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BIOACTIVE ISOMALTO-OLIGOSACCHARIDES SYNTHESIZED FROM *LEUCONOSTOC MESENTEROIDES* NRRL B-1426 DEXTRANSUCRASE WITH COLON CANCER CELLS INHIBITING AND FUNCTIONAL FOOD ADDITIVE PROPERTIES

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ABSTRACT

Isomalto-oligosaccharides (IMOs) are the major contributors to the prebiotic market. The present study is focussed on the optimization of the acceptor reaction and potential prebiotic application of IMOs. Optimum conditions of acceptor reaction for IMOs were 2 U of dextransucrase mL^{-1}, 30 °C, 7% (w/v) sucrose, 3% (w/v) maltose monohydrate as substrates after 24 h. The IMOs (DP ≥ 3) was used for the assessment of prebiotic activity and effect on mammalian cells. The IMOs showed that they were resistant to hydrolysis by simulated human gastric juice (pH 1) and human α-amylase (pH 7), exhibiting maximum hydrolysis of 20.9% and 16.2%, respectively. The IMOs also supported the growth of lactobacilli. The cytotoxicity assay indicated the selective inhibition of colon cancer cells' viability by 34% at 500 µg mL^{-1} after 36 h. The potential prebiotic activities of the synthesized IMOs further encouraged their applications in fast blooming food and nutraceutical industry.

Keywords Acceptor reaction; dextransucrase; isomalto-oligosaccharide; prebiotic; functional food.

INTRODUCTION

There has been a growing awareness among the consumers about the link between health and nutrition through the use of probiotics and prebiotics in diet (Saad et al. 2013; Chen et al. 2013). Recently, prebiotics tend to surpass probiotics due to various advantages such as resistance to digestive barrier, being cheaper, carrying less risks, providing new techno-functionalities, and being easier to incorporate into the diet (Goffin et al. 2011). Prebiotic is defined as "The selective stimulation of growth and/or activity(ies) of one or a limited number of microbial genus(era)/species in the gut microbiota that confer(s) health benefits to the host" (Roberfroid et al. 2010). Among various oligosaccharides functioning as prebiotics (Swennen et al. 2006), isomalto-oligosaccharides (IMOs) are also the major contributors to the oligosaccharide market (Seibel and Buchholz 2010; Bertrand et al. 2014). IMOs have been registered as a health-promoting prebiotic by the Korean Food and Drug Administration (http://www.foodnara.go.kr/hfoodi/). IMOs are marketed in Japan and United States of America as dietary supplements (Saad *et al.* 2013; Sharma *et al*. 2011). IMOs are primarily composed of α-(1→6) linkages and also a lower proportion of α-(1→4), α-(1→3) or α-(1→2) linkages (Goffin *et al*. 2011).

IMOs naturally occur in honey and also in various fermented foods viz. miso, sake or soy sauce (Goffin et al. 2011). IMOs have a great potential to improve the physico-chemical qualities of many foods as anti-fading agent for natural pigments, as food antioxidant and as sweetener (40% of the sweetness of sucrose with a calorific value of 2.8-3.2 kcal g^{-1}) (Kaneko *et al.* 1994; Chockchaisawasdee and Poosaran 2011) They have also been identified as low viscosity humectants with high moisture retaining capacity (Goffin et al. 2011). In addition, these oligosaccharides are recently reported to enhance the growth of selective intestinal microflora, signifying their role as potential prebiotics (Saad et al. 2013; Ketabi et al. 2011). However, the major concern in classifying IMOs as prebiotics is that only IMOs with high degree of polymerisation (DP), viz. isomaltotetraose and larger oligomers, are considered non-digestible (Patel et al. 2013).

The enzymatic synthesis of IMOs occurs via both hydrolytic and transfer activities (Chockchaisawasdee and Poosaran 2011). The conventional method for the production of IMOs from starch involves first the hydrolysis of starch into α-(1→4) linked dextrins using α-amylase (E.C. 3.2.1.1), pullulanase (E.C. 3.2.1.41) and β-amylase (E.C. 3.2.1.2) and then conversion to α-(1→6)-linked oligosaccharides using α-D-glucosidase (E.C. 3.2.1.20) (Delattre and Vijayalaksmi 2000; Goffin *et al.* 2011). Generally speaking, the commercially available IMOs, which is made by the above process, usually includes short-chain saccharides like isomaltose (DP2), panose (DP3), isomaltotriose (DP3) and isomaltotetraose

(DP4) (Ketabi et al. 2011) and thus synthesis of long-chain IMO is unachievable. The enzyme dextransucrase (E.C. 2.4.1.5), expressed by various species of the genus *Leuconostoc* catalyzes the synthesis of IMOs by its acceptor reaction with maltose in a single step (Cho et al. 2014). Maltose was determined as the best acceptor molecule and it was used for the production of molecular size-controlled dextrans and IMOs (Robyt and Eklund 1983; Lee et al. 2008; Kothari and Goyal 2013).

In the present study, the conditions for IMOs synthesis by acceptor reaction using dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426 were optimized. The IMOs were purified by gel permeation chromatography (GPC) and further characterized by HPLC and mass spectrometry. The purified IMOs (DP ≥ 3) were evaluated for their prebiotic potential in terms of gut digestibility, growth stimulation of probiotic bacteria and cytotoxicity activities.

MATERIALS AND METHODS

MICROORGANISMS AND CHEMICALS

Leuconostoc mesenteroides NRRL B-1426, *Bifidobacterium infantis* NRRL B-41661 and *Lactobacillus acidophilus* NRRL B-4495 were procured from ARS culture collection, National Center for Agricultural Utilization Research, Peoria, USA. The *L. mesenteroides* NRRL B-1426 was grown and maintained in modified de Man, Rogosa, and Sharpe (MRS) medium (Goyal and Katiyar 1996). *B. infantis* NRRL B-41661 and *L. acidophilus* NRRL B-4495 were grown and maintained in MRS medium (Deman et al. 1960) supplemented with 0.05% (w/v) cysteine-HCl (Vitali et al. 2012). Sucrose, maltose monohydrate, isomaltose, α-amylase from human saliva and all the cell culture related chemicals were purchased from Sigma-Aldrich, USA. The media components were purchased from Hi-media Pvt. Ltd., India. L-Cysteine, PEG-400 and TLC plates were purchased from Merck, India. Bio-Gel P-2 (fine mesh) was purchased from Bio-Rad Laboratories, Inc., USA. All the chemicals required for reducing sugar estimation, protein estimation and buffer preparation were of highest purity.

PREPARATION AND ASSAY OF DEXTRANSUCRASE

L. mesenteroides NRRL B-1426 was grown at 24 °C with 120 rpm for 6-8 h in enzyme production medium as described by Tsuchiya et al. (1952). The culture broth was centrifuged at 10,000 × *g* for 10 min at 4 °C to separate the cells and cell-free supernatant was subjected to 33% (v/v) PEG-400 fractionation for dextransucrase purification (Kothari and Goyal, 2015a). The dextransucrase activity was calculated by estimating fructose released using Nelson (1944) and Somogyi (1945) method. One unit (U) of dextransucrase activity was defined as the amount of enzyme that liberates 1 µmol fructose in 1 min at 30 °C and pH 5.6.

OPTIMIZATION OF REACTION CONDITIONS FOR IMO SYNTHESIS

EFFECT OF REACTION TIME

For the time course, the enzymatic reaction was carried out at 30 °C from 0 to 24 h in 10 mL mixture containing 5% (w/v) sucrose and 5% (w/v) maltose monohydrate, 1 U mL^{-1} of enzyme solution, 0.3 mM $CaCl_2$ and 15 mM NaN_3 in 20 mM sodium acetate buffer (pH 5.6).

EFFECT OF ENZYME ACTIVITY

The effect of enzyme activity on the synthesis of IMOs was determined by varying the enzyme activity from 0.1 to 8 U mL^{-1}. The enzymatic reaction was carried out at 30 °C for 24 h in a 1 mL mixture containing 5% (w/v) sucrose, 5% (w/v) maltose monohydrate, 0.3 mM $CaCl_2$ and 15 mM NaN_3 in 20 mM sodium acetate buffer (pH 5.6).

EFFECT OF TEMPERATURE

The effect of temperature on synthesis of IMOs was determined by varying the temperature from 20 to 40 °C. The enzymatic reaction was carried out for 24 h in a 1 mL mixture containing 5% (w/v) sucrose, 5% (w/v) maltose monohydrate, 2 U mL^{-1} of enzyme activity, 0.3 mM $CaCl_2$ and 15 mM NaN_3 in 20 mM sodium acetate buffer (pH 5.6).

EFFECT OF SUCROSE CONCENTRATION

To determine the optimum concentration of sucrose for IMOs synthesis, the enzymatic reaction was performed with varying concentrations of sucrose from 0 to 15% (w/v), maintaining the concentration of maltose monohydrate at 5% (w/v) and enzyme activity 2 U mL^{-1}. The enzymatic reactions were carried out at 30 °C for 24 h in 1 mL 20 mM sodium acetate buffer (pH 5.6) containing 0.3 mM $CaCl_2$ and 15 mM NaN_3.

EFFECT OF MALTOSE CONCENTRATION

To determine the optimum concentration of maltose for synthesis of IMOs, the enzymatic reaction was performed with varying concentrations of maltose from 0 to 15% (w/v), maintaining the concentration of sucrose at 7% (w/v) and enzyme activity 2 U mL^{-1}. The enzymatic reactions were carried out at 30 °C for 24 h in 1 mL 20 mM sodium acetate buffer (pH 5.6) containing 0.3 mM $CaCl_2$ and 15 mM NaN_3.

All the reactions were terminated by adding equal volumes of absolute ethanol and centrifuged at 16,000 × *g* for 10 min to remove polysaccharides (dextran). The supernatants were analyzed for IMO synthesis by TLC.

THIN LAYER CHROMATOGRAPHY (TLC)

The 1 µL of each fraction containing IMOs was analyzed by TLC using mobile phase of ethyl acetate:acetonitrile:H_2O:1-propanol (2/7/5.5/5, v/v/v/v) (Cote and Leathers 2005). The spots were visualized by dipping the TLC plate into an ethanol solution (0.5%, w/v α-naphthol and 5%, v/v H_2SO_4) and heating at 120 °C for

20 min (Kim and Day 2008). The quantification of IMOs was done on TLC plates by densitometric analysis using a Gel Logic 1500 Imaging System (KODAK, USA). Glucose, isomaltose and sucrose (0.1-20 µg) were used as standards.

SYNTHESIS OF DEXTRANS WITH DIFFERENT MOLECULAR WEIGHT (M_W) IN ACCEPTOR REACTION

The precipitated dextran was re-precipitated with three volumes of ethanol, centrifuged, re-dissolved in 1 mL of water, and this process was repeated thrice for the complete removal of sucrose, maltose and fructose. The resulting dextran was dissolved in water and the concentration was determined by phenol-sulfuric acid method (Fox and Robyt 1991). The reducing value of dextran was determined by using the copper bicinchoninate method (Fox and Robyt 1991). The number-average degree of polymerization (DP_n) and number average molecular weight (MW_n) of dextran were determined by the Equations (Eq. 1) and (Eq. 2) as described by Jane and Robyt (1984) as follows.

$$DP_n = \frac{\text{Total carbohydrate content in µg of D-glucose}}{\text{Reducing value in µg of D-maltose}} \times 1.9 \quad \text{E.q.1}$$

$$MW_n = [(DP_n)\times 162] + 18 \quad \text{Eq. 2}$$

PURIFICATION OF IMOS

The acceptor reaction of dextransucrase was carried out for 24 h under optimized conditions (sucrose 7%, w/v; maltose 3%, w/v; dextransucrase activity 2 U mL^{-1}; 30 °C) in 50 mL reaction mixture for purification of IMOs as described earlier. The reaction was terminated by adding equal volume of absolute ethanol after 24 h and centrifuged at 16,000 × *g* for 10 min at 4 °C to remove the polymeric dextran. The 100 mL supernatant containing IMOs, fructose, residual sucrose and maltose was concentrated to 50 mL using a rotary vacuum evaporator (IKA, HB 10). The supernatant containing oligosaccharides was purified by GPC on the basis of their degree of polymerization (DP) using XK16/70 column (GE Healthcare) packed with Bio-Gel P-2 (fine mesh) on AKTA purifier (GE Healthcare, model 100 Plus) (Kothari and Goyal 2015b). The fractions were analyzed for oligosaccharides by phenol-sulfuric acid method (Fox and Robyt 1991) and identified by TLC. The selected fractions under the peaks of purified IMOs of various DP were analyzed by HPLC and ESI-TOF MS. The purified IMOs of DP ≥ 3 were pooled, lyophilized and tested for their prebiotic activity and cytotoxicity.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

The selected IMO fractions obtained from GPC were analyzed by HPLC (Varian, ProStar). The HPLC system was equipped with a binary pump, refractive index detector and a 20 µL injection loop. Hi-Plex Na column (300 × 7.7 mm, ID) (Agilent Technologies, USA) was used for chromatographic separation at 37 °C. Water was used as eluent at the flow rate of 0.2 mL min^{-1}.

MASS SPECTROMETRY

The selected purified fractions of IMOs were analyzed by ESI-TOF MS in positive mode using an Agilent 6520 Accurate mass Q-TOF LC/MS system (Agilent Technologies, USA) under the conditions as described by Kothari and Goyal (2015b).

***IN VITRO* PREBIOTIC ACTIVITY ASSAY OF IMOS**

DIGESTIBILITY OF IMOS BY SIMULATED HUMAN GASTRIC JUICE

The digestibility of IMOs (DP ≥ 3) by simulated human gastric juice was tested in HCl buffer supplemented with 0.3% (w/v) pepsin by the method described by Kothari and Goyal (2015b). IMOs and inulin solutions (1 mL, 1%, w/v) were mixed with 1 mL of simulated human gastric juice at the pH 1, 2 and 3 separately and the reaction mixtures were incubated at 37 °C for 6 h. Aliquots (100 µL) of the reaction mixture from 0 to 6 h were collected to calculate the percent hydrolysis. Percent hydrolysis of samples was calculated by the following equation given by Korakli et al. (2002).

$$\text{Hydrolysis (\%)} = \frac{\text{Reducing sugar released}}{\text{Total sugar - Initial reducing sugar}} \times 100$$

Where, the reducing sugar released is the difference between its final and initial reducing sugar content. The total sugar (expressed in glucose equivalents) and reducing sugar (expressed in maltose equivalents) contents were determined by phenol sulfuric acid and copper bicinchoninate methods (Fox and Robyt 1991), respectively.

DIGESTIBILITY OF IMOS BY HUMAN A-AMYLASE

The IMOs (DP ≥ 3) and inulin were tested for digestibility by human α-amylase (Kothari & Goyal, 2015b). IMOs and inulin samples (1 mL, 1%, w/v) were mixed with 1 mL of α-amylase solution (2 U mL^{-1}, pH 7) and incubated at 37 °C for 6 h. Aliquots (100 µL) of the reaction mixture were collected at 0-6 h to calculate the percent hydrolysis as described above.

GROWTH STIMULATION OF PROBIOTIC BACTERIA BY IMOS

The effects of IMOs (DP ≥ 3) and inulin (standard prebiotic) on the growth of probiotic bacteria (*B. infantis* and *L. acidophilus*) were evaluated by the method as described by Kothari and Goyal (2015b). 1 mL of log phase probiotic cultures were harvested by centrifuging the cells at 3,200 × *g* for 15 min at 4 °C. The cell pellets were washed twice with phosphate buffered saline (PBS, pH 7.4), re-suspended in 1 mL PBS and inoculated into 5 mL MRS medium (1%, v/v) containing purified IMOs, inulin or glucose. The MRS medium, containing glucose (1%, w/v) was used as a positive control and without any

carbohydrate served as a negative control. The growth of probiotic bacteria was monitored as a function of absorbance at 600 nm (A_{600}) for the determination of prebiotic score (PS) of IMOs. PS is determined by the formula, PS = (A/B) × 100%, where A and B are the mean A_{600} values of a strain grown with prebiotics (IMOs or inulin) and glucose, respectively, after 24 h at 37 °C (Kneifel et al. 2000). The PS was correlated with the corresponding pH changes in the growth medium.

IN VITRO CYTOTOXICITY ASSAYS FOR IMOS

An early passage of human embryonic kidney (HEK-293), human cervical adenocarcinoma (INT-407) and human colon carcinoma (HT-29) cell lines were procured from National Center for Cell Sciences (NCCS), Pune, India. The cells were grown and maintained in T-25 and T-75 flasks (BD Biosciences, USA) as monolayers at 37 °C in a humidified incubator maintained at 5% CO_2 saturation (Kothari and Goyal 2015b). The effects of purified IMOs on viability of HEK-293, INT-407 and HT-29 cells were evaluated by MTT assay (Alley et al., 1988). The cell viability (%) was calculated as described by Patel et al. (2010).

STATISTICAL ANALYSIS

The prebiotic activity and cell culture experiments were performed in triplicate. Results were expressed as the mean values ± standard deviation. The data were statistically analyzed using paired t-test based on the confidence limits 95% ($p \leq 0.05$).

RESULTS AND DISCUSSION

OPTIMIZATION OF REACTION CONDITIONS FOR IMO SYNTHESIS

EFFECT OF REACTION TIME

The concentration of IMOs increased with increase in the time of acceptor reaction and consequently reached saturation after 12 h (Table 1). As the time proceeded from 1 to 2 h, the degree of polymerization (DP) of IMOs increased from DP3 to DP5 and thereafter, the DP of IMOs increased upto six (i.e., DP3-DP6). The concentration of IMOs and dextran ranged from 11.7 to 33.1 mg mL^{-1} and 2.3 to 5.2 mg mL^{-1}, respectively. The number average molecular weight (MW_n) of dextran also increased with time, ranging from 55 to 414 kDa (Table 1). Thus, 24 h was taken optimum time for the synthesis of IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase.

Table 1 Effect of time, enzyme activity, temperature, sucrose and maltose on the acceptor reaction of dextransucrase from *L. mesenteroides* NRRL B-1426.

Parameters	IMOs (mg mL^{-1})							Dextran (mg mL^{-1})	Dextran MW_n (kDa)
	DP3	DP4	DP5	DP6	DP7	DP8	Total		
Time (h)									
1	9.43	2.24	-	-	-	-	11.67	2.33	58.55
2	10.87	5.19	-	-	-	-	16.06	3.03	112.51
4	13.48	9.40	0.74	0.11	-	-	23.73	3.85	187.57
6	14.57	11.73	2.53	0.26	-	-	29.09	4.01	247.19
8	13.81	12.62	3.19	0.30	-	-	29.93	4.32	301.80
10	13.42	13.32	3.15	0.44	-	-	30.34	4.71	378.46
12	13.40	13.48	4.07	0.71	-	-	31.67	4.76	398.49
14	13.28	13.62	5.11	0.92	-	-	32.94	4.95	407.97
24	12.62	12.63	6.78	1.13	-	-	33.15	5.26	413.70
Dextransucrase actvity (U mL^{-1})									
0.1	9.49	1.22	-	-	-	-	10.71	1.85	604.75
0.2	9.32	10.71	1.68	-	-	-	21.71	2.74	477.29
0.5	10.49	12.35	3.70	0.34	-	-	26.88	4.26	393.10
1	11.33	12.59	8.08	0.46	-	-	32.46	4.30	351.84
2	11.99	12.66	8.43	0.53	-	-	33.62	6.09	346.41
3	12.79	12.39	8.78	0.57	-	-	34.52	7.89	285.24
4	13.10	11.56	9.18	0.55	-	-	34.39	9.12	273.89
5	13.69	11.37	9.03	0.53	-	-	34.62	10.36	227.99
6	13.35	12.37	9.75	0.61	-	-	36.08	10.89	181.32
8	14.13	12.59	9.56	0.33	-	-	36.61	12.29	146.74
Temperature (°C)									
20	11.02	9.31	3.81	-	-	-	24.14	3.13	143.98
25	11.95	9.22	4.08	-	-	-	25.25	3.94	177.49
30	12.75	11.64	6.92	0.54	-	-	31.85	4.43	367.52
35	10.97	9.22	4.17	0.21	-	-	24.57	3.59	331.30
40	10.68	7.29	1.78	-	-	-	19.75	2.51	157.56
Sucrose (%, w/v)									
1	8.07	2.64	-	-	-	-	10.71	1.36	102.99

2	10.63	4.11	-	-	-	-	14.75	2.33	254.31
3	12.29	9.98	1.13	-	-	-	23.41	2.95	385.86
4	13.07	10.77	2.66	-	-	-	26.51	4.19	448.44
5	13.52	11.37	7.34	0.46	-	-	32.69	4.52	375.21
6	13.23	12.45	8.06	0.60	-	-	34.34	4.19	184.18
7	11.82	12.98	9.63	0.97	0.39	-	35.79	2.23	132.10
8	12.83	14.96	9.07	1.62	0.51	-	39.01	2.27	139.99
9	11.59	16.42	10.24	1.73	0.72	-	40.70	3.06	168.18
10	9.62	13.11	12.65	8.99	1.60	0.53	46.50	3.48	213.66
12	9.63	12.66	10.88	9.07	3.29	0.55	46.06	3.81	340.92
15	10.61	11.69	8.39	6.90	6.83	0.52	44.96	3.92	415.78
Maltose (%, w/v)									
0	-	-	-	-	-	-	-	29.35	2170.55
1	1.25	5.76	5.82	3.91	0.66	-	17.40	9.11	303.10
2	9.01	11.34	9.15	6.51	0.99	-	36.99	5.66	231.40
3	10.44	12.03	13.59	9.50	4.46	3.19	53.21	0.76	49.71
4	11.46	11.01	9.24	6.09	1.28	1.12	40.19	1.44	75.69
5	11.74	10.18	9.00	3.57	0.51	-	35.00	1.71	94.74
6	12.37	10.30	8.72	2.26	0.95	-	34.60	1.99	81.42
7	12.90	11.18	8.64	1.33	-	-	34.05	1.92	116.87
8	13.97	10.06	9.26	0.77	-	-	34.07	1.79	109.86
9	14.56	13.33	7.10	0.44	-	-	35.43	1.72	98.85
10	15.20	13.82	4.96	0.35	-	-	34.33	1.99	95.75
12	18.01	11.42	4.65	0.25	-	-	34.33	1.75	91.48
15	18.98	11.59	4.26	0.12	-	-	34.95	1.54	64.03

EFFECT OF ENZYME ACTIVITY

The concentration of IMOs increased from 10.7 to 33.6 mg mL^{-1} as the enzyme activity was increased from 0.1 to 2 U mL^{-1} (Table 1). Higher than 2 U mL^{-1} dextransucrase activity showed little influence on the final IMOs yield. The enzyme activity above 1U mL^{-1} did not affect much the DP of IMOs and retained the number of acceptor products four, i.e., from DP3 to DP6. The concentration of dextran also increased from 1.8 to 12.8 mg mL^{-1} with increase in the dose of enzyme activity from 0.1 to 8 U mL^{-1}. However, an inverse relationship of the MW_n of the synthesized dextran from 605 to 147 kDa was observed as the dextransucrase activity increased from 0.1 to 8 U mL^{-1} (Table 1). These results were ascribed to the processive nature of the polymerization of dextran by *L. mesenteroides* NRRL B-1426 dextransucrase as also described for *L. mesenteroides* NRRL B-512FMC dextransucrase by Falconer et al. (2011). Processivity occurs when D-glucose from sucrose in the dextransucrase reaction is continuously added to the growing dextran chain that is covalently attached to the active site of the enzyme. At the lower enzyme concentration, fewer active sites will be available with covalently attached growing dextran chains (Falconer et al. 2011; Robyt et al. 2008). Therefore, these growing dextran chains will keep on adding more of D-glucose synthesizing longer chains of dextran resulting in higher MW_n.

EFFECT OF TEMPERATURE

The effect of temperature on acceptor reaction of dextransucrase was studied at 20, 25, 30, 35 and 40 °C (Table 1). The amount of IMOs increased as the temperature was increased from 20 °C to 30 °C and then decreased after that. The optimum temperature for synthesis of IMOs was found to be 30 °C. This might be due to the fact that the dextransucrase from *L. mesenteroides* NRRL B-1426 was reported to maximally active at 30 °C (Kothari et al. 2012). The number of acceptor products remained relatively constant at three or four (DP3-DP6) at different temperatures ranging from 20-40 °C. The concentration of IMOs first increased from 24.1 to 31.8 mg mL^{-1} with increasing temperature 20-30 °C and then decreased after that to 19.7 mg mL^{-1}. Similar trends of the concentration and MW_n of dextran were also observed (Table 1). The MW_n of dextran have also been reported to increase with the increase in temperature from 20 °C to 30 °C in case of *L. mesenteroides* NRRL B-512FMC dextransucrase (Falconer et al. 2011). Moreover, the higher temperature enhances the rate of polymerization reaction of dextransucrase and increases the branching of dextran resulting in a higher molecular weight of dextran (Falconer et al. 2011; Robyt and Taniguchi 1976).

EFFECT OF SUCROSE CONCENTRATION

The sucrose concentration played an important role in the synthesis of IMOs with wide variation in degree of polymerization. The concentration of IMOs increased from 10.7 to 46.1 mg mL^{-1} as the concentration of sucrose increases from 1 to 15% (w/v), with the formation of higher DP at higher sucrose concentration. The concentration of dextran ranged from 1.4 to 4.5 mg mL^{-1} and the MW_n of dextran ranged of 103 to 416 kDa (Table 1) under the same sucrose concentration range. The sucrose concentration affected the pattern of DP distribution. For instance, when the sucrose concentration increased from 1-2% (w/v), the number of acceptor products remained two or three only (DP3-DP4). The number of acceptor products became three to four (DP3-

DP6) till 5% (w/v) sucrose and beyond that the number increased to ten (DP3-DP10) (Table 1). However, in the present study, the concentrations of IMOs were calculated upto DP8 only, due the fact that DP > 8 had very low concentration. From these results, it could be inferred that when the sucrose concentration increases, the chain length of IMOs gets larger as also reported by Lee et al. (2008). In general, with increase in the concentration of sucrose there is an increase in the number of D-glucose units that are added to the growing reducing ends of the dextran chains giving higher dextran concentration and MW_n. This was primarily due to Michaelis-Menten kinetics in which the rate of the reaction is proportional to the concentration of the substrate, giving longer, higher molecular weight chains (Falconer et al. 2011). For industrial production of IMOs, an enzyme reaction at low substrate concentration is generally recommended resulting in lowering the cost of processing. Therefore, in the present study, the sucrose concentration of 7% (w/v) was chosen which also gave all the DP of IMOs and lower concentration of dextran.

EFFECT OF MALTOSE CONCENTRATION

Maltose concentration played a very important role in the synthesis of IMOs by acceptor reaction of dextransucrase (Table 1). The concentration of IMOs ranged from 17.4 to 53.2 mg mL^{-1} with varying maltose concentration (0-15%, w/v). The synthesis of IMOs was found to be maximum at 3% (w/v) maltose concentration. The number of acceptor products were higher (DP3-DP10) at lower maltose concentration (< 5%, w/v) and were restricted to four (DP3-DP6) at higher maltose concentration (> 5%, w/v) (Table 1). The dextran concentration decreased from 29.3 to 0.75 mg mL^{-1} as maltose concentration increased from 0 to 15% (w/v). The MW_n of dextran was also decreased from 2170 to 50 kDa on varying the maltose concentration from 0 to 15% (w/v). This may be due to the dominance of acceptor reaction of dextransucrase with maltose resulting in lower concentration and MW_n of dextran. The acceptor reaction of dextransucrase with maltose, a known best acceptor competes with the polymerization reaction to give low molecular weight dextrans and inhibiting the synthesis of dextran. The results of optimization of acceptor reaction conditions showed that the optimum conditions for synthesis of IMOs from DP3 to DP10 are 7% (w/v) sucrose, 3% (w/v) maltose, 2 U mL^{-1} dextransucrase and 30 °C for 24 h.

PURIFICATION AND CHARACTERIZATION OF IMOS

The IMOs of various DPs synthesized at optimum conditions were purified by gel permeation chromatography using Bio-Gel P-2 column. The chromatogram showed that the IMOs were eluted from 45 to 75 mL on the basis of carbohydrate content (Fig. 1A). The collected fractions corresponding to the peak (45-89 mL) in the chromatogram were analyzed by TLC (Fig. 1B), HPLC (Fig. 2) and ESI-TOF MS (Fig. S1) analyses. The TLC and HPLC analyses showed that with the increase in the fraction number, the DP of IMOs decreased (Fig. 1B and 2). The TLC and HPLC of the selected purified fractions of IMOs revealed that *L. mesenteroides* NRRL B-1426 dextransucrase synthesized IMOs from DP 3 to 10 as also confirmed by ESI-TOF MS (Fig. S1). The peaks at m/z 527.2, 689.2, 851.3, 1013.3, 1175.4, 1337.4, 1499.5 and 1661.6 corresponded to the $[M+Na]^+$ ions of IMOs from DP3 to DP10, respectively (Fig. S1). The findings confirmed that the acceptor products were a homologous series of IMOs that contained maltose at the reducing end and glucose units at the non-reducing end by α-(1→6) linkage. Similar maltose acceptor products have been reported for dextransucrase from *L. mesenteroides* NRRL B-512F (Robyt and Eklund 1983; Cote and Leathers 2005). It has been reported earlier that the strains producing low branched (10-12%) dextrans produced only one series of IMOs, while strains producing highly branched dextrans produced two series of acceptor products *viz.*, (i) linear isomaltodextrinyl series of oligosaccharides and (ii) linear isomaltodextrins plus branched oligosaccharides, with α-(1→2) or α-(1→3) or α-(1→4) linked branch points (Cote and Leathers 2005). In the present study, the IMOs from *L. mesenteroides* NRRL B-1426 belong to the first category containing only linear isomaltodextrinyl series of oligosaccharides, because the strain produces (i) only dextransucrase and no other glucansucrases (Kothari and Goyal 2015a) and (ii) low branched (~14.5%) dextran (Kothari and Goyal 2013).

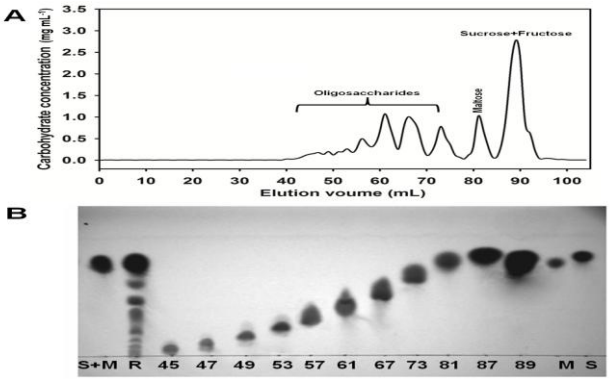

Figure 1.(A) Elution profile for the gel permeation chromatography of acceptor reaction mixture from *L. mesenteroides* NRRL B-1426 dextransucrase on Bio-Gel P-2 (XK 16/70) and (B) TLC of the selected fractions containing IMOs, S+M-sucrose and maltose; R-acceptor reaction mixture containing IMOs; 45 to 89-fraction numbers; M-Maltose and S-Sucrose.

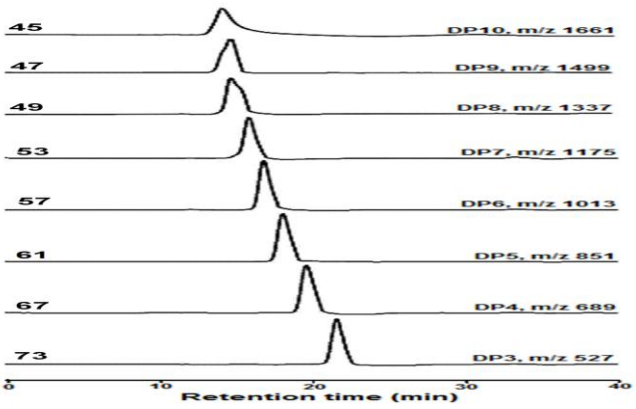

Figure 2. HPLC of the selected fractions (45 to 73) containing IMOs with their corresponding m/z values.

IN VITRO PREBIOTIC ACTIVITY ASSAY OF IMOS

DIGESTIBILITY OF IMOS BY SIMULATED HUMAN GASTRIC JUICE

Indigestibility is a one of the key criteria for oligosaccharide to act as a prebiotic (Wong and Jenkins 2007; Wang 2009; Saad et al. 2013). The indigestibility of prebiotics comes from the specific configuration of their linkage and the substrate specificity of the digestive gastrointestinal enzymes (Roberfroid 1997; Goffin et al. 2011). The IMOs (DP ≥ 3) from *L. mesenteroides* NRRL B-1426 dextransucrase were found resistant to hydrolysis by simulated human gastric juice (Fig. 3). The percent hydrolysis of IMOs and inulin increased with increase in incubation period and with decrease in the pH of simulated human gastric juice. The maximum hydrolysis of IMOs after 6 h at pH of 1, 2 and 3 was 20.9%, 12.5%, and 9.3%, respectively (Fig. 3A), whereas the inulin showed 39.7%, 19.4% and 13.8% of hydrolysis at the respective pHs (Fig. 3B). The IMOs showed significantly lower (1.9 to 20.9%) hydrolysis at pH 3 to 1 as compared with the standard inulin (4.7 to 39.7%).

IMOs were 1.9 fold higher resistant to hydrolysis at pH 1 and 1.5 fold higher resistant at pH 3 than inulin. Seo et al. (2007) reported that IMOs were acid stable at low pH ranging from 2 to 4. The IMOs were also not hydrolyzed by the *in vitro* digestive system (Kaneko et al. 1992). The main site of action for prebiotics is the colon where it is fermented by the probiotics. Thus, a prebiotic should resist the effects of gastric acidity and digestive enzymes in order to reach the colon intact (Das et al. 2014). Focussing on prebiotic properties, a large amount of the IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase will be available in the colon as compared with inulin for fermentation by probiotics. Therefore, IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase can be used as functional food supplement.

DIGESTIBILITY OF IMOS BY HUMAN A-AMYLASE

Utilization of oligosaccharides by probiotics, not otherwise digestible by human enzymes, has been recognized as an important attribute of prebiotics (Saad et al. 2013; Barrangou et al. 2003). The IMOs (DP ≥ 3) from *L. mesenteroides* NRRL B-1426 showed comparatively lower degree of resistance (16.2%) by α-amylase as compared with inulin (12.8%) after 6 h (Fig S2). This may be due to the fact that α-amylase cleaved α-(1→4) linkage of maltose in IMOs. However, more than 80% IMOs escaped the digestion by α-amylase and hence they are suitable for prebiotic application. Wichienchot et al. (2006) reported that at least 60% of gluco-oligosaccharides produced by *Gluconobacter oxydans* NCIMB 4943 could reach the colon for their prebiotic application.

GROWTH STIMULATION OF PROBIOTIC BACTERIA BY IMOS

The effect of IMOs on the growth of probiotics was studied by determining the prebiotic score (PS). The PS reveals the ability of a given substrate to support the growth of probiotic strains. IMOs showed higher prebiotic score (PS) for *L. acidophilus* as compared with *B. infantis* (Fig. 4). The PS of IMOs (95%) for *L. acidophilus* was higher as compared with the commercial prebiotic inulin (89%). However, IMOs (DP ≥ 3) from *L. mesenteroides* NRRL B-1426 dextransucrase did not significantly stimulate the growth of *B. infantis* as compared with inulin (Fig. 4).

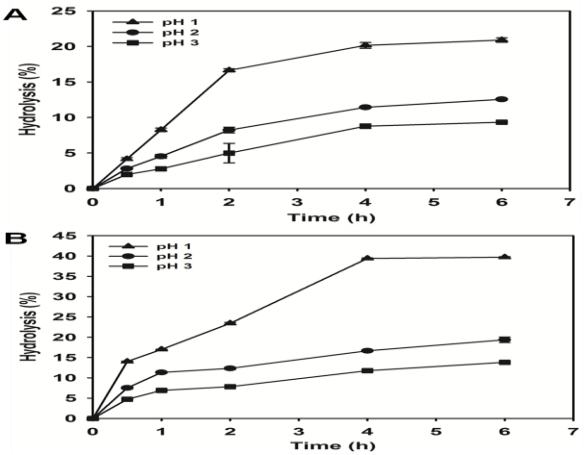

Figure 3. Hydrolysis of (A) IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase and (B) inulin by simulated human gastric juice at pH 1, 2 and 3 and 37 °C for 6 h. The hydrolysis of IMOs at pH 1 was significantly different vs. inulin at $p \leq 0.01$ and significantly different at pH 2 and 3 vs inulin at $p \leq 0.05$.

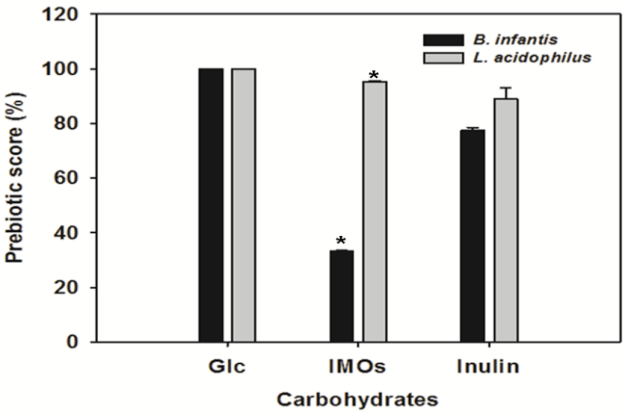

Figure 4. Prebiotic score of IMOs (DP ≥ 3) and inulin for *B. infantis* and *L. acidophilus.* *Significantly different ($p \leq 0.05$) vs inulin.

An increase in the number of bifidobacteria and lactobacilli resulting from the use of prebiotics has been demonstrated to protect against colonic DNA damage in animal models (Tuohy et al. 2005). The growth of probiotics was correlated with the change in pH of the fermentation medium. The fermentation of IMOs by *L. acidophilus* significantly reduced the pH of the growth medium as compared with inulin ($p \leq 0.01$) (Table S1). However, no significant change in the pH of medium containing IMOs was observed in case of *B. infantis*. Hence, it could be inferred that *L. acidophilus* used IMOs as carbon source during fermentation and produced short chain fatty acids (SCFA) such as lactic acid and acetic acid, which might have been responsible for lowering

significantly the pH of growth medium. SCFA are known to act as nutrients and growth signals for the intestinal epithelium and may play an important role in colon cancer prevention (Mai 2004; Mussatto and Mancilha 2007). The acidic pH condition in the human colon is also known to stimulate the growth of probiotics (Mussatto and Mancilha 2007) and suppress the intestinal pathogenic bacteria (Hongpattarakere *et al.* 2012).

IN VITRO CYTOTOXICITY ASSAYS FOR IMOS

The cell viabilities of HEK-293 (Fig. 5A) and INT-407 (Fig. 5B) were not affected by the IMOs, rather an increase in the growth of both the cell lines was observed with respect to the control cells. This increased growth may be attributed to the potential of IMOs serving as an additional carbon source in the medium. The oligosaccharides have also been reported to act as extra carbon or energy source for Chinese hamster ovary (CHO) cell line (Altamirano et al. 2000). The prebiotics should be safe for human consumption (Wang 2009). The results showed that the IMOs from *L. mesenteroides* NRRL-1426 dextransucrase are biocompatible in nature and hence could be safely used.

At the same time, IMOs were observed to negatively affect the viablility of cancerous HT-29 cells in a time- and dose-dependent manner. The HT-29 cells' growth was significantly inhibited by 14%, 21%, 24%, 29% and 34% at IMOs concentrations of 10, 50, 100, 200 and 500 µg mL^{-1}, respectively, at 36 h (Fig. 5C). Recently, the applications of nutraceuticals or functional foods as potential cancer prevention agents has gained significant attention in health- and cancer-related research (Li et al. 2013). One of the key physiological functions related to colon cancer risk includes control of epithelial cell proliferation (Tappenden and Deutsch 2007). A selective cytotoxic behaviour by IMOs, affecting only colon cancer cell lines (HT-29) can be used for the development of putative colon cancer chemo preventive agent. Although further in *vitro* and in *vivo* bioactivity studies are required for its confirmation. The chemo preventive effects on HT-29 cells were also reported for chito-oligosaccharides (Nam et al. 2007) and apple oligosaccharides (Altamirano et al. 2000). Therefore, IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase could act as bioactive oligosaccharides with potential pharmaceutical and functional food applications.

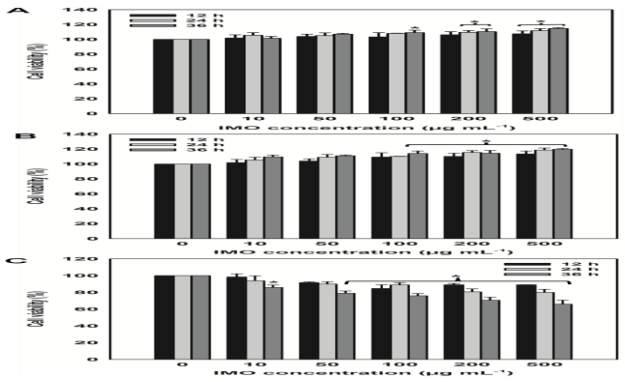

Figure 5.The effect of IMOs (DP ≥ 3) on the viability of (A) HEK-293, (B) INT-407 and (C) HT-29 cells at different concentrations of IMOs (0-500 µg mL^{-1}) for 12-36 h of incubation. *Significantly different ($p \leq 0.05$) vs control.

CONCLUSION

The optimum acceptor reaction conditions for the synthesis of IMOs (DP3-DP10) from *L. mesenteroides* NRRL B-1426 dextransucrase were determined to be 2 U mL^{-1} of dextransucrase, 7% (w/v) sucrose and 3% (w/v) maltose at 30 °C. Since the long-chain IMOs are preferred to short chain ones owing to the longer persistence in the colon, therefore, the present study will provide an alternative method for industrial production of IMOs. Moreover, the acceptor reaction of dextransucrase can be selelectively tuned to generate dextrans with different MWn. The purified IMOs (DP ≥ 3) also exhibited the potential prebiotic activity and selective inhibition of colon cancer cell lines without influencing the growth of normal cells (HEK-293). However, the detailed structural characterization of these IMOs and further confirmatory *in vitro* and *in vivo* experimental studies are need to be performed before their extension as functional food ingredient.

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