

Optimization of culture conditions for exopolysaccharides production in *Rhizobium* sp. using the response surface method

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Abbreviations: EPS: exopolysaccharides
HPLC: high performance liquid chromatography
YMA: yeast mannitol agar

The combined effects of the processing parameters for exopolysaccharides production by *Rhizobium* sp. was studied using the experimental design and response surface methodology. The experiments were carried out using a fermenter with 20 L capacity, as the reactor. All processing parameters were online monitored. The temperature [$(30 \pm 1)^\circ\text{C}$] and pH value (7.0 ± 0.1) were kept constant throughout the experimental time. As statistical tools, a complete 2^3 factorial planning with central point and response surface were used to study the interactions among three relevant variables of the fermentation process: calcium carbonate concentration, aeration and agitation. The processing parameters setup for reaching a maximum response for exopolysaccharides production was obtained when

applying the highest values for calcium carbonate concentration (1.1 g/L), aeration (1.3 vvm) and agitation (800 rpm). In addition, the combination of these optimum processing parameters yielded $Y_{P/S}$ (g/g) = 0.35.

Bacterial exopolysaccharides are extensively used as thickening and gelling agents in a wide range of industrial products and processes due to their structural and physical properties diversity (Copetti et al. 1997; Rinaudo, 2001). The structure, composition and viscosity of the microbial polysaccharides depend on several factors, such as the composition of the culture medium, carbon and nitrogen source, mineral salts, trace elements, type of strain, and fermentation conditions (pH, temperature, oxygen

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concentration, agitation) (Moreira et al. 1998; Weuster-Botz, 2000; Pinto et al. 2002; Duta et al. 2004).

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 bound protein peptidoglycan complexes in the outer membrane, as well as have a requirement for chemotaxis (Macció et al. 2002).

Table 1. Matrix of the factorial 2³ planning.

Experiments	X ₁	X ₂	X ₃
1	-1	-1	-1
2	+1	-1	-1
3	-1	+1	-1
4	+1	+1	-1
5	-1	-1	+1
6	+1	-1	+1
7	-1	+1	+1
8	+1	+1	+1
9	0	0	0
10	0	0	0
11	0	0	0

The reach of optimized fermentation conditions, particularly associated to physical and chemical parameters, is of primary and great importance for the development of any process, due to their impact upon its economics and practicability. The diversity of combinatory interactions among medium components, metabolism of cells and the large number of chemical requirements for processing

metabolic products, do not allow satisfactory detailed modelling. The one-dimensional search with successive changes on variables conditions is still employed, even though it is well accepted that it is practically impossible for the one-dimensional search to accomplish an appropriate optimum combination in a finite number of experiments. Single variable optimization methods are not only tedious, but can also lead to misinterpretation of results, especially taking into account that the interaction between different factors is overlooked (Abdel-Fattah et al. 2005). Statistical experimental designs have been used for many decades and can be adopted on several steps of an optimization strategy, such as for screening experiments or searching for the optimal conditions of a targeted response (Kim et al. 2005; Lee and Gilmore, 2005; Nawani and Kapadnis, 2005; Senthilkumar et al. 2005; Wang and Lu, 2005). Recently, the results analyzed by a statistical planned experiment are better acknowledged than those carried out by the traditional one-variable-at-a-time method. Some of the popular choices, applying statistical designs to bioprocessing, include the Plackett-Burman design (Liu et al. 2003; Wang and Lu, 2005) and response surface methodology with various designs (Abdel-Fattah, 2002; Abdel-Fattah and Olama, 2002; Tanyildizi et al. 2005). The response surface methodology is an empirical modelling system that assesses the relationship between a group of variables, which can be controlled experimentally, and the observed response. This methodology is applied mainly both in food science and in the optimization of fermentative processes. It is a 2-level factorial design, where contour plots are generated by linear or quadratic effects of key variables, and a model equation is derived, fitting the

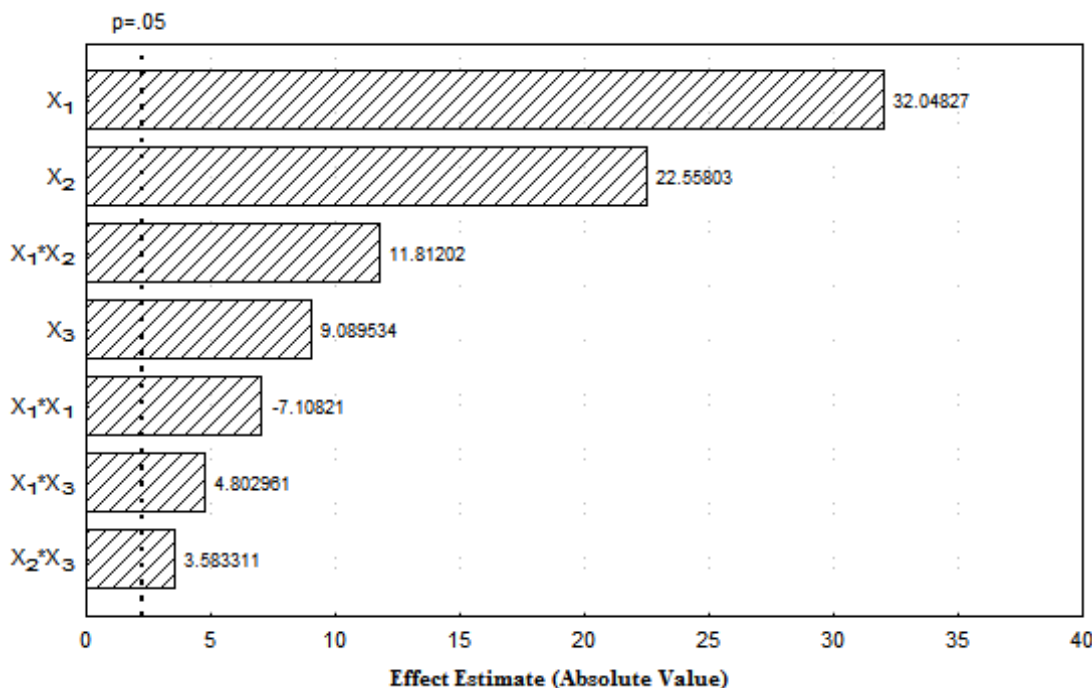


Figure 1. Pareto chart of standardized estimated effects of the different variables tested in the prospective experiment on exopolysaccharides production by *Rhizobium* sp. The variables tested were: calcium carbonate concentration (X₁), aeration (X₂) and agitation (X₃). The point at which the effect estimates were statistically significant (p = 0.050) is indicated by stippled line.

experimental data to the calculate system's optimal response (Lakshman et al. 2004; Cazetta et al. 2005; Khanna and Srivastava, 2005).

Fast growing rhizobia synthesize different extracellular polysaccharides, like acid exopolysaccharides (EPS) of high molecular weight (Zevenhuizen, 1986). Those microorganisms can also produce neutral glucans, formed by β -1,4 bonds, which are found as cellulosic microfibrils of low molecular weight (Zevenhuizen, 1986; Breedveld et al. 1990; Jain et al. 1990; Breedveld et al. 1993).

Table 2. The independent variables and their levels for the experimental design with central point.

Variables	Levels		
	+1	0	-1
X_1 – Calcium Carbonate Concentration (g/L)	1.0	0.8	0.5
X_2 – Aeration (vvm)	1.3	0.8	0.2
X_3 – Agitation (rpm)	800	500	200

Many rhizobia stains produced polysaccharides are not found freely dispersed in the medium, but attached upon the microbial cell as an amorphous viscous material. Among these, the curdlana homo-polymer is outstanding, in which the derived sulphated sites show anticoagulant and anti-thrombosis activities. Besides, they also present an inhibiting effect against the HIV-1 virus *in vitro* infection (Jagodzinski et al. 1994). Curdlana sulpho-alkylated sites present anti-tumour activity, inhibiting the development of the Sarcoma tumours 180 (Bohn, 1995).

The present study investigated the relationship among three variables, that is, calcium carbonate concentration, agitation, and aeration, in exopolysaccharide production by *Rhizobium* sp.

MATERIALS AND METHODS

Microorganism

The *Rhizobium* EQ1 strain was used in the experiments. This was isolated from a variety of beans culture, native from an arid region of the Northeast of Brazil, named as "Caupi". This strain has been kept in yeast mannitol agar (YMA), for several months, being catalogued, after its characterization, at the culture bank of the Biochemical Engineering Department from Federal University of Rio de Janeiro, under the code EQ1.

The microorganism was grown under $(30 \pm 1)^\circ\text{C}$ for 48 hrs, using laboratory tubes filled with YMA medium. After growth, the cultures were stored at $(5 \pm 1)^\circ\text{C}$.

Culture media

The microorganism stock culture was maintained in modified yeast mannitol agar extract, which presented the

following composition (g/L): mannitol (10.0); K_2HPO_4 (0.1); KH_2PO_4 (0.4); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.2); NaCl (0.1); yeast extract (0.4); agar (15.0). The medium pH was adjusted to 7.0 (Jordan, 1984).

The stock culture was used for preparing the inoculum, using 500 mL Erlenmeyer flasks, containing 100 mL of the YMA medium free from agar. Incubation was carried out in a rotary shaker at 200 rpm and $(30 \pm 1)^\circ\text{C}$, for 48 hrs, with the cells in the final of exponential growth phase.

The exopolysaccharides production assays were carried out using a similar culture medium, where the YMA medium was: supplemented with manganese ions ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 0.12 g/L); free from agar; and the calcium carbonate concentration ranged in accordance to the experimental design (CaCO_3 0.5-1.0 g/L). The pH of this medium was adjusted to 7.0 by the addition of NaOH (50% w/v). All culture media were sterilized at 121°C for 20 min.

The inoculation of the production medium was made in order to obtain an average cells concentration of 0.77 ± 0.02 mg/mL.

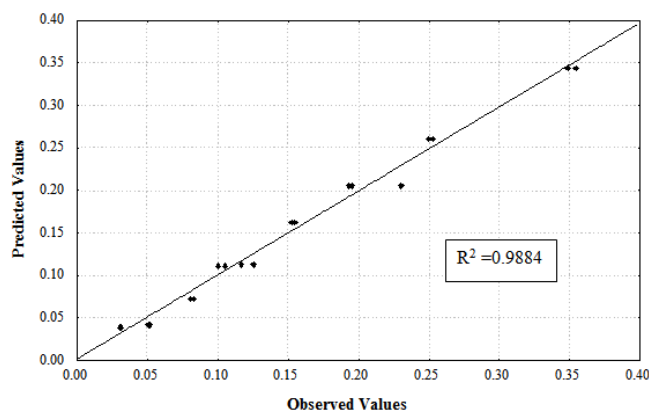


Figure 2. Parity plot showing the distribution of experimental versus predicted values by the mathematical model of the $Y_{p/s}$ values.

Production of exopolysaccharides in bioreactors

These experiments were conducted in a fermenter (Model BioFlow IV, New Brunswick Scientific), with 20 L capacity, equipped with disc impeller, oxygen and pH electrodes. The equipment also monitored temperature, agitation speed, gas purging flow rate, pumping rates, antifoam addition and the vessel level. All processing parameters were online monitored, with the aid of AFS 3.0 software (Advanced Fermentation Software, New Brunswick Scientific). The temperature $(30 \pm 1)^\circ\text{C}$ and pH value (7.0 ± 0.1) were kept constant during the experiments. Other parameters, like substrate concentration, aeration and agitation, were chosen as the most significant ones, considering the experimental design. After selecting those parameters, experiments were done in duplicate, for

Table 3. Results of the experimental design. Observed and predicted values.

Experiments	X ₁	X ₂	X ₃	Y _{p/s} (g/g) Observed	Y _{p/s} (g/g) Predicted
1	0.5	0.2	200	0.0305 ± 0.0007	0.0389
2	1.0	0.2	200	0.1210 ± 0.0056	0.1126
3	0.5	1.3	200	0.0815 ± 0.0011	0.0731
4	1.0	1.3	200	0.2513 ± 0.0020	0.2596
5	0.5	0.2	800	0.0507 ± 0.0005	0.0423
6	1.0	0.2	800	0.1534 ± 0.0019	0.1118
7	0.5	1.3	800	0.1023 ± 0.0031	0.1107
8	1.0	1.3	800	0.3515 ± 0.0037	0.3431
9	0.8	0.8	500	0.2301	0.2064
10	0.8	0.8	500	0.1934	
11	0.8	0.8	500	0.1956	

superior (+) and lower (-) levels of the experimental design, and in triplicate, for the central point (0).

For each experiment, 1000 mL of the inoculum was used, that is, 10% (v/v) of the initial working volume (10 L). The process was conducted throughout 48 hrs.

Analytical methods

During the process, microscopic examinations, using Gram method, were performed in order to detect possible microbial contaminations in the medium.

Prior for the quantitative determination of mannitol, the fermented broth was filtered through 0.2 µm Millipore membranes, in order to remove microbial cells. In the filtered fluid, the substrate was analyzed by high performance liquid chromatography (HPLC), in a Waters chromatograph, equipped with SHODEX SC1011 ion-exchange columns, at 75°C. Reagent water type I (ASTM, 2001) was used as eluent, and the elution rate applied was 0.8 mL/min.

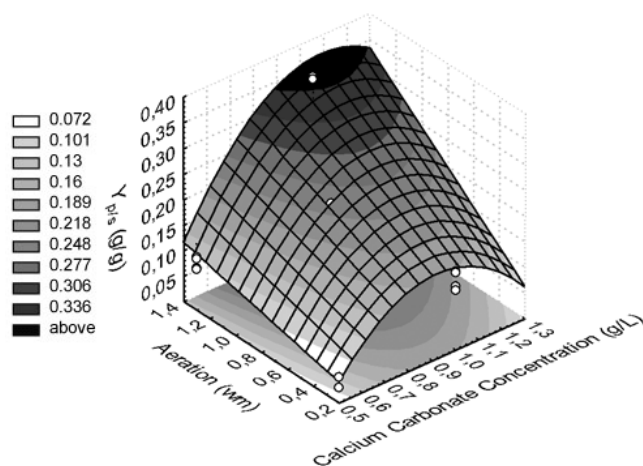


Figure 3. Effect of calcium carbonate concentration (X₁) and aeration (X₂) on the substrate/ exopolysaccharides factor, Y_{p/s}.

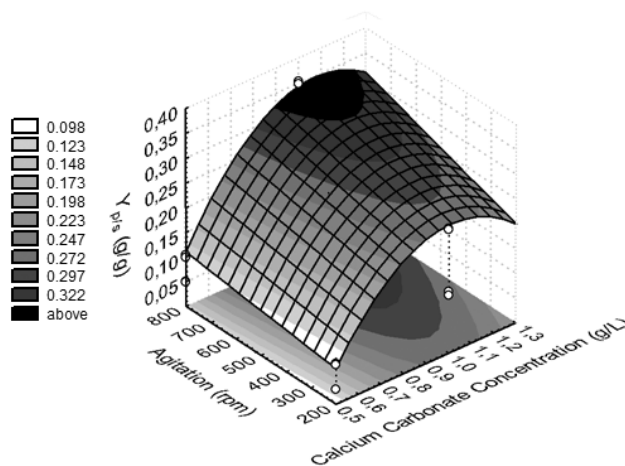


Figure 4. Effect of calcium carbonate concentration (X₁) and agitation (X₃) on the substrate/exopolysaccharides factor, Y_{p/s}.

The amount of fermented exopolysaccharide was determined by dry-weight measurements. The fermented broth was heated at (80 ± 1)°C, for 10 min, to ensure microbial inactivation. Afterwards, the microbial cells were removed by a filtration step. In order to precipitate the exopolysaccharides, a solution of ethanol P.A. and reagent water type I (ASTM, 2001) (3:1) was added to the fermented broth. After the exopolysaccharides total precipitation, the suspended material was filtered through 0.2 µm Millipore membrane, using Gouche crucible previously weighed. The obtained product was dried at (80 ± 1)°C until constant weight. All determinations were done in triplicate.

The exopolysaccharides extracted from the fermented broth was purified through successive washings with solutions of ethanol P.A. and reagent water type I (ASTM, 2001) at 70, 80 and 90% (v/v), respectively. The product was finally dried by a nitrogen gas purging flow, under controlled heating.

Experimental design and statistical analysis

This statistical technique is widely used as a tool to verify the efficacy of several processes. In the present work, it has been used for obtaining pieces of information about the

Table 4. Results of regression analysis and corresponding t and p-value of full second-order polynomial model for optimization of exopolysaccharides production of *Rhizobium* sp.

Factor	Regression coefficient	Standard deviation	t-statistic	p-value
β_0	-0.361612	0.053958	-6.70172	0.000054
β_1 (L)	1.147835	0.151855	7.55876	0.000019
β_{11} (Q)	-0.714687	0.100544	-7.10821	0.000033
β_2 (L)	-0.081844	0.015520	-5.27338	0.000361
β_3 (L)	-0.000081	0.000027	-2.96353	0.014202
β_1 (L) x β_2 (L)	0.205141	0.017367	11.81202	0.000000
β_1 (L) x β_3 (L)	0.000153	0.000032	4.80296	0.000720
β_2 (L) x β_3 (L)	0.000052	0.000014	3.58331	0.004984

Legend: L: linear; Q: quadratic; X_1 : Calcium Carbonate Concentration; X_2 : Aeration; X_3 : Agitation; β : terms coefficients.

exopolysaccharides production process; thus, a reduction in operational costs can be expected. A 2^3 factorial planning, in duplicate, with central point in triplicate, was used (Box et al. 1978; Neto et al. 1995). Additionally, each experiment was repeated once, around the central point neighbourhood, while the central point was repeated twice, leading to a set of 19 experiments (Table 1). Three central points were added to estimate the experimental error and to investigate the suitability of the proposed model. Table 2 lists the independent variables studied, X_1 calcium carbonate concentration (g/L), X_2 aeration (vvm), X_3 agitation (rpm). The manipulation responses of the input variables were evaluated as a function of the substrate conversion into exopolysaccharide, coded by $Y_{p/s}$ (g/g).

A mathematical model, describing the relationships among the process dependent variable, $Y_{p/s}$, and the independent variables in a second-order equation, was developed. Design-based experimental data were matched according to the following second-order polynomial equation:

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

[equation 1]

where, Y = substrate conversion into exopolysaccharides ($Y_{p/s}$),

β_0 = constant,

β_i = linear terms coefficients,

β_{ii} = quadratic terms coefficients,

β_{ij} = interaction coefficients.

All the calculations involved as well as the drawing of all three-dimensional surface (3D) have been obtained using the Statistica™ Software for Windows, Version 5.5 computer package, produced by Stat Soft. The model allowed the evaluation of the effects of linear, quadratic and combined effects of the independents variables upon the variable dependent variable. The Student's t -test was employed in order to check the statistical significance of the regression coefficients. The Fisher's F-test for analysis of variance (ANOVA) was performed on experimental data to evaluate the statistical significance of the model.

Three-dimensional surface (3D) plots were drawn to illustrate the main and interactive effects of the independent variables on exopolysaccharides production. The optimum values of the selected variables were obtained both by solving the regression equation and also by analyzing the response surface contour plots (Myers and Montgomery, 2002).

RESULTS AND DISCUSSION

The statistical technique is widely used as a tool for checking the efficiency of several processes. In the present work it has been used with the purpose of obtaining information about the exopolysaccharides production process; consequently, a reduction in the operational variability and a cut down in operational costs can be expected.

The experimental results ($Y_{p/s}$), associated to the processing set up of each independent variables are listed in Table 3. Using the designed experimental data presented in Table 3, the polynomial proposed model for $Y_{p/s}$ was regressed by only considering the significant terms. The expanded equation is shown below:

$$Y_{p/s} = -0.361612 + (1.147835 X_1) + (-0.714687 X_1^2) + (-0.081844 X_2) + (-0.000081 X_3) + (0.205141 X_1 X_2) + (0.000153 X_1 X_3) + (0.000052 X_2 X_3)$$

Besides the linear effect of the substrate/exopolysaccharides factor, $Y_{p/s}$, the response surface method also gives an insight about the parameters quadratic and combined effects. These analyses were done by using both Fisher's F- test and Student t -test statistical tools. The student t -test was used to determine the significance of the parameters regression coefficients. The p-values were used as a tool to check the significance of the interaction effects, which in turn may indicate the patterns of the interactions among the variables. In general, larger magnitudes of t and smaller of p , indicates that the corresponding coefficient term (Myers and Montgomery, 2002).

The regression coefficient, t and p values for all the linear,

quadratic and combined effects are given in the Table 4, with a 95% significance level. It was observed that the coefficients for the linear and quadratic effects of the factor calcium carbonate concentration parameter, the linear effect of the aeration parameter, the combined effects between calcium carbonate concentration with the aeration and agitation parameters ($p = 0.000$ for all) were highly significant. The value observed for the factor agitation was slightly less significant ($p = 0.014$).

The statistical significance of the ratio, between the of mean square variation, due to regression, and the mean square residual error, was tested using analysis of variance (ANOVA). ANOVA is a statistical technique that subdivides the total variation of a set of data into component associated to specific sources of variation for the purpose of testing hypotheses for the modelled parameters. According to the ANOVA (Table 5), the F-values for all regressions were high, what indicates that most of the variations on the response variable can be explained by the regression equation. The associated p-value is used to estimate whether F is large enough to indicate statistical significance. A p-value lower than 0.01 indicates that the model is considered to be statistically significant (Kim et al. 2003).

The p values of all of the regression were lower than 0.01. This means that at least one of the terms in the regression equation has a significant correlation with the response variable. The ANOVA table also shows a term for residual error, which measures the amount of variation in the response data left unexplained by the model. The type of the model, chosen to explain the relationship between the factors and response, is correct.

The analysis of variance (ANOVA), indicates that the second-order polynomial model (Equation 1) was highly significant and adequate to represent the actual relationship between the response and input variables, with very small p values ($p = 0.0000$).

Pareto chart (Figure 1) corroborates the data shown in

Table 5, and also enhances the understanding of this table. Figure 1 shows that all the linear terms of the model were significant for the set confidence level, as well as the quadratic term of X_1 variable (Table 5). It can also be seen that the interaction term among the three variables ($X_1X_2X_3$), was not statistically significant (Figure 1 and Table 5). Statistical data analysis shows that calcium carbonate concentration is the most important variable for the production process.

The matching quality, of the data obtained by the model proposed in Equation 1, was evaluated considering the correlation coefficient, R^2 , between the experimental and modelled data. The mathematical adjust of those values generated a $R^2 = 0.9884$, revealing that the model could not explain only 1.16% of the overall effects, showing that it is a robust statistical model.

Figure 2 shows the regression plot of the $Y_{p/s}$ experimental values against those predicted by Equation 1, revealing a linear mathematical relation among them. In addition, the mismatching analysis and further error terms found in Table 5, agree the adequacy of the predicted data, thus the reliability of the model proposed by Equation 1.

The 3D response surface plots described by the regression model were drawn to illustrate the effects of the independent variables, and combined effects of each independent variable upon the response variable. Figure 3 illustrates 3D response surface based on the $Y_{p/s}$ response against the variation of calcium carbonate concentration (X_1) and aeration (X_2) independent variables upon $Y_{p/s}$, while the third independent variable, agitation (X_3), was kept constant level (800 rpm). It can be observed that the maximum estimated $Y_{p/s}$ 0.3431 (g/g) was obtained using calcium carbonate concentration of 1.0 g/L and aeration of 1.3 vvm.

The data obtained by varying calcium carbonate concentration (X_1) and agitation (X_3), fixing aeration at 1.3 vvm, can be observed in Figure 4. The analysis of Figure 4 reveals that the maximum substrate conversion into

Table 5. Analysis of variance (ANOVA) for the fitted quadratic polynomial model for optimization of exopolysaccharides production of *Rhizobium* sp.

Factor	Sum of squares (SS)	Degrees of Freedom (DF)	Mean Square (MS)	F-value	p-value
X_1 (L)	0.093711	1	0.093711	1027.092	0.000000
X_1 (Q)	0.004610	1	0.004610	50.527	0.000033
X_2 (L)	0.046428	1	0.046428	508.865	0.000000
X_3 (L)	0.007538	1	0.007538	82.620	0.000004
X_1 (L) x X_2 (L)	0.012730	1	0.012730	139.524	0.000000
X_1 (L) x X_3 (L)	0.002105	1	0.002105	23.068	0.000720
X_2 (L) x X_3 (L)	0.001172	1	0.001172	12.840	0.004984
Lack of fit	0.001127	1	0.001127	12.353	0.005587
Pure error	0.000912	10	0.000091		
Total sum of squares	0.170333	18			

Legend: L: linear; Q: quadratic; X_1 : Calcium Carbonate Concentration; X_2 : Aeration; X_3 : Agitation.

exopolysaccharides was also obtained under the following condition: at calcium carbonate concentration of 1.0 g/L and agitation of 800 rpm.

Figure 3 and Figure 4 also show that an increase on agitation and aeration parameters promotes an increase on $Y_{p/s}$. For each combination of agitation and aeration values, the model generates a maximum value for the concentration of calcium carbonate. Thus, for the maximum values used in the experimental design, 1.3 vvm for the aeration and 800 rpm for agitation, the predicted value for of calcium carbonate concentration is 1.1 g/L.

The application of the regression model (Equation 1) for the substrate/exopolysaccharides factor, $Y_{p/s}$ was tested using calcium carbonate concentration of 1.1 g/L, agitation of 800 rpm and aeration of 1.3 vvm, with triplicate experiments. For this experimental condition, $Y_{p/s}$ mean value was of 0.3574 ± 0.008448 (g/g), which agrees with the predicted value, 0.3471 (g/g). This verification revealed a high accuracy of the model, that is, 97.12%, which is an evidence of the model validation, under the investigated conditions.

The process for exopolysaccharides production is carried out under aeration and agitation. The control of such parameters is of great importance for adequately conducting the fermentation process. According to Brock and Madigan (1991), for a rise in biomass from aerobic microorganisms, a vigorous aeration is required, what should be reached by forced aeration. This induced aeration is essential for getting high performance responses from the process, since oxygen is slightly soluble in water, not being quickly replaced by air diffusion, and worthwhile for microbial growth. Zevenhuizen (1986), using a mannitol-rich culture medium, has directed the polysaccharide synthesis towards exopolysaccharides by applying forced aeration.

The efficiency in conducting forced aeration is linked to agitation, which favours oxygen diffusion in the medium and its transfer to cells. Agitation also promotes a reduction in nutrient particles, favouring the nutrient homogenization in the culture medium, providing additionally a rise in mass transfer rates, this favouring microbial growth.

In the production medium used for obtaining biopolymers, several ions are added to propitiate the exopolysaccharides, and these shall be in appropriate amounts. The metallic ions perform catalytic and essential structural functions in proteins, being accumulated inside the cell by active transport (Macció et al. 2002).

The literature tells about the use of calcium carbonate to prevent the acidification of the bacterial broth (Macció et al. 2002). Jordan (1984) suggested the use of 4.0 g/L of calcium carbonate in the culture media, for controlling pH in the *Rhizobium* sp culture. O' Hara et al. (1989) previously reported that 1 and 2 mM of calcium was

necessary for cytoplasmic pH maintenance in *Rhizobium meliloti* acid-sensitive strain. The use of calcium ions can be intimately related to the stabilization of proteins involved in the exopolysaccharides synthesis process (Soto et al. 2004).

In agreement with the data presented in this study the yield income values of the product ($Y_{p/s}$), in a medium containing 1.0 g/L of calcium carbonate was 0.35 g/g, in average. These results are consistent with the biochemical processes involving polymerization reactions forming high-viscosity products.

CONCLUDING REMARKS

Most researches developed using *Rhizobium* genus bacteria are related to genetics and bacteria-host plant symbiotic interactions issues. Little is known about the production of extra-cellular polysaccharides by *Rhizobium*, as well as their properties in solution. In addition, no studies on monitoring medium composition, agitation and aeration parameters effects, on exopolysaccharides production, were found.

The analysis of the response surfaces obtained by the experimental design, with central and having $Y_{p/s}$ as response variable, shows the existence of a point of maximum production when agitation of 800 rpm, aeration of 1.3 vvm and calcium carbonate concentration of 1.1 g/L are applied.

The introduction of calcium carbonate, in the composition of the culture media, associated with high agitation and aeration, promoted a significant rise in the substrate/product yield ($Y_{p/s}$) (Duta et al. 2004).

Based on the present study, it is evident that the use of statistical optimization tools, response surface methodology, has helped to locate the optimum levels of the most significant parameters for exopolysaccharides production, with minimum effort and time.

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