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Simultaneous Production of Bioactive Compounds for Application in Enhanced Oil Recovery

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This research investigated the use of produced water (PW) and raw glycerine, waste products from the oil and biodiesel industry, respectively, as a growth medium and source of nutrients in a bioprocess employing *Xanthomonas* and *Pseudomonas* strains, in order to simultaneously produce xanthan gum and rhamnolipids, products already used in enhanced oil recovery (EOR) applications. The xanthan gum and rhamnolipid produced exhibited excellent viscosity and emulsifying activity characteristics, and better pseudoplasic rheological behaviour than conventional chemical EOR compounds. EOR benefits of incremental oil production, coupled with the use of waste products to produce the compounds employed, minimize the environmental impact of EOR methods employing such compounds.
Keywords: bioprocess, biopolymer; biosurfactant; EOR; rhamnolipid; xanthan gum;

1. Introduction

One of the main residues generated in the various stages of oil and gas production is produced water (PW) (Fakhru'l-Razi et al., 2009). PW represents a major environmental and processing problem for the oil and gas industry (Lee & Neff, 2011). This work describes bioprocesses that utilize PW to yield useful compounds, the biopolymer xanthan gum, a polysaccharide produced by the fermentation of glucose or fructose by bacteria of the *Xanthomonas* genera, and the biosurfactant rhamnolipid, produced by bacteria of the *Pseudomonas* genera. These compounds, produced either in situ or ex situ, are commonly utilized in enhanced oil recovery (EOR) methods in order to improve oil production (Al-Bahry et al., 2013).
Xanthan gum is a natural polysaccharide biopolymer able to form viscous solutions in water, even at low concentrations (García-Ochoa et al., 2000). In the oil industry, it is mainly used in drilling fluids and EOR, due to its highly pseudoplasic behaviour and the stability of the viscosity it confers at high salinity (170,000 ppm), temperature (80°C), and alkaline conditions (Han et al., 1999; Shah & Ashtaputre, 1999).
Rhamnolipids are biosurfactants that exhibit the same characteristics as chemical biosurfactants (Desai & Banat, 1997). They have hydrophilic and hydrophobic groups within the same molecule, and thus have the ability to reduce surface and interfacial tension, through the formation of a molecular film between two non-miscible phases, and also the ability to form stable emulsions of hydrocarbons (HC) in water, or water in HC.
Rhamnolipid applications in the oil industry are focused on EOR and HC remediation.
Combinations of xanthan gum with surfactants have also been studied, having been found to be beneficial, since their injection helps to increase sweep efficiency, thus better propagating the oil bank towards production wells (Nedjhioui et al., 2005).Furthermore, the compounds produced through the bioprocess described herein are known to exhibit lower toxicity and higher biodegradability than synthetic polymers and surfactants not produced by biological processes (Silva et al., 2014).
This research thus sought to utilize PW effluent and raw glycerine, a low-value waste by-product from biodiesel production, as a growth medium and source of nutrients, respectively, in a bioprocess employing

Xanthomonas and *Pseudomonas* strains, in order to simultaneously produce xanthan gum and rhamnolipids, products already used in EOR applications.

2. Methodology

2.1 Obtaining, maintaining and activating microorganisms

Strains obtained were *Xanthomonas axonopodis* pv. manihotis 1182, obtained from the culture collection of Instituto Biológico (Campinas, SP, Brazil), and *Pseudomonas* sp. CCMICS 109, from the culture collection of LABEM (Laboratório de Biotecnologia e Ecologia de Micro-organismos), of 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, they were activated overnight in a yeast malt (YM) broth, for *Xanthomonas* (Jeanes et al., 1974), and in a tryptic soy broth (TSB), for *Pseudomonas*, 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 *Xanthomonas axonopodis* pv. manihotis 1182 was prepared by aseptic transfer of 1 mL of the activated cells to 250 mL Erlenmeyer flasks containing 50 mL of sterile YM broth. The inoculum was then incubated in an orbital agitator at 120 rpm, at 30°C ± 1°C, until it reached an optical density of 2.5 (OD_{560nm} = 2.5), which corresponded to the optimal log-phase of the strain. That absorbency band is attained at 16-24 hours of incubation, which corresponds to a concentration of approximately 1011 CFU.mL^{-1}.
The preparation of the *Pseudomonas* sp. CCMICS 109 inoculum was the same as for *Xanthomonas*, but with a different growth medium, TSB, and incubated until OD_{560nm} = 2.7, which corresponded to the optimal log-phase of the strain. That absorbency band is attained at 12-20 hours of incubation, which corresponds to a concentration of approximately 10^{10} CFU. mL^{-1}.

2.3 Production of compounds

Production of the EOR compounds was carried out in 300 mL Erlenmeyer flasks containing 50 mL of the production medium. The production medium was composed of PW (pH 7.0) supplemented with the following nutrients: 5.0 $g.L^{-1}$ potassium phosphate monobasic; 0.5 $g.L^{-1}$ yeast extract, 1.0 $g.L^{-1}$ sodium citrate. Equal parts of sucrose and raw glycerine were added as carbon sources. The use of raw glycerine reduced the use of sucrose, of much higher cost. The production medium was subsequently pasteurized at 65 °C for 30 minutes. After pasteurization, the production media were inoculated with 5 mL each of bacterial suspension, then incubated in an orbital shaker at 180 rpm, at 30°C ± 1°C, for 120 hours. Every 24 hours, xanthan gum production, viscosity of the cell-free broth, rhamnolipid production, emulsification index of the cell-free broth, and bacterial biomass production were evaluated. Production runs were carried out in triplicate, and the Tukey test was applied in order to determine the differences in production of compounds obtained with the bioprocess, as a function of the time for production.

3. Analytical determinations

3.1 Xanthan gum

The quantification of xanthan gum 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 xanthan gum 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).

3.2 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.

3.3 Rhamnolipid

The biosurfactant produced was measured by the thioglycolic colorimetric method, expressed in rhamnose, according to Chandrasekaran & Bemiller (1980). The concentration of rhamnose was determined based on the difference between the two wavelengths (400 and 430 nm), using the calibration curve previously obtained with commercial rhamnose. The difference was used because the absorbency value obtained at 430 nm

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 80 mg.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).

3.4 Emulsifying activity

The emulsifying activity of the rhamnolipid was determined according to Cooper & Goldenberg (1987). In a test tube (1.8 x 15 cm), a mixture of 2 mL of mineral oil and 2 mL 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.

3.5 Bacterial biomass

For determining bacterial biomass, the cells were separated from the bacterial must by centrifuging at 5500 rpm for 40 minutes. Then, the cells were washed three times in 0.85% saline solution, in order to completely eliminate metabolites. The biomass was determined by measuring the dry weight of the cells, after oven drying at 90°C ± 1°C, until a constant weight was reached.

3.6 Total of Reducing Sugars (TRS)

The total sugars were determined by phenol-sulphuric acid method (DUBOIS et al., 1956) and reading was performed at a wavelength of 490 nm, using sucrose at concentrations of 0 to 30 g.L^{-1} as a standard solution.

4. Results

4.1 Produced Water

The pH of the PW was 6.2, which led to the need for pH correction in the production medium, since a neutral pH is considered ideal for *Xanthomonas campestris* growth (Garcia-Ochoa et al., 2000), and a pH between 6.0 and 7.0 is considered ideal for *Pseudomonas* sp. growth (Reiling et al., 1986; Cooper & Goldemberg, 1987). The redox potential (ORP) was -168.5 mV, suggesting that the environment from where the PW was collected exhibited reducing conditions, which are unfavourable for aerobic bacteria. Since the bioprocess developed involves producing the compounds under agitation, oxygen is incorporated into the medium, thus aiding the metabolic exchange of the bacterial cells.Bioprocesses often suffer from inadequate minerals in the production medium. This deficiency can be made up by the PW itself, since it contains trace elements required by microorganisms for the synthesis of desired products (Kampen, 1997).The PW utilized is essentially without cost, and the PW utilized in this application also reduces the need for treatment of these volumes.

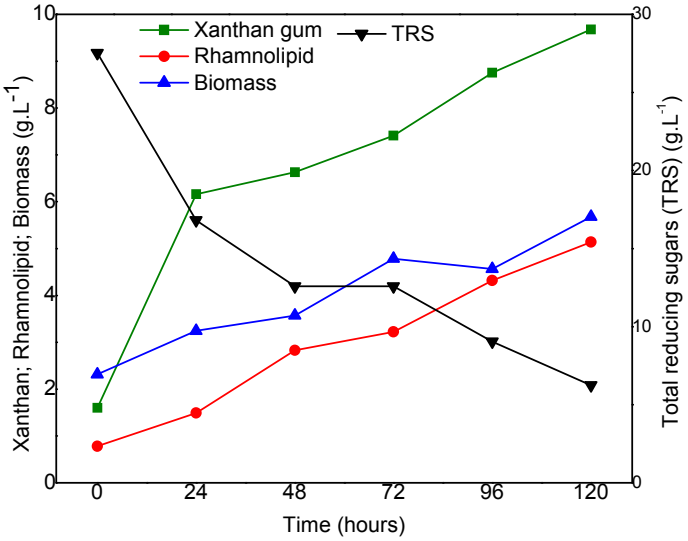

Figure 1. Average production xanthan gum, rhamnolipid and bacterial biomass, and nutrient consumption assessed in terms of total reducing sugars (TRS).

4.2 Production of EOR compounds

Figure 1 shows the result of xanthan gum production ($g.L^{-1}$) and rhamnolipid production ($g.L^{-1}$) over time. Bacterial biomass ($g.L^{-1}$) and the consumption of the substrate, as assessed in terms of total reducing sugars (TRS) ($g.L^{-1}$), are also shown over the 120 hours of fermentation. Table 2 shows the production ($g.L^{-1}$), productivity ($g.L^{-1}.h^{-1}$), yield ($g.g^{-1}$), yield in relation to substrate (%), biomass and TRS, as well as viscosity (cP at 1 s^{-1}) and emulsifying activity (%).

Table 2. Production, productivity, yield, and yield in relation to substrate, of bioactive compounds, biomass and TRS, as well as viscosity (cP at 1 s^{-1}) and emulsifying activity (%).

Time (hours)	0	24	48	72	96	120
Xanthan production - P_X ($g.L^{-1}$)	1.60±0.16	6.16±1.42	6.63±0,40	7.41±0.64	8.75±0.54	9.68±1.40
Xanthan productivity - P_X/h ($g.L^{-1}.h^{-1}$)	-	0.190	0.105	0.081	0.074	0.067
Xanthan yield - Y_X ($g.g^{-1}$)	-	0.43	0.34	0.33	0.39	0.38
Y_X/Substrate (%)	-	42.58	33.67	33.39	38.67	37.97
Rhamnolipid production - P_R ($g.L^{-1}$)	0.78±0.52	1.49±0.61	2.83±0.79	3.22±0.98	4.32±0.08	5.14±0.09
Rhamn. productivity - P_R/h ($g.L^{-1}.h^{-1}$)	-	0.030	0.043	0.034	0.037	0.036
Rhamn. yield - Y_R ($g.g^{-1}$)	-	0.07	0.14	0.14	0.19	0.20
Y_R/Substrate (%)	-	6.63	13.72	14.02	19.15	20.49
Biomass production - P_B ($g.L^{-1}$)	2.32±0.53	3.25±0.20	3.57±0.74	4.78±0.76	4.57±0.60	5.68±0.29
Biomass produtivity - P_B/h ($g.L^{-1}.h^{-1}$)	-	0.039	0.026	0.034	0.023	0.028
Biomass yield - Y_B ($g.g^{-1}$)	-	0.09	0.08	0.14	0.12	0.16
Tot. reducing sugars yield - Y_{TRS} ($g.L^{-1}$)	27.53±2.9	16.82±4.0	12.59±4.9	10.13±3.9	9.04±2.95	6.25±1.27
Y_{TRS}/Substrate (%)	-	8.68	8.37	14.14	12.17	15.79
Apparent viscosity - AV (cP at 1 s^{-1})	162	178	245	673	1059	1413
Emulsifying activity at 24h - EA_{E24} (%)	-	43.55	46.67	46.67	53.33	57.14
Emulsifying activity at 96h - EA_{E96} (%)	-	ND	ND	ND	50	57.14

ND Not determined

The EOR compounds obtained by the bioprocess were not subjected to any purification processes that could increase production costs.

4.3 Xanthan gum

Xanthan gum production reached 9.68 $g.L^{-1}$±1.40 at 120 hours of production, however the Tukey test suggests that there was no significant difference between 72, 96 and 120 hours ($p<0.05$). The productivity of xanthan gum fell throughout the production period, starting at 0.190 $g.L^{-1}.h^{-1}$ and reducing to 0.067$g.L^{-1}.h^{-1}$ after 120 hours. Maximum xanthan gum yield occurred in the first 24 hours (0.43 $g.g^{-1}$), then varied between 0.33 and 0.39 $g.g^{-1}$, until 120 hours.

At longer production times, the xanthan quality, as measured by the viscosity it imparts, is much better (higher viscosity). Despite the yield and productivity of xanthan gum being greater in the first 24 hours, other factors must be considered in determining the ideal time for production, such as the viscosity of the gum when used in drilling fluids, the production of rhamnolipids, and minimization of HC in the effluent generated by the process, which reduces treatment costs prior to disposal, and/or environmental impact of such disposal.

4.4 Rhamnolipid

Rhamnolipid production was 5.14 ± 0.09 $g.L^{-1}$ after 120 hours, but without a significant difference between 96 and 120 hours ($p<0.05$). Rhamnolipid productivity varied from 0.030 to 0.043 $g.L^{-1}.h^{-1}$.The rhamnolipid yield increased constantly during production, reaching a maximum after 120 hours of 0.20 $g.g^{-1}$.

Some authors suggest a fermentation time of 96 hours for the production of rhamnolipid (Lang, 2002; Ramana & Karanth, 1989), while others suggest up to 13 days (Sim, Ward & Li, 1997), which are much longer times than those used in this work. The shorter effective fermentation times achieved in this work should contribute towards low production costs.

Longer production times improved rhamnolipid quality, as measured by emulsifying activity (EA_{E96}), and rhamnolipid productivity remained constant even at longer production times, suggesting that rhamnolipid production could continue to increase with further increases in production times.

4.5 Bacterial biomass

Bacterial biomass production was 5.68 ± 0.29 $g.L^{-1}$ after 120 hours, without a significant change between 72 and 96 hours. The final productivity was 0.028 $g.L^{-1}.h^{-1}$ and the maximum yield was 0.16 $g.g^{-1}$. In the bioprocess industry, biomass production is directly associated with substrate consumption and the formation of end products. Bacterial biomass can either be processed and used as a nutrient source for animal feed, or it can be used as soil fertilizer, or even recovered and reutilized within the same bioprocess industry, in a process called cell recycling.

4.6 Viscosity

With longer production times, there was an increase in xanthan gum concentration, consequently there was an increase in the viscosity of the broth, with a maximum observed viscosity of 1,413 cP at 1 s^{-1} for the broth after 120 hours of fermentation.

4.7 Emulsifying activity

The most versatile property of tensoactive compounds for practical applications is their ability to stabilize a system with two immiscible liquids, promoting an emulsification of the mixture. This ability is measured by the emulsification index (EI), which is directly proportional to the production of biosurfactant (Rajeshwari, Prakash & Ghosh, 1995). The EI is the criterion used for the biosurfactant to be considered a good emulsifier, since it measures its capacity to form an emulsion with HC, and keep it stable (emulsified). An EI above 50% for 24 hours (E24) or more characterizes a good emulsifying agent (Willumsen & Karlson, 1997).The biosurfactant produced in this work after 96 or 120 hours of fermentation can be considered a good emulsifying agent, since the emulsifying activity (%) at 24 hours (EA_{24h}) and 96 hours (EA_{E96}) were above 50%.

4.8 Pseudoplastic behavior

The rheological behaviour of the compounds produced by the bioprocess developed in this work was very similar to that of the commercial xanthan used as the reference case. The solutions evaluated exhibited desired pseudoplastic behaviour, that is, the apparent viscosity decreases with increasing shear rate. The pseudoplasticity of the solutions allow low viscosity at drill bits, where the shear rate is high, and high viscosity in the annular space, where the shear rate is low.

5. Conclusion

The production of EOR compounds produced in this work (xanthan gum and rhamnolipids) was achieved in PW from the oil industry as the production medium, and raw glycerine from the biodiesel industry and sucrose as the carbon sources. At the end of the process, xanthan and rhamnolipid production were still increasing, but longer process times result in higher production costs, so that only a careful economic analysis could justify a longer production run.

The compounds produced by the bioprocess developed in this work exhibited excellent viscosity characteristics and emulsifying activity, and the pseudoplasic rheological behaviour was better than that of conventional synthetic equivalents.

The bioprocess developed in this work reduces costs and environmental impact of EOR methods employing such compounds.

There are no reported cases of simultaneous production of both compounds (xanthan gum and rhamnolipid) as were produced in this work, nor of a similarly effective bacterial consortium used in such a process.

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