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Raw Glycerol as an Alternative Carbon Source for Cultivation of Exopolysaccharide-Producing Bacteria

Renata Aguirre Trindade (Corresponding author)

School of Chemistry and Food, Federal University of Rio Grande

PO Box 474, Rio Grande, Brazil

Tel: 55-53-3232-6660 E-mail: renaguirretrindade@gmail.com

Adriel Penha Munhoz

School of Chemistry and Food, Federal University of Rio Grande

PO Box 474, Rio Grande, Brazil

Tel: 55-53-3232-6660 E-mail: adrielmunhoz@hotmail.com

Carlos André Veiga Burkert

School of Chemistry and Food, Federal University of Rio Grande

PO Box 474, Rio Grande, Brazil

Tel: 55-53-3232-6660 E-mail: burkert@vetorial.net

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Abstract

The large-scale use of biodiesel has shown significant environmental benefits as regards the reduction of global warming impacts. The increased generation of glycerol, the main byproduct of the reaction, makes necessary to propose alternatives to its use. In this context, the aim of this study was to evaluate raw glycerol (RG), a byproduct from biodiesel synthesis, as a carbon source for the cultivation of bacteria recognized as exopolysaccharides (EPSs) producers, compared with sucrose (S) and with a mixture of both components in a ratio of 1:1 w:w (SRG). The bacteria used were: *Xanthomonas campestris* pv. *mangiferaeindicae* IBSBF

1230, *Pseudomonas oleovorans* NRRL B-14683, *Sphingomonas capsulata* NRRL B-4261 and *Zymomonas mobilis* NRRL B-4286. All bacteria were capable of growing and producing EPSs using RG as the sole carbon source. For *X. campestris*, EPSs concentration of around 4.00 g L^{-1} was found for the different carbon sources tested. For *P. oleovorans*, only the medium composed by S (0.85 g L^{-1}) differed from the other media, with better results being found using RG and SRG. *S. capsulata* showed higher concentration in the medium containing S and SRG, around 3.40 g L^{-1}, and in the medium containing RG this value decreased to 1.70 g L^{-1}. *Z. mobilis*, on the other hand, showed a better result using SRG (1.41 g L^{-1}), and in the medium containing S and RG, these values were lower, reaching 0.27 and 0.77 g L^{-1}, respectively.

Keywords: *Xanthomonas, Pseudomonas, Sphingomonas, Zymomonas,* Crude glycerol, Reducing environmental impact

1. Introduction

Petroleum is the main source of energy in the world. As a fossil fuel, a decrease in the oil reserves in the near future is expected. Then, the use of renewable fuels, such as biodiesel, has been encouraged, leading to increased production (Silva et al., 2009a). According to the Global Status Report (2014), Brazil has excelled in the production of biodiesel, reaching 2.9 billion liters in 2013, corresponding to the 3rd position in the world rankings, behind the United States and Germany. The large-scale use of biodiesel has shown significant environmental benefits as regards the reduction of global warming impacts (European Biodiesel Board, 2014). Mainly obtained from vegetable oils, it contributes to the carbon cycle in the atmosphere, since the CO_2 emitted during burning is reabsorbed by plants that will produce it, thus reducing emissions in the long run, causing less impact on global warming (Mota et al., 2009).

One consequence of this production is the growing generation of glycerol, the main byproduct of the transesterification of vegetable oils, which leads to the formation of methyl or ethyl esters (biodiesel). This increase is accompanied by a significant reduction in the prices of glycerol. According to Amaral et al. (2009), the biodiesel industry converted glycerol into a commodity of low commercial value, with the supply increasing well above the increase related to its traditional uses. The resulting raw glycerol from biodiesel synthesis usually has 55-90% purity. The rest consists of unconverted triacylglycerols, unconverted methanol or ethanol, biodiesel, soaps and others (Amaral et al., 2009).

On the other hand, a high potential for application in various industrial segments has arisen for microbial biopolymers excreted by the cells, also called exopolysaccharides (EPSs). They are being used in food, pharmaceutical and chemical products, among others (Luvielmo & Scamparini, 2009; Freitas et al., 2011; Prasanna et al., 2012).

The most used carbon sources for the production of EPSs have been carbohydrates like glucose (Bajaj et al., 2006; Zhang et al., 2015) and sucrose (Rottava et al., 2009; Reis et al., 2010; Zhu et al., 2013; Silbir et al., 2014; Zhang et al., 2015). However, the high cost of these sources of carbon has a direct impact on production costs, limiting the market potential of these biopolymers. To reduce costs, byproducts and industrial wastes have been used. Berwanger et al. (2006) and Banik et al. (2007) used molasses as a carbon source for the cultivation of *Sphingomonas capsulata* and *Sphingomonas paucimobilis*, respectively, in order to produce EPSs. Mesomo et al. (2009) and Silva et al. (2009b) investigated whey as a carbon source for the production of xanthan gum from *Xanthomonas campestris*. Freitas et al. (2010) produced a biopolymer by *Pseudomonas oleovorans* and Reis et al. (2010) produced xanthan gum by *X. campestris* using raw glycerol as carbon source, however there are still few studies with this carbon source for the production of EPSs.

In our research group, raw glycerol has been proposed as a carbon source for microbial cultivation. Santos et al. (2013) investigated the possibility of using raw glycerol as a substrate for yeast biomass production as a source of proteins. Machado Junior et al. (2015) evaluated the effects of aeration and agitation on growth parameters and lipid content in the cultivation of *Yarrowia lipolytica* using raw glycerol as carbon source. In the work of Spier et

al. (2015), 12 different yeast strains were evaluated to gauge their ability to accumulate lipids using raw glycerol as the main carbon source

In this context, this paper proposes to evaluate the raw glycerol as a carbon source, in total or partial replacement of sucrose commonly used in cultivation of different bacteria known to produce EPSs of commercial importance.

2. Materials and Methods

2.1 Materials

2.1.1 Microorganisms

X. campestris pv. *mangiferaeindicae* IBSBF 1230 was obtained from the Culture Collection of Phytobacteria of the Biological Institute (IBSBF), Campinas, Brazil. *P. oleovorans* NRRL B-14683, *S. capsulata* NRRL B-4261 and *Zymomonas mobilis* subspecies *mobilis* NRRL B-4286 were obtained from the ARS Culture Collection (National Center for Agricultural Utilization Research, Peoria, United States). The microorganisms were supplied lyophilized.

2.1.2 Raw Glycerol

Raw glycerol was supplied by BS Bios Indústria e Comércio de Biodiesel Sul Brasil S/A (Passo Fundo, Brazil), with the following composition (%w/w): 81.92 glycerol, 11.29 moisture, 5.38 ash, 1.41 non-glyceridic organic matter, pH 5.39.

2.2 Cultivation Assays

2.2.1 Preparation of Inoculum

From the lyophilized and previously rehydrated cultures, successive transfers were performed in order to reactivate the microbial cultures. For *X. campestris* pv. *mangiferaeindicae* IBSBF 1230, it was used YM (Yeast Malt) agar, with the following composition (g L^{-1}): 3 yeast extract; 3 malt extract; 5 peptone; 20 glucose; 10 agar; and pH 7.2 (Mesomo et al., 2009). For *P. oleovorans* NRRL B-14683 and *S. capsulata* NRRL B-4261, the medium used contained (g L^{-1}): 20 glucose; 5 peptone; 3 yeast extract; 5 sodium chloride; 20 agar; and pH 6.8-7.0 (Bajaj et al., 2006). As for *Z. mobilis* NRRL B-4286, the medium used contained (g L^{-1}): 20 sucrose; 2.5 yeast extract; 1 KH_2PO_4; 1 $(NH_4)_2SO_4$; 0.5 $MgSO_4.7H_2O$; 20 agar (Oliveira et al., 2007).

Bacterial cultures were scraped from each slant tube with the aid of 10 mL of 0.1% peptone diluent, and the suspension was inoculated into 500 mL Erlenmeyer flasks with cotton plugs that contained 90 mL of the medium, and incubated in a rotary shaker (Tecnal TE-424, Brazil) at 28°C and 150 rpm until the optical density at 560 nm reached 1.9-2.1, corresponding to the logarithmic phase of microbial growth (Moreira et al., 2001; Mesomo et al., 2009).

2.2.2 Shaken Flasks Cultivation

The inoculum for each bacterial strain, representing 10% of the total volume (10 mL) was transferred to 500 mL Erlenmeyer flasks containing 90 mL of the culture medium. The flasks were kept in a rotary shaker (Tecnal TE-424, Brazil) at 28 °C and 200 rpm (Moreira et al., 2001).

Different culture media were used for each microorganism (Table 1). As carbon sources, sucrose (S), raw glycerol (RG) and a mixture of both (SRG), 1:1 w:w, were used, as suggested by Reis et al. (2010), since sucrose is the usual source of carbon for the production of the studied EPSs. The amount of raw glycerol used in the media considered its composition in order to result the concentration of substrate indicated in Table 1.

Table 1. Media composition for the different EPS-producing bacteria

Microorganism	Medium composition (g L^{-1})	Reference
X. campestris	50 carbon source; 2.5 $(NH_4)H_2PO_4$; 5.0 K_2HPO_4; 0.006 H_3BO_3; 2 $(NH_4)_2SO_4$; 0.0024 $FeCl_3$; 0.002 $CaCl_2.2H_2O$; 0.002 $ZnSO_4$; pH 7.0	Reis et al. (2010)
P. oleovorans	25 carbon source; 3.3 $(NH_4)_2HPO_4$; 5.8 K_2HPO_4; 3.7 KH_2PO_4; 10 mL solution $MgSO_4$ 100 mM; 1 mL solution of micronutrients*; pH 7.0	Freitas et al. (2010)
S. capsulata	20 carbon source; 10 Na_2HPO_4; 1 K_2SO_4; 1 NaCl; 0.15 $(NH_4)_2SO_4$; 0.2 $MgSO_4.7H_2O$; 0.01 $CaCl_2.2H_2O$; 0.001 $FeSO_4.7H_2O$; 0.5 yeast extract; pH 6.8-7.0	Bajaj et al. (2006)
Z. mobilis	20 carbon source; 2.5 yeast extract; 1 KH_2PO_4; 1 $(NH_4)_2SO_4$; 0.5 $MgSO_4.7H_2O$; pH 7.0	Oliveira et al. (2007)

*Solution of micronutrients (in g L^{-1} HCl 1N): 2.78 $FeSO_4.7H_2O$; 1.98 $MnCl_2.4H_2O$; 2.81 $CoSO_4.7H_2O$; 1.67 $CaCl_2.2H_2O$; 0.17 $CuCl_2.2H_2O$; 0.29 $ZnSO_4.7H_2O$.

2.3 Analytical Methods

Aliquots (10 mL) were taken at 0, 24 and 48 h, based on previous assays (data not shown), in order to determine biomass concentration. Samples were centrifuged for 12 min (Cientec CT-5000R, Brazil) and the pellet was recovered after washing with peptone diluent. The biomass concentration was estimated by measuring the absorbance at 560 nm in a spectrophotometer (Biospectro SP-22, China). A calibration curve between OD_{560} and the cell dry-weight concentration (g L^{-1}) was first established for each microorganism (Prieto et al., 2008).

The recovery of EPSs from the medium in 48 h cultivation was performed by centrifugation (Cientec CT-5000R, Brazil) of all content of the flasks at 3,400 × *g* for 30 min at 4 °C, followed by precipitation of EPS by the addition of ethanol 96.4 ºGL (1:4 v:v), standing for 24 h at 4 °C and centrifuged again for 30 min under refrigeration (4 °C) (Mesomo et al., 2009).

The EPS concentration was determined gravimetrically by drying of precipitates at 50 °C

(Quimis, Q314M242, Brazil) until constant weight (Sartorius, TE214S, Brazil), relating to the sample volume (Mesomo et al., 2009). The EPSs values shown correspond to the extracellular material precipitable by ethanol, as described by Staudt et al. (2012), since there was no further purification of the precipitate. The productivity was obtained by concentration of the EPS divided by the respective cultivation time (Rottava et al., 2009).

2.4 Statistical Analysis

All cultivations were performed in triplicate. The results were analyzed by analysis of variance and Tukey's test (Montgomery, 2004) in order to verify the existence of significant differences between the carbon sources for the same micro-organism, at 95% confidence level ($p < 0.05$).

3. Results and Discussion

3.1 Biomass

Figure 1 shows the monitoring of biomass in the cultivation of *X. campestris* pv. *mangiferaeindicae* IBSBF 1230, *P. oleovorans* NRRL B-14683, *S. capsulata* NRRL B-4261 and *Z. mobilis* NRRL B-4286, respectively, for the different carbon sources.

For almost all bacteria in the study, the highest biomass concentration was observed in 48 h cultivation. The highest biomass concentration for *X. campestris* was found using SRG (0.14 g L^{-1}), differing significantly ($p < 0.05$) from the other carbon sources (around 0.12 g L^{-1}). For *P. oleovorans*, the highest biomass concentration was obtained with the medium containing only RG (5.29 g L^{-1}) as a carbon source, differing significantly ($p < 0.05$) from other media. The concentration of biomass for *S. capsulata* in the three carbon sources did not differ significantly ($p > 0.05$), reaching around 0.95 g L^{-1}. In the cultivation of *Z. mobilis,* the highest concentration of biomass was reached with the medium S (0.77 g L^{-1}), differing significantly ($p < 0.05$) from the other media, with a sharp drop in the concentration of biomass (0.21 g L^{-1}) when RG was used as carbon source.

Mesomo et al. (2009), in the cultivation of *X. campestris mangiferaeindicae* IBSBF 1230 using cheese whey as carbon source, found a biomass concentration varying from 0.6 to 5 g L^{-1}, according to aeration and agitation conditions in a 2.5 L bench-scale bioreactor.

Freitas et al. (2010), using *P. oleovorans*, observed that using raw glycerol as carbon source, the highest biomass concentration was 9.55 g L^{-1} in 48 h when temperature was 30 ºC. When using a temperature of 25 ºC, a concentration around 3.00 g L^{-1} was reached. This result is in accordance with the present study (5.29 g L^{-1}), conducted at a temperature of 28 ºC.

West and Strohfus (1998) found for *S. paucimobilis* a biomass concentration of 0.82 g L^{-1} in 48 h when cultivated with sucrose as carbon source, a result similar to those found in this study for *S. capsulata* (0.96 g L^{-1}).

Oliveira et al. (2007), using 250 g L^{-1} sucrose (carbon source) in 24 h of cultivation, found a biomass concentration of 0.85 g L^{-1}, a result similar to that found in this study for *Z. mobilis* (0.77 g L^{-1}) where lower concentrations of sucrose were used.

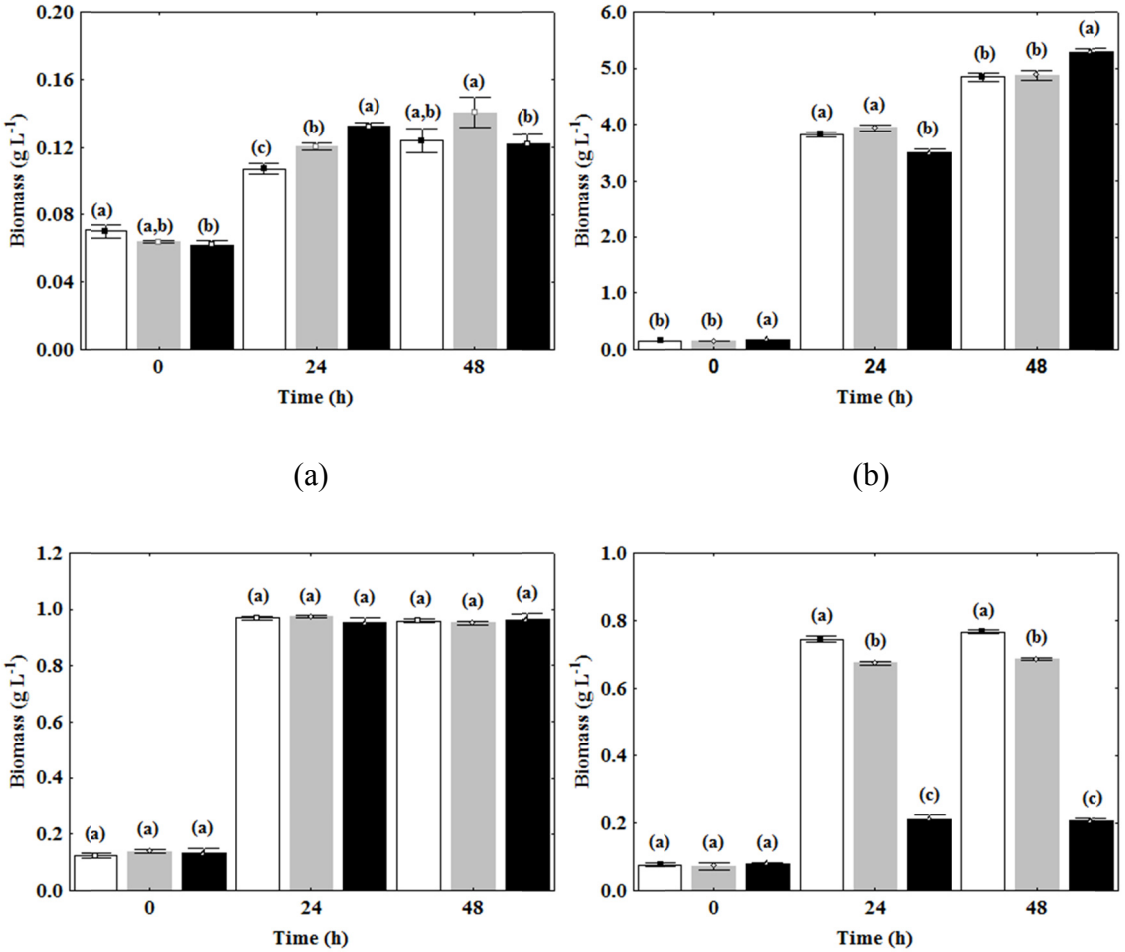

Figure 1. Biomass for *X. campestris* IBSBF 1230 (a), *P. oleovorans* NRRL B-14683 (b), *S. capsulata* NRRL B-4261 (c) and *Z. mobilis* NRRL B-4286 (d) cultivated in a medium containing S (□), SRG (■) and RG (■). Different lowercase letters indicate a significant difference between the media for the same bacteria at the same cultivation time ($p < 0.05$)

3.2 EPSs Concentration and Productivity

The EPSs concentration was evaluated at 48 h of cultivation (Table 2). The study was conducted in shaken flasks. In this case, the volume of sample collected (10 mL) did not permit the quantification of EPSs along time, because it is necessary a minimum weight for gravimetric determination. So, the EPSs concentration was determined at the end of cultivation (48 h), when it was possible to recovery all supernatant.

For *X. campestris*, 4.98 g L^{-1} of EPSs were found when RG was used as carbon source, not differing significantly from the EPSs produced with S (4.55 g L^{-1}). The medium containing only S was also not significantly different from medium containing SRG (4.07 g L^{-1}).

X. campestris is a gram-negative bacterium recognized as a xanthan gum producer, the

biopolymer most widely accepted commercially. It can be used in foods and other segments as a thickening, stabilizing and emulsifying agent (Rottava et al., 2009). Reis et al. (2010) evaluated the ability of *Xanthomonas* sp C1 and C9 to produce a biopolymer in a medium containing the same carbon sources used in this study (S, SRG and RG). The authors found that the use of sucrose and a mixture of sucrose and residual glycerol did not represent significant differences, producing xanthan gum at a concentration of around 0.33 g L^{-1} for S. As for the medium consisting only of RG, the concentration of this biopolymer was 0.157 and 0.186 g L^{-1} for C1 and C9, respectively, different from the other carbon sources. Moreira et al. (2001) evaluated the production of xanthan gum by 18 strains of *X. campestris* pv pruni, using sucrose as a carbon source, at 28 °C and 200 rpm agitation, yielding gum concentrations ranging from 2.3 to 8.3 g L^{-1} after 72 h of cultivation.

Table 2. Mean values* of EPSs concentration (standard deviation) for different bacteria at 48 h of cultivation (values are expressed in g L^{-1})

Carbon Source	*X. campestris* IBSBF 1230	*P. oleovorans* NRRL B-14683	*S. capsulata* NRRL B-4261	*Z. mobilis* NRRL B-4286
S	4.55(0.37)^{a,b}	0.84(0.05)^{b}	3.44(0.22)^{a}	0.27(0.03)^{c}
SRG	4.07(0.14)^{b}	4.11(0.13)^{a}	3.51(0.14)^{a}	1.42(0.16)^{a}
RG	4.98(0.36)^{a}	3.99(0.20)^{a}	1.87(0.19)^{b}	0.77(0.09)^{b}

*Different lowercase letters indicate a significant difference between the media for the same bacteria ($p < 0.05$).

Table 3. Mean values* of EPSs productivity (standard deviation) for different bacteria at 48 h of cultivation (values are expressed in g L^{-1} h^{-1})

Carbon Source	*X. campestris* IBSBF 1230	*P. oleovorans* NRRL B-14683	*S. capsulata* NRRL B-4261	*Z. mobilis* NRRL B-4286
S	0.09(0.01)^{a}	0.02(<0.01)^{b}	0.07(<0.01)^{a}	0.01(<0.01)^{c}
SRG	0.08(<0.01)^{b}	0.09(<0.01)^{a}	0.07(<0.01)^{a}	0.03(<0.01)^{a}
RG	0.10(0.01)^{a}	0.08(<0.01)^{a}	0.04(<0.01)^{b}	0.02(<0.01)^{b}

*Different lowercase letters indicate a significant difference between the media for the same bacteria ($p < 0.05$).

For *P. oleovorans,* it can be seen that a higher concentration of EPSs was found with SRG (4.11 g L^{-1}) and RG (3.99 g L^{-1}), not differing significantly from each other. The concentration of EPSs in the sucrose medium was rather low (0.84 g L^{-1}), differing significantly from the others.

P. oleovorans is mentioned as an EPS producer, but not available commercially. It is characterized by the presence of neutral sugars (such as galactose, glucose, mannose and rhamnose) and acyl groups substituents (pyruvil, acetyl and succinyl) (Freitas et al., 2010).

Hilliou et al. (2009), in the cultivation of *P. oleovorans* NRRL B-14682 using 25 g L^{-1} of glycerol as carbon source and 3.3 gL^{-1} $(NH_4)_2HPO_4$ as nitrogen source, at pH 6.75-6.85 and 0.125 vvm aeration, obtained 13.3 g L^{-1} of EPSs after 7 days of cultivation, however they used a 10 L bench-scale bioreactor, where the conditions of agitation and aeration are much more efficient.

When *S. capsulata* is evaluated, it can be seen that the highest concentration of EPSs was found when using S (3.44 g L^{-1}) and SRG (3.51 g L^{-1}) as a carbon source, not differing from each other, with the concentration found being rather low (1.87 g L^{-1}) when RG was used as carbon source.

A number of bacteria of the genus *Sphingomonas* produce polysaccharides called sphingans. The sphingans gellan, wellan, rhamsan and diutan are produced commercially for use in food, oilfield or personal care applications, presenting rheological properties similar to xanthan gum. They are composed by a generally conserved tetrasaccharide backbone structure and different side chains (Coleman et al., 2008).

For *Z. mobilis*, it can be seen that there was a higher concentration of EPSs in the medium containing a mixture of SRG (1.42 g L^{-1}), followed by RG (0.77 g L^{-1}) and S (0.27 g L^{-1}).

The levan is an EPS formed by fructose units that have a wide variety of applications. The levan is used in foods as color and flavor vehicle and as a source of fructose and fructooligosaccharides. It can be used in medicine as a hypo-cholesterol, antitumor, immune modulator, anti-inflammatory and plasma substitute agent (Ernandes & Cruz, 2011; Oliveira et al., 2007).

By comparing Figure 1 and Table 2, it can be seen that there is no relation between biomass and EPSs production in 48 h cultivation. A similar behavior was also observed by Kawai et al. (2006) for microalgae and by Maziero et al. (1999) for basidiomycetes.

Productivity is receiving a lot of attention, because it is fundamental to the success of a microbial process. As for productivity in EPSs, according to Table 3, it can be seen that for *X. campestris* the highest yield was in the medium with S (0.09 g L^{-1} h^{-1}) and RG (0.10 g L^{-1} h^{-1}), not differing significantly ($p > 0.05$) from each other. For *P. oleovorans*, a greater yield was found when SRG (0.09 g L^{-1} h^{-1}) and RG (0.08 g L^{-1} h^{-1}) were used as carbon sources, not differing significantly ($p > 0.05$) from each other. With *S. capsulata,* a greater yield was found when SRG (0.07 g L^{-1} h^{-1}) and S (0.07 g L^{-1} h^{-1}) were used as carbon sources, not differing significantly ($p > 0.05$) from each other. For *Z. mobilis,* the highest yield was

obtained when SRG mixture (0.03 g L^{-1} h^{-1}) was used as a carbon source, differing significantly ($p < 0.05$) from the other carbon sources.

Berwanger et al. (2006) evaluated the ability of *Sphingomonas capsulata* ATCC 14666 to produce a biopolymer, using raw and pretreated molasses and textured soybean protein (TSP) waste as carbon source by testing different concentrations (2.66, 4 and 6.08%), at 28 ± 2°C, 208 rpm and 72 h. The best productivity was found for pretreated molasses 8% (0.290 g L^{-1} h^{-1}), followed by the aqueous extract of the TSP residue 6% (0.240 g L^{-1} h^{-1}) and crude molasses 8 % (0.190 g L^{-1} h^{-1}). Freitas et al. (2010) evaluated the productivity of the bacterium *P. oleovorans* using RG as carbon source and found a yield of 0.0341 g L^{-1} h^{-1} at 25 °C and 0.083 g L^{-1} h^{-1} at 30°C, the latter being the same value found in this work, at a temperature of 28 °C.

Based on the results presented in this work, it was possible to use a low-cost substrate with high availability for different EPSs production, with potential to add value to the biodiesel production chain and reduce the environmental impacts of this industrial process.

4. Conclusions

By comparing *X. campestris* pv. *mangiferaeindicae* IBSBF 1230, *P. oleovorans* NRRL B-14683, *S. capsulata* NRRL B-4261 and *Z. mobilis* NRRL B-4286, all strains were able to grow and produce EPSs in medium containing only RG as a carbon source in 48 h cultivation, with the bacteria *X. campestris* and *P. oleovorans* being able to produce the same or higher concentrations of EPSs when only RG was used as a carbon source compared to the other carbon sources, not having a defined relationship between the concentration of biomass and production of EPSs. Thus, it was shown that RG is a potential alternative carbon source for substituting sucrose in the cultivation of some EPSs-producing bacteria.

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