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# THE ALGA *TRACHYDISCUS MINUTUS* (*PSEUDOSTAURASTRUM MINUTUM*): GROWTH AND COMPOSITION

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**Summary.** The growth and composition of xanthophycean *Trachydiscus minutus* (*Pseudostaurastrum minutum*) were studied. Algal density 3.5 g.dm$^{-3}$ of dry weight was achieved in laboratory, and high lipid content (26 % of dry weight) due to the abundance of triacylglycerols was found. The lipids of alga were characterized with about 26 % myristic and 30 % eicosapentaenoic acids. The first lipid is used in cosmetics, the second as a nutrient additive. Sterols were quantified and GC-MS identified, too.
Composition of *T. minutus* was: proteins (43 %), carbohydrates (25 %), and chlorophyll *a* (0.6 %). The cultivation was successfully run in pilot plant scale (330 dm$^3$, 2 m$^2$, in greenhouse), for the first time ever. Growth optimum about 26°C, resistance to contamination, easy centrifugation and drying on foil in a greenhouse, by sunlight, are the technological advantages.

***Key words:*** algae; fatty acids; pilot plant cultivation; PUFAs; sterols; *Trachydiscus minutus*.

***Abbreviations:*** Chl – chlorophyll; EPA – eicosapentaenoic acid; GC – gas chromatography; GC-MS – gas chromatography-mass spectrometry; MGDG – monogalactosyldiacylglycerol; PAR – photosynthetically active radiation; PG – phosphatidylglycerol; PUFAs – polyunsaturated fatty acids; RT – retention time; SQDG – sulphoquinovosyldiacylglycerol; TAG – triacylglycerols; TLC – thin layer chromatography.

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## INTRODUCTION

Domesticated cyanobacteria and microalgae, obviously possessing high potential of valuable properties, remain limited to several genus. Production of protein rich and cheap food, being the primary goal of mass cultivation, has been enlarged to the production of nutrient additives and valuable compounds, e.g. astaxanthin in *Haematococcus*, carotenes in *Dunaliella* (Borowitzka and Borowitzka, 1988), etc. Special attention has been paid to algal oils. The first experiments were done during World War II using Bacillariophyceae (Harder and Witsch, 1942a, b; Retovsky, 1946). The importance of quality oils in human diet, especially PUFAs is very well known (Borowitzka, 1988). This is also substantial in feeding fishes and zooplankton in aquacultures (Borowitzka, 1997; Brown, 2002; Krienitz and Wirth, 2006) and they support searching for new prospective producers of healthy oils rich of PUFAs. Especially EPA is recognized as a valuable nutrient additive improving many diseases like menthal problems, hearth troubles, immune system etc. (Calder, 2006). As producers of PUFAs there were proposed Bacillariophyceae (Kyle and Glaudule, 1993), marine diatom *Phaeodactylum tricornutum* (Ibanez et al., 1998; Patil et al., 2007), *Monodus subterraneus* (Cohen, 1994), *Porphyridium cruentum* (Cohen and Cohen, 1991), *Pavlova lutheri* (Meireles, 2003), *Spirulina* (Fournadzhieva et al., 2002a, b).

Prospective of exploitation of algae as biofuel is changing with oscillations of petroleum costs, olitics of self-reliance and security etc. The programme has been frozen at present (Pienkos and Darzins, 2009), nevertheless, in combination with treatment of waste waters by e.g. *Botryococcus* could become promising again (Shen et al., 2008; Li et al., 2008). Also necessary $CO_2$ can be supplied as flue gas from municipal waste incinerator, free of charge (Douskova et al., 2008). Some algae, as *Chlorella protothecoides* can be cultivated heterotrophically e.g. in sugar cane juice and oil content can reach 53 % (Cheng et al., 2009). Another improvements and progress can be expected from transgenics (Gressel, 2008). The alga *Trachydiscus minutus* Bourrelly (Ettl, 1964), Xanthophyceae, Heterokonta, found in a small pond in France, was described under the name *Pseudostaurastrum minutum* (Bourelly, 1951). The alga is wide-spread in the temperate zone, usually present in a low concentration amid the whole mass of phytoplankton (Amarasinghe et al., 1997; Zalocar et al., 1998). Probably *T. minutus* has often been mistaken for the ubiquitous species *Chlorella* or another green spheres. Routine methods normally applied in chemotaxonomy help to avoid such errors (Petkov and Garcia, 2007). The alga we deal with was isolated from an open shallow cooling pool of Temelin Nuclear Power Station, Czech Republic. The locality is characterised by a relatively constant temperature 18 - 22 °C year-round, application of biocides reducing bacteria and algae, and $ZnCl_2$ as an anti-corrosion substance. In this locality *T. minutus* was dominating in 2005-2006, but it is only scarce at present. There are no data concerning its physiology and biochemical composition with respect to biotechnology. In this paper we report the results of our study on the growth and general composition of the alga with an emphasis on lipids.

## MATERIALS AND METHODS

### Cultivation of the alga.

The alga *Trachydiscus minutus* Bourrelly is deposited in the algal collection CCALA Trebon, as strain Lukavsky and Pribyl 2005/1. The alga was grown in laboratory as mono-algal culture. Illumination of 35 $W.m^{-2}$ PAR, 24 h continuous light from luminescent tubes, temperature 32°C, bubbling with 3 $cm^3.s^{-1}$ air enriched with 0.5 % $CO_2$, and pH 7.3 - 7.8 were maintained. Cultivation vessels were big test tubes with a volume of 200 $cm^3$. The alga was cultivated in nutrition medium Z after Zehnder in Staub (1961) in normal concentration, as well as three and six times concentrated, to density 3.5 $g.dm^{-3}$ dry weight and centrifuged 20 min at 3000 x *g*, every 24 h, washed and dried at 105°C to constant weight. Pilot plant cultivation was tested in an orbiculate pond which was used for growing up inoculum of *Spirulina* (Fournadzhieva et al., 2002a, b; Arvanities et al., 2004). It had an area of 2 $m^2$, volume of 330 $dm^3$, continuous stirring by paddle wheel, bubbling with $CO_2$, in greenhouse. At noon, natural light intensity grew up to 240 - 260 $W.m^{-2}$ PAR, temperature of suspension was max 26°C.

### Chemical analyses.

Fresh algal biomass was extracted with chloroform/methanol 2:1, 3 times for 0.5 h under reflux. The extract was evaporated *in vacuo* and the residue was re-extracted with chloroform to obtain the total lipids, which were estimated gravimetrically. The main lipid classes were separated on preparative TLC as follows: TAG in hexane/ diethyl ether 3:1; glyco-, sulpho- and phospholipids in chloroform/methanol/water 65:25:4. Reference substances were used. The amount of triacylglycerols was determined gravimetrically as previously described elsewhere (Petkov and Ramazanov, 2003). Parts of the lipid samples were converted to fatty acid methyl esters by heating in methanol containing 6 % anhydrous HCl at 60°C for 1.5 h. The fatty acid methyl esters were extracted with hexane and purified by TLC on silica gel with hexane/diethyl ether 10:1. Solvents and TLC plates from Merck (Germany) were used. Fatty acids were analyzed using GC on a Perkin-Elmer instrument as previously described (Iliev and Petkov, 2006). Another portion of the lipids was saponified with 5% KOH in 96% ethanol for 2 h under reflux. Unsaponifiable matter was extracted with diethyl ether. Sterols were separated on TLC with hexane/diethyl ether 1:1, and identified GC-MS. GC-MS analysis was performed on a Hewlett-Packard gas chromatograph 6890 equipped with a Hewlett-Packard MS 5973 detector. An HP5-MS capillary column was used (30m x 0.25 mm, 0.25μm film thickness). The temperature was programmed from 40°C to 280°C at a rate 6°C.$min^{-1}$. Helium was used as a carrier gas at15 $mm^3.s^{-1}$. The ion source was set at 250°C and the ionization voltage was 70 eV. Identification was achieved using computer searches on an NIST98 MS data library. Total protein was analyzed according to Lowry's method, chlorophyll and carotenoids were determined spectrophotometrically after methanol extraction on Multiskan Spectrum REW instrument (Thermo electron Co), and calculated according to McKinney (1941). Analytical grade quality chemicals (Merck, D), distilled water and all-glass system in every step of the procedures were used.

## RESULTS

### Laboratory cultivation.

The alga has yielded to intensive cultivation in laboratory relatively easily. Its growth was rather satisfactory, keeping in mind its poor presence in wild nature: about 0.5 % of phytoplankton according to our observations and literature. In the cooling pool of the Nuclear Power Station Temelín it was dominating for 2 years. Three different concentrations of the nutrition medium were applied (Fig. 1). The culture reached its highest

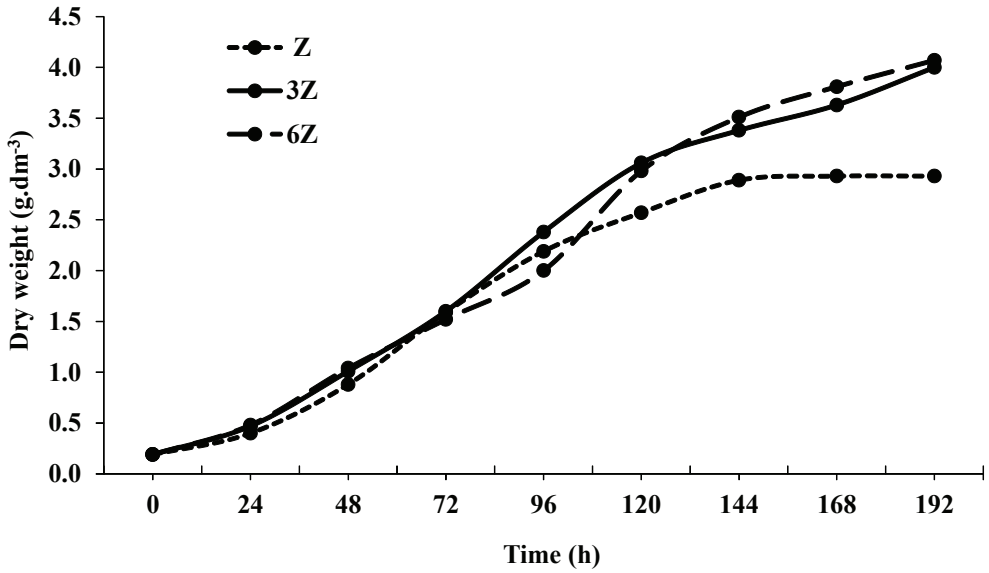


Fig. 1. Growth of *T. minutus* at 3 concentrations of the used nutrition medium (Z), in laboratory non-sterile monoalgal culture. Illumination at 35 $W.m^{-2}$ PAR 24-h uninterrupted light from luminescent tubes, temperature 32°C, bubbling with 3 $cm^3.s^{-1}$ air enriched with 0.5 % $CO_2$, and pH 7.3 - 7.8 were maintained.

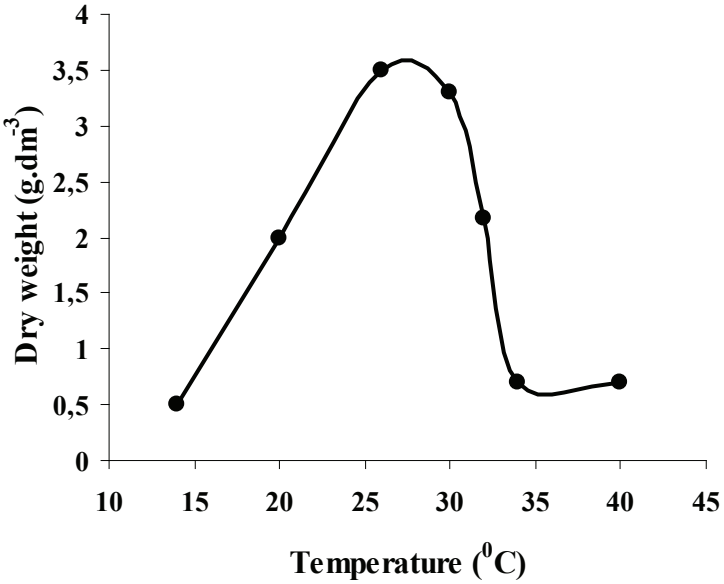


Fig. 2. Density of *T. minutus* after 96 h at light intensity of 35 $W.m^{-2}$ and different temperatures.

rate of growth at about 2 g.dm$^{-3}$ density of the alga. Extreme temperatures, 14°C and 40°C were characterized by very low growth, the maximum was observed at about 26°C, as demonstrated in Fig. 2.

**Biochemical composition.**

The lipids reached the relatively high value of 31 ± 3 % at 14°C, and protein was 48 ± 1 % at 40°C (Table 1). The percentage content of the main classes of substances could characterize *T. minutus* as an alga possessing valuable biomass with high protein and lipid contents. The lipids were extracted easily, and their smell was similar to that of fish oil. The main classes of substances presented in the total lipids were TAG, MGDG, SQDG, PG, according to TLC analyses. The content of TAG in the lipids of *Trachydiscus* was about 20%, which was high compared to cyanobacteria and green algae (Petkov and Furnadzieva, 1993). The percentage of fatty acids depends on the cultivation conditions and their values at the optimum are given in Table 2. A high percentage of myristic acid (26%) was found. In addition, EPA content (27 - 42%) was high and depending on cultivation conditions

Table 1. Main substances in *T. minutus* dry weight.

| Substances | % ± SD* |
|---|---|
| Protein | 43 ± 2 |
| Carbohydrates | 25 ± 5 |
| Lipids | 26 ± 5 |
| Chlorophyll *a* | 0.55 ± 0.09 |
| Chlorophyll *c* | 0.18 ± 0.04 |

*From 8 experiments in the temperature range 20-34°C.

Table 2. Fatty acid composition of *T. minutus*, % (w/w).

| Fatty acid | Total lipids | | | | Triacylglycerols |
|---|---|---|---|---|---|
| | 18 W.m$^{-2}$, 26°C | 35 W.m$^{-2}$, 26°C | [a]35 W.m$^{-2}$ | [b]264 W.m$^{-2}$ | 35 W.m$^{-2}$, 26°C |
| 12:0 | tr. | 0.8 ± 0.4 | 1.2 ± 0.5 | 0.6 ± 0.5 | 1.2 ± 0.1 |
| 14:0 | 28.0 ± 6.0 | 28.0 ± 3.0 | 26.0 ± 5.0 | 4.0 ± 2.0 | 20.3 ± 0.1 |
| 16:0 | 10.0 ± 2.0 | 8.0 ± 1.0 | 9.0 ± 4.0 | 33.0 ± 13.0 | 13.8 ± 0.9 |
| 16:1 | 5.0 ± 1.0 | 10.0 ± 3.0 | 10.0 ± 3.0 | 6.0 ± 1.0 | 14.8 ± 0.9 |
| 16:2 | tr. | 0.8 ± 0.3 | 1.5 ± 1.0 | 4.0 ± 2.0 | 0.8 ± 0.1 |
| 18:0 | 0.4 ± 0.1 | 0.4 ± 0.2 | 0.6 ± 0.5 | 3.0 ± 1.0 | 3.7 ± 0.4 |
| 18:1 | 3.0 ± 1.0 | 3.0 ± 0.5 | 3.0 ± 2.0 | 9.0 ± 3.0 | 9.9 ± 0.8 |
| 18:2 | 5.0 ± 1.0 | 7.0 ± 1.0 | 7.0 ± 1.0 | 23.0 ± 7.0 | 11.9 ± 0.5 |
| γ-18:3 | 0.2 ± 0.1 | 0.8 ± 0.4 | 0.6 ± 0.4 | 0.3 ± 0.3 | tr. |
| α-18:3 | 2.0 ± 1.0 | 7.0 ± 2.0 | 5.0 ± 3.0 | 6.0 ± 2.0 | 5.5 ± 0.3 |
| 20:4 | 4.0 ± 1.0 | 6.0 ± 1.0 | 6.0 ± 2.0 | 4.0 ± 2.0 | 10.7 ± 0.9 |
| 20:5 | 42.0 ± 6.0 | 27.0 ± 4.0 | 29.0 ± 7.0 | 3.0 ± 1.0 | 7.4 ± 0.8 |

[a] Mean value and SD from 8 experiments at 20, 26, 30, 34°C, 35 W.m$^{-2}$.
[b] Covered pond, 26 ± 4°C.

especially to irradiation intensity (Table 2). A relatively high percentage and abundance of sterols was found (Table 3), the main sterol being sitosterol (61.5 %).

Table 3. Percentage of sterols of *T. minutus*.

| RT [min] | Sterol | % |
|---|---|---|
| 40.97 | Cholesterol | 23.6 |
| 42.27 | 5,24(28)-ergostadien-3β-ol | 2.0 |
| 42.35 | 24-Methylcholesterol | 1.5 |
| 43.38 | Chondrillasterol | 0.6 |
| 43.64 | β-Sitosterol | 61.5 |
| 43.87 | Stigmasta-5,24(28)-dien-3-ol | 10.8 |

**Pilot plant cultivation.**

The alga cultivated in a 330 $dm^3$ orbicular, flat vat, in greenhouse, for 30 days, gave the average daily yield of 7 g. $m^{-2}$, under routine conditions for *Spirulina*, where natural light intensity grew up to 240 - 260 $W.m^{-2}$ PAR, the temperature of suspension max. 26°C. Centrifugation and drying by sun irradiation, on plastic foil, was successful too.

## DISCUSSION

**Taxonomy position of the studied strain.**

Beyond any doubt, *Trachydiscus* has often been mistaken for *Chlorella* because of morphologic likeness. The fatty acid composition of the alga, being very different from that of *Chlorella*, is a reliable taxonomic marker, together with lack of chlorophyll *b*. The taxonomic position of the genus *Trachydiscus* (*Pseudostaurastrum*) has been questioned. Its original place in Xanthophyceae (Heterokonta) was changed into Eustigmatophyceae by Hegewald et al. (2007). Spectrophotometrically, we found in our *Trachydiscus minutus* about 0.18 ± 0.04 of chl *c*, which must be confirmed chromatographycally. Schnepf et al. (1996) did not found chlorophyll *c*, in *Pseudostaurastrum limneticum* which confirms taxonomic membership of this alga in Eustigmatophyceae. Further research using cultures is obviously necessary to find out if *Trachydiscus limneticus* and *Trachydiscus minutus* belong to class Eustigmatophyceae and to class Tribonematophyceae, respectively. The molecular biology tree based on 18S rDNA reveals specific position of *Pseudostaurastrum* (Hegewald et al., 2007) unfortunately *Trachydiscus minutus* has not been studied, yet.

**Biochemical composition.**

Almost all fatty acids react to the temperature change and there was no clear pattern of temperature correlation. This is probably due to the fact that the fatty acids are diverse unlike cyanobacteria where fatty acid diversity is scarce (Iliev et al., 2006). Because of this diversity, the same membrane properties could be achieved by different fatty acid proportions, even at the same temperature. The changes at different light intensities were much more expressed, as is obvious from Table 2. The concentration of triacylglycerols as an absolute value and as the part of total lipids was high compared to most cyanobacteria, red, and green algae which contain about 3 % (Petkov and Furnadzieva, 1993). Triacylglycerols are the main reserve substances of the algae that help to survive unfavourable conditions, such as nitrogen insufficiency. For algal biotechnology,

they are the source of nutrient oils and biofuels. A high percentage of myristic acid (26 %) was found, which is less normal in algae. This saturated fatty acid is in use in cosmetics. Abundance of the highly unsaturated EPA characterized the total fatty acid composition. The values obtained were more than those found in many other species of microalgae (Pratoomyot et al., 2005). There was abundance of sitosterol (phytosterol) and cholesterol (zoosterol) in *T. minutus* (Table 3). It is worth mentioning here that we found 90 % ergosterol (mycosterol) amid total sterols of the green alga *Coelastrum sphaericum* (Petkov and Dang, 1999). This well known division of sterols may be tentative when characterize the organisms, but it is not very reasonable when algae are concerned: they obviously have extraordinary diversity of sterols. The content and composition of lipids, fatty acids, and sterols allows possible usage of the alga in artificial nutrition chains, and it could be recommended as a proper fodder in fish breeding. High content of polyunsaturated fatty acids and abundance of sterols are obligatory requirements in the artificial nutrition chain phytoplankton - zooplankton - fish in aquacultures (Krienitz and Wirth, 2006; Martin-Creuzburg and Elert, 2004). For this reason, its cultivation on larger scale was studied.

**Oil production prospective.**

The lipid content at intensive cultivation of *Trachydiscus* (26 %, Table 1) is higher than in Bacillariophyceae: 17 - 18 % in *Navicula* sp. (Retovsky, 1946). *Spirulina* had 9.4 % (Arvanitis et al., 2004) or 16.3 % (Ramadan et al., 2008). *Botryococcus* cultivated in livestock wastewater produced 19.8 % (Shen et al., 2008). Higher lipid content (40 - 50 %) was found in stationary cultures of Chlorophyta, where there was no algal growth at all. The lipid content at intensive growth was about 10 % (Harder and Witsch, 1942b). Similar results can be expected from another biotechnology research, e.g. by N-starvation. Lipid content in *Scenedesmus obliquus* increased from original 12.7 to 43 % of dry cell weight (Mandal and Mallick, 2009). Here is to mention that the growth of the alga was very limited. The alga *Trachydiscus* allows more successful approach, namely high lipid content at intensive growth.

**Pilot plant cultivation.**

The daily yield of 7 $g.m^{-2}$ is promising, having in mind that light intensity, rate of stirring, and nutrition medium has not been optimized yet. Cultivation conditions and equipments of harvesting were identical to the well known *Spirulina* (Fournadzhieva et al., 2002a, b). At noon, natural light intensity grew up to 240 - 260 $W.m^{-2}$ PAR under the plastic cover, which was 8-10-fold more than in laboratory. The changes in growth rate and percentage of polyunsaturated fatty acids proved that *Trachydiscus* required lower light intensity. That fact should be taken into consideration when cultivation device is constructed: the value of the area/volume ratio has to be higher when using natural light. Probably illumination must be of low intensity, and preferably through reflected or dissipated light, especially at lower algal density. Also resistance of *T. minutus* to higher temperature is promising. The yield was comparable at temperatures of 26 – 30°C (Fig. 2). Easy centrifugation of the biomass from the medium, and drying

of *Trachydiscus* on foil in greenhouse by sunlight only are further important technological advantages. Our results demonstrated that *T. minutus* possessed valuable features. Its composition as well as the behavior during cultivation, separation and drying makes it worth for further studies from the point of view of photoauthotrophic biotechnology. The high content of oils, myristic acid and EPA in the alga, is prospective for cosmetics and nutrient additive for humans and aquaculture.

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