Production and characterization of dextran and prebiotic isomalto-oligosaccharides from *Weissella cibaria* RBA12 isolated from Pummelo (*Citrus maxima*) for functional food applications

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by

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to the

Department of Biosciences and Bioengineering
Indian Institute of Technology Guwahati

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INDIAN INSTITUTE OF TECHNOLOGY GUWAHATI

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STATEMENT

I do hereby declare that the content embodied in this thesis is the result of investigations carried out by me in the Department of Bioscience and Bioengineering, 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.

June, 2017. **RWIVOO BARUAH**

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CERTIFICATE

It is certified that the work described in this thesis entitled **"Production and characterization of dextran and prebiotic isomalto-oligosaccharides from *Weissella cibaria* RBA12 isolated from Pummelo (*Citrus maxima*) for functional food applications."** by Rwivoo Baruah 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 Bioscience and Bioengineering, 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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Synopsis

Introduction

Nutraceutical, a dietary supplement, has a potential to deliver a concentrated form of a presumed bioactive agent from a food, presented in a non-food matrix and used with the purpose of enhancing health in dosages that exceed those that could be obtained from normal foods. Functional foods are the foods or dietary components consumption of which may have associated health benefits beyond the basic nutritional properties that the foods possess. Functional food should be a food similar in appearance to a conventional food (beverage, food matrix), consumed as part of a usual diet, contains biologically active components with demonstrated physiological benefits, and offers the potential of reducing the risk of chronic diseases beyond basic nutritional functions. Prebiotic is defined as "a selectively fermented ingredient that allows specific changes, both in the distribution 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. This beneficial action is due to the production of short chain fatty acids (SCFA) by the gastrointestinal microflora from the fermentation of prebioitcs. Most prebiotics identified today are non-digestible oligosaccharides and polysaccharides. Polysaccharides with prebiotic effect have several advantages over non digestible oligosaccharides which includes (i) tolerance to high ingestion which otherwise causes intestinal discomfort and flatulence, (ii) decrease calorific intake, (iii) mucosal damage from rapid acidification and (iv) laxative effect in the colon.

Lactic acid bacteria (LAB) of the genera *Leuconostoc*, *Streptococcus*, *Weissella*, *Pediococcus* and *Lactobacillus* synthesize dextrans using sucrose as a substrate (Falconer et al., 2011). Dextransucrase secreted by LABs hydrolyses sucrose to produce dextran and fructose as a by-product. Commercial applications of dextran from LAB are generally found in food and pharmaceutical industry; however, dextran also has several potential applications in photo film manufacturing, fine chemical, cosmetic, paper, petroleum, and textile industries. Due to the heterogeneity of dextran produced by various LAB, their application may depend on well-defined chemical and physicochemical properties. Long-chain, high molecular weight polysaccharides that dissolve or disperse in water to give improved rheological (gelling, thickening) or physicochemical (emulsion stabilization, particle suspension, etc.) properties are important for food product formulation. A current consumer trend towards healthy and additive-free food has made the dextran an attractive food ingredient. The microorganisms such as *Leuconostoc mesenteroides*, *Saccharomyces cerevisiae*, *Lactobacillus plantarum*, and *Lactobacillus sanfranciscensis* are used for the production of dextran for its application in food processing without any restriction.

Keeping the significance and applications of poly and oligo-saccharides in mind, dextran producing lactic bacteria was isolated from the pulp of pummelo (*Citrus maxima*). The isolated strain was phenotypically and biochemically characterized and was identified to be *Weissella cibaria* with the strain name given as RBA12. The production of enzyme dextransucrase was optimized from *Weissella cibaria* RBA12 and the enzyme was purified and characterized for the synthesis of isomalto-oligosaccharides. The dextran produced by *Weissella cibaria* RBA12 was structurally and physico-chemically characterized. The production of dextran by

Weissella cibaria RBA12 was optimized and the dextran production was scaled up to 2.5 l in batch fermentation followed by studies in dextran production in fed-batch fermentation. The functional food applications of the synthesized isomalto-oligosaccharides (IMO) and dextran were explored. The IMOs were purified by Bio-Gel P-2 chromatography and characterized by HPLC-RI and ESI-TOF MS analyses. The prebiotic IMOs and di-saccharides were produced by dextransucrase acceptor reaction using mango and pineapple juices utilizing their native sugars which acted as acceptors. The *in vitro* studies using simulated gastric juices, α-amylase and intestinal fluid were carried out on dextran for characterizing its prebiotic potential. The *in situ* production of dextran by *Weissella cibaria* RBA12 in whole wheat flour, wheat bran and rye bran was carried out in order to assess its application in the sourdough fermentation.

Present work

The present investigations are carried out on the **"Production and characterization of dextran and prebiotic isomalto-oligosaccharides from *Weissella cibaria* RBA12 isolated from Pummelo (*Citrus maxima*) for functional food applications."** 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 with emphasis to dextran and isomalto-oligosaccharides. A detailed literature on the importance of dextran, dextransucrase and isomalto-oligosaccharides along with their production, purification and applications were described. The chapter describes the production, purification, structure and application of the enzyme dextransucrase. The detailed production, purification and characterization methods for the dextran and

isomalto-oligosaccharides have also been discussed in this chapter. Various applications of dextran and isomalto-oligosaccharides are extensively reviewed.

Chapter 2 describes the detailed protocol of screening of the natural isolate of lactic acid bacterium, RBA12 from pommello. The morphological and biochemical characterization including Gram test, catalase test, carbohydrate fermentation profile and antibiotic susceptibility test was carried out on isolate RBA12. The isolate was Gram positive and catalase negative. Antibiogram analysis showed that the isolate RBA12 was resistant to the antibiotics nalidixic acid, nitrofurantoin, sulphamethoxazole, co-trimazine, co-trimoxazole, vancomycin, ciprofloxacin, norfloxacin and cefixime. Resistance to vancomycin is the common characteristic feature for most of the lactic acid bacteria. The isolate RBA12 was sensitive to ampicillin, ciprofloxacin, erythromycin, gentamicin, kanamycin, methicillin, pencillin and tetracycline. The bacterium RBA12 could ferment sucrose, mannose, salicin, arabinose, maltose, fructose xylose, cellobiose, galactose and glucose. The isolate could not utilize lactose, adonitol, inulin, inositol, melibiose, trehalose, dulcitol, sorbitol, mannitol, rhamnose and raffinose. The 16S rRNA gene sequence analysis revealed the identity of the isolate RBA12 which was found to be *Weissella cibaria* (Genbank Accession Number KF515952). The optimal conditions for glucansucrase production were 20 °C and 180 rpm, yielding a maximum enzyme activity of 7.2 U/ml after 12 h of incubation. The fermentation profile of *Weissella cibaria* RBA12 revealed that the production of glucansucrase was growth associated and the sucrose was completely utilized by the bacterium. The maximum glucan concentration was 8.3 mg/ml resulting in 83% efficiency. The results showed that *Weissella cibaria* RBA12 can be used as an efficient microorganism for glucan production. This is the

first report on isolation of any lactic acid bacteria from Pummelo (*Citrus maxima*) or from any citrus fruit. Further studies on the nature and properties of both glucansucrase and glucan from *Weissella cibaria* RBA12 are required to elucidate their potential applications.

Chapter 3 describes the purification, identification and characterization of dextransucrase from the isolate *Weissella cibaria sp.* RBA12. The dextransucrase was purified by using aqueous two phase system using different percentage of polyethylene glycol (PEG) of molecular weight 400 and 1500. The purification of crude dextransucrase from *Weissella cibaria* RBA12 with a specific activity of 1 U/mg by 25% (v/v) PEG-400 and 15% (w/v) PEG-1500 fractionation resulted in specific activity of 16.8 U/mg and 7.3 U/mg with 17 and 7 fold purification, respectively. Further purification of 25% (v/v) PEG-400 enzyme fraction by size exclusion chromatography using Sephacryl S-300HR resulted in specific activity of 24.6.0 U/mg with 25 fold purification. Silver staining and PAS staining analysis of dextransucrase under non denaturing SDS-PAGE showed a single distinct band with molecular size of 180 kDa. The enzyme was confirmed as dextransucrase by PAS staining. A bright magenta colour band was observed in the gel incubated with 5% (w/v) sucrose, and no band was detected in the gel incubated with raffinose which confirmed that the enzyme is dextransucrase not fructansucrase. The optimum conditions for enzyme were 40°C and pH 5.4. The dextransucrase showed maximum activity at 5% sucrose concentration with K_m of 19.2 mM and V_{max} of 29.3 µmole/mg/min. Mg^{2+} and Ca^{2+} ions enhanced the activity of enzyme by ~40% and 25%, respectively. The enzyme lost 97% and 50% of its activity at 5 M urea and 12 mM EDTA, respectively. Thermal and pH stability analysis showed that enzyme was

stable up to 40°C and in the pH range of 5.2 to 5.4. Among all the additives, glycerol provided maximum stabilization to dextransucrase with $t_{1/2}$ of 89.2 h as against 52h without any additives at 30°C. The purified enzyme can be exploited for enzymatic synthesis of dextran and of isomalto-oligosaccharides with potential applications in food and baking industry as also elaborated in the subsequent chapters.

Chapter 4 describes the synthesis, purification and characterization of dextran produced by the *Weissella cibaria* RBA12. Dextran from *Weissella cibaria* RBA12 was purified using ethanol precipitation yielding 8.7 mg/ml concentration. The monosaccharide composition of the dextran revealed the presence of only glucose, confirming its glucan nature. The FT-IR spectrum of dextran displayed the presence of α-(1→6) glycosidic bonds and the characteristic functional groups of dextran. ^{1}H NMR and ^{13}C NMR revealed the presence of 97% of α-(1→6) linkages and 3% of α-(1→3) branch linkages. The molecular weight of dextran was revealed to be of 26.3 MDa after 24h of fermentation by HPSEC analysis. The molecular size of dextran had increased with the fermentation time. The thermal stability analysis of dextran RBA12 displayed degradation temperature (T_d) of 292.2ºC. DSC analysis revealed the melting point of dextran from *Weissella cibaria* RBA12 to be 280ºC. Surface morphology by AFM of purified powder of dextran using SEM displayed a web-like architecture with several pores across its surface. The surface morphology revealed the presence of several spherical lumps with a maximum height of 56.3 nm. Dextran from *Weissella cibaria* RBA12 displayed solubility and water holding capacity 19.5% and 383%, respectively. Dextran retained 73.1% and 60.5% of the emulsification activity after 30 and 60 min, respectively which was greater than that of commercial stabilizers guar gum and sodium alginate. Dextran from *Weissella cibaria* RBA12 showed a

flocculating activity of 94.12% at concentration of 0.2 mg/ml. The structural and physicochemical characterization of dextran from *Weissella cibaria* RBA12 has shown properties that can enable it as an efficient food hydrocolloid that can be used in baking, diary and confectionery industries.

Chapter 5 describes the production and scale up of dextran from *Weissella cibaria* RBA12. The maximum dextran yield by *Weissella cibaria* RBA12 was obtained after 24h at 2% (w/v) sucrose, 2% (w/v) yeast extract and 2% (w/v) K_2HPO_4 and at growth conditions of 20°C and 180 rpm. In shake flask studies the dextran production was 8.9 mg/ml with 89% efficiency in the presence of 2% (w/v) sucrose. When this optimized medium was used for scale up in 2.5 L volume in a bioreactor, it yielded 9.32 mg/ml dextran with an 93% efficiency. The dextran formed in batch mode was utilized by the bacterium after the depletion of sucrose. In batch mode the specific growth rate (μ) of *Weissella cibaria* RBA12 was 0.19 h^{-1}, biomass yield coefficient ($Y_{X/Suc}$) 0.18 g/g of sucrose and dextran yield coefficient ($Y_{P/Suc}$) as 0.49 g/g of sucrose. In fed batch mode the maximum dextran concentration was 35.8 mg/ml which was approximately 4-fold higher than that of batch mode (9.32 mg/ml). Owing to the steady feed of sucrose, there was no decrease in dextran concentration. Therefore, the fed-batch mode can be used for large scale production of dextran from *Weissella cibaria* RBA12.

Chapter 6 describes the functional food applications using *Weissella cibaria* RBA12 by the production of IMOs and various applications of dextran-RBA12. Fermentation with *Weissella cibaria* RBA12 in the culture medium containing maltose as an acceptor produced IMOs, namely trisaccharide, tetrasaccharide and pentasaccharide. IMOs in the range of DP3–DP6 were produced *in vitro* using maltose as an acceptor

molecule by dextransucrase acceptor reaction. The mixture of IMOs produced was purified by GPC and individual IMO were obtained which on identification by TLC and by their mass analysis gave, DP3 (527.22), DP4 (689.42) and DP5 (851.25). The purified IMOs were used as the standard for HPLC analysis of IMOs produced in dextransucrase acceptor reactions with fruit juices. Dextransucrase was used to produce prebiotic juices using commercial mango and pineapple juice, which yielded IMOs in the range of DP3-DP5 along with disaccharides, leucrose and isomaltose. The reaction removed completely the native sucrose present in the juices lowering its calorific value. The significantly lower digestibility of dextran-RBA12 as compared with the commercial prebiotic inulin as well as its ability to be fermented by probiotic intestinal microbiota proved its effectiveness as an efficient soluble dietary fibre. The *in situ* formation of dextran in cereal matrices like wheat flour, wheat bran and rye bran after fermentation by *Weissella cibaria* RBA12 was observed to be very efficient. Among the three matrices, rye bran proved to be the best matrix producing maximum dextran of 3.26% d.w (32.6 g/kg). Further studies on the shelf-life and stability of dextransucrase as well as studies on the physio-chemical properties of dextran-RBA12 will throw light on its potential applications in food industry.

CONTENTS

Chapter 1

General Introduction

1.1 Introduction

Lactic acid bacteria (LAB) are contained in 11 genera of gram-positive bacteria *i.e. Carnobacterium, Oenococcus, Enterococcus, Pediococcus, Lactococcus, Streptococcus, Lactobacillus, Vagococcus, Lactosphaera, Weissella* and *Lecconostoc* within the phylum Firmicutes (Ercolini *et al.,* 2001; Holzapfel *et al.,* 2001). LAB are generally regarded as safe microorganism and have wide application in food fermentation where they contribute in acid production, proteolytic activity and aroma formation (Patel *et al.,* 2012). LAB produce a proteinaceous antimicrobial compound called bacteriocins, which display a high degree of specificity and display antimicrobial activity. Bacteriocins from LAB are the most studied due to the fact that most of bacteriocin-producing LAB are isolated from food and therefore are considered to be safe (Deegan, L. H., *et al.,* 2006; Zendo, T., 2013). The LAB are evidenced to be good probiotics as they accelerate the growth of beneficial microbial gut flora and have been proven effective against diarrhoea, irritable bowel disorder, allergies, stimulation of immunity and lactose intolerance (Patel *et al.,* 2012). LAB produce various types of exopolysaccharides which have numerous applications in food and pharmaceutical industry (Purama and Goyal, 2005; Das and Goyal, 2012).

Lactic acid bacteria are either homofermentative or heterofermentative based on the organism's hexose fermentation pathway. Homofermentative bacteria such as *Lactococcus, Enterococcus, Streptococcus, Pediococcus and Lactobacilli as L. acidophilus, L. delbrueckii, L. helveticus, L. salivarius* follows Embden Meyerhof Parnas pathway (EMP, or glycolysis) and yields two lactate molecule from one glucose molecule, whereas the heterofermentative bacteria such as *Leuconostoc, Oenococcus, Weissella and lactobacilli as L. brevis, L. buchneri, L. fermentum and L. reuteri.* transforms a glucose molecule into a lactate molecule, ethanol and carbon dioxide (Kandler and Wiess, 1986; Fugelsang and Edward, 2007) by following the pentose phosphate pathway.

1.2 Lactic acid bacteria in food industry

Lactic acid bacteria (LAB) have been utilized inadvertently since ancient times. The early practices of hand-milking of raw milk from livestock led to the introduction of a wide range of LAB in milk. These stains consequently converted the native lactose in milk to acid, mainly lactic acid, which inhibited the growth of undesirable bacteria (Vandenbergh, 1993). Lactic acid bacteria also produce acetic acid, aroma compounds, bacteriocins and exopolysaccharides and several important enzymes. For example, acetaldehyde provides the characteristic aroma of yoghurt (Leroy and Devuyst, 2004), while diacetyl imparts a buttery taste to other fermented milk which improve the taste and quality (Leroy and De vuyst, 2004; Todorov and Franco, 2010). The fermentation by lactic acid bacteria also produces lactic acid in fermented milk which gives slightly tart taste (Todorov and Franco, 2010). Several metabolites produced by LAB such as organic acids, fatty acids, hydrogen peroxide, carbon dioxide and bioactive peptides have antimicrobial effects (Galvez *et al.*, 2007). Some LAB are able to excrete high

molecular weight polysaccharides that can increase the viscosity of the liquid substrate. Exopolysaccharides (EPS) are formed through polymerization of sugar moities and can be either composed from repeating glucose or fructose moities (homopolysaccharides) or from two or more different subunits (heteropolysaccharides) (Galle and Arendt, 2014). Their contribution through *in situ* production of EPS is of particular interest to manufacturers of fermented cereal-based drinks aimed to imitate dairy products (Bernat, *et al.*, 2014).

1.3 Microbial diversity of India

Microbial diversity comprises the spectrum of variability among all types of microorganisms *viz*. bacteria, fungi, viruses and also mutated microorganisms that are altered at genomic level by human intervention. North eastern states of India is blessed with a wide range of physiographic and eco-climatic conditions and the geographical 'gateway' for much of country's endemic flora as well as fauna and hence is considered as biodiversity hot spot of India (Myers *et al*., 2000; Singh *et al*., 2009). The eastern Himalayas biodiversity 'hotspot' was modified to the 'Indo-Burma hotspot' covering central Nepal to whole of north east India, Andaman and Nicobar Islands, Hainan island in southern China, Myanmar, Thailand and southern Malaysian peninsula and the second largest with an area 2,20,60,000 sq km among the 25 identified globally (Myers *et al*., 2000). Western ghats and the north eastern region of India are the biodiversity rich areas and are a genetic treasure houses of plant, animal and microbial resources (Singh *et al.,* 2009; Jeyaram *et al*., 2009). The north eastern region comprising eight states *viz.* Assam, Arunachal Pradesh, Sikkim, Manipur, Mizoram, Meghalaya, Tripura and Nagaland are rich in numerous endemic species of microorganisms (Singh *et al*., 2009). Diverse microorganisms ranging from filamentous fungi to enzyme and alcohol producing yeast, LAB, bacilli and micrococci are associated with fermentation and

production of ethnic food alcoholic beverages in north eastern region of India (Tamang *et al*., 2012).

1.3.1 Importance of LAB in fruits and vegetables

Fruits and vegetables are an integral part of human diet and are consumed in large quantities all over the world. These products are rich in carbohydrates and poor in proteins with pH value from 7.0 to slightly acidic and provide a suitable niche to several bacteria, yeasts and moulds (Weissinger *et al*., 2000; Trias *et al*., 2010). In contrast to vegetables, fruits have good record from public health standpoint. Many fruits possess a natural defense mechanism as they contain organic acids in quantities adequate enough to contribute a pH value of 4.6 or lower. The pH and the type of the acid itself are the major influence that select for predominant microflora in fruits. Lactic acid bacteria are demonstrated as a part of autochthonous microflora of tomatoes owing to its low pH and organic acids (Brackett, 1988; Sajur *et al.*, 2007). The presence of different lactic acid bacteria (LAB) of varying genus are also seen in different fruits (Naeem *et al*., 2012). Food borne bacteria capable of causing human illness cannot grow at a pH less than 4.0, so edible portion of most fruits precludes the involvement as substrate for proliferation of human pathogen.

1.3.2 Pummelo

Shaddock (*Citrus grandis* or *Citrus maxima*) (Fig 1.1), also called pummelo or Chinese grapefruit, citrus tree of the family Rutaceae. Pummelo is allied to the orange and the lemon and is native to mainland Southeast Asia and the Malaysian portion of the island of Borneo. Pummelo is classified as one of the basal species of edible Citrus and is less acidic compared to grapefruit, with pigmented or non-pigmented flesh. The flowers are large and white and are succeeded by very large spheroid or almost pear-shaped fruits that resemble grapefruit, being lemon yellow and having a pungent, tart,

but agreeable flavour. The pulp segments are either pallid or red and shell out easily. The fruit is highly prized in Asia. Besides the identification of around 100 volatiles in the pummelo peel, 50 aroma-active compounds (mainly unsaturated aliphatic aldehydes, terpene aldehydes, esters, terpene alcohols and nootkatone) through sniffing via gas chromatography–olfactometry (GC–O) were found to play an important role in the complex pummelo flavor. Malaysian pummelo has a unique and strong aromatic peel oil, which could be utilized in both flavor and fragrance industries (Cheong, *et al.*, 2011).

Fig. 1.1 Shaddock (*Citrus grandis* or *Citrus maxima*).

1.4 Bacteria from genus *Weissella*

Bacteria belonging to the genus *Weissella* are Gram-positive, catalase-negative, non-endospore forming cells with coccoid or rod-shaped morphology (Collins *et al.*, 1993; Björkroth *et al.*, 2009, 2014). The *Weissella* species belong to the phylum *Firmicutes*, class *Bacilli*, order *Lactobacillales* and family *Leuconostocaceae* (Collins *et al.*, 1993). Bacteria belonging to the genus *Weissella* are difficult to separate from members of the genera *Leuconostoc* or the heterofermentative lactobacilli on the basis of phenotypic characteristics only. The genus *Weissella* was named after the German microbiologist Norbert Weiss, known for his many contributions in the field of lactic acid bacteria research (Collins *et al.*, 1993). Since the original description of the genus by Collins *et al.* (1993), various new species of *Weissella* have been described, so that currently the genus comprises 19 validated species (Fig. 1.2). The *Weissella* species grouped in five phylogenetic branches based on 16S phylogeny, with *W. soli*, *W. diestrammenae*, *W. koreensis*, *W. kandleri* and *W. oryzae* as members of the first branch, *W. cibaria* and *W. confusa* as members of a second and *W. thailandensis*, *W. hellenica* and *W. paramesenteroides* occurring in a third branch. *W. ceti*, *W. halotolerans*, *W. viridescens*, *W. minor* and *W. uvarum* are associated with the fourth branch, and *W. beninensis*, *W. fabalis*, *W. fabaria*, and *W. ghanensis* with the fifth (Fig.1.2).

Only *W. beninensis* was reported to be motile (Padonou et al., 2010) and all other species are non-motile. As in the original description of the genus *Weissella* (Collins *et al.*, 1993), the bacteria of this genus were described to be non-motile. The motile characteristic of *W. beninensis* is not in accordance with the description of general characteristics of bacteria in this genus and consequently the genus description

was amended by Padonou *et al.* (2010) to account for a typical motility of this particular species.

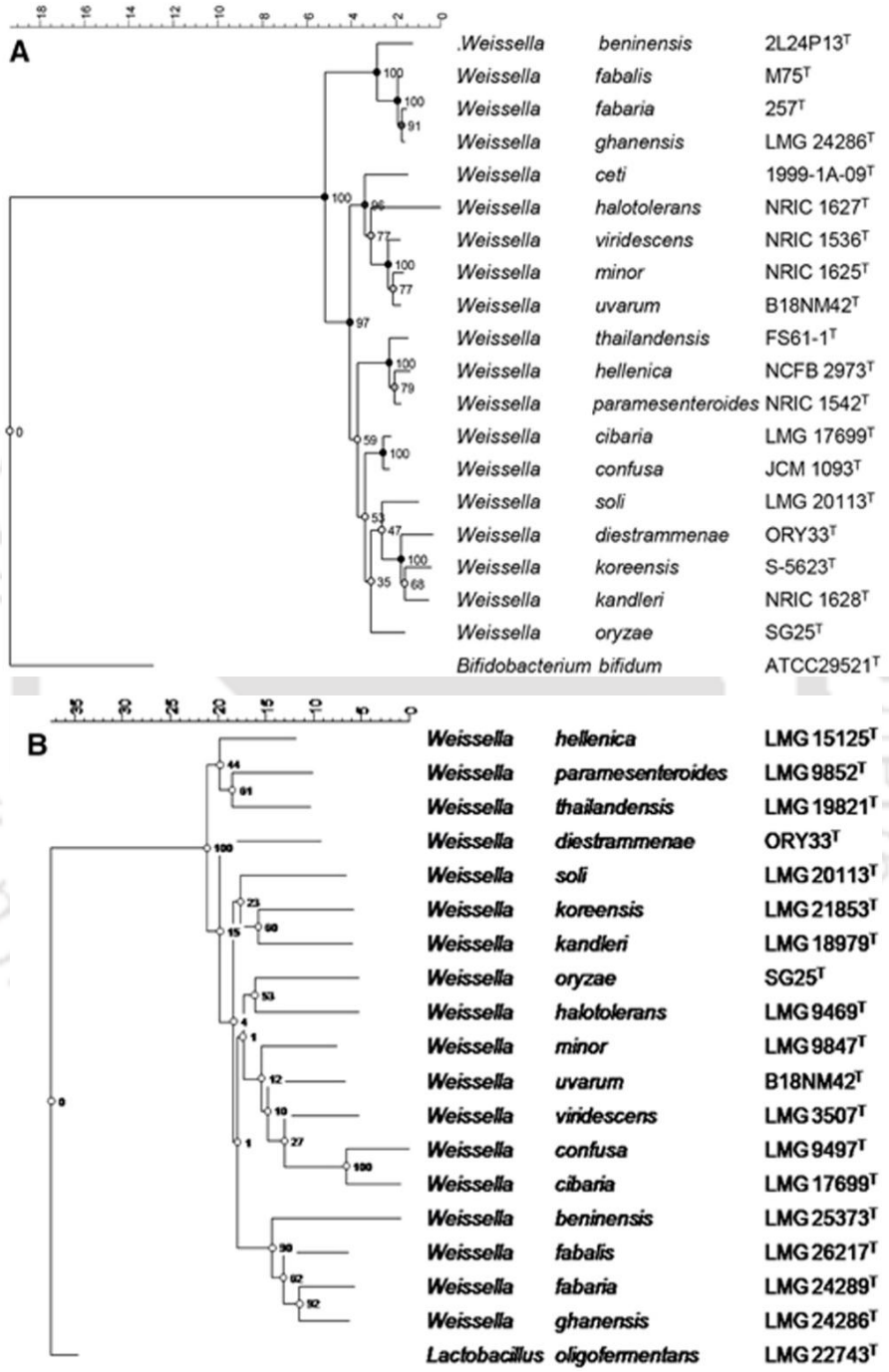

Fig. 1.2 Neighbor-joining phylogenetic tree based on **(A)**16S rRNAsequences and **(B)** *phe*S gene sequences of *Weissella* species type strains. The 16S rRNA sequence of *Bifidobacterium bifidum* was used as an out group sequence. Bootstrap values (%) derived from 1000 replicates are given at branch points. Bar indicates % sequence divergence. Adapted from Fusco *et al.,* (2015).

1.5 Glucansucrase from LAB

Glucansucrase (E.C. 2.4.1.5) catalyzes the polymerization of the glucopyranosyl moieties of sucrose to form glucan (Purama and Goyal, 2008). Glucansucrases have included in family 70 glycoside hydrolase (GH) in carbohydrate active enzyme database (http://www.cazy.org/Glycoside-Hydrolases.html) based on sequence similarity to GH13 α-amylases and GH77 amylomaltases (Cantarel *et al.,* 2009; Vujicic Zagar *et al*., 2010). Glucansucrases catalyze the synthesis of high molecular weight polymers of D-glucose units called glucans from sucrose by polymerization reaction, yielding dextran, mutan, alternan or reuteran. Dextransucrase (1,6-α-Dglucan- 6- α - glucosyltransferase, EC 2.4.1.5) catalyses the formation of the soluble dextran by a transfer of the D-glucosyl moieties of sucrose to a growing polymer chain (Patel *et al.*, 2011).

$$n C_{12}H_{22}O_{11} \xrightarrow{\text{Dextransucrase}} \underset{\text{(Dextran)}}{(C_6H_{10}O_5)n} + \underset{\text{(Fructose)}}{nC_6H_{10}O_6}$$

1.5.1 Molecular architecture of dextransucrase

The dextransucrase is a large protein consisting of approximately, 1600 - 1800 amino acid residues (Monchois *et al.*, 1999). The schematic structure of dextransucrase is shown in Fig. 1.3. Dextransucrase consists of encoding genes having A, signal peptide; B, variable region; C, N-terminal catalytic domain; D, C-terminal glucan binding domain (Russell, 1990; Giffard *et al.*, 1993; Simpson *et al.*, 1995; Vickermann *et al.*, 1997). N-terminal part contains typical signal peptides of Gram-positive bacteria (Monchois *et al.*, 1998). The main characteristic of the structure of glucansucrase signal peptides is that it is well conserved (Monchois *et al.*, 1998). The N-terminal signal

peptide of approximately, 32-34 amino acid residues aids in the translocation of dextransucrase across bacterial membrane. The signal peptide region is followed by a stretch of 121-129 amino acids which is highly variable. The non-conserved region located just downstream of the signal peptide tends to have no important role in the enzyme catalysis. Its deletion does not affect the enzyme activity (Abo *et al.*, 1991). As shown by protein sequence alignments of different dextransucrases, the N-terminal domain is highly conserved catalytic domain of about 1000 amino acids (Ferretti *et al.*, 1987). The catalytic domain is the glucan binding domain, capable of binding and cleaving sucrose. The C-terminal of glucansucrase covering about 500 amino acids is a functional glucan binding domain which is composed of a series of tandem repeats (Monchois *et al.*, 1999).

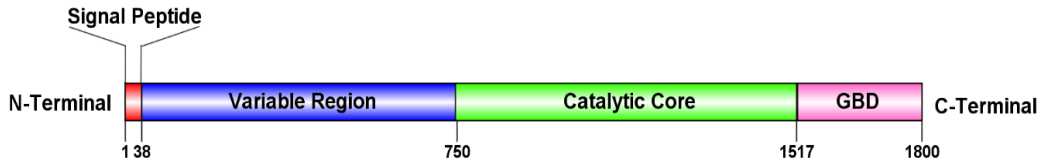

Fig. 1.3 General molecular architecture of dextransucrase.

1.5.2 Three dimensional (3D) structure of dextransucrase

The three-dimensional structures of GH70 glucansucrases (GSs) obtained with truncated forms of the enzyme are now available (Vujicic- Zagar *et al.*, 2010; Ito *et al.*, 2011; Brison *et al.*, 2012). Crystal structures of complete GS enzymes have not yet been reported. The crystal structure of a fully active, 1,031-residue fragment encompassing the catalytic and C-terminal domains of GTF180 from *Lactobacillus reuteri* 180 is shown in Fig. 1.4 as described earlier by Vujicic- Zagar *et al.*, 2010. The catalytic core of the GSs consists of three core domains, which resemble the A, B and C domains

which are also found in glycoside hydrolase family 13 (GH13) enzymes. There are two extra domains, called IV and V (Fig. 1.4A), attached to the core domains (Vujicic-Zagar *et al*., 2010; Leemhuis *et al.*, 2013).

Starting from residue 746 in, the A, B, IV and V domains in glucansucrase from *Lactobacillus reuteri* 180 (GTF180-_N) follows a U-shaped course which is arranged in two discontinuous segments as shown in Fig. 1.2B (Vujicic- Zagar *et al.*, 2010). On the other hand, the C domain consists of only one continuous polypeptide. Therefore, from N- to C-terminus the polypeptide chain follows" V, IV, B, A, C, A, B, IV, and V domains (Leemhuis *et al.*, 2013). The domain A comprises a $(\alpha/\beta)_8$ barrel which contain three proposed catalytic residues (the nucleophilic aspartate, the acid/base glutamate and the transition state stabilizing aspartate) at the bottom of a deep pocket (Vujicic-Zagar *et al.*, 2010). Domain A comprises four homology region *viz.* I, II, III and IV. Homology regions II, III and IV are located in the N-terminal part of domain A whereas, homology region I is located at the C terminal part of the domain A. Domain B forms a highly twisted antiparallel five or six-stranded β -sheet and lies next to the catalytic domain. Domain B contribute to shaping the groove near the catalytic site and the residues at the interface of domain B with domain A and forms a calcium binding site at about 10°A from the nucleophilic aspartate. Domain C is well conserved in all GS structures as well as in most GH13 enzymes, and is located next to the catalytic domain and 'opposite' of the domain B. The function of Domain C in glucansucrase has not yet been understood. The structure of domain IV revealed a novel fold with no similarity to any other known protein structure. Domain IV helps in connecting domains B and V. Ito *et al.*, 2011 propose that domain IV may act as a 'hinge' that brings domain V bound with glucan near or away from the catalytic site. Domain V, located next to

domain IV, is either built up of both N- and C-terminal segments or of an N-terminal segment alone.

Glucan binding domains of GSs facilitate the transfer of synthesized products from the catalytic site, but the detail mechanism is still unknown. However, it has been speculated that domain V may 'swing' between two conformations such as to bring bound intermediate glucan products away from or towards the active site (Ito *et al.*, 2011). The presence of the C-terminal glucan binding domain is necessary to keep an enzyme active (Abo *et al.*, 1991; Lis *et al.*, 1995). The clustered aromatic residues (tyrosine, tryptophane and phenylalanine) may stabilize the binding between sugar and protein by interacting with the sugar unit (Quiocho, 1986). The polar (lysine, glycine and phenylanine) or acid (aspartic acid) residues might be involved in the formation of hydrogen bonds with hydroxyl residues of the sugar (Quiocho, 1986). The amino acid residues such as lysine, glycine, asparagine or serine impart flexibility to the enzyme therefore, may allow the glucosyl residue to be correctly orientated to the binding sites (Lemieux, 1989). Glucansucrase are extracellular as well as found in a cell-associated form (Janeček *et al.*, 2000). Thus, the glucan binding domains (GBDs) of GSs may also anchor to carbohydrate moieties present in cell wall components in addition to the glucan products.

Dextransucrase gene from *Weissella confusa* Cab3 was cloned and expressed in *Lactococcus lactis* The modeled structure of WcCab3-rDSR using the crystal structures of dextransucrase from *Lactobacillus reuteri* (protein data bank, PDB id: 3HZ3) and *Streptococcus mutans* (PDB id: 3AIB) as templates depicted the presence of different domains such as A, B, C, IV, and V. The domains A and B are circularly permuted in nature having $(\beta/\alpha)_8$ triose phosphate isomerase-barrel fold making the catalytic core of

WcCab3-rDSR. The structure superposition and multiple sequence alignment analyses of WcCab3-rDSR with available structures of enzymes from family 70 GH suggested that the amino acid residue Asp510 acts as a nucleophile, Glu548 acts as a catalytic acid/base, whereas Asp621 acts as transition-state stabilizer and these residues are found to be conserved within the family (Shukla *et al.*, 2015).

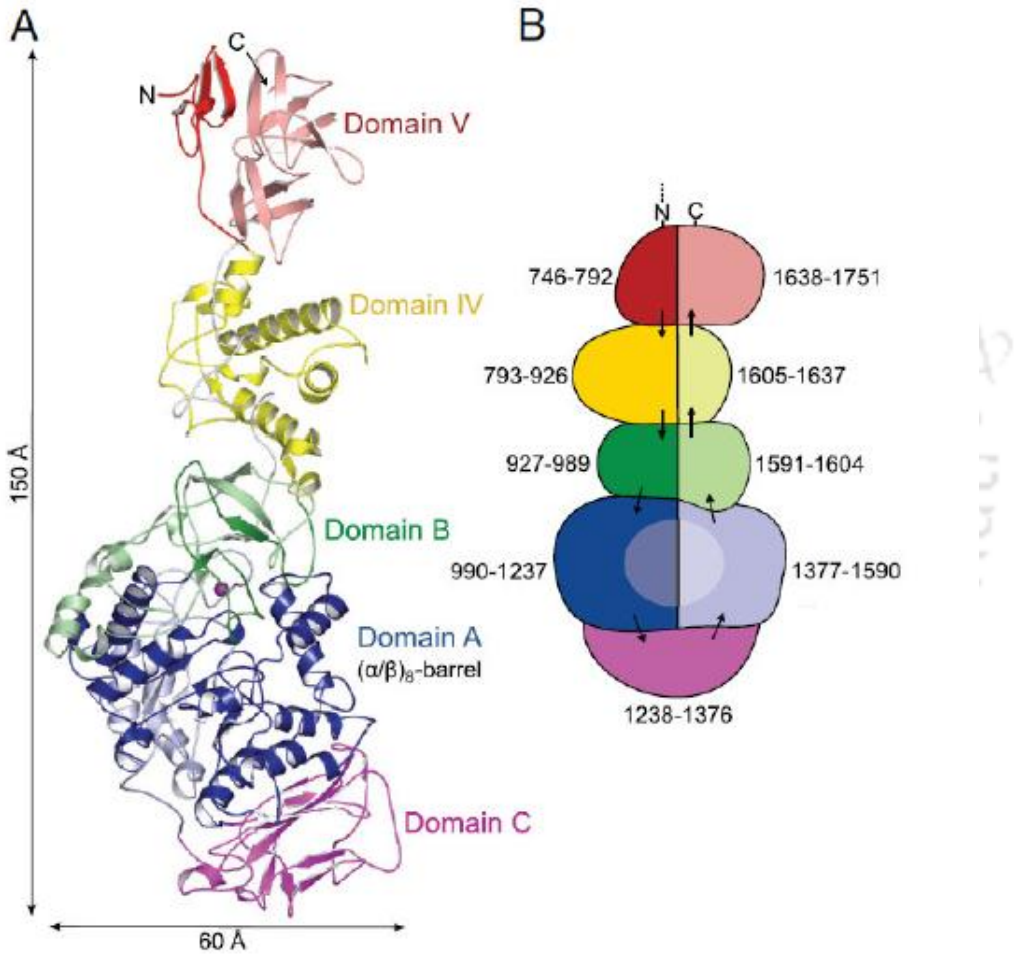

Fig. 1.4 Overall structure of *Lactobacillus reuteri* 180 GTF180-_N as reported by Vujicic-Zagar *et al.*, 2010. **(A)** Crystal structure of GTF180-_N, the N- and the C-terminal ends of the polypeptide chain are indicated; **(B)** Schematic presentation of the "U-shaped" course of the polypeptide chain. Domains A, B, C, IV, and V are coloured in blue, green, magenta, yellow, and red, respectively, with dark and light colors for the N- and C-terminal stretches of the peptide chain.

1.5.3 Production, purification and biochemical characterization of dextransucrase

Dextransucrase are produced by various *Leuconostoc* (Kim and Robyt, 1995a; Padmanabhan and Kim, 2002), *Pediococcus* (Patel *et al.*, 2011a), *Lactobacillus* (Hammond, 1969), *Weissella* (Maina *et al.*, 2008; Shukla and Goyal, 2011; Rao and Goyal 2013) and *Streptococcus* sp (Chludzinski *et al.*, 1974). Dextransucrase is an inducible enzyme requiring sucrose in the medium for the induction with the exception of *Streptococcus* sp and recently isolated constitutive mutant strains of *Leuconostoc mesenteroides viz.* B-512 FMC (Kitaoka and Robyt, 1998), B-742 (Kim and Robyt, 1995a), B-1299 (Kim and Robyt, 1995b) and B-1355 (Cote *et al.*, 1999). Dextransucrase production is affected by temperature, aeration and medium components (Cortezi *et al.*, 2005). Tsuchiya *et al.*, 1952 studied the effect of sucrose, corn steep liquor and phosphate on dextransucrase production from *Leuconostoc mesenteroides* strain NRRL B-512. Goyal and Katiyar, 1997 used low yeast extract and high K_2HPO_4 concentration for enhanced dextransucrase production from *Leuconostoc mesenteroides* NRRL B-512F. The *Leuconostoc mesenteroides* NRRL B-512F displayed increased production of biomass and enzyme with the increase in K_2HPO_4 concentrations (Rodrigues *et al.*, 2003). The aeration conditions were optimized for production and scale up of dextransucrase from *Leuconostoc mesenteroides* B/110-1-1 strain in a bioreactor (Michelena *et al.*, 2003). Purama and Goyal 2008a studied dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 in a bioreactor at different aeration rates and reported that the enzyme activity was 36% higher at 1.5 vvm aeration than that at 0 vvm.

Various purification methods such as salt, alcohol precipitation, fractionation by polyethylene glycol, ultra-filtration, chromatography and phase-partitioning have been

successfully used for purification of dextransucrase from different strains of lactic acid bacteria (Majumder *et al.*, 2007). Among all the reported purification methods, fractionation by polyethylene glycol (PEG) is an effective, rapid and single step purification method for dextransucrase from *Leuconostoc mesenteroides* (Purama and Goyal, 2008b). Polyethylene glycol (PEG) is a non-ionic hydrophilic polymer which selectively precipitates high molecular weight or aggregated form of proteins (Goyal and Katiyar, 1994). The dextransucrase from *L. mesenteroides* NRRL B-512F (Goyal and Katiyar, 1994), *Leuconostoc mesenteroides* NRRL B-640 (Purama and Goyal 2008b), *Leuconostoc dextranicum* NRRL B-1146 (Majumder *et al.*, 2008) and *Pediococcus pentosaceous* (Patel *et al.*, 2011a) have been purified by fractionation with PEG of different molecular weights.

Dextransucrases are large proteins existing in multiple forms ranging from molecular mass of 64-245 kDa (Willemot *et al.*, 1988). This variation is associated with the presence of dextran in the purified preparations, with the disassociation of subunits from a high molecular mass multimeric complex (Kobayashi and Matsuda, 1980; Kobayashi and Matsuda, 1986). The multiple molecular forms of dextransucrase could also result as the action of proteases (Miller and Robyt, 1986; Fu and Robyt, 1990a). Sanchez-Gonzalez *et al.*, 1999 showed that the presence of different molecular forms of dextransucrase from *L. mesenteroides* strains B-512F and B-512FMC are the result of proteolytic processing.

Divalent cations are associated with glucansucrases, therefore they provide stability to the enzymes (Goyal *et al.*, 1995). Miller and Robyt, 1986 reported the association of Ca^{2+} ions with the catalytic sites of glucansucrases. Alkaline earth metals are known to enhance the activity of dextransucrase whereas, EDTA inhibits their

activity significantly. Therefore, glucansucrases are associated with alkaline earth metals (Kobayashi and Matsuda, 1980). The presence of Ca^{2+} was shown to be essential for the activity of glucansucrase from *Lactobacillus reuteri* 121 (GTFA-ΔN) (Kralj *et al.*, 2004) and from *Lactobacillus reuteri* 180 (GTF180-ΔN) (Vujicic-Zagar *et al.*, 2010).

Dextransucrase form several *Weissella* species have been purified and characterized. Dextransucrase from *Weissella confusa* Cab3 isolated from fermented cabbage was purified and assay conditions were optimized to give specific acitivity of 11.7 U/mg at 35°C, pH 5.4, and ionic strength of 20 mM (Shukla and Goyal, 2011). Dextransucrase from *Weissella cibaria* JAG8 isolated from apple peel was purified and optimum assay conditions for dextransucrase were 35°C, pH 5.4, and 5.0% (w/v) sucrose concentration. The enzyme followed Michaelis–Menten kinetics with K_m of 13 m*M* and V_{max} 27.5 U/mg (Mohan Rao and Goyal, 2013).

1.6 **Dextran from LAB**

Lactic acid bacteria (LAB) are known through ages for their wide applications in food, pharmaceutical and chemical industries. But recently LABs have aroused interest for their ability to secrete extracellular polysaccharides. These exopolysaccharides (EPS) have immense commercial applications because of their industrially useful physico-chemical properties (Patel *et al.*, 2012). The exopolysaccharides from LAB are of two types;

A. Homopolysaccharides: polymers composed of glucose or fructose units, such as glucans which contain repetitive glucose units joined by α-(1→6) glycosidic linkages. *e.g.* dextran, mutan, alternan and reuteran.

B. Heteropolysaccharides: polymers composed of a variety of sugar residues, mainly glucose, galactose, fructose and rhamnose, such as kefiran that contains equal proportion of galactose and glucose. In some Heteropolysaccharides charged groups such as acetate, phosphate or glycerol phosphate are also present (De Vuyst and Degeest, 1999).

In 1861, Louis Pasteur initiated systematic scientific progress by explaining that these "viscous fermentations" resulted from microbial action. In 1878, van Tieghem named the causative bacteria *Leuconostoc mesenteroides* because its growth in colorless flocs resembled that of the green algae of the genus Nostoc. In 1880, Scheibler established this type of product as a glucan having positive optical rotation and named it dextran (Jeanes., 1977). Dextrans are homopolysaccharides of D-glucose units that are α-(1→6) linked in the main chains with a various degree of α-(1→2), α-(1→3), or α-(1→4) branched linkages (Capek *et al*., 2011). Lactic acid bacteria (LAB) of the genera *Leuconostoc*, *Streptococcus*, *Weissella*, *Pediococcus* and *Lactobacillus* synthesize dextrans using sucrose as a substrate (Falconer et al., 2011). Dextransucrase secreted by LABs hydrolyses sucrose to produce dextran and fructose as a by-product.

1.6.1 Mechanism of dextran biosynthesis

The mechanism of dextran biosynthesis involving a “two-site insertion mechanism” was first proposed for the synthesis of dextran by dextransucrase by Ebert and Schenk, 1968. Experimental evidence and further elaboration of the two catalytic site and insertion mechanism was provided by Robyt *et al.* (1974) and Robyt and Walseth, (1978). Robyt *et al.* (1974) by pulse-chase experiments demonstrated that glucan and glucosyl residues coming from sucrose cleavage were both covalently linked with enzyme through their reducing end. Robyt *et al.* 1974 proposed that the two

identical nucleophilic sites are involved for dextran biosynthesis without requiring the presence of exogenous primer at the beginning of the reaction. Moulis *et al.*, 2006 recently rejected the two-site insertion mechanism for B-512F dextransucrase. Moulis *et al.* (2006) concluded that the synthesis is a semi-processive mechanism, involves a single active site, and that the glucosyl residues are added to the non -reducing end of a growing dextranyl chain. According to Moulis *et al.* 2006, dextransucrase first hydrolyzes sucrose into glucose and fructose as a minor reaction. Then dextransucrase uses both glucose and sucrose, as initiator primers for the elongation of dextran, which continues by the addition of glucose to the non-reducing end of the isomaltodextrin chain. Robyt *et al.*, 2008 reinvestigated the dextransucrase reaction mechanism using pulse and chase experiments with soluble B-512FMC dextransucrase, as a conformational evidence. Robyt *et al.*, 2008 experimentally proved that the high molecular weight dextran does not require a primer and also that it does not follow a non-reducing-end synthesis mechanism. There experiments showed that glucose, sucrose or isomaltodextrins are not initiator primers for the synthesis of dextran by dextransucrase (Robyt *et al.*, 2008). In “two site insertion mechanism” D-glucose and the growing dextran chain are covalently attached to the active site of the dextransucrase (Robyt *et al.*, 2008). The D-glucose is transferred to the reducing end of the growing dextran chain.

However, studies on a 117 kDa crystal structure of a glucansucrase fragment (GTF180-_N) from *Lactobacillus reuteri* 180 have supported the non-reducing end growth mechanism (Vujicic-Zagar *et al.*, 2010). The crystal structure of the glucansucrase fragment (GTF180-_N) from *Lactobacillus reuteri* 180 has confirmed

that there was only one active site with no space for another covalently bound glucosyl residue or dextranyl chain (Vujicic-Zagar et al. 2010).

1.6.2 Mechanism for branching in dextran

Dextran can also be regarded as an acceptor resulting in an increase in the molecular weight of exogenous dextran (Mayer *et al.*, 1981). The type of glycosidic linkage synthesized is determined by the orientation, in which, the acceptor dextran attacks the covalent glucosyl-enzyme intermediate. Leemhuis *et al.*, 2012 stated that the architecture of the active site and acceptor subsite determine the glycosidic linkage specificity. The branching in the dextran biosynthesis results when a dextran chain acts as an acceptor and displaces either the D-glucose or the dextran chain from the active site, forming branched linkages (Robyt, 1995, Kitaoka and Robyt, 1999). The insolubilisation of exogenous dextran occurs by the formation of α-(1→3) linkages by different glucansucrases (McCabe and Smith, 1978; Mayer *et al.*, 1981; Sato *et al.*, 1982). Robyt and Taniguchi, 1976, reported that the α-(1→3) linkages are created between the anomeric carbon involved in one covalent glucosyl-enzyme complex and the OH-C3 of a glucosyl residue of the exogenous glucan. Cote and Robyt (1984) suggested that α-(1→3) linkages of a highly branched glucan may be synthesized by transfer of glucosyl residues from a glucosyl-enzyme site different from the two active sites to the glucan. However, this acceptor reaction mechanism is insufficient to explain the synthesis of highly branched glucan (Kim and Robyt, 1996).

1.6.3 Purification and characterization of dextran

Extracellular dextran extraction from culture medium usually involves cell removal, dextran precipitation and drying of the precipitated dextran (Ruas-Madiedo

and de los Reyes-Gavilan, 2005). Membrane-filtration, anion-exchange and gel permeation chromatography (Sanz and Martine, 2007) have been successfully employed to purify dextran. Precipitation of proteins with trichloroacetate and hydrolysis of proteins with proteases can be done to remove protein contamination from dextran. The molecular weight of dextran has been determined by high-performance size exclusion chromatography coupled with refractive index detection (HPSEC-RI) and with multi-angle laser light scattering (HPSEC-MALLS) (Picton *et al.*, 2000) field flow fractionation (FFF) and hydrodynamic chromatography (HDC) (Cave *et al.*, 2009; Isenberg *et al.*, 2010).The composition of dextran can be determined by acid hydrolysis followed by monomer detection using high-performance anion-exchange chromatography (HPAEC) with pulse amperometric detection (PAD) (Cataldi *et al.*, 2000). Structural analysis of the dextran has been studied by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy which provides information about the type of constituent monosaccharides, including ring size and anomeric configuration, and the position of glycosidic linkages. The FT-IR, NMR, scanning electron microscopy and rheological studies on dextrans of *Weissella cibaria* have been carried out (Rao and Goyal, 2013; Ahmed *et al.*, 2012). The dextran from *Weissella confusa* E392 containing α-(1→6) and α-(1→3) linked branches was characterized using NMR spectroscopic analysis (Maina *et al.*, 2008). *Leuconostoc mesenteroides* NRRL B-640 synthesizes α-(1→6) linked linear dextran (Purama *et al.*, 2009) and *Leuconostoc dextranicum* NRRL B-1146 synthesizes α-(1→6) linked dextran with α-(1→4) branching. The dextrans produced by *Weissella cibaria* and *Weissella confusa* have been reported to be highly linear. They contain only α-(1→6) linkages (Kang *et al.*, 2006) or with few (2.4–3.4%) α-(1→3) linked branches (Bounaix *et al.*, 2009, Ahmed *et al.*, 2012, Shukla *et al.*, 2014 and Maina *et al.*, 2008). Dextran containing 7% α-(1→3) linked branches, which is the

highest branching present among dextrans from *Weissella* sp was reported for *Weissella cibaria* JAG8 (Rao & Goyal 2013).

1.6.4 Physicochemical characterization of dextran

Dextran polymers have a remarkable diversity in chain length and in physicochemical properties due to the variation in degree of branching in their glucose backbone. In general, dextran is readily soluble in water, dimethyl sulfoxide, formamide, ethylene glycol, and glycerol but insoluble in monohydric alcohols, e.g., methanol, ethanol, and isopropanol, and also most ketones, e.g., acetone and 2-propanone. However, the water solubility of dextrans depends upon the branched linkage pattern (Kothari *et al.*, 2015). Linear dextrans have high water solubility, and the aqueous solutions behave as Newtonian fluids. However, some branched dextrans showed shear rate thinning effect, exhibiting non-Newtonian pseudoplastic behavior (Das and Goyal 2014). Viscosity of dextran solution depends on its concentration, temperature, and molecular weight. As dextran is a neutral polysaccharide, the viscosity is not significantly influenced by changes in pH or salt concentration. Dextrans with >43 % branching through α-(1→3) linkages are water insoluble. Dextrans have molecular weight in the range of 3–500,000 kDa. Dextrans with a molecular weight of 2,000–10,000 kDa exhibit the properties of an expandable coil, and at lower molecular weights (<2,000 kDa), dextran is more rod-like. Low molecular weight dextrans (40, 60, and 70 kDa) are generally preferred in clinical applications (Naessens et al. 2005). High molecular weight dextrans with few branched linkages are required for the application in sourdough (Lacaze *et al*. 2007). The surface morphological studies of dextran revealed a porous structure (Shukla and Goyal 2013; Das and Goyal 2014). The dextran has excellent thermal stability with degradation temperature ~300ºC (Das *et al*.

2014; Tingirikari *et al*. 2014a). Dextrans produced by genus *Weissella* have been reported to have similar structures, consisting of mostly α-(1→6) glycosidic bonds and a few α-(1→3) glycosidic bonds as branched linkages (2.4-7.0%) (Ahmed *et al*., 2012; Rao and Goyal 2013; Shukla *et al*., 2014). Dextran from genus *Weissella* are also attributed with high molecular weight ranging from 800 kDa in the case of *Weissella cibaria* JAG8 (Tingirikari *et al*. 2014b) to 18 MDa in the case of *Weissella confuse* CAB3 (Shukla *et al*., 2014).

1.6.5 Applications of dextran in foods

Dextran has been studied as a food ingredient since the 1950s. The US Food and Drug Administration (US FDA) currently lists dextran as GRAS (generally recognized as safe) additive for food and feed applications. In general, dextran is used as gelling, viscosifying, texturing, and emulsifying agent in various food products (Leemhuis *et al*. 2013). Commercial applications of dextran from LAB are generally found in food and pharmaceutical industry; however, dextran also has several potential applications in photo film manufacturing, fine chemical, cosmetic, paper, petroleum, and textile industries (Naessens *et al*. 2005; Leemhuis *et al*. 2013). Due to the heterogeneity of dextran produced by various LAB, their application may depend on well-defined chemical and physicochemical properties.

The incorporation of dextran in bread for the improvement of rheological properties and quality is gaining interest (Galle *et al*. 2012; Wolter *et al*. 2014). The increasing knowledge of sourdough fermentation generates new opportunities for its use in the bakery field. In situ dextran production from *Weissella* sp. and *Leuconostoc mesenteroides* improved the freshness, mouthfeel, texture, loaf volume, softness, and shelf life of sourdough wheat bread (Katina *et al*. 2009; Galle *et al*. 2012). It came forth

that dextran should have a high molecular weight and few branched linkages for the application in sourdough (Lacaze *et al*. 2007). The European Commission has approved the use of dextran in baked goods, up to the levels of 5%. The addition of 2% native dextran increases the water absorption of flour dough by about 12%. However, in situ formation of dextran in sourdough was reported to be more effective than external addition (Brandt *et al*. 2003).

Prebiotics as functional foods can be used to modulate the composition of the colonic microbiota to provide health benefits to the host (Saad *et al*., 2013). Foods containing prebiotic have also been associated with the protection against risk of several diseases, *viz*., bowel cancer, inflammatory bowel disease, diarrhoea, coronary heart disease, obesity, osteoporosis, cholesterolemia and type 2 diabetes. The α-(1→6) linkages are known to be resistant to hydrolysis by human intestinal enzymes, which results in the slow digestion of dextran in human. Dextran and dextran-derived oligosaccharides have also been reported to increase the distribution of *Bifidobacterium* species in an *in vitro* model of the fermentation process in the human colon exhibiting prebiotic activity (Olano-Martin *et al.* 2000). Dextran from *Weissella cibaria* JAG8 (Rao *et al.*, 2014) and *Lactobacillus plantarum* DM5 (Das *et al.*, 2014) showed promising prebiotic potential with very low gut digestibility and selective stimulation of probiotics.

Protein-polysaccharide conjugates *e.g.* protein-dextran conjugate, formed through the Maillard reaction, are carriers of active ingredients and act as agents that enhance techno-functional characteristics of food proteins. These include gelling properties, emulsifiers, stabilizers, antioxidants, and antimicrobial activity, as well as reduced protein allergenicity. Lysozyme-dextran conjugate produce by dry heating

method displayed improved emulsifying and antimicrobial properties of conjugates compared to pure protein (Nakamura *et al*., 1991). Conjugates of whey protein and dextran showed improved emulsifying properties of conjugates as well as improved solubility and thermal stability at acid pH (Jimenez-Castano *et al*., 2007). The conjugates of bovine serum albumin (BSA) and dextran were used in nanoparticle elaboration with high encapsulation capacity of doxorubicin. Conjugates showed potential use in controlled release systems (Deng *et al*., 2010).

1.7 Isomalto-oligosaccharides

Oligosaccharides are low molecular weight carbohydrates, containing sugar moieties with degree of polymerization (DP) between 3 and 10. The nondigestible oligosaccharides are resistant to human digestive enzymes *viz.* salivary and pancreatic amylases (Weijers *et al*., 2008). Thus, they pass through the upper digestive system intact and get fermented in the lower colon, producing short-chain fatty acids (SCFAs), which, in turn, nourish the resident beneficial microbiota (Mussatto and Mancilha 2007; Morris and Morris 2012). Since their recognition as important food additives in 1980s, these functional oligosaccharides have gained phenomenal popularity (Saad *et al*., 2013).

Isomaltooligosaccharides (IMOs) are known to have prebiotic potential. They structures consist of two to five glucose molecules joined together via α-(1→6) glycosidic linkages. IMOs are widely used in various foods and drinks as sweeteners, they have 40% of the sweetness of sucrose. IMOs have a lower calorific value of 2.8–3.2 kcal/g than to sucrose with a calorific value of 3.87 kcal/g (Chockchaisawasdee and Poosaran, 2013). IMOs are found naturally in various fermented foods such as miso, sake, soy sauce and also in honey. Commercial IMOs are produced enzymatically and are the market leader in the dietary carbohydrate sector of functional foods in Japan. The approaches for IMO production available to date are based on two major classes of enzymes *viz.*, glycosyltransferases and glycosidases (Kothari and Goyal 2015). In the presence of exogenous acceptor molecules along with sucrose, dextransucrase transfers the glucosyl moieties (produced by the hydrolysis of sucrose) to the acceptor molecule resulting in the formation of acceptor products (oligosaccharides) in addition to dextran. When maltose acts as an acceptor molecule, dextransucrase produces a homologous series of isomalto-oligosaccharides (IMOs) composed primarily of consecutive α-

(1→6) linkages (Robyt and Eklund, 1983). Dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426 showed the highest affinity towards maltose for the acceptor reaction with 83% efficiency of IMO production (Kothari and Goyal, 2013).

1.7.1 Acceptor molecules

The efficiency of the acceptor molecules varies depending upon their ability to compete with glucan synthesis or their effect on the reaction velocity (Sidebotham, 1974). A few examples of strong acceptors are maltose and isomaltose (Koepsell *et al.*, 1952; Robyt and Walseth, 1978; Robyt and Eklund, 1983). They strongly inhibit the yield of glucan synthesis by showing activator effect on the reaction velocity of the reaction (Robyt and Elkund, 1983). However, weak acceptors such as fructose or melibiose can have an inhibitory effect on the reaction (Koepsell *et al.*, 1953), therefore the yield of oligosaccharides can be low (Robyt and Elkund, 1983). Changing the acceptor molecule concentration in the dextransucrase reaction mixture along with sucrose can affect the yield, branching and length of the acceptor reaction products in the reaction. The yield of oligosaccharides decreases with increasing the ratio of sucrose concentration to maltose concentration (Monchois *et al.*, 1998b). However, by increasing the sucrose/maltose ratio (S/M), it is possible to catalyze the synthesis of oligosaccharides of increasing degree of polymerization (Vasileva *et al.*, 2010). For an S/M ratio of 7, both linear oligosaccharides (only composed of α-(1→6) linkages and a maltose residue at the reducing end) and branched oligosaccharides were produced (Vasileva *et al.*, 2010).

1.7.2 Kinetics and mechanism of acceptor reaction

Various mechanisms have been proposed to explain the kinetics of acceptor reaction of dextransucrase. Oligosaccharides may be synthesized by a nucleophilic attack of the hydroxyl group located at the non-reducing end of the acceptor to the C-1 of one of the two glucosyl residues involved in the two covalent glucosyl-enzyme complexes. This mechanism for oligosaccharide production is in accordance with the glucan biosynthesis mechanism proposed by Robyt *et al.,* 1974. There has been dispute for the acceptor binding site in the dextransucrase. Several studies established the fact that the acceptor binding site is really unique and is distinguished from the two active sites for glucan biosynthesis (Tanrivseven and Robyt, 1992; Su and Robyt, 1994). However, till date no direct evidence is available explaining the existence of a separate acceptor binding site. Moreover, one of the two sucrose binding sites may also be an acceptor binding site (Germaine and Schachtele, 1976; Kobayashi and Matsuda, 1978). Heincke *et al.,* 1999 explained the phenomenon of substrate inhibition and its elimination in the presence of acceptors on the basis of the mechanistic model. Heincke *et al.*, 1999 also proposed the existence of a separate acceptor binding site between the two active sites for dextran biosynthesis. It is assumed that occupation of the acceptor site by sucrose or an acceptor molecule causes hindrance to the dextran formation reaction. Both sucrose and acceptor molecule compete for the acceptor binding site (Heincke *et al.*, 1999). Therefore, increasing acceptor concentration with respect to sucrose enhances oligosaccharide production (Heincke *et al.*, 1999).

1.7.3 Prebiotic potential of oligosaccharides

The term prebiotics was coined by Gibson and Roberfroid (1995). Gibson *et al.* (2004) elaborated the prebiotics concept by certain criteria viz. resistance to gastric

acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption; fermentation by intestinal microflora and selective stimulation of the growth, and/or activity of intestinal bacteria associated with health and wellbeing. Typically, the prebiotics consist of dietary fibres and oligosaccharides. Prebiotics are being implicated in starter culture formulation, gut health maintenance, colitis prevention, cancer inhibition, immunopotentiaton, cholesterol removal, reduction of cardiovascular disease, prevention of obesity and constipation, bacteriocin production, use in fishery, poultry, pig, cattle feed and pet food. Among non-digestible carbohydrates, the functional oligosaccharides present important physicochemical and physiological properties beneficial to the health of consumers, and for this reason, their use as food ingredients has increased rapidly. The functional oligosaccharides are intermediate in nature between simple sugars and polysaccharides and are claimed to behave as dietary fibres and prebiotics (Kunz and Rudloff, 2006). The best known functional oligosaccharides include fructo-oligosaccharide, gluco-oligosaccharides (GOS), isomalto-oligosaccharides soybean oligosaccharides, xylo-oligosaccharides and maltitol (Chen, *et al*., 2000; Muzzarelli, 2009; Reis, *et al.*, 2004). The beneficial physiologic functions of the functional oligosaccharides in humans were summarized as follows:(1) they do not stimulate an increase in blood glucose or insulin secretion because they dissolve in the gut to form a viscous gel that lowers the absorption of released glucose; (2) they supply small amounts of energy, approximately 0–3 kcal/g of sugar substitute; (3) they are noncariogenic; (4) they improve the intestinal environment and change the intestinal microbiota so that it is dominated by salutary bacteria as a result of the acidic intestinal environment; (5) they improve and suppress diarrhea and symptoms of diarrhea and (6) they stimulate intestinal absorption of minerals, such as calcium, magnesium and iron (Qiang *et al*., 2009).

1.8 Functional foods and nutraceuticals

Functional foods are similar to conventional foods, which are consumed as part of a usual diet but are known to improve health status beyond primary nutritional function. However, nutraceuticals are the products produced from foods but sold in medicinal forms of either a capsule, tablet, powder, solution, or potion, which is not generally associated with the food and have demonstrated physiological benefits and/or provide protection against chronic diseases; these are now referred to as "natural health products" in Canada (Shahidi, 2004). Nutraceuticals and functional foods provide a means to reduce the increasing burden on the health care system by continuous preventive mechanisms, and the interest in nutraceuticals and functional foods continues to grow and is powered by progressive research efforts to identify properties and potential applications of nutraceutical substances, coupled with public interest and consumer demand. The principal reasons for the growth of the functional food market are current population and health trends (Gul *et al*., 2016).

1.8.1 Importance of functional food and nutraceuticals

Nutraceuticals, a hybrid term or a contraction of nutrition and pharmaceutical, was reportedly coined in 1989 by DeFelice and the Foundation for Innovation in Medicine (Espin *et al*., 2007). Nutraceutical, a dietary supplement, has a potential to deliver a concentrated form of a presumed bioactive agent from a food, presented in a non-food matrix and used with the purpose of enhancing health in dosages that exceed those that could be obtained from normal foods (Zeisel, 1999). These are sold in presentations similar to drugs: pills, extracts, tablets, etc. Functional foods are the foods or dietary components consumption of which may have associated health benefits beyond the basic nutritional properties that the foods possess. Health Canada defines

functional food as a product that resembles a traditional food but possess demonstrated physiological benefits (Shahidi, 2009). In South Korea, functional foods are defined as dietary supplements whose function is to supplement normal diet, and have to be marketed in measured doses, such as pills, tablets, etc. (Kim *et al*., 2007). Functional food should be a food similar in appearance to a conventional food (beverage, food matrix), consumed as part of a usual diet, contains biologically active components with demonstrated physiological benefits, and offers the potential of reducing the risk of chronic diseases beyond basic nutritional functions (Gul *et al*., 2016).

1.8.2 Prebiotic juices

The beverages contribute to weight gain because of their added-sugar content, low satiety, and potential incomplete compensation for total energy, leading to increased energy intake (DiMeglio and Mattes 2000). Because of the high amount of rapidly absorbable carbohydrates, such as various forms of sugars and high-fructose corn syrup and the large volume consumed, such drinks may increase risks of type 2 diabetes and cardiovascular problems independently of obesity and contribute to high dietary glycemic load, leading to inflammation, insulin resistance, and impaired b-cell function (Schulze *et al*., 2004). A simpler and cost-effective way is to consume foods with reduced energy while maintaining taste and nutrition to reduce the amount of energy consumed per day (Nguyen *et al*., 2015). The global market for functional foods has increased and fruit juices has been studied and suggested as proper raw material for functional food production because they are rich in nutrients and vitamins and they are accepted and consumed for a large group of people, including the ones with dairy products consumption restriction (Fontes *et al*., 2015). The use of dextransucrase in the preparation prebiotic fruit juices have recently gained popularity for its efficient use of

native sucrose to form IMOs and in turn lowering the calorific value of the fruit juices (Fontes *et al*., 2015). Prebiotics have been produced by dextransucrase acceptor reaction and included in fruit juices such as cashew apple juice (da Silva *et al*., 2012), apple and orange juices (Johansson *et al*., 2016), mandarin juice (Nguyen *et al*., 2015) and pineapple, cantaloupe melon and orange juices (Fontes *et al*., 2015).

1.8.3 Sourdough and lactic acid bacteria

The application of probiotic microbial strains for fermentation of cereals and legumes is a rational approach for the development of functional foods. Cereals contain high levels of carbohydrates, which act as a source of carbon and energy for microbes during fermentation. Most of the carbohydrates in cereals are present as starch and only available for microbes after amylolytic hydrolysis. Endogenous cereal enzymes, malt or selected enzymes can be used to break down the starch to simple fermentable sugars (*i.e.* maltose and glucose), which can be utilised by probiotics as a carbon source (Mishra and Mishra, 2012). Sourdough is ancient way to improve flavor, texture and shelf life of bread and is widely utilized in whole grain bread baking (Lorenz and Brummer, 2003). In the recent years, especially wheat and rye bran have been in the focus due to their nutritional properties such as dietary fiber, good quality proteins, source of minerals, vitamins and other phytochemicals and also due to technological challenges in the utilization of bran which are the negative influences on dough rheology, texture, and sensory quality (Kajala *et al*., 2016).

Sourdough is a process in which flour and water (and other ingredients) are mixed together and the fermentation takes place by microbes from preceding sourdough, commercial starter culture, bakery equipment or from flour. The most important quality characteristics for wheat breads are high volume, soft and elastic

crumb structure, good shelf-life and microbiological safety of the product (Cauvain, 2003). Sourdough utilization improves bread texture and prevents staling and has therefore been a prominent part of traditional whole grain baking. These positive effects are attributed to enzymatic modification of the dough components during fermentation and the production of functional metabolites such as organic acids and exopolysaccharides (EPS) (Arendt *et al*., 2007). Together with other sourdough lactic acid bacteria (LAB) species, food-derived *Weissella* spp. have gained attention especially due to their ability to produce technologically significant amounts of EPS, particularly dextran, which act as a hydrocolloid in the cereal systems. They also acidify sourdoughs to a lesser extent than conventional sourdough LAB species providing wider usability than conventional strongly acidifying LAB (Di Cagno *et al*. 2006; Katina *et al.,* 2009; Wolter *et al.,* 2014; Shukla *et al*., 2014). In gluten-free bread, dextran from *Weissella cibaria* improved nutritional and organoleptic properties of gluten-free bread (Schwab *et al.*, 2008). Dextran enriched *Weissella cibaria* MG1 sourdough increased loaf volume and improved crumb softness and provided mildly acidic bread with an improved shelf life of gluten-free sorghum sourdough bread (Galle *et al.*, 2012). *Weissella confusa* VTT E-90392 was analyzed for it's the growth, activity and *in situ* production of dextran in wheat sourdough. It efficiently produced dextran from the added sucrose in wheat sourdough without strong acid production. In addition to dextran, formation of shorter isomalto-oligosaccharides by W. *confusa* was also detected (Katina *et al.*, 2009). Two *Weissella confusa* strains (VTT E-90392 and Cab3) was to investigate dextran production in wheat and rye bran. Both strains produced more dextran in rye bran in which the fermentation induced acidification was slower than in wheat bran. *W. confusa* Cab3 produced slightly higher amounts of dextran than *W. confusa* VTT E-90392 in all raw materials (Kajala *et al*., 2016).

1.9 Significance of Investigation

Nutraceutical, a dietary supplement, has a potential to deliver a concentrated form of a presumed bioactive agent from a food, presented in a non-food matrix and used with the purpose of enhancing health in dosages that exceed those that could be obtained from normal foods. Functional foods are the foods or dietary components consumption of which may have associated health benefits beyond the basic nutritional properties that the foods possess. Functional food should be a food similar in appearance to a conventional food (beverage, food matrix), consumed as part of a usual diet, contains biologically active components with demonstrated physiological benefits, and offers the potential of reducing the risk of chronic diseases beyond basic nutritional functions. Prebiotic is defined as "a selectively fermented ingredient that allows specific changes, both in the distribution 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. This beneficial action is due to the production of short chain fatty acids (SCFA) by the gastrointestinal microflora from the fermentation of prebioitcs. Most prebiotics identified today are non-digestible oligosaccharides and polysaccharides. Polysaccharides with prebiotic effect have several advantages over non digestible oligosaccharides which includes (i) tolerance to high ingestion which otherwise causes intestinal discomfort and flatulence, (ii) decrease calorific intake, (iii) mucosal damage from rapid acidification and (iv) laxative effect in the colon (Roberfroid and Slavin, 2000).

Lactic acid bacteria (LAB) of the genera *Leuconostoc*, *Streptococcus*, *Weissella*, *Pediococcus* and *Lactobacillus* synthesize dextrans using sucrose as a substrate (Falconer *et al*., 2011). Dextransucrase secreted by LABs hydrolyses sucrose to produce dextran and fructose as a by-product. Commercial applications of dextran from LAB are generally found in food and pharmaceutical industry; however, dextran also has several potential applications in photo film manufacturing, fine chemical, cosmetic, paper, petroleum, and textile industries. Due to the heterogeneity of dextran produced by various LAB, their application may depend on well-defined chemical and physicochemical properties. Long-chain, high molecular weight polysaccharides that dissolve or disperse in water to give improved rheological (gelling, thickening) or physicochemical (emulsion stabilization, particle suspension, etc.) properties are important for food product formulation. A current consumer trend towards healthy and additive-free food has made the dextran an attractive food ingredient. The microorganisms such as *Leuconostoc mesenteroides*, *Saccharomyces cerevisiae*, *Lactobacillus plantarum*, and *Lactobacillus sanfranciscensis* are used for the production of dextran for its application in food processing without any restriction (Kothari et al., 2015).

1.10 Objectives of present study

The uses of dextran and isomalto-oligosaccharides have seen a phenomenal increase in the recent years. These applications are mostly observed in food industry and are improved with time. Fruits are a good source of novel lactic acid bacteria and are generally regarded as safe for human consumption. Indigenous fruits of Northeast India having potential microbial diversity will be used to isolated lactic acid bacteria with the potential to produce exopolysaccharides. Isolated strains will be screened on the basis of enzyme activity of exopolysaccharide producing enzyme. The production of exopolysaccharide producing enzyme will be optimized to obtain maximum possible enzyme activity. The isolated strain will be identified using 16S rRNA gene sequence analysis and several biochemical tests. The 16S rRNA gene sequence obtained will be submitted in NCBI genbank and phylogenetic tree will be constructed. The exopolysaccharide producing enzyme will be purified by polyethylene glycol fractionation and further by size exclusion chromatography. The enzyme will be biochemically characterized for temperature and pH optima, thermostability and pH stability. The influence of the physical factors such as temperature, shaking and media components on exopolysaccharide production will also be studied. Exopolysaccharide production will be scaled up in 2.5 l bioreactor and studied using batch and fed-batch fermentation. The polysaccharide structure will be characterized by ^{1}H-NMR, ^{13}C-NMR, FTIR spectroscopic techniques and also HPSEC analysis for molecular weight determination. The physicochemical properties of the exopolysaccharide will also be characterized by SEM, AFM, TGA and DSC. The applications of the exopolysaccharide in functional foods will be studied along with its application as a potential prebiotic compound.

1.11 Specific Objectives of the present study

1. Isolation and characterization of dextran producing lactic acid bacterium.
2. Purification and characterization of dextransucrase produced by the bacterium.
3. Structural and physicochemical characterization of dextran produced by the bacterium.
4. Production and scale up of dextran from *Weissella cibaria* RBA12 in bioreactor.
5. Applications of dextran and dextransucrase for functional food applications.

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Chapter 2

Screening, identification and characterization of exopolysaccharide producing *Weissella cibaria* RBA12 isolated from pummelo (*Citrus maxima*)

2.1 Introduction

Fruits and vegetables are an integral part of human diet and are consumed in large quantities all over the world. These products are rich in carbohydrates and poor in proteins with pH values from 4.6 to 7.0 and provide a suitable niche to several bacteria, yeasts and moulds (Weissinger *et al*., 2000; Trias *et al*., 2010). The type of microbiota are determined by the pH and the type of the acid present in fruits. Lactic acid bacteria are demonstrated as a part of autochthonous microflora of tomatoes owing to its low pH and organic acids (Brackett 1988; Sajur *et al*., 2007). Food borne bacteria capable of causing human illness cannot grow at a pH less than 4.0, so edible portion of most fruits precludes the involvement as substrate for proliferation of human pathogen (Naeem *et al*., 2012).

Weissella spp. are Gram positive, short rod, non-motile, non-spore forming, heterofermentative bacteria grouped under lactic acid bacteria (LAB) (Shukla and Goyal 2011). Phylogenetic classification of *Weissella cibaria* JAG8 has been reported by using 16S rRNA gene sequence analysis (Rao and Goyal 2013). *Weissella* spp. are

normally isolated from fermented foods and have the ability to produce glucan in the presence of glucansucrase using sucrose as substrate (Björkroth *et al*., 2002). α-D-Glucan production is a phenotypic criterion for the identification of bacteria under this genus (Bounaix *et al*., 2010; Shukla and Goyal 2011). α-D-Glucans are exopolysaccharides consisting of D-glucose repeating units. The α-D-glucans are classified into dextrans, mutans, alternans and reuterans depending on their glycosidic linkages composition and organization (Leemhuis *et al*., 2013). α-D-Glucans from LAB are regarded as natural food thickeners and can replace other hydrocolloid additives. The potential health benefits of α-D-glucan make it a product of high interest in food applications (Patel *et al*., 2012). The glucansucrases are named based on the α-glucans they synthesize, such as dextransucrases (DSR) and alternansucrases (ASR). *Weissella* spp. have received significant attention in the recent years due to their relatively higher dextran production ability, as compared to other lactic acid bacteria, especially in sourdough bread applications (Shukla *et al*., 2014). The α-D-glucan produced from *Weissella cibaria* species has been reported to act as a perfect hydrocolloid. It serves as a replacement for non-bacterial hydrocolloids such as guar gum and hydroxypropylmethyl cellulose for the generation of gluten-free soft bread with good texture, shelf life, hence holds potential application in baking industry for patients suffering from Celiac disease (Schwab *et al*., 2008; Galle *et al*., 2010). This study reports the isolation and characterization of a novel isolate *Weissella cibaria* RBA12 from Pummelo (*Citrus maxima*). The culture conditions for glucansucrase and glucan production by *Weissella cibaria* RBA12 were investigated.

2.2 Material and Methods

2.2.1 Chemicals and reagents

The components of MRS medium, enzyme production medium, antibiotics, carbohydrate discs, sodium carbonate, copper sulphate and ammonium molybdite were purchased from Himedia Pvt., Ltd. Phenol and sulphuric acid was purchased from Merck India Pvt. Ltd. Standard dextran (T40) was procured from Sigma Chemical Co., USA.

2.2.2 Isolation and culturing of the isolate

One gram of fresh pulp of pummelo was crushed separately using mortar pestle and mixed with 10 ml of MRS medium (DeMan et al. 1960) containing 2.0% (w/v) sucrose in culture tubes (in duplicates), under aseptic conditions and incubated at 28°C under static condition for 16 h. The MRS medium comprised (%, w/v): sucrose, 2; yeast extract powder, 0.5; beef extract and peptone, 1; K_2HPO_4, 0.2; tri-ammonium citrate, 0.2; sodium acetate, 0.5; Tween 80, 0.1 (v/v); $MgSO_4.7H_2O$, 0.02; $MnSO_4.4H_2O$, 0.02. 50 µl of the fermented culture was withdrawn from each of eight culture tubes and re-inoculated into 5.0 ml of MRS medium and incubated at 28°C for 16 h. The above grown cultures were serially diluted from 10^{-4} to 10^{-8} dilution and were spread on MRS medium containing 2.0% (w/v) sucrose 1.5% (w/v) agar and 0.5 % (w/v) $CaCO_3$ and incubated at 28°C for 24 h as described by Endo et al. (2009). Ten colonies were randomly picked from 10^{-8} dilution plate from each tube, based on the morphological differences like size, shape and zone of clearance formed by the production of lactic acid, causing the hydrolysis of $CaCO_3$. Each colony was transferred to 5.0 ml of MRS medium (pH 6.4) and incubated at 28ºC for 16 h. 1.0% (v/v) of the bacterial culture was inoculated in enzyme production medium as described by Tsuchiya *et al*., (1952).

2.2.3 Enzyme production medium

The enzyme production medium described by Tsuchiya et al. (1952) 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. The pH of the medium was adjusted to 6.9 with 0.1 M HCl solution. The medium was sterilized by autoclaving at a steam pressure of 10.3 kPa (15 lb/in^2) and at a temperature of 121°C for 20 min. For enzyme production, the cultures were grown in liquid enzyme production medium for 12h at 28°C under static condition. All inoculations and culture transfers were carried out under laminar air flow.

2.2.4 Screening and isolation of glucansucrase producing bacterial strain

Fresh pulp of Pummelo (1 g) was crushed and added to 100 ml of MRS medium (DeMan et al. 1960) containing 2 % (w/v) sucrose and incubated at 28ºC for 24 h. One percent of the culture was used for inoculation of 100 ml of MRS medium containing 2% (w/v) sucrose and incubated at 28ºC for 24 h. The culture samples were serially diluted up to 10^{-9} dilutions and samples from 10^{-5} to 10^{-9} were spread plated on MRS agar medium containing 0.5% (w/v) $CaCO_3$ and incubated at 28ºC for 24 h (Rao and Goyal 2013). The colonies showing the zone of clearance formed due to hydrolysis of $CaCO_3$ were selected based on their size and shape. The selected colonies were transferred to 5 ml MRS medium and incubated at 28ºC for 12 h. 1% (v/v) culture of the selected isolates were inoculated in 5 ml of enzyme production medium as described by Tsuchiya et al. (1952) and incubated at 28ºC under static condition for 12 h. The cultures were inoculated to 100 ml of enzyme production medium and cell free supernatant was analyzed for enzyme activity. The isolate which showed highest enzyme activity was chosen for further analysis. The selected isolate was denoted as

isolate RBA12 and was maintained in Tsuchiya medium (Tsuchiya *et al*., 1952) at final 20 % (v/v) glycerol and stored at -20ºC.

2.2.5 *Glucansucrase enzyme assay*

The enzyme assay was carried out in 1 ml reaction mixture containing 5% (w/v) sucrose, 20 mM sodium acetate buffer (pH 5.4) and 20 µl cell free supernatant containing enzyme. 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 in boiling water bath for 20 min. 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 to 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 (25 g), sodium potassium tartarate (25 g), sodium bicarbonate (20 g) and sodiumsulfate anhydrous (200 g) dissolved in distilled water and the volume made upto 1 ltr. Filtered and stored at a temperature between 30-37ºC.

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

Reagent C: Ammonium molybdate (25 g) in 450 ml, added 21 ml of concentrated sulphuric acid and mixed. To this was added sodium arsenate (3 g) dissolved in 25 ml of distilled water, mixed and stored at 37ºC for 24 h before use.

Reagent D: Prepared fresh, by mixing 25 ml of reagent A and 1 ml of reagent B.

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 30ºC and pH 5.4. The dextransucrase activity was calculated as

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

ΔA_{500} = Absorbance change at 500 nm.
C = 1 OD 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 Phenotypic characterization of the isolate

The morphological characterization of the isolate RBA12 was carried out using scanning electron microscopy (Zeiss, Sigma, Germany). The samples were prepared by centrifuging overnight grown cultures of the isolate and washed with crystal free Phosphate buffer saline (PBS) for five times and subsequently dehydrated in a desiccator. The phenotypic characterization of the isolate RBA12 was also done by Gram staining, catalase reaction and by motility, triple sugar iron agar and nitrate agar slant tests and were performed at 28ºC by incubating for 24 h. The production of ammonia from arginine was carried out in MRS medium containing 0.3% (w/v) arginine and 0.2% (w/v) sodium citrate replacing ammonium citrate by incubating at

28ºC for 24 h. The presence of ammonia was monitored by Nessler's Reagent (Patil *et al*., 2010). The *D*- or *L*-lactic acid isomers production from glucose metabolism were determined in MRS medium by using D/L lactic acid assay kit (Megazyme, Ireland) (Prasad *et al*., 2014).

The growth of isolate RBA12 was studied in 5 ml of MRS medium at 15ºC, 37ºC and 45ºC by incubating under static condition for 24 h. Similarly, growth of the isolate RBA12 in MRS medium was studied at pH 3.0, 4.0, 5.0, 8.0 and 9.0 by incubating culture at 28ºC for 24 h under static condition. Salt tolerance of the isolate RBA12 was determined in MRS medium containing 2.0%, 4.0%, 6.0%, 8.0% and 10.0% NaCl (w/v) by incubating at 28ºC under static condition for 24 h (Manca de Nadra and Saguir 2009).

2.2.7 Antibiotic sensitivity of isolate RBA12

The isolate RBA12 was tested for its susceptibility to thirty antibiotics using agar disc diffusion test (Fuchs *et al*., 1980). The antibiotic test was performed using commercially available antibiotic octo-discs impregnated with amoxyclav, cephalexin, ciproflaxacin, clindamycin, claxacillin, erythromycin, tetracyclin, ampicillin, carbenicilllin, cephatoxamine, chloramphenicol, co-trimazine, gentamicin, norflaxacin, oxacillin, amikacin, amoxycillin, bacitracin, cephalothin, novobiocin, oxytetracyclin, vancomycin, penicillin-G, tobramycin, cephaloridine, kanamycin, linomycin, methicillin, norfloxacin and oleandomycin purchased from Hi-Media Pvt. Ltd., India. The culture RBA12 growing in log phase was mixed in MRS-soft agar (0.8%, w/v agar) medium and poured over the MRS medium containing 1.8% (w/v) agar plate as described in Section 2.2.6. After 2 min, the antibiotic octodiscs were gently placed at the centre over the surface of the agar plates. The petri plates were incubated in inverted

position for overnight at 28°C and were observed for zone of inhibition around the discs next day.

2.2.8 Carbohydrate fermentation of isolate RBA12

The isolate RBA12 was analyzed for its carbohydrate fermentation ability (Kandler and Weiss 1986). The culture RBA12 growing in log phase in modified MRS medium was used for the analysis. The petri plates were first laid with MRS medium devoid of sugar source, containing 1.8%, (w/v) agar and Phenol red (0.05 g/l) as pH indicator. The isolate RBA12 was grown in modified MRS liquid medium, mixed in MRS-soft agar (0.8%, w/v agar) medium and poured over the MRS medium containing 1.8%, (w/v) agar devoid of sucrose. After 2 min, the carbohydrate octa discs were gently placed at the centre over the surface of the agar plates. The petri plates were incubated in inverted position for overnight at 28°C. The acid production was observed between 24-48 h. The acid production as a result of carbohydrate fermentation was indicated by a change in colour from red to yellow (Purama and Goyal 2008).

2.2.9 Molecular characterization of the isolate RBA12

The extraction of genomic DNA of isolate RBA12 was carried out by genomic DNA isolation kit (Hipura, Hi-Media Pvt. Ltd. India). The quality of the extracted DNA was checked by agarose gel (0.8 %, w/v) electrophoresis. The concentration of DNA was determined by nanodrop (BioSpectrometer kinetic, Eppendorf, Germany). The 16S rRNA gene fragment was amplified from the genomic DNA of the isolate using universal primers 27F (AGAGTTTGATCCTGGCTCAG) and 1429R (GGTTACCTTGTTACGACTT) (Dutta *et al.*, 2012) by PCR (Applied Biosystem, GeneAmp, USA). The 50 µl of reaction mixture contained 0.5 µM, each of 27F and

1429R primer, 2 mM each of dNTP (Merck, Germany), 2.5 mM of $MgCl_2$ (Bioline, UK), 5 µl of 10x reaction buffer (Bioline, UK), 1 U Taq DNA polymerase (Bioline, UK) and 10 ng of genomic DNA. The reaction conditions employed were one cycle at 94ºC for 4 min, 30 cycles of 94ºC for 30 sec, 55ºC for 1 min and 72ºC for 1 min. A final extension of 72ºC for 10 min was included. The amplicons were visualized on 0.8% (w/v) agarose gel. The amplicons were sequenced by SciGenom Labs Pvt. Ltd., Cochin, India. A sequence of 1372 bp of 16S rRNA gene was generated from the forward and reverse primer sequence data using Cap3 sequence assembly program. BLAST (Basic Local Alignment Search Tool) was used to analyze the sequence and submitted to Genbank, NCBI (http://www.ncbi.nlm.nih.gov/). Consensus sequences of representative members within the order imported from Genbank were aligned using ClustalW and phylogenetic tree was constructed by neighbor joining method using MEGA5.2 software. (http://www.megasoftware.net/mega.php).

2.2.10 Effect of temperature and shaking on glucansucrase production

The production of glucansucrase by the isolate RBA12 was studied under different culture conditions such as temperature and agitation speed. The effect of temperature on the enzyme production was studied by varying the temperature from 16ºC to 37ºC using 100 ml medium (Tsuchiya *et al*., 1952) under static condition. The effect of shaking on enzyme production was studied under different shaking conditions 80, 120, 150, 180, 200 rpm at 20ºC. The enzyme activity was determined every 3 h till 18 h after withdrawing 1 ml of culture, centrifuging at 10,500***g*** and 4ºC for 10 min. The cell free supernatant was used for the enzyme assay. The enzyme assay was carried out as mentioned in section 2.2.5. Activity measurements were done in triplicate and all data were reported the average of three independent experiment with ± standard error.

2.2.11 Fermentation profile of the isolate RBA12

The optimized conditions for glucansucrase production were used to study the fermentation profile of the isolate RBA12. Enzyme production medium of 100 ml was inoculated with 1% (v/v) isolate and incubated at 20ºC and 180 rpm. The optical density (600 nm) of the medium and pH profile were monitored every 2 h up to 36 h. The glucansucrase activity was assayed as mentioned earlier. Sucrose consumption in the broth was also monitored using dinitrosalicylic method (Miller 1959) using sucrose as a standard. Both glucansucrase activity and sucrose consumption was monitored every 4 h up to 36 h.

2.2.12 Glucan estimation by Phenol-Sulfuric acid method

The production of glucan by isolate RBA12 was studied in 100 ml enzyme production medium incubated at 20ºC and 180 rpm. The glucan concentration in the broth was determined every 4 h up to 36 h. For the estimation of glucan 200 µl of cell free supernatant was taken and glucan was precipitated by using 3 volume of 95% (v/v) chilled ethanol (Das and Goyal 2013). The solution was then centrifugated at 10,500***g*** and 4ºC for 30 min. The pellet collected was dissolved in 200 µl of de-ionised water and this process was repeated three times. The estimation of glucan concentration was done by using phenol-sulphuric acid method (Dubois *et al*., 1956). The estimation of glucan was carried out in a 175 µl of reaction mixture containing 25 µl precipitated glucan, 25 µl 5% (w/v) phenol and 125 µl concentrated sulphuric acid contained in wells of a microtitre plate and were mixed well. The microtitre plate was incubated at 80ºC for 30 min and the absorbance at 490 nm (A_{490}) was measured on a micro-plate reader. Dextran T40 (Sigma Aldrich, USA) was used as standard. The efficiency of glucan production was calculated as follows

$$Efficiency\ (\%) = \frac{Amount\ of\ glucan\ produced}{Maximum\ possible\ amount\ of\ glucan\ (theoretical)} \times 100$$

where maximum possible amount of glucan (theoretical) = 10 mg/ml at 2% (w/v) sucrose concentration.

2.3 Results and Discussion

2.3.1 Screening of glucansucrase producing isolates

Upon plating, a total of 20 colonies were isolated from MRS agar plates and analyzed for glucansucrase production. All the 20 isolates were grown in 10 ml enzyme production medium and subsequently in 100 ml enzyme production medium and screened on the basis of glucansucrase activity. Of the 20 isolates, one isolate gave maximum enzyme activity of 4.7 U/ml after screening (Fig. 2.3.1). This isolate was denoted as RBA12 and was selected for further characterization and identification.

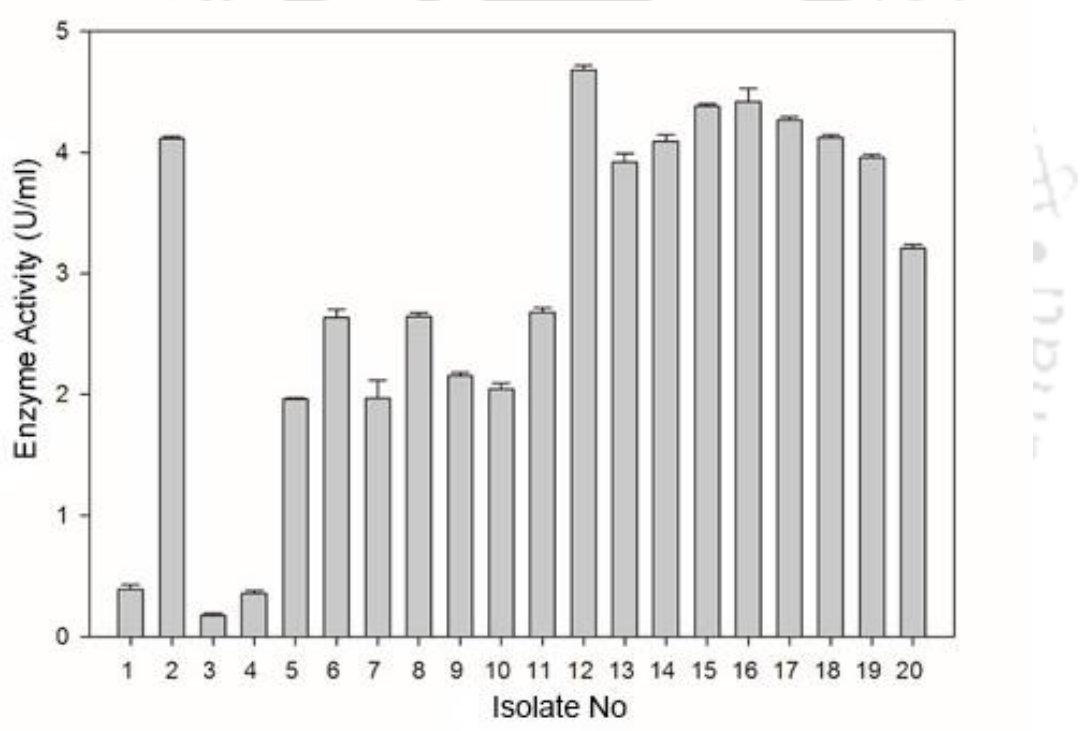

Fig. 2.3.1 Screening of isolates on the basis of glucansucrase activity

2.3.2 Identification of the selected isolate RBA12

The isolate RBA12 was identified based on various morphological, biochemical and physiological properties (Table 2.3.2). The gram staining of the isolate showed its Gram positive nature (Fig. 2.3.2 A). The SEM analysis revealed the isolate to be short rod of 0.6 µm in diameter and 1-1.2 µm in length (Fig. 2.3.2 B). The isolate RBA12 was found

catalase negative and hot loop test showed its hetero-fermentative nature by effervescence after the introduction of the hot loop in the culture medium (Rao and Goyal 2013). The motility test by indole lysine agar indicated the isolate RBA12 is non motile and tryptophanase negative. The isolate RBA12 was able to produce NH_3 from arginine and produced a mixture of D- and L- lactic acid (3.3 g/l and 2.8 g/l, respectively).

The isolate RBA12 could grow at temperatures 15ºC, 37ºC and 45ºC. The isolate RBA12 could not grow at pH range from 3.0 to 4.0 but could grow at pH 5.0, 8.0 and 9.0. Tolerance to NaCl was observed between 2 % (w/v) and 8 % (w/v) NaCl but the isolate RBA12 showed no tolerance to 10 % (w/v) NaCl.

Table. 2.3.2: Morphological, biochemical and physiological characterization of *Weissella cibaria* RBA12.

Characteristics		*Weissella cibaria* RBA12	*Weissella cibaria* JAG8[a]	*Weissella cibaria*[b]
Cell Shape		Short Rods	Short Rods	Short Rods
Cell arrangement		Single, pairs or chains	Single, pairs or chains	Single, pairs or chains
Gram Stain		+	+	+
Motility		-	-	nd
Indole test		-	-	nd
Nitrate Reduction		-	-	nd
Catalase		-	-	-
Gas production from glucose		+	+	+
NH_3 production form arginine		+	nd	+
Lactic Acid Isomer		DL	nd	nd
Growth at temp	15ºC	+	+	-
	37ºC	+	+	+
	45ºC	+	+	+
Growth at pH	3.0	-	nd	nd
	4.0	-	nd	-
	5.0	+	nd	nd
	8.0	+	nd	nd
	9.0	+	nd	-
Growth at NaCl	2 %	+	+	+
	4 %	+	+	+
	6 %	+	+	+
	8 %	+	-	nd
	10 %	-	-	-

nd= not determined. [a] Rao and Goyal (2013), [b]Patil *et al*., (2010)

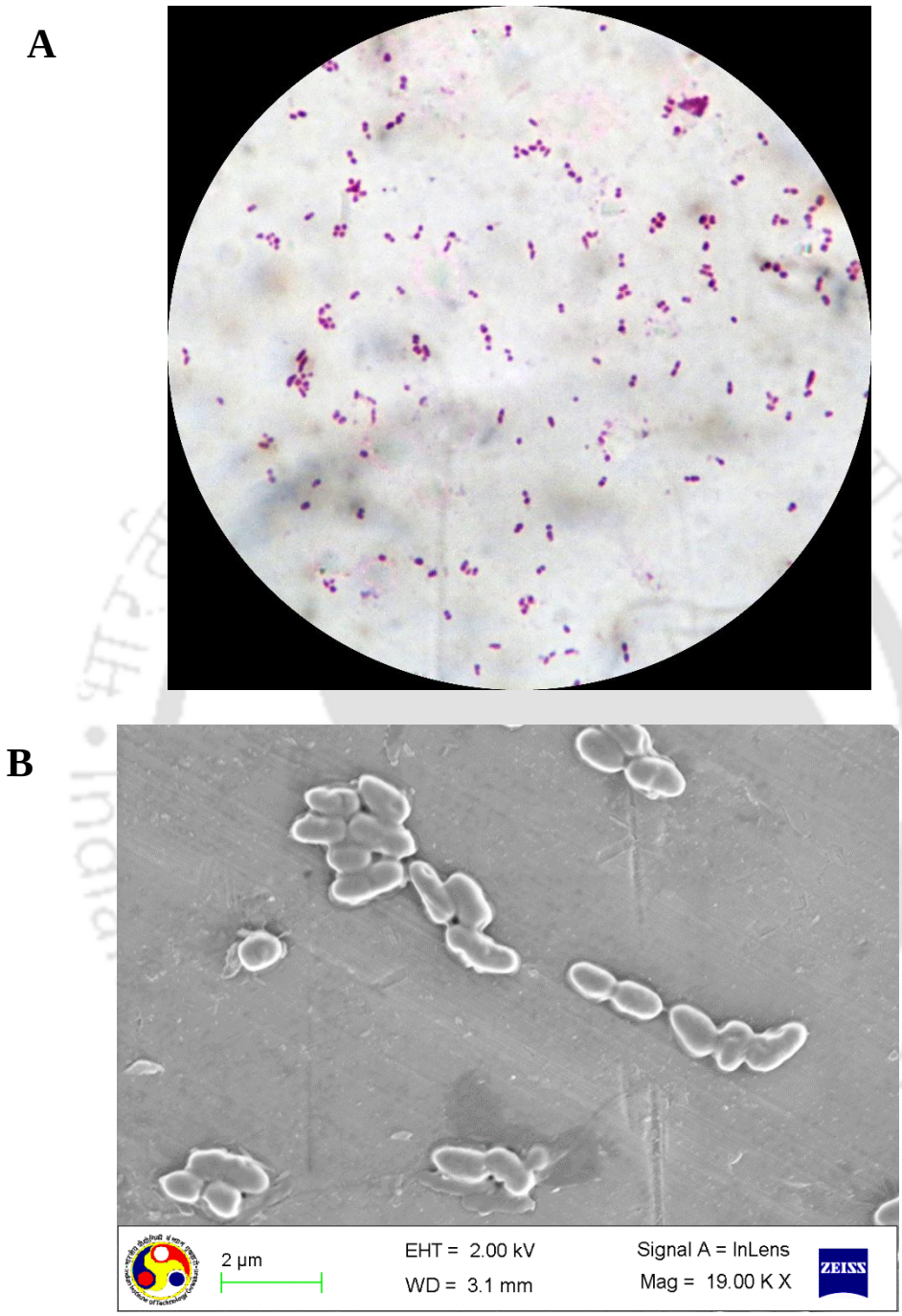

Fig. 2.3.2 A) Gram staining of *Weissella cibaria* RBA12. **B)** Scanning electron micrograph of *Weissella cibaria* RBA12.

2.3.3 Antibiotic sensitivity analysis of isolate RBA12

In order to elucidate the antibiotic susceptibility of the isolate RBA12, a standardized filter-paper disc-agar diffusion assay was carried out. This method determines the efficacy of the drug by measuring the diameter of the zone of inhibition which results from diffusion of the antibiotic from the disc into the medium. In this procedure, the filter-paper disc impregnated with specified concentrations of different antibiotics was placed centrally, on the surface of an agar plate seeded with the isolate RBA12. After the overnight incubation at 28ºC, the plates were examined for the zone of inhibition, surrounding the discs. The susceptibility of microorganism to a drug is determined by the size of this zone. The measurement of the length of the zone of inhibition was made. Based on this comparison, the test organism was classified as resistant, moderate or susceptible to the antibiotic and results were summarized in Table 2.3.3.

The isolate RBA12 was tested for susceptibility to thirty seven antibiotics. It was found resistant to nalidixic acid, nitrofurantoin, sulphamethoxazole, co-trimazine, co-trimoxazole, vancomycin, ciprofloxacin, norfloxacin and cefixime and was sensitive to amoxyclav, ampicillin, amikacin, amoxycillin, bacitracin, cephalexin, clindamycin, cloxacillin, carbenicillin, cephotaxime, cephalothin, ceftazidime, cephaloridine, cefaclor, erythromycin, gentamicin, kanamycin, lincomycin, methicillin, novobiocin, olaendomycin, oxytetracycline, pencillin-G, piperacillin, tobramycin, ticarcillin, tetracycline (Table 2.3.3). Vancomycin resistance is a characteristic feature of lactic acid bacteria. Similar results of vancomycin resistance displayed by other LAB strain *Weissella confusa* Cab3 were also reported by Shukla and Goyal (2011).

Table 2.3.3 Antibiogram of the isolate RBA12 using antibiotic octadisc on MRS agar.

Antibiotic	RBA12	Antibiotic	RBA12
Amoxyclav (10 µg)	S	Oxytetracycline (30 µg)	S
Cephalexin (10 µg)	S	Vancomycin (30 µg)	R
Ciprofloxacin (10 µg)	R	Cephaloridine (30 µg)	S
Cindamycin (2 µg)	S	Kanamycin (30 µg)	S
Cloxacillin (1 µg)	S	Lincomycin (2 µg)	S
Co-Trimoxazole (25 µg)	R	Methicillin (5 µg)	S
Erythromycin (15 µg)	S	Oleandomycin (15 µg)	S
Tetracycline (30 µg)	S	Penicillin-G (10 U)	S
Ampicillin (10 µg)	S	Tobramycin (10 µg)	I
Carbenicillin (100 µg)	S	Nitrofurantoin (50 µg)	R
Cephotaxime (30 µg)	S	Ticarcillin (75 µg)	S
Chloramphenicol	S	Nalidixic acid (30 µg)	R
Co-Trimazine (25 µg)	R	Trimetroprin (30 µg)	I
Gentamicin (10 µg)	S	Sulphamethoxazole (50 µg)	R
Norfloxacin (10 µg)	R	Cefactor (30 µg)	I
Oxacillin (5 µg)	S	Cefixime (5 µg)	R
Amikacin (10 µg)	S	Piperacillin (100 µg)	S
Amoxycillin (10 µg)	S	Ceftrazidime (30 µg)	S
Bacitracin (10 U)	S	Imipenam (10 µg)	S
Cephalothin (30 µg)	S	Cefoperazone (30 µg)	S
Novobiocin (30 µg)	I	Oxytetracycline (30 µg)	S

Legends: S= Sensitive, R= Resistant, I= Intermediate

2.3.4 Carbohydrate fermentation pattern of RBA12

The ability of the isolate RBA12 to utilize and ferment carbohydrates with the production of acid was tested. The isolate RBA12 utilized sucrose, mannose, salicin, arabinose, maltose, fructose xylose, cellobiose, galactose and glucose as colour changed from red to yellow but could not ferment lactose, adonitol, inulin, inositol, melibiose, trehalose, dulcitol, sorbitol, mannitol, rhamnose and raffinose. The majority of the carbohydrate fermentation profile of the isolate RBA12 was similar to other *Weissella* strains, as reported earlier by Bjorkroth *et al*., (2002) and Shukla and Goyal (2011). The extent of fermentation of carbohydrates was categorized and shown in Table 2.3.4.

Table 2.3.4 Carbohydrate fermentation by the isolate RBA12 after 24h incubation.

Sl no	Carbohydrate	RBA12
1	Sucrose	+
2	Adonitol	-
3	Inulin	-
4	Mannose	+
5	Salicin	+
6	Arabinose	+
7	Inositol	-
8	Maltose	+
9	Melibiose	-
10	Trehalose	-
11	Dulcitol	-
12	Lactose	-
13	Sorbitol	-
14	Mannitol	-
15	Fructose	+
16	Xylose	+
17	Glucose	+
18	Rhamnose	-
19	Cellobiose	+
20	Raffinose	-
21	Galactose	+

2.3.5 Sequence analysis of 16S rRNA gene of isolate RBA12

The 16S rRNA gene (1372 bp) sequence analysis by BLAST showed that the new isolate RBA12 from Pummelo (*C. maxima*) was *Weissella cibaria* of family *Leuconostocaceae* (Fig. 2.3.3). The 16S rRNA gene sequence was submitted to Genbank database and a genbank accession number KF515952 was obtained (http://www.ncbi.nlm.nih.gov/nuccore/536624219). The phylogenetic tree of isolate RBA12 showed common evolutionary relationship with *Leuconostoc* and *Lactobacillus* spp. (Fig. 2.3.4) and displayed highest sequence similarity (%) with *Weissella cibaria* AT6 (Fig. 2.3.5). The evolutionary relationship of *W. cibaria* RBA12 with other genus

is similar to those reported for *Weissella cibaria* MSS1, MSS2, MSS3 and MBP5 by Thongsanit *et al*., (2009) and for *Weissella cibaria* DFR3 by Patil *et al*., (2010).

5'-CGCTTTGTGGTTCAACTGATTTGAAGAGCTTGCTCAGATATGACGATGGACATTGCAAA
GAGTGGCGAACGGGTGAGTAACACGTGGGAAACCTACCTCTTAGCAGGGGATAACATTTG
GAAACAGATGCTAATACCGTATAACAATAGCAACCGCATGGTTGCTACTTAAAAGATGGT
TCTGCTATCACTAAGAGATGGTCCCGCGGTGCATTAGTTAGTTGGTGAGGTAATGGCTCAC
CAAGACGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACAC
GGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGAT
GGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGGCTCGTAAAACACTGTTGTAAGAGAAG
AATGACATTGAGAGTAACTGTTCAATGTGTGACGGTATCTTACCAGAAAGGAACGGCTAA
ATACGTGCCAGCAGCCGCGGTAATACGTATGTTCCAAGCGTTATCCGGATTTATTGGGCGT
AAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAAGCCCTCAGCTCAACTGAGGAAT
TGCTTTGGAAACTGGATGACTTGAGTGCAGTAGAGGAAAGTGGAACTCCATGTGTAGCGG
TGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAACT
GACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACACC
GTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTGCCGCAGCTAACGCATT
AAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGGAC
CCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTC
TTGACATCCCTTGACAACTCCAGAGATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTG
GTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTATTACTAGTTGCCAGCATTTAGTTGGGCACTCTAGTGAGACTGCCGGTGACAAAC
CGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTACACACGT
GCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTTAAAGTA
CGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCG
CGGATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCC-**3'**

Fig. 2.3.3 16S rRNA gene (1372 bp full length) sequence of *Weissella cibaria* RBA12.

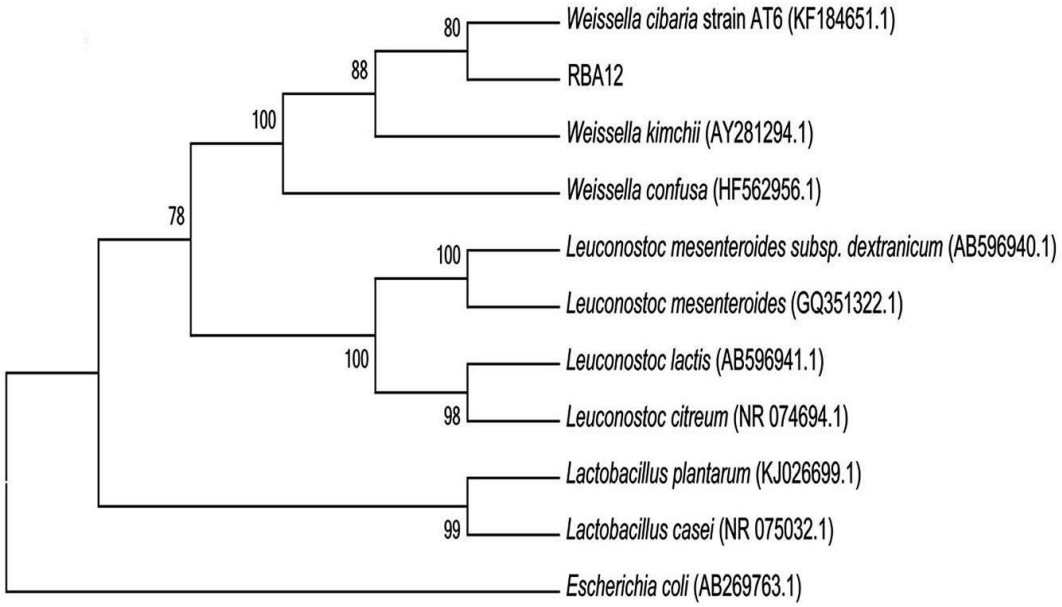

Fig. 2.3.4 Evolutionary relationship of *Weissella cibaria* RBA12 with other bacteria.

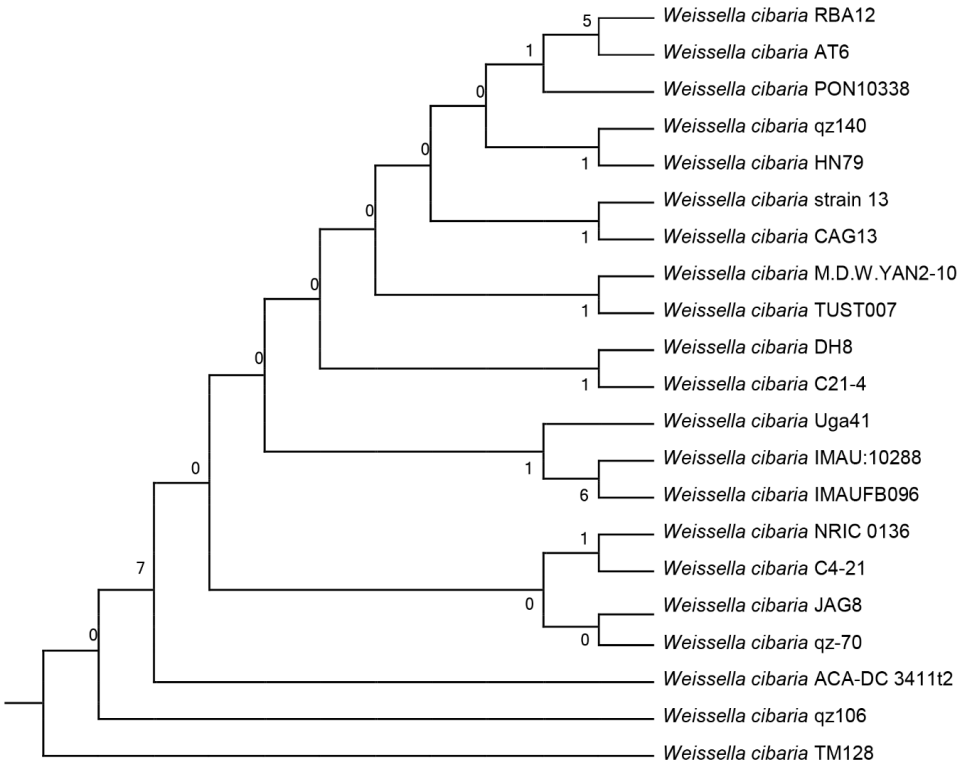

Fig. 2.3.5 Phylogenetic tree of *Weissella cibaria* RBA12 based on 16S rRNA gene sequences of other *Weissella cibaria*.

2.3.6 Effect of temperature and shaking on glucansucrase production from isolate RBA12

The production of glucansucrase from *Weissella. cibaria* RBA12 was greatly affected by the incubation temperature. The maximum enzyme activity, 6.15 U/ml was observed at 20ºC and at 12 h under static condition (Fig. 2.3.6). The enzyme activity decreased above 20ºC and it decreased by 83% at 37ºC and at 12 h due to the inactivation of enzyme at higher temperature as also reported by Das and Goyal (2014). The enzyme activity at 16ºC and 12 h was 59 % lower than that at 20ºC but later increased after 15 h of incubation. This might be due to the slower growth rate of cells at lower temperature which affects the production of enzyme glucansucrase. The effect of different shaking conditions from 80 to 200 rpm on glucansucrase production was studied and compared with the static condition at 20ºC (Fig. 2.3.7). The maximum enzyme activity of 7.2 U/ml was observed at 180 rpm displaying 14% higher than under static condition (6.15 U/ml). The lag phase under shaking at 180 rpm was reduced to 4 h against the prolonged lag phase of 6 h under static condition.

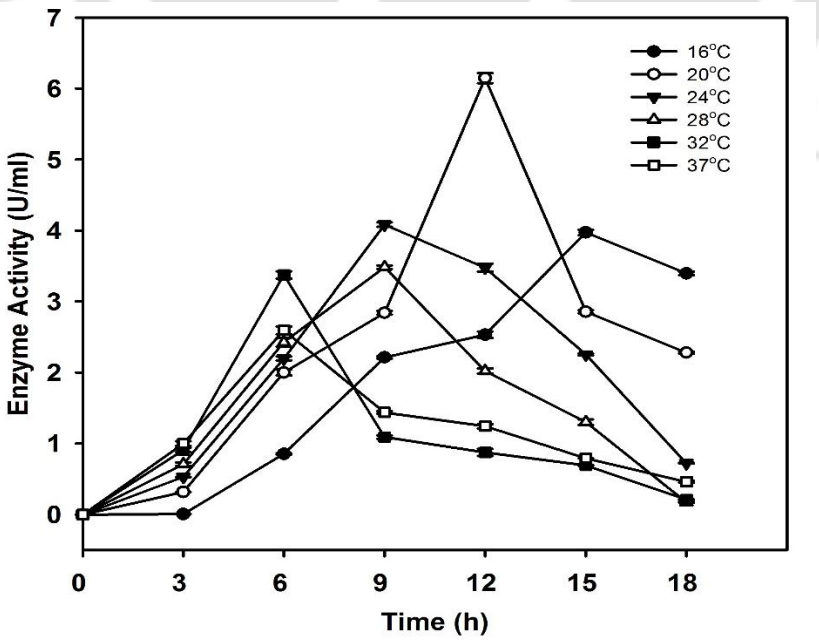

Fig. 2.3.6 Effect of temperature on glucansucrase production from *Weissella cibaria* RBA12.

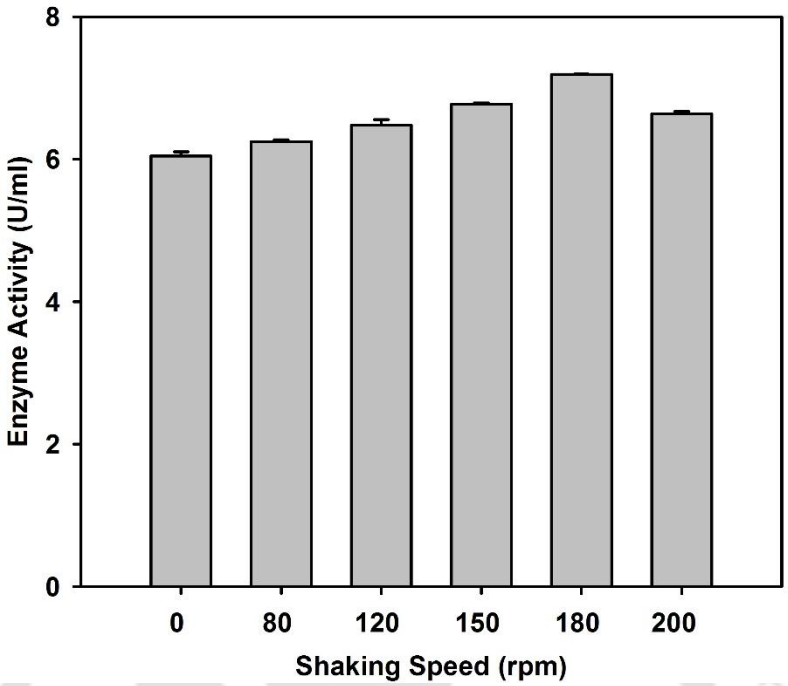

Fig. 2.3.7 Effect of shaking speed (rpm) on dextransucrase production from *Weissella cibaria* RBA12 at 20 ºC.

2.3.7 Fermentation profile of *Weissella cibaria* RBA12

The fermentation of *W. cibaria* RBA12 was studied by incubating the culture in 100 ml enzyme production medium at 20ºC and 180 rpm. The growth of cells was measured by taking absorbance at 600 nm (A_{600}). The cell growth, pH, enzyme activity, glucan concentration and sucrose utilization during fermentation were measured (Fig. 2.3.8). The lag phase continued up to 4 h followed by the exponential phase till 24 h followed by the stationary phase. The maximum enzyme activity of 7.2 U/ml was found at 12 h. The maximum enzyme activity of the isolate RBA12 was higher than commercial strain *Leuconostoc mesenteroides* NRRL B-512F (4.1 U/ml) (Shukla and Goyal 2011) and was higher also among other *Weissella* spp such as *Weissella cibaria* JAG8 (5.8 U/ml) (Rao and Goyal 2013) and *Weissella confusa* Cab3 (6.1 U/ml) (Shukla *et al*., 2014). The pH of the broth decreased from 6.9 to 5.1 after 36 h of incubation. Maximum enzyme activity was observed at pH 6.6. The decrease of pH was negligible after 24 h

due to the onset of the stationary phase. The decrease in pH was due to the formation of lactic acid which is a growth associated product of lactic acid bacteria and also responsible for the inactivation of the enzyme. A glucan concentration of 8.3 mg/ml was observed in the culture supernatant at 24 h. The efficiency of glucan production was 83%, which was quite high as compared with 32% efficiency of glucan production from *Weissella cibaria* CMGDEX3 as reported by Ahmed *et al*., (2012). The glucan concentration slowly decreased after 24 h which might be due to the utilization of glucan as a secondary substrate by the bacterium for its maintenance in its stationary phase after the depletion of its primary substrate sucrose. The utilization of sucrose by the isolate can be correlated with the growth, the exponential decrease of sucrose is observed within the log phase of the isolate. The sucrose was completely utilized after 24 h as the isolate entered the stationary phase.

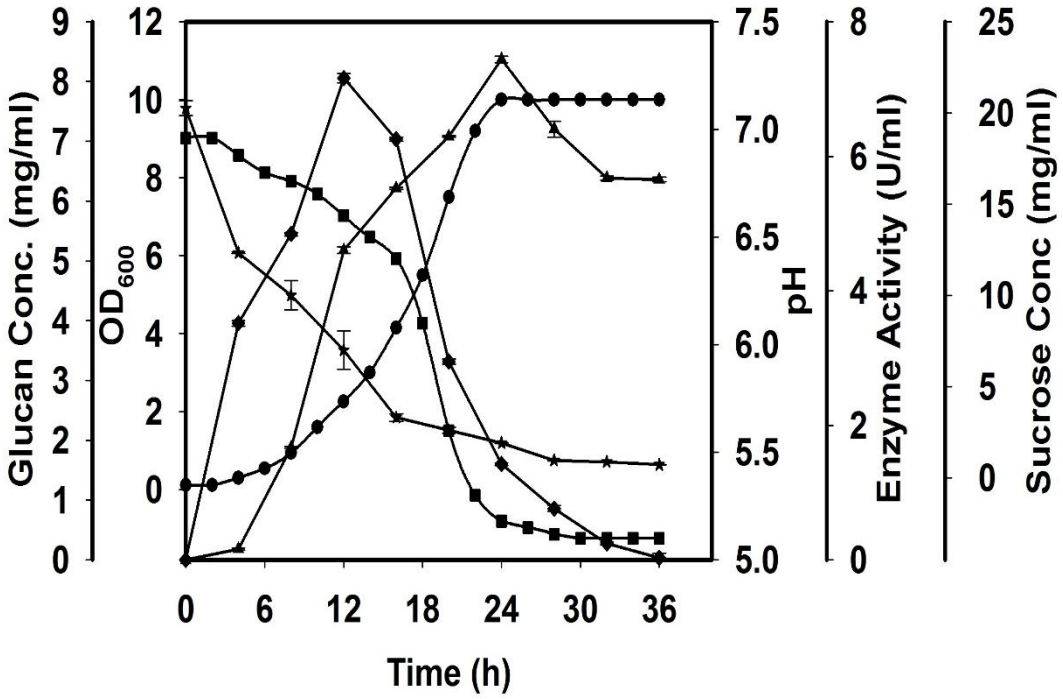

Fig 2.3.8 Fermentation profile of *Weissella. cibaria* RBA12 showing cell OD at 600 nm (OD_{600}) (●), pH (■), enzyme activity (♦), glucan concentration (▲) and sucrose utilization (★).

2.4 Conclusions

Weissella cibaria RBA12 was isolated from the pulp of Pummelo (*Citrus maxima*) on basis of maximum glucansucrase activity (4.7 U/ml). It was identified as Gram positive bacterium of short rods with average size of 0.6 µm diameter and 1.2 µm length. Biochemical characterization revealed the isolate RBA12 to be non-motile, catalase, indole and nitrate negative. The isolate RBA12 could grow between 15-45°C, which indicated the mesophilic nature of bacterium. The bacterium RBA12 could withstand the salt concentration up to 8.0% and pH range of 5-9 for its growth. Antibiogram analysis showed that the isolate RBA12 was resistant to the antibiotics nalidixic acid, nitrofurantoin, sulphamethoxazole, co-trimazine, co-trimoxazole, vancomycin, ciprofloxacin, norfloxacin and cefixime. Resistance to vancomycin is the common characteristic feature for most of the lactic acid bacteria. The isolate RBA12 was sensitive to ampicillin, ciprofloxacin, erythromycin, gentamicin, kanamycin, methicillin, pencillin and tetracycline. The bacterium RBA12 could ferment sucrose, mannose, salicin, arabinose, maltose, fructose xylose, cellobiose, galactose and glucose. The isolate could not utilize lactose, adonitol, inulin, inositol, melibiose, trehalose, dulcitol, sorbitol, mannitol, rhamnose and raffinose.

The 16S rRNA gene sequence analysis revealed the identity of the isolate RBA12 which was found to be *Weissella cibaria* (Genbank Accession Number KF515952). The optimal conditions for glucansucrase production were 20 °C and 180 rpm, yielding a maximum enzyme activity of 7.2 U/ml after 12 h of incubation. The fermentation profile of *Weissella cibaria* RBA12 revealed that the production of glucansucrase was growth associated and the sucrose was completely utilized by the bacterium. The maximum glucan concentration was 8.3 mg/ml resulting in 83% efficiency. The results showed that *Weissella cibaria* RBA12 can be used as an efficient microorganism for

glucan production. This is the first report on isolation of any lactic acid bacteria from Pummelo (*Citrus maxima*) or from any citrus fruit. Further studies on the nature and properties of both glucansucrase and glucan from *Weissella cibaria* RBA12 are required to elucidate their potential applications.

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Chapter 3

Purification, optimization of assay and stability studies of dextransucrase from *Weissella cibaria* RBA12

3.1 Introduction

Dextransucrase (E.C. 2.4.1.5) is an extra cellular enzyme belonging to glycoside hydrolase family 70 (GH70) according to the CAZy classification system (Cantarel *et al*., 2009). The dextransucrase (or) glucansucrase (GH70) are structurally, evolutionary and mechanically closely related to the GH13 and GH77 enzymes and together they form the GH-H clan of glycoside hydrolases (Stam *et al*., 2006). The common characteristic of GH-H clan enzymes is that they cleave the glycosidic linkage between a glucose moiety and another (glucose and fructose) moiety using a catalytic domain (Janecek *et al*., 2011). Dextransucrase from different lactic acid bacteria have been purified by ultrafiltration, salt precipitation, polyethylene glycol (PEG) fractionation, phase partition and size exclusion chromatography (Majumder *et al*., 2007). Among all the methods described PEG fractionation is commonly used because it can precipitate large molecular weight proteins. Dextransucrase exists in either single or multiple molecular forms with varying molecular weights ranging from 64-245 kDa (Kobayashi and Matsuda 1976). Metal ions such as Ca^{2+}, Mg^{2+}, Co^{2+} and inhibitors like urea and EDTA were reported to affect the activity of extracellular dextransucrase (Majumder *et al*., 2008; Goyal *et al*., 1995) and among stabilizers, the most widely used are glycerol,

Tween-80, polyethylene glycols, dextran, and glutaraldehyde on various enzyme systems (Kobayashi and Matsuda 1980; Miller and Robyt 1984). Dextran produced by dextransucrase has gained importance because of its commercial applications in clinical, pharmaceutical, food, photo film, fine chemical and other industries (Majumder *et al*., 2007) whereas oligosaccharides are used as prebiotics which help intestinal microflora by increasing the number of *Bifidobacteria* and lactic acid bacteria (Chen *et al*., 2000).

Stability of enzymes is a critical issue in the biotechnology industry. Enzymes are sensitive to change in the environmental conditions such as temperature, pH and ionic strength. Both operational and storage stabilities affect the production of enzyme-based products. Stabilizing an enzyme normally means suppressing the unfolding of the protein and retaining the catalytic activity (Kühlmeyer and Klein 2003). The stabilization can be achieved by the addition of additives like substrates, products, inhibitors, cofactors, metal ions. The use of enzymes for industrial purposes usually depends on their stability during isolation, purification and storage (Joo *et al*., 2005). There are several strains that have never been explored and yet they may produce dextransucrase with higher activity and resulting in a type of dextran that may find potential use in industries. *Weissella* species has gained importance because of its increasing applications in food industry and only few reports are available on the purification and stability studies of dextransucrase from *Weissella cibaria* (Schwab *et al*., 2008; Galle *et al*., 2010; Rao and Goyal 2013 a). Hence, it is important to study this industrially important enzyme for commercial exploitation.

3.2 Materials 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 chemicals required for reducing sugar estimation, protein estimation and buffer preparation were of high purity. PEG-400 from Merck, India and PEG-1500 from BDH Chemicals Ltd. UK were used for enzyme fractionation.

3.2.2 Maintenance of the isolate *Weissella cibaria* RBA12

Weissella cibaria RBA12 isolated from pumello (*Citrus maxima*) was maintained in Tsuchiya medium (Tsuchiya et al. 1952) with 20 % (v/v) glycerol at -20 °C. For the development of inoculum, a 1% (v/v) culture from glycerol stock was transferred to 5 ml of enzyme production medium Tsuchiya *et al*., (1952) as described in Chapter 2, Section 2.2.2. The culture was incubated at 20°C under static conditions for 12 h for production of dextransucrase.

3.2.3 Enzyme activity assay

The assay of dextransucrase was conducted in 1 ml of a reaction mixture containing 20 mM sodium acetate buffer (pH 5.4), 5% sucrose and 20 µl cell free supernatant containing enzyme. The reaction mixture was incubated at 30°C for 15 min. The enzyme activity was analyzed by estimating the reducing sugars by the Nelson-Somogyi protocol (Nelson, 1944; Somogyi, 1945) as described in Chapter 2, Section 2.2.10.2. Aliquots (0.1 ml) from the reaction mixture were analyzed for reducing sugar. The absorbance was recorded at 500 nm in a UV-visible spectrometer (Varian, model Cary 100) against a blank. D-fructose was used as a standard.

3.2.4 Determination of protein

The protein content of the cell free extract 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 from 25 (µg/ml) to 500 (µg/ml) was used to plot a standard curve.

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 B1 : 2% (w/v) sodium potassium tartarate.

Reagent B2 : 1% (w/v) copper sulfate.

Reagent C : prepared fresh by mixing 1.0 ml of reagent B1 and 100 ml of reagent A followed by addition of 1.0 ml of reagent B2.

Phenol reagent : 1 N phenol reagent (Folin's reagent).

3.2.5 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 optical density (OD) 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} = change in absorbance of the sample

C = 1 OD equivalent of BSA from standard plot

V = volume of the sample

3.2.6 Purification of dextransucrase from *Weissella. cibaria* RBA12 by PEG fractionation

One percent of *Weissella cibaria* RBA12 culture was inoculated in 100 ml of enzyme production medium as reported by Tsuchiya *et al*., (1952) and as described in Chapter 2, Section 2.2.3 and incubated at 20°C for 12 h. The grown culture was centrifuged at 10,500*g* at 4°C for 10 min. The cell free supernatant was analysed for enzyme activity as described in Chapter 2, Section 2.2.5.1 and for protein concentration as described in Section 3.2.4. The pre-chilled PEG 400 was added to 100 ml of cell free supernatant to obtain the final concentrations of 15, 20, 25, 33 and 40% (v/v). Similarly, in case of PEG 1500 the cell free supernatant was treated with 5, 10, 15 and 20% (w/v). The mixture was incubated for 12 h at 4°C to allow the dextransucrase to precipitate. The mixture was centrifuged at 17,200*g* at 4°C for 40 min to separate the fractionated dextransucrase. The enzyme pellet was dissolved in ice cold 20 mM sodium acetate buffer pH 5.4. The dissolved enzyme was subjected to dialysis using 14 kDa cut off membrane. Finally, the dialyzed enzyme samples were analysed for enzyme activity and protein concentration as described in Chapter 2, Section 2.2.5.1 and Chapter 3, Section 3.2.5, respectively. The dialyzed enzyme was characterised further by both denaturing and non-denaturing SDS-PAGE using silver staining as described in Chapter 3, Section 3.2.9 and Periodic acid Schiff (PAS) staining as described in Chapter 3, Section 3.2.10.

3.2.7 Purification of dextransucrase by gel filtration

3.0 ml of 33% (v/v) PEG-400 fractionated enzyme with specific activity of 20.0 (U/mg) and protein concentration of 0.44 (mg/ml) was applied to a glass column (50 cm length x 1.5 cm breadth) containing Sephacryl S-300HR with a bed volume of 40 ml. The

column was previously equilibrated with 20 mM sodium acetate buffer (pH 5.4). The partially purified enzyme was passed through the column connected to FPLC (Akta Prime, GE Healthcare). The enzyme was eluted using 20 mM sodium acetate buffer pH 5.4 at a flow rate of 0.5 ml/min. 3.0 ml fractions were collected and the protein was detected at by taking absorbance 280 nm. The enzyme activity and protein concentration of eluted fractions were determined as described earlier. The fractions containing the highest specific activity were analysed by non-denaturing Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining and Periodic acid Schiff (PAS) staining methods.

3.2.8 SDS-PAGE analysis of purified enzyme

3.2.8.1 Preparation of stock solutions

Table 3.2.1 Stock solutions preparation

Sl no	Solution	Preparation
1	**Acrylamide/Bis acrylamide solution (29.2%, w/v Acrylamide and 0.8% w/v Bisacrylamide)**	0.8 g of bis-acrylamide was weighed and transferred into an amber coloured bottle and dissolved in 50 ml of ultra-pure deionized 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 to it and stirred on a magnetic stirrer till the solution was clear. The final volume was adjusted to 100 ml with ultra-pure water as mentioned above by keeping the measuring cylinder (100 ml) wrapped with aluminium foil as acrylamide is light sensitive. The acrylamide solution was then filtered (Whatman No. 1) under dark condition and stored at 4°C.
2	**Tris-HCl (1.5 M, pH 8.8)**	54.45 g Tris base (121.14 g/mol) was first dissolved in 150.0 ml of distilled water. The pH of solution was adjusted to 8.8. Finally, the volume of the solution was made up to 300 ml and stored at 4°C.
3	**Tris-HCl (0.5 M, pH 6.8)**	6.0 g Tris base (121.14 g/mol) was first dissolved in 60.0 ml of distilled water. The pH of solution was adjusted to 6.8. Finally the volume of the solution was made up to 100 ml and stored at 4°C.
4	**Sodium Dodecyl Sulphate (SDS) (10%, w/v)**	10.0 g SDS was first dissolved in 60.0 ml of distilled water with gentle stirring. Finally the volume of the solution was made up to 100 ml.

5	**Ammonium persulphate (10%, w/v)**	100.0 mg ammonium persulphate (APS) was dissolved in 1.0 ml of distilled water.
6	**Sample buffer**	The protein sample buffer was prepared by mixing the components mentioned below in Table 3.2.2. A 5x stock solution of sample buffer was prepared and was mixed with 4 volumes of protein sample to make it to 1x before loading on to the gel.
7	**5x Running buffer**	Running buffer 5x by taking 45.0 g Tris base, 216.0 g glycine and 15.0 g SDS and dissolving in 200.0 ml of distilled water. Finally, the volume of the solution was made up to 300 ml.

Table 3.2.2 Composition of 5x sample loading buffer (Laemmli 1970).

Components	**Final concentration (5 x buffer)**
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)

Table 3.2.3 Composition of 5x Tris-Glycine running buffer.

Components	**Final concentration (5x buffer)**
Tris base	0.125 M
Glycine	1.25 M
SDS	0.5 % (w/v)

3.2.8.2 *Preparation of SDS-PAGE gels*

SDS-polyacrylamide gel electrophoresis was performed using a vertical slab mini gel unit (BioRad) using 1.5 mm thick gels, following the method of Laemmli (1970). 7.0% (w/v) acrylamide for resolving gel and 4% (w/v) for stacking gel were prepared as mentioned in Table 3.2.3 and Table 3.2.4, respectively. For running the enzyme samples under non denaturing conditions the sample buffer (5x) used did not contain β-mercaptoethanol (as described in Table 3.2.1) and the enzyme sample mixed with sample buffer was not subjected to heat denaturation. The samples from all the purified fractions with different molecular weights were loaded on 7.0% acrylamide gel under SDS non-denaturing conditions (without β-mercaptoethanol and heat treatment). Each

protein samples to be analyzed were loaded in duplicate on the 7.0% polyacrylamide gel. Electrophoresis was carried out with a current of 2 mA per lane using 1x running buffer (as described in Section 3.2.8.1). Following the run, the gel was cut in two parts. One part of the gel was subjected to silver staining as described in Section 3.2.10 and the other part of the gel was analyzed for in-situ activity detection by Periodic acid Schiff (PAS) staining protocol as described in Section 3.2.11. High range molecular mass marker proteins (from 10 kDa to 200 kDa) purchased from Fermentas, India, was used as standard for SDS-PAGE.

3.2.8.3 *Preparation of acrylamide 30% (w/v) solution*

Table 3.2.4 Composition of SDS-PAGE components for preparation of resolving gel.

Components	**7.5% (w/v) gel 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
Ammounium Persulphate (APS) (10%,w/v)	0.1
N,N,N,N Tetramethylethylenediamine TEMED	0.01

Table 3.2.5 Composition of SDS-PAGE components for preparation of stacking gel.

Components	**4% (w/v) gel 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
Ammounium Persulphate (APS) (10%,w/v)	0.05
N,N,N,N Tetramethylethylenediamine TEMED	0.01

3.2.9 Silver staining of protein

3.2.9.1 Preparation of buffers for silver staining

The solutions for silver staining were prepared as shown in table 3.2.6

Table 3.2.6 Composition of buffers for silver staining

Sl no	Solution	Composition
1	**Fixing solution**	40 (%, v/v) ethanol, 10 (%, v/v) acetic acid in water
2	**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.
3	**Silver nitrate solution**	0.1 (%, w/v) $AgNO_3$, 0.076 (%, w/v) formaldehyde
4	**Developer**	2.5 (%, w/v) Na_2CO_3, 0.05 (%, w/v) formaldehyde
5	**Stop solution**	1.46 (%, w/v) EDTA

3.2.9.2 Silver staining procedure

1. The gel was incubated with 50 ml of fixing solution for 30 min to fix the protein bands.
2. After fixing the protein bands, the gel was put in 50 ml of sensitizing solution for 30 min.
3. The gel was washed three times for 15 min with distilled water.
4. After washing, the gel was incubated in 50 ml of silver nitrate solution for 30 min in dark.
5. The protein bands were developed by putting the gel in developing solution until the protein bands appeared.
6. Finally, the reaction was stopped by adding stop solution.

3.2.10 Identification of dextransucrase by Periodic Acid Schiff staining protocol

The dextran synthesizing activity of dextransucrase was detected by conducting non-denaturing SDS-PAGE on 7.5% gels. The gel was cut in to equal half and both parts were subjected to activity staining protocol following the method of Vasileva *et al.*, (2009). The SDS removal was carried out by incubating the gels in 20 mM sodium acetate buffer (pH 5·4) containing 0.3 mM $CaCl_2$ and 0.1% Triton X-100 at 4°C for 30 min. One part of the gel was incubated in 20 mM sodium acetate buffer (pH 5·4) containing 0.3 mM $CaCl_2$ and 5% sucrose at 30°C for 48h and the other part of the gel was incubated with 5% raffinose. Following incubation the gel was washed twice with 75% (v/v) ethanol for 20 min and incubated in solution with 0.7% (w/v) periodic acid in 5% (v/v) acetic acid for 20 min at room temperature. The gel was then washed thrice with 0.2% (w/v) sodium meta bisulfate in 5% (v/v) acetic acid solution and finally stained with Schiff's reagent (0.5%, w/v basic fuschin, 1%, w/v sodium bisulfite and 0.1N HCl) until the discrete magenta bands appeared within the gel. The other gel was incubated in 5 (%, w/v) raffinose, which is a specific substrate for fructosyltransferases (inulansucrase and levansucrase), in order to exclude the presence of fructosyltransferases.

3.2.11 Optimization of reaction conditions and biochemical characterization of dextransucrase

3.2.11.1 *Effect of temperature, pH and ionic strength on dextransucrase activity*

The purified dextransucrase (specific activity 20.0 U/mg; 0.36 mg protein/ml) was used to study the effect of temperature. The enzyme was added in 1.0 ml of the reaction mixture was incubated at 7 different temperatures ranging from 10 to 50°C in 20 mM sodium acetate buffer pH 5.4 and 5.0% sucrose concentration for 15 min. In order to

determine the optimum pH of the enzyme, the pH of sodium acetate buffer was varied from 4.2 to 6.4, while keeping the ionic strength constant at 20 mM, temperature, 40°C and sucrose concentration, 5.0% (w/v). In the case of effect of ionic strength on dextransucrase, the concentration of sodium acetate buffer was varied from 10-500 mM, keeping the pH 5.4 and sucrose concentration 5% and a constant temperature of 40°C and the reaction time period 15 min. The enzyme activity was measured by quantifying the amount of reducing sugar released as described in Chapter 2, Section 2.2.4.1.

3.2.11.2 *Effect of sucrose concentration on dextransucrase activity*

To study the effect of sucrose concentration on the enzyme activity of the purified dextransucrase (specific activity 28.0 U/mg; 0.45 mg protein/ml), the enzyme assay was conducted at different sucrose concentrations ranging from 0.05-400 mM. The reaction was carried out in 1 ml mixture in 20 mM sodium acetate buffer pH 5.4 by adding 20 µl of enzyme and varying concentration of sucrose. The mixture was incubated at 40°C in water bath for 15 min and the activity was determined by estimating the released reducing sugar, as described earlier in Chapter 2, Section 2.2.5.

3.2.11.3 *Effect of metal ions on dextransucrase activity*

The effects of different divalent cations using $MgCl_2$, $CaCl_2$, $MnSO_4$, $CoCl_2$ and $NiSO_4$ salt solutions (0-12 mM) on dextransucrase from *Weissella cibaria* RBA12 was evaluated by incubating 20 µl dextransucrase preparation (28.0 U/mg; 0.45 mg protein/ml) in 1 ml of 20 mM sodium acetate buffer pH 5.4 containing 5% (w/v) sucrose solution and varying concentrations of salts at 40°C. The enzyme activity was measured

by quantifying the amount of reducing sugar released as described in Chapter 2, Section 2.2.5.

3.2.11.4 *Effect of denaturing agents on dextransucrase activity*

The effect of denaturing agents on the activity of dextransucrase from *Weissella cibaria* RBA12 was studied by using EDTA (0-12 mM) and urea (0-5 M). Dextransucrase from *Weissella cibaria* RBA12 was incubating 20 µl dextransucrase preparation (28.0 U/mg; 0.45 mg protein/ml) in 1 ml of 20 mM sodium acetate buffer pH 5.4 containing 5% (w/v) sucrose solution and varying concentrations of denaturing agents at 40°C. The enzyme activity was measured by quantifying the amount of reducing sugar released as described in Chapter 2, Section 2.2.5.

3.2.11.5 *Thermal and pH stability of dextransucrase*

To study the effect of temperature on enzyme stability, the enzyme (28.0 U/mg; 0.45 mg protein/ml) was incubated between 10-60°C for 1h. An aliquot of enzyme incubated at different temperatures was added to 1.0 ml of reaction mixture and assayed. In case of pH stability dextransucrase from *Weissella cibaria* RBA12 was incubated in the presence of different pH ranging from pH 3.5 to 8.0 for 1 h at 40°C. 20 µl aliquots of enzyme were assayed for residual enzyme activity in 1 ml reaction mixture containing 5% sucrose in 20 mM sodium acetate buffer, pH 5.4 by incubating at 40°C. The enzyme activity was measured by quantifying the amount of reducing sugar released as described in Chapter 2, Section 2.2.5.

3.2.11.6 *Effect of additives on stability of dextransucrase*

The effects of various additives on stability dextransucrase enzyme (28.0 U/mg; 0.45 mg protein/ml) from *Weissella cibaria* RBA12 at 30°C were tested. Aqueous solutions of glycerol, glutaraldehyde, acetonitrile, dextran T-40 (40 kDa), dextran T-500 (500 kDa), PEG-8000 and Tween 80 were added to dextransucrase in sodium acetate buffer, pH 5.4 to obtain the final concentrations of 0.5% (v/v), glycerol, 0.1% (v/v) glutaraldehyde, 2 µg/ml (w/v) dextran T-40 (40 kDa), 2 µg/ml (w/v) dextran (500 kDa), 10 µg/ml (w/v) PEG-8000 and 0.1% (v/v) Tween 80, respectively, in a final volume of 0.6 ml. The aliquots (20 µl) of enzyme were taken after 24h for activity assay and the amount of reducing sugar released was measured as described in Chapter 2, Section 2.2.5.

3.2.11.6.1 Determination of half life ($t_{1/2}$) of enzyme

The residual activity and half life ($t_{½}$) of dextransucrase were measured with respect to time without any additives by assuming that the decay followed first order rate kinetics. All the calculations were done according to Naidu et al. (2003). It was assumed that the active enzyme state *E* directly converts to inactive state *E*d without providing any significant amount of intermediates according to single step two-stage theory (Sadana, 1995). The two-state mechanism is as follows:

$$E \rightarrow E\mathrm{d} \quad ...(1)$$

The first-order deactivation can be represented as:

$$\mathrm{d}E/\mathrm{d}t = -k_{\mathrm{d}}[\mathrm{E}] \quad ...(2)$$

Integration of Eq. (2) leads to:

$$\alpha = \exp(-k_{\mathrm{d}}\, t) \quad ...(3)$$

where, $\alpha = Ed/E$.

From the plot of ln(α) versus t, the slope gives the value of deactivation rate constant k_d. The half-life of an enzyme is defined as the time required by the enzyme to lose half of its initial activity. After solving equation 3, for α =1/2 and t= $t_{½}$ we get:

$t_{½} = \ln 2 / k_d$...(4)

where,

$t_{½}$ = half-life of enzyme (time).

k_d = deactivation rate constant ($time^{-1}$)

3.3 Results and Discussion

3.3.1 Purification of dextransucrase from *Weissella cibaria* RBA12

The cell free extract having dextransucrase specific activity of 1.0 U/mg was subjected to purification by polyethylene glycol (PEG) fractionation. The enzyme purified by PEG-400 showed maximum specific activity of 16.8 U/mg at 25% (v/v) (Table 3.3.1). Further increase in PEG-400 concentration up to 40% (v/v) led to decrease in specific activity to 12.5 U/mg as displayed in Fig. 3.3.1. In case of PEG-1500 fractionation the enzyme showed a maximum specific activity of 7.3 U/mg at 15% (w/v) concentration (Table 3.3.1). The specific activity decreased to 6.9 U/mg and remained constant at 20% (w/v) of PEG-1500 concentrations (Fig. 3.3.1). 25% (v/v) of PEG-400 was found to be best condition that gave maximum enzyme activity with 17-fold purification and yield of 20.5%.

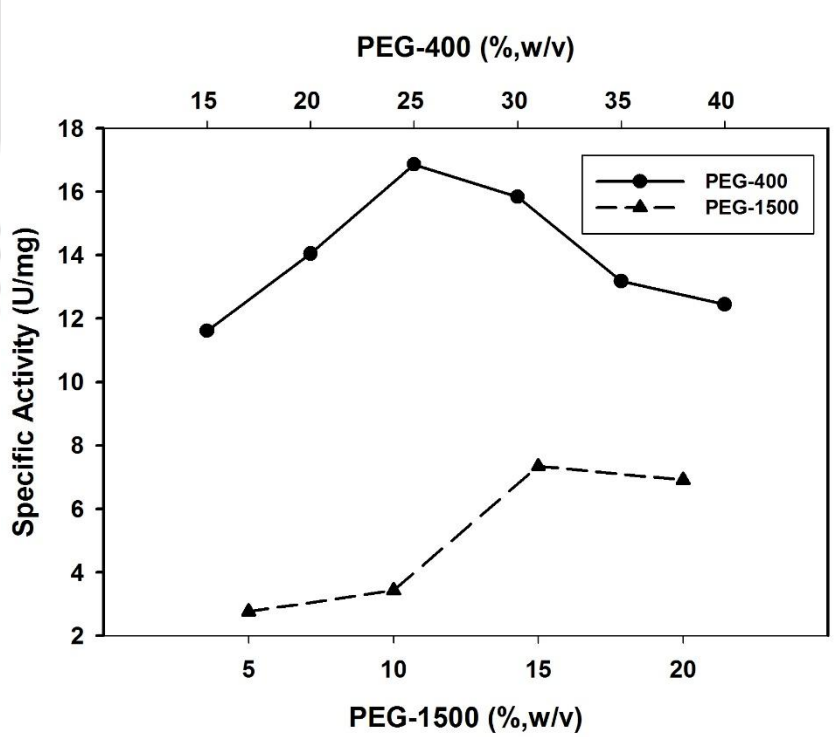

Fig. 3.3.1 Purification profile of dextransucrase by PEG fractionation. PEG 400 (●), PEG-1500 (▲).

Table 3.3.1. Purification of dextransucrase from *Weissella cibaria* RBA12 by PEG fractionation

Samples	Vol (ml)	Enzyme Activity (U/ml)	Total Units (U)	Overall activity yield (%)	Protein conc (mg/ml)	Specific Activity (U/mg)	Fold purification
Crude	40	7.2	288	-	7	1	-
PEG-400 (% v/v)							
15	1	1.33	1.33	0.4	0.12	11.61	12
20	5	7.80	39	13.54	0.56	14.05	14
25	**7**	**8.43**	**59.01**	**20.5**	**0.5**	**16.86**	**17**
30	8	9.03	72.24	25	0.57	15.84	16
35	7.5	4.22	31.65	10.9	0.32	13.18	13
40	6	4.98	29.88	10.3	0.4	12.45	12
PEG-1500 (% v/v)							
5	2.5	0.69	1.73	0.6	0.25	2.76	3
10	3	1.2	3.6	1.25	0.35	3.43	3
15	**2.8**	**7.34**	**20.6**	**7.14**	**1.0**	**7.34**	**7**
20	2.6	8.5	22.1	7.67	1.23	6.91	7

The specific activity of 17 U/mg and 20% yield achieved for dextransucrase from *Weissella cibraria* RBA12 in a single step by PEG 400 was much higher or comparable to those reported for other strains. The purification of dextransucrase by PEG 400 resulted from *Weissella confusa* cab3 with 10.4 U/mg with 26% yield (Shukla and Goyal 2011), from *Leuconostoc mesenteroides* NRRL B-640 with 5.5 U/mg with 3.1% yield (Purama and Goyal 2009) and from *Pediococcus pentosaceus* with 18 U/mg with 8.5% yield (Patel *et al*., 2011).

3.3.2 Purification of dextransucrase by size exclusion chromatography

The purified dextransucrase by 25% PEG-400 with maximum specific activity of 17 U/mg and 0.4 mg/ml was used further for purification by size exclusion

chromatography. Fig. 3.3.2 shows the elution profile of enzyme activity (U/ml) and protein absorbance at A_{280}. 6th to 14th fractions showed higher enzyme activity and it was maximum in the 8th fraction. The specific activity of 8th fraction was 24.5 U/mg, with 25-fold purification (Table 3.3.3).

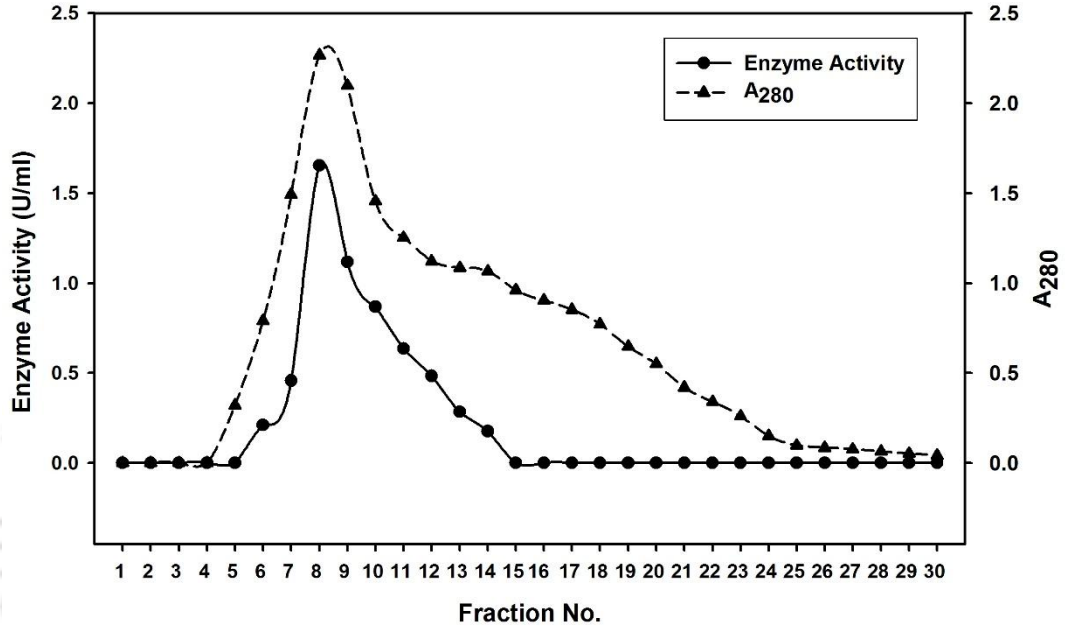

Fig. 3.3.2 Purification of dextransucrase by size exclusion chromatography using Sephacryl S300HR. Elution profile of dextransucrase showing (●) enzyme activity (U/ml) and (▲) A_{280}.

Table 3.3.2 Purification analysis of dextransucrase by PEG-400 and size exclusion chromatography.

	Vol (ml)	**Enzyme activity (U/ml)**	**Total Units (U)**	**Overall activity Yield (%)**	**Protein Conc (mg/ml)**	**Specific activity (U/mg)**	**Fold Purifi-cation**
Crude	40	7.2	288	-	7	1	-
PEG 400, 25% (v/v)	7	8.43	59.01	20.5	0.5	16.86	17
Sephacryl S-300HR	3.0*	1.65	4.95	0.02	0.067	24.62	25

**8th fraction of dextransucrase from Sepharcryl S300HR column*

3.3.3 SDS-PAGE analysis and PAS staining of dextransucrase from *Weissella cibaria* RBA12

The molecular mass of dextransucrase from cell free supernatant was determined by 7.5% (w/v) gel run on SDS polyacrylamide gel electrophoresis carried out under non-denaturing condition and *in-situ* activity detection was performed by PAS staining. The PAS staining of the crude dextransucrase from cell free supernatant showed one activity band at approximately, 180 kDa (Fig. 3.3.3, lane 2) when incubated with 10% (w/v) sucrose solution, which corresponded to the band obtained in 7.5% (w/v) SDS gel subjected to silver staining (Fig. 3.3.3, lane 1). No band was observed in the PAS staining of the enzyme when incubated with 10 % (w/v) raffinose (Fig. 3.3.3, lane 3). This excluded the possibility of the enzyme being fructansucrase. It has been reported that the molecular weight of extracellular dextransucrase are in the range of 120–200 kDa (Leemhuis *et al*. 2013) and can exist in multiple molecular forms (Purama and Goyal 2008; Patel *et al*. 2011). The presence of a single band after PAS staining showed that glucansucrase from *Weissella cibaria* RBA12 exists in single molecular form. Similar single molecular forms were reported for dextransucrase from *Weissella cibaria* JAG8 (Rao and Goyal 2013) and *Weissella confusa* Cab3 (Shukla *et al*. 2014).

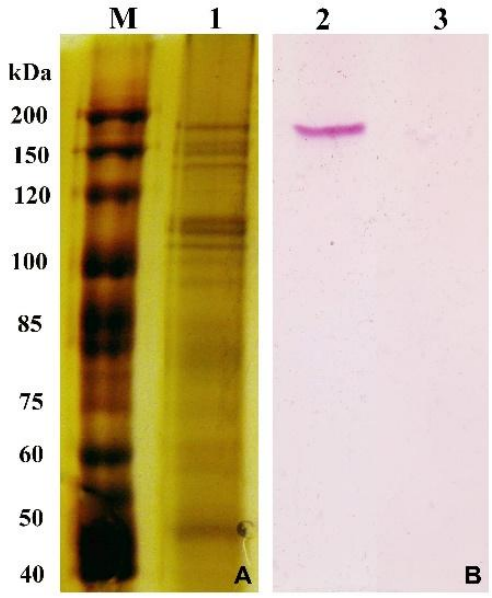

Fig. 3.3.3 Non-denaturing SDS-PAGE analysis of glucansucrase using 7.5 % acrylamide gel **A)** Lane M, protein molecular mass marker (40–200 kDa), lane 2, cell free supernatant; **B)** identification of glucansucrase by PAS staining lane 3, using sucrose; lane 4, using raffinose.

3.3.4 Identification and purity analysis of dextransucrase from 25% (v/v) PEG 400 and column purified fractions by Silver and PAS staining

The dextransucrase purified by 25% (v/v) PEG-400 fractionation (16.86 U/mg; 0.5 mg/ml) was subjected to non-denaturing SDS-PAGE (Lane 1) showed a multiple distinct band (Fig. 3.3.4) in silver staining and single magenta band at 180 kDa in PAS staining. In case of size exclusion chromatography, dextransucrase showing significant activity from 8^{th} to 10^{th} fractions were loaded on non-denaturing SDS-PAGE and detected by silver staining. Single distinct homogenous bands of 180 kDa molecular weight were observed in each of the three fractions (Fig. 3.3.4, lanes 2 to 4). The PAS staining of 8^{th} fraction showed single band at 180 kDa, the other fractions did not produce any band due to lack of sufficient enzyme activity No multiple molecular forms (or) isoforms of enzyme were detected in the gels. No other protein contaminants were present in the fractions which revealed the purity of enzyme and thus can be exploited for production of dextran and prebiotic oligosaccharides.

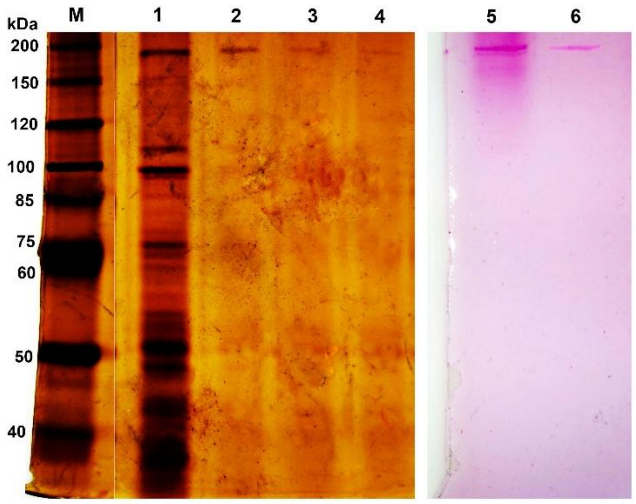

Fig. 3.3.4 Non-denaturing SDS-PAGE (7.5%) analysis after purification from Sephacryl S-300 for identification of dextransucrase using Silver staining and PAS staining. Lane M (molecular weight marker 40-200 kDa), Lane 1 PEG 25% (w/v) purified enzyme, Lane 2 (fraction 8), Lane 3 (fraction 9), Lane 4 (fraction 10), Lane 5 PAS staining of PEG 25% (w/v) purified enzyme, Lane 6 PAS staining (fraction 8) in 5.0% (w/v) sucrose solution.

3.3.5 Optimization of reaction conditions for dextransucrase activity

3.3.5.1 *Effect of temperature pH and ionic strength on dextransucrase activity*

The purified dextransucrase (specific activity 20 U/mg) was used to study the effect of temperature, pH and ionic strength on dextransucrase activity. The temperature of reaction mixture was varied between 10-50°C. The results showed 40°C was optimum temperature for dextransucrase activity as shown in Fig 3.3.5 A. Further increase in temperature beyond 40°C drastically decreased the enzyme activity, indicating that dextransucrase is thermo sensitive. The optimum temperature of dextransucrase from *Weissella cibaria* JAG8 (Rao and Goyal 2013) and *Weissella confusa* Cab3 (Shukla *et al*. 2014) was 35°C.

The pH profile of dextransucrase from *Weissella cibaria* RBA12 showed maximum enzyme activity at pH 5.4 (Fig 3.3.5 B). The activity of enzyme decreased gradually with further increase in pH of sodium acetate buffer. The above experiment clearly indicated that dextransucrase activity was significantly affected by pH of the reaction mixture.

In order to study the effect of ionic strength of sodium acetate buffer on dextransucrase activity the molar concentrations of buffer was varied between 10-500 mM. It was observed that ionic strength of buffer has no significant effect on enzyme activity up to 20 mM concentration (Fig 3.3.5 C). Beyond 50 mM concentration the decrease in enzyme activity was around 25% as compared to that at 20 mM. The enzyme was found to be stable between 10-50 mM sodium acetate concentrations. Similar results were reported in case of *Weissella cibaria* JAG8 (Rao and Goyal 2013).

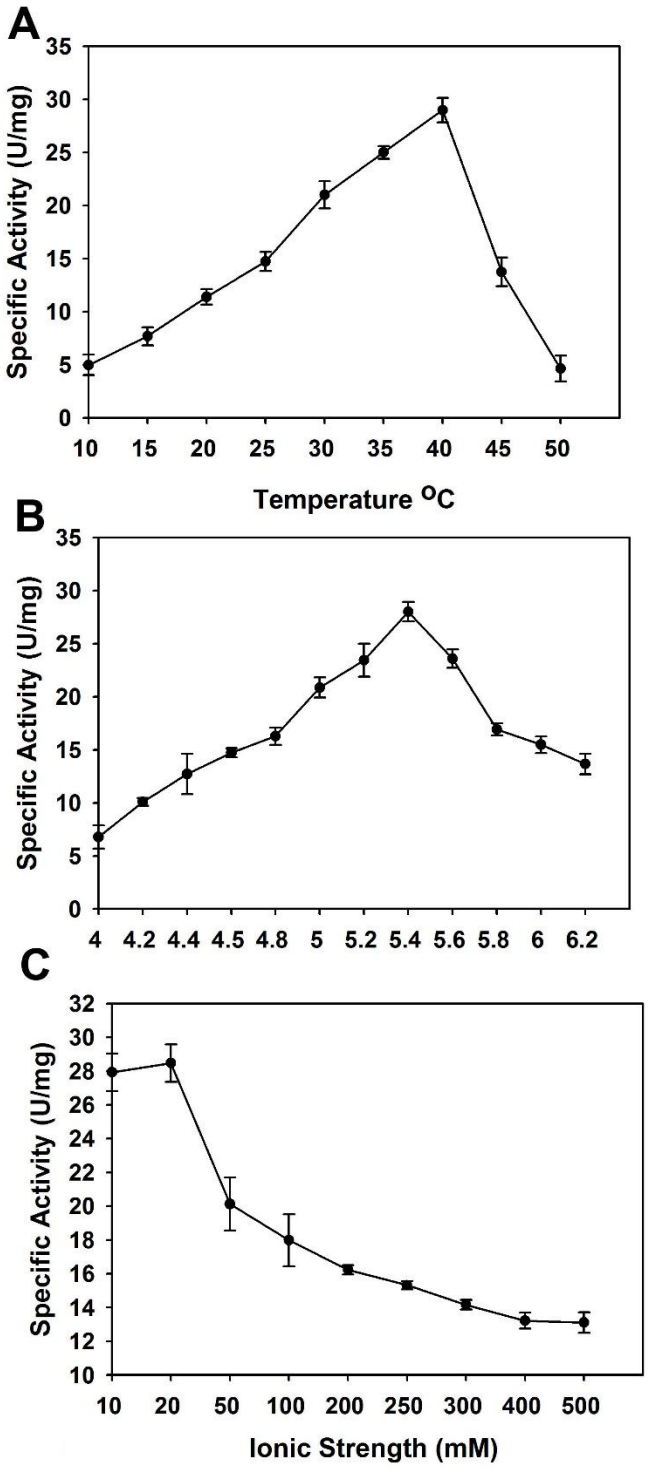

Fig 3.3.5 A) Effect of temperature on dextransucrase activity in the range of 10ºC to 50ºC **B)** Effect of pH on dextransucrase activity in the pH range of 3 to 8. **C)** Effect of ionic strength on dextransucrase activity in the range of 10 mM to 500 mM.

3.3.5.2 *Effect of sucrose concentration on dextransucrase activity*

The effect of sucrose concentration on the enzyme activity was studied with varying sucrose concentration from 0.05 mM to 400 mM. The PEG-400 (25%, v/v) purified enzyme with specific activity of 28.0 U/mg and protein concentration of 0.45 mg/ml was used for this study. The results showed that it follows the classical Michaelis-Menten kinetics and enzyme saturation was reached at 146 mM (5%, w/v) sucrose concentration (Fig. 3.3.6). The purified dextransucrase gave V_m of 29.3 ±0.75 µmole/mg/min and K_m of 19.2 mM ±2.6 mM. It was reported earlier that *L. mesenteroides* NRRL B-1146 (Majumder *et al* 2008) and *L. mesenteroides* NRRL B-512F (Goyal *et al* 1995) exhibited K_m of 18.7 and 14.9 mM, respectively. Similar result of 5% sucrose as an optimum concentration has been reported for glucansucrase from *Leuconostoc mesenteroides* NRRL B-640 (Purama and Goyal 2008).

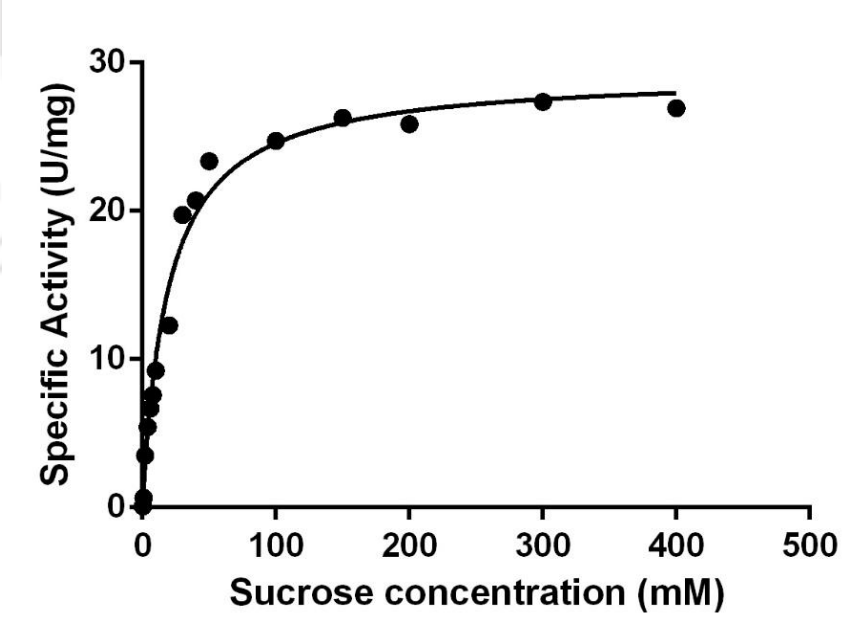

Fig. 3.3.6 Effect of sucrose concentration on dextransucrase from *Weissella cibaria* RBA12.

3.3.5.3 *Effect of metal ions on dextransucrase activity*

The effects of different divalent cations using $MgCl_2$, $CaCl_2$, $MnSO_4$, $CoCl_2$ and $NiSO_4$ salt solutions (0-12 mM) on dextransucrase from *Weissella cibaria* RBA12 were evaluated. The Mg^{2+} ions were the most effective for increasing the enzyme activity of dextransucrase. The enzyme activity increased by ~40% at 0.6 mM $MgCl_2$ (Fig. 3.3.7 A) and it increased by ~25% at 3 mM $CaCl_2$ (Fig. 3.3.7 B). This shows that the Mg^{2+} and Ca^{2+} ions might be stabilizing the enzyme structure thereby enhancing the catalytic activity. The addition of $MnSO_4$ (0.5 mM), $CoCl_2$ (0.5 mM) and $NiSO_4$ (1 mM) to the enzyme reaction resulted in the decrease in the enzyme activity by 16%, 20% and 29%, respectively (Fig. 3.3.7 C, D & E). Ni^{2+} ions adversely affected the enzyme activity as 8 mM $NiSO_4$ caused 95% inactivation of its initial enzyme activity (Fig. 3.3.7 E). It was reported that Ca^{2+} ions enhance the dextraunsucrase activity of *L. mesenteroides* NRRL B-1146 by 3 fold at 6 mM $CaCl_2$ (Majumder *et al*., 2008) and similar results were reported for *L. mesenteroides* NRRL B-512F (Kobayashi and Matsuda 1980).

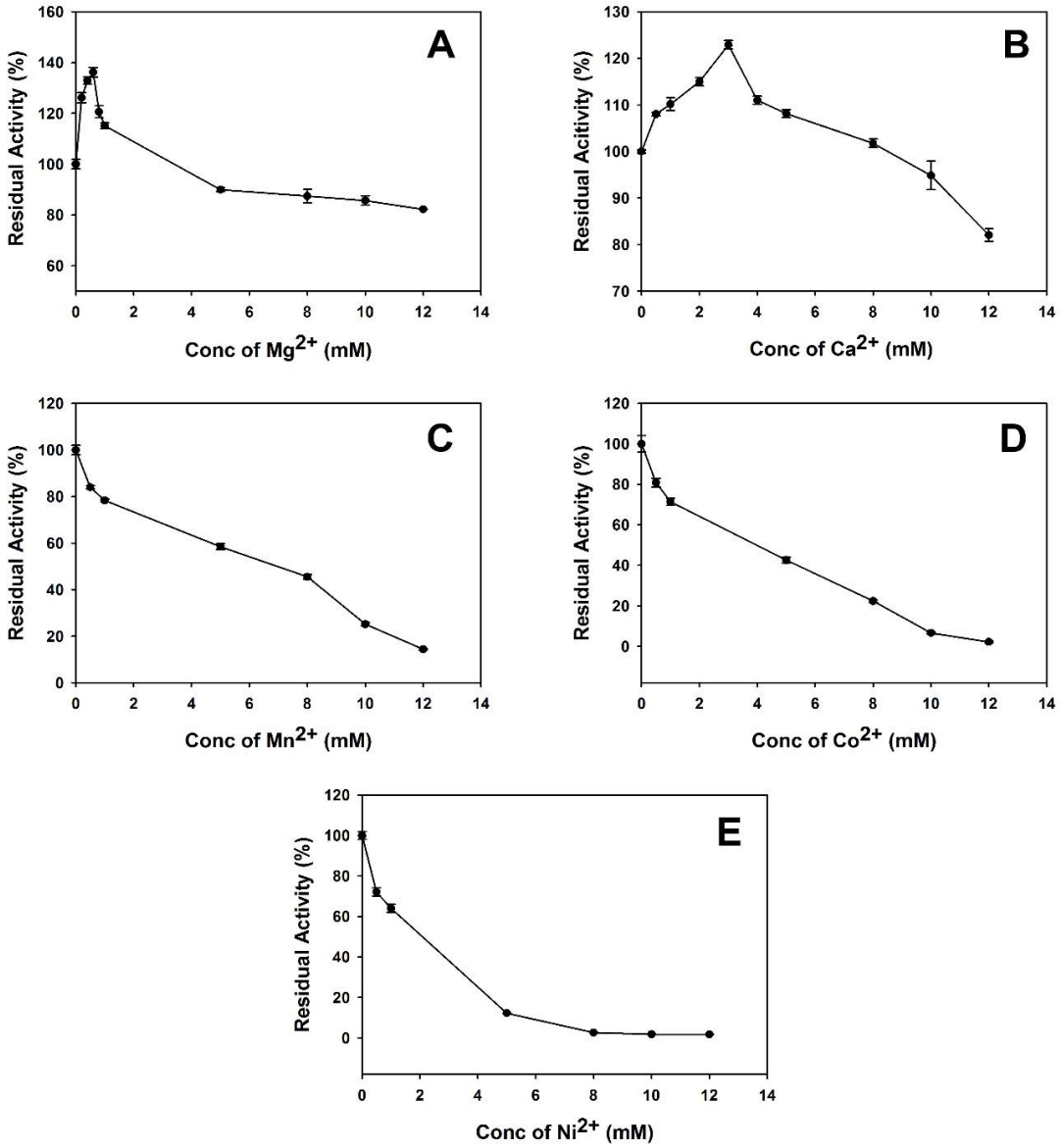

Fig. 3.3.7 Effect of **(A)** $MgCl_2$, **(B)** $CaCl_2$, **(C)** $MnSO_4$, **(D)** $CoCl_2$, **(E)** $NiSO_4$ on the activity of purified dextransucrase of *Weissella cibaria* RBA12.

3.3.5.4 *Effect of denaturing agents on dextransucrase activity*

The dextransucrase activity decreased in the presence of both EDTA or urea. EDTA (0.5 mM) decreased the dextransucrase activity by 31% and 12 mM EDTA decreased the activity by 50% (Fig 3.3.8 A). The decrease of activity by EDTA was due to the chelation of native divalent metal ions present in the enzyme by EDTA. Urea

decreased the dextransucrase activity by 47% and 3.2% at 0.5 M and 5 M, respectively (Fig 3.3.8 B). Similar results on the effect of urea were reported for dextransucrase from *Weissella cibaria* JAG8 (Rao and Goyal 2013). Similar results were reported in case of dextransucrase from *L. mesenteroides* NRRL B-1146 (Majumder *et al*., 2008) and *Pediococcus pentosaceus* (Patel *et al*., 2011) with respect to effect of urea.

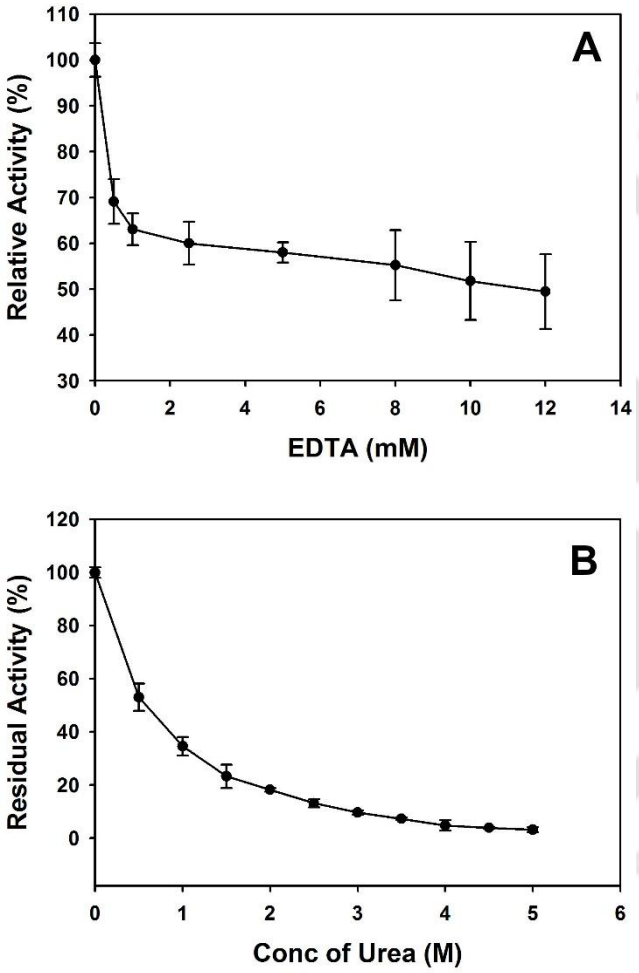

Fig. 3.3.8 Effect of denaturing agents EDTA **(A)** and Urea **(B)** on dextransucrase activity.

Table 3.3.3 Effects of metal salts and denaturing agents on dextransucrase activity

Reagents	Residual Activity (%)	
	Maximum	Minimum
$MgCl_2$	138 (0.6 mM)	82 (12 mM)
$CaCl_2$	125 (1mM)	81 (12mM)
$MnSO_4$	84 (0.5mM)	14 (12mM)
$CoCl_2$	80 (0.5mM)	3.7 (12mM)
$NiSO_4$	71 (1mM)	2.9 (12 mM)
EDTA	69 (0.5mM)	50 (12 mM)
Urea	53 (0.5M)	3.2 (5 M)

3.3.5.5 *Thermal and pH stability of dextransucrase*

Dextransucrase from *Weissella cibaria* RBA12 exhibited a mesophilic nature and showed stability up to 40°C retaining residual activity 92%. At a temperature above 40°C the enzyme rapidly lost the activity upon incubation for 1 h and was completely inactivated at 60°C (Fig. 3.3.9 A). Sodium acetate buffer was used in the range of pH 3.5 to 5.5 and sodium phosphate buffer was used in the range of pH 6.0 to 8.0 (Fig. 3.3.9 B). The enzyme was stable under acidic pH (5–6) range with residual activities of 68%, 88%, 93% and 83% at pH 4.5, 5.0, 5.5 and 6.0, respectively. The thermal and pH stability results were similar to the earlier results of dextransucrase from *L. mesenteroides* NRRL B-512F (Kobayashi and Matsuda 1980) and *L. mesenteroides* NRRL B-640 (Purama and Goyal 2010).

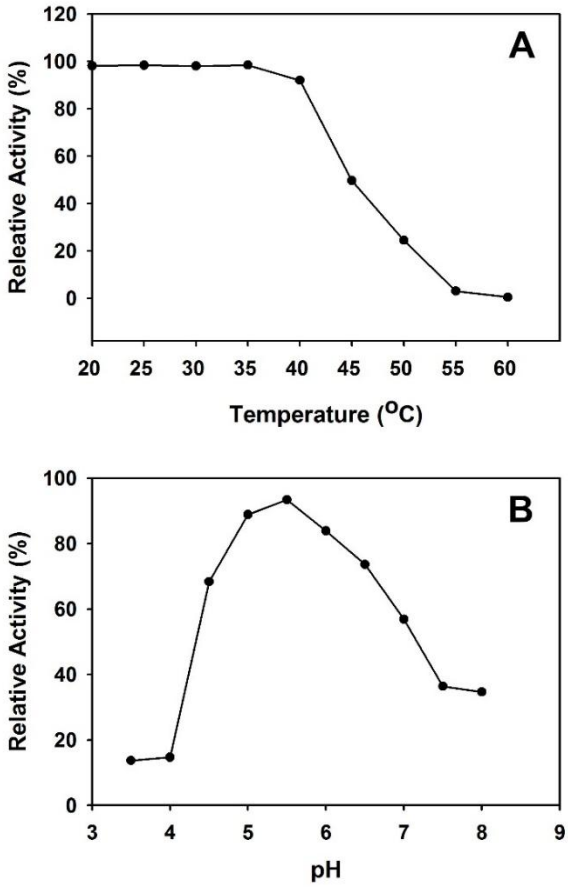

Fig. 3.3.9 Effect of temperature (**A)** and pH **(B)** on stability of dextransucrase from *Weissella cibaria* RBA12.

3.3.5.6 *Effect of additives on stability of dextransucrase*

The residual activity of dextransucrase at 30°C after 24 h with glycerol, acetonitrile, dextran T-40 (40 kDa), Tween 80, PEG 8000 and dextran T-500 (500 kDa) were 83%, 68%, 70%, 58%, 54% and 51%, respectively (Table 3.3.4). Glutaraldehyde did not stabilize the enzyme. The residual activity of dextransucrase from *Weissella cibaria* RBA12 with glutaraldehyde at 30°C after 24h was only 9% (Table 3.3.4). The residual activity of dextransucrase with glutaraldehyde at 30°C after 24h was lower than the residual activity of dextransucrase without any additive (52%). Thus glutaraldehyde negatively affected dextransucrase.

Among all the additives used glycerol and dextran T-40 (40 kDa) were the most efficient stabilizers giving stabilization with $t_{1/2}$ of 89.2 h and 46.6 h at 30°C, respectively (Table 3.3.4). A slight enhancement in the $t_{1/2}$ of 43 h and 30 h was observed with acetonitrile and Tween 80 as compared to the control (25.4 h). There was not much difference in the $t_{1/2}$ of dextransucrase with Dextran-500 (12 h) and PEG-8000. Glutaraldehyde addition negatively affected enzyme stability (6.9 h) when compared to dextransucrase with no additive ($t_{1/2}$ = 25.4 h) at 30°C.

Table 3.3.4 Effects of additives on purified dextransucrase from *Weissella cibaria* RBA12.

Stabilizers	**Residual Activity (%) at 24h**	**Half Life ($t_{1/2}$) (h)**	**Decay Constant (λ)**
Control	52	25.4	0.03
Glycerol (0.5%, v/v)	83	89.2	0.01
Glutaraldehyde (0.1%, v/v)	9	6.9	0.1
Acetonitrile (1%, v/v)	68	43	0.02
Dextran T-40 (2 µg/ml, w/v)	70	46.6	0.01
Dextran T-500 (2 µg/ml, w/v)	51	24	0.03
PEG-8000 (10 µg/ml, w/v)	54	26	0.03
Tween 80 (10 µg/ml, w/v)	58	30	0.02

3.4 Conclusions

The purification of crude dextransucrase from *Weissella cibaria* RBA12 with a specific activity of 1 U/mg by 25% (v/v) PEG-400 and 15% (w/v) PEG-1500 fractionation resulted in specific activity of 16.8 U/mg and 7.3 U/mg with 17 and 7 fold purification, respectively. Further purification of 25% (v/v) PEG-400 enzyme fraction by size exclusion chromatography using Sephacryl S-300HR resulted in specific activity of 29.3.0 U/mg with 25 fold purification. Silver staining and PAS staining analysis of dextransucrase under non denaturing SDS-PAGE showed a single distinct band with molecular size of 180 kDa. The enzyme was confirmed as dextransucrase by PAS staining. A bright magenta colour band was observed in the gel incubated with 5% (w/v) sucrose, and no band was detected in the gel incubated with raffinose which confirmed that the enzyme is dextransucrase not fructansucrase. The optimum conditions for enzyme were 40°C and pH 5.4. The dextransucrase showed maximum activity at 5% sucrose concentration with K_m of 19.2 mM and V_{max} of 29.3 μmole/mg/min. Mg^{2+} and Ca^{2+} ions enhanced the activity of enzyme by ~40% and 25%, respectively. The enzyme lost 97% and 50% of its activity at 5 M urea and 12 mM EDTA, respectively. Thermal and pH stability analysis showed that enzyme was stable up to 40°C and in the pH range of 5.2 to 5.4. Among all the additives, glycerol provided maximum stabilization to dextransucrase with $t_{1/2}$ of 89.2 h as against 52h without any additives at 30°C. The purified enzyme can be exploited for enzymatic synthesis of dextran and of isomalto-oligosaccharides with potential applications in food and baking industry as also elaborated in the subsequent chapters.

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Chapter 4

Structural and physicochemical characterization of dextran produced from *Weissella cibaria* RBA12

4.1 Introduction

Dextrans are homopolysaccharides of D-glucose units that are α-(1→6) linked in the main chains with varying degrees of α-(1→2), α-(1→3), or α-(1→4) branched linkages (Capek *et al*., 2011). Lactic acid bacteria (LAB) of the genera *Leuconostoc*, *Streptococcus*, *Weissella*, *Pediococcus* and *Lactobacillus* synthesize dextrans using sucrose as a substrate (Falconer *et al*., 2011). The structure of dextrans such as chain length and degree of branching is dependent on (i) the strain producing it, (ii) fermentation conditions to some extent and also (iii) available nutrients (Heinze *et al*., 2006). The biological activities of dextran are determined by their glycosidic linkages, monosaccharide composition and degrees of polymerization (Delattre & Vijayalakshmi 2009).

Dextrans have prolific usage in food, clinical, fine chemicals, cosmetics and agricultural industries (Kothari *et al*., 2015 a). Dextran has been shown suitable for cell-resistant coatings on biomaterial surfaces (Massia *et al*., 2000). Dextran has been used in microsurgery to reduce the risk of free tissue transfer loss and improve micro

circulation (Ridha *et al*., 2006). Dextran hydrogels have biomedical applications in contact lenses, cell encapsulation for drug-delivery, tissue engineering scaffolds, burn dressing and spinal cord regeneration (Hoffman 2002; Van Tomme and Hennink, 2007). Dextrans are used as viscosifying, texturizing, stabilizing, emulsifying or gelling agents in food formulations (Robyt, 1985; Majumder and Goyal, 2009).

The dextran-producing *Weissella* spp. have received significant attention in the recent years due to their relatively higher dextran production ability, as compared to other lactic acid bacteria, especially in sourdough bread applications (Di Cagno *et al*., 2006; Schwab, *et al*., 2008; Katina *et al*., 2009; Galle *et al*., 2010; Galle *et al*., 2012). The genus *Weissella* was proposed in 1993 after reclassification of some *Leuconostoc*-like bacteria (Collins, *et al*., 1993). They are rod-shaped heterofermentative LAB from the family *Leuconostocaceae*. Dextran production is a phenotypic criterion for the identification of bacteria under this genus. *Weissella* strains have been isolated from various sources, such as sourdough breads and vegetables (Bounaix *et al*., 2010; Shukla & Goyal, 2011; Ahmed *et al*., 2012). Dextrans produced by *Weissella* strains have been reported to possess highly similar structures, consisting of predominantly α-(1→6) linkages and only a few (2.4–7%), α-(1→3) branched linkages, with some of the branches elongated (Maina, *et al*., 2008; Bounaix *et al*., 2009; Maina, *et al*., 2011; Ahmed *et al*., 2012; Rao and Goyal, 2013). Dextrans produced by *Weissella* strains contain less branched linkages than the most commercially used *L. mesenteroides* NRRL B-512F dextran. In wheat sourdough, *Weissella confusa* VTT E-90392 synthesized a high yield of dextran, while *L. mesenteroides* NRRL B-512F produced almost none under the same conditions (Katina *et al*., 2009).

Celiac disease is a food-induced disorder caused by intolerance to wheat gluten and more or less similar proteins originated from barley and rye in genetically susceptible patients (Goggins and Kelleher, 1994). The dextran produced from *Weissella cibaria* species acts as a perfect hydrocolloid and serves as a replacement for non-bacterial hydrocolloids such as guar gum and hydroxypropylmethyl cellulose (HPMC) for the generation of gluten-free soft bread with good texture and shelf life, hence holds potential application in baking industry for the generation of gluten free food products for patients suffering from Celiac disease (Schwab *et al*., 2008; Galle *et al*., 2010). Gluten is an important structure building protein which contributes to appearance and crumb structure in many bakery products. The biggest challenge for food scientists and bakers is generation of high quality gluten free bread. Several impressive attempts have been made by the scientific community in developing potential therapeutic solutions for Celiac disease (Lerner 2010). But still the safe treatment is the dietary exclusion of grains containing gluten with parallel supplements of minerals and vitamins (Hopman *et al*., 2006). It was reported that the dextran and gluco-oligosaccharides produced from *W. cibaria* species are not digested by baker's yeast and are present in the produced bread, leading to significant intake of putative prebiotic gluco-oligosaccharides (Schwab *et al*., 2008). The higher percentage of branching in dextran imparts different chemical (resistance to enzyme hydrolysis) and physical properties (water solubility, viscosity and diffusion) (Vettori *et al*., 2012). Dextran from *Weissella cibaria* JAG8 was used as a coating for magnetic nanoparticles (MNPs), the *in vitro* effect of MNPs and dextran coated MNPs was performed on human colon cancer (HT-29) cell lines and the results showed that dextran coated: MNPs (2:1) displayed superior biocompatibility results over dextran coated: MNPs (1:1) and un-coated MNPs (Tingirikari *et al*., 2016). Dextran have been reported to

increase the population of *Bifidobacterium* species in an *in vitro* model of the fermentation process in the human colon exhibiting prebiotic activity (Olano-Martin *et al*., 2000). Dextran from *Weissella cibaria* JAG8 (Tingirikari *et al*., 2014) and *Lactobacillus plantarum* DM5 (Das *et al*., 2014) showed promising prebiotic potential with very low digestibility and the stimulation of probiotics. The present study describes the structural and physico-chemical properties of dextran from *Weissella cibaria* RBA12 which includes its monosaccharide analysis, linkage determination, molecular weight analysis, surface morphology and thermal stability.

4.2 Materials and Methods

4.2.1 Chemicals and reagents

Standard dextrans of molecular weights of 10, 20, 40, 70, 200, 270, 410 and 500 kDa, potassium bromide (KBr), guar gum, Sephacryl S-500HR matrix were purchased from Sigma Chemical Co., USA. Absolute ethanol, Trifluroacetic acid (TFA), phenol and sulphuric acid were from Merck, India. The hydrocarbon n-hexadecane was purchased from Spectrum Lab Pvt. Ltd. India. All the media components for culturing the bacteria are purchased from Hi-media Pvt. Ltd., India.

4.2.2 Microorganism and culturing condition

Weissella cibaria RBA12 isolated from pumello (*Citrus maxima*) was maintained in modified MRS medium (Goyal *et al*., 1995) as described in Section 2.2.1 of chapter 2. This isolate was maintained in Tsuchiya medium (Tsuchiya *et al*., 1952) with 20% (v/v) glycerol at -20°C. For the development of inoculum, a 1% (v/v) culture from glycerol stock was transferred to 5 ml of medium enzyme production medium (Tsuchiya *et al*., 1952) as described in Chapter 2, Section 2.2.2.

4.2.3 Production of dextran from *Weissella cibaria* RBA12

The production of dextran from *Weissella cibaria* RBA12 was carried out by inoculating 1 ml culture of *Weissella cibaria* RBA12 in sterile 100 ml enzyme production medium as reported by Tsuchiya *et al*., (1952) (as described in Chapter 2, Section 2.2.3). The culture was incubated at 20°C for 24 h under static condition and after 24 h of incubation; the cell free supernatant was collected by centrifugation at 10,000*g* and at 4°C for 10 min as mentioned in Chapter 2, Section 2.2.12. The cell free

supernatant was used subsequently for the purification of dextran as described in Section 4.2.4.

4.2.4 Purification of dextran from *Weissella cibaria* RBA12

4.2.4.1 *Purification of dextran by ethanol precipitation*

The cell free supernatant (100 ml) of *Weissella cibaria* RBA12 obtained by centrifugation at 10,000g at 4°C for 10 min (as described in Section 5.2.3), was used for purification of dextran by ethanol precipitation. The chilled ethanol (95%) was added to the cell free supernatant in a ratio of 3:1 and centrifuged at 10,000g at 4°C for 30 min (Purama *et al*., 2009). The pellet was collected and dissolved in 50 ml of de-ionised water and the process was carried out thrice. The dextran content was determined by phenol-sulfuric acid method (Dubois *et al*., 1956) in a micro titer plate (Fox and Robyt, 1991) as described in chapter 2, Section 2.2.12.

4.2.4.2 *Purification of dextran by gel filtration*

Dextran solution (50 ml) after purification by ethanol was freeze dried by using lyophilizer (Christ GmbH, model ALPHA 1-4 LD) at -51°C and at a vacuum pressure of $3.5x10^{-2}$ mbar for 24h and the dried powder of dextran was used for further purification by gel filtration using Sephacryl S-500HR (Sigma Aldrich, USA). The dried powder of dextran (10 mg) was dissolved in 1 ml of Milli-Q water and loaded onto a gel filtration column (1.5 cm x 50 cm) containing Sephacryl S-500HR, connected to Fast Protien Liquid Chromatography (FPLC) (Akta Prime, GE Healthcare). The dextran was eluted using Milli-Q water at a flow rate of 0.3 ml/min and fractions of 3 ml were collected (Das and Goyal, 2014). The total carbohydrate content was determined by phenol sulphuric acid method (Dubois *et al.*, 1956) as described in

Chapter 2, Section 2.2.12. The fractions showing higher absorbance at 490 nm were pooled and lyophilized for further physicochemical and structure analysis.

4.2.5 Structural characterization of dextran from *Weissella cibaria* RBA12

4.2.5.1 *Monosaccharide composition analysis of dextran*

Purified dextran (2 mg/ml) was hydrolyzed by 2 M trifluoroacetic acid (TFA) at 100°C for 2 h as reported earlier (Yang *et al*., 2009). After removing the TFA using hot-air oven at 80°C, the released monosaccharaides were analyzed by HPAEC-PAD using an ion-chromatography system (ICS-3000 system, Dionex Corporation, USA equipped with the ICS-3000 detector/chromatographic module,) using CarboPac PA-20 analytical column (3 mm x 150 mm) and the CarboPac PA-20 guard column (3 mm x 30 mm). The temperature of the column was adjusted to 30°C and the injection volume was 25 µl. The eluent 100 mM NaOH and 1M sodium acetate gradient were used at a constant flow rate of 1 ml/min with isocratic elution. The monosaccharide was detected with an electrochemical detector (ED 50). Glucose and fructose (Sigma–Aldrich, USA) were used as standards. Deoxygalactose (Sigma-Aldrich St. Louis, MO, USA) was used as an internal standard in a concentration of 50 µg/ml. The samples were analysed in triplicates.

4.2.5.2 *Fourier Transform Infrared Spectroscopic analysis of dextran*

The FTIR spectrum was recorded for column purified dextran in a KBR pellet using a spectrophotometer (Perkin Elmer Instruments, Spectrum Two Instruments, Waltham, MA, USA). The purified dextran (2 mg) was finely grinded with 400 mg of potassium bromide (KBr) powder and pressed into pellets of thickness 0.5 to 1 mm using hydraulic press. For a solid sample preparation in FTIR analysis, the

concentration of the sample in KBr should be in the range of 0.2% to 1% and the pellet thickness should be in the range of 0.5 mm to 1 mm for avoiding the noisy spectra (Davarcioglu, 2010). The pellet was then placed in a transmission holder and scanned in the region of 4000 cm^{-1} to 500 cm^{-1} with 20 scans per min.

4.2.5.3 *^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectral analysis of dextran*

^{1}H- and ^{13}C- NMR spectra for the purified dextran were recorded on a 600 MHz NMR spectrometer (Bruker Avance III HD equipped with Topspin software package from Bruker, MA, USA) with operating frequencies 600 MHz for ^{1}H- and 100 MHz for ^{13}C-NMR to determine the type of glycosidic linkage present. The purified dextran samples were dissolved in D_2O (99.96%) (Merck, Germany) at concentrations of 5 mg/ml (for ^{1}H NMR) and 30 mg/ml (for ^{13}C NMR).

4.2.5.4 *Molecular weight determination of dextran*

For the determination of molecular weight of dextran with high accuracy high performance size exclusion chromatography (HPSEC) was used. The HPSEC system consisted of Phenomenex Polysep-GFC-P6000 connected with a guard column Phenomenex Polysep-GFC-P and Prominence UFLC (Shimadzu, Japan) using Milli-Q water (18.2 MΩ cm) as eluent at a flow rate of 0.5 ml/min. The samples were detected using RI detector. The standard dextrans T10, T20, T40, T70, T200, T270, T410 and T500 of molecular weights (Mw) 10, 20, 40, 70, 200, 270, 410 and 500 kDa, respectively, were used for generation of a standard plot for the determination of molecular weight of dextran samples. Dextran from *Weissella confusa* Cab3 of molecular weight 18000 kDa (18 MDa) was used as a higher molecular weight standard (Shukla *et al*., 2014).

4.2.5.5 *Thermogravimetric Analysis (TGA) and Derivative Thermogravimetric Analysis (DTG) of dextran*

The TGA and DTG of purified dextran was carried out using thermal analyser (Hitachi STA7200, Japan) operating at atmospheric pressure under nitrogen gas flow rate of 100 ml/min. The purified dextran (6.5 mg) was placed in a Al_2O_3 crucible and heated at a linear heating rate of 10ºC min^{-1} over a temperature range, 30ºC-600ºC and the corresponding weight loss was determined.

4.2.5.6 *Differential scanning calorimeter (DSC) analysis of dextran*

The DSC analysis of purified dextran was carried out using thermal analyser (STA 449-F3, Netzsch, Germany) operating at atmospheric pressure under argon gas at a flow rate of 100 ml/min. The purified dextran (3 mg) was placed in an Al_2O_3 crucible and heated at a linear heating rate of 10ºC min^{-1} over a temperature range, 25ºC-400ºC and enthalpy changes were determined.

4.2.6 Physicochemical properties of dextran from *Weissella cibaria* RBA12

4.2.6.1 Scanning Electron Microscopic analysis of dextran

5 mg of purified dextran was fixed on the SEM stubs using double sided carbon tape, then coated with ~10 nm thick layer of gold in a sputter coater (SCH 620, Leo). The samples were observed in a scanning electron microscope (Zeiss, Sigma, Germany) at an accelerating voltage of 2.5 kV at magnifications from 750x to 2700x.

4.2.6.2 Solubility properties of dextran

Solubility of dextran from *Weissella cibaria* RBA12 in water was determined by the method established by Chang and Cho, (1997). The dextran (30 mg) was dissolved in 1 ml deionised water with continuous agitation at 25ºC for 24 h. The dextran suspension was then centrifuged at 5,000*g* at 25ºC for 15 min and the upper layer (0.2 ml) of supernatant was collected. The 0.6 ml of chilled ethanol (95%) was added to the 0.2 ml of the supernatant in a ratio of 3:1 and centrifuged at 10,000*g* at 4°C for 20 min. The resulting precipitate was vacuum dried at 80ºC and difference in weight loss was recorded. The solubility of dextran was calculated as follows;

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

4.2.6.3 Water holding capacity of dextran

The purified dextran was also characterized for water holding capacity (WHC) by following the method established by Ahmed *et al*., (2013). The dextran (0.2 g) was dissolved in 10 ml of deionised water and was centrifuged at 13,000g at 4ºC for 30 min. Unbound water that was not held by dextran was discarded and the precipitated dextran was placed on pre weighed filter paper to remove the moisture of the pellet. The wet weight of precipitated dextran in form of pellet was determined. The percentage of WHC was calculated as;

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

4.2.6.4 Atomic Force Microscopic (AFM) analysis of dextran

A stock solution of purified dextran (5 mg/ml) was prepared in Milli-Q water (18.2 MΩ cm). The dextran solution was diluted to make final concentrations of 0.1 mg/ml. 10 µl of dextran solution was dropped on the surface of mica sample carrier and allowed to dry at 25°C and analysed using semi-contact imaging mode. AFM analysis was performed using atomic force microscope (Agilent 5500, USA). The data obtained were processed using WSxM 5.0 Develop 7.0 software (Horcas *et al*., 2007).

4.2.6.5 Emulsion stability of dextran

The emulsifying activity of dextran from *Weissella cibaria* RBA12 was assayed by the method described by Bramhachari *et al*., (2007). Lyophilized dextran (0.5 mg) was dissolved in 0.5 ml deionised water by heating at 100ºC for 15 min and allowed to cool at 25ºC. The volume was then made up to 2 ml using 1x phosphate-buffered saline (PBS), pH 7.4. The sample was mixed on a vortex for 1 min after the addition of 0.5 ml *n*-hexadecane. The absorbance at 540 nm (A_0) was immediately measured after mixing (A_0). The sample was then incubated at 25ºC and decrease in absorbance was recorded at 30 and 60 min (A_t). A control was run simultaneously with only 2 ml of 1x PBS (pH 7.4) and 0.5 ml n-hexadecane. The emulsification activity was expressed as the percentage retention of emulsion during incubation for 30 or 60 min;

$$Emulsifying\ activity\ (\%) = \left(\frac{A_t}{A_0}\right) \times 100$$

Where, A_0 = absorbance (A_{540}) of the suspension at time t = 0 and A_t = absorbance (A_{540}) of the suspension at time t = 30 or 60 min.

4.2.6.6 Flocculating activity of dextran

The flocculating activity of dextran from *Weissella cibaria* RBA12 was determined by following the method of Lim *et al*., (2007) using activated charcoal carbon. In a test tube, 50 mg of activated charcoal carbon was added to 10 ml of deionised water and mixed with 0.1 ml of 6.5 mM $CaCl_2$ solution. The dextran and guar gum with various concentrations ranging from 0.1 to 0.8 mg/ml was added to the suspension and mixed on a vortex for 30 s. The reaction mixture was allowed to stand at 30ºC for 10 min and the absorbance (A_{550}) at 550 nm of the upper phase (1 ml) was measured. The absorbance of the control experiment (Ac) without the addition of dextran or guar gum was also measured. The flocculating activity (%) was measured as;

$$Flocculating\ activity\ (\%) = \left[\frac{A_c - A_s}{A_c}\right] \times 100$$

Where, A_s = absorbance of dextran or guar gum containing suspension; A_c = absorbance of control.

4.3 Result and Discussion

4.3.1 Purification of dextran from *Weissella cibaria* RBA12

The cell free supernatant of *Weissella cibaria* RBA12 obtained by centrifugation at 10,000*g* at 4°C for 10 min, was subjected to ethanol precipitation as described in Section 4.2.4.1. The crude dextran with slushy apple sauce like appearance was achieved after ethanol precipitation and it gave concentration of 8.7 mg/ml as determined by phenol sulphuric acid method (as described in Chapter 2, Section 2.2.12). The crude dextran was lyophilized to give fluffy white powder with gel like structure and was further purified by gel filtration using Sephacryl S-500 HR as matrix.

4.3.2 Structural characterization of dextran from *Weissella cibaria* RBA12

4.3.2.1 Monosaccharide composition analysis of dextran

The identification of released monosaccharide from hydrolyzed glucan by 2M TFA, was analyzed by HPAEC by comparing the retention time with the reference standard glucose, sucrose and fructose (Fig. 4.3.1A). The hydrolysate showed a single peak at 14.5 min (Fig. 4.3.1B) corresponding to that of standard glucose peak at 14.5 min (Fig. 5.3.1A) whereas the retention time of fructose was 17.5 min (Fig. Fig. 4.3.1A). Thus, the exopolysaccharide synthesized by *Weissella cibaria* RBA12 contained only glucose as building block, confirming its glucan nature.

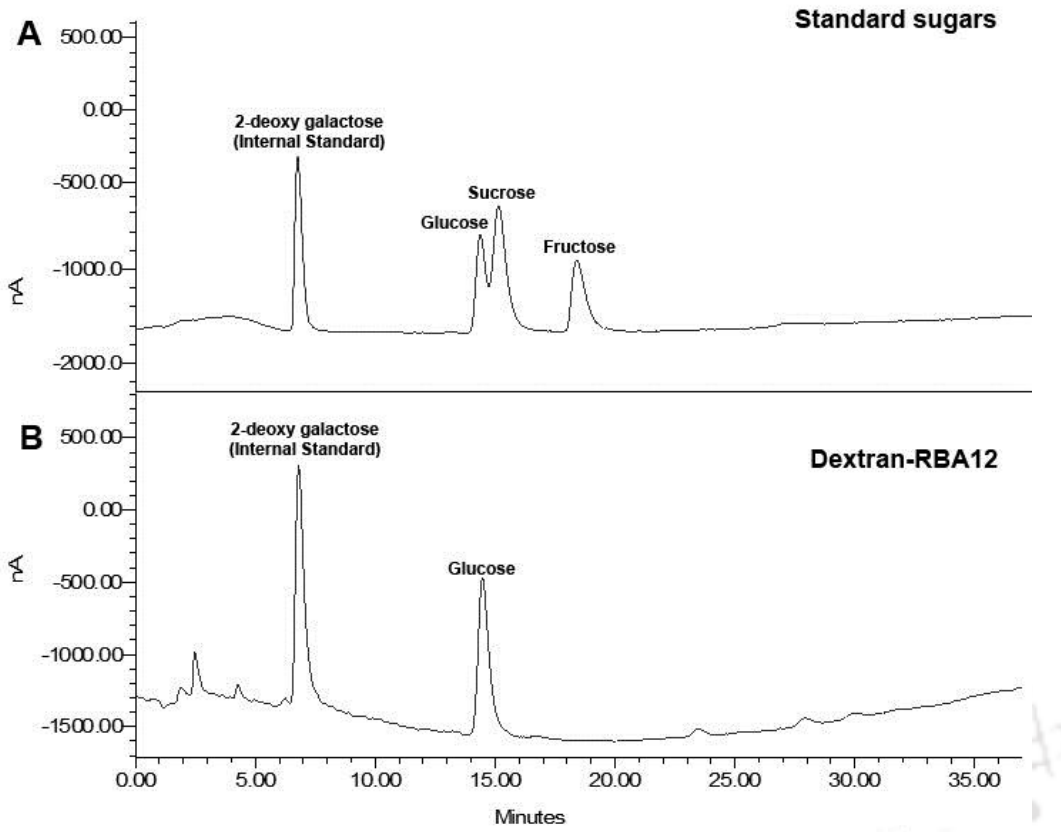

Fig. 4.3.1 Monosaccharide analysis of dextran from *Weissella cibaria* RBA12 by high performance anion exchange chromatography (HPAEC), using a Carbo-Pac P20 column by isocratic elution using 100 mM NaOH and 1 M sodium acetate gradient at a constant flow rate of 1 ml/min at 30°C. A) Standard Sugars, B) Hydrolysed dextran-RBA12.

4.3.2.2 Fourier Transform Infrared Spectroscopic analysis of dextran

The FT-IR spectrum of purified dextran from *Weissella cibaria* RBA12 is shown in Fig. 4.3.2, showing numerous peaks between 3406-531 (cm^{-1}). The peak in the region of 3400 (cm^{-1}) was due to the hydroxyl (-OH) stretching vibration of the polysaccharide as reported earlier by Purama *et al*., (2009). The peak in the region of 2930 (cm^{-1}) was due to C–H stretching vibration and the band in the region of 1639 (cm^{-1}) was due to the carboxyl group (Cao *et al*., 2006; Liu *et al*., 2007). The presence peaks in the 950–1200 (cm^{-1}) region is a fingerprint region for all polysaccharides (Černá *et al*., 2003). The peak at 1154 (cm^{-1}) is due to valent vibrations of C-O-C bond

and a glycosidic bridge. The broad band at 1107 (cm^{-1}) is due to the vibration of the C-O bond at the C-4 position of a glucose residue (Shingel 2002). The presence of a peak at 1020 (cm^{-1}) is due to the great chain flexibility present in dextran around α-(1→6) glycosidic bonds as shown earlier (Shingel, 2002). The peak at 915 (cm^{-1}) is a characteristic of α-glycosidic bond.

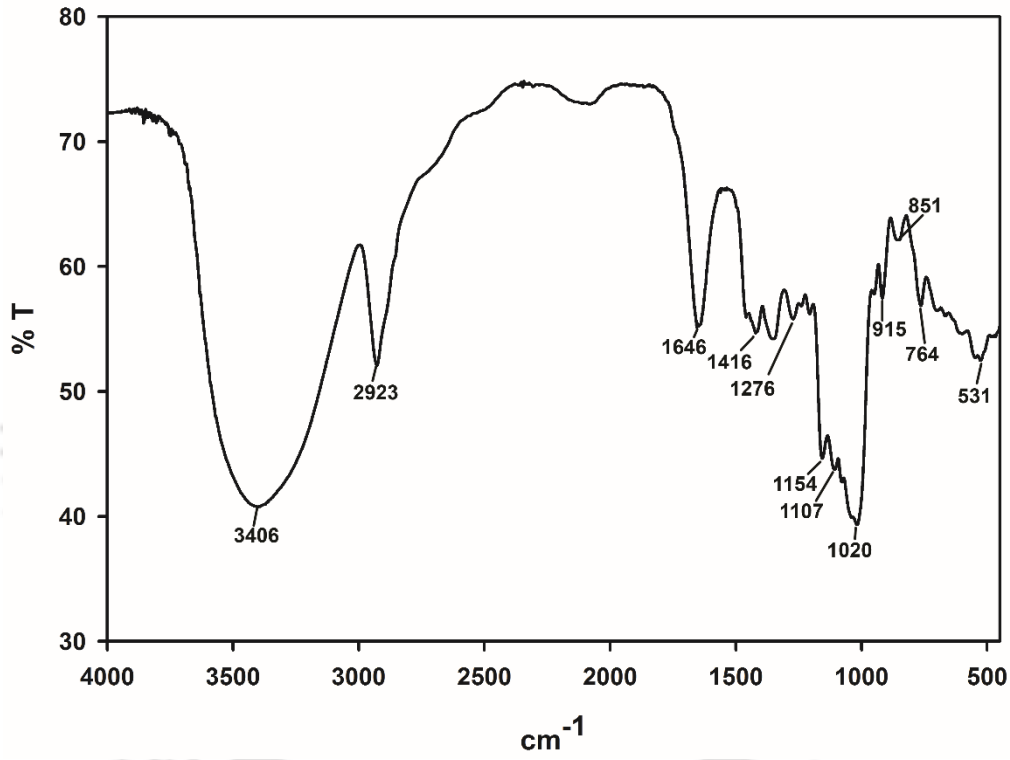

Fig. 4.3.2 FTIR (KBr) spectrum of *Weissella cibaria* RBA12 dextran showing the presence of α-(1→6) glycosidic linkages and other functional groups.

4.3.2.3 ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) spectral analysis of dextran

The ^{1}H NMR and ^{13}C NMR spectra of purified dextran-RBA12 are shown in Fig. 4.3.3A and 4.3.3B, respectively. The ^{1}H NMR spectrum of dextran-RBA12 showed an anomeric signal at 5.02 ppm as reported earlier (Maina *et al*., 2008; Ahmed *et al*., 2012) for α-(1→6) linked dextran. Another low-intensity signal at 5.36 ppm was

observed in the ^{1}H NMR spectrum which is attributed to the presence of α-(1→3) linked branches (Ahmed et al., 2012, Shukla et al., 2014). The percentage of α-(1→3) linkage calculated from integration analysis of the anomeric signals was 3%. The remaining five resonances in the ^{1}H NMR spectrum at 4.02, 3.96, 3.79, 3.76, 3.58 and 3.56 ppm (Fig. 5.3.3A), corresponded to H-6b, H-5, H-6a, H-3, H-2 and H-4 positions, respectively.

The ^{13}C NMR spectrum of purified dextran from *Weissella cibaria* RBA12 showed the major resonance in the anomeric region at 97.7 ppm and C-6 resonance occurred at 65.5 ppm (Fig. 4.3.3B), indicating the presence of C-1 and C-6 linkages typically seen in α-(1→6) glycosidic bonds in dextran as reported earlier by Ahmed *et al*., (2012). The other signals observed at 71.4, 73.4, 69.5 and 70.2 ppm corresponded to C-2, C-3, C-4 and C-5 positions, respectively.

The ^{1}H NMR and ^{13}C NMR spectra of purified dextran from *Weissella cibaria* RBA12 revealed the presence of 97% of α-(1→6) linkages and 3% of α-(1→3) branch linkages. The dextrans produced by *Weissella cibaria* and *confusa* have been reported to be highly linear. They contain only α-(1→6) linkages (Kang *et al*., 2006) or with few (2.4–3.4%) α-(1→3) linked branches (Bounaix *et al*., 2009, Ahmed et al., 2012, Shukla *et al*., 2014 and Maina *et al*., 2008). Dextran containing 7% α-(1→3) linked branches, which is the highest branching present among dextrans from *Weissella* spp was reported for *Weissella cibaria* JAG8 (Rao & Goyal 2013). The highly linear dextran from *Weissella* finds use in several food applications such as thickening, viscosifying and emulsifying agents and also as a potential soluble fibre which can act as a prebiotic compound (Kothari *et al*., 2015 b).

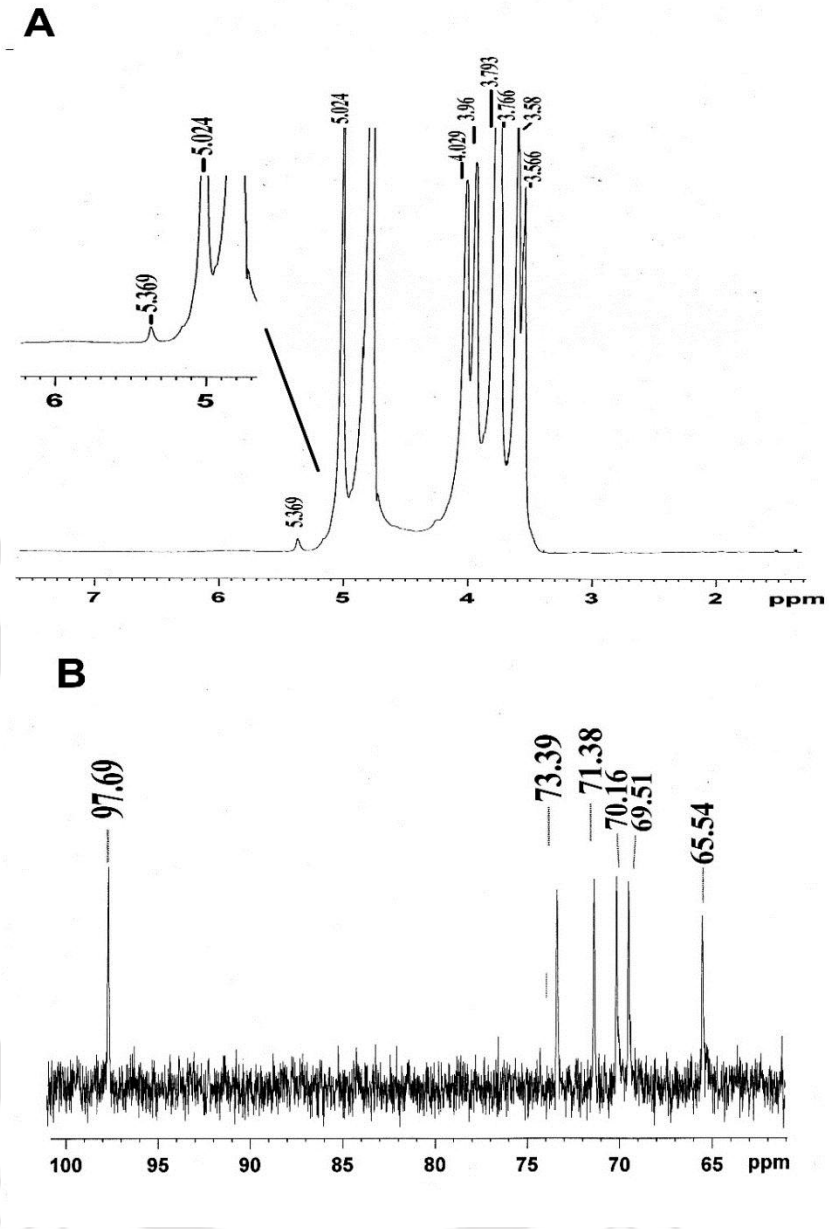

Fig. 4.3.3 **A)** ^{1}H NMR spectrum of dextran from *Weissella cibaria* RBA12; **B)** ^{13}C NMR spectrum of dextran from *Weissella cibaria* RBA12.

4.3.2.4 Molecular weight determination of dextran

It was observed that the higher the molecular weight, lesser is the retention time of the dextran (Fig. 4.3.4 & Fig. 4.3.5.) Therefore, molecular weights of the standards used were expressed as Log molecular weight (Mw) to obtain a linear equation for the determination of molecular weight of dextran samples. All subsequent molecular

weights of samples were determined using this equation to solve for the y variable using the retention time of sample and then calculating the Mw by taking the antilog of y.

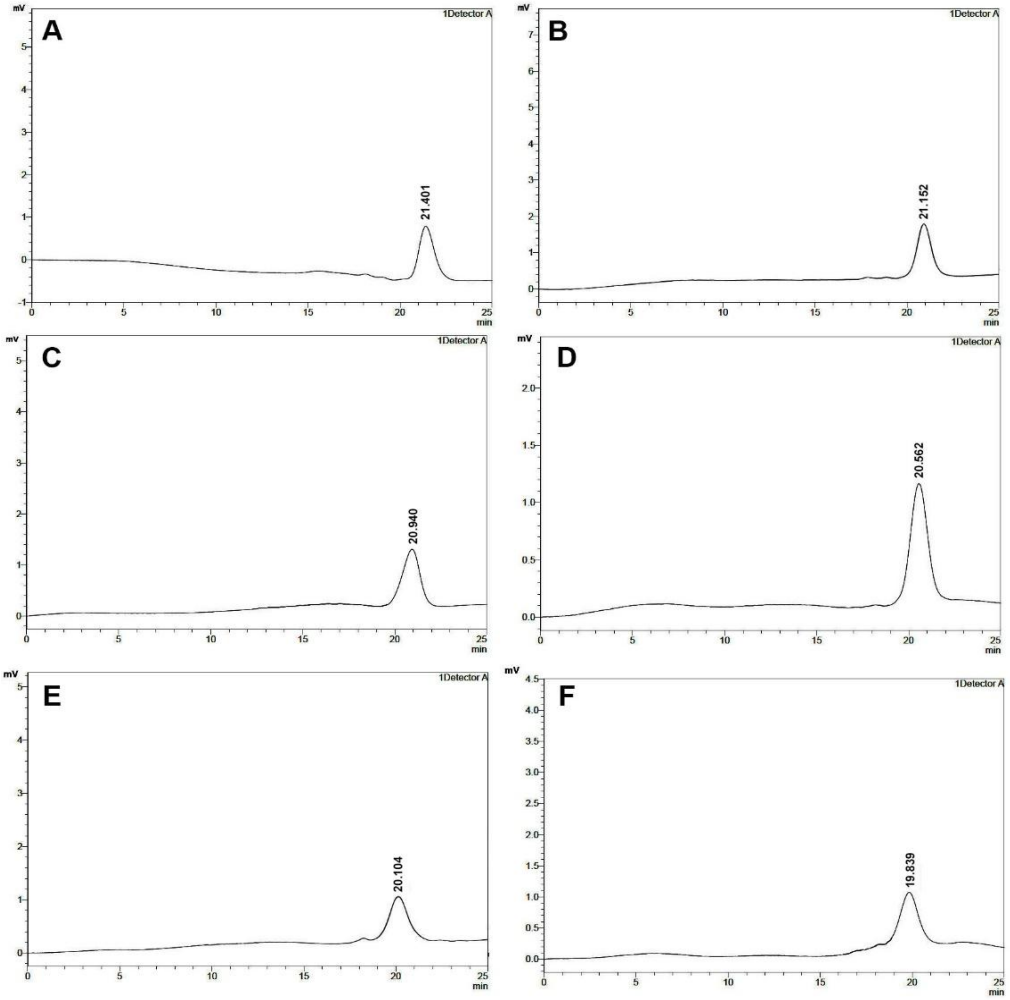

Fig. 4.3.4 HPSEC chromatogram of dextran standards of different molecular weights **(A)** 10kDa, **(B)** 20kDa, **(C)** 40kDa **(D)** 70kDa **(E)** 200 kDa **(F)** 270kDa

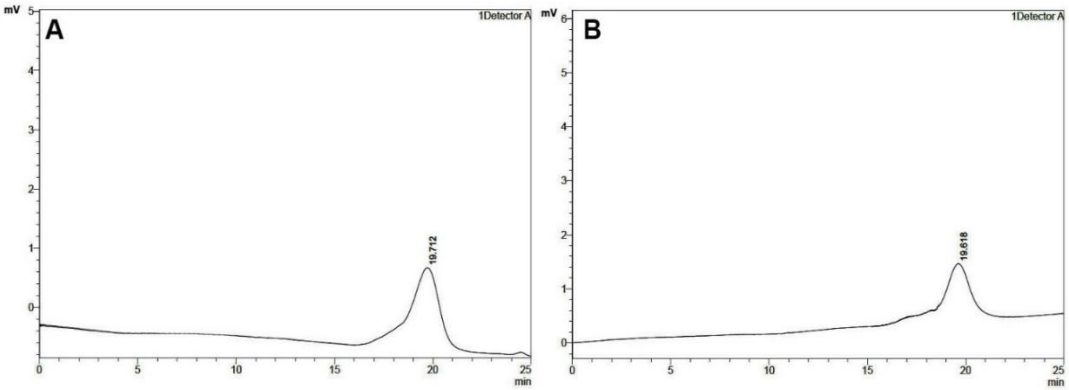

Fig. 4.3.5 HPSEC chromatogram of dextran standards of different molecular weights **(A)** 410kDa, **(B)** 500kDa.

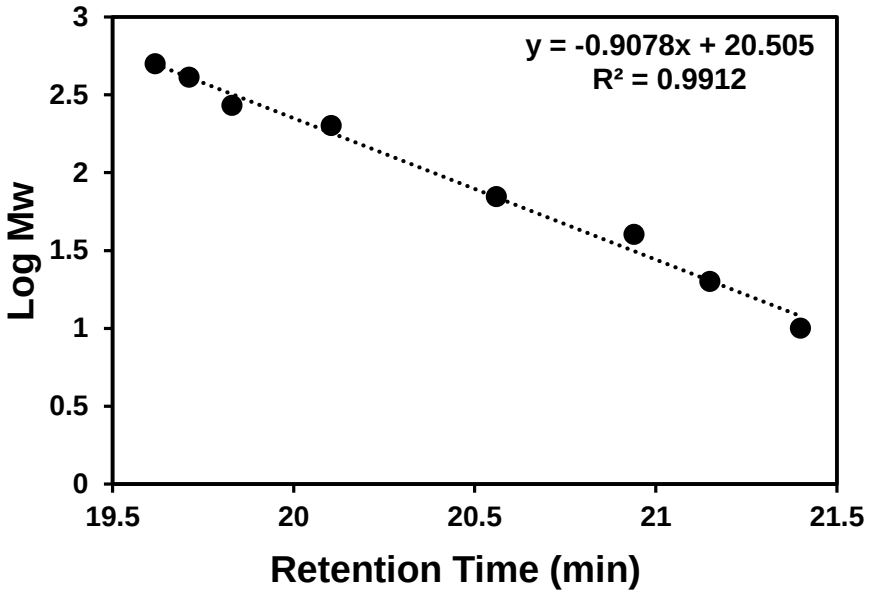

Fig. 4.3.4 Standard plot for the determination of molecular weight of dextran by high performance size exclusion chromatography (HPSEC) using column phenomenex Polysep-GFC-P6000 with RI detector.

Based on the calibration curve as shown in Fig. 4.3.6 (y = -0.9078x + 20.505, R^2 = 0.9912 where y = log molecular weight of the standard dextrans and x = retention time), the molecular weight (Mw) of the dextran was calculated at different time intervals of 12h, 18h and 24h. The Mw of the dextran increased with time. A Mw of 436 kDa (19.7 min), 8.15 MDa (18.2 min) and 26.3 MDa (17.6 min) were observed at 12h, 18h and 24h, respectively (Fig. 4.3.7 A, B and C).

Due to the higher range of Mw for the dextran i.e 26.3 MDa at 24h, a higher Mw dextran from *Weissella cibaria* Cab3 of Mw of 18 MDa (17.8 min) was used as a standard (Fig. 4.3.7 D). This experiment was done in triplicate. This is the first report of dextran of different molecular weight being produced at different time of fermentation. The presence of high molecular weight dextran over 10 MDa have been reported in the case of *L mesenteroides* KIBGE-IB22 and *L mesenteroides* KIBGE-IB22M20 producing dextran in the range between 15–20 MDa and 25–40 MDa,

respectively (Siddiqui *et al*., 2014). In a recent study, recombinant dextransucrase from *Weissella cibaria* 10M produced dextran with Mw of 118 MDa (Chen and Ganzle, 2016).

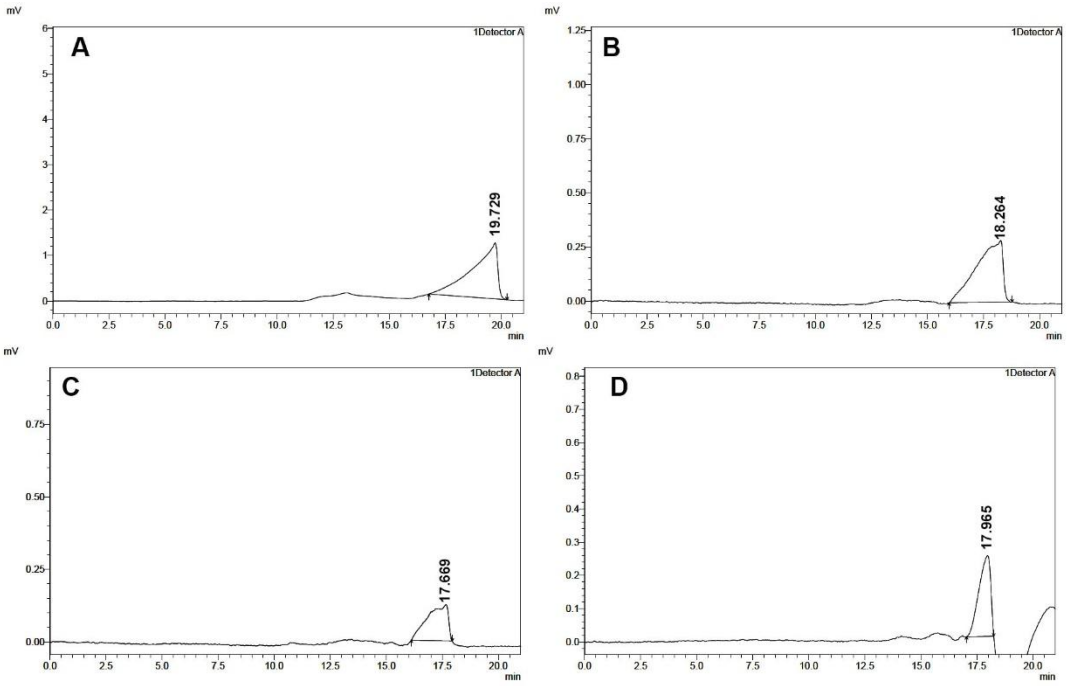

Fig. 4.3.7 HPSEC chromatogram of dextran from *Weissella cibaria* RBA12 at different fermentation time of **(A)** 12h, **(B)** 18h, **(C)** 24h and **(D)** dextran from *Weissella confusa* Cab3

4.3.2.5 Thermogravimetric Analysis (TGA) and Derivative Thermogravimetric Analysis (DTG) of dextran

The TGA of dextran displayed the weight loss by the exopolysaccharide with the increase in temperature (Fig. 4.3.8). The initial weight loss of approximately 10% between 40ºC to 100ºC was observed for dextran. This initial weight loss is attributed to the bound water molecules which is a characteristic of exopolysaccharide rich in carboxyl groups (Ahmed *et al*., 2013). The dextran showed a drastic weight loss of 60% from 270ºC to 320ºC. The degradation temperature (T_d) of dextran from *Weissella cibaria* RBA12 was 298ºC. The rapid weight loss of dextran decreased after 320ºC and

was completely decomposed at approximately, 600ºC. The degradation temperature (T_d) of dextran from *Weissella cibaria* RBA12 similar to that of dextran from *Lactobacillus plantarum* DM5 which had a degradation temperature of 292.2ºC. The high thermostability of dextran from *Weissella cibaria* RBA12 indicates its potential for use in food industry where a resistance to high temperature is desirable for food manufacturing and processing.

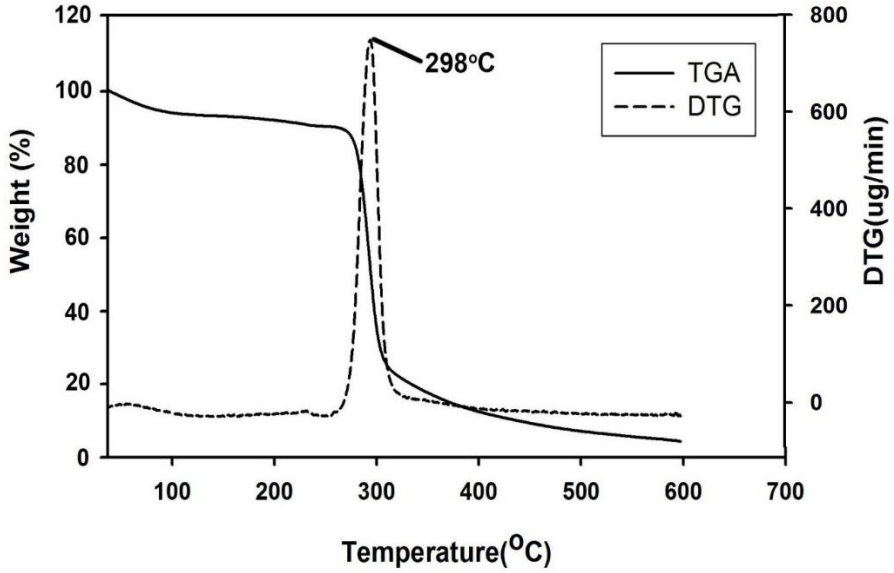

Fig. 4.3.8 Thermogravimetric analysis (TGA) showing weight loss till 600ºC and Derivative Thermogravimetric analysis (DTG) showing degradation temperature (T_d) of 298ºC.

4.3.2.6 Differential scanning calorimeter (DSC) analysis of dextran

DSC analysis of dextran from *Weissella cibaria* RBA12 showed an endothermic peak with heat flow from 10ºC to 400ºC (Fig. 4.3.9). The melting point of dextran from *Weissella cibaria* RBA12 was approximately, 280ºC. A similar melting peak at 220.7ºC was reported for dextran hydrogels after DSC analysis (Stenekes *et al*., 2001). Heteropolysaccharide from *Lactobacillus plantarum* YW11 showed a melting point of 146.6ºC (Wang *et al*., 2015).

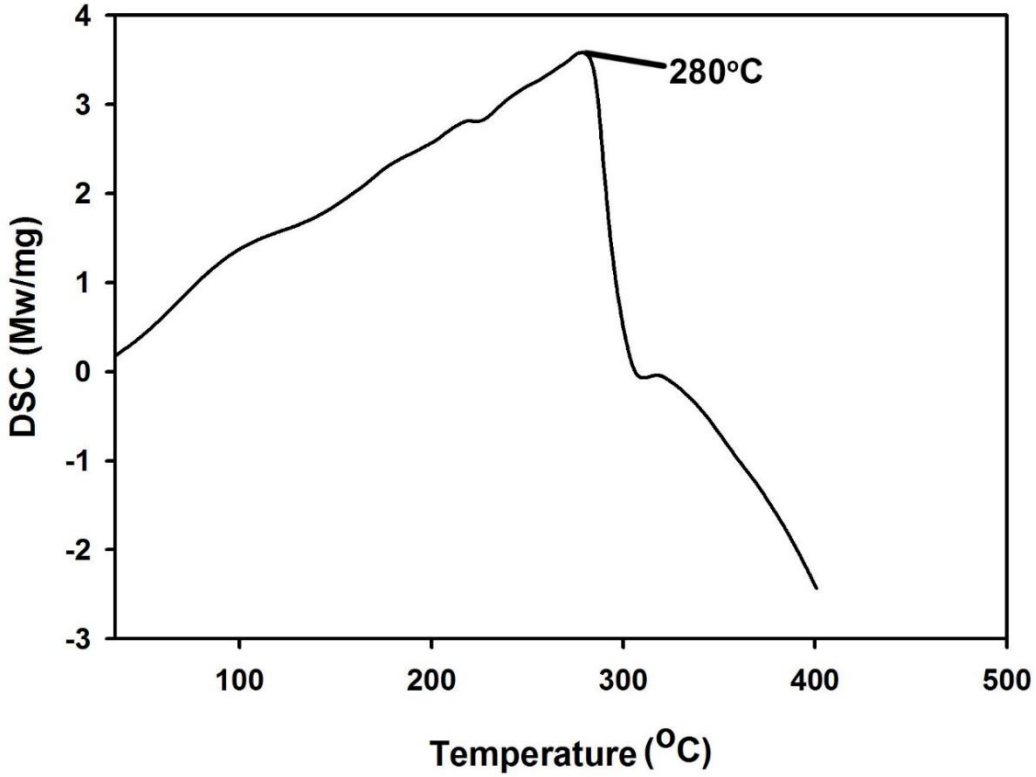

Fig. 4.3.9 Differential scanning calorimetry of dextran from *Weissella cibaria* RBA12 showing the melting point of dextran at 280ºC.

4.3.3 Physicochemical properties of dextran from *Weissella cibaria* RBA12

4.3.3.1 Scanning Electron Microscopic analysis of dextran

The lyophilized powder of purified dextran was reveal to be highly porous with gel like structures, upon analysis by scanning electron microscope. The pores were visible from 750x (20 µm) and displayed a web-like architecture under higher magnifications of 1750x (20 µm) and 2700x (10 µm) (Fig. 4.3.10). The hydroxyl groups present in the dextran chain facilitates high water holding capacity which make it a highly valuable hydrocollod for the food industry.

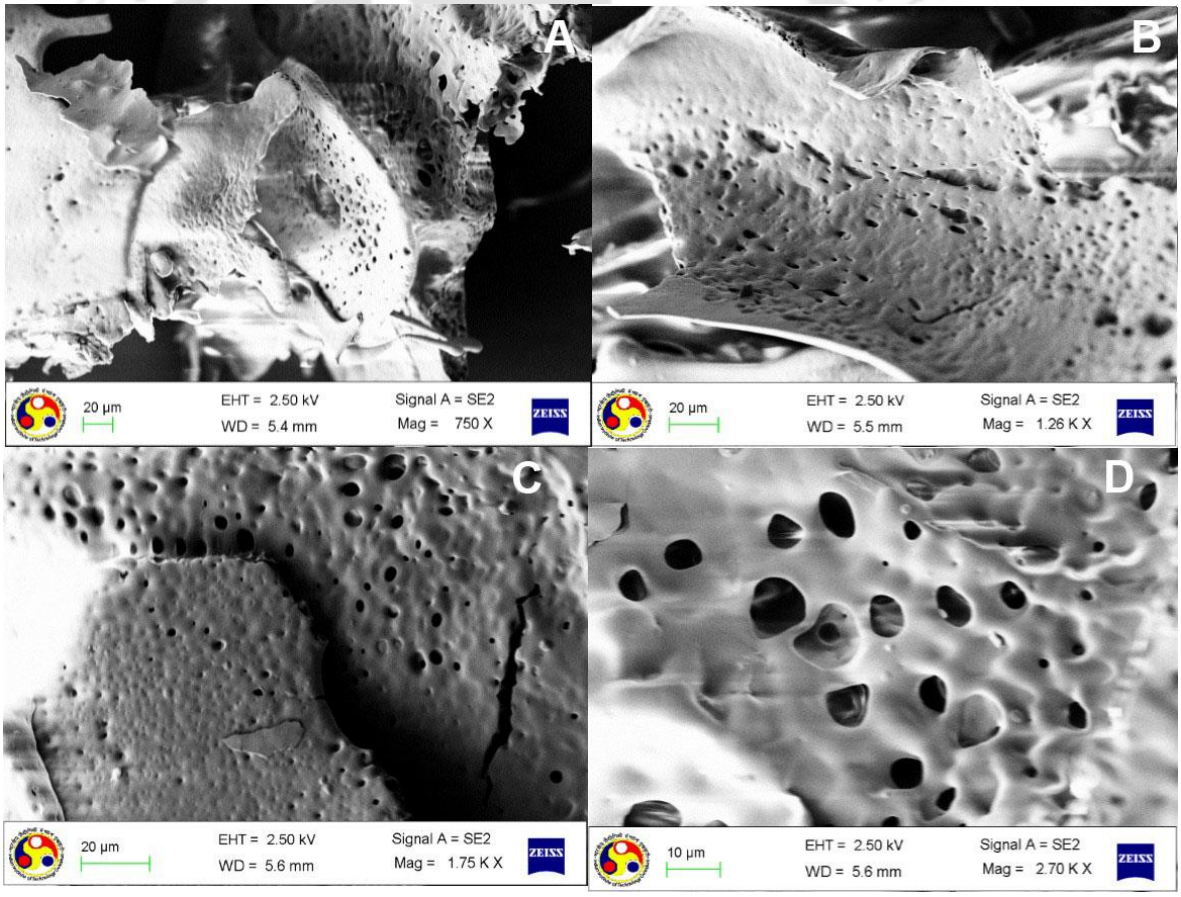

Fig. 4.3.10 Scanning Electron Micrograph (SEM) of dextran from *Weissella cibaria* RBA12 at magnifications **A)** 750x, **B)** 1260x, **C)** 1750 x and **D)** 2700x

4.3.3.2 Solubility and water holding capacity of dextran

The dextran from *Weissella cibaria* RBA12 displayed 19.5% solubility and 383% water holding capacity. These properties are associated with the porous matrix structure formed by polysaccharide chains which can hold large number of water molecules through hydrogen bonds (Das *et al*., 2014). Dextran from *Weissella cibaria* JAG8 displayed solubility of 24.5% and water holding capacity of 352% (Rao *et al*., 2014). It has been reported that the solubility of dextran polymer largely depends on its molecular weight and the percentage of branching (Cote and Robyt, 1995; Tususaki *et al.,* 2009). The good solubility and water holding abilities of dextran are attributed to its use as an emulsifier or stabilizer in the food industry.

4.3.3.3 Atomic Force Microscopic (AFM) analysis of dextran

AFM is a powerful tool that enables the study of surface morphology of hydrated and soft samples (Jacoboni *et al*., 1999). The images of dextran from *Weissella cibaria* RBA12 from 0.1 mg/ml aqueous solution were obtained by AFM (Fig 4.3.11 A & B). The surface morphology revealed the presence of several spherical lumps with a maximum height of 56.3 nm. The lumps appeared to form a network in certain regions and was relatively sparse in others. This arrangement was in agreement with the porous structures seen in the SEM image (Fig 4.3.8). Similar structural arrangement of EPS from *L. plantarum* YW11 displaying fibrous network of spherical lumps (Wang *et al*., 2015).

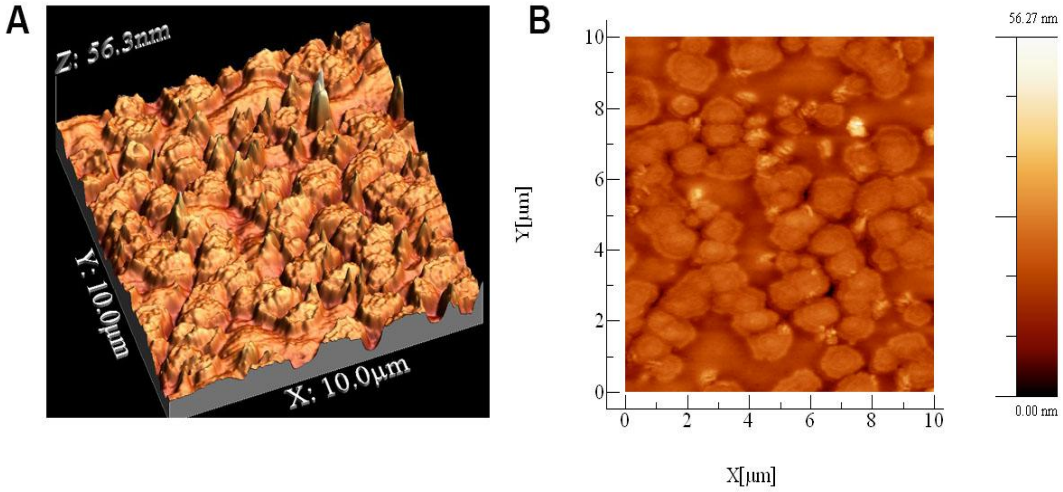

Fig. 4.3.11 Atomic force microscopy image of dextran from *Weissella cibaria* RBA12 **A)** 3D, **B)** Planar.

4.3.3.4 Emulsion stability of dextran

The emulsifying activity of EPS is determined by its strength in retaining the emulsion of the hydrocarbon in water. The emulsion stability of dextran from *Weissella cibaria* RBA12 was compared with guar gum, a natural polysaccharide and sodium alginate, a synthetic hydrocolloid, both being commercially used emulsifiers. The dextran retained 73.1% and 60.5% of the emulsification activity after 30 and 60 min, respectively however; the emulsion stability of guar gum was 72% and 37% after 30 and 60 min, respectively against n-hexadecane (Fig. 4.3.12). The emulsifying activity of sodium alginate was 69% and 35% after 30 and 60 min, respectively (Fig. 4.3.12) against n-hexadecane. To determine the emulsion stabilizing capacity of an emulsifier, it should be able to retain at least 50% of the emulsion after formation (Kanmani *et al*., 2013). A control experiment was run with 2 ml PBS (1x) mixed with 0.5 ml n-hexadecane without any added dextran or standard polymers. The control was unable to retain its 50% emulsion activity after 30 min of incubation, suggesting the emulsion activity of dextran, guar gum and sodium alginate. The emulsifying activity of dextran

was in accordance with dextran (80.6%) from *Lb. plantarum* DM5 (Das *et al*., 2014) and dextran (89%) from *Weissella cibaria* JAG8 (Rao *et al*., 2014) after 30 min of incubation.

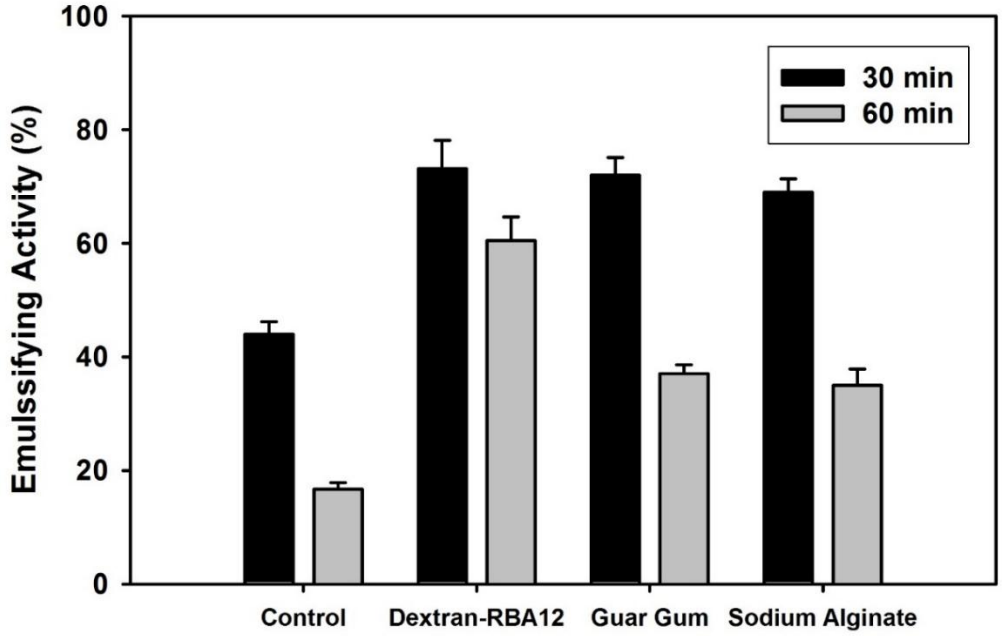

Fig. 4.3.12 Emulsifying activity of dextran from *Weissella cibaria* RBA12 after 30 or 60 min at 25ºC. A control was run with 2 ml PBS (1x), pH 7.4 mixed with 0.5 ml n-hexadecane without any added dextran or standard polymers and absorbance was measured at 540 nm. The mean value of three independent experiments is presented with ± S.D.

4.3.3.5 Flocculating activity of dextran

Flocculants are categorized into two groups, chemically synthesized flocculants (organic and inorganic flocculants) and natural flocculants (chitosan, alginate and microbial flocculants). Presently, the use of chemically synthesized flocculants is restricted, as they are hard to degrade and harmful to human, therefore, the screening of natural flocculants from microbial sources has been in focus as they are nontoxic, benign and biodegradable polymers (Shih *et al*., 2001; Liu *et al*., 2013). The flocculating activity of dextran from *Weissella cibaria* RBA12 ranging from 0.05 to 0.8 mg/ml in 5 mg/ml dispersion of activated charcoal containing 6.8 mM $CaCl_2$ solution was compared with guar gum and is shown in Fig. 4.3.11. It was observed that the flocculation activity of dextran initially increased with increasing the concentration of dextran and the maximum flocculating activity of 94.1% was achieved at concentration of 0.2 mg/ml. Above dextran concentration (0.2 mg/ml), the flocculating activity had decreased. The exopolysaccharide from *Lb. plantarun* DM5 showed similar trend of flocculating activity (Das *et al*., 2014), however; flocculating activity of the exopolysaccaride from *Enterococcus faecium* MC13 increased with increasing concentration of EPS (Kanmani *et al*., 2013). In case of guar gum, the maximum flocculating activity of 79.0% was observed at 0.2 mg/ml concentration. The flocculating activity of guar gum gradually increased up to 0.2 mg/ml concentration and subsequently decreased up to the concentration of 0.8 mg/ml (Fig. 4.3.13). The dextran from *Weissella cibaria* RBA12 showed 15% higher flocculating activity at the same concentration as compared to commercial hydrocolloid guar gum against activated charcoal. The aforementioned data imply that the dextran from *Weissella cibaria* RBA12 can be used as a bio-flocculant in dairy industry for making cheese

from curd. Apart from food industry, the dextran from *Weissella cibaria* RBA12 as a bio- flocculant can be used in a variety of industrial processes, such as wastewater treatment, drinking water purification and for harvesting microbial cells from culture broth in industrial downstream process.

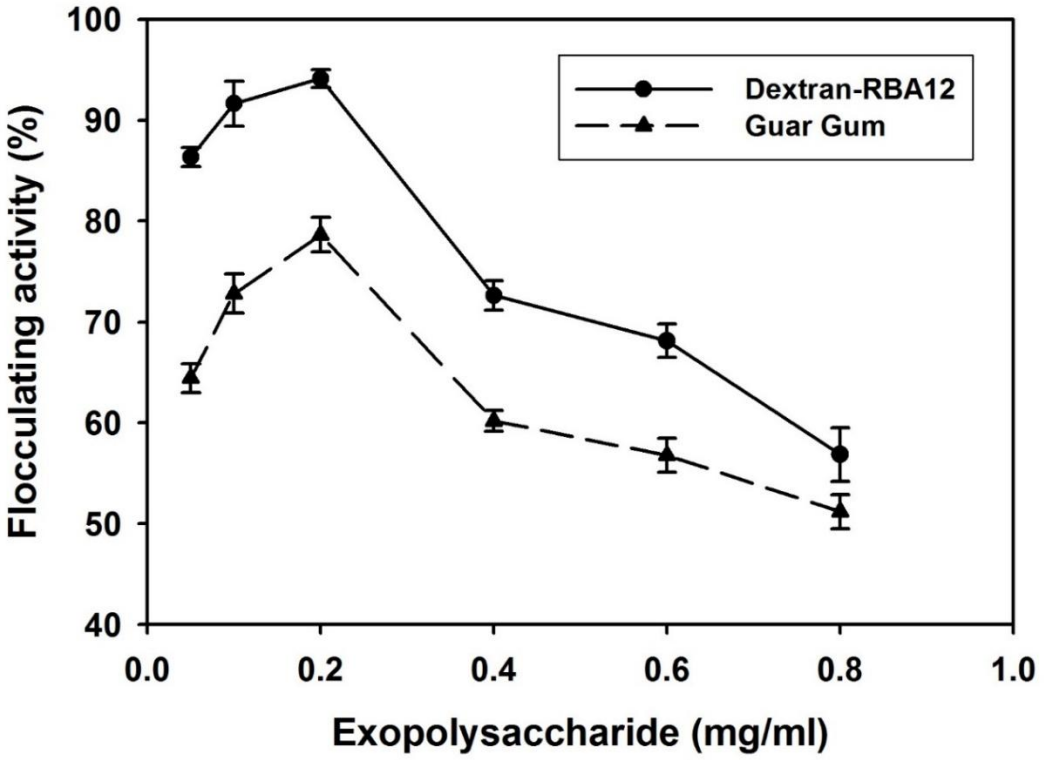

Fig. 4.3.13 Flocculating activity of dextran from *Weissella cibaria* RBA12 and guar gum against activated charcoal containing 6.8 mM $CaCl_2$ at room temperature (25°C). The mean value of three independent experiments is presented with ± S.D.

4.4 Conclusion

Dextran from *Weissella cibaria* RBA12 was purified using ethanol precipitation yielding 8.7 mg/ml concentration. The monosaccharide composition of the dextran revealed the presence of only glucose, confirming its glucan nature. The FT-IR spectrum of dextran displayed the presence of α-(1→6) glycosidic bonds and the characteristic functional groups of dextran. ^{1}H NMR and ^{13}C NMR revealed the presence of 97% of α-(1→6) linkages and 3% of α-(1→3) branch linkages. The molecular weight of dextran was revealed to be of 26.3 MDa after 24h of fermentation by HPSEC analysis. The molecular size of dextran had increased with the fermentation time. The thermal stability analysis of dextran RBA12 displayed degradation temperature (T_d) of 292.2ºC. DSC analysis revealed the melting point of dextran from *Weissella cibaria* RBA12 to be 280ºC. Surface morphology of purified powder of dextran using SEM displayed a web-like architecture with several pores across its surface. The surface morphology by AFM revealed the presence of several spherical lumps with a maximum height of 56.3 nm. Dextran from *Weissella cibaria* RBA12 displayed solubility and water holding capacity 19.5% and 383%, respectively. Dextran retained 73.1% and 60.5% of the emulsification activity after 30 and 60 min, respectively which was greater than that of commercial stabilizers guar gum and sodium alginate. Dextran from *Weissella cibaria* RBA12 showed a flocculating activity of 94.12% at concentration of 0.2 mg/ml. The structural and physicochemical characterization of dextran from *Weissella cibaria* RBA12 has shown properties that can enable it as an efficient food hydrocolloid that can be used in baking, diary and confectionery industries.

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Chapter 5

Production and scale up of dextran from *Weissella cibaria* RBA12 in bioreactor

5.1 Introduction

Lactic acid bacteria belonging to genera *Lactobacillus, Leuconostoc, Streptococcus, Pediococcus* and *Weissella*, have been reported to produce dextran, an extracellular homopolysaccharide using sucrose as substrate (Falconer *et al*., 2011). Dextran comprises of glucose monomers that are linked by α-(1→6) glycosidic bonds in the main chain with different degrees of α-(1→2), α-(1→3) and α-(1→4) glycosidic bonds as branched linkages (Capek *et al*., 2011). Among the genera mentioned above, genus *Weissella* has a distinctive phenotypic feature to produce dextran in the presence of sucrose (Malang *et al*., 2015, Fusco *et al*., 2015). Most notably *Weissella confusa* and *Weissella cibaria* are among the most prolific dextran producers (Baruah and Goyal 2017). Dextrans produced by Weissella spp. have been reported to have identical structure, consisting of mostly α-(1→6) glycosidic bonds and a few α-(1→3) glycosidic bonds as branched linkages (2.4-7.0%) (Ahmed *et al*., 2012, Rao and Goyal 2013, Shukla and Goyal 2014) and have high molecular weight ranging from 800 kDa (Tingirikari *et al*., 2014) to 36 MDa (Chen and Ganzle 2016). Presence of low degree of branching and high molecular weight confer a wide range of applications as a potent

food hydrocolloid (Li and Nie 2016). Dextran from *Weissella* spp. are widely used in bakeries to prepare sourdough breads with improved shelf-life and softness (Kajala *et al* 2015).

The production of dextran by lactic acid bacteria is influenced by several physical factors such as temperature, pH, agitation and medium composition (Shukla and Goyal 2012). Among the nutrients, sucrose concentration plays a crucial role in the dextran yield (Shukla and Goyal 2011). As the dextran concentration increases the viscosity of the medium also increases (Goyal *et al* 1995, Santos *et al* 2005). *Weissella cibaria* RBA12 employed in the present study was isolated from pummelo on the basis of dextransucrase activity, as reported earlier by us (Baruah and Goyal 2015). It produced 8.3 mg/mL of dextran with 83% efficiency under optimal conditions at 24h (Baruah and Goyal 2015). Dextran from *Weissella cibaria* RBA12 contained of 97% linear α-(1→6) linkages in the main chain and 3% α-(1→3) branched linkages and showed superior resistance to physiological barriers as compared to the standard prebiotic inulin (Baruah and Goyal 2017). In the present study the optimal conditions for the production of dextran by *Weissella cibaria* RBA12 were standardized. The production of dextran was then scaled up from 100 ml shake flask to 2.5 L in bioreactor using the optimized conditions. The kinetic parameters such as specific growth rate (μ), total biomass (X), biomass yield coefficient ($Y_{X/Suc}$) and dextran yield coefficient ($Y_{P/Suc}$) were also studied using data obtained from batch fermentation in bioreactor. Fed-batch fermentation of *Weissella cibaria* RBA12 was carried out to obtain higher dextran yield.

5.2 Materials and Methods

5.2.1 Microorganism and culturing condition

Weissella cibaria RBA12 isolated from pumello (*Citrus maxima*) was preserved and maintained in Tsuchiya medium (Tsuchiya *et al*. 1952) with 20% (v/v) glycerol at -20°C. For developing the inoculum, a 1% (v/v) culture from glycerol stock was transferred to 5 ml of enzyme production medium (Tsuchiya *et al*., 1952) as described in Chapter 2, Section 2.2.2.

5.2.2 Production and estimation of dextran

Dextran was precipitated from cell free supernatant (200 µl) of fermenting medium using ethanol (100%, v/v) in 3:1 ratio. The precipitated dextran was separated by centrifugation at 10,500 *g* and 4°C for 30 min as mentioned in Chapter 2, Section 2.2.12. The pellet was dissolved in 200 µl de-ionised water and the process of ethanol precipitation was repeated three times. The dextran content was estimated using phenol sulphuric acid method (Dubois *et al*. 1956) as described in chapter 2, Section 2.2.12. Dextran T40 (Sigma Aldrich, St. Louis, MO) was used as standard. Measurements were carried out in triplicate and the data expressed are the averages of three independent experiments with ± standard error.

5.2.3 Optimization of culture conditions for the production of dextran

5.2.3.1 Effect of temperature and aeration on dextran production

The effect of temperature on dextran production from *W. cibaria* RBA12 was studied by growing the culture in 100 ml medium (Tsuchiya *et al*., 1952). Fermentation medium was incubated at different temperatures of 15, 20, 24, 28 and 37°C for 24h under shaking condition at 180 rpm. The effect of aeration on dextran production was

studied by growing the 100 ml culture at different shaking speeds of 80, 120, 150 and 180 rpm by incubating at 20°C for 24h. The cell free supernatant after 24h of fermentation was used for the determination of dextran concentration as mentioned in the earlier section 5.2.2.

5.2.3.2 Effect of sucrose concentration on dextran production

The effect of sucrose concentration on dextran production from *W. cibaria* RBA12 was studied at 1, 2, 5, 10 and 15% (w/v) concentration in 100 ml fermentation medium (Tsuchiya *et al*., 1952) and incubating at 20°C and 180 rpm for 24h. The cell free supernatant after 24h of fermentation was used to estimate dextran concentration as mentioned in the earlier section 5.2.2. The efficiency of dextran production was calculated according to Baruah and Goyal (2015).

$$Efficiency\ (\%) = \frac{Amount\ of\ dextran\ produced}{Maximum\ possible\ amount\ of\ dextran\ (theoretical)} \times 100$$

Where, maximum possible amount of dextran (theoretical) =10 mg/ml at 2% sucrose concentration.

5.2.3.3 Effect of other medium components on dextran production

The effect of yeast extract on dextran production from *W. cibaria* RBA12 was studied by varying the yeast extract concentration in 100 ml fermentation medium (Tsuchiya *et al*., 1952) at 1, 1.5, 2, 2.5, 3, 3.5 and 4% (w/v) and incubating for 24h at 20°C and 180 rpm. Similarly, the effect of K_2HPO_4 was studied by varying its concentration in 100 ml fermentation medium (Tsuchiya *et al*., 1952) at 1, 1.5, 2, 2.5, 3 and 3.5% (w/v) and incubating for 24h at 20°C and 180 rpm. The cell free supernatant

after 24h of fermentation was used for the determination of dextran concentration as mentioned in the earlier section 5.2.2.

5.2.4 Scale up of dextran production to bioreactor level

5.2.4.1 Bioreactor

Dextran production from *Weissella cibaria* RBA12 was scaled up from 100 ml shake flask culture to 2.5 L working volume of optimized medium in a 5L bioreactor (New Brunswick, model BioFlo115). The bioreactor was equipped with pH probe, DO probe, and stirrer of two-six bladed Rushton turbines. The pH was maintained at 6.9 by addition of 2M NaOH and 2M H_3PO_4 solution. Temperature and aeration rate were kept at 20°C and 2 $vv^{-1}min^{-1}$, respectively. The Dissolved Oxygen (DO) was calibrated to 100% before inoculation and initial agitation rate was set to 200 rpm and later manipulated to maintain the DO above 30%. 10% inoculum from 12 h grown culture was inoculated in the bioreactor and silicon anti-foam was employed as anti-foam agent.

5.2.4.2 Batch fermentation for dextran production from Weissella cibaria RBA12

The optimized medium for dextran production contained 2% (w/v) each of sucrose, yeast extract and K_2HPO_4. The parameters like dextran concentration, sucrose concentration and cell optical density were monitored at every 2h intervals. The sucrose concentration was determined by estimating the reducing sugars by DNS method (Miller 1959) as mentioned in chapter 2 section 2.2.11. The kinetic parameters such as specific growth rate (μ), total biomass (X), biomass yield coefficient ($Y_{X/Suc}$) and dextran yield coefficient ($Y_{P/Suc}$) were calculated using the data from batch fermentation (Santos *et al*., 2005).

5.2.4.3 Bacterial growth measurement

Samples (2 ml) lifted at every 2h were centrifuged at 13,000 *g* for 10 min and cell pellet obtained was re-suspended in sterile culture medium (2 ml). Bacterial growth was analyzed by measuring cell OD at 600 nm. Cell dry weight was obtained by drying specific dilutions of re-suspended cell pellet using hot air oven (60°C) for 24h. The dried cell pellets were weighed and calibration curve of dry weight versus OD. (600 nm) was used to determine biomass concentration (g biomass/l) (Santos *et al*., 2005).

5.2.4.4 Specific growth rate (μ)

The specific growth rate is defined as the increase in cell mass per unit time, e.g., grams cells (g) per gram cells (g) per hour. The specific growth rate is commonly given by the symbol, μ, and the most common units are in reciprocal hours (h^{-1}). The growth rate of a microbial population is a measure of the increase in biomass over time and it is determined from the exponential phase. The specific growth rate was calculated by the ratio between the log of difference between the biomass at the start and end of the log phase to the change in time at the start and end of the log phase according to equation (1)

$$\mu = \frac{\ln X_2 - \ln X_1}{t_2 - t_1} \quad \ldots (1)$$

Where,
μ = Specific growth rate
X_2 = Biomass at end of the log phase
X_1 = Biomass at start of the log phase
t_2 = Time at end of the log phase
t_1 = Time at start of the log phase

5.2.4.5 Biomass yield ($Y_{X/Suc}$)

The biomass yield is defined as the gram of cells produced per gram of sucrose (substrate). The biomass yield was calculated with relation to the amount of sucrose used for cell growth and dextran production *i.e.* the initial sucrose concentration (20 mg/ml) minus the sucrose consumed at time *t* according to the equation (1). The maximum attained biomass yield was listed in Table 5.3.1.

$$Y_{X/Suc} = \frac{\Delta X}{\Delta S} \quad \ldots (1)$$

Where,

$Y_{X/Suc}$ = Biomass yield (gg^{-1})
ΔX = variation in the biomass ($X_t - X_0$)
ΔS = Sucrose consumption (S_0 - S_t)
t = 14 h.

5.2.4.6 Dextran yield ($Y_{P/Suc}$)

The dextran yield is defined as the grams of dextran produced per gram of sucrose (substrate). The dextran yield was calculated with relation to the amount of sucrose used for cell growth and dextran production *i.e.* the initial sucrose concentration (20 mg/ml) minus the sucrose consumed at time *t* according to the equation (2). However, the maximum attained dextran yield was listed in Table 5.3.1.

$$Y_{P/Suc} = \frac{\Delta P}{\Delta S} \quad \ldots (1)$$

Where,

$Y_{P/Suc}$ = Biomass yield (gg^{-1})
ΔP = variation in dextran concentration ($P_t - P_0$)
ΔS = Sucrose consumption (S_0 - S_t)
t = 14 h.

5.2.5 Fed-batch fermentation for dextran production from *Weissella cibaria*

The production of dextran from *Weissella cibaria* RBA12 was carried out by fed-batch fermentation (2.5 L working volume) in a bioreactor. The operation parameters and fermentation medium for fed-batch fermentation were kept same as in the batch fermentation as mentioned in the section 5.2.4.1. Temperature was controlled at 20°C and the and aeration rate was kept 2 $vv^{-1}min^{-1}$ with an initial agitation rate of 200 rpm, which was later increased. A constant feed rate of the sucrose was employed for the fed-batch mode. The constant feed rate was calculated using the following formula

$$\mathbf{F} = \frac{\boldsymbol{\mu} \times \mathbf{X} \times \mathbf{V}}{\mathbf{Y}(\boldsymbol{x}/\boldsymbol{s}) \times \mathbf{S}}$$

Where,

F = Feed rate (l/h) for fed-batch phase
μ = Specific growth rate (h^{-1}) in batch phase
X = Total Biomass (g/l) at the end of batch phase
V = Volume at the end of batch phase (l)
$Y_{(X/S)}$ = Biomass yield coefficient during batch phase
S = Feed concentration (g/l)

The volume at the end of batch phase was 2.7 L and the feed concentration employed was 500 g/L (mg/ml/ 50%, w/v) of sucrose. The feed rate (ml/h) for fed-batch phase was calculated to be 20 ml/h. The constant feed of sucrose was started at 10h of batch phase when the sucrose concentration was approximately, 5 mg/ml. The parameters like dextran concentration, sucrose concentration and cell optical density were analyzed at every 2h interval. The sucrose concentration was determined by estimating the reducing sugar by DNS method (Miller, 1959).

5.3 Results Discussion

5.3.1 Effect of temperature and orbital shaking speed on dextran production

The optimum temperature for dextran production from *Weissella cibaria* RBA12 was found to be at 20°C (Fig.5.3.1). It produced 8.9 mg/ml of dextran when incubated at 20°C which came down to 7.8 mg/ml dextran at 24°C. The cell growth of *Weissella cibaria* RBA12 was observed to be highest at 20°C with 2.7 g/l of cells (Fig.5.3.1). This can be attributed to increased growth rate at higher temperature leading to depletion of sugar in the medium and the bacterium starts utilizing the dextran as a secondary carbon source. Similar decrease in dextran concentration was also observed during the fermentation profile analysis when sucrose was completely exhausted after 24h of incubation at 20°C and 180 rpm (Baruah & Goyal, 2015). Optimum orbital shaking speed for dextran production from *Weissella cibaria* RBA12 was 180 rpm (Fig. 5.3.2). It produced 8.9 mg/ml dextran as compared to 4.6 mg/ml at 80 rpm which might have been caused by the creation of microaerophilic conditions which is suitable for lactic acid bacteria such as *Weissella cibaria* RBA12 as observed by the increase in cell growth with the increase in orbital shaking speed (Fig. 5.3.2).

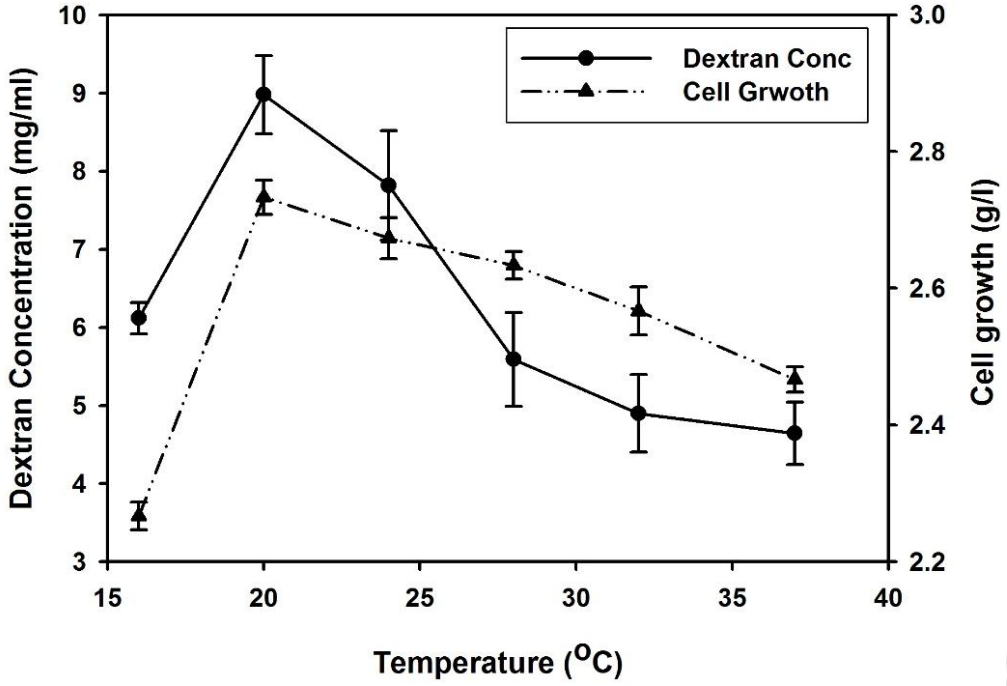

Fig. 5.3.1. Effect of temperature on the production of dextran and cell growth of *Weissella cibaria* RBA12.

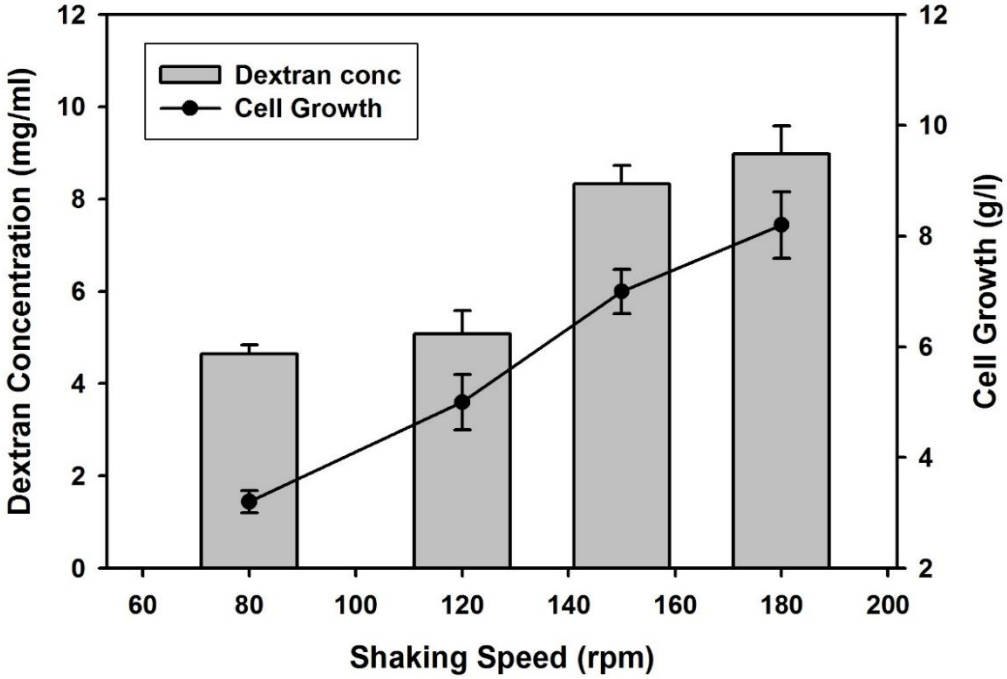

Fig. 5.3.2. Effect of shaking speed on the production of dextran and cell growth of *Weissella cibaria* RBA12.

5.3.2 Effect of sucrose concentration on dextran production

The optimum sucrose concentration for dextran production by *Weissella cibaria* RBA12 was 2% (w/v) producing 8.9 mg/ml dextran concentration amounting to 89% efficiency of conversion (Fig. 5.3.3). After 24h, at 10 and 15% (w/v) sucrose, dextran concentrations were 36.6 and 50.8 mg/ml, which made these samples highly viscous. Higher sucrose concentration resulted in markedly lower conversion efficiency at 73 and 67%, respectively as compared to 87% at 2% (w/v) sucrose (Fig. 5.3.3). This decrease in the efficiency was essentially the consequence of increase in viscosity of the fermenting medium caused by the higher dextran production (Santos *et al*., 2005). Therefore, subsequent experiments were carried out using 2% (w/v) sucrose.

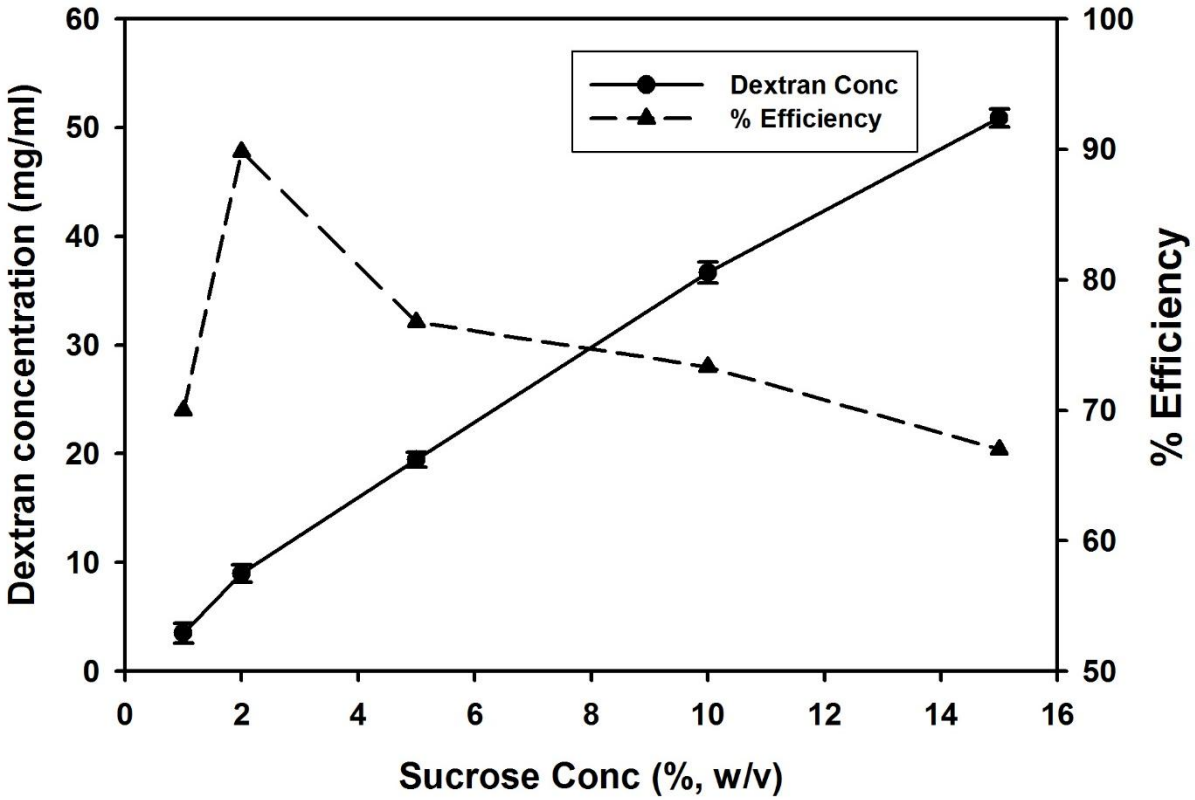

Fig. 5.3.3 Effect of sucrose concentration on dextran yield (-●-) and efficiency of dextran production (-▲-).

5.3.3 Effect of other medium nutrients on dextran production

Maximum production of 8.7 mg/ml dextran by *Weissella cibaria* RBA12 was found to be at 2% (w/v) of yeast extract (Fig. 5.3.4). Increase in yeast extract as nitrogen source from 2 to 4% (w/v) reduced the dextran production from 8.7 to 5.6 mg/ml dextran, respectively. Similar effect was also observed in case of dextran production by *Weissella confusa* Cab3 (Shukla and Goyal, 2011). At 2% (w/v), K_2HPO_4 supported maximum production of dextran (8.8 mg/ml) by *Weissella cibaria* RBA12. Further increase in K_2HPO_4 from 2 to 3.5% (w/v) decreased dextran from 8.8 to 4.2 mg/ml. K_2HPO_4 is an important phosphate source and buffering agent which helps in the growth of the bacterium and the production of dextran. Similar results from *Weissella confusa* Cab3 on dextran production by K_2HPO_4 was reported (Shukla and Goyal, 2011).

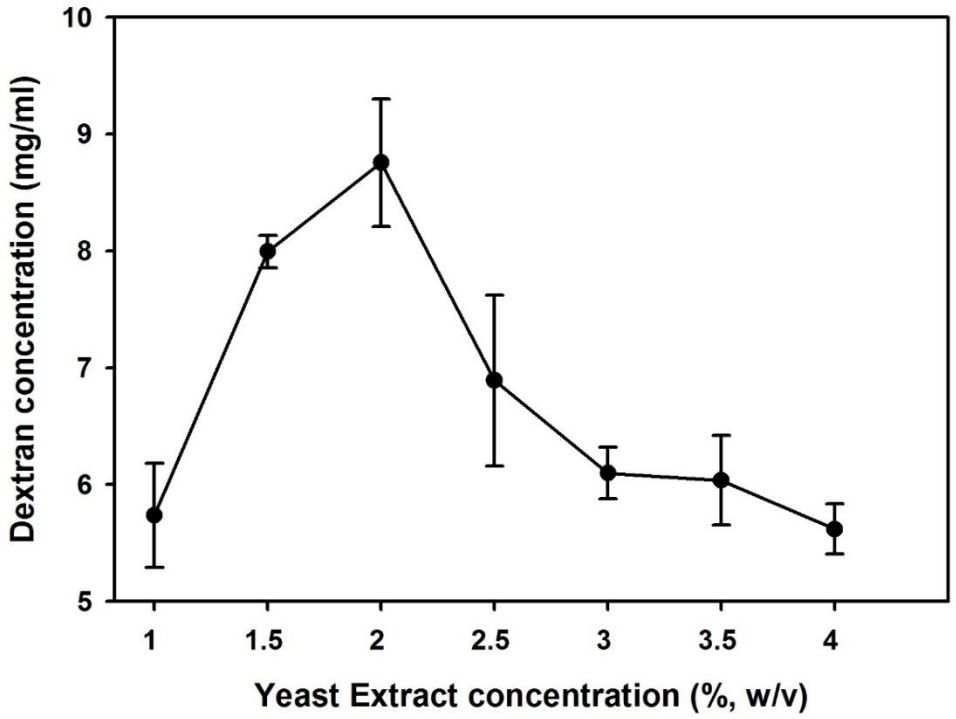

Fig. 5.3.4 Effect of nitrogen source on the production of dextran from *Weissella cibaria* RBA12.

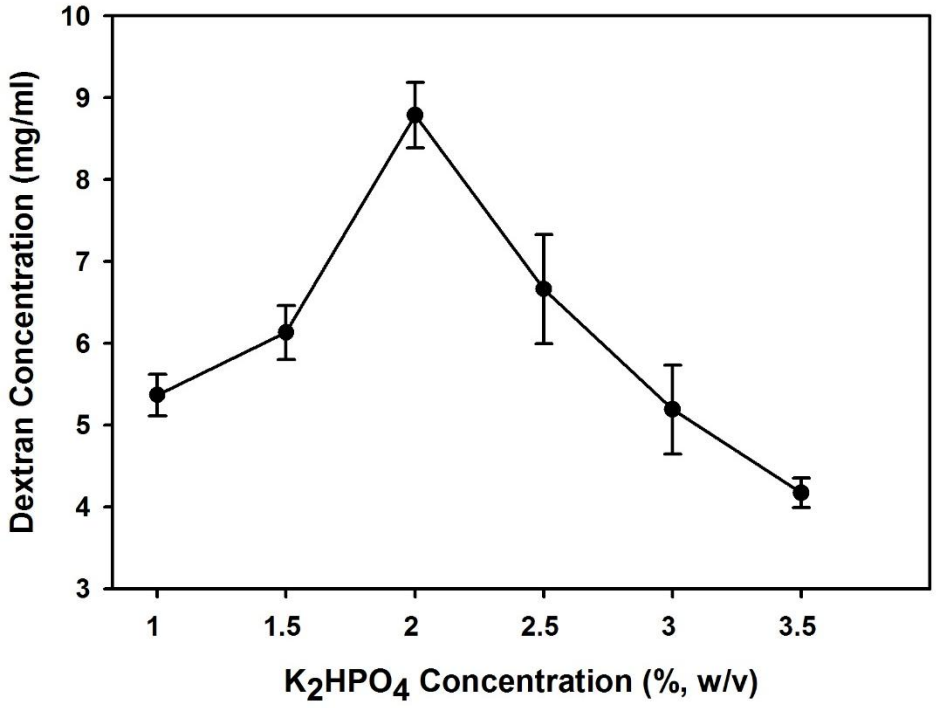

Fig. 5.3.5 Effect of K_2HPO_4 on the production of dextran from *Weissella cibaria* RBA12.

5.3.4 Determination of bacterial growth

The standard plot for the determination of bacterial growth was plotted between cell dry weight and OD at 600nm (Fig. 5.3.6). The OD was found to increase with the increase in the dry weight of the cells. Based on the calibration curve (Fig. 5.3.6) an equation was developed.

$$\mathbf{y = 3.093x,\ R^2 = 0.993,}$$

which was used in subsequent experiments to arrive at the x variable for calculating the cell dry weight at 2h intervals.

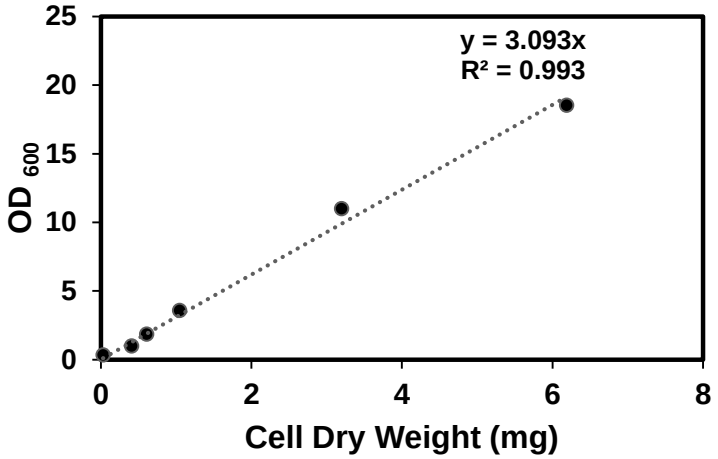

Fig. 5.3.5 Standard plot for the determination of bacterial growth was plotted between cell dry weight and OD at 600 nm.

5.3.5 Batch fermentation for dextran production from *Weissella cibaria* RBA12

Production of dextran from *Weissella cibaria* RBA12 by batch fermentation using 2.5 L working volume (Fig. 5.3.6) in a bioreactor reached 9.32 mg/ml after 14h attaining an efficiency of 93% (Fig. 5.3.7). The dextran levels decreased to 2 mg/ml after 24h, which was essentially because of decreased level of sucrose caused by rapid utilization by increased bacterial cell population, which in turn started utilizing dextran as carbon source. The concentration was further supported by the initiation of stationary phase in the growth after 14h.

Fig. 5.3.6 Dextran production from *Weissella cibaria* RBA12 in 5L bioreactor

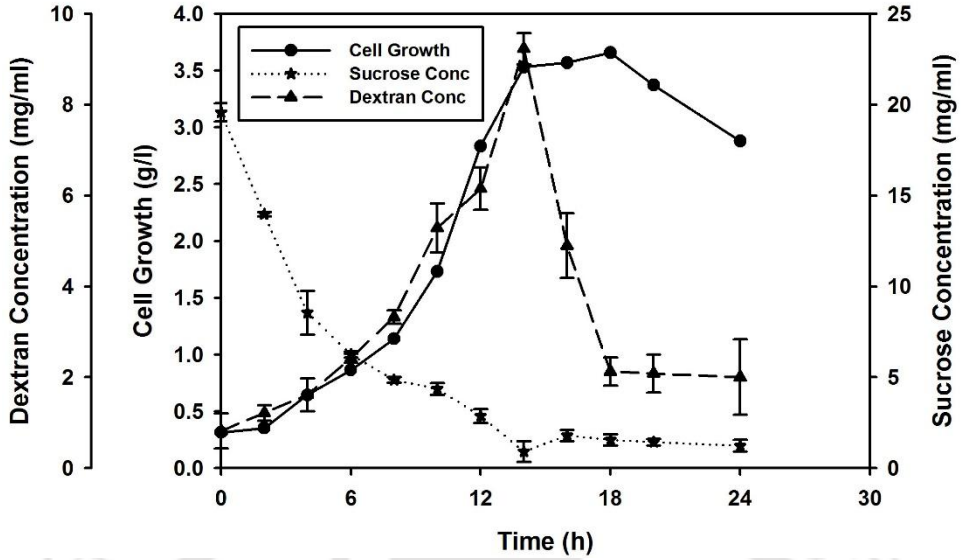

Fig. 5.3.7 Scale up of dextran production from *Weissella cibaria* RBA12 under batch fermentation in bioreactor.

5.3.6 Kinetics of dextran production from *Weissella cibaria* RBA12 under batch fermentation

The kinetic parameters for dextran production by *Weissella cibaria* RBA12 were determined for batch fermentation in a bioreactor at 20°C and pH 6.9. The total cell biomass produced was 3.53 g/l and the specific growth rate (μ) was 0.19 h^{-1} (Table 5.3.1). The cell biomass yield coefficient ($Y_{X/Suc}$) was 0.18 g/g of sucrose (Table 5.3.1). The dextran yield coefficient ($Y_{P/Suc}$) was 0.49 g/g of sucrose (Table 5.3.1), which was high and can be correlated with the low biomass yield ($Y_{X/Suc}$) due to the efficient utilization of sucrose for dextran formation. Earlier also a specific growth rate of 0.93 h^{-1} at 36.3°C and pH 6.6 has been reported in *Weissella cibaria* DBPZ1006 (Ricciardi *et al*., 2009).

Table 5.3.1. Kinetic parameters for the dextran production from *Weissella cibaria* RBA12.

Parameters	Calculated value
Total cell Biomass	3.53 g/l
Specific growth rate (μ)	0.19 h^{-1}
Biomass Yield coefficient ($Y_{X/Suc}$)	0.18 g/g of sucrose
Product Yield coefficient ($Y_{P/Suc}$)	0.49 g/g of sucrose

5.3.7 Fed-batch fermentation for production of dextran from *Weissella cibaria* RBA12

The production of dextran from *Weissella cibaria* RBA12 under fed-batch fermentation in a bioreactor was found to be 35.8 mg/ml after 32h (Fig. 5.3.8), which gradually increased throughout the fermentation, unlike the decrease observed in batch fermentation after 14h (Fig. 5.3.8). After the start of the feed (500 g/L/ 50%, w/v) at 10h, the sucrose concentration gradually increased. There was a sharp increase in sucrose concentration after 24h, which could be due to decrease in the bacterial growth after 24h. Maximum cell biomass was 8.4 g/l at 24h. The fed-batch mode for production of dextran from *Weissella cibaria* RBA12 proved to be better than the batch fermentation with 4.0-fold increase in dextran concentration from 9.32 mg/ml in batch mode to 35.8 mg/ml in fed-batch mode. The fed-batch mode further scored over the batch mode as there was no decrease in dextran concentration. Therefore, fed-batch fermentation can be employed for obtaining high dextran yields overcoming the possibility of dextran utilization.

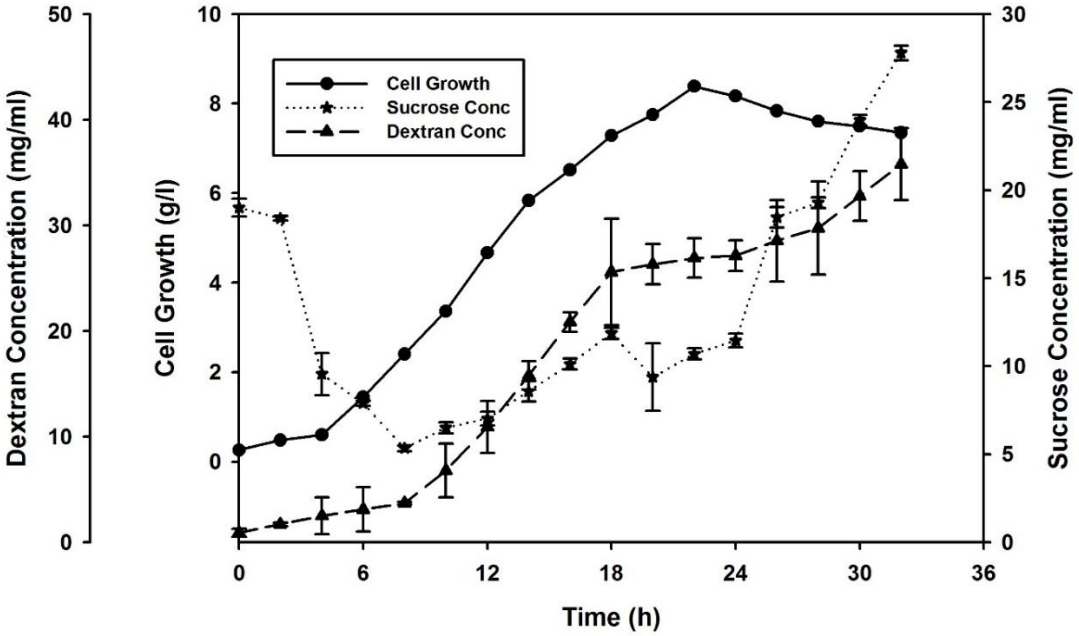

Fig. 5.3.8 Dextran production from *Weissella cibaria* RBA12 by fed-batch fermentation in bioreactor.

5.4 Conclusion

Maximum dextran yield by *Weissella cibaria* RBA12 was obtained after 24h at 2% (w/v) sucrose, 2% (w/v) yeast extract and 2% (w/v) K_2HPO_4 and at growth conditions of 20°C and 180 rpm. In shake flask studies the dextran production was 8.9 mg/ml with 89% efficiency in the presence of 2% (w/v) sucrose, yeast extract and K_2HPO_4. When this optimized medium was used for scale up in 2.5 L volume in a bioreactor, it yielded 9.32 mg/ml dextran with an 93% efficiency. The dextran formed in batch mode was utilized by the bacterium after the depletion of sucrose. In batch mode the specific growth rate (μ) of *Weissella cibaria* RBA12 was 0.19 h^{-1}, biomass yield coefficient ($Y_{X/Suc}$) 0.18 g/g of sucrose and dextran yield coefficient ($Y_{P/Suc}$) as 0.49 g/g of sucrose. In fed-batch mode the maximum dextran concentration was 35.8 mg/ml which was approximately 4-fold higher than that of batch mode (9.32 mg/ml). Owing to the steady feed of sucrose, there was no decrease in dextran concentration. Therefore, the fed-batch mode can be used for large scale production of dextran from *Weissella cibaria* RBA12.

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Chapter 6

Functional food applications of dextransucrase and dextran from *Weissella cibaria* RBA12

6.1 Introduction

Dextransucrase (EC 2.4.1.5) is an extracellular enzyme that synthesizes polysaccharide dextran from sucrose (Leemhuis *et al*., 2013). Dextransucrase belongs to family 70 glucoside hydrolase (GH) that exclusively contains glucansucrase also called glucosyltransferases (Stam *et al*., 2006). Dextransucrase is elaborated by lactic acid bacteria. The lactic acid bacteria of the genera *Lactobacillus*, *Leuconostoc*, *Streptococcus, Pediococcus* and *Weissella* are reported to produce dextransucrase (Leemhuis et al., 2013). Dextran is composed of glucose monomers linked by α-(1→6) bonds and α-(1→2), α-(1→3) and α-(1→4) branched linkages (Bounaix *et al*., 2009). Dextran is used as viscosifying and water binding agents in food and non-food applications (Badel *et al*., 2011). In the presence of exogenous acceptor molecules along with sucrose, dextransucrase transfers the glucosyl moieties (produced by the hydrolysis of sucrose) to the acceptor molecule resulting in the formation of acceptor products (oligosaccharides) in addition to dextran. When maltose acts as an acceptor

molecule, dextransucrase produces a homologous series of isomalto-oligosaccharides (IMOs) composed primarily of consecutive α-(1→6) linkages (Robyt & Eklund, 1983). Dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426 showed the highest affinity towards maltose for the acceptor reaction with 83% efficiency of IMO production (Kothari & Goyal, 2013). IMO are resistant to hydrolytic enzymes human gastrointestinal (GI) tract due to the absence of α-(1→6) linkage degrading enzymes (Kothari & Goyal, 2015). IMO can readily reach the lower GI tract and are used as a substrate for fermentation by colonic microflora to produce beneficial short chain fatty acid (SCFA) (Kothari *et al*., 2014).

Dextransucrase has been used to produce functional foods containing prebiotic oligosaccharides or dextran. The use of dextransucrase enables the *in-situ* production of the desired oligosaccharides and thereby reducing the cost of fortification of the products. Dextransucrase from *W. confusa* VTT E-90392 was used to produce dextran in bran containing wheat bread without any fermentation and the associated acid formation (Kajala *et al*., 2015a). The use of dextransucrase in the preparation prebiotic fruit juices have recently gained popularity for its efficient use of native sucrose to form IMOs and in turn lowering the calorific value of the fruit juices (Fontes *et al*., 2015). Prebiotics have been produced by dextransucrase acceptor reaction and included in fruit juices such as cashew apple juice (da Silva *et al*., 2012), apple and orange juices (Johansson *et al*., 2016), mandarin juice (Nguyen *et al*., 2015) and pineapple, cantaloupe melon and orange juices (Fontes *et al*., 2015).

Dietary fibres are the oligosaccharides, polysaccharides and their derivatives which cannot be digested by the human digestive enzymes into absorbable components in the upper alimentary tract (Thebaudin *et al*., 1997). Dietary fibres are a necessary

part of human nutrition with various benefits to health which includes a reduction in bowel transit time, reduction in risk of colorectal cancer, prevention of constipation, production of short chain fatty acids lowering of blood cholesterol and promotion of the growth of beneficial gut microbiota (Brennan & Cleary 2005). Probiotics are defined as a live microbial feed supplement which confers various beneficial affects to its host by improving the intestinal environment and native microbiota (Das & Goyal 2014). Prebiotics are the compounds which result in the selectively stimulate the growth and/or activity(ies) of probiotic microorganisms that confers health benefits to its host. Large polysaccharides such as dextran form a persistent source of fermentable carbohydrate throughout the colon for the resident microbiota rather than being completely fermented proximally. It was also reported that dextran and oligo-dextran which contain of α-(1→6) glycosidic backbones are less susceptible to attack by human or animal digestive enzymes (Kothari *et al*., 2015). Certain marine lactic acid bacteria, *Pediococcus pentosaceus, Weissella confusa, Weissella cibaria* and *Lactobacillus plantarum* producing dextran showed prebiotic properties (Hongpattarakere *et al*., 2012). These dextrans being resistant to hydrolysis by physiological barriers such as stomach acid and human pancreatic amylase are also utilized as a carbon source by probiotic bacteria *Bifidobacterium bifidum* (Hongpattarakere *et al*., 2012).

Sourdough is a pre-dough produced by the fermenting micro-organisms such as lactic acid bacteria (LAB) and yeasts. Dextran from several microbial sources have been used in sourdough baking (Kajala *et al*., 2015a). A low degree of branching and a high molecular weight are the key features of dextrans for the good impact on sourdough bread quality (Lacaze *et al*., 2007). Dextran present in liquid sourdough has been reported to improve the volume (up to 12%), mouthfeel and softness of rye breads

and rye mixed breads (Lacaze *et al*., 2007). In wheat breads, the presence of dextrans showed improvement in mouthfeel and freshness after storage and it also increased the bread volume as compared with the sourdough breads without dextran (Kajala *et al*. 2015b).

In the present study dextransucrase from *Weissella cibaria* RBA12 was used for the synthesis of IMOs using dextransucrase acceptor reaction *in vivo* and *in vitro* and the IMOs were purified by gel filtration. The prebiotic IMOs and di-saccharides were produced by dextransucrase acceptor reaction using mango and pineapple juices utilizing their native sugars which acted as acceptors. The *in vitro* studies using simulated gastric juices, α-amylase and intestinal fluid were carried out on dextran for characterizing its prebiotic potential. The *in situ* production of dextran by *Weissella cibaria* RBA12 in whole wheat flour, wheat bran and rye bran was carried out in order to assess its application in the sourdough fermentation.

6.2 Materials and Methods

6.2.1 Chemicals and reagents

The medium components for Tryptone-Glucose-Yeast extract (TGY) medium and Man Rogosa Sharpe (MRS) medium, the chemicals for carbohydrate estimation, reducing sugar assay, sucrose and standard prebiotic inulin were purchased from Hi-Media Pvt. Ltd (Mumbai, India). Sugar standards isomaltose, leucrose, sucrose and maltose were obtained from Sigma–Aldrich, (USA). The bile salts, pepsin and trypsin used in prebiotic assays were also obtained from Sigma–Aldrich, (USA). The separating matrix, Bio-Gel P2 was obtained from Bio-Rad Laboratories Pvt. Ltd (CA, USA).

6.2.2 Microorganisms

Weissella cibaria RBA12 (GenBank accession no: KF515952) producing dextransucrase and dextran isolated from the pulp of Pummelo (*Citrus maxima*) was used in this study (Baruah & Goyal 2015). Dextransucrase or dextran was produced by inoculating *W. cibaria* RBA12 in 100 ml of enzyme production medium (Tsuchiya *et al*., 1952) and was purified according the methods described for dextransucrase in section 3.2.6 and for dextran in section 5.2.4. Probiotic strain *Lactobacillus plantarum* DM5 (Genbank Accession Number KC020195) previously isolated (Das and Goyal, 2013) was available as glycerol stock in our laboratory was used. The other probiotic bacteria *Lactobacillus acidophilus* NRRL B-4495, *Bifidobacterium infantis* NRRL B-41661, *Bifidobacterium bifidum* NRRL 41410 and *Bifidobacterium animalis subsp. lactis* NRRL 41405 were procured from Agricultural Research Service Culture Collection (Peoria, USA). The enteric strain *Enterobacter aerogenes* MTCC 7016 was

procured from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India. *E. coli* DH5α were available as glycerol stock in our laboratory.

6.2.3 *In situ* production of IMOs using acceptor in the culture medium

Enzyme production medium (Tsuchiya *et al*., 1952) supplemented with 2 % (w/v) maltose was inoculated with 1% (v/v) of *Weissella cibaria* RBA12 and incubated at 20°C and 180 rpm for 24 h for IMO production. Samples (200 µl) were taken every 3 h up to 24 h from the broth and cell free supernatant were obtained by centrifugation at 10,000 rpm and 4ºC for 10 mins. The IMOs were purified from cell free supernatant by adding two volumes of absolute alcohol and centrifuged at 16000 *g* and 4ºC for 10 min to precipitate the polysaccharides (Côté and Leathers 2005). The 0.5 µl of supernatant containing oligosaccharides was analyzed by TLC at 25ºC using a silica gel 60 F_{254} TLC plate (Merck, Germany). The solvent system used was ethyl acetate/1-propanol/acetonitrile/water (4:10:14:11), by volume. The plate was visualized by spraying ethanol containing 0.5 % (w/v) α-naphthol and 5 % (v/v) H_2SO_4 and after heating at 120ºC (Kothari *et al*., 2012). The degree of polymerization (DP) of oligosaccharides was confirmed by ESI-TOF mass spectrometry analysis (Agilent, USA).

6.2.4 *In vitro* production and characterization of IMOs

6.2.4.1 Production of IMO using dextransucrase acceptor reaction

IMOs were produced using dextansucrase catalyzed acceptor reaction using maltose as an acceptor molecule (Kothari & Goyal, 2013). The 2 ml reaction mixture contained 200 µl of purified dextransucrase (10.5 U/mg, 0.2 mg protein/ml) from

Weissella cibaria RBA12, 5% (w/v) sucrose and 5% (w/v) maltose dissolved in 20 mM sodium acetate buffer, pH 5.4 and incubated at 30°C for 24h. The reaction mixture was also supplemented with 0.3 mM $CaCl_2$ and 15 mM NaN_3. After 24h, the reaction mixture was treated with 2 volumes of absolute ethanol and then the mixture was centrifuged at 16000 *g* for 10 min to precipitate the dextran formed. The supernatant (0.5 µl) containing IMOs was analyzed by thin layer chromatography (TLC) using silica gel 60 F254 TLC plate (Merck) with a solvent system, ethyl acetate/1-propanol/acetonitrile/water (4:10:14:11), by volume. The TLC plate was visualized by spraying ethanol containing 0.5% (w/v) α-naphthol and 5% (v/v) H_2SO_4 and after heating it at 120°C (Baruah and Goyal 2015). The IMOs were further purified by gel permeation chromatography (GPC) using XK16/70 column (GE Healthcare) packed with Bio-Gel P2 matrix (Bio-Rad, CA, USA) connected to FPLC (AKTA, GE Healthcare, USA). Milli-Q water (18.2 MΩ cm) was used for pre-equilibration of column and elution of IMOs, The flow rate of 0.2 ml/min was kept and 1 ml fractions were collected. The purified fractions were analyzed for IMO concentration by phenol sulphuric acid method (DuBois *et al*., 1956) as described in Chapter 2, Section 2.2.12 and identified by TLC as mentioned in section 6.2.3 and confirmed by ESI-TOF MS as mentioned in section 6.2.4.2.

6.2.4.2 Analysis of purified IMO using ESI-TOF MS and HPLC

The degree of polymerization (DP) of IMOs produced in purified fractions (67, 70 and 77) were analyzed by ESI-TOF MS in positive mode using an Agilent 6520 Accurate mass Q-TOF LC/MS system (Agilent Technologies, USA). Mass spectrum was recorded using a scan range of 100-1000 m/z per 1 min run and processed using Agilent Mass Hunter workstation software. The purified fractions were used as

standards for the quantification of IMO produced in other and fruit juice reactions. The purified fractions of IMO (single DP) and IMO mixture were analyzed by HPLC (UFLC, Prominence, Shimadzu, Japan) equipped with ion exclusion column Phenomenex Rezex RSO-Oligosaccharide (Phenomenex, CA, USA). The IMOs were analyzed using RI detector with an injection volume of 10 µl and degassed Milli-Q water (18.2 MΩ cm) as the eluent at a flow rate of 0.2 ml/min. The IMO fractions were run as standard in the range, 0.5 to 1.5 mg/ml (DP3), 0.2 to 1 mg/ml (DP4) and 0.1 to 0.5 mg/ml (DP5) for the quantification in subsequent applications.

6.2.5 Production of IMO using sucrose in fruit juices

Mango and pineapple juice concentrate of 200 ml packs (Real, Dabur India Pvt. Ltd.) were purchased from the local market and its pH and free sugars contents were determined. The free sugars present were quantified by HPLC equipped with Phenomenex Rezex ROA-Organic Acid (Phenomenex, CA, USA) and RI detector. Sucrose, glucose and fructose (Sigma Aldrich, USA) were used as standards. The production of IMO in mango and pineapple juice concentrates was carried out in 2.2 ml reaction mixture containing 200 µl of purified dextransucrase from *Weissella cibaria* RBA12 (10.5 U/mg, 0.2 mg protein/ml) and 2 ml of juice concentrate. The pH was adjusted to 5.4 using sterile NaOH and supplemented with 0.3 mM $CaCl_2$ and 15 mM NaN_3 and incubated for 24h at 30°C. The oligosaccharides produced were purified as mentioned in section 6.2.4.1 and quantified as mentioned in section 6.2.4.2.

6.2.6 Effect of dextran-RBA12 on the growth of probiotic bacteria

The growth profiles of probiotic bacteria (*L. plantarum* DM5, *L. acidophilus* NRRL B-4495, *B. infantis* NRRL B-41661, *B. bifidum*NRRL 41410 and *B. animalis*

NRRL 41405) along with non-probiotic enteric bacteria (*E. coli* DH5α and *E. aerogenes* MTCC7016) were studied in the presence of dextran-RBA12 and the standard inulin as prebiotic. The growth profiles of probiotic bacteria were studied by using modified-MRS medium, pH 6.4 (devoid of glucose or any carbon source), but supplemented with 0.5 mg/ml cysteine (Hongpattarakere *et al*., 2012). The probiotic bacterial cultures (~10^6 CFU/ml) were transferred to 5 ml MRS medium containing 1% (w/v) of glucose, dextran-RBA12 or standard prebiotic inulin and incubated anaerobically under static condition at 37°C for 24 h. The growth of enteric bacteria was evaluated in TGY medium (pH 7.0) containing tryptone (5.0 g/L), glucose (1.0 g/L), yeast extract (5 g/L) and di-potassium hydrogen phosphate K_2HPO_4 (1 g/L). The enteric mixture of non-probiotic *E. coli* DH5α and *E. aerogenes* MTCC 7016 was transferred to 5 ml of TGY medium supplemented with 1% (w/v) of glucose, dextran-RBA12 or standard prebiotic inulin and incubated under anaerobic conditions at 37°C for 24 h. The microbial growth of probiotic bacteria and enteric bacteria was enumerated by plate count method. MRS agar and TGY agar plate were used for the enumeration of probiotic bacteria and enteric bacteria, respectively. 20 µl of 10^{-7}, 10^{-8} and 10^{-9} dilutions were plated at 37°C for 12 h and 24 h. The growing colonies were counted and cell count were expressed as $\log_{10}$ cfu/ml. The prebiotic activity score of dextran-RBA12 and inulin was estimated by the method of Huebner *et al*., (2007) as shown below.

$$\text{Prebiotic activity score} = \left\{\frac{(\text{Probiotic log CFU } ml^{-1} \text{ on prebiotic at 24h}) - (\text{Probiotic log CFU } ml^{-1} \text{ on prebiotic at 0h})}{(\text{Probiotic log CFU } ml^{-1} \text{ on glucose at 24h}) - (\text{Probiotic log CFU } ml^{-1} \text{ on glucose at 0h})}\right\} - \left\{\frac{(\text{Enteric log CFU } ml^{-1} \text{ on prebiotic at 24h}) - (\text{Enteric log CFU } ml^{-1} \text{ on prebiotic at 0h})}{(\text{Enteric log CFU } ml^{-1} \text{on glucose at 24h}) - (\text{Enteric log CFU } ml^{-1} \text{on glucose at 0h})}\right\}$$

6.2.7 Effect of artificial gastric juice on hydrolysis of dextran

Dextran and commercial prebiotic inulin were analyzed for their hydrolysis in the presence of artificial gastric juice by the method as described by Korakli *et al*., (2002). The artificial gastric juice consisted of 1000 U/ml of pepsin in 1x Phosphate Buffer Saline (PBS) (Al-sheraji *et al*., 2012). The pH of artificial gastric juice was adjusted to 1, 2, 3 and 4 by 4 N HCl. The artificial gastric juice (10 ml) of each pH containing dextran or inulin were incubated at 37°C for 5 h. An aliquot of 500 µl from the reaction_mixture was collected at intervals of 0, 0.5, 1, 2, 3, 4 and 5 h. Phenol-sulphuric acid method (Dubois *et al*., 1956) was used for determining the total sugar content, using glucose as standard. The reducing sugar present in the sample was determined by the methods of Nelson (1944) and Somogyi (1945). The percent hydrolysis of the sample was estimated by taking ratio of the reducing sugar and total sugar content liberated from the samples (Korakli *et al*., 2002) as follows;

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

6.2.8 Hydrolysis of dextran by α-amylase and intestinal fluid

The hydrolysis of dextran and inulin by α-amylase was analysed by the method as described by Al-sheraji *et al*., (2012). Dextran and inulin (100 mg) were separately dissolved in 10 ml of α-amylase (100 U/ml) in 1x PBS buffer of pH in a range of pH 5, 6, 7 and 8 and incubated at 37°C for 5 h. The reducing sugar and total sugar content of the sample was analyzed at time intervals of 0, 0.5, 1, 2, 3, 4 and 5 h for calculating the percentage hydrolysis of dextran-RBA12 and inulin as described earlier in section 6.2.6. The intestinal fluid was composed of 0.5% (w/v) bile salt and 1000 U/ml of

trypsin solution in 1x PBS buffer at pH 8.0 (Fernandez *et al*., 2003). The 1.0 % (w/v) of each dextran and inulin were separately dissolved in 10 ml intestinal fluid and incubated at 37°C for 5 h. The reducing sugar and total sugar content of the sample was analyzed at time intervals of 0, 0.5, 1, 2, 3, 4 and 5 h and the percentage hydrolysis of dextran and inulin were calculated as described earlier in section 6.2.7.

6.2.9 *In vitro* dextran production in sourdough fermentation using *Weissella cibaria* RBA12

6.2.9.1 Sourdough fermentations

Weissella cibaria RBA12 was sub-cultured twice under static condition at 28°C for 24h in MRS-S medium (containing sucrose instead of glucose) (Goyal *et al*., 1995) before sourdough fermentations (Kajala *et al*., 2015a). Cells were harvested from 5 ml overnight culture by centrifugation at 10,000***g*** at 4°C for 10 min and re-suspended in sterile tap water. The stock for inoculation consisted of cell density of 10^9 cfu/ml.

Sourdough fermentations were carried out using whole wheat flour (Mylly-Matti, Helsingin Mylly, Finland), wheat bran and rye bran (Fazer, Finland) as cereal substrates. All the fermentations were carried out with 10% (w/w) sucrose supplementation using a total weight of 200 g. The samples for fermentation consisted of 160 g of sterile tap water, mixed with 0.2 ml of cell suspension ($5x10^5 – 1x10^6$ cfu/g) and 36 g of cereal substrate, a part of the matrix was replaced with the 4g of sucrose. The cells were mixed with the water and sucrose along with cereal matrix and incubated at 25°C for 20h (Katina *et al*., 2009). Microbial enumeration of the samples was carried out at 0 and 20 h using MRS agar plates. In addition, samples for pH, dextran and free sugar quantification were collected at 20 h. The samples were flash-frozen using liquid

nitrogen and later stored at -20°C. The samples were lyophilized for dextran and sugar analysis before analysis.

6.2.9.2 Enzyme-assisted method for dextran analysis of sourdough

Dextran produced in the bran fermentations was measured by the method described by Katina *et al*., (2009). Dextran was precipitated by ethanol precipitation using 100 mg of sample and centrifuged at 10000***g*** for 10 min at 4°C. The dextran formed was hydrolyzed to glucose monomers by enzyme hydrolysis using the combined activities of dextranase (25 µl, 60 U/mg from Sigma-Aldrich, St. Louis, MO, USA) and transglucosidase (30 µl, 6 U/mg from Megazyme, Ireland) incubated in 5 ml reaction mixture in sodium citrate buffer, pH 5.5 at 30°C for 48h. The glucose produced was quantified using with high performance anion exchange chromatography coupled with a pulsed amperometric detector (HPAEC-PAD) as described in Chapter 4, Section 4.2.5.1. The samples were filtered through a 0.45 mm membrane and the sample injection volume was 10 µl. The sum of anhydro-glucose was used to estimate the amount of dextran, which was corrected by a recovery factor of 1.45 taking into account the recovered dextran after purification from the cereal matrix. The recovery factor (1.45) was determined the method as described by Katina *et al*., (2009), using purified *W. confusa* VTT E90392 dextran and the cereal matrix used in this study.

Dextran (% d w) was calculated according to the equation of Katina *et al*., (2009).

$$(\%\, d.w) = \frac{Concentration\ of\ glucose\ \ (mg/ml)\ \times 5\ (ml)\ \times 0.9\ \times 1.45}{100\ mg} \times 100$$

5 ml is the total volume of the reaction mixture, 100 in denominator is the 100mg of sample used and the other 100 is for obtaining the % D.W. Glucose (mg/ml)

produced by hydrolysis of dextran was analyzed by HPAEC-PAD. In the formula, 0.9 is a correction factor for anhydro-glucose and 1.45 is a correction factor determined for the recovery of dextrans studied in cereal matrices (Katina *et al*., 2009).

6.2.9.3 HPAEC-PAD analysis of sugars from sourdough

Lyophilized sourdough samples (100 mg) dissolved in 5 ml of 0.05 M sodium citrate buffer, pH 5.5 was used for the quantification of free sugars (glucose, fructose, sucrose and maltose). The sourdough samples were vortexed and centrifuged at 10000***g*** at 25°C for 15 min and the supernatants were collected and boiled in a boiling water bath for 10 min (Kajala *et al*., 2015b). After cooling, 500 µl of the sample was transferred to centrifugal filter units (Amicon Ultra 0.5 ml, 10 K Merck, MA, USA) and centrifuged at 10000***g*** for 20 min. 400 µl of the filtrate was collected and diluted with Milli-Q water to a final volume of 1 ml. The sugar analyses were carried out using a solution of 100 mM NaOH and 1M sodium acetate gradient at a flow rate of 1 ml/min as described in Section 6.2.9.2. Deoxygalactose (Sigma-Aldrich St. Louis, MO, USA) was used as an internal standard at a concentration of 50 µg/ml. For the standards and sourdough samples were measured by HPAEC-PAD (Juvonen *et al*., 2015). The samples were filtered through a 0.45 mm membrane and the sample injection volume was 10 µl. A standard curve was prepared for quantification of glucose, fructose, maltose and sucrose (Sigma-Aldrich, St. Louis, MO, USA). Glucose and fructose concentration was in the range, 10-200 µg/ml and sucrose and maltose in 10-500 µg/ml. All the samples were analyzed in triplicates.

6.3 Result and Discussion

6.3.1 Production of IMOs using acceptor reaction in medium

The production of IMOs by acceptor reaction was carried out in the culture medium supplemented with maltose incubated at 20°C under shaking at 180 rpm. Time dependent analysis of acceptor reaction products were done at 3, 6, 9, 12 and 24 h of incubation by TLC (Fig. 6.3.1 A, lanes 1-5). There was gradual utilization of maltose and sucrose with time resulting in the formation of the homologous series of isomalto-oligosaccharides (IMO). Maltose was completely utilized and only the presence of IMOs was observed after 24 h (Fig. 6.3.1 A, lanes 5). The first product of the maltose acceptor reaction is panose (6-O-a-glucopyranosylmaltose), a trisaccharide. This trisaccharide further acts as an acceptor that resulted in the generation of a series of isomalto-oligosaccharides with an increasing degree of polymerization linked to the 6-hydroxyl group of the non-reducing end glucose residue of maltose as reported by Kothari *et al*. (2012). The series of IMOs produced in the acceptor reaction were tri-saccharide, tetra-saccharides and penta-saccharides, which were also confirmed by ESI-TOF-MS analysis. The peaks observed at m/z 527, 689, and 851 (as sodium adduct, $[M+Na]^+$) corresponded to tri-, tetra- and penta-saccharides (Fig. 6.3.1 B).

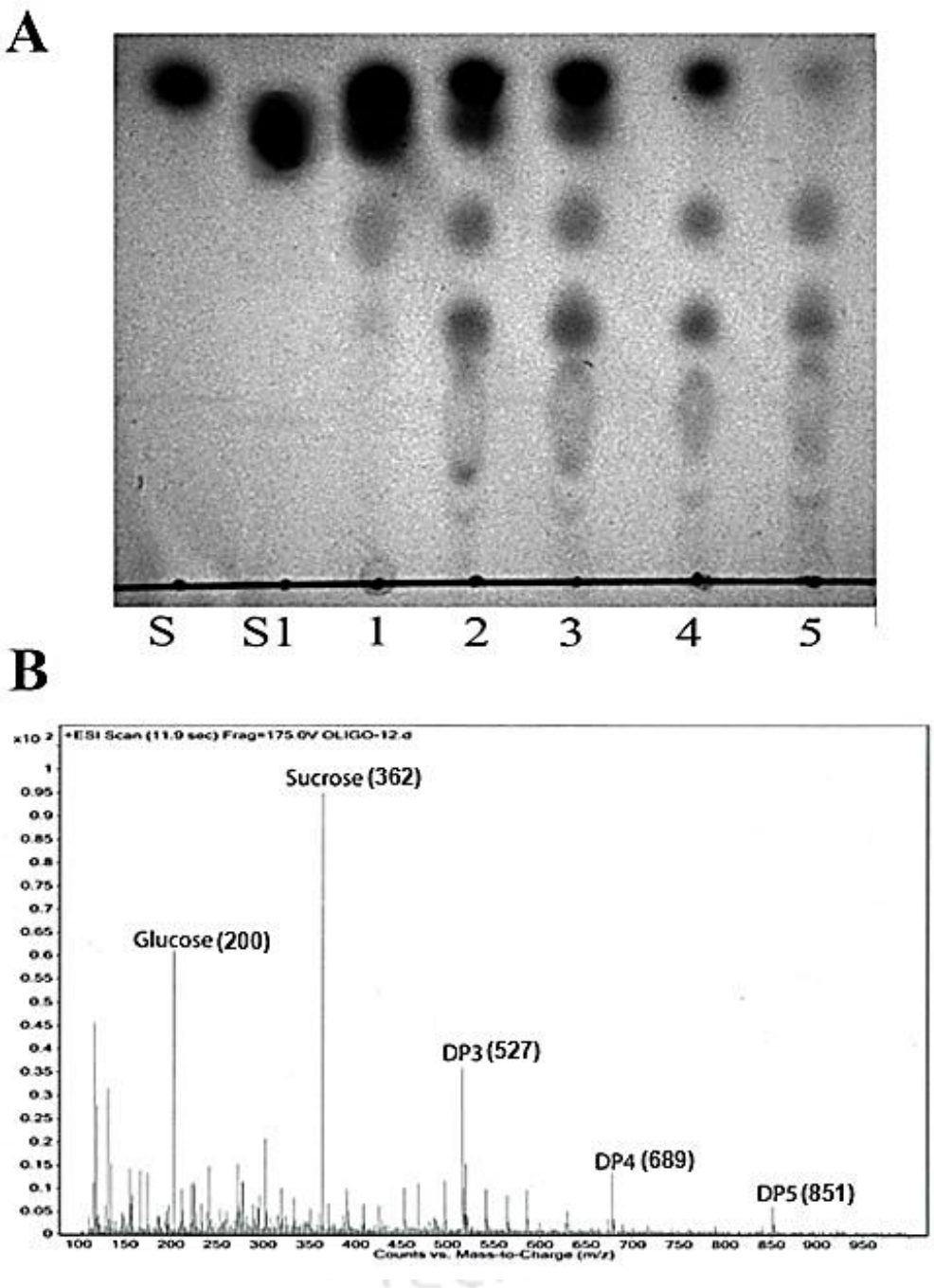

Fig. 6.3.1 A) Thin layer chromatography of time dependent acceptor reaction using fermentation medium containing maltose. Lane S, sucrose; lane S1, maltotriose; lane1, 3 h medium; lane 2, 6 h medium; lane 3, 9 h medium; lane 4, 12 h medium, lane 5, 24 h medium, B) ESI-TOF MS of isomalto-oligosaccharides (IMOs) produced using 12 h fermentation medium in positive ion mode.

6.3.2 *In vitro* production of IMO using dextransucrase acceptor reaction

The TLC analysis of IMO mixture revealed the presence of oligosaccharides from DP3 –DP6 (Fig 6.3.2 A *lane* R). The GPC purified fractions of IMO mixture was eluted between 60 to 95 fractions (Fig. 6.3.2 B). TLC analysis revealed the presence of isomalto-pentaose (DP5), isomalto-tetraose (DP4) and panose (DP3) in 67, 70 and 77 fractions, respectively (Fig. 6.3.2 A *lanes 1-5*). The unhydrolyzed sucrose and residual maltose were eluted in the 92 and 93 fractions, respectively (Fig. 6.3.2 A *lanes 6-7*). The molecular mass of IMO produced were confirmed by ESI-TOF MS. The peaks observed at m/z 527 for fraction 77, 629 for fraction 70 and 851 for fraction 67 revealed panose (DP3), isomalto-tetraose (DP4) and isomalto-pentaose (DP5), respectively (Fig. 6.3.2 C, D & E). The concentrations of DP3, DP4 and DP5 were quantified (Phenol-sulphuric acid assay) to be 17, 15 and 7 mg/ml, respectively.

IMO are used as food additives in functional foods and act as prebiotic in nature as they are resistant to hydrolysis by the enzymes of the human GI tract owing to the presence of α-(1→6) linkages, therefore, they are readily utilized by probiotic bacteria such as *bifiodobacterium* and *lactobacillus* and produce beneficial short chain fatty acids (SCFA) (Baruah *et al*., 2017). The fermentation of IMO by probiotic bacteria also produces vitamins of B complex (B1, B2, B6 and B12) (Mussatto & Mancilha, 2007). The daily requirement of IMO for human consumption is suggested to be 8-10 g per day (Goulas *et al*., 2004). IMOs in excessive doses could be responsible for intestinal discomfort, flatulence or even diarrhoea (an effect which is inversely related to chain length) and because of their high fermentation rate and production of gases (Roberfroid & Slavin, 2000).

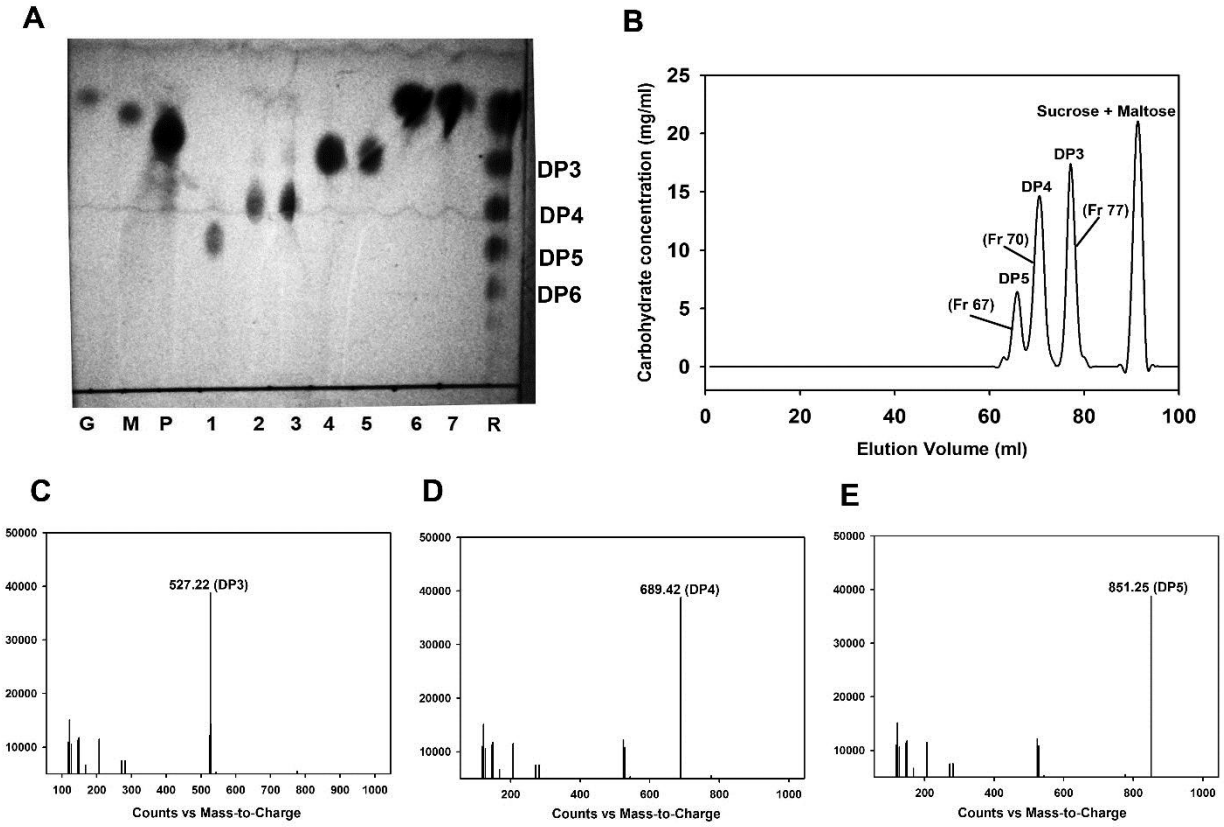

Fig 6.3.2 (**A**) TLC of G- Glucose, M- Maltose, P- Panose, selected Bio-Gel P2 fractions, 1-67, 2-69, 3-70, 4-77, 5-78, 6-92, 7-93 and C-IMO mixture. (**B**) Elution profile of IMO purification using Bio-Gel P2 by gel permeation chromatography (GPC). ESI-TOF mass spectrum of **C)** Fraction 77 (DP3) **D)** Fraction 70 (DP4) **E)** Fraction 67 (DP5).

6.3.3 Analysis of IMO produced using dextransucrase from *W. cibaria* RBA12 by HPLC

After the characterization of IMO, the purified fractions were subjected to HPLC analysis. The retention time for fractions containing DP3, DP4 and DP5 were 28.3, 24.7 and 22 min respectively (Fig. 6.3.3 A, B & C). The HPLC analysis of the IMO mixture revealed the presence of all the above mentioned peaks along with another peak at 20 min, which could correspond to DP6 as seen in the TLC analysis of the IMO mixture (Fig. 6.3.3 D). Due to its low concentration and subsequent dilution during GPC, the DP6 fraction was not detected by RI detector. The GPC purified fractions were used to plot standard curve to the detection of IMOs from DP3-DP5.

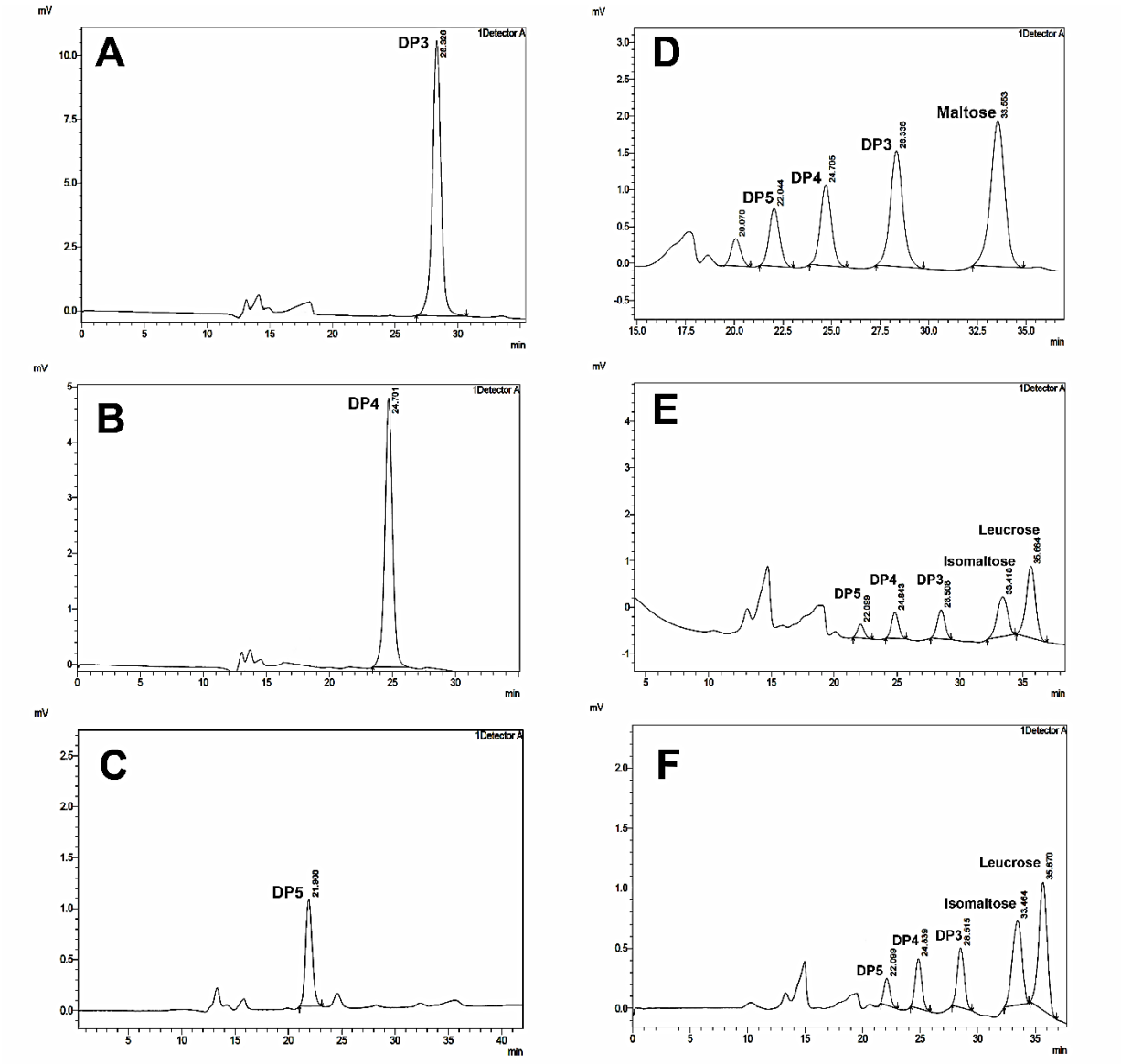

Fig. 6.3.3 HPLC analysis of (**A)** standard, DP3 (fraction 77), (**B)** standard DP4 (**f**raction 70 (**C)** standard DP5 **(f**raction 67) **(D)** standard IMO mixture showing DP2-DP5 **(E)** Mango and **(F)** Pineapple juice after dextransucrase acceptor reaction.

6.3.4 Production of IMO using sucrose in mango and pineapple juices

The mango and pineapple juice had a pH of 3.4 and 3.7, respectively and the total sugar was 124.7 mg/ml and 112.5 mg/ml, respectively. After the dextransucrase reaction the total sugar in mango and pineapple juice decreased to 103.6 mg/ml and

93.6 mg/ml, respectively resulting in decrease of their calorific values (Table 6.3.1). The dextransucrase reaction completely utilized the sucrose present in both the juices (Table 6.3.1). After the reaction there was a decrease in glucose concentration whereas the fructose concentration remained almost same as the initial value in both juices. In both juices the IMOs produced were panose (DP3), isomaltotetraose (DP4) and isomaltopentaose (DP5) along with the disaccharides leucrose and isomaltose (Fig. 6.3.3 E & F). In the dextransucrase reaction, fructose and glucose acts as acceptor molecules and isomaltose is formed when a glucose is transferred to another glucose whereas, leucrose is formed when the glucose is transferred to a fructose. After the dextransucrase acceptor reaction in mango juice the concentration of leucrose, isomaltose, DP3, DP4 and DP5 was 6.44, 4.03, 2.15, 1.4 and 0.94 mg/ml, respectively. Similarly, in pineapple juice after the dextransucrase acceptor reaction the concentration of leucrose, isomaltose, DP3, DP4 and DP5 was 4.38, 3.28, 1.72, 1.04 and 0.7 mg/ml, respectively (Table 6.3.1). The concentration of leucrose was highest amongst all oligosaccharides produced in both mango and pineapple juice followed by concentrations of isomaltose, DP3, DP4 and DP5. The production of oligosaccharides from mandarin juice using dextransucrase from *Leuconostoc mesenteroides* B-512FMCM also yielded oligosaccharides from DP3 to DP5 along with isomaltose (Nguyen *et al*., 2015). However, the concentrations of various oligosaccharides were not reported. Prebiotic juices are an important form of functional food as they have less calorific value due to lower sugar concentration. The value addition due to the oligosaccharides produced is especially important as they are not externally added and are synthesized with in the product (Fontes *et al*., 2015).

Table. 6.3.1. Oligosaccharide production in mango and pineapple juice using dextransucrase from *Wiessella cibaria* RBA12

Type of Juice	pH	Total Sugar (mg/ml)	Enzyme reaction	Sucrose (mg/ml)	Glucose (mg/ml)	Fructose (mg/ml)	Oligosaccharide produced (mg/ml)	
Mango	3.4	124.67	Before	33.22	44.90	46.55	-	
		103.64	After	0	41.49	62.15	**Leucrose**	6.44
							Isomaltose	4.03
							DP3	2.15
							DP4	1.40
							DP5	0.94
Pineapple	3.7	112.50	Before	28.10	40.71	43.69	-	
		93.60	After	0	38.60	55.00	**Leucrose**	4.38
							Isomaltose	3.28
							DP3	1.72
							DP4	1.04
							DP5	0.70

6.3.5 *In vitro* analysis of prebiotic properties of dextran

6.3.5.1 Effect of dextran on the growth of probiotic bacteria

The growth of probiotic bacteria namely *L. plantarum* DM5, *L. acidophilus* NRRL B-4495, *B. animalis* NRRL 41405, *B. bifidum* NRRL 41410 and *B. infantis* NRRL B-41661 in the presence of dextran-RBA12 or inulin was compared with the growth in the presence of glucose (Table 6.3.2). Dextran-RBA12 stimulated the growth of *L. plantarum* DM5, *L. acidophilus* NRRL B-4495, *B. animalis* NRRL 41405, *B. bifidum* NRRL 41410 and *B. infantis* NRRL B-41661 from 8.0, 7.4, 7.0, 5.4 and 7.6 to 9.2, 9.0, 9.5, 7.7 and 9.6 Log_{10} cfu/ml, respectively, after 24 h (Table 6.3.2). Similarly, the presence of inulin (commercial prebiotic) stimulated the growth of *L. plantarum* DM5, *L. acidophilus* NRRL B-4495, *B. animalis* NRRL 41405, *B. bifidum* NRRL 41410 and *B. infantis* NRRL B-41661 from 6.9, 6.8, 6.2, 5.4 and 6.6 to 9.7, 9.6, 9.8, 7.6 and 9.7 Log_{10} cfu/ml, respectively, after 24 h (Table 6.3.2). The prebiotic activity score signifies the ability of a substrate to stimulate the growth of probiotic strains as

compared to non-probiotic strains and also to that of non-prebiotic substrate glucose. The prebiotic potential of dextran-RBA12 was expressed using the prebiotic activity score. The prebiotic activity score of all the probiotic bacteria in the presence of both dextran-RBA12 and inulin are shown in Fig. 6.3.4. Dextran-RBA12 showed good prebiotic score from 0.26 (*L. plantarum* DM5) to 0.42 (*B. animalis* NRRL 41405) along with 0.3 (*L. acidophilus* NRRL B-4495), 0.42 (*B. bifidum* NRRL 41410) and 0.38 (*B. infantis* NRRL B-41661). Inulin showed a prebiotic score of 0.85 (*L. plantarum* DM5), 0.66 (*L. acidophilus* NRRL B-4495), 0.62 (*B. animalis* NRRL 41405), 0.33 (*B. bifidum* NRRL 41410) and 0.69 (*B. infantis* NRRL B-41661).

Table 6.3.2 Growth profile of probiotic and non-probiotic bacteria in the presence of glucose, inulin and dextran-RBA12.

Bacteria	Glucose			Inulin			Dextran-RBA12		
	0h	12h	24h	0h	12h	24h	0h	12h	24h
L. plantarum	7.3 ± 0.13	9.3 ± 0.1	9.8 ± 0.08	6.9 ± 0.11	9.8 ± 0.3	9.7 ± 0.05	8.0 ± 0.05	9.6 ± 0.01	9.2 ± 0.27
L. acidophilus	6.9 ± 0.04	9.8 ± 0.15	10 ± 0.04	6.8 ± 0.1	9.9 ± 0.11	9.6 ± 0.1	7.4 ± 0.21	10 ± 0.05	9.0± 0.17
B. animalis	7.2 ± 0.14	10.5 ± 0.08	11.3 ± 0.05	6.2± 0.17	10.6 ± 0.01	9.8± 0.2	7.0± 0.12	9.1± 0.09	9.5± 0.15
B. bifidum	7.5 ± 0.08	10.1 ± 0.06	11.3 ± 0.04	5.4 ± 0.1	7.9 ± 0.05	7.6 ± 0.18	5.4 ± 0.1	6.9 ± 0.03	7.7 ± 0.12
B. infantis	7.1 ± 0.08	10.2 ± 0.19	10.3 ± 0.09	6.6 ± 0.09	10.1 ± 0.16	9.7 ± 0.12	7.6 ± 0.1	9.1 ± 0.17	9.6 ±0.06
*Enteric Mixture**	6.9 ± 0.04	9.9 ± 0.17	10.7 ± 0.22	6.9 ± 0.04	7.5 ± 0.12	7.9 ± 0.02	6.9 ± 0.04	7.1 ± 0.17	7.6 ± 0.1

All experiments were done in triplicate sets. The growth profiles of bacteria were expressed in Log_{10}cfu/ml.

* Enteric mixture comprised a co-culture of *E. coli* DH5α and *Enterobacter aerogenes* MTCC 7016.

Prebiotic carbohydrates, by definition, can only be metabolized by selected microbiota of the gastrointestinal tract. Accordingly, these carbohydrates have the ability to influence the microbial population of the gastrointestinal tract due to their selective utilization. Organisms that rapidly ferment prebiotic carbohydrates are enriched, presumably at the expense of those that do not. The effectiveness of a prebiotic depends, therefore on its ability to i) be selectively fermented by and ii) support the growth of specifically targeted organisms (Huebner *et al.*, 2007). It has been

reported that anaerobic bacterial fermentation of prebiotics by several probiotic organisms produces lactate and many short-chain fatty acids (SCFA) such as acetate, propionate and butyrate, which results in the suppression of growth of several intestinal pathogenic bacteria (Topping and Clifton 2001). These SCFA are also involved in the modulation of cholesterol and lipid metabolism by supplying energy for the colonic epithelium (Das *et al*., 2014). The prebiotic score of dextran-RBA12 was low in *Lactobacillus* species but comparatively higher with the *Bifidobactrium*. In the case of *B. bifidum* NRRL 41410, dextran-RBA12 showed a higher prebiotic score of 0.42 as compared to a score of 0.33 in case of inulin. The prebiotic score of dextran-RBA12 was lower than inulin with other probiotic strains but was similar with a prebiotic score of another dextran from *Lactobacillus plantarum* DM5 (Das *et al*., 2014). Positive prebiotic activity score is exhibited by carbohydrates that are metabolized by only probiotic bacteria but not by any other enteric bacteria (Huebner *et al*., 2007). The exopolysaccharide (EPS) from *W. cibaria* A2 stimulated the growth of fecal microbiota and showed a significant increase in SCFA namely acetate, propionic acid and butyric acid (Hongpattarakere *et al*., 2012).

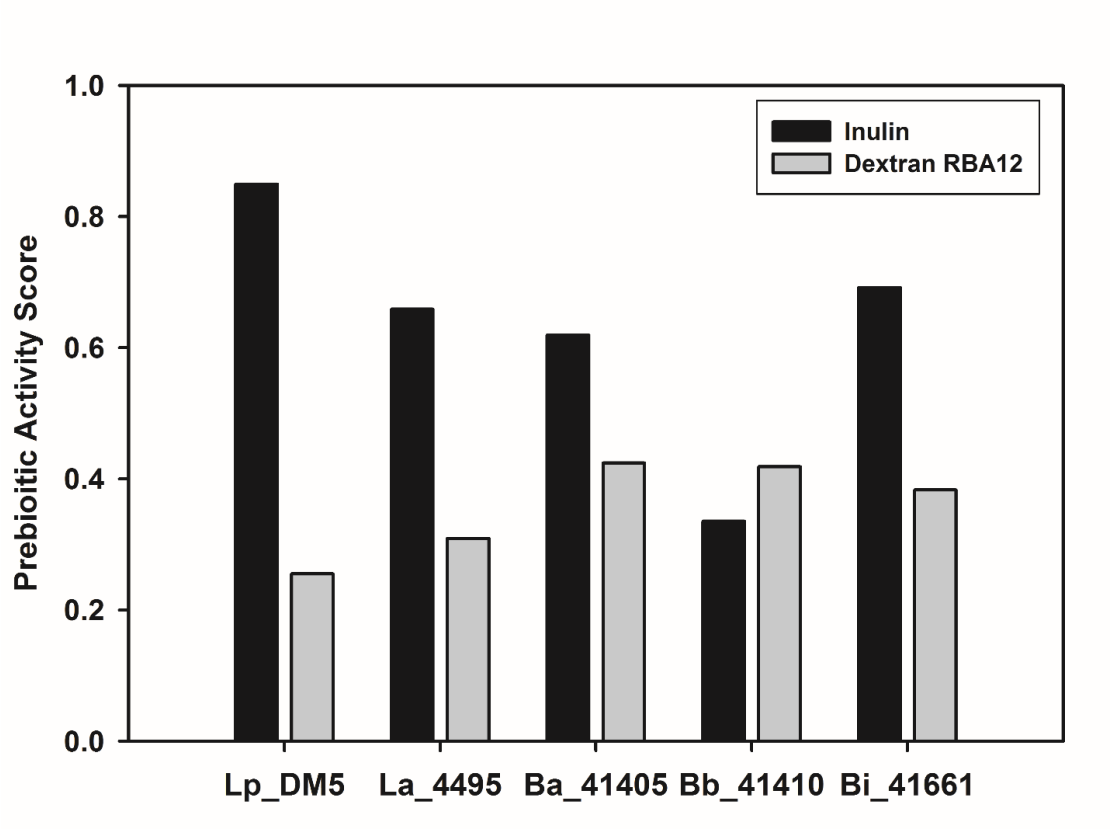

Fig. 6.3.4 Prebiotic activity score of dextran-RBA12 and commercial prebiotic inulin. The prebiotic activity score of the two EPS was determined by using the probiotic strains *L. plantarum* DM5, *L. acidophilus* NRRL B-4495, *B. animalis* NRRL 41405, *B. bifidum* NRRL 41410 and B. *infantis* NRRL B-41661 and a consortium of non-probiotic bacteria containing *E. coli* DH5α and *E. aerogenes* MTCC 7016.

6.3.5.2 Hydrolysis of dextran-RBA12 by artificial gastric juice, α-amylase and intestinal fluid

The hydrolysis of dextran by artificial gastric juice was studied at 37°C for different time intervals from 0.5 h to 5 h. The purified dextran from *Weissella cibaria* RBA12 proved to be resistant to hydrolysis by artificial gastric juice at different pH ranging from 1.0 to 4.0 as compared with standard prebiotic inulin. The percent hydrolysis of purified dextran-RBA12 steadily decreased with the increase of pH (Fig. 6.3.4). After 1 h of incubation, the percent hydrolysis was 0.32%, 0.28%, 0.26% and 0.17% at pH of 1, 2, 3 and 4, respectively (Fig. 6.3.4 A). After 5 h of incubation, the percent hydrolysis of purified dextran-RBA12 was 0.51%, 0.48%, 0.37% and 0.21% at pH 1, 2, 3 and 4, respectively (Fig. 6.3.4 A). The percent hydrolysis of inulin after 1 h of incubation also decreased with the increase in pH. The percent hydrolysis was 16.53%, 12.22%, 8.27% and 4.11% at pH 1, 2, 3 and 4, respectively (Fig. 6.3.4 B). The percent hydrolysis of inulin after 5 h was much higher i.e. 25.23%, 21.20%, 11.66% and 4.33% at pH 1, 2, 3 and 4, respectively (Fig. 6.3.4 B) when compared with dextran-RBA12.

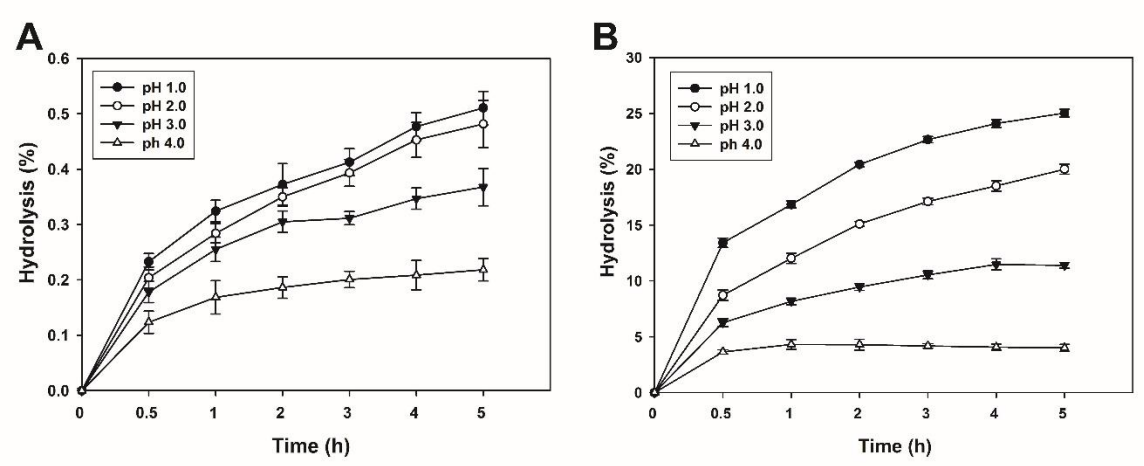

Fig. 6.3.4 **A)** Hydrolysis of dextran-RBA12 with artificial gastric of pH ranging from 1-4; **B)** Hydrolysis of prebiotic inulin with artificial gastric juice of pH ranging from 1-4

6.3.5.3 Hydrolysis of dextran-RBA12 by α-amylase

The hydrolysis of purified dextran-RBA12 and inulin at pH 5–8 by α-amylase showed that the percent of hydrolysis increased with the increase of pH. After 1 h of incubation, the percent hydrolysis of dextran-RBA12 was 0.08%, 0.10%, 0.13% and 0.2% at pH 5, 6, 7 and 8 respectively, at 37°C (Fig 6.3.5 A). After 5 h of incubation, the percent hydrolysis of dextran-RBA12 was 0.13%, 0.20%, 0.27% and 0.31% at pH 5, 6, 7 and 8 respectively. In case of inulin after 5 h of incubation, the hydrolysis was 8.1%, 12.7%, 15.8% and 19.1% at pH 5, 6, 7 and 8, respectively, at 37°C (Fig. 6.3.5 B).

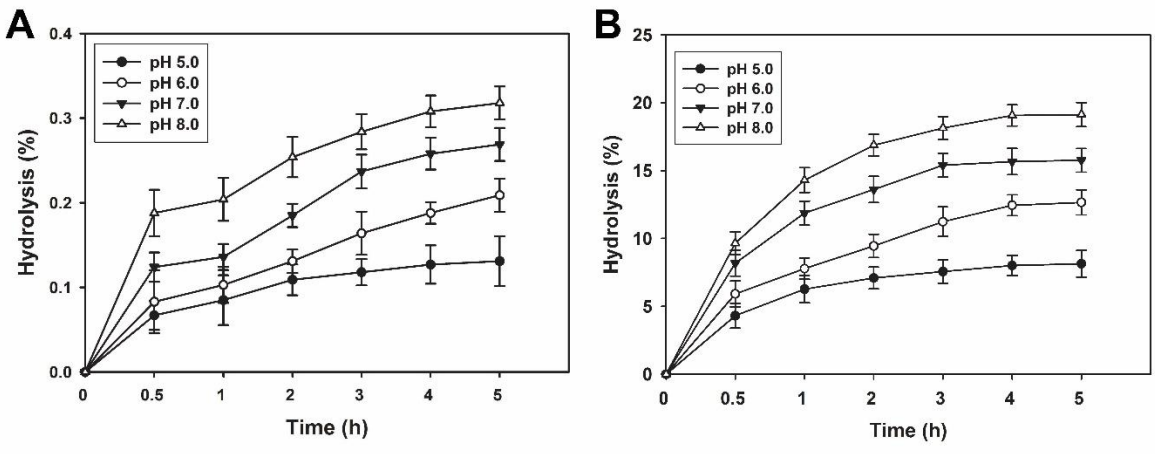

Fig. 6.3.5 **A)** Hydrolysis of dextran-RBA12 with α-amylase of pH ranging from 5-8; **B)** α-amylase of pH ranging from 5-8.

6.3.5.4 Hydrolysis of dextran-RBA12 by intestinal fluid

In the presence of intestinal fluid, dextran-RBA12 displayed a percent hydrolysis of only 0.24 % after 5 h of incubation, as compared to percent hydrolysis of 6.2% shown by commercial prebiotic inulin (Fig. 6.3.6). Dextran-RBA12 showed maximum hydrolysis of 0.51%, 0.31% and 0.24% by artificial gastric juices, α-amylase and intestinal fluid, respectively, whereas inulin showed maximum hydrolysis of 25.23%, 19.13% and 6%, respectively. Thus, dextran-RBA12 showed 50-fold higher

resistance to artificial gastric juice, 60-fold higher resistance to α-amylase and 25-fold higher resistance to intestinal fluid as compared to the prebiotic inulin. Similar results have been reported for exopolysaccharide (EPS) from *W. cibaria* A2 (Hongpattarakere *et al*., 2012), α-glucan from *Lactobacillus plantaum* DM5 (Das *et al*., 2014) and dextran from *Leuconostoc mesenteroides* NRRL B-1426 (Kothari *et al*., 2015). Inulin-type fructans resist hydrolysis by human digestive enzymes and reach the large intestine virtually intact where they are fermented by the intestinal microbiota. Dextran-RBA12 is clearly more resilient to the enzymes of the gastrointestinal tract of human than inulin and thus, can be considered as a soluble dietary fibre that is non-digestible, ferments in the large intestine and can act as a prebiotic for the stimulation of gut beneficial microbiota.

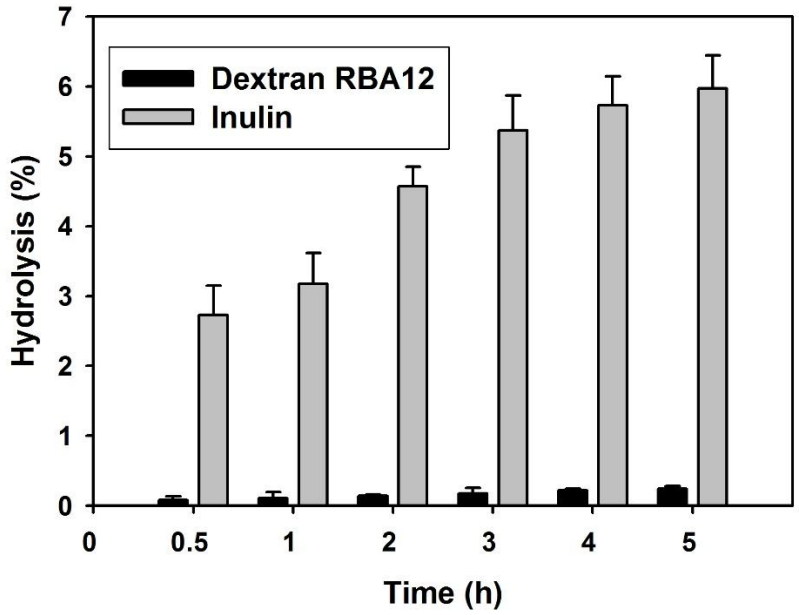

Fig. 6.3.6 Hydrolysis of dextran-RBA12 and commercial prebiotic inulin with intestinal fluid in pH 8.0.

6.3.6 Analysis of dextran and sugar from sourdough fermentations using cereal matrices

The cell count for the whole wheat flour, wheat bran and rye bran were 8.7, 9.3 and 9.5 Log_{10} cfu/g, respectively, after 20 h of incubation. The cell growth in the three cereals was almost the same with 3 Log_{10} cfu/g increase (Table 6.3.3). The pH of sourdough samples of the whole wheat flour, wheat bran and rye bran were 4.9, 4.2 and 4.4, respectively, after 20h of incubation. The acidification after 20h varied with matrix and was lowest in the wheat flour. The percentage of dextran formation varied with the type of matrix used. The dextran formation in the whole wheat flour, wheat bran and rye bran was 0.66 ±0.12, 1.28 ±0.14 and 3.26 ±0.12% dw of the matrix, respectively, after 20h of incubation. The free sugars present in the sourdough after 20h of fermentation were analyzed which showed 0.58, 0.05 and 1.05% (g/100g of matrix) glucose in whole wheat flour, wheat bran and rye bran respectively. Fructose present after 20h in whole wheat flour, wheat bran and rye bran was 1.25, 3.56 and 3.67% (g/100g of matrix), respectively. Maltose (0.27%, g/100g of matrix) was detected only in the whole wheat flour at the end of the fermentation. There was no residual sucrose left after 20h in any of the three matrices used.

Table 6.3.3 Analysis of sourdough fermentation using *Weissella cibaria* RBA12 in three different cereal matrices.

Sourdough	Log_{10} cfu/g 0h	Log_{10} cfu/g 20h	pH 20h	Dextran (%, dw)	Glucose (%)	Fructose (%)	Maltose (%)
Whole wheat flour	5.7	8.7	4.9	0.7 ± 0.12	0.6	1.3	0.3
Wheat Bran	6.1	9.3	4.2	1.3 ± 0.14	0.1	3.6	0
Rye Bran	6.1	9.5	4.4	3.3 ± 0.12	1.1	3.7	0

The positive technological effects of dextran on sourdough bread are correlated with high molecular mass and a low degree of branching of the dextran molecule (Lacaze *et al*., 2007). The ability of *W. cibaria* RBA12 to produce dextran and be able to grow in all three matrices was studied. There was an increase of approximately, 3 log cfu/g in all the three matrices. The pH after 20h for the whole wheat flour was 4.9 whereas for wheat bran and rye bran it was 4.2 and 4.4 respectively. Similar acidification is reported in buckwheat, quinoa, teff and wheat flour matrices fermented using *W. cibaria* MG1 (Wolter *et al.* 2014). The enzymatic hydrolysis method employed for dextran estimation from sourdough is most suitable with dextrans with few branched linkages, as the enzyme is not able to completely hydrolyze its more complex structural sections (Katina *et al*., 2009). The dextran produced by *W. cibaria* RBA12 was least in whole wheat flour with a 0.66% d.w (6.6 g/kg) dextran. The dextran was maximally produced in rye bran was the highest with 3.26% d.w (32.6 g/kg) followed by wheat bran with 1.28% d.w (12.8 g/kg). The low dextran yield in whole wheat flour can be attributed to higher maltose content in wheat flour as seen in the free sugar analysis. Maltose is formed in starch-rich matrices due to the activity of starch degrading enzymes (amylase) indigenously present in the matrix. Maltose acts as an acceptor molecule in the process of dextran synthesis by extracellular enzyme dextransucrase produced by the bacteria. In the presence of maltose homologous series of isomalto-oligosaccharides are formed which hinders the synthesis of dextran by dextransucrase. Similar observations regarding the formation of isomalto-oligosaccharides have been reported by Katina *et al.* (2009), Galle *et al.* (2010) and Wolter *et al.* (2014). The bran matrices used are an excellent source of dietary fibres and when coupled with the addition of dextran-RBA12 can increase the volume, shelf-life and the texture of bread. In the whole wheat flour matrix, the formation of isomalto-

oligosaccharides (IMO) was not quantified but the presence of maltose confirmed their formation. IMOs are by themselves prebiotic in nature and can work synergistically with prebiotic dextran-RBA12. The best cereal matrix for dextran production using *W. cibaria* RBA12 was rye bran followed by wheat bran and wheat flour. The superior performance of *Weissella* in rye bran was also reported earlier by Kajala *et al.* (2015a).

6.4 Conclusion

The functional food applications using *Weissella cibaria* RBA12 were explored by the production of IMOs and various applications of dextran-RBA12. Fermentation with *Weissella cibaria* RBA12 in the culture medium containing maltose as an acceptor produced IMOs, namely trisaccharide, tetrasaccharide and pentasaccharide. IMOs in the range of DP3–DP6 were produced *in vitro* using maltose as an acceptor molecule by dextransucrase acceptor reaction. The mixture of IMOs produced was purified by GPC and individual IMO were obtained which on identification by TLC and by their mass analysis gave, DP3 (527.22), DP4 (689.42) and DP5 (851.25). The purified IMOs were used as the standard for HPLC analysis of IMOs produced in dextransucrase acceptor reactions with fruit juices. Dextransucrase was used to produce prebiotic juices using commercial mango and pineapple juice, which yielded IMOs in the range of DP3-DP5 along with disaccharides, leucrose and isomaltose. The reaction removed completely the native sucrose present in the juices lowering its calorific value. The significantly lower digestibility of dextran-RBA12 as compared with the commercial prebiotic inulin as well as its ability to be fermented by probiotic intestinal microbiota proved its effectiveness as an efficient soluble dietary fibre. The *in situ* formation of dextran in cereal matrices like wheat flour, wheat bran and rye bran after fermentation by *Weissella cibaria* RBA12 was observed to be very efficient. Among the three matrices, rye bran proved to be the best matrix producing maximum dextran of 3.26% d.w (32.6 g/kg). Further studies on the shelf-life and stability of dextransucrase as well as studies on the physio-chemical properties of dextran-RBA12 will throw light on its potential applications in food industry.

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List of publications

Published/accepted

From Thesis:

1. **Rwivoo Baruah**, Barsha Deka and Arun Goyal* (2017) Purification and characterization of dextransucrase from *Weissella cibaria* RBA12 and its application in in vitro synthesis of prebiotic oligosaccharides in mango and pineapple juices. LWT Food Science and Technology. 84, 449–456. (JIF 2.3)
2. **Rwivoo Baruah**, Barsha Deka, Niharika Kashyap, and Arun Goyal. (2017)"Dextran Utilization during its synthesis by *Weissella cibaria* RBA12 can be overcome by Fed-Batch fermentation in a bioreactor." Applied Biochemistry and Biotechnology, doi: 10.1007/s12010-017-2522-4 (in press). (JIF 1.7)
3. **Rwivoo Baruah**, Ndegwa H. Maina, Kati Katina, Riikka Juvonen and Arun Goyal (2016) Functional food applications of dextran from *Weissella cibaria* RBA12 from Pummelo (*Citrus maxima*). *International Journal of Food Microbiology*. 242, 124-131 (JIF 3.3)
4. **Rwivoo Baruah** and Arun Goyal (2015). Hyper glucansucrase, glucan and oligosaccharide producing novel *Weissella cibaria* RBA12 isolated from Pummelo (*Citrus maxima*). *Annals of Microbiology*. 65, 2301-2310. (JIF 1.2)

Other Publications:

5. **Rwivoo Baruah**, Deeplina Das and *Arun Goyal (2016) Heteropolysaccharides from Lactic acid bacteria: Current trends and applications. *Journal of Probiotics and Health*, 4(2), 141. (JIF 1.7).
6. Aruna Rani, **Rwivoo Baruah** and Arun Goyal (2017) Physicochemical, antioxidant and biocompatible properties of chondroitin sulphate isolated from chicken keel bone for potential biomedical applications. *Carbohydrate Polymers* 159, 11-19. (JIF 4.8)
7. Shraddha Shukla, Anil Verma, Ilkka Kajala, Antti Nyyssolä, **Rwivoo Baruah**, Kati Katina, Riikka Juvonen, Maija Tenkanen and *Arun Goyal (2016) Structure modelling and functional analysis of recombinant dextransucrase from *Weissella confusa* Cab3 expressed in *Lactococcus lactis*. Preparative Biochemistry and Biotechnology, 46, 822-832. (JIF 1.3)
8. Deeplina Das, **Rwivoo Baruah** and *Arun Goyal (2014). A food additive with prebiotic properties of an α-D-glucan from *Lactobacillus plantarum* DM5. *International Journal of Biological Macromolecules*, 69: 20-26. (JIF 3.1)
9. Damini Kothari, **Rwivoo Baruah** and *Arun Goyal (2012) Immobilization of glucansucrase for the production of gluco-oligosaccharides from *Leuconostoc mesenteroides* NRRL B-1426. *Biotechnology Letters*, 34: 2101-2106. (JIF 1.8)

List of conference papers

International

1. **Rwivoo Baruah**, Barsha Deka and Arun Goyal (2017) Synthesis of in situ prebiotic isomalto-oligosaccharides in mango and pineapple juices using dextransucrase from *Weissella cibaria* RBA12. 12th Carbohydrate Bioengineering Meeting, April 23-26, 2017, Vienna, Austria.
2. **Rwivoo Baruah**, Barsha Deka, Niharika Kashyap, V.S. Moholkar and Arun Goyal (2016) Optimization and scale up of dextran production from *Weissella cibaria* RBA12. 57th International Annual Conference of The Association of Microbiologists of India (AMI-2016), Nov 24-27, 2016, Gauhati University and IASST, Guwahati, Assam India.
3. **Rwivoo Baruah**, Barsha Deka and Arun Goyal (2015) Purification and characterization of dextransucrase from *Weissella cibaria* RBA12 and production of isomalto-oligosaccharides. AMI 2015 at JNU New Delhi, India.
4. **Rwivoo Baruah** and Arun Goyal (2015) Characterization of glucan from *Weissella cibaria* RBA12 as a potential food additive and hydrocolloid. NHBT 2015 (Organized by BRSI) at Trivandrum Kerela, India.
5. **Rwivoo Baruah** and Arun Goyal (2014) Screening and identification of glucan producing lactic acid bacterium *Weissella cibaria* RBA12 from Pummelo (*Citrus maxima*). ICS 2014 at IISC Bangalore (India).
6. Aruna Rani, **Rwivoo Baruah** and Arun Goyal (2016) Biocompatible and antioxidant properties of chondroitin sulphate isolated from chicken keel bone for potential biomedical applications. 57th International Annual Conference of The Association of Microbiologists of India (AMI-2016), Nov 24-27, 2016, Gauhati University and IASST, Guwahati, Assam India.
7. Niharika Kashyap, **Rwivoo Baruah**, Vijay. S. Moholkar and Arun Goyal (2016) In situ production and analysis of *Weissella cibaria* RBA12 dextran in whole wheat sourdough. National Conference on Recent Advancement in Environmental Research, Center for the Environment, IIT Guwahati, 4-5 June, 2016.
8. Aruna Rani, **Rwivoo Baruah** and Arun Goyal (2015) Structural determination of chondroitin oligosaccharide isolated from Keel bone cartilage by recombinant chondroitin AC lyase from Pedobactor saltans and its prebiotic potential. 56th International Annual Conference of Association of Microbiologists of India (AMI), December 7-10, 2015, Jawaher Lal Nehru University, New Delhi.
9. Niharika Kashyap, **Rwivoo Baruah**, Vijay. S. Moholkar and Arun Goyal (2015) Production of dextran from sucrose containing industrial by-product using *Weissella cibaria* RBA12. 56th International Annual Conference of the Association of Microbiologists of India (AMI- 2015), Jawaharlal Nehru University (JNU), New Delhi, India.

National

1. Shraddha Shukla, **Rwivoo Baruah** and Arun Goyal (2013) Biochemical characterization of dextransucrase from *Weissella confusa* Cab3. MIBISEM 2013 at NBU Siliguri (India).
2. Rishikesh Shukla, **Rwivoo Baruah** and Arun Goyal (2012) Molecular identification of a probiotic strain *Pediococcus pentosaceus* CRAG3 isolated from fermented cucumber. Post ISCBC-2012 at IASST Guwahati (India).
3. Rishikesh Shukla, **Rwivoo Baruah** and Arun Goyal (2011) Production, purification and characterization of polysaccharides and oligosaccharides produced by hydrolysis from *Leuconostoc mesenteroides* NRRL B-1149. CARBO XXVI at IICB Kolkata (India).

Vitae

The author was born on April 19, 1989 in Nagaon, Assam India. He passed the All India Secondary School Examination (A.I.S.S.E) conducted by Central Board of Secondary Education (CBSE, 10th Class), Nagaon, Assam in 2004, and All India Senior Secondary School Examination (A.I.S.S.C.E) conducted by Central Board of Secondary Education (CBSE, 12th Class), Nagaon, Assam in 2006. He completed B. Sc. with Zoology as major subjects along with Botany and Chemistry from Nagaon College, Nagaon Assam, India, in 2009. He did his M. Sc. in Biotechnology from CMR Institute of Management Studies (affiliated to Bangalore University), Bangaluru, India in 2011.

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ORIGINAL ARTICLE

Hyper glucansucrase, glucan and oligosaccharide producing novel *Weissella cibaria* RBA12 isolated from Pummelo (*Citrus maxima*)

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Abstract *Weissella cibaria* RBA12 (GenBank accession no: KF515952) producing glucansucrase and glucan was isolated from pulp of Pummelo (*Citrus maxima*). The isolate RBA12 was characterized and identified on the basis of morphological, biochemical properties and 16S rRNA gene sequence analysis, and gave maximum glucansucrase activity of 7.2 U/mL at 20 °C and 180 rpm. The molecular mass of crude glucansucrase was approximately 180 kDa as determined by SDS-PAGE analysis and was confirmed as glucansucrase by periodic acid-Schiff staining using sucrose as substrate. Maximum glucan concentration of 8.3 mg/mL with an efficiency of glucan production of 83 % was observed in the cell-free supernatant under optimized conditions of growth of *W. cibaria* RBA12. The production of oligosaccharides by *W. cibaria* RBA12 was carried out by supplementing fermentation medium with maltose as an acceptor molecule. The time-dependent analysis of the acceptor reaction in the medium by TLC and ESI-TOF-MS revealed that *W. cibaria* RBA12 produced oligosaccharides ranging from trisaccharides to homologous penta-saccharides.

Keywords Lactic acid bacteria · *Weissella cibaria* · Glucan · Glucansucrase · Oligosaccharide

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Introduction

Fruits and vegetables are an integral part of the human diet and are consumed in large quantities all over the world. These products are rich in carbohydrates and poor in proteins, have pH values of 4.6 to 7.0, and provide a suitable niche for several bacteria, yeasts and moulds (Weissinger et al. 2000; Trias et al. 2010). The type of microflora are determined by the pH and the type of acid present in the fruits. Lactic acid bacteria (LAB) have been demonstrated to form part of the autochthonous microflora of tomato owing to its low pH and organic acids (Brackett 1988; Sajur et al. 2007). Food-borne bacteria capable of causing human illness cannot grow at a pH less than 4.0, so the acidic nature of the edible portion of most fruits precludes their use as substrates for proliferation of human pathogens (Naeem et al. 2012).

Weissella spp. are Gram positive, short rod, non-motile, non-spore forming, heterofermentative bacteria grouped under LAB (Shukla and Goyal 2011). Phylogenetic classification of *Weissella cibaria* JAG8 has been reported using 16S rRNA gene sequence analysis (Rao and Goyal 2013). *Weissella* spp. are normally isolated from fermented foods and have the ability to produce glucan in the presence of glucansucrase using sucrose as substrate (Björkroth et al. 2002). α-D-Glucans production is a phenotypic criterion for the identification of bacteria under this genus (Bounaix et al. 2010; Shukla and Goyal 2011). α-D-Glucans are exopolysaccharides that consist of D-glucose repeating units. The α-D-glucans are classified into dextrans, mutans, alternans and reuterans depending on their glycosidic linkage composition and organisation (Leemhuis et al. 2013). α-D-Glucans from LAB are regarded as natural food thickeners and can replace other hydrocolloid additives. The potential health benefits of α-D-glucan make it a product of high interest in food applications (Patel et al. 2012). The glucansucrases

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are named based on the α-glucans they synthesize, e.g. dextransucrases and alternansucrases. *Weissella* spp. have received significant attention in recent years due to their relatively high dextran production ability as compared to other LAB, especially in sourdough bread applications (Shukla et al. 2014).

Glucansucrase catalyses a primary reaction for synthesis of glucan from sucrose and a secondary reaction for oligosaccharide synthesis in the presence of acceptors like maltose or isomaltose (Patel and Goyal 2011). Oligosaccharides have been accorded prebiotic status and have evinced worldwide interest from the food and pharmaceutical sectors to match overwhelming consumer preference for healthier foods (Kothari et al. 2012). Oligosaccharides act as prebiotics, i.e. being non-digestible in nature they promote the growth of beneficial LAB in the colon and exert antagonism to *Salmonella* sp. or *Escherichia coli*, limiting their proliferation. This is possible due to the synthesis of short chain fatty acids, which are produced from oligosaccharide fermentation by native microflora of the colon (Patel and Goyal 2012). Oligosaccharides can be produced in fermentation medium containing LAB such as *Weissella cibaria* in the presence of the substrate sucrose and maltose as an acceptor. This study reports the isolation and characterisation of a novel isolate *Weissella cibaria* RBA12 from Pummelo (*Citrus maxima*). Culture conditions for glucansucrase production were investigated along with glucan and oligosaccharide production by *W. cibaria* RBA12.

Materials and methods

Screening and isolation of a glucansucrase-producing bacterial strain

Fresh pulp of Pummelo (1 g) was crushed and added to 100 mL MRS medium (DeMan et al. 1960) containing 2 % (w/v) sucrose and incubated at 28 °C for 24 h. An aliquot (1 %) of the culture was used for inoculation of 100 mL MRS medium containing 2 % (w/v) sucrose and incubated at 28 °C for 24 h. The culture samples were serially diluted up to 10^{-9} dilutions and dilutions from 10^{-5} to 10^{-9} were spread on MRS agar medium containing 0.5 % (w/v) $CaCO_3$ and incubated at 28 °C for 24 h (Rao and Goyal 2013). Colonies showing a zone of clearance formed due to hydrolysis of $CaCO_3$ were selected based on their size and shape. The selected colonies were transferred to 5 mL MRS medium and incubated at 28 °C for 12 h. A 1 % (v/v) culture of the selected isolates was inoculated in 5 mL enzyme production medium as described by Tsuchiya et al. (1952) and incubated at 28 °C under static condition for 12 h. The cultures were inoculated into 100 mL enzyme production medium and cell-free supernatant was analysed for glucansucrase activity. The isolate that showed highest enzyme activity was chosen for further analysis. The selected isolate was denoted as isolate RBA12 and was maintained in medium (Tsuchiya et al. 1952) with 20 % (v/v) glycerol at −20 °C.

Enzyme assay

The enzyme activity was determined using 20 μL cell-free supernatant and 5 % (w/v) sucrose as a substrate in 20 mM sodium acetate buffer (pH 5.4). The assay was carried out in a 1-mL reaction mixture incubated at 30 °C for 15 min; 100 μL of the reaction mixture was used for estimation of the reducing sugar following the method described by Nelson (1944) and Somogyi (1945). D-Fructose was used as a standard and 1 U glucansucrase activity was defined as the amount of enzyme producing 1 μmol reducing sugar per minute under the assay conditions of pH 5.4 and 30 °C.

Phenotypic characterisation of the isolate

Morphological characterisation of isolate RBA12 was carried out using scanning electron microscopy (SEM; Zeiss-Sigma, Jena, Germany). Samples were prepared by centrifuging overnight cultures of the isolate, washing the pellet five times with crystal-free phosphate-buffered saline and subsequently dehydrating in a desiccator. Phenotypic characterization of isolate RBA12 was also carried out by Gram staining, catalase reaction and by motility, triple sugar iron agar and nitrate agar slant tests performed at 28 °C and incubating for 24 h. The production of ammonia from arginine was carried out in MRS medium containing 0.3 % arginine and 0.2 % sodium citrate replacing ammonium citrate and incubating at 28 °C for 24 h. The presence of ammonia was monitored by Nessler's Reagent (Patil et al. 2010). The D- or L- lactic acid isomers production from glucose metabolism were determined in MRS medium by using D/L lactic acid assay kit (Megazyme, Bray, Ireland) (Prasad et al. 2014).

The growth of isolate RBA12 was studied in 5 mL MRS medium at 15 °C, 37 °C and 45 °C by incubating under static condition for 24 h. Similarly, growth of isolate RBA12 in MRS medium was studied at pH 3.0, 4.0, 5.0, 8.0 and 9.0 by incubating the culture at 28 °C for 24 h under static conditions. Salt tolerance of isolate RBA12 was determined in MRS medium containing 2.0 %, 4.0 %, 6.0 %, 8.0 % and 10.0 % NaCl (w/v) by incubating at 28 °C under static conditions for 24 h (Saguir et al. 2009). The carbohydrate utilization profile of isolate RBA12 was determined using a standard carbohydrate disc in MRS agar plates containing phenol red indicator along with the isolate and incubating at 28 °C for 24 h. The antibiotic resistance of the isolate RBA12 was determined using antibiotic octo-disc placed in MRS agar plates at 28 °C for 24 h (Rao and Goyal 2013). Carbohydrate discs

and antibiotic octo-discs were procured from Hi-Media (Mumbai, India).

Molecular characterization of the isolate

Extraction of genomic DNA of isolate RBA12 was carried out using a genomic DNA isolation kit (Hipura, Hi-Media). The purity of the extracted DNA was checked by agarose gel (0.8 %, w/v) electrophoresis. The concentration of DNA was determined by nanodrop (BioSpectrometer kinetic, Eppendorf, Germany). The 16S rRNA gene fragment was amplified from genomic DNA of isolate RBA12 using universal primers, 27F (AGAGTTTGATCCTGGCTCAG) and 1429R (GGTTACCTTGTTACGACTT) (Dutta et al. 2012) by PCR (Applied Biosystems, GeneAmp, Foster City, CA). The 50 μL reaction mixture contained 0.5 μM, 27F and 1429R primers, 2 mM each of dNTP (Merck, Darmstadt, Germany), 2.5 mM $MgCl_2$ (Bioline, London, UK), 5 μL 10x reaction buffer (Bioline), 1 U *Taq* DNA polymerase (Bioline) and 10 ng genomic DNA. The reaction conditions employed were 1 cycle at 94 °C for 4 min, 30 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min. A final extension of 72 °C for 10 min was included. The amplicons were visualized on a 0.8 % (w/v) agarose gel. The amplicons were sequenced by SciGenom Labs (Cochin, India). A 1,372 bp sequence of 16S rRNA gene was generated from the forward and reverse primer sequence data using Cap3 sequence assembly program. BLAST (Basic Local Alignment Search Tool) was used to analyse the sequence and submitted to GenBank, NCBI (http://www.ncbi.nlm.nih.gov/). Consensus sequences of representative members within the order imported from GenBank were aligned using ClustalW and phylogenetic tree was constructed by neighbour joining method using MEGA5. 2 software (http://www.megasoftware.net/mega.php).

Effect of temperature and agitation on glucansucrase production

The production of glucansucrase by isolate RBA12 was studied under different culture conditions, varying temperature and agitation speed (Das and Goyal 2014). The effect of temperature on enzyme production was studied by varying the temperature from 16 °C to 37 °C using 100 mL medium (Tsuchiya et al. 1952) under static conditions. The effect of agitation on enzyme production was studied under different shaking conditions (80, 120, 150, 180, 200 rpm) at 20 °C. Enzyme activity was determined every 3 h for 18 h, using 1 mL culture, after centrifuging at 10,500*g* and 4 °C for 10 min. Cell-free supernatant was used for the enzyme assay, which was carried out as described above. Measurements were done in triplicate and all data are the average of three independent experiments ±standard error.

Fermentation profile of the isolate

The optimized conditions for glucansucrase production were used to study the fermentation profile of isolate RBA12. Enzyme production medium (100 mL) was inoculated with 1 % (v/v) inoculum containing the isolate and incubated at 20 °C and 180 rpm. The optical density (600 nm) of the culture medium and pH profile were monitored every 2 h up to 36 h. Glucansucrase activity was assayed as mentioned earlier. Sucrose concentration in the broth was also monitored using the DNS method (Miller 1959) using sucrose as a standard. Both glucansucrase activity and sucrose consumption were monitored every 4 h up to 36 h.

Glucan estimation

The production of glucan by isolate RBA12 was studied in 100 mL enzyme production medium incubated at 20 °C and 180 rpm. The glucan concentration in the broth was determined every 4 h up to 36 h. For estimation of glucan, 200 μL cell-free supernatant was taken and glucan was precipitated by using 3 volumes of 95 % (v/v) chilled ethanol (Das and Goyal 2013). The solution was then centrifuged at 10,500*g* and 4 °C for 30 min. The pellet collected was dissolved in 200 μL de-ionised water and this process was repeated three times. Glucan concentration was estimated using the phenol-sulphuric acid method (Dubois et al. 1956). The estimation of glucan was carried out in 175 μL reaction mixture containing 25 μL precipitated glucan, 25 μL 5 % (w/v) phenol and 125 μL concentrated sulphuric acid mixed well in wells of a microtitre plate. The microtitre plate was incubated at 80 °C for 30 min and the absorbance at 490 nm (A_{490}) measured on a micro-plate reader. Dextran T40 (Sigma Aldrich, St. Louis, MO) was used as standard. The efficiency of glucan production was calculated as follows:

$$Efficiency(\%) = \frac{Amount\ of\ glucan\ produced}{Maximum\ possible\ amount\ of\ glucan(theoretical)} \times 100$$

where maximum possible amount of glucan (theoretical)= 10 mg/mL at 2 % sucrose concentration.

Analysis of glucansucrase by SDS-PAGE analysis and activity staining

The glucansucrase produced by isolate RBA12 was analysed by non-denaturing SDS-PAGE using a 7.5 % (w/v) gel following the method of Laemmli (1970). The cell-free supernatant obtained by centrifugation at 10,500*g* and 4 °C for 10 min was used as the enzyme source. Protein molecular mass markers (Fermentas, Loughborough, UK) were run on the gel with the enzyme samples. The samples were prepared in 0.0625 M Tris-HCl buffer (pH 6.8) containing 2.8 % (w/v)

SDS, 10 % (w/v) glycerol and 0.05 % (w/v) bromophenol blue, and loaded onto the gel without boiling. Tris-glycine buffer [200 mM glycine, 0.1 % (w/v) SDS, 50 mM Tris-HCl, pH 8.3] was used as running buffer. Electrophoresis was performed at 25 °C with a current of 2 mA per lane. After migration, the protein bands were stained using a silver staining protocol (Rabilloud 1992).

The activity of glucansucrase was determined in situ by the method described by Holt et al. (2001) with minor modifications (Purama and Goyal 2008). The cell free supernatant (40 μL) was loaded on a 7.5 % (w/v) polyacrylamide gel and run under non-denaturing conditions. For activity staining, the gel was washed three times in 20 mM sodium acetate buffer (pH 5.4) containing 0.3 mM $CaCl_2$ and 0.1 % (v/v) Tween 80, by incubating at 30 °C for 30 min. After washing, the gel was divided into two parts and subjected to periodic acid-Schiff (PAS) staining as follows: one part of the gel was incubated in 20 mM sodium acetate buffer (pH 5.4) supplemented with 10 % (w/v) sucrose at 30 °C for 16 h and the other part of the gel was incubated under the same conditions with 10 % (w/v) raffinose instead of sucrose. After incubation, both gel parts were washed twice with 75 % ethanol for 20 min and incubated in 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 for 1 h. Finally, the gels were stained with 15 mL Schiff reagent [0.5 % (w/v) basic Fuchsin, 1 % (w/v) sodium metabisulphite and 0.1 N HCl] until a discrete magenta color band was observed on the gel. The gels were washed in distilled water to remove the excess stain and stored in 10 % (v/v) acetic acid at 25 °C.

Production of oligosaccharides using acceptor in the culture medium

Oligosaccharides were produced by supplementing 2 % (w/v) maltose in the enzyme production medium (Tsuchiya et al. 1952). The medium was inoculated with 1 % (v/v) of isolate RBA12 and incubated at 20 °C and 180 rpm for 24 h. Samples (200 μL) were taken from the broth every 3 h up to 24 h. The oligosaccharides were purified from the cell-free supernatant by adding two volumes of absolute alcohol and centrifuging at 16,000*g* and 4 °C for 10 min to precipitate the polysaccharides (Côté and Leathers 2005). The 0.5 μL supernatant containing oligosaccharides was analysed by thin layer chromatography (TLC) at 25 °C using a silica gel 60 F_{254} TLC plate (Merck). The solvent system used was ethyl acetate/1-propanol/acetonitrile/water (4:10:14:11), by volume. The plate was visualised by spraying ethanol containing 0.5 % (w/v) α-naphthol and 5 % (v/v) H_2SO_4 and after heating at 120 °C (Kothari et al. 2012). The degree of polymerization (DP) of oligosaccharides was confirmed by ESI-TOF MS analysis (Agilent, Santa Clara, CA).

Results and discussion

Screening of glucansucrase-producing isolate

After growing Pummelo pulp in liquid MRS medium containing sucrose for 24 h and then plating on MRS agar plates containing $CaCO_3$, 20 colonies were isolated and analysed for glucansucrase production. All 20 isolates were grown in 10 mL enzyme production medium (Tsuchiya et al. 1952) and subsequently in 100 mL enzyme production medium as described in Materials and methods. The isolates were screened on the basis of glucansucrase activity. Of the 20 isolates, 1 gave maximum enzyme activity of 4.7 U/mL after screening (Fig. S1). This isolate was named RBA12 and selected for further characterisation and identification.

Identification of the selected isolate RBA12

Isolate RBA12 was identified based on various morphological, biochemical and physiological properties (Table 1). Gram staining of the isolate showed its Gram positive nature. SEM analysis revealed the isolate to be a short rod of 0.6 μm in diameter and 1–1.2 μm in length (Fig. 1a). The isolate RBA12 was catalase negative, and the hot loop test showed its heterofermentative nature by effervescence after introduction of a hot loop into the culture medium (Rao and Goyal 2013). The motility test on indole lysine agar indicated that isolate RBA12 is non motile and tryptophanase negative. Isolate RBA12 was able to produce NH_3 from arginine, and produced a mixture of D- and L-lactic acid (3.3 g/L and 2.8 g/L, respectively).

Isolate RBA12 could grow at temperatures 15 °C, 37 °C and 45 °C, and at pH range 3.0–4.0 but not at pH 5.0, 8.0 and 9.0. Isolate RBA12 grew in the presence of 2 % (w/v) and 8 % (w/v) NaCl but showed no tolerance to 10 % (w/v) NaCl. The carbohydrate utilization pattern of isolate RBA12 showed that it can ferment sucrose, mannose, salicin, arabinose, maltose, fructose xylose, cellobiose, galactose and glucose but not ferment lactose, adonitol, inulin, inositol, melibiose, trehalose, dulcitol, sorbitol, mannitol, rhamnose and raffinose (Table S1). The antibiogram of the isolate revealed that it is resistant to nalidixic acid, nitrofurantoin, sulphamethoxazole, co-trimoxazole, vancomycin, norfloxacin and cefixime; and sensitive to ampicillin, erythromycin, gentamicin, kanamycin, methicillin, penicillin and tetracycline (Table S2). Vancomycin resistance is a characteristic feature of LAB. The carbohydrate utilization pattern and antibiogram of isolate RBA12 was found to be quite similar to other *Weissella cibaria* isolates, with a few differences, e.g. the

Table 1 Morphological, biochemical and physiological characterization of *Weissella cibaria* RBA12

Characteristic		*Weissella cibaria* RBA12	*Weissella cibaria* JAG8[a]	*Weissella cibaria*[b]
Cell shape		Short rods	Short rods	Short rods
Cell arrangement		Single, pairs or chains	Single, pairs or chains	Single, pairs or chains
Gram stain		+	+	+
Motility		−	−	nd[c]
Indole test		−	−	nd
Nitrate reduction		−	−	nd
Catalase		−	−	−
Gas production from glucose		+	+	+
NH_3 production form arginine		+	nd	+
Lactic acid isomer		DL	nd	nd
Growth at temperature	15 °C	+	+	−
	37 °C	+	+	+
	45 °C	+	+	+
Growth at pH	3.0	−	nd	nd
	4.0	−	nd	−
	5.0	+	nd	nd
	8.0	+	nd	nd
	9.0	+	nd	−
Growth at NaCl	2 %	+	+	+
	4 %	+	+	+
	6 %	+	+	+
	8 %	+	−	nd
	10 %	−	−	−

[a] Rao and Goyal (2013)

[b] Patil et al. (2010)

[c] Not determined

Weissella cibaria isolates reported by Patil et al. (2010) could not ferment maltose and galactose.

Molecular characterization by 16S rRNA gene sequencing and phylogenetic analysis

BLAST analysis of 16S rRNA gene (1,372 bp) sequences showed that the new isolate RBA12 from Pummelo (*C. maxima*) was *Weissella cibaria* of the family Leuconostocaceae. The 16S rRNA gene sequence was submitted to the GenBank database and GenBank accession number KF515952 was assigned (http://www.ncbi.nlm.nih.gov/nuccore/536624219). The phylogenetic tree of isolate RBA12 showed a common evolutionary relationship with *Leuconostoc* and *Lactobacillus* spp. (Fig. 1b) and displayed the highest sequence similarity with *W. cibaria* AT6 (Fig. S2). The evolutionary relationship of *W. cibaria* RBA12 with other genera is similar to those reported for *W. cibaria* MSS1, MSS2, MSS3 and MBP5 by Thongsanit et al. (2009) and for *W. cibaria* DFR3 by Patil et al. (2010).

Effect of temperature and agitation on the production of glucansucrase

Production of glucansucrase from *W. cibaria* RBA12 was affected greatly by the incubation temperature. Maximum enzyme activity, 6.15 U/mL was observed at 20 °C and at 12 h under static conditions (Fig. 2a). Enzyme activity decreased above 20 °C, decreasing by 83 % at 37 °C and at 12 h due to deactivation of the enzyme at higher temperature, as also reported by Das and Goyal (2014). Enzyme activity decreased by 59 % at 16 °C and at 12 h but later increased after 15 h of incubation. This might be due to the slower growth rate of cells at lower temperatures, which would affect production of the enzyme glucansucrase. The effect of different shaking conditions from 80 to 200 rpm on glucansucrase production was studied and compared with the static conditions at 20 °C (Fig. 2b). The maximum enzyme activity of 7.2 U/mL, 14 % higher than under static conditions (6.15 U/ml), was observed at 180 rpm. The lag phase under shaking at 180 rpm was reduced to 4 h versus the prolonged lag phase of 6 h under static conditions (data not shown).

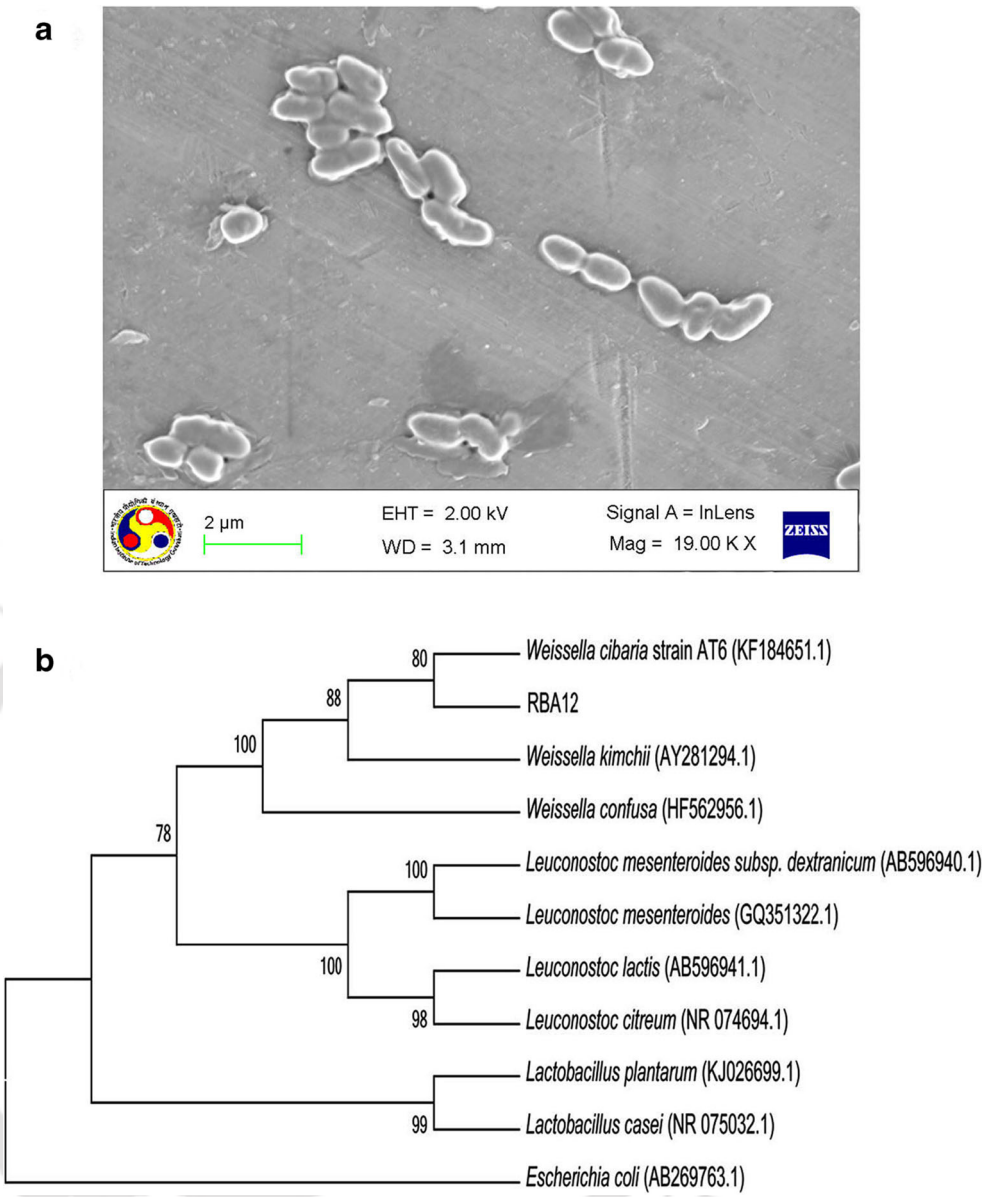

Fig. 1 **a** Scanning electron micrograph (SEM) of *W. cibaria* RBA12. **b** Phylogenetic tree based on 16S rRNA gene sequences

Fermentation profile of *Weissella cibaria* RBA12

Fermentation of *W. cibaria* RBA12 was studied by incubating the culture in 100 mL enzyme production medium at 20 °C and 180 rpm. The growth of cells was measured by measuring the absorbance at 600 nm (A_{600}). Cell growth, pH, enzyme activity, glucan concentration and sucrose utilisation during fermentation were also measured (Fig. 3). The lag phase continued up to 4 h followed by the exponential phase to 24 h followed by the stationary phase. The maximum enzyme activity of 7.2 U/mL was found at 12 h. The maximum enzyme activity of isolate RBA12 was higher than commercial strain *Leuconostoc mesenteroides* NRRL B-512 F (4.1 U/mL) (Shukla and Goyal 2011) and was also higher than other *Weissella* spp. such as *W. cibaria* JAG8 (5.8 U/mL) (Rao and Goyal 2013) and *W. confusa* Cab3 (6.1 U/mL) (Shukla et al. 2014). The pH of the broth decreased from 6.9 to 5.1 after 36 h of incubation. The pH at which maximum enzyme activity was observed was pH 6.6. The decrease in pH was negligible after 24 h due to the onset of stationary phase. The decrease in pH was due to the formation of lactic acid—a growth-associated product of LAB that is also responsible for inactivation of the enzyme. A glucan concentration of 8.3 mg/mL was observed at 24 h in the culture supernatant. The efficiency of glucan production was 83 %, which was quite high compared with the 32 % efficiency of glucan production from *W. cibaria* CMGDEX3 reported by Ahmed et al. (2012). The glucan concentration decreased slowly after 24 h, which might be due to the utilisation of glucan as a secondary substrate by the bacterium for maintenance of stationary phase

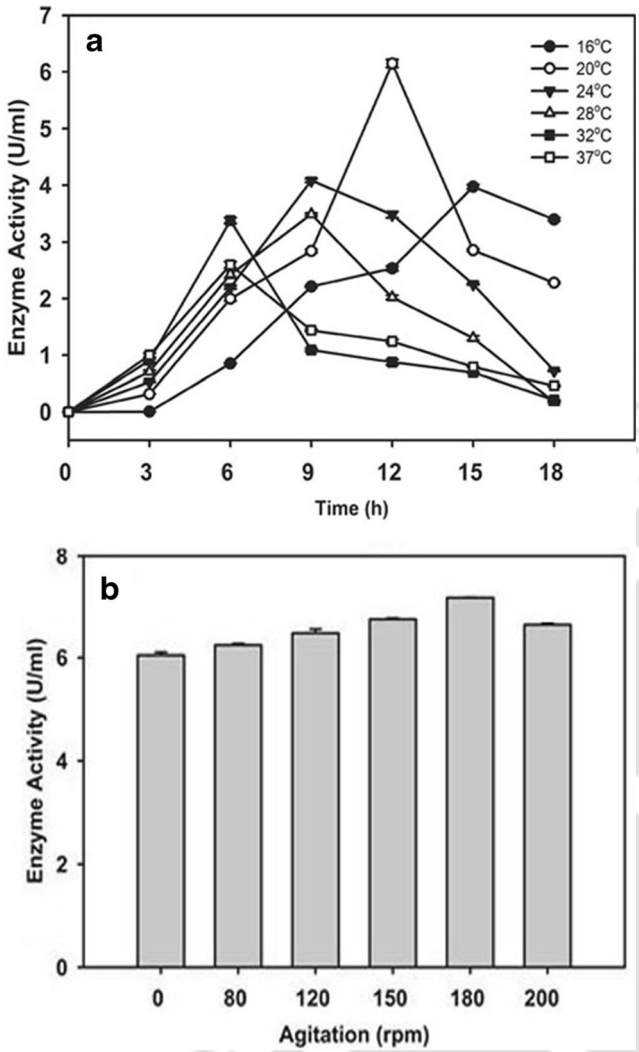

Fig. 2 **a** Effect of temperature under static conditions. **b** Effect of agitation speed (in rpm) on glucansucrase production from *W. cibaria* RBA12 at 20 °C

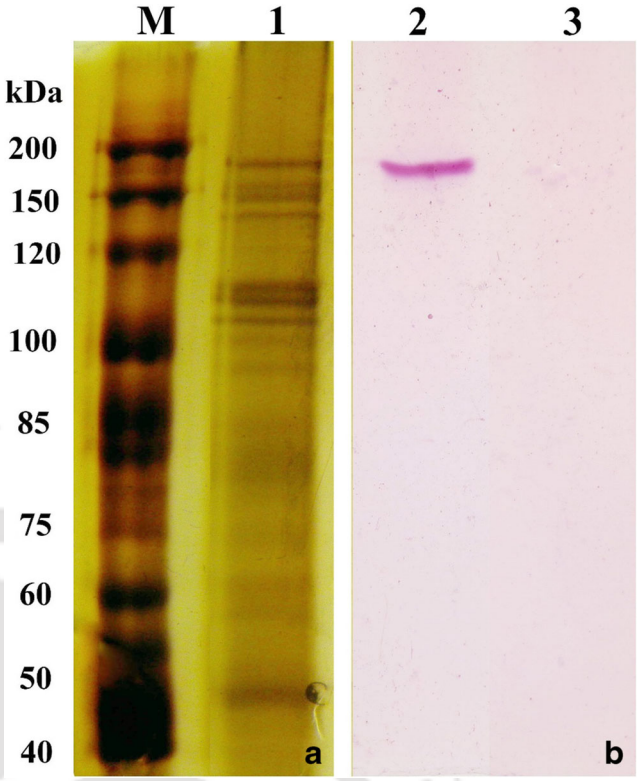

Fig. 4 **a** Non-denaturing SDS-PAGE analysis of glucansucrase on a 7.5 % acrylamide gel; **b** identification of glucansucrase by periodic acid-Schiff (PAS) staining. Lanes: *M* Protein molecular mass markers (40–200 kDa), *2* cell-free supernatant, *3* using sucrose, *4* using raffinose

after depletion of its primary substrate sucrose. The utilisation of sucrose by the isolate can be correlated with growth; an exponential decrease of sucrose is observed within the log phase of growth of the isolate. The sucrose was completely utilised after 24 h as the isolate entered stationary phase.

SDS-PAGE analysis and PAS staining of glucansucrase from *W. cibaria* RBA12

The molecular mass of glucansucrase from cell-free supernatant was determined by a 7.5 % (w/v) gel run on SDS poly-

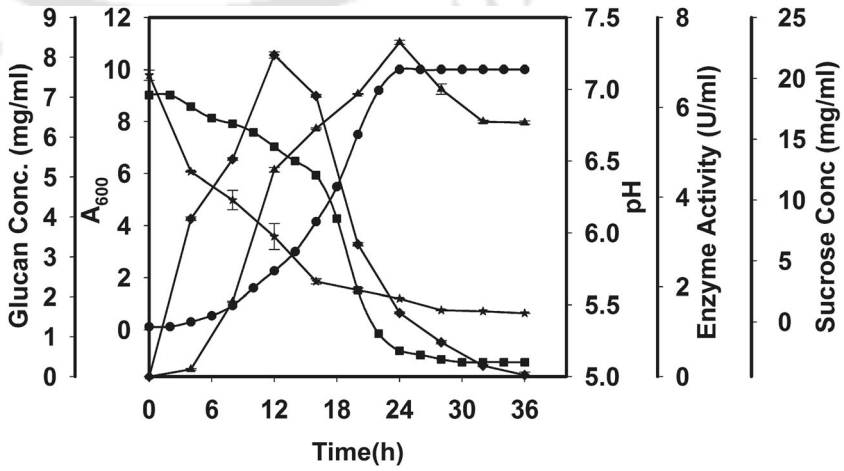

Fig. 3 Fermentation profile of *W. cibaria* RBA12 showing cell absorbance at 600 nm (A_{600}) (●), pH (■), enzyme activity (♦), glucan concentration (▲) and sucrose utilization (★)

acrylamide gel electrophoresis carried out under non-denaturing conditions, and in-situ activity detection. PAS staining of crude glucansucrase from the cell-free supernatant showed one activity band at approximately 180 kDa (Fig. 4, lane 2) when incubated with 10 % (w/v) sucrose solution, which corresponded to the band obtained in a 7.5 % (w/v) SDS gel subjected to silver staining (Fig. 4, lane 1). No band was observed in PAS staining of the enzyme when incubated with 10 % (w/v) raffinose (Fig. 4, lane 3). This excluded the possibility of the enzyme being fructansucrase. It has been reported that the molecular weight of extracellular glucansucrases are in the range of 120–200 kDa (Leemhuis et al. 2013) and that they can exist in multiple molecular forms (Purama and Goyal 2008; Patel et al. 2011). The presence of a single band after PAS staining showed that glucansucrase from *W. cibaria* RBA12 exists in a single molecular form. Similar single molecular forms were reported for glucansucrases from *W. cibaria* JAG8 (Rao and Goyal 2013) and *W. confusa* Cab3 (Shukla et al. 2014).

Production of oligosaccharides using an acceptor reaction in medium

The production of oligosaccharides by an acceptor reaction was carried out in culture medium supplemented with maltose and incubated at 20 °C under shaking at 180 rpm. Time-dependent analysis of acceptor reaction products was performed at 3, 6, 9, 12 and 24 h of incubation by TLC (Fig. 5a, lanes 1–5). There was gradual utilization of maltose and sucrose with time, resulting in the formation of a homologous series of isomalto-oligosaccharides (IMO). Maltose was utilised completely and the presence of isomalto-oligosaccharides was observed only after 24 h (Fig. 5a, lane

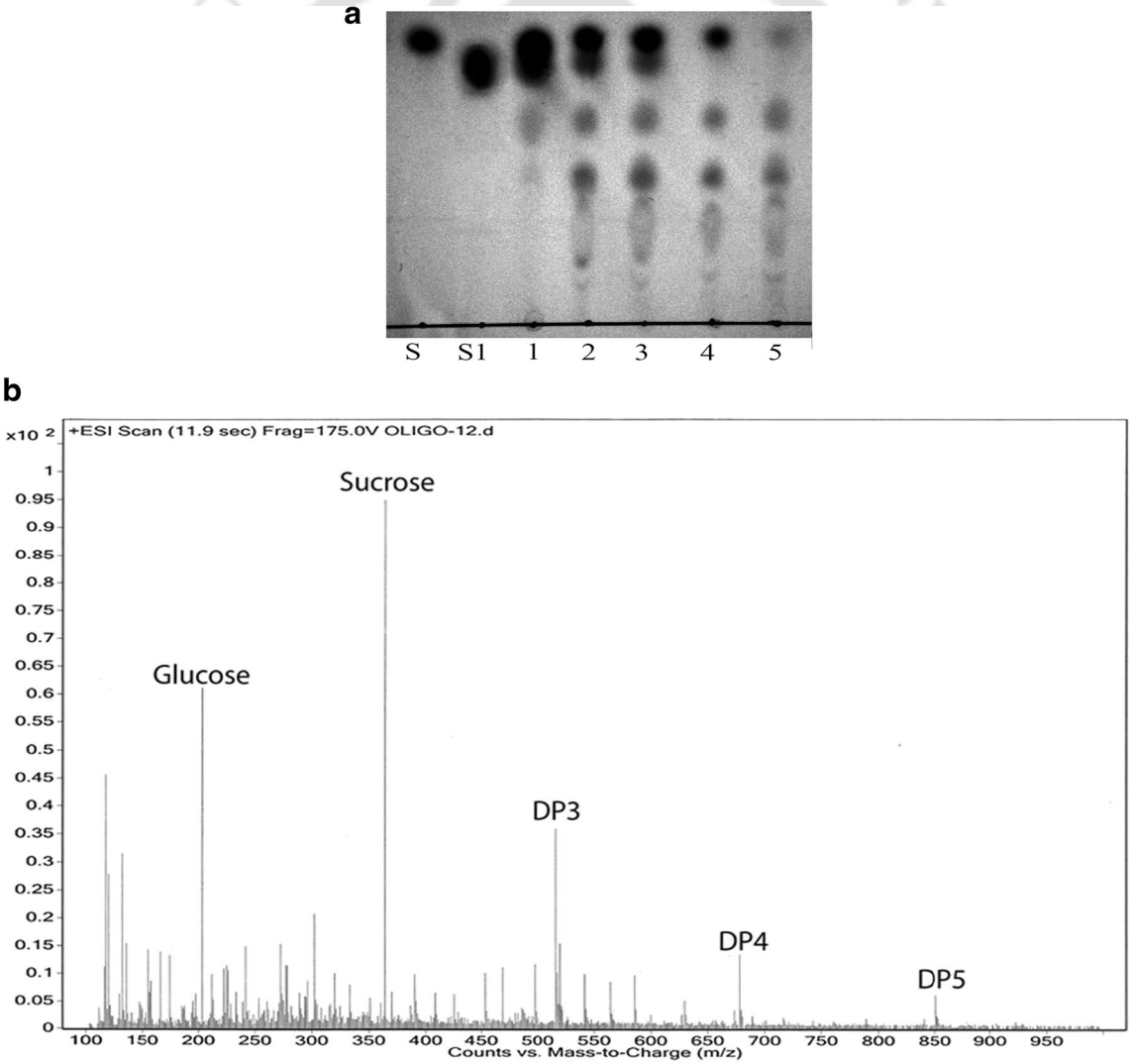

Fig. 5 **a** Thin layer chromatography (TLC) of time-dependent acceptor reaction using fermentation medium containing glucansucrase. Lanes: *S* sucrose, *S1* maltotriose, *1* 3 h medium, *2* 6 h medium, *3* 9 h medium, *4* 12 h medium, *5* 24 h medium. **b** ESI-TOF MS of gluco-oligosaccharides produced using 12 h fermentation medium in positive ion mode

5). The first product of the maltose acceptor reaction is panose (6-O-a-glucopyranosylmaltose)—a trisaccharide. This trisaccharide further acts as an acceptor, which results in the generation of a series of IMO with an increasing degree of polymerization linked to the 6-hydroxyl group of the non-reducing end glucose residue of maltose as reported by Kothari et al. (2012). The series of IMO produced in the acceptor reaction included tri-saccharide, tetra-saccharides and penta-saccharides, as also confirmed by ESI-TOF-MS analysis (Fig. 5b).The peaks observed at m/z 527, 689, and 851 (as sodium adduct, $[M+Na]^+$) corresponded to tri-, tetra- and penta-saccharides.

Conclusions

Weissella cibaria RBA12 was isolated from the pulp of Pummelo (*Citrus maxima*) on the basis of glucansucrase production (4.7 U/mL) and characterized on the basis of morphological, biochemical and physiological properties. 16S rRNA gene sequencing confirmed the isolate as *Weissella cibaria*. This is the first report on the isolation of any LAB from Pummelo (*C. maxima*). The optimal conditions for glucansucrase production were 20 °C and 180 rpm, yielding an enzyme activity of 7.2 U/mL after 12 h of incubation. The fermentation profile of *W. cibaria* RBA12 revealed that glucansucrase production was growth-associated and that sucrose was utilised completely by the bacterium. The maximum glucan concentration was 8.3 mg/mL at 24 h with 83 % efficiency, confirming *W. cibaria* RBA12 as an efficient strain for glucan production. Non-denaturing SDS-PAGE analysis and PAS staining of culture supernatant of *W. cibaria* RBA12 revealed that the enzyme is a glucansucrase with a molecular size of approximately 180 kDa. IMO, namely trisaccharide, tetrasaccharide and pentasaccharide, were produced in the culture medium containing maltose as an acceptor. Further studies on the nature and properties of both glucansucrase and glucan from *W. cibaria* RBA12 will be required to elucidate potential applications. The production of oligosaccharides by *W. cibaria* RBA12 could be further augmented by optimizing the concentration of medium components and purification by gel filtration.

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Functional food applications of dextran from *Weissella cibaria* RBA12 from pummelo (*Citrus maxima*)

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ABSTRACT

Weissella cibaria RBA12 isolated from pummelo from Northeast India produces a dextran composed of 97% α-(1 → 6) linkages in the main chain and 3% α-(1 → 3) branched linkages. The *in vitro* prebiotic activity of dextran-RBA12 was explored. Dextran-RBA12 displayed enhanced growth of probiotic *Bifidobacterium* and *Lactobacillus* spp., and controlled growth of non-probiotic enteric bacteria. Dextran-RBA12 showed superior resistance to physiological barriers with a maximum hydrolysis of 0.51%, 0.31% and 0.24% by artificial gastric juice, α–amylase and intestinal fluid, respectively, whereas compared to maximum hydrolysis of 25.23%, 19.13% and 6%, respectively after 5 h of incubation shown by commercial prebiotic inulin. The production of dextran from *Weissella cibaria* RBA12 in sourdough prepared from whole wheat flour, wheat bran and rye bran showed the highest dextran of 3.26 ± 0.12% d.w. in rye bran. The overall study summarized that dextran-RBA12 can be used as a prebiotic and also can be easily produced in sourdough.

1. Introduction

Dextrans are homopolysaccharides of D-glucose units that are α-(1 → 6) linked in the main chains with a various degree of α-(1 → 2), α-(1 → 3), or α-(1 → 4) branched linkages (Capek et al., 2011). Lactic acid bacteria (LAB) of the genera *Leuconostoc*, *Streptococcus*, *Weissella*, *Pediococcus* and *Lactobacillus* synthesize dextrans using sucrose as a substrate (Falconer et al., 2011). The structure of dextrans such as chain length and degree of branching is dependent on the strain producing it and along with the fermentation conditions to some extent and the available nutrients (Heinze et al., 2006). The biological activities of dextran are determined by their glycosidic linkages, monosaccharide composition and degrees of polymerization (Delattre and Vijayalakshmi, 2009).

Dietary fibres are the oligosaccharides, polysaccharides and their derivatives which cannot be digested by the human digestive enzymes into absorbable components in the upper alimentary tract (Thebaudin et al., 1997). Dietary fibres are a necessary part of human nutrition with various benefits to health which includes a reduction in bowel transit time, reduction in risk of colorectal cancer, prevention of constipation, production of short chain fatty acids lowering of blood cholesterol, and promotion of the growth of beneficial gut microbiota (Brennan and Cleary, 2005). Probiotics are defined as a live microbial feed supplement which confers various beneficial affects to its host by improving the intestinal environment and native microbiota (Das and Goyal, 2014). Prebiotics are the compounds which result in the selectively stimulate the growth and/or activity(ies) of probiotic microorganisms that confers health benefits to its host. Large polysaccharides such as dextran form a persistent source of fermentable carbohydrate throughout the colon for the resident microbiota rather than being completely fermented proximally. It was also reported that dextran and oligo-dextran which consist of α-(1 → 6) glycosidic backbones are less susceptible to attack by human and animal digestive enzymes (Kothari et al., 2015). Marine lactic acid bacteria including *Pediococcus pentosaceus*, *Weissella confusa*, *Weissella cibaria* and, *Lactobacillus plantarum* were able to produce dextran which showed prebiotic properties. These dextrans being resistant to hydrolysis by physiological barriers such as stomach acid and human pancreatic amylase are also utilized as a carbon source by probiotic bacteria *Bifidobacterium bifidum* (Hongpattarakere et al., 2012).

Sourdough is a pre-dough produced by the fermenting micro-organisms such as LAB and yeasts. Dextran from several microbial sources has been used in sourdough baking (Kajala et al., 2015a). A low degree of branching and a high molecular weight are the key features of dextrans for the positive impact on sourdough bread quality (Lacaze et al., 2007). Dextran present in liquid sourdough has been reported to improve the

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volume (up to 12%), moist feel in the mouth and softness of rye breads and rye mixed breads (Lacaze et al., 2007). In wheat breads, the presence of dextrans showed improvement in mouthfeel and freshness after storage and it also increased the bread volume as compared with the sourdough breads in absence of dextran (Kajala et al., 2015b).

The present study investigates the structural composition and functional applications of dextran from *Weissella cibaria* RBA12 that was isolated from pummelo of Northeast India (Baruah and Goyal, 2015). The structural characterization was carried out by FT-IR and NMR (^{1}H and ^{13}C) spectroscopy. The *in vitro* studies were carried out on dextran-RBA12 for its prebiotic applications. The *in situ* production of dextran by *Weissella cibaria* RBA12 in whole wheat flour, wheat bran and rye bran was studied in order to assess its application in the sourdough fermentation.

2. Materials and methods

2.1. Chemicals, media and microorganism

The media components for Tryptone-Glucose-Yeast extract (TGY) medium and Man Rogosa Sharpe (MRS) medium, the chemicals for carbohydrate estimation, reducing sugar assay and standard prebiotic inulin were purchased from Hi-Media Pvt. Ltd. (Mumbai, India). The bile salts and enzymes used were obtained from Sigma–Aldrich, (USA). *Weissella cibaria* RBA12 (GenBank accession no: KF515952) producing glucansucrase and glucan was isolated from the pulp of pummelo (*Citrus maxima*) (Baruah and Goyal, 2015). Probiotic strain *Lactobacillus plantarum* DM5 (GenBank accession number KC020195) previously isolated was available as glycerol stock in our laboratory. The other probiotic bacteria *Lactobacillus acidophilus* NRRL B-4495, *Bifidobacterium infantis* NRRL B-41661, *Bifidobacterium bifidum* NRRL 41410 and *Bifidobacterium animalis* subsp. *lactis* NRRL 41405 were procured from Agricultural Research Service Culture Collection (Peoria, USA). The enteric strain *Enterobacter aerogenes* MTCC 7016 was procured from Microbial Type Culture Collection (MTCC) and Gene Bank, Institute of Microbial Technology (IMTECH), Chandigarh, India. *E. coli* DH5α were available as glycerol stock in our laboratory.

2.2. Production and purification of dextran from Weissella cibaria RBA12

The production of dextran-RBA12 was carried out by transferring 1% (v/v) inoculum of *Weissella cibaria* RBA12 in 100 ml enzyme production medium containing 2% (w/v) sucrose, 2% (w/v) yeast extract, 2% (w/v) K_2HPO_4, 0.02% (w/v) $MgSO_4 \cdot 7H_2O$, 0.001 (w/v) $MnSO_4 \cdot 4H_2O$, 0.001% (w/v) $FeSO_4 \cdot 7H_2O$, 0.001% (w/v) $CaCl_2 \cdot 2H_2O$, 0.001% (w/v) NaCl (Tsuchiya et al., 1952) and incubated at 20 °C and 180 rpm for 24 h. The broth was centrifuged at 10,000 × *g* at 4 °C for 10 min to obtain the cell free supernatant. The cell pellet was discarded and the cell free supernatant containing dextran-RBA12 was precipitated by adding 95% (v/v) chilled ethanol in a ratio of 3:1 (ethanol: supernatant) as reported earlier by Das et al. (2014). The mixture was vortexed and then centrifuged at 10,000 × *g* at 4 °C for 30 min and the resulting pellet was resuspended in deionized water. The ethanol precipitation steps were repeated three times to eliminate any sugar contamination and further purification of dextran-RBA12 was carried out by gel filtration using Sephacryl S-500 HR (Sigma–Aldrich, USA) on a FPLC system (AKTA purifier 100 Plus, GE Healthcare, USA) using Milli-Q water (18.2 MΩ·cm) as the eluent at constant flow rate of 1 ml/min. Phenol-sulphuric acid method (Dubois et al., 1956) was used for determining the concentration of dextran-RBA12. The dextran-RBA12 was lyophilized for further analysis.

2.3. Monosaccharide analysis

Purified dextran-RBA12 (2 mg/ml) was hydrolyzed by 2 M trifluoroacetic acid (TFA) at 100 °C for 2 h as reported earlier (Yang et al., 2009). After removing the TFA using hot-air oven at 80 °C, the released monosaccharaides were analyzed by HPAEC-PAD using an ion-chromatography system (ICS-3000 system, Dionex Corporation, USA equipped with the ICS-3000 detector/chromatographic module) using CarboPac PA-20 analytical column (3 mm × 150 mm) and the CarboPac PA-20 guard column (3 mm × 30 mm). The temperature of the column was adjusted to 30 °C and the injection volume was 25 μl. The eluent 100 mM NaOH and 1 M sodium acetate gradient were used at a constant flow rate of 1 ml/min with isocratic elution. The monosaccharide was detected with an electrochemical detector (ED 50). Glucose and fructose (Sigma–Aldrich, USA) were used as standards. Deoxygalactose (Sigma-Aldrich St. Louis, MO, USA) was used as an internal standard in a concentration of 50 μg/ml. The samples were analyzed in triplicates.

2.4. FTIR and NMR spectroscopic analyses of dextran-RBA12

The Fourier Transform infra-red (FTIR) spectrum of the purified dextran-RBA12 was recorded by making a KBr pellet using FTIR spectrophotometer (Spectrum Two, Perkin Elmer Instruments, Waltham, MA, USA) for identification of functional groups, monomeric units and linkages.

^{1}H and ^{13}C NMR spectra for the purified dextran-RBA12 were recorded on a 600 MHz NMR spectrometer (Bruker Avance III HD equipped with Topspin software package from Bruker, MA, USA) with operating frequencies 600 MHz for ^{1}H- and 100 MHz for ^{13}C NMR to determine the type of glycosidic linkage present. The purified dextran samples were dissolved in D_2O (99.96%) (Merck, Germany) at concentrations of 5 mg/ml (for ^{1}H NMR) and 30 mg/ml (for ^{13}C NMR).

2.5. Effect of dextran-RBA12 on the growth of probiotic bacteria

The growth profiles of probiotic bacteria (*L. plantarum* DM5, *L. acidophilus* NRRL B-4495, *B. infantis* NRRL B-41661, *B. bifidum* NRRL 41410 and *B. animalis* NRRL 41405) along with non-probiotic enteric bacteria (*E. coli* DH5α and *E. aerogenes* MTCC 7016) were studied in the presence of dextran-RBA12 and inulin as standard prebiotic. The growth profiles of probiotic bacteria were analyzed using modified-MRS medium (pH 6.4) devoid of carbon source but supplemented with 0.5 mg/ml cysteine (Hongpattarakere et al., 2012). The probiotic bacterial cultures (~10^6 CFU/ml) were transferred to 5 ml MRS medium containing 1% (w/v) of glucose, dextran-RBA12 or standard prebiotic inulin and incubated under anaerobic conditions at 37 °C for 24 h. The growth of enteric bacteria was evaluated in TGY medium (pH 7.0) containing tryptone (5 g/l), glucose (1 g/l), yeast extract (5 g/l) and di-potassium hydrogen phosphate K_2HPO_4 (1 g/l). The enteric mixture of non-probiotic *E. coli* DH5α and *E. aerogenes*

MTCC 7016 was transferred to 5 ml of TGY medium supplemented with 1% (w/v) of glucose, dextran-RBA12 or standard prebiotic inulin and incubated under anaerobic conditions at 37 °C for 24 h. The microbial growth of probiotic bacteria and enteric bacteria was enumerated by plate count method and expressed as CFU/ml by growing on MRS agar and TGY agar plate, respectively, at 37 °C. The enumeration was done after for 12 h and 24 h.

The prebiotic activity of dextran-RBA12 and inulin was estimated by the method of Huebner et al. (2007).

$$\text{Prebiotic activity score} = \left\{\frac{\left(\text{Probiotic logCFU ml}^{-1}\text{ on prebiotic at 24 h}\right)-\left(\text{Probiotic logCFU ml}^{-1}\text{ on prebiotic at 0 h}\right)}{\left(\text{Probiotic logCFU ml}^{-1}\text{on glucose at 24 h}\right)-\left(\text{Probiotic logCFU ml}^{-1}\text{ on glucose at 0 h}\right)}\right\} - \left\{\frac{\left(\text{Enteric logCFU ml}^{-1}\text{ on prebiotic at 24 h}\right)-\left(\text{Enteric logCFU ml}^{-1}\text{ on prebiotic at 0 h}\right)}{\left(\text{Enteric logCFU ml}^{-1}\text{ on glucose at 24 h}\right)-\left(\text{Enteric logCFU ml}^{-1}\text{ on glucose at 0 h}\right)}\right\}$$

2.6. Hydrolysis of dextran-RBA12 by artificial gastric juice

Dextran-RBA12 and commercial prebiotic inulin were analyzed for their hydrolysis in the presence of artificial gastric juice by the method as described by Korakli et al. (2002). The artificial gastric juice consisted of 1000 U/ml of pepsin in 1× Phosphate Buffer Saline (PBS) (Al-Sheraji et al., 2012). The pH of artificial gastric juice was adjusted at 1, 2, 3 and 4 by 4 N HCl. The artificial gastric juice (10 ml) of each pH containing dextran-RBA12 or inulin was incubated at 37 °C for 5 h. An aliquot of 500 μl from the reaction mixture was collected at intervals of 0, 0.5, 1, 2, 3, 4 and 5 h (Das et al., 2014). Phenol-sulphuric acid method (Dubois et al., 1956) was used determine the total sugar content, using glucose as standard. The reducing sugar present in the sample was determined by the methods of Nelson (1944) and Somogyi (1945). The percent hydrolysis of the sample was estimated by the total sugar content and reducing sugar liberated from the samples (Korakli et al., 2002) as follows;

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

2.7. Hydrolysis of dextran-RBA12 by α-amylase and intestinal fluid

The hydrolysis of dextran-RBA12 and inulin by α-amylase was analyzed by the method as described by Al-Sheraji et al. (2012). Dextran-RBA12 and inulin (100 mg) were separately dissolved in 10 ml of α-amylase (100 U/ml) in 1× PBS buffer of pH in a range of pH 5, 6, 7 and 8 and incubated at 37 °C for 5 h. The reducing sugar and total sugar content of the sample was analyzed at time intervals of 0, 0.5, 1, 2, 3, 4 and 5 h for calculating the percentage hydrolysis of dextran-RBA12 and inulin as described earlier in Section 2.6 (Das et al., 2014).

The intestinal fluid was composed of 0.5% (w/v) bile salt and 1000 U/ml of trypsin solution in 1× PBS buffer at pH 8.0 (Fernandez et al., 2003). The 1.0% (w/v) of each dextran-RBA12 and inulin were separately dissolved in 10 ml intestinal fluid and incubated at 37 °C for 5 h. The reducing sugar and total sugar content of the sample was analyzed at time intervals of 0, 0.5, 1, 2, 3, 4 and 5 h and the percentage hydrolysis of dextran-RBA12 and inulin were calculated as described earlier in Section 2.6 (Das et al., 2014).

2.8. Sourdough fermentations

Weissella cibaria RBA12 was sub-cultured for 24 h in static condition at 28 °C twice in MRS-S medium before sourdough fermentations. Cells were harvested from 5 ml overnight culture by centrifugation in 10,000 × *g* at 4 °C for 10 min and re-suspended in sterile tap water. The stock for inoculation consisted of a cell density of 10^9 CFU/ml.

Sourdough fermentations were carried out using whole wheat flour (Mylly-Matti, Helsingin Mylly, Finland), wheat bran and rye bran (Fazer, Finland) as cereal substrates. All the fermentations were carried out with 10% (w/w) sucrose supplementation using a total weight of 200 g. The samples for fermentation consisted of 160 g of sterile tap water, mixed with 0.2 ml of cell suspension (5×10^5–1×10^6 CFU/g) and 36 g of cereal substrate, a part of the matrix was replaced with the 4 g of sucrose. The cells were mixed with the water and sucrose along with cereal matrix and incubated at 25 °C for 20 h (Katina et al., 2009). Microbial enumeration of the samples was carried out at 0 and 20 h using MRS agar plates. In addition, samples for pH, dextran and free sugar quantification were collected at 20 h. The samples were flash-frozen using liquid nitrogen and later stored at −20 °C. The samples were lyophilized for dextran and sugar analysis before analysis.

2.9. Enzyme-assisted method for dextran analysis of sourdough

Dextran produced in the bran fermentations was measured by the method described by Katina et al. (2009). The dextran formed was hydrolyzed to glucose monomers by enzyme hydrolysis using the combined activities of dextranase (Sigma-Aldrich, St. Louis, MO, USA) and transglucosidase (Megazyme, Ireland). The glucose produced was quantified with high performance anion exchange chromatography coupled with a pulsed amperometric detector (HPAEC-PAD). The sum of anhydro-glucose was used to estimate the amount of dextran, which was corrected by a recovery factor of 1.45 taking into account the recovered dextran after purification from the cereal matrix. The recovery factor (1.45) was determined the method as described by Katina et al. (2009), using purified *W. confusa* VTT E90392 dextran and the cereal matrix used in this study.

Dextran (% d w) was calculated according to the equation of Katina et al. (2009).

$$(\%\text{d.w.}) = \frac{\text{Concentration of glucose} \times 5 \times 0.9 \times 1.45}{100} \times 100$$

All amounts are percentages of dry weight bases (% d.w.). Glucose (mg/ml) produced by hydrolysis of dextran was analyzed. In the formula, 0.9 is a correction factor for anhydro-glucose and 1.45 is a correction factor determined for the recovery of dextrans studied in cereal matrices.

2.10. HPAEC-PAD analysis of sugars from sourdough

Lyophilized fermented samples (100 mg) dissolved in 5 ml of 0.05 M sodium citrate buffer, pH 5.5 was used for the quantification of free sugars (glucose, fructose, sucrose and maltose). The samples were vortexed and centrifuged at 10,000g for 15 min and the supernatants were collected and boiled in a boiling water bath for 10 min (Kajala et al., 2015a). After cooling, 500 μl of the sample was transferred to centrifugal filter units (Amicon Ultra 0.5 ml, 10 K Merck, MA, USA) and centrifuged at 10,000g for 20 min. 400 μl of the filtrate was collected and diluted with Milli-Q water to a final volume of 1 ml. Deoxygalactose (Sigma-Aldrich St. Louis, MO, USA) was used as an internal standard in a concentration of 50 μg/ml for the standards and sourdough samples which was measured by HPAEC-PAD (Juvonen et al., 2015). The sugar analyses were carried out using a solution of 100 mM NaOH and 1 M sodium acetate gradient at a flow rate of 1 ml/min as described by Katina et al. (2009). The samples were filtered through a 0.45 mm nylon membrane and the sample injection volume was 10 μl. A standard curve was prepared for quantification of glucose, fructose, maltose and sucrose (Sigma-Aldrich, St. Louis, MO, USA). Glucose and fructose concentration were in a range of 10–200 μg/ml and 10–500 μg/ml for sucrose and maltose. All samples were analyzed in triplicates.

3. Results

3.1. Production and characterization of dextran from Weissella cibaria RBA12

In present study the cell-free supernatant of *Weissella cibaria* RBA12 gave 8.3 mg/ml dextran concentration as reported earlier (Baruah and Goyal, 2015). The monosaccharide analysis of purified dextran from *Weissella cibaria* RBA12 after hydrolysis with 2 M TFA showed that it was composed of only D-glucose residues which indications its glucan nature (Fig. 1S).

3.2. FT-IR analysis

The FT-IR spectrum of purified dextran from *Weissella cibaria* RBA12 is shown in Fig. 1, showing numerous peaks between 3406 and 531 (1/cm). The peak in the region of 3400 (1/cm) was due to the hydroxyl (—OH) stretching vibration of the polysaccharide as reported earlier by Purama et al. (2009). The peak in the region of 2930 (1/cm) was due to C—H stretching vibration and the band in the region of 1639 (1/cm) was due to the carboxyl group (Cao et al., 2006; Liu et al., 2007). The presence peaks in the 950–1200 (1/cm) region is a fingerprint region for all polysaccharides (Černá et al., 2003). The peak at 1154 (1/cm) is due to valent vibrations of C—O—C bond and a glycosidic bridge. The broad band at 1107 (1/cm) is due to the vibration of the C—O bond at the C-4 position of a glucose residue (Shingel, 2002). The presence of a peak at 1020 (1/cm) is due to the great chain flexibility present in dextran around the α(1 → 6) glycosidic bonds as shown earlier (Shingel, 2002). The peak at 915 (1/cm) is a characteristic of α-glycosidic bond.

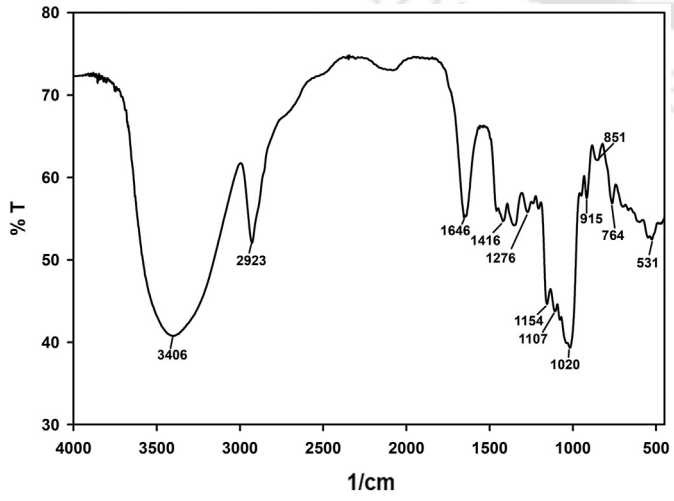

Fig. 1. FT-IR spectrum of dextran from *Weissella cibaria* RBA12.

3.3. NMR analysis

The ^{1}H NMR and ^{13}C NMR spectra of purified dextran-RBA12 are shown in Fig. 2A and B, respectively. The ^{1}H NMR spectrum of dextran-RBA12 showed an anomeric signal at 5.02 ppm as reported earlier (Maina et al., 2008; Ahmed et al., 2012) for α-(1 → 6) linked dextran. Another low-intensity signal at 5.36 ppm was observed in the ^{1}H NMR spectrum which is attributed to the presence of α-(1 → 3) linked

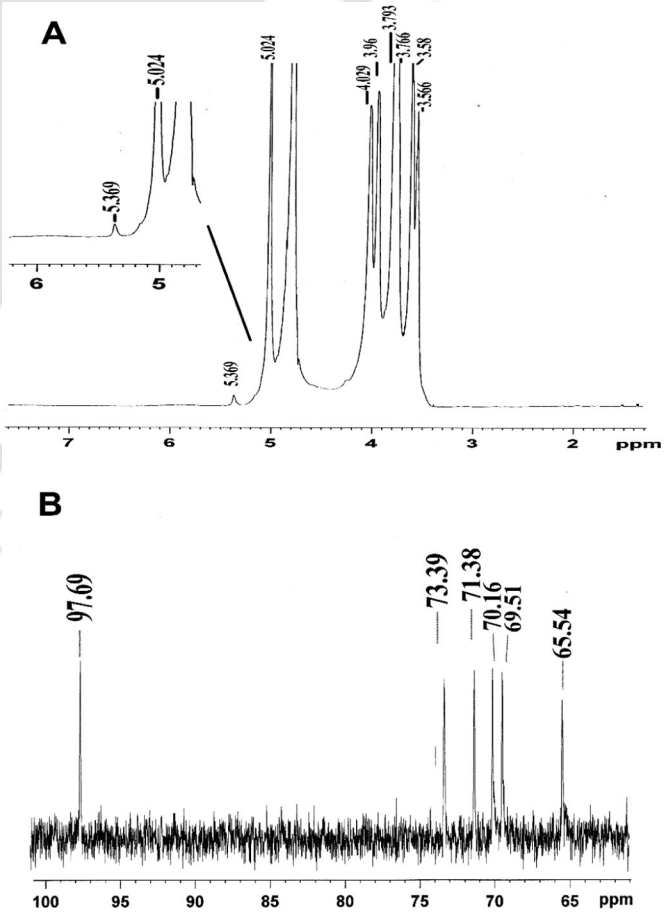

Fig. 2. A) ^{1}H NMR spectrum of dextran from *Weissella cibaria* RBA12; B) ^{13}C NMR spectrum of dextran from *Weissella cibaria* RBA12.

branches (Ahmed et al., 2012; Shukla et al., 2014). The percentage of α-(1 → 3) linkage calculated from integration analysis of the anomeric signals was 3%. The remaining five resonances in the ^{1}H NMR spectrum at 4.02, 3.96, 3.79, 3.76, 3.58 and 3.56 ppm (Fig. 2A), corresponded to H-6b, H-5, H-6a, H-3, H-2 and H-4 positions, respectively.

The ^{13}C NMR spectrum of purified dextran from *Weissella cibaria* RBA12 showed the major resonance in the anomeric region at 97.7 ppm and C-6 resonance occurred at 65.5 ppm (Fig. 2B), indicating the presence of C-1 and C-6 linkages typically seen in α-(1 → 6) glycosidic bonds in dextran as reported earlier by Ahmed et al. (2012). The other signals observed at 71.4, 73.4, 69.5 and 70.2 ppm corresponded to C-2, C-3, C-4 and C-5 positions, respectively.

3.4. In vitro prebiotic properties of dextran-RBA12

3.4.1. Effect of dextran-RBA12 on the growth of probiotic bacteria

The growth of probiotic bacteria namely *L. plantarum* DM5, *L. acidophilus* NRRL B-4495, *B. animalis* NRRL 41405, *B. bifidum* NRRL 41410 and *B. infantis* NRRL B-41661 in the presence of dextran-RBA12/inulin was compared with the growth in the presence of glucose (Table S1). Dextran-RBA12 stimulated the growth of *L. plantarum* DM5, *L. acidophilus* NRRL B-4495, *B. animalis* NRRL 41405, *B. bifidum* NRRL 41410 and *B. infantis* NRRL B-41661 from 8.0, 7.4, 7.0, 5.4 and 7.6 to 9.2, 9, 9.5, 7.7 and 9.6 $\log_{10}$ CFU/ml, respectively, after 24 h. Similarly, the presence of inulin (commercial prebiotic) stimulated the growth of probiotic bacteria *L. plantarum* DM5, *L. acidophilus* NRRL B-4495, *B. animalis* NRRL 41405, *B. bifidum* NRRL 41410 and *B. infantis* NRRL B-41661 from 6.9, 6.8, 6.2, 5.4 and 6.6 to 9.7, 9.6, 9.8, 7.6 and 9.7 $\log_{10}$ CFU/ml, respectively, after 24 h (Table S1). The prebiotic activity score signifies the ability of a substrate to stimulate the growth of probiotic strains as compared to non-probiotic strains and also to that of non-prebiotic substrate glucose. The prebiotic potential of dextran-RBA12 was expressed using the prebiotic activity score. The prebiotic activity score of all the probiotic bacteria in the presence of both dextran-RBA12 and inulin are shown in Fig. 3. Dextran-RBA12 showed good prebiotic score from 0.26 (*L. plantarum* DM5) to 0.42 (*B. animalis* NRRL 41405) along with 0.3 (*L. acidophilus* NRRL B-4495), 0.42 (*B. bifidum* NRRL 41410) and 0.38 (*B. infantis* NRRL B-41661). Inulin showed a prebiotic score of 0.85 (*L. plantarum* DM5), 0.66 (*L. acidophilus* NRRL B-4495), 0.62 (*B. animalis* NRRL 41405), 0.33 (*B. bifidum* NRRL 41410) and 0.69 (*B. infantis* NRRL B-41661).

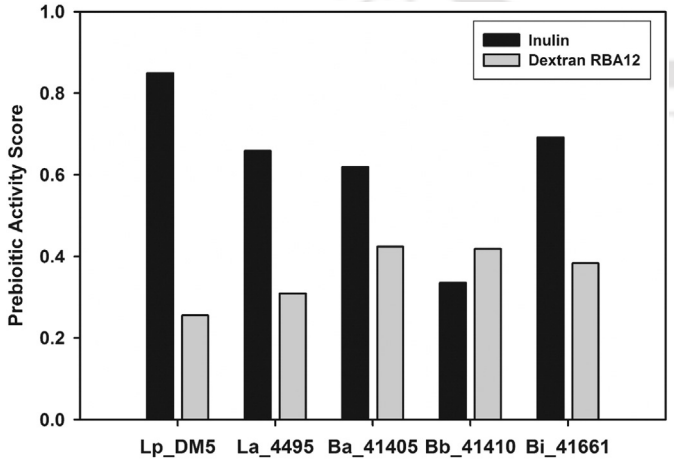

Fig. 3. Prebiotic activity score of dextran-RBA12 and commercial prebiotic inulin. The prebiotic activity score of the two EPS was determined by using the probiotic strains *L. plantarum* DM5, *L. acidophilus* NRRL B-4495, *B. animalis* NRRL 41405, *B. bifidum* NRRL 41410 and *B. infantis* NRRL B-41661 and a consortium of non-probiotic bacteria containing *E. coli* DH5α and *E. aerogenes* MTCC 7016.

3.4.2. Hydrolysis of dextran-RBA12 by artificial gastric juice

The percentage of hydrolysis of dextran artificial gastric juice was studied at different time intervals from 0.5 h to 5 h at 37 °C. The purified dextran from *Weissella cibaria* RBA12 proved to be very resistant to hydrolysis by artificial gastric juice at different pH ranging from 1.0 to 4.0 as compared with standard prebiotic inulin. The percent hydrolysis of purified dextran-RBA12 steadily decreased with the increase of pH. After 1 h of incubation, the percent hydrolysis was 0.32%, 0.28%, 0.26% and 0.17% at pH of 1, 2, 3 and 4, respectively (Fig. 4A). After 5 h of incubation, the percent hydrolysis of purified dextran-RBA12 was 0.51%,

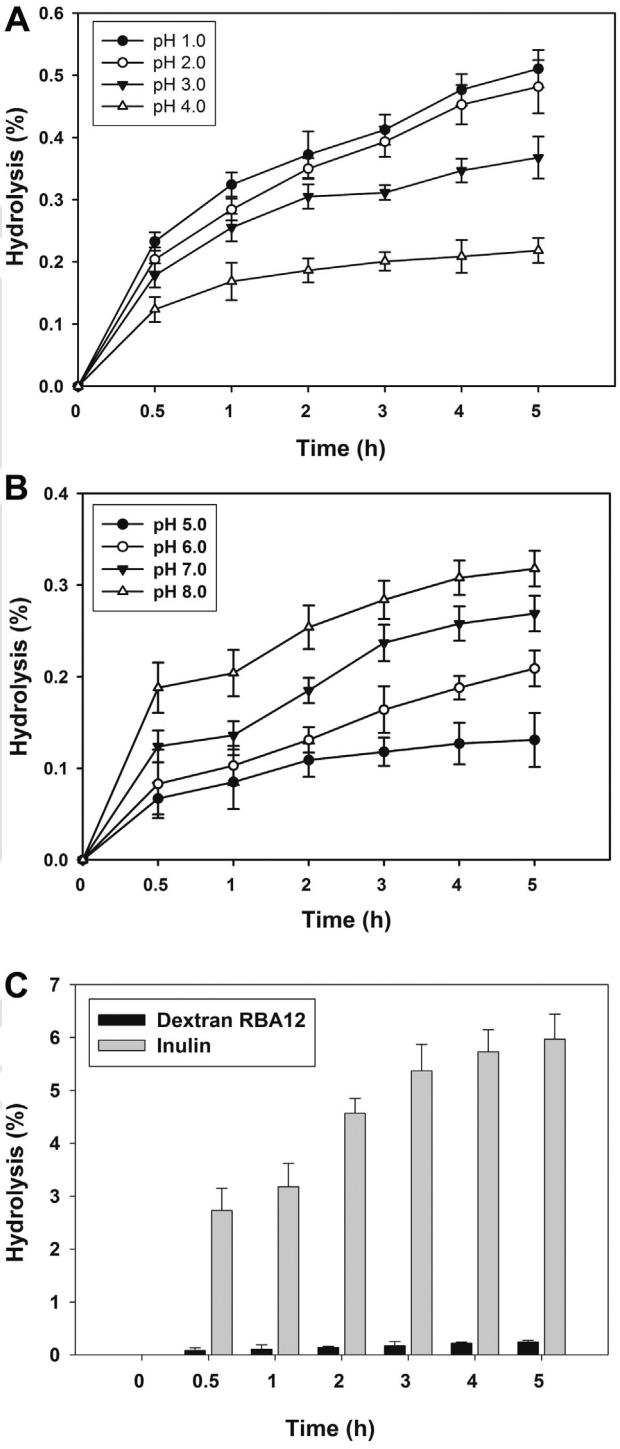

Fig. 4. A) Hydrolysis of dextran-RBA12 with artificial gastric of pH ranging from 1 to 4; B) hydrolysis of dextran-RBA12 with α-amylase of pH ranging from 5 to 8; C) hydrolysis of dextran-RBA12 and commercial prebiotic inulin with intestinal fluid in pH 8.0.

0.48%, 0.37% and 0.21% at pH 1, 2, 3 and 4, respectively (Fig. 4A). The percent hydrolysis of inulin after 1 h of incubation also decreased with the increase in pH. The percent hydrolysis was 16.53%, 12.22%, 8.27% and 4.11% at pH 1, 2, 3 and 4, respectively (Fig. S2A). The percent hydrolysis of inulin after 5 h was much higher *i.e.* 25.23%, 21.20%, 11.66% and 4.33% at pH 1, 2, 3 and 4, respectively (Fig. S2A).

3.4.3. Hydrolysis of dextran-RBA12 by α-amylase and intestinal fluid

The hydrolysis of purified dextran-RBA12 and inulin at pH 5–8 by α-amylase showed that the percent of hydrolysis increased with the increase of pH. After 1 h of incubation, the percent hydrolysis of dextran-RBA12 was 0.08%, 0.10%, 0.13% and 0.2% at pH 5, 6, 7 and 8 respectively, at 37 °C (Fig. 4B). After 5 h of incubation, the percent hydrolysis of dextran-RBA12 was 0.13%, 0.20%, 0.27% and 0.31% at pH 5, 6, 7 and 8 respectively. In case of inulin after 5 h of incubation, the hydrolysis was 8.1%, 12.7%, 15.8% and 19.1% at pH 5, 6, 7 and 8, respectively, at 37 °C (Fig. 2SB). The dextran-RBA12 showed its maximum hydrolysis of 0.31% at pH 8 after 5 h of incubation at 37 °C whereas commercial prebiotic inulin displayed 19.1% under the same conditions.

In the presence of intestinal fluid, dextran-RBA12 displayed a percent hydrolysis of only 0.24% after 5 h of incubation, as compared to percent hydrolysis of 6.2% shown by commercial prebiotic inulin (Fig. 4C).

3.5. Analysis of dextran and sugar from sourdough fermentations using cereal matrices

The cell count for the whole wheat flour, wheat bran and rye bran were 8.7, 9.3 and 9.5 $\log_{10}$ CFU/g, respectively, after 20 h of incubation. The cell growth in the three cereals was almost the same with 3 $\log_{10}$ CFU/g increase (Table 1). The pH of sourdough samples of the whole wheat flour, wheat bran and rye bran were 4.9, 4.2 and 4.4, respectively, after 20 h of incubation. The acidification after 20 h varied with matrix and was the lowest in wheat flour. The dextran formed in the whole wheat flour, wheat bran and rye bran was 0.66 ± 0.12, 1.28 ± 0.14 and 3.26 ± 0.12% d.w. of the matrix, respectively, after 20 h of incubation. The percentage of dextran formation varied with the type of matrix used. The free sugars present in the sourdough after 20 h of fermentation were analyzed which showed 0.58, 0.05 and 1.05% glucose in whole wheat flour, wheat bran and rye bran respectively. Fructose present after 20 h in whole wheat flour, wheat bran and rye bran was 1.25, 3.56 and 3.67% respectively. Maltose 0.27% was detected only in whole wheat flour at the end of the fermentation. There was no residual sucrose left after 20 h in any of the three matrices used.

4. Discussion

The maximum production of dextran from *Weissella cibaria* RBA12 was observed at 24 h of incubation during the early stationary phase. The dextran concentration decreased slowly after 24 h, which might be due to the utilization of dextran as a secondary substrate by the bacterium for maintenance of stationary phase after depletion of its primary substrate sucrose (Baruah and Goyal, 2015).

The monosaccharide analysis and FT-IR analysis of dextran-RBA12 indicated that it is composed of D-glucose units and possesses the typical functional groups and linkage peaks of dextran specifically the peak for α-(1 → 6) glycosidic bond. The two species of the genus *Weissella* namely *Weissella cibaria* and *Weissella confusa* are known for their high yields of dextran (Malang et al., 2015). They reported 123 *Weissella* isolates out of which 18 produced fructan along with the dextran.

The ^{1}H NMR and ^{13}C NMR spectra of purified dextran from *Weissella cibaria* RBA12 revealed the presence of 97% of α-(1 → 6) linkages and 3% of α-(1 → 3) branch linkages. The dextrans produced by *Weissella cibaria* and *confusa* have been reported to be highly linear. They contain only α-(1 → 6) linkages (Kang et al., 2006) or with few (2.4–3.4%) α-(1 → 3) linked branches (Bounaix et al., 2009; Ahmed et al., 2012; Shukla et al., 2014; Maina et al., 2008). Dextran containing 7% α-(1 → 3) linked branches, which is the highest branching present among dextrans from *Weissella* spp. was reported for *Weissella cibaria* JAG8 (Rao and Goyal, 2013). The highly linear dextran from *Weissella* finds use in several food applications such as thickening, viscosifying and emulsifying agents and also as a potential soluble fibre which can act as a prebiotic (Kothari et al., 2015)

Prebiotic carbohydrates, by definition, can only be metabolized by selected microbiota of the gastrointestinal tract. Accordingly, these carbohydrates have the ability to influence the microbial population of the gastrointestinal tract due to their selective utilization. Organisms that rapidly ferment prebiotic carbohydrates are enriched, presumably at the expense of those that do not. The effectiveness of a prebiotic depends, therefore on its ability to i) be selectively fermented by and ii) support the growth of specifically targeted organisms (Huebner et al., 2007). It has been reported that anaerobic bacterial fermentation of prebiotics by several probiotic organisms produces lactate and many short-chain fatty acids (SCFA) such as acetate, propionate and butyrate, which results in suppression of growth of several intestinal pathogenic bacteria (Topping and Clifton, 2001). These SCFAs are also involved in the modulation of cholesterol and lipid metabolism by supplying energy for the colonic epithelium (Das et al., 2014). The prebiotic score of dextran-RBA12 was low in *Lactobacillus* species but comparatively higher with the *Bifidobacterium*. In the case of *B. bifidum* NRRL 41410, dextran-RBA12 showed a higher prebiotic score of 0.42 as compared to a score of 0.33 in case of inulin. The prebiotic score of dextran-RBA12 was lower than inulin with other probiotic strains but was similar with a prebiotic score of another dextran from *Lactobacillus plantarum* DM5 (Das et al., 2014). Positive prebiotic activity score are exhibited by carbohydrates that are metabolized by only probiotic bacteria but not by any other enteric bacteria (Huebner et al., 2007). The exopolysaccharide (EPS) from *W. cibaria* A2 stimulated the growth of fecal microbiota and showed a significant increase in SCFA namely acetate, propionic acid and butyric acid (Hongpattarakere et al., 2012).

Dextran-RBA12 showed maximum hydrolysis of 0.51%, 0.31% and 0.24% by artificial gastric juices, α-amylase and intestinal fluid, respectively, whereas inulin showed maximum hydrolysis of 25.23%, 19.13% and 6%, respectively. Thus, dextran-RBA12 showed 50-fold higher resistance to artificial gastric juices, 60-fold higher resistance to α-amylase and 25-fold higher resistance to intestinal fluid as compared to prebiotic inulin. Similar results have been reported for exopolysaccharide (EPS) from *W. cibaria* A2 (Hongpattarakere et al., 2012), α-glucan from *Lactobacillus plantarum* DM5 (Das et al., 2014) and dextran from *Leuconostoc mesenteroides* NRRL B-1426 (Kothari et al., 2015). Inulin-type fructans resist hydrolysis by human digestive enzymes and reach the large intestine virtually intact where they are fermented by the intestinal microbiota. Dextran-RBA12 is clearly more resilient to the enzymes of the gastrointestinal tract of human than inulin and thus, can be considered

Table 1
Analysis of sourdough fermentation using *Weissella cibaria* RBA12 on three different cereal matrices.

Sourdough	$\log_{10}$ CFU/g 0 h	$\log_{10}$ CFU/g 20 h	pH 20 h	Dextran (%, d.w.)	Glucose (%)	Fructose (%)	Maltose (%)
Whole wheat flour	5.7	8.7	4.9	0.66 ± 0.12	0.6	1.3	0.3
Wheat bran	6.1	9.3	4.2	1.28 ± 0.14	0.1	3.6	0
Rye bran	6.1	9.5	4.4	3.26 ± 0.12	1.1	3.7	0

as a soluble dietary fibre that is non-digestible, ferments in the large intestine and can act as a prebiotic for the stimulation of existing beneficial microbiota.

The positive technological effects of dextran on sourdough bread are correlated with high molecular mass and a low degree of branching of the dextran molecule (Lacaze et al., 2007). The ability of *W. cibaria* RBA12 to produce dextran and be able to grow in all three matrices was studied. After 20 h of incubation, there was an increase of approximately 3 log CFU/g in all the three matrices. The pH after 20 h for the whole wheat flour was 4.9 whereas for wheat bran and rye bran it was 4.2 and 4.4 respectively. Similar acidification is reported by Wolter et al. (2014) in buckwheat, quinoa, teff and wheat flour matrices fermented using *W. cibaria* MG1. The enzymatic hydrolysis method employed for dextran estimation from sourdough is most workable with dextrans with few branch linkages, as the enzyme mixture is not able to completely hydrolyze the more complex structural sections present in dextrans (Katina et al., 2009). The dextran produced by *W. cibaria* RBA12 was least in whole wheat flour with a 0.66% d.w. (6.6 g/kg) dextran. The dextran produced in rye bran was the highest with 3.26% d.w. (32.6 g/kg) followed by wheat bran with 1.28% d.w. (12.8 g/kg). The low dextran yield in whole wheat flour can be attributed to higher maltose content in wheat flour as seen in the free sugar analysis. Maltose is formed in starch-rich matrices due to the activity of starch degrading enzymes (amylase) indigenously present in the matrix. Maltose acts as an acceptor molecule in the process of polymerization of dextran by extracellular enzyme dextransucrase produced by the bacteria. In the presence of maltose homologous series of isomaltooligosaccharides are formed which hinders the synthesis of dextran by dextransucrase. Similar observations have been reported by Katina et al. (2009), Galle et al. (2010) and Wolter et al. (2014). The bran matrixes used are an excellent source of dietary fibres and when coupled with the addition of dextran-RBA12 can increase the volume, shelf-life and texture of bread. In the whole wheat flour matrix the formation of isomalto-oligosaccharides (IMO) was quantified but the presence of maltose confirmed their formation. IMO's are by themselves prebiotic in nature and can work synergistically with prebiotic dextran-RBA12. Rye bran proved to be the best cereal matrix for dextran production using *W. cibaria* RBA12 followed by wheat bran and wheat flour. The better performances of *Weissella* in rye bran have also been reported earlier by Kajala et al. (2015a).

5. Conclusion

Dextran from *W. cibaria* RBA12 is composed of D-glucose units. FT-IR analysis revealed its characteristic functional group of a typical dextran. The ^{1}H and ^{13}C NMR analyses confirmed the presence of 97% α-(1 → 6) linkages in the main chain and 3% α-(1 → 3) branched linkages. The significantly lower digestibility of dextran-RBA12 as compared with the commercial prebiotic inulin as well as the ability to be fermented by probiotic intestinal microbiota proved its effectiveness as an efficient soluble dietary fibre. The formation of dextran in various cereal matrices during fermentation was observed to be very efficient. Among the three matrices, rye bran proved to be the best matrix. Further studies on the physio-chemical properties of dextran-RBA12 will throw light on its potential application in food industry.

Conflict of interest

The authors confirm that this article content has no conflicts of interest.

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Appendix A. Supplementary data

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Dextran Utilization During Its Synthesis by *Weissella cibaria* RBA12 Can Be Overcome by Fed-Batch Fermentation in a Bioreactor

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Abstract *Weissella cibaria* RBA12 produced a maximum of 9 mg/ml dextran (with 90% efficiency) using shake flask culture under the optimized concentration of medium components viz. 2% (*w*/*v*) of each sucrose, yeast extract, and K_2HPO_4 after incubation at optimized conditions of 20 °C and 180 rpm for 24 h. The optimized medium and conditions were used for scale-up of dextran production from *Weissella cibaria* RBA12 in 2.5-l working volume under batch fermentation in a bioreactor that yielded a maximum of 9.3 mg/ml dextran (with 93% efficiency) at 14 h. After 14 h, dextran produced was utilized by the bacterium till 18 h in its stationary phase under sucrose depleted conditions. Dextran utilization was further studied by fed-batch fermentation using sucrose feed. Dextran on production under fed-batch fermentation in bioreactor gave 35.8 mg/ml after 32 h. In fed-batch mode, there was no decrease in dextran concentration as observed in the batch mode. This showed that the utilization of dextran by *Weissella cibaria* RBA12 is initiated when there is sucrose depletion and therefore the presence of sucrose can possibly overcome the dextran hydrolysis. This is the first report of utilization of dextran, post-sucrose depletion by *Weissella* sp. studied in bioreactor.

Keywords *Weissella cibaria* · Dextran · Sucrose · Bioreactor

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Introduction

Lactic acid bacteria belonging to genera *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Pediococcus*, and *Weissella* have been reported to produce an extracellular homopolysaccharide named dextran using sucrose as a substrate [1]. Dextran is composed of glucose monomers that are linked by α-(1 → 6) glycosidic bonds in the main chain with different degrees of α-(1 → 2), α-(1 → 3), and α-(1 → 4) glycosidic bonds as branched linkages [2]. Among the several genera mentioned above, genus *Weissella* has a distinctive phenotypic feature to produce dextran in the presence of sucrose [3, 4]. Most notably, *Weissella confusa* and *Weissella cibaria* are among the most prolific dextran producers [5]. Dextrans produced by genus *Weissella* have been reported to have similar structures, consisting of mostly α-(1 → 6) glycosidic bonds and a few α-(1 → 3) glycosidic bonds as branched linkages (2.4–7.0%) [6–8]. Dextran from genus *Weissella* is also attributed with high molecular weight ranging from 800 kDa [9] to 36 MDa [10]. The presence of low degree of branching and high molecular weight of dextran from *Weissella* results in a wide range of applications as a potent food hydrocolloid [11]. Dextran from *Weissella* species has gained much importance in baking application such as its inclusion in sourdough bread results in improved shelf-life, softness, and softness of sourdough bread [12].

The production of dextran from lactic acid bacteria is influenced by several physiological factors such as temperature, pH agitation, and medium components [13]. Among the nutrients, sucrose concentration plays the most important role in the dextran yield [14]. As the dextran concentration increases, the viscosity of the medium also increases [15, 16]. *Weissella cibaria* RBA12 was isolated from pummelo on the basis of dextransucrase activity, as reported earlier by us [17]. *Weissella cibaria* RBA12 produced 8.3 mg/ml of dextran with 83% efficiency under optimal conditions at 24 h [17]. Dextran from *Weissella cibaria* RBA12 consisted of 97% linear α-(1 → 6) linkages in the main chain and 3% α-(1 → 3) branched linkages and showed superior resistance to physiological barriers as compared to the standard prebiotic inulin [5]. In the present study, the optimal conditions for the production of dextran by *Weissella cibaria* RBA12 were studied. The production of dextran was then scaled up from 100-ml shake flask to 2.5 l in bioreactor using the optimized conditions. The kinetic parameters such as specific growth rate (μ), total biomass (X), biomass yield coefficient ($Y_{X/Suc}$), and dextran yield coefficient ($Y_{P/Suc}$) were also studied using data obtained from batch fermentation in bioreactor. Fed-batch fermentation of *Weissella cibaria* RBA12 was carried out to study the influence of sucrose on the dextran production and compared with the batch mode.

Materials and Methods

Microorganism and Culture Medium

W. cibaria isolated from the pulp of pummelo (*Citrus maxima*), producing microbial exopolysaccharide "dextran" using sucrose as a substrate, was used [17]. The microorganism was grown and maintained in the medium described by Tsuhiya et al. (1952) [18], and it was preserved in 20% (*v*/*v*) glycerol and stored at −80 °C.

Production and Estimation of Dextran

Dextran was precipitated from cell-free supernatant (200 μl) of fermenting medium using ethanol (100%, *v*/*v*) in 3:1 ratio. The precipitated dextran was separated by centrifugation at 10,500*g* and 4 °C for 30 min. The pellet collected was dissolved in 200-μl de-ionized water and the process of ethanol precipitation was repeated three times. The dextran content was estimated using phenol sulfuric acid method [19]. Dextran T40 (Sigma Aldrich, St. Louis, MO) was used as standard. Measurements were carried out in triplicate, and all the data expressed were the average of three independent experiments with ±standard error.

Effect of Temperature and Aeration on Dextran Production

The effect of temperature on dextran production from *W. cibaria* RBA12 was studied by growing the culture in 100 ml medium [18]. Fermentation medium was incubated at different temperatures of 15, 20, 24, 28, and 37 °C for 24 h under shaking condition at 180 rpm. The effect of aeration on dextran production was studied by growing the 100-ml culture at different shaking speeds of 80, 120, 150, and 180 rpm by incubating at 20 °C for 24 h. The cell free supernatant after 24 h of fermentation was used for the determination of dextran concentration as mentioned in the earlier section.

Effect of Sucrose Concentration on Dextran Production

The effect of sucrose concentration on dextran production from *W. cibaria* RBA12 was studied by varying the sucrose concentration at 1, 2, 5, 10, and 15% (*w*/*v*) in 100-ml fermentation medium [18] and incubating at 20 °C and 180 rpm for 24 h. The cell free supernatant after 24 h of fermentation was used for the determination of dextran concentration as mentioned in the earlier section. The efficiency of dextran production was calculated according to Baruah and Goyal (2015) [17].

$$\text{Efficiency}\ (\%) = \frac{\text{Amount of dextran produced}}{\text{Maximum possible amount of dextran (theoretical)}} \times 100$$

where maximum possible amount of dextran (theoretical) = 10 mg/ml at 2% sucrose concentration.

Effect of Other Medium Components on Dextran Production

The effect of yeast extract on dextran production from *W. cibaria* RBA12 was studied by varying the yeast extract concentration in 100-ml fermentation medium [18] 1, 1.5, 2, 2.5, 3, 3.5, and 4% (*w*/*v*) and incubating for 24 h at 20 °C and 180 rpm. Similarly, the effect of K_2HPO_4 on dextran production from *W. cibaria* RBA12 was studied by varying the K_2HPO_4 concentration in 100-ml fermentation medium [18] at 1, 1.5, 2, 2.5, 3, and 3.5% (*w*/*v*) and incubating for 24 h at 20 °C and 180 rpm. The cell free supernatant after 24 h of fermentation was used for the determination of dextran concentration as mentioned in the earlier section.

Scale Up of Dextran Production to Bioreactor Level

Dextran production from *Weissella cibaria* RBA12 was scaled up from 100-ml shake flask culture to 2.5-l working volume of optimized medium in a 5-l bioreactor (New Brunswick, model BioFlo115). The optimized medium for dextran production contained 2% (*w*/*v*) sucrose, 2% (*w*/*v*) yeast extract, and 2% (*w*/*v*) K_2HPO_4. The bioreactor was equipped with pH probe, DO probe, and stirrer of two-six bladed Rushton turbines. For controlled pH cultivation, the pH was maintained at 6.9 by addition of 2 M NaOH and 2 M H_3PO_4 solution. During the experiment, temperature and aeration rate were controlled at 20 °C and 2 vv^{-1} min^{-1}, respectively. The dissolved oxygen (DO) was calibrated to 100% before inoculation. The initial agitation rate was set to 200 rpm, and it was changed accordingly to maintain the DO above 30%. A total of 10% inoculum from 12-h grown culture was inoculated in the bioreactor. Silicon anti-foam was simultaneously employed as anti-foam agent. The parameters like dextran concentration, sucrose concentration, cell optical density, and dry cell weight were analyzed at every 2-h interval. The sucrose concentration was determined by estimating the reducing sugars by DNS method [20]. The kinetic parameters such as specific growth rate (μ), total biomass (X), biomass yield coefficient ($Y_{X/Suc}$), and dextran yield coefficient ($Y_{P/Suc}$) were calculated using the above data from batch fermentation [16].

Bacterial Growth Measurement

Samples (2 ml) that were taken at every 2 h were centrifuged at 13,000*g* for 10 min, and cell pellet obtained was re-suspended in sterile culture medium (2 ml). Bacterial growth was analyzed by measuring cell OD at 600 nm. Cell dry weight was obtained by drying specific dilutions of re-suspended cell pellet using hot air oven (60 °C) for 24 h. The dried cell pellets were weighed, and calibration curve of dry weight versus OD (600 nm) was used to determine biomass concentration (g biomass/l) [16].

Fed Batch Fermentation for Dextran Production from *Weissella cibaria* RBA12

The production of dextran from *Weissella cibaria* RBA12 was carried out by fed-batch fermentation (2.5-l working volume) in a bioreactor. The operation parameters and fermentation medium for fed-batch fermentation were kept the same as in the batch fermentation as mentioned in the previous section. Temperature was controlled at 20 °C, and the aeration rate was kept 2 vv^{-1} min^{-1} with an initial agitation rate of 200 rpm, which was later increased. A constant feed rate of the sucrose was employed for the fed-batch mode. The constant feed rate was calculated using the following formula

Where,

$$\mathbf{F} = \frac{\boldsymbol{\mu} \times \mathbf{X} \times \mathbf{V}}{\mathbf{Y}\left(\boldsymbol{x}/\boldsymbol{s}\right) \times \mathbf{S}}$$

F	Feed rate (l/h) for fed batch phase
M	Specific growth rate (h^{-1}) in batch phase
X	Total biomass (g/l) at the end of batch phase
V	Volume at the end of batch phase (l)
$Y_{(X/S)}$	Biomass yield coefficient during batch phase
S	Feed concentration (g/l)

The volume at the end of batch phase was 2.7 l and the feed concentration employed was 500 g/l of sucrose. The feed rate for fed batch phase was calculated to be 20 ml/h. The constant feed of sucrose was started at 10 h of batch phase when the sucrose concentration was approximately, 5 mg/ml. The parameters like dextran concentration, sucrose concentration, and cell optical density were analyzed at every 2-h interval. The sucrose concentration was determined by estimating the reducing sugar by DNS method [20].

Results and Discussion

Effect of Temperature and Orbital Shaking Speed on Dextran Production

The optimum temperature for dextran production from *Weissella cibaria* RBA12 was observed to be at 20 °C (Fig. 1a). *Weissella cibaria* RBA12 produced 8.9 mg/ml of dextran when incubated at 20 °C and 7.8 mg/ml dextran at 24 °C. The cell growth of *Weissella cibaria* RBA12 was observed to be highest at 20 °C with 2.7 g/l of cells (Fig. 1a). The decrease in dextran concentration above 20 °C might be explained by the increase in growth rate of *Weissella cibaria* RBA12, and as a result, the sucrose present gets exhausted and the bacteria start utilizing the dextran as a secondary carbon source. Similar decrease in dextran concentration was also observed during the fermentation profile analysis when the sucrose was completely exhausted after 24 h of incubation at 20 °C and 180 rpm [17]. The optimum orbital shaking speed for dextran production from *Weissella cibaria* RBA12 was 180 rpm (Fig. 1b). *Weissella cibaria* RBA12 produced 8.9 mg/ml dextran when incubated at 180 rpm. The increase in dextran concentration from 4.6 mg/ml at 80 rpm to 8.9 mg/ml at 180 rpm was due to the micro-aerophilic nature of *Weissella cibaria* RBA12 which is common feature of lactic acid bacteria as observed by the increase in cell growth with the increase in orbital shaking speed (Fig. 1b).

Effect of Sucrose Concentration on Dextran Production

The optimum sucrose concentration for dextran yields from *Weissella cibaria* RBA12 was 2% (*w*/*v*) giving 8.9-mg/ml dextran concentration displaying 89% efficiency of conversion (Fig. 2a). The dextran production at 10 and 15% (*w*/*v*) sucrose concentrations was 36.6 and 50.8 mg/ml, respectively, after 24 h, due to which these samples were highly viscous. The higher sucrose concentrations after 24 h were 10 and 15% (*w*/*v*) resulting in lower efficiencies of conversion giving 73 and 67%, respectively, whereas 2% (*w*/*v*) sucrose gave 89% conversion (Fig. 2a). The dextransucrase activity increased with increase in the sucrose concentration till 5% (*w*/*v*), and no further increase in activity was observed beyond this concentration in case of *Weissella cibaria* JAG8 [21]. This decrease in the efficiency might be due to the increase in viscosity of the fermenting medium as a result of higher dextran yields as also reported earlier [16]. Further experiments were carried out using 2% (*w*/*v*) sucrose.

Effect of Other Medium Nutrients on Dextran Production

The optimum concentration of yeast extract was 2% (*w*/*v*) for dextran production from *Weissella cibaria* RBA12 resulting in 8.7 mg/ml dextran (Fig. 2b). On increasing the concentration of nitrogen source from 2% (*w*/*v*) to 4% (*w*/*v*), the dextran concentration gradually

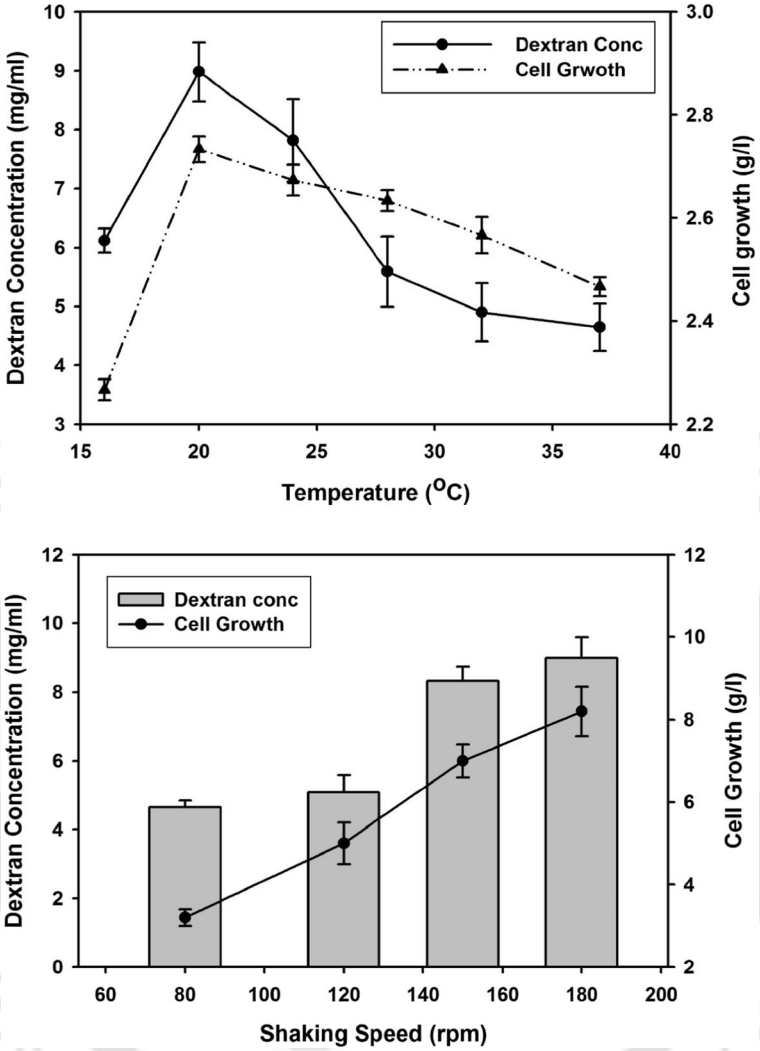
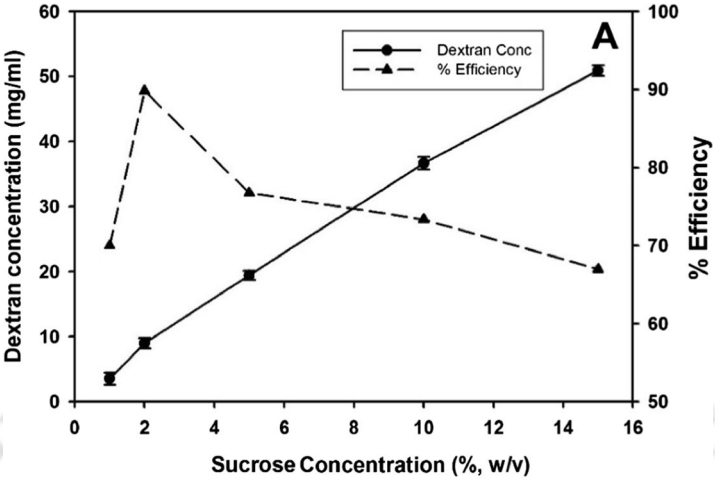
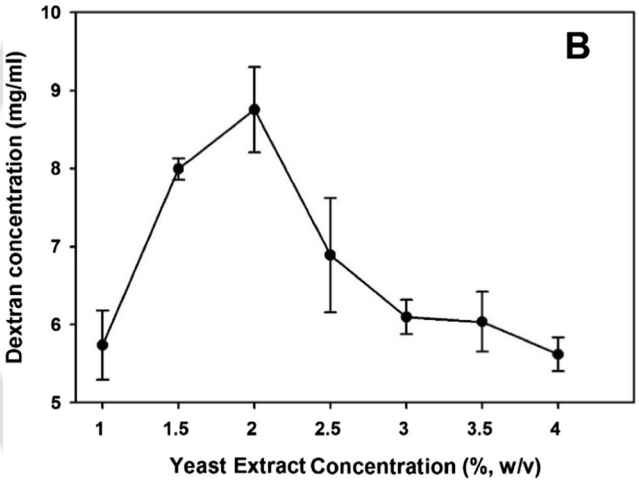


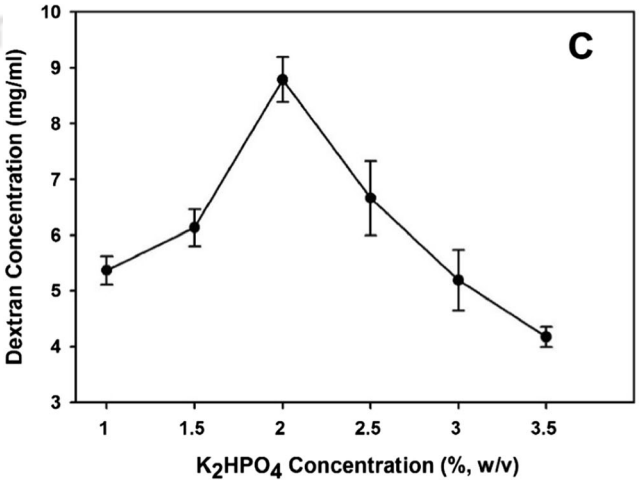
Fig. 1 **a** Effect of temperature. **b** Effect of orbital shaking speed on the production of dextran from *Weissella cibaria* RBA12

decreased from 8.7 to 5.6 mg/ml dextran, respectively. Similar effect of yeast extract on dextran production was reported for *Weissella confusa* Cab3 [14]. The optimum concentration of K_2HPO_4 for dextran production from *Weissella cibaria* RBA12 was 2% (*w*/*v*) (Fig. 2c) resulting in 8.8 mg/ml dextran concentration. On increasing the concentration of K_2HPO_4 from 2% (*w*/*v*) to 3.5% (*w*/*v*), the dextran concentration gradually decreased from 8.8 to 4.2 mg/ml dextran, respectively. K_2HPO_4 is an important phosphate source and buffering agent which

Fig. 2 **a** Effect of sucrose concentration on dextran yield (*black circle*) and efficiency of dextran production (*black triangle*). **b** Effect of nitrogen source. **c** Effect of K_2HPO_4 on the production of dextran from *Weissella cibaria* RBA12







helps in the growth of the bacterium and the production of dextran. Similar effect of K_2HPO_4 on dextran production from *Weissella confusa* Cab3 was reported [14].

Batch Fermentation for Dextran Production from *Weissella cibaria* RBA12

The production of dextran from *Weissella cibaria* RBA12 by batch fermentation in 2.5-l working volume in a bioreactor gave 9.32-mg/ml concentration after 14 h with a 93% efficiency (Fig. 3). The dextran concentration decreased from 9.32 mg/ml at 14 h to 2 mg/ml at 24 h. This decrease in dextran concentration can be correlated with the sucrose utilization by *Weissella cibaria* RBA12 (Fig. 3). The stationary phase of *Weissella cibaria* RBA12 growth started at 14 h with the depletion of sucrose in the medium, and during this phase, it started utilizing dextran as a secondary substrate and continued till the end of stationary phase (18 h) and also during the death phase (18–24 h). This is the first detailed report of dextran utilization observed in bioreactor by *Weissella* spp. In our previous study also, the dextran utilization in the stationary phase of *Weissella cibaria* RBA12 growth was observed under shake flask condition [17]. This dextran utilization could be due to dextranase activity, breaking down the high molecular weight dextran into sugars for easy assimilation by the bacterium. *Weissella* spp. producing high dextran content are of great importance in sourdough fermentation for baking industry [12, 22].

Kinetics of Dextran Production from *Weissella cibaria* RBA12 Under Batch Fermentation

The kinetic parameters for dextran production were determined for batch fermentation in a bioreactor that was carried out at 20 °C and pH 6.9. The total cell biomass produced was 3.53 g/l and the specific growth rate (μ) of *Weissella cibaria* RBA12 was 0.19 h^{-1} (Table 1). The cell biomass yield coefficient ($Y_{X/Suc}$) was 0.18 g/g of sucrose (Table 1). The dextran yield coefficient ($Y_{P/Suc}$) was 0.49 g/g of sucrose (Table 1), which was high and can be correlated

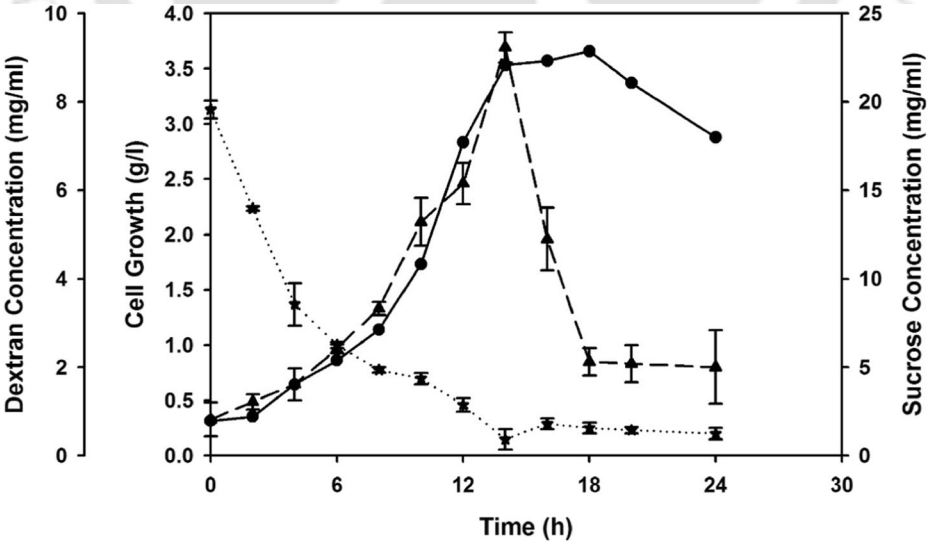


Fig. 3 Scale up of dextran production from *Weissella cibaria* RBA12 under batch fermentation in bioreactor

Table 1 Kinetic parameters for the dextran production from *Weissella cibaria* RBA12

Parameters	Calculated value
Total cell biomass	3.53 g/l
Specific growth rate (μ)	0.19 h^{-1}
Biomass yield coefficient ($Y_{X/Suc}$)	0.18 g/g of sucrose
Product yield coefficient ($Y_{P/Suc}$)	0.49 g/g of sucrose

with the low biomass yield ($Y_{X/Suc}$) due to the efficient utilization of sucrose for dextran formation. *Weissella cibaria* DBPZ1006 during batch fermentation displayed a specific growth rate of 0.93 h^{-1} at 36.3 °C and pH 6.6 [23].

Fed Batch Fermentation for Production of Dextran from *Weissella cibaria* RBA12

The effect of sucrose in the utilization of dextran was further studied by fed-batch fermentation with a constant sucrose feed. The production of dextran from *Weissella cibaria* RBA12 under fed-batch fermentation in a bioreactor resulted of 35.8 mg/ml concentration after 32 h (Fig. 4). The dextran concentration gradually increased throughout the fermentation, unlike the decrease in concentration observed in the batch fermentation after 14 h (Fig. 3). After the start of the feed (500 g/l/50%, *w*/*v*) at 10 h, the sucrose concentration gradually increased. There was a sharp increase in sucrose concentration after 24 h, which could be due to the decrease in the cell biomass after 24 h. The maximum cell biomass obtained was 8.4 g/l at 24 h. The fed-batch mode for production of dextran from *Weissella cibaria* RBA12 proved to be superior as compared with the batch fermentation displaying 4.0-fold increase in dextran concentration from 9.32 mg/ml in batch mode to 35.8 mg/ml in fed-batch mode. The fed-batch mode was efficient as there was no decrease in dextran concentration unlike the batch mode. It could be concluded that the sucrose depletion is a key factor in the utilization of dextran by *Weissella*

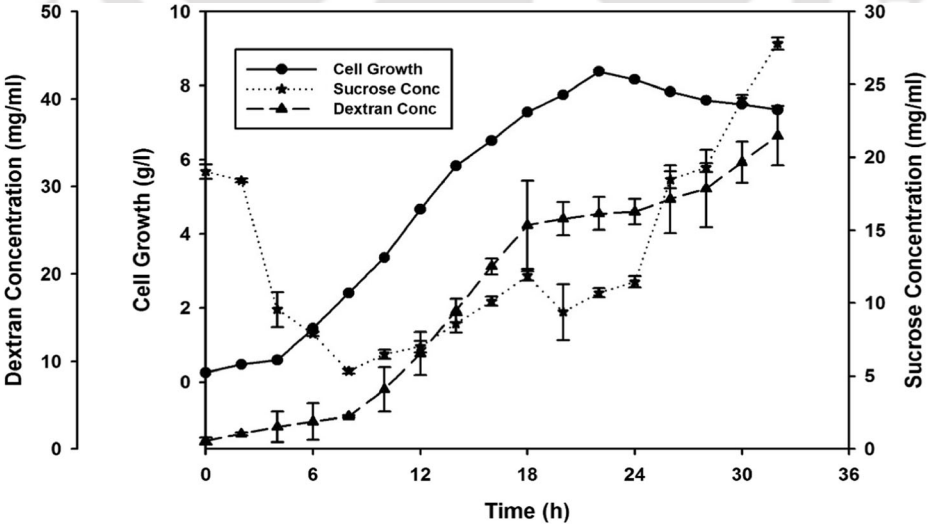


Fig. 4 Dextran production from *Weissella cibaria* RBA12 by fed-batch fermentation in bioreactor

cibaria RBA12. Therefore, fed batch fermentation can be employed for obtaining high dextran yields overcoming the possibility of dextran utilization.

Conclusion

The dextran production by *Weissella cibaria* RBA12 was optimized at 2% (*w*/*v*) sucrose, 2% (*w*/*v*) yeast extract, and 2% (*w*/*v*) K_2HPO_4 and at growth conditions of 20 °C and 180 rpm. In shake flask studies, the dextran production was 8.9 mg/ml with 89% efficiency using 2% (*w*/*v*) sucrose. The optimized medium concentration for shake flask was used for scale up in 2.5-l medium in a bioreactor for dextran production that yielded 9.32 mg/ml with a 93% efficiency. The dextran formed in batch mode was utilized by the bacterium after the depletion of sucrose. In batch mode, the specific growth rate (μ) of *Weissella cibaria* RBA12 was 0.19 h^{-1} along with biomass yield coefficient ($Y_{X/Suc}$) as 0.18 g/g of sucrose and dextran yield coefficient ($Y_{P/Suc}$) as 0.49 g/g of sucrose. In fed batch mode, the maximum dextran concentration was 35.8 mg/ml which was approximately, 4-fold higher than that of batch mode that gave 9.32 mg/ml of dextran. Owing to the steady feed of sucrose, there was no decrease in dextran concentration. Therefore, the fed-batch mode can be used for large-scale production of dextran from *Weissella cibaria* RBA12. Further studies on the utilization of dextran by *Weissella cibaria* RBA12 would reveal the enzymes involved in dextran degradation.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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Purification and characterization of dextransucrase from *Weissella cibaria* RBA12 and its application in *in vitro* synthesis of prebiotic oligosaccharides in mango and pineapple juices

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ABSTRACT

Dextransucrase produced by *Weissella cibaria* RBA12 isolated from pummelo was purified by PEG-400 and PEG-1500 fractionation, followed by gel filtration. The enzyme purified by 0.25 mL/L PEG 400 gave specific activity 410 μkat/g with 25-fold purification. Purified dextransucrase gave a single, homogeneous protein of molecular size ~180 kDa on analysis by SDS-PAGE. Purified dexransucrase was optimally active at 40 °C and pH 5.4. It gave maximum velocity (V_{max}) and Michaelis constant (K_m) of 488.3 μkat/g and 19.2 mmol/L, respectively. The enzyme was thermally stable up to 30 °C and highest pH stability at pH 5.5 for 1 h Mg^{2+} and Ca^{2+} ions enhanced the enzyme activity by 40% and 25%, respectively. The *in-situ* production of isomalto-oligosaccharide was carried out by dextransucrase using mango and pineapple juices. The native sugars (sucrose, glucose and fructose) present in both juices were confirmed by HPLC. The glucose and fructose present in juices acted as acceptor molecules. In both juices, isomalto-oligosaccharides from DP3 to DP5 along with isomaltose (DP2) and leucrose (DP2) were synthesized *in situ* by dextransucrase reaction utilizing the native sucrose. Sucrose content of the juices was eliminated resulting in its lower calorific value highlighting the potential of dextransucrase for production of functional foods.

1. Introduction

Dextransucrase (EC 2.4.1.5) is an extracellular enzyme that synthesizes polysaccharide dextran from sucrose (Leemhuis et al., 2013). Dextransucrase belongs to glucoside hydrolase (GH) family 70, containing exclusively glucansucrase/glucosyltransferases (Stam, Danchin, Rancurel, Coutinho, & Henrissat, 2006). Dextransucrases are elaborated by lactic acid bacteria. The lactic acid bacteria of genera *Lactobacillus*, *Leuconostoc*, *Streptococcus*, *Pediococcus* and *Weissella* are reported to produce dextransucrase (Leemhuis et al., 2013). Dextran is composed of glucose monomers containing consecutive α-(1 → 6) linkages and α-(1 → 2), α-(1 → 3) and α-(1 → 4) branch linkages (Bounaix et al., 2009). Dextrans are used as viscosifying and water binding agents in food and non-food applications (Badel, Bernardi, & Michaud, 2011). In the presence of exogenous acceptor molecules along with sucrose, dextransucrase transfers the glucosyl moieties (produced by the hydrolysis of sucrose) to the acceptor molecule resulting in the formation of acceptor products (oligosaccharides) in addition to dextran. When maltose acts as an acceptor molecule, dextransucrase produces a homologous series of isomalto-oligosaccharides (IMOs) composed primarily of consecutive α-(1 → 6) linkages (Robyt & Eklund, 1983). Dextransucrase from *Leuconostoc mesenteroides* NRRL B-1426 showed the highest affinity towards maltose for the acceptor reaction with 83% efficiency of IMO production (Kothari & Goyal, 2013). IMO are resistant to hydrolytic enzymes human GI tract due to the absence of α-(1 → 6) linkage degrading enzymes. IMO can readily reach the lower GI tract and are used as a substrate for fermentation by colonic microflora to produce beneficial short chain fatty acid (SCFA) (Kothari, Patel, & Goyal, 2014).

Dextransucrase has been used to produce functional foods containing prebiotic oligosaccharides or dextran. The use of dextransucrase enables the *in-situ* production of the desired oligosaccharides and thereby reducing the cost of fortification of the products. Dextransucrase from *W. confusa* VTT E–90392 was used to produce dextran in bran containing wheat bread without any

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fermentation and the associated acid formation (Kajala et al., 2015). The use of dextransucrase in the preparation prebiotic fruit juices have recently gained popularity for its efficient use of native sucrose to form IMOs and in turn lowering the calorific value of the fruit juices (Fontes, da Silva, Rabelo, & Rodrigues, 2015). Prebiotics have been produced by dextransucrase acceptor reaction and included in fruit juices such as cashew apple juice (da Silva, Rabelo, & Rodrigues, 2012), apple and orange juices (Johansson, Diehl, Christakopoulos, Austin, & Vafiadi, 2016), mandarin juice (Nguyen et al., 2015) and pineapple, cantaloupe melon and orange juices (Fontes et al., 2015). In the present study, dextransucrase from *Weissella cibaria* RBA12 was purified and characterized. The assay conditions for dextransucrase acceptor reaction were optimized. IMOs were produced using dextransucrase acceptor reaction and purified by gel filtration. Prebiotic such as IMOs and di-saccharides were produced by dextransucrase acceptor reaction from mango and pineapple juices utilizing their native sugars also acting as acceptors.

2. Materials and methods

2.1. Microorganism and culture medium

W. cibaria RBA12 isolated and screened from the pulp of pummelo was used (Baruah & Goyal, 2015). The microorganism was maintained in the medium described by Tsuchiya et al. (1952) and was stored as sterile 200 ml/L glycerol at −80 °C.

2.2. Production of dextransucrase from W. cibaria RBA12

Dextransucrase was produced by inoculating *W. cibaria* RBA12 in 100 ml of enzyme production medium (Tsuchiya et al., 1952). The composition of enzyme production medium was (g/L) sucrose, 20; yeast extract, 20; K_2HPO_4, 20; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot 4H_2O$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.01; $CaCl_2 \cdot 2H_2O$, 0.01; NaCl, 0.01 and the pH was adjusted to 6.9. The inoculated culture was incubated at 20 °C and 180 rpm for 12 h. The broth was then centrifuged at 10,000×*g* at 4 °C for 10 min to separate the cells and the cell free supernatant was analyzed for enzyme activity and protein concentration as described below.

2.3. Enzyme assay and protein estimation

The enzyme assay was carried out in 1 ml reaction mixture containing 50 g/L sucrose in 20 mmol/L sodium acetate buffer, pH 5.4 and 20 μl of cell free supernatant or purified enzyme. The reaction mixture was incubated at 30 °C for 15 min. An aliquot of 100 μl from the reaction mixture was taken and the released reducing sugar was determined by the method of Nelson and Somogyi (Nelson, 1944; Somogyi, 1945), using fructose as a standard. The absorbance at 500 nm was measured using spectrophotometer (Varian, Cary 100). Dextransucrase activity was expressed as micro katal, which is defined as the amount of enzyme that catalyzes the conversion of 1 μmole of substrate per second under the assay conditions of pH 5.4 and 30 °C. The protein concentration of the enzyme was determined using the method described by Lowry, Rosebrough, Farr, and Randall (1951), using Bovine serum albumin as a standard.

2.4. Purification of dextransucrase from W. cibaria RBA12

The purification of dextransucrase from *W. cibaria* RBA12 was carried out at different concentrations of polyethylene glycol (PEG) 400, 0.15–0.4 (mL/L) and polyethylene glycol (PEG) 1500, 0.05–0.2 (mL/L) using 40 ml cell free supernatant. After gentle mixing, the mixture was incubated at 4 °C overnight for the enzyme fractionation. The PEG fractionated enzyme was obtained by centrifugation at 10,000×*g* at 4 °C for 30 min (Das & Goyal, 2014). The different enzyme fractions were dissolved in 20 mmol/L sodium acetate buffer (pH 5.4) were subsequently dialyzed using 14 kDa cut-off membrane (Himedia Pvt. Ltd., India). The dialyzed enzyme with the highest enzyme activity was further purified by gel permeation chromatography (GPC) using Sephacryl S-300HR (Sigma Aldrich, USA) as the separating matrix. The column was pre-equilibrated and enzyme was eluted using 20 mmol/L sodium acetate buffer (pH 5.4) at a flow rate of 0.3 ml/min and 3 ml fractions were collected. The fractions showing higher specific activity were pooled and analyzed for enzyme activity and protein concentration. The purity of the enzyme was analyzed by non-denaturing SDS-PAGE and Periodic acid Schiff (PAS) staining (Baruah & Goyal, 2015).

2.5. Biochemical characterization of dextransucrase from W. cibaria RBA12

2.5.1. Effect of temperature, pH and ionic strength on dextransucrase from W. cibaria RBA12

The effect of temperature on the dextransucrase activity was studied by incubating the reaction mixture at different temperatures ranging from 10 to 50 °C for 15 min. One ml reaction mixture containing 20 μl of purified enzyme and 50 g/L sucrose in 20 mmol/L sodium acetate buffer, pH 5.4. The effect of pH on the dextransucrase activity was studied by incubating the reaction mixture containing 20 μl of purified enzyme and 50 g/L sucrose in 20 mmol/L sodium acetate buffer of pH ranging from 4 to 5.6 and in 20 mmol/L sodium phosphate buffer of pH ranging from 5.6 to 6.2 at 40 °C for 15 min. Similarly, the effect of ionic strength on the dextransucrase activity was studied by varying the ionic strength of sodium acetate buffer from 10 to 500 mmol/L at pH 5.4, while keeping the other parameters constant. The enzyme activity was calculated by quantifying the amount of fructose released as described in section 2.3.

2.5.2. Determination of kinetic parameters for dextransucrase from W. cibaria RBA12

The kinetic parameters, maximum velocity (V_{max}) and Michaelis constant (K_m) of dextransucrase from *W. cibaria* RBA12 were determined by incubating purified enzyme (467 μkat/g, 0.45 g/L) in the reaction mixture containing varying sucrose concentrations from 0.05 to 400 mmol/L in 20 mmol/L sodium acetate buffer, pH 5.4 at 40 °C for 15 min. The enzyme activity was measured by quantifying the amount of fructose released as described in section 2.3. The data of the specific activity *vs* substrate concentration were analyzed by Graph Pad Prism Software 6 for the determination of V_{max} and K_m using Lineweaver Burk plot.

2.5.3. Thermal and pH stability of dextransucrase from W. cibaria RBA12

The thermal stability of dextransucrase from *W. cibaria* RBA12 was studied by incubating the enzyme at varying temperatures from 10 to 60 °C and at pH 5.4 for 1 h. In the case of pH stability the enzyme was incubated at varying pH from 3.5 to 8.0 for 1 h at 40 °C. Sodium acetate buffer was used for pH range 3.5–5.5 and sodium phosphate buffer was used for pH range of 6.0–8.0. All the reactions were carried out by incubating 20 μl dextransucrase preparation (425 μkat/g, 0.4 g protein/L) with 980 μl of 50 g/L sucrose dissolved in 20 mmol/L sodium acetate buffer, pH 5.4 at 40 °C for 15 min. The enzyme activity was measured by determining the amount of reducing sugar released as described in section 2.3.

2.5.4. Effect of metal ions and denaturing agents on dextransucrase from W. cibaria RBA12

The effect of different divalent metal salts *viz*. $MgCl_2$, $CaCl_2$, $MnSO_4$, $CoCl_2$ and $NiSO_4$ on dextransucrase from *W. cibaria* RBA12 was evaluated by varying the salt concentrations between 0 and 12 mmol/L in the assay reaction mixture. The effect of denaturing agents such as Urea and EDTA were studied by varying their concentrations between 0 and 5 mol/L and 0–12 mmol/L, respectively. The reactions were carried out by incubating 20 μl dextransucrase preparation (425 μkat/g, 0.4 g protein/L) with 50 g/L sucrose dissolved in 980 μl 20 mmol/L sodium acetate buffer, pH 5.4 at 40 °C for 15 min. The enzyme activity was measured by determining the amount of reducing sugar released as described in section 2.3.

2.6. IMO production and characterization using dextransucrase from W. cibaria RBA12

2.6.1. Production of IMO using dextransucrase acceptor reaction

IMOs were produced using dextansucrase catalyzed acceptor reaction using maltose as an acceptor molecule (Kothari & Goyal, 2013). The 2 ml reaction mixture contained 200 μl of purified enzyme (175 μkat/g, 0.2 g protein/L), 50 g/L sucrose and 50 g/L maltose dissolved in of 20 mmol/L sodium acetate buffer, pH 5.4 and incubated at 30 °C for 24 h. The reaction mixture was also supplemented with 0.3 mmol/L $CaCl_2$ and 15 mmol/L NaN_3. The reaction mixture was treated with 2 vol of absolute ethanol and then the mixture was centrifuged at 16000×*g* for 10 min to precipitate the dextran formed. The supernatant (0.5 μl) containing IMOs was analyzed by thin layer chromatography (TLC) using silica gel 60 F254 TLC plate (Merck) with a solvent system, ethyl acetate/1-propanol/acetonitrile/water (4:10:14:11), by volume. The TLC plate was visualized by spraying ethanol containing 5 g/L α-naphthol and 50 ml/L H_2SO_4 and after heating it at 120 °C (Baruah & Goyal, 2015). The IMOs were further purified by GPC using XK16/70 column (GE Healthcare) packed with Biogel P2 matrix (Biorad, CA, USA) connected to FPLC (AKTA, GE Healthcare, USA). Milli-Q water (18.2 MΩ cm) was used for pre-equilibration of column and elution of IMOs was carried out at a flow rate of 0.2 ml/min, and 1 ml fractions were collected. The purified fractions were analyzed for IMO concentration by phenol sulphuric acid method (DuBois, Gilles, Hamilton, Rebers, & Smith, 1956) and identified by TLC and ESI-TOF MS (Kothari & Goyal, 2013).

2.6.2. Analysis of IMO using ESI-TOF MS and HPLC

The degree of polymerization (DP) of IMOs produced in different purified fractions were analyzed by ESI-TOF MS in positive mode using an Agilent 6520 Accurate mass Q-TOF LC/MS system (Agilent Technologies, USA). Mass spectrum was recorded using a scan range of 100–1000 m/z per 1 min run and processed using Agilent Mass Hunter workstation software. The purified fractions were used as standards for the quantification of IMO produced in other and fruit juice reactions. The purified fraction of IMO (single DP) and IMO mixture were analyzed by HPLC (UFLC, Prominence, Shimadzu, Japan) equipped with ion exclusion column Phenomenex Rezex RSO-Oligosaccharide (Phenomenex, CA, USA). The IMOs were analyzed using RI detector with an injection volume of 10 μl and degassed Milli-Q water (18.2 MΩ cm) as the eluent at a flow rate of 0.2 ml/min. The IMO fractions were run as standard in the range, 0.5–1.5 g/L (DP3), 0.2–1 g/L (DP4) and 0.1–0.5 g/L (DP5) for the quantification in subsequent applications.

2.7. Production of IMO using sucrose in fruit juices

Mango and pineapple juice concentrate were purchased from the local market and its pH and free sugars contents were determined. The free sugars present were quantified by HPLC equipped with Phenomenex Rezex ROA-Organic Acid (Phenomenex, CA, USA) and RI detector. Sucrose, glucose and fructose (Sigma Aldrich, USA) were used as standards. The production of IMO in mango and pineapple juice concentrates was carried out using 200 μl of purified enzyme (175 μkat/g, 0.2 g protein/L) in 2 ml of juice concentrate with pH adjusted to 5.4 using sterile NaOH and supplemented with 0.3 mmol/L $CaCl_2$ and 15 mmol/L NaN_3 for 24 h at 30 °C. The oligosaccharides produced were purified as mentioned in section 2.6.1 and quantified as mentioned in section 2.6.2.

3. Results and discussion

3.1. Production of and purification of dextransucrase from W. cibaria RBA12

The cell free supernatant from 12 h grown *W. cibaria* RBA12 gave an enzyme activity of 120 μkat/L with 7 g/L protein concentration resulting in specific activity of 17.1 μkat/g (Table 1). After purification by fractionation using different concentrations of PEG-400 and PEG-1500, it was found that 250 ml/L PEG-400 fraction gave the highest dextransucrase specific activity 281 μkat/g and 150 ml/L PEG-1500 gave the highest specific activity 122.3 μkat/g. The partially purified dextransucrase by 250 ml/L PEG-400 was obtained with 17-fold purification and with 20.5% overall yield (Table 1). This enzyme fraction was used for further purification by GPC using Shepacryl S-300HR matrix. The elution profile of dextransucrase-RBA12 showed an asymmetric peak where the maximum enzyme activity was limited to fractions 7 to 11 amongst which the fraction 8 showed highest specific activity, 410 μkat/g with 24-fold purification (Fig. 1S A, Table 1). A similar elution profile of dextransucrase purification from *L. plantarum* DM5 was observed (Das & Goyal, 2014). The fractions 8 and 9 having the highest activities were subjected to SDS-PAGE and PAS staining analysis where a single band of 180 kDa was observed in both GPC purified fractions (Fig. 1S B, *lanes 2-5*). The magenta color band confirmed the dextransucrase activity and purity of the enzyme. The molecular mass of crude dextransucrase-RBA12 in cell free supernatant was previously reported to be approx. 180 kDa and was also confirmed to be dextransucrase and not fructansucrase by PAS staining (Baruah & Goyal, 2015).

Table 1
Purification analysis of dextransucrase by PEG 400 and size exclusion chromatography.

	Vol (ml)	Enzyme activity (μkat/L)	Total Units (U)	Overall activity Yield (%)	Protein Conc (g/L)	Specific activity (μkat/g)	Fold Purification
Crude	40	120	288	–	7	17.1	–
PEG 400, 25% (v/v)	7	140.5	59.01	20.5	0.5	281	17
Sephacryl S-300HR	3.0[a]	27.5	4.95	0.02	0.067	410	24

[a] 8th fraction of dextransucrase from Sepharcryl S300HR column.

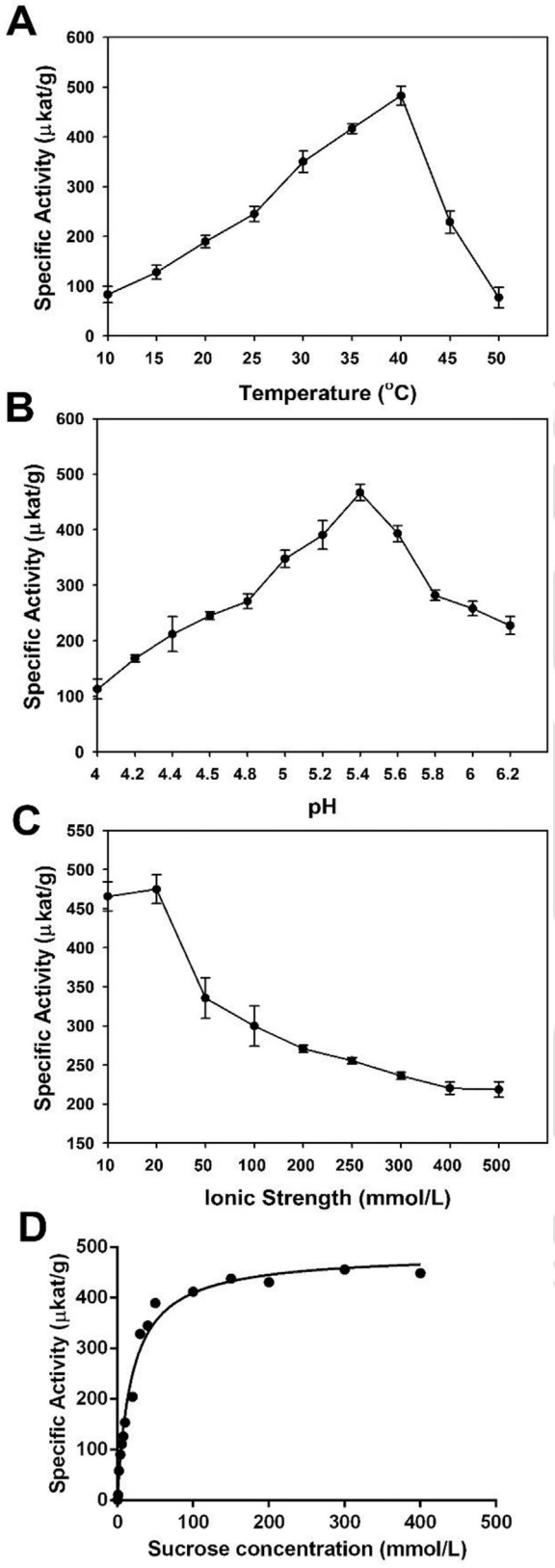

Fig. 1. Effect of temperature (**A**), pH (**B**), ionic strength (**C**) and sucrose concentration (**D**) on dextransucrase from *W. cibaria* RBA12.

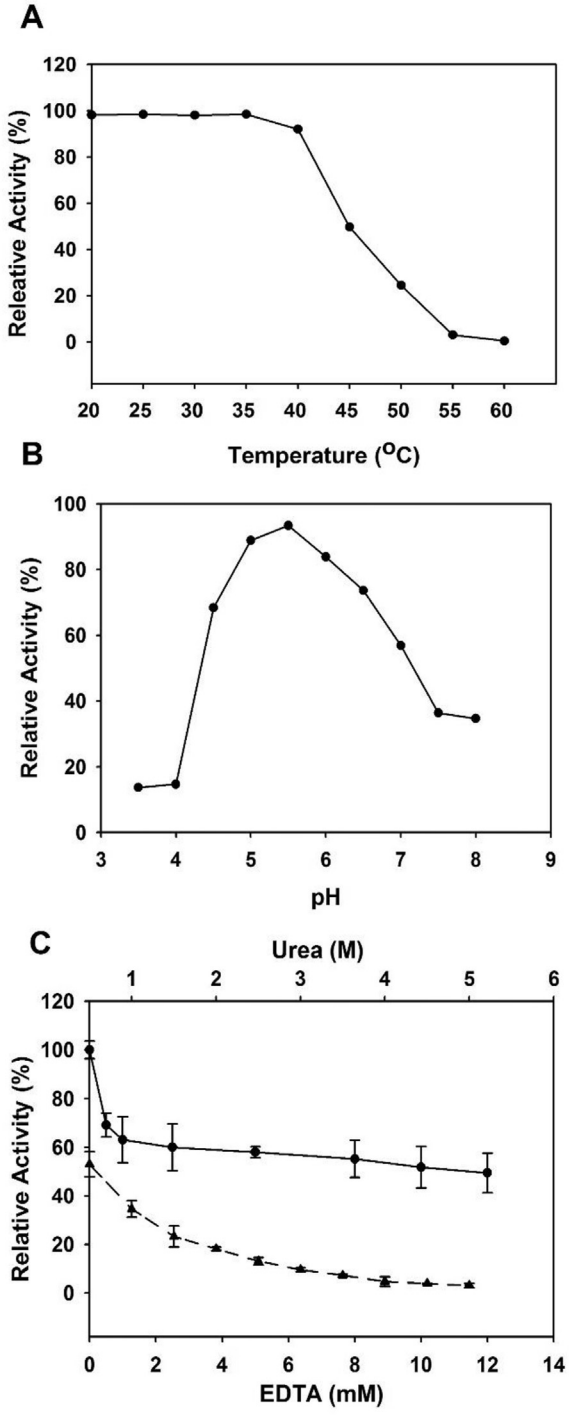

Fig. 2. Thermal stability study (**A**), pH stability study (**B**) and effect of denaturing agents, EDTA (-●-) and Urea (-▲-), (**C**) on dextransucrase from *W. cibaria* RBA12.

3.2. Biochemical characterization of dextransucrase from W. cibaria RBA12

3.2.1. Effect of temperature, pH and ionic strength on dextransucrase

The purified dextransucrase was used to study the effect of temperature, pH and ionic strength on its activity. Dextransucrase displayed maximum specific activity of 483 µkat/g at 40 °C (Fig. 1A). Increase in temperature beyond 40 °C drastically decreased the

enzyme activity, indicating the mesophilic nature of dextransucrase. The optimum temperature of 35 °C for dextransucrase from *Weissella cibaria* JAG8 (Rao & Goyal, 2013) and *Weissella confusa* Cab3 (Shukla, Shi, Maina, Juvonen, & Goyal, 2014) was reported. As per our knowledge, this is the only dextransucrase from genus *Weissella* having an optimum temperature 40 °C.

Dextransucase showed maximum specific activity of 467 μkat/g at pH 5.4 (Fig. 1B). The activity of enzyme decreased gradually with further increase beyond pH 5.4. The above experiment clearly indicated that dextransucrase activity was significantly affected by pH of the reaction mixture. The effect of ionic strength of sodium acetate buffer (pH 5.4) showed that there is no change in dextransucrase activity up to 20 mmol/L concentration. However, the activity beyond 20 mmol/L the enzyme activity decreased rapidly. The loss of enzyme activity at 500 mmol/L was 45% as compared with activity at 20 mmol/L (Fig. 1C). Similar results were reported for dextransucrase from *Weissella cibaria* JAG8 (Rao & Goyal, 2013).

3.2.2. Determination of kinetic parameters for dextransucrase from *W. cibaria* RBA12

The effect of sucrose concentration on dextransucrase activity showed that it follows the classical Michaelis-Menten kinetics and achieves the saturation at 146 mmol/L (50 g/L) sucrose concentration (Fig. 1D). The purified dextransucrase gave Vm of 488.3 ± 12.6 μkat/g and Km of 19.2 mM ± 1.9 mmol/L. Dextransucrase from *Weissella cibaria* JAG8 gave a Vm of 458.4 μkat/g and Km of 13 mmol/L (Rao & Goyal, 2013). It was reported earlier that dextransucrase from *L. dextranicum* NRRL B-1146 (Majumder, Mangtani, & Goyal, 2008) and *L. mesenteroides* NRRL B-512F (Goyal, Nigam, & Katiyar, 1995) exhibited Km of 18.7 and 14.9 mmol/L, respectively.

3.2.3. Thermal and pH stability of dextransucrase from *W. cibaria* RBA12

Dextransucrase from *W. cibaria* RBA12 showed thermal stability up to 40 °C retaining around 92% activity for 1 h. At temperatures above 40 °C the enzyme rapidly lost the activity upon incubation for 1 h and was completely inactive at 60 °C (Fig. 2A). Since the enzyme retained almost 100% activity at 30 °C for 1 h, all the reactions for extended time period were carried out at 30 °C. The enzyme was stable under mild acidic pH (5–6) range with residual activity of 68%, 88%, 93% and 83% after 1 h at pH 4.5, 5.0, 5.5 and 6.0, respectively (Fig. 2B). The thermal and pH stability results were similar to earlier reports of dextransucrase from *Weissella cibaria* JAG8 (Rao & Goyal, 2013) and *Weissella confusa* Cab3 (Shukla et al., 2014).

3.2.4. Effect of divalent metal salts on dextransucrase from *W. cibaria* RBA12

The Mg^{2+} ions were the most effective for enhancing the enzyme activity of dextransucrase. The enzyme activity increased by 40% at 0.6 mmol/L $MgCl_2$ (Fig. 2S A) and increased by 25% at 3 mmol/L $CaCl_2$ (Fig. 2S B). This shows that the Mg^{2+} and Ca^{2+} ions might be stabilizing the enzyme structure thereby enhancing the catalytic activity (Miller & Robyt, 1984). The addition of 1 mmol/L of $MnSO_4$, $CoCl_2$ or $NiSO_4$ to the enzyme reaction resulted in the

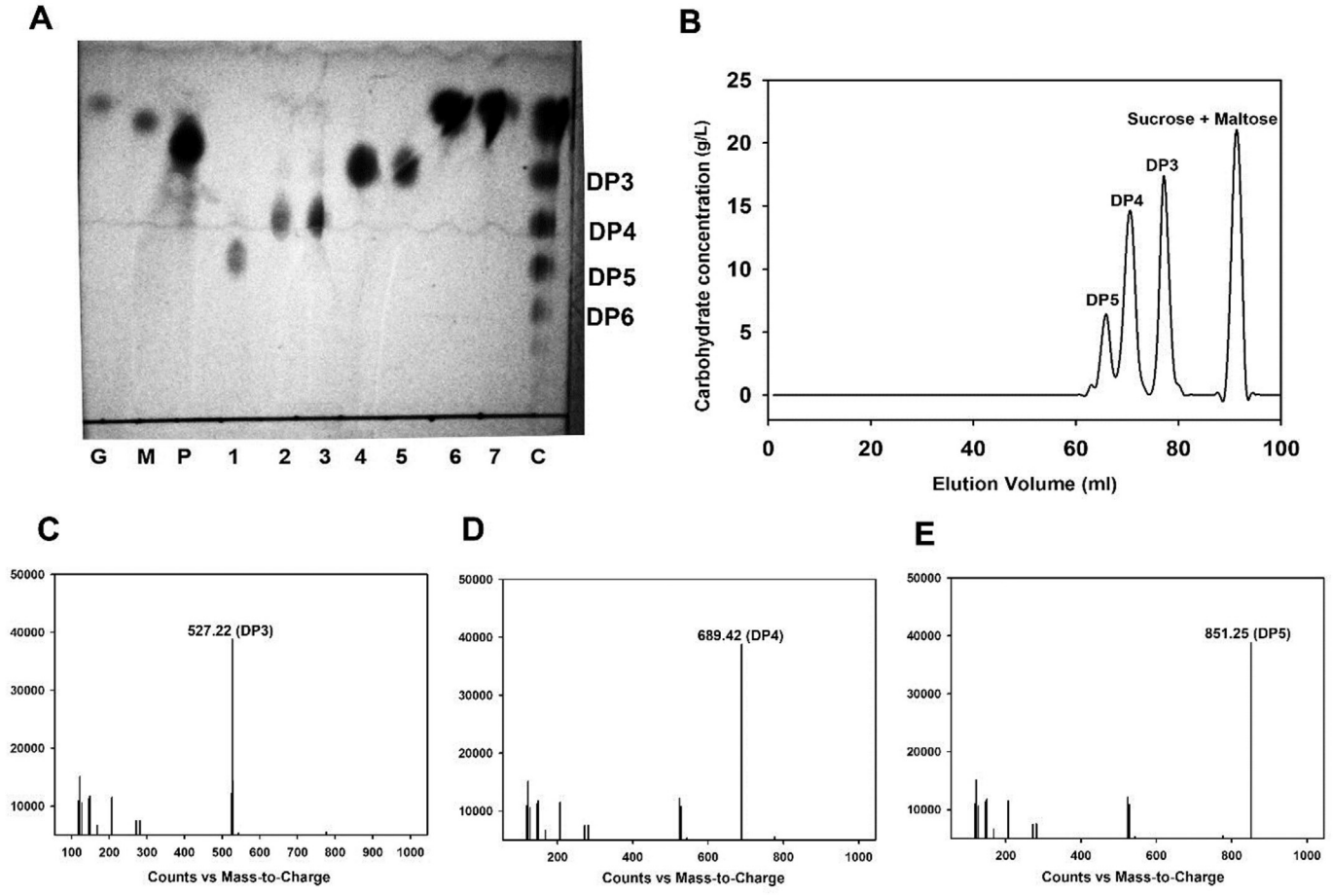

Fig. 3. (**A**) TLC of G- Glucose, M- Maltose, P- Pannose, selected Biogel P2 fractions, 1-67, 2-69, 3-70, 4-77, 5-78, 6-92,7-93 and C-IMO mixture. (**B**) Elution profile of IMO purification using Biogel P2 by gel filtration chromatography. ESI-TOF mass spectrum of **C)** Fraction 77 (DP3) **D)** Fraction 70 (DP4) **E)** Fraction 67 (DP5).

decrease of enzyme activity by 22%, 29% and 36%, respectively (Fig. 2S C, D & E). It was reported that Mg^{2+} and Ca^{2+} ions enhance the activity of dextransucrase from *Weissella cibaria* JAG8 by 22% and 13%, respectively (Rao & Goyal, 2013). In the case of *Weissella confusa* Cab3, the Mg^{2+} and Ca^{2+} ions enhanced the dextraunsucrase activity by 8% and 20%, respectively (Shukla et al., 2014).

3.2.5. Effect of denaturing agents on dextransucrase from W. cibaria RBA12

The dextransucrase activity decreased in the presence of both EDTA or urea. EDTA (0.5 mmol/L) decreased the dextransucrase activity by 31% and 12 mmol/L EDTA decreased the activity by 50% (Fig. 2C). The decrease of activity by EDTA was due to the chelation of native divalent metal ions present in the enzyme by EDTA. Urea decreased the dextransucrase activity by 47% and 3.2% at 0.5 mol/L and 5 mol/L, respectively (Fig. 2C). Similar results on the effect of urea were reported for dextransucrase from *Weissella cibaria* JAG8 (Rao & Goyal, 2013).

3.3. Production of IMO using dextransucrase acceptor reaction

The TLC analysis of IMO mixture revealed the presence of oligosaccharides from DP3–DP6 (Fig. 3A *lane C*). The GPC purified fractions of IMO mixture was eluted between 60 and 95 fractions (Fig. 3B). TLC analysis revealed the presence of isomalto-pentaose

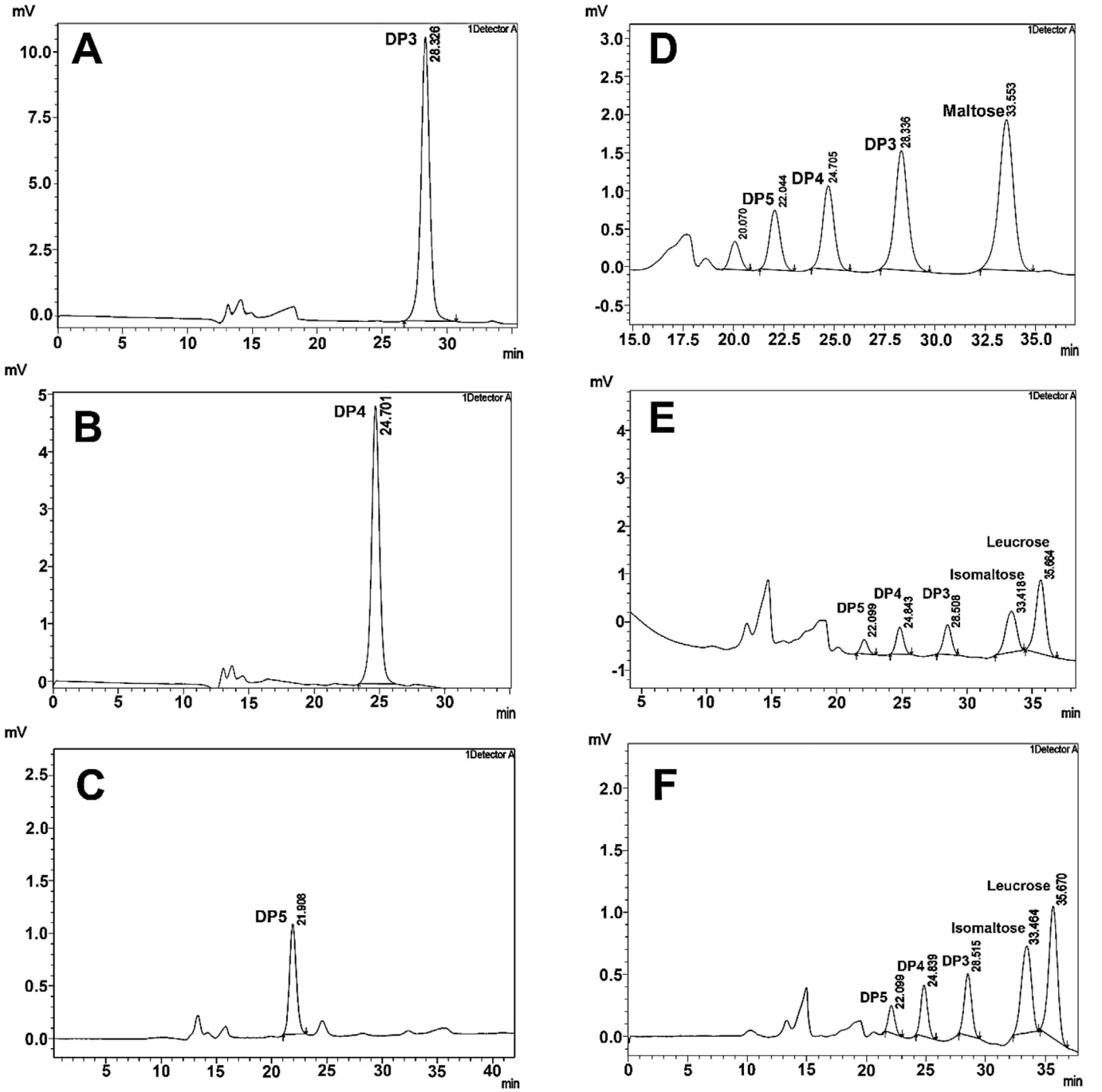

Fig. 4. HPLC analysis of (**A**) Fraction 77 (DP3) (**B**) Fraction 70 (DP4) (**C**) Fraction 67 (DP5). (**D**) IMO mixture, (**E**) Mango and (**F**) Pineapple juice after dextransucrase acceptor reaction.

Table 2
Oligosaccharide production in mango and pineapple juice using dextransucrase.

Type of Juice	pH	Total Sugar (g/L)	Enzyme reaction	Sucrose (g/L)	Glucose (g/L)	Fructose (g/L)	Oligosaccharide produced (g/L)	
Mango	3.4	124.67	Before	33.22	44.90	46.55		
		103.64	After	0	41.49	62.15	**Leucrose**	6.44
							Isomaltose	4.03
							DP3	2.15
							DP4	1.40
							DP5	0.94
Pineapple	3.7	112.50	Before	28.10	40.71	43.69		
		93.60	After	0	38.60	55.00	**Leucrose**	4.38
							Isomaltose	3.28
							DP3	1.72
							DP4	1.04
							DP5	0.70

(DP5), isomalto-tetraose (DP4) and panose (DP3) in 67, 70 and 77 fractions, respectively (Fig. 3A *lanes 1-5*). The unhydrolyzed sucrose and residual maltose were eluted in the 92 and 93 fractions (Fig. 3A *lanes 6-7*). The molecular mass of IMO produced were confirmed by ESI-TOF MS. The peaks observed at m/z 527 for fraction 77, 629 for fraction 70 and 851 for fraction 67 revealed panose (DP3), isomalto-tetraose (DP4) and isomalto-pentaose (DP5), respectively (Fig. 3C–E). The concentrations of DP3, DP4 and DP5 were quantified to be 17, 15 and 7 g/L, respectively.

IMO are used as food additives in functional foods and are prebiotic in nature as they are resistant to hydrolyzing enzymes of the human GI tract due to the presence of α-(1 → 6) linkages, therefore they are readily utilized by probiotic bacteria such as *bifiodobacterium* and *lactobacillus* and produce beneficial short chain fatty acids (SCFA) (Baruah, Maina, Katina, Juvonen, & Goyal, 2017). The fermentation of IMO by probiotic bacteria also produces vitamins of B complex (B1, B2, B6 and B12) (Mussatto & Mancilha, 2007). The daily requirement of IMO for human consumption is suggested to be 8–10 g per day (Goulas, Fisher, Grimble, Grandison, & Rastall, 2004). IMOs in excessive doses could be responsible for intestinal discomfort, flatulence or even diarrhea (an effect which is inversely related to chain length) and because of their high fermentation rate and production of gases (Roberfroid & Slavin, 2000).

3.4. HPLC analysis of IMO produced using dextransucrase from *W. cibaria* RBA12

After the characterization of IMO, the purified fractions were subjected to HPLC analysis. The retention time for fractions containing DP3, DP4 and DP5 were 28.3, 24.7 and 22 min respectively (Fig. 4A–C). The HPLC analysis of the IMO mixture revealed the presence of all the above mentioned peaks along with another peak at 20 min, which could correspond to DP6 as seen in the TLC analysis of the IMO mixture (Fig. 4D). Due to its low concentration and subsequent dilutions during GPC the DP6 fraction was not detected. The GPC purified fractions were used to plot standard curve to the detection of IMOs from DP3-DP5.

3.5. Production of IMO using sucrose in mango and pineapple juices

The mango and pineapple juice had a pH of 3.4 and 3.7, respectively. The total sugar present in mango and pineapple juice were 124.7 g/L and 112.5 g/L, respectively. After the dextransucrase reaction the total sugar present in mango and pineapple juice decreased to 103.6 g/L and 93.6 g/L, respectively, decreasing their calorific value (Table 2). The data in the Table 2 shows that dextransucrase reaction completely utilized the sucrose present in both the juices. After the reaction there was a decrease in glucose concentration whereas fructose concentration remained almost same in both juices. IMOs produced were panose (DP3), isomaltotetraose (DP4) and isomaltopentaose (DP5) along with the disaccharides leucrose and isomaltose (Fig. 4E and F). In this reaction, fructose and glucose acted as acceptor molecules, isomaltose is formed when glucose is transferred to another glucose and leucrose is formed when glucose is transferred to fructose. After the dextransucrase acceptor reaction in mango juice the concentration of leucrose, isomaltose, DP3, DP4 and DP5 was 6.44, 4.03, 2.15, 1.4 and 0.94 g/L, respectively. Similarly, in pineapple juice after the dextransucrase acceptor reaction the concentration of leucrose, isomaltose, DP3, DP4 and DP5 was 4.38, 3.28, 1.72, 1.04 and 0.7 g/L, respectively (Table 2). The concentration of leucrose was highest amongst all oligosaccharides produced in both mango and pineapple juice followed by concentrations of isomaltose, DP3, DP4 and DP5. The production of oligosaccharides from mandarin juice using dextransucrase from *Leuconostoc mesenteroides* B-512FMCM also yielded oligosaccharides from DP3 to DP5 along with isomaltose (Nguyen et al., 2015). However, the concentrations of various oligosaccharides were not reported. Prebiotic juices are an important form of functional food as they have less calorific value due to lower sugar concentration. The value addition due to the oligosaccharides produced is especially important as they are not added and synthesized with in the product (Fontes et al., 2015).

4. Conclusions

Dextransucrase from *W. cibaria* RBA12 on purification by 0.25 mL/L PEG-400 fractionation followed by gel filtration gave a specific activity of 410 μkat/g with 25-fold purification and showing single band of 180 kDa. The optimum assay conditions for purified enzyme was 40 °C, pH 5.4 and 20 mmol/L of sodium acetate buffer. The Vm amd Km values of dextransucrase were 488.3 μkat/g and 19.2 mmol/L, respectively. Dextransucrase was thermally stable at 30 °C for 1 h Mg^{2+} and Ca^{2+} ions enhanced the enzyme activity whereas Mn^{2+}, Co^{2+} and Ni^{2+} inactivated the enzyme. Urea and EDTA denatured the enzyme in concentrations as low as 0.5 mol/L and 0.5 mmol/L, respectively. IMOs in the range of DP3–DP6 were produced using maltose as an acceptor molecule by dextransucrase acceptor reaction. The mixture of IMOs produced was purified by GPC to obtain individual IMO which were identified by TLC and their masses, DP3 (527.22), DP4 (689.42) and DP5 (851.25) were confirmed by ESI-TOF MS. The purified IMOs were used as the standards for HPLC analysis of IMOs produced in fruit juice reactions using dextransucrase. Dextransucrase was used to produce prebiotic juices using commercial mango and pineapple juice, which yielded IMOs in the range of DP3-DP5 along with

disaccharides, leucrose and isomaltose. The reaction removed completely the native sucrose present in juices lowering its calorific value. Further studies on the production of prebiotic juices by dextransucrase, their shelf-life and stability of enzymes needs to be carried out for commercial applications.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2017.06.012.

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