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Screening of biocompatible organic solvents for enhancement of lipid milking from *Nannochloropsis* sp.

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ABSTRACT

To extract the microalgal lipid *in situ*, biocompatible solvents were screened for lipid milking of *Nannochloropsis* sp. in an aqueous–organic system. The effects of organic solvents on the microalgal growth, the lipid extractability, the dehydrogenases activity and the cell membrane integrity were investigated by UV–visible spectrophotometer, FT-IR spectroscopy, 2,3,5-triphenyltetrazolium chloride (TTC) and Evans Blue stain method, respectively. The results showed that alkane solvents with log $P > 5.5$ were biocompatible while the hydrophilic solvents with log $P < 5.5$ were toxic to *Nannochloropsis* sp. due to the deactivated dehydrogenase and increased cell membrane permeability. As 10% (v/v) hexadecane was used to establish biphasic system, the total lipid production of *Nannochloropsis* sp. was increased by 28.9% compared to the control. The screened biocompatible solvent hexadecane enhanced not only the algal growth but also the lipid accumulation, showing an effective way to facilitate the process for *in situ* lipid milking from *Nannochloropsis* sp.

1. Introduction

Recent soaring oil prices and diminishing world oil reserves, coupled with rise of greenhouse gases and the predicted climate change threat, have generated renewed interest in using algae as an alternative and renewable feedstock for energy production [1]. However, algal-oil price remained rather higher than that of fossil fuels, and the harvesting and extraction of fuel precursors from microalgal cultures was one of the most problematic areas for the algal biofuel production technology [2].

Current methods for lipid extraction had been reported including: (1) organic solvents extraction based on dry algal biomass [3]; (2) sub-critical water extraction based on wet algal biomass [4]; (3) supercritical CO_2 extraction based on dry algal biomass [5]. Since most microalgae could grow to the biomass density of only a few grams per liter water, processes built upon dry biomass were unlikely to be economical from the point of energy inputs involved [6].

Unlike the aforementioned methods, milking, as a method for simultaneous production and selective extraction of target products in an aqueous–organic biphasic system, had been reported previously in production of pigment [7,8], taxol [9] and hydrocarbon [10]. Compared with the aforementioned extraction methods, lipid milking from microalgae might possess several advantages including: (1) milking was generally conducted by organic solvent under atmospheric conditions; (2) microalgal lipid could be milked directly in the algal suspension without dewatering process; (3) milking of microalgae might provide a way to enhance lipid accumulation.

In the past published literatures, nevertheless, only two algal species had been experimentally tested to induce final metabolites production in the presence of organic solvents using the milking concept. For example, *Dunaliella salina*, cultured with biocompatible organic solvents of dodecane and hexadecane, could produce significantly higher amount of total β-carotene in a biphasic system compared with the control sample [7,11]. *Botryococcus braunii* could be induced to produce long-chain unsaturated hydrocarbons and the hydrocarbon production was enhanced by about 23.5% in an aqueous-dihexyl ether system after the incubation of 62 h [10]. It had indicated that the milking process might be used to integrate harvesting, dewatering and the following lipid extraction process. However, to the best of our knowledge and in the strict sense except for hydrocarbon, milking had not been adopted for the general lipid production from the microalgae. Although, diatom has been recently reviewed as the species to produce gasoline directly by this milking concept, no experimental study had been open published [12].

Screening the right algal species for maximization of lipid production, which was a product of lipid content and biomass productivity, had been intensively studied [13,14]. *Nannochloropsis*

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sp. was one of the most promising algal species with high lipid productivity of 37.6–61.0 mg L^{-1} d^{-1} [15]. Although it had been widely accepted that the lipid content of microalgae was dramatically increased under stress conditions, such as N-starvation [16] and high ferric ionic concentration [17], it was acknowledged that there was an inverse relationship between lipid content and algal growth, showing the necessity to increase both factors simultaneously. The tradeoff between the lipid content and algal growth, therefore, might be further overcome using this proposed lipid milking concept to selectively culture the microalgae and to alter their environment for maximization of both the rate and ratio of lipid secretion.

Although milking seemed to be an ideal method to extract lipid, three utmost important problems needed to be addressed for this process. Firstly, for the successful milking, microalgal cells were viable and generally recycled into the biphasic system to produce lipid continuously, therefore the right organic solvent must be biocompatible to the specific algal species [18]. The second problem was to obtain the lipid extractability as high as possible while the solvent could be recovered as completely as possible from the economic point of view. Finally, the possible mechanism for the interaction between organic solvent and algal cells needed to be investigated for the lipid milking process.

In this work, biocompatible organic solvents were screened for *in situ* lipid milking of *Nannochloropsis* sp. Organic solvents with different hydrophobicity ($2 < \log P < 9$) were added to form the aqueous–organic biphasic systems for the culture of *Nannochloropsis* sp. The effects of different solvents on algal growth and lipid extraction were investigated. The variations of biochemical compositions including lipids, proteins and carbohydrates of *Nannochloropsis* sp. in the presence of organic solvents were then analyzed. The influences of organic solvents on dehydrogenase activity of algal cells and cell membrane permeability were further studied. Finally, the possible toxic mechanism of organic solvents on algal cells was proposed.

2. Materials and methods

2.1. Microalgae and culture conditions

The marine microalga *Nannochloropsis* sp. was provided by the Key Laboratory of Marine Biotechnology, Ningbo University (Zhejiang, China). It was cultivated in artificial medium composed of 500 mg KNO_3, 10 mg KH_2PO_4, 10 mg Na_2EDTA, 2.5 mg $FeSO_4{\cdot}7H_2O$, 0.25 mg $MnSO_4$, 0.006 mg vitamin B1 and 0.00005 mg vitamin B12 in 1 L fresh water with the instant sea salt concentration of 34 g L^{-1} (Aquarium Systems, USA). The initial pH of the medium was adjusted to 7.5.

2.2. Chemicals and reagents

All chemical and reagents in the current work were of AR grade and purchased from Sigma (USA) or Sinopharm Chemical Reagent Co., Ltd. (China). Nile red and triolein (lipid standard) were used to determine lipid concentration in the solvent phase. Dehydrogenase activity of algal cells and cell membrane integrity were determined by 2,3,5-triphenyltetrazolium chloride (TTC), Evans Blue and sodium dodecyl sulfate (SDS), respectively. All the organic solvents were summarized in Table 1, which were selected according to their hydrophobicity (log *P*) and maximum solubility in water. The parameter of log *P* was defined as the partition coefficient of a given solvent in a mixture of octanol and water [19]. It was figured out the greater the polarity, the lower log *P* of the solvent [20].

Table 1
Physical properties of organic solvents.[a]

Solvent	log *P*	Molecular weight, M_w	Max. solubility in water (mg L^{-1})
Hexanol	2.03	102.174	5.90×10^3
Heptanol	2.62	116.201	3.27×10^3
Octanol	3.07	130.228	0.46×10^3
Hexane	4.00	86.175	9.80×10^3
Heptane	4.50	100.202	2.42×10^3
Octane	5.15	114.229	0.73
Nonane	5.65	128.255	0.17
Decane	6.25	142.282	1.50×10^{-2}
Dodecane	6.60	170.334	0.37×10^{-2}
Tetradecane	7.60	198.388	0.23×10^{-2}
Hexadecane	8.80	226.441	0.40×10^{-2}

[a] Data obtained from Handbook of Chemistry and Physics (90th edition, 2009–2010).

2.3. Experiment design

To investigate the effects of organic solvents on *Nannochloropsis* sp. growth and cell physiological properties, 11 samples were prepared according to the general experimental procedure as follows. The same algal seed was inoculated into 250 ml Erlenmeyer flask with 100 ml growth medium. Firstly, these algal cultures were statically cultivated under continuous illumination of 60 μmol photons m^{-2} s^{-1} at 25 °C for 96 h in an incubator (BoXun SPX-300B-G, China) until the cells growth entered into stationary phase. Afterwards, the cultures were transferred into a shaker (TaiCang THZ-22, China) with 100 rpm under the same light intensity at 25 °C, and the organic solvent of 10% (v/v) concentration was added into each sample according to Xu et al. [9]. Considering the mixing efficiency and hydrodynamics shear stress together, the rotation speed of 100 rpm was adopted [14,21]. The 11 samples, together with the control, i.e. equal volume algal suspension without the organic solvent, were used to compare their cell growth and other physiological indexes for another 96 h or longer time.

2.4. Algal biomass concentration assay

The effects of organic solvents on algal growth were then characterized by both the algal biomass concentration and the specific growth rate. The biomass concentration was evaluated by the dry weight (*DW*, g L^{-1}), and its relationship with the optical density (*OD*) at 450 nm was shown as:

$$DW = 0.28 \times OD_{450} \quad (1)$$

To measure the dry weight of biomass, 5 ml of microalgal culture was taken and centrifuged (1800 × *g*) for 20 min. The supernatant was removed and cells were then washed with distilled water and dried at 80 °C. The dried cells were weighed after 24 h. The optimal density was measured by using spectrophotometer at 450 nm in a quartz cuvette with 1 cm light path (LengGuang GS-54, China). The specific growth rate μ (d^{-1}) was calculated as:

$$\mu = \frac{\ln(C_t/C_0)}{\Delta T} \quad (2)$$

where C_t (g L^{-1}) and C_0 (g L^{-1}) were the final and initial biomass concentrations, respectively. ΔT (day) was the cultivation time.

2.5. Lipid analysis

2.5.1. Measurement of lipid concentration in solvent phase

After the microalga had been cultured in an aqueous–organic system for 96 h, the organic solvent in the upper phase was analyzed for the lipid concentration. Nile red fluorescence method was applied to measure the lipid which was extracted into organic sol-