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Article

A cold adapt and ethanol tolerant endoglucanase from a marine *Bacillus subtilis*☆

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ABSTRACT

The catalytic properties and thermodynamic kinetics of the endoglucanase from a marine *Bacillus subtilis* were analyzed. Optimum pH and temperature of the endoglucanase activity were 5.0 and 35 °C. The endoglucanase activity, melt point temperature was 1.13 folds (247.02 $U \cdot ml^{-1}$), 2.1 °C higher (39.2 °C) in 6% ethanol solution than that (218.60 $U \cdot ml^{-1}$), (37.1 °C) in free ethanol. At 40 °C–55 °C, Gibbs free energy, ΔG, and the content of α-helix was higher in 6% ethanol solution than that in ethanol free solution. The increasing of α-helix content led to higher activity and better thermostability in ethanol solution. The cold adapt ethanol tolerant endoglucanase was valuable for bioethanol product by simultaneous saccharification and fermentation process.

1. Introduction

Bioethanol production using carbon source released from cellulose has significance for sustainable development [1,2]. Hydrolyzing cellulose with cellulase is a promising method to transform cellulose into glucose, a widely used carbon source. Hydrolyzing cellulose with cellulase has the advantages of high hydrolysis efficiency, low environment pollution and mild conditions [3,4].

Separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) are two kinds of process of bioethanol production by hydrolyzing cellulose with cellulase. Separate hydrolysis and fermentation (SHF) process firstly adds cellulase into cellulose material. Cellulase hydrolyzes cellulose material into glucose. After cellulose hydrolysis, yeast is incubated [5,6]. Separate hydrolysis and fermentation (SHF) has some inadequacies. During the hydrolysis, high concentration of released glucose inhibits cellulase activity and hydrolysis efficiency. That inadequacies lead to increase the cost of cellulose hydrolysis [7–9]. In simultaneous saccharification and fermentation (SSF), cellulase, cellulose material and yeast are simultaneously added into the fermentation mixture. Cellulose material is hydrolyzed into glucose and glucose simultaneously was absorbed by yeast. Glucose maintained a low concentration. A low concentration of glucose cannot inhibit cellulase activity and hydrolysis efficiency. Cellulase can efficiently hydrolyze cellulose [10]. High cost is a bottleneck of industrial production of bioethanol. Bioethanol produced by simultaneous saccharification and fermentation (SSF) has a lower cost of bioethanol compared with separate hydrolysis and fermentation (SHF).

Bioethanol fermentation was carried out at the range of 23–37 °C [11–13]. The optimum temperature of common cellulase activity was above 50 °C [14,15]. The hydrolysis efficiency of common cellulase is low at the range of 23–37 °C. The optimum temperature of cold adapt enzyme activity was about 30–40 °C [16,17]. Cold adapt cellulalse could efficiently hydrolyze cellulose at the range of 23–37 °C. Cold adapt cellulase was valuable for bioethanol production by simultaneous saccharification and fermentation (SSF).

During the bioethanol fermentation, ethanol concentration continues to increase. High concentration of ethanol decreased the activity and hydrolysis efficiency of common cellulase. Ethanol tolerant cellulase is desired to bioethanol production by simultaneous saccharification and fermentation (SSF). Ethanol tolerant cellulase could remain high activity and thermostability in high concentration of ethanol. Ethanol tolerant cellulase is valuable for bioethanol production.

Cellulase is composed of exoglucanase, endoglucanase and β-glucosidase. Exoglucanase hydrolyzes macromolecular cellulose into small cellulose fragment. Endoglucanase hydrolyzes cellulose fragment into cellubiose. Cellulbiose is transformed into glucose by β-glucosidase [18–20]. Endoglucanase is indispensable for hydrolyzing cellulose [21, 22]. Ethanol tolerant endoglucanase is necessary for bioethanol production by simultaneous saccharification and fermentation (SSF).

Salt tolerant endoglucanase from *Stachybotrys microspore* [23], alkaline-stable endoglucanase from *Paenibacillus* sp. [24], and ionic liquid

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stable endoglucanase from *Stachybotrys microsporaInes* [23] have been reported. Cold adapt ethanol tolerant endoglucanase has not been reported.

A marine *B. subtilis* has been isolated from East China Sea. Endoglucanase from a marine *B. subtilis* was cold adapt and ethanol tolerant. The catalytic properties of the endoglucanase were analyzed under high concentration of ethanol conditions.

2. Material and Methods

2.1. Strain

The bacteria were isolated from amarine sediments. Based on the ITS DNA sequence and morphologies, the bacteria were identified as *B. subtilis*.

2.2. Media

Preservation medium was composed of 5.0 g yeast extract, 2.0 g glucose, 2.0 g peptide, 0.2 g $(NH_4)_2SO_4$, 0.01 g $MgCl_2$, 0.05 g KCl, 20.0 g agar, and 1.0 L tap water. The medium pH was adjusted to 6.5 and sterilized.

Fermentation medium was composed of 2.0 g starch, peptide 0.5 g, CMCNa 10.5 g, $(NH_4)_2SO_4$ 0.2 g, $MgCl_2$ 0.01 g, KCl 0.05 g, tap water 1.0 L.

2.3. Endoglucanase production and purification

B. subtilis was inoculated on preservation slants and cultivated for 2 d at 35 °C. Then *B. subtilis* was inoculated into fermentation medium. The cell concentration was 1×10^7 cells·ml^{-1}. 100 ml of fermentation medium was added into a 500 ml flask. Fermentation was carried out at 32 °C for 2 d. The fermentation broth was filtrated with filter paper. The filtrate was precipitated with ammonium sulphate (75%, w/v) and centrifugated at 10000 *g* for 10 min. The result precipitation was dissolved in 20 mmol·L^{-1} Tris-HCl buffer and dialyzed against 20 mmol·L^{-1} Tris-HCl. An ion-exchange column of Q-sepharose was equilibrated with 15 mmol·L^{-1} Tris-HCl buffer. The Q-sepharose column was eluted with 0–1.5 mol·L^{-1} NaCl. The elution fractions were collected and monitored at 280 nm. A fraction with high endoglucanase activity was purified by gel column. The fractions were pooled and concentrated by freeze-drying.

2.4. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

In order to measure the molecular weight of the endoglucanase, the fraction with high endoglucanase activity was analyzed by 12% gel SDS-PAGE. The fraction with high endoglucanase activity was loaded to 12% gel SDS-PAGE (containing 1.2% CMC) for zymogram analysis. The gel was soaking in wash buffer to remove SDS. The wash buffer contained 30 mmol·L^{-1} sodium phosphate buffer and 40% isopropanol (pH 7.2). The gel removed SDS was soaked in equilibrated buffer (containing 30 mmol·L^{-1} sodium phosphate buffer (pH 7.2)) for 1.5 h. Then the gel was transferred into renaturation buffer (containing 30 mmol·L^{-1} sodium phosphate buffer, 5 mmol·L^{-1} β-mercaptoethanol and 1 mmol·L^{-1} EDTA (pH 7.2)) at 4 °C for 16 h.

2.5. Non-denaturating gel electrophoresis and stainness

Native PAGE was performed with 7.5% separating gels and 5.0% stacking gels. Electrode buffer was tris-glycine buffer (pH 8.30). The native-PAGE gel was soaked in 1% carboxymethyl-cellulose in 50 mmol·L^{-1} acetate buffer, at pH 5.0 and 50 °C for 1.2 h. The gel was then transferred to 0.1% solution of Congo red and incubated at 25 °C, with constant shaking for 15 min. The gel was destained by washing with 1 mmol·L^{-1} sodium chloride. The activity band was clearly visible as yellowish clearances against a deep red background.

2.6. Effect of temperature and pH on endoglucanase activity

The optimum temperature of endoglucanase activity was analyzed at the temperature range from 25 °C to 55 °C with increments of 5 °C unit at pH 5.0.

The optimum pH of endoglucanase activity was analyzed at pH 4, 5 and 6 (50 mmol·L^{-1} Sodium acetate buffer), to 7 and 8 (50 mmol·L^{-1} Phosphate buffer) at 30 °C.

The endoglucanase activity was performed using 1% (w/v) β-glucan as a substrate. 5 ml buffer containing β-glucan and 0.5 ml diluted endoglucanase liquid was mixed. The mixture was incubated for 30 min at designed temperature. Released reduced sugar was measured by DNS method [25]. The amount of enzyme activity required to form an amount of reducing sugar corresponding to 1 μmol of glucose per min was regarded as one unit of enzyme activity.

2.7. Effect of ethanol on endoglucanase activity.

Endoglucanase activity was measure at different concentration of ethanol at pH 6.0 and 30 °C. The ethanol concentration range was from 2% (w/w) to 10% (w/w). The relative activity was calculated by the endoglucanase activity to the maximum activity.

2.8. Half life time of endoglucanase in different concentration of ethanol solution

Half life time of endoglucanase in different concentration of ethanol solution was determined according to the first order kinetics, which was a single step two stage theory [26]. The equation is the following,

$$\ln\left({}^{E_d}\!/_{E_0}\right) = K_d t \tag{1}$$

where E_0 is the initial endoglucanase activity. E_d is the endoglucanase activity after deactivation for some time in different concentrations of ethanol solution. K_d is first-order deactivation rate constant. t is the deactivation time. From the plot of $\ln(E_d/E_0)$ *versus* t, the slope was obtained. The slope was the value of K_d.

Half-life time of an enzyme is the time required by enzyme to lose half of the initial activity, which is calculated by,

$$t_{1/2} = {}^{\ln 2}\!/_{K_d} \tag{2}$$

where $t_{1/2}$ is half-life time of endoglucanase.

2.9. ΔG of thermodynamic kinetics of ethanol in different concentration of ethanol solution

Activation energy, E_a, is calculated from Arrhenius equation. It was the following [27]:

$$\ln k_d = \ln k_0 - \left(\frac{E}{R}\right)\frac{1}{T} \tag{3}$$

E_a were the slopes of plot of $\ln k_d$ *versus* $1/T$. Δ***G***, Gibbs free energy were calculated by the following equation:

$$\Delta G = -RT \ln\{(k_d h)/(k_B T)\} \tag{4}$$

where T, h, k_d, k_B, R and ΔG were temperature, Plank's constant, first-order deactivation rate constant, Boltzman's constant, universal gas constant and Gibbs free energy, respectively. Where h is 6.63×10^{-34} J·s^{-1} and k_B is 1.38×10^{-23} J·K^{-1} and R is 8.314 J·mol^{-1}·K^{-1}.

2.10. Melt temperature measurement

T_m was melt temperature. T_m of endoglucanase was determined by performing at 220 nm from 25 °C and 55 °C (1 °C·min^{-1}) using 1 mm quartz cells in an Aviv Model 400 spectrophotometer (AVIV Biomedical, USA). Endoglucanase samples were suitably diluted in citric sodium buffer (0.1 mol·L^{-1}, pH 5.0) to a working concentration of 0.31 mg·ml^{-1}. T_m values were determined by fitting the curves of circular dichroism signals *versus* temperature.

3. Results and Discussion

3.1. Endoglucanase purification

There was only one band in SDS PAGE (Fig. 1). The fraction with endoglucanase activity contained one kind of protein. Native PAGE gel was stained with congo red. A lighter clearance band in native PAGE gel was visible. The result suggested that the purified protein could hydrolyze CMC. (See Table 1.)

According to SDS PAGE, the molecular weight was about 31000. The endoglucanase from *B. subtilis* SB13 was 55000 [28]. Endoglucanase from *Thermotoga maritima* [29], microorganism from a salt-lake [30], *Aspergillus niger* [31], *Aspergillus flavus* [32], and *Mucor circinelloides* [33] was 70000, 61790, 33700, 35000, 27000. Different molecular weights indicate that amino acid sequences were not same. The difference of amino acid sequences leads to different catalysis properties.

3.2. Optimum pH and temperature of endoglucanase activity

Optimum pH and temperature of the endoglucanase from a marine *B. subtilis* was pH 5.0 and 35 °C (Figs. 2, 3). The endoglucanase from a marine *B. subtilis* was acidic. The optimum temperature of the enzyme activity was 35 °C. The endoglucanase from a marine *B. subtilis* was called as cold adapt enzymes [34]. It was suitable to hydrolyze cellulose under acidic and low temperature condition.

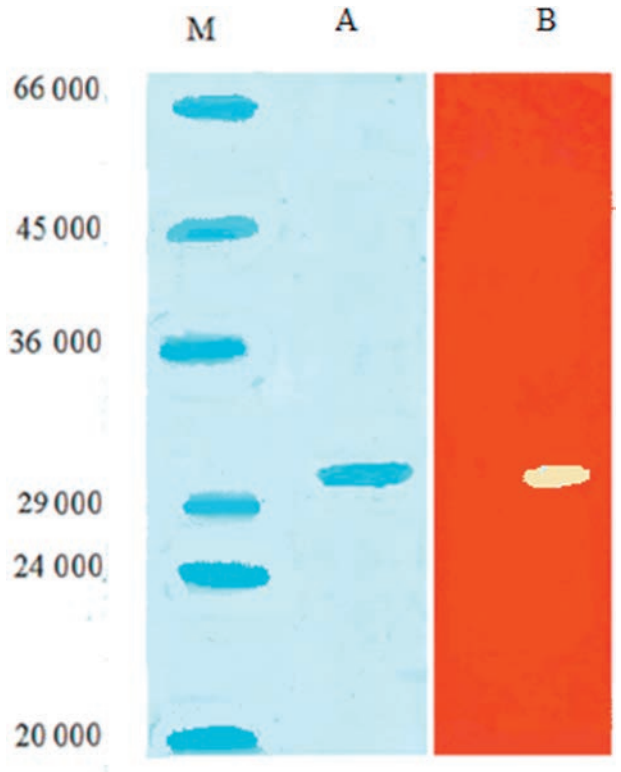

Fig. 1. SDS PAGE and native PAGE gel stained with congo red.

Table 1
Effect of reagents on endoglucanase activity

Reagents/ (10 mmol·L^{-1})	Relative activity/%	Reagents/ (10 mmol·L^{-1})	Relative activity/%
Control	100	Pb^{2+}	21 ± 1.2
K^+	102 ± 6.2	Cu^{2+}	86 ± 3.2
Mg^{2+}	102 ± 5.6	Zn^{2+}	29 ± 1.1
Cd^{2+}	78 ± 3.2	Na^+	96 ± 5.2
Ca^{2+}	110 ± 6.1	Fe^{2+}	93 ± 5.8
EDTA	101 ± 6.8	DTT	102 ± 6.6

The optimum pH and temperature of the endoglucanase from other strains of *B. subtilis* was pH 6.0 and 15 °C [28], pH 8.0 and 50 °C [35]. Optimum pH and temperature of endoglcanase from *A. niger, Aspergillus terreus NIH2624* were pH 4.0 and 70 °C [31], pH 5 and 55 °C [36]. The optimum activity temperature of the cold adapt endoglucanase from *Paenibacillus* sp. IHB B 3084 and *Eisenia fetida* was 40 °C [37,38].

The yeast produces bioethanol at about 32 °C [39]. The cold adapt endoglucanase from a marine *B. subtilis* was suitable for bioethanol production by simultaneous saccharification and fermentation process.

Optimum pH and temperature of the endoglucanases from different strains was different. Different amino acid sequences of the endoglucanase lead to different optimum pH and temperature of endoglucanase activity. The cold adapt endoglucanase probably had a flexible structure. The endoglucanase with a flexible structure could efficiently combine and catalyze the substrate at low temperature. At high temperature, the endoglucanase with a flexible structure easily changed the 3-dimension structure. The 3-dimension structure change made the endoglucanase not efficiently combine the substrate, so the endoglucanase activity decreased at high temperature.

3.3. Effect of ethanol on endoglucanase activity

In free ethanol solution, the endoglucanase from a marine *B. subtilis* showed 218.60 U·ml^{-1} activity. In 6% ethanol solution, the endoglucanase showed 247.02 U·ml^{-1} activity. In solution with over 6% ethanol, the endoglucanase remained over 70% activity (Fig. 4). The endoglucanase from *A. niger* was tolerant to 8% ethanol. It could efficiently hydrolyze cellulose in 8% ethanol solution. The concentration of ethanol produced by solid state fermentation was 6.47% [40]. The endoglucanase from a marine *B. subtilis* could efficiently hydrolyze cellulose in the solid state fermentation mixture.

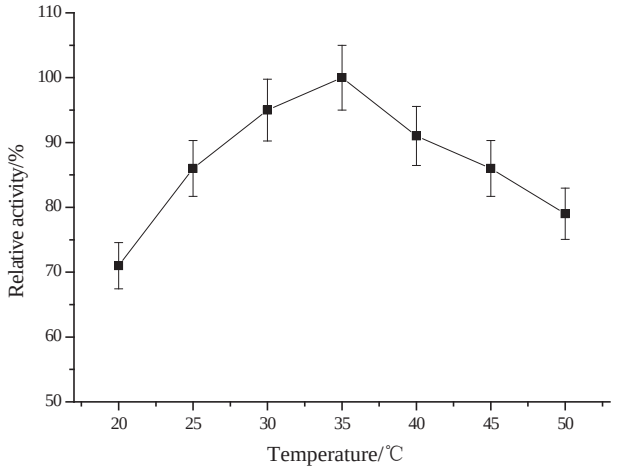

Fig. 2. Effect of temperature on endoglucanase activity.

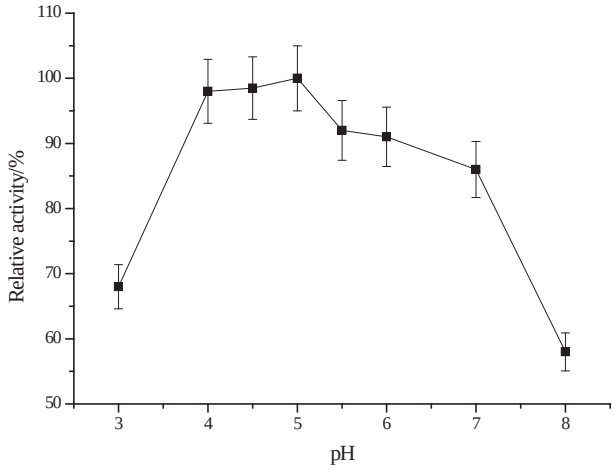

Fig. 3. Effect of pH on endoglucanase activity.

β-glucosidase from *Sporidiobolus pararoseus* was stable up to 15% in 15% ethanol solution [41]. Ethanol can act as an acceptor for the intermediate glycosyl cation during the substrate hydrolysis [42,43]. Increasing of endoglucanase activity probably lied on 3-dimension structure change in ethanol solution. 3-dimension structure changes probably lead to easier binding of substrate or easier releasing of product.

3.4. Effect of reagents on endoglucanase activity

Over 90% activity of the endoglucanase was remained in K^+, Na^+ Mg^{2+}, or Fe^{2+} solution. Ca^{2+} and Mg^{2+} slightly enhanced the endoglucanase activity. Ca^{2+} and Mg^{2+} were necessary to increase the substrate binding affinity of the enzyme and stabilize the conformation of the catalytic site [44]. The endoglucanase only remained 21% activity in Pb^{2+} solution. Some other works reported that heavy metals decreased endoglucanase activity [45].

The endoglucanase activity was enhanced by ethylenediaminetetraacetate (EDTA, 10 mmol·L^{-1}) or dithiothreitol (DTT, 10 mmol·L^{-1}). The endoglucanase activity was remained in EDTA solution. *Mucor circinelloides* endoglucanase activity was enhanced by EDTA or DTT [33]. The result indicated that the endoglucanase from a marine *B. subtilis* was not a metal ion enzyme.

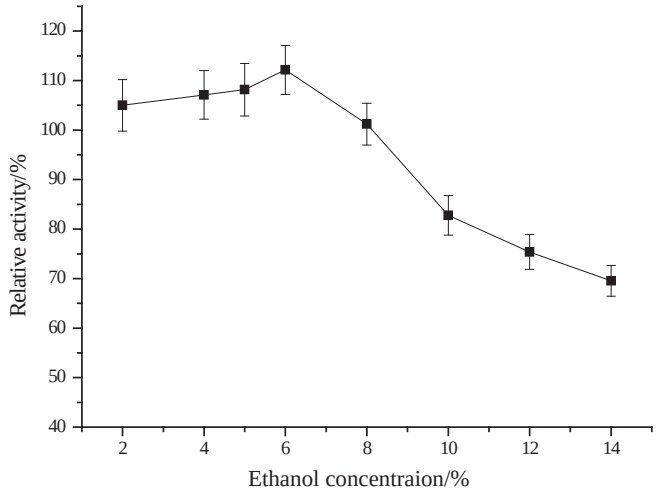

Fig. 4. Effect of ethanol concentration on endoglucanase activity.

Table 2
Substrates specificity of the endoglcanase at 35 °C and pH 6.0

Substrates	Relative activity/%	Substrates	Relative activity/%
Filter paper	23.01 ± 3.2	Avicel	0.00
CMCNa	100.00 ± 2.2	Cotton	0.32 ± 0.02
Cellobiose	0.00	Laminarin	0.00

3.5. Substrate specificity

The endoglucanase could hydrolyze CMCNa. It could not hydrolyze cellobiose, avicel, and laminarin (Table 2). The endoglucanase had the affinity to the substrate of CMC. The results concurred with the other reported results. The endoglcuanase from *B. subtilis* AS3 could only hydrolyze CMC [46].The endoglucanase from *Bacillus* sp. SV1 could hydrolyze CMC and cellobiose [47]. *Bacillus amyloliquefaciens* endoglucanase could hydrolyze CMC and avicel [48]. Hydrolyzing avicel needs exoglucanase activity. Unusual structure of bind domain and catalysis domain endowed some endoglucanase exoglucanase activity.

3.6. Melt points of the endoglucanase in ethanol solution

In ethanol free solution, the melt point of the endoglucanase was 37.1 °C. In 6% ethanol solution, the melt point of the endoglucanase was 39.2 °C (Fig. 5). The melt point of the endoglucanase was 1.9 °C higher in 6% ethanol solution than in ethanol free solution. The higher the melt point of the endoglucanase, the more stable the endoglucanase. The thermostability increasing in ethanol solution indicated that the structure of the endoglucanase changed in ethanol solution. The endoglucanase structure was more stable. It was valuable for bioethanol product by enzymatic hydrolyzing cellulose.

3.7. Thermodynamic kinetics parameters in solution with different concentration ethanol

Thermodynamic kinetics could be used to evaluate enzymes thermostability. Gibbs free energy, Δ*G*, was an effective parameter to analyze enzyme thermostability. The higher Δ*G*, is, the more stable enzyme is [49]. At 45 °C–60 °C, Δ*G* decreased with temperature increasing (Table 3). The enzyme thermostability decreased with temperature increasing. Δ*G* was higher in 6% ethanol solution than that in ethanol free solution (Table 2). The endoglucanase was more stable in 6% ethanol solution than in ethanol free solution. The endoglucanase probably changed to a more stable structure in 6% ethanol solution. Ethanol is a

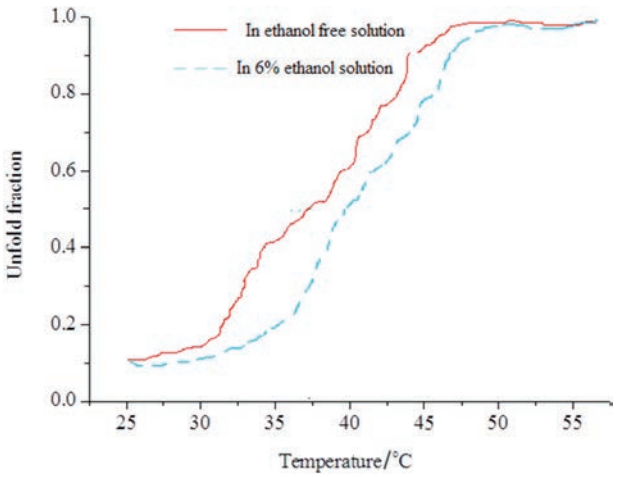

Fig. 5. Melt points of the endoglucanase in solution with different concentrations of ethanol.

Table 3
Gibbs free energy of endoglucanase in different concentrations of ethanol solution

T/K	ΔG/kJ·mol^{-1}	
	Ethanol concentration (v/v)	
	0	6%
318.15	58.700	59.112
323.15	56.068	56.968
328.15	54.202	54.937
333.15	52.666	53.017

polar compound. An oxhydryl of ethanol probably interacted with basic amino acids. The interaction probably made the endoglucanase have a different structures or molecular bridges. Structure or molecular bridges change could increase the stability.

The reason for the endoglucanase catalysis increasing in ethanol solution may be that ethanol could act as an acceptor for the intermediate glycosyl cation [43,50].

3.8. Secondary structure in different concentrations of ethanol solution

Secondary structure change can lead to 3-dimensional structure change. Secondary structures of enzymes include β-sheet, α-helix, unorder coil. The contents of secondary structures could be analyzed with FT-IR. The absorbance value of the amide peak at approximate 1670 cm^{-1} could be used to measure the content of the enzyme β-sheet [51]. The absorbance value of the amide peak at 1650–1640 cm^{-1} and could 1660 cm^{-1} be used to measure the content of α-helix content [52], unordered coil [53].

The absorbance at 1660 cm^{-1} and 1670 cm^{-1} was same in ethanol free solution with that in 6% ethanol solution (Fig. 6). The results indicated that the content of β-sheet and unordered coil was same in ethanol free solution with that in 6% ethanol solution. The absorbance at 1650–1640 cm^{-1} was higher in 6% ethanol solution than that in ethanol free solution. The results indicated that the content of α-helix increased. Many research results confirmed that the increasing of α-helix content could enhance the thermostability [18,54]. The α-helix content increasing made the endoglucanase thermostability increased. The α-helix content increasing probably led to the 3-dimension structure change. The 3-dimension structure change made the substrate more easily hydrolyzed. To systematically reveal the mechanism for ethanol tolerance of the endoglucanase, 3-dimension structure of the endoglucanase should be analysis by NMR in ethanol solution.

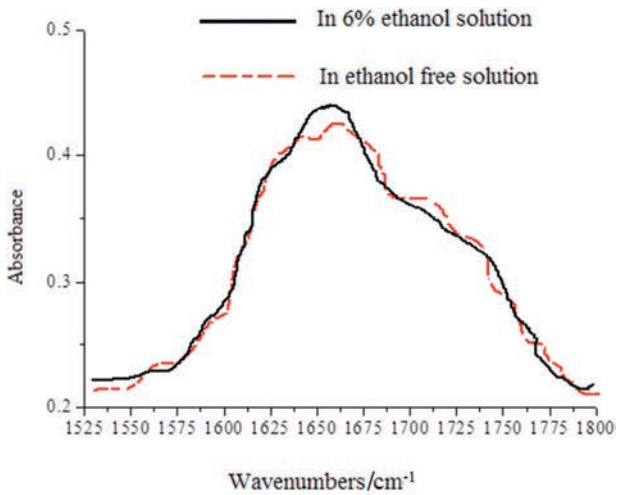

Fig. 6. FI-IR scan curves of the endoglucanase in different concentrations of ethanol solution.

4. Conclusions

Bottleneck of ethanol industrial scale product was high cost. Ethanol tolerant endoglucanase is valuable for decreasing bioethanol cost. The endoglucanase from a marine *B. subtilis* cellar showed higher activity and better thermostability in ethanol solution. The increasing of α-helix content probably led to better catalysis properties in ethanol solution. The acidic, thermostable and ethanol tolerant endoglucanase was valuable for bioethanol product by simultaneous saccharification and fermentation process.

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References

[1] A.S.O. Idris, A. Pandey, S.S. Rao, R.K. Sukumaran, Cellulase production through solid-state tray fermentation, and its use for bioethanol from sorghum stover, *Bioresour. Technol.* 242 (2017) 265–271.
[2] M. Linde, M. Galbe, G. Zacchi, Simultaneous saccharification and fermentation of steam-pretreated barley straw at low enzyme loadings and low yeast concentration, *Enzym. Microb. Technol.* 40 (2007) 1100–1107.
[3] L.P. Devendra, A. Pandey, Hydrotropic pretreatment on rice straw for bioethanol production, *Renew. Energy* 98 (2016) 2–8.
[4] L. Capolupo, V. Faraco, Green methods of lignocellulose pretreatment for biorefinery development, *Appl. Microbiol. Biotechnol* 100 (2016) 9451–9467.
[5] R.R. Singhania, R. Saini, R.M. Adsul, J.K. Saini, A. Mathur, D. Tuli, An integrative process for bio-ethanol production employing SSF produced cellulase without extraction, *Biochem. Eng. J.* 102 (2015) 45–48.
[6] F.M. Cunhaa, A.C. Badinoa, C.S. Farinasa, Effect of a novel method for in-house cellulase production on 2G ethanol yields, *Biocatal. Agric. Biotechnol.* 9 (2017) 224–229.
[7] M. Lever, G. Ho, R. Cord-Ruwisch, Ethanol from lignocellulose using crude unprocessed cellulase from solid-state fermentation, *Bioresour. Technol.* 101 (2010) 7083–7087.
[8] M. Narraa, J. Divechab, D. Shaha, V. Balasubramaniana, B. Vyasa, M. Harijana, K. Macwana, Cellulase production, simultaneous saccharification and fermentation in a single vessel: A new approach for production of bio-ethanol from mild alkali pre-treated water hyacinth, *J. Environ. Chem. Eng.* 5 (2017) 2176–2181.
[9] S. Baek, S. Kim, K. Lee, J. Lee, J. Hahn, Cellulosic ethanol production by combination of cellulase-displaying yeast cells, *Enzym. Microb. Technol.* 51 (2012) 366–372.
[10] A. Sørensen, M. Lübeck, P.S. Lübeck, B.K. Ahring, Fungal beta-glucosidases: A bottleneck in industrial use of lignocellulosic materials, *Biomol. Ther.* 3 (2013) 612–631.
[11] R. Cavicchioli, T. Charlton, H. Ertan, S.M. Omar, K. Siddiqui, T. Williams, Biotechnological uses of enzymes from psychrophiles, *Microb. Biotechnol.* 4 (2011) 449–460.
[12] W.C. Chen, Y.C. Lin, Y.L. Ciou, I.M. Chu, S.L. Tsai, J.C. Lan, Y.K. Chang, Y.H. We, Producing bioethanol from pretreated-wood dust by simultaneous saccharification and co-fermentation process, *J. Taiwan Inst. Chem. Eng.* 79 (2017) 43–48.
[13] A. Mahboubi, P. Ylitervo, W. Doyen, H.D. Wever, B. Molenberghs, M.J. Taherzadeh, Continuous bioethanol fermentation from wheat straw hydrolysate with high suspended solid content using an immersed flat sheet membrane bioreactor, *Bioresour. Technol.* 241 (2017) 296–308.
[14] A. Aguilar-Reynosa, A. Romaní, R.M. Rodríguez-Jasso, C.N. Aguilar, G. Garrote, H.A. Ruiz, Comparison of microwave and conduction-convection heating autohydrolysis pretreatment for bioethanol production, *Bioresour. Technol.* 243 (2017) 273–283.
[15] A.S. Bommarius, M. Sohn, Y. Kang, J.H. Lee, M.J. Realff, Protein engineering of cellulases, *Curr. Opin. Biotechnol.* 29 (2014) 139–145.
[16] M. Ueda, T. Goto, M. Nakazawa, K. Miyatake, M. Sakaguchi, K. Inouye, A novel cold-adapted cellulase complex from *Eisenia foetida*: Characterization of a multienzyme complex with carboxymethylcellulase, β-glucosidase, β-1,3 glucanase, and β-xylosidase, *Comp. Biochem. Physiol.* 157 (2010) 26–32.
[17] H.A. Cristóbala, A. Schmidtb, E. Kotheb, J. Brecciac, C.M. Abatea, Characterization of inducible cold-active-glucosidases from the psychrotolerant bacterium *Shewanella* sp. G5 isolated from a sub-Antarctic ecosystem, *Enzym. Microb. Technol.* 45 (2009) 498–506.
[18] L.Y. Liang, D.S. Xue, Kinetics of cellulose hydrolysis by halostable cellulase from a marine *Aspergillus niger* at different salinities, *Process Biochem.* 63 (2017) 163–168.
[19] R.C. Kuhad, D. Deswal, S. Sharma, A. Bhattacharya, K.K. Jain, A. Kaur, B.I. Pletschke, A. Singh, M. Karp, Revisiting cellulase production and redefining current strategies based on major challenges, *Renew. Sust. Energ. Rev.* 55 (2016) 249–272.
[20] K. Igarashi, T. Uchihashi, A. Koivula, M. Wada, S. Kimura, T. Okamoto, M. Penttilä, T. Ando, M. Samejima, Traffic jams reduce hydrolytic efficiency of cellulase on cellulose surface, *Science* 333 (2011) 1279–1282.
[21] M.K. Bhat, S. Bhat, Cellulose degrading enzymes and their potential industrial applications, *Biotechnol. Adv.* 15 (1997) 583–620.
[22] Y. Shi, Y.M. Tao, S.J. Ma, G. An, M.N. Long, Q.X. Chen, Purification and some properties of endoglucanase from Glyptotermes xiamenensis, *J. Appl. Entomol.* 134 (2010) 789–797.

[23] I.B. Hmad, M. Boudabbous, H. Belghith, A. Gargouri, A novel ionic liquid-stable halophilic endoglucanase from *Stachybotrys microspore*, *Process Biochem.* 54 (2017) 59–66.
[24] H. Dhara, M.K. Swarnkar, A. Rana, K. Kaushal, A.K. Singha, R.C. Kasanab, A. Gulatia, Complete genome sequence of a low-temperature active and alkaline-stable endoglucanase-producing *Paenibacillus* sp. strain IHB B 3084 from the Indian Trans-Himalayas, *J. Biotechnol.* 230 (2016) 1–2.
[25] L.Y. Liang, D.S. Xue, Kinetics of cellulose hydrolysis by halostable cellulase from a marine *Aspergillus niger* at different salinities, *Process Biochem.* 63 (2017) 163–168.
[26] K.S. Siddiqui, M.J. Azhar, M.H. Rashid, M.I. Rajoka, Stability and identification of active-site residues of carboxymethylcellulases from *Aspergillus niger* and *Cellulomonas biazotea*, *Folia Microbiol.* 42 (1997) 312–318.
[27] A. Karnchanatat, P. Sangvanich, J. Piaphukiew, A.J. Whalley, C.D. Reynolds, P. Sihanonth, Purification and biochemical characterization of an extracellular beta-glucosidase from the wood-decaying fungus *Daldinia eschscholzii* (Ehrenb: Fr.) Rehm, *FEMS Microbiol. Lett.* 270 (2007) 162–170.
[28] X.F. Guan, P.L. Chen, Q.X. Xu, L. Qian, J.Q. Huang, B. Lin, Expression, purification and molecular characterization of a novel endoglucanase protein from *Bacillus subtilis* SB13, *Protein Expr. Purif.* 134 (2017) 125–131.
[29] S.R. Chhabra, R.M. Kelly, Biochemical characterization of *Thermotoga maritima* endoglucanase Cel74 with and without a carbohydrate binding module (CBM), *FEBS Lett.* 531 (2002) 375–380.
[30] L. Lin, X.Z. Liu, Y.T. Zhou, L.Y. Guan, J.J. He, W.Q. Huang, A novel pH-stable, endoglucanase (JqCel5A) isolated from a salt-lake microorganism, *Jonesia quinghaiensis*, *Electron. J. Biotechnol.* 24 (2016) 56–62.
[31] J.J. Yan, W.D. Liu, Y.J. Li, H.L. Lai, Y.Y. Zheng, J.W. Huang, C.C. Chen, Y. Chen, J. Jin, H.Z. Li, R.T. Guo, Functional and structural analysis of *Pichia pastoris*-expressed *Aspergillus niger* 1,4-b-endoglucanase, *Biochem. Biophys. Res. Commun.* 475 (2016) 8–12.
[32] G. Gao, R.Q. Mao, Y. Xiao, J. Zhou, Y.H. Lin, G. Li, Efficient yeast cell-surface display of an endoglucanase of *Aspergillus flavus* and functional characterization of the whole cell enzyme, *World J. Microbiol. Biotechnol.* 33 (2017) 114.
[33] B.C. Saha, Production, purification and properties of endoglucanase from a newly isolated strain of *Mucor circinelloides*, *Process Biochem.* 39 (2004) 1871–1876.
[34] A.O. Smal, H.S. Leiros, V. Os, N.P. Willassen, Cold adapted enzymes, *Biotechnol. Annu. Rev.* 6 (2000) 1–57.
[35] S. Pandey, J. Kushwah, R. Tiwari, R. Kumarb, V.S. Somvanshi, L. Nain, A.K. Saxena, Cloning and expression of β-1,4-endoglucanase gene from *Bacillus subtilis* isolated from soil long term irrigated with effluents of paper and pulp mill, *Microbiol. Res.* 169 (2014) 693–698.
[36] F. Segato, B. Dias, G.L. Bertoa, D.M.D. Oliveira, F.H.M.D. Souza, A.P. Citadini, M.T. Murakami, A.R.L. Damásio, F.M. Squina, I. Polikarpova, Cloning, heterologous expression and biochemical characterization of a non-specific endoglucanase family 12 from *Aspergillus terreus* NIH2624, *Biochim. Biophys. Acta* 1865 (2017) 395–403.
[37] H. Dhara, R.C. Kasana, S. Duttb, A. Gulatia, Cloning and expression of low temperature active endoglucanaseEG5C from *Paenibacillus* sp. IHB B 3084, *Int. J. Biol. Macromol.* 81 (2015) 259–266.
[38] M. Uedaa, A. Ito, M. Nakazawa, K. Miyatake, M. Sakaguchi, K. Inouye, Cloning and expression of the cold-adapted endo-1,4-β-glucanase gene from *Eisenia fetida*, *Carbohydr. Polym.* 101 (2014) 511–516.
[39] S. Brethauer, C.E. Wyman, Review: continuous hydrolysis and fermentation for cellulosic ethanol production, *Bioresour. Technol.* 101 (2010) 4862–4874.
[40] A. Cavka, B. Alriksson, S.H. Rose, W.H.V. Zyl, L.J. Jönsson, Production of cellulosic ethanol and enzyme from waste fiber sludge using SSF, recycling of hydrolytic enzymes and yeast, and recombinant cellulase-producing *Aspergillus niger*, *J. Ind. Microbiol. Biotechnol.* 41 (2014) 1191–1200.
[41] M.A. Baffi, N. Martin, T.M. Tobal, A.L. Ferrarezi, J. Henrique, G. Lago, M. Boscolo, E. Gomes, R. Da-Silva, Purification and characterization of an ethanol-tolerant β-glucosidase from *Sporidiobolus pararoseus* and its potential for hydrolysis of wine aroma precursors, *Appl. Biochem. Biotechnol.* 171 (2013) 1681–1691.
[42] R.N. Barbagallo, G. Spagna, R. Palmeri, C. Restuccia, P. Giudici, *Enzym. Microb. Technol.* 35 (2004) 58–66.
[43] R. Leite, H. Alves, H. Cabral, F. Pagnocca, E. Gomes, R. Da-Silva, Production and characteristics comparison of crude β-glucosidases produced by microorganisms *Thermoascus aurantiacus* e *Aureobasidium pullulans* in agricultural wastes, *Enzym. Microb. Technol.* 43 (2008) 391–395.
[44] S.D. Mansfield, J.N. Saddler, G.M. Gubitz, Characterisation of endoglucanases from the brown rot fungi *Gloeophyllum sepiarium* and *Gloeophyllum traberum*, *Enzym. Microb. Technol.* 23 (1998) 133–140.
[45] Q. Gan, S.J. Allen, G. Taylor, Kinetic dynamics in heterogeneous enzymatic hydrolysis of cellulose: An overview, an experimental study and mathematical modelling, *Process Biochem.* 38 (2003) 1003–1018.
[46] D. Deka, S.P. Das, N. Sahoo, Enhanced cellulase production from *Bacillus subtilis* by optimizing physical parameters for bioethanol production, *ISRN Biotechnol.* (2013) https://doi.org/10.5402/2013/965310.
[47] L. Thomas, H. Hari, V.P. Singh, Inducible cellulase production from an organic solvent tolerant *Bacillus* sp. SV1 and evolutionary divergence of endoglucanase in different species of the genus *Bacillus*, *Braz. J. Microbiol.* 49 (2) (2018) 429–442.
[48] D.S. Xue, D.Q. Lin, C.J. Gong, C.L. Peng, S.J. Yao, Expression of a bifunctional cellulase with exoglucanase andendoglucanase activities to enhance the hydrolysis ability of cellulase from a marine *Aspergillus niger*, *Process Biochem.* 52 (2017) 115–122.
[49] A.G. Marangoni, Enzyme Kinetics A Modern Approach, John Wiley & Sons, Inc, NJ, 2003 146–150 (ISBN: 0–471–15985-9).
[50] R.N. Barbagallo, G. Spagna, R. Palmeri, C. Restuccia, P. Giudici, Selection, characterization and comparison of β-glucosidase from mould and yeasts employable for enological applications, *Enzym. Microb. Technol.* 35 (2004) 58–66.
[51] H. Susi, B.M. Michael, Protein structure by Fourier transform infrared spectroscopy: second derivative spectra, *Biochem. Biophys. Res. Commun.* 115 (1) (1983) 391–397.
[52] H. Torii, M. Tasumi, Three-dimensional doorway-state theory for analyses of absorption bands of many-oscillator systems, *J. Chem. Phys.* 97 (1992) 86–91.
[53] S. Spassov, M. Beekes, D. Naumann, Structural differences between TSEs strains investigated by FT-IR spectroscopy, *Biochim. Biophys. Acta* 1760 (2006) 1138–1149.
[54] D.S. Xue, L.Y. Liang, D.Q. Lin, S.J. Yao, Thermal inactivation kinetics and secondary structure change of a low molecular weight halostable exoglucanase from a marine *Aspergillus niger* at high salinities, *Appl. Biochem. Biotechnol.* 183 (2017) 1111–1125.