Effects of Hydrochloric Acid Pretreatment on Ethanol Yield of the Agarophyte, *Gracilaria tenuistipitata*

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ABSTRACT

Seaweed is a promising resource for bioethanol production because of its high carbohydrate content. This study utilized the red seaweed, *Gracilaria tenuistipitata*, as feed stock for the production of ethanol due to its high biomass in cultivation. This work aimed to improve ethanol production from algal species using different concentrations of hydrochloric acid during pretreatment. Samples of *Gracilaria* species were gathered from earthen ponds. The algal samples were pretreated with 0.5, 1.0, and 2 M HCl at 95°C for 15h. The yeast strain *Saccharomyces cerevisiae* TISTR No.5339 was used as the ethanol producer. Yield of ethanol was analyzed by gas chromatography. The highest yeast cell mass of 2.42×10^{8} cells mL^{-1} was obtained from fermentation of 1M HCl hydrolysate. Under the fermentation condition, pretreatment of the alga with 1M HCl gave a higher yield of ethanol (0.32 g ethanol g^{-1} sugars) than those with 0.5M HCl (0.29 g ethanol g^{-1} sugars) and 2M HCl (0.22 g ethanol g^{-1} sugars). Our study suggested that the acid pretreatment improved ethanol yield during algal fermentation.

Keywords: *Gracilaria tenuistipitata*, ethanol yield, agarophyte, acid pretreatment

INTRODUCTION

In Thailand, the demand for energy is drastically increasing every year. Thailand constructed dams to generate electricity, and has other power sources such as coal, oil and natural gas as fuel to produce electricity, as well as purchasing fuel from other countries. All of these entail high costs. Reports have revealed that the domestic energy consumption was more than 69%; while renewable energy was only 31% (Anonymous, 2012). In recent years, bioethanol has attracted our attention as an alternative renewable fuel to replace our dependency on fossil fuels. Unlike fossil fuels, ethanol is a renewable energy source produced through fermentation of sugars (Chandel *et al*. 2007). The fuel, ethanol, produced from corn, has been used in gasohol or oxygenated fuels production (Sun and Cheng, 2002). The majority of biomass used for ethanol production is from terrestrial sources. There has been a dramatic increase in ethanol production using terrestrial sources. However, using corn starch-based technology may not be practical, since their use for ethanol production will compete for the limited agricultural land needed for food and

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feed production. Thus, future limitations on terrestrial resources will require that our focus shifts from terrestrial-based resources to marine-based resources. In the last decade seaweeds have been considered as a promising feedstock for bioethanol production because of their high polysaccharides content. Among them, the red seaweeds possess a high amount of bioethanol-producible carbohydrates. The red seaweed, *Gracilaria tenuistipitata*, used in this study is mostly found in Pattani province, south of Thailand. This species can be cultured commercially in earthen ponds (Ruangchuay *et al*. 2010), which have high biomass and contain large amounts of agar.

Biological conversion from algal biomass to ethanol requires two steps: 1) hydrolysis of polysaccharides to sugars; and 2) fermentation by yeast or bacteria to convert fermentable sugars into ethanol (Feng *et al*. 2011). Several methods such as acid and/or enzymatic hydrolysis pretreatment, have been used for ethanol production (Sun and Cheng, 2002; Taherzadeh and Karimi, 2007; Meinita *et al*. 2013; Chirapart *et al*. 2014; Cho *et al*. 2014). Acid hydrolysis is the use of acid to break down the long chain polysaccharides into short chains or simple carbohydrates. The polysaccharides broken down by acid solution will occur randomly and most of the sugars produced are reducing sugars (Setyaningsih *et al*. 2012); in which some microorganism can be used as a carbon source. Hydrolysis of seaweed biomass by dilute sulfuric acid is a well-known method to obtain fermentable hydrolysates (Meinita *et al*. 2011; Meinita *et al*. 2012). The thermal acid hydrolysis pre-treatment has been used to improve the yield of saccharification in the seaweed (Horn *et al*. 2000; Jang *et al*. 2012; Cho *et al*. 2013; Meinita *et al*. 2013; Cho *et al*. 2014). Fermentation of the red alga *Gelidium amansii* after pretreatment with 0.2M H_2SO_4 gave ethanol yield of 0.23 g g^{-1} galactose or 45 % of the theoretical yield (Meinita *et al*. 2013). Another study reported the fermentation of *G. amansii* after the thermal acid hydrolysis and enzymatic saccharification using *Pichia stipitis* and *Saccharomyces cerevisiae* as an ethanol producer; which they can be produced the ethanol yields of 11.2 g L^{-1} and 6.9 g L^{-1}, respectively (Cho *et al*. 2014). Our recent work reported chemical composition and ethanol production from *Gracilaria salicornia*, *G. tenuistipitata*, *Ulva intestinalis*, and *Rhizoclonium riparium* (Chirapart *et al*. 2014). The study reported low ethanol production obtained from these seaweeds; and higher yield of ethanol was from *Gracilaria tenuistipitata*. Thus, this present study was done to improve ethanol production from the red seaweed *Gracilaria tenuistipitata* using different concentration of hydrochloric acid pretreatment.

MATERIALS AND METHODS

Sources of algae

The red seaweed, *Gracilaria tenuistipitata* was collected from earthen pond cultures. Contaminants, sand and mud were removed, and the sample was washed with tap water to remove salts, and then air-dried. The dried sample was ground, sieved, and stored in clean and dry Ziploc plastic bags at room temperature for further use.

Determination of monosugar

Algal powder (2 mg) was hydrolyzed with 0.5 mL of 2 M HCl at 100°C for 15 h. The hydrolysate was washed with distilled water to remove the acid, then evaporated in the rotary evaporator, and washed again until all the acid was removed. The hydrolysate was kept in a brown screw-capped vial before monosugar analysis. Five monosugar of xylose, fucose, arabinose, mannose, glucose and galactose, were used as standards. The sugar component was analyzed by high performance liquid chromatography (HPLC) equipped with a pump (Attech 626), detector (Attech ELSD 2000 ES), and column (LiChroCART NH2 250×4 mm); the mobile phase was 85% acetonitrile: 15% H_2O and a flow rate of 2 mL min^{-1} was used.

Ethanol determination

Preparation of algal hydrolysates

Two grams of algal powder was hydrolyzed at different concentrations (0.5, 1.0, and 2.0 M) of 50 mL HCl, at 95°C for 15 h. Each hydrolysate was volume-adjusted to 100 mL and pH-adjusted to 6.5 with 50 M NaOH. Then, 0.5 g of peptone and 0.3 g yeast extract were added then the mixture was sterilized in an autoclave at 121°C for 15 min for further use.

Starter preparation and alcohol fermentation

The starter preparation and alcohol fermentation protocols were modified from Pan-Utai (2010). *Saccharomyces cerevisiae* TISTR No.5339 was used as an ethanol producer. To prepare the starter, *S. cerevisiae* was cultured in 100 mL of yeast malt broth (YM) medium at 30°C and 120 rpm for 18 h. For ethanol fermentation, 10 mL of *S. cerevisiae* was added to the hydrolysate (from session above). Fermentation was conducted in 100 mL of liquid volume at 30°C with agitation at 120 rpm.

Sampling methodology

Slurry aliquots of 1.0 mL were removed during the fermentation period, with samples taken at 0, 2, 4, 6, 8, 10, 12, 15, 18, 24, 36, 48, and 96 h. Cells were counted at every sampling period. The supernatant was centrifuged at 3000 rpm for 10 min. Samples were pushed through a 0.45 μm syringe-driven filter unit into 0.5 mL glass vials, sealed with a crimp cap and stored at 5–8°C prior to gas chromatography (GC) analysis. Yield of ethanol was determined at 0, 48, and 96h. The ethanol yield of each sample was analyzed by gas chromatography (Shimadzu 7AG) equipped with a column (PQ 2 m×3 mm). Injection volume of samples was 1 μL, injection temperature 220°C, FID detector temperature 220°C, and using N_2 as carrier gas at flow rate of 50 mL min^{-1}. Yield of ethanol was presented as function of total sugar (g ethanol g^{-1} sugars).

Statistical analysis

The data are presented as means ± standard deviation (SD) and the differences in results between species were tested by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

The red seaweed, mainly composed of polysaccharides which are the major components of their extracellular matrix, are known as the source of unique sulfated galactans, such as agar, agarose, and carrageenan (Usov, 1998; Usov, 2011). The polysaccharide of the red algae *Gracilaria tenuistipitata* consist of agar which possesses the regular agarobiose repeating unit with partial methylation at the 6-position of the D-galactosyl residues (Lahaye *et al*. 1986; Montaño *et al*. 1999). In the present study, the acid hydrolysis was used to break down the long chain polysaccharides into short chains or simple carbohydrates. The hydrolysate of the *Gracilaria tenuistipitata* used in this study has total sugar of 98.2%; consist of 50.1% galactose, 25.1% mannose, 9.5% xylose, 6.6% glucose, and 0.1% fucose, as the main constituents (Table 1). The galactose and glucose components were lower than those reported in the red seaweed *Gelidium amansii* (31.3% Gal, 52.8% Glu), *Gracilaria tenuistipitata* (58.2% Gal, 74.4% Glu), and *Gracilaria chorda* (29.6% Gal, 60.6% Glu) (Meinita *et al*. 2013). The galactose component was also lower than that reported of 65.4 mol % in *Gracilaria birdiae* (Souza *et al*. 2012).

This study showed that the yeast *Saccharomyces cerevisiae* (TISTR No.5399) can grow during fermentation in all treatments (Figure 1). The highest number of cell mass was obtained from the fermentation of the hydrolysate which was pretreated with 1M HCl. In the 0.5M HCl pretreatment, the yeast cell mass tended to increase after 2 h and reached the maximum value of 2.34× 10^8 cells mL^{-1} after 10 h of fermentation. For the 1M HCl pretreatment, the cell mass gradually increased from the first hour of incubation and reached the maximum value of 2.42×10^8 cells mL^{-1} after 12h of fermentation. Whereas for those pretreated with 2M HCl, fermentation of the hydrolysate had lower maximum cell mass of 1.22x 10^8 cells mL^{-1} in 36 h, compared with 0.5 and 1M HCl pretreatments. In all acid pretreatments, *S. cerevisiae* cells reached the stationary phase after 30–36 h incubation This result corresponded with the study reported in the acid hydrolysis of *Eucheuma cottonii* from Indonesia (Setyaningsih *et al*.

Table 1. Monosugar components in the tissue of *Gracilaria tenuistipitata* as percentage of total carbohydrate.

Monosugar	% total carbohydrate
Xylose	9.5
Fucose	0.1
Arabinose	6.8
Mannose	25.1
Glucose	6.6
Galactose	50.1
Total sugar	98.2

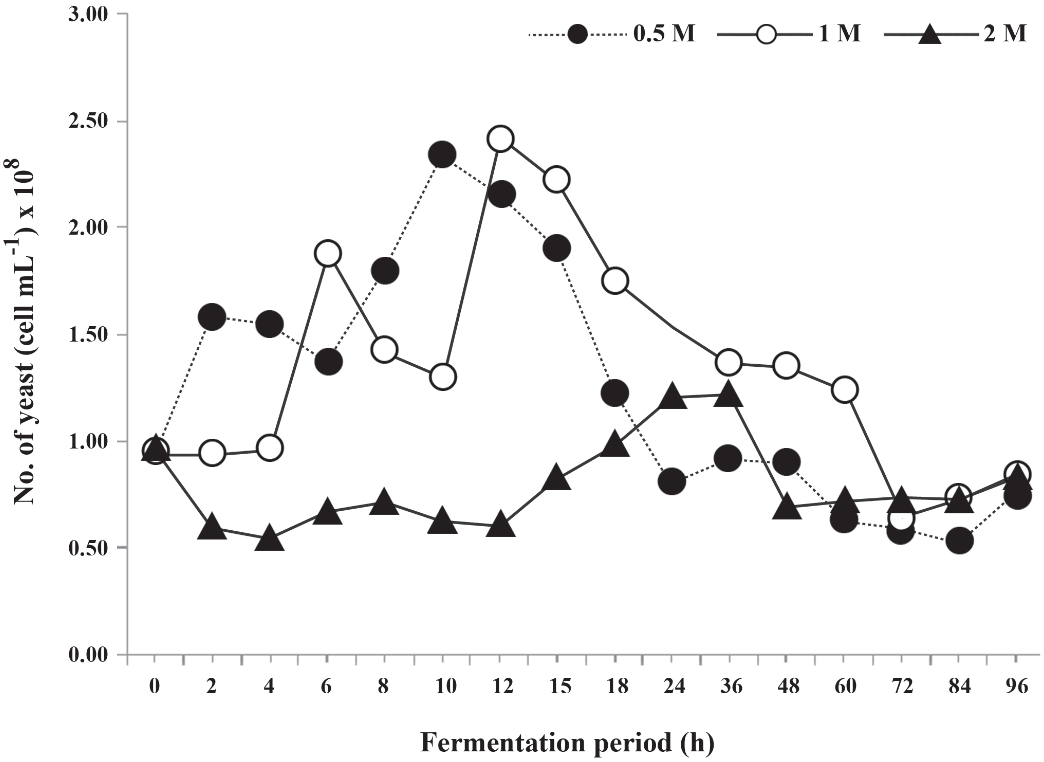

Figure 1. Yeast cell growth during fermentation of *Gracilaria tenuistipitata* after pretreatment with 0.5, 1, and 2 M HCl

2012). However, another study which used 0.1M HCl for the hydrolysis pretreatment of *G. tenuistipitata*, the growth of yeast reached stationary phase after 24 h of fermentation (Chirapart *et al*. 2014). In this study, the *S. cerevisiae* strain could grow in 2M HCl hydrolysate fermentation, but its cells were precipitated and mucous-like masses were formed which made cell counting difficult.

Hydrolysate of *Gracilaria tenuistipitata* had varieties of fermentable hexoses sugar (Chirapart *et al*. 2014), of which the yeast strain, *Saccharomyces cerevisiae* TISTR No. 5339 was able to use and convert to ethanol (van Zyl *et al*. 2007). Ethanol production has been taken into estimation depending upon the ratio of hexosans (glucan, galactan and mannan) and pentosans (xylan, arabinan) in each biomass source of the algae (Chandel *et al*. 2007). In the present study, differences in ethanol production were observed after pretreatment with different concentrations of hydrochloric acid (Table 2). Under the fermentation condition, ethanol yield was reached to the maximum value in 48h of fermentation. Pretreatment of the alga with 1M HCl gave a higher yield of ethanol (0.32 g ethanol g^{-1} sugars) than at 0.5M HCl (0.29 g ethanol g^{-1} sugars) and 2M HCl (0.22 g

Table 2. Ethanol yield obtained from fermentation of *Gracilaria tenuistipitata* after pretreatment with 0.5, 1, and 2 M HCl

Fermentation period (hr)	**Ethanol yield (g g^{-1} sugars)**		
	0.5 M HCl	**1 M HCl**	**2 M HCl**
0	0.05	0.04	0.03
48	0.29	0.32	0.22
96	0.05	0.18	0.12

ethanol g^{-1} sugars) ($p<0.05$). The ethanol yield obtained was higher than what was reported by Chirapart *et al*. (2014), in which ethanol yield from *G. tenuistipitata* fermentation after pretreatment with 0.1M HCl was 4.17×10^{-3} g ethanol g^{-1} sugars, and from hydrolysate of *G. salicornia* fermentation (0.031×10^{-3} g ethanol g^{-1} sugars).

In the current study, the ethanol concentration was lower than what was obtained in other studies. Yanagisawa *et al*. (2011) reported that ethanol produced from *Gelidium elegans* yielded 0.376 g ethanol. g^{-1} sugar. The enzymatic hydrolysate of *Gracilaria verrucosa* on fermentation with *Saccharomyces cerevisiae* HAU strain produced 0.43 g ethanol.g^{-1} sugars (Kumar *et al*. 2013). In addition, Meinita *et al*. (2013) reported that 0.2M H_2SO_4 hydrolysate with *Gelidium amansii* produced the most ethanol (0.83 g L^{-1}), followed by *Gracilaria tenuistipitata* (0.60 g L^{-1}) and *Gracilariopsis chorda* (0.5 g L^{-1}). Furthermore, Meinita *et al*. (2013) reported that levulinic acid and 5-hydroxy-methyl-furfural were found to be sugar degradation by-products in the hydrolysate samples. In our study, the low ethanol yield obtained in 2M HCl hydrolysate fermentation was thought to be a degradation of the sugars in hydrolysis reaction and formation of the undesirable by-products. These by-products could inhibit the formation of ethanol during the fermentation process (Taherzadeh and Karimi, 2007).

From the view point of each fermentable sugar, the acid hydrolysis pretreatment provided varieties of monosugar, in which the yeast strain, *Saccharomyces cerevisiae* TISTR No.5339 was able to use as carbon source. The sugar obtained could convert to 0.16 g ethanol g^{-1} of galactose, in the 1M HCl hydrolysate fermentation, at 48h. This was lower than that reported for *Kappaphycus alvarezii* (0.21 g g^{-1} galactose, Meinita *et al*. 2012), and for *Gelidium amansii* (0.23 g g^{-1} galactose, Meinita *et al*. 2013). Similarly the fermentation of 1M HCl hydrolysate could convert to higher yield of 0.08 g g^{-1} mannose, 0.03 g g^{-1} xylose, and 0.02 g g^{-1} glucose, compared to those obtained from the 0.5M HCl and 2M HCl hydrolysate. A comparison of ethanol production from other reports is shown in Table 3. The ethanol yield produced from the hydrolysate of *G. tenuistipitata* was comparable with those produced from other seaweeds.

Table 3. A comparison of ethanol production from various seaweed species

Seaweed sources	**Fermented organism**	**Pretreatment**	**Yield of ethanol**	**References**
Ulva pertusa	*S. cerevisiae* IAM 4178	2% H_2SO_4 at 121°C, 30min with enzymatic hydrolysis	0.381 g g^{-1} reducing sugar	Yanagisawa *et al.* (2011)
Ulva fasciata	*S. cerevisiae* MTCC No. 180	enzymatic hydrolysis	0.45 g g^{-1} reducing sugar	Trivedi *et al.* (2013)
Ulva intestinalis	*S. cereviceae* TISTR No. 5339	0.1 M HCl at 95°C, 15 h	9.98 µg g^{-1} glucose	Chirapart *et al.* (2014)
Rhizoclonium riparium	*S. cereviceae* TISTR No. 5339	0.1 M HCl at 95°C, 15 h	33.84 µg g^{-1} glucose	Chirapart *et al.* (2014)
Laminaria japonica	*E. coli* KO11	acid/enzyme hydrolysis	0.4 g g^{-1} sugars	Kim *et al.* (2011)
Alaria crassifolia	*S. cerevisiae* IAM 4178	enzymatic hydrolysis	0.281 g g^{-1} reducing sugar	Yanagisawa *et al.* (2011)
Undaria pinnatifida	*P. angophorae* KCTC 17574	75 mM H_2SO_4 at 121°C, 60 min	9.42 g L^{-1} 27.3% of theoretical yield from total CHO	Cho *et al.* (2013)
Eucheuma cottonii (*Kappaphycus alvarezii*)	*S. cereviceae*	5% H_2SO_4 at 100°C, 120 min	4.6% ethanol in fermentation broth	Candra *et al*. (2011)
Kappaphycus alvarezii	*S. cerevisiae*	0.2M H_2SO_4 at 130°C, 15 min	0.21 g g^{-1} galactose	Meinita *et al.* (2012)
Kappaphycus alvarezii	*S. cerevisiae* NCIM 3523	0.9 N H_2SO_4 at 100 °C, 1h	92.3% theoretical conversion	Khambhaty *et al.* (2012)
Gelidium elegans	*S. cerevisiae* IAM 4178	enzymatic hydrolysis	0.376 g g^{-1} reducing sugar	Yanagisawa *et al.* (2011)
Palmaria palmata	*S. cerevisiae*	0.4 M H_2SO_4 at 125°C, 25 min	17.3 mg g^{-1} seaweed	Mutripah *et al*. (2014)
Gracilaria verrucosa	*S. cerevisiae* HAU strain	enzymatic hydrolysis	0.43 g g^{-1} sugars	Kumar *et al*. (2013)
Gracilaria tenuistipitata	*S. cereviceae* TISTR No. 5339	0.1 M HCl at 95°C, 15 h	139.12 µg g^{-1} glucose	Chirapart *et al*. (2014)
Gracilaria salicornia	*S. cereviceae* TISTR No. 5339	0.1 M HCl at 95°C, 15 h	1.43 µg g^{-1} glucose	Chirapart *et al.* (2014)
Gracilaria sp.	*S. cerevisiae* Wu-Y2	sequential acid and enzyme hydrolysis	0.48 g g^{-1} sugars	Wu *et al*. (2014)
Gracilaria tenuistipitata	*S. cereviceae* TISTR No. 5339	0.5 M HCl at 95°C, 15 h	0.29 g g^{-1} sugars	this study
		1 M HCl at 95°C, 15 h	0.32 g g^{-1} sugars	this study
		2 M HCl at 95°C, 15 h	0.22 g g^{-1} sugars	this study

S. cereviceae = *Saccharomyce cerevisiae*, *E. coli* = *Escherichia coli*, *P. angophorae* = *Pichia angophorae*

CONCLUSION

In the present study, *Gracilaria tenuistipitata* was used as feedstock for the production of ethanol due to its high biomass in cultivation. The variety of sugar component found in *G. tenuistipitata* indicates the carbon source and ethanol production potential of this seaweed. Thermal acid hydrolysis pretreatment could improve yield of ethanol from fermentation. Our results reveal that the 1M HCl pretreatment can give higher yield of ethanol than the pretreatment with 0.5M HCl and 2M HCl. The findings of this study revealed that ethanol production from *Gracilaria tenuistipitata* can be improved using hydrochloric hydrolysis pretreatment. However, further work is needed to optimize pretreatment using a combination of acid with enzymes to increase hydrolysis efficiency and optimize fermentation to increase ethanol production from this seaweed species.

ACKNOWLEDGEMENT

This work was done under the research project, “Study on yield and chemical component for ethanol production from seaweed in Thailand”, partially supported by the Kasetsart University Research and Development Institute. Special thanks to anonymous reviewers whose remarks helped to improve this paper.

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