ARTÍCULO ORIGINAL

Influence of agitation and aeration in xanthan production by *Xanthomonas campestris* pv pruni strain 101

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ABSTRACT

Production, viscosity, and chemical composition of xanthan synthesized by bacterium *Xanthomonas campestris* pv pruni strain 101 were evaluated in bioreactor systems. During the process, the volumetric oxygen mass transfer coefficient (k_La) and the biomass were determined and the pH was monitored. The cultures were grown in a 3 l bioreactor, with aeration and agitation varying as follows: conditions (A) 300 rpm, 3 vvm and (B) 200 rpm, 2 vvm, at 28 °C. Our results showed that gum production was dependent on k_La, with a maximum yield of 8.15 g/l at 300 rpm, 3 vvm, 54 h of fermentation, k_La 21.4/h, while biomass was not affected. All aqueous solutions of 3% (w/v) xanthans synthesized showed a pseudoplastic behavior. The highest viscosity was reached under the strongest aeration/agitation conditions. All xanthan samples contained glucose, mannose, rhamnose, and glucuronic acid as their main components. The highest agitation and aeration rates used under condition A (300 rpm and 3 vvm) favorably influenced the yield and viscosity of the xanthan produced by bacterium *X. campestris* pv pruni 101 at different fermentation times.

Key words: *Xanthomonas campestris* pv pruni, xanthan gum, aeration/agitation

RESUMEN

Influencia de la agitación y la aireación en la producción de xantano por *Xanthomonas campestris* pv. pruni cepa 101. Se evaluó la producción, viscosidad y composición química del xantano sintetizado por la bacteria *Xanthomonas campestris* pv. pruni cepa 101 en un fermentador. Durante el proceso se controló el pH y se determinaron el coeficiente de transferencia de masa de oxígeno (k_La) y la producción de masa celular seca. Los cultivos se realizaron en un fermentador de 3 l variando la aireación y la agitación, en las siguientes condiciones: (A) 300 rpm, 3 vvm y (B) 200 rpm, 2 vvm; a 28 °C. Nuestros resultados mostraron que la producción de goma fue dependiente del k_La, con un rendimiento máximo de 8,15 g/l a 300 rpm y 3 vvm a las 54 h de fermentación, k_La de 21,4/h, mientras que la producción de biomasa no se afectó. Todas las soluciones acuosas de xantano al 3% (m/v) sintetizadas presentaron comportamiento pseudoplástico. La mayor viscosidad se alcanzó en la condición de aireación/agitación más intensa. Todas las muestras de xantano contenían glucosa, manosa, ramnosa y ácido glucurónico como constituyentes principales. La mayor tasa de agitación y aireación utilizada en la condición A (300 rpm y 3 vvm) influyó favorablemente en el rendimiento y la viscosidad del xantano producido por la bacteria *X. campestris* pv. pruni 101 a diferentes tiempos de fermentación.

Palabras clave: *Xanthomonas campestris* pv pruni, goma xantano, aireación/agitación

INTRODUCTION

Xanthomonas campestris is one of the most important microorganisms, not only from an agricultural point of view as phytopathogenic bacteria, but also for many branches of industry as the producer of a very useful polymer affecting the physico-mechanical conditions of products through thickenning, stabilizing, suspending, gelifying and emulsifying (10).

Xanthan production is a strictly aerobic process which can be carried out in either semi-solid or liquid media. When performed in a liquid medium, an increase in the medium viscosity occurs by the production of the extracellular polymer during fermentation. The oxygen transfer depends on medium viscosity and varies during the process, influenced by the air flow rate and the stirrer speed (7).

In general, agitation has a significant effect on oxygen transfer in non-Newtonian systems more so than aeration, because agitation decreases the medium viscosity, influencing the oxygen transfer (16). Most of the literature regarding different aeration conditions is based on the variation of stirring rates. The effect of aeration or low agitation together with high aeration on xanthan production has been scarcely studied.

A fundamental understanding of the key fermentation parameters is necessary in order to optimize production (16). The microorganism growth, production, structure, and rheological quality of xanthan are influenced by fac-

tors such as strain, bioreactor design, operation mode (in batches or continuous operation, medium composition, culture conditions (temperature, pH, concentration of dissolved oxygen, agitation and aeration rates), and by fermentation time (2, 5, 7, 11, 22).

According to Moreira (11), aeration/agitation and fermentation time influenced the yield, quality and chemical composition of xanthan produced by strain 06 of *X. campestris* pv pruni. The interaction between time and culture conditions highlights the need for this type of study in order to determine the optimal culture parameters for each strain.

The present work aimed at evaluating the influence of the aeration rate together with the agitation speed on production, viscosity, and chemical composition of xanthan synthesized by bacterium *X. campestris* pv pruni strain 101 and at assessing pH, the volumetric oxygen mass transfer coefficient (k_La), and the cell dry weight of the medium at different fermentation times.

MATERIAL AND METHODS

Microorganism
X. campestris pv pruni strain 101 was used in this study. The strain was isolated at Centro de Pesquisa Agrope-cuaria de Clima Temperado (EMBRAPA, Pelotas, Brazil) and selected based on xanthan production and viscosity results from a screening made among 30 strains at the Biopolymers Laboratory of the Federal University of Pelotas (UFPel, Pelotas, Brazil). Stocks of the bacterial strain were maintained by lyophilization.

Commercial xanthan
Commercial xanthan was used for comparing the viscosity results.

Media
a) Inoculum (g/l): yeast malt 3 g, malt extract 3 g, peptone 5 g and glucose 10 g (9).
b) Production medium (per liter): $NH_4H_2PO_4$ 1.5 g, K_2HPO_4 2.5 g, $MgSO_4.7H_2O$ 0.1 g and sucrose 50 g (3, 23).

Xanthan production
The fermentation of sugars by *X. campestris* pv pruni was performed in submerged culture. Two hundred and fifty ml Erlenmeyers containing 50 ml of YM liquid medium (9) were inoculated with 10^9 CFU/ml. The culture was incubated in an orbital agitator (Nova Tecnica model NT 711) at 150 rpm, 30 °C for 24 h.

A total of 300 ml of this culture was transferred to a 5 l bioreactor (B. Braun model Biostat B) containing 2700 ml of production medium (3, 23) and 0.5 ml of anti-foaming agent. The process was conducted at 28 °C, controlling the dissolved oxygen by means of an oxygen probe (Mettler Toledo model PN52200102) and the pH was monitored (Mettler Toledo model P/N 104054482).

Two combinations of agitation and aeration rates were tested: (A) 300 rpm, 3 vvm and (B) 200 rpm, 2 vvm. Samples were collected at 0, 6, 12, 24, 48, 54, 66, and 72 h, centrifuged for cell removal (Hiyechi model CR-21E) at 23,000 *g*, 30 min at 4 °C.

Ethanol was added to the supernatants at 4:1 ratio (v/v). Precipitated polymers were collected, dried in a stove at 56 °C until constant weight, and subsequently ground with a disc (Fritsch model Pulverisette) to a particle size of 0.5 μm. Xanthan production was measured in grams of dry polymer per fermented broth liter (g/l).

The cell dry weight concentration was determined by the gravimetric method. The broth was centrifuged at 23,000 *g* for

20 min, at 4 °C. The supernatant was discarded and the biomass was washed once with 0.89% saline solution and centrifuged again. The biomass was dried at 56 °C to constant weight, and expressed as grams of cells (dry weight) per liter of fermented broth (g/l).

Volumetric oxygen mass transfer coefficient (k_La)
The dynamic gassing-out method was used to determine the volumetric oxygen mass transfer coefficient that was obtained by the following equation:

$$k_La = \frac{\ln\left(\dfrac{C_i - C_0}{C_i - C}\right)}{t - t_0}$$

Where k_La is given in h^{-1}; C, C_i, C_0 are determined oxygen concentrations of the medium during the experiment expressed as mgO_2/l, and t, t_0 are interval time of measurements, expressed in h.

Viscosity
For the determination of the rheological characteristics of gum, 3% (w/v) xanthan solutions were prepared in distilled water, agitated for 2 h at room temperature, heated at 60 °C for 20 min, and finally maintained at room temperature for 24 h previous to test (25).

The viscosity of solutions was measured with a rheometer in the rotary mode (HAAKE model RS150), at 25 °C and 65 °C. A plate-plate system was used with a PP35Ti sensor and a shear rate of 0.01-100 s^{-1}.

Chemical composition
The polymers were hydrolyzed using 2 N HCl [3:100 (w/v)] at 80 °C for 16 h in a temperature controlled water bath (12).

The comparative thin-layer chromatography (TLC) technique on silica gel 60 F_{254} aluminum sheets was used. A volume of 3 μl of hydrolyzed samples and standards was applied. The eluent used was chloroform: methanol: acetic acid: water, at a ratio of 40:40:10:10 (12).

The chemical composition of biopolymers was determined by comparing with samples of rhamnose, mannose, glucose, and glucuronic acid. A sulphuric-anisaldehyde reagent was used for detection (24).

Statistics
All experiments were run in triplicates and results were subjected to a variance analysis, with comparison of means by the Tuckey's test at 5% significance level.

RESULTS AND DISCUSSION

Production of xanthan
Xanthan production by *X. campestris* pv pruni strain 101 was influenced by agitation speed/aeration rate and by fermentation time (Figure 1).

There was a significant difference in xanthan production between treatments at 54 h and 66 h. The production under higher agitation/aeration (A) was higher except at 48 h, when a higher production was achieved with lower agitation/aeration (B), reaching 7.15 g/l. Under condition A, the highest production occurred at 66 h, reaching 8.15 g/l. Under condition A, there was no significant difference between 48 h and 72 h, or between 54 h and 66 h. Under condition B, there was no difference among the 48 h, 54 h, and 66 h time intervals.

Moreira (11) observed a similar behavior for strain 06 of the same bacteria, using two combinations of agitation
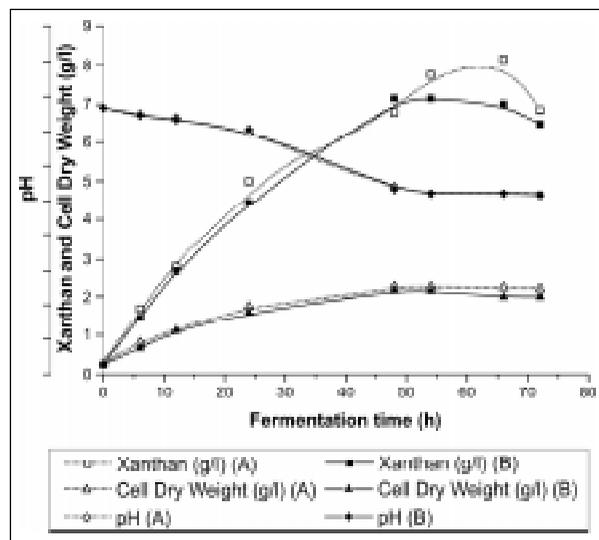
Figure 1: Effect of fermentation time on xanthan production by *X. campestris* pv pruni 101, cell dry weight and pH of the broth in relation to the agitation speed and aeration rate (condition A: 300 rpm, 3 vvm and condition B: 200 rpm, 2 vvm).

and aeration rates (250 rpm, 1.5 vvm and 350 rpm, 2 vvm). In her study, there was no significant difference in production between treatments during the first 24 h; the condition with lower agitation/aeration reached 5.5 g/l at 48 h, and that with higher agitation/aeration arrived at 6.5 g/l at 66 h. At 72 h, there was a lower yield for both treatments.

The results of cell dry weight were similar in both treatments (Figure 1), only significantly differing at 66 h. However, under condition A, the results were slightly better. In both treatments, the log phase extended from zero to 48 h, followed by the stationary phase, which extended until the process was stopped at 72 h, before the decline phase could be observed with the technique used. In general, the literature shows that for most biopolymer producing bacteria, maximum production is achieved towards the end of the exponential phase (14, 21). However, for strain 101, maximum xanthan production occurred during the stationary phase for both treatments, similarly to the results reported by Amanullah et al.; García-Ochoa *et al.* and Koníček *et al.* (1, 7, 10). For García-Ochoa *et al.* (7), this behavior occurs due to uncontrolled pH.

Different aeration conditions based on variation of agitation speed have been studied by several authors. Casas *et al.* (4) analyzed different agitation speeds (100, 300, 500, and 800 rpm) with an airflow of 1 l/min at a volume of 1.5 l. For them, xanthan production is partly associated with metabolic growth; up to 500 rpm, an increase in xanthan production and biomass concentration occurred, but at 800 rpm there was a decrease in xanthan production as well as in biomass, probably due to cells damaged by hydrodynamic stress.

Peters *et al.* (15) also analyzed the influence of agitation speed (200, 400, 600, and 800 rpm) at a lower aera-

tion rate, 0.33 vvm in a 10 l medium. In this case, xanthan production and cell growth increased with a rise in agitation speed; as a lower aeration rate was used, the increase in agitation speed was more important than the potential damage to the cells.

The effect of aeration by increase of the air flow rate on xanthan production has been scarcely studied. Roukas *et al.* (18) studied the effect of aeration (1 vvm to 3 vvm) on the production of pullulan, a biopolymer produced by *Aureobasidium pullulans*. The maximum pullulan concentration occurred at 2 vvm, and the authors attributed the decrease in pullulan concentration at high aeration rates to the likelihood of morphological modifications of the microorganism in the course of fermentation.

According to Li *et al.* (8), the increase in agitation speed and/or increase in oxygen supply decreases the medium viscosity, increasing the volumetric oxygen mass transfer coefficient ($k_L a$). Such an effect was observed in this experiment: under condition A, $k_L a$ was higher than under condition B, 21.4/h and 8.6/h, respectively. With this fact, our results agree with García-Ochoa *et al.* (6), who mentioned that the increase in the concentration of dissolved oxygen favours xanthan production. In this study, the best results of xanthan production were achieved under the condition of higher agitation and aeration rates.

There was no significant difference in pH decline between conditions A and B. Beginning with pH 6.9 at 0 h, there was a decline to pH 4.8 at 48 h, and then pH remained unchanged up to 72 h, when it reached 4.6 (Figure 1). According to García-Ochoa *et al.* (7) during xanthan production pH decreases from neutral to values close to 5 due to acid groups present in xanthan.

Viscosity

The viscosity results at 25 °C and 65 °C, presented in Table 1, show that all 3% (w/v) aqueous solutions of xanthan achieved under the combinations of agitation/aeration tested (A and B) at different fermentation times had a pseudoplastic behavior. The viscosity of the solutions was influenced by fermentation time, agitation/aeration rate, and temperature of analysis (Table 1).

The xanthans produced under higher aeration/agitation were more viscous at all fermentation times, and all samples produced showed a reduction in viscosity with the elevation of temperature. Under condition A, from 24 h to 54 h, viscosity increased, reaching 7.100 mPa.s at 25 °C and 4.700 mPa.s at 65 °C, both at a shear rate of 10 s^{-1}; after this period, there was a decrease in viscosity. Under condition B, at 25 °C and 65 °C, viscosity increased up to 48 h, remaining stable up to 66 h, reaching 5.400 mPa.s at 25 °C and 3.370 mPa.s at 65 °C, at the same shear rate. At 72 h, there was a decline in viscosity values. For Moreira (11), while studying strain 06, the opposite took place, that is, higher viscosity values were

Table 1. Viscosity (mPa.s) at 25 °C and 65 °C at a shear rate of 10, 30, 60 and 100 s^{-1} of 3% (w/v) aqueous solutions of commercial xanthan and xanthan synthesized by *X. campestris* pv pruni strain 101 at different fermentation times, under the following conditions: (A) 300 rpm, 3 vvm and (B)* 200 rpm, 2 vvm

Polymer	Temperature (°C)	Viscosity (mPa.s) Shear rate (s^{-1})			
		10	30	60	100
Xp-24h[1]	25	4.820 (3.770*)	1.830 (1.520*)	1.030 (0.880*)	0.660 (0.570*)
	65	3.560 (2.140*)	1.410 (0.940*)	0.800 (0.560*)	0.500 (0.380*)
Xp-48h[1]	25	5.940 (5.150*)	2.240 (2.000*)	1.250 (1.130*)	0.790 (0.720*)
	65	3,990 (3.150*)	1.650 (1.410*)	0.940 (0.820*)	0.580 (0.530*)
Xp-54h[1]	25	7.100 (5.200*)	2.620 (2.130*)	1.450 (1.230*)	0.910 (0.800*)
	65	4.710 (3.320*)	1.820 (1.430*)	1.010 (0.840*)	0.620 (0.540*)
Xp-66h[1]	25	6.480 (5.410*)	2.410 (2.110*)	1.340 (1.200*)	0.840 (0.770*)
	65	4.450 (3.370*)	1.800 (1.440*)	1.000 (0.840*)	0.620 (0.530*)
Xp-72h[1]	25	5.460 (4.820*)	2.070 (1.890*)	1.150 (1.080*)	0.730 (0.690*)
	65	3.860 (3.170*)	1.520 (1.270*)	0.840 (0.720*)	0.530 (0.460*)
X[2]	25	3.400	1.190	0.650	0.430
	65	3.310	1.130	0,610	0,380

[1]Xp: Xanthan produced by *X. campestris* pv pruni strain 101
[2]X: Commercial Xanthan

achieved under the condition of lower agitation/aeration, at all fermentation times. This distinct behavior seems to result from the strain used. The dependence on the strain is also clear in the correlation between viscosity and fermentation time, as shown by Souza *et al.* (19) when studying *X. campestris* pv pruni strain 24, where the xanthan synthesized at 72 h of fermentation was the most viscous.

Comparing the apparent viscosity of the 3% (w/v) aqueous solution of xanthan synthesized by *X. campestris* pv pruni 101 at 54 h, under condition A (7.100 mPa.s), with the 3% (w/v) aqueous solution of a commercial xanthan (3.400 mPa.s), both at 25 °C at 10 s^{-1}, it can be concluded that the polymer synthesized by strain 101 is of higher quality, despite its yield having failed to reach the optimal amount for industrial scale production. Although the polymer synthesized by strain 101 (4.710 mPa.s) is still more viscous than the commercial one (3.310 mPa.s), it did not show the same stability at 65 °C, as expected for the xanthan polymer.

According to Rocks (17), most aqueous gum solutions show a sharp decrease in viscosity with an increase in temperature, as observed with the aqueous solution of the xanthan synthesized by *X. campestris* pv pruni 101. Nevertheless, there are exceptions. For instance, the commercial xanthan usually sold with addition of ions, because it facilitates solubilization, increases viscosity, and also maintains its structure (double helix), stabilizing its viscosity up to approximately 100 °C (13).

Chemical composition

All samples from treatments A and B contained glucuronic acid, rhamnose, glucose, and mannose (Figure 2), as expected for pathovar pruni (2, 19, 23). The xanthan produced by *X. campestris* pv pruni has a different chemical composition (in sugar type) from that of the commercial xanthan gum (from *X. campestris* pv campestris) (23).

The qualitative composition of the biopolymers was similar, although the difference in size and intensity of the spots on the chromatograms suggests variation in the quantity of its components.

Up to 54 h, no difference was observed in the intensity of the bands between treatments, but from 66 h onwards, the mannose content decreased for both, coinciding with the reduction observed in viscosity. In treatment A, the high mannose content at 54 h, may have been responsible for the increase in viscosity, because at 66 h and 72 h there was a reduction in this monosaccharide. In treatment B, the mannose content was stable up to 54 h; however, at 72 h, an increase was observed in the amount of glucose in relation to mannose. This may account for the decline in viscosity noted after this period. The rhamnose content increased up to 66 h, when it reached its peak, under both treatments, which indicates a positive relationship between rhamnose and viscosity.

According to Antunes *et al.* (2) the differences in the intensity and size of spots could explain why some strains produce biopolymers with higher viscosity than others. According to these authors, the polymers with higher mannose and glucuronic acid showed higher thickening properties.

Moreira (11) also determined the chemical composition for TLC of xanthan synthesized by *X. campestris* pv pruni strain 06 under different agitation/aeration conditions (250 rpm, 1.5 vvm and 350 rpm, 2 vvm). The poly-
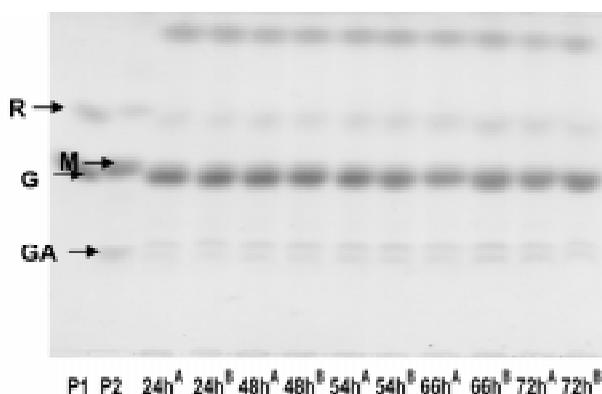
Figure 2. Comparative thin-layer chromatographic analysis (TLC) of polymers produced by *X. campestris* pv pruni strain 101 under conditions A and B at different fermentation times. (P1) standard of glucuronic acid (GA) and mannose (M); (P2) standard of glucose (G) and rhamnose (R).

mer produced under lower agitation/aeration showed a higher amount of mannose, being the most viscous. Our results agree with Sutherland (20), who reported that the chemical structure of the exopolysaccharides depends upon culture conditions and, especially, on the strain used.

The higher agitation and aeration rates used under condition A (300 rpm and 3 vvm) favorably influenced the yield and viscosity of the xanthan produced by bacterium *X. campestris* pv pruni 101 at different fermentation times. For strain 101, the end of fermentation must occur at 54 h, when the best combination of viscosity and yield is achieved, with lower energy expense and at a shorter time.

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