Lipid production and mixotrophic growth features of cyanobacterial strains isolated from various aquatic sites

Sima Modiri,1 Hakimeh Sharafi,1,2 Leila Alidoust,1 Hamidreza Hajfarajollah,1 Omid Haghighi,1 Aisan Azarivand,1 Zahra Zamanzadeh,1 Hossein Shahbani Zahiri,1 Hojatollah Vali3 and Kambiz Akbari Noghabi1

1Division of Industrial & Environmental Biotechnology, National Institute of Genetic Engineering and Biotechnology (NIGEB), PO Box 14155-6343, Tehran, Iran
2Department of Microbiology, Faculty of Sciences, Alzahra University, Tehran, Iran
3Facility for Electron Microscopy Research, McGill University, 3640 Street, Montreal, Quebec, Canada

The present study was conducted to determine the potential of five cyanobacteria strains isolated from aquatic zones to induce lipid production. The phylogenetic affiliation of the isolates was determined by 16S rRNA gene sequencing. Amongst the isolates, an efficient cyanobacterium, Synechococcus sp. HS01 showing maximal biomass and lipid productivity, was selected for further studies. In order to compare lipid productivity, the HS01 strain was grown in different media to screen potential significant culture ingredients and to evaluate mixotrophic cultivation. Mixotrophic cultivation of the strain using ostrich oil as a carbon source resulted in the best lipid productivity. GC analysis of fatty acid methyl esters of the selected cyanobacterial strain grown in media supplemented with ostrich oil showed a high content of C16 (palmitoleic acid and palmitic acid) and C18 (linoleic acid, oleic acid and linolenic acid) fatty acids of 42.7 and 42.8%, respectively. Transmission electron micrographs showed that the HS01 cells exhibited an elongated rod-shaped appearance, either isolated, paired, linearly connected or in small clusters. According to initial experiments, ostrich oil, NaNO 3 and NaCl were recognized as potential essential nutrients and selected for optimization of media with the goal of maximizing lipid productivity. A culture optimization technique using the response surface method demonstrated a maximum lipid productivity of 56.5 mg l⁻¹ day⁻¹. This value was 2.82-fold higher than that for the control, and was achieved in medium containing 1.12 g l⁻¹ NaNO 3, 1 % (v/v) ostrich oil and 0.09 % (w/v) NaCl.

INTRODUCTION

Over the last few years, increasing petroleum costs and, more importantly, environmental concerns have driven scientists to develop new technical knowledge and skills about renewable energy sources. Biodiesel is currently receiving considerable attention due to its great potential as a sustainable and environmentally friendly alternative to petroleum-based fossil fuels (Griffiths & Harrison, 2009).

Global concern about exhausting petroleum reservoirs is one reason for the increasing level of research on biodiesel production (Schenk et al., 2008). Therefore, searching for renewable energy sources is necessary and finding new clean energy resources has become one of the most daunting challenges of the world.

Cyanobacterial lipid could be one of the promising feedstocks for biodiesel production (Rittmann, 2008; Yang et al., 2011). As common photosynthetic prokaryotic organisms, cyanobacteria can be technologically developed as an excellent microbial cell factory that can harvest solar energy and transform atmospheric carbon dioxide to useful organic compounds (de Loura et al., 1987; Parmar et al., 2011). Lipids, carotenoids, pigments, vitamins and aromatic compounds are amongst the bioproducts of cyanobacteria.
Accumulated lipids in the thylakoid membranes of cyanobacteria are of definite interest and can be used as lipid feedstock for biodiesel production (Karatay & Dönmez, 2011; Costa & de Morais, 2011).

Cyanobacteria have basic growth requirements, and use carbon dioxide, light and other inorganic nutrients effectively (Parmar et al., 2011). However, some cyanobacteria and microalgae could use both organic carbon sources and carbon dioxide under mixotrophic conditions, and exhibit higher biomass and lipid yields (Wan et al., 2011). Insufficient information is available on the use of appropriate carbohydrates as carbon sources to support cyanobacterial heterotrophic metabolism (Francisco et al., 2014). The growth rate, cell density and productivity of heterotrophic Chlorella sorokiniana were found to be 3.0, 3.3 and 7.4 times higher than their phototrophic counterparts, respectively (Zheng et al., 2012; Francisco et al., 2014). Abreu et al. (2012) also observed higher specific growth rates, final biomass concentrations and productivities of lipids, starch and proteins by Chlorella vulgaris under mixotrophic conditions using cheese whey powder as a carbon source when compared with photoautotrophic conditions.

To develop an effective process, a significant step is to search, sort and identify hyper-lipid-producing strains of cyanobacteria. Successful isolation can lead to the selection of appropriate cyanobacterial strains with relevant properties for specific culture conditions and products. Culture condition optimization of the selected strains is of fundamental importance to the success of the process. Patel et al. (2014) obtained 130.76% more lipid production compared with the control by Synechocystis sp. PCC 6803 using optimized culture conditions. In another work, Karatay & Dönmez (2011) found maximum lipid contents of 42.8% for Synechocystis sp. PCC 6803 using BG-11 fermentation medium supplemented with NaNO3 (1.5 g), MgSO4.7H2O (75 mg), CaCl2.2H2O (36 mg), citric acid (6 mg), H3BO3 (2.86 mg), MnCl2.4H2O (1.81 mg), ZnSO4.7H2O (222 µg), Na2MoO4.2H2O (390 µg), CuSO4.5H2O (79 µg), Co(NO3)2.6H2O (49.4 µg), ferric ammonium citrate (6 mg), Na2CO3 (20 mg), KH2PO4 (30.5 mg) and Na2-EDTA (10 mg) (Rippka et al., 1979). Erlenmeyer flasks were sparged with sterile filtered air and incubated at 25-27°C under continuous illumination by cool white fluorescent lamps for 10 days. After purification of the cyanobacterial isolates using agar plates, molecular identification of the isolated purified cyanobacteria was carried out using 16S RNA gene sequencing with primers CYA106F (5’-CGGACCGGTTGATACGGTGTA-3’) and CYAN1281R (5’-GCAATTACTAGCGATTCCTCC-3’) (Valério et al., 2009). The reaction was carried out in a 25 µl volume containing 1× PCR buffer, 1.5 mM MgSO4, 200 µM each dNTP, 1 µM each primer, 1 U Pfu (Fermentas) DNA polymerase and 1 ng template DNA. PCR amplification was performed as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1.5 min; and a final extension at 72°C for 10 min. The amplified 1260 bp product, which appeared as a single band, was purified using a High Pure PCR Product Purification kit (Roche) and sequenced using an ABI Prism 377 automated sequencer (Applied Biosystems). Sequence homologies with other reference strains were examined using BLAST 2.2.12 (Altschul et al., 1990). Multiple sequence alignments were carried out using CLUSTAL W software and a consensus neighbour-joining tree was constructed using MEGA 4.0 (Saitou & Nei, 1987).

**METHODS**

**Sampling, isolation and molecular identification.** In order to isolate cyanobacterial strains, lake water samples were collected from southern areas of Iran and also an urban lake in the north of Iran. Samples were immediately transferred to the laboratory and stored under appropriate conditions for further study. Aliquots of 5 ml were transferred into 250 ml Erlenmeyer flasks containing 100 ml BG-11 fermentation medium [NaNO3 (1.5 g), MgSO4.7H2O (75 mg), CaCl2.2H2O (36 mg), citric acid (6 mg), H3BO3 (2.86 mg), MnCl2.4H2O (1.81 mg), ZnSO4.7H2O (222 µg), Na2MoO4.2H2O (390 µg), CuSO4.5H2O (79 µg), Co(NO3)2.6H2O (49.4 µg), ferric ammonium citrate (6 mg), Na2CO3 (20 mg), KH2PO4 (30.5 mg) and Na2-EDTA (10 mg)] (Rippka et al., 1979). Erlenmeyer flasks were sparged with sterile filtered air and incubated at 25-27°C under continuous illumination by cool white fluorescent lamps for 10 days. After purification of the cyanobacterial isolates using agar plates, molecular identification of the isolated purified cyanobacteria was carried out using 16S RNA gene sequencing with primers CYA106F (5’-CGGACCGGTTGATACGGTGTA-3’) and CYAN1281R (5’-GCAATTACTAGCGATTCCTCC-3’). The amplification was performed as follows: initial denaturation at 95°C for 5 min; 30 cycles of denaturation at 94°C for 1 min, annealing at 50°C for 1 min and extension at 72°C for 1.5 min; and a final extension at 72°C for 10 min. The amplified 1260 bp product, which appeared as a single band, was purified using a High Pure PCR Product Purification kit (Roche) and sequenced using an ABI Prism 377 automated sequencer (Applied Biosystems). Sequence homologies with other reference strains were examined using BLAST 2.2.12 (Altschul et al., 1990). Multiple sequence alignments were carried out using CLUSTAL W software and a consensus neighbour-joining tree was constructed using MEGA 4.0 (Saitou & Nei, 1987).

**Growth pattern and lipid productivity of cyanobacterial isolates.** High lipid productivity and appropriate growth rates are key desirable characteristics of cyanobacteria for biodiesel production. The lipid productivity, lipid content and growth rate of cyanobacterial isolates were evaluated initially. In these series of experiments, an appropriate amount of activated culture was inoculated into 2000 ml Erlenmeyer flasks containing 400 ml BG-11 medium until the final OD750 of the culture reached 0.1. The growth curve of the strains was obtained by measuring dry cell weight (DCW) and OD750 using a Lambda25 spectrophotometer (PerkinElmer) during the incubation period. All experiments were performed at 27°C and under a continuous light intensity of 70 µE (m²s)⁻¹. Subsequently, biomass productivity was determined as the difference between the dry weights at the start and at the end of the experiment per unit time (days). All runs were performed in triplicate. Finally, according to the lipid productivity and growth rate results, one strain with the best characteristics was selected for further study.

**Evaluation of phototrophic and mixotrophic cultivation.** To investigate the effect of carbon source, nitrogen source and other nutrients on lipid production, the selected strain was grown in BG-11 medium supplemented with different potential nutrients. After a number of primary experiments using a one-factor-at-a-time method and comparing the effect of different media with control medium, we selected three potential essential nutrients. The media evaluated were BG-11 (see above) supplemented with (a) 0.5 g NaNO3 1⁻¹, (b) 3 % (w/v) NaCl, (c) 3 % (w/v) glucose and (d) 3 % (v/v) ostrich oil. Simple BG-11 medium was considered as control. Biomass and lipid productivity of selected strain in the stationary growth phase were compared. Based on these preliminary experiments, three factors that were identified as significantly affecting lipid production were selected for optimization experiments.
Analysis

Lipid extraction and quantification. The total lipid content of cyanobacterial cells was estimated gravimetrically. Cyanobacterial cells were harvested at the stationary growth phase by centrifugation (4500 g, 4 °C, 10 min), followed by immediate freezing at −80 °C and then lyophilized. Total lipid content was extracted from lyophilized cells according to the Bligh–Dyer method (Bligh & Dyer, 1959) with minor modifications. Dry biomass of cyanobacterial strains (100 mg) was mixed with 5 ml chloroform/methanol (2:1) solvent mixture and sonicated for 30 s. The mixture was heated at 65 °C for 1 h followed by addition of methanol and NaCl solution (1% w/v) to a final volume ratio of 1:1:0.9 (chloroform/methanol/NaCl solution). The organic layer containing lipid and chloroform at the bottom of the tube was recovered by centrifugation (4000 g, 4 °C, 5 min) and then transferred into a weighed vial. The remainder was then dried by an evaporator. Dried vials were weighed and lipid content was measured gravimetrically. Based on the lipid content, the lipid productivity and lipid production were then calculated. Lipid productivity (mg l⁻¹ day⁻¹) was calculated from the difference in lipid concentration on two consecutive days, whilst lipid production (mg l⁻¹) was determined only at the end of the culture period.

GC analysis. The extracted lipids were transesterified to fatty acid methyl esters (FAMEs) by 2 N potassium hydroxide solution in methanol according to ISO 5509 (Animal and Vegetable Fats and Oils — Preparation of Methyl Esters of Fatty Acids; ISIRI 4091). The FAMEs were further extracted by n-hexane and prepared for GC analysis according to ISO 5509 (ISIRI 4091). A GC analyser (Younlig 6000) equipped with a CP-Sil 18 Varian column (60 m × 0.25 mm) and flame ionization detector was used for the analysis of the fatty acid composition of lipid samples. The column temperature was set at 175 °C for isothermal analysis. The injector and detector temperatures were 280 and 300 °C, respectively. Standard samples were initially injected to obtain the exact retention time for each compound. All samples were injected three times and means were reported.

Scanning electron microscopy (SEM). SEM (Hitachi SU-70, 1 nm resolution) was employed to investigate the morphology of the cyanobacterial cells. Samples of cyanobacteria were stuck with a conducting adhesive on the metallic substrate holder and introduced directly into the chamber after preparation.

Transmission electron microscopy (TEM). To evaluate the morphological characteristics of Synechococcus sp. HS01, a small pellet of the bacterial cells collected in the late exponential growth phase was exposed to 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer and kept at 4 °C for 24 h to fix the bacteria. The fixative solution was then carefully removed and the pellet was washed three times for 10 min with 0.1 M cacodylate washing buffer. The pellets comprising the bacteria were dehydrated with ethanol in percentage increases of 10% from 30 to 90%, with a 5 min interval between each increment. The specimens were then further dehydrated twice with 100% ethanol over 10 min intervals. Following dehydration, the pellets were infiltrated by adding increasing amounts of epon/ethanol (from 1:1 to 3:1), allowing a 30 min interval between each subsequent addition. Pure epon, deaerated under vacuum [constant pressure <25 p.s.i. (172 kPa)], was then added to the pellets, and allowed to stand for 1 h under vacuum to remove ethanol residue and air bubbles in the epon. Finally, the pellet-containing wells were refilled with new epon and placed in an oven at 60 °C for 48 h to polymerize the epon. For TEM observation, ultrathin sections (70 nm thick) were cut and transferred to TEM grids (200 mesh copper grids; EMS Sciences). Digital images were obtained using a Gatan 792 239 Bioscan 1k×1k Wide Angle Multiscan charge-coupled device camera in an FEI Tecnai 12 transmission electron microscope at 120 kV.

Fourier transform IR spectroscopy (FTIR). In order to gain a general insight into the chemical nature of the extracted lipids, their FTIR spectra were analysed. FTIR spectra were recorded in the 4000–400 cm⁻¹ region on an FTIR system (PerkinElmer) with the sample dispersed in pellets of KBr. The wavenumber accuracy and resolution of the spectra were 0.01 and 4 cm⁻¹, respectively.

Experimental design. The response surface method (RSM) was employed to optimize the nutrient concentrations that maximized lipid production. The Box–Behnken approach is an example of the RSM. It streamlines the economics of experimentation as it allows reducing a classical 3³ setup, requiring 27 replications, to 15 replications. The linear, quadratic effects and first-order interactions can be estimated elegantly, which is in most cases satisfactory (Hajifarajollah et al., 2014). The regression equation for this model can be presented as:

\[ Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_1^2 + \beta_5 X_2^2 + \beta_6 X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \]  

where \( Y \) is the final response, \( X_i \) are the variables and \( \beta_i \) are the model coefficients.

Therefore, the Box–Behnken design was used to determine the optimum levels of the significant variables and the effects of their mutual interactions on lipid productivity. In total, 15 experiments were carried out for the three test variables. Each independent variable was studied at three different levels (low, medium and high, coded as −1, 0 and +1, respectively). The centre point of the design was replicated two times for the estimation of error. A centre point is a point at which all variables are set at their mid-value.

RESULTS AND DISCUSSION

Phylogenetic analysis and features of the isolated strains

Phylogenetic trees of five isolated strains of cyanobacteria were reconstructed based on 16S rRNA gene sequences. Phylogenetic analysis was performed on a concatenated dataset obtained from a BLAST search. The sequence was then subjected to multiple sequence alignment using CLUSTAL_W and MEGA 4.0. Finally, the aligned sequences were subjected to phylogenetic tree reconstruction, estimation and validation using neighbour joining. Results from 16S RNA gene sequencing identified our strains as Synechococcus sp. HS01, Pseudanabaena sp. SK03, Nodosilinea sp. AK01, Pseudanabaena sp. SK02 and Pseudanabaena sp. SK01. Fig. 1 shows the phylogenetic tree for all of the isolated strains using MEGA 4.0 with 1000 times bootstrap replication and a substitution model \( p \) distance. As can be observed, strain HS01 had near 100% similarity to Synechococcus sp. in the 16S rRNA gene. Bright-field microscopic observations of the cyanobacterial isolates demonstrated that HS01 was unicellular with a rod-shaped appearance, similar to Synechococcus elongatus sp. PCC 7942 shown by Mackey et al. (2008). Furthermore, strain HS01 and Synechococcus sp. PCC 7942 showed 99% sequence similarity in their 16S rRNA genes. Further morphological examination was performed using SEM along with TEM. Table 1 shows the
related information, including SEM images of isolated cyanobacterial strains, their GenBank accession numbers, the source of isolation and geological location of the sampling sites as well as the lipid content of each cyanobacterial isolate. Cyanobacteria represent one of the most morphologically diverse groups of prokaryotic phyla, with morphotypes ranging from unicellular to multicellular filamentous forms. In this study, cyanobacterial species were investigated based on morphological traits, including cell shape and width. Cell measurements were made based on the optical images. As illustrated in the SEM images, Synechococcus sp. HS01 is a unicellular cyanobacterium with a rod-shaped appearance and a mean thickness of 0.86 μm. The other strains were filamentous cyanobacteria with trichomes of

![Phylogenetic tree of Synechococcus sp. HS01](image-url)
different widths. *Nodosilinea* sp. AK01 was characterized by its relatively thin trichomes (0.75 μm). In the strains recognized to be from the family *Pseudanabaenaceae*, trichome diameters were greater (0.94–1.35 μm) and cell lengths were often much longer.

**Table 1. SEM images, isolation source and lipid content of five cyanobacterial isolates**

<table>
<thead>
<tr>
<th>Cyanobacterium (GenBank accession no.)</th>
<th>SEM image</th>
<th>Isolation source and location</th>
<th>Lipid content (% DCW)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pseudanabaena</em> sp. SK01 (KF834988)</td>
<td><img src="image1" alt="SEM image" /></td>
<td>Marsh, Kelachay City, Rudsar County, Gilan Province, Iran 37° 4’ 0.12” N, 50° 24’ 0” E (37.06 N, 50.4 E)</td>
<td>12.85</td>
</tr>
<tr>
<td><em>Pseudanabaena</em> sp. SK02 (KF834989)</td>
<td><img src="image2" alt="SEM image" /></td>
<td>Marsh, Ramsar City, Ramsar County, Boarder Gilan Province, Iran 49° 24’ 12” N, 50° 24’ 0” E (30.02 N, 51.4 E)</td>
<td>7.4</td>
</tr>
<tr>
<td><em>Synechococcus</em> sp. HS01 (KF946940)</td>
<td><img src="image3" alt="SEM image" /></td>
<td>Oil-contaminated water, Dezful County, Khuzestan Province, Iran 22° 51.96” N, 48° 23’ 50.64” E (32.38 N, 48.39 E)</td>
<td>12.33</td>
</tr>
<tr>
<td><em>Pseudanabaena</em> sp. SK03 KF834990</td>
<td><img src="image4" alt="SEM image" /></td>
<td>Urban lake, Javaherdeh village, Ramsar County, Mazandaran Province, Iran 36° 51’ 20” N, 50° 28’ 28” E (36.85 N, 50.47 E)</td>
<td>15.66</td>
</tr>
<tr>
<td><em>Nodosilinea</em> sp. AK01 (KJ957191)</td>
<td><img src="image5" alt="SEM image" /></td>
<td>Maharloo Lake, Shiraz City, Fars Province, Iran 29° 28’ 24.18” N, 52° 46’ 1.21” E (29.47 N, 52.76 E)</td>
<td>8.33</td>
</tr>
</tbody>
</table>

**TEM analysis**

TEM images of ultrathin sections prepared from *Synechococcus* sp. HS01 show the typical shape of this bacterium grown in BG-11 medium. In general, *Synechococcus* sp. HS01 is widely regarded as having rod-shaped cells. Thylakoids of the cells...
were characteristically arranged in parallel in the cell membrane (Fig. 2). As can be seen, a number of cells divided and some daughter cells remained in pairs. TEM micrographs of the HS01 cells showed a normal appearance and different numbers of visible electron-dense areas known as carboxysomes can be seen. The bacteria showed a clearly layered structure of the Gram-negative cell envelope. Dense black spots and white halos in the cytoplasmic space suggested the presence of polyphosphate and poly-β-hydroxybutyrate granules, respectively (Fig. 2).

Selection of appropriate lipid-producer strain

As stated above, lipid productivity is a key desirable parameter for biodiesel production by cyanobacteria. However, biomass productivity is also important because it can be an indication of how fast the growth rate of cyanobacteria is. Therefore, all things considered, a comprehensive study was conducted to analyse the behaviour of all cyanobacterial isolates in terms of biomass and lipid productivity as well as lipid content. Fig. 3 shows maximum biomass and lipid productivity during the growth of cyanobacterial isolates. In addition, the lipid contents are presented in Table 1. As can be seen from Fig. 3, the five cyanobacterial isolates showed strain-to-strain variation in biomass and lipid productivity. However, *Synechococcus* sp. HS01 showed much higher productivity in terms of both lipid and biomass compared with other strains. *Nodosilinea* sp. AK01 showed the lowest lipid and biomass productivities at 3.43 and 40.28 mg l\(^{-1}\) day\(^{-1}\), respectively. However, the lipid content of *Synechococcus* sp. HS01 was lower than *Pseudanabaena* spp. SK01 and SK03 (Table 1). Higher lipid content is almost associated with lower biomass productivity (Chen et al., 2011). Consequently, lipid content cannot be the only factor in determining the oil-producing capability of cyanobacteria, and a simultaneous evaluation of lipid content and biomass production is desired. As a result, lipid productivity, representing the combined effects of oil content and biomass production, is a more suitable performance index to indicate the oil-producing capability of cyanobacteria. Biomass and lipid productivities of 193.08 and 23.42 mg l\(^{-1}\) day\(^{-1}\), respectively, were attained by *Synechococcus* sp. HS01, which were higher than the reported data (52.7 and 14.2 mg l\(^{-1}\) day\(^{-1}\), respectively) for *Synechococcus* sp. PCC 7942 (Da Róis et al., 2013).

Considering that productivity is a time-dependent factor, the higher lipid productivity of *Synechococcus* sp. HS01 in comparison with filamentous strains may be due to the high growth rate of this unicellular strain. In view of the results obtained, *Synechococcus* sp. HS01 is certainly an efficient lipid producer and was selected for further experiments.

Evaluation of culture conditions

To evaluate the potential benefits of media components for the cultivation of *Synechococcus* sp. HS01 and to compare mixotrophy and phototrophy, media (a)–(d) and control (see Methods) were prepared, and biomass and lipid productivity were compared over time. Fig. 4 shows maximum biomass and lipid productivities of *Synechococcus* sp. HS01 in the different media. Figs S1–S5 (available in the online Supplementary Material) represent variations of biomass and lipid production in these media over time. According to Figs S1–S5, in the exponential growth phase, both lipid and biomass production increased in parallel in media (a), (c) and (d), whereas lipid production increased with
biomass in the early and late exponential phases in medium (b) and control, but lipid production remained constant during the mid-exponential phase in medium (b) and control. Maximum levels of lipid production were achieved during the mid-exponential phase in medium (b) and control, but lipid production remained constant biomass in the early and late exponential phases in medium (b) and control. Maximum levels of lipid production were achieved during the mid-exponential phase in medium (b) and control, but lipid production remained constant.

Furthermore, as shown in Figs S3 and S4, mixotrophic cultivation of Synechococcus sp. HS01 can lead to faster growth, which in turn results in improved productivity (a value dependent on time).

The maximum lipid productivity was obtained in under mixotrophic conditions using ostrich oil as a carbon source. Lipid productivity increased 2.1-fold (to 43.14 mg l⁻¹ day⁻¹) in the medium containing ostrich oil in comparison with the control. With a rapid 9 h heterotrophic doubling time, Chlorella sorokiniana UTEX 1230 maximally accumulated 39 % total lipid extract (by dry weight) during heterotrophic metabolism compared with 18 % autotrophically (Rosenberg et al., 2014). In the past decade, considerable progress has been made in our understanding of the mechanisms by which cyanobacteria regulate the expression of their genes in order to adapt to changes in light intensity and spectral quality, and in response to nutritional conditions, especially carbon, nitrogen and sulfur sources (Tandeau de Marsac & Houmard, 1993). It was shown that incorporation of fatty acids into cyanobacterial lipids occurred when exogenous fatty acids were added to both Anacystis nidulans R2 and Synechocystis sp. 6803 culture media (Williams et al., 1990). The growth of A. nidulans was stimulated after 120 h of incubation in low concentrations of 18:1 fatty acids (Williams et al., 1990). This was the first report of addition of lipid compounds to the culture media of cyanobacteria. Our preliminary data presented here suggest that cyanobacterial cells were very adaptable to changing levels of ostrich oil.

The approximate cost of the organic carbon substrate is estimated to be ~80 % of the whole cultivation medium cost (Bhatnagar et al., 2011). As a result, less expensive organic sources need to be found to overcome the high carbon cost resulting from mixotrophic culture conditions. Under nitrogen deficiency conditions in medium (a) (in which the nitrogen source was lower than the control), in spite of biomass productivity reduction, lipid productivity was improved. In agreement with previous studies, the lipid productivity of Chlorella zofingiensis cultivated in nitrogen-deficient media was higher than that obtained from cells grown in complete medium, whilst biomass productivity was much lower compared with the control (Feng et al., 2012). It can be speculated that the better lipid productivity under nitrogen deficiency conditions could be due to the conditional utilization of nitrogen by cells for the synthesis of essential cell constitutions. Hence, any carbon dioxide subsequently fixed is therefore converted into lipid or carbohydrate rather than protein. In a similar manner, for the selected cyanobacterial isolate Synechococcus sp. HS01 grown in medium (b) (supplemented with NaCl), in which the biomass productivity was reduced in comparison with control, the lipid productivity increased from 19.97 to 32.18 mg l⁻¹ day⁻¹. A recent study showed that increasing the concentration of NaCl could lead to higher lipid production by Chlorella sp. PCH90 (Abdelaziz et al., 2014).

Fig. 4. Biomass and lipid productivity of Synechococcus sp. HS01 in BG-11 medium with (a) 0.5 g l⁻¹ NaNO₃, (b) 3 % (w/v) NaCl, (c) 3 % (w/v) glucose and (d) 3 % (v/v) ostrich oil.

- Biomass productivity
- Lipid productivity

Culture medium

0 50 100 150 200 250

Biomass productivity (mg l⁻¹ day⁻¹)

a b c d Control

0 10 20 30 40 50 60

Lipid productivity (mg l⁻¹ day⁻¹)

a b c d Control

Additional relevant information:
- Our preliminary data presented here suggest that cyanobacterial cells were very adaptable to changing levels of ostrich oil.
- The approximate cost of the organic carbon substrate is estimated to be ~80 % of the whole cultivation medium cost (Bhatnagar et al., 2011).
- Under nitrogen deficiency conditions in medium (a) (in which the nitrogen source was lower than the control), in spite of biomass productivity reduction, lipid productivity was improved.
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et al., 2014). It can be hypothesized that NaCl induces some metabolic pathways through which the cells increase the production of their lipid components. This hypothesis is supported by our results showing an increase in lipid productivity upon the addition of NaCl into BG-11 medium. However, further studies are needed to elucidate the mechanisms underlying the apparent effect of NaCl on lipid production.

From another point of view, the mean specific growth rates, \( \mu (\text{day}^{-1}) \), of Synechococcus sp. HS01 grown in media (a)–(d) were calculated using \( \mu = (\ln N_n - \ln N_1)/(t_n - t_1) \), where \( N_n \) is the final dry weight of cyanobacterial cells at time \( n \), \( N_1 \) is the dry weight of cyanobacterial cells at time 1 and \( t_n \) is the time (days) after the initiation of the experiment (Guclu & Ertan, 2012). In addition, the doubling time of Synechococcus sp. HS01 was also calculated in media (a)–(d). Specific growth rates for media (a)–(d) and control were 0.52, 0.38, 0.78, 0.7 and 0.39 day\(^{-1}\), and the doubling times were 1.3, 1.76, 0.88, 0.99 and 1.76 days, respectively. Under mixotrophic conditions, we obtained growth rates of 0.78 and 0.7 day\(^{-1}\) in media supplemented with glucose and ostrich oil, respectively. The growth rate of Synechocystis PCC 6803 reported in the literature was 0.89 ± 0.06 day\(^{-1}\) (Knoop et al., 2010). Although Synechococcus sp. HS01 exhibited a slightly lower growth rate compared with this model cyanobacterium, lipid production of Synechococcus sp. HS01 in medium supplemented with ostrich oil (215 mg l\(^{-1}\)) was higher than for Synechocystis sp. PCC 6803 (197 mg l\(^{-1}\)) (Machado & Atsumi, 2012).

Thus, mixotrophic cultivation of Synechococcus sp. HS01 using ostrich oil as a carbon source produced an appropriate lipid yield. In addition, NaNO\(_3\) and NaCl also seemed to be effectual nutrients that influenced lipid productivity. Therefore, ostrich oil, NaNO\(_3\) and NaCl were selected for further optimization experiments with the objective of maximizing lipid productivity in under mixotrophic cultivations.

**Fatty acid analysis**

The composition and distribution of FAMEs of the lipids accumulated in Synechococcus sp. HS01 grown in media (a)–(d) and control are presented in Fig. 5(a). Table S1 also shows the data in detail. The GC profile of the lipid sample extracted from medium (d) using ostrich oil as a carbon source is presented in Fig. 5(b). Most fatty acids present in the samples grown in the five media studied were unsaturated, although saturated fatty acids were detected as well. As can be seen, cis-palmitoleic acid (hexadecanoic acid) was the major fatty acid in the lipids extracted from media (b), (d) and control, whilst lipids extracted from media (a) and (c) contained a major proportion of oleic acid and linoleic acid, respectively.

Patil et al. (2011) noted that algal biodiesel contains a major proportion of palmitoleic acid (C16:1, 30–33 %) and oleic acid (C18:1, 35–38 %). As can be seen in Fig. 5(a) and Table S1, the proportion of palmitoleic acid decreased against control in all four tested media. The oleic acid content was increased in all the test cyanobacteria under the conditions tested. Overall, high yields of methyl esters with 16 and 18 carbon atoms were achieved by transesterification of the cellular crude lipids. The proportions of total C16 and C18 fatty acids increased in almost cases, except in medium (b). These results indicated that each of the crude lipids of cyanobacterial cells cultivated in BG-11 medium could be used as a raw material for biodiesel production (Karataay & Dönmez, 2011). According to the fatty acid composition of Synechococcus sp. HS01, this strain could be a promising feedstock for biofuel production, especially when cultured in the presence of ostrich oil. Moreover, the monounsaturated/polysaturated fatty acid ratios were increased in media (a) and (b), which is desirable for good quality biodiesel as these offer oxidative stability to the fuel. These results are in agreement with increasing oleic acid content and monounsaturated/polysaturated fatty acid ratio in nitrogen-deprived cells of Synechocystis PCC 6803 (Singh &
Mallick, 2014). Oxidative stability is one of the most important issues affecting the use of biodiesel as a consequence of the presence of polyunsaturated methyl esters in algal oil (Knothe, 2006). Accordingly, in this study, ostrich oil as a carbon source is more suitable for biofuel production when compared with glucose, for the reason that polyunsaturated fatty acids were increased less when using ostrich oil. Polyunsaturated fatty acids have valuable benefits to human health and nutritional markets (Patnaik et al., 2006). Therefore, polyunsaturated fatty acids derived from mixotrophic cultivation of *Synechococcus* sp. HS01 using glucose as a carbon source could be employed in such applications.

**FTIR analysis of extracted lipids**

Chromatography and Nile red fluorescent staining have been used to analyse lipid droplets in cyanobacteria (Volkman et al., 1998; Wase et al., 2014). However, these methods are time-consuming and technically challenging. Dean et al. (2010) determined lipid accumulation in two species of freshwater microalgae, *Chlamydomonas reinhardtii* and *Scenedesmus subspicatus*, by FTIR. Laurens & Wolfrum (2011) examined the FTIR spectra of the pure lipid compounds. Three distinctive absorption bands were noticeable, of which the CH3 and CH2 (3025–2954 cm−1) lipids and important functional groups of the lipids. The FTIR spectrum of the lipid produced in ostrich oil-containing medium is shown in Fig. 6. As can be seen, all of the important peaks associated with a lipid-like compound are detectable. CH3 and CH2 (3025–2954 cm−1) of the lipid chain, the C=O group for the ester group (1746–1654 cm−1), and OH of carboxylic acid (3450 cm−1) were detected.

![FTIR spectrum of the lipid extracted from Synechococcus sp. HS01 grown in ostrich oil-containing medium.](image)

**RSM**

In order to determine the nature of the response surface in the optimum region, a Box–Behnken design with three levels was performed (Table 2). The protocol applied in this study involved 15 experiments conducted according to Table 3. The response surface graphs were obtained to understand the effect of variables individually and in combination, to determine their optimum levels for maximum lipid production.

ANOVA of the model along with the corresponding *P* values and the parameter estimated for lipid production are shown in Table 4. The value of the correlation coefficient, *R*2 (99.74%), shows that the regression model provides an accurate description of the experimental data. A reasonable agreement between predicted (97.5%) and adjusted (99.29%) values was observed. As a rough rule of thumb, *R*2 values of >80% are desirable to have confidence that the response model provides useful information on the experiment. In addition, there is no evidence of lack of fit (*P*=0.59 > 0.05). The *P* value for the lack of fit is very important and it should be insignificant (range 0.5–0.99). If it is significant, then the whole model will be insignificant and of no value. All these evaluations confirmed that the model could be used for the prediction of lipid production within the given range of variables. Equation (2) shows the relative lipid concentration (*Y*) as a function of the test variables (*X*1) in uncoded units:

\[
Y = 14.055 + 71.072X_1 + 6.158X_2 + 26.945X_3
- 28.93X_1^2 - 3.072X_2^2 - 245.54X_3^2
- 5.38X_1X_2 - 6.016X_1X_3 + 25X_2X_3
\]  

(2)

The parameter estimate and the corresponding *P* values suggested that, amongst the test variables, all the factors were significant and had important effects on lipid productivity (Table 4). This was expected from our previous experiments using these three compounds. Interestingly, mutual interactions between ostrich oil and both NaCl and NaNO3 were very significant (*P*<0.001). However, interaction of NaCl with NaNO3 was insignificant (*P*=0.28).

When we use a full second-order model we get main interaction and quadratic effects, so that it becomes harder

**Table 2. Experimental range and levels of independent test variables used in the Box–Behnken design**

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>-1</th>
<th>0</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaNO3 (g l−1)</td>
<td>0.25</td>
<td>0.875</td>
<td>1.5</td>
</tr>
<tr>
<td>Ostrich oil (%)</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>NaCl (g l−1)</td>
<td>0.05</td>
<td>0.175</td>
<td>0.3</td>
</tr>
</tbody>
</table>
to differentiate which variables have the most influence on yield. We can get more information by looking at the response surfaces. The response surface is an \( n \)-dimensional surface in \( n+1 \)-dimensional space. In this case, it will be a graph of lipid productivity as a function of NaNO\(_3\), ostrich oil and NaCl concentration. The response surfaces from all of the interactions between the compounds are illustrated in Fig. 7. *Synechococcus* sp. HS01 showed an increase of lipid productivity as NaCl concentration was increased; however, this relationship was not linear and, as expected, curvature along the NaNO\(_3\) axis occurred. The nature of the surface plot indicated that the interaction between NaNO\(_3\) and ostrich oil was significant.

In order to determine the optimum conditions, a solution was given by the ‘optimizer’ option of Minitab software. The concentrations of NaNO\(_3\), ostrich oil and NaCl were adjusted to be in range when the lipid productivity was assumed to be at its maximum. The RSM model predicts that a medium containing 1.12 g l\(^{-1}\) NaNO\(_3\), 1 % (v/v) ostrich oil and 0.09 % (w/v) NaCl gives the maximum lipid productivity of 56.5 mg l\(^{-1}\) day\(^{-1}\), which is 2.82-fold higher than the control (Fig. 7a–c). This could represent considerable lipid productivity when compared with Silva et al. (2014), who obtained maximum lipid productivity of 45.6 mg l\(^{-1}\) day\(^{-1}\) by *Synechococcus* sp. PCC 7942 under optimized conditions. These results suggest that HS01 might be a suitable candidate for lipid production.

Table 3. Box–Behnken design matrix with experimental values for lipid productivity

<table>
<thead>
<tr>
<th>Standard order</th>
<th>Run order</th>
<th>Point type</th>
<th>Blocks</th>
<th>NaNO(_3) (g l(^{-1}))</th>
<th>Ostrich oil (g l(^{-1}))</th>
<th>NaCl (g l(^{-1}))</th>
<th>Lipid productivity (mg l(^{-1}) day(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0.875</td>
<td>3</td>
<td>0.05</td>
<td>35.05</td>
</tr>
<tr>
<td>13</td>
<td>2</td>
<td>0</td>
<td>1</td>
<td>0.875</td>
<td>2</td>
<td>0.175</td>
<td>50.1</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>2</td>
<td>1</td>
<td>0.875</td>
<td>1</td>
<td>0.05</td>
<td>54.77</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1.5</td>
<td>1</td>
<td>0.175</td>
<td>50.1</td>
</tr>
<tr>
<td>6</td>
<td>5</td>
<td>2</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
<td>0.05</td>
<td>42.11</td>
</tr>
<tr>
<td>8</td>
<td>6</td>
<td>2</td>
<td>1</td>
<td>1.5</td>
<td>2</td>
<td>0.3</td>
<td>38.23</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>2</td>
<td>1</td>
<td>1.5</td>
<td>3</td>
<td>0.175</td>
<td>30.98</td>
</tr>
<tr>
<td>1</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>0.25</td>
<td>1</td>
<td>0.175</td>
<td>32.99</td>
</tr>
<tr>
<td>3</td>
<td>9</td>
<td>2</td>
<td>1</td>
<td>0.25</td>
<td>3</td>
<td>0.175</td>
<td>27.33</td>
</tr>
<tr>
<td>15</td>
<td>10</td>
<td>0</td>
<td>1</td>
<td>0.875</td>
<td>2</td>
<td>0.175</td>
<td>48.77</td>
</tr>
<tr>
<td>5</td>
<td>11</td>
<td>2</td>
<td>1</td>
<td>0.25</td>
<td>2</td>
<td>0.05</td>
<td>30</td>
</tr>
<tr>
<td>7</td>
<td>12</td>
<td>2</td>
<td>1</td>
<td>0.25</td>
<td>2</td>
<td>0.3</td>
<td>28</td>
</tr>
<tr>
<td>12</td>
<td>13</td>
<td>2</td>
<td>1</td>
<td>0.875</td>
<td>3</td>
<td>0.3</td>
<td>37.11</td>
</tr>
<tr>
<td>11</td>
<td>14</td>
<td>2</td>
<td>1</td>
<td>0.875</td>
<td>1</td>
<td>0.3</td>
<td>44.33</td>
</tr>
<tr>
<td>14</td>
<td>15</td>
<td>0</td>
<td>1</td>
<td>0.875</td>
<td>2</td>
<td>0.175</td>
<td>50.3</td>
</tr>
</tbody>
</table>

In order to determine the optimum conditions, a solution was given by the ‘optimizer’ option of Minitab software. The concentrations of NaNO\(_3\), ostrich oil and NaCl were adjusted to be in range when the lipid productivity was assumed to be at its maximum. The RSM model predicts that a medium containing 1.12 g l\(^{-1}\) NaNO\(_3\), 1 % (v/v) ostrich oil and 0.09 % (w/v) NaCl gives the maximum lipid productivity of 56.5 mg l\(^{-1}\) day\(^{-1}\), which is 2.82-fold higher than the control (Fig. 7a–c). This could represent considerable lipid productivity when compared with Silva et al. (2014), who obtained maximum lipid productivity of 45.6 mg l\(^{-1}\) day\(^{-1}\) by *Synechococcus* sp. PCC 7942 under optimized conditions. These results suggest that HS01 might be a suitable candidate for lipid production.

Table 4. ANOVA results obtained from response surface optimization

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>Degrees of freedom</th>
<th>Sequential sum of squares</th>
<th>Adjusted sum of squares</th>
<th>Adjusted mean square</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>9</td>
<td>1196.33</td>
<td>1196.33</td>
<td>132.926</td>
<td>217.22</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Linear</td>
<td>3</td>
<td>591.99</td>
<td>536.32</td>
<td>178.772</td>
<td>292.13</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NaNO(_3)</td>
<td>1</td>
<td>232.2</td>
<td>527.82</td>
<td>527.816</td>
<td>862.51</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ostrich oil</td>
<td>1</td>
<td>334.37</td>
<td>6.97</td>
<td>6.972</td>
<td>11.39</td>
<td>0.02</td>
</tr>
<tr>
<td>NaCl</td>
<td>1</td>
<td>25.42</td>
<td>3.03</td>
<td>3.035</td>
<td>4.96</td>
<td>0.046</td>
</tr>
<tr>
<td>Square</td>
<td>3</td>
<td>519.1</td>
<td>519.1</td>
<td>173.035</td>
<td>282.76</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NaNO(_3) × NaNO(_3)</td>
<td>1</td>
<td>436.12</td>
<td>471.61</td>
<td>471.61</td>
<td>770.66</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ostrich oil × ostrich oil</td>
<td>1</td>
<td>28.63</td>
<td>34.84</td>
<td>34.837</td>
<td>56.93</td>
<td>0.001</td>
</tr>
<tr>
<td>NaCl × NaCl</td>
<td>1</td>
<td>54.35</td>
<td>54.35</td>
<td>54.351</td>
<td>88.82</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Interaction</td>
<td>3</td>
<td>85.24</td>
<td>85.24</td>
<td>28.413</td>
<td>46.43</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NaNO(_3) × ostrich oil</td>
<td>1</td>
<td>45.29</td>
<td>45.29</td>
<td>45.293</td>
<td>74.01</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>NaNO(_3) × NaCl</td>
<td>1</td>
<td>0.88</td>
<td>0.88</td>
<td>0.884</td>
<td>1.44</td>
<td>0.283</td>
</tr>
<tr>
<td>Ostrich oil × NaCl</td>
<td>1</td>
<td>39.06</td>
<td>39.06</td>
<td>39.063</td>
<td>63.83</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Residual error</td>
<td>5</td>
<td>3.06</td>
<td>3.06</td>
<td>0.612</td>
<td>0.692</td>
<td></td>
</tr>
<tr>
<td>Lack of fit</td>
<td>3</td>
<td>1.68</td>
<td>1.68</td>
<td>0.559</td>
<td>0.81</td>
<td>0.594</td>
</tr>
<tr>
<td>Pure error</td>
<td>2</td>
<td>1.38</td>
<td>1.38</td>
<td>0.692</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>1199.39</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CONCLUSION

Considering the fact that fast-growing strains with high biomass productivity and lipid content are the most important requirements for the provision of bioenergy generation from cyanobacteria, the results presented here confirm that lipid extracted from *Synechococcus* sp. can be used as a promising feedstock for biodiesel production. Ostrich oil has been valued for its numerous benefits to human health for a long time. To be able to reduce production costs, whilst increasing lipid extraction yield, ostrich oil, as a low-priced byproduct of the ostrich industry, might be a promising alternative substance for the production of clean fuels. We are continuously striving to isolate efficient lipid-producing cyanobacterial strains by using cheaper alternative byproducts, with our main aim being to improve biofuel production yield.

ACKNOWLEDGEMENTS

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