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Evaluation of the saccharification and fermentation process of two different seaweeds for an ecofriendly bioethanol production

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ABSTRACT

The study is aimed to produce bioethanol and more specifically to provide a process for producing ethanol by the fermentation of macroalgal biomass. The seaweeds were evaluated for their hydrolysis process (acid and enzyme treatment). The hydrolysates obtained from acid and enzyme saccharification process were analysed for ethanol production to show the efficiency of hydrolysis processes. The content of reducing sugar after acid hydrolysis was found to be 60.6 ± 1.7 and 71 ± 1.6 mg/g biomass for *Sargassum* sp. and *Gracilaria* sp. respectively. Likewise, the biomass obtained from two stage hydrolysis, showed 110 ± 1.6 and 140.6 ± 1.7 mg/g biomass reducing sugar for the above mentioned two seaweeds. The yeast used for fermentation of ethanol was isolated from grape juice and identified by sequencing of large subunit (LSU) ribosomal DNA D1/D2 region of the yeast isolate. Maximum ethanol production (19.9 ± 0.3 and 28.7 ± 0.4 gL^{-1} for *Sargassum* sp. and *Gracilaria* sp. respectively) was significantly observed after two stage hydrolysis using *Saccharomyces cerevisiae* rather than *Hanseniaspora opuntiae* GK01 (18.37 ± 0.3 and 27.0 ± 0.6 gL^{-1} for *Sargassum* sp. and *Gracilaria* sp. respectively). This study is obviously proved that *S. cerevisiae* MTCC174 was found to be a better strain for ethanol production in both the seaweeds than the yeast strain isolated from grape juice *H. opuntiae* GK01.

1. Introduction

The world population is increasing at an alarming rate, so there is a demand for liquid fuel in the transport sector. Global warming, depletion of fossil fuels and increasing price of petroleum based fuels are reasons to for alternative, sustainable, renewable, efficient and cost effective energy sources with lower emission of green house gases (Nigam and Singh, 2010). Biomass is one of the best candidates to serve as an excellent alternative resource to meet the current and upcoming fuel demand. The fuel generated from any type of biomass is termed biofuel. The most successful and common biofuels are biodiesel, bioethanol and biogas. The biodisel would replace the use of conventional liquid fuels like diesel and petrol. The biofuel most widely used around the globe is bioethanol which can be produced from biomass (Chiaramonti, 2007). Since biomass crops assimilate atmospheric carbon dioxide, their growth for bioethanol production can reduce green house gas levels. Bioethanol is environmentally friendly and generate less air pollutants (Champangne, 2007). It can be produced from several different biomass feedstock such as sugar or starch crops (sugar cane, sugar beet, corn and wheat) and lignocellulosic biomass. The extensive use of this biomass may lead to starvation in developing countries and the impact may reflect in planning conditions and public opinion, in some other countries (Chiaramonti, 2007).

Algae based technologies could be a key tool for reducing green house gas emission. Macroalgae or seaweeds are multi cellular plants growing in salt or fresh water (Demirbas and Demirbas, 2011). Microalgae are the dominant algae being researched for biodiesel production (Walker, 2008). Like microalgae, macroalgae, the large sized algae, can also be utilized for ethanol fermentation by converting their storage material to fermentable sugars. The absolute absence or near absence of lignin makes the enzymatic hydrolysis of algal cellulose (Adams et al., 2009). Macroalgae can be grown on nets or string, and can be seeded onto thin weighed strings suspended over a larger horizontal rope (Adams et al., 2009). Among the macro algae brown algae is an evolutionary diverse and abundant in the world's ocean and coastal waters. It has a high content of easily degradable carbohydrates,

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making it a potential substrate for the production of liquid fuels. The mainly composed carbohydrates of brown algae are alginate, laminaran, mannitol and fucoidan and small quantity of cellulose (Horn et al., 2000).

In general, two methods are normally implemented for bioethanol production from biomass. The first one is biochemical process (fermentation) and other one is thermo-chemical process (gasification). Bioethanol production from algae could be a solution for alternative energy sources. Therefore research is needed to obtain the potential of bioethanol from algae by means of fermentation process. Fermentation is the decomposition of organic compounds into simpler compounds with the help of microorganisms that produce energy (Hogg, 2005). Most commercial scale ethanol fermentation is by yeast, *Saccharomyces cerevisiae* (Hutkins, 2006). *S. cerevisiae*, baker yeast is able to metabolize nearly 90% of glucose to ethanol (Elevri and Surya, 2006).

Although there are numerous efforts on developing core technologies (production, harvest, storage, depolymerization, and biochemical conversion) for producing biofuels from terrestrial plant biomass, production of biofuels from marine plant biomass has received less attention. Macroalgae in fact contain high amount of carbohydrates which can be utilized for the production of bioethanol. Thus, the aim of this study is to highlight the possibility, perspective and challenges of production of bioethanol from seaweeds in Mandapam by using *S. cerevisae*.

2. Materials and methods

2.1. Sample collection and processing

The red and brown seaweed (*Gracilaria* sp. and *Sargassum* sp.) used in this study were collected from Mandapam coastal regions, Southeast coast of India. The samples were picked with hand and immediately washed with sea water in order to remove the foreign particles and epiphytes. The samples were transported to laboratory by keeping in ice box and washed thoroughly with tap water to remove the salt on the surface of the sample. Then the seaweeds were shade air dried to remove excess water. The dried samples were cut into small pieces to prepare powder form of seaweeds and stored in precleaned polythene containers for the further use.

2.2. Analysis of seaweeds composition

2.2.1. Estimation of moisture content

The known quantity of wet seaweeds was dried in hot air oven at 60 °C until moisture content dried. The seaweeds were weighed properly before and after drying process. The percentage of moisture content was calculated by the formula

$$\%\ \ of\ \text{moi}sture\ content = \frac{Dry\ weight\ of\ \text{the}\ sample}{Wet\ weight\ of\ the\ sample} \times 100$$

2.2.2. Protein, lipid and carbohydrate estimation

The total protein content of the seaweeds was estimated by Biurette method (Raymont et al., 1964). The quantity of seaweeds lipid content was evaluated by chloroform- methanol mixture method (Folch et al., 1956). The total carbohydrate of the seaweeds was estimated by phenol-sulfuric acid method (Dubois et al., 1956).

2.2.3. Ash estimation

The known quantity of sample was heated over low flame and it was heated at 550 °C overnight. The lid was placed after complete heating to prevent loss of fluffy ash. It was then cooled in a desiccator. Finally the ash content was weighed when the sample turns to gray (A.O.A.C, 2000). The percentage of ash content was calculated by

$$Ash(\%) = \frac{Weigth\ of\ the\ ash}{Weight\ of\ the\ sample} \times 100$$

2.3. Fermentation

2.3.1. Mechanical pre-treatment

The powered form of seaweeds was desalinated to avoid salinity problem during purification. The samples were treated with hot water and alkali to extract the polysaccharides (Luo et al., 2009). The extract was purified by filtration or centrifugation. The water content of the purified extract was removed by spray drying on steam heated drums.

2.3.2. Acid hydrolysis

About two gram powder of *Sargassum* sp. and *Gracilaria* sp. were added individually to 3% and 4% H_2SO_4 (80 mL) respectively, and heated in an autoclave at 121 °C for 30 min for sterilization. Then each sample was kept in shaking incubator (Remi, India) at 150 rpm for 1 h at 30 °C and neutralized with sodium bicarbonate to adjust the pH to 6.5–7 (Hwa et al., 2011).

2.3.3. Enzyme hydrolysis

The hydrolysates obtained from acid hydrolysis were used for enzymatic hydrolysis. The enzymes used for hydrolysis of *Sargassum* sp. were cellulase (53 FPU/g dry substrate) and pectianse (20 U). The enzymes used for *Gracilaria* sp. were cellulase (53 FPU/g dry substrate) and β- glucosidase (30 U/g dry substrate). All the enzymes used in this study were purchased from Sigma Aldrich. The enzymatic hydrolysis was performed in shaking incubator (pH 5.0, 50 °C, 150 rpm) for 4 h. After hydrolysis each sample was centrifuged at 8000 rpm for 10 min and the supernatant was separated and used for ethanol production. Hydrolysates of each sample (acid and two stage hydrolysis) were separately used for estimation of reducing sugar by dinitrosalicylic acid (DNS) method using glucose as standard (Miller, 1959).

2.4. Isolation and identification of yeast for bioethanol production

The yeast isolate for the production of ethanol was isolated from sugar cane juice and identified by sequencing of large subunit (LSU) ribosomal DNA D1/D2 region. These regions were amplified by PCR (Eppendorf master cycler, Germany) using primer UL18F: 5' TGTACA CACCGCCCGTC 3'. PCR products were purified for sequencing and the sequences were further used for taxonomic identification using BLAST program and submitted to NCBI.

2.5. Microorganisms

Two yeast strains used in this work are *Saccharomyces cerevisiae* MTCC174 obtained from Microbial Type Culture Collection Centre, Chandigarh, India and a new strain was isolated from grape juice (Senthilkumar et al., 2014). Both the strains were maintained in yeast extract peptone dextrose (YPD) agar slants and were stored at 4 °C by periodic subculture.

2.6. Preparation of inoculum

One loop of each pure stock culture was transferred from YDP agar slant to a 100 mL Erlenmeyer flask was plugged with cotton wool and contained 50 mL YPD broth medium. The culture was incubated under aerobic condition in rotary shaker with shaking speed of 120 rpm and 30 °C for 18 h. The obtained culture was then used as inoculum.

2.7. Fermentation process

The production of bioethanol was carried out in an 500 mL Erlenmeyer flask containing 100 mL of fermentation broth (yeast

extract-5 g, $(NH_4)2SO_4$ − 5 g, K_2HPO_4 −0.125 g, KH_2PO_4 − 0.875 g, NaCL − 0.1 g, $MgSO_4{\cdot}7H_2O$ − 0.5 g, $CaCL_2{\cdot}2H_2O$ 0.1 g, $CuSO_4{\cdot}5H_2O$-0.1 g, hydrolysate − 100 mL). After adjusting the pH to 5.0 with 3 N NaOH and 3 N HCL, the fermentation broth was autoclaved at 121 °C for 20 min. The *S. cerevisiae* and *Hanseniaspora opuntiae* GK01 yeast cell suspension (2% v/v) were inoculated into the medium of *Sargassum* sp. and *Gracilaria* sp. Both the medium were incubated in shaking incubator at 30 °C with mild agitation (125 rpm) for 96 h. Aliquots were centrifuged (14,000 rpm for 4 min) at 4 °C to get cell free supernatants for the distillation of ethanol.

2.8. Product recovery

The product obtained from four different fermentation medium was a mixture of ethanol, cell mass and water. The first step to recover the ethanol is distillation column, where most of the water content remains with the solid part. The ethanol was then concentrated in a rectifying column and was estimated by dichromate oxidation method (Neish, 1952).

2.9. Statistical analysis

The experiments were repeated thrice and the average data were submitted to analysis of variance using ANOVA (Minitab version 15). The significance of the differences was determined at ($p \leq .05$). The figures were analysed using origin 6.0 (Microcal Software Inc,).

3. Result and discussion

The interest towards alternative energy sources pushes the scientific community to implement technologies that use aquatic biomass for the production of biofuel. Production of bioethanol from macroalgae is regarded as one of the promising alternative to food crops for biofuel production. In this study macroalgae macroalgae, *Gracilaria* sp. (red seaweed) (Fig. 1a) and *Sargassum* sp. (brown seaweed) (Fig. 1b) collected from Southeast Coast of India were used for ethanol production. They were all processed and pre-treated for bioethanol production.

3.1. Biochemical composition analysis of seaweeds

The moisture content of *Sargassum* sp. and *Gracilaria* sp. was found to be 46% and 30.5% respectively. It showed that dry matter of *Sargassum* sp. is higher when compared with *Gracilaria* sp. The protein content of *Sargassum* sp. and *Gracilaria* sp. was found to be 2.5% and 9.5% respectively. The result suggested that brown algae have less protein content than red seaweed. This result is correlated with an earlier study which found low protein fraction (3–15% of the dry weight) in brown seaweeds when compared to that of green and red seaweeds (10–47% of the dry weight) (Fleurence, 1999). Likewise, lipid

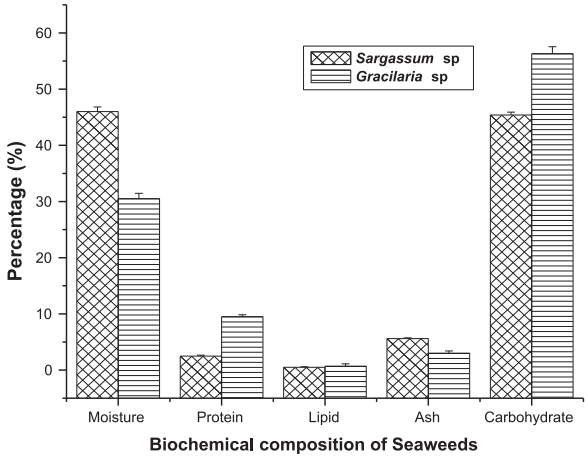


Fig. 2. Biochemical composition of seaweeds.

content was estimated in both the seaweeds (0.5% and 0.7% for *Sargassum* sp. and *Gracilaria* sp. correspondingly). This result is concurrent with some previous study, which also found the similar result (5% of lipid content in macroalgae) (McDermid and Stuercke, 2003). Because of low lipid levels, production of biofuels from macroalgae is expected to depend on conversion of carbohydrate feedstock, rather than the lipids conversion (McDermid and Stuercke, 2003).

The percentage of ash content of *Sargassum* sp. and *Gracilaria* sp. was found to be 5.6% and 3.0% respectively. About 45% and 56% of the dry weight of the brown and red seaweed was found to be their total carbohydrate content (Fig. 2). These results significantly proved that both seaweeds contain low content of protein and lipid and nearly half of their dry weight is the content of total carbohydrates ($p \leq .05$). Both the seaweeds are composed of higher carbohydrates than other compounds like protein and lipids. Seaweed carbohydrates are constituted of microscopic crystal structure of fibre, covered by mucilaginous polysaccharides layer and storage polysaccharides contained in cells. Our report is agreed with Jang et al. (2013) who found the similar composition of *G. amansii* (carbohydrate-71.4%, protein- 10.5%, lipid-0.74%, ash- 2.82% and water- 14.6%).

3.2. Acid hydrolysis

The seaweeds (brown and red) were hydrolysed by diluted acid hydrolysis and the converted reducing sugar was estimated by DNS method. It was found to be 60.6 ± 1.7 and 71 ± 1.6 mg/g biomass of *Sargassum* sp. and *Gracilaria* sp. respectively (Fig. 3). The amount of reducing sugar was significantly higher in *Gracilaria* sp. than the *Sargassum* sp. ($P \leq .05$) and this is due to increased availability of fermentable carbohydrates in *Gracilaria* sp. The result proved that acid



Fig. 1. Seaweeds a) *Gracilaria* sp b) *Sargassum* sp.

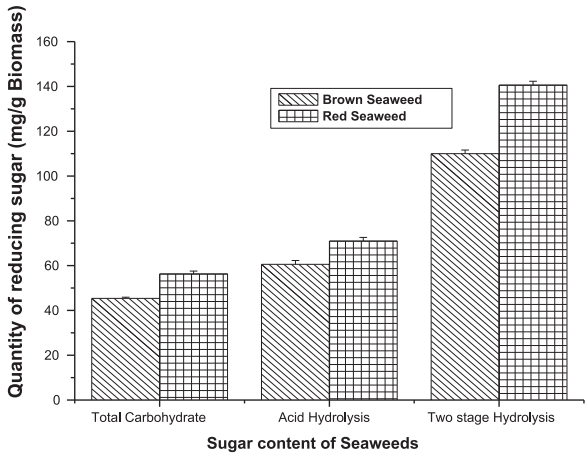


Fig. 3. Conversion of reducing sugar from the biomass of hydrolysates obtained from acid hydrolysis and two stage hydrolysis (acid and enzyme treatment).

hydrolysis could effectively convert the carbohydrate content of both the seaweeds and this is due to acid hydrolysis of alginate and other carbohydrates. Previous report stated that alginate can be depolymerised chemically by acid and alkali hydrolysis, by oxidative reductive depolymerization or enzymatically (Moen et al., 1997). The major component of *Ulva pertusa* (green seaweed), *Laminaria japonica* (brown seaweed) and *Gelidium amansii* (red seaweed) is carbohydrate which can be effectively converted into monosugars using diluted sulfuric acid hydrolysis treatment (Jang et al., 2012). In brown seaweeds, alginate is the main structural compound while mannitol and laminarian are common storage materials (Kloareg and Quatrano, 1988) and the cell wall of red seaweeds is mainly composed of cellulose and combined with agar and carrageenan. Thus, the absence of lignin and the low content cellulose in brown algae makes them a less considered material for bioconversion than land plants (Wei et al., 2013).

3.3. Two stage hydrolysis (acid hydrolysis followed by enzyme hydrolysis)

Cellulose hydrolysis is carried out by cellulase which is highly specific and the resulting products are usually reducing sugars including glucose (Sun and Cheng, 2002). In the present study, the biomass acid pretreated was hydrolysed with cellulase and β- glucosidase. The conversion of reducing sugar due to enzyme saccharification was shown in Fig. 3. It was found to be 141 ± 1.7 and 110 ± 1.6 mg/g biomass of *Gracilaria* sp. and *Sargassum* sp. The present study result indicates that pretreatment with acid significantly play important role during enzyme saccharification ($P \leq .05$). The present study result is correlated with previous works (Kim et al., 2011; Kumar et al., 2013; Borines et al., 2013) showed the effectiveness of combining acid hydrolysis and enzymatic hydrolysis for saccharification of seaweed.

3.4. Phylogenetic analysis

On the basis of BLAST and phylogenetic results strains GK01 was identified as *Hanseniaspora opuntiae* GK01 (NCBI GenBank Accession No. KC870065) (Fig. 4). The phylogenetic data described were obtained by using MEGA4 package using maximum likelihood and neighbour-joining. A direct analysis of the genetic distance and the phylogenetic tree was determined by sequencing of large subunit (LSU) ribosomal DNA D1/D2 region. Various strains have been examined for the phylogenetic relationships. The evolutionary history was inferred using the unweighted pair group method with arithmetic mean (UPGMA) method (Sneath and Sokal, 1973).

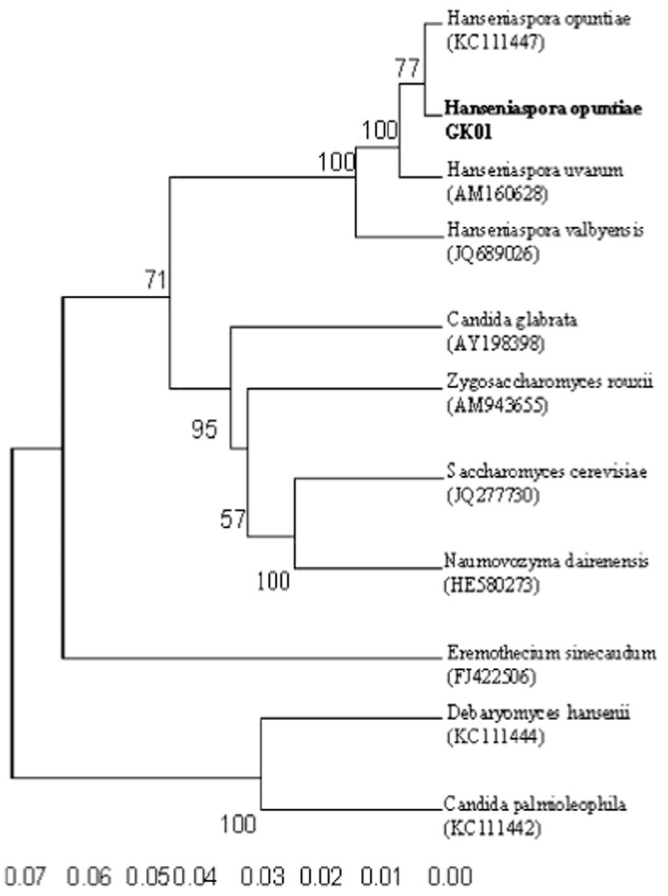


Fig. 4. Phylogenetic tree showing evolutionary relationship of *H. opuntiae* GK01.

3.5. Comparison of ethanol fermentation of two different hydrolysates and by two different yeast strains

The two different yeast cell suspensions were obtained by centrifuging the yeast broth culture and used for fermentation process. The hydrolysates obtained from acid treatment and two stage hydrolysis (acid and enzyme treatment) were used separately as substrate for fermentation of ethanol production. The hydrolysates were removed for their insoluble salts through filtration or centrifugation, inoculating with yeast and fermenting it to form a fermented broth. After fermentation time (96 h), the fermented broth was removed, distilled, concentrated and finally estimated for the amount of ethanol.

A comparison of ethanol production from two different seaweeds by two different yeast strains was shown in Fig. 3. Significant difference in final ethanol concentration was observed between seaweeds and yeast strains. The acid hydrolysates of *Sargassum* sp. added with *S. cerevisiae* MTCC174 and *H. opuntiae* GK01 as inoculum produced 8.6 ± 0.2 and 6.6 ± 0.2 gL^{-1} ethanol respectively, while 11 ± 0.4 and 9.6 ± 0.3 gL^{-1} of ethanol production was observed in acid hydrolysates of *Gracilaria* sp. inoculated with *S. cerevisiae* MTCC174 and *H. opuntiae* GK01 respectively. Likewise *S. cerevisiae* MTCC174 showed significantly increased ethanol production in the biomass of two stage hydrolysis of *Sargassum* sp. (19.9 ± 0.3 gL^{-1}) and of *Gracilaria* sp. (28.7 ± 0.4 gL^{-1}) than *H. opuntiae* GK01 ($P \leq .05$). (Fig. 5). However, *H. opuntiae* GK01 showed lower production of ethanol than *S. cerevisiae* MTCC174 in all the samples, it showed considerable productivity in all the biomass. *Saccharomyces cerevisiae* NSE-3 reported higher efficiency of ethanol production than *S. cerevisiae* NSE-1 and *Hanseniaspora opuntiae* NSE-2 but the two strains produced significant amount of ethanol (Praneetrattananon and Kitpreechavanich, 2011). In

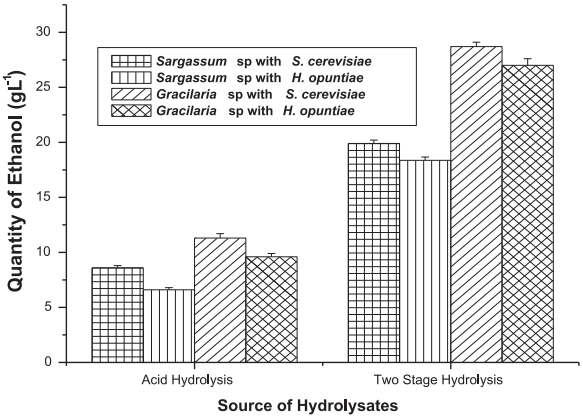


Fig. 5. Fermentation comparison data of two different hydrolysates of two seaweeds using *S. cerevisiae* and *H. opuntiae* GK01.

simultaneous saccharification and fermentation process using NaOH-HC pretreatment sugarcane bagasse, 62.33% of total carbohydrate fractions were hydrolysed and 17.26 gL^{-1} of ethanol production (0.48 g of ethanol/g of glucose and xylose consumed) was achieved (Teran Hilares et al., 2017).

This study also showed that production of ethanol was found to be higher in hydrolysates of two stage hydrolysis and this may be due to high conversion rate of sugars from seaweed extracts. A parallel result is obtained in an earlier study that showed the efficiency of two stage hydrolysis (combination of acid and enzyme hydrolysis) in ethanol production (Wang et al., 2011). Several researches on ethanol production from seaweed has been focussed on acid hydrolysis of seaweeds rather than enzyme hydrolysis (Chien-Wei, 2010; Hwa et al., 2011; Jang et al., 2013; Park et al., 2012; Dawei et al., 2011; Ravikumar et al., 2011). Among the two seaweeds, the red seaweed *Gracilaria* sp. (28.7 ± 0.4 gL^{-1}) significantly gave maximum ethanol production by *S. cerevisiae* MTCC174 than the *Sargassum* sp. (19.9 ± 0.3 gL^{-1}; $P \leq .05$) and this may be due to complex composition of brown seaweed (Wargacki et al., 2012).

4. Conclusion

This study represents the report on third generation biofuel production from invasive macroalgae, suggesting for the production of renewable energy using marine biomass as it has great potentiality. The two seaweeds (*Sargassum* sp. and *Gracilaria* sp.) were analysed for their composition and revealed that higher percentage of total carbohydrates (45.4 ± 0.5 and 56.3 ± 1.25 in *Sargassum* sp. and *Gracilaria* sp.) make them as feed stock for bioethanol production. The reducing sugar was markedly higher in the biomass after obtained from two stage hydrolysis (acid and enzyme treatment) than from acid hydrolysis only. This study clearly suggested that pretreatment of biomass with diluted acid greatly improve the efficiency of enzyme saccharification. The ethanol produced from the biomass of *Gracilaria* sp. was found to be marginally higher than that of produced from *Sargassum* sp. Likewise *S. cerevisiae* (MTCC174) showed markedly increased production of ethanol than that of *H. opuntiae* GK01 isolated from grape juice. In general, the comparative study highlights the possibility of bioethanol production with minimal cost and effort which results in the promotion of industrial scale up processing ecofriendly.

Conflict of interest

The authors declare that they have no conflict of interest.

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