

ORIGINAL ARTICLE

Characterization of biofilm-forming cyanobacteria for biomass and lipid production

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biodiesel, biofilm-forming cyanobacteria, lipid content, polyphasic approach.

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Abstract**Aims:** This work reports on one of the first attempts to use biofilm-forming cyanobacteria for biomass and lipid production.**Methods and Results:** Three isolates of filamentous cyanobacteria were obtained from biofilms at different Italian sites and characterized by a polyphasic approach, involving microscopic observations, ecology and genetic diversity (studying the 16S rRNA gene). The isolates were grown in batch systems and in a semi-continuous flow incubator, specifically designed for biofilms development. Culture system affected biomass and lipid production, but did not influence the fatty acid profile. The composition of fatty acids was mainly palmitic acid (>50%) and less amounts of other saturated and monounsaturated fatty acids. Only two isolates contained two polyunsaturated fatty acids.**Conclusions:** Data obtained from the flow-lane incubator system would support a more economical and sustainable use of the benthic micro-organisms for biomass production. The produced lipids contained fatty acids suitable for a high-quality biodiesel production, showing high proportions of saturated and monounsaturated fatty acids.**Significance and Impact of the Study:** Data seem promising when taking into account the savings in cost and time derived from easy procedures for biomass harvesting, especially when being able to obtain the co-production of other valuable by-products.**Introduction**

With the shortage of fossil fuels, there is increasing effort to find alternative, renewable and therefore sustainable energy sources, such as solar and wind energy or energy from plant biomass. In particular, biofuels are now currently gaining considerable attention, especially bioethanol and biodiesel (Rodolfi *et al.* 2009; Vieira Costa and Greque de Moraes 2011). Biodiesel has been reported as a possible substitute or extender for conventional petrol-based diesel, being compatible with conventional diesel and offering several interesting and attractive properties, including biodegradability and non-toxicity (Demirbas 2007). Currently, the first generation of biofuels is being produced from soybean, coconut,

corn or palm (Chisti 2007; Lu *et al.* 2010). However, the use of agricultural products for energy results in an increased competition for fertile lands. Many studies are exponentially focusing on microalgae and cyanobacteria, considered one of the most promising feedstock for biofuels (Wijffels and Barbosa 2010; Singh *et al.* 2011; Machado and Atsumi 2012) because of their high growth rates and high photosynthetic efficiency. Moreover, amongst other advantages, the productivity of these photosynthetic micro-organisms in converting carbon dioxide into carbon-rich lipids greatly exceeds that of agricultural oleaginous crops, without competing for arable land (Stephens *et al.* 2010; Wijffels and Barbosa 2010). As for lipid composition, according to biodiesel standard published by the American Society for Testing

Materials (ASTM), biodiesel from microalgal oil is similar in properties to the standard biodiesel and is also more stable according to the flash point value (Demirbas and Demirbas 2011). Algae biodiesel contains no sulphur and performs as well as petroleum diesel, whilst reducing emissions of particulate matter, CO, hydrocarbons and SO_x (Mata *et al.* 2010). Sources for biofuel production include the heterotrophic green alga *Chlorella protothecoides* (Lu *et al.* 2010), the autotrophic microalgae *Botryococcus braunii* (Choi *et al.* 2011), *Dunaliella* sp. (Takagi and Karseno Yoshida 2006), *Nannochloropsis* spp. (Rodolfi *et al.* 2009; Wiyarno *et al.* 2011) and a limited number of diatoms (Ramachandra *et al.* 2009; Popovich *et al.* 2010). However, despite their potential, the production capacity of microalgae is presently limited in comparison with land-based energy crops (Wijffels and Barbosa 2010; Singh *et al.* 2011) because of the high investment costs and energy requirements for the biomass and oil production. In particular, the biomass harvesting, mainly performed by means of centrifugation, typically contributes to 20–30% of the total production costs (Kumar *et al.* 2010; Christenson and Sims 2012). Although alternative harvesting processes/methods have been proposed, large-scale application is still limited (Kumar *et al.* 2010). Previous studies were, in fact, focused on biodiesel production from planktonic microalgae, mainly grown in open ponds and in enclosed photobioreactor systems where the cells are in suspension. Very little attention has been given to date to using benthic species requiring a surface to grow on (Esson *et al.* 2011) and easily removed from the substrates. Moreover, for the production of lipid-based biofuels, cyanobacteria have received less attention than microalgae, in spite of their unique properties, which make them a promising model to transform C sources into valuable fuels and in spite of the fact that they are already successfully grown commercially (Quintana *et al.* 2011).

In this study, we report on the potential use of biofilm-forming cyanobacteria in biodiesel production. Phototrophic biofilms are matrix-enclosed, attached microbial communities of phototrophs and heterotrophs that can develop highly differentiated architectures and occur on surfaces exposed to light in a wide range of natural and anthropized environments. These complex communities produce biomass easily removable from substrates, because of their benthic habitus. Three filamentous cyanobacteria were isolated from biofilms at different Italian sites and characterized by a polyphasic approach, as strongly suggested for modern cyanobacterial taxonomy (Komárek 2011) in which the cytology, the ecology and the genetic diversity were assessed. The biomass productivity, the lipid content

and profile of these biofilm-forming cyanobacteria were also investigated. As culture conditions affect cyanobacterial lipid content, isolates were grown in batch cultures and in a semi-continuous flow system, appositely made for biofilm development.

Materials and methods

The three cyanobacterial isolates used in this study, namely VRUC162, VRUC163 and VRUC164 (Table 1), belong to the 'Univ. Roma Tor Vergata Culture Collection' (Castenholz 2001). The three isolates were characterized at the cytological and molecular levels. Samples were grown in two different culture systems, in batch culture and in a flow-lane incubator prototype, especially constructed for biofilm growth. The biomass obtained by each sample was evaluated and used for lipid extraction. Results were then compared with those obtained for the planktonic marine alga *Nannochloropsis oculata* CCAP849/1, an eustigmatophyceae commonly used as biodiesel producer (Rodolfi *et al.* 2009) which was grown in batch culture.

Microscopy

Morphological investigations were performed using an optical light microscope (LM; Zeiss Axioskop, Thornwood, NY, USA) equipped with a device for differential interferential contrast. Images were acquired using a digital camera Coolpix995 (Nikon Corp., Tokyo, Japan). For a phenotypic identification of the cyanobacterial isolates, the diacritical traits used for description of botanical species were considered (Komárek and Anagnostidis 1989, 2005). To estimate cell size, 100 measurements were

Table 1 Designation, origin and growth medium of the tested isolates

Isolate designation	Origin	Medium
VRUC162	Eroded soils Valle dell'Esaro (CZ, Italy)	BG11 ₀
VRUC163	Cabras lagoon sediments (OR, Italy)	BG11 ₀
VRUC164	Wastewater Treatment Plant of Fiumicino Airport (Rome, Italy)	Modified BG11*
<i>Nannochloropsis oculata</i> CCAP849/1	CCAP (Scotland)	F ½†

*BG11 modified using only nitrates as nitrogen source and increasing the concentration of PO₄³⁻ to obtain a final N : P ratio of 15 in the medium (K₂HPO₄ 16 mg l⁻¹) (Guzzon *et al.* 2005).

†Guillard and Ryther 1962.

made for each biometrical character. The isolates were also observed after staining with alcian blue (AB) at pH 0.5, specific for sulphated polysaccharides and at pH 2.5, specific for acidic polysaccharides (Bellezza *et al.* 2003).

Samples were also observed with confocal laser scanning microscope (CLSM), FV1000 (Olympus Corp., Tokyo, Japan), using chlorophyll *a*-based autofluorescence channels (λ excitation: 488 and 635 nm, 650–750 nm emission range) and after labelling with different fluorochromes (500–590 nm emission range). Samples were stained with 100 $\mu\text{g ml}^{-1}$ concanavalin A-Alexa Fluor 488 conjugate (Zippel and Neu 2005), selectively binding α -glucopyranosyl and α -mannopyranosyl residues in the exopolysaccharidic envelopes of the micro-organisms and with 3 $\mu\text{g ml}^{-1}$ acridine orange (AO) (Bellezza *et al.* 2003) to label nucleic acids and polysaccharides. CLSM data consisted of a set of two-dimensional (2D), cross-sectional images in the *x-y* plane obtained with IMARIS 6.2 software (Bitplane AG, Zurich, Switzerland).

Molecular analyses

The culture pellets were subjected to five cycles of freeze–thawing, and then, DNA was extracted from the three cyanobacterial isolates using the GeneMATRIX Plant and fungi DNA purification kit (EURx) according to the manufacturer's instructions. The 16S rRNA gene was amplified using the cyanobacterial specific primer CYA359 and the prokaryotic universal primer C (5'-AC-GGGCGGTGTGTAC-3') (Nübel *et al.* 1997). Amplifications were run in a GeneAmp PCR system 2700 (Applied Biosystem, Foster City, CA) using the condition described in Bruno *et al.* (2009). After purification from the agarose gel using the Wizard[®] SV Gel and PCR Clean-Up System (Promega Corp., Madison, WI), the PCR products (\approx 1100 bp) were cloned into pGEM-T Easy vector (Promega) and sequenced using primers CYA359 and C. The 16S rRNA gene sequences of the isolates were first analysed by a similarity search using the BLAST function of GenBank at the National Center NCBI electronic site (<http://www.ncbi.nlm.nih.gov/>). Phylogenetic analysis was made by comparing the sequences obtained with others from GenBank (16S rRNA gene sequences longer than 900 nucleotides), and in addition, multiple sequence alignments were conducted using the CLUSTAL W program from the European Bioinformatics Institute (<http://www.ebi.ac.uk/>). To infer the phylogenetic position of the studied taxa, different analytical methods were employed. Maximum-parsimony and distance trees were generated using a heuristic search constrained using the PAUP* 4.0 b10 software package (Swofford 2002). A maximum-likelihood tree was constructed using the PAUP* software with the computation of likelihood scores obtained with

PHYML by JMODELTEST 0.1.1 (Posada 2008) with the estimation of the GTR+I+G model. The robustness of the inferred phylogenies was determined by bootstrap analysis based on 500 re-samplings of data. The 16S rRNA gene sequence of *Gloeobacter violaceus* PCC7421 was used as an outgroup for the construction of trees.

Cultures

Each nonaxenic culture was grown (Table 1) in 500-ml flasks under static conditions in a controlled chamber at 18°C, 60% relative humidity (r.h.), 18 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ PPF (photosynthetic photon flux density) and at light/dark regime of 14:10 h. Growth was estimated by measuring optical density (Kontron Uvikon 860 spectrophotometer; Interlink Scientific Services Ltd, Dartford, Kent, UK) at 730 nm. When the stationary phase was reached, the obtained biomass was used for biomass and lipid analysis.

Each isolate was also cultivated in a semi-continuous culture system, a flow-lane incubator prototype, illuminated at 90 $\mu\text{mol photon m}^{-2} \text{s}^{-1}$ following a light/dark regime of 16:8 h and with flow rate of 25 l h^{-1} in a controlled chamber at 25°C at 60% r.h. The flow-lane incubator prototype (Fig. 1) (Zippel and Neu 2005; Zippel *et al.* 2007) was realized within the framework of the EU-project PHOBIA (PHototrophic Biofilms and their potential Application). It consisted of four separate chambers (LCs), each having a 798 cm^2 growing area, where medium was continuously pumped through the inlet device within each lane. Medium was changed twice a week. Polycarbonate slides were used as artificial substrata for the biofilm adhesion. Light was provided by fluorescent lamps (True-light 36 W; Auralight, Karlskrona, Sweden), and four light sensors were integrated per lane: one for the control of incident light (IL, Fig. 1) and the other three, located directly

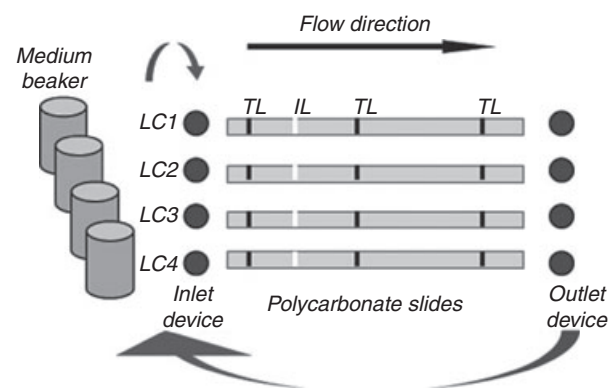


Figure 1 Scheme of the continuous flow-lane incubator prototype used to culture biofilm-forming cyanobacteria strains. LC: light chamber; TL: transmitted light sensor; IL: incident light sensor.

under three selected slides (TL, Fig. 1), for monitoring the transmittance (the percentage of incident light attenuation through biofilm biomass) during the experiments. Growth curves of the biofilms were obtained from daily mean values of light transmittance of each subsurface sensor converted into light absorption. Samples were collected at the mature/stationary stage by scraping biofilms off of polycarbonate substrates (<10% of transmittance) (Zippel *et al.* 2007) to perform, along with the biomass obtained by batch culture, the analyses below.

Productivity estimation, lipid content determination and fatty acid profiles

The daily biomass productivity was calculated by dividing the difference between the dry weights ($T = 105^{\circ}\text{C}$, overnight) at the end and at the start of the experiment by its duration (days). The lipid extraction was carried out according to Kochert (1978) protocol as follows: 50 mg of biomass produced by each isolate grown in each condition was treated for 5 min with 1 ml $1 \text{ mol l}^{-1} \text{ HClO}_4$ at 0°C , and then, the suspension was extracted three times with 45 ml of $\text{CHCl}_3/\text{MeOH}$ (2:1 v/v). Each time the organic layer was separated by centrifugation (Biofuge pico Haereus) at 754 g for 5 min at room temperature, rinsed with water and brine (saturated NaCl solution), and after solvent removal, the crude products were obtained. 30 mg of crude product was boiled for 5 min with 1 ml of MeONa in $0.25 \text{ mol l}^{-1} \text{ MeOH/diethylether}$ (1 : 1). Then, 5 ml of brine and 3 ml of esane/diethylether (1 : 1) were added and mixed. The phases were separated, and the fatty acid methyl esters (FAMES) were obtained from the organic one (Bannon *et al.* 1982).

The FAMES were analysed with a gas chromatograph – mass spectrometry GC-17A (Gas chromatograph QP-5000 mass spectrometer; Shimadzu Corp., Kyoto, Japan) equipped with split injector (sample size, $1.0 \mu\text{l}$) and fitted with fused Silica Capillary column Supelco SLB-5ms (length 30 m, i.d. $0.25 \mu\text{m}$). The split ratio 1 : 40 was used. The oven temperature was programmed at 170°C for 3 min and increased, at a rate of $3^{\circ}\text{C min}^{-1}$, to 220°C . Injector and detector temperatures were 250 and 230°C , respectively. The identification of fatty acids was performed by comparing the obtained mass spectra with NIST Mass Spectral Data Base (<http://webbook.nist.gov/chemistry/>).

Nucleotide sequence accession numbers

The sequences determined in this study have been submitted to the GenBank database and assigned accession no. JQ390606 (VRUC163), JQ390607 (VRUC162) and JQ390608 (VRUC164).

Results

Identification

The blue-green mucous colonies formed by the filamentous cyanobacteria VRUC162 were characterized by elongated and irregularly coiled trichomes with deep constrictions. The uniseriate and isopolar trichomes, without a firm sheath and any branching (Fig. 2a,b), were formed by vegetative cells $3.81 \mu\text{m}$ long ($\text{SD} = 0.64$) and $5.19 \mu\text{m}$ wide ($\text{SD} = 0.82$). In later stages of development, several solitary, intercalary heterocytes were present, with constrictions at the cross-wall clearly evident. Completely formed heterocytes were of ellipsoid shape, $5.34 \mu\text{m}$ wide ($\text{SD} = 0.43$) and $7.18 \mu\text{m}$ long ($\text{SD} = 0.5$), whilst those of immature trichomes were smaller and with terminal location. When examined with CLSM, at 650–750 nm emission range, vegetative cells emitted the fluorescence owing to the chlorophyll *a*, whilst mature heterocytes partially lacked the auto-fluorescence. Moreover, CLSM observations showed trichomes containing mature, separated cells and young, recently divided cells, still in close pairs (Fig. 2b). The observations at CLSM of VRUC162 stained with acridine orange and the separation of the two channels showed a well distinguished nucleoid region in the centre of the cell and underlined the presence of a polysaccharidic envelope surrounding the heterocytes (Fig. 2c,d). In general, no akinetes were found close to heterocytes in this isolate, possibly due to their apoheterocytic development. Trichomes were surrounded by very thin, anionic mucous sheaths, and heterocytes were coated by a bigger and more complex envelope, as shown by stained samples with alcian blue dyes (Fig. 2e), which also underline the presence of diffuse mucilage surrounding akinetes (Fig. 2f). On the basis of the morphological observations, the isolate could be assigned to the genus *Trichormus*.

The isolate VRUC163 consisted in more or less parallel oriented trichomes, forming macroscopic mucilaginous bright blue-green mats. The unbranched trichomes were characterized by the presence of heterocytes and akinetes (Fig. 3a). The isopolar trichomes were formed by vegetative cells $4.17 \mu\text{m}$ long ($\text{SD} = 0.42$) and $4.79 \mu\text{m}$ ($\text{SD} = 0.98$) wide, intercalary heterocytes slightly greater than vegetative cells ($4.57 \pm 0.41 \mu\text{m}$ long and $5.39 \pm 0.71 \mu\text{m}$ wide) and akinetes $13.63 \mu\text{m}$ long ($\text{SD} = 1.14$) and $8.18 \mu\text{m}$ wide ($\text{SD} = 0.63$). In general, akinetes were found close to heterocytes, probably because of their paraheterocytic development (Fig. 3b). Although under light microscope trichomes appeared without sheaths, CLSM observations after staining with concanavalin A-Alexa Fluor 488 conjugated and the separation of the two used channels revealed the presence of

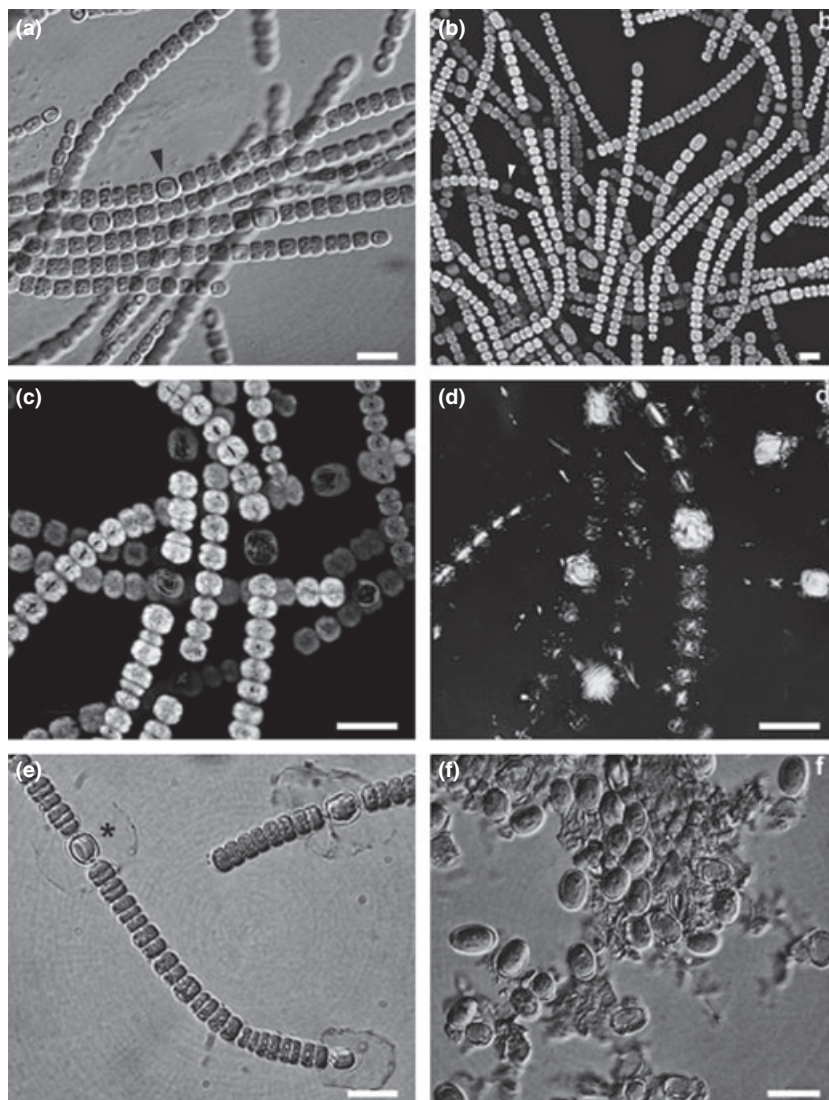


Figure 2 Photomicrographs illustrating the morphological features of the biofilm-forming cyanobacteria VRUC162. Light microscope (a) and CLSM (b) images showing the uniseriate and isopolar trichomes with deep constrictions at the cross-walls and the intercalary heterocytes (head arrows). CLSM images after staining with acridine orange (c, d) and light microscope photographs after staining samples with alcian blue dyes illustrating the acidic nature of heterocyte envelope (e, asterisk) and of the matrix surrounding akinetes (f). Bars: 10 μm .

a fine polysaccharidic envelope surrounding the trichomes (Fig. 3c,d). On the basis of these data, the isolate could be a representative of the *Anabaena* genus.

The cyanobacterial isolate VRUC164 formed macroscopic mats with blackish velvet surface. Microscopical observation showed isopolar, unbranched filaments without heterocytes and/or akinetes (Fig. 4). The isolate had trichomes with cells 5.72 μm wide (SD = 0.61) and 4.37 μm long (SD = 0.71), un-constricted at the cross-walls, with shortly narrowed apical cells, usually with a calyptra (Fig. 4a,b). On the basis of these morphological observations, the isolate can be assigned to the *Phormidium* group VII sensu Komárek and Anagnostidis (2005).

Cells were mostly shorter than wide, and most of the filaments were surrounded by colourless and thin sheaths, containing always a single trichome. Trichomes were dirty green and granulated (Fig. 4d). On the basis of these observations and, in particular, on the cell dimensions and the ecology of this sample, *Phormidium* group VII VRUC164 was presumptively assigned to the species *Phormidium autumnale*.

Phylogenetic analyses

Almost 1000 bp of the 16S rRNA gene were sequenced for the three isolates. The maximum-likelihood tree

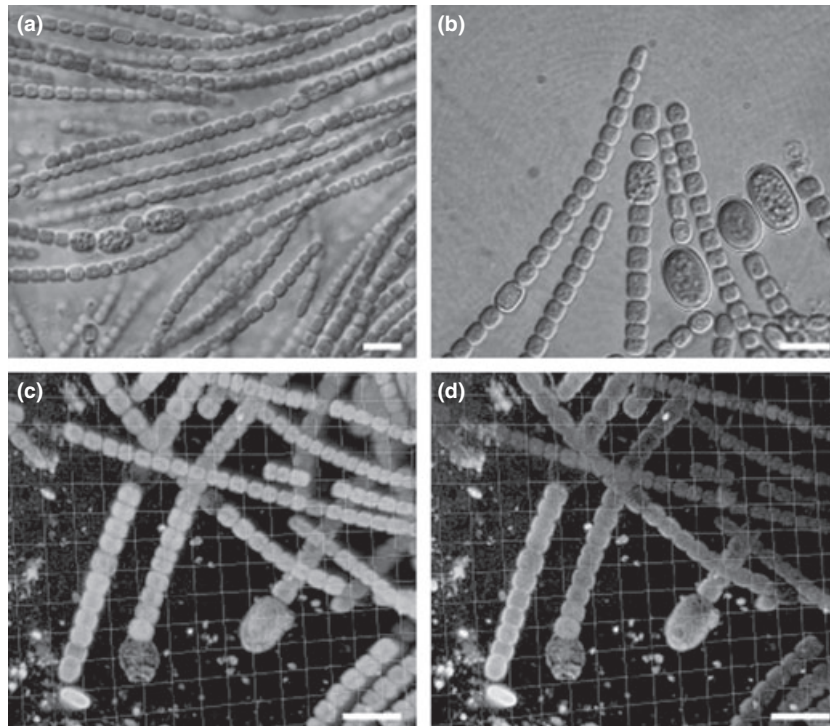


Figure 3 Photomicrographs showing the main morphological characteristics of the isolate VRUC163 observed at LM (a, b). CLSM images after staining the samples with concanavalin A-Alexa Fluor 488-conjugated using two channels together (c) or alone (d), see text for details. Bars: 10 μm .

(Fig. 5) is based on a data set of 28 16S rRNA gene sequences, having the same topology of the trees inferred by the distance and the maximum-parsimony methods.

Two clusters were consistently obtained by genetic analysis (Fig. 5): cluster 1 contained the isolates VRUC162 and VRUC163 and cluster 2 contained isolate VRUC164. VRUC162 and VRUC163 were genetically heterogeneous showing low sequence identity (97.9%). VRUC162 formed a subcluster (B), supported by 99–94% of bootstrap values, with two benthic isolates: *Trichormus variabilis* HINDAK 2001/4, from soil, and Ms2 identified as *Anabaena* sp. from lake sediments. The sequence similarity between VRUC162 and these two isolates was 99.8 and 99.5%, respectively. Another subcluster (A) was formed by isolates of *T. variabilis* and *Anabaena* sp., with unknown ecological origin and with 99.2% of sequence similarity with VRUC162. Lastly, VRUC163 formed a third subcluster (C) with three *Anabaena* spp. isolates and had a sequence identity of 99.5% with the benthic isolate of *Anabaena augstumalis* SCMIDKE JANKE/4a. The sequence identities of VRUC163 with the other isolates in this subcluster were lower than 99% (bootstrap values 97–71%). In cluster 2, different *Phormidium* spp. isolates were grouped. VRUC164 grouped in a subcluster E together with two isolates of *P. autumnale*, SAG 35.90 and SAG 78.79 with high sequence identity (99.9%) and

a bootstrap value of 100%. The sequence identities of VRUC164 and the isolates in cluster D ranged from 98.7 to 98.9%.

Growth evaluation, biomass production and lipid analysis

The growth curves (not shown) obtained for samples cultured in batch were characterized by an initial exponential growth phase at around the fifth and seventh day, whilst the stationary phase of growth was reached at around the 25th and the 30th day. *N. oculata* CCAP849/1 reached the same phase at the 18th day.

All isolates grown in the incubator prototype showed an uneven substrate adhesion. At the initial stage of development, a thin film covered the slides, though not uniformly, followed by a gradual development of the biofilm in the flow direction up to the mature phase of growth with was reached in 30–33 days when the biofilm covered the entire surface homogeneously.

Table 2 reports on the productivity attained by the three isolates grown in batch cultures and in the incubator prototype compared to that of *N. oculata*. In batch system, VRUC164 showed the highest productivity, whilst in the flow-lane incubator system, VRUC164 and VRUC163 resulted as the best producers of biomass.

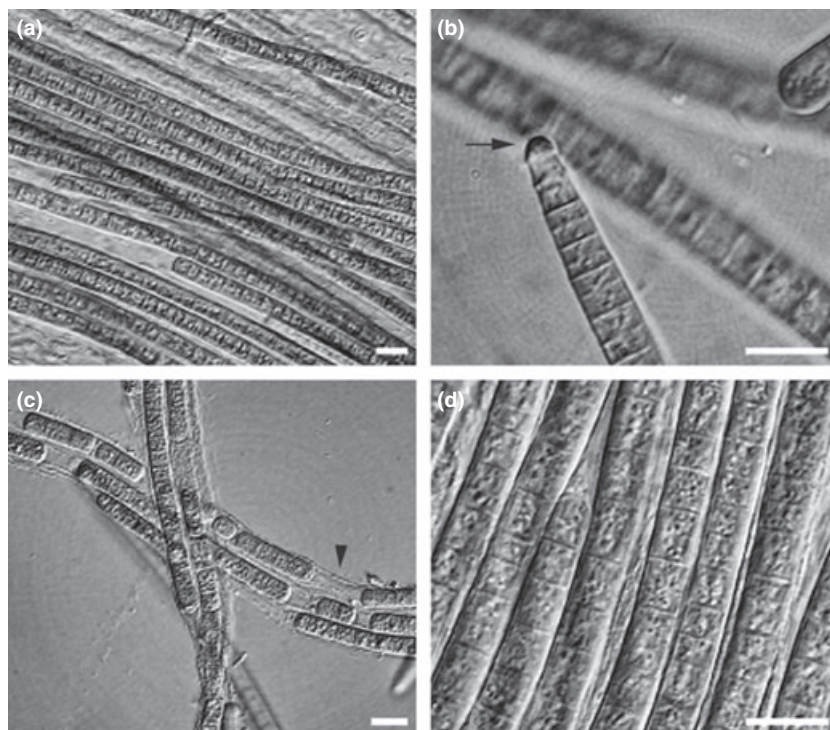


Figure 4 LM photomicrographs showing the main morphological characteristics of the isolate VRUC164. Trichomes unstricted at the cross-walls, with rounded or elongated apical cells (a, b), sometimes with a calyptra (b, arrow), presence of a colourless sheath (c, head arrow) and granules (d). Bars: 10 μm

Table 2 also shows the biomass, expressed as g DWd^{-1} , obtained by the three isolates in the two different culture conditions to compare results. Isolates clearly produced higher quantities of biomass when grown in the flow-lane incubator prototype. The lipid content obtained for each isolate is also reported in Table 2. All three cyanobacterial isolates growing in batch culture had lower lipid quantities than those produced by *N. oculata*, whilst the VRUC164 and VRUC162 cultured in the incubator had higher values. Comparing the culture systems, the three cyanobacterial species showed higher lipid content values when grown in the incubator system than in batch cultures.

Fatty acids in the three isolates of cyanobacteria and in *N. oculata* were primarily esterified and then identified through GC/MS analysis. We obtained the same FA profile (Table 3) for each isolate grown at the two different culture conditions (data not shown). At least five fatty acids were detected in the extracts of the four isolates. The most abundant fatty acid detected in all the isolates was the saturated FA hexadecanoic acid (palmitic acid), reaching the maximum percentage in VRUC162 (65–87%). Other saturated FAs such as myristic acid (C14:0), stearic acid (C18:0) and arachidic acid (C20:0), were found. The monounsaturated FAs also present were myristoleic acid (C14:1), palmitoleic acid (C16:1), oleic

acid (C18:1), 11-eicosenoic acid and the short-chain 11-dodecenoic acid (C12:1). Only two polyunsaturated fatty acids (PUFAs), linoleic acid (C18:2) and linolenic acid (C18:3) were found in VRUC163 and VRUC164, and eicosapentaenoic acid (C20:5) was found only in *N. oculata*. The ratio from the unsaturated FAs to the saturated FAs obtained for the three isolates ranged between 0.17 (VRUC162) and 0.73 (VRUC163) and was lower than that of *N. oculata* (1.37).

Discussion

Three isolates of biofilm-forming cyanobacteria were studied for potential use in biodiesel production. These benthic isolates were taken from natural environments and identified by a polyphasic approach. All the isolates were filamentous and two of them, VRUC162 and VRUC163, were also heterocytous. Morphological observations of the two heterocytous isolates allowed us to identify them to the genus level. Both of the isolates had diacritical features, such as isopolar trichomes, a lack of branching, terminal cells were rounded or conical-rounded and the presence of akinetes, features which are attributable to the Nostocaceae family (Komárek and Anagnostidis 1989). On the basis of morphological characteristics, such as the position of heterocytes, the

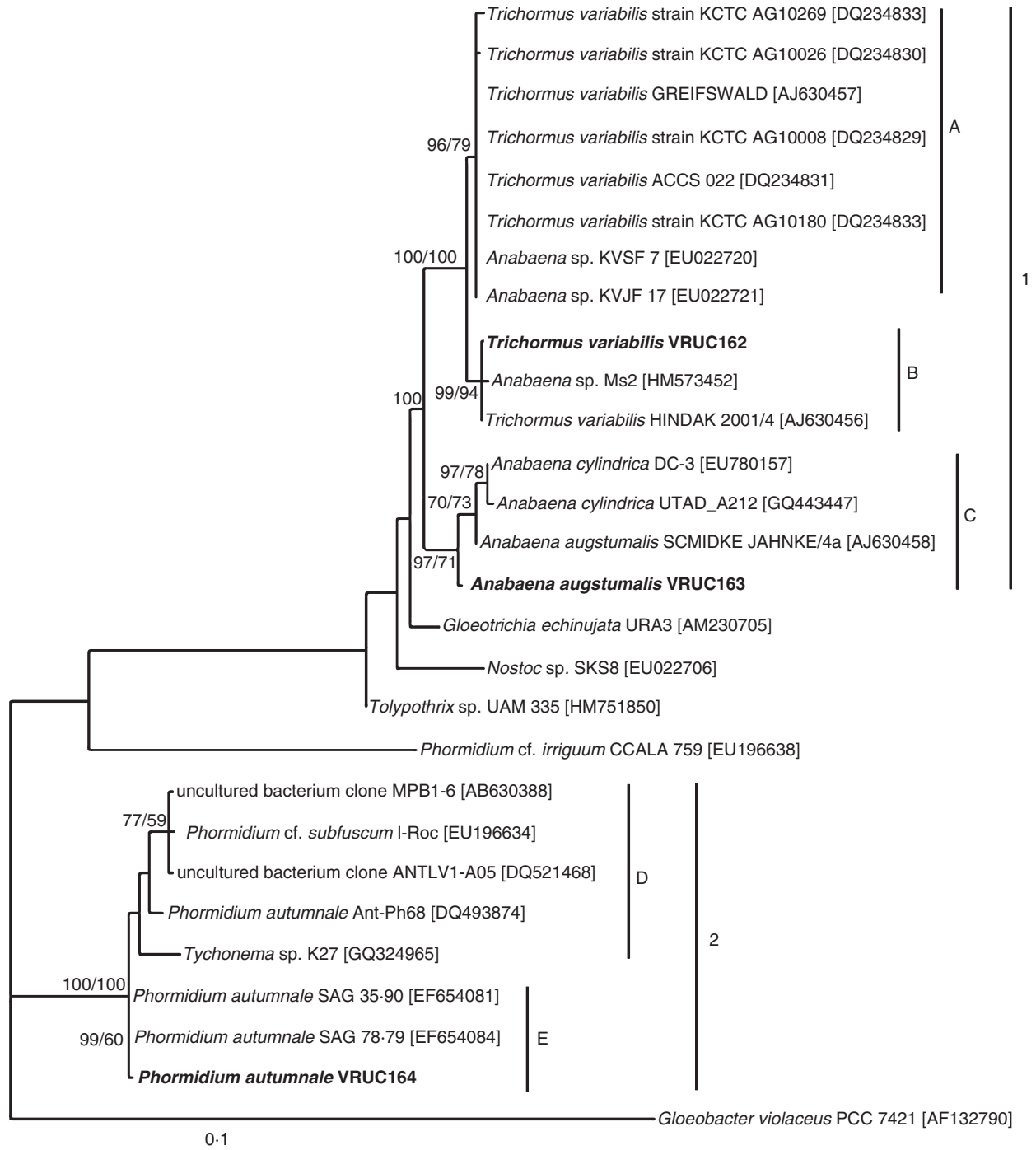


Figure 5 Phylogenetic relationships (based on maximum-likelihood method) of the three cyanobacterial isolates (in bold) and related cyanobacteria based on 16S rRNA gene sequences (923 bp). Numbers near nodes indicate bootstrap values >50% for parsimony/neighbour joining analyses. *Gloeobacter violaceus* PCC7421 is the outgroup taxa. GenBank accession numbers in square brackets.

morphology of filaments and the position of the akinetes, VRUC163 was identified as *Anabaena* sp. and the VRUC162 as *Trichormus* sp. according to the classification combining botanical and bacteriological approaches (Komárek and Anagnostidis 1989). The morphological

observations of the two isolates were in agreement with molecular data that identified them to species level as *A. augstumalis* Schmidle 1899, isolate VRUC163, and as *T. variabilis* Kützing ex Bornet et Flahault (Komárek and Anagnostidis 1989) isolate VRUC162. *A. augstumalis*

Table 2 Biomass productivity, production, lipid and biodiesel content of the three biofilm-forming cyanobacteria strains cultivated in two different culture system

Isolate	Productivity		Production		Lipid (biomass%)	
	Batch (g DW l ⁻¹ d ⁻¹ ±SD)	Incubator (g DW m ⁻² d ⁻¹ ±SD)	Batch (g DWd ⁻¹)	Incubator (g DWd ⁻¹)	Batch	Incubator
VRUC 162	0.040 ± 0	1.082 ± 0.010	0.020	1.298	12.1	24
VRUC 163	0.017 ± 0.001	3.3 ± 0.023	0.008	3.960	3.2	13.2
VRUC 164	0.055 ± 0.003	3.332 ± 0.021	0.027	8.750	2.4	18.4
<i>Nannochloropsis oculata</i> CCAP84971	0.004 ± 0.000	–	0.002	–	15.1	–

Table 3 Fatty acid methyl ester composition of cyanobacterial isolates compared to that obtained by *Nannochloropsis oculata*

Systematic name	Common name	Number of carbon atoms: double bond(s)	Family	Fatty acid content (% total)			
				VRUC162	VRUC163	VRUC164	<i>N. oculata</i> CCAP84971
11-Dodecenoic acid		C12:1	Monounsaturated	–	–	5.6	–
Tetradecanoic acid	Myristic acids	C14:0	Saturated	–	2.00	2.67	6.03
9-Tetradecenoic acid	Myristoleic acid	C14:1	Monounsaturated	–	–	13.76	–
Hexadecanoic acid	Palmitic acid	C16:0	Saturated	65.87	32.87	40.38	32.88
9-Hexadecenoic acid		C16:1	Monounsaturated	–	5.99	–	27.04
Octadecanoic acid	Stearic acid	C18:0	Saturated	6.29	6.77	2.34	–
9-Octadecenoic acid	Oleic acid	C18:1	Monounsaturated	6.23	6.5	–	17.38
9,12-Octadecadienoic acid	Linoleic acid	C18:2	Polyunsaturated	–	6.06	6.64	–
9,12,15-Octadecatrienoic acid	Linolenic acid	C18:3	Polyunsaturated	–	11.64	4.00	–
Eicosanoic acid	Arachidic acid	C20:0	Saturated	3.78	–	–	–
11-Eicosenoic acid	Gondoic acid	C20:1	Monounsaturated	6.4	–	–	–
Eicosapentanoic acid		C20:5	Polyunsaturated	–	–	–	9.03
Other FAs				11.43	28.17	24.61	7.64
Unsaturated FAs/saturated FAs*				0.17	0.73	0.66	1.37

*Ratio between the unsaturated FAs and the saturated FAs.

VRUC163 that was isolated from microphytobenthic communities growing in sediments of the Cabras Lagoon had 99.5% of sequence identity with a benthic isolate of *A. augustumalis*, SCMDKE JAHNKE/4a (Rajaniemi *et al.* 2005) isolated from sediments in the lake of Rostock (Germany). They also shared morphological features including the absence of gas vesicles and the presence of a diffuse mucilaginous sheath, which are characteristic of benthic *Anabaena* species (Rajaniemi *et al.* 2005). Isolate VRUC162 of *T. variabilis*, isolated from eroded soil in Valle dell'Esaro (Cosenza, Italy), had 99.8% of sequence identity with an isolate of *T. variabilis* HINDAK 2001/4 (Rajaniemi *et al.* 2005) isolated from soil in Dombay valley (Caucasus mountains) and had in common the shape of terminal cells and the akinetes. Dimensions of akinetes resulted to be different in the two isolates, but Rajaniemi

et al. (2005), working on 51 Nostocacean isolates, showed that length and width of akinetes resulted to be the most variable characteristics (Rajaniemi *et al.* 2005). The genus *Trichormus* was separated from the traditional genus *Anabaena* on the basis of akinete development, being apoheterocytic in *Trichormus* and paraheterocytic in *Anabaena* (Komárek and Anagnostidis 1989). According to this revision, some species, including *Anabaena variabilis*, were assigned to the genus *Trichormus* (Komárek and Anagnostidis 1989). This could explain why *T. variabilis* VRUC162 clustered with an *Anabaena* isolated from sediment samples from Lake Taihu, but for which no morphological information is available. Isolate VRUC164 was assigned to the genus *Phormidium* Kützing ex Gomont 1892 on the basis of morphological features such as a thin, partially diffluent sheath that causes filaments to

stick together in mat-like layers. The genus *Phormidium* includes a large number of species from freshwater and marine environments and belongs taxonomically to one of the most difficult cyanoprokaryotic genera (Palinska *et al.* 2011). Komárek and Anagnostidis (2005) divided it into eight nontaxonomic groups based on the differences in their apical morphologies. On the basis of the calyptrate apical cells, we recognized the isolate VRUC164 to belong to the *Phormidium* group VII. Moreover, on the basis of cell dimensions, granulation and ecological features, we assigned the isolate VRUC164 to the species *P. autumnale* (Agardhi) Trevisan ex Gomont 1892. This result was confirmed by the molecular analysis. In fact, isolate VRUC164 of *P. autumnale*, isolated from a wastewater treatment plant of Fiumicino Airport (Rome, Italy), had 99.9% sequence identity with two isolates of *P. autumnale* SAG 35.90 and 78.79 (Siegesmund *et al.* 2008), the first one isolated from the sides of the brook Bachlein in the valley Verzascatal (Switzerland), which is considered to be polluted (Palinska and Marquardt 2008).

All cyanobacterial isolates grown in batch culture reached the stationary stage of growth at around the 25th day, showing a slower growth than that of the eustigmatophyta *N. oculata* CCAP849/1. Moreover, all three filamentous cyanobacteria isolates grew in aggregates that were hardly separable and unequally distributed in the culture medium. However, the initial nonuniform colonization of substrates by each of the three isolates grown in the incubator prototype was clearly indicated by the gradual increase in biofilm light absorbance as directly registered by light sensors integrated in the system. Moreover, the active/exponential development of biofilms in the flow direction to the attainment of mature stage at around 29–30 days and covering uniformly the entire surface was indicated by absorbance values reaching around 5–10% (Zippel *et al.* 2007). These data showed similarities between the dynamics of the development of multi-species biofilms previously grown in the same incubator (Zippel and Neu 2005; Di Pippo *et al.* 2011), an important assumption when considering the possibility of using monospecific cultures for biotechnological applications. Another important prerequisite for the application of micro-organisms in biotechnology should also be the ability, through the optimization of culture conditions, to obtain sufficient biomass that can be used for different purposes for which they are grown. The biomass volumetric productivity of micro-organisms grown in batch culture is quite promising because the values were within the ranges of those obtained for marine and freshwater microalgae previously studied (Griffiths and Harrison 2009; Rodolfi *et al.* 2009; Mata *et al.* 2010). In particular, the noteworthy *P. autumnale* VRUC164 had biomass values comparable to those obtained for the cyanobacterium

Spirulina platensis (Mata *et al.* 2010), which is by far the most widely used cyanobacterium as a source of protein and vitamin and is usually grown in large outdoor ponds under controlled conditions. The areal productivity values of biofilm-forming cyanobacteria grown in the incubator were in the ranges obtained for other marine and freshwater microalgae grown in open ponds (Mata *et al.* 2010). Our isolates also showed values comparable to those obtained from the microalga *Chlorella* grown in an attached microalgal growth system (Johnson and Wen 2010).

The comparison between biomass values (g DWd^{-1}) of micro-organisms developed in the two different culture systems showed that the benthic isolates were able to produce higher amounts of biomass when grown in the flow-lane incubator, where they formed thick biofilms at the mature stage and could be easily removed by scraping them off the slides. This was mainly because of the presence of a laminar flow and to the higher irradiance and temperature tested in this flow-lane system. These parameters are known to be key factors in phototrophic biofilm development and biomass production. Light has been shown to affect biofilm growth, physiology and biomass production (Hill 1996; Stevenson 1996; Sabater *et al.* 2002) as it is the principle energy source and fuels the entire metabolism. Temperature affects the photosynthetic process and the biomass accrual. Even though the flow effect cannot be discussed here, we hypothesize that the presence of a laminar flow in the incubator system may have affected the biomass production. It is in fact known that flow rate influences colonization, biomass, structure and spatial distribution of benthic algae. (Stevenson 1996; Sabater *et al.* 2002; Battin *et al.* 2003). More experimentation is required to test different experimental conditions, however, these preliminary data from the flow-lane incubator system are interesting showing the potential for using biofilms as viable alternative for biomass production on the basis of harvesting alone. It is in fact known that harvesting of algal biomass is a significant operating cost and it was previously suggested to select algae with properties, such as large cell size, high specific gravity or autoflocculating species, which simplify and then reduce the cost of the harvest (Borowitzka 1997; Griffiths and Harrison 2009).

The biomass produced by each isolate grown in the two systems was then used to extract, quantify and characterize lipids. The lipid content of the three isolates grown in the incubator was similar or higher than that of *N. oculata* strain 849/1 grown in batch culture, especially for VRUC164 of *P. autumnale* and VRUC162 of *T. variabilis*. Moreover, lipid content values were similar to those obtained in other studies focused on microalgae (Rodolfi *et al.* 2009) and on cyanobacteria (Sharathchandra and

Rajashekhar 2011). These results are interesting, taking into account that the lipid production can be improved by manipulating the culture conditions. It is in fact known that nutrient starvation for a short period and/or CO₂ supplementation may increase the lipid yield (Rodolfi *et al.* 2009). Together with lipid content, biodiesel quality is also an important factor as it should meet standard criteria, according to the European or American standards (UNE-EN 14214 and US ASTM D6751) (Chisti 2007; Quintana *et al.* 2011). In fact, not all oils result to be satisfactory for making biodiesel because oxidation stability and cold-flow properties are important parameters that have to be considered (Chisti 2007; Quintana *et al.* 2011). Because these properties are closely related to the fatty acid composition and are determined by their degree of saturation, investigation of the lipid profile is important when selecting microalgal or cyanobacterial species for biodiesel production. The fatty acid profiles obtained for the three cyanobacterial isolates were in agreement with published data (Lang *et al.* 2011; Sharathchandra and Rajashekhar 2011). The main fatty acid detected in the studied isolates was palmitic acid (C16:0), present in all cyanobacteria and in many other microalgae studied to date (Lang *et al.* 2011; Quintana *et al.* 2011; Sharathchandra and Rajashekhar 2011). Fatty acids and fatty acid methyl esters (FAMES) with four and more double bonds are susceptible to oxidation during storage, and this reduces their acceptability for use in biodiesel. The high proportion of saturated fatty acids found in the three cyanobacterial isolates (unsaturated/saturated FAs < 0.73) along with the prevalence of monounsaturated FAs with respect to the PUFAs is considered optimal from a fuel quality standpoint. Fuel polymerization during combustion should be substantially less than what would occur with polyunsaturated fatty acid-derived fuel as those from Plantae and many other algae (Demirbas and Demirbas 2011). Moreover, the obtained lipid profiles showed low percentages of saturated long-chain fatty acids, and it is believed that lipids of this type that are present in high proportions will result in biodiesel with poor cold-flow properties (Quintana *et al.* 2011).

This study showed that different culture systems affected cyanobacterial biomass and lipid production but did not influence the fatty acid profile. Comparing the different culture systems, the three cyanobacterial species showed higher biomass and lipid production when grown in the flow-lane incubator. Benthic cyanobacteria are able to produce fatty acids suitable for a high-quality third-generation biodiesel production and, even if more experiments need to be performed on other different cyanobacterial species, our results show that feasible biomass and lipid production from cyanobacteria could be achieved using new and innovative techniques as reported

here. These processes can be further optimized by manipulating the experimental conditions (i.e. irradiance, temperature and flow rate). Biodiesel from cyanobacteria seems to be particularly promising considering the possibility of combining cyanobacterial growth with bioremediation as might be the case for the isolate of *P. autumnale* VRUC164 from an Italian wastewater treatment (this study) or as might the case with other cyanobacteria (Quintana *et al.* 2011; Christenson and Sims 2012). Lastly, it could be possible to obtain other valuable by-products such as exopolysaccharides that are mostly produced by cyanobacteria (De Philippis *et al.* 2011), high protein feed for livestock, and residual biomass can be used for thermal conversion. Although algae are highly suitability as a source of biodiesel, the co-production of multiple products from microalgae remains a challenge (Quintana *et al.* 2011).

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References

- Bannon, C.D., Breen, G.J., Craske, J.D., TrongHai, N., Harper, N.L. and O'Rourke, K.L. (1982) Analysis of fatty acid methyl esters with high accuracy and reliability. III. Literature review of and investigations into the development of rapid procedures for the methoxide-catalysed methanolysis of fats and oils. *J Chrom* **247**, 71–89.
- Battin, T.J., Kaplan, L.A., Newbold, J.D. and Hansen, C.M.E. (2003) Contributions of microbial biofilms developing under various external factors. *Eur J Phycol* **40**, 1–8.
- Bellezza, S., De Philippis, R., Paradossi, G. and Albertano, P. (2003) *Leptolyngbya* sp. strains from Roman hypogea: cytochemical and physico-chemical characterisation of exopolysaccharides. *J Appl Phycol* **15**, 193–200.
- Borowitzka, M.A. (1997) Microalgae for aquaculture: opportunities and constraints. *J Appl Phycol* **9**, 393–401.
- Bruno, L., Billi, D., Bellezza, S. and Albertano, P. (2009) Cytomorphological and genetic characterization of

- troglobitic *Leptolyngbya* strains isolated from Roman hypogea. *Appl Environ Microbiol* **75**, 608–617.
- Castenholz, R.W. (2001) Phylum BX. Cyanobacteria. Oxygenic Photosynthetic Bacteria. In *Bergey's Manual of Systematic Bacteriology*, 2nd edn, Vol 1 ed. Boone, D.R., Castenholz, R. W. and Garrity, G.M. pp. 473–487. New York, NY: Springer.
- Chisti, Y. (2007) Biodiesel from microalgae. *Biotechnol Adv* **25**, 294–306.
- Choi, G.G., Kim, B.H., Ahn, C.Y. and Oh, H.M. (2011) Effect of nitrogen limitation on oleic acid biosynthesis in *Botryococcus braunii*. *J Appl Phycol* **23**, 1031–1037.
- Christenson, L.B. and Sims, R.C. (2012) Rotating algal biofilm reactor and spool harvester for wastewater treatment with biofuels by-products. *Biotechnol Bioeng* **109**, 1674–1684.
- De Philippis, R., Colica, G. and Micheletti, E. (2011) Exopolysaccharide-producing cyanobacteria in heavy metal removal from water: molecular basis and practical applicability of the biosorption process. *Appl Microbiol Biotechnol* **92**, 697–708.
- Demirbas, A. (2007) Importance of biodiesel as transportation fuel. *Energy Policy* **35**, 4661–4670.
- Demirbas, A. and Demirbas, M.F. (2011) Importance of algae oil as a source of biodiesel. *Energy Convers Manag* **52**, 163–170.
- Di Pippo, F., Ellwood, N.T.W., Guzzon, A., Siliato, L., Micheletti, E., De Philippis, R. and Albertano, P.B. (2011) Effect of light and temperature on biomass, photosynthesis and capsular polysaccharides in cultured phototrophic biofilms. *J Appl Phycol* **24**, 211–220.
- Esson, D., Wood, S.A. and Packer, M. (2011) Harnessing the self-harvesting capability of benthic cyanobacteria for use in benthic photobioreactors. *AMB Express* **1**, 19.
- Griffiths, J.M. and Harrison, T.L. (2009) Lipid productivity as a key characteristic for choosing algal species for biodiesel production. *J Appl Phycol* **21**, 493–507.
- Guillard, R.R.L. and Ryther, J.H. (1962) Studies of marine planktonic diatoms. *I. Cyclotella nana* Husted and *Detonula confervacea* Cleve. *Can J Microbiol* **8**, 229–239.
- Guzzon, A., Congestri, R. and Albertano, P. (2005) Light-induced changes in photosynthesis and structure of cyanobacterial cultured biofilms from an Italian wastewater treatment plant. *Arch Hydrobiol Suppl Algol Stud* **117**, 223–238.
- Hill, W.R. (1996) Effects of light. In *Algal Ecology – Freshwater Benthic Ecosystems* ed. Stevenson, R.J., Bothwell, M.L. and Lowe, R.L. pp. 121–148. San Diego, CA, USA: Academic Press.
- Johnson, M.J. and Wen, Z. (2010) Development of an attached microalgal growth system for biofuel production. *Appl Microbiol Biotechnol* **85**, 525–534.
- Kochert, G. (1978) Quantitation of the macromolecular components of microalgae. In *Handbook of Phycological Methods: Physiological and Biochemical Methods* ed. Hellebust, J.A. and Craigie, J.S. pp 189–195. Cambridge: Cambridge University Press.
- Komárek, J. (2011) Introduction to the 18th IAC Symposium in České Budějovice 2010, Czech Republic. Some current problems of modern cyanobacterial taxonomy. *Fottea* **11**, 1–7.
- Komárek, J. and Anagnostidis, K. (1989) Modern approach to the classification system of cyanophytes. 4 –Nostocales. *Arch Hydrobiol Suppl Algol Stud* **43**, 157–226.
- Komárek, J. and Anagnostidis, K. (2005) Süßwasserflora von Mitteleuropa. In *Cyanoprokaryota 2 (Oscillatoriales)* ed. Büdel, B., Krienitz, L., Gärtner, G. and Schagerl, M. pp. 7–107. München, Germany: Elsevier GmbH.
- Kumar, A., Ergas, S., Yuan, X., Sahu, A., Zhang, Q., Dewulf, J., Malcata, F.X. and Langenhove, H.V. (2010) Enhanced CO₂ fixation and biofuel production via microalgae: recent developments and future directions. *Trends Biotechnol* **28**, 371–380.
- Lang, I., Hodac, L., Friedl, T. and Feussner, I. (2011) Fatty acid profiles and their distribution patterns in microalgae: a comprehensive analysis of more than 2000 strains from the SAG culture collection. *BMC Plant Biol* **11**, 124.
- Lu, Y., Zhai, Y., Liu, M. and Wu, Q. (2010) Biodiesel production from algal oil using cassava (*Manihot esculenta* Crantz) as feedstock. *J Appl Phycol* **22**, 573–578.
- Machado, I.M.P. and Atsumi, S. (2012) Cyanobacterial biofuel production. *J Biotechnol*, in press, doi:10.1016/j.jbiotec.2012.03.005
- Mata, T.M., Martins, A.A. and Caetano, N.S. (2010) Microalgae for biodiesel production and other applications: a review. *Renewable Sustainable Energy Rev* **14**, 217–232.
- Nübel, U., Garcia-Pichel, F. and Muyzer, G. (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. *Appl Environ Microbiol* **63**, 3327–3333.
- Palinska, K.A. and Marquardt, J. (2008) Genotypic and phenotypic analysis of strains assigned to the widespread cyanobacterial morphospecies *Phormidium autumnale* (Oscillatoriales). *Arch Microbiol* **189**, 325–335.
- Palinska, K.A., Deventer, B., Hariri, K. and Lotocka, M. (2011) Taxonomic study on *Phormidium*-group (cyanobacteria) based on morphology, pigments, RAPD molecular markers and RFLP analysis of the 16S rRNA gene fragment. *Fottea* **11**, 41–55.
- Popovich, C.A., Damiani, C., Constenla, D. and Leonardi, P.I. (2010) Lipid quality of the diatoms *Skeletonemacostatum* and *Naviculagregaria* from the South Atlantic Coast (Argentina): evaluation of its suitability as biodiesel feedstock. *J Appl Phycol* **24**, 1–10.
- Posada, D. (2008) jModelTest: phylogenetic model averaging. *Mol Biol Evol* **25**, 1253–1256.
- Quintana, N., Van der Kooy, F., Van de Rhee, M.D., Voshol, G.P. and Verpoorte, R. (2011) Renewable energy from Cyanobacteria: energy production optimization by metabolic pathway engineering. *Appl Microbiol Biotechnol* **91**, 471–490.

- Rajaniemi, P., Hrouzek, P., Kastovska, K., Willame, R., Rantala, A., Hoffmann, L., Komárek, J. and Sivonen, K. (2005) Phylogenetic and morphological evaluation of the genera *Anabaena*, *Aphanizomenon*, *Trichormus* and *Nostoc* (Nostocales, Cyanobacteria). *Int J Syst Evol Microbiol* **55**, 11–26.
- Ramachandra, T.V., Mahapatra, D.M. and Karthick, B. (2009) Milking diatoms for sustainable energy: biochemical engineering versus gasoline-secreting diatom solar panels. *Ind Eng Chem* **48**, 8769–8788.
- Rodolfi, L., ChiniZittelli, G., Bassi, N., Padovani, G., Biondi, N., Bonini, G. and Tredici, M.R. (2009) Microalgae for oil: strain selection, induction of lipid synthesis and outdoor mass cultivation in a low-cost photobioreactor. *Biotechnol Bioeng* **102**, 100–112.
- Sabater, S., Guasch, H., Romani, A. and Munõz, I. (2002) The effect of biological factors on the efficiency of river biofilms in improving water quality. *Hydrobiologia* **469**, 149–156.
- Sharathchandra, K. and Rajashekhar, M. (2011) Total lipid and fatty acid composition in some freshwater cyanobacteria. *J Algal Biomass Util* **2**, 83–97.
- Siegesmund, M.A., Johansen, J.R., Karsten, U. and Friedl, T. (2008) *Coleofasciculus* Gen Nov (Cyanobacteria): morphological and molecular criteria for revision of the Genus *Microcoleus* Gomont. *J Phycol* **44**, 1572–1585.
- Singh, A., Singh Nigam, P. and Murphy, J.D. (2011) Renewable fuels from algae: an answer to debatable land based fuels. *Bioresour Technol* **102**, 10–16.
- Stephens, E., Ross, I.L., King, Z., Mussgnug, J.H., Posten, C., Borowitzka, M.A. and Hankamer, B. (2010) An economic and technical evaluation of microalgal biofuels. *Nat Biotechnol* **28**, 126–128.
- Stevenson, R.J. (1996) An introduction to algal ecology in freshwater benthic habitats. In *Algal Ecology-Freshwater Benthic Ecosystems* ed. Stevenson, R.J., Bothwell, M.L. and Lowe, R.L. pp 321–340. San Diego, CA, USA: Academic Press.
- Swofford, D.L. (2002) *PAUP_Phylogenetic Analysis Using Parsimony (and Other Methods)*. Version 4. Sunderland, MA: Sinauer Associates
- Takagi, M. and Karseno Yoshida, T. (2006) Effect of salt concentration on intracellular accumulation of lipids and triacylglyceride in marine microalgae *Dunaliella* Cells. *J Biosci Bioeng* **101**, 223–226.
- Vieira Costa, J.A. and Greque de Morais, M. (2011) The role of biochemical engineering in the production of biofuels from microalgae. *Bioresour Technol* **102**, 2–9.
- Wijffels, R.H. and Barbosa, M.J. (2010) An outlook on microalgal biofuels. *Science* **329**, 796–799.
- Wiyarno, B., Yunus, R.M. and Mel, M. (2011) Extraction of algae oil from *Nannochloropsis* sp.: a study of soxhlet and ultrasonic-assisted extractions. *J Appl Sci* **11**, 3607–3612.
- Zippel, B. and Neu, T.R. (2005) Growth and structure of phototrophic biofilms under controlled light conditions. *Water Sci Technol* **52**, 203–209.
- Zippel, B., Rijstenbil, J. and Neu, T.R. (2007) A flow-lane incubator for studying freshwater and marine phototrophic biofilms. *J Microbiol Methods* **70**, 336–345.