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Potential Use of *Gelidium amansii* Acid Hydrolysate for Lactic Acid Production by *Lactobacillus rhamnosus*

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Summary

Galactose and glucose are the main monosaccharides produced from the saccharification of *Gelidium amansii*. They were hydrolysed with 3 % (by volume) H_2SO_4 at 140 °C for 5 min and obtained at concentrations of 19.60 and 10.21 g/L, respectively. *G. amansii* hydrolysate (5 %, by mass per volume) was used as a substrate for L(+)-lactic acid production by *Lactobacillus rhamnosus*. The maximum lactic acid yield ($Y_{P/S}$) was 42.03 % with optical purity of 84.54 %. Lactic acid produced from *G. amansii* hydrolysate can be applicable, among others, for the production of lactic acid esters, like ethyl or methyl lactate, and disinfectant in seaweed cultivation.

Key words: Gelidium amansii, galactose, lactic acid fermentation, *Lactobacillus rhamnosus*, acid hydrolysis

Introduction

Lactic acid has many pharmaceutical and cosmetic applications as formulations in topical ointments, lotions, anti-acne solutions, humectants, parenteral and dialysis solutions or as anticaries agent (*1*). The properties and applications of lactic acid, its derivatives and polymers have been discussed previously. Many studies have reported on the production of lactic acid from renewable feedstock and its usage in the production of a variety of biodegradable plastics (*2–4*), but high concentrations and high optical purity are required. Lactic acid esters like ethyl and methyl lactate are high-boiling, non-toxic and degradable components (*5*), and can be used as green (environmentally benign) solvents, as they do not need to be of high optical purity. In addition, lactic acid has a strong disinfectant effect, so it is used as a disinfectant of seaweed culture in the treatment of laver culture (*6*).

The selection of suitable raw materials is an effective approach to reduce the cost involved in the production of lactic acid. Marine biomass (marine algae) has recently received a lot of attention as a renewable source of bioethanol (*7*), biomethanol (*8*), biobutanol (*9*), biohydrogen (*10*) and biodiesel (*11*). Generally, marine algae are categorized into macroalgae (seaweed) and microalgae, whereas seaweed is divided into three categories according to the colour: green, brown, and red seaweed (*12*). Among the seaweeds indigenous to Japan, we focused on *Gelidium amansii* in this study. *G. amansii* is an economically important species of red seaweed commonly found in the shallow coastal waters of many East and Southeast Asian countries. It is applied in the food and chemical industries or sometimes even served in salads. Approximately 3.0–3.3·10^4 tonnes (dry mass) of *G. amansii* is produced in Japan annually (*13*). *G. amansii* agar can be used to obtain galactose. Agar is a gelati-

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nous substance derived from red seaweed, and it can be divided into two fractions as follows: a neutral polymer (agarose) and a sulphate polysaccharide (agaropectin). Therefore, galactose is obtained from agarose and agaropectin hydrolysates (*14,15*).

Although many studies on the production of biofuels from seaweed have been reported, thus far there are only a few studies about lactic acid production from seaweeds (*16*). The purpose of this work is to investigate lactic acid production by *Lactobacillus rhamnosus* using *Gelidium amansii* hydrolysate as renewable feedstock or a synthetic medium (glucose, galactose or their mixture).

Materials and Methods

General analysis of Gelidium amansii

G. amansii used in this study was obtained from a supermarket in Munakata city, Japan. It was thoroughly washed to remove salt and then dried in an oven at 50 °C. After drying, the seaweed was milled to less than a 100 mesh size using a coffee mill. Protein, lipid, ash and water were analyzed using the method of AOAC (*17*). Carbohydrate content was determined by calculating the percentage remaining after all the other constituents had been measured (*18*).

Saccharification of Gelidium amansii by sulphuric acid hydrolysis

Saccharification of 3–5 % (by mass per volume) biomass of *G. amansii* was conducted using 3 % (by volume) H_2SO_4 as a catalyst at the reaction temperature of 140 °C and reaction time of 15 min, according to our previous work (*19*). After hydrolysis, the mixture was neutralized with 10 % (by volume) NH_4OH and centrifuged at 8000 rpm for 10 min to remove the precipitated salt.

Microorganism and culture condition

Lactobacillus rhamnosus KY-3 was used in this study as L(+)-lactic acid producer and seeded at an initial cell density of 10^6 cells/mL. The stock cultures were maintained at –80 °C in nutrient supplement (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) with 30 % glycerol. A stock culture was transferred to 100 mL of general LAB medium as follows: yeast extract 5.5 g/L, peptone 12.5 g/L, glucose 11 g/L, KH_2PO_4 0.25 g/L, K_2HPO_4 0.25 g/L, CH_3COONa 10 g/L, $MgSO_4$ 0.1 g/L, $MnSO_4$ 0.05 mg/L and $FeSO_4$ 0.05 mg/L. Before fermentation, the precultivation of inoculum was carried out for 24 h in an incubator at 37 °C under anaerobic conditions.

Fermentation parameters

The fermentation was conducted in 1-litre bioreactor (MDL-1 L, B.E. Marubishi, Tokyo, Japan, with a working volume of 700 mL) equipped with temperature, agitation and pH controllers. The bioreactor was autoclaved at 121 °C for 15 min and then nutrient supplement (in g/L) was added to the neutralized *G. amansii* hydrolysate: yeast extract 5.5, peptone 12.5, KH_2PO_4 0.25, K_2HPO_4 0.25, CH_3COONa 10 and $MgSO_4$ 0.1. The 10 % (by volume) inoculum of *L. rhamnosus* was aseptically inoculated into the bioreactor. At the initial stage of fermentation, the pH was set at 6.8 by the addition of 5 % (by volume) aqueous ammonia. The fermentation broth was incubated at 37 °C and agitated at 100 rpm for 6 days. At given fermentation times, samples were withdrawn from the medium and centrifuged. The supernatant was then kept in HPLC vials and stored at –20 °C prior to the analysis. Fermentation inhibitors were added to the fermentation broth in 300-mL conical flask containing 100 mL of broth. All the incubation conditions were the same as above except for the pH control, which was done with $CaCO_3$. The absorbance (*A*) of the sample was measured at 600 nm using spectrophotometer F-4500/U-3310 (Hitachi, Tokyo, Japan) to determine the bacterial growth.

Analytical methods

The acid hydrolysis and fermentation process were monitored by measuring the concentration of glucose and galactose, and lactic acid, respectively, by HPLC. The Rezex ROA-Organic Acid H^+ (8 %) cation-exchange column (Phenomenex, Torrance, CA, USA) at 40 °C with UV-detector and Shim-pack SPR-Ca column (Shimadzu, Kyoto, Japan) at 80 °C with IR detector were used to analyze lactic acid, and glucose and galactose concentrations, respectively. The mobile phase was 0.005 M H_2SO_4 and water at a flow rate of 0.5 mL/min. The D- and L-enantiomers were separated with MCI GEL™ CRS10W column (Mitsubishi Chemical, Tokyo, Japan) at 30 °C and eluted with 1 mM $CuSO_4$ solution (flow rate 0.6 mL/min). The samples were filtered with 0.45 μm of cellulose acetate filter, and 10 μL of injection volume were added. To determine the concentration of fermentation inhibitors (5-hydroxymethyl-2-furfural, furfural and phenol) in *G. amansii* hydrolysate, a colorimetric assay was done (*20–22*).

Results and Discussion

Composition of Gelidium amansii

G. amansii was composed of (in %): carbohydrates 71.43, dietary fibers 67.10, protein 10.47, lipids 0.74, ash 2.82 and water 14.55. Overall composition of *G. amansi* sample in this study was in agreement with the values previously reported for red seaweed (*23*). *G. amansii* is composed of higher carbohydrates and has a microscopic crystal structure of fibres covered by mucilaginous polysaccharide layer and storage polysaccharides (*24*).

G. amansii carbohydrates were converted to galactose and glucose. The conversion ratio was estimated to be 59.62 % (39.20 % galactose and 20.42 % glucose) and concentrations of galactose and glucose in *G. amansii* hydrolysate (5 %, by mass per volume) were 19.60 and 10.21 g/L, respectively.

Lactic acid production by Lactobacillus rhamnosus on Gelidium amansii hydrolysate and on carbohydrates

Fig. 1 shows the time course of lactic acid production on *G. amansii* hydrolysate using *L. rhamnosus*. The lactic acid production gradually increased and achieved the maximum value at 72 h, which was maintained until the end of fermentation. The time required to reach the maximum levels of lactic acid production may be affect-

ed by the reduction of sugars consumed by *L. rhamnosus* throughout the fermentation (*25*). Lactic acid was produced simultaneously with the consumption of galactose and glucose for 24 h. After all glucose was consumed, *L. rhamnosus* grew and lactic acid was produced until the remaining galactose was consumed. However, galactose had a lower consumption rate and productivity than glucose during fermentation on *G. amansii* hydrolysate.

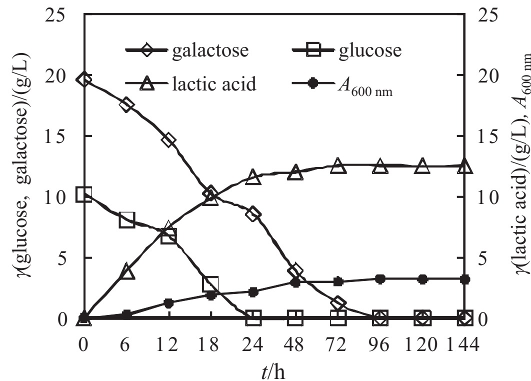

Fig. 1. Lactic acid production on *Gelidium amansii* hydrolysate by *Lactobacillus rhamnosus*

Fig. 2 and Table 1 show the results of lactic acid production on the synthetic medium, *i.e.* the mixture of galactose and glucose compared to *G. amansii* hydrolysate. It can be seen from Fig. 2 that both glucose and galactose in the mixture were consumed faster by *L. rhamnosus* (in 18 and 48 h, respectively), compared to *G. amansii* hydrolysate, which was consumed in 24 and 90 h, respectively. In the fermentation using the mixture of galactose and glucose, the consumption of galactose and production of lactic acid came to an end simultaneously (Fig. 2), although lactic acid was not produced after 70 h despite the consumption of the remaining galactose in *G. amansii* hydrolysate (Fig. 1).

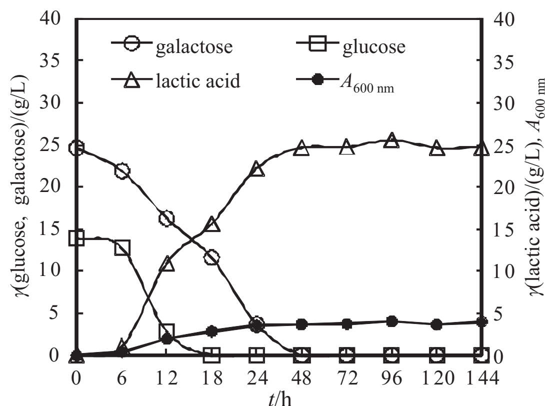

Fig. 2. Lactic acid production on the mixture of galactose and glucose by *Lactobacillus rhamnosus*

Results in Table 1 show that product yield on *G. amansii* hydrolysate was 42.03 %, on the mixture of galactose and glucose was 64.05 % and on galactose only was 66.07 %. The lactic acid yield on only galactose or glucose was higher than on *G. amansii* hydrolysate and on their mixture. In *G. amansii* hydrolysate and the mixture, it was difficult to determine which was the main carbon source for the lactic acid production because galactose and glucose were consumed simultaneously. Higher yield was obtained on glucose compared to galactose (Table 1), so it can be assumed that lactic acid is mainly produced from glucose. According to the results, it was found that sugar consumption rate, lactic acid production rate, bacterial growth rate and lactic acid production yield on *G. amansii* hydrolysate were lower compared to the mixture of galactose and glucose.

Galactose fermentation and metabolism have been studied by many lactic acid bacteria (LAB) so far (*26*), but it was difficult to find literature about lactic acid production on galactose by *Lactobacillus rhamnosus*. As illustrated in Table 1, the lactic acid yields of 66.07 and 95.66 % were obtained when only galactose or glucose were used respectively, with 100 % of substrate consumption by *L. rhamnosus*. The yield of lactic acid on galactose was lower than on glucose, possibly due to different metabolism pathways. The inducible enzyme system required for galactose utilization in LAB is a complex of structural and regulatory genes that includes a galactose permease and three enzymes of the Leloir pathway: galactokinase, galactose-1-phosphate uridyltransferase and uridine diphosphoglucose-4-epimerase. The enzyme phosphoglucomutase converts glucose-1-phosphate to glucose-6-phosphate, which then enters the glycolytic pathway (*27*). Although galactose might be expected to be fermented in a similar way as glucose since it enters the path-

Table 1. Parameters of lactic acid production by *Lactobacillus rhamnosus* on *Gelidium amansii* hydrolysate and on synthetic media

Parameter	*G. amansii* hydrolysate			Galactose and glucose mixture			Sole chemical	
	Total	Galactose	Glucose	Total	Galactose	Glucose	Galactose	Glucose
$\gamma(\text{sugar})_{\text{initial}}$/(g/L)	29.81	19.6	10.21	38.53	24.61	13.92	30.15	30.79
$\gamma(\text{sugar})_{\text{final}}$/(g/L)	0	0	0	0	0	0	0	0
sugar consumption rate/%	100	100	100	100	100	100	100	100
γ(lactic acid)/(g/L)		12.53			24.68		19.92	29.45
$Y_{P/S}$/%		42.03			64.05		66.07	95.66

Hydrolysate obtained from 5 % (by mass) of *G. amansii* treated with 3 % H_2SO_4 (by volume) at 140 °C for 15 min
Sugar consumption rate=$(\gamma(\text{sugar})_{\text{final}}/\gamma(\text{sugar})_{\text{initial}})\cdot 100$
The fermentations were conducted in a 1-litre bioreactor with working volume of 700 mL at 37 °C and 100 rpm for 6 days
$Y_{P/S}=(m(\text{lactic acid})/m(\text{substrate}))\cdot 100$

way of glycolysis as glucose-6-phosphate (*28*), there is a possibility that it enters another pathway like tagatose pathway. In addition, when pyruvate enters the course of fermentation, there is a possibility that some pyruvate enters tricarboxylic acid (TCA) cycle. The reported results showed that yields of lactic acid on galactose were in the range from 23.3 to 69.5 % (*27,29–32*). *L. rhamnosus* showed considerably high yield of 66.07 % in this study (Table 1).

Fermentation inhibitors in G. amansii hydrolysate and their effect on lactic acid fermentation

This study focused on the determination of concentrations of fermentation inhibitors 5-hydroxymethyl-2-furfural (5-HMF, 1.15 g/L), furfural (0.26 g/L) and phenol (0.47 g/L) in *G. amansii* hydrolysate using colourimetric method, and their effect on lactic acid production by *L. rhamnosus*. To investigate the effect of inhibitors on the lactic acid production, fermentation broth was prepared by the addition of 5-HMF, furfural and phenol to the mixture of galactose and glucose. The fermentation broth without any inhibitors was used as a control. Table 2 shows the effects of inhibitors on lactic acid fermentation by *L. rhamnosus*. From the experimental results, it was found that lactic acid yield decreased in the presence of fermentation inhibitors compared to the control. The results clearly show that fermentation inhibitors in *G. amansii* hydrolysate affect the fermentation process.

In addition, more research needs to be done to identify toxic compounds in the acid hydrolysates such as furans, aliphatic acids (*33*) and phenolic compounds (*34*). The removal of these inhibitors is very important for the improvement of the fermentability of seaweed hydrolysate (*35*).

Optical purity of L(+)-lactic acid produced on G. amansii hydrolysate and on the mixture of galactose and glucose

Optical purity of lactic acid is important for the quality of final products such as polylactic acid (PLA), so that the selection of microorganisms that produce high-purity L(+)-lactic acid was essential (*36*). Table 3 shows the optical purity of L(+)-lactic acid obtained on *G. amansii* hydrolysate and on the mixture of galactose and glucose. The optical purity of L(+)-lactic acid produced from *G. amansii* hydrolysate by *L. rhamnosus* was 84.54 %, which was lower than when using glucose only. L(+)-lactic acid produced from *G. amansii* hydrolysate is not suitable for PLA production because of its low optical purity; however, alternative applications for the production of lactic acid esters, like ethyl or methyl lactate, or as a disinfectant of seaweed culture would be possible.

End products of fermentation on G. amansii hydrolysate and on the mixture of glucose and galactose

Lactic acid bacteria can belong to the genera *Aerococcus*, *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Carnobacterium*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Tetragenococcus*, *Vagococcus* and *Weissela*, and they metabolize carbohydrates *via* different pathways resulting in homo-, hetero-, or mixed acid fermentations (*37*). The homofermentation routes give only lactic acid as a major end product of glucose catabolism through Embden–Meyerhof–Parnas pathway, while the hetero- or mixed acid fermentation routes give not only lactic acid, but also formic and acetic acids as by-products. Only a few lactic acid bacteria, such as *L. bulgaricus*, *L. helveticus*, *L. amylophilus* and *L. delbrueckii*, could produce optically pure lactic acid (*18*, *38*). Table 4 shows the end products of fermentation by

Table 2. Lactic acid yield influenced by the presence of fermentation inhibitors

	Control (without inhibitor)			Chemical inhibitor		
	Total	Galactose	Glucose	Total	Galactose	Glucose
γ(sugar)$_{initial}$/(g/L)	30.23	20.2	10.13	31.57	21.68	9.89
γ(sugar)$_{final}$/(g/L)	0	0	0	0	0	0
sugar consumption rate/%	100	100	100	100	100	100
γ(lactic acid)/(g/L)		19.95			16.99	
$Y_{P/S}$/%		65.99			53.82	

Chemical inhibitors: 5-HMF (1.15 g/L), furfural (0.26 g/L), phenol (0.47 g/L)
Sugar consumption rate=(γ(sugar)$_{final}$/γ(sugar)$_{initial}$)·100
$Y_{P/S}$=(m(lactic acid)/m(substrate))·100

Table 3. The optical purity of L(+)-lactic acid produced on *Gelidium amansii* hydrolysate and on synthetic media

Optical purity ratio	Fermentation on *G. amansii* hydrolysate	Fermentation on synthetic media		
		Galactose and glucose mixture	Sole chemical	
			Galactose	Glucose
L(+)-lactic acid/%	84.54	85.62	83.43	97.15
D(–)-lactic acid/%	15.46	14.38	16.57	2.85

Table 4. End product of the fermentation by *Lactobacillus rhamnosus* on *Gelidium amansii* hydrolysate and on synthetic media

Product	γ/(g/L)			
	Sole chemical		Chemical mixture (galactose and glucose)	*G. amansii* hydrolysate*
	Galactose	Glucose		
galactose	30.15	0	24.61	19.60
glucose	0	30.79	13.92	10.21
lactic acid	19.92	29.45	24.68	12.53
formic acid	0.43	n.d.	0.33	0.24
acetic acid	1.61	n.d.	1.34	0.98
ethanol	0.82	n.d.	0.62	0.37

*5 % (by mass) of *G. amansii* was treated with 3 % H_2SO_4 (by volume) at 140 °C for 15 min
The fermentations were conducted in a 1-litre bioreactor with working volume of 700 mL at 37 °C and 100 rpm for 6 days
n.d.= not detected

L. rhamnosus on *G. amansii* hydrolysate and on the mixture of glucose and galactose. *L. rhamnosus* ferments carbohydrates *via* homofermentative pathways. However, in the case of galactose, formic acid, acetic acid and ethanol were produced as end products, although the cell growth was similar as in case of glucose fermentation.

Conclusion

In this study, sulphuric acid hydrolysate of *Gelidium amansii*, a major species of red seaweed was used for lactic acid production. *G. amansii* has more than 70 % of carbohydrates, and its hydrolysate contains galactose and glucose as the main components originating from agar and cellulose. These fermentable monosaccharides were utilized as a substrate for lactic acid production by *Lactobacillus rhamnosus* KY-3. In *G. amansii* hydrolysate, fermentation inhibitors were detected as follows: 5-HMF, furfural and phenol. Inhibitor removal is necessary to improve lactic acid production. Although *L. rhamnosus* is homofermentative bacterium, a small amount of formic acid, acetic acid and ethanol as end products was also produced from *G. amansii* hydrolysate. Lactic acid produced from *G. amansii* hydrolysate can be applied for the production of lactic acid esters, like ethyl and methyl lactate, or as disinfectant of seaweed culture, even though its optical purity is lower. Among the seaweed, *G. amansii* hydrolysate could be a good potential substrate for the production of lactic acid as shown in this study.

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