Production of L(+)-Lactic Acid from Mixed Acid and Alkali Hydrolysate of Brown Seaweed

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Note

Production of L(+)-Lactic Acid from Mixed Acid and Alkali Hydrolysate of Brown Seaweed

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The species of brown seaweeds, *Laminaria japonica* is commercially cultivated in Japan. Mannitol and uronic acid were the main component of mono sugar produced from the saccharification of *L. japonica* which hydrolysed with H_2SO_4 or NH_4OH. The mannitol concentration of *L. japonica* (5 w/v %) hydrolysate using 0.5 v/v % H_2SO_4 or 1 v/v % NH_4OH were 15.84 g/L and 13.87 g/L, respectively. Hydrolysates from both acid and alkali hydrolysis were mixed together for neutralization as well as to obtain higher mannitol concentration of 15.18 g/L. Among the mono sugar in the hydrolysate, Mannitol was the main substrate for the lactic acid fermentation by *Lactobacillus rhamnosus*. L(+)-Lactic acid with 97.9% of optical purity was successfully produced at the yield of 14.42 g/L ($Y_{p/s}$ = 94.99%).

Keywords: brown seaweed, *Laminaria japonica*, mannitol, L(+)-lactic acid, *Lactobacillus rhamnosus*

Introduction

L(+)-lactic acid is a typical organic acid and has been used in various industries, such as brewing, food processing, pharmaceutical and chemical industries. L(+)-lactic acid is also a key component of poly-lactic acid (PLA), with biodegradable, biocompatible and resorbable characteristics. Production of L(+)-lactic acid at high concentrations and optical purity from renewable resources would be of great interest. Several studies have been reported on the production of lactic acid from renewable feedstock (Praneetrattananon *et al.*, 2005; Schmidt *et al.*, 1997; Thomas, 2000).

Marine biomass (marine algae) has recently received considerable attention as a renewable source of bioethanol (Okamoto *et al.*, 1994), biomethanol (Bird *et al.*, 1990), biobutanol (i), biohydrogen (Rupprecht *et al.*, 2006) and biodiesel (ii). Generally, marine algae are categorized into macroalgae (seaweed) and microalgae, whereas seaweed is divided into three categories according to coloration: green, brown, and red seaweed (McHugh *et al.*, 2003).

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Among the variety of seaweed, *Laminaria japonica* (brown seaweed) has great potential as a resource for bioconversion of value-added products, both in its quantity and quality as a carbon source. This seaweed grows faster than any other, exhibiting productivity of around 2.7 kg/m^2/yr (Yokoyama *et al.*, 2007). Approximately 3 × 10^5 ton (wet weight) *L. japonica* is produced annually in Japan, with cultivation comprising the largest part (Luning and Pang, 2003). In addition, this seaweed has high carbohydrate content, greater than 50%.

Although many studies on the production of biofuels from seaweed have been reported, thus far, there is little research on lactic acid production from seaweeds (Uchida, 2005). Therefore, the purpose of this work was to investigate L(+)-lactic acid fermentation by *Lactobacillus rhamnosus* using a mixture of *L. japonica* acid and alkali hydrolysates.

Materials and Methods

Raw materials *L. japonica* originating from Hokkaido was used in this study and was obtained from a supermarket in Fukuoka, Japan. *L. japonica* was washed under running water 5 times to remove salts and then dried in an oven at

50°C. Dried *L. japonica* was milled to less than a 100-mesh size using a coffee mill.

General analysis of Laminaria japonica Dietary fibre was determined according to the Association of Official Analytical Chemist (A.O.A.C.) method (iii). Carbohydrate content was determined by calculating the percentile difference from all the other constituents, and protein, lipid, ash and water were analyzed using the method of A.O.A.C (1990) (AOAC, 1990).

Saccharification of Laminaria japonica In order to determine the appropriate biomass concentration for sulfuric acid hydrolysis of *L. japonica*, approximately 3-10 w/v % of seaweed was treated with 0-25 v/v % H_2SO_4 at 120−150°C by hot-compress treatment (Jang *et al.*, 2010). Alkali hydrolysis using NH_4OH was carried out using 5% w/v dried *L. japonica* and 0.2 to 20% concentration of NH_4OH at 120°C for 30 min by autoclave (HA-300M, Hirayama, Japan). Once the extraction was completed, neutralization to pH 7.0 was accomplished by mixing the H_2SO_4 and NH_4OH hydrolysates. After neutralization, centrifugation was conducted at 3500 rpm for 5 min to remove the solid residue, followed by centrifugation at 8000 rpm for 10 min to remove salt and inhibitor. The supernatant was then stored at −20°C before use in the fermentation process for L(+)-lactic acid production.

Microorganism *Lactobacillus rhamnosus* (*L. rhamnosus*), a type of L-lactic acid producer, was used in this study. The stock culture was maintained at −80°C in 30% glycerol. Inoculum was incubated at 37°C under anaerobic conditions for 24 hr twice. The inoculum was then added to a nutrient supplement (*Nissui* Pharmaceutical Co. Ltd. Japan) as follows (per liter); yeast extract: 5.5 g; peptone: 12.5 g; glucose: 11 g; KH_2PO_4: 0.25 g; K_2HPO_4: 0.25 g; CH_3COONa: 10.0 g; $MgSO_4$: 0.1 g; $MnSO_4$: 0.05 mg; $FeSO_4$: 0.05 mg.

Mannitol and uronic acid were the main mono sugars in *L. japonica* hydrolysate. The effect of the mono sugars on the yield of lactic acid production was investigated. Mannitol was obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan), while uronic acid was derived from digestion of alginate (Wako) through enzymatic hydrolysis by alginate lyase. *Alginate lyase S* (specific activity, 29,000 U/g) was purchased from Nagase ChemteX (Japan). The degradation reaction was carried out in 0.2 M acetate buffer solution, pH 6.3, at 37°C for 30 min.

Lactic acid fermentation Fermentation was conducted using a 1L-bioreactor (MDL-1L, B.E.MARUBISHI, Japan) equipped with temperature, agitation and pH controller. The bioreactor was autoclaved at 121°C for 15 min and the following nutrient supplement (per liter) in the neutralized *L. japonica* hydrolysate was added: YE: 5.5 g; peptone: 12.5 g; KH_2PO_4: 5 g; KH_2PO_4: 0.25 g; K_2HPO_4: 0.25 g; CH_3COONa: 10.0 g; $MgSO_4$: 0.1 g. A 10 v/v % inoculum of *L. rhamnosus* was aseptically inoculated into the bioreactor. At the initial stage of fermentation, pH was adjusted to 6.8 by the addition of 5 v/v % NH4OH. The fermentation broth was incubated at 37°C and agitated at 100 rpm for 5 days. Aseptic sampling was carried out every 12/24 h using a sterile sampler and centrifuged at 4500 rpm for 10 min. The supernatant was then kept in High Pressure Liquid Chromatograhy (HPLC) vials and stored at 4°C prior to analysis.

Analyses The acid and alkali hydrolyses and fermentation process were monitored by the amount of mannitol and lactic acid. The concentrations of lactic acid and mannitol were measured by HPLC. A Rezex ROA-Organic Aic H^+ (8%) cation-exchange column (Phenomenex, USA) at 40°C with UV-detector and a Shim-pack SPR-Ca column (Shimadzu, Japan) at 80°C with IR-detector were used to analyze lactic acid and mannitol, respectively. The mobile phase was carried out at 0.005 N H_2SO_4 and water at a flow rate of 0.5 ml min^{-1}. The D- and L- enantiomers were separated with the column (MIC GEL. CRS IOW) and eluted with l mM $CuSO_4$ solution (flow rate 0.6 ml min^{-1}) for the analysis of optical purity. The samples were filtered with a 0.45 μm cellulose acetate filter and approximately 10 μL of injection volume was added. Determination of the total uronic acid was measured by colorimetric assay (Laurence and Bronwen, 2001). The optical density (OD) of the samples was measured at 600 nm in a Coleman® model 35 spectrophotometer (Beckman) to determine bacterial growth.

Results and Discussion

Composition of Laminaria japonica The composition of *L. japonica* is shown in Figure 1. In general, the composition of seaweed components varies according to the season, age

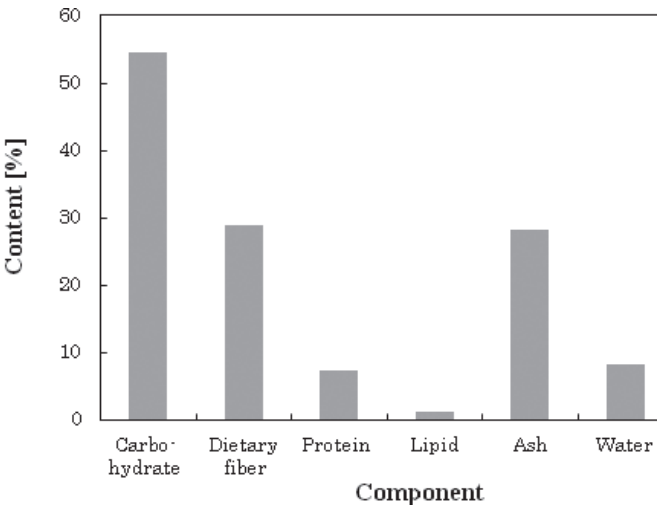

Fig. 1. Composition of *Laminaria japonica*.

of population, species, geographic location and temperature, (Kaehler *et al.*, 1996; iii). The overall composition of the seaweed sample in this study agreed with the values previously reported for seaweed (Manivannan *et al.*, 2009). Figure 1 shows that *L. japonica* is composed of higher carbohydrate content than dietary fiber content. Plant carbohydrates are composed of soluble (starch and others) and insoluble (cellulose, hemicelluloses) dietary fiber, which can be converted to glucose, xylose and other reducing sugars. However, seaweed carbohydrate is comprised of fibers with microscopic crystal structures, covered by a mucilaginous polysaccharide layer and as storage polysaccharides contained in cells (Indergaad, 1982). These seaweed carbohydrates are converted to various sugars, like glucose, galactose, xylose, rhamnose, arabinose, fucose and uronic acid. The conversion ratio from biomass to sugar is shown in Table 1. It was estimated that approximately 45.15% mono sugars (32.47% mannitol and 12.68% uronic acid) can be produced from *L. japonica*. This suggested that *L. japonica* could be considered a good source of mono sugars for further bioconversion. In addition, *L. japonica* also contains approximately 7.4% protein, which is a very important element for microbial growth.

Saccharification of Laminaria japonica by H_2SO_4 hydrolysis In the previous study, the production of mono sugars from acid hydrolysis of seaweed was investigated (Jang *et al.*, 2010). From the results, 3−5 w/v % biomass concentration and 0.5−1 v/v % H_2SO_4 catalyst concentration were the optimum conditions for mannitol production. Mannitol and uronic acid could easily be extracted from the milled seaweed under optimal conditions.

Ammonium hydroxide hydrolysate as a neutralizer of sulfuric acid hydrolysate After sulfuric acid hydrolysis, the hydrolysate needs to be neutralized for the fermentation process. In order to minimize sugar reduction during the neutralization process, acid and alkali hydrolysates were mixed together. At first, the appropriate ammonium hydroxide (NH_4OH) concentration for *L. japonica* hydrolysis was investigated (Fig. 2). 1% v/v NH_4OH showed the highest mannitol conversion among the range of NH_4OH concentrations examined. However, no conversion was achieved over 5 v/v % NH_4OH because the sample dried up and only solid residue remained.

Table 2 shows that the mannitol concentration in the mixed H_2SO_4 and NH_4OH hydrolysate(E) was higher than H_2SO_4(C) or NH_4OH(D) hydrolysate neutralized by individual counter reagents. It was suggested that a mixture of acid and alkali hydrolysates is the best approach for neutralization purposes and for further utilization as a fermentation substrate.

L(+)-Lactic acid production from the hydrolysate mixture Based on the above results, the *L. japonica* hydrolysate which mainly consisted of mannitol and uronic acid was fermented with *L. rhamnosus*. In brown seaweeds, alginate is the main structural compound (Kloareg and Quatrano, 1988), while mannitol is a common storage material. Enzymatic degradation of alginate is catalysed by alginate lyases, which cleave the alginate polymer, resulting in unsaturated uronic

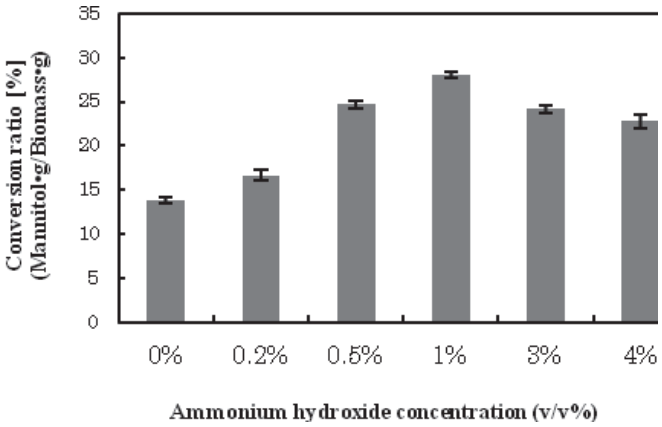

Fig. 2. Effect of NH_4OH concentration on saccharification of 5 w/v % *Laminaria japonica*.

Table 1. Sugar composition of *Laminaria japonica* acid hydrolysate.

	Concentration (g/L)	Conversion Ratio[1] (w/w %)
Biomass	30	•
Total sugar	13.54	45.13
Mannitol	9.74	32.47
Uronic acid	3.8	12.67
Other mono sugars	ND	ND

[1] (sugar amount (g) / biomass amount (g)) × 100.
Seaweed was treated with H_2SO_4 at 120°C for 30 min by autoclave.
ND is not detected.

Table 2. Mannitol concentration of *Laminaria japonica* hydrolysate before and after neutralization.

	Concentration of catalysts	Neutralizer	Concentration of mannitol before neutralization	Concentration of mannitol after neutralization
H_2SO_4	0.5 v/v %	10 v/v % NH_4OH	(A) 15.84 g/L	(C) 14.96 g/L
NH_4OH	1 v/v %	10 v/v % H_2SO_4	(B) 13.87 g/L	(D) 13.17 g/L
Hydrolysate mixture of (A) and (B)				(E) 15.18 g/L

Reaction temperature was 120°C, reaction time was 30 min and *Laminaria japonica* concentration was 5 w/v %.
The volume ratio to hydrolysate and neutralizer; (C) 50:1.9, (D) 50:1.8, and (E) as mixed acid and alkali hydrolysate; 50 (A):64.9 (B).

acids such as D-mannuronic and L-guluronic acid (Sutherland, 1995; Haugen *et al.*, 1990).

Figure 3 shows the comparison of lactic acid fermentation from chemicals and the hydrolysate of *L. japonica*. It was difficult to produce lactic acid from alginate pre-treated with alginate lyase(B), and uronic acid is not a suitable sugar for lactic acid production by *L. rhamnosus*. The mixture of mannitol and alginate pre-treated with alginate lyase(C) had higher production of lactic acid than mannitol(A) alone. On the other hand, lactic acid production from *L. japonica*(D) hydrolysate was higher than chemicals(A, C). The reason behind these results will be investigated further.

Figure 4 shows a time course of the lactic acid production by *L. rhamnosus* grown in mixed acid and alkali hydrolysate. Lactic acid production gradually increased and achieved the maximum value at 48 h, maintaining the maximal value until the end of the fermentation. The time required to reach the maximum level of lactic acid production may be affected by the reduction of mannitol, which is consumed by *L. rhamnosus* throughout the fermentation process. As shown in Figure 4, mannitol concentration decreased at the initial stage of fermentation and this correlates with the increase in turbidity, indicating bacterial growth; however, the concentration of uronic acid did not change. Therefore, it was confirmed that uronic acid was not utilized for lactic acid production. The highest lactic acid production of 14.42 g/L ($Y_{p/s}$ = 94.99%) was achieved after 48 h of fermentation, and mannitol was completely consumed by the bacteria.

Although little research has been reported on lactic acid fermentation of seaweed, Uchida undertook detailed investigation, specifically with respect to microorganisms involved as effective starters for marine silage production (Uchida, 2005). Our objective is whole utilization of seaweed as a feedstock to produce lactic acid for industrial application. From this point of view, lactic acid yield is of great concern

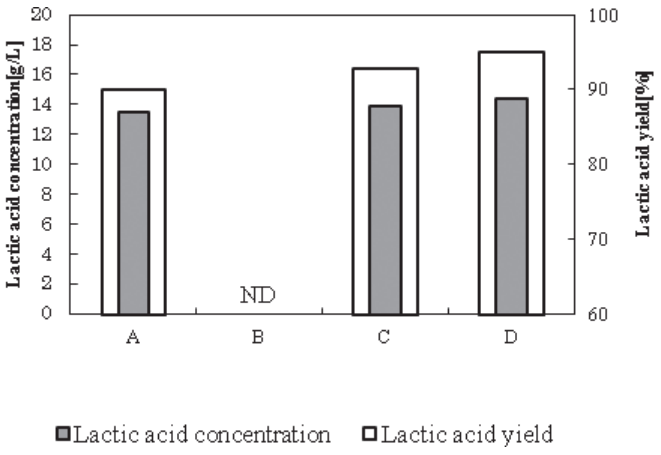

Fig. 3. Comparison of lactic acid fermentation from chemicals and *Laminaria japonica* hydrolysate. (A) 15 g/L mannitol, (B) 15 g/L alginate (after degradation by alginate lyase), (C) Mixture of 15 g/L mannitol and 15 g/L alginate (mixture is pre-treated by alginate lyase), (D) *Laminaria japonica* hydrolysate included mannitol (15.18 g/L) and uronic acid (3.86 g/L). ND is not detected.

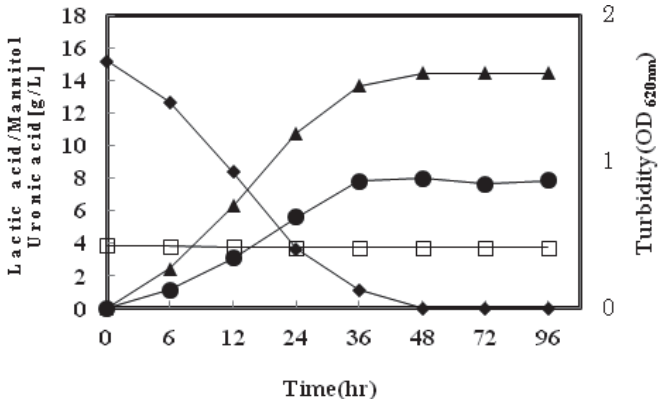

Fig. 4. Lactic acid production by *L. rhamnosus* using a mixture of H_2SO_4 and NH_4OH hydrolysates. Mannitol (◆), Uronic acid (□), Lactic acid (▲), Turbidity (●).

and effective processing, hydrolysis through to fermentation, should be developed.

The optical purity of lactic acid is important for the quality of final products such as PLA (Narayanan *et al.*, 2004). Therefore, selection of microorganisms producing high-purity L(+)-lactic acid was essential in this study. The optical purity of L(+)-lactic acid produced from *L. japonica* hydrolysate by *L. rhamnosus* was 97.9%. L(+)-lactic acid optical purity greater than 95% is usually recommended for polymerization. The results obtained in this study suggest that L(+)-lactic acid in high concentrations and of high purity can be produced from *L. japonica* using *L. rhamnosus*.

Conclusions

Mono sugars can easily be extracted from milled *L. japonica* by H_2SO_4 hydrolysis under optimal conditions: 3−5 w/v % biomass concentration and 1.0 v/v % H_2SO_4 catalyst concentration under 120°C reaction temperature and 30 min reaction time. *L. japonica* hydrolysate contained high mono sugars (45.15 wt %), which consisted of mannitol (71.92%) and uronic acid (28.08%). In this study, a mixture of acid and alkali hydrolysates was utilized to minimize sugar reduction during neutralization. Cost benefits would also be achieved by utilizing these nutrients as the nitrogen or mineral source for fermentation. The results of this study prove that *L. japonica* hydrolysate could be used as an good substrate for L(+)- lactic acid production. *L. rhamnosus* was successfully grown in the mixed acid and alkali hydrolysate, with mannitol as the main substrate for the fermentation. The maximum lactic acid concentration of 14.42 g/L was achieved after 48-hr fermentation, which corresponded to 94.99% yield with 97.9% optical purity. The present study indicates that mannitol from brown seaweed is suitable for L-lactic acid production.

In addition to brown seaweed, green and red seaweed are also good potential resources for bioconversion of value added products. They contain a variety of mono sugars, like glucose, galactose and rhamnose, although their component composition differs from each other. Further study is expected to reveal this great potential.

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