Use of Green Coconut Shells as an Alternative Substrate for the Production of Xanthan Gum on Different Scales of Fermentation

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Abstract: Xanthan, a biopolymer with extensive industrial applications, is commercially produced by fermenting glucose or sucrose using the bacteria Xanthomonas. Green coconut shells, rich in nutrients, could be an alternative substrate to obtain xanthan. This study aimed to evaluate the production and rheological properties of xanthan obtained on different fermentation scales using green coconut shells as the substrate, using its production from sucrose for comparison. Media containing minimal nutritional requirements (carbon, urea, phosphate) were prepared. Upon changing from the conventional medium to the alternative medium there was a 30% increase in production using the shaker and 81% increase using the bioreactor. Increasing the fermentation scale resulted in an increased yield of xanthan and a 30% increase in apparent viscosity. Coconut shells deserve special attention, constituting a possibility for the large scale production of xanthan with cost reduction and application of a residue.

Keywords: *Xanthan gum, rheology, green coconut shells, Xanthomonas campestris.*

Introduction

Xanthan is an exo-polysaccharide produced by the anaerobic microorganism *Xanthomonas campestris* by fermenting simple carbohydrates generally extracted from sugarcane, corn or beetroot, and is widely used in the food, cosmetic and pharmaceutical areas, with a potential use by the petrochemical industry in the exploitation of petroleum. It is a polymer of the poly-β-(1→4)-D-glucopyranose type, similar to cellulose, but with alternating branches at the C3 position, normally constituted of two molecules of mannose and one of glucuronic acid, in addition to the acetyl and pyruvic groups substituting the hexose, thus presenting a high molecular weight and highly viscous aqueous solutions[1].

The commercial fermentation media used in the production of xanthan gum contains glucose as the carbon source, and the conventional media used in research contains both this source and sucrose. If xanthan were produced commercially in Brazil as from sucrose, this source would represent a relatively small percentage of the production costs, but since the imported polymer is obtained from glucose, the cost of the carbon source represents a critical factor in the fermentation from a commercial point of view[2-5]. This reality makes it feasible to use xanthan for food and pharmaceutical ends, but not in the petroleum area, where large amounts would be required for the oil exploration process.

Thus the petroleum industry does not use large amounts of xanthan on account of its high cost, and ends up using other polysaccharides derived from plants or synthetic polymers, which are cheaper but have less specific properties for the tertiary extraction of petroleum from deep wells[2,6]. Xanthan gum is imported for about US$ 10-18/Kg, but if manufactured in Brazil from sucrose, the cost is estimated to decrease by 40 to 60%[2,7]. This cost would further decrease if the gum were produced from a low cost carbon source. Although the majority of the literature cites the use of glucose and sucrose as the preferred carbon sources, other alternative sources have been suggested, aimed principally at minimizing the production costs of this biopolymer[2,5].

Brazil is the fourth world producer of coconut, after Indonesia, the Philippines and India[8]. The green coconut shell is composed of a high concentration of directly fermentable carbohydrates (cellulose, fibers, hemicellulose, lignin etc.), which can be bio-converted into free sugars. Basically this conversion occurs by way of a chemical pre-hydrolysis during the sterilization of the medium, followed by the enzymatic action of the bacteria themselves during fermentation[4,9]. According to data provided by the Urban Rubbish Collection Company of Rio de Janeiro, green coconut shells correspond to about 80% of all rubbish collected along the marine shoreline[10]. The increase in consumption of coconut water has produced about 6.7 million tons of shells/ year[11], transforming this item into a serious environmental problem. The simple disposal of green coconut shells in the environment represents

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a loss of biomass that could be of great use due to the great volume produced, including a large amount of directly fermentable and highly biodegradable organic matter, which could therefore be considered as a viable alternative for testing as a substrate in the production of biopolymers such as xanthan gum, principally due to the ease of storage after drying and transformation into powder.

The objective of this study was to test the use of green coconut shell in the form of a dry powder, as the main raw material in the fermentation medium for the production of xanthan, both in a shaker and in a 2 L bioreactor, comparing the yields and rheological properties of the products with those of xanthan produced from sucrose.

Methodology

Microorganisms

The native bacteria nº 1866 of *Xanthomonas campestris campestris*, donated by the Phytobacterial Culture Collection of the Biology Institute – IBSBF, Campinas, SP, Brazil, was used.

The culture was maintained under refrigeration on YM-agar slopes and re-streaked periodically. The steps used to obtain the gum were as follows: inoculum preparation; cultivation and recovery (precipitation) of the gum.

Green coconut shell

The green coconut shells used were donated by *Embrapa Agroindústria Tropical*, Fortaleza, CE, Brazil. The material was obtained by processing the green coconut shells in a helicoidal grinder with a 30 HP triphasic motor. The green coconut shell powder was dried in an incubator at 30-40 °C to constant weight and stored in a dry place.

The moisture, crude protein and ash contents were determined according to AOAC methodology[12]. The total lipid content was determined by the Bligh & Dyer method[13], and the percent carbohydrates calculated from the difference between 100 and the sum of the percentages of water, protein, total lipids and ash.

Culture media

YM-agar was used for replication of the *Xanthomonas* culture (Table 1). Aliquots of 10 mL of YM-agar were transferred to test-tubes and autoclaved at 121 °C/15 min. After sterilization the tubes were cooled in a slightly inclined position. Streaking was carried out using a loopful of the *Xanthomonas* culture and incubation at 28 °C for 48h.

YM (Yeast-Malt) broth was used to prepare the inoculum (Table 1). Fifty milliliter volumes of broth were added to 250 mL conical flasks and autoclaved (121 °C/15 min), cooled and inoculated with a loopful of the culture *Xanthomonas campestris*, and incubated in a shaker at 28 °C/ 150 rpm for 20 h.

Production of xanthan gum

The green coconut shell powder was weighed and stirred in water for 30 minutes to extract the soluble compounds. The broth obtained was filtered through a 0.35 mm mesh filter, and urea and phosphate added in the proportions indicated in Table 1. The pH value was then adjusted to 7.0 using NaOH, and for the trials in the bioreactor, 1.5 mL of an anti-foaming agent were also added. The tests in the shaker were carried out using 250 mL conical flasks containing 80 mL of the broth obtained from the green coconut shell powder supplemented with urea and phosphate. The material was autoclaved (121 °C/ 15 min), cooled, inoculated with 8 mL of inoculum and incubated in the shaker at 28 °C, 250 rpm for 60 h.

The tests in the 2 L bioreactor were carried out using 1500 mL of the broth obtained from the green coconut shell powder, supplemented with urea, phosphate and 1.5 mL of anti-foaming agent. The reactor flask, its connections, electrodes and tubing were all autoclaved (121 °C/ 15 min), cooled and the flask inoculated with 150 mL of inoculum. The initial conditions adopted were agitation at 400 rpm, temperature of 28 ± 1 °C, aeration at 0.5 vvm and pH value of 7. Fermentation was continued for up to 60 hours and dissolved oxygen was monitored throughout the process and not allowed to go below 20%.

Samples of fermented broth were taken at regular intervals (roughly every 6 to 8 hours to determine the yield in xanthan (X_p) and cell growth (X_c).

The same production tests carried out in the shaker and bioreactor with the standard fermentation medium described in Table 1 were also carried out using the same control conditions but substituting the green coconut shell powder by sucrose.

Table 1. Composition of the culture media used for the replication and inoculum of the *Xanthomonas* cultures and for the fermentative process to produce xanthan gum.

Component	YM broth	YM agar	Standard Fermentation medium	Alternative Fermentation medium
Glucose (gL⁻¹)	10.0	10.0	-	-
Peptone (gL⁻¹)	5.0	5.0	-	-
Yeast extract (gL⁻¹)	3.0	3.0	-	-
Malt extract (gL⁻¹)	3.0	3.0	-	-
Agar (gL⁻¹)	-	1.5	-	-
Sucrose (%)	-	-	2.0	-
Green coconut shell (g)	-	-	-	80.0
Urea (%)	-	-	0.01	0.01
K₂HPO₄ (%)	-	-	0.10	0.10

Determination of product formation (Xp)

The cells were first separated from the fermented medium by centrifugation at 9625 x g at 4 °C for 15 minutes. Ethyl alcohol was then added to the supernatant at a rate of 3 parts of alcohol to 1 part of supernatant (fermented broth). Precipitated gum was collected and transferred to previously weighed Petri dishes. The gums obtained were dried in an incubator at (30 ± 2 °C) to constant mass, and the xanthan gum yields measured by a gravimetric method, the values being expressed in g.L^{-1}.

Determination of cell growth (Xc)

After centrifugation, the cells were re-suspended in distilled water to determine cell growth, reading the absorbance at 560 nm in a UV/ VIS spectrophotometer. The absorbance readings were compared with those of a calibration curve to estimate cell density. The calibration curve was determined as from a dry mass of cells, determining absorption of the solution at various dilutions. A linear relation was obtained between absorption and cell density.

Viscosity

The apparent viscosity was calculated from the measurement of shear stress at pre-fixed shear rates. Aqueous solutions of the gums were prepared at concentrations of 1%, and left to rest for 12 h at room temperature before measuring the viscosity. The viscosity was measured using a Brookfield digital rheometer, model LV-DVIII, with a cone and plate sensor, shear range from 50 to 450 s^{-1}, with temperature variation from 25 to 85 °C controlled by a Brookfield TC-501 water bath.

The dependency of the viscosity of the xanthan gum solutions on the shear rate can be described by the Ostwald-de Waele kinetic model or power law, as shown in Equation 1:

$$\mu_\alpha = \frac{\tau}{\gamma} = K(\gamma)^{n-1} \qquad (1)$$

where: K is the consistency index; n is the flow behavior index; μ_a is the apparent viscosity; τ is the shear stress and γ is the shear rate.

The Ostwald-de Waele model was fitted to the experimental data as from the linear regression to confirm the pseudoplastic behavior of the gum solutions using Excel 2003 software. The log γ was plotted against log μ_a in order to obtain the values for K and n and the equations of the straight lines.

Results and Discussion

Table 2 shows the results obtained for the proximate composition of the green coconut shell used as an alternative fermentation medium in the biosynthesis of xanthan gum. The *in nature* green coconut shell contained 10.44% total solids, of which the carbohydrates were the main constituent (75.23% dwb), consisting of fibers which were subsequently hydrolyzed by *Xanthomonas* and bio-converted into the hetero-polysaccharide xanthan gum.

Table 2. Composition of the green coconut shell used as the fermentation medium in the biosynthesis of xanthan gum.

Parameters	Composition (%)	
	Wet weight basis	Dry weight basis
Humidity	89.56 ± 0.02	-
Ash	0.36 ± 0.01	3.40 ± 0.01
Crude protein	2.00 ± 0.02	19.00 ± 0.02
Total lipids	0.18 ± 0.01	1.70 ± 0.01
Carbohydrates	7.90 ± 0.01	75.23 ± 0.01

The values found in the dry matter for protein, ash and lipids are of great importance, considering their use as substrates in fermentations by *Xanthomonas*, since they represent part of the nutrients required for both cell growth and xanthan gum synthesis during the fermentative process.

According to Barroso[11] and Noguera et al.[14], the green coconut shell is composed of a variety of nutrient and micronutrient sources favorable to microbial development, varying from 50-142 mg.L^{-1} of P, 115-803 mg.L^{-1} of K, 0.5-28.0 mg.L^{-1} of Ca, 0.5-18 mg.L^{-1} of Mg, 12.5-37 mg.L^{-1} of Na, 11.4-19.7 mg.L^{-1} of Fe, 2.2-31.8 mg.L^{-1} of Zn, 8-23.3 mg.L^{-1} of Mn and 2.3-6.6 mg.L^{-1} of Cu.

The green coconut shell consists of 19.9% cellulose, 68.7% hemicellulose and 30.1% lignin[15]. It contains the following sugars: L-arabinose, galactose, L-rhamnose, xylose and glucose, and glucose represents about 26% of the green coconut shell powder[7].

According to Sutherland[16], an elevated nitrogen concentration is required for rapid cell growth, but on the other hand, when the nitrogen content in the culture medium is very high, the xanthan produced shows inadequate rheological properties, whereas media with high carbon contents and low nitrogen contents favor accumulation of the polymer. In general industrial processes aim to use media with good conditions for both growth and gum accumulation. Noguera et al.[14], found a C/N ratio of 117 in coconut shells.

Figure 1 shows the results obtained for cell growth and xanthan gum yield in both the shaker and bioreactor, using the alternative fermentation medium (0.01% urea, 0.1% phosphate and 2% dried green coconut shell) and the standard fermentation medium (2% sucrose, 0.01% urea and 0.1% phosphate) throughout the fermentation time. It can be seen that the peak of product formation occurred at the end of the exponential phase and beginning of the stationary phase for cell growth, between 40 and 50 hours of fermentation.

For biopolymers, enzymes and some antibiotics, product formation occurs in an associated way, that is, the rate of formation of the product is proportional to the increase in biomass, and when cell growth stops, product formation also stops[17]. This could also be seen for the *Xanthomonas* strain used (Figure 1). The cell growth rate decreased after 50 h of fermentation, and after this product formation still continued for a further five hours.

During the process, the temperature of the system was maintained at 28 °C with the aid of a temperature controlled water bath connected to the bioreactor. This

control was necessary since heat was released during the substrate consumption reactions, and thus the temperature of the medium tended to rise. The initial agitation rate of the bioreactor system was 400 rpm, but this varied during fermentation in order to maintain the dissolved oxygen concentration between 20 and 30% of saturation (Figure 2). This control was not possible in the shaker which was submitted to constant shaking of 250 rpm. This could justify the greater xanthan gum production in the bioreactor (Figure 1).

A comparison of the values obtained for the production of xanthan gum from green coconut shell in a shaker (250 mL, 28 °C and 250 rpm) and in the bioreactor (1.5 L, 28 °C, variation in O$_2$ saturation between 20-30% and rotation between 400-640 rpm), showed that higher yields were obtained in the bioreactor. There was a 5.4 times increase in xanthan production comparing fermentation in the shaker with that in the bioreactor (Figure 1).

These results indicate that it is possible to obtain better yields of xanthan gum on larger scales using controlled parameters such as dissolved oxygen optimized for production, since variations in this parameter have a direct influence on the final product.

However, the production values for xanthan gum from coconut shells were greater than those obtained from the fermentation of sucrose (conventional carbon source), both in a shaker and in a bioreactor (Figure 1).

Figure 3 shows the viscosity curves for aqueous solutions of the xanthan gums obtained from green coconut shells in the shaker.

The values obtained for viscosity at the different temperatures for the gums obtained in a shaker with sucrose as the fermentative medium were lower than those obtained at the same temperatures with green coconut shell as the fermentative medium.

Figure 4 shows the apparent viscosity curves for aqueous solutions of the xanthan gums obtained from green coconut shells in a 2.0 L bioreactor.

The values obtained for viscosity for the gums obtained in a bioreactor with green coconut shell as the fermentative medium were also higher, showing a 22% increase when compared with those obtained with sucrose as the fermentative medium (Figure 4).
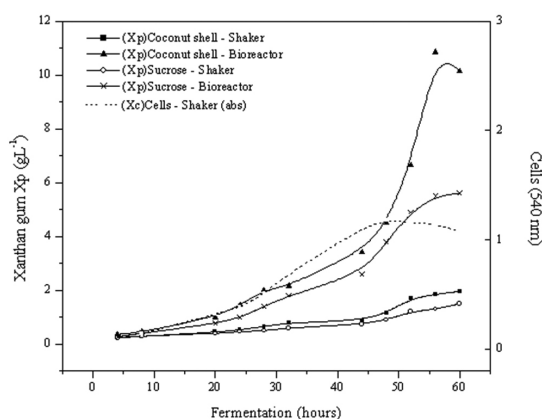

Figure 1. Cell growth (Xc) curve in the shaker; and formation of xanthan gum (Xp) curves in the shaker and in the bioreactor, for gums obtained from coconut shell and from sucrose during 60 hours of fermentation.
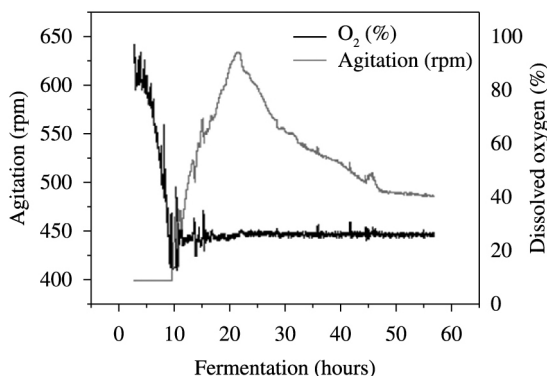

Figure 2. Curve of the rate of dissolved oxygen maintained between 20% and 30% of saturation at a pressure close to 1 atm., during the 60 hours of fermentation in a bioreactor using green coconut shell.
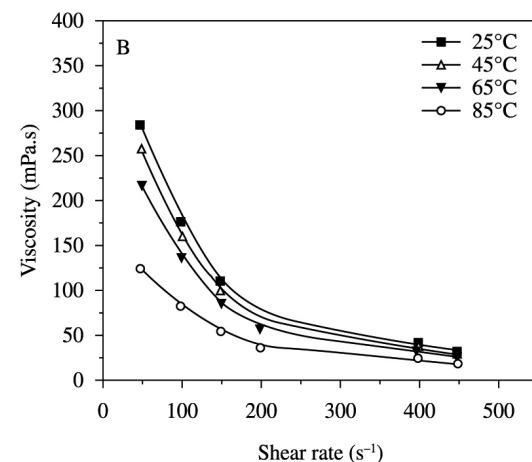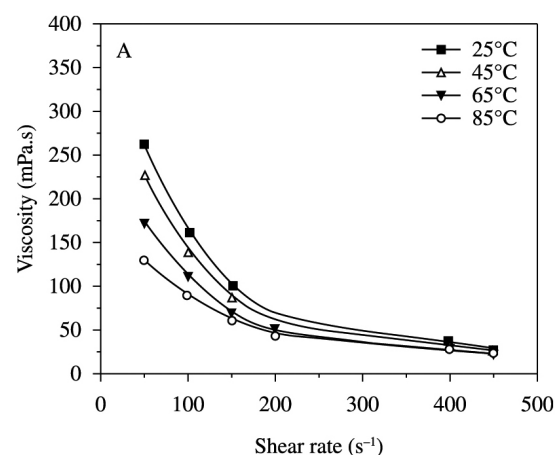

Figure 3. Curves of the apparent viscosity at different temperatures for 1% solutions of the xanthan gum obtained in a shaker from sucrose (A) and from green coconut shell (B), all dissolved in distilled water.

Independently of the shear rate, as the temperature of the gum solutions increased, so the apparent viscosity decreased, although this decrease was more accentuated with the higher shear rates (Figures 3 and 4). The highest values for viscosity were obtained at temperatures of 25 and 45 °C. According to Correia et al.[18], xanthan gum jellifies at temperatures close to 45 °C, explaining the higher values for viscosity at this temperature. There was an accentuated decrease in the viscosity at 85 °C, which can be explained by the conformational changes occurring in the xanthan molecules[16]. The conformation of xanthan gum suffers a transition from an ordered molecule (helical form) to a disordered one (like a ball of wool) with the action of heat, decreasing the effective hydrodynamic volume and, consequently, the viscosity[1,3,4].

Knowledge of the rheological behavior of polymeric solutions is essential for their processing, evaluation, quality control and industrial acceptability. In addition to being a direct measurement of the quality of a fluid, the viscosity can provide important information concerning fundamental changes in the structure of the fluid during a determined process, such as polymerization, emulsification and homogenization[19].
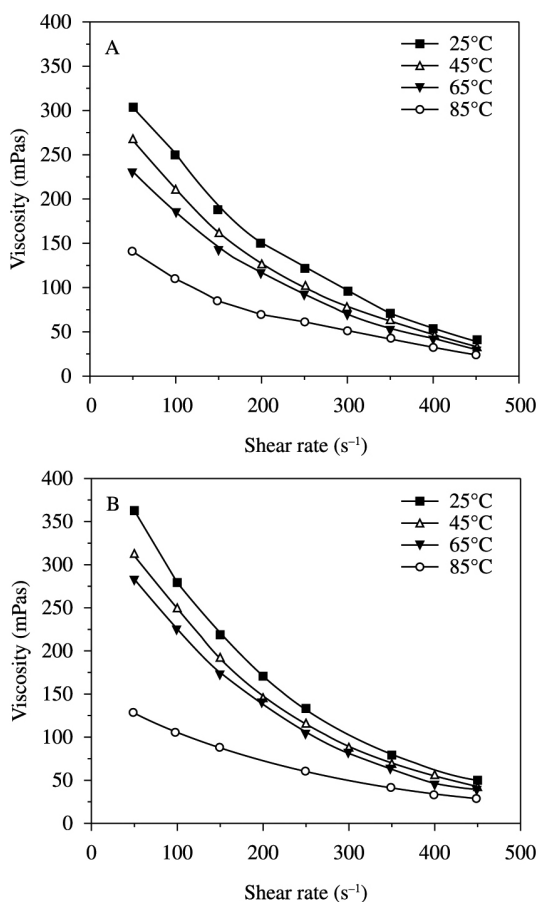
The effect of the shear rate on the viscosity of xanthan gum solutions can be described by the Ostwald-de Waele kinetic model. Figures 3 and 4 show the effect of the shear rate on the viscosity of the xanthan gums produced from sucrose and from green coconut shells in the shaker and in the 2 L bioreactor, according to Equation 1. Independently of the substrate and scale used, the viscosity of the xanthan gums decreased with increase in shear rate. The solutions prepared with the polysaccharides produced from the green coconut shells also showed pseudoplastic behavior typical of xanthan solutions[19].

The experimental data were fitted to the Ostwald-de Waele model as from the regression of the power to confirm the pseudoplastic behavior of the xanthan gum solutions (Table 3).

The 1% xanthan gum solutions obtained from the green coconut shells followed the model described, all the correlation coefficients (R^2) being equal to 0.99 for the different temperatures, as also for the gums obtained from sucrose, independently of the fermentative scale on which they were produced. In all cases, the value for *n* was smaller than unity, implying pseudoplastic behavior according to Xuewu et al.[20], varying between 0.27 and 0.47 for the gum produced with sucrose, and between 0.27 and 0.40 for that obtained from green coconut shells. According to Speers[21], the magnitude of *n* and the changes in the values with variation in concentration of the aqueous solutions, are highly dependent on the size of the molecules.

It was shown that increases in temperature from 25 to 85 °C caused decreases in the value for *K* of from 2.1 to 3.1 times for the aqueous solutions obtained from green coconut shell, and from 3.1 to 5.5 times for those obtained from sucrose, depending on the scale used (Table 3). Independently of the fermentation medium, the values for *K* of the xanthan gums obtained in the bioreactor was less influenced by the temperature.

According to the Petrobras norm n° N-2604[22], xanthan gum should have a maximum value for *n* of 0.5 and minimum value for *K* of 1000 for application as a perforation fluid, thus the xanthan gum produced from the green coconut shell could be used for application in water based perforation fluids.

Figure 4. Curves of the apparent viscosity at different temperatures for 1% solutions of the xanthan gum obtained in a 2.0 L bioreactor from sucrose (A) and from green coconut shell (B), all dissolved in distilled water.

Table 3. Values for the flow (*n*) and consistency (*K*) indexes of the xanthan gum produced from green coconut shells by *Xanthomonas campestris* 1866 at temperatures from 25 to 85 °C.

Sucrose (Shaker)			Coconut shells (Shaker)		
T (°C)	*n*	*K*	*R²*	*n*	*K*	*R²*
25	0.29	2545	0.99	0.31	2585	0.99
45	0.30	2133	0.99	0.31	2345	0.99
65	0.34	1409	0.99	0.33	1878	0.99
85	0.47	459.6	0.99	0.40	839.9	0.99

Sucrose (Bioreactor)			Coconut shells (Bioreactor)		
T(°C)	*n*	*K*	*R²*	*n*	*K*	*R²*
25	0.27	3206	0.99	0.27	3806	0.99
45	0.27	2854	0.99	0.28	3141	0.99
65	0.29	2352	0.99	0.28	2883	0.99
85	0.29	1520	0.99	0.32	1834	0.99

Even without carrying out an economic analysis, the bioconversion of green coconut shell into xanthan gum appears to be a feasible alternative. Sucrose, which is one of the alternatives for producing the gum in Brazil costs approximately $720 per ton, compared to $250 per ton for coconut shell powder[8], with the additional advantage that the gum produced is innocuous to human health, since the green coconut shell residues are discarded in the *in natura* form, and thus the xanthan gum obtained can be destined for food, cosmetic or pharmaceutical ends.

Conclusions

It is possible to produce xanthan gum by the bioconversion of green coconut shells by *Xanthomonas campestris campestris* 1866. The production of xanthan gum from green coconut shells was higher than that obtained from sucrose on both scales tested, and with the alternative substrate the production was greater in the bioreactor.

The xanthan produced from green coconut shells and from sucrose showed pseudoplastic behavior. The maximum values for viscosity of the gums obtained from green coconut shells in the shaker and in the bioreactor were higher than those obtained from sucrose.

In addition to the reduction in production costs as compared to the use of traditional carbon sources, the bioconversion of green coconut shells to xanthan represents an alternative to reduce environmental problems caused by discarding this residue.

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