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Fructo-oligosaccharide synthesis by whole cells of *Microbacterium paraoxydans*

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ABSTRACT

The synthesis of fructo-oligosaccharides was carried out using whole cells of *Microbacterium paraoxydans*. Reactions were carried out using un-induced, inulin-induced and sucrose-induced cells displaying different amounts of invertase and inulinase activities out of which the best transfructosylation occurred using sucrose-induced cells displaying 12 I.U. invertase/0.75 I.U. inulinase activities. Using 40% w/v sucrose and in the sucrose-induced cells, a maximum fructo-oligosaccharide yield of 155 g/l (corresponding to product yield of 0.38 g/g initial substrate) was obtained. The major products synthesized were the tri-saccharide, 1-kestose [1^F (1-β-D-fructofuranosyl)sucrose] and the tetrasaccharide, nystose [1^F (1-β-D-fructofuranosyl) kestose]. A Box–Behnken design was used to optimize the factors affecting the fructo-oligosaccharide synthesis and these were at 31.5% sucrose, 10.96 I.U. invertase/0.69 I.U. inulinase and 14.32 h incubation time leading to an overall yield of 0.44 g/g initial substrate. The synthesized 1-kestose and nystose were purified to homogeneity by preparative TLC and structurally characterized by ESI-MS and 2D NMR.

1. Introduction

Fructo-oligosaccharides are in high demand in functional food market as prebiotics. These are non-digestible sugars with low caloric values, as well as being non-cariogenic and having low sweetness intensity. Fructo-oligosaccharides prevent gastro-intestinal disorders and help in mineral absorption (calcium and magnesium). They have also been reported to decrease levels of phospholipids, triglycerides, cholesterol and have an anti-cancerous effect.[1,2] Generally, fructo-oligosaccharides comprise of 1-kestose (GF_2, G and F stand for glucose and fructose), 1-nystose (GF_3) and fructofuranosyl nystose (GF_4), in which the fructose units are bonded at the β-2,1 position of the sucrose.

Fructo-oligosaccharides can be synthesized either by hydrolysis of inulin or by transfructosylation of sucrose. Since the hydrolytic process may contain long chain oligomers compared to the small transfructosylation products, the transfuctosylation process is preferred. The enzymes capable of transfructosylation are (i) fructosyl-transferases and (ii) fructosyl-hydrolases. Fructosyl-transferases, such as inulosucrase (EC 2.4.1.9) and levansucrase (EC 2.4.1.10), are mainly responsible for the 'in vivo' conditions of the synthesis. Although there are several reports on the use of fructosyl-transferases for the synthesis of fructo-oligosaccharides,[1–3] their non-availability and requirement of expensive cofactors makes them a less attractive option compared to the fructosyl-hydrolases, such as inulinase (EC 3.2.1.7) and invertase (EC 3.2.1.26), which are more readily available. It is also important to note that the chirality of the product synthesized is as important as the method employed. While in the chemical route, the protecting groups have to be manipulated to obtain the desired linkages; the enzymes do the same by virtue of their specificity. The methods employed should be such that product isolation is also taken care of. In recent years, solid-phase techniques[4] have been employed for oligosaccharide synthesis and while this works well for peptide and oligonucleotide syntheses, its general advantage in oligosaccharide synthesis has yet to be demonstrated. The whole cells, induced for specific enzyme systems[5] can be a good inexpensive alternative for the synthesis of oligosaccharides with specific linkages. The easy separation of cells, their reusability, cultivation on inexpensive medium make this route very attractive.

Industrially, the synthesis of fructo-oligosaccharides is carried out by fungal transfructosylations from *Aspergillus niger*, *Aspergillus japonicus*, *Aureobasidium pullulans* and *Fusarium oxysporum*.[6–9] This requires purification of the enzyme followed by its use for synthetic reactions. There are few reports on the use of whole cells wherein either free[10] or immobilized cells of *A. pullulans* have been used for synthesis of fructo-oligosaccharides. A maximum productivity of 180 g fructo-oligosaccharides/l. h was reported using sucrose in a continuous set-up. Herein we report on the

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use of free cells of the bacterium *Microbacterium paraoxydans* as effective catalytic agents for transfructosylation of sucrose (Fig. 1) resulting in synthesis of specific oligosaccharides. The optimal conditions (time profile, substrate and enzyme units) for the synthesis of fructo-oligosaccharides were investigated and a designed experiment was applied for enhancing the yield.

2. Results and discussion

2.1. Synthesis of fructo-oligosaccharides using differentially induced cells

Efficient transglycosylation with the cell bonded α-glucosidase of *M. paraoxydans* has been previously reported for the synthesis of hexyl-glucoside and polyglucosides[11] and also for synthesis of prebiotic isomalto-oligosaccharides.[12] In view of the observed selective induction of enzymes on the cell surface, based on the carbon source used during cell cultivation,[5] cells were cultivated on inulin and sucrose, which could act as potential inducers of transfructosylation activities. Both invertase and inulinase activities have been reported[6,13] to be responsible for transfructosylation, and were measured in the three cell systems (un-induced, sucrose- and inulin-induced). The products obtained with sucrose were also identified. As observed in Table 1, constitutive levels of invertase and inulinase were detected in un-induced cells with ~4-fold higher invertase levels compared to inulinase activity. In the inulin-induced cells, a two-fold increase in invertase and a seven-fold increase in inulinase activity was observed, thus indicating some induction of invertase activity also. This was attributed to the hydrolysis of inulin resulting in the formation of compounds that enhance invertase activity. In sucrose-induced cells, induction of invertase activity was observed (compared to the control) but a repression of inulinase activity was also observed, which could be attributed to an accumulation of glucose, released as a consequence of sucrose hydrolysis. The repression of inulinase activity by glucose has been reported.[14] Analysis of the synthesized products (Table 2) indicated maximum relative intensities for trioses and tetraoses using sucrose-induced cells, clearly showing efficacy of invertase for carrying out transfructosylation reactions. There are few reports on invertases displaying good transfructosylation activity.[13,15] The inulin-induced activities were ineffective for transfructosylation reactions while large amounts of glucose were detected with un-induced cells. A total fructo-oligosaccharide yield of 0.31 g/g initial substrate was obtained with sucrose-induced cells and this was studied in detail as described below.

Table 1
Enzyme activity by using three different cell systems

Dry lyophilized cell system	Invertase activity (IU/g dcw)	Inulinase activity (IU/g dcw)
Un-induced cell	3366.9	924.3
Inulin-induced cell	7182.5	6739.8
Sucrose-induced cell	7147.1	442.9

2.2. Effect of various parameters on fructo-oligosaccharide yield

In order to evaluate the effect of the process conditions on fructo-oligosaccharide yield, an efficient HPLC based system was developed first for resolution and quantitation of the products. Among the different parameters studied, a solvent ratio of 60:40, v/v (for ACN:water) and a column temperature of 35 °C was found to be optimal for the resolution of glucose, sucrose, 1-kestose and nystose (retention time in min: 4.23, 4.82, 5.73 and 6.24, Fig. 2). This solvent ratio was previously reported to be effective for the separation of isomaltooligosaccharides.[12]

A time course profile of the reaction was carried out using sucrose-induced cells and the results (Fig. 3a) indicated a linear increase in product concentration to 27 g/l until 24 h, after which there was a decrease, thus indicating hydrolysis of the products. Generally, a time period of 24 h has been considered optimal for the synthesis of fructo-oligosaccharides using inulosucrase from *Lactobacillus gasseri,*[16] but times as short as 3–5 h have also been reported using a commercial fructosyl-transferase.[17] While long incubation times (up to 264 h) have been reported for the synthesis of fructans under in vitro conditions using roots extract of *Vigiera discolor*[18] this may not always result in an increase in the yield.[19] The fructosyl transfer may occur either in a processive or in a non-processive manner. In the first case, long polymers are formed as the fructan chain remains bonded to the enzyme as reported for levansucrase of *Bacillus subtilis.*[20] In the second case, the fructan chain is released after every transfer of fructosyl moiety resulting in the formation of short β, 2–1 linked products such as kestose and nystose. This has been reported in *Gluconoacetobacter diazotrophicus*, *Zymomonas mobilis* and *Lactobacillus sanfranciscensis.*[21–24] Our results with *M. paraoxydans* indicate non-processive reactions as higher polymers were not detected at all. An increase

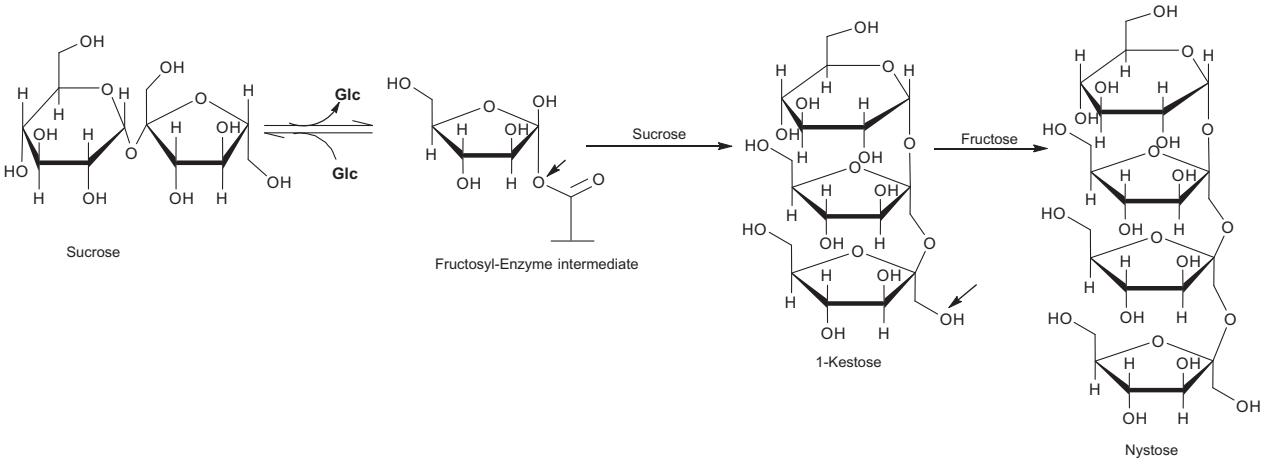

Figure 1. Reaction scheme for conversion of sucrose to 1-kestose and nystose by transfructosylation activities on the cell surface of *M. paraoxydans*. The arrow indicates the position of the nucleophilic attack by the incoming sugar.

Table 2
Mass spectrometry analysis of synthesized products by using three different cell types

Cell system	Glucose (203[a])	Sucrose (365)	Triose GF_2 (527)	Tetraose GF_3 (689)	Total product (GF_2 + GF_3) (%)
Un-induced cell	100[b]	69	21	2	23
Inulin-induced cell	22	100	13	<1	14
Sucrose-induced cell	62	100	29	2	31

[a] The number in parenthesis indicate the M+Na ion adduct.
[b] Relative intensities of various peaks.

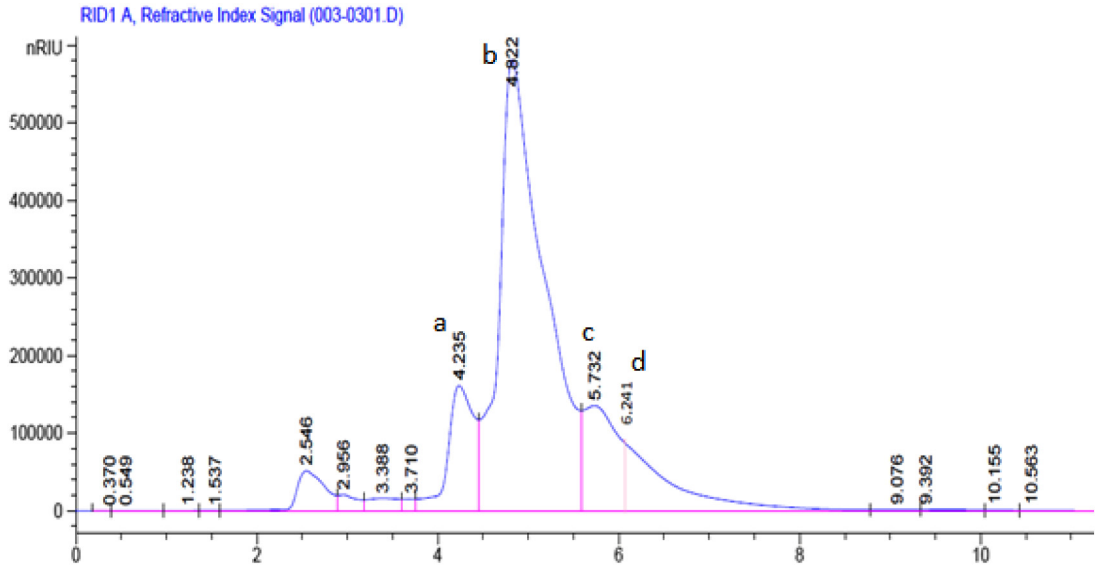

Figure 2. HPLC chromatogram of synthesized products using sucrose induced cells, (a) glucose; (b) sucrose; (c) 1-kestose; (d) nystose.

in sucrose concentration to 40% did not result in a significant increase in the yield of the products (Fig. 3b), thus indicating a possible enzyme limitation in the system. The addition of higher number of cells resulted in an increase in the fructo-oligosaccharide yield to ~39%. However, since hydrolysis occurs at high enzyme units resulting in the formation of glucose, this effect was only seen up to a certain level (Fig. 3c). The initial concentration of the substrate may also change the product profile, as shown by Caputi et al.[19] wherein glucose and fructose were detected at low sucrose concentrations (1 mM), and kestose and nystose were formed at 10 mM sucrose. Herein, an increase in tetraose was observed as the substrate concentration increased.

A statistical design experiment was used to arrive at a combination of three parameters (time of incubation, substrate and enzyme concentrations) to optimize fructo-oligosaccharide yield by Response Surface Modelling. From Design Matrix software, a total of 17 experiments were designed, carried out in duplicates, and the results obtained (data not shown) were fitted onto a quadratic model regression equation for the prediction of fructo-oligosaccharide yield as a function of the three selected parameters. The selected statistically significant model equation (shown below) displayed a low noise level.

$$\text{Yield } (\%) = +36.48 + 2.63 * A + 10.39 * B + 2.37 * C + 2.29 * A * B - 0.02 * A * C - 1.19 * B * C - 4.10 * A^2 - 3.49 * B^2 - 3.34 * C^2$$

where A is the substrate concentration (%, w/v), B is time (h) and C is enzyme units (I.U).

The synthesis of fructo-oligosaccharide was carried out under the model predicted conditions and the analysis of variance data are shown in Table 3. The results indicated that the quadratic model was effective. An R^2 value of 0.9918 was obtained, thus indicating that 99.18% of the experimental data could be explained by the model. The model F-value of 93.64 implied that the model was significant. Values of Prob >F of 0.0001 also lend validity to the model. The 'Lack of Fit-F value' of 26.38 indicated all other coefficients or terms of model equation such as A, B, C, A^2, B^2, C^2, AC, and BC to significantly influence the fructo-oligosaccharide yield (Table 3). The observed response was analysed and compared to the predicted values and the results are shown in Fig. 4a–d. As observed (Fig. 4d), only 4 points were outside of the diagonal line, thus indicating the reliability of the other 13 points. The predicted R^2 value of 0.97 was in agreement between the experimental and the predicted values of fructo-oligosaccharide yield. The optimum conditions obtained from the model were as follows: substrate concentration 31.55% (w/v), time 14.32 h, and enzyme unit 10.96 I.U invertase activity/0.69 I.U inulinase activity. The synthesis of fructo-oligosaccharide was carried out under the model predicted optimal conditions and a maximum concentration of 139 g/l fructo-oligosaccharide (corresponding to yield of ~0.44 g/g substrate, i.e. 31.55% initial sucrose) was obtained. The lower usage of sucrose (31.5% compared to 40%) also makes this an attractive option. The three dimensional response surface curves indicated the superposed effects of time and substrate concentration, enzyme units and time and substrate and enzyme concentrations. The response indicated that the fructo-oligosaccharide synthesis was sensitive to all three parameters. A higher yield was obtained at high substrate concentrations but the time of incubation had to be longer (Fig. 4a). At all cell loadings, time of incubation had to be longer for increasing the yield, thus indicating that time was a crucial factor. An optimum ratio of substrate/cells was also seen to be important (Fig. 4c). The yield of 0.44 g/g initial substrate becomes significant (when compared with the yield of 0.55–0.6 g/g initial substrate obtained with purified enzymes.[25]

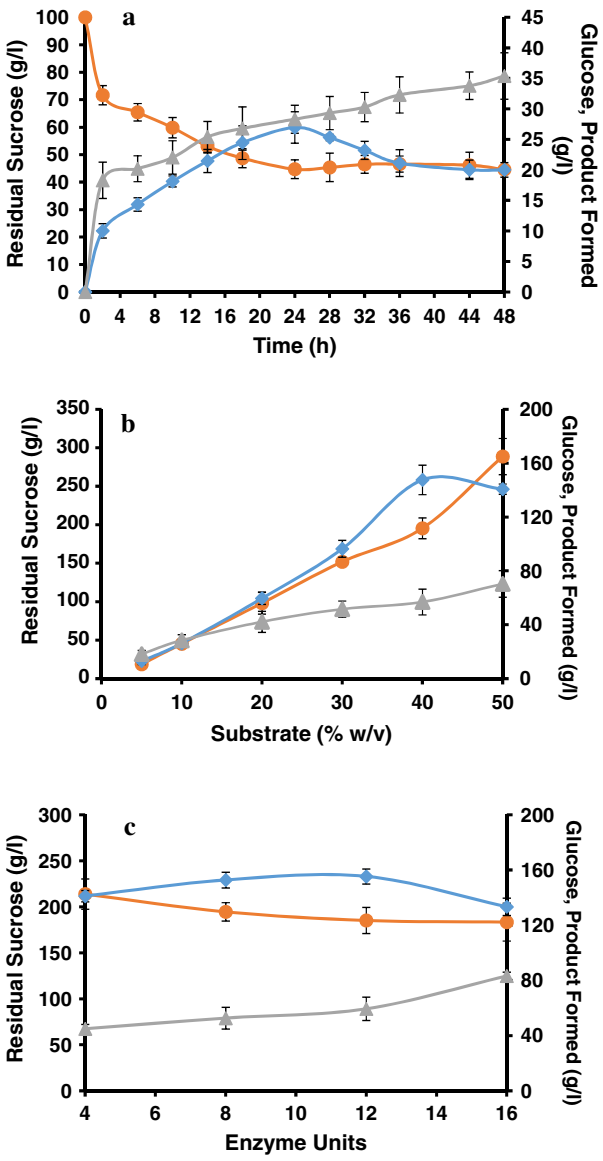

Figure 3. Fructo-oligosaccharide synthesis as a function of (a) time, (b) substrate concentration and (c) enzyme units. (◆) Total fructo-oligosaccharide; (▲) Glucose; (●) Residual Sucrose.

2.3. Detailed mass profile, purification and NMR analysis of synthesized products

The mass profile of products (Fig. 5) displayed a monomodal mass distribution with a mass difference of 162 Da (corresponding to glucose/fructose residue) between consecutive peaks. The *m*/*z* values of 203.01, 365.06, 527.12, and 689.18 represented sodium ion adducts $(M+Na)^+$ of glucose or fructose, sucrose, triose and tetraose respectively suggesting cells of *Microbacterium* to be capable of synthesizing fructo-oligosaccharides in much the same way as reported with fructosylating enzyme systems.[16,19,26] Similarly, higher DP (DP < 6) was observed when inulin was hydrolysed by endo-inulinase for fructo-oligosaccharide synthesis.[27]

Various purification strategies have been attempted to remove glucose or fructose so as to enrich fructo-oligosaccharides. These include the use of glucose oxidase, activated charcoal and microbial treatment.[10] Membrane technology (including cross-flow filtration) has been suggested for the fractionation and purification of oligosaccharides.[10,28] Herein, preparative TLC was used and the products identified based on their R_f values. The identified spots were scraped off the plate, extracted with water and lyophilized. These were directly used for structure determination by 1D and 2D NMR. The complete assignment of ^1H and ^{13}C NMR spectrum and the coupling constant ($J_{H,H}$) is presented in Table 4. Inspection of the ^1H NMR spectrum showed the presence of trisaccharide/tetrasaccharide units, as shown by the signals for anomeric protons at δ 5.25, 3.84 and 3.66 in case of GF_2 and at δ 5.39, 3.83, 3.72 and 3.78 in case of GF_3 reflecting the presence of one glucopyranosyl and two/three fructofuranosyl residues. In ^{13}C spectrum, the anomeric glucose linkage was observed at 93.20 (GF_2) and 93.71 (GF_3) ppm respectively, indicating that the linkage is α. The C2′, C2″ and C2‴ signals (for both of them) were observed at approximately 102 ppm indicating β-linkage. The signal for C1 and C6 of each fructose residues lied in the range of 60.49–61.92. These spectral assignments were in complete agreement with the previously reported data.[29,30] In order to further confirm the linkage and the structure of these residues, HMBC (Heteronuclear Multiple Bond Connectivity) was carried out. In 2D NMR, the chemical shifts of the ^1H and ^{13}C of the residues were assigned based on the literature.[26,30] A cross peak of C2 (of fructose unit) at 103.68 and H1 of glucose at 5.42 ppm characterized this C2 to be of Fructose 1 moiety (fructose unit directly linked to glucose is mentioned here as fructose 1) .The assignments of other cross peaks are shown in Fig. 6. This data clearly indicated that the fructofuranosyl units were of β-configuration.

Table 3
Analysis of variance data set of quadratic model influencing the selected variables on fructo-oligosaccharide yield

Source	Sum of squares	df	Mean square	*F* value	*p*-Value (Prob > *F*)	
Model	1178.94	9	130.99	93.64	<0.0001	Significant
A-Substrate	55.39	1	55.39	39.6	0.0004	
B-Time	863.2	1	863.2	617.08	<0.0001	
C-Enzyme Unit	45.08	1	45.08	32.22	0.0008	
AB	20.93	1	20.93	14.96	0.0061	
AC	1.60E-03	1	1.60E-03	1.14E-03	0.974	
BC	5.64	1	5.64	4.03	0.0846	
A^2	70.92	1	70.92	50.7	0.0002	
B^2	51.18	1	51.18	36.59	0.0005	
C^2	46.94	1	46.94	33.56	0.0007	
Residual	9.79	7	1.4			
Lack of fit	9.32	3	3.11	26.38	0.0043	Significant
Pure error	0.47	4	0.12			
Corrected total	1188.73	16				

R-Squared-0.9918.

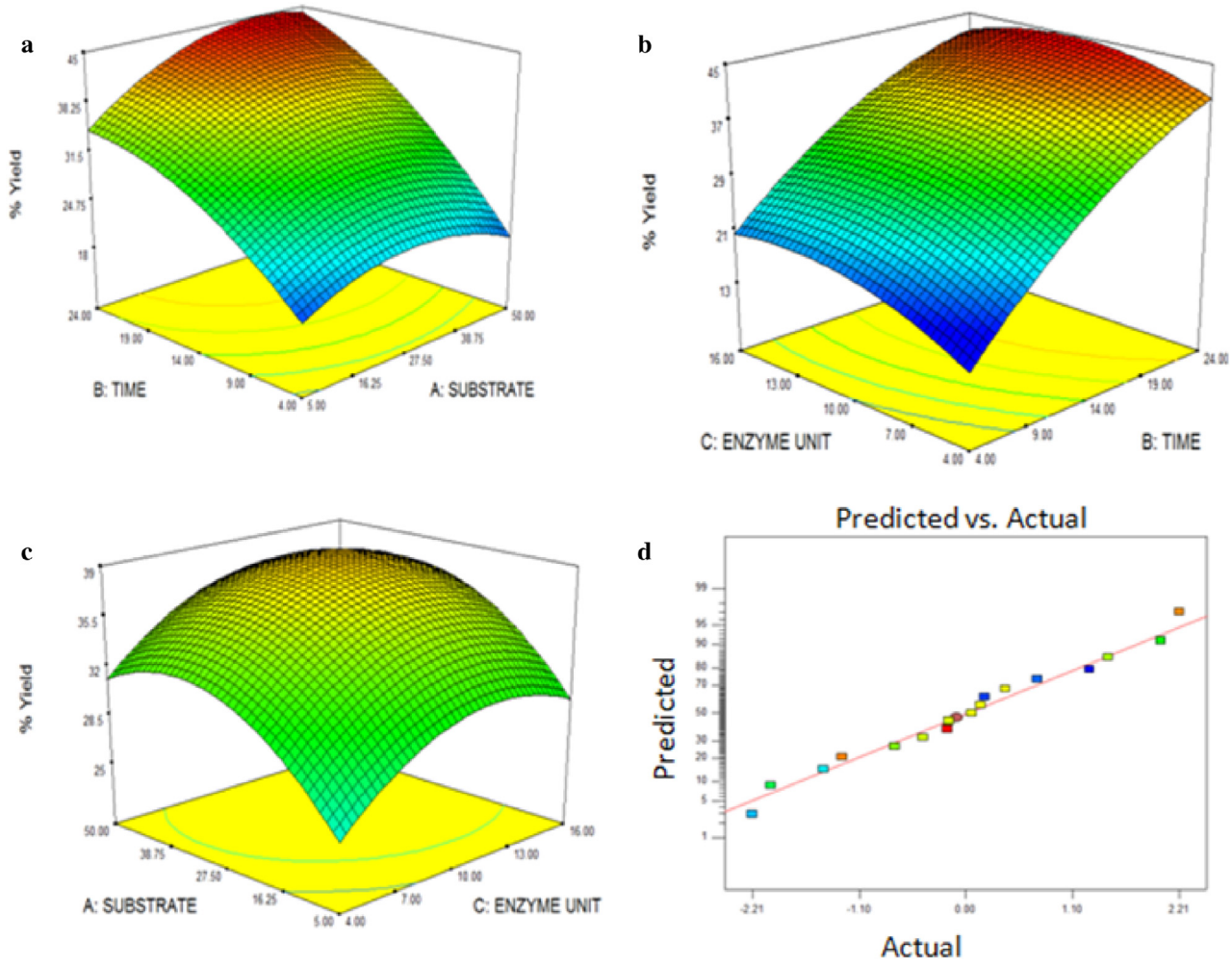

Figure 4. Response surface graphs of fructo-oligosaccharide yield showing the effect of variables (a) time and substrate (b) enzyme units and time and (c) substrate and enzyme units (d) comparison between actual and predicted values.

3. Conclusion

In conclusion, *M. paraoxydans* cells were used as biocatalysts for synthesis of fructo-oligosaccharides using sucrose as the fructosyl donor. Through the non-processive route adopted by cells, 1-kestose and nystose were identified as the major products. Optimization of the incubation time, substrate concentration and enzyme units through statistical design experiment led to a final conversion of ~44%. Cell surface displayed enzymes, which induced selectively, were demonstrated to be effective biocatalysts for synthesis of short chain oligosaccharides.

4. Experimental

4.1. General

Inulin, Sucrose, Fructose and Glucose were purchased from Merck, India. Analytical grade 1-Kestose and Nystose were obtained from Sigma–Aldrich. HPLC grade Acetonitrile, water, pre-coated silica gel 60 F_{254} Aluminium plates and Glucose-Oxidase Peroxidase (GOD-POD) assay kit were from Merck. All other chemicals were of analytical grade.

4.2. Preparation of cell catalysts

Lyophilized cells of *M. paraoxydans* were used as biological catalysts. The cells were grown in Luria Bertani medium at 37 °C with shaking at 220 rpm. The induction of fructosylation activities was carried out by addition of (i) sucrose (2%, w/v) or (ii) inulin (2%, w/v) at the beginning of experiment. Un-induced cells were used as control. The cells were allowed to grow overnight (20 h) during which samples were removed every 4 h for the measurement of growth and enzyme activities. For long term storage, cells were lyophilized and stored at −20 °C.

4.3. Fructo-oligosaccharide synthesis using whole cells

Transfructosylation reactions were carried out using un-induced, inulin-induced and sucrose-induced cells in a reaction volume of 5 ml. The reaction mixture contained 100 mg lyophilized cells and 10% (w/v) sucrose in 5 ml phosphate citrate buffer (pH 7.0). The mixture was incubated at 37 °C for 24 h at 220 rpm in a reciprocating shaker (SI-300R Jeo Tech). At the end of the reaction, cells were separated out by centrifugation and the vials heated in a boiling water bath. The clear supernatant was analysed for residual sucrose, glucose and synthesized fructo-oligosaccharides.

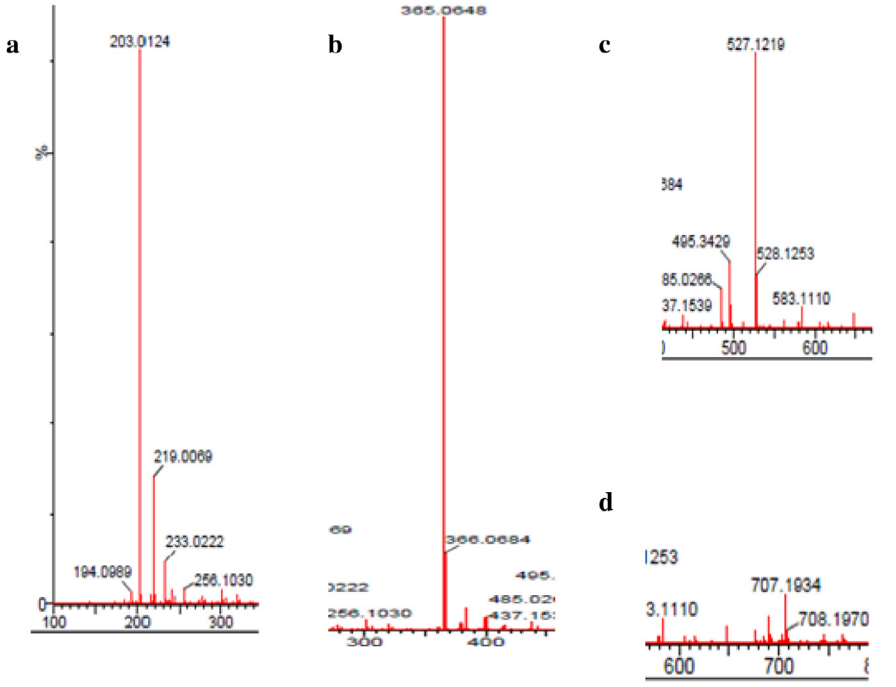

Figure 5. ESI-MS analysis of reaction mixture; (a) Glucose/fructose, *m*/*z* 203 $(M+Na)^+$; (b) Sucrose, *m*/*z* 365 $(M+Na)^+$; (c) Triose, m/z 527 $(M+Na)^+$; (d) Tetraose, *m*/*z* 689 $(M+Na)^+$.

Table 4
^1H and ^{13}C spectral assignments for purified triose and tetraose

		Triose		$J_{(H,H)}$ (HZ)	Tetraose		$J_{(H,H)}$ (HZ)
		δ_C	δ_H		δ_C	δ_H	
Glc	**1**	93.20	5.25; 5.42	3.8	93.71	5.39; 5.41	3.4
	2	72.44	3.64	8.8	71.81	3.54	6.3
	3	72.13	3.62	13.6	73.96	3.69	13.2
	4	71.01	3.44	–	69.93	3.41	9.2
	5	72.13	3.85	–	73.05	3.83	–
	6	60.49	3.80	–	60.80	3.80	–
Fru′	**1**	62.32	3.84	–	61.92	3.83; 3.74	–
	2	103.68	–	–	102.46	–	–
	3	77.24	4.15	7.2	77.31	4.20	8.2
	4	–	4.12	7.2	–	4.01	9.5
	5	81.10	3.87	–	80.55	–	–
	6	65.80	3.81	–	64.16	3.80	–
Fru″	**1**	60.49	3.66	–	–	3.83; 3.72	–
	2	107.65	3.96	–	102.46	–	–
	3	77.24	4.13	8.0	79.02	4.18	7.9
	4	–	3.98	8.2	–	4.03	7.5
	5	81.10	3.87	–	85.33	–	–
	6	65.80	3.85	–	64.16	3.72	–
Fru‴	**1**				60.95	3.78; 3.69	–
	2				102.46	–	–
	3				77.04	4.10	6.9
	4				76.14	4.06	7.0
	5				85.33	–	–
	6				65.82	3.74	–

–: cannot be determined.

4.4. Fructo-oligosaccharide synthesis using sucrose induced cells and effect of various parameters on yield

The time course profile of fructo-oligosaccharide synthesis was determined using sucrose-induced cells. For this, the reaction mixture (as described in Section 4.3) was incubated at 37 °C for 48 h. Aliquots were removed every 2 h, processed as described above, and analysed for fructo-oligosaccharides. Effect of substrate concentration on fructo-oligosaccharide yield was evaluated by varying sucrose from 5 to 50% (w/v). Enzyme units were varied from 4 to 16 I.U. invertase +0.25 to 1 I.U. equivalent inulinase (corresponding to 40–160 mg of lyophilized sucrose-induced cells) at the fixed sucrose concentration of 40% (w/v). All reactions were carried out in duplicate. Reported results are an average of the duplicates with standard deviation between 6 and 9%.

The interaction of parameters (time, sucrose concentration and enzyme units) on fructo-oligosaccharide yield was studied by Box–Behnken design. This design works well for three factorial level and

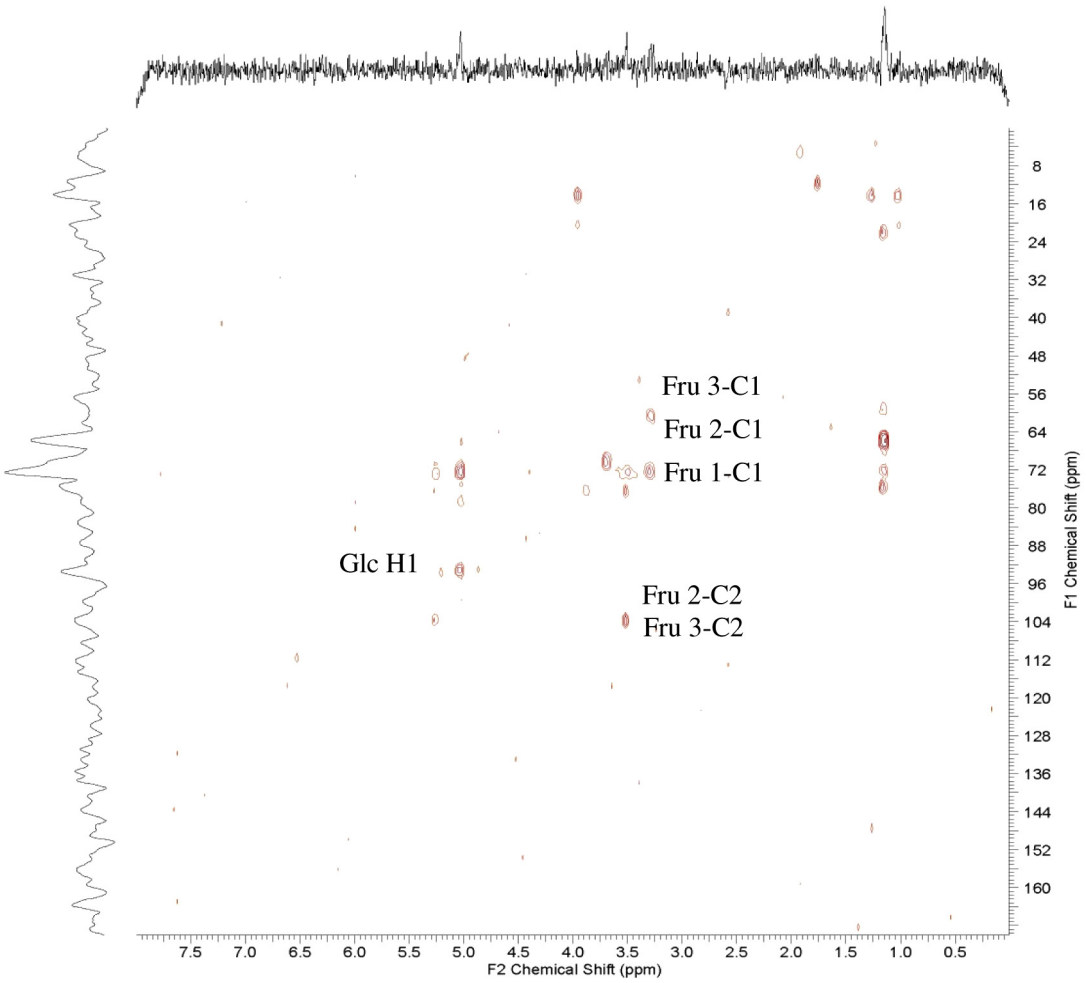

Figure 6. HMBC spectrum of 1-kestose dissolved in D_2O.

independent factors were studied at three levels (−1, 0, and +1). Duplicates experiments were designed. This design gives the optimal values with less number of designed experiments. Quadratic model was prepared and polynomial coefficients were calculated using Design Expert (Trial version 8.0, Stat- Ease Inc. Minneapolis, USA) for which the general equation is as follows:

$$Y = \beta_O + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_{ii} + \Sigma\beta_{ij} x_{ij}$$

where Y was the predicted response; β_O was the constant coefficient; β_i was ith linear coefficient; β_{ii} was the iith quadratic coefficient, and β_{ij} was the ijth interaction coefficient. The x_i and x_j were the input variables which influence response Y. The statistical analysis included correlation coefficient R, determination coefficient R-square (indicates the wellness of fit of the model), Fischer's F test, and its probability p (F). Response surface was generated for each variable using quadratic model.

4.5. Analytical methods

The cell bonded β-fructofuranosidase (i.e., inulinase and invertase) activities were assayed by measuring reducing sugars released from inulin and sucrose respectively.[31] Enzyme activities were also measured in the lyophilized cells by re-suspending in phosphate citrate buffer (pH 7.0).

Preliminary qualitative analysis of the synthesized fructo-oligosaccharide was carried out by thin layer chromatography (TLC). An aliquot (1 µl) of the clear supernatant was loaded on pre-coated silica gel 60 F_{254} aluminium plates (Merck, Germany) along with the respective standards. The products were resolved using ethyl acetate/2-propanol/water solvent mixture in a ratio of 4:3:1 (v/v/v).[32] The bands of formed products were visualized by spraying with 0.2% naphthoresorcinol reagent in ethanol: H_2SO_4 (95:5), followed by heating at 120 °C for 5 min.

Quantitative analysis of fructo-oligosaccharide was carried out by developing an HPLC based method, where several operating parameters such as eluent ratio, flow rate (varied from 0.5 to 1 ml/min), and column temperature (varied from 25 to 40 °C) were optimized in order to attain a complete resolution of the synthesized product peaks. An acetonitrile: water (60:40 v/v) solvent mixture was used as eluent. Shodex Asahipak NH2P-50 4E (4.8 × 250 mm) silica based amino column was used for proper separation of fructo-oligosaccharide residues. A 20 µl sample of the centrifuged and filtered reaction mixture was injected directly into the automatic sample injector loop and residual substrate and the products were detected by refractive index detector (RID, Agilent 100 series).

Mass analysis of the synthesized products was carried out in Qstar Electro-spray Ionization Mass Spectrometer equipped with quadruple and TOF (time of flight) mass analyzers in tandem. Centrifuged, filtered and appropriately diluted sample in 1 ml of methanol:water (1:1) was injected in mass spectrometer at a flow rate of 5 µl/min. The dilutions were made so that the samples may lie in the range of 10–100 ppm. The conditions set for analyses were: ion spray voltage: 5500 V, nebulizer gas: 20 lb in^{-2}, curtain

gas: 25 lb in^{-2}, declustering potential: 60 V, focusing potential: 265 V. Spectral acquisitions were done in the mass range of 100–2000 m/z value in a positive ion mode. Collision energy was varied in the range of 10–30 V to analyze the product ion spectra of tri- and tetra-oligosaccharide of fructo-oligosaccharide.

4.6. Nuclear magnetic resonance of synthesized products

NMR studies of purified extracted products were recorded on Bruker FT AM 500 (500 MHz and 125 MHz resonance frequencies respectively) in deuterated methanol solvent (MeOD). About 40 mg of purified trisaccharide and tetrasaccharide was dissolved in 0.5 ml of MeOD. For ^{1}H NMR, the region between 0 and 10 ppm was recorded for all the samples while for ^{13}C, a range of 0–200 ppm was recorded. Chemical shifts (δ) were expressed in parts per million (ppm) relative to tetramethylsilane as the internal standard. The coupling constant (J) was reported in Hertz (Hz).

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