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RESEARCH ARTICLE

PHYSICAL PRETREATMENT OF ULVA FASCIATA FOR ENHANCINGBIODIESEL PRODUCTION AND QUALITY

Nihal Galal Shams El-Din[1], Nayrah A. Shaltout[2], Mustafa A. Ghazal[1], Alaa E. Ali[3] and Doha M. Beltagy[3]

1. Marine Hydrobiology Dep., National Institute of Oceanography and Fisheries, Alexandria, Egypt.
2. Marine Chemistry Dep., National Institute of Oceanography and Fisheries, Alexandria, Egypt.
3. Chemistry Dep. Faculty of Science, Damanhour University, Damanhour, Egypt.

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Abstract

The green algae Ulva faciata was subjected to different physical pretreatments comprising thermal and mechanical techniques at different experimental conditions to state the most appropriate method of cell disruption for increasing the quantity of the extracted lipid and hence improve the quality of the produced biodiesel with low cost. Thermal pretreatment was autoclaving of either wet or dry algal biomass, while mechanical pretreatments include microwave and ultrasonication at different time intervals. The control was the alga without pretreatment extracted at optimum conditions: 60 min, 55^{o}C, shaking speed at 250 rpm, < 0.16 mm particle size with 25:1 v/w solvent to solid ratio. The results showed that the quantity of extracted lipids in case of using all physical pretreatments increased the Total fatty acids yield significantly by about 2-folds of the control for wet algae in hydrothermal treatment with optimum time of treatment 40 minutes, and 1.4 folds for dry algae in thermal pretreatment of the dried alga for 60minutes autoclaving period. The sharp increase by 2.2 folds of extracted lipids was recorded by microwave pretreatment for radiation period (5 min), while ultrasonication showed 2.1-fold increase in lipid yield at 15minutes ultrasound exposure time. Concerning the physical properties of the produced biodiesel after all physical pretreatments, the results indicated that the produced biodiesel had very high quality as all its properties are almost complied with the ASTM D6751 and EN14214 standards. These results were confirmed statistically; where all physical pretreatments had high significant effect on fatty acids yield and Biodiesel properties.

Introduction:-

The increase in energy demand and decrease in fossil fuel resources in addition to the climate change crises arises from greenhouse gases produced in accordance of using fossil fuel, were the main reason for seeking renewable energy resources (Zhu et al 2014).Algae wererecognized as one of the renewable energy sources for biodiesel production due to their advantages. Firstly, they are not used as a primary food source for humans, so that it can be used solely for fuel production and there would be little effect on the food industry. Secondly, many of the waste-product extracts produced during the processing of algae for biofuel can be used as a sufficient animal feed.

Corresponding Author:- Nayrah A. Shaltout
Address:- Marine Chemistry Dep., National Institute of Oceanography and Fisheries, Alexandria, Egypt.

Moreover, they aremore productive than terrestrial plants and can succeed in salty or brackish water with only sunlight and available nutrients. The most important, is that they do not need any chemical fertilizers. For all these reasons, recent researches around the world focused on algae for biofuel production; either bioehanol(Ghaza et al 2016), biogas (Montingelli et al., 2016), or biodiesel (Shaltout and Shams El-Din, 2015).

However, the lipid extraction from algal cells is difficult reference to cell membrane morphology's in addition to lipid types, those arelinked to the cell membranes. Thus, the algal biomass pretreatment prior to direct lipid extraction is necessary to break the cells and shatter the cell walls to maximize the lipid recovery. Disintegration of the cellular structure before the lipid extraction has many advantages, such as faster extraction time, less solvent consumption, greater solvent penetration into the cell, and increasing the release of the cell content (Lee et al., 2017).Currently, numerous attempts have been made to disrupt effectively algal cell wall for lipid extraction. Physical pretreatments can disrupt the cell wall and break algal cells through a physical force (Paoss et al., 2015). The physical pretreatment methods of U. fasciatacomprise thermal and mechanical techniques. The thermal pretreatments include autoclaving of algal biomass, while mechanical pretreatments include sonication and microwave.Actually, the physical pretreatments are regarded as the most effective on microalgae cell disruption (Lee et al., 2012). They are effective and they are preferred methods for lipid extraction, where they do not require chemicals, do not induce unwanted chemical reactions such as the saponification of free fatty acids, which may contaminate the produced biodiesel(Paoss et al., 2015). The total fatty acid concentration readings of different cell disruption methods were used to indicate the efficiency of cellular wall disruption in this study. A higher degree of disruption causes increased breakdown of the cells and more released intracellular materials. However, the main disadvantage of physical pretreatments is high energy consumption, where they require a higher energy input if they are compared with chemical and biological methods (Lee et al., 2012; Paoss et al., 2015).

Noticeably, mechanical pretreatments of algal biomass disrupt the cell wall and enhance the efficiency of the lipid extraction process by enhancing the solvent/lipid contact. The disruption of the cellular wall allows for easier recovery of the intracellular lipids, resulting in rapid and higher efficiencies in lipid extraction (Al-Hattab and Ghaly, 2015).

Autoclaving of algal biomass is a form of thermal treatment operating at a temperature of 121°C and pressure of 15 lbs(Surendhiranand Vijay, 2014; USGS, 2015). However, high thermal stress causes disruption of cell walls and membranes, forcing the release of the intracellular lipids (Prabakaran and Ravindran, 2011). The positive aspects of this approach are that it is very effective, as this technique is used commonly to destroy bacterial cells and sterilize laboratory and medical equipment. The main limitation of this method is the cost involved to generate the heat and pressure required. Most autoclaves have a fixed volume which means that any large scale lipid extraction would be batch type; also autoclave run times can be quite lengthy.

Microwave technology has allowed the development of rapid, safe, and economical methods for extracting lipids and does not require dewatering of algal biomass(Kumar et al., 2015). Microwaves are short waves of electromagnetic energy varying in frequency from 300 MHz to 300 GHz. Generally, microwave frequencies are around 2450 MHz. It is a consequence of the rapidly oscillating electric field of a polar or dielectric material, which induces heat by the frictional forces of molecules in movement. The increase of kinetic energy leads water to a boiling state. The quantum energy applied by microwave irradiation is not capable of breaking down chemical bonds, except hydrogen bonds. In this manner, induction heating and dielectric polarization result in changes in the secondary and tertiary structure of proteins (Paoss et al., 2015). This method is quite efficient as the exposure time needed to disrupt cells is generally quite short, around 5-10 minutes

Ultrasonicationis another physical method that can be used for pretreatment of algae prior to lipid extraction, where it is an emerging powerful tool to accelerate many physical operations(Suganya and Renganathan, 2012;Rokicka et al., 2020).In this method, algae are exposed to high intensity ultrasonic waves, creating tiny cavitations bubbles around the cells. The bubbles collapse and emit shockwaves that shatter the cell walls causing the intracellular lipids to enter the bulk of the solution. Ultrasound-assisted extraction devoid the difficulties associated with the conventional mechanical disruption methods. The process is simple with easy working set-up conditions, imparting higher purity to the final product and eliminating treatment of wastewater generated during the process. Furthermore, the technique is more economical and eco-friendly and can be completed in a very short time with high reproducibility. The energy input is very little when compared to that in conventional methods, and can be operated at lower temperatures (Chemat et al., 2011).

In the previous work we have carried out optimization and kinetic studies of biodiesel production from Ulva fasciata(Shaltout et al., 2019)for seek of reducing biodiesel production cost, increasing yield and enhancing the quality. Also, we have carried out a study on different chemical pretreatment methods to find out the most effective one that enhance lipid extraction and increase the yield and properties of the produced biodiesel(Shams El-Din et al., 2020). The aim of the present work is to continue our investigation to achieve this goal by using different physical pretreatment methods on the dried form of Ulva faciata for lipid recovery and to find the most appropriate method of cell disruption that produce the highest quality biodiesel by the lowest cost using an environmentally friendly technique.

Materials and Methods:-

Collection of Ulvafasciata:

The green alga UlvafasciataDelilewas 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 latitudes 31o 18` 36.049`` N andlongitudes 30o 04` 18.732``E. The specieswas identified according to Aleem (1993). It belongs to the class Chlorophyceae, order Ulvales, family Ulvaceae.

Healthy specimens of the alga werehandpicked whole, from their bases, scraping thesubstrata on which they were adhered, and then kept at 4 °C in icebox.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 (25oC) 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 (25oC). 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.

Physical pretreatments:

Different physical pretreatments of the grained alga (<0.16 mm) were pretreatedas follows: thermal, microwave and ultrasonicationpretreatments.

Thermal pretreatments of algal biomass (autoclaving):

Thermal pretreatment methods of U. fasciata include hydrothermal autoclaving for wet algae or thermal autoclavingfor dry algae. In case of hydrothermal autoclaving the dried alga was immersed in distilled water before autoclaving (hydrothermal), while in case of the thermal autoclaving the dried alga was directly autoclaved.

Hydrothermal pretreatment (thermal pretreatment of the wet alga):

the algal biomass was mixed with distilled waterin a ratio (1:10 w/v dry alga /DW) in a 100 ml screw top bottles and then was autoclaved (Sturdy Automatic Autoclave: SA-260FA) at 121ºC, 15 lbs pressure at different time intervals20 min, 40 min and 60 min for optimization of hydrothermal pretreatment. Thereafter the biomass was filtered and dried in an oven at 60□ C (Surendhiran and Vijay, 2014).

Thermal pretreatment of dried algal biomass:

In this experiment, the dried alga was autoclaved at 121ºC, 15 lbs pressure for 20 min, 40 min and 60 min for seekof identifying optimization of thermal pretreatment time(Trivedi et al., 2013).

Microwave (electromagnetic radiation) pretreatment of algal biomass:

This was conducted with the microwave oven (Model-Daewoo electronics KOG-391) for 1, 3 and 5 min at 100□ C, 900W and 2450MHz for optimization of microwave pretreatment time, and then the treated biomass was dried in an oven at 60□ Caccording to Surendhiran and Vijay (2014).

Ultrasonication (mechanical) pretreatment

In this method, the alga was exposed to high intensity ultrasonic waves by using the ultrasonic bath (Model: Ultrasons-HD, 3000866), creating tiny cavitation bubbles around the cells. The bubbles collapse and emit shockwaves that shatter the cell walls.

The pretreatment process for algal cell wall destruction was performed in the ultrasound water bath. Dry algal biomass along with distilled water in a ratio (water to biomass as 3:1) was taken into a 100 ml screw top bottles and sonicated for 5 min, 10 min, 15 minand 30 min at a temperature at (50 ± 1 ºC), 180W for optimization of

Ultrasonication pre-treatment time. The treated biomass was filtered and dried in an oven at 60 C(Suganya and Renganathan, 2012).

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 modified Folch et al. (1957) (Shaltout and Shams El-Din, 2015)

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 (Afifyet al.,2010). The extract was shaken vigorously for one minute and allowed to undergo phase separation for 15 min in a separating funnel. 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 60oC to constant weight. The total extracted lipid yield (%w/w) was then quantified gravimetrically bysubtractingtheweight of the empty tube from the weight of the tube and the residue as in the following equation:

Total extracted lipid yield (%) = weight of lipid extracted (g) x 100
weight of algal biomass (g)

Esterification of fatty acids to biodiesel:
The extracted total lipid was reacted directly with a freshly prepared mixture of methanol, chloroform and HCl (10:1:1 v/v/v) at 90oC 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 fatty acids concentration was characterized gas chromatography (GC-QqQ/MS tripleQuade). Analysis system was an Agilent 7890A series GC system coupled with an Agilent 7000B QqQMS (Agilent Technologies Inc., USA). 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 Saravananand 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 using Scanning Electron Microscope (Jeol JSM-5300 scanning electron microscope, Tokyo, Japan) operated between 15 and 20 KeVatmagnification 10000 (a) and 20000 (b)(Surendhiran and Vijay, 2014).

Statistical analysis:
The evaluationstudy between the efficiency of the four physical pretreatments ofUlvafasciatafor enhancement of biodiesel production was conducted by applying statistical analysis that 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 cumulative total fatty acids were improved from 1148.94 µg g-1 (control) to a maximum of 2269.70 µg g-1and 1644.89 µg g-1 by using hydrothermal and thermal pretreatment of dried algal biomass for incubation period 40 min and 60 min, respectively(Table 1&Fig.1).In the case of using of microwave, TFAs improved to a maximum of 2497.89 µg g-1 after pretreating algal biomass for 5 min. while after 30 min of ultrasound waves' exposure of algal biomass, the TFAs improved gradually above (control) to a maximum of 2439.51 µg g-1 (Table 1&Fig.1).

By hydrothermal and thermal pretreatments, ΣSFAs improved from 979.43 µg g-1 (control) to a maximum of 1861.15 µg g-1 and to1311.82 µg g-1 for 40 min and 60 min, respectively. Considering ΣMUFAs, they increased from 136.98 µg g-1 (Control) to a maximum of 383.02 µg g-1and 301.70µgg-1by hydrothermal and thermal pretreatment for 60 min each (Table1). Moreover, PUFAs increased from 32.53 µg g-1 (control) to a maximum of 183.74 µg g-1 by hydrothermal pretreatment for 60 min, while in case of thermalpretreatment it was comparable to that of control for all exposure periods (Table1).

On the other hand, the contents of SFAs, MUFAs, and PUFAs increasedto 1554.66, 783.51 and 159.72 µg g-1using microwave for 5 min, respectively, while applyingultrasonication pretreatment for 30 min cause an increase to 1783.01 µg g-1, 587.23 µg g-1 and 69.33µgg-1, respectively(Table1).

Among SFAs group, palmitic acid C16:0 was the dominant fatty acid in the four pretreatments at all exposure times, recording a concentration of 965.66 and 687.89 µg g-1 by using hydrothermal and thermal pretreatments of U. fasciata for 40 min and 60 min, respectively (Fig. 2A&B), while it attained a maximum of 973.09 µg g-1and 907.10 µg g-1by using microwave for 3 min and ultrasonication for 30 min, respectively (Fig.2C&D). Behenic Acid (C22:0) was the second dominant acid, where it improved from 89.67 µg g-1 (control) to a maximum of 561.56 µg g-1and 374.17µgg-1by hydrothermally and thermally pretreating U. fasciata for 40 min and 60 min, respectively(Fig. 2A&B).By using microwave pretreatment for 5 min, its concentration upgraded to 391.52 µg g-1, while it attained a maximum of 579.46 µg g-1 by using ultrasonication for 15min (Fig.2C&D). The third dominant fatty acid; stearic acid (C18:0) increased from 29.61 µgg-1 (control) to a maximum of 106.02 and 55.79 µg g-1by hydrothermal and thermal pretreatment for 40 min and 60 min, respectively(Fig. 2A&B), The concentration of the acid raised to a maximum of 90.64 and 108.70 µg g-1 by using microwave for 3min and ultrasonication for 30 min, respectively (Fig.2C&D).

The long chain SFA, Lignoceric acid (C24:0) upgraded from 11.46 µg g-1 (control) to a maximum of 48.41 µg g-1and 32.80 µg g-1using hydrothermal and thermal pretreatments for 40 min and 60 min, respectively(Fig.2A&B). By using microwave for 3 min and ultrasonication for 15 min, lignoceric acid attained a maximum of 40.67µg g-1 and 54.63 µg g-1, respectively (Fig.2C&D).

Considering MUFAs, the oleic acid (C18:1c) was the predominant oneacid in the four pretreatments at all exposure times. The concentration of Oleic acid attained a maximum of 328.49 µg g-1and 233.58µgg-1by using hydrothermal and thermal pretreatments for autoclaving period 60 min each, respectively(Fig.2A&B). Using microwave for 5 min and ultrasonication for 15 min, its concentration attained a maximum of 572.72 µgg-1 and 499.47µgg-1, respectively (Fig.2C&D). Palmitoleic acid (C16:1) concentration decreased with autoclaving periods in the two pretreatments and by using ultrasonication(Fig. 2A&B&D), while it was comparable to control (39.90 µg g-1) in the case of microwave pretreatment (Fig.2C). Likewise, cis-10-heptadecenoic acid (C17:1) slightly increasedfrom 7.03 µg g-1 (control) to a maximum of 16.92,19.93 and 48.23 µg g-1µg g-1, after thermal, microwave and ultrasonication pretreatment for 60, 5 and 30 min, respectively (Fig. 2B&C&D). On the other hand, there was no effect on this acid by using the hydothermal pretreatment (Fig.2A).

The prevailing PUFA was cis- 4,7,10,13,16,19-Docosahexaenoic acid (C22:6) which improved by 30 folds from 4.53 µg g-1 (control) to 119.74 µg g-1 by using hydrothermal pretreatment for 60 min, while it slightly increased to a maximum of 11.35µg g-1 by thermally pretreating dried alga for 20 min(Fig.2A&B). Whileusing microwave for 3minand ultrasonication for 10 min, thisfatty acid concentration attained a maximum of 137.62 µg g-1and 57.35 µg g-1, respectively (Fig.2C&D). The second dominant acid was α- Linolenic acid (C18:3-α), which increased from 2.77 µg g-1 (control) to a maximum of 10.70 µg g-1 by using hydrothermal pretreatment for 20 min (Fig.2A). On the other hand, either thermal, microwave or ultrasonicationpretreatments had no significant effect on Linolenic acid concentration at all autoclaving periods (Fig. 2B&C&D).

It was obvious that the concentration of the other PUFAs was meager and the change between the control sample and that each pretreatmentwas in very narrow ranges for all exposure period.

SEM analysis showed that after hydrothermal and thermal pretreatments of Ulva fasciata, the cell walls of the algal cells were altered, creating pores in the cell wall (Fig.3&4). It was noticed from the SEM pictures that the created pores in case of Hydrothermal pretreatment were larger in size than that in thermal processes confirming the highest extraction efficiency recorded in case of hydrothermal pretreatment. On the other hand, SEM analysis showed that

after electromagnetic radiation (microwave) and ultrasonication pretreatments, there was degradation in algal cell wall (Fig5&6). Sharp cracked were noticed confirming the rupture of the cellular wall bonds and facilitating fatty acids extraction from the algae.

As far as the properties of the produced biodiesel are concerned, the CN from hydrothermally and thermally pretreated U. fasciata improved from 73.21 (control) to its maximum value (76.34) and (74.27), both after 40 min (Table1). The increase in CN was parallel to the increase in LCSF, that increased from 23.06 (control) to a maximum of 49.70 and 48.69, after hydrothermal and thermal pretreatments for 40 min,respectively(Table1). It was apparent that the value of CN using microwave pretreatment for 1 min (73.55) was comparable to that of control, while increasing radiation period showed no significant effect on CN. In the same trend, maximum LCSF (41.75) was attained after 1 min microwave pretreatment(Table1). By using ultrasonication, the maximum value (75.27 and 75.28) was attained after 5 and 10 min exposure, which was parallel to the increase in LCSF from 23.06 (control) to 50.19 and 49.93, respectively(Table1). All CN values after all pretreatments at different exposure periods complied with the cetane number standards (ASTM D6751 and EN14214), which indicates good ignition quality(Table1).

The Kinematic viscosity of biodiesel produced from U. fasciatashowed an increase from the control (4.68 mm2 s-1) to 5.32 mm2 s-1after hydrothermal pretreatment for 40 min, while it slightlyincreased for the other three pretreatments, but with no significant differences (4.96-5.09). The values of all the pretreatments are in accordance with the limits of ASTM6751-02 standard(Table1).

From data in Table (1), it is obvious that the there was no effect of the four pretreatments of U. fasciataon biodiesel density (ρ), where the values were comparable to that of the control (0.87 g cm-3)which was complied with the EN14214 standard range that ensure a good engine performance.

The HHVs of biodiesel produced from hydrothermally and thermally pretreated U. fasciata for 60 min and 40 min was 40.12 and 39.65 MJ kg−1, respectively, which were comparable to control (39.85 MJ kg-1) (Table1)and closed to the range set for regular biodiesel that indicates high energy content of produced biodiesel(Sivaramakrishnan and Ravikumar, 2012). The HHVs attained a maximum value of 40.12 MJ kg-1 after microwave pretreatment for 5 min, while its values were comparable to that of control ultrasonicationfor the different exposure periods(Table1).

Minimum IV values of produced biodiesel were recorded in case of hydrothermally and thermally pretreated U. fasciata was 31.36 and 18.67gI2 100g1fat, both after 40 min autoclaving (Table1) which was in consistence with almost minimum DU (23.68& 18.64), where IV increases with increasing degree of unsaturation of extracted fatty acids(Table1). Degree of unsaturation changed as the extracted fatty acids were either the cell wall phospholipids or the glycolipids or the inner cellular lipids.On the other hand, iodine values of biodiesel produced from microwave attained a maximum of 50.39 by pretreating algal biomass for 5 min, that synergized with increase in DU from 17.58 (control) to 44.16(Table1).The same pattern was recordedwhen using ultrasonicationfor 30 min, where the increase in value of IV (31.27gI2 100g-1fat) coupled with that of DU attaining a maximum of 29.76. At all incubation periods of all pretreatments, IV values were compatible with the limit of EN 14214 standard(Table1), which indicates less susceptibility to oxidation by oxygen and hence good ignition of the produced fuels with high power efficiency(Duarte and Maugeri, 2014).

The C18:3% in biodiesel produced from hydrothermally and thermally pretreated U. fasciatadecreased from 0.68% (control) to a minimum of 0.36 and 0.34%, for 40min and 60min exposure respectively. By using microwavefor 3 min andultrasonication for 15min, C18:3% decreased to the lowest percentage of 0.33 and 0.26%, respectively. All recorded C18:3% values of the four treatments confirm the good quality of the produced biodiesl. Inaddition it is important to highlight that all these values were in acceptance with EN 14214 standard limits(Table1).

On the other hand the polyunsaturated fatty acid methyl esters containing ≥ 4d.b% in the biodiesel produced were highly increased after the four pretreatments for all incubation periods, except forthermal pretreatment of dried U. fasciata for 40 and 60 min, they were improved to 0.68 and 0.61%, respectively (Table1). This may be attributed to the extraction of unsaturated fatty acids which mainly constitute the cellular wall and the pretreatment made it easily to be extracted, so it became a large portion of the produced methylesters.

The highestsaponification value (SV) of biodiesel produced from hydrothermal and thermal pretreatments was 196.41 and 199.06 mg KOHg−1 for 20 min exposure,respectively, which were comparable with SV of control

(200.66 mg KOHg-1). By pretreating algal biomass with microwave for 1 min and ultrasonication for 30 min, SV recorded a maximum of 195.48 and 194.05 mg KOHg-1, respectively (Table1). All recorded values by using the four pretreatments were withinlimits of UNI 10635 standard which confirm the high efficiency of burning the biodiesel and in avoiding misfire by increasing biodiesel volatility and decreasing its density (Azeem et al., 2015).

Statistical analysis of physical pretreatments of U. fasciata:

The results of comparison between the effect of the four pre-treatments methods on produced biodiesl quantity and quality showed great differences between them (Fig.1), which were confirmed statistically. The values obtained by analysis of variance (ANOVA) were used to evaluate this effect of physical pretreatments.It is easily noticed that, all physical pretreatments had high significant effect (at 0.01 level of probability) on the values of ΣTFAs, ΣSFAs, ΣMUFAs, SFAs/ MUFAs, C18:1/C18:3, C16:0, C18:0, C22:0, C24:0, C16:1 and C18:1c, except for ΣPUFAs (Table 2). Moreover, there was high significant difference between control and each pretreatment and among pretreatments, since all calculated F-values were higher than that of corresponding tabulated ones (Table 3).

It is also recorded that the biodiesel properties were also greatly affected by physical pretreatments of the alga before extraction, except density (Table 4).The statistical analysis revealed a significant difference between all physical pretreatments on LCSF, SFAs%, MUFAs%, PUFAs%, Db<1% values, while IV was significantly different between all pretreatments, except for dried thermal pretreatments at 40 and 60 min.Likewise, DU values were significantly different between all pretreatments, except hydrothermal pretreatment for 40 min and ultrasonication for 5 min (Table5).

Table (1):- Biodiesel properties of U. fasciata biodiesel after hydrothermal pretreatment, thermal pretreatment, microwave and utrasonication of dried U.fasciata for different autoclaving periods compared with ASTM D 6751-02 and EN 14214).

Biodiesel properties	DU	LCSF	IV (gI2100g-1fat)	SV						
(mg KOH g-1) CN	TFA wt (µg g-1)	SFA								
MUFA										
PUFA										
Kinematic viscosity (υ)										
(mm2 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	-	-							
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										
Hydrothermal										
pretreatment 20min 26.75	43.94	33.56	196.41	73.80	1776.44	1394.17	289.42	92.85	5.06	
0.87 39.59	0.80	3.65								
40min 23.68 49.70	31.36	193.19	76.34	2269.70	1861.15	279.69	128.87	5.32	0.87	
40.08 0.36	3.75									
60min 33.72 39.91	45.76	195.28	74.03	2225.83	1659.07	383.02	183.74	5.08	0.88	
40.12 0.66	5.64									
Thermal pretreatment of dried U. fasciata	20min	18.54	48.72	20.04	199.06	73.79	1255.63	1054.66		
169.15 31.82	5.05	0.87	39.47	0.41	1.43					
40min 18.64	48.69	18.67	198.35	74.27	1427.35	1195.02	198.59	33.74	5.09	0.87

	39.65	0.43	0.68									
	60min	22.16	45.63	21.77	197.97	73.51	1644.89	1311.82	301.70	31.38	5.03	0.87
	39.54	0.34	0.81									
Microwave	1 min	31.26	41.75	41.69	195.48	73.55	2056.08	1547.30	374.84	133.94	5.05	
0.87	39.61	0.35	5.48									
	3 min	34.75	38.19	47.19	195.27	73.14	2369.94	1716.86	482.72	170.37	5.00	0.87
	39.64	0.33	6.27									
	5 min	44.16	32.61	50.39	195.07	71.50	2497.89	1554.66	783.51	159.72	4.96	0.88
	40.12	0.42	4.16									
Ultrasonication	5 min	23.70	50.19	26.52	193.82	75.27	2088.80	1658.83	364.96	65.01	5.26	
0.87	39.69	0.30	2.18									
	10 min	25.78	49.93	30.26	193.10	75.28	2251.04	1756.95	407.78	86.31	5.28	0.87
	39.73	0.30	2.90									
	15 min	29.32	47.42	30.75	192.63	74.74	2414.81	1780.86	559.97	73.98	5.27	0.87
	39.88	0.26	1.93									
	30 min	29.76	44.28	31.27	194.05	73.91	2439.58	1783.01	587.23	69.33	5.16	0.87
	39.69	0.27	2.01									

Table (2):- Analysis of variance for fatty acids from physical 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	13	631374.40**	245173.60**	95828.81**	17579555 7	7.22**	1397.93**	51449.52**	1578.65**	51672.37**	410.80**	453.37**	61497.40**
Error	28	0	0.853	0	17587291 6	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0004	0.0001

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

Physical pretreatments	ΣTFAs	ΣSFAs	ΣMUFAs	ΣPUFAs	SFAs/							
MUFAs	C18:1/											
C18:3	C16:0	C18:0	C22:0	C24:0	C16:1	C18:1c						
Control	1148.94n	979.43n	136.98n	32.53a	7.15a	9.91 8n	768.42j	29.61n	89.67n	11.46n	39.90a	74.66n
Hydrothermal 20 min	1776.44j	1394.17j	289.42j	92.85a	4.82e	17.28m	757.16k	68.59j	382.65j	34.13j	23.95i	241.14j
Hydrothermal 40 min	2269.70e	1861.15a	279.69k	128.87a	6.65b	29.75i	965.66b	106.02b	561.56c	48.41e	10.88k	241.96i
Hydrothermal 60 min	2225.83g	1659.07f	383.02f	183.74a	4.33g,h	22.56l	944.73c	75.63h	433.54f	36.90i	29.75g	328.49k
Dried	1255.6	1054.6	169.1	31.82	6.24	26.7	538.2	43.19	306.6	27.2	5.80	136.9

thermal 20 min	3m	6m	5m	a	c	9j	8n	m	9m	7m	n	1m
Dried thermal 40 min	1427.35l	1195.02l	198.59l	33.74a	6.02d	26.06k	624.58m	46.65l	349.43l	30.29l	17.71j	160.411
Dried thermal 60 min	1644.89k	1311.82k	301.70i	31.38a	4.35g	42.48h	687.89l	55.79k	374.17k	32.80k	37.98c	233.58k
Microwave 1 min	2056.08i	1547.30i	374.84g	133.94a	4.13i	44.48g	848.28h	76.84g	415.57h	40.45g	30.09f	320.71g
Microwave 3 min	2369.94d	1716.86e	482.72d	170.37a	3.56j	53.53d	973.09a	90.46c	432.05g	40.67f	38.46b	408.62d
Microwave 5 min	2497.89a	1554.66h	783.51a	159.72a	1.98m	67.90c	888.88f	70.53i	391.52i	36.94h	30.45e	572.72a
Ultrasonication 5 min	2088.80h	1658.83g	364.96h	65.01a	4.55f	50.43e	837.53i	80.26f	528.54e	49.19d	25.34h	316.73h
Ultrasonication 10 min	2251.04f	1756.95d	407.78e	86.31a	4.31h	49.98f	870.90g	86.91d	570.20b	51.41b	8.041	339.28e
Ultrasonication15 min	2414.81c	1780.86c	559.97c	73.98a	3.18k	79.63a	891.64e	83.77e	579.46a	54.63a	6.70m	499.47b
Ultrasonication 30 min	2439.58b	1783.01b	587.23b	69.33a	3.04l	73.02b	907.10d	108.70a	533.35d	50.91c	33.90d	461.21c
General mean	1990.50	1518.13	379.97	92.40	4.59	42.41	821.72	73.07	424.89	38.96	24.21	309.71
L.S.D 0.01	0.0226	2.0837	0.0226	29921	0.0226	0.0226	0.0226	0.0226	0.0226	0.0226	0.0457	0.0226
CV	0.0005	0.0608	0.0026	619.96	0.2159	0.0236	0.0012	0.0137	0.0024	0.0257	0.0836	0.0032

Table (4):- Analysis of variance for properties of biodiesel form physical 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	13	187.20**	181.63**	366.43**	17.64**	5.22**	118.60**	81.68**	14.60**	0.08**	0.00004	0.14**	0.09**	10.12**
Error	28	0.0001	0.0001	0.0232	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001

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

Physical pretreatments	DU	LCSF	IV	SV	CN	SFAs					
(%) MUFA											
(%) PUFA (%)	Kinematic viscosity										
(υ) Density											
(ρ) HHV	C18:3										
(wt%) Db≥4											
(wt%)											
Control	17.58m	23.06n	18.48k	200.66a	73.21j	85.25a	11.92n	2.83h	4.68j	0.87a	39.85d	0.68b	0.98k
Hydrothermal 20 min	26.75f	43.94i	33.56e	196.41e	73.80g	78.48g	16.29j	5.23f	5.06e,f	0.87a			

39.59h	0.80a	3.65f									
Hydrothermal 40 min		23.68h	49.70c	31.36f	193.19k	76.34a	82.00d	12.32m	5.68e	5.32a	0.87a
40.08b	0.36d	3.75e									
Hydrothermal 60 min		33.72c	39.91k	45.76c	195.28g	74.03e	74.54j	17.21i	8.26a	5.08d,e	0.88a
40.12a	0.66b	5.64b									
Dried thermal 20 min		18.54l	48.72d	20.04k	199.06b	73.79g	83.99b	13.47l	2.53l	5.05f,g	0.87a
39.47j	0.41c	1.43l									
Dried thermal 40 min		18.64k	48.69e	18.67l	198.35c	74.27d	83.72c	13.91k	2.36m	5.09d	0.87a
39.65g	0.43c	0.68n									
Dried thermal 60 min		22.16j	45.63g	21.77l	197.97d	73.51i	79.75e	18.34e	1.91n	5.03g	0.87a
39.54i	0.34d,e	0.81m									
Microwave 1 min	31.26d	41.75j	41.69d	195.48f	73.55h	75.25i	18.23f	6.51d	5.05f,g	0.87a	39.61h
0.35d,e	5.48c										
Microwave 3 min	34.75b	38.19l	47.19b	195.27g	73.14k	72.44m	20.37d	7.19c	5.00h	0.87a	39.64g
0.33e	6.27a										
Microwave 5 min	46.83a	32.05m	54.00a	195.01h	70.60l	61.17n	30.83a	8.00b	4.93i	0.88a	40.11a
0.42c	4.16d										
Ultrasonication 5 min		23.70h	50.19a	26.52i	193.82j	75.27b	79.42f	17.47h	3.11i	5.26b	0.87a
39.69f	0.30f	2.18h									
Ultrasonication 10 min		25.78g	49.93b	30.26h	193.10l	75.28b	78.05h	18.12g	3.83g	5.28b	0.87a
39.73e	0.30f	2.90g									
Ultrasonication 15 min		23.19i	47.42f	30.75g	192.63m	74.74c	73.75k	23.19c	3.06j	5.27b	0.87a
39.88c	0.26g	1.93j									
Ultrasonication 30 min		29.76e	44.28h	31.27f	194.05i	73.91f	73.09l	24.07b	2.84k	5.16c	0.87a
39.69f	0.27g	2.01i									
General mean	26.88	43.11	32.24	195.73	73.96	77.21	18.27	4.53	5.09	0.87	39.76
0.42	2.99										
L.S.D 0.01	0.0226	0.0226	0.7018	0.0226	0.0226	0.0226	0.0226	0.0226	0.0226	0.0226	0.0226
0.0226	0.0226										
C.V	0.0372	0.0232	0.4711	0.0051	0.0135	0.0129	0.0549	0.2193	0.1964	1.1475	0.0252	2.3689
0.3304											

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.

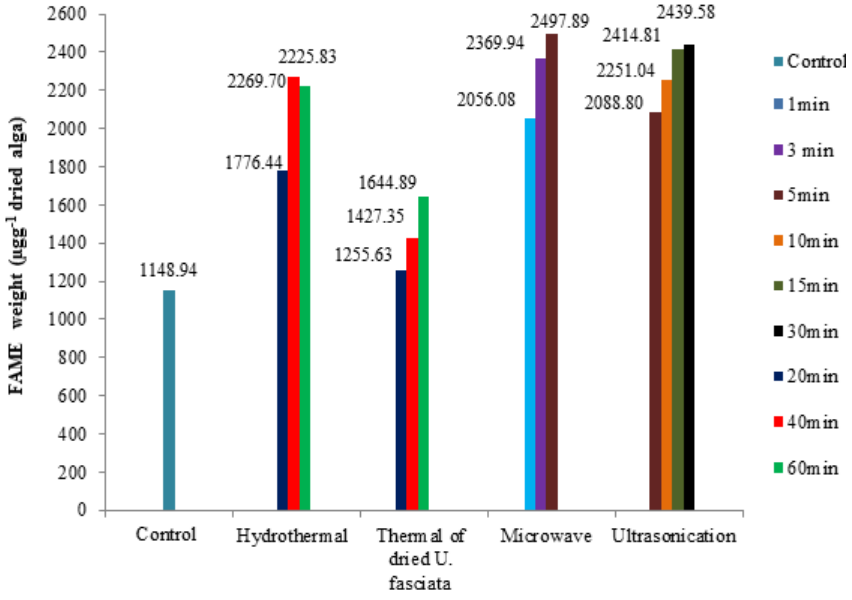

Figure (1):- The effect of different physical pretreatments of U. fasciataon total fatty acids weight (µg g-1dried alga).

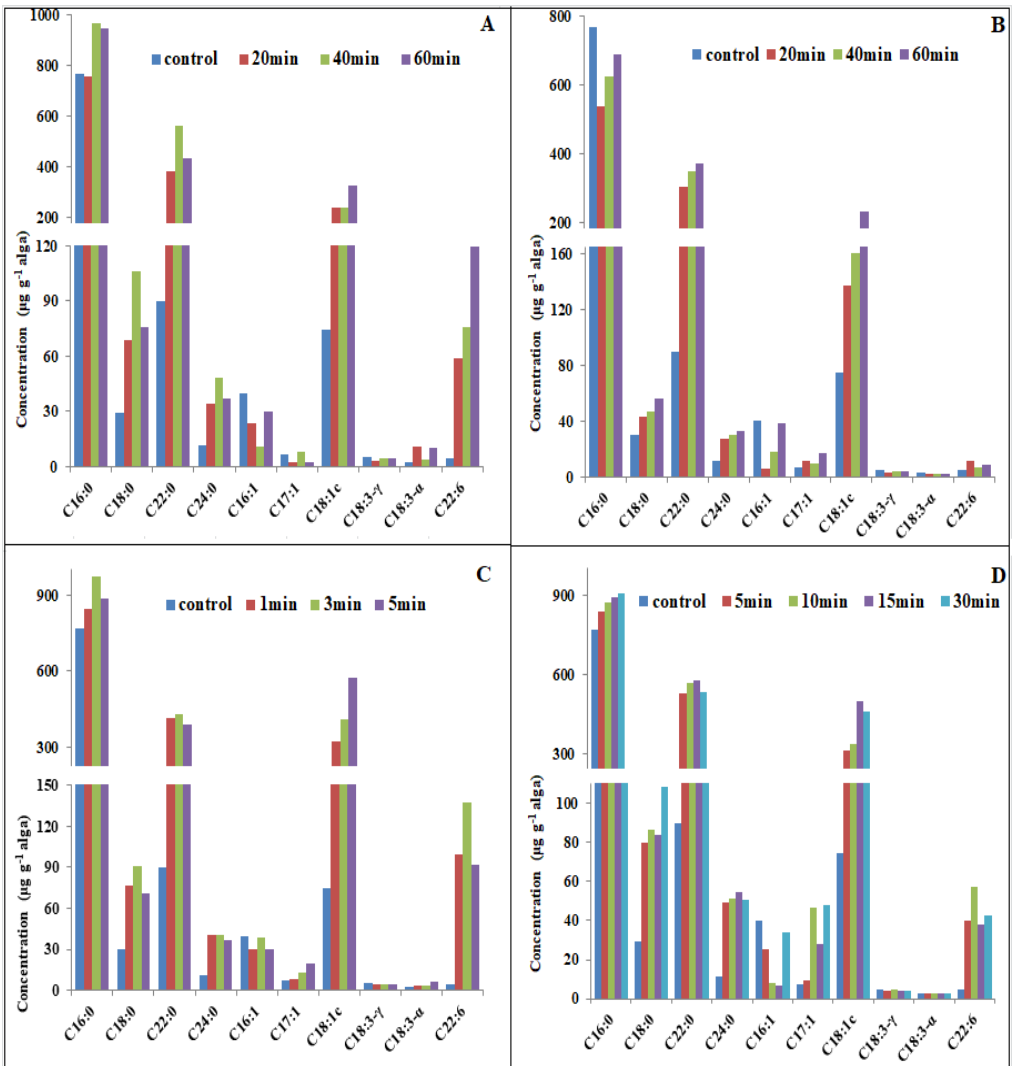

Figure (2):- Effect of physical pretreatments:hydrothermal (A),thermal (B), microwave (C) and ultrasonication(D)for different periods on concentration of FAMEs (µg g-1alga).

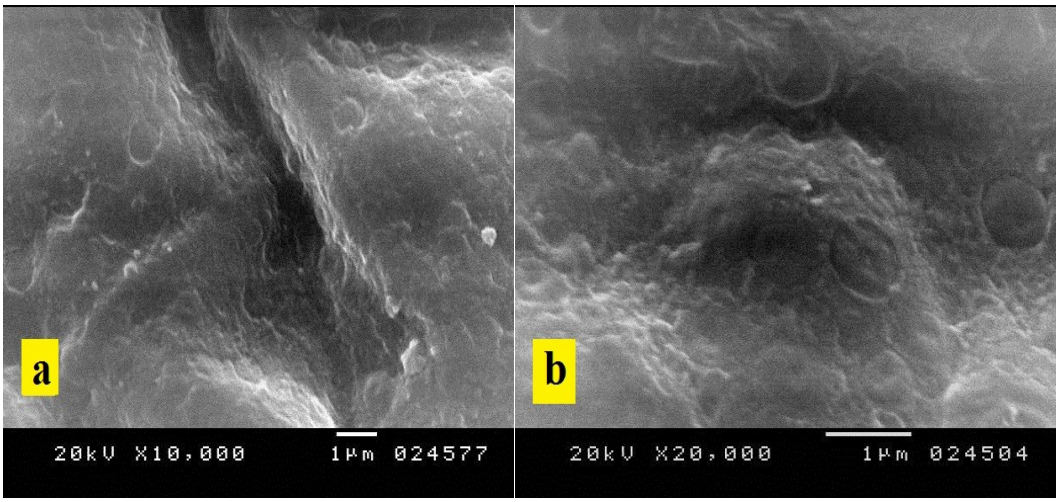

Figure (3):- Scanning electron microscope (SEM) images (Morphological analysis) of Ulva fasciata before treatment.

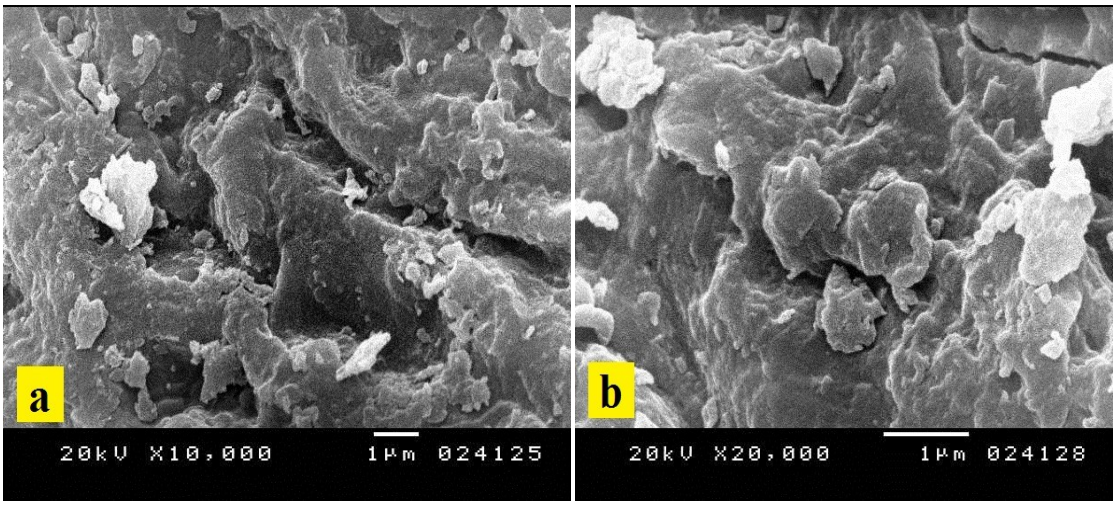

Figure (4):- Scanning electron microscope (SEM) images (Morphologicalanalysis) of Ulva fasciata after hydrothermal pretreatment for 40 min.

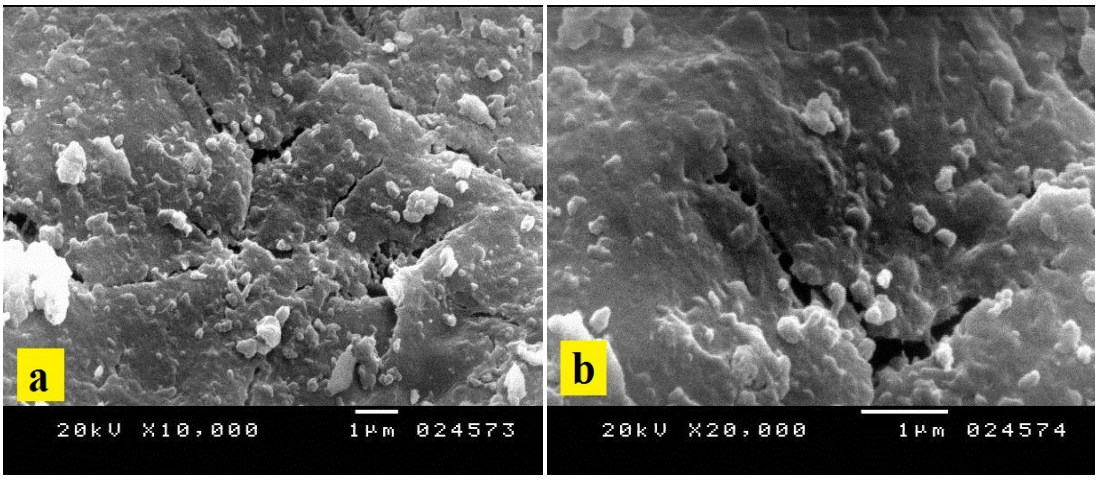

Figure (5):- Scanning electron microscope (SEM) images (Morphological analysis) of Ulva fasciata after thermalpretreatment of dried biomass for 60 min.

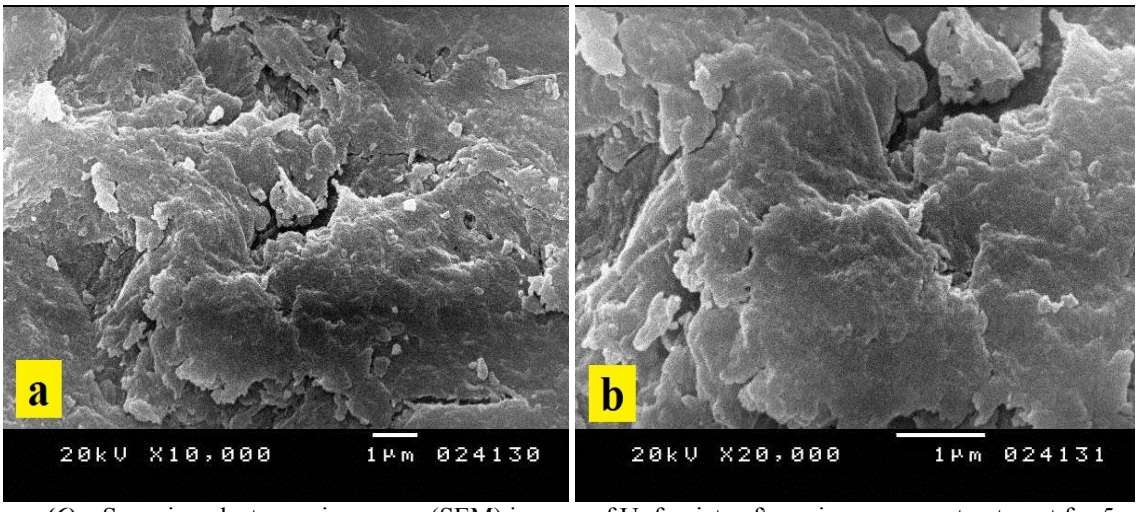

Figure (6):- Scanning electron microscope (SEM) images of U. fasciata after microwave pretreatment for 5 min.

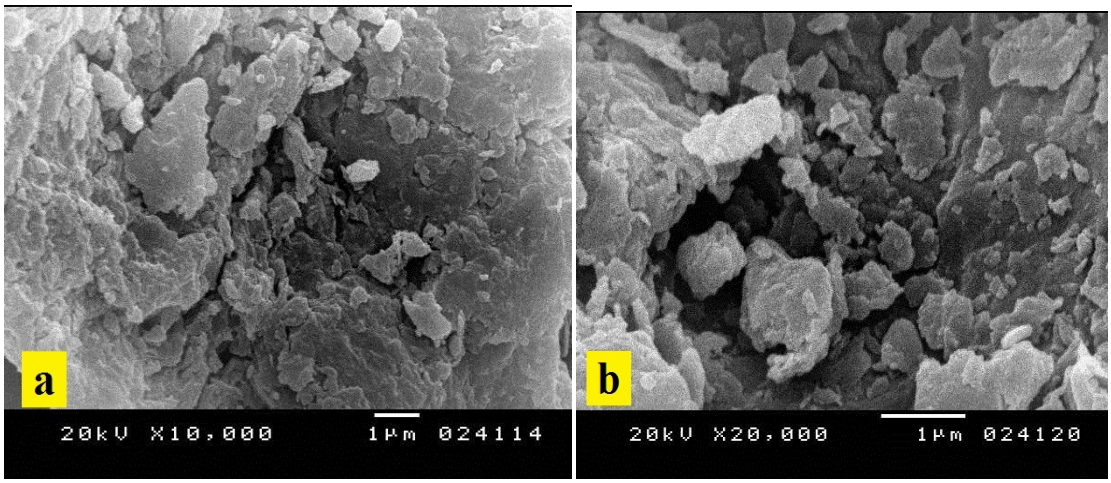

Figure (7):- Scanning electron microscope (SEM) images of U. fasciata after ultrasonication pretreatment for 30 min.

Discussion:-

In the present study, autoclaving of Ulva fasciata biomass was carried out for improving lipid extraction at various time intervals 20, 40, and 60 min. It was clearly noted that the effect of hydrothermal pretreatment is more efficient than thermal pretreatment of dried biomass.As recorded the20 min hydrothermal pretreatment produce higher TFAs yield than 60 min thermal pretreatment of dried algal biomass. In fact, the hydrothermal pretreatment process is considered as auto-hydrolysis of cellulosic linkages in the presence of hydronium ions [H+], generated from water and acetic groups released from hemicelluloses. Since the H+ ions produced by water ionization act as a catalyst in higher concentrations at high temperatures than in ambient liquid water, providing an effective medium for acid hydrolysis. In this trend,Lei et al.(2013) attributed the physical disruption of the cellulose structure to the high pressures, which are involved in autoclave; resulting in decreased cristallinity of cellulose as well as the degree of polymerization. However, algal biomass pretreatment by using hot water was considered as a clean and environmentally benign process. It was found that hydrothermal pretreatments maximize physical changes of cellulose and produce sugar degradation products during pretreatmentwhich leads to increase in the cellular wall pore size(Lei et al. 2013). Consequently, this will enhance solvent penetration into algal cells, and facilitate lipid extraction.Conclusively, the usage of water and high temperatures is a promising alternative to utilization of chemicals (e.g. acid or base hydrolyses)(Zu et al., 2006; Lei et al. 2013).This was confirmed in the present study by SEM analysis which showed that after hydrothermal pretreatment the cell walls of the alga were altered.There were pores in the wall and the cells weredisrupted and breaks appeared, leaving hollow areas where cells have been removed, and the inner parts of the cell were exposed. Prabakaran and Ravindran (2011)attributed this change to that autoclaves employ extreme heat and pressure to disrupt algal cells.

On the other hand, results showed that changing the autoclave pretreatment period affected the effectiveness of algal fatty acids recoveries. Our results agreed with Prabakaran and Ravindran(2011) who noted an increase in lipid content of 22% in Nannochloropsisoculatawith autoclave pretreatment at 121°C for 5 min, but higher yields were achieved using microwave pretreatment. In this trend, Surendhiran and Vijay (2014)pretreated Nannochloropsisoculatabiomass by using autoclave at 10, 20 and 30 min and achieved the highest lipid recovery of 29.34% at 30 min treatment which were higher than those without initial treatment.

Thus, the produced biodiesel has high yield and high quality, which confirm the high efficiencyof thermal pretreatment for enhancing the biodesiel production from U. fasciata. It is noteworthy to mention that the use of autoclave pretreatment of U. fasciata prior to lipid extraction is advantageous because it disturbs the extracellular cell membrane allowing for easier recovery of lipids due to increased penetration, although it gave lower TFAs yield than chemical pretreatments. No hazardous substances are used like chemical and no release of harmful compounds or disruption to the environment. Furthermore,this method can be easily reused and costs associated with maintenance can be restively low. However, this technique has somewhat disadvantages, where it is difficult to upscale; long duration of time required for effective disruption, and large scale use would require high costs due to high energy consumptions required for high heat and pressure(Al-Hattab and Ghaly, 2015).

The variation ofexposure time to microwave radiation showedsignificant influence on the effectiveness in the destruction of the cell wall, and hence had significant effect on lipid extraction. In the current study, it is clearly indicated from SEM analysis the effect of microwave pretreatment of Ulva fasciata which changed the cellular morphology. The cell wall degradation showed as presence of several micro cracks. This could be explained as radiation penetrated through the cell wall structure and increase in porosity which facilitate lipid extraction and hence biodiesel production. Our results agreed with that of Sostaricet al. (2012), where they attributed the higher oil yields by pretreatment biomass using microwave irradiation to micro-cracks present in the cell wall.

However, Lee et al.(2010) tested the effect of bead milling and microwave cell disruption techniques on lipid extraction and found that microwave pretreatment method resulted in higher lipid yield for the three microalgal species (Botryococcus sp., Chlorella vulgaris and Scenedesmussp.).Dejoyeet al.(2011)reported that extraction of oil from pretreated microalgae with microwave showed higher yields. In contrast, Surendhiran and Vijay (2014)recordedthat microwave pretreatementmethod did not significantly affect cell disruption, with a decline in oil content as the time of exposure was prolonged. Also, Zheng et al.(2011) explained the ineffeiciency of the microwave pretreatment techniqueas the lipid extracted from algae in this method becamevolatile during cell wall disruption and lipid extraction process.On the other hand, Patilet al. (2011) noted that the reaction time has a significant effect on the effectiveness of the microwave pretreatment and that increased duration of microwave exposure increased the oil yields.

It was recorded in the current studythat TFAs yield of U. fasciata was enhanced by total amount (1.8-fold) after ultrasonic pretreatment for 5min compared to untreated algae. By increasing exposure time to 10, 15 and 30 min, TFAs yield increased about 1.95, 2.10 and 2.12-fold, respectively, which indicates more effective cell disruption. From the economic point of view, the ultrasonication for 15 min could be considered the optimum pretreatment time. Also, the high SFAs content of the algae after ultrasonication pretreatment make it a potential feedstock for biodiesel production. Moreover, the predicted biodiesel properties also confirm the suitability of ultrasonication pretreatment of the algae for biodiesel production, especially the exposure time of 15 min.The major advantage of the sonication process is that it generates relatively low temperatures when compared to microwave reactors and autoclaves, thereby leading to less thermal denaturation of biomolecules. Furthermore, it does not require the addition of beads or chemicals, which have to be removed later in the process, which in turn will incur more cost. It is noteworthy to mention that prolonged ultrasonication leads to the production of free radicals, which may be detrimental to the quality of the extracted oil(Kumar et al., 2015).

In a good agreement with our results, Suganya and Renganathan (2012) reported that, through ultrasonication of green macroalgaUlva lactuca, extraction efficiency was achieved 2.25 times higher than that of direct extraction of oil. They observed that the increase in the ultrasonication pretreatment time increased the oil yield from 2 to 5 min. At 5 min the yield was found to be 8.25% and the maximum oil extraction yield of 8.49% was obtained at 6 min duration of pretreatment. After 6 min, the amount of oil yield was found to be constant.Moreover,Menendez et al., 2013, indicated that ultrasonication has been noted to significantly increase the lipid and FAMEs yields and reduce the extraction time.

Lee et al.(2009) found that the sonication method showed the highest efficiency for Chlorella sp., for Scenedesmus sp., followed by microwaves, bead beating, osmotic shock and autoclave. Koberget al. (2011) reported a lipid yield of 18.9% and 32.8% in Nannochlorpsissp. by using microwave and ultrasonication pre-treatment, respectively. In this trend, Prabakaran and Ravindran (2011) described ultrasonication as the most efficient among five cell disruption methods tested for extracting lipids from Chlorella sp.and the most applicable for large-scale lipid extraction from microalgae. In fact, there are some contradictions in the literature regarding scale up. Mercer and Armenta (2011) stated that ultrasound maybe difficult for upscale, whereas, Halim et al.(2012b) noted that this technique is moderately suitable for scale up.

In contrast to the present study, De Souza Silvaet al.(2014) tested the pre-treatment of microalgae culture by using microwave, autoclaving and ultrasonication technology for lipid extraction and found that ultrasound resulted in the lowest yields. Similarly, Ranjanetal.(2010) reported that ultrasound assisted microalgae lipid extraction demonstrated more distorted clusters of biomass on micrographs, in comparison to cells with solvent penetration.

Considering the extraction time, Adam et al.(2012) noted that increasing the treatment time resulted in higher lipid recovery efficiencies. Similarly, McMillan et al.(2013) found that increasing the sonication time resulted in greater

cell disruption efficiencies. However, Mendez et al.(2014) reported also that an increase in extraction time from 5 to 20 min increased the lipid yield from 31 to 36%, respectively.In contrast, Tang et al.(2011) found that increasing the ultrasonic treatment time over the range of 15 to 90 min has no significant effect on the microalgae lipid recovery. Moreover, Jeon et al.(2013)and Menendez etal.(2013)stated that disruption of microalgae biomass by using horn sonicators is not suitable for microalgae and lipid yield didn't increase compared to conventional extraction techniques and this was attributed to the cavitationsare localized.

As shown in Figure (6), SEM analysis showed that after ultrasound waves (ultrasonication)pretreatment of Ulva fasciata, the cellular morphology changed and degradation in algal cell wall was noted, presence of several cracks in the cell wall were due to the cavitation's effect.The ultrasound waves penetrated through the cell wall structure, increased porosity and shattered cell wall, therefore facilitatinglipid extraction and hence fatty acids extraction and consequentlybiodiesel production.

By comparing the four treatments, it was obvious that all physical pretreatments of U. fasciata had significant effect on TFAs yield, where ÓFA was 1148.94 µg g-1 (control), but when using hydrothermal pretreatment for optimum time period (40 min) before extraction it resulted in about 2-fold increase in ÓFA yield. While the thermal pretreatment of the dried alga atoptimum autoclaving time(60 min) resulted in increase in about 1.4-fold in ÓFA yield. Regarding the microwave pretreatment atoptimum radiation period (5 min), it resulted in about 2.2-fold increase in ÓFA yield, while ultrasonication gave about 2.1-fold increase in ÓFA yield at optimum ultrasound exposure time (15 min). These results indicate the effect of these pretreatments on the TFAs yield as a results of different effects on cell membrane, such as disrupting, or degradation and cracking, or shattering the cell wall.They all enhanced the lipid extraction and fatty acid yield processes by different percentages but they all are efficient.

Comparing the four physical pretreatments, the negative aspects of the Ultrasonication technique are outweighed by its effectiveness, rapidness and relatively low costs when compared to thermal pretreatments techniques which downrated due its high operational costs, lengthy treatment times, high maintenance costs and the scale up difficulty. On the other hand, autoclave techniques were deemed unsuitable for U. fasciata pretreatment because of the high costs and scale up.

Noticeably, the current study highlighted microwaves as thebestof the physical pretreatment options from biofuel production than other processes. In addition to be industrially applicable due its short reaction time, low-operating costs, and efficient extraction of algal oils. However, the disadvantage with the microwave-assisted process is the maintenance cost particularly on a commercial scale.Microwave algae assisted lipid extractions have been noted to be the most applicable method for large scale use due to its simplicity and effectiveness(Lee et al., 2010). The rapid extraction time, high heating rates, low operating costs, environmentally friendly nature, lesser solvent requirements, high product purity and high efficiency make it an attractive method for microalgae lipid recovery(Al-Hattab and Ghaly, 2015).

Regarding the physical properties of the produced biodiesel after all physical pretreatments, it had a high quality and its properties are complied with the ASTM D6751 and EN14214 standards, except ≥ 4d.b%, which was slightly higher than the limit of EN14214 standard, indicating that the produced biodiesel has high yield and high quality.

Thus, it could be concluded that microwave pretreatment is the most suitable pretreatment method for U. fasciata for biodiesel production followed by ultrasonication.

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