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# Processing Parameters Matching Effects upon *Rhizobium tropici* Biopolymers' Rheological Properties

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**Abstract** The combined effects of the processing parameters upon rheological properties of biopolymers produced by *Rhizobium tropici* were studied as a function of the $Ca^{+2}$ ions' concentration variation, yeast extract concentration added to the medium, aeration, and agitation, maintaining the mannitol concentration in 10 g/L. The experiments were carried out using a fermenter with 20-L capacity as a reactor. All processing parameters were monitored online. The temperature [(30±1) °C] and pH values (7.0) were kept constant throughout the experimental time. As a statistical tool, a complete $2^3$ factorial design with central point and response surface was used to investigate the interactions between relevant variables of the fermentation process: calcium carbonate concentration, yeast extract concentration, aeration, and agitation. The processing parameter setup for reaching the maximum response for rheological propriety production was obtained when applying mannitol concentration of 10.0 g/L, calcium carbonate concentration 1.0 g/L, yeast extract concentration 1.0 g/L, aeration 1.30 vvm, and agitation 800 rpm. The viscosimetric investigation of polysaccharide solutions exposed their shear-thinning behavior and polyelectrolytic feature.

**Keywords** *Rhizobium tropici* · Biopolymer · Exopolysaccharides · Experimental design · Rheological properties · Shear-thinning behavior

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## Introduction

Microbial polysaccharides represent a class of biopolymers that have been the object of many studies in biotechnology. Obtained from renewable sources, they present peculiar characteristics, such as biocompatibility, biodegradability, nontoxicity, wide availability, and low cost. Due to their large diversity of structures and physicochemical and rheological properties, microbial polysaccharides have found a wide range of applications in the food, pharmaceutical, chemical, petroleum, and cosmetics industries, as well as in some sectors of the textile industry [1–3]. These microbial gums may serve as emulsifiers, stabilizers, binders and jellifying agents, coagulant agents, flocculating agents, film-forming agents, lubricants, and thickeners [2, 4].

The structure and composition of microbial polysaccharides depend on a number of factors, such as medium-culture composition, type of carbon source, type of microbial system employed, and fermentation conditions (pH, temperature, oxygen concentrations) [5–7]. Through the control of the fermentation conditions, one may control not only the amount of exopolysaccharide (EPS) produced, but also the biopolymer properties and composition [8, 9]. For many microbial species, calcium is essentially required in small amounts: it is essential in maintaining cell wall rigidity, it stabilizes oligomeric proteins, and covalently binds protein peptidoglycan complexes in the outer membrane, also being required for chemotaxis [10].

The industrial value of polysaccharides, among the most used so far, lies in their properties of changing the rheological characteristics of fluids, either through gelation or through alteration on the flowing characteristics. The behavior of polysaccharide solutions may be Newtonian, shear-thinning behavior, or shear-thickening behavior; some of them may even exhibit tixotropy; in other words, they are characterized by high viscosities and low tensions and by decreasing viscosities for increasing tensions [11, 12].

The knowledge on the rheological properties of polysaccharides is a valuable contribution for their physicochemical and molecular characterization, promoting their potential application in a large number of industrial processes [13, 14]. Gums produced by microorganisms are frequently used in several products due to their capacity of changing the viscosity of water and aqueous systems, providing high viscosity when used at low concentrations [15]. Thus, the present study investigated the relation of variables aeration, agitation, and composition of the production medium with the rheological properties of the EPSs produced by *Rhizobium tropici*.

## Material and Methods

### Microorganism

The *R. tropici* strain was used in the experiments, and it was isolated from a variety of bean culture, native from an arid region of northeastern Brazil, called “Caupi.” This strain was generously granted by the Recife Agronomic Institute and has been kept in agar–mannitol–yeast extract (YMA) for several months, being cataloged after its characterization at the culture bank of the Department of Biochemical Engineering from the Federal University of Rio de Janeiro, under the code EQ RT.

## Culture Media

### *Medium for the Maintenance of the Culture*

The microorganism was kept in modified YMA extract with the following composition in grams per liter: mannitol 10.0 [16], $K_2HPO_4$ 0.10, $KH_2PO_4$ 0.40, $MgSO_4.7H_2O$ 0.20, NaCl 0.10, yeast extract 0.40, and agar 15.0. The medium pH was adjusted to 7.0 [17].

### *Medium for Inoculum Preparation*

For the inoculum preparation, two culture mediums (M1 and M2) were used according to the experiment design:

| | |
|---|---|
| Medium M1 | Its composition is similar to the maintenance medium (YMA), but without agar addition. $CaCO_3$ concentration varying from 0.50 to 1.0 g/L, according to experimental design 1 |
| Medium M2 | Similar to the maintenance medium (YMA), with yeast extract concentration changing from 0.4 to 1.0 g/L and $CaCO_3$ concentration proposed by experimental design 2 |

The culture mediums were sterilized at 121 °C for 20 min and the pH was adjusted to 7.0 with NaOH solution at 50% (w/v) before medium sterilization.

### *Medium for the Polysaccharides Production*

The production of extracellular polysaccharides was performed by using a culture medium similar to M1 or M2, added of ions $Mn^{+2}$ ($MnCl2.\ 4H_2O$ 0.12 g/L). The pH of the mediums was adjusted to 7.0±0.1 with NaOH solution at 50% (w/v) and sterilized at 121 °C for 20 min at the fermenter. The inoculation of the production medium was performed to obtain an average cell concentration of 0.77±0.02 mg/mL.

## Maintenance of Microorganisms

The microorganism was cultivated in laboratory tubes containing YMA medium at 30± 1 °C for 48 h. After growth, the cultures were stored at 5±1 °C.

## Production of EPS in Bioreactors

These experiments were conducted in a fermenter (New Brunswick Scientific, Edison, NJ, USA, Model BioFlow IV) with 20-L capacity equipped with disc impeller (outer diameter 3.75 in., type six-bladed 316 L stainless steel, Rushton blade, standard) and oxygen and pH electrodes. The equipment also monitored temperature, agitation speed, purging gas flow rate, pumping rates, antifoam addition, and the vessel level. All processing parameters were monitored online with the aid of AFS 3.0 software (Advanced Fermentation Software, New Brunswick Scientific). The temperature (30±1 °C) and pH value (7.0±0.1) were kept constant during the experiments. For each experiment, 1,000 mL of the inoculum was used, that is, 10% (v/v) of the initial working volume (10 L). The process was conducted through 48 h.

Qualitative and Quantitative Determinations During the Fermentation Process (During the Fermentation Process and Immediately After, in the Broth)

*Culture Purity*

During the process, microscopic examinations were performed in laboratory preparations through the Gram method to detect possible contaminations in the medium.

*Broth Viscosity*

Viscosity measurements of the fermented broth were performed in a Brookfield Synchro-Lectric, model LVT rheometer, with the accessory small sample adapter. Viscosities were determined at various shear rates. Temperature was kept constant at 20 °C.

*Mannitol Determination*

For the quantitative mannitol determination, the fermented broth was initially filtered through 0.2-μm Millipore membranes to remove cells. In the filtrate, the substrate was analyzed through high-performance liquid chromatography in a Waters chromatograph equipped with SHODEX SC1011 ion-exchange columns, at 75 °C. Ultrapurified water (Milli-Q, Millipore, Bedford, MA, USA) was used as eluent, and elution rate was 0.8 mL/min. The final result was obtained through a differential refractometer detector type, model Waters 410, and an integrator–registrator Waters 746 (data module).

*EPS Extraction and Purification*

The amount of EPSs produced after fermentation was determined through dry-weight measurements. The fermentation broth was heated at 80±1 °C, during 10 min, to ensure cell inactivation. After this stage, a filtration was conducted to remove cells. To precipitate the EPSs, ethanol Pro Analysis (3:1) was added to the fermented broth. After total precipitation of the EPS present in the medium, the mixture (broth plus ethanol) was filtered through 0.2 μm Millipore membrane, using Gouche crucible, previously weighed.

The biopolymers extracted from the fermented broth were purified through successive washings with ethanol Pro Analysis, 70, 80, and 90% (v/v), respectively. The drying of the biopolymer was performed by introduction of nitrogen gas under controlled heating. The product obtained was dried at 80±1 °C until constant weight. All determinations were performed in triplicate.

Rheological Characterization

Ultrapurified water and sodium chloride solutions of polysaccharide samples obtained at the best experimental design conditions were analyzed in relation to their rheological behavior. The viscosity measurements were performed in rheometer RHEO STRESS 600 (HAAKE), coupled to a bath equipped with a thermostat. Several rheological measurements were conducted with the objective of obtaining the relative viscosity of samples, as well as the study of their behavior in function of concentration and shear rate.

The responses regarding inlet variable manipulations were evaluated in relation to the apparent viscosity of gums produced at 0.50 g/L in ultrapurified water. Due to the low viscosity values of some gums produced at the different experimental conditions, no

response was obtained when submitted to different shear rates. Thus, the results for apparent viscosity response of gums were obtained at a shear rate of 20.4 $s^{-1}$.

The shear-thinning behavior of the purified biopolymers samples produced was verified through the relative decrease on the relative viscosity ($\eta_{relative}$) in function of the increase on the shear rate. Relative viscosity is calculated through the following equation:

$$\eta_{relaive} = \frac{\eta}{\eta^0}$$

where $\eta$ is the apparent viscosity of the polymer solution, expressed in Pa s, and $\eta^0$ is the solvent apparent viscosity. According to the equation showed above, the relative viscosity is a dimensionless parameter.

Viscosity measurements have been carried out at 20 °C and the rheological characteristics of the samples have been done in NaCl solution. The viscosity of the ultrapurified water was 0.00086 Pa s and the viscosity of NaCl 0.1 mol/L solution was 0.00092 Pa s.

## Experimental Design and Statistical Analysis

Recent studies have demonstrated the importance of the use of experimental design and the response surface methodology in fermentative processes with the objective of optimizing process parameters [18–20]. Through this technique, it is possible to save time and reduce cost during the research phases. The selection of variables (influence factors) and the higher (+), lower (−), and central (0) levels employed in the experimental design were defined based on reports from literature [21] and on knowledge acquired during the time that defined these parameters as significant for the EPS production with enhanced rheological properties. Thus, for the production of EPS through *R. tropici*, the following strategies were adopted:

| | |
|---|---|
| Experimental design 1 | Experimental design 1 was performed with the objective of studying interactions between aeration, agitation, and $CaCO_3$ concentration and of identifying the relevance of the addition of $CaCO_3$ in the viscosimetric properties, based on a fixed substrate concentration of 10.0 (g/L) [16]. Table 1 presents the control factors and levels employed in the development of experimental design 1. |
| Experimental design 2 | Experimental design 2 was performed with the objective of studying interactions between aeration, agitation, and yeast extract concentration. In this design, the $CaCO_3$ concentration was fixed based on result obtained in experimental design 1. Table 2 presents the control factors and levels employed in the development of experimental design 2. |

The best way to treat more than one parameter is to conduct a factorial experiment. This experimental strategy consists of varying all factors together, which enables identifying the influence of each factor on the system and also any possible interaction between them [22]. Thus, a complete $2^K$ factorial design with a central point was performed, where $K$ is the number of factors and 2 is the number of levels, resulting in the following condition: $2^3$= 8 developed in duplicate, and one central point performed in triplicate, summing up 19 experiments [22–25].

The inclusion of the central point aimed to allow the calculation of the experimental error and, hence, the verification of the lack of adjustment for the model selected, with the

**Table 1** Summary of experimental results and apparent viscosity empirical model predictions corresponding to experimental design 1.

| Experiments | Factors | | | Apparent viscosity (Pa s) | |
|---|---|---|---|---|---|
| | $X_1$ calcium carbonate concentration (g/L) | $X_2$ aeration (vvm) | $X_3$ agitation (rpm) | Observed value | Predicted value |
| 1a and 1b | 0.50 (−) | 0.20 (−) | 200 (−) | 0.0472<br>0.0496 | 0.0561 |
| 2a and 2b | 1.00 (+) | 0.20 (−) | 200 (−) | 0.06450<br>0.06250 | 0.0558 |
| 3a and 3b | 0.50 (−) | 1.30 (+) | 200 (−) | 0.0535<br>0.0551 | 0.0466 |
| 4a and 4b | 1.00 (+) | 1.30 (+) | 200 (−) | 0.0785<br>0.0763 | 0.0851 |
| 5a and 5b | 0.50 (−) | 0.20 (−) | 800 (+) | 0.0353<br>0.0415 | 0.0675 |
| 6a and 6b | 1.00 (+) | 0.20 (−) | 800 (+) | 0.0798<br>0.0823 | 0.0888 |
| 7a and 7b | 0.50 (−) | 1.30 (+) | 800 (+) | 0.0578<br>0.0525 | 0.0629 |
| 8a and 8b | 1.00 (+) | 1.30 (+) | 800 (+) | 0.1700<br>0.1652 | 0.1599 |
| 9a, 9b, and 9c | 0.80 (0) | 0.80 (0) | 500 (0) | 0.0565<br>0.0500<br>0.0490 | 0.0507 |

lowest number of assays as possible. According to Benício et al. [26], two repetitions for the central point would be sufficient for the calculation of the experimental error, and the selection of three repetitions was aimed at obtaining a more precise estimation of the error. The experiments were developed based on a random sequence to assure the equanimous distribution of all factors, which were later analyzed at once.

The design matrix presents assays in the so-called standard order. All columns start with level (−), and then the signs alternate. One by one in the first column, −+−+…, and then two by two, −−++…, and finally, four negative signs and four positive signs in the last column [22]. A mathematical model, describing the relationships between the process indices (apparent viscosity) and the variable study contents in second-order equation, was developed. Design-based experimental data were matched according to the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{3} \beta_i X_1 + \sum_{i=1}^{3} \beta_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{i \neq j} \beta_{ij} X_i X_j + \beta_{123} X_1 X_2 X_3$$

where $Y$=apparent viscosity (Pa s)

$\beta_0$ Constant
$\beta_i$ Linear term coefficients
$\beta_{ii}$ Quadratic term coefficients
$\beta_{ij}$ Interaction coefficients

**Table 2** Summary of experimental results and apparent viscosity empirical model predictions corresponding to experimental design 2.

| Experiments | Factors | | | Apparent viscosity (Pa s) | |
|---|---|---|---|---|---|
| | $X_1$ yeast extract concentration (g/L) | $X_2$ aeration (vvm) | $X_3$ agitation (rpm) | Observed value | Predicted value |
| 1a and 1b | 0.40 (−) | 0.20 (−) | 200 (−) | 0.0655<br>0.0615 | 0.0570 |
| 2a and 2b | 1.00 (+) | 0.20 (−) | 200 (−) | 0.0738<br>0.06370 | 0.0752 |
| 3a and 3b | 0.40 (−) | 1.30 (+) | 200 (−) | 0.0738<br>0.0562 | 0.0715 |
| 4a and 4b | 1.00 (+) | 1.30 (+) | 200 (−) | 0.1735<br>0.01815 | 0.1710 |
| 5a and 5b | 0.40 (−) | 0.20 (−) | 800 (+) | 0.0613<br>0.0814 | 0.0803 |
| 6a and 6b | 1.00 (+) | 0.20 (−) | 800 (+) | 0.1920<br>0.2126 | 0.1958 |
| 7a and 7b | 0.40 (−) | 1.30 (+) | 800 (+) | 0.0785<br>0.0879 | 0.0767 |
| 8a and 8b | 1.00 (+) | 1.30 (+) | 800 (+) | 0.2704<br>0.2640 | 0.2737 |
| 9a, 9b, and 9c | 0.70 (0) | 0.8 (0) | 500 (0) | 0.0724<br>0.0695<br>0.0705 | 0.0708 |

All calculations involved, as well as the drawing of all three-dimensional surfaces, have been obtained using the Statistica™ Softsware for Windows, version 5.5 computer package, produced by StatSoft. The model allowed the evaluation of the effects of linear and quadratic coefficients and combined effects of the independents variables upon the dependent variable. The $p$ value was employed to check the statistical significance of the regression coefficients. The $F$ test for analysis of variance (ANOVA) was performed on experimental data to evaluate the statistical significance of the model.

Three-dimensional surface plots were drawn to illustrate the main and interactive effects of the independent variables on the apparent viscosity. The optimum values of the selected variables were obtained both by solving the regression equation and by analyzing the response surface contour plots [27].

## Results and Discussion

### Experimental Design

The optimization of fermentative processes has been in use to improve the productivity of a number of bioprocesses for a long time. Fermentation processes have been optimized for the implementation of the variation of one component at a time. This approach considers that the process variables do not interact and that the process response is a function of a simple parameter, which is varied. Recent studies have demonstrated the importance of the

experimental design and the response surface methodology utilization in fermentative processes with the objective of optimizing process parameters [18–20]. Through this methodology, factors and interactions that affect the desired response and may be experimentally controlled are tested according to their efficiencies in the optimization of important process parameters in fermentation, with a limited number of experiments [28–31]. Through the control of the fermentation conditions, one may control not only the amount of EPS produced, but also the biopolymer properties and composition.

*Experimental Design 1*

The results of experimental design 1 and the experimental values of independent variables are presented in Table 1. The increase on the EPS production with the addition of $CaCO_3$ to the culture medium [32] was verified through the increase on the viscosity of the fermented broth 48 h after the process started.

The polynomial mathematical correlation for the apparent viscosity of gums produced in experimental design 1 (0.50 g/L in ultrapurified water) was adjusted considering significant conditions only, for a confidence level of 95% ($\alpha$=0.05), as described below:

$$\begin{aligned}\text{Apparent viscosity} = {} & 0.3372 + (-0.7780\,X_1) + \left(0.4828\,X_1^2\right) + (-0.0567\,X_2) \\ & + (-0.0001\,X_3) + (0.0707\,X_1X_2) + (0.0002\,X_1X_3) \\ & + (0.0001\,X_2X_3) \end{aligned} \quad (1)$$

Unfortunately, for any model that is chosen, there will be lack of fit that could be caused, at least partially, by the random errors. In our research, the total lack of fit left by the model can be decomposed into two parts: the first one caused by the errors and the other one due to the lack of fit of the model. The portion caused by the lack of fit can be reduced by improving the model. This cannot be done with the other portion.

The *F* test of the mean square of the lack of fit (MSLF) by the mean square of the pure error (MSPE) – (MSLF/MSPE) – was used to evaluate if the chosen model is well adjusted to the observation or not. High values of MSLF/MSPE will mean high lack of fit. Table 3 (ANOVA) also shows a term for the residual error that measures variation of response data that could not be explained in the model.

The variance analysis of experimental designs 1 and 2 for this adjustment is shown in Tables 3 and 4. For experimental design 1, the percentage of variation explained by the regression, that is, the quadratic sum due to the regression divided by the total quadratic sum, corresponds to 95.80%, but this value should not be compared with 100% because of the pure error contribution. As there is no model that is able to reproduce the square sum of the pure error (SSPE), the maximum accountable value is the difference between the total sum of squares (SST) and the SSPE. In our research, SST−SSPE=0.025401−0.000111= 0.02529. This corresponds to 0.02529/0.025401=99.56%, and with this value, the effective explained variation, 95.80%, should be compared. For the proposal model, the percentage values indicate a significant regression if it were not the lack of fit. For experimental design 1, the value MSLF/MSPE is 86.82, which is much higher if compared with $F$ 1,10=4.96 (with a level of confidence of 95%), evidencing a high lack of fit.

One may verify that, for the apparent viscosity response in experimental design 1, all terms were statistically significant ($p \leq 0.05$). The calcium carbonate concentration for apparent viscosity response was the most statistically significant term. One may also verify that the interaction between the three variables ($X_1X_2X_3$) was not statistically significant.

**Table 3** ANOVA for significant terms of the model in $\alpha$=0.05 of experimental design 1 with apparent viscosity (Pa s) of gums in aqueous solution as response (0.50 g/L in ultrapurified water), at a shear rate of 20.4 $s^{-1}$.

| Factor | Sum of squares | Degrees of freedom | Mean square | *F* value | *p* Value |
|---|---|---|---|---|---|
| $X_1$ (L) | 0.009341 | 1 | 0.009341 | 844.1627 | 0.000000 |
| $X_1$ (Q) | 0.002104 | 1 | 0.002104 | 190.1004 | 0.000000 |
| $X_2$ (L) | 0.003788 | 1 | 0.003788 | 342.3565 | 0.000000 |
| $X_3$ (L) | 0.002430 | 1 | 0.002430 | 219.6424 | 0.000000 |
| $X_1$ (L)×$X_2$ (L) | 0.001513 | 1 | 0.001513 | 136.7482 | 0.000000 |
| $X_1$ (L)×$X_3$ (L) | 0.003416 | 1 | 0.003416 | 308.7390 | 0.000000 |
| $X_2$ (L)×$X_3$ (L) | 0.001743 | 1 | 0.001743 | 157.5199 | 0.000000 |
| Lack of fit | 0.000955 | 1 | 0.000955 | 86.2858 | 0.000003 |
| Pure error | 0.000111 | 10 | 0.000011 | | |
| SST | 0.025401 | 18 | | | |

L = linear, Q = quadratic, $X_1$ = calcium concentration, $X_2$ = aeration, $X_3$ = agitation

Figures 1 and 2 present response surface curves for apparent viscosity of gums produced in experimental design 1 (0.50 g/L in ultrapurified water) at a shear rate of 20.4 $s^{-1}$. The analysis of response surfaces in function of the apparent viscosity of gum at 0.50 g/L in ultrapurified water presented in Figs. 1 and 2 shows that the maximum estimation for the apparent viscosity of 0.1599 Pa s was estimated for $CaCO_3$ concentration of 1.00 g/L, 1.30 vvm, and 800 rpm. The increase in the apparent viscosity for higher aeration, agitation, and $CaCO_3$ concentration values may be reasonable if it is based on the fact that, for these conditions, an increase in the production estimation is observed [32]. In this case, the high aeration and agitation promotes better oxygen diffusion.

The introduction of $CaCO_3$ in the culture medium composition promoted an increase of 316% in the apparent viscosity maximum estimation, a phenomenon that might be associated with the intermolecular complexation of calcium ions with available carboxylic groups [33]. The calcium ions form ionic bonds between carboxylic groups at the adjacent units of the same chain, and for the formation of crossed bonds between chains, hydroxyl groups must be available for the formation of coordinate bonds with the calcium atom. This bond with calcium forms a three-dimensional net of chain called the “egg box” [34].

**Table 4** ANOVA for significant terms of the model in $\alpha$=0.05 of experimental design 2 with apparent viscosity (Pa s) of gums in aqueous solution as response (0.50 g/L in ultrapurified water), at a shear rate of 20.4 $s^{-1}$.

| Factor | Sum of squares | Degrees of freedom | Mean square | *F* value | *p* Value |
|---|---|---|---|---|---|
| $X_1$ (L) | 0.037008 | 1 | 0.037008 | 127.3083 | 0.000001 |
| $X_1$ (Q) | 0.006635 | 1 | 0.006635 | 22.8258 | 0.000748 |
| $X_2$ (L) | 0.013231 | 1 | 0.013231 | 45.5139 | 0.000051 |
| $X_3$ (L) | 0.010635 | 1 | 0.010635 | 36.5837 | 0.000124 |
| $X_1$ (L)×$X_2$ (L) | 0.010842 | 1 | 0.010842 | 37.2966 | 0.000115 |
| $X_1$ (L)×$X_3$ (L) | 0.005569 | 1 | 0.005569 | 19.1570 | 0.001384 |
| $X_2$ (L)×$X_3$ (L) | 0.000023 | 1 | 0.000023 | 0.0784 | 0.785138 |
| Lack of fit | 0.000010 | 1 | 0.000010 | 0.0336 | 0.858236 |
| Pure error | 0.002907 | 10 | 0.000291 | | |
| SST | 0.08686 | 18 | | | |

L = linear, Q = quadratic, $X_1$ = yeast extract, $X_2$ = aeration, $X_3$ = agitation

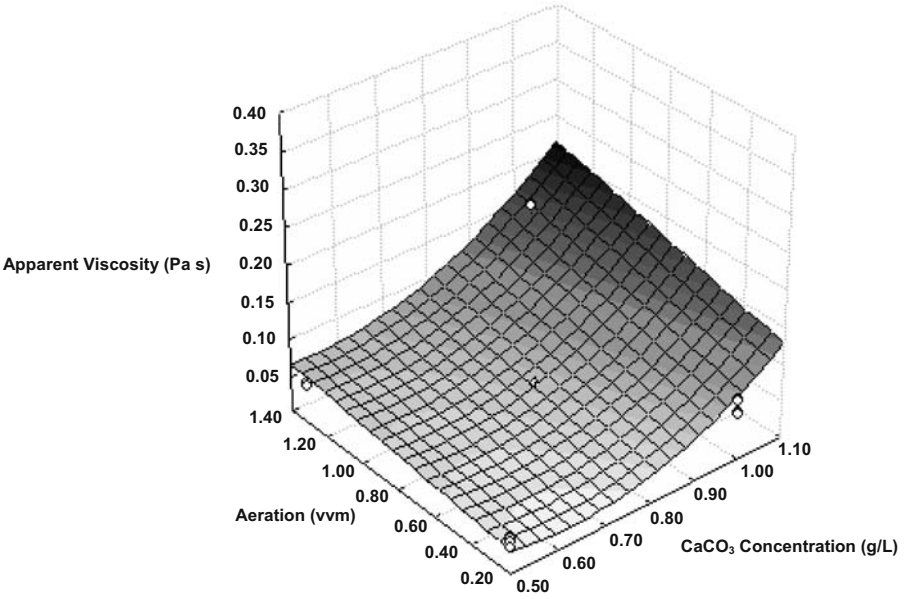


**Fig. 1** Apparent viscosity response surface: effect of the variation of $CaCO_3$ concentration and aeration, considering agitation value as constant agitation of 800 rpm

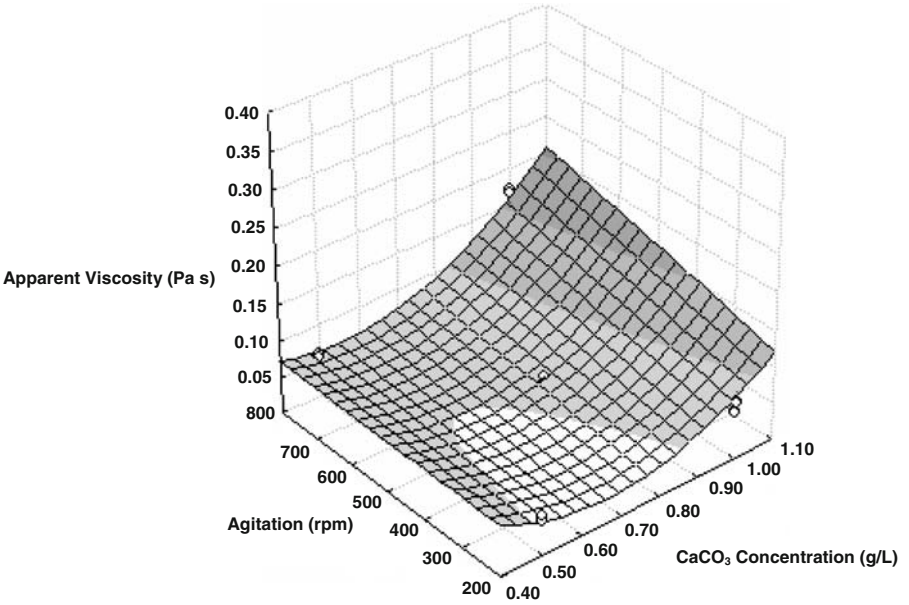


**Fig. 2** Apparent viscosity response surface: effect of the variation of $CaCO_3$ concentration and agitation, considering aeration value as constant aeration of 1.30 vvm

*Experimental Design 2*

The results of experimental design 2 and the experimental values of independent variables are presented in Table 2. The polynomial mathematical correlation for the apparent viscosity of gums produced in experimental design 2 (0.50 g/L in ultrapurified water) was adjusted considering significant conditions only. The model equation for apparent viscosity response for a confidence level of 95% ($\alpha$=0.05) may be described as shown below:

$$\begin{aligned}\text{Apparent viscosity} = {} & 0.3111 + (-0.8594\,X_1) + \left(0.5698\,X_2^1\right) + (-0.0618\,X_2) \\ & + (0.0001\,X_3) + (0.1578\,X_1X_2) + (0.0002\,X_1X_3) \\ & + (0.00001\,X_2X_3) \end{aligned} \quad (2)$$

Table 4 presents the ANOVA with results of $p$ and $F$ tests for all terms of the model with confidence level of 95%, $\alpha$=0.05. For experimental design 2, the variation explained by the regression corresponds to 96.64%, and it indicates a significant regression supported by the evidence of low lack of fit. The value MSLF/MSPE is 0.03436, which is small if compared with $F$ 1,10=4.96 (with a level of confidence of 95%), eliminating, therefore, the evidence of lack of fit.

The analysis of Table 4 indicates that the second-order polynomial mathematical correlation (Eq. 2) showed good suitability to represent the current relation between the apparent viscosity response and the variables, with very small estimated $p$ values ($p$=

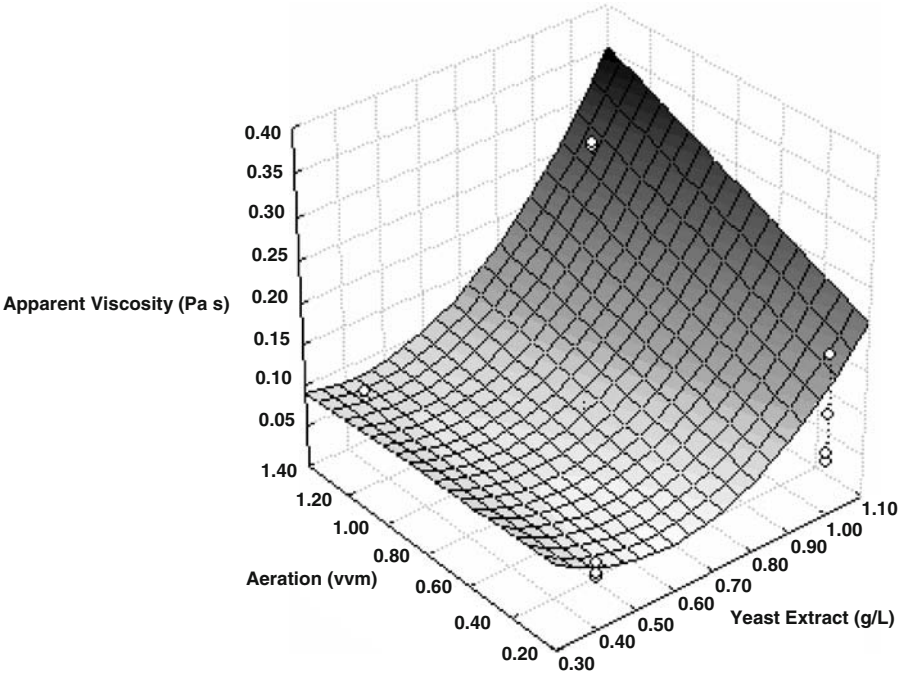


**Fig. 3** Apparent viscosity response surface: effect of yeast extract concentration and aeration variations considering agitation value as constant agitation of 800 rpm

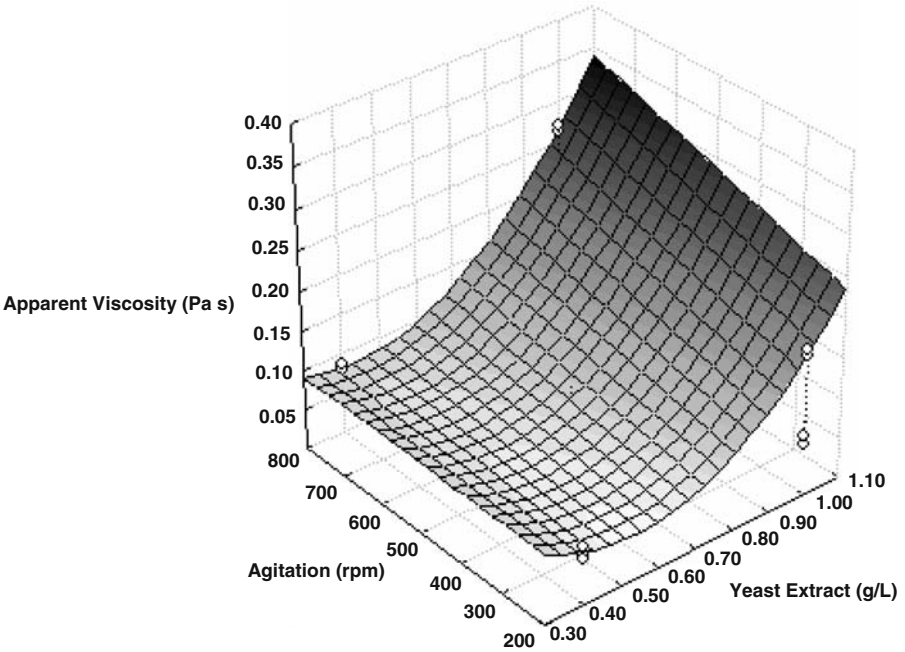


**Fig. 4** Apparent viscosity response surface: effect of yeast extract concentration and agitation variations considering aeration value as constant aeration of 1.30 vvm

0.000). So, the best adjustment of the experimental data to the proposed model is obtained for experimental design 2.

The analysis of Table 4 indicates that all linear terms were significant at the established confidence level. The calcium carbonate concentration for apparent viscosity response in experimental design 2 was the most statistically significant term. One may also verify that the aeration/agitation interaction had little statistical significance and that the interaction between all variables ($X_1X_2X_3$) was not statistically significant.

Figures 3 and 4 present surface curves for apparent viscosity response of gums produced in experimental design 2 (0.50 g/L in ultrapurified water) at a shear rate of 20.4 $s^{-1}$. The analysis of response surfaces obtained through experimental design 2 with response to apparent viscosity of gum at 0.50 g/L in ultrapurified water as variable shows that the apparent viscosity maximum value of 0.2737 Pa s will occur for higher aeration and yeast extract concentration values, with agitation value of 800 rpm as constant. Therefore, for the simultaneous attainment of maximum production and viscosity in medium enriched with 1.0 g/L of $CaCO_3$, the use of aeration of 1.30 vvm, vigorous agitation of 800 rpm, and yeast extract concentration of 1.0 g/L is recommended.

The increase on the yeast extract concentration in medium previously enriched with $CaCO_3$ enabled an increase on the apparent viscosity probably in function of the increase on the EPS production. The yeast derivatives are used in the composition of culture mediums for the growth of several microorganisms because it is composed of protein from 31% to 48%; high vitamin contents, especially those from B complex ($B_1$, $B_2$, $B_6$, niacin, pantothenic acid, folic acid, and biotin); minerals, including macroelements (Ca, P, Mg, K, Na, Al, and Fe) and microelements (Mn, Cu, B, Zn, Mo, Cd, Cr, Ni, Pb, Si, and Se); and carbohydrate content ranging from 25% to 35% [35].

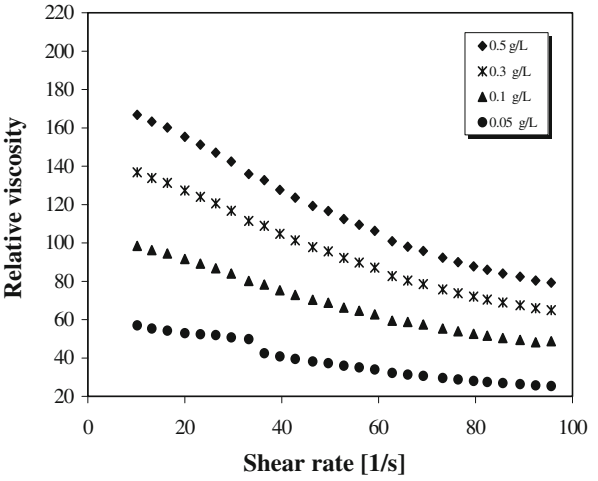


**Fig. 5** (Relative viscosity × shear rate [1/s]): viscosimetric behavior in function of the shear rate of solutions at different concentrations of EPS produced by *R. tropici* in experiment 8b from experimental design 1 in ultrapurified water at 20 °C

The main yeast derivatives are self-lysed and produced through the self-digestion of cells, yeast extract, and yeast cell wall, fractions obtained by centrifuging the self-lysed portion [36]. The yeast extract contains all soluble material of the self-lysed portion, including proteins and peptides [35, 36]. The insoluble fraction, mainly composed of cell wall, is rich in mannoproteins glucan and mannan. Both mannan and glucan, when incorporated in food, play the role of transporting taste and flavor, and also performing as thickeners and stabilizers [37]. Glucan and mannan polymers may originate oligosaccharides, which are carbohydrates with 3 to 10 sugar monomers, and these oligosaccharides have been widely studied as functional ingredients in the composition of culture mediums.

## Rheological Characterization

Viscosity is associated with the polymer structure, the molecular weight and its distribution, the conformation that the macromolecule presents in solution and its interactions with the

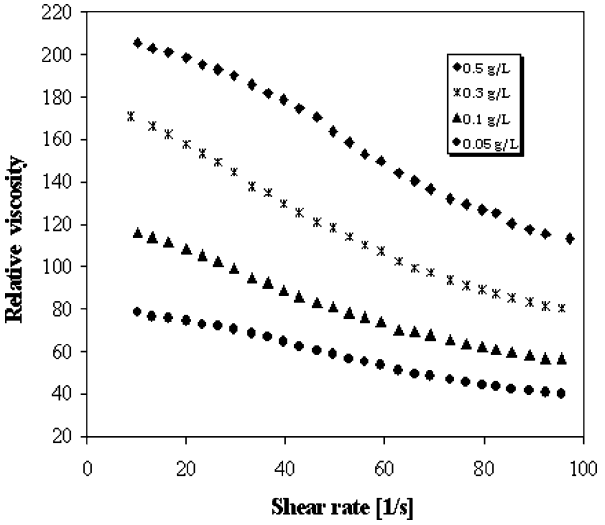


**Fig. 6** (Relative viscosity × shear rate [1/s]): viscosimetric behavior in function of the shear rate of solutions at different concentrations of EPS produced by *R. tropici* in experiment 8a from experimental design 2 ultrapurified water at 20 °C

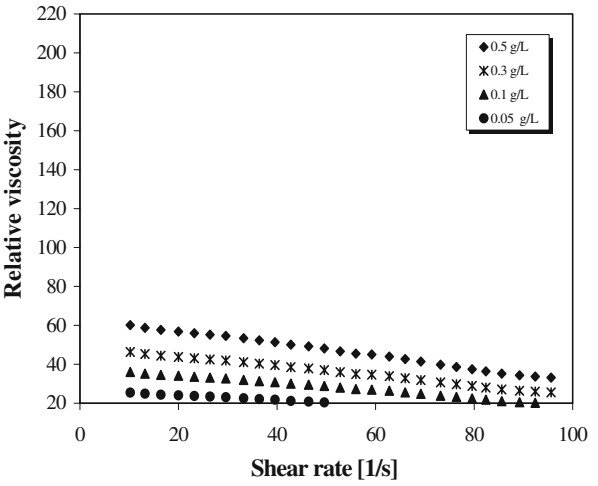


**Fig. 7** (Relative viscosity × shear rate [1/s]): viscosimetric behavior in function of the shear rate of solutions at different concentrations of EPS produced by *R. tropici* in experiment 8b from experimental design 1 in 0.10 mol/L NaCL aqueous solution

solvent, the macromolecules aggregation, the polymer concentration, and the flexibility of chains in relation to temperature [38, 41, 42]. Therefore, the study on the viscosity of macromolecule compound solutions allows characterizing their hydrodynamic behavior.

The objective of the viscosimetric evaluation was to verify the rheological behavior of EPS synthesized by *R. tropici* at the best experimental design conditions. The viscosity measurements were performed at 20 °C and the samples were submitted to the rheological characteristics study both in water and in NaCl solution. The viscosity of the ultrapurified water is $8.60\times10^{-4}$ Pa s, while the viscosity in 0.10 mol/L NaCl aqueous solution is $9.20\times10^{-4}$ Pa s.

Figures 5 and 6 show the viscosimetric behavior of purified samples obtained from experiments 8b and 8a of experimental designs 1 and 2, respectively, at different concentrations in aqueous solution. The figures show the reduction on the viscosity with the increase in the shear rate characteristic of a shear-thinning behavior that may be justified

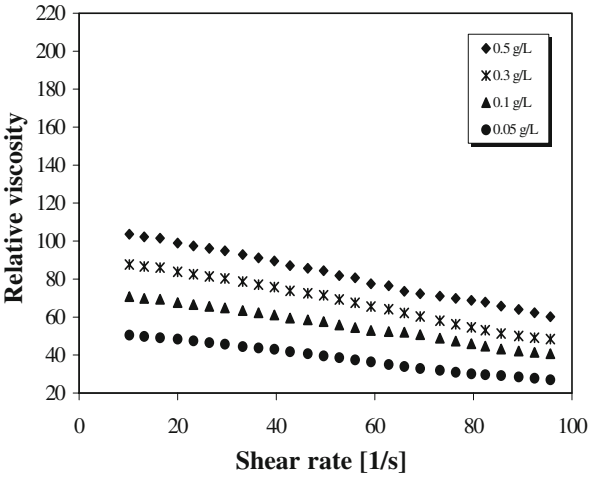


**Fig. 8** (Relative viscosity × shear rate [1/s]): viscosimetric behavior in function of the shear rate of solutions at different concentrations of EPS produced by *R. tropici* in experiment 8a from experimental design 2 in 0.10 mol/L NaCL aqueous solution

through the orientation of molecules towards the flow direction, thus reducing the flow resistance [43].

The modification of the shape of flexible molecules with the shear rate and the effect of the flow in the rupture of intermolecular interactions are factors that also collaborate for the shear-thinning properties [44]. These characteristics encourage the employment of biopolymers such as the xanthan gum as thickener in liquid and pasty food products. The xanthan gum is considered the industrial gum presenting the highest pseudoplasticity level both due to its high molecular weight and to its rigid secondary structure when in solution [42]. The xanthan gum when dissolved in distilled/deionized water (low ionic force) or at high temperature presents disorganized conformation [45–47], being characterized by the rupture of interactions between side chains and the polymeric skeleton. As a result of the separation of branches in relation to the main chain, an increase on the hydrodynamic volume is observed, granting a higher viscosity to the disorganized species [48].

The figure analyses also show that the higher values of relative viscosity were observed for the EPS sample obtained through experimental design 2. These results can be related to differences in the molecular mass of the EPS produced in the different experimental conditions. Figures 7 and 8 show the viscosimetric behavior in function of the shear rate of solutions at different concentrations of EPS produced by *R. tropici* at conditions 8b and 8a from experimental designs 1 and 2 in 0.10 mol/L of NaCl aqueous solution.

The analysis of Figs. 5, 6, 7, and 8 shows the shear-thinning profile for EPSs produced at the different culture mediums of experimental designs 1 and 2. Lower relative viscosity values are also observed in electrolyte solutions. The decrease on the saline solution viscosity is a result of the reduction on the hydrodynamic volume of the macromolecule, leading to the formation of more compact structures. This alteration is promoted by the electrostatic repulsion between ionic groups as a result of the neutralization of charges present in the molecule [49, 50]. It is reasonable supposing that the dissolution of EPSs produced in experiments through ultrapurified water would favor a more expanded macromolecular structure.

## Conclusion

Most research developed using *Rhizobium* genus bacteria is related to genetics and bacteria–host plant symbiotic interaction issues. Little is known about the production of extracellular polysaccharides by *Rhizobium*, as well as their properties in solution. In addition, no studies on monitoring medium composition, agitation and aeration parameter effects, or apparent viscosity were found [39, 40].

The analysis of the response surfaces obtained by the experimental design, with apparent viscosity as response variable, shows the existence of a maximum point when agitation of 800 rpm, aeration of 1.30 vvm, yeast substrate concentration of 1.0 g/L, and calcium carbonate concentration of 1.0 g/L are applied. Based on the present study, it is evident that the use of experimental design has helped to locate the optimum levels of the most significant parameters for EPS apparent viscosity, with minimum effort and time.

Viscosimetric studies of solutions of EPS produced by *R. tropici* revealed a shear-thinning behavior, which allows flexibility in relation to its applicability, including that as a thickener. However, further studies on the rheological behavior of these substances must be carried out to characterize this material and its properties when in solution. Further chemical characterization of the EPS produced by *R. tropici* is necessary to determine its specific application. The use of *Rhizobium* cells for the production of EPS relies on their

easy manipulation and cultivation and the previous knowledge about their potential ability to produce EPS with enhanced rheological properties.

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## References

1. Kumar, C. G., Joo, H.-S., Choi, J.-W., Koo, Y.-M., & Chang, C. S. (2004). *Enzyme and Microbial Technology*, *34*, 673–681.
2. Rinaudo, M. (2001). *Food Hydrocolloids*, *15*, 433–440.
3. Renaud, M., Belgacem, M. N., & Rinaudo, M. (2005). Polymer, *46*, 12348–12358.
4. Copetti, G., Grassi, M., Lapasin, R., & Pricl, S. (1997). *Glycoconjugate Journal*, *14*, 951–961.
5. Chang, M.-Y., Tsai, G.-J., & Houng, J. Y. (2005). *Enzyme and Microbial Technology*, *38*, 407–414.
6. Liew, S. L., Ariff, A. B., Raha, A. R., & Ho, Y. W. (2005). *International Journal of Food Microbiology*, *102*, 137–142.
7. Margaritis, A., & Pace, G. W. (1985). *Comprehensive Biotechnology*, *49*(3), 1005–1004.
8. Sutherland, I. W. (1990). *Biotechnology of microbial exopolysaccharides*. New York: Cambridge University Press.
9. Breedveld, M. W., Zevenhuizen, L. P. T. M., Canter Cremers, H. C. J., & Zehnder, A. J. B. (1993). *Antoine van Leewenhoek*, *64*, 1–8.
10. Macció, D., Fabra, A., & Castro, S. (2002). *Soil Biology & Biochemistry*, *34*, 201–208.
11. Iagher, F., Reicher, F., & Ganter, J. L. M. S. (2002). *International Journal Macromolecules*, *31*, 9–17.
12. Bueno, S. M., & Garcia-Cruz, C. H. (2001). *Journal Food Engineering*, *50*, 41–46.
13. Gargallo, L. G., Radic, D. F., Abuin, E. S., & Lissi, E. G. (1987). in Propriedades Hidrodinâmicas - Macromoléculas en solución, chapter IV, Santiago, PNUD-UNESCO (CHI-84/006), pp. 92–106.
14. Bobbio, P. A., & Bobbio, F. O. (1992). *Química do Processamento de Alimentos à Química de Alimentos* (2nd ed.). São Paulo: Livraria Varela.
15. Paul, F., Morin, A., & Monsan, P. (1986). *Biotechnology Advenced*, *4*, 874–879.
16. Duta, F. P., França, F. P., Servulo, E. F. C., Lopes, L. M. A., Costa, A. C. A., & Barros, A. (2004). *Applied Biochemistry and Biotechnology*, *113–116*, 639–652.
17. Jordan, D. C. (1984). *Bergey's manual of systematic bacterilogy* (vol. I). Baltimore: Williams & Wilkins.
18. Abdel-Fattah, Y. R. (2002). *Biotechnology Letters*, *38*(14), 1217–1222.
19. Abdel-Fattah, Y. R., & Olama, Z. A. (2002). *Process Biochemistry*, *38*, 115–122.
20. Tanyildizi, M. S., Özer, D., & Elibol, M. (2005). *Process Biochemistry*, *40*, 2291–2296.
21. Chaves, N. A. (2000). Doctor thesis, CCS, Universidade Federal do Rio de Janeiro.
22. Barros Neto, B., Scarmínio, I., & Bruns, R. E. (1995). *Planejamento e otimização de experimentos*. Campinas: Editora Unicamp.
23. Calado, V., & Montgomery, D. C. (2003). *Planejamento de Experimentos utilizando o Statistica*. E-papers Serviços Editoriais Ltda.
24. Montgomery, D. C. (1997). *Design in analysis of experiments* (4th ed.). New York: Wiley.
25. Box, G. E. P., Hunter, W. G., & Hunter, J. S. (1978). *Statistics for experimenters*. New York: Wiley.
26. Benício, B. N., Scarminio, I. S., & Bruns, R. E. (2001). *Como fazer experimentos*. São Paulo: Editora da Unicamp.
27. Myers, R. H., & Montgomery, D. C. (2002). *Response surface methodology: Process and product optimization using designed experiments*. New York: Wiley.
28. Khanna, S., & Srivastava, A. S. (2005). *Process Biochemistry*, *40*, 2173–2182.
29. Lakshman, K., Rastogi, N. K., & Shamala, T. R. (2004). *Process Biochemistry*, *39*, 1977–1983.
30. Cazetta, M. L., Celligoi, M. A. P. C., Buzato, J. B., Scarmino, I. S., & da Silva, R. S. F. (2005). *Process Biochemistry*, *40*, 747–751.
31. Abdel-Fattah, Y. R., Saeed, H. M., Gohar, Y. M., & El-Baz, M. A. (2005). *Process Biochemistry*, *40*, 1707–1714.
32. Duta, F. P., França, F. P., & Lopes, L. M. A. (2006). *Electronic Journal of Biotechnology*, *9*, 391–399.
33. Yang, B. Y., Ding, Q., & Montgomery, R. (2003). *Carbohydrate Research*, *338*, 2673–2771.
34. Tavares, A. P. M. (2000). Master dissertation, Escola de Química, Universidade Federal do Rio de Janeiro.

35. Halász, A., & Lasztity, R. (1991). *Use of yeast biomass in food production*. Boca Raton: CRC.
36. Kollar, R., Sturduk, E., & Sajbidor, J. (1992). *Food Biotechnology*, *6*(3), 225–237.
37. Kelly, M. (1983). *In industrial enzymology: The application of enzymes in industry.* New York: Nature.
38. Cinquini-Dantas, L. A. (1992). Doctor these, Centre de Recherches sur les Macromolécules Végétales, CNRS.
39. Gil-Serrano, A., Gonzálea-Jiménez, I., Tejero-Mateo, P., Del Junco, A. S., Megias, M., & Romero-Vázquez, M. J. (1992). *Carbohydrate Research*, *225*, 169–174.
40. Canter Cremers, H. C. J., Stevens, K., Lugtenberg, B. J. J., Wiffelman, C. A., Batley, M., Redmond, J. W., et al. (1991). *Carbohydrate Research*, *218*, 185–200.
41. Dea, I. C. M., Mckinnon, A. A., & Rees, D. A. (1972). *Journal of Molecular Biology*, *68*, 153–172.
42. Lopes L. M. (1989). Master dissertation, Instituto de Macromoléculas Universidade Federal do Rio de Janeiro.
43. Severs, E. T. (1962). *Rheology of polymers, Chapter 1*. New York: Reinhold.
44. Mitchell, J. R. (1979). *Polisaccharides in food, Part 1, Chapter 4*. London: Butterworths.
45. Holzwarth, G., & Prestridge, E. B. (1977). *Science*, *197*, 757–759.
46. Norton, I. T., Goodall, D. M., Frangou, S. A., Morris, E. R., & Rees, D. A. (1984). *Journal of the Molecular Biology*, *175*, 371–394.
47. Morris, V. J., & Belton, P. S. (1982). *Progress in Food and Nutrition Science*, *6*, 55–66.
48. Launay, B., Cuvelier, G., & Martinez-Reyes, S. (1984). *Gums and stabilisers for the food industry 2, Chapter, 2*. Oxford: Pergamon.
49. Carrington, S., Odell, J., & Fisher, L. (1996). *Polymer*, *37*(13), 2871–2875.
50. Goycoolea, F. M., Richardson, R. K., Morris, E. R., & Gidley, M. J. (1995). *Macromolecules*, *28*, 8308–8320.