The Isolation and Performance Studies of an Alginate Degrading and Ethanol Producing Strain

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Bioethanol production from brown algae is a promising way to help solve the energy problem. Alginate is a major component of brown algae, but it cannot be utilized for ethanol fermentation. In order to improve the utilization rate of algae, high alginate fermentation strains should be obtained. In this research, alginate fermentations were carried out and 6 alginate degrading strains were obtained from different samples. The ethanol yield of strain 5 was the highest, which was 0.154 g g^{-1} (ethanol to alginate). Identification of strain 5 revealed that it was 99 % identical to *Meyerozyma guilliermondii*. The transform procedure from alginate to ethanol by *M. guilliermondii* was studied. The crude enzymes of alginate lyase and ethanol dehydrogenase were extracted, and the enzyme activity was measured. The main polysaccharides of brown algae are alginate, laminaran and mannitol. So the fermentation experiments were carried out with different substrates, such as laminaran, mannitol, *Laminaria japonica* and acid hydrolysate of *L. japonica*. The ethanol yield rate of *L. japonica* was the highest, reaching 0.237 g g^{-1} ethanol to *L. japonica*. This showed that strain 5 might be a promising strain for ethanol production from algae.

Key words:

alginate fermentation strains, ethanol production, ethanol dehydrogenase, alginate lyase

Introduction

With the limited fossil fuel resources and aggravating energy crisis, coupled with the concern about the climate change caused by greenhouse gases, many people hope that renewable fuels will be developed as an alternative to fossil fuels, with special attention being paid to bioethanol.[1,2] Compared to fossil fuels, biofuels emit less ozone, benzene, carbon dioxide and other harmful pollutants. For a long time, bioethanol has been raising world-wide attention and many researchers are searching for alternative biomass sources for the production of bioethanol, such as corn,[4] wood,[5] sugarcane[6] switch grass,[7] rice straw,[8] corn straw[9] and wheat straw.[10]

Today, about 30 % of the corn currently grown is used for ethanol production, and more corn is needed to meet the increasing demand for bioethanol.[11] The higher amounts of corn turned to biofuel production could have devastating effects on food supply around the world and cause conflicts in the food vs. fuel dilemma.

Ethanol production from lignocellulose is a promising alternative but the current technologies for lignocellulose fermentation have to overcome the cost of the complex processes needed to release simple sugars from recalcitrant polysaccharides.[12] With limited land area, pretreatment technical difficulties and low conversion rate, much more needs to be done in bioethanol production from lignocellulose. And the increasing need for energy consumption is expected to continue as the world's population is expected to increase. In order to meet the expected increasing demand for bioethanol, there is a need to find alternative biomass sources, particularly those that do not rely on using large amounts of agricultural land.

Marine algae are attractive renewable energy resources due to their abundance, high photosynthetic efficiency and production rate. Algae contain a low concentration of lignin and sugars can be easily released by simple operations such as milling or crushing, so seaweeds are proposed as one of the most promising biomass materials for ethanol production.[13] Marine algae are classified into three groups by their colors: green, brown, and red. Brown algae, as the second most abundant marine biomass, have several key features of an ideal feedstock for biofuel production. They do not require arable land, fertilizer, or fresh water, they are of

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high photosynthetic efficiency and production rate, and free of land management and adverse impacts on food supplies.[14] All these features make them an attractive alternative resource.

Brown algae are a fast growing biomass with production cycles of 4–6 times annually.[15] According to a report, the optimum bioethanol productivity from algae can be approximately twice higher than ethanol productivity from sugarcane and 5 times higher than that from corn.[16] Brown algae contain about 30–67 % carbohydrate by dry weight, and the main components of polysaccharides are alginate, laminaran, and mannitol.[17] Laminaran and mannitol can be easily used by microbes and converted into bioethanol, but industrial microbes cannot degrade alginate as a carbon source. The potential of brown algae to produce bioethanol cannot be fully exhibited.[18,19]

Alginate is a linear block copolymer of two uronic acids,[20,21] β-D-mannuronate (M) and α-L-guluronate (G), arranged in varying sequences, with uniform regions of M (poly-M) or G (poly-G), and a mixture of M and G (poly-MG). To utilize alginate, a series of lyases are needed, such as alginate lyase and ethanol dehydrogenase. But present ethanol fermentation microbes cannot degrade alginate. Gene engineering might be a good way of improving the conversion rate of alginate. Takeda *et al.* developed an integrated bacterial system for converting alginate to ethanol using a metabolically modified, alginate-assimilating, pit-forming bacterium, *Sphingomonas sp.* A1, which accumulated 13.0 g L^{-1} ethanol in 3 d using alginate as the sole carbon source.[11] But there were still drawbacks in the genetically modified strain, because ethanol from this strain was produced under aerobic conditions, and the oxygen supply should be strictly controlled to maintain a balance between energy and ethanol production, since an oversupply of oxygen would lead to low ethanol production. Wargacki integrated a fragment of gene to *E. coli* for alginate transport and metabolism, together with an engineered system for extracellular alginate depolymerization, generating a microbial platform that can simultaneously degrade, uptake, and metabolize alginate. The ethanol yield reached 0.281 g ethanol g^{-1} dry macroalgae.[14] These genetically modified microbes could achieve the goal of utilizing alginate for bioethanol production, and researches on the metabolic pathway of alginate were conducted.

Now many researchers are focused on the genetically modified microbe for alginate fermentation, but research on the naturally existing microorganism for alginate fermentation is scarce. In order to achieve effective fermentation, a careful selection of naturally existing microorganisms needs to be made, and it is necessary to study the metabolic mechanism. However, no relative reports are available at present. The aim of this research was to isolate one or more strains that can utilize alginate as the only carbon source and convert alginate into bioethanol in fermentation, and more researches on the metabolic mechanism and modification should be conducted carefully.

Materials and methods

Materials

All chemicals of analytical reagent grade were purchased from Beijing Chemical Factory (Beijing, China). All the biochemical reagents were bought form Beijing Biological Technology Factory (Beijing, China). The strains were stored in the tube culture at 4 °C. General biological equipment was purchased from Shanghai Precision Instrument Co., LTD (Shanghai, China).

Culture media and microorganism culture

Yeast Extract Peptone Dextrose Medium (YPD medium) was used as the enrichment medium containing 10 g L^{-1} yeast extract, 20 g L^{-1} peptone and 20 g L^{-1} glucose. The pH of YPD medium was adjusted to 4–5. The agar (2 %) was added to the YPD medium to make YPD solid culture medium. The composition of solid selection culture medium was as follows: 10 g L^{-1} alginate, 1.0 g L^{-1} KNO_3, 0.5 g L^{-1} K_2HPO_4, 1.5 g L^{-1} NaCl, 0.5 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.01 g L^{-1} $FeSO_4$, 2 % agar, and the pH was adjusted to 4–5. Triphenyltetrazolium chloride (TTC) upper-part culture medium consisted of 0.5 g L^{-1} TTC, 10 g L^{-1} alginate, 20 g L^{-1} agar; and the TTC lower-part culture medium was the same as solid selection culture medium. The composition of agar slant culture medium was: 2 % peptone, 1 % yeast extract, 2 % alginate, and 2 % agar. The composition of fermentation culture medium was: 10.8 g L^{-1} $(NH_4)_2SO_4$, 5.0 g L^{-1} KH_2PO_4, 1.1 g L^{-1} $MgSO_4 \cdot 7H_2O$, and 20 g L^{-1} alginate. Different carbon sources such as laminaran, mannitol, *L. japonica* and *L. japonica* acid hydrolysate (all at 20 g L^{-1}) were then tested to examine the fermentation performance.

Isolation and identification of efficient alginate-fermentation strains

Different samples were obtained from the Bohai Sea (Qinhuangdao, Hebei province, China), the wine lees of China Great Wall Wine Company (Shacheng, Hebei province, China), and the soil (Yanshan University, Qinhuangdao, Hebei province, China).

Samples were marked and stored at 4 °C. After enrichment in YPD culture medium for 48 h at 30 °C, 1 mL inoculum was diluted 10 times to 10^8 times, then dilution from 10^{-5} to 10^{-8} was smeared onto the solid selection culture medium and cultured in the incubator at 30 °C for 3 d with alginate as the only carbon source. The strains with different morphologies were purified and examined by microscope, and stored at 4 °C.

The strains obtained from the alginate selection were cultured on the TTC lower-part culture medium, each strain was made into two copies and cultured in the incubator at 30 °C for 3 d. One of the copies was then covered by TTC upper-part culture medium, and cultured for about 3 h; then the color of the colonies turned red, and the redder the colony was, the more dehydrogenase with high activity the strain produced. If all the colonies turned red also meant that the strain was purified. Strain weed from the other dish, which was not covered with TTC upper-part culture medium, were stored in agar slant culture medium at 4 °C.

The genomic DNA of strain 5 was extracted by the fungi DNA extraction kit. The 26S rDNA[22] was amplified using the primers: ITS1: TCCGTAGGTGAACCTGCGG, ITS4: TCCTCCGCTTATTGATATGC. PCR amplification was performed under the following conditions: 3 minutes at 95 °C; 30 cycles of 30 s at 95 °C, 30 s at 55 °C and 40 s at 72 °C, plus an additional 5 minutes cycle at 72 °C. The automatic sequence was carried out by Beijing Sun Biotech Co., Ltd. The 26S rDNA sequence was checked in GeneBank.

Ethanol production experiments from alginate

Ethanol production experiments were carried out with alginate as the only carbon source. Strains with high dehydrogenase activity were first activated in YPD broth for 24 h in a shaking incubator at 150 rpm, 30 °C, and then inoculated to the fermentation medium with inoculum amount of 5 %. All the fermentation experiments were carried out in 150 mL flasks with 50 mL working medium, with an initial pH from 3.0 to 7.0. The flasks were autoclaved at 121 °C for 15 minutes, inoculated with various strains after cooling to room temperature, and then capped with butyl rubber covers to create an anaerobic condition. All the flasks were cultured in a shaking incubator, with culture conditions as follows: 150 rpm for 96 h. The effect of temperature on ethanol production was studied. Experiments were performed in the fermentation medium at temperature varied from 20 °C to 40 °C. Fermentation conditions were as follows: inoculation amount of 5 %; initial pH 6.0; rotation speed of 150 rpm and initial sugar concentration of 20 g L^{-1}. The other components remained unchanged as in the fermentation medium; it was also the same with the other experiments below. Ethanol concentration was determined after fermentation for 96 h. To determine the optimum carbon source concentration, the effect of alginate concentration on ethanol production was investigated at initial pH 6.0, 30 °C, 150 rpm, and inoculation amount of 5 %. The alginate concentration was varied from 5 g L^{-1} to 70 g L^{-1}. Ethanol concentration was determined after fermentation of 96 h. In order to optimize the pH value in the ethanol fermentation process, fermentation experiments were conducted with fermentation conditions as follows: inoculation amount, 5 %; fermentation temperature, 30 °C; rotation speed of 150 rpm, and initial sugar concentration of 20 g L^{-1}. The pH was from 3 to 7. Ethanol concentration was determined after fermentation of 96 h.

Alginate lyase and dehydrogenase activity

Alginate is a major polysaccharide in brown algae, but no known ethanol producing strain can utilize alginate for ethanol production. In order to convert alginate to bioethanol, alginate fermentation strains should express alginate lyase and dehydrogenase at the same time.

Alginate lyase was obtained and the enzyme activity was measured according to the reference.[23] After culturing for 3 d in the shaking incubator (30 °C, 150 rpm), the fermentation liquor was centrifuged at 5000 rpm for 10 min, the supernatant was crude alginate lyase. 0.1 mL; 0.2 mL; 0.3 mL; 0..4 mL; 0.5 mL crude alginate lyase was added to 0.9 mL; 0.8 mL; 0.7 mL; 0.6 mL; 0.5 mL alginate substrate (dissolved in 0.05 mol L^{-1} K-phosphate buffer) respectively, mixed and kept in 40 °C water for 10 minutes and then put into boiling water to deactivate the enzyme. The reducing sugar produced was measured by DNS method, with alginate lyase replaced by ionized water in the control group.

Dehydrogenase was obtained and the enzyme activity was measured according to a refined method based on reference.[24] After culturing for 3 d in the shaking incubator (30 °C, 150 rpm), 40 mL fermentation liquor was centrifuged at 5000 rpm for 5 minutes, the cells were washed and suspended in 20 mL K-phosphate buffer (0.05 mol L^{-1}, pH8.0). Added with 200 μL 32 mg mL^{-1} lysozyme, and kept at 37 °C for 30 minutes. The beaker containing the solution was put in iced water and the cells were disrupted by ultrasonic cell disintegrator. The procedure was: ultrasonic disruption for 1 s, pause 3 s, and the entire procedure lasted 15 minutes at power of 320 W. The solution was then centrifuged at 10000 rpm for 15 minutes; the supernatant was kept and stored at 4 °C. Enzyme activity was measured according to the reference.

Fermentation with different substrates

The purpose of this research was to isolate strains that could ferment alginate into ethanol, but it was also important to utilize other components of the brown algae. *L. japonica,* for example, contains mainly alginate, laminaran, and mannitol, and it is important to degrade all the components. Fermentation was carried out with different substrates, such as laminaran, mannitol, *L. japonica* and *L. japonica* acid hydrolysate (all 20 g L^{-1}), while the other components (10.8 g L^{-1} $(NH_4)_2SO_4$, 5.0 g L^{-1} KH_2PO_4, 1.1 g L^{-1} $MgSO_4 \cdot 7H_2O$) remained the same.

L. japonica was obtained in dry state from a market in Qinhuangdao, China. The sample was finely grinded down with a ball miller and stored at 4 °C. *L. japonica* acid hydrolysate was obtained with 1 % concentrated sulfuric acid in the solution, and heated to 121 °C for 15 minutes.

All experiments were carried out in duplicate and in the same conditions as the alginate fermentation (150 rpm, 30 °C, 4 d, and pH 6.0), with 50 mL working medium in 150 mL flasks. The ethanol concentrations were measured and compared with other strains.

Analytical methods

The fermentation liquor was centrifuged at 5000 rpm for 10 minutes, and the supernatant diluted 50 times and analyzed to determine the reducing sugars by the DNS (dinitrosalicylic acid) method.[25] The content of soluble sugar was measured by the commonly used phenol-sulfate method. The solution was determined through the 722S spectrophotometer (Shanghai Precision Instrument Co. Ltd. (Shanghai, China)) at 520 nm with distilled water as control group. The fermentation broth was distilled first and then ethanol concentration was determined by sulfuric acid-potassium bichromate method.[26] All experiments were carried out four times.

The pH was measured in a pH-meter (PHS-3B, Shanghai Precision & Scientific Instrument Co. Ltd, Shanghai, China). Cell growth in the YPD culture medium was measured with a spectrophotometer (Shanghai Precision Instrument Co. Ltd. (Shanghai, China)) at 600 nm. The identification of strain 5 was carried out through 26S rDNA sequence by Beijing Sunbiotech Co. Ltd. The sugar to ethanol yield in the ethanol fermentation experiments was calculated as:

$$E_r = \gamma_e/\gamma_1$$

where γ_1 was sugar concentration (g L^{-1}) before fermentation, γ_e was the ethanol concentration (g L^{-1}) after fermentation.

Results and discussion

The isolation of effective alginate fermentation strain

Different samples were obtained from the Bohai Sea, the wine lees of China Great Wall Wine Company, and the soil. The sample were first enriched in YPD medium for 48 h at 30 °C, and then cultured with alginate as the only carbon source in an incubator at 30 °C for 3 d, strains colonies showed up and were purified. Nine strains were obtained in this part and stored at 4 °C.

The nine strains were then cultured on TTC lower-part culture medium at 30 °C for 3 d in an incubator, each strain was made into two copies. One of the copies was covered by TTC upper-part culture medium, and cultured for 3 h at 30 °C. Colonies of six strains turned red indicating that the strains were purified and could express dehydrogenase.

Identification and growth curve of alginate fermentation strain

Six strains were first enriched in YPD medium and then inoculated to fermentation medium in the amount of 5 %, all the fermentation experiments were carried out in 150 mL flasks with 50 mL working medium, with an initial pH of 6.0 without additional pH control during fermentation. All the flasks were cultured in a shaking incubator, with culture conditions as follows: 150 rpm, 30 °C for 96 h.

The reducing sugar and ethanol concentration after fermentation were measured and the results are shown in Table 1. According to the results, strain 5 and strain 2 had higher ethanol concentration under the same conditions, strain 1 and strain 4 had higher reducing sugar concentration, while strain 5 had the highest ethanol concentration and yield of 0.139 g g^{-1} (ethanol to alginate). However,

Table 1 – *Reducing sugar and ethanol content in the fermentation broth of different strains (numbers in parentheses correspond to the number of measurements performed for the determination of the mean values and standard deviations)*

Strain	Original alginate concentration (g L^{-1})	Reducing sugar content (g L^{-1})	Ethanol concentration (g L^{-1})
Strain 1	20	0.835±0.064(4)	0.312±0.049(4)
Strain 2	20	0.740±0.053(4)	2.169±0.115(4)
Strain 3	20	0.599±0.088(4)	1.224±0.104(4)
Strain 4	20	0.905±0.093(4)	1.912±0.067(4)
Strain 5	20	0.222±0.058(4)	2.783±0.127(4)
Strain 6	20	0.104±0.062(4)	1.628±0.125(4)

the fermentation broth of strain 5 had a low reducing sugar concentration, maybe the reducing sugar was converted to ethanol or the alginate lyase activity was relatively low, which affected the ethanol production. Strain 2 had a relatively high reducing sugar concentration, but the ethanol yield was low. Strain 5 was chosen as the optimal strain and stored at 4 °C. Strain 5 was taken from the sample of the wine lees of China Great Wall Wine Company.

The identification of strain 5 was carried out through 26S rDNA sequence by Beijing Sunbiotech Co. Ltd., which showed that strain 5 was 99 % identical to *M. guilliermondii* and the Accession No. was EF375700.

The growth curve of strain 5 was measured and created. As shown in Fig. 1, the lag phase was 0~4 h, and the exponential phase 4~24 h. After 50 h, the amount of cells declined. The best period for activation of the weed was 12~24 h, so the yeast suspension cultured overnight could be used for inoculation. The relationship between fermentation time and ethanol yield was also measured and the ethanol concentration increased when the fermentation time was prolonged. The best fermentation time was 96 h, which was the same as the reference used.[19] The ethanol yield of 24 h, 48 h, 96 h and 108 h was respectively 0.059 g g^{-1}, 0.09 g g^{-1}, 0.139 g g^{-1} and 0.136 g g^{-1}.

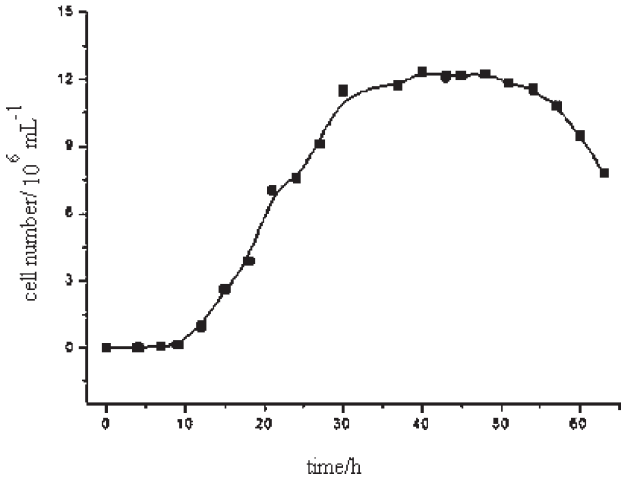

Fig. 1 – *Growth curve of strain 5*

Ethanol fermentation from alginate by strain 5

In our research, strain 5 was isolated from the wine lees and it could transform alginate to ethanol. Different influencing factors on ethanol yield were studied. There were many factors that influenced the ethanol yield and fermentation rate in the fermentation process from sugar with microorganism, such as pH, sugar content, and temperature.

Effect of temperature on ethanol fermentation

The effect of temperature on ethanol production was studied. Experiments were performed in the fermentation medium at temperatures varied from 20 °C to 40 °C. Fermentation conditions were as follows: inoculation amount of 5 %; initial pH 6.0; rotating speed of 150 rpm, and initial sugar concentration of 20 g L^{-1}. The other components remain unchanged as in the fermentation medium, and it was also the same with the other experiments below. Ethanol concentration was determined after 96 h fermentation. The results are shown in Fig. 2. In this research, the ethanol yield rate was the highest at temperature of 30 °C.

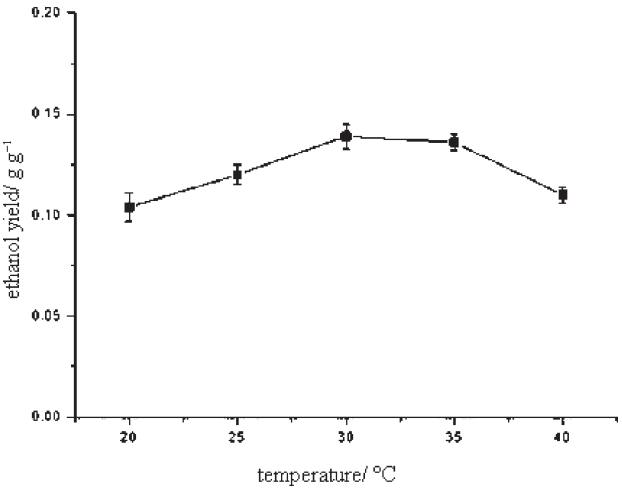

Fig. 2 – *Effect of temperature on ethanol fermentation performance (inoculation amount 5 %; initial pH 6.0; rotating speed 150 rpm, and initial sugar concentration 20 g L^{-1}, temperature varied)*

Temperature affects the activity of the enzyme and changes the rate of catalytic reaction. For instance, the dehydrogenase activity was affected by temperature and the optimum temperature was from 29 °C to 35 °C,[27] which may have brought about higher ethanol yield. At the same time, the heat resisting property of yeast was always poor.[9] At the appropriate temperature, the microorganism can achieve the fastest growth speed and reproduction rate. The temperature can also affect the fluidity of the cell membrane and the transport of substances, therefore the change in temperature has some effect on the absorption of nutrients and secretion of metabolites.

Effect of nutrient conditions on ethanol fermentation

Nutrient conditions played an important role in the production of ethanol. To determine the optimum carbon source concentration, the effect of alginate concentration on ethanol production was in-

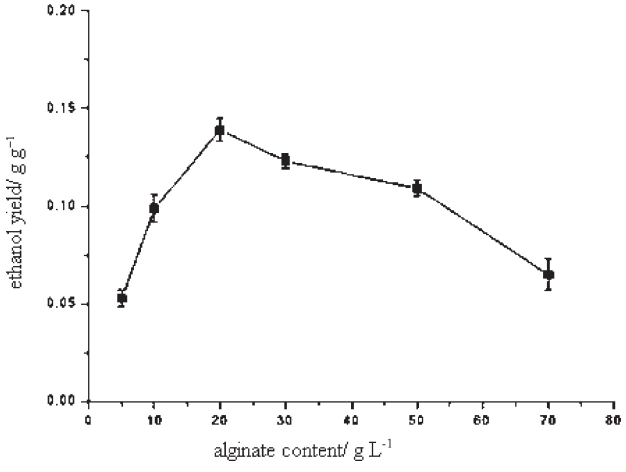

Fig. 3 – *Effect of alginate content on ethanol fermentation performance (initial pH 6.0, 30 °C, 150 rpm, and inoculation amount 5 %, alginate content varied)*

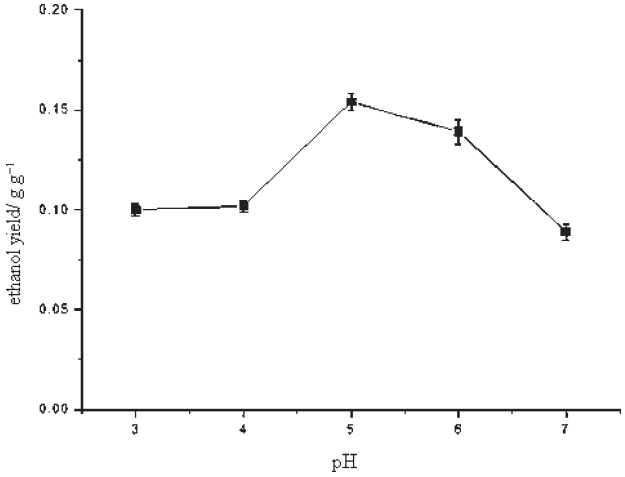

Fig. 4 – *Effect of pH on ethanol fermentation performance (inoculation amount 5 %; 30 °C; rotating speed 150 rpm, and initial sugar concentration 20 g L^{-1}, pH varied)*

vestigated at initial pH 6.0, 30 °C, 150 rpm, and inoculation amount of 5 %. As shown in Fig. 3, ethanol production rose at first with initial alginate concentrations less than 20 g L^{-1}, and at higher concentrations, utilization of alginate was inhibited, the ethanol production decreased. The optimal concentration of alginate content for ethanol production was nearly 20 g L^{-1}. The solubility of alginate was low and therefore, the increase in SS in the fermentation broth affected the respiration of yeast.[28] A high concentration of alginate would cause high viscosity in the broth, thus leading to the high transfer resistance and the heterogeneity of cell metabolism.

Effect of pH on ethanol fermentation

In order to optimize the pH value in the ethanol fermentation process, fermentation experiments were conducted with fermentation conditions as follows: inoculation amount 5 %; fermentation temperature 30 °C; rotating speed of 150 rpm, and initial sugar concentration of 20 g L^{-1}. The pH ranged from 3 to 7. The influence of pH on ethanol production is shown in Fig. 4. Ethanol concentration was determined after ethanol fermentation. Judging by Fig. 4, a pH of 5.0–6.0 was considered suitable for ethanol fermentation. At pH 5.0, the highest ethanol yield could be obtained. The reason for this was that the pH could affect the activity of the enzyme which could inhibit the fermentation.[9] At the same time, high pH increased the opportunity of contamination.

According to the experiments, the optimum alginate fermentation conditions were as follows: the fermentation medium with 20 g L^{-1} alginate, initial pH at 5.0, and 30 °C. At pH 5.0, the highest ethanol concentration was obtained, which reached 3.073 g L^{-1}, and a yield rate of 0.154 g g^{-1}.

Activity of alginate lyase and dehydrogenase

The metabolism procedure of alginate for ethanol production in yeast was studied. The crude enzymes of alginate lyase and ethanol dehydrogenase were extracted, and the enzyme activity measured.

Alginate is a major polysaccharide in brown algae, but no known natural hydrolase depolymerizes alginate. Although several bacteria express alginate lyase and can assimilate alginate, existing ethanol fermentation microbes cannot degrade alginate as carbon sources.[29] In order to convert alginate to bioethanol, alginate fermentation strains should express alginate lyase and dehydrogenase at the same time.

The alginate lyase activity was about 40.00 U mL^{-1}, compared to 12.79 U mL^{-1} in the reference,[23] alginate lyase of strain 5 was much higher indicating that strain 5 might be a promising strain for the conversion of alginate to bioethanol.

Enzyme activity of ethanol dehydrogenase was 126.25 U mL^{-1}, in our experiments the dehydrogenase was obtained from cells in 40 mL fermentation culture, and prepared in 20 mL K-phosphate buffer (0.05 mol L^{-1}, pH 8.0). While in the inference 20 g abandoned beer yeast was suspended in 40 mL K-phosphate buffer (0.08 mol L^{-1}, pH 8.5), and according to the reference, the highest activity was 1806 U mL^{-1}.[24] Cells in 40 mL fermentation culture were far less than 20 g, so the activity of dehydrogenase of strain 5 was actually higher.

Ethanol fermentation from different substrates by strain 5

In order to study the fermentation performance of strain 5 by different substrates, ethanol fermentation experiments were carried out. Table 3 shows the results of ethanol production. The ethanol pro-

Table 2 – *Ethanol yield rates of different substrates of strain 5 and comparison with other strains*

Ethanol yield rate (g g^{-1})	Substrate				
	alginate	laminaran	mannitol	*L. japonica*	acid hydrolysate of *L. japonica*
Meyerozyma guilliermondii (strain 5)	0.139	0.076	0.014	0.237	0.034
Pichia stipitis (KCTC7228)	0.0002	0.0002	0.0278	0.029	
Debaryomyces occidentalis (KCTC7196)	0.0006	0.0022	0.0308	0.1086	

Table 3 – *Soluble sugar and reducing sugar content of L. japonica and acid hydrolysate of L. japonica*

	L. japonica (20 g L^{-1})	Acid hydrolysate of *L. japonica* (20 g L^{-1})
soluble sugar (mg L^{-1})	130.12	327.75
reducing sugar (mg L^{-1})	0.14	109.50
soluble sugar (autoclaved) (mg L^{-1})	418.75	747.28
reducing sugar (autoclaved) (mg L^{-1})	21.31	20.13

duction of strain 5 with different substrates is shown. The maximum ethanol production yield was 0.237 g per 1 g substrate. While in the former report, fermentation for different components of brown-algae using various yeasts gave us a look into the bioethanol production capacity, two strains with relatively higher ethanol yield rates are shown in Table 2; ethanol yield rates of *Pichia stipitis* (KCTC7228) and *Debaryomyces occidentalis* (KCTC7196) from *L. japonica* was 0.029 and 0.1086, respectively.[19]

As shown in Table 2, *P. stipitis* (KCTC7228) *and D. occidentalis* (KCTC7196) had better fermentation performance with mannitol. But *M. guilliermondii* (strain 5) could utilize alginate and *L. japonica* at a relatively higher ethanol yield rate, indicating *M. guilliermondii* (strain 5) might be a promising ethanol-producing strain. In our research, strain 5 could utilize alginate and laminaran, but the yield of mannitol was poor; however, the ethanol yield of *L. japonica* was higher. The same phenomenon was observed in the reference.[14,19] That was because the catabolic pathway of alginate provides both an additional source of sugars and a counterbalance to the excess-reducing equivalents produced by mannitol catabolism, enabling ethanol fermentation from all three sugar components in macro-algae simultaneously.[14] Table 3 shows that alginate was partly degraded in the autoclaved procedure, and acid hydrolysate could depolymerize alginate, but the reducing sugar was damaged under the high temperature and certain toxic materials may have been produced, which made it difficult to produce ethanol. Softer sterilization conditions should be applied.

Others researchers have previously reported ethanol production with alginate as substrate. Wargacki generated an engineered microbial platform encoding enzymes for alginate transport and metabolism, achieving a titer of 4.7 % volume/volume and a yield of 0.281 weight ethanol/weight dry macroalgae.[14] Takeda developed an integrated bacterial system for converting alginate to ethanol under aerobic conditions, but with strictly controlled oxygen supply, ethanol accumulated to 13.0 g L^{-1} in 3 d with 0.05 g mL^{-1} sodium alginate as the sole carbon source.[18] Compared to these gene-modified strains, ethanol production rate of strain 5 reached 0.237 g per 1 g substrate, and it might be a promising strain for ethanol production of brown algae.

Conclusion

In general, brown algae is a promising biomass for bioethanol production, but one of the most important components, alginate, cannot be utilized by industrial microbes to improve the utilization rate of brown algae and the ethanol production concentration, high alginate fermentation performance strains should be available. In this research, alginate degrading strains were isolated from wine lees with alginate as the only carbon source; several strains that showed high fermentation ability were obtained through TTC selection experiments and fermentation experiments. The total reducing sugars and ethanol concentrations were measured, and the ethanol yield rate of strain 5 was the highest, which was 0.154 g g^{-1} (ethanol to alginate). The identification of strain 5 was carried out through 26S rDNA sequence, which showed that strain 5 was 99 % identical to *M. guilliermondii* and the Accession No. was EF375700. The growth curve of strain 5 was measured and created. The crude enzymes of alginate lyase and ethanol dehydrogenase were extracted, and enzyme activity measured at 40.00 U mL^{-1} and 126.25 U mL^{-1}, respectively. Fermentation experiments were carried out with different substrates, such as laminaran, mannitol, *L. japonica* and *L. japonica* acid hydrolysate. The ethanol yield rate of *L. japonica* was the highest, which reached

0.237 g ethanol per substrate. This showed that strain 5 could converse alginate to ethanol at a relatively high yield rate, and might be a promising bioethanol producing strain. We believe more research should be carried out on this strain.

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