Producción y caracterización de la goma xantana por diferentes cepas de Xanthomonas campestris aislados de fuentes naturales en cultivo sumergido

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Resumen.-

La goma xantano es un exopolisacárido (EPS) de origen microbiano, producida por la bacteria X. campestris. El objetivo de esta investigación fue evaluar la producción de goma xantano de diferentes cepas de X. campestris aisladas de varias fuentes naturales en cultivo sumergido. Se aislaron cuatro cepas bacterianas de X. campestris de lechuga americana, romana (Lactuca sativa) y brócoli (Brassica oleácea var. itálica). Los parámetros cinéticos de crecimiento celular de las cepas aisladas y de la cepa de referencia se determinaron en cultivo discontinuo. Se evaluó el efecto del oxígeno y calcio en el medio de cultivo sobre el crecimiento celular de la cepa de referencia, demostrándose su influencia en el crecimiento celular del microorganismo y en la producción del exopolisacárido. Los parámetros cinéticos de las cepas estudiadas mostraron tendencias congruentes. La goma xantano obtenida con la cepa LR1-UCIN tuvo un rendimiento de 8,84 g/L, viscosidad de 1814 cP y un contenido de restos de piruvato de 5,10 %.

Palabras clave: Exopolisacárido, Bacteria fitopatógena, Xanthomonas campestris, Goma xantano

Production and characterization of xanthan gum by different strains of Xanthomonas campestris isolated from various natural sources in submerged culture

Abstract.-

The xanthan gum is an exopolysaccharide (EPS) of microbial origin, produced by the bacteria of the X. campestris. The objective of this research was to evaluate production of xanthan gum by different strains of X. campestris isolated from various natural sources in submerge culture. Four bacterial strains of X. campestris were isolated from American and Romaine lettuce (Lactuca sativa) and broccoli (Brassica oleácea var. italic). Kinetic parameters of growth were determined in discontinuous cultivation of the isolated strains and one of reference. The effect of the oxygen and calcium in the cultivation media was evaluated about the growth of the reference strain demonstrating their influence in the growth of the microorganism and in the production of the exopolysaccharide. Kinetic parameters of studied strains showed appropriate tendencies. Xanthan gum was obtained from LR1-UCIN strain with yield of 8.84 g/L, viscosity of 1814 cP, and content of remains of piruvato of 5,10 %.

Keywords: Exopolysaccharide, Phytopathogenic bacteria, Xanthomonas campestris, Xanthan gum

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1. Introduction

Biopolymers are polysaccharides of microbial origin, synthesized by bacteria, moulds and yeasts. These polysaccharides of complex chain as well known gums due to their capacity to form viscous solutions or gels in aqueous media. The importance and potentiality of the use of gums in the industry are guided to the pharmaceutical, chemistry and petrochemistry industry [1].

Xanthan gum is an exopolysaccharide produced by Gram-negative bacteria of the gender Xanthomonas through aerobic submerged fermentation, methods which it involves those processes that are carried out with the biological agents (bacteria and moulds generally) submerged in the aqueous phase and in the presence of molecular oxygen. It was discovered in the Regional Northern Research Laboratory (NRRL) of the Department of Agriculture of USA in 1961 as a product of fermentation of the bacteria Xanthomonas campestris NRRL B-1459 and it was denominated xanthan [2]. In our country, notwithstanding the importance that represents for the petroleum and oil industry, few are the studies that have been carried out, and for that reason the initiative arises of producing xanthan gum in submerged fermentation.

Azuaje and Sánchez [2] determined that X. campestris vp. ocumo exhibits bigger capacity to grow and to produce xanthan gum that other varieties analyzed with a wide diversity of sources of carbohydrates. Under these conditions a maximum production of 23 g/L xanthan gum was obtained.

In contrast, Papagianni et al. [3] have reported researches about the kinetics of growth and xanthan gum production from *X*. campestris ATCC 1395 in discontinuous cultivation. The maximum yield obtained of xanthan gum was 6.60 g/L. Nitschke and Rodrigues [4] studied the virulence of six Xanthomonas campestris isolates utilizing the percentage of lesion area in Brassica oleraceae host plant, compared the diameter of colonies, xanthan production and gum viscosity.

Based upon the above–described backgrounds, the objective of this investigation reached over the isolation of pure strains of X. campestris of natural resources of national precedence. Subsequently, the cell growth was evaluated and compared through kinetic studies of the isolated microorganisms and one bacteria of reference. Consecutively the best isolated producer strain was selected and a laboratory procedure for the production of the biopolymer was elaborated. Finally obtained xanthan gum was characterized of physicochemical and rheological form.

2. Materials and methods

2.1. Isolation of strains of X *campestris of alternative cultures*

2.1.1. Obtaining of pure strains of X *campestris*

Initially the obtaining of strains isolated from various natural sources came true: American and Romaine lettuce (Lactuca sativa) and broccoli (Brassica oleácea var. italic), which show the typical symptomatology in plants that, are affected by *X* campestris [5].

2.1.2. Characterization of X *campestris*

Pure strains of X. campestris were identified with the macroscopic typical characteristics (light yellow and smooth) [5, 6, 7].

2.2. Reference strain of X *campestris pv. zantedeschiae*

The bacterial strain was donated by Bacteriology Laboratory of Faculty of Agronomy of UCV and it was determined as *X* campestris pv. zantedeschiae.

2.3. Parameters of growth of the bacterium of reference X *campestris pv. zantedeschiae*

2.3.1. Production of bacterial cellules

Table 1: Yeast calcium carbonate agar composition (YCD).

Yeast Calcium Carbonate Media YCD	
Composition	1L
Yeast extract	10.0 g
Dextrose	5.0 g
Calcium carbonate (0.02 %p/v)	0.2 g

Inoculum preparation was by means of microorganism transfer from the original cultures

of UCV to yeast calcium carbonate dextrose agar (YCDA) (Table 1) on the plate surface and incubated at room temperature from 28 to 30°C for 48 h [8]. Bacterial formed cellules were cultivated in Erlenmeyer flasks with yeast calcium carbonate media (YDC) modified with calcium carbonate 0,02 % p/v at 28°C for 20 h in a shaker incubator at 250 rpm. At a later time the fermentation broth was centrifugated at 9000 rpm during 20 min. The obtained cells were re-dissolved in NaCl 0.75 % p/v solution to get a maximum biomass concentration of 0.2 g humid cells·mL^{-1} in solution [9, 10].

2.3.2. Standard curve

Three 500 mL Erlenmeyer flasks were inoculated with previous bacterial suspension to obtain a 0.05 OD and incubated at 28°C and 250 rpm for 20 h. A graph of cell biomass (g/L) and optical density (OD) units was constructed [9].

2.4. Determination of parameters of growth of isolated bacteria

Microorganism cultures were realized in 250 mL Erlenmeyer flasks with yeast calcium carbonate media (YDC) modified with calcium carbonate 0,02 % p/v at 28°C and 250 rpm for 72 h. At several reaction times optical densities of bacterial culture were determined for estimate biomass concentrations [9].

2.5. Spontaneous production of xanthan gum from a strain of reference X. campestris pv. zantedeschiae utilizing a conventional cultivation media in discontinuous cultivation

Microorganism cultures of *X*. campestris pv. zantedeschiae were realized in 250 mL and 500 mL Erlenmeyer flasks with yeast calcium carbonate media (YDC) at 28°C and 250 rpm for 72 h. Once each reaction time was finalized biomass concentration was determined from standard curve [9]. Biopolymer determination was realized using the supernatant with addition of 1:3 (v/v) ethanol and then xanthan gum was precipitated.

2.6. Evaluation of the effect of the oxygen dissolved in the media for the growth of the strain of reference X. campestris pv. zantedeschiae and production of gum xantano

Bath fermentations were carried in 250 mL and 500 mL Erlenmeyer flaks containing 120 mL of YDC. The OD for the inoculum of *X*. campestris pv. zantedeschiae was in a range from 0.02 to 0.05 [9]. Experiments were conducted twice in shaker incubator at 28°C and 250 rpm for 36 h. Cellular biomass was determined for several reaction times using standard curve. In order to determine xanthan gum the fermentation broth was centrifugated and the formed gum was precipitated with addition of ethanol 1:3 (v/v) [7].

2.7. Evaluation of the effect of the calcium in the media for the growth of the strain of reference X campestris pv. zantedeschiae and production of xanthan gum

Bath fermentations were carried in 250 mL and 500 mL Erlenmeyer flaks containing 100 mL of bacterium cellular suspense (0.2 g humid cells·mL^{-1}) with a media constituted of: 20 g/L glucose and 20 g/L yeast extract and variations of calcium consisted of: 20 ppm $CaCO_3$, 20 ppm $CaCl_2$, 100 ppm $CaCl_2$ and 1500 ppm $CaCl_2$ [6]. Biomass and xanthan gum concentrations were determined at several times by the same previous procedures.

2.8. Comparison of conventional sources isolated strains of X. campestris with the reference strain

Once results from the morphologic characteristics and biochemistries of the isolated strains and of the strain of reference were obtained, these were compared to the end to get explanatory proposals.

2.9. Production of the xanthan gum with the selected strain

Two experimental phases were realized, firstly a preliminary phase which the best-adequate strain for the production of the xanthan gum and the obtaining of parameters to use in the process and that second phase involved in executing the productive process.

2.10. *Characterization of the xanthan gum produced in cultivation media selected by rheological assays*

For rheological analysis, apparent viscosity was determined using a Brookfield viscometer accomplish at water bath with xanthan gum solutions of concentration of 3 wt % and 25°C of temperature [7].

2.11. *Determination of pyruvate content*

Pyruvate content was used as parameter of quality of the xanthan gum. It was determined following the methodology of colorimetric analysis used by Sloneker and Orentas (1962) [8].

3. Results and discussion

3.1. *Obtaining of pure strains of X. campestris*

The presence of yellow colonies could observe through its isolation, characteristic of the bacterium X. campestris, for that proceeded to evaluating itself for his confirmation. Two colonies were obtained from lettuce and broccoli and they were Gram-negatives [5] and mucoid, convex and yellow [6].

3.2. *Characterization of the bacteria isolated of X. campestris*

Table 2 shows fermentation results obtained at laboratory and compared with what reported by Schaad *et al* [6] for *X*. campestris. Small variations were found in any one of the realized tests owed principally to the existence of over 140 pathovars for the *X*. campestris [5].

3.3. *Determination of parameters of growth of isolated bacteria*

Cellular growth was evaluated using a culture media consisted of yeast, dextrose and calcium carbonated (YDC) (Table 2) because it is adequate for this type of phytopathogenic bacteria. A low concentration of calcium carbonated of 0.02 % p/v was used in order to avoid depositions of the same in media and that it may interfere in the measurements of optical density (OD). The cell growth curves of cell biomass over time for each of the isolated strains showed a typical behavior

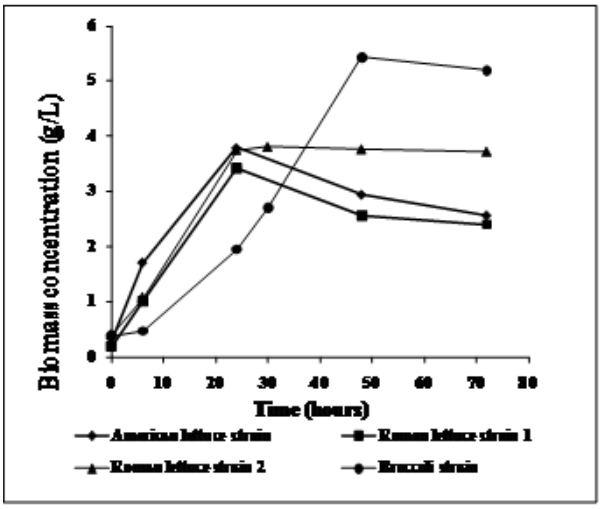

Figura 1: Biomass concentration time-courses in fermentations of isolate bacteria of *X*. campestris.

for a bacterial growth, caning to observe lag, exponential, stationary and death phases in each theirs, standing out some of another one because they were different strain (Figure 1) [5].

The equations of Malthus's law and Ricatti's equation were used for the determination of the kinetic parameters relative to the cell growth (Table 3) [11, 12]. Malthus's law (1), measure the bacterial growth like the increment of the cell mass expressed like cell concentration (X, g/L) over time (t, h). Being α specific growth rate in death phase (h^{-1}) y μ specific growth rate in exponential phase (h^{-1}).

$$\frac{dX}{dt} = \mu X - \alpha X. \tag{1}$$

Ricatti's equation (2) is a generic model and it is adapted to the microorganism's majority right now than model so much the exponential phase like stationary phase having like parameters K and T (h^{-1} y mL/g).

$$\begin{aligned} r_X &= \frac{dX}{dt} = kX - kTX^2 \\ &= kX(1 - TX). \end{aligned} \tag{2}$$

3.4. *Growth parameters of strain of reference X. campestris pv. zantedeschiae*

Came true firstly revision of references as to the cultivation media for the maintenance of strain,

Table 2: Morphologic studies and biochemical tests more relevant realized to strains *X*. campestris isolated from natural sources of lettuce and broccoli.

Realized tests (Biochemical, morphological and taxonomic tests)	American Lettuce–Leaf Strain (ALLS)	Romaine Lettuce–Leaf Strain (RLL1)	Romaine Lettuce–Leaf 2 Strain (RLL2)	Broccoli–Leaf Strain (BLS)	Xanthomona campestris
Color in (nutritive agar) AN	Light yellow	Light yellow	Light yellow	Light yellow	Light yellow
Form	Short rod	Short rod	Short rod	Short rod	Short rod
Growth at 35°C	+	+	+	+	+
Hugh Leifson media, OF (Oxidation – Fermentation)					
Oxidation, SG	+	+	+	+	+
Fermentation , CG	-	-	-	-	-
Mucoid growth in YDCA at 30°C	+	+	(+)	+	+
Gram tintion	-	-	-	-	-
Starch hydrolysis	+	+	(+)	+	+

(+), slightly positive; * Data according to Schaad et al. (2001) [6].

Table 3: Kinetic parameters of cellular growth for different isolated strains.

X. campestris strain	Specific growth rate μ (h^{-1})	Parameter K of Ricatti's equation, K (h^{-1})	Parameter T of Ricatti's equation, T $(g/L)^{-1}$
American lettuce, LA	0.0442	0.3799	0.3
Romaine lettuce, LR1	0.0684	0.4859	0.3
Romaine lettuce, LR2	0.0694	0.2683	0.3
Broccoli, B	0.0789	0.1153	0.2

same growth of it's and production of the xanthan gum [2, 13, 14].

3.5. Spontaneous production of xanthan gum in a conventional media and evaluation of the effect of oxygen dissolved of the strain of reference X. campestris pv. zantedeschiae

The cell growth curves of X. campestris pv. zantedeschiae in the utilized media and the applied environmental conditions (Figure 2) shows a typical tendency of growth. There is a notable difference between both fermentations as to the bacterium's growth because the space exists bigger upside down in 500 mL's Erlenmeyer exists than in 250 mL's Erlenmeyer, so, there are bigger available oxygen and bigger level of saturation or oxygen dissolved in the middle in 500 mL's Erlenmeyer.

3.6. Evaluation of the effect of the calcium dissolved in the cultivation media

Results of optic density and biomass concentrations over time in *X*. campestris pv. zantedeschiae fermentations with different concentrations of calcium added to cultivation media are showed in Figure 3.

There are not observe loud differences themselves even approximately when comparing the two salts of calcium studied ($CaCO_3$ y $CaCl_2$) until 40 h of fermentation, although as from this time with the chloride ion gets bigger quantity from biomass itself than with the ion carbonate (Figure 3).

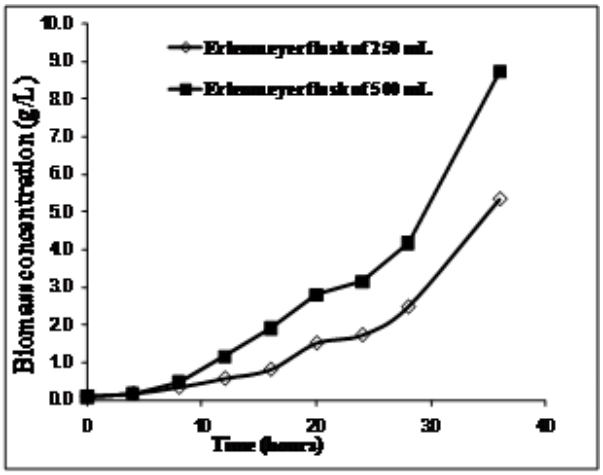

Figura 2: Biomass concentration time-courses of X. campestris pv. zantedeschiae in fermentations for Erlenmeyer flasks of 250 mL and 500 mL.

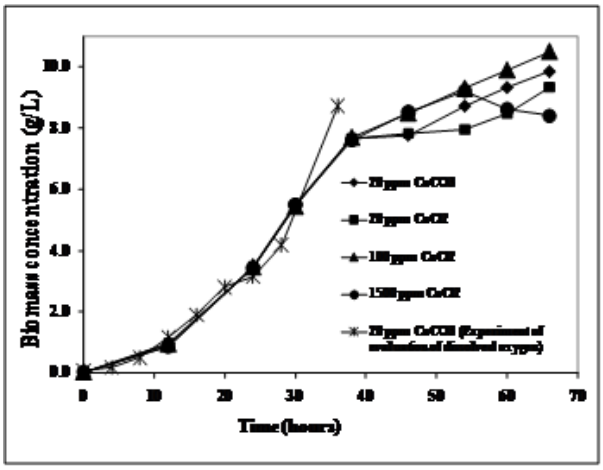

Figura 3: Biomass concentration time-courses of *X*. campestris pv. zantedeschiae in fermentations with several concentrations of calcium.

Calcium seems to be one of the ions with bigger incidence in this bioprocess, and according references various concentrations and presentations of the calcium in the cultivation media were evaluated [6, 15]. The previous results suggest than in the cultivations gone into with variations of 20 ppm of $CaCO_3$, 20 ppm of $CaCl_2$ and 100 ppm of $CaCl_2$, *X*. campestris pv. zantedeschiae bacterium finds to the 66 h even in exponential phase of growth (Figure 3).

In contrast, for the cultivation gone into with 1500 ppm of $CaCl_2$, the bacterium *X*. campestris pv. zantedeschiae to that time of incubation tends to reach the stationary phase. In this case, calcium at that concentration seems to effort a loud favorable effect on growth of bacterium because of that element is required by enzymes for its stability and bigger activity in the cell metabolism. Besides, calcium carbonate acts as a buffer maintaining the close pH to neutrality being beneficial to the phytopathogenic bacteria, however this salt becomes very loud concentrations little soluble (0.00066g/100g a 20°C; Lide [16]) and form deposits, contrary to calcium chloride (CaCl2) that it is much more soluble in watery media (81.3 g/100 ml a 25°C) (Lide [16]) (Figure 3). In addition, comparing these results to the obtained in the previous experiment of evaluation of the effect of oxygen (20 ppm of $CaCO_3$, Figure 3), obtains it a similar tendency in the growth plus no in the proportion due to the differences of the start inoculums.

3.7. Comparison of the isolated strains with the reference strain

Table 4: Kinetic parameters of adjusts of Malthus's law and Ricatti's equation of cellular growth for the reference strain using 1500 ppm of CaCl2 added to the cultivation media.

Specific growth rate μ (h^{-1})	Parameter K of Ricatti's equation, K (h^{-1})	Parameter T of Ricatti's equation, T $(g/L)^{-1}$
0.0863	0.2224	0.1

Specific growth rates (μ) of isolate strains were minor that values obtained for the reference strain (Table 4), this ends was measured to the two significant concentrations of more calcium in the biopolymer's production. These results are similar with the obtained in experiments of Rottava [7]. In kind another investigations yielded higher values of specific growth rates in the production of xanthan gum from *X*. campestris [3, 17, 18].

3.8. Selection of the process for xanthan gum production

The utilized production media was the proposed by García–Ochoa *et al*. [19] and it was modified

Table 5: Xanthan gum productivities.

Inoculums' volume mL of sucrose	Productivities, $\frac{g(\text{xanthan gum})}{g(\text{biomass})} \cdot h$
120	0.0177
120	0.0264
50	0.0511

in order to substitute the carbon source by another more effective [7], for which the sucrose for D-glucose was substituted. Calcium chloride ($CaCl_2$) was used in concentration of 1500 ppm because it was the one that yielded better results in previous experiments was utilized. Erlenmeyer flasks of 250 mL were used with 2 different work volumes of cultivation broth (120 mL and 50 mL) with 10 replicas, taking into account the previous results to see than the incidence of an optimal relation volume of the inoculate in relation to the bioreactor's free volume it is significant. In this process was used Romaine Lettuce–Leaf 1 Strain (RLL1) according different factors of xanthan gum production in previous experiments. Xanthan gum productivities with each one of modifications and origin conditions, shows in Table 5, being congruent with the reported by Rottava [7] and being majors with other authors [3, 17, 18].

3.9. Characterization of xanthan gum

3.9.1. Viscosity tests

Table 6: Comparative behavior of the viscosity of solutions of xanthan gum.

Xanthan gum	Viscosity, μ(cP)
Produced by (RLL1)	1684
Commercial	1814

The obtained xanthan gum (G–UC) exhibited elders values of apparent viscosity in relation to the commercial xanthan gum, and both found themselves in the range considered by García et al [19] (Table 6).

3.9.2. Determination of pyruvate content

Following the methodology described by Sloneker and Orentas [8] and suggested for Mata [13], piruvato contents of the gum was determined in the commercial gum (G–C) and obtained xanthan gums (G–UC). The obtained xanthan gum was submitted to a process of purification, where an increment in the concentration of pyruvate contents of a 3.81 % to 5.10 % became evident, what in practice is translated that the gum is more effective like thickener (Table 7) [20].

Table 7: Pyruvate content in xanthan gum samples.

Commercial	3.74
Unpurified	3.81
Purified	5.10

4. Conclusions

Isolated strains were identified as *X.* campestris. Evaluation of growth cellular trough kinetic studies of isolated and the reference strains had congruent tendencies. In experiments of evaluation of dissolved oxygen effect, productivities and quantities of xanthan gum in Erlenmeyer flasks of 250 mL were minor than values obtained from Erlenmeyer flaks of 500 mL. There were no significant differences ($p > 0{,}05$) in the microorganism's growth with variations of concentrations of calcium in the cultivation media until 38 h of fermentation and the stationary phase of growth was reached with 1500 ppm of $CaCl_2$. The maximum productivity of xanthan gum with the isolated strain of Romaine Lettuce–Leaf 1 (RLL1) was 0.0177 $g.L^{-1}$. Xanthan gum obtained had bigger apparent viscosity in relation to the commercial gum and an increase from 3.81 % to 5.10 % in pyruvate contents was achieved.

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