Characterization of dextransucrase and dextran from *Weissella cibaria* JAG8 and *in vitro* analysis of dextran as prebiotic

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DEPARTMENT OF BIOTECHNOLOGY

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 Biotechnology, Indian Institute of Technology Guwahati, Guwahati, 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.

May, 2013

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CERTIFICATE

It is certified that the work described in this thesis entitled **"Characterization of dextransucrase and dextran from *Weissella cibaria* JAG8 and *in vitro* analysis of dextran as prebiotic"** by Mr. T. Jagan Mohan Rao for the award of degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision mainly in the Department of Biotechnology, Indian Institute of Technology Guwahati, Guwahati, India. The work embodied in this thesis has not been submitted elsewhere for a degree.

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Jagan Mohan Rao Tingirikari
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Synopsis

Introduction

Dextran is a biopolymer produced by some of the selected strains of lactic acid bacteria *viz. Lactobacillus, Leuconostoc, Streptococcus, Weissella* species. The formation of this biopolymer is dependent upon a glucosyltransferase, belonging to glycoside hydrolases family (GH70) and is known as dextransucrase (E. C. 2.4.1.5). This inducible extracellular enzyme catalyzes the synthesis of high molecular weight dextran from sucrose as well as low molecular weight oligosaccharide in the presence of maltose and isomaltose. Dextrans may be linear or branched, with variable degree of branching comprising of α(1→6) glycosidic linkages in the main chains and α(1→2) or α(1→3) or α(1→4) branched glycosidic linkages. It is used as food syrup stabilizers, matrix of chromatography columns, plasma substitute, anti-thrombogenic agent, biomaterials, paper and metal-plating processes, oil recovery. Porous dextran scaffolds have tissue engineering applications as drug delivery vehicles. Recently the application of dextran in food and baking industry has increased significantly, as an alternative for non bacterial hydrocolloids like guar gum and hydroxypropylmethyl cellulose for the generation of gluten-free cereal food products for patients suffering from celiac disease. Microbial polysaccharides represent only a small fraction of the current biopolymer market. Factors limiting the use of microbial exopolysaccharides are their production, which requires a thorough knowledge of their biosynthesis and an adapted bioprocess technology, and the high cost of their recovery. Thus, there is a

great need for isolation of high yielding strains and strain improvement for enhanced production of industrially important enzyme. 16s rRNA sequencing identified the isolate to be *Weissella cibaria sp.,* belonging to lactic acid bacteria family. The dextran produced from certain strains of *Weissella cibaria sp* acts as a putative hydrocolloid and serves as a replacement for non bacterial hydrocolloids such as guar gum and hydroxypropylmethyl cellulose for the generation of gluten free food products for patients suffering from celiac disease. The present work reports the high dextran producing ability of the newly isolated *Weissella cibaria sp.* The molecular, morphological and physiological characterization of the isolate *Weissella cibaria* JAG8 was conducted. Production, purification and characterization of both dextransucrase and dextran were carried out. Prebiotic analysis of dextran from this isolate was studied and found to be a putative prebiotic ingredient in food industry to modulate intestinal microbiota for healthcare. While *in vitro* study of dextran as food additive indicated the promising potential of dextran as food ingredient to improve the textural and rheological properties of sucrose-supplemented dairy products.

Present work

The present investigations are carried out on the "**Characterization of dextransucrase and dextran from *Weissella cibaria* JAG8 and *in vitro* analysis of dextran as prebiotic**". The thesis work comprises 6 Chapters.

Chapter 1 is the General Introduction which embodies the brief review of literature dedicated to the importance of lactic acid bacteria, their classification,

characterization and the importance of isolation of new strains. This chapter elaborates about the production, purification and characterization methods of the enzyme dextransucrase and the exopolysaccharide dextran. This chapter also elaborates about the key amino acid residues involved in the active site of dextransucrase and the effect of different metal ions, chelating agent and stabilizers on the stability of dextransucrase were also reviewed. The application and importance of prebiotics in maintaining human health has been emphasized in this chapter. Role of dextran as food additive and biomaterial are extensively reviewed. The importance of lactic acid bacteria in sourdough fermentation and generation of gluten free cereal food products has been described. This chapter reviews about the potential application of the *Weissella cibaria* dextran as prebiotic and includes the significance of present study and specific objectives.

Chapter 2 describes the detailed protocol of screening of the natural isolate of lactic acid bacterium, JAG8 from apple. The morphological and biochemical characterization including Gram test, catalase test, carbohydrate fermentation profile and antibiotic susceptibility test was carried out on isolate JAG8. The isolate was Gram positive and catalase negative. Antibiotic susceptibility test showed its resistance to the antibiotics norflaxacin, vancomycin, cotrimaxazole, co-trimize, nalidixic acid, nitrofurantoin, oxacillin, sulphamethoxazole. Vancomycin resistant is the unique characteristic feature of lactic acid bacteria. The isolate could ferment arabinose, cellobiose, dextrose, fructose, mannose, maltose, sucrose and xylose was found to be efficient. The isolate could not metabolize adonitol, dulcitol, lactose, melibiose, trehalose, inulin, rhamnose and raffinose. The 16S rRNA gene sequencing

based identification of the isolate and its phylogenetic tree was described. The isolate JAG8 was identified as *Weissella cibaria* (Gen Bank Accession no KC110687) belonging to lactic acid bacteria family. The dextran production capacity of this genus was reported for the first time. The culture conditions such as temperature and shaking or static were optimized for dextransucrase and dextran production. The mesophilic and micro-aerophilic nature of the isolate was observed. The maximum dextransucrase activity 5.8 (U/ml) and maximum dextran yield of 7.8 mg/ml was observed at 24°C under static condition. The shaking flask culture gave dextransucrase activity of 3.3 (U/ml) which was 40% lower than the static flask culture. Various parameters such as change in pH, cell optical density and dextransucrase activity of *W. cibaria* JAG8 were studied. The cell density and dextransucrase activity of the isolate was highest at 12 h incubation, which confirmed that the enzyme production was growth associated.

Chapter 3 describes the purification, identification and characterization of dextransucrase from the isolate *Weissella cibaria sp.* JAG8. The dextransucrase was purified by using aqueous two phase system using different percentage of polyethylene glycol (PEG) of molecular weight 400 and 1500. The cell free extract with specific activity of 1.0 U/mg was subjected to fractionation by PEG-400 and PEG-1500. 33% (v/v) PEG-400 gave a specific activity of 20.0 U/mg with 20 fold purification and 18.2% overall yield in a single step. PEG-1500 fractionation gave a maximum specific activity of 10.2 U/mg with 10.2 fold purification and 7.2% overall yield at 15% (w/v) concentration. Partially purified 33% of PEG-400 fraction was further subjected to purification by gel filtration using Sephacryl S-300 matrix. The

enzyme was eluted from 8th to 12th fractions with maximum enzyme activity of 37 (U/mg) in the 11th fraction. The PEG-400 and gel filtration purified fractions were analyzed by *in situ* activity staining by the formation of dextran by dextransucrase in presence of sucrose. No band was detected when the gel containing the enzyme dextransucrase was incubated with raffinose which confirmed the absence of fructansucrase. The samples were run on SDS-PAGE under non-denaturing condition and subjected to Periodic Acid Schiff staining. The single magenta colour activity band on the gel corresponded to approximately, 177 kDa band on the Coomassie Brilliant Blue stained and Silver stained SDS-PAGE gel, this confirming the presence of dextransucrase. The conditions for dextransucrase activity were optimized using PEG-400 purified enzyme (20.0 U/mg). The reaction conditions as temperature, pH, ionic strength, sucrose concentration were optimized for maximum dextransucrase activity. A temperature of 35°C, a pH 5.4, and the concentration of 5.0% sucrose were optimum for activity of dextransucrase from *Weissella cibaria* JAG8. It was observed that an ionic strength up to 50 mM concentration of sodium acetate buffer has no significant effect on enzyme activity, with increase in ionic strength to 500 mM has led to 20% decrease in enzyme activity. The effects of different divalent metal ions on the activity of dextransucrase were studied. The activity of enzyme increased by 22, 14, and 13% by the addition of 2 mM $MgCl_2$, 2 mM $CoCl_2$, and 4 mM $CaCl_2$ to dextransucrase. The inactivation was observed with increase in the concentration of EDTA and 10 mM caused 64% decrease in enzyme activity. Urea at all concentrations displayed inhibitory effect on dextransucrase. With increase in each concentration of urea there was a drastic decrease in enzyme activity. The enzyme lost 43%, 75%, 94% and 98% of its activity at 1, 2, 3, and 4M urea respectively, in 15min.

The effect of stabilizers on storage stability of dextransucrase were studied in presence of Tween 80, dextran (100 kDa). The residual activity of dextransucrase at 30°C at 24h was 18%, 81% and 43% with control, Tween 80 and dextran (100 kDa). The addition of Tween 80 to dextransucrase provided stabilizing effect at all the three temperatures 30°C, 4°C and -20° C with half life of 50 h, 104 days and 345 days against controls 10.8 h, 24.5 days and 173 days.

Chapter 4 describes active site mapping of novel dextransucrase from *Weissella cibaria* JAG8, for the identification of essential amino acid residues present at the active site by using lysine specific inhibitor and *viz.* pyridoxal-5'-phosphate (PLP), 2,4,6-trinitrobenzenesulphonic acid (TNBS) and cysteine specific inhibitors *viz.* 5, 5'-dithiobis (2-nitrobenzoic acid) DTNB, iodoacetic acid and *o*-phthalaldehyde a bifunctional lysine and cysteine specific inhibitor. The enzyme inactivation caused by lysine specific reagents PLP (25 mM) and TNBS (25 mM) was 98.5% and 98.7%, respectively. The ε-NH_2 lysine derivative of enzyme-inhibitor complex with PLP and TNBS gave absorbance maxima at 325 nm and 369 nm, respectively. PLP modified dextransucrase on reduction with sodium borohydride led to the formation of N^{ε}-phosphopyridoxyllysine complex which showed fluorescence maximum at 397 nm at excitation wavelength of 325 nm. The above results clearly indicated that one or more lysine residues present near or at the active site are essential for enzyme activity. The substrate sucrose, provided protection to the enzyme against inactivation by PLP. The cysteine specific reagents DTNB (10 mM) and iodoacetic acid (25 mM) caused 98.7% and 98.9% enzyme inactivation, respectively. The formation of enzyme-inhibitor complex with DTNB (thio nitro benzoate) and with iodoacetic acid (thio

acetate) were confirmed by absorbance maxima at 406 and 323 nm, respectively. The bifunctional inhibitor *o*-phthalaldehyde is used to know the involvement of lysine and cysteine residues in the activity. The enzyme lost 97% of its activity in presence of 10 mM *o*-phthalaldehyde. The enzyme inhibitor complex gave absorbance maxima at 334 nm and fluorescence emission maxima at 418 nm, due to the formation of isoindole derivative. These results showed that one or more cysteine residues present near or at the active site are essential for enzyme activity. The presence of one or more essential cysteine residue at the active site is reported for the first time for dextransucrase.

Chapter 5 describes the synthesis, purification and characterization of dextran produced by the *Weissella cibaria* JAG8. The dextran was enzymatically synthesized using dextransucrase and substrate sucrose. The dextran was purified by ethanol precipitation and the structure of the lyophilized dextran was analyzed by surface morphology study by SEM, FT-IR, ^{1}H-NMR, ^{13}C-NMR spectroscopic techniques. The Scanning Electron Microscopy revealed the porous, web like structure displaying water holding capacity of the dextran. With increase of shear rate, shear stress increased and viscosity decreased confirming the typical polymeric non-Newtonian pseudoplastic behavior. The monosaccharide analysis of dextran was carried out using High Performance Anion Exchange Chromatography (HPAEC). The monosaccharide analysis revealed that the dextran is composed of only glucose units. The dextran was purified and its molecular weight was determined by using Sephacryl S-500 matrix. The average molecular weight of the dextran from *W. cibaria* JAG8 was found to be around 800 kDa. The FT-IR spectrum of the dextran showed bands at 3493, 2917,

1646, 1157 and 1013 cm^{-1}. The band in the region of 3493 cm^{-1} represents -OH group, 2917 cm^{-1} represents the C-H group and 1646 cm^{-1} represents -COOH group. The absorption peak at 913 and 857 cm^{-1} indicates the α-glycosidic bond. The characteristic bands at 1157 and 1013 cm^{-1} found in the spectra of dextran are due to vibrations of C-O and C-C bonds and deformational vibrations of the CCH, COH and HCO bonds. As shown by the ^{1}H-NMR spectrum, the resonance at 4.96 ppm was assigned to the C-1 of the α (1→6) glucosyl residues of main chain of dextran. The peak at 5.3 ppm confirms the branched nature of the dextran with α (1→3) linkage. ^{13}C-NMR spectrum elucidates the major resonance in the anomeric regions occurs at 97.8 ppm indicating that the C-1 is linked. The signal at 65.7 ppm indicates that most of the C-6s are also linked. The absence of peaks at 102.2 ppm and 78.5 ppm suggested the absence of α (1→4) linkage in the dextran. The FT-IR, ^{13}C-NMR and ^{1}H-NMR data showed that the dextran produced by the isolate *Weissella cibaria* JAG8 is soluble, branched and main chain has 93.0 % α (1→6) linear linkage and 7.0 % of α (1→3) branched linkage. As branched polymers are resistant to glucosidases, thus holds potential application in baking and food industry. The above illustrated structural features make dextran a very valued candidate for food, pharmaceutical and tissue engineering scaffold applications. The dextran produced by isolate *Weissella cibaria* JAG8 has potential use in food industry as gelling or stabilizing agent. It is a well known fact that dextran produced by the bacteria are biocompatible in nature, so it can be a potential candidate to be used as carrier for drug delivery.

Chapter 6 describes the prebiotic potential of enzymatically synthesised dextran from *Weissella cibaria* JAG8. The enzymatically synthesized dextran was treated with

artificial human gastric juice with pH ranging from 1-4 to detect the percentage of hydrolysis. The biopolymer showed resistance to acid with only 1.09%, 0.85%, 0.77% and 0.64% hydrolysis at pH 1, 2, 3 and 4 respectively, at 37°C. In case of standard prebiotic (inulin) maximum hydrolysis of 33.6%, 27.5%, 9.6% and 7.3% was observed at pH 1, 2, 3 and 4 respectively, indicating greater degree of hydrolysis. The dextran showed 0.86% and 0.83% hydrolysis when treated with α-amylase at pH 5 and 7 at 37°C. The degree of hydrolysis of inulin was 13.2% and 11.5% at pH 5 and 7 with α-amylase which was significantly higher than that of dextran. Based on the above hydrolysis experiments it is confirmed the non-digestible nature of dextran at both acidic and alkaline pH. Thus promoting the growth of probiotic bacteria and it could be a potent candidate as prebiotic. Maillard reaction was carried out by incubating the dextran, standard prebiotic (inulin) and control (glucose) at 85°C. The rate of hydrolysis (browning) in dextran was lower than commercially available prebiotics such as NutraFlora P-95 and Raftilose P95. This indicated the stability of dextran at higher temperatures for prolonged period of time. Thus, the dextran can be exploited in food and baking industry. The growth profile of probiotic bacteria *viz.* *Bifidobacterium infantis* NRRL B-41661, *Lactobacillus acidophilus* NRRL B-4495 and *Bifidobacterium animalis lactis* NRRL B-41405 in presence of 1% (w/v) dextran, inulin and control (without sugar) was carried out for 48 h. It was observed that the dextran significantly supported the growth of probiotic bacteria. Based on the above *in vitro* studies the dextran produced from *Weissella cibaria* JAG8 could be used as potential substrate for prebiotic applications. The application of dextran as food additive was carried out by incubating dextransucrase with varying concentrations of sucrose *viz*. 2%, 4%, 6% and 8% in skimmed milk for 8 h at 30°C. Skimmed milk

containing the dextransucrase but not sucrose was used as positive control and only milk was used as negative control. While Appreciable degrees of solidification were achieved when sucrose was used at concentrations ranging from 2% to 8% (w/v). The enzyme-mediated milk solidification was due to the synthesis of dextran from sucrose. The dextransucrase and the dextran from *Weissella cibaria* JAG8 has a promising potential for application as a safe food additive to improve the textural properties of sucrose-supplemented dairy products. Earlier reports have suggested that the modification of the textural properties of dairy products by exopolysaccharides (EPS) was the consequence of interactions between these polymers and the proteins present in milk. The micro-structure of milk-derived products depends on physical characteristics of EPS, including the nature of glycosidic linkages, charge, branching linkages, and molecular mass, as well as the type of the proteins present in the milk and the EPS (or) milk protein ratio. These structural features offered by EPSs from LAB can explain their extensive application in the food industry to enhance the rheological properties of dairy and bakery products.

CONTENTS

Chapter 1

General Introduction

1.1 Exopolysaccharides

Bacteria produce a variety of polysaccharides with varied chemical properties by utilization of simple to complex substrates. Some of these polysaccharides perform the same function whereas others serve distinct biological functions (Anderson *et al*., 1990; Rehm *et al*., 1997). With respect to cellular location, biopolymers could either be intracellular or extracellular. Majority of the polysaccharides produced by bacteria are extracellular in nature. Technological advancement has led to the discovery of the usefulness of bacterial exopolysaccharides to man, consequently ensuring a myriad of industrial and medical applications. The inherent biocompatibility and apparent non-toxic nature of some of these bacterial exopolysaccharides has prompted their uses in numerous medical applications as scaffolds or matrices in tissue engineering, drug delivery and wound dressing, thus making them more attractive compared to polysaccharides from plants and microalgae origin (Rehm 2010; Sutherland 1998; Otero 2003). Some biopolymers are gradually degraded *in vivo*, making them well

suited for use in tissue replacement and controlled drug release (Rehm 2010). The history of bacterial exopolysaccharides began during the mid-19th century with the discovery of an exopolysaccharide in wine, which was later known as dextran and the bacterium responsible for the production was identified as *Leuconostoc mesenteriodes* (Rehm 2010; Linker and Jones 1966). Over the course of time, other exopolysaccharides *viz*. cellulose, alginate and xanthan were discovered (Nwodo *et al*., 2012). The addition of hydrocolloids such as xanthan gum, guar gum and hydroxy propylmethylcellulose (HPMC) was essential for generation of gluten-free (GF) baking food products in terms of texture, volume and shelf life. Many lactic acid bacteria (LAB) produce a wide variety of long-chain sugar polymers called exopolysaccharides (EPS), which vary in chemical composition, structure and physical properties (De Vuyst *et al*., 1999). These polysaccharides are synthesized extracellularly by glycosyltransferase using sugar as precursor molecule. The LAB, generally produce oligo- and homopolysaccharides, which improve the nutritional property of gluten free cereal food products by acting as prebiotics and hydrocolloides. Recently, Schwab *et al*., (2008) have showed the applicability of *Lactobacillus reuteri* LTH5448 and *Weissella cibaria* 10M; whereas the implication potential of *W. cibaria* MG1 and *W. kimchii* in GF sourdoughs has been investigated by Galle *et al*., (2010).

1.2 Exopolysaccharides produced from Lactic acid bacteria

Lactic acid bacteria (LAB) are used in many fermented foods particularly fermented dairy products such as cheese, buttermilk, and fermented milks. LAB produce lactic acid, carbon dioxide, and diacetyl (or) acetoin that contribute to the flavor, texture, and shelf life of fermented foods. Some LAB produce exopolysaccharide (EPS), which play a major role as natural texturizer in the

industrial production of yoghurt, cheese, and milk-based desserts. Recently, EPS produced by LAB have received increasing attention, mainly because of their health benefits. In particular, immune stimulation, antimutagenicity, and the antitumor activity of fermented dairy products prepared with EPS-producing LAB or EPS themselves have been investigated (Chabot *et al*., 2001; Kitazawa *et al*., 1998; Sreekumar and Hosono 1998; Tsuda *et al*., 2008). EPS are polysaccharides secreted from the cell induced by extracellular enzymes. The genes encoding the EPS synthesis is located on the plasmid, such as in *Lactococcus lactis* and *Lactobacillus casei* (Van Kranenburg *et al*., 1997) or on the chromosome, as in all thermophilic LAB (De Vuyst *et al*., 2001). The EPS from LAB are divided into two classes, homo- and hetero-EPS. Homo-EPS are composed of one type of monosaccharide, whereas hetero-EPS consist of regular repeating units of 3-8 different carbohydrate moieties synthesized from intracellular sugar nucleotide precursors (Ganzle *et al*., 2005). The biosynthesis of homo-EPS and hetero-EPS are different. Homo-EPS are made from sucrose using glucansucrase or levansucrase (Kralj *et al*., 2004; Van Hijum *et al*., 2004) whereas the synthesis of hetero-EPS involves four major steps, sugar transportation, sugar nucleotide synthesis, repeating unit synthesis, and polymerization of the repeating units (De Vuyst *et al*., 2001). The major physiological function of EPS is believed to be the biological defense against various stresses such as phage attack, toxic metal ions, and desiccation (Ruas-Madiedo *et al*., 2002) and it is very unlikely that bacteria use EPS as an energy source. However, Ruijssenaars *et al*., (2000) and Korakli *et al*., (2002) proved that some potentially probiotic LAB strains have been reported to degrade EPS produced by the other LAB strains.

1.3 Production of dextransucrase

Dextransucrase production is affected by several factors like temperature, aeration and medium components (Cortezi *et al*., 2005). Tsuchiya *et al*., (1952) intensively studied the culture medium composition for dextransucrase production from *Leuconostoc mesenteroides* NRRL B-512F and found the conditions 27°C and pH 6.7 best for efficient growth without enzyme denaturation. Goyal *et al*., (1995) reported the maximum production of dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F at 23°C under static condition. Santos *et al*., (2000) studied the same strain and observed maximum dextransucrase activity at 20°C and decrease in enzyme activity with increase in temperature. Purama and Goyal (2009) reported 25°C temperature and shaking condition was optimum for dextransucrase production from the *Leuconostoc mesenteroides* NRRL B-640. For glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146, 28°C temperature and static condition was optimum (Majumder and Goyal 2008). However, the regulation of pH and aeration conditions have little effect on the enzyme production by strain *Leuconostoc mesenteroides* NRRL B-1299 (Dols *et al*., 1997). Tsuchiya *et al*., (1952) assessed the effect of sucrose concentration on dextransucrase production from *Leuconostoc mesenteroides* NRRL B-512F and concluded that 2% (w/v) sucrose was the optimum level. Tsuchiya *et al*., (1952), Barker *et al*., (1993) and Lazic *et al*., (1993) showed that when medium pH drops to 5.0-5.5, dextransucrase was more active in transforming sucrose to dextran. Majumder and Goyal (2007a) studied the glucansucrase production from *Leuconostoc dextranicum* NRRL B-1146 in a bioreactor. Recently, the dextransucrase production from *Weissella* species has been increasing tremendously because of high-yielding nature and unique properties of the dextran produced. The optimum condition for *Weissella cibaria* CMU dextransucrase

was found to be pH 5.4 and 20°C (Kang *et al*., 2009). Shukla and Goyal (2011) has reported that dextransucrase from *Weissella confusa* Cab3 showed maximum production at pH 5.2-5.6 and 25°C under shaking conditions. In case of *Weissella cibaria* JAG8 the optimum production of dextransucrase was pH 6.0 and 24°C under static conditions (Tingirikari and Goyal 2013a). For *Weissella* sp TN610 the optimum conditions for dextransucrase production was pH 5 and 37°C (Bejar *et al*., 2013).

1.3.1 *Purification of dextransucrase*

Various methods such as precipitation by ammonium sulphate, ethanol or polyethylene glycol, phase partitioning, ultrafiltration and chromatography are used to purify the enzyme (Goyal and Katiyar 1995; Majumder *et al*., 2007b; Pijning *et al*., 2008). Polyethylene glycol (PEG) fractionation is a reasonably successful and cost-effective method for purification of dextransucrase. PEG is a non-ionic hydrophilic detergent known to selectively precipitate the proteins of high molecular weights or in aggregated forms. PEG fractionation method is inadequate for removal of the associated polysaccharides from the dextransucrase. For characterization of the enzyme, it should be essentially free of the carbohydrate content and the yields of purification should be high. This is achieved by a combination of dextranase treatment, ion-exchange and affinity chromatography after PEG precipitation (Majumder *et al*., 2007b). Dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F was purified by concentration and dialysis of the culture supernatant followed by treatment with dextranase and chromatography on Bio-Gel A-5m, which resulted in 240 fold purification with a specific activity of 53 (U/mg), (Robyt and Walseth 1979). Kobayashi *et al*., (1986) reported the efficacy of DEAE-cellulose and Sephadex G-100 column chromatography of dextransucrase from the same strain,

which resulted in 679 fold purification with a specific activity 26 (U/mg). Kang *et al*., (2009) purified the cloned dextransucrase rDSRWC from *Weissella cibaria* CMU strain using Ni-NTA super flow column and Centricon Ultracel YM-100 column which showed 5 fold purification with specific activity of 12.1 (U/mg). Where as in case of *Weissella cibaria* JAG8, dextransucrase purified by Sephacryl S-300 HR gave 37 fold purification with a specific activity of 37 U/mg (Tingirikari and Goyal 2013b).

1.3.2 *Biochemical characterization of dextransucrase*

The properties of dextransucrase have been extensively studied and reviewed (Kim and Robyt 1995b). The molecular sizes of dextransucrase was in the 160–180 kDa range in several LAB strains belonging to the *Leuconostoc*, *Lactobacillus*, and *Weissella* genera (Bounaix *et al*., 2010a; Bounaix *et al*., 2010b; Kralj *et al*., 2004). Some other LAB strains were described to produce extracellular glycosyl transferase with a molecular mass over 180 kDa. In case of *L. reuteri* 180 and *L. reuteri* ML1, the molecular mass of enzyme was about 200 kDa (Kralj *et al*., 2004). The highest molecular mass of dextransucrase reported so far was of about 313 kDa, produced by *L. mesenteroides* NRRL B-1299 (Bozonnet *et al*., 2002). The variation in the molecular size has been associated with the presence of dextran in the purified dextransucrase preparations, disassociation of high molecular mass multimeric complex (Kim and Robyt 1995b) or action of proteases (Miller and Robyt 1986). Dextrasucrase activity depends on pH, temperature and the dilution (Miller and Robyt 1984). A purified glucansucrase from *Leuconostoc mesenteroides* B512F enzyme exhibited maximum activity at 30°C and pH 5.2 (Goyal *et al*., 1995). The glucansucrase elaborated by *Leuconostoc mesenteroides* NRRL B-640 displayed a maximum enzyme activity when assayed in the temperature range of 30-35°C and at

pH of 5.4 (Purama and Goyal 2008a). In case of *Weissella confusa* Cab3 (Shukla and Goyal 2011) and *Weissella cibaria* JAG8 (Tingirikari and Goyal 2013b), the enzyme exhibited maximum activity at 35°C and pH 5.4 Divalent cations are associated with glucansucrase, hence they stabilize the activity of enzymes (Goyal *et al*., 1995). The activity of enzyme was enhanced by the addition of alkaline earth metals and inhibited by chelating agent such as EDTA, indicating that glucansucrases are associated with alkaline earth metals (Kobayashi and Matsuda 1980). Miller and Robyt (1986) reported the association of Ca^{2+} ions with the catalytic sites of glucansucrase.

1.4 The genus *Weissella*

Weissella are gram positive, non spore forming, non motile, short rod and hetero fermentative lactic acid bacteria (Martinez-murcia and Collins 1990). Nine species are recognized so far, comprising of *Weissella confusa*, *W. halotolerans*, *W. hellenica*, *W. kandleri*, *W. minor*, *W. paramesenteriodes*, *W. thailadensis* (Tanasupawat *et al*., 2000), *W. viridescens* and *W. cibaria* (Bjorkroth *et al*., 2002). It was observed that *Weissella* strains have been isolated from a variety of sources. *W. paramesenteroides* is predominantly found in fresh vegetables and plays an important role in the first phase of silage fermentation (Dellaglio *et al*., 1984; Dellaglio and Torriani 1986). *W. halotolerans*, *W. hellenica* and *W. viridescens* have been associated with meat and its products (Niven *et al*., 1957; Milbourne 1983; Collins *et al*., 1993), *W. kandleri* have originated from a desert spring and desert plants (Holzapfel *et al*., 1982). *W. confusa* strains have been detected in sugar cane, carrot juice, raw milk and sewage (Hammes and Vogel 1995), while *W. minor* was isolated from the sludge of milking machines (Kandler *et al*., 1983). Recent studies on lactic acid bacteria (LAB) associated with

traditional fermented foods have been characterized and many of these foods contain *Weissella* species (Hancioglu and Karapinar 1997; Ampe *et al*., 1999; Paludan-Muller *et al*., 1999). The best example was *W. thailandensis* which was isolated from fermented fish product in Thailand (Tanasupawat *et al*., 2000). Members of the *Weissella* genus are of economic importance in the fermented food and baking industry for the *in situ* production of exopolysaccharides which are of prebiotic potential. *Weissella kimchii* PL9023 was selected as prebiotic as it produces mostly hydrogen peroxide which inhibited the growth and adherence of vaginal isolates of *candida albicans*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus agalactiae* as reported by Lee (2005) and Maina *et al*., (2008) has reported the industrial useful of dextran producing *Weissella confusa* E392 in the production of linear dextran. The exopolysaccharides produced by *W. cibaria* 10M was investigated in generation of gluten free sourdough (Schwab *et al*., 2008). The glucooligosaccharides produced by *W. cibaria* 10M during fermentation were not digested by baker's yeast as a result there is a significant intake of prebiotic glucooligosaccharides (Schwab *et al*., 2008). It was observed that *W. kinchii* F28 and *W. cibaria* MG1 produced dextran in concentrations high enough to be use as potential replacers of non bacterial hydrocolloids, such as guar gum, and (hydroxyl propyl methylcellulose) HPMC in gluten-free sourdough (Galle *et al*., 2010). The glucan produced by *W. cibaria* CMU strain has strong inhibitory activity against the formation of *Streptococcus mutans* biofilms (Kang *et al*., 2006) as the latter causes dental caries. So *W. cibaria* CMU can be used as potential probiotic strain in oral health. The prebiotic potential of dextran produced from *W. cibaria* A2 and *W. confusa* A9 has been reported very recently (Hongpattarakere *et al*., 2012). Recently the application of dextransucrase produced from *Weissella* sp. TN610 (Bejar *et al*.,

2013) has been reported to have a promising potential application as a safe food additive to improve the textural properties of sucrose-supplemented dairy products. This enzyme-mediated milk solidification was attributed to the synthesis of dextran from sucrose as previously observed by Kim *et al*., (2008) in case of *W. hellenica SKKimchi* 3 strain.

1.5 Dextran producing lactic acid bacteria

The chemical structure of the dextran is highly specific to the dextransucrase producing strain (Dols *et al*., 1997). *L. mesenteroides* NRRL B-512F is the commercial dextran producing strain (Goyal *et al*., 1995). *L. mesenteroides* NRRL B-640 (Purama and Goyal 2008a), *L. dextranicum* NRRL B-1146 (Majumder and Goyal 2009a), *L. mesenteroides* NRRL B-1299 (Edward *et al*.,1974), NRRL B-523 (Padmanabhan and Kim 2002), *L. mesenteroides* NRRL B-742 (Kim and Robyt 1995a) are some other well-known dextran producing lactic acid bacteria. The cariogenic *Streptococcus mutans* (Kang *et al*., 2006), *Streptococcus* OMZ 51 and the non-cariogenic *Streptococcus* ATCC 10558 produce dextrans (Sidebotham *et al*., 1971). A human oral strain of *Lactobacillus casei* is reported to produce dextran-like, extracellular polysaccharide (Hammond 1969). Bjorkroth *et al*., (2002) reported *Weissella cibaria* species for the first time and later the probability of dextran production by *W. cibaria* CMU species was reported by Kang *et al.,* (2006). Later *W. kinchii* F28 and *W. cibaria* MG1 (Galle *et al*., 2010), *W. cibaria* 10M (Schwab *et al*., 2008), *W. cibaria* A2 and *W. confusa* A9 (Hongpattarakere *et al*., 2012), *Weissella* sp TN610 (Bejar *et al*.,2013), *W. cibaria* CMGDEX3 (Rifat *et al*., 2012) were reported for dextran production. While the other strains of *Weissella* are *W. confusa* E392 (Maina *et al*., 2008), *W. confusa* VTTE-90392 (Katina *et al*., 2009) and *W. confusa*

Cab3 (Shukla and Goyal 2011). Recently, the applications of dextran and gluco-oligosaccharides produced by *Weissella* species has been increasing tremendously in food and baking industry.

1.6 Active site mapping studies of dextransucrase by chemical modification

1.6.1 *Dextran biosynthesis and reaction mechanism by dextransucrase*

Dextransucrases are extracellular enzymes produced by *Leuconostoc*, oral *Streptococcus*, *Lactococcus*, *Lactobacillus* and *Weissella* species respectively (Sidebotham 1974; Barker and Ajongwen 1991; Kang *et al*., 2006). Glucansucrases (GSs) are classified as glycoside hydrolase family 70 (GH70) enzymes according to the CAZy classification system (Cantarel *et al*., 2009) based on amino acid sequence similarity. Within a GH family the catalytic mechanism and the catalytic residues are conserved. The GSs (GH70) are evolutionary, structurally and mechanistically closely related to the GH13 and GH77 enzymes and together they form the GH-H clan (Stam *et al*., 2006) of glycoside hydrolases. The common characteristic of GH-H clan enzymes is that they cleave the α-glycosidic linkage between a glucose moiety and another (glucose, fructose, etc.) moiety using a catalytic (β/α)8 barrel domain. The catalytic domain is decorate with N and (or) C-terminal domains. The type and number of other domains are different for each type of GH-H enzyme, indicating that the additional domains determine the reaction specificity of GH-H enzymes. However, the reaction specificity is largely determined by the catalytic domain. Many of the additional domains are, however, essential for catalytic activity of the enzymes. Moreover, they are often carbohydrate binding modules that provide the enzymes with carbohydrate binding functionality (Janecek *et al*., 2011). GS genes have been

cloned mainly from LAB strains belonging to the *Leuconostoc*, *Lactobacillus*, *Streptococcus* and *Weissella* genera (Kang *et al*., 2009; Ferretti *et al*., 1987; Funane *et al*., 2000; Bozonnet *et al*., 2002; Kralj *et al*., 2002) They encode large proteins with approximately 1400–1800 amino acid residues (Van Hijum *et al*., 2006). The cloning of dextransucrase encoding genes in addition to structure-function relationship studies have allowed the identification of important amino acid residues and have shown that dextransucrases are composed of two functional domains: a N-terminal core region (1000 amino acids) involved in sucrose binding and splitting and a C-terminal domain (500 amino acids) composed of a series of tandem repeats involved in dextran binding (Funane *et al*., 2005). In the highly-conserved core region, about 700 amino acids make up a circularly permuted (β/α)8 barrel (MacGregor *et al*., 1996), catalytic Asp (Tsumori *et al*.,1997) and essential substrate-binding Gln (Monchois *et al*., 1999) exist in this region. A striking feature of the amino acid analysis of these dextransucrase is that they are virtually devoid of cysteine residues and are rich in acidic amino acid residues. Less information is available on the nature of amino acids present at the active site of dextransucrase. In a two-site mechanism for dextran synthesis, it was shown that the two nucleophiles at the active site attack the two bound sucrose molecules to give two covalent intermediates (Robyt *et al*., 1974). Later, Remaud-Simeon *et al*.,(2006) cloned the N- and C-terminal truncated dextransucrase of *L. mesenteroides* B-512F in *E. coli* and stated that dextran is synthesized by a non processive or semi processive reaction in which D-glucose and sucrose act as initiator primers and the D-glucose moiety of sucrose is added to the C-6–OH of D-glucose and to the C-6–OH of the D-glucose moiety of sucrose to give dextran polymerization from a single active site by the addition to the non-reducing-

ends of isomaltodextrins and not by the two-site insertion mechanism (Robyt *et al*., 2008).

Apart from synthesis of dextrans, the dextransucrases also catalyze secondary trans-glycosylation reactions in which the D-glucose moiety of sucrose is transferred to mono- and oligosaccharides present or added to the digest to give oligosaccharide products (Koepsell *et al*., 1952; Tsuchiya *et al*., 1955; Robyt and Walseth 1978). This is called an 'acceptor reaction' and occurs at the expense of dextran synthesis (Robyt and Eklund 1983). The acceptor reactions also involve (a) the transfer of D-glucose to an acceptor monosaccharide or oligo-saccharide (Robyt and Walseth 1978) (b) the transfer of D-glucose to a dextran chain to give D-glucosyl branch linkages, and the transfer of the dextranyl chain to a dextran chain to give dextranyl branched dextran chains (Robyt and Taniguchi 1976) (c) a very minor acceptor reaction in which the D-glucose moiety of sucrose is transferred to water to give the hydrolysis of sucrose (Luzio and Mayer 1983) (d) and the transfer of the dextranyl chain to water and/or to an acceptor saccharide (D-glucose, D-fructose, sucrose, or maltose) to release the dextran from the active site and terminate polymerization (Robyt and Walseth 1978; Luzio and Mayer 1983).

1.6.2 *Chemical modification studies*

The mode of action of enzyme on different substrates has attracted many investigators in understanding their structural organization and reaction mechanism. The main characteristic feature of the enzymes is their ability to catalyze a reaction at a very high rate under mild conditions. Several methods such as site-directed mutagenesis (Wagner and Benkovic 1990), chemical modification (Anderson and Morgenstern 1990), reaction kinetics (Jabalquinto *et al*., 1983) X-ray diffraction

(Hajdu and Johnson 1990) and nuclear magnetic resonance (Wuthrich 1989) have been applied to understand the reaction mechanism. By studying reaction kinetics with chemical modification techniques, a great deal of information regarding the structural and functional aspects of the enzyme can be obtained. Chemical modification is one of the versatile and rather simple techniques employed not only in identification of functional groups in enzyme but also in understanding the role of amino acids in stability of enzyme. Generally, the modification of enzyme involves binding of chemical moiety to the side chain of amino acid residues in it leading to change in its properties. Amino acids with polar side chain are normally the targets for chemical modification. Chemical modification studies revealed that the inhibitor binds to the functional group belonging to a particular amino acid residue only without affecting other functional groups or the conformation of enzyme molecule.

1.6.3 *Specificity of enzymes*

Generally, all the enzymes are highly substrate specific in nature. The non-covalent forces through which substrates and other molecules bind to the enzyme are identical to the forces that confers the conformation of the enzymes which involve vander waals force, hydrogen bonding, electrostatic interactions, ionic and non ionic interaction (Voet and Voet 1990). It is a known fact that amino acids present at the active site are arranged in a particular fashion, which allows only the substrate molecules to bind or interact in a specific manner.

1.6.4 *Essential amino acid residues and their behavior*

The functional side chains of amino acids located at or near the active site are involved in the substrate binding and catalysis. These amino acid residues are very

crucial for the enzyme activity and the modification of their functional groups will lead to loss in enzyme activity. The amino acid residues which participate in the catalytic activity of enzyme are called essential amino acid residues. These include acidic amino acids such as glutamate and aspertate, basic amino acids such as lysine, arginine and histidine, polar uncharged amino acids like serine, cysteine, tyrosine and side chains of methionine (with a nucleophilic sulfur) and tryptophan (with a heterocyclic indole side chain) (Eyzaguirre 1987). The above residues are generally present in a specific arrangement so that substrate molecules can easily interact and form enzyme substrate complex. There are several factors which are responsible for different behavior of active site residues *viz*. polarity which affects dissociable side chain, hydrogen bonding which stabilize the neutral or ionic species, electrostatic effects (presence of charges in the vicinity of the group) and the steric effect by the adjacent side chains (Cohen 1970). All the above factors differentiate the essential amino acid residues from non essential ones.

1.6.5 *Group specific reagents*

Generally the chemical reagents are highly specific to particular amino acids. They interact by binding covalently with side chain of amino acid residues present at or near the active site (Bell and Bell 1988; Creighton 1989). The binding of these chemical modifiers greatly depend on the specificity of amino acid residues. Reagents and reaction conditions for chemical modification of enzyme are chosen depending on the properties of enzyme and the purpose of investigation. The essential residues involved in the catalysis can be selectively modified by a variety of reagents. The absorbance maxima of various chemical reagents are mentioned in Table 1.1.

Table 1.1 Represents the absorbance maxima of different chemical reagents

Chemical reagents	**Absorbance maxima (nm)**
Pyridoxal 5**'**-phosphate	325
2,4,6-Trinitro benzene-1-sulphonic acid	367
Iodoacetic acid	323
5,5**'**-Dithiobis (2-nitro-benzoic acid)	412
o-Phthalaldehyde	337

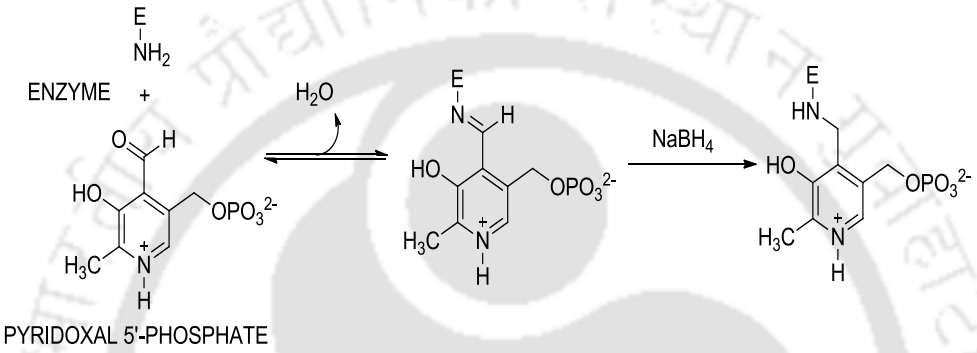
1.6.6 *Lysine specific reagents*

The lysine specific reagents include Pyridoxal 5'-phosphate (PLP) and 2, 4, 6-trinitro phenyl benzene sulphonic acid (TNBS). These chemical modifiers are highly specific in nature. PLP has been extensively used to study the role of lysine as active site residues of enzymes (Talbot *et al*., 1977; Katiyar and Porter 1982; Dong and Fromm 1990). The reaction of PLP with lysine residues results in the formation of schiff's base which on reduction with sodium borohydride gives an irreversible Nε-phosphopyridoxyl lysine derivative. The group introduced in the reaction product is a chromophore with absorbance maxima at 325 nm. The above chromophore can be detected by fluorescence spectroscopy on excitation at wavelength of 325 nm which gives characteristic fluorescence maxima near 400 nm depending on the nature of active sire of enzyme. The inactivation (or) modification of enzyme with PLP can be made reversible by simple dialysis of enzyme PLP complex (or) Schiff's base (Fig 1.1) at 4°C for 24 h (Yost and Harrison 1971; Katiyar and Porter 1982). The reversibility is dependent on the nature and orientation of catalytic residues present at the active site of the enzymes.

2,4,6-Trinitrobenzene-1-sulphonic acid (TNBS) specifically binds with amino group of lysine and has been used for chemical modification studies of various

enzymes. The reaction of enzyme with TNBS is irreversible (Freedman and Radda 1969; Carlberg and Mannerwick 1979). It reacts with ε- amino group of lysine to give a trinitrophenyl derivative (Fig 1.1) with absorbance maxima around 367 nm (Bell and Bell 1988) and can be quantified by absorbance measurement at this wavelength.

(A)



(B)

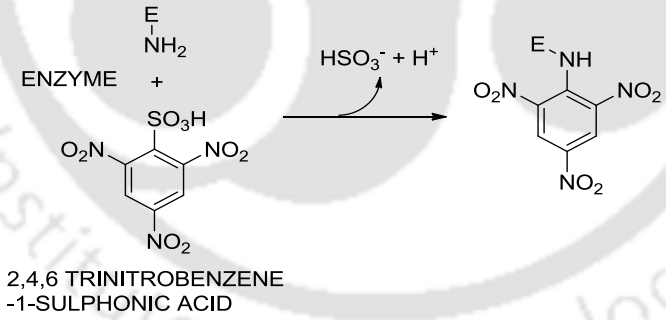


Fig 1.1. Modification of enzyme in presence of lysine specific reagent

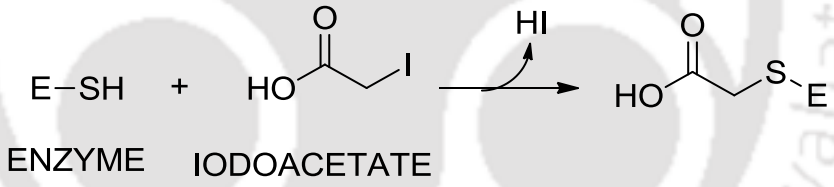
1.6.7 *Cysteine specific reagents*

A wide range of reagents are available for the modification of sulfhydryl group of cysteine. Some of them are Iodoacetic acid and 5, 5'-dithiobis (2-nitro-benzoic) acid (DTNB) which plays important roles in the identification of cysteine

residues in the enzyme, owing to their high selectivity. Iodoacetic acid reacts with sulfhydryl group of cysteine to give acid stable derivative (i.e) thioacetate complex with absorbance maxima around 323 nm which further depends on the amino acids residues present at the active site. The formation of thio acetate derivative is shown in Fig (1.2).

DTNB is a widely used chemical reagent for the detection of cysteine residues at (or) near the active site. This reagent forms a mixed disulfide with cysteine and releases the thio-nitrobenzoate anion (Fig 1.2), which can be quantified spectroscopically by absorbance maxima around 412 nm depending on the nature of active site residues present in the enzyme (Bulaj *et al*., 1998).

(A)



(B)

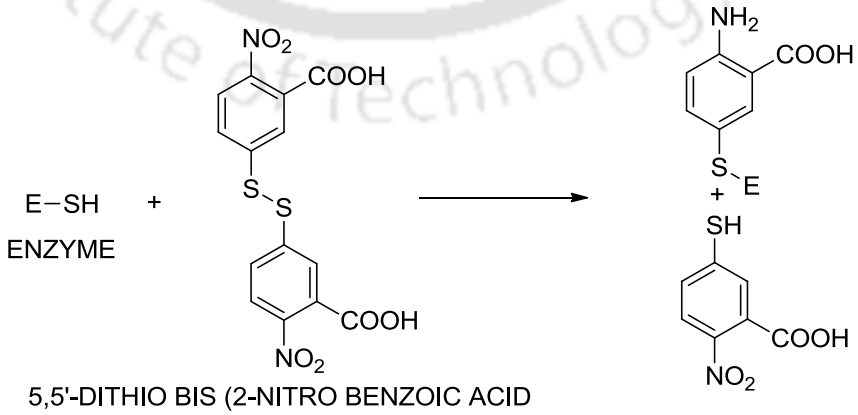


Fig 1.2. Modification of enzyme in presence of cysteine specific reagent.

1.6.8 *Modification of enzymes by o-phthalaladehyde, a fluorogenic bifunctional chemical reagent*

A fluorogenic bifunctional reagent, *o*-phthalaladehyde is amongst very few compounds which have been used for the characterization of the nature of amino acids present at the active site of the enzyme. *o*-phthalaldehyde is regarded as a bifunctional reagent because it specifically binds to the amino group of lysine and sulfhydryl group of cysteine. The chemical reagent can form fluorogenic isoindole derivative only when the distance between the two active site residues (i.e) lysine and cysteine is in the range of 2.6-3.4A°. The isoindole derivative formation will not occur if the distance between the two residues is less than 2.6A° and greater than 3.4A°. Palczewski *et al*., (1983) reported the isoindole derivative formation involving lysine and cysteine residues in aldolase enzyme. The fluorogenic property of *o*-phthalaladehyde with amino acids in presence of reducing agents like β-mercaptoethanol was reported by Roth (1971). Fluorescence studies help in understanding the nature of the catalytic environment because maxima of emission spectra is red or blue shifted depending on the environment present at the active site. The molar transition energies (E_T) can be calculated from the fluorescence emission maximum (λ_{em}) of an isoindole derivative with the linear free energy relationships as described by the equation (Palczewski 1983).

$$E_T = 2.985\ \lambda_{em} - 1087.28$$

Where λ_{em} corresponds to the wavelength of the fluorescence emission maximum of an isoindole derivative in solvents of different polarities and was employed to calculate the values of E_T for reactants. The reaction of dextransucrase with *o*-phthalaldehyde is shown in Figure 1.3.

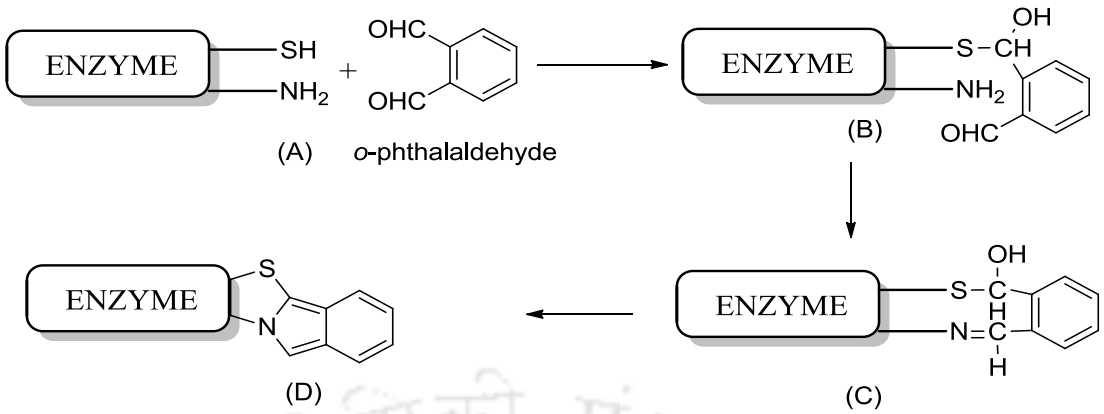


Fig.1.3. The reaction of *o*-phthalaldehyde with sulfhydryl group of cysteine and ε-amino group of lysine residues of an enzyme.

1.7 Production and purification of dextran

Conventional fermentation used for the production of dextran involves three phases: cell growth, enzyme production phase and glucan synthesis (Alsop 1983). Extensive work has been done on optimization and modification of the fermentation processes for improved production of glucan (Stacey 1942; Hehre 1946, Koepsell and Tsuchiya 1952; Jeanes *et al*., 1957; Jeanes 1965; Lawford *et al*., 1979; Barker *et al*., 1987; Landon and Webb 1990; Brown and McAvoy 1990; Lazic *et al*., 1993; Ajongwen and Barker 1993; Barker *et al*.,1993). Since, glucan synthesis takes place outside the cell in presence of glucansucrase, decoupling of the enzyme and glucan production was explored for optimizing the glucan synthesis (Landon and Webb 1990).

1.7.1 *Structure of dextran*

Dextrans $(C_6H_{10}O_5)_n$ are a class of homopolysaccharide consisting of D-glucopyranosyl units polymerized predominantly in α(1→6) linkage (Padmanabhan and Kim 2002). The α(1→2), α(1→3) and α(1→4) glycosidic linkages form

branching (Kim *et al*., 2003; Kang *et al*., 2003). The degree of branching involving α-(1→2), α(1→3) and α(1→4) linkages in dextrans vary according to the origin of dextransucrase (Seymour and Knapp 1980). Dextran from *Leuconostoc mesenteroides* NRRL B-512F consists of 95% of α(1→6) osidic linkages and 5% of α(1→3) branched linkages (Bertrand *et al*., 2006). *L. mesenteroides* NRRL B-1299 produces a highly branched dextran containing between 27-35% of α(1→2) branch linkages (Dols *et al*., 1997). It was reported earlier that dextran produced from *Weissella* species such as *Weissella* sp TN610, *Weissella cibaria* CMGDEX3 and *Weissella confusa* E392 are linear in nature with α (1→6) linkage and have lower percentage of α(1→3) branching *i.e* 4%, 3.4% and 2.7%, respectively (Bejar *et al*., 2013; Maina *et al*., 2008; Bounaix *et al*., 2009; Rifat *et al*., 2012). In case of *W. cibaria* CMU, the dextran synthesized was linear (Kang *et al*., 2009). The molecular weights and the degrees of branching of dextrans depend on the sucrose concentration, temperature and pH conditions (Kim *et al*., 2003). The bio-funtionality of dextrans hinge on their physico-chemical attributes. So, the analytical characterizations of the dextrans are of utmost importance. The differences in dextran structure are determined by Fourier-transform infrared spectroscopy, ^{1}H and ^{13}C nuclear magnetic resonance spectrometry (Holt and Cote 1998). The FT-IR, NMR, scanning electron microscopy and rheological studies on dextrans of *L. mesenteroides* NRRL B-640 and *L. dextranicum* NRRL B-1146 have been carried out. *L. mesenteroides* NRRL B-640 synthesizes a α (1→6) linked linear dextran (Purama *et al*., 2009) and *L. dextranicum* NRRL B-1146 synthesizes a α(1→6) linked dextran with α(1→4) branching (Majumder and Goyal 2009b). Misaki *et al*., (1980) studied the structure of dextran from *L. mesenteroides*

B-1355, which revealed the branched structure containing α(1→6) and α(1→3) glucosidic linkages (Seymour 1980). While the dextran from *W. cibaria* CMU synthesizes a α(1→6) linked linear dextran (Kang *et al.,* 2006) and *W. cibaria* CMGDEX3 shows 96.6% of α(1→6) and 3.4% of α(1→3) glucosidic linkages. In case of *W. cibaria* JAG8, 93% of α(1→6) and 7% α(1→3) glucosidic linkages was reported by Tingirikari and Goyal (2013a).

1.7.2 *Applications of dextran*

Dextrans have many industrial applications due to their non-ionic character and good stability under normal operating conditions. These are used for the matrix preparation of chromatography columns such as sephadex (Shamala and Prasad 1995). They are also used for preparing blood plasma substitutes, plasminogen activators and antithrombogenic agents (Soetaert *et al*., 1995; Purama and Goyal 2005). Iron dextran is used to treat iron deficiency anaemia (Thayu and Mamula 2005). Dextrans come handy in microsurgery to reduce the risk of free tissue transfer loss. They are used as lubricant in eye drops and to increase blood sugar levels. By coupling with dextran T40, plasma half-life of trichosanthin, a low molecular weight plant protein, capable of suppressing the replication of human immunodeficiency virus (HIV-1) can be prolonged as it escapes glomerular filtration (Ko *et al*., 1991). Sodium salt of dextran sulfate is also reported to inhibit AIDS virus (Baba *et al.,* 1990). Dextran sulfates are highly active inhibitors of encephalomyocarditis hemagglutination (Kunin 1967). Dextran hydrogels are used in various pharmaceutical and biomedical applications such as contact lenses, cell encapsulation for drug delivery, burn wound dressing and in spinal cord regeneration (Aumelas *et al*., 2007). The dextran produced from

Weissella species has got tremendous and wide application in dental, food and baking industry. It was observed that *W. cibaria* CMU isolated from human saliva have strong inhibitory activity against the formation of *Streptococcus mutans* biofilms (Kang *et al*., 2006). Glucan is the principal constituent of oral biofilms and also constitutes a potential site for the formation of caries. The use of *W. cibaria* as a probiotics in oral health may be considered. Probiotics may offer an attractive alternative to chemicals (including chlorhexidine, triclosan, sanguinarine, fluoride and a variety of antibiotics) with regard to the inhibition of oral biofilm formation (Shapiro *et al*., 2002). The water soluble polymers generated by the *W. cibaria* strain inhibited the synthesis of water-insoluble glucans from *S. mutans* by converting the glucosyltransferase activity for the production water-soluble glucans (Robyt and Martin 1983; Kang *et al*., 2006). Dextrans provide a stabilizing coating for protecting metal nanoparticles against oxidation (Bautista *et al*., 2005). Dextrans have been frequently employed as drug carriers (Ulbrich and Subr 2004). These are used in nanotechnology as tool for antigen delivery in vaccination (Sahoo *et al*., 2007). Dextran-conjugated dendritic nanoconstructs are being evaluated as potential vectors for anti-cancer agents (Agarwal *et al*., 2009). Colloidal iron oxide formulated with dextran is clinically used as MRI contrast agents (Koo *et al*., 2005). Encapsulated dextran acts as conjugate of cancer drug doxorubicin for tumour targeted delivery (Oh *et al*., 2009). Low molecular weight dextran sulfate acts as endothelial and cyto-protectant in solid organ and islet transplantation, preventing complement-mediated damage of the donor graft endothelium (Spirig *et al*., 2008). Generally hydrocolloids such as xanthan gum, guar gum and hydroxy-propylmethylcellulose (HPMC) are essential in gluten-free (GF) baking to obtain acceptable product quality in terms of volume, texture, and shelf life (Lazaridou *et al*., 2007). Recently, the applicability of

the dextran from *Weissella cibaria* 10M was investigated in GF sourdoughs (Schwab *et al*., 2008). The strain was suitable as sourdough fermentation starters for quinoa and sorghum, concomitantly producing dextran (gluco-oligosaccharides-GOS). These were softer than the ones without EPS. Moreover, GOS produced by the LAB were not digested by baker's yeast and they were still present in the final bread. Recently, Galle *et al*., (2010) screened EPS-forming *Weissella* strains for their potential use as starter strains in sorghum and wheat sourdoughs. In particular, the strains *W. kimchii* and *W. cibaria* MG1 produced dextrans in concentrations high enough to be used as potential replacers of non-bacteria hydrocolloids, such as guar gum and HPMC in gluten-free sourdoughs bread. All these studies indicate that EPS-producing Weissella strains in sourdough could play a promising role for the production of GF products with improved quality characteristics, reduced additives content and as a substitute of hydrocolloids in gluten-free cereal products. Dextran (EPS) from *W. cibaria* A2 and *W. confusa* A9 exhibited high potential prebiotic property, with resistance to gastric and intestinal digestions, selective enhancement of beneficial gut bacteria particularly bifidobacteria group indicating their prebiotic potentials as reported by Hongpattarakere *et al*., (2012).

1.8. Prebiotics

1.8.1 *Concept of prebiotics*

The concept of functional foods was introduced in Japan during the 1980s, and it could be defined as "any food or ingredient that may provide a health benefit beyond the traditional functions hitherto known" (Hasler 1998). Prebiotics can be defined as non-digestible food ingredients that beneficially affect the body by selectively stimulating the growth and/or activity of a limited number of bacteria in

the colon (Kaur and Gupta 2002; Anjo 2004). To be considered a prebiotic, the compound must reach the colon without degradation or alteration and must stimulate the normal gut flora (Saier and Mansour 2005; Bosscher *et al*., 2006). Food ingredients with prebiotic characteristics generally exhibit certain unique characteristics, such as limited hydrolysis and absorption in the upper gastrointestinal tract, selective augmentation of beneficial bacteria in the colon, potential to suppress pathogens and limit virulence by immuno-stimulation (Urgell *et al*.,2005; Huebner *et al*., 2007; Roberfroid 2007). The consumption of prebiotics has been associated with reduced risks of certain diseases. These include the suppression of diarrhea associated with intestinal infections; averting osteoporosis because inulin promotes the uptake of calcium and thereby increases bone mass; lowered threats of obesity and Type 2 diabetes; neutralization of toxic products and decreased frequency of colon cancer; stimulation of immunity and protection of the urogenital system (Cao and Fernandez 2005; Quera *et al*., 2005).

1.8.2 *Prebiotics: Origin and chemical nature*

Prebiotics are substances, capable of modifying the colonic microflora and stimulating the proliferation of non-pathogenic bacteria with health promoting potential, particularly *Lactobacilli* and *Bifidobacteria* (Kaur and Gupta 2002; Silva and Nornberg 2003; Huebner *et al*., 2007). Some examples of prebiotics include fructooligosaccharides, galactooligosaccharides, arabinose, galactose, inulin, raffinose, mannose, lactulose, stachyose, mannanoligosaccharides, xylooligosaccharides, palatinose, lactosucrose, glycooligosaccharides, isomaltooligosaccharides, soybean oligosaccharides, etc (Gibson and Fuller 2000; Saier and Mansour 2005; Bosscher *et al*., 2006).

1.8.3 *Criteria for classifying a food as a prebiotic*

1) It should not undergo hydrolysis or absorption in the upper gastrointestinal tract.
2) When it reaches the colon, it should be selectively metabolized by a limited number of beneficial bacteria.
3) It should be able to alter the colonic microflora to a healthier bacterial flora.
4) It should be capable of inducing a physiological effect that is beneficial to health (Manning *et al*., 2004).

1.8.4 *Properties exhibited by prebiotics*

1) Maintenance of intestinal flora and stimulation of intestinal transit (Arabbi 2001).
2) Change in colonic microflora, contributing to normal stool consistency, preventing diarrhea and constipation (Bosscher *et al*., 2006; Ouwehand *et al*., 2005; Macfarlane *et al*., 2006).
3) Elimination of excess substances such as glucose and cholesterol, favoring only the absorption of substances needed (Kaur and Gupta 2002).
4) Stimulation of the growth of bifidobacteria (Losada and Olleros 2002).
5) Stimulation of the absorption and production of B vitamins (B1, B2, B3, B6, B9, B12) (Wang and Gibson 1993).
6) Support of the immune system (Silva and Nornberg 2003).
7) Contribution to the control of obesity (Manning and Gibson 2004).
8) Contribution to the decrease of the risk of osteoporosis (Kaur and Gupa 2002).

Many non-digestible polysaccharides have been widely proven to have "prebiotic" potential, but only a few reports regarding the use of EPSs produced by LAB as prebiotics, apart from other beneficial effects mentioned above. EPSs from *W. cibaria* A2, *W. confusa* A9, *L. plantarum* A3 and *P. pentosaceus* 5S4

exhibited high potential prebiotic property of high resistance to gastric and intestinal digestions, selective enhancement of beneficial gut bacteria particularly bifidobacteria group indicating their prebiotic potentials enhanced growth of beneficial *Bifidobacterium* and *Lactobacillus* (or) *Enterococcus* groups, which were generally considered as the main bacterial target of prebiotic activity, (Gibson and Roberfroid 1995; Reid 2008).

1.8.5 *Applications of prebiotics*

Prebiotics can be found in some vegetables, such as leeks, onions, chicory, tomatoes, asparagus, artichokes, bananas, and alfalfa. It can also be added to industrial products such as foods for children, dairy and confectionery products, beverages, light mayonnaise and low-fat cheese, and they can be used as dietary supplements (Saier and Mansour 2005; Arabbi 2001). Prebiotics are being used in the food industry as functional ingredients in beverages (fruit juices, coffee, cocoa, tea, soft drinks and alcoholic beverages), milk products (fermented milk, milk powder and ice cream), probiotic yogurts and symbiotic products (Gibson and Roberfroid 1995; Mussatto and Mancilha 2007). Other applications include desserts (e.g. jellies, puddings, fruit-flavored ice cream), confectionery items (e.g. sweets), biscuits, breakfast cereals, chocolates, breads and pastas, meat products (e.g., fish paste) and tofu. Prebiotics can also be used in cosmetics, pharmaceuticals and products for people with diabetes (Mussatto and Mancilha 2007).

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Chapter 2

Screening, identification and characterization of high dextran yielding *Weissella cibaria* JAG8 isolated from apple

2.1 Introduction

Lactic acid bacteria (LAB) have been exploited for thousands of years for the production of fermented foods for their ability to produce desirable changes in the taste, flavor and texture as well as for their capability of inhibiting pathogenic and spoilage microorganisms. Fresh products like fruits and vegetables are rich in carbohydrates and poor in proteins with pH values ranging from 7.0 to slightly acidic. Microorganisms grow faster in wounded, damaged (or) cut vegetables and fruits than intact surfaces (Ponce *et al*., 2002). Due to high carbohydrate content and slightly acidic or neutral pH, fruits serve as perfect host for several LAB such as *fructobacillus* and *lactobacillus* species as reported by Endo *et al*., (2009).

LAB produce a wide variety of structurally different exopolysaccharides (EPSs). These EPSs include homopolysaccharides that consist of only one monosaccharide, such as glucose (glucans), fructose (fructans) or galactose (galactans) and heteropolysaccharides that consist of repeating units of different monosaccharides,

including glucose, galactose, fructose and rhamnose (De Vuyst and Degeest 1999). Some of these EPSs have also been reported to be beneficial to human health due to their anti-tumoral, immunomodulatory, cholesterol lowering and biofilm formation inhibiting activities (Ruas-Madiedo *et al*., 2002). Novel EPSs may replace a traditional polymer product in terms of the improved rheological and stability characteristics. Now, microbial polysaccharides represent only a small fraction of the current biopolymer market. The factors limiting the use of microbial EPSs are the production, which requires a thorough knowledge of their biosynthesis, an adapted bioprocess technology and the high cost of their recovery. Xanthan is a microbial EPS approved in the food industry, mainly because of its unique rheological properties in foods and the possibility of low-cost production. It is produced in high amounts by *Xanthomonas campestris*, a phyto-pathogenic bacterium that is not generally recognized as safe (GRAS) (De Vuyst and Degeest 1999). Strains of GRAS, food grade microorganisms in particular lactic acid bacteria that are able to produce EPSs in large enough quantities could be the solution to many of the above-mentioned disadvantages. Unlike the dextran produced from the species of *Weissella*, the dextran from *Leuconostoc* and *Lactobacillus* species are GRAS. The dextran produced by these species are widely used in pharmaceutical industry as blood volume expander and in food industry as conditioning, stabilising and bodying agent replacing natural gums (Koepsell and Tsuchiya 1952). It is used against iron deficiency anemia, in open wound healing and intermediate carrier for drug targeting (Robyt and Walseth 1979).

Weissella species are Gram positive, short rod, non-motile, non-spore forming, hetero fermentative bacteria grouped under LAB. They are normally isolated from fermented foods and have the ability to produce dextran in presence of dextransucrase using sucrose as substrate (Bjorkroth *et al*., 2002; Kang *et al*., 2006). LAB associated

with traditional fermented foods has been characterized and many of them have been found to be *Weissella sp.* (Schwab *et al*., 2008; Shukla and Goyal 2011). The dextran produced from *Weissella cibaria* species has been reported to act as a perfect hydrocolloid and 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 the generation of gluten-free food products for patients suffering from Celiac disease (Schwab *et al*., 2008; Galle *et al*., 2010). In this study, a high dextran yielding bacterium growing on the apple was isolated. The microorganism was identified as *Weissella cibaria* JAG8 based on morphological, biochemical, physiological characterization and 16S rRNA gene sequence analysis.

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 India 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

1 gram of fresh peels of apple, banana, rind of orange and bark of sugar cane were crushed separately using mortar pestle and mixed with 10 ml of MRS medium (DeMan *et al*., 1960) containing 2.0% (w/v) glucose 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): glucose, 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) Glucose 1.5% (w/v) agar with 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 28°C 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 aseptic condition.

2.2.4 Screening of the isolates based on enzyme activity

Ten colonies were picked from 10^{-8} dilution agar plate from each source *viz.* apple, orange, banana and sugar cane. Each culture was inoculated in 5.0 ml of enzyme production medium by Tsuchiya *et al*., (1952) as described in Chapter 2, Section 2.2.3. The enzyme activity of all the isolates were determined by measuring the amount of reducing sugar released as described in Chapter 2, Section 2.2.5 and the isolate with higher enzyme activity was selected for further studies.

The isolate showing the highest enzyme activity was selected for further study and named as JAG8 after the author's name. The isolate JAG8 was grown in modified MRS-agar stabs at 24°C for 16 h and maintained at 4°C. The isolate was propagated by sub-culturing every 2 weeks. The culture was preserved as glycerol stock using 22% (v/v) final glycerol concentration and frozen at -80°C for long term storage.

2.2.5 *Enzyme assay*

The enzyme assay was carried out in 1 ml reaction mixture containing 5% (w/v) sucrose, 20 mM sodium acetate buffer (pH 5.4) 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 for 20 min in boiling water bath. It was cooled to room temperature and then 100 µl of reagent C (Section 2.2.5.1) was added. The colour developed rapidly and simultaneously with the evolution of CO_2. The mixture was diluted by adding 700 µl distilled water. The absorbance of colour developed was measured at 500 nm on a UV-visible spectrophotometer (Varian, Cary 100). Fructose (0.5 µg/ml 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 30-37°C.

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 37°C 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 30°C 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} = Optical Density (OD) 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 Morphological and biochemical characterization of isolate JAG8

The phenotypic characterization of the isolate JAG8 was carried out by Gram staining (Millere *et al*., 1989). The Gram staining of the isolate JAG8 was performed by spreading the bacterial smear on the glass slide with the help of loop and was air dried. Later the glass slide was flooded with crystal violet solution and left it for 30s, the stain was decanted and it was rinsed with running water. The smear was treated with alcohol and then the slide was flooded with iodine solution for 30s, excess of this mordant was washed off with water and flooded with counter stain safranin for 30s. It was washed with water and the smear was observed under compound microscope.

The cell shape, size and their arrangement was studied by Scanning Electron Microscopy. The cell sample was prepared by taking the cell pellet after centrifuging 1ml of 12 h grown culture at 5,000 rpm and 4°C for 10 min. The cell pellet was resuspended in 1.0 ml of saline (0.85%, w/v). The sample was fixed with equal volume of glutaraldehyde (2.5%, v/v) for 4h. One drop of this bacterial smear was dehydrated using different percent of alcohol ranging from 30-100% (v/v) and dried in a vacuum desiccator. This dried sample was attached to the SEM stub with double-sided tape and coated with 10 nm Au in a sputter coater (SCH 620, Leo). The surface of the sample was viewed at various magnifications in Scanning Electron Microscope (Leo1330 VP) operated at 10.0 kV (Patel and Goyal 2010).

The catalase test was performed by adding few drops of 5.0% (v/v) H_2O_2 to 1.0 ml of overnight grown isolate JAG8 culture and *E. coli* DH5α cells were used as positive control (Millere *et al*., 1989). The agar slants procured from Hi-Media Pvt. Ltd, Mumbai, India, were used for motility test; triple sugar iron test and nitrate test were performed at 28°C, incubated for 24 h to detect the presence of *Enterobacterium*. Temperature tolerance was judged by growing 1.0% of the isolate JAG8 in modified MRS medium (Goyal and Katiyar 1996). The composition of modified MRS medium contains sucrose instead of glucose while rest of the components of the medium are similar to that of MRS medium described in Section 2.2.1, at temperature range of 15°C-45°C for 24 h. Halo tolerance of the isolate was determined by adding NaCl ranging from 4-9% (w/v) in modified MRS medium and growing at 28°C for 24 h, as described by Bjorkroth *et al*., (2002). The growth of the isolate was measured by culturing the isolate in modified MRS medium at initial pH of 2.0-10.0 to determine its pH tolerance as described by Manes-Laazaro (2008).

2.2.7 Antibiotic sensitivity of isolate JAG8

The isolate JAG8 was tested for its susceptibility to thirty antibiotics using agar disc diffusion test (Barry and Thornsberry 1980). The antibiotic test was performed using commercially available antibiotic octadiscs 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 JAG8 growing in log phase was mixed in modified MRS-soft agar (0.8%, w/v agar) medium and poured over the modified 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 JAG8

The isolate JAG8 was analyzed for its carbohydrate fermentation ability (Kandler and Weiss 1986). The culture JAG8 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 JAG8 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 *et al*., 2008).

2.2.9 Sequencing of 16S rRNA gene of isolate JAG8

The isolate JAG8 isolated from apple was sent to Bioaxis DNA Research Pvt. Ltd., Hyderabad, India, for 16S rRNA gene sequence analysis. The forward primer used was (27F) 5'-AGAGTTTGATCCTGGCTCAG-3' and (1525R) reverse primer 5'-AAGGAGGTGATCCAGCC-3'. The sequence generated from the test was subjected for Basic Local Alignment Search Tool (BLAST) analysis with non redundent database of National Centre for Biotechnological Information (NCBI) GenBank database. Based on maximum identity score, the sequences were selected and aligned using multiple sequence alignment software program ClustalW. Distance matrix was generated using Ribosomal Database Project (RDP) database and the phylogenetic tree was constructed by using Tree View software.

2.2.10 Effect of temperature and shaking on dextransucrase production from isolate JAG8

The effect of temperature on enzyme production was studied between 20°C and 40°C under static condition, using 100 ml of enzyme production medium as described in Section 2.2.3, in 250 ml Erlenmeyer conical flask. The effect of shaking condition on dextransucrase production was analyzed by varying the rpm from 100 to 175 at 24°C. 1.0 ml of culture was withdrawn from each conical flask at every 4 h interval

and centrifuged at 10,500*g* and at 4°C for 10 min. The cell free supernatant obtained was analyzed for dextransucrase activity as described in Section 2.2.5.

2.2.11 Fermentation profile of isolate JAG8

The growth parameters of isolate JAG8 such as pH, cell density, enzyme activity and dextran production were studied at every 4 h interval up to 24h under static condition at 24°C. The pH change was measured by using pH meter. The growth of culture was determined by measuring the optical density of cells at 600 nm on UV-Vis spectrophotometer (Varian, Cary 100) using the sterile enzyme production medium (Tsuchiya *et al*., 1952) as described in Section 2.2.3 as blank. 20 µl of the cell free supernatant was used for determining the enzyme activity by centrifugation of 1.0 ml of the culture broth at 10,500*g* at 4°C for 10 min, and the enzyme activity was determined as described in Section 2.2.5. The amount of dextran produced by isolate JAG8 was analyzed by precipitation of 200 µl cell free supernatant, by the addition of 3 volume of 95% (v/v) ethanol pre-chilled at 4°C and centrifuged at 13,700*g* for 20 min. The process of precipitation was repeated three times to remove any trace impurities or free reducing sugars. Finally the precipitate was dissolved in 200 µl of Milli-Q (18 MΩ) water.

2.2.12 Determination of dextran concentration by Phenol-Sulfuric acid method

The total carbohydrate content was determined by phenol-sulfuric acid method (Dubois *et al*., 1956) in a micro-titer plate (Fox and Robyt 1991). 25 µl of sample containing dextran was added to micro-titre plate, to this 25 µl of 5% (w/v) phenol was added and the content were mixed by shaking the plate at slow speed on a vortex mixer for 30s. The plate was then placed onto ice bath and 125 µl of concentrated

sulphuric acid was added to each well containing sample and phenol. The content was mixed by gently shaking the plate by vortex for 30s. The plate was wrapped with cling film followed by incubating at 80°C in water bath for 30 min. The plate was finally cooled and the absorbance was measured at 490 nm on a microtitre plate reader (Tecan Microplate Reader, M1000 PRO) The standard curve was prepared using dextran (40 kDa) from Sigma Chem. Co., USA, in the concentration range 0.05-0.5 mg/ml.

$$\text{Dextran (mg/ml)} = \Delta A_{490} \times C$$

ΔA_{490} = Optical Density (OD) change at 490 nm.

C = 1 OD equivalent fructose concentration (mg/ml) from standard plot.

2.3 Results and Discussion

2.3.1 Selection of isolate based on maximum dextransucrase activity

Ten distinct colonies were picked from 24 h incubated 10^{-8} diluted MRS agar plates from each source *viz.* apple, banana, sugarcane and orange. Screening of the isolates was done under static condition at 28°C. Maximum enzyme activity of 5.2 (U/ml) was observed from the strain isolated from apple and was named as "JAG8" as described in Table 2.3.1. The culture conditions for dextransucrase production from the isolate JAG8 were optimized and used for further studies.

Table 2.3.1. Enzyme activity profile of isolates from fruit samples.

S. No.	Source	Enzyme activity (U/ml)	S. No.	Source	Enzyme activity (U/ml)
1	Apple	0.9	21	Orange	1.2
2	Apple	1.02	22	Orange	0.9
3	Apple	1.0	23	Orange	1.8
4	Apple	0.5	24	Orange	4.12
5	Apple	1.67	25	Orange	1.17
6	Apple	2.14	26	Orange	2.64
7	Apple	0.86	27	Orange	1.86
8	**Apple**	**5.20**	28	Orange	3.10
9	Apple	2.12	29	Orange	2.62
10	Apple	1.35	30	Orange	1.05
11	Banana	0.9	31	Sugar Cane	1.97
12	Banana	2.52	32	Sugar Cane	3.11
13	Banana	1.0	33	Sugar Cane	1.07
14	Banana	0.85	34	Sugar Cane	0.88
15	Banana	2.47	35	Sugar Cane	2.67
16	Banana	3.14	36	Sugar Cane	3.06
17	Banana	1.92	37	Sugar Cane	1.76
18	Banana	2.36	38	Sugar Cane	0.70
19	Banana	0.56	39	Sugar Cane	2.68
20	Banana	2.05	40	Sugar Cane	2.55

2.3.2 Morphological and biochemical characterization of the isolate JAG8

The bacterium was selected based on maximum dextransucrase activity and characterized morphologically using scanning electron microscopy. Gram staining

showed that the isolate was Gram positive, short rod with grayish white color colonies arranged in group (Fig. 2.3.1). The morphology of cells observed by scanning electron microscopy revealed short rod shape and random arrangement in groups or chains (Fig. 2.3.2) with 0.6 µm diameter and 1-1.2 µm in length. The isolate was found to be catalase and nitrate negative, hot loop test confirmed the hetero fermentative nature of JAG8 as the culture created a stream of bubbles immediately after inoculation of the red hot loop (Table 2.3.2). The bacterium JAG8 was non-motile, as it grew along the stab line of agar (Table 2.3.2). No colour change was observed in triple sugar iron test indicated that the isolate did not belong to *Enterobacteriace* family (Table 2.3.2). The morphological and biochemical characteristic features of JAG8 are enlisted in Table 2.3.2. The isolate could grow between 15°C to 45°C but, no growth was observed at 4°C or 50°C, indicating the mesophilic nature of the bacterium JAG8. The bacterium could grow in the presence of 7.0% (w/v) NaCl but, further increase in salt concentration inhibited the growth of the bacterium. The bacterium JAG8 could grow within pH range 4-8. The initial pH of 2.0 and 10 did not support the growth of the isolate. If the isolate is catalase negative, gram-positive and short rod shaped they belong to lactic acid bacteria family (Lin *et al*., 2006).

Table 2.3.2 Morphological and Biochemical Characteristic features of isolate JAG8

Parameter	**Isolate (JAG8)**
Gram staining	Gram Positive
Cell morphology	Small rods
Size	0.5-0.6 µm(diameter), 1-1.2 µm (length)
Colony Characteristics	White smooth small colonies
Catalase Test	Negative
Motility Test	Non Motile
Indole Test	Negative
Nitrate Test	Negative
H_2S Gas Production	Negative

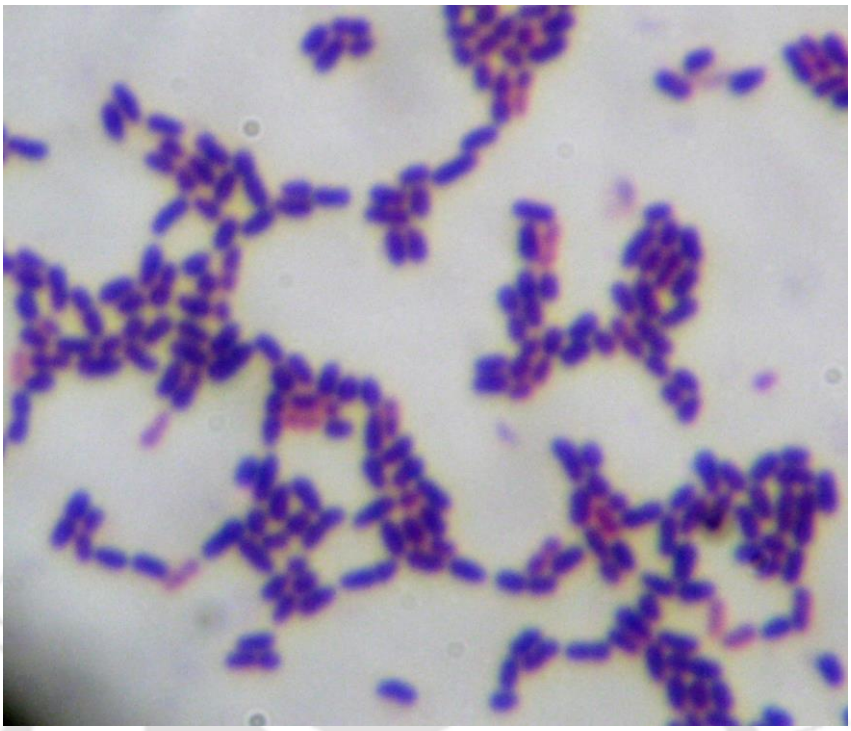

Fig. 2.3.1 Gram staining of the isolate JAG8 showing purple colour cells, indicating their Gram positive nature.

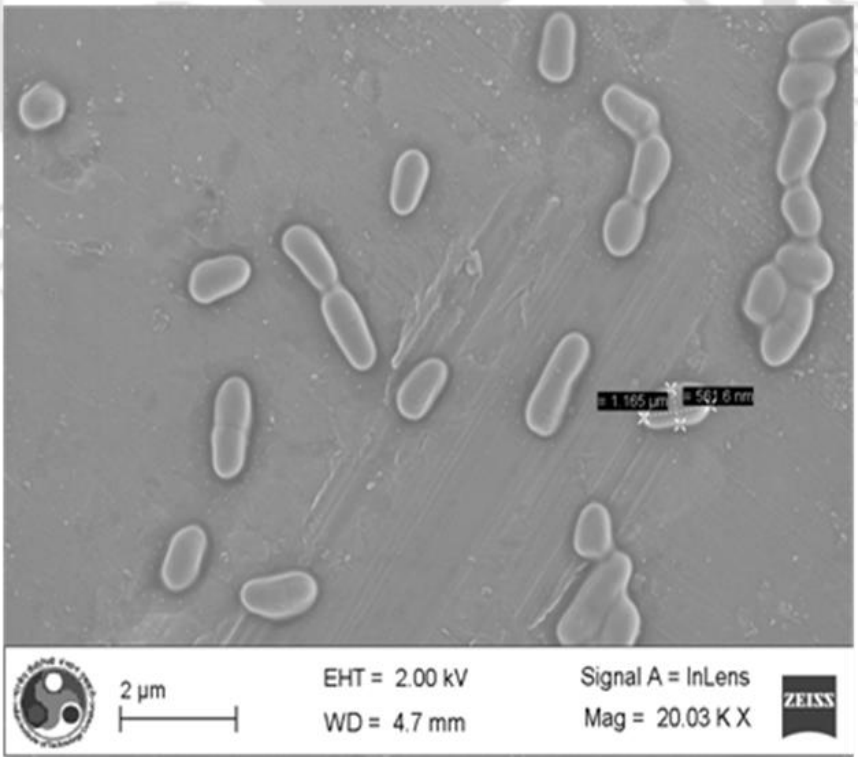

Fig. 2.3.2 Scanning Electron Microscopic analysis of the isolate JAG8 showing rod shape and random arrangement of cells.

2.3.3 Antibiotic sensitivity analysis of isolate JAG8

In order to elucidate the antibiotic susceptibility of the isolate JAG8, 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 JAG8. After the overnight incubation at 28°C, 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 JAG8 was tested for susceptibility to thirty seven antibiotics. It was found resistant to norflaxacin, vancomycin, cotrimaxazole, co-trimize, nalidixic acid, nitrofurantoin, oxacillin, norfloxacin, sulphamethoxazole and was sensitive to amoxyclav, ampicillin, amikacin, amoxycillin, bacitracin, cephalexin, ciprofloxacin, clindamycin, cloxacillin, carbenicillin, cephotaxime, cephalothin, ceftazidime, cephaloridine, cefaclor, cefexime, erythromycin, gentamicin, kanamycin, lincomycin, methicillin, novobiocin, olaendomycin, oxytetracycline, pencillin-G, piperacillin, tobramycin, ticarcillin, tetracycline (Table 2.3.3). Similar results with vancomycin resistance were also reported from other LAB strain *Weissella confusa* Cab3 by Shukla and Goyal (2011).

Table 2.3.3 Antibiogram of the isolate JAG8 using antibiotic octadisc on MRS agar.

S. No.	Antibiotic	Conc.	JAG8	S. No.	Antibiotic	Conc.	JAG8
1	Amoxyclav (Ac)	10 µg	S	20	Gentamicin (G)	10 µg	S
2	Ampicillin (A)	10 µg	S	21	Kanamycin (K)	30 µg	S
3	Amikacin (Ak)	10 µg	S	22	Lincomycin (L)	2 µg	S
4	Amoxycillin (Am)	10 µg	S	23	Methicillin (M)	5 µg	S
5	Bacitracin (B)	10 U	S	24	Nalidixic acid (Na)	30 µg	R
6	Cephalexin (Cp)	10 µg	S	25	Nitrofurantoin (Nf)	50 µg	R
7	Ciprofloxacin (Cf)	10 µg	S	26	Norfloxacin (Nx)	10 µg	M
8	Clindamycin (Cd)	2 µg	S	27	Novobiocin (Nv)	30 µg	S
9	Cloxacillin (Cx)	1 µg	S	28	Olaendomycin (OL)	15 µg	S
10	CoTrimaxazole(Co)	25 µg	R	29	Oxacillin (Ox)	5 µg	M
11	Carbenicillin	100µg	S	30	Oxytetracycline (O)	30 µg	S
12	Cephotaxime (Ce)	30 µg	S	31	Pencillin-G (P)	10 U	S
13	Co-Trimazine (Cm)	25 µg	R	32	Piperacillin (Pc)	100µg	S
14	Cephalothin (Ch)	30 µg	S	33	Sulphamethoxazole (Sx)	50 µg	R
15	Ceftazidime (Ca)	30 µg	S	34	Tobramycin (Tb)	10 µg	S
16	Cephaloridine (Cr)	30 µg	S	35	Ticarcillin (Ti)	75 µg	S
17	Cefaclor (Cj)	30 µg	S	36	Tetracycline (T)	30 µg	S
18	Cefexime (Cfx)	5 µg	S	37	Vancomycin (Va)	30 µg	R
19	Erythromycin (E)	15 µg	S				

R- Resistant (0-0.1 cm*); M- Moderate (0.2-0.8 cm*); S- Sensitive (0.9-2.5 cm*).
**Values in centimeters are the distance of zone of inhibition of growth of microorganism.*

2.3.4 Carbohydrate fermentation pattern of JAG8

The ability of the isolate JAG8 to utilize and ferment carbohydrates with the production of acid was tested. The isolate JAG8 utilized arabinose, cellobiose, dextrose, fructose, mannose, maltose, sucrose, xylose as colour changed from red to yellow but could not ferment adonitol, dulcitol, lactose, melibiose, trehalose, inulin, rhamnose and raffinose. The majority of the carbohydrate fermentation profile of isolate JAG8 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 of the isolate JAG8 after 24h incubation.

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

Symbols: (+++) strongly positive; (++) fairly positive; (+) weakly positive; (-) negative.

2.3.5 Sequence analysis of 16S rRNA gene of isolate JAG8

The isolate was identified as *Weissella cibaria* JAG8 by BLAST analysis of 1440 bp sequence (Fig. 2.3.3) of 16S rDNA encoding gene and by phylogenetic analysis using neighbour-joining method. The isolate JAG8 sequence was subjected to sequence alignment with bacterial genome database which showed 96.0% similarity with *Weissella cibaria* 105 and was assigned the Genbank accession number KC110687. The evolutionary distances between the isolate JAG8 and *Weissella cibaria* 4712 and 1-19 showed common ancestry (Fig. 2.3.4) whereas, the

sequence showed common evolutionary relationship with *Bifidobacterium* PG13 (Fig. 2.3.5).

5'-GTCGAACGCTTTGTGGTTCAACTGATTTGAAGAGCTTGCTCAGATATGACGATGGACAT
TGCAAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAACCTACCTCTTAGCAGGGGATA
ACATTTGGAAACAGATGCTAATACCGTATAACAATAGCAACCGCATGGTTGCTACTTAAA
AGATGGTTCTGCTATCACTAAGAGATGGTCCCGCGGTGCATTAGTTAGTTGGTGAGGTAAT
GGCTCACCAAGACGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTG
AGACACGGCCCATACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGCGAAA
GCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGGCTCGTAAAACACTGTTGTA
AGAGAAGAATGACATTGAGAGTAACTGTTCAATGTGTGACGGTATCTTACCAGAAAGGAA
CGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGTTCCAAGCGTTATCCGGATTTAT
TGGGCGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAAGCCCTCAGGCTCAAC
TGAGGAATTGCTTTGGAAACTGGATGACTTGAGTGCAGTAGAGGAAAGTGGAACTCCATG
TGTAGCGGTGAAATGCGTAGATATATGGAAGAACACCAGTGGCGAAGGCGGCTTTCTGGA
CTGTAACTGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACAGGATTAGATACCCTGGTAG
TCCACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTGCCGCAGCT
AACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGA
CGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTA
CCAGGTCTTGACATCCCTTGACAACTCCAGAGATGGAGCGTTCCCTTCGGGGACAAGGTG
ACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTTATTACTAGTTGCCAGCATTCAGTTGGGCACTCTAGTGAGACTGCCGGTG
ACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGACCTGGGCTAC
ACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTT
AAAGTACGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTA
GTAATCGCGGATCAGCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGT
CACACCATGAGAGTTTGTAACACCCAAAGCCGGTGGGGTAACCTTCGGGAGCCAGCCG-**3'**

Fig. 2.3.3 16S rRNA gene sequence (1440 bp full length) of isolate *Weissella cibaria* JAG8.

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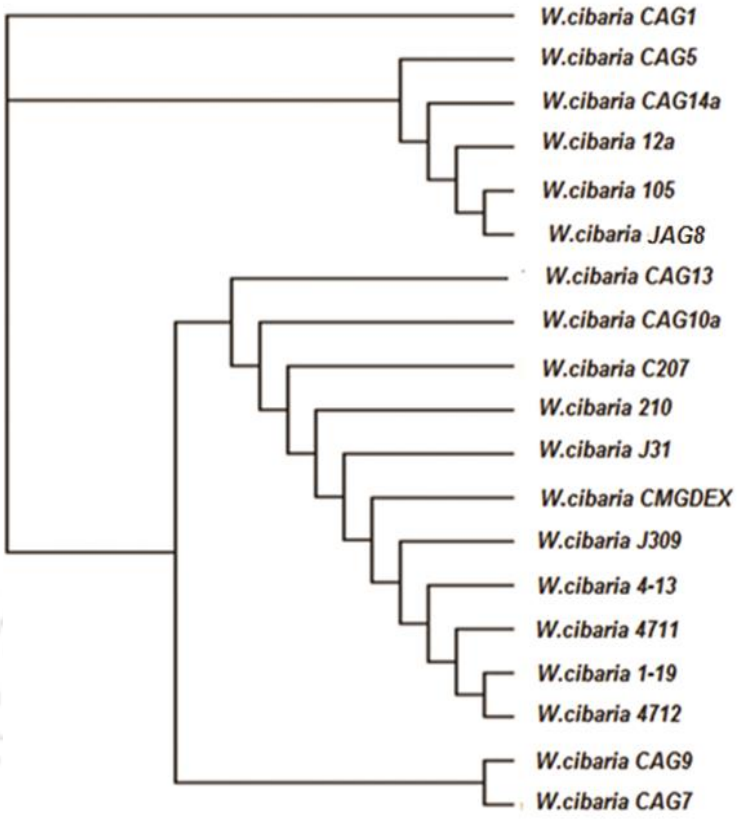

Fig. 2.3.4 Phylogenetic relationship of isolate *Weissella cibaria* JAG8.

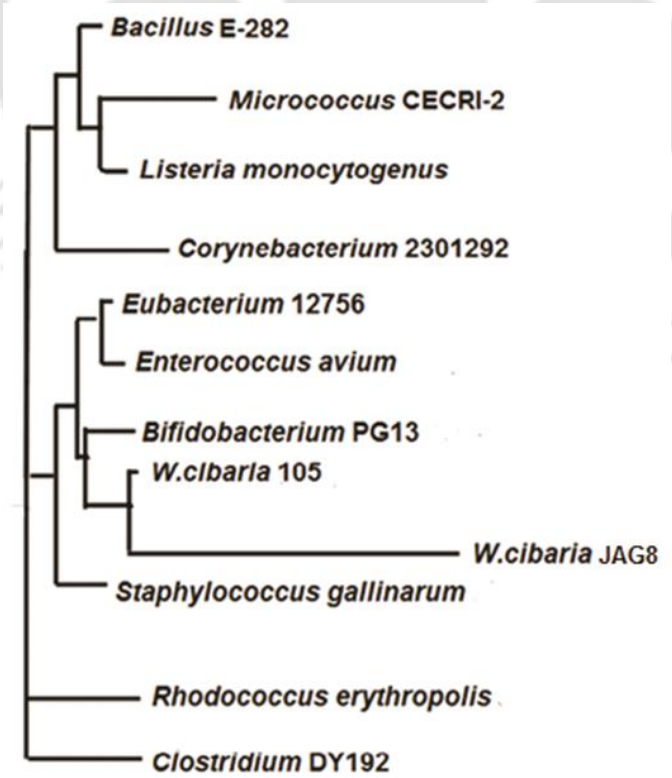

Fig. 2.3.5 Evolutionary relationship of *Weissella cibaria* JAG8 with other bacteria.

2.3.6 Effect of temperature and shaking on dextransucrase production from isolate JAG8

The maximum dextransucrase activity of 5.8 (U/ml) for the isolate JAG8 was observed at 24°C and 12 h under static condition at pH 6.0 (Fig. 2.3.6). The enzyme activity was significantly low at 20°C, because of slow growth of the bacterium. The loss of enzyme activity at higher temperature (40°C) was due to deactivation of enzyme. This indicated the mesophilic nature of isolate JAG8 as also described in section 2.3.2. The isolate JAG8 showed 40% less dextransucrase activity i.e. 3.3 U/ml under shaking condition as compared with static condition at 24°C (Fig. 2.3.7). Higher dextransucrase acrtivity obtained under static condition indicates the micro-aerophilic nature of the isolate JAG8.

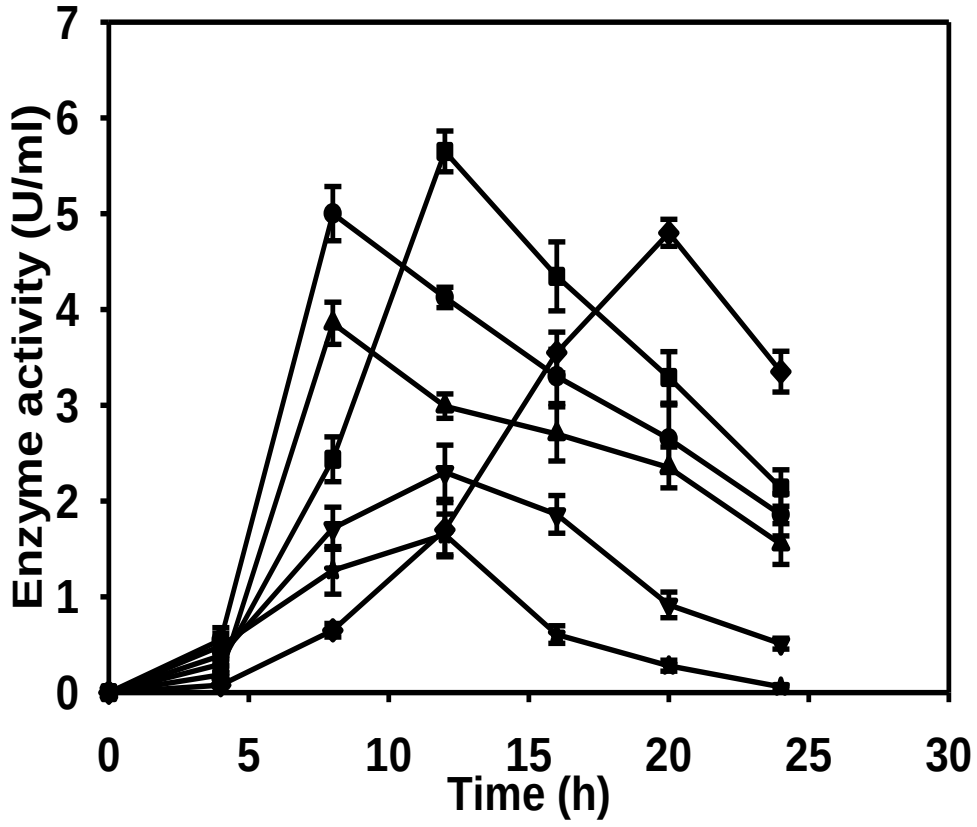

Fig. 2.3.6 Effect of temperature on dextransucrase production from *W. cibaria* JAG8. (♦) 20°C (■) 24°C, (●) 28°C, (▲) 32°C, (▼) 36°C, (►) 36°C, (∗) 40°C.

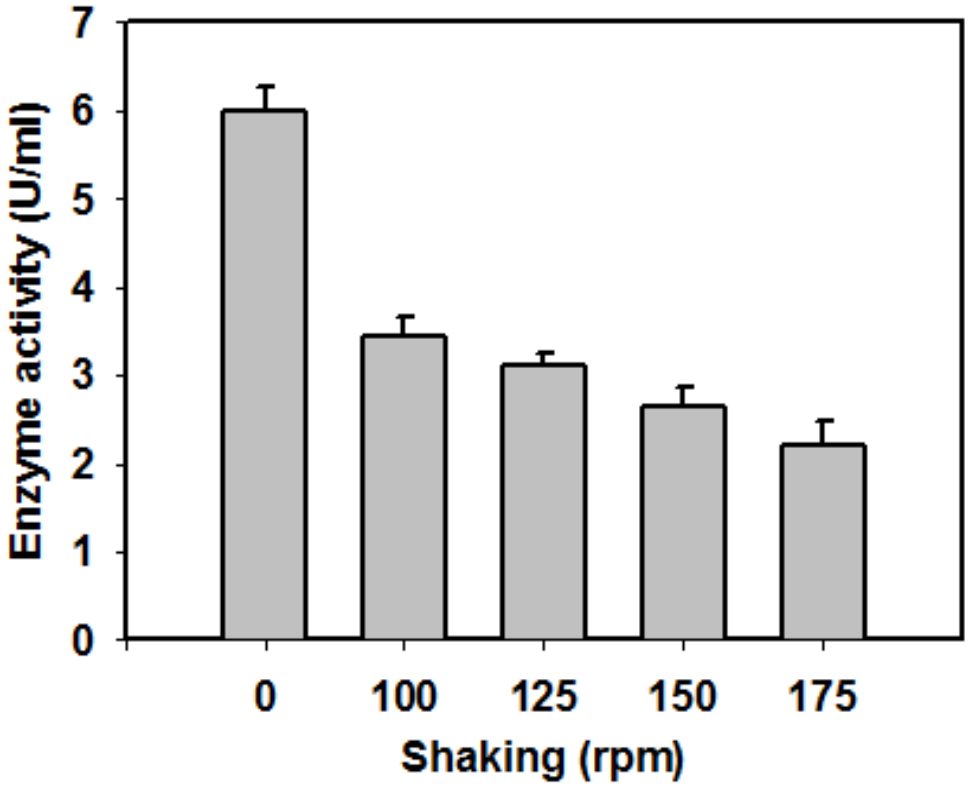

Fig. 2.3.7 Effect of shaking (rpm) on dextransucrase production from *Weissella cibaria* JAG8 production at 24ºC.

2.3.7 Fermentation profile of *Weissella cibaria* JAG8

The fermentation profile of *Weissella cibaria* JAG8 showed maximum enzyme activity of 5.8 (U/ml) at 12 h and 24°C under static condition. The pH range for maximum dextransucrase production was 6.0-6.4 with maximum dextran yield of 7.8 mg/ml (Fig. 2.3.8). The cell density was maximum at 12 h and remained almost constant up to 16 h and no further increase in growth was observed because of depletion of medium components. The pH decreased from initial 6.9 to 5.0 at 24 h because of production of lactic acid.

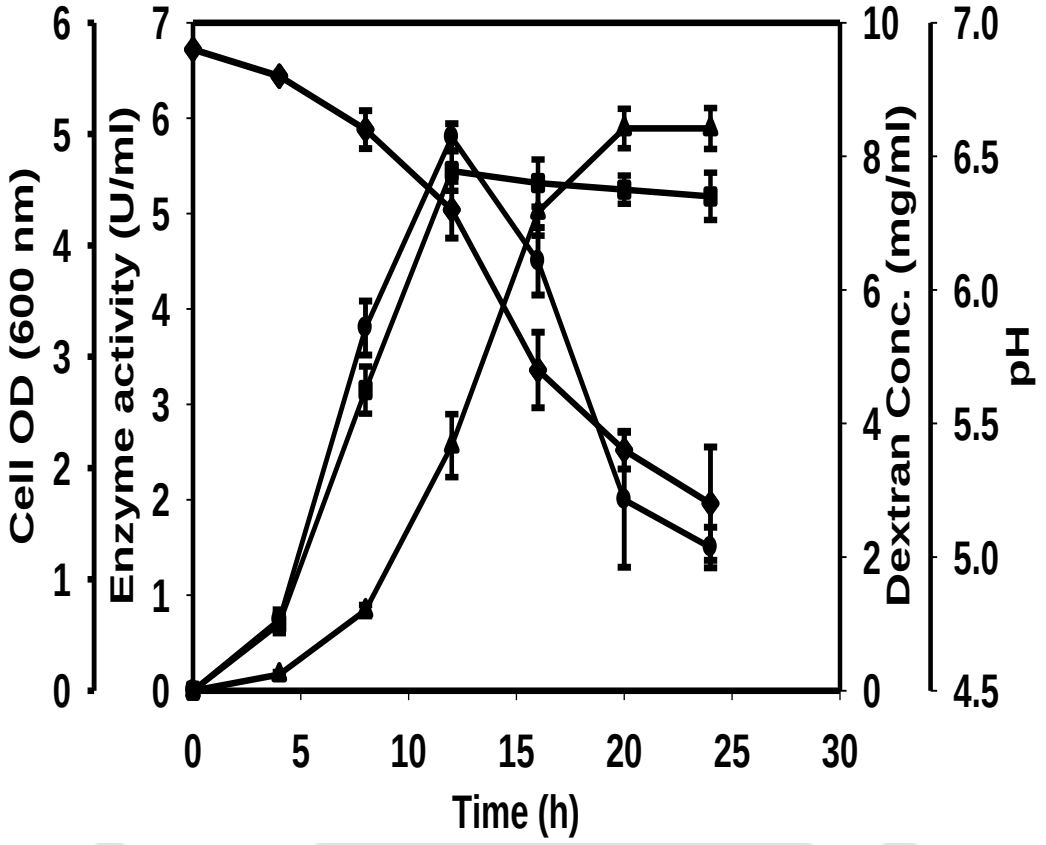

Fig. 2.3.8 Fermentation profile of *Weissella cibaria* JAG8 showing the enzyme activity (●), dextran concentration (■), pH (▲) and Cell OD at 600 nm (♦).

Table 2.3.5 Comparison of fermentation characteristics of *W. cibaria* JAG8, *L. mesenteroides* NRRL B-512F and *P. pentosaceus* in flask culture.

Fermentation Parameters	***W. cibaria* JAG8**	***L. mesenteriodes* NRRL-B512F**	***P. pentosaceus* SPA**
Duration (h)	12	12	16
Maximum enzyme activity (U/ml)	**5.8**	4.1	3.1
pH range for maximum enzyme production	6.0-6.4	6.0-6.5	4-5
Temperature (°C)	24	23	25
Static/Shaking	Static	Static	Shaking
Optical density (OD 600 nm) of cells	5.2	5.8	8.1
Dextran concentration (mg/ml)	**7.8**	6.9	7.0

In case of commercial strain *Leuconostoc mesenteroides* NRRL B-512F maximum dextransucrase activity of 4.1 (U/ml) was obtained at 23°C in the pH range 6.0-6.5, under static condition (Table 2.3.5.), and in *Pediococcus pentosaceus* SPA it was 3.1 (U/ml) at 25°C, pH 4.0-5.0 and 180 rpm (Table 2.3.5). The comparative fermentation profile of *Weissella cibaria* JAG8 with other dextransucrase producing lactic acid bacteria is shown in Table 2.3.5. The dextran production from *Weissella cibaria* JAG8 was 7.8 mg/ml, which was 10% and 11% higher than *Pediococcus pentosaceus* SPA, 7.0 mg/ml (Patel and Goyal 2010) and *Leuconostoc mesenteroides* NRRL B-512F, 6.9 mg/ml (Goyal *et al*.,1995).

2.4 Conclusions

A bacterial isolate JAG8 from apple displaying dextransucrase activity of 5.2 (U/ml) was screened and characterized. Based on morphological analysis it was identified as Gram positive, short rod with average size of 0.6 µm diameter and 1.2 µm length. Biochemical characterization revealed the isolate JAG8 as non motile, catalase, indole and nitrate negative. The isolate JAG8 could grow between 15-45°C, and no growth was observed at 4 and 50°C that indicated the mesophilic nature of bacterium. The bacterium JAG8 could withstand the salt concentration of up to 7.0% and pH range of 4-8 for its growth. Antibiogram analysis showed that the isolate JAG8 was resistant to the antibiotics norfloxacin, ampicillin, amikcin, vancomycin, kanamycin, tobramycin, cephaloridine and norflaxacin. Resistance to vancomycin is the common characteristic feature for most of the lactic acid bacteria. The isolate JAG8 was sensitive to ampicillin, ciprofloxacin, erythromycin, gentamicin, kanamycin, methicillin, pencillin and tetracycline. The bacterium JAG8 could ferment arabinose, cellobiose, dextrose, fructose, mannose, maltose, sucrose and xylose. The isolate could not utilize adonitol, dulcitol, lactose, melibiose, trehalose, inulin, rhamnose and raffinose.

The 16S rRNA gene sequence analysis revealed the identity of the isolate JAG8 which was found to be *Weissella cibaria* (Genbank Accession Number KC110687). The maximum dextransucrase activity of 5.8 (U/ml) was observed at 24°C and 12 h under static condition. Higher enzyme production under static condition as compared to shaking condition indicated the micro-aerophilic nature of the bacterium. This is the first report of *Weissella cibaria* producing high dextran of 7.8 mg/ml under unoptimized medium condition. The dextran production can be enhanced through the manipulation of the medium components.

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Chapter 3

Purification, optimization of assay and stability studies of dextransucrase from *Weissella cibaria* JAG8

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 Triton X-100, glycerol, Tween-80, polyethylene glycols, dextran, glutaraldehyde and polyvinyl alcohols 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 (Kuhlmeyer 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. One such strain could be a newly isolated *Weissella cibaria* JAG8. *Weissella* species is gaining importance because of its increasing applications in food industry (Schwab *et al*., 2008) and only few reports are available on the purification and stability studies of dextransucrase from *Weissella cibaria*

(Kang *et al*., 2006). *Weissella* species is gaining 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). Hence, it is important to study this industrially important enzyme for commercial exploitation. In the present study an efficient method of purification of dextransucrase from *W. cibaria* JAG8 isolated from apple using polyethylene glycol followed by gel-filtration chromatography using Sephacryl S-300HR. The purity of enzyme was analyzed by non-denaturing SDS-PAGE using Coomossie Brillent Blue (CBB) staining and silver staining. The dextran producing ability was confirmed by periodic acid Schiff (PAS) staining. The effects of certain metal ions (Ca^{2+}, Mg^{2+} and Co^{2+}), additives such as, dextran 100 kDa and Tween 80 and different storage temperatures were investigated.

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. Sephacryl S-300HR was purchased from Sigma Chemicals., USA, and Bovine serum albumin was from Sigma Aldrich Pvt. Ltd., USA.

3.2.2 Maintenance of the isolate *Weissella cibaria* JAG8

Weissella cibaria JAG8 isolated from apple and maintained in modified MRS medium (Goyal and Katiyar 1995) as described in Section 2.2.1 of chapter 2. This isolate was maintained as stab in modified MRS agar (containing 2% sucrose, w/v) at 4°C and sub-cultured every two weeks. For the development of inoculum, a loopful of culture from modified MRS agar stab was transferred to 5 ml of medium enzyme production medium Tsuchiya *et al*., (1952) as described in Chapter 2, Section 2.2.2. The culture was incubated at 24°C under static conditions for 12 h for production of dextransucrase.

3.2.3 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.

3.2.4 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

3.2.5 Purification of dextransucrase from *W. cibaria* JAG8 by PEG fractionation

One percent of *W. cibaria* JAG8 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 24°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 20, 25, 33, 40 and 50% (v/v). Similarly, in case of PEG 1500 the cell free supernatant was treated with 5, 10, 15, 20 and 25% (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 3.2.4, respectively. The dialyzed enzyme was characterised further by both denaturing and non-denaturing SDS-PAGE using Commassie Brillant Blue (CBB) staining as described in Chapter 3, Section 3.2.6 and Periodic acid Schiff (PAS) staining as described in Chapter 3, Section 3.2.7.

3.2.6 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 280 nm. The enzyme activity and protein concentration of

eluted fractions were determined as described earlier. The fractions containing the highest specific activity were lyophilized and re-dissolved in minimum amount of 20 mM sodium acetate buffer pH 5.4 and were analysed by non-denaturing Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis (SDS-PAGE) followed by silver staining and Periodic acid Schiff (PAS) staining methods.

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

3.2.7.1 *Preparation of SDS-PAGE gels*

The polyacrylamide gels are prepared by copolymerization of acrylamide and bis-acrylamide (bisN,N'-methylene-bisacrylamide). The copolymerization reaction is basically a vinyl addition reaction initiated by a free radical-generator *viz.* Ammonium per sulphate (APS) in presence of N,N,N',N'-tetramethylethane-1,2-diamine (TEMED) which acts as a catalyst (Chrambach 1985). SDS-Polyacrylamide gel electrophoresis was performed following the method of Laemmli (1970). The resolving gel containing 7.5% (w/v) acrylamide and stacking gel containing 4% (w/v) acrylamide was prepared as described in Table 3.2.1 and Table 3.2.2, respectively. The sample loading buffer was prepared as described in Table 3.2.3. In case of non-denaturing conditions β-mercaptothanol was not added in the loading dye. The purified enzyme sample was mixed with 5x loading dye buffer in the ratio 4:1. The sample mixture was subjected to heat denaturation for 5 min and centrifuged for 1 min. The sample was loaded on 7.5% acrylamide gel and the electrophoresis was carried out using 1x running buffer (200 mM glycine, 0.1% SDS, 50 mM Tris-HCl pH 8.3, as described in Table 3.2.4) with a current of 2.5 mA per lane. The protein bands were visible by staining the gel in Coomassie Brilliant Blue staining solution (which was prepared as described in Section 3.2.7.3) for 40 min. The gel was destained by using destaining solution (which

was prepared as described in Section 3.2.7.3). High range protein marker, 10-200 kDa (Fermentas Pvt. Ltd., India) was used as standard.

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

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.

Table 3.2.1 Composition of SDS-PAGE components for preparation of resolving gel.

Components	**7.5% 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
APS (10%,w/v)	0.1
TEMED	0.01

Table 3.2.2 Composition of SDS-PAGE components for preparation of stacking gel.

Components	**4% 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
APS (10%,w/v)	0.05
TEMED	0.01

3.2.7.3 *Preparation of sample buffer*

The sample loading buffer (5x) was prepared by dissolving the components as described in Table 3.2.3 and the pH of the buffer was adjusted to 6.8. The final concentration while loading to a SDS-PAGE gel was always kept to 1x by mixing 4 volumes of sample (protein) with 1 volume of 5x sample buffer.

Table 3.2.3 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)

3.2.7.4 *Preparation of SDS-PAGE running buffer*

The SDS-PAGE gels were run using a 1x running or tank buffer prepared from the 5x stock solution as described in Table 3.2.4. 15.14 g of Tris free base and 94 g of glycine were dissolved in 800 ml of deionized water. To this 50 ml of 10% (w/v) SDS was added and the final volume was adjusted to 1 litre. The final pH of the buffer was adjusted to 8.3. The 5x buffer was filtered (Whatman, Filter No. 1) and stored at 4°C.

Table 3.2.4 Composition of 5xTris-Glycine running buffer.

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

3.2.7.5 *Preparation of staining and destaining solutions*

The proteins on the SDS-PAGE gel were visualized using a staining solution that contained Coomassie Brilliant Blue (CBB) R-250 dye. The CBB R-250 dye (detection range of 100-1000 ng of protein) forms a non-covalent complex with proteins, based on a combination of vander waals forces and electrostatic interactions (Neuhoff *et al*., 1985). The negatively charged anionic form of the dye is stabilized by formation of a blue colour protein-dye complex which may then be seen on gel (Meyer and Lambert 1965). The staining solution (100 ml) was prepared by dissolving 250 mg, of CBB R-250 dye in 50 ml of deionized water in an amber colour bottle by keeping on a magnetic stirrer for overnight. The solution was filtered through Whatman, Filter No. 1 and 40 ml of methanol and 10 ml of glacial acetic acid were added. The destaining solution was prepared by mixing 40 ml of methanol and 10 ml of glacial acetic acid and making up the volume by deionized water to 100 ml. The gels were destained by immersing the gel in destaining solution under gentle shaking condition with change of buffer every 30 min, until the protein bands were clearly visible.

3.2.8 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 loading dye buffer contained 0.0625 M Tris-HCl buffer (pH 6.8), 2.3% (w/v) SDS, 10% (w/v) glycerol and 0.05% (w/v) bromophenol blue, but did not contain β-mercaptothanol. The enzyme sample was mixed with 5x sample buffer in the ratio 4:1. The heat denaturation by boiling step was omitted. The sample was loaded on 7.5% acrylamide gel and the electrophoresis

was carried out using 1x running buffer (200 mM glycine, 0.10% SDS, 50 mM Tris-HCl, pH 8.3) with a current of 2.5 mA per lane.

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 0·10% Tween 80 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 once with a solution of methanol: acetic acid (50:10) in water for 30 min, then with water for 30 min, and incubated in a periodic acid solution (1.0% (w/v) periodic acid and 3% (v/v) acetic acid) at 30°C for 45 min. After the periodic acid treatment, the gel was washed with water for 2h, three times. The gel was then stained with 15 ml Schiff reagent composed of 0.5% (w/v) Fuchsin basic, 1.0% (w/v) sodium bisulphite and 0·1N HCl, and detected for magenta colour band.

3.2.9 Identification and molecular size analysis of dextransucrase purified from size exclusion chromatography by non-denaturing SDS-PAGE using silver staining and PAS staining

The fractions 8^{th} to 12^{th} showing high dextransucrase activity were lyophilised and then resuspended in 20 mM sodium acetate buffer (pH 5.4) and subjected to non-denaturing SDS-PAGE for identification and molecular size analysis. 30 µl of each of the fraction from 8^{th} to 12^{th} was loaded onto the lanes from 1-5 and run on non-denaturing SDS-PAGE (7.5%) along with the unstained protein marker, 10-200 kDa (Fermentas Pvt. Ltd.). The 11^{th} fraction was also loaded onto 6^{th} and 7^{th} lanes. The electrophoresis was carried out in mini gel unit (BioRad, USA) using 1.5 mm thick

gels. The gel was cut into three parts, one containing lanes 1-5 was subjected to silver staining (Mortz *et al*., 2001) as described in Section 3.2.9.1. The second part of gel containing 6th and the third part containing 7th lane was subjected to Periodic acid Schiff (PAS) staining as described in Section 3.2.8.

3.2.9.1 ***Silver staining analysis of dextransucrase***

Silver Staining was performed following the method of Mortz *et al*., (2001). Here the non-denaturing gel was incubated in fixing solution for 30 min. The fixing solution comprised 40% absolute ethanol, 10% glacial acetic acid and 50% distilled water. The gel could be stored up to one week in fixing solution. The gel was transferred to sensitizing solution and incubated for 30 min. The sensitizing solution consisted of absolute ethanol 30% (v/v), sodium acetate 6.8% (w/v), sodium thiosulphate 0.2% (w/v) in deionized water. To this 125 µl (v/v) of glutaraldehyde was added and make up to 100 ml before use. Sensitizing solution can be stored up to 2 months with out adding glutaraldehyde. Now the gel was washed with double distilled water thrice at every 10 min interval. The gel was transferred to silver nitrate solution and incubated for 30 min. The silver nitrate solution was prepared by adding 0.25% (w/v) silver nitrate and 40 µl of 37% (v/v) formaldehyde and make up to 100 ml. The silver nitrate solution was added just before adding the silver nitrate solution to the gel in double distilled water. The gel was washed with double distilled water for 2 min. The developing solution was added to the gel and incubated for 10 min or till the bands appear. The developing solution comprised anhydrous sodium carbonate 2.5% in double distilled water and 20 µl of 37% (v/v) formaldehyde was added just before adding the solution to gel. Finally stop solution was added which comprised 1.5% (w/v) disodium EDTA in double distilled water. The gel was washed three times with

double distilled water for 10 min. The gel was preserved in solution of absolute ethanol 30% (v/v) and 4% (v/v) glycerol prepared in double distilled water.

3.2.10 Optimization of reaction conditions and biochemical characterization of dextransucrase

3.2.10.1 *Effect of sucrose concentration on dextransucrase activity*

To study the effect of sucrose concentration on the enzyme activity of the purified dextransucrase (specific activity 20.0 U/mg; 0.44 mg protein/ml), the enzyme assay was conducted at different sucrose concentrations ranging from 0.1-10% (w/v). 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 35°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.

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

The purified dextransucrase (specific activity 20.0 U/mg; 0.44 mg protein/ml) was used. To study the effect of temperature the enzyme in 1.0 ml of the reaction mixture was incubated at 7 different temperatures ranging from 20 to 50°C in 20 mM sodium acetate buffer pH 5.4 and 5.0% sucrose concentration for 15 min. In order to know optimum pH for the enzyme reaction, the pH of sodium acetate buffer by varied from 4.2 to 6.4, while keeping the ionic strength at 20 mM, temperature at 35°C and 5.0% sucrose concentration. In case of effect of ionic strength on dextransucrase, the concentration of sodium acetate buffer was varied from 10-500 mM, keeping pH 5.4 and sucrose concentration 5% and a constant temperature of 35°C for 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.

3.2.10.3 *Effect of metal ions and denaturing agents on dextransucrase activity*

The purified dextransucrase (20.0 U/mg and protein concentration of 0.44 mg/ml) was incubated with 0-10 mM concentrations of divalent metal ions such as $CaCl_2$, $MgCl_2$ and $CoCl_2$ for 1h at 35°C. In case of urea and EDTA the concentrations were varied between 0-5 M and 0-10 mM respectively, for 1h at 35°C. The enzyme assays were carried out by taking an aliquot of enzyme in 1 ml reaction mixture containing 5% (w/v) sucrose in 20 mM sodium acetate buffer (pH 5.4) at 35°C. The enzyme activity was measured by quantifying the amount of reducing sugar released as described in Chapter 2, Section 2.2.5.

3.2.10.4 *Thermal and pH stability of dextransucrase*

To study the effect of temperature and pH on enzyme stability, the enzyme (20.0 U/mg; 0.44 mg/ml) was incubated at 20-50°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 the partially purified enzyme with specific activity of 20.0 U/mg (0.44 mg/ml) was subjected to lyophilisation. 20 µl of enzyme was added to 20 mM sodium acetate buffer with pH range from 4.2-6.0 and incubated for 1 h at 35°C. The enzyme activity was measured by quantifying the amount of reducing sugar released as described in Chapter 2, Section 2.2.5.

3.2.10.5 ***Effect of storage temperature and additives on stability of dextransucrase***

Storage stability was studied by incubating the dextransucrase at different temperatures (30°C, 4°C and -20°C). The samples at different time intervals were analysed for dextransucrase activity. Aqueous solutions of dextran (100 kDa) and Tween 80 were added to dextansucrase solution (20 U/mg specific activity, 0.44 mg/ml) in sodium acetate buffer, pH 5.4 to obtain the final concentrations of 2 µg/ml dextran (100 kDa) and 0.5% (v/v) Tween 80, respectively. The enzyme with and without Tween 80 was incubated at three different temperatures at 30°C, 4°C and -20°C. The aliquots (20 µl) of enzyme were taken periodically for activity assay at regular time intervals and the amount of reducing sugar released was measured as described in Chapter 2, Section 2.2.5.

3.3 Results and Discussion

3.3.1 Purification of dextransucrase from *Weisella cibaria* JAG8

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 20.0 U/mg at 33% (v/v) (Table 3.3.1). Further increase in PEG 400 concentration up to 50% (v/v) led to decrease in specific activity to 14.9 U/mg as displayed in Fig. 3.3.1. In case of PEG 1500 fractionation the enzyme showed a maximum specific activity of 10.6 U/mg at 15% (w/v) concentration (Table 3.3.2). The specific activity decreased to 7.2 U/mg and remained constant at 20 and 25% (w/v) of PEG 1500 concentrations (Fig. 3.3.1). 33% (v/v) of PEG 400 was found to be best condition that gave maximum enzyme activity with 20 fold purification and yield of 18.2%.

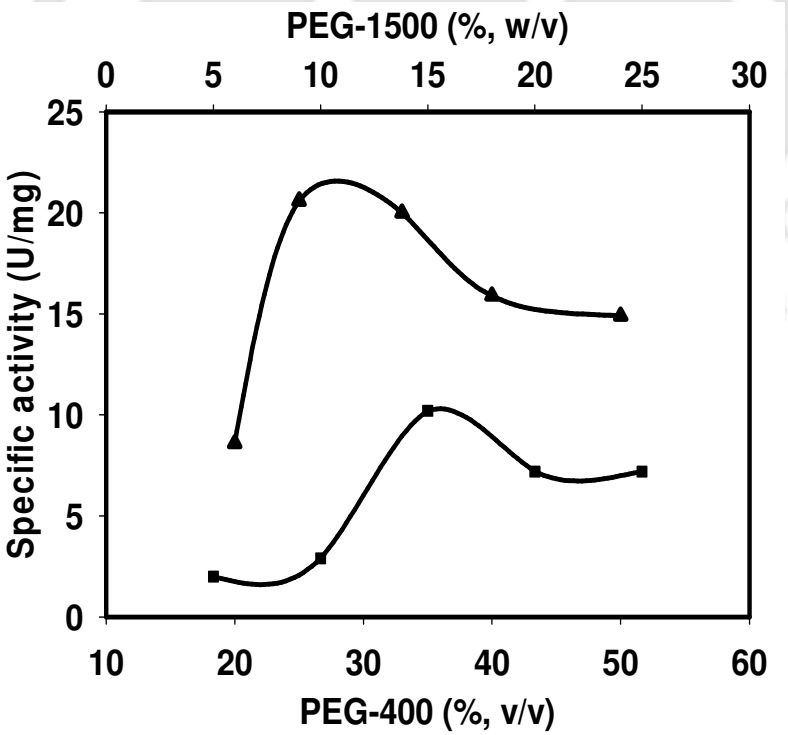

Fig. 3.3.1 Purification profile of dextransucrase by PEG fractionation. PEG 400 (▲), PEG-1500 (■).

Table 3.3.1 Purification of dextransucrase from *W. cibaria* JAG8 by PEG 400.

PEG 400 (%)	Vol (ml)	Enzyme activity (U/ml)	Total Units (U)	Overall activity Yield (%)	Protein Conc (mg/ml)	Specific activity (U/mg)	Fold Purific-ation
Crude	100	5.8	580	-	5.5	1.0	-
20	0.5	2.2	1.1	0.189	0.25	8.6	8.6
25	9.5	9.7	92	15.8	0.47	19.6	19.6
33	12	8.8	105.6	18.2	0.44	**20.0**	**20.0**
40	12	6.7	80.4	13.8	0.42	15.9	15.9
50	14.5	6.3	91	15.6	0.42	14.9	14.9

The specific activity of 20 U/mg and 18.2 yield achieved for dextransucrase from *Weisella cibraria* JAG8 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).

Table 3.3.2 Purification of dextransucrase from *W. cibaria* JAG8 by PEG 1500.

PEG 1500 (%)	Vol (ml)	Enzyme activity (U/ml)	Total Units (U)	Over all activity Yield (%)	Protein Conc (mg/ml)	Specific activity (U/mg)	Fold Purifi-cation
Crude	100	5.8	580	-	5.5	1.0	-
5	0.9	1.9	1.7	0.29	0.93	2.0	2.0
10	2.5	2.8	7.0	1.2	0.98	2.9	2.9
15	3.6	11.7	42.0	7.2	1.1	**10.2**	**10.2**
20	6.2	11.2	69.5	12.0	1.5	7.19	7.19
25	5.9	7.9	46.6	8.0	1.1	7.19	7.19

3.3.2 Purification of dextransucrase by size exclusion chromatography

The 33% PEG-400 purified dextransucrase with maximum specific activity of 20.0 (U/mg) and 0.44 (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 280 nm. The enzyme eluted from 8th to 12th fractions showed

higher enzyme activity and it was maximum in the 11th fraction. The specific activity of 11th fraction was 37 U/mg, with 37 fold purification (Table 3.3.3).

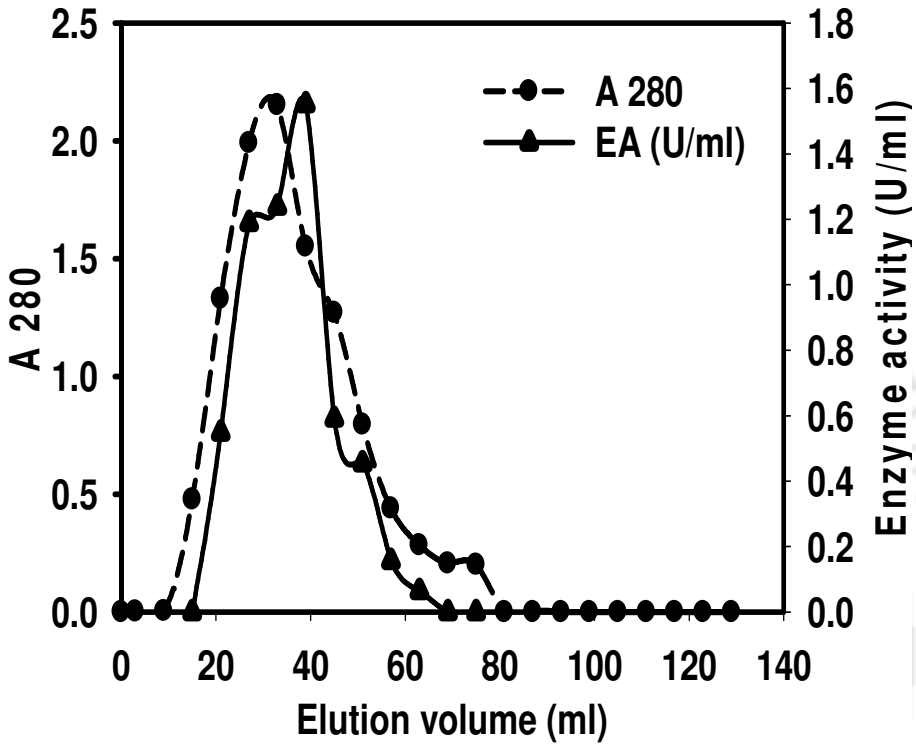

Fig. 3.3.2 Purification of dextransucrase by size exclusion chromatography using Sephacryl S300HR. Elution profile of dextransucrase showing (●) A_{280} and (▲) enzyme activity (U/ml).

Table 3.3.3 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	100	5.8	580	-	5.5	1.0	-
PEG 400, 33% (v/v)	12	8.8	105.6	18.2	0.44	20.0	20.0
Sephacryl S-300HR	3.0*	1.55	4.6	0.08	0.042	37.0	37.0

**11th fraction of dextransucrase from Sepharcryl S300HR column*

3.3.3 Identification and purity analysis of dextransucrase from 33% (v/v) PEG 400 and column purified fractions by Coomassie brillant blue, Silver and PAS staining

The dextransucrase purified by 33% (v/v) PEG-400 fractionation (20 U/mg; 0.44 mg/ml) was subjected to both denaturing (Lane 2) and non-denaturing SDS-PAGE (Lane 3) showed a single distinct band (Fig. 3.3.3). In case of size exclusion chromatography, dextransucrase showing significant activity from 8th to 12th fractions were loaded on non-denaturing SDS-PAGE and detected by silver staining method. Single distinct homogenous bands of 177 kDa molecular weight were observed in each of the five fractions (Fig. 3.3.4, lanes 1 to 5). 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.

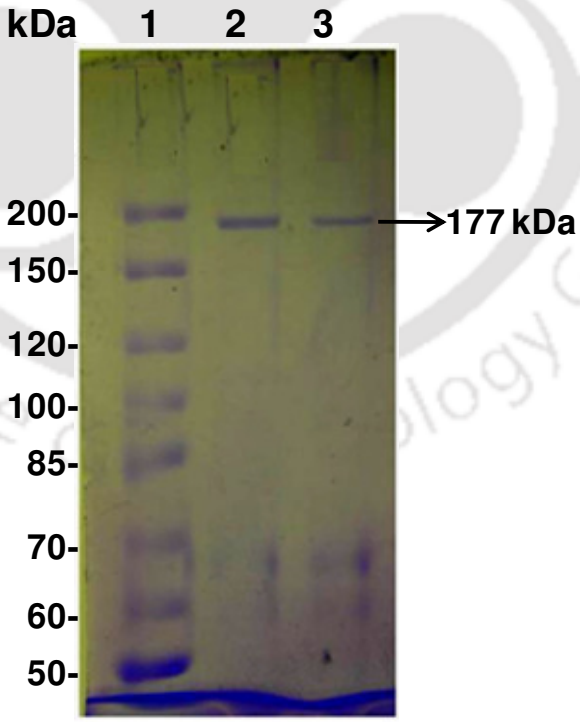

Fig. 3.3.3 SDS-PAGE analysis of purified dextransucrase using 7.5% gel. Lanes; (1) protein molecular weight marker (10–200 kDa), (2) 33% (v/v) PEG-400 purified enzyme (20 U/mg; 0.44 mg/ml) under denaturating condition (3) non-denaturing condition.

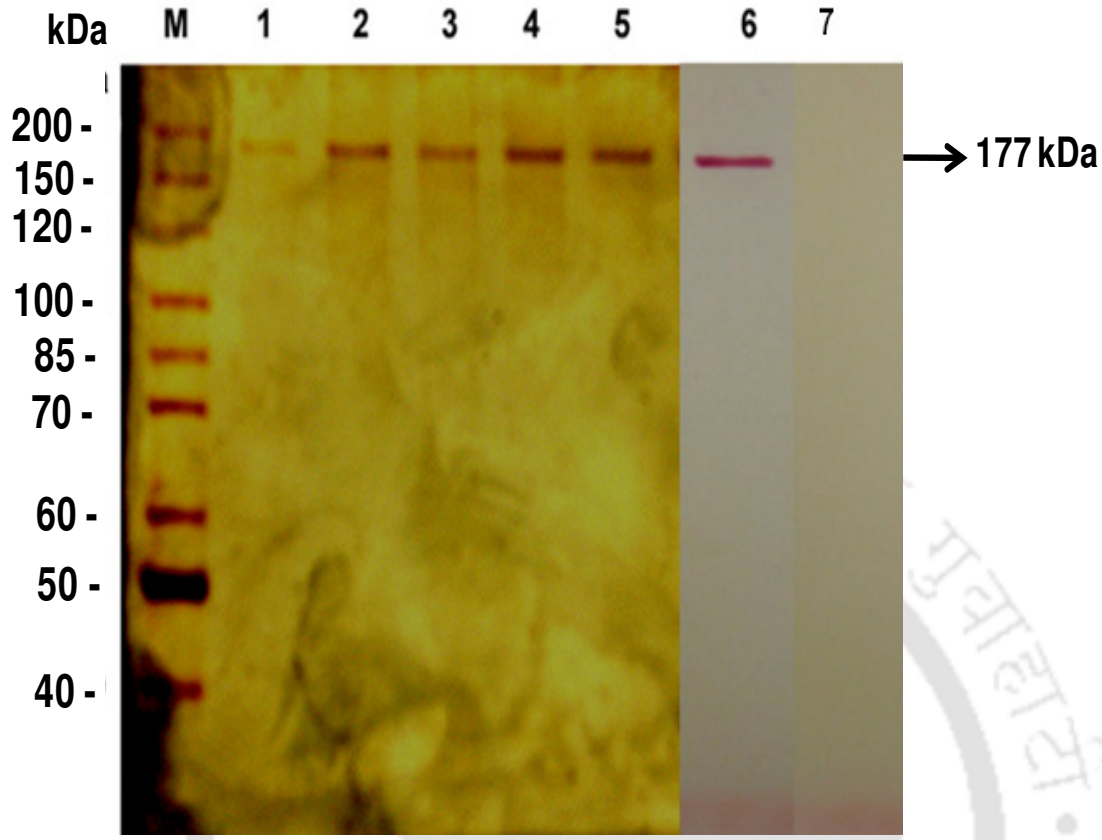

Fig. 3.3.4 Non-denaturing SDS-PAGE (7.5%) analysis after purification from Sephacryl S-300 for identification of dextransucrase using Silver staining and PAS staining. Lane M (molecular weight marker 10-200 kDa), Lyophilized fractions. Lane 1 (fraction 8), Lane 2 (fraction 9), Lane 3 (fraction 10), Lane 4 (fraction 11), Lane 5 (fraction 12). Lane (6) PAS staining (fraction 11) in 5.0% (w/v) sucrose solution. Lane (7) PAS staining (fraction 11) in 5.0% (w/v) raffinose solution.

A bright magenta colour band was observed in the gel incubated with 5.0% (w/v) sucrose solution in PAS staining showing that the enzyme was dextransucrase which formed the dextran by utilizing sucrose. The magenta colour band appeared at the same level as that in the silver staining confirming again the molecular size as 177 kDa (Fig. 3.3.4, Lane 6). No band was detected in the gel incubated in raffinose using PAS staining (Fig. 3.3.4, Lane 7) confirming that the enzyme was not fructansucrase. All these results confirmed the presence of single molecular form of dextransucrase with dextran forming ability.

3.3.4 Optimization of reaction conditions for maximum dextransucrase activity

3.3.4.1 *Effect of sucrose concentration on dextransucrase activity*

The PEG 400 (33% v/v) purified enzyme with specific activity of 20.0 U/mg and protein concentration of 0.44 mg/ml was used to study the effect of sucrose concentration on dextransucrase activity by varying sucrose concentration between 0.1-10% final concentrations in the assay mixture. The results showed that it follows the classical Michaelis-Menten kinetics as it gave a K_m of 13 mM and V_{max} of 27.5 U/mg, and the saturation reached at 5% (Fig. 3.3.5). The final sucrose concentration of 5% was taken as optimum concentration for further dextransucrase activity assay. Similar result of 5% sucrose as an optimum concentration has been reported for glucansucrase from *Leuconostoc mesenteroides* NRRL B-640 (Purama and Goyal 2007).

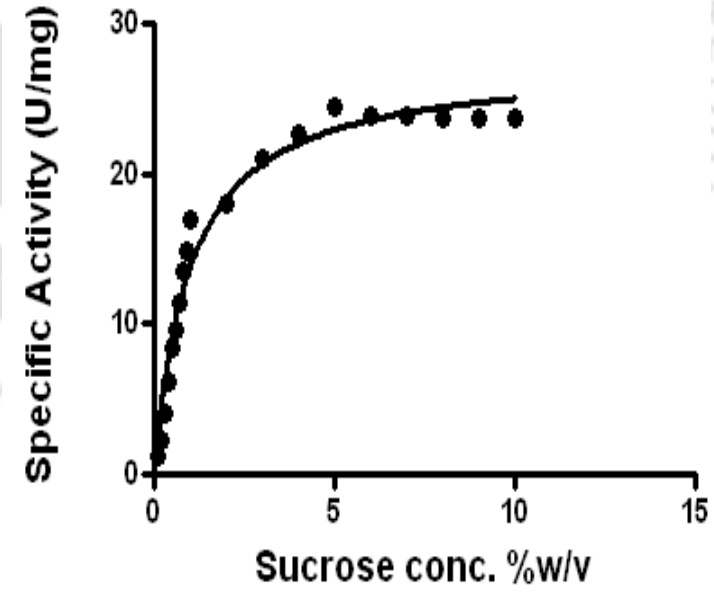

Fig. 3.3.5 Effect of sucrose concentration on dextransucrase activity. The assay was performed at 35°C in 20 mM sodium acetate buffer, pH 5.4.

It was reported earlier that *L. mesenteroides* NRRL B-1146 and *L. mesenteroides* NRRL B-512F exhibited K_m of 18.7 mM and 14.9 mM, respectively (Majumder *et al.*,

2008; Goyal *et al*., 1995). From the above analysis it was confirmed that dextransucrase from *Weissella cibaria* JAG8 exhibited low K_m value when compared to reported strains indicated that enzyme high affinity for substrate, as a result the enzyme activity is more. Higher the K_m, lesser will be the affinity towards substrate, which indicates dextransucrase isolated from *Weissella cibaria* JAG8 is more efficient in its activity when compared to other commercial strains.

3.3.4.2 *Effect of temperature and ionic strength on dextransucrase activity*

The purified dextransucrase (specific activity 20 U/mg) was used to study the effect of temperature and ionic strength on dextransucrase activity. The temperature of reaction mixture was varied between 20-50°C. The results showed 35°C was optimum temperature for dextransucrase activity as shown in Fig 3.3.6 A. It was observed that the optimum temperature for enzyme activity of *Leuconostoc mesenteroides* strains IBT-PQ, B-512F and B-1355 was in the range 30-35°C (Chellapandian *et al*.,1998; Kobayashi and Matsuda 1980; Lopez *et al*.,1993).

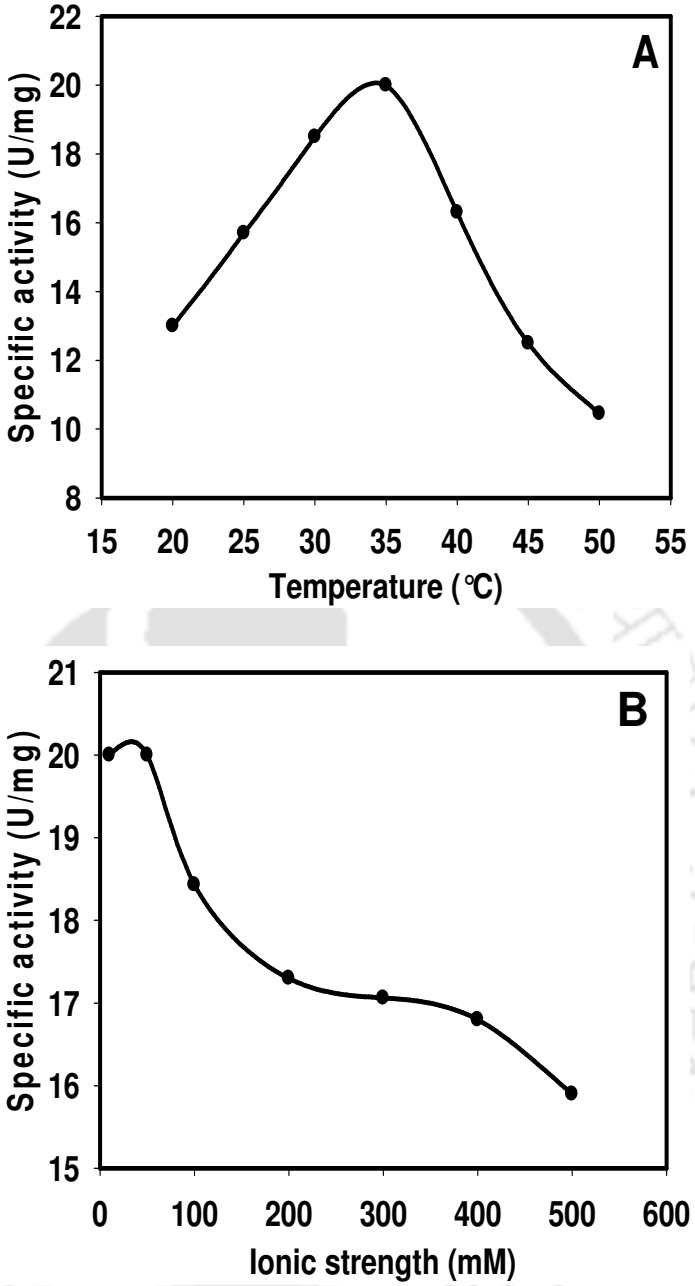

Fig. 3.3.6 (**A**) Effect of temperature (**B**) ionic strength on dextransucrase activity. Assay was carried out at 35°C in presence of 20 mM sodium acetate buffer pH 5.4.

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 impact on enzyme activity up to 50 mM concentration. At 100 mM concentration the decrease in enzyme

activity was around 10%. The loss of enzyme activity at 500 mM was 20% as shown in Fig 3.3.6 B. The enzyme was found to be quite stable between 10-500 mM sodium acetate concentrations. Similar results were reported in case of *L. mesenteroides* NRRL B-640 (Purama and Goyal 2009).

3.3.4.3 *Effect of salts on the activity of dextransucrase*

It was earlier reported that divalent cations have a significant role in maintaining the enzyme activity. Generally metal ions act as cofactors and maintain the stable conformation of enzyme (Majumder *et al*., 2008). The effects of different divalent metal ions Mg^{2+}, Co^{2+} and Ca^{2+} on activity of dextransucrase were studied. The addition of 2 mM $MgCl_2$, 2 mM $CoCl_2$ and 4 mM $CaCl_2$ increased the activity of dextransucrase by 22%, 14% and 13% respectively (Figure 3.3.7). It was reported that Ca^{2+} ions enhances 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). In case of *L. mesenteroides* NRRL B-640, 4 mM $CoCl_2$ enhanced the enzyme activity by 22% (Purama and Goyal 2009). From this study it can be inferred that dextransucrase activity is greatly influenced by different metal ions.

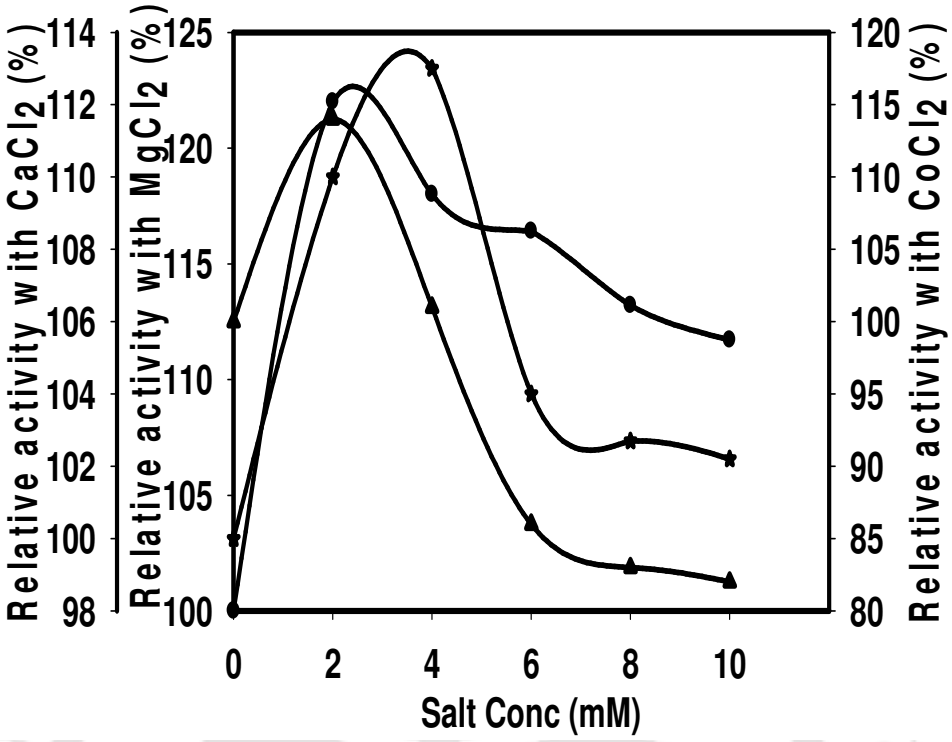

Fig. 3.3.7 Effect of metal ions on dextransucrase activity. $MgCl_2$ (●), $CoCl_2$ (▲), $CaCl_2$ (⁎). The assay was carried out at 35°C in 20 mM sodium acetate buffer pH 5.4.

3.3.4.4 *Effect of denaturing agents on the activity of dextransucrase*

The addition of EDTA inhibited the enzyme activity of dextransucrase by 52% at 4 mM concentration. Greater extent of inactivation was observed with further increase in the concentration of EDTA to 10 mM with 64% decrease in enzyme activity (Fig. 3.3.8). Urea also displayed inactivation effect on dextransucrase at all concentrations. With increase in concentration of urea there was a drastic decrease in enzyme activity. The enzyme lost 43%, 75%, 94% and 98% of its activity at 1, 2, 3 and 4 M urea, respectively in 15 min (Fig. 3.3.8). 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.

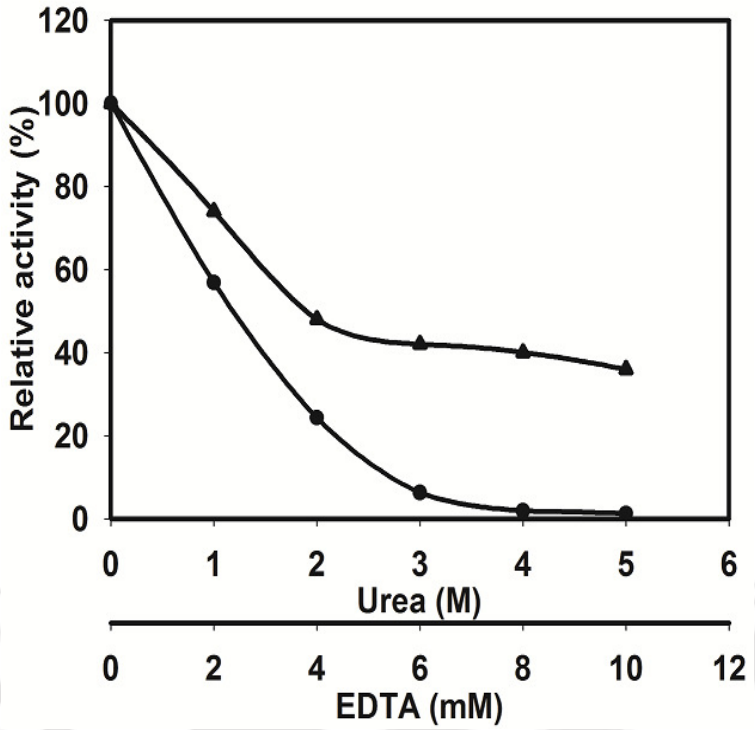

Fig. 3.3.8 Effect of Urea (●) and EDTA (▲) on dextransucrase activity. The assay was carried out at 35°C in 20 mM sodium acetate buffer, pH 5.4.

3.3.4.5 *Effect of thermal and pH stability of dextransucrase*

The enzyme was stable up to 35°C with further increase in temperature there was a constant decrease in relative activity of enzyme. It was observed that the enzyme nearly lost 50% of its relative activity at 50°C as shown in Fig. 3.3.9 A. The enzyme showed maximum stability at pH 5.4 with 25% increase in relative activity, as the enzyme activity was affected with constant increase in pH from 5.4 to 6.0 with 21% decrease in relative activity as shown in Fig. 3.3.9B. From the above analysis it was inferred that the enzyme was stable between 20-35°C at pH 5.4. The thermal and pH stability results were similar to earlier reports of dextransucrase from *L. mesenteroides* NRRL B-512F (Kobayashi and Matsuda 1980) and *L. mesenteroides* NRRL B-640 (Purama and Goyal 2010).

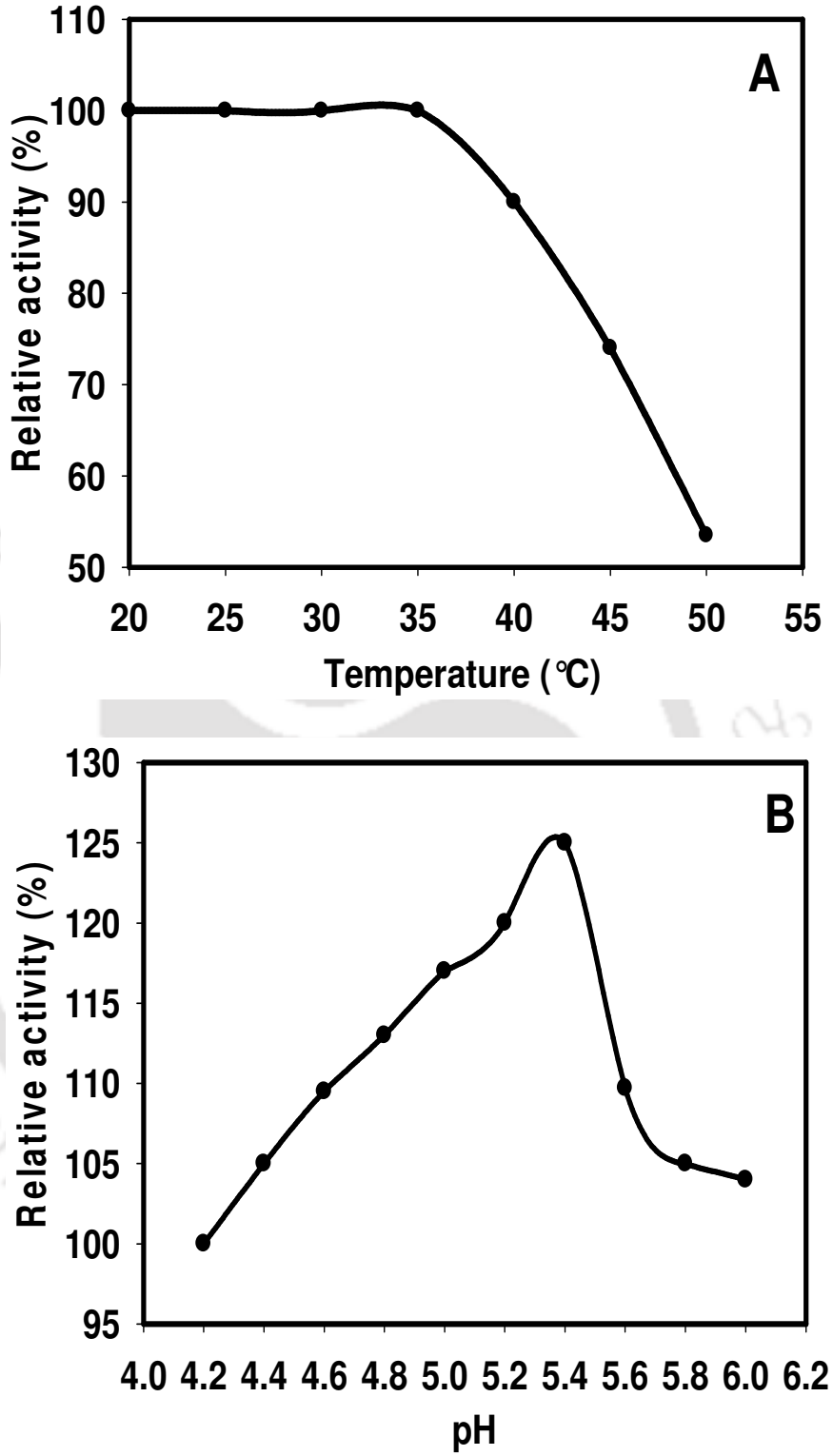

Fig. 3.3.9 **(A)** Thermal stability (**B**) pH stability of dextransucrase. Assay was carried out in 20 mM sodium acetate buffer, pH 5.4 at 35°C.

3.3.4.6 ***Effect of storage temperature and additives on stability of dextransucrase***

The effects of various additives on the stability effect on dextransucrase were studied. Tween 80 had significant stabilising effect on the enzyme activity whereas dextran did stabilize the enzyme but to a much lesser extent. The residual activity of dextransucrase with control, Tween 80, dextran (100 kDa) at 30°C after 24h was 18%, 81% and 43%, respectively (Fig. 3.3.10).

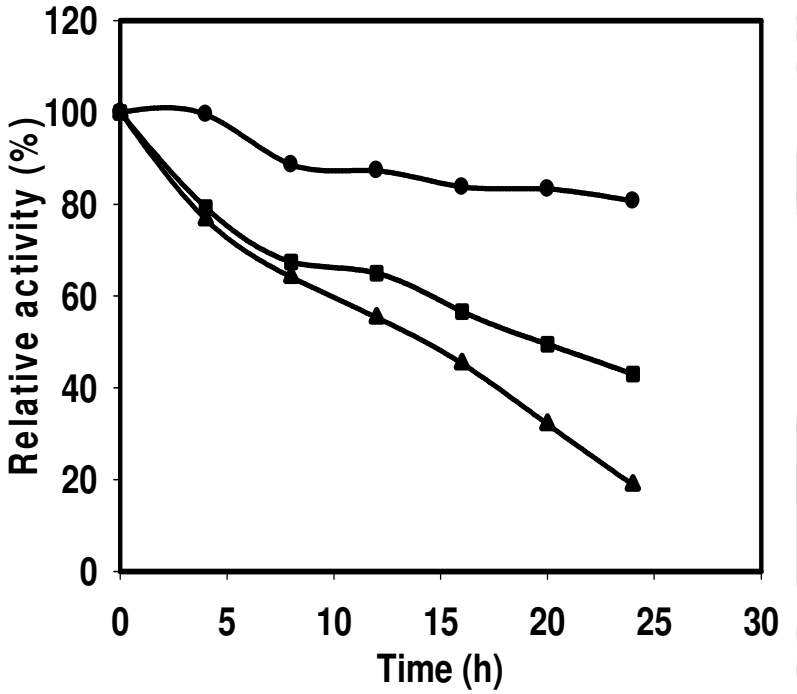

Fig. 3.3.8 Effect of different stabilizers on dextransucrase stability at 30°C. Control (—▲—), Tween-80 (—●—), Dextran 100 kDa (—■—).

Tween 80 was the best stabilizer for dextransucrase from *Weisella cibaria* JAG8 at 30°C followed by Dextran (100 kDa). Similar results were also reported earlier for dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 (Purama *et al*., 2010). The storage of dextransucrase at 4°C showed a loss of 70% enzyme activity in 40 days of incubation, whereas in presence of Tween 80, the enzyme lost only 22% of its activity in 40 days as shown in Fig 3.3.9.

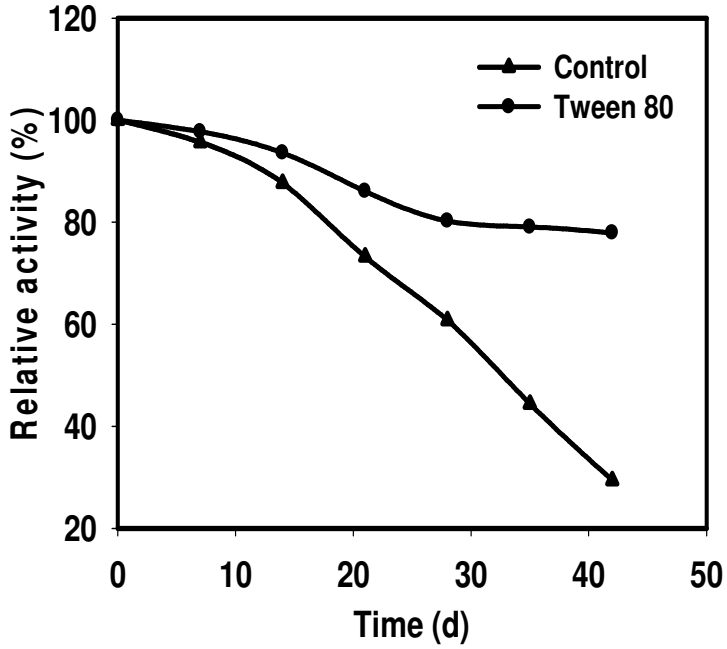

Fig. 3.3.9 Effect of Tween 80 on dextransucrase stability at 4°C.

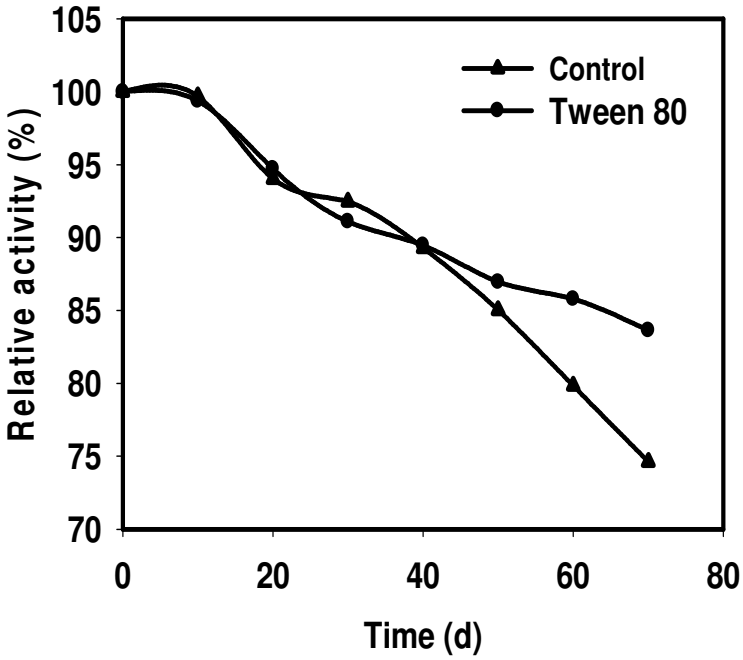

Fig. 3.3.10 Effect of Tween 80 on dextransucrase stability at -20°C.

Dextransucrase when stored at -20°C it lost 25.4% of its activity, whereas in the presence of additive Tween 80 it lost 16.4% of its activity in 70 days, as shown in Fig

3.3.10. The addition of Tween 80 to dextransucrase provided stabilizing effect at all the three temperatures 30°C, 4°C and -20°C.

3.3.4.7 Half-life of dextransucrase and additive-treated dextransucrase

The residual activity of dextransucrase was measured at various temperatures with respect to time with and without additives. The enzyme deactivation followed first order rate kinetics. The half-life ($t_{½}$) of dextransucrase and additives treated dextransucrase was calculated by assuming that the decay followed first order kinetics and are listed in Table 3.3.4.

Table 3.3.4 Half life ($t_{½}$) of dextransucrase with and without additive at various temperatures.

	Half-life ($t_{1/2}$) h/d		
Dextransucrase + Additives	**30°C**	**4°C**	**-20°C**
Control (Dextransucrase)	10.8 h	24.5 d	173 d
Tween 80	50 h	104 d	345 d
Dextran (100 kDa)	16 h	nd	nd

nd - not determined; h - hour; d - day

The $t_{½}$ of enzyme (control) increased from 10.8 h to 24.5 d when stored at 4°C and increased to 173 d when stored at -20°C instead of 30°C. This showed that the dextransucrase could be stored for long term at -20°C. In presence of Tween 80 at -20°C, the $t_{½}$ of enzyme was 345 d and in case of control it was 173 d. Among the two additives used Tween 80 gave higher stabilization of dextransucrase with $t_{½}$ of 50 h at 30°C as compared with dextran with $t_{½}$ of 16 h (Table 3.3.4).

3.4 Conclusions

The purification of crude dextransucrase from *Weissella cibaria* JAG8 with a specific activity of 1 U/mg by 33% (v/v) PEG 400 and 15% (w/v) PEG 1500 fractionation resulted in 20 and 10 fold purification with specific activity of 20.0 U/mg and 10.2 U/mg respectively. Further purification of 33% (v/v) PEG 400 enzyme fraction by size exclusion chromatography using Sephacryl S-300HR resulted in specific activity of 37.0 U/mg with 37 fold purification. Coomassie brillent blue staining analysis of dextransucrase under denaturing, non denaturing SDS-PAGE showed a single distinct band with molecular weight of 177 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 dextransucrase showed maximum activity at 5% sucrose concentration with K_m of 13 mM and V_{max} of 27.5 U/mg. The optimum conditions for enzyme reaction were 35°C and pH 5.4. Mg^{2+}, Co^{2+}, Ca^{2+} ions enhanced the activity of enzyme by 22%, 14% and 13%, respectively. The enzyme lost 98% and 64% of its activity at 4 M urea and 10 mM EDTA, respectively. Thermal and pH stability analysis showed that enzyme was stable up to 35°C and in the pH range of 5.2 to 5.4. Among the additives Tween 80 provided higher stabilization to dextransucrase than dextran. The storage of dextransucrase at different temperatures showed that best temperature for storage of enzyme is -20°C. In presence of Tween 80 at -20°C, the $t_{½}$ of enzyme was 345 d and in case of control it was 173 d. The results suggested that storage of dextransucrase at -20°C was best and Tween 80 can be used as additive for storage of enzyme. The purified enzyme can be exploited for enzymatic synthesis of

dextran and of oligosaccharides with potential applications in food and baking industry.

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Chapter 4

Active site mapping of dextransucrase from *Weissella cibaria* JAG8: Identification of essential cysteine and lysine amino acid residues

4.1 Introduction

Dextransucrase from *Weissella* species has recently gained importance for its high yielding dextran with unique properties which can be exploited in food and bakery industry (Schwab *et al*., 2008). It was reported that dextran from *Weissella cibaria* act as a perfect hydrocolloid with potential application in generation of gluten free cereal food products which can be used as food source for celiac disease patients (Galle *et al*., 2010). Dextransucrase (EC 2.4.1.5) is an extracellular enzyme that catalyses the formation of dextran from sucrose as depicted in equation 1 (Eq. 1). Dextransucrases have been included in the glycoside hydrolase family and, based on their sequence homologies, they has been grouped into 104 families (http://afmb.cnrs-mrs.fr/CAZY/). The enzyme dextransucrase belongs to the glycoside hydrolase family 70 (Goyal *et al*., 2007).

$$nC_{12}H_{22}O_{11} \xrightarrow{\text{dextransucrase}} (C_6H_{10}O_5)_n + n(C_6H_{12}O_6) \quad \text{Eq. 1}$$

(Sucrose) (Dextran) (Fructose)

Although much work has been carried out on the mechanism of dextran synthesis and structural organization of catalytic site of dextransucrase (Fu and Robyt 1988; Su and Robyt 1994) not much information is available on the nature of amino acids present at the active site. One-site and two-site mechanisms have been proposed for the synthesis of dextran from sucrose. In one-site mechanism it was proposed that sucrose and dextran bind to distinct sites of the enzyme (Kobayashi *et al*., 1984). Whereas, in case of two-site mechanism it was shown that the two nucleophiles at the active site attack the two bound sucrose molecules to give two covalent intermediates (Robyt *et al*., 1974). It was shown that two histidine residues are present at the active site by chemical modifications and proposed that the imidazolium groups of histidine residues facilitate the formation of α(1→6) glucosidic linkage (Fu and Robyt 1988). It was reported by Goyal and Katiyar 2007 that lysine is the key amino acid present at the catalytic site of the dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F and is virtually devoid of cysteine residues and rich in acidic amino acid at the active site. DTNB reacts with thiol group of cysteine to form thinitrobenzoate which was detected by characteristic absorbance at 412 nm (Bulaj *et al*., 1998).

In the present study dextransucrase isolated from *Weissella cibaria* JAG8 was subjected to active site mapping analysis by chemical modification. The enzyme was modified with lysine specific reagents such as pyridoxal-5'-phosphate (PLP), 2,4,6-trinitrobenzene sulphonic acid (TNBS) and cysteine specific reagents such as 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB) and iodoacetic acid as active site residues. The Chemical modification was also carried out using fluorogenic bifunctional irreversible

inhibitor *o*-phthalaldehyde (Placzewski *et al*., 1983) for the identification of lysine and cysteine as active site residues (Puri *et al*.,1985; Goyal and Katiyar 1995c; Goyal and Katiyar 1998b). The present investigation reveals for the first time that one of more cysteine residues are also present apart from lysine residues, at the active site of dextransucrase from *Weissella cibaria* JAG8.

4.2 Material and Methods

4.2.1 Chemicals and reagents

All the reagents such as pyridoxal-5'-phosphate (PLP), 2,4,6-trinitrobenzenesulphonic acid (TNBS), 5, 5'-dithiobis 2-nitrobenzoic acid (DTNB), iodoacetic acid, *o*-phthalaldehyde, urea, β-mercapatoethanol, EDTA, lysine and cysteine were purchased from Sigma Chem. Co., (USA).

4.2.2 Enzyme and activity assay

Dextransucrase produced from *Weissella cibaria* JAG8 was purified by fractionation with 33% (v/v) polyethylene glycol 400 as described in Chapter 3, Section 3.2.5. The purified enzyme with specific activity of 20 (U/mg) and 0.44 (mg/ml) protein was used for the inactivation reactions in 20 mM sodium acetate buffer pH 5.4 was used at 35°C unless otherwise stated. The enzyme activity and protein concentration was determined as described in Chapter 2, Section 2.2.5 and Chapter 3, Section 3.2.4, respectively.

4.2.3 Modification of dextransucrase with pyridoxal-5'-phosphate

The enzyme was incubated with varying concentrations of pyridoxal-5'-phosphate (PLP) ranging from 5-25 mM concentration at 35°C. The reaction of enzyme with PLP was stopped at different time intervals by transferring 40 µl of enzyme incubated with PLP to 10 µl of 100 mM lysine to stop the reaction. 20 µl of the above mixture was used to determine the enzyme activity as described in Chapter 2, Section 2.2.5. Since, the reaction of PLP with enzyme is reversible, in order to obtain an irreversible modification after PLP treatment of enzyme, the mixture was reduced by adding 50 mM sodium borohydride (prepared freshly in ice cold 20 mM

sodium acetate buffer pH 5.4). The reaction mixture, after reduction with sodium borohydride was incubated at 4°C for 30 min and then dialyzed with ice cold 20 mM sodium acetate buffer pH 5.4 for 24 h. The resulting N^{ε}- phosphopyridoxyl lysine complex was detected by measuring the absorbance in the range of 200-600 nm. (Goyal and Katiyar 1995a; Goyal and Katiyar 1998a) and was further confirmed by fluorescence emission spectrum of the sample at the wave length where maximum absorbance was achieved.

4.2.4 Effect of sucrose on dextransucrase inactivation by pyridoxal-5'-phosphate

The enzyme was incubated with 5 mM EDTA in 20 mM sodium acetate buffer pH 5.4 at 35°C for 30 min. Sucrose (150, 300 mM) was added and incubated for another 15 min at 35°C, prior to the addition of 25 mM PLP and incubated for 1 h followed by treatment with 5 mM $CaCl_2$ and incubated for 1 h. The enzyme activity was determined before and after Ca^{2+} against control. After 1 h the fluorescence emission of the N^{ε}-phosphopyridoxyl lysine complex was detected by measuring the fluorescence emission spectrum with excitation at wavelength 325 nm (Goyal and Katiyar 1998a). A control was run with the enzyme treated with EDTA without sucrose treatment in presence of PLP.

4.2.5 Modification of dextransucrase with 2, 4, 6-trinitrobenzenesulphonic acid

The enzyme was incubated with varying concentrations of 2,4,6-trinitrobenzenesulphonic acid (TNBS) ranging from 5-25 mM concentration at 35°C. The reaction of enzyme with TNBS was stopped at different time intervals by transferring 40 µl of enzyme incubated with TNBS to 10 µl of 100 mM lysine to stop

the reaction. 20 µl of the above reaction mixture was subjected for enzyme activity assay as described in Chapter 2, Section 2.2.5. The inactivation by TNBS was confirmed by incubating dextransucrase with 25 mM TNBS that forms an irreversible trinitrophenyl derivative of amino group of lysine (ε-TNP-lysine) complex (Goyal and Katiyar 1995b) which was detected by measuring the absorbance in the range of 200-600 nm. The complex formation was further confirmed by measuring the fluorescence emission spectra by excitation at wavelength were maximum absorbance was achieved.

4.2.6 Time dependent inactivation of dextransucrase with 5, 5'-dithiobis-2-nitrobenzoic acid

The enzyme was incubated with varying concentrations of 5, 5'-dithiobis-2-nitrobenzoic acid (DTNB) ranging from 2-10 mM concentration at 35°C. The reaction of enzyme with DTNB was stopped at different time intervals by transferring 40 µl of enzyme incubated with DTNB to 10 µl of 100 mM cysteine to stop the reaction. 20 µl of the above mixture was used to determine the enzyme activity as described in Chapter 2, Section 2.2.5. The formation of thionitrobenzoate complex (Bulaj *et al.*, 1998) as a result of enzyme-DTNB reaction was detected by incubating the enzyme with 10 mM DTNB for 60 min at 35°C and by measuring the absorbance in the range of 200-600 nm. The complex formation was further confirmed by measuring the fluorescence emission spectra by excitation at wavelength were maximum absorbance was achieved.

4.2.7 Time dependent inactivation of dextransucrase with Iodoacetatic acid

The enzyme was incubated with varying concentrations of iodoacetic acid ranging from 5-25 mM concentration at 35°C. The reaction of enzyme with iodoacetic acid was stopped at different time intervals by transferring 40 µl of enzyme incubated with iodoacetic acid to 10 µl of 100 mM cysteine to stop the reaction. 20 µl of the above mixture was used to determine the enzyme activity as described in Chapter 2, Section 2.2.5. The formation of thioacetate complex as a result of enzyme-iodoacetic acid reaction was detected by incubating the enzyme with 25 mM iodoacetic acid for 1h at 35°C and taking the absorbance scan in the range of 200-600 nm. The complex formation was further confirmed by fluorescence spectroscopy by exciting the sample at the wavelength were maximum absorbance was achieved.

4.2.8 Modification of dextransucrase with *o*-phthalaldehyde

The enzyme was treated with varying concentrations of *o*-phthalaldehyde ranging from 2-10 mM concentration at 35°C. The reaction of enzyme with *o*-phthalaldehyde was stopped at different time intervals by transferring four parts (40 µl) of enzyme incubated with *o*-phthalaldehyde to one part (10 µl) of 100 mM cysteine to stop the reaction. 20 µl of the above mixture was used to determine the enzyme activity as described in Chapter 2, Section 2.2.5.

4.2.8.1 *UV-visible absorbance of dextransucrase-o-phthalaldehyde complex*

Dextransucrase with 20 U/mg and 0.44 mg/ml was incubated with 10 mM *o*-phthalaldehyde at 35°C for 1h and the absorbance scan was carried out from 200-600

nm. *o*-phthalaldehyde specifically binds to the sulphydryl group of cysteine and amino group of lysine and results in the formation of isoindole derivative (Goyal and Katiyar 1995c; Goyal and Katiyar 1998b) which can be detected by the absorbance maxima at 334 nm.

4.2.8.2 *Fluorescence spectroscopy analysis of dextransucrase-o-phthalaldehyde complex*

The enzyme was inactivated by incubating with 10 mM *o*-phthalaldehyde in 20 mM sodium acetate buffer (pH 5.4) for 1 h at 35°C. The absorbance scan showed absorbance maxima at 334 nm. The fluorescence emission spectrum of dextransucrase-*o*-phthalaldehyde complex was recorded at excitation wavelength 334 nm (Goyal and Katiyar 1998b).

4.2.8.3 *Effect of substrate sucrose and denaturants on the binding of by o-phthalaldehyde with dextransucrase*

The enzyme (0.44 mg protein/ml; 20 U/mg) was pre-incubated with 5 mM EDTA in 20 mM sodium acetate buffer (pH 5.4) at 35°C for 30 min, followed by treatment with 150 mM sucrose solution for 15 min at 35°C prior to the addition of 10 mM *o*-phthalaldehyde for 60 min and the residual enzyme activity was determined. The assay mixture contained 5 mM of Ca^{2+} ions for reactivation of the enzyme. The enzyme pre incubated with EDTA followed by treatment with Ca^{2+} ions without *o*-phthalaldehyde was used as control. In case of denaturant, the enzyme was treated with 4 M urea followed by treatment with 10 mM *o*-phthalaldehyde. A control was kept which contained only dextransucrase. All the reactions were carried out using 20

mM sodium acetate buffer (pH 5.4) at 35°C. The fluorescence emission spectra of the above samples were carried out at excitation wavelength 334 nm.

4.2.8.4 *Spectral analysis of o-phthalaladehyde treated dextransucrase in presence and absence of β-mercaptoethanol*

Dextransucrase was incubated with 10 mM β-mercaptoethanol for 30 min followed by incubating with 10 mM *o*-phthalaldehyde at 35°C for 30 min. A control was run in the absence of β-mercaptoethanol. The fluorescence emission spectra of the above samples were recorded at excitation wavelength of 334 nm (Goyal and Katiyar 1998b).

4.2.8.5 *Effect of PLP pretreated dextransucrase on fluorescence emission of dextransucrase-o-phthalaldehyde adduct*

The enzyme was incubated with 25 mM PLP for 30 min prior to incubation with 10 mM *o*-phthalaldehyde at 35°C for 1 h. The incubation of enzyme with two inhibitors was monitored by fluorescence emission spectra on excitation at 334 nm before and after dialysis along with control containing only the enzyme (Goyal and Katiyar 1998b). Fluorescence emission spectra were recorded on Spectrofluorometer (Jobin Yvon Horiba; Model Fluoro Max-3). Absorption spectra were recorded on spectrophotometer (Carry-100, Varian). Both, the fluorescence and absorbance were recorded in cuvettes of 1 cm path length and 1 ml capacity.

4.3 Results and Discussion

4.3.1 Time dependent inactivation of dextransucrase by pyridoxal-5'-phosphate

Inactivation of dextransucrase was augmented with increase in concentration of PLP showing 98.5% loss of enzyme activity at 25 mM concentration. The absorbance maxima of enzyme-PLP complex was observed at 325 nm (Fig. 4.3.1A). The inactivation of dextransucrase by PLP was reversed completely by dialysis with 20 mM sodium acetate buffer pH 5.4 at 4°C for 24 h. However, the reduction of enzyme-PLP complex by sodium borohydride led the inactivation irreversible and the resulting complex exhibited an absorbance maximum at wavelength of 325 nm (Fig. 4.3.1B). Since PLP inactivation is reversible, the enzyme activity could be retained by dialysis. However, reduction with sodium borohydride after PLP treatment rendered the enzyme inactivation irreversible. This indicated that inhibition of enzyme activity was due to specific modification of ε-NH_2 group of lysine residues and not due to non covalent interactions between PLP and enzyme (Goyal and Katiyar 1995a). In the present study 25 mM concentration of PLP which was quite high for causing maximum or more than 98% inactivation. This is because the lysine residue(s) at the active site are less accessible or due to presence of dextran associated with the enzyme. The inactivation of dextransucrase by PLP was due to the reaction of ε-NH_2 group of lysine residues with aldehyde group of PLP and results in formation of Schiff's base which, on reduction with sodium borohydride gives N^{ε}-phosphopyridoxyl lysine complex (Fig. 4.3.2). These results were similar to those reported earlier for dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F (Goyal and Katiyar 1995a; Goyal and Katiyar 1998a).

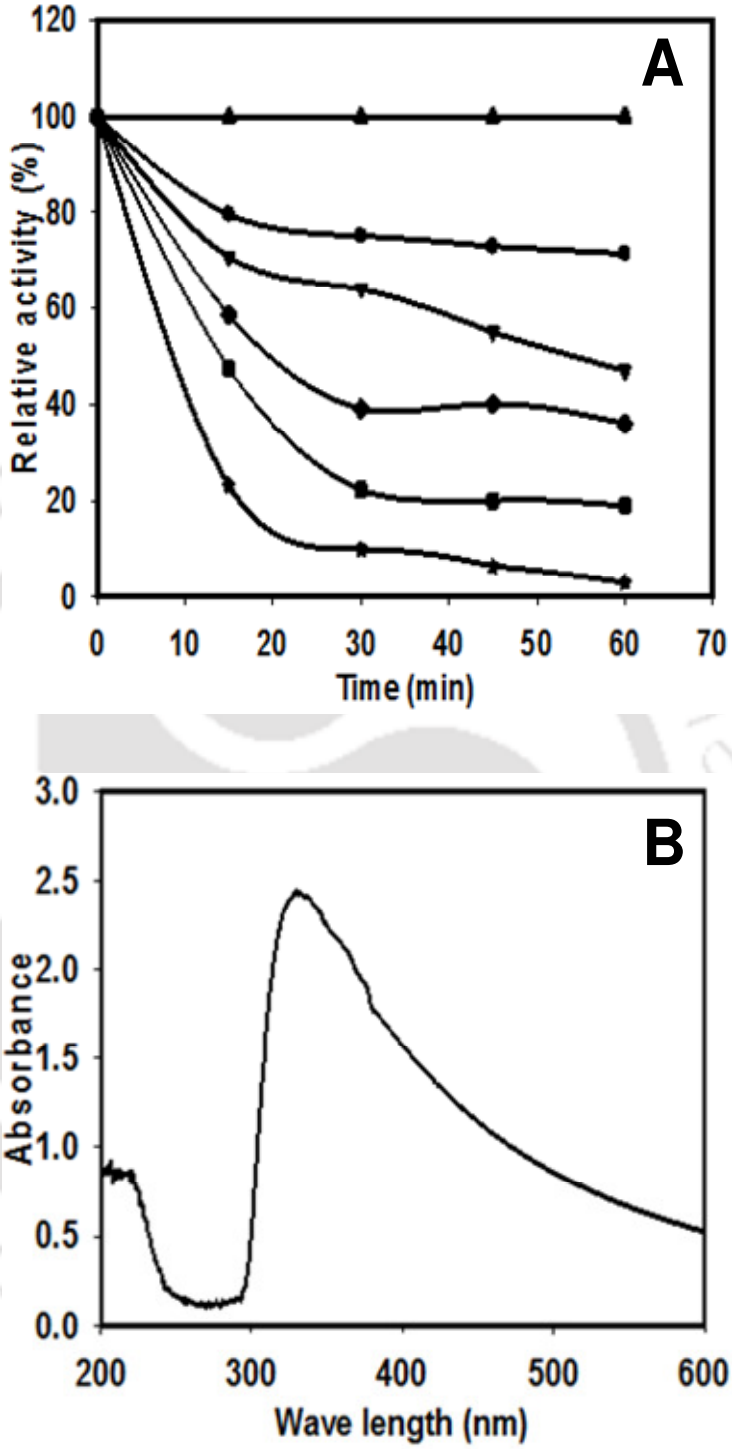

Fig. 4.3.1. Effect of PLP on dextransucrase isolated from *Weissella cibaria* JAG8. (**A**) Time dependent inactivation of dextransucrase by PLP. The enzyme (0.44 mg/ml; 20 U/mg) was incubated with 0 (▲), 5 (●), 10 (▼), 15 (♦), 20 (■), 25mM (*) PLP at 35°C for 60 min. Aliquots were withdrawn at the indicated time intervals and the relative activity was determined. (**B**) Absorption spectrum of dextransucrase-PLP complex with 25 mM PLP. The enzyme was treated with 25 mM PLP and incubated for 1h at 35°C.

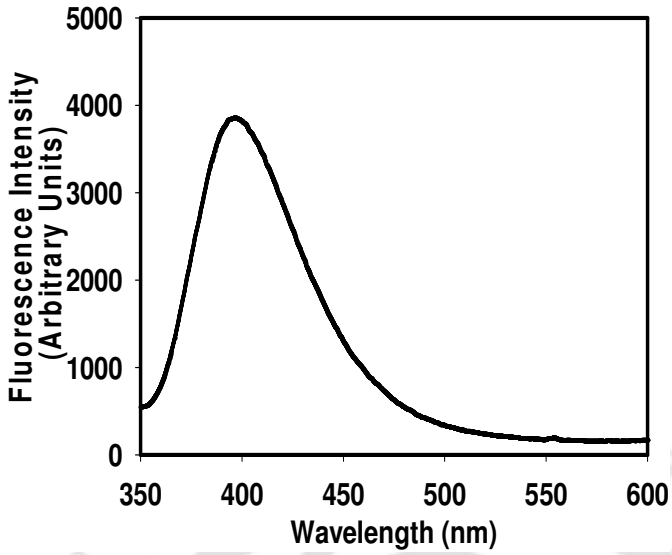

Fig. 4.3.2 Fluorescence emission spectra of dextransucrase-PLP complex after reduction with sodium borohydride. The enzyme (0.44 mg/ml; 20 U/mg) was treated with 25 mM PLP for 60 min at 35°C and reduced with 50 mM sodium borohydride for 30 min. The mixture was dialyzed extensively. The resulting N^{ε}-phosphopyridoxyllysine complex characterized by fluorescence emission spectrum with excitation at wavelength of 325 nm.

4.3.2 Effect of sucrose on dextransucrase inactivation by PLP

Dextransucrase from *Weissella cibaria* JAG8 pre-incubated with 5 mM EDTA followed by treatment with 25 mM PLP and reduction with sodium borohydride in the absence of sucrose gave a fluorescence emission maxima at 397 nm (Fig.4.3.3). Whereas in case of enzyme pre-incubated with 5 mM EDTA followed by treatment with 150 mM sucrose for 15 min and finally treated with PLP and sodium borohydride caused decrease in fluorescence intensity (Fig.4.3.3). Treatment of dextransucrase with 5 mM EDTA at 35°C led to 75% inactivation in 30 min, (Table 4.3.1) any further increase in EDTA concentration significantly decreased the enzyme activity. The addition of 5 mM $CaCl_2$ led to around 45% of enzyme reactivation and no significant increase in the reactivation was observed with increase in concentration of Ca^{2+} ions (Fig.4.3.4).

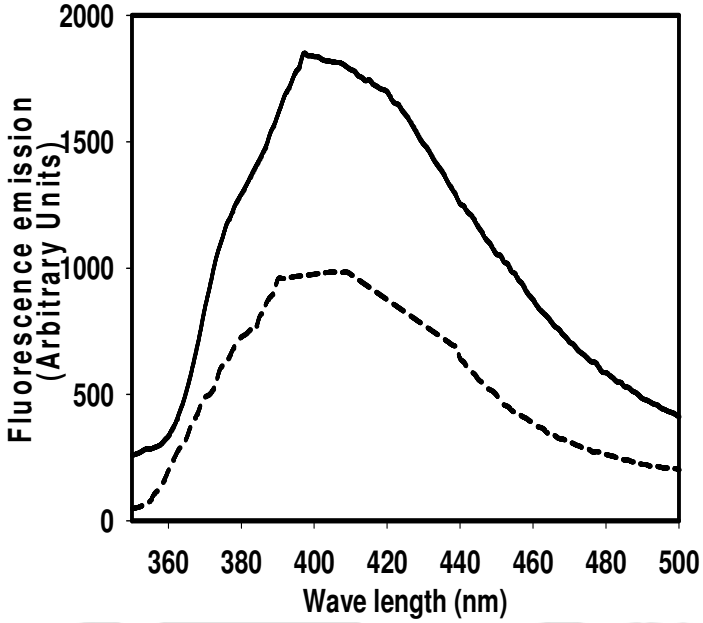

Fig. 4.3.3 Effect of substrate sucrose on the binding of PLP with dextransucrase. The enzyme (0.44 mg/ml; 20 U/mg) was preincubated with 50 mM EDTA for 30 min, followed by incubation with 150 mM sucrose for 15 min and finally with 25 mM PLP in 20 mM in sodium acetate buffer pH (5.4), at 35°C for 60 min. A control was run parellely without sucrose. The fluorescence spectra of control **(—)** and with 300 mM sucrose **(- - -)** were recorded at excitation wavelength, 325 nm.

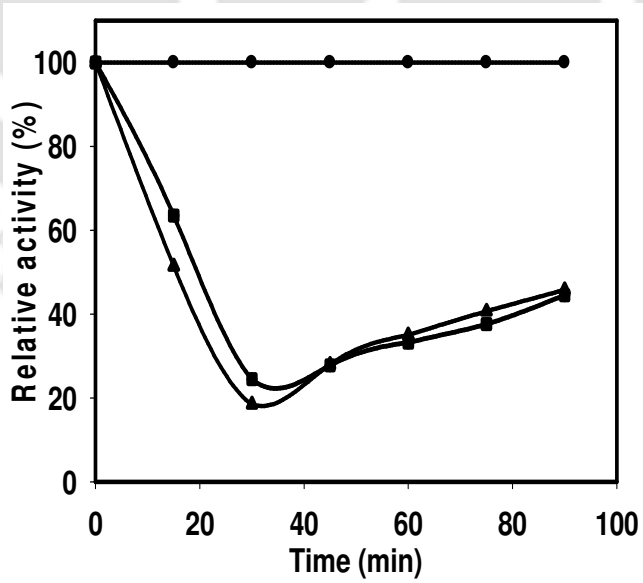

Fig. 4.3.4 Dextransucrase inhibition by EDTA and reactivation by Ca^{2+} ions. The enzyme (0.44 mg/ml; 20 U/mg) was incubated with 5 mM (■) and 10 mM (▲) EDTA at 35°C for 30 min followed by the addition of 5 mM and 10 mM Ca^{2+} ions, respectively. Aliquots were withdrawn at indicated time intervals and the relative activity was determined.

The effect of sucrose on dextransucrase inactivation by PLP is shown in Table 4.3.1. A concentration of 300 mM sucrose solution provided maximum protection to enzyme with 92.8% of reactivation. Sucrose has more affinity to bind the dextransucrase, when compared to PLP, as sucrose auto-polymerizes in presence of dextransucrase and forms dextran (Goyal and Katiyar 1998a). In case of control the enzyme pre-treated with EDTA caused inactivation but it still allowed the binding of PLP with the free lysine residues that led to the formation of Schiff's base and thus giving higher fluorescence intensity. In presence of sucrose the fluorescence intensity was very less, indicating that sucrose has blocked the inhibitor PLP from binding to the enzyme. As sucrose was readily hydrolyzed by dextransucrase and led to the formation of dextran which formed a protective covering around the enzyme and prevented the binding of PLP which resulted in reduced fluorescence intensity as reported by Goyal and Katiyar (1995a). Similar reactivation of dextransucrase was reported earlier from *Leuconostoc mesenteroides* NRRL B-512F (Goyal and Katiyar 1998a).

Table 4.3.1 Effect of substrate sucrose on inactivation of dextransucrase by PLP. The reagents shown in the table were incubated with 0.44 mg protein/ml of dextransucrase for indicated time period followed by incubation with 25 mM PLP for 60 min at 35°C. Appropriate controls in each case without PLP were run in parallel.

Reagent	Relative Activity (%)
Control	100
Enzyme + EDTA 5 mM (30 min) at 35°C	24.5
Enzyme + EDTA 5 mM (30 min) + $CaCl_2$ 5 mM (60 min) at 35°C	44.5
Enzyme + EDTA 5 mM (30 min) + Sucrose 150 mM (15 min) + $CaCl_2$ 5 mM (30 min) +PLP (25 mM) 60 min at 35°C	89.3
Enzyme + EDTA 5 mM (30 min) + Sucrose 300 mM (15 min) + $CaCl_2$ 5 mM (30 min) + PLP 25 mM (60 min) at 35°C	92.8

4.3.3 Modification of dextransucrase with TNBS

Dextransucrase inactivation was augmented with increase in concentration of TNBS which displayed 98.7% loss in enzyme activity at 25 mM concentration in 60 min (Fig. 4.3.5A). The absorbance maximum of enzyme-TNBS (ε-amino-TNP) complex (Goyal and Katiyar 1995b) was observed at 369 nm (Fig. 4.3.5B). The TNBS reaction with enzyme is irreversible and forms ε-NH_2-TNP (Trinitrophenyl) complex. The above complex does not emit fluorescence when excited at 369 nm. These results showed that one or more lysine residues present at active site are entailed for enzyme activity.

4.3.4 Time dependent inactivation of dextransucrase with DTNB

The extent of inactivation of dextransucrase increased with increase in concentration of DTNB which displayed 98.7% loss of enzyme activity at 10 mM concentration in 60 min (Fig. 4.3.6A). The absorbance of thionitrobenzoate complex was found to be maximum at 406 nm (Fig. 4.3.6B). It has been reported that thionitrobenzoate complex has absorbance maxima at 412 nm (Bulaj *et al*., 1998). The inactivation of dextransucrase by DTNB was caused by the formation of enzyme-DTNB (thionitrobenzoate) complex at 406 nm. The above complex does not emit fluorescence when excited at 406 nm. These results showed that one or more cysteine residues are present at the active site, which are essential for enzyme activity of dextransucrase.

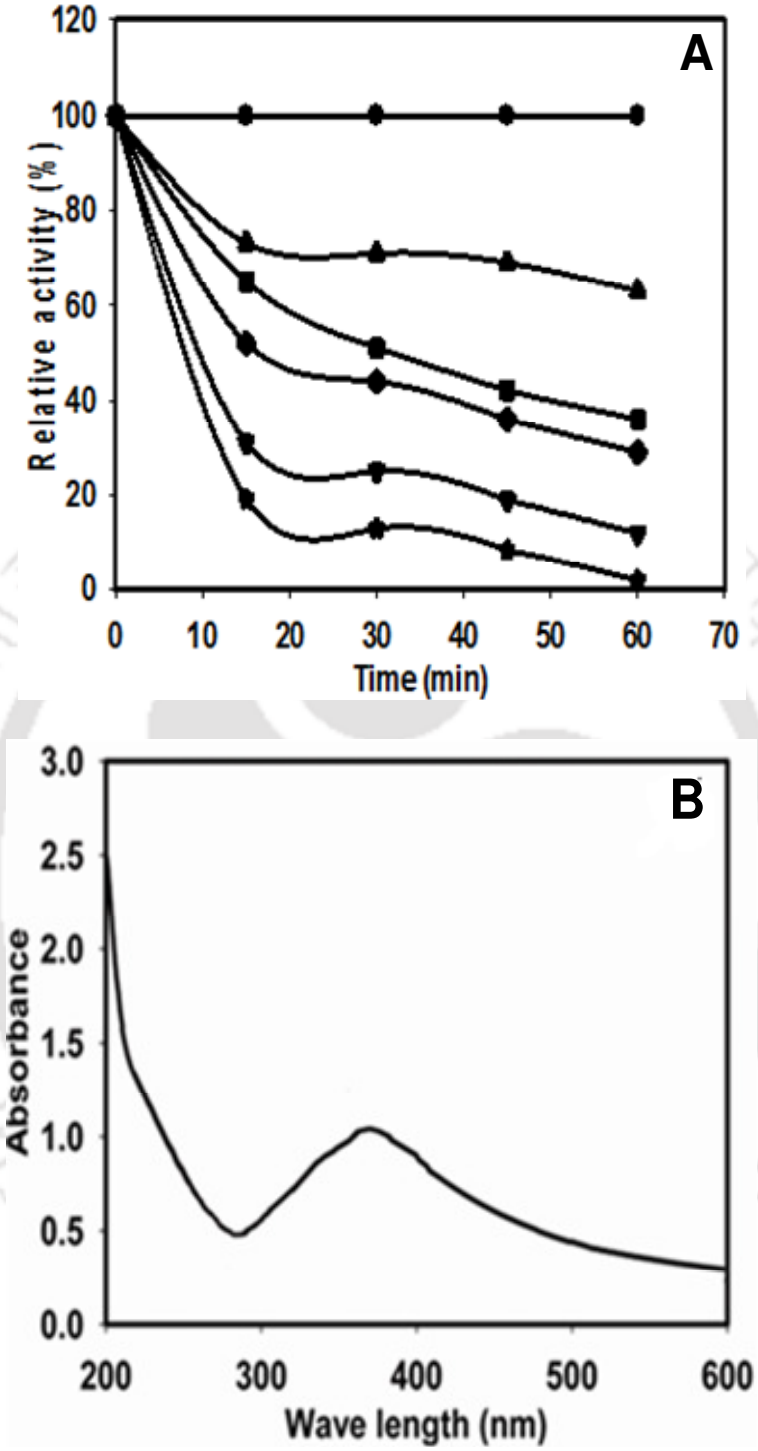

Fig. 4.3.5 Effect of TNBS on dextransucrase isolated from *Weissella cibaria* JAG8. (**A**) Time dependent inactivation of dextransucrase by TNBS. The enzyme (0.44 mg/ml, 20 U/mg) was incubated with 0 (●), 5 (▲), 10 (■), 15(♦), 20 (▼), 25 mM (*) TNBS in 20 mM sodium acetate buffer pH 5.4 at 35°C for 60 min. Aliquots were withdrawn at the indicated time intervals and the relative activity was determined. (**B**) Absorption spectrum of dextransucrase-TNBS complex. The enzyme was treated with 25 mM TNBS and incubated for 1h at 35°C.

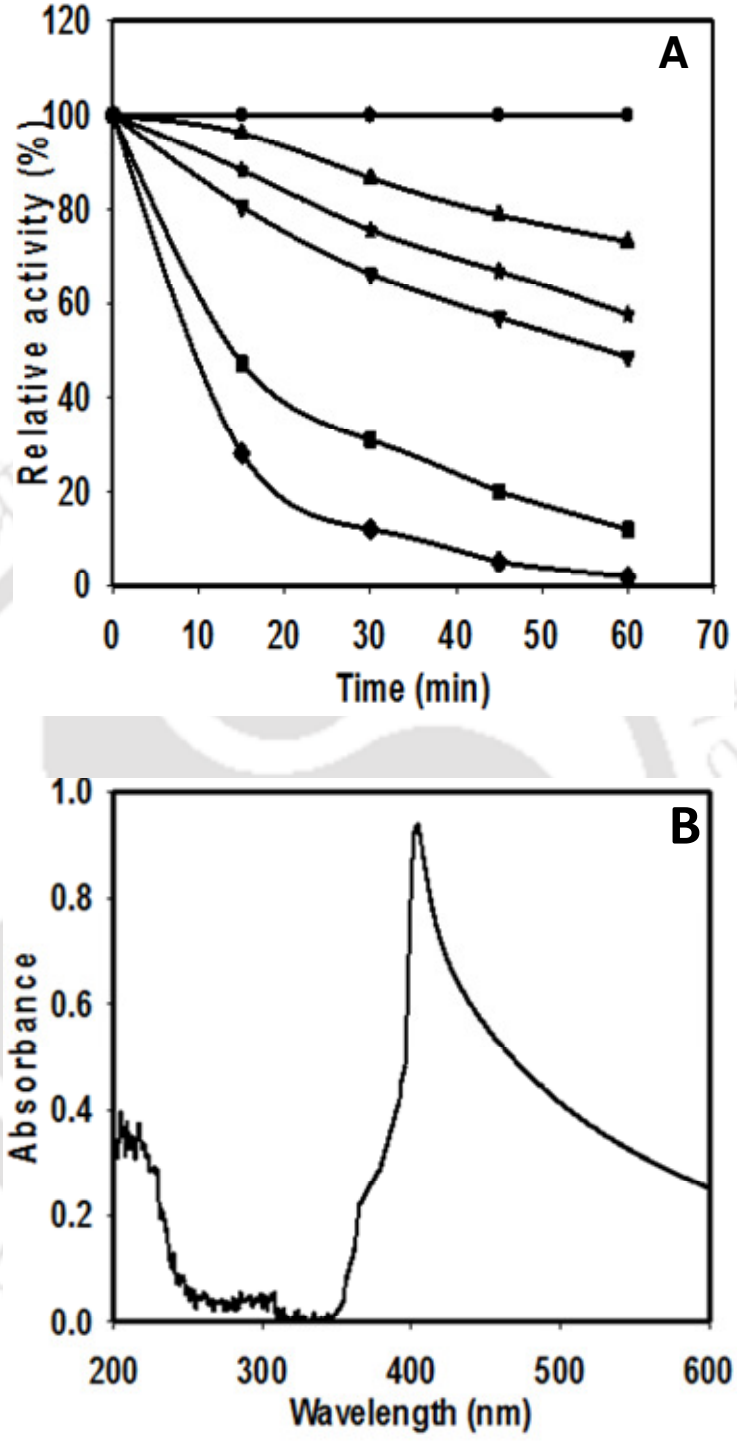

Fig. 4.3.6. Effect of DTNB on dextransucrase isolated from *Weissella cibaria* JAG8. (**A**) Time dependent inactivation of dextransucrase by DTNB. The enzyme (0.44 mg/ml; 20 U/mg protein) in 20 mM sodium acetate buffer pH (5.4) was incubated with 0 (●), 2 (▲), 4 (*), 6 (▼), 8 (■), 10 mM (♦) DTNB at 35°C for 60 min. Aliquots were withdrawn at the indicated time intervals and the relative activity was determined (**B**) Absorption spectrum of dextransucrase-DTNB complex. The enzyme was treated with 10 mM DTNB and incubated for 1h at 35°C.

4.3.5 Time dependent inactivation of dextransucrase with Iodoacetatic acid

Iodoacetic acid on treatment with dextransucrase led to the loss of enzyme activity as the extent of inactivation of dextransucrase increased with increase in concentration of iodoacetic acid. The enzyme lost 98.9% of its activity at 25 mM concentration of iodoacetic acid in 60 min (Fig. 4.3.7A). The absorbance of thioacetate complex was maximum at 323 nm (Fig. 4.3.7B). The inactivation of dextransucrase by iodoacetic acid was due to formation of thioacetate complex that gave absorbance maxima at 323 nm, and the complex did not emit any fluorescence when excited at 323 nm. The enzyme inactivation by iodoacetic acid confirmed that one or more cysteine residues are present at the active site of dextransucrase and are essential for enzyme activity.

4.3.6 Modification of dextransucrase with *o*-phthalaldehyde

Dextransucrase on treatment with 10 mM concentration of *o*-phthalaldehyde displayed 97.0% loss in activity in 60 min (Fig. 4.3.8A). The absorbance maxima of the enzyme-*o*-phthalaldehyde (isoindole derivative) complex (Goyal and Katiyar 1995c) was observed at 334 nm (Fig. 4.3.8B). This showed that the inactivation of enzyme is due to the formation of isoindol derivative which was detected by increase in fluorescence emission maxima at 418 nm, when excited at 334 nm (Fig. 4.3.9). The fluorescence intensity did not change when recorded even after 24h. The isoindole ring is formed only, when there is an interaction between sulfhydryl group of cysteine and ε-amino group of lysine are involved in presence of *o*-phthalaldehyde. And the distance between the two functional groups (i. e) cysteine and lysine should be around

2.5-3 A° apart. If the distance between these two residues are less than 2.5 A° or greater than 3 A° the isoindole ring is not formed (Goyal and Katiyar 1998b).

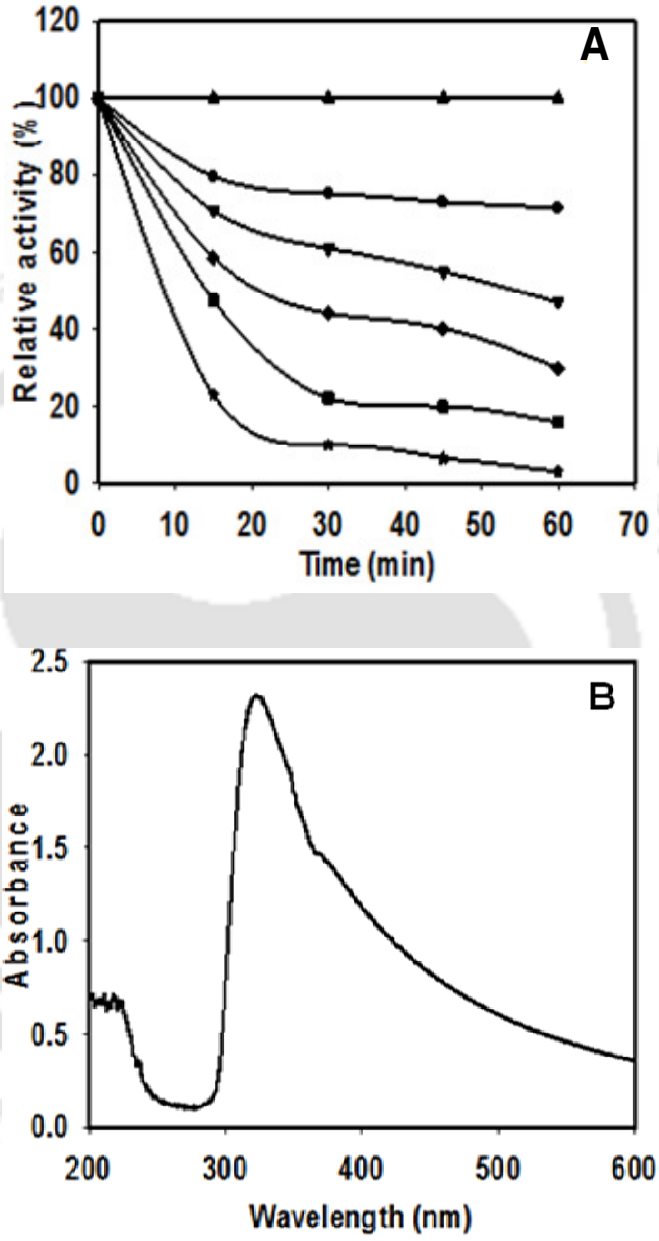

Fig. 4.3.7. Effect of Iodoacetic acid on dextransucrase isolated from *Weissella cibaria* JAG8. (**A**) Time dependent inactivation of dextransucrase by Iodoacetic acid. The enzyme (0.44 mg/ml, 20 U/mg protein) in 20 mM sodium acetate buffer pH (5.4) was incubated with 0 (●), 5 (▲), 10 (▼), 15 (■), 20 (♦), 25 mM (*) Iodoacetic acid at 35°C for 60 min. Aliquots were withdrawn at the indicated time intervals and the relative activity was determined. (**B**) Absorption spectrum of dextransucrase-thioacetate complex. The enzyme (0.44 mg/ml, 20 U/mg) was treated with 25 mM Iodoacetic acid and incubated for 1h at 35°C.

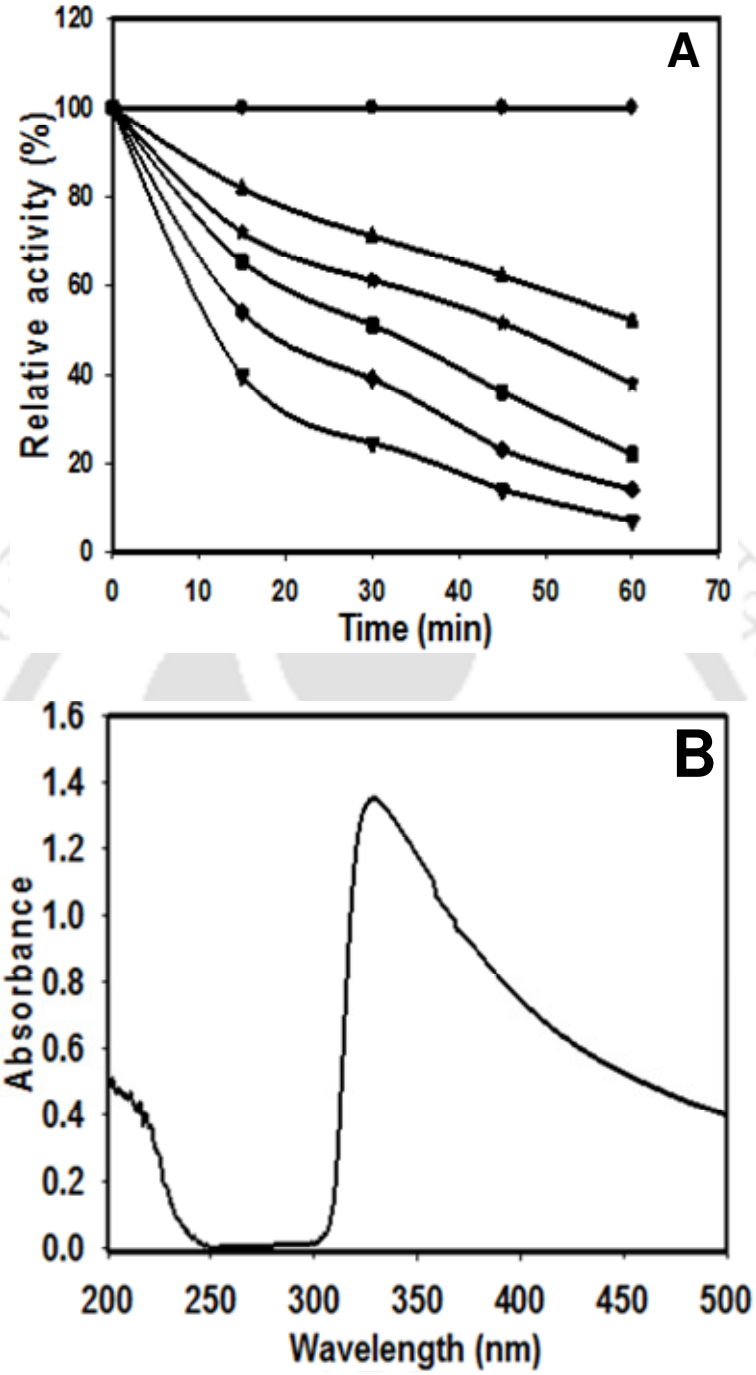

Fig. 4.3.8 Effect of *o*-phthalaldehyde on dextransucrase from *Weissella cibaria* JAG8. (**A**) Time dependent inactivation of dextransucrase by *o*-phthalaldehyde. The enzyme (0.44 mg/ml, 20 U/mg) was incubated with 0 (●), 2 (▲), 4 (*), 6 (■), 8 (♦), 10 mM (▼) *o*-phthalaldehyde at 35°C for 60 min. Aliquots were with drawn at the indicated time intervals and the residual activity was determined. (**B**) Absorption spectrum of dextransucrase-*o*-phthalaldehyde complex. The enzyme (0.44 mg/ml, 20 U/mg) was treated with 10 mM *o*-phthalaldehyde. The resulting isoindole derivative was characterized by absorption spectrum at wavelength of 334 nm**.**

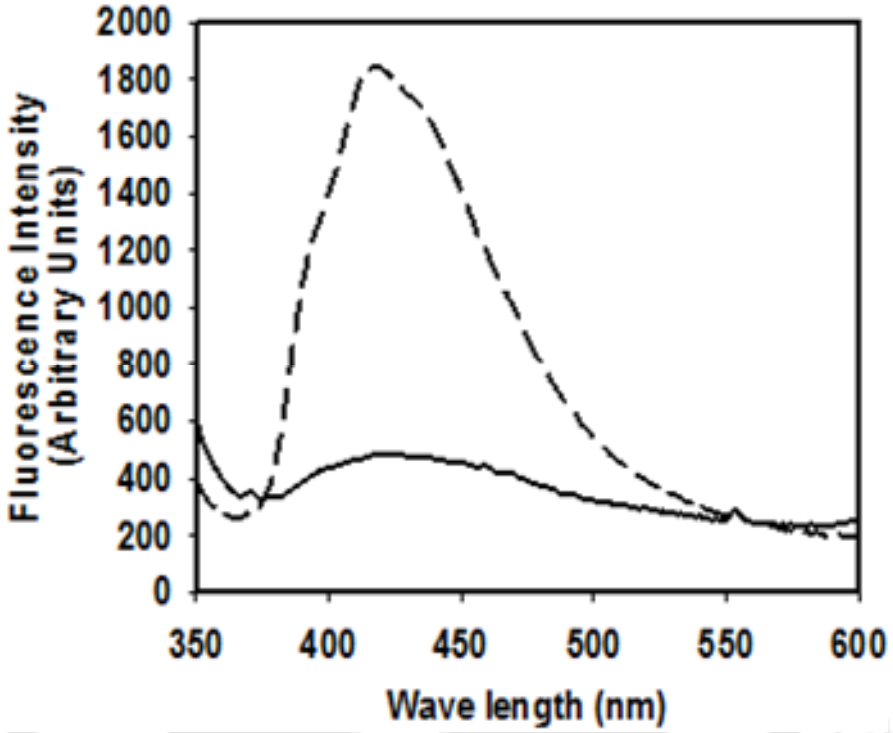

Fig. 4.3.9. Dextransucrase enzyme (0.44 mg/ml; 20 U/mg) in 20 mM sodium acetate buffer pH (5.4) was incubated with 10 mM *o*-phthalaldehyde at 35°C for 60 min. Fluorescence emission spectra of dextransucrase (——) and dextransucrase-*o*-phthalaldehyde complex (— — —) on excitation at 334 nm.

4.3.7 Effect of sucrose and denaturants on binding of *o*-phthalaldehyde with dextransucrase

Dextransucrase on treatment with *o*-phthalaldehyde led to the formation of isoindole derivative which was detected by increase in fluorescence intensity at 418 nm. The enzyme pre treated with EDTA, followed by incubation with sucrose and then with *o*-phthalaldehyde led to decrease in fluorescence intensity as compared with the control (in absence of sucrose) (Fig. 4.3.10). Inactivation of dextransucrase with 5 mM EDTA at 35°C, led to 75% inactivation in 30 min, (Table 4.3.2). Further increase in EDTA significantly decreased the inactivation of enzyme. The addition of 5 mM $CaCl_2$ to EDTA treated enzyme led to reactivation of enzyme with increase in activity

of 46.5% and no further significant increase in the reactivation was observed with increase in the concentration of Ca^{2+} ions (Table 4.3.2). The effect of sucrose on dextransucrase inactivation by *o*-phthalaldehyde is shown in Table 4.3.2. A concentration of 300 mM sucrose solution has provided maximum protection to enzyme with 94.3% of reactivation and 76.5% in case of 150 mM sucrose concentration. In case of control the enzyme pre-treated with EDTA caused inactivation, but it still allowed the binding of *o*-phthalaldehyde with free lysine and cysteine residues that led to the formation of isoindole derivative and thus giving fluorescence intensity (Fig. 4.3.10). Whereas in presence of sucrose blocked the inhibitor *o*-phthalaldehyde from binding to the enzyme which resulted in reduced fluorescence intensity (Fig. 4.3.10). In case of enzyme treated with urea followed by treatment with *o*-phthalaldehyde led to decrease in fluorescence emission, when compared with control indicating that proximal integrity of lysine and cysteine residues at the active site of native enzyme is essential for isoindole complex formation (Goyal and Katiyar 1998b).

Table 4.3.2. Effect of substrate sucrose on the inactivation of dextransucrase by *o*-phthalaldehyde. The reagents shown in the table were incubated with 0.44 mg protein/ ml of dextransucrase for indicated time period followed by incubation with 10 mM *o*-phthalaldehyde for 60 min. Appropriate controls in each case without *o*-phthalaldehyde were run in parallel.

Reagent	Relative Activity (%)
Control	100
Enzyme + EDTA 5 mM (30 min)	24.9
Enzyme + EDTA 5 mM (30 min) + $CaCl_2$ 5 mM (60 min)	44.5
Enzyme + EDTA 5 mM (30 min) + Sucrose 150 mM (15 min) + $CaCl_2$ 5 mM (60 min) + *o*-phth 10 mM (60 min)	76.5
Enzyme + EDTA 5 mM (30 min) + Sucrose 300 mM (15 min) + $CaCl_2$ 5 mM (60 min) + *o*-phth 10 mM (60 min)	94.3

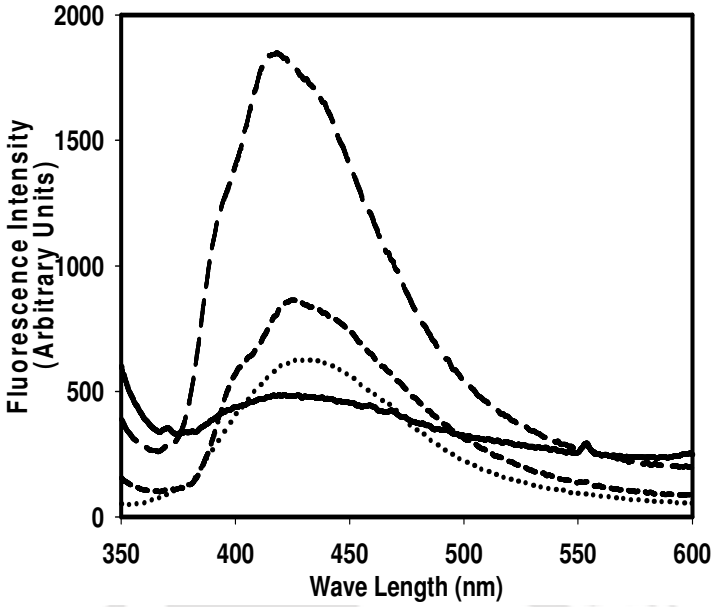

Fig. 4.3.10. Effect of substrate sucrose and denaturants on the binding of *o*-phthalaldehyde with dextransucrase. The enzyme (0.44 mg/ml; 20 U/mg) was pre incubated with 50 mM EDTA for 30 min followed by incubation with 300 mM sucrose for 15 min and finally with 10 mM *o*-phthalaldehyde in 20 mM sodium acetate buffer (pH 5.4) for 30 min at 35°C. The fluorescence spectra of control was run without sucrose (— — —) and with 300 mM sucrose (· · ·) were recorded with excitation wavelength 334 nm. The emission spectra of pure enzyme (—) and the enzyme denaturated by 4M urea (- - -) followed by *o*-phthalaldehyde treatment.

4.3.8 Spectral analysis of modified dextransucrase by *o*-phthalaladehyde in presence and absence of β-mercaptoethanol

Dextransucrase modified by *o*-phthalaladehyde showed a fluorescence emission maxima at 418 nm (λ_{em}) upon excitation at 334 nm was due to the formation of isoindole derivative which involved the participation of proximal thiol and ε-amino groups of cysteine and lysine respectively. The molar transition energy (E_T) was calculated by the following equation (Goyal and Katiyar 1998b).

$$E_T = 2.985\ \lambda_{em} - 1087.28$$

The molar transition energy of dextransucrase and *o*-phthalaladehyde adduct (Fig. 4.3.11) was found to be 160.4 kJ/mol which is close to synthetic isoindole in

dioxane (157.5 kJ/mole) indicating that the microenvironment around lysine and cysteine residues is relatively in hydrophobic environment (Goyal and Katiyar 1998b). Fluorescence emission spectra of enzyme modified with *o*-phthalaladehyde in presence of β-mercaptoethanol showed a shift in peak which was maximum at 457 nm (λ_{em}) upon excitation at 334 nm (Fig. 4.3.11). The E_T was found 276.8 kJ/mole to which is close to isoindole in water (Placzewski 1983) indicating that the isoindole derivatives are relatively in hydrophilic environment.

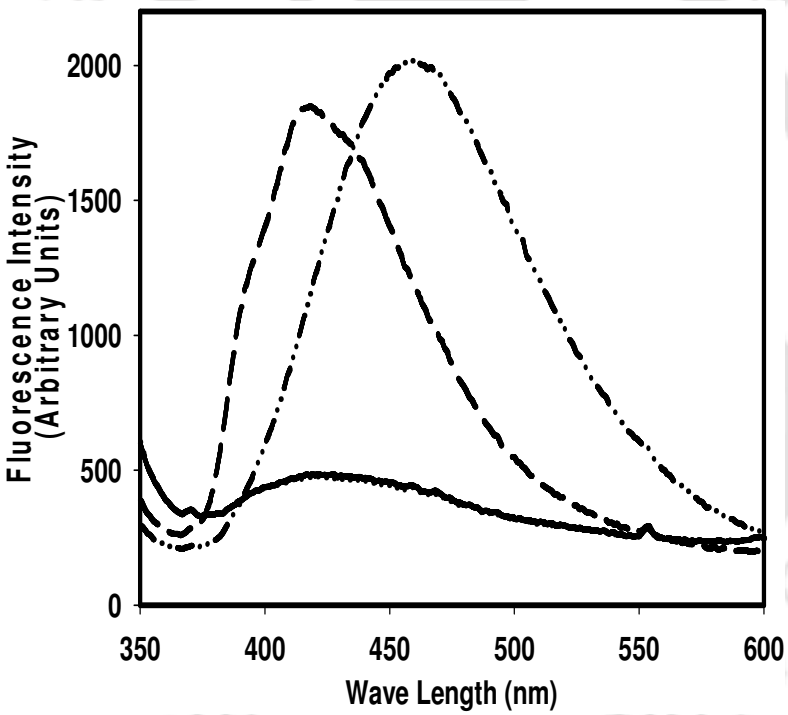

Fig. 4.3.11. Fluorescence emission spectra of dextransucrase-*o*-phthaladehyde adduct in the presence and absence of β-mercaptoethanol. The enzyme (0.44 mg/ml; 20 U/mg) in 20 mM sodium acetate buffer pH (5.4) was incubated with 10 mM *o*-phthalaldehyde for 30 min at 35°C. In another set of experiment the enzyme was pre incubated with 10 mM β-mercaptoethanol was treated with 10 mM *o*-phthaladehyde. The resulting isoindole derivatives were characterized by fluorescence emission spectrum in the presence (— ·· — ·· —) and absence of β-mercaptoethanol (— — —) with excitation wavelength of 334 nm. The emission spectra of pure enzyme (—) were recorded with excitation wave length of 334 nm.

4.3.9 Effect of PLP pretreated dextransucrase on fluorescence emission spectra of dextransucrase-*o*-phthalaldehyde adduct

Dextransucrase pre-incubated with 25 mM PLP followed by the treatment with *o*-phthalaldehyde before dialysis did not showed any fluorescence. This happened because the active site of enzyme was blocked by PLP and there was no free lysine available to the *o*-phthalaladehyde to bind and to form isoindole derivative. But after dialysis there was increase in the fluorescence intensity at 418 nm as shown in Fig. 4.3.12. PLP being a reversible inhibitor gets dissociated from dextransucrase during dialysis leaving behind the active enzyme thus providing the chance to unbound *o*-phthalaldehyde to interact with the lysine and cysteine residues to form isoindole derivative which led the enhanced fluorescence intensity (Fig. 4.3.12).

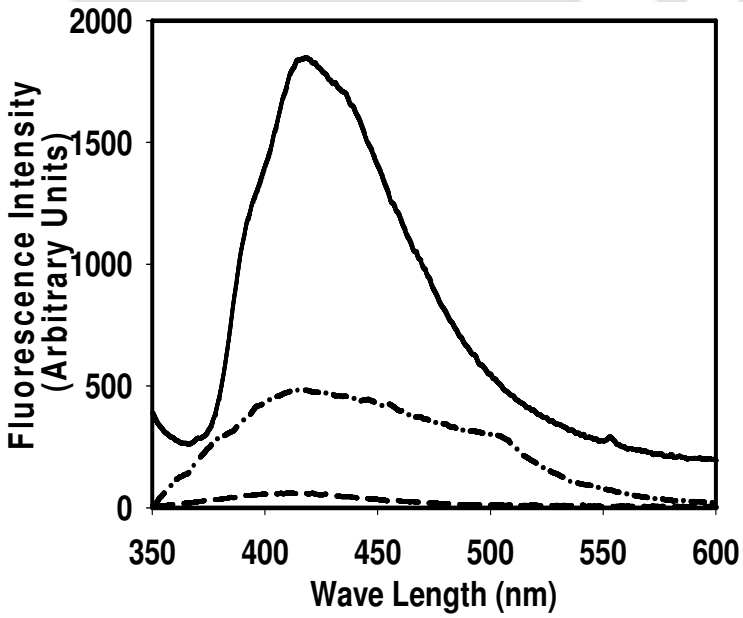

Fig. 4.3.12 Effect of PLP pre treatment of dextransucrase on fluorescence emission spectra of dextransucrase-*o*-phthalaldehyde adduct. The enzyme (0.44 mg/ml; 20 U/mg) in 20 mM sodium acetate buffer pH (5.4) was incubated with 25 mM PLP for 1 h followed by treatment with 10 mM *o*-phthalaldehyde for 30 min at 35°C. The fluorescence emission spectra were recorded with excitation wavelength, before dialysis (— — —) and after dialysis (— · — · —). A control (—) with the enzyme treated with 10 mM *o*-phthalaldehyde for 30 min without PLP treatment was also run.

In the case of control the enzyme when was treated only with *o*-phthalaldehyde exhibited maximum fluorescence emission at 418 nm when excited at 334 nm (Fig. 4.3.12). This indicated that PLP and *o*-phthalaldehyde are binding to the same lysine residues at the active site of dextransucrase.

4.4 Conclusions

Dextransucrase of *Weissella cibaria* JAG8 was strongly inhibited by both lysine specific inhibitors *viz.* PLP and TNBS confirming that lysine residue is essential for the activity of the enzyme. 25 mM PLP resulted gave 98.5% inhibition of enzyme activity and led to the formation of ε-amino-PLP complex, which gave absorbance maxima at 325 nm. PLP was the only reversible inhibitor among the reagents used, where the activity of enzyme could be regained by dialysis. However, the inactivation could be made irreversible on reduction with sodium borohydride to give N^{ε}-phosphopyridoxyl lysine complex that showed fluorescence maxima at 397 nm when exited at 325 nm. The substrate sucrose protected dextransucrase against PLP inactivation indicating the presence of lysine residues at active site of enzyme. In case of TNBS 25 mM concentration gave 98.7% inhibition in enzyme activity and led to the formation of ε-amino-TNP complex, which gave absorbance maxima at 369 nm.

The essential cysteine residue present at or near the active site of dextransucrase from *Weissella cibaria* JAG8 was discovered by cysteine specific reagents *viz*. DTNB and iodoacetic acid. 10 mM concentration of DTNB caused 98.7% inactivation of enzyme and led to the formation of thionitrobenzoate complex with absorbance maxima at 406 nm. In case of iodoacetic acid 98.9% inhibition of enzyme activity was obtained and the reaction led to the formation of thioacetate complex with absorbance maxima at 323 nm**.** All these results showed that one or more cysteine residues are present at or near the active site and are essential for enzyme activity. Cysteine residue was reported for the first time at the active site of dextransucrase from *Weissella cibaria* JAG8.

The presence of lysine and cysteine residues at the active site of dextransucrase was further confirmed by treating the enzyme by bifunctional reagent *o*-phthalaldehyde. It was observed that 10 mM concentration of *o*-phthalaldehyde gave 97% of inhibition in enzyme activity and led to the formation of isoindole derivative with absorbance maxima at 334 nm and fluorescence maxima at 418 nm. The fluorescence study of *o*-phthalaldehyde reaction with dextransucrase indicated the hydrophobic environment at the active site. The substrate sucrose protected the enzyme against *o*-pthalaldehyde inactivation confirming that both lysine and cysteine residues are present at the active site. It was observed that lysine residues modified in presence of β-mercaptoethanol do not play any role in the enzyme inactivation. Dextransucrase pretreated with PLP followed by *o*-phthalaldehyde incubation showed that both these inhibitors are binding to same specific lysine residues that are essential for the enzyme activity. From the above results it was confirmed that essential lysine and cysteine residues are present at or near the active site of enzyme. However, the present study does not elucidate whether these residues are directly associated with catalysis or with binding of substrate or with maintaining the conformational state of the active site of the enzyme.

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Chapter 5

Synthesis, purification and characterization of dextran from *Weissella cibaria* JAG8

5.1 Introduction

Dextran is a generic term given to a group of bacterial polysaccharides and are synthesized by the enzymatic hydrolysis of sucrose by dextransucrases (E.C 2.4.1.5) belonging to family 70 of glycoside hydrolases (Henrissat and Davies 1997). These dextransucrases produce three types of exopolysaccharides based on the linking pattern. They are dextran, alternan and mutan with a general formula of $C_n (H_2O)_{n-1}$, where "*n*" is usually a large number varying from 200-2500. The dextran is homopolysaccharide mainly composed of chains of D-glucose units linked mainly by α(1→6) bonds forming the linear part of the molecule and the branches result from α(1→3), α(1→2) or α(1→4) bonds (Sidebotham 1974). Alternansucrase synthesizes alternan which contains alternating α(1→6) and α(1→3) glucosidic linkages with some degree of α(1→3) branchings (Jeanes *et al*., 1954; Cote and Robyt 1982).

Mutan is a type of insoluble glucan having more than 50% α(1→3) linkages (Sidebotham 1974).

Dextrans have prolific usage in food, clinical, fine chemicals, cosmetics and agricultural industries (Patel *et al*., 2010). Dextran has been investigated 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 (Majumder and Goyal 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). It was reported that Celiac disease is a major health problem affecting around 1% of population in western world (Mustalahti *et al*., 2010). 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 bakers 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). Considering the immense industrial and pharmaceutical applications of dextrans, their production, purification, structural and functional study are of great interest. In this regard, the physico-chemical attributes and application potentials of dextran from a isolate *Weissella cibaria* JAG8 was reported by Tingirikari and Goyal (2013). The present study describes the synthesis, purification, molecular weight determination and structure characterization of dextran from *Weissella cibaria* JAG8 by SEM, FT-IR, ^{1}H and ^{13}C NMR analyses.

5.2 Materials and Methods

5.2.1 Chemicals and reagents

Standard dextran with molecular weight of 2000 kDa, 200 kDa, 70 kDa, bichinconinic acid and serine was purchased from Sigma Chemicals Co., USA. Sephacryl S-500HR matrix was from (Sigma Chemical Co., USA). Absolute ethanol, copper sulphate, sodium carbonate and sodium bicarbonate were from Merck, Pvt. Ltd., Germany. All the media components for culturing the bacteria are purchased from Himedia Pvt. Ltd., India.

5.2.2 Microorganism and culturing condition

Natural isolate *Weissella cibaria* JAG8, isolated from peal of apple as described in Chapter 2, Section 2.2.2 was used for dextransucrase production. *Weissella cibaria* JAG8 is a micro-aerophilic lactic acid bacterium growing at 24°C. A loopful of the culture was transferred to modified MRS agar medium as stabs (Goyal and Katiyar 1995), grown at 28°C for 16h and stored at 4°C and sub-cultured every 15 days.

5.2.3 Enzymatic synthesis of dextran

The 33.0% (v/v) PEG-400 purified dextransucrase produced by the natural isolate of *Weissella cibaria* JAG8 as described in Chapter 3, Section 3.2.5 was used for dextran synthesis. For dextran synthesis, 1.0 ml of enzyme (0.44 mg protein/ml of specific activity 20.0 U/mg) was incubated in 10 ml of 20 mM sodium acetate (pH 5.4) containing 5% sucrose, 0.3 mM $CaCl_2$ and 15 mM sodium azide. The reaction mixture was incubated at 28°C for 24h.

5.2.4 Purification of dextran from *Weissella cibaria* JAG8

The polymeric mass of dextran produced by enzymatic treatment was centrifuged at 17,200*g* for 20 min. The reducing sugar fructose and the left over sucrose was discarded by washing three times with 3 volumes of ethanol (100%, v/v) and the pellet was finally resuspended in 2 ml of deionized water. The jelly-like mass was frozen at -20°C. The solidified sample was then freeze dried using a lyophilizer (Christ GmbH, model ALPHA 1-4 LD) at -51°C at a vacuum pressure of $35x10^{-3}$ mbar for 24h. The sample was stored at 4ºC for further physical, spectroscopic, microscopic and rheological characterization.

5.2.5 Molecular mass distribution of dextran by gel filtration using Sephacryl S-500HR

The average molecular weight of dextran was determined by fast protein liquid chromatography (FPLC, Akta Prime, GE Healthcare). 3 ml of purified dextran (2 mg/ml) was applied to column (40 x 16 cm) and 40 ml volume packed with Sephacryl S-500HR. The matrix was equilibrated with degassed milli-Q water (18 MΩ). 50 fractions of 3 ml each were at a flow rate of 1 ml/min at room temeprature. Dextran 70 kDa, 200 kDa and 2000 kDa of 2 mg/ml concentration were used as standards and run under similar conditions as mentioned above (Sarwat *et al*., 2008). The eluted fractions were analyzed for total carbohydrate (dextran) content using Phenol-Sulphuric acid method as described in Chapter 2, Section 2.2.12.

5.2.6 Determination of number average molecular weight (MWn) and degree of polymerization (Dpn) of dextran by Bichinconinate (BCA)method

The number average molecular weight (MWn) and degree of polymerization (DPn) of the dextran were determined by the measurement of the reducing

value using the copper bicinchoninate method as described by Vettori *et al*., (2012) and the measurement of total carbohydrates using the phenol-sulfuric acid method (Fox and Robyt 1991) as described in Chapter 2, Section 2.2.12.

$$\text{Degree of polymerization (DPn)} = \frac{\text{(total carbohydrate in µg of D-glucose)}}{\text{(reducing value in µg of maltose)}} \times 1.9$$

$$MWn = [(DPn) \times 162] + 18.$$

BCA-A and BCA-B reagents were dissolved in 1:1 ratio to form reagent C. 100 µl of reagent C and 100 µl of sample were added to the well in ELISA titre plate. The plate was covered with cling film and was incubated at 80°C in water bath for 45 min. Read the absorbance at 560 nm. Maltose was used as standard (2-20 µg/ml).

BCA-A	**in 50 ml**
Na_2CO_3	2.713 g
$NaHCO_3$	1.209 g
Bichinconinic acid	0.1015 g
BCA-B	
$CuSO_4$	62.4 mg
Serine	63.05 mg

Note: Place the reagent in amber colour bottle.

5.2.7 Spectroscopic analyses of dextran

5.2.7.1 *FT-IR spectrum of dextran from Weissella cibaria JAG8*

The polysaccharide dextran produced from the isolate was characterized using FT-IR spectrophotometer (Perkin-Elmer Instruments, model Spectrum One FT-IR Spectrometer, California, USA). The dried powder of dextran polymer (5 mg) was

grinded with potassium bromide (KBr) powder and pressed into pellets. The FT-IR analysis was carried out in the frequency range of 4000-400 cm^{-1} with 20 scans per min.

5.2.7.2 *NMR analyses of dextran from Weissella cibaria JAG8*

The NMR spectroscopic analysis of dextran was conducted using NMR spectrometer (Varian, Model AS400, California, USA) 400 MHz equipped with VnmrX for Sun Microsystems Ver. 6.1 software. NMR analyses helps in finding out the type of glycosidic linkage. The purified dextran was dissolved in D_2O to a final concentration of 5 (mg/ml) for ^{1}H NMR analysis at operating frequency of 400 MHz and to a final concentration of 20 (mg/ml) for C^{13} NMR at operating frequency of 100 MHz.

5.2.8 Monosaccharide analysis of dextran from *Weissella cibaria* JAG8

1 ml of dextran 2 (mg/ml) was hydrolysed by using 1 ml of 2M Trifluoroacetic acid (TFA) at 100°C for 4 h. The sample mixture was passed through 0.2 µm membrane filter. The released monosaccharide was analyzed by High Performance Anion Exchange Chromatography (HPAEC) using ion chrome system (ICS 3000 system, Dionex corporation, USA) using a Carbo-Pac P20 Column (15 x 0.3 cm) by isocratic elution using 0.1 N NaOH at a constant flow rate of 0.5 (ml/min) at 30°C. The monosaccharide detection was carried out with in Pulse Amperometric Detection (PAD) by detector ED50. Glucose and fructose (100 µg/ml) were used as standards.

5.2.9 Scanning electron microscopic analysis of dextran from *Weissella cibaria* JAG8

1 mg of lyophilized dextran was applied to the SEM stub by means of an adhesive tape and coated with 10 nm Au in a sputter coater (Leo, model SCH 620). The surface of the sample was viewed in Scanning Electron Microscope (Leo, model 1330 VP) at magnification of 1.05 kx operated at 10.0 kV.

5.2.10 Rheological analysis of dextran from *Weissella cibaria* JAG8

The change in steady shear viscosity and shear stress with change in shear rate were measured using the viscous colloid of dextrans (5 mg/ml) at 25°C by rheometer (Thermo Electron, model Haake rheostress RSI) interfaced with a HAAKE RheoWin 323 software. The applied shear rate was in the range of 0.05-500 s^{-1}. The experiment was carried out in duplicate to ensure accuracy of results.

5.3 Result and Discussion

5.3.1 Partial purification of dextran from *Weissella cibaria* JAG8

Enzymatically synthesized dextran was purified by precipitation using ice cold absolute ethanol in 3 cycles as described in Section 5.2.3. The purified dextran was lyophilized as white flakes and was used for structural studies.

5.3.2 Determination of molecular mass of dextran from *Weissella cibaria* JAG8 by gel filtration using Sephacryl S-500HR

The dextran from *Weissella cibaria* JAG8 was purified by gel filtration using Sephacryl S-500HR. The fractions (3 ml) were collected at a flow rate of 1 ml/min at room temperature. It was observed that dextran started eluting from fraction no. 16 and maximum concentration of dextran eluted at fraction no. 19, (i.e.) 57 ml (19 x 3 ml). The standard dextran 2000 kDa started eluting from fraction no. 12 and the maximum concentration was observed at fraction no. 15, (i.e.) at 45 ml (15 x 3 ml). While, 200 kDa standard dextran started eluting from fraction no. 21 and the peak was observed at fraction no. 25, (i.e.) 75 ml (25 x 3 ml) and 70 kDa was eluted from fraction no. 27 and peak was observed at fraction no. 29, (i.e.) 87 ml (29 x 3 ml). The elution profile of dextran from *Weissella cibaria* JAG8 and standard dextrans are displayed in Fig. 5.3.1. The results clearly suggested that dextran produced by *Weissella cibaria* JAG8 is of high molecular weight. Molecular mass of dextran produced from *Weissella cibaria* JAG8 was eluted earlier than standard dextran 200 kDa and 70 kDa indicating that its molecular mass is more than 200 kDa and less than 2000 kDa. Based on elution profile of dextran from *Weissella cibaria* JAG8 that molecular weight was approximately, in the range of 500-1000 kDa. The average

molecular weight was confirmed further by bichinconinate method as described in Section 5.3.3.

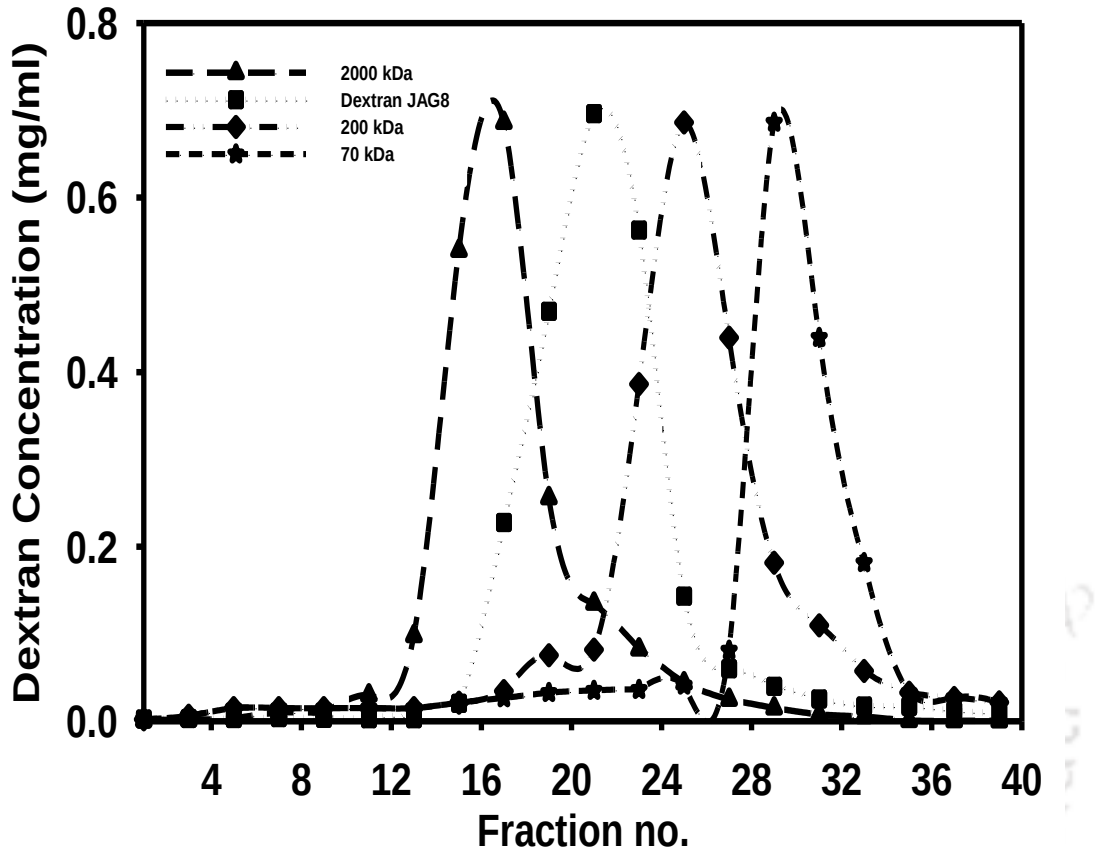

Fig. 5.3.1 Gel filtration of dextran from *Weissella cibaria* JAG8 using Sephacryl S-500HR matrix.

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5.3.3 Determination of number average molecular weight (MWn) and degree of polymerization (Dpn) of dextran by Bichinconinate (BCA) method

The molecular weight of dextran from *Weissella cibaria* JAG8 was further confirmed by BCA method and it was found to be approximately, 800 kDa. The molecular mass determined by gel filtration and BCA method were in agreement. Dextran eluted in the fractions from 16-26 were pooled after determining the total carbohydrate content by phenol sulphuric acid method and was subjected for freezing at -20°C for 3-4 h and finally lyophilized to powder.

5.3.4 Structure analysis of dextran *Weissella cibaria JAG8* by spectroscopy

5.3.4.1 *FT-IR Spectroscopic analysis of dextran*

The FT-IR spectrum of dextran provided information on the functional groups, monomeric units and linkages present in dextran. Generally carbohydrates show high absorbance in the region of 1200-950 cm^{-1}. The bands obtained in the FT-IR spectrum of dextran are shown in Table 5.3.1 and Fig. 5.3.2. The band in the region 2447 cm^{-1} indicated C-H stretching vibration and the band in the 1638 cm^{-1} was due to carboxyl group stretching and the results were in agreement with the earlier report of Liu *et al*., (2007). The absorption peaks at 865 and 920 cm^{-1} indicated the existence of α-glycosidic bond and the results were in accordance with the earlier report of Majumder *et al*., (2009). The absorption at 1084 and 988 cm^{-1} indicated C-O, C-C bond and deformational vibration of CCH, COH and HCO bonds. The band at 1084 cm^{-1} was assigned to be valent vibrations of C-O-C bond and glycosidic bond as reported earlier by Purama *et al*., (2009). The band at 988 cm^{-1} in polysaccharide indicated α(1→6) linkage and can be considered as the characteristic for the type of inter-unit link as also reported by Shingel (2002). The band obtained in the region of 3402 cm^{-1} was due to the hydroxyl stretching vibration of the polysaccharide as also reported by Purama *et al*., (2009).

Table 5.3.1 Characterization of functional groups present in dextran by FT-IR.

Wave length (cm^{-1})	**Functional Group**
3402	Represents hydroxyl stretching vibration.
2447	C-H group stretching vibration.
1638	Carboxyl group.
1401, 1306	C=C (Aromatic/Cyclic) group.
1084, 988	C-O, C-C bond and deformational vibration of CCH, COH and HCO bonds.
920, 866	α-Glycosidic linkage.
540	C-Br stretching vibrations.

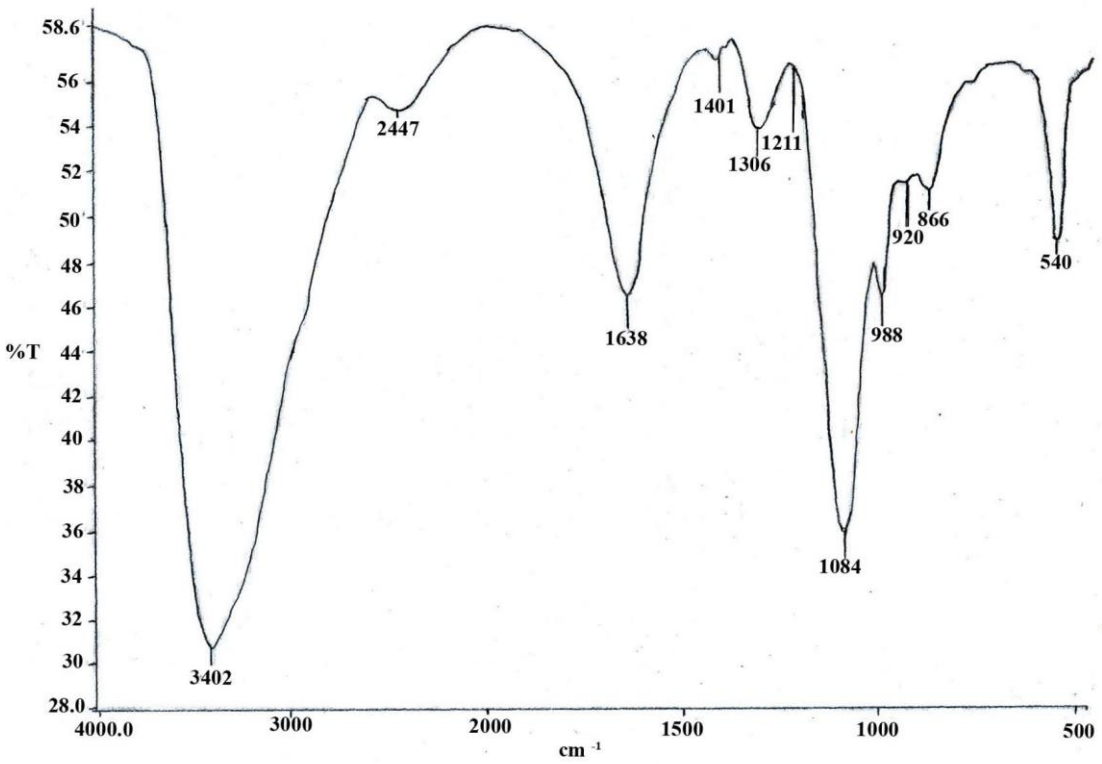

Fig. 5.3.2 FT-IR spectrum of dextran produced from the purified dextransucrase of isolate *Weissella cibaria* JAG8.

5.3.4.2 *^{1}H NMR spectroscopic analysis of dextran*

The structural characterization of dextran was performed by NMR spectroscopic analysis. The ^{1}H data is shown in Fig. 5.3.3 and Table 5.3.2. Based on the data obtained from ^{1}H NMR, the anomeric α(1→6) proton appeared at 4.96 ppm and a low intensity peak at 5.3 ppm indicated the presence of α(1→3) branching (Maina *et al*., 2008). No peaks were detected in the range 4.9-5.3 ppm indicating the presence of no branching other than α(1→3) as shown in Fig. 5.3.3. It was reported earlier that the dextran produced from *Weissella* species such as *W. cibaria* and *W. confusa* are linear in nature with lower percentage (2.4% to 3.4%) of α(1→3) branching (Maina *et al*., 2008; Bounaix *et al*., 2009 and Rifat *et al*., 2012). Based on integration analysis of ^{1}H NMR, it was observed that *Weissella cibaria* JAG8 produces dextran with 93.0% of

α(1→6) and 7.0% of α(1→3) branching with average chain length of 14 glucose units between branched linkages. There are 69 branch linkages for every 1000 glucose units in case of dextran produced from *Weissella cibaria* JAG8 as analysed based on the report of Vettori *et al*., (2012). Dextran from *Weissella cibaria* JAG8 has 2-fold more α(1→3) branching than the dextran from *W. cibaria* CMGDEX3 (Rifat *et al*., 2012). The branched dextrans are resistant to enzyme hydrolysis by exodextranases and glucosidases indicating its applications for the production of prebiotic oligosaccharides (Remaud-Simeon *et al*., 2000). This is the first report on *Weissella cibaria* JAG8 with 7.0% of α (1→3) branching thus giving a different structure and characteristic feature to the dextran.

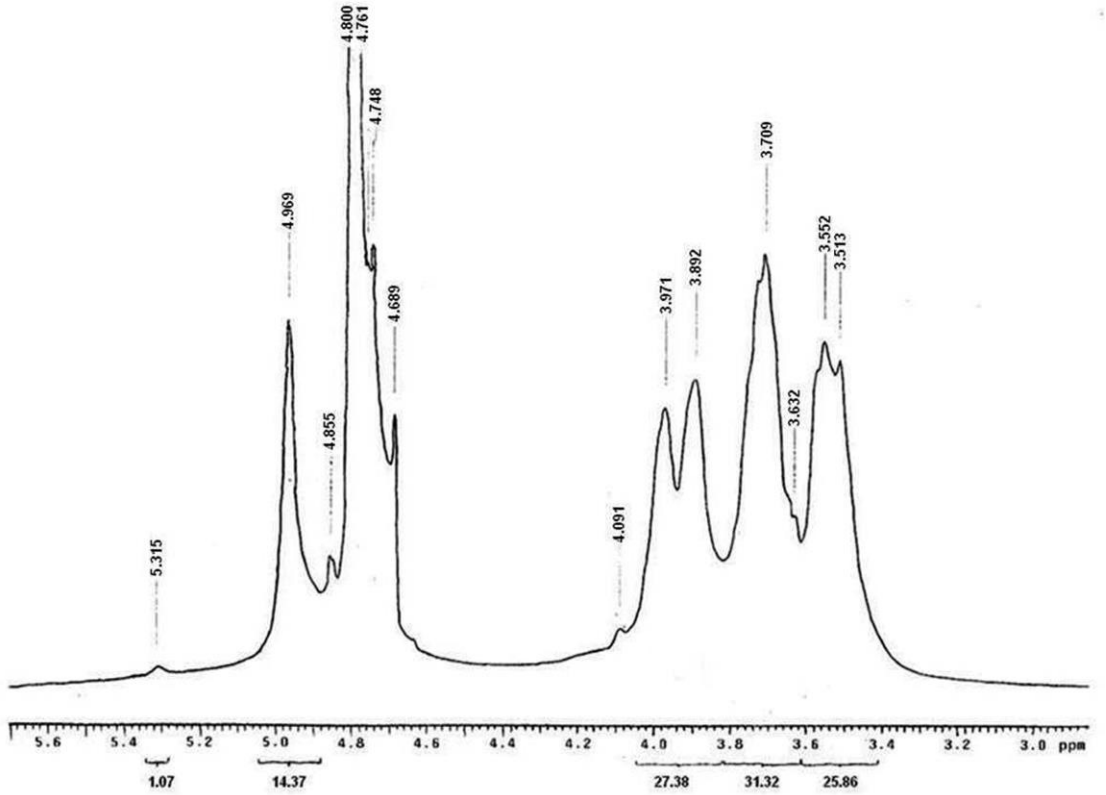

Fig. 5.3.3 ^{1}H NMR (400 MHz, D_2O) spectrum of dextran produced by the purified dextransucrase from *Weissella cibaria* JAG8.

5.3.4.3 *^{13}C NMR analysis of dextran*

The structure of dextran was further confirmed by 100 MHz C^{13} NMR. The resonances at 97.832, 71.543, 73.541, 69.652, 70.315 and 65.655 ppm were obtained, which are the characteristic peaks of linear dextran as reported by Seymour (1979a) and Uzochukwu *et al*., (2002). The C^{13} NMR spectra displayed two prominent regions (a) the 95-105 ppm region, which is the anomeric region and (b) the 75 to 85 ppm for dextran branched at C-2, C-3 (or) C-4. C^{13} NMR resonances with in the 70-75 ppm region are associated with free positions at C-2, C-3 and C-4 residues. As no additional peaks were observed in the 75-85 ppm region indicated the absence of branched linkages (Seymour 1979a). The major resonance in the anomeric region occurs generally at 98.7 (97.8) showing the C-1 is linked. An equal intensity peak at 66.5 (65.6 ppm), indicated that the most of the C-6 are also linked as reported by Uzochukwu *et al*., (2002). The equal peak intensity at 97.8 and 65.6 ppm confirmed that glucose residues in dextran are linked by α(1→6) glycosidic bond and no peaks at 75-85 ppm confirmed the linear nature of dextran from *Weissella cibaria* JAG8 as reported in *Weissella cibaria* CMU (Kang *et al*., 2006). The resonance data of ^{13}C NMR is represented in Table 5.3.2 and Fig 5.3.4.

Table 5.3.2 ^{1}H and ^{13}C NMR chemical shift of dextran from *W. cibaria* JAG8

Atoms	H1/C1	H2/C2	H3/C3	H4/C4	H5/C5	H6/C6
^{1}H	4.96	3.55	3.70	3.51	3.89	3.97
^{13}C	97.83	71.54	73.54	69.65	70.31	65.65

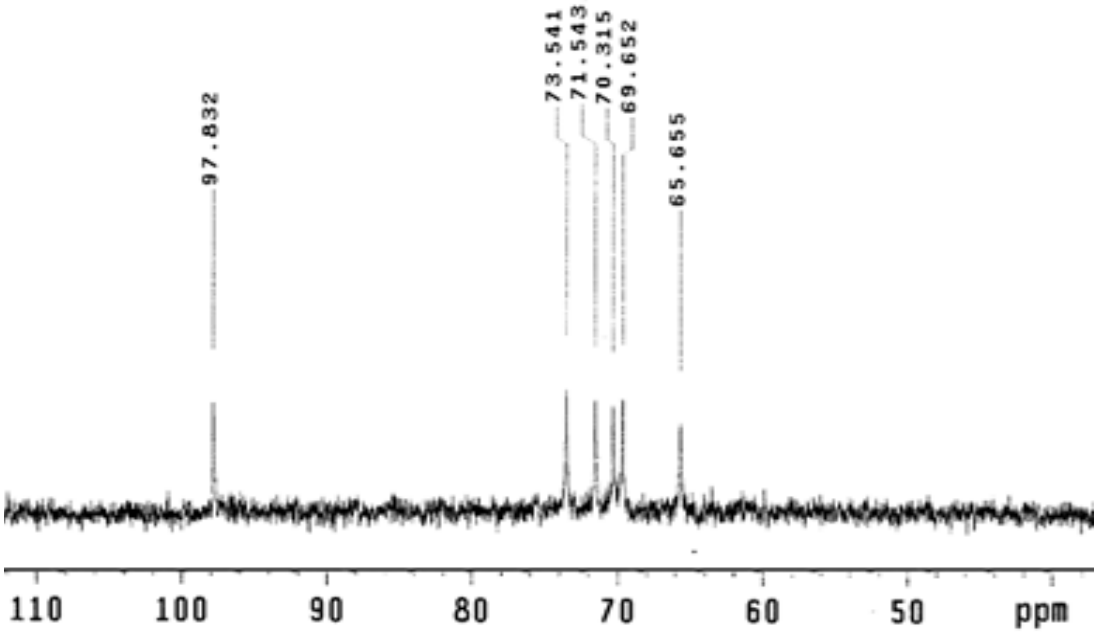

Fig. 5.3.4 ^{13}C NMR (100 MHz, D_2O) spectrum of dextran produced by the purified dextransucrase from *Weissella cibaria* JAG8.

5.3.5 Monosaccharide analysis of dextran from *Weissella cibaria* JAG8

The monosaccharide analysis of polysaccharide from *Weissella cibaria* JAG8 by HPAEC showed that the retention time of polymer was observed at 4.28 min as shown in Fig 5.3.5C. The retention time for standard glucose was observed to be at 4.22 min as shown in Fig 5.3.5B. In case of fructose the retention time was 5.4 min as shown in Fig 5.3.5A. From the above analysis it was confirmed that the retention time of polymer produced from *Weissella cibaria* JAG8 was close to that of standard glucose indicated that the polymer is comprised of only glucose units confirming the dextran nature of the polymer (Wang *et al*., 2012).

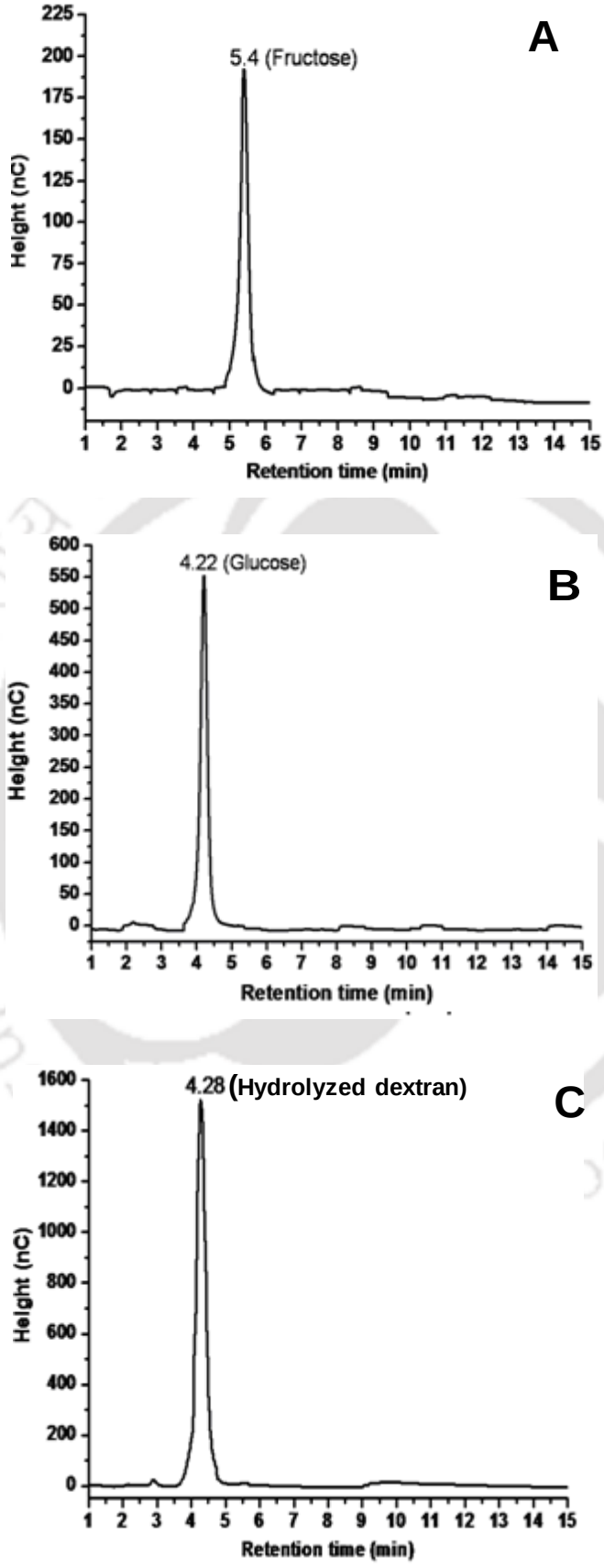

Fig 5.3.5 Monosaccharide analysis of dextran from *Weissella cibaria* JAG8.

5.3.6 Scanning electron microscopic analysis of dextran from *Weissella cibaria* JAG8

The surface morphology dextran of *Weissella cibaria* JAG8 was studied by scanning electron microscopy at various magnifications. Numerous pores were observed in the reticular surface of dextran (Fig. 5.3.6). The porous structure revealed high water holding capacity and consequently can have potential applications in the food industry as additives. From the porous dextrans, hydrogels can be created by either physical or chemical crosslinking by taking the advantage of the abundant hydroxyl groups present on the α(1→6) linked D-glucose residues (Levesque *et al*., 2005).

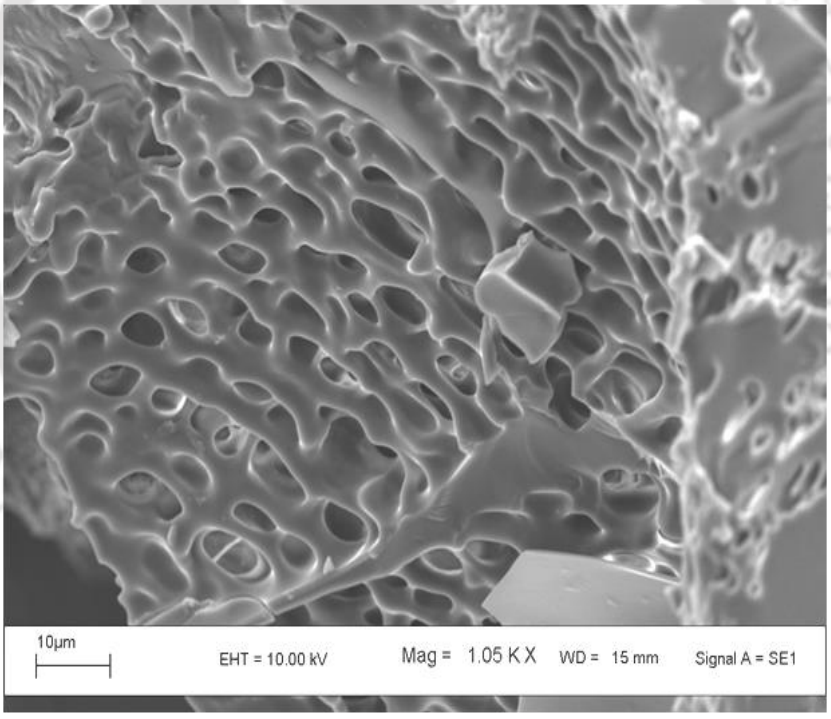

Fig. 5.3.6 Scanning Electron Micrograph of dextran from *W. cibaria* JAG8 at magnification 1.05Kx showing the porous reticular surface morphology.

Hydrogels composed of hydrophilic polymeric networks can absorb considerable amount of water and exhibit compatibility with proteins and living

tissues (Hoffman 2002). Hydrogels designed for tissue scaffolds should also contain pores large enough to allow the migration, penetration and proliferation of living cells into the wound bed (Maire *et al*., 2005). The key factors controlling the pore size, volume fraction and the interconnections between the polymer network are the composition of the chains and crosslink density (Hoffman 2002). Polysaccharides are widely used in foods as thickening, gelling, stabilizing, emulsifying and water-binding agents (Khan *et al*., 2007).

5.3.7 Rheological property of dextran from *Weissella cibaria* JAG8

On analysis of the experimental data obtained from the influence of shear rate on the apparent viscosity and shear stress, the results showed that the viscosity is inversely proportional to the shear rate and the semi liquid dextran exhibited a typical non-Newtonian behavior (Fig. 5.3.7).

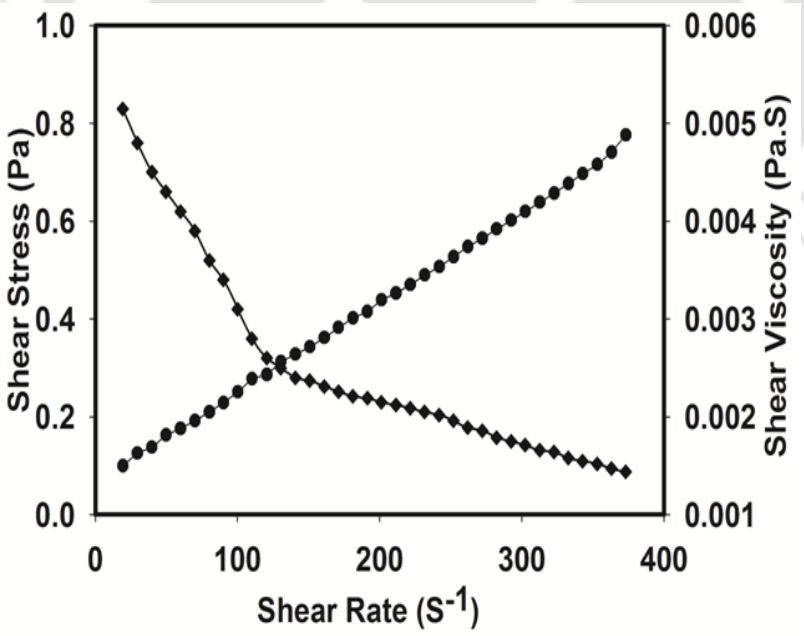

Fig. 5.3.7. Correlation of shear viscosity (■) and shear stress (●) of dextran from *Weissella cibaria* JAG8 with shear rate.

This is a typical characteristic of pseudoplastic fluid showing a shear thinning behavior, normally expected in polymer solutions, where the fluid molecular weight is high. In these systems, as the shear rate increases, the stress also increases, but its dependence on shear rate is less than linear; therefore viscosity decreases (Padmanabhan *et al*., 2003). The non-Newtonian fluids formed by the polymers are employed in the food industry as gelling, stabilizers or thickening agents (Feddersen and Thorp 1993).

5.4 Conclusions

Dextran was synthesized by *Weissella cibaria* JAG8 dextransucrase (20 U/mg) purified by PEG-400. The enzymatically synthesized dextran was purified by alcohol precipitation. The partially purified dextran was analyzed for molecular mass by gel filtration using Sephacryl S-500HR matrix. The average molecular mass of dextran from *Weissella cibaria* JAG8 determined by gel filtration and BCA method was approximately, 800 kDa.

FT-IR analysis of dextran of *Weissella cibaria* JAG8 revealed the functional groups present and the peaks at 913 cm^{-1} and 857 cm^{-1} indicated the α-glycosidic bond and pyranose ring respectively. The peaks at 1013 cm^{-1} and 1157 cm^{-1} indicated the -C-O-, -C-C- and -C-O-H stretching vibrations.

The proton NMR analysis revealed a peak near 5.33 ppm which signifies α(1→3) branching and peak at 4.96 ppm confers the α(1→6) proton. Based on integration analysis it was observed that dextran from *W. cibaria* JAG8 has 93.0% of linear α(1→6) linkage and 7.0% of α(1→3) branched linkages. The equal peak intensity at 97.8 and 65.6 ppm confirmed that glucose residues in dextran are linked by α(1→6) glycosidic bond.

Monosaccharide analysis of dextran confirmed that the polymer comprised of only glucose units which confirmed that it is dextran. The surface morphology of dextran was observed by scanning electron micrograph, which revealed its network like highly porous structure. The rheological studies revealed that viscosity of dextran from *Weissella cibaria* JAG8 decreased with increase in shear rate which indicated a typical non-Newtonian hydrocolloidal nature. The low branched nature of the dextran from *Weissella cibaria* JAG8 can be exploited as starter culture in sourdough fermentation or the dextran produced by this bacterium can be exploited as food

additive by adding directly to the sourdough for generating cost effective gluten-free food products with significant amount of putative prebiotic gluco-oligosaccharides and as stabilizing agent in pharmaceutical and cosmetics industries emphasizes the importance of exploration of the new strains and characterization of their traits. Thus the bacterium *Weissella cibaria* JAG8 can be a potential candidate for cereal food products.

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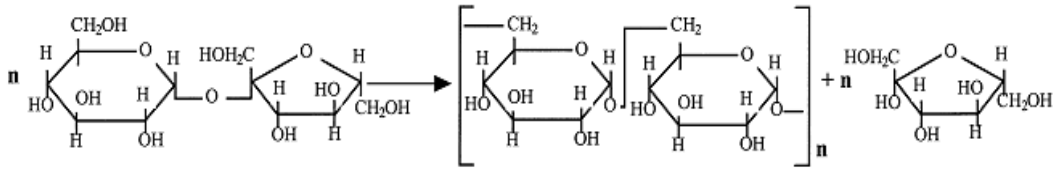
Chapter 6

In vitro studies on dextran produced from *Weissella cibaria* JAG8 as prebiotic and food additive

6.1 Introduction

Dextransucrase (E.C. 2.4.1.5) are large extracellular enzymes capable of synthesizing various exopolysaccharides using sucrose as substrate. Exopolysaccharides (EPSs) are long-chain, high-molecular-mass polymers that dissolve or disperse in water to give thickening or gelling properties in food product formulation (Khan *et al*., 2007). Microbial exopolysaccharides are extracellular polysaccharides which are either associated with the cell surface in the form of capsules or secreted into the extracellular environment in the form of slime (Sutherland 1972). EPSs occur widely among bacteria (Sutherland 1977; 1982; 1985; 1990). EPSs in their natural environment are considered to play an important role in the protection of the microbial cell against desiccation, phagocytosis and phage attack, antibiotics or toxic compounds (e.g. toxic metal ions, sulfur dioxide, ethanol), predation by protozoans, osmotic stress, adhesion to solid surfaces and biofilm formation, and also in cellular recognition (e.g. via binding to a lectin), (De Vuyst and Degeest 1999). The health-promoting effect of EPSs include antitumor, anti-

ulcer and cholesterol-lowering activities, has resulted to their valuable ingredients for application in food industry (Dalbello *et al*., 2001; Kitazawa *et al*., 1991; Oda *et al*., 1983; Ruas-Madieido *et al*., 2002). It is not likely that EPS serve as a food reserve, since most slime-forming bacteria are not capable of catabolising the EPS they produce (Cerning 1990).



n sucrose dextran *n* fructose

Lactic acid bacteria (LAB) are classified as generally regarded as safe (GRAS) by US-FDA. LAB produces variety of exopolysaccharides (EPSs) which play an important role in protection against desiccation, toxic compounds, bacteriophages and to permit adhesion to solid surfaces and biofilm formation (De Vuyst and Degeest 1999). Bacterial EPSs exhibited high resistance to human gastrointestinal digestion, and selective enhancement of beneficial bacteria colonized in the colon function in the same way to other prebiotics, non-digestible polysaccharides such as inulin, fructo-oligosaccharides and galacto-oligosaccharides by acting as carbon source acquired by gut bacteria (Topping and Clifton 2001). Many non-digestible polysaccharides have been widely proven to have "prebiotic" potential (Patel and Goyal 2012), meanwhile only a few reports regarding the use of EPSs produced by LAB as prebiotics, (Hongpattarakere *et al*., 2012). Prebiotics are defined as non digestible, fermentable,

foods that beneficially affect the host by selectively stimulating the growth and activity of health-promoting species already residing in the colon like, *lactobacilli* and *bifidobacteria* species (Huebner *et al*., 2007). Meanwhile only a few reports regarding the use of EPSs produced by LAB as prebiotics, apart from other beneficial effects mentioned above. Biodegradability of EPSs differs greatly, hence the diverse variation of their physiological function. One such exopolysaccharides produced by LAB is dextran. 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). Dextran produced from *Weissella cibaria* A2 strain has recently been reported for its prebiotic property (Hongpattarakere *et al*., 2012)

Food ingredients with prebiotic characteristics generally exhibit certain unique characteristics, such as limited hydrolysis and absorption in the upper gastrointestinal tract, selective stimulation of the multiplication of beneficial bacteria in the colon, potential to suppress pathogens and limit virulence by processes such as immunostimulation and the stimulation of the beneficial microflora, which promote resistance to colonization by pathogens (Urgell *et al*., 2005). In the present study the prebiotic potential of dextran from *Weissella cibaria* JAG8 was studied by subjecting it to hydrolysis by gastric juice and α-amylase, by browning reaction, by supplementing medium with dextran for growth of probiotic bacterial strains and by *in vitro* study as food additive.

6.2 Materials and Methods

6.2.1 Chemicals and reagents

Serine, di-sodium phosphate, citric acid, glycine, bichinconinic acid, and α-amylase were purchased from Sigma Chemical Co., USA. Copper sulphate, calcium chloride, hydrochloric acid, sodium chloride, sodium carbonate, sodium bicarbonate, sodium phosphate monobasic, sodium phosphate dibasic, magnesium chloride, L-cysteine-HCl, potassium chloride, sulphuric acid were from Merck, Pvt. Ltd., Germany. Inulin and all the media components, anaero bag system for culturing the bacteria were purchased from Himedia Pvt. Ltd., India. Skimmed milk was purchased from Amul Pvt. Ltd., India.

6.2.2 Determination of the digestibility of dextran from *Weissella cibaria* JAG8 by artificial human gastric juice

Dextran obtained from *Weissella cibaria* JAG8 and inulin as a standard prebiotic were dissolved in milli-Q water to give a 1% (w/v) solution and tested for digestibility by acid according to the method by (Korakli, *et al*., 2002). Artificial human gastric juice was prepared using a hydrochloric acid buffer containing the following in (g/l) NaCl, 8; KCl,0.2; $Na_2HPO_4.2H_2O$, 8.25; $NaHPO_4$, 14.35; $CaCl_2.2H_2O$, 0.1 and $MgCl_2.6H_2O$, 0.18; at pH 1, 2, 3 and 4. The pH of the buffer was adjusted to 1, 2, 3, 4 and 5 using 5 M HCl. The dextran sample (1.0 ml, 1% w/v) was mixed with 1.0 ml of above artificial human gastric juice of all pHs and the reaction mixture was incubated in a water bath at a controlled temperature of 37 ± 1°C for 6 h. 100 µl of the reaction mixture was collected at 0, 0.5, 1, 2, 4 and 6 h to determine the reducing and total sugar. The bichinconinic acid (BCA) method as described in Chapter 5, Section 5.2.6, was used to determine reducing sugar content in the sample

(Vettori *et al*., 2012), and the phenol-sulphuric acid method as described in Chapter 2, Section 2.2.12, was used to determine the total sugar (Fox and Robyt 1991). The percentage hydrolysis of the dextran sample was calculated based on the reducing sugar that liberated and the total sugar content of the sample as described by Korakli *et al*., (2002).

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

6.2.3 Determination of digestibility of dextran from *Weissella cibaria* JAG8 by α-amylase

Dextran from *Weissella cibaria* JAG8 and inulin, as a standard prebiotic, were dissolved in sodium phosphate buffer to give a 1% (w/v) solution and were tested for digestibility by α-amylase according to the method of Wichienchot *et al*., (2010). The enzyme (2 U/ml) was prepared in a solution containing sodium phosphate buffer (20 mM) containing sodium chloride (6.7 mM); in addition, the pH of the buffer was adjusted to pH 5.0 and 7.0. 1 ml of the polysaccharide sample was mixed with 1 ml enzyme solution, and the reaction mixture was incubated in a water bath at a controlled temperature of 37 ± 1°C for 6 h. 100µl of the reaction mixture was collected at 0, 0.5, 1, 2, 4 and 6 h to determine the reducing and total sugar content to calculate the percentage hydrolysis as described previously in Section 6.2.2.

6.2.4 Effect of Maillard reaction on dextran from *Weissella cibaria* JAG8

1% (w/v) of dextran from *Weissella cibaria* JAG8, standard prebiotic inulin and glucose were added to 20 mM citrate-phosphate buffer at pH 7.0 with 1% glycine. The solutions were heated at 85°C in water bath. The samples were removed at 0, 1, 2

and 3 h and the browning of each sample was determined against control glucose by measuring the reducing and total sugar content to calculate the relative browning (Huebner *et al.*, 2007). With progress of time the browning of the polysaccharide increases, and as a result the reducing sugar concentration also increases. The total sugar content was measured by phenol-sulfuric acid method as described in Chapter 2, Section 2.2.12, and the reducing sugar content was measured by bichinconinic acid (BCA) method as described in Chapter 5, Section 5.2.6.

6.2.4.1 *Composition of citrate phosphate buffer*

0.1M Citric acid, 1.921 g in 100 ml (x)

0.2 M Disodium hydrogen phosphate (Na_2HPO_4), 2.8392 g in 100 ml (y)

6.5 ml of (x) + 43.5 ml of (y). Finally make up the volume to 100 ml. Prepare 20 mM concentration of Citrate-Phosphate buffer (pH 7.0) from the above stock solution.

$$\text{Relative browning (\%)} = \frac{\text{Absorbance at 420 nm of prebiotic at time } t_a}{\text{Absorbance at 420 nm of glucose at time } t_b} \times 100$$

6.2.5 Effect of *Weissella cibaria* JAG8 dextran on growth of probiotic bacteria

Growth of probiotic bacteria *Bifidobacterium animalis* sub species *lactis* NRRL B-41405, *Bifidobacterium infantis* NRRL B-41661 and *Lactobacillus acidophilus* NRRL B-4495 were analyzed by culturing 1% (v/v) of above cultures in MRS medium as described in Chapter 2, Section 2.2.1. In the present study MRS medium without carbon source and supplemented with 0.5 g/ 100 ml of L-cysteine-HCL was used for analysis following the method of Huebner *et al*., (2007) and Su *et al*., (2007).

The cysteine acts as oxygen scavenger and maintains low redox potential for making conditions suitable for the extended viability of probiotic bacteria (Talwalkar and Kailasapath 2004). 4 ml of MRS medium described above was autoclaved separately in each test tube and to this 1 ml of 5% (w/v) of autoclaved inulin was added (positive control), as a result the final concentration of inulin is 1% (w/v). Similarly 1 ml of 5% (w/v) of autoclaved dextran was added (test sample), from *Weissella cibaria* JAG8 to the 4 ml of autoclaved MRS medium as described above, to get the final concentration of dextran as 1% (w/v). In case of control, 5 ml of above described medium without carbon source was used as negative control under sterile conditions. The above test tubes supplemented with inulin, dextran and control was inoculated with 1% (v/v) of overnight grown cultures and incubated at 37°C under anaerobic conditions by placing the tubes in anaero bag. Bacterial growth was determined by measuring absorbance at 600 nm at regular intervals of 0, 6, 12, 24 and 48 h.

6.2.6 Effect of polymer synthesis on solidification of sucrose-supplemented milk

The sterile skimmed milk was supplemented with 2, 4, 6, and 8% (w/v) sucrose. 1.0 ml (0.44mg/ml and 20 U/mg) of partially purified dextransucrase from *Weissella cibaria* JAG8 was then added to 9.0 ml each of the sucrose-supplemented milk samples and mixtures were incubated for up to 8 h at 30°C. The solidified milk was then visually compared to that of the controls (milk containing the dextransucrase but not sucrose as positive control and only milk as negative control) following the method reported by Bejar *et al*., (2013).

6.3 Results and Discussion

6.3.1 Effect of artifical human gastric juice on hydrolysis of dextran from *Weissella cibaria* JAG8

The effect of stimulated gastric juice on exopolysaccharide (dextran) produced from *Weissella cibaria* JAG8 showed high resistance to digestion. The hydrolysis of dextran caused by the artificial gastric juice at pH 1, 2, 3 and 4 was much low and 1.1%, 0.85%, 0.77% and 0.64%, respectively, whereas with the standard prebiotic inulin it was much higher and 33.6%, 27.5%, 9.6% and 7.3%, respectively after 6 h (Fig 6.3.1). Dextran from *Weissella cibaria* JAG8 was more stable to acid hydrolysis up to 6 h. It was also reported earlier by Hongpattarakere *et al*., (2012) that exopolysaccharides produced from *Weissella cibaria* A2, *Weissella confusa* A9, *Lactobacillus plantarum* A3 and *Pediococcus pentosaceus* 5S4 showed high resistance to simulated gastric juice with 0.35%, 2.51%, 0.55% and 1.59%, respectively. Several evidences demonstrate that the administration of different prebiotics such as inulin and fructo-oligosaccharides induce significant modification in intestinal microflora (Kruse *et al*., 1999; Bouhnik *et al*., 1999). Prebiotic carbohydrates are metabolized only by selected members of the gastrointestinal tract. Accordingly, these sugars have the ability to influence the population of the gastrointestinal tract due to their selective utilization. Hydrolysis of prebiotics to their component sugars would no longer offer selective stimulation. These findings are especially relevant for acidic foods that may be supplemented with prebiotics and then processed at elevated temperature. Examples include salad dressings, crackers, bakery foods, and other products containing yogurt or cultured dairy products (Huebner *et al*., 2008).

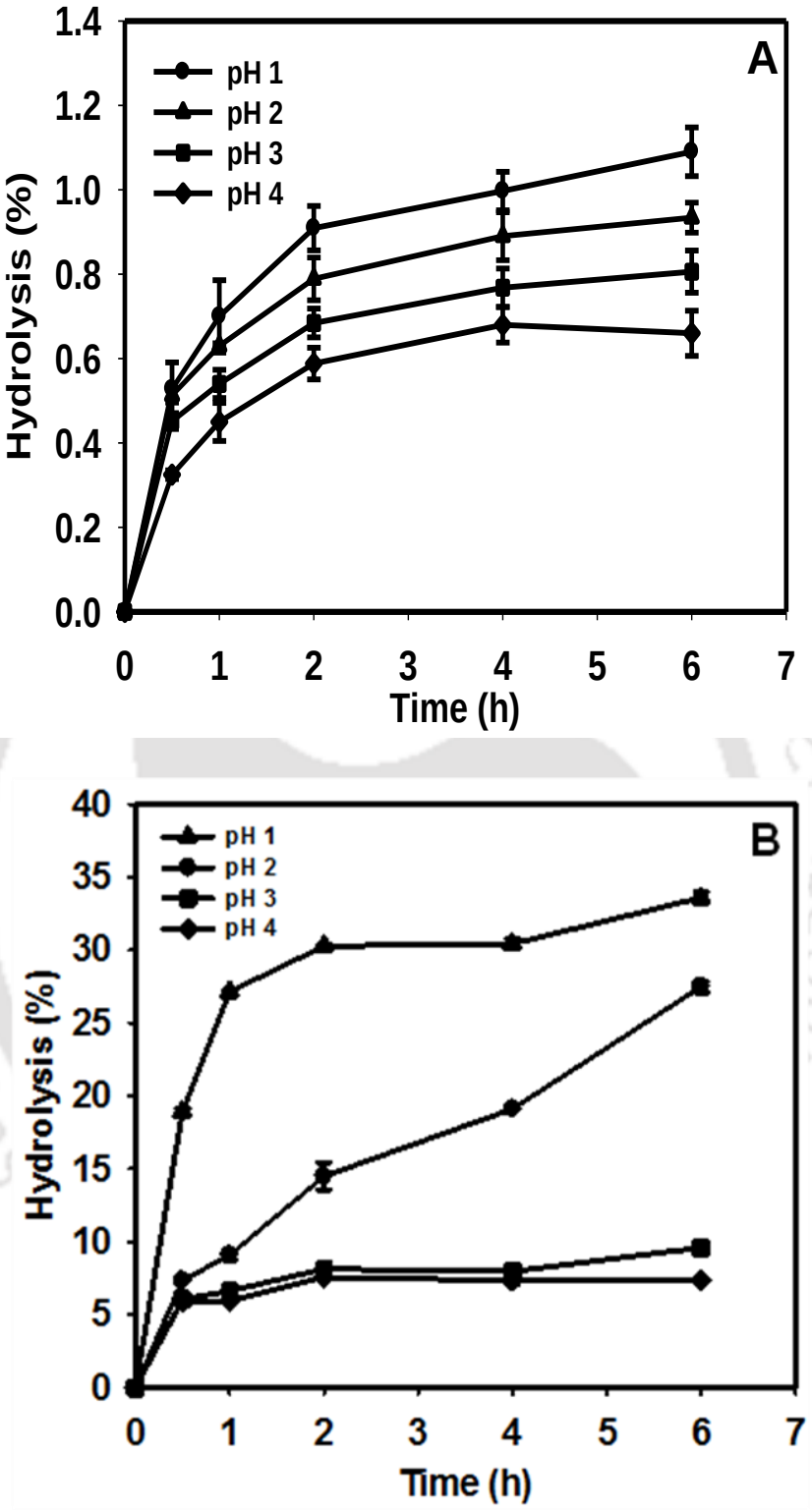


Fig. 6.3.1 Acidic hydrolysis of 1% (w/v) (**A**) dextran from *Weissella cibaria* JAG8 and (**B**) inulin to artificial gastric juices at 37°C.

6.3.2 Effect of α-amylase on hydrolysis of dextran from *W. cibaria* JAG8

Dextran produced from *W. cibaria* JAG8 showed high resistance to α-amylase treatment. The extent of hydrolysis of dextran at pH 5 and 7 was found to be only 0.86% and 0.83%, respectively, whereas with the standard prebiotic inulin it was 13.2% and 11.51%, respectively, up to 6 h (Fig 6.3.2). Hongpattarakere *et al*., (2012) reported earlier that exopolysaccharides produced from *Weissella cibaria* A2, *Weissella confusa* A9, *Lactobacillus plantarum* A3 and *Pediococcus pentosaceus* 5S4 showed high resistance to α-amylase hydrolysis with 0.17%, 0.0%, 0.14% and 0.03%, respectively under simulated intestinal condition. The exopolysaccharide, dextran produced by *Weissella cibaria* JAG8 exhibited high potential prebiotic property with higher resistance to α-amylase. Stability to processing conditions is essential if prebiotics are to confer beneficial effects to the host. If the prebiotic gets degraded to its component mono- and disaccharides then the prebiotic is not available to bacteria. In contrast, prebiotics should be stable enough to processing conditions such that it can selectively enrich the growth of beneficial colonic bacteria (Bohm *et al*., 2005). It is possible that after the processing, the exopolysaccharides, fructo-oligosaccharides (FOS) and inulin could be completely or partially degraded to fructose or glucose or could lead to formation of alternate compounds (Bohm *et al*., 2006).

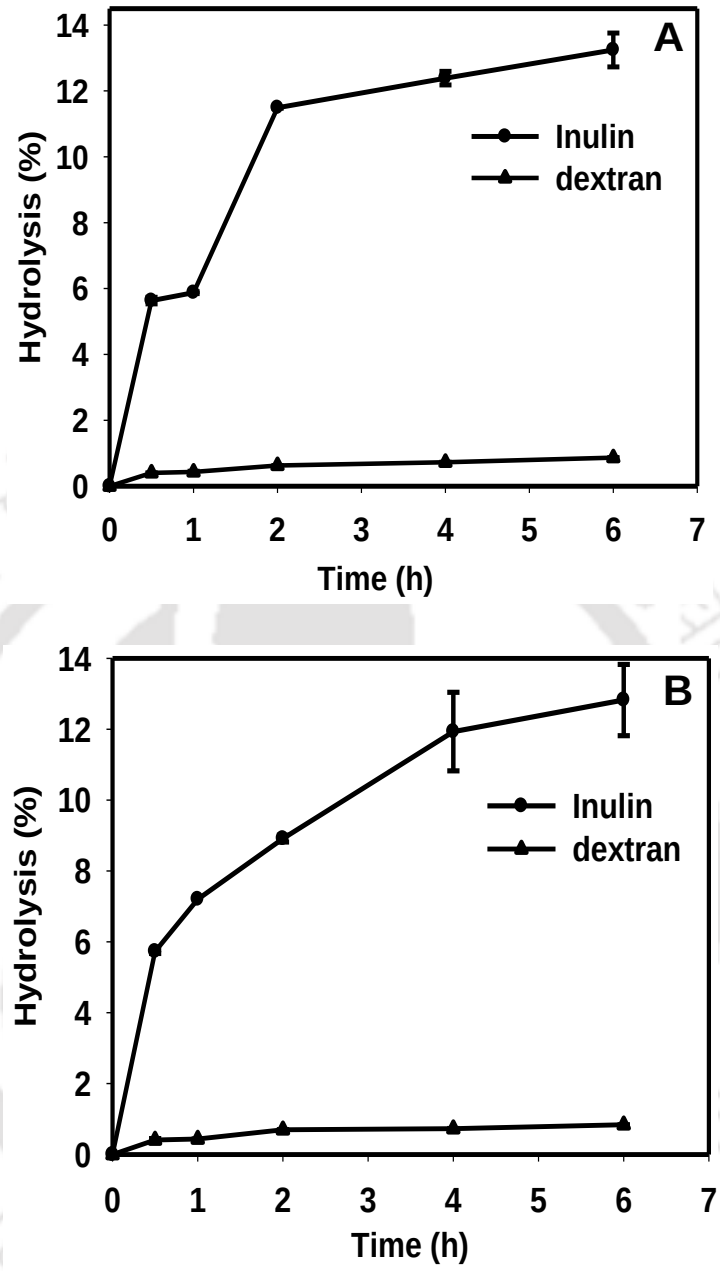


Fig. 6.3.2 Enzymatic hydrolysis of 1% (w/v) of dextran from *Weissella cibaria* JAG8 and inulin by α-amylase at 37°C and at (**A**) pH 5 and (**B**) pH 7.

6.3.3 Effect of Maillard reaction condition on prebiotic activity of dextran from *Weissella cibaria* JAG8

The prebiotic activity of exopolysaccharides (dextran) before and after exposure to Maillard reaction conditions showed positive response. Dextran from *Weissella cibaria* JAG8, standard prebiotic inulin and glucose was compared from 0 to 3h

interval and was further compared with already reported commercial prebiotics Nutraflora P-95 and Raftilose P-95 (Huebner *et al*., 2008). The relative browning for dextran from *Weissella cibaria* JAG8 was found to be around 8.9% and of glucose was 56.12%. The percentage of browning in standard prebiotic inulin was found to be 2.64%. The percentage of browning with time has been described in Table 6.3.1. The relative browning of commercial prebiotics such as Nutraflora P-95 (11.98%) and Raftilose P-95 was found to be 11.98% and 47.79% as described previously by Huebner *et al*., (2008). The relative browning of dextran from *Weissella cibaria* JAG8 was found to be 1.35 fold lower than Nutraflora P-95 and 5.5 fold lower than Raftilose P-95. The formation of Maillard reaction products could also reduce the prebiotic activity of a carbohydrate. During the Maillard reaction, reducing sugars react with amino acids to produce higher molecular weight compounds that can influence the flavor, aroma, and color of the food (Huebner *et al*., 2008). The products formed are chemically stable and are most likely not be available for metabolism by microorganisms. However, only those carbohydrates that contain reducing ends are reactive. Of the prebiotics tested, dextran from *Weissella cibaria* JAG8, standard prebiotic inulin and glucose. The relative browning of dextran from *Weissella cibaria* JAG8 was compared with earlier reported commercial prebiotic browning. It was observed that the percent browning of dextran from *Weissella cibaria* JAG8 was relatively less than Nutraflora P-95 and Raftilose P-95. The stability of prebiotic is greatly dependent on structural composition of the exopolysaccharide .The commercial prebiotics are composed of 95% FOS, with the remaining carbohydrates being glucose and fructose (Huebner *et al*., 2008), while the exopolysaccharides from *Weissella cibaria* JAG8 is composed of only glucose units with 93% of α (1→6) linkage and 7% of α (1→3) branching (Tingirikari and Goyal 2013).

Table 6.3.1 Effect of Maillard browning reaction conditions on prebiotic activity. *(The values in the table indicate percentage of browning with time.)*

Prebiotic	Time (h)			
	0	1	2	3
Inulin	1.34 ± 0.043	1.87 ± 0.26	2.42 ± 0.12	2.64 ± 0.14
Dextran	0.92 ± 0.06	3.21 ± 0.18	6.52 ± 0.27	8.9 ± 0.24
NutraFlora P-95	0.19 ± 0.25*	3.30 ± 0.20*	7.24 ± 0.34*	11.98 ± 0.26*
Raftilose P-95	0.47 ± 0.57*	13.54 ± 0.66*	30.12 ± 0.95*	47.79 ± 0.88*
Glucose	1.35 ± 0.025	7.87 ± 0.96	16.73 ± 0.43	56.12 ± 0.29

Note: *"*" indicates values taken for comparison from earlier report of Huebner et al., 2008. Prebiotics were exposed to reaction conditions of 1% glycine in pH 7.0. 20 mM citrate-phosphate buffer heated at 85°C for 0-3 h.*

6.3.4 Effect of dextran on growth of probiotic bacteria

The growth of various probiotic bacteria in presence of exopolysaccharides (dextran) from *Weissella cibaria* JAG8 was studied by culturing *Bifidobacterium animalis lactis* NRRL B-41405, *Bifidobacterium infantis* NRRL B-41661 and *Lactobacillus acidophilus* NRRL B-4495 in MRS medium using 1% of dextran as carbon source. Similarly, a positive control containing 1% of inulin and a negative control containing MRS medium devoid of any carbon source was used. It was observed that exopolysaccharides (dextran) produced from *Weissella cibaria* JAG8 not only supported but enhanced the growth of all three probiotic cultures (Fig.6.3.3). Growth curves were obtained by measuring the OD at 600 nm at each of the time points as mentioned in the materials and methods Section 6.2.4. Of the three probiotic cultures the maximum growth was observed in MRS medium substituted with inulin, followed by dextran from *Weissella cibaria* JAG8 and the least growth of probiotic cultures were observed in control. The order of probiotic cultures which showed

maximum growth was *Bifidobacterium infantis* NRRL B-41661, followed by *Lactobacillus acidophilus* NRRL B-4495 and *Bifidobacterium animalis lactis* NRRL B-41405 as displayed in Fig 6.3.3. Maximum growth of probiotic bacteria was observed up to 24 h, there after no significant growth was observed. Prebiotic carbohydrates are metabolized only by selected members of the gastrointestinal tract. Accordingly, these sugars have the ability to influence the population of the gastrointestinal tract due to their selective utilization. The effectiveness of a prebiotic depends, on its ability to be selectively fermented and to support growth of specific targeted organisms such as *Lactobacillus, Bifidobacterium* and other probiotic bacterial species (Jaskari *et al*., 1998).

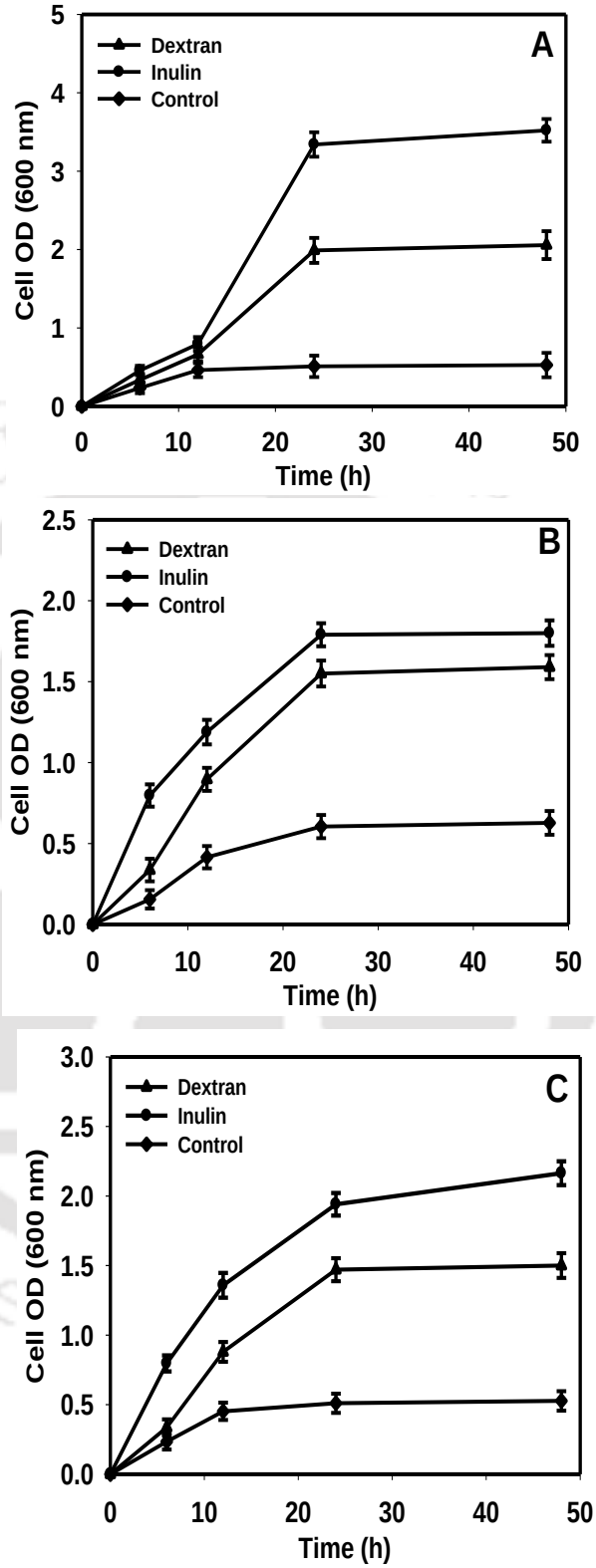


Fig. 6.3.3 The growth curves of (**A**) *Bifidobacterium infantis* NRRL B-41661 (**B**) *Lactobacillus acidophilus* NRRL B-4495 (**C**) *Bifidobacterium animalis* sub species *lactis* NRRL B-41405 grown in MRS medium supplemented with 1% dextran, 1% inulin and control devoid of carbon sources. The cultures were incubated for 48 h at 30°C.

6.3.5 Application of dextransucrase from *Weissella cibaria* JAG8 for solidification of sucrose-supplemented milk

The solidification of sucrose-free skimmed milk was not observed in the presence of the dextransucrase from *Weissella cibaria* JAG8 (Fig. 6.3.4, A). Solidification was, however, notably activated by the addition of sucrose in the skimmed milk after 8 h of incubation. Appreciable degrees of solidification were achieved when sucrose was used at concentrations ranging from 2% to 8% (w/v) (Fig. 6.3.4). While no solidification of milk was observed in the skimmed milk substituted with dextransucrase, indicated that the enzyme-mediated milk solidification was attributed to the synthesis of dextran from sucrose as previously observed by Kim *et al*., (2008) in *Weissella hellenica* SKKimchi3 strain, when cultured in a medium where 10% sucrose was added to skim milk. Later Bajer *et al*., (2013) used crude dextransucrase from *Weissella* sp. TN610 for the solidification of skimmed milk. The dextransucrase from *Weissella cibaria* JAG8 has a promising potential for application as a safe food additive to improve the textural properties of sucrose-supplemented dairy products. Earlier reports have suggested that the modification of the textural properties of dairy products by EPS was the consequence of interactions between these polymers and the proteins present in milk (Ayala-hernandez *et al*., 2008). The micro-structure of milk-derived products depends on physical characteristics of EPS, including the nature of glycosidic linkages, charge, branching linkages, and molecular mass, as well as the type of the proteins present in the milk and the EPS/milk protein ratio (Ayala-hernandez *et al*., 2008). These structural features offered by EPSs from LAB can explain their extensive application in the food industry to

enhance the rheological properties of dairy and bakery products (Katina *et al*., 2009; Behare *et al*., 2010).

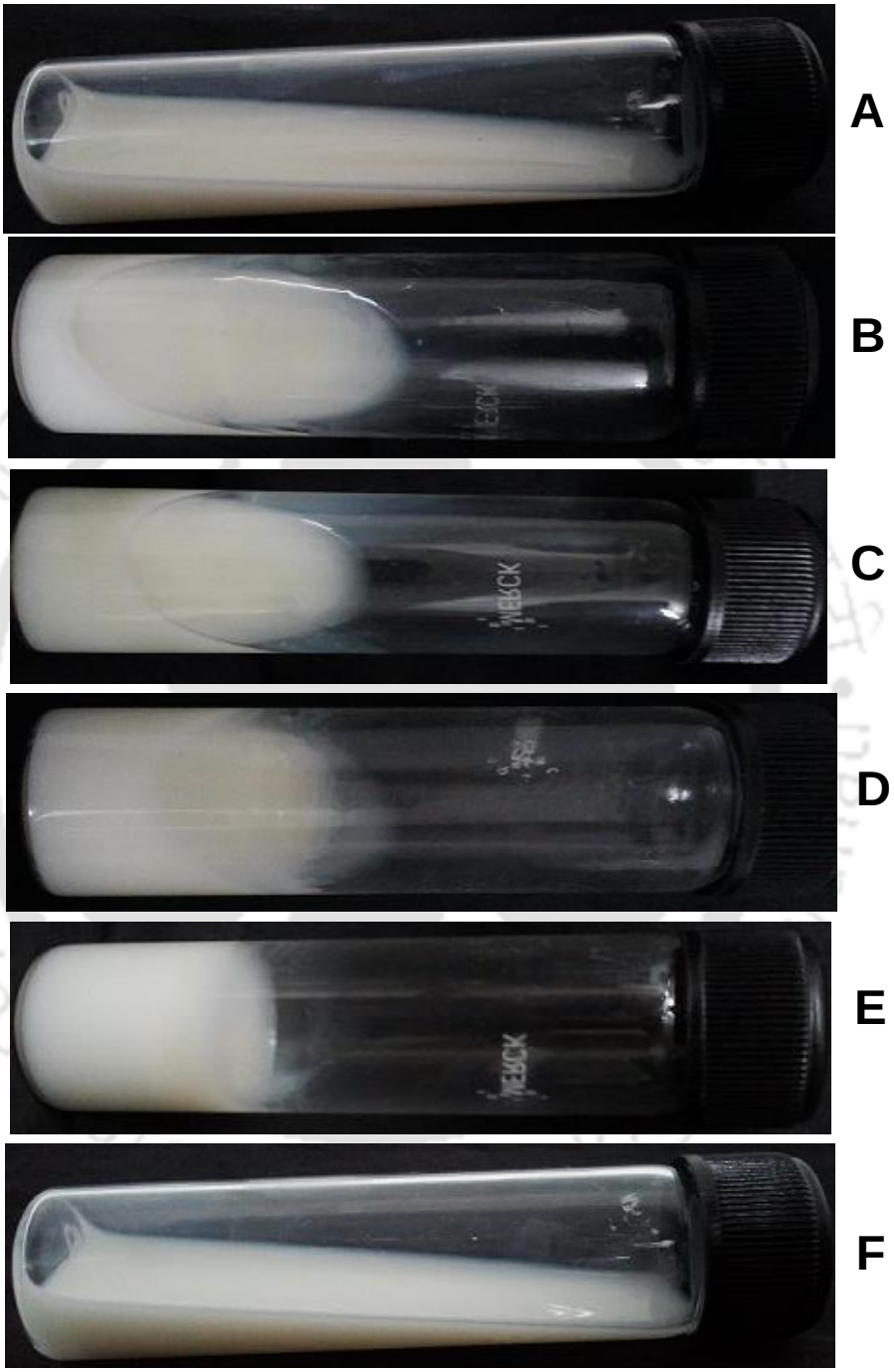


Fig. 6.3.4. Effect of dextran produced by dextransucrase from *Weissella cibaria* JAG8 on the solidification of skimmed milk after incubation at 30°C for 8 h. Skimmed milk containing only dextransucrase as positive control (**A**). Skimmed milk containing dextransucrase and varying concentrations of sucrose; (**B**) 2% sucrose, (**C**) 4% sucrose, (**D**) 6% sucrose, (**E**) 8% sucrose, (**F**) Skimmed milk only as negative control.

6.4 Conclusions

The goal of this study was to quantify the functional stability of prebiotic exopolysaccharide, dextran from *Weissella cibaria* JAG8 by comparing the prebiotic activity before and after exposure to simulated physiological conditions. Acidic pH conditions, as might occur in fermented dairy products, had little influence on prebiotic activity. Dextran from *Weissella cibaria* JAG8 showed significantly low extent of hydrolysis by the artificial human gastric juice at acidic pHs and was around 1% or less whereas, with standard prebiotic inulin it was several fold higher. This indicated the greater stability of dextran at low pH, when compared with inulin. Similar results were observed when the exopolysaccharides were subjected to α-amylase treatment. The extent of hydrolysis of dextran by α-amylase was less than 1%, whereas the prebiotic inulin showed more than 10%. The relative browning for dextran from *Weissella cibaria* JAG8 was 8.9% when exposed to Maillard reaction conditions for 3 h. The relative browning of dextran from *Weissella cibaria* JAG8 was 1.3 fold and 5.5 fold lower than earlier reported commercial prebiotics Nutraflora P-95 and Raftilose P-95, respectively. The dextran from *Weisella cibraria* JAG8 significantly supported the growth of probiotic bacterial cultures, *Bifidobacterium infantis* NRRL B-41661, *Lactobacillus acidophilus* NRRL B-4495 and *Bifidobacterium animalis lactis* NRRL B-41405. Dextransucrase-mediated milk solidification was attributed to the synthesis of dextran from sucrose clearly indicated the promising potential of dextran as food additive to improve the textural and rheological properties of sucrose-supplemented dairy and bakery products. This study strongly proved that (dextran) from *Weissella cibaria* JAG8 can be a putative prebiotic ingredient in food industry to modulate intestinal microbiota for healthcare.

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List of publications

Published/accepted

1. **Jagan Mohan Rao Tingirikari** and Arun Goyal (**2013**). A novel high yielding dextran from *Weissella cibaria* JAG8 for cereal food application. *International Journal of Food Science and Nutrition.* 64, 346-354

2. **Jagan Mohan Rao Tingirikari** and Arun Goyal. (**2013**). Purification, optimization of assay and stability studies on dextransucrase isolated from *Weissella cibaria* JAG8. *Preparative Biochemistry and Biotechnology*. 43, 1-13.

3. **Jagan Mohan Rao Tingirikari** and Arun Goyal (**2013**). Active site mapping of novel dextransucrase isolated from *Weissella cibaria* JAG8: identification of essential amino acid residues by lysine and cysteine specific inhibitors. *Current enzyme inhibition* (in Press)

4. **Jagan Mohan Rao Tingirikari** and Arun Goyal (**2013**). Identification of active site residues in novel dextransucrase isolated from *Weissella cibaria* JAG8 by fluorescence spectroscopy. *Journal of Proteins and Proteomics* (in press).

Submitted/to be submitted

5. **Jagan Mohan Rao Tingirikari**, Damini Kothari, Rishikesh Shukla and Arun Goyal (**2013**). *In vitro* analysis of biocompatible *Weissella cibaria* JAG8 dextran as food additive. (Submitted).

6. **Jagan Mohan Rao Tingirikari** and Arun Goyal (**2013**). Superior prebiotic and physicochemical properties of novel dextran from *Weissella cibaria* JAG8 for potential food applications. (Submitted).

7. Arabinda Ghosh, **Jagan Mohan Rao. T,** Rishikesh Shukla and Arun Goyal (**2013**). Production of manno-oligosaccharides from copra meal by recombinant endo β-mannanase: Their potential role as prebiotics and anti-tumorigenic agent.(Submitted)

8. **Jagan Mohan Rao. T** and Arun Goyal (**2013**). Synthesis and characterization of dextran coated super paramagnetic (Fe_3O_4) nanoparticles with potential biomedical application (to be submitted).

9. Sowmyadeep Chakraborty, **Jagan Mohan Rao. T** and Arun Goyal (**2013**). Immobilization of recombinant endo pectate lyase of family 1 Polysaccharide lyase (PL1) from *Clostridium thermocellum* ATCC 27405 and its application in bioscouring of cotton fabric (to be submitted).

Book Chapter

- **Jagan Mohan Rao Tingirikari** and Arun Goyal (**2013**) Production and Application of Dextransucrase in Book Series -New and Future Developments In Biotechnology And Bioengineering, Vol. VB: Production, Isolation and Purification of Industrial Products. Volume Editors- Ashok Pandey (India), Sangeeta Negi (India), Poonam Nigam (UK), Carlos Ricardo Soccol (Brazil), Elsevier. (to be submitted)

List of conference papers

National

1. **Jagan Mohan Rao, T.** and Arun Goyal (**2012**). Inhibition studies of dextransucrase from *Weissella cibaria* JAG8 by UV and Fluorescence Spectroscopy. Conference on Photochemistry and Luminescence, (CPL 2012), Mar 9-10, 2012. Department of Chemistry, Indian Institute of Technology Guwahati.

International

1. **Jagan Mohan Rao, T** and Arun Goyal (**2013**) Analysis of prebiotic potential of dextran from *Weissella cibaria* JAG8. 10th Convention of Biotech Research Society and International Conference on Advances in Biotechnology and Bioinformatics, Nov 25-27, 2013, D.Y. Patil Institute of Biotechnology and Bioinformatics, Pune, India.
2. **Jagan Mohan Rao**, **T.** and Arun Goyal (**2012**). Dextran produced from *Weissella cibaria* JAG8 with potential application in gluten free cereal products. V International symposium on Sourdough-Cereal Fermentation for Future Foods, Oct 10-12, 2012, Helsinki, Finland.
3. **Jagan Mohan Rao**, **T.** and Arun Goyal (**2012**). Screening and Identification of glucansucrase producing lactic acid bacterium *Weissella cibaria* isolated from apple, 18th (Post ISCBC-2012) International Conference. Perspective and Challenges in Chemical and Biological Sciences Innovation Cross roads, Jan 28-30, 2012, Institute of Advanced Study in Science and Technology (IASST), Guwahati, India.
4. **Jagan Mohan Rao**, **T.** and Arun Goyal (**2011**). Optimisation of culture and assay conditions of glucansucrase from *Weissella cibaria* isolated from apple, 52ndAMI Convention and International Conference on Microbial BioTechnology for Sustainable development Nov 3-6, 2011, Panjab University, Chandigarh, India.
5. **Jagan Mohan Rao**, **T**. Shraddha Shukla and Arun Goyal (**2010**). Optimisation of culture conditions for production and assay conditions of glucansucrase from *Weissella confusa* isolated from fermented cabbage, 7th BRSI Convention and International Conference on Genomic Sciences, Nov 12-14, 2010, Madurai Kamraj University, Madurai, India.

Vitae

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