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Enhanced lipid selective extraction from *Chlorella vulgaris* without cell sacrifice

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ABSTRACT

This study examines the performance of biocompatible organic solvents for lipid milking without cell sacrifice utilizing microalgal cells up to four cycles. Simultaneous production and extraction, is an integrated process of milking that involves cultivation of *C. vulgaris* under the influence of dodecane and tetradecane within the aqueous-organic biphase system and the extraction of intracellular lipids. Different concentrations (10% v/v, 15% v/v, and 20% v/v) of dodecane and tetradecane were used for the extraction of lipid. The highest lipid amount (1175 ± 34 mg/L) within the solvent phase was achieved with maximum lipid recovery ratio of 47 ± 5% during first cycle when 20% concentration of dodecane was used as the solvent. In addition maximum cell dehydrogenase activity (4.5 ± 0.5 OD_{492} g^{-1} cell) and growth rate (1.8 d^{-1}) were also observed under 20% dodecane. The presence of higher concentration of tetradecane lowers the dehydrogenase activity thus resulting in lower lipid extraction under milking. Solvent recovery ratio was 12% higher under 20% dodecane as compared to 10% of tetradecane. Growth profile study of *C. vulgaris* showed an overall increase in the lipid content when the cultures were extended up to four cycles. The analyzed fatty acid composition suggests that this technique could yield lipids that are suitable for conversion to biodiesel. According to the present results, a hypothetical scheme of milking technique under biocompatible organic solvents is proposed.

1. Introduction

Although substantial advances in microalgae biotechnology have taken place, the biodiesel production cost is still higher as compared to fossil fuels [1]. Major hindrance in the successful industrialized commercialization of microalgae biotechnology is the downstream processing which involves harvesting, dewatering, drying, extracting the lipids and conversion of lipids to fuel. Since most of the lipid molecules are synthesized and stored inside the microalgae cells, generally; metabolic, enzymatic, osmotic shock, weakening of cell walls, lysis and electrical degradation of the cell walls and membranes are the frequent methods used to excrete these bio-lipids molecules out of the cell [2,3,4]. An alternative approach is to use biocompatible organic solvents that can milk or export intracellular lipids to outside the cell. "Milking" is akin to the milking of cows without killing or weakening them. The term refers to the extraction of lipids from microalgae without cell sacrifice or nondestructive extraction of lipids from the live cells in the presence of biocompatible organic solvent [5]. Milking can be a cost effective technique for biodiesel production from microalgae.

Milking (simultaneous production and extraction) integrates current harvesting and post harvesting processes to reduce the production cost of biodiesel from microalgae. Chaudry et al., reported that the fertilizer requirement, water consumption and energy inputs in the milking process can be decreased by 90%, 30% and 70%, respectively [6]. It is a potential technique to enhance the concept of recycling the microalgal cells for further lipid accumulation so that production of fuel can be cost effective. The aim of the study is to develop an ecofriendly solvent extraction technique to milk the lipid without cell sacrifice. The advantages of lipid milking lies in several aspects such as; it occurs under aqueous organic biphasic system utilizing biocompatible organic solvent, it does not require energy intensive dewatering process, (like hydrothermal liquefaction that converts wet algae biomass directly into biocrude oil), it enhances the lipid productivity by recycling the algal cells [7]. For successful milking, the organic solvent must be biocompatible with minimum inhibition of biocatalytic algal cell viability. The second concern is the recovery and separation of the solvent from the final product (lipid) so as to reduce the overall cost of fuel [8].

Selection of biocompatible organic solvent is critical in milking of lipids from microalgae. Hydrophobic organic solvents with log $p > 5.5$, due to their relatively lower solubility in the cell culture medium are biocompatible. Organic solvent with log $p < 5.5$ in critical concentration causes the denaturation and destruction of dehydrogenase that adversely affect the viability of cells [9].

In the previous related literature, the lucrative milking had been reported for the extraction of hydrocarbons and beta-carotene from

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Botryococcus braunii and *Dunaliella salina* by using the biocompatible organic solvents, dihexyl ether and dodecane respectively [10,11]. Recently milking had been induced for the successful extraction of secondary metabolite "lipid" from *Nannochloropsis* sp. and *Botryococcus braunii* by employing the biocompatible organic solvents. The maximum percentage of lipids obtained in those studies were 415.5 mg/L and 117.3 mg/L respectively under the influence of 10% v/v of tetradecane (log p 7.60) and hexadecane (log p 8.80) in a single cycle [8,12]. To the best of our knowledge, this study is among one of the very few attempts to evaluate the effectiveness of various concentrations of biocompatible organic solvent when applied to extraction of lipids without cell sacrifice from *C. vulgaris* up to four cycles.

In this study, we adopted a novel method using biocompatible organic solvents to extract maximum amount of lipids without cell sacrifice. The effectiveness of various concentrations of biocompatible organic solvent when applied to microalgae lipid milking for four cycles was examined *via* cell viability and percentage of lipid content. The biocompatible organic solvents include dodecane and tetradecane using three different concentrations of 10% v/v, 15% v/v, and 20% v/v, for enhanced lipid selective extraction from *C. vulgaris*.

2. Materials and methods

2.1. Microalgae media and cultivation

Fresh water microalgae *C. vulgaris* was selected in this study because of its high growth rate and survived well in normal growth conditions. *C. vulgaris* was obtained from Algae Tech research institute Kuala Lumpur, Malaysia. *C. vulgaris* was cultivated in the modified Bolds Basal medium of composition: KNO_3, 1.25; KH_2PO_4, 1.25; $MgSO_4 \cdot 7H_2O$, 1; $CaCl_2$, 0.0835; H_3BO_3, 0.1142; $FeSO_4 \cdot 7H_2O$, 0.0498; $ZnSO_4 \cdot 7H_2O$, 0.0882; $MnCl_2 \cdot 4H_2O$, 0.0144; MoO_3, 0.0071; $CuSO_4 \cdot 5H_2O$, 0.0157; $Co(NO_3)_2 \cdot 6H_2O$, 0.0049; EDTA, 2; Na, 0.5 g/L in 500 mL. 30 mL/L of soil extract was added to the culture medium in order to obtain maximum growth of *C. vulgaris*. Batch cultivation of *C. vulgaris* was carried out in a 500 mL Erlenmeyer flask, aerated with fresh air through a 0.2 μm filter. The flask was placed under blue LED with illumination intensity adjusted to 200 μmol m^{-2} s^{-1} and a photoperiod of 12:12 light and dark cycles [13]. Temperature was controlled at 25 °C and the initial pH of the media was adjusted to 6.8 before autoclaving. All the chemicals and reagents were of analytical grade and purchased directly from Sigma Aldrich Malaysia.

2.2. Milking experiments

Batch experiments consisting of two sets of milking culture were conducted in a 500 mL of Erlenmeyer flasks with the experimental conditions described in Section 2.1. The whole set up were placed in an incubator. A set of controlled flasks without organic solvents were also incubated under the same cultivation conditions. Cultivation was done for the period of 4 days until it reached the appropriate cell density and culture entered into the stationary phase. After four days, cultures were placed on magnetic stirrers attaining the mixing speed of 120 rpm. Three different concentrations 10% v/v, 15% v/v and 20% v/v of the solvent-aqueous volume ratio of dodecane and tetradecane were loaded into the prefixed flask. After eight days of cultivation, the upper organic layer containing lipid and organic solvent were drawn carefully from each flask and stored for lipid analysis for the first cycle. For the second cycle, the cultures were again reloaded with the 10% v/v, 15% v/v and 20% v/v of organic solvents and allowed to milk the lipids in each respective flask. At the 12th day, the organic phase of the culture in each flask was removed and organic solvents of the same concentrations (10% v/v, 15% v/v and 20% v/v) were added to milk the culture for the third cycle. The same procedure was repeated after the next four days up to 20 days for two more milking cycles until the microalgae cultures were no more able to secrete lipids into the aqueous phase. The control without organic solvent was cultivated to indicate culture conditions. Each flask was labeled with the codes for analysis factors.

2.3. Biocompatible organic solvents

Biocompatible organic solvents used were dodecane and tetradecane. These organic solvents were selected on the basis of their hydrophobic nature and maximum solubility in the water. The hydrophobic nature is mainly defined in the terms of log p value. The parameter of log p is defined as the partition coefficient of a given solvent in a mixture of octanol and water [14]. The greater the log p value, the lower the polarity and the higher the biocompatibility of that solvent. Physical properties of the biocompatible solvents used in this study are given in the Table 1.

2.4. Microalgae growth

Optical density (OD) was employed to monitor the effects of organic solvents on microalgae growth. Effect of biocompatible organic solvent concentrations on optical density and growth rates were determined after every four days by using spectrophotometer at a wavelength of 680 nm (Shimadzu UV MINI 1241) for 24 days. 1 mL sample of culture was taken after each four days and diluted in distilled water. Culture samples were shaken for 30 s before readings were taken. Measurement of cell dry weight was done by taking 15 mL microalgae culture in a preweighed centrifuge tube, centrifuged at (1800*g*) for 20 min. Cells were washed with distilled water after removing the supernatant and dried at 80 °C. The dried cells were weighed after 4 days. Growth rates were measured by using the following equation:

$$\mu = \frac{\ln(N_2 - N_1)}{t_2 - t_1} \quad (1)$$

where N_2 and N_1 are the final and initial cell concentrations at time t_2 and t_1 respectively.

2.5. Measurement of crude lipid content

Lipid content of the control was determined by the conventional method utilizing water bath assisted extraction. 100 mg of microalgae biomass was mixed with chloroform- methanol in the ratio of 2:1 v/v and vortexed for 1 min. The mixture was heated and agitated in a water bath at 150 rpm for 40 min at 65 °C and then left to cool to room temperature. The mixture was then filled with deionized water, vortexed for 5 s and centrifuged at 4000 rpm for 5 min to achieve separation of the aqueous and organic phase. The solvent containing extracted lipid was evaporated by a rotary evaporator. The mass of the lipid obtained was determined gravimetrically.

2.6. Nile red fluorescence method

Nile red fluorescence method was applied for the determination of lipid content in microalgae as well as in solvent phase [15] by employing Perkin Elmer LS-55 fluorescence spectrophotometer. For the determination of microalgae lipid content, microalgae samples were obtained from the second set of experiments for milking under the same concentrations of organic solvents and same cultivation period. Appropriate amount of samples were taken and diluted with distilled water to achieve an OD680 of 0.4. The samples were pushed through syringe filter with 0.22 μm pore-size membrane so as to remove any microalgae cell residues. For the Nile red staining, 50 μL of Nile red (9-diethylamino-5H-benzo [a] phenoxazine-5-ones; Sigma, USA) in acetone representing a concentration of 0.1 mg/mL was added to the 1 mL of sample. The mixtures were pretreated using a microwave oven for 1 min. The excitation and emission wavelengths for the fluorescence were 490 and 585 nm respectively. The lipids used for calibration