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Synthesis of Fructooligosaccharides from Sucrose Using Inulinase from *Kluyveromyces marxianus*

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Summary

Fructooligosaccharides (FOS) from sucrose, new alternative sweeteners with functional properties, also called soluble fibers, have a number of desirable characteristics such as low calories, no cariogenicity, and safety for diabetics and Bifidus stimulating factor. Fructooligosaccharides are also known as prebiotics, since they stimulate probiotic organisms. The production, as well as the application of food-grade fructooligosaccharides, has increased rapidly during last years. In this work, experimental factorial design has been applied to optimize the fructooligosaccharide synthesis conditions by inulinase from *Kluyveromyces marxianus* var. *bulgaricus*. The studied variables were: temperature, pH, sucrose and enzyme concentrations. According to the results, only temperature and sucrose concentrations have shown to be significant parameters. The syntheses of the fructooligosaccharides were carried out on stirred reactor and packed bed reactors, using free and immobilized enzymes, with the best conditions obtained from the experimental design. It has been shown that there is no significant difference between these processes. The final sugar concentrations can be tailor made by varying residence time in the reactor to cope with the different standard needs in food industries. A typical solution product consists of a mixture of fructose (155 g/L), glucose (155 g/L), sucrose (132 g/L) and fructooligosaccharides (50 g/L). These concentrations are suitable for applications in most food industries, in products such as sweets, candies, chocolates and yogurts. Besides, the prebiotic function of fructooligosaccharides as stimulants of the beneficial intestinal flora will give the product a functional and differentiated feature.

Key words: Kluyveromyces marxianus, fructooligosaccharides, inulinase, functional foods, experimental design

Introduction

Fructooligosaccharides represent one of the major classes of bifidogenic oligosaccharides in terms of their production volume (*1*). They have been defined as a combination of three sugars: 1-kestose (β-D-fru-$(2\rightarrow1)_2$-α-D-glucopyranoside, GF_2), nystose (β-D-fru-$(2\rightarrow1)_3$-α-D-glucopyranoside, GF_3) and fructofuranosylnystose (β-D-fru-$(2\rightarrow1)_4$-α-D-glucopyranoside, GF_4), in which fructosyl units (F) are bound at the β(2→1) position of sucrose (*2–4*).

Many carbohydrates are reported to be prebiotic, including fructooligosaccharides (FOS), galactooligosaccharides (GOS), isomaltooligosaccharides (IMO) and lactulose (*5,6*). The term prebiotic has been defined as a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of a limited number of bacteria in the colon (*7,8*). FOS represent a selective nutrient for beneficial microorganisms and they have the potential to increase the effectiveness of probiotic products. Furthermore, studies

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carried out in Japan indicate that consumption of FOS shifts the balance of microflora in the intestine towards greater populations of *Bifidobacteria* and other beneficial microorganisms even in the absence of a probiotic added to the diet. To obtain the efficiency of FOS in intestine, the administration of about 8 g of FOS per day to adult is necessary (*8,9*).

Recent developments in industrial enzymology have made possible the large-scale production of FOS by enzymatic synthesis. It appears that industrial process for the production of FOS can be divided in two classes: the first is the batch system using a soluble enzyme, and the other is the continuous process using immobilized enzyme or whole cells (*4–6*).

The synthesis of fructooligosaccharides is studied using enzymes with high transfructosylation activity, where the best enzymes are from fungi such as *Aspergillus niger, Aspergillus japonicus, Aureobasidium pullulans,* and *Fusarium oxysporum* (*10–14*).

In this work, the production of fructooligosaccharides by inulinase from *Kluyveromcyes marxianus* var. *bulgaricus* is described. This process implies the possibility of employment of inulinase in industrial production of FOS from sucrose using stirred or packed bed reactors, in batch or continuous process, with free or immobilized inulinase (*15,16*).

Materials and Methods

Enzyme production

Inulinase was produced in shake flasks, in 72 h of fermentation at pH=3.0 and 30 °C. The medium composition was (in g/L): sucrose 30, yeast extract 20, peptone 20 and K_2HPO_4 5. The fermented medium was centrifuged at 10 000 × *g* for 15 min, at 4 °C (Sorvall RC-26 Plus). The precipitation of the enzyme was carried out by adding ethanol up to 70 % final concentration to the supernatant. The temperature was kept as low as possible, using ethanol at –20 °C, with mild agitation. After the addition of ethanol, the solution was centrifuged at 10 000 × *g* for 15 min, at 4 °C. The precipitated enzyme was diluted in 0.1 M phosphate buffer, pH=5.2, and stocked at –20 °C.

Immobilization in calcium alginate

The immobilization of inulinase was carried out by adding 10 mL of enzyme solution to 10 mL of 3.5 % (by mass per volume) sodium alginate solution. The enzyme solution was then dropped into a 0.2 M calcium chloride solution under agitation, forming pellets that were kept in this solution for 2 h. The pellets were washed and stored in a 0.05 M calcium chloride solution.

Enzyme activity

Inulinase activities were measured according to the concentration of reducing sugars released from sucrose, as fructose equivalent, after incubation of 9.0 mL of sucrose solution (2 % by mass per volume in 10 mM sodium acetate buffer, pH=5.0), with 1.0 mL of enzyme solution or 1 mL of particles for the immobilized enzyme, at 50 °C. In the latter case, the volume of particles was measured according to the volume of water placed in a graduated tube. Reducing sugars were measured according to the dinitrosalicylic acid (DNS) method (*17*). One unit of inulinase activity was defined as the amount of enzyme that hydrolyses 1.0 µmoL of sucrose per minute. The volumetric activity (µmol/(min·mL)) was defined as the enzyme activity per mL of enzyme solution for free enzyme, and per mL of particle volume for immobilized enzyme.

Experimental design

Factorial design and response surface analysis are important tools to determine the optimal process conditions. Factorial design of a limited set of variables is advantageous compared to the conventional methods, which handle a single parameter per trial, and they frequently fail to locate optimal conditions due to the fact that they do not consider the effect of possible interactions between factors (*18*). For most multivariable processes, such as biochemical systems, in which numerous potentially influential factors are involved, it is important to test as many variables as possible. Hence, it is necessary to submit the process to an initial screening. Fractional factorial design is a methodology that makes it possible to establish an initial selection of the significant variables that influence the process, using a few numbers of trials, instead of using more extensive factorial design, which would furnish more complete information, but which involves unfeasible complexity (*18,19*).

This work used the methodology of fractional design to select the reaction condition leading to the highest production of FOS from sucrose by the inulinase of *Kluveromyces marxianus* var. *bulgaricus*. The variables were sucrose, enzyme concentration, temperature and pH. Real values and coded levels used in the fractional design are shown in Table 1. The 2^{4-1} factorial design is shown in Table 2.

Table 1. Real values and coded levels used in the factorial design

Variables	–1	0	+1
pH	5.0	5.5	6.0
t/°C	40	45	50
γ_S/(g/L)	450	550	650
γ_E/(IU/mL)	4	5	6

γ_S=sucrose concentration, γ_E=enzyme concentration

Fructooligosaccharide synthesis

Stirred reactor

The fructooligosaccharide production was carried out in jacket reactors with total volume of 250 mL. In reactors containing 150 mL of 500 g/L sucrose solution in 0.1 M sodium acetate buffer (pH=5.0), 6 IU/mL of enzyme preparation, free or immobilized, were added. All processes were carried out at 50 °C.

Packed bed reactor

The packed bed reactor consisted of a glass column measuring 2.3 × 25 cm, containing 60 cm^3 of 6 IU/mL of

Table 2. Real (r) and coded (cd) values for the fractional (2^{4-1}) factorial design and glucose, fructose, sucrose and fructooligosaccharide production

Run	Independent variables								Dependent variables/%			
	pH		t/°C		γ_S/(g/L)		γ_E/(IU/mL)		Glucose	Fructose	Sucrose	FOS
	r	cd	r	cd	r	cd	r	cd				
01	5.0	–1	40	–1	450	–1	4.0	–1	38.31	29.83	25.88	5.98
02	6.0	+1	40	–1	450	–1	6.0	+1	21.14	12.16	59.99	5.92
03	5.0	–1	50	+1	450	–1	6.0	+1	35.48	28.77	26.92	8.81
04	6.0	+1	50	+1	450	–1	4.0	–1	38.56	31.33	17.46	12.65
05	5.0	–1	40	–1	650	+1	6.0	+1	32.27	29.59	33.17	5.00
06	6.0	+1	40	–1	650	+1	4.0	–1	16.34	14.76	66.64	4.26
07	5.0	–1	50	+1	650	+1	4.0	–1	32.00	30.88	33.24	3.87
08	6.0	+1	50	+1	650	+1	6.0	+1	32.62	30.13	31.29	4.63
09	5.5	0	45	0	550	0	5.0	0	31.59	31.48	22.59	4.95
10	5.5	0	45	0	550	0	5.0	0	33.96	35.17	26.39	4.47
11	5.5	0	45	0	550	0	5.0	0	33.71	35.41	26.50	5.21

FOS=fructooligosaccharides: kestose+nystose+1-fructosylnystose

immobilized enzyme preparation. The column was fed with 500 g/L of sucrose solution in 0.1 M sodium acetate buffer, pH=5.0, at 50 °C, at different flow rates.

The column residence time (τ) was calculated using the following equations:

$$\tau = \frac{V_{bed}}{F} \qquad /1/$$

$$V_{bed} = V_{column} - V_{particles} \qquad /2/$$

where V_{bed} is bed volume (cm^3), V_{column} is column total volume (cm^3), $V_{particle}$ is particle total volume containing the immobilized inulinase (cm^3), and F is feed flow rate to the column (cm^3/h).

Sugar analysis by HPLC-PAD

Samples were taken at regular time intervals and analyzed by high-performance liquid chromatography with pulsed amperometric detection (HPLC-PAD) method to identify and quantify sugars and fructooligosaccharides using standard curve of kestose, nystose and 1-fructosylnystose (Wako Industry, Japan) and sucrose, glucose and fructose (Merck). A Dionex DX500 system (Sunnyvale, CA, USA) ion chromatograph was utilized. This equipment consists of a GP50 gradient pump, ED40 electrochemical detector and CarboPac PA100 analytical anion exchange column (250 × 4 mm) equipped with guard column CarboPac PA100 (50 × 4 mm). Gradient elution was applied using two solvents: 0.1 M aqueous sodium hydroxide (A) and 0.1 M aqueous sodium hydroxide containing 0.6 M sodium acetate (B). Both eluents were prepared with ultrapure water and degassed by nitrogen bubbling. The volume of injection was 25 μL. Total run time per sample was 36 min. The elution began with 100 % of solvent A for 10 min at a flow rate of 1 mL/min. This was followed by linear gradient from 0–90 % of solvent B in solvent A for 15 min. Then the column was washed for 5 min with 100 % of B. The next injection was performed after equilibrating the column with 100 % solvent A for 5 min.

Results and Discussion

The syntheses of fructooligosaccharides were carried out according to the fractional factorial design shown in Table 2, in order to identify the appropriate reaction conditions. Temperature, pH, sucrose and enzyme concentrations were the studied factors, while the FOS concentration was the main response variable. The experimental design, with independent and dependent (glucose, fructose, sucrose, and FOS (kestose, GF_2; nystose, GF_3; and 1-fructosylnystose, GF_4)) variables, is shown in Table 2. The estimated effects are presented in Table 3. All data were obtained at 12 h of synthesis.

The main effects are estimated by evaluating the differences in process performance caused by a change from low (–1) to high (+1) levels of the corresponding factor (*18*). The process was evaluated by the production

Table 3. Estimated effects for fructooligosaccharide production from fractional design at 12 h of synthesis

	Estimated effect	Standard error	T	p	Conf. limit –95 %	Conf. limit +95 %
Mean	5.977	0.113	52.807	0.004	5.490	6.464
pH	0.950	0.265	3.578	0.069	–0.192	2.092
t/°C*	2.200	0.265	8.287	0.142	1.057	3.342
γ_S/(g/L)*	–3.900	0.265	–14.692	0.004	–5.042	–2.757
γ_E/(IU/mL)	–0.600	0.265	–2.260	0.152	–1.742	–0.542

*significant factors

of fructooligosaccharides, glucose, fructose and sucrose hydrolysis as the response. As it can be seen in Table 3, only temperature and sucrose concentration are significant factors. Temperature presents a positive effect on fructooligosaccharide production when an increase from level –1 (40 °C) to +1 (50 °C) is performed. The best result was achieved in run 4 (pH=6.0, 50 °C, 450 g/L and 4 IU/mL).

Synthesis of the fructooligosaccharides in stirred reactors

The syntheses were carried out, initially, in a glass jacketed stirred reactor. The production of the fructooligosaccharides is shown in Table 4 and Fig. 1. In all cases, kestose, nystose, glucose and fructose were detected. As it can be seen in Fig. 1, the concentration of fructooligosaccharides (kestose, nystose) as well as of glucose and fructose increased up to 3–4 h of reaction. However, at this time, fructooligosaccharides started being hydrolyzed, probably due to an inhibitory effect on the transfructosylation activity caused by the gradual increase of glucose and fructose concentrations. It is clear that the process should be stopped at a precise time in order to obtain the maximum concentration of fructooligosaccharides. As it can be seen in Table 4, the maximum concentration is achieved at 3–4 h (about 50 g/L of FOS). This kind of mechanism has also been verified in other works (*20–23*). After 7 h, as shown in Fig. 1, almost all fructooligosaccharides are hydrolyzed.

Inulinase from *Kluyveromyces marxianus* var. *bulgaricus* is well known for its hydrolytic activity, rather than for its transfructosylation behaviour. Similar results have been shown with the invertase from *Saccharomyces cerevisae*. This enzyme, when in high sucrose concentration solutions, synthesizes non-reductor trisaccharides (1-kestose, 6-kestose, and neokestose), and reductor monosaccharide (D-glucose and D-fructose) (*24*).

Synthesis of the fructooligosaccharides in fixed bed reactors

The FOS was produced continuously by immobilized inulinase in fixed bed reactor. Inlet medium composition was always 50 % sucrose solution at pH=5.0. The feed flow was changed in order to have different residence time. The FOS profile as a function of residence time (Table 5 and Fig. 2) is similar to the one in batch and continuous stirred reactor, as well as the maximum concentration of oligosaccharides. Also, the residence time for maximum oligosaccharide concentration

Table 4. Fructooligosaccharide production by immobilized inulinase in stirred reactor

Time/h	γ/(g/L)					
	Glucose	Fructose	Sucrose	Kestose	Nystose	FOS
0	0	0	500.0	0	0	0
0.5	19.5	24.3	407.1	11.1	7.0	18.1
1.0	24.9	36.5	317.0	17.6	12.4	30.0
2.0	94.7	87.3	246.3	25.2	13.5	38.6
3.0	128.9	116.8	183.7	26.0	24.2	50.2
4.0	146.9	140.0	125.3	23.7	26.3	50.0
5.0	171.9	159.1	67.3	6.0	29.8	35.8
6.0	199.2	190.1	48.0	1.3	9.0	10.3
7.0	215.2	211.5	41.7	1.2	7.8	9.0

FOS=fructooligosaccharides: kestose+nystose

Table 5. Fructooligosaccharide production from sucrose using immobilized enzyme in calcium alginate on continuous packed bed reactor

Residence time τ/h	γ/(g/L)			
	Glucose	Fructose	Sucrose	FOS
0	0	0	500.00	0
1.5	42.46	46.16	355.56	4.44
2.0	142.88	120.99	151.96	27.33
2.5	196.23	178.94	100.00	24.88
4.0	157.01	158.49	107.86	44.32
5.0	167.64	160.37	129.86	18.76
12.0	227.59	211.19	39.66	17.23

FOS=fructooligosaccharides: kestose+nystose

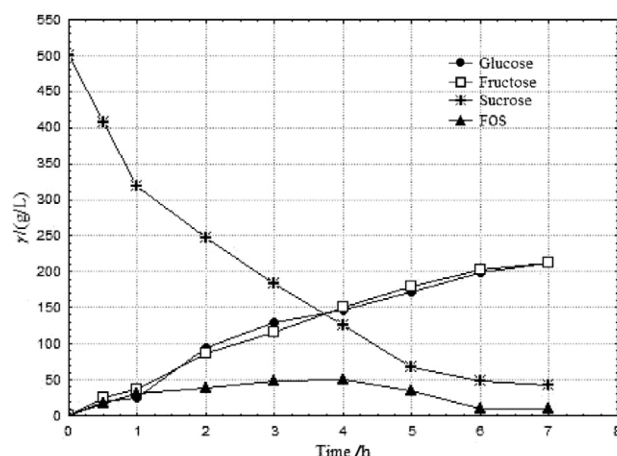

Fig. 1. Fructooligosaccharide production by immobilized inulinase in stirred reactor from 500 g/L of sucrose solution at pH=5.0 and 50 °C

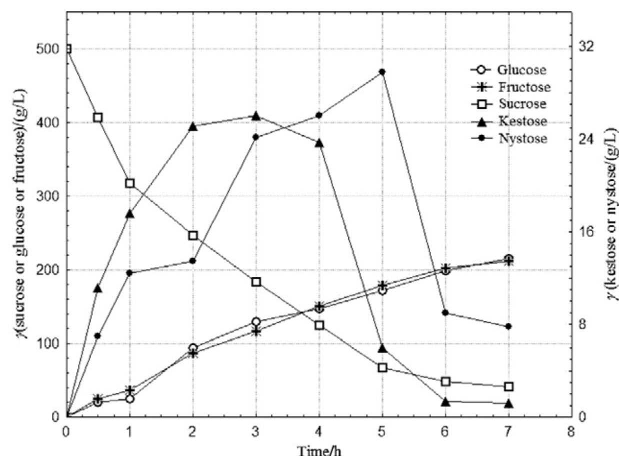

Fig. 2. Fructooligosaccharide production by immobilized inulinase in packed bed reactor from 500 g/L of sucrose solution at pH=5.0 and 50 °C

was about 4 h, which was similar to batch reactor reaction time, achieving 44.32 g/L of FOS. This represents a feed rate of 0.18 mL/min. When considering residence times greater than 4 h, a more accentuated hydrolysis of FOS can be expected.

In Fig. 3, all the producing methods are put together (free, immobilized on batch and packed bed reactor). It can be seen that there is a slight difference among all processes, with a maximal production of about 50 g/L of FOS. These processes allow the production of fructooligosaccharides in form of syrup, whose composition and concentration are convenient for products such as soda, fruit juices, yogurts, candies and desserts, which will give them an additional functional property due to the prebiotic oligosaccharides. Although the yields of fructooligosaccharides in these processes are lower than those obtained by other microorganisms, the FOS concentration in the syrup is enough for a daily administration to a human being, which is about 8 grams per adult (*8,9*). Besides, the possibility of producing different FOS concentrations at the outlet of the packed bed reactor by varying the residence time gives to this process an interesting feature, which is the production of a tailor made sugar solution.

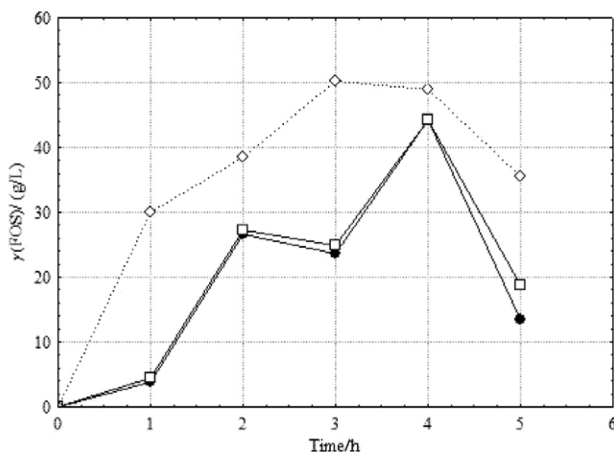

Fig. 3. Fructooligosaccharide production using three different reactors: batch stirred with free enzyme (–●–), batch stirred with immobilized enzyme (··◇··) and continuous packed bed reactor with immobilized enzyme (–□–)

Conclusion

The main goal of this work was to explore the inulinase capability of producing fructooligosaccharides from sucrose, using the inulinase from *Kluyveromyces marxianus* var. *bulgaricus*. The methodology of experimental design helped to find the significant process parameters. It has been shown that temperature and sucrose concentration have the most important roles in the synthesis of fructooligosaccharides. The best results were achieved at pH=6.0, temperature of 50 °C, 450 g/L of sucrose concentration and 4 IU/mL of enzyme activity. Different reactor processes (stirred batch with free and immobilized enzyme and continuous packed bed reactor with immobilized enzyme) were compared, with no significant differences among them.

The final composition of the product is a mixture of the fructose and glucose or a mixture of the glucose, fructose, sucrose and fructooligosaccharides, which can be tailor made, according to the reactor parameters, to fulfill the standards for applications in food products, such as sweets, candies, chocolates, yogurts, *etc*. Besides, the prebiotic properties of the fructooligosaccharides, which are beneficial stimulants of the intestinal flora, will give a functional and differentiated feature to the product.

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