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Screening high oleaginous *Chlorella* strains from different climate zones

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HIGHLIGHTS

- 23 Oil-producing *Chlorella* strains from different climate zones were obtained.
- Four *Chlorella* strains with high triacylglycerol content were cultured at 5–40 °C.
- *Chlorella* sp. NJ-18 displayed higher triacylglycerol productivity at 5–30 °C.
- Accumulation of triacylglycerols in strain NMX35N changed a little from 30 to 40 °C.
- NJ-18 and NMX35N are promising as feedstock of biodiesel at various temperatures.

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ABSTRACT

In outdoor cultivation, screening strains adapted to a wide temperature range or suitable strains for different environmental temperatures is of great importance. In this study, triacylglycerol (TAG) content of 23 oil-producing *Chlorella* strains from different climate zones were analyzed by thin layer chromatography. Four selected *Chlorella* strains (NJ-18, NJ-7, NMX35N and NMX139N) with rather high TAG content had higher total lipid content compared with *Chlorella vulgaris* SAG 211-11b. In particular, NJ-18 displayed the highest TAG productivity among the four high oil-producing *Chlorella* strains. Accumulation of TAGs in strain NMX35N changed a little from 30 to 40 °C, showing a desirable characteristic of accumulating TAGs at high temperatures. Our results demonstrated that NJ-18 and NMX35N had suitable fatty acid profiles and good adaption to low and high temperatures respectively. Therefore, cultivation of the two *Chlorella* strains together might be a good option for economic biodiesel production during the whole seasons of the year.

1. Introduction

Since fossil fuel is not sustainable and its usage may cause serious environmental problems, more and more attention has been attached to clean renewable energy (Borowitzka and Moheimani, 2013; Chisti, 2008; Hu et al., 2008b; Huang et al., 2010; Jones and Mayfield, 2012). Biodiesel (fatty acid methyl esters), derived from triacylglycerols (TAGs) by transesterification with methanol, is a renewable, biodegradable, and nontoxic fuel (Chisti, 2007). Currently, it is produced mainly from vegetable oils and animal fats, however, the large-scale production of biodiesel from them is unsustainable and uncompetitive in cost (Chisti, 2008). Microalgae as feedstock for biodiesel can serve as an alternative and potential candidate, based on the high photosynthetic efficiency and lipid productivity, and their ability to grow rapidly, tolerate and adapt to a variety of environmental conditions (Chisti, 2007; Hu et al., 2008b).

Screening an optimal oleaginous microalga for outdoor cultivation is the very first and most important step toward the utilization of microalgae as feedstock for biodiesel production (Scott et al., 2010). Temperature is an important factor for the outdoor cultures, and it has a great influence on the growth and lipid content of microalgae (Li et al., 2013). Therefore, the ability of microalgae to acclimate to variable outdoor temperatures should be taken into account in the screening process.

Chlorella strains are regarded as competent candidates for biodiesel production due to their fast growth and easier cultivation (Mata et al., 2010). They distribute all over the world including polar areas (Ahn et al., 2012; Hoek et al., 1995; Hu et al., 2008a), showing an extensive adaptability to changing temperatures (2–42 °C) (Ahn et al., 2012; Hu et al., 2008a; Huss et al., 1999; Kessler, 1985), and the optimal growth temperature and the maximum lipid productivity are strain-specific. For example, *Chlorella vulgaris* tended to stop growing at temperature above 30 °C (Kessler, 1985), while *Chlorella sorokiniana* exhibited a better growth with an increase of temperature from 28 to 42 °C (de-Bashan et al., 2008). In addition, lipid content and fatty acid composition are influenced greatly by temperature (Converti et al., 2009; Li et al.,

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2013). Therefore, to reduce the cultivation expenditure it is very important to screen strains with a capacity of adapting to a wide range of temperature or find suitable strains for different environmental temperatures. In this study, *Chlorella* strains isolated from Antarctica and different climate zones in China were screened by thin layer chromatography (TLC) for a rough identification of high lipid productive stains on the basis of the TAG content. Selected high oleaginous stains were grown under different cultivation temperatures and evaluated by Nile Red dye to define those with high lipid productivity, hence obtaining *Chlorella* strains that can serve as feedstock for biodiesel production at different seasons.

2. Methods

2.1. Strains

Water samples from subtropical (Hubei Province) and warm temperate zone (Jiangsu Province and Inner Mongolia Autonomous Region) in China were collected and plated on BG11 medium (Stanier et al., 1971) solidified with 1.5 % agar. Single green algal colonies were obtained and further purified by repeated streaking on solid BG11 medium. The unialgal cultures of genus *Chlorella* were used in this study. *C. vulgaris* NJ-7 and *Chlorella* sp. NJ-18 were isolated from the Zhongshan Station of Antarctica by our lab in January 1999 (Hu et al., 2008a). *C. vulgaris* SAG 211-11b was purchased from the Culture Collection of Algae at the University of Texas (Austin, TX, USA). FACHB3, FACHB31, FACHB270 and FACHB275 were obtained from the FACHB-Collection (Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China).

2.2. Phylogenetic analysis

The total genomic DNA was extracted from algal cells using a glass milk DNA isolation kit (Fermentas, Vilnius, Lithuania) according to the manufacturer's instructions. Polymerase chain reaction (PCR) was performed by using the general primers 18S-1 (5′-tggttgatcctgccagtagtc-3′) and 18S-2 (5′-tgatccttctgcaggttcacc-3′) to amplify 18S rDNA gene as previously described (Hu et al., 2008a). The PCR products were excised from agarose gel and recovered with the DNA isolation kit, cloned into pMD18-T (Takara, Dalian, China) and sequenced. All "true" *Chlorella* species (Huss et al., 1999; Krienitzi et al., 2004) and some oil-producing *Chlorella* species *sensu lato* were included in phylogenetic analysis. Phylogenetic analyses were performed by PAUP4.0b (Swofford, 1998), with 1000 bootstrap replicates for neighbor-joining and parsimony analyses, 100 replicates for the maximum likelihood analysis using *Gloeotilopsis planctonica* (Z28970) and *Ulothrix zonata* (Z47999) as the outgroup.

2.3. Preliminary screening of oleaginous strains

All *Chlorella* strains were grown in 50 ml flasks containing 20 ml BG11 liquid medium (the starting optical density OD_{750} = 0.05) at 25 °C under continuous illumination of 100 μmol photons m^{-2} s^{-1} for 20 d, then total lipid was extracted from the cell pellet and analyzed using TLC for the comparison of the TAG content. The diatom *Phaeodactylum triconutum* with high TAG content was used as control. TLC was performed as described by Reiser and Somerville (1997) by one-dimensional TLC on silica gel plates 60 F254 (Merck KgaA, Darmstadt, Germany). Standard triolein was purchased from Sigma–Aldrich Corporation (St. Louis, MO, USA).

2.4. Growth of high oil-producing Chlorella at different temperatures

Four high oil-producing *Chlorella* stains (*C. vulgaris* NJ-7, *Chlorella* sp. NJ-18, *Chlorella* sp. NMX35N and *Chlorella* sp. NMX139N) determined by preliminary screening and the type species of *Chlorella*, *C. vulgaris* SAG 211-11b, were grown in 250 mL flasks containing 150 mL BG11 ($NaNO_3$ concentration was reduced to 880 μM) liquid medium (the starting optical density OD_{750} = 0.05) at 5, 15, 20, 25, 30, 35 and 40 °C under continuous illumination of 100 μmol photons m^{-2} s^{-1}. Each was performed in triplicate. Cell density was determined by measuring OD_{750} every 2 days and specific growth rates (μ) were calculated with the formula $\mu = (\ln X_t - \ln X_0)/t$, in which X_0 is the initial cell density and X_t is the cell density after t days.

2.5. Total lipid extraction and fatty acid analysis

Four high oil-producing *Chlorella* stains and *C. vulgaris* SAG 211-11b were grown in 2 L flasks containing 1.5 L BG11 liquid medium bubbled with aseptic air at 25 °C under continuous illumination of 150 μmol photons m^{-2} s^{-1}. Cells were harvested during the stationary phase by centrifugation (6000*g*, 5 min) and washed for three times with deionized water, then freeze-dried.

Total lipids of each sample were extracted from approximately 100 mg dry cells and lipid contents (dry weight) were measured as described by Bligh and Dyer (1959). TAGs were separated and quantified by one-dimensional TLC on silica gel plates 60 F254 (Merck KgaA, Darmstadt, Germany), and the bands were identified by staining with iodine and then scraped off the plates.

TAGs fractionated from the TLC plate and total lipids were methylated according to Hu et al., (2008a). Fatty acid methyl esters were identified and quantified by gas chromatography (TRACE GC, Thermo Scientific, Milan, Italy) equipped with a split/splitless injector, a flame ionization detector (FID) and a capillary column (60 m × 0.25 mm) (DB-23, J&W Scientific, USA). Two microliters of each sample were injected in the splitless injection mode. The

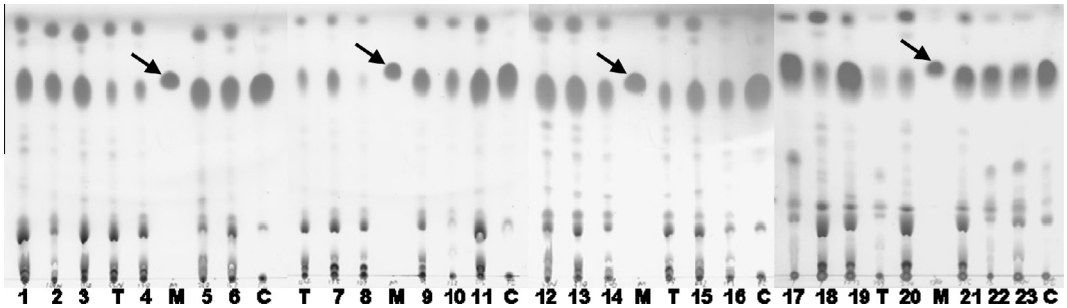

Fig. 1. Analysis of the total lipids extracted from microalgal cultures by thin layer chromatography (TLC). The diatom *P. triconutum* with high TAG content was used as control. 1–23: *Chlorella* strains (1: NXM2, 2: NXM44, 3: NMX139N, 4: THX130, 5: THX193, 6: THX129, 7: FACHB275, 8: HBX169, 9: FACHB31, 10: THX132, 11: HBX356, 12: NMX35N, 13: NMX310, 14: HBX326, 15: HBX402, 16: YEL, 17: NJ-7, 18: NMX1, 19: NJ-18, 20: FACHB270, 21: FACHB3, 22: NMX37N, 23: NMX36); M: triolein (0.02 mg); C: *P. triconutum*; T: *C. vulgaris* SAG 211-11b. TAGs were indicated by arrows.