J. Microbiol. Biotechnol. (2014), 24(9), 1245–1253
http://dx.doi.org/10.4014/jmb.1402.02038

jmb

Sulfuric Acid Hydrolysis and Detoxification of Red Alga *Pterocladiella capillacea* for Bioethanol Fermentation with Thermotolerant Yeast *Kluyveromyces marxianus*

Chien-Hui Wu[1], Wei-Chen Chien[2], Han-Kai Chou[2], Jungwoo Yang[3*], and Hong-Ting Victor Lin[2,4*]

[1]*Department of Seafood Science, National Kaohsiung Marine University, Nan-Tzu, Kaohsiung 81143, Taiwan, R.O.C.*
[2]*Department of Food Science, National Taiwan Ocean University, Keelung 202, Taiwan, R. O. C.*
[3]*School of Life Science & Biotechnology for BK21 Plus, Department of Biotechnology, Korea University, Seoul 136-713, Republic of Korea*
[4]*Center of Excellence for the Oceans, National Taiwan Ocean University, Keelung 202, Taiwan, R. O. C.*

Received: February 19, 2014
Revised: May 20, 2014
Accepted: May 23, 2014

First published online
May 23, 2014

*Corresponding authors
H.-T.V.L.
Phone: +886-2-24622192 ext 5121;
Fax: +886-2-24634203;
E-mail: HL358@ntou.edu.tw
J.Y.
Phone: +82-61-450-0148;
E-mail: dbl3jwy@korea.kr

pISSN 1017-7825, eISSN 1738-8872

One-step sulfuric acid saccharification of the red alga *Pterocladiella capillacea* was optimized, and various detoxification methods (neutralization, overliming, and electrodialysis) of the acid hydrolysate were evaluated for fermentation with the thermotolerant yeast *Kluyveromyces marxianus*. A proximate composition analysis indicated that *P. capillacea* was rich in carbohydrates. A significant galactose recovery of 81.1 ± 5% was also achieved under the conditions of a 12% (w/v) biomass load, 5% (v/v) sulfuric acid, 121°C, and hydrolysis for 30 min. Among the various detoxification methods, electrodialysis was identified as the most suitable for fermentable sugar recovery and organic acid removal (100% reduction of formic and levulinic acids), even though it failed to reduce the amount of the inhibitor 5-HMF. As a result, *K. marxianus* fermentation with the electrodialyzed acid hydrolysate of *P. capillacea* resulted in the best ethanol levels and fermentation efficiency.

Keywords: Biothanol, fermentation, *Pterocladiella capillacea*, *Kluyveromyces marxianus*, acid saccharification, electrodialysis

Introduction

Energy shortages and greenhouse gas emissions resulting in global warming are forcing countries worldwide to seek renewable energy sources, such as wind power, solar power, hydrogen power, and biofuels [11, 18]. Bioethanol is one of the most promising renewable energy sources, and is defined as a liquid biofuel obtained from plant biomasses through the saccharification and fermentation of fermentable sugars by yeasts. According to the type of biomass used, bioethanol from sugar and starch crops is considered a "first-generation fuel," whereas bioethanol from a lignocellulosic biomass is considered a "second-generation fuel."

Although the production of first-generation bioethanol is already well-established, the use of food-related biomasses for energy purposes is raising concerns owing to limited cultivable land and increasing food prices. Conversely, even though the production of second-generation bioethanol using low-cost lignocellulosic biomass waste is unrelated to high food prices, the high cost of the saccharification of a lignin-containing biomass and a reliable supply of land plants make the production of second-generation bioethanol very challenging [11]. Thus, the use of marine algae as a third-generation biomass is now attracting considerable attention due to the following advantages: First, marine algae have a short generation cycle and can be easily cultivated in various aquatic environments. Second, marine algae have no lignin, which makes saccharification more feasible, and they are not considered a primary food crop. Third, marine algae are rich in carbohydrates. Therefore, all these reasons indicate their great potential as a source of

renewable energy [8, 11].

The production of biofuel from a nonsugar biomass requires saccharification, which can be performed enzymatically or chemically, to generate sugars to be fermented by bioethnol-producing yeasts. During enzymatic hydrolysis, the carbohydrates in the biomass are broken down into fermentable sugars by enzymes. Although environmentally friendly, the high cost of enzymes and lengthy hydrolysis procedure over several days are major obstacles to practical application [10]. During chemical hydrolysis, the carbohydrates in the biomass are degraded into fermentable sugars using an acid, base, or organic solvent [25]. Among these, sulfuric acid is widely used owing to its low cost and rapid reaction time. However, sulfuric acid can decompose sugars into undesirable byproducts, such as furans and organic acids, which are known to inhibit the ethanol-producing microorganisms in the fermentation process [1, 12]. Thus, routine selective detoxification processes, such as ion exchange resins, laccase treatment, and overliming with calcium hydroxide, have been proposed to remove these inhibitors from the acid hydrolysates of the biomass to improve the ethanol fermentation efficiency. Yet, this involves extra costs for additional filtration steps and more importantly sugar loss.

Accordingly, this study analyzed the proximate composition of the red alga *Pterocladiella capillacea* (Rhodothyta), which is found along the northeast coast of Taiwan, and optimized the sulfuric acid saccharification and detoxification processes for *P. capillacea* that consists of chains of repeating alternative units of fermentable simple sugar galactose and non-fermentable 3,6-anhydro-galactose [21]. The acid hydrolysate derived from *P. capillacea* was then fermented using the yeast *Kluyveromyces marxianus* for biothanol production. *K. marxianus* is a Crabtree-negative yeast [6] that can ferment a wide variety of monosaccharides, including hexoses and pentoses, into bioethanol. *K. marxianus* has several advantages over *Saccharomyces cerevisiae*, including thermotolerance, a higher growth rate, and broader substrate spectrum [22], all of which make it an effective alternative to *S. cerevisiae* as a bioethanol producer.

Thus, the main objectives of this study were to optimize the acid hydrolysis conditions for *P. capillacea* using sulfuric acid; analyze the effects of the inhibitors hydroxymethylfurfural (5-HMF), formic acid (FA), and levulinic acid (LA), all of which are the degradation products of 3,6-anhydro-galactose, on the cell growth and ethanol production by *K. marxianus*; and evaluate the detoxification approaches of neutralization, overliming, and electrodialysis on inhibitor removal and ethanol production.

Materials and Methods

Source of *P. capillacea* and Determination of Its Proximate Composition

Dried *P. capillacea* was purchased from a local market in Chao-Ching Park, Taiwan. The seaweed was ground, sieved (1 mm pore size), and stored at −20°C before use. The proximate composition, including the moisture, crude protein, crude lipid, crude fiber, and ash of *P. capillacea,* was analyzed according to AOAC official methods of analysis [2]. The *P. capillacea* was also acid hydrolyzed and analyzed, as described previously [24], to determine the percentage of monosaccharide galactose and glucose, respectively.

Preparation of Sulfuric Acid Hydrolysate of *P. capillacea*

The sulfuric acid treatment of *P. capillacea* was investigated using different acid hydrolysis parameters: biomass loading (4%, 7%, 10%, 12%, and 15% (w/v)), acid concentration (0.5%, 1%, 2%, 5%, and 10% (v/v)), and hydrolysis time (30, 60, and 90 min). The dried, crushed, and sieved *P. capillacea* powder was loaded in distilled water to obtain different biomass/water ratios (w/v), followed by the addition of different concentrations (v/v) of sulfuric acid. The biomass/sulfuric acid mixtures were then incubated for different times at 121°C for acid hydrolysis. Thereafter, the samples were cooled to room temperature, adjusted to approximately pH 6, and filtered through a 0.22 μm membrane filter prior to fermentation.

Analyses of Sugars, Inhibitors, and Ethanol Using HPLC

The sugars glucose and galactose, inhibitors 5-HMF, formic acid (FA), and levulinic acid (LA), and ethanol in the acid hydrolysate of *P. capillacea* were all analyzed using an Aminex HPX-87H column (Bio-Rad, Sunnyvale, CA, USA), along with a refractive index detector with a 5 mM H_2SO_4 eluent at a flow rate of 0.6 ml/min at 60°C. All the samples were filtered through a 0.22 μm membrane filter prior to the HPLC analysis.

Inhibitory Test of Inhibitors on Yeast *K. marxianus*

The *K. marxianus* was cultured for 24 h in a YPG medium (1% yeast extract, 2% bactopeptone, and 2% dextrose) containing various concentrations of the inhibitors 5-HMF, FA, and LA, respectively, in order to evaluate the tolerance of *K. marxianus* to these inhibitors. The cell growth and ethanol production were monitored using the aerobic plate count and HPLC, respectively.

Detoxification of Acid Hydrolysate of *P. capillacea*

The detoxification of the acid hydrolysate was carried out using three different approaches: neutralization, overliming, and electrodialysis. The neutralization was accomplished by adjusting the pH of the acid hydrolysate to approximately pH 6. The overliming of the acid hydrolysate was performed according to the procedure described previously, with certain modifications [19]. Briefly, the acid hydrolysate was adjusted to pH 10–11 by adding calcium hydroxide and incubated at 60°C for 30 min. The

hydrolysate was then cooled to room temperature, filtered through a 0.22 μm membrane filter, and adjusted to approximately pH 6 before use. The electrodialysis of the acid hydrolysate was achieved using an electrodialyzer from the ASTOM Corporation (Tokyo, Japan). The sample was loaded and circulated in the machine at room temperature until the conductivity was lower than 5 mS/cm.

Fermentation of Acid Hydrolysate of *P. capillacea* by *K. marxianus*

The yeast *K. marxianus* ATCC 36907 was purchased from the Bioresource Collection and Research Center, Hsinchu, Taiwan. *K. marxianus* was cultured in a YPD medium (1% yeast extract, 2% bactopeptone, and 2% dextrose) and then inoculated into 100 ml of the detoxified acid hydrolysate of *P. capillacea* containing 1% yeast extract and 2% bactopeptone. Thereafter, the acid hydrolysate was incubated at 40°C and 150 rpm for 48 h, and samples were taken every 12 h for an HPLC analysis of the sugars, ethanol, and inhibitors.

Statistical Analysis

The data were analyzed statistically using SPSS ver. 12.0 (SPSS Inc., Chicago, IL, USA). A one-way analysis of variance (ANOVA) was used to determine the statistical differences between the sample means, where the level of significance was set at $p < 0.05$ or 0.01. Multiple comparisons of the means were conducted using a Tukey test. All the data are expressed as the mean ± SD.

Results and Discussion

Proximate Composition of *P. capillacea*

The proximate composition of the dried *P. capillacea*, as presented in Table 1, included 14.5% moisture, 1.8% protein, 0.2% lipids, 4.5% ash, and 80% carbohydrates, including galactose (34.9%) and glucose (26.4%). Jang *et al.* [8] previously reported that the red alga *Gelidium amansii* and brown alga *Sargassum fulvellum* had carbohydrate contents of 74.4% and 44.5%, respectively. Thus, the current data for the proximate composition of the red alga *P. capillacea* indicated that it was abundant in carbohydrates, making it a promising candidate biomass for bioethanol production.

Table 1. Proximate composition (%) of *P. capillacea*.

Compound	Content (%)
Moisture	14.5
Crude protein	1.8
Crude lipid	0.2
Ash	3.5
Carbohydrate	80.0[a]
Galactose[b]	34.9
Glucose[b]	26.4

[a]One hundred percent subtracted moisture, crude protein, crude lipid, and ash.
[b]Determination of sugar content in biomass using high-performance liquid chromatography.

Effect of Biomass Concentration on *P. capillacea* Acid Hydrolysis

Meinita *et al.* [17] previously reported that the acid concentration, raw materials used, reaction temperature, and reaction time all affected the yield of monosaccharides released from the acid hydrolysis of polysaccharides. Accordingly, 4%, 7%, 10%, 12%, and 15% *P. capillacea* (w/v) were hydrolyzed by 2% sulfuric acid at 121°C for 15 min. The theoretical yields (%) of the fermentable sugars galactose and glucose presented in Table 2 indicate that the recovery yields were biomass quantity dependent. All the preparations, except for the 15% (w/v) biomass, presented statistically similar theoretical yields of galactose production, with the highest at 68.2% ± 4.3%. The theoretical yield of galactose with 15% biomass decreased to 55 ± 5.4%, which may have been due to an overload, as the highly viscous *P. capillacea* suspension was difficult to acid hydrolyze. However, since the 4%, 7%, 10%, and 12% biomass loadings presented no significant statistical differences in their galactose recovery yields, a 12% (w/v) biomass loading

Table 2. Effect of biomass loading on production of sugars and byproducts during acid hydrolysis of *P. capillacea*.

P. capillacea (% w/v)	Galactose (g/l) (% of theoretical yield)	Glucose (g/l) (% of theoretical yield)	5-HMF (g/l)	FA (g/l)	LA (g/l)
4	8.7 ± 0.8 (62.4 ± 5.9%)[a]	2.0 ± 0.1 (20.0 ± 1.0%)[a]	1.5 ± 0.1[e]	3.3 ± 0.4[b]	1.4 ± 0.2[b]
7	16.7 ± 1.0 (68.2 ± 4.3%)[a]	2.7 ± 0.2 (15.2 ± 1.1%)[b]	2.9 ± 0.2[d]	1.1 ± 0.1[c]	1.6 ± 0.1[b]
10	23.7 ± 1.9 (67.9 ± 5.4%)[a]	3.2 ± 0.2 (12.3 ± 0.8%)[c]	4.3 ± 0.2[c]	1.1 ± 0.1[c]	1.5 ± 0.1[b]
12	28.2 ± 2.3 (67.5 ± 5.5%)[a]	3.4 ± 0.3 (11.0 ± 1.0%)[c]	5.5 ± 0.3[b]	1.1 ± 0.1[c]	1.5 ± 0.1[b]
15	28.8 ± 2.8 (55.0 ± 5.4%)[b]	4.2 ± 0.3 (10.8 ± 0.8%)[c]	6.4 ± 0.3[a]	4.1 ± 0.2[a]	3.2 ± 0.4[a]

5-HMF, 5-hydroxymethylfurfural; FA, formic acid; LA, levulinic acid.
Various biomass loadings (w/v) of *P. capillacea* were hydrolyzed by 2% (v/v) sulfuric acid at 121°C for 15 min.
Data are expressed as the mean ± SD ($n = 3$). Different letters in a column show significant differences.

Table 3. Effect of sulfuric acid concentration on production of sugars and byproducts during acid hydrolysis of *P. capillacea*.

H_2SO_4 (% v/v)	Galactose (g/l) (% of theoretical yield)	Glucose (g/l) (% of theoretical yield)	5-HMF (g/l)	FA (g/l)	LA (g/l)
0.5	9.6 ± 0.5 (23.4 ± 2.6%)^{c}	1.0 ± 0.1 (3.2 ± 0.3%)^{d}	3.3 ± 0.1^{b}	0.5 ± 0.0^{b}	0.1 ± 0.0^{d}
1.0	18.8 ± 1.0 (45.9 ± 2.4%)^{b}	2.1 ± 0.2 (6.8 ± 0.6%)^{c}	5.5 ± 0.2^{a}	0.8 ± 0.1^{b}	0.5 ± 0.1^{d}
2.0	24.9 ± 1.8 (60.7 ± 4.3%)^{a}	3.4 ± 0.3 (11.0 ± 1.0%)^{b}	5.9 ± 0.3^{a}	1.3 ± 0.1^{b}	2.1 ± 0.1^{c}
5.0	26.1 ± 2.5 (63.4 ± 6.0%)^{a}	4.5 ± 0.3 (14.4 ± 1.0%)^{a}	2.3 ± 0.1^{c}	5.8 ± 0.4^{a}	7.0 ± 0.3^{b}
10.0	24.8 ± 3.1 (60.4 ± 7.4%)^{a}	4.4 ± 0.4 (14.2 ± 1.3%)^{a}	0.3 ± 0.1^{d}	6.2 ± 0.7^{a}	8.7 ± 0.8^{a}

5-HMF, 5-hydroxymethylfurfural; FA, formic acid; LA, levulinic acid.

Various concentrations (v/v) of H_2SO_4 were used for acid hydrolysis of 12% (w/v) *P. capillacea* at 121°C for 15 min.

Data are expressed as the mean ± SD (n = 3). Different letters in a column show significant differences.

was used to obtain the maximum galactose production from acid hydrolysis during the remainder of the study.

The recovery yields for glucose production with all the preparations ranged from 10.8% to 20%, and this yield decreased as the quantity of the biomass used was increased (Table 2). The present data indicated that the glucose recovery ratio from this alga biomass was not efficient using sulfuric acid hydrolysis. A similar result was also reported by Jang *et al*. [9] with a recovery ratio of 12.6% for glucose. As the proximate composition data for *P. capillacea* showed that the carbohydrates in *P. capillacea* were hexoses, the major inhibitors 5-hydroxymethylfurfural, formic acid, and levulinic acid were formed during the acid hydrolysis. 5-HMF is the degradation product of galactose and glucose, whereas FA and LA are the breakdown products of 5-HMF. As shown in Table 2, the generation of the inhibitor 5-HMF and the biomass quantity load were positively correlated; 15% *P. capillacea* resulted in the highest production of 5-HMF at 6.4 g/l. However, the other two inhibitors, FA and LA, were not related to the biomass quantity load.

Effect of Acid Concentration on *P. capillacea* Acid Hydrolysis

As shown in Table 3, H_2SO_4 concentrations of 0.5%, 1%, 2%, 5%, and 10% (v/v) were used for the acid hydrolysis of 12% (w/v) *P. capillacea* at 121°C for 15 min. For the H_2SO_4 range of 0.5%–5% (v/v), the higher the concentration of acid used, the higher the amount of fermentable sugars obtained. Thus, 5% (v/v) H_2SO_4 produced the best galactose yield of 26.1 ± 2.5 g/l (63.4 ± 6.0% of theoretical yield) and a glucose yield of 4.5 g/l (14.4 ± 1.0% of theoretical yield). Meanwhile, 10% H_2SO_4 resulted in lower yields of fermentable sugars, indicating that this acid concentration likely caused the maximum degradation of the fermentable sugars.

H_2SO_4 at 2% (v/v) resulted in most 5-HMF, which may have been because H_2SO_4 at >2% caused the subsequent transformation of 5-HMF into FA and LA. Obvious increases in FA and LA were also observed when using 2% to 5% (v/v) H_2SO_4. A similar result was previously observed [20], where 10% (w/v) of the red alga *P. capillacea* was hydrolyzed using 2% (v/v) sulfuric acid at 121°C for 59 min, and approximately 5 g/l of 5-HMF, 5 g/l of formic acid, and 7 g/l of levulinic acid were produced.

Effect of Hydrolysis Time on *P. capillacea* Acid Hydrolysis

P. capillacea at 12% (w/v) was hydrolyzed using 5% (v/v) sulfuric acid at 121°C for 15, 30, 60, or 90 min. As shown in Table 4, 30 min of acid hydrolysis gave the best recovery yield of galactose at 35.3 ± 2.3 g/l (81.4 ± 5% of theoretical yield) and glucose at 6.2 ± 0.3 g/l (19.9 ± 1%). Meanwhile,

Table 4. Effect of acid hydrolysis time on production of sugars and byproducts during acid hydrolysis of *P. capillacea*.

Time (min)	Galactose (g/l) (% of theoretical yield)	Glucose (g/l) (% of theoretical yield)	5-HMF (g/l)	FA (g/l)	LA (g/l)
15	26.0 ± 2.3 (62.0 ± 5%)^{b}	4.5 ± 0.3 (14.5 ± 1%)^{b}	1.6 ± 0.2^{a}	4.1 ± 0.2^{b}	4.9 ± 0.2^{c}
30	34.3 ± 2.3 (81.1 ± 5%)^{a}	6.2 ± 0.3 (19.9 ± 1%)^{a}	0.6 ± 0.1^{ab}	6.6 ± 0.5^{a}	5.8 ± 0.3^{c}
60	27.9 ± 3.1 (66.8 ± 7%)^{b}	5.3 ± 0.3 (16.9 ± 1%)^{ab}	0.4 ± 0.1^{bc}	6.8 ± 0.4^{a}	9.2 ± 0.6^{b}
90	25.9 ± 2.6 (62.0 ± 6%)^{b}	4.3 ± 0.6 (14.0 ± 2%)^{b}	0.2 ± 0.0^{c}	4.8 ± 0.9^{b}	11.2 ± 1.1^{a}

5-HMF, 5-hydroxymethylfurfural; FA, formic acid; LA, levulinic acid.

P. capillacea at 12% (w/v) was hydrolyzed using 5% (v/v) sulfuric acid at 121°C for different hydrolysis times.

Data are expressed as the mean ± SD (n = 3). Different letters in a column show significant differences.

hydrolysis for 60 and 90 min resulted in lower recovery yields of these sugars, possibly because these extended acid hydrolysis times resulted in the transformation of the galactose into inhibitors.

The concentration of 5-HMF increased as the reaction time decreased. In contrast, the FA and LA production increased as the reaction time increased. This may have been because the sugar breakdown product 5-HMF was further transformed into FA and LA as the acid hydrolysis time was extended. Jeong *et al*. [10] previously investigated different sulfuric acid concentrations and reaction times for the acid hydrolysis of *G. amansii* and made similar observations.

Effect of Inhibitors 5-HMF, FA, and LA on Cell Growth and Ethanol Production by *K. marxianus*

Since inhibitors are invariably formed during the acid hydrolysis of a biomass, the present optimized acid saccharification of *P. capillacea* resulted in 34.3 ± 2.3 g/l of galactose, 6.2 ± 0.3 g/l of glucose, 0.6 ± 0.1 g/l of 5-HMF, 6.6 ± 0.5 g/l of FA, and 5.8 ± 0.3 g/l of LA. Thus, the effect of these inhibitors on *K. marxianus* was then investigated. The thermotolerant yeast *K. marxianus* can grow and ferment at temperatures of 40°C or higher [14], which makes it a suitable yeast for biofuel fermentation without air conditioning in Taiwan's subtropical climate. As shown in Fig. 1, all three inhibitors decreased the cell growth and ethanol production of *K. marxianus* in a concentration-dependent manner. 5-HMF was the most significant when all three inhibitors were used at similar concentrations. 5-HMF at 0.5 g/l, reduced the cell growth and ethanol production of *K. marxianus* to 98 ± 2.5% and 88 ± 6.1%, respectively, after 24 h, when compared with the control without 5-HMF (Fig. 1A). No ethanol production was observed at 5-HMF concentrations >6 g/l, and 8 g/l of 5-HMF completely inhibited cell growth after 24 h (Fig. 1A). Liu *et al*. [15] previously reported that 5-HMF inhibited the growth of yeasts in a dose-dependent manner and showed that 60 mM (approximately 10 g/l) 5-HMF caused an extended growth lag phase of 25 to 125 h for selected *S. cerevisiae* strains.

As shown in Figs. 1B and 1C, the organic acid inhibitors FA and LA did not significantly inhibit the cell growth of *K. marxianus*, where 10 g/l of FA and 13 g/l of LA only reduced the cell growth to 89% and 92%, respectively. However, 6 g/l of FA and 13 g/l of LA significantly decreased the bioethanol production of *K. marxianus* to 42% (Fig. 1B) and 51% (Fig. 1C), respectively. The influx of undissociated acid likely uncoupled the ATP conservation

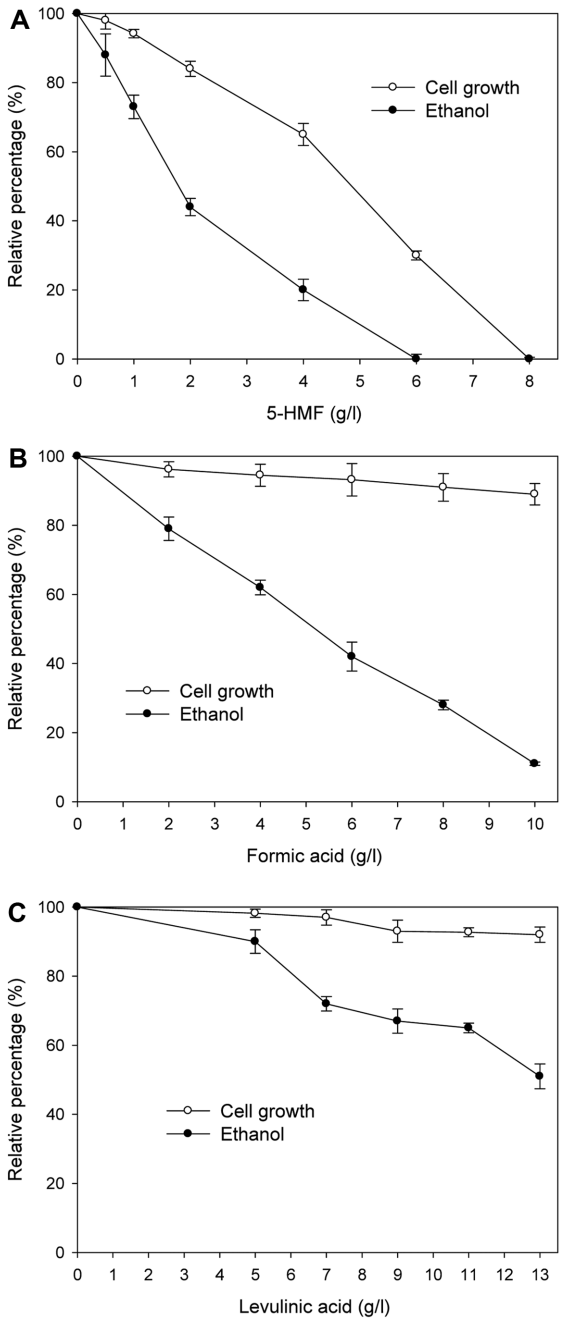

Fig. 1. Effect of inhibitors (**A**) 5-HMF, (**B**) formic acid, and (**C**) levulinic acid on cell growth and ethanol production by *K. marxianus*.

K. marxianus was cultured in a YPG medium containing 1% yeast extract, 2% bactopeptone, and 2% galactose at 40°C and 150 rpm for 48 h prior to the analysis using HPLC.

by disrupting the proton-motive force across the cell membrane and thereby decreasing the bioethanol production [13]. FA has a smaller pK_a value than LA, which indicates

that the concentration of undissociated FA was less than that of LA when they were at equal concentrations and pH levels.

Notwithstanding, as shown in Fig. 1, FA was more inhibitory than LA, which may have been because the smaller FA was efficiently diffused through the cell membrane and likely had a higher anion toxicity. Larsson *et al*. [13] previously reported that FA and LA marginally increased the ethanol production of *S. cerevisiae* at concentrations of <100 mM. Yet, this phenomenon was not observed in the present study, probably because *S. cerevisiae* and *K. marxianus* have different tolerances to FA and LA.

According to the present inhibition data, all the inhibitors from the acid hydrolysis step significantly reduced the bioethanol production by *K. marxianus*, and a combined effect on reducing the bioethanol production was also observed [13]. The present optimized acid saccharification of *P. capillacea* resulted in 34.3 ± 2.3 g/l galactose, 6.2 ± 0.3 g/l glucose, 0.6 ± 0.1 g/l 5-HMF, 6.6 ± 0.5 g/l FA, and 5.8 ± 0.3 g/l LA. According to Fig. 1, the actual concentrations of 5-HMF, FA, and LA decreased the growth of *K. marxianus* by 3%, 8%, and 2%, respectively, and lowered the ethanol fermentation by 15%, 64%, and 18%, respectively. Thus, further detoxification steps to remove these inhibitors from the acid hydrolysate of *P. capillacea* are needed to improve the bioethanol production when using *K. marxianus*.

Effect of Detoxification Method on Reducing Amount of Fermentable Sugars and Inhibitors 5-HMF, Formic Acid, and Levulinic Acid

Without any detoxification step, the acid hydrolysate had a pH of <0.1 and contained various inhibitors, which made it too toxic for *K. marxianus* to grow and produce ethanol for 96 h (data not shown), confirming that detoxification of the acid hydrolysate is necessary for *K. marxianus* fermentation. As shown in Table 5, neutralization reduced the amount of 5-HMF, FA, and LA by 42.9 ± 14.2%, 8.7 ± 2.9%, and 11.5 ± 4.5%, respectively, and reduced the amount of fermentable sugars by 24.9 ± 4.5%. Meanwhile, overliming reduced the amount of 5-HMF, FA, and LA by 57.1 ± 14.3%, 52.2 ± 10.1%, and 47.5 ± 9.8%, respectively; a similar result was also previously reported by Chandel *et al*. [4]. However, unexpectedly, overliming also reduced the amount of fermentable sugars by 42.1 ± 2.8%, which may have been because some ionized sulfated algae monosaccharides were captured and precipitated by calcium hydroxide [23].

Even though electrodialysis failed to reduce the amount of 5-HMF, it completely removed the FA and LA and reduced the amount of fermentable sugars by 16.5 ± 3.9%. Electrodialysis can transport charged ions, such as salts and acids, from the main circuit channel through ion-exchange membranes under the influence of an applied electric potential difference. Therefore, this could explain why the charged organic acids FA and LA were completely removed, while the 5-HMF remained. Organic acids formed during acid hydrolysis have always been an obstacle in ethanol fermentation by yeasts [16].

Bellido *et al*. [3] previously reported that ethanol productivity and the complete inhibition of *Pichia stipitis* were observed with 3.5 g/l of acetic acid. The total removal of organic acids from an acid hydrolysate can be beneficial

Table 5. Effect of detoxification approach on reduction of fermentable sugars and inhibitors 5-HMF, formic acid, and levulinic acid.

	Concentration (g/l)			
	Acid hydrolysate (Control)	Neutralization (% reduction)	Overliming (% reduction)	Electrodialysis (% reduction)
Fermentable sugars	38.2 ± 1.2	28.7 ± 1.7 (24.9 ± 4.5%)	22.1 ± 0.7 (42.1 ± 2.8%)	31.9 ± 1.5 (16.5 ± 3.9%)
Galactose	33.3 ± 0.6	25.3 ± 1.2	19.7 ± 0.4	27.8 ± 0.8
Glucose	4.9 ± 0.5	3.4 ± 0.5	2.4 ± 0.3	4.1 ± 0.8
5-HMF	0.7 ± 0.1	0.4 ± 0.1 (42.9 ± 14.2%)	0.3 ± 0.1 (57.1 ± 14.3%)	0.7 ± 0.1 (0.0 ± 0.0%)
FA	6.9 ± 0.4	6.3 ± 0.2 (8.7 ± 2.9%)	3.3 ± 0.7 (52.2 ± 10.1%)	N.D. (100 ± 0.0%)
LA	6.1 ± 0.4	5.4 ± 0.6 (11.5 ± 4.5%)	3.2 ± 0.6 (47.5 ± 9.8%)	N.D. (100 ± 0.0%)

5-HMF, 5-hydroxymethylfurfural; FA, formic acid; LA, levulinic acid.

N.D.: not detected.

Data are expressed as the mean ± SD (n = 3).

to fermentation by yeasts. In addition, an industrial electrodialyzer is capable of continuously processing large volumes of liquid, which is beneficial for large-scale detoxification. Thus, electrodialysis has drawn considerable attention in the case of acid recovery due to its simplicity and potential for large-scale manipulation [7]. Furthermore, the FA and LA removed by an electrodialyzer from an acid hydrolysate can be easily collected for further purification and for pharmaceutical and chemical uses as a concept for biorefinery [5].

Effect of Detoxification Method for *P. capillacea* Acid Hydrolysates on Bioethanol Production by *K. marxianus*

The effects of the three detoxification methods, neutralization, overliming, and electrodialysis, on the acid hydrolysates of *P. capillacea* were evaluated by monitoring the sugar usage, cell growth, and ethanol production by *K. marxianus* during fermentation. The inhibitor and sugar (galactose and glucose) concentrations varied according to the detoxification method. *K. marxianus* was cultured in various media at 40°C and 150 rpm for 48 h.

Fig. 2A shows that when *K. marxianus* was cultured in a YPG medium, the highest cell count of 9.1 ± 0.1 log CFU/ml was reached after 12 h. Moreover, 95.8 ± 0.5% of the sugar was used after 24 h and 12.5 ± 0.5 g/l of ethanol was produced after 24 h.

Fig. 2B shows that when *K. marxianus* was fermented with the acid hydrolysate of *P. capillacea* following neutralization, it achieved the highest cell count of 8.0 ± 0.1 log CFU/ml after 36 h. Moreover, 90.9 ± 0.8% of the sugar was used after 36 h and 3.9 ± 0.2 g/l of ethanol was produced after 36 h.

Fig. 2C shows that when *K. marxianus* was fermented with the acid hydrolysate of *P. capillacea* following overliming, the highest cell count of 8.3 ± 0.2 log CFU/ml was reached after 24 h. Moreover, 73 ± 4.9% of the sugar was used after 24 h and 5.7 ± 0.1 g/l of ethanol was produced after 24 h.

Fig. 2D shows that when *K. marxianus* was fermented with the acid hydrolysate of *P. capillacea* following electrodialysis, the highest cell count of 8.5 ± 0.2 log CFU/ml was reached after 24 h. Moreover, 87 ± 4.2% of the sugar was used after 24 h and 10.6 ± 0.4 g/l of ethanol was produced after 24 h.

The kinetic parameters for *K. marxianus* when fermented

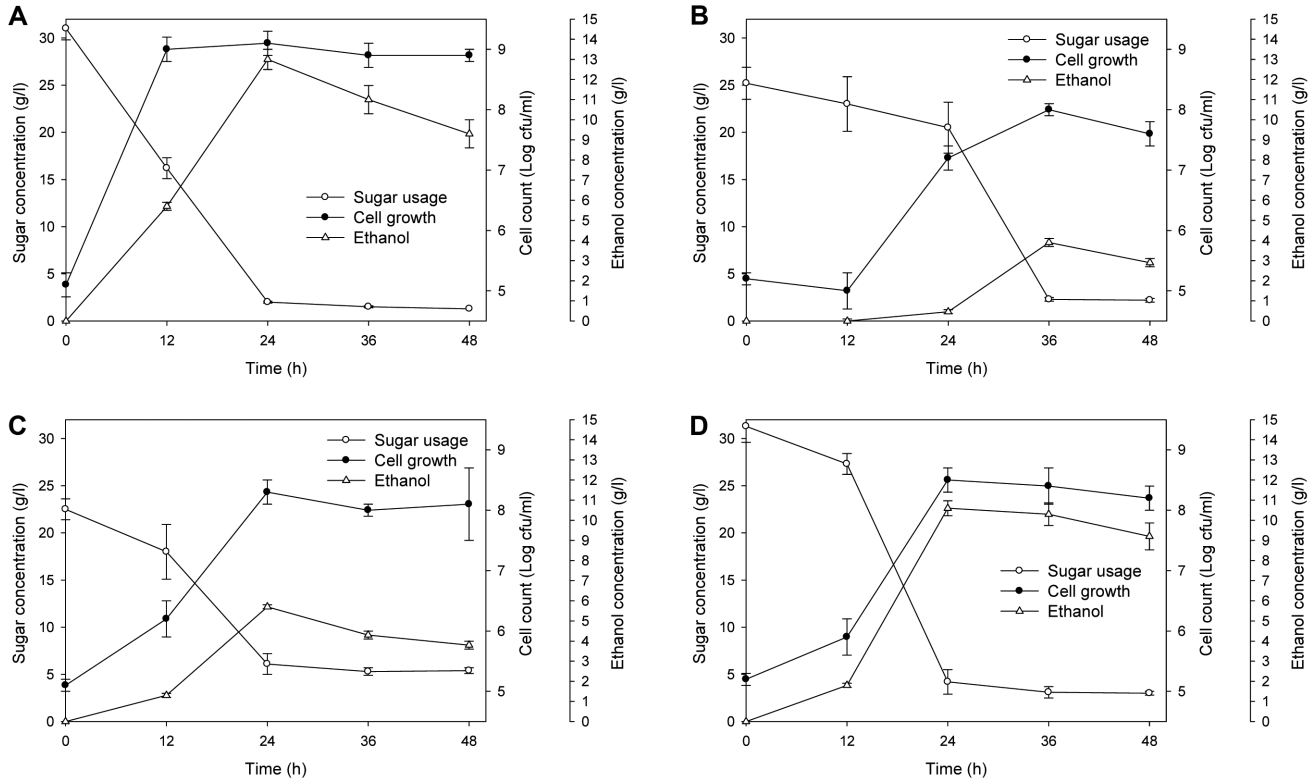

Fig. 2. Measurements of cell counts and bioethanol production by *K. marxianus* fermented with (**A**) YPG, (**B**) acid hydrolysate (following neutralization), (**C**) acid hydrolysate (following overliming), and (**D**) acid hydrolysate (following electrodialysis).
K. marxianus was inoculated into the YPG or acid hydrolysate supplemented with 1% yeast extract and 2% bactopeptone at 40°C and 150 rpm.

Table 6. Effect of detoxification method on bioethanol production efficiency.

Medium	C_{EtOH} (g/l)	r P_{EtOH} (g/1-h)	Max. $Y_{P/S}$ (g/g)
YPG	12.5 ± 0.5	0.52 ± 0.02 (24 h)	0.42 ± 0.02
Hydrolysate	3.9 ± 0.2	0.11 ± 0.01 (36 h)	0.17 ± 0.01
Overliming	5.7 ± 0.1	0.17 ± 0.00 (24 h)	0.35 ± 0.01
Electrodialysis	10.6 ± 0.37	0.44 ± 0.02 (24 h)	0.39 ± 0.02

r P_{EtOH}, initial ethanol production rate in g ethanol/l-h; Max. Yp/s, maximum ethanol yield in g ethanol/g consumption sugar; C_{EtOH}, ethanol concentration in g/l.

with the acid hydrolysates of *P. capillacea* following different detoxification methods are summarized in Table 6. The fermentation of *K. marxianus* in a YPG medium without any inhibitors resulted in the best cell count, sugar usage, and max. $Y_{P/S}$ (maximum ethanol yield 0.42 ± 0.02 g/g). Neutralization of the acid hydrolysate allowed the *K. marxianus* to grow, and the C_{EtOH} (ethanol concentration, g/l), r P_{EtOH} (initial ethanol production rate, g/h), and max. $Y_{P/S}$ (g/g) values were 3.9 ± 0.2, 0.11 ± 0.01 (36 h), and 0.17 ± 0.01, respectively, which were considerably low when compared with the other conditions. This was also consistent with the previous data (Table 5), which indicated that neutralization did not efficiently remove the inhibitors.

The overliming process for the acid hydrolysate removed half of the 5-HMF, LA, and FA, which enabled the fermentation to achieve the maximum ethanol concentration after 24 h. However, the C_{EtOH} (5.7 ± 0.1 g/l), r P_{EtOH} (0.17 ± 0.00 g/h), and max. $Y_{P/S}$ (0.35 ± 0.01 g/g) values were not promising. This may have been due to a significant loss of monosaccharides during the overliming. Meanwhile, although the electrodialysis did not remove any 5-HMF, the present data indicated that it was the most efficient detoxification method for the acid hydrolysate of *P. capillacea*, as it achieved the highest c_{EtOH} (10.6 ± 0.37 g/l), r P_{EtOH} (0.44 ± 0.02 g/h, in 24 h), and max. $Y_{P/S}$ (0.39 ± 0.02 g/g) values among the three detoxification approaches.

Electrodialysis gave a max. $Y_{P/S}$ of 0.39 ± 0.02 g/g, which was 93% of the max. $Y_{P/S}$ (0.42 ± 0.02 g/g) of *K. marxianus* when it was fermented in the YPG medium. This may have been due to the absence of the inhibitors FA and LA, plus the effects on the yeast metabolism by furans at a concentration of 1.0 g/l or lower were limited [3]. Furthermore, the low concentration of 5-HMF was likely rapidly assimilated by the fermenting yeast before its growth and ethanol production [26].

Acknowledgments

The financial and equipment support from the Mackay Medicine, Nursing and Management College (MKC100R14) and from the Center of Marine Bioenvironment and Biotechnology, National Taiwan Ocean University (NTOU-RD-AA-2013-2-02012) are gratefully acknowledged.

References

1. Almeida JRM, Modig T, Petersson A, Hahn-Hagerdal B, Liden G, Gorwa-Grauslund MF. 2007. Increased tolerance and conversion of inhibitors in lignocellulosic hydrolysates by *Saccharomyces cerevisiae*. *J. Chem. Technol. Biotechnol.* **82:** 340-349.
2. AOAC. 1998. *Offical Methods of Analysis of the Association of Official Analytical Chemists.* Association of Official Analytical Chemists, Washington, DC.
3. Bellido C, Bolado S, Coca M, Lucas S, Gonzalez-Benito G, Garcia-Cubero MT. 2011. Effect of inhibitors formed during wheat straw pretreatment on ethanol fermentation by *Pichia stipitis*. *Bioresour. Technol.* **102:** 10868-10874.
4. Chandel AK, Kapoor RK, Singh A, Kuhad RC. 2007. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. *Bioresour. Technol.* **98:** 1947-1950.
5. De Jong W, Marcotullio G. 2010. Overview of biorefineries based on co-production of furfural, existing concepts and novel developments. *Int. J. Chem. React. Eng.* **8**.
6. Gonzalez-Siso MI, Freire-Picos MA, Ramil E, Gonzalez-Dominguez M, Torres AR, Cerdan ME. 2000. Respirofermentative metabolism in *Kluyveromyces lactis*: insights and perspectives. *Enzyme Microb. Technol.* **26:** 699-705.
7. Huang CH, Xu TW, Zhang YP, Xue YH, Chen GW. 2007. Application of electrodialysis to the production of organic acids: State-of-the-art and recent developments. *J. Membr. Sci.* **288:** 1-12.
8. Jang JS, Cho Y, Jeong GT, Kim SK. 2012. Optimization of saccharification and ethanol production by simultaneous saccharification and fermentation (SSF) from seaweed, *Saccharina japonica*. *Bioprocess Biosyst. Eng.* **35:** 11-18.
9. Jang SS, Shirai Y, Uchida M, Wakisaka M. 2012. Production of mono sugar from acid hydrolysis of seaweed. *Afr. J. Biotechnol.* **11:** 1953-1963.
10. Jeong TS, Kim YS, Oh KK. 2011. Two-stage acid saccharification of fractionated *Gelidium amansii* minimizing the sugar decomposition. *Bioresour. Technol.* **102:** 10529-10534.

11. John RP, Anisha GS, Nampoothiri KM, Pandey A. 2011. Micro and macroalgal biomass: a renewable source for bioethanol. *Bioresour. Technol.* **102:** 186-193.
12. Jonsson LJ, Alriksson B, Nilvebrant NO. 2013. Bioconversion of lignocellulose: inhibitors and detoxification. *Biotechnol. Biofuels* **6**.
13. Larsson S, Palmqvist E, Hahn-Hagerdal B, Tengborg C, Stenberg K, Zacchi G, Nilvebrant NO. 1999. The generation of fermentation inhibitors during dilute acid hydrolysis of softwood. *Enzyme Microb. Technol.* **24:** 151-159.
14. Limtong S, Sringiew C, Yongmanitchai W. 2007. Production of fuel ethanol at high temperature from sugar cane juice by a newly isolated *Kluyveromyces marxianus*. *Bioresour. Technol.* **98:** 3367-3374.
15. Liu ZL, Slininger PJ, Dien BS, Berhow MA, Kurtzman CP, Gorsich SW. 2004. Adaptive response of yeasts to furfural and 5-hydroxymethylfurfural and new chemical evidence for HMF conversion to 2,5-*bis*-hydroxymethylfuran. *J. Ind. Microbiol. Biotechnol.* **31:** 345-352.
16. Martin C, Jonsson LJ. 2003. Comparison of the resistance of industrial and laboratory strains of *Saccharomyces* and *Zygosaccharomyces* to lignocellulose-derived fermentation inhibitors. *Enzyme Microb. Technol.* **32:** 386-395.
17. Meinita MDN, Hong YK, Jeong GT. 2012. Comparison of sulfuric and hydrochloric acids as catalysts in hydrolysis of *Kappaphycus alvarezii* (cottonii). *Bioprocess Biosyst. Eng.* **35:** 123-128.
18. Nahak S, Nahak G, Pradhan I, Sahu RK. 2011. Bioethanol from marine algae: a solution to global warming problem. *J. Appl. Environ. Biol. Sci.* **1:** 74-80.
19. Okuda N, Soneura M, Ninomiya K, Katakura Y, Shioya S. 2008. Biological detoxification of waste house wood hydrolysate using *Ureibacillus thermosphaericus* for bioethanol production. *J. Biosci. Bioeng.* **106:** 128-133.
20. Park JH, Hong JY, Jang HC, Oh SG, Kim SH, Yoon JJ, Kim YJ. 2012. Use of *Gelidium amansii* as a promising resource for bioethanol: a practical approach for continuous dilute-acid hydrolysis and fermentation. *Bioresour. Technol.* **108:** 83-88.
21. Quemener B, Lahaye M. 1998. Comparative analysis of sulfated galactans from red algae by reductive hydrolysis and mild methanolysis coupled to two different HPLC techniques. *J. Appl. Phycol.* **10:** 75-81.
22. Rodrussamee N, Lertwattanasakul N, Hirata K, Suprayogi, Limtong S, Kosaka T, Yamada M. 2011. Growth and ethanol fermentation ability on hexose and pentose sugars and glucose effect under various conditions in thermotolerant yeast *Kluyveromyces marxianus*. *Appl. Microbiol. Biotechnol.* **90:** 1573-1586.
23. Silva RO, dos Santos GMP, Nicolau LAD, Lucetti LT, Santana APM, Chaves LDS, *et al*. 2011. Sulfated-polysaccharide fraction from red algae *Gracilaria caudata* protects mice gut against ethanol-induced damage. *Mar. Drugs* **9:** 2188-2200.
24. Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. 2008. Determination of structural carbohydrates and lignin in biomass. Technical Report NREL/TP-510-42618. National Renewable Energy Laboratory.
25. Sun Y, Cheng JY. 2002. Hydrolysis of lignocellulosic materials for ethanol production: a review. *Bioresour. Technol.* **83:** 1-11.
26. Taherzadeh MJ, Gustafsson L, Niklasson C, Liden G. 2000. Physiological effects of 5-hydroxymethylfurfural on *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **53:** 701-708.