Synthesis, purification, characterization and prebiotic applications of dextran and oligosaccharides from *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase

A Thesis

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by

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Under the supervision of

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STATEMENT

I do hereby declare that the content embodied in this thesis entitled "**Synthesis, purification, characterization and prebiotic applications of dextran and oligosaccharides from *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase"** is the result of investigations carried out by me in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, Assam, India under the guidance of Professor Arun Goyal.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

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CERTIFICATE

It is certified that the work described in this thesis entitled "**Synthesis, purification, characterization and prebiotic applications of dextran and oligosaccharides from *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase"** by **Damini Kothari (Roll No. 10610614)** for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision mainly in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, Assam, India. The work embodied in this thesis has not been submitted elsewhere for a degree.

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Damini Kothari
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SYNOPSIS

Introduction

The modern world faces the new challenges in 21st century, the exponentially growing costs of health care, increasing life expectancy, improving scientific knowledge and developing new technologies owing to major changes in lifestyles. Nutrition has to adapt to these new challenges by developing new concepts. 'Optimized nutrition' is one of these new concepts that aimed at maximizing physiological functions since it is now well established that the gut microbiota act as a key player in health with a minimum risk of disease throughout life. In this context, "functional food" is a new and stimulating concept, which has been deeply ingrained in Asia and is a fast growing segment of the food industry in the United States and Europe. Thus, there is clearly a need to increase our knowledge of gut microbiota and interactions with functional food. Regulation of the microbial ecology of the colon through the use of prebiotics as functional foods, has for decades gained special interest in the scientific consortium as well as among consumers. Prebiotic is defined as "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well being and health". Health benefits attributed to prebiotics include protection against bowel cancer, inflammatory bowel disease, pathogenic agents, coronary heart disease, obesity, low caloric content, and stimulation of growth and metabolism of specific colonic microbiota. Most prebiotics identified today are non-digestible oligosaccharides except inulin. Although a number of oligosaccharides have been proposed as prebiotics, fructo-oligosaccharide (FOS), galacto-oligosaccharide (GOS) and lactulose have achieved the

prebiotic status. The other most commonly studied oligosaccharides for prebiotic activity are gluco-oligosaccharide (GLOS), isomalto-oligosaccharide (IMO), xylo-oligosaccharide (XOS), lactosucrose, arabinoxylose oligosaccharide (AXOS), soybean meal oligosaccharide (SMO), mannan oligosaccharide (MOS), pectin-derived acidic oligosaccharide (pAOS), chito-oligosaccharide (COS), konjac glucomannan oligosaccharide, xanthan-derived oligosaccharide, alginate derived oligosaccharide (ADO), laminarin oligosaccharide, agaro-oligosaccharide (AOS), palatinose and human milk oligosaccharide (HMO). Many non-digestible polysaccharides have also been found to be excellent prebiotics. An important polysaccharide being used as prebiotic is inulin (DP 11-65). Polysaccharides with prebiotic effect have several advantages over non digestible oligosaccharides: (i) decrease caloric intake, (ii) tolerance to high ingestion which otherwise causes intestinal discomfort and flatulence, (iii) mucosal damage from rapid acidification and (iv) laxative effect in the colon. In addition, large polysaccharides afford a persistent source of fermentable carbohydrate throughout the colon rather than being completely fermented proximally, which may prevent colon cancer.

Oligosaccharides and polysaccharides are key biomolecules in essentially all living organisms. They serve multiple functions such as structural materials of cell walls, energy storage, cell recognition, regulation of signalling, cell differentiation, cell proliferation, and immune responses. They have also found manifold interests in the fields of food, feed, pharmaceuticals and cosmetics. The biosynthesis of naturally occurring oligo- and polysaccharides is a complex process that involves formation of glycosidic bonds between their constituent monosaccharide units and side chain modifications to produce specific functional group derivatizations. Therefore, the

efficient strategies for synthesis of the oligo- and polysaccharides have been in high demand due to their structural and functional diversity.

Keeping the significance and applications of poly- and oligosaccharides in mind, dextransucrase was isolated from *Leuconostoc mesenteroides* NRRL B-1426. The dextransucrase was explored for the synthesis of dextran and oligosaccharides. The structural characterization of dextran was carried out. The specificity of the acceptor reactions of *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase with sixteen different acceptors has been examined. The isomalto-oligosaccharides (IMOs) and gentio-oligosaccharides (GnOS) were synthesized by the acceptor reaction of dextransucrase. The oligosaccharides (IMOs and GnOS) were purified by Bio-Gel P-2 chromatography and characterized by HPLC-RI and ESI-TOF MS analyses. *In vitro* applications of the purified dextran and oligosaccharides as prebiotics were investigated.

Present work

The present investigations are carried out on the **"Synthesis, purification, characterization and prebiotic applications of dextran and oligosaccharides from *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase"**. The thesis work comprises 6 Chapters.

Chapter 1 is the General Introduction representing a brief review of literature on the importance of poly- and oligosaccharides in food and pharmaceutical industries. A detailed literature on the sources, criteria and application of prebiotics was described. The chapter describes the link between health and nutrition through the use of prebiotics in diet. Various production methods for the synthesis of prebiotics were also described. The chapter also elaborates the production, purification, immobilization and applications of the dextransucrase. The detailed production, purification and characterization methods for the dextran and oligosaccharides have also been discussed in this chapter. Various applications of dextran and oligosaccharides are extensively reviewed.

Chapter 2 deals with the production, purification, characterization and immobilization of dextransucrase from *L. mesenteroides* NRRL B-1426 for the synthesis of isomalto-oligosaccharides. The maximum dextransucrase production from *L. mesenteroides* NRRL B-1426 was observed at 24°C and 120 rpm after 6 h (specific activity, 0.74 U/mg). The cell-free supernatant containing dextransucrase (specific activity of 0.74 U/mg) was subjected to purification by various concentrations of polyethylene glycol 400 and 1500 fractionation. The maximum specific activity of 10.14 U/mg and 6.40 U/mg was achieved at 33% (v/v) PEG-400 and 10% (w/v) PEG-1500, respectively. The dextransucrase showed multimeric nature with molecular sizes of approximately, 240, 178 and 165 kDa under SDS non-denaturing condition. However, the native-PAGE analysis of dextransucrase revealed that it existed in a single molecular form. The zymogram analysis of PEG purified enzyme confirmed that it was dextransucrase as it showed magenta colour band only

in presence of sucrose. The purified dextransucrase was optimally active at 30ºC and pH 5.6 and was not affected much by the ionic strength of the buffer. The V_{max} and K_m of purified dextransucrase for sucrose as substrate was 13.2±0.51 U/mg and 12.7±0.67 mM, respectively, under optimized conditions. Partially purified dextransucrase was successfully immobilized with 1% (w/v) sodium alginate showing 93% immobilization yield. The immobilized enzyme had 40% activity after ten batch reactions at 30°C and retained 75% activity after a month of storage at 4°C, which was 6 times more stable than the free enzyme. Immobilized enzyme was more stable at lower (3.5-4.5) and higher (6.5-7.0) pH ranges and higher temperatures (35-40°C) as compared with the free enzyme. Immobilized dextransucrase was used for the production of IMO ranging from trisaccharides to homologous pentasaccharides, showing 90% efficiency when compared with the free enzyme. Hence, immobilized dextransucrase by sodium alginate can be used for oligosaccharide synthesis at commercial level.

Chapter 3 describes the physico-chemical characterization and prebiotic potential of dextran from *L. mesenteroides* NRRL B-1426 dextransucrase. The dextran was enzymatically synthesized by dextransucrase and purified by ethanol precipitation. The structure analyses by FTIR, ^{1}H and ^{13}C NMR spectroscopy demonstrated that it contains ~85.5% α-(1→6) linear and ~14.5% α-(1→3) branched linkages. The molecular mass determination by gel permeation chromatography showed that it is a high molecular mass dextran of size > 2000 Da. The dextran exhibited high solubility (32%), water holding capacity (290%) and viscosity (5.9 cp), indicating various applications in food industry as a thickener, an emulsifier and a stabilizer. Rheological

studies of dextran showed non-Newtonian, pseudoplastic, shear thinning properties, which may be valuable for use in different food industries. The high thermal stability of dextran with degradation temperature ~290°C can have potential application in the high temperature mediated manufacturing and processing of food products. The prebiotic potential of dextran from *L. mesenteroides* NRRL B-1426 was also evaluated. The dextran showed significantly low degree of hydrolysis (0.2-0.9%) by the simulated human gastric juice at acidic pHs whereas, with inulin (commercial prebiotic) it was several fold higher (1.5-28%). This indicated the greater stability of dextran at low pH, when compared with inulin. Similar results were observed when the dextran was subjected to α-amylase treatment. The digestibility of dextran and inulin by α-amylase was 0.8% and 13%, respectively. The dextran supported the growth of probiotics, bifidobacteria and lactobacillus. The dextran did not encourage the growth of unwanted coliforms like *E. coli*. The dextran exhibited a nontoxic effect on human embryonic kidney (HEK-293), human cervical adenocarcinoma (INT-407) and human colorectal carcinoma (HT-29) cell lines upto a concentration of 1000µg/ml, displaying its biocompatible nature. With these desirable attributes, the dextran from *L. mesenteroides* NRRL B-1426 can be used as a prebiotic and as an ingredient in functional food and nutraceutical products in food industry for health benefits.

Chapter 4 describes the determination of acceptor specificity of dextransucrase from *L. mesenteroides* NRRL B-1426 with sixteen different sugars. The sugars tested were: L-arabinose, D-arabinose, D-cellobiose, D-fructose, D-galactose, D-gentiobiose, D-glucose, D-isomaltose, D-lactose, D-maltose, D-mannose, D-melibiose, D-raffinose, D-

rhamnose, D-trehalose and D-xylose. The TLC analysis of the acceptor reaction mixture showed that cellobiose, gentiobiose, glucose, isomaltose, lactose, maltose, melibiose and trehalose were effective acceptors for oligosaccharide synthesis. Notable differences in the oligosaccharide pattern with the eight acceptors were observed. However, no acceptor reaction products were obtained with arabinose, fructose, galactose, mannose, rhamnose, raffinose and xylose. Dextran production was also observed in the reaction of dextransucrase with all eight acceptors. The sodium or potassium adducts of oligosaccharides ranging from DP3 to DP6 (527-1013 or 543-1029) were observed in case of all the acceptors. However, the peak intensities of sodium or potassium molecular ions of DP > 4 (689-1013 or 705-1029) varied with the type of acceptor molecule used. Oligosaccharides of DP > 4 were present in considerable amount in case of gentiobiose, isomaltose, glucose, cellobiose, lactose and maltose. However, DP3 and DP4 were the main acceptor products with melibiose and trehalose. The isomaltose was found to be the best acceptor having 89% efficiency followed by gentiobiose (64%), glucose (30%), cellobiose (25%), lactose (22.5%), melibiose (17%) and trehalose (2.3%) with reference to maltose, a known best acceptor. Since β-linkages are stronger than α-linkages and are highly resistant to the attack of digestive enzymes, cellobiose (β-1→4 linked), gentiobiose (β-1→6 linked) and lactose (β-1→4 linked) can be used as acceptors for synthesis of prebiotic oligosaccharides.

Chapter 5 deals with the synthesis, purification and prebiotic applications of isomalto-oligosaccharides (IMOs) from the acceptor reaction of dextransucrase from *L. mesenteroides* NRRL B-1426. The reaction conditions for synthesis of IMOs were

optimized by changing the concentration of enzyme, sucrose and maltose and temperature. The 30°C, 5 U/ml of dextransucrase, 7% (w/v) sucrose and 3% (w/v) maltose were found to be optimum for synthesis of IMOs. The acceptor reaction mixture containing IMOs, fructose and residual sucrose and maltose was chromatographed on Bio-Gel P-2 column. The collected fractions corresponding to the peak in the separation profile were analyzed by TLC and HPLC for their purity. The ESI-TOF MS of the pooled fractions revealed the *L. mesenteroides* NRRL B-1426 synthesized IMOs from DP 3 to 10. The peaks at m/z 527.1, 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. The findings confirmed that the acceptor products were a homologous series of IMOs that contain maltose at the reducing end and glucose units at the non-reducing end by α-(1→6) linkage. 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 by using the acceptor reaction of dextransucrase. The purified IMOs of DP ≥ 3 from *L. mesenteroides* NRRL B-1426 dextransucrase were pooled and tested for their prebiotic activity in terms of gut digestibility and stimulation of probiotic bacteria. IMOs showed lower degree of hydrolysis (9.3-20.8%) by the simulated human gastric juice (supplemented with pepsin) at acidic pHs as compared with inulin (13.8-39.7%), indicating greater stability. The IMOs supported the growth of *Lactobacillus acidophilus*. The effects of purified IMOs (DP ≥ 3) were evaluated on HEK-293, INT-407 and HT-29 cell lines in the concentration range 10-500 µg/ml. The cell viability of HEK-293 and INT-407 were not affected by the purified IMOs. However, the cell viability of HT-29 was inhibited in time- and dose-dependent

manner in response to increasing concentrations of IMO (10-500 µg/ml). The IMOs treated HT-29 cells were significantly inhibited by 14%, 21%, 24%, 29% and 34% at concentrations of 10, 50, 100, 200 and 500 µg/ml, respectively, at 36 h. The purified IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase showed a very selective cytotoxic behaviour, affecting only colon cancer lines and hence, can be used for the development of colon cancer chemopreventive agent.

Chapter 6 deals with the synthesis, purification and prebiotic applications of gentio-oligosaccharides (GnOS) from the acceptor reaction of dextransucrase from *L. mesenteroides* NRRL B-1426. The acceptor reaction of dextransucrase using gentiobiose as an acceptor was carried out for the synthesis of GnOS. The synthesized GnOS were purified by Bio-Gel P-2 chromatography and characterized by TLC, HPLC and mass spectrometry. TLC and HPLC analyses of the pooled fractions revealed the *L. mesenteroides* NRRL B-1426 dextransucrase synthesized GnOS from DP 3 to 10. However, ESI-TOF MS of the pooled fractions showed the peaks at m/z 527.1, 689.2, 851.3 and 1013.3 corresponding to the $[M+Na]^+$ ions of GnOS from DP3 to DP6, respectively. The GnOS of DP7-DP10 in TLC and HPLC analysis were not detected in the ESI-TOF MS analysis under the conditions used. The purified GnOS (DP ≥ 3) were tested for their prebiotic activity in terms of gut digestibility and selective stimulation of probiotics. GnOS showed lower degree of hydrolysis (10.1 to 18.1%) by the simulated human gastric juice (supplemented with pepsin) at acidic pHs as compared with inulin (13.8 to 39.7%) indicating greater stability. The enzymatic hydrolysis of GnOS and inulin at pH7 by α-amylase was 7.1% and 12.8%, respectively, at 6 h. The GnOS stimulated the growth of probiotics, *Bifidobacterium*

infantis and *Lactobacillus acidophilus*. The effects of purified GnOS (DP ≥ 3) were also evaluated on HEK-293, INT-407 and HT-29 cell lines in the concentration range 10-500 µg/ml. The GnOS did not affect the cell viability of HEK-293 and INT-407 cells. However, the cell viability of HT-29 was inhibited in time- and dose-dependent manner by GnOS. The viability of GnOS treated HT-29 cells were significantly inhibited by 25%, 31%, 32%, 40% and 44% at concentrations of 10, 50, 100, 200 and 500 µg/ml, respectively, at 36 h. A selective cytotoxicity was observed by the purified GnOS, affecting only colon cancer cell lines, HT-29. The prebiotic activity and selective cytotoxicity suggest promise for the use of GnOS as food or feed ingredients.

CONTENTS

Chapter 5. Synthesis, purification and prebiotic applications of isomalto-oligosaccharides from the acceptor reaction of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426

Chapter 6. Synthesis, purification and prebiotic applications of gentio-oligosaccharides from the acceptor reaction of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426

Chapter 1

General Introduction

1.1 Introduction

Oligosaccharides and polysaccharides are key biomolecules in essentially all living organisms (Faijes and Planas, 2007). They serve multiple functions such as structural materials of cell walls, energy storage, cell recognition, cell differentiation, cell proliferation, regulation of signalling and immune responses (Descroix *et al.*, 2006). They have also found manifold interests in the fields of food, feed, pharmaceuticals and cosmetics (Seibel and Buchholz, 2010). The biosynthesis of naturally occurring oligo- and polysaccharides is a complex process that involves formation of glycosidic bonds between their constituent monosaccharide units and side chain modifications to produce specific functional group derivatizations (Faijes and Planas, 2007). Glycosyltransferases, transglycosidases and phosphorylases are the enzymes responsible for formation of glycosyl bonds, whereas accessory enzymes such as estearases, epimerases and sulfotransferases (Crout and Vic, 1999; Silbert and Sugumaran, 2002; Kusche-Gullberg and Kjellen, 2003) modify the carbohydrate structures to produce the functional biomolecules. The efficient strategies for synthesis of the oligo- and polysaccharides have been in high demand due to their applications (Faijes and Planas, 2007).

The modern world faces the new challenges in 21st century, the exponentially growing costs of health care, increasing life expectancy, improving scientific knowledge and developing new technologies owing to major changes in lifestyles. Nutrition has to adapt to these new challenges by developing new concepts. 'Optimized nutrition' is one of these new concepts that aimed at maximizing physiological functions since it is now well established that the gut microbiota act as a key player in health with a minimum risk of disease throughout life (Roberfroid *et al.*, 2010). In this context, "functional food" is a new and stimulating concept, which has been deeply ingrained in Asia and is a fast growing segment of the food industry in the United States and Europe (Goffin *et al.*, 2011). Thus, there is clearly a need to increase our knowledge of gut microbiota and interactions with functional food. Furthermore, a better understanding of the recent development and safety assessment of prebiotics in food science and technology is also required.

1.2 Prebiotics

1.2.1 Prebiotic concept

Regulation of the microbial ecology of the colon through the use of probiotics and prebiotics as functional foods, has for decades gained special interest in the scientific consortium as well as among consumers (Fooks *et al.*, 1999; Douglas and Sanders, 2008; Wang, 2009). In recent years, prebiotics tend to supersede 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 (Roberfroid, 2002; Manning and Gibson, 2004; Goffin *et al.*, 2011). The prebiotic concept was first introduced by Gibson and Roberfroid (1995), who exchanged ''pro'' to ''pre'', which means ''before'' or ''for''. A prebiotic is a

non-viable food component that confers a health benefit on the host associated with modulation of the microbiota. The prebiotic concept is being continuously updated and expanded by various researchers (Gibson *et al.*, 2004; Roberfroid, 2007; Roberfroid *et al.*, 2010) (Table 1.1).

Table 1.1 Developing definitions of the prebiotic concept.

Definition	Reference
A non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health.	Gibson and Roberfroid, 1995
A selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well being and health.	Gibson *et al.*, 2004
A dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health.	ISAPP (2008) 6th Meeting of the International Scientific Association of Probiotics and Prebiotics, London, Ontari
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

1.2.2 Criteria of prebiotics

In general terms, intestinal bacteria can be divided on the basis of whether they can exert health promoting or potentially harmful activities in their host (Gibson and Roberfroid, 1995; Manning and Gibson, 2004). Thus the factors that influence the microflora composition need to be studied. A prebiotic substrate is selectively utilized by beneficial components of the indigenous gut flora *viz.*, bifidobacteria and lactobacilli but does not promote potential pathogens such as toxin-producing

clostridia, proteolytic bacteroides and toxigenic *Escherichia coli* (Gibson *et al.*, 2004). The prebiotics must be able to withstand digestive processes before they reach the colon and preferably persist throughout the large intestine to exert health-promoting effects distally (Gibson *et al.*, 2004; Wang, 2009). The resistance to digestive processes includes their resistance to gastric acidity, hydrolysis by mammalian enzymes, and gastrointestinal absorption. (Ellegard *et al.*, 1997; Roberfroid, 2007). The criterion which allows the classification of a food ingredient as a prebiotic also includes selective fermentation by potentially beneficial bacteria in the colon. Furthermore, selective stimulation of the growth and activity of intestinal bacteria potentially associated with health and well-being is required as one of the criteria (Fooks *et al.*, 1999). Candidate prebiotics are reported to be particularly suited to the growth and activities of the probiotics, bifidobacteria and lactobacilli, which have been known to exert therapeutic and prophylactic influences on the health of infants and adults (Salminen *et al.*, 1998; Gibson *et al.*, 2004; Wang, 2009). Moreover, the prebiotics must be stable to food processing treatments and have a history of safe use in the target host, such as GRAS or its equivalents (Wang, 2009). Thus, the criteria for classification of a food ingredient as prebiotic are illustrated in Fig. 1.1.

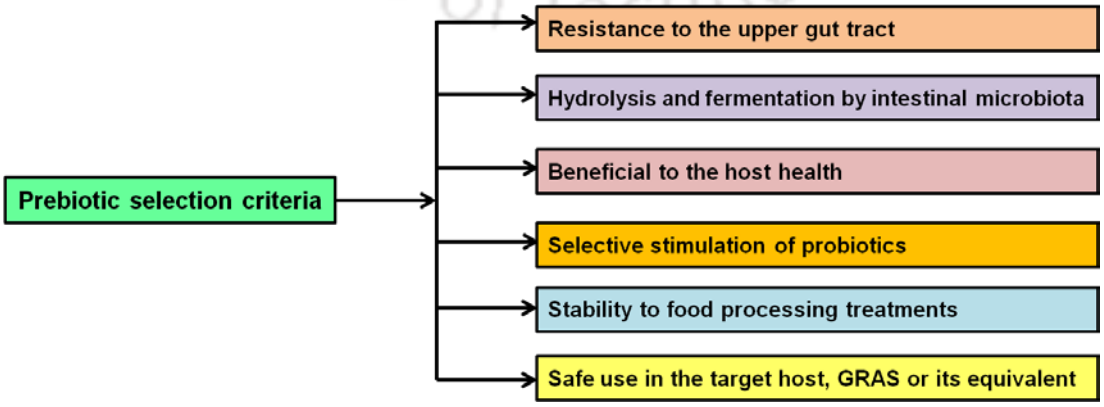

Fig. 1.1 Criteria for selection of a food ingredient as a prebiotic.

1.2.3 Health benefits of prebiotics

The majority of the effects claimed by the prebiotics are associated with optimized colonic function and metabolism (Saad *et al.*, 2013). These benefits include an increase in the expression or change in the composition of short-chain fatty acids (SCFAs), increased fecal weight, a reduction in luminal colon pH, nutrient production, such as vitamin B complex (B1, B2, B6 and B12), nicotinic and folic acids, a decrease in nitrogenous end products and reductive enzymes, an increased expression of the binding proteins or on certain biomarkers in the field of lipid and mineral metabolism and immune system modulation (Manning and Gibson, 2004; Mussatto and Mancilha, 2007; Qiang *et al.*, 2009; Saad *et al.*, 2013). All these effects support the logic of the use of prebiotics for promoting health benefits (Fig. 1.2).

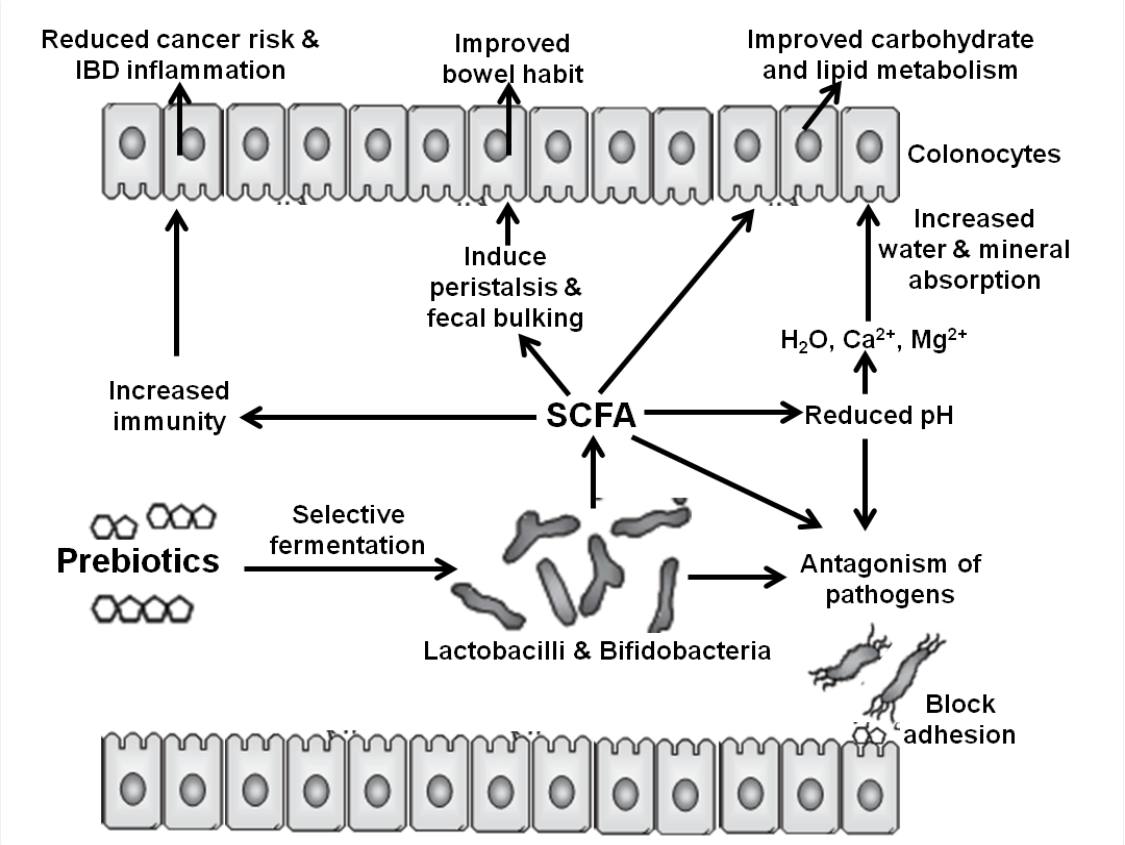

Fig. 1.2 Health benefits of prebiotics (adopted from Crittenden, 2006).

A reduction in the risk of gut cancer was observed by the use of prebiotic in diet on a daily basis (Wang, 2009). The anticarcinogenic effect appears to be related to an increase in cellular immunity, fecal physiological parameters such as pH, ammonia, *p*-cresol and indole. It has been demonstrated in a human study that the intake of transgalactosylated disaccharides reduces the fecal pH as well as ammonia, *p*-cresol and indole concentrations with an increase in probiotics (bifidobacteria and lactobacilli) and a decrease in bacteroidaceae populations. These alterations may be considered to be beneficial in reducing the risk of cancer development (Mussatto and Mancilha, 2007). The gut microbiota, with its metabolic, trophic and protective functions, is able to positively affect the integrity of the intestinal barrier. Loss of the integrity (*i.e.*, intestinal barrier dysfunction) results in a progressive increase of

intestinal, which leads to intestinal bowel disease (IBD) (Frank *et al.*, 2007; Lambert, 2009).

1.2.4 Source of prebiotics

It is becoming more common to properly distinguish between prebiotic substances and the food that contains them. No plant or food is a prebiotic. Foods contain prebiotics in varying concentrations in milk, honey, sugarcane juice, soyabean, lentils, mustard, fruits and vegetables such as onion, asparagus, sugar beet, artichoke, chicory, leek, garlic, banana, rye, barley, yacon, wheat, tomato and bamboo shoots (Mussatto and Mancilha, 2007). Most prebiotics and prebiotic candidates identified today are non-digestible oligosaccharides except inulin. Oligosaccharides are short-chain of carbohydrates of 3-10 monomers. Monosaccharide composition, glycosidic linkage and degree of polymerization are important in influencing the prebiotic properties (Bournet *et al.*, 2002; Manning and Gibson, 2004; Sanz *et al.*, 2006; Mussatto and Mancilha, 2007). Glucose, galactose, fructose and xylose are the most common building blocks (Mussatto and Mancilha, 2007; Patel and Goyal, 2011). Although a number of oligosaccharides have been proposed as prebiotics, only inulin, fructo-oligosaccharide (FOS), galacto-oligosaccharide (GOS) and lactulose have achieved the prebiotic status (Gibson *et al.*, 2004; Roberfroid, 2007; Sarbini *et al.*, 2013). The other most commonly studied oligosaccharides for prebiotic activity are gluco-oligosaccharide (GLOS), isomalto-oligosaccharide (IMO), xylo-oligosaccharide (XOS), lactosucrose, arabinoxylose oligosaccharide (AXOS), soybean meal oligosaccharide (SMO), mannan oligosaccharide (MOS), pectin-derived acidic oligosaccharide (pAOS), chito-oligosaccharide (COS), konjac glucomannan oligosaccharide, xanthan-derived oligosaccharide, alginate derived

oligosaccharide (ADO), laminarin oligosaccharide, agaro-oligosaccharide (AOS), palatinose and human milk oligosaccharide (HMO) (Patel and Goyal, 2011; Kothari *et al.*, 2014). Many non-digestible polysaccharides have also been found to be excellent prebiotics (Ruijssenaars *et al.*, 2000; Dal-Bello *et al.*, 2001; Hongpattarakere *et al.*, 2012). An important polysaccharide being used as prebiotic is inulin (DP 11-65) (Gibson and Roberfroid, 1995). Polysaccharides with prebiotic effect have several advantages over non digestible oligosaccharides: (i) decrease caloric intake, (ii) tolerance to high ingestion which otherwise causes intestinal discomfort and flatulence, (iii) mucosal damage from rapid acidification and (iv) laxative effect in the colon. In addition, large polysaccharides afford a persistent source of fermentable carbohydrate throughout the colon rather than being completely fermented proximally, which may prevent colon cancer (Ellegard *et al.*, 1997). Therefore, there has been a considerable increase in the demand for new and novel sources of prebiotics (Chen *et al.*, 2013).

1.2.5 Production of prebiotics

Most prebiotics and prebiotic candidates identified today are nondigestible oligosaccharides (Wang, 2009; Saad *et al.*, 2013). Oligosaccharides can be obtained from natural sources or synthesised (Courtois, 2009). Commonly employed production methods are controlled hydrolysis of polysaccharides or synthesis from disaccharide substrates by enzymatic and chemical treatments (Mussatto and Mancilha, 2007). Various methods for the commercial production of oligosaccharides are summarized in Table 1.2.

Table 1.2 Various methods for the commercial production of prebiotics.

Methods	Sources/Processes	Oligosaccharides	Reference
Extraction	Soybean seeds	Soybean oligosaccharides	Mussatto and Mancilha, 2007
	Leguminous vine peas (*Pisum sativum L.*)	Raffinose family oligosaccharides	Ekvall *et al.*, 2007
	Dried roots of *Morinda officinalis*	Inulin type oligosaccharides	Yang *et al.*, 2011
	Chichory roots	Inulin	Van Loo *et al.*, 1995
Controlled hydrolysis	**Physical**		
	Autohydrolysis of brewery's spent grain with water at 190°C	Xylo-oligosaccharides	Carvalheiro *et al.*, 2004
	Hydrolysis of Xylan	Xylo-oligosaccharides	Mussatto and Mancilha, 2007
	Hydrolysis of rice bran by trifluoroacetic acid	Feruloylated oligosaccharides	Li *et al.*, 2008
	Microwave induced acid hydrolysis of glucans	Gluco-oligosaccharides	Majumder *et. al.*, 2009
	Hydrothermal processing of wheat bran	Feruloylated arabino-xylo-oligosaccharides	Rose and Inglett, 2010
	Enzymatic		
	Degradation of arabinan with arabinohydrolases	Arabino-oligosaccharides	Westphal *et al.*, 2010
	Hydrolysis of dextran using endodextranase from *Penicillium* sp.	Isomalto-oligosaccharides	Vettori, *et al.*, 2012
Enzymatic	Enzymatic transglucosylation of glucose syrup	Gentio-oligosaccharides	Crittendan and Playne, 1996
	Action of β-galactosidases on lactose	Galacto-oligosaccharides	Ganzle, 2012
	Transglycosylation of sucrose	Leucrose	Reh *et al.*, 1996
	Transfructosylation of lactose and sucrose	Lactosucrose	Avigad, 1957
	Using dextransucrase from *Leuconostoc mesenteroides* B-512	Isomalto-oligosaccharides	Kubik *et al.*, 2004
	Using an engineered levansucrase	Fructo-oligosaccharides	Beine *et al.*, 2008
	Action of fusion enzyme, dextransucrase and dextranase	Isomalto-oligosaccharides	Kim *et al.*, 2009
	Using alternansucrase by acceptor reaction	Gentio-oligosaccharides	Cote, 2009

The isolation of oligosaccharides from natural sources is extremely difficult due to their structural complexity and besides requiring unique biochemical and analytical apparatus and the yields are also low (Zhou *et al.*, 2011). Therefore, access to pure oligosaccharides for research, and in particular for development of carbohydrate-based therapies, fully relies on the chemical and enzymatic synthesis of oligosaccharides (Koeller and Wong, 2000; Pukin *et al.*, 2008). However, despite the recent advances, the synthesis for large-scale production of oligosaccharides still cannot be performed by the classical chemical procedures which require long protection and deprotection steps for selectivity control and also give low final yields (Moracci *et al.*, 2001). Hence, the enzyme-catalyzed of oligosaccharides represents, nowadays, a feasible alternative to chemical synthesis that allows high stereo- and regioselectivity under mild aqueous conditions and high yields (Wymer and Toone, 2000; Koeller and Wong, 2001; Moracci *et al.*, 2001). Enzymes for the synthesis of oligosaccharides available to-date are: glycosyltransferases, glycosidases and glycosynthases-specifically mutated glycosidases. Each group of enzymes has certain advantages and disadvantages for the synthesis of oligosaccharides. Glycosidases catalyze oligosaccharides synthesis by reactions of reverse hydrolysis (equilibrium-controlled synthesis) or transglycosylation (kinetically controlled process) (Koshland, 1953). Reverse hydrolysis is straightforward approach, but provides poor yields, not exceeding 15%. Also, the formation of side product makes the purification difficult. While, the transglycosylation approach must be monitored carefully in order to minimize glycoside hydrolysis (Hendrix and Wong, 1996). Glycosidase are abundant in nature, easily available and capable of using simple and cheap substrates; however, the concomitant hydrolysis of the synthetic products results in low yields. This

problem can be largely overcome by glycosynthase, a genetically engineered glycosyl hydrolase, in which a catalytic nucleophile (glutamate or aspartate) is altered by small, neutral amino acids, alanine, serine or glycine (Mackenzie *et al.*, 1998). These enzymes efficiently synthesize oligosaccharides, but do not hydrolyze them. However, their stringent substrate specificities prohibit the synthesis of more diverse structures (Wei *et al.*, 2013). Another important class of enzymes involved in oligosaccharide synthesis are glycosyltranferases (GTFs). Glycosyltransferases are a ubiquitous group of enzymes that catalyse the transfer of a sugar moiety from an activated sugar donor onto a saccharide or non-saccharide acceptor (Coutinho *et al.*, 2003; Leemhuis *et al.*, 2012). GTFs are highly specific for their substrates as well as for the stereo- and regio-chemistry *in vivo* (Beyer *et al.*, 1981). Transglycosylation catalyzed by these enzymes from various bacteria has been used to improve physicochemical properties such as water solubility and oxidative stability of various compounds (Robyt *et al.*, 2008). This enzymatic method of oligosaccharide synthesis has the advantage of high efficiency and selectivity. Moreover, this class of enzyme do not act on the products they synthesize (Leemhuis *et al.*, 2012). Therefore, the following sections are focussed on GTFs, particularly glucansucrases. Glucansucrases are bifunctional enzymes that can synthesize glucan polymers from sucrose and oligomers when an efficient acceptor molecule is introduced (Demuth *et. al.*, 2002).

1.3 Glucansucrase

Glucansucrases (E.C. 2.4.1), extracellular glycosyltransferases (GTFs) have been classified in family 70 glycoside hydrolase (GH70) from their amino acid sequence similarities (Henrissat, 1991). They are evolutionarily closely related to the enzymes such as amylosucrase, cyclodextrin glucanotransferase, amylomaltase and α-

amylase from GH13 and GH77 according to the Carbohydrate-Active Enzymes (CAZy) (http://www.cazy.org) classification (Cantarel *et al.*, 2009). Till date over 239 different glucansucrases from various lactic acid bacteria: *Leuconostoc* sp., *Streptococcus* sp., *Lactobacillus* sp., *Pediococcus* sp. and *Weissella* sp. (CAZy database) have been reported. Glucansucrases catalyze three types of reaction, *viz.*, polymerization (*i.e.*, α-glucan formation), hydrolysis and an acceptor reaction (Fig. 1.3). All three reactions start with cleavage of α-(1→2) bond of sucrose and the formation of the glycosyl-enzyme intermediate, and it is the nature of the incoming acceptor substrate, which determines the type of reaction that is catalysed. Hydrolysis is the simplest reaction, converting sucrose to glucose and fructose using water as acceptor. In the polymerization reaction, the glycosyl intermediate is transferred to a growing α-glucan chain. Glucansucrases can also transfer the glycosyl intermediate to a wide variety of hydroxyl-group containing molecules in an acceptor reaction, which is useful in (chemo-) enzymatic synthesis, because it prevents all kinds of difficulties regarding stereo- and regio-specificity associated with the chemical synthesis of carbohydrate compounds (Remaud-Simeon *et al.*, 2000; Monsan *et al.*, 2010; Leemhuis *et al.*, 2012). Four distinct types of GH70 glucansucrases have been identified based on the polysaccharides produced by them (Andre *et al.*, 2010).

- Dextransucrase (E.C. 2.4.1.5): Dextran, containing above 50% α-(1→6) linkages.
- Mutansucrase (E.C. 2.4.1.5): Mutan, containing above 50% α-(1→3) linkages.
- Reuteransucrase (E.C. 2.4.1.5): Reuteran, containing above 50% α-(1→4) linkages.

- Alternansucrase (E.C. 2.4.1.140): Alternan, containing alternating α-(1→3) and α-(1→6) linkages.

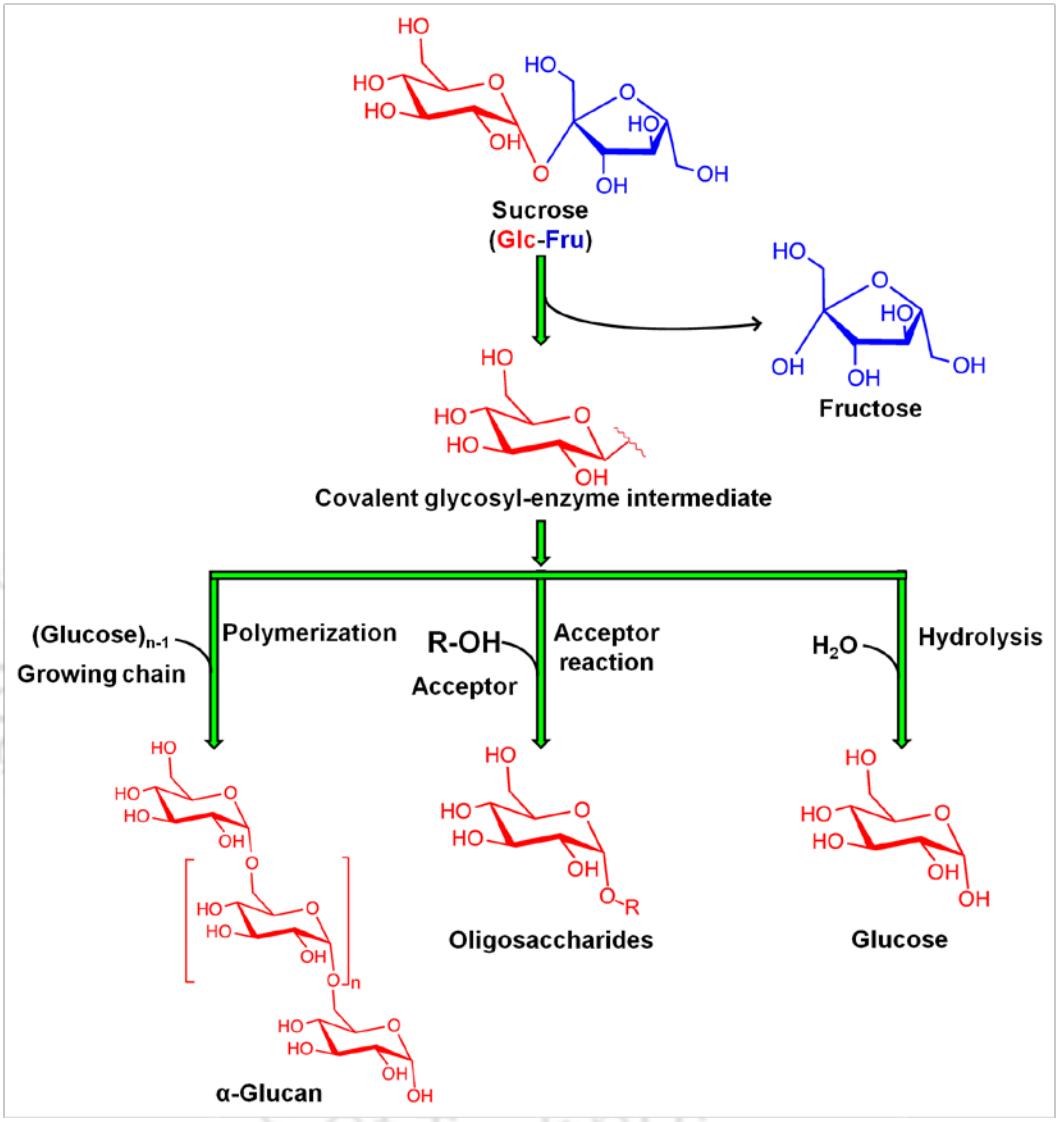

Fig. 1.3 Reactions catalyzed by glucansucrase.

Glucansucrases exist in either single or multiple molecular forms with varying molecular weights ranging from 64-313 kDa (Monsan *et al.*, 2001), which play an important role in the control of glucan synthesis *in vivo* (Kobayashi and Matsuda, 1986). This variation has been ascribed to the presence of glucan in the purified glucansucrase preparations, dissociation of high molecular mass multimeric complex

(Kim and Robyt, 1994) or action of proteases (Miller and Robyt, 1986). Glucansucrases (dextransucrases) have p*I* value of 4.1and Michaelis-Menten constant (K_m) for sucrose in the range ~3-16 mM. The purified enzyme has an optimum pH of 5.0-5.6 and a temperature optimum of 30°C. Low levels of calcium are necessary for optimal enzyme production and activity (Robyt and Walseth, 1979; Purama *et al.*, 2010). Recently, the three-dimensional structure of GH70 glucansucrases in their truncated forms has been reported. These structures were GTF180-ΔN from *Lactobacillus reuteri* 180 (PDB: 3KLK; Vujicic-Zagar *et al.*, 2010), GTF-SI from *Streptococcus mutans* (PDB: 3AIE; Ito *et al.*, 2011), and ΔN_{123}-GBD-CD2 from *Leuconostoc mesenteroides* NRRL B-1299 (PDB: 3TTQ; Brison *et al.*, 2012). However, till date no crystal structures of complete glucansucrases have yet been reported.

1.3.1 Purification of glucansucrase

Various purification methods such as precipitation by ammonium sulphate, ethanol or polyethylene glycol, phase partitioning, ultrafiltration and chromatography have been used to purify glucansucrases (Majumder *et al.*, 2007; Pijning *et al.*, 2008). Fractionation by polyethylene glycol (PEG) is a reasonably successful and cost-effective method for purification of glucansucrase. PEG, a non-ionic hydrophilic detergent selectively precipitates the proteins of high molecular weights or in aggregated forms. PEGs of different molecular weight are used for precipitation of glucansucrase, but high molecular weight PEGs such as PEG 6000 and 8000 also allow other proteins to precipitate (Purama and Goyal, 2008). Hence, PEG 400 is usually preferred for purification due to its ability to get removed easily after dialysis

(Purama and Goyal, 2008; Rao and Goyal, 2013). However, PEG fractionation method is inadequate for removing the associated polysaccharides from the glucansucrase. This can be achieved by a combination of dextranase treatment, ion-exchange, affinity and gel permeation chromatography after PEG fractionation (Majumder *et al*., 2007). Various matrices such as hydroxyapatite, DEAE-cellulose, DEAE-Sephadex, Sephadex, ultrogel AcA 34 (Majumder *et al.*, 2007), Sephacryl S-200HR (Purama and Goyal, 2008), Sephacryl S-300HR (Rao and Goyal, 2013) have been used for the purification of glucansucrase.

1.3.2 Immobilization of glucansucrase

An effective immobilization method is required to ensure continuous and economical production of glucan and oligosaccharides, and reuse of glucansucrase. Studies on immobilization of glucansucrases have not been successful with respect to yield and stability. One of the reasons is that the dextran associated with dextransucrase masks the residues such as lysine, aspartate and glutamate residing at the active site of the enzyme and leading to inactivation of the enzyme (Funane *et al.*, 2005; Parlak *et al.*, 2013). Kaboli and Reilly (1980) studied the immobilization of glucansucrase using 13 different carriers and concluded that "glucansucrase is an extraordinarily difficult enzyme to immobilize". Different methods have been proposed to immobilize glucansucrases, based on adsorption, entrapment in polymers or covalent binding (Petronijevic *et al.* 2007). In a study by Monsan and Lopez (1981) glucansucrase was attached covalently onto silica support with 3.1 and 4.3% immobilization yields in the absence and presence of maltose, respectively. Alcalde *et al.* (1999) also immobilized native and dextran-free dextransucrase covalently onto glutaraldehyde activated silica, giving rise to 0.6% and 13% immobilization yields,

respectively. The removal of glucan associated with glucansucrase facilitates the reaction between the amino acid residues of the enzyme and the matrix. The immobilization yield was not higher than 13%, which could be due to the reaction of lysine residue at the active site of glucansucrase with aldehyde groups of gluteraldehyde. Another study was conducted by de Segura *et al.* (2004) on covalent immobilization of dextran-free dextransucrase onto Eupergit C. The removal of dextran layer made the reactive groups of the enzyme surface more accessible to the support so that the immobilization resulted in about 72% immobilization and 22% activity yields. However, the immobilized enzyme lost more than 55% of the recovered activity in 2 h in stability tests carried out at 30°C. Recently, a novel dextransucrase (truncated at N- and C-terminals and fused to glutathione S-tranferase) was successfully covalently immobilized onto Eupergit C 250L, giving rise to 83.3% activity yields (Parlak *et al.*, 2013). Adsorption of dextransucrase onto phenoxy acetylcellulose or hydroxyapatite gave to improved immobilization yields, however the enzyme desorbed rapidly from the supports (Chang *et al.*, 1981; Parnaik *et al.*, 1983). Ionic attachment of the enzyme onto DEAE-Sephadex or SP-Sephadex yielded a 10% immobilization yield under the best condition (Kaboli and Reilly, 1980). Affinity immobilization of dextransucrase on Sephadex G-200 was also studied for the production of leucrose (Han *et al.*, 2005). Activity yield was 80% and the half-life of the enzyme was 15 days. Encapsulation of dextransucrase in alginate is the only successful immobilization method. There are several studies on immobilization of glucansucrase by alginate, resulting in immobilization yields up to 90% (Reischwitz *et al.*, 1995; Alcalde *et al.*, 1999; Tanriseven and Dogan, 2002; Kothari *et al.*, 2012). However, the immobilization by alginate can only be used for the production of

oligosaccharides because the high molecular weight dextran cannot diffuse out of the beads (Tanriseven and Dogan, 2002).

1.4 Dextran

Dextran are extracellular α-glucan poymers with a linear backbone made of α-(1→6)-linked D-glucopyranosyl units with varying percentage of α-(1→2), α-(1→3), α-(1→4) branching (Robyt, 1995; Naessens *et al.*, 2005; Capek *et al.*, 2011). Dextransucrases (E.C. 2.4.1.5) are the sole industrial enzymes used in the commercial production of dextran (Parlak *et al.*, 2013). Dextrans produced by different strains vary in the percent of linear glycosidic linkages, degree and type of branching, molecular mass and physical and chemical characteristics (Maina *et al.*, 2008; Siddiqui *et al.*, 2014). A survey of dextrans from 96 strains (primarily *Leuconostoc mesenteroides*) demonstrated that the amount of α-(1→6) linkages in a specific dextran can vary from 50 to 97% of the total glycosidic linkages (Jeanes *et al.*, 1954). Dextran production is influenced by a number of factors: physical and chemical properties (Jeanes, 1965), such as solubility, viscosity, specific optical rotation and the content of nitrogen, phosphorus and ash in the medium (Jeanes *et al.*, 1954). A single dextransucrase can catalyze the synthesis of several types of dextran linkages, thereby permitting the formation of a branched polymer (Neely and Nott, 1962; Smith *et al.*, 1994). On the other hand, certain bacterial strains have been shown to produce dextrans of different structures due to the excretion of different dextransucrases (Figures and Edwards, 1981; Cote and Robyt, 1982; Zahnley and Smith, 1995). Thus, the structure of each dextran is a characteristic of the specific dextransucrase produced by a specific microbial strain (Jeanes *et al.*, 1954, Vettori *et al.*, 2012).

1.4.1 Applications of dextran

Due to the heterogeneity of dextran produced by various LAB, their application may depend on well-defined chemical and physicochemical properties (Table 1.3). In general, dextran is used as gelling, viscosifying and emulsifying agent in various food products (Leemhuis *et al.*, 2013). Dextran is used for viscosity, moisture retention, inhibition of sugar crystallization and used as gelling agents in gum and jelly candies in confectioneries (Maina *et al.*, 2011). *In situ* dextran production improved the texture, volume, softness and shelf life of sourdough wheat bread (Katina *et al.*, 2009). Moreover, the dextran should have a high molecular weight and few branch linkages for the application in sourdough (Lacaze *et al.*, 2007). Dextran is a good candidate for making reduced-fat cheese by increasing moisture content in the non-fat substance (Awad *et al.*, 2005). Dextran is also used as a cryoprotectant in ice cream (Naessens *et al.*, 2005). Dextran and dextran derived oligosaccharides have also been reported to increase the fraction of *Bifidobacterium* species in an *in vitro* model of the fermentation process in the human colon exhibiting prebiotic activity (Olano-Martin *et al.*, 2000). A low molecular weight dextran containing α-(1→2) branched linkages also reported to act as prebiotic with selective effect on the gut microbiota (Sarbini *et al.*, 2013). Therefore, a current consumer trend toward healthy and additive free food has made dextran an attractive solution.

The clinical applications of dextran are vast due to their biocompatible and biodegradable nature. Low molecular weight dextran containing 5% α-(1→3) branched linkages produced by *L. mesenteroides* B-512F is generally preferred in clinical applications, due to its low antigenicity, high water solubility and high biological stability in the human blood stream (Naessens *et al.*, 2005; Siddiqui *et al.*,

2014). Clinical fractions of dextran with molecular weights of 70, 60 and 40 kDa are presently used for replacing moderate blood losses (De Belder *et al.*, 1996). Intravenously administered iron dextran is used to alleviate iron deficiency anaemia (Blanshard and Mitchell, 1979). Special iron dextran preparations have also been developed to enhance magnetic resonance imaging (MRI) techniques (De Belder *et al.*, 1996). The sulfate ester of dextran has anticoagulant properties similar to that of heparin (Alsop, 1983). Dextran sulphate has also been studied as an antiviral agent, particularly in the treatment of the human immunodeficiency virus (HIV) (Naessens *et al.*, 2005). As there are plenty of primary and secondary hydroxyl groups present on dextran backbone which provide potential functional sites for drug conjugation through direct or indirect methods. Various anticancer drugs, such as doxorubicin (DOX), CPT, mitomycin C (MMC) and methotrexate (MTX) have been conjugated to dextrans to form cytotoxic drug conjugates (Goodarzi *et al.*, 2013). Three derivatives of dextran have been used in drug delivery including amino-dextran (Shih *et al.*, 1991), oxidized dextran (Danhauser-Riedl *et al.*, 1993) and carboxymethyl dextran (Chau *et al.*, 2004). These conjugates had a prolonged effect and reduced toxicity and immunogenicity in many *in vitro* and *in vivo* studies. Cathepsin B, a cysteine proteinase acts as a peptidyl linker for indirect conjugation of dextran and has a profound role in determining the conjugates success in murine xenograft models and in clinical studies (Veltkamp *et al.*, 2008). Dextrans are also used in several formulations for the eye and skin care market. Dextrans and their derivatives offer many advantages as ingredients for cosmetics, where excellent biocompatibility, moisturising properties and stability are high on the list of requirements (Bhavani and

Nisha, 2010). High molecular weight dextrans can act as osmotic agents to treat hypovolemia (Alpar and Killampalli, 2004).

Different types of esters and ethers of dextran provide macromolecules having diverse properties with negative, positive or neutral charges. The most widely used dextran derivative is “Sephadex”, obtained by the reaction of an alkaline solution of dextran with epichlorohydrin. This cross-linked dextran revolutionised the purification and separation of biochemically important macromolecules *viz.*, proteins, nucleic acids and polysaccharides. Dextrans are also an essential component of Sephacryl, Superdex and other Sephadex derivatives, including carboxymethyl (CM) Sephadex, diethylaminoethyl (DEAE) Sephadex, diethyl(2-hydroxypropyl)aminoethyl (QAE) Sephadex, sulfopropyl (SP) Sephadex (Naessens *et al.*, 2005). Many other potential uses for dextrans include in secondary recovery of petroleum, oil drilling muds, soil conditioners, surgical sutures and developing X-ray and other photographic emulsions (Naessens *et al.*, 2005). Dextran also protects steel against corrosion in both humid and acidic environments (Finkenstadt *et al.*, 2011).

Table 1.3 Industrial applications of dextran.

Industry	Product	Applications	Reference
Food	Native dextran	**Bakery:** Improves softness, crumb texture, loaf volume and shelf life.	Katina *et al.*, 2009
		Confectionary: Improves moisture retention, viscosity and inhibit sugar crystallization and as gelling agents in gum and jelly candies.	Maina *et al.*, 2011
		Ice cream: Cryoprotectant.	
		Frozen and Dried foods: Protects from oxidation and chemical changes and preservation in texture and flavour.	Bhavani and Nisha, 2010
	Oligosaccharides by dextran hydrolysis; native dextran	**Prebiotic.**	Olano-Martin *et al.*, 2000
	Native dextran	**Cheese making:reduced-fat cheese:** Improves water binding and increase moisture content in the non-fat substances.	Awad *et al.*, 2005
Health	Low molecular mass native dextran	Replacement of blood loss, plasma substitution, thrombosis prophylaxis, volume expansion, rheological improvement.	Naessens *et al.*, 2005
	Native dextran	Lubricant in eye drops.	Naessens *et al.*, 2005
	Iron dextran	Iron deficiency anaemia, enhances MRI techniques.	Naessens *et al.*, 2005
	Carboxymethyl dextran Oxidized dextran Amino dextran	Drug delivery with prolonged effect, reduced toxicity and immunogenicity.	Goodarzi *et al.*, 2013
	Dextran sulfate	Heparin substitute for anticoagulant therapy, antiviral agent, particularly in the treatment of the human immunodeficiency virus (HIV), cure to arteriosclerosis.	Alsop, 1983; Naessens *et al.*, 2005
Environment	Mercaptodextran	Cleanup of heavy metal contamination.	Naessens *et al.*, 2005
	Native dextran	Waste water management.	Bhavani and Nisha, 2010
Paper	Native dextran	Deflocculants.	Murphy and Whistler, 1973
Cosmetic	Cationic dextran	Moisturizing effects, anti-ageing and anti-wrinkle effects.	Bhavani and Nisha, 2010
Research	Dextran derivative: Sephadex, Sephacryl and Superdex	Size exclusion chromatography matrices.	Naessens *et al.*, 2005
	Native dextran	Immobilization in biosensors.	Bhavani and Nisha, 2010
	Native dextran	Stabilizes coating of metal nano-particles from oxidation and improves biocompatibility.	Bhavani and Nisha, 2010

1.4.2 Characterization of dextran

The structural characterization of dextran is an important factor for its utilization. The structural analysis of dextran starts with its isolation in pure form in such a way that the chemical and physical properties are not affected (Leemhuis *et al.*, 2013). Dextran recovery from culture medium or enzymatic reaction mixture generally involves the following steps: (i) cell removal by centrifugation or filtration in case of culture medium, (ii) dextran precipitation from the cell-free supernatant/enzymatic reaction mixture by the addition of water-miscible organic solvents (*e.g.*, ethanol, acetone, *etc.*), (iii) re-precipitation and dialysis (Ahmed *et al.*, 2012). Purification of dextran can also be achieved by membrane filtration, anion-exchange or gel permeation chromatography (Sanz and Martinez-Castro, 2007).

The molecular weight (MW) of dextran is generally determined by viscometry, hydrodynamic chromatography (HDC), high performance size-exclusion chromatography coupled with refractive index detector (HPSEC-RI) or with multi-angle laser light scattering (HPSEC-MALLS) (Leemhuis *et al.*, 2013). However, higher branched polysaccharides are not well fractionated by means of classical SEC due to their shear scission, low exclusion limit (Cave *et al.*, 2009) and limited resolution (Vilaplana and Gilbert, 2010). To avoid these problems, asymmetrical flow field flow fractionation (AF4) has emerged as a very powerful technique for the determination of the macromolecular structure of high molar weight branched biopolymers upto10^8 Da (Rolland-Sabate *et al.*, 2011). Recently, AF4 coupled with MALLS have been used to determine the molecular mass of hyperbranched α-glucans ($2x10^6$ to $4.3x10^7$ Da) (Rolland-Sabate *et al.*, 2014).

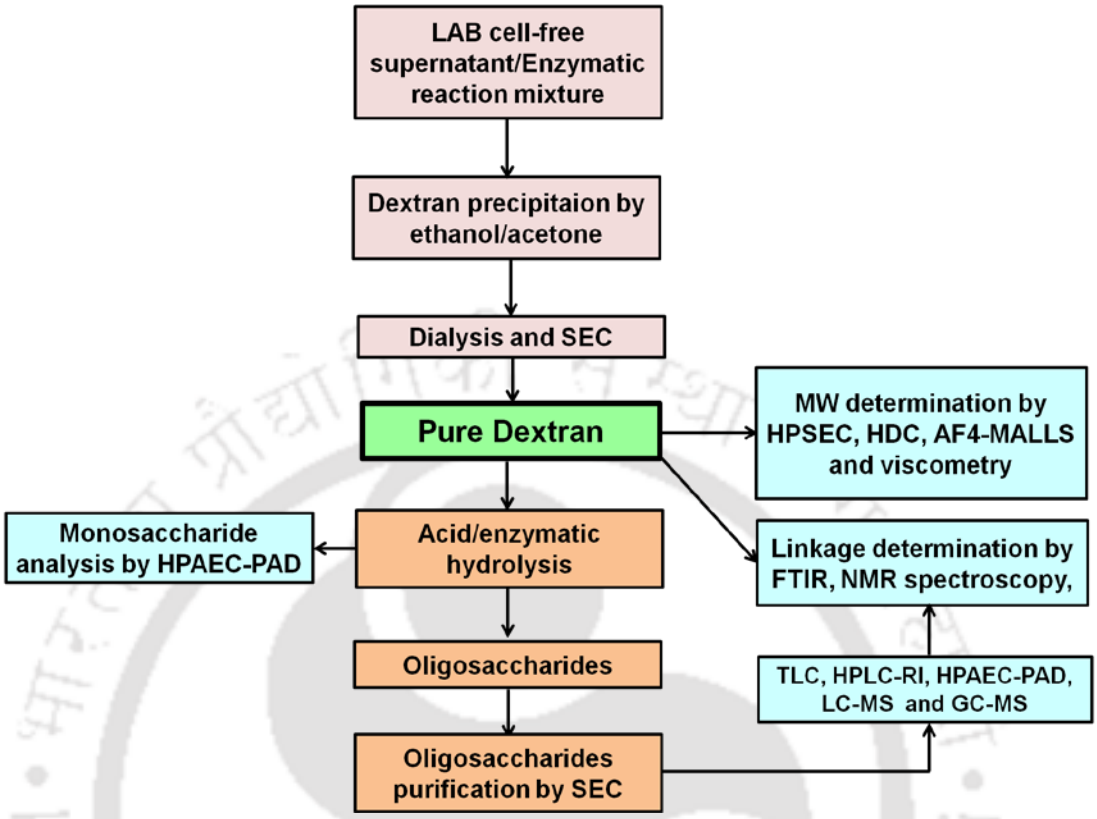

Fig. 1.4 A general strategy for the dextran characterization.

The monosaccharide composition of dextran can be determined by total acid hydrolysis followed by high-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Kothari and Goyal, 2013; Shukla *et al.*, 2014). The structural characterization of dextran is accomplished using well known advance techniques such as one dimensional (^{1}H and ^{13}C) or two dimensional (TOCSY, NOESY, ROESY and HMQC) nuclear magnetic resonance spectroscopy (NMR), fourier transform infrared (FTIR) and scanning electron microscopy (SEM) (Maina *et al.*, 2008; Bounaix *et al.*, 2010; Vettori *et al.*, 2012; Shukla *et al.*, 2014). To elucidate the structure of large glucans, it is often necessary to prepare oligosaccharides from the native polysaccharide by mild acid or enzymatic hydrolysis (Leemhuis *et al.*, 2013). These oligosaccharides are then identified by thin layer

chromatography (TLC), high performance liquid chromatography with refractive index detector (HPLC-RI), HPAEC-PAD, mass spectrometry coupled with liquid or gas chromatography (LC-MS or GC-MS), and FTIR and NMR spectroscopy (Naessens *et al.*, 2005).

1.5 Oligosaccharides

Oligosaccharides, the non-digestible carbohydrates with degree of polymerization (DP) 3-10 have emerged as valuable components of food and dietary supplements (Mussatto and Mancilha, 2007). The acceptor reactions of dextransucrase (glucansucrase) offer the potential for a targeted synthesis of a wide range of di-, tri- and higher oligosaccharides by the transfer of a glucosyl group from sucrose to the acceptor. For many acceptors, the glucose from sucrose is either transferred to 6-hydroxyl group of the monosaccharide or to the 6-hydroxyl group of non-reducing end of the disaccharide to give a homologous series of oligosaccharides of DP 2 to 7 attached to the acceptor (Robyt, 1995). The properties of oligosaccharides are highly dependent on the linkages of dextran produced by the dextransucrase (glucansucrase) and the type of acceptor molecule used (Kothari and Goyal, 2013). The efficiency and the extent of the acceptor reaction also vary with the particular acceptor molecule used (Demuth *et al.*, 2002). So far, maltose and isomaltose have been found to be the best acceptors of dextransucrase for oligosaccharide synthesis (Robyt and Eklund, 1983; Kothari and Goyal, 2013). However, the synthetic potential of this enzyme is not restricted to 'normal' saccharides. Additionally, functionalized saccharides, such as alditols, aldosuloses, a sugar acid, alkyl saccharides and D-glycals, nigero-oligosaccharides, flavonoids, hydroquinones, salicin and phenol and primary alcohols may act as acceptors

(Leemhuis *et al.*, 2012). Oligosaccharides known to have prebiotic properties and hence, they have been increasingly used by the food industry (beverages, sweets) for modifying viscosity, emulsification capacity, gel formation, freezing point and colour of foods (Saad *et al.*, 2013). They also provide nutrition- and health-benefits by modulating the composition of the colonic microbiota as described in the Section 1.2.2 (Fig. 1.2). The schematic illustration of the oligosaccharide production and its application for prebiotics has been presented in Fig. 1.5.

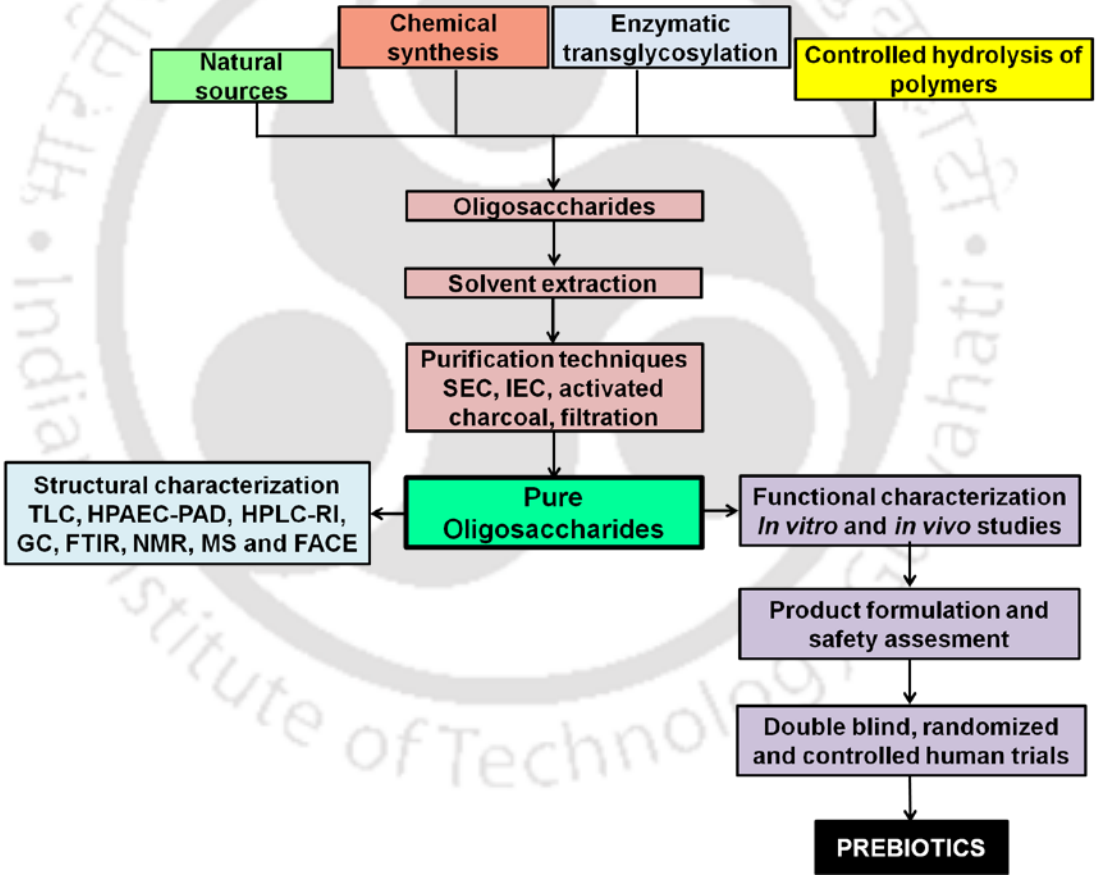

Fig. 1.5 General strategy for the oligosaccharide production and application.

1.5.1 Characterization of oligosaccharides

The application of oligosaccharides for human consumption requires a high degree of product purity (Pinelo *et al.*, 2009). Nowadays, production of

oligosaccharides at competitive prices is a challenge since the well-established production processes involve many labour-intensive and expensive purification steps (Montanes *et al.*, 2012). Purification of oligosaccharides is even more complex when they are synthesized via enzymatic transglycosylation containing high concentrations of glucose and unreacted sugars. These carbohydrates may mask the functional properties of oligossacharides *in vitro* and *in vivo* studies (Ruiz-Matute *et al.*, 2008). The oligosaccharide purification represents the most expensive operation in food manufacturing involving these types of compounds, accounting for around 90% of costs of food production (Montanes *et al.*, 2012). Several methods like use of ion exchange resins (Hernandez *et al.*, 2009), size exclusion chromatography (Ballance *et al.*, 2009), activated charcoal (Nobre *et al.*, 2012), supercritical fluid extraction (SFE) (Montanes *et al.*, 2009), immobilized yeast cells (Lu *et al.*, 2013), ultrafiltration and nanofiltration (Akin *et al.*, 2012) have been employed to purify oligosaccharides from sugar mixtures. Microbes such as *Saccharomyces cerevisiae* (Goulas *et al.*, 2007) and *Zymomonas mobilis* (Crittenden and Playne, 2002) selectively consume up to 90% of monosaccharides present in saccharide mixtures, effectively enriching lactose and oligosaccharides. Oligosaccharides can also be purified by ethanol precipitation, exploiting the differences in solubility (Sen *et al.*, 2011).

The structural characteristics of oligosaccharides, including the types of monosaccharides, substitutions, linkages, molecular weight-encompassing degree of polymerization and extent of branching influence their physico-chemical properties and functionality (Pinelo *et al.*, 2009; Patel and Goyal, 2011). Thin Layer Chromatography (TLC) (Reiffova and Nemcov, 2006) can reveal the degree of polymerization of oligosaccharides. Among the existing analytical methods, mass

spectrometry (MS) is the most favoured because of its precise results, analytical versatility and high sensitivity (Patel and Goyal, 2011; Maina *et al.*, 2013). Over the past decade, the fast atom bombardment (FAB) technique has been replaced by the more sensitive techniques of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (Cmelik and Chmelik, 2010). ESI-MS/MS especially in negative ion mode has been used successfully for the structure elucidation of longer linear mixed-linkage gluco-oligosaccharides. In combination with liquid chromatography this method will enable rapid profiling of the structural variation of gluco-oligosaccharides (Maina *et al.*, 2013). Methylation analysis, MALDI-TOF MS, NMR and FTIR spectroscopy, X-ray diffraction are some other well known methods for structural characterization of oligosaccharides (Okada *et al.*, 2010; Zhao *et al.*, 2013). The most common techniques employed to quantify oligosaccharides are: capillary electrophoresis with a UV-diode array detector (Wahlstrom *et al.*, 2013), high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD) (Ammeraal *et al.*, 1991), gas chromatography (GC) with flame ionization detection (FID) (Low, 1996), high-performance liquid chromatography (HPLC) with evaporative light scattering detection (ELSD) or refractive index (RI) detection (Hernandez *et al.*, 1998), TLC with densitometry (Reiffova and Nemcova, 2006), mass spectrometry (MS) (Jiang and Cole, 2005), fluorophore-assisted carbohydrate electrophoresis (FACE) (O'Shea *et al.*, 1998) and UV spectrophotometry combined with artificial neural networks (ANN) (Dias *et al.*, 2009).

The structural characterization of oligosaccharides must be followed by some functional analysis to claim them as prebiotics. The prebiotic potential of

oligosaccharides in terms of gut acidic and enzymatic resistance and selective stimulation of probiotic bacteria (bifidobacteria and lactobacilli) need to be evaluated both *in vitro* and *in vivo* (Ellegard *et al.*, 1997; Wang, 2009). Dietary patterns, foods, nutrients and other dietary constituents are closely associated with the risk for several types of cancer (Manson, 2003). Colon cancer is being one of the most common causes of cancer-related death world-wide (Jemal *et al.*, 2011). However, the side effects of the chemotherapy for the treatment of cancer led to the development of a new approach to cancer prevention, termed "chemoprevention", which aims to block, inhibit or reverse the development and progression of precancerous cells through use of non-cytotoxic nutrients and/or pharmacological agents (Sporn and Newton, 1979). The chemopreventive effects of various oligosaccharides from different sources have also been evaluated on human colorectal adenocarcinoma cells, HT-29 (Nam *et al.*, 2007; Li *et al.*, 2013). Accordingly, the validation and utilization of dietary components as potential cancer chemoprevention agents in the form of functional foods or nutraceuticals has become an important area in health- and cancer-related research (Li *et al.*, 2013).

The functional characterization for any food component must be followed by the biological risk evaluation of the safety parameters based on the international regulations. The alterations in gut microflora by prebiotics could result in adverse effects, depending on the bacterial populations stimulated. Therefore, safety evaluation involves the analysis of inherent properties of the prebiotic compound and their effects on the gut microflora (Wang, 2009). However, to confirm any clinical benefits and to demonstrate the absence of harmful consequences from their use, well designed, randomized clinical trials are needed (Bruzzese *et al.*, 2006).

1.6 Significance and objectives of the present study

A new era in medical science has dawned with the realization of the critical role of the “forgotten organ”, the gastrointestinal (GI) microbiota, in generating a variety of functions which sustain health and, when disrupted, lead to a disease. The development of prebiotics has been considered as a means of influencing the gut microbiota and risk of allergy. The hunt for new, novel sources of prebiotics seems to be an interesting quest. The aim of the present study was to explore *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase for the production of prebiotic dextran and oligosaccharides. The culture conditions for maximum enzyme production will be optimized. The purification of dextransucrase will be carried out using the polyethylene glycol fractionation and characterized by SDS-PAGE and zymogram analyses. The reaction conditions for enzyme such as sucrose concentration, temperature, pH and ionic strength of the buffer will be optimized. The immobilization of dextransucrase using sodium alginate will be carried out for synthesis of oligosaccharides. The purified dextransucrase will also be used to synthesize dextran and oligosaccharides. The dextran will be purified by alcohol precipitation and subjected to lyophilization. The dextran structure will be analyzed by optical rotation, acid and enzymatic hydrolysis, gel filtration, thermo-gravimetry, FTIR, NMR and SEM studies. Gel filtration will be carried out to determine the molecular mass of dextran. The oligosaccharides will be synthesized with sixteen different acceptors *via* acceptor reaction of dextransucrase and will be characterized by HPAEC-PAD and ESI-TOF MS analyses. The synthesis of isomalto-oligosaccharides (IMOs) and gentio-oligosaccharides (GnOS) will be carried out. The reaction conditions for synthesis of IMOs will be optimized and correlated with

dextran production and their number average molecular mass. The oligosaccharides (IMOs and GnOS) will be purified by Bio-Gel P-2 chromatography and characterized by HPLC-RI and ESI-TOF MS analyses. *In vitro* applications of the purified dextran and oligosaccharides (IMOs and GnOS) as prebiotics will also be investigated.

1.7 Specific objectives of the present study

1. Production, purification, characterization and immobilization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426.
2. Synthesis, purification, characterization and prebiotic application of dextran.
3. Synthesis and characterization of oligosaccharides with different acceptors.
4. Synthesis, purification and prebiotic application of isomalto-oligosaccharides.
5. Synthesis, purification and prebiotic application of gentio-oligosaccharides.

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Chapter 2

Production, purification, characterization and immobilization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426 for synthesis of isomalto-oligosaccharides

2.1 Introduction

Dextransucrase (E.C. 2.4.1.5; sucrose: 1,6-α-D-glucan 6-α-D-glucosyltransferase) synthesizes dextran using the glucosyl part of sucrose and fructose is formed as a byproduct. Dextransucrase is placed in family 70 glycoside hydrolase (GH70) based on sequence similarity in the CAZy classification system (Henrissat, 1991). Dextransucrase is the sole industrial enzyme used in the commercial production of dextran. Dextrans have many uses including production of sephadex, prebiotics and blood plasma substitute (Parlak *et al.*, 2014). In addition to dextran biosynthesis dextransucrase catalyzes acceptor reactions by transferring glucosyl units from sucrose onto acceptors to produce oligosaccharides, when an efficient acceptor molecule is introduced into the medium (Koepsell *et al.*, 1953; Alcalde *et al.*, 1999; Kubik *et al.*, 2004; Kothari *et al.*, 2012; Parlak *et al.*, 2014). Recently, the oligosaccharides have evinced worldwide interest from food and pharmaceutical sector to match the overwhelming consumer preference for healthier food, as they act as prebiotics (Patel and Goyal, 2011). Prebiotics are “food components/ingredients/supplements which result in 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). The production efficiency of oligosaccharides through chemical synthesis is very low because of tedious and selective protecting and deprotecting group manipulations as compared with the enzymatic approach (Wang *et al.*, 2007), which has been found to be more selective. In this context, dextransucrases have emerged as attractive tools for the production of functional oligosaccharides. Unlike chemical synthesis, the acceptor reaction is of special interest, as it enables the synthesis of new oligosaccharide derivatives depending on the regio-specificity (*i.e.*, formation of 1-2 or 1-3 or 1-4 or 1-6 bonds) and stereo-specificity (*i.e.*, formation of only one anomer) of dextransucrase for the acceptor (Heincke *et al.*, 1999). With rapid advances in enzymology and fermentation technology, newer strains of *Leuconostoc mesenteroides* capable of producing commercially important dextransucrase and oligosaccharides, are being searched and explored (Aman *et al.*, 2012). The most important parameters for dextransucrase production are pH, temperature and shaking or static conditions (Tsuchiya *et al.*, 1952; Goyal *et al.*, 1995; Purama and Goyal, 2009). Different dextransucrases have been purified by salt or alcohol precipitation, fractionation by polyethylene glycol, ultrafiltration, size exclusion chromatography and phase partitioning (Majumder *et al.*, 2007). Among all these, polyethylene glycol (PEG) fractionation is an effective and rapid purification method, because it can precipitate large molecular weight proteins and can be readily removed by dialysis (Purama and Goyal, 2008). Dextransucrase exists in either single or multiple molecular forms with varying molecular sizes ranging from 64-313 kDa (Monsan *et al.*, 2001).

Economical production of dextran and oligosaccharides requires the usage of immobilized dextransucrase. Different methods have been proposed to immobilize dextransucrases based on covalent binding (Kaboli and Reilly, 1980; Alcalde *et al.*, 1999; de Segura *et al.*, 2004; Parlak *et al.*, 2013) adsorption (Kobs, 1991) and entrapment in polymers (Reischwitz *et al.*, 1995; Tanriseven and Dogan, 2002; Kubik *et al.*, 2004; Kothari *et al.*, 2012). Studies on immobilization of dextransucrases have not been successful with respect to yield and stability. One of the reasons is that the dextran associated with dextransucrase masks the residues such as lysine, aspartate and glutamate residing at the active site of the enzyme. These residues could react with aldehyde and epoxy groups of the immobilization matrix leading to inactivation of the enzyme (Funane *et al.*, 2005; Robyt *et al.*, 2008; Olcer and Tanriseven, 2010; Parlak *et al.*, 2013, 2014). However, encapsulation of dextransucrase in alginate has been the only successful immobilization method. There are several studies on immobilization of dextransucrase in alginate, resulting in immobilization yields up to 90% (Reischwitz *et al.*, 1995; Alcalde *et al.*, 1999, Tanriseven and Dogan, 2002, Kothari *et al.*, 2012). However, the alginate immobilization method can only be used for the production of oligosaccharides. This is due to the fact that high molecular weight dextran cannot diffuse out of the beads. Moreover, the enzyme can also be recovered easily from the reaction mixture for its reuse (Tanriseven and Dogan, 2002). The present study is focused on production, purification, characterization and immobilization of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426 for the production of isomalto-oligosaccharides.

2.2 Materials and Methods

2.2.1 Chemicals and reagents

All the media components for maintenance and enzyme production were purchased from Hi-Media Pvt. Ltd., India. All the organic solvents were purchased from Qualigens, India. PEG-400 and 1500 from Merck, India were used for enzyme fractionation. Bovine serum albumin, maltotriose and dextranase from *Penicillium* sp., were from Sigma Aldrich, USA. All the chemicals required for reducing sugar estimation, protein estimation and buffer preparation were of high purity.

2.2.2 Microorganism and culture conditions

Leuconostoc mesenteroides NRRL B-1426 was procured from Agricultural Research Service (ARS) culture collection, National Centre for Agricultural Utilization Research, Peoria, USA. The culture was grown, maintained in modified de Man, Rogosa, and Sharpe (MRS) medium (de Man *et al.*, 1960; Goyal and Katiyar 1996) as stab at 4°C and sub-cultured every 2 weeks. The MRS medium contained in (g/l): sucrose, 20; yeast extract, 5; beef extract, 10; K_2HPO_4, 2; sodium acetate, 5; tri-sodium citrate, 2; Tween 80, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.2.

2.2.3 Inoculum preparation

Inoculum was prepared by transferring a loopful from stab culture of *L. mesenteroides* NRRL B-1426 to 5 ml enzyme production medium as described by Tsuchiya *et al.* (1952) which contained (g/l) sucrose, 20; yeast extract, 20; K_2HPO_4, 20; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.01; NaCl 0.01 and incubated at 28°C. The pH of the medium was adjusted to 6.9 with 0.1

M HCl solution. Then, 1 ml of log phase culture (1%, v/v) was transferred to 250 ml Erlenmeyer flask containing 100 ml of sterile enzyme production medium.

2.2.4 Production of dextransucrase

The dextransucrase from *L. mesenteroides* NRRL B-1426 was produced using the enzyme production medium as described in Section 2.2.3. The culture was incubated at 24°C and 120 rpm for 6-8 h under static condition. The cell-free supernatant was obtained by centrifugation at 10,000*g* and 4°C for 10 min and analyzed for enzyme activity and protein concentration as described in the Sections 2.2.4 and 2.2.5, respectively. The cell-free supernatant was used subsequently for the purification of dextransucrase as described in Section 2.2.7.

2.2.5 Enzyme assay

The enzyme assay was carried out in 1 ml reaction mixture containing 5% (w/v) sucrose, 20 mM sodium acetate buffer (pH 5.6) and 20 µl of enzyme sample. The enzymatic reaction was performed at 30°C for 15 min. To 100 µl aliquot from the reaction mixture, 100 µl of reagent D (Section 2.2.5.1) was added for reducing sugar estimation. The solution was mixed and heated for 20 min in a boiling water bath. It was cooled to room temperature and then 100 µl of reagent C (Section 2.2.5.1) was added. The colour developed rapidly and simultaneously with the evolution of CO_2. The mixture was diluted by adding 700 µl distilled water. The absorbance of colour developed was measured at 500 nm on a UV-visible spectrophotometer (Varian, Cary 100). Fructose (0.5 µg/ml-500 µg/ml) was used to plot the standard graph.

2.2.5.1 Preparation of reagents for reducing sugar estimation

The reagents for estimation of reducing sugar were prepared as described by Nelson (1944) and Somogyi (1945).

Reagent A: Sodium carbonate anhydrous (2.5 g), sodium potassium tartarate (2.5 g), sodium bicarbonate (2.0 g) and sodium sulfate anhydrous (20 g) dissolved in distilled water and the volume made upto 100 ml. Filtered and stored at a temperature between 30-37°C.

Reagent B: 15% copper sulphate containing one or two drops of concentrated sulphuric acid.

Reagent C: Ammonium molybdate (2.5 g) dissolved in 45 ml distilled water, then 2.1 ml concentrated sulphuric acid added and mixed. To this was added sodium arsenate (0.3 g) dissolved in 2.5 ml of distilled water, mixed and stored at 37°C for 24 h before use.

Reagent D: Prepared fresh by mixing reagent A and reagent B in the ratio 25:1.

2.2.5.2 Calculation of enzyme activity

One unit (U) of dextransucrase activity is defined as the amount of enzyme that liberates 1 µmole of reducing sugar (fructose) in 1 min at 30°C and pH 5.4. The dextransucrase activity was calculated as follows:

$$\text{Enzyme activity (U/ml)} = \frac{\Delta A_{500} \times C \times V}{180 \times t \times v} \quad (\mu\text{mole/min/ml})$$

ΔA_{500} = Absorbance change at 500 nm.
C = 1 absorbance equivalent fructose concentration (mg/ml) from standard plot.
V = volume of the reaction mixture (ml).
t = time of reaction (min).
180 = molecular weight of fructose.
v = volume of the enzyme source (ml) for reducing sugar estimation.

2.2.6 Determination of protein content

The protein content of the cell-free supernatant containing dextransucrase and partially purified dextransucrase was estimated by the method of Lowry *et al.* (1951). Bovine serum albumin (BSA) was used as a reference and a concentration range 25-500 µg/ml was used to plot a standard curve.

2.2.6.1 Reagents for Lowry method

Reagent A : Sodium hydroxide (0.4 g) and sodium carbonate (2.0 g) were dissolved in water and the volume made up to 100 ml.

Reagent B_1 : 2% (w/v) sodium potassium tartarate.

Reagent B_2 : 1% (w/v) copper sulfate.

Reagent C : Prepared fresh by mixing reagent B1, A and B2 in the ratio 1:100:1.

Phenol reagent : 1 N phenol reagent.

2.2.6.2 Estimation of protein

To 0.2 ml of sample containing protein or BSA, 1 ml of reagent C was added. After 15 min, 0.1 ml of Folin's reagent was added and mixed and the absorbance was measured after 30 min at 660 nm against a blank. The concentration of protein was calculated as follows:

$$\text{Protein concentration (mg/ml)} = \frac{\Delta A_{660} \times C}{V} \text{ (mg/ml)}$$

ΔA_{660} = Absorbance change at 660 nm.
C = 1 absorbance equivalent of BSA concentration (mg/ml) from standard plot.
V = volume of the sample (ml).

2.2.7 Effect of temperature and aeration on dextransucrase production from *L. mesenteroides* NRRL B-1426

The effect of temperature on dextransucrase production from *L. mesenteroides* NRRL B-1426 was studied by varying temperature from 20 to 40°C under static condition, using 100 ml of enzyme production medium (as described in Section 2.2.3) in 250 ml Erlenmeyer flask. The effect of aeration on dextransucrase production was analyzed by varying the shaking speed from 90 to 180 rpm at 24°C. 1.0 ml of culture was withdrawn from each conical flask at every 3 h interval and centrifuged at 10,000*g* and 4°C for 10 min. The cell-free supernatant obtained was analyzed for dextransucrase activity as described in Section 2.2.5 and for protein concentration as described in Section 2.2.6.

2.2.8 Partial purification of dextransucrase from *L. mesenteroides* NRRL B-1426 by PEG fractionation

1% (v/v) of *L. mesenteroides* NRRL B-1426 culture was inoculated in 100 ml of enzyme production medium as described in Section 2.2.4 and incubated at 24°C and 120 rpm for 6 h. The grown culture was centrifuged at 10,000*g* at 4°C for 10 min. The cell-free supernatant was analyzed for enzyme activity as described in Section 2.2.5 and for protein concentration as described in Section 2.2.6. The cell-free supernatant was subjected to polyethylene glycol (PEG) fractionation. Different percentages of the ice cold PEG-400 in the range of 15-45% (v/v) and PEG-1500 in the range of 5-20% (w/v) were added to 50 ml of cell-free supernatant. They were incubated at 4°C for 12 h to allow the dextransucrase to fractionate. The mixture was centrifuged at 17,200*g* for 30 min at 4°C to separate the fractionated dextransucrase. The obtained enzyme pellets were dissolved in 1 ml of 20 mM sodium acetate buffer, pH 5.6. The enzyme samples were subjected to dialysis using 14 kDa cut-off

membranes (Hi-Media Pvt. Ltd., India). Purified enzyme samples were analyzed for enzyme activity and protein content as described in Sections 2.2.5 and 2.2.6, respectively. The dialyzed enzyme samples further were characterized by non-denaturing and denaturing SDS-PAGE using silver staining as described in Sections 2.2.9 and 2.2.10, respectively, and periodic acid Schiff (PAS) staining under non-denaturing and native condition as described in Section 2.2.11.

2.2.9 Denaturing and non-denaturing SDS-PAGE analysis of dextransucrase

2.2.9.1 Preparation of SDS-PAGE gels

The polyacrylamide gels are prepared by copolymerization of acrylamide and bis-acrylamide (bis-N,N'-methylene-bisacrylamide). The copolymerization is a vinyl addition reaction initiated by a free radical-generator ammonium per sulphate (APS) in presence of a catalyst, N,N,N',N'-tetramethylethane-1,2-diamine (TEMED) (Chrambach, 1985). Sodium docdecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed following the method of Laemmli (1970). The resolving gel containing 7.5% (w/v) acrylamide and stacking gel containing 4% (w/v) acrylamide were prepared as described in Table 3.2.1 and Table 3.2.2, respectively. The sample loading buffer was prepared as described in Table 3.2.3. In case of non-denaturing conditions β-mercaptothanol was not added in the loading dye. The purified enzyme sample was mixed with 5x loading dye buffer in the ratio 4:1. The sample mixture was subjected to heat denaturation by boiling for 4 min. The sample was loaded on 7.5% acrylamide gel and the electrophoresis was carried out using 1x running buffer (200 mM glycine, 0.1% SDS, 50 mM Tris-HCl pH 8.3), as described in Table 3.2.4 with a current of 2.5 mA per lane. High range protein marker, 10-200 kDa (Thermo Fisher Scientific, Fermentas) was used as standard.

2.2.9.2 Preparation of acrylamide 30% (w/v) solution

0.8 g of bis-acrylamide was weighed and transferred into an amber colour bottle and dissolved in 50 ml of deionised water collected at 18 MΩcm (Millipore, Milli-Q water purification system) on a magnetic stirrer (IKA, C-MAG HS7). After completely dissolving bis-acrylamide, 29.2 g of acrylamide was added and stirred on a magnetic stirrer till the solution was clear. The final volume was adjusted to 100 ml with deionised water. The acrylamide solution was then filtered (Whatman No. 1) and stored in amber colour bottle at 4°C.

Table 2.2.1 Composition of SDS-PAGE components for preparation of 7.5% resolving gel.

Components	Volume (ml)
Acrylamide solution (30%,w/v)	2.5
Deionized water	2.2
SDS (10%,w/v)	1.0
Glycerol (50%,v/v)	1.0
1.5 M Tris-HCl (pH 8.8)	3.3
APS (10%,w/v)	0.1
TEMED	0.01
Total	**10 ml**

Table 2.2.2 Composition of SDS-PAGE components for preparation of 4% stacking gel.

Components	Volume (ml)
Acrylamide solution (30%, w/v)	0.7
Deionized water	2.8
SDS (10%,w/v)	0.5
0.5 M Tris-HCl (pH 6.8)	1.0
APS (10%,w/v)	0.05
TEMED	0.01
Total	**10 ml**

2.2.9.3 Preparation of sample buffer

The sample loading buffer (5x) was prepared by dissolving the components as described in Table 3.2.3 and the pH of the buffer was adjusted to 6.8. The final

concentration while loading to a SDS-PAGE gel was always kept to 1x by mixing 4 volumes of sample (protein) with 1 volume of 5x sample buffer.

Table 2.2.3 Composition of 5x sample loading buffer.

Components	Final concentration
Tris-HCl (pH 6.8)	62.5 mM
Glycerol	20.0% (v/v)
SDS	2.0% (w/v)
Bromophenol blue	0.025% (w/v)
β-mercaptoethanol	5.0% (v/v)

2.2.9.4 Preparation of SDS-PAGE running buffer

The SDS-PAGE gels were run using a 1x running or tank buffer prepared from the 5x stock solution as described in Table 2.2.4. 15.14 g of trizma free base and 94 g of glycine were dissolved in 800 ml of deionised water. To this 50 ml of 10% (w/v) SDS was added and the final volume was adjusted to 1 litre. The final pH of the buffer was adjusted to 8.3. The 5x buffer was filtered (Whatman, Filter No. 1) and stored at 4°C.

Table 2.2.4 Composition of 5x Tris-glycine running buffer.

Components	Final concentration
Trizma base	0.125 M
Glycine	1.25 M
SDS	0.5 % (w/v)

2.2.10 Silver staining

2.2.10.1 Preparation of reagents for silver staining

Silver staining after gel electrophoretic separation provides excellent sensitivity for protein detection (0.2-2 ng range) (Rabilloud, 1992). The solutions used in silver staining are listed in Table 2.2.4 and the staining procedure is described in Section 2.2.10.2.

Table 2.2.5 Composition for preparation of silver staining reagents.

Reagents	Components
Fixing solution	40% (v/v) ethanol, 10% (v/v) acetic acid in water
Sensitizing solution	0.2% (w/v) $Na_2S_2O_3$, 6.8% (w/v) sodium acetate, 30% (v/v) ethanol and 0.025% (v/v) glutaraldehyde
Silver nitrate solution	0.1% (w/v) $AgNO_3$, 0.076% (w/v) formalin
Developing solution	2.5% (w/v) Na_2CO_3, 0.05% (w/v) formaldehyde
Stop solution	1.46% (w/v) EDTA
Preserving solution	30% (v/v) ethanol, 4% (v/v) glycerol

2.2.10.2 Silver staining procedure

Silver staining of the gel was carried out by following the method reported by Rabilloud (1992). After the electrophoresis, the gel was immersed in 50 ml of fixing solution at 25ºC for at 30 min to fix the protein bands and placed on a shaker at a very gentle speed. After 30 min, the solution was discarded and the gel was washed in 20% ethanol for 20 min to remove the remaining detergent as well as fixing solution from the gel. The gel was then incubated in 100 ml sensitizing solution at 25°C for 20-30 min. After the incubation, the sensitizing solution was discarded and the gel was washed thrice by deionised water at every 10 min intervals. The silver nitrate solution of 100 ml was added to the gel and the gel was incubated for 20 min in dark to allow the silver ions to bind with proteins. After staining, the gel was rinsed with 100 ml of deionised water for 2-3 min and 100 ml of developing solution was added to the gel. The reaction was stopped as soon as the protein bands appeared in desired intensity by adding the 50 ml of stop solution. The gel was stored in preserving solution.

2.2.11 Identification of dextransucrase by Periodic Acid Schiffs (PAS) staining

The PEG-400 (33%, v/v) purified dextransucrase was loaded on a 7.5% SDS-polyacrylamide gels and run under denaturing and non-denaturing condition. In non-

denaturing condition, the sample buffer (Table 2.2.3) did not contain β-mercaptoethanol and the enzyme sample was not subjected to heat denaturation. After the run, gel was cut into four parts. One part of the gel containing denatured and non-denatured samples was subjected to silver staining as described in Section 2.2.10.2. The other three parts containing non-denatured enzyme samples were analyzed for *in situ* activity detection by periodic acid Schiff (PAS) staining (Vasileva *et al.*, 2009). All the three parts were treated thrice in 20 mM sodium acetate buffer (pH 5.6) containing 0.3 mM $CaCl_2$ and 0·1% (v/v) Triton X-100 at 25°C for 20 min to remove SDS. Then, one part of the gel was incubated in 20 mM sodium acetate buffer (pH 5·6) containing 0·3 mM $CaCl_2$ and 10% (w/v) sucrose and the other two parts of the gel were incubated with 10% (w/v) raffinose instead of sucrose at 30°C for 12-18 h. One part of the raffinose incubated gel was then treated with dextranase (1 U/ml) at 37°C for 12 h. The treatment of raffinose incubated gels with dextranase prior to staining will eliminate the possibility any GTF bands including alternansucrase and fructansucrase (Smith and Zahnley, 1999). Thereafter, all the gels were washed with 75% (v/v) ethanol for 40 min to remove the substrates (sucrose and raffinose) and incubated in periodic acid solution (0.7%, w/v periodic acid and 5%, v/v acetic acid) for 1 h at 25°C. After the periodic acid treatment, the gels were washed with a solution containing 0.2% (w/v) sodium metabisulphite and 5% (v/v) acetic acid. Finally, the gels were stained with 15 ml Schiff reagent (0.5%, w/v Fuchsin basic, 1% sodium bisulphite and 0.1 N HCl) until the discrete magenta colour band within the gel matrix appeared.

The PEG purified dextransucrase was also run under native condition on a 7.5% gel and subsequently analyzed by PAS staining with sucrose as a substrate to

determine monomeric or multimeric nature of the enzyme. For native condition, SDS was omitted from all the steps including gel, sample buffer and tank buffer as described in Section 2.2.9.

2.2.12 Optimization of reaction conditions of dextransucrase

2.2.12.1 Effect of pH, temperature and ionic strength on dextransucrase activity

The purified dextransucrase (10.14 U/mg, 0.76 mg/ml) was used for optimization of reaction conditions. In order to know optimum pH for the enzyme reaction, the pH of sodium acetate buffer by varied from 4.2 to 6.4, while keeping the ionic strength at 20 mM, temperature at 30°C and 5% (w/v) sucrose concentration. To study the effect of temperature the enzyme was incubated at different temperatures ranging from 20 to 40°C in 20 mM sodium acetate buffer pH 5.6 and 5.0% (w/v) sucrose concentration for 15 min. In case of effect of ionic strength on dextransucrase, the concentration of sodium acetate buffer was varied from 10-500 mM, keeping pH 5.6, sucrose concentration 5% (w/v) and a constant temperature of 30°C for 15 min. The enzyme activity and protein content were determined as described in Sections 2.2.5 and 2.2.6, respectively.

2.2.12.2 Effect of sucrose concentration on dextransucrase activity

To study the effect of sucrose concentration on the enzyme activity of the purified dextransucrase (10.14 U/mg, 0.76 mg/ml), the enzyme assay was conducted at different sucrose concentrations ranging from 2.92-234 mM (0.1-8%, w/v). The reaction was carried out in 1 ml mixture in 20 mM sodium acetate buffer pH 5.6 by adding 20 µl of enzyme and varying concentration of sucrose. The mixture was incubated at 30°C in water bath for 15 min and analyzed for enzyme activity and protein content as described in Sections 2.2.5 and 2.2.6, respectively.

2.2.13 Immobilization of dextransucrase from *L. mesenteroides* NRRL B-1426

2.2.13.1. Optimization of sodium alginate concentration and reaction conditions

L. mesenteroides NRRL B-1426 dextransucrase (1 ml, 10.14 U/mg, 0.76 mg/ml) was mixed with 4 ml of 2% (w/v) sodium alginate solution. This solution was added drop wise through a micropipette (50-200 µl) to 100 ml of 2% (w/v) $CaCl_2$ solution prepared in 20 mM sodium acetate buffer (pH 5.6). The beads formed were allowed to harden in $CaCl_2$ solution for 2 h with continuous stirring and then transferred to 20 mM sodium acetate buffer (pH 5.6) containing 0.2 M $CaCl_2$ and 0.02% NaN_3. The immobilized enzyme was stored at 4°C. Optimization of sodium alginate concentration for immobilization of dextransucrase was carried out. Dextransucrase was immobilized by using different concentration of sodium alginate from 0.5 to 3% (w/v) using the method as mentioned above and immobilization yield was determined. Immobilization yield is defined as the ratio of activity of immobilized enzyme to the activity of soluble enzyme used in immobilization (Tanriseven and Dogan, 2002). The enzyme activity was measured by quantifying the amount of reducing sugar released as described in Section 2.2.5.

$$\text{Immobilization yield} = \frac{\text{Activity of immobilized enzyme}}{\text{Activity of soluble enzyme}} \times 100\%$$

The effect of agitation for assay of immobilized enzyme was studied by varying the shaking speed from 50 to 350 rpm. All the assays of immobilized dextransucrase were done at the optimum conditions, which were not affected by immobilization. The activity of dextransucrase in alginate beads was also determined as described in Section 2.2.5 after crushing the beads in a mortar to determine the

whole activity retained in beads (Tanriseven and Dogan, 2002). The protein content of immobilized enzyme in crushed beads was estimated by the method as described in Section 2.2.6.

2.2.13.2 Operational stability of immobilized dextransucrase

Operational stability of immobilized enzyme was carried out by repeating 10 batch of reactions in which the immobilized enzyme was reacted with sucrose (5%, w/v) in 20 mM sodium acetate buffer (pH 5.6) at 30°C for 15 min, and the relative activity was determined by quantifying reducing sugar as described in the Section 2.2.5. At the end of each cycle, the enzyme was washed with 20 mM sodium acetate buffer (pH 5.6) and the reaction was restarted.

2.2.13.3 Storage stability of free and immobilized dextransucrase

Storage stability of free and immobilized enzyme at 4°C was tested for 30 days by determining the residual activity, quantifying reducing sugar as described in Section 2.2.5.

2.2.13.4 Thermal and pH stability of free and immobilized dextransucrase

Free and immobilized enzyme in 20 mM sodium acetate buffer (pH 5.6) were kept at temperatures ranging from 20 to 40°C for 60 min and the residual activity was determined by quantifying the amount of reducing sugar released as described in Section 2.2.4. Similarly, the effect of pH ranging from 3.5 to 7.5 was examined at 30°C for 60 min on the stability of free and immobilized enzyme.

2.2.13.5 Production of oligosaccharides using free and immobilized dextransucrase

The oligosaccharides were produced by incubating free (1 U/ml) and immobilized enzyme (1 U/ml) in 5 ml of 5% (w/v) sucrose and 5% (w/v) maltose

solution in 20 mM sodium acetate buffer (pH 5.6, containing 0.3 mM $CaCl_2$ and 15 mM NaN_3) at 30^oC for 48 h. Equal volume of absolute ethanol was added to the reaction mixture and centrifuged at 16,000*g* for 10 min in order to precipitate polysaccharides. The supernatant containing oligosaccharides was then analyzed by thin layer chromatography (TLC) at 25°C using a silica gel 60 F_{254} TLC plate (Merck, Germany). The reaction digest (0.5 µl) was spotted onto a silica gel plate and the plate was developed using a solvent mixture of ethyl acetate:1-propanol:acetonitrile:water (4:10:14:11) (Cote and Leathers, 2005). The plate was visualized with ethanol solution containing 0.5% (w/v) α-naphthol and 5% (v/v) H_2SO_4 and heating at 120°C until the spots became visible (Kim and Day, 2008). The relative concentration of oligosaccharides was determined using a Scion program from National Institute of Health (NIH, USA) (Kim and Day, 2008). The oligosaccharides were also identified by ESI-TOF MS (Waters, Q-TOF Micromass HAB273) using the following ion source parameter conditions: source-desolvation gas temperatures set at 100ºC and 250ºC; sampling, capillary and extraction cone voltages were set 35 V, 3 KV and 3 V, respectively, with the ion guide of 1 V. Flow injection rate was set at 10 µl/min. Mass spectrum was acquired in positive ion mode and processed using MassLynx software (Kothari *et al.*, 2012).

2.3 Results and Discussion

2.3.1 Effect of temperature and aeration on dextransucrase production from *L. mesenteroides* NRRL B-1426

Dextransucrase from *L. mesenteroides* NRRL B-1426 showed maximum specific activity of 0.65 U/mg at 24°C (Fig. 2.3.1A). The enzyme activity decreased as the temperature increased above 24°C, and decreased by 50% at 32°C due to thermal denaturation of the enzyme at higher temperatures. The enzyme activity decreased by 32.5% at 20°C, which might be due to the slower growth rate of cells consequently resulting in lower enzyme activity.

The maximum specific activity of 0.74 U/mg was observed at 120 rpm after 6 h (Fig. 2.3.1B). Dextransucrase showed 14% higher activity (0.74 U/mg) under shaking condition, whereas under static condition it was 0.65 U/mg when grown at 24°C and 9 h (Fig. 2.3.1B). Although, Leuconostoc strains are known to be microaerophilic (Barker *et al.*, 1991), some reports revealed that oxygen positively affects their growth (Lebrun *et al.*, 1994; Cortezi *et al.*, 2004). The extracellular dextransucrase production is positively affected by bacterial growth and the oxygen mass transfer rates (Purama *et al.*, 2007). This might be the reason for the maximum enzyme production by *L. mesenteroides* NRRL B-1426 at 120 rpm. These results suggested that low aeration rate is an important factor for dextransucrase production. Shaking condition was also favourable for production of dextransucrases from *L. mesenteroides* NRRL B-640 (Purama and Goyal, 2009).

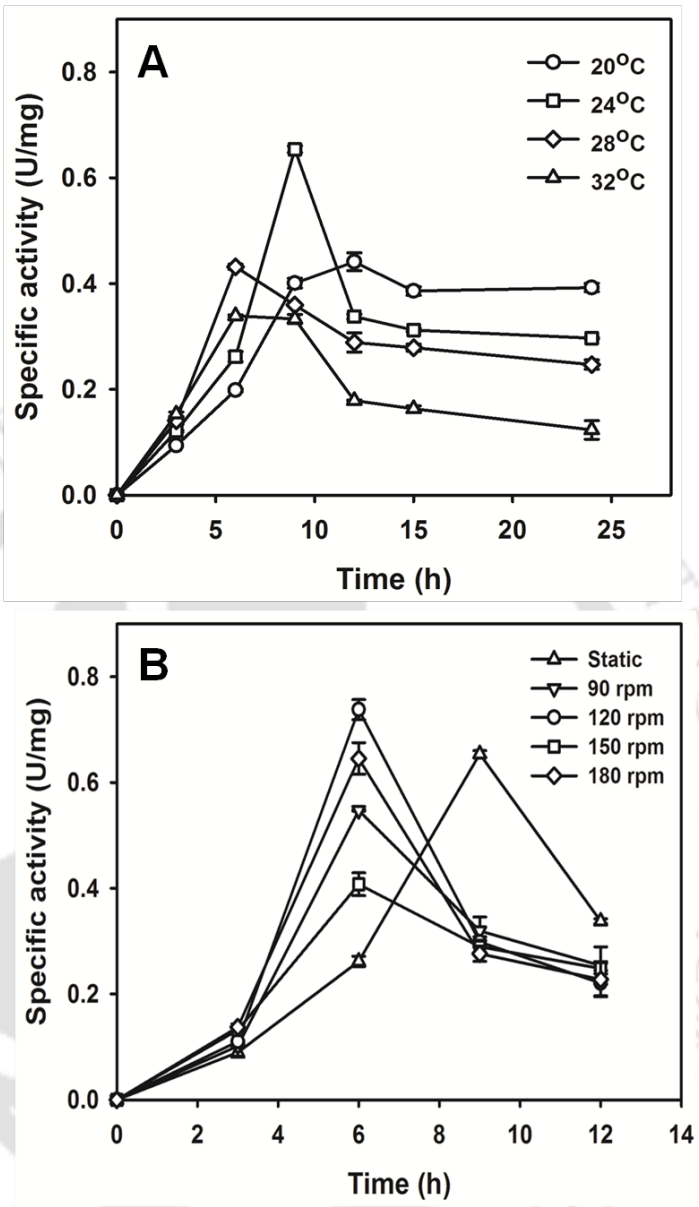

Fig. 2.3.1 Effect of (A) temperature (B) aeration on dextransucrase production from *L. mesenteroides* NRRL B-1426.

2.3.2 Partial Purification of dextransucrase from *L. mesenteroides* NRRL B-1426

The cell-free supernatant containing dextransucrase (specific activity of 0.74 U/mg) was subjected to purification by PEG fractionation. The specific activity of dextransucrase increased with increase in the concentration of PEG-400 from 15 to 33% (v/v) (Fig. 2.3.2).

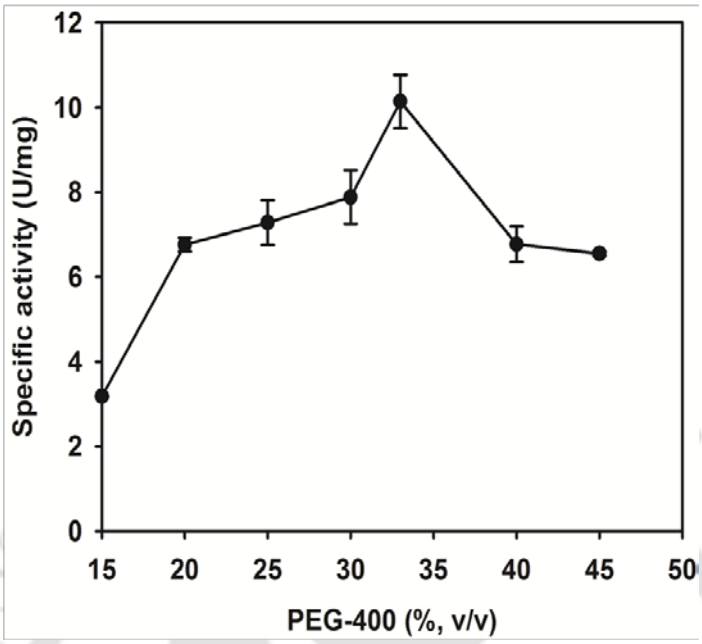

Fig. 2.3.2 Purification dextransucrase from *L. mesenteroides* NRRL B-1426 by PEG-400 fractionation.

Table 2.3.1 Purification of dextransucrase from *L. mesenteroides* NRRL B-1426 by PEG-400 fractionation.

PEG-400 (%, v/v)	Vol. (ml)	Enzyme activity (U/ml)	Total units (U)	Overall activity yield (%)	Protein conc. (mg/ml)	Specific activity (U/mg)	Fold Purification
Crude	40	4.12	164.8	-	5.49	0.74	-
15	1.3	0.70	0.91	0.42	0.22	3.18	4.29
20	3.8	3.65	13.87	8.42	0.54	6.76	9.10
25	3.3	6.84	22.57	13.69	0.94	7.28	9.84
30	3.6	6.94	24.98	15.15	0.88	7.88	10.00
33	**3.6**	**7.71**	**27.76**	**16.84**	**0.76**	**10.14**	**13.70**
40	3.4	5.35	18.80	11.41	0.79	6.77	9.14
45	3.1	5.24	16.24	9.85	0.80	6.55	8.85

The specific activity and the extent of purification achieved with PEG-400 are listed in Table 2.3.1. The maximum specific activity of 10.14 U/mg was achieved at 33% (v/v) PEG-400 with 14-fold purification and 17% overall yield in a single step,

which was much higher to those reported for *L. mesenteroides* NRRL B-1146 (4.5 U/mg and 10% yield) (Majumder *et al.*, 2008) and *L. mesenteroides* NRRL B-640 (5.5 U/mg and 3.1% yield) (Purama and Goyal, 2008). The specific activity of dextransucrase decreased with increase in the concentration of PEG-1500 from 10 to 20% (w/v) as shown in Fig. 2.3.3. The maximum specific activity of 6.40 U/mg was achieved at 10% (w/v) PEG-1500. The specific activity and the extent of purification achieved with PEG-1500 are listed in Table 2.3.2.

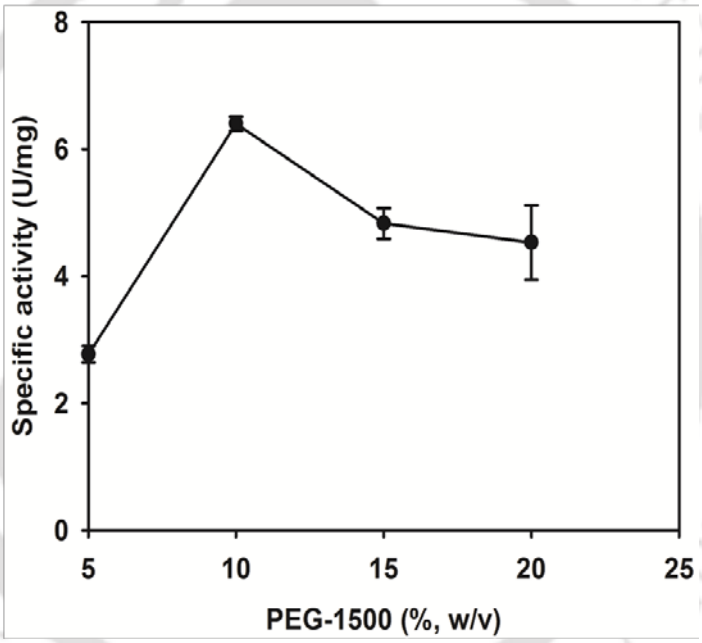

Fig. 2.3.3 Purification dextransucrase from *L. mesenteroides* NRRL B-1426 by PEG-1500 fractionation.

Table 2.3.2 Purification of dextransucrase from *L. mesenteroides* NRRL B-1426 by PEG-1500 fractionation.

PEG-1500 (%, w/v)	Vol. (ml)	Enzyme activity (U/ml)	Total units (U)	Overall activity yield (%)	Protein conc. (mg/ml)	Specific activity (U/mg)	Fold Purification
Crude	40	4.12	164.8	-	5.49	0.74	-
5	2.5	2.72	6.80	4.13	0.98	2.77	3.74
10	**3**	**6.66**	**19.98**	**12.12**	**1.04**	**6.40**	**8.65**
15	2.4	5.17	12.4	7.52	1.07	4.83	6.53
20	2.6	5.71	14.85	9.01	1.26	4.53	6.12

The PEG-400 (33%, v/v) gave higher specific activity (10.14 U/mg) of dextransucrase from *L. mesenteroides* NRRL B-1426 as compared with PEG-1500 (6.40 U/mg) at 10% (w/v). PEG-400 is known to give higher specificity of dextransucrase precipitation as compared with higher molecular weight PEG. It has been also reported that the higher concentration of PEG precipitated other non-dextransucrase proteins, while lower concentration of PEG did not fractionate most of the enzyme and therefore a decrease in specific activity was observed (Russell, 1979; Majumder *et al.*, 2008).

The dextransucrase fractions obtained with PEG-400 and 1500 were also analyzed by SDS-PAGE as described in Sections 2.2.9 and 2.2.10 to check the purity. The SDS-PAGE results of PEG-400 and 1500 showed the presence of multiple protein bands for dextransucrase, but the pattern of bands were different in both the cases (Fig. 2.3.4A and 2.3.5A). The active form of the enzyme was confirmed by PAS activity staining (Fig. 2.3.4B and 2.3.5B) as described in Section 2.2.11. The molecular weight of the active form of the purified dextransucrase was analysed and described in the next Section 2.3.3.

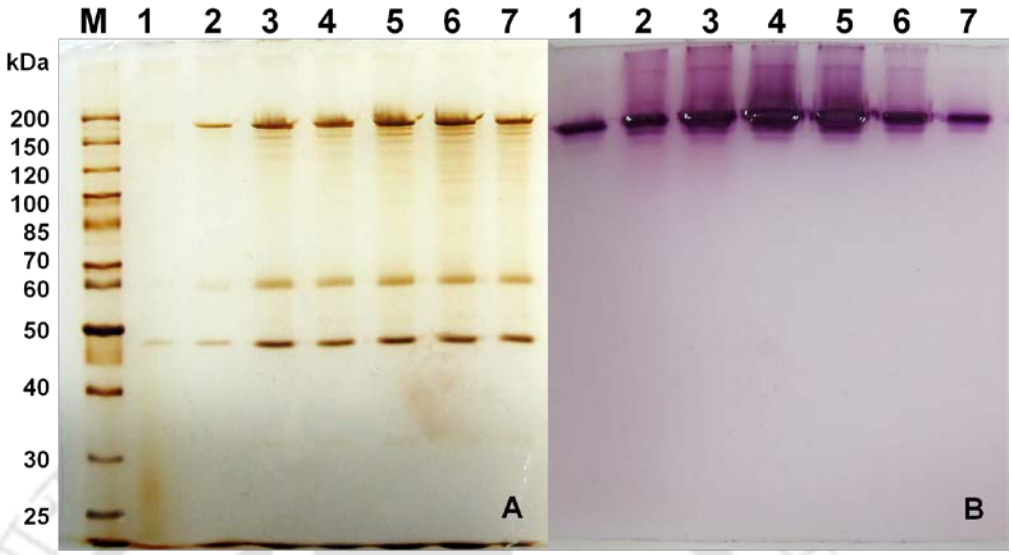

Fig. 2.3.4 Non-denaturing SDS-PAGE analysis of purified dextransucrase fractions by PEG-400 (A) Silver staining, Lanes: M-Protein molecular weight marker: 10-200 kDa; 1-15%; 2-20%; 3-25%; 4-30%; 5-33%; 6-40%; 7-45% (v/v) PEG-400 fractions. (B) PAS staining with sucrose as substrate, Lanes: 1-15%; 2-20%; 3-25%; 4-30%; 5-33%; 6-40%; 7-45% (v/v) PEG-400 fractions.

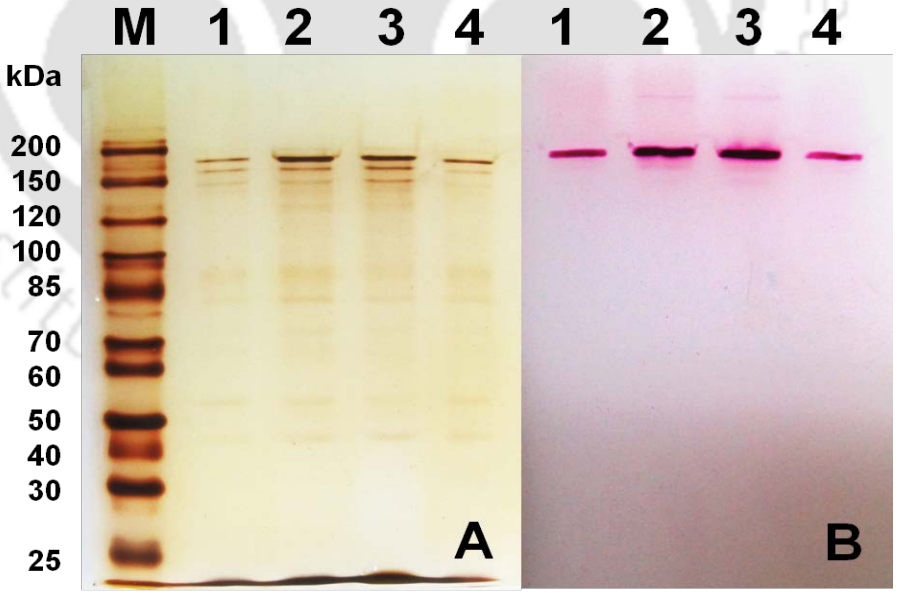

Fig. 2.3.5 Non-denaturing SDS-PAGE of purified dextransucrase fractions by PEG-1500 (A) Silver staining, Lanes: M-Protein molecular weight marker: 10-200 kDa; 1-5%; 2-10%; 3-15%; 4-20% (w/v) PEG-1500 fractions. (B) PAS staining with sucrose as a substrate, Lanes: 1-5%; 2-10%; 3-15%; 4-20% (w/v) PEG-1500 fractions.

2.3.3 Identification and molecular size analysis of dextransucrase by non-denaturing and denaturing SDS-PAGE using silver staining and PAS staining

L. mesenteroides NRRL B-1426 dextransucrase purified by 33% (v/v) PEG-400 fractionation (10.14 U/mg, 0.76 mg/ml) was subjected to both non-denaturing (Fig. 2.3.6A, Lane 1) and denaturing SDS-PAGE (Fig. 2.3.6A, Lane 2). The dextransucrase showed multimeric nature with molecular sizes of approximately, 240, 178 and 165 kDa (Fig. 2.3.6A, Lane 1 and 2) corresponding to the three activity bands on sucrose incubated gel (Fig. 2.3.6B, Lane 3). The faint band of 240 kDa was observed only in PAS staining but not in silver staining, which might be due to active form of enzyme was present in very low amounts (picogram level) and therefore, could not be stained by the silver staining. As the active form of enzyme in picogram levels also synthesized dextran in presence of sucrose, it was easily detected by PAS staining. The most active form was found to be of approximately 178 kDa molecular size (Fig. 2.36B, Lane 3). Dextransucrases are reported to exist in either single or multiple molecular forms with varying molecular sizes ranging from 64-313 kDa. This may be due to the proteolytic degradation of high multimeric complex or to dextran contamination during protein preparation (Monsan *et al.*, 2001). There is a good correlation between the type of synthesized dextran and dextransucrase band pattern (Kobyashi and Mastuda, 1986; Bounaix *et al.*, 2010). The multimeric nature of dextransucrase from *L. mesenteroides* NRRL B-1426 resembled in number with dextransucrases from *Leuconostoc citreum* NRRL B-742 (Holt *et al.*, 2001) and *Leuconostoc mesenteroides* NRRL B-1149 (Shukla *et al.*, 2011), but the molecular size of bands were different. However, several Leuconostoc strains also displayed similarity in the molecular sizes of activity bands (Kim and Robyt, 1995; Smith and

Zahnley, 1999). *L. mesenteroides* NRRL B-1426 produced a 240 kDa band of dextransucrase that was similar to that of *L. mesenteroides* NRRL B-1299 (Kim and Robyt, 1995), *L. mesenteroides* NRRL B-1355 and *L. mesenteroides* NRRL B-1501. The 178 kDa band of *L. mesenteroides* NRRL B-1426 dextransucrase was similar to that of *L. mesenteroides* NRRL B-512F (Smith and Zahnley, 1999). A single band was also detected in the raffinose incubated gel (Fig. 2.3.6B, Lane 4) with PAS staining. However, no magenta colour band was detected in the raffinose incubated gel treated with dextranase, confirming that enzyme produced by *L. mesenteroides* NRRL B-1426 was not alternansucrase or fructansucrase (Fig. 2.3.6B, Lane 5). Therefore, the band appeared in the gel incubated in raffinose only might due to the dextran associated with the enzyme.

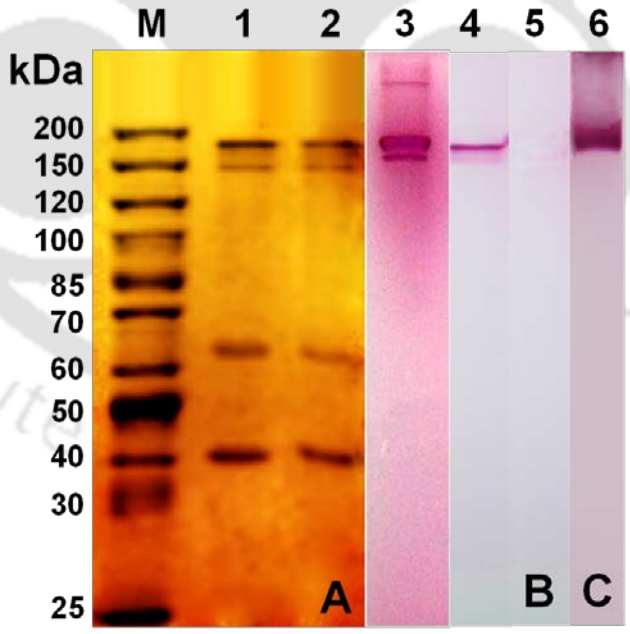

Fig. 2.3.6 PAGE analysis of partially purified dextransucrase fractions by PEG-400 (33%, v/v) (A) Silver staining of SDS-PAGE under non-denaturing and denaturing conditions, Lanes: M-Protein molecular weight marker: 10-200 kDa; 1-non-denaturing; 2-denaturing; (B) PAS staining of dextransucrase under non-denaturing condition; 3-sucrose; 4-raffinose; 5-raffinose treated with dextranase; (C) Native-PAGE analysis of dextransucrase.

The native-PAGE analysis of dextransucrase revealed that it existed in a single molecular form (Fig. 2.3.6C, Lane 6). Similarly, dextransucrase from *L. mesenteroides* NRRL B-640 was reported to exist as a single homogeneous protein in native condition (Purama and Goyal, 2009).

2.3.4 Optimization of reaction conditions for dextransucrase

2.3.4.1 Effect of pH, temperature and ionic strength on dextransucrase activity

The partially purified dextransucrase (10.14 U/mg, 0.76 mg/ml) was used to study the effect of pH, temperature and ionic strength on its activity. The optimum pH of dextransucrase was 5.6 (Fig. 2.3.7A), which is slightly higher than that of the dextransucrases from *L. mesenteroides* NRRL B-512F (Goyal *et al.*, 1995), *L. dextranicum* NRRL B-1146 (Majumder *et al.*, 2008), *L. mesenteroides* NRRL B-640 (Purama and Goyal, 2009), *P. pentosaceus* (Patel *et al.*, 2011), *Weissella confusa* (Shukla and Goyal, 2011) and *Weissella cibaria* (Rao and Goyal, 2013), whose optimum pH was 5.4. The temperature of reaction mixture was varied between 20-45°C. The results showed that 30°C was the optimum temperature for dextransucrase activity (Fig 2.3.7B). The optimum temperature for dextransucrase activity of *Leuconostoc mesenteroides* strains, B-512F (Kobayashi and Mastuda, 1980), B-1355 (Lopez *et al.*, 1993) and IBT-PQ (Chellapandian *et al.*, 1998) was in the range 30-35°C; The ionic strength of buffer has no significant impact on enzyme activity up to 50 mM concentration (Fig 2.3.7C.). The decrease in enzyme activity at 100 mM concentration was only 7.6%. The loss of enzyme activity at 500 mM was 36%. Similar trends were reported for dextransucrases from *L. mesenteroides* NRRL B-640 (Purama and Goyal, 2009), *P. pentosaceus* SPA (Patel *et al.*, 2011) and *W. cibaria* JAG8 (Rao and Goyal, 2013).

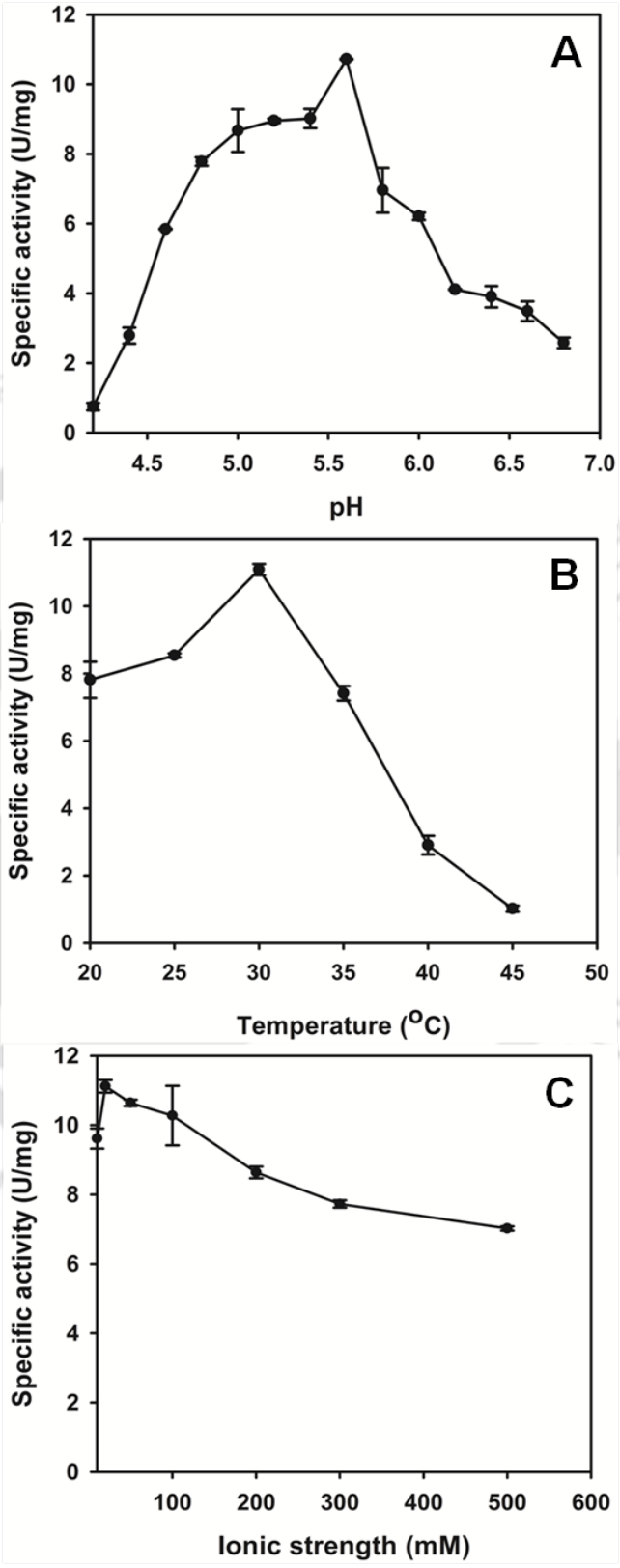

Fig. 2.3.7 Effect of (A) pH, (B) temperature and (C) ionic strength on activity of dextransucrase from *L. mesenteroides* NRRL B-1426.

2.3.4.2 Effect of sucrose concentration on dextransucrase activity

The final sucrose concentration of 146 mM (5%, w/v) was optimum for assay of dextransucrase activity (Fig. 2.3.8). Similar result of 5% sucrose as an optimum concentration was reported for dextransucrase from *L. mesenteroides* NRRL B-640 (Purama and Goyal, 2009) and *P. pentosaceus* (Patel *et al.*, 2011). The data were analysed using GraphPad Prism software and the results showed that dextransucrase followed the classical Michaelis-Menten kinetics with K_m = 12.7±0.51 mM and V_{max} = 13.2±0.67 U/mg, reaching the saturation at 146 mM (5%) (Fig. 2.3.8). Dextransucrase from *L. mesenteroides* NRRL B-1426 exhibited slightly lower K_m value as compared with commercial dextransucrase from *L. mesenteroides* NRRL B-512F, which has K_m = 14.9 mM (Goyal *et al.*, 1995), indicating its higher affinity for sucrose.

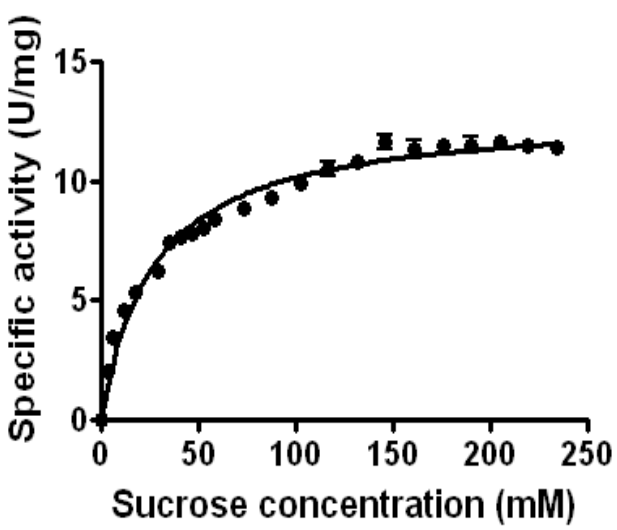

Fig. 2.3.8 Effect of sucrose concentration on dextransucrase activity.

2.3.5 Immobilization of dextransucrase from *L. mesenteroides* NRRL B-1426

2.3.5.1 Optimization of sodium alginate concentration for immobilization

Sodium alginate concentration played an important role in the yields of dextransucrase immobilization. During entrapment method of immobilization, internal diffusion of the enzyme may be a limiting step due to the difference in pore sizes of the beads. In the present study, the maximum immobilization was achieved at 1% (w/v) sodium alginate concentration that displayed 93% yield (Fig. 2.3.9). The immobilization of dextransucrase with alginate might be due to some special structures: a stable complex of dextransucrase and dextran of very high molecular weight, an aggregate of enzyme and dextran or a supramolecular cluster preventing the effusion of the enzyme as described by Reischwitz *et al.* (1995).

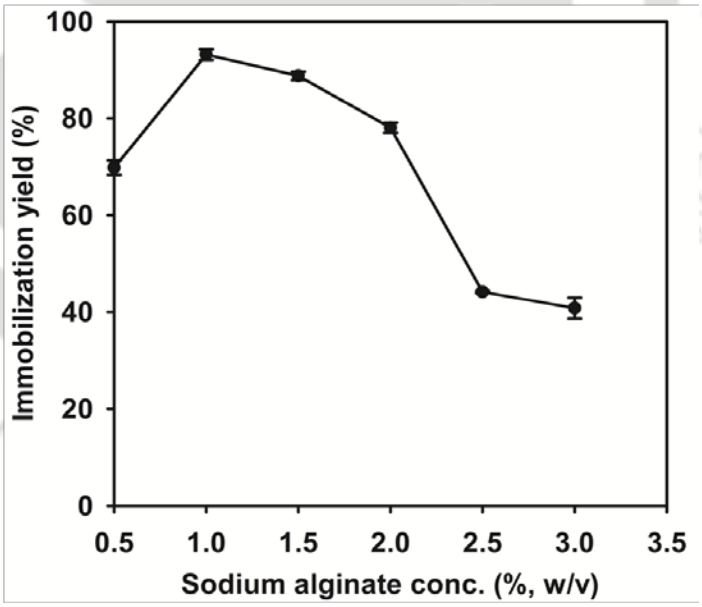

Fig. 2.3.9 Effect of sodium alginate concentration on immobilization yield of dextransucrase from *L. mesenteroides* NRRL B-1426.

The effect of agitation on the activity of immobilized dextransucrase was studied in the range of 50-200 rpm. The immobilized enzyme showed maximum

enzyme activity at 150 rpm (Fig. 2.3.10). The shaking condition might have increased the chances of enzyme substrate interaction, resulting in higher activity.

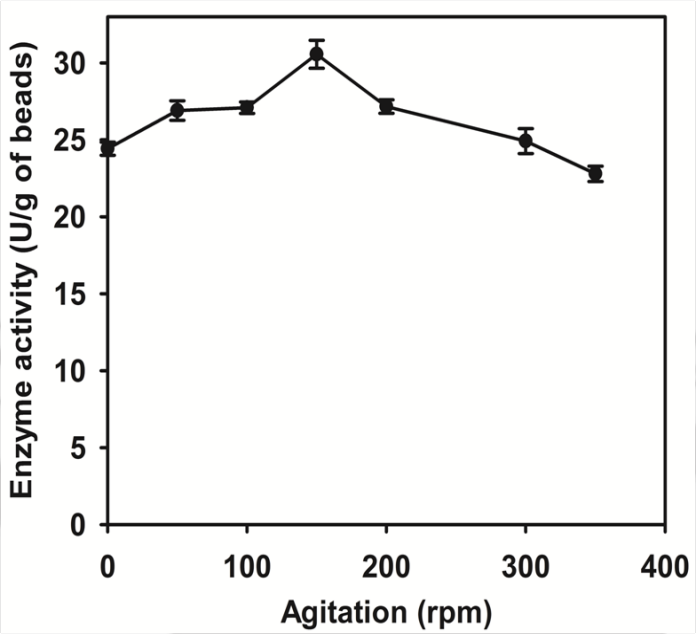

Fig. 2.3.10 Effect of agitation on activity of immobilized dextransucrase from *L. mesenteroides* NRRL B-1426.

2.3.5.2 Operational stability of immobilized enzyme

The possibility of the reuse of immobilized enzyme preparations is important, because this is a key feature for the economic viability of bioprocesses anchored in immobilized enzyme systems (Goradia *et al.*, 2005). Immobilized dextransucrase retained 40% activity after ten consecutive batch reactions at 30°C, each lasting for 15 min (Fig. 2.3.11). The loss of enzyme activity was probably due to the diffusion hindrance of substrate and product as reported by Kubik *et al.* (2004).

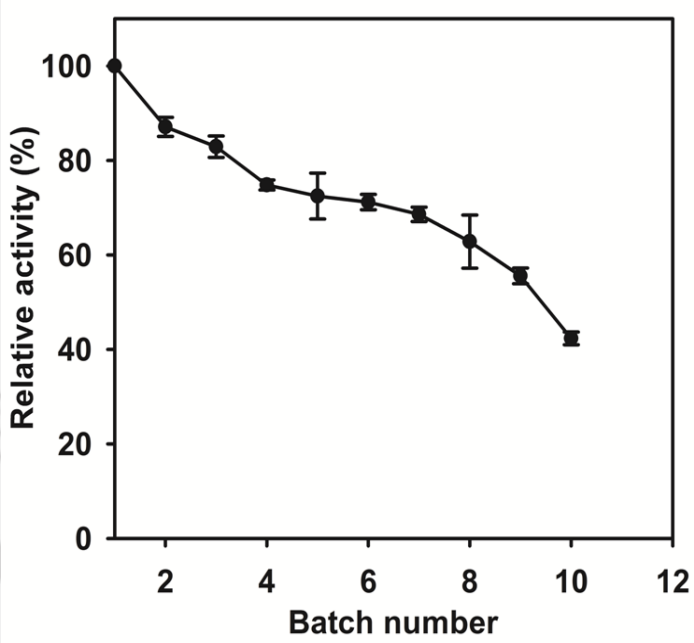

Fig. 2.3.11 Operational stability of immobilized dextransucrase from *L. mesenteroides* NRRL B-1426 in ten consecutive batch reactions.

2.3.5.3 Storage stability of free and immobilized enzyme

The immobilized dextransucrase lost only 1% and 25% activity after 20 and 30 days of storage at 4°C, respectively, whereas, free enzyme lost about 70% and 88% activity after the same period of storage at 4°C (Fig. 2.3.12). The half-life ($t_{1/2}$) of the immobilized dextransucrase was 64.41 d, 7 times higher than that of the free enzyme (9.84 d) (Table 2.3.4). The major drawback to the extensive use of many enzymes compared with chemical catalysts is their relatively low stability in their native state as compared with their immobilized form and their often prohibitive cost (Mateo *et al.*, 2007). The immobilized dextransucrase displayed higher storage stability as compared with that of the free enzyme, which can be harnessed for industrial applications.

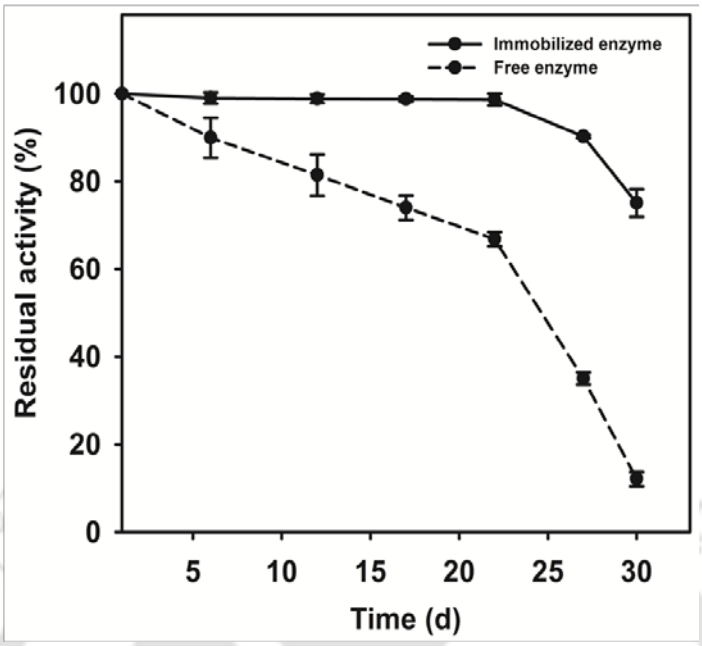

Fig. 2.3.12 Storage stability of free and immobilized dextransucrase from *L. mesenteroides* NRRL B-1426 for 30 days at 4°C.

Table 2.3.4 Half-life of free and immobilized dextransucrase at 4°C.

Dextransucrase	Half life ($t_{1/2}$) (d)
Free	9.84±1.26
Immobilized	64.41±6.69

2.3.5.4 Thermal stability and pH stability of free and immobilized enzyme

Immobilization enhanced the thermal stability of the dextransucrase. The immobilized enzyme lost only 23% and 75% activity at 35°C and 40°C, respectively, whereas, the free enzyme lost about 88% and 93% activity at the same temperatures (Fig. 2.3.13A). Similarly, immobilized enzyme was more stable at lower pH (3.5-4.5) and higher pH (6.5-7.0) ranges as compared with free enzyme. The immobilized enzyme lost 81%, 67%, 36%, 31% and 34% activity at pH 3.5, 4.0, 4.5, 6.5 and 7.0, respectively, whereas, the free enzyme lost 96%, 95%, 77%, 39% and 58% activity at the respective pHs (Fig. 2.3.13B). Immobilization affects the conformational

flexibility of enzyme and increases the enzyme rigidity and stability towards thermal and pH denaturation (Lloret *et al.*, 2012). This can be ascribed to the protective role of the microenvironment surrounding the biocatalyst (Figueira *et al.*, 2011).

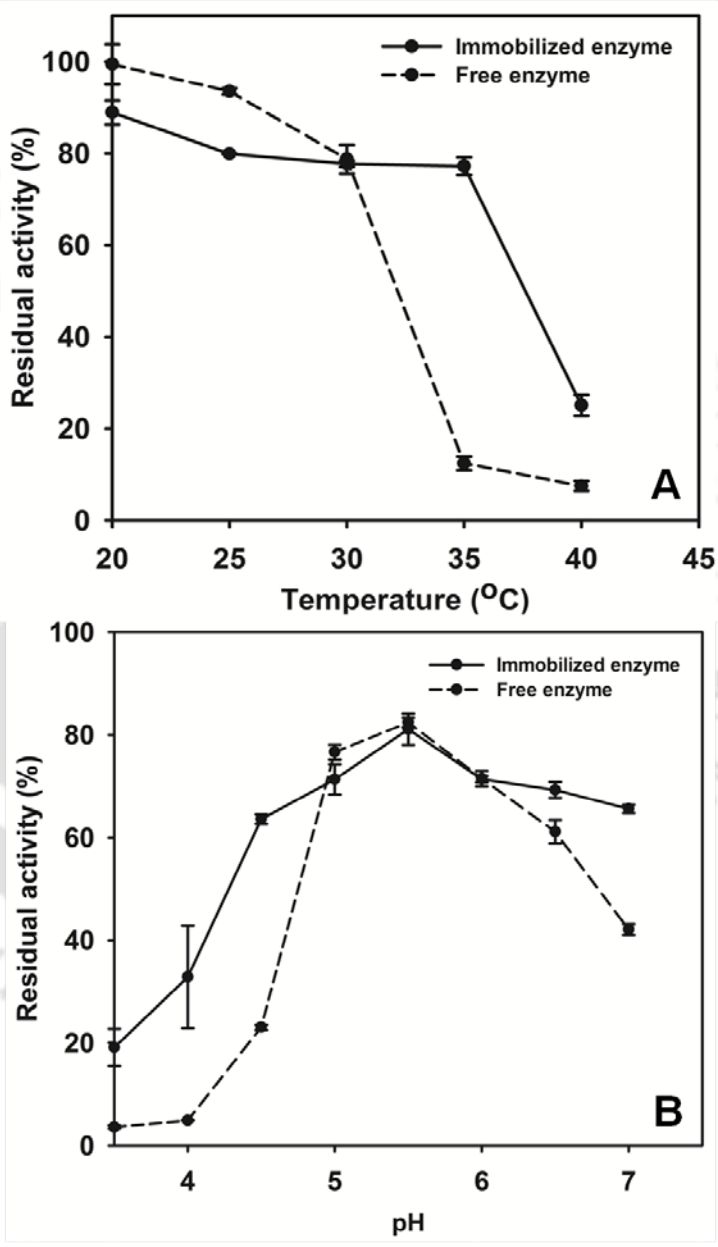

Fig. 2.3.13 Thermal and pH stabilities of free and immobilized dextransucrase from *L. mesenteroides* NRRL B-1426 **(**A) Thermal stability at temperatures ranging from 20 to 40°C at pH 5.6 for 60 min and (B) pH stability ranging from 3.5-7.5 at 30°C for 60 min.

2.3.5.5 Production of oligosaccharides using free and immobilized enzyme

The acceptor reaction of dextransucrase with sucrose as a glucosyl donor and maltose as a glucosyl acceptor, synthesizes isomalto-oligosaccharides (IMOs). IMOs are glucose oligomers with α-(1→6) linkages and a low percentage of α-(1→2) or α-(1→3) or α-(1→4) linkages (Goffin *et al.*, 2011). The acceptor reactions of free and immobilized dextransucrase with maltose were carried out for 48 h.

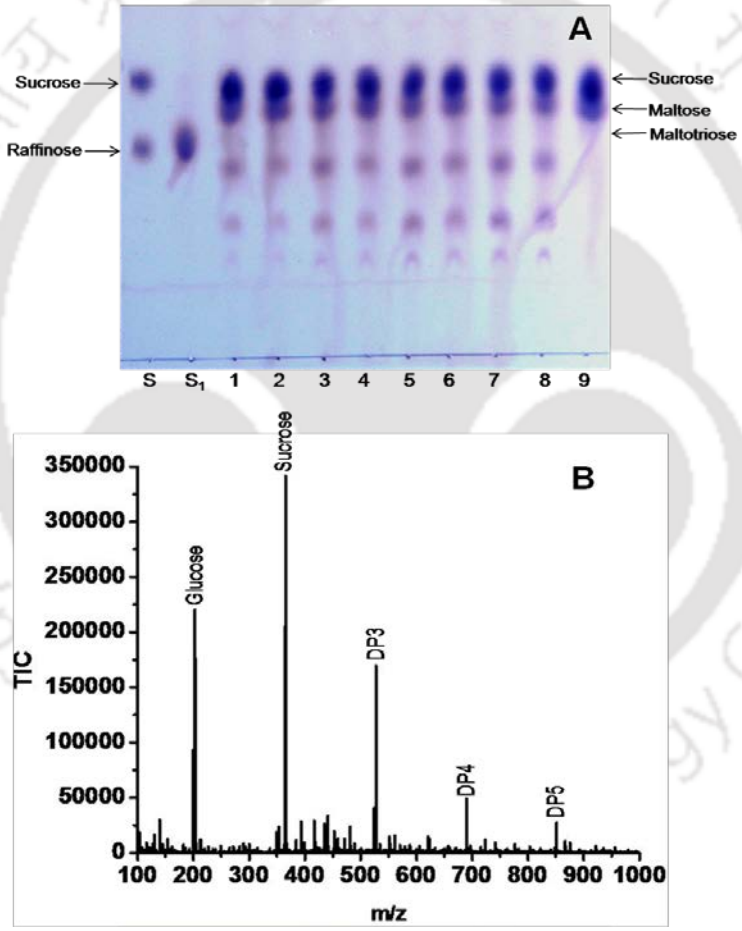

Fig. 2.3.14 (A) TLC of time dependent acceptor reaction of immobilized and free dextransucrase from *L. mesenteroides* NRRL-1426 with maltose. Lane S, sucrose and raffinose; Lanes: S_1-maltotriose; 1-12 h free enzyme; 2-12 h immobilized enzyme; 3-24 h free enzyme; 4-24 h immobilized enzyme; 5-36 h free enzyme; 6-36 h immobilized enzyme; 7-48 h free enzyme; 8-48 h immobilized enzyme; 9-sucrose and maltose. (B) ESI-TOF MS of IMOs produced using immobilized dextransucrase.

The behavior of acceptor reaction products using maltose at different time intervals (12-48 h) with free (Fig. 2.3.14A, Lane-1, 3, 5 and 7) and immobilized (Fig. 2.3.14A, Lane-2, 4, 6 and 8) dextransucrase was similar. Dextransucrase showed the production of IMOs ranging from trisaccharides to pentasaccharides (Fig. 2.3.14A) as confirmed by ESI-TOF MS analysis with m/z 527, 689 and 851, respectively as shown in Fig. 2.3.14B. The ion responses were in sodium adduct [M + Na] form in positive ion mode. The efficiency of IMO synthesis (tri- to pentasaccharides) using immobilized enzyme was 90% as compared with the free enzyme as determined by calculating the relative concentration of IMOs using Scion program from NIH (Fig. 2.3.15). Hence, immobilized dextransucrase by sodium alginate can be used for IMO synthesis at commercial level.

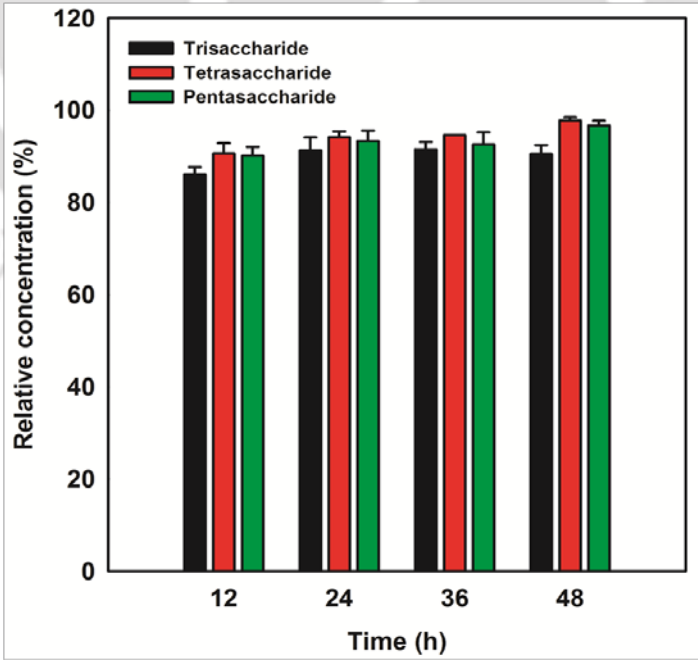

Fig. 2.3.15 Efficiency of IMO production from immobilized dextransucrase from *L. mesenteroides* NRRL B-1426.

2.4 Conclusions

L. mesenteroides NRRL B-1426 was explored for its ability to produce extracellular dextransucrase during fermentation. The bacterium displayed maximum dextransucrase activity of 4.1 U/ml (0.74 U/mg) at 24°C and 120 rpm after 6h. The cell-free supernatant of *L. mesenteroides* NRRL B-1426 containing extracellular dextransucrase (0.74 U/mg, 5.53 mg/ml) was subjected to fractionation with various concentrations of PEG-400 and PEG-1500. The maximum specific activity of 10.14 U/mg and 6.40 U/mg was achieved at 33% (v/v) PEG-400 and 15% (w/v) PEG-1500, respectively. SDS-PAGE analysis of dextransucrase under denaturing, non-denaturing SDS-PAGE showed its multimeric nature with molecular sizes of approximately 240, 178 and 165 kDa. However, a single band of active dextransucrase was found on native-PAGE analysis as well as followed by PAS staining. The zymogram analysis of purified enzyme also confirmed that it was dextransucrase. The purified dextransucrase was optimally active at 30ºC and pH 5.6 and was not affected much by the ionic strength of the buffer. The V_{max} and K_m of purified dextransucrase for sucrose as substrate was 13.2 U/mg and 12.7 mM, respectively, under optimized conditions. Partially purified dextransucrase was successfully immobilized with 1% (w/v) with sodium alginate showing 93% immobilization yield. The immobilized enzyme had 40% activity after 10 batch reactions at 30°C and retained 75% activity after a month of storage at 4°C, which was 6 times more stable than the free enzyme. Immobilized enzyme was more stable at lower (3.5-4.5) and higher (6.5-7.0) pH ranges and higher temperatures (35-40°C) as compared with the free enzyme. Immobilized dextransucrase was used for the production of IMO ranging from trisaccharides to homologous pentasaccharides, showing 90% efficiency when

compared with the free enzyme. Hence, immobilized dextransucrase by sodium alginate can be used for oligosaccharide synthesis at commercial level. Moreover, the linkage selectivity of oligosaccharides could be regulated by varying dextransucrase producing strain and acceptor molecules in the reaction mixture. Therefore, the production of oligosaccharides of commercial interest using *L. mesenteroides* NRRL B-1426 dextransucrase can be studied by manipulating the acceptors.

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Chapter 3

Synthesis, purification, characterization and prebiotic applications of dextran from *Leuconostoc mesenteroides* NRRL B-1426

3.1 Introduction

The progressively increasing demand of natural polymers for various industrial applications has led to the development of microbial exopolysaccharide (EPS) in recent years. Lactic acid bacteria (LAB) are generally regarded as safe (GRAS) (Holzapfel and Schillinger, 1992) and hence, the EPS excreted by LAB can be regarded as safe bio-polymer offering an alternative source of industrial microbial polysaccharides (Wang *et al.*, 2010). Among several EPS, dextran has gained worldwide recognition due to its biodegradability and biocompatibility properties (Siddiqui *et al.*, 2014). Dextran synthesized by *Leuconostoc mesenteroides* was one of the first biopolymers produced at industrial scale and has numerous applications in pharmaceutical, food and biotechnology industries (Sandford, 1979; Naessens *et al.*, 2005; Bounaix *et al.*, 2010). Dextran has also been reported to show prebiotic properties (Olano-Martin *et al.*, 2000), besides viscosifying, texturizing and gelling properties (Purama *et al.*, 2009; Patel *et al.*, 2010). Dextran is a homopolysaccharide of D-glucopyranose, with contiguous α-(1→6) glycosidic linkages in the main chain and a variable degree of α-(1→2), α-(1→3) or α-(1→4) branched linkages (Jeanes *et al.*, 1954; Sidebotham, 1974; Kothari and Goyal, 2013). Dextransucrase (E.C.2.4.1.5)

is the sole industrial enzyme used in the commercial production of dextran (Monchois *et al.*, 1997; Parlak *et al.*, 2014). The degree of branching in dextran depends on the specificity of the particular dextransucrase produced by a specific microbial strain (Jeanes *et al.*, 1954; Robyt, 1995). A survey of dextrans from 96 strains (primarily *L. mesenteroides*) demonstrated that the amount of α-(1→6) linkages in a specific dextran can vary from 50 to 97% of the total glycosidic linkages (Jeanes *et al.*, 1954). Based on such studies, dextrans are divided into three classes. Class 1 dextrans have contiguous α-(1→6)-linked D-glucopyranosyl units in the main chain and α-(1→2), α-(1→3) or α-(1→4) branched linkages. Class 2 dextrans (alternans) contain alternating α-(1→3) and α-(1→6) linkages with α-(1→3) branched linkages, while class 3 dextrans (mutans) have consecutive α-(1→3) linkages and α-(1→6) branched linkages (Robyt, 1986). Various LAB of the genera *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Weissella* are known to synthesize extracellular dextran (Bounaix *et al.*, 2010; Shukla and Goyal, 2011; Ahmed *et al.*, 2012). Most of the studies have been carried out on production and structural analysis of dextrans from *Leuconostoc* species particularly the strains of *L. mesenteroides* (Maina *et al.*, 2008; Sarwat *et al.*, 2008; Majumder *et al.*, 2009; Purama *et al.*, 2009). Commercial production of dextrans by *L. mesenteroides* and their use in the biochemical and pharmaceutical industry has been carried out for more than 50 years (Alsop, 1983; Sutherland, 1996; Ahmed *et al.*, 2012). The most widely used dextran is produced by *L. mesenteroides* NRRL B-512F which contains 95% of linear α-(1→6) linkages in the backbone and ~5% of α-(1→3) branched linkages (Kim and Robyt, 1995; Vettori *et al.*, 2012). Dextrans have variable structural features, exhibiting different properties such as solubility, rheology, and other physico-chemical characteristics (Monchois *et al.*,

1997; Capek *et al.*, 2011).Therefore, the discovery and characterization of new dextrans with potentially different properties resulting from different structures is important for improvement and new applications (Vettori *et al.*, 2012). Keeping the significance and the applications of dextran in mind, the aim of the current study was synthesis, purification and structural characterization of a water-soluble dextran produced from *L. mesenteroides* NRRL B-1426 dextransucrase. The *in vitro* prebiotic potential of dextran in term of gut digestibility and selective stimulation of probiotic bacteria were evaluated. The effects of dextran on human embryonic kidney (HEK-293), human cervical adenocarcinoma (INT-407) and human colon carcinoma (HT-29) cell lines were also studied.

3.2Materials and Methods

3.2.1 Chemicals and reagents

All the media components for maintenance and enzyme production were purchased from Hi-Media Pvt. Ltd., India. All the organic solvents were purchased from Qualigens, India. The enzymes (dextranase from *Penicillium* sp., and α-amylase from human saliva), dextran standards (100 kDa, 500 kDa and 2000 kDa), potassium bromide (KBr), disodium bicinchoninate, Sephacryl S-500HR, Dulbecco's Modified Eagle's Medium (DMEM), minimum essential medium (MEM),Roswell Park Memorial Institute (RPMI 1640) medium, fetal bovine serum (FBS), sodium bicarbonate, sodium pyruvate, antibiotic antimycotic solution, trypsin EDTA and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT)were purchased from Sigma Aldrich, USA. All the chemicals required for reducing sugar estimation, protein estimation and buffer preparation were of high purity.

3.2.2 Microorganisms and culture conditions

Leuconostoc mesenteroides NRRL B-1426 and probiotic bacteria, *Bifidobacterium animalis* sub species *lactis* NRRL B-41405, *Bifidobacterium infantis* NRRL B-41661 and *Lactobacillus acidophilus* NRRL B-4495 were procured from ARS culture collection, National Centre for Agricultural Utilization Research, Peoria, USA. *L. mesenteroides* NRRL B-1426 was grown and maintained in modified MRS medium as described in Chapter 2, Section 2.2.2. The probiotic cultures were grown and maintained in MRS medium, which contained in (g/l): glucose, 20; yeast extract, 5; beef extract, 10; K_2HPO_4, 2; sodium acetate, 5; tri-sodium citrate, 2; Tween 80, 1; $MgSO_4 \cdot 7H_2O$, 0.2 and $MnSO_4 \cdot 4H_2O$, 0.2 (De Man *et al*. 1960) amended with 0.05% cysteine-HCl for maintaining chemically induced anaerobic conditions (Vitali *et al*.,

2012). *Escherichia coli* DH5α was procured from Novagen, India and maintained in Tryptone Glucose Yeast Extract (TGY) medium, which contained in g/l: tryptone, 5; glucose, 1; yeast extract, 3 (Deak *et al*., 1998).

3.2.3 Enzymatic synthesis of dextran

L. mesenteroides NRRL B-1426 dextransucrase was partially purified by 33% (v/v) PEG-400 as described in Chapter 2, Section 2.2.8 was used for dextran synthesis. Dextran was synthesized by incubating 1 ml of partially purified dextransucrase (0.76 mg of protein/ml and specific activity 10.14 U/mg) in 50 ml of 146 mM sucrose in 20 mM sodium acetate, pH 5.6, containing 0.3 mM $CaCl_2$ and 15 mM NaN_3 at 30°C for 24 h. The dextran produced was purified by ethanol precipitation following the method reported by Purama *et al.* (2009). Three volume of cold absolute ethanol (v/v) was added to the reaction mixture and dextran was allowed to precipitate for 12-16 h at 4°C. The reaction mixture was then centrifuged at 13,000*g* and 4°C for 20 min and re-suspended in deionised water. This process was repeated two more times to ensure complete removal of fructose and unreacted sucrose. Finally, the precipitated dextran was dissolved in minimum volume of deionised water and freeze-dried using a lyophilizer (Labconco Corporation, FreeZone 6) for further analysis. The purity of dextran was determined by measuring the protein content, total and reducing sugar content as described in Sections 2.2.6 (Chapter 2), 3.2.4 and 3.2.5 (Chapter 3), respectively.

3.2.4 Estimation of total carbohydrate content

The total carbohydrate concentration was determined by phenol-sulfuric acid method as described by Dubois *et al.* (1956) using a micro-titer plate (Fox and Robyt,

1991). A 25 µl sample containing dextran or inulin was added to 25 µl of 5% (w/v) phenol in a micro-titer plate and mixed by gentle shaking on a vortex for 30 s. Then the plate was placed on an ice bath and 125 µl of concentrated sulfuric acid was added to each well containing the mixture. The plate was again shaken for 30 s to ensure proper mixing. Then the plate was wrapped in cling film and incubated in water bath at 80°C for 30 min. After cooling to room temperature, the absorbance was measured at 490 nm on a micro-titer plate reader (Tecan, Infinite 200 Pro). A standard graph was plotted using glucose in the concentration range, 20-200 µg/ml.

3.2.5 Estimation of reducing sugar content

The reducing sugar content of dextran or inulin was determined by the copper bicinchoninate method (Fox and Robyt, 1991). A 100 µl of sample containing dextran or inulin was added to 100 µl working reagent C in a micro-titer plate and mixed by gentle shaking on a vortex for 30 s. The plate was then wrapped in cling film and incubated in water bath at 80°C for 45 min. After cooling to room temperature, the absorbance was measured at 560 nm on a micro-titer plate reader (Tecan, Infinite 200 Pro). A standard graph was plotted using maltose in the concentration range, 2-20 µg/ml.

3.2.5.1Preparation of reagents for reducing sugar estimation

Reagent A : Disodium bicinchoninate (0.19 g), sodium carbonate (5.43 g) and sodium bicarbonate (2.42 g) were dissolved in water and the volume made up to 100 ml.

Reagent B : Copper sulfate (0.12 g) and L-serine (0.13 g) were dissolved in water and the volume made up to 100 ml.

Reagent C : Working reagent was prepared freshly by mixing equal volumes of reagents A and B.

3.2.6 Physico-chemical characterization of dextran

3.2.6.1 Optical rotation of dextran

The optical rotation of dextran (50 mg/ml) was measured in a polarimeter (Perkin-Elmer, 343) using a sodium D-line (589 nm) at 25ºC.

3.2.6.2 Acid hydrolysis of dextran

The monosaccharide composition of dextran was determined by acid hydrolysis (Yang *et al.*, 2009). Dextran (2 mg/ml) was hydrolysed in 2 M trifluoroacetic acid (TFA) at 100°C for 4 h. After removing TFA, the hydrolysed dextran was filtered with 0.2 µm filter membrane (Pall, USA). The released monosaccharides were analyzed by high pH anion exchange chromatography (HPAEC) system (Dionex Corporation, ICS 3000) equipped with CarboPac PA-20 analytical column (3x150 mm) and CarboPac PA-20 guard column (3x30 mm). An isocratic elution with 0.1 N NaOH at a constant flow rate of 0.5 ml/min was used for chromatographic separation at 30°C. The injection volume was 25 µl. The released monosaccharides were detected with an electrochemical detector (ED 50). Glucose and fructose at a concentration of 50 µg/ml in deionised water were used as standard monosaccharides.

3.2.6.3 Hydrolysis of dextran by dextranase

The susceptibility of dextran towards dextranase from *Penicillium* sp. was analysed. 40 mg of dextran was dissolved in 950 µl of 20 mM sodium acetate buffer (pH 5.5) and then 50 µl of dextranase (final concentration of 1 U/ml) was added for 48 h at 37°C. Aliquots of 100 µl were taken at 0.5, 1, 2, 4, 8, 12, 24 and 48 h and equal volumes (100 µl) of absolute ethanol was added to stop the reaction. The

sample was finally centrifuged at 13,000*g* for 10 min to remove the unhydrolysed dextran. The supernatant was used for the analysis of hydrolysis products by TLC as described in Chapter 2, Section 2.2.3.15.

3.2.6.4 Spectroscopic analysis of dextran

3.2.6.4.1 Fourier transform infrared (FTIR) spectroscopy

The FTIR spectrum of dextran was recorded by making a KBr pellet using a spectrometer (Perkin Elmer, Spectrum One) for the identification of functional groups and glycosidic linkages. The dextran (2 mg) was finely grinded with 400 mg of KBr powder and pressed into pellets of thickness 0.5 to 1 mm using hydraulic press. The pellet was then placed in a transmission holder and scanned in the region of 4000 cm^{-1} to 450 cm^{-1} with 20 scans per min.

3.2.6.4.2 ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy

^{1}H and ^{13}C NMR spectra of dextran were recorded with a 400 MHz NMR spectrometer (Varian, AS400) equipped with VnmrX for Sun Microsystems v.6.1 software. The operating frequencies were 400 MHz for ^{1}H and 100 MHz for ^{13}CNMR to determine the type of glycosidic linkage in dextran. The samples were dissolved in D_2O (99.96%) at concentrations of 10 mg/ml (for ^{1}HNMR) and 30 mg/ml (for ^{13}C NMR).

3.2.6.5 Average molecular mass determination of dextran

The average molecular mass of dextran from *L. mesenteroides* NRRL B-1426 was determined by gel permeation chromatography (GPC) on an AKTA purifier (GE Healthcare, 100 Plus) using an XK16/40 column (GE Healthcare) packed with

Sephacryl S-500HR. The dextran was eluted using deionised water at a constant flow rate of 1 ml/min. Dextran 100, 500 and 2000 kDa were used as standards.

The number average molecular mass (MM_n) of dextran was also determined by the bicinconinate method as described by Jane and Robyt (1984) following the equations (1) and (2).

$$MM_n = [(DP_n) \times 162] + 18 \qquad \text{Eq. 1}$$

Where, DP_n is the number average degree of polymerization.

$$DP_n = \frac{\text{Total carbohydrate content in µg of D-glucose}}{\text{Reducing value in µg of D-maltose}} \times 1.9 \qquad \text{Eq. 2}$$

The total carbohydrate and reducing sugar contents were measured using phenol sulfuric acid (Section 3.2.4) and copper bicinchoninate (Section 3.2.5) methods,respectively.

3.2.6.6 Scanning electron microscopy of dextran

The surface morphology of dextran was studied by field emission scanning electron microscopy (FE-SEM) (Carl Zeiss, Σigma). Dextran (1 mg) was attached to the SEM stub with double-sided carbon tape and sputter-coated with ~10 nm gold films using a sputter coater (Quorum Technologies, SC7620 Polaron). The surface of the sample was analyzed in FE-SEM operated at 2.5 kV.

3.2.6.7 Solubility and water holding capacity of dextran

The solubility of dextran in water was determined by the method described by Chang and Cho (1997). 50 mg of dextran was dissolved in 1 ml of deionised water with continuous stirring at 25ºC for 24 h. The dextran suspension was then

centrifuged at 5,000*g* and 25ºC for 15 min. Three volume of ethanol (95%) was added to 0.2 ml collected supernatant and centrifuged again at 10,000*g* and 4°C for 20 min. The precipitate obtained was vacuum dried at 80ºC and difference in weight was measured. The solubility of dextran was calculated as follows:

$$\text{Solubility (\%)} = \frac{\text{Total dextran concentration in supernatant}}{\text{Initial dry weight of dextran}} \times 100$$

Water holding capacity (WHC) of dextran was determined by the method described by Ahmed *et al.* (2013). 0.2 g of dextran was dissolved in 10 ml of deionised water and was centrifuged at 13,000*g* and 4ºC for 30 min. The unbound water not held by dextran was discarded. The precipitated dextran was placed on pre weighed filter paper for complete removal of water. The wet weight of precipitated dextran was determined. The percentage of WHC was calculated as follows:

$$\text{WHC (\%)} = \frac{\text{Wet weight of dextran after water absorption}}{\text{Initial dry weight of dextran}} \times 100$$

3.2.6.8 Viscosity and rheology of dextran

Dextran solution (0.5%, w/v) was used for viscosity measurement at 25ºC using a digital viscometer (Brookfield, DV-II) with a speed of 100 rpm. The rheological measurement (viscosity versus shear rate) of aqueous solution of dextran (0.5%, w/v) was performed at 25°C in a rheometer (Thermo Electron, Haake ReoStress 1) interfaced with a HAAKE RheoWin 323 software. The applied shear rate was in the range of 10-340 s^{-1}.

3.2.6.9 Thermo-gravimetric analysis of dextran

The measurement of thermal stability and mass changes of dextran was carried out using a differential scanning calorimeter/thermo-gravimetric analyzer (DSC/TGA) (STA 449 F3 *Jupiter*, NETZSCH) with Proteus software. 5 mg of dextran was placed on alumina (Al_2O_3) crucible and heated at a constant rate 10°C/min over a temperature range 20-600°C in nitrogen atmosphere (60 ml/min).

3.2.7 *In vitro* application analysis of dextran as prebiotic

3.2.7.1 Effect of simulated human gastric juice on digestibility of dextran

The dextran from *L. mesenteroides* NRRL B-1426 and inulin (commercial prebiotic) were separately dissolved in deionised water to give 1% (w/v) solutions. The hydrolysing effect by simulated human gastric juice was tested according to the method of Korakli *et al*. (2002). The simulated human gastric juice was prepared using hydrochloric acid buffer, which contained in g/l: NaCl, 8; KCl, 0.2; $Na_2HPO_4 \cdot 2H_2O$, 8.25; NaH_2PO_4, 14.35; $CaCl_2 \cdot 2H_2O$, 0.1 and $MgCl_2 \cdot 6H_2O$, 0.18. The pH of the buffer was adjusted to 1, 2, 3 and 4 by 5 M HCl. Dextran and inulin samples (1.0 ml, 1%, w/v) were mixed with 1.0 ml of simulated human gastric juice at the four pH levels separately and the reaction mixtures were incubated at 37°C for 6 h. Aliquots (100 µl) of the reaction mixture were collected from each treatment at 0, 0.5, 1, 2, 4 and 6 h intervals. The total and reducing sugar contents before and after digestion were determined by phenol sulfuric acid and copper bicinchoninate methods, as described in the Sections 3.2.4 and 3.2.5, respectively. 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.

3.2.7.2 Effect of human α-amylase on digestibility of dextran

Digestibility of dextran from *L. mesenteroides* NRRL B-1426 and inulin by human α-amylase was ascertained by following the method of Wichienchot *et al*. (2010). The dextran and inulin were separately dissolved in deionised water to give 1% (w/v) solution and tested for digestibility by α-amylase. Solution of α-amylase (2 U/ml) was prepared in 20 mM sodium phosphate buffer containing 6.7 mM sodium chloride with varying pH 5, 6, 7 and 8. Dextran and inulin samples (1.0 ml, 1%, w/v) were mixed with 1.0 ml of α-amylase solution at the four pH levels separately and the reaction mixtures were incubated at 37°C for 6 h. Aliquots (100 µl) of the reaction mixture were collected from each treatment at 0, 0.5, 1, 2, 4 and 6 h intervals to calculate the percent hydrolysis as described in the Section 3.2.7.1.

3.2.7.3 Effect of dextran on the growth of human gut bacteria

The growth stimulatory effects of dextran and inulin on *B. animalis* sub species *lactis, B. infantis, L. acidophilus* and *E. coli* DH5α were evaluated. The log phase cultures (1%, v/v) of *Bifidobacteria* and *Lactobacillus* were inoculated in 10 ml MRS medium whereas *E. coli* DH5α into 10 ml TGY medium contained in a test tube (25x150 mm) as described in Section 3.2.2. Chemically induced anaerobic conditions were obtained with 0.05% cysteine-HCl (filter sterilized by 0.2 µm membrane, Pall, USA), which acts as oxygen scavenger and maintains low redox potential (Vitali *et al*., 2012).The cultures were treated with 1% (w/v) of dextran from *L. mesenteroides* NRRL B-1426 and commercial inulin as carbon source. Dextran and inulin were

separately autoclaved and was added to the media. The respective growth media (MRS and TGY) without any carbon source were maintained as negative controls. The bacterial cultures were incubated at 37°C under anaerobic conditions in anaero bags (Hi-Media Pvt. Ltd., India). The bacterial growth was monitored by measuring absorbance at 600 nm (A_{600}) using UV-visible spectrophotometer (Varian, Cary 100). The bacterial growth was correlated with the pH changes and carbohydrate utilization in the medium. The pH was measured using a digital pH meter (Sartorious, PP-20). The carbohydrate utilized was estimated by phenol sulfuric acid method as described in the Section 3.2.4 and calculated by the following equation:

$$\text{Carbohydrate utilization (\%)} = \frac{\text{Residual carbohydrate content}}{\text{Initial carbohydrate content}} \text{x100}$$

Where, initial carbohydrate content was 10 mg/ml for both dextran and inulin and it was considered as 100%.

3.2.8 *In vitro* analysis of effect of dextran on mammalian cells

3.2.8.1 Culturing and maintenance of mammalian cells

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 Centre for Cell Sciences (NCCS), Pune, India. HEK-293 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Dulbecco, 1976) containing 2.0 mM L-glutamine and supplemented with 1.5 g/l sodium bicarbonate, 1.0 mM sodium pyruvate, 10% fetal bovine serum (FBS) and 1% antibiotic antimycotic solution (penicillin 10,000 U, streptomycin 10 mg and amphotericin B 25 µg). The INT-407 cells were cultured in minimum essential medium (MEM) (Darnell

et al., 1970) containing 2.0 mM L-glutamine and supplemented with 2 g/l sodium bicarbonate, 10% FBS and 1% antibiotic antimycotic solution. HT-29 cells were cultured in Roswell Park Memorial Institute (RPMI 1640) (Moore *et al.*, 1967) medium containing 2.0 mM L-glutamine and supplemented with 2 g/l sodium bicarbonate, 10% FBS and 1% antibiotic antimycotic solution. The serum added medium, designated as complete medium was used for maintaining and culturing the cell lines.These cells were grown in T-25 and T-75 flasks (BD Biosciences, USA) at 37ºC in a humidified incubator maintained at 5% CO_2 saturation.The medium was changed at regular interval of 36 h and cell splitting was done after 90% confluency by 1x Trypsin EDTA solution (0.25% trypsin and 0.2% EDTA) as described in the next Section 3.2.8.2. All the three cell lines were stored at -80ºC in freezing medium (Invitrogen, USA) for long term storage.

3.2.8.2 Sub-culturing of cells

The used cell culture media from the culture vessels was carefully removed and discarded. The adhered cells (HEK-293, INT-407 or HT-29) were then washed with 1x PBS (pH 7.4) gently by adding it to the side of the vessel opposite the attached cell layer to avoid disturbing the cell layer. The PBS was discarded and then the cells were treated with pre-warmed 1x Trypsin EDTA solution for 3-4 min at 37°C and observed under microscope (Nikon Instruments Inc., TS100-F) at 40x magnification for detachment. After ≥ 90% of the cells have detached, 1 ml of complete medium was added to the detached cells and centrifuged at 200*g* at 4°C for 5 min. The supernatant was discarded and the cells were re-suspended in 5 ml of complete medium. A split ratio of 1:10 (1 ml of above cell suspension in 9 ml complete medium) or a seeding density of $4x10^4$ viable cells/cm^2 was used for sub-culturing cells.

3.2.8.3 Cell viability assay

The effect of dextran on viability of HEK-293, INT-407 and HT-29 cells were studied by a colorimetric 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) cell viability assay (Alley *et al.*, 1988). The evaluation of the metabolic viability by MTT assay is based on the ability of the mitochondria succinate tetrazolium reductase system to convert the yellow compound MTT to a purple formazan dye. The amount of dye produced is proportional to the number of viable cells. The purified dextran powder was dissolved in sterile 1x PBS to prepare a stock solution of 5 mg/ml. The effect of dextran on mammalian cells was tested at a concentration range, 50-1000 µg/ml for 36 h. A 200 µl of respective medium for HEK-293, INT-407 and HT-29 containing $1x10^4$ cells/well were seeded separately into 96 well micro-titre plates. The plates were incubated at 37ºC for 12 h for cell adherence in CO_2 incubator (5%). After the incubation, the medium was completely removed and the adhered cells were exposed to dextran of varying concentrations in complete growth media for respective cells for 12, 24 and 36 h. After 12 h intervals, the media containing different concentrations of dextran from each well were removed gently, cells were washed with 1x PBS (pH 7.4) and 100 µl of MTT solution (0.5 mg/ml in PBS) was added into each well and incubated for 4 h at 37°C. After 4 h, the MTT solution of each well was replaced with equal volume of dimethyl sulfoxide (DMSO). The plate was then vortexed gently to dissolve the precipitate completely and the absorbance was detected at 570 nm with reference wavelength of 660 nm using a microplate reader (Tecan, Infinite 200 Pro). The viability (%) was calculated as described by Patel *et al.* (2010).

$$\text{Cell viability (\%)} = (N_t/N_c)x100$$

Where, N_t is absorbance of cells treated with dextran and N_c is the absorbance of untreated cells.

3.2.9 Statistical analysis

The prebiotic application 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% ($p \leq 0.05$).

3.3 Results and Discussion

3.3.1 Physico-chemical characterization of dextran from *L. mesenteroides* NRRL B-1426

3.3.1.1 Purification of dextran

The enzymatically synthesized dextran from *L. mesenteroides* NRRL B-1426 dextransucrase was purified by precipitating reaction mixture with absolute ethanol as described in Section 3.2.3. The purified dextran (20 mg/ml) showed 0.06% protein content (12 µg/ml) and 0.2% reducing sugar content (40 µg/ml). The low protein content may be due to the dextransucrase associated with dextran (Majumder and Goyal, 2009).

3.3.1.2 Optical rotation of dextran

The dextran synthesized by *L. mesenteroides* NRRL B-1426 dextransucrase showed an optical rotation

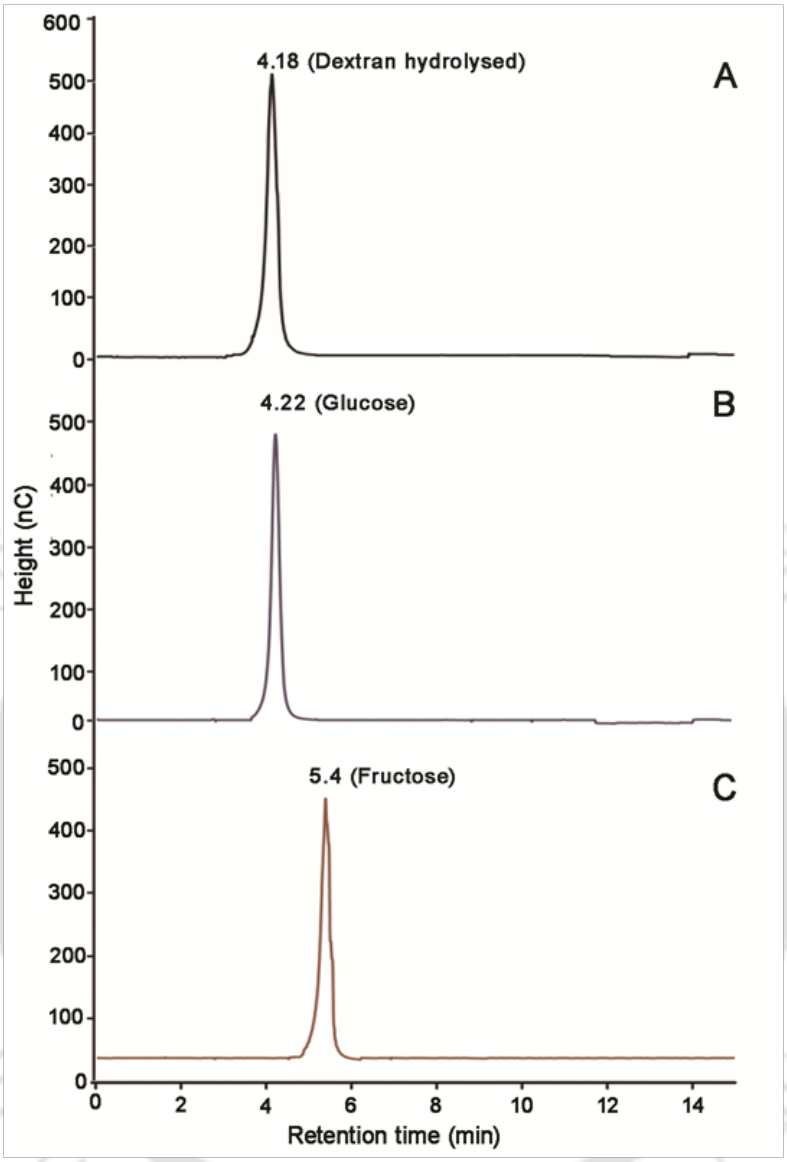

Fig. 3.3.1 Monosaccharide analysis of dextran from *L. mesenteroides* NRRL B-1426 by HPAEC.

3.3.1.4 Hydrolysis of dextran by dextranase

The hydrolysis of dextran by dextranase requires a minimum of six or seven non-substituted contiguously linked α-(1→6) glucose residues (Boune *et al.*, 1962; Vettori *et al.*, 2012). So, enzymatic hydrolysis of dextran with dextranase from *Penicillium* sp. was carried out to ascertain the presence of predominantly α-(1→6)

glucopyranosidic linkages within the main chain. The major products after 48 h of hydrolysis of dextran with dextranase were isomaltose and isomaltotriose (Fig. 3.3.2, lane 48). These were derived from the linear regions of α-(1→6) backbone dextran chains. However, presence of higher oligosaccharides (DP> 4) produced indicated the presence of significant branching in dextran from *L. mesenteroides* NRRL B-1426. The type of branching in dextran was further analyzed by NMR spectroscopy.

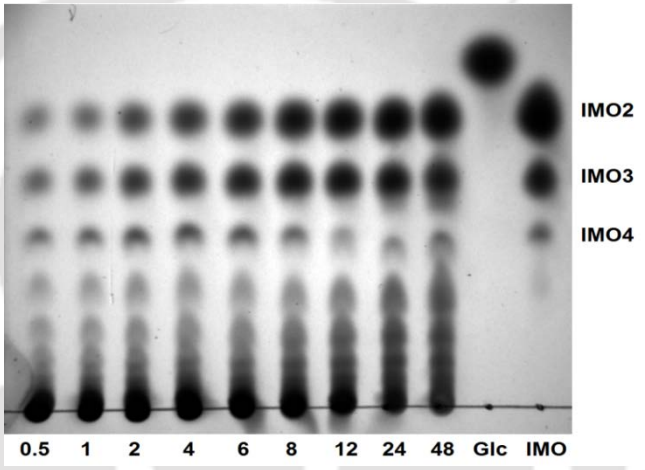

Fig. 3.3.2 TLC analysis of hydrolysis of dextran from *L. mesenteroides* NRRL B-1426 using *Penicillium* sp. dextranase (1 U/ml) at indicated time intervals from 0.5 to 48 h at 37°C. Lanes: Glc-Glucose; IMO-Standards of isomalto-oligosaccharides, IMO2-Isomaltose, IMO3-Isomaltotriose and IMO4-Isomaltotetraose.

3.3.1.5 FTIR spectroscopy of dextran

The type of linkages and the functional groups of the dextran from *L. mesenteroides* NRRL B-1426 were characterized by FTIR spectroscopic analysis (Fig. 3.3.3). The fingerprint region for polysaccharide is considered to be from 1200-950 cm^{-1} for its possible identification based on the position and intensity of the bands (Cerna *et al.*, 2003). The dextran showed a peak at 1018 cm^{-1}, which is characteristic for α-(1→6) linkages as reported by Shingel (2002). The presence of α-glycosidic bond was confirmed by the presence of a peak at 916 cm^{-1}. The band at 1153 cm^{-1} was

due to covalent vibrations of the C–O–C bond and glycosidic bridge. There was no characteristic peak at 890 cm^{-1}, indicating the absence of β-configuration. The hydroxyl and the C-H stretching vibrations corresponded to the bands in the region of 3440 and 2922 cm^{-1}, respectively. The band in the region of 1651 cm^{-1} was due to bound water. Similar results were reported by Purama *et al.* (2009) for dextran from *L. mesenteroides* NRRL B-640. Thus, the FTIR spectrum revealed that the dextran from *L. mesenteroides* NRRL B-1426 contained α-(1→6) linkages, which was further confirmed through ^{1}H and ^{13}C NMR analyses as described in Sections 3.3.1.6 and 3.3.1.7, respectively.

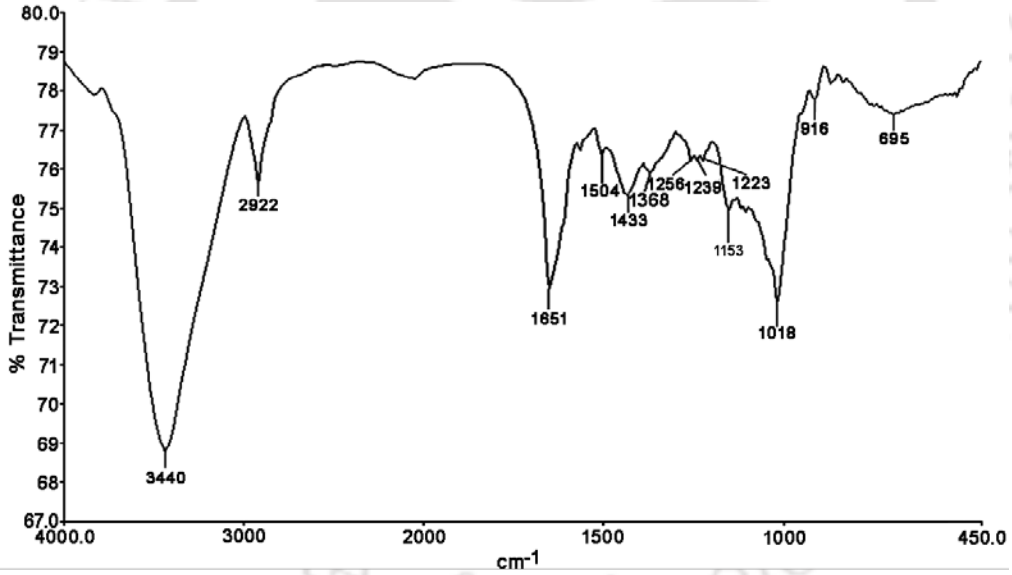

Fig. 3.3.3 FTIR spectrum of dextran from *L. mesenteroides* NRRL B-1426.

3.3.1.6 ^{1}H NMR spectroscopy of dextran

The ^{1}H NMR spectrum provides the convincing facts for the glycosidic linkages of dextran (Vettori *et al.*, 2012). Sidebotham (1974) reported that various dextrans have ^{1}H NMR spectral resonances (C-2, C-3, C-4, C-5, and C-6) in the 3-4 ppm region and the hemiacetal C-1 resonance in the 4-6 ppm region. The ^{1}H NMR

spectrum of dextran from *L. mesenteroides* NRRL B-1426 and their individual assignments of the signals are shown in Fig. 3.3.4 and Table 3.3.1, respectively. The dextran showed ^{1}H chemical shifts of anomeric signals at 4.99 ppm and a low intensity peak at 5.33 ppm, which are due to α-(1→6) and α-(1→3) glycosidic linkages, respectively (Fig. 3.3.4 and Table 3.3.1). Similar results were obtained for dextran from *L. mesenteroides* NRRL B-1355 (Seymour *et al.*, 1979) and *Lactobacillus reuteri* (van Leeuwen *et al.*, 2008). Other protons appear as a complex series of overlapping signals ranging from 3.4 to 4 ppm. The peak recorded at 4.8 ppm in the spectrum was from D_2O (Fig. 3.3.4).

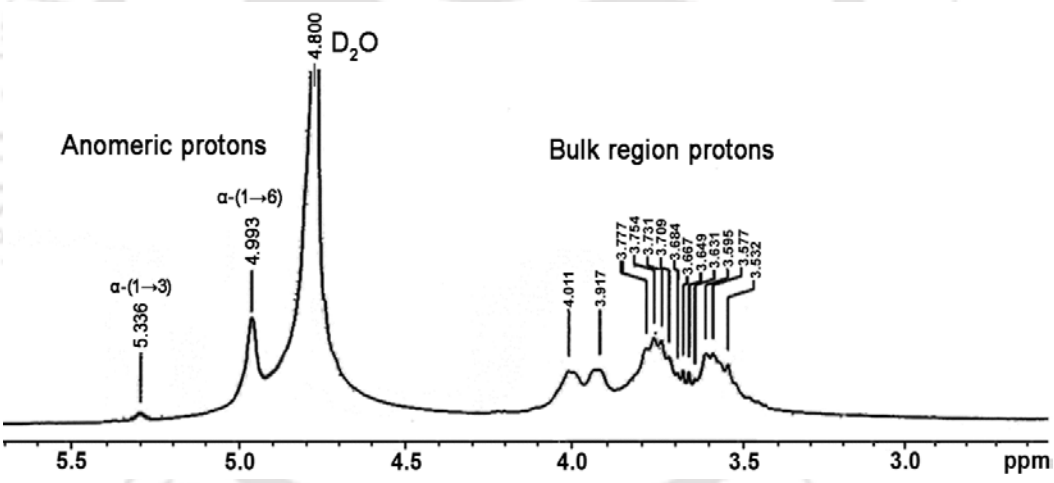

Fig. 3.3.4 ^{1}H NMR spectrum of dextran from *L. mesenteroides* NRRL B-1426.

Based on integration analysis of the ^{1}H NMR signal, it was inferred that the dextran from *L. mesenteroides* NRRL B-1426 contained ~85.5% α-(1→6) linked D-glucosyl linkages and ~14.5% α-(1→3) linked D-glucosyl linkages. There are 145 branched linkages for every 1000 glucose units of glucan. The presence of no other signal in the region 4.9-5.3 ppm, except at 5.33 ppm in the ^{1}H-NMR spectrum,

indicated the absence of any branching other than α-(1→3) in the dextran from *L. mesenteroides* NRRL B-1426.

Table 3.3.1 Assignment of chemical shifts in ^{1}H NMR (ppm) spectrum of dextran from *L. mesenteroides* NRRL B-1426.

Atoms	*L. mesenteroides* NRRL B-1426	*L. mesenteroides* NRRL B-512F*
[a]H-1	4.99	4.99
H-2	3.57	3.6
H-3	3.72	3.76
H-4	3.53	3.52
H-5	3.91	3.88-4.04
H-6	4.01	3.88-4.04
[b]H-1	5.33	5.32

**Seymour et al., 1979.*
[a]H-1 Anomeric proton corresponding to α-(1→6) linkage.
[b]H-1 Anomeric proton corresponding to α-(1→3) linkage.

3.3.1.7 ^{13}C NMR spectroscopy of dextran

Dextrans display ^{13}C NMR anomeric signals downfield at ~90 ppm, C-2, C-3, C-4, and C-5 appear in the 70-85 ppm range, and C-6 is normally upfield at ~60 ppm (Ahmed *et al.*, 2012). The ^{13}C NMR spectrum of *L. mesenteroides* NRRL B-1426 dextran showed six prominent resonances as shown in Fig. 3.3.5 and Table 3.3.2, which are characteristics of linear dextrans as reported by Seymour *et al.* (1979). Apart from these six peaks, which correlate with six signals of linear dextran, the spectrum also contains minor peaks (60.5, 99.5 and 80.8 ppm) indicative of branching. The signal at 60.5 ppm assigned to the C-6 atom on the non-reducing glucose units is of considerable interest as it corresponds to branching (Colson *et al.*, 1974). The resonance peaks at 99.5 and 80.8 ppm indicated branched linkage at C-3, which was also supported by resonance at 5.33 ppm in the ^{1}H NMR spectrum. Similar results of branching of α-(1→3) linkages were also reported for dextran from *L. mesenteroides* NRRL B-512F at 99.5 ppm by Colson *et al.* (1974) and for dextran

from *L. citreum* NRRL B-742 at 81.66 ppm by Seymour and Knapp (1980). The ^{1}H and ^{13}C NMR spectral analyses confirmed that the *L. mesenteroides* NRRL B-1426 dextran is composed of ~85.5% α-(1→6) linear and ~14.5% α-(1→3) branched linkages. Several studies have previously reported that the content of α-(1→6) glycosidic linkages in dextrans originating from LAB ranges from 50 to 97% (Jeanes *et al.*, 1954; Naessens *et al.*, 2005; Maina *et al.*, 2011; Ahmed *et al.*, 2012) due to the differences in the type of dextransucrases (Bejar *et al.*, 2013). Dextran containing α-(1→3) branched linkages can escape digestion in the mouth and stomach and reach the lower gastrointestinal tract for utilization by probiotic bacteria and thus can act as prebiotic.

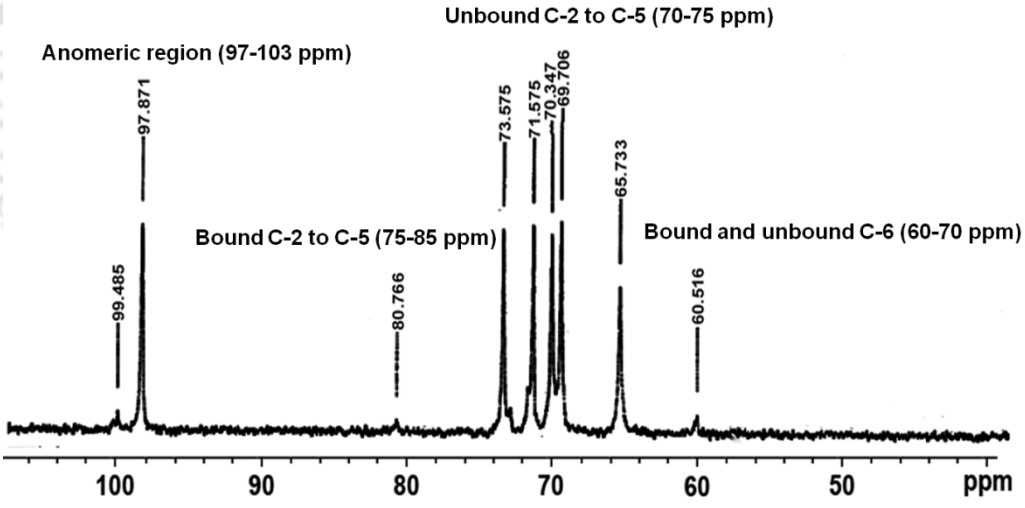

Fig. 3.3.5 ^{13}C NMR spectrum of dextran from *L. mesenteroides* NRRL B-1426.

Table 3.3.2 Assignment of individual signals in ^{13}C NMR spectrum of dextran from *L. mesenteroides* NRRL B-1426.

Atoms	*L. mesenteroides* NRRL B-1426	*L. mesenteroides* NRRL B-512F*
C-1	97.87	97.8
C-2	71.57	71.5
C-3	73.57	73.5
C-4	69.70	70.0
C-5	70.35	70.3
C-6	65.73	66.1
C-6’	60.51	61.0

**Seymour et al., 1979.*

3.3.1.8 Molecular mass determination of dextran

The applications of dextran in various foods and medicines depend on its molecular mass. The average molecular mass of the dextran from *L. mesenteroides* NRRL B-1426 was >2,000 kDa by GPC (Fig. 3.3.6). The average number molecular mass as determined by bicinchoninate method was found to be 2,910 kDa with DP_n of 17,963. Irague *et al.* (2012) also reported significantly high molar mass (~1,00,000 kDa) for enzymatically synthesized dextran by dextransucrase from *L. mesenteroides* NRRL B-512F mutant. The high molecular mass dextran can be used in oil drilling industries as dextran-aldehyde complex or as protective colloid during mud drilling operations (Aman *et al.*, 2012).

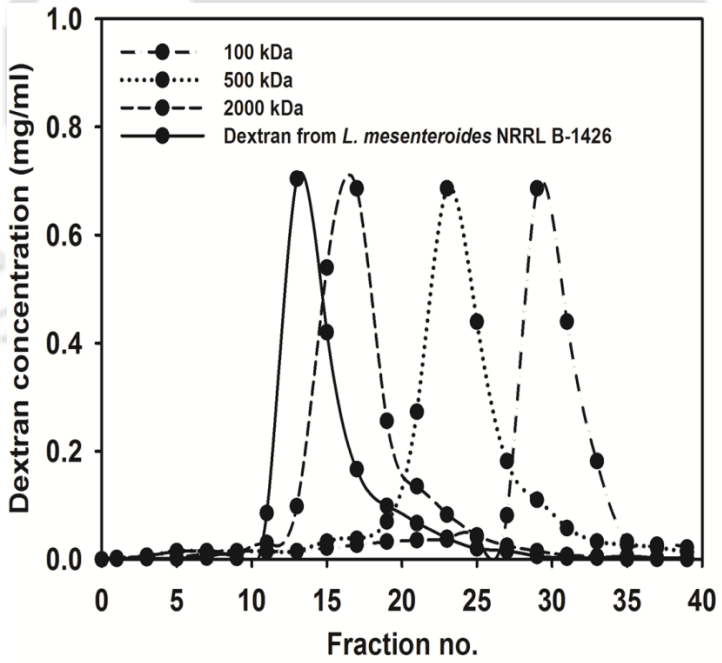

Fig. 3.3.6 Gel permeation chromatography of dextran from *L. mesenteroides* NRRL B-1426 using Sephacryl S-500HR.

Moreover, dextrans of high molecular mass, 1000-2000 kDa have been approved by the European Union as food ingredients in bakery products (Naessens *et*

al., 2005). The required molecular mass ranging from 2000 to 4000 kDa of dextrans for bakery products have been reported by Katina *et al.* (2009). Relatively high molecular mass dextrans have also been cross-linked to varying extents to give different size-exclusion chromatography materials (Sephadex products) that have been used as gel filtration materials for over 50 years (Falconer *et al.*, 2011). The key utility of dextran in different industries depends on its molecular mass (Aman *et al.*, 2012). Therefore, the dextran synthesized by *L. mesenteroides* NRRL B-1426 dextransucrase can be tailored to meet the requirement for industrial application.

3.3.1.9 Scanning electron microscopy of dextran

Scanning electron microscopy is a powerful tool to study the surface morphology of polymers, which could be used to elucidate their physical properties (Wang *et al.*, 2010). The FE-SEM analysis of dextran from *L. mesenteroides* NRRL B-1426 revealed the porous structure present all over the surface (Fig. 3.3.7).

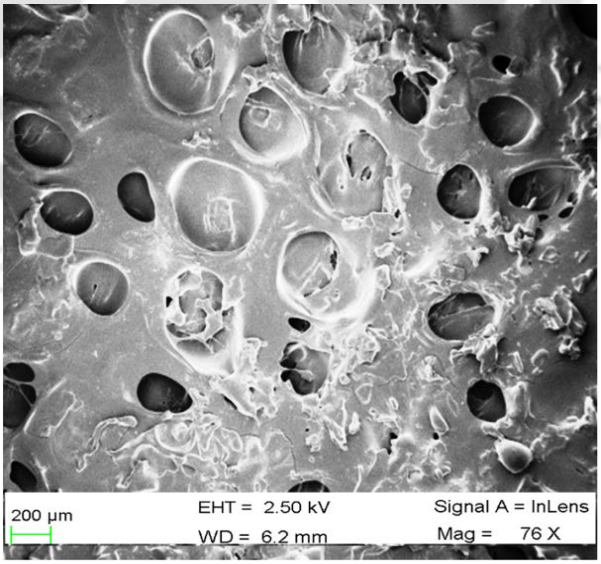

Fig. 3.3.7 Field emission scanning electron microscopic (FE-SEM) image of dextran showing surface morphology.

Similar porous structures were reported for dextrans from *L. dextranicum* NRRL B-1146 (Majumder and Goyal, 2009) and *L. mesenteroides* NRRL B-1149 (Shukla *et al.*, 2011). The porous structure of dextran could impart a higher water holding capacity, which is a desirable characteristic for texturing agent in the food industry.

3.3.1.10 Viscosity and rheology of dextran

The viscosity measurements and rheology were used to find the behaviour of dextran in solution and to know its conformation. The viscosity of dextran (0.5%, w/v) from *L. mesenteroides* NRRL B-1426 was 5.9 cp. The high viscosity of dextran suggested its flexible and extended nature with high molecular mass. The viscous behavior of polymers is dependent on its structure and mass (Freitas *et al.*, 2009). It has been also reported that a linear relationship exists between the molecular mass of the polysaccharide and the viscosity, in which the higher molecular mass of the polymer leads to higher viscosity (Prasanna *et al.*, 2012).

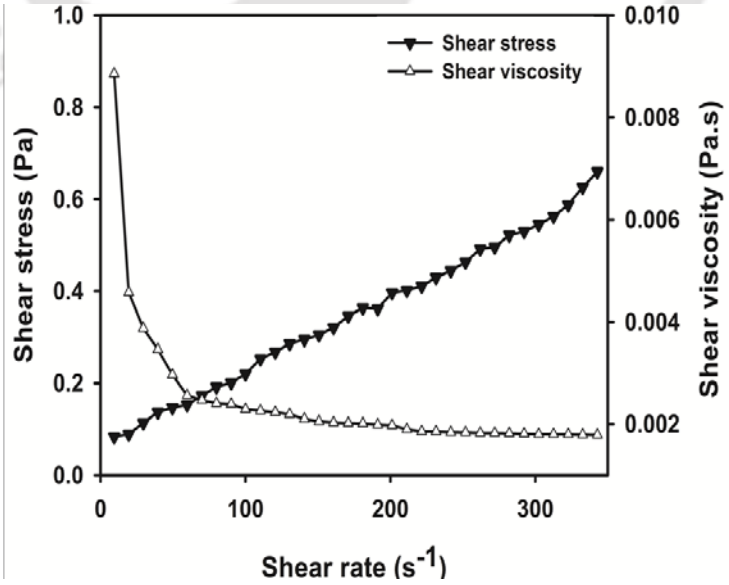

Fig. 3.3.8 Correlation of shear viscosity and shear stress of dextran with respect to shear rate.

The rheology data showed a low shear plateau and viscosity decrease with increasing shear rate, corresponding to a shear thinning, non-Newtonian (pseudoplastic) behaviour of *L. mesenteroides* NRRL B-1426 dextran (Fig. 3.3.8). This shear thinning behaviour of dextran is caused by the hydrodynamic forces generated during the shear breakdown of its structural units (Khattar *et al.*, 2010). A similar pseudoplastic behaviour of viscosity was observed in case of dextrans from *L. mesenteroides* NRRL B-640 (Purama *et al.*, 2009), *L. dextranicum* NRRL B-1146 (Majumder and Goyal, 2009) and *P. pentosaceus* SPA (Patel *et al.*, 2010). The pseudoplastic property of dextran is important to yield good sensory properties such as mouth feel and flavour release properties of foods (Moreno *et al.*, 2000). Furthermore, this property is important for various processes involved in food processing, such as mixing, pouring and pumping, where different operative shear rates are applied. In addition, it is reported that polysaccharides with pseudoplastic, non-Newtonian, shear thinning behaviour are suitable for manufacturing different food products like cake, salad dressings, relishes, sauces, syrups, puddings and dairy products. These may also be used in cosmetics, *i.e.*, in toothpaste, creams and eye contour gels (Jindal *et al.*, 2011).

3.3.1.11 Solubility and water holding capacity of dextran

Solubility and water holding capacity are important criteria for evaluating the industrial application of microbial polymers. Dextran from *L. mesenteroides* NRRL B-1426 displayed 32% solubility. This may be due to the fact that the high proportion of α-(1→6) linkages imparts high water solubility to the dextran molecules (Robyt, 1986; Tirtaatmadja *et al.*, 2001; Bejar *et al.*, 2013). Water holding capacity (WHC) of dextran was found to be 290%, which may be due to the porous matrix structure

formed by dextran chains as reported by Zhu *et al.* (2010). The high solubility and WHC of dextran make it a suitable candidate for various applications in food industry as a thickener, an emulsifier and a stabilizer (BeMiller, 2003; Kasaai, 2012).

3.3.1.12 Thermo-gravimetric analysis of dextran

The thermal stability of dextran was investigated by thermo-gravimetric analysis (TGA) in a nitrogen stream at a heating rate of 10°C/min (Fig. 3.3.9). Dextran exhibited three degradation stages. In the first stage, weight loss of ~5% occurred at 100°C-150°C due to vaporization of bound water. The second decomposition occurred at ~290°C due to the degradation of main chain with a weight loss of 13%. In the third stage, combustion of the degraded products began at ~310°C with a weight loss of 67% and complete decomposition occurred at 600°C.

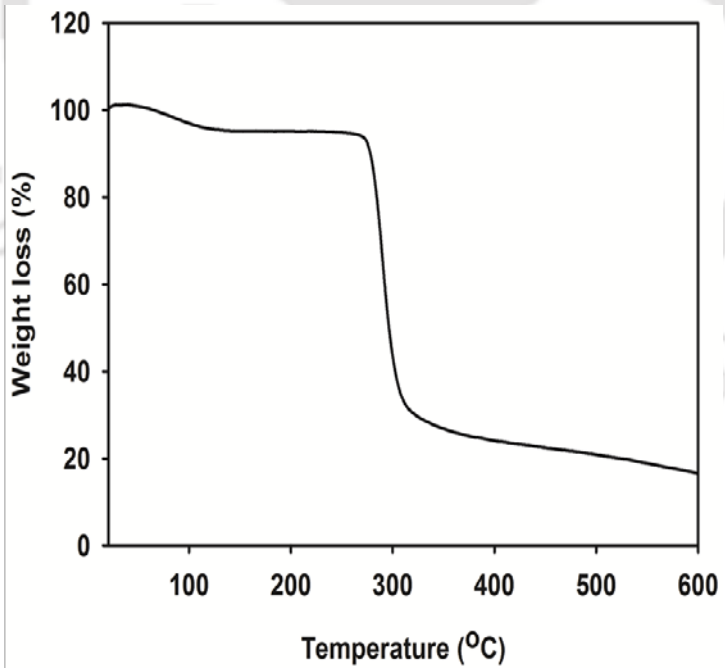

Fig. 3.3.9 Thermo-gravimetric analysis of dextran from *L. mesenteroides* NRRL B-1426 over a temperature range 20-600°C.

The high thermal stability of dextran from *L. mesenteroides* NRRL B-1426 finds its potential application in the high temperature mediated manufacturing and processing of food products.

3.3.2 *In vitro* application analysis of dextran as prebiotic

3.3.2.1 Effect of simulated human gastric juice on digestibility of dextran

The dextran from *L. mesenteroides* NRRL B-1426 was found highly resistant to hydrolysis by simulated human gastric juice (Fig. 3.3.10). The percent hydrolysis of dextran and inulin increased with increase in incubation period and with decrease in pH of simulated human gastric juice. The maximum hydrolysis of dextran after 6 h at pH of 1, 2, 3 and 4 was 0.92%, 0.60%, 0.44% and 0.23%, respectively (Fig. 3.3.10A), whereas the inulin showed 27.7%, 21.6%, 3.7% and 1.5% of hydrolysis at the respective pHs (Fig. 3.3.10B). The dextran showed significantly lower (0.2 to 0.9%) hydrolysis at pH 4 to 1 after 6 h (Fig. 3.3.10A) as compared with standard inulin (1.5 to 28%) (Fig. 3.3.10B). Dextran was 31-fold higher resistant to hydrolysis at pH 1 and 7.5-fold higher resistant at pH 4 than inulin at corresponding pHs. The dextran from *L. mesenteroides* NRRL B-1426 showed better resistance than the exopolysaccharides from *Weissella confusa* A9 (2.55%) and *Pediococcus pentosaceus* 5S4 (1.59%) in simulated gastric juice (pH 1) after 4 h of incubation (Hongpattarakere *et al*., 2012). As a result, dextran from *L. mesenteroides* NRRL B-1426 would reach the colon in more or less intact form for the use by probiotics. These findings are especially relevant for acidic foods such as yoghurt and dairy products that can be supplemented with prebiotics as reported earlier by Huebner *et al*. (2008).

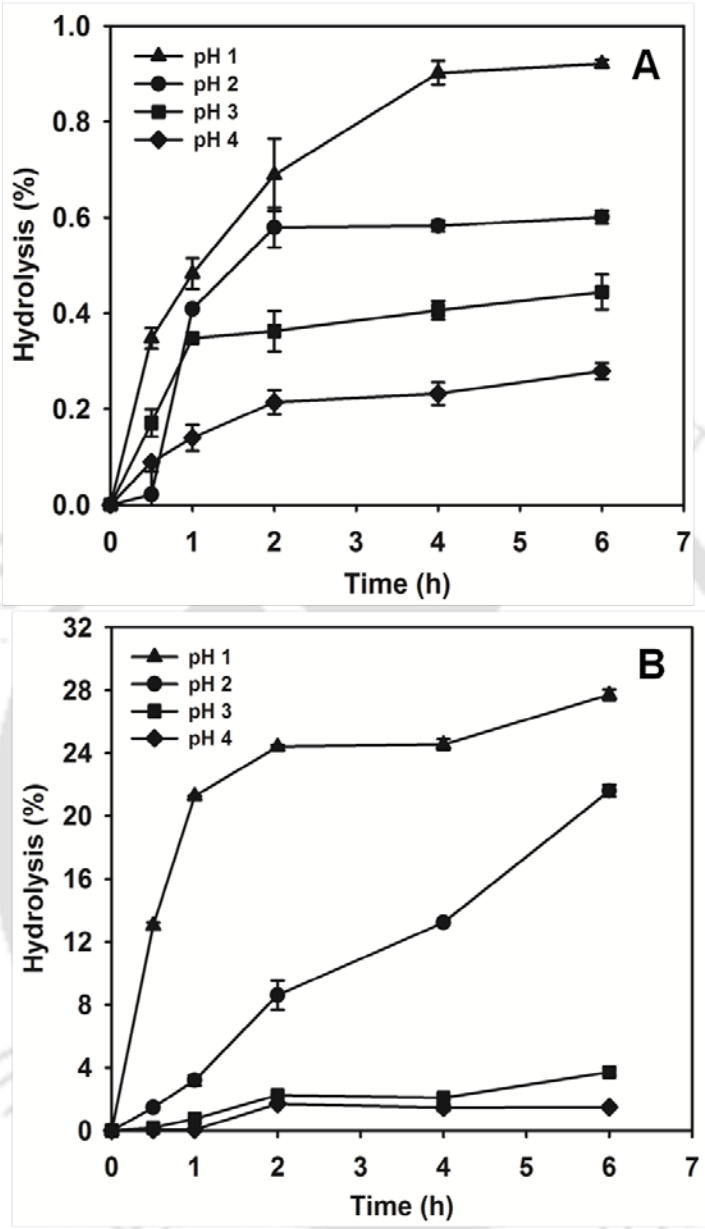

Fig. 3.3.10 Acid hydrolysis by simulated human gastric juice at pH 1, 2, 3 and 4 at 37 °C for 6 h, (A) dextran from *L. mesenteroides* NRRL B-1426 and (B) inulin. The hydrolysis of dextran was significantly different *vs* inulin ($p \leq 0.01$).

3.3.2.2 Effect of human α-amylase on digestibility of dextran

The dextran from *L. mesenteroides* NRRL B-1426 showed high resistance to digestion byhuman α-amylase as compared with inulin (Fig. 3.3.11). The variation in pH from 5 to 8 displayed no significant change on the digestibility of dextran and

inulin (Fig. 3.3.11). The maximum hydrolysis of dextran at pH 5, 6, 7 and 8 after 6 h was 0.83%, 0.82%, 0.87% and 0.87%, respectively, (Fig. 3.3.11A), whereas, the maximum hydrolysis of inulin was 13.2%, 12.7%, 12.8% and 12.8% at the respective pHs (Fig. 3.3.11B). The degree of hydrolysis of dextran was only 0.87% (Fig. 3.3.11A), as compared with 10% for inulin at pH 8 after 6 h (Fig. 3.3.11B).

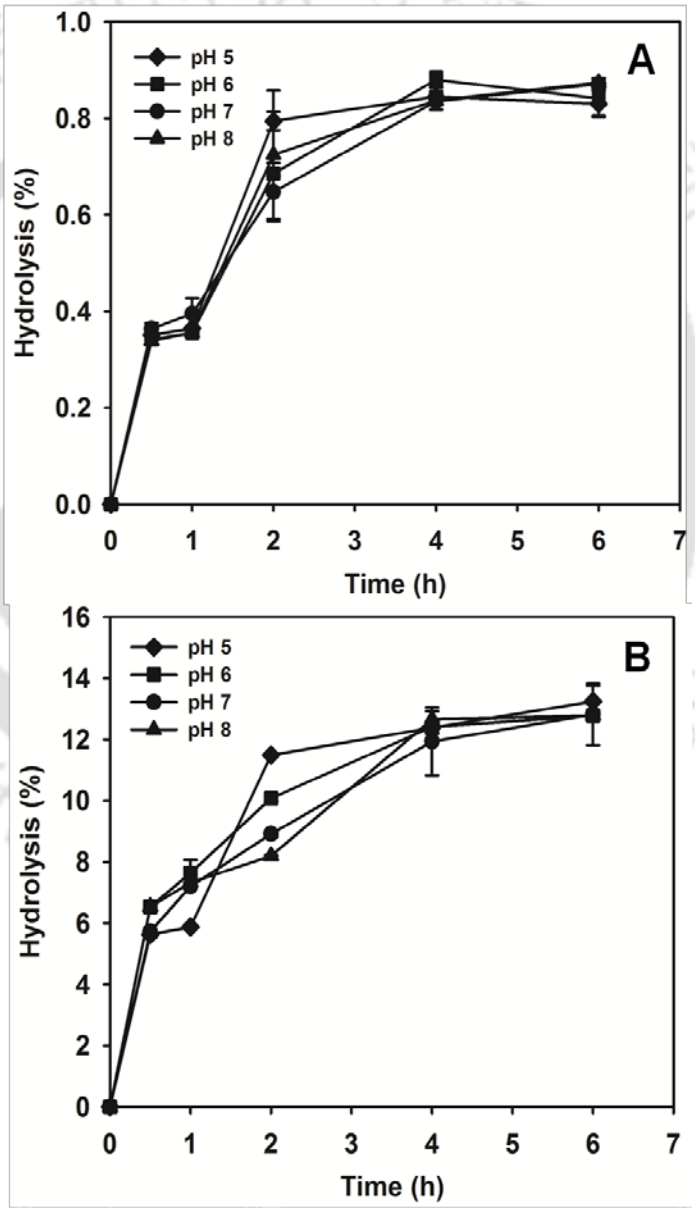

Fig.3.3.11 Enzymatic hydrolysis by human α-amylase at pH 5, 6, 7 and 8 at 37°C for 6 h. (A) dextran from *L. mesenteroides* NRRL B-1426 and (B) inulin. The hydrolysis of dextran was significantly different *vs* inulin ($p \leq 0.01$).

These results are in good agreement with other reported prebiotics, *viz.*, 88% of inulin (Cummings and Macfarlane, 2002) and 60% of gluco-oligosaccharide (Wichienchot *et al.*, 2010) resisted the enzymatic hydrolysis. It has been also reported that the enzymes such as glucoamylase, sucrase and maltase present in the small intestine, hydrolyze α-(1→4) and α-(1→6) linkages of polysaccharides to yield monosaccharides (Johnson and Schmit, 2005). Presence of α-(1→3) linkages in dextran from *L. mesenteroides* NRRL B-1426 might be providing resistance to α-amylase hydrolysis making it ideal prebiotic candidate.

3.3.2.3 Effects of dextran on the growth of human gut bacteria

The dextran from *L. mesenteroides* NRRL B-1426 stimulated the growth of *B. infantis*, *B. animalis* subspecies *lactis* and *L. acidophilus* and was comparable with that displayed by inulin (Fig. 3.3.12). The growth of probiotics *B. animalis* subspecies *lactis*, *B. infantis* and *L. acidophilus* was significantly higher than non-probiotic *E. coli* DH5α in presence of dextran and commercial prebiotic inulin (Fig. 3.3.12). The maximum absorbance (A_{600}) with dextran and inulin for *B. animalis* subspecies *lactis* was within 1.6-1.9 (Fig. 3.3.12A), for *B. infantis* the growth was within 2.5-2.9 (Fig. 3.3.12B), and for *L. acidophilus* it was within 1.7-1.9 (Fig. 3.3.12C), whereas for *E. coli* DH5α, it was only within 0.4-0.5 after 24 h (Fig. 3.3.12D).

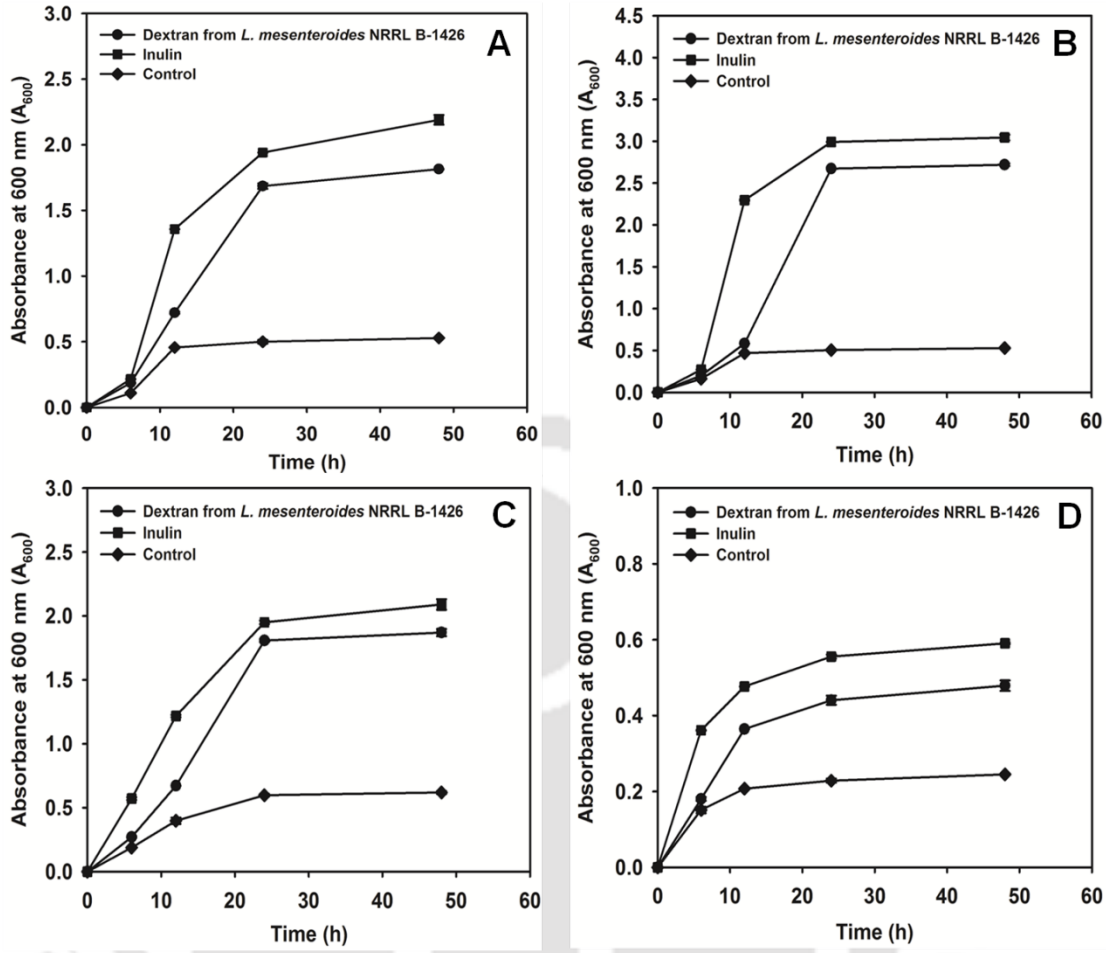

Fig. 3.3.12 Growth profiles of (A) *B. animalis* subspecies *lactis* (B) *B. infantis* (C) *L. acidophilus* and (D) *E. coli* in the presence of dextran (1%, w/v) from *L. mesenteroides* NRRL B-1426, inulin (1%, w/v)at 37°C. MRS medium without carbon source as control.The growth of probiotics was significantly different *vs* control ($p \leq 0.01$).

The growth profile of bacteria was correlated with carbohydrate utilization profiles (dextran and inulin) as shown in Table 3.3.3. All the probiotics utilized dextran and inulin as the sole carbon source. However, the maximum utilization was observed with *B.infantis* with 47% and 52.8% for dextran and inulin, respectively, followed by *L. acidophilius* and *B. animalis* subspecies *lactis*. However, *E. coli* showed insignificant consumption of supplemented carbohydrate with only 9.65% and 10.7% for dextran and inulin, respectively, even after 48 h (Table 3.3.3). The

carbohydrate fermentation profile clearly indicated that dextran could be used as sole carbon source by probiotic bacteria similar to the commercial prebiotic, inulin.

Table 3.3.3 Carbohydrate utilization (%) of dextran from *L. mesenteroides* NRRL B-1426 and inulin by human gut bacteria.

Carbohydrate utilization (%)				
Prebiotic	***B. animalis* subspecies *lactis***		***B. infantis***	
	Time (h)		**Time (h)**	
	24	**48**	**24**	**48**
Dextran[§]	21.8±0.4	23.2±0.5	44.6±1.6	46.8±2.4
Inulin	25.4±1.6	26.3±0.6	51.9±0.2	52.8±1.0
	L. acidophilus		***E. coli***	
Dextran[§]	25.9±2.3	26.5±2.30	10.0±1.82	10.1±0.5
Inulin	38.4±1.8	39.8±1.50	9.22±0.25	10.7±0.4

[§]*From L. mesenteroides NRRL B-1426.*

Change in the pH of the medium fermented by *B. animalis* subspecies *lactis*, *B. infantis*, *L. acidophilus* and *E. coli* is shown in Table 3.3.4. The pH values in the growth media were significantly reduced by the probiotic cultures tested, except for *E. coli*, where no significant decrease in the pH was observed. The pH decreased from 6.4 to 5.8 in dextran supplemented media by *B. animalis* subspecies *lactis*, respectively, after 48 h. The reduction in pH by *B. infantis* and *L. acidophilus* was 5.0 and 5.5 for dextran, respectively (Table 3.3.4). No perceptible change in the pH by *E. coli* was observed when dextran or inulin was used as sole carbon source. It was inferred that *Bifidobacteria* spp. and *Lactobacillus* sp. used dextran as carbon source during fermentation and produced organic acids like lactic acid and acetic acid by bringing down significantly the pH of growth medium. The acidic pH condition in the human colon is known to stimulate the growth of probiotics (Mussatto and Mancilha, 2007) and suppress the intestinal pathogenic bacteria (Hongpattarakere *et al.*, 2012).

Thus, these results strongly suggest that the dextran from *L. mesenteroides* NRRL B-1426 can be an alternative and a better prebiotic.

Table 3.3.4 pH changes of selected bacterial genera during fermentation of medium.

Prebiotic	***B. animalis* subspecies *lactis*** Time (h)			***B.infantis*** Time (h)		
	0	**24**	**48**	**0**	**24**	**48**
Dextran[§]	6.4±0.0	5.8±0.04*	5.8±0.01*	6.4±0.0	5.1±0.02**	5.0±0.02**
Inulin	6.4±0.0	5.7±0.02*	5.7±0.01*	6.4±0.0	4.9±0.02**	4.9±0.03**
Control	6.4±0.0	6.2±0.01	6.0±0.04	6.4±0.0	6.3±0.00	6.2±0.12
	L. acidophilus			***E. coli***		
Dextran[§]	6.4±0.0	5.5±0.01*	5.5±0.04*	7.0±0.0	6.9±0.04	6.8±0.01
Inulin	6.4±0.0	5.8±0.01*	5.8±0.03*	7.0±0.0	6.9±0.01	6.8±0.03
Control	6.4±0.0	6.3±0.02	6.2±0.03	7.0±0.0	6.9±0.03	6.9±0.06

[§]*From L. mesenteroides NRRL B-1426.*
*Significantly different ($p \leq 0.05$) *vs* control.
**Significantly different ($p \leq 0.01$) *vs* control.

3.2.3*In vitro* analysis of effect of dextran on mammalian cells

The *in vitro* analysis of effect of dextran from *L. mesenteroides* NRRL B-1426 on human embryonic kidney (HEK-293), human cervical adenocarcinoma (INT-407) and human colon carcinoma (HT-29) cell lines was studied by the MTT assay (Fig. 3.3.13). The cell viability of HEK-293, INT-407 and HT-29 cells remained constant as a function of both time and dose. The data revealed that even at higher concentrations of dextran (1000 µg/ml) no significant effect on the viability of cells was observed after 36 h. The percentage of cell viability of dextran treated cells was similar to that of the respective untreated cells at all concentrations (50-1000 µg/ml) as shown in Fig. 3.3.13. This demonstrated the non-toxic and biocompatible nature of dextran from *L. mesenteroides* NRRL B-1426. The biocompatibility of dextran was also reported for dextran from *Pediococcus pentosaceus* (Patel *et al.*, 2010),

Lactobacillus plantarum DM5 (Das and Goyal, 2014) and *Weissella cibaria* JAG8 (Rao *et al.*, 2014). Hence, the dextran from *L. mesenteroides* NRRL B-1426 can be considered as biocompatible and safe for consumptionas function food.

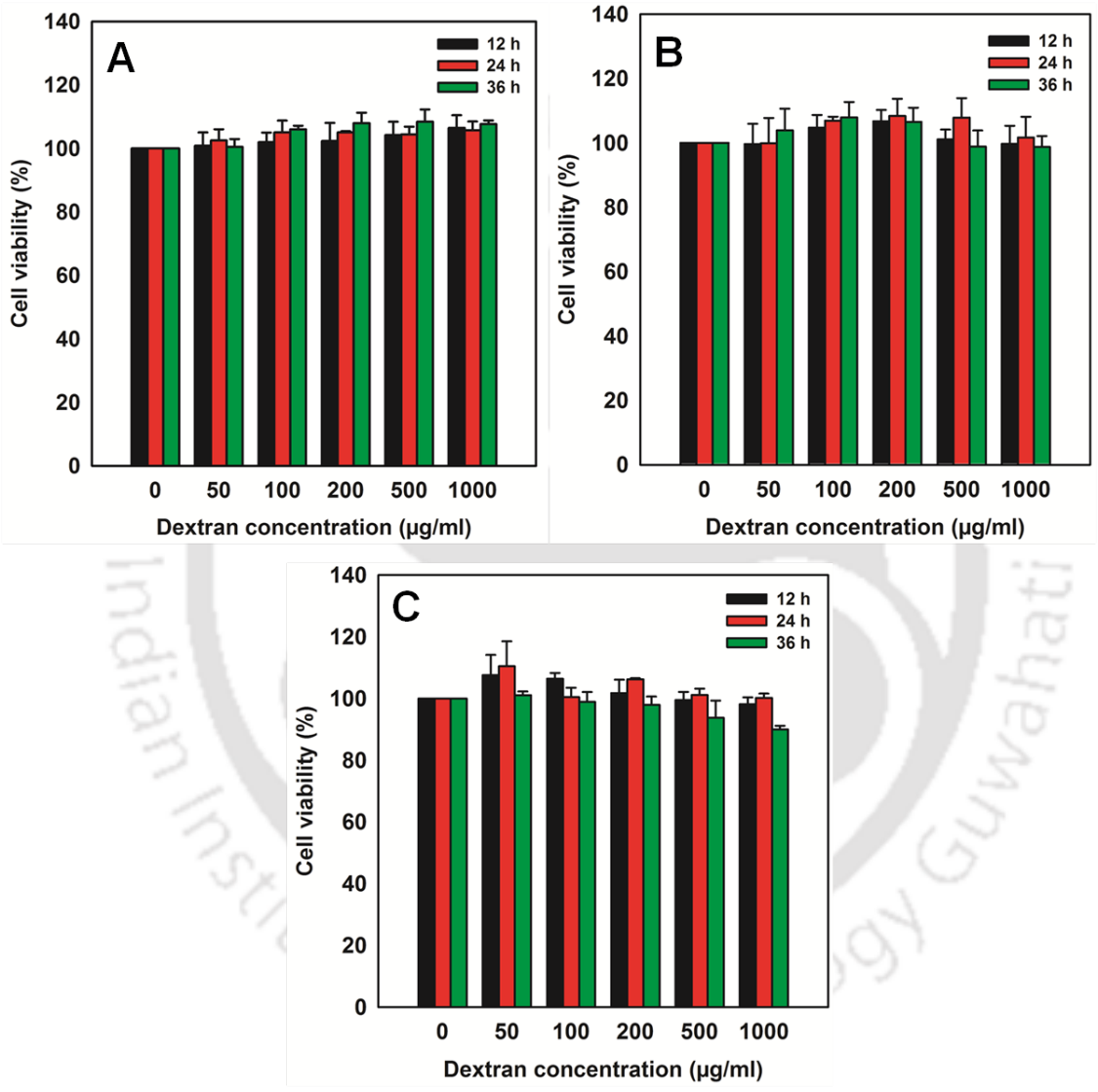

Fig. 3.3.13 Cell viability (%) of (A) HEK-293, (B) INT-407 and (C) HT-29 cells treated with various concentrations of dextran (0-1000 µg/ml) over a period of 12-36 h of incubation.

3.4 Conclusions

The physico-chemical characterization and prebiotic potential of dextran from *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase was explored for the first time. The dextran was enzymatically synthesized by dextransucrase and purified by ethanol precipitation. The structure analyses by FTIR, ^{1}H and ^{13}C NMR spectroscopy demonstrated that it contains~85.5% α-(1→6) linear and ~14.5% α-(1→3) branched linkages. The molecular mass determination by gel permeation chromatography showed that it is a high molecular mass dextran of size >2000 kDa. This high molecular mass dextran containing branched α-(1→3) linkages can be hydrolysed for the production of enzyme resistant isomalto-oligosaccharides. Scanning electron microscopy of dextran demonstrated the porous structure present all over the surface. The dextran exhibited high solubility, water holding capacity and viscosity, indicating various applications in food industry as a thickener, an emulsifier and a stabilizer. Rheological studies of dextran showed non-Newtonian, pseudoplastic, shear thinning properties, which may be valuable for use in different food industries. The high thermal stability of dextran with degradation temperature ~290°C can have potential application in the high temperature mediated manufacturing and processing of food products. The prebiotic potential of dextran from *L. mesenteroides* NRRL B-1426 was also evaluated. The dextran showed significantly low degree of hydrolysis (0.2-0.9%) by the simulated human gastric juice at acidic pHs whereas, with inulin (commercial prebiotic) it was several fold higher (1.5-28%). This indicated the greater stability of dextran at low pH, when compared with inulin. Similar results were observed when the dextran was subjected to α-amylase treatment. The digestibility of dextran and inulin by α-amylase was 0.8% and 12%, respectively. The dextran supported the

growth of probiotic bacteria, bifidobacteria and lactobacillus. The dextran did not encourage the growth of unwanted coliforms like *E. coli*. The dextran exhibited the nontoxic effect on human embryonic kidney (HEK-293), human cervical adenocarcinoma (INT-407) and human colorectal carcinoma (HT-29) cell lines upto a concentration of 1000µg/ml, displaying its biocompatible nature. With these desirable attributes the dextran from *L. mesenteroides* NRRL B-1426 can be used as prebiotic and as an ingredient of functional food for health benefits.

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Chapter 4

Acceptor reactions of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426 for synthesis of oligosaccharides

4.1 Introduction

The nutritional importance of oligosaccharides with prebiotic effect (Monsan and Ouarne, 2010), has been recently underlined by the demonstration of key role of the human intestinal microbiota in obesity and diseases (Holmes *et al.*, 2008; Turnbaugh *et al.*, 2009). However, the chemical access to oligosaccharides is difficult due to their complex structure. Many protecting and deprotecting group manipulations are needed to control the stereo-specificity (*i.e.*, formation of only one anomer) and regio-specificity (*i.e.*, formation of 1-2 or 1-3 or 1-4 or 1-6 bonds), which eventually result in low chemical synthesis yields (Heincke *et al.*, 1999; Perugino *et al.*, 2004; Wang *et al.*, 2007). The stereo- and regio-specificity of oligosaccharides are needed for biological applications (Crout and Vic, 1998). The development of efficient synthetic methods, for the production of carbohydrate derivatives is thus of prime interest (Monsan *et al.*, 2010). The enzymatic synthesis of oligosaccharides has been achieved by the transfer reactions between donor and various types of acceptors (Robyt and Eklund, 1983; Heincke *et al.*, 1999, Kitaoka and Robyt, 1999). Among various enzymes available, dextransucrases can efficiently catalyze the synthesis of oligosaccharides by the transfer of glucosyl units from sucrose to other carbohydrates.

This reaction is called acceptor reaction and the added carbohydrates are called acceptors (Koepsell *et al.*, 1953). This acceptor reaction has gained attraction for the synthesis of new oligosaccharides owing to its high efficiency and selectivity (Leemhuis *et al.*, 2012). The efficiency of an acceptor reaction varies with particular dextransucrase and acceptor molecule used (Demuth *et al.*, 2002; Kothari and Goyal, 2013). Transglycosylation catalyzed by these enzymes from various bacteria has also been used to improve physicochemical properties such as water solubility and oxidative stability of various compounds (Robyt *et al.*, 2008). Dextransucrase displayed significant flexibility towards a diverse array of saccharide or non-saccharide acceptors (Leemhuis *et al.*, 2012). So far, maltose and isomaltose are known to be the best acceptors of dextransucrase for oligosaccharide synthesis (Robyt and Eklund, 1983; Demuth *et al.*, 2002; Leemhuis *et al.*, 2012; Kothari and Goyal, 2013). However, the synthetic potential of this enzyme is not restricted to 'normal' saccharides. Additionally functionalized saccharides, such as alditols, aldosuloses, a sugar acid, alkyl saccharides and D-glycals (Demuth *et al.*, 2002), salicin and phenol (Seo *et al.*, 2005), primary alcohols (Seibel *et al.*, 2006), hydroquinones (Seo *et al.* 2009), ascorbic acid (Kim *et al.*, 2010) nigero-oligosaccharides (Komatsu *et al.*, 2011), flavonoids (Kim *et al.*, 2012), may also act as acceptors. In the present study, the specificity of acceptor reactions of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426 was examined. A series of acceptor reactions was carried out with sucrose as a glucosyl donor and sixteen different saccharides as acceptors. The synthesized oligosaccharides were analyzed by thin layer chromatography (TLC), high performance anion exchange chromatography (HPAEC) and electrospray ionization-time of flight mass spectrometry (ESI-TOF MS).

4.2 Materials and Methods

4.2.1 Chemicals and reagents

All the media components for maintenance and enzyme production were purchased from Hi-Media Pvt. Ltd., India. All the organic solvents were purchased from Qualigens, India. L-arabinose, D-arabinose, D-cellobiose, D-fructose, D-galactose, D-gentiobiose, D-glucose, D-isomaltose, D-lactose, D-maltose, D-mannose, D-melibiose, D-raffinose, D-rhamnose, D-trehalose, D-xylose and maltotriose were purchased from Sigma Aldrich, USA. All the chemicals required for reducing sugar estimation, protein estimation and buffer preparation were of high purity.

4.2.2 Microorganism and culture conditions

Leuconostoc mesenteroides NRRL B-1426 was procured from Agricultural Research Service (ARS) culture collection, National Centre for Agricultural utilization Research, Peoria, USA. The culture was grown and maintained in modified MRS as described in Chapter 2, Section 2.2.2.

4.2.3 Enzymatic synthesis of oligosaccharides

L. mesenteroides NRRL B-1426 dextransucrase was partially purified by 33% (v/v) PEG-400 as described in Chapter 2, Section 2.2.8 and was used for synthesis of oligosaccharides. The oligosaccharides were synthesized via acceptor reaction of dextransucrase by incubating 100 µl of PEG-400 purified enzyme (0.76 mg of protein/ml and specific activity 10.14 U/mg) with 900 µl of sixteen different acceptors: arabinose (L-Ara), D-arabinose (D-Ara), D-cellobiose (Cel), D-fructose (Fru), D-galactose (Gal), D-gentiobiose (Gnt), D-glucose (Glc), D-isomaltose (Imal), D-lactose (Lac), D-maltose (Mal), D-mannose (Man), D-melibiose (Mel), D-raffinose

(Raf), D-rhamnose (Rha), D-trehalose (Tre) and D-xylose (Xyl). The acceptor-to-sucrose ratio was taken as 1:1 at 146 mM final concentration in 20 mM sodium acetate buffer (pH 5.6) containing 0.3 mM $CaCl_2$ and 15 mM NaN_3 at 30°C for 24 h. Equal volume of absolute ethanol was added to the reaction mixtures and centrifuged at 16,000*g* for 10 min to remove polysaccharides (dextran). Polysaccharide (dextran) production was analyzed by phenol sulfuric acid method as described in Chapter 3, Section 3.2.4 and compared with the reaction with sucrose (5%, w/v) without any acceptor. The supernatant containing oligosaccharides was analyzed using TLC, HPAEC and ESI-TOF MS. The supernatant was diluted 200 times with Milli Q water and filtered with 0.2 µm membrane before analysis by HPAEC and ESI-TOF MS.

4.2.4 Analysis of oligosaccharides

4.2.4.1 Thin layer chromatography

An aliquot of 0.5 µl of each reaction mixture containing oligosaccharides was analyzed by TLC as described in Chapter 2, Section 2.2.3.15.

4.2.4.2 High pH anion exchange chromatography

The quantitative analysis of oligosaccharides was done by HPAEC system (Dionex Corporation, ICS 3000) equipped with CarboPac PA-200 analytical column (3x250 mm) and CarboPac PA-20 guard column (3x50 mm). An isocratic elution with 0.2 N NaOH at a constant flow rate of 0.5 ml/min was used for chromatographic separation at 30°C. The injection volume was 25 µl. The oligosaccharides were detected by an electrochemical detector (ED 50). The concentrations of oligosaccharides were calculated from the peak areas using glucose as the standard in the range 10-100 µg/ml (Rabelo *et al.*, 2008). Oligosaccharide production was compared with maltose acceptor reaction (100%).

4.2.4.3 Mass spectrometry

The oligosaccharides were identified by ESI-TOF MS (Waters Q-TOF, micromass HAB273) using the following ion source parameter conditions: source-desolvation gas temperatures set at 100°C and 250°C; sampling, capillary and extraction cone voltages were set 35 V, 3 KV and 3 V, respectively, with the ion guide of 1 V. Flow injection rate was set at 10 µl/min. Mass spectra were acquired in positive ion mode in the range of m/z 500-1200 and processed using MassLynx software.

4.3 Results and Discussion

4.3.1 Synthesis of oligosaccharides

The acceptor specificity of dextransucrase from *L. mesenteroides* NRRL B-1426 with sixteen different sugars was investigated. In the dextransucrase acceptor reaction, sucrose was used as the donor, where the glucosyl group is transferred by tranglycosylation to the acceptor and fructose is released as a product. Dextran is formed in major or minor amounts, depending on the strength of the acceptor. Table 4.3.1 shows the structure of different acceptors used.

Table 4.3.1 Structure of sixteen different acceptors used for oligosaccharide synthesis.

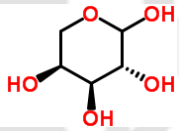
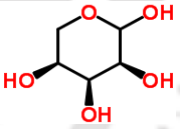
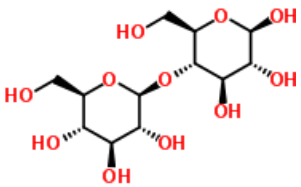
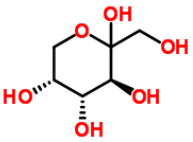
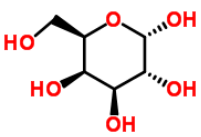
Acceptor	IUPAC name	Structure
L-Arabinose	L-Arabinopyranose	
D-Arabinose	L-Ribopyranose	
Cellobiose	4-O-β-D-Glucopyranosyl-β-D-glucopyranose	
Fructose	D-Fructopyranose	
Galactose	α-D-Galactopyranose	

Table 4.3.1 Continued

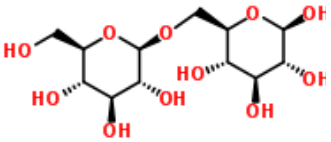
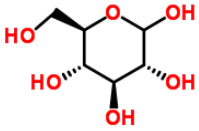
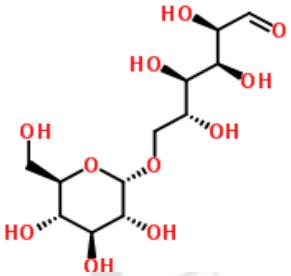
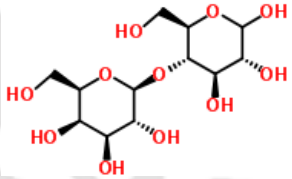
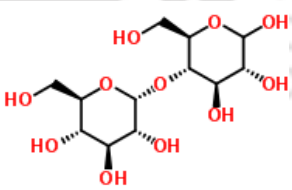
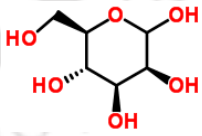
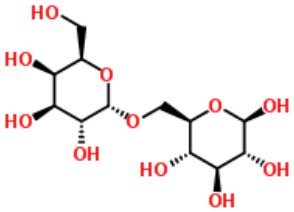
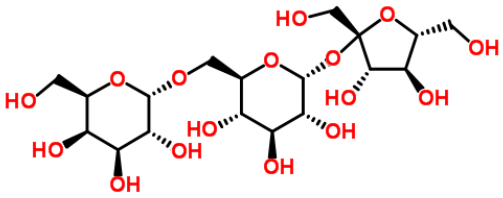
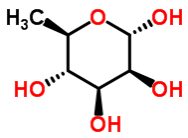
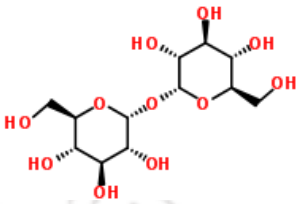
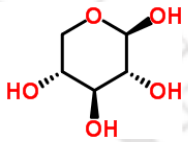
Gentiobiose	6-O-β-D-Glucopyranosyl-β-D-glucopyranose	
Glucose	D-Glucopyranose	
Isomaltose	6-O-α-D-Glucopyranosyl-D-glucopyranose	
Lactose	4-O-β-D-Galactopyranosyl-D-glucopyranose	
Maltose	4-O-α-D-Glucopyranosyl-D-glucopyranose	
Mannose	D-Mannopyranose	
Melibiose	6-O-α-D-Galactopyranosyl-β-D-glucopyranose	
Raffinose	β-D-Fructofuranosyl α-D-galactopyranosyl-(1→6)-α-D-glucopyranoside	

Table 4.3.1 Continued

Rhamnose	6-Deoxy-α-D-mannopyranose	
Trehalose	α-D-Glucopyranosyl α-D-glucopyranoside	
Xylose	β-D-Xylopyranose	

The TLC analysis of the acceptor reaction mixture showed that cellobiose, gentiobiose, glucose, isomaltose, lactose, maltose, melibiose and trehalose were effective acceptors for oligosaccharide synthesis (Fig. 4.3.1). Notable differences in the oligosaccharide pattern with these eight acceptors were observed (Fig. 4.3.1). However, no acceptor reaction products were obtained with arabinose, fructose, galactose, mannose, rhamnose, raffinose and xylose. This may be due to the conformational differences in the acceptor region or the differences in the amino acid residues in the acceptor region (responsible for the precise orientation of an acceptor subsite +1) (Leemhuis *et al.*, 2012). The effective acceptors were further compared for their efficiency towards acceptor reaction by monitoring the oligosaccharide concentrations.

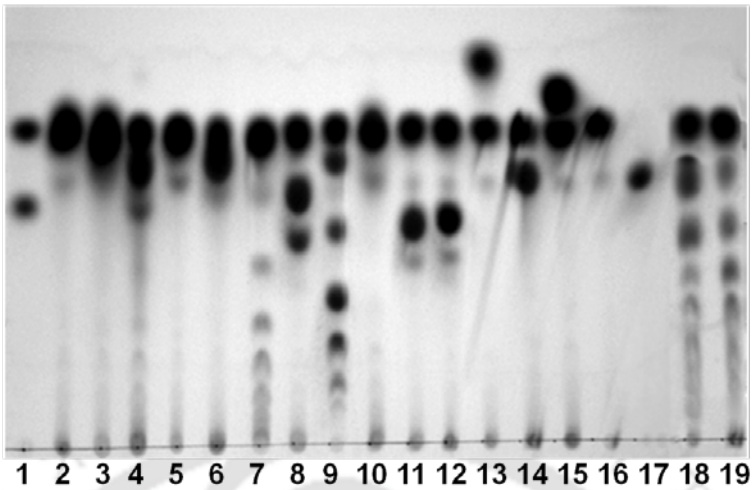


Fig. 4.3.1 TLC of representative types of acceptor reaction mixtures. Lanes: 1-sucrose and raffinose; 2-D-arabinose; 3-L-arabinose; 4-cellobiose; 5-fructose; 6-galactose; 7-glucose; 8-lactose; 9-maltose; 10-mannose; 11-melibiose; 12-raffinose; 13-rhamnose; 14-trehalose; 15-xylose; 16-sucrose; 17-maltotriose; 18-gentiobiose and 19-isomaltose.

4.3.2 Analysis of oligosaccharides

The oligosaccharides synthesized with the effective acceptors were analyzed by HPAEC and ESI-TOF MS. In a typical acceptor reaction, the transfer of the glucosyl moiety from sucrose onto the non-reducing end of the acceptor occurs and a series of oligosaccharides are consequently formed (Arguello Morales *et al.*, 2001). In general, α-(1→6) glycosidic linkage is formed but all of the other possible glycosidic linkages *viz.*, (1→1), (1→2), (1→3) and (1→4) have also been observed depending on the structure of particular acceptor (Demuth *et al.*, 2002).

Cellobiose is a disaccharide of two glucose molecules linked by a β-(1→4) glycosidic bond produced from enzymatic hydrolysis of cellulose. The HPAEC chromatogram of the acceptor reaction mixture with cellobiose showed the presence of oligosaccharides eluting between 6.4 and 10.8 min (Fig. 4.3.2A).

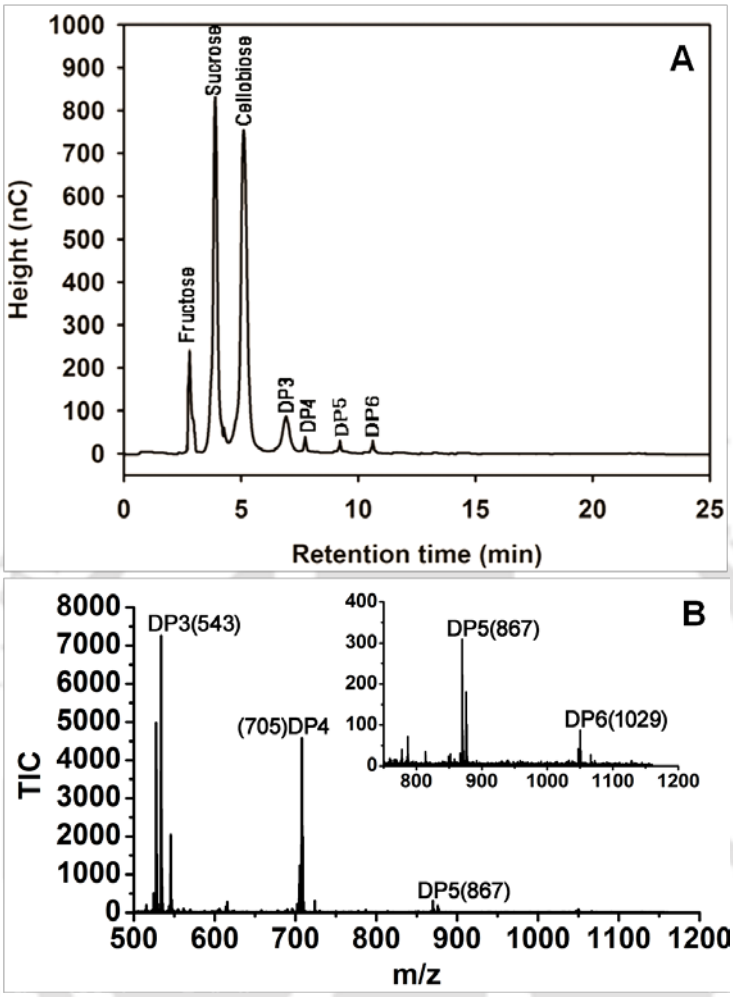


Fig. 4.3.2 Acceptor reaction products synthesized by dextransucrase from *L. mesenteroides* NRRL B-1426 using sucrose and cellobiose at a concentration of 146 mM (1:1). (A) HPAEC and (B) ESI-TOF MS analyses of the acceptor reaction products with cellobiose.

The mass spectrometry analysis confirmed the synthesis of oligosaccharides by the acceptor reaction of dextransucrase with cellobiose. The mass spectrum showed that DP3 and DP4 were the major products with peak values of the pseudo-molecular ion $[M+K]^+$ at m/z 543 and 705, respectively. However, the peak intensities of potassium molecular ions of DP > 4 were not observed in considerable amount (Fig. 4.3.2B). The dextransucrase from *L. mesenteroides* B-512FMCM also reported to produce a series of oligosaccharides using cellobiose as an acceptor and sucrose as a

donor (Kim and Day, 2008). It has been reported earlier that cellobiose was glucosylated both at position O-2 of the reducing unit and at O-6 of the non-reducing unit forming α-(1→2) linkage, thus differing from a typical acceptor reaction (Arguello Morales *et al.*, 2001; Cote and Sheng, 2006). Oligosaccharides containing α-(1→2) linked residues are highly resistant to the digestive enzymes (Remaud-Simeon *et al.*, 2000) and hence can be used as prebiotics.

Gentiobiose is a disaccharide composed of two units of glucose linked with a β-(1→6) linkage. The HPAEC chromatogram of the acceptor reaction mixture with gentiobiose revealed the presence of oligosaccharides eluting from 7.5 to 18 min (Fig. 4.3.3A). The mass spectrometry analysis confirmed the synthesis of oligosaccharides. The mass spectrum showed that the acceptor reaction products of dextransucrase with gentiobiose ranged from DP3 to DP6. The peak values of the pseudo-molecular ion $[M+Na]^+$ at m/z 527, 689, 851 and 1013, for DP3, DP4, DP5 and DP6, respectively, were observed (Fig. 4.3.3B). Gentiobiose has also been reported to serve as an acceptor for *L. mesenteroides* NRRL B-512F dextransucrase for the synthesis of oligosaccharides of DP3 only (Yamauchi and Ohwada, 1969). Gentiobiose and gentio-oligosaccharides have shown promising potentials as prebiotics (Rycroft *et al.*, 2001) and low-glycemic sweetener (Cote, 2009). Hence, it can be hypothesized that the oligosaccharides derived through the transglucosylation of gentiobiose by dextransucrase can act as food or feed ingredients.

The HPAEC chromatogram of the acceptor reaction mixture with glucose revealed the presence of oligosaccharides eluting from 4.8 to 19 min (Fig. 4.3.4A). When glucose was used as an acceptor, the glucose from sucrose is transferred to the 6-hydroxyl group forming α-(1→6) glycosidic linkage.

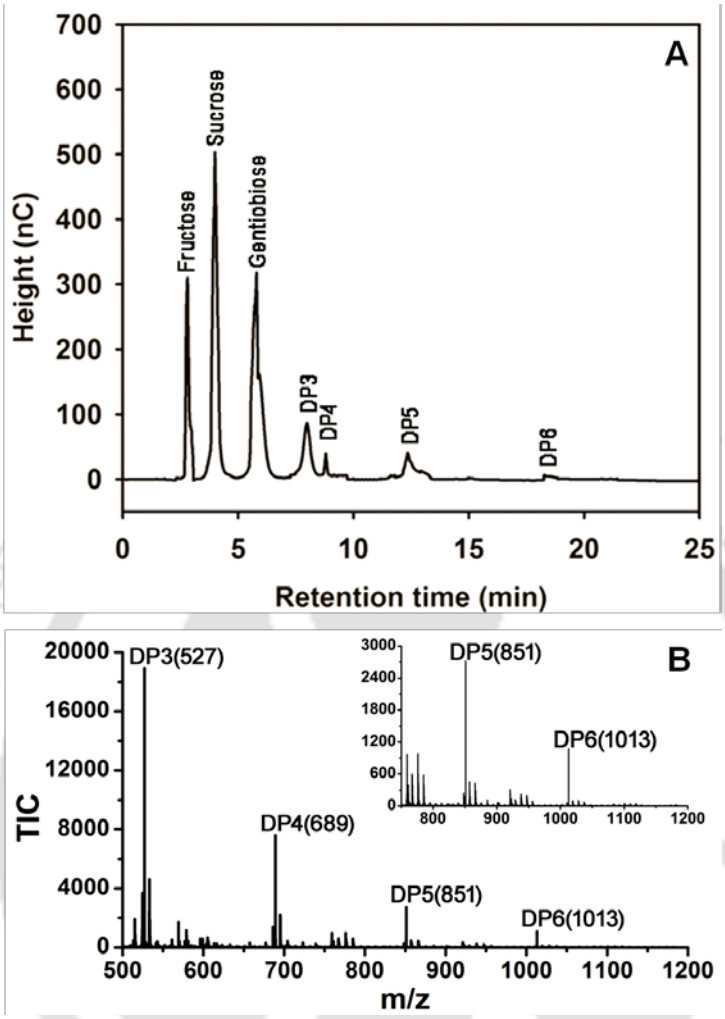


Fig. 4.3.3 Acceptor reaction products synthesized by dextransucrase from *L. mesenteroides* NRRL B-1426 using sucrose and gentiobiose at a concentration of 146 mM (1:1). (A) HPAEC and (B) ESI-TOF MS analyses of the acceptor reaction products with gentiobiose.

The chromatographic analysis revealed the synthesis of oligosaccharides ranging from DP3 to DP6 (Fig. 4.3.3A). However, the mass spectrometry analysis showed oligosaccharides of DP3 and DP4 only with m/z 527 and 689, respectively. The acceptor products of DP > 4 were not observed in mass spectrum, which might be due to the lesser ionization of the acceptor products (Fig. 4.3.4B). Glucose was also reported to act as acceptor for dextransucrase from *L. mesenteroides* NRRL B-512F

and synthesize small chain oligossaccharides (Pereira *et al.*, 1998). The price of glucose is considerably lower than that of maltose, so it may be a better choice for production of oligosaccharides (Pereira *et al.*, 1998).

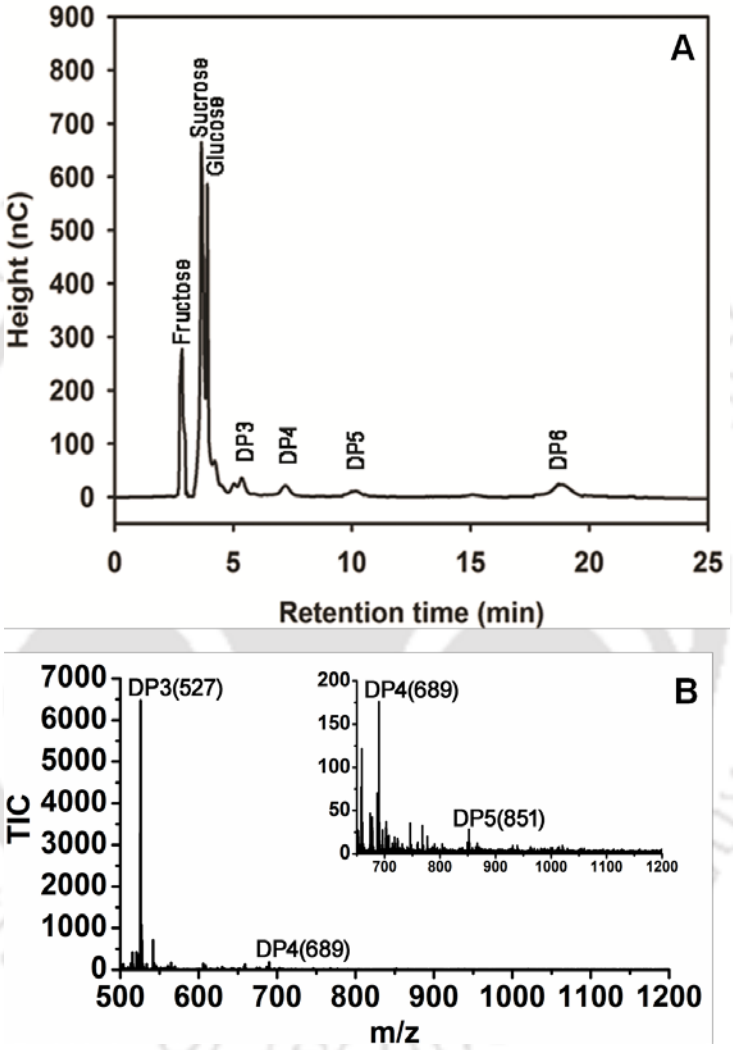


Fig. 4.3.4 Acceptor reaction products synthesized by dextransucrase from *L. mesenteroides* NRRL B-1426 using sucrose and glucose at a concentration of 146 mM (1:1). (A) HPAEC and (B) ESI-TOF MS analyses of the acceptor reaction products with glucose.

Isomaltose is a disaccharide composed of two units of glucose linked by an α-(1→6) glycosidic linkage. The HPAEC chromatogram of the acceptor reaction mixture with isomaltose revealed the presence of oligosaccharides eluting at time from

6.5 to 19 min (Fig. 4.3.5A). The chromatographic analysis demonstrated the synthesis of oligosaccharides ranging from DP3 to DP6 (Fig. 4.3.5A).

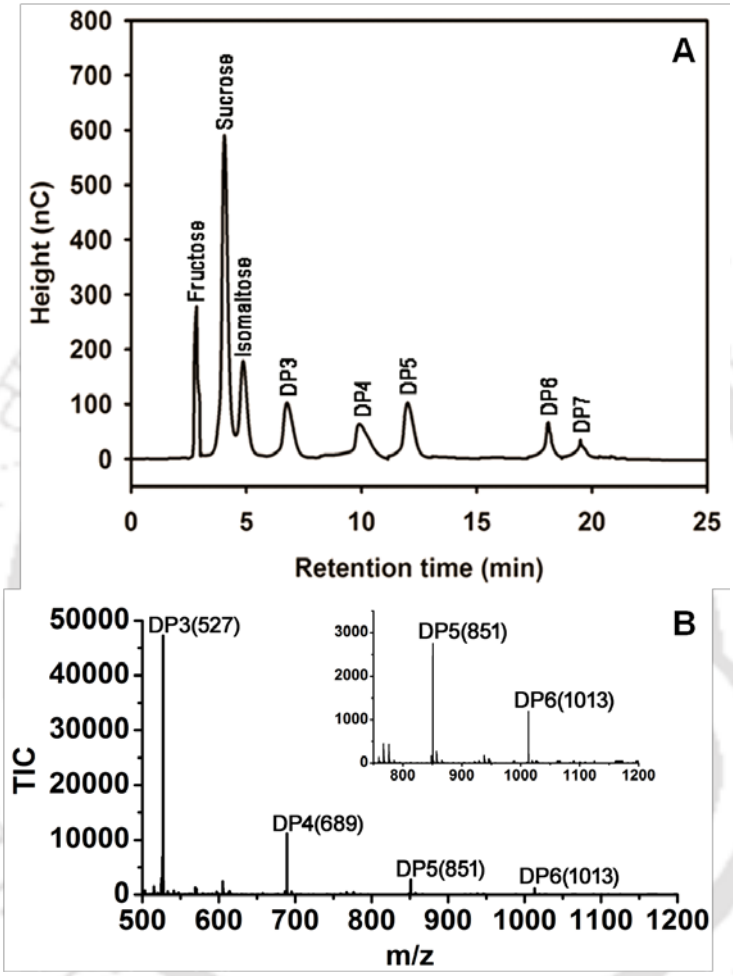


Fig. 4.3.5 Acceptor reaction products synthesized by dextransucrase from *L. mesenteroides* NRRL B-1426 using sucrose and isomaltose at a concentration of 146 mM (1:1). (A) HPAEC and (B) ESI-TOF MS analyses of the acceptor reaction products with isomaltose.

When isomaltose was used as an acceptor, the glucosyl unit from sucrose is transferred to the 6-hydroxyl group of the non-reducing end of the glucose residue of the isomaltose (Demuth *et al.*, 2002). Each acceptor product contains an isomaltose unit at the reducing end and α-(1→6)-linked D-glucopyranosyl units at the non-reducing end of the isomaltose. The mass spectrometry analysis confirmed that the

acceptor reaction products of dextransucrase with isomaltose ranged from DP3 to DP6. The peak values of the pseudo-molecular ion $[M+Na]^+$ at m/z 527, 689, 851 and 1013, for DP3, DP4, DP5 and DP6, respectively were observed (Fig. 4.3.5B). Isomaltose is known to be the second best acceptor for dextransucrase (Robyt and Eklund, 1983; Fu *et al.*, 1990; Demuth *et al.*, 2002; Kothari and Goyal, 2013). Human intestinal enzymes readily digest α-(1→4) glycosidic linkages but α-(1→6) linkages are not easily hydrolyzed exhibiting a digestion-resistant property, an important criterion for prebiotics (Remaud-Simeon *et al.*, 2000). The oligosaccharides containing exclusively α-(1→6) glycosidic bonds thus can be synthesized by the transglucosylation of isomaltose by dextransucrase acceptor reaction.

Lactose is a disaccharide derived from the condensation of galactose and glucose, which forms a β-(1→4) glycosidic linkage. The HPAEC chromatogram of the acceptor reaction mixture with lactose revealed the presence of oligosaccharides eluting at time from 5 to 11 min (Fig. 4.3.6A). The chromatographic analysis demonstrated the synthesis of oligosaccharides ranging from DP3-DP6 (Fig. 4.3.6A). The mass spectrometry analysis also confirmed that the acceptor reaction products of dextransucrase with lactose ranged from DP3 to DP6. The peak values of the pseudo-molecular ion $[M+Na]^+$ or $[M+K]^+$ at m/z 527, 705, 851 and 1013, for DP3, DP4, DP5 and DP6, respectively, were observed (Fig. 4.3.6B). However, only one acceptor product was reported from lactose acceptor reaction using dextransucrase from *L. mesenteroides* NRRL B-512F (Yamauchi and Ohwada, 1969). Like cellobiose, lactose was also glycosylated both at position O-2 of the reducing unit and at O-6 of the non-reducing unit forming α-(1→2) linkage (Robyt and Eklund, 1983).

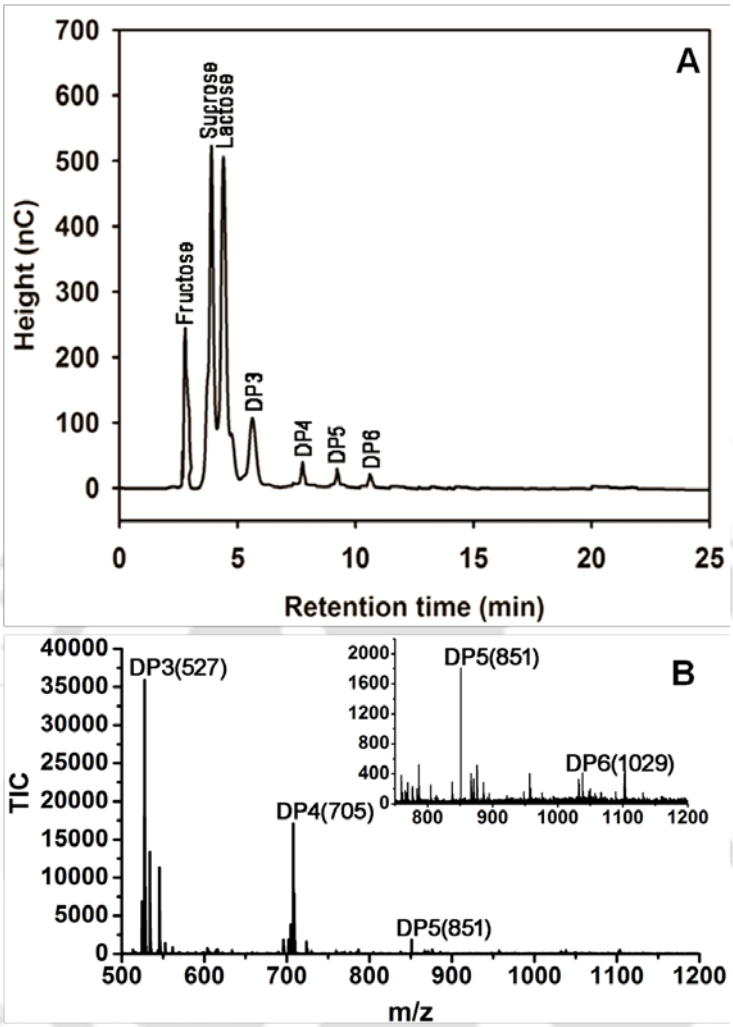


Fig. 4.3.6 Acceptor reaction products synthesized by dextransucrase from *L. mesenteroides* NRRL B-1426 using sucrose and lactose at a concentration of 146 mM (1:1). (A) HPAEC and (B) ESI-TOF MS analyses of the acceptor reaction products with lactose.

Lactose is the precursor for a number of bioactive compounds derived by chemical, physical or enzymatic conversion that have an established and expanding place in the pharmaceutical and food industries (Playne and Crittenden, 2009). Moreover, lactose derived oligosaccharides are indigestible in the human digestive tract, hence are of low calorific value. They are also putative growth stimulators of

intestinal bifidobacteria (Arakawa *et al.*, 2002). Thus, lactose derived oligosaccharides could potentially be employed as prebiotic oligosaccharides.

Maltose is a disaccharide composed of two units of glucose linked with an α-(1→4) bond. The HPAEC chromatogram of the acceptor reaction mixture with maltose revealed the presence of oligosaccharides eluting at the time from 7.8 to 19 min (Fig. 4.3.7A). The chromatographic analysis revealed the synthesis of oligosaccharides ranging from DP3 to DP6 (Fig. 4.3.7A).

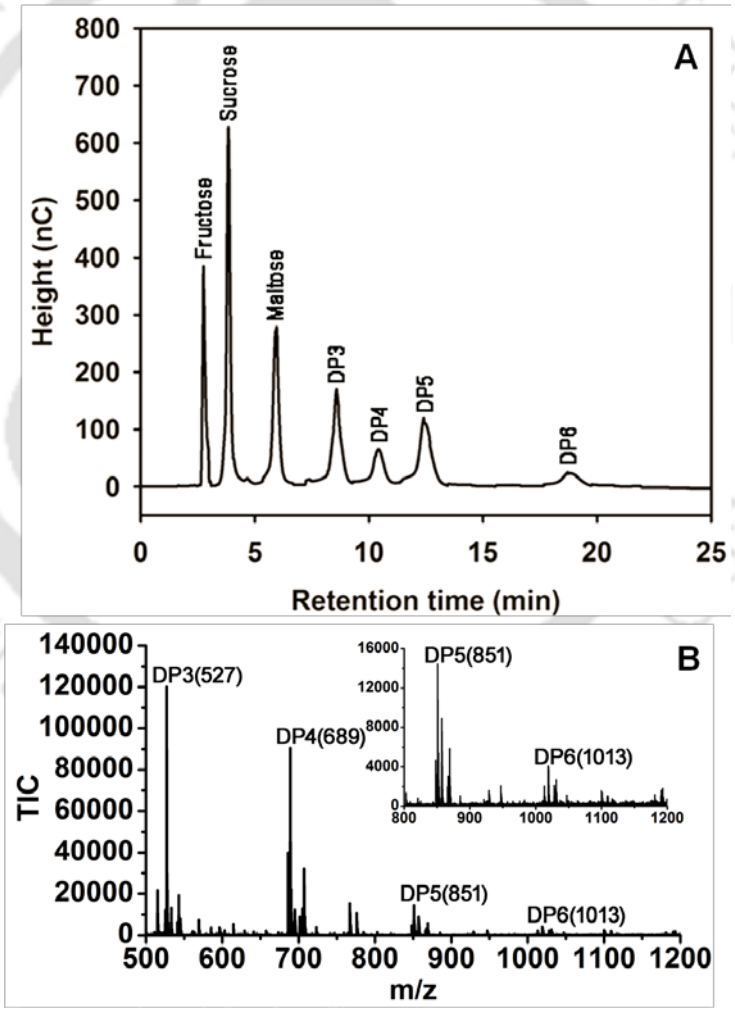


Fig. 4.3.7 Acceptor reaction products synthesized by dextransucrase from *L. mesenteroides* NRRL B-1426 using sucrose and maltose at a concentration of 146 mM (1:1). (A) HPAEC and (B) ESI-TOF MS analysis of the acceptor reaction products with maltose.

The mass spectrometry analysis confirmed that the acceptor reaction products of dextransucrase with maltose ranging from DP3 to DP6. The peak values of the pseudo-molecular ion $[M+Na]^+$ at m/z 527, 705, 851 and 1013, for DP3, DP4, DP5 and DP6, respectively were observed (Fig. 4.3.7B). When maltose was used as an acceptor, the glucose moeity from sucrose is transferred to the 6-hydroxyl group of the non-reducing end of glucose residue (Demuth *et al.*, 2002). Each acceptor product contains a maltose unit at the reducing end and α-(1→6)-linked D-glucopyranosyl unit at the non-reducing ends of the maltose. The first product of maltose acceptor reaction is panose, which again acts as an acceptor for the synthesis of homologous oligosaccharides of DP > 3 (Cote and Leathers, 2005). The maltose is known to be the best acceptor for dextransucrase (Robyt and Eklund, 1983; Fu *et al.*, 1990; Demuth *et al.*, 2002; Kothari and Goyal, 2013). The high efficiency of this acceptor reaction can be used for the commercial production of isomalto-oligosaccharides.

Melibiose is a disaccharide formed by α-(1→6) linkage between galactose and glucose. The HPAEC chromatogram of the acceptor reaction mixture with melibiose revealed the presence of oligosaccharides eluting at the time from 4.8 to 7 min (Fig. 4.3.8A). The chromatographic analysis demonstrated the synthesis of oligosaccharides ranging from DP3 to DP4 (Fig. 4.3.8A). When melibiose is used as an acceptor, the glucosyl moeity from sucrose is transferred to the 6-hydroxyl group of the non-reducing end glucose residue of melibiose. The acceptor products will contain a melibiose unit at the reducing end and α-(1→6)-linked D-glucopyranosyl units at the non-reducing end of melibiose. The mass spectrometry analysis confirmed that the acceptor reaction products of dextransucrase with melibiose were only DP3 and DP4. The peak values of the pseudomolecular ion $[M+Na]^+$ at m/z 543 and 705, for DP3

and DP4, respectively were observed. However, the peak intensities of oligosaccharides, DP > 4 were not observed in considerable amount (Fig. 4.3.8B). Melibiose contains a galactose residue and hence can be used for the synthesis of galacto-oligosaccharides, which is used as commercial prebiotic.

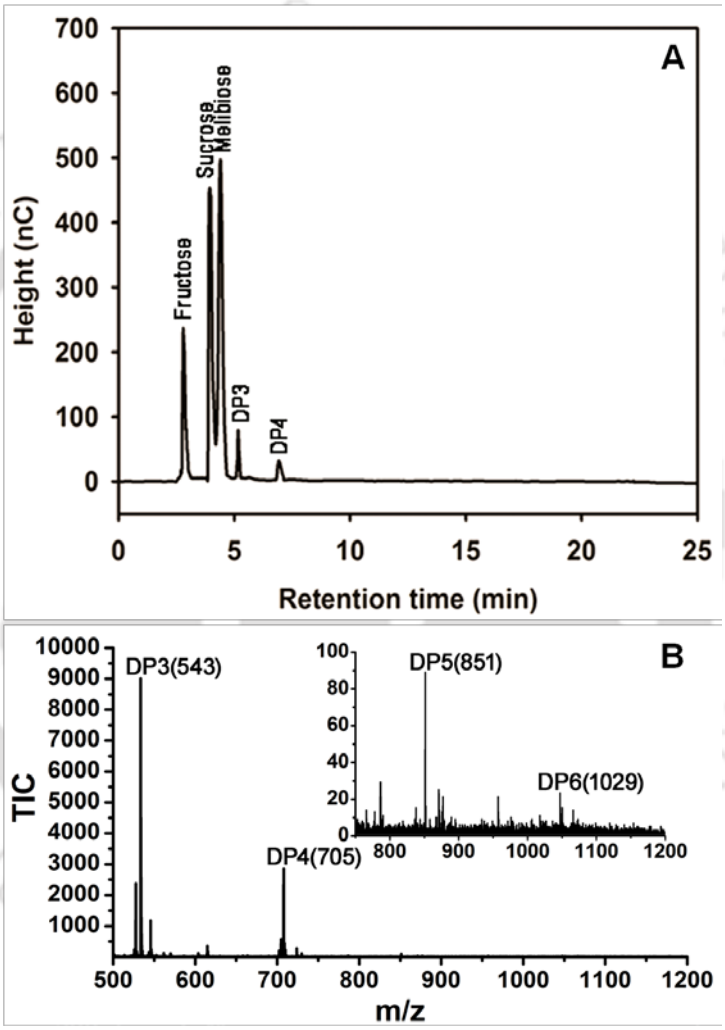


Fig. 4.3.8 Acceptor reaction products synthesized by dextransucrase from *L. mesenteroides* NRRL B-1426 using sucrose and melibiose at a concentration of 146 mM (1:1). (A) HPAEC and (B) ESI-TOF MS analyses of the acceptor reaction products with melibiose.

Trehalose is a disaccharide formed by an α-(1→1) glycosidic bond between two glucose units. The HPAEC chromatogram of the acceptor reaction mixture with sucrose and trehalose revealed the presence of oligosaccharide eluting at the time from 4.9 to 5 min (Fig. 4.3.9A). The chromatographic analysis revealed the synthesis of oligosaccharides of only DP3 (Fig. 4.3.9A).

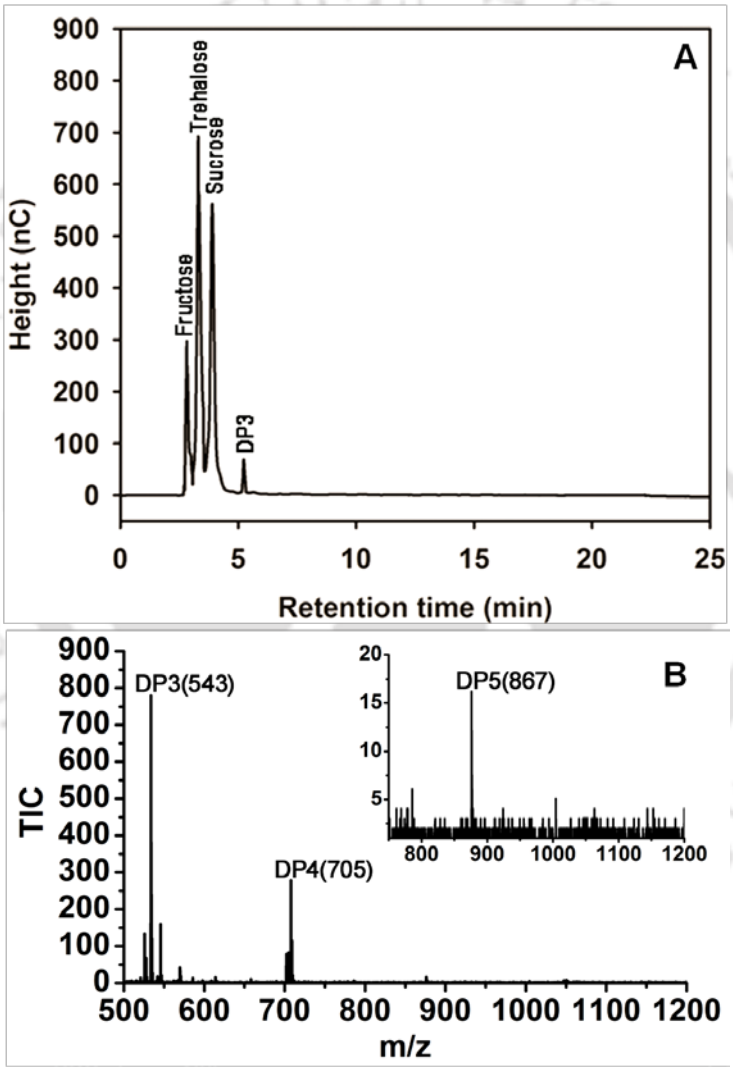


Fig. 4.3.9 Acceptor reaction products synthesized by dextransucrase from *L. mesenteroides* NRRL B-1426 using sucrose and trehalose at a concentration of 146 mM (1:1). (A) HPAEC and (B) ESI-TOF MS analyses of the acceptor reaction products with trehalose.

The peak values of the pseudo-molecular ion $[M+Na]^+$ at m/z 543, 705 and 867 for DP3, DP4 and DP5, respectively were observed (Fig. 4.3.9B). However, the mass spectrometry analysis showed very low peak intensities of the acceptor reaction products of dextransucrase with trehalose ranging from DP3 to DP5. The unique feature of trehalose is its ability to sustain and preserve a wide array of biological molecules in nature. Hence, trehalose has found its use in several daily use food and cosmetic products (Ohtake and Wang, 2011). Therefore, trehalose derived oligosaccharides can be suitable for many commercial applications.

4.3.4 Efficiency of acceptors for oligosaccharide synthesis

The eight effective acceptors for dextransucrase were further compared for their efficiency towards acceptor reaction by monitoring the oligosaccharide concentrations (Fig. 4.3.10). The concentration of oligosaccharides was determined by HPAEC using glucose as standard due to the non-availability of commercial standards. The concentration of oligosaccharides synthesized by dextransucrase with eight acceptors using sucrose was varied from 0.73 to 31.8 mg/ml (Table 4.3.2). Dextran production was also observed and it could not be avoided in the reaction of dextransucrase with any of the acceptor (Fig. 4.3.10). The dextran production was significantly varied with different acceptors. The dextran concentration with cellobiose, 20.44 mg/ml; gentiobiose, 13.40 mg/ml; glucose, 19.19 mg/ml; isomaltose, 8.64 mg/ml; lactose, 16.64 mg/ml; maltose, 3.01 mg/ml; melibiose, 16.00 mg/ml and trehalose, 24.14 mg/ml were obtained (Fig. 4.3.10).

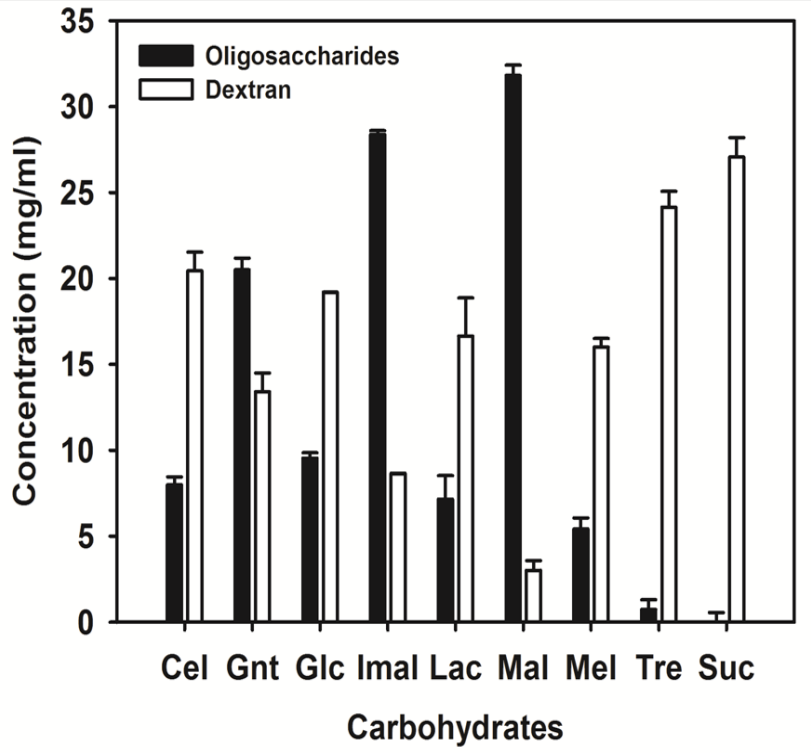


Fig. 4.3.10 Enzymatic synthesis of oligosaccharides and dextran from *L. mesenteroides* NRRL B-1426. Cel-cellobiose; Gnt-gentiobiose; Glc-glucose; Imal-isomaltose; Lac-lactose; Mal-maltose; Mel-melibiose; Tre-trehalose and Suc-sucrose. Data are expressed as the mean of triplicates ± standard deviation.

Oligosaccharide synthesis with the effective acceptors was compared with maltose acceptor reaction (100%), the best known acceptor. Table 4.3.2 shows the efficiency of the eight acceptors for oligosaccharides synthesis. The best acceptor for oligosaccharide synthesis after maltose (100%) was isomaltose (89%) followed by gentiobiose (64%), glucose (30%), cellobiose (25%), lactose (22.5%), melibiose (17%) and trehalose (2.3%).

Table 4.3.2 Efficiency of the eight acceptors for oligosaccharides synthesis.

Acceptors	Oligosaccharide concentration (mg/ml)	Oligosaccharide concentration (%)
Cellobiose	7.99	25
Gentiobiose	20.50	64
Glucose	9.54	30
Isomaltose	28.37	89
Lactose	7.15	22.5
Maltose	31.81	100
Melibiose	5.42	17
Trehalose	0.73	2.3

Thus, gentiobiose, glucose, cellobiose and lactose could also be used as efficient acceptors other than maltose and isomaltose for oligosaccharide production using dextransucrase from *L. mesenteroides* NRRL B-1426. The product composition of acceptor reactions can also vary with the donor/acceptor ratio (Kim *et al.*, 2001). Since β-linkages are stronger than α-linkages and are highly resistant to the attack of digestive enzymes (Mussatto and Mancilha, 2007), cellobiose (β-1→4 linked), gentiobiose (β-1→6 linked) and lactose (β-1→4 linked) can act as efficient acceptors for synthesis of prebiotic oligosaccharides.

4.4 Conclusions

The acceptor specificity of dextransucrase from *L. mesenteroides* NRRL B-1426 with sixteen different sugars was investigated. The synthesized oligosaccharides were analyzed by TLC, HPAEC and ESI-TOF MS. The TLC analysis of the acceptor reaction mixture showed that cellobiose, gentiobiose, glucose, isomaltose, lactose, maltose, melibiose and trehalose were the effective acceptors for oligosaccharide synthesis. Dextran production was also observed in the reaction of dextransucrase with all eight acceptors. The mass spectrum of acceptor reaction products revealed predominant ion species as sodium or potassium adducts $[M+Na]^+$ or $[M+K]^+$. The sodium or potassium adducts of oligosaccharides ranging from DP3 to DP6 (527-1013 or 543-1029) were observed in case of all the acceptors. However, the peak intensities of sodium or potassium molecular ions of DP > 4 (689-1013 or 705-1029) varied with the type of acceptor molecule used. Oligosaccharides of DP > 4 were present in considerable amount in case of isomaltose, gentiobiose, glucose, cellobiose, lactose and maltose. However, DP3 and DP4 were the main acceptor products with melibiose and trehalose. The isomaltose was found to be the best acceptor having 89% efficiency followed by gentiobiose (64%), glucose (30%), cellobiose (25%), lactose (22.5%), melibiose (17%) and trehalose (2.3%) with reference to maltose, a known best acceptor. Thus, gentiobiose, glucose, cellobiose and lactose can also be used as efficient acceptors other than maltose and isomaltose for oligosaccharide production using dextransucrase from *L. mesenteroides* NRRL B-1426. Since β-linkages are stronger than α-linkages and are highly resistant to the attack of digestive enzymes, cellobiose (β-1→4 linked), gentiobiose (β-1→6 linked) and lactose (β-1→4 linked) can be used as acceptors for synthesis of prebiotic oligosaccharides.

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Chapter 5

Synthesis, purification and prebiotic applications of isomalto-oligosaccharides from the acceptor reaction of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426

5.1 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). Most of the marketed prebiotics are inulin-based fructose oligomers or galacto-oligosaccharides (Saad *et al.*, 2013; Chen and Huang, 2014). 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). 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 and in functional foods (Sharma *et al.*, 2011). IMOs contain α-(1→6) linkage and a lower proportion α-(1→4), α-(1→3) or α-(1→2) linkages (Goffin *et al.*, 2011). IMOs naturally occur in various fermented foods such as miso, sake or soy sauce but also in honey (Goffin *et al.*, 2011). IMOs have a great potential to improve the physiochemical quality of many foods as anti-fading agent for food pigments, as food antioxidant and as sweetener (40% of the sweetness of

sucrose and a calorific value of 2.8-3.2 kcal/g) (Kaneko *et al.*, 1994; Chockchaisawasdee and Poosaran, 2013). They have also been identified as good humectants with low viscosity and water activity but high moisture retaining capacity (Goffin *et al.*, 2011). In addition, these oligosaccharides have physiological functions such as the improvement of intestinal microflora based on the selective stimulation of growth of bifidobacteria in humans and lactobacilli in rats (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, 2013). 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) (Kaneko *et al.*, 1994; Delattre and Vijayalakshmi, 2009). Generally speaking, the commercially available IMO, 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 (Robyt and Eklund, 1983; Kothari and Goyal, 2013) and it was used for the production of molecular size-controlled dextrans and IMOs. This acceptor reaction also allows the control of chain

lengths of IMOs by changing the concentration of sucrose and maltose (Lee *et al.*, 2008). Moreover, the synthesis of IMOs is mainly governed by the type of dextransucrase derived from a specific microorganism (Kothari and Goyal, 2013). However, the operational variables, *viz.*, pH, temperature and enzyme concentration have been shown to have little influence on final IMOs yields.

In the present study, the conditions for IMO synthesis by acceptor reaction of *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase were optimized. The IMOs were purified by gel permeation chromatography and characterized by HPLC and mass spectrometry. The purified IMOs (DP ≥ 3) were tested for their prebiotic activity in term of gut digestibility and selective stimulation of probiotic bacteria. The effects of purified IMOs were evaluated on human embryonic kidney (HEK-293), human cervical adenocarcinoma (INT-407) and human colon carcinoma (HT-29) cell lines.

5.2 Materials and Methods

5.2.1 Chemicals and reagents

All the media components for maintenance and enzyme production were purchased from Hi-Media Pvt. Ltd., India. All the organic solvents were purchased from Qualigens, India. D-glucose, D-isomaltose, D-sucrose, D-maltose monohydrate, α-amylase from human saliva and all the chemicals required for cell culture were purchased from Sigma Aldrich, USA. 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.

5.2.2 Microorganisms and culture conditions

Leuconostoc mesenteroides NRRL B-1426, *Bifidobacterium infantis* NRRL B-41661 and *Lactobacillus acidophilus* NRRL B-4495 were procured from ARS culture collection, National Centre for Agricultural Utilization Research, Peoria, USA. *L. mesenteroides* NRRL B-1426 was grown and maintained in modified MRS agar stab as described in Chapter 2, Section 2.2.2. The probiotic cultures were grown and maintained in MRS medium as described in Chapter 3, Section 3.2.2.

5.2.3 Optimization of reaction conditions for synthesis of IMOs

L. mesenteroides NRRL B-1426 dextransucrase (10.14 U/mg, 0.76 mg/ml) was partially purified by 33% (v/v) PEG-400 as described in Chapter 2, Section 2.2.8 and was used for optimization of reaction conditions for synthesis of IMOs. 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) as described in

Chapter 4, Section 4.2.3. The supernatants were analyzed for IMO synthesis by TLC as described in Chapter 2, Section 2.2.3.15.

5.2.3.1 Effect of time

For the time course, the enzymatic reaction was carried out at 30°C from 0 to 24 h in 1 ml mixture containing 5% (w/v) sucrose and 5% (w/v) maltose monohydrate, 0.5 U/ml of enzyme solution, 0.3 mM $CaCl_2$ and 15 mM NaN_3 in 20 mM sodium acetate buffer (pH 5.6).

5.2.3.2 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. 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).

5.2.3.3 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 of enzyme activity, 0.3 mM $CaCl_2$ and 15 mM NaN_3 in 20 mM sodium acetate buffer (pH 5.6).

5.2.3.4 Effect of sucrose concentration

To determine the optimum concentration of sucrose for IMO 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. 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.

5.2.3.5 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. 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.

5.2.4 Quantification of IMO

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.

5.2.5 Synthesis of dextrans with different molecular mass in acceptor reaction

The precipitated dextran was re-precipitated with three volumes of ethanol for the complete removal of sucrose, maltose and fructose as described in Chapter 3, Section 3.2.3. The resulting dextran was dissolved in water and the concentration was determined as described in Chapter 3, Section 3.2.4. The number average molecular mass (MM_n) of dextran was also determined as described in Chapter 3, Section 3.2.6.5.

5.2.6 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; 30°C) in 50 ml reaction mixture for purification of IMOs as described in the Section 5.2.3. 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 on the basis of their degree of polymerization (DP) by gel permeation chromatography (GPC) using XK16/70 column (GE Healthcare) packed with Bio-Gel P-2 (fine mesh) on AKTA purifier (GE Healthcare, model 100 Plus). The acceptor product solution (2 ml) was filtered through 0.45 µm membrane filters (Pall Corporation, USA) and injected into the column through a 2-ml injection loop. The elution was carried out using Milli Q water at a constant flow rate of 0.2 ml/min and fraction volume of 1 ml was collected. The fractions were analyzed for oligosaccharides by phenol-sulfuric acid method (as described in Chapter 3, Section 3.2.4) and identified by TLC (as described in Chapter 2, Section 2.2.13.5). The selected fractions under the peaks of purified IMOs of various DP were analyzed HPLC and ESI-TOF MS. The purified IMOs of DP ≥ 3 were pooled, lyophilized and tested for their prebiotic activity and cytotoxicity.

5.2.6.1 High performance liquid chromatography

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 (300x7.7 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.

5.2.6.2 Mass spectrometry

The molar masses of the selected fractions of IMOs obtained from GPC were determined by mass spectrometry with electron spray ionization (ESI-MS) in positive mode using an Agilent 6520 Accurate mass Q-TOF LC/MS system (Agilent Technologies, USA). The sample was mixed with a few drops of formic acid, filtered through 0.2 µm membrane (Pall Corporation, USA) and then directly introduced into the electrospray ionizer at a flow rate of 0.3 ml/min with water:acetonitrile (40:60, v/v) solvent system. The nebulizer gas (N_2) pressure was 30 psi, the drying gas flow rate was 5 l/min, the gas and vaporizer temperatures were 300°C and 200°C, respectively. The capillary, skimmer, fragmentor and charging voltages were set at 2500 V, 65 V, 175 V and 2000 V, respectively. The mass spectrum was recorded using a scan range of 100-2000 m/z per 1 min run and processed using Agilent MassHunter workstation software.

5.2.7 *In vitro* analysis of prebiotic application of IMOs

5.2.7.1 Effect of simulated human gastric juice on digestibility of IMOs

The hydrolyzing effect of simulated human gastric juice on IMOs (DP ≥ 3) was tested in HCl buffer supplemented with 0.3%, w/v pepsin as described in Chapter 3, Section 3.2.7.1. The pH of the buffer was adjusted to 1, 2 and 3 by 5 M HCl. The purified IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase acceptor reaction and inulin (commercial prebiotic) were separately dissolved in deionised water to give 1% (w/v) solutions. IMOs or inulin solutions (1.0 ml, 1%, w/v) were mixed with 1.0 ml of simulated human gastric juice at the three pH levels separately and the reaction mixtures were incubated at 37°C for 6 h. Aliquots (100 µl) of the reaction mixture at

0, 0.5, 1, 2, 4 and 6 h intervals were collected to calculate the percent hydrolysis as described in Chapter 3, Section 3.2.7.1.

5.2.7.2 Effect of human α-amylase on digestibility of IMOs

The IMOs (DP ≥ 3) and inulin were separately dissolved in deionised water to give 1% (w/v) solution and tested for digestibility by human α-amylase. Solution of α-amylase (2 U/ml) was prepared in 20 mM sodium phosphate buffer containing 6.7 mM sodium chloride, pH 7.0. IMOs and inulin samples (1.0 ml, 1%, w/v) were mixed with 1.0 ml of α-amylase solution and the reaction mixtures were incubated at 37°C for 6 h. Aliquots (100 µl) of the reaction mixture were collected from each treatment at 0, 0.5, 1, 2, 4 and 6 h intervals to calculate the percent hydrolysis as described in as described in Chapter 3, Section 3.2.7.1.

5.2.7.3 Effect of IMOs on the growth of probiotics

The growth stimulatory effects of IMOs (DP ≥ 3) and inulin on *B. infantis* and *L. acidophilus* were evaluated. 1 ml of overnight grown probiotic cultures (as described in Chapter 3, Section 3.2.2) were harvested by centrifuging the cells at 3,200*g* for 15 min at 4°C and the cell pellets were twice washed 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 or inulin. Aqueous stock solutions (10%, w/v) of purified IMOs or inulin were filter sterilized using 0.2 µm membrane and added to sterile 5 ml MRS medium to get a final concentration of 1% (w/v). The MRS medium without any carbohydrate was maintained as a negative control. The bacterial cultures were incubated at 37°C under anaerobic conditions in anaero bags.

The bacterial growth was monitored by measuring absorbance at 600 nm (A_{600}) using UV-visible spectrophotometer (Varian, Cary 100).

5.2.8 *In vitro* analysis of effect of IMOs on mammalian cells

5.2.8.1 Culturing and maintenance of mammalian cells

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 Centre for Cell Sciences (NCCS), Pune, India. The HEK-293, INT-407 and HT-29 cells were cultured and maintained in DMEM, MEM and RPMI, respectively, at 37ºC in a humidified incubator at 5% CO_2 saturation as described in Chapter 3, Section 3.2.8.1.

5.2.8.2 Cell viability assay

The effects of purified IMOs (DP ≥ 3) at concentrations ranging from 10-500 µg/ml on cell viability of HEK-293, INT-407 and HT-29 were evaluated by MTT assay for 36 h as described in the Chapter 3, Section 3.2.8.3.

5.2.9 Statistical analysis

The prebiotic application 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% ($p \leq 0.05$).

5.3 Results and Discussion

5.3.1 Optimization of reaction conditions for synthesis of IMOs

5.3.1.1 Effect of time

The concentration of IMOs increased with increase in the time of acceptor reaction and consequently reached saturation after 12 h (Fig. 5.3.1). 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) (Fig. 5.3.1A and Table 5.3.1).

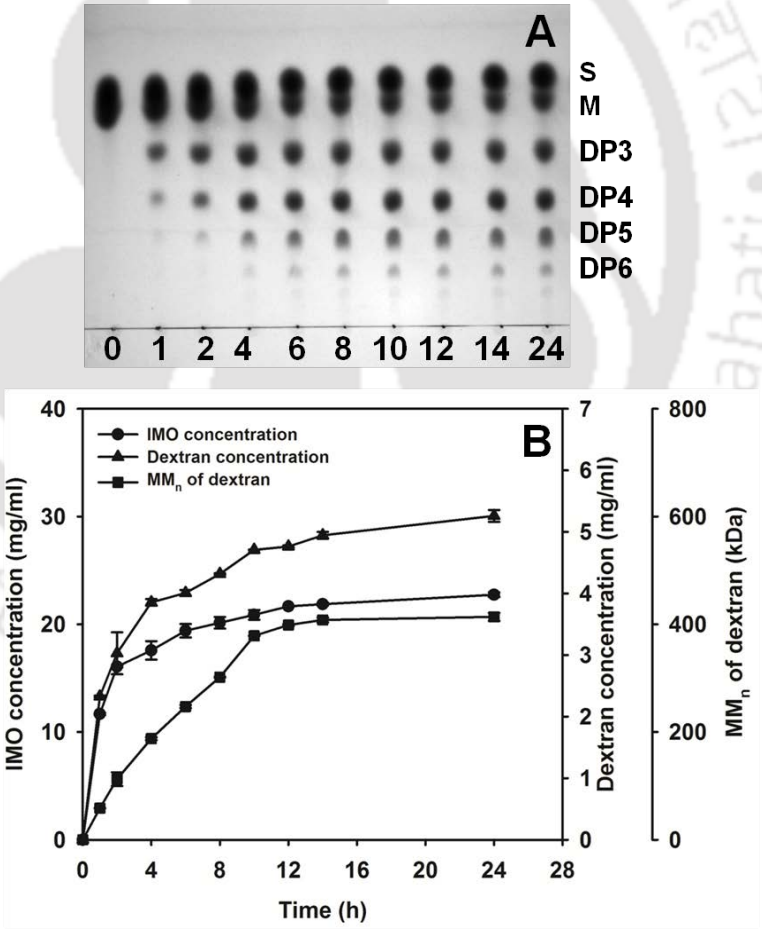

Fig. 5.3.1 (A) TLC of the acceptor reaction mixture of *L. mesenteroides* NRRL B-1426 dextransucrase (0.5 U/ml) using sucrose (5%, w/v) and maltose (5%, w/v) at pH 5.6 and 30°C from 0-24 h. (B) Synthesis of IMOs and dextran with their number average molecular mass (MM_n) at different time intervals (0-24 h).

The concentration of IMOs and dextran ranged from 11.7 to 22.7 mg/ml and 2.3 to 5.2 mg/ml, respectively. The number average molecular mass (MM_n) of dextran also increased with time, ranging from 55 to 414 kDa (Fig. 5.3.1B). Thus, 24 h was taken optimum time for the synthesis of IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase.

Table 5.3.1 Effect of time (0-24 h) on the degree of polymerization of IMOs. The acceptor reaction was carried out using 0.5 U/ml dextransucrase (10.14 U/mg, 0.76 mg/ml), 5% (w/v) sucrose and 5% (w/v) maltose at pH 5.6 and 30°C from 0-24 h.

Time (h)	IMOs (mg/ml)						
	DP3	DP4	DP5	DP6	DP7	DP8	Total
1	9.43	2.24	-	-	-	-	11.67
2	10.87	5.19	-	-	-	-	16.06
4	15.13	9.40	0.74	0.11	-	-	17.57
6	14.57	11.73	2.53	0.26	-	-	19.40
8	14.09	12.62	3.19	0.30	-	-	20.14
10	13.42	14.29	3.15	0.44	-	-	20.87
12	13.82	13.61	4.35	0.71	-	-	21.66
14	13.28	13.48	5.11	0.92	-	-	21.86
24	12.76	12.75	7.47	1.13	-	-	22.74

5.3.1.2 Effect of enzyme activity

The concentration of IMOs increased from 10.7 to 33.6 mg/ml as the enzyme activity was increased from 0.1 to 2 U/ml (Fig. 5.3.2). No further increase in the concentration of IMOs was observed beyond 2 U/ml enzyme activity (Fig. 5.3.2B). Higher than 2 U/ml dextransucrase activity showed little influence on the final IMOs yield. The enzyme activity above 0.5 U/ml did not affect much the DP of IMOs and retained the number of acceptor products four, *i.e.*, from DP3 to DP6 (Fig. 5.3.2A and Table 5.3.2). The concentration of dextran also increased from 1.8 to 12.8 mg/ml with increase in the dose of enzyme activity from 0.1 to 8 U/ml. However, an inverse

relationship of the MM_n of the synthesized dextran from 605 to 147 kDa was observed as the dextransucrase activity increased from 0.1 to 8 U/ml (Fig. 5.3.2B).

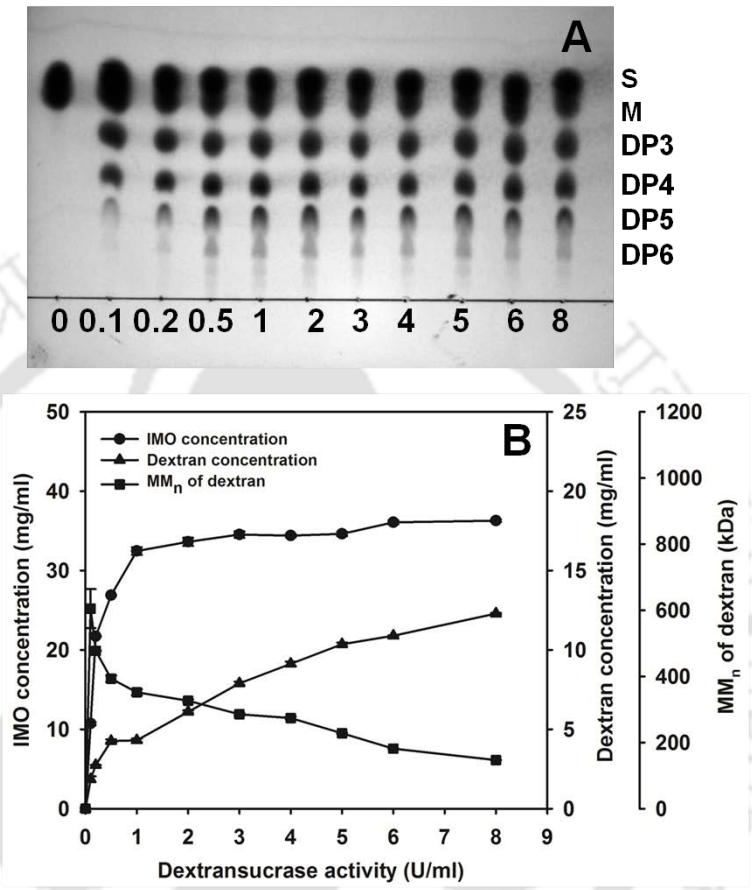

Fig. 5.3.2 (A) TLC of the acceptor reaction mixture of *L. mesenteroides* NRRL B-1426 dextransucrase (0.1-8 U/ml) using sucrose (5%, w/v) and maltose (5%, w/v) at pH 5.6, 30°C for 24 h. (B) Synthesis of IMOs and dextran with their number average molecular mass (MM_n) at different enzyme activity (0.1-8 U/ml).

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 (Robyt *et al.*, 2008; Falconer *et al.*, 2011). Therefore, these growing dextran chains will keep on adding more of D-glucose synthesizing longer chains of dextran resulting in higher MM_n.

Table 5.3.2 Effect of dextransucrase activity (0.1-8 U/ml) on the degree of polymerization of IMOs. The acceptor reaction was carried out using 0.1-8 U/ml dextransucrase (10.14 U/mg, 0.76 mg/ml), 5% (w/v) sucrose and 5% (w/v) maltose at pH 5.6 and 30°C for 24 h.

Dextransucrase activity (U/ml)	IMOs (mg/ml)						
	DP3	DP4	DP5	DP6	DP7	DP8	Total
0.1	9.49	1.22	-	-	-	-	10.71
0.2	9.32	10.71	1.68	-	-	-	21.71
0.5	10.49	12.35	3.70	0.34	-	-	26.88
1	11.33	12.59	8.08	0.46	-	-	32.46
2	11.99	12.66	8.43	0.53	-	-	33.62
3	12.79	12.39	8.78	0.57	-	-	34.52
4	13.10	11.56	9.18	0.55	-	-	34.39
5	13.69	11.37	9.03	0.53	-	-	34.62
6	13.35	12.37	9.75	0.61	-	-	36.08
8	14.13	12.59	9.56	0.33	-	-	36.27

5.3.1.3 Effect of temperature

The effect of temperature on acceptor reaction of dextransucrase was studied at 20, 25, 30, 35 and 40°C. The amount of IMOs increased as the temperature was increased from 20°C to 30°C and then decreased after that (Fig. 5.3.3). The optimum temperature for synthesis of IMOs was found to be 30°C. The number of acceptor products remained relatively constant at three or four (DP3-DP6) at different temperatures ranging from 20-40°C (Table 5.3.3). The concentration of IMOs first increased from 24.1 to 31.8 mg/ml with increasing temperature 20-30°C and then decreased after that to 19.7 mg/ml. Similar trends of the concentration and MM_n of dextran were also observed (Fig. 5.3.3B). The MM_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). 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). Moreover, the higher temperature enhances the rate of polymerization reaction of dextransucrase (Falconer *et al.*, 2011) and increases the branching of dextran (Robyt and Taniguchi, 1976), resulting in a higher molecular mass of dextran.

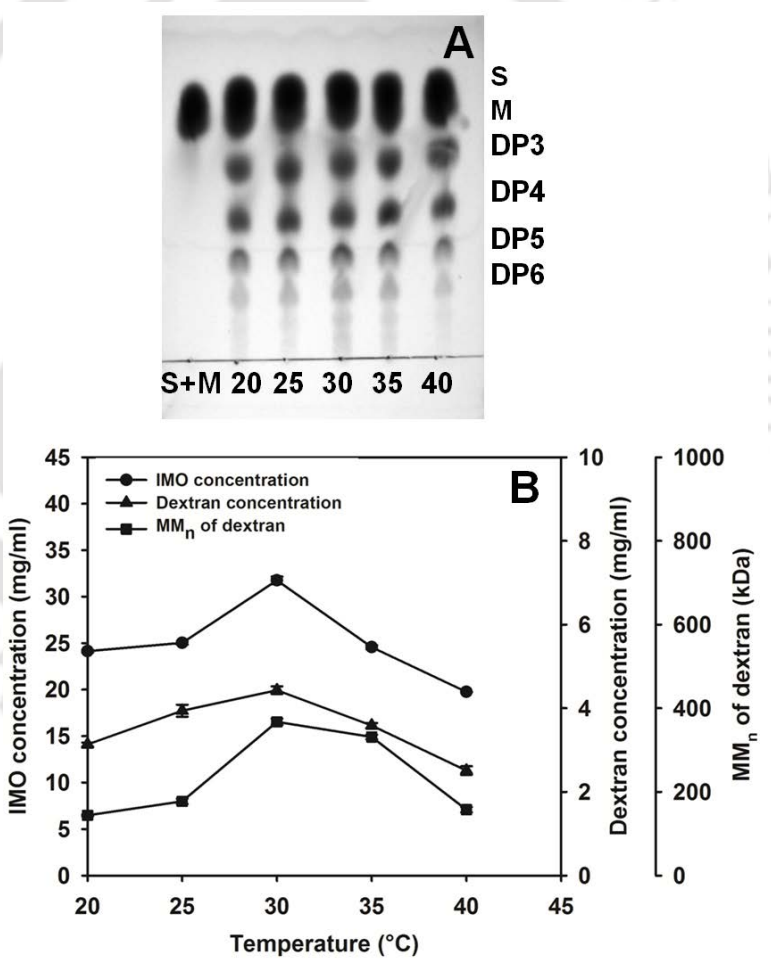

Fig. 5.3.3 (A) TLC of the acceptor reaction mixture of *L. mesenteroides* NRRL B-1426 dextransucrase (2 U/ml) using sucrose (5%, w/v) and maltose (5%, w/v) at 20-40°C and pH 5.6 for 24 h. (B) Synthesis of IMOs and dextran with their number average molecular mass (MM_n) at different temperatures (20-40°C).

Table 5.3.3 Effect of temperature (20-40°C) on the degree of polymerization of IMOs. The acceptor reaction was carried out at 20-40°C and pH 5.6 using 2 U/ml dextransucrase (10.14 U/mg, 0.76 mg/ml), 5% (w/v) sucrose and 5% (w/v) maltose for 24 h.

Temperature (°C)	IMOs (mg/ml)						
	DP3	DP4	DP5	DP6	DP7	DP8	Total
20	11.02	9.31	3.81	-	-	-	24.14
25	11.95	9.22	4.08	-	-	-	25.03
30	12.75	11.64	6.92	0.54	-	-	31.85
35	10.97	9.22	4.17	0.21	-	-	24.57
40	10.68	7.29	1.78	-	-	-	19.75

5.3.1.4 Effect of sucrose concentration

The sucrose concentration played an important role in the synthesis of IMOs with wide variation in degree of polymerization (DP). The concentration of IMOs increased from 10.7 to 46.1 mg/ml 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 and the MMn of dextran ranged of 103 to 416 kDa (Fig. 5.3.4) 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) (Fig. 5.3.4A and Table 5.3.4). 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). The dextran concentration (from 1.4 to 4.2) and its

MM_n (from 103 to 448 kDa) increased with the increase in the sucrose concentration from 1 to 4% (w/v) and then decreased till 7% (w/v) and further increased up to 15% (w/v) (Fig. 5.3.4B). Therefore, 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 MM_n (Falconer *et al.*, 2011). 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.

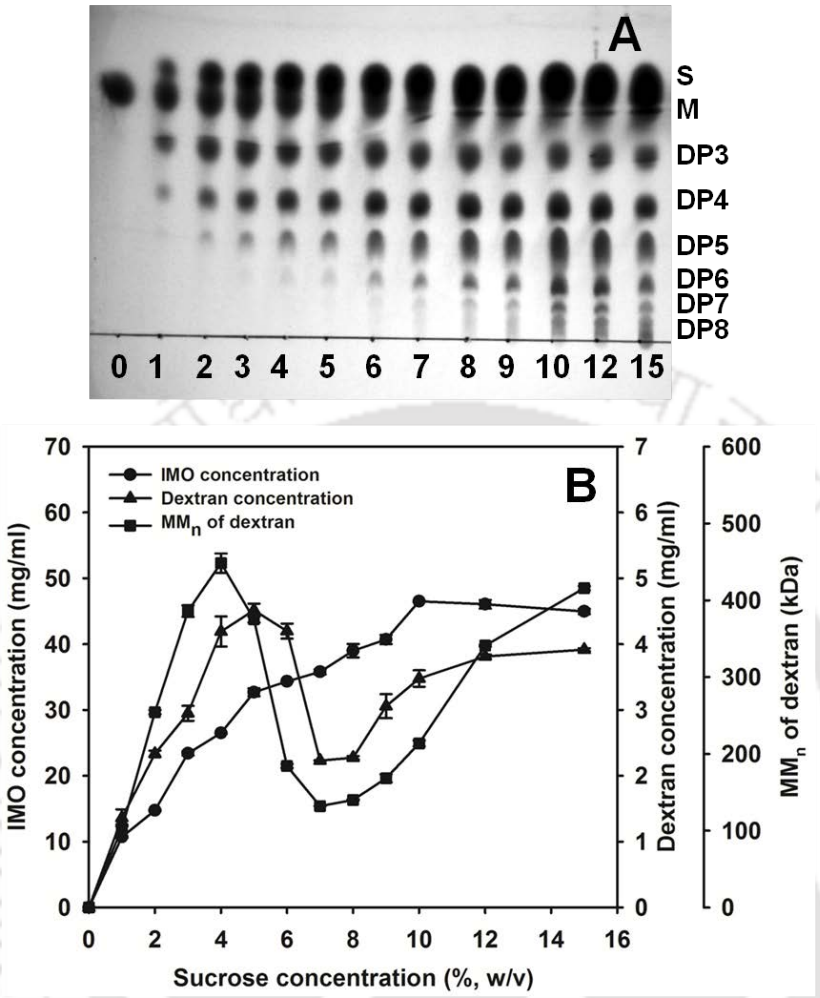

Fig. 5.3.4 (A) TLC of the acceptor reaction mixture of *L. mesenteroides* NRRL B-1426 dextransucrase (2 U/ml) using sucrose (1-15%, w/v) and maltose (5%, w/v) at pH 5.6 and 30°C for 24 h. (B) Synthesis of IMOs and dextran with their number average molecular mass (MM_n) at different sucrose concentration (1-15%, w/v).

Table 5.3.4 Effect of sucrose concentration (1-15%, w/v) on the degree of polymerization of IMOs. The acceptor reaction was carried out using 2 U/ml dextransucrase (10.14 U/mg, 0.76 mg/ml), 1-15% (w/v) sucrose and 5% (w/v) maltose at pH 5.6 and 30°C for 24 h.

Sucrose (%, w/v)	IMOs (mg/ml)						
	DP3	DP4	DP5	DP6	DP7	DP8	Total
1	8.07	2.64	-	-	-	-	10.71
2	10.63	4.11	-	-	-	-	14.75
3	12.29	9.98	1.13	-	-	-	23.41
4	13.07	10.77	2.66	-	-	-	26.51
5	13.52	11.37	7.34	0.46	-	-	32.69
6	13.23	12.45	8.06	0.60	-	-	34.34
7	11.82	12.98	9.63	0.97	0.39	-	35.79
8	12.83	14.96	9.07	1.62	0.51	-	39.01
9	11.59	16.42	10.24	1.73	0.72	-	40.70
10	9.62	13.11	12.66	8.99	1.60	0.53	46.50
12	9.63	12.66	10.86	9.07	3.29	0.55	46.06
15	10.61	11.69	8.39	6.90	6.83	0.52	44.96

5.3.1.5 Effect of maltose concentration

Maltose concentration played a very important role in the synthesis of IMOs by acceptor reaction of dextransucrase (Fig. 5.3.5). The concentration of IMOs ranged from 17.4 to 53.2 mg/ml with varying maltose concentration (0-15%, w/v) as shown in Fig. 5.3.5B. 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) (Fig. 5.3.5A and Table 5.3.5). The dextran concentration decreased from 29.3 to 0.75 mg/ml as maltose concentration increased from 0 to 15% (w/v). The number average molecular mass (MM_n) of dextran was also decreased from 2170 to 50 kDa on varying the maltose concentration from 0 to 15% (w/v) (Fig. 5.3.5B). This may be due to the dominance of acceptor reaction of dextransucrase with maltose resulting in lower concentration and MM_n of dextran.

The acceptor reaction of dextransucrase with maltose, a known best acceptor competes with the polymerization reaction to give low molecular mass 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 dextransucrase and 30°C for 24 h.

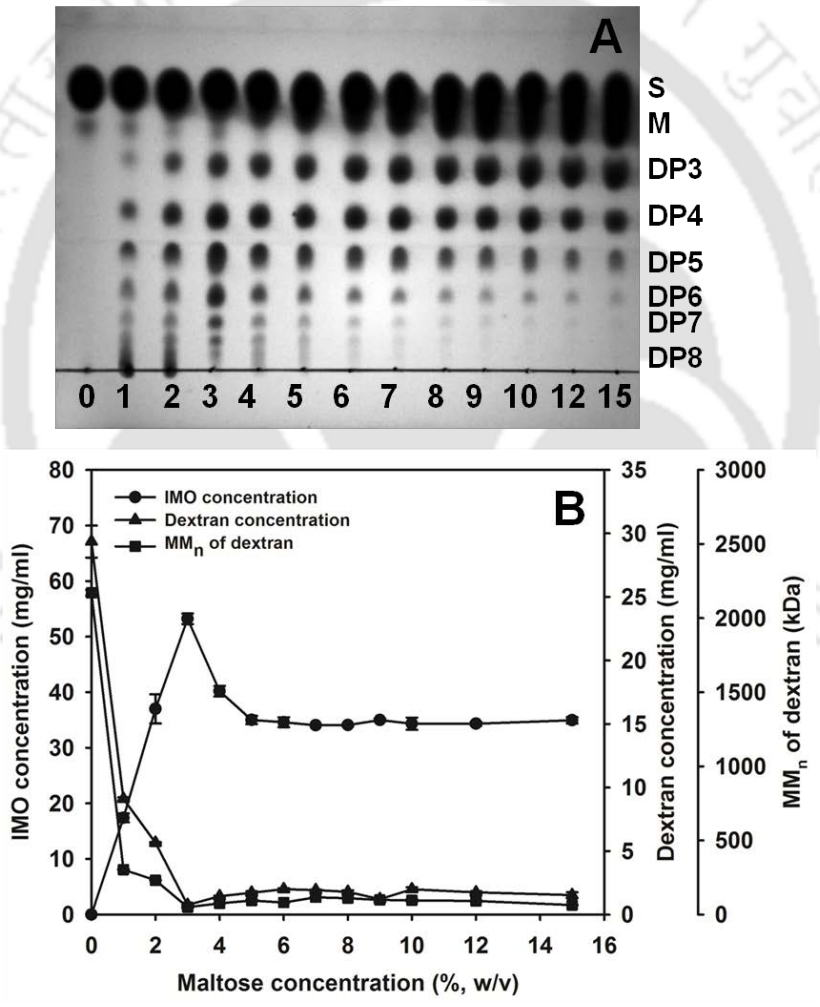

Fig. 5.3.5 (A) TLC of the acceptor reaction mixture of *L. mesenteroides* NRRL B-1426 dextransucrase (2 U/ml) using sucrose (7%, w/v) and maltose (1-15%, w/v) at pH 5.6 and 30°C for 24 h. (B) Synthesis of IMOs and dextran with their number average molecular mass (MM_n) at different maltose concentration (1-15%, w/v).

Table 5.3.5 Effect of maltose concentration (1-15%, w/v) on the degree of polymerization of IMOs. The acceptor reaction was carried out using 2 U/ml dextransucrase (10.14 U/mg, 0.76 mg/ml), 7% (w/v) sucrose and 1-15% (w/v) maltose at pH 5.6 and 30°C for 24 h.

Maltose (%, w/v)	IMOs (mg/ml)						
	DP3	DP4	DP5	DP6	DP7	DP8	Total
1	1.25	5.76	5.82	3.91	0.66	-	17.40
2	9.01	11.34	9.15	6.51	0.99	-	36.99
3	10.44	12.03	13.59	9.50	4.46	3.19	53.21
4	11.46	11.01	9.24	6.09	1.28	1.12	40.19
5	11.74	10.18	9.00	3.57	0.51	-	35.00
6	12.37	10.30	8.72	2.26	0.95	-	34.60
7	12.90	11.18	8.64	1.33	-	-	34.05
8	13.97	10.06	9.26	0.77	-	-	34.07
9	14.56	13.33	7.10	0.44	-	-	34.99
10	15.20	13.82	4.96	0.35	-	-	34.33
12	18.01	11.42	4.65	0.25	-	-	34.33
15	18.98	11.59	4.26	0.12	-	-	34.95

5.3.2 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. 5.3.6A). The collected fractions corresponding to the peak (45-89 ml) in the chromatogram were analyzed by TLC (Fig. 5.3.6B), HPLC (Fig. 5.3.7) and ESI-TOF MS (Fig. 5.3.8) analyses. The TLC and HPLC analyses showed that with the increase in the fraction number, the DP of IMOs decreased (Fig. 5.3.6B and 5.3.7). 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. 5.3.8).

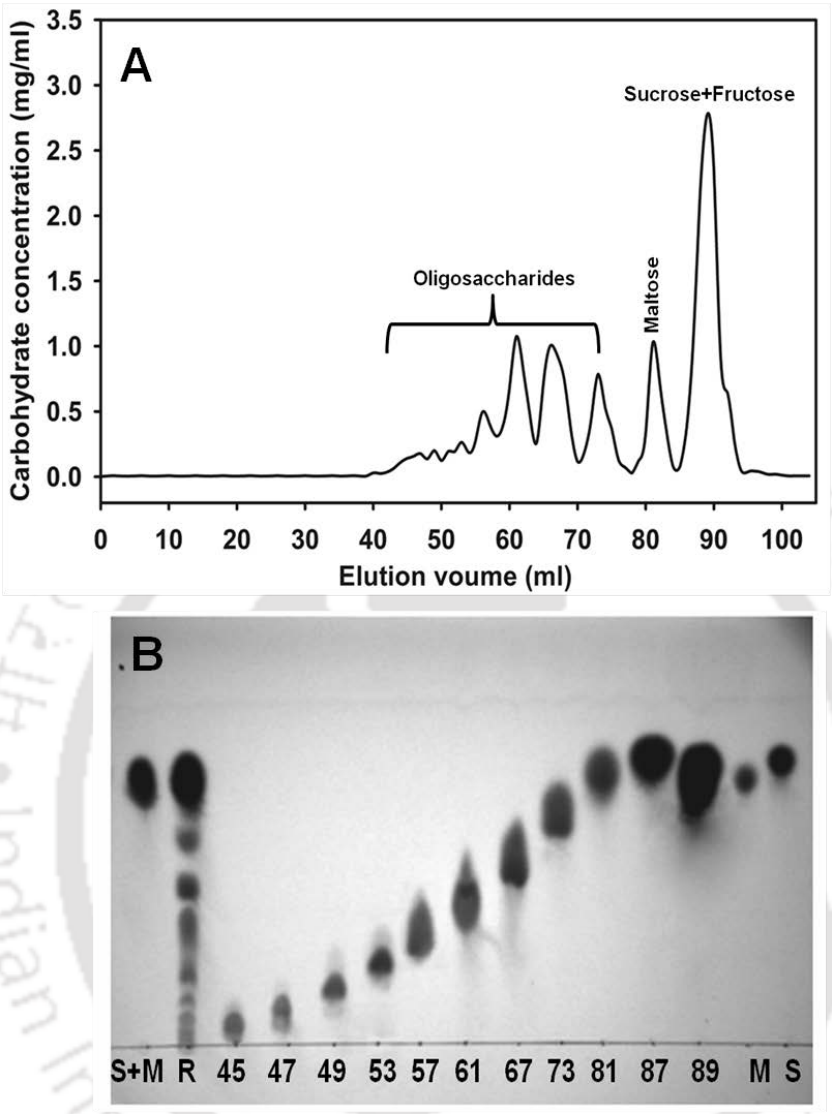

Fig. 5.3.6 (A) Bio-Gel P-2 chromatogram from GPC of acceptor reaction mixture from *L. mesenteroides* NRRL B-1426 dextransucrase using sucrose and maltose and (B) TLC of the selected Bio-Gel P-2 fractions containing IMOs, S+M-sucrose and maltose; R-acceptor reaction mixture containing IMOs; 45 to 89-fraction numbers; M-Maltose and S-Sucrose.

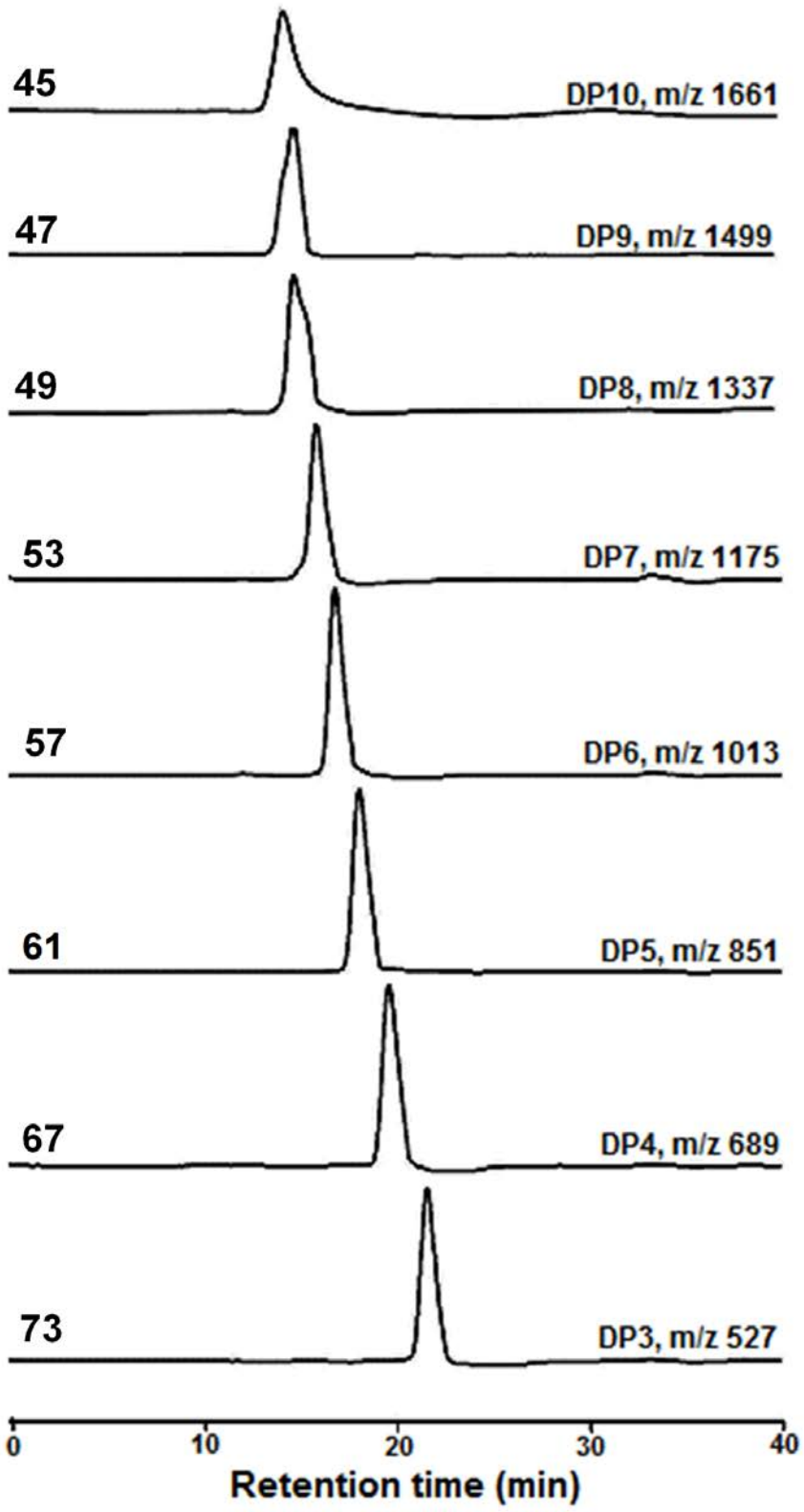

Fig. 5.3.7 HPLC of the selected Bio-Gel P-2 fractions (45 to 73) containing IMOs from acceptor reaction of *L. mesenteroides* NRRL B-1426 dextransucrase.

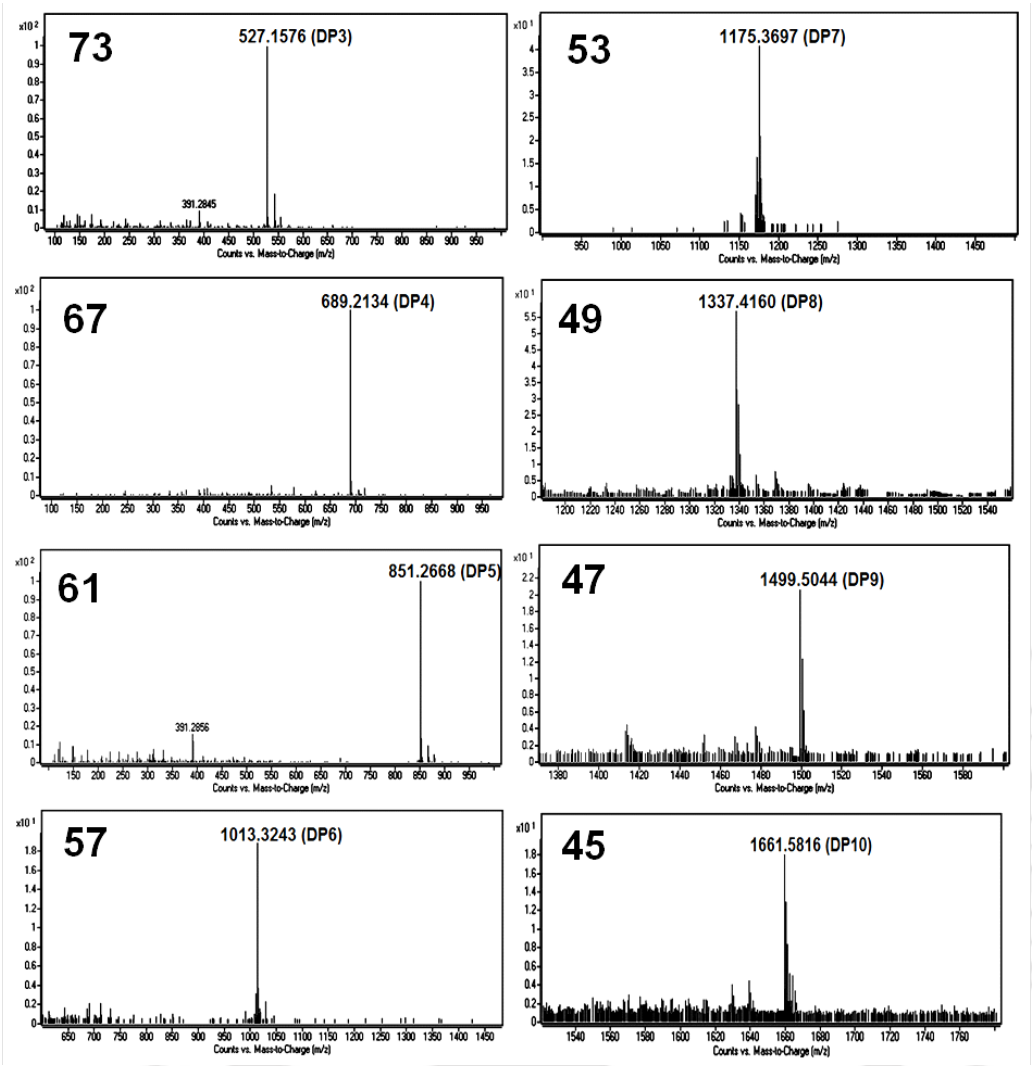

Fig. 5.3.8 ESI-TOF MS of the selected Bio-Gel P-2 fractions (45 to 73) containing IMOs from acceptor reaction of *L. mesenteroides* NRRL B-1426 dextransucrase.

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. 5.3.8). 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 as described in the Chapter 2, Section 2.3.3 and (ii) low branched (~14.5%) dextran as described in the Chapter 3, Section 3.3.1.6.

5.3.3 *In vitro* analysis of prebiotic application of IMOs

5.3.3.1 Effect of simulated human gastric juice on digestibility of IMOs

Indigestibility is a one of the key criteria for oligosaccharide to act as a prebiotic (Wong and Jenkins, 2007; Wang, 2009). 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. 5.3.9). 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. 5.3.9A), whereas the inulin showed 39.7%, 19.4% and 13.8% of hydrolysis at the respective pHs (Fig. 5.3.9B). The IMOs showed significantly lower (1.9 to 20.9%)

hydrolysis at pH 3 to 1 (Fig. 5.3.9A) as compared with the standard inulin (4.7 to 39.7%) (Fig. 5.3.9B).

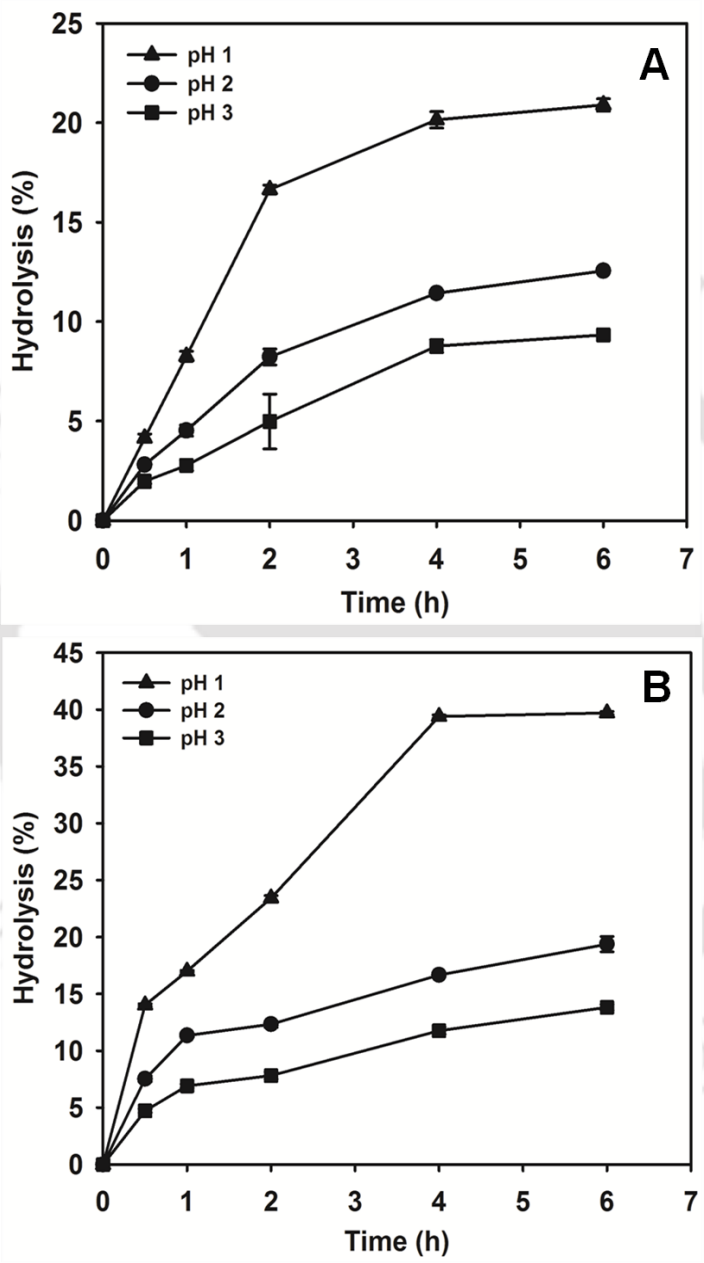

Fig. 5.3.9 Hydrolysis of (A) IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase and (B) inulin by simulated human gastric juice at pH 1, 2 and 3 at 37°C for 6 h. The hydrolysis of IMOs at pH 1 was significantly different *vs* inulin at $p \leq 0.01$ and significantly different at pH 2 and 3 *vs* inulin at $p \leq 0.05$.

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). Focusing 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.

5.3.3.2 Effect of human α-amylase on digestibility of IMOs

Utilization of oligosaccharides by probiotics, not otherwise digestible by human enzymes, has been recognized as an important attribute of prebiotics (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. 5.3.10). 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.

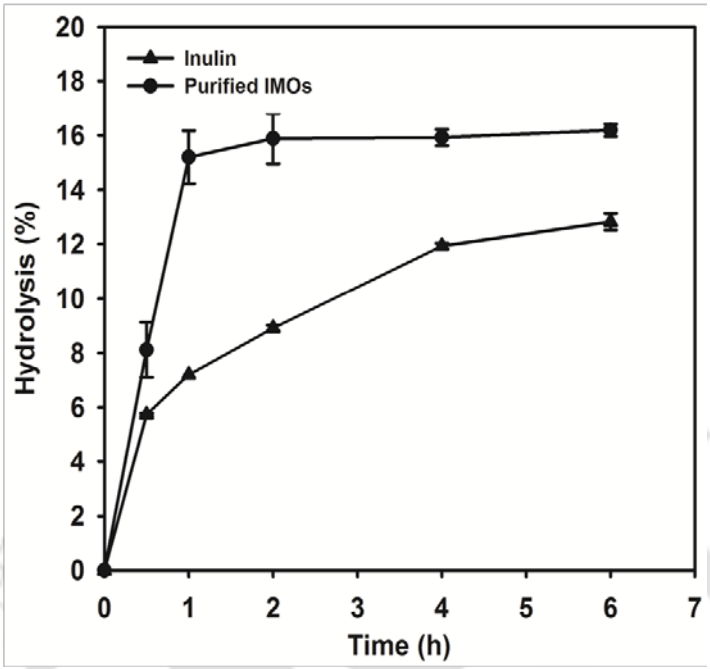

Fig. 5.3.10 Enzymatic hydrolysis of IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase and inulin by human α-amylase (pH 7) at 37°C for 6 h. The hydrolysis of IMOs was significantly different *vs* inulin ($p \leq 0.05$).

5.3.3.3 Effect of IMOs on the growth of probiotics

The effect of purified IMOs (DP ≥ 3) on the growth of probiotics is shown in Fig. 5.3.11. The IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase acceptor reaction stimulated the growth of *L. acidophilus* and was similar to that displayed by inulin (Fig. 5.3.11A). The maximum absorbance (A_{600}) with IMO and inulin for *L. acidophilus* was within 1.4-1.6 after 24 h (Fig. 5.3.11A). 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. 5.3.11B).

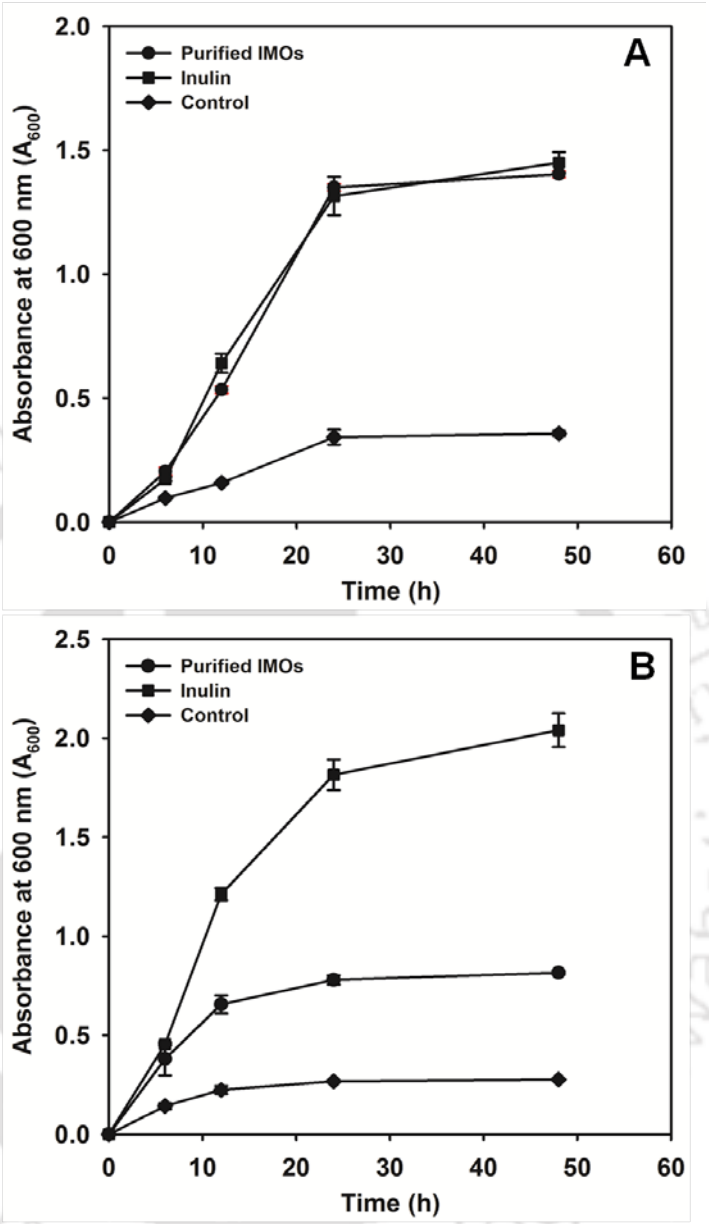

Fig. 5.3.11 Growth profile of (A) *L. acidophilus* and (B) *B. infantis* in presence of IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase and inulin. MRS medium without any carbon source as control. The growth of *L. acidophilus* was significantly different *vs* control ($p \leq 0.01$).

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.*, 2007). The growth of probiotics was correlated with the

change in pH of the fermentation medium. The changes in the pH of the medium fermented by *B. infantis* and *L. acidophilus* are shown in Table 5.3.6.

Table 5.3.6 pH changes of probiotics during fermentation of medium.

Prebiotic	*L. acidophilus* Time (h)		*B. infantis* Time (h)	
	0	**24**	**0**	**24**
IMOs (DP ≥ 3)[§]	6.4±0.0	4.7±0.02**	6.4±0.0	5.9±0.04
Inulin	6.4±0.0	5.2±0.03**	6.4±0.0	4.8±0.02**
Control	6.4±0.0	6.4±0.12	6.4±0.0	6.1±001

[§]From *L. mesenteroides* NRRL B-1426.
**Significantly different ($p \leq 0.01$) *vs* control.

The fermentation of IMOs by *L. acidophilus* significantly reduced the pH of the growth medium as compared with inulin ($p \leq 0.01$). The reduction in pH by *L. acidophilus* was 4.7 and 5.2 for IMOs and inulin, respectively, after 24 h (Table 5.3.6). However, no significant change in the pH of medium containing IMOs was observed in case of *B. infantis*. Hence, it could be infered 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).

5.3.4 *In vitro* analysis of effect of IMOs on mammalian cells

The use of nutraceuticals or functional foods as potential cancer prevention agents has become important in current health- and cancer-related research (Li *et al.*, 2013). The effects of IMOs (DP ≥ 3) from *L. mesenteroides* NRRL-1426 dextransucrase on human embryonic kidney (HEK-293), human cervical adenocarcinoma (INT-407) and human colon carcinoma (HT-29) cell lines were investigated using the colorimetric MTT assay (Fig. 5.3.12 and Fig. 5.3.13). The cell viability of HEK-293 (Fig. 5.3.12A) and INT-407 (Fig. 5.3.12B) were not affected by the IMOs, rather a slight increase in the growth of both was also observed as compared with the control cells. This increased growth may be attributed to the potential of IMOs serving as an additional carbon source. The oligosaccharides have 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.

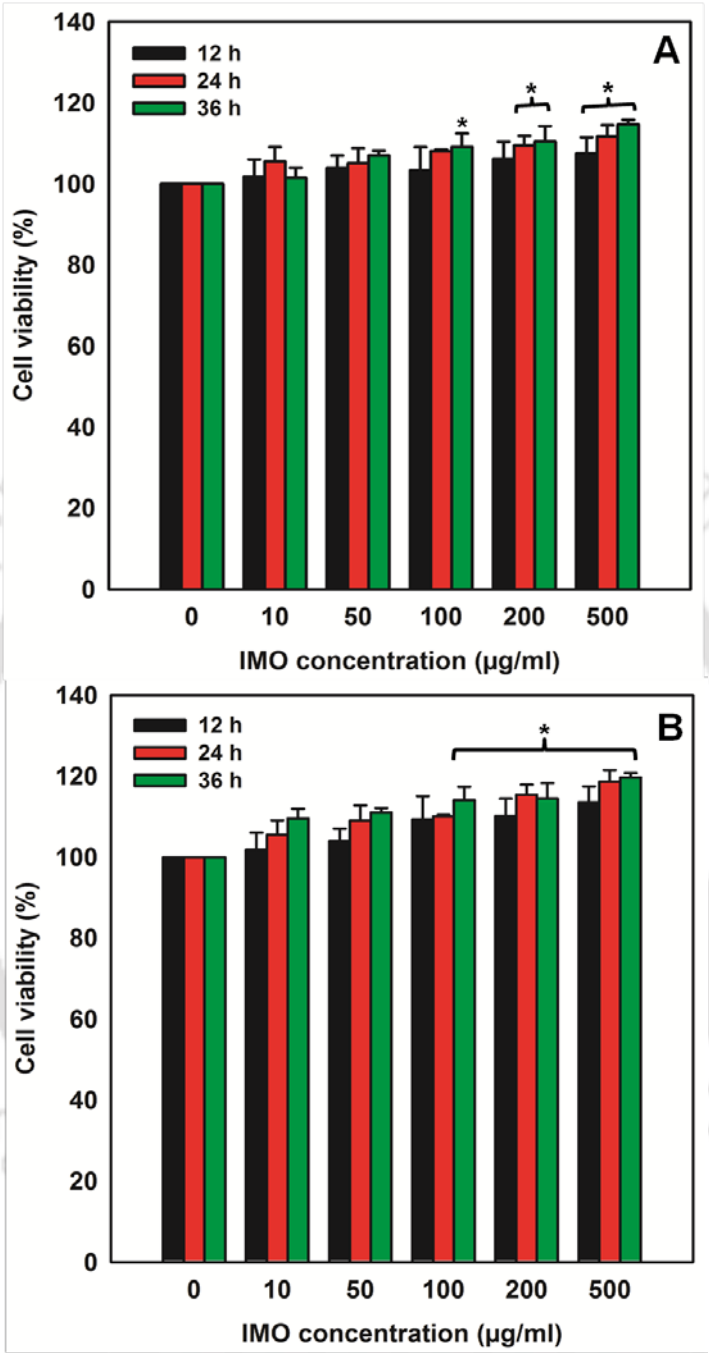

Fig. 5.3.12 The effect of IMOs on the viability of (A) HEK-293 and (B) INT-407 cells at different concentrations of IMOs (0-500 µg/ml) for 12 h, 24 h and 36 h of incubation. *Significantly different ($p \leq 0.05$) *vs* control.

The inhibition in the viability of HT-29 cells was time- and dose-dependent with increasing concentrations of IMOs from 10 to 500 µg/ml. The viability of HT-29

cells were significantly inhibited by 14%, 21%, 24%, 29% and 34% at IMOs concentrations of 10, 50, 100, 200 and 500 µg/ml, respectively, after 36 h (Fig. 5.3.13). 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) was observed and hence, can be used for the development of colon cancer chemopreventive agent. Although further in *vitro* and in *vivo* bioactivity studies are required for its confirmation. The chemopreventive effects on HT-29 colon cancer cells were also reported for chito-oligosaccharides (Nam *et al.*, 2007) and apple oligosaccharides (Li *et al.*, 2013). Therefore, IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase could act as bioactive oligosaccharides with potential pharmaceutical and functional food applications.

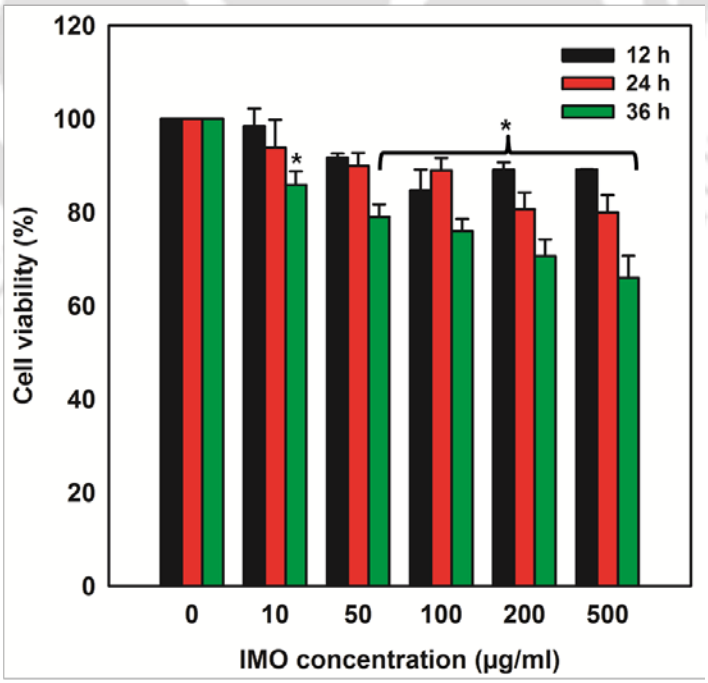

Fig. 5.3.13 The effect of IMOs on the viability of HT-29 cells at different concentrations of IMOs (0-500 µg/ml) for 12 h, 24 h and 36 h of incubation. *Significantly different ($p \leq 0.05$) *vs* control.

5.4 Conclusions

The acceptor reaction conditions for the synthesis of IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase were optimized by studying the effect of time, enzyme activity, temperature, concentration of sucrose and maltose. The optimum conditions for synthesis of IMOs were found to be 24 h, 2 U/ml of dextransucrase, 30°C, 7% (w/v) sucrose and 3% (w/v) maltose. The synthesis of IMOs was also correlated with the concentration and number average molecular mass (MM_n) of dextran. The dextran concentration ranged from 0.8 to 29 mg/ml and MM_n of dextran varied from 50 to 2170 kDa. IMOs were purified by gel permeation chromatography using Bio-Gel P-2 column based on their degree of polymerization. TLC and HPLC analyses revealed the *L. mesenteroides* NRRL B-1426 dextransucrase synthesized IMOs from DP3 to 10, as also confirmed by ESI-TOF MS. Since the long-chain IMOs are preferred over short chain ones owing to the longer persistence in the colon. Therefore, the present study provides an alternative method for large scale production of IMO by using the acceptor reaction of *L. mesenteroides* NRRL B-1426 dextransucrase. The purified IMOs (DP $\geq$ 3) showed lower degree of digestibility of 20% by simulated human gastric juice at pH 1.0 as compared with commercial prebiotic, inulin (40%). The IMOs supported the growth of *Lactobacillus acidophilus* similar to that by inulin. The purified IMOs showed nontoxic effect on HEK-293 and INT-407 cell lines, displaying biocompatible nature. However, selective inhibition of viability of HT-29 cells by IMOs (DP $\geq$ 3) proved their potential for the development of colon cancer chemopreventive agent. With these desirable characteristics, the IMOs from *L. mesenteroides* NRRL B-1426 dextransucrase can act as potential ingredient for functional foods.

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Chapter 6

Synthesis, purification and prebiotic applications of gentio-oligosaccharides from the acceptor reaction of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426

6.1 Introduction

There is a growing interest in the synthesis of oligosaccharides because these carbohydrates confer functional and prebiotic activities to animals as well as human and provide them health benefits (Rabelo *et al.*, 2009). Gentiobiose, a β-(1→6) linked glucodisaccharide, is a well known sugar that possesses bitter taste. This bitterness makes gentiobiose a useful taste improver for certain beverages (Cote *et al.*, 2003). The β-(1→6) linkages occur frequently in the glucans of yeast and filamentous-fungal cell walls (Manners *et al.*, 1973). However, there have been only a limited number of reports of free gentiobiose as oligosaccharin in xylem sap (Adamas and Haq, 1962) and in tomato (Dumville and Fry, 2003) and as a structural component of crocin, a saffron constituent (Escribano *et al.*, 1994). The gentiobiose contained in the structure of crocin is regarded as a determinant moiety that plays an important role in the antitumor activity of saffron (Sugiura *et al.*, 1994; Abe and Saito, 2000; Ghadrdoost *et al.*, 2011).

Fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS) and inulin are well established as prebiotics (Gibson *et al.*, 2004; de Vrese and Schrezenmeir, 2008;

Goffin *et al.*, 2011; Moller *et al.*, 2012). However, many oligosaccharides are still under investigation for their prebiotic potential. One of them is gentio-oligosaccharides (GnOS), a β-(1→6) linked gluco-oligosaccharide (Sanz *et al.*, 2006). In general, GnOS are not prevalent as free molecules (Fujimoto *et al.*, 2009). GnOS are conventionally obtained by the partial hydrolysis of the lichen polysaccharide, pustulan (Nanzo *et al.*, 1984), which gives low yields of the desired higher oligosaccharides. Chemical methods for GnOS synthesis have been also developed, but they are multistep involving a number of protection, glycosylation and deprotection reactions (Zhu and Kong, 2001). In general, enzyme-catalyzed synthesis of oligosaccharides is a useful method because it allows the formation of well-defined oligosaccharides with high regio- and stereo-selectivity (Kothari and Goyal, 2013). Promising sources of GnOS are those obtained by enzymatic synthesis using alternansucrases (Sanz *et al.*, 2006). Dextransucrase through its acceptor reaction using sucrose and gentiobiose can also be used for the synthesis of GnOS (Robyt and Eklund, 1983; Kothari and Goyal, 2013). GnOS have shown promising potentials as prebiotics by showing bifidogenic and lactobacilli-promoting effect *in vitro* (Rycroft *et al.*, 2001; Sanz *et al.*, 2006) and as low-glycemic sweetener (Cote, 2009). The bitterness of gentiobiose makes GnOS useful as a taste improver for certain beverages (Cote *et al.*, 2003; Fujimoto *et al.*, 2009). Moreover, the gentiobiose derivatives, bearing different alkyl groups at C-1, are also reported to act as antitumor agents against human leukemia (HL-60) cells (Marrero *et al.*, 2012). Such characteristics suggest the further potential of the gentiobiose derived oligosaccharides in food industry.

In the present study, the transglycosylation of gentiobiose was mediated by *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase for the synthesis of GnOS. The GnOS was purified by gel permeation chromatography and characterized by TLC, HPLC and ESI-TOF MS. The prebiotic potential of purified GnOS (DP ≥ 3) in terms of gut digestibility (acidic and enzymatic) and stimulation of growth of probiotics was investigated. In addition, the effects of GnOS (DP ≥ 3) on human embryonic kidney (HEK-293), human cervical adenocarcinoma (INT-407) and human colon carcinoma (HT-29) cell lines were studied.

6.2 Materials and Methods

6.2.1 Chemicals and reagents

All the media components for maintenance and enzyme production were purchased from Hi-Media Pvt. Ltd., India. All the organic solvents were purchased from Qualigens, India. D-Gentiobiose, D-sucrose, α-amylase from human saliva and all the chemicals required for cell culture were purchased from Sigma Aldrich, USA. 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.

6.2.2 Microorganisms and culture conditions

Leuconostoc mesenteroides NRRL B-1426, *Bifidobacterium infantis* NRRL B-41661 and *Lactobacillus acidophilus* NRRL B-4495 were procured from ARS culture collection, National Centre for Agricultural Utilization Research, Peoria, USA. *L. mesenteroides* NRRL B-1426 was grown and maintained in modified MRS agar stab as described in Chapter 2, Section 2.2.2. The probiotic cultures were grown and maintained in MRS medium as described in Chapter 3, Section 3.2.2.

6.2.3 Synthesis and purification of GnOS

L. mesenteroides NRRL B-1426 dextransucrase was partially purified by 33% (v/v) PEG-400 as described in Chapter 2, Section 2.2.8 and was used for synthesis of GnOS. The acceptor reaction of dextransucrase was carried out in 25 ml reaction mixture containing 3 ml enzyme (10.1 U/mg, 0.76 mg/ml), gentiobiose-to-sucrose ratio as 1:1 at 146 mM, 0.3 mM $CaCl_2$ and 15 mM NaN_3 in 20 mM sodium acetate buffer (pH 5.6) and by incubation at 30°C as described in the Chapter 4, Section

4.2.3. The reaction was terminated by adding equal volume of absolute ethanol after 24 h and centrifuged at 16,000*g* and 4°C for 10 min to remove the polymeric dextran. The 50 ml supernatant containing GnOS, fructose, residual sucrose and gentiobiose was concentrated to 25 ml using a rotary vacuum evaporator (IKA, HB 10). The supernatant containing oligosaccharides was purified on the basis of their degree of polymerization (DP) by gel permeation chromatography (GPC) using XK16/70 column (GE Healthcare) packed with Bio-Gel P-2 (fine mesh) on AKTA purifier (GE Healthcare, model 100 Plus) as described in the Chapter 5, Section 5.2.6. The fractions were analyzed for the concentration of oligosaccharides by phenol-sulfuric acid method (as described in Chapter 3, Section 3.2.4) and identified by TLC (as described in Chapter 2, Section 2.2.13.5). The selected fractions of purified GnOS of various DP were analyzed by HPLC and identified by ESI-TOF MS as described in Sections 5.2.6.1 and 5.2.6.2 of Chapter 5, respectively. The purified GnOS of DP ≥ 3 were pooled, lyophilized and tested for their prebiotic and cytotoxic activity.

6.2.4 *In vitro* analysis of GnOS application as prebiotic

6.2.4.1 Effect of simulated human gastric juice on digestibility of GnOS

The effect of digestibility of simulated human gastric juice on GnOS (DP ≥ 3) was studied as described in the Chapter 5, Section 5.2.7.1.

6.2.4.2 Effect of human α-amylase on digestibility of GnOS

The effect of digestibility of human α-amylase on GnOS (DP ≥ 3) was studied as described in the Chapter 5, Section 5.2.7.2.

6.2.4.3 Effect of GnOS on the growth of probiotics

The growth stimulatory effects of GnOS (DP ≥ 3) and inulin on *B. infantis* and *L. acidophilus* were evaluated as described in the Chapter 5, Section 5.2.7.3. The growth profile of probiotics was correlated with the corresponding pH changes in the medium and pH was measured using a digital pH meter (Sartorious, PP-20).

6.2.5 *In vitro* analysis of effect of GnOS on mammalian cells

6.2.5.1 Culturing and maintenance of mammalian cells

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 Centre for Cell Sciences (NCCS), Pune, India. The HEK-293, INT-407 and HT-29 cells were cultured and maintained in DMEM, MEM and RPMI, respectively at 37ºC in a humidified incubator at 5% CO_2 saturation as described in Chapter 3, Section 3.2.8.1

6.2.5.2 Cell viability assay

The effects of purified GnOS (DP ≥ 3) at concentrations ranging from 10-500 µg/ml on viability of HEK-293, INT-407 and HT-29 cell lines were evaluated by MTT assay for 36 h as described in the Chapter 3, Section 3.2.8.3

6.2.6 Statistical analysis

The prebiotic application and mammalian 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% ($p \leq 0.05$).

6.3 Results and Discussion

6.3.1 Synthesis and purification of GnOS

The acceptor reaction of dextransucrase from *L. mesenteroides* NRRL B-1426 using sucrose and gentiobiose led to the synthesis of GnOS (Fig. 6.3.1). The acceptor reaction mixture containing GnOS collected after ethanol precipitation was separated on Bio-Gel P-2 column based on their DP.

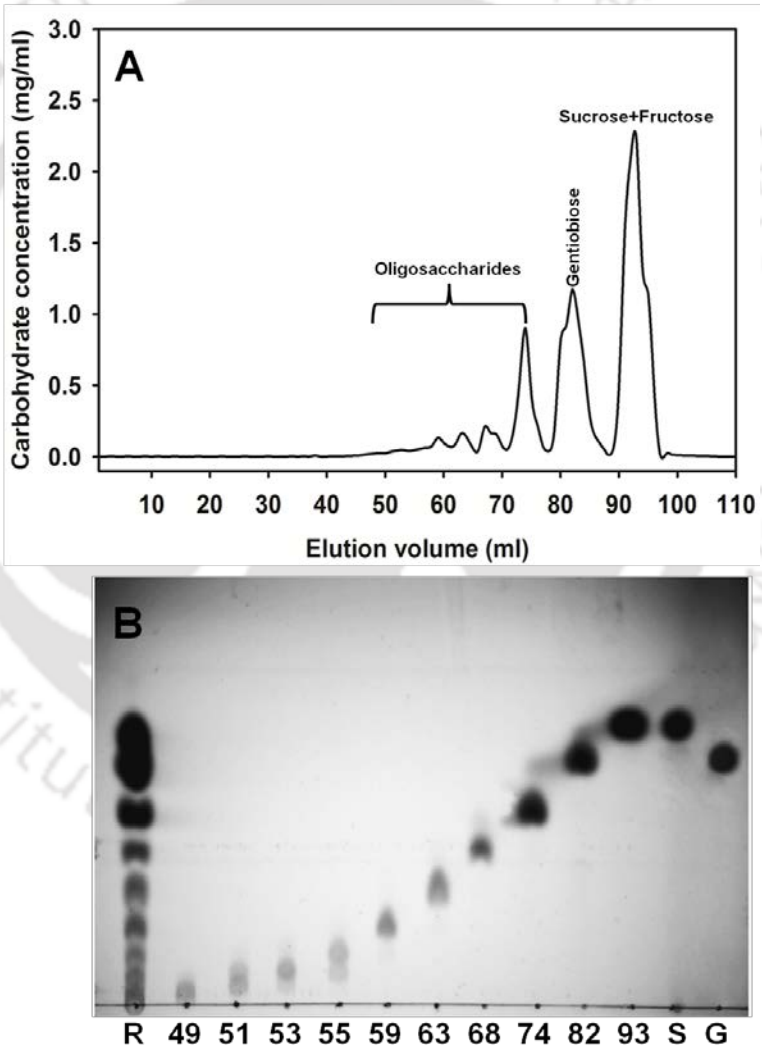

Fig. 6.3.1 (A) Gel permeation chromatography of acceptor reaction mixture from *L. mesenteroides* NRRL B-1426 dextransucrase using Bio-Gel P-2 (XK 16/70) and (B) TLC of the selected Bio-Gel P-2 fractions containing oligosaccharides, R-acceptor reaction mixture containing oligosaccharides; 49 to 93-fraction numbers; S-Sucrose and G-Gentiobiose.

The separation profile showed that the oligosaccharides were eluted from 49 to 78 ml based on their carbohydrate content (Fig. 6.3.1A). The selected fractions corresponding to the peak in the separation profile were analyzed by TLC (Fig. 6.3.1B), HPLC (Fig. 6.3.2A) and ESI-TOF MS (Fig. 6.3.2B) analyses. The TLC and HPLC analyses showed that with the increase in the fraction number, the DP of oligosaccharides decreased (Fig. 6.3.1B and 6.3.2A). The chromatography of the selected purified fractions of GnOS revealed that *L. mesenteroides* NRRL B-1426 dextransucrase synthesized GnOS from DP 3 to 10. However, ESI-TOF MS of the selected fractions showed the peaks at m/z 527.1, 689.2, 851.3 and 1013.3 corresponding to the $[M+Na]^+$ ions of GnOS from DP3 to DP6, respectively (Fig. 6.3.2B). The fractions 59, 63, 68 and 74 contained DP6, DP5, DP4 and DP3, respectively. The GnOS of DP7-DP10 observed in TLC and HPLC analysis was not detected in the ESI-TOF MS analysis under the conditions used (Fig. 6.3.2B). The ESI-TOF MS analysis of GnOS confirmed that they belong to a homologous hexose series like maltose and isomaltose acceptor products of dextransucrase. Gentiobiose has also been reported serving as an acceptor for *L. mesenteroides* NRRL B-512F dextransucrase (Yamauchi and Ohwada, 1969) and *L. mesenteroides* NRRL B-21297 alternansucrase (Cote, 2009) for the synthesis of oligosaccharides. In both the above cases, the first acceptor product synthesized trisaccharide is same, which is a gentiobiose bearing a α-D-glucopyranosyl unit at the free 6-position [α-D-Glcp-(1→6)-β-D-Glcp-(1→6)-D-Glc] (Cote, 2009). The higher DP products (DP > 3) from dextransucrase acceptor reactions with gentiobiose have not yet been isolated and identified. The product distribution in an acceptor reaction depends on relative rates

of formation of acceptor products from each immediate precursor, rather than on an absolute structural specificity of dextransucrase (Cote, 2009).

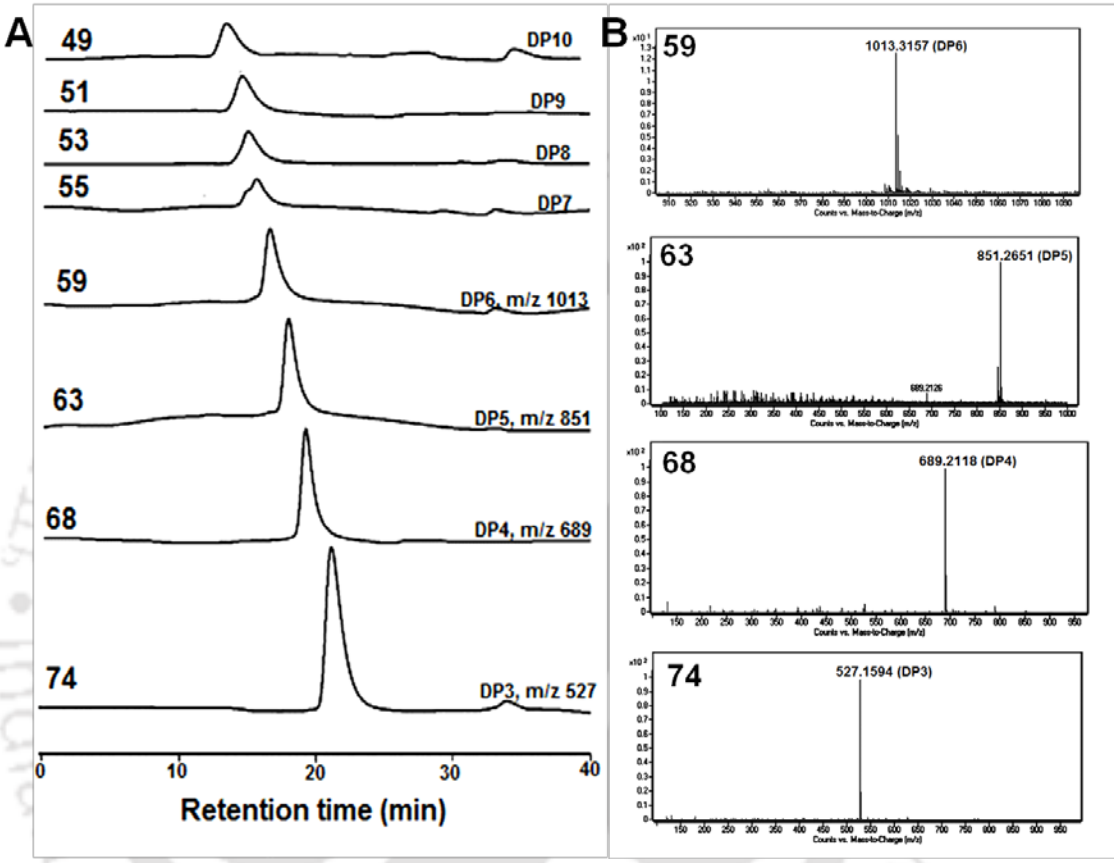

Fig. 6.3.2 (A) HPLC and (B) mass spectrometry of selected Bio-Gel P-2 fractions containing GnOS from acceptor reaction of *L. mesenteroides* NRRL B-1426 dextransucrase.

6.3.2 *In vitro* analysis of application of GnOS as prebiotic

6.3.2.1 Effect of simulated human gastric juice on digestibility of GnOS

Colon is the main site for action of prebiotics, where they are fermented by the probiotic bacteria. Thus, a prebiotic must withstand the effects of gastric acidity and digestive enzymes in order to reach the colon intact (Das *et al.*, 2014). The GnOS (DP ≥ 3) from *L. mesenteroides* NRRL B-1426 dextransucrase was found resistant to hydrolysis by simulated human gastric juice. The percent hydrolysis of GnOS and

inulin increased with increase in incubation period and with decrease in the pH of simulated human gastric juice (Fig. 6.3.3).

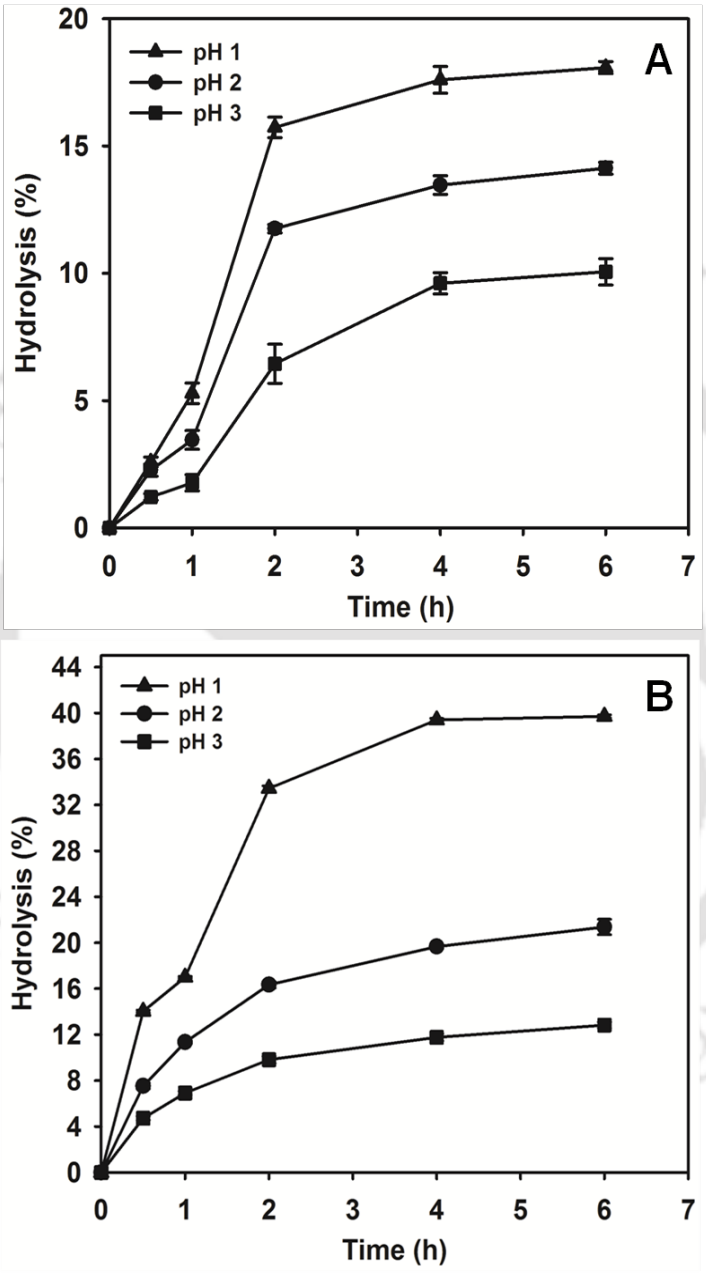

Fig. 6.3.3 Acidic hydrolysis of (A) GnOS (DP ≥ 3) from *L. mesenteroides* NRRL B-1426 dextransucrase and (B) inulin by simulated human gastric juice at pH 1, 2 and 3 at 37 °C for 6 h. The hydrolysis of GnOS at pH 1 was significantly different *vs* inulin at $p \leq 0.01$ and significantly different at pH 2 and 3 *vs* inulin at $p \leq 0.05$.

The maximum hydrolysis of GnOS after 6 h at pH of 1, 2 and 3 was 18.1%, 14.1%, and 10.1%, respectively (Fig. 6.3.3A), whereas the inulin showed 39.7%, 21.4% and 12.8% of hydrolysis at the respective pHs (Fig. 6.3.3B).The GnOS showed significantly lower (10.1 to 18.1%) hydrolysis at pH 3 to 1 after 6 h (Fig. 6.3.3A) as compared with standard prebiotic, inulin (12.8 to 39.7%) ($p \leq 0.01$) (Fig. 6.3.3B). The GnOS exhibited 2.2, 1.5 and 1.3 fold higher resistance than inulin at pH 1, 2 and 3, respectively, after 6 h of incubation. This was due to the presence of β-(1→6) linkages as β-linkages are reported to be stronger than α-linkages (Mussatto and Mancilha, 2007). As a result, GnOS (DP ≥ 3) from *L. mesenteroides* NRRL B-1426 dextransucrase would reach the colon in more or less intact form to be utilized by probiotics. These findings are also pertinent for acidic foods such as yoghurt and dairy products that can be supplemented with prebiotics as reported earlier by Huebner *et al*. (2008).

6.3.2.2 Effect of human α-amylase on digestibility of GnOS

The GnOS (DP ≥ 3) from *L. mesenteroides* NRRL B-1426 dextransucrase showed significantly lower degree of digestibility (7.1%) by human α-amylase (pH 7) as compared with inulin (12.8%) after 6 h (Fig. 6.3.4) ($p \leq 0.01$). The brush border enzymes in small intestine *viz.*, isomaltase, glucoamylase, maltase, sucrase and lactase hydrolyse the carbohydrates α-(1→4) and α-(1→6) linked gluco-saccharides, yielding monosaccharides as end products (Johnson and Schmit, 2005; Wichienchot *et al.*, 2010). It has also been reported that at least 60% of gluco-oligosaccharide produced by *Gluconobacter oxydans* NCIMB 4943 reaches the colon (Wichienchot *et al.*, 2006). The GnOS (DP ≥ 3) used in the present study showed higher acid and

enzymatic resistance as compared with inulin and hence possesses significant potential as prebiotics.

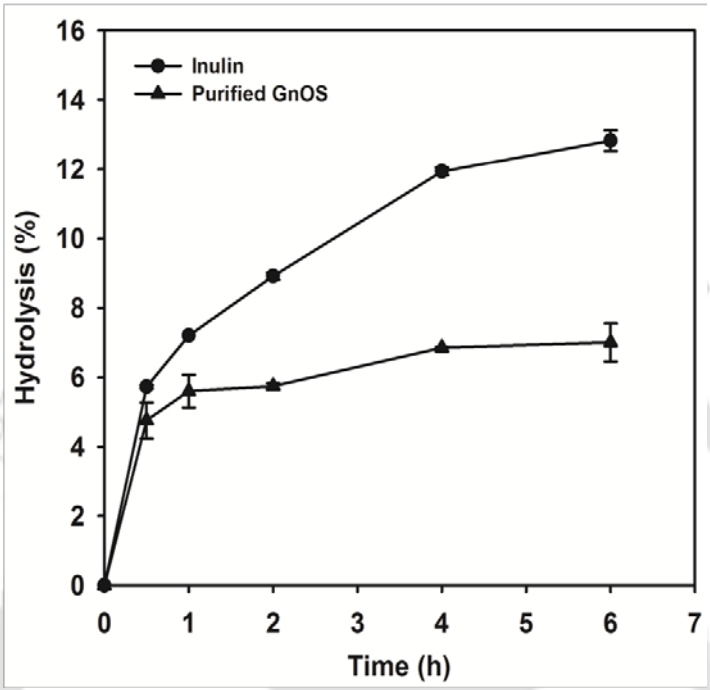

Fig. 6.3.4 Enzymatic hydrolysis of GnOS (DP ≥ 3) from *L. mesenteroides* NRRL B-1426 and inulin by human α-amylase (pH 7) at 37°C for 6 h. The hydrolysis of GnOS was significantly different *vs* inulin ($p \leq 0.01$).

6.3.2.3 Effect of GnOS on the growth of probiotics

The GnOS from *L. mesenteroides* NRRL B-1426 dextransucrase stimulated the growth of *B. infantis* and *L. acidophilus* and was comparable with that displayed by inulin (Fig. 6.3.5). The maximum absorbance (A_{600}) with GnOS and inulin for *B. infantis* was within 1.6-2.0 (Fig. 6.3.5A), and for *L. acidophilus* it was within 1.4-1.6 (Fig. 6.3.5B). The growth of *L. acidophilus* in GnOS was higher than that with commercial prebiotic, inulin.

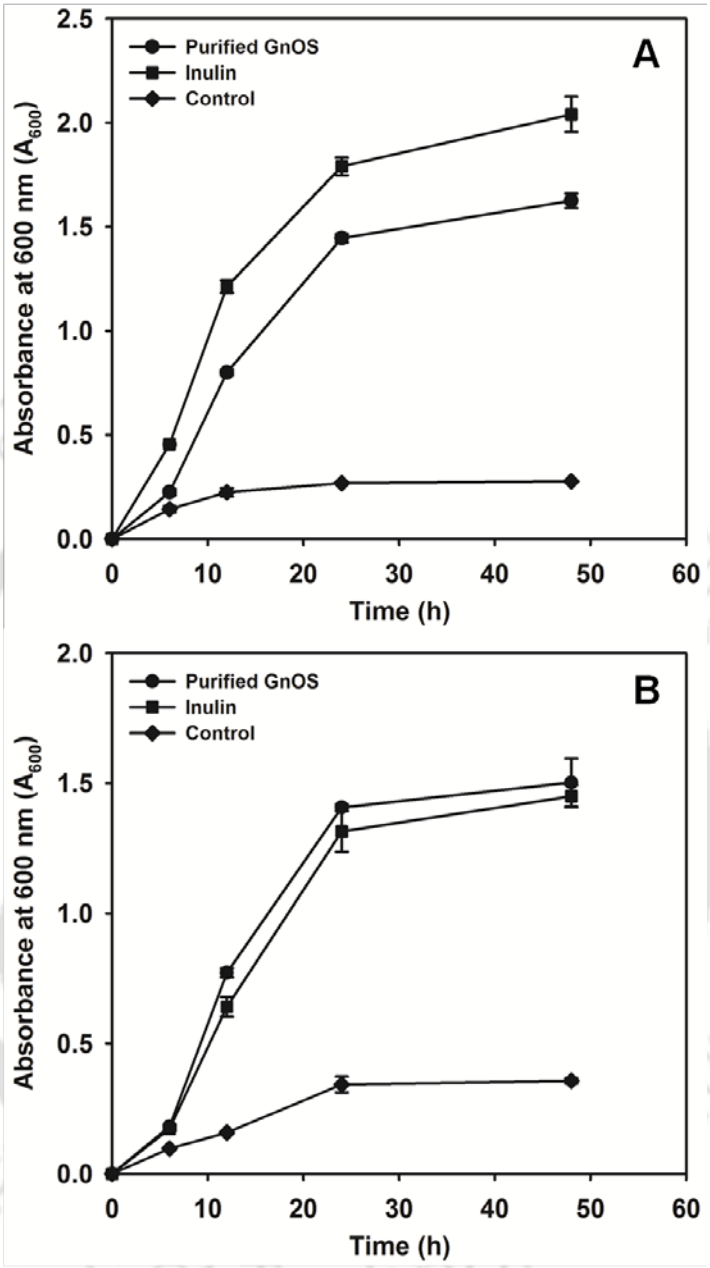

Fig. 6.3.5 Growth profile of (A) *B. infantis* and (B) *L. acidophilus* in presence of GnOS and inulin at 37°C. MRS medium without any carbon source as control. The growth of probiotics was significantly different *vs* control ($p \leq 0.01$).

The growth of probiotics was correlated with the change in pH of the fermentation medium. The changes in the pH of the medium fermented by *B. infantis* and *L. acidophilus* are shown in Table 6.3.1. The pH values in the growth media were

significantly reduced by the probiotic cultures tested ($p \leq 0.01$). The reduction in pH by *B. infantis* and *L. acidophilus* was 4.9 and 5.1 for GnOS, respectively (Table 6.3.1). It was inferred that *B. infantis* and *L. acidophilus* used GnOS 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. The production of SCFA is one of the important factor responsible for the role of dietary fibre in carcinogenesis (Campos-Vega *et al.*, 2010). 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). GnOS have also been shown to stimulate the growth of bifidobacteria and lactobacilli and resulted in the production of short chain fatty acids (Rycroft *et al.*, 2001; Sanz *et al.*, 2006). However, *in vivo* studies are necessary to confirm further the functional properties of GnOS.

Table 6.3.1 pH changes of probiotics during fermentation of medium.

Prebiotic	*B. infantis* Time (h)		*L. acidophilus* Time (h)	
	0	24	0	24
GnOS(DP ≥ 3)[§]	6.4±0.0	4.9±0.04**	6.4±0.0	5.1±0.02**
Inulin	6.4±0.0	4.8±0.02**	6.4±0.0	5.2±0.03**
Control	6.4±0.0	6.1±001	6.4±0.0	6.4±0.12

[§]From *L. mesenteroides* NRRL B-1426.

**Significantly different ($p \leq 0.01$) *vs* control.

6.3.4 *In vitro* analysis of effect of GnOS on mammalian cells

The use of prebiotics is increasingly being promoted as a strategy to improve gut health by stimulating the survival and growth of probiotics (Goetze *et al.*, 2008; Grimoud *et al.*, 2010). The effects of GnOS (DP ≥ 3) produced from *L. mesenteroides*

NRRL-1426 dextransucrase on human embryonic kidney (HEK-293), human cervical adenocarcinoma (INT-407) and human colon carcinoma (HT-29) cells were investigated by the colorimetric MTT assay.

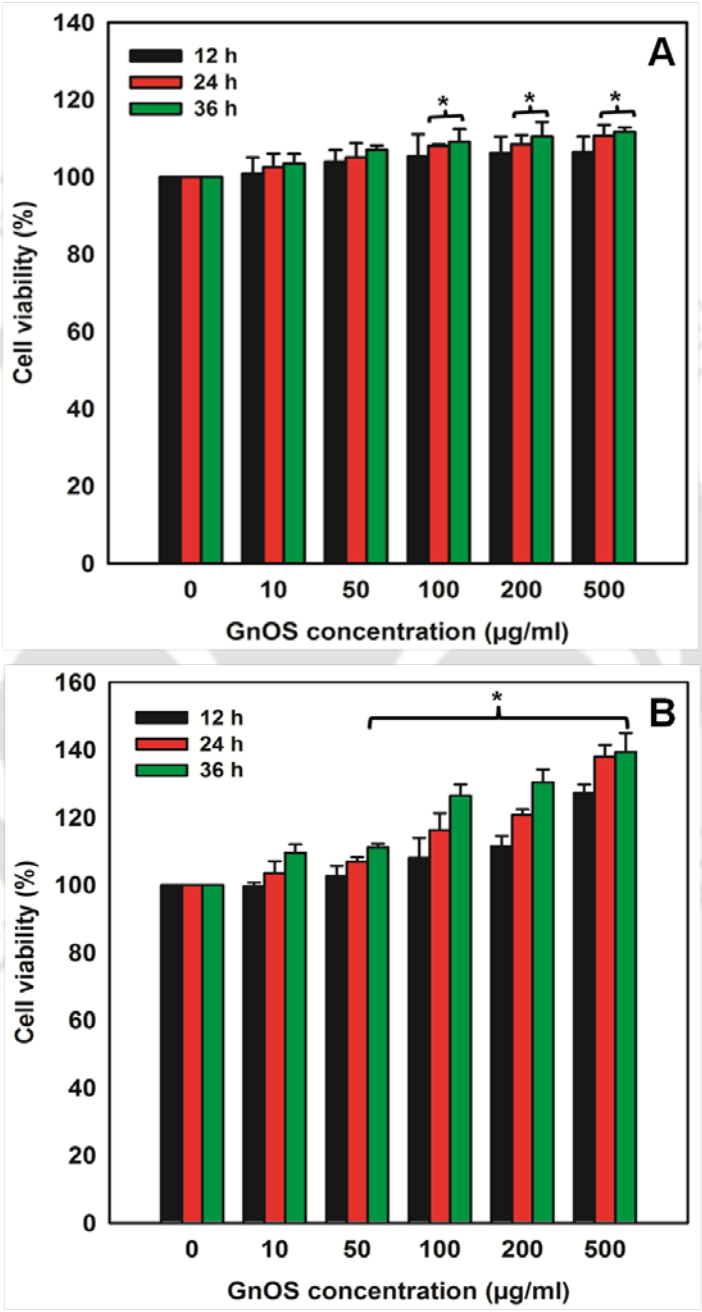

Fig. 6.3.6 Cell viability (%) of (A) HEK-293 and (B) INT-407 cells treated with various concentrations of GnOS (0-500 µg/ml) over a period of 12-36 h of incubation. *Significantly different ($p \leq 0.05$) *vs* control.

The viability of HEK-293 and INT-407 cells was not affected by the GnOS rather a slight increase in the growth of both was also observed as compared with the control cells (Fig. 6.3.6A and 6.3.6B). This increased growth may be attributed to the potential of GnOS serving as an additional carbon or energy source. The oligosaccharides have been reported to act as extra carbon or energy source (Altamirano *et al.*, 2000). Thus, GnOS (DP ≥ 3) were observed to be biocompatible in nature and hence could be safely used by the host, satisfying one of the criteria of prebiotics. However, The viability of HT-29 cells was significantly inhibited in time- and dose-dependent manner in response to increasing concentrations of GnOS (10-500 µg/ml). The GnOS treated HT-29 cells were inhibited by 25%, 31%, 32%, 40% and 44% at concentrations of 10, 50, 100, 200 and 500 µg/ml, respectively, after 36 h (Fig. 6.3.7).

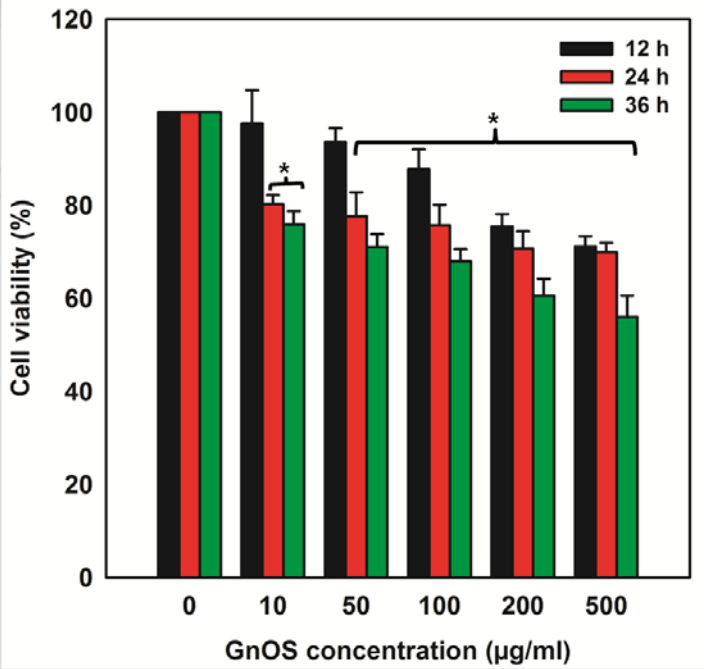

Fig. 6.3.7 Cell viability (%) of HT-29 cells treated with various concentrations of GnOS (0-500 µg/ml) over a period of 12-36 h of incubation. *Significantly different ($p \leq 0.05$) *vs* control.

Dietary ingredients such as prebiotics are known to modify the gut microflora (probiotics) and hence potentially reduce the risk of colorectal cancer (Nam *et al.*, 2007). The fermentation of prebiotics by probiotics produces SCFA that are known to act as anti-proliferative agents of colonic cells (Olmo *et al.*, 2007). The cytotoxic activity against HT-29 cells was also reported for chito-oligosaccharides (Nam *et al.*, 2007) and apple oligosaccharides (Li *et al.*, 2013). The GnOS in the present study showed a very selective cytotoxic behaviour, affecting only colon cancer lines and hence, can be used as food supplement for cure or control of colon cancer.

6.4 Conclusions

Gentio-oligosaccharides (GnOS) were synthesized by the acceptor reaction of dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426 using gentiobiose and sucrose. GnOS were purified by gel permeation chromatography using Bio-Gel P-2 column based on their degree of polymerization and characterized by TLC, HPLC and mass spectrometry. TLC and HPLC analyses revealed the *L. mesenteroides* NRRL B-1426 synthesized GnOS from DP3 to 10. However, ESI-TOF MS of the fractions showed the peaks at m/z 527.1, 689.2, 851.3 and 1013.3 corresponding to the $[M+Na]^+$ ions of GnOS from DP3 to DP6, respectively. The GnOS of DP7-DP10 in TLC and HPLC analysis were not detected in the ESI-TOF MS analysis under the conditions used. The purified GnOS of (DP ≥ 3) were pooled and explored for *in vitro* prebiotic and cytotoxic activity. GnOS exhibited significantly lower degree of digestibility of 18.1 % by simulated human gastric juice (pH 1.0) and 7.1 % by human α-amylase (pH 7.0) after 6 h, whereas inulin, a standard prebiotic showed 39.7 % and 12.8 % of digestibility, respectively, suggesting longer persistence of GnOS in the colon. GnOS significantly supported the growth of probiotics such as *Bifidobacterium infantis* and *Lactobacillus acidophilus* and was comparable to that of inulin. The effect of purified GnOS on HEK-293 and INT-407 cell lines revealed its nontoxic biocompatible nature. However, a selective inhibitory effect of GnOS on human colon carcinoma (HT-29) cells was observed revealing its potential as a functional food additive for the benefit of human health. Thus, the present study provides an assessment of gentiobiose-derived oligosaccharides for predicting the prebiotic potential and inhibitory effect on mammalian cells.

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SUMMARY

- Glucansucrase from *Leuconostoc mesenteroides* NRRL-1426 exist as a single active molecular form of dextransucrase.
- *L. mesenteroides* NRRL B-1426 dextransucrase synthesized high molecular mass dextran (>2000 kDa) containing 85% α-(1→6) linear and 15% α-(1→3) branched linkages.
- Dextran showed potential prebiotic activity and biocompatible nature.
- IMOs and GnOS of DP3-DP10 synthesized by acceptor reaction of *L. mesenteroides* NRRL B-1426 dextransucrase showed prebiotic potential and selective inhibition against colon cancer cells indicating their applications as functional food supplement.

FUTURE PROSPECTS

- Detailed structure characterization of IMOs and GnOS synthesized by *L. mesenteroides* NRRL B-1426 dextransucrase using NMR spectroscopy and tandem mass spectrometry analyses shall be carried out.
- More detailed *in vitro* and *in vivo* studies of dextran, IMOs and GnOS as prebiotics are required.

LIST OF ABBREVIATIONS

A_{600}	Absorbance at 600 nm
ARS	Agricultural Research Service
BCA	Copper bicinchoninic acid method
BSA	Bovine serum albumin
CAZy	Carbohydrate active enzymes
DMEM	Dulbecco's Modified Eagle's medium
DMSO	Dimethyl sulfoxide
DP	Degree of polymerization
DP_n	Number degree of polymerization
DSC/TGA	Differential scanning calorimeter/thermo-gravimetric analyzer
ED	Electrochemical detector
EPS	Exopolysaccharides
ESI-TOF MS	Electrospray ionization-time of flight mass spectrometry
FBS	Fetal bovine serum
FE-SEM	Field emission-scanning electron microscopy
FTIR	Fourier transform infrared spectroscopy
GH	Glycoside hydrolase
GnOS	Gentio-oligosaccharides
GPC	Gel permeation chromatography
GTF	Glycosyltranferase
HEK-293	Human embryonic kidney cells
HPAEC	High pH anion exchange chromatography
HPLC-RI	High performance liquid chromatography with refractive index detector
HT-29	Human colon carcinoma cells
INT-407	Human cervical adenocarcinoma cells
IMOs	Isomalto-oligosaccharides
LAB	Lactic acid bacteria
MRS	de Man, Rogosa, and Sharpe medium
MEM	Minimum essential medium
MM_n	Number average molecular mass
MTT	3-(4,5-dimethylthia-zol-2-yl)-2,5-diphenyl tetrazolium bromide
NMR	Nuclear magnetic resonance spectroscopy
NRRL	Northern Regional Research Laboratory
PAS	Periodic acid Schiffs staining
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
RPMI	Roswell Park Memorial Institute medium
SCFA	Short chain fatty acids
TFA	Trifluoroacetic acid
TLC	Thin layer chromatography
WHC	Water holding capacity

LIST OF PUBLICATIONS

Published/accepted

Thesis

1. **Damini Kothari,** Deeplina Das, Seema Patel and Arun Goyal **(2014)** Dextran and food applications. In Polysaccharides: Bioactivity and Biotechnology, Jean-Michel Merillon and Kishan G. Ramawat (eds.) **Springer International Publishing Switzerland**, DOI 10.1007/978-3-319-03751-6_66-1.
2. **Damini Kothari** and Arun Goyal **(2014)** Gentio-oligosaccharides from *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase as prebiotics and as supplement for functional foods with anti-cancer properties. ***Food and Function***, DOI: 10.1039/c4fo00802b.
3. **Damini Kothari,** Seema Patel and Arun Goyal **(2014)** Therapeutic spectrum of non-digestible oligosaccharides: Overview of current state and prospect. ***Journal of Food Science***, 79, R1491-R1498.
4. **Damini Kothari** and Arun Goyal **(2013)** Structural characterization of enzymatically synthesized dextran and oligosaccharides from *Leuconostoc mesenteroides* NRRL B1426 dextransucrase. ***Biochemistry (Moscow)***, 78(10), 1483-1490.
5. **Damini Kothari,** Rwivoo Baruah and Arun Goyal **(2012)** Immobilization of glucansucrase for the production of gluco-oligosaccharides from *Leuconostoc mesenteroides*. ***Biotechnology Letters***, 34, 2101-2106.

Others

6. Jagan Mohan Rao Tingirikari, **Damini Kothari** and Arun Goyal **(2014)** Superior prebiotic and physicochemical properties of novel dextran from *Weissella cibaria* JAG8 for potential food applications. ***Food and Function***, 5, 2324-2330.
7. Jagan Mohan Rao Tingirikari, **Damini Kothari**, Rishikesh Shukla and Arun Goyal **(2014)** Structural and biocompatibility properties of dextran from *Weissella cibaria* JAG8 as food additive. ***International Journal of Food Sciences and Nutrition***, 15, 1-6.
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10. Seema Patel, **Damini Kothari**, Rishikesh Shukla, Debasish Das and Arun Goyal **(2011)** Scale up of dextran production from a mutant of *Pediococcus pentosaceus* (SPAm) using optimized medium in a bioreactor. ***Brazilian Archives of Biology and Technology***, 54, 1125-1134.
11. Seema Patel, **Damini Kothari** and Arun Goyal **(2011)** Enhancement of dextransucrase activity of *Pediococcus pentosaceus* mutant SPAm1 by response surface methodology. ***Indian Journal of Biotechnology***, 10, 346-351.

Submitted

12. **Damini Kothari** and Arun Goyal **(2014)** Enzyme resistant isomalto-oligosaccharides produced from *Leuconostoc mesenteroides* NRRL B-1426 dextran for functional food application. ***Biotechnology and Applied Biochemistry*. (Under Revision)**
13. **Damini Kothari,** Cedric Delattre and Arun Goyal **(2014)** Synthesis and purification of prebiotic isomalto-oligosaccharides from *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase. ***Journal of the Science of Food and Agriculture*.**
14. **Damini Kothari,** Jagan Mohan Rao Tingirikari and Arun Goyal **(2014)** Non digestible, prebiotic and biocompatible dextran from *Leuconostoc mesenteroides* NRRL B-1426 for potential functional food application. ***Applied Biochemistry and Biotechnology*.**

LIST OF CONFERENCES

International

1. **Damini Kothari** and Arun Goyal **(2014)** Synthesis, purification and cytotoxicity of prebiotic gentiobio-oligosaccharide from *Leuconostoc mesenteroides* NRRL B-1426 dextransucrase. XI Convention of Biotech Research Society of India (BRSI), November 6-9, 2014, Jawaharlal Nehru University, New Delhi, India.
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3. **Damini Kothari** and Arun Goyal **(2013)** Enzymatic synthesis of glucan and oligosaccharides by glucansucrase from *Leuconostoc mesenteroides* NRRL B-1426. 5th Congress of European Microbiologists, FEMS, July 21-25, 2013, Leipzig, Germany.
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VITAE

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