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EMULSIFYING PROPERTIES OF THE GUM PRODUCED BY *PROSOPIS LAEVIGATA* (HUMB. & BONPL. EX WILLD) M.C. JOHNST (MESQUITE) CELLS SUSPENSION CULTURE IN BIOREACTOR

PROPIEDADES EMULSIFICANTES DE LA GOMA PRODUCIDA POR CULTIVO DE CÉLULAS EN SUSPENSIÓN DE *PROSOPIS LAEVIGATA* (HUMB. & BONPL. EX WILLD) M.C. JOHNST. (MEZQUITE) EN UN BIORREACTOR

J.L. Trejo-Espino[1,2], M. Rodríguez-Monroy [2*], E.J. Vernon-Carter[3] and F. Cruz-Sosa[1]

[1] *Departamento de Biotecnología, Universidad Autónoma Metropolitana-Iztapalapa, Av. San Rafael Atlixco No. 186, Col. Vicentina, CP 09340, México DF, México*

[2] *Departamento de Biotecnología, Centro de Desarrollo de Productos Bióticos del Instituto Politécnico Nacional, PO Box 24, CP 62730, Yautepec, Morelos, México*

[3] *Departamento de Ingeniería de Procesos e Hidráulica, Universidad Autónoma Metropolitana-Iztapalapa, Av. San Rafael Atlixco No. 186, Col. Vicentina, CP 09340, México DF, México*

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Abstract

The mesquite gum (MG) production from *Prosopis laevigata* cells suspension culture in a stirred tank type bioreactor, with characteristics similar to those of MG obtained from wild trees are reported. The cells showed a specific growth rate (μ) of 0.08 1/d and a viability of over 60 %, reaching a maximum biomass of 16.6 g dry mass (dm)/L after 14 d. The cells produced a gum made up by 83.5% of carbohydrates (L-arabinose, D-galactose and glucuronic acid) and 8.1% of protein, also detected arabinogalactan-proteins (AGPs) as constituents of the gum. Oil-in-water emulsions were prepared using as continuous phase aqueous solutions of MG obtained from wild trees and that produced in bioreactor (MGb). The emulsifying capacity of MGb was slightly superior, but the emulsion stability lower, than that obtained with MG.

Keywords: *Prosopis laevigata*, mesquite gum, stirred tank type bioreactor, arabinogalactan-proteins, emulsifying properties.

Resumen

Se reporta la producción de goma de mezquite (GM) en un cultivo de células en suspensión de *Prosopis laevigata* a nivel de biorreactor, cuyas características son similares a la GM obtenida de árboles silvestres . Las células mostraron una velocidad específica de crecimiento (μ) de 0.08 1/d y una viabilidad superior al 60%, alcanzando una biomasa máxima de 16.6 g en masa seca (ms)/L a los 14 d. Las células produjeron una goma compuesta de 83.5% de carbohidratos (L-arabinosa, D-galactosa y ácido glucurónico) y 8.1% de proteína, además se detectó la presencia de arabinogalactano-proteínas (AGPs) como constituyentes de la goma. Se prepararon emulsiones aceite-en-agua utilizando como fase continua soluciones acuosas de goma obtenida de árboles silvestres (GM) y producida en el biorreactor (GMb). La capacidad emulsificantede GMb fue ligeramente mayor, pero la estabilidad de la emulsión menor, que las obtenidas con GM.

Palabras clave: *Prosopis laevigata*, goma de mezquite, biorreactor tipo tanque agitado, arabinogalactano-proteínas, propiedades emulsificantes.

* *Corresponding author. E-mail*: mrmonroy@ipn.mx

1 Introduction

Several studies have established that mesquite gum (MG), the exudate from *Prosopis laevigata*, possesses emulsifying and microencapsulating properties (Beristain and Vernon-Carter, 1995; Vernon-Carter *et al.*, 1996, 1998; Rodríguez-Huezo *et al.*, 2004), forms dense films upon drying that limit oxygen diffusion through its matrix (Báez-González *et al.*, 2004), and mechanical properties (Villagómez-Zavala *et al.*, 2008) comparable or superior to those of gum arabic (GA), the exudate from *Acacia senegal*, which is considered as a benchmark commodity worldwide (Dickinson, 2003). MG is exudated in response to different environmental stress conditions, wounds, pathogen infection, or attack by insects (Verbeken *et al.*, 2003). MG is a complex proteoglycan, of which 90% is made up by a glycosidic part characterized by a central backbone of β-(1-3)-D-galactose, which has attached side chains of (1-6) L-arabinose (in both, pyranose and furanose forms), L-rhamnose, β-D-glucuronic acid and 4-O-methyl-β-D-glucuronic acid. It also contains a protein fraction that varies between 0.7 and 5.8 % of the total molecule (Vernon-Carter *et al.*, 2000).The main aminoacids of the protein fraction are: hydroxyproline, proline y serine (Anderson *et al.*, 1985). Thus, the MG molecule is considered a type II (protein attached covalently to polysaccharide) arabinogalactan-protein (AGP) (López-Franco *et al.*, 2004). MG has been fractionized by hydrophobic affinity chromatography (Orozco-Villafuerte *et al.*, 2003; Román-Guerrero *et al.*, 2009). Mesquite gum was separated in three main fractions (F_1, F_2, and F_3), whose average molecular masses ranged from 1.81×10^{-4} to 5.23 $\times$ Da; F_1 had 90% polysaccharide and 1% protein contents, while F_2 had 16% and F_3 46% protein, respectively, using hydrophobic affinity chromatography (Román-Guerrero *et al.*, 2009). F_2 produced films at the oil-water interface with higher interfacial viscosity and higher instantaneous elastic modulus, and provided orange peel oil-in-water emulsions with lower coalescence rates than whole MG, and the latter than F_1 and F_3. Thus, the fraction imparting best emulsion stability and interfacial rheological properties was that having a relatively high protein/high polysaccharide balance, and was also mainly responsible for the functionality of whole MG.

Guaranteeing the supply of natural products is hazardous, as periodic shortages occur due to complex geo-global environmental, sociological, and political factors. Thus, an ongoing research topic is how to obtain natural products through novel biotechnological approaches such as the use of plant cell and tissue culture (PCTC) techniques (Kieran *et al.*, 1997; Pan *et al.*, 2000). The efforts in this sense regarding *P. laevigata* have been few. Orozco-Villafuerte *et al.* (2003) established a nodal explants culture from *P. laevigata* stems and found that it was possible to induce secretion of a gum-like substance using elicitation. Later it was demonstrated that the gum-like secretion produced *in vitro* had similar chemical composition than the gum exuded by wild *P. laevigata* trees, and that it could be considered as a true gum (Orozco-Villafuerte *et al.*, 2005). Production of AGPs from several vegetable cells tissue cultures in Erlenmeyer flasks has been successful (Günter and Ovodov, 2007; Sánchez-Sanpedro *et al.*, 2008; Capataz-Tafur *et al.*, 2010), but very few reports have been published about their production in bioreactors. AGPs production in bioreactor has been reported for *Beta vulgaris* (Rodríguez-Monroy and Galindo, 1999) and tobacco (Xu *et al.*, 2005) cells cultures, among others.

The objective of this work was to establish *Prosopis laevigata* cells suspension culture in bioreactor, to chemically characterize the gum production and the presence of arabinogalactan-proteins, and to evaluate the emulsifying properties of the gum produced in bioreactor in comparison the gum produced by *P. laevigata* wild trees.

2 Materials and methods

2.1 Plant cell culture

P. laevigata cell suspension culture was initiated from 4 weeks old friable callus obtained previously from hypocotyls segments of *P. laevigata* plantlets. 3-5 g of callus were transferred to 250 mL Erlenmeyer flasks containing 50 mL of Murashige and Skoog (MS) medium (Murashige and Skoog 1962) supplemented with 3% (w/v) of sucrose, 5.0 μM of kinetin (KIN) and 5.0 μM of 2,4,5-trichlorophenoxy acetic acid (2,4,5-T). The cultures were maintained at 25 $\pm$ 2 °C under cool white fluorescent light at irradiance of 200 μmol

m^{-2} s^{-1} and 16 h light/8 h dark photoperiod cycle, on an orbital shaker at 110 rpm. When the biomass increased, the cells were sieved through 200 μm nylon mesh filters in order to obtain a homogeneous and fine cell suspension culture. The culture was then maintained during 6 months, by sub-culturing every 12-15 days, using a cell inoculum size of 10% (v/v) in 500 mL Erlenmeyer flasks containing 100 mL of MS medium.

2.2 Stirred tank bioreactor cultures

A 2-L stirred tank bioreactor (Applikon, Schiedam, Netherlands) with a glass vessel, two baffle plates, multiport stainless heat plate and six-bladed disk turbine impeller operating at 400 rpm was used. The stirred tank bioreactor with a working volume of 1L was inoculated with cells (10% w/v) from 10-day-old suspension cultures. The stirred tank bioreactor was aerated through stainless-steel tube with sparger holes. The airflow rate was set at 0.1 vvm and the pH was maintained at 5.8 ± 0.1 with 0.1N NaOH. The pH and dissolved oxygen tension were monitored online by specific electrodes connected to a biocontrol ADDI 1030 (Applikon, Schiedam, Netherlands). Every 3 days over a 21 days period, aliquots of 30 mL from 5 independent runs were harvested for analytical measurements. The oxygen transfer rate (OTR) and the oxygen uptake rate (OUR) were calculated according to the procedure described by Orozco-Sánchez (2009). First, the volumetric oxygen transfer coefficient (K_La) using the dynamic method (Doran, 1995) was determined and with the experimental data of dissolved oxygen (DO) and biomass, the following equations were applied:

$$OTR = K_La\,(C^* - C_L) \tag{1}$$

Where OTR is the oxygen transfer rate (Kg/m^3d), K_La is the volumetric oxygen transfer coefficient (1/d), C^* is the saturated DO concentration (Kg/m^3) (˜0.008Kg/m^3at 25°C and 1 atm) and C_L is the actual DO concentration in the liquid (Kg/m^3)

$$\frac{dCO_2}{dt} = OUR = Q_{O_2}X \tag{2}$$

Where dCO_2/dt is the change in O_2 over time, OUR is the oxygen uptake rate (Kg/m^3d), Q_{O_2} is the specific oxygen uptake rate by the cells (Kg O_2/(Kg(viable biomass) d) and X is the biomass (Kg/m^3). Viable biomass (vm) is the dry mass multiplied by the cell viability.

2.3 Biomass

The biomass was filtered using pre-weighed cellulose filters (Whatman No. 1) and determined by dry mass (dm) measurement, oven drying the filters at 70 °C for 24 h. Data from stirred tank bioreactor (5 runs) was used to calculate means and standard error (SE) of the means. The specific cell growth rate (μ) was calculated by plotting cell growth data in the form of natural logarithm versus time. The slope of the linear part of the plot corresponds to the specific cell growth rate (Capataz-Tafur *et al.*, 2010). The time required for biomass to double (doubling time, t_d) was computed from the μ experimental data.

2.4 Cells viability

The cells viability was determined using Evan's blue staining test (Rodríguez-Monroy and Galindo 1999). A 2 mL sample from stirred tank bioreactor was incubated into 0.25% Evan's blue stain for 5 min and then at least 700 cells were counted. The viability was calculated taking in count the non-staining cells (viable cells).

2.5 Mesquite gum (MG) and Gum from cell suspension culture (MGb) purification

Mesquite gum (MG) from *P. laevigata* wild trees, and the gum from stirred tank bioreactor (MGb) were purified according the procedure reported by Orozco-Villafuerte *et al.* (2003). First, the MG samples were dissolved in deionized water and solutions were filtered through nitrocellulose membranes with a pore size of 0.45 μm. On the other hand, cells were separated from the culture broth by filtration (Whatman No. 1). Subsequently samples of MG and MGb, were dialyzed separately using cellulose membranes (Sigma) with a cutoff molecular weight of 12 KDa. Samples were finally concentrated by lyophilization and stored at −20 °C for further characterization.

2.6 Total protein, total and neutral sugars composition

The Bradford (Bradford, 1976) and phenyl-sulphuric (Dubois *et al.*, 1956) methods were used for determining total protein and total sugars contents of MG and MGb. Neutral sugar composition was determined by HPLC. The samples were prepared according to Vázquez-Ortíz *et al.* (2006). Aliquots of 0.05 g of the MG and MGb were weighed out accurately into tare 15 mL stopped Pyrex test tubes and 4% (w/w) sulphuric acid was added. The tubes were placed in a water bath at 100 °C for 4 h and then reweighed and made up to the own weight by addition of distilled water. The solutions were neutralized by adding 2.0 g $BaCO_3$ and shaking overnight. The filtered hydrolyzates were analyzed by HPLC (Varian Pro Star) fitted with a refraction index detector and a sugar SC 1011 column, using water (HPLC grade) as a mobile phase at a flow rate of 0.25 mL/min and a temperature of 85 °C.

2.7 AGPs quantification

AGPs contents in MGb were measured using the methodology reported by Van Holst and Clark (1985). This method is based on the specific interaction and precipitation of AGPs by the β-glucosyl Yariv reagent. Agarose gel (AG, Sigma-Aldrich. St. Louis, MO) at 1% (w/w) containing NaCl (0.15 M), $NaNO_3$ (0.02%) and 10 μg/mL-β-glucosyl Yariv reagent was prepared and wells of uniform diameter (1.2 mm) were punched in the gel using a Pasteur pipet. A standard curve (0.15 0.6 g/L) was obtained by filling the gel wells with 1 μL of AG sample with different concentrations. Defined red halos were formed around the test wells. There is a linear relationship between the concentration of AGPs and the area of the halo formed in the gel. Gels were incubated overnight at room temperature in a wet chamber and the standard curve was used to calculate the AGPs content of the MGb samples. The MG from trees was assayed only the qualitative presence of AGPs by determining if the red halo in the gel occurred or not.

2.8 Emulsifying properties

Oil-in-water emulsions were prepared by mixing 50 mg of a 1:1 blend of white mineral oil/orange oil into 5 mL of aqueous solutions of MG and MGb (100 mg/mL) respectively. Mixing was done with a VCX 130PB ultrasonic processor (Sonics and Materials Inc., Newtown, CT, USA) during 5 min at 70% amplitude. The emulsification properties of MG and MGb were measured as emulsifying activity and emulsifying stability according to the method of Pearce and Kinsella (1978). Emulsifying activity usually refers to a single chemical species that promotes emulsion formation and short-term stabilization by interfacial action. Emulsifying stability refers to a single chemical component conferring long-term emulsion stability, possibly by an adsorption mechanism, but not necessarily so (Dickinson 2003). The optical density of the diluted emulsions (500x) were measured at 650 nm with a spectrophotometer (Spectronic Genesys 2) immediately after preparation (time $(t) = 0h$) for the emulsifying activity and after 24 h for the emulsifying stability.

2.9 Emulsions rate of coalescence

The Oil-in-water emulsions were observed at 100x magnification with an Eclipse 80i optical microscope (Nikon, Tokyo, Japan) and filmed with a video camera (DC330 DAGE-MTI, Inc., Michigan IN, USA) coupled to the microscope and linked to a PC. The image processing of the micrographs was done using the free software image J 1.4 (National Institutes of Health, USA). Ten different images of each emulsion were analyzed providing 600-700 droplets for each data point. A micrometer was used for determining the mean number-length droplet size ($d_{1,0}$).

The emulsions droplet rate of coalescence (C) can be represented by the following equation (Ruiz-Ramos *et al.*, 2006):

$$N_t/N_o = e^{-Ct} \tag{3}$$

Where N_t is the number concentration of droplets a time $= t$, N_o is the number concentration of freshly formed droplets ($t = 0$), and C is the rate constant. The relationship between the emulsion droplet number, N, and the mean volume average droplet diameter ($d_{3,0}$) is given by:

$$N = 6\phi/\pi d_{3,0} \tag{4}$$

where ϕ is the volume fraction of disperse phase, and $d_{3,0} = (\Sigma n_i d_{1,0}/\Sigma n_i)^{1/3}$, where n_1, n_2, n_3,

..., n_i are the number of droplets with diameters $(d_{1,0})_1$, $(d_{1,0})_2$, $(d_{1,0})_3$, ..., $(d_{1,0})_i$.

If the kinetic plots of $\ln(N_t/N_0)$ versus t for the emulsions give a straight line, then the slope is $-C$.

3 Results and discussion

3.1 Bioreactor culture

The kinetics of *P. laevigata* cells suspension culture in the bioreactor did not show a lag phase, but an exponential growth was observed between 1 and 14 days. The culture displayed a specific growth rate (μ) of 0.08 1/d, reaching a maximum biomass of 16.6 gdm/L after 14 d (Fig. 1a). Cells viability at the beginning of culture was 65%, and was maintained throughout growth kinetics above 60% up to 18 d, time taken by cells to completely consume the medium sucrose (Fig. 1b). The yield of biomass per consumed sucrose ($Y_{X/S}$) was 0.45 g dm/g consumed sucrose. These results compare favorably with those for *P. laevigata* cultures grown in Erlenmeyer flasks where maximum biomass obtained was 12 g dm/L after 16 d, with a 70% viability (Trejo-Espino *et al.*, 2009), but differ with those reported for other vegetable species such as *Taxus chinensis* (Pan *et al.*, 2000) and *Centaura calcitrapa* (Raposo and Lima-Costa, 2006), where a significant decrease in the above mentioned parameters occurred when scaling up the culture from flasks to bioreactor. These authors attributed their scaling up results to the cells fragility when exposed to the hydrodynamic stress in the bioreactor. On the other hand, some authors have reported significant increases in dry mass yield and survivability of cells when scaling up from flasks to bioreactor such as that occurring for *Beta vulgaris* (Rodríguez-Monroy and Galindo 1999), *Azadirachta indica* (Prakash and Srivastava, 2007) and *Uncaria tomentosa* (Trejo-Tapia *et al.*, 2007). Some authors have suggested that probably some vegetable cells are resistant to hydrodynamic stress conditions occurring in bioreactors, while others are not (Meijer *et al.*, 1994; Zhao and Verpoorte, 2007).

P. laevigata cells suspension secreted AGPs into the bioreactor culture medium (Fig. 1a). This phenomenon has also been documented for cell cultures of *Rosa spp.* (Serpe *et al.*, 1994) and *Malva sylvestris* (Classen and Blaschek, 2002). As cells growth continued, so did AGPs production, reaching a maximum of 64.5 mg/L after 18 d.

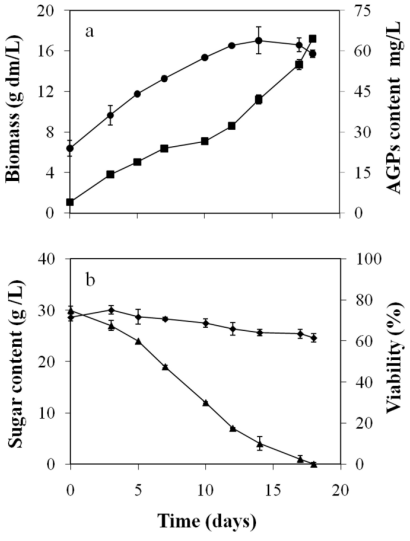

Fig. 1. Kinetics of cells growth and arabinogalactan-proteins (AGPs) secretion of the *P. laevigata* cells suspension culture in a 2-L bioreactor. (a) Biomass production (•); Extracellular AGPs content (■); and (b) Sugar content in the medium (▲); Cell viability (♦).

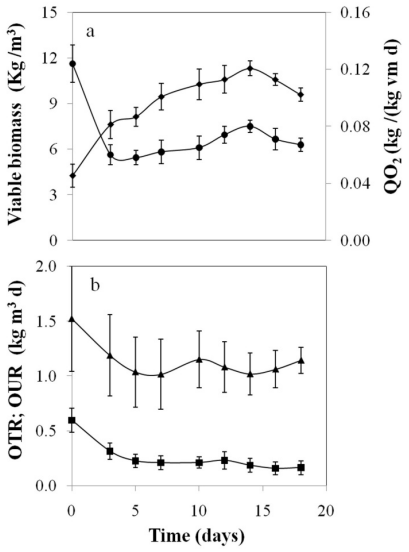

Fig. 2. Oxygen transfer and oxygen requirements by *P. laevigata* cells suspension in bioreactor: a) Viable biomass (vm) (♦) and specific oxygen consumption (QO_2) (•); b) Oxygen transfer rate (OTR) (▲) and oxygen uptake rate (OUR) (■).

Yield of produced AGPs per consumed sucrose ($Y_{AGPs/S}$) was of 5.43 mg AGPs/g sucrose. These results suggest that a direct relationship existed between cells growth and AGPs secretion, which has also been observed for other species cultured in flasks, such as *Arabidopsis thaliana* (Darjania *et al.*, 2002) and *Beta vulgaris* (Capataz-Tafur *et al.*, 2010). AGPs accumulation in culture medium may keep an analogy with what occurs in plant tissues, where AGPs are secreted to the apoplast and act as signaling molecules (Classen, 2007). Nevertheless, the definition of the biological function of AGPs in the growth and development of plants still requires more research (Ellis *et al.*, 2010), and is beyond the scope of this study.

Dissolved oxygen tension was maintained above 30% saturation throughout culture time, due to the constant air flow of 0.1 vvm fed to the bioreactor (Fig. 2). At the beginning of the growth kinetics the cells showed a maximum QO_2 of 0.12 Kg O_2/Kg vm d, indicative that the cells are consuming a great amount of oxygen in response to the hydrodynamic stress to which they were exposed when transferring the culture from flask to bioreactor. QO_2 diminished to a minimum of 0.055 Kg O_2/Kg vm d after about 3 d of culture, and then tended to increase at a similar rate as viable biomass increased, up to about 14 d culture, followed by a drop of both parameters after 15 d culture. Similar behavior was observed for several cells cultures when transferred from flask to bioreactor, attributed to an adaptive mechanism of cells for overcoming the drastic change in environmental conditions (Zhong, 2001). OTR was always higher than OUR, confirming that oxygen supply to the bioreactor was adequate for cells growth. Schlatmann *et al.* (1994) reported that an adequate oxygen supply should fall between 29 and 43% of saturated air, and Pareilleus and Vinas (1983) reported that the critical concentration of dissolved oxygen should be 20% of saturated air, for achieving an adequate growth of *C. roseus* cells suspension culture in bioreactor.

The chemical compositions of mesquite gum obtained from *P. laevigata* wild trees (MG) and that produced in bioreactor (MGb) are presented in Table 1. AGPs presence was detected in both cases. Both gums presented similar carbohydrate content, but MGb had higher protein content than MG. Furthermore, the main carbohydrates making up the gums were the same (L-arabinose, D-galactose, and glucuronic acid). However, the L-arabinose content in MGb (15.4 %) was considerably lower than in MG (62.2 %).

Table 1. Chemical comparison of MG from *P. laevigata* wild trees and produced in bioreactor (MGb).

	MG	MGb
AGPs	+	+
Total protein (%)	6.9 ± 0.28	8.1 ± 0.31
Total sugars (%)	86.2 ± 3.22	83.8 ± 2.7
Glucuronic acid (%)	11.8 ± 0.73	9.0 ± 0.67
D-Galactose (%)	22 ± 3.06	30.3 ± 2.69
L-Arabinose (%)	65.2 ± 2.06	15.4 ± 0.84
Rhamnose (%)	ND	ND

ND: Not detected by the method
+: Positive for Yariv's reagent; *: Obtained from growth kinetics after 20 days of culture
All percentages are expressed in dm basis
MG = mesquite gum from wild trees; MGb = mesquite gum produced in bioreactor; AGPs = arabinogalactan-proteins
± Standard error

3.2 Emulsifying properties

The emulsifying properties of GM and GMb were compared as they largely depend on chemical composition and molecular configuration (Xu *et al.*, 2005). The emulsifying activity, measured as the absorbance of the emulsions immediately after formation was slightly higher for MGb than for MG, indicating that the surface activity of both gums was directly related to protein content (Table 2). However, the emulsion stability, estimated from the absorbance after 72 h of emulsion formation, was substantially higher for MG than for MGb. This result was confirmed by the higher droplet coalescence rate displayed by the emulsion stabilized with MGb compared to that stabilized by MG (Table 2). Once an emulsion of small droplets has been successfully prepared, considerations of surface activity or interfacial tension gradients are no longer relevant. What matters for long-terms stability it show well the molecular characteristics of the adsorbed biopolymer conform to the requirements of producing a robust molecular barrier at the interface. Droplets are prevented from aggregating or coalescing when strong steric

Table 2. Emulsifying properties of MG and MGb; Mean volume average droplet diameter ($d_{3,0}$) and coalescence rate (C).

Emulsifier	Absorbance t = 0 h	Absorbance t = 24 h	$d_{3,0}$ (μm) t = 0 h	$d_{3,0}$ (μm) t = 72 h	C (s^{-1})	R^2
MG	0.65 ± 0.032	0.48 ± 0.025	1.6 ± 0.09	2.1 ± 0.17	6.02×10^{-6}	0.961
MGb	0.70 ± 0.047	0.31 ± 0.014	1.4 ± 0.11	3.6 ± 0.21	3.81×10^{-5}	0.879

Absorbance at 650 nm
MG = mesquite gum from wild trees; MGb = mesquite gum produced in bioreactor
± Standard error

and electrostatic stabilization terms arise (Dickinson, 2003). From our results, it seems that although both gums had similar total carbohydrate content, the considerably higher amount of L-arabinose in MG, contributed to the formation of a thicker steric stabilizing layer formed around the oil droplets, and to increased emulsion stability.

Conclusions

In this work it was established that *P. laevigata* cells suspension culture in bioreactor operated under proper conditions was capable to produce a gum whose composition was chemically very close to that of the gum exuded by *P. laevigata* wild trees. Furthermore, the gum produced in bioreactor had an emulsifying activity slightly superior, but provided less long-term stability to oil-in-water emulsions than the gum from wild trees. This work provides the ground work for producing mesquite gum at an industrial level, contributing to encourage the use of this gum in diverse industrial applications, as for the moment the supply of this commodity cannot be ensured though wild tree production.

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