Biopolymer Synthesized by Strains of *Xanthomonas* sp Isolate from Brazil Using Biodiesel-Waste

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Summary: The future supplies and usage of glycerol are expected to increase as biodiesel plants increase production, and the output will greatly outpace demand. Biodiesel production has already had a significant impact on the price of refined glycerol. A major concern of glycerol producers is the reduced price of glycerol resulting from the increased production of biodiesel. Some alternative uses for this glycerol that have been investigated are substrates for fermentation process or the production of biosurfactants, fatty acids, biopolymers, and others products. This work had as objective to evaluate two strains of *Xanthomonas* sp isolate from Brazil for xanthan gum in orbital agitator, analizing the apparent viscosity of aqueous solutions and selecting viscosity. The experiments of xanthan gum production were realized in orbital agitator with 120 rpm agitation, for cells production, and 180rpm, for biopolymer production, under a 28 °C temperature. The rheology of the fermentation broth was analyzed by apparent viscosity and the polymer was recovered with ethanol (1:3, v/v). After its recovery, the productivity evaluation was performed. The productivity were 0.157 and 0.363 gL^{-1} for C1 and 0.186 and 0.363 gL^{-1} for C9 to media glycerol or glycerol and sucrose, respectively. The viscosity analysis was performed for aqueous solutions 3%, at 25 °C, the best apparent viscosity was obtained using gum synthesized with glycerol and sucrose (50:50) at 25 °C, 143 $mPa.s^{-1}$ from *Xanthomonas* sp C1.

Keywords: biodiesel; rheology; xanthan gum; *Xanthomonas*

Introduction

The production of microbial biopolymers, also known as extracellular polymeric substances (EPS), is an alternative to gums extracted from plants, since they present physico chemical properties of high industrial interest, which are essential to define their applications. These compounds have the ability to form gels and viscous solutions in aqueous medium, even at low concentrations. In the last decades significant progress was observed, concerning the identification, characterization, and utilization of microbial polysaccharides.[1,2]

Among the biodegradable polymers, xanthan gum is well- known. This is commercially produced by fermentation and employed as a tickening agent and emulsifier in the nutritional, pharmaceutical, and oil drilling industries. The production has been the subject of numerous publications in the many aspects involved in its manufacture, because your produced has estimated to grow continuously at an

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annual rate of 5–0%, so that it now exceeds 86,000 tons per annum. Due to the highly competitive market of xanthan gum, more efficient processes are permanently needed. Many works is begin realized in the area of the selection of improved *Xanthomonas* strains and the manipulation of culture conditions such as the use of optimized culture media.[3–10]

The xanthan is relative expensive due to glucose or sucrose being used as the sole carbon source. However, the high cost of the carbon sources used which have direct impact on production costs, limits the market potential of these biopolymers.[11]. Therefore, it is critical to search for less expensive carbon sources in order to decrease the production costs. The use of low-cost substrates, such as agroindustry wastes and byproducts in fermentation processes, may favor the reduction of production costs and minimize environmental problems. Some alternative sources have been suggested, such as sugarcane molasses, soybean industry wastes, cheese whey, and others.[12–15] Industrial media are very complex, and some of their components may be responsible for inhibiting polysaccharide production or hindering its recovery and purification. Contaminants, such as, heavy metals and specific inhibitors may be removed with special pre-treatments. These pre-treatments may clarify the medium without affecting fermentation performance and then guarantee better product extraction and purification.[6]

Glycerol, a byproduct of many industrial processes, mainly from biodiesel production, is generated in large quantities, far beyond current consumption in traditional applications, thus making it a residue for which interesting applications are lacking. During the biodiesel production process, oils/fats (triglycerides) are mixed with methyl alcohol and alkaline catalysts to produce esters of free fatty acids, with glycerol as a primary by-product. In general, production of 100 kg of biodiesel yields approximately 10 kg of glycerol, which is impure and of low economic value.[16] Considering the fact that the projected production volume of crude glycerol will exceed the present commercial demand for purified glycerol, and that purification for sales of medical glycerol will not be a viable option for the biodiesel industry; some alternative uses for the glycerol will need to be found. Converting glycerol into value-added products provides an alternative for glycerol disposal and for its surplus problems. The use of glycerol as carbon source may contribute for the reduction of production costs, thus making the process more cost effective.

Thus, the purpose of this study was to investigate *Xanthomonas* sp C1 and C2, both Brazil isolated, as a novel potential xanthan gum producer using sucrose, glycerol or glycerol:sucrose in submerged culture.

Materials and Methods

Bacterial Strains, Media and Growth Conditions

Strains of the genus *Xanthomonas* sp C1 and C9 were used. The strains were obtained from Phytobacteria Culture Collection of Rural Federal Pernambuco University, Pernambuco, Brazil, all having been isolated in Brazil. The strains were maintained at 4 °C, in YM (Yeast Malt) medium containing (g.L^{-1}): yeast extract 3.0, malt extract 3.0, peptone 5.0, glucose 10.0, and agar 20.0.[17] The organisms were replicated every 15 days and stored at a temperature of 4 °C. The morphological characteristics of the colonies were determined by applying the Gram stain test and incubating streak plates in YM agar.

Inoculum

The production of cells was carried out in 14 mL liquid YM medium in 300 mL Erlenmeyers, in two steps.[17] Firstly a pre-inoculum was prepared, inoculating a loopful of stock culture in 50 mL of YM medium and incubating at 120 rpm, 28 °C, for 24 h. Secondly, the inoculum was prepared by the addition of 1 mL of pre-inoculum culture to 14 mL of YM medium incubated in an orbital shaker, at 120 rpm, 28 °C, for 24 h, when cell concentration reached 10^{11} CFU.mL^{-1}.

Xanthan Gum Production

A 14 mL inoculums, according to the strain was added to 86 mL of biopolymer production medium containing (g.L^{-1}): 2.5 $NH_4H_2PO_4$; 5.0 K_2HPO_4; 0.006 H_3BO_3; 2.0 $(NH_4)_2SO_4$; 0.0024 $FeCl_3$; 0.002 $CaCl_2.2H_2O$; 0.002 $ZnSO_4$; 50.0 carbon source (sucrose 50; or sucrose:glycerol 25:25; or glycerol 50), pH 7.0.[24] The inoculated medium was incubated in 250 mL erlenmeyer flasks in an orbital shaker at 28 ± 2 °C and 180 rpm for 96 h. The experiments were carried out in triplicate. The fermentations were started with an inoculums concentration of 10% (v/v), at initial pH of 7.0, 28 °C and stirring rate of 180 rpm, in an orbital shaker. For each experimental condition, three replicates were used and productivity was used as the response. Results were statistically evaluated using Tukey's test.

After fermentation, the broth was centrifuged at 4700 x g for 40 minutes at 4 °C, for cell separation. The polysaccharide was precipitated from the supernatant with the addition 92.6° Gay Lussac (GL) ethanol (1:3 v/v), followed by refrigeration for 12 h. After, samples were centrifuged at 4700 x g for 40 minutes at 4 °C to recover the precipitated biopolymer. The precipitate was dried in an oven at 50 °C ± 5 °C for 24 hours. The polysaccharide was then dialyzed (cut off 12000 Da), 48 h against sterile Milli-Q® water, lyophilized, and stored in hermetical flasks until further analyses.

Rheological Analysis of Xanthan Gum

Rheological measurements of the different aqueous solutions (3% w/v) of the xanthan gum were carried out using a controlled stress rheometer (MCR 301, Anton Paar Physica, Austria) calibrated and certificated, with a parallel plate fixture (diameter 25 mm with gap of 1 mm) at 25 °C ± 0.01 °C controlled by means of a Peltier system. Flow curves were plotted from the corresponding transient tests (apparent viscosity, η (Pa.s), vs. time at constant shear rate, γ (s-1) at different shear rates) in a wide range from 0.01 to 300 s^{-1}.

Flow curves were made in duplicate at each tested storage time. Experimental apparent viscosity data were correlated by means of Ostwald model given by Eq. (1):

$$\sigma = K\,\gamma^{n} \tag{1}$$

Viscosity data was fitted for increasing and decreasing shear rate, using the Ostwald-de-Waele model ($\sigma = K\ \gamma^n$), where σ is shear stress, and γ is shear rate, K and n are the consistency index and power law exponent, respectively, for to confirm the psudoplastic behaviour of the gum solutions. For Newtonian flow n is equal to one and K equals the viscosity.

Results and Discussion

Xanthan Gum of Production

Xanthan gum produced by genus *Xanthomonas* fermentation depends on many parameters and variables, including medium composition, temperature, pH, strains, and oxygen transfer.[3–8,13,18] With base in these variables, it was studied production of xanthan gum with glycerol obtained from biodiesel indystry and avaliable the quality of the gum for analysis of apparent viscosity.

Table 1 showed the results of the production xanthan gum from *Xanthomonas* sp C1 e C9 in three different fermentation media using different carbon source (glycerol; glycerol:sucrose and sucrose). The productivity was carried through being based on the triplicate and express in g.L^{-1} and g.L^{-1}.h^{-1}.

It can be verified that the highest yield of xanthan gum (0.0038 g.L^{-1}.h^{-1}) among the three media was obtained using glycerol with supplementation of the sucrose (25:25, w/w) by the wild-type isolate *Xanthomonas* C1 and C9. However, the glycerol:sucrose ratio did not present a significant effect ($p < 0.05$) on the gum production with sucrose. In this sense and considering the

Table 1.
Available of the productivity (g.L^{-1} or g.L^{-1}.h^{-1})* of xanthan gum synthesized in different carbon sources from *Xanthomonas* sp C1 and C9

Strain	Sucrose		Sucrose:Glycerol		Glycerol	
	g.L^{-1}	g.L^{-1}.h^{-1}	g.L^{-1}	g.L^{-1}.h^{-1}	g.L^{-1}	g.L^{-1}.h^{-1}
C1	$0.300 \pm 0.07^{a,**}$	0.0031	0.363 ± 0.04^{a}	0.0038	0.157 ± 0.04^{b}	0.0016
C9	0.314 ± 0.05^{a}	0.0032	0.363 ± 0.02^{a}	0.0038	0.186 ± 0.01^{b}	0.0019

*The men of three replicates.**Same letters superscripts means that there is no significant difference ($p < 0.05$) by the Tukey test.

low cost and high amounts of glycerol produced in biodiesel industry, this by-product was used as supplement carbon source. The glycerol was not a source carbon good when used without any supplementation.

Table 1 presents the statistical analysis regarding the influence of carbon source (glycerol and sucrose) on xanthan gum production. Same letters superscript means that there is no significant diference ($p < 0.05$) by the Tukey test. Significant differences for xanthan gum produced were verified only with the medium containing glycerol as unique carbon source.

In the production of xanthan gum using molasses was achieved 0.165 g.L^{-1}, using a wild type isolated in the work and a medium containing 5% (w/v) sucrose on the molasses.[19] Psomas et al.[20] studied the xanthan gum optimization by response surface methodology and maximum xanthan gum production 0.09 g.L^{-1}.h^{-1} was obtained in a synthetic medium at 25 or 35 °C in 72h. Moreira et al.[21], obtained higher polymer productivity in synthetic medium (0.16 g.L^{-1}.h^{-1}) with xanthan gum synthesized by *X. c.* pv pruni. Kalogiannis et al.[6] obtained higher polymer productivity (2.21 g.L^{-1}.h^{-1}) using molasses. The production of xanthan gum by *X. campestris* mangiferaeindicae 1230 used cheese whey was evaluated by salts concentrations and cheese whey:sucrose ratio, where the better result was 0.33 g.L^{-1}.h^{-1}.[13] The other work used glucose as source carbon was obtained 0.29 g.L^{-1}.h^{-1} using bioreactor[22]. The results suggest that the productivity is affected by microbial strain, nutrient requirement, fermentation parameters, and other factors. Further studies are needed for increase the productivity as others strains, and optimization medium.

Viscosity Measurements

Rheological properties of xanthan gum produced by *Xanthomonas* sp C1 and C9 under different condition were determined at different shear rates. All xanthan gum solutions at 3% show a similar pseudoplastic behavior. This behavior is waited in polymeric solutions of microbial polysaccharides, where a flow behavior index lower than unity, indicates the fluid has a pseudoplastic behavior. Viscosities measured in these solutions were fitted to the Ostwald-de-Waele model ($\sigma = K \quad \gamma^n$), where σ is shear stress, and γ is shear rate, K and n are the consistency index and power law exponent, respectively.[9,23–25]

Figure 1 can be observed a comparative graph of gotten apparent viscosities of the aqueous solution 3% (w/v) at 25 °C, At 0.257 s^{-1} was obtained following results for the sucrose, glycerol:sucrose and glycerol media: 941, 143 and 140 mPa.s^{-1}, respectively, when used *Xanthomonas* sp C1 for xanthan gum production. The aqueous solutions of xanthan gum produced by *Xanthomonas* C9 (Figure 2) showed lower viscosity. This results were statistically different when compared with a xanthan gum synthesized by C1.

Comparing the values for apparent viscosity of the aqueous solutions of the gums obtained with the values reported in other papers, it can be seen that the xanthan gums obtained from the strains *Xanthomonas* C1 (143 mPa.s^{-1}) and *Xanthomonas* C9 (93 mPa.s^{-1}) in medium glycerol supple-

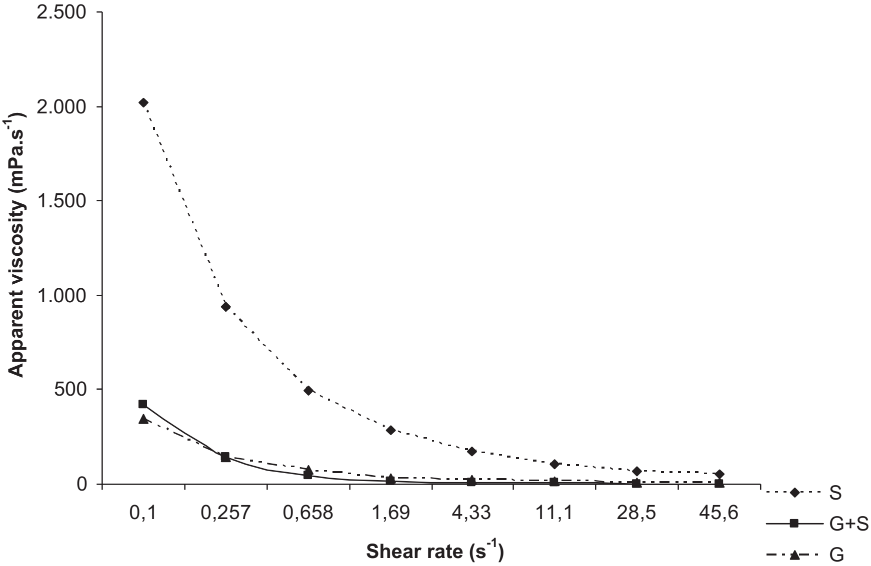

Figure 1.

Apparent viscosity of the aqueous solution at 3% of the gum synthesized by *Xanthomonas* sp C1 using sucrose (S), glycerol:sucrose (G + S) and glycerol (G) in rheometer MCR 301, Anton Paar Physica, with a parallel plate fixture, diameter 25 mm with gap of 1 mm at 25 °C controlled by means of a Peltier system.

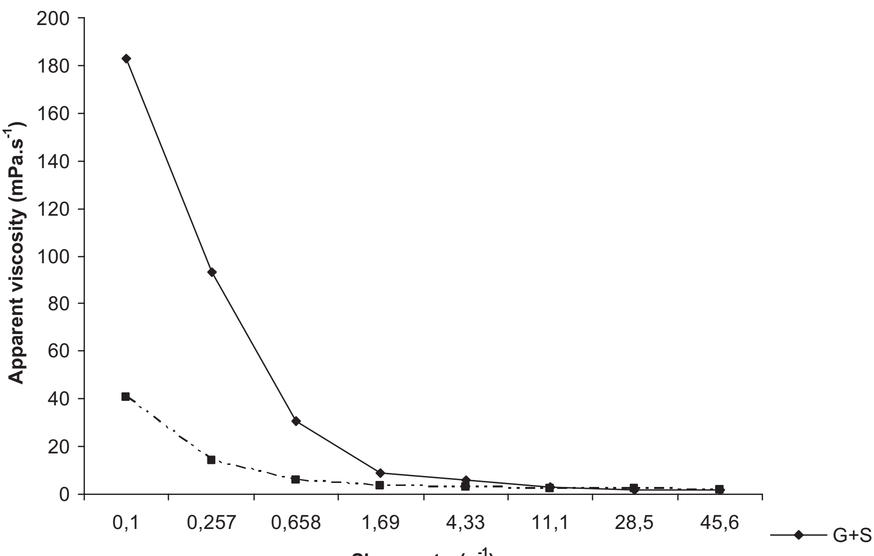

Figure 2.

Apparent viscosity of the aqueous solution at 3% of the gum synthesized by *Xanthomonas* sp C9 using glycerol:sucrose (G + S) and glycerol (G) in rheometer MCR 301, Anton Paar Physica, with a parallel plate fixture, diameter 25 mm with gap of 1 mm at 25 °C controlled by means of a Peltier system.

mented with sucrose gave low values for viscosity, considering that the biopolymer concentration used in the aqueous solutions in the present study (3% w/v) and shear rate was lower than the used in the other studies. Rottava et al.[14] obtained an apparent viscosity between 1800 mPa.s^{-1} at 71 mPa.s^{-1} for *Xanthomonas campestris* pv magiferaeindicae 1230 and *X. c.* pv arracaciae 1198, respectively, at 1.32 s^{-1} for 3% solutions of xanthan gum synthesized with sucrose.

However, the rheologic properties of the polysaccharides in solution do not depend only on the concentration of the aqueous solution analyzed, but also of its characteristics physical-chemical intrinsic, that is, molecular weight, polidispersivity and degree of substitution. In the case of the microbial polysaccharides these properties physical-chemical, as well as the efficiency of the production are related to the fermentative process, strain, medium composition, pH, temperature and other ambient parameters of the fermentation.[13–15]

Conclusion

This work investigated the possibility of using glycerol, obtained of biodiesel industry as substrate for xanthan gum production by *Xanthomonas* C1 and *Xanthomonas* C9 isolated from Brazil. The results showed that the glycerol as unique carbon source not is a media adequate for xanthan gum. However, when supplemented with sucrose were not detectable significance differences with the sucrose medium. The best productivity and apparent viscosity was with *Xanthomonas* C1 when compared with the media containing glycerol.

Therefore, the results obtained showed that the raw glycerol, by produced discharged after biodiesel production process, was an adequate substrate for the production of xanthan gum. As for the economic significance of the results obtained from the present study, it should be stressed that the utilization of glycerol in new integrated bioprocess to valorization of this produces a valuable bioproduct.

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