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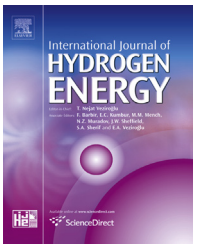

Selection and identification of microorganisms present in the treatment of wastewater and activated sludge to produce biohydrogen from glycerol

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ABSTRACT

One of the major challenges for the coming years is to develop alternative forms of producing sustainable energy. Biodiesel has shown to be an option in substituting fossil fuels. It is produced by transesterification of a fat and a monoalcohol, thus releasing glycerol, which corresponds to 10% of the reaction volume. Large increments in biodiesel production will result in proportional volumes of crude glycerol. Studies have shown that strict anaerobic bacteria and fermentative bacteria are able to produce biohydrogen, a high-energy fuel, which does not generate gaseous pollutants during its combustion. Given the importance of increasing the added value of crude glycerol, the purpose of this study was to isolate and characterize bacteria found in reactors of wastewater and activated sludge treatment able to produce biohydrogen from glycerol. Fifteen bacterial species able to grow in medium with glycerol were identified by sequencing of the 16S rRNA gene, of which nine species were found to be able to produce biohydrogen: *Enterobacter ludwigii*, *Shigella sonnei*, *Bacillus licheniformis*, *Bacillus amyloliquefaciens*, *Staphylococcus warneri*, *Alcaligenes faecalis*, *Bacillus subtilis*, *Bacillus atrophaeus*, and *Citrobacter freundii*. The isolates of *Bacillus amyloliquefaciens* showed higher yield in biohydrogen production with values of 0.50 ± 0.20 mol H_2/mol of glycerol. The results indicate that there is a great potential for selecting biohydrogen-producing bacteria in the wastewater evaluated.

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Introduction

One of the major challenges for the coming years is to find alternatives for the production of clean and sustainable energy, aiming at replacing fossil fuels from non-renewable sources, such as coal, oil, and natural gas. In this point, the hydrogen appears how a very friendly and sustainable fuel for some reasons, like a wide range of substrate namely solid or liquid waste and industrial subproducts. A typical industrial subproduct in Brazil is the glycerol due to biodiesel production program like an alternative option to the gasoil from the fossil source. According Santos (2012) [24] the Brazilian biodiesel production in 2035 will be around 40 million cubic meter, only with transesterification process and the worldwide production will overpass 100 million cubic meter. With the worldwide increased use of biofuels, products such as glycerol have accumulated in the environment [8,36]. For every nine tonnes of biodiesel produced, around one tonne of crude glycerol is formed [10,14]. For 2020, it is estimated that the world production of glycerol will reach three million tonnes, while its demand will not exceed 500 thousand tonnes [20]. Therefore, we have to look for alternative ways to utilize the glycerol in order to ensure an increase in the production of biodiesel [18].

The biotechnological processes have become an option for the production of energy using industrial waste such as glycerol. The anaerobic microbiological fermentation requires little energy for generating byproducts that can be used as a source of energy, e.g., hydrogen gas. Studies show that the use of glycerol as carbon source in fermentations could be more advantageous compared to the substrates traditionally used [21].

The production of biohydrogen by bioconversion of crude glycerol may be a good alternative for using glycerol from biodiesel production, which tends to accumulate in the environment. Hydrogen has a high-energy content and is a source of clean energy and a potential alternative to fossil fuels, which are becoming more and more depleted [5,25].

In sludge and effluents, it is easy to find microorganisms well adapted to extreme survival conditions, e.g., pH extremes, high levels of organic matter, high temperatures, etc. These microorganisms have a natural ability to convert organic matter into products of industrial interest. Few studies have revealed the content of these communities in tropical countries such as Brazil. In this context, the purpose of this study was to isolate and characterize bacteria from reactors treating wastewater and activated sludge able to produce biohydrogen from glycerol.

Materials and methods

Substrates

The substrates used in the study were crude glycerol obtained from the production of biodiesel by an oil plant (donated by Ergostech Renewable Energy Solutions) and commercial glycerol of the brand *Simoquímica*®.

Physical and chemical analysis of the substrates

The physical chemical analysis of the substrates was performed according to the *Standard Methods for Examination of Water and Wastewater* [33], analyzing total COD (chemical oxygen demand), calcium, iron, magnesium, potassium, and sodium.

Microorganisms isolation

The microorganisms were isolated from six different samples of aerobic sludge and effluents: two sludge samples from an oil refinery, one sample of agro-industrial sludge, one composting sample, and two sludge samples from a pig farm.

The different samples were centrifuged for 2 min at 10,000 rpm, and 1 mL of the supernatant was transferred with sterile syringes to 20-mL glass bottles, closed with rubber covers and aluminum seals, containing 10 mL of culture medium (previously autoclaved at 120 °C for 20 min at 1 atm) (g/L): 4 $(NH_4)_2SO_4$; 0.52 K_2HPO_4; 0.25 KH_2PO_4; 0.2 $MgSO_4.7H_2O$; 1.5 of yeast extract; 1 of bacteriological peptone, 30 of commercial glycerol or crude biodiesel glycerol, 1 mL of solution containing trace elements (g/L): 1 $MnCl_2.4H_2O$; 0.06H_3BO_3; 0.037 $CuSO_4.5H_2O$; 0.2 $CoCl_2.6H_2O$; 0.025 $NiCl_2.6H_2O$; 0.035 $Na_2MoO_4.2H_2O$; 0.14 $ZnSO_4.7H_2O$ and 0.9 mL HCl (37%). The pH of the medium was kept at 6.8. To ensure an anaerobic environment, nitrogen gas was bubbled in the culture medium for 5 min. The flasks were kept overnight in an orbital shaker (140 rpm) at 37 °C and then transferred to an incubator for 24 h at 37 °C. After the growing time, the direct sowing was done using a platinum loop in the same medium mentioned above, with the addition of 20 g/L of agar. The plates were maintained in an anaerobic jar, with an anaerobic generator (Anaerobac, *Probac do Brasil*) for 48 h at 37 °C. Samples of microbiological culture underwent Gram staining and were observed under optical microscope (Zeizz® – Axiostrar Binocular Microscope).

Extraction and amplification of the genetic material

The extraction of the genetic material of the bacteria was performed with the Illustra bacteria genomicPrep Mini Spin kit (*GE Healthcare*), according to the manufacturer's protocol. The extraction was quantified by spectrophotometry (260 nm and 280 nm), confirmed by electrophoresis in agarose gel (1.5% w/v) with the addition of GelRed™ nucleic acid gel stain (Biotium, 5x concentrated solution) and visualized under ultraviolet light.

The amplification of genetic material was performed by Polymerase Chain Reaction (PCR) with the Phusion™ High-Fidelity PCR kit (Finnzymes), according to the manufacturer's protocol. We used standard primers for the domain *Bacteria* of the subunit 16S rRNA: ″16S-F 5′ – CCTGGCTCAGGACGAACGCTGG-3′ and ″16S-R 5′ – CTG CGCTCGCTTTACGCCAAT-

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