New Culture Medium to Xanthan Production by *Xanthomonas campestris* pv. *campestris*

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Abstract Xanthan is a important biopolymer for commercial purpose and it is produced in two stages by *Xanthomonas campestris*. In the first one, the bacterium is cultivated in the complex medium enriched in nitrogen and the biomass produced is used as inoculum for the next stage in which the gum is produced in another medium. In this work a new medium for the first stage is proposed in place of currently used YM medium. Different formulated growth media were studied and the correspondent biomass produced was analysed as inoculum for the second stage. The inoculum and gum were produced by batch process in shaker at 27°C in pH 6.0 and at 30°C in pH 7.0, respectively. The gum was precipitated with ethanol (3:1 v/v). The dried biomass and xathan gum produced were determined by drying in oven at 105 and 40°C, respectively. The viscosity of the fermentation broth and 1% gum solution in water were determined in Brookfield viscometer. The formulated medium presented the increase in gum production (30%), broth (136%) and 1% gum solution viscosity (60%) compared to YM, besides the inferior cost. The results showed the importance of the quality of the inoculum from the first stage of the culture which influenced on the gum viscosity in the second stage.

Keywords Development · Growth medium · *Xanthomonas campestris* · Xanthan · Viscosity

Introduction

The xanthan gum, a biopolymer synthesized by *Xanthomonas campestris*, is mainly used as thickener, stabilizer and friction reducer for respectively food, pharmaceutical and petroleum industry [1]. The xanthan market is about US \$270 million and it is expected to reach US \$400 million with a production of 80,000 tons/year in 2015 [2]. In the industrial production is used 2–4% of carbon source as sucrose or glucose, and 0.05–0.1% of nitrogen source as yeast extract, peptone, ammonium nitrate or urea [3]. The cost, rheological properties and yield of this gum depends on the culture medium used in the fermentation process [4–9]. Some authors reported that sucrose is the best carbon source [10, 11]. There are controversies about the best nitrogen source to be organic or inorganic for the growth of *X. campestris* and xanthan production. Cadmus et al. [12] produced gum more viscous using $(\text{NH}_4)_2\text{HPO}_4$, while Souw and Demain [11] and Garcia-Ochoa et al. [10] used glutamate.

The ratio C/N should also be considered in the process of production of the biopolymer. The biomass is limited by concentration of available nitrogen and the xanthan production rate is influenced by concentration of carbon [13]. Therefore, the requested nutrients by *Xanthomonas campestris* pv. *campestris* are different during the bioprocess, becoming necessary its division in two stages: cell growth and gum production. It was verified the importance of using a concentration of yeast extract relatively high in the medium to reach a high cellular density before culture to enter in the stationary phase. Several reports verified that the yield and the rate of production of the biopolymer are favored with the increase of the proportion glucose/yeast extract in the medium [14–16]. Some organic acids have been used as nutrients for the bacterium *Xanthomonas*

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campestris. Souw and Demain [11] studied the addition of succinate, pyruvate, and α -ketoglutarate and verified that excess concentrations of these organic acids inhibited xanthan formation. The same authors concluded that citrate (0.09–0.18%) stimulates the production of xanthan when the pH is not controlled in defined media [17].

Many nutritional studies have been focused in the production stage to improve yield and viscosity gum, but not in the first stage where the inoculum is produced. The YM medium is internationally described as growth medium for *X. campestris* [10–12, 18–20]. However it is expensive (US \$248.00/Kg) to be used in the production of xanthan gum with costs on average US \$5.5/kg [21, 22].

In this study is evaluated a new culture medium as alternative to YM to produce an inoculum resulting in cost reduction and improve in xanthan technology.

Materials and Methods

Microorganism and Fermentation

Xanthomonas campestris pv. *campestris* FCLA n. 26 was obtained from Culture Collection of the University of São Paulo State-UNESP, in Assis, SP, Brazil. This strain was maintained by plating in YM agar [23]. The experiments were conducted in shaker in four fermentations and each one with two stages and in three replications. In the first stage was produced the inoculum for the second one in which xanthan was produced.

The Inoculum Production

The culture of *Xanthomonas campestris* pv. *campestris* was started with a aseptic inoculation in plates containing YM agar medium and incubated in stove at 27°C for 24–48 h. From these plates, a cell suspension was obtained mixing the biomass with water solution. The cell concentration (10^7 cells/ml) was adjusted by sterilized water dilution and quantification by microscopy in a Neubauer Chamber. This cells suspension, an aliquot of 7.5 ml was inoculated in 75 ml of each formulated medium in 500 ml Erlenmeyer flask, and incubated in orbital shaker (Mod. TE 421-Tecnal, Piracicaba, Brazil) at 27°C and 160 rev/min for 24 h.

Formulated Medium

The growth medium was formulated testing different concentrations of the following nutrients: nitrogen source, sucrose, citrate and glutamate, in this sequence. It was selected for each experiment the condition in which was obtained the best quality of the gum through the viscosity in 1% gum solution. In the Table 1 is described the

formulated medium for nitrogen source and the other nutrients. Sucrose with several concentrations (0, 1.0, 1.5, 2.0, 2.5 and 2.5% w/v) was studied using 0.5% yeast extract and the other nutrients used in the nitrogen source test. Citrate and glutamate with the same concentrations (0.05, 0.1, 0.15, 0.20% w/v) were formulated as the same previous reported to the others. All media also contained 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.15% K_2HPO_4 . The media pHs were adjusted to 6.0 with 1 M sodium hydroxide solution before sterilization at 121°C for 15 min.

Gum Production

In the second stage of the fermentation process, 25 ml of the culture from the first stage were inoculated aseptically in 300 ml of medium described by Souw and Demain [11] in 1,000 ml Erlenmeyer flask. The culture was incubated in orbital shaker for 72 h at 30°C and 160 rev/min. In all media the pH was adjusted to 7.0 with 1 M sodium hydroxide solution before sterilization by autoclaving at 121°C for 15 min.

Analytical Methods

The cell and xanthan concentration were determined by dry weight. The samples were taken of the fermented broth at initial and final time of the first stage (0 and 24 h) and second one (0 and 72 h). These samples were centrifuged under $5310 \times g$ for 40 min at 4°C for cells separation. The washed biomass was obtained after mixed cells and distilled water and other centrifugation. The dried biomass was determined by drying in oven at 105°C until constant weight. Supernatant of the first centrifugation was mixed with ethanol (1:3 v/v) to precipitate the gum. The bio-polymer was separated and dried in oven at 40°C until constant weight. The dried gum was ground with mortar and pistil and its weight determined.

The viscosity of the fermentation broth and 1% gum solution in water were determined in Brookfield viscometer

Table 1 Formulation of culture media of *Xanthomonas campestris* pv. *campestris* with different nitrogen sources

Medium ^a	Yeast extract (%)	(NH_4) ₂ HPO ₄ (%)
1	0.30	0
2	0.30	0.25
3	0.50	0
4	0.50	0.25
5	0.70	0
6	0.70	0.25

^a Percentage (w/v)—all culture media including 2.5% sucrose, 0.01% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 0.15% K_2HPO_4

(model LVDVII +) at 25°C. The total reduced sugars were analyzed by Nelson's method [24], after biopolymer recovery in initial and final time of growth and production stages. Cell yield and xanthan yield were calculated according Pirt [25].

Results and Discussion

Influence of Nitrogen Source in Xanthan and Biomass Production

The nitrogen source in the first stage of fermentation influenced all parameters studied (Table 2). The cell yield was superior ($Y_{x/s} = 0.06$ g cell/g sugar) in medium 4 with yeast extract (0.5%) and $(\text{NH}_4)_2\text{HPO}_4$ (0.25%). The medium 3 showed superior gum viscosity (245 cP) and it was intermediate in nitrogen source (0.5% yeast extract). Media with higher values showed inferior viscosity (ex. medium 5 and 6). The same occurred with YM medium, with more nitrogen sources (0.5% peptone, 0.3% malt extract and 0.3% yeast extract). The quality of the inoculum produced in the first stage influenced the gum production and its viscosity. Flores and Deckwer [26] observed that low concentrations of NH_4Cl improved rheological properties of xanthan. According Souw and Demain [11] this fact could be explained due to nitrogen excess to stimulate the metabolism of cellular growth in detriment of the xanthan production.

Influence of Sucrose and Organic Acid on Xanthan and Biomass Production

The effect of the sucrose and organic acids on biomass and gum production in the first and second stage of fermentation is showed (Table 3). A direct correlation between sucrose concentration and produced biomass was verified. The maximum value of 1.26 g/l was obtained with 2.0%

(w/v) sucrose. This value was 72.6% superior to YM medium. Citrate in the growth medium not only serves as a buffering agent but also improve the production of biomass as demonstrated in this work (Table 3). In the fermentation with 0.2% citrate and 2.5% sucrose in the growth medium was obtained higher level of biomass (2.44 g/l), causing further increase in the difference in relation to YM medium (3.3 times superior). Whereas the glutamate did not cause increase in the biomass comparing with the medium without this nutrient.

Increasing sucrose concentration in the medium also stimulated xanthan production in both stages of fermentation (Table 3). There was a gum increase of 5.86 times in the first stage and 61.3% in the second stage, when the medium with 2.5% sucrose was compared with the condition without sucrose addition, and 14.9% in the second stage when it was compared with YM medium. A linear correlation between sucrose and produced gum in the first stage of growth ($R = 0.9772$ and $P < 0.0001$) and second stage ($R = 0.8575$ $P < 0.01$) were determined showing the importance of these concentrations in gum production (Fig. 1).

According to Gandhi et al. [27] the carbon source influences both cell growth and the production of the biopolymer; however, given carbon source can support a good growth without significant production of polysaccharide. In this work it was observed that 2.5% sucrose in the medium of the first stage provided a more efficient inoculum for the second stage improving biomass and gum production.

The addition of the 0.05% citrate in the formulation of the medium increased the amount of gum produced especially in the gum production step, about 9% superior to the medium without citrate and 25.3% superior to YM medium. For this reason citrate was incorporated into the growth medium for *X. campestris* pv. *campestris* and it was used in the test of glutamate. The glutamate did not contribute to an expressive increase in xanthan production in none of the steps (Table 3).

Table 2 Influence of different nitrogen source on xanthan production and viscosity, cell and biopolymer yield

Media	Gum (g/l) (24 h) $\bar{A}$	Gum (g/l) (72 h) $\bar{A}$	Cell yield ^a (g/g) $\bar{A}$	Xanthan yield ^b (g/g) $\bar{A}$	Gum viscosity (cP) $\bar{A}$
YM	1.1 ± 0.2	8.4 ± 0.9	0.015 ± 0.001	0.206 ± 0.079	124 ± 19
1	5.3 ± 1.0	9.2 ± 1.5	0.043 ± 0.006	0.162 ± 0.037	194 ± 72
2	4.8 ± 0.5	9.8 ± 1.4	0.024 ± 0.002	0.404 ± 0.204	207 ± 94
3	5.1 ± 2.3	9.9 ± 1.8	0.027 ± 0.002	0.345 ± 0.236	245 ± 109
4	3.6 ± 0.5	9.6 ± 1.4	0.060 ± 0.007	0.212 ± 0.094	171 ± 66
5	3.7 ± 1.0	7.1 ± 2.9	0.037 ± 0.005	0.154 ± 0.097	121 ± 109
6	3.3 ± 1.2	9.5 ± 0.6	0.038 ± 0.004	0.334 ± 0.179	124 ± 53

A Average 24 h e 72 h correspond to the growth and production phases, respectively

^a g biomass/g sucrose

^b g xanthan/g sucrose

Table 3 Influence of sucrose, citrate and glutamate in the formulated medium for growth of *Xanthomonas campestris* pv. *campestris* and biomass and xanthan production

Nutrients (%)	Growth step (g/l)		Gum production step (g/l)	
	Biomass $\bar{A}$	Gum $\bar{A}$	Biomass $\bar{A}$	Gum $\bar{A}$
Sucrose^a				
0.0	0.34 ± 0.06	0.56 ± 0.28	1.58 ± 0.51	6.2 ± 1.2
1.0	0.76 ± 0.14	1.80 ± 0.40	1.39 ± 0.22	5.7 ± 1.6
1.5	1.19 ± 0.19	2.10 ± 0.44	1.34 ± 0.43	7.5 ± 0.9
2.0	1.26 ± 0.22	3.49 ± 1.18	2.83 ± 0.69	7.7 ± 0.9
2.5	1.25 ± 0.16	3.28 ± 0.89	3.03 ± 0.54	10.0 ± 1.4
Citrate^b				
0.0	1.25 ± 0.16	3.28 ± 0.89	3.03 ± 0.54	10.0 ± 1.4
0.05	2.16 ± 0.13	2.86 ± 0.43	1.70 ± 0.33	10.4 ± 0.7
0.10	1.94 ± 0.21	2.76 ± 0.43	1.78 ± 0.33	11.7 ± 1.8
0.15	2.35 ± 0.45	2.73 ± 0.73	2.10 ± 0.27	10.4 ± 1.8
0.20	2.44 ± 0.21	2.35 ± 0.36	2.24 ± 0.41	10.9 ± 0.3
Glutamate^c				
0.0	2.16 ± 0.13	2.86 ± 0.43	1.70 ± 0.33	10.4 ± 0.7
0.05	1.87 ± 0.31	2.47 ± 0.31	2.11 ± 0.56	10.6 ± 1.3
0.10	2.02 ± 0.29	2.45 ± 0.19	1.98 ± 0.21	10.4 ± 2.3
0.15	2.09 ± 0.47	2.86 ± 0.51	2.22 ± 0.21	11.5 ± 0.3
0.20	1.92 ± 1.02	2.07 ± 1.26	2.08 ± 0.42	10.4 ± 1.0
YM Medium	0.73 ± 0.23	1.54 ± 0.41	1.96 ± 0.38	8.7 ± 1.3

^a Including: 0.5% (w/v) yeast extract, 0.15% (w/v) K₂HPO₄, 0.01% (w/v) MgSO₄·7H₂O

^b Including 2.5% sucrose

^c Including 0.05% citrate

Fig. 1 Effect of sucrose concentration in the formulated medium on the gum production in the first (24 h) and second stage (72 h) of fermentation

Influence of the Nutrients in the Viscosity of Biopolymer

An increase of 70% of the viscosity was observed in the medium with 2.5% sucrose compared with medium without sugar. It was observed (Fig. 2) that the formulation containing 2.5% sucrose significantly (Tukey $P < 0.01$)

Fig. 2 Viscosity of the fermentation broth and xanthan solution (1%) from inoculum obtained in medium with different sucrose concentration (25°C)

increased the viscosity of the fermentation broth (532 cP) and 1% gum solution (1,464 cP) in relation to culture with YM medium (1% glucose), respectively increase of 111 and 92.6%.

Comparative studies of glucose and sucrose showed that the highest yields and viscosities were obtained in fermentation using sucrose as carbon source [11]. The direct correlation verified in sucrose and gum viscosity in the first stage in the present work was not observed with the increase of sucrose in the medium of second stage with *X. campestris* pv. *pruni* [28]. In the medium of gum production (second stage) with sucrose concentrations of 4, 6, 8 and 10% (w/v) in fermentation of *Xanthomonas campestris* pv. *campestris* occurred decrease of the viscosity and gum production with the increase of sugar [29]. According to the author, the increase of osmotic pressure due to the increase in sucrose concentration may have inhibited the enzymes of xanthan biosynthesis and consequently the viscosity.

In this present work, a higher osmotic pressure caused by increased sucrose concentration (2.5%) in the medium influenced positively the gum production and viscosity. Probably the increase in the osmotic pressure due to higher sucrose concentration in the first stage of fermentation may be responsible for induction of biosynthesis of gum enzymes in the produced biomass, which are responsible for the production and quality of xanthan gum.

Fig. 3 The viscosity of the fermentation broth and xanthan solution (1%) from inoculum obtained in medium with different citrate concentration (25°C)

Fig. 4 Viscosity of the fermentation broth and xanthan solution (1%) from inoculum obtained in medium with different glutamate concentration (25°C)

The addition of citrate in the medium did not contribute to increase significantly (Anova $P > 0.05$) in the viscosity of the gum (Fig. 3). However with 0.05% (w/v) citrate in the medium provided a superior broth viscosity (702 cP). Also as previously reported this increase in the broth viscosity was not due to concentration of the gum because it did not increase (Table 3). So it can be attributed to other factors such as the presence of salts in the medium influencing the behavior of viscosity.

The addition of 0.15% (w/v) sodium glutamate in the culture medium to the growth of *X. campestris* pv. *campestris* improved the quality of the produced gum. There was an increase of 24.6% (Fig. 4) in the viscosity of 1% gum solution compared with the medium without glutamate. A significant (Anova $P < 0.05$) difference was

verified among the formulated medium containing 0.15% glutamate (422 cP) and YM medium (263 cP).

The proposed medium with 2.5% sucrose, 0.05% (w/v) citrate, 0.15% (w/v) glutamate, 0.5% (w/v) yeast extract, 0.15% (w/v) K_2HPO_4 and 0.01% (w/v) $MgSO_4 \cdot 7H_2O$ contributed to the formation of gum with a chemical structure in favor of higher viscosity, which is probably associated with high molecular mass and with favorable chemical composition. The literature shows that the conformation and molecular size influence gum viscosity. Xanthan with higher molecular mass shows higher viscosity [4, 26]. Acetyl and pyruvate present in the xanthan may also have influence on the viscosity. Casas et al. [4] demonstrated that xanthan gum with high levels of this groups had higher viscosity. According Shatwell et al. [30] the rates of acetyl and pyruvate affect the stability of the chain. Pyruvate creates an effect of instability in the helix due to increased repulsion between the internal chemical ramifications. According to these authors, the acetyl influence on stability due to apolar interactions between acetyl and methyl groups or because the acetyl group act as hydrogen acceptor. Thus, probably, the chemical structure of gum produced in the medium formulated contributed to an adequate intramolecular interaction between the gum and the aqueous solution.

The Table 4 shows a comparison for the new formulated medium and YM. The formulated medium shows smaller cost (57%) than YM for the growth of *Xanthomonas campestris* pv. *campestris*. Besides this new medium shows superior quality of the gum (60%), increase in

Table 4 Comparison of YM and formulated medium—cost medium per liter and fermentation parameters for gum production

Reagents	Formulated medium		YM medium		Difference (%)
	Weight (g)	Cost (US \$)	Weight (g)	Cost (US \$)	
Components					
Yeast extract	5	3.58	3	2.15	
K ₂ HPO ₄	1.5	0.090			
MgSO ₄ ·7H ₂ O	0.1	0.002			
Sucrose	25	0.45			
Citrate	0.5	0.010			
Glutamate	1.5	0.086			
Malt extract			3	1.43	
Peptone			5	3.51	
Glucose			10	0.34	
Total cost		4.21		7.43	−57
Fermentation parameters					
Biomass (g/l)		2.09		0.89	135
Gum (g/l)		11.5		8.8	30
Broth viscosity (cP)		648		274	136
Gum viscosity (cP)		422		263	60

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broth viscosity (136%), biomass (135%) and gum production (30%). These results indicate the proposed medium can contribute for the improvement of the xanthan technology by *Xanthomonas campestris*.

Conclusions

This work showed the importance of the quality of the inoculum from the first step of the culture which influenced in the gum and viscosity produced on the second step.

The growth medium used for production of inoculum is a factor impacting the gum production. A relatively high concentration of sucrose (2.5%), acetate (0.05%), glutamate (0.15%) and yeast extract (0.5%) produced an appropriated biomass as inoculum for the xanthan production. This new medium for the growth of *Xanthomonas campestris* pv. *campestris* was superior to YM technically and economically.

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