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Full Length Research Paper

High production of fructooligosaccharides by levansucrase from *Bacillus subtilis* natto CCT 7712

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Fructooligosaccharides (FOSs) are fructose oligomers known as prebiotics that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improving host's health. FOS can be produced by the action of fructosyltranferases from microorganisms, including the *Bacillus subtilis* natto. The objective of the present study was applying response surface methodology in order to optimize the FOS production by levansucrase from *B. subtilis* natto CCT 771. The variables evaluated were sucrose concentration, pH and temperature. From the analysis of the results, the significant variables were pH and sucrose concentration ($p < 0.05$). Levansucrase of *B. subtilis* natto synthesized fructooligosaccharides in all experimental conditions, showing its potential for use in an industrial process of producing FOS from sucrose. The use of response surface methodology allowed the determination of the concentration of sucrose (334 $g \cdot L^{-1}$), pH (6.0) and temperature (45.8°C) for a maximum yield of 54.86 $g \cdot L^{-1}$ FOS.

Key words: Fructooligosaccharides, *Bacillus subtilis*, levansucrase, prebiotics.

INTRODUCTION

Fructooligosaccharides (FOSs) are low molecular weight carbohydrates containing fructose residues with a degree of polymerization from 3 to 9; fructosyl units are bound at the β-(2 → 1) position of sucrose. FOSs are mainly composed of 1-kestose (GF2), nystose (GF3) and fructofuranosyl nystose (GF4) (Yun, 1996). FOSs are part of non-digestible fibers and they act as prebiotics. In the digestive system they serve as a carbon source for colon bacteria, such as lactobacilli and bifidobacteria, which are beneficial to human health (Matulová et al., 2011). The growth and stimulated activity of these bacteria act in the removal of potentially pathogenic bacteria (Gibson and Roberfroid, 1995; Yun, 1996). During microorganism growth, short-chain fatty acids (SCFAs) are produced

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as the end products of oligosaccharide fermentation. SCFAs stimulate the growth of colorectal mucosal cells, retard mucosal atrophy, and decrease the risk of malignant transformation in the colon (Ningegowda and Gurudutt, 2011). Other important properties of FOS are that they have low caloric value, effects in reducing phospholipid, triglycerides and cholesterol levels and help the absorption of calcium and magnesium in gut (Mussato and Teixeira, 2010).

FOS can be obtained from inulin hydrolysis by acid treatment at high temperatures which is expensive and makes it worthwhile to search for new ways of obtaining FOS by either microbial synthesis or enzymatic means (Nemukula et al., 2009). FOSs were produced by the action of fructosyltransferases present in plants and microorganisms (Sánchez et al., 2008). Several microorganisms have β-fructofuranosidases (EC 3.2.1.26) which are responsible for producing FOS obtained from sucrose, such as *Aspergillus oryzae*, *Aureobasidium pullulans*, *Bacillus subtilis* and *Zymomonas mobilis* (Bekers et al., 2002; Euzenat et al., 1996; Sangeetha et al., 2004; Shin et al. 2004). Among them, *Bacillus subtilis* has been the most extensively studied for production of levansucrase, and strains producing high levels of constitutive levansucrase have been isolated. When incubated with sucrose, *B. subtilis* levansucrase catalyses the formation of high and low molecular weight levans. *B. subtilis* natto has shown to have capacity to produce levan by levansucrase extracellular, in sucrose medium and to be able to synthesize FOS by the action of this enzyme (Abdel-Fattah et al., 2005).

Some authors have focused in FOS production by microorganism and its respective enzymes, for example B. circulans (El-Refai et al. 2009), B. subtilis 33 NRC (Abdel-Fattah et al., 2005), B.subtilis C4 (Euzenat et al. 1997), and *Zymomonas mobilis* (Bekers et al., 2002). These authors point out the influence of parameters such as sucrose concentration and temperature in the production and size of the oligofructose chains. Increasing the reaction temperature interferes with FOS synthesis because it accelerates the transfructosylation rate converting 1-kestose rapidly to nystose (Vega-Zuniga and Hansen 2011). Abdel-Fattah et al. (2009) studied the effect of sucrose concentration, temperature and found that increasing the sucrose concentration to 40% favored the formation of FOS and temperature increase was accompanied by a reduction in the oligofructose molecular weight. Other factors such as pH, enzyme concentration and reaction time are parameters that act in FOS production (Sangeetha et al., 2005).

There are many factors that influence FOS production as temperature, pH, medium composition, agitation and time of cultivation. An alternative is the use of response surface methodology, a method used in the optimization of the composition of media and other critical variables responsible for biomolecule production. This method allows testing multiple variables with a minimum number of experiments and evaluates the interaction among factors (Liu et al. 2010).

In this work, response surface methodology was used as an optimization tool for the production of fructooligosaccharides by levansucrase from *Bacillus subtilis* natto CCT 7712. Three independent variables, pH, temperature and concentration of sucrose were tested in the reaction medium.

MATERIALS AND METHODS

Microorganisms

B. subtilis natto CCT 7712 was maintained at 4°C on slant medium containing ($g \cdot L^{-1}$): peptone 50, yeast extract 30 and agar 20. This strain was isolated from soybeans fermented, a Japanese food called “natto” at the Department of Biochemistry and Biotechnology of State University of Londrina (Brazil) and identified by Fundação André Tosello (Campinas- Brazil). The product “natto” was purchased at a health food store. The soybeans were macerated and were diluted with distilled water. Serial dilutions were made, inoculated on Petri dishes containing standard medium and incubated at 37.5°C for 48 h.

Crude enzyme production

First stage of enzyme production was cultivation of the microorganism source of levansucrase. *B. subtilis* was activated in inoculum medium with the following composition: 100 $g \cdot L^{-1}$ of sucrose, 2 $g \cdot L^{-1}$ of yeast extract, 2 $g \cdot L^{-1}$ of KH_2PO_4, 1 $g \cdot L^{-1}$ of $(NH_4)_2SO_4$ and 0.5 $g \cdot L^{-1}$ of $MgSO_4.7H_2O$.

The second step was the fermentation, this medium contained 300 $g \cdot L^{-1}$ of sucrose, 2 $g \cdot L^{-1}$ of yeast extract, 1 $g \cdot L^{-1}$ of KH_2PO_4, 3 $g \cdot L^{-1}$ of $(NH_4)_2SO_4$, 0.6 $g \cdot L^{-1}$ of $MgSO_4.7H_2O$, 0.2 $g \cdot L^{-1}$ of $MnSO_4$, H_2O and 0.25 $g \cdot L^{-1}$ of ammonium citrate. The pH was adjusted to 7.7; the culture was incubated at 37°C in a rotary shaker at 150 rpm for 48 h. The cells obtained in the first stage were used as an inoculum to fermentation medium, were transferred 0.2 g of cells for each liter of fermentation medium. At the end of the incubation period, the culture was centrifuged (4°C, 9056 x *g*) using a refrigerated centrifuge (Hetic V 320R), and the supernatant and the cells were separated. The supernatant was considered as source of levansucrase.

Enzyme assay

Levansucrase enzyme was assayed by estimating the reducing sugar released during exopolymer hydrolysis. The reaction mixture, containing 250 µL enzyme extract and 250 µL 1 M sucrose in acetate buffer (pH 5.0) was incubated at 30°C for 2 h (Ananthalakshmy and Gunasekaran 1999). After 2 h, the exopolysaccharide was precipitated by adding chilled ethanol. The precipitate was hydrolyzed in 1 mL 1 M HCl at 100°C for 1 h, the solution was then neutralized by adding NaOH 2 M and the reducing sugars were determined by the Somogyi (1952) and Nelson (1944) methods.

One unit of activity (UA) was defined as the amount of enzyme that released 1 µMol reducing sugar per mL per minute under the experimental conditions. All experiments used standardized crude enzyme extract at 6 U.

Table 1. Fructooligosaccharide production using levansucrase of B. *subtilis* natto with three independent variables at three levels after 12 h reaction time and enzymatic activity = 6 u/ml.

Run	x_1 (X_1, $g \cdot L^{-1}$)	x_2 (pH)	x_3 (X_3, °C)	FOS ($g \cdot L^{-1}$)
1	-1 (150)	-1 (5)	-1 (45)	47,86
2	-1 (150)	-1 (5)	1 (55)	22,12
3	-1 (150)	1 (7)	-1 (45)	37,62
4	-1 (150)	1 (7)	1 (55)	30,69
5	1 (350)	-1 (5)	-1 (45)	50,53
6	1 (350)	-1 (5)	1 (55)	39,32
7	1 (350)	1 (7)	-1 (45)	48,08
8	1 (350)	1 (7)	1 (55)	33,69
9	-1,68 (81,8)	0 (6)	0 (50)	40,31
10	1,68 (418,18)	0 (6)	0 (50)	67,73
11	0 (250)	-1,68 (4,3)	0 (50)	22,55
12	0 (250)	1,68 (7,7)	0 (50)	18,57
13	0 (250)	0 (6)	-1,68 (41,6)	57,99
14	0 (250)	0 (6)	1,68 (58,4)	53,49
15	0 (250)	0 (6)	0 (50)	51,15
16	0 (250)	0 (6)	0 (50)	64,21
17	0 (250)	0 (6)	0 (50)	54,38
18	0 (250)	0 (6)	0 (50)	59,38
19	0 (250)	0 (6)	0 (50)	60,40

X1: Sucrose concentration, X2: pH and X3: temperature.

FOS enzymatic synthesis using response surface methodology

Response surface methodology with a central composite design was used to determine the optimal condition of sucrose concentration (X_1), pH (X_2) and temperature (X_3) for maximal FOS formation (Table 1). The variables were coded as in Equation 1 1:

$$x_i=(X_i-X_{cp})\Delta X_i \quad (1)$$

Where, x_i is the coded level of the variable, X_i is the real level of the variable, X_{cp} is the real level of the variable at the center point, and ΔX_i is the step change value in the real level. The parameters that were simultaneously varied for FOS production in the response surface methodology (RSM) were sucrose concentration (81.8 $g \cdot L^{-1}$ to 418.18 $g \cdot L^{-1}$), pH (4.3 - 7.7) and temperature (41.6 to 58.4°C). The optimal response of the FOS formation was predicted by the following quadratic Equation 2:

$$\hat{Y} = b_0 + \sum b_i x_i + \sum b_{ii} x^2_{ii} + \sum b_{ij} x_i x_j \quad (2)$$

Where, $\hat{Y}$ is the response variable, b_0 is the constant, b_i is the coefficient for the linear effect , b_{ii} is the coefficient for the quadratic effect, b_{ij} is the coefficient for the interaction effect, and x is the coded level of the variable.

The experiments were performed in 1.5 mL of standardized sucrose solution in 0.1 M citrate buffer or 0.1 M phosphate buffer with the addition of 0.5 mL crude enzyme extract. The reaction was carried out in a water bath for 12 h. After stopping the reaction by incubating the samples in a boiling water bath for 15 min, the FOS production was analyzed by high-performance liquid chromategraphy - HPLC (Sageentha et al., 2004). Sucrose concentration, pH and temperature were studied.

Analytical procedures

FOS production was analyzed with HPLC using Shimadzu equipment with a Shimadzu RID-10A refractive index detector. The column used was AMINEX Carbohydrate HPX-87C (300 mm x 7.8 mm Biorad). The column temperature was maintained at 80°C. The samples (20 µL) were eluted with 0.6 $mL \cdot min^{-1}$ Milli-Q water and the FOS standards 1-kestose (GF_2 - 504.44 Da) and 1- nystose (GF_3 - 666.58 Da) were from Sigma-Aldrich. The total FOS production was calculated as the sum of 1-kestose and 1-nystose expressed in $g \cdot L^{-1}$.

Statistical and data analysis

All statistical analyses were conducted using STATISTICA (data analysis software system), Version 7.0 (StatSoft, Inc. 2004, EUA). Differences were considered significant at p-values < 0.05.

RESULTS

Nineteen assays were performed to define the best condition for enzymatic synthesis of FOS. Total FOS produced was analyzed by HPLC, in the chromatograms obtain from aliquots of the reactions.

There were five peaks which correspond to fructose, glucose, sucrose and two types of FOS (1-kestose and 1-nystose). Therefore, it can be concluded that this levansucrase only synthesized small chains of FOS with molecular weight of 556 and 666 Da.

Table 2. Analysis of variance (ANOVA) for the second-order model of levansucrase FOS production.

Factor	Sum of squares	d.f.	Mean square	p-value
Sucrose (L)	462.389	1	462.389	0.0092*
pH (L)	19.79	1	1979	0.5125
Temperature (L)	317.613	1	317.613	0.0231*
Sucrose (Q)	65.389	1	65.389	0.246
pH (Q)	2682.78	1	2682.78	0.000024*
Temperature (Q)	34.116	1	34.116	0.394
x_1x_2	5.136	1	5.136	0.736
x_1x_3	6.224	1	6.224	0.711
x_2x_3	30.531	1	30.531	0.419
Lack of fit	27696	5	55.393	0.248
Pure error	106.218	4	26.554	

R^2=0.9020; *Significant for p-values < 0.05.

The optimum sucrose concentration (X_1), pH (X_2) and temperature (X_3) for maximum FOS formation were investigated by response surface methodology with a central composite factorial design. The results are shown in Tables 1 and 2. Regression analysis of the experimental data shows that the response producing FOS can be predicted by the following second order polynomial equation:

$$Y= 55.3762+5.8187x_1-13.7505x_2^2-4.8225x_3 \quad (3)$$

Where, Y is the response for the FOS production, x_1, x_2 and x_3 are coded values for sucrose, pH and temperature, respectively.

The model coefficient of determination (R^2) was 0.902, which indicates that 90.2% of the variability of the responses can be explained by the model. This value was considered acceptable, because according to Joglekar and May (1990), the coefficient of determination must be at least 80%. The lack of fit was not significant (p = 0.248), indicating that the model equation was suitable for predicting FOS production.

The analysis of the regression equation showed that intercept, the linear term of sucrose, the quadratic term of the temperature and pH were significant at 5% (Table 2). Among the tests performed, the highest FOS production was achieved in the experiment 10. In this point, the FOS production was 67.73 g. L^{-1}, the three variables were set in the following parameters: sucrose concentration of 418.18 $g{\cdot}L^{-1}$, pH 6 and 50°C of temperature. The FOS production significantly decreased to 18.57 $g{\cdot}L^{-1}$ with sucrose at a concentration of 250 $g{\cdot}L^{-1}$, pH 7.7 and 50°C (Table 1; Test 12).

The effect of the sucrose concentration can be seen by comparing the experiments presented at Table 1. In the assay 9, the sucrose content was 81.8 $g{\cdot}L^{-1}$ and in the assay 10, the sucrose content was 418.18 $g{\cdot}L^{-1}$, in both the pH were 6.0 and temperature, 50°C. The yield was 40.31 $g{\cdot}L^{-1}$ in assay 9 and 67.73 $g{\cdot}L^{-1}$ in assay 10, with an increase of about 68 %, indicating a positive effect of this variable, which can be seen in Equation 3.

The temperature was significant for FOS production (p = 0.0231) (Table 2) and the yield was reduced when increasing the temperature from 45 to 55°C for assays 1 to 8 (Table 1). In assay 1, yield was 47.86 $g{\cdot}L^{-1}$ FOS and with an increase of 10°C in experiment 2 it was 22.12 $g{\cdot}L^{-1}$. The optimum pH for FOS production was around 6.0.

Figure 1 shows the response surface for FOS production by levansucrase of *B. subtilis* natto as function of sucrose concentration. Increase in sucrose promoted FOS production while temperature above 45°C reduced the synthesis.

In order to determine the best conditions to synthesis of FOS, a predictive analysis was done. The statistical analysis indicated the theoretical maximum value of 63.62 $g{\cdot}L^{-1}$, which could be obtained setting the variables in the following parameters: sucrose concentration of 334 $g{\cdot}L^{-1}$, pH 6.0 and temperature of 45.8°C (Figure 2). A validation of predictive model (Equation 3) was necessary, therefore four tests were performed using the conditions cited above. The average yield of experiments was 54.86 $g{\cdot}L^{-1}$, which was compared statically with theorical value. The analysis shows that the predicted value and experimental validation were not statistically different at 5% level of significance. The optimization of FOS production shown in this work demonstrates the potential of levansucrase from *B. subtilis* for this application.

DISCUSSION

Recently, many studies have focused on the production of fructooligosaccharides. These works investigated

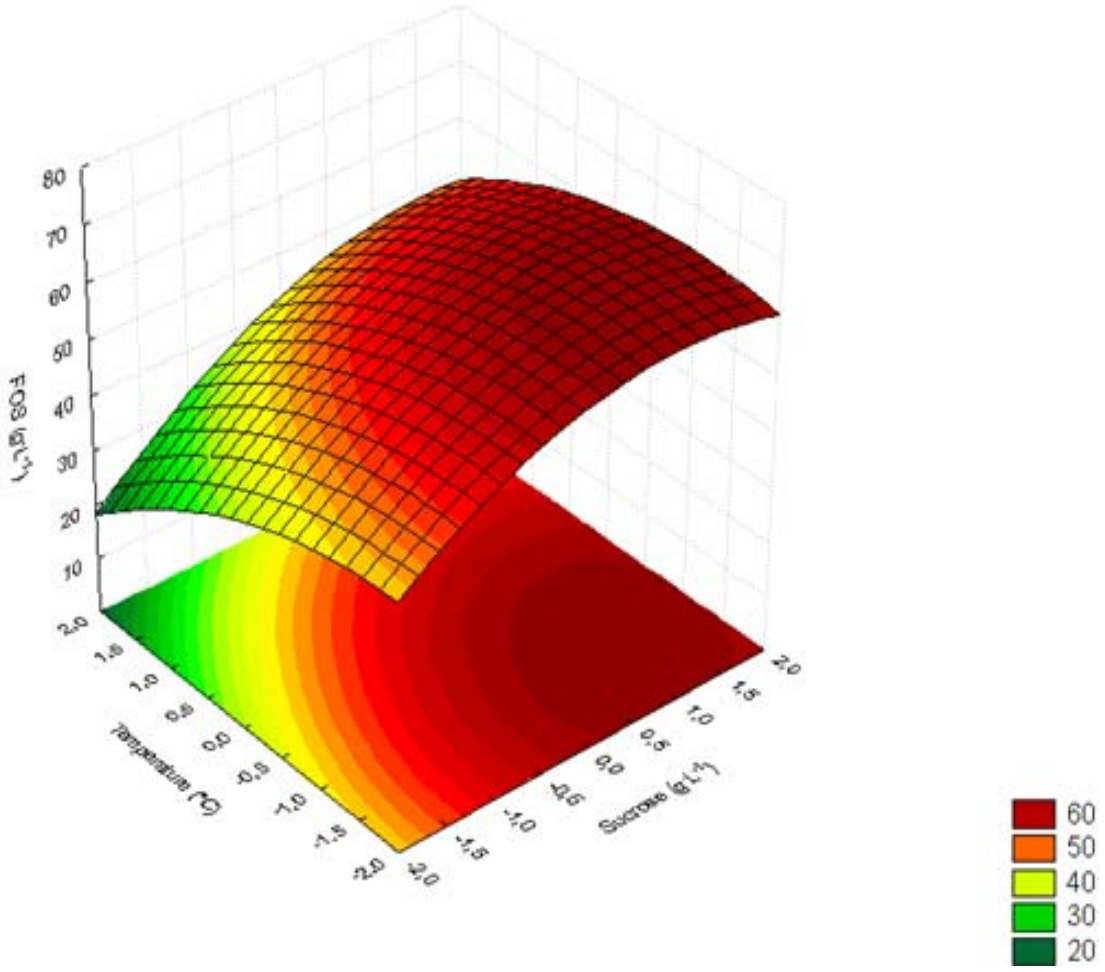

Figure 1. Response surface and contour plots for fructooligosaccharides production by levansucrase of *Bacillus subtilis* natto as a function of sucrose and temperature, pH=6.0.

microbial FOS production in sucrose medium by the action of fructosyltransferases and β-fructofuranosidades from fungi and bacteria (Park et al., 2003). The best FOS productions were obtained in the assays in which the sucrose concentrations were among the highest. Silva et al. (2011) noticed similar effect for fructosyltransferase from Aureobasidium pullulan, the sucrose concentration was positively related to FOS, once the fructosyltransferase and transfructosylation performance more efficiently in media with high concentrations of this substrate.

This positive effect may be due to the fact that activity of levansucrase increases with the increase of sucrose in medium (Belghith et al., 2012). According to Vega-Paulino and Zuniga-Hansen (2012), the initial sucrose concentration is the major factor in FOS formation because it increases the availability of fructosyl acceptors and reduces water availability; so that this condition can improve the formation of FOS. Hettwer and Rudolph (1995) reported that at higher sucrose concentrations, levan production at the beginning is high but later its synthesis is inhibited. Then, the hydrolase activity increases, accumulating oligosaccharides and increasing glucose concentration that continues inhibition of levansucrase. Temperature was an important factor, as soon as it increases; FOS production improves until a specific level, after this specific point the production decreases. Similar behavior was observed for fructosyltransferase from *Aspergillus aculeatus* (Ghazi et al., 2007) and FOS synthesis decreased from 60°C and the catalytic activity was reduced after 2 h of reaction due to thermal inactivation. The levansucrase of *B. subtilis* natto in the present work showed to be more active at 45.8°C.

The pH of medium plays an important role in fructosyltransferase activity. The optimum pH 6.0 verified in this study corresponds to the optimum pH for the activity of the levansucrase enzyme from *Bacillus* sp. described by Ammar et al. (2002). In fact, most of the levansucrases reported so far have showed optimum pH

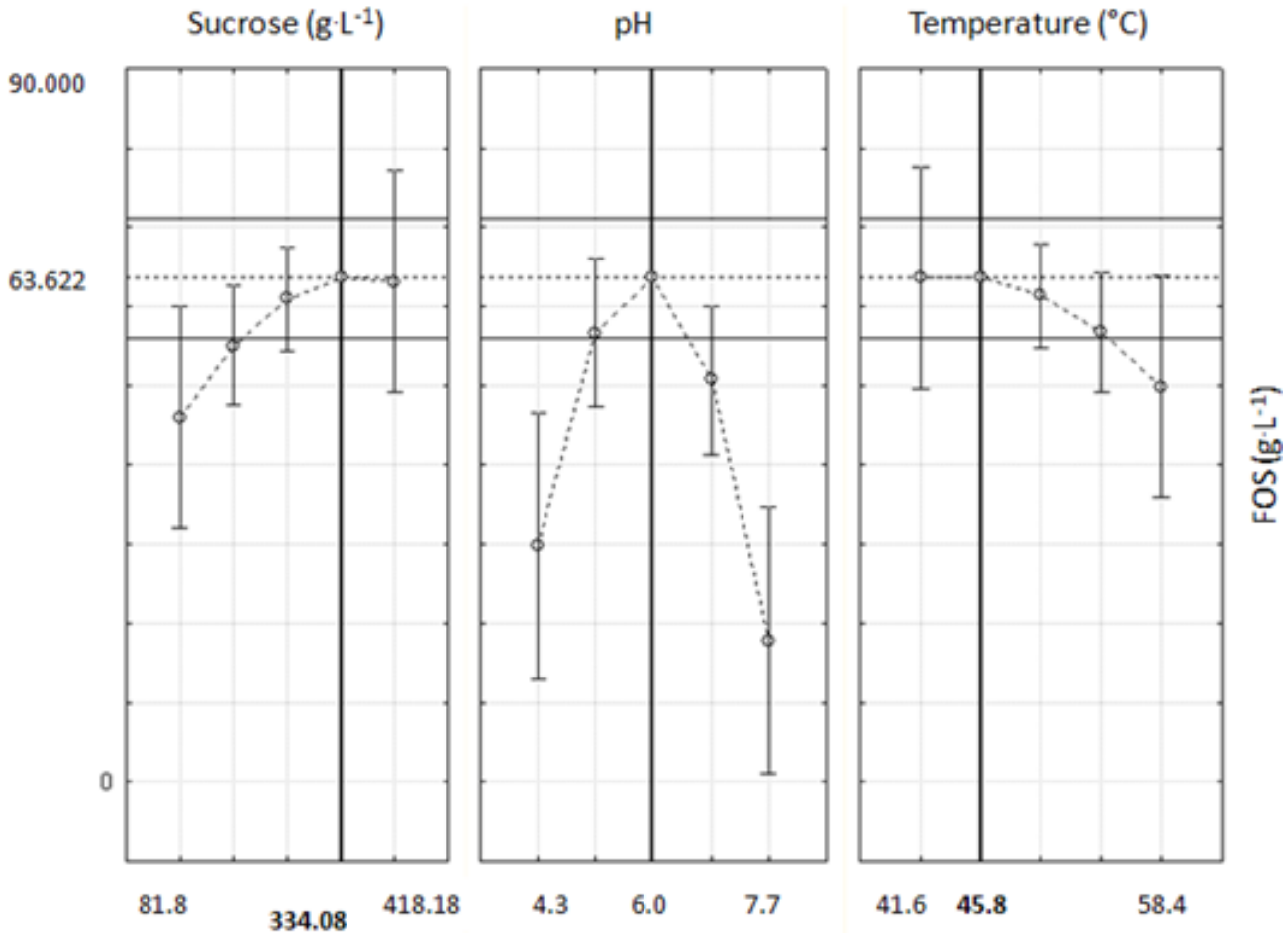

Figure 2. Optimization of the production of fructooligosaccharides . (Statistca Software 7.0).

values ranging between 5.0 and 6.5, as reported by Vega- Paulino and Zúniga (2012).

Levansucrase from *B. subtilis* natto synthesized fructooligosaccharides in all experimental conditions, showing its potential for use in an industrial process of producing FOS from sucrose. The response surface methodology allowed the determination of the best values of sucrose (334 $g.L^{-1}$), pH (6.0) and temperature (45.8°C) for a maximum yield of 54.86 $g.L^{-1}$ of FOS.

Conflicts of Interest

The authors declare that they have no conflict of interest.

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