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Biodiesel and Bioethanol Production from *Ulva fasciata* Delie Biomass via Enzymatic Pretreatment using Marine-Derived *Aspergillus niger*

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ABSTRACT

In this context, a green seaweed Ulva fasciata was selected as a potential feedstock for cellulase hydrolysis for the aim of producing bioethanol and the remaining treated algae were subjected to lipid extraction for biodiesel production. Five marine-derived fungal strains (Aspergillus niger, Aspergillus flavus, Penicillium oryzae, Penicillium chrysogenum, and Rhizopus oryzae) were screened to produce cellulase for breaking down the algal cell wall. The extracted cellulase obtained by A. niger showed the highest cellulase activity (mm, Uml^{-1}), specific activity (Umg^{-1} protein) and protein content ($mgml^{-1}$) as; 25mm, 3.24Uml^{-1}, 0.121Umg^{-1} protein and 18.6 $mgml^{-1}$, respectively. In addition, the crude cellulase showed higher activity (2.5 Uml^{-1}) and specific activity (20.83Uml^{-1}) than semi purified enzyme with acetone (0.42Uml^{-1}) and with ammonium sulfate (0.14Uml^{-1}). Moreover, commercial cellulose (CMCase; EC 3.2.1.4) also applied for algal cell wall hydrolysis and its efficiency was compared to the Aspergillus niger crude enzyme. Different pre-treatment schemes were applied in order to achieve the highest cell wall degradation, and then lipid extraction. The highest quantity and the greatest quality of biodiesel was achieved by applying the following treatment, thermal pretreatment with 1% H_2SO_4 pre to biological treatment either with 3% commercial cellulase or with 1% crude cellulase enzyme. The produced biodiesel in both cases showed a great improvement in total fatty acid ΣFA content (3605.44 and 3528.11 µg/g for 3% commercial and 1% crude enzymes respectively), and total saturated fatty acids ΣSUFA content jumped to 3259.93 and 3282.38 µg/g for 3% commercial and 1% crude enzymes respectively. It is worth identifying that this scheme (thermal with 1% H_2SO_4 pre to 1% crude enzyme) transcends the other scheme for the coast value factor which let crude enzyme surpass commercial enzyme. In later step, S. cerevisiae fermented wasted reducing sugars with concentration 51.75gl^{-1} produce bioethanol of 24.77mgg^{-1}. The efficiency of conversion process improved upon time intervals, it reached 93.88% after 7 days with ethanol yield 3.14%.

Key words: *Ulva fasciata; Cellulase; Biodiesel; Bioethanol; Enzymatic pretreatment.*

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INTRODUCTION

Marine floras, such as bacteria, actinomycetes, cyanobacteria, fungi, yeasts, microalgae, seaweeds, mangroves and other halophytes are extremely important oceanic resource, constituting over 90% of the oceanic biomass. By the biological processes, the marine biomass has been converted to usable energy forms[21,43]. Recently, marine macroalgal species have gained considerable global attention as source of third generation biofuels[22,37]. The major advantages offered by seaweeds 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 depolymerization as it does not contain lignin in their cell wall[20]. The green seaweed *Ulva* which proliferates fast and occurs abundantly worldwide was used as a feed-stock for production of ethanol following enzymatic hydrolysis[29]. Water extract of this alga contains carbohydrates, sulfate, uronic acid, proteins etc[50]. Also, *Ulva* was proved to be a suitable source for the biodiesel production[54]. The enzymatic hydrolysis of cellulosic materials involves synergistic actions of the three components of cellulase enzyme complex[1]. However, the enzymatic reaction is safe, less contaminating and seems to produce higher yield of the biofuel compared to the chemical reaction[29]. Even though these enzymes are produced by several such as; bacteria, fungi and actinomycetes, but fungi are known for secretion of cellulase in copious amounts. Fungi are the most widely studied for biological pretreatment since they can degrade cellulose more effectively and more specifically[1,26]. Among fungi *Aspergillus* and *Trichoderma* spp. are being exploited for commercial production of cellulases[17].

The purpose of this work was to compare the efficiency of the enzymatic pretreatment of *Ulva fasciata* using crude cellulase from local *Aspergillus niger* strain against commercial celullase (0.3 U/mg, Sigma Aldrich, USA). In addition the investigation of the effect of this biological cell wall pretreatment on biodiesel production will be conducted for improving the biodiesel and bioethanol production.

MATERIALS AND METHODS

Collection of *Ulva fasciata*

Ulva fasciata was collected during May (2014) from the beach of the touristic site "Bardiss" located at the extremely western head of Abu Qir Bay at longitudes 30° 04` 18.732``E and latitudes 31° 18` 36.049`` N; Mediterranean Sea and identified as a green algae by helping Prof. Nihal Galal Shams El-Din. It was dried, grinded and then used as substrate for natural lipid through microbial hydrolysis process.

The green alga *Ulva fasciata* Delile belongs to the family: Ulvaceae, genus: *Ulva*, class: Ulvaphyceae. *Ulva fasciata* consists of wide blades, 10-15 cm wide at base, 2.5 cm wide at tip. The upper portion is divided deeply into many ribbons like segments, up to 1 meter long.

Indicator fungi & commercial cellulose

Five marine-derived fungi were kindly provided by Dr Hassan A.H. Ibrahim; Marine Microbiology Dep., National Institute of Oceanography and Fisheries, Alexandria, Egypt. These fungi were; *Aspergillus niger, Aspergillus flavus, Penicillium oryzae, Penicillium chrysogenum,* and *Rhizopus oryzae*. Commercial cellulase was purchased from Sigma Aldrich, USA, with activity 0.3U/mg.

Screening of marine-derived fungi for producing cellulase

The CMC broth medium was used for detecting cellulase production, containing cellulose instead of sole carbon source. This medium was inoculated with different isolates of fungi and incubated at 28ºC for 7 days. Furthermore, the well-cut diffusion technique was used to test the ability of the fungal isolates to produce cellulase[36].

Cellulase activity was measured by cellulose hydrolysis test (carboxy methyl cellulase activity) according to Mandels and Weber (1969)[35], while protein content was estimated according to Lowry's method[58] for determination of specific activity as Unit/mg protein.

After selection of Fungi which produce enzyme of highest activity and specific activity (*A. niger*), isolates of *A. niger* were inoculated into the conical flasks. The flasks were incubated at 28°C on shaking incubator at 120 rpm for incubation period adjusted at time intervals, 3, 5, and 7 days.

Partial purification of crude enzym

Partial Purification was achieved by Salting - out with ammonium sulphate and by fractional precipitation with acetone. The crude culture supernatant obtained from 7 days incubation with CMC broth medium was precipitated by each precipitant. Thereafter, the precipitate was dissolved in a certain amount of distilled water and dialyzed against distilled water in a refrigerator for one day. After dialysis, the protein content and enzyme activity were determined.

Pretreatment of *Ulva fasciata* cell wall

Using crude *A. niger* cellulase

Dried algal biomass (5 g) was hydrolyzed with 100 ml of crude cellulase Enzyme in 250 ml Erlenmeyer flask and incubated for 36 h at 45^{o}C on an orbital shaking incubator with a speed of 150 rpm, then followed by rinsing of biomass with distilled water [34].

Using commercial *A. niger* cellulase

The algal biomass was disrupted with commercial CMCase (0.3U/mg, EC 3.2.1.4, Sigma, Aldrich, USA). Dried algal biomass (5g) was hydrolyzed with different concentration of cellulase, 1, 3 and 5% wt/v in a fix volume (100 ml) of sodium acetate buffer (pH 4.8) and incubated for 36 h at 45^{o}C on an orbital shaking incubator at 150 rpm[34].

Using different pre-enzymatic treatments

Both thermal pretreatment using autoclave (Sturdy Automatic Autoclave: SA-260FA) only and thermal with 1% H_2SO_4 pretreatments using autoclave for 60 min were investigated comparatively to the absence of pre -enzymatic treatment crude *A. niger* cellulose and commercial *A. niger* cellulases.

The residual biomass from hydrolysis with different treatments (crude and commercial *A. niger* cellulase enzymes) was rinsed with distilled water and dried in an oven at 60°C.

Extraction and purification of total lipids

The dried pretreated algal biomass (less than 0.16 mm particle size) was weighted (1 g ± 0.001). A total of 25 ml solvent was added in a predetermined sequence for oil extraction according to Folch *et al*[11]., with some modifications[49]. The total extracted lipid yield (%w/w) was then quantified gravimetrically by calculating difference between the weight of tube with the residue and the weight of the empty tube as in the following equation[15]

$$\text{extraction yield (\%)} = \frac{\text{Weight of lipid extracted (g)}}{\text{Weight of algal biomass (g)}} X\ 100$$

Determination of fatty acids

The extracted total lipid was reacted directly with a freshly prepared mixture of methanol, chloroform and HCl (10:1:1 v/v/v) at 90^{o}C for 120 min for esterification reaction[31]. FAMEs were then extracted using hexane/ chloroform (4:1, v/v), hexane layer with extracted FAMEs and evaporated till dryness, then FAMEs redissolved in 1 ml of hexane at time of measurement then characterized via gas liquid chromatographic analysis[8]. A gas chromatography (GC-QqQ/MS triple Quade) analysis system was an Agilent 7890A series GC system coupled with an Agilent 7000B QqQMS (Agilent Technologies Inc., USA) was run to identify the fatty acid fraction of the lipid extract.

Calculation of biodiesel properties from fatty acid profiles

The physical properties of biodiesel products were calculated to investigate the quality of the biodiesel extracted from *U. fasciata*. The fatty acids methyl ester profiles were used to estimate the Degree of Unsaturation (DU), Long Chain Saturation Factor (LCSF), Iodine Value (IV), Saponification Value (SV), the Cetane Number (CN), kinematic viscosity (υ), density (ρ), the Higher Heating Value (HHV), C18:3% (wt%) and weight percent of fatty acids with double bond higher than 4 Db≥4

(wt%) according to Islam *et al*[18]., and Saravanan *et al*[47].

Fermentation process of *U. faciata* reducing sugars for bioethanol production

Preparation of sugar solution

The resulted waste from the saccharification process by *A. niger* and other pretreatments were collected and then concentrated by rotary evaporator to obtain final reducing sugar concentration (40 mgm^{-1}).

Estimation of reducing sugars

The amount of reducing sugars was estimated by dinitrosalicylic acid (DNSA) method[38]. The absorbance in terms of optical density of the standards and sample were measured at λ510 nm using 6800UV/VIS Spectrophotometer (JENWAY). Glucose standards concentration ranged from 200-1000µg.

Bioethanol production by *Saccharomyces cerevisiae*

The commercial live bakers' yeast (*S. cerevisiae*) was obtained, in the forms of active dry yeast, from the Egyptian Company for Advanced Foodstuff Industries. Active dry yeast (5 g) was dispersed in 99 ml of 0.1% sterile peptone water pre-warmed at 34°C for 20 min. The yeast solution contained 2×10^7 viable cells per ml and used as inoculum for fermentation medium[61].

The production of bioethanol from sugar solution obtained from saccharification process was screening by using *S. cerevisiae* by following the method, 1 ml of the yeast isolates were inoculated into fermentation medium which contains; 10 gl^{-1} yeast extract, 20 gl^{-1} peptone and 20 gl^{-1} glucose. The fermentation was carried out in 250 ml Erlenmeyer flasks using 100 ml of sterilized media at pH 5.5. The flask was kept for fermentation at 30°C for 48 h under static condition.

Estimation of bioethanol

Quantitative estimation of ethanol was carried out by potassium dichromate method[6]. Ethanol standard was prepared by dissolving absolute ethanol 99.9% (HPLC grade) in water to get 20%. Standard curve with different concentrations of prepared ethanol was drawn. The optical density was measured at λ_{660} nm. Standard graph was used for the calculation of concentration of unknown samples[2].

RESULTS AND DISCUSSION

Biochemical composition of *U. fasciata*

Macroalgae are gaining some attention as a source of biomass for the production of biodiesel[53] and bioethanol[5]. Some algae were represented to have high contents of carbohydrate that can be used as substrate for bioethanol production[51]. They also contain a low concentration of lignin[62] or no lignin at all[13]. Therefore, marine algae can be converted economically sugars and then to bioethanol[7,19]. Also, they can be converted to biodiesel via transesterification of algal lipids[49].

The chemical analysis for algal species (*U. fasciata*) was carried out and the content the carbohydrates, lipids, proteins, and cellulose percentages were detected. The carbohydrate was 48.57 ± 0.50%, lipid content was (7.08 ±0.22%), total protein content was (20.51%) and cellulose content 11.9±0.1%. The high recorded carbohydrate content during current study is in a good agreement with the findings of Moustafa and Moustafa and Saeed[40], which were (45.45% and 48.91%) at Al-Muntazah and Ras ElTin, Alexandria respectively in *Ulva sp*. The measured cellulose is approximately in consistence with measurement of Trivedi *et al*[58]., with content of 15% in *U. fasciata*. These high contents of both carbohydrate and cellulose give the advantage of using *U. fasciata* as a promising substrate in biodiesel and bioethanol production.

Screening some marine-derived fungi for secreting hydrolytic enzymes

The studied five marine-derived fungal strains (*A. niger*, *A. flavus*, *P. oryzae*, *P. chrysogenum*, and *R. oryzae*) showed great variability in production of cellulase enzyme and its specific activity as presented in Table:1.

It is shown that the cellulase activity based on clearance zone (mm) ranged from 15-25 mm. recalling that the highest fungal enzymatic activity for cellulase was (25 mm)

produced by *A. niger*, followed by *P. chrysogenum* (20 mm).

Luckily, the highest recorded cellulase activity, specific cellulase activity and protein content (extra cellular protein) were also obtained by *A. niger;* 3.24 Uml^{-1}, 0.121 U/mg protein and 18.6 $mgml^{-1}$, respectively. The data in the table also showed that, *P. chrysogenum* exhibited valuable activity (2.25 Uml^{-1}), specific activity (17.58 Umg^{-1} protein) and protein content (0.128 $mgml^{-1}$), followed by *R. oryzae*, while *A. flavus* had the lowest cellulase activity. Therefore, these results supported the hydrolytic activity of fungal strain; *A. niger* for further investigation.

Screening the optimum incubation period of *A. niger*

The incubation period of *A. niger* producing the maximum enzyme activity was investigated at four different time intervals and the results represented in Table 2. The obtained results confirmed that enzyme activity increased gradually from 3 to 7 days, then decreased after 10 days. The activity and specific activity of cellulase reached the maximum peaks after 7 days of incubation (2.91 Uml^{-1} and 24.87 Umg^{-1} protein) respectively, while the protein content ($mgml^{-1}$) was rather in the same range of the rest intervals.

Comparison between crude and semi-purified cellulases from *A. niger*

Data presented in Table 3 reveal that the crude cellulase showed the highest activity (2.5 Uml^{-1}) and specific activity (20.83 Uml^{-1}), while the cellulase activity by acetone (0.42Uml^{-1}) was effective than that obtained by precipitation with ammonium sulfate (0.14 Uml^{-1}).

Comparison between commercial and crude *A. niger* cellulases under different conditions

This experiment was carried out to compare the commercial and crude *A. niger* cellulase activity and glucose liberated after 36 h of incubation. The results (table 4) confirmed that the best conditions in which cellulase activity and glucose concentration were; untreated *U. fasciata* + 5% commercial enzyme (15.1 Uml^{-1}), yielding; 43.38 mg/ml, and 60 min autoclave + 1% H_2SO_4 + 3% commercial cellulase enzyme (9.3 Uml^{-1}), yielding; 44.31 $mgml^{-1}$.

Table 1: Screening five marine-derived fungi for secreting cellulase enzyme expressing in activity, specific activity and protein content

Fungus	Cellulase activity (Uml^{-1})	Protein content ($mgml^{-1}$)	Specific cellulase activity (Umg^{-1} protein)	Clearance zone (mm) on plates
Aspergillus niger	3.24	0.121	18.6	25
Aspergillus flavus	0.33	0.093	3.5	17
Penicillium oryzae	0.71	0.118	6.02	15
Penicillium chrysogenum	2.25	0.128	17.58	20
Rhizopus oryzae	1.92	0.113	16.99	19

Table 2: Cellulase from *A. niger* expressing in activity (U/ml), specific activity (U/mg protein) and protein content

Time (day)	Activity (Uml^{-1})	Protein ($mgml^{-1}$)	Specific activity (Umg^{-1} protein)	Clearance zone (mm)
3	1.35	0.113	13.64	15
5	1.72	0.117	16.08	21
7	2.91	0.118	24.87	25
10	1.46	0.115	15.55	20

Table 3: Activity, specific activity and protein concentration of crude and semi-purified cellulase from *A. niger*

Treatment	Activity (Uml^{-1})	Protein ($mgml^{-1}$)	Specific activity (Uml^{-1})
Crude cellulase	2.5	0.120	20.83
Precipitation with ammonium sulfate	0.14	0.096	1.46
Precipitation with acetone	0.42	0.100	2.43

Acharya *et al*[1]., examined the effects of enzymatic hydrolysis on saw dust by using *A. niger* in optimizing enzyme production. They found that alkaline pretreated (2 N NaOH) saw dust at 9.6% concentration gave 0.1813 IU/mL cellulase activity. Also, Begum and Alimon[4] pretreated three lignocellulosic substrates viz. sugarcane bagasse, sawdust and water hyacinth with alkali and enzyme and studied their effect on bioconversion agricultural and industrial wastes to chemical feedstock. They also observed that the maximum degree of substrate conversion by *A. oryzae* ITCC (0.415%) and improved specific substrate consumption (0.99 g substrate/g dry biomass) was exhibited in sugarcane bagasse after alkali treatment at 96 h. They observed that the alkali-treatment and enzyme-treatment, water hyacinth was the best for cellulase induction and showed maximum endoglucanase activity of 11.42 U/ml. Reducing sugar yield ranged from 1.12 mg/ml for enzyme treated sawdust at 48 h to 7.53 mg/ml for alkali treated sugarcane bagasse at 96 h. Alkali-treated sugarcane bagasse gave the highest saccharification rate of 9.03% after 96 h.

Trivedi *et al*[58]., investigated different cellulases for efficient saccharification, cellulase 22119 showed the highest conversion efficiency of biomass into reducing sugars than Viscozyme L, Cellulase 22086 and 22128. Pre-heat treatment of biomass in aqueous medium at 120°C for 1 h followed by incubation in 2% (v/v) enzyme for 36 h at 45°C gave a maximum yield of sugar $206.82 \pm 14.96\ mgg^{-1}$.

In recent study, Trivedi *et al*[59]., investigated cellulase produced from the marine fungus *Cladosporium sphaerospermum* using *U. fasciata*. They observed that the seaweed substrate, containing inoculated fungus with 60% moisture content, cultured at 25°C and pH 4 for four days, showed optimum enzyme production. The enzyme, assayed for carboxymethyl cellulase and filter paper assay, showed an activity of $10.20 \pm 0.40\ Ug^{-1}$ and $9.60 \pm 0.64\ Ug^{-1}$ on a dry weight basis, respectively. Further, ionic liquid tolerance of the enzyme was studied in the presence of 1-ethyl-3-methylimidazolium acetate, 1-butyl-3-methylimidazolium chloride, 1-butyl-3-methylimidazolium trifluoromethanesulfonate and 1-butyl-1-methylpyrrolidinium trifluromethanesulfonate. At 10% v/v concentration, the enzyme retained 72.17 to 85.04% activity in all the ionic liquids. The pre-incubation of enzyme in the same ionic liquids for 24 h, the activity got slightly enhanced and ranged between 73.80 and 93.70%. The hydrolysis of *U. fasciata* feedstock with enzyme ($10\ Ug^{-1}$) for 24 h at 40°C and pH 4 gave maximum yield of sugar $112 \pm 10\ mgg^{-1}$ dry weight. On fermentation, an ethanol yield of $0.47\ gg^{-1}$ reducing sugar was obtained, corresponding to 93.81% conversion efficiency.

Effect of different enzymatic treatments on total extracted lipids

Algal cell wall disruption is one of the most important challenges involved while processing this type of biomass. There are

several enzymes that can digest the cell wall and weaken this defense for the purpose of lipid extraction [30].

Enzymatic pretreatments were carried out using commercial and crude cellulase enzyme produced from *A.niger* to facilitate cell disruption and increase lipid extraction yield. Different commercial cellulase dosage of 1%, 3% and 5% (w/v) resulted into approximately very little increase in lipid yield of 4.22, 4.92 and 4.85%,corresponding to cellulase dosage respectively, it is worth noted that this enzymatic pretreatment produced lipid in about two folds lipid yield of the untreated sample (1.82%). It is well identified[9,12] that Ulvophyceae cell wall is made up of polysaccharide mainly comprising cellulose that can be hydrolyzed by the enzyme, cellulase. So that the increase in extracted lipid as the cellulase concentration increase till 3%, can be explained on the bases that cellulase enzyme degraded the cell wall and hence enable the intracellular lipids to be extracted easily after degradation[56]. Moreover the cause why the further increasing of enzyme concentration (>3%) has no more effect confirm complete cell wall degradation and identify this concentration as an optimum enzyme concentration for *U. fasciata* biological cell wall eruptions.

The other pretreatment tools were to apply only thermal pretreatment or thermal with 1% H_2SO_4 that employed to increase cellulose degradation before biological treatment with 3% commercial enzyme resulted into lipid yield of 4.17 and 7.08%, respectively. It was clear that the lipid extraction increased with thermal with acid pretreatment.

Crude enzyme treatment instead of commercial one resulted in lipid extraction yield of 3.41%. While in case of thermal with 1% H_2SO_4 pre-treatment followed by crude enzyme the extracted lipid was 6.42% which means doubling the amount extracted. On the other hand, the lipid yield in case of only thermal pre enzymatic treatment showed no effect and the extracted lipid was 3.24% approximately similar to that without pretreatments.

These results were in a good agreement with the findings of Fu *et al*[12]., who reported that cellulase enzyme hydrolyses of *Chlorella sp.* increased the lipid recovery to 56% compared to 32% prior to treatment. Also the study of Liu *et al*[33]., was in a good consistence with our results since the using cellulase assisted enzyme hydrolysis increases lipid recovery from *Chlorella vulgaris* by more than two folds and final outcome was 65%.

Characterization of algal biodiesel

The TFA, SFA, MUFA, and PUFA contents are shown in Table 4. It was observed that the cumulative TFAs were progressively improved from 1141.16 µg/g dried algae to 3011.71 µg/g by pretreating algal biomass with 1% commercial cellulase. From the data, One can noted that increasing the enzyme concentration to 3 and 5% had no significant impact on the TFAs, it increase to 3193.72 and 3274.36 $\mu g g^{-1}$, respectively. This could explained on the cause that greater enzyme concentration allows for rapid cell wall degradation by increasing the enzyme to biomass content[32]. Only thermal Pre- enzymatic treatment wasn't effective, while thermal treatment with 1% H_2SO_4 increased yield to 3605.44$\mu g g^{-1}$. Also, TFAs were progressively improved to 2321.68µg/g by treating algal biomass with Crude cellulase enzyme and to 2712.70 and 3528.11 $\mu g g^{-1}$ by treating algal biomass with only thermal and thermal with 1% H_2SO_4, respectively. This could be attributed to the fact that high thermal stress causes the cell walls to rapture which enhances the release of the intracellular lipids[16]. Also, the thermal treatment with 1% H_2SO_4 causes algal cell wall lysed leading to increase the porosity of cell wall, and intracellular lipid got released[55].

Table 4: Commercial and crude *A. niger* cellulase activity, extracted lipids and glucose concentration after 36 h of incubation with treated and untreated *U. fasciata*

Cellulase type	Condition	Activity (Uml^{-1})	Conc. of produced glucose ($mgml^{-1}$)	Lipid extracted %	Fatty acids group			
					ΣSUFA	ΣMUFA	ΣPUFA	ΣFA
Commercial	Untreated *U. fasciata* + 1% enzyme	3.2	23.64	4.22	2354.23	575.02	82.46	3011.71
	Untreated *U. fasciata* + 3% enzyme	9.1	31.08	4.92	2476.30	585.22	132.21	3193.72
	Untreated *U. fasciata* + 5% enzyme	15.1	43.38	4.85	2619.74	553.85	100.77	3274.36
	60 min autoclave + 3% enzyme	9.2	34.46	4.17	2401.39	465.31	52.45	2919.15
	Autoclave for 60 min + 1% H_2SO_4 + 3% enzyme	9.3	44.31	7.08	3259.93	273.08	72.43	3605.44
Crude	Untreated *U. fasciata* + 1% enzyme	2.5	3.22	3.41	1972.52	310.93	38.22	2321.68
	Autoclave for 60 min + 1% enzyme	2.5	2.51	3.24	2089.51	538.04	85.16	2712.70
	Autoclave for 60 min + 1% H_2SO_4 + 1% enzyme	2.5	6.19	6.42	3282.38	132.51	113.22	3528.11

Saturated fatty acids (ΣSFAs) were improved progressively from 2354.23 to 2619.74 μgg^{-1} by increasing enzymatic concentration from 1 to 5%, while they increased to 3259.93 μgg^{-1} by thermal with 1% H_2SO_4 pre 3% enzyme treatment. On the other hand in case of using crude enzyme the (ΣSFAs) 1972.52 which increased to 3282.38 μgg^{-1} when pretreated with-heated1% H_2SO_4.

Among treatments, maximum ΣMUFA of 585.22μgg^{-1} produced with using 3% commercial enzyme, while minimum values of 132.51 and 273.08 µg/g were obtained using thermal and thermal with 1% H_2SO_4 pre enzymatic treatment with crude and 3% commercial enzymes respectively. Maximum ΣPUFA of 132.21 μgg^{-1} was obtained by using 3% commercial enzyme, while minimum value of 38.22 μgg^{-1} was obtained using 1% crude enzyme.

Palmitic acid C16:0 was the dominant fatty acid in all treatments, except in case of thermal with 1% H_2SO_4 pre enzymatic treatment with crude and 3% commercial enzymes, Behenic acid C22:0 was the dominant fatty acid. Palmitic acid recorded the highest concentration (1163.13 μgg^{-1}) (36.42%) at treatment with 3% commercial enzymes, while Behenic acid highest concentration was 1620.20 (45.92%) μgg^{-1} in case of using thermal with 1% H_2SO_4 pre crude enzyme treatment.

The second dominant saturated fatty acid was behenic acid, followed by Stearic acid (C14:0) in all treatments, except in case of thermal with 1% H_2SO_4 pre enzymatic treatment with crude and 3% commercial

enzymes, palmitic acid was the second dominant saturated fatty acid followed by Stearic acid. Among MUFAs, Oleic acid (C18:1c) was the dominant in all treatments, except in case of thermal with 1% H_2SO_4 pre enzymatic treatment with crude and 3% commercial enzymes, while cis-10-Heptadecenoic acid (C17:1) was the dominant in case of thermal with 1% H_2SO_4 pre 3% commercial enzyme treatment with concentration of 182.47 μgg^{-1} (5.06%) and Palmitoleic acid (C16:1) was the dominant in case of thermal with 1% H_2SO_4 pre 1% crude enzyme treatment with concentration of 57.37 μgg^{-1} (1.63%).

Based on the fact that Good biodiesel is the one which comprises high content of saturated long chain fatty acids. From this study we can conclude that using crude enzyme surpass using commercial one not only for the cause of economic value but also for better produced biodiesl quality. Moreover the best pretreatment was to use the thermal with 1% H_2SO_4 pre 1% crude enzyme treatment.

Among PUFAs, α- linolenic acid (C18:3-α) was dominant in case of thermal with 1% H_2SO_4 pre 1%crude enzyme treatment and 5% commercial enzyme with concentrations of 73.09 and 64.43 μgg^{-1}, respectively, while cis-4,7,10,13,16,19-Docosahexaenoic acid (C22:6) was 33.04 and 29.59 μgg^{-1} at 1 and 3% commercial enzymes, respectively.

Suganya[53] found that from the analysis off fatty acid profile of *C. peltata*, showed higher saturated fatty acids content 67.43% compared to the unsaturated fatty acids (32.57%). Among the saturated fatty acids, palmitic acid was determined as a prominent fatty acid (36.82%) in algal oil. Also the study of Satpati and Pal[48] on macroalgae *Ulva rigide* found that palmitic acid was the dominant fatty acid *(*12.2%*)*, Oleic acid (18:1) was the dominant MUFA (4.2%), where Linolenic acid (C18:3) was found to be the dominant PUFA (10.3%).

Physicochemical characterization of *U. fasciata* biodiesel

The results of the physicochemical properties of produced biodiesel are shown in Table 5 , including the degree of saturation of fatty acids methyl ester (DU), Long Chain Saturation Factor (LCSF), Iodine Value (IV), Saponification Value (SV), the Cetane Number (CN), Kinematic viscosity (υ), density (ρ), the Higher Heating Value (HHV), C18:3 (wt%) and double bond Db ≥4 (wt%).

It was observed that the biodiesel properties play a significant role in the combustion process and delay period, which in turn affect the engine performance[52]. The Cetane Number (CN) of the fuel is such an important parameter which is responsible for the delay period. A fuel of higher cetane number gives lower delay period and provides smoother engine operation. Both the British biodiesel standard EN (14214) and the American biodiesel standard ASTM D6751-02 stated that a Cetane number CN should be (≥51 and ≥47), respectively. In fact, biodiesel has a higher CN than petro-diesel because of its higher oxygen content[52]. In the present study, the biodiesel has a CN (based on molecular weight and degree of unsaturation) that exceeded 68 and its value varied between treatments. The highest CN was recorded in case of thermal with 1% H_2SO_4 pre enzymatic treatment with crude enzyme (81.99) and 3% commercial enzyme (82.44), whereas the lowest CN (75.52) was recorded in case of treatment with 3% commercial cellulose. Factors that affect the CN in the biodiesel are the number of carbon atoms of the original fatty acids, the number of double bonds, the highest amounts of SFA in addition to the type of feed stock[14,57].

Shaltout and Shams El-Din[49] recorded that the CN of biodiesel extracted from *Ulva fasciata* collected during summer from Abu Qir and Eastern harbor was 68.96 and 72.62, respectively, while it was 72.36 and 72.15 during winter, respectively, and 59.17 and 62.52, respectively during spring.

The degree of saturation plays a significant role in fuel properties. It has been suggested that, the higher the degree of unsaturation of the FAMEs of a biodiesel, the higher the tendency of the biodiesel to oxidize[21]. It also influences largely the kinematic viscosity (υ) and density (ρ)[39]. The shorter and more unsaturated chains increase the viscosity and flow characteristics at low temperatures, which are undesirable

characteristics of fuels. To obtain a biodiesel with appropriate quality characteristics, an appropriate ratio between saturated and unsaturated fatty acids should be maintained[25]. In the present study, the percentage of saturated FAs was almost high, against MUFA% and PUFA%.

The maximum degree of unsaturation was recorded in case of using 3% commercial CMCase pretreatment (26.60), whereas, the minimum was recorded in case of using thermal with 1% H_2SO_4 pre crude enzyme treatment (10.17). Islam *et al*[18]., found that DU of *Picochlorum* sp. and *Amphidinium* sp. was 136 and 87 respectively, which were higher than DU at all treatments.

Kinematic viscosity is a measure of the internal friction or resistance of oil to flow and is the most important physical property of biodiesel since it affects the operation of fuel injection equipment, particularly at low temperatures when the increase in viscosity affects the fluidity of the fuel. High viscosity leads to poorer atomization of the fuel spray and less accurate operation of the fuel injectors[52]. Kinematic viscosity limits are set by international biodiesel standards to be in the ranges of 1.9-6.0 mm^2s^{-1} and 3.5-5.0 mm^2s^{-1} as per ASTM6751-02 and EN 14214, respectively[18]. In the present study, the produced biodiesel after all treatments were in the prescribed viscosity range with 5.5-5.79 mm^2s^{-1} and meet ASTM6751-02 standard except that of thermal with 1% H_2SO_4 pre crude and 3% commercial enzymes that were 6.19 and 6.20 mm^2s^{-1}, respectively. Suganya[53] found that *E. compressa* algal biodiesel kinematic viscosity was found to be 4.35 mm^2s^{-1}. Islam *et al.* (2013)[21] recorded in some microalgal species viscosity range between 3.44 and 4.20 mm^2s^{-1}, whereas Sivaramakrishnan and Ravikumar[52] recorded viscosity values of biodiesel between 3.6 and 5.7 mm^2s^{-1}.

Density (ρ) is another important parameter for biodiesel quality because the fuel injection system supplies fuel by volume not by mass which means denser biodiesel will be injected with greater mass into the combustion chamber, consequently affecting the stoicheometric ratio of air and fuel[42]. For density, a standard value has been set at 0.86-0.90 gcm^{-3} according to EN 14214. Our results are consistent with the standard value, where the density of produced biodiesel in the collected algae was 0.87 gcm^{-3}. In a good agreement of our results, Suganya[53] found that the density of *E. compressa* biodiesel was 0.88 gcm^{-3}, while Wagutu *et al*[60]., found that the densities of *Calodendrum capense* oil, *Croton megalocarpus* oil, *Jatropha curcas* oil and *Cocos nucifera* oil were 0.908, 0.918, 0.939 and 0.911 g cm^{-3}, respectively which were slightly higher than the density of *Ulva fasciata* biodiesel with different treatments.

The Heat of combustion refers to the measure of energy content in the fuel[52]. Heating value of fuels is an important measure of its releasing energy for producing work. According to Sivaramakrishnan and Ravikumar[52], the lower heating value of biodiesel is attributed to the decrease in engine power and the energy content of oils which depends on the place where they are grown, the season, the composition and other factors. In fact, the HHV values in the present study (39.86-40.39 $MJkg^{-1}$) were closed to that of biodiesel blend Jatropha (39.17- 41.52 $MJkg^{-1}$), blend Karanja (35.9-41.5 MJ kg^{-1}) fuel and the values of some macroalgae (39.41-42.98 $MJkg^{-1}$)[49]. Ramirez-Verduzco *et al*[45]., found that the range of regular biodiesel was between 39.8 and 40.4 $MJkg^{-1}$, which is normally 10 to 12% less than obtained for petroleum-derived diesel (46 $MJkg^{-1}$).

The Iodine Index (IV) refers to the tendency of biodiesel to react with oxygen at room temperature. The low iodine index indicates less susceptibility to oxidation by oxygen[10], which is desirable for biodiesel. According to the biodiesel standard EN (14214), iodine value is ≤120 gI2100g^{-1}fat. In the present study, all IV values are within the limits of the standards. It ranged between minimum 12.08 g$I^2$100g^{-1}fat in case of using thermal with 1% H_2SO_4 pre 3% commercial enzyme to maximum 28.17 gI2100g^{-1} fat for using 3% commercial enzyme. In agreement with our results, Nascimento *et al*[41]., conducted that the IV of *Chlamydomonas* sp. and *Scenedesmus obliquus* were 26 and 34 g $I_2$100 g^{-1} respectively, whereas Ramachandran[44] mentioned that, IV of oil extracted from *Adenanthera pavonina* was 89.1.

Regarding C18:3 wt% and the weight percentage of fatty acid esters containing number of double bonds higher than 4, they are of the most important property that govern the biodiesel stability. A limit of 12 wt% for this FAME has been set in the European B100 biodiesel standard (EN 14214), which also limits the amount of FAMEs with four or more double bonds to 1 wt%[3]. All treatments in this study gave C18:3 wt% much lower than the EN14214 threshold. The minimum value (0.23%) was achieved from thermal 3% commercial enzyme pretreatment and maximum value (1.03%) was from treatment with 3% commercial enzyme. Whereas, the values of wt% of ≥ four double bonds complied with that of biodiesel standard EN (14214) (≤1), except treatments with 1 and 3% commercial enzymes and thermal pre crude and 3% commercial enzymes. Minimum recorded value of wt% of ≥ 4 double bond (0.38%) was in case of using thermal with 1% H_2SO_4 pre 3% commercial enzyme, while maximum value (1.46%) in case of using 1% commercial enzyme.

Presently, commercial enzymatic cell wall degradation is not widely practiced in industry because cell lysing enzymes have traditionally been cost prohibitive. Though cost intensive, enzyme degradation is important because the rigid cell wall of algal cell is resistant to mechanical methods that will require excess energy usage and multiple passes through disruption equipment. Therefore, the use of enzymes to degrade algal cell walls may be advantageous as compared to other methods[30]. In the present study, we overcome the cost factor by using crude cellulase enzyme from local marine isolate of *A. niger* which is cheap and improve the quantity and quality of the produced biodiesel. Moreover, an alga (*U. fasciata*) does not have lignin in its cell wall, which is a major advantage to perform enzymatic hydrolysis of its polysaccharide components[46].

Bioethanol production

To achieve the fermentation process, the commercial yeast (*S. cerevisiae*) was activated upon yeast extract peptone dextrose agar medium (YEPD). Its ability to ferment glucose in reducing sugars was then examined. From results shown in Table 6, *S. cerevisiae* fermented glucose with concentration 51.75 gl^{-1} and produced 24.77 mgg^{-1} of bioethanol. The efficiency of conversion process improved upon time intervals, it reached 93.88% after 7 days with ethanol yield (3.14%).

Several investigations were carried out on the fermentation of seaweeds reducing sugar involving that obtained from *U. fasciata*. The fermentation efficiencies of reducing sugars into ethanol reported for different macroalgal feedstock. However, the fermentation efficiency obtained in this study was found to be comparatively lower than those values reported by previous work[23,24,34].

The obtained bioethanol from *U. lactuca* biomass using acidic + enzymatic pretreatment by Kim *et al*[24]., releasing 0.194 gg^{-1} sugar. While Trivedi *et al*[58]., produced bioethanol from *U. fasciata* biomass pretreated with hot buffer + enzyme through the fermentation of hydrolysate with yield of 0.45 gg^{-1} reducing sugar accounting for 88.2% conversion efficiency. These values are substantially higher than those of reported so far for both agarophytes and carrageenophytes.

Recently, Korzen *et al*[26]., conducted that sonication provided a faster way for the simultaneous release of glucose from *Ulva rigida* and its conversion into bioethanol. Within 3 h, 196 ± 2.5 mg glucose per gram of dry weight of biomass that converted to 333.3 ± 4.7 mg bioethanol per gram of glucose. In addition to being fast, the process was devoid of any chemical pretreatment and involves only a single stage of sonication for the release of glucose from algae by the action of enzymes and also for the simultaneous fermentation of glucose to ethanol using *S. cerevisiae*.

Khan and Hussain[23] produced bioethanol from the marine green alga *U. fasciata* from the Karachi coast fermented by *S. cerevisiae*. The carbohydrates was extracted by distilled water and subjected to different biochemical tests. It was hydrolyzed by various methods including acidic and basic as well as by using ammonia, hydrogen peroxide and mixture of organic solvents in the presence of $AlCl_3$. The comparative study of these methods revealed their efficiency order as NaOH (10%)/heating (method-2) > NaOH (10%)/H_2O_2 in microwave oven (method-5) > ammonia (50%)/reflux (method- 6).

Table 5: properties of biodiesel produced from transestification of *U. faciata* fatty acids

Condition	**DU**	**LCSF**	**IV ($gI2100g^{-1}fat$)**	**SV ($mgKOHg^{-1}$)**	**CN**	**TFA wt (µg/g)**	**SFAs (%)**	**MUFA (%)**	**PUFA (%)**	**Kinematic viscosity (υ) ($mm^2\ s^{-1}$)**	**Density (ρ) ($g\ cm^{-3}$)**	**HHV (MJ kg−1)**	**C18:3 (wt%)**	**Db ≥ 4 (wt%)**
Biodiesel Standard EN (14214)	-	-	≤120	-	≥51	-	-	-	-	3.5–5.0	0.86–0.9	NA	≤12	≤1
Biodiesel Standard ASTM D6751−02	-	-	NA	-	≥47	-	-	-	-	1.9–6.0	NA	NA	-	-
min/max	max	max	max	max	min	min	max	Max	max	max	max	min	max	max
Threshold value	-	-	120	-	47	-	-	-	-	-	0.9	-	12	1
Treatments:														
Control	17.07	23.23	17.75	200.64	73.33	1141.16	85.70	11.53	2.77	4.69	0.87	39.86	0.70	0.88
1% commercial E	24.57	59.26	25.69	188.32	77.18	3011.71	19.09	19.09	2.74	5.59	0.87	39.96	0.79	1.46
3% commercial E	26.60	54.77	28.17	190.20	76.52	3193.72	18.32	18.32	4.14	5.50	0.88	40.35	1.03	1.33
5% commercial E	23.07	62.08	22.99	188.17	77.09	3274.36	16.91	16.91	3.08	5.62	0.87	39.96	2.09	0.50
Autoclave 60min +3% commercial E	19.53	60.69	19.87	189.35	77.79	2919.15	15.94	15.94	1.80	5.58	0.87	39.90	0.24	1.09
Autoclave 60min + 1% $H_2SO_4^+$ 3% commercial E	11.59	84.30	12.08	183.39	82.44	3605.44	7.57	7.57	2.01	6.20	0.87	40.39	0.48	0.38
1% crude E	16.69	70.73	16.43	187.76	79.02	2321.68	13.39	13.39	1.65	5.79	0.87	39.90	0.35	0.71
Autoclave 60min + 1% crude E	26.11	61.87	27.15	187.95	77.72	2712.70	19.83	19.83	3.14	5.68	0.87	40.29	0.23	1.43
Autoclave 60min + 1% H_2SO_4 +1% crude E	10.17	86.65	12.16	183.27	81.99	3528.11	3.76	3.76	3.21	6.19	0.87	40.12	2.18	0.51

***CN*1: Cetane number 58; *CN*2: Cetane number 53; Db: Double bond; A: entirely within both biodiesel standards (EN 14214; ASTM D6751-02)59 except the number of double bond ≥ 4; B: Within biodiesel standard ASTM D6751-02 59; C: not compliant with any of the two biodiesel standards; a: Oxidation stability was not considered for PROMETHEE analysis.**

Table 6: Bioethanol production from reducing sugars by *S. cerevisiae* obtained from treatment of *U. fasciata* during the biodiesel production steps

Incubation period (day)	Theoretical yield (g)	Ethanol yield (g)	Ethanol yield (%)	Efficiency (%)	Total reducing sugar
5	26.39	-----	-----	-----	51.75
6	26.39	22.10	2.81	83.71	51.75
7	26.39	24.77	3.14	93.88	51.75

CONCLUSION

The present study provides valuable information about the enzymatic treatment of algal cell wall to extract lipids efficiently. It demonstrated the utilization of the green seaweed *U. fasciata* as potential marine feedstock for the biodiesel and then bioethanol production. The local isolate of *A. niger*, herein, was able to efficiently utilize algal wastes for production of cellulase enzymes. The results strongly supported recycling the *U. fasciata* reducing sugars resulting from lipid extraction step after be concentrated for the biothanol production. Finally, the biological pretreatment appears to be a promising technique and has very clear advantages, including no chemical requirement, low energy input, and mild environmental.

All these findings indicate that cellulase produced from a marine-derived *A. niger* can be employed for saccharification of algal polysaccharides for the production of renewable biofuels from marine macroalgal feedstock.

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