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PURIFICATION AND CHARACTERIZATION OF A CELLULASE FROM *BACILLUS SUBTILIS* YJ1

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Key words: cellulase, *Bacillus subtilis*, purification, character.

ABSTRACT

Broth incubated with *Bacillus subtilis* YJ1 at 37°C for 36 h was collected and removed bacterial cells by passing through a 0.45 μm membrane. A cellulase was purified to electrophoretical homogeneity by ammonium sulfate precipitation, Macro-Prep ion exchange and Bio-Gel P-100 chromatography. The recovery and purification fold were 9.7% and 289, respectively, after Bio-Gel P-100 chromatograph. The purified cellulase, with a molecular mass (*M*) of 32.5 kDa, had an optimal pH and temperature at 6.0 and 50-60°C, respectively. It was stable at pH 6.0-7.5 and <50°C. The purified cellulase was activated by Mn^{2+} and Co^{2+}, but inhibited by SDS, *p*-CMB, DTT, Hg^{+}, Cd^{2+}, Fe^{2+} and Fe^{3+}. According to substrate specificity, the purified cellulase has high specificity to CMC and was considered to be an endo-1,4-glucanase.

I. INTRODUCTION

It is well known that plants are the most common source of renewable carbon and energy on the earth. They annually produce about 4×10^9 tons of cellulose which is a highly stable polymer consisting of β-1,4-linked glycosyl residues, along with other polysaccharides [6]. The potential of these biological resources are possible substitutes for diminishing fossil energy resources and becoming increasingly important.

The biological degradation of cellulose has been studied for many years, and a number of cellulolytic enzymes, especially cellulases produced by fungi and bacteria, have been isolated and characterized [32]. These enzymes, which cleave the β-1,4 bond of cellulose, belong to the large family of glycosyl hydrolases. On the basis of sequence comparisons and hydrophobic cluster analysis, the catalytic domains of all glycosyl hydrolases have been classified into 63 families of homologous folds [8, 9, 10]. The catalytic domains of cellulases and related xylanases have been identified in 14 of these families. According to their mechanism of cellulose degradation, cellulases are subdivided into either non-processive cellulases (endocellulases) or processive cellulases (including different exocellulases and some new processive endocellulases) [3, 26]. Endocellulases can randomly cleave the cellulose at exposed positions and produce new reducing ends, however, the processive cellulases remain attached to the chain and release mainly cellobiose or cellotetraose units from one end of the chain [29]. These mechanisms are similar to those degradation of amylase [27]. During the degradation of cellulose, non-processive cellulases and processive cellulases have been found to work synergistically [7, 11, 12]. It has been generally accepted that effective biological hydrolysis of cellulose into glucose requires synergistic actions of three enzymes including endo-β-1,4-glucanase (EC 3.2.1.4, EG, randomly cleaving internal linkages), cellobiohydrolase (EC 3.2.1.91, CBH, specifically hydrolyzing cellobiosyl units from non-reducing ends), and β-*D*-glucosidase (EC 3.2.1.21, hydrolyzing glucosyl units from cellooligosaccharides) [25]. Currently, cellulase is commonly used in many industrial applications, especially in animal feed, textile, waste water, brewing and wine-making [4, 18]. With the shortage of petroleum fuels, increase of greenhouse gases and air pollution due to the incomplete combustion of fossil fuel, there has been increasing worldwide interest in the production of bioethanol from lignocellulosic biomass [33]. To utilize these materials and to avoid waste pollution, one of the most important approaches is to find applicable cellulase and hemicellulase to hydrolyze the lignocellulosic biomass. The cost of related enzymes has been considered to be critical to the success of bioethanol industry. Accordingly, a highly stable, efficient and also economic cellulase or hemicellulase for the bio-fuel industry has been attracted many scientists concerns.

Among bacteria, *Bacillus* species can produce numbers of extracellular polysaccharide hydrolyzing enzyme [5]. However, these carboxymethylcellulase (endoglucanase) can not hydrolyze crystalline cellulose [24]. These polysaccharide hydrolyzing enzymes also include alkaline cellulase with high potential as laundry detergent additives. There are, however, some studies on *Bacillus* and fungal endoglucanases which had shown detectable activity on microcrystalline cellulose [22]. A cellulase producing *Bacillus* strains have isolated from fermented food and optimized the culture conditions for en-

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zyme production [15]. In this study, the cellulase was purified and characterized from *Bacillus subtilis* YJ1, which were found to be taxonomically similar to *Bacillus* strains [15].

II. MATERIALS AND METHODS

1. Materials

Rice bran from a local rice husking factory in southern Taiwan was pre-treated by the milling machine. All media for bacteria cultivation were from Difco Laboratories (BD Co., MD, USA). All chemicals including metals, inhibitors, reducing agents and substrates were the products of Sigma-Aldrich Inc. (MO, USA). Marco-Prep ion exchanger and Bio-Gel P-100 were obtained from Bio-Rad (CA, USA). Low molecular weight marker was obtained from Amersham Biosciences (GE Healthcare BioSciences Corp., MA, USA).

2. Microorganism and Culture

The *Bacillus subtilis* YJ1, isolated from fermented foods in our laboratory, was cultured in a medium containing 2% rice bran, 1% casein, 1% soytone and 0.1% sodium chloride. After 36 h incubation at 37°C with shaking at 150 rpm, the cultured broth was centrifuged at 8000 × *g* for 20 min (High speed refrigerated centrifuge, CR21G, Hitachi, Japan) and passed through a 0.45 μm membrane (Gelman Sciences, MI, USA) to remove the cells. The resulted samples were used for the further cellulase purification.

3. Enzyme Activity Assay

1) Endo-β-1,4-glucanase

Endo-β-1,4-glucanase activity was determined by incubation of 900 μL of 1% CMC in 20 mM phosphate buffer (pH 7.0) with 100 μL of appropriate concentration of enzyme at 50°C. After 30 min reaction, 1 mL of dinitrosalicylic acid (DNS) was added and boiled in a water bath for 5 min to stop the reaction. The resulted samples were then cooled to room temperature and measured the absorbance at 540 nm (A_{540}). One unit of endo-β-1,4-glucanase activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 μg of glucose within 1 min reaction at 50°C [20].

2) Avicelase

Avicelase activity was determined by incubation of 900 μL of 1% avicel in 20 mM phosphate buffer (pH 7.0) with 100 μL of appropriate concentration of enzyme at 50°C. After 30 min reaction, the activity was measured according to Miller [20]. One unit of avicelase activity was defined as the amount of enzyme that could hydrolyze avicel and release 1 μg of glucose within 1 min reaction at 50°C [1].

3) β-glucosidase

β-glucosidase activity was determined by the hydrolysis of *p*-Nitrophenol-β-D-glucopyranoside (*p*-NPG) in 100 mM acetate buffer (pH 5.0) at 37°C for 20 min. The reaction was terminated by the addition of 1 mL of 0.25 M Na_2CO_3. The absorbance at 405 nm (A_{405}) was measured. One unit of β-glucosidase activity was defined as the amount of enzyme that could hydrolyze *p*-NPG and release 1 μmol of *p*-nitrophenol within 1 min reaction at 37°C [16].

4. Determination of Protein Concentration

Protein concentration was determined by Lowry's method, using bovine serum albumin as a standard [16].

5. Purification of Cellulase

After the broth, incubated with *Bacillus subtilis* YJ1, was centrifuged at 8,000 × *g* for 20 min and passed through a 0.45 μm membrane to remove cells, the crude cellulase was precipitated by 60-80% saturation of ammonium sulfate. The precipitate was centrifuged at 8,000 × *g* for 15 min and dialyzed against 20 mM Tris-HCl buffer (pH 8.0) overnight. The resulted crude enzymes were eluted by Macro-Prep ion exchange chromatography (2.6 × 20 cm) with 20 mM Tris-HCl buffer (pH 8.0) at a flow rate of 30 mL/h. Fractions with cellulase activity were collected and concentrated by ultrafiltration with a membrane (MW cutoff: 5 kDa). The concentrated crude enzymes were then chromatographed on a gel-filtration of Bio-Gel P-100 column (1.6 × 100 cm) with 20 mM Tris-HCl buffer (pH 8.0) at a flow rate of 9 mL/h. Fractions with cellulase activity were collected and subjected to enzyme activity assay and electrophoresis (SDS-PAGE).

6. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Electrophoresis was performed mainly according to the method described by Laemmli [14]. To the purified enzyme, equal volume of sample buffer (0.05% bromophenol blue, 5% β-mercaptoethanol, 10% glycerol, and 2% SDS in 0.25M Tris–HCl buffer; pH 6.8) was added and then boiled at 100°C for 2 min. The resulted samples were then subjected to SDS-PAGE (resolving gel: 12.5%; stacking gel: 4%) by using a Mini-Protein II system (Bio-Rad, USA). Electrophoresis was performed at room temperature for 1.5 h with a 100 Volt.

7. Molecular Mass

The molecular mass (*M*) of purified cellulase was determined using SDS-PAGE. Phosphorylase B (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20.1 kDa) and α-lactalbumin (14.4 kDa) were used as markers.

8. Influence of Temperature and pH

1) Temperature Optimum

To 900 μL of 1% CMC in 20 mM phosphate buffer (pH 7.0), 100 μL of appropriate concentration of enzyme were added and incubated at various temperatures (0, 20, 30, 40, 50, 60, 70, 80, 90°C) for 30 min. The activity was then measured according to Miller [20].

2) Temperature Stability

Purified cellulase in 20 mM phosphate buffer (pH 7.0) was incubated at various temperatures (0, 20, 30, 40, 50, 60, 70, 80, 90°C) for 30 min. The residual activity was measured according to Miller [20].

3) pH Optimum

To 900 μL of 1.0% CMC in various pHs (pH 3.0-7.0: 50 mM citrate buffer; pH 6.0-9.0: 50 mM phosphate buffer; pH 8-11: 50 mM carbonate buffer), 100 μL of cellulase was added and measured the activity according to Miller [20].

4) pH Stability

Purified cellulase was incubated in buffers with various pHs (pH 3.0-7.0: 50 mM citrate buffer; pH 6.0-9.0: 50 mM phosphate buffer; pH 8-11: 50 mM carbonate buffer) at 25°C for 30 min. The residual activity was measured according to Miller [20].

9. Effecf Metals and Inhibitors

Purified cellulase in 20 mM Tris-HCl buffer (pH 7.0) with various metals (Ag^+, Hg^+, K^+, Li^+, NH_4^+, Sr^+, Ba^{2+}, Ca^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Fe^{3+}) or inhibitors (β-mercaptoethnol, β-Me; dithiothreitol, DTT; ethylenediamine-tetraacetic acid, EDTA; iodoacetic acid, IAA; *p*-chloromercuribenzoate, *p*-CMB; sodium dodecyl sulfate, SDS; urea) were incubated at 25°C for 30 min. The final concentrations of metals and inhibitors were 1.0, 5.0, 10.0 mM. After incubation, the residual activity was measured according to Miller [20].

10. Substrate Specificity

The hydrolytic ability against 1% CMC, avicel, cotton, filter paper, xylan and *p*-nitrophenol-β-D-glucopyranoside (*p*-NPG) in 20 mM phosphate buffer (pH 7.0) were determined to evaluate the substrate specificity of purified cellulase.

III. RESULTS AND DISCUSSION

1. Enzyme Production

Bacillus subtilis YJ1 was cultivated in the medium and only produced the Endo-β-1,4-glucanase at 37°C for 36 h. However, the activities of other 2 enzymes, Avicelase and β-glucosidase were very low during cultivation, they did not show in the manuscript (data not shown). After removing the cells, the sample was subjected to the further Amicon ultrafiltration, ion exchange and gel filtration chromatography. Most of the popular ion exchangers and gel filtration beads could not be used, since they could be easily hydrolyzed by the crude and purified cellulases in this study. Accordingly, the crude enzymes were firstly precipitated by 60-80% saturation of ammonium sulfate and further purified by Micro-Prep ion exchange (made by polyacrylamide) equilibrated with buffer A and eluted with 0.0~1.0 M NaCl in buffer A. Most of contaminant was excluded after Micro-Prep ion exchange

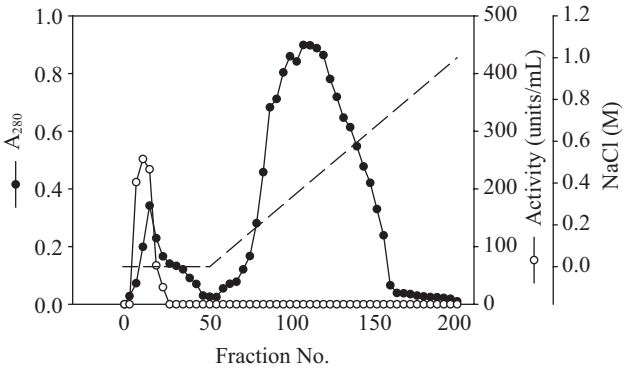

Fig. 1. Chromatogram of cellulase from *Bacillus subtilis* YJ1 on Macro-Prep ion exchange. [column: 2.6 × 20 cm, equilibrated with 20 mM Tris-HCl buffer (pH 8.0, buffer A) and eluted with 0.0~1.0 NaCl in buffer A].

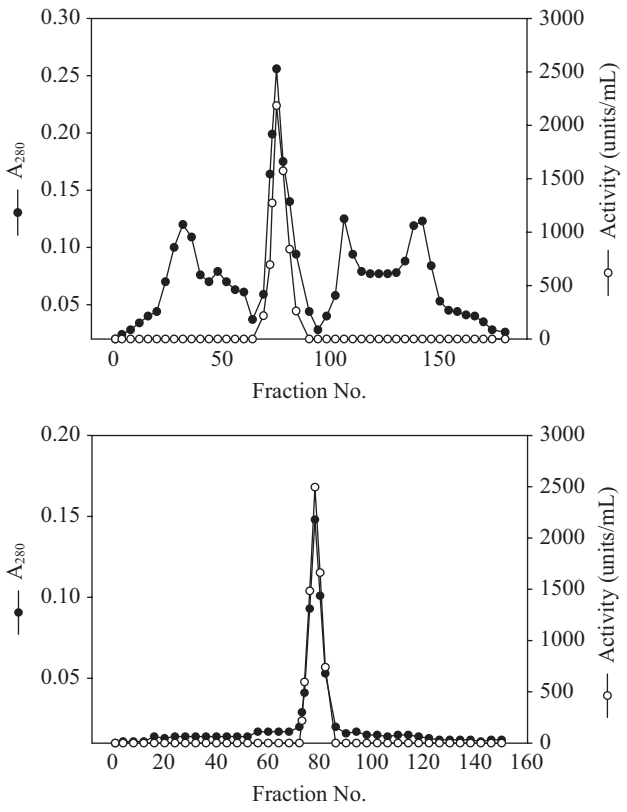

Fig. 2. Chromatogram of cellulase from *Bacillus subtilis* YJ1 on Bio-Gel P-100. [column: 1.6 × 100 cm, equilibrated with 20 mM Tris-HCl buffer (pH 8.0); upper: 1st Bio-Gel P-100 chromatography; bottom: 2nd Bio-Gel P-100 chromatography].

chromatography (Fig. 1). Fractions with cellulase activity was collected and further purified by being chromatographed on Bio-Gel P-100 twice (Fig. 2). As indicated on this figure, the contaminants were almost removed after the 1st Bio-Gel P-100 chromatography. The cellulase was purified to electrophoretical homogeneity after 2nd size exclusion of Bio-Gel P-100 chromatography (Fig. 3). The recovery and purification fold were 9.7% and 289, respectively (Table 1).

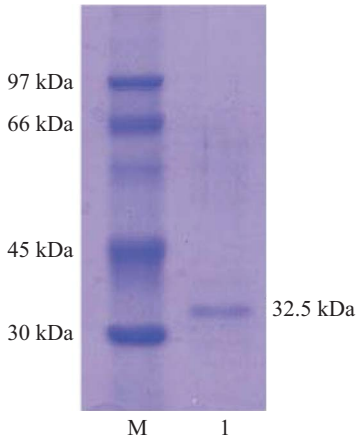

Fig. 3. SDS-PAGE of purified cellulase from *Bacillus subtilis* YJ1. *Note:* The enzyme was electrophoretically run at pH 8.6 on a 10% acrylamide gel and stained with Coomassie Brilliant Blue R-250; M: protein markers [phosphorylase B (97 kDa), albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa)]; 1: purified cellulose.

Table 1. Summary of the purification of cellulase from *Bacillus subtilis* YJ1.

Procedure	Total activity	Total protein	Specific activity	Purification	Yield
	(U)	(mg)	(U/mg)	(fold)	(%)
Crude enzyme	150296.0	6211.0	24.2	1.0	100.0
Ammonium sulfate precipitation (60-80%)	64180.0	52.6	1,220.0	50.4	42.7
Macro-Prep Ion Exchange chromatography	30907.0	16.8	1,842.0	76.1	20.6
Bio-Gel P-100 (2^{nd})	14633.0	2.1	7,001.0	289.3	9.7

2. Molecular Characteristics of Purified Cellulase

The molecular mass (*M*) of purified cellulase was 32.5 kDa, determined by SDS-PAGE and 2nd Bio-Gel P-100 chromatography (Figs. 2 and 3). This *M* was similar with some other low *M* endo-glucanases (25-45 kDa) obtained from *Bacillus* spp. such as 27 kDa from *Mucor circinelloides* [28], 35 kDa from *Chalara paradoxa* [17], 40 kDa from *Bacillus* strains [19], and 40 kDa from *Aspergillus niger* [2].

3. Effect of Temperature on Purified Cellulase Activity and Stability

The optimal temperature for the purified cellulase was found to be 60°C at pH 7.0 (Fig. 4). The purified cellulase was stable at temperatures under 50°C, while the temperature for I_{50} was 60°C (Fig. 4). The optimum temperature of purified cellulase was lower than some of other *Bacillus* strains [65°C (CH43) and 70°C (RH68)] [19], but higher than *Mucor circinelloides* (55°C) [28]. The thermal stability of purified cellulase (0-50°C)

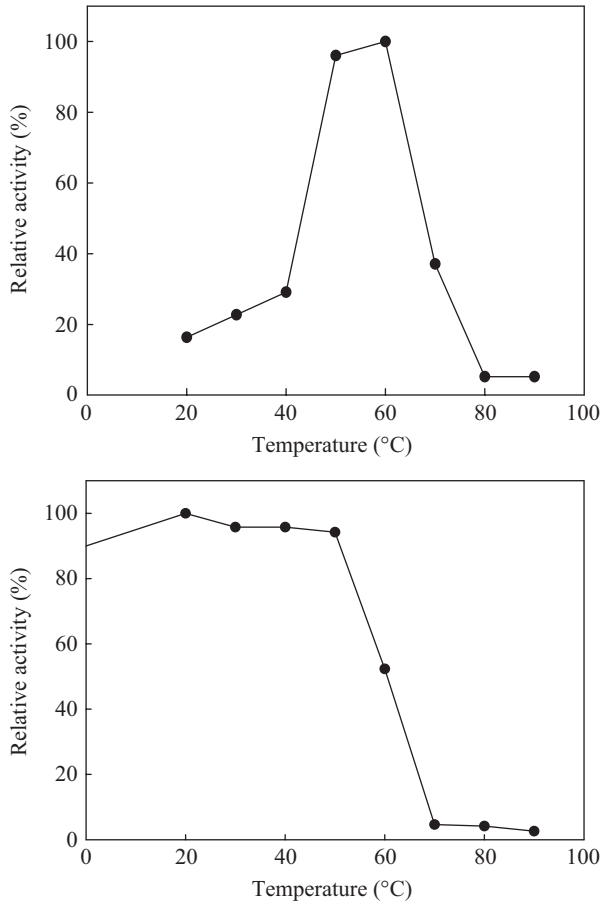

Fig. 4. Effect of temperature on purified cellulase from *Bacillus subtilis* YJ1. (Upper: Optimal Temperature; Bottom: Thermostability).

was similar to those from other *Bacillus* strains 0-50°C [19], but lower than that from *Mucor circinelloides* (0-70°C) [28].

4. Effect of pH on Purified Cellulase Activity and Stability

Bacillus subtilis YJ1 cellulase exhibited highest activity at pH 6.0-6.5 and was stable between pH 6.5 and 7.5 (Fig. 5). According to some previous studies, the optimal pH was 5.0-6.5 for those from *Bacillus* strains [19], 6.0-7.0 from *Aspergillus niger* [2], and 5.0-7.0 from *Lysobacter* sp. [23]. The purified cellulase was stable at pH 6.5-7.5, which was higher than those from *Mucor circinelloides*, 4.0-7.0 [28] and *Bacillus circulans*, 4.5-7.0 [13].

5. Effect of Inhibitors and Other Reagents

As indicated in Table 2, 10 mM SDS, IAA, PCMB inhibit about 95, 67, 44% of the enzyme activity, while 10 mM β-Me and DTT activated its activity. Since the IAA and PCMB can bind with the SH group with different degree interaction and subsequently inhibit the activity. However, the β-Me and DTT can reduce the disulfide bonds and renature their activity, if the oxidation or aggregation of these enzyme proteins occurs during purification and storage. These phenomena suggested that the active site of the enzyme contains -SH group [30].

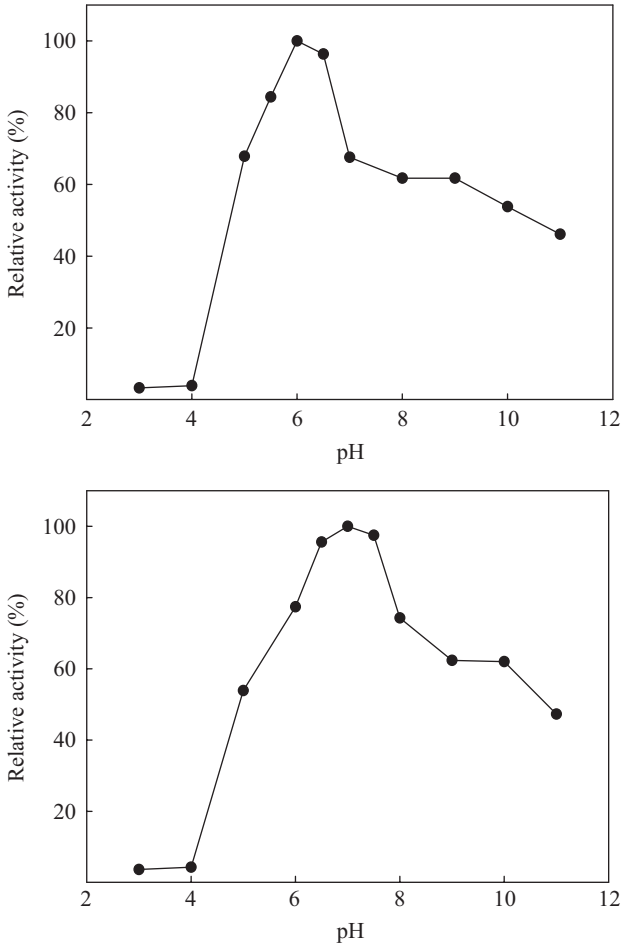

Fig. 5. Effect of pH on purified cellulase from *Bacillus subtilis* YJ1. (Upper: Optimal pH; Bottom: pH stability; 50 mM citrate buffer: pH 3.0-7.0, 50 mM phosphate buffer: pH 6.0-9.0, and 50 mM carbonate buffer: pH 8-11).

Table 2. Effect of various inhibitors and reductants on cellulase from *Bacillus subtilis* YJ1.

Inhibitors and reductants	Relative activity (%)		
	Concentration (mM)		
	1	5	10
None	100.0	100.0	100.0
Sodium dodecyl sulfate (SDS)	100.0	71.0	5.0
Urea	100.0	106.0	102.0
Ethylenediaminetetraacetic acid (EDTA)	100.0	101.0	101.0
Iodoacetic acid (IAA)	100.0	86.0	33.0
p-chloromercuribenzoate (*p*-CMB)	100.0	98.0	56.0
Sodium azide (NaN_3)	100.0	96.0	97.0
Dithiothreitol (DTT)	100.0	113.0	123.0
Cysteine	100.0	103.0	106.0
β-Mercaptoethnol (β-Me)	100.0	122.0	140.0
Glutathione (GSH)	100.0	106.0	114.0

Table 3. Effect of metal ions on the cellulase from *Bacillus subtilis* YJ1.

Metal ions	Relative activity (%)		
	Concentration (mM)		
	1	5	10
None	100.0	100.0	100.0
Na^+	99.0	101.0	101.0
K^+	114.0	119.0	123.0
Li^+	105.0	107.0	113.0
Sr^+	109.0	109.0	110.0
Ag^+	107.0	108.0	108.0
NH_4^+	111.0	108.0	110.0
Hg^{2+}	6.0	5.0	7.0
Ca^{2+}	91.0	106.0	107.0
Mn^{2+}	160.0	184.0	240.0
Mg^{2+}	104.0	104.0	106.0
Co^{2+}	120.0	151.0	158.0
Cd^{2+}	51.0	31.0	44.0
Fe^{2+}	106.0	52.0	26.0
Fe^{3+}	55.0	55.0	6.0

The counter ion of these metals is chloride

Table 4. Substrate specificity of the purified cellulase.

Substrates	Activity (%)
Carboxy methyl cellulose (CMC)	100
Avicel	34
Cotton	8
Filter paper	5
Xylan	14
p-nitrophenol-β-D-glucopyranoside (*p*-NPG)	0

6. Effect of Metal Ions

As shown in Table 3, most metal ions such as K^+, Na^+, NH_4^+ did not affect the activity, while Co^{2+} and Mn^{2+} greatly activated the purified cellulase. However, Cd^{2+}, Fe^{2+} and Hg^{2+} inactivated the purified cellulase activity. This phenomenon further confirmed that the active site of the purified cellulase contained SH group. These results are almost similar to that from *Catharanthus roseus* [31]. According to the studies by Saha [28], Lucas *et al*. [17] and Murashima [21], K^+, Na^+, NH_4^+ did not affect the cellulase from *Rhizopus oryzae* [21], while Co^{2+} and Mn^{2+} activated that from *Mucor circinelloides* [28] and *Chalara paradoxa* [17], respectively.

7. Substrate Specificity

The purified enzyme showed highest activity against CMC (Table 4). There was almost no hydrolysis ability against crystalline substrates of avicel, cotton fiber, filter paper, xylan or *p*-NPG. It was, accordingly, considered the purified cellulase to be an endo-1,4-glucanase.

IV. CONCLUSION

From the results obtained in this study, this novel *Bacillus subtilis* YJ1 could utilize natural wastes such as rice bran as substrate for growth and produce high levels of cellulase. Hence, this cellulase was purified to electrophoretical homogeneity by ammonium sulfate precipitation, Macro-Prep ion exchanger and Bio-Gel P-100 chromatography. The produced cellulase was considered to be endoglucanase and highly benefits to the industrial application. The further optimization on the commercial scale production for cellulase using this strain is on-going currently.

REFERENCES

1. Acebal, C., Castillon, M. P., Estrada, P., Mata, I., Costa, E., Aguado, J., Romero, D., and Jimenez, F., "Enhanced cellulase production from *Trichoderma reesei* Qm-9414 on physically treated wheat straw," *Applied Microbiology and Biotechnology*, Vol. 24, No. 3, pp. 218-223 (1986).
2. Akiba, S., Kimura, Y., Yamamoto, K., and Kumagai, H., "Purification and characterization of a protease-resistant cellulase from *Aspergillus niger*," *Journal of Fermentation and Bioengineering*, Vol. 79, No. 2, pp. 125-130 (1995).
3. Barr, B. K., Hsieh, Y. L., Ganem, B., and Wilson, D. B., "Identification of two functionally different classes of exocellulases," *Biochemistry*, Vol. 35, No. 2, pp. 586-592 (1996).
4. Beguin, P. and Aubert, J. P., "The biological degradation of cellulose," *FEMS Microbiology Reviews*, Vol. 13, No. 1, pp. 25-58 (1994).
5. Bhat, M. and Bhat, S., "Cellulase degrading enzymes and their potential industrial applications," *Biotechnology Advances*, Vol. 15, No. 3-4, pp. 583-620 (1997).
6. Coughlan, M., "Cellulose degradation by Fungi," in *Microbial Enzymes and Biotechnology*, Fogarty, W. and Kely, C. (Eds.), Elsevier Applied Science: London, UK, 1990; pp 1-36.
7. Creuzet, N., Berenger, J. F., and Frixon, C., "Characterization of exoglucanase and synergistic hydrolysis of cellulose in *Clostridium stercorarium*," *FEMS Microbiology Letters*, Vol. 20, No. 3, pp. 347-350 (1983).
8. Henrissat, B., "A new cellulase family," *Molecular Microbiology*, Vol. 23, No. 4, pp. 848-849 (1997).
9. Henrissat, B. and Bairoch, A., "New families in the classification of glycosyl hydrolases based on amino acid sequence similarities," *Biochemical Journal*, Vol. 293, No. Pt 3, pp. 781-788 (1993).
10. Henrissat, B. and Bairoch, A., "Updating the sequence-based classification of glycosyl hydrolases," *Biochemical Journal*, Vol. 316, No. Pt 2, pp. 695-696 (1996).
11. Henrissat, B., Driguez, H., Viet, C., and Schulein, M., "Synergism of cellulases from *Trichoderma reesei* in the degradation of cellulose," *Biotechnology*, Vol. 3, No. 8, pp. 722-726 (1985).
12. Irwin, D. C., Spezio, M., Walker, L. P., and Wilson, D. B., "Activity studies of eight purified cellulases: Specificity, synergism, and binding domain effects," *Biotechnology and Bioengineering*, Vol. 42, No. 8, pp. 1002-1013 (1993).
13. Kim, C. H., "Characterization and substrate specificity of an endo-beta-1,4-D-glucanase I (Avicelase I) from an extracellular multienzyme complex of *Bacillus circulans*," *Applied and Environmental Microbiology*, Vol. 61, No. 3, pp. 959-965 (1995).
14. Laemmli, U. K., "Cleavage of structural proteins during the assembly of the head of bacteriophage T4," *Nature*, Vol. 227, No. 5259, pp. 680-685 (1970).
15. Li, X., Lin, W., Gao, P., and Chen, F., "Endoglucanase S, a novel endocellulase exhibiting exocellulase activity from a newly isolated *Streptomyces* sp. LX.," *Journal of Applied Microbiology*, Vol. 85, No. 2, pp. 347-356 (1998).
16. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., "Protein measurement with the Folin phenol reagent," *Journal of Biological Chemistry*, Vol. 193, No. 1, pp. 265-275 (1951).
17. Lucas, R., Robles, A., Garcia, M. T., Alvarez De Cienfuegos, G., and Galvez, A., "Production, purification, and properties of an endoglucanase produced by the hyphomycete *Chalara* (syn. *Thielaviopsis*) paradoxa CH32," *Journal of Agricultural and Food Chemistry*, Vol. 49, No. 1, pp. 79-85 (2001).
18. Mandels, M., "Applications of cellulases," *Biochemical Society Transactions*, Vol. 13, No. 2, pp. 414-416 (1985).
19. Mawadza, C., Hatti-Kaul, R., Zvauya, R., and Mattiasson, B., "Purification and characterization of cellulases produced by two *Bacillus* strains," *Journal of Biotechnology*, Vol. 83, No. 3, pp. 177-187 (2000).
20. Miller, G. L., "Use of dinitrosalicylic acid reagent for determination of reducing sugar," *Analytical Chemistry*, Vol. 31, No. 3, pp. 426-428 (1959).
21. Murashima, K., Nishimura, T., Nakamura, Y., Koga, J., Moriya, T., Sumida, N., Yaguchi, T., and Kono, T., "Purification and characterization of new endo-1,4-beta-D-glucanases from *Rhizopus oryzae*," *Enzyme and Microbial Technology*, Vol. 30, No. 3, pp. 319-326 (2002).
22. Ogawa, K., Toyama, D., and Fujii, N., "Microcrystalline cellulose-hydrolyzing cellulase (endo-cellulase) from *Trichoderma reesei* CDU-11," *Journal of General and Applied Microbiology*, Vol. 37, No. 3, pp. 249-259 (1991).
23. Ogura, J., Toyoda, A., Kurosawa, T., Chong, A. L., Chohnan, S., and Masaki, T., "Purification, characterization, and gene analysis of cellulase (Cel8A) from *Lysobacter* sp. IB-9374," *Bioscience, Biotechnology, and Biochemistry*, Vol. 70, No. 10, pp. 2420-2428 (2006).
24. Ozaki, K. and Ito, S., "Purification and properties of an acid endo-1,4-beta-glucanase from *Bacillus* sp. KSM-330," *Journal of General Microbiology*, Vol. 137, No. 1, pp. 41-48 (1991).
25. Perez, J., Munoz-Dorado, J., de la Rubia, T., and Martinez, J., "Biodegradation and biological treatments of cellulose, hemicellulose and lignin: an overview," *International Microbiology*, Vol. 5, No. 2, pp. 53-63 (2002).
26. Reverbel-Leroy, C., Pages, S., Belaich, A., Belaich, J. P., and Tardif, C., "The processive endocellulase CelF, a major component of the *Clostridium cellulolyticum* cellulosome: purification and characterization of the recombinant form," *Journal of Bacteriology*, Vol. 179, No. 1, pp. 46-52 (1997).
27. Robyt, J. F. and French, D., "Multiple attach hypothesis of alpha-amylase action: action of porcine pancreatic, human salivary, and *Aspergillus oryzae* alpha-amylases," *Archives of Biochemistry and Biophysics*, Vol. 122, No. 1, pp. 8-16 (1967).
28. Saha, B. C., "Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*," *Process Biochemistry*, Vol. 39, No. 12, pp. 1871-1876 (2004).
29. Sakon, J., Irwin, D., Wilson, D. B., and Karplus, P. A., "Structure and mechanism of endo/exocellulase E4 from *Thermospora fusca*," *Nature Structural Biology*, Vol. 4, No. 10, pp. 810-818 (1997).
30. Singh, A., Agrawal, A. K., Abidi, A. B., and Darmwal, N. S., "Properties of cellobiase from *Aspergillus niger*," *Applied Microbiology and Biotechnology*, Vol. 34, No. 3, pp. 356-358 (1990).
31. Smriti, G. G. Sanwal, "Purification and characterization of a cellulase from *Catharanthus roseus* stems," *Phytochemistry*, Vol. 52, No. 1, pp. 7-13 (1999).
32. Tomme, P., Warren, R. A., and Gilkes, N. R., "Cellulose hydrolysis by bacteria and fungi," *Advances in Microbial Physiology*, Vol. 37, No. 1, pp. 1-81 (1995).
33. Zaldivar, J., Nielsen, J., and Olsson, L., "Fuel ethanol production from lignocellulose: A challenge for metabolic engineering and process integration," *Applied Microbiology and Biotechnology*, Vol. 56, No. 1-2, pp. 17-34 (2001).