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Optimization of levansucrase/endo-inulinase bi-enzymatic system for the production of fructooligosaccharides and oligolevans from sucrose

Feng Tian, Maryam Khodadadi, Salwa Karboune*

Department of Food Science and Agricultural Chemistry, McGill University, 21,111 Lakeshore, Ste-Anne de Bellevue, Quebec, Canada H9X 3V9

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ABSTRACT

A bi-enzymatic system based on the combined use of levansucrase (LS) from *Bacillus amyloliquefaciens* and endo-inulinase from *Aspergillus niger* in a one-step reaction was investigated for the synthesis of fructooligosaccharides (FOSs) and oligolevans using sucrose as the sole substrate. Sucrose concentration was the most important independent variable, whilst LS to endo-inulinase ratio exhibited significant effects on the end-product profiles. The interaction between sucrose concentration and reaction time exhibited significant effect on all responses. At the initial stage of time course, short chain FOSs (scFOSs, 1-kestose, nystose, 1^F-fructosylnystose) were the major products, whilst 6-kestose, medium chain fructooligosaccharides (mcFOSs, levanohexaose, levanopentaose) and oligolevans became the dominant ones at the late stage. The optimal conditions leading to a high yield of scFOSs (1:1 ratio, 0.5 h, 0.6 M) were different from those resulting in a high yield of mcFOSs and oligolevans (1.85:1 ratio, 1.77 h, 0.6 M). The bi-enzymatic system has a great potential for the production of FOSs and oligolevans at a large scale because of its high yield (57–65%, w/w) and productivity (65.8–266.8 g/L h), and its uses of low temperature (35 °C) and low concentration of sucrose. To the best of our knowledge, this is the first study on the optimization of a LS/endo-inulinase bi-enzymatic system.

1. Introduction

Fructooligosaccharides (FOSs) constitute a class of bioactive molecules whose potential health benefits in terms of supporting intestinal health and reducing the risk of cancers are increasingly being recognized. In addition, FOSs have been recognized by their low caloric value and non-carcinogenic properties [1,2]. Enzymatic strategies for the synthesis of FOSs are generally based on the transfructosylation of sucrose or the hydrolysis of inulin [3]. The hydrolysis strategy is limited by the seasonal availability of inulin. However, the production of FOSs from sucrose, by the action of fructofuranosidase or fructosyl-transferase through transfructosylation reaction, is a more cost effective and convenient route [4].

Levansucrases (LSs) (E.C.2.4.1.10), a subclass of fructosyltransferases, have recently gained more interest, because of their ability to directly use the free energy of cleavage of non-activated sucrose to transfer the fructosyl group to a variety of acceptors including mono- (exchange), oligosaccharides (FOS synthesis) or the growing fructan chain (polymer synthesis) [5]. In fact, β-(2→6)-levan-type-FOSs, obtained through LS-catalyzed transfructosylation, have shown beneficial prebiotic effects that surpass current commercial β-(2→1)-inulin-type FOSs [6]. In addition to their excellent water-holding capacity and protecting effect, β-(2→6)-levan polymers have shown antitumor and antidiabetic activities [7–9]. Hence, microbial LSs are of high interest as biocatalysts for the catalytic synthesis of novel type FOS prebiotics as well as levan for food, cosmetics, and pharmaceutical fields [10,11].

LSs differ widely with respect to their reaction specificity (hydrolysis/transfructosylation) and oligo-/polymerization ratio, and thus result in different product spectrum (i.e. levan or FOSs) [12–16]. Recently, some hypotheses and structural features have been put forward to describe the polymerization/oligomerization ratio [12,13,17,18]. In our previous studies, a bi-enzymatic system, based on the synergistic actions of LS from *Bacillus amyloliquefaciens* and endo-inulinase from *Aspergillus niger*, was, for the first time, investigated for the synthesis of FOSs and oligolevans using sucrose as an abundant substrate [19]. LS catalyzes the synthesis of levan from sucrose, whilst the endo-inulinase hydrolyses levan into FOSs and oligolevans. These transfructosylation products may be further hydrolyzed by endo-inulinase or serve as fructosyl acceptors for LS. The bi-enzymatic system showed higher yield and productivity of FOSs and oligolevans (67%, w/w; 96 g/L h) than the LS enzymatic system (3.0%, w/w; 0.8 g/L h) alone. As compared to other biocatalytic systems [20–22], the developed

* Corresponding author. Tel.: +1 514 398 8666; fax: +1 514 398 7977.
E-mail address: salwa.karboune@mcgill.ca (S. Karboune).

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bi-enzymatic system showed superior FOSs productivity and a broad product spectrum, whilst it used lower temperature and sucrose concentration. It has also been shown that the synthesis of transfructosylation products (FOSs and oligolevans) can be regulated by modulating the substrates and acceptors availabilities and the equilibrium of the involved reactions. Indeed, the contribution of endo-inulinase to the formation of FOSs and oligolevans through its hydrolytic activity was higher than that of LS and endo-inulinase through their acceptor reactions. However, the production of intermediate levans with appropriate molecular weight (MW) by LS was prerequisite for the production of FOSs and oligolevans. Investigation of the interactive effects of reaction parameters of bi-enzymatic system is, therefore, needed for better understanding of the synergistic interaction and the thermodynamic relationships between LS and endo-inulinase. As part of the ongoing research work in our laboratory, the specific objectives of the present study were (1) the investigation of the interactive effects of selected reaction parameters of bi-enzymatic system on the production of total transfructosylation products and, in particular, of short chain fructooligosaccharides (scFOSs: 1-kestose, 6-kestose, nystose and 1^F-fructosylnystose), medium chain ones (mcFOSs: levanopentaose, levanohexaose) and oligolevans using response surface methodology (RSM), (2) the development of mathematical models to produce transfructosylation products with targeted yield, specific end-product profile and structural properties, and (3) the determination of the conditions for the optimum production of scFOSs, mcFOSs and oligolevans and the comparison of the efficiency of bi-enzymatic system with current available ones. To the best of our knowledge, there is no report on the optimization of LS/endo-inulinase bi-enzymatic system. RSM is an effective tool for modeling and optimizing any complex process that is affected by the levels of more than one factor [23]. The advantage of RSM is the low number of experimental trials required to study the linear or quadratic effects of the factors and also their cross product effects.

2. Materials and methods

2.1. Chemicals and materials

D-(−)-fructose, D-(+)-glucose, D-(+)-raffinose, and sucrose were purchased from Sigma–Aldrich (St-Louis, MO). Carbohydrate standards 1-kestose, nystose, and 1^F-fructosylnystose were purchased from Wako Pure Chemical (Japan). Chemical reagents, including, 3,5-dinitrosalicylic acid (DNS), K_2HPO_4, KH_2PO_4, NaOH, polyethylene glycol (PEG) 200, and triton X-100 were also obtained from Sigma–Aldrich (St-Louis, MO). Endo-inulinase (EC 3.2.1.7) from *A. niger* was purchased from Sigma–Aldrich (St-Louis, MO).

2.2. Levansucrase preparation

B. amyloliquefaciens (ATCC 23350) was obtained from American type culture collection (Manassas, USA). *B. amyloliquefaciens* was grown aerobically at 150 rpm and 35 °C for 11 h in a mineral based medium supplemented with yeast extract (10 g/L) and sucrose (10 g/L) as described previously by Tian et al. [16]. Intracellular LS was recovered by ultrasonication (2 kHz, cycle 25/50 s, 550 Sonic Dismembrator, Fisher Scientific) of cell suspension in 50 mM potassium phosphate buffer (pH 6.0) containing Triton X-100 (1%). After centrifugation (9800 × *g*, 20 min) to remove cell debris, the intracellular LS extract was further purified by PEG 200 fractionation (30%, v/v) [24]. The partially purified LS extract was dialyzed against 5 mM potassium phosphate buffer (pH 6.0) through a membrane with a cutoff of 5–6 kDa at 4 °C and then lyophilized. PEG-200 precipitation selectively purified LS with a purification factor of 76-fold and a high yield of 57% [24].

2.3. Levansucrase activity assay

LS activity was assayed using sucrose as substrate as described previously [24]. The assay was initiated by adding 0.25 mL of LS extract (6–25 μg) to 0.25 mL of sucrose solution (1.8 M), prepared in potassium phosphate buffer (50 mM, pH 6.0). The reaction mixtures were incubated at 30 °C for 20 min, and then heated in 100 °C water bath for 5 min to stop the reaction. The concentrations of glucose and fructose were quantified using high-pressure-anionic-exchange chromatography equipped with pulsed amperometric detection (HPAEC-PAD, Dionex), the Chromeleon Software and a CarboPac PA20 column (3 mm × 150 mm) set at a temperature of 32 °C. Isocratic elution was applied with 10 mM NaOH as the mobile phase at a flow rate of 0.5 mL/min. Subtracting the total amount of fructose from that of glucose provides the amount of glucose resulting from transferring fructose. One transfructosylation unit of LS is defined as the amount of the biocatalyst that releases 1 μmol of glucose as a result of transferring fructose, per min. Specific activity was expressed as the transfructosylation units per mg of protein.

2.4. Endo-inulinase activity assay

The hydrolytic activity of endo-inulinase was investigated using low MW levan (5.5 ± 0.5 kDa) as substrate, which was prepared as previously described through LS-catalyzed transfructosylation reaction [19]. Only very minor activity of exo-inulinase (<8%) was detected in the investigated endo-inulinase product. The enzymatic assay consisted of 0.25 mL endo-inulinase solution (0.36–0.52 mg proteins) and 0.25 mL of 2% (w/v) levan as substrate in potassium phosphate buffer (50 mM, pH 6.0). The reaction mixtures were incubated at 30 °C for 20 min, and then heated in 100 °C water bath for 5 min to deactivate the enzyme. The reducing fructose end-groups of FOSs were quantified using the dinitrosalicylic acid method. After adding 0.75 mL of 1% (w/v) dinitrosalicylate reagent, prepared in 1.6% (w/v) NaOH, the reaction mixtures were then placed in a boiling water-bath for 5 min, for the development of reducing ends color. 0.25 mL of potassium sodium tartrate solution (50%, w/v) was, thereafter, added to the mixtures. The absorbance of the resulting mixture was measured spectrophotometrically at 540 nm, against reagent blank. The amount of the released reducing fructose end-groups was determined from the standard curve, constructed with fructose. One unit of endo-inulinase was estimated as the amount of the biocatalyst that released 1 μmol of reducing fructose end-groups per min of reaction.

2.5. Bi-enzymatic system

The combined use of LS and endo-inulinase in one-step bi-enzymatic system was investigated using an initial sucrose concentration of 0.4 M and an enzymatic ratio of 1:1 (0.6 U/ml:0.6 U/ml). The reactions were carried out at 35 °C in 0.1 M potassium phosphate buffer (pH 6.0) and at 70 rpm using an orbital incubator shaker (New Brunswick Scientific Co, Inc, Edison, NJ). At selected reaction times, aliquots were withdrawn, and methanol was added at a ratio of 1:1 (v/v) followed by boiling for 5 min. The analysis of the reaction components was carried out by HPAEC and high performance size exclusion chromatography (HPSEC).

2.6. Identification and characterization of product spectrum

The product spectrum of the bi-enzymatic system was characterized by HPAEC using a Dionex (ICS-3000) system equipped with pulsed amperometric detector (PAD), the Chromeleon Software, and a CarboPac PA200 (3 mm × 250 mm) column set at 32 °C. The elution of the reaction components was carried out at 0.5 mL/min using a linear gradient of sodium acetate from 0 to

Table 1
Central composite rotatable design arrangement of the actual and coded independent variables and the estimated responses.

Run	Independent variables			Responses[e]								
	X_1[a]	X_2[b]	X_3[c]	Total products (mM)	OL[f] (mM)	Levan (mg/ml)	LH[g] (mM)	LP[h] (mM)	1-kestose (mM)	6-kestose (mM)	Nystose (mM)	GF_4[i] (mM)
1	0.40 (0)	1.50 (0)	1:1 (0)	117.0 (47.1%)[d]	19.4	1.3	7.1	20.0	3.6	32.4	5.1	7.4
2	0.40 (0)	1.50 (0)	1:1 (0)	117.9 (52.1%)	19.5	1.4	7.2	19.9	3.6	33.3	4.9	7.3
3	0.40 (0)	1.50 (0)	1:1 (0)	116.6 (51.5%)	18.8	1.3	7.0	20.5	3.6	33.4	4.9	7.2
4	0.40 (0)	1.50 (0)	1:1 (0)	123.3 (54.5%)	20.1	1.4	7.4	21.3	3.7	34.7	5.3	7.7
5	0.40 (0)	1.50 (0)	1:1 (0)	118.1 (52.2%)	19.4	1.5	7.1	20.1	3.6	33.3	4.9	7.3
6	0.40 (0)	1.50 (0)	1:1 (0)	118.4 (52.8%)	20.1	1.6	7.4	20.3	4.3	31.6	6.2	8.3
7	0.20 (−1.68)	1.50 (0)	1:1 (0)	38.1 (30.9%)	3.3	0.1	1.0	3.7	0.14	16.8	0.4	1.1
8	0.60 (1.68)	1.50 (0)	1:1 (0)	193.5 (59.5%)	38.1	0.02	12.3	30.4	11.6	34.4	20.4	20.1
9	0.40 (0)	0.50 (−1.68)	1:1 (0)	129.0 (59.1%)	13.6	0.004	4.2	16.1	13.9	14.7	33.5	14.1
10	0.40 (0)	1.50 (0)	3:1 (1.68)	113.9 (47.2%)	23.2	1.4	7.1	19.4	2.7	31.5	5.0	7.0
11	0.40 (0)	1.50 (0)	1:3 (−1.68)	123.3 (55.7%)	22.1	1.3	8.8	21.7	3.8	32.6	7.6	9.8
12	0.40 (0)	2.50 (1.68)	1:1 (0)	119.9 (50.2%)	17.2	0.3	6.4	16.5	2.1	42.8	2.2	3.6
13	0.28 (−1)	0.91 (−1)	1:1.85 (−1)	73.1 (45.9%)	9.1	0.04	3.8	13.3	1.9	24.7	4.1	4.8
14	0.52 (1)	2.09 (1)	1:85:1 (1)	175.1 (58.4%)	36.9	0.3	12.1	26.0	6.3	45.3	7.4	9.5
15	0.28 (−1)	2.09 (1)	1:1.85 (−1)	70.4 (40.6%)	6.2	1.4	2.01	6.9	0.6	29.1	0.6	1.6
16	0.28 (−1)	2.09 (1)	1.85:1 (1)	69.6 (39.9%)	7.8	1.4	2.1	7.2	0.6	27.9	0.7	1.6
17	0.52 (1)	0.91 (−1)	1.85:1 (1)	169.7 (60.6%)	28.6	0.2	7.8	25.8	14.1	24.7	29.5	20.1
18	0.28 (−1)	0.91 (−1)	1.85:1 (1)	75.0 (46.3%)	10.6	0.07	3.8	13.3	1.6	25.3	3.7	4.5
19	0.52 (1)	0.91 (−1)	1:1.85 (−1)	166.7 (59.8%)	25.3	0.1	7.8	24.7	15.0	23.8	29.6	21.0
20	0.52 (1)	2.09 (1)	1:1.85 (−1)	175.7 (56.6%)	34.5	0.3	12.6	27.8	6.6	45.8	7.6	10.0

[a] Sucrose concentration (M).
[b] Reaction time (h).
[c] LS to endo-inulinase ratio.
[d] Total products weight percentage (%, w/w) as compared to the initial sucrose concentration.
[e] Responses are expressed in mM, expect for levan, which is expressed in mg/ml.
[f] Oligolevans.
[g] Levanohexaose.
[h] Levanopentaose.
[i] 1^F-fructosylnystose.

0.2 M in 0.1 M NaOH for 20 min. To assess the molecular weight distribution of end-products, HPSEC was carried out using Waters HPLC system equipped with 1525 binary pump, refractometer 2489 detector and Breeze™ 2 software. Three size exclusion columns (7.8 mm × 30 cm), TSKgel G3000PWXL-CP, TSKgel G5000PWXL-CP and TSKgel G6000PWXL-CP, were sequentially used with an isocratic elution of de-ionized water at a flow rate of 0.5 mL/min.

2.7. Experimental design of bi-enzymatic system

A five-level, three-factor central composite rotatable design (CCRD) was employed to evaluate the effects of selected independent variables on the synthesis of total transfructosylation products, FOSs and oligolevans by the combined use of LS and endo-inulinase in the bi-enzymatic system. Levels of the independent variables, including sucrose concentration (X_1, 0.2–0.6 M), reaction time (X_2, 0.5–2.5 h), and LS to endo-inulinase ratio (X_3, 1:1–3:1), were pre-determined. The design consisted of 8 factorial points, 6 axial points (2 axial points on the axis of each design variable at a distance of 1.68 from each design center), and 6 center points, leading to 20 runs (Table 1). Each of these runs, except the central points, was performed in duplicate. The response variables were the concentrations of total transfructosylation products, oligolevans, levan, levanopentaose, levanohexaose, 1-kestose, 6-kestose, nystose and 1^F-fructosylnystose. The variables were coded according to Eq. (1):

$$x_i = (X_i - X)/\Delta X_i \quad i = 1, 2, 3, \ldots, j \tag{1}$$

where x_i and X_i are the dimensionless coded and actual values of the independent variable i, X is the actual value of the independent variable i at the central point, and ΔX_i is the step change of X_i corresponding to a unit variation of the dimensionless value [25].

2.8. Statistical analysis

For the regression analysis, the experimental data, obtained based on the described design, were fitted to quadratic equations using the software Design-Expert 8.0.7:

$$Y = \beta_0 + \sum_{i=1}^{3}\beta_i x_i + \sum_{i=1}^{3}\beta_{ii}x_i^2 + \sum\sum_{i<j=1}^{3}\beta_{ij}x_i x_j \tag{2}$$

where Y are the predicted responses for total transfructosylation products, oligolevans, levanopentaose, levanohexaose, 1-kestose, 6-kestose, nystose and 1^F-fructosylnystose, concentrations; β_0, β_i, β_{ii}, and β_{ij} are constant, linear, quadratic, and interaction coefficients, respectively; $x_{i(i=1-3)}$, are the coded independent variables. The variability of the fit of the polynomial model equation was expressed by the coefficient of determination R^2 and its statistical significance was checked using an F-test. Contour plots were obtained using the fitted model, by keeping the least effective independent variable at a constant value while changing the other two variables.

2.9. Purification of selected FOSs and structural analyses

Produced FOSs were fractionated using BioGel P2 Column (2.5 cm × 50 cm, Bio-Rad). The elution was carried out at a flow rate of 0.6 mL/min with de-ionized water. Each fraction (3 mL) was analyzed by HPAEC-PAD. The major FOSs were recovered and further analyzed structurally by NMR and MS.

Prior to NMR analysis, the lyophilized pure FOSs were dissolved in D_2O and the ^1H and ^{13}C spectra were recorded at room temperature on a Varian VNMRS-500 operating at 125 MHz for ^{13}C and 400 MHz for 1H. The ^{13}C spectrum is the accumulation of 448 transients with a 45° pulse width, acquisition time of 1.3 s and a recycle delay of 1 s. Lorentzian broadening of 1.0 Hz was applied before

Fourier transformation. The ^{1}H spectrum is the accumulation of 4 transients with a 45° pulse width, acquisition time of 2.0 s and a recycle delay of 1 s.

The mass spectra of FOSs were analyzed by electrospray ionization mass spectrometry (ESI-MS) using a triple quadrupole mass spectrometer equipped with a Surveyor LC pump, an LCQ advantage mass spectrometer (ion trap) and with Xcalibur® software to control the system acquisition and data processing. Samples were infused into the spectrospray ion source (fused silica capillary of 100 μm i.d.) at a rate of 1 μL/min from a low-pressure infusion pump (model 22, Harvard Apparatus, South Natick, MA).

3. Results and discussion

3.1. Total products, oligolevans and mcFOSs: model fitting and statistical analyses

Our previous study [24] has shown that *B. amyloliquefaciens* LS synthesized mainly levan (47%, w/w) and low amount of FOSs (16 mM; 3%, w/w). Only 9.6–10.7% (w/w) of scFOSs were produced by endo-inulinase using 0.6 M sucrose and 0.6–1.2 U/ml (Data not shown). These preliminary results revealed the efficiency of the LS/endo-inulinase bi-enzymatic system as compared to each of LS and endo-inulinase enzymatic system alone. In order to better understand the effects of reaction parameters and their interactions on the efficiency and the product profile of bi-enzymatic system, RSM has been used. Table 1 summarizes the concentrations of total transfructosylation products, oligolevans, levanopentaose, and levanohexaose at selected reaction conditions. The highest concentrations of total transfructosylation products (193.5 mM, 81.3 g/L h, 59.5%, w/w), oligolevans (38.1 mM, 25.2 g/L h, 18.4%, w/w), levanopentaose (30.4 mM, 16.8 g/L h, 12.3%, w/w) and levanohexaose (12.3 mM, 8.1 g/L h, 5.9%, w/w) were all obtained at sucrose concentration of 0.6 M and LS to endo-inulinase ratio of 1:1 after 1.5 h of reaction (run #8). As expected, the amount of levan was low when the concentrations of the transfructosylation products reached their maximum values.

The best-fitting model was determined by multiple regression analyses of the experimental data and they were statistically checked by the coefficients of determination (R^2) and adjusted *R*-squared (*Adj* R^2) values, model lack of fit test, and *P* value. The analyses of variance (ANOVA) are summarized in Table 2. The response of oligolevans was transformed to square root according to the recommended transformation suggested by the Box–Cox plot. The results show that the quadratic model was statistically the most suitable for the description of the synthesis of total transfructosylation products, oligolevans, levanopentaose, and levanohexaose in the LS/endo-inulinase bi-enzymatic system, with $P < 0.0001$ and high F_{model} value of 634.97, 581.57, 213.31, and 319.69, respectively.

Upon the regression analyses, R^2 values of 0.9941, 0.9971, 0.9870, and 0.9947 were obtained for total transfructosylation products, oligolevans, levanopentaose, and levanohexaose models, respectively (data not shown); these results indicate that more than 98.70% of the variabilities in the responses can be explained by the established models. In addition, the non-significant "lack of fit" indicates that the quadratic polynomial models are adequate for representing the experimental data. The Adj R^2, which adjusts the R^2 for the sample size and the number of variables in the model, are 0.9926, 0.9953, 0.9824, and 0.9916 for total transfructosylation products, oligolevans, levanopentaose and levanohexaose models, respectively. The predicated *R*-squared (Pred R^2) values, which indicate how well the models predict responses for the new observations, are close to the R^2 value (data not shown). In addition, the small difference between Adj R^2 and Pred R^2 reveals the good predictive ability of the established models. Adequate precisions (51.96–91.51) for total transfructosylation products, oligolevans, levanopentaose, and levanohexaose predictive models, which measure the signal to noise ratio, are greater than 4 and therefore desirable. Similarly, Zhao et al. [26] have reported that the production of extracellular polysaccharides by *Pseudomonas fluorescens* PGM37 can be described by a quadratic model. In addition, Cui and Qiu [27] have applied central composite design to evaluate and optimize the production of polysaccharide, curdlan, by a *Pseudomonas* sp.

Table 2 shows that linear and quadratic terms of sucrose concentration (x_1, *F* value of 7.45–3745.328, $P < 0.02$) and reaction time (x_2, *F* value of 6.27–77.4, $P < 0.025$) had significant effects on the concentrations of synthesized oligolevans, levanopentaose, and levanohexaose. In the total transfructosylation product predictive model, the linear term of sucrose concentration (x_1, *F* value of 2526.85, $P < 0.0001$), and the quadratic term of reaction time (x_2, *F* value of 7.57, $P < 0.0149$) were the significant factors. In all established models, the interaction between sucrose concentration and reaction time ($x_1 x_2$, *F* value of 34.6–216.6, $P < 0.0001$) exhibited significant effects on the responses; in addition, the positive sign of all cross-product coefficients (x_1x_2) reveals their positive synergistic interaction. Furthermore, the linear and quadratic effects of LS to endo-inulinase ratio (x_3, *F* value of 9.5–34.7, $P < 0.0001$) were only significant in oligolevans and levanohexaose predictive models. The final predictive equations for total transfructosylation products, oligolevans, levanopentaose, and levanohexaose in terms

Table 2
Analysis of variance (ANOVA) for total transfructosylation products, oligolevan, levanopentaose, and levanohexaose response models.

Source	Total products			Oligolevans			Levanopentaose			Levanohexaose		
	Sum of squares	*F* value	Prob > *F*	Sum of squares	*F* value	Prob > *F*	Sum of squares	*F* value	Prob > *F*	Sum of squares	*F* value	Prob > *F*
Model	32,099.1	634.97	<0.0001	26.16	581.57	<0.0001	940.97	213.31	<0.0001	202.01	319.69	<0.0001
x_1[a]	31,934.4	2526.85	<0.0001	24.07	3745.28	<0.0001	862.62	977.75	<0.0001	166.24	1841.5	<0.0001
x_2[b]	5.78	0.46	0.5090	0.13	20.14	0.0007	5.53	6.27	0.0253	6.38	70.63	<0.0001
x_3[c]				0.11	17.35	0.0013	–	–	–	0.86	9.53	0.0094
x_1x_2	63.29	5.01	0.0408	0.83	129.42	<0.0001	30.50	34.57	<0.0001	19.55	216.60	<0.0001
x_1^2				0.32	49.43	<0.0001	18.41	20.86	0.0004	0.67	7.45	0.0183
x_2^2	95.64	7.57	0.0149	0.42	65.97	<0.0001	27.65	31.34	<0.0001	6.99	77.41	<0.0001
x_3^2				0.22	34.73	<0.0001	–	–	–	0.94	10.46	0.0072
Residual	189.57			0.08			12.35			1.08		
Lack of fit	160.67	2.78	0.1354	0.06	2.71	0.1450	11.03	4.65	0.0527	0.94	4.59	0.0563
Pure error	28.90			0.02			1.32			0.15		
Cor Total	32,288.71			26.24			953.32			203.09		

[a] Sucrose concentration (M).
[b] Reaction time (h).
[c] LS to endo-inulinase ratio (U:U).

of coded variables are given here below. For comparison purpose, the hierarchical term of reaction time (x_2,) was added in the predictive equation of the total transfructosylation products (3) after backward elimination regression.

$$Y_{TP} = 118.47 + 48.36x_1 - 0.65x_2 + 2.81x_1x_2 + 2.55x_2^2 \quad (3)$$

$$Y_{OLs}^{1/2} = 4.42 + 1.33x_1 + 0.097x_2 + 0.090x_3 + 0.32x_1x_2 - 0.15x_1^2 - 0.17x_2^2 + 0.12x_3^2 \quad (4)$$

$$Y_{LP} = 20.45 + 7.95x_1 - 0.64x_2 + 1.95x_1x_2 - 1.12x_1^2 - 1.38x_2^2 \quad (5)$$

$$Y_{LH} = 7.20 + 3.49x_1 + 0.68x_2 - 0.25x_3 + 1.56x_1x_2 - 0.22x_1^2 - 0.7x_2^2 + 0.26x_3^2 \quad (6)$$

where Y_{TP}, $Y_{OLS}^{1/2}$, Y_{LP} and Y_{LH} represent total transfructosylation products, oligolevans, levanopentaose, and levanohexaose, respectively.

3.2. Short chain fructooligosaccharides: model fitting and statistical analyses

Table 1 shows the experimental conditions and the experimental responses of scFOSs concentration, including 1-kestose, 6-kestose, nystose and 1^F-fructosylnystose. Among the various treatments, the maximum concentration of 1-kestose (15.0 mM, 8.3 g/L h, 4.3%, w/w) and 1^F-fructosylnystose (21.0 mM, 19.1 g/L h, 9.8%, w/w) was obtained at run #19 (sucrose concentration of 0.52 M; reaction time 0.91 h; LS to endo-inulinase ratio of 1:1.85). Higher maximum concentration of nystose (33.5 mM, 44.7 g/L h, 16.3%, w/w) was obtained at sucrose concentration of 0.4 M and enzymatic ratio of 1:1 after 0.5 h of reaction (run #9). In addition, the highest concentration of 6-kestose (45.8 mM, 11.1 g/L h, 11.1%, w/w) was achieved at sucrose concentration of 0.52 M and enzyme ratio of 1:1.85 after longer reaction time of 2 h (run # 20). As expected, under the experimental conditions of runs #9, 19 and 20, the amounts of levan present in the bi-enzymatic system was low (<0.3 g/L).

The analyses of variance (ANOVA) and the adequacy of scFOSs models are summarized in Table 3. To find the best models that can fit the data, the responses of 1-kestose, nystose and 1^F-fructosylnystose concentrations were transformed to logarithm according to the recommended transformation suggested by the Box–Cox plot; while the response of 6-kestose was transformed to 1.98 power. The results show that within the investigated range, the quadratic model did statistically fit the description of the synthesis of scFOSs in the LS/endo-inulinase bi-enzymatic system with $P < 0.0001$ and high F_{model} value of 356.7, 207.9, 397.6, and 450.8, respectively.

The regression analyses resulted in R^2 values of 0.9896, 0.9823, 0.9930 and 0.9938 for 1-kestose, 6-kestose, nystose and 1^F-fructosylnystose models, respectively; these results indicate that only less than 1.77% of the variabilities in the responses cannot be explained by the predictive models. The non-significant "lack of fit" indicates that the quadratic polynomial models are adequate for predicting the experimental data. The Adj R^2 are 0.9868, 0.9776, 0.9905 and 0.9916 for 1-kestose, 6-kestose, nystose and 1^F-fructosylnystose models, respectively. The Pred R^2 values are close to the R^2 value (data not shown). In addition, the small difference between Adj R^2 and Pred R^2 suggests the good predictive ability of the established models. Adequate precisions (45.60–72.40) for 1-kestose, 6-kestose, nystose and 1^F-fructosylnystose prediction models are greater than 4 and therefore are desirable for the prediction. Lim et al. [28] have shown that the production of *neo*-FOSs

Table 3
Analysis of variance (ANOVA) for 1-kestose, 6-kestose, nystose, and 1^F-fructosylnystose response models.

Source	1-Kestose			6-Kestose			Nystose			1^F-fructosylnystose		
	Sum of squares	*F* value	Prob > *F*	Sum of squares	F value	Prob > *F*	Sum of squares	*F* value	Prob > *F*	Sum of squares	F value	Prob > *F*
Model	5.03	356.65	<0.0001	4.13E + 006	207.88	<0.0001	5.28	397.63	<0.0001	2.42	450.81	<0.0001
x_1 [a]	3.90	1107.0	<0.0001	9.83E + 005	197.82	<0.0001	3.38	1270.91	<0.0001	1.84	1707.3	<0.0001
x_2 [b]	0.67	191.17	<0.0001	2.29E + 006	460.91	<0.0001	1.64	617.81	<0.0001	0.48	449.10	<0.0001
x_3 [c]	–	–	–				–	–	–	0.007	6.45	0.024
x_1x_2	–	–	–	7.19E + 005	144.75	<0.0001	0.02	6.25	0.0254	0.01	9.23	0.0088
x_1^2	0.36	100.92	<0.0001	1.39E + 005	28.06	<0.0001	0.16	60.45	<0.0001	0.09	81.90	<0.0001
x_2^2	0.07	18.61	0.0006				0.07	24.94	0.0002	–	–	–
x_3^2	–	–	–				–	–	–	–	–	–
Residual	0.05			74,544.19			0.04			0.015		
Lack of fit	0.05	4.66	0.0516	53,564.07	1.28	0.4158	0.03	1.99	0.2322	0.012	2.54	0.1586
Pure error	0.005			20,980.12			0.008			0.003		
Cor total	5.08			4.21E + 006			5.32			2.44		

[a] Sucrose concentration (M).
[b] Reaction time (h).
[c] LS to endo-inulinase ratio (U:U).

from sucrose using *Penicillium citrinum* was suitably described by a quadratic model. Moreover, Sangeetha et al. [29] have demonstrated that the synthesis of FOSs by using a two stage continuous process could be optimized using quadratic models. In addition, Nemukula et al. [30] have used a classic factorial design (i.e. 2^3 standard Box–Wilson central composite design) to evaluate the scFOSs (i.e. 1-kestose, nystose, and 1^F-fructosylnystose) production by fructosyl-transferase from *Aspergillus aculeatus*.

According to the established models (Table 3), sucrose concentration (x_1, F value of 1107.0–1707.4, $P<0.0001$) and reaction time (x_2, F value of 191.2–617.8, $P<0.0001$) were significant linear terms, affecting importantly the synthesis of 1-kestose, nystose and 1^F-fructosylnystose in the bi-enzymatic system. For the synthesis of 6-kestose, the linear term of reaction time (x_2, F value of 460.9, $P<0.0001$) seems to be more important than the linear and quadratic terms of sucrose concentration (x_1, F value of 197.8 and 28.1, $P<0.0001$). The results also show that the quadratic effects of sucrose concentration (x_1^2, F value > 60.4, $P<0.0001$) and reaction time (x_2^2, F value > 18.6, $P<0.05$) were significant terms in 1-kestose and nystose predictive models; however, the linear and quadratic terms of LS to endo-inulinase ratio (x_3) had no significant effect on these models. In addition to the quadratic effect of sucrose concentration (x_1^2, F value > 81.9, $P<0.0001$), the linear effect of LS to endo-inulinase ratio (x_3) was only significant in the 1^F-fructosylnystose concentration predictive model (F value of 6.45, $P<0.024$). No significant interaction effect between the variables could be detected in the 1-kestose concentration model. However, the sucrose concentration (x_1) and the reaction time (x_2) showed significant interaction effect in 6-kestose (F value of 144.75, $P<0.0001$), nystose (F value of 6.25, $P<0.025$) and 1^F-fructosylnystose (F value of 9.23, $P<0.0088$) models. In addition, the positive sign of this interaction term (x_1x_2) indicates the positive synergistic effects of the variables. Considering the significant terms, the syntheses of 1-kestose, 6-kestose, nystose and 1^F-fructosylnystose by the combined use of LS and endo-inulinase in a one-step bi-enzymatic system can be described by the following quadratic equations in terms of coded variables:

$$\log_{10}(Y_{1-Kes}) = 0.56 + 0.53x_1 - 0.22x_2 - 0.16x_1^2 + 0.0067x_2^2 \quad (7)$$

$$Y_{6-Kes}^{1.98} = 1014.84 + 268.30x_1 + 409.54x_2 + 299.86x_1x_2 - 97.48x_1^2 \quad (8)$$

$$\log_{10}(Y_{Nys}) = 0.73 + 0.50x_1 - 0.35x_2 + 0.046x_1x_2 - 0.11x_1^2 + 0.067x_2^2 \quad (9)$$

$$\log_{10}(Y_{GF4}) = 0.88 + 0.37x_1 - 0.19x_2 - 0.023x_3 + 0.035x_1x_2 - 0.077x_1^2 \quad (10)$$

where Y_{1-Kes}, Y_{6-Kes}, Y_{Nys}, and Y_{GF4} represent 1-kestose, 6-kestose, nystose, and 1^F-fructosylnystose concentration, respectively.

3.3. Effect of bi-enzymatic system parameters

The relationships between the reaction parameters and the concentrations of synthesized total transfructosylation products, oligolevans, mcFOSs and scFOSs can be better understood by studying the planned series of two dimensional (2D) contour plots of fitted models. In the 2D contour plot, the curves of equal response values are drawn on a plane. In fact, each contour represents a specific value for the height of the surface. The 2D contour plots presented in Fig. 1 illustrate the interaction effect of sucrose concentration and reaction time on the predicted concentrations of total transfructosylation products, oligolevans, and mcFOSs (e.g. levanopentaose, levanohexaose) at constant LS to endo-inulinase ratio of 1:1. The vertical lines obtained in the contour plot of total transfructosylation products (Fig. 1A) reveal the negligible effect of reaction time on their predicted concentration as compared to the sucrose concentration. The concentration of the total transfructosylation products increased as the sucrose concentration was increased within the investigated design space, and the highest predicted concentration was achieved at around 0.6 M sucrose after 0.5–2.5 h reaction time. It should be pointed out that although the concentration of total transfructosylation products was maintained more or less constant over the reaction time course, their composition profile varied depending on the reaction time. At the initial stage of reaction, scFOSs were the major transfructosylation products (Fig. 2), whilst mcFOS and oligolevans became the dominant products at the last stage (Fig. 1B–D).

As compared to the total transfructosylation products, the inclination of lines in the contour plots of mcFOSs and oligolevans (Fig. 1 B–D) reveals the effect of reaction time on their predicted concentrations; however, the sucrose concentration exhibited higher effect as compared to the reaction time. The highest predicted concentrations of mcFOSs and oligolevan could be achieved at high sucrose concentrations (i.e. 0.5–0.6 M) and in prolonged reaction times (i.e. from midpoint, 1.5 h,) within the investigated design space region. The possible rational explanation of these contour plot trends could be that sufficient amount of levan needed to be initially produced in the bi-enzymatic system (e.g. 0.5–1 h) as intermediates of mcFOSs and oligolevans. The slopes of contour lines at the last stage of reaction time course also reveal the high affinity for the use of levanopentaose as fructosyl acceptor by LS and/or as substrate by endo-inulinase than levanohexaose or oligolevans. Contrary to levanopentaose, the predicted concentrations of levanohexaose and oligolevans increased when LS to endo-inulinase ratio was decreased or increased from 1:1 ratio (data not shown). Similar results were reported in our previous work [19] and attributed to (a) the high levels of levan as a result of the high extent of the transfructosylation reaction at high LS to endo-inulinase ratio of 2:1 and to (b) the high availability of acceptors at lower enzyme ratio of 1:2.

Fig. 2A, C and D shows that at constant LS to endo-inulinase ratio of 1:1, predicted 1-kestose, nystose and 1^F-fructosylnystose concentrations increased with an increase in the sucrose concentration from 0.2 to 0.6 M, and they decreased as the reaction proceeded from 0.5 to 2.5 h. The 6-kestose contour plot (Fig. 2B) displays a different elliptic trend in that the predicted concentration increased with an increase in the sucrose concentration and the reaction time. Varying the LS to endo-inulinase ratio did not have a noticeable effect on the predicted concentration of scFOSs (data not shown). From the contour plots, sucrose concentration seems to be a more important variable as compared to the reaction time in all scFOSs predictive models. However, the sucrose concentration had a more critical effect on the synthesis of 1-kestose and 6-kestose (a steep slope) than nystose and 1^F-fructosylnystose. Overall, the effect of high sucrose concentrations can be attributed to the mass action effect favoring the formation of the product and/or to their ability to stabilize the active conformation of LS and endo-inulinase biocatalysts. In addition, higher sucrose concentrations have been reported to increase the availability of fructosyl acceptor and decrease the availability of water [31]. Sucrose concentration has also been identified as one of the key factors effecting enzymatic FOS synthesis [16,32]. Fig. 2 also indicates that when the sucrose concentration was lower, the reaction time did not have a significant effect on the predicted concentrations of scFOSs (i.e. 1-kestose, 6-kestose, nystose and 1^F-fructosylnystose). These results can be due to the fact that the reaction equilibrium may have been reached faster at low sucrose concentrations. The low predicted concentrations of scFOSs at low sucrose concentrations (<0.35 M) are also consistent with

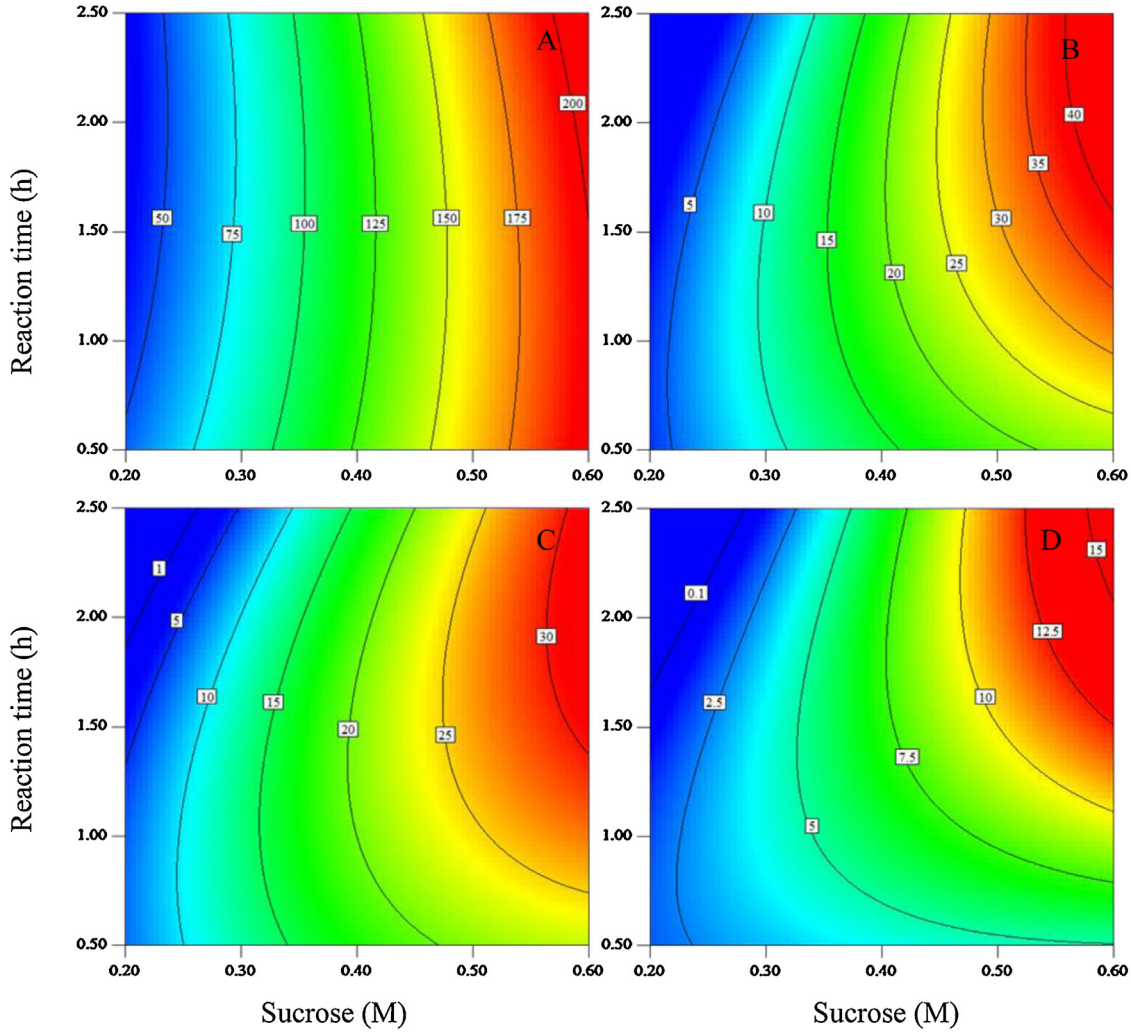

Fig. 1. Contour plots of predictive models of total transfructosylation products (A), oligolevans (B), levanopentose (C), and levanohexaose (D) produced in the LS/endo-inulinase bi-enzymatic system. The numbers inside contour plots indicate concentration (mM) under given conditions and at LS to endo-inulinase ratio of 1:1.

high hydrolytic activity of LS (~74% of total activity) in this range [19]. The increase in the reaction time had a significant negative effect on the predicted concentrations of scFOSs, with the exception of 6-kestose (Fig. 2). This effect can be attributed to the shift of the reaction toward their further hydrolysis by endo-inulinase and/or to the use of scFOSs as acceptors by LS. These results are in agreement with our previous findings in which the optimum production of 1-kestose, nystose and 1^F-fructosylnystose was achieved in the initial reaction period (i.e. 0.5–1 h) [19]. As compared to other FOSs, the accumulation of 6-kestose over the reaction time course may reveal that LS was unable to use it efficiently as a fructosyl acceptor.

3.4. Model verification, optimization and structural characterization

The optimal conditions for the synthesis of each of scFOSs (Opt A) and mcFOSs (Opt B) were estimated via the numerical optimization using the Design-Expert 8.0.7 software. The non-coded optimal conditions are reported in Table 4. The confirmations of these optimal conditions and of the liability of the model predictions were investigated through additional runs. The predicted and the experimental maximum concentrations of the products are shown in Table 4.

The output suggests that scFOSs (1-kestose, nystose, 1^F-fructosylnystose) could reach their optimum concentrations at the initial stage of the reaction (0.5 h) using a LS to endo-inulinase ratio of 1:1 (Opt A); while mcFOSs (levanopentaose and levanohexaose) and 6-kestose could achieve their optimum concentrations upon a prolonged reaction time (1.77 h) and using a high ratio of LS to endo-inulinase (1.85:1) (Opt B). Table 4 shows that all findings of the maximum concentrations of the targeted transfructosylation products are within the statistically significant range of the estimated optimum values with 95% prediction intervals (PIs). These results indicate that there is no significant ($P < 0.05$) differences between the experimental data and the predicted values. As an overall, validation of the RSM models was confirmed.

Commercially available β-(2→1)-FOSs are mainly composed of short chain ones (degree of polymerization, DP of 2–4) and are produced from sucrose (60%, w/v, 1.75 M) by microbial enzymes with transfructosylating activity (fructofuranosidases, EC.3.2.1.26; fructosyltransferases, inulosucrase, EC.2.4.1.9) with an approximate yield of 50% (w/w) [33,3,34]. Using the developed bi-enzymatic system, transfructosylation products (β-(2→1)-/β-(2→6)-FOSs and β-(2→6)-oligolevans) with high DP (3–10) were produced with higher yield of 57–65% (w/w) and productivity of 65.8–266.8 g/L h. Using the optimal conditions determined for the synthesis of scFOSs (Opt A),1-kestose (34.6 g/L h, 8.4% w/w), nystose (GF_3) (76.8 g/L h, 18.7%, w/w) and 1^F-fructosylnystose (GF_4) (43.3 g/L h, 10.6%, w/w) exhibited the highest productivity and yield (Table 4). Under the optimal conditions identified for the synthesis of

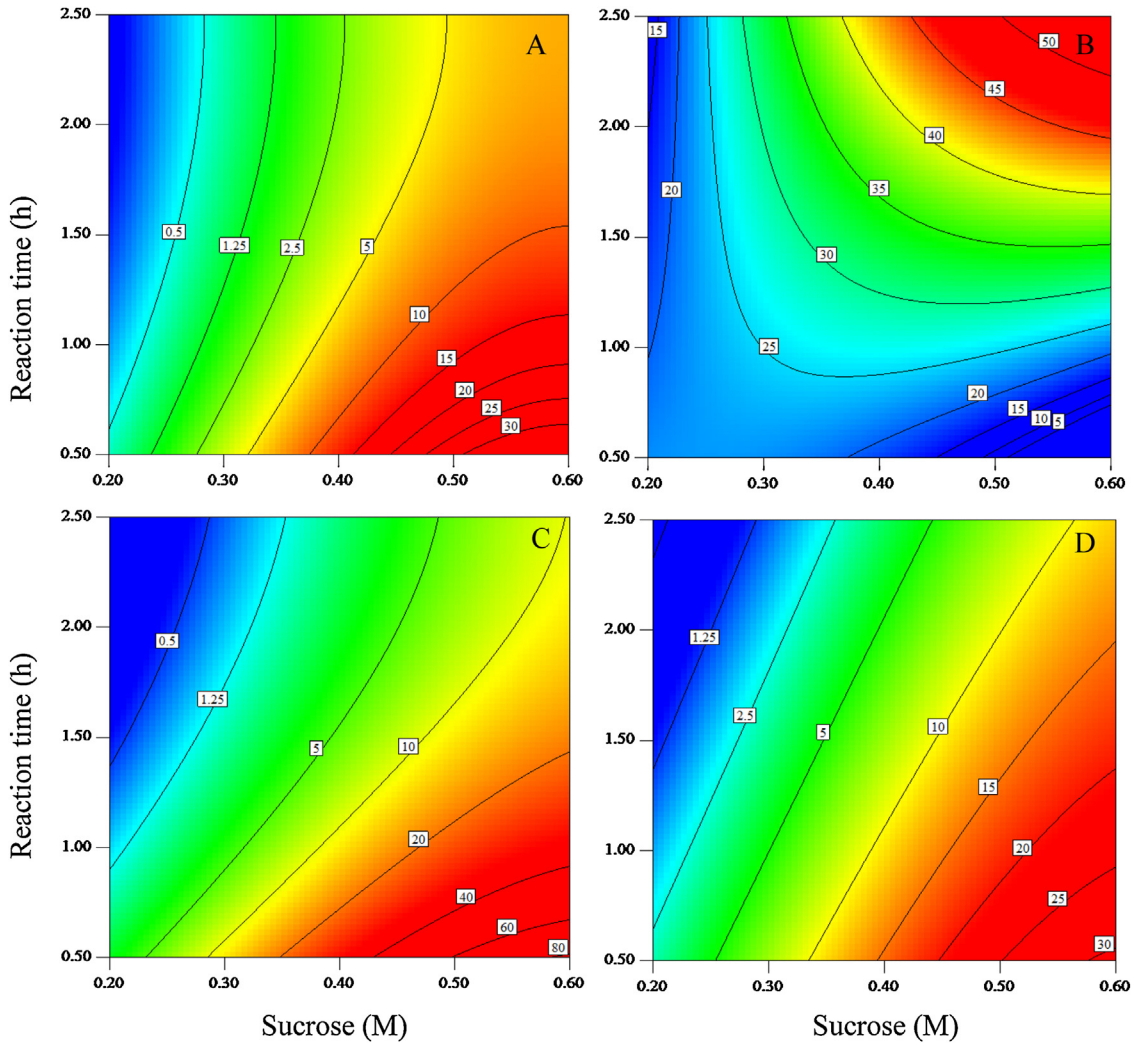

Fig. 2. Contour plots of predictive models of 1-kestose (A), 6-kestose (B), nystose (C), and 1^F-fructofranosylnystose (D) produced in the LS/endo-inulinase bi-enzymatic system. The numbers inside contour plots indicate concentration (mM) under given conditions and at LS to endo-inulinase ratio of 1:1.

Table 4
Model verification and optimization of scFOSs and mcFOSs yield.

	Opt A[a]					Opt B[b]				
	Predicted conc[c]	95% PI[a]	Experimental conc[c]	Yield[d]	Productivity[e]	Predicted conc[c]	95% PI[b]	Experimental conc[c]	Yield[d]	Productivity[e]
TP[f]	200.2	187.5–212.8	203.2	65.0	266.8	–	–	186.0	56.7	65.8
OL[g]	21.8	19.0–24.8	19.2	7.8	31.8	–	–	26.2	10.6	12.3
1-Kestose	37.9	24.7–58.1	34.3	8.4	34.6	–	–	5.7	1.4	1.6
6-Kestose	–	–	15.9	3.9	16.0	41.6	39–40.1	48.3	11.9	13.8
Nystose	82.4	52.4–129.5	57.6	18.7	76.8	–	–	7.7	2.5	2.9
GF_4[h]	32.5	23.6–40.5	26.1	10.6	43.3	–	–	9.1	3.7	4.3
LP[i]	–	–	16.3	6.6	27.0	31.5	29.0–34.2	31.3	12.7	14.7
LH[j]	–	–	6.6	3.2	13.0	13.8	13.0–14.7	13.9	5.8	6.7

[a] Opt A conditions are 0.6 M sucrose, 0.5 h, and LS to endo-inulinase ratio of 1:1.
[b] Opt B conditions are 0.6 M sucrose, 1.77 h, and LS to endo-inulinase ratio of 1.85:1.
[c] Concentrations are expressed in mM.
[d] Yield (%) represents the weight percentage (w/w) of the end-product as compared to the initial sucrose concentration.
[e] Productivity is expressed in g/L h.
[f] Total transfructosylation products.
[g] Oligolevans.
[h] 1^F-fructosylnystose.
[i] Levanopentaose.
[j] Levanohexaose.

Table 5
^{1}H and ^{13}C NMR analysis of levan, levanopentaose and levanohexaose.

Product	Sugar unit	^{1}H and ^{13}C chemical shifts/d					
		1, 1′	2	3	4	5	6,6′
Levan	2,6-β-Fru	59.8 3.87, 3.570	104.1	76.2 4.168	75.13 4.085	80.2 3.849	63.2 3.788, 3.3530
Levanopentaose	[-6-)β-Fru(2-]$_5$	60.01 3.77, 3.61	103.5	76.6 4.18	73.1 4.454	80.9 3.832	60.3 3.917, 3.620
Levanohexaose	[-6)-β-Fru(2-]$_6$	60.058 3.787, 3.62	104.6	76.4 4.182	74.1 4.354	80.54 3.932	62.3 3.895, 3.625

mcFOSs (Opt B), 6-kestose (13.8 g/L h, 11.9%, w/w), levanopentaose (14.7 g/L h, 12.7%, w/w) and levanohexaose of (6.7 g/L h, 5.8%, w/w) showed the highest productivity and yield values. The molecular mass of levanopentaose and levanohexaose were confirmed by MS. The spectral pattern of NMR spectra of levanopentaose and levanohexaose was similar to that of β-(2-6)-levan [16], revealing the presence of only 2,6-β-linked Fru residues in the mcFOSs (Table 5).

As compared to other approaches, the new investigated bi-enzymatic system requires lower sucrose concentration (0.6 M) and reaction temperature (35 °C), whilst achieving high volumetric productivity of FOSs. As far as the authors are aware, the highest volumetric productivity (163 g/L h) of FOSs (mainly 1-kestose and nystose products) was reported for β-fructofuranosidases from *Aspergillus oryzae* KB-catalyzed transfructosylation of sucrose (60%, w/v, 1.75 M, 55 °C) [21]. Mussatto and Teixeira [35] have reported lower productivity of 10.76 g/L h for the production of FOSs from agroindustrial residues, by solid-state fermentation with *Aspergillus japonicus*. On the other hand, Fernandez et al. [20] have obtained comparable productivity (35.5–58.5 g/L h) for the production of 1-kestose and nystose by fructofuranosidase from *Aspergillus* sp 27H using high sucrose concentration of 615 g/L (1.8 M). Similarly, the productivity of *Xanthophyllomyces dendrorhous* cell in the neo-FOSs production was in the same range of 56.93 g/L h using 400 g/L (1.16 M) sucrose concentration [23]. Lim et al. [22] have taken a new approach for the neo-FOSs production by co-immobilization of *P. citrinum* and neo-fructosyl-transferase, and reported lower productivity of 18.06 g/L h.

4. Conclusion

The combined use of LS and endo-inulinase in a one-step bi-enzymatic system was proven to be a potential efficient approach for the synthesis of FOSs with high DP. This new bi-enzymatic system requires low substrate concentration and short reaction time. The application of RSM showed that sucrose concentration and reaction time were the most significant independent variables, affecting importantly the yield and the product profiles of the end-products. In addition, sucrose concentration and reaction time showed the most significant interaction effect. At the initial stage of reaction time course, scFOSs were the major transfructosylation products with 6-kestose being the most abundant one, whilst mcFOS (levanopentaose and levanohexaose) and oligolevans become the dominant transfructosylation products at the last stage. LS to endo-inlunase ratio has only limited effect on the end product profile, but not on the yield. Comparison of optimal predicted and experimental values showed very good correspondence, implying that the established predicted models can effectively be used to describe the relationship between the reaction parameters and the yield.

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