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Preparation of Bioethanol from Brown Seaweed (*Sargassum* Sp.)

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ABSTRACT

In this study, brown seaweed (*Sargassum* sp.) was used to produce bioethanol by using enzymatic liquefaction and saccharification method. Bioethanol from brown seaweed (*Sargassum* sp.) was more commercial than using any other starch based raw materials because it can be easily collected on Chaung Tha beach in Myanmar without any impact on environment. In this regard, the productivity of bioethanol from brown seaweed (*Sargassum* sp.) was determined by separate hydrolysis and fermentation (SHF) with yeasts. Two types of yeasts were used. *Saccharomyces cerevisiae* was used for glucose fermentation in brown seaweed and selected nitrogen fixing yeast isolate (N3,N18,N24) were used for mannitol fermentation which consist plenty in brown seaweed. The effects of enzymatic liquefaction, enzymatic saccharification and fermentation on this sample were studied. From the fermentation studies, brown seaweed (*Sargassum* sp.) gave the ethanol percent by weight of 2.56% using *Saccharomyces cerevisiae* only and 4.1% by using mixture of yeast *Saccharomyces cerevisiae* and selected nitrogen fixing yeast isolate. The maximum yield of crude ethanol was 32.5% by fermentating yeast mixture of *Saccharomyces cerevisiae* and nitrogen fixing yeast isolate. When it was fermented by just only *Saccharomyces cerevisiae,* yield of crude ethanol percent was 20.3%.

KEYWORDS: *Brown seaweed (Sargassum sp.), bioethanol, enzymatic liquefaction, saccharification, fermentation*

I. INTRODUCTION

As crude oil resources are becoming depleted there is significantly more effort being placed into researching viable alternatives to petroleum based fuels [1]. Since people learned that utilizing renewable resources was essential for sustainable development, energy policies had slowly shifted to renewable resources such as biofuel. In particular, liquid biofuel has become the priority since 40% of total energy consumption in the world is in the form of liquid fuel [2]. Bioethanol is derived via fermentation process of renewable biomass containing sugar and starch materials such as sugar cane, corn or grains [1].

The production and use of biofuels have entered a new era of global growth. The two primary biofuels in use today are ethanol and biodiesel. Ethanol is readily blended with gasoline, and biodiesel is blended with petroleum-based diesel for use in conventional diesel-fueled engines. Ethanol currently accounts for more than 90 percent of total biofuels production, with biodiesel making up the rest. Ethanol has a potential market as big as the oil market. It can potentially replace the entire fuel market for gasoline. Methanol or ethanol is also used for manufacture of biodiesel, during the process of transesterification [3]

While development of fuels from biomass continues apace, first generation biofuel based on edible crops has raised morality and ethics issues as there are millions of people around the world still suffer from malnutrition and hunger. In order to overcome this issue, bioethanol refined from lignocellulosic biomass, namely second generation bioethanol (SGB) offers a great option which is compatible with economic growth and morality issues. However, although SGB is attractive with its non-edible feedstock, it is much debated because the cultivation of terrestrial plants requires the resources that could otherwise be used for food. Furthermore, separation of lignin content from lignocelluloses has become an obstacle to be combated. In this context, third generation biofuel based on marine algae and seaweeds offers an excellent alternative to displace fossil fuels. From the point of view of ecology, macroalgae assist in reducing carbon dioxide in the atmosphere and supplying oxygen to the sea. Third-generation bioethanol (TGB) represents fuel ethanol produced from algal biomass [2].

Macroalgae in fact contain high amount of carbohydrates which can be utilized for the production of bioethanol [2]. Seaweed was proposed as one of the most promising biomass materials that can be easily converted to ethanol because seaweeds are known to contain a low concentration of lignin or no lignin at all [4]. The production of bioethanol from biomass involves the following process steps; biomass pre-treatment, saccharification, fermentation and product recovery. Saccharification is one of the most crucial steps as fermentable sugars such as glucose and mannose are released and metabolised in the presence of yeast to produce bioethanol [1].

Although land plant biomass is more easily converted to bioethanol than is marine algae biomass, bioethanol production from land plants causes rising of food prices through competition with food sources. So, this work aimed at improving its yield by using source of biomass namely: *Sargassum* sp. brown seaweed from Chaung Tha Beach, Myanmar. This study focused on the technology to convert brown macroalgae *Sargassum* sp. to ethanol.

II. MATERIALS AND METHODS

MATERIALS

The raw material used for this work was *Sargassum* sp., member of brown seaweed. Its function is a primary building units of the framework. *Sargassum* sp. was collected from Chaung Tha Beach, Ayeyarwaddy Division in Myanmar.

Trichoderma spp. and α-amylase Enzyme were used in liquefaction step and Glucoamylase Enzyme was used in saccharification step respectively. *Saccharomyces cerevisiae* and selected nitrogen fixing yeast were used in fermentation step.

Pretreatment of brown seaweed (*Sargassum* sp.)

Before *Sargassum* sp. was pretreated by liquid hot water pretreatment method, it was characterized to determine the compositions. The content of moisture, total ash, crude fiber content and crude fat content were analyzed by R.Lee's food analysis method. Moreover protein content was also analyzed by Kjeldahl method . The component of starch and cellulose were analyzed by R.Lee's food analysis method and heat of dilution dichromate method. The experimental results of compositions are shown in Table 1.

Brown seaweed (*Sargassum* sp.) can be collected from Chaung Tha Beach of Myanmar during the month of February to May. They were washed by using tap water in order to remove adhering debris and sand. After that, the cleaned *Sargassum* sp. were dried under sunshine for three days and ground by using grinder to get the size under 425 µm. The ratio of ground *Sargassum* sp. and distilled water was 1:10 and it was heated to the temperature at 100˚ C for 40 minutes by using magnetic stirrer with heater. The purpose of this process is in order to be easily degraded by enzymes in liquefaction step and saccharification step

First Liquefaction Process

Pretreated brown seaweed (*Sargassum* sp.) slurry was liquefied by using *Trichoderma* spp. as cellulase enzyme to degrade cellulose content in *Sargassum* sp. *Trichoderma* spp.was from the help of Department of Biotechnology, Mandalay Technological University. The pH of pretreated slurry was set at 5 and temperature was controlled at 50˚ C, 55˚ C, 60˚ C, 65˚ C and 70˚ C respectively as the specifications of most of cellulase enzyme. The sample was drawn every half hour period to determine the glucose concentration in this process.

Second Liquefaction Process by using α-amylase enzymes

After first liquefaction process, continued to second liquefaction process by using α-amylase enzyme. The liquefied slurry which has the highest glucose content in first liquefaction process was used in this process. GC 262 SP enzyme and SPEZYME ALPHA enzyme were tested in using as α-amylase enzyme to breakdown α-(1, 4) bond of starch in brown seaweed (*Sargassum* sp.) and to reduce the viscosity of the gelatinized starch and ease the next saccharification process.

When GC 262 SP enzyme was tested in second liquefaction step, the pH of liquefied slurry was adjusted at 6, 6.5, 7 and 7.5 respectively and temperature was set at 68±2˚ C according to the specifications of the enzyme. The sample was drawn every 0.5 hr period to determine the highest glucose content.

When the SPEZYME ALPHA enzyme was tested in second liquefaction step, the pH and temperature were adjusted as the specifications of SPEZYME ALPHA enzyme. Therefore the pH of liquefied slurry was set at 6 and temperature was controlled at 85±2˚ C. The sample was taken every 0.5 hr to determine the glucose content.

Determination of Glucose Concentrations after Liquefaction Process

After first liquefaction process, the slurry was centrifuged to get the supernatant sample. Then the supernatant sample was determined glucose concentration by using DNS method to find out the optimum condition of liquefaction process. After second liquefaction process, to determine the glucose concentration the slurry was done like above the procedure.

Saccharification Process

After second liquefaction process, the resultant liquefied slurry was saccharified by using gluco-amylase. In this saccharification process, OPTIDEX ® L-400 and DISTILLASE ASP were tested in using as gluco-amylase enzyme to breakdown α-(1,6) bond of starch in brown seaweed (*Sargassum* sp.). The liquefied slurry was mixed with the gluco-amylase enzyme to produce more glucose from starch of brown seaweed (*Sargassum* sp.).

When OPTIDEX ® L-400 enzyme was tested in saccharification process, the pH of the liquefied slurry was adjusted at 6, 6.5 and 7 respectively and the temperature was controlled at 60±2˚ C according to the specification of enzyme. The sample was taken one time per hour to know the glucose content.

When DISTILLASE ASP enzyme was tested in this process, pH and temperature were adjusted as the specifications of enzyme. So the pH of liquefied slurry was set at 5 and temperature was controlled at 62±2˚ C. The sample was drawn every one hour period to determine the glucose content by DNS method.

Fermentation Process

After saccharification process, produced glucose and mannitol which includes plenty in brown seaweed were fermented by using yeast *Saccharomyces Cerevisiae* and three isolated nitrogen fixing yeasts N3, N18 and N24 which is from the help of department of Biotechnology, Taw Twin, Kyaukse. These four kinds of yeast were also symbiotic tested if these mixed yeasts could be used or not. Fermentation was done at pH 5 and temperature 37˚ C. The fermentation was allowed until not evolving of CO_2 gas bubbles. Therefore, total fermentation time for *Saccharomyces cerevisiae* were 72 hrs and for nitrogen fixing yeasts were 144 hrs.

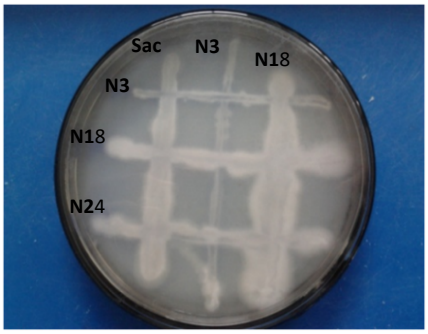

Figure1. Symbiotic Test

According to symbiotic test, yeast *Saccharomyces Cerevisiae* and three nitrogen fixing yeast isolated can be cultured in the same medium. So that yeasts can be used together in the same fermentation broth in the same time.

The ethanol fermentation process was controlled automatically, at pH 5, temperature 37°C, and 150 rpm. 100 ml samples were taken from the fermentation broth at planned intervals and kept at -20°C freezer. The samples were then distilled and analyzed using FTIR to study the ethanol fermentation processes and yields of the crude ethanol.


Figure2. Ethanol fermentation by fermenter

Distillation Process to Produce Crude Ethanol

The amount of crude ethanol produced from fermented solution was determined by using simple distillation. The solution (100 ml) obtained from fermentation process was taken and was added to 100 ml of water. This solution was put in a three neck round bottomed flask and heated to 78-100̊ C by heating mental. The condensate containing crude ethanol was collected to get in a 100 ml measuring cylinder.

III. RESULTS AND DISCUSSIONS

Table 1 shows the experimental results of the compositions of protein, fat, fiber, ash, moisture and carbohydrate in brown seaweed (*Sargassum* sp.). Table 2 shows the experimental results of total carbohydrate compositions of brown seaweed (*Sargassum* sp.).

Table1. The Compositions of Brown Seaweed (*Sargassum* sp.) Found in Chaung Tha Beach

Component	Composition (wt %)
Moisture	10.20
Ash	16.09
Protein	7.34
Crude Fiber	11.59
Crude Fat	0.89
Carbohydrate	53.89

Table2. Total Carbohydrate Compositions of Brown Seaweed (*Sargassum* sp.) Found in Chaung Tha Beach

Component	Composition (wt %)
Starch	24.257
Cellulose	2.976
Others (such as mannitol) Source; [5]	26.657
Total	53.89

When the slurry was heated over 50˚ C the more water was absorbed and the starch granules start to swell. This is due to an aqueous suspension of starch is heated the hydrogen bonds with the molecules weak and water is absorbed and then starch granules swell [6]. When the temperature was increased, the gelatinization of starch was also increased as the starch granules release their amylase and amylopectin. These large molecules quickly thicken and its viscosity also rises. It is no need to use other pre-treatment method because of no lignin content in brown seaweed (*Sargassum* sp.).

The pretreated sample was liquefied by using *Trichoderma* spp. as cellulase enzyme to degrade cellulose in the raw *Sargassum* sp. Although the temperature range of cellulase enzyme is 50-60̊ C, the temperature was controlled 50-70˚ C because local strain of *Trichoderma* spp.was used in this research. The glucose content were determined at 50˚ C, 55˚ C,60˚ C,65˚ C and 70˚ C respectively and the results were shown in Figure 3. It might be *Trichoderma* spp performed the best activity at 60̊ C, pH 5 and reaction time 1 hr and it is the best condition in first liquefaction process as the more yield of ethanol content can be get when the more glucose content formed.

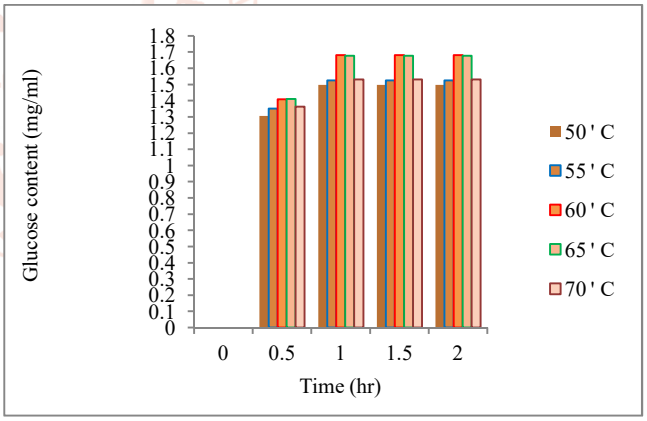

Figure3 Glucose Content of First Liquefaction Step by Using *Trichoderma* spp.

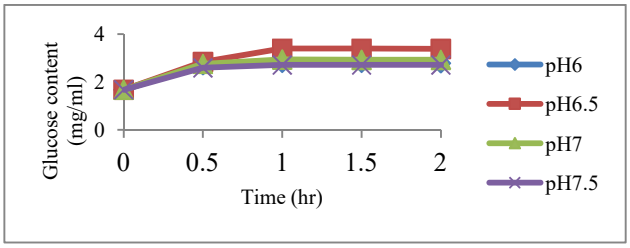

Figure4. Glucose Content of Second Liquefaction Step by Using GC 262 SP Enzyme

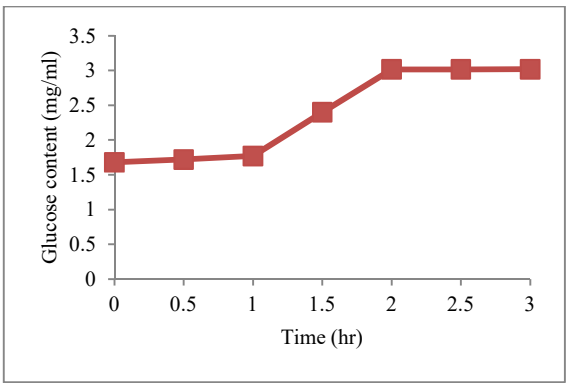

Figure5. Glucose content of second liquefaction step by using SPEZYME ALPHA enzyme

In second liquefaction process, two types of α-amylase name of GC 262 SP enzyme and SPEZYME ALPHA enzyme were tested to produce glucose.

According to Tap et al (2005), the enzyme was stable over a wide pH range but not stable below pH 3.5 or above pH 10.0. Although the given pH range of GC 262 SP enzyme is 6-7.5, the most favorable pH value where the enzyme is most active should be checked. The glucose content was not significantly changed at pH 6,7 and 7.5. But at pH 6.5, the glucose content of first half hour gave 2.84 mg/ml and increased to 3.39 mg/ml after next half hour. The glucose content did not changed appreciably change and followed by a period of stability after getting 3.39 mg/ml of glucose. Therefore it might be at the condition of pH 6.5 and reaction time 1 hr , GC 262 SP enzyme work best. Figure 4 shows changing in glucose content by using GC 262 SP enzyme.

Figure 5 also shows changing in glucose content of second liquefaction step by using SPEZYME ALPHA enzyme. The produced glucose content by using SPEZYME ALPHA enzyme was low compared to glucose content by using GC 262 SP enzyme. Therefore GC 262 SP enzyme was chosen to use in the second liquefaction process. In this second liquefaction step, the viscosity of slurry decreased as the presence of amylase enzymes that will break down amylose and amylopectin molecules of starch granules. The glucose content of second liquefaction process was low compared to saccharification process. This is due to α-amylase enzyme which is used in second liquefaction step can break down (α, 1-4) bond and cannot break down (α, 1-6) bond of starch granule. So this process can produce short chain dextrin and some glucose [7].

In the saccharification process, two types of gluco-amylase enzymes, OPTIDEX ® L-400 and DISTILLASE ASP enzymes were tested. Figure 6 and 7show the glucose content of saccharification process by using OPTIDEX ® L-400 enzyme and DISTILLASE ASP enzymes respectively.

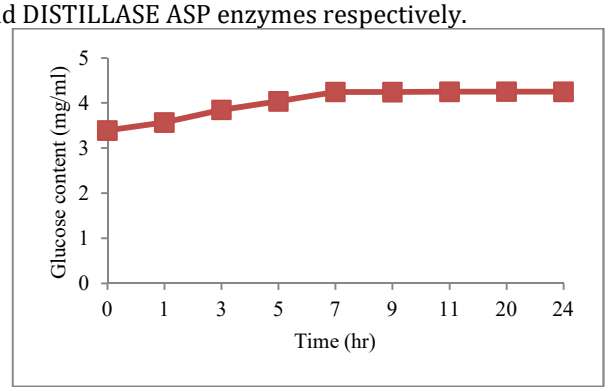

Figure6. Glucose content of saccharification step by using OPTIDEX ® L-400

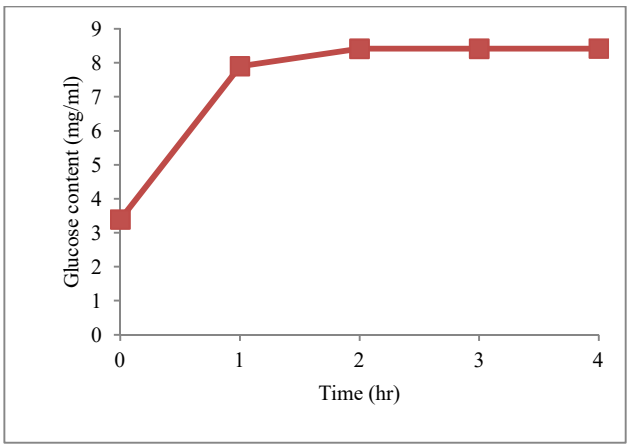

Figure7. Glucose Content of Saccharification Step by Using DISTILLASE ASP Enzyme

According to the figures, the activity of DISTILLASE ASP enzyme was better than OPTIDEX ® L-400 that the glucose content increased nearly twice when DISTILLASE ASP enzyme was used. Therefore DISTILLASE ASP enzyme was more suitable to use than OPTIDEX ® L-400 in this saccharification process.

The glucose concentration of saccharification process was high compared to second liquefaction process. This is because gluco-amylase enzyme can break down (α, 1-6) bond of starch granule which cannot be degraded by α-amylase enzyme [7]. It can also be noted that the higher temperature the faster the amylase enzyme will be denatured and the less fermentable sugars are produced. At lower temperature these enzymes will be able to work for a longer time and will produce more fermentable sugars.

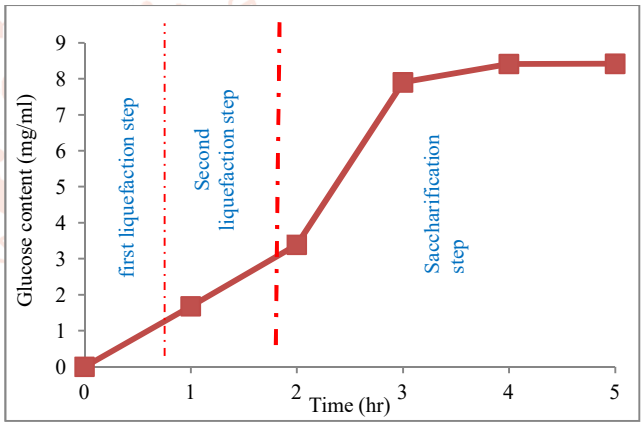

Figure8 Glucose Content of Brown Seaweed (*Sargassum* sp.) by Enzymatic Saccharification

Figure 8 represents the changes in glucose concentration with first liquefaction, second liquefaction and saccharification for brown seaweed (*Sargassum* sp.). The concentration of glucose produced from first liquefaction by *Trichoderma* spp., second liquefaction by GC 262 SP enzyme and saccharification by DISTILLASE ASP enzyme were 1.68 mg/ml, 3.39 mg/ml and 8.41mg/ml respectively.

Starch can be saccharified much more easily than cellulose since cellulose is a straight chain polymer without coiling or branching which contributes to its high crystallinity. However, the brown seaweed (*Sargassum* sp.) has very low cellulose content and no lignin content. The glucose concentration after saccharification was about two times higher than glucose concentration after second liquefaction

process. The conversion of starch to glucose for brown seaweed (*Sargassum* sp.) was 31.5%. Finally, it can be concluded that the starch in the seaweed are easily hydrolyzed by enzymes without any acid or alkaline pretreatment.

Two types of yeast, *Saccharomyces cerevisiae* and selected nitrogen fixing yeast isolate, were tested in sugar fermentation. Although sugars are vital in living organisms, yeasts are capable of using some sugars but not all sugars as a food source. Bromothymol blue (BTB) is the indicator and the color is blue at pH7. When the yeast is inoculated into the tube, the yeast which ferments the sugar will result in the production of acid that will change the color of BTB.

When the yeast ferments sugars, acidic organic byproduct is accumulated and it turns the medium into yellow color with the reduction in the pH (acidic). In the absence of fermentation, the broth retains the blue color and shows as gram negative yeast.

After the tubes with *Saccharomyces cerevisiae* were incubated for 3 days, the medium turned into yellow color with the reduction in pH as the yeasts ferment sugars and acidic organic byproducts were accumulated. But *Saccharomyces cerevisiae* cannot ferment mannitol as the broth with mannitol and *Saccharomyces cerevisiae* retain the blue color.

The color of the broth with mannitol and selected nitrogen fixing yeasts isolate turned into yellow color after 6 days. It shows that selected nitrogen fixing yeasts isolate can ferment not only glucose but also mannitol. All of the three nitrogen fixing yeasts isolate (N3, N18 and N24) are gram positive yeasts. Results are shown in Table 3.

Table3. Results of Sugar Fermentation Test

Sugar 2%	Selected nitrogen fixing yeasts isolate			*Saccharomyces cerevisiae*
	N3	N18	N24	
Glucose	+	+	+	+
Mannitol	+	+	+	–
Dextrose	+	+	+	+
Sucrose	+	+	+	+

Fermentation period of 72 hrs gave the maximum possible concentration having 2.56 (v/v%) for *Saccharomyces cerevisiae*. When mixed yeasts was used in fermentation, maximum possible concentration of 4.1 (v/v%) was produced after 144 hrs.

The concentration of ethanol by using mixed yeasts was higher than that by using *Saccharomyces cerevisiae*. It was suggesting that selected nitrogen fixing yeast isolate enhanced the ethanol concentration. *Saccharomyces cerevisiae* was used in fermentation for glucose content of brown seaweed (*Sargassum* sp.). And selected nitrogen fixing yeast isolate were used in fermentation for mannitol which is plenty consist in brown seaweed (*Sargassum* sp.). The crude ethanol samples by using *Saccharomyces cerevisiae* and by using mixed yeast (*Saccharomyces cerevisiae* and selected nitrogen fixing yeast isolate) were analysed with FTIR spectrophotometer Genesis II, Maltson instruments. Inc 1001, Fourier Drive, Nadison, USA. The FTIR spectra of crude ethanol samples and ethanol sample from local market are described in figure 9 and 10.

According to the figures the spectra shows typical absorption bands at 3000-3700 cm^{-1} correspond to O-H stretching in the region and the bands at 2700-3300cm^{-1} correspond to C-H stretching in the region.

Then the band at 1600-1700cm^{-1} correspond to C=C stretching in the region. The bands at 1600-1900 cm^{-1} correspond to rough calculation of primary alcoholic C=O stretching. The band in the 1260-1000 cm^{-1} region of the spectrum corresponds to C-O stretching vibrations in alcohols and phenols. The band at 480 cm^{-1} corresponds to bending vibration due to C-C. The impurities having boiling point less than 100̊ C could be contained because these ethanol samples were distilled by simple distillation method.

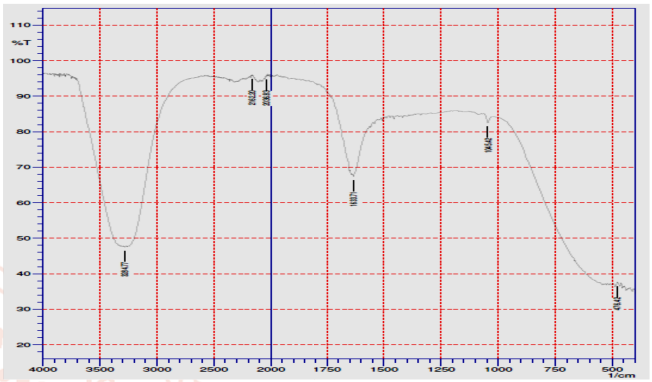

Figure9. FTIR spectra of crude ethanol by using *Saccharomyces cerevisiae*

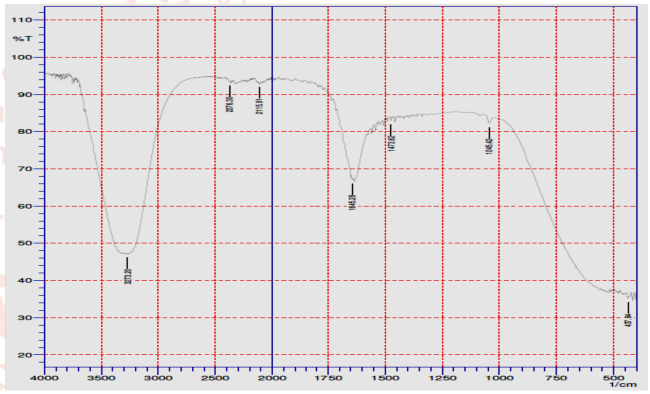

Figure10. FTIR spectra of crude ethanol by using mixed yeast

IV. CONCLUSION

In this research, bioethanol was prepared from brown seaweed (*Sargassum* sp.) by using enzymatic saccharification method. Total composition, carbohydrate composition and chemical compositions were determined. It was sure that brown seaweed (*Sargassum* sp.) is carbohydrate rich material for bioethanol production. Ethanol percent of 4.1 v/v% was produced by using mixed yeasts (*Saccharomyces cerevisiae* and selected nitrogen fixing yeast isolate (N3, N18, N24)). When only yeast *Saccharomyces cerevisiae* was used, ethanol percent of 2.56 v/v% was produced. In this research, although theoretical yield (%) of ethanol (100%) is 13.63% which is calculated based on starch content of brown seaweed, the experimental yield (%) of ethanol (100%) by using mixed yeasts is 32.5 % and using yeast *Saccharomyces cerevisiae* is 20.3%.

The experimental yield percent of ethanol was higher than the theoretical yield percent because of the low boiling point impurities. Furthermore, theoretical ethanol yield percent was calculated based on theoretical glucose yield percent

and after saccharification process the produced sugars might be not only glucose but also another sugars which can be fermented by yeasts. Therefore experimental ethanol yield percent was higher than theoretical ethanol yield percent in this study.

In Myanmar, brown seaweed (*Sargassum* sp.) is only the waste material and it can be found plenty at Rakhine and Tanintharyi costal region, Ayeyarwady Delta and Mottama costal region. If bioethanol can be produced efficiently by using *Sargassum* sp., this will be beneficial to environmental sector and energy sector. Therefore, this investigation has good potential for near future and it should be continued to get better results for bioethanol production.

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