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Chemical Characterization of Algae Oil and Optimization of Transesterification Parameters for Boosting of Algal Biodiesel Production from *Spirulina* Wild Stuff Explored in the Natural Habitation

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Abstract

The investigation was carried-out on standardizing the parameters for achieving biodiesel production from a distinctive approach where the identified wild stuff of algal biomass (*Spirulina maxima*) grown at its natural conditions. The composed algal wild stuff was subjected to extraction of the crude lipids using a flawless solvent system coupled with the soxhlet method. The accumulation of lipid contents was expansively analyzed and the oil obtained from lipid content was subjected to transesterification using both acid (H_2SO_4) and base (KOH) catalysts for its analogous fatty ester which is the potential way out to the high viscosity elements that ultimately converted algal oil into biodiesel. Further, the optimization of transesterification parameters for boosting algal biodiesel production at variable reaction factors was carried-out with respect to; molar ratio, temperature, reaction time, stirring speed, and amount of promising catalysts were also evaluated. In the acid esterification process, the acid value (AV) was reduced from 11.43 to 0.52 mg KOH/g of the feedstock and, the most favorable conditions for the highest yield of esterified oil were found at the molar ratio 8:1, temperature 65^0C, catalyst concentration 1% (wt%) H_2SO_4 and the stirring speed of 600rpm for a reaction time of 90th minute. Whereas in the alkali transesterification, the optimum biodiesel (96.6%) was achieved, and promising conditions were recorded at molar ratio 6:1, temperature 60^OC, stirring speed of 500 rpm, catalyst concentration 0.3% (wt%) of KOH for a reaction time of 60th minute. The produced algal biodiesel was evaluated in contrast to ASTM standard specification and all the results within standards limit were observed. The Physico-chemical properties of biodiesel-derived crude glycerol samples were also determined and the compositional analysis of crude glycerol was found to be significant in crude glycerol. Besides, the work-out on the effectiveness of capital and operating costs are found to be rational and this certainly makes algae fuel production commercially viable. However, the novelty of this study is to explore the wild stuff of *S.maxima* algae from its natural habitat for biodiesel production is significantly cost-effective which is possible by omitting the algal culturing segment *in vitro*.

Keywords: *Spirulina maxima* (wild stuff); Chemical characterization; Transesterification; Methyl esters, GC-MS & FTIR; Optimization of Biodiesel; Techno-economic feasibility.

1. Introduction

The exploitation of various resources for the production of energy has now arrived at the level that the whole world is depending on the same. Fuels are considered to be the most important sources of energy production. For a decade, there is an alarming situation regarding overutilization with the hankering of all the available sources are being diminished due to increased biofuel production [1,2]. Therefore, algae (an undifferentiated mass like primitive plant community) gaining the most attention and preferred for the production of biofuels; by keeping the food crops oriented problems. The exercise on food crops oriented bio-fuels and its outcome demonstrating that the utilization of crop and food-producing plants will not be the best deal on a real-time approach for bio-

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fuel production as they can certainly disturb the food needs [3]. Thus, the precedence on algal sources and asserting its diverse category found to be most promising based on its efficiency and production competence of all types of biofuels [4, 5]. In general, algae have 20%-80% oil contents that could be successfully converted into different types of fuels such as kerosene oil and biodiesel. Besides, the biodiesel production from the source of algae has been established with techno-economic feasibility [6]. The different algal species accomplished as superior and potential for the production of biodiesel are *Chlorella, Ocillatoria, Navicula, Spyrogyra, Scenedesmus, Pinullaria, Tribonema, Ulothrix*, and *Euglena*, etc [7, 8].

Even though a lot of works have been carried out on algal sources the selection of appropriate species having potential characteristics explicitly on growth parameters as well as biofuel production is of great interest. Moreover, algal growth needs a suitable environment and the factors relating to its cultivation and harvesting on a large scale are the need of the hour. Besides, the scientific and technical experience for developing of most promising algal species under controlled conditions is a big deal and the cost-effectiveness in not synchronized yet especially on its cultivation and harvesting aspects followed by producing requisite amounts of biodiesel in a short time and less energy utilization is also a massive unit operation [9].

1.1. Algae to Biofuels

However, the alternative ideology needs to be functioning that, the microalgae should make obvious that in particular the exploration of wild stuff material as most potential resources for the superfluity of bio-fuels owing to their trouble-free, low-cost and simple fostering system at its natural habitat [9,10]. The biodiesel production from algae uncultivated stuff has been an area of substantial interest which is because of superior output in corresponding with other desired crop plants. Interestingly, some algal species having doubling times in a few hours considerably at its natural environment as compared to in vitro conditions. The marketable production of biodiesel from some microalgae types is still not feasible due to the culturing difficulties in vitro [11]. Also, the selection of the right media, organizing suitable reactors in line with low biomass concentration, and costly downstream processes are practically not workable. However, keeping the above verity, the viability of microalgae biodiesel production can be achieved by focusing on the feed-stock collection directly at its natural habitat such as tanks, ponds, ditches, river/stream sides, lakes respectively. Moreover, it is a real-time to develop a low-cost technology for algal biomass harvesting conventionally through its natural habitat would be

most beneficial in facilitating low-cost courses of action such as material processing, drying of biomass, and oil extraction respectively [12]. In addition, Algae-based bio-fuels are technically and economically feasible and cost-competitive, require no additional lands for its cultivation practices at its natural condition, require minimal water usage, and mitigate atmospheric CO_2. Besides, algae are having a challenging attitude with respect to growth, very resistant trait, and can rise virtually at diversified environmental conditions like, in salt and fresh water, and even in the salt & wastewater. Furthermore, algae can reproduce quickly by utilizing CO_2 for photosynthesis and a reduced amount of water than other crop plants, and additionally, biofuel from algae is biodegradable and contains no sulfur and it is not toxic [13].

1.2. Algae and its diverse nature

Many algal strains like *Chlamydomonas, Chlorella, Scenedesmus, Botryococcus braunii*, and some species of *Spirulina* have also been reported to produce bio-fuels. The selection of algal strains is exclusively dependent on various factors like oil content, production yield, and downstream processing and also on the adaptability of microalgae towards high oxygen concentration, temperature variations, and water chemistry. The efficiency of microalgae in converting captured solar energy into biomass exceeds the potential of terrestrial plants [14]. Microalgae do not compete with terrestrial plants for land or water supply as they can be grown in wastewater, leading to their remediation coupled with biomass production. The acumen of microalgae to inhabit diverse habitats could be exploited to allow for the production of compounds near the site of use, which could reduce the transportation costs. They grow easily with basic nutritional requirements like air, water, and mineral salts with light as the only energy source. They grow on liquid media, so diverse wastewater can also be utilized, which can be efficiently remediated by algae coupled with biofuel production [15].

Algae possess higher photosynthetic levels and growth rates that can be used for the production of desired bio-fuels. They can contain considerable amounts of lipids that are mainly present in the thylakoid membranes so, their bio-fuels are non-toxic and highly biodegradable. They are essentially free-living chloroplasts and are the pinnacle of minimizing structural components. They have high carbon dioxide sequestering efficacy thereby, reducing GHG emissions. The cultivation of algae is self-governing of seasons as they can be cultivated around the year and have a minimal environmental impact. The cultures can be facilitated to produce high yields through technological interventions of genetic engineering, synthetic biology, metabolic

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engineering, and so on as algal systems are readily adaptable. The biofuels from algae are multifarious irrespective of environmental conditions. The Carbohydrate component of biomass is used for bio-ethanol production, while algal oil for biodiesel and the residual biomass can be utilized for methane, fuel gas, or fuel oil production.

1.3. Algae to Biodiesel

The biomass after biofuel production can further be used as a source of many value-added products like Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA), nutraceuticals, protein supplements, therapeutics, bio-control agents, fertilizers, animal feed, and aquaculture. The exploration of biofuels from microalgae is under their unique potential characteristic features which are evidenced through its eco-friendly nature, non-toxic characteristics, biodegradability, and lower net carbon cycle compared to conventional diesel fuels [16]. Further, biodiesel has comparable engine performance to petroleum diesel fuel, while reducing sulfur and particulate matter emissions. However, biodiesel is a biodegradable alternative fuel derived from renewable sources and is nontoxic with respect to its characteristic features. During the manufacturing process, triacylglycerols (TAGs) are transesterified with an acid or alkali catalyst to produce biodiesel and glycerol [17].

The algal biodiesel production processes fatty acid methyl esters (FAME). The chemical composition of biodiesel is generally produced by transesterification of algal oil in the presence of acid or alkali as a catalyst. The biodiesel from algae can be derived directly from the transesterification of algal biomass [18, 19]. Alternately, it can also be produced by a two-step process wherein the lipids are initially extracted and later on transesterified, though either of the processes involves lipid extraction through solvents and alcohols like methanol, isopropanol, and petroleum ether. The process of direct transesterification is fast and cost-effective technology [20]. Biodiesel generated from microalgae can be an excellent alternative to the current diesel crisis, but to efficiently produce biodiesel from microalgae, strains with a high growth rate and oil content have to be selected.

1.4. Gas chromatography (GC) analysis of Biodiesel

Classification and identification of biodiesels and petroleum fuels are a crucial challenge in the scientific investigation for its purity and have high importance in cases of commercial, industrial, and forensic criminal acts. Several methods have been proposed to identify and discriminate fuel samples, based on gas chromatography-mass spectrometry. The Gas Chromatographic (GC) method was employed in particular to know-how the fatty acid methyl ester (FAME) composition in the produced biodiesel by comparing with the standard diesel fuel sample. The main difference between the chromatographic profiles is the variability in the number of the peaks. The diesel has a higher number of peaks corresponding to long chain hydrocarbons.

1.5. The value-added perspective of crude glycerol

In biodiesel, the most significant residues and by-products are glycerol, biodiesel washing waste waters, methanol, and solid residues respectively. But, glycerol is a major by-product generate in the biodiesel manufacturing process. It can involve the largest revenue for the biodiesel industry. Therefore, the analysis of crude glycerol is important for the biodiesel industry future for its enhanced utilization.

1.6. Glycerol and Glycerides in Biodiesel

Fatty acid methyl ester (FAME) biodiesel is produced from the transesterification of triglycerides in oils and a fat through a reaction that uses methanol in the presence of a catalyst is explained in Figure 1. The main byproduct of this reaction is glycerol and, after the reaction is complete, most of the glycerol can be washed away with water. Any remaining glycerol is measured and reported as free glycerol contamination.

The biodiesel reaction also has a few intermediate steps during which mono- and diglycerides are formed. Mono-, di- and unreacted triglyceride contaminants are water-insoluble, which makes them more problematic to remove. The amount of residual glycerides depends on how successful the transesterification reaction was, and a poor reaction results in fuel with insufficient stability that can significantly affect engine performance [18].

Glycerol is a colorless, odorless, hygroscopic, and sweet-tasting viscous liquid and because of its humectants properties. On the whole, biodiesel is produced by the transesterification process by involving plant oil or animal fat using alcohol characteristically; methanol is performed in presence of a catalyst frequently sodium hydroxide or potassium hydroxide. The resulting product not only contains ester but also by-product and residual alcohol and catalyst respectively. Hence, the traces of glycerol can be generated apart from biodiesel. Generally, the crude glycerol yield of about 10pounds may be generated for every 100 pounds of biodiesel production [21]. On the quick expansion of the biodiesel industry, a surplus of crude glycerol is also being generated which is considerably expensive to purify the same for use in various sectors such as, pharmaceutical, cosmetic, soap manufacturing, and food industries. Therefore, the priority has to be made

for the sustainable utilization of glycerol during biodiesel production.

The different methods are accomplished for characterizing the glycerol separated from biodiesel to obtain value-added products. One of the promising approaches for assessing glycerol has been presented in the Figure 2.The global production of biodiesel has also facilitating glycerol generation from 250000 tonnes to 1.225 million tonnes.

Therefore, it is of great importance for scientists to find new applications for refined and crude glycerol [21, 22]. Therefore, the search is on for documentation on diversified algae and their respective species at their natural origin and also monitoring of the conservation of algal diversity at their natural habitat. This database will make it possible to obtain desired algal types for biodiesel production [23].

1.7. About Spirulina algae

Spirulina is profusely available in the clusters of algal blooms at the natural ecosystem and regarded as wild stuff belonging to the group Cyanobacteria (blue-green algae). For a long time, *Spirulina* was regarded as algae, on the other hand, it is now known that it is a bacterium, thereby it can be able to perform photosynthesis; subsequently, it is classified as Cyanobacteria. This alga is found in ponds, lakes with increased pH, therefore, it can survive in alkaline waters, with a pH between 10 and 11. The algae *Spirulina* was found to be most viable for biodiesel production as it is having significant lipid contents in its biological organization [23, 24].

Apart from these, *Spirulina* distinctively has been used as an additive in a variety of human foods and animal feeds. The current use of this resource has three precedents: traditional, scientific, and technological development, and the so-called green tendency. *Spirulina* is being cultured in vitro by employing idiosyncratic factors in controlled conditions. But, it has been focused to maintain and monitor the wild stuff culture at its natural habitat followed by attempts with the cultural approach in large explicitly at its natural environment such as outdoor ponds, lakes, river streams, trough/gulley; other desired waterway channels under moderate-controlled conditions [25, 26].

On the other hand, it is now widely cultured throughout the world as an additive in a variety of human foods and animal feeds. The current production of *Spirulina* worldwide is estimated to be about 3000 metric tons. Currently, more than 70 percent of the *Spirulina* market is for human consumption, mainly as a health food because of its- -rich content of protein, essential amino acids, minerals, vitamins, and essential fatty acids [27, 28, 29]. The Open System of Natural habitat (OSNH) was most favorable for the exploration of algal

biomass in particular with S. maxima. The production and the minimum technical strategies can contribute to the significant production of *S. maxima* in its natural region like controlling the culture temperature, reducing production cost and retrospective climatic data-based 'Habitation Information Modeling' (HIM) cluster in their natural environment [30, 31, 32]. Hence, the proposed decisive objectives can be a distinctive approach to reduce the burden on culturing constraints of algae in vitro. However, the main focus of the present study is to evaluate the efficiency in the wild stuff of algae collected directly at its natural origin for biodiesel recovery, characterization, and optimization of transesterification parameters with variable reaction parameters to achieve maximum yield of biodiesel.
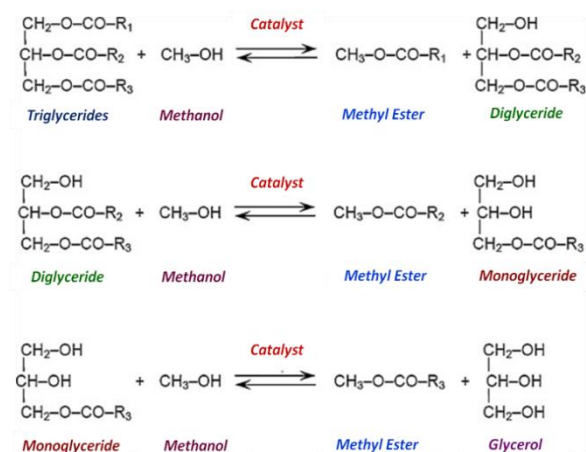

Fig.1. Transesterification reaction process with three-step equations for the production of Bio-diesel from triglycerides

2. Experimental Section

The *Spirulina maxima* algal material was collected productively in its natural origin at different locations of Devarayana Durga, Tumakuru district, Karnataka during 2018-2019 has been presented in Figure 6A, B & C. The growth expressions of algal samples were compared in different water bodies distributed in the study area to assess the variability.

2.1. Materials

Pure potassium/sodium hydroxide as the alkaline catalyst, anhydrous sodium sulfate, chloroform, and methanol was procured from Merck Co. (Mumbai). A commercial Egyptian petro-diesel sample was obtained from a local fueling station. Besides, highly pure analytical grade chemicals were selected for this investigation: 99.8 % pure CH_3OH (methanol), 96 % pure H_2SO_4 (sulfuric acid), 86 % pure KOH (potassium hydroxide), diethyl ether, methylene chloride, n-hexane.
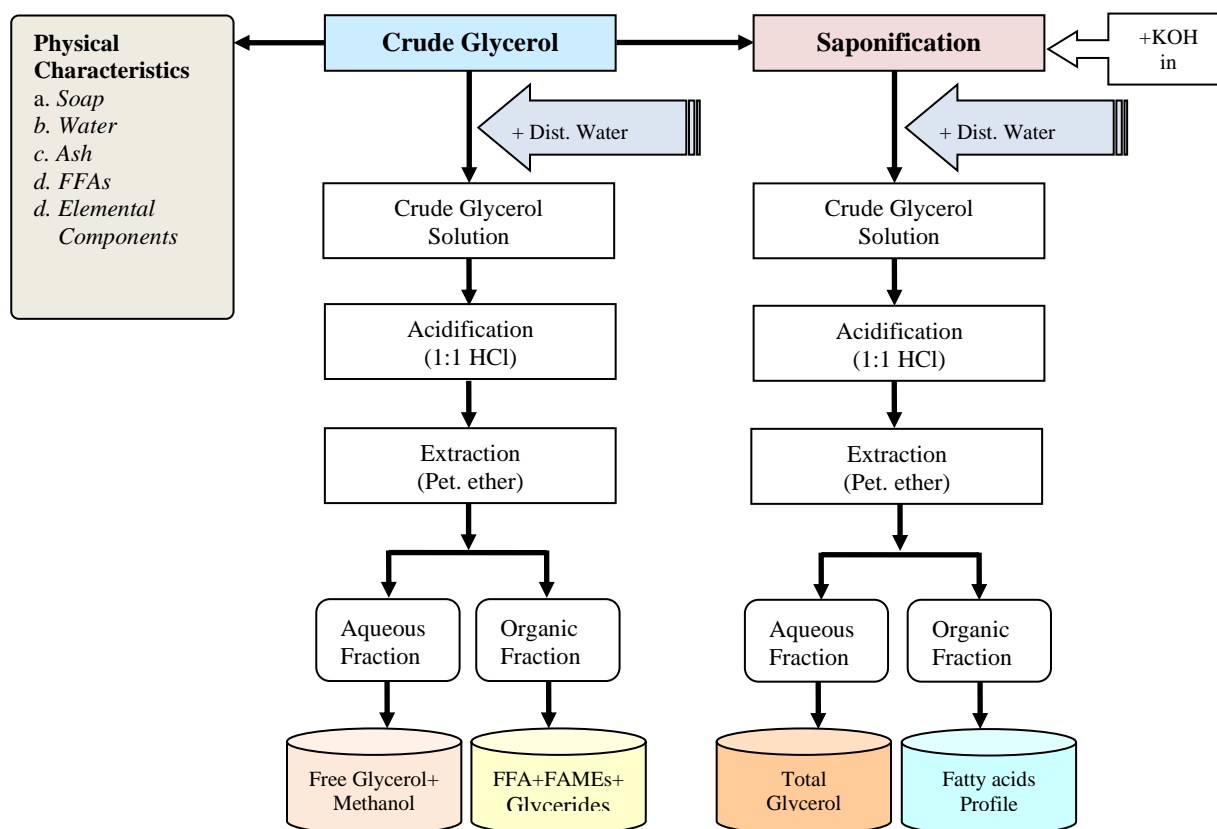
Fig.2. Schematic approach on Fractionation and characterization of Crude Glycerol

2.2. Taxonavigation of collected Microalgae

The algae are placed in a diverse group of species which can be broadly described as oxygen-producing, photosynthetic, unicellular or multicellular organisms excluding embryophyte terrestrial plants and lichens; they were subjected for classification by dividing under main taxonomic groups based on their pigmentation *i.e.,* Phaeophyta (brown algae), Cyanophyta (Blue-green), Chlorophyta (green algae), and Rhodophyta (red algae-13). Consequently, the collected algal species were studied under a light microscope and identified with the help of standard references which is presented in Figure 6C&D. Besides, the quantitative analysis was also made using a plankton-counting cell (Sedgwick rafter) and identification and enumeration of algae were done as per the standard methods [33, 34].

2.3. Collection of Algal Wild feedstock

The water samples with a visible micro-algal population of *Spirulina maxima* were collected from different water bodies such as ponds, lakes, temple tanks, and river streams that are located in and around the Devarayana Durga biodiversity area of Tumakuru

district (Karnataka), India. In the samples, the identified micro-algae, a *Spirulina maxima* was collected directly from its natural habitat shown in Figure 6B & C [25]. Further, the collected algal wild stuff biomass was allowed for drying and then the dried biomass and stored for further oil extraction processes in association with biofuel division, Hamsageetha Research Foundation, Tumakuru, Karnataka state, India represented in Figure 6E & F and Table 1.

2.4. Growth analysis of Algae

The growth of algal biomass was monitored on a regular interval of days up to 30 days in its natural habitation. The growth of biomass was recorded with respect to days and the lipid content was also assessed in the specified days of interval.

2.5. Preliminary phytochemical screening and Nutritional composition of Algae

The dried powdered extract of the algal sample was subjected to qualitative tests for the identification of phytochemical constituents such as carotenoids, alkaloids, flavonoids, glycosides, phenols, lignins,

saponins, sterols, tannins, reducing sugars, volatile oil, fats, amino acids, and carbohydrates according to standard procedures [35]. In particular, the chief composition of *Spirulina maxima* with respect to amino acids and vitamins was also congregated from the chemical profile presented in Table 2.

2.6. Pre-treatment

At first, *Spirulina* algae was cleaned properly in fresh water, dried in shade condition, and heated in an oven at 70 °C for removing moisture. Secondly, the dry biomass was prepared into fine-grained powder by a mechanical crusher and mixed with water in 1:3 (v/v) ratios. Finally, an ultra-sonication process was employed for cell destruction of the algae biomass, where 24 kHz frequency and 50^0C temperature were maintained for 5 minutes and their physic-chemical properties are represented in Figure 6E, F, G, H and Table 1. Then the sample was prepared to extract oil effectively for biodiesel production.

2.7. Lipid Extraction from Micro Algae

Initially, the biomass of *Spirulina maxima* wild stuff was subjected for drying and ground into a homogenous fine powder using a mechanical blender and the dried cells of algae were mixed with the solvent system, Hexane and Iso-propanol with the ratio of 3:2 (v/v). Then, the homogenate mixture was subjected to a soxhlet apparatus connecting to a round bottom flask and coupled with a magnetic stirrer at 30^0C for 2hr and the cell residue was detached by the filtering process. The filtrate material was transferred into a separating funnel and sufficient water was added to induce biphasic layering. After settling, the solvent mixture was partitioned into two distinct phases, the top dark green hexane layer containing most of the extracted lipids and the bottom light green layer containing most of the co-extracted non-lipids. The hexane layer was collected in a pre-weighed flask and evaporated using a rotary evaporator [36, 37].

2.8. The distinctive equation for Algal oils to Biodiesel production

The production of biodiesel was performed through time and temperature based receptive reactions called esterification and transesterification processes. The esterification reaction was carried out using 98% acid catalyst, (H_2SO_4) and 100% mass of lipid-derived from the algal wild feedstock, in the

interim, Methanol (CH_3OH) was added and the ratio of alcohol/lipid was 50:2 (volume/weight). The methanol (50% of the assigned volume) was added earlier to facilitate the dissolution of Oil and the remaining 50% of the Methanol was mixed with acid catalyst followed by total reaction component. The reaction was carried out at 60°C for 3 hours duration in constant stirring using a water bath under the reflux approach. After the complete reaction, the surplus alcohol is removed by evaporation using a rotary evaporator. The transesterification reaction will be carried-out using esterified oil with a base catalyst (NaOH/KOH) using Methanol and the rest of the reaction will be performed with algal feed-stock as explained above is accessible in Figure 3.

2.9. Esterification of S. maxima algae crude Oil

In the two-step reaction processes, initially, the esterification was performed with the acid catalyst (H_2SO_4), using a suitable glass reactor for the production of biodiesel has been revealed in Figure 4. This has been recommended when the algal oil contains a high content of Free Fatty Acid (FFA) which is above 2%. The esterification process facilitates the reduction of FFA in algal Oil which will be preventing the formation of emulsified soap as it will be inhibiting the yield of biodiesel at the end. Hence, the two-step processes for the production of biodiesel have been carry-out. Therefore, in the esterification reaction, H_2SO_4 (1.5%v/v) and 12:1 methanol to oil molar ration with 500rpm stirring speed at 60^0C temperature for about 90 min duration were monitored [38, 39] is shown in Figure 4. Finally, the esterified oil was separated through a separating funnel and the FFA (%) was found to be reduced from 5.9% to 0.450%. Further, the esterified oil will be subjected to the transesterification process using an alkali catalyst (NaOH/KOH).

2.10. Transesterification of S. maxima algae Oil

In the second stage of reaction, the Transesterification process is performed and which is the major step of biodiesel production is reflected in Figure 3. The esterified oil was transferred into the glass reactor and heated at 60^0C. The catalyst KOH was prepared in a separate flask with methanol at different concentrations and the molar ratio was taken.

Subsequently, the methanolic KOH was subjected to heating up to 60^0C and this was mixed with the

algal oil. The mixture was allowed for the period of 60min to facilitate the reaction and settle down for 2 h in a different funnel to separate the layer of both glycerol and biodiesel is showed in Figure 5. Meanwhile, the reaction parameters such as yield of biodiesel, molar ratio, the concentration of catalyst, temperature, reaction time with stirring speed were recorded at variable conditions [40, 41, 42]. At last, the upper layer containing biodiesel and the lower-- layer having glycerol was successfully divided. The Biodiesel was then, subjected to the purification process and the glycerine was stored in a suitable container as it is having a value addition in soap industries.
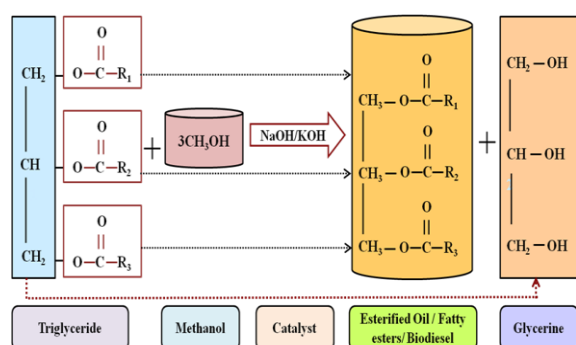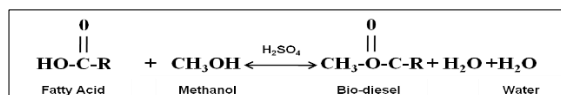

Fig.4. Esterification reaction with the Acid catalyst

2.11. Post-treatment

After completing the reaction, the mixture of reactant and product was poured into a separation funnel and glycerine was settling down at the bottom due to gravity. Concurrently, methyl ester (biodiesel) was taken out from the funnel (top layer) and waited until separation appeared not to be advancing anymore. The glycerine and other impurities were drawn off at the bottom of the separation funnel. It was washed with 60°C temperature warm water for removing the soap and other impurities like salt, free fatty acid. The process was repeated until raising the biodiesel pH range flanked by 6-7 and no soap bubbles appeared in it or the washed water became crystal in color. Then the biodiesel was evaporated with a rotary evaporator to remove dissolved methanol and water. The excess sodium sulfate (Na_2SO_4) anhydrous was utilized for chemical treatment, and finally, pure biodiesel was obtained by filtering with filter paper is indicated in Figure 6H.
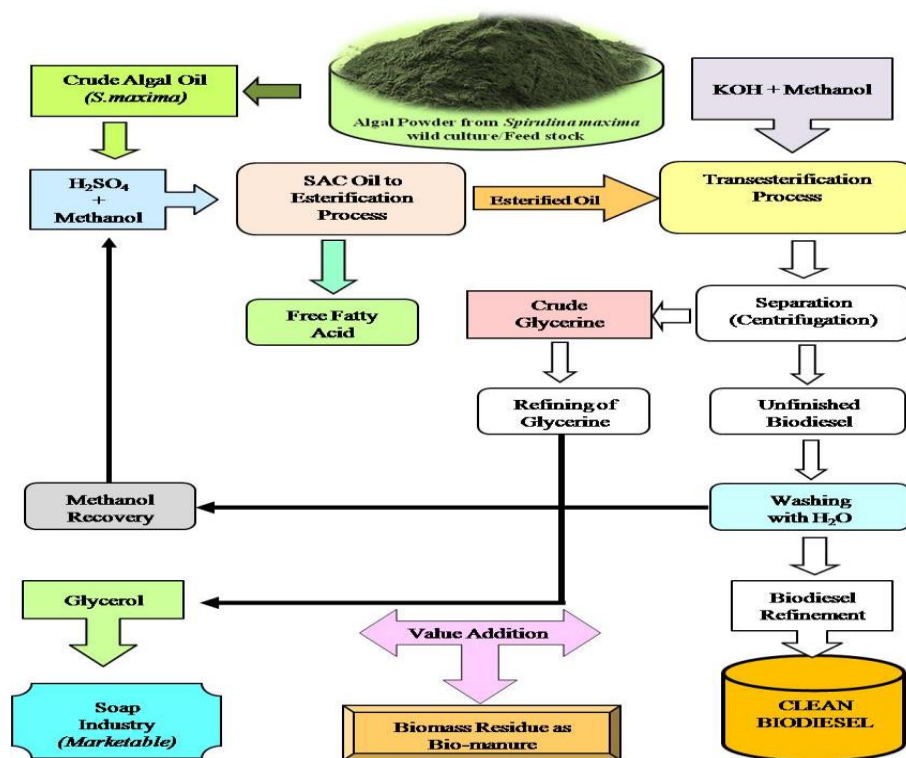

Fig.**3**. Flow diagram showing Biodiesel production from *Spirulina* algal feedstock

Fig.5. Transesterification reaction (Methanolysis of Triglycerides) with the base catalyst

2.12. Optimization parameters of Biodiesel production

The treatment was done to reduce the free fatty acid of algal oil to a static level where the transesterification is executed. This step will facilitate to remove the impurities in the oil through variable reaction parameters for optimizing the yield of biodiesel. The reaction parameters are, methanol to oil molar ratio, catalyst concentration, temperature, stirring speed and reaction speed respectively.

2.13. Analytical calculation of Biodiesel Yield (%)

Biodiesel yield (% w/w) was calculated for different conditions by using the following Eq. (3) [41].

$$\text{The yield of biodiesel (\%)} = \frac{\text{Weight of the biodiesel produced (g)}}{\text{Weight of the algal oil used (g)}} \times 100$$

2.14. Fatty Acid composition in S. maxima algal biodiesel

Fatty acids are a major constituent of micro-algal biomass. These fatty acids are present in different acyl-lipid classes; in particular the fatty acids present in triacylglycerol (TAG) which are of commercial interest, because they can be subjected to the production of biofuels. Microalgae mainly produce fatty acids with chain lengths of 16 and 18 carbon atoms, but some species can make fatty acids of up to 24 carbon atoms in length is presented in Table 2 & 3. Both saturated as well as highly unsaturated fatty acids are produced by microalgae. The (distribution of) fatty acid chain length and degree of saturation also determines the properties and quality of algae-derived biofuels and edible oils. The fatty acid composition in the wild stuff of S. maxima algal biodiesel has been enumerated [42].

2.15. Gas chromatography (GC) analysis

The analysis was carried out with the experimental design comprising of Agilent 6890 plus, equipped with an HP-50 capillary column (0.53 mm x30 m, 0.5 lm film) and a flame ionization detector (FID). The carrier gas used was pure Nitrogen (4.5ml min-1) at 250^0C injector temperature, 280ºC detector temperature, split ratio (1:50), sample size 1 µL, and the temperature program used was 80–240ºC at a fixed rate of 5ºC min-1. The detection of the fatty acid esters was accomplished by chromatography using a prepared reference mixture of fatty acid methyl esters [43].

2.16. FT-IR analysis of Biodiesel

Fourier transform infrared spectroscopy (FTIR) was used to determine biodiesel content in the reaction mixture to monitor the transesterification reaction. The method has also been used to determine the oil content and biodiesel content in the biodiesel-diesel blends.

2.17. Physicochemical characterization of Algal biodiesel

The purified product obtained from oil esterification was tested for estimating and evaluating its fuel properties as compared to an Egyptian petro-diesel sample, using the standard methods of analysis for petroleum products, ASTM Standard Methods. The results were compared with the petro-diesel oil followed by European and American standards of biodiesel (EN14214 and D-6751) respectively. The blends of Egyptian petro-diesel with different percent (2%, 5%, 10%, and 20%) of the produced biodiesel were prepared on a volume basis and their physicchemical characteristics were also studied. All the parameters were analyzed in three replicates and the final results given below were obtained as the mean values [44].

2.18. Evaluation of Physical Properties of Crude Glycerol

The physical properties were measured in crude Glycerol on the parameters such as the density of glycerol (Volume and weight) of crude Glycerol at room temperature 24°C. The pH was determined in crude glycerol of1gm was dissolved in 50ml of de-ionized (DI) water, then the pH was analyzed using Elico-digital pH meter at room temperature (24°C). Further, the viscosity of crude glycerol was calculated at 25°C using a Viscometer coupled with a temperature probe, and a temperature control unit.

2.19. Analysis of Glycerol content generated during the production of Algal Biodiesel

The glycerol is an abundant by-product produced during biodiesel production *via* transesterification reaction that takes place between the fatty acids with methanol or ethanol to produce methyl esters. Crude glycerol represents about 10 % of the product output in biodiesel production. Hence, the determination of the free glycerol content of crude glycerol by the iodometric-periodic acid method was conducted as per the AOCS Official Method [45].

2.20. Fractionation of Crude Glycerol

Crude glycerol was fractionated into aqueous and organic fractions according to the procedures shown in Figure 2. For fractionation without saponification, the crude glycerol sample was weighed and well dissolved in 50 mL of DI water in a 120-mL pressure tube. The solution was then acidified and fractionated as per AOCS method, complete conversion of soap in crude glycerol to FFAs was achieved by acidifying a crude glycerol solution using a sufficient amount (5 ml) of 1:1 (v/v) HCl solution, and then the recovery of the organic fraction was achieved by petroleum ether extraction. To ensure the complete recovery of the organic fraction, petroleum ether extraction was repeated until the obtained top petroleum ether layer became colorless.

The organic fraction was obtained after the removal of petroleum ether by rotary-evaporation and vacuum-drying until a constant weight was reached. For fractionation with saponification, the crude glycerol sample was weighed into a 120-mL pressure tube and dissolved in 10 mL of 50 g/L KOH-ethanol solution. The tightly capped tube was heated in an oil bath at 90 °C for 1 h with constant magnetic stirring (200 rpm). Upon completion, the tube was removed from the bath and cooled to room temperature (23 ± 0.5 °C) in a fume hood, followed by the addition of 50 mL of DI water. The solution was then acidified and fractionated using the procedures as described in fractionation without saponification. All aqueous and organic fractions obtained from the fractionation of crude glycerol were kept for later analyses [46,47].

2.21. Determination of Total Glycerol

After the completion of the transesterification reaction, the reaction mixture is set in motion by adding HCL 5 wt % solution. This set up not only neutralizes the residue catalyst but also promotes a separating process of glycerol from the ester phase. Later two additional washing steps using HCl 2.5 wt% and distilled water are performed to accelerate the recovery of glycerol at the maximum level. The analysis of total glycerine was done as per the following standard method [39].

2.22. Procedure for Total glycerol determination

1. Weigh a quantity of biodiesel samples of 50-100grams and carry-out transesterification reactions using a batch reactor method fixed with a reflux condenser.

2. The experimental set-up consisting of a three-necked flask placed in an oil bath (60-65^{0}C) on a magnetic stirrer with a speed of about 800rpm.

3. The weighed biodiesel sample was transferred to the three-necked flask and to this, 1.6 wt. % of CH_3ONa and 40 wt. % of CH_3OH was added.

4. The contents were subjected for heating by keeping the temperature between 60-65^{0}C with a fixed stirring speed

5. The generated glycerol was separated from the biodiesel layer during the production and the extraction of glycerol by the washing process was done in the following way.

6. In the experimental set-up, remove the reflux condenser, and add an amount of HCL (5wt. %) equal to the amount (used in step no.3) into the flask by maintaining the temperature 60-65^{0}C with a stirring speed between 800-1000rpm for approximately 20min.

7. Transfer the whole reaction mixture carefully to the separating funnel without cooling and wait for about5-10min then separate & collect the aqueous phase into an Erlenmeyer flask of about 250mL capacity.

8. Repeat the washing process by returning to the biodiesel phase at three-necked flask by adding HCl of 2.5wt. % equivalent to half of the weight as admitted under step 6.

9. Wait 5 min. and the aqueous phase was separated & transferred the same to Erlenmeyer flask as executed above.

10. Repeat the same now by adding distilled water instead of HCl of 2.5wt. %

11. Repeat steps 8 and 9 but using distilled water instead of HCl 2.5 wt%. Now, rinse the three-necked flask with distilled water (20 ml) and add it to the funnel containing the biodiesel phase. Transfer the aqueous phase to the Erlenmeyer flask containing the previous washing phase.

12. Now, the biodiesel phase was discarded and the separating funnel was rinsed by distilled water (20ml) and collected it in the same Erlenmeyer flask.

13. Finally, the total amount of aqueous phase weighed exactly and the total glycerol content was measured in the aqueous phase is as follows ($\sum$m).

14. Weigh the sample for about 50g of aqueous phase (m_1), to this, 5-6 drops of bromo-thymol blue which is an indicator; during the addition of indicator the color of the mixture was yellow.

15. This was neutralized by the addition of NaOH 0.1 mol l^{-1} (drop by drop) until the color turns to blue (magnetic stirrer can be used if necessary) and the color was tested using pH paper

16. Repeat the same process but using 10-15 g of distilled water (equal weight of the aqueous phase used in step 14, which is treated as blank.

17. Further, add 50 ml NaIO$_4$ solution (60g NaIO$_4$, 60 ml H$_2$SO$_4$ 0.1 mol l^{-1} in 1000 ml solution) into both samples (aqueous phase and blank sample), check and keep it in the dark for 30 min.

18. The process was continued by adding 10 ml C$_2$H$_4$(OH)$_2$ 50% (in H$_2$O), later, check and keep it in the dark for 20 min.

19. Titrate the solution by NaOH 0.125 mol l^{-1} (the concentration can be lower than) with bromo-thymol blue as an indicator (5-7 droplets) until the color changes to blue.

Calculation:

$$\%G_T = \frac{9.209 \times NNaOH \times (V1-V2) \times \sum m}{m\, 1\, XW}$$

Where; % GT- g of glycerol/100 g of biodiesel sample, V1-volume titrated of NaOH (ml) for the aqueous phase, V2-volume titrated of NaOH (ml) for blank sample, NNaOH-exactly concentration of NaOH solution, $\sum m$- the total amount of aqueous phase in step 13, m1-weight of the aqueous phase in step 14, W- the weight of biodiesel (g).

3. Results And Discussion

The analytical study was carried out in Engineering Chemistry laboratory, Maharaja Institute of Technology, Thandavapura (Nanjanagud taluk), Mysuru district in association with the Bio-energy research division of Hamsageetha Research Foundation, Tumkur during 2017-2018.the investigative experiment was done at Azyme Biotechnologies, Bengaluru, and, Karnataka, India and the qualitative parameters were ascertained. The compilation of findings *via* aggregating the research itinerary, methods, research data, data visualizations, and insights etc., were done at University Department of Chemistry, Ranchi University, Ranchi-834 001, Jharkhand, India.

3.1. Taxonavigation, Culturing & Growth of Microalgae

The algal feedstock collected during the baseline survey was identified as *Spirulina* maxima in its natural habitat (water bodies located near Devrayana Durga, Tumakuru district, Karnataka). The identification of algal species normally necessitates a concoction of both morphological and genetic description. The onsite morphological identification was done for the wild culture sample using a dissection microscope followed by a compound light microscope which was done using the standard method [48, 49]. The collected sample was intact mass with lush green and found to a significant algal wild stuff material are detailed in Figure 6A,B,C&D and Table 1.

The cultivation of microalgae *Spirulina* maxima was performed productively in the close pond system as per the standard procedure by employing the functional operating conditions of light exposure, light intensity, and culture aeration with respect to the growth rate of biomass to obtain the maximum yield of algae. The basic data has been summarized in Table 1.

3.2. Growth analysis of Algal biomass

The increase ratio of algal biomass was continuously monitored and as per the growth kinetics between 22nd to 25th period of days the yield of algal biomass was maximum and beyond this period the algal growth was gradually declined with respect to the external factors like pH, temperature exists in the natural environment are showed in Figure 6A, B and 10. Then, the biomass sample was harvested and subjected to drying in the shadow region for 4-5 days. Later, the dried algal material was ground to make powder for further analysis presented in Figure 6E&F.

3.3. Preliminary phytochemical evaluation

Different crude organic extracts of S. maxima were different in color based on the components presenting in the extracts. The algal sample was subjected for qualitative determination of preliminary phytochemicals [50]. The presence of concentrations of carotenoids referred to as coloring pigments and various fatty acids are present. Sterols, phenols, and saponins are also present in good concentrations. Sugars, amino acids, and flavonoids are also identified. Tannins were absent in all extracts. Chloroform and hexane extracts showed the presence of a larger group of molecules followed by ethyl acetate and methanol extracts. The presence of carbohydrates, amino acids, and lipids was confirmed in almost all the organic extracts [51]. Petroleum ether extract showed less significance in the preliminary Phyto-chemical analysis as compared to other solvent extracts. In addition, the main components of the *S.maxima* sample have been assessed with respect to protein, Amino acids and vitamins which are enlisted in Table 4.

3.4. Fatty acid composition

The yield of the generated biodiesel along with crude glycerol was subjected for evaluation by taking their mass (wt) with respect to the weight of the microalgae biomass and recorded approximately 16% and 3.45% (wt%), respectively. The fatty acid profile of *Spirulina* maxima wild stuff is presented in Table III. The saturated (33%) and unsaturated fatty acids (67%) were detected and the results revealed [52] that, percentage of myristic acid (14:0) 3%, palmitic acid was (C16:0) 23.4% and Stearic acid was (C18:0) 3.4%. However, the unsaturated fractions were detected as C16:1, (Palmitoleic acid methyl ester, 7.1%), C18:1 (Oleic acid methyl ester, 17.4%), C18:2 (Linoleic acid methyl ester 19.4%), and C18:3 (Linolenic acid methyl ester 23.1%) are represented in Table 4. The increased viscosity of the oil facilitates the increasing fatty acids chain length and degree of saturation. The atomization of fuel in the combustion chamber is highly affected by the viscosity of the biofuel and resulted in deposits formation. It could be concluded that the saturated and long-chain length fatty acids produce biodiesel with good stability and a higher cetane number.

The FAME profile is remarkable compared to the common fatty acid profile of other biodiesels. It was characterized by a high concentration of saturated FAMEs and low concentrations of unsaturated and polyunsaturated FAMEs; around 90%, 8%, and 2%, respectively. The high concentration of saturated FAMEs guarantees good oxidation resistance for biodiesel [53]. The major FAMEs were stearic, and palmitic acid methyl esters, recording approximately 28%, and 15%, respectively. The presence of behenic and lignoceric acids methyl esters with concentrations of approximately 7% and 4%, respectively was also recorded. The high concentration (81%) of FAMEs having a carbon chain of P18 carbons would impart high viscosity for biodiesel [54].

3.5. Production and Characteristics of Biodiesel
Algal biomass yield

The collected huge mass of *Spirulina* maxima wild stuff was processed as per the standard procedure [55] and after drying, a substantial reduction in the weight of algal feedstock was noticed. The estimated dry weight of the S. maxima algae was 1.25 to 1.60 kg for every 10 kg of wet algal biomass is shown in Table 1.

3.6. Oil generation form Algal biomass

The fine-tuned and weighed algal biomass was subjected for oil extraction and the generated oil in weight per kilogram of dried biomass was calculated using the standard formula.

$$\text{Oil yield} = \frac{\text{Oil extracted}}{\text{Algal dried biomass taken}} \times 100$$

The oil from algal biomass was obtained through solvent extraction (hexane-9%) where the volume of hexane was higher out of two solvents used in the extraction process. In addition, acetone was also preferred to extract algal oil (8.5%) as it is economically viable compared to hexane and the quantity of oil has been indicated in Table. 1.

3.7. Transesterification and Biodiesel production

The extracted oil from algal biomass was processed into biodiesel by the process of transesterification after the significant reduction of free fatty acid (FFA) by the pre-esterification process respectively. The evaluation for the yield of biodiesel and crude glycerol was carried-out by their weight relative to the weight of the algal biomass in the later part respectively [56]. The FAME composition of the biodiesel achieved and its profile was compared with the common fatty acid outline that has been given Table 1. The produced biodiesel was evaluated based on its distillation characteristics as well as its fuel properties compared to petro-diesel sample and international biodiesel standards as shown in Table 5. The characteristics of biodiesel with respect to viscosity were compared with petro-diesel and international biodiesel standards and all the properties of the produced biodiesel are completely acceptable and meet most of the specifications. So it can be ranked as a realistic fuel and as an alternative to petro-diesel.

3.8. Appraisal between Algal Biodiesel with Petro-diesel

The algal biodiesel was then subjected to blending with petroleum diesel for increasing the efficiency in the engine. Besides, to overcome the dependency and burden on petro-fuel, the produced biodiesel from a novel approach (using wild stuff of algal biomass in the natural habitat) was compared with petro-fuel with respect to their Characteristic features [57] are detailed in Table 5. It is evident from the laboratory tests that the properties of the biodiesel are found to

3.9. Physical and Chemical properties of Biodiesel

The data of Table 5 showed that the physical and chemical properties of algal biodiesel fractions like density, viscosity, flash point, cetane index, and heating values were comparable with those of diesel fuel. The listed main fuel properties and ASTM standards of petro- diesel fuel were compared. The viscosity of algal biodiesel was higher than diesel fuel; however, it was fit with ASTM standards values. More viscous fuel is generally unsuitable for use in diesel engines due to inefficient atomization. However, the heating value of algal biodiesel was within the acceptable limit of diesel fuel. The flashpoint of S. maxima biodiesel is higher than diesel fuel and its value was 189 and 73°C respectively. This makes the biodiesel fuel safer during handling and storage than diesel fuel. Besides, the cetane number of biodiesel was higher than the minimum requirement of diesel fuel [58].

Therefore, it is expected that fuel quality and combustion efficiency will be higher than that of diesel fuel. It could be concluded that the fuel properties of S. maxima biodiesel are almost within the acceptable recommended limits [32]. The obtained biodiesel from *S. maxima* algal oil was analyzed for the viscosity of 4.82 mm2s-1, the flashpoint of 189°C, the specific gravity of 0.91 g/ml, and cetane number of 47 and the properties of S. maxima biodiesel were compared with ASTM standards and it was found with high-quality biodiesel.

3.10. GC Analysis and Physico-chemical properties in Algal oil

The algal lipids consisted of fatty acids between 14 and 18 carbon chain length and the higher concentration fatty acids were palmitic, oleic, and linolenic acid are indicated in Table 2 and Figure 7. The physicochemical properties of algal oil has a lower carbon content while at the same time having a larger amount of oxygen, which both justify the significant heating value of algal oil compared to petro-diesel [59]. The difference in the composition of the algal oil is explained by the higher amount of phospholipids, pigments (like chlorophyll) and waxes in algal lipids compare to other vegetable oils which have higher purity in triglycerides. The cetane

number was calculated based on the saponification number and iodine value of the oil is showed in Table 5. The results suggest that algal oil demonstrates higher cetane number, density, and viscosity (both dynamic and kinematic) respectively.

3.11. FT-IR Spectroscopic analysis of Biodiesel

The detection of the assemblage of crucial compositions in the esterified algal oil was attested by a prevailing investigative tool called Fourier Transform Infrared (FTIR) spectroscopy. Likewise, the functional group that exists in the algal oil and in comparison with petro-fuel was ascribed by this approach of FTIR spectroscopic analysis are depicted in Figure 8 & 9. The distinctive absorption bands for the vibrations of the C-H bond were around 3156.20 & 2966.85 cm-1 analogous to the asymmetric and symmetric vibration approach of methyl groups respectively.

The presence of alkane in this group was very powerful and the subsequent peaks 1810.29 cm-1 and 1670 cm-1 characterizes the stretching of C=0 (Aldehyde/ketone). Further, the transmittance peaks of 1086 cm-1 and 965.80 cm-1 correspond to the alcohol which is a functional group of stretching carbohydrates. The absorbance peaks of 1469.40 cm-1 and 815.60 cm-1 for the vibrations of C-H stands for an alkane. In the spectra of biodiesel the peak higher than 3000 cm-1 similar to -OH of carboxylic acid indicates absolute transesterification. Concisely, the transmittance of main pointed peaks of FTIR spectra is an alkane, indicating that, biodiesel is a saturated hydrocarbon which can be proposed as an incredible and prospective alternative fuel to the existing engine system [60].

3.12. Optimization Parameters Of Biodiesel Production

3.12.1. Optimization of the Transesterification process

The effect of the temperature on the transesterification process has been evaluated and the sample consisting of 500 ml of algal oil, 90 ml of methanol, 8 g of potassium hydroxide, and a reaction time between 60-90min have shown properties fairly close to diesel fuel. The optimized parameters of the esterification process with acid catalyst were assessed with the effect of the reaction time, catalyst, temperature, mixing intensity and reaction time respectively are presented in Table 9. Further, the

transesterification process was admitted with alkali (base) catalyst with respect to methanol-to-molar ratio, catalyst concentration, temperature, mixing intensity and reaction time were found to be optimal for the sample of esterified algal oil which are represented in Table 9 & 10. In addition, other parameters for achieving optimized biodiesel have been detailed below.

3.12.2. Methanol to Molar ratio

The most imperative factor for the optimized production of biodiesel is methanol to oil molar ratio which gave assorted impression between the molar ratios of 4:1 to 8:1. The preliminary approach of reactions was set-up with 1% KOH catalyst concentration at 50^0C temperature and 500 rpm mixing intensity was monitored in the reaction time duration of 90 minutes. The outcome of molar ratio on biodiesel production is well illustrated; where it displayed an increased yield of Biodiesel with an increase in molar ratio. Whereas, the formation of glycerol and soap noticed was at variance with respect to the formation of biodiesel. When the molar ratio increased from 4:1 to 6:1, biodiesel yield significantly increased by 16.5 %, while, the formation of glycerol and soap was decreased by 21.5 % and 42.25 % respectively are presented in Figure 11.

The optimized yield of biodiesel (55.2 %) at a molar ratio of 6:1 with a minimum amount of formation of soap was observed. Later, biodiesel was turn down with an increase in molar ratio of beyond 6:1, which resulted in an increased tendency of formation of soap and glycerol due to emulsification. Therefore, the little excess of methanol than the requisite amount can enhance the solubility factor of glycerol, which slowdowns the separation of biodiesel from its by-products layer. Thus, the soluble glycerol presented in the methyl ester phase may lead to the formation of foam which finally hampers the optimization of biodiesel which is also in conformity with the earlier reports [48]. The optimized yield (94 %) was obtained with the reaction condition of methanol to oil molar ratio was 6:1 in tune with 1 % NaOH catalyst loading, 35^0C reaction temperature followed by mixing intensity of 200 rpm was monitored for the reaction time duration to 66 min which is also supporting the present findings [64].

3.12.3. Catalyst concentration

The consecutive essential parameter with respect to catalyst concentration for optimized biodiesel production is represented in Figure 12; where, the effect of catalyst concentration was assessed by varying the KOH concentrations (0.25, 0.5, 1 & 1.5 % wt) respectively. The reaction was operated at a temperature of 50^0C with a fixed ratio (6:1) of methanol to oil by monitoring a mixing intensity at 500rpm for the time duration of 90 minutes. The biodiesel yield was increased with an increase in catalyst concentration up to a certain level, beyond that decreased the yield. The catalyst concentration of 0.25 % KOH was insufficient to complete the reaction; thus, the yield of 42.5 % was very low, whereas soap formation was 6.12 %, which was very high.

The highest yield of biodiesel (63.7 %) was attained at 1.0 % KOH with the formation of 5 % glycerol and 2.1 % soap. Further, the increased level of catalyst concentration (1.5% of KOH) caused the decreased trend of the yields to 56 % but with an increasing level of soap formation. This was due to large amounts of soap formed by saponification when high concentrated KOH was added resulting in a reduction of biodiesel yield [41]. A similar trend for maximum biodiesel yield of 96 % at methanol: oil 6:1, temperature 65 °C, 1 % KOH, and stirring speed 600 rpm from Algal samples was also noticed in the previous report [65].

3.12.4. Temperature

The succeeding vital factor of optimized biodiesel production is the effect of temperature which is represented shown in Figure 13. The reaction was performed at variable temperatures (45, 55, 65, and 75 °C) with 1 % KOH & molar ratio of 6:1 followed by the mixing intensity of 500 rpm in the time duration of 90 minutes. The temperature parameter had a constructive influence on the reaction where the conversion into biodiesel was optimized with increasing temperatures. The reason behind that, the amalgamation of oil and methanol enhanced the solubility level amongst molecules, thereby, the maximum amount was achieved.

The maximum biodiesel yield (81.38 %) was attained at 65^0C, but there was no further enhancement in biodiesel production when it goes beyond the above temperature. This was mainly due to the solubility of the solvent reached at a saturated

level with decreased diffusion level and the lower boiling point of methanol (64.7^{0}C), which has a tendency to escape from the reactor. The concurrent research results stating that the maximum biodiesel production (90.6 %) was achieved in rapeseed oil at a temperature of 65^{0}C with a molar ratio of 6:1, followed by the addition of 1 % KOH at a stirring speed rate of 600 rpm [66].

3.12.5. Mixing intensity/Stirring speed

Further, the very crucial factor is 'Mixing intensity' which can have a better influence on the production of biodiesel as it increases the interaction area between the catalyst and alcoholic oil. The effect of mixing speed on the production of biodiesel was shown in Figure 14. The reaction does not take place at the interface between the two layers when the improper mixing is caused and therefore it may reduce the yield of biodiesel**.** To evaluate the effect of mixing intensity on the production of biodiesel, the stirring speed was varied between 400 to 700 rpm. The operation conditions of the reaction comprising of fixed at temperature 65^{0}C with a ratio of methanol: oil 6:1 and 1% KOH for 90 minutes was monitored.

The yield of Biodiesel was increased with an increase in mixing intensity due to an increase in the homogenization of the reactant**.** At the variable stirring rate of 400, 500, 600, and 700 rpm, the value of biodiesel yield was 60, 70.5, 85, 84.2 %, glycerol formation was 6.4, 7, 9, 6 %, and soap formation was 3, 2.22, 1.12, 1.9 %, respectively. When stirring speed was increased from 400 to 600 rpm, biodiesel yield increased from 60 to 85 %. With a further increase in stirring speed, there was no improvement in the biodiesel yield. The maximum biodiesel yield of 85 % was at 600 rpm. The concurrent outcome was also evidenced in the literature report [67] where; the maximum biodiesel (97%) yield was achieved from *Brassica carinata* at 600 rpm, 6:1 molar ratio, 1.2 % KOH, and 25 °C reaction temperature for 60 minutes.

3.12.6. Reaction time

The decisive factor of Reaction time was the most essential part required to complete a transesterification reaction with maximum biodiesel production. The time and temperature-oriented reaction was carried out at variable time durations, such as 50, 60, 70, and 80 minutes with 1% KOH, methanol to oil ratio 6:1 at temperature 65 °C and

600 rpm. After passing 50 minutes, biodiesel yield was 70 %, glycerine was 5.1 % and soap formation was 3% indicated in Figure 15.

The optimized biodiesel yield of 87.75 % was achieved after 70 minutes with the formation of 9% glycerol and 1% soap. While, the subsequent increase of reaction time duration beyond 70 minutes, there was no significant improvement in biodiesel yield which may due to decreased diffusion rate. Similar findings were noticed [68] where the optimum time of 70 minutes was fixed to obtain maximum biodiesel yield of 90.6 % from microalgae *E. compressa* and the associated parameters were fixed at 1 % KOH & 55 ^{0}C followed by a 9:1 molar ratio for the time duration of 90 minutes. Eventually, the optimized parameters of transesterification process has been standardized for *S. maxima* algal oil are given Table 11 and the maximum yield of biodiesel was achieved out of this routine approach.

3.13. Analysis of algal biodiesel with ASTM standard limits

Finally, the elementary analysis of the production of biodiesel with a maximum yield condition is represented in Table 5. The algal biodiesel have a higher viscosity than diesel fuel. In addition, the higher calorific value (HCV) of the algal biodiesel was 44.6 MJ/kg, and it is almost closer to petro-diesel. The Cetane number (CN) of the algal biodiesel was also comparatively higher (47) than diesel fuel. Similarly, the acid value (0.52mg KOH/g) of the biodiesel was higher than diesel fuel. The moisture and carbon residue content was 0.061 wt% and 0.044 %wt., respectively. The measured biodiesel and diesel properties were within ASTM standard limits which are found to be most significant.

3.14. Physical Properties of Crude Glycerol

As a matter of course, the mass of crude glycerol in S maxima was found to be 1.28 gcm-3 which is lower than that of pure glycerol (commercial-1.46 gcm-3). This was due to the presence of some wispy impurities like fatty acid methyl ester (FAMEs), fatty acids, methanol, and water in the crude glycerol. In addition, the crude sample of glycerol will have a pH value closer to 10 due to the subsistence of residual alkalies like NaOH or KOH derived during the production of biodiesel.

Similar findings were also made in the previous reports where, the pH value of 11 caused the crude glycerol samples from different biodiesel plants

varied from 2.0 to 10.8. 29. The viscosity of crude glycerol sample was found to be 85 mPa.s-1, due to their diverse compositions [61] are presented in Table 6.

3.15. Determination of Total Glycerol content

The total content of glycerol in the unfinished glycerol sample was carried out. The determination of glycerol was explored using aqueous fractions obtained devoid of the saponification process was correspondingly referred to as total glycerol in the total crude glycerol sample [62]. Therefore, the total glycerol contents in the crude glycerol generated during biodiesel production by S. maxima has been given Table 7.

3.16. Chemical compositions of crude glycerol

Essentially, the chemical profile of crude glycerol derived from wild stuff of S. maxima was accurately illustrated into the specialized components such as glycerol, methanol, water, soap, FAMEs, glycerides, FFAs, and ash respectively. Captivatingly, the crude glycerol comprising of the above components will be accounted for more than 85% of the total mass of crude glycerol; whereas, total glycerides, free fatty acids, and ash contents were less than 15% represented in Table 8.

The compositions of crude glycerol samples will be varied considerably from each other as the glycerol content on average ranged between 30 to 65%. Hence, the crude glycerol has to be exemplified before subjecting into their conversions with value additions [63].

3.17. Cost-effectiveness Magnitude (CEM) of Spirulina biodiesel

Further, the Cost-effectiveness Magnitude of Spirulina biodiesel production in an array of medium-scale was found to excellent as it is explored from its natural habitat as wild stuff feedstock which can facilitate the sustainable production of algal biodiesel to fulfil the gap of the energy crisis. The proposed scheme of Cost-effectiveness Magnitude (CEM) of Spirulina maxima biodiesel production was found that the wild stuff biomass available round the year was estimated was 1.21 tons (little variable due to climatic fluctuations) per year which can be placed under medium scale production in the R&D or industry which are given in Table 11. Hence, the wild stuff feedstock is estimated to obtain from its natural

ecosystem thus; the cost-effectiveness is very much economical and is technically more feasible.

However, with the generated data of results it was found that, the microalgae are the only potential sources of alternative renewable fuel in the future and are available in a wide range of ecological conditions. They are very petite aquatic organisms that convert sunlight into energy and some of these algae store energy in the form of natural oils called Algae Fatty Acid Methyl ester (AFME). Under the right conditions, algae can make a lot of oil that can be converted into biofuels. Besides, algae need carbon dioxide to grow and that's good for the environment, since it takes CO_2 out of the atmosphere, making it a nearly carbon-neutral fuel source [69-72]. Therefore the outcomes are emphatically discussed on the exploration of algal wild stuff in the natural eco-system, Fatty acid analysis, lipid extraction, oil recovery and biodiesel production.

In the subsequent section, the optimization of transesterification reaction, and cost effectiveness magnitude for medium scale production of S. maxima biodiesel has been enunciated hereunder. The algae are considered to be the fastest-growing primitive plant and hypothetically, validated that their functional properties are efficacious to produce a significant amount of oil as compared to other oilseed crops and plants.

However, the ratio with respect to energy efficiency and carbon along with water footprint for algae-based bio-fuels are evaluated that, they are the prospective fuel sources that can replace the petroleum diesel [73-75].

3.18. Algal lipid content and production of Biodiesel (AFME)

Theoretically, the algal lipids would be a superlative feedstock for generating transportation fuels with high energy density such as biodiesel, green jet fuel, and green gasoline, etc. This in view of the fact that algae do not inclusive with food products, agriculture land and it exhibits the extreme ability to sequester large quantities of carbon dioxide in the environment. In addition, the practical analysis indicated that biodiesel is more focused on its power output due to higher oxygen content over the petro-diesel [76-78].

Generally, biodiesel is a mixture of fatty acids of diverse lengths esterified with an alcohol, typically methanol. The hypothesis elucidates that, the algae oil with a high free fatty acid content will be

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Egypt. J. Chem. Vol. 64, No. 9 (2021)

converted to Bio-diesel by employing both pre-esterification and transesterification respectively. The microalgae biomasses are driven by the higher growth rates and lipid content of several algae species when compared to other conventional crops. The suitability of microalgal biomass as a biofuel feedstock is closely related to the length and degree of saturation of its fatty acids as specified by the key characters of iodine value, oxidation stability, cetane number, etc. This has been justified apparently through physical properties comprising viscosity, density, heating value, and melting temperature, etc. [79, 80].

This observation can be interrelated with the oxygen content of the fuel and the biodiesel is composed of only a few diverse compounds and consequently, their properties are largely influenced by the fatty acid composition of the fuel, which is identical to that of feedstock oil (triglycerides from vegetable/biomass).

Interestingly, the biodiesel produced from microbially derived oils especially, algal oils gaining large attention concerning its habitual diversity in all geographical locations that leads to the possibility of exploring oils along with considerable yields throughout the year. Besides, the fatty acid composition in the plant-based esterified oils is similar to one another. These analogous expressions principally include C16 and C18 fatty acids with varying degrees of unsaturation; however, the oils from micro-algae can differ significantly and may contain exceptional fatty acids that diverge in both chain length and structure. The algal sources of oil preferred in the present study are indicating considerable difference to a certain extent as compared to previous reports as the cultural protocols are followed for a varied habitual feedstock for biodiesel production [81].

Further, the biodiesel derived from the *S. maxima* contains a substantial amount of C16:1 (palmitoleic) methyl ester and C14:0 (myristic) methyl ester, which are rarely found in vegetable oils. The presence of both fatty acids is beneficial to biodiesel fuels by improving oxidative stability without sacrificing cold flow performance in the case of palmitoleic acid and improved NOx emissions in the case of shorter chain fatty acids such as myristic acid.

The biodiesel from the algal oils contains a supplementary class of fatty acids predominantly C16 and C18 fatty acids but lacks the polyunsaturated fatty acids that are also found in plant oils. The high amount of oleic acid in this oil should be advantageous for oxidative stability without greatly affecting cold flow properties [82].

Nevertheless, the presence of potential fatty acid composition in the obtained esterified oils can have an influence relating to the overall performance of biodiesel fuel. Besides, it is imperative to determine the physical properties of the biodiesel from the selected algal types towards evaluating their effectiveness in an existing diesel engine. The main physical properties of the algal fuel were compared with petro-fuel that are found to be within the ASTM standard.

3.19. Biodiesel yield via Optimization of the transesterification parameters

Further, the optimization of biodiesel from algae is of great interest, thus, it is imperative to explore the algae oil having a high content of free fatty acid can yield a promising amount of biodiesel by two step reactions *i.e.,* pre-esterification and transesterification. Methodically, the conversion rate of triacylglycerols reached an optimum level under the influence of methanol to oil molar ratio during catalysis with sodium/potassium hydroxide at a standardized temperature. Therefore, a factorial experiment was performed to determine the relationship between the yield of methyl esters and the technological parameters (methanol/oil molar ratio, amount of catalyst, reaction temperature, and time) that affect the biodiesel fuel production process [83, 84].

The momentous parameters of the process were optimized to obtain the highest yield of biodiesel wherein the evaluation of each parameter under optimal rate/level was performed. In the optimization process, assessment of individual parameters was considered with respect to yield while the other parameters were kept constant and each optimal value arrive at the optimization of the next parameter was retained. Later, the overall production was accomplished using the optimal parameters obtained. The methanol concentration is one of the significant factors facilitating the conversion of triglycerides into methyl esters. The variable molar ratio of methanol to oil was analyzed from 4:1 to 8:1. The yield of biodiesel mainly depends on the molar ratio of methanol to oil resulted in an optimum yield of 68.2 wt% at a 6:1 molar ratio. The ratio of methanol ratio is relatively lower than that of algal bio-oil which was due to the collective effect of acid pre-treatment

and the unique characteristic of *S. maxima* algae oil [85-87].

Subsequently, the reaction distinctly needs a catalyst for assisting the conversion of triglycerides ultimately into esters. The varied concentration of catalyst was used between 1 to 4 wt% by a molar ratio of 6:1 and with a reaction temperature of 65^0C, a reaction time of 90 min, and a stirring rate of 600 rpm. The increased yield was recorded with a maximum of 66.56 wt% at 6 wt% catalyst concentration. Theoretically, this may be due to the presence of active CH_3O- species dissociated from methanol that collides with nucleophiles attached to the triglyceride molecule [88]. Increasing the catalyst beyond 5 wt% does not improve the yield which might be due to the poor diffusion of methanol oil system on the catalyst surface when the catalyst dosage is increased [89,90]. A similar expression was also reported [91] where the algae oils need a higher amount of catalyst required for achieving an optimum yield. This is due to the variation in the physicochemical properties of the feedstock.

Further, the Reaction time plays a very crucial role in the conversion of biodiesel through the transesterification process. The variable time duration was employed between 50–90 min for the reactions showed that, the effect of reaction time on biodiesel yield which facilitates a maximum yield of 84.50 wt% at 70 min. On the other hand, a report reveals that a similar yield was obtained for algal oil by taking the reaction time of 180 min [92,93].

Another pivotal factor is temperature which plays a major role in achieving the significant yield of biodiesel through the transesterification reaction. The variable reaction temperature from 45^0C to 75^0C was used with methanol to oil molar ratio of 6:1, by a catalyst concentration of 6 wt%, and keeping a reaction time of 70 min. Under this standardized approach, a maximum yield of 86.66 wt% was achieved at 65^0C, but subsequently, the yields were affected by an increase in temperature which is due to a part of the methanol is in the vapour phase at boiling temperature. This is in accord with many of the research reports since the conversion is maximum near the boiling temperature of methanol and it is self-governing with respect to feedstock [94,95]

The vital cofactor during transesterification is the stirring rate which can have a substantial effect on the yield of biodiesel. The appropriate mixing of oil with methanol is very essential for enhancing the yield of biodiesel. In the analysis, the variable stirring rate between 400 and 700 rpm was adopted by keeping the other parameters in the optimized condition [96-98]. Finally, the optimized yield of biodiesel (86.85 wt%) was obtained at a stirring rate of up to 600 rpm but on increasing the stirring rate beyond 600 rpm, the constant yield was noticed.

The produced biodiesel eventually showed that it is a saturated hydrocarbon as the alkane was displayed through the main sharp transmittance peaks of FTIR spectra. Hence, the algal biodiesel is confirmed as the most potential hydrocarbon and can be recommended as an alternative fuel or the same may be associated with petro-fuel in terms of promising blends [99-101].

In the study, the scheme on Cost-effectiveness Magnitude (CEM) of *Spirulina* biodiesel production was projected. Since the wild stuff, algal biomass is available round the year; it can be accessed in natural habitations. It has been estimated that CEM can be positioned under medium scale production in the R&D or industry as the possible exploration of algal biomass quantity was about 1.21 ton (little variable due to climatic fluctuations) per year. Therefore, the cost-effectiveness magnitude is found to be very much economical and is technically more viable [102,103].

Table 1. Preliminary Physico-chemical analysis of S. maxima algae (wild stuff) Oil and Biodiesel production

SL. No.	Algae type	Appearance	Moisture (Wt %)	Temperature (^{O}C)	Algal biomass (Kg)	Time taken for max Temp.(hr)	Quantity of Oil (Kg)	Optimized Biodiesel (%)
1.	*Spirulina* maxima (Wild stuff)	Thin & slightly dark green liquid	0.061	60-90	1.5	2.50	0.915	96.56
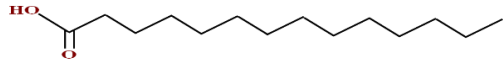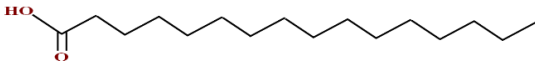
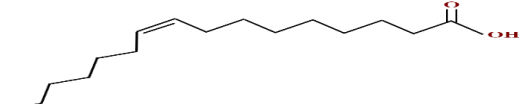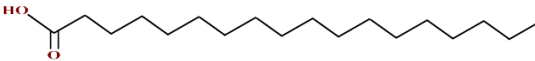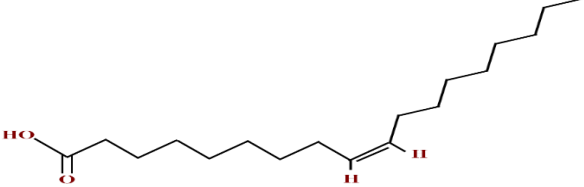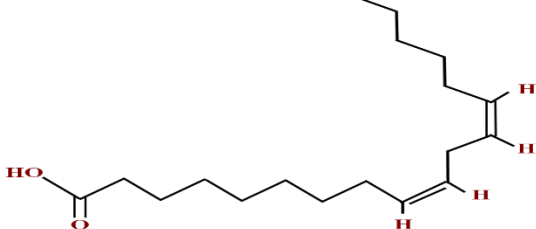Table 2. Fatty Acid composition in *S. maxima* algal biodiesel

SL. No.	Fatty acids	Structure (FA Chain length)	% (w/w)
1.	Myristic acid	C14:0	01.35
2.	Palmitic acid	C16:0	38.24
3.	Palmitoleic	C16:1	10.21
4.	Stearic acid	C18:0	06.19
5.	Oleic acid	C18:1	18.43
6.	Linoleic acid	C18:2	19.21
7.	Others	C18:3	7.65

Fig.6. (A-H). The saga of *Spirulina* wild stuff biodiesel
A. *S. maxima* at Natural condition, **B.** *S. maxima* Sample at the closer view at its habitat,
C. Algal sample air dried, **D.** Microscopic view of *S. maxima*, **E.** Pre-processing of *S.maxima*, **F.** Powder sample of *S.maxima*, **G.** Algae Oil for Transesterification reaction and **H.** Biodiesel sample of *S. maxima*

Table 3. Fatty Acid compositions present in *S.maxima* algal biodiesel

SL. No.	Type of Fatty Acid	Molecular Formula	Molecular Weight
01.	Myristic acid	$C_{14}H_{28}O_2,$	228.37 g/mol
02.	Palmitic acid	$C_{16}H_{32}O_2$	256.43 g/mol
03.	Palmitoleic acid	$C_{16}H_{30}O_2,$	254.414
04.	Stearic acid	$C_{18}H_{36}O_2$	284.48 g/mol
05.	Oleic acid	$C_{18}H_{34}O_2$	282.47 g/mol
06.	Linoleic acid	$C_{18}H_{32}O_2$	280.4472 g/mol
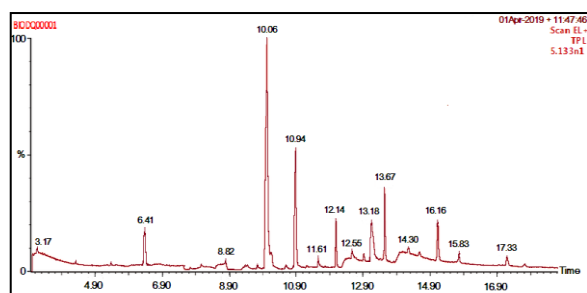
Figure 7. GC-MS Spectra of *Spirulina* algal Oil

Figure 9. FT-IR of Biodiesel obtained from wild stuff feedstock of *Spirulina*

Figure 8. FT-IR of Petro-diesel obtained from authorized sources
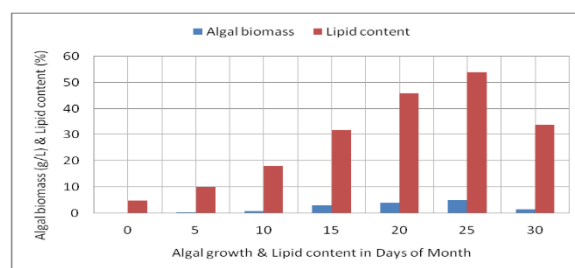
Figure 10. Growth of Algal biomass with respect days in the natural habitation

Table 4. Composition of *Spirulina* with respect to main components of Amino acids and Vitamins

| SL. No.	*Spirulina* maxima	Nutritional Composition (g 100 g⁻¹ dry wt)
	Protein	
1.	Crude protein (N × 6.25)	55–71
2.	True protein	48–61
	Amino acids	
3.	Alanine	5.0–6.1
4.	Arginine	4.5–9.3
5.	Aspartic acid	6.0–15.2
6.	Cystine	0.6–2.2
7.	Glutamic acid	8.2–21.8
8.	Glycine	3.2–4.0
9.	Histidine	0.9–1.6
10.	Isoleucine	3.7–4.5
11.	Leucine	5.6–7.7
12.	Lysine	3.0–4.5
13.	Methionine	1.6–2.2
14.	Phenylalanine	2.8–4.0
15.	Proline	2.7–3.2
16.	Serine	3.2–4.3
17.	Threonine	3.2–4.5
18.	Tryptophan	0.8–1.2
19.	Tyrosine	3.9
20.	Valine	4.2–6.0
21.	Lipids	4–7
22.	Carbohydrates	13–16
23.	Minerals	4–9
	Vitamins	
24.	Thiamin	5.5
25.	Riboflavin	4.0
26.	Pyridoxine	0.3
27.	Nicotinic acid	11.8
28.	Pantothenic acid	1.1
29.	Folic acid	0.05
30.	Biotin	0.04
31.	Cyanocobalamin	0.02
32.	Ascorbic acid	--
33.	β-Carotene	0.17
34.	γ-Tocopherol	19.0
35.	Chlorphyll a (mg/g)	14.4
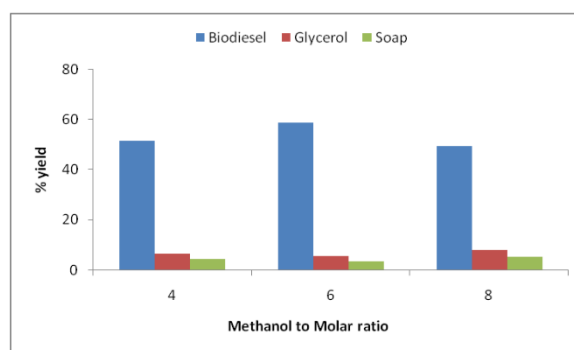
Figure 11. Showing the effect of Molar ratio on biodiesel yield

Table 5. Properties of Biodiesel derived from *S. maxima* algae

SL. No.	Property	Crude Oil	Algal diesel	Limits (ASTM) Std.
1.	Density (kg/m3) at 15⁰C	0.966	0.882	0.86-0.89
2.	Kinematic Viscosity (mm²/s) at 40⁰C	37.45	4.82	1.9-6.2
3.	Specific gravity (g/ml)	nd	0.93	na
4.	Higher calorific value (MJ/kg)	nd	44.60	46.56
5.	Cetane Number (CN)	nd	47	na
6.	Acid value (mg KOH/g)	11.44	0.52	0.50 max.
7.	Iodine value (IV)	84.24	nd	na
8.	Saponification Value (mgKOH/g)	188.92	nd	na
9.	Flash Point (0C)	nd	189	130 max.
10.	Oxygen content (%wt)	nd	20.04	na
11.	Carbon Residues (wt%)	nd	0.044	0.050 max.

[Source: SINGLE-CELL PROTEIN Algae: M. García-Garibay, E. Bárzana, in Encyclopedia of Food Sciences and Nutrition (Second Edition), 2003]

Table 6. Physical Properties of Crude Glycerol

SL. No.	Samples	Physical Parameters				
		Glycerol content	Color	Density (g/cm3)	pH	Viscosity (mPa·s)
1.	*S. maxima* Crude Glycerol	36.75	Light greenish brown	1.28± 0.01	8.6± 0.0	85± 0.1
2.	Pure Glycerol (Commercial)	99.98	Clear	1.46± 0.00	7.0± 0.0	840± 0.2

Data expressed as mean of three replicates ± standard deviation

Table 7. Composition of Crude Glycerol generated during Biodiesel production from *S maxima*

SL. No.	Compositions of Crude Glycerol (wt %)	Amount
1.	Free glycerol	22.9 ± 0.2
2.	Methanol	10.9 ± 0.2
3.	Water	18.2 ± 0.1
4.	Soap	26.2 ± 0.2
5.	FAME	21.3 ± 0.2
6.	Glycerides	1.2 ± 0.2
7.	FFA	1.0 ± 0.1
8.	Ash	3.0 ± 0.0
9.	Total	99.4± 0.0

Data are expressed as the mean of three replicates ± standard deviation.[Source: SINGLE-CELL PROTEIN Algae: M. García-Garibay, E. Bárzana, in Encyclopedia of Food Sciences and Nutrition (Second Edition), 2003]

Table 8. Total Glycerol Content in crude glycerol sample generated during production of Biodiesel from *S. maxima*.

Replicates	Glycerol content (wt%)
1	89.5±0.7
2	95.8±0.3
3	87.5±0.5

Data are expressed as the mean of three replicates ± standard deviation.

Table 9 Optimized parameters of Transesterification with Acid catalyst

SL. No.	Acid Treatment	Value
1.	Methanol-to-oil-molar ratio	8:1
2.	Catalyst (H2SO4) Concentration	1.5wt%
3.	Reaction temperature	65^{0}C
4.	Reaction time	90 min
5.	Stirring speed	600rpm
6.	Settling time	180 min

Table 10. Optimizing Parameters of Transesterification Process for Biodiesel production

SL. No.	Parameters	Values
1.	oil (mL)	500
2.	methanol (mL)	90
3.	KOH (g)	6
4.	Reaction time (min)	60
5.	Reaction temperature (°C)	65
6.	Yield (mL)	460
7.	Viscosity (CST)	4.80
8.	HCV (MJ/kg)	44.60

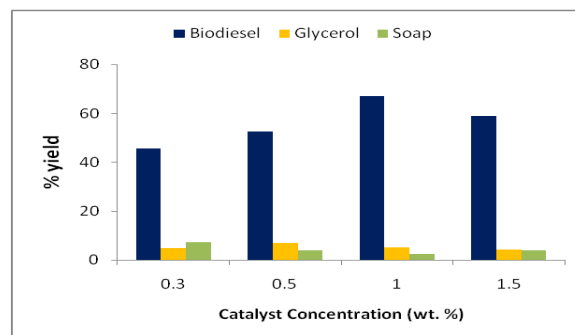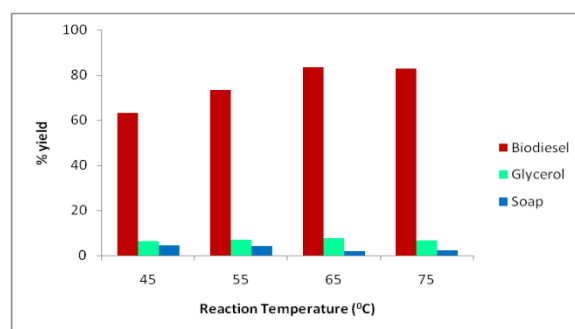
Figure 12. Showing the effect of Catalyst concentration on biodiesel yield

Figure 13. Showing the effect of Reaction temperature on biodiesel yield

Table 11. Cost-effectiveness Magnitude (CEM) of *Spirulina* biodiesel production at Medium-scale production in the R&D/Industry

SL. No.	Parameter	*Value in ₹	Outline in the present study
1.	Cost of Algal biomass and other contents	30,000/1T *(By In vitro* Lab culture)	₹10000/1T [Collection cost] *(Biomass explored from natural habitat as Algae wild stuff)*
2.	Price for 1kg biomass (commercial)	155.25	Nil
3.	Price for 1kg oil (commercial)	299.46	Nil
4.	Price for 1L oil (commercial)	269.10	Nil
5.	Oil recovery	720 L/T	590L/T
6.	CO$_2$ mitigation	fractional	--
7.	Residue use for biogas/fertilizer	Significant	--
8.	By-product/co-product	need to be produced	--
9.	Chemicals for extraction	2000.00	₹1500.00
10.	Electricity (Pumps, centrifuges, driers, etc.)	1000.00	₹750.00
11.	Maintenance (Annual)	3000.00	₹1500.00
	Total Cost	**36,723.81**	**₹13,750.00**

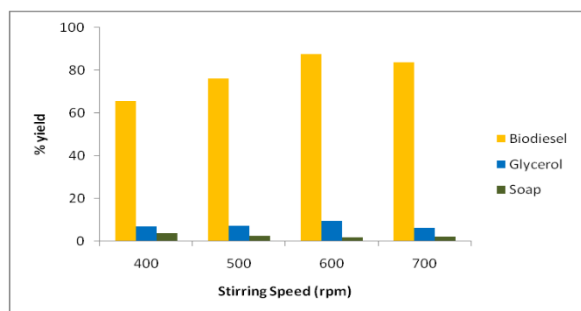***From confidential industrial sources*

Figure 14. Showing the effect of Stirring speed on biodiesel yield.

Figure 15. Showing the effect of Reaction time on biodiesel yield

4. Conclusion

In the present study, the production of biodiesel from the oil derived from wild stuff of *Spirulina maxima* algae, confirms that it's a potential alternative renewable fuel source which can associate with the diesel fuel through blending at the proper ratio. Furthermore, the technological parameters for optimized biodiesel production such as, methanol/oil molar ratio, amount of catalyst, reaction temperature, and time are found to be cost-effective with an eco-friendly approach and *Spirulina maxima* wild stuff biomass being a low-cost feedstock with high FFA makes the process techno-economically viable. Besides, the presence of high FFA content in the feedstock was reduced by appropriate pre-treatment which is evident through optimum production biodiesel *via* subjected to transesterification with a pertinent catalyst. As a whole, all the crucial parameters influencing the process of reactions were optimized to achieve a optimum yield of (96.65%) biodiesel. Captivatingly, the produced biodiesel with significant physical and chemical properties meet the terms with ASTM specifications. As a whole, the direct exploration of *Spirulina maxima* wild stuff algal sample from its natural habitation is found to be significant and the algal *in vitro* culturing part can be omitted.

5. Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

6. Acknowledgment

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