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Chemical Analysis and Utilization of *Sargassum* sp. as Substrate for Ethanol Production

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Abstract: The brown seaweed *Sargassum sp.* is a promising feedstock for ethanol production because of its relatively high content (41.6% dry basis) of holocellulose. Chemical pretreatment of the seaweed was carried out with 1% (v/v) H_2SO_4 solution at 5% solid loading for 15 min at 121°C prior to saccharification with commercial cellulase. The cellulose-to-glucose conversion efficiency after 72 hours incubation was 49.84% and 61.54% for the untreated and chemically treated biomass, respectively.The algal residue after pretreatment with dilute acid was subjected to either simultaneous enzymatic saccharification and fermentation (SESF) or separate enzymatic hydrolysis and fermentation (SEHF). *Saccharomyces cerevisiae* was used for ethanol fermentation. The cellulose to ethanol conversions forSESF and SEHF were 66.9 and 34.1%, respectively.

Key words: Bioethanol · *Sargassum* sp. · Ethanol fermentation · Algal saccharification · Brown seaweed

INTRODUCTION

Due to escalating price of petroleum-based fuels, extensive research has been done towards the development of alternative, renewable and cost-effective energy sources with lower greenhouse gas emissions [1, 2]. One promising energy source is biofuel, which is derived from plant biomass. One the most important biofuel is ethanol which can be produced from abundant supplies of cellulosic biomass, both terrestrial and marine. The leading countries in the world in terms of bioethanol production are Brazil, US and Canada [3]. Bioethanol production can reduce green house gas levels since biomass assimilation by feedstock crops can utilize atmospheric carbon dioxide. In addition, ethanol is less toxic and is readily biodegradable and its use producesless air borne pollutants compared to petroleum fuel.

Ethanol fuel can be produced from terrestrial feedstocks that produce sugar, starch and cellulose. In addition, use of agricultural wastes and palm trees residual wastes to ethanol in tropical regions created great potential for ethanol production from wastes and outcomes are encouraging as alternative sources of energy [4,5].However, the use of feedstock crops for ethanol production has raised issues on its possible impact on food supply and security. Because of these drawbacks on the use of food crops as bioethanol feedstock, several species of algae with high content of fermentable carbohydrates are now being investigated. Thus, algae are now gaining wide attention as biomass source for the production of bioethanol and biodiesel, which are known asthird-generation of biofuels.

Marine algae consist of macroalgae (seaweeds) and microalgae. Seaweeds are classified as green, brown and red. They are good sources of colloidal substances which are useful as gelling agents, emulsifiers and stabilizers in pharmaceutical, cosmetics and food products. Most seaweed research has dealt with chemical analysis because of seaweeds contain unique carbohydrates. *Sargassum*is one of the important brown seaweed that is distributed in temperate and tropical oceans of the world. It is utilized as animal feed, fertilizer and as source of alginate which has various used in industries. In Philippines, 72 *Sargassum* species have been recorded [6].

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Production of ethanol from biomass requires two steps: (1) hydrolysis of polysaccharides to sugars; and (2) fermentation by yeast or bacteria to convert fermentable sugars into ethanol. At present, hydrolysis has been done using acid or enzyme. One advantage of the latter is high specificity of the enzyme and the saccharification can be done under mild conditions with minimal formation of side products that can be potential inhibitors in the fermentation process[7].

The present study deals with chemical characterization of the brown seaweed, *Sargassum* sp., as source of fermentable sugars for bioethanol production. Proximate chemical analysis was done on the seaweed and its contents of holocellulose, alpha-cellulose and alginate were also determined. Dilute acid pretreatment was conducted on the dried seaweed followed by enzymatic hydrolysis and yeast fermentation of the resulted sugars into ethanol. The efficiencies for saccharification and sugar to ethanol conversion were determined.

MATERIALS AND METHODS

Preparation of Biomass: *Sargassum sp*. was obtained from Real, Quezon; it was washed with running water to remove salts, sun-dried and then oven-dried at 50°C. The ground samples were stored in plastic containers at room temperature.

Proximate Analysis: Contents of crude protein, crude fat, crude fiber, ash and moisture of the ground algal biomass were determined according to standard methods. The moisture content was determined by drying to constant weight in an oven at 105°C. Crude protein was analyzed by the Kjeldahl method and ash was determined gravimetrically. Crude lipid was extracted using petroleum ether in a Soxhlet extractor and measured gravimetrically after oven-drying.

Determination of Alginate and Holocellulose

Alginate: The extraction procedure for alginate was adapted from Fenoradosoa, *et al*. [8]. The seaweed (25 g) was suspended and stirred in 800mL of 2% formaldehyde for 24 hours, then washed with deionized water prior to acidification (800 mL 0.2M HCl, 24 hours). The algal sample was then washed with deionized water prior to alginate extraction by stirring in 800mL 2% Na_2CO_3 for 3 hours at room temperature. After filtration, the alginate was precipitated with 95% ethanol (1:2 v/v) as sodium salt. The precipitate was washed with ethanol, then acetone and dried for 24 hours at 40°C.

Holocellulose and alpha-cellulose. Cellulose was isolated from the seaweed using the method of Siddhanta with some modifications [9]. Dried algal powder was placed in a Soxhlet extractor with ethanol for a period of 16 hours to remove the extractives. The holocellulose portion of the biomass was isolated using sodium chlorite, $NaClO_2$. Alpha-cellulose was then obtained as the residue which was insoluble in 17.5% sodium hydroxide solution from the isolated holocellulose.

Enzymatic Hydrolysis: Enzymatic hydrolysis was carried out on the chemically-pretreated samples. Chemical pretreatment is carried out with 1.0 % v/v H_2SO_4 at a solid-to-liquid ratio of 1:20. The effect of pretreatment was assessed in order to determine the potential sugar yield prior to enzymatic hydrolysis. Chemical treatment was done in an autoclave at 121°C (15 psi) for 15 min. After treatment, the pretreated solids were filtered and washed with distilled water until neutral pH prior to store in the refrigerator.

A commercial enzyme mixture was used in the experiments. The activity of the enzyme was determined prior to saccharification of the pretreated substrates. The procedure for measurement of cellulase activity was based on the NREL method [10]. Cellulase activity was expressed in terms of "filter paper units" (FPU) per milliliter of original (undiluted) enzyme solution. One unit of FPU is defined as the amount of enzyme that produces 1μmol of glucose per minute under the assay conditions.

The enzymatic digestibility of the pretreated sample was examined at constant enzyme loading of 25 FPU/g and substrate loading of 5%. All enzymatic hydrolysis experiments were done at 50°C for 72 h in 125 mL Erlenmeyer flasks using an incubator shaker. Samples were withdrawn after 0 h, 24 h, 48 h and 72 h and analyzed for reducing sugars content, which was determined using the DNS method.

Fermentation: Ethanol fermentation was carried out in flask experimentson the enzymatic hydrolysates obtained as described above. *Saccharomyces cerevisiae,* which was used in all fermentation processes. The organism was inoculated into fresh slant culture medium in a test tube and cultivated at 30°C for 1 day, until yeast growth appeared on the slant culture and was distributed evenly. To maintain the purity and activity of the *S. cerevisiae*, the sample was streaked on a slant of sterile YEPG (1% yeast extract, 2% peptone, 2% glucose and 2% agar dissolved in distilled water). After 24 hours of incubation at room temperature, the slant was inoculated into the pre-culture medium.

Two fermentation processes were performed, namely (1) separate enzymatic hydrolysis and fermentation (SEHF) and (2) simultaneous enzymatic saccharification and fermentation (SESF). In both SEHF and SESF experiments, enzyme hydrolysates obtained from untreated (raw) and acid-pretreated biomass were used. Samples were withdrawn after 0 h, 24 h, 48 h and 72 h and analyzed for ethanol content which was determined by Gas Chromatography with a Flame Ionization Detector.

RESULTS AND DISCUSSION

Proximate Composition of *Sargassum sp:* The results of proximate analysis of *Sargassum* sp. used in present study are presented in Table 1. It should be noted that seaweed composition generally varies with algal age and species, season, geographic location and temperature [11].

The appreciable amount of ash in the seaweed sample indicates residual mineral salts in the sample that were not completely removed by washing with water. The varying ash content of each type of seaweed shows differences in the amount of minerals salts that attach to the algal surface [12].The mineral salt content of seaweed depends on the type, age and condition of hydrology. Based on the results, it was found that the biomass used in present work was a good source of carbohydrates for the commercial production of monosaccharides. These rich sources of carbohydrates could be utilized as substrates for the production of bioethanol.

Alginate and Holocellulose Contents of Seaweed: Alginate is the major structural polysaccharide present in brown seaweeds. The seaweed was soaked first in a formaldehyde solution overnight before it was extracted with alkali. Formaldehyde kills microorganisms in seaweeds which could contribute to the degradation of alginates [13]. Also, the presence of transition metal ions such as Fe^{2+} or Fe^{3+} together with phenolic compounds enhances free radical formation which causes depolymerization of the phycocolloids. Therefore, removal of phenolic compounds prior to extraction by Na_2CO_3 solution was done in order to reduce the degradation of alginates.

After the sample was soaked overnight in formaldehyde solution, it was mixed with HCl solution. The alginic acid was extracted with Na_2CO_3 (with stirring for 3 hours) that gave a thick brownish slurry. Addition of 95 % ethanol (1:2 v/v) precipitated the alginic acid as sodium alginate which was jelly-like. The sodium alginate obtained was yellowish and the calculated yield was 32.2%; this is the same as that obtained by Omar *et al.* [13].

Table 1: Proximate composition* of *Sargassum sp*

Composition	Values (%)
Moisture	13.93 ± 0.06
Ash	27.11 ± 0.62
Crude Fiber	12.50 ± 0.39
Crude Fat	0.54 ± 0.02
Crude Protein	6.99 ± 0.03
Nitrogen-free extract	51.43 ± 0.73

*Based on dry-weight basis and average of three replicates.

Table 2: Holocellulose, alpha-cellulose and alginate contents* of *Sargassum sp*

Component	Values (%)
Alginate	32.2± 0.4
Holocellulose	41.6± 0.3
Alpha-cellulose	22.0± 0.8
Hemicellulose	19.6± 0.9

*On dry-weight basis and average of three replicates.

The amounts of holocellulose and alpha-cellulose fractions in the algal sample are listed in Table 2. Holocellulose consists of cellulose and hemicellulose after all the extractives have been dissolved by ethanol in a Soxhlet extractor for 16 hours. Then the holocellulose in the sample was extracted with sodium chlorite. Traditionally, acidified sodium chlorite is commonly used to delignify wood as an initial step in the isolation of pure cellulose [14]. Sodium chlorite is a moderately strong oxidizing agent; its use does not allowheavy metal contamination and yields pure cellulose with no residual lignin. The alpha-cellulose was then obtained by adding 17.5% NaOH to the isolated holocellulose. Alpha-cellulose (or true cellulose) is the insoluble residue after treatment with alkali. The hemicellulose in the biomass was not isolated but was calculated as the difference between the holocellulose and alpha-cellulose contents.

The relatively high cellulose content of *Sargassum* makes it a promising source of fermentable sugars for ethanol production. However, the alginate, which cannot be fermented to ethanol, is a valuable by-product with useful applications.

Enzymatic Hydrolysis: Enzymatic hydrolysis using commercial cellulase was done to yield sugars prior to fermentation into ethanol. Chemical pre-treatment was done in order to enhance enzymatic saccharification because of the recalcitrance of native cellulose to the enzyme due to the crystalline structure and low surface area of the cellulose matrix [15].

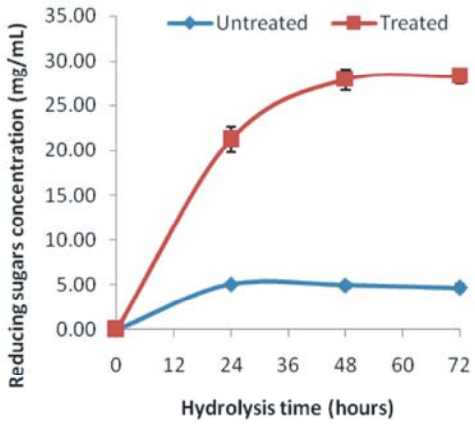

Fig. 1: Enzymatic saccharification of *Sargassum sp.* using a commercial enzyme.

Enzymatic saccharification was done at a substrate loading of 5% (w/v). An increased substrate loading allowed a higher sugar yield. Figure 1 showsthe concentration profiles with respect to time of enzymatic saccharification for the untreated and pretreated algal samples. The reducing sugar yield for the untreated sample was appreciably lower than that of the treated sample. The amount of sugars released increased up to 48 hours and remained constant until 72 hours of hydrolysis for the pretreated sample. The maximum amount of sugars released (326.89 mg/g dry biomass) was observed after 72 hours hydrolysis for the acid-pretreated sample. The untreated sample gave only 91.93 mg/g dry biomass.

Various factors have been found to affect the enzymatic hydrolysis of the pretreated material including (a) accessibility and adsorption characteristics of the cellulose, (b) reactivity of the cellulose and (c) adsorption characteristics of the lignin, if present [16].

These factors are influenced by biomass composition and pretreatment method [17]. Previous studies have reported that cellulose hydrolysis improved with increasing lignin removal, although differences have been reported in the degree of lignin removal needed [18]. Since seaweeds have no lignin, the pretreatment step can be omitted prior to saccharification. However, in the present study acid pretreatmentwas observed to improvesaccharification of the material; this is probably due to the alginate-cellulose matrix present in the cell wall of brown seaweeds. The cellulose matrix may be exposed upon acid pretreatment making it more accessible to the enzyme resulted in greater saccharification.

The cellulose to glucose conversion efficiencies for the untreated (raw) and pretreated *Sargassum sp*. after 72 hours incubation were 49.84% and 61.54%, respectively. Generally, pretreated biomass had higher saccharification efficiencies than the untreated ones.

Fermentation: Ethanol fermentation was done on the enzymatic hydrolysates of the acid-pretreated algal sample. Two types of fermentation experiments were considered: (1) Simultaneous Enzymatic Saccharification and Fermentation (SESF) and (2) Separate Enzymatic Hydrolysis and Fermentation (SEHF).

Simultaneous Enzymatic Saccharification and Fermentation (SESF): Simultaneous production of ethanol and consumption of glucose was observed during the first 12 hours of fermentation (Figure 2). A sudden drop in total sugar concentration was observed during the first 24 hours of fermentation which remained almost constant for the 48th up to the 72nd hour. This indicated that there was no end-product inhibition of the enzyme

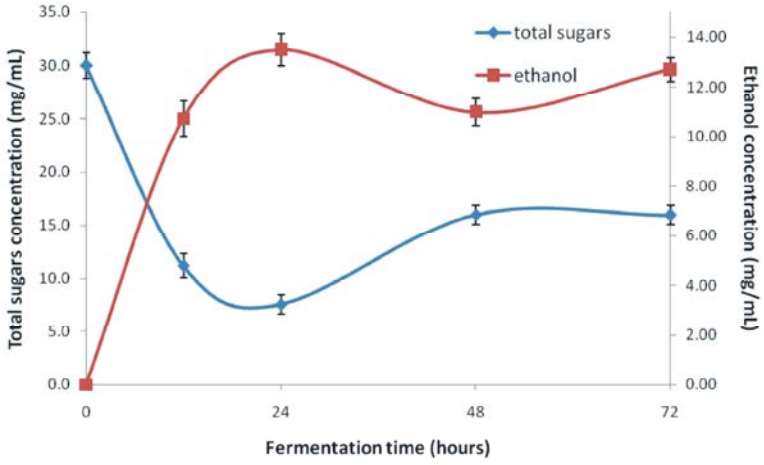

Fig. 2: Time profile of simultaneous enzymatic saccharification and fermentation (SESF) for pretreated*Sargassum sp*.

Table 3: Ethanol concentration obtained from SESF.

		Final Ethanol Concentration*	
Treatment	Initial Conc. of Total Sugars, mg/mL	% v/v	mg/mL
Untreated	5.63 ± 0.92	0.52 ± 0.12	4.10 ± 0.26
1% H_2SO_4, 15 min, 121°C	30.01 ± 1.03	1.60 ± 0.25	12.70 ± 0.54

* Concentration obtained after 72 hours fermentation based on three replicates

Table 4: Ethanol concentration obtained from SEHF.

		Final Ethanol Concentration*	
Treatment	Initial Conc. of Total Sugars, mg/mL	% v/v	mg/mL
Untreated	8.95 ± 0.26	0.53 ± 0.16	4.14 ± 0.73
1% H_2SO_4, 15 min, 121°C	29.41 ± 1.41	0.82 ± 0.17	6.47 ± 0.94

* Concentration obtained after 72 hours fermentation based on three replicates

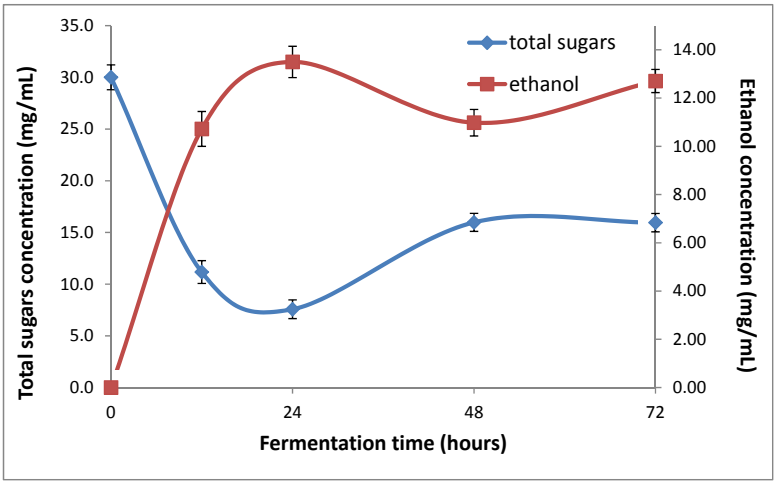

Fig. 3: Time profile of separate enzymatic hydrolysis and fermentation (SEHF) for pretreated *Sargasssum sp*.

as the glucose produced was immediately converted to ethanol.The ethanol concentrations of the untreated and pretreated biomass are presented in Table 3.

The maximum ethanol concentration for SESF of acid-pretreated *Sargassum sp*. after 72 hours of fermentation was 12.70 mg/mL.

There are two fundamental problems with Simultaneous Enzymatic Saccharification and Fermentation. Hydrolysis and fermentation both require specific temperature ranges for optimal operation. *Saccharomyces cerevisiae* ferments best at about 30°C and pH range of 4-5. A temperature during fermentation which is lower than 20 °C or greater than 40°C results in a lower concentration of ethanol for most yeast strains. The enzymatic hydrolysis process, however, performs best at temperatures of about 50°C and at 30°C enzymatic hydrolysis proceeds at a much lower rate.

Ethanol produced from glucose during SESF could inhibit fermentation of the glucose by yeast. Both enzyme and yeast undergo plasma degradation as the ethanol concentration increases. Eventually, a high ethanol concentration could cause enzyme denaturation and yeast cell death.

Separate Enzymatic Hydrolysis and Fermentation (SEHF): Concentrated enzymatic hydrolysates of the untreated and pretreated biomass were used as substrates (at 8% substrate loading) for the separate enzymatic hydrolysis and fermentation (SEHF). The initial total sugar concentrations are listed in Table 4 and the time profile for fermentation of the pretreated alga is shown in Figure 3. A rapid drop in total sugar concentration was observed after 24 hours which was accompanied by an increase in ethanol concentration. After 24 hours, the amount of ethanol produced remained constant which may be due to

Table 5: Cellulose to ethanol conversion efficiency for pretreated algae using different strategies

Fermentation Strategy	Cellulose to Ethanol Conversion Efficiency (%)
SESF	66.93
SEHF	34.10

the low amount of available sugars present in the fermentation broth. The maximum ethanol concentration for SEHF of pretreated biomass after 72 hours of fermentation was 6.47 mg/mL.

The advantage of the separate process (SEHF) over the simultaneous one (SESF) is the possibility of separate optimization, namely enzymatic hydrolysis at 50°C and fermentation at about 32°C. However, the disadvantage of this method is the inhibition of cellulase and α-glucosidase by glucose which is released during hydrolysis; this requires lower solids loadings and higher enzyme loadings in order to achieve reasonable yields [19].

The calculated cellulose to ethanol conversion efficiencies for the two bioconversion strategies are given in Table 5. The efficiency value depends on the glucose yield after saccharification. SESF gave a higher efficiency for the fermentation step compared to SEHF.

The cellulose-to-ethanol conversion efficiency value was based on glucose liberated from cellulose; the conversion of pentose (resulting from hydrolysis of hemicellulose) was not considered. Also, the yeast (*S. cerevisiae*) is not capable of fermenting pentose into ethanol. A fermentation strategy wherein both five-carbon and six-carbon sugars can be converted into ethanol involves simultaneous saccharification and co-fermentation using *S. cerevisiae* and a pentose-fermenting microorganism. Alternatively, a genetically-engineered yeast or bacterium can be used which can ferment both hexose and pentose into ethanol.

The ethanol yields (g ETOH/g dry biomass) for the treated *Sargassum* sp. are 0.112 and 0.057 for SEHF and SEHF, respectively.

CONCLUSION

Production of ethanol from brown seaweeds has received little attention because of alginate, the main carbohydrate fraction in brown seaweed, cannot be utilized directly as a substrate for ethanol production. Nonetheless, results of the present studyshowed that *Sargassum* sp. is a promising raw material for bioethanol production in addition to being a source of valuable by-products, especially alginate.

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Persian Abstract

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چکیده

جلبک دریایی قهوه ای، گونه ی سارگاسوم به خاطر محتوای نسبتاً غنی از هالوسلولوز (۴۱/۶٪ بر اساس وزن خشک)، یک ماده اولیه مناسب برای تولید اتانول می‌باشد. پیش از ساکاریفیکاسیون توده جلبک با سلولاز تجاری، پیش تیمار شیمیایی جلبک با محلول H_2SO_4 ۱٪(v/v) و میزان ماده جامد ۵٪ به مدت ۱۵ دقیقه در ۱۲۱°C صورت گرفت. بازده تبدیل سلولوز به گلوکز بعد از ۷۲ ساعت انکوباسیون برای بایومس هیدرولیز نشده و هیدرولیز شده به ترتیب ۴۹/۸۴ و ۶۱/۵۴٪ می‌باشد. باقی مانده های جلبک پس از پیش تیمار با اسید رقیق با تخمیر همزمان با ساکاریفیکاسیون آنزیمی (SESF)، و تخمیر مجزا پس از هیدرولیز آنزیمی (SEHF) مورد آزمایش قرار گرفت. ساکارومایسیس سرویسیه برای تخمیر اتانول مورد استفاده گرفت و تبدیل سلولز به اتانول با فرآیند SESF وSEHF به ترتیب ۶۶/۹ و ۳۴/۱٪ به دست آمد.