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Microcystis aeruginosa lipids as feedstock for biodiesel synthesis by enzymatic route

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ABSTRACT

The cyanobacterium *Microcystis aeruginosa* strain NPCD-1, isolated from sewage treatment plant and characterized as a non-microcystin producer by mass spectrometry and molecular analysis, was found to be a source of lipid when cultivated in ASM-1 medium at 25 °C under constant white fluorescent illumination (109 μmol photon m^{-2} s^{-1}). In these conditions, biomass productivity of 46.92 ± 3.84 mg L^{-1} day^{-1} and lipid content of 28.10 ± 1.47% were obtained. Quantitative analysis of fatty acid methyl esters demonstrated high concentration of saturated fatty acids (50%), palmitic (24.34%) and lauric (13.21%) acids being the major components. The remaining 50% constituting unsaturated fatty acids showed higher concentrations of oleic (26.88%) and linoleic (12.53%) acids. The feasibility to produce biodiesel from this cyanobacterial lipid was demonstrated by running enzymatic transesterification reactions catalyzed by Novozym® 435 and using palm oil as feedstock control. Batch experiments were carried out using *tert*-butanol and iso-octane as solvent. Results showed similarity on the main ethyl esters formed for both feedstocks. The highest ethyl ester concentration was related to palmitate and oleate esters followed by laurate and linoleate esters. However, both reaction rates and ester yields were dependent on the solvent tested. Total ethyl ester concentrations varied in the range of 44.24–67.84 wt%, corresponding to ester yields from 80 to 100%. Iso-octane provided better solubility and miscibility, with ester yield of 98.10% obtained at 48 h for reaction using the cyanobacterium lipid, while full conversion was achieved in 12 h for reaction carried out with palm oil. These results demonstrated that cyanobacterial lipids from *M. aeruginosa* NPCD-1 have interesting properties for biofuel production.

1. Introduction

Cyanobacteria have significant potential for producing a wide range of products including lipids, carotenoids, pigments, vitamins and aromatic compounds. The production of lipids is of particular interest since they can be used as feedstock for biodiesel synthesis [1–3].

Microalgae and cyanobacteria have simple cellular structure, higher rates of biomass and oil production than conventional crops. According to Chisti [4], algae have been claimed to be up to 20 times more productive per unit area than the best oil-seed crop. Other advantages of algae and cyanobacteria are that they can be grown in marginal areas such as on arid land or potentially in the ocean, reducing competition with food crops for agricultural land and freshwater [1–3]. Regarding algae, some species produce large quantities of vegetable oil as a storage product, regularly achieving 50–60% dry weight of lipid [2,3,5]. Depending on species, algae produce many different groups of lipids, hydrocarbons and other complex oils [2,6]. However, not all algal oils are satisfactory as feedstock for biodiesel. The quality and characteristics of biodiesel are strongly dependent on the composition of the oil source used in its production. Raw material that contains a greater amount of saturated fatty acids, which makes transesterification difficult, resulted in problems, such as high melting point and high viscosity [7]. According to the European Standard EN 14214, a limit of 1–12% of unsaturated fatty acids, with minimal of polyunsaturated, is appropriate for good biodiesel quality [8]. Comparing to algae, cyanobacteria have a much simpler lipid and fatty acid composition. Murata and Nishida [9] have divided cyanobacteria into four groups based on the original fatty acids classification of Kenyon [10]. The group of *Anacystis nidulans* synthesizes only saturated and monounsaturated acids. The other three groups (*Anabaena variabilis*, *Synechocystis* sp. 6714 and *Tolypothrix tenuis*) synthesize polyunsaturated linoleate and, in addition, accumulate γ-linolenate, α-linolenate, and (*n*−3) octadecatetraenoate, respectively [11].

Although cyanobacteria have potential as feedstock for biodiesel, many of them produce bioactive natural compounds,

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such as microcystins [12], saxitoxin [13], anatoxins [14,15], and lyngbyatoxins [16], that can be harmful to human health. In this way, screening studies for non-toxin producer must be conducted before recommending cyanobacterial strains as feedstock source for biodiesel.

In a previous work, five cyanobacterial strains were screened for their lipid production by evaluating biomass productivity and lipid content [17]. According to these results the strain *Microcystis aeruginosa* NPCD-1 isolated from sewage treatment plant was found to be a good source of lipid. In addition, mass spectrometry and molecular analysis were performed to verify the ability of *M. aeruginosa* NPCD-1 to synthesize microcystin, a common hepatotoxin found in *Microcystis* genus. Microcystin is a cyclic peptide and is the most studied cyanotoxin worldwide. Nearly 90 structural variants of microcystin have been isolated and characterized so far [18]. Microcystins are powerful inhibitors of protein phosphatases 1 and 2A in eukaryotic cells, causing fatal hepatic hemorrhage in mammalians [19]. These compounds have been implicated in human deaths, animal poisonings, and linked to liver cancer in humans [20,21].

To check the feasibility to produce biodiesel from this cyanobacterial lipid, enzymatic transesterification analysis was carried out using palm oil as control under the same reaction conditions. Palm oil was chosen for control parameter since this oil is the third most widely produced edible oil, representing 10% of the global biodiesel raw material source, after rapeseed (59%) and soybean oil (25%) [22]. In addition this feedstock has been already successfully used in biodiesel synthesis employing different catalysts, including also immobilized lipases [23].

2. Experimental

2.1. Materials

The experiments were carried out with the cyanobacterium strain *M. aeruginosa* NPCD-1 maintained in the culture collection of the Molecular Ecology of Cyanobacteria Laboratory (CENA/USP – Piracicaba, SP, Brazil). The enzyme Novozym® 435 manufactured by Novozymes and acquired from Sigma–Aldrich was used as catalyst for biodiesel synthesis. Refined palm oil kindly provided by Agropalma (Para, Brazil) was used in the reaction control, having the following composition in fatty acids (%, w/v): 0.1 lauric, 1.2 myristic, 46.8 palmitic, 3.8 stearic, 37.6 oleic and 10.5 linoleic, with 849.0 g mol^{-1} average molecular weight. All other reagents were of analytical grade.

2.2. Cyanobacterium growth conditions

Cyanobacterium cells were grown in ASM-1 liquid medium [24] sparged with sterile air and maintained at 25 °C under constant illumination by white fluorescent light (40 μmol photon m^{-2} s^{-1}) for 20 days. The cyanobacterium culture was inoculated at 10% (v/v) in flasks of 9 L containing ASM-1 liquid medium sparged with sterile air and maintained at 25 °C under constant white fluorescent illumination (109 μmol photon m^{-2} s^{-1}) during 8 days. Nitrogen ($NaNO_3$) and phosphorus (K_2HPO_4/Na_2HPO_4) concentrations in the ASM-1 medium were modified as shown in Table 1 to improve biomass productivity and lipid accumulation.

2.3. Harvesting and drying

Cyanobacterial cells were harvested by centrifugation at 6000 × *g* for 3 min. Cell pellets were washed with distilled water and then lyophilized at −40 °C and a pressure of 50 mmHg.

Table 1
Culture medium composition for growth of the *M. aeruginosa* NPCD-1.

Cultivation run	N-source $NaNO_3$ (mg L^{-1})	P-source K_2HPO_4/Na_2HPO_4 (mg L^{-1})
1	85	22
2	85	66
3	255	22
4	255	66
5[a]	170	44

[a] Values according to ASM-1 standard medium.

2.4. Mass spectrometry (MS) analysis of microcystin

The lyophilized biomass (0.2 g) of *M. aeruginosa* NPCD-1 was resuspended in 2 mL of methanol and glass beads (Ø6 mm) were added and vortexed for 1 min in order to disrupt the cells. After that the tube was centrifuged at 9000 × *g* at 4 °C for 5 min. The supernatant was collected and evaporated. The MS analysis was carried out following the procedure described by Silva-Stenico et al. [25].

2.5. Search for microcystin synthetase genes

Total genomic DNA was isolated from cells of cultured *M. aeruginosa* NPCD-1 using a modified cetyl-trimethyl-ammonium bromide (CTAB) based extraction method adapted for cyanobacteria [26]. The presence of *mcyA*, *mcyD*, *mcyE* and *mcyG* genes involved in microcystin biosynthesis was searched in the genome of *M. aeruginosa* NPCD-1 using specific oligonucleotide primer sets MSF/MSR [27], mcyDF/mcyDR and mcyEF2/mcyER4 [28] and mcyGF/mcyGR [29]. The PCRs and thermal cycles used were the same described by the primer authors. The *M. aeruginosa* SPC777 was used as a reference strain for microcystin production.

2.6. Lipid extraction

Total lipids were extracted using the methodology described by Folch et al. [30]. The lipid extracted was dried in a rota-evaporator to remove remaining residues of solvent and subsequently dried at 60 °C to constant weight. The total lipids were measured gravimetrically, and then lipid contents and yields were calculated. Analysis of fatty acid composition was performed in a capillary gas chromatography (CGC Agilent 6850 Series GC System) and fatty acid composition was determined according to AOCS procedure 2-66 [31].

2.7. Biodiesel synthesis

Biodiesel syntheses were performed in closed glass reactors with a capacity of 25 mL containing the cyanobacterial lipids and anhydrous ethanol at fixed molar ratio of 1:12. The mixtures were incubated with the commercial preparation of lipase from *Candida antarctica* Novozym® 435 at proportions of 10% (w/w) related to the total weight of reactants involved in the reaction medium. Iso-octane and *tert*-butanol were tested as solvents: 1:1 (w/w) in relation to the reaction medium. The ethanolysis of palm oil under the same reaction conditions was taken as control. Reactions were performed for a maximum period of 48 h under constant magnetic agitation of 150 rpm at 50 °C. An aliquot of reaction medium was taken at various time intervals and analyzed by gas chromatography. The analyses were performed in duplicate.

2.8. Monitoring ethyl esters

The ethyl esters formed in the ethanolysis reaction were analyzed in FID gas chromatography (Varian CG 3800, Inc. Corporate Headquarters, Palo Alto, CA, USA) using a 5% DEGS CHR-WHP