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Lipid production by microalga *Micractinium* sp. IC-76 in a flat panel photobioreactor and its transesterification with cross-linked enzyme aggregates of *Burkholderia cepacia* lipase

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ABSTRACT

In this study, the high-lipid biomass accumulation of the microalga *Micractinium* sp. IC-76 in a 110-L flat panel photobioreactor was studied in detail. The productivity of microalgae biomass during phototrophic cultivation was 35.6 ± 1.5 mg L^{-1} d^{-1}. The maximum content of neutral lipids with a high saturated and monounsaturated fatty acid content (44.1%) was observed on the 17th day of cultivation, which was optimal for processing into biodiesel. The main fatty acids of lipids were C16:0, C16:2, and C18:2, with contents of 26.0, 15.6 and 29.8%, respectively. The enzymatic transesterification of *Micractinium* sp. IC-76 lipids using cross-linked enzyme aggregates (CLEAs) of *Burkholderia cepacia* lipase as biocatalysts was optimized by response surface methodology (RSM) to produce fatty acid methyl esters (FAMEs). This model determined that the optimum conditions for enzymatic transesterification of microalgal lipids with a maximal FAME yield of 92.3 ± 1.5% were a temperature of 38 °C, a methanol to oil molar ratio of 1:3.1, a CLEA of 9.1% and a water content of 2.5% relative to the oil weight. This model has a high correlation with the experimental data and can be used to model the production of FAMEs from the lipids of *Micractinium* sp. IC-76 during process scaling.

1. Introduction

Due to the depletion of fossil fuels and climate change, the most important directions of sustainable development include the rational utilization of natural resources and the reduction of the environmental burden [1,2]. Thus, the development of industrial processes for biofuel production from the renewable biomass is of high priority. Microalgae with a high lipid content represent a promising source of such biomass [3,4]. Some microalgae strains are capable of accumulating up to 20–50% of lipids by weight within their cells, making them suitable for biodiesel production. Several factors influence lipid accumulation, and lipid productivity varies depending on the strain [5]. The chemical composition of the lipid fraction of the microalgal biomass depends on the phase of growth [6], which subsequently affects the quality of the biofuel. According to the international standards ASTM D6584 and EN 14105, the main criterion for the production of high-quality biodiesel is the high degree of fatty acid saturation in the feedstock. Therefore, the study of the physiological and metabolic properties of microalgae strains is essential for the development of scaled production of renewable microalgal biomass and high-quality biodiesel [7,8].

Currently, the processing of the microalgal biomass into fatty acid methyl esters (FAMEs) is mainly performed by transesterification of the lipid fraction using alkaline [9] or acid [10] catalysts. However, this approach has a number of problems. For alkaline catalysts, an oil with a low content of free fatty acids (< 0.5% by weight) is required for transesterification due to the formation of soap [11]. In addition, the ideal oil must contain a low concentration of water. This leads to the deactivation of catalysts due to the appearance of emulsions at high levels of glycerol, which is produced as a by-product of hydrolysis. This approach also requires the separation of the catalyst from the reaction products, thus resulting in the generation of a significant amount of wastewater [12,13]. A more environmentally friendly and energy efficient method to produce biodiesel fuel involves the application of immobilized lipases as biocatalysts. Because thermostable bacterial lipases are most active at 30–60 °C [14,15], it is possible to perform transesterification at temperatures that are, on average, only half of those required for the homogeneous catalysts. In this respect, the transesterification process proceeds at a lower pressure, thus allowing for the repeated use of the biocatalyst during several cycles of operation [16,17]. Previously, a number of studies on the production of biodiesel were performed with various types of biocatalysts based on lipases of the bacteria *Burkholderia cepacia* and *Pseudomonas fluorescens*, the yeast

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Candida antarctica, and the fungi *Thermomyces lanuginous*, *Rhizopus oryzae*, *Penicillium expansum*, and *Geotrichum* sp. [18]. Among them, the commercial lipases from *B. cepacia* and *C. antarctica* are often used for the transesterification of vegetable oils due to their high activity and stability.

Lipases are preliminarily immobilized on a suitable carrier, thereby improving their stability and facilitating their separation from the reaction products after transesterification. The most common approaches for lipase immobilization include adsorption, covalent bonding, cross-linking and entrapment [19]. At the same time, the application of the cross-linked enzyme aggregates (CLEAs) is sometimes preferable to other methods of immobilization. Their advantages include high catalyst performance, lower cost due to a lack of need to use solid carriers [20] and a high stability (the activity of CLEAs can be maintained at a high level during several cycles of operation [21]). CLEAs of lipase were investigated mainly to produce biodiesel from vegetable oils. Studies of the transesterification of microalgal lipids with this type of biocatalyst are limited to the work of Lai [22], where the CLEAs of *P. expansum* lipase were used for lipid transesterification in the microalga *Chlorella pyrenoidosa*. The maximum yield of FAMEs was 85.7%, indicating that this approach shows great promise for the production of biodiesel from microalgal lipids. A number of studies have shown that CLEAs of *B. cepacia* lipase can also be effectively used for the production of biodiesel from vegetable oils due to the high activity and stability of these biocatalysts [23–25]. However, CLEAs of *B. cepacia* lipase have not been used for the transesterification of microalgal lipids.

Here, for the first time we show a combined approach to optimize lipid production by the microalga *Micractinium* sp. IC-76 and to process the resulting lipids into biodiesel by enzymatic transesterification using the CLEAs of *B. cepacia* lipase. The aim of this work was to study the process of lipid accumulation in the biomass of *Micractinium* sp. IC-76 in a 110-L flat panel photobioreactor and to optimize the enzymatic transesterification with CLEAs of *B. cepacia* lipase. The biomass and lipid contents, along with the fatty acid composition, and changes in their levels were studied. The optimal conditions for obtaining the maximum yield of neutral lipids were also assessed. After extraction, lipids were transesterified with the CLEA biocatalyst to produce FAMEs. Response surface methodology (RSM) was used to influence the temperature, the molar ratio of methanol:oil, and the amount of water, as well as the effect of the catalyst on the yield of FAMEs during the enzymatic transesterification of lipids of microalgae to obtain biodiesel. This study demonstrates the importance of harvesting microalgae when it has reached its optimal biomass composition, when it produces the maximal yield of FAMEs with CLEAs.

2. Materials and methods

2.1. Microalgae biomass production in the photobioreactor

Microalga *Micractinium* sp. strain IC-76 (GenBank accession number: MF629793) was isolated from freshwater samples from the Novosibirsk region (Russia). A flat panel glass photobioreactor (0.2 m × 1.5 m × 1.0 m) with an effective volume of 110 L was used for batch cultivation under indoor conditions (Fig. 1).

An air mixture with 1.5% CO_2 was supplied with a rotameter at a constant flow rate of 220 L h^{-1} through a porous tube in the bottom of the chamber. The inoculum culture was grown in flasks (1000 mL) aerated with air mixed with 1.5% CO_2 until the exponential growth phase was reached. Then, the culture was inoculated in a photobioreactor at an initial concentration of 0.1 g L^{-1} biomass (dry weight). *Micractinium* sp. IC-76 was cultured in Chu-13 medium [26] for 19 days at a temperature of 28 ± 1 °C. The photobioreactor was illuminated from both sides with six fluorescent lamps (cool light, TL-D 36 W/54-765, Philips, Netherlands) at 60 μmol m^{-2} s^{-1}. Microalgae cells were used for the subsequent enzymatic transesterification and were precipitated with polyaluminum chloride to a final concentration of

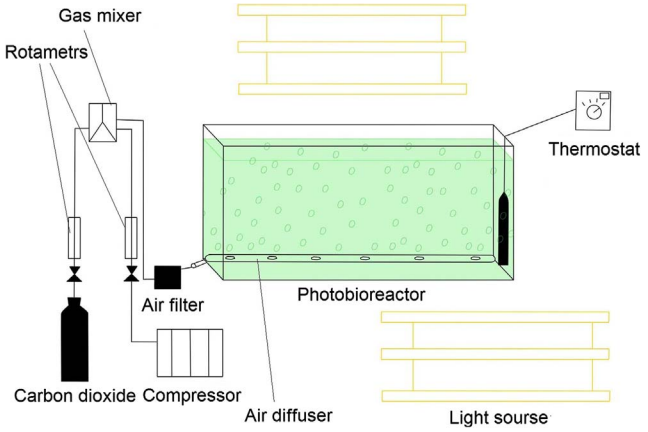

Fig. 1. The flat 110-L panel photobioreactor used for cultivation of *Micractinium* sp. IC-76.

120 mg L^{-1}. They were then washed twice with deionized water and separated by centrifugation on a Beckman Coulter Allegra X-12 centrifuge in a bucket rotor at 3000*g*. The resulting biomass was dried at 105 °C until a constant weight was reached.

2.2. Determination of the biomass dry weight

To estimate the accumulation of the biomass of the microalgae cells throughout the experiment, 500 mL of culture was washed twice with deionized water and then centrifuged at 3000*g* for 10 min. The resulting precipitate was dried for 24 h at 105 °C until a constant weight was reached. Then, the biomass dry weight was determined.

2.3. Estimation of growth rate and productivity

The specific growth rate was calculated using the equation: $\mu\ (d^{-1}) = (\ln X_2 - \ln X_1) \cdot (t_2 - t_1)^{-1}$, where X_1 and X_2 are the biomass concentrations (g L^{-1}) per day on t_1 (the beginning of the exponential phase) and t_2 (the end of the exponential growth phase), respectively. The doubling time was calculated based on the specific growth rate (μ): $T = \ln 2 \cdot (\mu)^{-1}$. The productivity of biomass and lipids was calculated in accordance with the equation: $P_{\text{biomass, lipids}}\ (\text{mg L}^{-1}\ \text{d}^{-1}) = (X_2 - X_1) \cdot (t_2 - t_1)^{-1}$, where X_1 and X_2 are the biomass (lipids) concentrations (mg L^{-1}) on day t_1 (the beginning of the exponential phase) and t_2 (the end of the exponential growth phase), respectively.

2.4. Extraction of lipid fraction

For lipid extraction, 1 g of dry biomass was added to 20 mL of a chloroform:methanol mixture (2:1 by volume). The mixture was sonicated for 10 min in an ultrasonic bath (100 W, 35 kHz) at 25 °C, followed by the addition of 0.25 vol of 0.9% NaCl solution with vigorous stirring. After phase separation, the organic phase was evaporated in a stream of nitrogen. The resulting lipids were further extracted with hexane to reduce the content of the unsaponifiable lipid fraction. To this end, 1 mL of hexane was added to the lipids after chloroform-methanol extraction. The mixture was shaken vigorously, and the whole suspension was centrifuged for 1 min at 1000*g*. After centrifugation, the liquid phase was transferred to a new tube and evaporated in a stream of nitrogen to a constant weight.

2.5. CLEAs preparation

CLEAs of *B. cepacia* lipase (Amano) were prepared as described [27], with slight modifications. Briefly, 2 mL of enzyme solution (25 mg mL^{-1} concentration, in 0.025 M phosphate buffer, pH 7.0) was mixed with 5 mg BSA and 0.008 mL of Triton X-100. Then, 4 mL of