Vol. 26 (2) 43–49 30 June 2014

Biokemistri

An International Journal of the Nigerian Society for Experimental Biology

Original Article

Characterization of a cellulolytic enzyme from wood degrading bacteria, *Bacillus circulans*

[a]Gabriel S. Aruwajoye, [b]Leonard O. Ehigie and [a]Femi K. Agboola

[a]Department of Biochemistry, Obafemi Awolowo University, Ile-Ife 220005. Osun-State. Nigeria

[b]Department of Biochemistry, Ladoke Akintola University of Technology, Ogbomoso, Oyo-State. Nigeria

*Corresponding author: Femi K. Agboola. E-mail: fkagbo@oau.ife.edu; Tel.: +2348034738078

Received: 04 April 2014: Revised 28 May 2014; Accepted: 30 May 2014

ABSTRACT: This report describes the purification and characterization of an enzyme that exhibits cellulase activity produced by the wood degrading bacteria, *Bacillus circulans*. The enzyme was purified by ion-exchange chromatography using CM-Sepharose CL-6B, and shown to exhibit hydrolytic activity on carboxymethylcellulose. The molecular weight of the purified enzyme was determined to be 43 KDa by means of SDS-PAGE. The kinetic parameters, and the effects of pH and temperature on the purified enzyme were determined. The enzyme was 4.37 fold and showed a specific activity of 29.13 µg of glucose produced/min/mg protein. The apparent K_m value for the hydrolysis of carboxymethylcellulose was 1.061 ± 1.17 mg/ml with a V_{max} of 13.75 ± 1.51 µg of glucose produced/ml/min. The enzyme showed an optimum pH value of 9.0 and the optimum temperature was 50 °C. Alkalophilicity and moderate thermostability of this enzyme are some of its essential characteristics that may make it suitable for industrial and biotechnological applications.

KEYWORDS: *Bacillus circulans*, cellulase, decayed wood, optimum, bacteria, enzyme.

INTRODUCTION

Wood is very rich in cellulose which is a homopolymer of β-D-glucose units linked by β-1,4-glycosidic bonds. Cellulose is the most abundant renewable natural product in the biosphere with an estimated annual production of $4.0x10^7$ tons (Bakare *et al.,* 2005). Its crystalline structure is stabilized by intramolecular and intermolecular hydrogen bonds (Hikaru *et al.,* 2008). Various chemical or bioconversion processes can be used to transform this polymer into glucose units that can be used for industrial fermentation. Some of the conversion methods are acid hydrolysis, pyrolysis and enzymatic hydrolysis, but the latter being environmentally safe and can be performed at normal temperature and pH is the most preferred method (Bakare *et al.*, 2005). Therefore most studies focus on enzymatic hydrolysis (Lynd, 1996; Mawazda *et al.,* 2000; Lee *et al.,* 2008; Adeleke *et al.,* 2012).

Cellulase refers to a complex of enzymes that works by synergy to catalyze the conversion of cellulose to glucose units. There are three major groups of enzymes in this system, endo-β-1,4-glucanase (EC 3.2.1.4, EG), exo-β-1,4-glucanase (cellobiohydrolases) (EC 3.2.1.21, CBH) and β-1,4-glucosidase (EC 3.2.1.91). Endoglucanases, cleave β-glucosidic bonds at random in the middle of cellulose molecules; cellobiohydrolases attack cellulose molecules stepwise from the non reducing end, liberating cellobiose subunits and β-glucosidases hydrolyzes cellobiose and low molecular weight cellodextrins into glucose (Beguin, 1990). Cellulases can be found in some animals, plants, protozoans and microorganisms but are obtained basically from fungal and bacterial sources. Bacteria, yeast, fungi among other microorganisms produce cellulases (Bhat, 2000: Camassola *et al.,* 2004: Haakana *et al.,* 2004: Roberto *et al.*, 2005; Bischoff *et al.,* 2006:). Cellulolytic enzymes have great industrial and biotechnological applications in food, brewery,

wine, pulp and paper, textile, detergent, feed industries, and in agriculture (Bhat, 2000). High natural diversity is one of the major characteristics that confer on bacteria the capability to serve as highly potent sources of industrially important enzymes (Bhat, 2000: Camassola *et al.,* 2004). The characteristics of the cellulase produced by these microorganisms such as optimum pH, temperature, kinetic parameters among others do contribute to their activity in various industrial applications. Here, we report that a wood-degrading strain, *Bacillus circulans,* produced an enzyme with cellulase activity. In addition, we purified the enzyme and characterized its biochemical properties.

MATERIALS AND METHODS

Isolation and identification of bacterial strain

The isolation, screening and identification of the cellulolytic bacteria used in this study have recently been described in detail (Aruwajoye *et al.*, 2014).

Crude enzyme preparation

The organism was cultured on modified liquid basal medium containing peptone (2% w/v), KH_2PO_4 (0.1% w/v), $Mg_2SO_4.7H_2O$ (0.5% w/v), NaCl (0.075% w/v), carboxymethylcellulose (CMC) (0.2% w/v) and 0.1 M phosphate buffer (pH 7.0) (Kotchoni and Shonukan, 2002). Aqueous suspension of pure bacteria isolates was made in sterile distilled water and the medium containing 0.2% (w/v) CMC was inoculated with an aqueous suspension of the organism from a 24 hours old culture. The mixture was incubated at 37 °C for 48 hours on a rotatory shaker at 140 rpm.

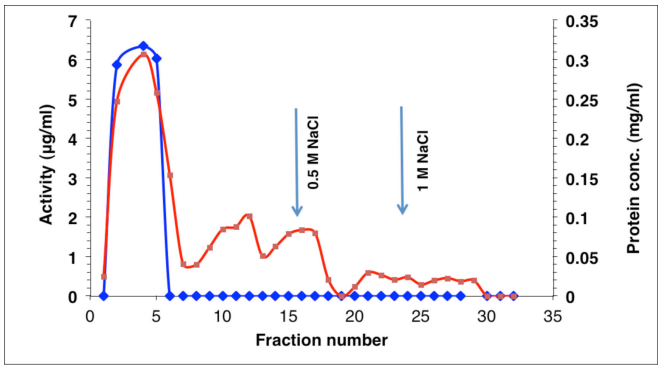

Figure 1: Elution profile of the enzyme obtained from *Bacillus circulans* on CM-Sepharose CL – 6B ion-exchange column.

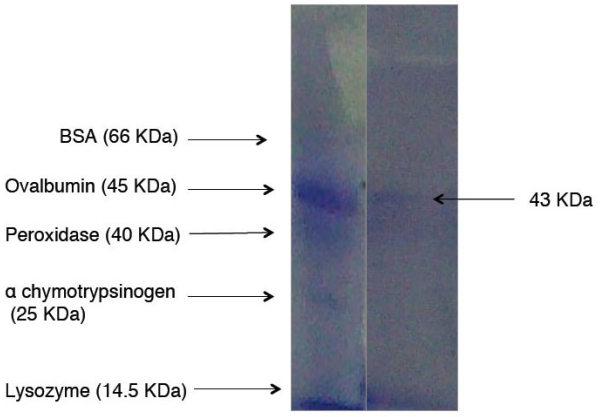

Figure 2: Photograph of SDS-PAGE of the purified enzyme from *Bacillus circulans.* The pure enzyme was found to be 43 KDa after interpolation from the calibration curve showing the molecular weight of marker proteins.

Assay of cellulase activity

Cellulase activity was measured by the presence of reducing end groups released by the action of the enzyme on the substrate using Dinitrosalicylic acid (DNSA) method (Miller, 1959). The reducing ends released were determined after incubating 0.75 ml of 1% w/v CMC (in 0.05 M Tris.HCl buffer, pH 8.0) with 0.25 ml of crude enzyme. Heat-inactivated (boiled) crude enzyme was used as blank (Bailey *et al.,* 1992). The reaction mixture was incubated at 40 °C for 20 minutes and the reaction terminated by addition of 1 ml DNSA reagent. The reaction mixture was heated at 100 °C for 15 minutes in boiling water. The tubes were then cooled under running water. The optical density (OD) was read at 540 nm. The amount of glucose released was determined by interpolation from a glucose standard curve. 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 activity was expressed as the unit of enzyme activity per mg of protein.

Determination of protein concentration

Protein concentration was determined using the Bradford (1976) method with bovine serum albumin used as the standard.

Dialysis of crude enzyme extract

Activation and acetylating of dialysis bag was done according to the method of Whitaker *et al.* (1963). Dialysis was done in 0.1 M phosphate buffer for 8 hours at room temperature.

Table 1: Summary of the purification of the cellulolytic enzyme obtained from *Bacillus circulans*.

Purification Steps	Volume (ml)	Total Enzyme Activities (U)	Total protein concentration (mg/ml)	Specific activity (U/mg protein)	Yield (%)	Purification Fold
Crude	275	585.80	88	6.66	100	1.00
CM-Sepharose CL-6B followed by dialysis against 50% glycerol	57	880.08	30.21	29.13	150	4.37

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

The dialysate obtained above was layered on a 1–cm x 10–cm column of CM–Sepharose CL–6B that had previously been equilibrated with 0.1 M phosphate buffer, pH 7.0.

Fractions (2 ml) were collected at a flow rate of 12 ml/h and bound proteins were eluted by stepwise elution with 0.5 M and 1.0 M NaCl. The cellulase activity in the fractions and the protein concentration were determined, and active fractions were pooled and dialyzed against 50% glycerol in the elution buffer.

Determination of subunit molecular weight

The subunit molecular weight of purified protein was determined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Weber and Osborn (1975) in 4% stacking gel and 15% separating gel. 100 microlitre (100 µl) of the purified enzyme was mixed with 100 µl 25 mM Tris buffer, pH 8.3 containing 4% SDS, 5% mercaptoethanol, 0.01 bromophenol blue and 20% (v/v) glycerol. The mixture was boiled for 5 min and cooled prior to loading. An aliquot (20 µl) of the denatured enzyme and the low molecular weight calibration kit standard marker proteins were loaded separately on slab gel. The standard marker proteins used were lysozyme, α-chymotrypsinogen, peroxidase, ovalbumin and bovine serum albumin. The proteins were allowed to stack at 100 V and run at 150 V for 2 hours in a Bio-Rad electrophoresis pack. The gel was fixed for an hour in a fixing solution composed of 10 % (v/v) acetic acid and 40 % (v/v) methanol and stained and destained according to the method of Weber and Osborn (1975).

Determination of kinetic parameters

The apparent kinetic parameters (K_m and V_{max}) were determined by incubating an aliquot of the enzyme with varied concentrations of CMC (6.5–11.5 mg/ml).

Effect of temperature on enzyme activity

The effect of temperature was studied by incubating aliquots of the enzyme with the substrate at temperatures ranging from 30–70 °C. The enzyme activity was then assayed.

Thermostability of the enzyme

The thermal stability of the purified enzyme was determined by assaying the enzyme at various temperatures (30–60 °C) for 60 min. The residual activities were expressed as a percentage of the activity at the optimum temperature, which was taken to be 100%. The percentage residual activity was plotted against the various temperatures.

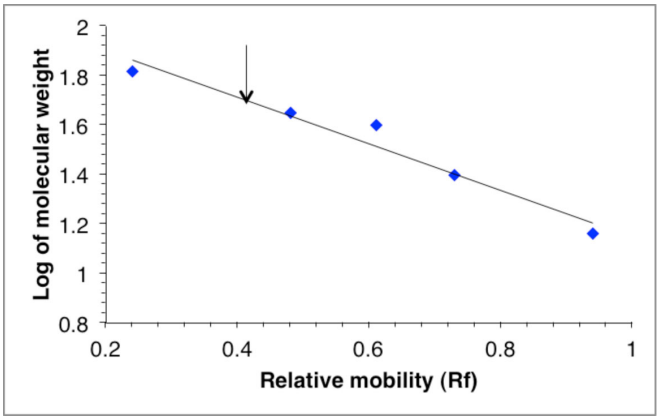

Figure 3: Calibration curve for subunit molecular weight determination on SDS-PAGE. The relative mobility (R_f) was calculated by measuring the distance moved by each of the band relative to that of the dye front. The R_f of the purified enzyme is indicated by the arrow.

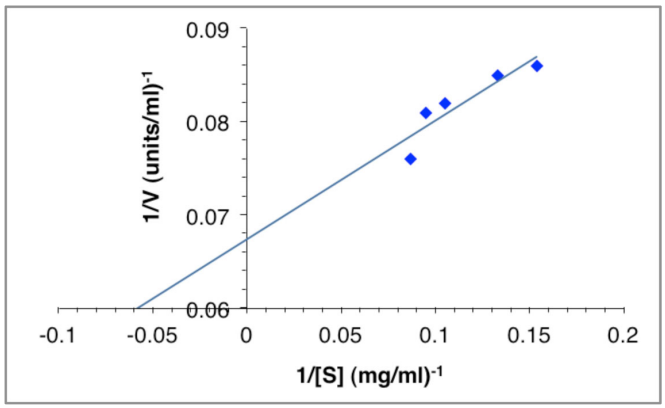

Figure 4: Lineweaver-Burk plot for the determination of kinetic parameters of the purified enzyme.

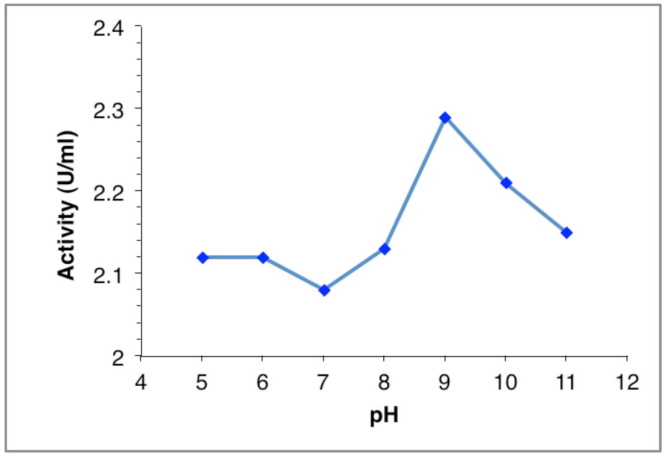

Figure 5: Effect of pH on purified cellulolytic enzyme of *Bacillus circulans* isolated from decayed wood. The enzyme showed highest activity at pH 9. The different buffers at the various pH was used to incubate the assay mixture.

Effect of pH on enzyme activity

The effect of pH on the enzyme was studied in the pH range of 5.0 to 11.0. The optimum pH was determined by carrying out the reactions 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) and 50 mM Glycine-NaOH (pH 9.0-11.0).

Effect of pH on enzyme stability

The enzyme was incubated at various pH ranging from 4.0 to 11.0 for 60 minutes. The residual CMC hydrolytic activity of each sample was then assayed. The residual activities were expressed as a percentage of the activity at the optimum pH, which was taken to be 100%. The percentage residual activity was plotted against the various pH.

RESULTS

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

When applied to the ion-exchange column, the protein did not bind to the column, and was eluted under high-salt conditions. A single peak (Figure 1) of activity was observed with a yield of 150%. The purification step increased the specific activity from 5.61 to 29.13 units/mg protein. The purification fold of the enzyme was 4.37 (Table 1).

Molecular weight determination

The calibration curve for the determination of the subunit molecular weight is shown in Figure 3. The slab gel indicated a single major band and the molecular weight was estimated to be 43 KDa.

Determination of kinetic parameters

Figure 4 shows the Lineweaver-Burk plot used in estimating the kinetic parameters of the cellulase enzyme from *B. circulans*. From this plot, the K_m for CMC was determined to be 1.061 ± 1.17 mg/ml. The maximum rate (V_{max}) for CMC hydrolysis was determined to be 13.75 ± 1.51 µg of glucose produced/ml/min.

Effect of pH on enzyme activity and its stability

The effect of pH on the activity of the enzyme is shown on Figure 5. The optimum pH for the purified cellulase was found to be 9.0. The activity of purified cellulase was relatively stable throughout the period of incubation at the different pH values (Figure 6). Indeed, less than 20% of the activity of the enzyme was lost when the enzyme was incubated in buffers on both sides of the optimum pH.

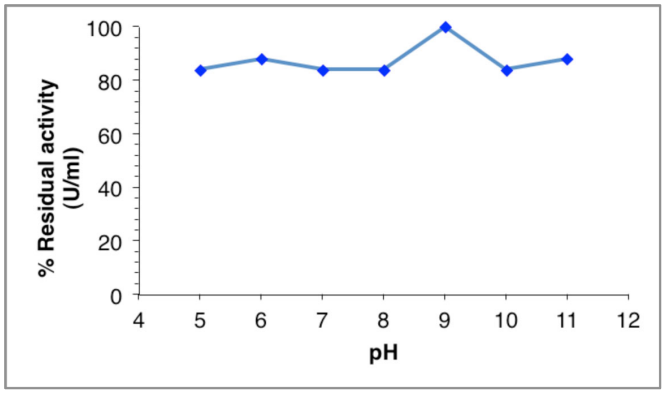

Figure 6: Effect of pH on enzyme stability. The enzyme was incubated for 60 min at various pH indicated before carrying out the assay. The activity obtained for the optimum pH was taken

Effect of temperature on enzyme activity and stability

The effect of temperature on the purified enzyme is shown in Figure 7. There was a gradual increase in the activity of the enzyme as the temperature was increased up to 50 °C, after which there was a steep decrease in the activity observed. Figure 8 shows the effect of incubating the enzyme at different temperatures prior to determining its residual activity. The enzyme was relatively stable at a lower temperature of 40 ^{0}C and a higher temperature of 60 °C such that only less than 5% of the activity was lost. However, at a much lower temperature of 30 °C almost 50% of the enzyme activity was lost in comparison with the optimum temperature.

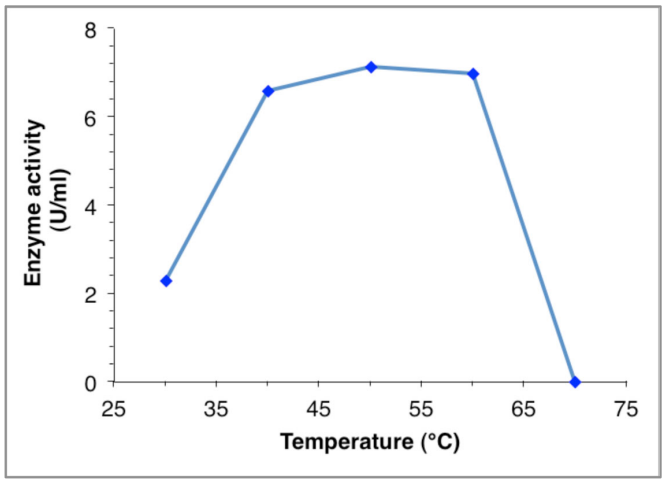

Figure 7: Effect of Temperature on enzyme activity. Purified enzyme showed highest activity at 50° C. The enzyme was added to the reaction mixture and the reaction was carried out at indicated temperatures.

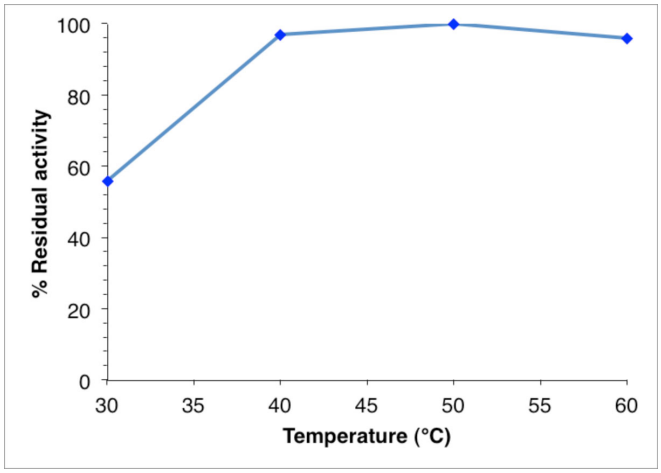

Figure 8: Effect of Temperature on enzyme stability. The enzyme was incubated for 60 min at indicated temperatures. Then samples were measured under the same conditions for activity assay.

DISCUSSION

Growth of the isolated bacterium, *Bacillus circulans,* in the culture medium led to the expression of cellulose-degrading enzyme in the culture supernatant. The organism was isolated from decayed wood sample and was purified from a range of cellulolytic bacteria that tested positive to carboxymethylcellulose agar (CMCA). Initial attempt at precipitating the crude cellulase obtained from *Bacillus circulans* with ammonium sulfate proved ineffective necessitating the use of dialysis against 50% glycerol. Purification gave just one peak with a specific activity of 29.13 µg of glucose produced/ml/min/mg protein and a purification fold of 4.37. The subunit molecular weight of the cellulolytic enzyme of 43 KDa was comparable with the reported values range of 20 to 60 KDa for cellulase from different cellulolytic organisms (Cole, 1980). Chen *et al.* (2004) reported a higher molecular weight of 94 KDa for carboxymethylcellulase (CMCase) obtained from *Sinorhizobium fredii* but a similar molecular weight of 42 KDa has been reported for *T. viridae* by Ogawa (1989).

The K_m value of this enzyme is lower than that of the enzyme from thermophilic fungus *M. thermophilia* (3 mg/ml). However, the K_m is relatively higher than that of the cellulase from *Bacillus coagulans* Co4 (0.18 mg/ml) as reported by Adeleke *et al.* (2012). The V_{max} value is higher than 3.33 units/ml reported for *Pseudomonas fluorescens* by Bakare *et al.* (2005).

The optimum temperature for the purified enzyme in this study was 50 °C. The temperature optimum was similar to that of cellulase produced by *Bacillus sp.* and that of *Bacillus* CH43 and HR68 with the values 1.5 and 1.7 mg/ml respectively (Mawazda *et al.,* 2000; Vijayaraghavan and Vincent, 2012). The enzyme was relatively stable at a lower temperature of 40 °C and a higher temperature of 60 °C such that less than 5% of the activity was lost (Figure 8). However, at a much lower temperature of 30 °C, almost 50% of the enzyme activity was lost in comparism with the optimum temperature. Also, almost all the activity was lost at a much higher temperature of 70 °C. This pattern is consistent with the general effects of temperature on enzyme activity. The initial rate of enzyme activity increases as temperature rises and then decreases abruptly (Fructon and Simmonds, 1963).

The purified enzyme had optimum activity at pH of 9.0, which is similar to what was obtained for the cellulase from *Bacillus sp.* cellulase (pH 9.0) (Fukumori *et al.,* 1985). However, several reports have shown that cellulases tend to have optimum pH of 7.0–7.5. These have been shown in the case of cellulases produced by *Sinorhizobium fredii*, *Bacillus amyoliquefaciens* and *Bacillus cogulans* Co4 (Chen *et al.,* 2004; Lee *et al.,* 2008; Adeleke *et al.,* 2012). The activity of purified cellulase was relatively stable throughout the period of incubation at the different pH values (Figure 7). The

enzyme displayed a broad pH activity profile over a range of 5–11. *Bacillus amyoliquefaciens* cellulase also showed activity in a pH range of (3.0-11.0) and retained more than 40% of the original purified cellulase activity when maintained in a pH ranging from pH 4.0 to pH 9.0 (Lee *et al.,* 2008). The purified enzyme in this study can therefore be classified as an alkaline enzyme. Enzymatic activities are usually sensitive to changes in pH values. This may be due to ionic composition of the medium, which may contribute to the stability of the enzyme.

In conclusion, this study has shown the characteristics of the cellulolytic enzyme present in wood degrading bacteria, *Bacillus circulans*. The optimum pH and temperature are essential characteristics of the cellulolytic enzyme that may make it suitable for industrial applications. Further biochemical, genetic and biophysical works are required in order to engineer the enzyme for biotechnological and industrial uses.

REFERENCES

Adeleke EO, Omafuvbe BO, Adewale IO, Bakare MK (2012) Purification and characterization of a cellulase obtained from cocoa (*Theobroma cacao*) pod-degrading *Bacillus coagulans* Co4. *Turkish Journal of Biochemistry* 37: 222–230.

Aruwajoye GS, Omoboye OO, Daramola IO, Agboola FK (2014) Extracellular cellulase production by *Bacillus circulans* isolated from decayed wood. *International journal of academic research in applied science.* 3: 1–8.

Bailey MJ, Biely P, Poutanen K (1992) Inter-Laboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology* 23: 257–270.

Bakare MK, Adewale IO, Ajayi A, Shonukan OO (2005) Purification and characterization of cellulase from the wild type and two improved mutants of Pseudomonas fluorescens. *African Journal of Biotechnology* 4: 898–904.

Beguin P (1990) Molecular biology of cellulose degradation. *Annual Review of Microbiology* 44: 219–248.

Bhat MK (2000) Cellulases and related enzymes in biotechnology. *Biotechnology Advances* 18: 355–383.

Bischoff KM, Rooney AP, Li XL, Liu S, Hughes SR (2006) Purification and characterization of a family five endoglucanase from a moderately thermophilic strain of *Bacillus licheniformis, Biotechnology Letters* 28: 1761–1765.

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72: 248–254.

Camassola M, De Bittencourt LR, Shenem NT, Andreaus J, Dillon AJP (2004) characterization of the cellulose complex of *Penicillium echinulatum Biocatalysts Biotransformation* 22: 391–396.

Chen P-J, Wei T-C, Chuang Y-T, Lin L-P (2004) Purification and characterization of carboxymethylcellulase from *Sinorhizobium fredii.* Botanical Bulletin of Academia Sinica 45: 111–118.

Cole OO (1980) Studies on the extracellular carbohydrates produced by bacteria from the gut of the termite *Amitermes evuncifer* Fellowship Thesis, Institute of Science Technology, London, p. 160.

Fructon JS, Simmond S (1963) General Biochemistry. 2nd Ed. John Wiley and Son Inc. New York, London. pp 1077.

Fukumori F, Kudo T, Horikoshi K (1985) Purification and Properties of a cellulase from Alkalophilic *Bacillus sp.* No 1139. *Journal of General Microbiology* 131: 3339–3345.

Haakana H, Miettinen-Oinonen A, Joutsjoki V, Mantyla A, Suominen P, Vahmaanpera J (2004) Cloning of cellulase genes from *Melenocarpus albomyces* and their efficient expression in *Trichoderma reesei*. *Enzyme Microbial Technology* 34: 159–167.

Hikaru N, Katsunori O, Ryota K, Tetsuya K, Tomoko O, Nobuhiro O, Naoki O, Wataru O, Hirofumi O, Yasushi M (2008) Characterization of the catalytic domains of *Trichoderma reesei* endoglucanase I, II, and III expressed in *Escherichia coli*. *Applied Microbiol. and Biotech.* 81: 681–689.

Kotchoni SO, Shonukan OO (2002) Regualtory mutations affecting the synthesis of cellulase. *World Journal of Microbiology and Biotechnology* 18: 487–491.

Lee Y-J, Kim B-K, Lee B-H, Kang I-J, Lee N-K, Chung C-H, Lee Y-C, Lee J-W (2008) Purification and characterization of cellulase produced by *Bacillus amyoliquefaciens* DL-3 utilizing rice hull. *Bioresource Technol*ogy 99: 378–386.

Lynd LR (1996) Overview and evaluation of fuel ethanol from cellulosic biomass: technology economics, the environment, and policy. *Annual Review of Energy and Environment* 21: 403–465.

Mawadza C, Hatti-Kaul Zvauya R, Mattiasson B. (2000) Purification and characterization of cellulases produced by two *Bacillus strains. Journal of Biotechnology* 83: 177–187

Miller GL (1959) Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry* 31: 426–428

Ogawa K (1989) Fractionation and purification of cellulases from *Tichoderma viridae. Bulletin of Faculty of Agriculture Myazaki University* 36: 271–280.

Roberto DS, Ellen SL, Carolina W, Mariana M, Youg KP, Eleni G (2005) Production of xylanase and CMCase on solid-

state fermentation in different residues by *Theroascus aurantiacus* MIEHE. *Brazilian Journal of Microbiology* 36: 235–241.

Vijayaraghavan P, Vincent P (2012) Purification and characterization of carboxymethylcellulose from Bacillus sp. Isolated from a Paddy field. *Polish Journal of Microbiology* 61: 51–55.

Weber K, Osborn M (1975) Protein and Sodium dodecyl sulphate: Molecular weight determination on polyacrylamide gel and related procedures. In: The proteins. Neurath, H. and Hill, R.L., eds., vol. I., pp. 179-223. Academic Press, New York.

Whitaker DR, Hamson KR, Dalta PK (1963). Improved procedure for preparation and characterization of *Myrothecium* cellulase Part 2. Purification procedures. *Canadian Journal of Biochemistry and Physiol*ogy 41: 671–696.