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Xanthan gum production and rheological behavior using different strains of *Xanthomonas* sp.

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ABSTRACT

The proposal of the present study was to select and carry out the molecular characterization of strains of *Xanthomonas* sp. in order to correlate with gum production and determine possible genetic alterations during the study. The gums produced were also evaluated rheologically. Ten strains of *Xanthomonas* were used in the screening and the best ones in terms of productivity were *Xanthomonas campestris* pv. *mangiferaeindicae* 1230 (8.93 g/L), *X. campestris* pv. *campestris* 254 (9.49 g/L) and *X. campestris* pv. *campestris* 1078 (9.67 g/L). The gum produced by *X. campestris* pv. *mangiferaeindicae* presented the best apparent viscosity. The results for the profiles of the bands produced by RAPD showed considerable genetic variability amongst the evaluated strains, making not possible to neither group the strains according to pathovar or species, nor correlate the band profile with the productivity obtained. According to the RAPD analysis, no detectable mutations occurred in these bacteria during the study.

1. Introduction

In a wide sense a gum can be defined as any long chain polysaccharide (slightly, considerably, or not branched) that is soluble in water, can be extracted from land or marine vegetables or produced by different microorganisms, and has the capacity, in solution, of increasing the viscosity and/or form gels (Pasquel, 1999).

The areas of interest for microbial exo-polysaccharides (EPS), or biopolymers as they are commonly referred to are considerably varied, including: food industry, agro-chemistry, crude oil recovery, medical and pharmaceutical, and chemical and cosmetic industries (Rosalan & England, 2006). Their application in numerous industrial segments is due mainly to their rheological properties that allow the formation of viscous solutions at low concentration (0.05–1%), and a wide range of pH and temperature stability, characteristics resulting from their ramified structure and high molecular weight (Boza, 2002; García-Ochoa, Santos, Casas, & Gómez, 2000; Silva et al., 2009; Sutherland, 2002).

Xanthan gum, the microbial exo-polysaccharide produced by *Xanthomonas campestris*, has been reported as being the biopolymer most widely accepted commercially. It can be used in foods and other segments as a thickening, stabilizing and emulsifying agent and, in synergism with other gums, can act as a gelling agent (López, Moreno, & Ramos-Cormenzana, 2001).

The main characteristic of xanthan gum is its ability to modify the rheology or flow behavior of solutions (Margaritis & Pace, 1985). These properties are determined by its chemical composition, arrangements and molecular bonds (Pace, 1980). The culture medium and operational conditions influence the yield and structure of the xanthan gum produced (García-Ochoa et al., 2000).

The screening of microorganisms polysaccharides producers with economically interesting functional properties, and studies to optimize the yields and productivity in the fermentative processes used to obtain them, represent a constant challenge (Boza, 2002).

Several authors have cited RAPD as an ideal methodology to study genomic polymorphism. This method has been used to compare specific intra and inter differences in bacteria, and can be used both for purified DNA and for cell extracts cultivated in broth or agar (Silveira, Oliveira, Carvalho, Carvalho, & Pilon, 2000; Williams, Hanafey, Rafalski, & Tingey, 1993; Williams, Kubelik, Livak, Rafalski, & Tingey, 1990).

Although the use of RAPD to study variability in microorganisms is fairly common, its use associated with screening, especially in relation to the production of xanthan gum production, has practically not been reported in the literature.

Based on these aspects the objectives of the present study were the screening and molecular characterization of *Xanthomonas* sp.

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strains for the production of xanthan gum, aiming to correlate with production and determine possible mutations during the successive culture replications. The rheological behavior of the gum produced by the microorganisms tested was also evaluated.

2. Experimental

2.1. Microorganism

Ten strains of the genus *Xanthomonas* were used: *Xanthomonas* sp. (1537); *X.* campestris pv. *mangiferaeindicae* (1230); *X.* campestris pv. *campestris* (254); *X.* campestris pv. *arracaciae* (1198); *Xanthomonas axonopodis* pv. *manihotis* (1182); *X.* campestris pv. *campestris* (1078); *Xanthomonas melonis* (68); *X.* campestris pv. *campestris* (729); *X.* campestris pv. *campestris* (607); *X.* campestris pv. *campestris* (1167). The strains were obtained from the Culture Collection of the Institute of Biology (Campinas-SP), all having been isolated in Brazil. The strains were first evaluated with respect to the morphology and pigmentation of the colonies.

The microorganisms were maintained in YM (Yeast Malt) agar containing (g L^{-1}): 3.0 yeast extract; 3.0 malt extract; 5.0 peptone; 10.0 glucose; 20.0 agar; q.s.p. distilled water, pH 7.0. For cell growth the agar was not added (Jeannes, Rogovin, Cadmus, Silman, & Knutson, 1976).

The organisms were replicated every 30 days for a period of 12 months and stored at a temperature of ±4 °C. Some of the morphological characteristics of the colonies were determined by applying the Gram stain test and incubating streak plates in YM agar.

2.2. Replication and morphological characterization of the microorganisms

To preserve the cultures and diminish the risk of genetic profile alterations, the strains were frozen at −80 °C and maintained for 12 months. The freezing procedure included incubation of the culture in YM broth at 28 ± 2 °C until an absorbance of between 2.5 and 5.5 (depending on the strain) at 560 nm was reached, the addition of a sterile cryoprotector, 13%(w/v) glycerol, and homogenization of the mixture. The suspension was then distributed into duly labeled sterile microtubes (1.5 mL) and immediately frozen at −80 °C. All the procedures were carried out under aseptic conditions (Stanbury, Whitaker, & Hall, 2000).

2.3. Production of xanthan gum

A 14 mL inoculum (cell concentration of about 10^{11} CFU/mL, according to the strain) was added to 86 mL of biopolymer production medium, MPI + II, containing (g L^{-1}): 2.5 $NH_4H_2PO_4$; 5.0 K_2HPO_4; 0.006 H_3BO_3; 2.0 $(NH_4)_2SO_4$; 0.0024 $FeCl_3$; 0.002 $CaCl_2{\cdot}2H_2O$; 0.002 $ZnSO_4$; 50.0 sucrose, pH 7.0 (Cadmus, Knutson, Lagoda, Pitsley, & Burton, 1978). The inoculated medium was incubated in 300 mL conical flasks in an orbital shaker at 28 ± 2 °C and 180 rpm for 96 h. The experiments were carried out in triplicate.

2.4. Recovery of xanthan gum

The fermentation broth was centrifuged at a velocity of 5500 rpm for 40 min at a temperature of 4 °C to remove the cells, and ethanol (1:3(v/v)) added to precipitate the gum, the formation of a precipitate being observed. The mixture was stored under refrigeration (±4 °C) for 12 h and then centrifuged again at 7000 rpm for 30 min at 4 °C to recover the precipitated biopolymer, which was dried in an oven (50 ± 5 °C/24 h) to constant weight. The polysaccharide was stored in a sealed flask for later analysis.

2.5. Genetic characterization of the microorganisms by RAPD (Random Amplified Polymorphic DNA)

2.5.1. DNA extraction

Isolation of DNA from each microorganism was carried out using the procedure described by Sambrook, Fritsch, and Maniatis (1989), quantified at 260 nm and checked for integrity and purity at 280 nm and in 0.8% agarose gel.

2.5.2. RAPD amplification reaction

Decamer primer kits from Operon Technologies Inc. (Alameda, CA): OPA-03, OPA-12, OPA-13, OPA-20, OPF-05, OPF-09, OPH-18, OPW-19, OPY-03 and OPY-17 were chosen based on the best results in relation to the intensity and reproducibility of the bands obtained.

Amplification was carried out using the method described by Williams et al. (1990), adding the following components and completing to a final volume of 25 μL: reaction buffer (50 mM Tris–HCl pH 9.0; 50 mM KCl), dNTPs (200 mM each), 0.2 mM of primer, 3 mM $MgCl_2$, 0.25 mM TRITON, 1.5 U of Taq DNA polymerase Gibco BRL (Life Technologies, São Paulo, Brasil) and approximately 40 ng of DNA. Amplification was carried out in a thermocycler (model PTC 100, MJ Research Inc., Watertown, MA). The amplification process was as follows: 3 min at 92 °C, 40 cycles of 1 min at 92 °C, 1 min at 35 °C, 2 min at 72 °C and finally 3 min at 72 °C before cooling to 4 °C.

The electrophoretic separation was run in 1.4% agarose gel in TBE 1× buffer (0.089 M Trisma, 0.089 M boric acid and 0.008 M EDTA) in a horizontal electrophoresis chamber. Runs were carried out at a constant voltage of 90 V. Phage Lambda DNA was used as the molecular weight marker and the fragments were visualized with ethidium bromide under UV light. The gels were photographed using a GEL-PRO system (Media Cybernetics, Silver Spring, MD).

To determine the genetic variability, the data obtained by determining the presence or absence of bands, formed a matrix that was analyzed with the help of the NTSYS computer program, version 1.7 (Numerical Taxonomy System of Multivariate Analysis System). Tree diagrams were built using the UPGMA algorithm (Unweighted Pair Group Method Using Arithmetic Averages), developed by Sokal and Michener (1958), and Jaccard's similarity coefficient. The confidence limits for the groups formed were calculated by the randomization of 100 samples of the results using the Winboot program (Yap & Nelson, 1996). To check the non-occurrence of mutations during the successive replications to which the microorganisms were submitted, for each microorganism, a sample of the initial replication (maintained at −80 °C) and another of the final replication (after 12 replications) were analyzed, giving a total of 20 samples.

2.6. Rheological analysis of the xanthan gum

The apparent viscosity was determined using 3% aqueous solutions of the gums produced by the 10 strains of *Xanthomonas* sp. at 25 °C with spindles 18 and 31. A Brookfield model LVDV III+ digital rheometer was used, connected to a Brookfield model TC-502P water bath. Readings were taken at 10-s intervals, varying the shear rate (0.264–0 s^{-1}) according to the characteristics of each sample. The units used were: centipoise (cP) = mPas s^{-1} for apparent viscosity, 1/second (s^{-1}) for the shear rate and dyna/centimeter squared (D/cm^2) for shear tension.

3. Results and discussion

3.1. Morphological characteristics of the colonies

The morphological characteristics of the colonies were determined using the Gram stain test and plating in YM agar. All the strains studied were in the form of gram negative rods.

According to the literature, *Xanthomonas* colonies are usually yellow, smooth and viscous (Bradbury, 1984; García-Ochoa et al., 2000). However, the strain 1167 showed less intense or even no pigmentation. Throughout the study there was no visual alteration in the pigmentation of any of the strains.

The pigmentation of the colonies is due to xanthomonadins, which are yellow pigments characteristic of the genus *Xanthomonas*, and can be absent due to degradation or mutation. According to some works related, this group of pigments is of elevated scientific interest, since they are apparently related to low molecular weight diffusibility factors (pheromones), and are also involved in the regulation of various bacterial physiological processes (Poplawski, Chun, Slater, Daniels, & Dow, 1998; Poplawski, Urban, & Chun, 2000). These processes include the regulation of the synthesis of extracellular enzymes and the synthesis of extracellular polysaccharides. However, the authors affirm that the literature related to these factors is incipient. Up to the present moment, the relationship between these pigments and the plant/host pathogenic process has been under study.

3.2. Production of xanthan gum

Table 1 shows the mean productivities of three fermentation processes carried out in triplicate. Microorganism 6 (1078) showed the greatest productivity, whilst microorganisms 2 (1230), 3 (254), 5 (1182), 6 (1078), and 8 (729) presented higher productivities than the remaining strains studied, there being no statistically significant difference ($p < 0.05$) between them.

In the present study the fermentations were carried out in the medium MPI + II, which is a medium widely reported in the literature, using sucrose as carbon source (Cadmus et al., 1978). Although it is known that productivity is influenced by the microbial strain, the time and the fermentation medium, up to the present moment, no alternative medium has substituted the use of sucrose with a significant effect on the quality and productivity of the xanthan gum (Antunes, Moreira, Vendruscolo, & Vendruscolo, 2000; Padilha, 2003; Souza & Vendruscolo, 2000; Torres, Brito, Galindo, & Chopin, 1993).

Table 1
Productivity in terms of xanthan gum produced in the medium MPI + II with 10 strains of *Xanthomonas* sp.

Microorganism	*Productivity ($g\,L^{-1}\,h^{-1}$)	*Production ($g\,L^{-1}$)
1. *Xanthomonas* sp (1537)	0075 ± 0.003	7.20[c]
2. *X. campestris* pv *mangiferaeindicae*(1230)	0.092 ± 0.002	8.93[a b]
3. *X. campestris* pv *campestris* (254)	0.098 ± 0.004	9.49[a]
4. *X. campestris* pv *arracaciae* (1198)	0.064 ± 0.004	6.20[d]
5. *X. axonopodis* pv *manihotis* (1182)	0.083 ± 0.009	7.99[b c]
6. *X. campestris* pv *campestris* (1078)	0.100 ± 0.009	9.67[a]
7. *Xanthomonas melonis* (68)	0.073 ± 0.009	7.09[c]
8. *X. campestris* pv *campestris* (729)	0.085 ± 0.006	8.21[b c]
9. *X. campestris* pv *campestris* (607)	0.078 ± 0.007	7.53[c]
10. *X. campestris* pv *campestris* (1167)	0.061 ± 0.002	5.90[d]

* Mean of three experiments. Mean followed by equal letters do not differs by Duncan test ($p < 0.05$).

Some other works related to xanthan gum production have been reported in the literature. López et al. (2001) tested four strains of *X. campestris* to produce xanthan gum using olive mill wastewaters (OMW). The most valuable strain was *X. campestris* NRRL B-1459 S4LII because of its ability to produce xanthan using 7% of OMW as the nutrient source, producing 7 $g\,L^{-1}$ of xanthan gum. Kalogiannis, Iakovidou, Liakopoulou-Kyriakides, Kyriakidis, and Skaracis (2003) studied the xanthan gum production by *X. campestris* ATCC 1395 using pre-treated sugar beet molasses as carbon source, supplemented with K_2HPO_4, yeast extract, Triton 80, and tap water. Addition of K_2HPO_4 to the medium had a significant positive effect on xanthan gum production. Maximum xanthan gum production was 53 $g\,L^{-1}$ after 24 h at 175 $g\,L^{-1}$ molasses, 4 $g\,L^{-1}$ K_2HPO_4 and at the neutral initial pH. Cheese whey was also used in an earlier study to produce xanthan gum, but a very lower concentration of this polymer was obtained, reaching a maximum xanthan gum production (1.2 g/100 mL of cheese whey) with *X. campestris* XLM 1521 in a medium containing 50 wt% of cheese whey (Papoutsopoulou, Ekateriniadou, & Kyriakidis, 1994). Another work by Fialho et al. (1999) evaluated the gellan gum production by *Sphyngomonas paucimobilis* in media containing lactose, glucose, and sweet cheese whey as substrates. The authors reported that a maximum gum production obtained was 7.9 $g\,L^{-1}$.

3.3. Genetic characterization of the microorganisms by RAPD

From the results obtained in the morphological characterization, a genetic analysis of the strains could be carried out in a search for the occurrence of mutations and the possibility of grouping the strains according to genetic similarity from an analysis of the random fragments.

Considering the 10 strains under study, a total of 93 fragments were identified, of which 70 (75.27%) were polymorphic. The amplified fragments presented between 50 and 2200 bp, and the

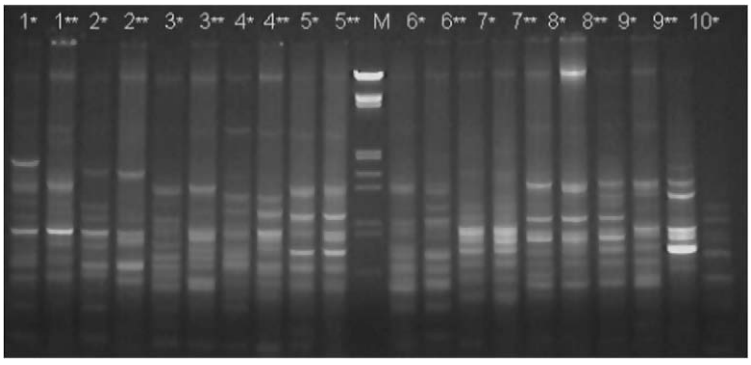

Fig. 1. Demonstrative agarose gel obtained with the primer OPH-18.

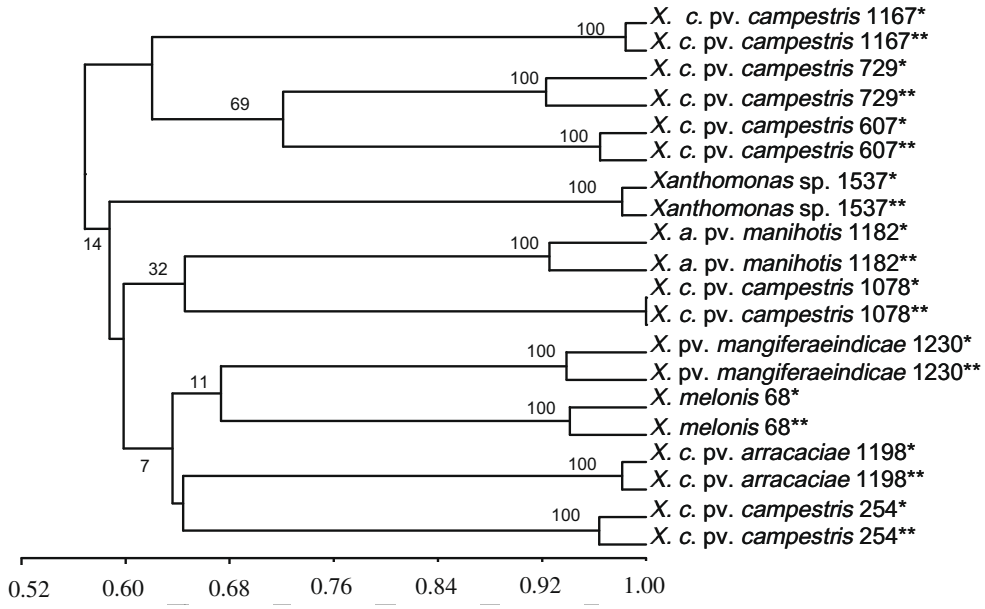

Fig. 2. Tree diagram based on the cluster analysis (UPGNA) of the estimate of genetic similarity (Jaccard's coefficient) by RAPD, between the initial and final replications of the different strains of *Xanthomonas* The numbers on the tree diagram refer to the limits of confidence of the clusters, calculated by the Winboot program. *Initial replication; **final replication.

mean number of fragments per primer was 9.3. Fig. 1 shows the variability observed within the different tested species, using the primer OPH-18.

Gonçalves and Rosato (2000) characterized the genotype of 55 strains of *Xanthomonas* isolated from passion fruit plants (*Passiflora* sp.). They were identified as *X. campestris* pv. *passiflorae* and were initially evaluated using the RAPD analysis. The strains showed a high level of polymorphism with well differentiated fingerprints. All the *Xanthomonas* species gave a differentiated RAPD profile and no consistent report for the *Pseudomonas syringae* pv. *passiflorae* strains were observed.

The similarity of 0.47–0.71 between the strains analyzed in this work can be considered low. These results indicate the existence of considerable variability between the strains. Low similarity be-

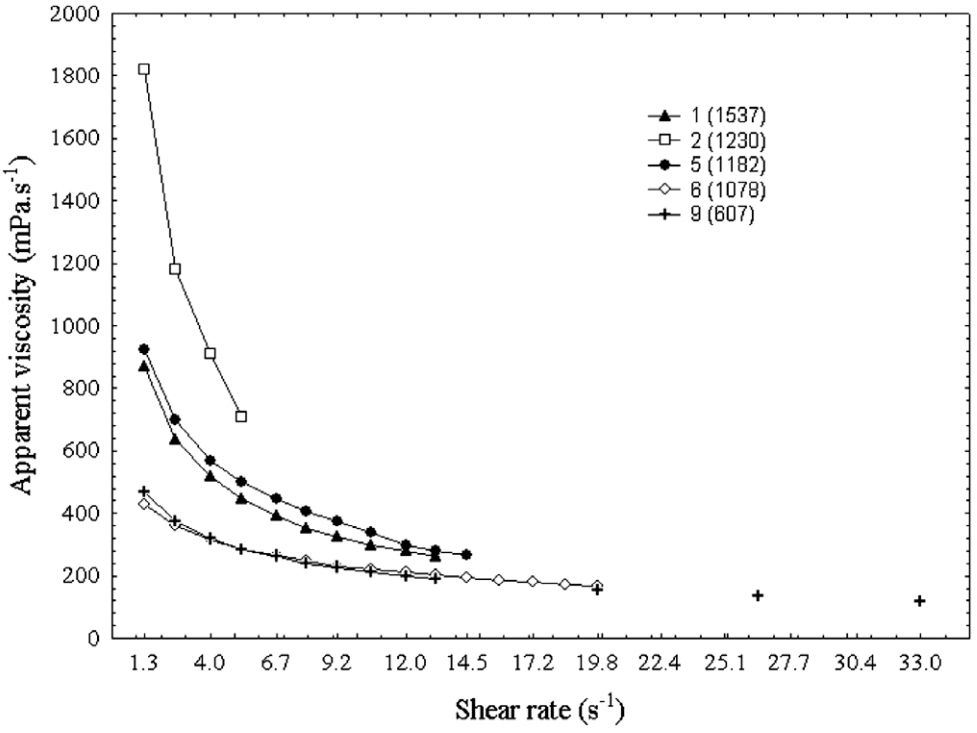

Fig. 3. The apparent viscosity of 3% aqueous solutions of gums produced by the strains of *Xanthomonas* sp. (1), *Xanthomonas campestris* pv. *mangiferaeindicae* (2), *Xanthomonas axonopodis* pv. *manihotis* (5), *Xanthomonas campestris* pv. *campestris* (6), and *Xanthomonas campestris* pv. *campestris* (9), determined using a model LVDV III+ Brookfield viscometer, *spindle* 18 at 25 °C.

Table 2
Similarities between the initial and final replications of the microorganisms analyzed.

Control	Strain number	Microorganism	Similarity between the subcultures
01	1537	*Xanthomonas* sp.	0.98
02	1230	*X. campestris* pv. *mangiferaeindicae*	0.94
03	254	*X. campestris* pv. *campestris*	0.96
04	1198	*X. campestris* pv. *arracaciae*	0.98
05	1182	*X. axonopodis* pv. *manihotis*	0.93
06	1078	*X. campestris* pv. *campestris*	1.00
07	68	*X. melonis*	0.94
08	729	*X. campestris* pv. *campestris*	0.92
09	607	*X. campestris* pv. *campestris*	0.96
10	1167	*X. campestris* pv. *campestris*	0.198

tween *Xanthomonas* of different species is to be expected, but such variation in the genotypic profile between strains of the same species and merely from different pathovars, was not expected (Fig. 2).

The RAPD analysis between the strains failed to allow for a total grouping of the strains according to pathovar or species (Fig. 2).

The genetic differences found between the microorganisms analyzed suggest that no isolate can be discarded when desiring to carry out a screening of these microorganisms in a search for a specific characteristic. This analysis also showed that the similarity between the DNA samples of the different replications of *Xanthomonas* (initial and final) analyzed by the UPGMA cluster method, with Jaccard's similarity coefficient, was above 90% with a confidence limit of 100%, proving that no detectable mutations occurred in these bacteria during the study.

Although high (0.92–1.00) the similarities between the initial and final replications showed small differences between the initial and final DNA (Table 2). This could be attributed to the fact that the initial cultures were not pure, being originated from various initial cells that could each contain small genetic differences.

An absence of detectable mutations was also verified from an observation of the morphological characteristics of the colonies and the Gram test, where no differences were detected, demonstrating that the bacteria maintaining their characteristics.

3.4. Rheological behavior of xanthan gum

Figs. 3 and 4 show the results obtained in the evaluation of the rheological behavior of the xanthan gums produced by the 10 strains of microorganism tested. The initial analysis of these samples showed evidence of pseudoplastic behavior in the solutions analyzed, that is, the apparent viscosity decreased with an increase in shear rate. This behavior is to be expected in polymeric solutions of microbial polysaccharides (Cacik, Dondo, & Marqués, 2001; Rao, Suresh, & Suraishkumar, 2003). In these systems with non-Newtonian fluids, some models that predict and adjust the experimentally obtained data, with the possibility of predicting the effect of shear rate on the apparent viscosity by applying the power-law model, are cited in the literature, as the one presented in Eq. (1), used in this work.

$$\eta = K\gamma^{n-1} \quad (1)$$

where K is the consistency index and η is the behavior index (Kiosseoglou, Papalamprou, Makri, Doxastakis, & Kiosseoglou, 2003; Xuewu, Xin, & Deixang, 1996).

From the comparative graph of the apparent viscosity of aqueous solutions of the different gums produced, it can be seen that samples 2 (1230) and 5 (1182) gave the highest values for viscosity (Figs. 3 and 4).

Duncan's test was applied to statistically prove the behaviors presented by the aqueous solutions of the different gums produced, comparing the readings of the samples at a shear rate of 1.32 s^{-1}, this being the only value possible to carry out the readings, since the gums presented very different apparent viscosity

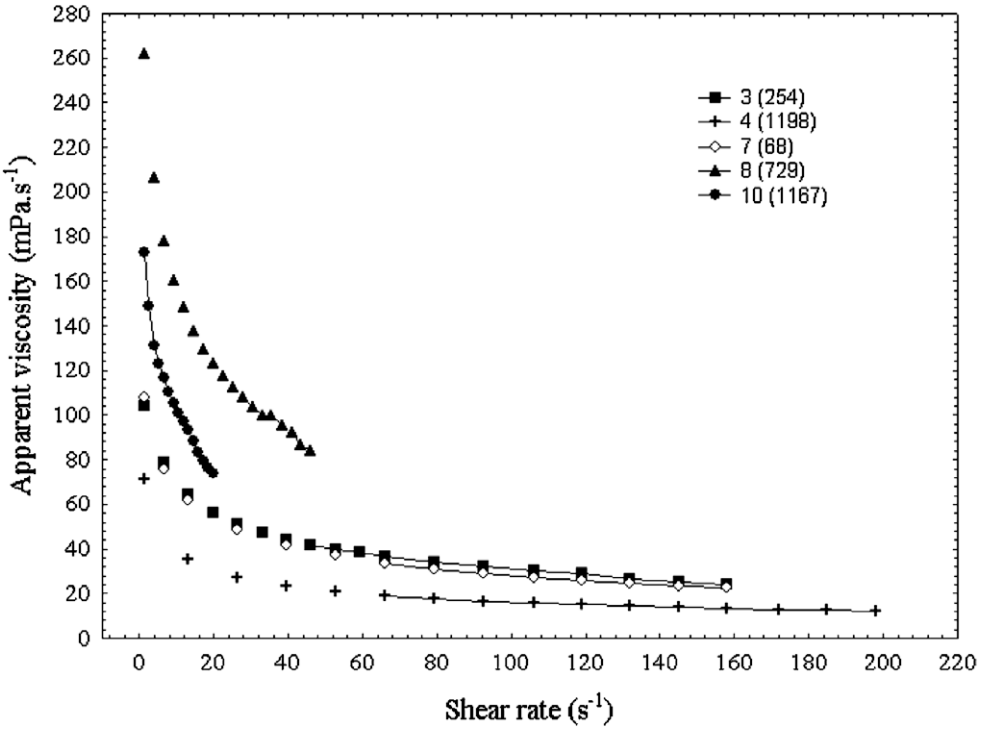

Fig. 4. The apparent viscosity of 3% aqueous solutions of gums produced by the strains of *Xanthomonas campestris* pv. *campestris* (3), *Xanthomonas campestris* pv. *arracaciae* (4), *Xanthomonas melonis* (7), *Xanthomonas campestris* pv. *campestris* (8) and *Xanthomonas campestris* pv. *campestris* (10), determined using a model LVDV III+ Brookfield viscometer, *spindle* 18 at 25 °C.

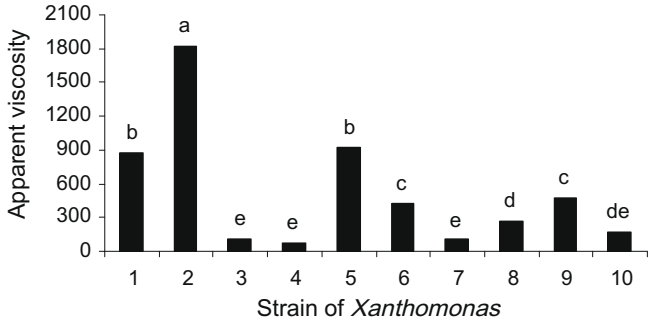

Fig. 5. The apparent viscosity (mPa s^{-1}) of aqueous solutions of the different xanthan gums, representing the mean of three readings of each solution analyzed, determined using a model LVDV III+ Brookfield viscometer, *spindle* 18 at 25 °C (1, 1537; 2, 1230; 3, 254; 4, 1198; 5, 1182; 6, 1078; 7, 68; 8, 729; 9, 607; 10, 1167). Measurements followed by the same letter did not differ from each other according to Duncan's test ($p < 0.05$).

values within the group. Fig. 5 presents the results obtained in this test.

Making an individual analysis of the apparent viscosity readings of the various 3% aqueous solutions of the gums obtained from the 10 microorganisms studied, determined using spindle no. 18 and a shear rate of 1.32 s^{-1}, it can be seen that the aqueous solution of the gum from *X. campestris* pv. *mangiferaindicae* (1230) gave the best viscosity and was statistically different from the others, according to Duncan's test ($p < 0.05$). The aqueous solutions of the gums from microorganisms 5 (1182) and 1 (1537) also showed high viscosities and were statistically equal to each other.

Since the apparent viscosities of the aqueous solutions of the gums produced by microorganisms 2 (1230) and 5 (1182) could not be carried out at a minimum of 10 different shear rates with spindle 18, the readings of these solutions were also carried out with spindle 31, since this is more indicated for solutions with higher apparent viscosity values (Fig. 6).

Comparing the values for apparent viscosity of the aqueous solutions of the gums obtained with the values reported in other papers, it can be seen that the xanthan gums obtained from the strains *X. campestris* pv. *campestris* (3), 104cP; *X. campestris* pv. *arracaciae* (4), 71cP; *X. melonis* (7), 108cP; and *X. campestris* pv. *campestris* (10), 173cP gave low values for viscosity, considering that the biopolymer concentration used in the aqueous solutions in the present study (3% w/v) was higher than that used in the other studies at the shear rate of 1.32 s^{-1}. For example, for a shear rate of approximately 10 s^{-1}, Navarrete and Shah (2001) obtained an apparent viscosity of approximately 100 cP for 1.4×10^{-4}% solutions of diutane at 24 °C, and Ashtaputre and Shah (1995) obtained an apparent viscosity of approximately 200 cP for 0.5% solutions of a biopolymer at 30 °C.

4. Conclusions

The results obtained in the present study permit us to conclude that of the 10 strains studied, the best microorganisms for the production of xanthan gum were *X. campestris* pv. *mangiferaeindicae* (1230), *X. campestris* pv. *campestris* (254), and *X. campestris* pv. *campestris* (1078), in terms of productivity. Considerable variability was found between the strains of *Xanthomonas* sp. studied in the genetic evaluation, according to the RAPD analysis. The RAPD analysis between the strains did not allow for a total grouping of the strains according to pathovar or species or even with respect to productivity. According to the RAPD analysis, there were no detectable mutations of these bacteria during the study (initial and final replications of the different strains of *Xanthomonas* sp.). The strain showing the best productivity and apparent viscosity was *X. campestris* pv. *mangiferaeindicae*.

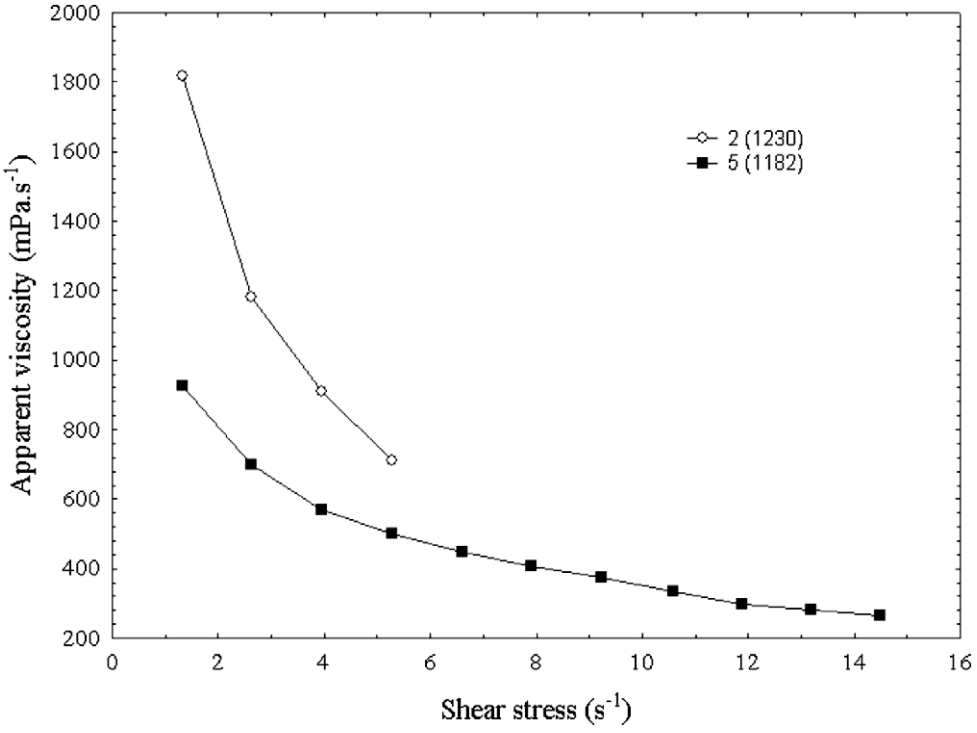

Fig. 6. The apparent viscosity of 3% aqueous solutions of gums produced by the strains of *Xanthomonas campestris* pv. *mangiferaeindicae* (2) and *Xanthomonas axonopodis* pv. *manihotis* (5), determined using a model LVDV III+ Brookfield viscometer, *spindle* 31 at 25 °C.

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