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Microalgae cultivation for biofuel production: optimization of environmental conditions for the intensive culture of *Neochloris oleoabundans* (Chlorophyta)

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Abstract

Microalgae have the potential to produce environmental friendly biofuels, in view of the growing need for alternatives to fossil resource exploitation. Indeed microalgae grown under nitrogen stress may accumulate significant amount of lipids, which are the raw material for biodiesel. The aim of this study was to optimize culture conditions to increase lipid production on the freshwater green alga *Neochloris oleoabundans.* In a first experiment, we supplied growth medium in two different modes, fed batch and batch; for this latter we used increasing salt concentration. In the second part of the study, we tested 4 different operational modes of cultivation (batch, semicontinuous, N-limitation, N-starvation). Experiments have been performed in glass cultivation tubes. We obtained higher concentrations using batch than fed-batch, in term of maximum algal biomass (4-4.7 and 3.8 g L^{-1} respectively). These results showed that the fed-batch micronutrient depletion limited the growth, while high salt concentration did not affect the algal growth in the batch cultures. The second part of the study showed that the maximum biomass concentration was reached in batch N-limited condition. N-limitation resulted as the preferable culture condition in term of lipid concentration, without a substantial difference between semicontinuous and batch. These findings highlight that *N. oleoabundans* is a halotolerant strain thus being potentially suitable for substrates with high electrolytic concentration, including wastewater and brackish water. Moreover, our results show that N-limitation combines a good increase in the percentage of lipids with adequate biomass productivity, thus being a valid option in microalgae feeding system for energy production.

KEYWORDS: green algae / lipids / algal nutrients / nitrogen limitation / nitrogen starvation

Microalghe per la produzione di biocarburanti: ottimizzazione delle condizioni di coltura di *Neochloris oleoabundans* (Chlorophyta)

Le microalghe hanno delle ottime potenzialità come produttori di biocarburanti, in un'ottica di favorire fonti energetiche alternative allo sfruttamento di idrocarburi fossili. Infatti, le microalghe coltivate in carenza di azoto possono accumulare notevoli quantità di lipidi, che sono la materia prima per il biodiesel. Lo scopo di questo studio è stato quello di ottimizzare le condizioni di coltura per aumentare la produzione di lipidi dell'alga verde *Neochloris oleoabundans*. In un primo esperimento abbiamo fornito terreno di coltura in due modalità differenti, *fed batch* (sistema statico con aggiunta periodica di nutrienti) e *batch* (sistema statico senza aggiunta successiva di nutrienti). Nel *batch* abbiamo testato concentrazioni saline crescenti. Nella seconda parte dello studio abbiamo testato 4 diverse modalità operative di coltura (*batch*, semicontinuo, N-limitazione, N-deprivazione).

Gli esperimenti sono stati condotti in tubi di coltura in vetro aerati con una miscela di aria e CO_2. Abbiamo ottenuto valori più alti di biomassa algale utilizzando colture *batch* piuttosto che *fed-batch* (rispettivamente 4-4,7 g L^{-1} e 3,8 g L^{-1}). Questi risultati hanno mostrato che l'esaurimento dei micronutrienti nel *fed-batch* ha limitato la crescita, mentre l'alta concentrazione di sali non influenza la crescita delle alghe nelle colture *batch*. La seconda parte dello studio ha evidenziato che la concentrazione massima della biomassa è stata raggiunta in condizioni *batch* N-limitate. L'N-limitazione è quindi risultata la condizione di coltura preferibile in termini di concentrazione di lipidi, senza sostanziali differenze tra semicontinua e *batch*. Questi risultati sottolineano che *N. oleoabundans* è un ceppo alotollerante quindi adatto anche a mezzi di coltura con salinità medio-elevata, come acque reflue e salmastre. Inoltre, i nostri risultati dimostrano che l'N-limitazione combina una buona percentuale di lipidi con una adeguata produttività di biomassa, rappresentando così una valida modalità di coltura finalizzata alla produzione di energia.

PAROLE CHIAVE: alghe verdi / lipidi / nutrienti algali / azoto limitazione / carenza di azoto

INTRODUCTION

Microalgae are considered a source for the production of biodiesel with great potential, thanks to some characteristics: a) high lipid content of some strains; b) their high rate of growth without employment of agricultural land, high quality water or pesticides as for plants; c) their high convert capacity (more than 10% of solar radiation into biomass, compared to plants (less than 0.5%) (Zhou *et al*., 2015). The third generation of biodiesel production (Tab. I) involves the use of those microalgae strains that have the potential for high lipid accumulation. Microalgae grown under nitrogen stress may address their metabolism towards lipid accumulation, especially triacylglycerols (TAG), which are the ideal raw material for the production of biodiesel. The total lipid content of various species varies from 1% to 85% of the dry weight, with increases of more than 40% under conditions of nutrient limitation (Chisti, 2007).

However, a lot of challenges have to be overcome before allowing large-scale microalgae production. For example, it is necessary to develop competitive and sustainable exploitation systems (Wijffels & Barbosa, 2010; Pruvost *et al*., 2011), and to select appropriate microalgae strains combining features affecting in a positive manner any step of the global process from biomass production to lipid extraction and valorisation as biofuel (Rodolfi *et al*., 2009).

It is also necessary to clarify the role of two of the main limiting factors for lipid and biomass productivity: a) the cell density, that can cause self-shading, and b) the way nutrients are supplied. Indeed different cultivation conditions may affect the cell lipid content. Under nitrogen deficiency the protein and chlorophyll content decreases, while carbohydrates and lipid increase (Sun *et al*., 2014). The cultivation of microalgae under nitrogen stress may be carried out by nitrogen starvation or nitrogen limitation. Under N-starvation, microalgae grow in a medium lacking nitrogen supply, while under N-limitation algae are cultivated with a constant but insufficient nitrogen supply. Using N-starvation to increase algae lipid content, is a well-known process: indeed, under nitrogen-starvation conditions, many algae alter their lipid biosynthetic pathways for the formation and accumulation of neutral lipids, mainly in the form of TAGs (Hu *et al*., 2008; Gouveia & Oliveira, 2009; Pruvost *et al*., 2009; Rodolfi *et al*., 2009; Pruvost *et al*., 2011; Breuer *et al*., 2012; Sun *et al*., 2014; Baldisserotto *et al*., 2014), while the N-limitation is less studied or it is meant as progressive starvation (Rodolfi *et al*., 2009; Feng *et al*., 2011; Griffiths *et al*., 2012; Harwati *et al*., 2012).

In this study we selected *Neochloris oleoabundans* as test organism for its well known potential in biodiesel production (Tornabene *et al*., 1983; Li *et al*., 2008; Gouveia *et al*., 2009; Pruvost *et al*., 2009; Wang & Lan, 2011; Sun *et al*., 2014; Giovanardi *et al*., 2014; Silva *et al*., 2016). We carried out a series of laboratory experiments to test *Neochloris* growth by varying a) the medium concentration and b) the operational mode to supply it (batch vs. semicontinuous; N-limitation vs. N-starvation). The ultimate goal of this work was to optimize culture conditions to obtain an adequate balance between lipid and biomass productivity.

MATERIALS AND METHODS

Culture conditions of microalgae inoculum

N. oleoabundans UTEX #1185 was cultivated in 250 ml flasks maintained in sterile condition in an CO_2 incubator (Sanyo MCO-19AIC) flushed with air/CO_2 (97/3, v/v) to support growth and maintain pH within a desired range. In the incubator the temperature was 28 ± 2 °C and the continuous artificial illumination of 200 µmol m^{-2} s^{-1} (expressed as PPFD - Photosyntetic Photon Flux Density) was provided by daylight LED. To allow the mixing of the culture an orbital shaker with 150 rpm rotation speed was used.

Algae were cultured in BG11 medium (Rippka *et al.*, 1979). pH was adjusted with HCl 1 M to pH 7.0 and the medium was autoclaved.

After six days of culture, microalgae were pelleted by centrifugation at 2000 rpm for 30 min (centrifuge ALC CWS 4236), re-suspended with 200 mL of physiological solution, followed by a second centrifugation and resuspension with fresh medium for the experiments. Initial biomass was between 0.2 and 0.7 g L^{-1} depending on the specific experiment (see after).

General experimental conditions

Experiments have been performed in glass tubes of working volume of 0.4 L and diameter of 5 cm, placed in a thermostat (ASAL Climatic Hood 810) to maintain

Tab. I. Classification of biofuels.

1st generation	biofuels produced by fermentation of usually edible biomass: wheat, barley, corn, potato or sugars from sugarcane, sugar beet, etc.
2nd generation	fuels produced from a wide array of different feedstocks, especially but not limited to non-edible lignocellulosic biomass, such as agricultural and forest residues or even municipal solid wastes
3rd generation	fuels produced from microalgal biomass, which has a very distinctive growth yield as compared with classical lignocellulosic biomass. Production of biofuels from algae usually relies on their lipid content

a temperature of 28±2°C. The lighting was continuously supplied through six cool daylight fluorescent tubes that provided the overall culture 400 μmol m^{-2} s^{-1}. Each experimental condition was performed in triplicate. Biomass was estimated experimentally by measuring a) the optical density (OD) at 750 nm using a spectrophotometer (LKB Biochrom Ultrospec 4050); b) concentration (dry weight) according to Chini-Zittelli *et al.* (2000). Daily productivity was estimated as the difference between two subsequent biomass concentrations. Growth rate was calculated as the difference between two subsequent logarithmic biomass concentrations.

Algal growth in different nutrient supply modes

The aim of a first series of experiments is to detect the maximum biomass obtained under the experimental conditions described above, assuming that a high biomass concentration was the main limiting factor. For this purpose, we used the fed-batch as nutrient supply mode: BG11 medium was used at the beginning of the experiment, then, in order to avoid limitation of macronutrients, they were periodically added. The timing and the amount of macronutrients supplied depended on the uptake by algae. The latter was estimated considering the theoretical elementary biomass composition (N:P:Mg:Fe:Ca, 10:1:0.5:0.2:0.5), combined with the experimental daily productivity. Macronutrients were added on day 5 and 9. This experiment lasted for 14 days starting with an initial biomass concentration of 0.3 g L^{-1}.

We performed another experiment to detect a possible growth inhibition due to high salt concentration. Three different culture media were prepared: a) BG11 with the composition reported above; b) 2-BG11: where nutrient concentrations were doubled; c) 3-BG11 where nutrient concentrations were tripled. These tests were carried out in batch without addition of nutrients. Initial biomass was 0.2 g L^{-1}, indeed a low value of this parameter allow to obtain a growth curve that well describes all the growth phases. Analyses of biomass were carried out periodically, following the methods described above.

Algal growth and lipid production in different operational modes

A second series of experiments was designed to compare algal growth and lipid production obtained with different operational modes. N-limitation and N-starvation were tested in batch and in semicontinuous operational mode (Tab. II). In the semicontinuous we daily removed 50% of the culture volume by replacing the correspondent volume of fresh medium. In all experiments an higher initial biomass concentration was chosen, in order to sustain the daily dilution (0.7 g L^{-1}).

In the N-limitation experiment, the culture conditions were as follows: a) semicontinuous nitrogen limitation (SL). A modified BG11 medium with a nitrogen concentration of 24.75 mg L^{-1} was used as medium for the culture and for daily dilutions (10% of nitrogen in replete medium); b) batch nitrogen limitation (BL). The medium used was a modified 2-BG11 with an initial nitrogen concentration of 24.75 mg L^{-1} as SL. The same proportion of nitrogen (10%) that was added daily for the dilution in SL using a $NaNO_3$ concentrate sterile stock solution.

In the N-starvation experiment, the corresponding culture conditions were the following: a) also for the

Tab. II. Design of the experiments.

Experiment	Factor	Mode	Initial conditions	Nutrient addition
Supply modes	High algal density	Fed-batch	BG11	Addition of macronutrients
	Medium concentration	Batch	BG11	No
		Batch	2-BG11	No
		Batch	3-BG11	No
Operational modes	N-Limitation	Semicontinuous	BG11 with N at 10% of standard concentration	Dilution using BG11 with N at 10% of standard concentration
		Batch	2-BG11 with N at 10% of standard concentration	Daily addition of N
	N-Starvation	Semicontinuous	BG11 with N at 10% of standard concentration	Dilution using BG11 without N
		Batch	2-BG11 with N at 10% of standard concentration	No addition

semicontinuous nitrogen starvation (SS), the modified BG11 medium was used with a initial nitrogen concentration of 24.75 mg L^{-1}, but the medium used for the daily dilution was without a nitrogen source, therefore nitrogen consumption during culture growth leads to a progressive nitrogen starvation; b) in the batch nitrogen starvation (BS), the modified 2-BG11 medium with a nitrogen concentration of 24.75 mg L^{-1} was used as for SS.

Tab. II reports the factor investigated and the operational modes for each experiment.

Lipid extraction and analysis

In the semicontinuous/batch tests, samples for lipid analysis were centrifuged at 3500 rpm for 20 minutes. Biomass were re-suspended with physiological solution, followed by a second centrifugation. Pelletized biomass was frozen and lyophilized. The total lipids were extracted from microalgae biomass using the method of Bligh & Dyer (1959). Lipid content was measured spectrophotometrically after carbonization of the extracted material with a 2:1 methanol/chloroform solution, according to Marsh & Weinstein (1966). Tripalmitin (Sigma-Aldrich, Milan, Italy) was used as standard (Holland & Gabbott, 1971). Lipid content has been calculated as percentage of dry weight and lipid concentration as the product of the lipid content and biomass concentration.

RESULTS AND DISCUSSION

Algal growth in different nutrient supply modes

In the first series of experiments, the supply mode was tested in order to ascertain what could be the role a) of high algal density with possible self-shading or accumulation of toxic metabolites and b) of medium nutrient concentration on algal biomass growth. Regarding the first issue, in the fed-batch the maximum biomass was 3.8 g L^{-1}, which can be considered an appreciable production value. Indeed, according to the literature, *N. oleoabundans* growth in synthetic freshwater media in batch mode ranges from 0.5 to 3.15 g L^{-1} (Li *et al.*, 2008; Gouveia & Oliveira, 2009; Gouveia *et al.*, 2009; Pruvost *et al.*, 2009; Sun *et al.*, 2014), depending on different culture conditions.

Data on algal growth at three different initial BG11 concentrations are reported in Fig. 1 and Tab. III. Growth showed the same trend in the three modes until day 7. This means that a higher nutrient concentration did not affect the algal growth. Data obtained measuring optical density were very similar (data not shown). This finding agrees with some previous papers (Vazquez-Duhalt & Arredondo-Vega, 1990; Arredondo-Vega *et al.*, 1995; Renimel *et al.*, 2008) in which *N. oleoabundans* was defined as a brackish and halotolerant species. This feature makes it extremely versatile and suitable to be cultivated using different water resources (from fresh to seawater), with advantages in terms of environmental and economical sustainability.

The highest biomass (4.6 g L^{-1}) was reached in 3-BG11 at day 9; 2-BG11 reached its peak (4.1 g L^{-1}) at day 8 and BG11 at day 7 (4.0 g L^{-1}). In 3-BG11 nutrients are higher promoting an increase in algal growth for a longer period, while at lower concentrations after 7-8 days growth slowed possibly due to nutrient depletion. In the fed-batch the highest biomass was lower than in the enriched BG11: we can assert that the addition of only macronutrients is non effective to promote a higher growth. These results indirectly show that the limiting factor in the fed-batch is not only the high algal density but also the micronutrient depletion.

Average daily biomass productivity (Tab. III) in batch ranged between 0.54 and 0.56 g L^{-1} d^{-1}.

These data are similar to those obtained in other studies: Li *et al.* (2008) found a biomass productivity of 0.63 g L^{-1} d^{-1}, while Pruvost *et al.* (2009) showed a slightly

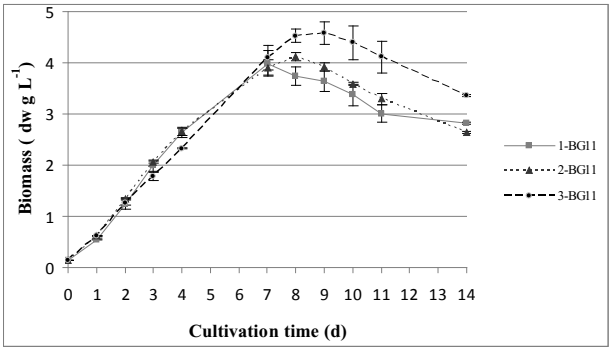

Fig. 1. Algal growth (g L^{-1} dry weight) at three different BG11 concentrations. Error bars are standard deviations.

Tab. III. Average daily productivity, growth rate and biomass doubling time of *Neochloris oleoabundans* at three BG11 concentrations; the first seven days were taken into account for the calculation. Each experimental condition was performed in triplicate.

	Productivity (g L^{-1} d^{-1})		Growth rate		Biomass Doubling time (d^{-1})	
	mean	max	mean	max	mean	min
BG11	0.55 ± 0.04	0.74 ± 0.02	0.47	1.29	1.48	0.53
2-BG11	0.54 ± 0.02	0.73 ± 0.01	0.47	1.41	1.49	0.54
3-BG11	0.56 ± 0.03	0.64 ± 0.04	0.47	1.44	1.47	0.48

lower value (0.53 g L^{-1} d^{-1}). Otherwise, our productivity data are much higher than those obtained by Gouveia *et al.* (2009), Griffiths *et al.* (2012), and by Sun *et al*. (2014) who reported values up to 0.29 g L^{-1} d^{-1}.

These results can have important operational implications in the setting-up of batch culture systems: it seems preferable in terms of biomass productivity to supply all the necessary nutrients at the beginning, rather than adding them during algae growth. *N. oleoabundans* growth was not affected by high nutrients concentration present in 2 and 3-BG11, while the absence of the micronutrients addition lowered the biomass production. In a strictly freshwater strain it should be expected a growth reduction in environment characterized by high salt concentrations, but this seems not the case with *N. oleoabundans*. These findings are applicable when using nitrate as N source. On the other hand *N. oleoabundans* was proven to be a promising strain for wastewater treatment (Wang & Lan, 2011; Franchino *et al.*, 2013; Giovanardi *et al*., 2013), where high ammonia concentration can occur. In this case, the fed-batch mode can be preferred as nutrient supply mode (Levine *et al.*, 2011).

Comparison among different operative conditions for the optimization of lipid production

Fig. 2 shows the algal growth in N-limited medium, in batch and semicontinuous modes. The maximum biomass concentration in batch was 4.24 g L^{-1} (day 10). Semicontinuous reached the steady state from day 2 until the end of the experiment. The maximum productivity for the semicontinuous was 0.725 g L^{-1} d^{-1} (starting day), while the batch attained 0.603 g L^{-1} d^{-1} (day 1).

Fig. 3 shows the algal growth (expressed as dry weight) in N-starvation for both operational modes. The cultivation in batch reaches a maximum concentration of 3.20 g L^{-1} after 8 days; the semicontinuous mode supported the algal growth only for the first 2 days, as from the third day the deficiency of N combined with the dilution of the medium did not allow the maintenance of the steady state and the biomass decreased. Data expressed as OD follow the same trend (data not shown).

Tab. IV summarizes data concerning the average daily productivity, growth rate and doubling time for all the operational modes. In N-limited cultures, daily

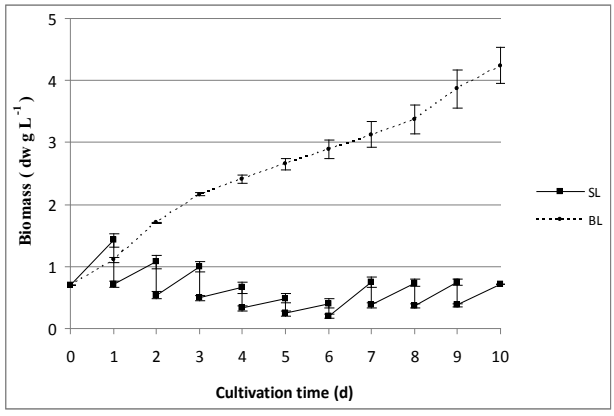

Fig. 2. Algal growth (g L^{-1} dry weight) in semicontinuous N-limitation (SL) and batch N-limitation (BL). Error bars are standard deviations.

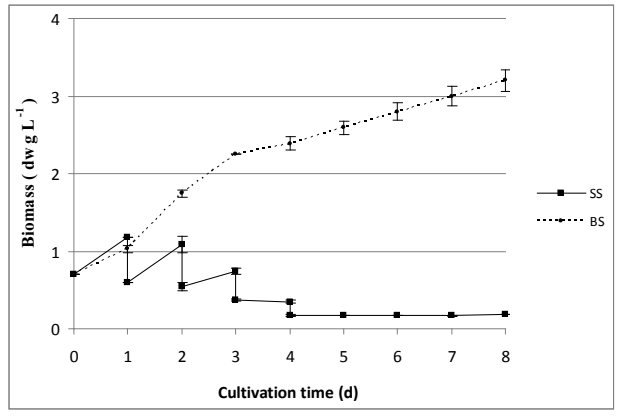

Fig. 3. Algal growth (g L^{-1} dry weight) in semicontinuous N-starvation (SS) and batch N-starvation (BS). Error bars are standard deviations.

Tab. IV. Average daily productivity, growth rate and doubling time of *Neochloris oleoabundans* in the four operational modes. **SL**= semicontinuous N-limited; **BL**= batch N-limited; **SS**=semicontinuous N-starved; **BS** = batch N-starved. In SS, during the last days of the experiment, *N. oleoabundans* didn't grow, therefore only the first five days were taken into account for the calculation of the starved cultures, while the whole duration of the tests was considered for the limitation mode. Each experimental condition was performed in triplicate.

	Productivity (g L^{-1} d^{-1})		Growth rate		Doubling time (d^{-1})	
	mean	max	mean	max	mean	min
SL	0.37 ± 0.02	0.70 ± 0.11	0.55	0.71	1.20	1.00
BL	0.35 ± 0.03	0.66 ± 0.05	0.20	0.46	5.50	1.50
SS	0.23 ± 0.02	0.50 ± 0.11	0.28	0.60	4.50	1.20
BS	0.38 ± 0.02	0.68 ± 0.03	0.26	0.51	4.80	1.40

Tab. V. Maximum total lipids (% on biomass and concentration in the culture) in the four cultivation procedures. Maximum values were obtained in day 4 for starved cultures and in day 7 for limited ones. Data are expressed as mean ± standard deviation. **SL** = semicontinuous N-limited, **BL** = batch N-limited, **SS** = semicontinuous N-starved and **BS** = batch N-starved

	SL	**BL**	**SS**	**BS**
Lipid content (%)	28.6 ± 0.18	30 ± 0.112	46 ± 0.069	32.8 ± 0.085
Lipid concentration (g L^{-1})	0.95 ± 0.109	0.932 ± 0.066	0.295 ± 0.018	0.366 ± 0.014

productivity is comparable in batch and semicontinuous; it must be noticed that to achieve these productivities, the culture in the semicontinuous must have a higher growth rate due to the lower biomass. In both cases, the highest productivity was attained in the first part of our experiment. In N-starved cultures it can be noticed a higher biomass productivity in batch (0.38 g L^{-1} d^{-1} in BS and 0.23 g L^{-1} d^{-1} in SS) while the growth rate and doubling time were almost the same. From the comparison of all of the culture conditions, it can be noted that despite the stress conditions, BS reaches biomass productivities comparable with those of SL and BL, while the extreme conditions of SS does not allow to maintain an acceptable growth.

For batch and N-starved conditions, a comparison can be made with data obtained by Gouveia *et al.* (2009), who showed a mean biomass productivity of 0.03 g L^{-1} d^{-1}, therefore one grade of magnitude less. For N limitation, the most similar operational mode is the progressive starvation adopted by Li *et al.* (2008) and Griffiths *et al.* (2012), who obtained a biomass productivity of approximately 0.30 g L^{-1} d^{-1}.

The comparison in terms of total lipids production (Tab. V) allows further considerations. For each cultivation mode, we chose the day in which the maximum in lipid production has been attained. For N-limited conditions the maximum was reached after 7 days, while the harsher conditions of N-starvation led to an earlier peak in lipid production, attained at day 4. The higher value in terms of lipid content (%) on dry weight was achieved in SS (46%). In general, the N-starved cultures showed a higher lipid content in comparison with N-limited ones. In terms of commercial production, it may be more significant to analyze lipid production as lipid concentration in the culture. With this regard, N-limitation resulted as the preferable culture condition, without a substantial difference between semicontinuous and batch (0.95 g L^{-1} in SL and 0.93 g L^{1} in BL).

These values are consistent with recent literature, mostly referring to starvation and to batch conditions, while for semicontinuous and limitation data are scarce. *N. oleabundans* has been proved to be a good lipid producer (Li *et al.,* 2008; Pruvost *et al.,* 2009; Griffiths *et al.,* 2012). Our results are higher than those obtained by Klok *et al.* (2013), who reached a lipid content of 16.6% and biomass productivity of 0.26 g L^{-1} d^{-1} under N-limitation condition. Sun *et al*. (2014) obtained a content of TAGs of only 25% and a biomass productivity of 0.205 g L^{-1} d^{-1}, even adding Fe^{3+} to the medium.

CONCLUSION

The first series of experiments confirm the wide tolerance range of *N. oleoabundans* for the medium saline concentration and therefore its potential to be cultured in wastewater and brackish water. This enlarges the cultivation possibilities for different purposes (biodiesel, biomass production, wastewater treatment). Moreover, our results suggest that it is preferable to supply the adequate amount of nutrients (in accordance with the goal of the cultivation) since the first cultivation day, instead of adopting a fed-batch. This study is the first that has compared four possible operational modes in order to optimize biomass and lipid production for the selected strain. In particular, the most innovative aspect is that N-depletion is a good alternative to N-starvation in view of an increase of lipid production. N-starvation determines a significant increase of the percentage of lipid content but also a significant reduction of biomass productivity, for this reason lipid concentration should be always used to compare different studies, since it combines lipid and biomass data. With this regard, our results highlight the higher lipid concentration obtained using N-limitation, which can be considered as a valid option in microalgae feeding system from energy production. This operational mode has the advantage of matching a good increase in the percentage of lipids with adequate biomass productivity.

In conclusion, *N. oleoabundans* is a suitable species for production due to its high lipid productivity under standardized conditions and for its plasticity towards culture media.

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