Enhancement of biodiesel production from the green microalga *Micractinium reisseri* via optimization of cultivation regimes

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**ABSTRACT**

The present work introduces a new strategy for enhancing a sustainable biodiesel from *Micractinium reisseri* by combining mixotrophic nutrition with nutrients stresses. Cultivating of *Micractinium reisseri* in 25 mg/l promoted lipid yield reaching 206%. While mixotrophic nutrition by 0.1 M glycerol promoted dry weight by 2.8 folds, 0.05 M glycerol enhanced lipid yield by 26% only. However, supplementation algal culture by 1 g/l sugarcane molasses promoted lipid content by 61% and total lipid yield by 31%. While polyunsaturated fatty acids (PUSFA) reduced from 48% to 31%, monounsaturated fatty acids (MUSFA) increased from 14% to 30% and Oleic and Stearic acids increased by 383% and 284%, respectively under optimized conditions. Evaluation of the produced biodiesel by monetary standard EN 14214 and ASTM D-6751 analysis exhibited high-quality products. Combining mixotrophic nutrition with salts and nitrogen stresses led to encouraging switching the metabolic pathways towards lipid output that used as a biodiesel source.

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1. Introduction

Energy stands alone to be the 1st key for the development of human welfare. The ascension in crude prices and controlling greenhouse gas emissions has seen a renewed interest in large-scale biodiesel production [1]. Since fossil fuel (traditional oils) is non-renewable, it is more likely that fuel expenses will face a steep hike in the near future. So there is no doubt that huge problems will appear unless renewable energy sources are found and developed [2].

For this reason, the conversion of solar energy into chemical energy within algal cells is considered logically and competitively approaches for its sustainability and economically [3]. Biodiesel is a renewable, clean, non-toxic, and sustainable alternative to petroleum-based fuels, so it can reduce toxic emissions when it is burned in a diesel engine [4]. Moreover, microalgae are capable of producing much more lipids than any cultivated crops Chisti [5], Schenk et al. [6]. The large-scale algal cultivation is not the only way for promoting biodiesel yield, but also improve the cultivation conditions lead to switching the metabolic pathway through which the relative and mesh biodiesel production will be enhanced. Many cultivation conditions (e.g. light intensity, availability of nutrients, salinity, heavy metals, pH scale, temperature, and carbon enrichment) can strongly affect the growth and metabolism of microalgae, including lipid accumulation. Under optimal growth conditions, large algal biomass could be gained but with relatively low lipid contents, which constitute about 5–20% of their cell dry weight (CDW) [7]. Under unfavourable environmental or stress conditions, many microalgae alter their fat biosynthetic pathways towards the formation and accumulation of neutral lipids, mainly in the form of Triacylglycerides (TAGs). In short circuit, biomass productivity and lipid output are inversely related [7]. Triacylglycerides (TAGs) generally serve as energy depots in microalgae that, once extracted, can be easily converted into biodiesel through transesterification reactions [8]. Nutrients availability strongly affects algal metabolism, that consequently influences algal growth and lipid production [9–11]. Switching the nutrition pattern to heterotrophic and/or mixotrophic behaviour may also improve biodiesel production [12–14]. Palmitic, Stearic, Oleic, Linoleic, and Linolenic fatty acids are the most abundant fatty acid methyl esters present in algae [15,16], which are known to be the predominant ingredient of biodiesel. Since the fatty acids profile of certain oil could be an indicator of biodiesel quality, the
fuel properties of biodiesel are logically influenced by the properties of the individual fatty esters in biodiesel. Fatty acids can considerably influence on fuel properties via cetane number (CN), cold flow, oxidative stability, viscosity, and lubricity. Generally, cetane number, the heat of combustion, melting point, and viscosity of fatty acids increase with chain length, while they decrease with increasing unsaturation [18]. The present work shows enhancement of the production of high-quality biodiesel from green microalga, *Micractinium reisseri*, through exposing it to nitrogen starvation, salt stress, and switch cultivation pattern to mixotrophic nutrition.

2. Material and methods

2.1. Cultivation and growth condition

*Micractinium reisseri* was obtained from the Algal Collection at Algae Research Unit, Tanta University. Three replicates of investigated microalga were cultivated in 1000 ml Erlenmeyer flasks filled with 500 ml of Bold Basal medium (BB medium) [19]. 10 ml of an algal suspension (16 days old of investigated alga) was inoculated into the new medium reaching an optical density of 0.05 at 680 nm [9]. The light intensity of 80 μmol photons⁻¹ m⁻¹ s⁻¹ was adjusted using tubular white fluorescent lamps. Cultures were incubated at 25 ± 1°C and stirred gently by air bump stream. Algal growth was monitored daily by optical density at 680 nm using Visible Spectrophotometer (721–100 VISIBLE LW, Scientific). Algal biomass was collected in logarithmic phase (after 16 days). For nitrogen stress, stock solution of NaNO₃ was used for adjusting the final concentration of 0%, 25% (0.73 mM), 50% (1.45 mM), 75% (2.18 mM), 100% (2.9 mM) and 150% (4.35 mM) of recommended NaNO₃ concentration in BB medium. Concerning sodium chloride stress, *M. reisseri* was exposed to sodium chloride concentration of (0, 12.5, 25, 37.5, 50, 62.5 mg/l).

2.2. Carbon enrichment

Two different carbon sources, glycerol and sugarcane molasses, were performed in different concentrations. In the case of glycerol, investigated alga was supplemented by glycerol concentrations of (0.0, 0.05, 0.1, 0.2 M), while in the case of sugarcane molasses, the final concentrations were (0, 1, 3 and 5 g/l). pH value was adjusted at 6.5 before autoclaving.

2.3. Estimation of dry weight, lipid content and lipid yield

30 ml of an algal culture was sediment by centrifugation at 5000 g for 5 min. followed by 3 washing cycles by distilled water. Algal cells were dried at 60°C till constant weight. Total lipid content was extracted from investigated algal cells according to modified protocols of Folch et al. [20] and Egan et al. [21]. One g algal dry cells were suspended in 16 ml distilled followed by adding 60 ml methanol: chloroform mixture (2:1) was added. After 2 min incubation period, 20 ml of chloroform was added. The mixture was incubated for an additional 30 min and then 20 ml distilled water was added and shaken for 30 s. Two layers were distinguished after centrifugation at 1000 g for 10 min. The lower layer (chloroform layer) was collected and filtrated through a coarse filter paper followed by evaporation till dryness.

Lipid content was estimated by dividing the weight of extracted total lipid from 30 ml of an algal culture by the algal dry weight of the same culture volume (mg/g – lipid/dry weight). Lipid content was estimated by dividing the weight of extracted lipids by the culture volume and expressed as (mg/ml) (w/v).

2.4. Cultivation of *Micractinium reisseri* in optimum condition

The optimum level of nitrogen stress and carbon enrichments were mixed into the combined condition and signed as an optimum condition for further experiment and analysis.

2.5. Determination of fatty acids profiles

Fatty acid methyl ester (FAMES) was prepared according to the modified method reported by Jumat et al. [22]. Esterified fatty acids were injected into gas chromatography (HP, Hewlett Packard, 6890 GC at Alexandria University). Reference and investigated fatty acids algal were run under the following conditions: Detector: FID (Flame Ionization Detector), Detector temperature: 240°C, Injectortemperature: 220°C, injection volume 3 μl, split ratio 50:1, Column: DB-23 (50% – Cyanopropyl-methylpolysiloxane), 30, 0.32 mm ID, 0.25μm film thickness, Carrier gas: Nitrogen, gas flow: 1 ml/min, Oven Program: Initial temp. 140°C for 5 min., Ramps = 1, Rate °C/min = 4, Final temp. = 240°, Hold time = 0.

2.6. Theoretical properties of produced biodiesel

Produced biodiesel properties were analyzed based on fatty acids profiles obtained via GC-analysis using computer software, the Biodiesel Analyzer software, version 2.2 (2016) http://www.brteam.ir/analysis/ or http://www.brteam.ir/biodieselanalyzer [23–25].

2.7. Statistical analysis

Results are presented as the mean ± standard error (SE) from three replicates. The statistical analyses were carried out using IBM SPSS (Statistics 20). Obtained data
were analyzed statistically to determine the degree of significance using one-way analysis of variance (ANOVA).

3. Results

3.1. Effect of salinity on lipid content and lipid yield

Salinity stress showed a direct effect on growth, lipid content, and lipid yield of *M. reisseri*. High sodium chloride concentration enhanced the dry weight, lipid content and lipid yield, where 64% and 152% rise in dry weight and lipid content were recorded, which consequently promoted the lipid yield to 206% compared to control. Statistical analysis pointed to the significant effect of NaCl concentration on growth, lipid content, and lipid yield of both microalgae (Table 1).

3.2. Effect of different nitrogen concentrations on lipid content

Obtained results in Table 2 reveal that in spite of high nitrogen supply (150% and 100%) promoted biomass production of *M. reisseri*, nitrogen starvation (75%) enhanced lipid induction. *M. reisseri* exhibited the highest lipid content at 75% N, where the lipid content increased to 133% and lipid yield to 123% compared to control. In contrast, lipid content and lipid yield reduced for tested microalga at 150% nitrogen content compared to control.

3.3. Effect of glycerol addition on lipid content and yield

As shown in Table 3, glycerol supplementation of *M. reisseri* enhanced both growth and lipid content at different concentrations, where raising glycerol concentration to 0.1 M enhanced the OD<sub>680nm</sub> and dry weight reaching 277% and 199%, respectively. Also, the lipid content of investigated alga was significantly affected by glycerol supplementation at 0.05 M. Cultures supplemented by 0.05 M glycerol promoted lipid content by 110%, while lipid yield increased by 22.4%. The statistical analysis of obtained results showed the significant impact of glycerol on both biomass production and lipid yield.

| Table 1. Effect of different sodium chloride concentrations on growth (OD<sub>680nm</sub>), dry weight (g/l), lipid content (mg/g) and lipid yield (mg/ml) *Micractinium reisseri* after 16 days incubation period. |
|---------------------------------------------------------------|
| **OD<sub>680nm</sub>**                                      | 0 | 12.5 mg/l | 25 mg/l | 37.5 mg/l | 50 mg/l | 62.5 mg/l |
| Average            | 3.332 | 3.818 | 4.837 | 4.58 | 4.53 | 4.255 |
| S. Error           | 0.07 | 0.013 | 0.013 | 0.018 | 0.017 | 0.014 |
| **Dry weight g/l**                                      | 0 | 50% | 100% | 150% | 200% | 250% |
| Average            | 1.65 | 1.8 | 2.717 | 2.55 | 2.4 | 2.08 |
| S. Error           | 0.104 | 0.1258 | 0.1856 | 0.18 | 0.153 | 0.264 |
| **Lipid content mg/g**                                   | 0 | 0% | 25% | 50% | 75% | 100% |
| Average            | 0.192 | 0.43 | 0.48 | 0.23 | 0.217 | 0.183 |
| S. Error           | 0.016 | 0.016 | 0.016 | 0.016 | 0.044 | 0.016 |
| **Lipid yield mg/ml**                                    | 0 | 0% | 25% | 50% | 75% | 100% |
| Average            | 0.118 | 0.18 | 0.244 | 0.094 | 0.094 | 0.088 |
| S. Error           | 0.008 | 0.0256 | 0.017 | 0.019 | 0.017 | 0.012 |

Data are statically analyzed using ONE-WAY ANOVA. Significant result is obtained at *P* = 0.05. M.S.: Mean square between groups and F: F-Value between groups.

| Table 2. Effect of different nitrogen concentrations on growth (OD<sub>680nm</sub>), dry weight (g/l), lipid content (mg/g) and lipid yield (mg/ml) of *Micractinium reisseri* after 16 days incubation period. |
|---------------------------------------------------------------|
| **OD<sub>680nm</sub>**                                      | 0 | 0.73 mM | 1.45 mM | 2.18 mM | 2.9 mM | 4.35 mM |
| Average            | 1.21 | 2.93 | 2.9767 | 3.47667 | 3.26 | 2.97 |
| S. Error           | 0.003 | 0.003 | 0.222 | 0.0145 | 0.0033 | 0.0058 |
| **Dry weight g/l**                                      | 0 | 0% | 25% | 50% | 75% | 100% |
| Average            | 2.1 | 2.333 | 2.6 | 3.23 | 3.0667 | 2.9333 |
| S. Error           | 0.058 | 0.033 | 0.058 | 0.088 | 0.088 | 0.067 |
| **Lipid content mg/g**                                   | 0 | 0% | 25% | 50% | 75% | 100% |
| Average            | 0.1667 | 0.2 | 0.22 | 0.41 | 0.31 | 0.28 |
| S. Error           | 0.02 | 0.017 | 0.01 | 0.0296 | 0.02 | 0.0236 |
| **Lipid yield mg/ml**                                    | 0 | 0% | 25% | 50% | 75% | 100% |
| Average            | 0.079 | 0.08533 | 0.085 | 0.127 | 0.1027 | 0.0877 |
| S. Error           | 0.008 | 0.008 | 0.0029 | 0.0064 | 0.0075 | 0.019 |

Data are statically analyzed using ONE-WAY ANOVA. Significant result is obtained at *P* = 0.05. M.S.: Mean square between groups and F: F-Value between groups.
that 18% reduction in dry weight, while a remarkable enhancement in total lipid content and lipid yield were observed in investigated microalga. Lipid content and lipid yield of *M. reisseri* increased by 65 and 51%, respectively.

These results support previously obtained results and could be used as optimum culture media for tested microalga. The statistical analysis of present results reveals that combination nitrogen deficiency, low salt stress, and supplementation of aleg media with glycerol and molasses have a significant role in the lipid yield of investigated microalga, which supports the hypothesis of the switch the pathway toward lipid.

### 3.6. Gas chromatography (GC) analysis of fatty acids

Not all fatty acids are suitable to be used as a biodiesel source; for this reason, fatty acids analysis is considered important parameters for qualification the biodiesel quality. The given data in Table 5 show significant differences in fatty acids profiles (quantity and quality level). Palmitic acid (C16:0) found to be the highest abundant fatty acid under control conditions, and it dramatically increased by 45% when culture cultivated in optimized conditions. Palmitoleic acid (C16:1) slightly increased by 3.5% (1.71–1.77%), Stearic acid (C18:0) remarkably increased by 183.94%. Moreover, Oleic acid (C18:1) increased by 283.41% (6.09% – 23.35%), while Linoleic acid (C18:2) reduced by 46.17% (41.85% – 28.63%). Although Linolenic acid (C18:3) exists in a low amount (1.82%), it decreased by reaching 0.33%. It could be believed that subjecting *M. reisseri* to optimized conditions could be the main reason for producing some new fatty acids like; γ-Linoleic acid (C18:3γ) 0.23%, Arachidic acid (C20:0) 0.14%, Arachidonic acid (C20:4) 0.14%, Eicosapentaenoic acid (EPA) (C20:5) 1.32%, Docosahexaenoic acid (DHA) (C22:6) 0.35%. While the only fatty acid which disappeared due to optimized conditions was cis-11,14-Eicosadienoic acid C20:2 (appeared under control conditions 4.35%). It should be pointed to the fact that almost all of these mentioned fatty acids were recognized as the most common fatty acids contained in biodiesel. The ratio of both saturated to unsaturated was slightly changed. Where cultivating *M. reisseri* in optimized conditions, the percentage of saturated fatty acids increased from 36.96% to 38.67%. Meanwhile, unsaturated fatty acids amount reduced from 63.04% to 61.33% (Table 5).

### 3.7. Biodiesel properties

Results in Table 6 show that the theoretical CN were ranged from 57.114 to 62.8, which are all in the accepted range (more than 47). Additionally, IV data were ranged from 64.224 to 87.462, which came in an accepted range of EN 14214 (Maximum 120). Regard-

### Table 3. Effect of inducing different concentration of glycerol on growth (OD_{680nm}), dry weight (g/l), lipid content (mg/g) and lipid yield (mg/ml) of *Micractinium reisseri* after 16 days incubation period.

|          | 0 g/l | 1 g/l | 3 g/l | 5 g/l | M.S. | F  |
|----------|-------|-------|-------|-------|------|----|
| OD_{680nm} | Average | 2.35 | 3.9 | 6.3 | 5.067 | 0.422 | 400.4 |
|           | S. Error | 0.0289 | 0.0289 | 0.0167 | 0.123 |
| Dry weight g/l | Average | 1.6283 | 2.825 | 3.233 | 3.0067 | 6.116 | 493.46 |
|           | S. Error | 0.142 | 0.00289 | 0.022 | 0.0088 |
| Lipid content mg/g | Average | 0.222 | 0.467 | 0.367 | 0.233 | 0.037 | 31.34 |
|           | S. Error | 0.01 | 0.025 | 0.02 | 0.02 |
| Lipid yield mg/ml | Average | 0.1363 | 0.1668 | 0.1135 | 0.0775 | 0.004 | 35.39 |
|           | S. Error | 0.005 | 0.004 | 0.0038 | 0.0014 |

Data are statically analyzed using ONE-WAY ANOVA. Significant result is obtained at *p* = 0.05. M.S.: Mean square between groups and F: F-Value between groups.

### Table 4. Effect of inducing different concentration of sugarcane molasses on growth (OD_{680nm}), dry weight (g/l), lipid content (mg/g) and lipid yield (mg/ml) of *Micractinium reisseri* after 16 days incubation period.

|          | 0 g/l | 1 g/l | 3 g/l | 5 g/l | M.S. | F  |
|----------|-------|-------|-------|-------|------|----|
| OD_{680nm} | Average | 3.145 | 3.28667 | 3.60333 | 4.9367 | 1.54 | 2648 |
|           | S. Error | 0.0087 | 0.0044 | 0.0192 | 0.0104 |
| Dry weight g/l | Average | 2.083 | 2.567 | 2.65 | 2.9333 | 3.244 | 291 |
|           | S. Error | 0.076 | 0.076 | 0.05 | 0.076 |
| Lipid content mg/g | Average | 0.217 | 0.35 | 0.2 | 0.15 | 0.206 | 0.802 |
|           | S. Error | 0.016 | 0.029 | 0.029 | 0.029 |
| Lipid yield mg/ml | Average | 0.104 | 0.1367 | 0.0756 | 0.05087 | 0.006 | 124.5 |
|           | S. Error | 0.0078 | 0.0128 | 0.0112 | 0.009 |

Data are statically analyzed using ONE-WAY ANOVA. Significant result is obtained at *p* = 0.05. M.S.: Mean square between groups and F: F-Value between groups.

### 3.4. Effect of sugarcane molasses addition on lipid content and yield

Results in Table 4 show the various effects of sugarcane molasses on investigated microalga. Although the optical density and dry weight of *M. reisseri* were enhanced by 57 and 40% at 5 g/l molasses, at 1 g/l sugarcane molasses, lipid content and lipid yield were increased to be 161 and 131% respectively.

### 3.5. Cultivation of algae under optimized conditions (combined nitrogen stress with carbon enrichments s conditions)

According to the previously obtained results, the optimum level of each single stress factor was applied (optimized media), where *M. reisseri* was cultivated in BB medium containing 25 mg/ml sodium chloride, 2.18 mM nitrogen concentration, 0.05 M glycerol, and 1 g/l molasses. Obtained results in Figure 1 show...
Figure 1. Dry weight (mg/mL*100), lipid content (mg/g) and lipid yield (mg/l) of M. reisseri grown under control and optimum conditions. Represented data were recorded after 16 days incubation periods. Data are statically analyzed using ONE-WAY ANOVA. Significant result is obtained at $P = 0.05$.

Table 5. Fatty acids profiles analysis of M. reisseri cultivated under control and optimized conditions (Data are expressed as relative percentage).

| FA%   | Fatty acid C-number FA | Controlled conditions | Optimized conditions |
|-------|------------------------|-----------------------|---------------------|
| SFA   |                        |                       |                     |
| Capric acid C10:0 | — | 0.137 |
| Undecanoic acid C11:0 | 1.968 | 0.462 |
| Lauric acid C12:0 | 0.363 |
| Myristic acid C14:0 | 0.374 |
| Pentadecanoic acid C15:0 | 0.389 |
| Palmitic acid C16:0 | 19.871 |
| Heptadecanoic acid C17:0 | 13.382 |
| Stearic acid C18:0 | 1.372 |
| Arachidic acid C20:0 | 3.892 |
| MUSFA |                        |                       |                     |
| Myristoleic acid C14:1 | — | 0.25 |
| Palmitoleic acid C16:1 | 1.718 |
| Cis-10-Heptadecenoic acid C17:1 | 7.185 |
| Oleic acid C18:1| — | 4.625 |
| PUSFA |                        |                       |                     |
| Linoleic acid C18:2| 41.85 | 28.632 |
| Linolenic acid C18:3| 1.828 |
| γ-Linolenic acid C18:3| — | 0.228 |
| Eicosadienoic acid C20:2 | 4.355 |
| Arachidonic acid C20:4| — | 0.299 |
| Eicosapentaenoic acid C20:5| 1.4 |
| Arachidonic acid C20:4| — | 1.326 |
| Docosahexaenoic acid C22:6 | — | 0.358 |

Total PUSFA 48.03 | 31.32 |
Total MUSFA 14.99 | 30.25 |
Total USFA 63.03 | 61.57 |
Total SFA 36.97 | 38.67 |

SFA = saturated fatty acids, USFA = unsaturated fatty acid, MUSFA = Mono-unsaturated fatty acid, PUSFA = Poly-unsaturated fatty acid.

Table 6. Evaluation of theoretical biodiesel properties of detected fatty acids under controlled and optimized conditions using Biodiesel Analyzer software, version 2.2 (2016) for M. reisseri.

| Property                  | M. reisseri control | M. reisseri optimized | Accepted range Ref. |
|---------------------------|---------------------|-----------------------|---------------------|
| CN_min                    | 57.521              | 57.114                | 47 ASTM D-6751      |
| IV_max                    | 87.462              | 78.307                | 120 EN 14214        |
| CP_min                    | 5.46                | 10.199                | 3.12 ASTM D-6751    |
| PP                        | −0.894              | 4.25                  | −15:10 ASTM D-6751  |
| $V_{mm2/s}$               | 3.045               | 3.4                   | 1.96 EN 14214       |
| $OS_{min}$                | 5.29                | 6.629                 | 3 ASTM D-6751       |

4. Discussion

Algal biodiesel starts to be one of the sustainable energy sources that can be an alternative to fossil sources. Although microalgae are the main biodiesel source, the cost of produced biodiesel is still quite high. Presently, several challenges try to overcome this problem by enhancing the productivity of biodiesel. This may occur by switching the metabolic pathways to promote both biomass and lipid content. The obtained results reveal promoting biomass production and lipid content at a low salt concentration (25 mg/L). This enhancement may be due to the presence of salt-tolerant enzymes that support the growth and metabolic activities of some microalgae due to the [26] and/or enhancement of ATPase activity [27]. The main role of sodium chloride in algal media may be oxidative stress leading to increment in the triacylglycerol content [28–30] and/or changes in lipid amount and composition [27]. The type and concentration of salts are strain-dependent [31].

Due to the incorporation of nitrogen within almost all cell components, it is considered one of the essential macronutrients that represent 7–20% of cell dry weight [32]. For this reason, under nitrogen limitation, algal cells switch the metabolism to lipid accumulation [12,33,34]. Decreasing nitrogen concentration to 1.8...
mM (75% of recommended concentration) promoted the lipid content and lipid yield 133% and 123%, respectively. These results agree with that of Hu and Gao [35], who observed a four-fold increase in lipid content of *Nannochloropsis* sp. under nitrogen starvation. Sheehan *et al.* attributed the reason for raising the lipid content under N-deficiency to lowering of cell protein components with remaining the lipid content that finally leads to an accumulation of oil in the cells [36]. The present results showed promoting effect of mixotrophic nutrition by glycerol on dry weight (199%) and lipid accumulation (210%) of *M. reisseri*. These data are supported by those published by EL-Sheekh et al. [13,37]. Choi and Yu and El-Mohsnawy *et al.* reported that supplementing *Chlorella vulgaris*, *Scenedesmus obliquus*, and *Botryococcus braunii* by glycerol promoted the growth and lipid production of investigated algae. Algae may also use glycerol as an artificial carbon source to support the growth and lipid content [14,37].

Sugarcane molasses promoted the dry weight to 140% and lipid induction to 161% of *M. reisseri*. The main reason for lipid induction may be due to the utilization of some part of the energy in cell division, while the excess energy could be stored in the form of lipid granules [38,39]. The presence of some vitamins, trace elements, and many other kinds of ingredients promote the metabolism, including lipid production [40,41]. Since the main chemical composition of molasses is soluble sugar (sucrose 29%, glucose 12%, and fructose 13%), a lot of vitamins (Thiamin, Riboflavin, Niacin, Pantothenic acid, Vitamin B-6, Vitamin B-6), several minerals and few amount of lipids [40], both biomass and lipid content should be promoted at low concentration, where the soluble sugars are easily incorporated within different metabolic pathways including lipid accumulation [41]. Additionally, vitamins and minerals may enhance the activity of the cell leading to high lipid yield.

Fatty acid profile differs among photosynthetic organisms; fatty acids produced by microalgae usually contain mainly C16 and C18 fatty acids, which are suitable for biodiesel production [42].

Obtained results showed high fatty esters are Palmitic acid (16:0), Stearic acid (18:0), Oleic acid (18:1), Linoleic acid (18:2), and γ-Linolenic acid (18:3) that enhanced by applied stress and mixotrophic nutrition. These fatty acids are a common component of biodiesel [15]. Changing fatty acids profile after exposing stress and carbon enrichment was supported by data reported by Wang *et al.* [43], who found that cultivating of *Chlorella zofingiensis* heterotrophically enhanced the production of Palmitic acid, Oleic acid, and Linoleic acid. Jena *et al.* [44] observed high produced 34% saturated fatty acid and 66% unsaturated fatty acid of *Chlorella vulgaris* by mixotrophic nutrition. The present results showed agreement with that of Karpagam *et al.* [45] who found that of C-14, C-16:0, C-18:0, C-18:1, and C-18:2 were the most abundant fatty acids of *M. reisseri* that exposed to stress conditions.

Since the most common obtained fatty acid methyl esters present in our biodiesel are C16–C18 (Palmitic acid, Stearic acid, Oleic acid, Linoleic acid, and Linolenic acid) which are known to be the highest predominant components of biodiesel [17], determination the properties should support the application of using present investigated microalga as a source of renewable energy (biodiesel). Cetane number (CN), oxidation stability (OS), cloud point (CP), iodine value (IV), and viscosity (V) are considered limiting factors for the quality of biodiesel, so by comparing obtained data with that of American standardization (ASTM D-6751) and European standardization (EN 14214), it comes in the acceptable range [23]. Moreover, obtained biodiesel is very similar to conventional diesel fuel, so it does not require any engine modifications [15,23,46]. The most important features of obtained biodiesel are their Viscosity, Density, Cetane Number, Cloud, Pour Points, Flash Point came in an accepted range of either ASTM D-6751 or EN 14214 [15,46]. These properties depend upon the proportion of long-chain and short-chain of constituent fatty acids and the presence of one or more double bonds (saturated or unsaturated) [23].

Generally, Cetane Number (CN), the heat of combustion, and viscosity increase with increasing chain length, which means that long-chain fatty acid (C16–18) is preferable [42]. The CN increases in fuels with high amounts of saturated fatty acids [47], the higher CN, the better ignition quality.

5. Conclusion

Through the present work, *Micractinium reisseri* was exposed to chemical stresses and mixotrophic nutrition. Statistical analysis of low salt treatment and nitrogen limitation were found to be key parameters for lipid yield in *M. reisseri*. Soluble sugars, vitamins, and minerals present in sugarcane molasses saved additive carbon sources required for lipid yield. Also, low glycerol-containing media (0.05 M) promoted induction of lipid content and yield, fatty acids profile analysis showed no remarkable changes of saturated fatty acids, while the polysaturated fatty acids remarkably reduced and monosaturated fatty acids increased. Properties evaluation of produced biodiesel of investigated microalga under control or optimized condition with a standard level of EN 14214 and ASTM D-6751 proved the high quality of produced biodiesel, enabling it to be used in industrial applications without additional modifications.

Disclosure statement

No potential conflict of interest was reported by the author(s).
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