Increasing of activity and thermostability of cold active butanol-tolerant endoglucanase from a marine *Rhodococcus* sp. under high concentrations of butanol condition

Xuhao Zeng¹ · Dongsheng Xue¹

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Abstract

Optimum pH and temperature of the endoglucanase from *Rhodococcus* sp. was 5.0 and 40 °C. The endoglucanase activity was 1.18 folds higher in 5% butanol solution than that in butanol-free solution. Melt point temperature was 2.5 °C higher in 5% butanol solution (50.0 °C) than that in butanol-free solution (47.5 °C). At 45–60 °C, Gibbs-free energy change, ΔG , was higher in 5% butanol solution than that in butanol-free solution. The content of α -helix and β -sheet increased in 5% butanol solution. The increasing of α -helix and β -sheet content led to higher activity and better thermostability in butanol solution. The cold active butanol-tolerant endoglucanase was valuable for biobutanol production by a simultaneous saccharification and fermentation process.

Keywords Endoglucanase · Butanol tolerant · Cold active · *Rhodococcus* sp

Introduction

Renewable energy production is important for sustainable development. Bioethanol and biobutanol is two kinds of major renewable energy. Biobutanol with superior to bioethanol is a better replace of fossil fuel. Compared with bioethanol, biobutanol has high energy content, low vapor pressure, high octane numbers, flexible fuel blend and so on (Nanda et al. 2017). A biobutanol market is increasing at the rate of 3% each year (Lee et al. 2008).

Cellulose is the most abundant renewable resource. It can be hydrolyzed into glucose, a carbon source to produce biobutanol (Hiden et al. 2011). Cellulose hydrolysis is a key step to produce biobutanol. Enzymatic hydrolysis of cellulose has advantages of high hydrolysis efficiency, low environment pollution, compared with chemical hydrolysis.

There are two kinds of process of bioethanol production by hydrolyzing cellulose with cellulase. Cellulase is

added into cellulose material without inoculum of butanol-producing microorganism. The mixture of cellulose material and cellulase is incubated at designed temperature to hydrolyze cellulose. After cellulose hydrolysis, butanol-producing microorganism, such as *Clostridium acetobutylicum*, *Clostridium beijerinckii*, is added into the hydrolyzed mixture to metabolize glucose to produce butanol (Nanda et al. 2017; Hou et al. 2017; Ndaba et al. 2015). Hydrolysis and fermentation process of the process is separate. This kind of process has some inadequacies. During the hydrolysis, high concentrations of glucose decreased cellulase activity and hydrolysis efficiency (Sathe et al. 2017; Andric et al. 2010; Uchima et al. 2012). That leads to increased biobutanol production cost. In another kind of process to produce butanol, cellulase and cellulose, butanol-producing microorganism is simultaneously added into the fermentation mixture. During the process, glucose released by cellulase from cellulose is simultaneously metabolized by biobutanol-producing microorganism. The process is a simultaneous saccharification and fermentation process. Glucose in the fermentation system maintained a low concentration. Low concentration of glucose cannot inhibit cellulase activity. Cellulase can more efficiently hydrolyze cellulose (Sørensen et al. 2013; Ibrahim et al. 2015). High cost is a bottleneck of utilization of cellulose to produce biobutanol. Simultaneous saccharification and fermentation process can decrease hydrolysis cost

✉ Dongsheng Xue
xdsf1bm@hbut.edu.cn

¹ Key Laboratory of Fermentation Engineering (Ministry of Education), Hubei Key Laboratory of Industrial Microbiology, Hubei Provincial Cooperative Innovation Center of Industrial Fermentation, Hubei University of Technology, Wuhan 430068, People's Republic of China

and increase product yield (Su et al. 2015). Simultaneous saccharification and fermentation process has advantage of lower hydrolysis cost, compared with a separate hydrolysis and fermentation process.

Butanol-tolerant cellulase has significance for producing biobutanol by simultaneous saccharification and fermentation process. High concentrations of butanol decreased the activity and hydrolysis efficiency of common cellulase. Butanol-tolerant cellulase is a desirable enzyme to decrease biobutanol-producing cost. Butanol-tolerant cellulase could show high activity and thermostability under high concentrations of butanol.

Cellulase consists of exoglucanase, endoglucanase and β -glucosidase. Exoglucanase hydrolyzes macromolecular cellulose into small fragments. Endoglucanase hydrolyzes small fragments into cellubiose. Cellulbiase is transformed into glucose by β -glucosidase (Kuhad et al. 2016; Igarashi et al. 2011). Endoglucanase is indispensable for hydrolyzing cellulose (Vlasenko et al. 2010; Xue et al. 2017a, b, c). Butanol-tolerant endoglucanase is necessary for hydrolyzing cellulose in high concentrations of butanol.

Halostable or halophilic endoglucanase showed higher activity or better thermostability under high salinity conditions. Green ion liquid-tolerant endoglucanase showed the maximum activity in green ion liquid solution (Hmad et al. 2017). Alkali-tolerant endoglucanase was expressed in *Pichia pastoris* (Phadtare et al. 2017). Butanol-tolerant endoglucanase has not been reported.

Rhodococcus sp. was isolated from China East Sea sediments. Endoglucanase from *Rhodococcus* sp. was butanol tolerant. The catalytic properties of butanol-tolerant endoglucanase from *Rhodococcus* sp. was analyzed in high concentrations of butanol.

Materials and methods

Strain

An endoglucanase-producing bacterial strain was isolated from China East Sea. Based on the 16S rDNA sequence and morphologies of the colonies, the bacterial strain was identified as *Rhodococcus* sp.

Media

Preservation medium was composed of 1.0 g yeast extract, 2.0 g glucose, peptide 2.0 g, $(\text{NH}_4)_2\text{SO}_4$ 0.2 g, MgCl_2 0.01 g, KCl 0.05 g agar 20.0 g, tap water 1.0 L.

Fermentation medium was composed of 0.5 g yeast extract, peptide 0.3 g, wheat bran 1.2 g, CMCNa 10.5 g, $(\text{NH}_4)_2\text{SO}_4$ 0.2 g, MgCl_2 0.01 g, KCl 0.05 g, and tap water 1.0 L.

Endoglucanase preparation

Rhodococcus sp. was inoculated on preservation slants and cultivated for 5 days at 28 °C. *Rhodococcus* sp. was inoculated into fermentation medium. *Rhodococcus* sp. was 1×10^7 spores/mL. 100 mL of fermentation medium was added into a 500 mL flask. Fermentation was carried out at 28 °C for 3 d. Fermentation broth was filtrated with filter paper. The filtrate was precipitated with ammonium sulphate (75%, w/v). The precipitates were collected by centrifugation at $10,000 \times g$ for 10 min. The result precipitation was dissolved in a small amount of 20 mM Tris–HCl buffer and dialyzed against 20 mM Tris–HCl. An ion-exchange column of Q-Sepharose equilibrated with 20 mM Tris–HCl buffer was used to purify the endoglucanase. An ion-exchange column was eluted with 0.5–2.5 M NaCl. The elution fractions were collected and monitored at 280 nm. The fraction with high endoglucanase activity was purified by gel column, pooled and concentrated by freeze-drying.

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis

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 soaked in wash buffer to remove SDS. The wash buffer contained 30 mM sodium phosphate buffer and 40% isopropanol (pH 7.2). The gel-removed SDS was soaked in equilibrated buffer containing 30 mM of sodium phosphate buffer (pH 7.2) for 1.5 h. The gel was transferred into renaturation buffer containing 30 mM of sodium phosphate buffer, 5 mM β -mercap and 1 mM EDTA (pH 7.2) at 4 °C for 16 h. The gel was renatured. The renaturation gel was stained with 1% congo red solution and washed with 1.0 M NaCl solution. The band of endoglucanase was visualized as a clear zone against background.

Non-denaturing gel electrophoresis and stain

Native PAGE was performed with 7.5% separating gels and 5.0% stacking gels. Electrode buffer was tris–glycine buffer (pH 8.3). The native PAGE gel was soaked in 1% carboxymethyl-cellulose in 50 mM of acetate buffer (pH 5.0) at 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 20 min. The gel was destained by washing with 1M sodium chloride. The activity band was

clearly visible as yellowish clearances against a deep red background at the end of 10 min for destaining.

Effect of temperature and pH on endoglucanase activity

The optimum temperature of endoglucanase activity was analyzed at the temperature range from 30 to 65 °C at pH 6.0.

The optimum pH of endoglucanase activity was analyzed at pH 4, 5, 6 (50 mM of sodium acetate buffer), 7 and 8 (50 mM of phosphate buffer) at 50 °C.

Effect of butanol concentration on endoglucanase activity

Endoglucanase activity was measured at different concentrations of butanol at pH 6.0 and 50 °C. Butanol concentration range was from 0 to 10% (w/w). The relative activity was calculated by the endoglucanase activity to the maximum activity.

Thermodynamic kinetics of the endoglucanase at different concentrations of butanol

Half-life time of the endoglucanase at different concentrations of butanol was determined according to first-order kinetics, which was a single step two-stage theory (Siddiqui et al. 1997). The equation is the following:

$$\ln \left(\frac{E_d}{E_0} \right) = K_d t \quad (3)$$

where E_0 is the initial endoglucanase activity. E_d is the endoglucanase activity after deactivation for some time in different concentrations of butanol 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 the enzyme to lose half of the initial activity, which is calculated by the following equation:

$$t_{1/2} = \ln 2 / K_d \quad (4)$$

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

Activation energy, E_a , is calculated from Arrhenius equation. It was the following (Karnchanatat et al. 2007):

$$\ln(k_d) = \ln k_0 - \left(\frac{E}{R} \right) \frac{1}{T} \quad (5)$$

E_a were the slopes of plot of $\ln(k_d)$ versus $1/T$. Gibbs-free energy change, ΔG , was calculated by the following equation (Xue et al. 2017a, b, c):

$$\Delta G = -RT \ln \left\{ (k_d h) / (k_B T) \right\} \quad (6)$$

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

Measurement of melt point temperature

T_m represents for melt temperature. T_m of the endoglucanase was determined by performing at 220 nm from 25 and 60 °C (1 °C/min) using 1 mm quartz cells in an Aviv Model 400 spectrophotometer (AVIV Biomedical, USA). Endoglucanase samples were suitably diluted to 0.38 mg/ml with citric sodium buffer (0.1M, pH 5.0). T_m values were determined by fitting the curves of circular dichroism signals versus temperature.

Analysis of endoglucanase activity

The endoglucanase activity was performed using 1% (w/v) CMCNa (Carboxy methyl cellulose sodium) as a substrate. 5 mL of buffer containing CMCNa and 0.5 ml of diluted endoglucanase liquid was mixed. The mixture was incubated for 30 min at designed temperatures. The amount of an enzyme activity required to release an amount of reducing sugar corresponding to 1 μmol of glucose per min was regarded as one unit of an enzyme activity. Released reduced sugar was measured by DNS method.

Results and discussion

Endoglucanase purification

The endoglucanase from a marine *Rhodococcus* sp. was purified. One blue band was visualized in SDS-PAGE (Fig. 1). The fraction with an endoglucanase activity only contained one kind of protein. Staining with congo red, a clearance band was visualized in native gel. The purified protein could hydrolyze CMC. It was an endoglucanase. The molecular weight of the endoglucanase from *Rhodococcus* sp. was about 38 kDa. *Mucor circinelloides* endoglucanase was 27 kDa (Saha 2004). The molecular weight of an endoglucanase from *Aspergillus niger*, *Escherichia coli*, *Bacillus subtilis* SB13 and *Trichoderma reesei* was 33.7 kDa (Yan et al. 2016), 41 kDa (Lin et al. 2016), 54 kDa (Guan et al. 2017), 70 kDa (Akcipinar et al. 2012). Different molecular weights suggested that amino acids sequences were not same. Difference of amino acids sequences leads to different catalysis properties.

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

Optimum pH and temperature of the endoglucanase activity

Optimum pH and temperature of the endoglucanase from a marine *Rhodococcus* sp. was pH 5.0 and 40 °C (Fig. 2). The optimum temperature of cold-adapt enzyme activity is 40 °C or lower than 40 °C. The typical properties of cold active enzymes are relatively high activity at low temperature. The endoglucanase from a marine *Rhodococcus* sp. showed significant activity at 10 °C. The endoglucanase from a marine *Rhodococcus* sp. belong to a cold active enzymes. The properties of the endoglucanase from a marine *Rhodococcus* sp. was similar with that of a cold-adapted endoglucanase from *Eisenia fetida* (optimum temperature of 40 °C, exhibited significant activity at 10 °C) (Ueda et al. 2014). Optimum pH and temperature of the endoglucanase from a marine *Rhodococcus* sp. was different from that from *Aspergillus niger* (Yan et al. 2016), *Aspergillus terreus* NIH2624 (Segato et al. 2017). Different amino acid sequences of the endoglucanases probably led to different optimum pH and temperature of an endoglucanase activity.

Fig. 2 Effect of pH and temperature on endoglucanase activity

Effect of butanol on the endoglucanase activity

The endoglucanase from a marine *Rhodococcus* sp. was 1.18 folds higher in 5% butanol solution than that in butanol-free solution (Fig. 3). The endoglucanase from a marine *Rhodococcus* sp. could tolerate to 5% butanol. It could efficiently hydrolyze cellulose in 5% butanol solution.

The concentrations of butanol in fermentation broth was 0.33% (Ou et al. 2017). Typically, butanol only accounts for 13–18 g/L in fermentation broth (Ezeji et al. 2004). Increasing concentration of butanol is necessary to decrease cost of butanol production. The energy savings in distillation can be achieved if butanol yield is augmented from 10 to 40 g/L (Nanda et al. 2017). The endoglucanase showed higher activity in 5% butanol solution. It was valuable for high concentrations of butanol production by simultaneous sacchrification and fermentation process. Butanol could act as an acceptor for the intermediate glycosyl cation during the substrate hydrolysis. Action as an acceptor for the intermediate cation increased the hydrolysis activity (Barbagallo et al. 2004; Leite et al. 2008). Butanol probably acted as an acceptor for the intermediate glycosyl cation during a substrate hydrolysis. The action as an acceptor for the intermediate glycosyl cation probably increased the activity of the endoglucanase in high concentrations of butanol solution.

Effect of metal ions, EDTA and DTT on the endoglucanase activity

The endoglucanase from a marine *Rhodococcus* sp. remained over 95% activity in K^+ , Na^+ or Fe^{2+} solution. Ca^{2+} and Mg^{2+} enhanced the activity of the endoglucanase from *Rhodococcus* sp., showed in Table 1. Ca^{2+} and Mg^{2+}

Fig. 3 Effect of butanol concentration on the endoglucanase activity

Table 1 Effect of metal ions, EDTA and DTT on the endoglucanase activity

Reagents (10 mM)	Relative activity (%)	Reagents (10 mM)	Relative activity (%)
Control	100	Pb ²⁺	35 ± 1.3
K ⁺	95 ± 3.5	Cu ²⁺	78 ± 2.3
Mg ²⁺	105 ± 3.3	Zn ²⁺	26 ± 1.2
Cd ²⁺	86 ± 2.6	Na ⁺	98 ± 1.8
Ca ²⁺	103 ± 3.7	Fe ²⁺	96 ± 1.9
EDTA	101 ± 3.8	DTT	102 ± 4.1

was necessary to increase the substrate-binding affinity of the endoglucanase and stabilize the conformation of the catalytic site (Mansfield et al. 1998). The endoglucanase from a marine *Rhodococcus* sp. only remained over 26% activity in Pb²⁺ or Zn²⁺ solution. Another work reported these heavy metals also decreased endoglucanases activity (Gan et al. 2003).

Mucor circinelloides endoglucanase activity was enhanced by ethylenediaminetetraacetate (EDTA, 10 mM) or dithiothreitol (DTT, 10 mM) (Saha 2004). EDTA or DTT also enhanced the activity of endoglucanase from a marine *Rhodococcus* sp.

Substrate specificity

The endoglucanase from a marine *Rhodococcus* sp. could hydrolyze CMCNa, avicel and cotton. It could not hydrolyze cellobiose and laminarin (Table 2). Endoglucanase

Table 2 Substrate specificity of the endoglucanase

Substrates	Relative activity (%)	Substrates	Relative activity (%)
Filter paper	18.00 ± 2.3	Avicel	0.58 ± 0.01
CMCNa	100.00 ± 3.2	Cotton	0.92 ± 0.02
Cellobiose	0	Laminarin	0

from Thai higher termite bacteria (Seneesrisakul et al. 2017) and *Bacillus amyloliquefaciens* (Xue et al. 2017a, b, c) could hydrolyze CMC and avicel. Hydrolyzing avicel needs exoglucanase activity. Some endoglucanases have a bind domain to bind avicel, which was necessary to perform exoglucanase activity (Xue et al. 2017a, b, c).

Melt points of the endoglucanase in butanol solution

In butanol-free solution, the melt point of the endoglucanase was 47.2 °C. In 5% butanol solution, the melt point of the endoglucanase was 49.7 °C (Fig. 4). The melt point of the endoglucanase was 2.2 °C higher in 5% butanol solution than that in butanol-free solution. The higher the melt point of the endoglucanase was, the more stable the endoglucanase was. The thermostability increasing in butanol solution suggested that the structure probably changed to be more stable in high concentrations of butanol solution. Thermostable endoglucanase in high concentrations of butanol was valuable for biobutanol production by enzymatic hydrolyzing cellulose.

Fig. 4 Melt points of the endoglucanase in free or 5% butanol solution

Thermodynamic kinetics parameters in solution with different concentration butanol

Gibbs-free energy change, ΔG , was an effective parameter to analyze enzyme thermostability. The higher the Gibbs-free energy change, ΔG , was, the more stable enzyme was (Marangoni 2003). At the temperature range of 45–60 °C, Gibbs-free energy change, ΔG , decreased with temperature increasing (Table 3). The thermostability decreased with temperature increasing. Gibbs-free energy change, ΔG , was higher in 5% butanol solution than that in butanol-free solution (Table 3). The endoglucanase was more stable in 5% butanol solution than in butanol-free solution. The endoglucanase probably changed to a more stable structure in 5% butanol solution. Butanol is a polar compound. An oxyhydril of butanol probably interacted with basic amino acids. The interaction probably made the endoglucanase form a different structure or more molecular brigdes. Secondary structure or molecular bridges change probably increased the stability of the endoglucanase.

Secondary structures in different concentrations of butanol solution

Secondary structure contents change leads to three-dimensional structural variety. Secondary structures of enzymes

Table 3 Gibbs-free energy of the endoglucanase in different concentrations of butanol solution

T (K)	ΔG (kJ/mol)	
	Butanol concentration (v/v)	
	0%	8%
318.15	53.329	54.188
323.15	52.991	53.285
328.15	52.253	52.676
333.15	51.021	51.331

Fig. 5 FI-IR scan curves of the endoglucanase in different concentrations of butanol solution

include β -sheet, α -helix, and 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 analyze a β -sheet content. The absorbance value of the amide peak at 1650–1640 cm^{-1} could be used to analyze a α -helix content. The absorbance value of the amide peak at 1660 cm^{-1} could be used to analyze an unordered coil content (Spasov et al. 2006).

The absorbance at 1650–1640 and 1670 cm^{-1} increased in 5% butanol solution (Fig. 5). The results indicated that the content of β -sheet and α -helix was higher in 5% butanol-free solution than that in butanol-free solution. Many research results confirmed that the increasing of α -helix or β -sheet content could enhance the thermostability (Xue et al. 2017a, b, c). The α -helix and β -sheet content increase probably led to three-dimensional structure change. three-dimensional structure change provided the endoglcanase higher catalysis ability.

Conclusions

The endoglucanase from a marine *Rhodococcus* sp. showed higher activity and better thermostability in butanol solution. The increasing of α -helix content or butanol acting as an acceptor probably led to more efficiently hydrolyze the substrate in butanol solution. The acidic, thermostable butanol-tolerant endoglucanase was valuable for butanol production by simultaneous saccharification and fermentation process.

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Compliance with ethical standards

Conflict of interest There are no conflict of interest exists for all participating authors. And the article also complies with the ethical standard.

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