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Research Article

Immobilization of *Erwinia* sp. D12 Cells in Alginate-Gelatin Matrix and Conversion of Sucrose into Isomaltulose Using Response Surface Methodology

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Isomaltulose is a noncariogenic reducing disaccharide and also a structural isomer of sucrose and is used by the food industry as a sucrose replacement. It is obtained through enzymatic conversion of microbial sucrose isomerase. An *Erwinia* sp. D12 strain is capable of converting sucrose into isomaltulose. The experimental design technique was used to study the influence of immobilization parameters on converting sucrose into isomaltulose in a batch process using shaken Erlenmeyer flasks. We assessed the effect of gelatin and transglutaminase addition on increasing the reticulation of granules of *Erwinia* sp. D12 cells immobilized in alginate. Independent parameters, sodium alginate concentration, cell mass concentration, $CaCl_2$ concentration, gelatin concentration, and transglutaminase concentration had all a significant effect ($P < 0.05$) on isomaltulose production. *Erwinia* sp. D12 cells immobilized in 3.0% (w/v) sodium alginate, 47.0% (w/v) cell mass, 0.3 $molL^{-1}$ $CaCl_2$, 1.7% (w/v) gelatin and 0.15% (w/v) transglutaminase presented sucrose conversion into isomaltulose, of around 50–60% in seven consecutive batches.

1. Introduction

In the food industry, sucrose is the most commonly used sweetener as a result of its physical and chemical and sensory characteristics. However, because of its high caloric value and cariogenic properties, alternative sweeteners are being studied.

In the past two decades, there has been an increasingly high interest in the production of isomaltulose (also known as Palatinose or Lylose). Isomaltulose is found naturally in small amounts in both honey and sugarcane and has been considered a promising sucrose replacement. Isomaltulose is a reducing disaccharide, a sucrose isomer obtained by microbial enzymatic conversion of sucrose [1, 2]. Isomaltulose has low cariogenic potential and reduced rates of hydrolysis and monosaccharide formation in the body, and it is therefore recommended for use in beverages and foods intended for diabetics and athletes [1, 3].

Many isomaltulose-derived products have potential industrial applications. Such items as intermediate disaccharides, polymers such as biodegradable detergents, and surfactants for industrial use may be obtained [4, 5]. Isomaltulose can also be applied to produce isomaltulose oligomers, which act as prebiotics, stimulating proliferation of intestinal microbiota bifidobacteria [6]. The main isomaltulose derivative is isomalt, a sugar alcohol obtained by hydrogenation resulting in an equimolar mixture of [6-*O*-(*α*-D-glucopyranosido)-D-sorbitol] and [1-*O*-(*α*-D-glucopyranosido)-D-mannitol], a noncariogenic compound with low caloric value.

Microbial conversion of sucrose into isomaltulose has attracted great commercial interest due to its complexity of chemical synthesis. Isomaltulose is currently produced in large scale by using stable continuous columns of immobilized cells [7]. Alginate is one of the most commonly used supports for immobilizing whole microbial cells [8], because

its use is simple and cheap; it is also a reproducible technique and gentle during the immobilization process [9, 10].

Some papers have recently reported the use of transglutaminase to immobilize cells and enzymes [11–15]. Transglutaminase is a microbial enzyme that catalyzes the formation of protein cross-links by creating a link between the carboxyl group of glutamine and the ε-amino group of lysine. In this paper, experimental design technique and response surface analysis were used to assess both immobilization parameters and effects of gelatin and transglutaminase addition to a calcium alginate immobilization process on the stability and conversion of sucrose into isomaltulose.

2. Materials and Methods

2.1. Microorganism and Culture Maintenance. The microorganism used in this study was the *Erwinia* sp. D12 strain that produces the intracellular enzyme sucrose isomerase, capable of converting sucrose into isomaltulose. The culture was grown in slant test tubes containing 6.0% sucrose (w/v), 4.0% peptone (w/v), 0.4% meat extract (w/v), and 2.0% agar (w/v), for 15 hours, at 30°C. After incubation, sterile Vaseline was added to the test tubes and the culture was kept at 5°C, with transfers every 2 months.

2.2. Erwinia sp. D12 Cell Production in a 6.6-Liter Fermenter. An *Erwinia* sp. D12 strain cell mass was obtained by fermenting the microorganism in a culture medium containing 150 g/L of sugarcane molasses, 20 g/L of corn steep liquor, and 15 g/L of yeast hydrolysate (Prodex Lac SD), a commercially available yeast extract; pH was adjusted to 7.5 [16].

2.3. Preinoculum and Fermentation. The 15-hour culture of that microorganism, as described previously, was inoculated into 250 mL Erlenmeyer flasks, containing 50 mL of the above-mentioned culture medium. These flasks were incubated in a shaker (model Series 25, New Brunswick Scientific, Edison, NJ, USA) at 200 rpm and kept at 30°C, for 15 hours. A 300 mL aliquot of preinoculum, prepared following the above description, and 3 mL of Dow Corning FG-10 (D'altomare Química, São Paulo, SP, Brazil), an antifoam agent, were aseptically added to a New Brunswick Bioflo IIc 6.6-liter fermenter (New Brunswick Scientific, Edison, NJ, USA) containing 2700 mL of that culture medium. Shaking and aeration were maintained at 200 rpm and 1 vvm. After 8 hours of fermentation at 27°C, that cell mass was retrieved by centrifuging at 9600 $\times$ *g* for 15 minutes (centrifuge model J2-21, Beckman Coulter, Inc, Fullerton, Calif, USA), at 5°C, and washing twice under aseptic conditions, using presterilized distilled water.

2.4. Converting Sucrose into Isomaltulose by Cells Immobilized in Calcium Alginate in a Batch Process. The experimental design technique was used to assess the effect of immobilization parameters on converting sucrose into isomaltulose. The parameters assessed were sodium alginate concentration, wet cell mass concentration, and $CaCl_2$ concentration. The granules containing immobilized cells, following the conditions described in Tables 1 and 4, were transferred to Erlenmeyer flasks containing a sucrose solution (granulated sugar) and maintained in a New Brunswick Scientific Series 25 shaker incubator (New Brunswick Scientific, Edison, NJ, USA). The sugar solutions in each flask were replaced with new sucrose solution samples after every 24 hours of reaction time. The conversion of sucrose into isomaltulose was analyzed as described above.

2.5. Cell Immobilization with Calcium Alginate. The *Erwinia* sp. D12 wet cell mass was obtained as described previously. For immobilization, a cell suspension of wet cells in sterilized distilled water was mixed with sterilized solution (autoclaved at 121°C for 15 minutes) of Sigma sodium alginate (Sigma Chemical Co., St. Louis, Mo, USA) at a 1 : 2 ratio (v : v). The mixture was then dripped with a MasterFlex L/S peristaltic pump (Cole-Parmer Instruments Co., Vernon Hills, Ill, USA) into a previously sterilized $CaCl_2$ solution in order to form small granules, which were kept immersed in the same $CaCl_2$ solution, at 5°C, for 12 hours. These granules were later washed with distilled water to remove excess $CaCl_2$. All steps were performed under aseptic conditions.

2.6. Determining Reducing Sugars. The reducing sugars formed were determined by the Somogyi [17] method, using glucose as a standard. A "blank" was used to adjust the spectrophotometer at 540 nm (DU-70, Beckman Coulter, Inc., Fullerton, Calif, USA), replacing 50 μL of the sample with 50 μL of distilled water in the reaction mixture.

2.7. Carbohydrate Analysis with Dionex Liquid Chromatography. Carbohydrate analysis was performed using a DIONEX DX-600 chromatograph (Dionex Corporation, 1228 Titan way Sunnyvale, Calif, USA) equipped with an IP25 isocratic pump and an ED50 gold electrochemical detector. Sugars were separated using a CarboPac PA 1 column (4 mm $\times$ 250 mm), a CarboPac PA 1 guard column (4 mm $\times$ 50 mm), and 250 mM sodium hydroxide solution as the mobile phase, with 1 mL/min flow, at 20°C. Carbohydrates were analyzed for retention time, by comparison with fructose, glucose, sucrose, and isomaltulose standards (Sigma Ultra, Sigma Chemical Co., St. Louis, Mo, USA).

2.8. Converting Sucrose into Isomaltulose by Cells Immobilized in Calcium Alginate and Gelatin-Transglutaminase during a Batch Process. Gelatin and transglutaminase concentrations were tested in cell immobilization, using experimental design technique and response surface analysis. These tests were performed as described previously.

2.9. Cell Immobilization with Calcium Alginate and Gelatin-Transglutaminase. A gelatin and alginate solution was prepared in shaken distilled water at 50°C. This solution was autoclaved at 121°C for 15 minutes and cooled to room temperature. After cooling, the Activa TG transglutaminase enzyme (Ajinomoto Interamericana Indústria e Comércio Ltda) was added to that gelatin and alginate solution to

Table 1: Coded and decoded 2^3 central composite rotatable design matrix (actual values in parentheses) for study of immobilization parameters of *Erwinia* sp. D12 cells in calcium alginate and their influence on converting sucrose into reducing sugars in a batch process.

Test	Parameter			Reducing sugar* (mg/mL)
	Sodium alginate* (%-w/v)	Cell mass (%-w/v)	$CaCl_2$ ($molL^{-1}$)	
1	−1 (2.00)	−1 (20.00)	−1 (0.10)	381.21
2	+1 (4.00)	−1 (20.00)	−1 (0.10)	391.36
3	−1 (2.00)	+1 (40.00)	−1 (0.10)	438.24
4	+1 (4.00)	+1 (40.00)	−1 (0.10)	422.91
5	−1 (2.00)	−1 (20.00)	+1 (0.50)	413.71
6	+1 (4.00)	−1 (20.00)	+1 (0.50)	505.75
7	−1 (2.00)	+1 (40.00)	+1 (0.50)	647.02
8	+1 (4.00)	+1 (40.00)	+1 (0.50)	688.42
9	−1.68 (1.30)	0 (30.00)	0 (0.30)	426.97
10	+1.68 (4.70)	0 (30.00)	0 (0.30)	477.83
11	0 (3.00)	−1.68 (13.00)	0 (0.30)	384.29
12	0 (3.00)	+1.68 (47.00)	0 (0.30)	812.93
13	0 (3.00)	0 (30.00)	−1.68 (0.04)	399.09
14	0 (3.00)	0 (30.00)	+1.68 (0.54)	582.20
15	0 (3.00)	0 (30.00)	0 (0.30)	419.69
16	0 (3.00)	0 (30.00)	0 (0.30)	441.84
17	0 (3.00)	0 (30.00)	0 (0.30)	447.11
18	0 (3.00)	0 (30.00)	0 (0.30)	441.39

* Total reducing sugar formed in seven batches.

Table 2: Main effects and interactions of immobilization parameters of *Erwinia* sp. D12 cells in calcium alginate and their influence on converting sucrose into reducing sugars in a batch process.

	Effect	Standard error	t (3)	P
(1) Sodium alginate (L)*	31.3234	6.5836	4.7578	0.0176
Sodium alginate (Q)	−3.6308	6.8480	−0.5302	0.6327
(2) Cell mass (L)*	179.5066	6.5836	27.2655	0.0001
Cell mass (Q)*	99.9771	6.8480	14.5996	0.0007
(3) $CaCl_2$ (L)*	136.1412	6.5836	20.6787	0.0002
$CaCl_2$ (Q)*	23.4725	6.8480	3.4277	0.0416
(1)L × (2)L	−19.0278	8.5982	−2.2130	0.1138
(1)L × (3)L*	34.6544	8.5982	4.0304	0.0275
(2)L × (3)L*	81.8471	8.5982	9.5191	0.0025

* Statistically significant parameters at a 95% confidence level.
L: linear parameter; Q: quadratic parameter.

increase reticulation of the alginate gel. The cell suspension and gelatin+alginate+transglutaminase solution mixture, at a 1 : 2 (v : v) ratio, was dripped in $CaCl_2$ with a MasterFlex L/S peristaltic pump, in order to form small granules, which were kept immersed in the same solution at 5°C, for 12 hours. These granules were later washed with distilled water to remove excess $CaCl_2$. All steps were performed under aseptic conditions. Conversion of sucrose into isomaltulose was analyzed as described previously.

3. Results and Discussion

The conversion of sucrose into isomaltulose in a batch process by *Erwinia* sp. D12 immobilized cells was assessed using experimental design technique. This study showed that the independent parameters assessed had a significant impact on isomaltulose stability and conversion.

3.1. Converting Sucrose into Isomaltulose by Cells Immobilized in Alginate during a Batch Process. The parameters assessed in both immobilization and conversion of sucrose into isomaltulose were sodium alginate concentration, wet cell mass concentration, and $CaCl_2$ concentration. These tests were performed in 250 mL Erlenmeyer flasks and kept in an incubator, shaken at 100 rpm and at 30°C. Each flask contained 50 mL of 35% sucrose solution (w/v) and 10 g of granules containing immobilized cells. The sucrose solution in the flasks was replaced with a new 35% sucrose solution (w/v) every 24 hours. The granules containing immobilized cells were reused for 7 batches. Samples containing such

Table 3: Analysis of variance in the study of immobilization parameters of *Erwinia* sp. D12 cells in calcium alginate and their influence on converting sucrose into reducing sugars in a batch process.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F test
Regression	227670,51	7	32524,36	17,63
Residuals	18444,10	10	1844,41	
Lack of Fit	18000,53	7		
Pure Error	443,57	3		
Total	246114,60	17		

Coefficient of determination: $R^2 = 0.92$; $F_{0.95;7;10} = 3.014$.

reducing sugars as isomaltulose, trehalose, glucose, and fructose were analyzed using the Somogyi-Nelson method [17].

A 2^3 central composite rotatable design (CCRD-2^3) was used to assess the following independent parameters: sodium alginate concentration, wet cell mass concentration and $CaCl_2$ concentration. Table 1 presents the CCRD-2^3 matrix of 18 tests, including 8 tests for the complete factorial design 2^3 (± 1), 8 tests for the axial points ($\alpha = \pm 1.68$), the central point quadruplicate (0), and the reducing sugars (isomaltulose, trehalose, glucose and fructose) as the dependent parameter as well as the levels studied with their decoded values. Table 1 shows the total of sucrose conversion into isomaltulose in each of the 18 tests, reusing those immobilized cells in 7 batches. It was found that most tests presented high conversion in the batches. Those granules containing immobilized cells in tests 7, 8, 12, and 14 were more stable in comparison with the other tests and 647.02 mg/mL, 688.42 mg/mL, 812.93 mg/mL, and 582.20 mg/mL were obtained, respectively.

Table 2 presents effects of independent parameters on converting sucrose into reducing sugars. Regarding the main effects, it was observed that the independent parameters, sodium alginate (L), cell mass (L; Q), and $CaCl_2$ (L; Q) had positive and significant effects within the range studied, with a 95% ($P < 0.05$) confidence level. Only the interaction between sodium alginate and cell mass did not present any significant effect in the levels studied. These results indicate that increasing the concentrations of those independent parameters within the studied range would result in an increased conversion of sucrose into reducing sugars. It was observed that the interactions between sodium alginate and $CaCl_2$ and also between cell mass and $CaCl_2$ had positive effects, indicating some synergism between those parameters.

Table 2 also presents the t and P values used to create the quadratic polynomial equation of a sucrose conversion into reducing sugars based on those studied parameters. The P values were used to check the significance of each coefficient and also to indicate how important each independent parameter or interaction between parameters in the equation result is. The greater the t value and the lower the P value, the greater the significance of the coefficient, as observed in Table 2, where only sodium alginate (Q) and interactions between sodium alginate and cell mass and also between cell mass and $CaCl_2$ were not significant at a 95% confidence level ($P < 0.05$). Cell mass and $CaCl_2$ presented the strongest effects and regression coefficient values, indicating a greater influence of such parameters on the conversion of sucrose into reducing sugars.

The analysis of variance (ANOVA) is shown in Table 3. The correlation measurements used to estimate that equation were the correlation coefficient (R) and the coefficient of determination (R^2). The closer R is to one [18], the stronger the correlation between the equation-predicted conversion values and the observed values. A 0.96 value of R was obtained, indicating a satisfactory correlation. A determination coefficient of 0.92 was obtained, indicating that only 8% of the total variation in responses obtained is not explained by the equation. The F value obtained from ANOVA was 17.63 (5.61 times greater than the value of $F_{\text{tabulated}} = F_{0;95;7;10} = 3.14$), indicating that the equation to convert sucrose into reducing sugars may be considered statistically significant at a 95% confidence level. The quadratic polynomial equation (1) was obtained after these studied parameters were validated. The statistically non significant parameters were eliminated from the equation and added to lack of fit

$$\begin{aligned} y = {} & 439.14 + 15.66x_1^2 + 49.99x_2^2 \\ & + 89.75x_2 + 11.74x_3^2 + 68.07x_3 \\ & + 17.33x_1x_3 + 40.92x_2x_3, \end{aligned} \tag{1}$$

where x_1, x_2, and x_3 are sodium alginate concentration, cell mass concentration, and $CaCl_2$ concentration, respectively, and y refers to the dependent parameter, reducing sugars (%).

Figures 1(a) and 1(b) were created based on that equation. After analyzing these figures, it can be verified that the concentration of sodium alginate in which the greatest conversion of sucrose into isomaltulose would be obtained was above 2.5% (w/v). Regarding the $CaCl_2$ variable, a concentration above 0.50 M would be beneficial for cell immobilization, resulting in greater conversion into reducing sugars. Figure 1(b) shows that a cell mass concentration above 40% (w/v) would increase conversion by *Erwinia* sp. D12 cells immobilized in calcium alginate in a batch process.

To confirm those results, a second test was performed with parameters previously used from tests 7, 8, 12, and 14, which achieved the best results in converting sucrose into reducing sugars from Table 1 (2^3 central composite rotatable design matrix). A test designated as A (alginate 4.7% (w/v), cell mass 47.0% (w/v), and $CaCl_2$ (w/v) solution

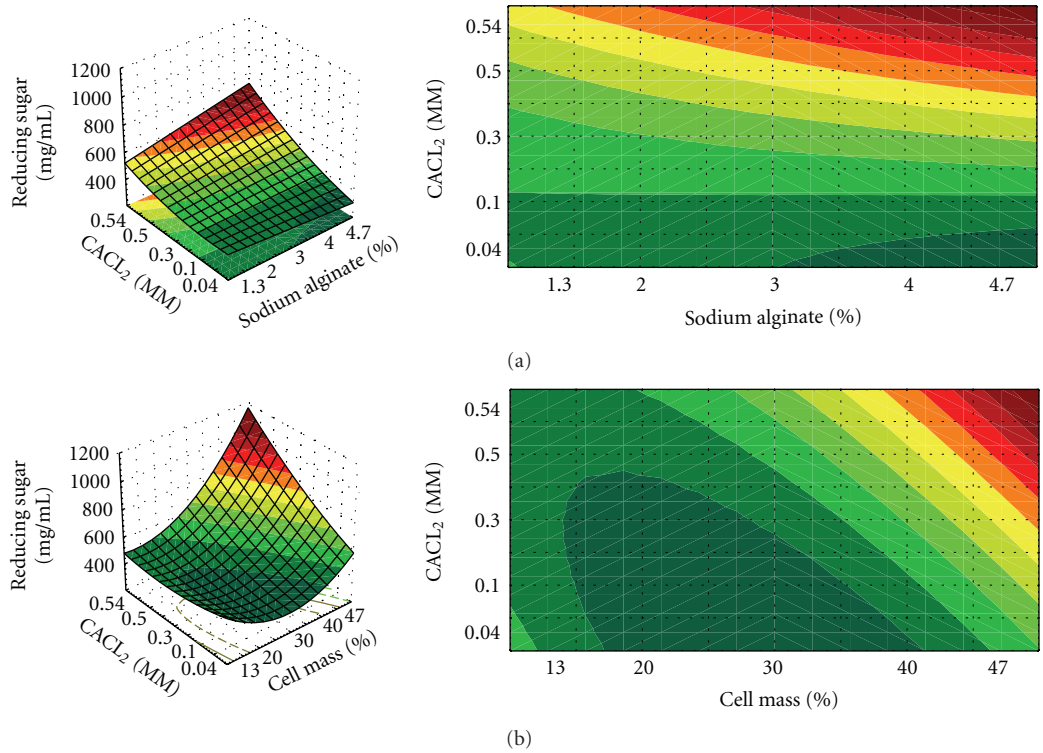

Figure 1: Response surfaces and isocurves in a study of immobilization parameters of *Erwinia* sp. D12 cells in calcium alginate and their influence on converting sucrose into reducing sugars in a batch process: (a) as a function of sodium alginate and $CaCl_2$ concentrations; (b) as a function of cell mass and $CaCl_2$ concentrations.

Table 4: Coded and decoded 2^2 central composite rotatable design matrix (actual values in parentheses) for a study of gelatin and transglutaminase concentrations when immobilizing *Erwinia* sp. D12 cells in calcium alginate and influence on converting sucrose into isomaltulose in a batch process.

Test	Parameter		
	Gelatin (%-w/v)	Transglutaminase (%−w/v)	Isomaltulose* (%)
1	−1 (0.30)	−1 (0.15)	63.07
2	+1 (1.70)	−1 (0.15)	62.86
3	−1 (0.30)	+1 (0.85)	62.37
4	+1 (1.70)	+1 (0.85)	59.52
5	−1.41 (0)	0 (0.50)	58.65
6	+1.41 (2.00)	0 (0.50)	59.16
7	0 (1.00)	−1.41 (0)	60.86
8	0 (1.00)	+1.41 (1.00)	59.24
9	0 (1.00)	0 (0.50)	56.75
10	0 (1.00)	0 (0.50)	57.58
11	0 (1.00)	0 (0.50)	56.75
12	0 (1.00)	0 (0.50)	57.89

*Values refer to the mean rate of sucrose conversion into isomaltulose in 5 batches.

0.54 $molL^{-1}$) was included to represent the immobilization parameters obtained by (1) and Figures 1(a) and 1(b), by which the best cell immobilization and isomaltulose conversion results would be obtained in a batch process. These tests were performed in duplicate 250 mL Erlenmeyer flasks and kept in a shaker incubator, at 100 rpm and 30°C, as described above.

Figure 2 shows the values of sucrose conversion into reducing sugars by *Erwinia* sp. D12 immobilized cells used in 6 consecutive batches. Tests A and 7 achieved the best conversion and cell stability results. Parameters for test 7 (3.0% sodium alginate concentration (w/v), 47.0% cell mass concentration (w/v), and 0.3 $molL^{-1}$ $CaCl_2$ concentration) were chosen for cell immobilization and for continuous

Table 5: Main effects and interactions of gelatin and transglutaminase concentrations when immobilizing *Erwinia* sp. D12 cells in calcium alginate, and influence on converting sucrose into isomaltulose in a batch process.

	Effect	Standard Error	t (3)	P
(1) Gelatin (L)	−0.5894	0.4131	−1.4269	0.2489
Gelatin (Q)*	2.9157	0.4631	6.2965	0.0081
(2) Transglutaminase (L)*	−1.5830	0.4131	−3.8321	0.0313
Transglutaminase (Q)*	4.0698	0.4631	8.7888	0.0031
(1) L × (2)L	−1.3174	0.5833	−2.2587	0.1091

* Statistically significant parameters at a 95% confidence level.
L: linear parameter; Q: quadratic parameter.

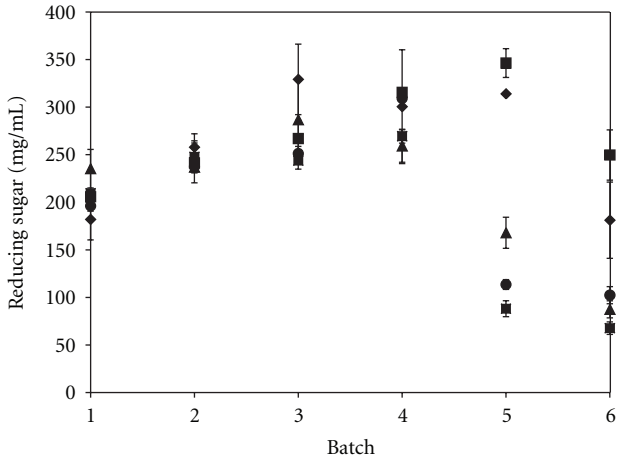

Figure 2: Converting sucrose into reducing sugars in a study of the effect of immobilization parameters of *Erwinia* sp. D12 cells immobilized in calcium alginate, in a batch process: ♦ Test A; ■ Test 7; ▲ Test 8; ● Test 12; ∗ Test 14.

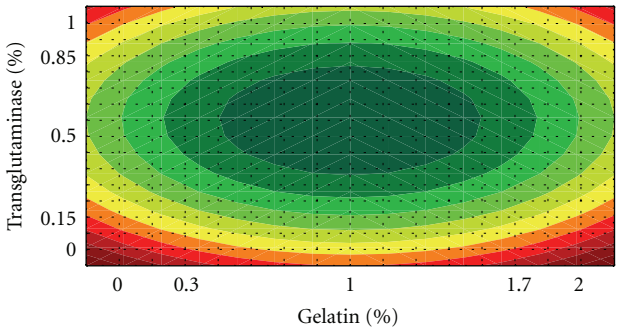

Figure 3: Isocurve in a study of the effects of gelatin and transglutaminase concentration on immobilizing *Erwinia* sp. D12 cells in calcium alginate and its influence on converting sucrose into isomaltulose in a batch process.

testing, as a result of the need for lower sodium alginate and $CaCl_2$ concentrations and, consequently, a lower immobilization process cost.

3.2. Converting Sucrose into Isomaltulose by Cells Immobilized in Calcium Alginate and Gelatin-Transglutaminase in a Batch Process. Effects of adding gelatin and transglutaminase

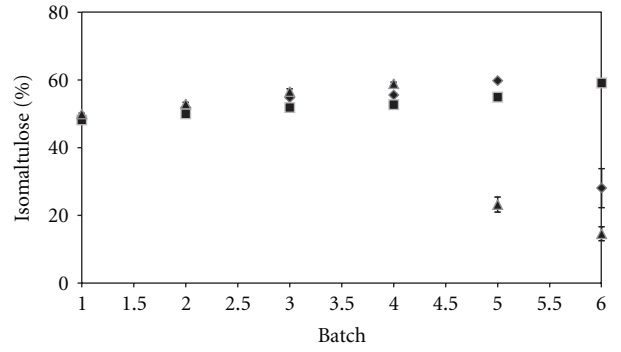

Figure 4: Converting sucrose into isomaltulose by *Erwinia* sp. D12 cells immobilized in calcium alginate with gelatin and transglutaminase, in a batch process: ♦ Test 1; ■ Test 2; ▲ Test 7.

were assessed when converting sucrose into isomaltulose by *Erwinia* sp. D12 cells immobilized in calcium alginate. Tests were performed in shaken Erlenmeyer flasks in a batch process. The *Erwinia* sp. D12 cell mass used in these tests was obtained as described previously. The immobilization process for a mixture containing both cell mass suspension and alginate solution plus gelatin and transglutaminase was performed as described previously.

A 2^2 central composite rotatable design (CCRD-2^2) was used to assess effects of a gelatin and transglutaminase concentration on increased reticulation of the granules of cells immobilized in calcium alginate and on conversion of sucrose into isomaltulose. Table 4 presents the CCRD-2^2 matrix of 12 tests, including 4 tests for a complete factorial 2^2 (±1), 4 tests for axial points (α = ±1.41), the central point quadruplicate (0), and the dependent parameter, isomaltulose (%), as well as the studied levels with their decoded values.

These tests were performed in 250 mL Erlenmeyer flasks and kept in a shaker incubator at 75 rpm and 25°C. Five grams of granules containing immobilized cells in 25 mL of 35% sucrose solution (w/v) were added to each flask. This sucrose solution in those flasks was replaced with a new 35% sucrose solution (w/v) every 24 hours. Granules containing immobilized cells were reused for 5 batches. Carbohydrate analysis was performed using a DIONEX DX-600 chromatograph.

Table 6: Analysis of variance in a study of gelatin and transglutaminase concentrations when immobilizing *Erwinia* sp. D12 cells in calcium alginate and their influence on converting sucrose into isomaltulose in a batch process.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F test
Regression	44.7670	3	14.9223	10.2996
Residuals	11.5906	8	1.4488	
Lack of fit	10.5699	5		
Pure error	1.0207	3		
Total	56.3575	11		

Correlation coefficient: $R = 0.83$; $F_{0.95;3;8} = 4.07$.

The main effects and parameter interactions that were studied are found in Table 5. Gelatin (Q) and transglutaminase (Q) significantly affected ($P < 0.05$) the conversion of sucrose into isomaltulose; effects were positive, suggesting that their increased concentration within the range studied would have resulted in increased isomaltulose conversion.

Table 6 shows the analysis of variance (ANOVA) to convert sucrose into isomaltulose. The equation obtained can be considered predictable and significant at a 95% confidence level, as shown by the F-test, in which the F value obtained in our tests ($F_{\text{experimental}} = 10.30$) was greater than $F_{\text{tabulated}} = 4.07$. Fit of the equation was verified by the correlation coefficient ($R = 0.83$). Pure error was 1.02, a low value, indicating good reproducibility of the obtained values. These results were sufficient and satisfactory to obtain an equation representing the actual relationship between the parameters studied and the conversion of sucrose into isomaltulose.

After ANOVA and validation of the studied parameters, the quadratic polynomial equation representing isomaltulose production by cells immobilized in calcium alginate plus gelatin and transglutaminase was used to create a response surface and isocurve (Figure 3). Equation (2) predicts the conversion of sucrose into isomaltulose within the studied parameter range:

$$y = 57.23 + 1.48x_1^2 + 2.03x_2^2 - 0.79x_2, \quad (2)$$

where x_1 and x_2 are gelatin and transglutaminase concentrations, respectively, and y is the dependent parameter, isomaltulose (%).

Both response surface and contour curve are represented in Figure 3. Note that an increased rate of sucrose conversion into isomaltulose by immobilized cells would take place at the (maximum and minimum) extremities of gelatin and transglutaminase concentration, within the studied range.

Three duplicate tests were performed, with concentrations used in tests 1, 2, and 7 of CCRD-2^2, in order to confirm the best gelatin and transglutaminase concentrations for immobilization of *Erwinia* sp. D12 cells in calcium alginate, in a batch process. The chosen parameter values were based on responses presented in Table 4 and Figure 3, in which greater isomaltulose conversion was obtained when lower transglutaminase concentrations were used. New tests with high transglutaminase concentration were not performed due to the high cost of this enzyme. Tests were performed as described above, and cells were reused in six batches.

Figure 4 shows the conversion of sucrose into isomaltulose by *Erwinia* sp. D12 cells immobilized in calcium alginate plus gelatin and transglutaminase, in a batch process at 25°C. The sucrose conversions into isomaltulose obtained in all three tests were similar, around 50–60%. However, from the sixth batch on there was a large decrease in conversion of tests 1 and 7, in which 28.07% and 14.59% of isomaltulose were obtained, respectively. Granules containing immobilized cells in test 2 were more stable, maintaining a constant isomaltulose conversion of about 50%. Our results indicate that adding gelatin and transglutaminase may favor cell immobilization.

Comparative studies have shown that *Erwinia rhapontici* NCPPB 1578 cells immobilized in 5% sodium alginate (w/v) and 0.1 molL^{-1} $CaCl_2$ solution, kept in stable continuous columns at 30°C, and fed with sucrose solution 1.6M were 350 times more stable than free cells, reaching a half-life of around 8,600 hours [18]. It was found that activity of immobilized cells decreased with increasing cell concentration, something that normally occurs in diffusionally limited systems, resulting also in less mechanical resistance.

Protaminobacter rubrum cells immobilized in calcium alginate have been used to obtain isomaltulose [19]. The maximum immobilized cell activity, at a pH of 5.5, was observed after 3 hours and was proportional to the amount of cells, yielding conversions of 18%, 30%, and 44% for 10, 20, and 40 mg of cells/mL of solution, respectively.

The bacterium *Klebsiella planticola* MX 10 was used by Tsuyuki et al. [20] to convert sucrose into isomaltulose. The cell mass was mixed with a 4% sodium alginate solution (w/v) at a 1 : 1 ratio (v : v), and cell suspension was dripped into a shaken 0.25 molL^{-1} $CaCl_2$ solution, in order to form immobilized cell granules. These granules were kept in the solution for 1 hour and were later washed with distilled water and placed in a 2% polyethyleneimine solution (w/v), at pH 5.6. After 5 minutes, these granules were separated and mixed with a 0.5% glutaraldehyde solution (v/v) at 5°C, for 20 minutes. The immobilized cells were separated from the glutaraldehyde solution and washed with distilled water. The polyethyleneimine- and glutaraldehyde-treated immobilized cells completely converted the 25% sucrose solution, yielding 65.4% of isomaltulose and 29.7% of trehalose.

The experimental design technique and response surface analysis have been used and were considered useful tools to study the conversion of sucrose into isomaltulose by immobilized cells. Moraes et al. [21] immobilized a 20% cell suspension of *Erwinia* sp. (w/v) in 1% sodium alginate

(w/v) and 2% $CaCl_2$ solution (w/v). Using stable continuous columns, they achieved a yield of about 50% of isomaltulose from sucrose solutions at 20–30% concentration (w/v) and 35°C. Mundra et al. [22] studied the effect of various immobilization parameters on converting sucrose into isomaltulose by immobilized cells of *Erwinia rhapontici* NCPPB 1578. Using a 30% sucrose solution (w/v), the maximum production of 140 mg/mL was obtained in a batch process when cells were suspended at 5 g/L and immobilized in 5% sodium alginate (w/v).

4. Conclusions

The experimental design technique was used to assess the effect of parameters when immobilizing *Erwinia* sp. D12 cells. The following independent parameters: sodium alginate concentration, cell mass concentration and $CaCl_2$ concentration, had significant effect on the stability and amounts of sucrose converted into isomaltulose. Cells immobilized in 3.0% sodium alginate (w/v), 47.0% cell mass (w/v), 0.3 $molL^{-1}$ $CaCl_2$, 1.7% gelatin (w/v), and 0.15% transglutaminase (w/v) presented greater rates of isomaltulose conversion, achieving 50–60% in seven consecutive batches. New studies must be performed to ascertain the influence of the parameters studied in this paper on stability and conversion of sucrose into isomaltulose in a continuous process.

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