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Whole cell three phase bioreactors allow for effective production of fatty acid alkyl esters derived from microalgae lipids

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HIGHLIGHTS

- Feasible whole cell catalysis process to produce FAAE from microalgae lipids.
- The efficiency of the process is related to the complexity of carbon source used (lipids).
- The hyphae have an important role in mass transfer since they may acts as pipeline.
- Temperature affected the quantity of alcohol added, not the fungi activity.

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ABSTRACT

Lipids of the microalgae *Botryococcus braunii* and *Nannochloropsis gaditana* were used in a novel whole cell three-phase bioreactor (WCTB) using *Rhizopus oryzae* as biocatalyst for biodiesel (fatty acid alkyl esters, FAAE) production. The effects varying running conditions (the microalgae lipid, type of acyl acceptor, temperature, nutrient availability, alcohol to lipid ratio, and agar surface area exposed to gas-phase alcohol of varying convective fluxes) on the FAAE yield were studied and optimal conditions for the reactor identified. We found that the complexity of carbon source used (lipids) was related to the efficiency of the process, since it is used for both, growth and FAAE production, hence *N. gaditana* lipids were a more suitable raw material for FAAE production compared to *B. braunii* lipids due their higher content of fatty acid. The optimal operational conditions for our work were 20 °C and 8:1 alcohol to lipid volumetric ratio, where the best results was obtained using *N. gaditana* lipids as raw material and ethanol as acyl acceptor, reaching a FAAE yield of 92%. The results of our work indicate that both mass transfer process and the availability of nutrients are the most important variables in the performance of WCTB, where the hyphae have an important role in mass transfer since they may acts as 'pipeline', connecting different phases with the different components of the three phase bioreactor. These results should be considered as key design factors for developing the three phase bioreactor configuration.

1. Introduction

The synthesis of fatty acid alkyl esters (FAAE) from microalgae lipids remains to be a challenge [1]. Pertinent problems of FAAE production include the high water content in biomass, the necessity of biomass pretreatment, the high free fatty acid content (FFA), low lipids content and the high complexity of microalgae lipids [2]. Up to date the main efforts have focused on the development of chemical procedures where extreme operational conditions such as high methanol to lipids molar ratios are employed [3,4] restricting their economic application in large scale processes. It is therefore necessary to explore new avenues for the production of FAAE such as the application of enzymatic catalysts. The use of enzymatic catalyst can help (i) to reduce the quantity of methanol, (ii) to avoid problems associated to the presence of free fatty acids (FFA), and (iii) to circumvent environmental problem

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caused for the application of chemical catalyst [5]. Despite of such advantages, only few have explored enzymatic catalysts for FAAE production based on microalgae lipids up to date [6,7]. One reason may be that the use of highly purified lipase enzymes (glycerol-ester-hydrolases, E.C.3.1.1.3.) in FAAE production, is still limited by the high cost of the enzyme due to its complex isolation, purification and immobilization [8]. To circumvent this challenge and the restriction of high toxicity of the alcohols used Ciudad et al. [9,10], have developed a novel three phase bioreactor concept using *Rhizopus oryzae* as a whole cell biocatalyst. The WCTB consist of: (i) a solid agar phase to support the nutrients for growth and well-being of the biocatalyst, (ii) a lipid layer to promote lipase production in cells and (iii) an alcohol saturated gas flow to provide an acyl acceptor to the transesterification system. In this system is promoting the interaction between the different phases to support the microorganism growth and at the same time the FAAE production. This concept was proven to produce FAAE from vegetable oil reaching FAAE yields as high as 70 wt.%.

In the study presented we aim at demonstrating the feasibility of expanding the three phase bioreactor concept to FAAE production from microalgae lipids extracted from two different well kwon strains for biodiesel production, i.e. the fresh water microalgae *Botryococcus braunii* and the marine microalgae *Nannochloropsis gaditana*. The main process parameters of the system such as temperature, and alcohol to lipid ratio were evaluated in a broad range to provide useful information towards the scaling up of the operation. The effect of the type of acyl acceptor, surface area and nutrient availability in the performance of the WCTB was evaluated.

2. Material and methods

2.1. Production, extraction and characterization of microalgae and their lipids

The two microalgae *B. braunii* and *N. gaditana* were obtained from Desert Bioenergy SA, Chile. Both microalgae were cultivated according to Bazaes et al. [11]. In order to facilitate lipid extraction, the biomass was dried in a dried tunnel until 70–80 wt.% and finally milled. The dry matter content of the initial biomass was approximately 20 wt.%. Lipid extraction was performed during 6 h by using a Soxhlet systems [12] with petroleum ether (PE) as a solvent. The organic fraction (lipid) was concentrated by solvent removal in a rotatory evaporator and stored in darkness at 4 °C for its characterization and further experiments.

The lipids extracted were characterized regarding fatty acids composition. The fatty acid profile was determined by GC–MS, where the lipids were previously hydrolyzed and esterified as described in Araujo [13]. The acid value was determined by colorimetric titration [13].

2.2. Whole cell three phase bioreactor

2.2.1. Design and microorganism used

The WCTB consisted in a Petri dish (90 mm of diameter) with nutrient agar (solid phase) covered by a thin microalgae lipids layer (liquid phase) (Fig. 1A). The alcohol was added in gaseous phase using a small vessel with alcohol inside each Petri dish, where the alcohol evaporated during the experience and formed a saturated environment of alcohol inside the bioreactor [9,10]. For this study the fungus *R. oryzae* 4697 [8,14,15] (NBRC cultures, Japan) was used as whole cell biocatalyst. In order to support the fungus growth, the bioreactor included a solid phase, consisting in a nutrient agar adapted for Ciudad et al. [9,10] from Ban et al. [14,16] (70 g/L peptone, 1 g/L $NaNO_3$, 1 g/L KH_2PO_4, 0.5 g/L $MgSO_4$ $7H_2O$ and 1.5% agar, pH 7.4).

The fungus was inoculated 5 days before to start the experiment, for this, a thin layer of 250 μL of microalgae lipid was previously spread over the solid culture media inside each Petri dish; then a portion of 1 cm diameter of *R. oryzae* inoculums was cut and put into the bioreactor in sterile conditions. Once the Petri dish was completely colonized, additional 250 μL of microalgae lipids were distributed over the mycelia.

2.2.2. Operation conditions and calculation of FAAE yield

The experimental design included variations of temperature and alcohol to lipid volumetric ratio (v/v). The temperature was evaluated at 20, 30 and 40 °C. The alcohol to lipid ratio was evaluated at 4:1, 8:1 and 12:1(v/v). This design was applied to both different acyl acceptors (methanol and ethanol) and different microalgae lipids (*B. braunii*, and *N. gaditana*), respectively. All experiments were performed in triplicate. To evaluate FAAE production, samples were taken during the bioreactor operation. Hydrolysis of lipids was determined by measuring the acid value, whereas the occurrence of esterification was evaluated in terms of the behavior of acid value. Additionally, set of experiments at time zero were prepared in order to analyze both FAAE and FAA evolution. The experiment was carried out in triplicate under destructive sampling mode each 24 h using *B. braunii* and *N. gaditana* lipids. The experiment was evaluated using ethanol at 20 °C.

For methyl ester determination was used GC–MS methods. The results were compared based on the reaction yield. FAAE production yield was calculated using Eq. (1):

$$\mathrm{FAAE}_{yield}(\%\mathrm{wt}) : \frac{\mathrm{FAAE}(\%\mathrm{wt}) x L(\mathrm{g})}{\mathrm{TFA}(\%\mathrm{wt})} \quad (1)$$

where FAAE (wt.%) represents the FAAE percent calculated by GC–MS, L(g) corresponds to the gram of lipids used in the analysis and TFA corresponds to the total fatty acid content in microalgae lipid.

2.3. Design of WCTB operated at constant alcohol–air fluxes

To improve mass transfer in the process, a three phase bioreactor with alcohol addition by air stripping was implemented using the following operational parameters for FAAE production: 20 °C; 96 h duration, *N. gaditana* lipids and ethanol.

To supply the alcohol to the bioreactor, an air flow was injected through a diffuser to the bottom of a graduated test tube filled with alcohol producing alcohol saturated-air bubbles accumulating in the headspace (Fig. 1B). The alcohol–air containing headspace was introduced in the three phase bioreactor through an inlet parallel to the agar surface in order to produce a tangential gas flow over the liquid phase. The flow rate of alcohol added to the system was calculated by mass balance from the alcohol volume removed from the test tube and adjusted by the flow rate of air fed to the system. All experiments were evaluated with methanol at 3 different alcohol flow rates, i.e.0.05, 0.2 and 0.3 mL/h. The alcohol flow rates were established according to the results obtained on alcohol evaporation experiments.

The evaluation of both agar surface area and nutrients availability on the performance of the whole cell bioreactor was carried out by a set of four experiments where the amount of microalgae lipid supplied was identical in order to avoid carbon source limitation effects. The first experiment (E1) consisted in a sterile polystyrene Petri dish covered completely with enriched agar (56.7 cm^2 of total area and 15,670 mg of enriched agar media) inoculated with one patch *R. oryzae* (Ø: 1 cm, *h*: 4 mm). In the second experiment (E2) one patch of *R. oryzae* inoculum (Ø: 1 cm, *h*: 4 mm) was placed to one side of a sterile polystyrene Petri dish, and the rest of the Petri dish was filled with 130 cylinder of enriched agar (Ø: 5 mm, *h*: 4 mm) embedded in microalgae lipids (1327 cm^2 of total area