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Comparison between several methods of total lipid extraction from *Chlorella vulgaris* biomass

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ABSTRACT

The use of lipids obtained from microalgae biomass has been described as a promising alternative for production of biodiesel to replace petro-diesel. It involves steps such as the cultivation of microalgae, biomass harvesting, extraction and transesterification of lipids. The purpose of the present study was to compare different methods of extracting total lipids. These methods were tested in biomass of *Chlorella vulgaris* with the solvents ethanol, hexane and a mixture of chloroform:methanol in ratios 1:2 and 2:1. The solvents were associated with other mechanisms of cell disruption such as use of a Potter homogenizer and ultrasound treatment. The percentage of triglycerides in the total lipids was determinated by the glycerol-3-phosphate oxidase-p-chlorophenol method (triglycerides monoreagent K117; Bioclin). Among the tested methods, the mixture of chloroform:methanol (2:1) assisted by ultrasound was most efficient, extracting an average of 19% of total lipids, of which 55% were triglycerides. The gas chromatographic analysis did not show differences in methyl ester profiles of oils extracted under the different methods.

1. Introduction

Given the current technological process and increased exploitation of new unconventional reserves (i.e., natural gas), it is probable that fossil fuels will continue to be available for a considerable period of time, although there may be variations in the supply and in the cost arising from geopolitical developments over time [1].

The steadily growing costs of petroleum, a desire for energy security in countries with limited petroleum resources and the inevitable depletion of fossil fuels are common concerns that have increased worldwide interest in biofuels [2]. In addition to these concerns, burning fossil fuels causes numerous environmental problems, including greenhouse gas (GHG) effects, which significantly contribute to global warming [3].

Microalgae are prokaryotic or eukaryotic photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to their simple cellular structure [4]. Known as one of the oldest life forms on the Earth, these microorganisms have a diversity of forms and ecological functions [5]. This diversity creates the capability for microalgae to be a valuable source in a multitude of products, such as value-added products for pharmaceutical purposes, food crops for human or animal consumption and as energy sources [4].

Microalgae biomass is considered a promising feedstock for producing a variety of renewable fuels, such as biodiesel, bioethanol, biohydrogen and methane [6,7]. Microalgae lipids have attracted attention as future raw materials for biodiesel synthesis, among others, because (1) microalgae have potential to attain higher lipid productivity in relation to oilseed crops [8]; (2) the biochemical composition of the microalgae biomass can be modulated by varying growth conditions, so the oil yield can be significantly increased [9]; (3) the microalgae biomass production can result in biofixation of waste CO_2 (1 kg of dry microalgae biomass utilizes about 1.83 kg of CO_2) [10]; (4) microalgae can be cultivated in brackish water or on non-arable land [11]; (5) the microalgae cultivation does not require application of herbicides or pesticides [12].

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The key processes involved in biodiesel production from microalgae are cultivation, harvesting, lipid extraction (cell disruption) and the transesterification of the lipids. Although all of these steps are essential, extraction is particularly important, as the contents of the extracted lipids are determined according to the disruption method and device. Therefore, the appropriate cell disruption method and device are keys to increasing the lipid extraction efficiency [13].

There are several reports of different methods for extracting lipids from microalgae, such as mechanical pressing, milling, supercritical fluid extraction, enzymatic extraction, microwave-assisted extraction, osmotic shock, homogenization, solvent extraction and ultrasonic-assisted extraction. The last three are evaluated here. While homogenization essentially involves using pressures to rupture cell walls, the solvent extraction entails extracting lipids by repeated washing or percolation with an organic solvent [14]. Some methods are usually used in combination with some kind of organic solvent. The application of ultrasound can enhance the extraction process due to a cavitation phenomenon. Ultrasonic waves create bubbles in the solvent, the bubbles burst near the cell walls, which produce shock waves, causing the release of lipid in the solvent [15]. All of these methods have their individual benefits and drawbacks.

According to numerous reports in the literature about lipid extraction from microalgae biomass, the method's efficiency depends on the species studied. But due to small number of studies with comparative analysis between these different methods, there have been no reports of the most efficient method of lipid extraction from *Chlorella vulgaris* biomass. The objective of the present study was to compare different methods of lipid extraction in relation to the total lipids and triglycerides.

2. Materials and methods

2.1. Microalga strain

The microalga *C. vulgaris* was kindly donated by the Dr. Armando Augusto Henriques of São Carlos University Federal (UFSCar). Brazil. The strain was preserved in tubes containing 8 mL of sterile WC medium [16] for each 2 drops of culture, which was removed with a sterile Pasteur pipette. The tubes were kept in a germination chamber under 20 μmol of photons m^{-2} s^{-1} and 21 ± 1 °C manually shaken every 48 h. The WC medium was composed of TRIS buffer (0.5 g L^{-1}), $NaNO_3$ (0.085 g L^{-1}), $NaHCO_3$ (0.0126 g L^{-1}), $CaCl_2{\cdot}2H_2O$ (0.03676 g L^{-1}), $MgSO_4{\cdot}7H_2O$ (0.03697 g L^{-1}), K_2HPO_4 (0.00871 g L^{-1}), 1% H_3BO_3 (0.1 mL L^{-1}), vitamin solution (1 mL L^{-1}) and trace metals solution (1 mL L^{-1}). The initial pH was adjusted to 8.5 with HCl 1 M. The vitamin solution was composed of thiamine (0.1 g L^{-1}), cyanocobalamin (0.0005 g L^{-1}) and biotin (0.0005 g L^{-1}), being filtered through a 0.22-μm membrane. The trace metals solution was composed of $CuSO_4{\cdot}5H_2O$ (0.0098 g), $ZnSO_4{\cdot}7H_2O$ (0.022 g), $CoCl_2{\cdot}6H_2O$ (0.01 g), $MnCl_2{\cdot}4H_2O$ (0.18 g), $NaMoO_4{\cdot}2H_2O$ (0.0063 g) and chelated iron (1 L). The chelated iron was composed of Na_2EDTA (4.36 g L^{-1}) and $FeCl_3{\cdot}6H_2O$ (3.5 g L^{-1}).

2.2. Obtaining microalgae biomass

To obtain the inoculum, the cells were grow in 500-mL Erlenmeyer flasks containing 300 mL of WC medium. The flasks were kept under constant agitation of 180 rpm, 100 μmol of photons m^{-2} s^{-1} and 25 ± 2 °C. The culture was cultivated until it achieved an optical density at 730 nm (OD_{730nm}) of approximately 0.8 (exponential phase). The inoculum obtained was transferred for clear 6-L bottles containing 5 L of WC medium. The cultures the feed batch were kept under pneumatic stirring, light intensity of 100 μmol of photons m^{-2} s^{-1} and room temperature of 25 ± 2 °C until they achieved an OD_{730nm} around 1.0 (stationary phase). The biomass obtained was harvested by centrifugation at 3500 rpm for 10 min, then lyophilized and stored at 4 °C until extractions.

2.2.1. Monitoring of biomass in culture medium

The microalgae grown in culture medium were monitored by the optical density of the culture at 730 nm. The growth curve was plotted from the biomass value measurement in OD_{730nm} and the growth phase determined when the growth curve was constructed in logarithmic scale. In order to maintain the inoculum, the microalgae were kept in exponential phase while the biomass obtained from extraction was harvested in stationary phase (about 25 days).

2.3. Lipid extraction

Four methods of extraction were tested: ethanol [17], hexane [3], chloroform:methanol (1:2) [18] and chloroform:methanol (2:1) [19]. All solvents used in the extractions were of HPLC grade and were obtained from commercial source (Tedia Brazil). In the methods using ethanol and hexane, we added a mass of 0.5 g of dry microalgae for each 20 mL of solvent (ethanol or hexane) at room temperature (25 °C). This mixture was submitted to ultrasonic bath working at 40 kHz and producing an ultrasonic intensity of 34.74 W/L (Unique model 1800 USC – Indaiatuba, Brazil, 3.8 L, internal dimensions: 30 × 15.1 × 10 cm) during 20 min. The flask containing the mixture was submitted to ultrasonic bath with the help of a metal support to be centralized and not touch in the bottom tank. Later, the sample was centrifuged at 2000 rpm for 5 min. The organic phase was carefully collected and the solvent evaporated with a rotary evaporator at 60 °C. The lipid fraction was dried to constant weight in an oven with air circulation at 30 °C.

In the method using chloroform:methanol (1:2) it was necessary to add 2 mL of distilled water for each 0.5 g of dry microalgae. A volume of 7.5 mL of the solvent mixture chloroform:methanol (1:2) was added to the wet biomass, and then 2.5 mL of chloroform and 2.5 mL of distilled water were added. This mixture was manually shaken during 3 min at room temperature. The biomass was harvested by centrifugation at 3500 rpm for 8 min at 4 °C. The organic phase was carefully collected and the solvent evaporated with a rotary evaporator at 50 °C. The lipid fraction was dried to constant weight in an oven with air circulation at 30 °C.

The extraction using chloroform:methanol (1:2) was realized again replacing the manual agitation by a Potter homogenizer or ultrasound at room temperature. In the first case, the mixture was processed in a Potter homogenizer (Tecnal model TE-099 – Piracicaba, Brazil, internal dimensions: 30 × 35 × 54 cm) at medium speed during 3 min, while in the second case the mixture was submitted to ultrasonic bath during 20 min.

In the methods using chloroform:methanol (2:1), we added a mass of 0.5 g of dry microalgae for each 36 mL of the solvent mixture at room temperature. This mixture was processed in a Potter homogenizer at medium speed during 3 min at room temperature. Later the sample was centrifuged at 3500 rpm for 8 min at 4 °C. The organic phase was carefully collected and transferred for another tube to which 9 mL of 0.88% KCl was added. At this moment, there were two phases and the upper phase was discarded with a pipette. Then 4.5 mL of chloroform:methanol:water (3:48:47) was added to the lower phase, so that two phases formed again. Again the upper phase was discarded with pipette. The washing with chloroform:methanol:water was repeated two times. The organic phase was carefully collected and the solvent evaporated