Algal Research 33 (2018) 358–368

Contents lists available at ScienceDirect

Algal Research

journal homepage: www.elsevier.com/locate/algal

Description of a new species of soil algae, *Parietochloris grandis* sp. nov., and study of its fatty acid profiles under different culturing conditions

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ARTICLE INFO

Keywords:
Trebouxiophyceae
Taxonomy
New species
Fatty acid profile
Nutrient starvation

ABSTRACT

The new species *Parietochloris grandis* sp. nov. is described from forest soil in the Dnipropetrovsk region, Ukraine. The description is based on morphological features and the phylogenetic analysis of partial SSU rDNA and *rbcL* genes. Phylogenetic analysis places *P. grandis* in the *Parietochloris* clade, within the family Trebouxiophyceae. The novel strain formed a strongly supported monophyletic lineage with the type species of *Parietochloris*, *P. alveolaris*. *P. grandis* differed from other species in the *Parietochloris* clade by the size and form of vegetative cells and the large number of zoospores in zoosporangia. A number of experiments with different phosphates and nitrates concentrations were conducted to evaluate changes in fatty acid profile and biomass. The dominant fatty acids during cultivation on standard BG-11 medium, as well as with the phosphates concentrations ranged from 0.22 to 2 mM, were linoleic acid (24–25%), palmitic acid (12–14%), linolenic acid (9–12%), and oleic acid (7–11%). The content of arachidonic acid and eicosapentaenoic acid ranged from 3.5 to 4.5% and 0.7% to 0.8%, respectively. The fatty acid profile and total fatty acids varied significantly under different nutrient deficiency. The greatest variation was found for oleic acid (9–46%) and linolenic acid (2–13%). The percentage of arachidonic acid was the highest with a standard nitrates concentration in the medium (5%) and the lowest in the absence of phosphates and nitrates (1.3–1.5%), but the absolute content in dry biomass was similar in all variants of the experiment (6.5–9.3 mg g^{-1} dry weight). The absence of nitrogen and both nitrogen and phosphorus led to a 3–fold increase in TFA in comparison with the control. Thus, this strain can be considered in biotechnological application as a potential producer of the essential linoleic acid or oleic acid.

1. Introduction

Parietochloris Shin Watanabe et Floyd is one of many poorly known genera of green algae (Chlorophyta). It was separated from the genus *Neochloris* Starr with its included species having mononuclear cells and naked zoospores with counter-clockwise absolute orientation of the flagellar basal bodies [1]. *Parietochloris alveolaris* (H.C. Bold) Shin Watanabe et Floyd in Deason et al. (basionym: *Neochloris alveolaris* H.C. Bold), the type species of the genus, was isolated from a spring-fed calcareous pool (USA, Tennessee) [2]. From an ecological point of view, species in *Parietochloris* are found in terrestrial environments, such as soils. They have also been identified from habitat types such as steppe, meadow, forest and floodplain at different climatic zones: equatorial, subequatorial, tropical, subtropical and temperate zone [3–5]. According to modern taxonomic concepts, the genus belongs to the algal class Trebouxiophyceae but does not form a monophyletic group [6,7]. The numerous phylogenetic studies of the coccoid green algae now available confirm the presence of hidden diversity within the group.

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https://doi.org/10.1016/j.algal.2018.06.008
Received 10 November 2017; Received in revised form 9 June 2018; Accepted 9 June 2018

This has been clearly demonstrated in the classes Chlorophyceae and Trebouxiophyceae with numerous generic level revisions, which include the separation, revision and description of new taxa [6,8–12]. Trebouxiophyceae is characterized by the great variability in the morphological structure of the thallus (unicellular, filamentous, multicellular and colonial representatives) and by the wide range of habitats it occupies (aquatic, terrestrial and symbiotic) [7]. Currently, the phylogeny of Trebouxiophyceae includes a large number of lineages, most of which include several genera (for example, Chlorellales, Trebouxiales, Microthamniales, the *Prasiola* and *Watanabea* clades), but there are also lineages represented by a single genus or species (*Xylochloris* Neustupa, Eliás et Skaloud, *Leptosira* Borzì, *Lobosphaera* Reisigl) [7]. Within Trebouxiophyceae, several genera are recognized as polyphyletic, such as *Chlorella* Beijerinck [Beijerinck] [13] and *Parietochloris* [6,7].

Green algae continue to attract attention because of their biotechnological potential in the production of biofuels or high-value biological products (e.g. fatty acids) [14,15]. A number of studies have shown that the Trebouxiophycean species *Lobosphaera incisa* (Reisigl) Karsten (previously known as *Parietochloris incisa* (Reisigl) Shin Watanabe and *Myrmecia incisa* Reisigl) is one of the most effective producers of arachidonic acid among algae [16,17]. Polyunsaturated fatty acids are important for the adaptation of algae to stress conditions [18]. Artificial stressing of algae often leads to an increase in the content of arachidonic acid in *Lobosphaera incisa* [19,20]. *Parietochloris* includes strains known as producers of various lipids [21].

During a study of the soils of the Samara forest (Dnipropetrovsk region, Ukraine), we isolated a strain, which matched to the description of the genus *Parietochloris*, but according to a number of its morphological characteristics, was not identical to any of the already described species and we therefore conducted a molecular study to ascertain the phylogenetic position of our isolate. The main purpose of this work was to describe this new species of *Parietochloris*, which formed a monophyletic lineage with other members of the *Parietochloris* clade. In addition, we performed experiments to find the optimal growth conditions and analyzed the composition of fatty acids and its variability, depending on these growth conditions.

2. Materials and methods

2.1. Isolation and cultivation

The strain CAMU MZ–Ch5 was isolated from soil of the artificial pine plantation within the territory of the Samara forest (coordinates N 48°38′10.15″ E 35°40′41.47″). The Samara forest is one of the most southerly of natural forests in the steppe zone of Ukraine, which grows in the valley of the Samara river (Dnipropetrovsk region) within the herb-fescue-feather grass steppes [22]. The climate of the area is arid with an annual amount of precipitation not exceeding 425–450 mm. The monoclonal strain CAMU MZ–Ch5 was isolated by micropipeting individual cells under an inverted Zeiss Axio Vert microscope A1. The culture was maintained in a liquid WC medium [23] under illumination with 12 h light/12 h dark cycle. Light microscopy and photography were carried out using a Zeiss Scope microscope A1, equipped with an oil immersion objective (×100/na.a 1.4, DIC). Cells were stained with Lugol solution for the presence of starch granules, with 0.1% methylene blue solution and 1% ink solution for the determination of the structure of mucus. The time of observation of the strain was from 24 h to 12 months. The culture was deposited in the Collection of Algae at Bohdan Khmelnytskyi Melitopol State Pedagogical University CAMU and in the culture collection of Molecular systematics of aquatic plants laboratory at Institute of Plant Physiology Russian Academy of Sciences. The culture was maintained on the BG–11 medium [24,25]. 1 L of BG–11 medium contained 17.6 mM $NaNO_3$; 0.22 mM K_2HPO_4; 0.18 mM Na_2CO_3; 0.2 mM $CaCl_2 \times 2H_2O$; 0.03 mM Citric Acid; 0.02 mM Ferric Ammonium Citrate, 0.003 mM Na_2EDTA, 0.03 mM $MgSO_4 \times 7H_2O$ and trace elements solution ($4.63 * 10^{-5}$ mM H_3BO_3; $9.15 * 10^{-6}$ mM $MnCl_2 \times 4H_2O$; $7.65 * 10^{-7}$ mM $ZnSO_4 \times 7H_2O$; $1.61 * 10^{-6}$ mM $Na_2MoO_4 \times 2H_2O$; $3.16 * 10^{-7}$ mM $CuSO_4 \times 5H_2O$; $1.70 * 10^{-7}$ mM $Co(NO_3)_2 \times 6H_2O$).

Two series of experiments were conducted. First, the effect of different phosphorus concentration on the total biomass and fatty acid profile of the alga was evaluated. Four different final concentrations of K_2HPO_4 were used in standard BG–11 medium: 0.22 mM, 0.5 mM, 1 mM, 2 mM. In the second experiment five types of cultivation conditions were used for analysis of changes in fatty acids profile at the different nitrogen and phosphorus concentrations: BG–11 medium with 1 mM of K_2HPO_4 and 17.6 mM $NaNO_3$ (“control”), BG–11 medium with 1 mM of K_2HPO_4 and 5.9 mM $NaNO_3$ (“Low N”), BG–11 medium with 1 mM of K_2HPO_4 and without $NaNO_3$ (“–N”), BG–11 medium with 17.6 mM $NaNO_3$ without K_2HPO_4 (“–P”), BG–11 medium without $NaNO_3$ and K_2HPO_4 (“–N, –P”).

Microalgae were cultured in laboratory incubator shaker Multitron (Infors HT) at a temperature of 24 °C, with constant shaking at 150 rpm and 5% CO_2 in the air supply for 14 days. Light intensity was 120 μmol photons $m^{-2} s^{-1}$ with 16:8 h light/dark photoperiod. During the experiments, the cultures were cultivated in 125 mL flasks with 40 mL of medium and 10 mL of inoculum.

Nitrate and phosphate concentrations were measured by Nitrate UV screening method and Permachem PhosVer 3 Phosphate Reagent using UV–Vis spectrophotometer DR 6000 (HACH–Lange). To obtain dry mass data, the biomass was harvested at the 14th day of cultivation, centrifuged (2900 ×*g*, 15 min) and lyophilized using freeze-dryer FreeZone 2,5 L (Labconco). Dry weight was recorded and biomass was stored in fridge at −70 °C until extraction.

2.2. Molecular analysis

The DNA of the investigated strain CAMU MZ–Ch5 was extracted using the InstaGene™ Matrix kit according to the manufacturer's protocol. Amplification of a fragment of the 18S rDNA gene with a length of 1661 bp. was performed with a pair of primers 18S–FA2 (5′–ACC TGG TTG ATC CTG CCA GTA–3′) and 18S–RB2 (5′–GAT CCT TCT GCA GGT TCA CCT ACG–3′) [26]. Amplification conditions: initial denaturation – 5 min at 95 °C, followed by 32 cycles (denaturation at 94 °C – 30 s, annealing of primers – 40 s at 64 °C, elongation – 1.5 min at 72 °C), final elongation – 5 min at 72 °C. Amplification of a fragment of the chloroplast gene *rbcL* with a length of 1071 bp. was performed with a pair of primers *rbcL*–F9 (5′–CGT GAC AAA CTA AAC AAA TAT GG–3′) and *rbcL*–R8 (5′–AAG ATT TCA ACT AAA GCT GGC A–3′) [26]. Amplification conditions: initial denaturation – 5 min at 95 °C, followed by 45 cycles (denaturation at 94 °C – 30 s, annealing of primers – 40 s at 60 °C, elongation – 1.2 min at 72 °C), final elongation – 5 min at 72 °C. Amplification of the site of ITS1–5.8S–ITS2 rDNA with a length of 680 bp. was performed with a pair of primers ITS1 (5′–TCC GTA GGT GAA CCT GCG G–3′) and ITS4 (5′–TCC TCC GCT TAT TGA TAT GC–3′) [27]. Amplification conditions: initial denaturation – 5 min at 95 °C, followed by 35 cycles (denaturation at 94 °C – 30 s, annealing of primers – 30 s at 60 °C, elongation – 1 min at 72 °C), final elongation – 5 min at 72 °C.

The PCR products were visualized by horizontal agarose gel electrophoresis (1%) and stained with SYBR® Safe (Life Technologies, USA). Purification of PCR products was carried out with a mixture of FastAP, 10 × FastAP Buffer, Exonuclease I (Thermo Fisher Scientific, USA) and water. Sequencing was performed using the Genetic Analyzer 3500 sequencer (Applied Biosystems, USA). Internal primers were also used: for the 18S rDNA gene, 18S–MZ–F (5′–GGC TTC ACT GTC TGG GAC TC–3′) and 18S–MZ–R (5′–ATC AAC CTG ACA AGG CAA CC–3′) and the primer 5′–ATG GTT CCA CAA ACA GAA AC–3′ [28] for *rbcL*.

Editing and assembling of the consensus sequences were carried out by comparing the direct and reverse chromatograms using the Ridom TraceEdit program (ver 1.1.0) and Mega6 [9,29]. In addition to these