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Rapid neutral lipid accumulation of the alkali-resistant oleaginous *Monoraphidium dybowskii* LB50 by NaCl induction

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HIGHLIGHTS

- 0.25 g L^{-1} urea and 0.1 M $NaHCO_3$ are optimal nutritions for *M. dybowskii* LB50.
- Neutral lipid productivity is enhanced by 20 g L^{-1} NaCl.
- Induction and cultivation time are shortened at optimum NaCl addition.
- Induction efficiency in 140 L reactor is the same as that in 5 L flasks.
- Membrane remodeling contributes to NL accumulation under salt stress.

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ABSTRACT

NaCl is an effective inducer of lipid accumulation in freshwater microalgae, but little is known on whether the enhanced lipid components are desired. To address this issue, *Monoraphidium dybowskii* LB50 from a freshwater habitat was selected, cultivated, and subjected to NaCl induction at different scales outdoors. Results showed that the optimal salt concentration reduced glycolipid (GL) content, as well as enhanced neutral lipid (NL) and phospholipid (PL) contents. Moreover, GL was preferentially converted to NL at 20 g L^{-1} NaCl. Total lipid and NL contents respectively increased to 41.7% and 17.48% in 1 d. The highest NL productivity was also achieved at both the 5 L (24.13 mg L^{-1} d^{-1}) and 140 L (13.05 mg L^{-1} d^{-1}, 3.43 g m^{-2} d^{-1}) scales. These results suggest that NL accumulated effectively and rapidly at different scales, indicating that this strategy has broad application prospects for the scale-up cultivation of oily algae.

1. Introduction

Microalgae have numerous advantages as feedstock for third-generation bioenergy. These advantages include photosynthetic efficiency, high lipid content, and capability to grow in extreme environments (Hu et al., 2008). Microalgae can also be cultivated as an integrated approach for wastewater treatment to optimize the energy and financial input for the production process (Sharma et al., 2012). However, to achieve the sustainable industrial production of bioenergy from microalgae, the crucial technological bottleneck of providing sufficient oleaginous feedstock must be addressed. Given that microalgae with high lipid contents are often confronted with significantly low lipid contents when grown indoors, polluted by other miscellaneous algae, or preyed upon by protozoa (Hu et al., 2008; Moheimani, 2013a,b), large-scale culture in outdoor environments is recommended. Therefore, an essential prerequisite to achieve the industrial-scale application of microalgal biofuel is the selection of robust and highly productive microalgal strains with relatively high lipid content.

To accumulate large amounts of lipids and neutral lipids, microalgae are usually subjected to stress conditions, such as nutrient and phosphorus deficiency (Lacour et al., 2012), because lipid contents can be significantly enhanced by the absence of nutrients. Triacylglycerols (TAG) of *Chlamydomonas reinhardtii* increased in the absence of sulfur (Cakmak et al., 2012). However, high biomass cannot be obtained in this way. Therefore, the photoautotrophic two-stage process naturally becomes a very promising approach (Xia et al., 2013). However, the use of the method from nutrient-rich to nitrogen-strarvation medium is difficult for outdoor large-scale cultures and changes in light and temperature are limited to outdoor cultures (Rodolfi et al., 2009; Su et al., 2011). Thus, a

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promising idea is to incorporate the inducer directly into the medium. The effectiveness of this type of inducer mainly depends on the use of iron salt, zinc salt, and sodium salt (NaCl, $NaHCO_3$, NaAC) etc. (Einicker-Lamas et al., 2002; Herrera-Valencia et al., 2011; Liu et al., 2008; Xia et al., 2013, 2014). However, the effects are not evident in numerous microalgae strains when iron salt and zinc salt are used for induction. Moreover, these salts can harm the environment when used at a large scale. Therefore, sodium salt is widely used to stimulate lipid accumulation in microalgae, with NaCl proven to be very effective. However, efficiency and induction time are directly related to production cost (Xia et al., 2014). Therefore, induction efficiency should be enhanced to shorten the culture period.

Under adverse environmental or stress conditions, many microalgae form and accumulate neutral lipids (20–50% DW) in the form of TAG by altering the lipid biosynthesis pathway (Sharma et al., 2012). Although the mechanism of lipid transformation under the NaCl-induced stress is unclear, the case is sure to differ from that of nitrogen and phosphorus nutrient deficiency. Approximately 60% lipid was synthesized from de novo carbon fixation (from CO_2 fixation) in cases of nitrate deprivation. The remaining lipids came from the transformation of pigment, protein, starch, and other components of lipid membranes (Burrows et al., 2012; Li et al., 2014). Under NaCl stress, the adaptability of some amino acids (proline, homoserine, etc.), carbohydrates (sucrose, trehalose, etc.) increases, which reduces the pyruvate content, which is a precursor of TAG synthesis (Bromke et al., 2013). To resist the osmotic pressure, an increase in membrane lipid content naturally reduces the TAG percentage from membrane lipid transformation (Chen et al., 2008), and the increased total lipid mainly comprise polar lipids, not TAG (Azachi et al., 2002; Chen et al., 2008). However, this condition is inconsistent with the transformation relationships among NL, PL and GL (Chen et al., 2008; Zhila et al., 2011). The effect of NaCl induction is closely related to the growth stage of microalgae. When *Dunaliella tertiolecta* was cultured with different concentrations of NaCl (0.5–1.0 M), high salt increased lipid content by 7%, TAG by 15.8%. Adding NaCl at mid-log phase or at the end of log phase did not cause a significant increase in lipid and TAG contents (Takagi et al., 2006). Notably, few studies have been conducted under relatively large-scale outdoor conditions, which is the key to using NaCl as an inducer.

This study aims to screen the alkali-resistant oleaginous *Monoraphidium dybowskii* LB50 with high lipid productivity and to obtain the maximum neutral lipid productivity within a short time of NaCl induction in a 140 L bioreactor situated outdoors. Microalgal strains were first screened and then induced with different NaCl concentrations. Lipid productivity and the transformation relationships were investigated among NL, PL, and GL. Based on the above concentrations, the NL accumulation of the selected strains at different outdoor scales were induced by the optimal NaCl content. Finally, a quick and effective culture method was established.

2. Methods

2.1. Organisms

The six oleaginous microalgae used in this study were provided by Prof. Xudong Xu of the Institute of Hydrobiology the Chinese Academy of Sciences. These strains were *Micractinium* sp. XJ-2, *Westella botryoides* XJ-4, *M. dybowskii* XJ-2, *Monoraphidium* sp. 1, *M. dybowskii* LB50, and *Monoraphidium* sp. LB59. *M. dybowskii* LB50 was isolated from an alkaline (pH 8.48–9.04) reservoir in Inner Mongolia of China. The stock cultures were maintained indoors in a sterilized BG11 medium containing 1.5 g $NaNO_3$, 40 mg K_2HPO_4, 75 mg $MgSO_4 \cdot 7H_2O$, 20 mg Na_2CO_3, 36 mg $CaCl_2 \cdot 2H_2O$, 6 mg ammonium ferric citrate, 6 mg ammonium citrate monohydrate, 1 mg EDTA, 2.86 μg H_3BO_3, 1.81 μg $MnCl_2 \cdot 4H_2O$, 0.222 μg $ZnSO_4 \cdot 7H_2O$, 0.39 μg $Na_2MoO_4 \cdot 2H_2O$, 0.079 μg $CuSO_4 \cdot 5H_2O$, 0.050 μg $CoCl_2 \cdot 6H_2O$ in 1 L water.

2.2. Experimental design

2.2.1. Strain selection

Six freshwater microalgal strains were screened indoors. These strains were cultivated in modified BG11 medium with 0.15 g L^{-1} $NaNO_3$ in 400 mL of the culture medium in 500 mL Erlenmeyer flasks. The algal cultures underwent continuously bubbling with filter-sterilized air (from the bottom) through the transparent glass tube. A light density of 60 μmol m^{-2} s^{-1} (24 h) was provided by cool white fluorescent tubes (400–750 nm), and the temperature was maintained at 25 °C by an air conditioner.

Six freshwater microalgal strains were screened in 5 and 140 L bioreactors in a green house in Beijing, China (40°220′, 116°200′ E). Gases were supplied to each bioreactor through the pipage mixed with 2% CO_2 (v/v) during daytime. At night only pure air was supplied. The medium was thoroughly compounded with tap water to realize the microalgae resistance. The strains were cultivated in modified BG11 medium with 0.15 g L^{-1} $NaNO_3$ in 4 L of the culture medium in 5 L flasks. To keep cells in suspension, a 5 cm magnetic stirring bar (mixing at 150 rpm) was used to stir the middle of the 5 L flasks. *Micractinium* sp. XJ-2, *M. dybowskii* LB50, and *M. dybowskii* XJ-2 were cultivated in modified BG11 medium with 0.25 g L^{-1} urea in the 140 L photobioreactor. The 140 L bioreactor was used to test whether the microalgae can grow well and accumulate the neutral lipid under large-scale culture conditions. This bioreactor was composed of two connected 70 L polyvinylchloride hanging bags (1800 mm in height and 220 mm in diameter) (Dalian Huixin Titanium Equipment Development Co., Ltd., China) (Xia et al., 2013).

2.2.2. Optimization of microalgal nutrition

M. dybowskii LB50 was cultivated in 400 mL of culture medium in 500 mL Erlenmeyer flasks indoors. The concentrations of $NaNO_3$ and urea were respectively set at 0.15, 0.30, 0.9, 1.50 g L^{-1} and 0.05, 0.10, 0.25, 0.50 g L^{-1}. Under suitable concentration of $NaNO_3$ and urea, the $NaHCO_3$ concentrations were set to 0, 0.1, and 0.2 M.

2.2.3. Induction experiments

M. dybowskii LB50 was cultivated under optimal nutrition conditions in 4 L of the culture medium in 5 L flasks outdoors. On the 12th day (the late-exponential growth phase) NaCl was added for final concentration of 0, 20, 40, and 60 g L^{-1} in the 5 L flasks. In the 140 L bioreactor, the NaCl concentration was 20 g L^{-1}. The induction experiments were conducted from June to August 2013. The greenhouse temperature was 34.44 ± 6.10 °C during day time and 22.17 ± 3.05 °C at night.

2.3. Analytical procedures

2.3.1. Biomass measurement

The biomass productivity (BP, mg L^{-1} d^{-1}) was calculated according to the following equation:

$$BP = (B2 - B1)/t$$

where $B2$ and $B1$ represent the dry weight biomass density at the time t (days) and at the start of the experiment, respectively.

The algal density was determined by measuring the OD680. The relationships between the dry weight (DW, g L^{-1}) and the OD680 values of the algae can be described using the following equations: