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Exploration of *Rhizoclonium* sp. algae potential under different ethanol production strategies with SEM analysis of biomass and detoxification of hydrolysate

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Abstract: One of the most important challenges for the new global economy is to find out new sources of bioenergy. Microalgae play an important role as a source of renewable biomass fuel due to its photosynthetic efficiency and the possibility of biotransformation of its carbohydrates into bioethanol. This paper aims to assess first time ever the role of *Rhizoclonium* sp. (green algae) as a potential substrate for bioethanol production by using different monoculture and co-culture combination of yeast cells under different modes of fermentation SHF and SSF. It also throws light on biomass structure analysis after different pretreatment conditions through Scanning Electron Microscopy (SEM) and analysis of different inhibitors generated after enzymatic hydrolysis of biomass. Activated charcoal and overliming strategies were employed for detoxification of hydrolysates and 5-Hydroxy methyl furfurals as well as sugars were quantified with the help of HPLC. When compared, the maximum ethanol of 23.70 g/l with fermentation efficiency of 46.37% was observed from pretreated biomass under Separate hydrolysis and fermentation (SHF) with S*accharomyces cerevisiae* I.
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1. Introduction

Rapid growth in population and industrialization increasing worldwide energy demand continuously (Sharma and Sharma, 2018). 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 sugars by yeasts. 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 (John *et al*., 2014)**.** Currently algae are being promoted as an ideal third generation biofuel feedstock because of their rapid growth rate, greenhouse gas fixation ability (net zero emission balance) and high production capacity of carbohydrates and lipids. The major advantages offered by algae over terrestrial biomass are (1) higher biomass production rate per unit area, (2) do not compete with agricultural plants for land, (3) require no agricultural input such as fertilizer, pesticides and water, and (4) easier depolymerisation as it does not contain lignin in their cell wall (Jones and Mayfield, 2012). Microalgae algae-based fuels are ecofriendly, nontoxic and with strong potential of fixing global CO_2 (Khan et al., 2018). Therefore, all these reasons indicate their great potential as a source of renewable energy (Jang et al., 2012). Phototrophic algae convert carbon dioxide in atmosphere to nutrients such as carbohydrate. Conversely, heterotrophic algae continue their development by utilizing organic carbon sources (Wen and Chen, 2014). Algae can grow in every season and everywhere such as salty waters, fresh waters, lakes, deserts and marginal fields etc. However for their cultivation, generally open systems like ponds and photobioreactors as closed systems are used. Algae are classified as microalgae and macroalgae. Microalgae as their name implies, are prokaryotic or eukaryotic photosynthetic microorganisms. They can survive in hard conditions with their unicellular or simple colony

structures (Mata *et al.*, 2013). Because of being photosynthetic organism, they can produce high amount of carbohydrate, lipid and protein in a short time. Chemical composition of algae can change according to the cultivation type and cultivation conditions. The marine ecosystem has vast resources of algal biomass with high to very high carbohydrate percentage. Many researchers have studied the temperate environment around the world; however, in India especially the North Western Himalayas are still poorly understood. Himachal Pradesh has not thoroughly been explored, as yet for the occurrence and distribution of algal species, a very little information is available on the species composition and potential of algae in different water streams (Gour *et al.*, 2014). Due to its abundancy and negative impact on the environment, its management presents a great environmental challenge. Thus, it is use as a source of 3rd generation biofuel and seems highly promising by bioconverting it to ethanol using suitable microbial technology. Accordingly, this study analyzed the structure of green alga *Rhizoclonium* sp. by Scanning Electron Microsopy (SEM) after enzymatic hydrolysis and microwave pretreatment and detoxification process by using overliming and activated charcoal were studied. Quantification of inhibitor 5- Hydroxymethy furfural (5-HMF) using High Performance Liquid Chromatography (HPLC) and different ethanol production strategies under monoculture and co-culture were explored and significant research efforts have been put into utilizing *Rhizoclonium* sp. algal biomass as a feedstock for bioethanol production

2. Material and Methods

2.1 Biomass collection and processing

The fresh water green algae were collected in sterile polythene bags from different districts viz. Bilaspur, Hamirpur and Kangra of Himachal Pradesh, India. The algal samples were washed, dried and grinded to make a powder. The algae was identified with the help of Algae Identification Field Guide and identified as *Rhizoclonium* sp.

2.2 Pretreatment of Biomass

2.2.1 Microwave

1 g untreated dried algal biomass was taken and subjected to different dose of microwave irradiation i.e. 150, 300, 450 W for different time intervals of 30 and 60 sec.

2.2.2 Enzymatic hydrolysis of biomass

1 g of microwave pretreated algal powder was taken and to this 10 ml of phosphate buffer (0.1 M, pH 7.0) was added and autoclaved. The inhouse enzymes which were prepared i.e. cellulase from *Bacillus stratosphericus* N_{12}(M), amylase from *Bacillus aureus* GC6 and xylanase from *Bacillus altitudinis* Kd_1(M) and pectinase from *Brevibacillus parabrevis* C1 used in the ratio 5:3:1:1 (cellulase: amylase: xylanase: pectinase) at 45°C for 48 h to undergo enzymatic hydrolysis.

2.3 Morphological characterization of native and pretreated algal biomass by scanning electron microscopy (SEM) (Mc Mullan, 2006)

The degree of effectiveness of pretreatment/ enzymatic hydrolysis on the structure of *Rhizoclonium* sp. algal biomass was analyzed by comparing the structures of native, microwave pretreated, enzyme pretreated and both microwave cum enzyme pretreated biomass. For this, samples obtained after the pretreatment were centrifuged at 10,000 rpm for 10 min. The mounted samples were then spatter coated with gold using fine coat, JEOL ion sputter, Model JFC-100. The gold coated stubs were examined at different magnification under scanning electron microscope; model Hitachi S-3400N field emission SEM (Hitachi High-Tech, Japan).at 10 kV.

2.4 Estimation of inhibitors i.e. Furfurals and 5-Hydroxy Methyl Furfurals generated during pretreatment and fermentation

2.4.1 Detoxification of algal biomass

(a) Overliming with Ca (OH) $_2$ (Chandel et al., 2007)

Calcium hydroxide was added to the microwave pretreated and enzyme saccharified hydrolyzate (45°C, pH-7.0, 48 h) to increase the pH to 10.5. At high pH inhibitors were precipitated out with calcium hydroxide. The whole mixture was stirred for 30 min at 90°C, allowed to cool slowly to room temperature and then adjusted back to pH 6.0 with HCl. It was then centrifuged (10,000 rpm × 30 min) to remove precipitate formed before using as substrate for fermentation. After removing the precipitates sugar estimation was done (Miller, 1959).

(b) Detoxification by Activated Charcoal (Ra et al., 2015)

5% activated charcoal (granular 1.0-5.0 mm) was added to 5 ml of microwave pretreated and enzyme saccharified hydrolysate (45°C, pH-7.0, 48 h) taken in 100 ml conical flasks. The hydrolysate was subjected to different adsorption times i.e. for 0, 2, 4, 6, 8, 10 min in shaking water bath at 50°C on 100 rpm. After

treatment, solids were removed by centrifugation (10,000 rpm × 10 min) and sugars were estimated. Sample with and without detoxification was withdrawn for qualitative and quantitative analysis.

2.4.2 Qualitative estimation of furfurals and 5-Hydroxy Methyl Furfural

Presence of furfurals and 5- HMF was checked by performing Molisch's test (Thimmaiah, 2004) by adding 1 drop of Molisch's reagent (10% α-napthol in ethanol) in sample solution (2 ml). To which 2 ml of conc. H_2SO_4 poured down the side of the test tube, so that it forms a layer at the bottom of the tube. The color at the interface between two layers was observed and compared with a control test.

2.4.2 Quantitative estimation of 5-HMF using High performance Liquid Chromatography (HPLC)

5-HMF and reducing sugars were quantified by HPLC using following standard conditions.

HPLC Conditions (Sharma, 2013):

Column:	Ultra C18 (Restek Corp.), 250mm × 4.6 mm, 5µm
Mobile Phase A:	90: 10 water: methanol, 10mM ammonium formate
Mobile phase B:	10: 90 water: methanol, 10mM ammonium formate
Gradient:	0-5 min at 100% A, to 100% B at 10 min, 10 min hold
Flow:	0.5 ml /min
Temperature:	Ambient
Detector:	UV@ 280 nm
Injection volume:	10 µl
Standard dilution:	500 ppm

2.5 Bioconversion of algal biomass into ethanol under Simultaneous Hydrolysis and Fermentation (SHF)

2.5.1 Preparation of fermentation media: After enzymatic hydrolysis, to untreated/ pretreated supernatant, 0.5 % yeast extract and 0.5% peptone was added and autoclaved at 121°C, 15 lbs for 20 min.

2.5.2 Inoculum preparation: The inoculum of above mentioned microorganisms was prepared by growing cells aerobically in 250 ml flask containing 100 ml of the growth medium as mentioned below in a rotary shaker incubator for 24 h at 25±2°C to make culture 1 OD.

2.5.3 Fermentation: To the fermentation media 10 % (1 OD) inoculum *Saccharomyces cerevisiae*-I, *Saccharomyces cerevisiae*-II, *Pichia stipitis*, *Candida shehatae, Zymomonas mobilis*, *S. cerevisiae*-I + *P. stipitis*, *S. cerevisiae*-I + *C. shehatae*, *S. cerevisiae*-II + *P. stipitis* and *S. cerevisiae*-II + *C. shehatae* were added and kept for 72 h at 25°C. Ethanol estimation was done by using Caputi method (Caputi et al., 1969).

2.6 Bioconversion of algal biomass into ethanol under Simultaneous Saccharification and Fermentation (SSF)

1 g of untreated/pretreated *Rhizoclonium* algal biomass was taken in each of 18 sets of 100 ml flasks and to these flasks, 10 ml phosphate buffer was added. The pH was maintained 6.0 and 0.5% yeast extract and 0.5% peptone was added, autoclaved at 121°C, 15 lbs for 20 min. To the cooled autoclaved slurry in each of the flask, hydrolytic enzymes @12.5 ml/g were added, simultaneously with fermenting microorganisms (24 h, 1.0 OD) i.e. *Saccharomyces cerevisiae*-I, *Saccharomyces cerevisiae*-II, *Pichia stipitis*, *Candida shehatae, Zymomonas mobilis*, *S. cerevisiae*-I + *P. stipitis*, *S. cerevisiae*-I + *C. shehatae*, *S. cerevisiae*-II + *P. stipitis* and *S. cerevisiae*-II + *C. shehatae* and these flasks were kept for fermentation at 32°C for 72 h. Ethanol estimation was done [18].

$$\text{Fermentation efficiency} = \frac{\text{Ethanol produced (g/g)}}{\text{Theoretical yield of ethanol}} \times 100$$

Theoretical yield was referred as standard value of 0.511 g/g of sugars.

3. Results and Discussion

3.1 Collection and identification of algal sample

Himachal Pradesh is situated in north western Himalayas. It extends from the latitudes 30°22'40" North to 33°12'40" North and longitudes 75°45' 55" East to 79°04' 20" East located in the northern part of India. In the present study, the algae samples were collected from different water bodies of Himachal Pradesh i.e. from Mandh khad and Khabli khad (Distt. Kangra), Sunail Khad (Distt. Bilaspur) and Sheer Khad (Distt. Hamirpur). The alga was identified as *Rhizoclonium* sp. algal biomass based upon its morphological characteristics with the help of algal monographs (Anand, 1998) and classified as *Rhizoclonium* sp. Analytical studies for estimation of starch, cellulose, pectin, were performed by Sadasivam and Manickam (1991) and protein, ash content and moisture content were determined by AOAC (2007). It showed starch 13.50%, cellulose 19.40%, hemicelluloses 2.85%, and pectin 2.98% (total= 38.73%) on dried weight basis of algal biomass.

3.2 Surface structure analysis of *Rhizoclonium* sp. algal biomass using Scanning Electron Microscope (SEM)

SEM analysis was conducted to determine the surface structure changes and surface characteristic of *Rhizoclonium* sp. algal biomass. Plate 1 represents the images of algal biomass examined by SEM for verification of the structural changes caused by microwave pretreatment, enzymatic hydrolysis and by combined effect of microwave with enzymes. Initially its was found that the native substrate had an even and compact structure (Plate 1a) and surface morphology of the untreated substrate serve as a major barrier for enzymes to penetrate the surface and access cellulose, starch, hemicelluloses and pectin for production of sugars. Plate 1b shows the structure of microwave pretreated algal biomass where the compact structure of algal biomass was disrupted and uneven fragmented ribbon shape structures were formed due to breakage of large cellulose fibrils. The cracks and uneven structures resulted in higher surface area facilitating more rapid accessibility for the degrading enzymes to attack the inner structure of the biomass during saccharification. After the enzymatic hydrolysis with inhouse enzymes, the fragments of biomass structures were more disrupted and segregated as compared to its intact assembly thus exposing it more for further reaction. This would provide better digestibility of biomass, hence accelerating the degradation process (Plate 1c). As shown in Plate 1d, the combined effect of microwave with enzymes had severely damaged the structure of biomass, leading to formation of uneven and small crystal like structures. The cell wall of microwave pretreated biomass appeared to be thinner and broken after enzymatic hydrolysis, indicating the release of carbohydrate constituents of cell wall into the medium. Kassim *et al.* (2014) examined the SEM images of microalgal biomass *Tetraselmis suecica* before and after alkaline pre-treatment. Untreated *Tetraselmis suecica* biomass seemed to have actual cell structure form, while uneven distribution and rough surface were observed in pretreated *Tetraselmis suecica* biomass. Eldalatony *et al.* (2015) used Scanning electron microscopy to observe the cell integrity after the pretreatments. Scanning electron microscopy (SEM) analysis revealed ultrastructural changes in *Chlamydomonas mexicana* during sonication and enzymatic hydrolysis. The surface of untreated sample was smooth and continuous. The sonicated samples showed partially ruptured cell wall while enzymatic hydrolysis increased the rupturing of cell wall. The cell wall of sonicated-hydrolyzed cells appeared to be thinner after enzymatic hydrolysis, indicating the release of carbohydrate constituents of cell wall into the medium.

3.3 Qualitative and quantitative estimation of inhibitor and reducing sugars

3.3.1 Qualitative estimation of inhibitors and reducing sugars

Qualitative estimation of inhibitors was performed by applying Molish's test which showed ring formation for the presence of inhibitors. In our study we tried two detoxification methods to remove or overcome inhibitors generated during fermentation processes. The microwave pretreated algal biomass was detoxified by using $Ca(OH)_2$ and activated charcoal. Table 1 shows the reducing sugar yield of non-detoxified and detoxified pretreated algal biomass after enzymatic hydrolysis. In case of Ca $(OH)_2$, a thin ring of inhibitors was formed but sugar production was significantly reduced up to 85.13 mg/g. Activated charcoal at different adsorption times of 0, 2,4,6,8,10 min was used for detoxification of inhibitors. At adsorption time of 6 min, activated charcoal retained maximum sugars i.e. 135.97 mg/g but this was significantly lower than non-detoxified sample i.e. 190.08 mg/g. The quantity of inhibitors was overliming, a significant loss of sugars was observed but overliming resulted into greater loss of sugars than activated charcoal. Therefore, due to a dip in the yield of reducing sugars during detoxification, both the approaches were discarded. Ra *et al.* (2015) carried out the detoxification of *Eucheuma spinosum* hydrolysates with activated carbon for ethanol production by the salt-tolerant yeast *Candida tropicalis.* Monosaccharides from *Eucheuma spinosum* slurry were obtained by thermal acid hydrolysis and

enzymatic hydrolysis. Addition of activated carbon at 2.5% (w/v) and the adsorption time of 2 min were used in subsequent adsorption treatments to prevent the inhibitory effect of HMF. The adsorption surface area of the activated carbon powder was 1,400-1,600 m^2/g and showed selectivity to 5-hydroxymethyl furfural (HMF) from monosaccharides. Wu *et al.* (2016) studied various modes of detoxification for alga *Pterocladiella capillacea* in which neutralization not only reduced the amount of 5-HMF and Levulinic acid by 42.9 ± 14.2% and 11.5 ± 4.5% respectively, but also reduced the amount of fermentable sugars by 24.9 ± 4.5%. Meanwhile, overliming reduced the amount of 5-HMF and Levulinic acid by 57.1 ± 14.3% and 47.5 ± 9.8% respectively but overliming also reduced the amount of fermentable sugars by 42.1 ± 2.8% . The results in our study are in accordance with these earlier reports.

3.3.2 Quantitative estimation of 5-HMF by HPLC

The detection of most of inhibitors generated during hydrolysis can be accomplished through HPLC, but with different combinations of detector wavelength. 5-HMF are one of the important inhibitors generated during the degradation of sugars resulting in the toxicity of fermenting liquor and thus limiting the fermentation. In the present study, concentration of 5-HMF of different samples collected after enzymatic hydrolysis process were quantified to find out the effect of these inhibitors on sugar production. The quantitative analysis of 5-HMF of 4 different samples ranging from non-detoxified untreated and pretreated, detoxified untreated and pretreated have been compared in table 2. Fig. 1 (a, b) depicted the chromatograms of 5-HMF in non-detoxified untreated and pretreated enzymatically hydrolysed syrup at retention times of 14.92 min and 14.94 min respectively whereas Fig. 1 (c, d) represented the chromatographic peak of 5-HMF in detoxified untreated and pretreated algal biomass with 14.92 and 14.97 min of retention time. In the table 2, non-detoxified untreated and pretreated algal hydrolysate expressed the high concentration, 5-HMF i.e. 36.35 and 58.96 mg/lt. On the other hand, detoxified untreated and pretreated algal hydrolysate concentration of 5-HMF was found to be relatively low i.e. 8.00 and 20.89 mg/l in comparison to non-detoxified samples. Other peaks found in HPLC chromatograms may be due to presence of some different components i.e. Oligosaccharides, levulinic acid and acetic acid etc. present in hydrolysate. In a similar study the concentration of 5- HMF generated during each step of separate hydrolysis and fermentation (SHF) by detoxification as well as non detoxification of *Populus deltoides* wood hydrolysate were detected by using HPLC technique (Sharma and Sharma, 2017). Hydrothermal and wet oxidation pretreatments of macroalgae *Ulva* yielded high concentrations of formic acid (0.7 and 1.8 g/100 g DM, respectively) and acetic acid (0.2 g/100 g and 1.0 g/100 g DM, respectively). A high amount of furfural (0.2 g/100 g) obtained after acid and hydrothermal pretreatments were detected by HPLC (Ross et al., 2014). High amounts of furfural and formic acid formed through pretreatment with 7 % acid or at a high temperature in *Saccharina japonica* were detected by HPLC (Lee et al., 2013).

3.3.3 Estimation of reducing sugars by HPLC

Using HPLC the quantitative analysis of sugars in four different samples i.e. non-detoxified untreated and pretreated, detoxified untreated and pretreated enzymatically hydrolysed syrup has been shown in table 3. Fig. 2 depicted the chromatograms of four samples taken for quantitative estimation of sugars. Fig. 2 (a, b) presented the chromatogram of sugars in untreated and pretreated non-detoxified algal syrup after enzymatic hydrolysis. As presented in table 3, untreated and pretreated non-detoxified algal syrup after enzymatic hydrolysis showed retention time of 6.44 and 6.55 with glucose sugar concentration of 163.8 and 287.78 mg/g respectively. As indicated by HPLC chromatogram (Fig 2c, d) in detoxified untreated and pretreated algal biomass, the concentration of glucose sugar had dipped to 80.29 and 164.79 mg/g respectively. While their chromatographic peaks were reported in 5.74 min and 5.24 min of retention time. This was probably due to adsorption of sugars by detoxifying agent along with adsorption of inhibitors generated in this process. Therefore detoxification of inhibitors was avoided for further ethanol fermentation experiments. The maximum yield of glucose from the macro-alga *Nizimuddinia zanardini* by enzymatic saccharification (45°C, pH 4.8, 24 h), using cellulase and *β*-glucosidase, was 70.2 g/kg (70.2% yield based on total glucan content) quantified by HPLC (Yazdani *et al.*, 2011). Quantification of various reducing sugars was done by HPLC after acidic hydrolysis and found yielding reducing sugar concentration of 0.079 g/g in *Ulva lactua* (EI Sayed *et al.,* 2017).

3.4 Bioconversion of *Rhizoclonium* sp. algal biomass into bioethanol under SHF and SSF

In the present study, the bioconversion of untreated and microwave pretreated *Rhizoclonium* sp. algal biomass into bioethanol was taken under two different modes of fermentation i.e. SHF and SSF by using different ethanologens in monoculture and co-culture combinations. Table 4 and 5 represented bioethanol production by using different ethanologenic microorganisms (monoculture and co-culture) under SHF using untreated and pretreated algal biomass. As the results presented in table 4**,** the maximum ethanol

i.e. 14.22 g/l was fermented by culture of *S. cerevisiae* I followed by 10.27 g/l by *S. cerevisiae* I + *P. stipitis*, while the minimum ethanol production i.e. 3.16 g/l was noticed in monoculture of *Pichia stipitis*. The maximum fermentation efficiency (27.83%) was obtained in *S. cerevisiae* I. During SSF of untreated biomass, *S. cerevisiae* I produced maximum amount of ethanol i.e. 9.41 g/l with fermentation efficiency of 18.55 %. The lowest ethanol yield of 3.16 g/l with fermentation efficiency of 6.18 % was shown by *P. stipitis* (Table 6**).** An approach was applied for bioethanol production using same monoculture and co-culture combinations of ethanologens and yielded maximum ethanol of 11.26 g/l with fermentation efficiency of 44.16% by co-culture combination of *Saccharomyces cerevisiae* + *Pichia stipitis* (Sharma and Sharma, 2016b).

In pretreated biomass, *S. cerevisiae* I also produced maximum amount of ethanol i.e. 23.7 g/l with fermentation efficiency of 46.37% and ethanol yield of 0.24 g/g (Table 5) under SHF. Least amount of ethanol yield was noticed in *P. stipitis* attributed due to its inability to utilize hexose sugars while pretreated algal biomass was rich in hexose sugars only. Similarly in case of SSF the maximum ethanol yield of 0.19 g/g was also obtained by *S. cerevisiae* I which showed fermentation efficiency of 40.19% as presented in table 7**.** The least ethanol yield obtained was 0.08 g/g with fermentation efficiency of 15.45% by *P. stipitis*.

S. cerevisiae I yielded highest ethanol as compared to other co-cultures and monocultures. *S. cerevisiae* is widely known for hexose utilization due to its high affinity for hexose sugars and have utilized most of the fermentable sugars present in algal biomass producing highest ethanol concentration. A comparative study of bioethanol production was carried out by using different methods of non- detoxification and detoxification under SHF by different monoculture as well as co-culture combinations, where yielded maximum of 18.47 g/l ethanol by Saccharomyces cerevisiae II + Pichia stipitis (Sharma and Sharma, 2016a). Bioethanol Production from seaweed *Gracilaria chilensis* obtained ethanol yield of 0.18 g ethanol/g dry seaweed with a yield of 86.64% w/w under SHF (Seguel et al., 2015). Bioethanol Production from *Ulva fasciata* Delie Biomass via enzymatic Pretreatment using marine-derived *Aspergillus niger* was studied by Mustafa and Saeed **(**Mustafa and Saeed, 2016**).** *S. cerevisiae* fermented reducing sugars with concentration 51.75 g/l to produce bioethanol of 24.77 mg/g. The efficiency of conversion process improved upon time intervals, it reached 93.88% after 7 days with ethanol yield 3.14%.

In a study, red macroalga *Gelidium amansii* was used for bioethanol fermentation under SSF and achieved an ethanol concentration of 3.78 g/l and an ethanol conversion yield of 84.9% after 12 h (Kim et al., 2015). *Chaetomorpha linum*, green macroalgae species, used in SSF process by *S. cerevisiae* ATCC 96581 for ethanol fermentation and yielded 77.2% of the theoretical ethanol yield (Wang et al., 2016). Ho *et al.* (2013) evaluated the potential of a carbohydrate-rich microalga *Chlorella vulgaris* FSP-E as feedstock for bioethanol production under SSF and gained maximum ethanol concentration and yield of 4.27 g/l and 92.3%, respectively.

The overall ethanol production by two modes of fermentation i.e. SHF and SSF have been compared, out of which the maximum ethanol yield as well as fermentation efficiency was observed in separate hydrolysis and fermentation (SHF). Thus it has been recommended as the best mode of fermentation for improved ethanol yield. In SHF, hydrolysis and fermentation is carried out in completely separate steps, where enzymes added and saccharification is executed to completion, after that yeast is added for fermentation. So each step is allowed to perform at its optimum conditions (Sharma and Sharma, 2016a). Where as in case of SSF, all the steps occur at the same conditions. Both in case of SHF and SSF, highest amount of ethanol was produced by monoculture of *S. cerevisiae* I.

Conclusion

Last three decades of vigorous developments in pretreatment technologies, microbial biotechnology, and downstream processing have made it reality to harness the algal biomass for the production of bioethanol. Biomass recalcitrance is a main challenge toward the successful exploitation of biomass. To overcome the biomass recalcitrance, pretreatment is an inevitable process to ameliorate the accessibility of carbohydrate for the subsequent enzymatic hydrolysis reaction to generate fermentable sugars. As the enzymatic hydrolysis is conducted at low temperature and does not cause the decomposition of monosaccharides. Therefore, enzymes for the hydrolysis of polysaccharides with high conversion rates are required for the efficient production of ethanol from algal biomass. The goal of this research was to determine the physical and inhibitor profiles of selected alga *Rhizoclonium* sp. in order to prove its worth as biofuel feedstock. SEM images proved that the methods used for pretreatment and hydrolysis caused significant changes in algal cell structure. Using an enzyme cocktail of inhouse enzymes, improved ethanol yields at higher yeast titres of *S. cerevisiae* I could be achieved using SHF mode of fermentation. Detection of main inhibitor 5-HMF was done by using HPLC technique and the results demonstrated that algae could be an alternative to other well-known biofuel

feedstocks. Therefore, in order to produce bioethanol from algal biomass in a cost-effective manner, microorganisms that possess the ability to directly convert polysaccharides into ethanol must be employed for better fermentability.

Table 1. Qualitative estimation of inhibitors and reducing sugars generated during enzymatic hydrolysis

Sample	Reducing Sugars (mg/g)	Molish Test
Control (Without detoxification)	190.08	Thick ring
$Ca(OH)_2$	85.13	Thin ring
Activated Charcoal (0 min)	114.74	Thick ring
Activated Charcoal (2 min)	109.78	Thick ring
Activated Charcoal (4 min)	110.82	Thin ring
Activated Charcoal (6 min)	**135.97**	**Thin ring**
Activated Charcoal (8 min)	107.55	Thin ring
Activated Charcoal (10 min)	100.55	Thin ring
$CD_{0.05}$	0.361	
S.E.(m)	0.118	

Table 2. Quantitative estimation of 5- HMF in untreated and pretreated *Rhizoclonium* sp. algal biomass after enzymatic hydrolysis by HPLC

Sample	Quantity of HMF (ppm or mg/lt)	
	NDT* Hydrolysate	DT** Hydrolysate
Untreated *Rhizoclonium* sp. algal hydrolysate	36.35	8.00
Pretreated *Rhizoclonium* sp. algal hydrolysate	58.96	20.89

NDT*: Non detoxified
DT**: Detoxified

Table 3. Estimation of reducing sugars from untreated and pretreated *Rhizoclonium* sp. algal biomass after enzymatic hydrolysis by HPLC

Sample	Retention time	Area	Area %	Glucose (mg/g)
Glucose	6.03	277811	45.26	Standard
Non-detoxified untreated *Rhizoclonium* sp. algal biomass	6.40	2022946	50.67	163.8
Non-detoxified pretreated *Rhizoclonium* sp. algal biomass	6.55	3555074	80.33	287.78
Detoxified untreated *Rhizoclonium* sp. algal biomass hydrolysate	5.74	2034772	98.44	80.29
Detoxified pretreated *Rhizoclonium* sp. algal biomass	5.24	991381	97.19	164.79

Table 4. Ethanol fermentation of untreated *Rhizoclonium* sp. algal biomass under SHF

Yeast culture	Ethanol (%)	Ethanol (g/l)*	Ethanol (g/g)	Fermentation Efficiency** (%)
Saccharomyces cerevisiae I	1.80	14.22	0.14	27.83
Saccharomyces cerevisiae II	1.10	8.69	0.87	17.02
Candida shehatae	0.90	7.11	0.07	13.91
Pichia stipitis	0.40	3.16	0.03	6.184
Zymomonas mobilis	1.25	10.22	0.10	19.95
S. cerevisiae I + *P. Stipitis*	1.30	10.27	0.10	20.09
S. cerevisiae I + *C. Shehatae*	1.20	9.48	0.09	18.55
S. cerevisiae II + *P. Stipitis*	1.30	10.27	0.10	20.09
S. cerevisiae II + *C. Shehatae*	1.10	8.69	0.87	17.02
$CD_{0.05}$	0.35	0.82	0.04	0.97
SE(m)	0.12	0.27	0.01	0.32

*Ethanol (g/l) = ethanol (%) × absolute density of ethanol

$$\text{**Fermentation efficiency} = \frac{\text{ethanol produced (g/g)}}{\text{theoretical yield of ethanol}} \times 100$$

Table 5. Ethanol fermentation of microwave pretreated *Rhizoclonium* sp. algal biomass under SHF

Yeast culture	Ethanol (%)	Ethanol (g/lt)*	Ethanol (g/g)	Fermentation Efficiency** (%)
Saccharomyces cerevisiae I	3.00	23.7	0.24	46.37
Saccharomyces cerevisiae II	2.20	17.38	0.17	34.01
Candida shehatae	1.50	11.85	0.12	23.18
Pichia stipitis	0.40	7.11	0.07	13.91
Zymomonas mobilis	1.30	10.27	0.10	20.09
S. cerevisiae I + *P. Stipitis*	1.80	14.22	0.14	27.82
S. cerevisiae I + *C. Shehatae*	2.00	15.80	0.16	30.91
S. cerevisiae II + *P. Stipitis*	2.10	16.59	0.17	32.48
S. cerevisiae II + *C. Shehatae*	2.00	15.80	0.16	30.91
$CD_{0.05}$	0.42	0.67	0.08	1.30
S.E.(m)	0.14	0.22	0.03	0.44

*
** } Same as in Table 4

Table 6. Ethanol fermentation of untreated *Rhizoclonium* sp. algal biomass under SSF

Yeast culture	Ethanol (%)	Ethanol (g/l)*	Ethanol (g/g)	Fermentation Efficiency** (%)
Saccharomyces cerevisiae I	1.20	9.41	0.09	18.55
Saccharomyces cerevisiae II	1.05	8.29	0.08	16.23
Candida shehatae	0.80	6.32	0.06	12.37
Pichia stipitis	0.40	3.16	0.03	6.18
Zymomonas mobilis	0.70	5.53	0.06	10.82
S. cerevisiae I + *P. Stipitis*	1.00	7.9	0.08	15.45
S. cerevisiae I + *C. Shehatae*	1.15	9.09	0.09	17.78
S. cerevisiae II + *P. Stipitis*	0.80	6.32	0.06	12.27
S. cerevisiae II + *C. Shehatae*	0.90	7.11	0.07	13.91
$CD_{0.05}$	0.18	0.99	0.03	0.91
SE	0.06	0.33	0.01	1.41

*

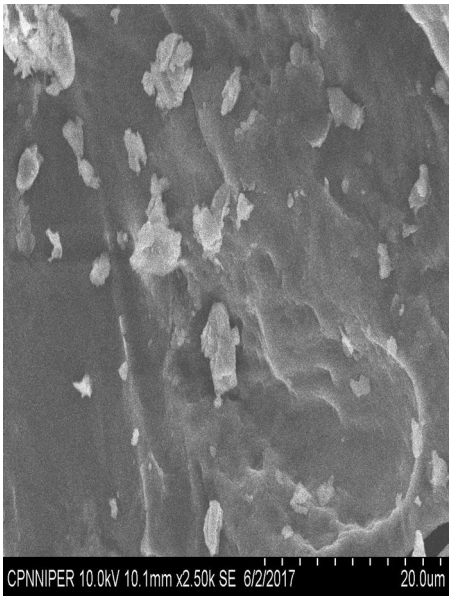
** Same as in Table 4

Table 7. Ethanol fermentation of microwave pretreated *Rhizoclonium* sp. algal biomass under SSF

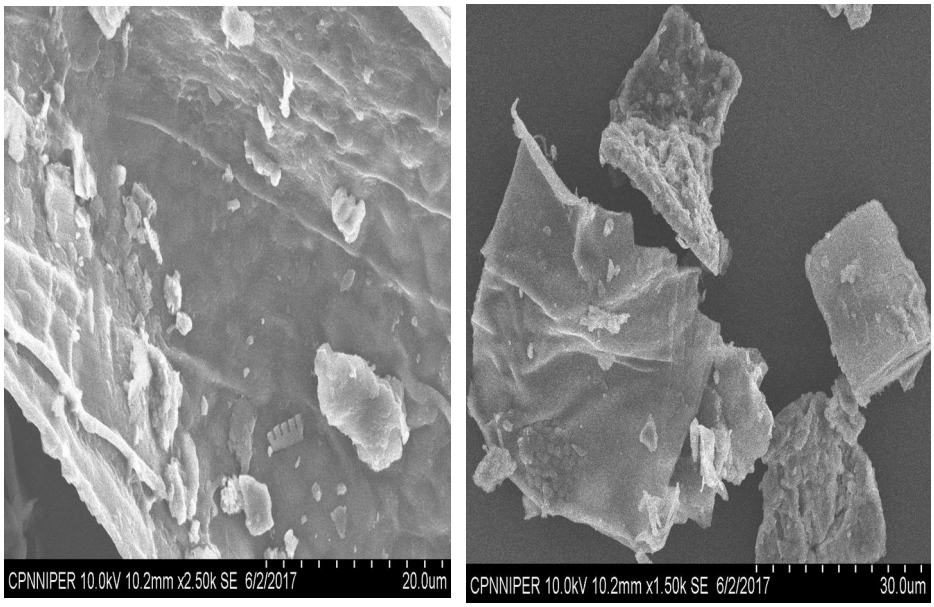
Yeast culture	Ethanol (%)	Ethanol (g/l)*	Ethanol (g/g)	Fermentation Efficiency** (%)
Saccharomyces cerevisiae I	2.60	20.51	0.19	40.19
Saccharomyces cerevisiae II	2.00	15.80	0.16	30.92
Candida shehatae	1.25	9.88	0.09	19.33
Pichia stipitis	1.00	7.90	0.08	15.45
Zymomonas mobilis	1.30	10.27	0.10	20.09
S. cerevisiae I + *P. Stipitis*	1.90	15.01	0.15	29.37
S. cerevisiae I + *C. shehatae*	2.10	16.59	0.17	32.48
S. cerevisiae II + *P. Stipitis*	1.30	10.27	0.10	20.09
S. cerevisiae II + *C. Shehatae*	1.40	11.06	0.11	21.64
$CD_{0.05}$	0.41	0.87	0.06	1.07
SE	0.14	0.29	0.02	0.36

*

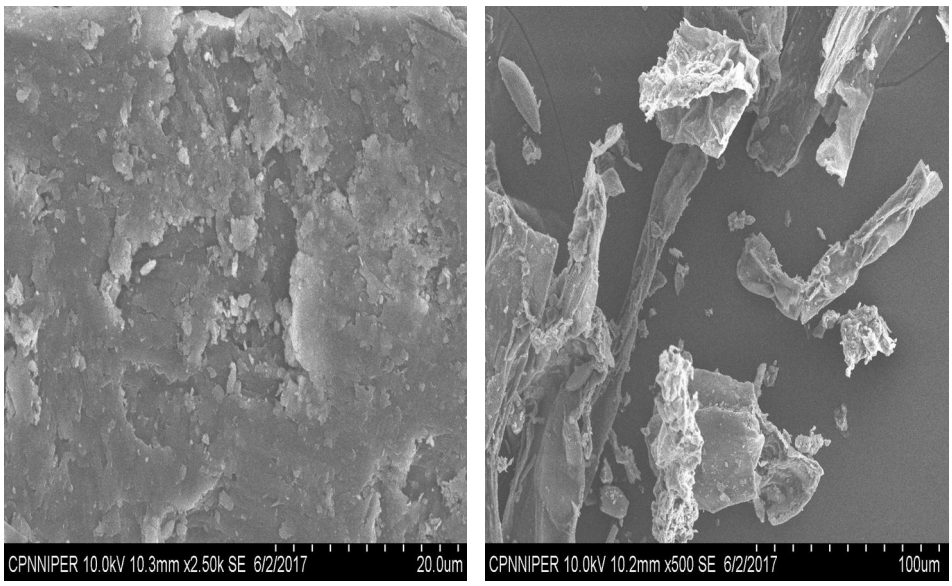
** Same as in Table 4



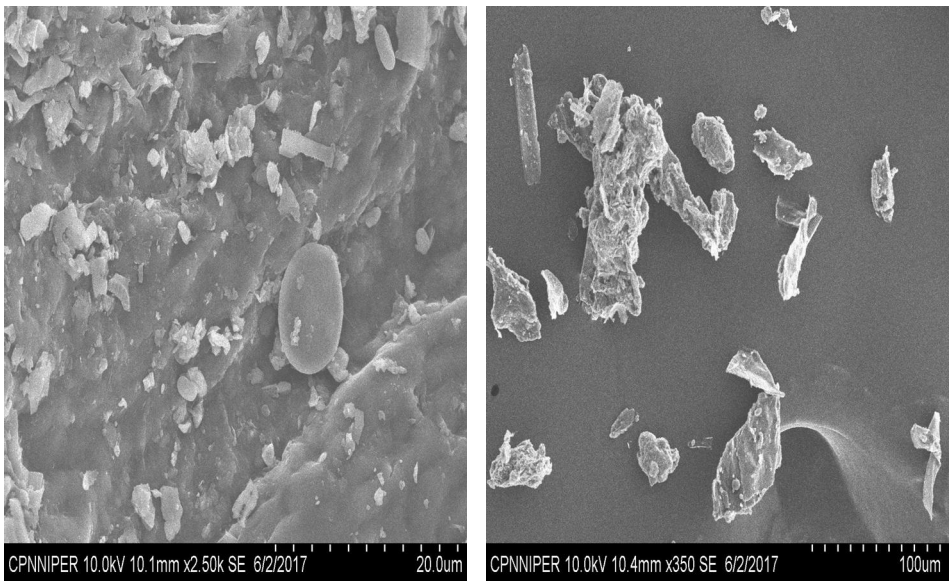
(a). Native substrate



(b) Microwave pretreated algal biomass



(c) Enzyme treated algal biomass



(d) Microwave + enzyme pretreated algal biomass

Plate 1(a-d) Surface analysis of *Rhizoclonium* sp. algae biomass using Scanning Electron Microscope×1000 magnification clearly depicting the effect of pretreatment and hydrolytic enzymes on carbohydrate solubilization

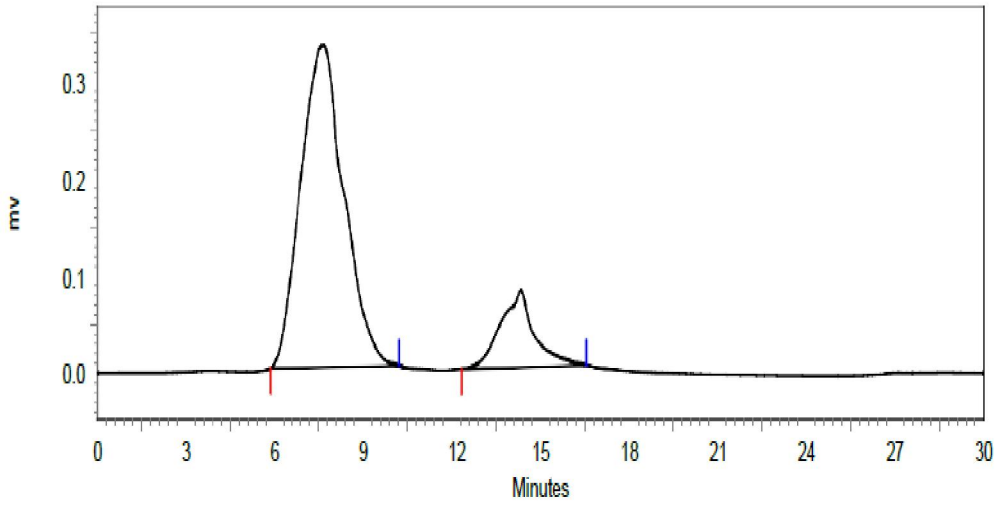


Fig. 1(a) Chromatogram of concentration of 5-HMF in non- detoxified untreated *Rhizoclonium* sp.

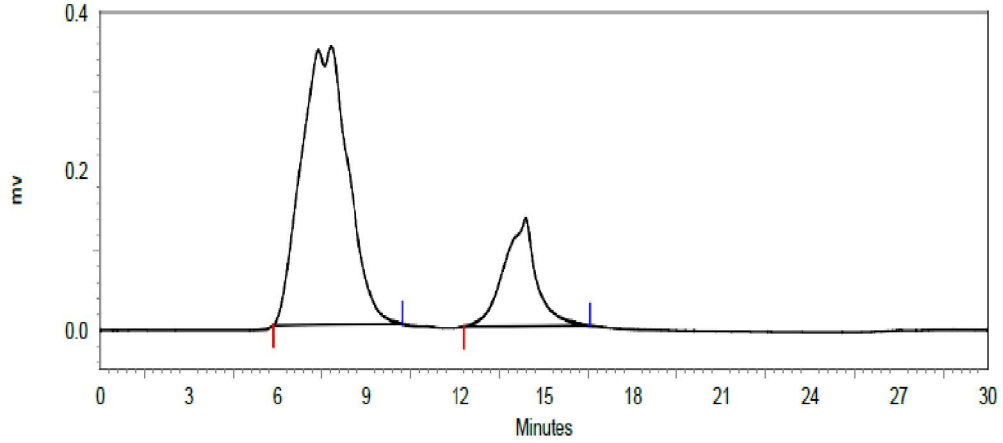


Fig. 1(b) Chromatogram of concentration of 5-HMF in non- detoxified pretreated *Rhizoclonium* sp.

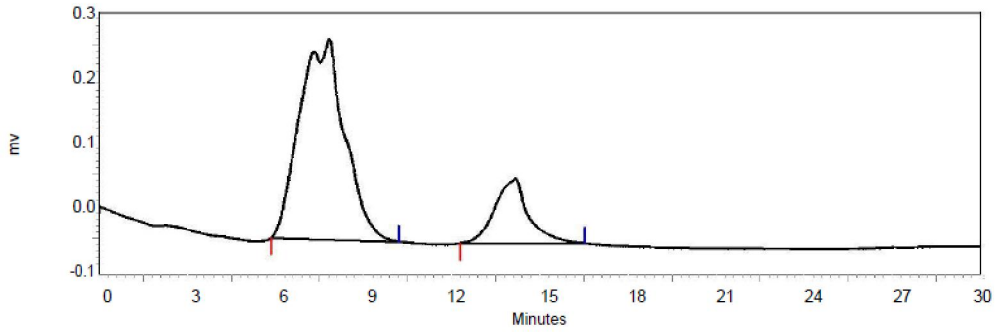


Fig. 1(c) Chromatogram of concentration of 5-HMF in detoxified untreated *Rhizoclonium* sp.

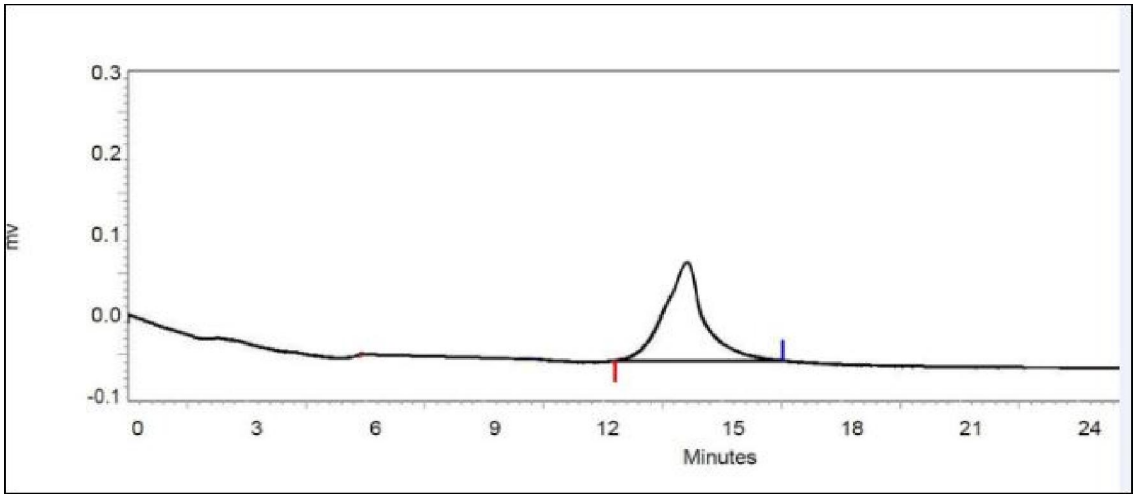


Fig. 1(d) Chromatogram of concentration of 5-HMF in detoxified pretreated *Rhizoclonium* sp.

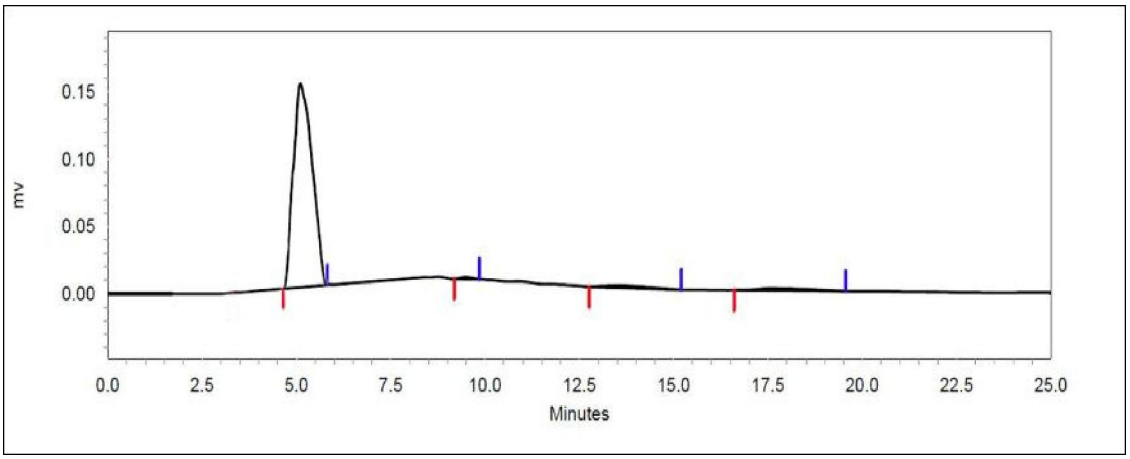


Fig. 2(a) Chromatogram of reducing sugars in non-detoxified untreated *Rhizoclonium* sp.

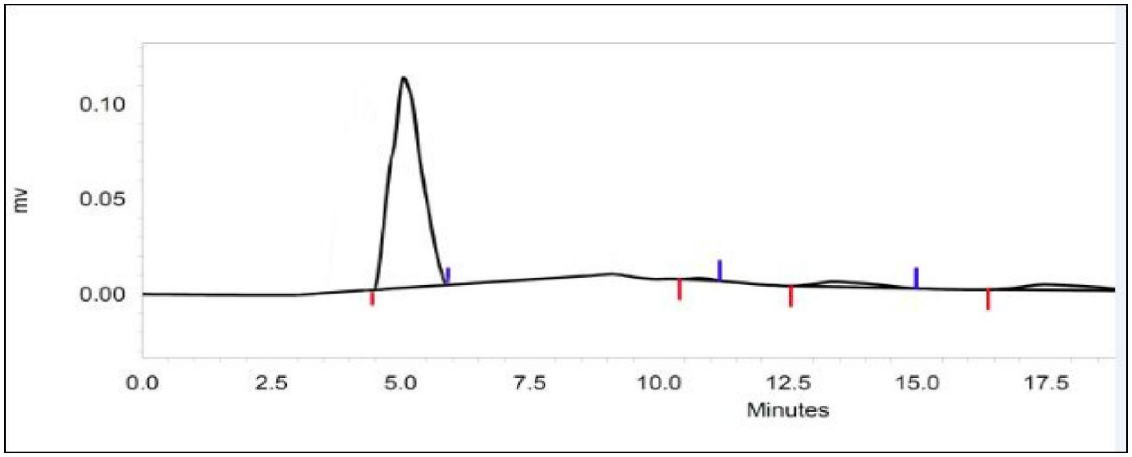
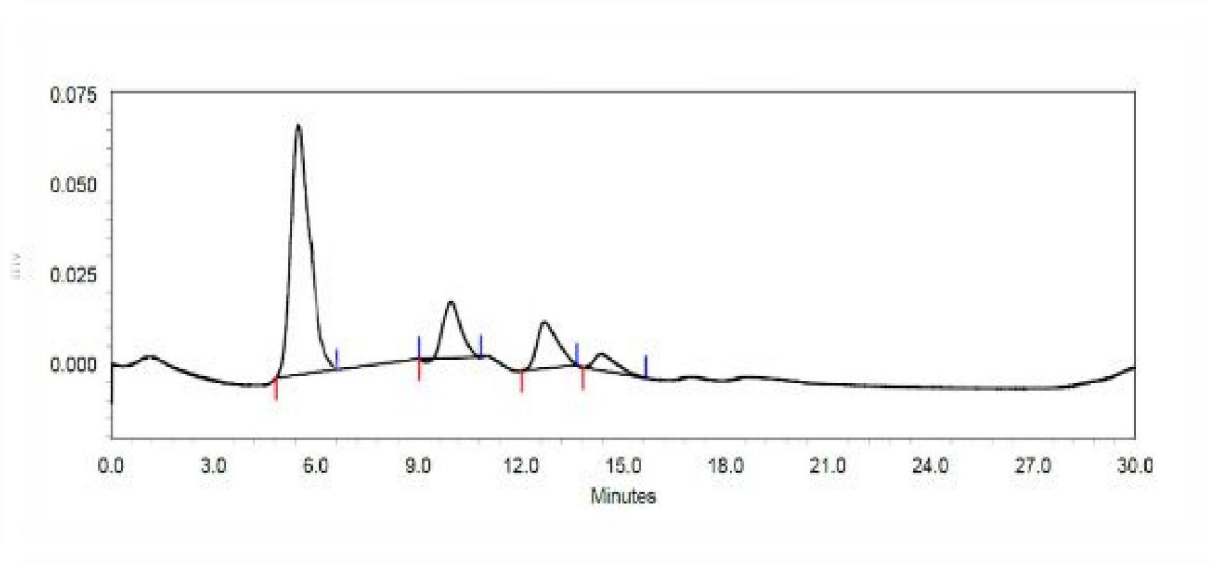


Fig. 2(b) Chromatogram of reducing sugars in non-detoxified pretreated *Rhizoclonium* sp.



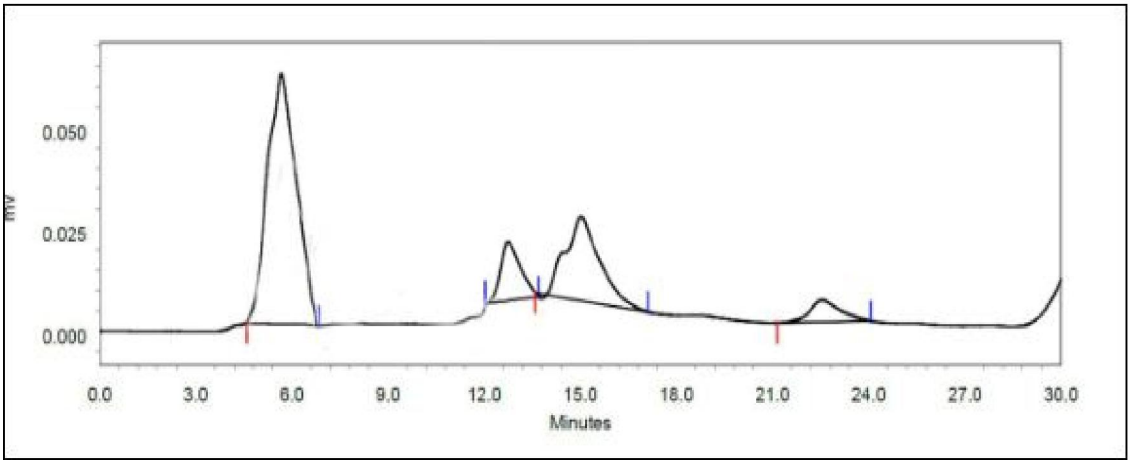


Fig. 2(d) Chromatogram of reducing sugars in detoxified pretreated *Rhizoclonium* sp.

References

[1]. Anand N. 1998. Indian freshwater microalgae. 1st ed. Bishen Singh Mahendra Pal Singh, Dehradun. 94p.

[2]. Caputi A, Ueda M and Brown J. 1969. Spectrophtometric determentaion of ethanol in wine. *American Journal of Enology and Viticulture* 19:160-65.

[3]. Chandel AK, Kapoor RK, Singh AK and Kuhad RC. 2007. Detoxification of sugarcane bagasse hydrolysate improves ethanol production by *Candida shehatae* NCIM 3501. *Bioresource Technology* 98:1947-50

[4]. Eldalatony MM, Kabra AN, Hwang JH, Govindwar SP, Kim KH, Kim H and Jeon BH. 2015. Pretreatment of microalgal biomass for enhanced recovery/extraction of reducing sugars

and proteins. *Bioprocess and Biosystems Engineering* 39:95–103.

[5]. El-Sayed AB, Nashwa AHF and El-Fakharany MK. 2017. Bioethanol Production from Egyptian alga *Enteromorpha* sp. *Middle East Journal of Applied Sciences* 1:216-25.

[6]. Gour RK, Kant A and Chauhan RS. 2014. Screening of micro algae for Growth and lipid accumulation properties. *Journal of Algal Biomass Utilization* 5:38-46.

[7]. Ho SH, Huang SW, Chen CY, Hasunuma T, Kondo A and Chang JS. 2013. Bioethanol production using carbohydrate-rich microalgae biomass as feedstock. *Bioresource Technology* 135:191–98.

[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]. John RP, Anisha GS, Nampoothiri KM, Pandey A. 2014. Micro and macroalgal biomass: a renewable source for bioethanol. Bioresour. Technol. 102: 186-193.

[10]. Kassim MA, Potumarthi A, Tanksale A, Srivatsa SC, Bhattacharya C. 2014. Enzymatic saccharification of dilute alkaline pre-treated microalgal (*Tetraselmis suecica*) biomass for biobutanol production. *International Journal of Biological, Biomolecular, Agricultural, Food and Biotechnological Engineering* 8:1-9.

[11]. Khan M I, Shin J H and Kim J D. 2018. The promising future of microalgae: current status, challenges and optimization of a sustainable and renewable industry or biofuels, feed and other products. Microbial Cell Factories. 17: 36.

[12]. Kim HM, Seung GW, Jung S and Bae HJ. 2015. Efficient approach for bioethanol production from red seaweed *Gelidium amansii*. *Journal of Biotechnology* 5:12-16.

[13]. Lee JY, Kim YS, Um BH and Oh K. 2013. Pretreatment of *Laminaria japonica* for bioethanol production with extremely low acid concentration. *Renewable Energy* 54:196–200.

[14]. Mata TM, Almeida R and Caetano NS. 2013. Effect of the culture nutrients on the biomass and lipid productivities of microalgae *Dunaliella tertiolecta*. *Chemical Engineering Transactions*. 32:973 – 78.

[15]. McMullan D. 2006. SEM- past, present and future. *Journal of Microscopy* 155:373-92.

[16]. Miller GH. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Annals of Chemistry* 31:426–29.

[17]. Mustafa YTA and Saeed SM. 2016. Nutritional evaluation of green macroalgae, Ulva sp. and related water nutrients in the Southern Mediterranean Sea coast, Alexandria shore, Egypt. *Egyptian Academy Journal of Biological Sciences* 5:1-19.

[18]. Ra CH, Jung JH, Sunwoo IY, Kang CH, Jeong GT and Kim AK. 2015. Detoxification of *Eucheuma spinosum* hydrolysates with activated carbon for ethanol production by the salt-tolerant yeast *Candida tropicalis*. *Journal of Microbiology and Biotechnology* 25:856-62.

[19]. Ross AB, Jones JM, Kubacki ML, Bridgeman T. 2014. Classification of macroalgae as fuel and its thermochemical behaviour. *Bioresource Technology* 99:6494-04.

[20]. Sadasivam S and Manickam A. 1991.Biochemical methods. 1st ed.New Age International Ltd., New Delhi. 103p.

[21]. Seguel CG, Soto E and Rojas J. 2015. *Gracilaria chilensis*: Bioethanol production and by-product characterization. *Journal of Coastal Zone Management* 18:2-7.

[22]. Sharma N. 2013. Bioconversion of cellulosic waste into bioethanol as biofuel. Ph.D. Thesis. Department of Basic Sciences, Dr YS Parmar University of Horticulture and Forestry, Solan. 279p.

[23]. Sharma N. and Sharma N. 2016a. Bioethanol production from NaOH+H2O2 pretreated Populus deltoides wood using cocktail of inhouse and commercial enzymes under four different modes of Separate hydrolysis and fermentation. World Journal of Pharmaceutical Research. 5(2): 661-676.

[24]. Sharma N. and Sharma N. 2016b. Bioethanol production under simultaneous saccharification and fermentation (SSF) and simultaneous saccharification and Co-fermentation (SSCF) as well as its scale up in a stirred tank bioreactor. International Journal of Innovative Research in Science, Engineering and Technology. 5(2): 1572-1576.

[25]. Sharma N. and Sharma N. 2017. Determination of 5-Hydroxy methyl fufurals generated during bioethanol production from lignocellulosic waste by high performance liquid chromatography technique. World Journal of Pharmacological Research and Technology. 6(1): 1-17.

[26]. Sharma N. and Sharma N. 2018. Second generation bioethanol production from lignocellulosic waste and its future perspectives: A review. International Journal of Current

Microbiology and Applied Sciences.7 (5): 1285-1290.

[27]. Thimmaiah SR. 2004. Standard methods of biochemical analysis. 3rd ed. Kalyani Publishers, New Delhi. 545p.

[28]. Todhanakasem T, Sangsutthiseree A, Areerat K, Young GM and Thanonkeo P. 2014. Biofilm production by *Zymomonas mobilis* enhances ethanol production and tolerance to toxic inhibitors from rice bran hydrolysate. *New Biotechnology* 31:451-59.

[29]. Wang Y, Ho SH, Cheng CL, Guo WQ, Nagarajan NQ, Lee DJ and Chang JS. 2016. Perspectives on the feasibility of using microalgae for industrial wastewater treatment. *Bioresource Technology* 222:485-97.

[30]. Wen ZY and Chen F. 2014. Heterotrophic production of eicosapentaenoic acid by microlagae. *Biotechnology advances* 21:273-94

[31]. Wu CH, Chien WC, Chou HK, Yang J and Lin HT. 2016. Sulfuric acid hydrolysis and detoxification of red alga *Pterocladiella capillacea* for bioethanol fermentation with thermotolerant yeast *Kluyveromyces marxianus*. *Journal of Microbiology and Biotechnology* 24:1245-53.

[32]. Yazdani P, Karimi K and Taherzadeh MJ. 2011. Improvement of enzymatic hydrolysis of a marine macro-alga by dilute acid hydrolysis pretreatment. World Renewable Energy Congress (Bioenergy Technology), 8-13 May, ed. Moshfegh B., editor. (Linkoping, Sweden) p.186–191.

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