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Xanthan gum production by *Xanthomonas axonopodis pv. mangiferaeindicae* from glycerin of biodiesel in different media and addition of glucose

Tayssa Santos Gondim[1*], Roberto Guimarães Pereira[2] and Sorele Batista Fiaux[3]

[1]Programa de Pós-Graduação em Engenharia de Biossistemas, Universidade Federal Fluminense, Rua Passo da Pátria, 156-209, 24210-240, Niterói, Rio de Janeiro, Brazil. [2]Escola de Engenharia, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil. [3]Faculdade de Farmácia, Universidade Federal Fluminense, Niterói, Rio de Janeiro, Brazil. *Author for correspondence. E-mail: tayssasantos@hotmail.com

ABSTRACT. Biodiesel production has been increasing yearly in Brazil. A large amount of glycerin is generated in this process and needs a correct destination. One possible use of this glycerin in crude form is in biotechnological processes. Xanthan gum is a commercial gum used primarily in the pharmaceutical and food industries as thickener, emulsifier and stabilizer. It is synthetized by species of the bacterium *Xanthomonas* generally from glucose. However, current research shows that species of this bacterium have the capacity to grow and synthesize the gum using glycerin from biodiesel. The aim of this study was to produce xanthan gum using glycerin from biodiesel production in medium with different nitrogen content, named complex and simple media. The kinetics of fermentation in simple medium showed a two-fold increase in gum production (3.16 $kg.m^{-3}$) compared to the one in complex medium (1.46 $kg.m^{-3}$) after 120 hours. The gum generated in this study showed chemical and rheological characteristics of xanthan gum. Glucose supplementation did not show an increase in xanthan production but did increase the consistency index and the behavioral index of solutions of this gum.

Keywords: residue; biopolymer; biotechnological process.

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Introduction

Biodiesel production in Brazil is increasing every year due to the need of its addition to the common diesel (Agência Nacional do Petróleo, Gás Natural e Biocombustíveis [ANP], 2017). The production of biodiesel generates a large amount of glycerin, the main by-product of this process (10 m^3 glycerin for 90 m^3 biodiesel) (Pagliaro, Ciriminna, Kimura, Rossi, & Pina, 2009). Although it can be destined to the industrial sector, a costly previous purification is necessary. A possible use, without the need for purification, is as a source of carbon in microbial processes to obtain products with higher commercial value.

Xanthan gum is a commercial gum that has special rheological characteristics compared to other gums, like high viscosity at low concentrations, pseudoplasticity and stability over a wide range of pH. Therefore, it is used as a thickener, stabilizer and emulsifier in the pharmaceutical, food and oil industries (Luvielmo & Scamparini, 2009; Palaniraj & Jayaraman, 2011). Brazil is a large importer of xanthan gum, spending a lot to acquire the product. In 2016, it spent 16 million dollars importing 5.3 million kg gum (AliceWeb, 2017). The gum is industrially synthesized from glucose by the bacterial species *Xanthomonas campestris*.

Glucose is usually the most widely used carbon source in the production of xanthan gum (Rosalam & England, 2005), but other sources have been successfully studied to substitute and cheapen the process, such as whey (Nitschke, Rodrigues, & Schinatto, 2001), apple juice residue (Druzian & Pagliarini, 2007), soluble compounds of the green coconut shell (Nery, Cruz, & Druzian, 2013), cassava whey (Brandão, Esperidião, & Druzian, 2010), sugarcane juice (Brandão, Nery, Machado, Esperidião, & Druzian, 2008), lignocellulosic agro-industrial wastes (Silva et al., 2018) and glycerin from biodiesel (Brandão et al., 2013). Since glycerin is low in cost and represents an environmental problem, it is a good option, but further research is needed to determine optimum growth conditions.

This paper aimed to study the production of xanthan gum by *Xanthomonas axonopodis* bacterium in two different growing medium (simple and complex) using crude glycerin as carbon source.

Material and methods

Microorganism and glycerin

Xanthomonas axonopodis pv. mangiferaeindicae 1230, previously *X. campestris mangiferaeindicae* 1230, acquired from the collection of Phytobacteria of the Biological Institute of Campinas, was used.

Glycerin from soybean oil biodiesel production was used. The glycerol content was measured by high resolution gas chromatography (Column temperature ramp Initial: 80°C Stabilization: 1 min. Final: 250, 10°C $min.^{-1}$ Stabilization: 1 min. Initial mass: 50 UA - Final mass: 500 UA; Initial acquisition time: 2.1 min. - Final acquisition time: 19 min. Injector: Split/Split less Temperature: 150°C; Stabilization: 20 min. Split Ratio: 10; Sample volume: 1 microliter. Column Bruker - BR-5 ms FS 30 m 0.25 mm D 0.25 um. Detector Masses (MS – TQ)). The pH was measured using a pHmeter.

Inoculum

The inoculum was grown in Erlenmeyer flasks (250 mL) containing 50 mL YM broth (1 kg m^{-3}): peptone, 5, yeast extract, 3, malt extract, 3, glucose, 20, distilled water qsp 1000 mL) from slant cultures of the microorganism. The flasks were incubated at 30°C and 180 rpm for 72 hours.

Production of xanthan gum:

Experiments were carried out using two different growth media: Medium 1, complex: modified YM broth (1 kg m^{-3}): peptone, 5, yeast extract, 3, malt extract, 3, glycerol, 40, KH_2PO_4, 0.1 and urea, 0.01; Medium 2, simple (1 kg m^{-3}): glycerol, 40, KH_2PO_4, 0.1 and urea, 0.01. The pH was adjusted to 7 in both media. The experiments were done in triplicates in Erlenmeyer flasks (250 mL) with 50 mL medium, inoculated at room temperature with 10% (v v^{-1}) volume of inoculum culture (5 mL).

The flasks were incubated at 180 rpm and 26ºC± 0.1ºC (Luvielmo & Scamparini, 2009). All replicates of each experiment were made at the same time. The supplementation of glucose was done by adding 5 kg m^{-3} glucose to the culture after 48 and 96 hours. Samples represented the entire contents of the flask at 8, 24, 48, 96 and 120 hours. Cells and gum were recovered from the culture for analysis.

Analytical Methods

Biomass and gum recovery and analysis

Cells were separated by centrifugation (Eppendorf 5804R rotor S-4-72) in 50 mL Falcon tubes and rcf = 2934g at 25°C for 30 minutes, and washed twice with distilled water. The cell concentration was determined by dry weight at 80°C for 24 hours.

Gum was recovered by precipitation after cell harvest using ethanol 98% (v v^{-1}) added to the culture medium in a 3:1 ratio, followed by 48 hours of rest in the refrigerator at 4°C. The gum was centrifuged in 50 mL Falcon tubes (Eppendorf 5804R rotor S-4-72), rcf = 2934 g at 5°C for 30 minutes, dried at 37°C for 72 hours and weighed.

Rheological analysis and chromatography

Rheological analyses for each replicate were performed after resuspension of the gum in distilled water at a concentration of 1% on a HaakerRheometer (Model RS 50 - Rheostress, with a Haake K20 thermostatic bath). The cone - plate geometry (sensor: C28/1° Ti) was used. Flow curves of the xanthan gum samples (5,000 ppm) were determined at room temperature (26°C) with a tension ranging from 0.01 to 0.5 Pa, during 800 seconds. Chromatography of xanthan gum was done using the method described by Moreira, Souza, and Vendrusculo (1998). The power law model was used to verify consistency (k) and behavior (n) (Machado, 2002). The calculations were performed in the software Origin.

Results and discussion

Production of xanthan gum

The pH and the content of glycerol in the soybean glycerin used in this study was 8.3 and 55.71% (v v^{-1}), respectively. Glycerin from the production of biodiesel usually has a content of 40-88% (v v^{-1}) of glycerol (Mota, Silva, & Gonçalves, 2009; Quispe, Coronado, & Carvalho, 2013).

Previous experiments with glycerin from soybean and beef tallow in different concentrations showed better results with the first one at the concentration of 40 kg m^{-3}. Thus, the concentration of 40 kg m^{-3} was chosen for the present study. Figure 1 illustrates the cell and gum concentrations over time in both media tested. In medium 1, the gum concentration decreased after 36 hours but the cell concentration increased until 120 hours. In medium 2, the growth rate decreased after 36 hours but the concentration of the gum continued to increase until 96 hours. The production of gum in medium 2 (simpler, less nitrogen content) was twice as the production in medium 1 (more complex, higher nitrogen content). On the contrary, the growth in medium 1 was approximately five times greater.

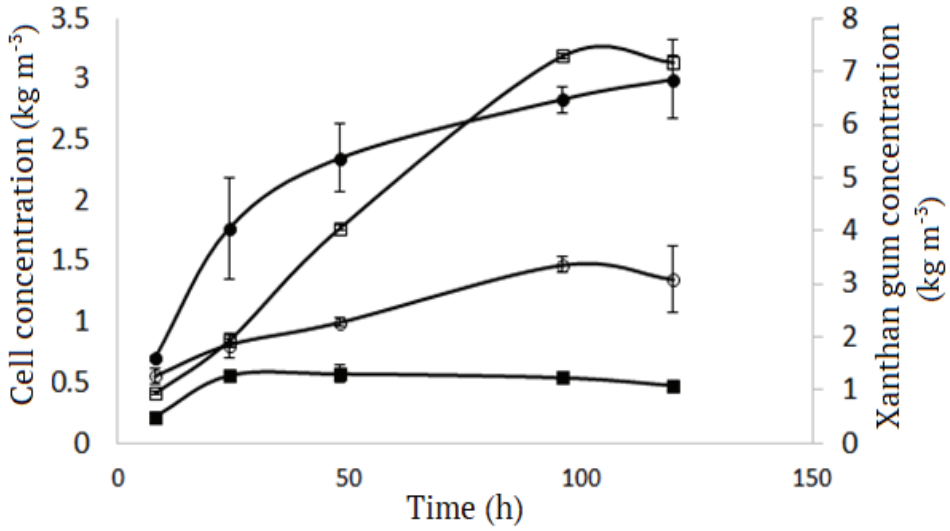

Figure 1. Cell growth in complex (medium 1) (•) and simple (medium 2) (o) medium; Xanthan gum production in complex (medium 1) (■) and simple (medium 2) (□) medium. Incubation at 180 rpm and 26ºC, n = 3.

The carbon/nitrogen ratio in addition to having an effect on xanthan gum production also affects bacterial growth (Assis et al., 2014). An excess of carbon and low concentrations of nitrogen are essential for production of xanthan gum (Sutherland, 1983; Lo, Yang, & Min, 1997). The production of gum was two times higher in the simple medium, in which the carbon/nitrogen ratio was high. In this medium, a well-defined stationary phase was observed, and the highest production of gum coincided with this phase. In the complex medium (with peptones and extracts), the cell concentration increased throughout the time, as well as the concentration of xanthan gum, showing that high concentrations of nitrogen in this case were detrimental for gum production but led to a greater cellular growth. Lo et al. (1997) showed that a moderate concentration of nitrogen source at the beginning of the fermentation process combined with a high concentration of carbon source leads to an optimization of xanthan gum production, since it accelerates fermentation and increases production of gum.

In order to verify if the addition of glucose in the culture medium could provide higher production or quality of the gum, glucose additions at 48 and 96 hours culture were performed. The results are shown in Figure 2.

The fermentation profile was essentially the same as the fermentation without supplementation (Figure 1). The addition of glucose in both media did not lead to an increase in xanthan gum production. Funahashi, Yoshida, and Taguchi (1987) showed that a good way to increase the production of xanthan gum is through the constant feeding of glucose throughout the process. They observed an increase in gum yield from 30 to 42 kg m^{-3} when glucose was constantly fed to the medium.

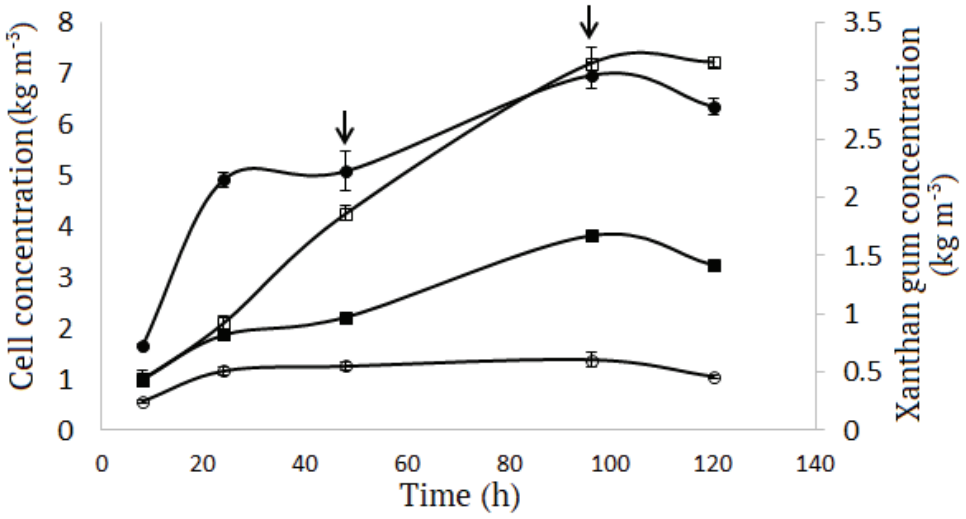

Figure 2. Cell growth in complex (medium 1) (•) and simple (medium 2) (o) medium; Xanthan gum production in complex (medium 1) (■) and simple (medium 2) (□) medium.With 5 kgm^{-3} glucose addition in 48 and 96 hours of culture (indicated by arrows). Incubation at 180 rpm and 26°C, n = 3.

It has been reported xanthan gum production from 1.3 to 43.3 kg m^{-3} depending on the carbon source (sucrose, glucose, cassava whey, glycerin, etc) (Brandão et al., 2010; Ghashghaei, Soudi, & Hoseinkhani, 2016). In the present study, the gum concentration obtained at the best condition (3.2 kg m^{-3}) is in good agreement with the literature. Brandão et al. (2013) used the same strain of the present study to produce xanthan gum from glycerin of biodiesel in a peptone and extracts free medium. They observed a cell concentration of 5 kg m^{-3} and 7 kg m^{-3} xanthan at the end of 120 hours cultivation. The lower gum production in the present study can be associated with the glycerin used. Although Brandão et al. (2013) have used the same microbial strain of the present study, the glycerin was not the same. During biodiesel production, some factors such as low conversion, accumulation of impurities from the reaction and a poor separation of the esters produced, may lead to a low glycerol and high impurity content in the glycerin. The different impurities present in the glycerin used in this study could lead to lower production. Impurities in glycerin showed an inhibitory effect on cell growth and production in fermentation process (Ardi, Aroua, & Awanis Hashim, 2015). The higher pH of this glycerin (pH 8) compared to that in Brandão et al. (2013) study (pH 6) shows that at least the content of salts is different, besides the content of glycerol.

Initial pH also shows influence on xanthan gum production. The gum production by *Xanthomonas campestris* NRRL B – 1459 was maximum at initial pH 7, having a huge fall in more acidic pH (pH 6) or more basic pH (pH 8) (Salah, Chaari, Besbes, Blecker, & Attia, 2011). In the present study, glycerin presented pH 8, which could be another possible explanation for the lower production compared to Brandão et al. (2013).

Agitation and aeration rates are other parameters that influence gum production. The production by *X. campestris mangiferaeindicae* 2103, cultivated in peptone and extracts free medium and biodiesel glycerin (20 kg m^{-3}), was studied by Assis et al. (2014). Better results (5.47 kg m^{-3}) were obtained at aeration of 1 vv m and agitation of 500 rpm than in other two combinations (0.5 vv m and 300 rpm or 1.5 vv m and 300 rpm). This was associated with the cell damage caused by shear stress and the differences in oxygen transfer to the culture medium. Gum production at low aeration and low rotation (2.02 – 2. 89 kg m^{-3}) are close to that found in the present study (3.19 kg m^{-3}), which may indicate that the lack of oxygen dispersion in the medium may have led to low productivity of gum when compared to the literature.

Characterization of xanthan gum

The chromatography of the hydrolyzed polysaccharide obtained (Figure 3) showed that its composition is compatible with that of xanthan gum (Moreira et al., 1998).

Xanthan gum is composed of pentasaccharide units that present in their main chain units of D-glucose and trisaccharide side chains containing glucuronic acid between two mannose units (Borges & Vendruscolo, 2008). Other sugars have been reported in this composition, such as rhamnose, depending on the microbial strain (Luvielmo & Scamparini, 2009).

The presence of glucose, mannose and glucuronic acid and the absence of rhamnose in all the samples were verified (Figure 3). The production of rhamnose-containing gum was reported in some species of genetically related *Xanthomonas* (Moreira, Vendruscolo, Gil-Turnes, & Vendruscolo, 2001).

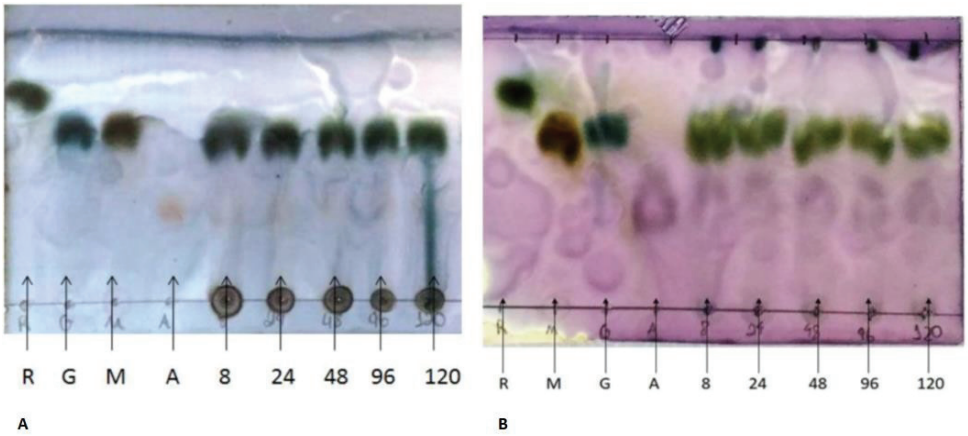

Figure 3. Thin layer chromatography of xanthan gum produced from soybean glycerin. A – Complex medium. B – Simple medium R = Rhamnose; G = Glucose; M = Mannose; A = Glucuronic acid; 8, 24, 48, 96 and 120 = Culture time in hours relative to each sample.

The spots related to glucuronic acid were very tenuous and were not very visible. The literature shows that the percentage of glucuronic acid present in xanthan gum is almost five times lower than the percentage of mannose and glucose (Assis et al., 2014), which explains the observed. Glucose and mannose, despite having bands at the same retention value (Rf), have different colors which allowed their visualization, indicating the presence of both in the gum obtained (Moreira et al., 2001). The addition of glucose did not change the composition of the produced polysaccharide.

The flow curves of the obtained xanthan indicated a gum with low viscosity (Figure 4). The investigated aqueous xanthan gum solutions exhibit the behavior of pseudoplastic fluid that is expected for xanthan gum (Diaz, Vendruscolo, & Vendruscolo, 2004; Rottava et al., 2009).

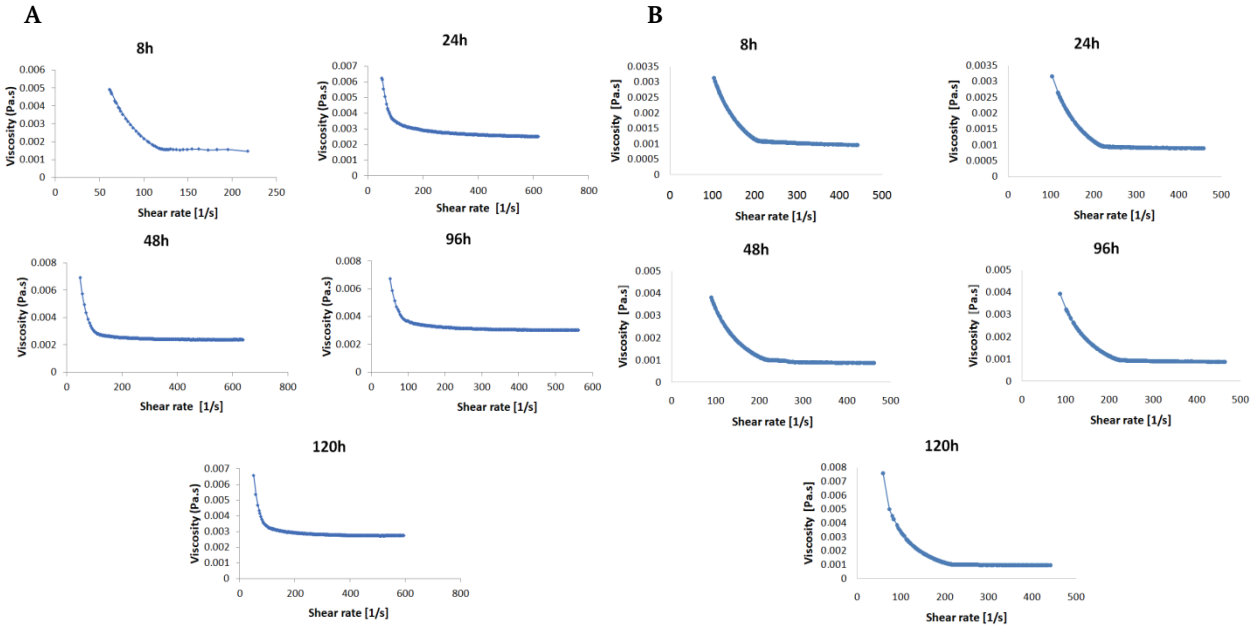

Figure 4. Flow curves at room temperature of aqueous solutions (5000ppm) of xanthan gum produced from biodiesel glycerin. A – Complex medium and B – Simple medium at 8, 24, 48, 96 and 120 hours of cultivation.

The viscosity of the gum did not vary along the cultivation remaining between 0.001 and 0.004 Pa.s for shear rates higher than 200 s^{-1}. The viscosity showed an increase of 25% after 96 hours in the experiments using complex medium with glucose supplementation, which was not observed in simple medium, it kept the same viscosity (data not shown).

The power law model showed that the consistency index (k) increased significantly in the medium 2 (simple) when compared to medium 1 (complex) (Table 1). Higher consistency index means a fluid with better consistency.

Table 1. Results of power law index k and n.

	medium 1 (Complex)		medium 2 (Simple)	
Glucose	n	k (Pa.s^n)	n	k (Pa.s^n)
Without supplementation	0.1054	0.223	0.5173	3.640
With supplementation	0.1054	0.223	0.6647	7.588

The behavior index (n) also increased in medium 2 (simple), showing a reduction in the pseudoplastic behavior. Glucose supplementation caused no change in the behavior and consistency indices in the complex medium. However, in the simple medium, glucose supplementation increased both indices.

Conclusion

The results showed that the excess of nitrogen can be detrimental to the production of xanthan gum, once the production was higher in simple medium. The relationship between cell growth and gum production showed that excess cell growth may impair gum production. The polysaccharide produced showed the chemical and rheological characteristics expected. The later addition of glucose affected the rheology of xanthan by increasing the consistency and behavior index. The production of xanthan gum can be favored in simpler medium with higher carbon/nitrogen ratio with later addition of glucose to enhance gum properties.

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