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Author: Esperanza Del Río Ana Armendáriz Elena García-Gómez Mercedes García-González Miguel G. Guerrero

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Continuous culture methodology for the screening of microalgae for oil

Esperanza Del Río, Ana Armendáriz, Elena García-Gómez, Mercedes García-González, Miguel G. Guerrero

Instituto de Bioquímica Vegetal y Fotosíntesis, Universidad de Sevilla-Consejo Superior de Investigaciones Científicas, Centro de Investigaciones Científicas Isla de la Cartuja, Avenida Américo Vespucio 49, 41092 Sevilla, Spain.

Author for correspondence:

Dr. Esperanza Del Río

Instituto de Bioquímica Vegetal y Fotosíntesis

Centro de Investigaciones Científicas Isla de la Cartuja

Avenida Américo Vespucio 49, 41092 Sevilla, Spain

Phone: +34 954489520

Fax: +34 954460065

Email: esperanza@ibvf.csic.es

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Abstract

A basic criterion in the selection of microalgae suitable as source of oil for biodiesel should be their actual capacity to produce lipids or, more properly, the fatty acid yield. Performance assessment of ten preselected microalgae under both batch and continuous culture points to the latter approach as the most adequate for evaluating fatty acid productivity. Differences were patent in continuous culture among strains that otherwise had analogous oil accumulation potential under batch culture. Some promising strains under batch culture (like *Muriella aurantiaca* and *Monoraphidium braunii*) exhibited, however, values for actual fatty acid productivity lower than 40 mg $L^{-1}d^{-1}$ in continuous regime. The analysis performed in photochemostat under continuous culture regime revealed the great potential of *Chlorococcum olefaciens*, *Pseudokirchneriella subcapitata* and *Scenedesmus almeriensis* as oil producing microalgae. Fatty acid productivity levels over 90 mg $L^{-1}d^{-1}$ were recorded for the latter strains under moderate nitrogen limitation, conditions which led to an enrichment in saturated and monounsaturated fatty acids, a more suitable profile as raw material for biodiesel. The continuous culture methodology employed represents a sound procedure for screening microalgae for biofuel production, providing a reliable evaluation of their fatty acid production capacity, under conditions close to those of outdoor production systems.

Keywords: Microalgae, continuous culture, photochemostat, fatty acid productivity, biodiesel

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1. Introduction

The use of microalgae as source of oil for biodiesel production is an issue of current general interest (Chisti, 2007; Hu et al., 2008). Since strain selection represents the first key step towards the development of an oil production process from microalgae (Griffiths et al 2012; Rodolfi et al., 2009), the establishment of suitable selection criteria and appropriate experimental systems for screening are of outmost relevance.

Most of the information regarding lipid accumulation in microalgae is solely based on the lipid content and derives from studies performed in batch cultures, being highly diverse and heterogeneous with regard to both strains and experimental conditions used. Reported cellular lipid levels generally correspond to those found in the stationary phase, which is characterized by severe limitation in nutrients and light availability.Values as high as 70% of the dry biomass have been reported, although 20-50% are more common (Chisti, 2007).

The selection of algal strains for the purpose of oil production must consider not only the lipid content (more properly, that of fatty acids) but also the biomass generation capacity, as to define the oil yield or productivity (Griffiths et al., 2012; Pruvost et al., 2011; Rodolfi et al., 2009). Being essential the adoption of this parameter as key criterion, care has to be taken on how it is evaluated. A reliable and precise measurement of actual lipid/fatty acid productivity can be performed in the steady state situation of a continuous culture, in which the productivity of biomass and its composition (including lipid/fatty acid content) keep stable. Nevertheless, such a situation does not apply to the batch culture, in which growth rate and biomass composition change as growth progresses.

In the present work, a screening strategy for the selection of oil-producing microalgae is derived from experimentation with ten strains, for which fatty acid

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productivity is evaluated. The continuous culture, especially operating under moderate N limitation, emerges clearly as the option of choice. The screening conditions include daylight cycle illumination, as to mimic those prevailing outdoors, in which production systems operate.

2. Materials and methods

2.1. Microalgae and culture conditions

The following ten microalgae were used: *Chlorella fusca* (SAG 211-8b), *Chlorococcum oleofaciens* (SAG 213-11), *Monoraphidium braunii* (SAG 202-7d), *Muriella aurantiaca* (SAG 249-1), *Muriella decolor* (SAG 249-2), *Muriellopsis* sp. (Del Campo et al., 2000), *Neochloris oleoabundans* (UTEX 1185), *Pseudokirchneriella subcapitata (*SAG 61-81*), Scenedesmus almeriensis* (CCAP 276-24) and *Tetraselmis suecica (ICMAN, Cádiz 03-0203).*

Cells were grown photoautotrophically on the medium described by Arnon et al. (1974) modified to contain 4 mM K_2HPO_4, with the exception of *T. suecica*, for which f/2 medium (Guillard and Ryther, 1962) was used. The media were supplied with $NaNO_3$, as to reach the final nitrate concentrations indicated in each case.

Batch culture was performed at 25ºC, in 4.5 cm diameter 0.2 L capacity cylindrical flasks containing 0.15 L of culture, laterally illuminated with fluorescent lamps providing 115 μE $m^{-2}s^{-1}$ on the surface of the flasks. The culture was continuously sparged with air (40 L (L $culture^{-1}$) h^{-1}) supplemented with 1% CO_2. The culture medium and the vessels were sterilized in autoclave at 120ºC for 20 min.

Continuous culture was performed in 2.0 L capacity (0.07 m diameter, 0.50 m height) jacketed sterilized photochemostat (bubble columns) containing 1.8 L of cell suspension, continuously sparged with air (33 L (L $culture^{-1}$) h^{-1}). Temperature was maintained at 25°C, and pH at 7.5 by on demand injection of CO_2 into the air stream

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entering the culture. The photochemostat was illuminated following a light cycle of 12 h light/12 h dark, with simulated solar daylight cycle during the light period, using six Phillips PL-32 W/840/4p white-light lamps, which provided a maximal incident irradiance of 2500 μE m^{-2} s^{-1} on the reactor surface. Initially, the reactors were inoculated with batch-grown cells and operated on batch mode for about 3-4 d, with incident irradiance being progressively increased, until becoming close to stationary phase. Then, it was switched to operate on continuous mode, fresh medium being continuously fed during the light cycle at a flow rate of 45 mL h^{-1} (dilution rate (D), 0.3 d^{-1}), with withdrawal of culture at the same rate. Once steady state conditions were achieved, analytical determinations were performed. The data presented correspond to stabilized situations, being average values of 4 to 8 determinations of each steady state. Measurement of photosynthetically active irradiance was carried out using a 4π quantum scalar irradiance sensor QSL-100 (Biospherical Instrument, San Diego, CA), inside the vessel (without cells).

2.2. Analytical procedures

Biomass concentration was determined by dry weight measurement. Ten mL aliquots of the cell suspension were filtered through pre-weighed filters (1.2 μm or 0.45 μm, depending on the cell size). Cells were washed with either distilled water or 1% ammonium formate, for freshwater or marine microalgae, respectively. The filters containing the algae were weighted after drying in an oven at 80ºC during 24 h.

Fatty acids were analyzed on 10 mg of freeze-dried biomass, after transesterification of the dry lipid fraction (Rodriguez Ruiz et al., 1998). The lipid fraction was obtained by mechanical disruption of cells employing a Mini Bead Beater (Biospec Products), using chloroform:methanol (2:1, v/v) as extracting solvent and 0.5 mm diameter glass beads. Ten μL of an internal standard, containing 10 mg of either

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heptadecanoic or nonadecanoic acid in 1 mL toluene, was included in the disruption step. For transesterification, 2 mL of methylation mixture (methanol:acetyl chloride, 20:1 v/v) and 1 mL hexane were added to the dry lipid fraction and heated at 100°C for 20 min. After cooling to room temperature, 2 mL distilled water were added. Following centrifugation (5 min, 1500 x *g*), the upper hexane phase was injected into the gas chromatograph (GC-2010, Shimadzu) equipped with a flame ionization detector. A Suprawax-280 (15 m x 0.1 mm x 0.1 µm) capillary column (Teknokroma) was used, with He as carrier gas. Temperature was programmed to increase from 150 (hold 4 min) to 200ºC at 15ºC min^{-1} (hold 21 min), then to 250ºC at 15ºC min^{-1} (hold 7 min). Injector and detector were kept at 250ºC and 270ºC, respectively. For determination of fatty acid content (% of dry biomass), peaks between C14 and C24 were integrated. Retention times for individual fatty acids were previously calibrated with a mixture of methyl esters (SupelcoTM 37 Fame mix, nº 47885U; PUFA-3 Matreya LLC, nº 1177). The percentage of each fatty acid was expressed in relation to the total sample (% of total).

2.3. Productivity calculation

Productivity of biomass (Pb) and that of fatty acids (P_{FA}) were estimated in steady state situations of continuous cultures: $P_b = Cb*D$; and $P_{FA} = Pb*X_{FA}$; where; Cb is the concentration of biomass; D is the dilution rate; and X_{FA} the mass fraction of fatty acid in the biomass.

3. Results and discussion

3.1. Batch culture

The potential of ten preselected microalgae for lipid accumulation has initially been evaluated in 7-day old batch cultures, when nitrate in the culture (10 mM at t = 0 h) was exhausted (data not shown). At this stage of the batch culture, *C. oleofaciens*, *M. braunii*, *M. aurantiaca*, *P. subcapitata* and *S. almeriensis* exhibited a fatty acid content

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of at least 20% of the dry biomass, outstanding *C. oleofaciens*, with 34%. Under the same conditions, the fatty acid levels of *M. decolor*, *T. suecica*, *C. fusca*, *Muriellopsis* sp. and *N. oleoabundans* were between 11 and 17% (Table 1). According to the prevalent trend, the relative potential for fatty acid accumulation of the different strains could be predicted on the basis of the fatty acid content alone, or considering as an additional reference the biomass accumulation capacity. Total biomass accumulated in the stationary phase (day 7) was over 5 g L^{-1} for *C. oleofaciens* and *M. aurantiaca*, with the highest accumulation of biomass (around 6 g L^{-1}) corresponding to *C. fusca* and *Muriellopsis sp*. From the results of batch growth experiments, the group of most promising strains would thus include *C. oleofaciens*, *M. aurantiaca*, *Muriellopsis* sp. and *M. braunii*, whereas *T. suecica*, *M. decolor*, *P.subcapitata* or *N. oleoabundans* would appear as the less favorable. *C. fusca* and *S. almeriensis* could be considered as of moderate expectancy.

It is worth stating at this point that the generally high lipid content (including fatty acids) of cells in the stationary phase of batch cultures is concomitant with severely restricted growth. Lipid productivity (i.e. the product of lipid content and growth rate) would therefore approach zero in this situation. So, whereas the maximal lipid or fatty acid content of a particular strain can be assessed at the stationary phase of a batch culture, no accuracy is to be expected for lipid (or fatty acid) productivity values estimated from this parameter, unless a careful analysis of instant productivity throughout the whole growth curve is performed (Griffiths et al., 2012). Notwithstanding, such estimates are not sustained in time as the batch culture proceeds towards the stationary phase. Conversely, actual lipid or fatty acid productivity can be easily and unequivocally assessed in a continuous culture from values of flow rate, cell density and lipid (fatty acid) level determined under steady state conditions. Evaluation

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in continuous culture thus emerges as the strategy of choice for most proper assessment of oil production capacity of microalgae.

3.2. Screening of microalgae under continuous culture: evaluation of productivity

Biomass and fatty acid productivity of the ten microalgae have been estimated in continuous culture in photochemostat, under conditions of both N sufficiency (20 mM $NaNO_3$ in the feed medium) and moderate N limitation (2 mM $NaNO_3$), confirming that N availability affects both productivity and biomass composition.

N-sufficient continuous cultures of *C. fusca, C. oleofaciens, M. decolor*, *Muriellopsis, P. subcapitata* and *S. almeriensis* reached steady biomass concentration values over 2 g L^{-1} with *M. aurantiaca, M. braunii* and *T. suecica* exhibiting about 1.2 g L^{-1}, the corresponding biomass productivity values ranging between 0.35 and 0.80 g L^{-1} d^{-1} (Table 2). Values in this range have been reported for *T. suecica* (0.44 g L^{-1} d^{-1}, San Pedro et al. 2013), *N. oleoabundans* (0.53 g $L^{-1}d^{-1}$, Pruvost et al., 2011); *P. subcapitata* (0.63 g $L^{-1}d^{-1}$, Patil et al., 2007) and *S. almeriensis* (0.87 g $L^{-1}d^{-1}$, Sánchez et al., 2008), for continuous cultivation under nitrogen sufficiency. Under moderate N limitation, biomass productivity (between 0.14 and 0.59 g $L^{-1}d^{-1}$) was always markedly lower than under N sufficiency (Table 2).

Under moderate N limitation in continuous culture, fatty acid accumulation takes place in parallel with stable growth. The fatty acid content under these conditions was overall superior as compared to those of N sufficiency, standing out the cases of *C. oleofaciens*, *M. aurantiaca* and *P. subcapitata*, with 2.5-fold higher levels in 2 mM $NaNO_3$. A converse situation, with slightly lower fatty acid content in N limitation was found for *M. braunii* and *T. suecica* (Table 3). Under the unfavorable conditions for growth of reduced N availability, modifications in photosynthetic carbon partitioning occur, leading to the accumulation of either or both, lipids or carbohydrates, the

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compounds being stored preferentially varying with the microalgal strain. Although the fatty acid content of *M. braunii* and *T. suecica* under N limitation were not higher than under N sufficiency, their carbohydrate content under limiting N were markedly high, reaching values of 35 and 42%, respectively (data not shown).

Regarding fatty acid productivity in continuous culture, *C. oleofaciens*, *Muriellopsis, N. oleoabundans, P. subcapitata* and *S. almeriensis* exhibited values around 80 mg L^{-1} d^{-1} in N sufficiency, whereas *M. aurantiaca* and *M. braunii* did only reach less than 30 mg fatty acid L^{-1} d^{-1} (Table 3). The latter microalgae had shown high fatty acid production potential in batch culture (Table 1), being therefore preliminary considered within the most promising strains according to their performance in batch. Notwithstanding, the actual fatty acid productivity data determined in continuous regime recommend to disregard these two species as putative oil producers. Conversely, *N. oleoabundans* and *P. subcapitata*, which were considered as of low potential according to the batch culture approach, are outstanding producers according to their actual fatty acid productivity determined in continuous regime. Altogether, these results raise serious concern on the adequacy of batch cultures for screening procedures addressed to the selection of oil producing microalgae. They should rather be based on the continuous culture approach.

Under moderate N limitation, improved levels of fatty acid productivity where obtained for *C. oleofaciens P. subcapitata* and *S. almeriensis*, since the lowered biomass productivity (Table 2) was more than compensated by the enhanced fatty acid level subsequent to N limitation, to result in fatty acid productivity values of 109, 93 and 92 mg $L^{-1}d^{-1}$, respectively (Table 3). Continuous culture under controlled N limitation has in fact proven effective for optimized production of astaxanthin by *Haematococcus pluvialis* (Del Río et al., 2005) in a one-step approach (alternative to the

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conventional two-step strategy), and Wen et al. (2014) have recently applied it to lipid production by *Chlorella pyrenoidosa*, achieving a productivity of 145 mg lipids L^{-1} d^{-1}. Under N sufficiency, yields of about 10 mg fatty acids L^{-1} d^{-1} have been reported for continuous cultures of *C. minutissima* and *D. tertiolecta* (Tang et al., 2012), as well as 126 mg lipids L^{-1} d^{-1} for *N. oleoabundans* (Pruvost et al. 2009).

Since conditions set for the screening, including a daylight cycle illumination, are close to those in which outdoor systems operate, the oil productivity values obtained should provide valuable information to consider in the scaling up of the corresponding production process. It should be noted that since the main purpose of this work was to devise a reliable screening procedure, the operating conditions were aimed to this end but not to maximize fatty acid productivity. Further optimization of culture conditions for those highlighted microalgae species could result in improved yields.

3.3. Influence of nitrogen availability on fatty acid profile

The properties of biodiesel are largely determined by those of its component fatty acid esters (Knothe, 2005). In general, a raw material rich in saturated and monounsaturated fatty acids is more suitable as biodiesel feedstock (Chisti, 2007; Hu et al., 2008; Stansell et al., 2012). Thus, fatty acid profile thus emerges as a relevant criterion when evaluating the suitability of a microalgal oil for biodiesel.

The influence of N availability on fatty acid profile of the 10 microalgae growing in continuous culture has been analysed. In all cases, the profile in N sufficiency was different to that in N limitation, with higher levels of saturated and monounsaturated fatty acids in the latter situation, as it might be expected (Griffiths et al. 2012; Piorreck et al. 1984). Higher levels of palmitic and oleic and lower of linolenic acid were found in the biomass produced under N-limiting conditions (Table 4), with overall lower levels of C16:4 (C16:3 in the case of *N. oleoabundans*). For *C.*

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oleofaciens, M. aurantiaca, *M. decolor*, *Muriellopsis* and *P. subcapitata* the aggregate of C16:0 and C18:1 accounted for more than 60% of total fatty acids under N-limiting conditions, against only about 20-30% under N sufficiency. The N-limited microalgal biomass was more suitable as biodiesel feedstock also because of its lower content in PUFA and linolenic acid (in some cases below 12%). This must be taken into consideration when establishing conditions for production of microalgal oil for biodiesel.

4. Conclusions

The continuous culture approach is a most appropriate methodology for the screening of microalgae for the purpose of biodiesel production, as it allows the determination of real fatty acid productivity. Ten strains have been evaluated in photochemostat, under both sufficient and limiting N availability, with higher yield of fatty acids apposite as raw material for biodiesel being found in N limitation. Particularly high potential capacity for oil production has been established for *C. oleofaciens*, *P. subcapitata* and *S. almeriensis*. The proposed continuous system for evaluating actual oil production capacity is reliable and accurate, the corresponding operating conditions –that include daylight cycle illumination- being similar to those of outdoor production systems.

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Table 1. Biomass concentration and fatty acid content of microalgae in batch culture (7th day, stationary phase)

Strain	Biomass concentration ($g\ L^{-1}$)	Fatty acids (% of dry biomass)
Chlorella fusca	5.88±0.17	11.67±1.06
Chlorococcum oleofaciens	5.51±0.22	33.62±1.90
Monoraphidium braunii	4.08±0.41	20.39±0.86
Muriella aurantiaca	5.29±0.19	21.96±2.04
Muriella decolor	3.02±0.06	11.21±0.21
Muriellopsis sp.	6.34±0.34	14.16±0.33
Neochloris oleoabundans	1.74±0.01	16.66±3.10
Pseudokirchneriella subcapitata	2.42±0.11	20.49±0.91
Scenedesmus almeriensis	3.22±0.09	20.43±2.36
Tetraselmis suecica	2.76±0.29	11.35±0.96

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Table 2. Influence of nitrate availability on biomass concentration and productivity of microalgae in continuous culture

Strain	Biomass concentration ($g\ L^{-1}$)		Biomass productivity ($g\ L^{-1}\ d^{-1}$)	
	NO_3^- sufficiency*	NO_3^- limitation**	NO_3^- sufficiency*	NO_3^- limitation**
Chlorella fusca	2.20±0.13	1.09±0.05	0.66±0.04	0.33±0.02
Chlorococcum oleofaciens	2.53±0.07	1.36±0.08	0.76±0.02	0.41±0.03
Monoraphidium braunii	1.19±0.17	0.47±0.07	0.36±0.05	0.14±0.02
Muriella aurantiaca	1.18±0.18	0.83±0.11	0.35±0.05	0.25±0.03
Muriella decolor	2.30±0.10	1.62±0.07	0.69±0.03	0.49±0.02
Muriellopsis sp	2.42±0.11	1.34±0.18	0.73±0.03	0.40±0.05
Neochloris oleoabundans	1.91±0.09	1.11±0.11	0.57±0.04	0.33±0.03
Pseudokirchneriella subcapitata	2.09±0.10	0.96±0.06	0.63±0.03	0.29±0.02
Scenedesmus almeriensis	2.61±0.09	1.97±0.11	0.80±0.03	0.59±0.03
Tetraselmis suecica	1.19±0.21	0.78±0.07	0.36±0.06	0.23±0.02

*20 mM $NaNO_3$ in the feed medium

**2 mM $NaNO_3$ in the feed medium

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Table 3. Influence of nitrate availability on fatty acid content of the biomass and fatty acid productivity of microalgae in continuous culture

Strain	Fatty acids (% of dry biomass)		Fatty acid productivity (mg $L^{-1}d^{-1}$)	
	NO_3^- sufficiency*	NO_3^- limitation**	NO_3^- sufficiency*	NO_3^- limitation**
Chlorella fusca	8.3±0.7	10.4±0.2	54.7±4.8	34.2±0.6
Chlorococcum oleofaciens	10.5±0.5	26.8±2.5	79.2±4.2	109.2±2.3
Monoraphidium braunii	7.5±0.2	5.7±0.2	27.0±0.7	8.0±0.27
Muriella aurantiaca	5.7±0.7	14.5±1.4	19.8±2.6	36.2±3.4
Muriella decolor	9.6±0.6	16.5±1.2	66.2±3.8	80.6±6.0
Muriellopsis sp.	11.0±1.2	15.5±0.6	80.7±8.7	61.8±2.4
Neochloris oleoabundans	13.6±1.5	17.4±1.4	77.9±4.9	57.8±6.1
Pseudokirchneriella subcapitata	12.7±0.8	32.2±1.6	79.8±4.8	92.6±4.5
Scenedesmus almeriensis	9.9±1.3	15.6±1.4	79.0±10.4	92.0±8.2
Tetraselmis suecica	9.6±2.7	8.2±0.4	34.6±9.7	18.7±0.9

*20 mM $NaNO_3$ in the feed medium

**2 mM $NaNO_3$ in the feed medium

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Table 4. Profile of major fatty acids (% of total FA) in the biomass of microalgae in continuous culture, under nitrate sufficiency (S) or limitation (L). (Cf: *Chlorella fusca*; Col: *Chlorococcum oleofaciens;* Mb: *Monoraphidium brauni;* Ma: *Muriella aurantiaca*; Md: *Muriella decolor*; Mur: *Muriellopsis*; Nol: *Neochloris oleoabundans;* Ps: *Pseudokirchneriella subcapitata;* Sca: *Scenedesmus almeriensis*; Ts: *Tetraselmis suecica*). Relative standard deviation ≤ 10%.

Strain	Nitrate	C16:0	C16:3	C16:4	C18:1	C18:2	C18:3
Cf	S	13.5	3.2	21.3	3.0	5.9	36.8
	L	19.9	3.3	4.7	37.1	7.7	17.4
Col	S	13.9	3.4	13.6	16.7	7.0	25.0
	L	20.7	3.2	3.6	42.4	8.8	9.7
Mb	S	14.9	1.8	17.9	7.4	8.2	33.1
	L	21.7	1.3	8.9	28.9	5.2	24.3
Ma	S	16.4	6.3	13.9	9.3	9.6	29.1
	L	18.7	3.3	3.8	47.6	6.5	10.2
Md	S	14.8	5.0	15.9	3.6	4.8	41.2
	L	25.1	2.0	3.5	42.3	5.9	14.3
Mur	S	15.9	4.2	14.3	4.9	4.9	42.1
	L	23.1	2.7	3.4	40.6	7.3	15.2
Nol	S	20.9	13.9	0.5	2.5	20.5	25.2
	L	30.3	7.0	-	13.2	21.2	22.7
Ps	S	18.9	1.0	14.5	15.1	4.4	29.0
	L	20.5	0.9	2.8	54.1	1.9	8.9
Sca	S	13.8	4.8	8.0	15.7	17.1	16.9
	L	16.9	6.1	3.6	34.6	11.7	13.4
Ts	S	23.6	1.4	12.5	19.5	5.9	18.5
	L	27.5	1.5	9.7	24.0	5.4	15.5

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Highlights

1. A continuous culture methodology is established for the screening of microalgae for oil

2. This reliable system allows actual evaluation of fatty acid productivity

3. Moderate nitrogen limitation results in improved fatty acid profile

4. Actual fatty acid productivity has been determined for ten microalgae

5. Values of up to 109 mg fatty acid L^{-1} d^{-1} have been recorded

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