American Journal of Food Technology

ISSN 1557-4571

Academic Journals Inc.

www.academicjournals.com

American Journal of Food Technology 9 (3): 144-150, 2014
ISSN 1557-4571 / DOI: 10.3923/ajft.2014.144.150

Optimization of High Production of Fructooligosaccharides by Sucrose Fermentation of *Bacillus subtilis* Natto CCT 7712

[1]Patricia Bittencourt da Silva, [2]Dionisio Borsato and [1]Maria Antonia Pedrine Colabone Celligoi

[1]Department of Biochemistry and Biotechnology, State University of Londrina, Rodovia Celso Garcia Cid, PR 445 Km 380, Londrina, Parana, Brazil

[2]Department of Chemistry, State University of Londrina, Londrina, Parana, Brazil

Corresponding Author: Patricia Bittencourt da Silva, Department of Biochemistry and Biotechnology, State University of Londrina, Rodovia Celso Garcia Cid, PR 445 Km 380, Londrina, Parana, Brazil

ABSTRACT

Fructooligosaccharides (FOS) are fructose oligomers containing a glucose residue at the end of the chain and its production takes place by the action of fructosyltransferase in plants and micro-organisms. FOS have functional properties that make them important food ingredients, with functions of regulating lipid metabolism, increased intestinal calcium absorption and being prebiotic selectively stimulating the growth and/or activity of beneficial bacteria to the body. *Bacillus subtilis* natto produces levan by fermentation in medium rich in sucrose. Levan is synthesized by the enzyme levansucrase which is also responsible for the synthesis of FOS. The aim of this study was to evaluate by statistical methodology, using a central composite factorial design, the influence of varying sucrose concentration (X_1), pH (X_2) and agitation (X_3) in the formation of FOS by *B. subtilis* natto CCT 7712. Fermentations were conducted in 125 mL Erlenmeyer flasks containing 25 mL of culture medium, the media were inoculated with 0.2 g L^{-1} cells and incubated on orbital shaker at 37°C for 24 h, the other parameters were adjusted according to the experimental design. The production of FOS was accompanied by High Performance Liquid Chromatography (HPLC) using kestose and nystose as standards. The analysis showed that the pH was significant. The optimum conditions were determined sucrose concentration of 300 g L^{-1}, pH 7.7 and agitation 234 rpm, where production was 98.86 g L^{-1} and did not differ statistically from the expected value of 106.98 g L^{-1}.

Key words: *Bacillus subtilis* natto, levansucrase, fructooligosaccharides, prebiotics

INTRODUCTION

The concern with a healthy lifestyle has increased the search for functional foods. The global market moved back to this area, at least 33 billion dollars in 2001 and only in the U.S. market demand for functional foods was approximately $15 billion (Menrad, 2003). Among the products that confer health benefits are some specific types of dietary carbohydrates, especially Fructooligosaccharides (FOS) which has become popular due to the physiological effects provided to consumers (Patel and Goyal, 2011).

FOS are carbohydrates of low molecular mass containing sugar residues with a degree of polymerization between 3 and 9, in which the fructosyl units are linked by β-position (2>1) sucrose,

among the most frequent oligofructoses are 1-kestose (GF2), nystose (GF3) and fructofuranosyl nystose (GF4) (Yun, 1996). The connection type this makes them not susceptible to hydrolytic activity of the human digestive enzymes, possessing important physiological function as prebiotics (Biedrzycka and Bielecka, 2004). Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of a limited number of bacteria present in the colon, improving host health (Gibson and Roberfroid, 1995).

Besides stimulating the growth of bifidobacteria and lactobacilli, there is the production of short chain fatty acids as end products of fermentation. These acids stimulate the growth of colorectal mucosal cells, retarding atrophy of the mucosa and reducing the risk of harmful changes to the colon (Ningegowda and Gurudutt, 2012). Other features of the FOS have had special attention as the low calorific value, the effects in reducing levels of phospholipids, triglycerides and cholesterol, aid in the absorption of calcium and magnesium from the gut (Mussatto and Teixeira, 2010).

Although the FOS can be found in various natural sources such as asparagus, beets, onions, garlic and chicory, its concentration in these sources is low and its production is limited by climatic conditions (Yun, 1996). Industrially, the FOS is mainly produced from sucrose by microbial enzymes with activity transfructosilation (β-frutofuranosidases, also called fructosyltransferases) (Park *et al*., 2003). Conventionally, the industrial production of FOS by microorganisms includes a two-stage process and another process in a single step. At first, is initially taken enzyme production by microbial fermentation, followed by reaction with the enzyme substrate for the production of FOS. The second process is carried out with the enzyme production and enzymatic reaction in a single fermentation process. The latter process does not depend on the purification of the enzyme, enabling the production of FOS with only one step, coupled with the lower cost of production (Ning *et al*., 2010).

There are several micro-organisms that possess transfructosilation enzymes among fungi *Aspergillus japonicus* (Mussatto *et al*., 2009; Mussatto and Teixeira, 2010), *Aspergillus oryzae* (Sangeetha *et al*., 2005a), *Aureobasidium pullulans* (Shin *et al*., 2004) and bacteria such as *Mycobacterium laevaniformans* (Park *et al*., 2003), *Zymomonas mobilis* (Bekers *et al*., 2002), *Bacillus circulans* (El-Refai *et al*., 2009) and *B. subtilis* (Abdel-Fattah *et al*., 2005; Euzenat *et al*., 1997).

B. subtilis natto has the enzyme levansucrase (EC 2.4.1.10), one fructosyltransferase that performs the formation of levan, a homopolymer linked β-(2>6) branched β-(1>2). Besides the formation of this polymer, levansucrase catalyzes the formation fructo-oligosaccharides (Abdel-Fattah *et al*., 2005).

The FOS production is affected by several variables such as the sucrose concentration in the fermentation medium, pH, time of cultivation, among others (Sangeetha *et al*., 2005b) which makes the response surface methodology a tool to optimize the conditions of culture medium and other critical variables for production using a smaller number of experimental tests (Liu *et al*., 2010).

This study aimed to select the best conditions for the production of fructooligosaccharides by fermentation by *B. subtilis* natto CCT7712 using response surface methodology.

MATERIALS AND METHODS

Microorganism and media: *Bacillus subtilis* natto CCT 7712 was maintained at 4°C on slant medium containing (g 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 Londrina State University (Brazil) and identified by Fundacao Andre 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 in a for 48 h.

The inoculum was prepared from stock culture which was transferred to Erlenmeyer flask (125 mL) containing 25 mL of medium, maintained at 37°C, agitation 150 rpm for 48 h. After incubation, the medium was centrifuged at 9056 g and the cells were resuspended in saline solution 0.9% (w/v) was then used to inoculate 0.2 g L^{-1} cells in different fermentation media. Fermentations were conducted in Erlenmeyer flasks (125 mL) containing 25 mL of fermentation medium, being carried out at 37°C for 24 h, sucrose, pH and stirring speed were adjusted according to the statistical design (Table 1). The cultures were stopped by centrifugation at 9056 g for 15 min at 4°C, then was determined FOS production in the supernatant of the fermentation.

Determination of fructooligosaccharides: 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 (300×7.8 mm Biorad). The column temperature was maintained at 80°C. The samples (20 μL) were eluted with 0.6 mL Milli-Q water/min 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 L^{-1}.

Central composite design: To check the best conditions for producing FOS by *B. subtilis* natto, we performed a factorial central composite design with the variables: sucrose concentration (X_1), pH (X_2) and agitation (X_3) for the production of fructooligosaccharides. The variables were coded by Eq. 1:

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

where, x_i is the coded level of variable X_i is the actual value of the variable, X_{cp} is the actual value of the variable at the center point and ΔX_i is the change in value at the actual levels. The parameters for the production of FOS sucrose concentrations were 216-384 g L^{-1}, pH 4.3 to 7.7 and agitation from 66 to 234 rpm (Table 1). The optimal response for the production of FOS was predicted following the quadratic model Eq. 2:

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

where, $\hat{Y}$ is the dependent variable, b_0 is the intercept, 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, x is the coded level of the variable.

Statistical analysis: Statistical analyses were performed using the software Statistica 7.0 (2004) and values were considered significant when $p<0.05$.

RESULTS

Production of fructooligosaccharides by fermentation by *Bacillus subtilis* natto was assessed in different ways. The optimization of the production of FOS was performed using a factorial central composite design. The results of the design are shown in Table 1 and 2.

Table 1: Coded and actual levels of factorial central composite design for fructooligosaccharides (FOS) production by *Bacillus subtilis* natto. The temperature set at 37°C and cultivation time 24 h

Assays	X_1	X_2	X_3	FOS production (g L^{-1})
1	-1 (250)	-1 (5)	-1 (100)	12.61
2	-1 (250)	-1 (5)	1 (200)	0.00
3	-1 (250)	1 (7)	-1 (100)	58.28
4	-1 (250)	1 (7)	1 (200)	91.98
5	1 (350)	-1 (5)	-1 (100)	10.77
6	1(350)	-1 (5)	1 (200)	0.00
7	1(350)	1 (7)	-1 (100)	53.61
8	1(350)	1 (7)	1 (200)	77.23
9	-1.68 (216)	0 (6)	0 (150)	44.89
10	1.68 (384)	0 (6)	0 (150)	0.00
11	0 (300)	-1.68 (4,3)	0 (150)	0.00
12	0 (300)	1.68 (7,7)	0 (150)	69.97
13	0 (300)	0 (6)	-1.68 (66)	11.39
14	0 (300)	0 (6)	1.68 (234)	62.78
15	0 (300)	0 (6)	0 (150)	64.86
16	0 (300)	0 (6)	0 (150)	82.01
17	0 (300)	0 (6)	0 (150)	48.56

X_1: Sucrose concentration, X_2: pH, eXx_3: Agitation

Table 2: Analysis of variance (ANOVA) for the quadratic model of fructooligosaccharides (FOS) production by *B. subtilis* natto in sucrose medium

Parameters	Sum of squares	Degree of freedom	Mean square	p-value
Sucrose (L)	684.96	1	684.96	0.1693
pH (L)	10296.05	1	10296.05	0.0005*
Agitation (L)	1060.19	1	1060.19	0.0983
Sucrose (Q)	1969.51	1	1969.51	0.0355*
pH (Q)	872.09	1	872.09	0.1274
Agitation (Q)	729.75	1	729.75	0.1578
x_1x_2	38.63	1	38.63	0.7267
x_1x_3	8.49	1	8.49	0.8694
x_2x_3	814.06	1	814.06	0.1388
Pure error	559.57	2		
Lack of fit	1467.02	5	279.79	0.5541

$R^2 = 0.88$

Results were statistically analyzed by the coefficient of determination (R^2) was 0.882, this indicates that 88.20% of the variability of the responses can be explained by the model, this value was considered acceptable as the authors describe (Joglekar and May, 1990) that the coefficient of determination must be at least 80%. The lack of fit of the model was evaluated, presenting not significant ($p = 0.5541$), indicating that the model equation was adequate for predicting the production of FOS, under any combination of variable values.

B. subitlis natto has shown promise in producing FOS oligofructose reaching values of 91.98 g L^{-1} in trial 4 (250 g L^{-1} sucrose, pH 7 and 200 rpm) Table 1. From the regression variables, it was found that the negative effect of sucrose while agitation and pH showed a positive effect on the production of FOS.

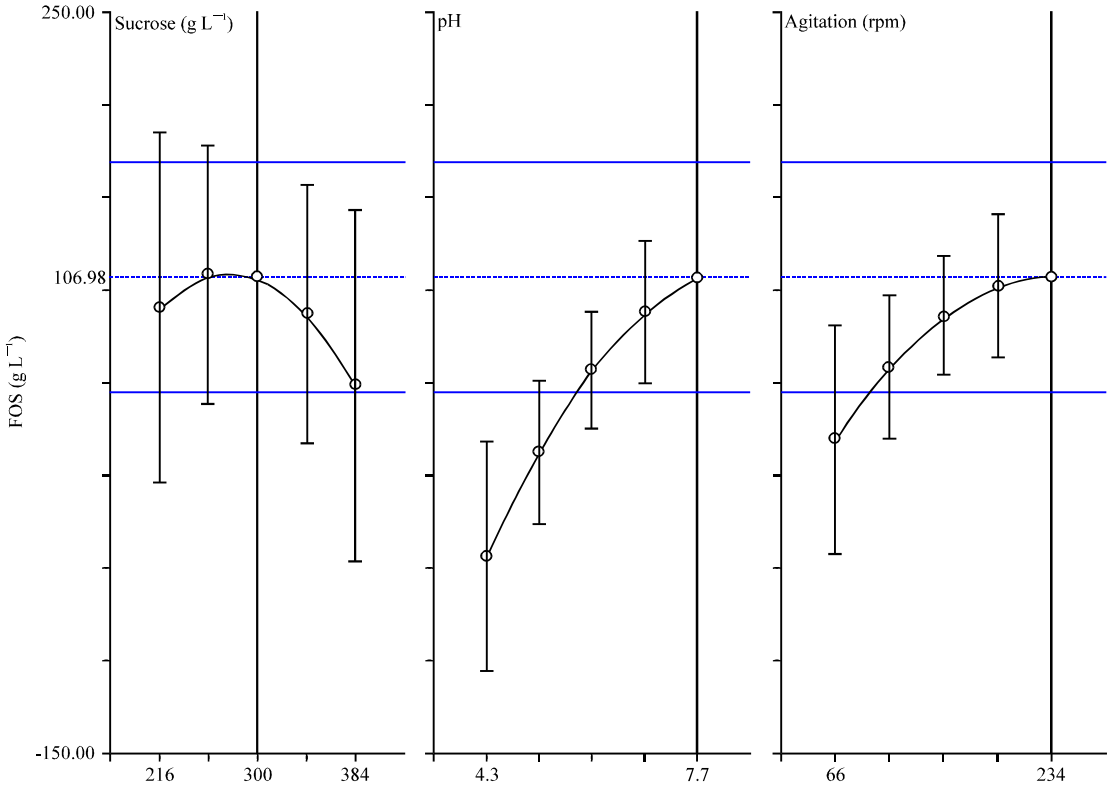

Fig. 1: Optimization of the production of FOS by *B. subtilis* natto using statistca software 7.0

The analysis of the regression Eq. 3 shows that only the intercept of the linear term and quadratic term pH of sucrose were significant at the 5% level, these variables had no interactions (Table 2). Comparing the tests 2 and 4, the same concentration of sucrose (250 g L^{-1}) and the same agitation (200 rpm), there is not a production of FOS when the pH was changed from 7 to 5, demonstrating the strong effect this parameter in the production of oligofructose.

The variable acts on sucrose FOS production but in very high values of 384 g L^{-1} (test 10) the null and has been output in the tests 15, 16 and 17 with 300 g L^{-1} (core) The average yield was 65.14 g L^{-1}.

Figure 1 optimizes the best conditions for the production of FOS obtained by the predictive model, indicating that the concentration of 300 g L^{-1} of sucrose, pH 7.7 and agitation of 234 rpm allows the maximum theoretical production of 106.98 g L^{-1} of FOS. With the aim of validating the model equation, three additional tests under optimum conditions described were performed. The average value of FOS was 98.86 g L^{-1} which is very close to the predicted, when compared by Tukey test at 5%, were not statistically different.

From the regression coefficients was obtained the following second order polynomial equation for predicting the production of FOS:

$$Y = 64{,}59-7{,}08x_1+27{,}49x_2+8{,}81x_3-13{,}18x_1^2-8{,}75x_2^2-8{,}01x_3^2-2{,}19x_1x_2-1{,}03x_1x_3+10{,}08x_2x_3 \quad (3)$$

where, Y is the predicted response to FOS (g L^{-1}), x_1, x_2 and x_3 are the coded values for sucrose, pH and agitation, respectively.

DISCUSSION

The production of FOS was performed by extracellular enzyme levansucrase (EC2.4.1.10) that presents the free fermentation medium. Thus, the production of levan and FOS is subject to environmental conditions of fermentation, it was verified by sucrose concentration and pH variables that affect the activities of the two levansucrase, hydrolysis and transfructosilation.

The concentration of sucrose to FOS production was better with 342 g L^{-1} close to the result found by Abdel-Fattah *et al.* (2005). These authors observed that concentrations that high concentrations of sucrose stimulated the production of fructo-oligosaccharides by levansucrase from *Bacillus subtilis* NRC 33. Da Silva (2008) studied the FOS production by fermentation by *Kluyveromyces marxianus* and *Saccharomyces cerevisiae* which reached a maximum of 2.38 and 0.73 g L^{-1}, respectively. Coimbra *et al.* (2005) achieved a production of FOS 32.5 g L^{-1}in the fermentation with *Zymomonas mobilis* conducted for 24 h at 40°C, 100 rpm and 150 g L^{-1} sucrose by the microorganism.

In the present study, the *Bacillus subtilis* natto has been shown to produce large amounts of fructooligosaccharides and the results obtained indicate that the maximum yield of 98.86 g L^{-1} of FOS can be achieved in 300 g L^{-1} of sucrose, pH 7.7 and agitation 234 rpm. These results make this promising microorganism for the production of industrially oligofructose.

ACKNOWLEDGMENT

The authors acknowledge the financial support provided by CAPES-Brazil.

REFERENCES

Abdel-Fattah, A.F., D.A.R. Mahmoud and M.A.T. Esawy, 2005. Production of levansucrase from *Bacillus subtilis* NRC 33a and enzymic synthesis of levan and fructo-oligosaccharides. Curr. Microbiol., 51: 402-407.

Bekers, M., J. Laukevics, D. Upite, E. Kaminska, A. Vigants, U. Viesturs, L. Pankova and A. Danilevics, 2002. Fructooligosaccharide and levan producing activity of *Zymomonas mobilis* extracellular levansucrase. Process Biochem., 38: 701-706.

Biedrzycka, E. and M. Bielecka, 2004. Prebiotic effectiveness of fructans of different degrees of polymerization. Trends Food Sci. Technol., 15: 170-175.

Coimbra, C.G.O., A.V. Lobato and G.M.T. Calazans, 2005. Perfil da producao de oligossacarideos na fermentacao de sacarose por zymomonas mobilis. Proceedings of the 15th Simposio Nacional de Bioprocessos, August 2-5, 2005, Recife, Brasil.

Da Silva, C.E.V., 2008. Producao enzimatica de frutooligossacarideos (FOS) por leveduras a partir de melaco de cana-de-acucar [Production of fructooligosaccharides from sugarcane molasses by Saccharomyces cerevisiae and Kluveromyces marxianus cells]. Master's Thesis, Escola Superior de Agricultura Luiz de Queiroz, Universidade de Sao Paulo, Piracicaba, Brazil.

El-Refai, H.A., A.F. Abdel-Fattah and F.A. Mostafa, 2009. Enzymic synthesis of levan and fructo-oligosaccharides by *Bacillus circulans* and improvement of levansucrase stability by carbohydrate coupling. World J. Microbiol. Biotecnol., 25: 821-827.

Euzenat, O., A. Guibert and D. Combes, 1997. Production of fructo-oligosaccharides by levansucrase from *Bacillus subtilis* C4. Process Biochem., 32: 237-243.

Gibson, G.R. and M.B. Roberfroid, 1995. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J. Nutr., 125: 1401-1412.

Joglekar, A.M. and A.T. May, 1990. Product Excellence through Design of Experiments. In: Food Product Development: From Concept to the Marketplace, Graf, E. and I.S. Saguy (Eds.). Springer, New York, ISBN: 0-8342-1689-2, pp: 211-229.

Liu, J., J. Luo, H. Ye, Y. Sun, Z. Lu and X. Zeng, 2010. Medium optimization and structural characterization of exopolysaccharides from endophytic bacterium *Paenibacillus polymyxa* EJS-3. Carbohydr. Polym., 79: 206-213.

Menrad, K., 2003. Market and marketing of functional food in Europe. J. Food Eng., 56: 181-188.

Mussatto, S.I., C.N. Aguilar, L.R. Rodrigues and J.A. Teixeira, 2009. Colonization of *Aspergillus japonicas* on synthetic material and application to the production of fructooligosaccharides. Carbohydr. Res., 344: 795-800.

Mussatto, S.I. and J.A. Teixeira, 2010. Increase in the fructooligosaccharides yield and productivity by solid-state fermentation with *Aspergillus japonicas* using agro-industrial residues as support and nutrient source. Biochem. Eng. J., 53: 154-157.

Ning, Y., J. Wang, J. Chen, N. Yang, Z. Jin and X. Xu, 2010. Production of neo-fructooligosaccharides using free-whole-cell biotransformation by *Xanthophyllomyces dendrorhous*. Bioresour. Technol., 101: 7472-7478.

Ningegowda, M.A. and P.S. Gurudutt, 2012. *In vitro* fermentation of prebiotics by *Lactobacillus plantarum* CFR 2194: Selectivity, viability and effect of metabolites on β-glucuronidase activity. Word J. Microbiol. Biotechnol., 28: 901-908.

Park, H.E., N.H. Park, M.H. Kim, T.H. Lee. H.G. Lee, J.Y. Yang and J. Cha, 2003. Enzymatic synthesis of fructosyl oligosaccharides by levansucrase from *Microbacterium laevaniformans* ATCC 15953. Enzyme Microb. Technol., 32: 820-827.

Patel, S. and A. Goyal, 2011. Functional oligosaccharides: Production, properties and applications. World J. Microbiol. Biotechnol., 27: 1119-1128.

Sangeetha, P.T., M.N. Ramesh and S.G. Prapulla, 2005a. Fructooligosaccharide production using fructosyl transferase obtained from recycling culture of *Aspergillus oryzae* CFR 202. Process Biochem., 40: 1085-1088.

Sangeetha, P.T., M.N. Ramesh and S.G. Prapulla, 2005b. Recent trends in the microbial production, analysis and application of fructooligosaccharides. Trends J. Food Sci. Technol., 16: 442-457.

Shin, H.T., S.Y. Baig, S.W. Lee, D.S. Suh, S.T. Kwon and Y.B.Lim, 2004. Production of fructo-oligosaccharides from molasses by *Aureobasidium pullulans* cells. Bioresour. Technol., 93: 59-62.

Yun, J.W., 1996. Fructooligosaccharides-occurrence, preparation and application. Enzyme Microbial Technol., 19: 107-117.