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Chemical pretreatment of *Ulva fasciata* cell wall for enhancing biodiesel yield and properties

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ABSTRACT

In the present study, the dried biomass of *Ulva faciata* was subjected to different chemical pretreatments at different incubation periods to find the most appropriate method of cell disruption that increase extracted lipid, fatty acids and improve the quality of the produced biodiesel with low cost. These pretreatments were as follows: acid (HCl and 1% H_2SO_4) for 1, 2, and 3 hrs, alkaline (NaOH, pH=12) for 1, 2, and 3hrs and osmotic shock (36% solution of NaCl) for 24, 48 and 72 hrs. The control was kept at optimum conditions without pretreatment: 60 min extraction time, 55^oC, shaking speed at 250 rpm, < 0.16 mm particle size with 25:1 v/w solvent to solid ratio, using chloroform: methanol: H_2O (2: 2: 1) solvent mixture. The results showed that all chemical pretreatments have a significant effect on total fatty acids yield, where ÓFA was 1148.94µgg^{-1} dried alga without any treatment, while using acid pretreatment with HCl (pH=2) for an optimum time period (1h) resulted in an about 2.9-fold increase in ÓFA yield, while it resulted in an about 2.4-fold increase in ÓFA yield when pretreated with 1% H_2SO_4 for an optimum time period (2h). On the other hand, alkaline pretreatment with NaOH (pH=12) for optimum incubation period (2h), resulted in an about 2.84-fold increase in ÓFA yield, while the osmotic shock of NaCl for 72h, gave the about 3-fold increase, but it needs longer incubation periods, which cause time-wasting. The produced biodiesel after all chemical pretreatments had a high quality and its properties complied with the ASTM D6751 and EN14214 standards, except in the case of 1% H_2SO_4 and NaOH; they were slightly higher than the limit of EN14214 standard. These results were confirmed statistically; where it was observed that all chemical pretreatments had a high significant effect on fatty acids yield and on all properties of the produced biodiesel.

INTRODUCTION

Increasing population and industrialization has created serious problems of energy requirement. The International Energy Agency (IEA) has reported in the reference scenario that world's primary energy need is projected to grow by 55% between 2005 and 2030 and there will be no oil reserves beyond 2050 **(Yen *et al.*, 2013)**. In addition to the energy crisis, using fossil fuels for energy generation also had a significant negative impact on the environment.

Combustion of fossil fuel leads to accumulation of greenhouse gases (GHG) in the atmosphere (carbon dioxide, nitrous oxide and methane), which result in Global Warming (GW)**(Martins *et al.*, 2019)**. Thus, a search for new alternative renewable forms of fuel has to be generated against fossil fuels to fulfill our needs.

Biodiesel is a kind of bioenergy as a substitute for conventional petrodiesel fuel **(Foster *et al.*, 2017)**. Its renewability and better combustion performance are the most attractive properties **(Jianbing *et al.*, 2006)**. However, environment-friendliness is the second shining point of biodiesel. The application of biodiesel can greatly reduce greenhouse gas emissions by 78%, hydrocarbon emissions by 56% **(Jianbing *et al.*, 2006)** and carcinogenic properties by 94% compared to conventional diesel fuel. Moreover, the presence of oxygen in biodiesel (~10%) improves combustion and reduces carbon monoxide and hydrocarbon emissions. Unlike petrodiesel, biodiesel is free of sulfur impurities and aromatics components **(Suganya, 2013)**. The third highlight of biodiesel is that it can be used in late-model diesel engines without any modifications **(Bilgin *et al.*, 2015)**.

One alternative to the conventional fuels is algae (third generation biodiesel) which are considered as one of the most efficient method. They represent an economical and environmentally sustainable, renewable source of biomass for the production of biofuels **(John and Anisha, 2011)** and they have many advantages over first and second generations. In addition, they are capable to fix CO_2 from the atmosphere, while simultaneously capturing solar energy with efficiency 10–50 times greater than terrestrial plants; a golden opportunity for carbon credit program **(Khan *et al.*, 2009)**.

Lipids in macroalgae are contained as small spherical droplets in the chloroplast adjacent to the plasma membrane, where they function in the structural support for the cell, the metabolic organelles in photosynthesis metabolism, growth process of the cell and in the synthesis of lipoprotein membranes contained in the chloroplast **(Clayton, 1992)**. As cell wall and membrane present in algae are formidable barriers to permeation by extraction solvents, cells have to be disrupted prior to extraction, which enhances lipid recovery **(Jin *et al.*, 2012**).

However, methods of cell wall disruption should be established to ensure a low operating cost, high product recovery, and high quality of the recovered lipids **(Zheng *et al.*, 2011)**. These pretreatment methods are significantly different from one another in terms of reaction conditions, efficiency and complexity **(Suganya, 2013)**. An efficient extraction requires that the solvent penetrates completely into the biomass and has a connection corresponding to the polarity of the target compound, thus physical contact between the lipid and the solvent is related to the successful extraction. Cell disruption methods are classified based on the manner in which they achieve algal cellular disintegration: mechanical or non-mechanical **(Halim *et al.*, 2012)**. Mechanical methods include ultrasonication, autoclave and microwave, while non-mechanical methods often involve chemical or biological (enzymatic) lysing of algal cells **(Halim *et al.*, 2012)**.

Among pretreatment methods, chemical pretreatments (acid, alkali and NaCl) are demonstrated to effectively modify the chemical structures of lignocellulose. Acidic pretreatment

by using protic acids such as sulfuric and hydrochloric acids was studied for a long time and is considered to be close to commercialization due to their efficiency in weakening the cell wall through the dissolution of cellulosic materials **(Schell *et al.,* 2003)**. Hence, lipid droplets inside the cell become more accessible and easier to extract with solvents such as chloroform and hexane **(Yoo *et al.,* 2015)**. Similarly, alkaline treatment using a strong base such as sodium hydroxide can also hydrolyze cellulose and delignify biomass **(Sindhu *et al.,* 2015)**. The commonly used alkalis for pretreatment are, calcium hydroxide ($Ca(OH)_2$), ammonium hydroxide (NH_4OH), potassium hydroxide (KOH) and sodium hydroxide (NaOH), which is highly used in the pretreatment of lignocelluloses. The controlling variables of these hydrolysis processes are acidity or alkalinity (pH or dosage of acid or base) and treatment duration. On the other hand, the osmotic pressure brought about by osmotic shock method (using saturated NaCl solution; 36%) is usually sufficient to burst algal cells **(Lee *et al.,* 2010)**.

In the previous work we have carried out optimization and kinetic studies of biodiesel production from *Ulva fasciata* **(Shaltout *et al.,* 2019)** for the sake of reducing biodiesel cost, increasing yield and enhancing the quality. The aim of the present work is to continue our investigation to achieve this goal by using different chemical pretreatment methods on the dried form of *Ulva faciata* for lipid recovery and to find the most appropriate method of cell disruption that increase extracted lipid and fatty acids.

Collection of *Ulva fasciata*:

The green alga *Ulva fasciata* Delile was collected during May (2014) from the beach of the touristic site "Bardiss" located at the extremely western head of Abu Qir Bay on the Egyptian Mediterranean Sea at longitudes 30^o 04` 18.732``E and latitudes $31^o$ 18` 36.049`` N. The species was identified according to **Aleem (1993)**. It belongs to the class Chlorophyceae, order Ulvales, family Ulvaceae.

Healthy specimens of the alga were handpicked whole, from their bases, scraping the substrata on which they were adhered, and then kept at 4 °C in icebox **(Hardisson *et al.* 1998)**. The collected alga was brought to the laboratory and was washed with tap water to separate epiphytes and impurities. Algal biomass was dried at room temperature (25^oC) in shade for about four days, then dried in a drying oven (Model: DX302) at 60°C, to remove the water content from the biomass as it will interfere with lipid extraction **(Jegathese and Farid, 2014)**. Thereafter, it was desiccated at room temperature (25^oC). The dried seaweed was hand crushed, grinded as coarse powder with a mixer grinder, and particle size distribution was determined using a sieve shaker (Cisa - BA 200N), following ASTM standards.

Chemical pretreatments of algal biomass:

Different chemical pretreatments of the grained alga (<0.16 mm) were done as follows: acid pretreatment, alkaline pretreatment and osmotic shock pretreatment with saturated NaCl solution. The control was kept at optimum conditions without pretreatments: 60 min extraction time, 55^oC, shaking speed at 250 rpm,< 0.16 mm particle size with 25:1 v/w solvent to solid (the grained alga) ratio, using chloroform: methanol: H_2O (2: 2: 1) solvent mixture)**(Shaltout *et al.,* 2019**).

Acid pretreatment of algal biomass:

This treatment was done by applying two different schemes by changing the used acid pH and the time intervals of the treatment as follows:

A) Adjustment at pH = 2:

The dried algal biomass was added to filtrated seawater in a ratio (1:10 w/v dry alga /seawater) into 100 ml screw top bottles, where the pH of the seawater was reduced to 2.0 by using 1M HCl, then the bottles were shaken by using orbital shaker (Unimax 1010) at 250 rpm. Shaking time optimization was adjusted at different time intervals, 1, 2, and 3 hrs. The liquid was adjusted to pH 7 by using 1M NaOH solution and the treated biomass was rinsed with distilled water till neutrality, then filtered and dried in an oven at 60°C **(Surendhiran and Vijay, 2014)**.

B) H_2SO_4 (1% v/v) lysis

The dried algal biomass was acidified by 1% (v/v) H_2SO_4 in a ratio (1:10 w/v dry alga /acid) into 100 ml screw top bottles, then shaken for 1 hr, using orbital shaker (Unimax 1010) at 250 rpm. The treatment was repeated at different shaking time; 2 and 3 hrs for the purpose of studying the optimum time of the treatment. The lysed alga was filtrated and brought to neutrality by washing; using distilled water then was dried in an oven at 60°C.

Alkaline pretreatment of algal biomass:

The dried algal biomass was added to filtrated sea water into 100 ml screw top bottles in a ratio (1:10 w/v dry alga / seawater). The pH was raised to 12.0 with 1N NaOH, and the solution was shaken for time intervals (1hr, 2 hrs, and 3hrs) by using orbital shaker (Unimax 1010) at 250 rpm in order to identify the optimum time for alkaline pretreatment. The liquid was adjusted to pH 7 by using 1M HCl solution and the treated algal biomass was then rinsed with distilled water till neutrality and then dried in an oven at 60°C.

Osmotic shock pretreatment with saturated (36%) NaCl solution:

The algal dried biomass was treated by osmotic shock with 36% NaCl solution in a ratio (1:15 w/v dry alga /solution) in an Erlenmeyer flask and kept at 250 rpm in an orbital shaker (Unimax 1010) for time intervals of (24, 48 and 72 hrs) in order to identify the optimum time for osmotic shock pretreatment. Thereafter, the biomass was washed with distilled water many times and was dried in an oven at 60°C for using in lipid extraction **(Surendhiran and Vijay, 2014)**.

Extraction and purification of total lipids:

The dried algal biomass (<0.16 mm particle size) was weighted (1 g ± 0.001) into 100 ml screw top bottles. A total of 25 ml solvent was added in a predetermined sequence according to **Folch *et al.* (1957)** with some modifications **(Shaltout and Shams El-Din, 2015)**, lipids were extracted from algae with 15 ml of chloroform/ methanol (2/1, v/v) by shaking at 250 rpm in orbital shaking incubator (model: JSSI-100T) and 55°C for 30 minutes. This was followed by the addition of a mixture of methanol/water (10 ml, 1:1, v/v) to achieve a final solvent mixture ratio of 2:2:1 for chloroform: methanol: water. The bottles were well caped and re-shacked for another 30 minutes.

Thereafter, the mixture was filtered by using Whatman filter paper No. 1 (Whatman, USA). The supernatants were collected and the residues were re-extracted with 5 ml chloroform **(Afify *et***

***al.*, 2010)**. The extract was shaken vigorously for one minute and allowed to undergo phase separation for 15 min in a separating funnel **(Doan et al., 2011)**. The lower organic phases were collected by using the separating funnel in pre-weighted 25 ml dried clean screw top tubes and the chloroform-methanol mixture was evaporated on a water bath until dryness leaving a residue at the bottom of the tube and then dried in an oven at 60^{o}C to constant weight. The total extracted lipid yield (%w/w) was then quantified gravimetrically by subtracting the weight of the empty tube from the weight of the tube and the residue as in the following equation:

$$\text{Total extracted lipid yield (\%)} = \frac{\text{weight of lipid extracted (g)}}{\text{weight of algal biomass (g)}} \times 100$$

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 **(Lewis *et al.*, 2000)**. The fatty acid methyl esters (FAMEs) were then extracted using hexane/ chloroform (4:1, v/v), where hexane layer with extracted FAMEs was evaporated till dryness, then FAMEs were re-dissolved in 1 ml of hexane at time of measurement then characterized via gas liquid chromatographic analysis **(Doan *et al.*, 2011)**. 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 concentration of fatty acid fractions of the lipid extract. Individual peaks of FAMEs were identified by the comparison of the retention times and equivalent chain length values, using the standard Supelco 37 component FAME Mix, (C4-C24) and quantified by area normalization.

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*.,(2013)** and **Saravanan and Chandrasekar (2013)**.

Morphological identification by using scanning electron microscope (SEM):

The analysis was carried out for a small amount of dried algal biomass without pretreatments as control and samples after each pretreatment to identify the changes in the surface morphology caused by each pretreatment by using Scanning Electron Microscope (Jeol JSM-5300 scanning electron microscope, Tokyo, Japan) operated between 15 and 20 KeV at magnification 10000 (a) and 20000 (b)**(Surendhiran and Vijay, 2014)**.

Statistical analysis:

The comparison study between the four pretreatments for *Ulva fasciata* before lipid extraction was conducted using different chemical pretreatments and control. Statistical analysis

was performed using analysis of variance (ANOVA) using **SAS v.9.1.3. (2007)** to determine means and least significant difference test for comparison between pretreatments ($\alpha = 0.01$).

RESULTS

The results showed that the contents of total fatty acids (TFAs), saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs) were improved after acid lysis of *U. fasciata* biomass, where the TFAs increased from 1148.94 µg g^{-1} (control) to a maximum of 3307.03 µg g^{-1} using HCl (pH=2) pretreatment for 1 hr. By pre-treating with 1% H_2SO_4 for 2 h, they attained the highest concentration (2773.43 µg g^{-1}) (Table1&Fig.1). TFAs were improved to 3145.51 µg g^{-1} by using NaOH (pH=12) for 1 h, increasing to its maximum concentration (3267.03µg/g) for 2 h, while there was no significant effect more than 2 h (Table1& Fig.1). By osmotic shock pretreatment of algal biomass, TFAs were improved from (control) to a maximum of 3411.72 µg g^{-1} using 36% NaCl pretreatment for 72 h (Table1 &Fig.1).

Considering saturated Fatty acids (ΣSFAs), they were improved from 979.43 µg g^{-1} (control) to 2375.08 µg g^{-1} after HCl (pH=2) pretreatment for 1 hr, while increasing incubation period (>1 hr) had no remarkably effect (Table 1). By pre-treating algal biomass with 1% H_2SO_4 for 2hrs, ΣSFAs was improved from (control) to a maximum of 2040.38 µg g^{-1}, while there was no considerable effect on SFAs yield by further increasing incubation period (Table 1). Similarly, ΣSFAs attained a maximum of 2461.11 µg g^{-1} by using NaOH for 1h.On the other hand, ΣSFAs was improved from (control) to a maximum of 2596.28 µg g^{-1} by using NaCl pretreatment for 72 h (Table 1).

As well, monounsaturated fatty acids (ΣMUFAs) increased from 136.98 µg g^{-1} (control) to 891.82 µg g^{-1} using HCl (pH=2) pretreatment for 1 hr, with no considerable improvement by increasing incubation period, while it increased to a maximum of 591.99 µg g^{-1} by pre-treating with 1% H_2SO_4 for 2 hrs. Using NaOH, ΣMUFAs improved from (Control) to a maximum of 854.61 µg g^{-1} for 3 h (Table1). By using NaCl pretreatment for 72 h, ΣMUFAs recovered from (Control) to a maximum of 767.86 µg g^{-1} (Table 1).

Regarding the polyunsaturated fatty acids ΣPUFAs, they increased from 32.53 µg g^{-1} (control) to 40.13 µg g^{-1} after 1 h HCl pretreatment, with further increase to 45.15 and 50.17 µg g^{-1}at higher time intervals of 2 and 3 hrs, respectively. On the other hand, they increased from (control) to 97.30 µg g^{-1} at 1 hr pretreatment and 141.10 µg g^{-1}at 2 hrs by using 1% H_2SO_4, with no significant effect by increasing time to 3 hrs (Table 1).

Table (1): Biodiesel properties of *U. fasciata* after pretreatment with HCl, 1% H_2SO_4, NaOH and 36% NaCl solution for different periods compared with ASTM D 6751-02 and EN 14214:

Biodiesel properties		DU	LCSF	IV ($gI_2100g^{-1}fat$)	SV (mg KOH g^{-1})	CN	TFA wt (µg/g)	SFA (µg/g)	MUFA (µg/g)	PUFA (µg/g)	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					max	max	min	max	max
Threshold value		-	-	120	-	47					-	0.9	-	12	1
Treatments															
Control		17.58	23.06	18.48	200.66	73.21	1148.94	979.43	136.98	32.53	4.68	0.87	39.85	0.68	0.98
HCl (pH=2)	**1h**	29.39	44.44	26.93	192.58	74.22	3307.03	2375.08	891.82	40.13	5.22	0.87	39.81	0.21	0.56
	2h	28.89	44.33	26.65	192.79	74.37	3231.55	2343.03	843.37	45.15	5.22	0.87	39.86	0.21	0.57
	3h	28.37	44.22	26.35	193.00	74.54	3156.08	2310.98	794.92	50.17	5.22	0.87	39.91	0.20	0.59
1% H_2SO_4	**1h**	25.42	54.30	30.75	191.01	76.26	2366.51	1862.14	407.07	97.30	5.42	0.87	39.81	0.43	3.14
	2h	31.52	55.95	34.86	189.17	76.77	2773.47	2040.38	591.99	141.10	5.57	0.88	40.61	0.36	2.28
	3h	26.65	59.48	29.55	189.14	77.48	2624.15	2038.78	471.44	113.92	5.62	0.88	40.40	0.38	2.01
NaOH (pH=12)	**1h**	23.74	44.57	23.95	194.23	74.83	3145.51	2461.11	622.10	62.30	5.17	0.87	39.75	0.37	1.16
	2h	29.53	46.21	29.56	192.53	74.56	3267.03	2392.59	784.25	90.19	5.25	0.87	39.95	0.72	1.16
	3h	39.03	36.06	35.92	194.52	71.78	2429.94	1528.46	854.61	46.87	5.02	0.87	39.82	0.40	0.68
Osmotic shock (36% NaCl solution)	**24h**	27.83	46.92	31.70	193.57	74.74	2089.92	1591.14	415.88	82.90	5.23	0.87	39.85	0.59	2.59
	48h	25.16	53.11	23.79	190.02	76.32	2996.56	2284.78	669.75	42.02	5.45	0.87	39.88	0.23	0.75
	72h	25.30	53.53	23.89	189.89	76.36	3411.72	2596.28	767.86	47.58	5.46	0.87	39.89	0.24	0.73

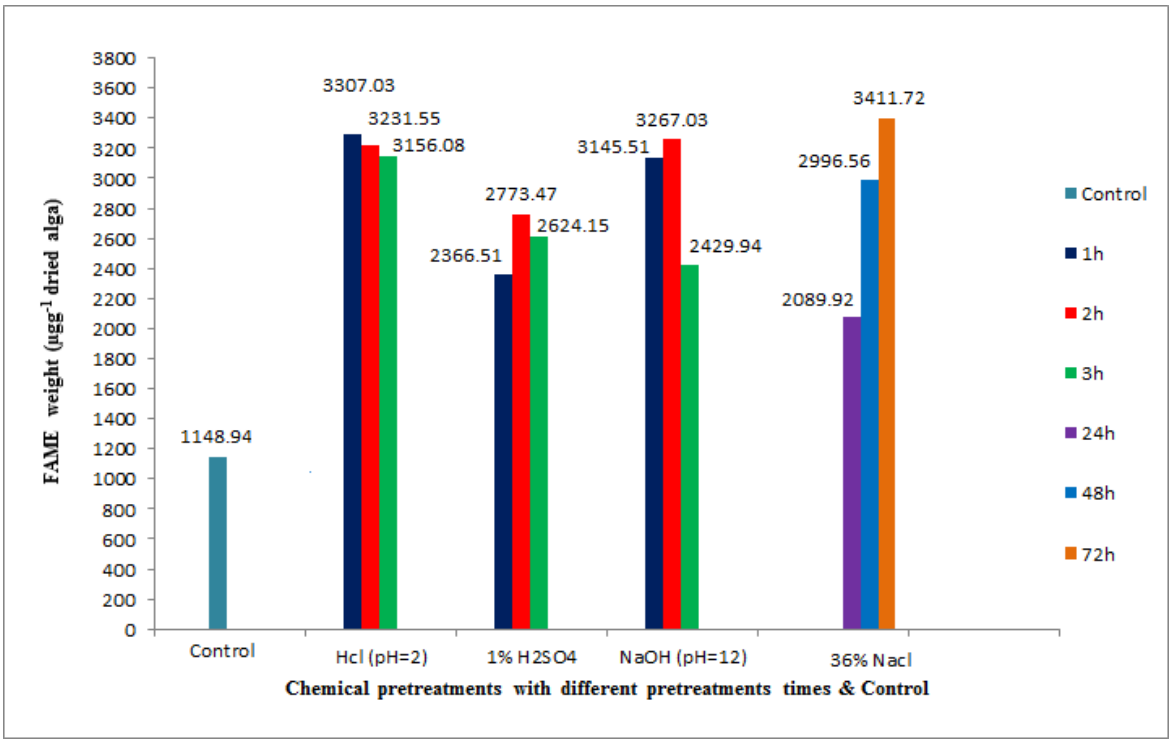

Figure (1): Effect of different chemical pretreatments of *U. fasciata*on total FAME weight (µgg^{-1}dried alga).

Similarly, ΣPUFAs increased from the (control) to 62.30 and 90.19 µgg^{-1} by using NaOH for 1h and 2h, respectively, while there was no significant effect on ΣPUFAs by further increasing the incubation period (Table 1). Furthermore ΣPUFAs upgraded from (control) to 82.90 µg g^{-1} by using NaCl pretreatment for 24 h, decreasing to 42.07 and 47.58 µg g^{-1},by increasing incubation period to 48 and 72 hrs, respectively (Table 1). It was observed that concentrations of SFAs> MUFAs >PUFAs at the four pretreatments at different times (Table 1).

The fatty acid profile investigated in Figure (2) showed that, among SFAs, palmitic acid C16:0 was the dominant at all incubation periods, its concentration was improved from 768.42 µg g^{-1} (control) to a maximum of 1247.54 and 917.99 after µg g^{-1}HCl (pH=2) for 1h (Fig.2A) and 1% H_2SO_4 for 1h (Fig.2B), respectively. By using NaOH pretreatment for 1 h, palmitic acid was improved from the control to its maximum concentration (1362.16 µg g^{-1}), but further increasing incubation period, resulted in decrease in its concentration (Fig. 2C).The same pattern was followed when applying the osmotic shock pretreatment, where palmitic acid was improved from (Control) to a maximum of 1252.15 µgg^{-1}for incubation period 72 h (Fig.2D).

The second dominant SFA was the behenic acid (C22:0), followed by stearic acid (C18:0) at all incubation periods for all chemical pre-treatments. The former increased from 89.67 µg g^{-1}(control) to a maximum of 746.88 µg g^{-1}after 1 hr HCl (pH=2) pretreatment, while the latter increased from 29.61 µg g^{-1} to its maximum concentration, 95.92 µg g^{-1} (Fig. 2A). By using 1% H_2SO_4 for 2 hr, behenic Acid (C22:0) increased about 10 folds from (control) to 826.36 µg g^{-1}, while stearic acid increased to a maximum of 90.32 µg g^{-1} (Fig. 2B). Applying alkaline pretreatment with NaOH for 2 h, the concentration of behenic acid and stearic acid

increased to a maximum of 762.94 µg g^{-1} and 122.74 µg g^{-1}, respectively (Fig. 2C).By applying osmotic shock for 72 h, behenic and stearic acids increased from (control) to a maximum of 971.72 µg $g^{-1}$and 106.43 µg g^{-1}, respectively (Fig.2D).

As well, lignoceric acid (C24:0) increased from 11.46 µg g^{-1} (control) to a maximum of 64.54 µg g^{-1} after 1 hr HCl pretreatment (Fig.2A), while it increased to a maximum of 68.15 µg g^{-1} by 1% H_2SO_4 pre-treatment for 2h (Fig.2 B). By using NaOH pretreatment for 2 h, the lignoceric acid increased to a maximum of 65.75 µg g^{-1}, with no further increase for longer period (Fig.2C). Similarly, it increased to the highest concentration (81.46 µg g^{-1}) by applying the osmotic shock for 72h (Fig.2D).

Considering MUFAs, the oleic acid (C18:1c) was dominant in the control sample and all incubation periods with HCl, H_2SO_4, NaOH solution and osmotic shock (36%NaCl solution). It improved from (control) 74.66 µg $g^{-1}$to a maximum of 775.92 and 493.93 µg $g^{-1}$by using HCl for 1 hr and 1% H_2SO_4 for 2 h, respectively (Fig. 2 A& B). By pretreatment the biomass with NaOH solution, Oleic acid was improved from (control) to a maximum of 786.29 µg g^{-1} for 3 h (Fig. 2C), while it improved to a maximum of 661.55 µg g^{-1} by pre-treating with NaCl solution for 72 h (Fig.2D).

Also, palmitoleic acid (C16:1) improved from 39.90µg g^{-1} (control) to a maximum of 55.39 and 76.90 µg $g^{-1}$by using HCl for 1 h (Fig.2A) and NaOH for 2h, respectively (Fig.2C). In contrast, it decreased than the control by using 1% H_2SO_4 and NaCl solution for the three periods (Fig. 2B & D), whereas cis-10-Heptadecenoic acid (C17:1) increased from 7.03 µg g^{-1} (control) to its maximum value, 56.02 µg $g^{-1}$and 64.31 µg g^{-1} using 1% H_2SO_4 for 2 h and NaCl for 72 h, respectively (Fig. 2B&D).

Among PUFAs, cis- 4,7,10,13,16,19-Docosahexaenoic acid (C22:6) was the dominant one. It increased from 4.53 µg g^{-1} (control) to a maximum of 11.86 µg g^{-1} and 65.49 µg $g^{-1}$by using HCl for 3hrs and 1% H_2SO_4 pretreatment for 1 h, and to 31.40µg $g^{-1}$and 48.22 µg $g^{-1}$by using NaOH pretreatment for 2h and 36% NaCl solution for 24 h, respectively (Fig. 2A & B & C& D). It was obvious that the other PUFAs were very low and there was negligible difference between them by applying each of the four treatments.

By investigating the biodiesel properties, it was clear that the CN of biodiesel produced from HCl pretreatment for 1h improved from 73.21 (control) to 74.22, whereas it improved to 76.77 by pre-treating with 1% H_2SO_4 for 2h. Similarly, by alkali treatment the CN improved to 74.56 for 2 h, while it upgraded to a maximum of 76.36 by osmotic shock pretreatment for 72 h (Table 1). This was associated with increase in LCSF from 23.06 (control) to 44.44, after pretreatment with HCl for 1h, and to 55.95 using 1% H_2SO_4 pretreatment for 2 h. On the other hand, LCSF improved to 46.21and 53.53 after alkali pretreatment for 2 h and osmotic shock for 72h, respectively (Table1). The results of CN at all incubation periods for all treatments obey the CN standards (the ASTM D6751 and EN14214 standards). Kinematic viscosity of biodiesel produced from *U. fasciata* after the four treatments slightly increased from the control (4.68 mm^2 s^{-1}) to >5 mm^2 s^{-1} at all incubation periods (Table1).

From the data interpreted in Table (1), it is obvious that the four chemical pretreatments of *U. fasciata* had no influence on density of the produced biodiesel.

The Higher Heating Value (HHVs) of biodiesel produced from HCl, NaOH, NaCl pretreated *U. fasciata* for the three incubation periods were close to that of control (39.85 MJ kg^{-1}). The same pattern was followed using 1% H_2SO_4 pretreatment for 1 h, while it improved to 40.61 and 40.40 MJ kg^{-1} by increasing incubation period to 2 and 3h, respectively. All the results complied with the set range of regular biodiesel (Table 1).

As shown in Table (1), Iodine values of biodiesel produced from HCl (pH=2) pretreated *U. fasciata* for 1 h increased from that of control (18.48 gI_2 $100g^{-1}$fat) to a maximum of 26.93, gI_2 $100g^{-1}$fat, in consistence with increasing DU from 17.58 (control) to 29.39. Iodine value (IV) of 1% H_2SO_4 attained a maximum of 34.86 g I_2 $100g^{-1}$fat at 2 h pretreatment, in consistence with maximum degree of unsaturation (DU) (31.52), while the values obtained from NaOH pretreatment was 23.95, which increased to 29.56 and 35.92 gI_2 $100g^{-1}$fat by increasing incubation period to 2 and 3 h, respectively, that were in consistence with the corresponding DU values. By applying the osmotic shock, maximum IV was 31.70 gI_2 $100g^{-1}$fat while minimum IV was 23.79 gI_2 $100g^{-1}$fat, that were in consistence with maximum DU (27.83), and minimum DU (25.16) at 24 h and 48 h incubation period, respectively. Iodine values at all incubation periods were lower than the maximum limit identified by the EN 14214.

As interpreted in Table (1), C18:3% in biodiesel produced from HCl (pH=2) pretreated *U. fasciata* improved from 0.68% (control) to the best value 0.21% for all incubation periods. Similarly, it improved by pretreating with 1% H_2SO_4 for 2 h, recording 0.36 %. The C18:3% in biodiesel produced from NaOH pretreatment for 2h attained a maximum of 0.72 %. On the other hand, it improved from (control) to 0.24%, when applying osmotic shock for 72 h. All these results are accepted with the European B100 biodiesel standard, EN 14214 limit (≤12%). Furthermore, polyunsaturated fatty acid methyl esters containing ≥ 4 double bonds % recovered from 0.98% (control) to a minimum of 0.56 % and 0.73 by pretreating algal biomass with HCl (pH=2) for 1 h and NaCl for 72h, respectively, while using 1% H_2SO_4 for 1 h it increased to 3.14%, decreasing to 2.28 and 2.01%, by further increasing incubation period to 2 and 3 h, respectively (Table1). The pretreatment by alkali followed the same pattern, where the values slightly increased from control to 1.16% after both 1 and 2 h pretreatments, while it decreased to 0.68% by increasing incubation period to 3 h (Table1). All the values of HCl and NaCl pretreatments were exactly within the acceptable limits of EN14214 standard, except that of NaCl for 24h incubation period. On the other hand, the values of 1% H_2SO_4 and NaOH were higher than these limits (Table 1).

The saponification values of biodiesel produced from HCL (pH=2) pretreated *U. fasciata* for 1, 2 and 3 h, slightly decreased from 200.66 mg $KOHg^{-1}$ (control) to 192.58, 192.79 and 193.00 mg $KOHg^{-1}$ Table (43), respectively. The same trend was followed by applying1% H_2SO_4, NaOH and NaCl solution. These values were in limits of UNI 10635 standard which indicated the high volatility and low density of produced biodiesel that could be helpful in burning the biodiesel smoothly and in avoiding misfire **(Azeem *et al.*, 2015)**.

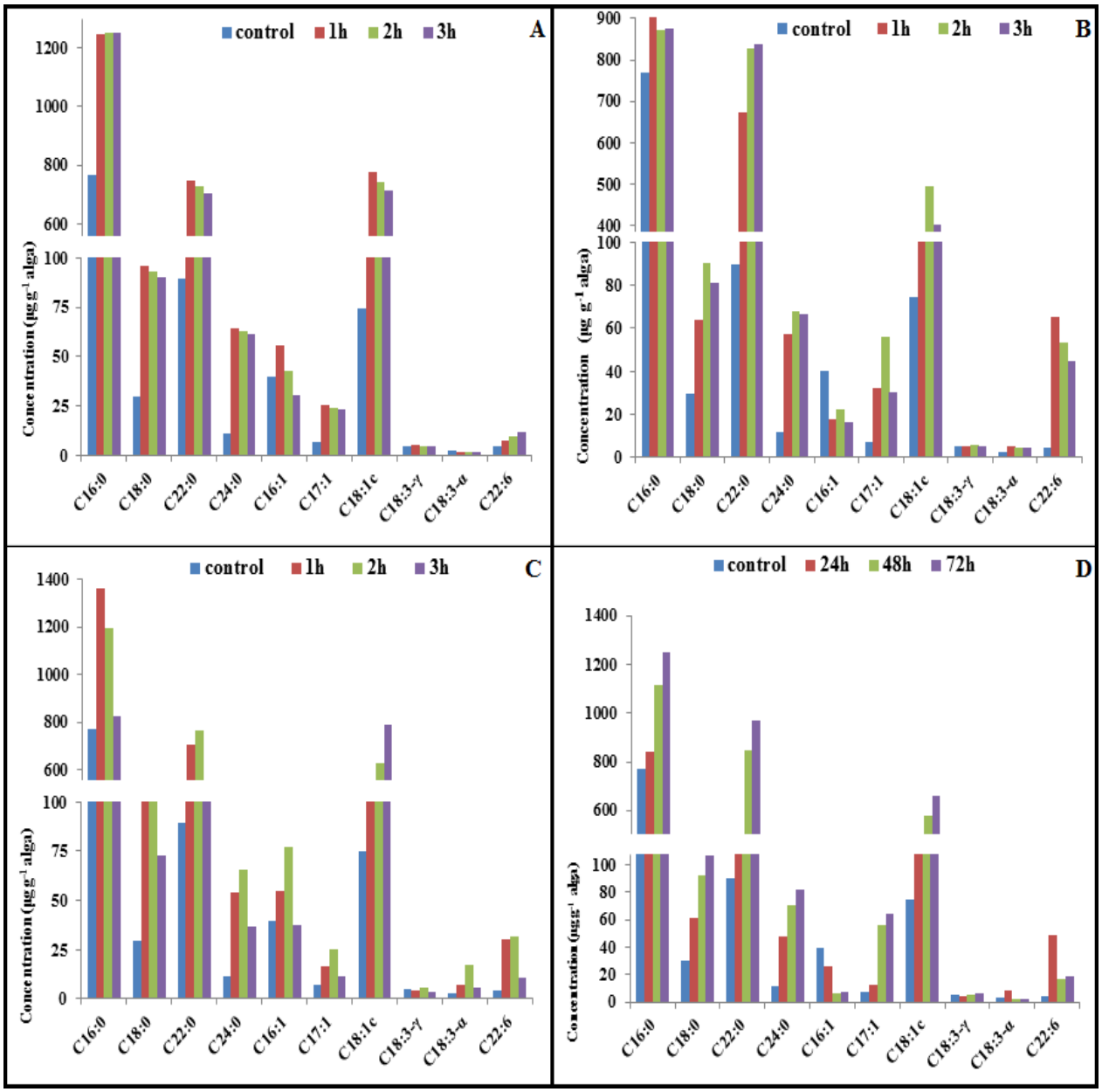

Figure (2): Effect of pretreatment with HCl (A), 1% H_2SO_4 (B), NaOH (C) and 36% NaCl solution (D) **for different periods on concentration of FAMEs (µg g^{-1}alga).**

Compared with cell wall morphology of the untreated *Ulva fasciata* by SEM analysis (Fig. 3a&b), the morphological structure of the alga changed after HCl (pH=2) for 1 h (Fig.4 a&b), 1% $H_2SO_4$for 2h pretreatment (Fig. 5 a&b), alkaline pretreatment with NaOH for 2h (Fig.6 a&b) and osmotic shock for 72 h (Fig.7 a&b), where there was degradation in algal cell wall and the porosity of cell wall increased giving greater surface area.

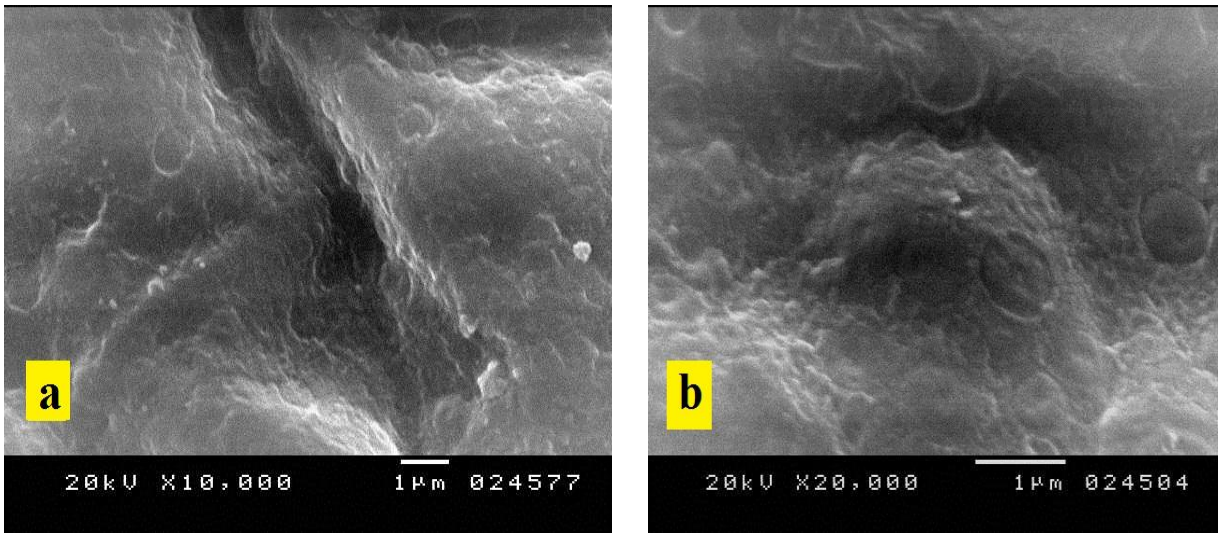

Figure (3): Scanning electron microscope (SEM) images (Morphological analysis) of *Ulva fasciata* before treatment

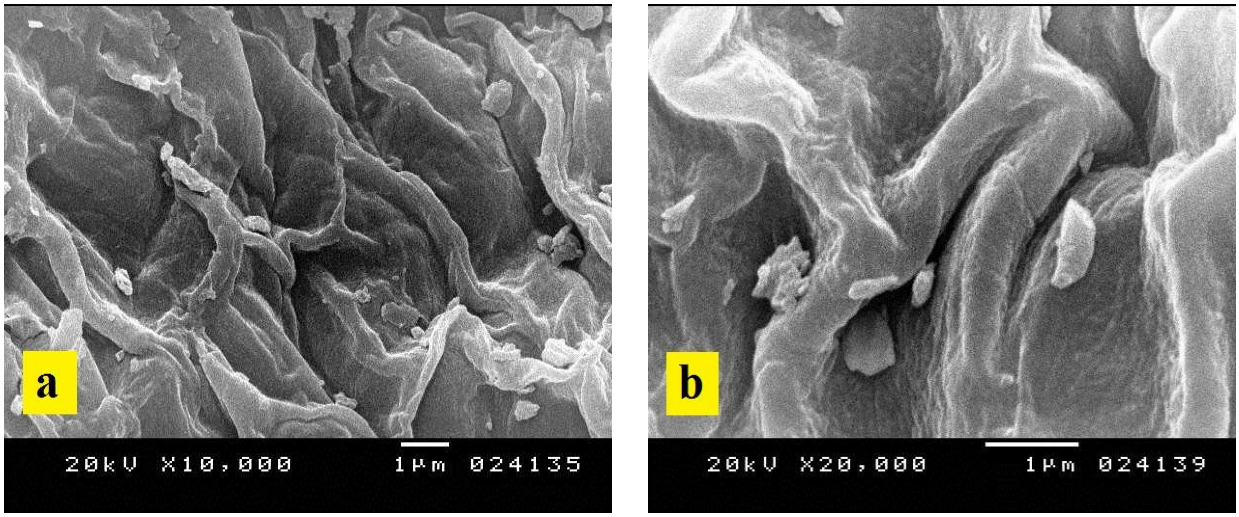

Figure (4): Scanning electron microscope (SEM) images (Morphological analysis) of *U. fasciata* after acid lysis with HCl (pH=2) for 1h.

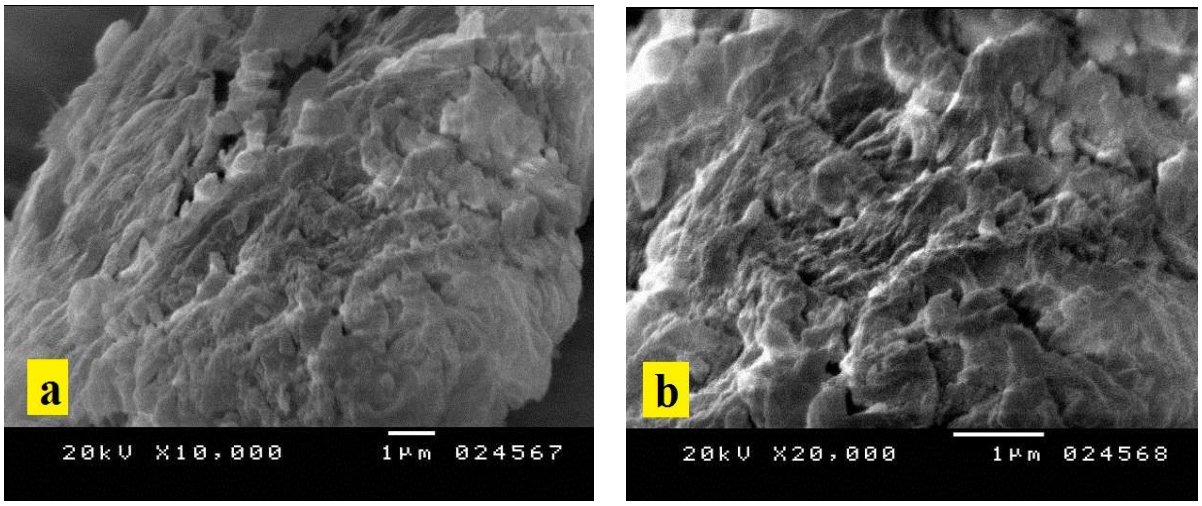

Figure (5): Scanning electron microscope (SEM) images of *U. fasciata* after acid lysis with 1% H_2SO_4 for 2h.

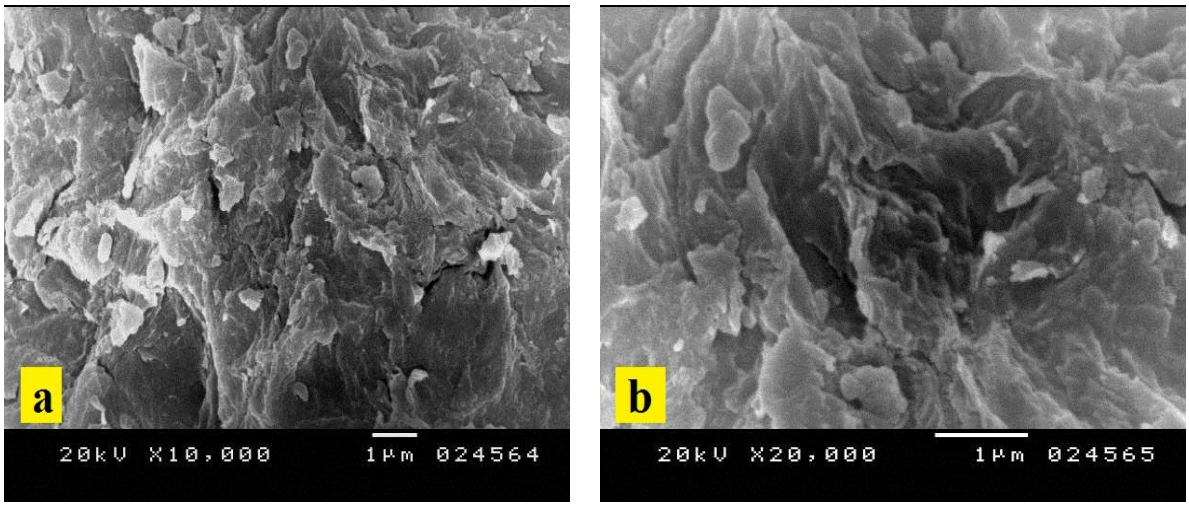

Figure (6): Scanning electron microscope (SEM) images of *U. fasciata* after alkaline pretreatment with NaOH (Adjustment at pH = 12) for 2h.

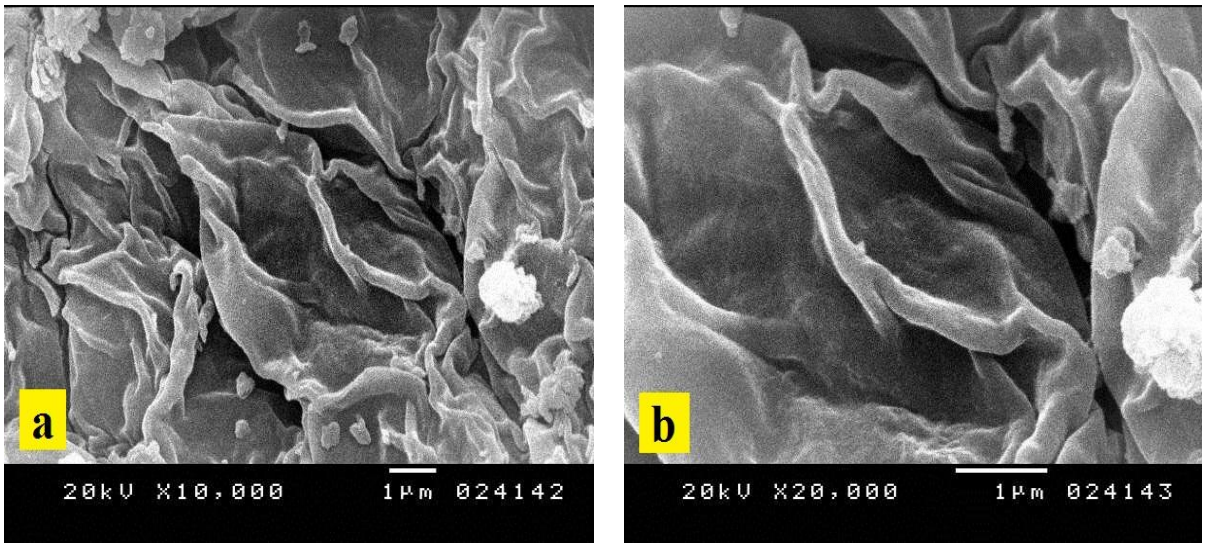

Figure (7): Scanning electron microscope (SEM) images of *Ulva fasciata* after osmotic shock pretreatment for 72h.

The results of comparison between the four pre-treatments methods showed great differences between them (Fig.1), which were confirmed statistically. By evaluating the chemical pretreatments effects from the values obtained by analysis of variance (ANOVA) (Table 2), it was observed that all chemical pretreatments had high significant effect on ÓTFAs, ÓSFAs and ÓPUFAs, SFAs/MUFAs, C18:1/C18:3, saturated fatty acids (C16:0, C18:0, C22:0 and C24:0), and MUFAs (C16:1 and C18:1c) yields at 0.01 level of probability, where all values were highly significant (Table 2). In addition, there were highly significant differences between control and each chemical pretreatment and between chemical pretreatments, where all calculated F-values were higher than that of corresponding tabulated ones (Table 3). These results were shown by using the least significant difference (L.S.D), where the most significant effect on both ÓTFAs, ÓSFAs was pretreatment with osmotic shock for 72 hrs as reflected by the 3 and 2.7 folds increase, respectively, while pretreatment with 1% H_2SO_4 for 2 h showed the highest effect on ÓPUFAs yield (141.10 µg g^{-1}). Also, there were significant differences between all chemical pretreatments on C16:0, C18:0, C22:0 and C24:0, C16:1 and C18:1c (Table 3). Although, there were no significant differences between different pretreatments ($P \leq 0.01$) on ÓMUFAs yield (Table 2), the values were higher than that of the control after all chemical pretreatments (Table 3). Furthermore, the analysis of variance (ANOVA) and the least significant difference (L.S.D) showed the highly significant effect of chemical pretreatments on all properties of the produced biodiesel, except density, which considered not changed from control and all pretreatments (Table 4& 5).

DISCUSSION

Among pretreatment methods of biomass before lipid extraction, are chemical pretreatments (acid, alkali and NaCl) which demonstrated to modify effectively the chemical

structures of lignocellulose. Luckily, macroalgae contain a large amount of polysaccharides and protein, which are easily degraded by chemical pretreatment **(Jung *et al.*, 2013)**. Moreover, the absence of lignin along with three-dimensional structures of cell wall appears to contribute to the successful application of the conventional pretreatment methods to algal biomass for biofuel production **(Vergara-Fernandez *et al.*, 2008)**. Due to the simplicity, low cost and effectiveness, dilute acid pretreatment is considered close to commercialization. It is regarded as suitable either for hydrolysis of lignocellulose or to enhance enzymatic digestibility **(Jung and Kim, 2015)**. When diluted acid is used, hemicellulose is mostly converted to monosaccharides **(Jung and Kim, 2015)**. The treatment of algae at low pH values is in fact similar to those found in the stomachs of herbivorous fishes, causing damages to the algal cells, thus allowing digestive enzymes to enter the cell **(Zemke-White *et al.*, 2000)**.

In consistence with our results, **Zemke-White *et al*. (2000)** analyzed 4 macroalgae, namely, *Enteromorpha intestinalis, Ulva rigida, Porphyra sp.*, and *Polysiphonia strictissima* and found that the cell wall pore size of all algal species increased up to at least 13.5 nm after pretreatment at pH 2.0 for 60 min more than the normal cell wall size (8.8 nm).

While, **Surendhiran and Vijay (2014)** showed that the most efficient cell disruption occurred with HCl (pH=2) after 2 h of incubation, giving 33.18% lipid yield for nitrogen supplied culture and 54.26% for nitrogen depleted condition. **Gupta *et al.* (2011)** recorded that compared to untreated substrates (control), the higher residual lignin and lower residual holocellulose in the pretreated substrates may be due to the removal of acid soluble carbohydrate fraction (hemicellulose). The easily degraded hemicellulose is released by diluted acid pretreatment **(Jung and Kim, 2015)**. In fact, hemicellulose removal increases porosity and improves enzymatic digestibility, with maximum enzymatic digestibility usually coinciding with complete hemicellulose removal **(Chen *et al.*, 2007)**. This method is especially suitable for biomass with low lignin content such as algae. However, the cellulose hydrolysis catalyzed by acid was extensively studied, with the focus on the scission of β-1, 4-glycosidic bonds that connect hemiacetal and hydroxyl terminal groups **(Jung and Kim, 2015)**. The cleavage of β-1, 4-glycosidic bonds starts by the diffusion of protons through lignocellulosic matrices and rapid protonation of glycosidic oxygen bonds between sugar monomers **(Unhasirikul *et al.*, 2012)**. The positive charges from the breakage of C-O bonds are transferred to other carbons, and this forms carbocations (cyclic or acyclic, depending on the site of protonation) as intermediate complexes. In the presence of water, free saccharides and protons are released by slow splitting of the glycosidic bonds **(Jung and Kim, 2015)**. Similar to the hydrolysis of cellulose, hemicellulose is hydrolyzed by acid through the selective cleavage of C-O bonds between sugar molecules via the protonation of glycosidic bonds or pyranic oxygens **(Dutta *et al.*, 2012)**. Since the hydrogen bonds in hemicellulose are not strong enough, hemicellulosic sugars are preferentially converted to 2 furaldehydes and then to 2,5-anhydride intermediates in the following order of reactivity **(Ximenes *et al.*, 2013)**: xylose > arabinose > mannose >galactose> glucose.

Table (2): Analysis of variance for fatty acids from chemical pretreatments

Source of variation	Degree of freedom	Mean square											
		ΣTFAs	ΣSFAs	ΣMUFAs	ΣPUFAs	SFAs/ MUFAs	C18:1/ C18:3	C16:0	C18:0	C22:0	C24:0	C16:1	C18:1c
Model	12	1226212.18**	640694.81**	146543.13**	3270.46**	5.98**	3949.92**	134408.24**	1732.33**	154397.67**	942.26**	1281.82**	133198.61**
Error	26	0	0	6410.64	0.0001	0.0001	0.0001	0	0.0001	0	0.0001	0.0001	0

** Denote significant at 0.01 level of probability.

Table (3): Values, general mean, least significant difference (L.S.D) and coefficient variance (C.V) of fatty acids for different traits from chemical pretreatments:

Chemical pretreatments	ΣTFAs	ΣSFAs	ΣMUFAs	ΣPUFAs	SFAs/ MUFAs	C18:1/ C18:3	C16:0	C18:0	C22:0	C24:0	C16:1	C18:1c
Control	1148.9^{m}	979.43^{m}	136.98^{h}	32.53^{m}	7.15^{a}	9.918^{m}	768.42^{m}	29.61^{m}	89.67^{m}	11.46^{m}	39.90^{e}	74.66^{m}
HCl (pH 2) for 1h	3307.03^{b}	2375.08^{d}	891.80^{a}	40.13^{l}	2.66^{l}	112.0^{c}	1247.54^{e}	95.92^{d}	746.88^{f}	64.54^{f}	55.39^{b}	775.92^{b}
HCl (pH 2) for 2h	3231.55^{d}	2343.03^{e}	$843.37^{a,b}$	45.15^{j}	2.78^{k}	112.9^{b}	1250.52^{d}	93.05^{e}	726.08^{g}	63.04^{g}	43.08^{d}	744.94^{c}
HCl (pH 2) for 3h	3156.08^{e}	2310.98^{f}	$794.92^{a,b,c}$	50.17^{g}	2.91^{j}	113.9^{a}	1253.51^{b}	90.17^{h}	705.27^{h}	61.54^{h}	30.76^{g}	713.96^{d}
H2SO4 (1%) for 1h	2366.51^{k}	1862.14^{j}	407.07^{g}	97.30^{c}	4.57^{b}	34.66^{j}	917.99^{h}	64.01^{k}	674.80^{j}	57.14^{i}	17.45^{j}	343.78^{l}
H2SO4 (1%) for 2h	2773.47^{h}	2040.38^{h}	$591.99^{d,e,f}$	141.10^{a}	3.45^{f}	49.15^{g}	872.30^{j}	90.32^{g}	826.36^{d}	68.15^{c}	22.22^{i}	493.93^{i}
H2SO4 (1%) for 3h	2624.15^{i}	2038.78^{i}	$471.44^{e,f,g}$	113.92^{b}	4.32^{c}	40.44^{i}	876.84^{i}	81.13^{i}	838.35^{c}	66.70^{d}	16.40^{k}	402.65^{j}
NaOH (pH 12) for 1h	3145.51^{f}	2461.11^{b}	$622.10^{c,d,e}$	62.30^{f}	3.96^{d}	46.90^{h}	1362.16^{a}	107.70^{b}	703.13^{i}	53.90j	54.90^{c}	517.71^{h}
NaOH (pH 12) for 2h	3267.03^{c}	2392.59^{c}	$784.25^{c,d,e}$	90.19^{d}	3.05^{i}	28.01^{l}	1195.46^{f}	122.74^{a}	762.94^{e}	65.75^{e}	76.90^{a}	626.89^{f}
NaOH (pH 12) for 3h	2429.94^{j}	1528.46^{l}	854.61^{a}	46.87^{i}	1.79^{m}	81.81^{f}	822.95^{l}	72.79^{j}	434.26^{l}	36.76^{l}	37.60^{f}	786.29^{a}
36% NaCl for 24h	2089.92^{l}	1591.14^{k}	$415.88^{f,g}$	82.90^{e}	3.83^{e}	28.70^{k}	838.90^{k}	61.30^{l}	492.59^{k}	47.97^{k}	26.44^{h}	352.84^{k}
36% NaCl for 48h	2996.56^{g}	2284.78^{g}	$669.75^{b,c,d}$	42.025^{k}	3.41^{g}	84.47^{d}	1115.73^{g}	92.59^{f}	845.40^{b}	70.87^{b}	6.36^{m}	575.55^{g}
36% NaCl for 72h	3411.72^{a}	2596.28^{a}	$767.86^{a,b,c,d}$	47.58^{h}	3.38^{h}	82.15^{e}	1252.15^{c}	106.43^{c}	971.72^{a}	81.46^{a}	7.31^{l}	661.55^{e}
General mean	2765.26	2061.86	634.77	68.63	3.64	63.48	1059.57	85.21	678.27	57.64	33.44	543.90
L.S.D 0.01	0.0227	0.0227	181.66	0.0227	0.0227	0.0227	0.0227	0.0227	0.0227	0.0227	0.0227	0.0227
CV	0.0004	0.0005	12.8886	0.0145	0.2720	0.0158	0.0009	0.0117	0.0015	0.0174	0.0300	0.0018

Values followed by the same letter (s) in columns are not significantly different, but values with different letter (s) are highly significant at 0.01 level of probability according to L.S.D procedure.

Table (4): Analysis of variance for properties of biodiesel form chemical pretreatments

Source of variation	Degree of freedom	Mean square												
		DU	LCSF	IV	SV	CN	SFAs (%)	MUFA (%)	PUFA (%)	Kinematic viscosity(υ)	Density(ρ)	HHV	C18:3 (wt%)	Db≥4 (wt%)
Model	12	70.67**	271.08**	63.91**	27.45**	7.28**	79.28**	98.88**	5.51**	0.18**	0.000042	0.19**	0.096**	2.36 **
Error	26	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0066

** Denote significant at 0.01 level of probability.

Table (5): Values, general mean, least significant difference (L.S.D) and coefficient variance (C.V) of biodiesel properties for different traits from chemical pretreatments:

Chemical pretreatments	DU	LCSF	IV	SV	CN	SFAs (%)	MUFA (%)	PUFA (%)	Kinematic viscosity(υ)	Density(ρ)	HHV	C18:3 (wt%)	Db≥4 (wt%)
Control	17.58^{m}	23.056^{m}	18.48^{l}	200.66^{a}	73.21^{k}	85.25^{a}	11.92^{m}	2.83^{e}	4.68^{i}	0.87^{a}	39.85^{g}	0.68^{b}	0.98^{e}
HCl (pH 2) for 1h	29.39^{d}	44.44^{i}	26.93^{f}	192.58^{g}	74.22^{j}	71.82^{k}	26.97^{b}	1.21^{k}	5.22^{f}	0.87^{a}	39.81^{h}	$0.21^{i,h}$	0.56^{h}
HCl (pH 2) for 2h	28.89^{e}	44.33^{j}	26.65^{g}	192.79^{f}	74.37^{i}	72.50^{j}	26.10^{c}	1.40^{j}	5.22^{f}	0.87^{a}	$39.86^{f,g}$	$0.21^{i,h}$	$0.57^{g,h}$
HCl (pH 2) for 3h	28.37^{f}	44.22^{k}	26.35^{h}	193.00^{e}	74.54^{h}	73.22^{i}	25.19^{d}	1.59^{i}	5.22^{f}	0.87^{a}	39.91^{d}	0.20^{i}	0.59^{g}
H_2SO_4 (1%) for 1h	25.42^{i}	54.30^{c}	30.75^{d}	191.01^{i}	76.26^{e}	78.69^{b}	17.20^{l}	4.11^{c}	5.42^{d}	0.87^{a}	39.81^{h}	0.43^{d}	3.14^{a}
H_2SO4 (1%) for 2h	31.52^{b}	55.95^{b}	34.86^{b}	189.17^{l}	76.77^{b}	73.57^{h}	21.34^{k}	5.09^{a}	5.57^{b}	0.88^{a}	40.61^{a}	0.36^{f}	2.28^{c}
H_2SO4 (1%) for 3h	26.65^{h}	59.48^{a}	29.55^{e}	189.14^{m}	77.48^{a}	77.69^{d}	17.97^{h}	4.34^{b}	5.62^{a}	0.88^{a}	40.40^{b}	$0.38^{e,f}$	2.01^{d}
NaOH (pH 12) for 1h	23.74^{l}	44.57^{h}	23.95^{i}	194.23^{c}	74.83^{f}	78.24^{c}	19.78^{j}	1.98^{g}	5.17^{g}	0.87^{a}	39.75^{i}	0.37^{f}	1.16^{f}
NaOH (pH 12) for 2h	29.53^{c}	46.21^{g}	29.56^{e}	192.53^{h}	74.56^{h}	73.23^{i}	24.00^{e}	2.76^{f}	5.25^{h}	0.87^{a}	39.95^{c}	0.72^{a}	1.16^{f}
NaOH (pH 12) for 3h	39.03^{a}	36.06^{l}	35.92^{a}	194.52^{b}	71.78^{l}	62.90^{l}	35.17^{a}	1.93^{h}	5.02^{e}	0.87^{a}	39.82^{h}	0.40^{e}	0.68^{g}
36% NaCl for 24h	27.83^{g}	46.92^{f}	31.70^{c}	193.57^{d}	74.74^{g}	76.13^{f}	19.90^{i}	3.97^{d}	$5.23^{e,f}$	0.87^{a}	39.85^{g}	0.59^{c}	2.59^{b}
36% NaCl for 48h	25.16^{j}	53.11^{e}	23.79^{k}	190.02^{j}	76.32^{d}	76.25^{e}	22.35^{g}	1.40^{j}	5.45^{c}	0.87^{a}	$39.88^{e,f}$	$0.23^{g,h}$	0.75^{g}
36% NaCl for 72h	25.30^{k}	53.53^{d}	23.89^{j}	189.89^{k}	76.36^{c}	76.10^{g}	22.51^{f}	1.39^{j}	5.46^{c}	0.87^{a}	$39.89^{d,e}$	0.24^{g}	0.73^{g}
General mean	27.57	46.63	27.88	192.55	75.03	75.05	22.34	2.62	5.27	0.87	39.95	0.39	1.32
L.S.D 0.01	0.0227	0.0227	0.0227	0.0227	0.0227	0.0227	0.0227	0.0227	0.0227	0.022	0.0227	0.0227	0.1848
C.V	0.0362	0.0214	0.0357	0.0052	0.0133	0.0133	0.0449	0.3767	0.1897	1.147	0.0200	2.5896	6.0341

Values followed by the same letter (s) in columns are not significantly different, but values with different letter (s) are highly significant at 0.01 level of probability according to L.S.D procedure.

On the other hand, **Martin *et al.* (2007)** showed that using concentrated acid is less preferable than dilute acid because of forming high amount of inhibiting components and causing corrosion in the equipments. They preferred application of dilute acids at moderate temperatures to convert lignocellulosic structures to soluble sugars. In the same trend, **Sun and Cheng (2002)** showed that dilute acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. Similarly, **Singh and Trivedi (2013)** observed that sulphuric and hydrochloric acids are the most commonly used catalysts for hydrolysis of lignocellulosic biomass.

Alkaline pretreatment of algal biomass is considered to be a promising pretreatment method that alters structural properties, such as accessible surface area and crystallinity of pretreated substrate, thereby enhancing lipid extraction **(Sindhu *et al.*, 2015)**. The alkali pretreatment processes utilize lower temperatures and pressures compared with some other pretreatment technologies.

However, **Sun and Cheng (2002)** reported that the mechanism of alkaline hydrolysis is believed to be saponification of intermolecular ester bonds cross linking xylan hemicelluloses and other components such as lignin and other hemicellulose. The porosity of the lignocellulosic materials increases with the removal of the cross links. Dilute NaOH treatment of lignocellulosic materials caused swelling, leading to an increase in internal surface area, a decrease in the degree of polymerization, a decrease in crystallinity, separation of structural linkages between lignin and carbohydrates, and disruption of the lignin structure. On the other hand, **Zhao *et al.* (2007)** reported that pretreatment with NaOH could obtain a higher enzymatic conversion ratio of cellulose, compared with acid or oxidative reagents, since alkali treatment appears to be the most effective method in breaking the ester bonds between hemicellulose and cellulose, and avoiding fragmentation of the hemicellulose polymers **(Gaspar *et al.*, 2007)**. **Singh and Trivedi (2013)** found that alkaline pretreatment with NaOH is more optimum than KOH and $Ca(OH)_2$. They also found that dilute NaOH pretreatment was found to be effective for the hydrolysis of substrates with relatively low lignin contents. In this trend, **Sindhu *et al.* (2014)** found that the reducing sugar yield from NaOH pretreated biomass was seven times higher when compared with untreated one. This indicates the effect of alkali pretreatment with NaOH of hemicellulose degradation, which facilitates lipid extraction and biodiesel production.

After NaOH pretreatment of *Ulva fasciata*, the cellular morphology changed, there was degradation in algal cell wall, increase in porosity and greater surface area, which facilitates lipid extraction and hence fatty acids and biodiesel production. This is due to the removal of hemicelluloses, which in turn destroyed the cellulose-hemicellulose network, leading to the disruption of the hydrogen bond between the cellulose. The loose structure as well as an increase in surface area of the NaOH pretreated *U. fasciata* allows solvent to easily penetrate cell wall and extract lipid more easily, thus increasing biodiesel production **(Sindhu *et al.*, 2015)**.

Osmotic shock is considered an innovative cost-effective way to compete with other extraction methods **(Adam *et al.*, 2012)**. Osmotic pressure can disturb algal cell walls through a hasty increase and decrease in the salt concentration of the aqueous media, which can disturb the

balance of osmotic pressure between the interior and exterior of the algal cells. Algal cell damage can occur by two osmotic stresses; hyper-osmotic and hypo-osmotic. When the salt concentration is higher in the exterior, the cells suffer from hyper-osmotic stress. As a result, the cells shrink as the fluids inside the cells diffuse outwards, and damage is caused to the cell envelopes. In contrast, hypo-osmotic stress occurs when the salt concentration is lower in the exterior; thus the fluid flows into the cells to balance the osmotic pressure, and the cells swell or burst if the stress is too high **(Kumar *et al.*, 2015)**. However, a recent study by **Kar and Singhal, (2015)** reported that the osmotic shock was most suitable method in extracting intracellular products, which can also be industrially scaled up.

Kim and Yoo (2013) found that the osmotic shock was efficient method for lipid extraction from microalgae. In contrast, **Lee *et al.* (2010)** showed that this method was not effective against all types of microalgae. Species such as *Botryococcus braunii* and *Nannochloropsis oculata* have thick cell walls which are not easily disrupted using this method **(Lee *et al.*, 2010)**. In the current study, SEM analysis demonstrated that after osmotic shock pretreatment of *Ulva fasciata*, cells lysed and the cellular morphology changed because of disturbance of osmotic pressure balance between the interior and exterior of the algal cells, which makes the cells shrink as the fluids inside the cells diffuse outwards, and damage is caused to the cell envelopes, which facilitates lipid extraction and hence fatty acids and biodiesel production.

In the current study, all chemical pretreatments have significant effect on total fatty acids yield, where ÓFA was 1148.94µgg^{-1} dried alga without any treatment, while using acid pretreatment with HCl (pH=2) for optimum time period (1h) resulted in about 2.9-fold increase in ÓFA yield. On the other hand, it resulted in about 2.4-fold increase in ÓFA yield when pretreated with 1% H_2SO_4 for optimum time period (2h). On the other hand, alkaline pretreatment with NaOH (pH=12) for optimum incubation period (2h), resulted in about 2.84-fold increase in ÓFA yield, while osmotic shock with saturated (36%) solution of NaCl for 72h, gave about 3-fold increase, but it needs longer incubation periods, which cause time wasting. The produced biodiesel after all chemical pretreatments had a high quality and its properties complied with the ASTM D6751 and EN14214 standards, except in the case of 1% H_2SO_4 and NaOH; they were slightly higher than the limit of EN14214 standard. These results were confirmed statistically; where it was observed that all chemical pretreatments had high significant effect. In addition, there were highly significant differences between control and each chemical pretreatment and between chemical pretreatments themselves, where all calculated F-values were higher than that of corresponding tabulated ones. Also, chemical pretreatments had highly significant effect on all properties of the produced biodiesel.

CONCLUSION

The different chemical pretreatments at different incubation periods improved the quality of the produced biodiesel, compared with the untreated biomass of *Ulva fasciata*. The acid

pretreatment with HCl (pH=2) for optimum time period (1h) can be considered the most effective one, resulted in about 2.9-fold increase in ÓFA yield, since osmotic shock with saturated (36%) solution of NaCl for 72h, gave about 3-fold increase, but it needs longer incubation periods which cause time wasting.

REFERENCES

Adam, F.;Abert-Vian, M.; Peltier, G. and Chemat, F. (2012). Solvent-free" ultrasound assisted extraction of lipids from fresh microalgae cells: a green, clean and scalable process. Bioresour.Technol., 114: 457–465.

Afify, A.M.R.; Shalaby, E. A. and Shanab, S.M.M. (2010).Enhancement of biodiesel production from different species of algae. Journal of Grasasyaceites, 61 (4): 416-422. DOI: 10.3989/gya.021610.

Aleem, A. A. (1993). Marine algae of Alexandria, Egypt.University of Alexandria publications, Alexandria, 135 pp.

Azeem, M.W.; Hanif, M. A.; Al-Sabhi, J. N.; Khan, A. A.; Naz, S. and Ijaz, A. (2015). Production of biodiesel from low priced, renewable and abundant date seed oil. Renewable Energy, 86: 124-132.

Bilgin, A.; Gülüm, M.; Koyuncuoglu, İ.; Nac, E.; and Cakmak, A. 2015. Determination of transesterification reaction parameters giving the lowest viscosity waste cooking oil biodiesel. Procedia - Social and Behavioral Sciences, 195: 2492-2500.

Chen, Y.; Sharma-Shivappa, R.R.; Keshwani, D. and Chen, C. (2007). Potential of agricultural residues and hay for bioetha-nol production. Applied Biochemistry and Biotechnology 142(3): 276-290.

Clayton, M. N. (1992). Propagules of marine macroalgae: structure and development. British Phycological Journal, 27 :219–232.

Doan, T.Y.; Sivaloganathan, B. and Obbard, J.P. (2011). Screening of marine microalgae for biodiesel feedstock. Journal of Biomass and Bioenergy, 35: 2534-2544.https://doi.org/10.1016/j.biombioe.2011.02.021**.**

Dutta, S.; De, S.; Saha, B. and Alam, M.I. (2012). Advances in conversion of hemicellulosic biomass to furfural and upgrading to biofuels. Catal. Sci. Technol., 10: 2025-2036.

Folch, J.; Lees, M; and Sloane-Stanley, G.H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. Journal of Biological Chemistry, 226: 497-509.

Foster, E.; Contestabile, M.; Blazquez, J.; Manzano, B.; Workman, M.; Shah, N. (2017). The unstudied barriers to widespread renewable energy deployment: Fossil fuel price responses Energy Policy, 103: 258. https://doi.org/10.1016/j.enpol.2016.12.050

Gaspar, M.; Kalman, G. and Reczey, K. (2007). Corn fiber as a raw material for hemicellulose and ethanol production. Process Biochem., 42: 1135-1139.

Gupta, R.; Khasa, Y.P. and Kuhad, R.C. (2011). Evaluation of pretreatment methods in improving the enzymatic saccharification of cellulosic materials. Carbohydrate Polymers, 84: 1103–1109.

Halim, R.; Danquah, M.K. and Webley, P.A. (2012). Extraction of oil from microalgae for biodiesel production: A review. Biotechnol. Adv., 30: 709-732.

Hardisson, A.; Frìas, I.; Lozano, G. and Baéz, A. (1998). Mercury in algae of the Canary Islands littoral. Environment International, 24(8).

Islam, M.A.; Magnusson, M.; Brown, R.J.; Ayoko, G.A.; Nabi, M. N. and Heimann, K. (2013). Microalgal species selection for biodiesel production based on fuel properties derived from fatty acid profiles. Journal of Energies, 6: 5676-5702. DOI: 10.3390/en6115676.

Jegathese, S. J. P. and Farid, M. (2014). Microalgae as a renewable source of energy: A niche opportunity. Journal of Renewable Energy, ID 172461, 10 pp.

Jianbing, J.; Wang, J.; Yongchao, L.; Yunliang, Y. and Zhichao, X. (2006). Preparation of biodiesel with the help of ultrasonic and hydrodynamic cavitation. *Ultrasonics*, 44 Suppl. 1:e 411-e 414. doi:10.1016/j.ultras.2006.05.020.

Jin, G.; Yang, F.; Hu, C.; Shen, H. and Zhao, Z. K. (2012).Enzyme assisted extraction of lipids directly from the culture of the oleaginous yeast *Rhodosporidium toruloides*. Bioresource Technology, 111: 378-382.

John, R.P. and Anisha, G.S. (2011). Macroalgae and their potential for biofuel, CAB Reviews: Perspectives in agriculture, veterinary science, nutrition and natural resources, 6: 1-15.

Jung, K.A.; Lim, S.R.; Kim, Y. and Park, J.M. (2013). Potentials of macroalgae as feedstocks for biorefinery. Bioresour. Technol., 135: 182-190.

Jung, Y. H. and Kim, K. H. (2015). Acidic Pretreatment. In Pretreatment of Biomass: Processes and Technologies. (pp. 27-50). Elsevier Inc. DOI: 10.1016/B978-0-12-800080-9.00003-7.

Kar, J.R. and Singhal, R.S. (2015). Investigations on ideal mode of cell disruption in extremely *halophilic Actinopolyspora halophila* (MTCC 263) for efficient release of glycine betaine and trehalose. Journal of Biotechnology Reports, 5: 89-97.

Khan, S.A.; Rashmi; Hussain, M.Z.; Prasad, S. and Banerjee, U.C. (2009). Prospects of biodiesel production from microalgae in India, Renewable and Sustainable Energy Reviews, 13: 2361-2372.

Kim, J., and Yoo, G. (2013).Methods of downstream processing for the production of biodiesel from microalgae. Biotechnol. Adv. ,31: 862–876.

Kumar, R.R.; Rao, P.H. and Arumugam, M. (2015). Lipid extraction methods from microalgae: a comprehensive review. Frontiers in Energy Research- Bioenergy and Biofuels, 2 (61): 1-9.

Lee, J. Y.; Yoo, C.; Jun, S.Y.; Ahn, C.Y. and Oh, H.M. (2010).Comparison of several methods for effective lipid extraction from microalgae. Bioresource Technology, 101 (1): S75-S77.

Lewis, T.; Nichols, P.D. and McMeekin, T. A. (2000). Evaluation of extraction methods for recovery of fatty acids from lipid-producing microheterotrophs. Journal of Microbiology Methods, 43: 107-116.https://doi.org/10.1016/S0167-7012 (00) 00217-7.

Martin, C.; Klinke, H. B. and Thomsen, A. B. (2007).Wet oxidation as a pretreatment method for enhancing the enzymatic convertibility of sugarcane bagasse. Enzyme and Microbial Technology, 40 (3): 426-432.

Martins, F.; Felgueiras, C.; Smitkova, M. and Caetano, N. (2019). Analysis of Fossil Fuel Energy Consumption and Environmental Impacts in European Countries. Journal of Energies, 12, 964; doi: 10.3390/en12060964

Saravanan, S. and Chandrasekar, S. (2013). Predictive equations for viscosity and cetane number of biodiesel. German Journal of Renewable Sustainable Energy Research, 1: 23-28.

SAS Institute, Inc. (2007). SAS Technical Report AS/STAT Software: Changes and Enhancements User's Guide, Volume 2, Version 9.1.3, Fourth Edition, Cary, NC: SAS Institute,Inc.

Schell, D.J.; Farmer, J.; Newman, M. and McMillan, J.D. (2003). Dilute-sulfuric acid pretreatment of corn stover in pilot scale reactor: investigation of yields, kinetics, and enzymatic digestibilities of solids. Appl. Biochem. Biotechnol., 105-108: 69-85.

Shaltout, N.A. and Shams El-Din, N.G. (2015).Investigation of the fatty acid profile in some macroalgae in relation to the environmental conditions for biodiesel production.American Journal of Environmental Sciences, 11 (6): 402-419. DOI: 10.3844/ajessp.2015.402.419.

Shaltout, N.A.; Ghazal, M.A.; Shams El-Din, N.G.; Ali, A. E. and Beltagy, D.M. (2019).Optimization and Kinetic studies of biodiesel production from the green alga *Ulva fasciata* Delile. Egyptian Journal of Aquatic Biology & Fisheries, 23(4): 301- 322.

Sindhu, R.; Kuttiraja, M.; Binod, P.; Sukumaran, R.K. and Pandey, A. (2014). Physicochemical characterization of alkali pretreated sugarcane tops and optimization of enzymatic saccharification using response surface methodology. Renew Energy, 62: 362-368.

Sindhu, R.; Pandey, A. and Binod, P. (2015). Alkaline pretreatment. In Pretreatment of Biomass: Processes and Technologies. (pp. 51-60). Elsevier Inc. DOI: 10.1016/B978-0-12-800080-9.00004-9.

Singh, D. P. and Trivedi, R.K. (2013). Acid and alkaline pretreatment of lignocellulosic biomass to produce ethanol as biofuel. IJCRGG, 5 (2): 727-734.

Suganya, T. (2013). Studies on production of biodiesel from marine macroalgae using homogeneous catalysis. Ph.D Thesis, Department of chemical engineering, Faculty of Technology, Anna University, Chenni, 235pp.

Sun, Y. and Cheng, J. (2002). Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresource Technology, 83 (1): 1-11.

Surendhiran, D. and Vijay, M. (2014). Effect of various pretreatment for extracting intracellular lipid from *Nannochloropsis oculata* under nitrogen replete and depleted conditions. ISRN Chem. Eng., 2014: 536310-536318. DOI: 10.1155/2014/536310.

Unhasirikul, M.; Naranong, N. and Narkrugsa, W. (2012). Reducing sugar production from durian peel by hydrochloric acid hydrolysis. World Acad. Sci. Eng. Technol., 69: 444-449.

Vergara-Fernandez, A.; Vargas, G.; Alarcon, N. and Velasco, A. (2008). Evaluation of marine algae as a source of biogas in a two-stage anaerobic reactor system. Biomass Bioenerg, 32: 338-344.

Ximenes, E.; Kim, Y. and Ladisch, M.R. (2013). Biological conversion of plants to fuels and chemicals and the effects of inhibitors. In: Wyman CE, editor. Aqueous pretreatment of plant biomass for biological and chemical conversion to fuels and chemicals. John Wiley & Sons; 2013.

Yen, H.W.; Hu, I.C.; Chen, C.Y.; Ho, S.H.; Lee, D.J. and Chang, J.S. (2013). Microalgae based biorefinery from biofuels to natural products. Bioresource Technology, 135: 166-174.

Yoo, G.; Park, M.S. and Yang, J.W. (2015).Chemical pretreatment of algal biomass, 12th chapter in Pretreatment of Biomass. Elsevier Inc. DOI: 10.1016/B978-0-12-800080-9.00012-8.

Zemke-White, W.L.; Clements, K.D. and Harris, P.J. (2000). Acid lysis of macroalgae by marine herbivorous fishes: effects of acid pH on cell wall porosity, Journal of Experimental Marine Biology and Ecology, 24, 57-68.

Zhao, X.; Zhang, L. and Liu, D. (2007). Comparative study on chemical pretreatment methods for improving enzymatic digestibility of Crofton weed stem. Bioresource Technol., 99: 3729-3736.

Zheng, H.; Yin, J.; Gao, Z.; Huang, H.; Ji, X. and Dou, C. (2011). Disruption of *Chlorella vulgaris* cells for the release of biodiesel producing lipids: a comparison of grinding, ultrasonication, bead milling, enzymatic lysis and microwaves. Applied Biochemistry and Biotechnology, 164 (7): 1215-1224.