cellulase in the culture supernatant. Initial attempt at precipitating the crude cellulase with ammonium sulphate proved ineffective necessitating the use of cold acetone. Purification was subsequently done using ion exchange chromatography on CM Sepharose CL-6B and gel filtration on Sephadex G-100. The first run on CM Sepharose resulted in just one peak. This peak was pooled and re-run on a fresh column which thereafter yielded two peaks (X and Y) of activity. Purification of each of the peaks on a column of Sephadex G-100 produced a single homogeneous peak with CMCase activity. Though the specific activity of peak Y was lower than that of X, (47 and 290 Units/mg

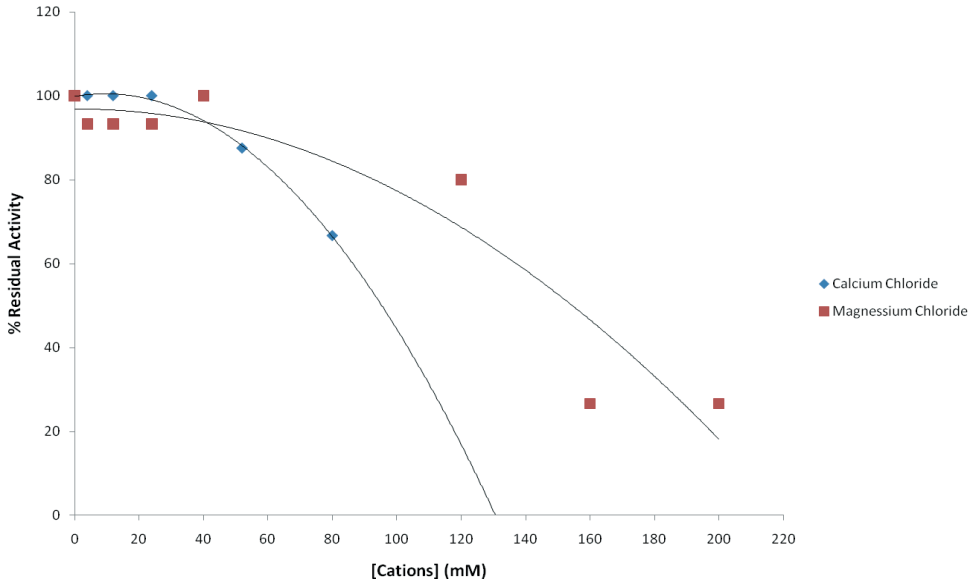


Figure 6. Effect of Ca^{2+} and Mg^{2+} on purified cellulase obtained from *Bacillus coagulans Co4* isolated from a heap of cocoa pods. The activity of the purified cellulase at the indicated concentrations of the metal ions were determined and compared with the activity in the absence of the metal ions which served as the control and taken to have 100% residual activity. 5µg of the purified protein was used in each assay.

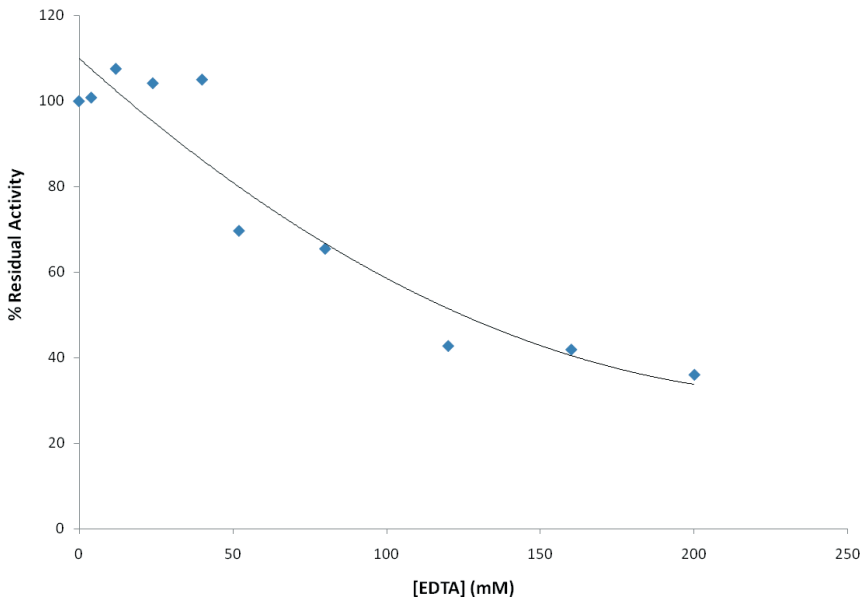


Figure 7. Effect of EDTA on purified cellulase obtained from *Bacillus coagulans Co4* isolated from a heap of cocoa pods. The enzyme (5 µg) was incubated with the substrate at the indicated concentrations of EDTA for 1 hr, and the amount of reducing ends released was quantified as stated in the text. Control had noEDTA and was taken to have 100% residual activity.

protein, respectively) the two peaks appear to contain only purified cellulase, as they produced a single band each on SDS-PAGE. They also share identical molecular characteristics. The removal of endogenous inhibitors probably accounted for a sharp increase in the purification fold towards the end of purification.

In contrast to the high V_{max} value, the K_m of the purified cellulase was quite low (0.18 ±0.06 mg/ml of CMC). This Km is lower than some values that had been previously reported for bacteria cellulase, isolated from *Coptotermes formosanus* [16]; and *Pseudomonas fluorescens* [17]. Since K_m value can to some extent be defined as a measure of the apparent affinity of an enzyme for its substrate, it follows that the cellulase purified in this study has more affinity than some others that had been previously reported. The optimum pH for the purified cellulase was 7.5 which is in agreement with the findings of Dasilva *et al.* [18] who reported an optimum pH value of 7.0 to 8.0 at 60 °C. However, this value is higher than 6.5, and 6.5 to 7.0 pH values

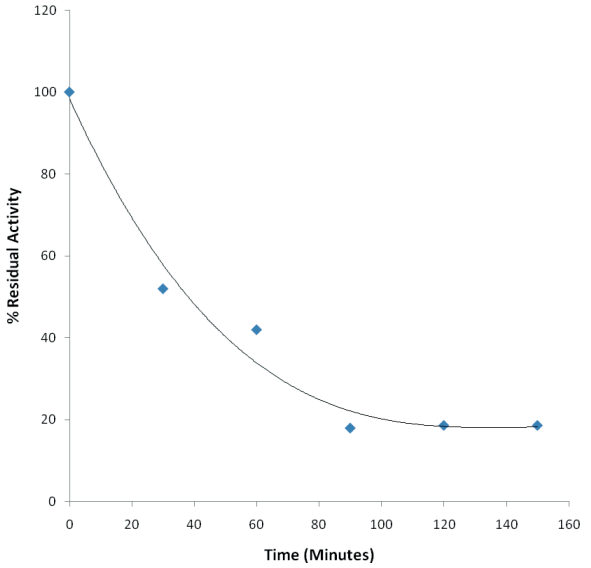


Figure 8. Effect of heat on stability of purified cellulase obtained from *Bacillus spp.* at 60°C. The stability of the enzyme to heat at 60°C was measured by incubating 100 µg of a solution of the enzyme in a bath at this temperature. At the indicated time intervals, aliquots were withdrawn and assayed for residual activity. An unincubated control was taken to have 100% residual activity.

of CMCase of *Clostridium* sp. and *Pseudomonas fluorescens* respectively [17,19]. The *Bacillus coagulans Co4* used in this study can therefore be classified as a neutrophile or facultative alkalophile.

The activity of the purified cellulase was inhibited by Ca^{2+} and Mg^{2+} at concentrations above 25 mM and 40 mM respectively. The other cations (Na^{+}, K^{+}) had no significant effect. Cations have been suggested as an intermediate which may help the enzyme to bind properly to the substrate by ionic bonds (enzyme-metal-substrate) holding the substrate in the appropriate position for an efficient action of the active site within the enzyme [20]. The size of the cation may determine the activation of an enzyme, and the metal may form an essential part of the active centre of the enzyme [21]. The cation may also help in removing the enzyme inhibitors that may be present in the enzyme preparation by forming a complex with such inhibitors [21]. The ability of both monovalent and divalent cations to stimulate or inhibit cellulose degrading enzymes has been reported by some researchers [22,23]. Yoon *et al.* [22] and some other workers had reported the stimulation of cellulase, by Ca^{2+} and $Mg,^{2+}$ which is in contrary to the findings in this study. Bakare *et al.* [17] reported stimulation of cellulase activity by Na^{+} and Mg^{2+}. However, all monovalent cations had no significant effect on the activity of this cellulase even up to 200 mM concentration of the cations. On the other hand, the divalent cations employed inhibited the activity of the purified cellulase at concentrations above 25 mM. Also, in agreement with the work of Osagie, and Oikawa *et al*., [24, 25], EDTA was found to be inhibitory to the activity of cellulase used in this study. EDTA as a metal chelating agent probably acts by inactivating the cellulase either by removing metal ions from the enzyme through the formation of coordination complex or by binding inside the enzyme as a ligand, as had been noted by Schmid, [26]. The optimum temperature was 60°C. The sharp decline in the rate of reaction above the optimum temperature is probably due to thermal inactivation of the enzyme (denaturation). Incubation of the enzyme at the optimum temperature for 30 minutes resulted in a loss of about 50% of the residual activity. This showed that the purified cellulase in this work is more stable than the cellulase obtained by Shikata *et al.* [27] from *Bacillus sp.* However, Hreggvidsson *et al*. [28] reported a more stable enzyme which when preheated at 85°C lost only 10% activity after 8 hours of incubation.

In conclusion the study has shown that there exists *Bacillus sp* capable of producing cellulase in the heap of cocoa pod wastes. The purified cellulase from this organism may be adapted for large scale industrial applications in the bioconversion of agricultural wastes such as cocoa pods; cassava peels etc. into economically useful products. The optimum pH and temperature of 7.5 and 60°C respectively; relatively low K_m and high V_{max} values are indices that may make the enzyme suitable for such applications. The enzyme may however have to be used at low substrate concentrations, since high substrate concentration is inhibitory to the enzyme.

Conflict of interest: The authors declare that there was no conflict of interest in this work.

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