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Microbiological Strategies for Sustainable Production of Bioactive Compounds Using Oil Produced Water Effluent with Sucrose and Crude Glycerine as Carbon Sources

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Biopolymers and biosurfactants are bioactive compounds of great commercial interest. They are used for changing fluids viscosity and emulsifying properties of several types of solutions, characteristics necessary for the performance of enhanced oil recovery (EOR) strategies applied at distinct rock formations. The present work evaluated the production of *Enterobacter amnigenus* exopolysaccharide (EPS) of the strain grown alone or in consortium with *Pseudomonas aeruginosa*. Culturing was carried out using produced water amended with a mixture of crude glycerin and sucrose as carbon sources. *E. amnigenus* exopolysaccharide production was low when grown on pure culture, on the other hand, in the presence of *P. aeruginosa*, the amount of EPS was of 14.74 gL^{-1}. The produced biopolymer and biosurfactant exhibited excellent viscosity and emulsifying activity characteristics for the application in EOR strategies, and better pseudoplastic rheological behavior (was detected) than conventional chemicals used for this purpose. Statistical analysis showed that EPS production was influenced by the interaction between sucrose and glycerin amendments. Infrared absorption spectroscopy showed similar molecular structure to the *E. amnigenus* FucoPoL. This research shows that a recovering oil fluid can be prepared using produced water as main medium, contributing for reducing costs and increasing environmental gains.
Keywords: Exopolysaccharide; biosurfactant; produced water; oil recovery.

1. Introduction

Due to rising energy demands, it becomes a challenge to recover oil from declining productive fields considered mature. Currently, most of the techniques used for this purpose are expensive, unsustainable, and technically (technologically) difficult to deploy because the oil is located in regions of difficult access, most of which remain inside the reservoir (Sarafzadeh et al., 2014). Special technologies for advanced oil recovery (EOR) with environmental concerns have many advantages compared to other recovery processes because they are efficient, ecologically correct and versatile. By also using products with physicochemical properties equal to or higher than products of synthetic origin, it is easier for implementation and there is an increasing interest in improving the productive efficiency (Patel et al., 2015; Varjani and Upasani, 2017).
Based on the advantages of EOR, there are different types of microorganisms capable of producing a variety of bioactive bioproducts including biosurfactants, biopolymers, acids, gases and solvents. They are capable of reducing interfacial tension (IFT) between brine and petroleum and therefore reduce the capillary forces that capture oil in rock pores. Additionally, they decrease the IFT between the oil and the aqueous phases necessary for the mobilization of residual hydrocarbons, induce the alteration of the wettability and bonding permeable zones and reduce the viscosity of the oil, among other things (Deng et al., 1999; AL-Wahaibi et al., 2014).

Among the most used bioactive are biopolymers and biosurfactants, that have been used for several industrial applications in recent years, which has led to a growing interest in the production of exopolysaccharide and rhamnolipid produced by microorganisms such as *Enterobacter amnigenus* and *Pseudomonas aeruginosa*, respectively.
This technology refers to recovery methods that involve the manipulation of the functions and / or structures of mixed populations of environmental microorganisms existing in oil environments that facilitate the transport of oil and extend the life of the reservoirs for prolonged exploration of one of the largest sources of energy on the planet (Almeida et al., 2004, Lazar et al., 2007). Furthermore, it is also based on the metabolic versatility of microorganisms, which may be isolated or in consortia (Almeida et al., 2004).
The use of bioactive for oil recovery is not yet fully feasible due to the cost of basic inputs such as sucrose and alcohol. In Brazil and in other countries, the cost of the fermentation represents a critical factor in the commercial aspect of production (Pradella, 2006), although there are also many byproducts.
Because of results like this, the use of residues as low cost alternative substrates in bioprocesses has been increasing (Henkel et al., 2014; Deepika et al., 2016). The use of these residues allows for the reduction of production costs and minimizes environmental problems, since their use can lower the generation of effluents, such as the use of crude glycerin as a source of carbon, helping to reduce sucrose and produce water at the same time.
With a cost-efficient potential, this research shows a good alternative to increase oil recovery in a sustainable way, since its process can substitute techniques and chemicals used as well as incorporate produced water, recoverable oil fluid and crude glycerin, a by-product of the energy industry. As a substrate and nutrient used in the environment for the production of bioactive, it contributes to reducing costs in the production process and increasing environmental gains.

2. Methodology

2.1 Obtaining, maintaining and activating microorganisms

Strains obtained were *Enterobacter amnigenus* CCMICS *549* and *Pseudomonas aeruginosa* CCMICS 109, from the culture collection of LABEM (Laboratório de Biotecnologia e Ecologia de Micro-organismos), of the Instituto de Ciências da Saúde (ICS-UFBA, Salvador, BA, Brazil). The strains were preserved through freezing, and stored at -70°C in a Thermolectron (2006) ultra-freezer. At the time of use, both strains were activated separately overnight in a in a tryptic soy broth, incubated at 30°C for 24 hours. After this period, the bacterial inocula were prepared.

2.2 Bacterial inocula

After activation, the bacterial inoculum of *Enterobacter amnigenus CCMICS 549* was prepared by aseptic transfer of 1 mL of the activated cells to 250mL Erlenmeyer flasks containing 50mL of sterile TSB broth. The inoculum was then incubated in an orbital agitator at 120 rpm, at 30°C ± 2°C, until it reached an optical density of 2.2 (OD_{560nm} = 2.2), which corresponded to the optimal log-phase of the strain. That absorbance band is attained at 04-12 hours of incubation, which corresponds to a concentration of approximately 10^8 $CFU.mL^{-1}$.
The preparation of the *Pseudomonas aeruginosa* CCMICS 109 inoculum and the media were the same as for *Enterobacter*, and incubated until OD_{560nm} = 2.7, which corresponded to the optimal log-phase of the strain. That absorbance band is attained at 12-20 hours of incubation, corresponding to a concentration of approximately 10^{10} CFU. mL^{-1}.

2.3 Production of bioactive compounds

Production of the bioactive compounds was carried out in 250mL Erlenmeyer flasks containing 50mL of the production medium. The production medium was composed of produced water (PW) (pH 7.0) supplemented with the following nutrients: $5.0g.L^{-1}$ potassium phosphate monobasic; $0.5g.L^{-1}$ ammonium carbonate; $0.1g.L^{-1}$ yeast extract; 0.1 $g.L^{-1}$ sodium glutamate. Equal parts of sucrose and raw glycerine were added as carbon sources. The use of raw glycerin reduced the amount of sucrose. The production medium was subsequently pasteurized at 65°C for 30 minutes. After pasteurization two flasks were inoculated, one with 5.0mL of the *E. amnigenus* for isolated production and the other to the simultaneous production of the bioactive compounds was inoculated with 5.0mL each of bacterial suspension. Incubation for both flasks was conducted in an orbital shaker at 180 rpm, at 30°C ± 2°C, for 72 hours. After 72 hours, exopolysaccharide production, viscosity of the cell-free broth, rhamnolipid production and emulsification index of the cell-free broth were evaluated. Production runs were carried out in triplicate, and the statistical method of response surface was performed using ANOVA in order to optimize the production from operational conditions.

Analytical determinations

2.4 Exopolysaccharide

The quantification of exopolysaccharide was performed gravimetrically: 96°GL ethanol was added (1:3 v/v) to the cell-free fermented broth, then centrifuged at 7000 rpm for 30 minutes at 4°C, and the exopolysaccharide recovered and weighed. The biopolymer recovered was dried in an oven at 50°C, in order to obtain the dry weight (García-Ochoa et al., 2000).

2.5 Viscosity

The viscosity of the cell-free broth was measured in a Brookfield model LVDV III + rheometer, in a double boiler, utilizing the adapter for small samples, spindle 18, which allows the shear rate to be varied from 0 to 264 s^{-1}, and the viscosity measured from 1.3 to 30,000 cP. The readings were made by fixing the shear rate at 1.0 s^{-1}. The measurement units utilized were: centipoise (cP=mPa.s), for the apparent viscosity, 1/second (s^{-1}) for the shear rate, and dyne/square centimetre (D/cm^2) for the shear stress.

2.6 Rhamnolipid

The biosurfactant produced was measured by the thioglycolic colorimetric method, expressed in rhamnose, according to Chandrasekaran and Bemiller (1980). The concentration of rhamnose was determined based on the difference between the two wavelengths (400 and 430nm), using the calibration curve previously obtained with commercial rhamnose. The difference was used because the absorbance value obtained at 430nm suggests interference from other sugars. The type of calibration curve utilized was the standard addition method, since the medium was complex, with known quantities of commercial rhamnose (0 to 80mg.L^{-1}) added to the cell-free broth. The determination of the rhamnose was achieved by extrapolation of the analytical curve to the x axis (concentration), obtaining the scalar value. The value obtained was multiplied by 3.4, which furnishes the rhamnolipid value in terms of rhamnose, according to Raza et al. (2007).

2.7 Emulsifying activity

The emulsifying activity of the rhamnolipid was determined according to Cooper and Goldenberg (1987). In a test tube (1.8 x 15cm), a mixture of 2mL of mineral oil and 2mL of cell-free broth was agitated in a vortex agitator for 2 minutes, and the emulsion formed was left to stand. The resulting emulsion index obtained corresponds to the height of the emulsified layer divided by the total height of the liquid, times 100.

Partial characterization

2.8 FTIR

The FTIR spectrum was determined using a KBr insert method and Fourier transform infrared spectroscopy operating in a spectral window of 500-4000cm^{-1} presented in absorbance mode.

2.9 Viscosity

The apparent viscosity of the aqueous solutions was obtained with a Brook field LVDV III+(Brook field, USA)+rheometer, coupled to a water bath and small spindle adapter 18, which allows varying shear rates from 0 to 264s^{-1} and evaluation of the viscosity in the range of 1.3 to 30000cP. All readings were performed at 25ºC and solutions with concentrations of 1%. The kinetic model of Ostwald-de Waele: $\mu=K.(\gamma)n^{-1}$ was used to evaluate the effect of the shear rate on the viscosity of the solutions.

2.10 Rheological experiments

The effect of the rheological behavior of the samples was measured by the Physicometer MCR 501 - ANTON PAAR, equipped with a Peltier temperature control device configured to simulate the measurements at 60°C, at a shear rate of 0 to 1000s^{-1}.

3. Results and discussion

3.1 Production of bioactive compounds

The production of exopolysaccharides from the E. amnigenus after optimization through the statistical analysis surface response, as shown in Figure 1, reached 4.51g.L^{-1} with 72 hours of production. After analyzing the operational conditions that showed a direct influence on the cellular metabolism, increasing the productive performance of the isolated production of the strain under study and simultaneous production with the *P. aeruginosa* strain was achieved reaching 14.74gL^{-1} of exopolysaccharides and 1.75gL^{-1} of ramnolipid. This presented a positive correlation for the interaction of co-culture of bacteria producing bioactive compounds.

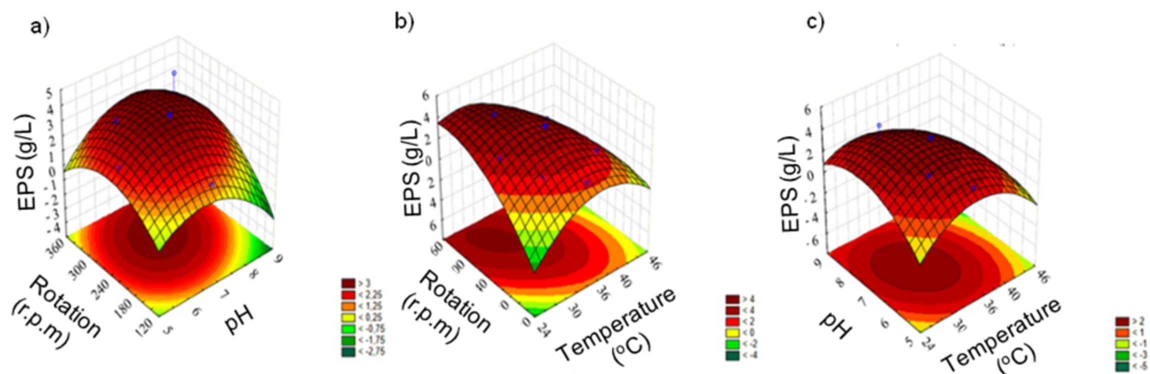

Figure 1 - Three-dimensional response surface graphs for the production of EPS by Enterobacter amnigenus as a function of: (a) rotation and pH; (b) rotation and temperature; (c) pH and temperature.

3.2 FTIR spectrum analysis

In Figure 2, the FTIR spectrum shows the well defined peaks found between 1250-980cm-1 represent skeletal vibrations of C-O and C-C bands of glycosidic bonds (Synytsya et al., 2003), commonly found in carbohydrate and lipid molecules. According to Daverey and Pakshirajan (2009) and Wadecar et al. (2010) the presence of a characteristic band of carbonyl stretch with medium intensity can also be observed in many spectra of biosurfactants and esters. The two bands observed around 1648-1418cm^{-1} can be attributed to the asymmetric and symmetric stretches of carboxylates, respectively (Synytsya et al., 2003). According to Freitas et al. (2011) these same bands were also observed in commercial FucoPoL spectra and fucose-containing EPS spectra obtained through an *Enterobacter* A47 strain.

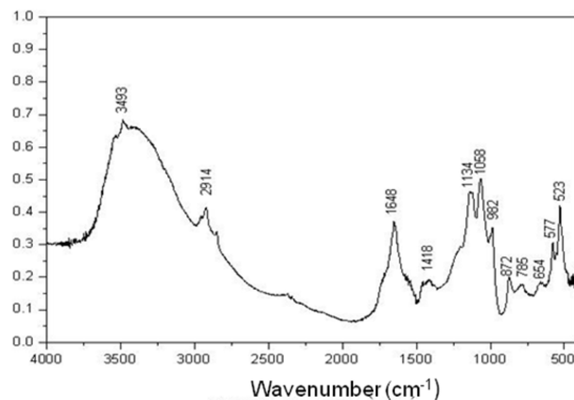

Figure 2 - Infrared absorption spectrum of the bioactives produced by P. aeruginosa and E. amnigenus.

3.3 Emulsifying activity

The emulsifying activity is a property that allows the formation of emulsions in which it is established that a stable emulsion must present results of the emulsification index (EI) after 24 hours, greater than 50%, for efficient application in industrial processes (Willumsen and Karlson, 1997; Haba et al., 2000). Emulsification index (EI) is also directly associated with the production of biosurfactant (Rajeshwari, Prakash and Ghosh, 1995).The results presented in this study show values of I.E. varying between 57 and 66% for more than 48 hours, for the tests involving simultaneous production with *E. amnigenus* and *P. aeruginosa* strains. In the literature consulted, no reports of the emulsifying activity of the simultaneous production by different microorganisms were found. This indicates a possible pioneer character found in this study.

3.4 Viscosity

According to Figure 3, the solutions of the bioactives such as exopolysaccharide and rhamnolipid analyzed in this work, behave according to the described model in which all values of n were smaller than one unit, which confirms the pseudoplastic behavior. The value of k indicates the degree of resistance of the fluid to the flow, so the higher the k value, the more viscous the fluid will be.

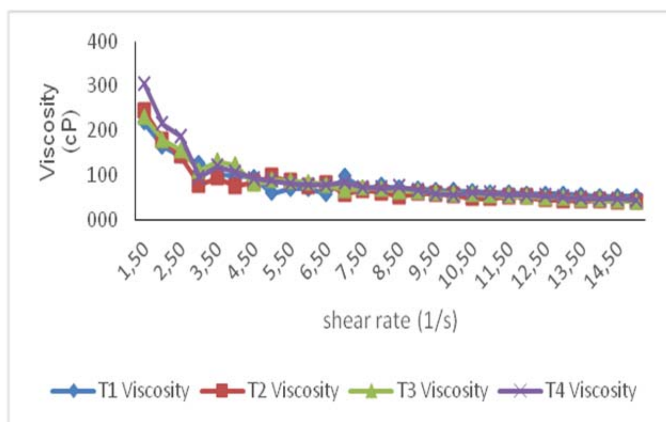

Figure 3 - Apparent viscosity versus shear rate (25°C, 25 sec-1.1% (m/v) of the bioproducts produced from the optimized conditions in the base water and crude glycerin medium by E. amnigenus and P. aeruginosa.

3.5 Rheological analysis

Through Figures 4 and 5, it is possible to identify a zone of linear viscoelasticity for voltages close to 5 Pa for commercial xanthan and bioactive solutions at 60°C. Both solutions present a mechanical spectrum with a behavior normally attributed to polymer solutions with interlaced polymer chains. This same viscoelastic behavior was observed by Torres et al. (2011) when studying a biopolymer rich in fucose.

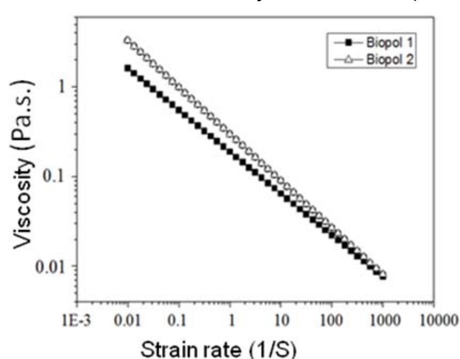

Figure 4 - Apparent viscosity versus strain rate of fluids with commercial xanthan (Biopol 1) and bioactive solution (Biopolymer and RL) (Biopol 2) at 60 ° C.

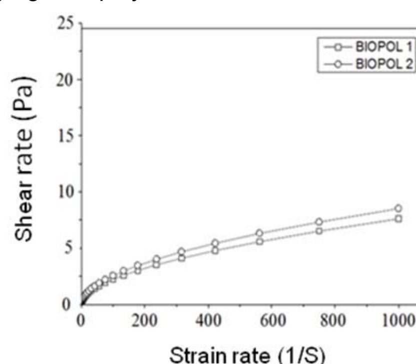

Figure 5 - Shear stress versus shear rate deformation of the fluids with commercial xanthan and solution (Biopolymer and RL) at 60°C.

4. . Conclusion

Under optimized operating conditions, bioactive compounds (which can be used in EOR technology) produced in the present study by *Enterobacter amnigenus* and *Pseudomonas aeruginosa* strains, in production medium based on by-products such as produced water and crude glycerin, showed higher production with *E. amnigenus*, confirming the metabolic synergy between the two microorganisms.

The bioactive compounds produced showed similar viscosity and pseudoplastic rheological behavior similar to commercial xanthan used as synthetic equivalent. In addition, the FTIR showed similarity between the spectra of the biocomposites produced with commercial FucoPoL spectra and EPS containing FucoPoL, highlighting the bioprocesses as competitive alternatives and advantageous to the chemically produced products, because they are low cost and sustainable in character. In view of the obtained results, new biocomposites with potential for EOR applications are currently under study in our group.

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