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Effect of Process Parameters on Production of a Biopolymer by *Rhizobium* sp.

Flávia Pereira Duta,[1] Francisca Pessôa De França,[*,1] Eliana Flávia Camporese Sérvulo,[1] Léa Maria De Almeida Lopes,[2] Antonio Carlos Augusto Da Costa,[3] and Ana Barros[4]

[1]Universidade Federal do Rio de Janeiro Departamento de Engenharia Bioquímica, Bloco E, Ilha do Fundão, Rio de Janeiro, RJ CEP: 21949-900 Brazil, and [2]Instituto de Macromoléculas Professora Eloisa Mano, Rio de Janeiro-RJ, Brazil, E-mail: fpfranca@eq.ufrj.br and [3]Universidade do Estado do Rio de Janeiro, Departamento de Tecnologia de Processos Bioquímicos, and [4]Universidade de Aveiro, Portugal

Abstract

The production of biopolymers by a *Rhizobium* strain was studied under batch and bioreactor conditions. The best viscosity levels were obtained under low mannitol concentrations as well as low agitation and aeration conditions. Infrared spectra indicated the presence of chemical groups characteristic of microbially produced biopolymers, including C = O and O-acetyl groups. Thermogravimetric analysis showed the characteristic degradation profiles of the exopolysaccharide produced (T_{onset} = 290°C). The experimental design showed that a low substrate concentration (10.0 g/L), and low aeration (0.2 vvm) and agitation (200 rpm) levels should be used. The maximum yield of the process was a Yp/s (g/g) of 0.19 ± 0.1, obtained under optimized conditions.

Index Entries: Biopolymer; exopolysaccharides; *Rhizobium* sp; viscosity; conversion factor.

Introduction

Polysaccharides constitute an important class of natural macromolecules present in all living organisms, with a wide range of functions, some

*Author to whom all correspondence and reprint requests should be addressed.

of them still under investigation. Polysaccharides are formed by the condensation of monosaccharides or their derivatives through glucosidic bonds. They are usually substances with a high molecular weight, reaching values up to 1 million. Polysaccharides differ from high molecular weight oligosaccharides, not only by the size of the molecule, but also by their common ability to combine during biosynthesis; this property contributes to the formation of chains with different species of monosaccharides, linked through glucosidic bonds under distinct configurations. Those polysaccharides can have linear, branched, or cyclic chains *(1,2)*.

Microbial polysaccharides constitute a specific class of biopolymers. These biopolymers are formed during the growth of the living organisms, and are thus, called natural polymers. Their synthesis usually involves enzymatic catalysis and an increase in the chain through polymerization reactions of the monomers, typically inside the cells, mediated by complete metabolic processes *(3,4)*.

Because of the diversity of their structural and physical properties, microbial polysaccharides are widely used in the food, pharmaceutical, chemical, and petroleum industries. These microbial gums can act as emulsifying agents, stabilization and ligand agents, gelifying agents, flocculating agents, and film-forming and lubrifying compounds *(1,5)*. The structure and composition of the microbial polysaccharides depend on several factors, such as the composition of the culture medium, carbon source, type of strain, and fermentation conditions (pH, temperature, oxygen concentration) *(6)*.

Most of the research on bacteria from the genus *Rhizobium* is conducted in the field of genetics and bacteria-host plant simbiotic interactions. Little is known about the production of extracellular polysaccharides produced by *Rhizobium* as well as their properties in solution; in particular, no studies have been conducted on the effect of substrate concentration, agitation, and aeration as relevant parameters to monitor in the process of exopolysaccharide (EPS) production. The present work aimed to determine the extent to which some variables affect the production of polysaccharides by *Rhizobium* sp.

Materials and Methods

Microorganism

The strain *Rhizobium* EQ1 was used. This strain was isolated from culture of the beans variety "Caupi" from an arid region of the northeast of Brazil. This strain was kept in agar-mannitol-yeast extract (YMA) for several months, and, after its characterization was cataloged, at the culture bank of the School of Chemistry from Federal University of Rio de Janeiro, under the code EQ1.

Culture Media

All culture media were autoclaved at 121°C for 20 min.

Medium for Maintenance of Culture

The microorganism was kept in modified YMA with the following composition: 10–50 g/L of mannitol, with the concentration changed according to the experimental design; 0.1 g/L of K_2HPO_4; 0.4 g/L of KH_2PO_4; 0.2 g/L of $MgSO_4 \cdot 7H_2O$; 0.1 g/L of NaCl; 0.4 g/L of yeast extract; 15 g/L of agar. The pH of the medium was adjusted to 7.0 *(7)*.

Medium for Preparation of Inoculum

For preparation of the inoculum, the YMA medium was used without the addition of agar.

Medium for Production of Polysaccharide

The production of extracellular polysaccharides was done using a culture medium similar to YMA, supplemented with manganese ions (0.12 g/L of $MnCl_2 \cdot 4H_2O$) and omitting agar. The pH of this medium was adjusted to 7.0 by the addition of NaOH (50%, w/v).

Maintenance of Microorganisms

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

Preparation of Inoculum

The inoculum was prepared from the stock culture in 500-mL Erlenmeyer flasks containing 100 mL of the corresponding medium. Incubation was performed in a rotary shaker at 200 rpm and 30 ± 1ºC for 48 h with the cells in the exponential phase of growth.

Production of EPS in Bioreactors

The experiments were conducted in a fermentor (Model BioFlow IV); New Brunswick Scientific, with a 20-L capacity. All process parameters were monitored on-line, with the help of the software AFS 3.0 (Advanced Fermentation Software, New Brunswick Scientific). The temperature (30 ± 1ºC) and pH value (7.0) were kept constant during the experiments. The parameters of substrate concentration, aeration, and agitation were chosen as the most significant ones for the experimental design. After selecting these parameters, experiments were conducted in duplicate for superior (+) and lower (–) levels of the experimental design, and triplicate for the central point (0). The inoculum was added at 1% of the initial working volume (10 L); thus, 1000 mL of the grown inoculum was used. The process was conducted for 48 h.

Qualitative and Quantitative Determinations During Fermentation Process

The determinations were made during the fermentation process and immediately after in the broth.

Purity of Culture

During the process, microscopic examinations were performed in laboratory preparations (with the addition of dyes) through the method of Gram, in order to detect possible contaminations in the medium.

Absolute Viscosity of Broth

Measurements of viscosity of the fermented broth were done in a Brookfield Synchro-Lectric, model LVT rheometer with the accessory Small Sample Adapter. Absolute viscosity was determined at various shear rates. The temperature was kept constant at 20°C.

Determination of Mannitol

For the quantitative determination of mannitol, the fermented broth was initially filtered through 0.2-µm Millipore membranes in order to remove the 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. Milli-Q water was used as the eluent and the elution rate was 0.8 mL/min. The final result was obtained through a detector-type Waters 410 model differential refractometer, and Waters 746 integrator-registrator (data module).

Extraction and Purification of EPS

The quantity of EPS produced after fermentation was determined through dry weight measurements. The fermentation broth was heated at 80° ± 1°C for 10 min, to ensure microbial inactivation. A filtration was then conducted to remove the cells. To precipitate the EPS, ethanol P.A. (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 a 0.2-µm Millipore membrane using a Gouche crucible previously weighed. The obtained product was dried at 80 ± 1°C until constant weight. All determinations were done in triplicate.

The biopolymer extracted from the fermented broth was purified through successive washings with 70, 80, and 90% (v/v) ethanol P.A., respectively. The biopolymer was dried by introducing nitrogen gas under controlled heating.

Molecular Characterization

Infrared Spectrometry

Infrared-spectrometry (IR) was used for the investigation of substituent acetyl groups in the molecules of the polysaccharides. A Fourier transform IR Perkin-Elmer spectrometer, Model 1720-X, was used. The

presence of the substituent acetyl groups is observed in 1700- and 1200-cm^{-1} peaks, owing to axial deformation of the carbonyl groups of esters and to assymetric axial deformation of the C-O-C bonds, respectively. KBr pastilles were prepared in a proportion of 2 mg of polysaccharide/200 mg of KBr.

Thermal Analysis Through Thermogravimetry

Thermogravimetric analysis can measure the amount and ratio of changes of mass in a material, as a function of temperature or time, in a controlled atmosphere. The measurements are used to determine the composition and thermal stability of a material. This technique is used to determine the purity, humidity, volatile content, and residues in polymeric materials *(8)*. To determine the thermal stability and humidity levels of the biopolymer synthesized by *Rhizobium* sp. EQ1 in a specific experimental condition, thermogravimetric analysis was conducted using DSC 2910 TA instruments. The loss of weight was registered from 25 to 700°C at a heating rate of 10°C/min. The analysis of the sample was conducted in a nitrogen atmosphere at a flow rate of 20 cm^3/min.

Experimental Design

This experimental design technique is widely used as a tool to verify the efficiency of several processes. In the present work, it was used for the purpose of obtaining information from the EPS production process; consequently, a reduction in the variability, as well as in operational costs can be expected. The choice of the variables (factors that affect the process), as well as the superior (+), lower (–), and central (0) levels used in the design, was defined from preliminary studies that defined the parameters as the most significant for the production of EPS. The selected variables were aeration, agitation, and initial substrate concentration (*see* Table 1).

This way, a factorial 2^k design is thus obtained, with a central point, in which k is the number of factors and 2 is the number of levels, resulting in $2^3 = 8$; the central point 1 is, done in duplicate, in a total of 11 experiments, according to Table 1.

The data obtained were processed using the software Statistica™,'99 for Windows, Version 5.5, produced by StatSoft. From the software, the values of the effects of each parameter were determined as well as their interactions.

Results

Viscosity Analysis

Table 1 presents the results obtained from the fermented broth in the different experimental conditions selected after 48 h. Determination of the viscosities indicated that at conditions below the control factors, higher viscosity values were obtained for the fermented broth; low values of substrate concentration, aeration, and agitation promoted higher viscosity values in the cultivation broth after 48 h of process.

Table 1
Control Factors and Levels Used in Matrix for Experimental Design 2^3 with Central Point, Viscosity Values for Fermented Broth, and Substrate/Product Yield ($Y_{P/S}$) at Distinct Conditions after 48 h at 20°C

	Factor			Apparent viscosity (cP)			
				Shear rate (s^{-1})			$Y_{P/S}$
Expt	X_1 (substrate, g/L)	X_2 (aeration, vvm)	X_3 (agitation, rpm)	20,400	10,200	4080	(g/g)
1	10 (–)	0.2 (–)	200 (–)	56.5 ± 1.2	42.5 ± 1.0	28.5 ± 1.2	0.19 ± 0.10
2	10 (–)	0.2 (–)	800 (+)	28.0 ± 1.0	***	***	0.13 ± 0.10
3	10 (–)	1.3 (+)	200 (–)	47.5 ± 0.8	3.5 ± 0.8	***	0.18 ± 0.10
4	10 (–)	1.3 (+)	800 (+)	35.0 ± 0.5	***	***	0.16 ± 0.10
5	50 (+)	0.2 (–)	200 (–)	25.0 ± 1.1	***	***	0.06 ± 0.10
6	50 (+)	0.2 (–)	800 (+)	25.0 ± 0.2	***	***	0.07 ± 0.11
7	50 (+)	1.3 (+)	200 (–)	7.5 ± 0.4	***	***	0.03 ± 0.30
8	50 (+)	1.3 (+)	800 (+)	8.5 ± 1.2	***	***	0.03 ± 0.22
9	30 (0)	0.8 (0)	500 (0)	31.5	***	***	0.06
10	30 (0)	0.8 (0)	500 (0)	32.0	***	***	0.06
11	30 (0)	0.8 (0)	500 (0)	32.5	***	***	0.06

***Not detected

On-line Monitoring

pH

During the on-line monitoring of the pH of the medium, no significant changes in the values of pH were observed in the time course of the process.

Concentration of Dissolved Oxygen

Monitoring of dissolved oxygen concentration indicated that in the first 12 h of the process, the concentration remained constant. Afterwards between 12 and 24 h of the process, a slight decrease in the concentration of oxygen was observed, which was kept constant up to 48 hours of monitoring.

Molecular Characterization

IR Spectrometry

Figure 1 presents the IR spectra of different samples of the EPS obtained at different conditions of the process. The polysaccharides produced in the fermented broth presented the same chemical composition. These spectra did not show significant differences in relation to the characteristic bands.

Around 3400 cm^{-1} a band could be observed characteristic of axial deformation of hydroxyl groups that participate in interactions of bridge-type hydrogen bonds. Close to 2900 cm^{-1} another band appeared characteristic of axial deformation of a C-H bond. At 1246 cm^{-1}, a band indicating an axial deformation of ester C-O bonds could also be detected *(9)*.

The characteristic bond for axial deformation of ester carbonyl (C=O), around 1730 cm^{-1}, was observed in the three samples of the biopolymer. The absorption of that band demonstrates the presence of substituent O-acetyl groups in the molecule of the biopolymer. In the 400- to 1400-cm^{-1} region, the differences between the biopolymers synthesized by *Rhizobium* sp could be clearly observed *(9)*.

Thermal Analysis Through Thermogravimetry

The variation in the total mass of the polymer registered between 25 and 700 °C is presented in Fig. 2. The thermogravimetric analysis of the EPSs produced are presented for both purified and nonpurified EPS from *Rhizobium* sp. EQ1.

Experimental Design

Table 1 presents the matrix from the experimental design and the results obtained for substrate/product yield ($Y_{P/S}$). After 48 h of process the maximum value for $Y_{P/S}$ was 0.19 (g/g), relatively low, considering biochemical processes involving polymerization reactions.

A direct analysis of the results in Table 1 cannot be made; the data presented do not enable verification of the significance of the parameters studied. The use of a Pareto chart is necessary. From the results of the conversion factor ($Y_{P/S}$), the main effects of the interactions were calculated

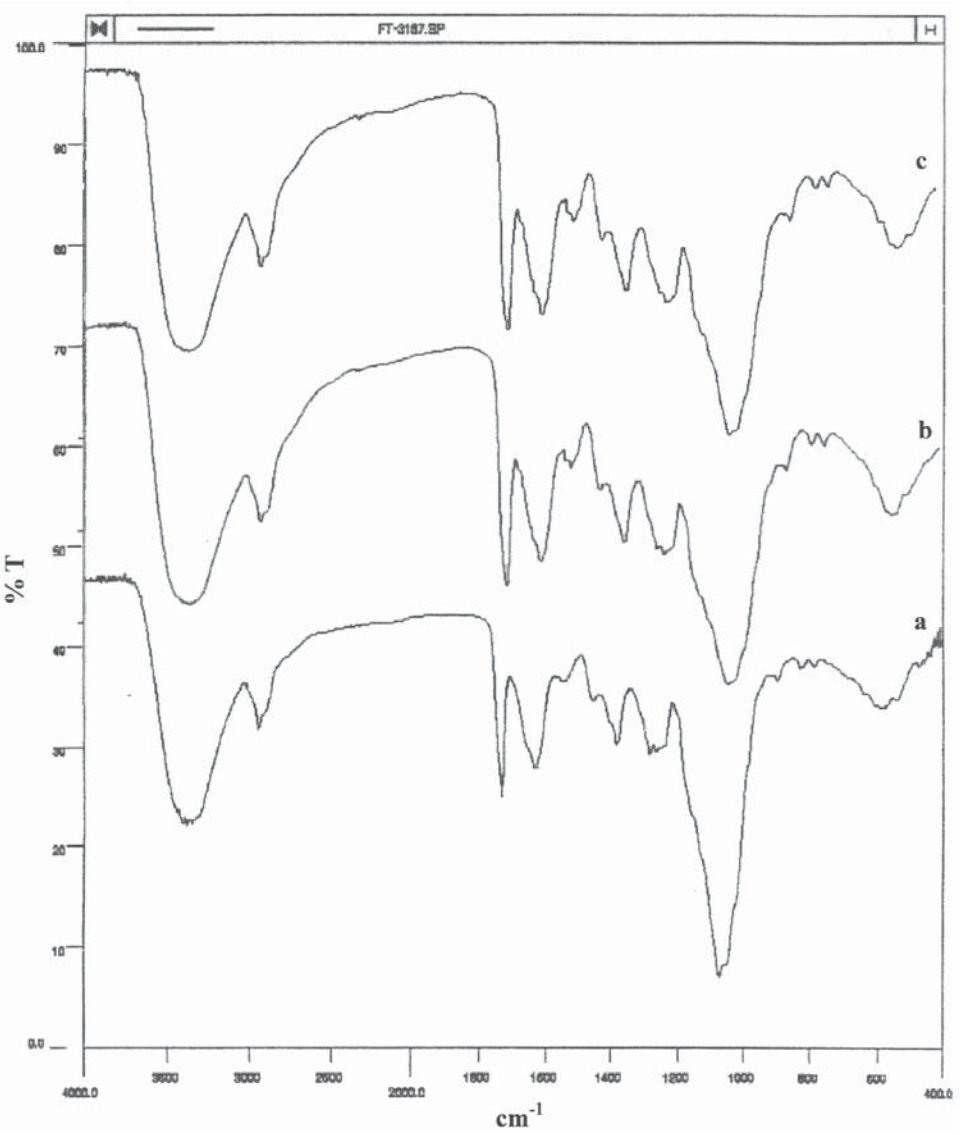

Fig. 1. IR spectra of samples of EPS produced by *Rhizobium* sp. EQ1 at different conditions: **(A)** expt. 1; **(B)** expt. 2; **(C)** expt. 4.

through use of the matrix from Table 1. Analysis of the main effects and interactions was done through a Pareto chart (*see* Fig. 3) from data obtained from analysis of variance, and surface graphs (Figs. 4 and 5).

The stippled line in the Pareto chart in Fig. 3 indicates the minimal magnitude of the statistically significant effect for a 95% confidence level. Values presented in the horizontal columns correspond to the values of the student's *t*-test for each factor studied. Figures 4 and 5 present a better visualization of the effect of the factors studied on $Y_{P/S}$. Analogously,

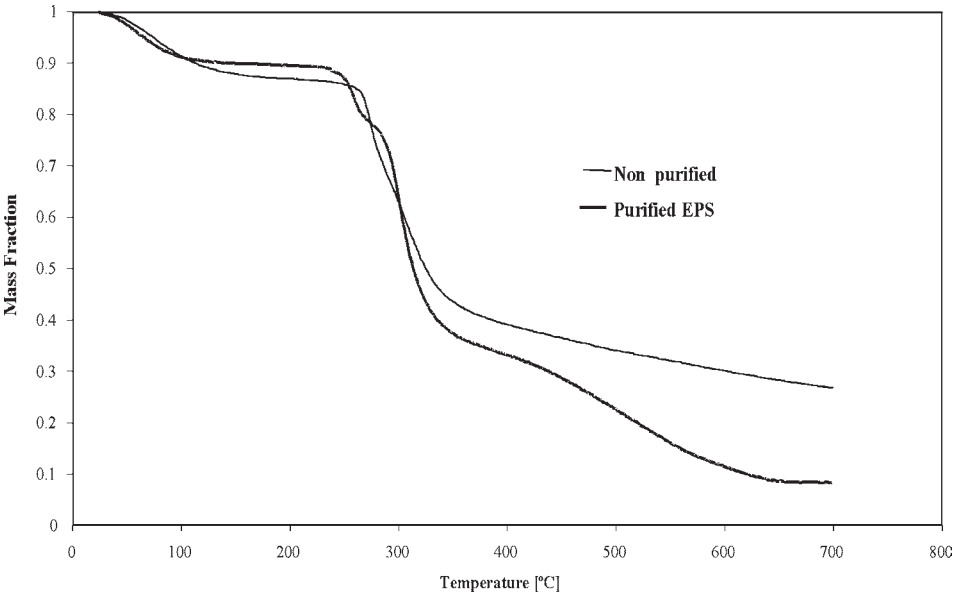

Fig. 2. Thermogravimetric curves obtained for purified and nonpurified EPS produced by *Rhizobium* sp. EQ1 (experiment 1).

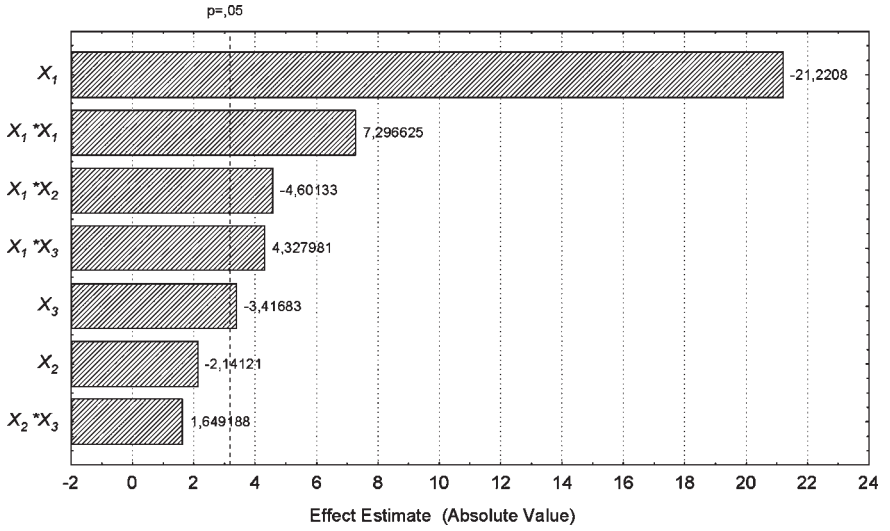

Fig. 3. Pareto chart: X_1, concentration of substrate; X_2, aeration; X_3, agitation.

Fig. 5 shows the effect of substrate concentration and agitation on substrate/EPS factor, $Y_{P/S}$. Figure 6 shows the correlation between experimental results and values predicted by the mathematical model generated by *Statistica*, indicating a good correlation between experimental and predicted values.

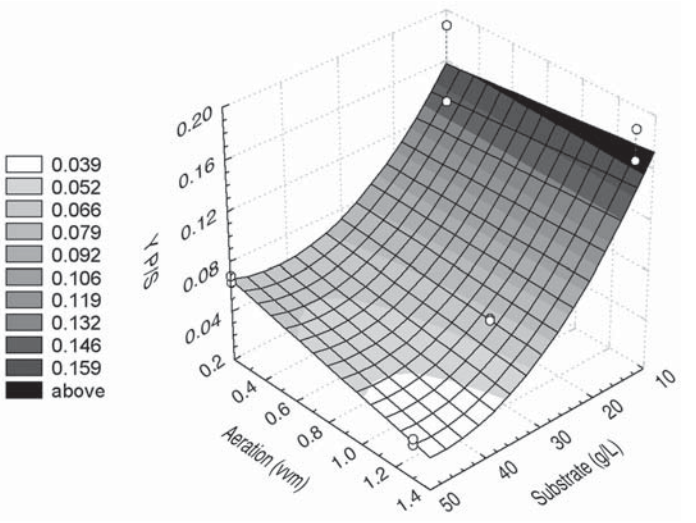

Fig. 4. Effect of X_1 and X_2 on substrate/EPS factor, $Y_{P/S.}$

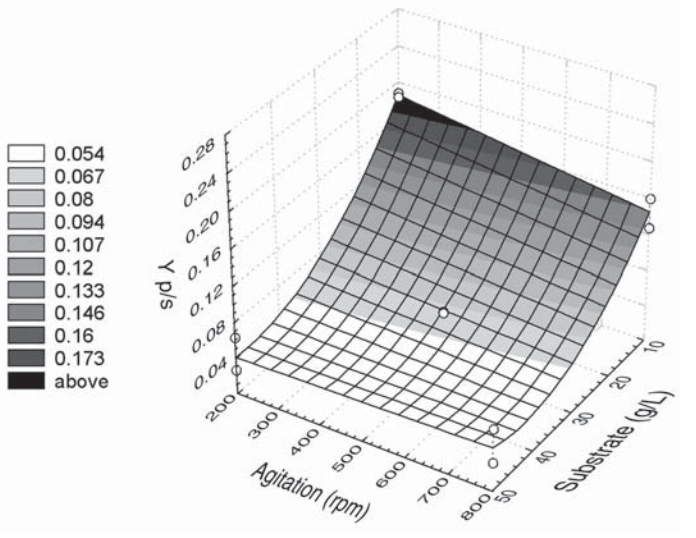

Fig. 5. Effect of X_1 and X_3 on substrate/EPS factor, $Y_{P/S.}$

Discussion

As described, production of the biopolymer was accompanied by the determination of the viscosity of the fermented broth. Viscosity measurements under different conditions described in the experimental design were done as a function of shear rate. In order to obtain suitable conditions that

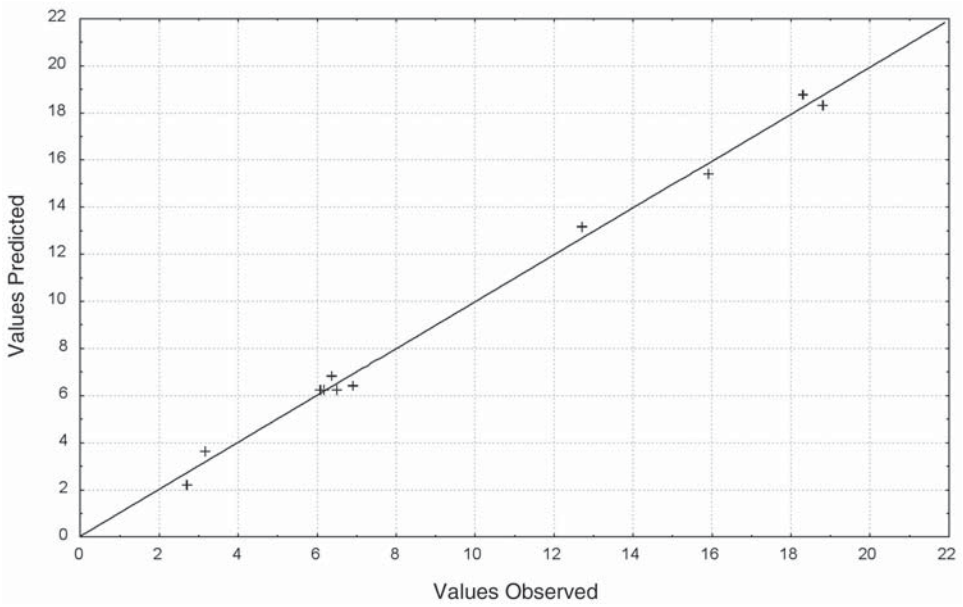

Fig. 6. Correlation between experimental values and values predicted by mathematical model.

could enhance the apparent viscosity of the medium corresponding to a higher production of extracellular polysaccharides. In some cases, the low values of viscosity found suggest that the production medium must be improved, in order to improve its nutrition value, thus enhancing EPS production by the strain.

pH was constant during the process, probably owing to the formation of phosphate-buffering substances. The results indicate that the product synthesized was neutral.

It is widely known that oxygen dissolution in a medium is highly dependent on the medium's viscosity. The decrease observed in the dissolved oxygen concentration is probably associated with the increase in the viscosity observed along the process. The two observations, the decrease in oxygen concentration and the increase in viscosity, were simultaneously observed during the production of EPS by *Rhizobium* sp. EQ1.

Zevenhuizen and Scholten-Koerselman *(10)* studied the carbohydrates on the surface of *Rhizobium* (β-1,2-glucans) and determined the nature of the glucosidic bonds by IR spectrometry in KBr pastilles based on the methodology proposed by Barker et al. *(11)*. This methodology distinguishes among various types of polymers, indicating the predominating type of bond present, as well as the existence of side chains in the molecule. The authors *(10)* observed that in the region between 960 and 730 cm^{-1}, IR spectra of several derivatives of pyranosidic rings could be related to some stereochemical characteristics of the molecule.

D-Glucopiranose derivatives presenting α-type bonds absorbed at 844 cm^{-1}. This characteristic band, named 2a type, was attributed to the deformation of equatorial $C_{(1)}$-H. D-Glucopiranose derivatives containing β bonds present an absorption band (2b type) at 891 ± 7 cm^{-1}, attributed to the deformation of equatorial $C_{(1)}$-H bond. Derivatives of α-D-galactopiranose and α-D-manopiranose present absorption bands (2a type) at 825 ± 11 and 833 ± 8 cm^{-1}, respectively.

Absorption from 2b type was observed at 895 ± 9 and 893 ± 6 cm^{-1} in α-D-galactopiranose and β-D-manopiranose derivatives, respectively. An absorption band (type 1) related to the vibration of a piranose ring in α-D-glucopiranose was observed (peak with a strong to moderate intensity at 917 ± 13 cm^{-1}). On the other hand, for β-D-glucopiranose a peak was generated at 920 ± 5 cm^{-1}.

A band close to 880 cm^{-1}, can be attributed to a β-glycosidic bond. Another band in the region of 846 cm^{-1} could indicate the presence of an α type involving galactose and/or mannose units. The principles of the method proposed by Barker et al. *(11)* proved to be a useful tool for the interpretation of IR spectra obtained from EPS from *Rhizobium* sp. EQ1.

The curves presented in Fig. 2 show the thermal degradation of the polysaccharide; a decomposition stage for both samples tested, with the initial temperature of degradation (T_{onset}) of 290°C, can be clearly observed. This fact implies that the material should not be submitted to temperature ranges close to 290°C, in order not to compromise the physical integrity of the material evaluated. Regarding the purified sample, there was a less intense decrease, probably associated with some degree of heterogeneity. This means that it is possible for aggregate and disperse types to exist, considering that the material contains only polymers.

The results showed that for the nonpurified sample, humidity levels were 14% and final residue was 27%, whereas for the purified sample those values were 10 and 8.5%, respectively.

The Pareto chart (*see* Fig. 3) showed that the substrate concentration was the most significant parameter affecting the process. The interaction $X_1 \cdot X_2$ and $X_1 \cdot X_3$ also presented a significant effect over the process, although less marked than the one caused by the substrate concentration. The effect of agitation on the conversion factor substrate/EPS ($Y_{P/S}$) showed a less significant action over the process, whereas the effect of aeration and agitation was clearly negligible.

From the analysis of surface graphs, it can be observed that the conversion factor substrate/EPS ($Y_{P/S}$) changed from 3.9 to 15.9%, representing an increase of 24.5% in the efficiency of the process. Smaller values of substrate concentration (X_1) increased in the conversion factor, $Y_{P/S}$, whereas aeration did not represent a significant effect.

From Fig. 6, it can be concluded that small values of X_1 and X_2 produced an increase in the conversion factor, changing from 5.4 to 17.3%, representing an increase of 31.2% in the efficiency of the process.

Considering the conversion factor as the response variable, the mathematical regression obtained indicated that 99.49% of the total variation around the mean could be explained by the regression at a 95% significance level; this demonstrates a good agreement between the mathematical model generated by *Statistica* indicating a good fitting of experimental data to the model.

The decrease in the conversion factor with the increase in substrate concentration was also observed by Stredansky et al. *(3)*, who compared the EPS production through solid-state fermentation with *Xanthomonas campestris, Rhizobium hedysari* and *Agrobacterium tumefaciens*. A comparison of submerged and solid-state fermentation indicated that the yield obtained in solid-state conditions was 2 to 4.7 times higher than for submerged fermentation. The polymer yields obtained from solid-state fermentation, as referred to impregnating liquid volumes, were as follows: 38.8 g/L of xanthan from *X. campestris*, 21.8 g/L of succinoglycan from *R. hedysar* and 20.3 g/L of succinoglycan from *A. tumefaciens* PT45.

Selbmann et al. *(12)* used starch as the source of carbon for the production of EPS with the fungal species *Sclerotium glucanicum* and *Botryosphaeria rhodina*. They observed a maximum EPS production of 30.6 (*B. rhodina*) and 19.8 g/L (*S. glucanicum*).

Further characterization of the EPS produced by *Rhizobium* sp. EQ1 is necessary in order to determine its specific application. The use of *Rhizobium* cells for the production of EPS relies on their easy manipulation, cultivation, and the previous knowledge about their potential ability to produce EPS. This work makes the unique contribution of the knowledge of the metabolic activity of these nitrogen-fixing bacteria for the production of EPS, which is still incipient in the published literature.

Acknowledgments

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