Immobilization of Cold-Active Cellulase from Antarctic Bacterium and Its Use for Kelp Cellulose Ethanol Fermentation

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Immobilization is an effective way to solve the problem associated with the application of cold-active cellulase in industrial processes. In this study, a cold-active cellulase from the Antarctic psychrophilic bacterium *Pseudoalteromonas* sp. NJ64 was obtained, immobilized, and analyzed for optimal immobilization conditions. Then it was used in kelp cellulose ethanol fermentation, achieving a higher purity level of kelp cellulose ethanol. The enzymatic activity of this cold-active cellulase was 49.7 U/mL. The optimal immobilization process conditions were as follows: sodium alginate, 30 g/L; calcium chloride, 5 g/L; glutaraldehyde, 0.4%; and cross-linking time, 5 h. Under these conditions, the activity recovery rate was 51.58%. The optimum reaction temperature was at 40 °C, the optimum initial pH was 9.0, and the relative enzyme activity was 58.37% after being recovered seven times. A higher purity level of kelp cellulose ethanol has reached (37.37%). Immobilized cold-active cellulase can effectively hydrolyze the cellulose of kelp residue, which is a valuable component of cellulose bio-ethanol production and will have broad implications in the development of the ethanol industry in China.

Keywords: Cold-active cellulase; Antarctic bacteria; Cellulose ethanol fermentation; Immobilized enzyme; Kelp cellulose

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INTRODUCTION

Bio-ethanol is increasingly receiving global attention as a clean and green renewable energy source (Kemppainen and Shonnard 2005; Sticklen 2008). Cellulose is the most abundant renewable resource and source of renewable energy on planet Earth, but also has the lowest utilization ratio. Cellulase provides a key opportunity for exploiting the tremendous benefits of cellulose utilization (Kasana and Gulati 2011; Teugjas and Väljamäe 2013). Compared with corn ethanol, which is the most common bio-ethanol, the costs of cellulosic ethanol production are high; however, more energy can be produced while releasing less CO_2 (Duff and Murray 1996; Kemppainen and Shonnard 2005).

Marine macroalgae are considered important feedstocks for biofuels production (Wei *et al.* 2013). China's scale of kelp cultivation and production ranks first in the world.

There is a lot of kelp residue produced during the industrial kelp production processes, which provide a rich cellulose resource and a quality raw material source of cellulosic ethanol fermentation (Park *et al*. 2009; Jang *et al*. 2012). These wastes have not been thoroughly utilized for a long time, and this has caused the waste of resources and also environmental pollution problems.

Cold-active enzymes found in cold-adapted organisms thriving in polar regions and other areas with the mean annual temperature below 10 °C offer a potential for the development of new industrial applications (Gerday *et al*. 2000; Gomes and Steiner 2004; Marx *et al*. 2007). Compared with the mesophilic enzymes, cold-active enzymes are more advantageous as proven by many studies and development achievements. For example, the application of these enzymes offers considerable potential to the biotechnology industry (Gerday *et al*. 2000; Cavicchioli *et al*. 2002; Marx *et al*. 2007; Cavicchioli *et al*. 2011; Kasana and Gulati 2011). Cold-active enzymes isolated from psychrophilic (cold-living) or psychrotolerant (cold-adapted) microorganisms are characterized by a higher specific activity than that of their mesophilic counterparts at low and moderate temperatures (generally below 40 °C); this is associated with rather high thermo-sensitivity, which makes them valuable tools for biotechnology (Feller and Gerday 1997; Gerday *et al*. 2000; Feller 2003; Marx *et al*. 2007; Berlemont *et al*. 2009). On the other hand, these features are also shortcomings, because it would be very easy to lose activity when temperature is higher.

Immobilizing enzymes is an effective way to solve a series of problems regarding the application of cold-active cellulase in industrial production processes (Ahmed *et al*. 1997). Immobilization can improve the efficiency of the enzyme effectively, increase the yield of the product while reducing production costs, simplify the purification process, and improve the quality of the product at the same time. Immobilization of cellulase is the focus of much scientific attention (Takeuchi and Makino 1987; Chakrabarti and Storey 1988; Pan *et al*. 2007; Zhao *et al*. 2007; Lupoi and Smith 2011; Ungurean *et al.* 2013), but the reports about immobilization of cold-active cellulase are very few. Only limited work has occurred in this field.

To date, investigators have found that many marine bacteria can produce cold-active cellulase, with most belonging to the genus *Pseudoalteromonas* sp.; such enzymes have been screened from polar regions and marine sediments (Ryu *et al*. 2001; Garsoux *et al*. 2004; Gomes and Steiner 2004; Ciemlijski *et al*. 2005; Zeng *et al*. 2006; Marx *et al*. 2007; Kasana and Gulati 2011). Previous researchers have isolated and purified a psychrophilic strain named *Pseudoalteromonas* sp. NJ64 from Antarctic seawater which can efficiently produce cold-active cellulase at low-temperatures (Gao *et al*. 2012).

In this manuscript, the enzymatic properties of cold-active cellulase isolated from *Pseudoalteromonas* sp. NJ64 were studied. This cellulase was first immobilized by the sodium alginate-glutaraldehyde cross-linking-entrapment method. The influence of sodium alginate, $CaCl_2$, glutaraldehyde, and cross-linking time regarding the efficiency of immobilization were determined. The enzymatic activity of immobilized cold-active cellulase was measured, and the recovery rate after immobilization was analyzed. Finally, this immobilized cellulase was used in kelp residue cellulose bio-ethanol fermentation, optimizing the fermentation conditions and obtaining a higher purity kelp cellulosic ethanol.

EXPERIMENTAL

Materials

The bacterial strain *Pseudoalteromonas* sp. NJ64 was isolated and purified from Antarctic seawater, which was collected during the Chinese 18th Antarctic Science Exploration during 2001 to 2002. The optimum growth temperature of NJ64 is between 10 and 15 °C, and it can secrete cellulase efficiently at low-temperatures. The enzymatic activity of this cold-active cellulase was the highest at pH 9.0 and a temperature of 40 °C (Gao *et al*. 2012).

Yeast (*Saccharomyces cerevisiae*), species number 1525, was provided by the Biological Resources Centre Institute of Microbiology, Chinese Academy of Sciences (IMCAS-BRC).

The kelp residue was processing waste from the Qingdao MingYue Seaweed Company.

Reagents and culture media

The bacteria cultures were incubated in 2216E liquid medium, which was prepared by mixing 1 g of yeast powder and 5 g of tryptone with 1 mL of 0.5 % (w/v) carboxymethyl cellulose sodium (inducer of cold-active cellulase) and 1 L of seawater, final pH 7.0~7.5.

The ethanol fermentation medium was composed of 60 g/L glucose, 3 g/L yeast extract, 0.53 g/L $(NH_4)_2SO_4$, 2.5 g/L KH_2PO_4, and 0.5 g/L $MgSO_4 \cdot 7H_2O$ (Sharma *et al*. 2004).

Methods

Preparation and activity analysis of cold-active cellulase

The bacteria cultures of NJ64 were incubated in a shaker-flask containing 2216E liquid medium at 100 rpm and 10 °C in culture for 72 h. The fermentation solution was centrifuged at 4 °C, 4900 xg for 15 min, and the supernatant was filtered (pore size 0.45 μm) to provide a crude solution of cold-active cellulase.

The crude enzyme solution (200 mL) was precipitated by salting with different concentrations of ammonium sulphate (*i.e.*, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90%). The enzyme activity of the crude cellulase solution and the protein content in the precipitate were determined and the influence of ammonium sulfate saturation on salting, and enzyme activity of cold-active cellulase was analyzed. The enzyme solution was desalted by dialysis at 10 °C after salting (dialysis bag: Solarbio-MD34, molecular weight cutoffs 8000-14000, solution was 1/2 in full), frozen at -20 °C for 12 h, and freeze-dried at -50 °C under a vacuum of 100 Pa for 8 h, resulting in lypholyzed cold-active cellulase powder. Cellulase activity was measured by the 3,5-dinitrosalicylic acid (DNS) colorimetric method (Horikoshi *et al*. 1984), and the unit of enzyme activity (U) was defined as the reference (Horikoshi *et al*. 1984), one unit (U) being the amount of enzyme that catalyzed the release of 1 μg glucose equivalent per min, by hydrolysis of CMC-Na, in the given conditions. The measurement was repeated five times.

The enzyme activity and protein content of the fermentation solution, crude enzyme liquid after salting out and desalting, and dissolving solution of the cold-active cellulase powder were measured. Next, the specific activity values of cellulase were calculated .

Immobilization of cold-active cellulase

The cold-active cellulase was immobilized by the sodium alginate-glutaraldehyde cross-linking entrapment method (Catana *et al*. 2005; Pan *et al*. 2007). Using sodium alginate, calcium chloride, and glutaraldehyde as experimental factors, orthogonal tests were designed to obtain the optimal process conditions of immobilization (Table 1). The experimental results were verified under optimal conditions. The measurement was repeated three times.

Table 1. Factors and Levels of Orthogonal Test

Level	Sodium alginate (g/L)	Calcium chloride (g/L)	Glutaraldehyde (v/v; %)	Cross-linking time (h)
1	25	5	0.3	3
2	30	10	0.4	4
3	35	15	0.5	5

The crude enzyme solution was fully mixed with the sodium alginate solution at a ratio of 1:2, glutaraldehyde was added. The mixture was shaken for 30 min at room temperature and left in a refrigerator at 4 °C. The mixture was added to the $CaCl_2$ solution dropwise to form smooth gel beads. The enzymatic activity before and after entrapment in the gel beads was determined by the DNS method, and the recovery rate after immobilization by sodium alginate-glutaraldehyde cross-linking entrapment method was calculated by Eq. (1):

$$\text{Recovery rate of cellulase} = \frac{\text{enzymatic activity of immobilized cellulase (U)}}{\text{enzymatic activity of free cellulase (U)}} \times 100\% \quad (1)$$

Enzymatic kinetics of immobilized cold-active cellulase

The enzymatic activities of immobilized cold-active cellulase were measured at different temperatures (*i.e.*, 10, 20, 30, 40, 50, 60, and 70 °C) and different initial pH (*i.e.*, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, and 11.0) of the reaction system to obtain the optimum reaction temperature and the optimum initial pH of the immobilized cellulase.

The immobilized cellulase under the optimal immobilization conditions was used in the enzymatic hydrolysis reaction that reacted at optimum initial pH and temperature, and carboxymethyl cellulose sodium was used as a substrate for the enzymatic reaction. The immobilized cellulase gel beads were removed by filtration and washed with distilled water after each reaction; the enzymatic reaction was repeated under the same conditions seven times. The relative enzymatic activity in the first enzymatic reaction was defined as 100% and compared to the relative enzymatic activities of each recovered sample to test the stability of immobilized cellulase. The measurement was repeated five times.

Pretreatment of kelp cellulose and low-temperature enzymatic hydrolysis

The pretreatment process of kelp cellulose was performed as follows: Kelp was soaked in sodium hydroxide solution (0.02 g/mL) at a ratio of 1:20 for 48 h at room temperature, and washed to neutral with distilled water. The kelp solution was mixed with water at a ratio of 1:25 and broken up by steam explosion at 121 °C under a pressure of 0.14 MPa for 20 min.

After the pretreatment step, the immobilized cold-active cellulase gel beads were added in to the kelp suspension at 20 U/g. The enzymatic reaction was carried out in a 35 to 40 °C water bath for 72 h.

A thin layer chromatography (TLC) method (Zhang *et al*. 2005) was adopted to measure the results of enzymatic hydrolysis. Enzymolysis liquid was concentrated by rotary concentration at 50 °C after filtration until the concentration of reducing sugars reached 150 mg/mL. Disodium hydrogen phosphate (0.03 M) was used as plate in TLC. The plate was then dried naturally and activated for 1 h in an oven at 100 °C. The concentration of glucose standard solution was 150 mg/mL, and the measurement was repeated five times.

Ethanol fermentation of kelp cellulose

Ethanol fermentation of kelp cellulose used yeast fermentation, with the inoculation amount of yeast was 10% (v/v) with the density of 10^8 per mL. Three influence factors were chosen to analyze their effects on the fermentation process. An optimal combination was obtained to optimize fermentation conditions.

The initial concentrations of glucose were 10, 30, 50, 70, or 90 g/L, the volumes of culture medium were 20%, 30%, 40%, 50%, 60%, or 70% fermented at 30 °C, and the fermentation times were 24, 36, 48, 60, or 70 h.

The ethanol concentrations in the fermentation broth were measured with a GC900A gas chromatograph (GC; Shanghai Tianpu Co., China) under the following conditions: column temperature, 100 °C; FID chamber temperature, 140 °C; injection port temperature, 140 °C; nitrogen gas velocity, 100 mL/min; hydrogen gas velocity, 60 mL/min; air velocity, 500 mL/min; and injection volume, 0.5 μL. The measurement was repeated three times. The ethanol yield was calculated according to Eq. (2):

$$\text{Ethanol yield}\,(\%) = \frac{\text{ethanol production}\,(\text{g}) \times 1000}{\text{glucose concentration in medium}\,(\text{g/L}) \times \text{volume of medium}\,(\text{mL})} \times 100\% \quad (2)$$

RESULTS AND DISCUSSION

Enzymatic Activity of Cold-Active Cellulase

The enzymatic activity of cold-active cellulase that was prepared was 49.7 U/mL, which was measured using the DNS method. This was very close to the cellulase activity from *Trichoderma reesei* CCN 03116 (Ungurean *et al*. 2013).

The protein contents in the precipitate increased continuously when the saturation of ammonium sulphate was being raised from 20% to 40%. The enzyme activity was very low at first, but had no obvious increase (Fig. 1). This shows that the proteins precipitated in this saturation range were mainly superfluous proteins with only a small amount of cellulase. The activity increased faster when the saturation was at 50%, meaning that cellulase had started to precipitate. When the saturation of ammonium sulphate was in the range of 60% to 80%, the enzyme activity was at a high value and increased rapidly, showing that a large amount of cellulase was precipitated from the fermentation broth. When the saturation of ammonium sulphate reached 70%, the protein contents in the precipitate and the change of cellulase activity remained constant, signifying that the cellulase had precipitated sufficiently.

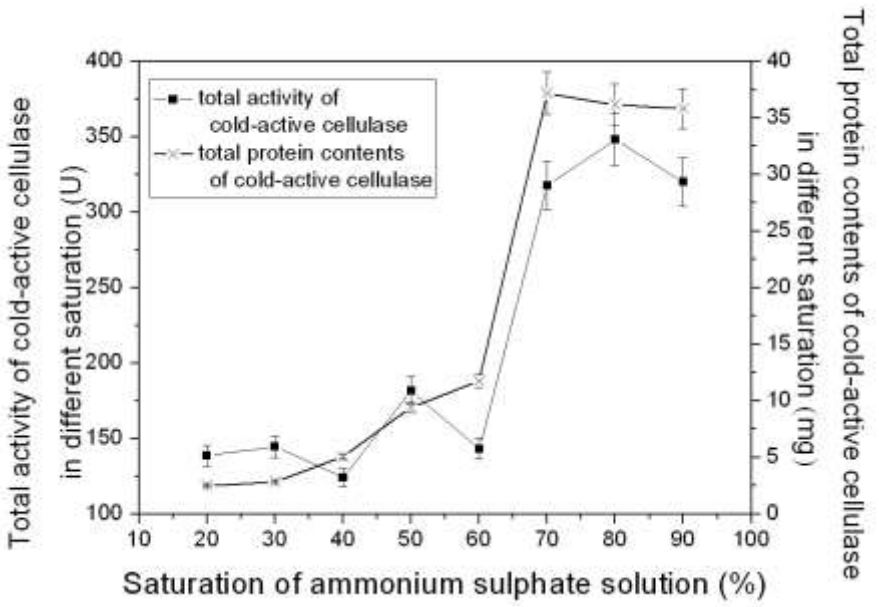

Fig. 1. Salting-out curve of cold-active cellulose. Data presented as average ± standard deviation

By comprehensive consideration, the fermentation liquid was salted out in 50% saturated ammonium sulfate the first time, and the precipitate was centrifuged. After centrifugation, the supernatant was salted out in 80% saturated ammonium sulfate for the second time, and the precipitates were combined and desalted by dialysis after salting out.

Table 2. Activity Recovery Results of the Various Processing Steps

Steps	Total enzyme activities (U)	Total protein Content (mg)	Specific activity of cellulase (U/mg)	Purification fold	Recovery rate (%)
Original broth	4560.89	493.00	9.3	1.00	100
Salting out	3929.35	169.82	23.1	2.50	86
Freeze drying	3831.19	90.06	42.5	4.60	84

Table 2 shows the activity recovery results of the various processing steps. The volume of crude enzyme solution was 200 mL. After preliminary separation and purification, the activity of cellulase increased to 4.6 times that of the original fermentation broth, and the recovery rate reached 84%. Therefore, this separation process improved the activity of cellulase and the recovery rate of enzyme activity appears optimal.

Optimization of Immobilization Process Conditions

As can be seen by the results of the orthogonal experiment (Table 3), the influence order of each factor on the immobilized ratio and recovery rate of enzyme activity during the immobilization process was as follows: concentration of glutaraldehyde > concentration of sodium alginate > concentration of calcium chloride > cross-linking time. The optimal immobilization process conditions of cold-active cellulase were: concentration of sodium alginate, 30 g/L; concentration of calcium chloride, 5 g/L; concentration of glutaraldehyde, 0.4% (v/v); and cross-linking time, 5 h.

Under the above conditions, the recovery rate of activities of immobilized enzyme was 51.58%, and the recovery rate in three validation experiments was 51.58 ± 1.7%.

Table 3. Result of Orthogonal Experiment and Activity Recoveries

Factors	Sodium alginate (g/L)	Calcium chloride (g/L)	Glutaraldehyde (v/v, %)	Cross-linking time (h)	Recovery rate (%)
1	25	5	0.3	3	41.64
2	25	10	0.4	4	49.03
3	25	15	0.5	5	37.33
4	30	5	0.4	5	51.58
5	30	10	0.5	3	41.12
6	30	15	0.3	4	42.87
7	35	5	0.5	4	35.64
8	35	10	0.3	5	41.93
9	35	15	0.4	3	44.56
K1	42.667	42.953	42.147	42.440	
K2	45.190	44.027	48.390	42.513	
K3	40.710	41.587	38.030	43.613	
R	4.480	2.440	10.360	1.173	

The results for immobilized cellulases that used similar immobilization methods (Pan *et al*. 2007; Zhao *et al*. 2007) were analyzed and compared. It was found that the activity of immobilized cellulase was limited by the performance of the immobilization carrier, cross-linking agent, amount of coagulant, and the ratio between enzyme and carrier. There was positive correlation between activities of the enzyme and the concentration of glutaraldehyde, and also between activities of the enzyme and the concentration of sodium alginate.

Sodium alginate is very important as the carrier for enzyme immobilization. The surface of the gel was loose when the concentration of sodium alginate was low, so that the cold-active cellulase could seep out of the gel. On the other hand, the surface of the gel was dense when the concentration was higher, affecting the combination of cellulase and substrate after embedding, thus reducing the activity of cold-active cellulase (Catana *et al*. 2005; Zhao *et al*. 2007). Furthermore, many materials have been used as the carrier in the immobilization of cellulose, such as poly-L-glutamic acid, polyurethane foam, and nanoparticles (Takeuchi and Makino 1987; Chakrabarti and Storey 1988; Li *et al*. 2007; Lupoi and Smith 2011).

Calcium chloride is the coagulant which congealed the colloids of immobilized cellulose, and the concentration of calcium chloride affected the mechanical strength of gels (Pan *et al*. 2007). The pore diameter of mesh on the colloid was large when concentration was low and the cold-active cellulase could seep out of the gel very easily. Alternatively, the pore diameter of mesh on the colloid was small when the concentration was higher so that it affected the combination of cellulase and substrate.

Glutaraldehyde was the cross-linking agent and protein denaturant for the immobilization process (Zhao *et al*. 2007). The activity of cellulase was easy to deactivate when the concentration of glutaraldehyde was high, but there were many free enzymes and the binding rates were lower when the concentration was low so that the

recovery rate of enzyme activity was lower. This was disadvantageous for the recovery of the immobilized cellulase.

The cold-active enzymes were characterized by having a high specific activity at low temperatures with a high catalytic efficiency while having poor thermal stability at the same time (Feller and Gerday 1997; Feller 2003). Therefore, use of the sodium alginate-glutaraldehyde cross-linking-entrapment method was judged to be better for the immobilization of cold-active cellulase, and the effect of the enzymatic reaction at lower temperatures would improve.

Optimum Reaction Temperature, Initial pH, and Thermal Stability of Immobilized Cellulase

As shown in Fig. 2, the optimum reaction temperature of immobilized cold-active cellulase was 40 °C, consistent with that for free cold-active cellulase (Gao *et al*. 2012). The activity of cellulase decreased when the temperature increased beyond 40 °C, but the activity decreased slowly when the temperature was below 60 °C. Compared with the free cellulase, the immobilized cellulase had a better thermal stability. The activity of immobilized cellulase had a high catalytic activity at 60 °C, and the activity was roughly equivalent to that at 30 °C, while the enzyme activity of free cellulase decreased rapidly when the temperature was above 40 °C.

The optimum initial pH of immobilized cold-active cellulase was pH 9.0, and there was higher activity of the cellulose in the pH range of 8.0 to 10.0. Compared with free cellulase, the immobilized enzyme exhibited a wider optimum pH.

In the immobilization, the sodium alginate gel played a protective role for the cellulase, improving the stability of the spatial structure of the enzyme molecules to some extent, improving the tolerance of temperature changes of cold-active cellulase, and reducing the sensitivity to the pH.

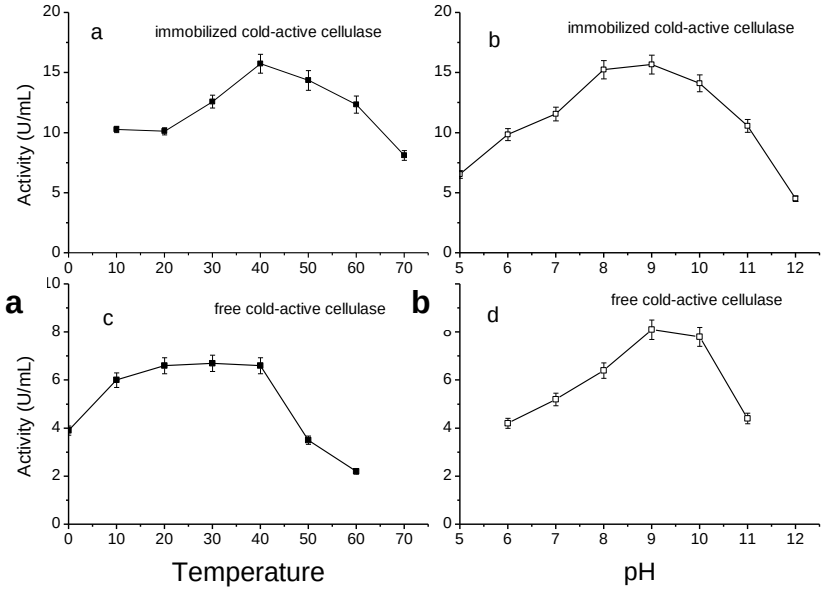

Fig. 2. Effects of temperature (a and c) and pH (b and d) on the activity of immobilized cold-active cellulase and free cold-active cellulase. Data presented as average ± standard deviation

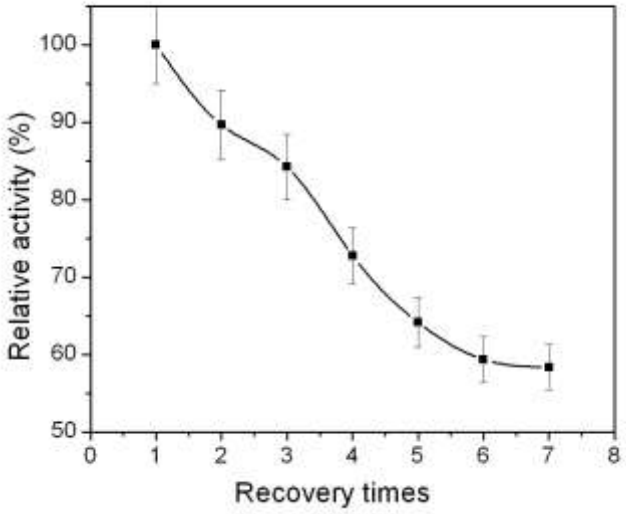

Fig. 3. Stability studies of immobilized cold-active cellulase. Data presented as average ± standard deviation

The relative enzymatic activity of the immobilized cold-active cellulase was 58.37% after having been recovered seven times (Fig. 3). The relative enzymatic activity was maintained at over 80% with three recoveries, after which activity began to decrease with increasing recoveries; the recoveries did remain stable at around 60%. The results of Pan *et al*. (2007) and Zhao *et al*. (2007) showed that the relative enzymatic activity of immobilized β-glucosidase was maintained above 90% after six recoveries. This also explains why a certain gap exists on the recovery of relative enzymatic activity between cold-active enzyme and mesophilic enzymes, which may be related to the stability of the enzyme.

Enzymatic Hydrolysis Results of Kelp Cellulose and Optimized Fermentation Conditions

As shown in the thin layer chromatography results (Table 4), the kelp cellulose was generated to glucose by the enzymatic hydrolysis of cold-active cellulase.

Table 4. Analysis of TLC Results

	1	2	3	4	5	Average Rf values	Standard deviation
Glucose	0.47	0.49	0.49	0.5	0.48	0.486	0.0102
Enzymolysis liquid	0.49	0.47	0.48	0.47	0.50	0.482	0.0117

Influence of Initial Glucose Concentration on Ethanol Yield

As shown in Fig. 4, the ethanol yield increased with an increase of glucose concentration, and it reached the maximum yield when the glucose concentration was 70 g/L. Ethanol yield decreased when the concentration of glucose was too high. This was caused by the metabolism and growth of the yeast, which was inhibited in a high concentration solution.

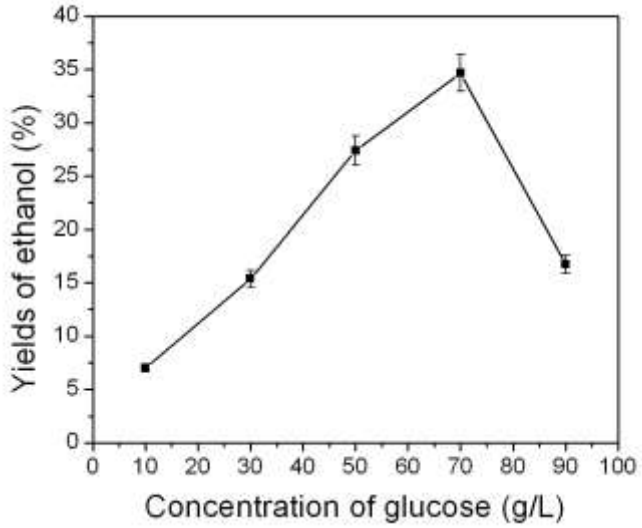

Fig. 4. Influence of glucose concentration on ethanol yields. Data presented as average ± standard deviation

Influence of Medium Volume on Ethanol Yield

The ethanol fermentation medium was prepared with a glucose concentration of 70 g/L. The ethanol production increased with an increase of medium volume, reached maximum yield when the volume was 50% (v/v) (Fig. 5), and then started to decline. Because there was more dissolved oxygen in the medium when the medium volume was lower, the yeast could carry out anaerobic respiration which affected the ethanol production. The dissolved oxygen concentration was too low when the medium volume was too full, so it was not conducive to the growth of yeast, thereby affecting the ethanol yield so that it was reduced.

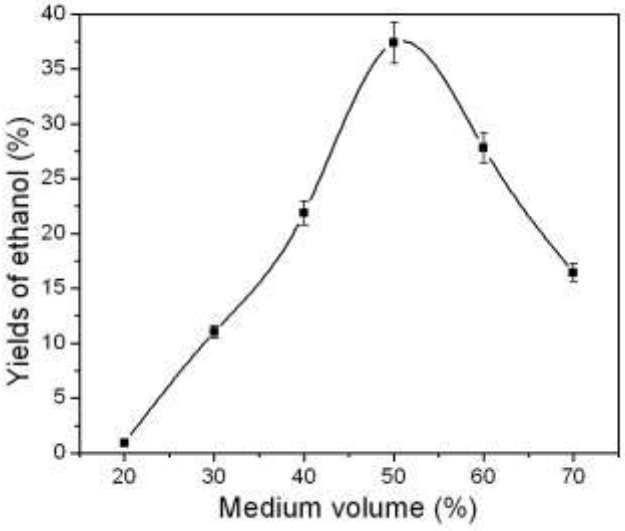

Fig. 5. Influence of medium volume on ethanol yield. Data presented as average ± standard deviation

Influence of Fermentation Time on Ethanol Yield

The ethanol fermentation medium was prepared with a 50% volume of culture medium and a glucose concentration of 70 g/L. The influence of fermentation time on the ethanol yield was also analyzed.

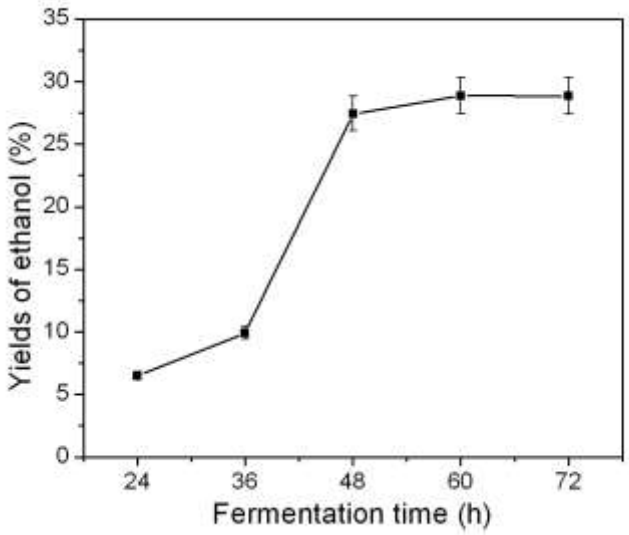

Fig. 6. Influence of fermentation time on ethanol yield. Data presented as average ± standard deviation

Figure 6 shows that the ethanol yields increased with the extension of the fermentation time before 48 h, and they increased more slowly after 48 h, maintaining a certain yield. Glucose and other nutrients were present in sufficient quantities for the rapid growth of yeast at the beginning of fermentation, and thus produced a lot of ethanol. The glucose and other nutrients in the medium were then reduced, causing the growth of yeast to reach an equilibrium level in fermentation anaphase. Therefore, the ethanol yield was gradually reduced and the ethanol content remained stable in the medium. In summary, a higher ethanol yield was reached at 37.37% when the glucose concentration was 70 g/L, the volume was 50% (v/v), and fermentation time was 48 h.

It was shown that the immobilized cold-active cellulase isolated from NJ64 could hydrolyze the kelp cellulose into glucose by this fermentation experiment. The kelp cellulose that was prepared by saccharification from kelp residue contributed to high quality for the kelp cellulosic ethanol, and it provided a new source of raw materials for the future development in the bio-ethanol industry.

A large problem in the simultaneous saccharification and fermentation reactions was that the fermentation temperature of the yeast was low (the optimum temperature of the yeast growth was 25 to 30 °C) but the hydrolysis temperature of the mesophilic cellulase was high (45 to 65 °C). Most of the proposed solutions have been to select an intermediate temperature that not only reduces the activity of cellulase but also affects the efficiency of fermentation. Therefore, studying cold-active cellulase is a good way to solve this problem because the optimum temperature for their activity generally ranges from 20 to 40 °C (Kasana and Gulati 2011), and this is very suitable for yeast growth. Typically, the specific activity of cold-active cellulase is higher than that of their mesophilic counterparts at temperatures of approximately 0 to 30 °C. The mesophilic

cellulase was almost no activity at this low temperature, but at higher temperatures, denaturation of the cold-active cellulase occurs.

The yield of ethanol can be effectively improved by using cold-active cellulase and mesophilic cellulase complexes in cellulosic ethanol production. Lupoi and Smith (2011) studied the use of immobilized cellulase for simultaneous saccharification and fermentation of the cellulosic ethanol process, and their results showed that immobilized cellulase on silica gel can increase the conversion rate of lignocellulosic materials and improve production of cellulose ethanol. So cold-active cellulase can effectively solve the large temperature disparity issue of simultaneous saccharification and fermentation reactions between ethanol yeast fermentation and cellulase hydrolysis.

The use of kelp residue for the production of cellulosic ethanol, which can make full use of the kelp resources, reduces the waste and can also reduce costs to the kelp industry. At the same time, environmental problems, such as the eutrophication of water bodies and red tides, can be avoided by the direct discharge of this industrial residue. At present, most of the materials for the production of cellulosic ethanol come from crop straws and have realized industrial production (Sticklen 2008; Brethauer and Wyman 2010; Wi *et al*. 2013). Compared to the crop straw cellulose ethanol, although the studies of cellulose ethanol from kelp and other algae started relatively late, development is fast and has a broad prospects (Park *et al*. 2009; Jang *et al*. 2012; Van der Wal *et al*. 2013). In addition to the kelp, there are many types of seaweed such as *Ulva* that are also used in the preparation of bio-ethanol (Van der Wal *et al*. 2013). The pretreatment process of kelp residue makes it simpler and easier to hydrolyze the cellulose into glucose (Park *et al*. 2009; Jang *et al*. 2012). Compared to the crop straw, the effect of hydrolysis of kelp was completely and the ethanol concentration was significantly higher under the same condition.

The production of corn ethanol in the U.S. reached 39.49 million tons in 2013, which reduced the pressure of oil supplication effectively (Renewable Fuels Association 2014). Currently, the production of fuel ethanol in the United States and Brazil have accounted for about 10% and 50% of the national gasoline fuel consumption, respectively (Renewable Fuels Association 2010). This solves the problem of energy demand effectively. There are many kelp resources in China, the production of which accounts for about 50% of the global output. The shortage of liquid fuels at present can be solved by using kelp residue for cellulosic ethanol production, which is also beneficial for the conservation of resources, environmental protection, and sustainable development. Therefore, the kelp cellulosic ethanol industry has a large development potential in China.

CONCLUSIONS

1. In this study, cold-active cellulase from the Antarctic psychrophilic bacterium *Pseudoalteromonas* sp. NJ64 was obtained and immobilized by the sodium alginate-glutaraldehyde cross-linking entrapment immobilization method.
2. An enzyme activity of this cold-active cellulase of 49.7 U/mL was realized, and the optimal immobilization conditions were determined. Under the optimal conditions, the recovery rate of the immobilized enzyme activity was 51.58%.

3. The optimum reaction temperature of the immobilized cold-active cellulase was 40 °C and the optimum initial pH was 9.0. Compared to the free cellulase, the immobilized cellulase exhibited a wider optimum and reduced sensitivity to pH.
4. This immobilized cold-active cellulase was used to optimize the kelp residue cellulose bio-ethanol fermentation process, resulting in a higher-purity kelp cellulose ethanol that reached 37.37% when conducted with a glucose concentration of 70 g/L, a 50% (v/v) volume of culture medium, and fermentation time of 48 h. Immobilized cold-active cellulase can effectively hydrolyze the cellulose of kelp residue and therefore, a valuable component of cellulose bio-ethanol production.

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