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Biodiesel properties of *Neochloris oleoabundans* grown in sludge waste☆

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ABSTRACT

Within the bioremediation framework, this study was aimed at finding out the optimal amount of sludge waste consumption and microalgal growth conditions of *Neochloris oleoabundans* in order to reach a significant fat production and chemical transformation of microalgal lipids into biodiesel. The effects of the lipid extraction methods – using ionic liquids and chloroform – on the total amount of fat, the individual fatty acid concentrations, and the biodiesel properties were assessed and compared to the EN 14214 and ASTM D6751−02 biodiesel standards. The microalgae strains grown in the optimized sludge waste medium with a dilution of 0.65 g/L wet sludge, and extracted by ionic liquid:chloroform mixed solvent, resulted being the most significant strains to obtain the highest quality of biodiesel with a density of 0.887 g cm^{-3}, kinematic viscosity of 4.7 mm^2 s^{-1}, higher heating value of 40.21 MJ kg^{-1}, iodine value of 107.3 g I_2 100 g^{-1} fat, and cetane number of 50.

1. Introduction

Growing energy demand leads to a necessary evaluation of sustainable and renewable energy sources, thus limiting the erosion of fossil fuels. Population increase and environmental pollution have a critical impact on the ecological balance, along with the increase of greenhouse gases (Al-Ghussain, 2019). To tackle these problems, focusing on biodiesel production could bring about important benefits. Biodiesel is, indeed, the most common biofuel due to its high similarity with conventional diesel being compatible with the current engine models.

Oleaginous microorganisms (OM) are very attractive as they do not compete with food crops. The OM are species of bacteria, algae, molds, and yeasts with fatty acid compositions similar to vegetable oils (Arora et al., 2019) which are able to utilize or bio-convert different types of agro-industrial waste and by-products into cellular lipids (Altunoz et al., 2020; Mandotra et al., 2020).

Algae are capable of efficiently producing primary metabolites such as vitamins (Ljubic et al., 2020), pigments (Saini et al., 2020), lipids (Veronesi et al., 2020), proteins and carbohydrates (Hussain et al., 2020), over short periods of time. In addition, algae can also be conveniently used for the bioremediation of polluted wetlands (Allesina et al., 2017; Altunoz et al., 2017).

Microalgae have gained increasing attention as a third-generation biodiesel source (Naeini et al., 2020) due to their high specific growth rates (Chisti, 2007) and lipid/fatty acid productivities (Wahidin et al., 2018). *Neochloris oleoabundans* is a fast-growing microalga characterized by a high triacylglycerols' production (Tornabene et al., 1983), up to 40% of the total dried microalgal biomass (Li et al., 2008). For this reason, it is considered a promising candidate for biofuel production. Further, it has a good heterotrophic growth capability (Altunoz et al., 2017). The successful commercial production of biodiesel from microalgal biomass is directly related to the lipid extraction methods, some of which are currently at starting stage except for lab applications (Bligh and Dyer, 1959; Folch et al., 1957). Other methods employed for microalgal lipid extraction exploit mechanical disruption (Harris et al., 2018), homogenization, enzymatic extractions (Mercer and Armenta, 2011), supercritical fluids (CO_2, as well as alcohols, such as methanol or ethanol) (Jafari et al., 2021), and ultrasonic-assisted extraction (Goh et al., 2019).

Ionic liquids are an interesting alternative to organic solvents due to their unique characteristics such as thermal stability, tunable viscosity, very low vapor pressure, miscibility with water and organic solvents, and good extractability for a wide range of compounds (Han and Row, 2010). In addition, they require less energy than organic solvents and, finally, are easily recyclable and reusable (Bahadur et al., 2016).

Specific modifications of the ionic liquid extraction process can

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increase the lipid yield and, in turn, the biodiesel quantity and quality. Biodiesel properties can also be affected by the feedstock used for algal growth and by the use of catalysts. Properties of biodiesel must comply with EN-14214 specifications for Europe (Automotive fuels, 2003), and with the American society for testing and materials' specifications (ASTM, D-6751) for the USA, in order to be used with diesel engines (Gerpen, 2005). The main parameters of biodiesel are related to the molecular weight (Ramírez-Verduzco et al., 2012) and the number of double bonds of the fatty acids methyl esters (FAMEs) (Giakoumis and Sarakatsanis, 2018), as well as the long-chain saturated factor (LCSF) (Deshmukh et al., 2019a). In addition, biodiesel density plays a direct role in the performance of diesel engines (Deshmukh et al., 2019b), specifically on the injection systems and pumps to provide convenient combustion by delivering a suitable amount of fuel (Rajak et al., 2019).

The experimental project focused on a complete biodiesel production chain (Gouveia et al., 2009), starting with oleaginous microalgae *N. oleoabundans* cultivation (Deason et al., 1991), and finishing with the analysis of fatty acid (FA) profiles (Hwang and Maier, 2019) and the determination of biodiesel properties (Kalaimurugan et al., 2020).

This specific study aimed at sludge waste valorization as feedstock as well as at evaluating the optimal microalgal growth medium, based on the microalgae lipid yield and FA profile. The main biodiesel parameters have been determined to assess the biodiesel quality according to the different growth media and lipid extraction methods. In particular, the lipid extraction efficiency of 1-butyl-3-methylimidazolium trifluoromethanesulfonate [BMIM] [CF_3SO_3] was compared to the extraction efficiency of [BMIM] [CF_3SO_3] mixed with chloroform.

2. Materials and methods

2.1. Chemicals

Chloroform, hexane, and methanol were all of the analytical grade (VWR International Ltd, Milan, Italy). Ionic liquid (IL), specifically 1-butyl-3-methylimidazolium trifluoromethanesulfonate [BMIM] [CF_3SO_3], hydrochloric acid (HCl), sodium hydroxide (NaOH), Supelco 37 Component FAME mix, and undecanoic acid methyl ester (internal standard) were purchased from Merck-Sigma-Aldrich® (Milan, Italy). Deionized water was obtained through an Elix 3UV purification system (Merck-Millipore, Milan, Italy).

2.2. Microalgal growth conditions and mediums

N. oleoabundans (Sin. *Ettlia oleoabundans*) from the University of Modena and Reggio Emilia, Interdepartmental Research Centre BIOGEST - SITEIA (Reggio Emilia, IT) was used. Fourteen days old microalgal cells were inoculated in 1-L Erlenmeyer flasks containing 800 mL of growth medium for each sample (10%, v/v).

The sludge was provided by the wastewater treatment company Montagna2000 Spa (Borgo Val di Taro, Parma, Italy). The company manages the integrated water service of a large area that includes the Taro and Ceno valleys in the Province of Parma (Italy), giving service of the collection and treatment of the wastewater/sewerage, and distribution of the water in the territories of the municipalities. The sludge waste composition was obtained from the wastewater treatment company, and it was analyzed according to the 'Regional provisions on the use of sewage sludge for the benefit of agriculture' (D.G.R. Lombardia 1 luglio 2014, n. X/2031).

Four different amounts of wet sludge waste dilutions were prepared: 0.65 g/L (S1), 1.30 g/L (S2), 2.60 g/L (S3), and 5.20 g/L (S4), while the strains for the control group (Ctrl) were cultivated using BG_{11} microalgal growth medium. Each culture medium was autoclaved at 121 °C for 15 min. The pH of the mediums was adjusted to 7.3 ± 0.1 using NaOH and HCl solutions, and airflow was supplied with an aeration rate of 0.5 vvm. The temperature of the cultivation was adjusted to 28 ± 1 °C, under the 8/16 h cold white LED light (avr. 555 nm) photoperiod.

The microalgal growth medium was optimized based on the content ratio of the trace elements, carbon, nitrogen, or phosphorus of the sludge waste. The BG_{11} growth medium was taken as a reference, while the sludge waste medium was optimized based on the BG_{11} growth medium composition (Rippka et al., 1979).

2.3. Pigment extraction, optical density, and growth rate

The samples were periodically collected from each strain and transferred into Eppendorf tubes (2 mL). The tubes were centrifuged at 4000 rpm for 5 min and washed three times. The supernatant was removed, and methanol solution (90%) was added into the tubes which were vortexed for 15 s and left at 70 °C for 20 min. Then, the samples were kept under dark conditions for 5 min and centrifuged at 4000 rpm for 5 min. Finally, the supernatants were collected to be measured using a UV–Vis spectrophotometer (Jasco V-500/V-600 Series Instruments, Jasco Europe, Cremella, Italy). Absorbance spectrum was scanned from 350 to 800 nm for analytical determinations of chlorophyll *a* (Chl *a*), chlorophyll *b* (Chl *b*) and total carotenoids. The pigment contents were calculated using Eq. A.1 (Lichtenthaler and Buschmann, 2001). The growth rate was calculated according to each sample's optical density (665 nm) values within 9 days from the beginning of the growth period (Eq. A.2).

2.4. Lipid extraction using ionic liquids

At the end of the photoautotrophic growth phase, samples were centrifuged, washed, and dried using a vacuum-centrifuge concentrator (Eppendorf Concentrator 5301, Hamburg, Germany) up to a moisture content <10%.

After weighing the samples, lipids were extracted using IL according to two different methods:

i) with IL. A sample of dried microalgae (200 mg) was obtained from each strain and suspended in IL with a ratio of 1:5 (g/g) in a 10 mL screw-cap glass tube that was kept at 105 °C for 2 h (IL strains);
ii) with IL:chloroform. The extraction solvent was prepared by mixing IL and chloroform (IL:C) with a ratio of 1:1 (V/V). Each sample of dried microalgae (200 mg) was obtained from each strain and transferred into 10 mL screw-cap glass tubes containing IL:C. The extractions were performed at 70 °C for 20 min (IL:C strains). The method was modified based on the Folch method (Folch et al., 1957).

Both extracts were homogenized through an Ultra-Turrax® T25 homogenizer (IKA, Königswinter, Germany) and then centrifuged at 2000 rpm for 15 min (Montevecchi et al., 2020). Finally, the supernatants with IL and IL:C (total lipids) were transferred into Eppendorf tubes and dried using a vacuum concentrator. The remaining cell debris was centrifuged again (4000 rpm, 10 min) to separate residual IL, and finally, IL was recovered from the supernatant.

2.5. Fatty acids analysis

2.5.1. Acid-catalyzed esterification and transesterification for FA determination

Acid-catalyzed transesterification was carried out on the samples to determine total FAs using the method described by Christie (1982). This specific transesterification method, which is widely used in the analytical field, can convert all fatty acids (free and bound fractions) into fatty acid methyl esters.

2.5.2. GC-MS determinations of fatty acids

A gas chromatograph (HP 6890 Series, Hewlett-Packard, Waldbronn, Germany) with a split/splitless injection port and coupled with a mass spectrometer (HP 5973 Mass Selective Detector, Hewlett-Packard, Waldbronn, Germany) was used to determine FAs as FAMEs as already

described by Hadj Saadoun et al. (2020).

Peaks were identified by comparing the retention times and mass spectra of pure standards (Supelco 37 Component FAME Mix, Merck) and by comparing the mass spectra with those ones present in libraries focused on fatty acids methyl esters (Famedb23.l and Famedbwax.l; Agilent Technologies). Quantification was performed using the internal standard (undecanoic acid methyl ester) method. Each sample was analyzed twice, and the fatty acids were expressed as mg/100 $mg_{algal\ biomass}$.

2.6. Fuel properties from fatty acid profiles

Fuel characteristics and performances were calculated with the methods described by Ramírez-Verduzco et al. (2012) for the fuel properties, Ramos et al. (2009) for the biodiesel quality, and Park et al. (2008) for biodiesel properties and the fatty acid compositions (see Appendix).

2.7. Data analysis

Parametric and non-parametric statistical processing were used to analyze the significance of the obtained data. Since parametric ANOVA tests, (such as normality, equal variance and equal or near-equal sample size) were not satisfied (Zar, 2007), non-parametric Kruskal-Wallis ANOVA and Friedman's 2-factor ANOVA tests were performed to observe the differences in median values among the five samples. The tests were performed using Matlab R2016a software (Math-Works, Natick, Massachusetts, USA).

3. Results

3.1. Elemental composition of the sludge waste

The elemental composition of the sludge waste was: 27.2 g/100 g dry matter ($_{dm}$) organic carbon, 4.5 g/100 g_{dm} total nitrogen, 1.0 g/100 g_{dm} total phosphorus, 4.1 g/kg_{dm} potassium, 3.1 mg/kg_{dm} arsenic, <1.0 mg/kg_{dm} cadmium, 87.9 mg/kg_{dm} chromium, <1.0 mg/kg_{dm} mercury, 62.6 mg/kg_{dm} nickel, 34.8 mg/kg_{dm} lead, 266 mg/kg_{dm} copper, 388 mg/kg_{dm} zinc, 20.5 g/100 g fresh weight moisture content.

The amount of trace metals (such as copper and zinc) is crucial since high concentrations of them can inhibit algal growth. Indeed, microalgal cells need a balanced and accurate mechanism to regulate uptake, transportation, and allocation of these elements (Xiao et al., 2018). Furthermore, the photosynthesis apparatus is sensitive to high levels of heavy metals (Yong et al., 2020).

3.2. Optical density and growth rate determinations of N. oleoabundans cells

Optical density and growth rate parameters are shown in Fig. 1a and b, respectively. According to the optical density determinations, after the 4th day, all the strains grown in sludge waste media showed higher values than those shown by Ctrl strains ($p < 0.05$). The highest values were recorded in S1 and S2 strains at the end of the 9th day (Fig. 1a). According to the growth rate determination, S1 strains showed a sharp increase on the 2nd and on the 7th days (Fig. 1b). It is known that *N. oleoabundans* cells exposed to various growth stress factors, such as illumination (Altunoz et al., 2017) or growth medium alterations (Altunoz et al., 2020), show a high peak of growth rate at the initial days of the growth period, followed by a decrease, and then slightly increases till around the 9th (±3) day depending on the initial cell concentration and the volume of the cultivation (Loera-Quezada et al., 2011). Similar behavior was observed in the present study. In short, stress factors cause a rapid increase in cell concentration in parallel with the cell division, thus assuring the continuance of the population after the initial cell deaths occurred due to the stress factor. Consequently, high cell concentration values provide a duration of adaptation for the cultivation exposed to stress factors where the remaining living cells are capable of continuing microalgal growth with success and stability.

On the 9th day of the cultivation, S1 and S2 strains showed lower growth rates in comparison with the 7th day (Fig. 1b). This behavior is explained by the accumulation of lipids in the cells. Indeed, during the decreasing growth rate phase, the mature cells stopped cell division and started to accumulate lipids under the growth conditions in question. According to growth rate determinations, it is recommended to refresh the sludge waste medium in a concentration of 0.65 g/L (which is S1 strains) at the 9th day.

The control strains showed a steady growth rate and only started to decrease after the 4th day. This behavior was predictable considering the low optical density values of the strain. According to the growth rate observed within the entire growth period, even S4 strains, which contained the highest amount of sludge waste (5.20 g/L), were able to tolerate and metabolize the sludge contaminant. The highest values of the growth rate were recorded in S1 and S4 strains after 7 days. This data suggested that both the lowest (0.65 g/L) and the highest concentrations (5.20 g/L) of wet sludge waste were suitable to obtain an optimal biomass yield from microalgal cultivation.

Kruskal-Wallis one-way ANOVA applied on the optical density and growth rate data showed the following results for χ^2 and p values: 21.23 and 0.0003 for optical density, 13.83 and 0.0031 for growth rate, respectively, while Friedman's ANOVA tests showed the following results for χ^2 and p values: 18.72 and 0.0009 for optical density, 10.68 and

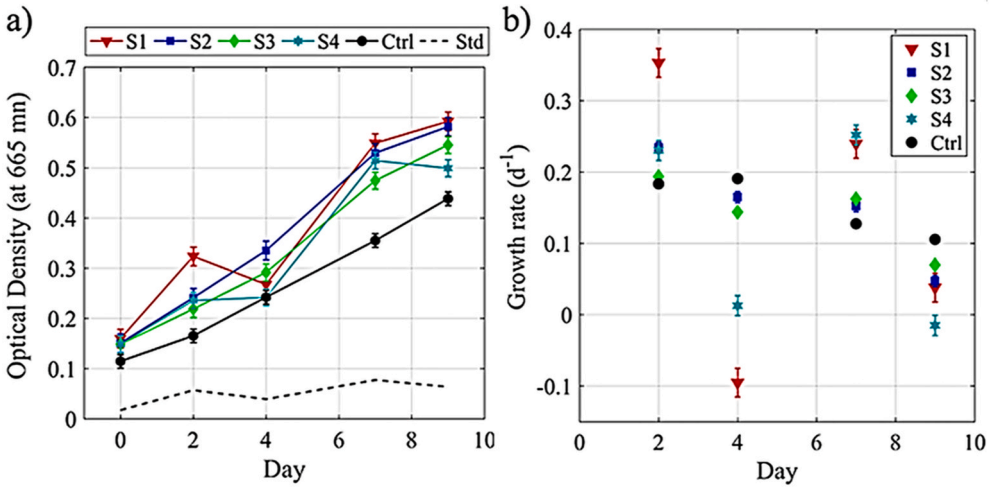

Fig. 1. a) Optical density vs. time response (day); **b)** Growth rate vs. time response (day) of *N. oleoabundans* within 9 days of growth period.
In **1a)**, the filled markers and five lines represent the *N. oleoabundans* strains grown in five different microalgal growth mediums. Dashed line represents the standard deviation. The strains are represented as: **S1:** 0.65 g/L, **S2:** 1.30 g/L, **S3:** 2.60 g/L, **S4:** 5.20 g/L sludge waste concentrations used in the microalgal growth medium, while **Ctrl** are the control strains grown in BG 11 medium. All the data reported in the figures are the mean values of three biological samples ± SD. Matlab R2017a (MathWorks, Natick, Massachusetts, USA) was used to create the figures.

0.0136 for growth rate, respectively. Both tests showed significant differences among the samples due to $\chi^2 > 5$ and $p < 0.05$.

3.3. Photosynthetic pigment content

Photosynthetic pigment contents are shown in Fig. 2 and statistical analysis was applied to assess the different treatments. All strains grown in the sludge waste medium showed higher values of Chl *a* in comparison with the Ctrl group (Fig. 2a). S1 strains had the highest amount of Chl *a* content (6.9 μg/mL) followed, in decreasing order, by S2 (6.7 μg/mL), S3 (6.3 μg/mL), and S4 (5.8 μg/mL) strains. The highest amount of Chl *b* was observed in the S2 strains (2.2 μg/mL) followed by S1 (2.1 μg/mL) and S3 (2.1 μg/mL) strains, while Ctrl (1.9 μg/mL) and S4 (1.9 μg/mL) strains showed lower values (Fig. 2b). The chlorophyll content of the S1 strain was 9.01 μg/mL, thus showing the successful growth of the strain. In a previous study performed under the nitrogen replete and depleted conditions, the chlorophyll contents of *Nannochloropsis oculata* were 9.40 and 7.38 μg/mL, respectively (Surendhiran and Vijay, 2014).

S1 strains showed the highest amount of carotenoids content (1.7 μg/mL), followed by S2 and S3, while S4 (1.4 μg/mL) and control (1.3 μg/mL) strains showed lower values and similarities between each other (Fig. 2c). Carotenoid contents of *Chlorella vulgaris* and *Nannochloropsis* sp. cultivated in secondary treated municipal wastewater were determined as 0.75 and 0.80 mg/$g_{algal\ biomass}$, respectively (Fallahi et al., 2020).

The microalgal pigment content analyses were performed periodically to monitor the growth dynamics of the microalgal strains during the cultivation period. The weight ratios of pigments provide information about the microalgal growth conditions. For example, low values for the ratio [Chl (*a*+*b*)/Carotenoids (x + c)] are a chemical marker of stress (Lichtenthaler and Buschmann, 2001). Moreover, the pigment and the lipid concentrations play an important role in the monitoring of microalgal cell content production and metabolism in general.

Kruskal-Wallis one-way ANOVA applied on Chl *a*, Chl *b*, and carotenoids data showed the following results for χ^2 and *p* values: 21.23 and 0.0003, 21.84 and 0.0002, 20.44 and 0.0004, respectively; while Friedman's ANOVA tests showed the following results for χ^2 and *p* values: 18.72 and 0.0009, 19.36 and 0.0007, 18.72 and 0.0009, respectively. Both tests showed significant differences among the samples due to $\chi^2 > 5$ and $p < 0.05$.

3.4. Dried biomass, total lipid determination, and fatty acid profiles

The dried biomass content of the strains was determined (Table 1) with a moisture content ≤10%. According to Raoult's law, which is only applicable to ideal diluted solutions, water activity commonly used to determine the stability of a product is equal to free water content of the specific material. The final moisture content ≤10% wet basis was selected since it prevents microbial growth, enzymatic and non-enzymatic reactions and therefore it guarantees safe storage.

Table 1
Biomass dry weights and lipid contents of *N. oleoabundans* after the growth period.

Strains	Dry weight (g/L)	Lipid contents of dried microalgal biomass (mg/100 $mg_{algal\ biomass}$)	
		IL	IL_C
S1	0.300 ± 0.001	38.5 ± 1.4	41.8 ± 1.4
S2	0.290 ± 0.001	32.7 ± 0.8	40.5 ± 1.2
S3	0.340 ± 0.001	30.7 ± 0.6	27.2 ± 0.7
S4	0.360 ± 0.001	28.7 ± 0.7	30.8 ± 0.8
Ctrl	0.270 ± 0.001	31.1 ± 0.4	39.6 ± 0.6

Lipid extraction solvents were represented as: **IL,** Ionic liquid [BMIM] [CF_3SO_3]; **IL:C,** Ionic liquid [BMIM] [CF_3SO_3] and chloroform mixed solvent. The strains are represented as: **S1,** 0.65 g/L; **S2,** 1.30 g/L; **S3,** 2.60 g/L; **S4,** 5.20 g/L sludge waste concentrations used in the microalgal growth medium and **Ctrl** are the control strains grown in BG 11 medium.

In a study performed using *N. oleoabundans* fed with wastewater, the highest dried biomass value was determined as 0.4 ± 0.1 g/L (Valev et al., 2020), which corresponds to the dried biomass content found in the present study. Further, the dried biomass amount of *N. oleoabundans* could be increased by adding a heterotrophic growth phase after the photoautotrophic growth one (Altunoz et al., 2020).

Ionic liquids were applied for the extraction of lipids after considering the composition and structure of the cell walls in microalgal species *N. oleoabundans* belonging to Chlorophyta division (Baudelet et al., 2017). The ionic liquid [Bmim][CF_3SO_3] had already proven to be a promising solvent for the extraction of lipids from microalga *Nannochloropsis oceanica* compared to other ionic liquids such as [Emim][Ac], [Emim][$DEtPO_4$], and [Bmim][HSO_4] (Lee et al., 2015) due to its low viscosity (90 mPI at 25 °C) at room temperature (Huddleston et al., 2001). Accordingly, in this study, [Bmim][CF_3SO_3] was selected as ionic liquid to be used for the extraction of lipids from *N. oleoabundans* microalgal cells.

The lipid percentages of dried microalgal biomass showed that lipids accumulated more in strains S1 (41.8 mg_{lipid}/100.0 $mg_{dried\ algal\ biomass}$) and S2 (40.5 mg_{lipid}/100.0 $mg_{dried\ algal\ biomass}$) extracted with IL:C, compared to S3 and S4 (Table 1). Chi-square (χ^2) and statistical significance (*p*) tests were applied on lipid content data using Friedman's ANOVA. χ^2 and *p* values were determined as 18.57 and 0.0291,

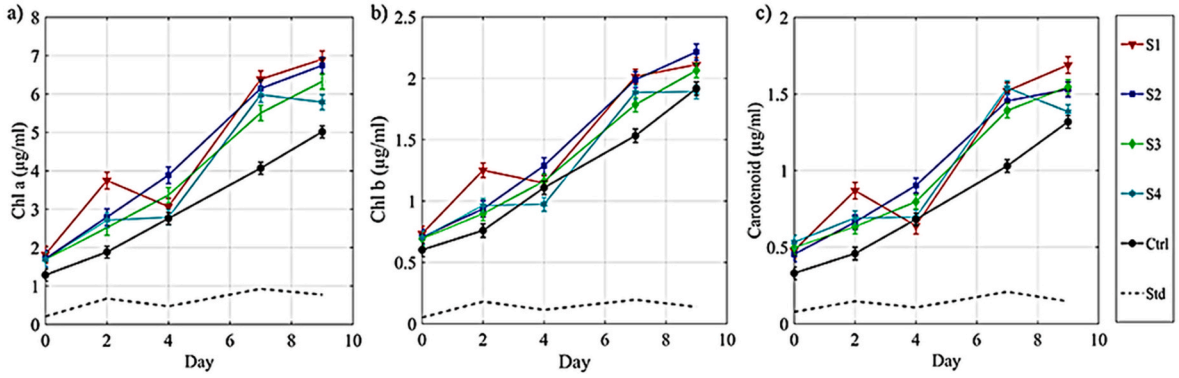

Fig. 2. Photosynthetic pigment content. **a)** Chl-a content vs. time response (day); **b)** Chl-b content vs. time response (day); **c)** carotenoids' content vs. time response (day) of *N. oleoabundans* within 9 days of growth period.
In each figure, the five lines represent the *N. oleoabundans* strains grown in five different microalgal growth mediums. Dashed lines represent the standard deviation. The strains are represented as: **S1:** 0.65 g/L, **S2:** 1.30 g/L, **S3:** 2.60 g/L, **S4:** 5.20 g/L sludge waste concentrations used in the microalgal growth medium and **Ctrl** are control the strains grown in BG 11 medium. All the data reported in the figures are the mean values of three biological samples ± SD. Matlab R2017a (MathWorks, Natick, Massachusetts, USA) was used to create the figures.

respectively, which showed significant differences among the samples due to $\chi^2 > 5$ and $p < 0.05$. In addition, all the lipid extractions performed using [Bmim][CF_3SO_3] and chloroform mixed solvent have proven to be more effective (on average by 3.6% higher) than the extractions performed using IL alone.

A total of 15 FAs were quantified as FAMEs (Table 2). Palmitic acid ($C_{16:0}$), oleic acid ($C_{18:1}$), linoleic acid ($C_{18:2}$); and α-linolenic ($C_{18:3}$) showed the highest concentrations. In particular, oleic acid content showed the highest concentrations in the S1 strains extracted using both IL and IL:C. However, its concentration progressively decreased when the sludge amount in the growth medium was increased. The concentrations of linoleic acid and α-linolenic acid were in the order of 5–6 mg/100 $mg_{algal\ biomass}$. Also in this case, the highest concentrations were shown by the S1 and S2 samples. The combined use of ionic liquid and chloroform provided higher values of fatty acid concentrations than ionic liquid alone and showed higher difference between S1–S2 and S3–S4 strains.

As for saturated fatty acids, the highest concentrations of palmitic acid and stearic acid were found in S1 and S2 strains using IL:C and IL alone. The other FAs showed lower concentrations, however the highest concentrations were found in the S1 and S2 strains in many cases.

The FAs profiles extracted using IL:C showed on average higher values in comparison with FAs extracted using IL alone, although this behavior was not regular (Table 2).

Chi-square (χ^2) and statistical significance (p) tests were performed on the fatty acid concentrations by Kruskal-Wallis one-way ANOVA and Friedman's ANOVA and the resulting $\chi^2 > 5$ and $p < 0.0001$ both showed significant differences among the samples.

3.5. Properties of biodiesel obtained from N. oleoabundans biomass

Biodiesel properties of the extracted microalgae samples were compared to the EN 14214 and ASTM D6751−02 biodiesel standards (Table 3). Density ($0.86 \leq \times \leq 0.90$), kinematic viscosity ($3.5 \leq \times \leq 5.0$; $1.9 \leq \times \leq 6.0$), and higher heating values ($39 \leq \times \leq 41$) of all the samples complied with the optimal ranges suggested by EN 14214 and ASTM D6751−02 biodiesel specifications.

As for IV, the highest values were observed in the S4 strains extracted using IL:C followed by S3 strains extracted using IL:C, and by S4 and S3 strains extracted using IL alone. These samples exceeded the critical threshold of 120 g I_2 100 $g^{-1}_{BIODIESEL}$ (EN-14214, 2012) and for this reason, S3 and S4 strains proved to be the least efficient ones for biodiesel production.

The cetane number (CN) values of S1 and Ctrl strains extracted using IL were 48 and 49, respectively; and both complied with the ASTM D6751−02 biodiesel standards. S1 strains extracted through IL:C had the highest CN value (50) and complied with the ASTM D6751−02 biodiesel standard (Table 3).

In a previous study (Al-lwayzy and Yusaf, 2017), the biodiesel obtained from *Chlorella protothecoides* showed a density value of 0.900 g cm^{-3}, which was higher – and therefore less efficient – than the density value of *N. oleoabundans* (S1 strain, 0.886 g cm^{-3}) observed in the present study. However, in the same study the cetane number of *C. protothecoides* was determined as 52, while it was determined as 50 in the S1 strain analyzed in this study. In another study (Islam et al., 2015), the CN and density of *Crypthecodinium cohnii* were determined as 46.5 and 0.912 g cm^{-3}, respectively, which were less efficient values than the ones observed in the present study for the S1 strain of *N. oleoabundans*. Accordingly, *N. oleoabundans* is a promising species for future biofuel production, especially in microalgal cultivation systems and biorefineries fed with sludge waste. Total lipid content of *N. oleoabundans* during nitrogen-depleted growth can vary between 25% (Pruvost et al., 2011) and 54% lipids (w/w) (Tornabene et al., 1983), which makes it an efficient species in biodiesel production.

The microalgal growth parameters comply with the same specifications as S1 and S2 that are more efficient strains compared to S3 and S4,

Table 3
EN 14214, ASTM D6751−02 Standards and biodiesel properties of the strains derived by FAME contents. IV, iodine value; CN, cetane number.

		Density (ρ) ($g\ cm^{-3}$)	Kinematic viscosity (υ) ($mm^2\ s^{-1}$)	Higher heating values ($MJ\ kg^{-1}$)	IV ($gI_2\ 100\ g^{-1}\ fat$)	CN
EN 14214		$0.86 \leq \times \leq 0.90$	$3.5 \leq \times \leq 5.0$		≤120	≥51
ASTM D6751−02			$1.9 \leq \times \leq 6.0$			≥47
Lipid extraction using IL	S1[a]	0.886	4.83	40.24	112.6	48
	S2	0.887	4.72	40.22	119.1	46
	S3	0.886	4.81	40.24	123.6	45
	S4	0.885	4.87	40.25	126.9	45
	Ctrl[a]	0.886	4.80	40.24	110.5	49
Lipid extraction using IL:C mix	S1[a]	0.887	4.70	40.21	107.3	50
	S2	0.888	4.64	40.20	120.5	46
	S3	0.887	4.76	40.23	134.4	42
	S4	0.880	5.23	40.34	136.6	41
	Ctrl	0.889	4.57	40.18	122.1	46

[a] *Strains which comply with CN value ≥ 47.*

Table 2
Fatty acid concentrations expressed as mg/100 $mg_{algal\ biomass}$ through the lipid extraction process using IL only, and IL:C mixed solvent.

	IL					IL:C				
	S1	S2	S3	S4	$Ctrl_{IL}$	S1	S2	S3	S4	$Ctrl_{IL:C}$
$C_{12:0}$	0.67 ± 0.04	0.78 ± 0.06	0.90 ± 0.06	0.79 ± 0.05	0.71 ± 0.04	0.44 ± 0.03	0.62 ± 0.06	0.66 ± 0.05	0.66 ± 0.05	0.81 ± 0.07
$C_{14:0}$	0.39 ± 0.05	0.39 ± 0.07	0.48 ± 0.06	0.44 ± 0.04	0.25 ± 0.04	0.21 ± 0.04	0.24 ± 0.03	0.40 ± 0.04	0.31 ± 0.05	0.23 ± 0.03
$C_{14:1}$	0.07 ± 0.05	0.15 ± 0.02	0.30 ± 0.02	0.34 ± 0.04	0.04 ± 0.01	0.66 ± 0.05	1.95 ± 0.09	1.85 ± 0.12	1.26 ± 0.10	1.36 ± 0.11
$C_{14:2}$	0.10 ± 0.01	0.19 ± 0.01	0.17 ± 0.02	0.17 ± 0.02	0.12 ± 0.01	0.07 ± 0.01	0.16 ± 0.01	0.33 ± 0.03	0.44 ± 0.04	0.18 ± 0.02
$C_{15:0}$	0.20 ± 0.03	0.36 ± 0.04	0.32 ± 0.03	0.30 ± 0.02	0.24 ± 0.03	0.15 ± 0.02	0.24 ± 0.02	0.31 ± 0.03	0.20 ± 0.02	0.29 ± 0.02
$C_{16:0}$	7.45 ± 0.42	6.38 ± 0.38	6.02 ± 0.43	6.06 ± 0.36	6.67 ± 0.62	8.54 ± 0.63	7.30 ± 0.64	5.57 ± 0.43	6.77 ± 0.37	7.32 ± 0.54
$C_{16:1\ cis\text{-}9}$	2.86 ± 0.35	1.81 ± 0.14	1.85 ± 0.12	1.39 ± 0.10	1.22 ± 0.07	3.40 ± 0.26	2.32 ± 0.15	1.26 ± 0.13	1.20 ± 0.05	1.58 ± 0.13
$C_{16:1\ cis\text{-}6}$	1.39 ± 0.12	1.37 ± 0.13	1.18 ± 0.11	1.36 ± 0.14	1.60 ± 0.15	1.54 ± 0.14	1.72 ± 0.16	0.95 ± 0.07	1.25 ± 0.10	1.70 ± 0.15
$C_{16:2}$	0.45 ± 0.03	0.40 ± 0.04	0.60 ± 0.05	0.64 ± 0.06	0.35 ± 0.03	0.16 ± 0.01	1.16 ± 0.10	1.70 ± 0.15	2.17 ± 0.15	1.39 ± 0.12
$C_{18:0}$	3.40 ± 0.32	3.10 ± 0.28	2.47 ± 0.23	2.27 ± 0.22	3.65 ± 0.29	2.74 ± 0.24	3.23 ± 0.29	1.81 ± 0.08	1.61 ± 0.07	4.33 ± 0.24
$C_{18:1}$	8.80 ± 0.47	6.22 ± 0.38	5.35 ± 0.44	4.26 ± 0.31	4.39 ± 0.49	11.38 ± 0.83	8.22 ± 0.73	3.89 ± 0.37	2.83 ± 0.24	5.14 ± 0.52
$C_{18:2}$	6.01 ± 0.48	5.08 ± 0.38	4.93 ± 0.42	5.00 ± 0.39	5.11 ± 0.37	5.85 ± 0.55	5.78 ± 0.43	3.70 ± 0.32	3.81 ± 0.33	6.28 ± 0.56
$_{\gamma}C_{18:3}$	0.39 ± 0.02	0.33 ± 0.02	0.37 ± 0.03	0.35 ± 0.02	0.23 ± 0.02	0.52 ± 0.04	0.48 ± 0.02	0.43 ± 0.03	0.35 ± 0.02	0.45 ± 0.05
$_{\alpha}C_{18:3}$	5.58 ± 0.32	5.51 ± 0.49	5.11 ± 0.42	4.82 ± 0.37	5.97 ± 0.52	5.24 ± 0.43	6.14 ± 0.60	3.77 ± 0.25	3.49 ± 0.24	7.62 ± 0.38
$C_{20:0}$	0.72 ± 0.04	0.64 ± 0.04	0.60 ± 0.03	0.53 ± 0.02	0.52 ± 0.04	0.86 ± 0.06	0.90 ± 0.08	0.54 ± 0.03	0.45 ± 0.04	0.90 ± 0.07

Lipid extraction solvents were represented as: **IL,** Ionic liquid [BMIM] [CF_3SO_3]; **IL:C,** Ionic liquid [BMIM] [CF_3SO_3] and chloroform mixed solvent. The strains are represented as: **S1,** 0.65 g/L; **S2,** 1.30 g/L; **S3,** 2.60 g/L; **S4,** 5.20 g/L sludge waste concentrations used in the microalgal growth medium and **Ctrl** are the control strains grown in BG 11 medium.

respectively. Kruskal-Wallis one-way ANOVA test was applied to obtain the p values of fatty acids and biodiesel properties data, and the result was $p < 0.0001$ for both of them. The p values derived by Friedman's ANOVA test both resulted in $p < 0.0001$. All these statistical results highlighted significant differences among the samples.

4. Discussion

At the end of the experimental period, the samples grown with lower concentrations of wet sludge (0.65 g/L, S1 and 1.30 g/L, S2) in the microalgal growth medium showed high values of OD and photosynthetic pigment contents which decreased using a concentration of wet sludge waste higher than 2.60 g/L up to 5.20 g/L. However, the highest amount of sludge waste per liter of medium did not affect the microalgal growth rate nor the whole cultivation process until the 7th day of the photoautotrophic growth period which is not indicating a long-term growth inhibition effect. The growth rate of *C. vulgaris* and *N. oleoabundans* grown in the wastewater have been determined up to 0.61 day^{-1} and 0.41 day^{-1} in a previous study (AlMomani and Örmeci, 2016), where the wastewater has been added in the algal cultivation. In the present study, the most significant growth rate (up to 0.35 day^{-1}) was achieved with sludge waste only, without adding any commercial microalgal growth medium, thus revealing the effectiveness of sludge waste bioconversion technology which can be applied to microalgal biorefineries integrated with wastewater plants.

The moisture content of the samples represents a critical factor for their susceptibility to spoilage and for the success of the transesterification reaction. In a previous study, microalgal biomass samples with moisture contents of 68.5%, 40.6%, 20.5% and 4.5% were prepared and it was observed that a higher biomass moisture content reduces the lipid extraction efficiency, which has a significant effect on the lipid yield ($p < 0.05$) (Balasubramanian et al., 2013). Other studies highlighted that values of moisture content higher than 10% negatively affect the efficiency of transesterification by making it decrease (Ehimen et al., 2010; Sathish et al., 2014). Unfortunately, the drying process prior to lipid extraction carries a high economic burden. However, the higher quality of microalgal biomass is undeniable, since thanks to this process the algal biomass is converted into a stable product, which in industrial applications reduces the costs of handling, transportation, packaging, and storage. Further, once the drying process and lipid extraction from the algal biomass have been carried out, the high-value remaining residue can be used for feed, biofertilizers or other types of applications (depending on the content composition of the feedstock source).

S1 strains with a sludge waste concentration of 0.65 g/L resulted as the most efficient in terms of both total lipid content and FA specific profile, in comparison with microalgae grown with higher concentrations of sludge waste in the growth medium in which these characteristics translate into a higher biodiesel quality. Sludge waste concentrations of 2.60 and 5.20 g/L resulted in lower lipid yields.

The derivatization method used in the present study is the transesterification catalyzed from an acidic environment and it is capable of transforming into methyl esters both free fatty acids and fatty acids present in the structures of triglycerides and phospholipids. This method was specifically adopted to obtain a complete picture of the fatty acids' presence in the samples. Conversely, the transesterification in alkaline environment would not have permitted to take into account the fraction of free fatty acids, potentially available to be transformed into methyl esters and, therefore, into biodiesel. However, the transesterification in acidic environment is essentially an analytical method of evaluation from which it is possible to extrapolate the indexes relating to the quality of the biodiesel produced. It was not the purpose of this work to investigate and set up biodiesel purification methods.

ILs are salts in the liquid state which have excellent solvent properties currently exploited in many applications of the metabolite extraction from vegetal material, including microalgae (Shankar et al., 2017). As for strains grown in the optimized sludge waste growth medium, IL:C mixed solvent yielded higher values of total lipid content and individual FA than IL alone, while the strains grown in the commercial BG_{11} medium showed higher lipid contents when IL alone was used for lipid extraction. Algal cells grown under stress conditions, like the ones caused by the presence of sludge waste, likely develop cell walls more resistant to the solvent deconstructing action (Teixeira, 2012). As a consequence, the choice of combining chloroform to IL, with a parallel reduction of working temperature up to 70 °C, caused a more effective cell membrane breaking and therefore a higher lipid yield.

Chloroform:methanol is a mixture that has been widely used for a long time in lipid extraction through the Bligh-Dyer method as well as the Folch method. Chloroform is effective on neutral lipid extraction, while methanol permits the extraction of more polar membrane phospholipids. The combination of IL with methanol has already been used for lipid extraction from *Chlorella vulgaris* with a yield of up to 19.0% dried biomass of total lipid employing [Bmim][CF_3SO_3] mixed with methanol. The lipid extractions carried out using the Bligh and Dyer method (11.1%) and [Bmim][$MeSO_4$] mixed with methanol (17.4%) yielded lower percentages of total lipids (Kim et al., 2012). The present study proved that the combination of IL with chloroform is equally effective in lipid extraction of microalgal biomass. Chemical treatment methods are effective and consume relatively less energy in the cell lysis process, which is essential for lipid extraction, although effluent management and disposal represents a disadvantage. However, it has been proven that the mixtures of organic solvents still succeed in breaking the microalgal cell wall, in terms of benefit-cost ratio (Miazek et al., 2017).

In this study, reducing the IL amount by half and mixing it with chloroform yielded a more efficient FA extraction in comparison with IL alone, also reducing the total cost of the reagents.

There are two main consequences of the results obtained:

i) as for environmental purification, especially in waste management facilities, *N. oleoabundans* is effective for the bioremediation of the wastewater containing sludge waste up to a concentration of 5.20 g_{sludge}/L_{water}. However, the quality and amount of lipids and fatty acids extracted from the microalgal biomass depend on the sludge concentration in the optimized microalgal growth medium during the cultivation period and depend on the solvent selection for the lipid extraction process.
ii) as for the yield of microalgal biomass with a high content of lipids, *N. oleoabundans* only required a low amount of sludge waste feedstock (0.65 g/L) to grow efficiently which represents a saving of feedstock for large scale microalgal biomass production in biorefineries. Moreover, such a low concentration of wet sludge waste in the growth medium yielded a higher amount of lipids compared to other strains grown in high concentrations of sludge waste (between 1.30 and 5.2 g/L).

The different cellular processes of *N. oleoabundans* are due to different growth parameters, such as light penetration which is dependent on microalgal cell concentration and medium opacity, metabolized contaminants, and the ratio between the amount of nutrients contained in the feedstock and the organic material as a whole.

Heterotrophic cultivation of microalgae eliminates the need for light. Moreover, growth rates and cell biomass, as well as protein and lipid production, can significantly increase due to the higher energy density of the carbon source compared with the carbon dioxide utilized during the photoautotrophic cultivation (Perez-Garcia et al., 2011). There is a limited number of microalgal species that can grow heterotrophically and *N. oleoabundans* is one of them (Altunoz et al., 2020); it is also able to rapidly adapt to environmental changes and it is efficient in the conversion of waste into biomass applications, which enhances its value in the biofuel production sector of the future.

As for the biodiesel main parameters, CN is one of the main indexes of diesel quality. It can be calculated from IV and it is crucial for the delay periods of the compression-ignition engines. Indeed, the higher

the CN the lower the delay periods, therefore, the smoother the engine functioning (Hocking, 2005). S1 strains cultivated in the optimized sludge waste medium with a sludge concentration of 0.65 g/L showed the lowest value of IV as 107.3 and the highest value of CN as 50 using IL [BMIM] [CF_3SO_3] and chloroform mixed lipid extraction solvent with a ratio of 1:1 (V/V). Specifically, EN 14214 advises CN higher than 51, while ASTM D6751−02 advises CN higher than 47. However, biodiesel CN is on average higher than in the fossil diesel due to its higher oxygen content (Sivaramakrishnan and Ravikumar, 2012). A similar behavior was found in fat extracted from animal sources (Hadj Saadoun et al., 2020), pointing that biodiesel production is based on both animal fat and vegetable oil (Giakoumis, 2013).

The biorefineries are the future solutions of increasing energy demand, and the integration of biorefineries with wastewater treatment plants is the subject to be clarified together with the innovations to increase the efficiency of lipid production, accordingly quality and quantity of biodiesel to be obtained. Resource recovery from wastewater is a desired goal of "waste to energy" applications which is mentioned in this study.

5. Conclusions

The present study has proven that *N. oleoabundans* microalgal biomass grown in optimized sludge waste feedstock is suitable to produce biodiesel by means of innovative processes which exploit ionic liquid [BMIM] [CF_3SO_3] mixed with chloroform. The optimizations applied to the sludge waste in the municipal treatment centers to obtain microalgal biomass are an efficient strategy to convert waste into biomass in the framework of future microalgal biorefinery technologies. This new research aimed at the reduction of pollutant in the water treatment facilities as well as at obtaining algal biomass in a cost-effective and eco-friendly way which represents a prerequisite for sustainable biofuel production. Microalgae cultivation is, therefore, an effective way to produce fuel for the automotive sector and to replace petrodiesel with biodiesel.

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Appendix A. Supplementary data

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