Full Length Research Paper

Purification and characterization of an endo-1,4-β-glucanase from *Bacillus cereus*

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A cellulase produced by *Bacillus cereus (B. cereus)*, isolated from the local soil, was purified to homogeneity from culture broth. The enzyme had a molecular mass of 51.3 kDa. The optimal cellulase activity was at pH 8 and at the temperature of 55°C. The enzyme was stable at 50°C in the pH range of 5 to 9. The enzyme was stable up to 55°C above which stability decreased rapidly after incubation for 1 h. The enzyme showed the highest activity with carboxymethyl cellulose. Slight activity was also observed towards cellulose in the filter paper and xylan. For carboxymethyl cellulose, the cellulase had a K_m of 2.12 mg/ml and V_{max} of 5.37 µg/ml·min. The activity of the enzyme was not influenced by Fe^{2+}, Zn^{2+}, urea, EDTA, Ca^{2+}, Co^{2+}, K^+ and Na^+, but it was increased by 13% in the presence of Mn^{2+}. Ba^{2+}, $C_2O_4^{2-}$, Mg^{2+} and Cu^{2+} caused a loss in the enzyme activity. The FT-IR spectrum of this carboxymethyl cellulase (CMCase) showed the characteristic cellulase peaks. Infrared spectrum of the amide I and II bands of the CMCase showed that secondary structure of the CMCase mainly consists of α-helix structures in solid phase. This enzyme (the cellulase produced by *B. cereus*) is of high value in the industry applications (such as laundry) in the future.

Key words: Cellulase, *Bacillus cereus,* purification, characterization.

INTRODUCTION

Cellulose, an abundant and renewable energy resource, can be converted into useful products such as soluble sugars, alcohols and other industrially important chemicals by enzymatic degradation (Ryu and Mandels, 1980; Mandels, 1985). Cellulolytic microorganisms are found among extremely variegated taxonomic groups. Most belong to eubacteria and fungi, such as aerobic and anaerobic bacteria (Gilkes et al., 1991), white rot fungi (Uzcategui et al., 1991), soft rot fungi (Wood et al.,1988) and anaerobic fungi (Barichievic and Calza, 1990), however, anaerobic, cellulose-degrading protozoa have also been identified in the rumen (Coleman, 1978).

Microorganisms capable of hydrolyzing this biopolymer secrete cellulase, include three types of enzymes, namely endo-1,4-β-D-glucanase [(1, 4)-β-D-glucan gluca-nohydrolase, EC3.2.1.4], exo-1,4-β-D-glucanase [(1, 4)-β-D-glucan cellobiohydrolase, EC3.2.1.91] and β-glucosidase [β-D-glucoside glucohydrolase, EC3.2.1.21] (Wood, 1985).

Cellulases have attracted much attention because of their diverse practical applications and the need to understand the mechanisms of their hydrolysis of plant carbohydrate polymers (Bhat and Bhat, 1997). The major industrial applications of cellulases are in the textile industry for "bio-polishing" of fabrics and producing stonewashed look of denims, as well as in household laundry detergents for improving fabric softness and brightness (Cavaco-Paulo, 1998). Besides, they are used in animal feeds for improving the nutritional quality and digestibility, in the processing of fruit juices and in baking. De-inking of paper is yet another emerging application

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(Tolan and Foody, 1999). An area where cellulases would have a central role is the bioconversion of renewable cellulosic biomass to commodity chemicals (Gilbert and Hazlewood, 1993; Lark et al., 1997; Gong et al., 1997; Gong et al., 1999; Himmel et al., 1999). The cellulases that are used for these industrial applications are generally from fungal sources (Tolan and Foody, 1999).

Historically, *Bacillus* species are a rich source of industrial enzymes (Horikoshi and Alkaliphiles, 1999; Priest, 1977), but the study of *Bacillus* cellulase has, until recently, lagged behind that of fungal enzymes. This is largely due to the fact that most *Bacillus* cellulase hydrolyzes synthetic carboxymethyl cellulose (CMC), but barely hydrolyzes the crystalline form of cellulose. These *Bacillus* cellulases also include the alkaline cellulases, which has the potential to be used as laundry detergent additives.

The main purpose of this study was to investigate the purification procedures and the properties of the cellulase produced by *Bacillus cereus*. The biochemical properties of the purified enzyme and its application in the industry for the cellulase produced by *B. cereus* were studied.

MATERIALS AND METHODS

Xylan (from beechwood), *p*-nitrophenyl-β-D-cellobioside (pNPC), *p*-nitrophenyl-β-D-galactopyranoside (pNPG) and mannosan were purchased from Sigma (St. Louis, MO, USA). Filter paper was from Whatman (Kent, UK). Sephadex G-75 and protein MW marker was purchased from Pharmacia (Now Pfizer, NY, USA). D(-)-salicin was obtained from Aldrich (Milwaukee, MI, USA). Other chemicals used were of the purest grade commercially available.

Microorganisms and culture conditions

B. cereus was isolated from the local soil by the laboratory of Green Chemical Technology of College of Heilongjiang Province. The minimal medium contained (per liter): Na_2HPO_4 3.0 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $CaCl_2$ 0.5 g, $MnSO_4 \cdot H_2O$ 2.5 mg, $ZnSO_4$ 2.0 mg, $CoCl_2$ 3.0 mg and $FeSO_4 \cdot 7H_2O$ 7.5 mg. Wheat bran (30 g/l) and bean cake (10 g/l) were add to supplement the minimal medium as the carbon source and nitrogen source for maximum cellulase production (unpublished data). The bacterium was grown in a 19 L stirred bioreactor (working volume was 10 L) at 30°C and pH 5 for 60 h. Oxygen concentration was kept at a minimum of 50% saturation by varying the agitation speed.

Enzyme purification

The culture broth (every time 200 ml) was centrifuged in a J2-21M centrifuge (Beckman, Palo Alto, CA) at 3000 rpm for 15 min at 4°C for 10 L culture broth. Ammonium sulfate was added to the supernatant at 90% saturation and the solution was stirred for 30 min in an ice bath. The precipitate formed was collected by centrifugation (5000 rpm, 15 min) and dissolved in a small amount of distilled water. The solution was then dialyzed against the distilled water overnight and applied to a Sephadex G-75 column (80 × 1.6 cm) that had been pre-equilibrated with 0.02 M sodium acetate (NaAc) buffer (pH 4.8). The flow rate was 12 ml/h. The fractions showing CMCase activity were pooled and concentrated by ultrafiltration through a 10 kDa cut off membrane. This purifica-

tion procedure yielded one endoglucanase active fraction and its homogeneity was determined by SDS-PAGE and HPLC.

Enzyme assay

Endo-β-1,4-glucanase activity was assayed by incubating 1 ml of the isolated enzyme (suitably diluted) with 1 ml of 1% (W/V) carboxymethyl cellulose in NaAc buffer (0.02 M, pH 4.8) at 50°C for 30 min (Ghose, 1987). The amount of reducing sugar produced was measured by the 3, 5-dinitrosalicylic (DNS) reagent method (Miller, 1959).

Hydrolytic activities of the enzyme towards salicin, mannan, xylan, *p*-nitrophenyl-β-D-cellobioside (pNPC) (Desphander, 1984) and *p*-nitrophenyl-β-D-galactopyranoside (pNPG) (Tilbeurog and Claeysseus, 1985), were determined by replacing carboxymethyl cellulose with 1% (W/V) of the respective substrate in the same buffer.

To determine the enzyme activity towards the cellulose in filter paper (FPA), 50 mg Whatman No. 1 filter paper, 1 ml of 0.02 M NaAc buffer (pH 4.8) and 1 ml of the enzyme solution (suitably diluted) were mixed in a tube, which was incubated for 1 h at 50°C by the method of Eriksson et al. (1990). The reducing sugars produced during incubation were assayed with the same method as described.

To determine the enzyme activity towards the cellulose in cotton, 50 mg absorbent cotton, 1 ml of 0.02 M NaAc buffer (pH 4.8) and 1 ml of the enzyme solution (suitably diluted) were mixed in a tube, which was incubated for 24 h at 50°C according to the method of Vallander and Eriksson (1985). The reducing sugars produced during incubation were assayed with the same method as described.

The cellulase activity towards microcrystalline cellulose was assayed by incubating 1 ml of 2% (W/V) microcrystalline cellulose diluted in 0.02 M NaAc buffer at pH 4.8 with 1 ml of enzyme solution (suitably diluted) at 50°C for 2 h according to the method of Coudray et al. (1982). The samples were centrifuged at 5000 rpm for 10 min to remove the insoluble substrates. Reducing sugar in the supernatant was assayed with the DNS method.

The protein concentration was examined by the Coomassie bright blue procedure (Bradford, 1976) using bovine serum albumin as the standard. One unit of enzyme activity is defined as the amounts of enzyme that liberated 1 μg of glucose in 1 min at 50°C, except for cotton; one unit of enzyme activity is defined as the amount of enzyme that liberated 1 μg of glucose in 1 h at 50°C. All the activity measurements were performed three times.

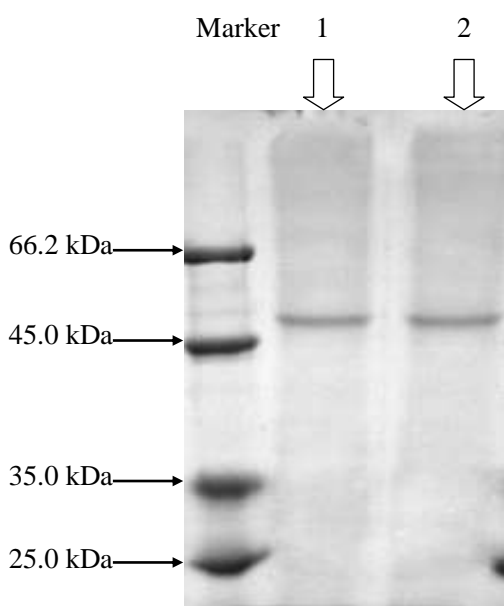### Determination of purity and molecular weight

The purity and molecular weight of the purified endoglucanase were determined by SDS-PAGE and gel filtration chromatography. Purity was assessed on an Agilent 1100 HPLC instrument with a multiple wavelength detector. Specifically, 5 μl was injected onto a ODS column (4.6 × 100 mm) and acetonitrile and water (70/30; V/V) were pre-mixed, degassed, filtered and used as the mobile phase at a flow rate of 1.0 ml/min.

Effect of pH and temperature on the activity and stability of endoglucanase

The enzyme activity was measured in the pH range of 3 to 12 with CMC as the substrate. The temperature effect was studied between 35 and 80°C in 0.02 M NaAc buffer, pH 4.8 with CMC as the substrate. For stability measurements, the enzyme was incubated at different pH or temperature for a period of time before used in the activity assay.

Table 1. Summary of the purification steps of endoglucanase from *B. cereus.*

Purification step	Total protein (mg)	Total activity (IU/ml)	Specific activity (IU/mg)	Purification fold	Yield (%)
Culture broth	181.73	6357.00	34.98	1.00	100.00
Precipitation	42.52	3688.68	86.75	2.48	58.03
SephadexG-75	7.93	1005.24	126.83	3.63	15.81

Figure 1. SDS-PAGE of purified endoglucanase. Lane 1 and 2: purified sample.

Effects of metals, surfactants and chelating agents

Metals ions, surfactants and chelating agents (10 mM) were added to endoglucanase solutions and the remaining activity was measured in 0.02 M NaAc buffer (pH 4.8) at 50°C for 30 min with CMC as the substrate.

FTIR spectrum of the endoglucanase

Fourier-transform infrared spectra (FT-IR) were obtained with a Magna-IR 560 E.S.P spectrometer. The wave number scanning range was 4000 and 600 cm^{-1}. After homogenizing, KBr disks containing the freeze-dried enzyme were made for the IR measurement.

RESULTS AND DISCUSSION

Purification of endoglucanase

After cultivating *B. cereus* in a medium containing wheat bran and bean cake as the respective carbon source and nitrogen source for 60 h, the extracellular cellulase (FPA) was detected at 16.94 IU/ml in the culture broth. The typical purifica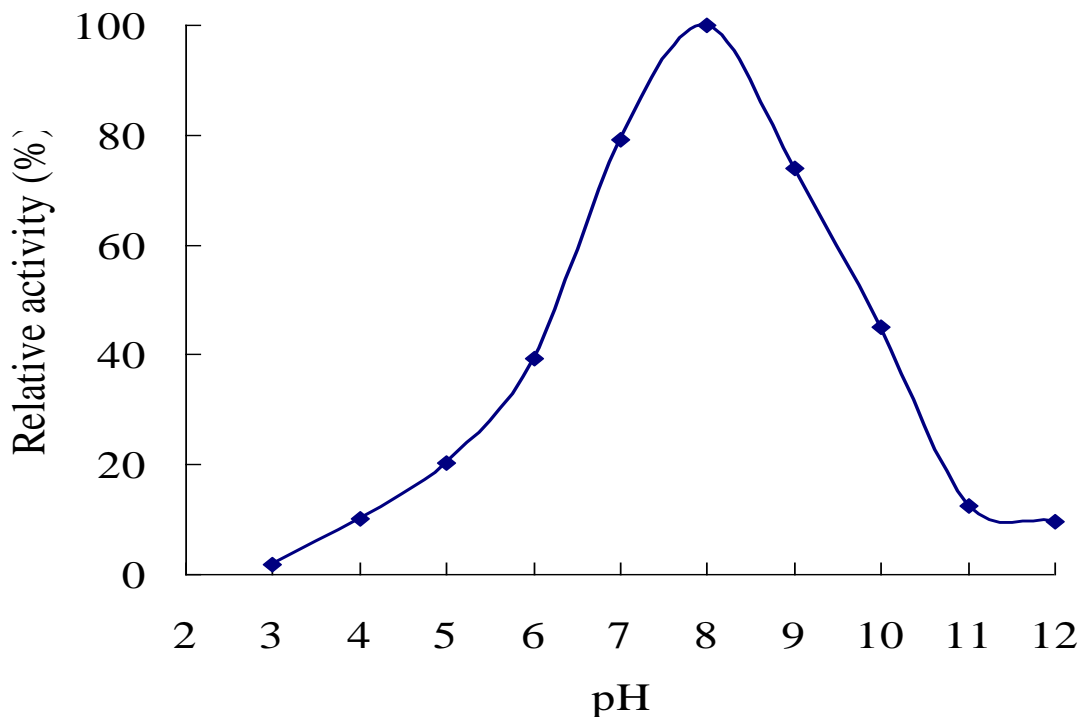tion steps of endoglucanase are summarized in Table 1. The crude extract from the culture medium underwent one-step purification by gel filtration. The purified endoglucanase proteins showed a single band on SDS-PAGE and had a molecular mass of 51.3 kDa (Figure 1), which is higher than that of the previously reported endoglucanases (23 to 43 kDa) produced by *Bacillus* sp. (Chan and Au, 1987; Ozaki and Ito, 1991; Hakamada et al., 2002; Kim and Pack, 1988), but it is lower than that produced by *Bacillus pumilus* (Christakopoulos et al., 1999). The purity of the purified enzyme was determined to be 92.83% by HPLC (Figure 2). About 15.81% of the enzyme activity, with an overall purification of 3.63 fold, was recovered (Table 1).

Substrate specificity

The enzyme was active towards the hydrolysis of CMC, but not active in the hydrolysis of microcrystalline cellulose, cotton cellulose, mannan, pNPC and pNPG (Table 2). Using CMC as the substrate at concentrations of 2 to 10 mg/ml, K_m and V_{max} of the enzyme were determined through the Lineweaver-Burk double reciprocal plot of the initial reaction rates at different substrate concentrations. The cellulase from *B. cereus* exhibited a K_m of 2.12 mg/ml for CMC at pH 4.8 and 50°C. This value (2.12 mg/ml) is significantly higher than those reported earlier for other *Bacillus* endoglucanases (0.59 to 1.60 mg/ml) (Paul et al., 1999; Kim and Pack, 1988; Sharma et al., 1990). The V_{max} of the reaction was 5.37 μg/ml·min.

The purified enzyme exhibited low levels of activity towards filter paper and xylan, indicating that the enzyme has weak activity in the hydrolysis of crystalline cellulose and hemicellulose. The substrate specificities of various cellulases is important (Crispen et al., 2000), because it is now generally accepted that the synergism between different enzymes is required to achieve cellulose hydrolysis (Wood and Garcia-Campayo, 1990). Enzymes with overlapping specificities have been reported (Gilbert and Hazlewood, 1993). The ability to degrade crystalline cellulose is commonly regarded as the synonymous with exoglucanases (Beldman et al., 1985). It has been shown that the endoglucanases from *Bacillus* sp. and fungi exhibited filter paper hydrolyzing activity in addition to the CMCase activity, because these endoglucanases also had exoglucanase activity in the same molecule (Han et al., 1995; Kim, 1995). Among the endoglucanases from

Figure 2. HPLC chromatogram of the purified endoglucanase.

Table 2. Activity of the cellulase from Bacillus cereus towards various substrates.

Substrate	Activity (IU/ml)
Whatman filter paper	0.13
Microcrystalline cellulose	0
Cotton	0
Xylan	0.05
Mannosan	0
Carboxymethyl cellulose	4.38
p-Nitrophenyl-β-D-cellobioside (pNPC)	0
p-Nitrophenyl-β-D-galactopyranoside (pNPG)	0

Bacilli, such property characteristic has been found in endoglucanases with sizes varying from 35 to 82 kDa (Beldman et al., 1985; Han et al., 1995). Therefore, it is likely that the purified endoglucanase from *B. cereus* also has weak exoglucanase activity.

Effect of pH on the activity and stability of the endoglucanase

The isolated CMCase exhibited optimal activity in the pH range of 7 to 9. The highest activity of the CMCase was observed at pH 8 (Figure 3). The enzyme was stable

between pH 5 and 9 (Figure 4) and retained 83% activity at pH 9. The stability over a broad pH range seems to be one of the characteristics of many *Bacillus* endoglucanases (Christakopoulos et al., 1999; Crispen et al., 2000; Hakamada et al., 2002).

Effect of temperature on the activity and stability of endoglucanase

The activities of the enzyme were determined at different temperatures in the range of 35 to 80°C at pH 4.8 by the method of Miller (1959). The optimal temperature of the
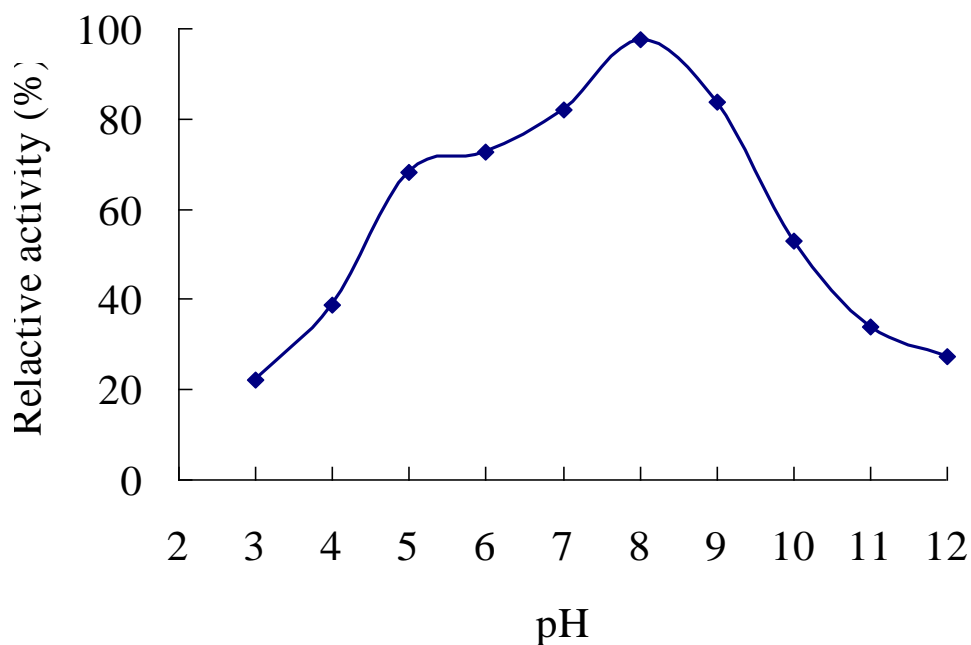
Figure 3. Effect of pH on the activity of purified CMCase. Enzyme was incubated in 0.02 M buffer for 30 min at 50°C. The buffers used were citrate (pH 3.0 to 6.0), phosphate (pH 7.0 to 8.0), borax-NaOH (pH 9.0), NaHCO$_3$-NaOH (pH 10.0) and Na$_2$HPO$_4$-NaOH solution (pH 11.0 to 12.0). The highest enzyme activity in various pH buffers was taken as 100%.
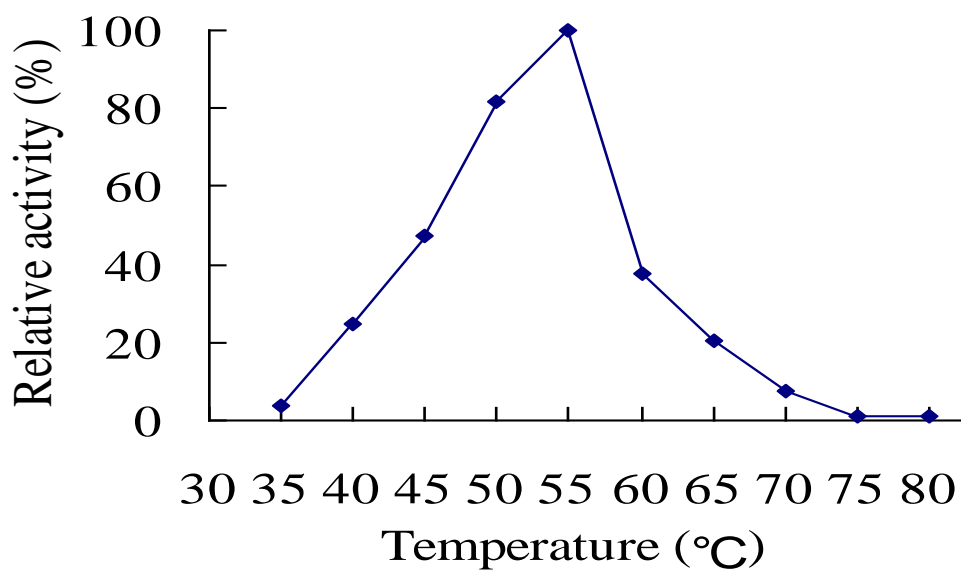

Figure 4. Effect of pH on the stability of purified CMCase. The enzyme was incubated in 0.02 M buffer for 1 h at 30°C prior to measuring the residual endoglucanase activity under standard assay conditions. The buffers used were citrate (pH 3.0 to 6.0), phosphate (pH 7.0 to 8.0), borax-NaOH (pH 9.0), NaHCO$_3$-NaOH (pH 10.0) and Na$_2$HPO$_4$-NaOH solution (pH 11.0 to 12.0). The original activity without incubation in various pH buffers was taken as 100%.

enzyme was 55°C (Figure 5). The enzyme was stable up to 55°C. A rapid decrease in stability was observed above 55°C after incubation for 1 h (Figure 6) and the enzyme was almost completely inactive at 80°C.

Effect of metal ions and other reagents on the endoglucanase
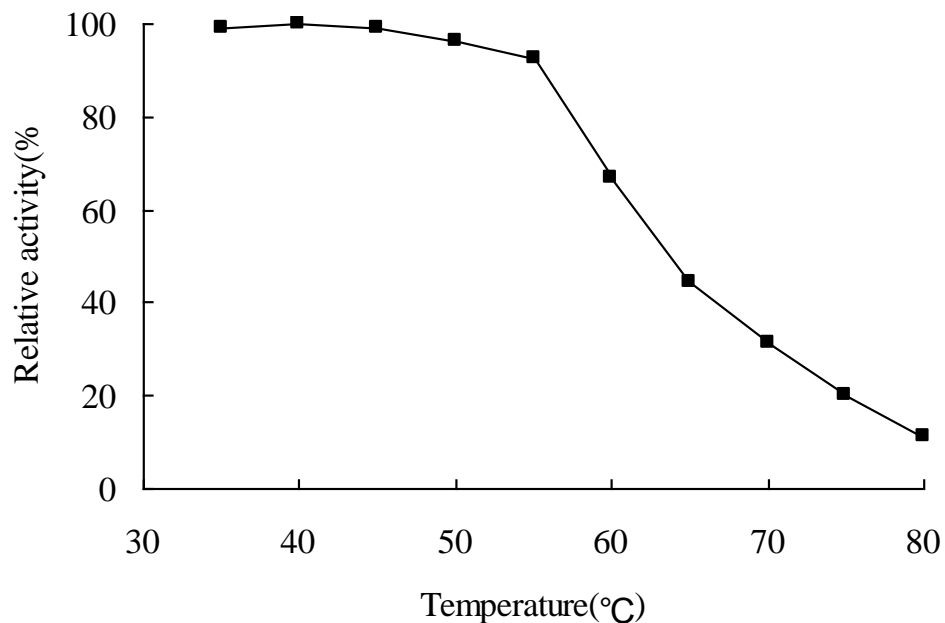
The purified enzyme and 10 mM metal ions and other

Figure 5. Effect of temperature on the activity of purified CMCase. Enzyme activity was determined by incubating the enzyme in NaAc buffer (0.02 M, pH 4.8) containing 1% (W/V) carboxymethyl cellulose at respective temperature and assaying the reducing sugars released. The 100% enzyme activity was the maximum enzyme activity in NaAc buffer (0.02 M, pH 4.8) between 35 and 80°C.
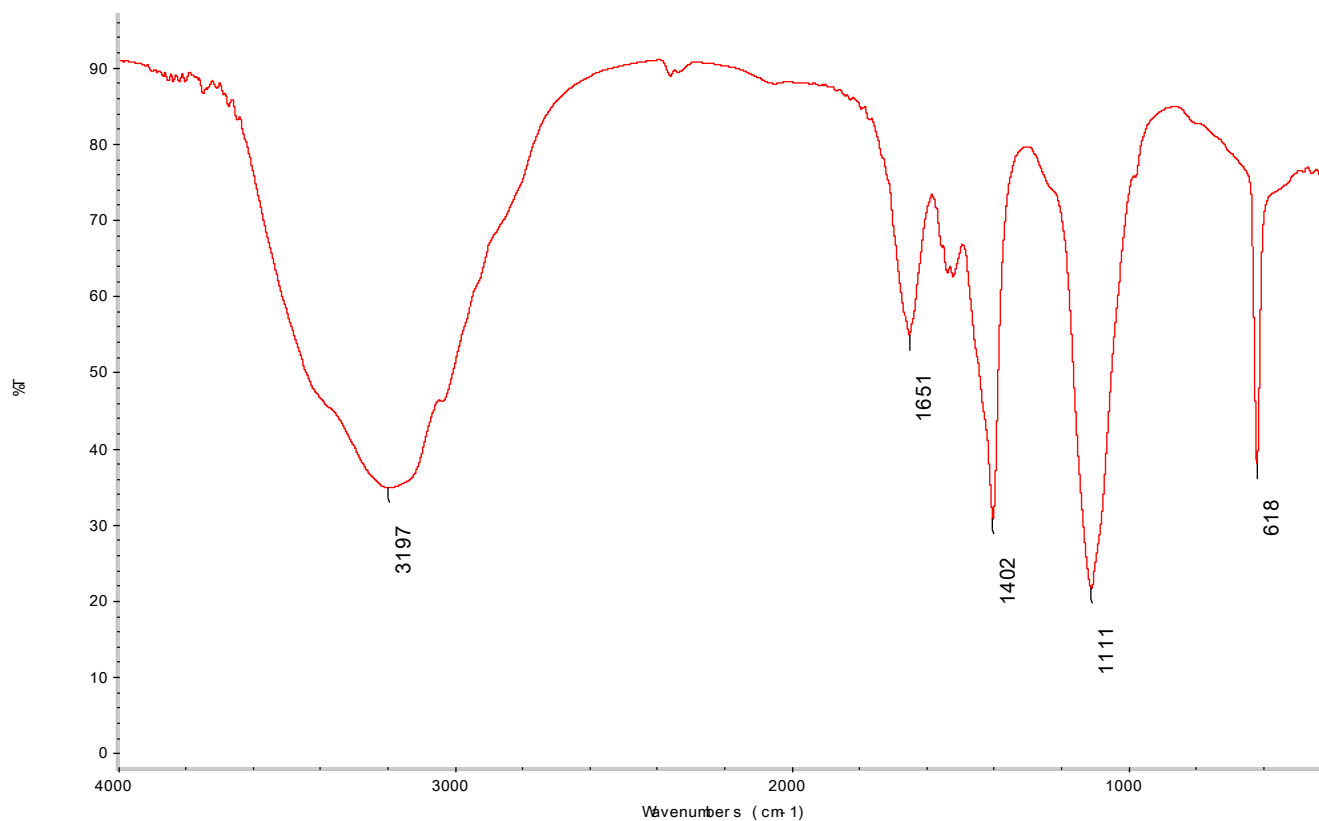

Figure 6. Effect of temperature on the stability of purified CMCase. The enzyme was incubated in 0.02 M NaAc buffer (pH 4.8) at respective temperature for 30 min, prior to measuring the endoglucanase activity under standard assay conditions. The original activity without incubation in 0.02 M NaAc buffer (pH 4.8) at 50°C was taken as 100%.

Table 3. Effect of metal ions and other reagents on the activity of purified CMCase.

Additive (10 mM)	Residual activity (%)
Original activity	100.00
Fe^{2+}	102.01
Zn^{2+}	100.67
Urea	97.82
SDS	94.97
EDTA	101.84
Mn^{2+}	113.40
Ba^{2+}	60.97
$C_2O_4^{2-}$	62.98
Mg^{2+}	81.91
Ca^{2+}	97.82
Cu^{2+}	70.85
Co^{2+}	99.16
K^+	98.49
Na^+	97.82

reagents were incubated at 50°C for 30 min and the residual endoglucanase activity was assayed with carboxymethyl cellulose as the substrate. As shown in Table 3, incubation with Fe^{2+}, Zn^{2+}, urea, EDTA, Ca^{2+}, Co^{2+}, K^+ and Na^+ did not affect the activity of the endoglucanase. Mn^{2+} ions (113.40%) caused an increase in the activity, while SDS (94.97%) caused a slight decrease. However, Ba^{2+} (60.94%), $C_2O_4^{2-}$ (62.98%), Mg^{2+} (81.91%) and Cu^{2+} (70.85%) caused significant losses in enzyme activity. The response of the activities of CH43 and HR68 enzyme to metal ions seemed to follow a pattern resembling other *Bacillus* endoglucanases, most metal ions such as K^+, Na^+, Mg^{2+}, Cu^{2+}, Ca^{2+}, Ni^{2+}, Zn^{2+} and Fe^{3+} did not influence the activity (Chan and Au, 1987). While Hg^{2+} and Mn^{2+} caused a decrease in the activity (Aa et al., 1994). The significant stability of the enzyme in the presence of surfactant (SDS) and chelating agent (EDTA) argues well for this endoglucanase to be used as an additive in laundry detergents.

FT-IR spectrum of the endoglucanase

The characteristic region of FT-IR spectrum, namely the 4000 to 1330 cm^{-1} region (Figure 7), was analyzed. The CMCase showed a group of wide absorption peaks in the range of 3600 to 3000 cm^{-1}, which was attributed to both O-H stretching vibration and N-H asymmetric vibration. The width of the absorption peak is attributed to hydrogen bonding. The presence of this broad peak indicates that the CMCase exists as a complex of individual molecules associated by hydrogen bonds in the solid phase. The absorption peak of the CMCase near 1650 cm^{-1} is attributed to the amide band I and amide band II in the main amide chain, which are mainly caused by both N-H bending vibration in plane and C=O symmetric stretching vibration. The absorption peak near 1400 cm^{-1} is amide band III, which is attributed to both C-N stretching vibration and N-H bending vibration in plane. The FT-IR spectrum of CMCase shows characteristic cellulase peaks. The FT-IR spectroscopic data of the CMCase also showed peaks in the fingerprint region 1330 and 400 cm^{-1}. The absorption peak near 1100 cm^{-1} is caused by skeleton stretching vibration of C-N of the amino acid residues with aliphatic carbon chains and the absorption peak near 600 cm^{-1} is caused by O=C-N bending vibration in plane. Amide band I displayed the characteristic α-helix absorption at 1650 to 1660 cm^{-1} and characteristic β-sheet absorption at 1630 to 1640 cm^{-1} have been reported (Li et al., 1998); amide band I displayed that characteristic α-helix absorption at 1653±4 cm^{-1} have been reported (Elliot and Amobrose, 1950). The FT-IR spectrum of the CMCase reveals that the absorption peak at 1651 cm^{-1} is the characteristic α-helix peak, which is caused by both N-H bending vibration in plane and C=O symmetry stretching vibration. We found that the characteristic peak of β-sheet was not obvious in the IR spectrum of the CMCase. Infrared spectrum of the amide I and II bands of CMCase showed that the absorption peaks at 1630 to 1640 cm^{-1} were weak, indicating the lack of the characteristic β-sheet structure. Therefore, it is likely that secondary structure of the CMCase mainly consists of α-helixes in the solid phase (Figure 8).

Conclusions

The work presented a newly isolated *B. cereus*. Purification and characterization of an endo-1,4-β-glucanase from *B. cereus* were investigated. The optimal cellulase activity was at pH 8 and 55°C. The enzyme showed the highest activity with carboxymethyl cellulose. Slight activity was also observed towards cellulose in the filter paper and xylan. The applications of this enzyme in the industry (such as laundry) (the cellulase produced by *B. cereus*) are importance in future.
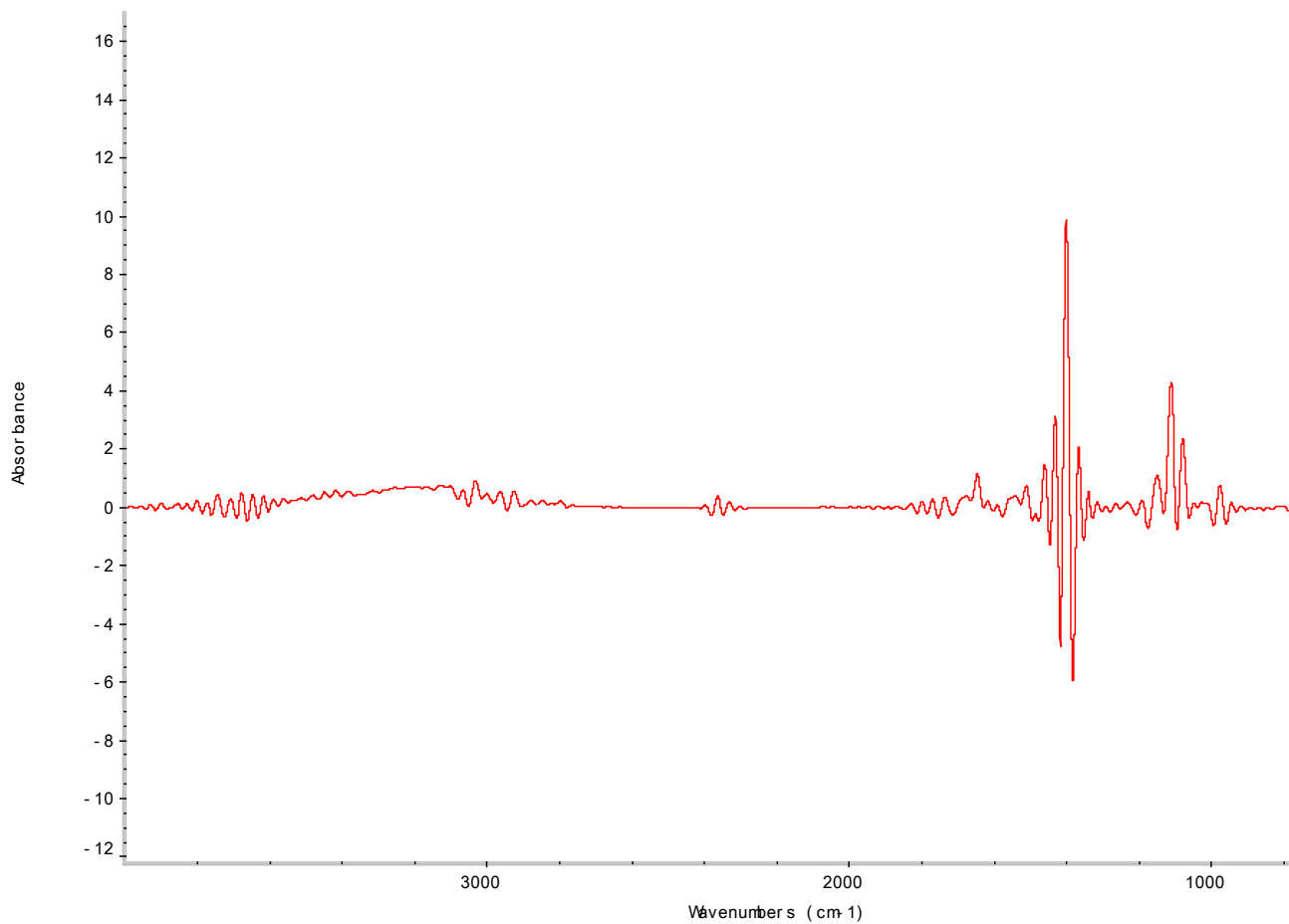
ACKNOWLEDGEMENTS

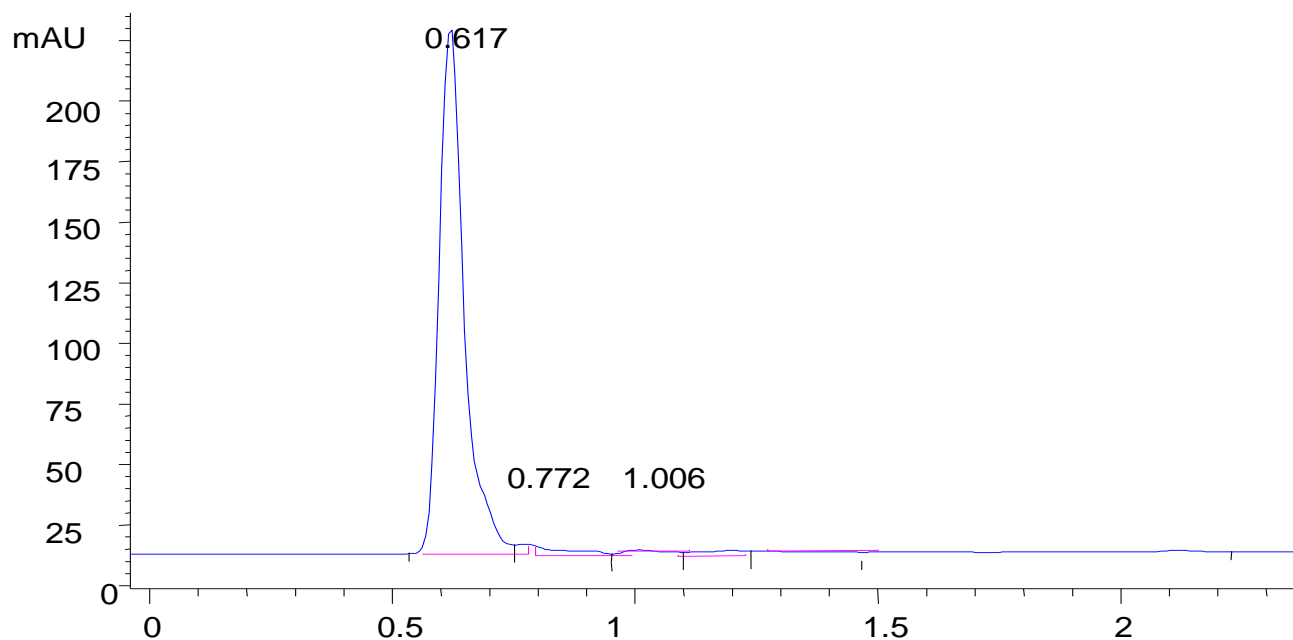**Figure 7.** FT-IR spectrum of purified CMCase.

Figure 8. Infrared spectrum showing the amide I and II bands of purified CMCase.

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