academicJournals

Vol. 15(20), pp. 835-842, 18 May, 2016
DOI: 10.5897/AJB2015.14464
Article Number: 83457B058515
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Isomaltulose production using free and immobilized *Serratia plymuthica* cells

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Received 2 February, 2016,Accepted 20 April, 2016.

Isomaltulose is a low cariogenic sweetener used as a substitute for sucrose in the food industry. In this study, isomaltulose production by *Serratia plymuthica* ATCC 15928 was performed using free and immobilized cells. Response Surface Methodology was employed to evaluate the influence of temperature, wet cell mass concentration and sucrose concentration during the conversion of sucrose into isomaltulose by free cells. After 2 h of reaction time in shake flasks, a high production of isomaltulose (85.23%) was obtained with a temperature of 25ºC, wet cell mass of 20% (w/v) and sucrose solution of 25% (w/v). The free cells were reused during seven successive batches and resulted in efficient isomaltulose conversion between 83.74 and 67.37%. The production of isomaltulose by immobilized cells in calcium alginate was studied in a packed bed bioreactor during seven days in a continuous process. A conversion yield of sucrose into isomaltulose between 81.26 and 70.89% was obtained, using immobilized cells in calcium alginate Synth® 2% (w/v), sucrose solution of 35% (w/v), wet cell mass of 30% (w/v) and temperature of 25ºC. The conversion of sucrose into isomaltulose remained high using free cells and using immobilized cells in calcium alginate during the period of execution of the experiments.

Key words: Isomaltulose, glucosyltransferase, free cells, immobilized cells, *Serratia plymuthica.*

INTRODUCTION

The use of new sweeteners as sucrose substitutes in food composition has attracted much interest in recent decades. Alternative sweeteners are used in non-cariogenic and low-calorie food production, one segment of the food industry that is growing fastest (Kroger et al., 2006). Isomaltulose or palatinose® (6-*O*-α-D-glucopyranosyl-D-fructofuranose) is considered a promising replacement alternative sweetener for sucrose. It is a reducing sugar and a structural isomer of sucrose, naturally present in honey in small quantities (Rhimi et al., 2008; Kawaguti and Sato, 2010; Mundra et al., 2007). Isomaltulose is a low-cariogenic sugar (Hamada, 2002; Mundra et al., 2007), selectively promotes growth of beneficial bifido-bacteria among human intestinal microflora (Cummings et al., 2001; Birch and Wu, 2007) and it has a sweet taste and bulk similar to sucrose

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(Rhimi et al., 2008; Irwin and Strater, 1991). This alternative sugar is already used commercially in Japan as a sucrose replacement in the production of chocolate, chewing gum, cookies, drinks and pharmaceutical products (Rhimi et al., 2008; Kawaguti and Sato, 2010; Irwin and Strater, 1991). Also, isomaltulose, by hydrogenation, can be transformed into Isomalt®, a sugar-alcohol used as a dietetic non-cariogenic sweetener (Kroger et al., 2006; Kawaguti and Sato, 2010; Irwin and Strater, 1991).

Isomaltulose is obtained from food grade sucrose by enzymatic conversion of the glucosyl linkage from (1-2) fructoside to (1-6) fructoside. The microorganisms *Protaminobacter rubrum* (Oliva-Neto and Menão, 2009), *Erwinia* sp. (Kawaguti and Sato, 2010; Korneeva et al., 2008; Mundra et al., 2007), *Serratia plymuthica* (Krastanov and Yoshida, 2003) and *Klebsiella* sp. (Orsi et al., 2009; Zhang et al., 2003) are known to produce the intracellular enzyme α-glucosyltransferase (EC 5.4.99.11), also called sucrose isomerase, responsible for sucrose conversion into isomaltulose. In a recent work, Goulter et al. (2012) showed by molecular analyses that the most widely used bacterial strain for industrial production of isomaltulose referred to as *Protaminobacter rubrum* CBS 574.77 was genetically identified as *S. plymuthica*.

Isomaltulose production is documented in literature using free enzymes (Korneeva et al., 2008), free cells (Kawaguti et al., 2007) and immobilized cells (Kawaguti and Sato, 2010; Oliva-Neto and Menão, 2009; Krastanov and Yoshida, 2003; Orsi et al., 2009). The industrial use of free enzymes for isomaltulose production is complicated, because glucosyltransferase is an intracellular enzyme. The process of cell disruption required to release the enzyme is costly and difficult to plan on a large scale. There are many methods of cell disruption on a lab scale (Brown and Audet, 2008), but industrially only few methods of cell disruption are used extensively due to difficulties in scale up of the operations and cost effectiveness (Kar and Singhal, 2015).

The industrial production of isomaltulose is done by Südzucker AG (Germany industry), Cerestar (North American industry) and Shin Mitsui Sugar Co. (Japanese industry). The *P. rubrum* CBS 574.44 non-viable cells are used as the source of the biocatalyst for the production of isomaltulose by both industries. The cells are killed using formaldehyde before they are added to the sucrose. Cerestar industry uses free cells of *P. rubrum* in its process of isomaltulose production and removes the residual *P. rubrum* material by filtration. Südzucker AG industry utilizes immobilized cells of *P. rubrum* in its process of isomaltulose production. Similarly to Südzucker industry, Shin Mitsui Sugar Co. industry immobilizes the biocatalyst. The process utilized in cell immobilization by the industries is not detailed (Bail-Collet, 2003).

Sucrose is a raw material abundant and cheap in Brazil, which is the world's leading sugar producer, accounting for approximately 25% of global production. During the most recent years (2013-2014), national sugar output reached 37.8 million metric tons (Conab, 2013). However, until now there is no Brazilian industry that produces isomaltulose. Therefore, the aim of the present study was to describe the experimental results for isomaltulose production by *S. plymuthica* ATCC 15928 using free and immobilized cells (whole and viable) and show that the two processes are possible to use in isomaltulose production with high conversion rates of sucrose into isomaltulose during the period of execution of the experiments.

MATERIALS AND METHODS

Microorganism and culture conditions

S. plymuthica ATCC 15928 was obtained from a culture collection of the Foundation André Tosello, Campinas, São Paulo, Brazil. The cell biomass was obtained from fermentation in a 6.6 L bioreactor New Brunswick Bioflo IIc (New Brunswick Scientific, Edison, NJ, USA), with a 3.0 L working volume. It used the medium composed by sugar cane molasses (40 g L^{-1}), bacteriological peptone (15 g L^{-1}) and yeast extract Prodex Lac SD® (20 g L^{-1}). The temperature was maintained at 26°C, with constant agitation and aeration at 200 rpm and 1 vvm, respectively. After 12 h of fermentation, the cell mass was separated by centrifugation for 15 min at 9650×*g* at 5°C and then washed twice with sterile water. The wet cell obtained was used in the following experiments.

Study of the conversion of sucrose into isomaltulose by free cells using response surface methodology

Response Surface Methodology using a two level rotatory central composite design (2^3-RCCD) was employed to evaluate the variables that affected isomaltulose production by free cells of *S. plymuthica* (Neto et al., 2001). The variables studied were temperature (25-45°C), wet cell mass concentration (10-30%, w/v) and sucrose solution concentration (15-35%, w/v). These variables and their values are listed in Table 1. All data were treated with the aid of STATISTICA® version 7.0 from Statsoft Inc. (2325 East 13th Street, Tulsa, OK, 74104, USA). The significance of the model was verified by applying the analysis of variance (ANOVA) combined with the Fischer test to evaluate if a given term has a significant effect ($p< 0.05$). Additionally R^2 value was calculated to measure the good fit of the regression model. Experiments were performed in an orbital shaker (New Brunswick Scientific, Edison, NJ, USA) at 180 rpm, using 250 mL Erlenmeyer flasks containing a mixture of wet cell mass and sucrose solution, whose concentrations were varied according to the experimental design. Samples were collected after 2 h of reaction and the carbohydrates formed were determined by high performance anion exchange chromatography.

Isomaltulose production by free cells using repeated batch process

The free cells of *S. plymuthica* were reused during repeated batch process for isomaltulose production. Experiments were carried out in 250 mL Erlenmeyer flasks containing 100 mL of a mixture of wet cell mass 20% (w/v) and sucrose solution 25% (w/v). The conversion of sucrose into isomaltulose occurred in an orbital

Table 1. Experimental design (2^3-RCCD) and results for isomaltulose production by *Serratia plymuthica* free cells (real values in parentheses).

Assay	Variable levels			
	Temperature (ºC)	Wet cell mass concentration (%)	Sucrose solution concentration (%)	Isomaltulose yield (%)
1	-1 (30)	-1 (15)	-1 (20)	85.02
2	+1 (40)	-1 (15)	-1 (20)	50.09
3	-1 (30)	+1 (25)	-1 (20)	84.52
4	+1 (40)	+1 (25)	-1 (20)	60.97
5	-1 (30)	-1 (15)	+1 (30)	76.49
6	+1 (40)	-1 (15)	+1 (30)	71.50
7	-1 (30)	+1 (25)	+1 (30)	77.45
8	+1 (40)	+1 (25)	+1 (30)	78.09
9	-1.68 (25)	0 (20)	0 (25)	85.23
10	+1.68 (45)	0 (20)	0 (25)	29.01
11	0 (35)	-1.68 (10)	0 (25)	75.02
12	0 (35)	+1.68 (30)	0 (25)	81.35
13	0 (35)	0 (20)	-1.68 (15)	82.08
14	0 (35)	0 (20)	+1.68 (35)	78.46
15	0 (35)	0 (20)	0 (25)	83.41
16	0 (35)	0 (20)	0 (25)	83.45
17	0 (35)	0 (20)	0 (25)	83.46

shaker (New Brunswick Scientific, Edison, NJ, USA) at 180 rpm and 25ºC. After 2 h, the reaction mixture was centrifuged for 15 min at 9650×*g* at 5°C to separate the cell mass and the free cells were reused for the next batch process with fresh substrate. At the end of the incubation of each batch, samples were collected and the carbohydrates formed were determined by high performance anion exchange chromatography. This process was repeated for seven times.

Cell immobilization in alginate

The cells of *S. plymuthica* were immobilized by entrapment in calcium alginate beads. The cell mass solution (30%) (wet weight/v) was mixed with sodium alginate solution, to give a volumetric ratio of 1:2. The resulting mixture was extruded, drop wise, through a peristaltic pump (ColeParmer Instrument Co., Vernon Hills, Il, USA) into 2% calcium chloride solution (2% $CaCl_2$ w/v) to form gel beads of 3.0 mm diameter. The beads were allowed to harden in the $CaCl_2$ solution for 12 h at 5ºC and subsequently washed with sterile distilled water to remove the excess of $CaCl_2$. Two types of sodium alginate were tested: Alginate Synth® 2% (w/v) and high viscosity alginate Sigma® 1% (w/v). All stages were carried out under aseptic conditions.

Isomaltulose production by immobilized cells using packed bed reactor

For continuous isomaltulose production, 100 g of the beads were packed in a column reactor (150 mm × 30 mm) maintained at 25°C. Sucrose solution of 35% (w/v) was passed through the reactor in an ascending flow of approximately 0.3 mL $min.^{-1}$.

The reactor was continuously operated for seven days. Samples were collected at time-defined intervals and the carbohydrates formed were determined by high performance anion exchange chromatography.

Carbohydrate analysis

High performance anion exchange chromatography was performed on a Dionex System DX-600 (Dionex Corporation, Sunnyvale, CA), consisting of an eluent degassing device module, an isocratic pump IP 25 and an ED50 gold electrochemical detector. Carbohydrate separation was carried out by a Carbopac PA-1 pellicular anion-exchange resin (4 mm×270 mm) connected to a Carbopac PA-1 guard column (4 mm×50 mm) at 20°C. The elution was a solution of NaOH 400 mM and the flow rate was constant at 1 mL/min. The carbohydrates were analyzed comparing their retention times with those of the fructose, glucose, sucrose and isomaltulose standards (Sigma Ultra®, Sigma Chemical Co., St. Louis, MO, USA).

RESULTS AND DISCUSSION

Response surface methodology

In this study, response surface methodology was used to evaluate the combined effects of temperature, wet cell mass concentration and sucrose concentration in the conversion of sucrose into isomaltulose by free cells of *S. plymuthica*. As can be seen in Table 1, the isomaltulose production showed a minimum of 29.10% for assay 10 (temperature 45ºC, wet cell mass 20% and sucrose concentration 25%) and a maximum of 85.23% for assay 9 (temperature 25ºC, wet cell mass 20% and sucrose concentration 25%). It was observed that the temperature of 25ºC was favorable for a higher production of isomaltulose. Statistical evaluation of the model was performed by Fisher's test, obtained from ANOVA, whose results are shown in Table 2. The model was significant at a confidence level of 95% ($p < 0.05$). The calculated

Table 2. ANOVA for the experimental design (2^3-RCCD) used to evaluate the significance of the model.

Variation source	Quadratic sum	Freedom level	Quadratic mean	F test
Regression	3245.69	8	405.71	10.04
Residues	323.37	8	40.42	
Lack of fit	323.37	6		
Pure error	0.00	2		
Total	3569.06	16		

Coefficient of determination $R^2 = 0.91$; F 0.95,8,8 = 3.44.

F-value should be higher than the tabulated F-value if the model has a good prediction of the experimental results. In this work the calculated F-value was 2.92 times greater than the tabulated F-value. The adequacy of the model was checked by the coefficient of determination (R^2), whose value was 0.91, indicating that only 9% of the total variation was not explained by the model.

After the analysis of variance and validation of the studied parameters, the model of response surface for isomaltulose production is represented by Equation 1:

$$Y = 83.30 - 11.51.X_1 + 2.08.X_2 + 1.22.X_3 - 8.93.X_1^2 - 1.43.X_2^2 - 0.69.X_3^2 + 2.15.X_1.X_2 + 6.79.X_1.X_3 - 0.33.X_2.X_3 \quad (1)$$

Where, Y is the isomaltulose production, X_1 is the temperature, X_2 is the wet cell mass concentration and X_3 is the sucrose concentration, respectively.

The contour curves, presented in Figure 1, indicated that the best conditions for isomaltulose production by free cells were: temperature between 25 to 35°C, wet cell mass concentration of 20% and sucrose solution concentration between 15 to 25%. The free cells of *S. plymuthica* showed a high conversion rate of sucrose into isomaltulose (85.23%) using temperature of 25°C, wet cell mass concentration of 20% (w/v) and sucrose solution concentration of 25% (w/v), and that was the chosen condition for isomaltulose production by free cells using repeated batch process.

Isomaltulose production by free cells using repeated batch process

The isomaltulose production by free cells of *S. plymuthica* using a repeated batch process is shown in Figure 2. The free cells were active and could be reused for seven times during the batch process. The highest conversion of sucrose into isomaltulose was 83.74% (first batch). During the first four batches a total consumption of substrate sucrose and a high isomaltulose production of 80.40% occurred. In the fifth batch, a little percentage of sucrose (3.53%) was not converted and 77.31% of isomaltulose, 7.28% of trehalulose, 6.85% of fructose and 5.00% of glucose was obtained. A conversion yield of sucrose into isomaltulose between 83.74 and 67.37%, using free cells repeatedly in 7 batches operations, from 25% sucrose solution, 20% wet cell mass and temperature of 25ºC was obtained.

Comparing the results of this study with the work of Kawaguti et al. (2007), higher conversion rates of sucrose into isomaltulose using free *S. plymuthica* cells was obtained. Kawaguti et al. (2007) evaluated the stability of the glucosyltransferase from *Erwinia* sp. D12 during the conversion of sucrose into isomaltulose using the free cells repeatedly during batch operations. The conversion of sucrose into isomaltulose after eight batches was higher than 60% and the highest conversion of sucrose into isomaltulose (72.11%) was verified in the first batch.

The production of isomaltulose using free cells is less documented in literature than the production of isomaltulose using immobilized cells, but there are some studies that obtained an efficient sucrose conversion into isomaltulose using free cells. Heikkila et al. (2000) obtained 85.90% of isomaltulose using free cells of *P. rubrum* after 13 h of reaction. Krastanov and Yoshida (2003) obtained a total conversion of a sucrose solution (40%, w/v) into 79.95% of isomaltulose, 7.04% of trehalulose, 5.84% of fructose and 2.90% of glucose, using free cells of *S. plymuthica* after 4 h of reaction. These studies (Heikkila et al., 2000; Krastanov and Yoshida, 2003) had high initial conversion rates of sucrose into isomaltulose, but they did not performed tests to keep the conversion for long periods.

Isomaltulose production by immobilized cells using packed bed bioreactor

Continuous production of isomaltulose by immobilized cells of *S. plymuthica* in alginate was studied in a packed bed bioreactor. Figure 3a and b illustrates the conversion of sucrose into isomaltulose using cells immobilized in alginate Synth® 2% (w/v) and in alginate of high viscosity Sigma® 1% (w/v). The production of isomaltulose during seven days in a continuous process was between 81.26

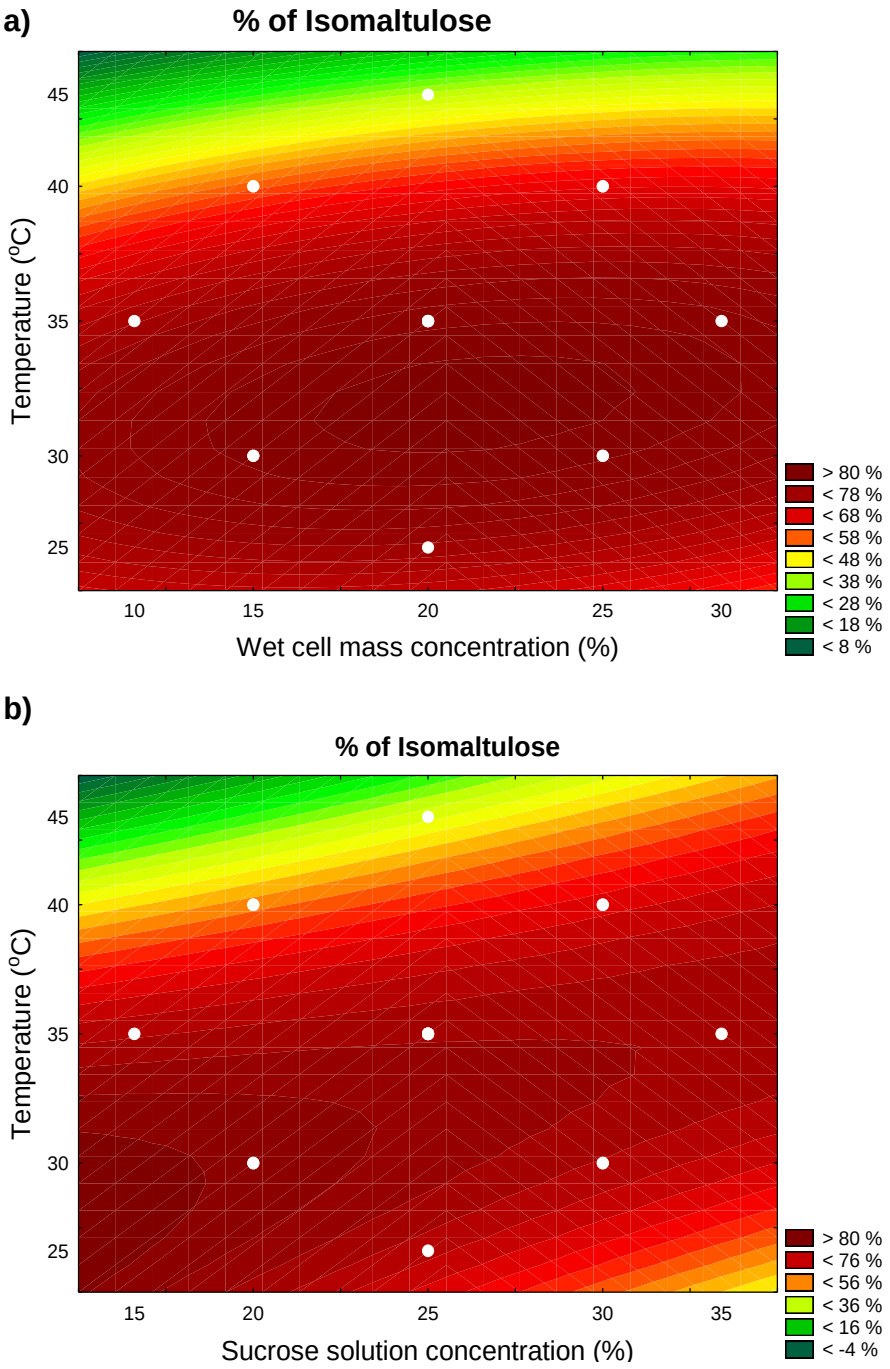

Figure 1. Contour curves for isomaltulose production from *Serratia plymuthica* free cells: (a) as a function of wet cell mass concentration and temperature and (b) as a function of sucrose solution concentration and temperature, according to the experimental design.

and 70.89% using alginate Synth® 2% (w/v) and 77.56 and 64.84% using alginate Sigma® 1% (w/v). Based on these results, the alginate Sigma® 1% can be substituted by a cheaper one, the alginate Synth® 2%, since the immobilized cells in alginate Synth® 2% resulted in higher conversion rates of sucrose into isomaltulose. Using immobilized cells in calcium alginate Synth® 2% the results obtained were 81.26 to 70.89% of isomaltulose, 9.01 to 8.04% of trehalulose, 6.43-11.42% of fructose and glucose and 3.30-9.02% of sucrose not converted, during the seven days of continuous process, using sucrose solution of 35% (w/v), wet cell mass of 30% (w/v) and temperature of 25°C. Comparing the results of this study with the work of Kawaguti and Sato

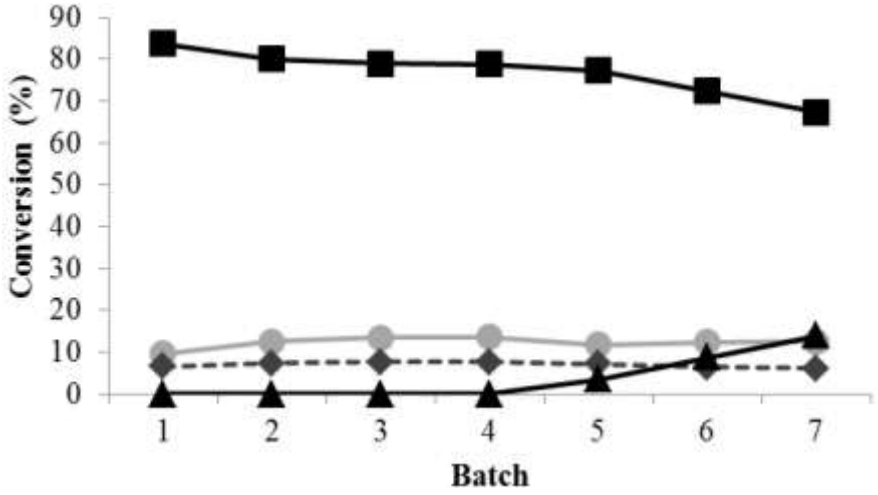

Figure 2. Repeated batch conversion of sucrose into isomaltulose using free *S. plymuthica* cells (■ Isomaltulose, ●Glucose and Fructose, ♦ Trehalulose, ▲Sucrose).

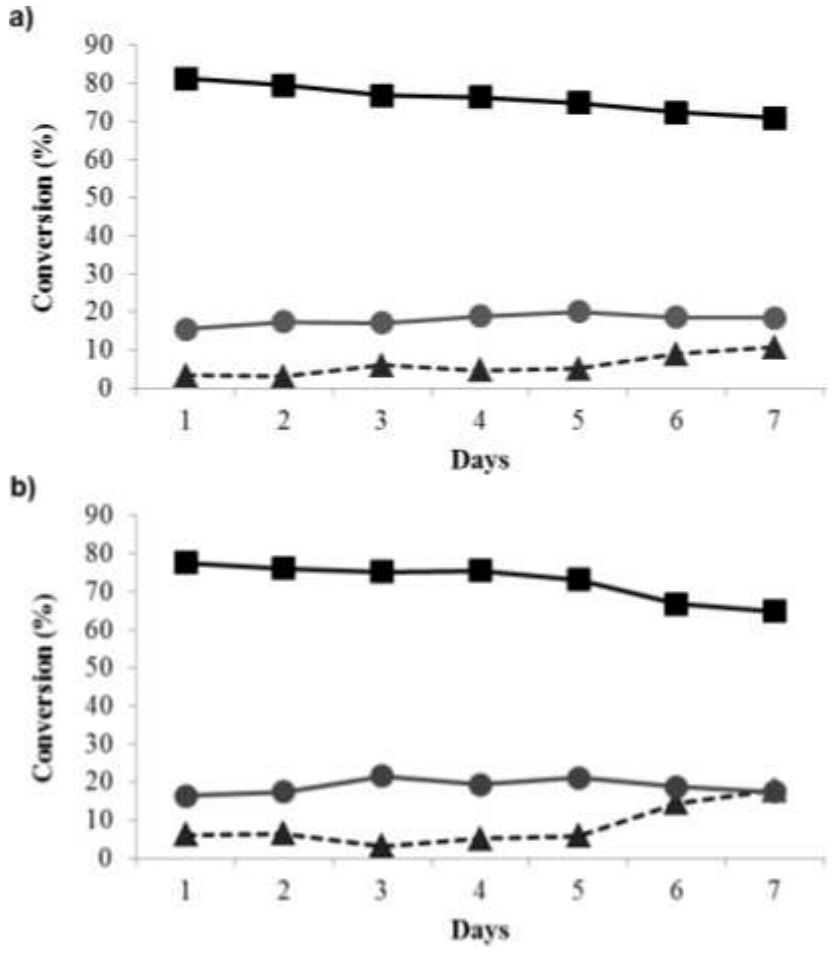

Figure 3. Continuous production of isomaltulose in a packed bed bioreactor using immobilized *S. plymuthica* cells: a) cells immobilized in alginate Synth® 2% (w/v) and b) cells immobilized in alginate Sigma® 1% (w/v) (■ Isomaltulose, ●Glucose, Fructose and Trehalulose, ▲Sucr

(2010), higher conversion rates of sucrose into isomaltulose using immobilized *S. plymuthica* cells was obtained. Kawaguti and Sato (2010) obtained 75 to 53% of isomaltulose in a continuous process during eight days using similar conditions of this study: A packed-bed reactor containing immobilized *Erwinia* sp. cells in alginate of low viscosity Sigma® 3% (w/v), at 25°C and using sucrose solution of 35% (w/v).

In this work the production of isomaltulose by immobilized cells was studied using alginate concentration of 1 to 2% (w/v) and wet cell mass concentration of 30% (w/v). The mechanical strength of the gel beads may decrease due to low concentration of alginate or high cell concentration. Ogbonna et al. (1989) studied the influence of cell mass concentration and alginate concentration (0.5-4.0%) in the process of *Saccharomyces cerevisiae* cells immobilization in calcium alginate. It was found that very high concentrations of cell mass resulted in a high percentage of the beads which developed cracks. If a high population of cells is formed at the center of the beads (which often occurs when a high initial cell population is used), the rate of CO_2 evolution may be more than the rate of diffusion of the gas out of the beads. This may lead to the building up of gas pressure at the center of the beads, which eventually may lead to the development of cracks in the beads (gas outlet). It was found that the beads obtained with alginate 1 to 2% had the lowest number of disruptions. Beads prepared with 0.5% alginate were much softer, thus a high number of cracked beads resulted. High concentrations of alginate (4.0%) caused high viscosity problems and resulted in beads with deficient diffusion, due to diffusional limitations imposed by the porosity of the gel matrix.

Temperature is an important factor that influences the growth of microorganisms and metabolites biosynthesis. It was observed in this study that the temperature of 25°C was important for a higher production of isomaltulose. Other works that studied the conversion of sucrose into isomaltulose using cells of *S. plymuthica* (Krastanov et al., 2007) or *P. rubrum* (Oliva-Neto and Menão, 2009; Heikkila et al., 2000) used temperatures of 20 to 30°C. Krastanov et al. (2007) used temperature of 20°C to study the operational stability of a hollow-fibre membrane reactor of *S. plymuthica* cells in a continuous process of transformation of sucrose into isomaltulose.

In this work the production of isomaltulose by immobilized cells was studied using sucrose solution of 35% (w/v). The use of high sucrose concentrations tends to inhibit division of immobilized cells and to prevent microbial contamination, while at the same time making it easier to recover the product and decreasing the volume of liquid to be processed (Oliva-Neto and Menão, 2009; Krastanov et al., 2006). Krastanov et al. (2006) using immobilized *S. plymuthica* cells in chitosan achieved the highest isomaltulose content when 40% sucrose solution was used. In the case of more concentrated sucrose solutions, lower isomaltulose content was determined, presumably due to substrate inhibition of the glucosyltransferase in higher sucrose concentrations. Kawaguti and Sato (2010) studied the effects of sucrose concentration (35-55%, w/v) on the conversion of sucrose into isomaltulose by immobilized cells of *Erwinia* sp. in alginate. It was observed that there was a tendency towards a higher conversion rate when a lower amount of sucrose was used, below 40%, with optimum concentration at approximately 35%.

Conclusion

This experimental study showed two possible processes to use in the isomaltulose production. The conversion of sucrose into isomaltulose by *S. plymuthica* ATCC 15928 remained high during the seven repeated batch processes using free cells and during seven days in a continuous process using immobilized cells in calcium alginate. The productivity and the operational stability of the biocatalyst were successful and the results could be used for scale up of suitable reactors for an industrial plant to start production of isomaltulose, a sugar higher added value than sucrose.

Conflict of interests

The authors hereby declare that no conflict of interest exists among them.

ACKNOWLEDGEMENTS

The authors acknowledge the financial support from Coordenação de Aperfeiçoamento de Pessoal de Nível Superior-Brazil (CAPES).

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