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Continuous production of bioethanol using microalgal sugars extracted from *Nannochloropsis gaditana*

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Abstract−We developed a continuous production process of bioethanol from sugars extracted from *Nannochloropsis gaditana*. To improve algal sugar production, the reaction conditions of acid-thermal hydrolysis were investigated based on five different types of acid and their concentrations (1-4%), and the loading ratio of solid/liquid (S/L). As a result, the maximum hydrolysis efficiency (92.82%) was achieved under 2% hydrochloric acid with 100 g/L biomass loading at 121 °C for 15 min. The hydrolysates obtained from *N. gaditana* were applied to the main medium of *Bretthanomyces custersii* H1-603 for bioethanol production. The maximum bioethanol production and yield by the microalgal hydrolysate were found to be 4.84 g/L and 0.37 g/g, respectively. In addition, the cell immobilization of *B. custersii* was carried out using sodium alginate, and the effect of the volume ratio of cell/sodium alginate on bioethanol productivity was investigated in a batch system. The optimal ratio was determined as 2 (v/v), and the immobilized cell beads were applied in the continuous stirred tank reactor (CSTR). Continuous ethanol production was performed using both free cells and immobilized cells at 1 L CSTR. In both groups, the maximum bioethanol production and yield were achieved at dilution rate of 0.04 h^{-1} (3.93 g/L and 0.3 g/g by free cell, and 3.68 g/L and 0.28 g/g by immobilized cell, respectively).

Keywords: Bioethanol, CSTR, Fermentation, Hydrolysis, *Nannochloropsis gaditana*

INTRODUCTION

Research on the development of renewable energy sources is attracting worldwide attention due to the environmental damage and inevitable depletion caused by the indiscriminate use of fossil fuels. Bioenergy, as an alternative and renewable energy, has gained global attention for its benefits of both environmental protection and agricultural economy. In various types of bioenergy, bioethanol has been received high attention as an alternative to gasoline, the fossil fuel with the highest usage [1-5].

The use of the third generation biomass has become a popular topic in recent studies on the bioethanol process, especially, for the development of technologies to produce fuel from marine biomass instead of lignocellulosic biomass [6]. Among the many types of marine biomass available, microalgae have been recognized as the next generation biomass for the production of biofuels, which reduces carbon dioxide through biotransformation [7,8]. Compared with the first and second generation biomass, microalgae have shown higher performance with large amounts of lipids and polysaccharide, which are two major feedstock candidates for biofuel conversion. In particular, microalgae are considered as a sustainable biomass because of the large amounts around the world, as well as the high growth rate [9,10]. Due to these advantages, biofuel conversion using microalgae has recently attracted attention, and many studies have focused on the production of biodiesel by extracting lipids from microalgae. However, since the polysaccharide among the cell debris of the microalgae remaining after the lipid extraction can be converted into sugars, a new possibility that can be applied to the bioethanol fermentation has been suggested, and researches on this field are being conducted actively.

Microalgae for biofuels are cultivated in an incubator or large outdoor, and the efficiency of growth, harvesting, dewatering, and extraction depends on the composition and chemical structure of the cell wall. The cell wall serves as a buffering barrier between the external environment and the living protoplast, which not only protects the cell from external environmental pressure, but also acts as a mass transfer barrier to dehydration and extraction. According to Scholz et al. [11], the cell wall of *Nannochloropsis gaditana* is composed of a bilayer structure protected by a cellulosic inner wall (~75% of the mass balance) and an outer hydrophobic algaenan layer. They determined the inner layer to be mainly cellulose and identified as 1,4-linked glucopyranosyl (78%) and terminal glucopyranosyl (8%) linkages. The outer wall contains algaenan, an enzymatically and chemically resistant aliphatic material, which can be derived from distinct biochemical pathways in other organisms. Therefore, thermo-chemical treatment processes are necessary to obtain fermentable sugars from microalgae with high efficiency since various components constituting the cell wall are linked in complex.

We used *N. gaditana* was a microalgal biomass to produce bioeth-

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anol by a biological conversion process. To improve the sugar production from *N. gaditana*, the conditions of acid-thermal reaction such as the type of acid and ratio of solid/liquid (S/L) were investigated. In addition, the hydrolysate by acid hydrolysis was utilized as a carbon source for bioethanol production in a cultural medium, and the fermentation was carried out using *Bretthanomyces* strain, which converts the substrates of glucose and galactose to bioethanol. In particular, a continuous stirred tank reactor (CSTR) system using cell encapsulated in sodium alginate is the original application toward the continuous production of bioethanol from microalgal hydrolysate.

EXPERIMENTAL

1. Algal Biomass and Reagents

Algal biomass (*Nannochloropsis gaditana*) dried powder was obtained from WUDI LV QI Bioengineering Co., LTD (China). For the acid hydrolysis process sulfuric acid (95% H_2SO_4), hydrochloric acid (37% HCl), nitric acid (70% HNO_3), phosphoric acid (85% H_3PO_4), and peracetic acid (39% CH_3CO_3H), were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of reagent grade.

2. Analysis of Algal Biomass Composition

The solid algal biomass was analyzed to determine its absolute composition (carbohydrate, fiber, protein, lipid, and ash), according to the standard procedures of the National Renewable Energy Laboratory (NREL, USA) and the Official Methods of Analysis of AOAC International (OMA) [12,13].

3. Diluted-acid Hydrolysis of Algal Biomass

To investigate the optimum conditions for the acid hydrolysis of *N. gaditana*, the solid/liquid (S/L, microalgae/dilute acid) ratio and acid concentration were evaluated in 20 ml vials within the range of 20-140 g/L and 0.5-10% (w/w), respectively. Acid concentration was determined according to specific gravity using a gravimeter. Heat treatment was performed in an autoclave for 15 min at 121 °C. After acid hydrolysis, the mixture was neutralized to pH 7.0 using 50% (w/v) NaOH. The neutralized specimen was then centrifuged for 10 min at 12,000 rpm. The concentration of glucose and galactose in the separated supernatant liquid was analyzed by high-performance liquid chromatography (HPLC). The efficiency (E_p, %) of acid hydrolysis was calculated using Eq. (1).

$$E_p\,(\%) = \frac{\Delta S}{CP_{TC}} \times 100 \quad (1)$$

Here, ΔS is the concentration (g/L) of the sugar produced during the acid-catalyzed thermal hydrolysis, and CP_{TC} is the total carbohydrate (total carbohydrate) content (g/L) of *N. gaditana*.

4. Fermentation Process

For bioethanol fermentation, *Bretthanomyces custersii* H1-603 was used, which is a mutant strain of *B. custersii* KCCM 11490. Seed culture involved using YPD medium with a working volume of 100 ml in a 250 ml flask for 24 h. The main medium was composed of yeast extract (1%, w/v), peptone (2%, w/v) and fermentable sugars, which were obtained from the hydrolysate of *N. gaditana* under optimal conditions or pure chemical reagents (basal medium), respectively. The fermentation was in 250 ml flasks with a working volume of 50 ml and 2% (v/v) inocula at 30 °C for 30 h. The concentration of bioethanol and sugars was analyzed by HPLC. Ethanol yield (Y_{EtOH}, g/g) was calculated using Eq. (2).

$$Y_{[EtOH]}\,(g/g) = \frac{[EtOH]}{[Sugar]_{initial}} \quad (2)$$

Here, [EtOH] is the concentration (g/L) of the bioethanol, and $[Sugar]_{initial}$ is the initial concentration (g/L) of monosaccharide (glucose+galactose). The maximum theoretical ethanol yield (Y_{EtOH}, g/g) is 0.51.

5. Cell immobilization and Continuous Fermentation

The entrapping method by sodium alginate was used for cell immobilization. The seed culture of *B. custersii* H1-603 was performed using YPD medium with a working volume of 100 ml in a 250 ml flask for 24 h. The cells were recovered by centrifugation at 8,000 rpm for 25 min, and then collected cells were mixed with 2.5% (w/v) sodium alginate. The mixed solution was dropped into 4% $CaCl_2$ (w/v) solution to make a spherical immobilized cell. To increase the hardness, the immobilized cell was stored at 4 °C for 24 h. A 1 L continuous stirred tank reactor (CSTR) was prepared for continuous ethanol production (Fig. 1). The inner temperature was maintained at 30 °C by water circulation, and the dilution rate was controlled by a peristaltic pump.

6. Analytical Methods

To analyze the concentrations of ethanol and sugars from the biomass, HPLC was utilized with a Shodex SUGAR SH1011 H^+ ion exclusion column (300 mm×8 mm, Shodex, Japan) and a refractive index detector (RID-10A, Shimadzu, Japan), using a mobile phase of 0.005 N H_2SO_4 at a flow rate of 0.6 ml/min. The temperature of the column and detector was maintained at 50 °C, and the injection volume was 20 μl.

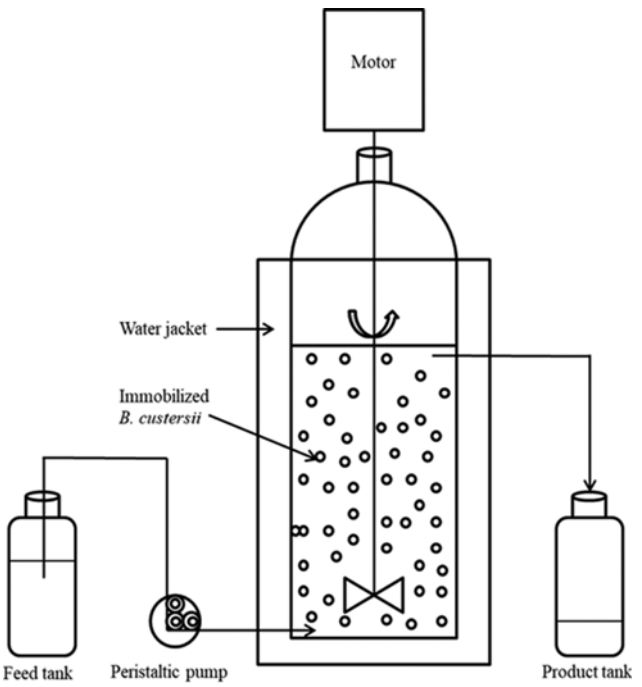

Fig. 1. Schematic diagram of continuous stirred tank reactor for bioethanol production.

Table 1. Effect of various acid types on acid-thermal hydrolysis of *N. gaditana*

Con. (%)	Sulfuric acid		Peracetic acid		Phosphoric acid		Hydrochloric acid		Nitric acid	
	Sugars con. (g/L)	E_p (%)	Sugars con. (g/L)	E_p (%)	Sugars con. (g/L)	E_p (%)	Sugars con. (g/L)	E_p (%)	Sugars con. (g/L)	E_p (%)
1	0.92±0.01	8.36	0.76±0.01	6.90	0.84±0.01	7.63	7.59±0.07	68.94	1.37±0.01	12.44
2	2.86±0.18	25.98	0.89±0.02	8.08	1.01±0.01	9.17	10.22±0.17	92.82	6.19±0.19	56.22
3	3.80±0.01	34.51	0.87±0.04	7.90	1.11±0.01	10.08	9.52±0.04	86.47	8.98±0.16	81.56
4	4.38±0.01	39.78	0.80±0.01	7.27	1.25±0.01	11.35	9.43±0.01	85.65	9.37±0.09	85.10

RESULTS AND DISCUSSION

1. Composition Analysis of Algal Feedstock

The composition analysis of *Nannochloropsis gaditana* was performed by NREL standard procedures, and the biomass consisted of 11.01% carbohydrate, 1.15% fiber, 56.41% protein, 5.13% lipid, and 5.74% ash. The carbohydrate contained about 65% glucose and 35% galactose.

Microalgal carbohydrate can be stored in various forms, and the monosaccharide contained in microalgae exists in various forms. Typically, carbohydrate can be used by dissolving it into each monosaccharide form. According to the cellulose structure, glucose exists in the shape of β-1,4 glycosidic bonds. Galactan, a polymer with galactose as the basic frame, is based on a crosslinked structure of 3-linked β-D-galactopyranosyl units (D-galactose) and 4-linked α-3,6-anhydrogalactopyranosyl units (3,6-anhydrogalactose, 3,6-AHG). The carbohydrates included not only fermentable sugars such as glucose and galactose, but also non-fermentable sugar such as 3,6-anhydro-L-galactose [14,15].

2. Acid-thermal Hydrolysis of *N. gaditana*

Because the characteristics of microalgae depend on the environment they inhabit, their taxonomic group and species composition, the components of microalgae differ, and thus the conditions of extraction also vary. The major purpose of this study was to achieve efficient bioethanol conversion using the fermentable sugars obtained from microalgae with limited inhibition of by-products. The efficiency of hydrolysis needs to be promoted to produce reducing sugars for bioethanol production. To produce fermentable sugars using microalgae, both chemical and biological methods can be used. In biological methods, while sugar can be obtained using various enzymes, the efficiency of hydrolysis is slow and such enzymes are expensive. The feasibility of chemical hydrolysis on microalgae has previously been proven. Generally, acid and alkali solvents are used for the hydrolysis, while acid tends to be preferred because it raises the conversion rate of the reducing sugars in the cellulose materials [16]. To measure the degree of hydrolysis obtained by various solvents, hydrolysis reactions were conducted using distilled water, 1% sulfuric acid and 1% sodium hydroxide under 50 g/L (S/L ratio) at 121 °C for 15 min. As a result, the amount of released glucose obtained by sulfuric acid was 1 g/L, and 3.34 g/L and 0.01 g/L were obtained by sodium hydroxide and distilled water, respectively. This result indicates that sulfuric acid is the most effective for the conversion of reducing sugar (data not shown) [17].

Subsequently, the optimum conditions of acid-catalyzed thermal hydrolysis were investigated to achieve maximum production of reducing sugars from microalgae. The effects of acid-thermal hydrolysis using various types and concentrations of acid are shown in Table 1. The acid-thermal reaction was performed in the autoclave with an S/L ratio of 100 g/L at 121 °C for 15 min. Five types of acids (sulfuric acid, peracetic acid, phosphoric acid, hydrochloric acid and nitric acid) were utilized as chemical reagents, and the effect of acid concentration (1-4%) on the hydrolysis efficiency was investigated. In the five chemical reagents, sulfuric acid and nitric acid showed a positive performance, in which the hydrolysis efficiency was increased from 8.36-39.78% and 12.44-85.10%, respectively, with an increased acid concentration from 1% to 4%. However, the hydrolysis efficiency was less than 15% when using peracetic acid and phosphoric acid, which were not appropriate for the hydroly-

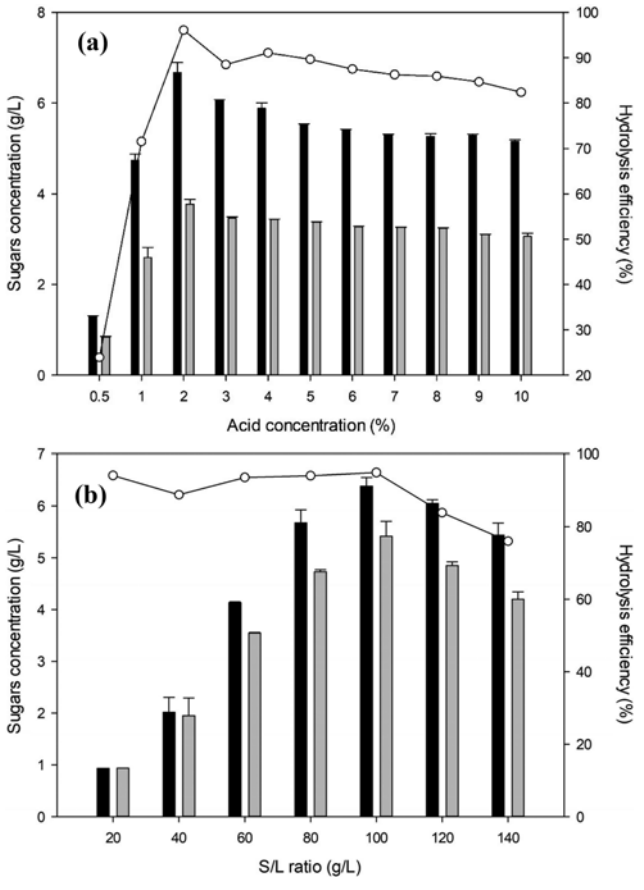

Fig. 2. Effect of acid-thermal hydrolysis using *N. gaditana*: (a) Concentrations of acid, (b) solid/liquid ratio (glucose (■), galactose (■), and hydrolysis efficiency (○)).

sis reaction. The maximum efficiency of hydrolysis (92.82%) was achieved using 2% hydrochloric acid.

Hydrogen ions can be easily extracted from an aqueous solution of hydrogen chloride, including hydrochloric acid, nitric acid, and sulfuric acid; thus, the hydrolysis efficiency was improved. Hydrochloric acid has been found to be the most efficient acid for hydrolysis since it has the highest ionization constant [18]. Hydrolysis by the selected acid was performed at a concentration of 0.5% to 10% to measure the reducing sugar produced at various concentrations using hydrochloric acid. The amount of reduced sugar rapidly increased by 2% hydrochloric acid, which showed the highest hydrolysis efficiency (Fig. 2(a)). The hydrolysis efficiency decreased at hydrochloric acid concentrations higher than 2%. Many studies have reported that this is due to the excessive degradation of the polysaccharide of biomass in severe reactions [19,20].

Subsequently, the effect of the S/L ratio (*N. gaditana* and hydrochloric acid) on sugar concentration and hydrolysis was investigated based on a biomass concentration of from 20 g/L to 140 g/L (Fig. 2(b)). As the content of *N. gaditana* increased from 20 g/L to 100 g/L, it was observed that the reducing sugar concentration almost linearly increased. However, the reducing sugar concentration and hydrolysis efficiency decreased gradually over 100 g/L biomass concentration. The maximum hydrolysis efficiency of about 92.82% was at the S/L ratio of 100 g/L. Finally, the optimal reaction condition for the acid-thermal hydrolysis of *N. gaditana* was determined as 2% hydrochloric acid with 100 g/L biomass loading at 121 °C for 15 min.

3. Ethanol Fermentation

As is well known, the theoretical maximum production of bioethanol is 0.51 g per 1 g glucose [21]. The hydrolysates from microalgal biomass also contain non-reducing sugars and oligosaccharides would be another reason to make the reaction formula for ethanol conversion by yeast fermentation much more complex. However, the maximum ethanol conversion rate of reducing sugars is expected to be approximately 50%. Based on this result, bioethanol fermentation was carried out using microalgal hydrolysate as the carbon source, which was obtained from *N. gaditana* by 2% hydrochloric acid hydrolysis. Fig. 3 shows the fermentation profiles of *B. custersii* H1-603 based on the results of the microalgal hydrolysate containing glucose and galactose and the basal medium with pure chemical glucose and galactose. As shown in Fig. 3(a), the

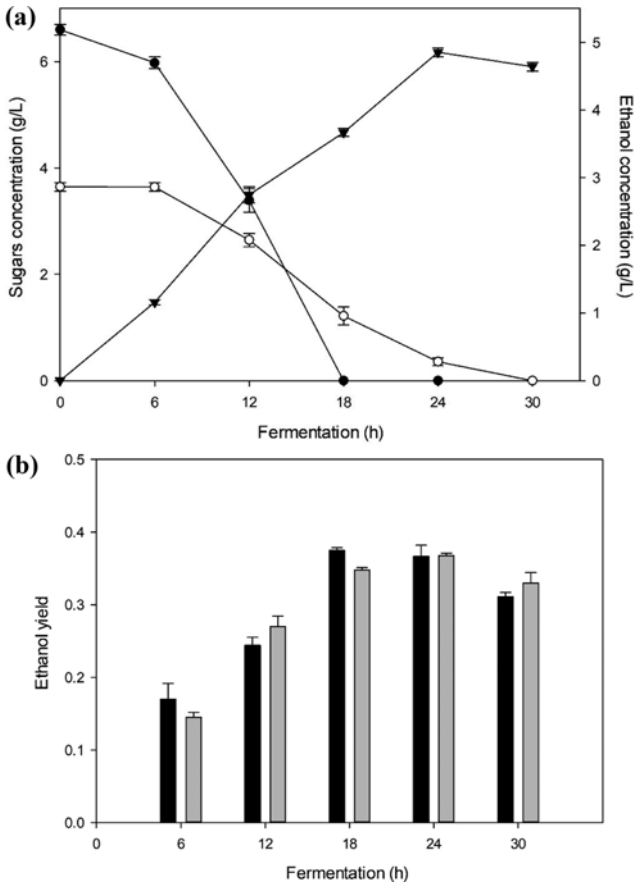

Fig. 3. Bioethanol production from (a) *N. gaditana* (glucose (●), galactose (○) and ethanol concentration (▼)), (b) comparison of bioethanol yield between basal medium (■) and carbohydrates from *N. gaditana* (■).

consumption of reducing sugars from hydrolysates was observed after 6 h, and the maximum amount of bioethanol was achieved after 24 h fermentation. The ethanol productivity decreased in both the basal medium and reducing sugar after 24 h fermentation, which might be the result of ethanol consumption by strain. As shown in Fig. 3(b), 4.91 g/L of ethanol was produced in the basal medium at 18 h, with an ethanol yield of 0.38. However, 4.84 g/L of ethanol was produced from algal reducing sugar at 24 h, with the yield of 0.37,

Table 2. Bioethanol production from various algal biomass

Algal feedstock	Hydrolysis conditions	Fermentation conditions	Yields	References
Tribonema sp.	3% (v/v) H_2SO_4 at 121 °C for 45 min	*Saccharomyces cerevisiae*, SHF, 16 h	0.38	[22]
Chlorella	2% HCl and 2.5% $MgCl_2$ at 180 °C for 10 min	*Zymomonas mobilis* Y01, SHF, 48 h	0.19	[23]
Gelidium amansii	2% (w/w) H_2SO_4 at 121 °C for 59 min	*Bretthanomyces custersii* KTCT18154P, SHF, 56 h	0.13	[24]
Nannochloropsis gaditana	2% HCl at 121 °C for 15 min	*Bretthanomyces custersii* H1-603, SHF, 24 h	0.37	This study

Yield: g ethanol/g $sugar_{ini}$, SHF: separate hydrolysis and fermentation

which is close to the results of the control group (basal medium).

Table 2 shows the results obtained by other studies conducted on bioethanol production using acid and alkali hydrolysis [22-24]. In particular, *N. gaditana* used in our study shows relatively fast growth compared with other species and has a high content of cellulose, which is advantageous for bioethanol production. Most researches on third generation of bioethanol fermentation have been carried out using glucose from algal hydrolysate, as the carbon source, because the algal feedstock has a high content of carbohydrate. Thus, fermentation was performed with glucose metabolizing yeast, which led to high yield. However, the hydrolysate also contains sufficient amounts of galactose, together with glucose. Park et al. [24] reported that it is possible to extract reducing sugars from *Gelidium amansii*, which contains large amounts of glucose and galactose, both of which can be metabolized by *B. custersii* to produce bioethanol. However, the ethanol yield of that reference was not high. To increase the ethanol production and yield by using all of the reducing sugars obtained from microalgae, *B. custersii* H1-603, a mutant strain, was employed.

4. Fermentation in CSTR Using Immobilized Cell

The use of immobilized cell on bioethanol production has the advantage of increasing the ethanol tolerance of microorganisms and reducing the fermentation time. In addition, the separation process of the immobilized cell from the bioethanol product is very simple and can reduce the production costs for bioethanol production [25,26]. In the current study, *B. custersii* H1-603 was immobilized with sodium alginate, and the diameter of the immobilized cell was fixed at 2 mm. The cell concentration was found to be 6.0 $OD_{600\,nm}$ at 24 h cultivation. Various volume ratios of cell/sodium alginate (0.33, 0.5, 1 and 2) were used to determine the highest bioethanol productivity in the batch system. The effect of the cell/sodium alginate ratio on bioethanol production is shown in Table 3. The sugars from microalgal hydrolysate were consumed in all experimental groups after 24 h fermentation. The highest ethanol concentration and yield were achieved at about 3.10 g/L and 0.36 g/g, respectively, at the cell/sodium alginate ratio of 2 (v/v). The high concentration of cells in the sodium alginate beads might have resulted in a higher consumption of reducing sugars than in the other groups, making it possible to produce higher bioethanol. Therefore, the cell/sodium alginate ratio of 2 was finally determined for continuous bioethanol production in CSTR.

A CSTR of 1 L scale was used to compare the ethanol production between free cell and immobilized cell (Table 4). In both experimental groups, an equal concentration of cells (about 6.8×10^9 *B. custersii* H1-603) was inoculated into the main medium (microalgal hydrolysate). After 24 h activation in batch mode, continuous fermentation was operated according to different dilution rate (0.08, 0.06 and 0.04 h^{-1}). Both experimental groups showed the highest bioethanol production at the lowest dilution rate of 0.04 h^{-1}. As the dilution rate decreases, more time is available for the cell to uptake the sugar in the main medium, since the dilution rate is the reciprocal of the residence time in the reactor. Table 4 shows that when the dilution rate was 0.08 h^{-1}, the use of immobilized cells resulted in a higher sugar consumption than that of the free cells. The fermentation by the immobilized cell therefore showed a higher production of bioethanol than that by the free cells since more sugar was consumed with the higher cell concentration of immobilized cells than with the free cells. The immobilized cells showed a higher cell concentration under the same conditions because the immobilized cells in the CSTR are maintained without loss. On the other hand, washout of cells occurs in the case of free cells in the CSTR depending on the flow rate of the supplied medium.

When immobilized cell is used in CSTR, the ethanol production and yield are lower than those of free cell at 0.04 h^{-1} and 0.06 h^{-1}, respectively; however, this has several advantages. Because an immobilized cell is encapsulated in alginate beads, it is not only easily

Table 3. Effect of cell/sodium alginate ratio on the bioethanol production in batch fermentation for 24 h

Parameters	Ratio of cell/sodium alginate (v/v)			
	0.33	0.5	1	2
Ethanol concentration (g/L)	2.23±0.22	2.51±0.00	2.93±0.04	3.10±0.09
Ethanol yield (g/g)	0.25±0.05	0.29±0.09	0.33±0.01	0.36±0.04
Productivity (g/L·h)	0.09±0.01	0.10±0.00	0.12±0.00	0.13±0.00

Table 4. Comparison of fermentation between free and immobilized *B. custersii* in CSTR

Types	Parameters			
	Dilution rate (h)	Sugars consumption (g)	Ethanol concentration (g/L)	Ethanol yield (g/g)
Free cell	0.08	5.84±0.10	3.01±0.05	0.23±0.01
	0.06	6.52±0.05	3.70±0.08	0.29±0.02
	0.04	7.47±0.19	3.93±0.10	0.30±0.02
Immobilized cell	0.08	6.82±0.07	3.49±0.08	0.27±0.02
	0.06	6.99±0.07	3.53±0.06	0.27±0.01
	0.04	7.27±0.04	3.68±0.03	0.28±0.02

*About 6.8×10^9 cell of *B. custersii* were inoculated into both CSTRs, and the fermentation was performed after 24 h activation

separated from the medium but also easily recycled [27]. Therefore, it is more suitable for continuous culture.

Overall, the hydrolysate of *N. gaditana* could be a promising carbon source for bioethanol production.

CONCLUSIONS

Efficient bioethanol production was successfully performed using acid-thermal hydrolysis of microalgae, *N. gaditana*. The maximum hydrolysis efficiency of 92.82% was achieved. The bioethanol production and yield were 4.84 g/L and 0.37 g/g, respectively. These results are close to that of fermentation with pure chemical reagents (4.91 g/L and 0.38 g/g, respectively). Furthermore, *B. custersii* H1-603 was immobilized by sodium alginate and the volume ratio of cell/sodium was finally determined as 2 (v/v). The immobilized and free cell were utilized in the CSTR for continuous bioethanol production, and the equal concentration of cells was inoculated into the microalgal medium. Both groups showed the highest bioethanol production (3.68 g/L by immobilized cell and 3.93 g/L by free cell, respectively) at the dilution rate of 0.04 h^{-1}. This study contributes in both optimization of the acid-thermal hydrolysis of *N. gaditana* and demonstration of continuous bioethanol production from microalgae.

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