

**Production, structure analysis and sourdough applications of
dextran and gluco-oligosaccharides from hyper-dextran
producing *Weissella confusa* Cab3 isolated from sauerkraut**

A Thesis

***Submitted in Partial Fulfillment of the
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DOCTOR OF PHILOSOPHY

by

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Under supervision of

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STATEMENT

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

March, 2013

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CERTIFICATE

It is certified that the work described in this thesis entitled **“Production, structure analysis and sourdough applications of dextran and gluco-oligosaccharides from hyper-dextran producing *Weissella confusa* Cab3 isolated from sauerkraut”** by Ms. Shraddha Shukla 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. However some part of the thesis work has been carried out at Department of Botany, North Eastern Hill University (NEHU) Shillong and Department of Food and Environmental Sciences, University of Helsinki, Helsinki, Finland. The work embodied in her thesis has not been submitted elsewhere for a degree.

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SYNOPSIS

Introduction

Dextranase (1,6- α -D-glucan-6- α -glucosyltransferase, EC 2.4.1.5) catalyses the formation of the soluble dextran by a transfer of the D-glucosyl moieties of sucrose to a growing polymer chain. Dextran is a class of homopolysaccharides composed of α -(1 $\rightarrow$ 6) glycosidic linkages in the main chains and α -(1 $\rightarrow$ 2), α -(1 $\rightarrow$ 3) and α -(1 $\rightarrow$ 4) branched glycosidic linkages. Dextran may be linear or branched, with variable degree of branching. Dextran has been used as adjuvant, emulsifier, carrier and stabilizer in food and pharmaceutical industries. Dextran is also used as matrix of chromatography column, anticoagulant, metal-plating processing, for enhanced oil recovery, biomaterials. Dextran has been investigated suitable for cell-resistant coatings on biomaterial surfaces. *In situ* production of dextran during sourdough fermentation improves the volume, moist feel in the mouth and softness of rye breads and rye mixed breads. Dextran derivatives as oligosaccharides have applications as nutraceuticals, prebiotics, immune stimulatory and anti cancer agents. Functionality of dextran depends on the strain of lactic acid bacteria synthesizing it. Dextran producing lactic acid bacteria have recently attracted much attention due to immense industrial potential of dextran. The commonly known dextran producing lactic acid bacteria are *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Weissella*. Molecular weight of dextran plays a key role in determining their application. High molecular weight dextran with linear structure finds their application in food industries as hydrocolloids. Whereas, low molecular weight dextran is important in medical field. Dextranase has also been known for years to catalyze secondary, transglycosylation reactions in

which glucose is transferred primarily to the C6-OH at the nonreducing-ends of acceptor molecules such as mono-, di-, and tri-saccharides. As the result of transglycosilation reaction, a series of glucooligosaccharides is produced. The acceptor efficiency of various sugar molecules and their corresponding acceptor reaction products have been studied. The efficiency of the acceptor molecules vary depending upon their ability to compete with glucan synthesis or their effect on the reaction velocity.

The screening of industrially important lactic acid bacteria from the untapped Indo-Burma biodiversity hot spot, the Northeast India, deserves special attention. The hot and humid tropical climate of Assam is conducive for isolation of microbes from the soil producing rare and novel industrial enzymes and bioactive exopolysaccharides. With this objective, a high dextransucrase yielding strain of lactic acid bacterium “Cab3” was isolated from the sauerkraut. 16s rRNA sequencing identified the isolate to be *Weissella confusa*, belonging to lactic acid bacteria family. *Weissella confusa* shows multiple metabolic traits viz. lactic acid fermentation, exopolysaccharide production from sucrose and antifungal activity which can be used widespread for diverse biotechnological applications. The molecular, morphological and physiological characterization of the isolate *Weissella confusa* Cab3 was conducted. Production, purification and characterization of both dextransucrase and dextran were carried out. Biotechnological utility of dextran from this isolate was studied and found suitable for food formulation and biomaterial fabrication. Statistical methods were employed to enhance the dextransucrase and dextran production from the *Weissella confusa* Cab3. The *Weissella confusa* Cab3 was explored for its potential *in situ* dextran production in sourdough fermentation using wheat bran. The purified dextransucrase from *Weissella confusa* Cab3 was used for the production of

gluco-oligosaccharides using maltose as the acceptor molecule. The gluco-oligosaccharides produced were purified and characterized using various techniques.

Present work

The present investigations are carried out on the “**Production, structure analysis and sourdough applications of dextran and gluco-oligosaccharides from hyper-dextran producing *Weissella confusa* Cab3 isolated from sauerkraut**”. The thesis work comprises 8 Chapters.

Chapter 1 is the General Introduction which embodies the brief review of literature dedicated to the importance of lactic acid bacteria, their classification and characterization. It describes the molecular architecture, the three dimensional structural of dextransucrase and the mechanism of dextran production. The production, purification and characterization methods of the enzyme dextransucrase and the exopolysaccharide dextran have been described in this chapter. The chapter also elaborates the potential applications of the dextran. Application of dextran as food additives and biomaterial are extensively reviewed. Statistical optimization of medium composition by applying the Taguchi's orthogonal methodology and response surface methods (RSM) for maximizing dextransucrase activity and dextran concentration is also described. Chapter 1 also describes the acceptor reaction mechanism and kinetics of dextransucrase and the production of gluco-oligosaccharides. Sourdough is ancient way to improve flavor, texture and microbiological shelf life of bread, and is widely utilized in whole grain bread baking. The importance of the lactic acid bacteria in sourdough fermentation has been described. The microorganism *Weissella confusa* and their phylogenetic and

metabolic traits, dextranase and dextran production capacity and their potential applications in sourdough fermentation are discussed.

Chapter 2 describes the detailed protocol of screening of the natural isolate of lactic acid bacterium, Cab3 from Sauerkraut. The physiological and biochemical characterization were carried out to distinguish the isolate from other closely related lactic acid bacteria. Based on biochemical and physiological studies the strain showed its relation with other *Weissella* sp. The Cab3 was Gram's positive short rod shaped. The cell morphology of Cab3 was studied by scanning electron microscopic study which revealed it to be short rod shaped. Being a lactic acid bacterial strain Cab3 was catalase negative. The Cab3 produced slimy colony when streaked on modified MRS medium which showed the production of polysaccharide. The antibiogram and carbohydrate fermentation profile study enabled the identification of isolated bacterium as it exhibited the typical characteristic features of *Weissella* sp. *Weissella confusa* Cab3 was sensitive towards chloramphenicol, erythromycin, tetracycline, a common trend in *Weissella* sp. The strain was resistant to gentamicin, kanamycin, norfloxacin and vancomycin. The isolate *Weissella confusa* Cab3 could not ferment melibiose, raffinose and trehalose, a common trait of *Weissella confusa* Cab3. The isolate Cab3 fermented cellobiose, galactose, maltose, ribose, sucrose, xylose but, poorly utilized melibiose, raffinose and trehalose. The carbohydrate fermentation profile of the isolate also supported its similarity with *Weissella* sp. The isolated bacterium Cab3 was identified at species and genus level based on 16S rDNA sequencing to be *Weissella confusa* and assigned the Genbank Accession Number JX649223. The culture conditions for maximum enzyme production were optimized. The 25°C temperature and shaking condition were optimum for enzyme production.

At optimum culture conditions *Weissella confusa* Cab3 produced 6.1 U/ml enzyme activity. The enzyme activity of the isolate *Weissella confusa* Cab3 was quite high as compared to other lactic acid bacterial strains. The production of dextransucrase could be increased further by optimizing the fermentation process. Till date not much attention has been paid to the dextran production capacity of *Weissella confusa* which has recently drawn attention for its very high dextran yield and more linearity in linkage pattern, which makes it very potent candidate for dextran production at industrial level. Wide applications of dextran in food, pharmaceutical and cosmetics industries emphasize the importance of exploration of the new strains and characterization of their traits. This study reveals a novel high dextran producing bacterial isolate which could be a good alternative for dextran production in the future.

Chapter 3 deals with the statistical optimization of medium composition for enhancement of dextransucrase from the *Weissella confusa* Cab3. The influence of medium ingredients on extracellular dextransucrase production by a new bacterial strain *Weissella confusa* Cab3 was evaluated. *Weissella confusa* Cab3 produced substantially high dextransucrase in basic unoptimized enzyme production medium. The effects of various macronutrients such as sucrose, yeast extract, beef extract, peptone, sodium acetate, K_2HPO_4 and Tween 80 were studied on dextransucrase production from *Weissella confusa* Cab3 using one factor at a time approach (OVAT). The medium used as control gave 6.0 U/ml enzyme activity. Sucrose (5%), Tween 80 (0.1%), yeast extract (1.5%), peptone (1.5%) and K_2HPO_4 (1.5%) were effective nutrients displaying higher dextransucrase production giving 18.2, 7.0, 6.4, 6.2 and 6.4 U/ml enzyme activity, respectively. Sodium acetate and beef extract were not

effective for enzyme production. The effect of medium ingredients on dextransucrase production was further evaluated using fractional factorial design of Taguchi's orthogonal array. The basic principle of this method serves as screening filters, which examine the effects of many process variables and identify those factors which have major effects on process using a few experiments. Metabolism influencing four factors viz. sucrose, yeast extract, K_2HPO_4 and Tween 80 were selected to optimize dextransucrase production by *Weissella confusa* Cab3 using fractional factorial design of Taguchi method. Based on the influence of interaction components of fermentation, least significant factors of individual level have higher interaction severity index and *vice versa* for enzyme production from *Weissella confusa* Cab3. Sucrose and yeast extract were most significant factors which positively influenced the dextransucrase production. The optimized medium composition consisted of sucrose-5% (w/v); yeast extract-2% (w/v); K_2HPO_4 -1.0% (w/v); Tween 80-0.5% (v/v), based on Taguchi orthogonal array method. The optimized composition gave an experimental value of dextransucrase activity of 17.9 U/ml at shake flask level which corresponded well with the predicted value of 17.54 U/ml by the model. After scaling up the dextransucrase production in a lab scale bioreactor 22.0 U/ml enzyme activity was obtained. The optimized medium gave 3.0 and 3.7 fold higher dextransucrase production at shake flask and bioreactor level, respectively from *Weissella confusa* Cab3 as compared with unoptimized medium. The result displayed successful effort in understanding the effects of various medium components on *Weissella confusa* Cab3, which is a microorganism with a potential of the production of dextransucrase and dextran at the industrial level.

Chapter 4 describes the purification, identification and characterization of dextransucrase from the isolate *Weissella conusa* Cab3. Crude dextransucrase (6.1 U/mg and 6.0 mg/ml) from *Weissella confusa* Cab3 was purified by PEG fractionation. The concentration of 30% (v/v) PEG-400 gave the highest specific activity of 10.5 U/mg with 10 fold purification and 25% overall yield. The partially purified dextransucrase was subjected to gel filtration which resulted in the specific activity of 33 U/mg with 33 fold purification. The purified dextransucrase showed a single band of approximately 180 kDa molecular size on the non-denaturing gel with silver staining. The dextransucrase was identified by zymogram analysis using activity staining protocol. The partially purified dextransucrase was used for further biochemical characterization. 35°C temperature, pH 5.4 and 20 mM ionic strength were optimum for the dextransucrase assay. The reaction kinetics of dextransucrase fitted a Michaelis Menten model, with a K_m of 13.2 mM and V_m of 11.2 $\mu\text{mol/mg/min}$. The dextransucrase activity was enhanced by Co^{2+} , Ca^{2+} , Zn^{2+} Mg^{2+} and Ni^{2+} ions by 21%, 20%, 9%, 8% and 7%, respectively. EDTA inhibited activity of dextransucrase which showed the requirement of metal ion for the catalytic efficiency of dextransucrase from *Weissella confusa* Cab3. The thermo-stability results showed that dextransucrase was stable at lower temperatures (10-40°C) and loses rapidly the enzyme activity at temperatures higher than 4°C. Glycerol and Tween 80 significantly enhanced the stability of dextransucrase at 30°C increasing half life ($t_{1/2}$) to 74.12 h and 59.26 h, respectively as compared with half life ($t_{1/2}$) of dextransucrase without any additive (10.33 h). The storage stability of purified enzyme at -20°C, 4°C and 30°C was studied. The half life ($t_{1/2}$) of enzyme increased from 10.33 h to 69.4 d with decreasing the storage temperature from 30°C to 4°C. Thus 4°C was selected for storage of dextransucrase.

Chapter 5 deals with the optimization of medium composition by response surface methodology for maximization of dextran production from *Weissella confusa* Cab3. *Weissella confusa* Cab3 was used for the dextran production. The stoichiometric parameters were determined to study the kinetics of dextran production. The dextran concentration (**P**), dextran yield ($Y_{\text{Dex/suc}}$) and specific dextran production rate (q_{dextran}) from *Weissella confusa* Cab3 were 9.0 g/l 0.49 gg⁻¹ and 0.09 gg⁻¹h⁻¹, respectively. The dextran concentration from *Weissella confusa* Cab3 was compared with the lactic acid bacterial strain *Leuconostoc mesenteroides* NRRL B-512F which has been used for dextran production at industrial scale. *Weissella confusa* Cab3 produced significantly higher dextran 9.0 mg/ml than *Leuconostoc mesenteroides* NRRL B-512F (4.5 mg/ml) using the same medium. The medium component for the dextran production was optimized using statistical methods. First step towards medium optimization for enhanced dextran production by *Weissella confusa* Cab3 was to screen the medium components by one variable at a time approach. The effects of various macronutrients such as sucrose yeast extract, beef extract, peptone, sodium acetate, K₂HPO₄ and Tween 80 were studied on dextran production from *Weissella confusa* Cab3. The medium used as control gave 9.0 mg/ml dextran concentration. Sucrose (5%), Tween 80 (0.1%), yeast extract (1.5%), peptone (1.5%) and K₂HPO₄ (1.5%) were effective nutrients displaying higher dextran production giving 22.4, 10.0, 10.0, 9.9 and 9.8 mg/ml dextran concentration. Sodium acetate and beef extract were not effective for dextran production. One variable at a time approach does not depict the interaction among nutrients, making it necessity to fine tune the medium components using statistical approach for better and enhanced dextran production by *Weissella confusa* Cab3. Therefore, a 2-level Plackett-Burman factorial design and 5-level central composite design were applied for the optimization experiments. The

statistical method was applied to optimize fermentation medium for dextran production by *Weissella confusa* Cab3. Six nutrients were tested using Placket-Burman Design for their significance. Based on the results of the Placket-Burman Design three nutrients viz. sucrose, K_2HPO_4 and Tween 80 showing significant effect on dextran production were selected for further optimization in order to increase dextran production of *Weissella confusa* Cab3 by response surface methodology. A quadratic polynomial equation obtained by the CCD with $R^2=0.9808$ was used for the determination of the optimal concentrations of constituents having significant effects on dextran production. Under the optimal condition, 28.7 mg/ml dextran production was predicted by the software which upon validation at shake flask resulted in 28.0 mg/ml dextran production. In an attempt to approximate industrial conditions for dextran production, scale-up was carried out in a lab scale bioreactor by using optimized medium. At lab scale bioreactor level (3 l) 29.0 mg/ml dextran was produced. The dextran concentration of *Weissella confusa* Cab3 was enhanced by 3.2 fold in the optimized medium. These results are encouraging for pilot scale or industrial scale production of dextran from hyper producing *Weissella confusa* Cab3.

Chapter 6 describes production, purification and structural characterization of dextran produced by the lactic acid bacterium isolate *Weissella confusa* Cab3. The dextran from *Weissella confusa* Cab3 was enzymatically synthesized and purified by ethanol precipitation. The purified dextran contained less than 2% protein concentration. The dextran was physiochemically and structurally characterized. The *dextro* rotatory property of the biopolymer from *Weissella confusa* Cab3 was established by polarimetry. From the rheological study it was observed that dextran at low concentration followed a typical non Newtonian pseudoplastic behavior. Such

fluids can be employed in the food industry as gelling, stabilizers or thickening agents. The scanning electron microscopic study revealed the highly porous structure of the dextran which aid in the water holding capacity of dextran. Therefore, dextran can be used as potent hydrocolloid in sourdough and food industries as texturizing and viscosifying agent. Dextran supplementation improves dough stability and gas retention through a structure build up of the dextran and interaction with gluten network. HPSEC analysis of dextran from *W. confusa* Cab3 in DMSO based system gave M_w values of 1.77×10^7 g/mol. The hydrodynamic radius (R_h) and radius of gyration (R_g) of dextran molecule from *Weissella confusa* Cab3 in DMSO based solvent as determined by HPSEC were 63.65 nm and 90.8 nm, respectively. Dynamic light scattering (DLS) analysis of dextran in water based system showed that the dextran was monodispersed with hydrodynamic radius (R_h) 121.4 ± 25 nm. The monosaccharide analysis of the dextran from *Weissella confusa* Cab3 showed only glucose moiety as monomer suggested that the biopolymer produced was glucan. FT-IR analysis helped to identify the functional groups present in the dextran. The FT-IR spectrum of the dextran showed bands at 3394, 2930, 1639, 1418, 1237, 1154, 1101, 1050 and 1022 cm^{-1} . The band in the region of 3394 cm^{-1} represents -OH group, 2930 cm^{-1} represents the C-H group and 1639 cm^{-1} represents -COOH group. The absorption peak at 906 cm^{-1} indicates the α -glycosidic bond. The peak at 1022 cm^{-1} indicates great chain flexibility around the α -(1 $\rightarrow$ 6) glycosidic bonds. The characteristic bands at 1150, 1101 and 1022 cm^{-1} found in the spectra of dextran are due to valent vibrations of C-O and C-C bonds and deformational vibrations of the CCH, COH and HCO bonds. The $^1\text{H-NMR}$ spectrum of dextran from *Weissella confusa* Cab3, showed the resonance at 4.97 ppm which was assigned to the C-1 of the α -(1 $\rightarrow$ 6) glucosyl residues of main chain of dextran. The peak at 5.32 ppm at low

intensity confirms the linearity of the dextran of *Weissella confusa* Cab3. The 2D heteronuclear single quantum correlation spectroscopy (HSQC) spectrum of dextran from *Weissella confusa* Cab3 (Fig. 6.3.8) showed two anomeric ^{13}C chemical shifts at 99.1 ppm and 100.5 ppm corresponding to ^1H anomeric signals at 4.97 ppm (Spin system A), and 5.32 ppm (Spin system B), respectively. The anomeric ^{13}C chemical shift at 100.5 ppm was due to the presence α -(1 $\rightarrow$ 3) linkages. The dextran from *Weissella confusa* Cab3 was analyzed to be linear dextran made up of 96.8% linear chain of α -(1 $\rightarrow$ 6) linked glucose residues with low degree (3.2%) of α -(1 $\rightarrow$ 3) branching based on ^1H NMR spectroscopy.

The dextran from the isolate *Weissella confusa* Cab3 was non-toxic and biocompatible as it was evident from the *in vitro* contact based cytotoxicity assay. Therefore, the non-toxic and biocompatible dextran from *Weissella confusa* Cab3 can be used as a biomaterial for drug delivery, tissue engineering and various other biomedical, pharmaceutical and biotechnological applications. The properties like high yield, linear chain with low degree of branching, high-molecular-weight, monodispersity, pseudoplastic behavior and biocompatibility of dextran from *Weissella confusa* Cab3 can be efficiently used to improve rheological (gelling, thickening) or physico-chemical (emulsion stabilisation, particle suspension etc.) properties which are important for food product formulation such as sourdough bread.

Chapter 7 The impact of *in situ* dextran production from *Weissella confusa* Cab3 on wheat bran sourdough fermentation was studied in Chapter 7. This study established the potential of *Weissella confusa* Cab3 to produce significant amounts of *in situ* dextran in wheat bran without strong acidification. The *Weissella confusa* Cab3 grew well in wheat bran to a final cell count of 2.1×10^8 cfu/g after 20 h fermentation. The

pH of the wheat bran sourdough decreased from pH 6.65 to pH 5.43 showing the production organic acids in sourdough. After 20 h of fermentation of the wheat bran sourdough, the texture of the sourdough was improved with increased viscosity. The *Weissella confusa* Cab3 efficiently sucrose (11.2 %, w/w) and consequently produced significantly higher dextran (3.7 g/g) and released fructose (3.8%, w/w) after 20 h of fermentation at 25°C. *Weissella confusa* Cab3 did not produce gluco-oligosaccharides in wheat bran because of the absence of any acceptor molecule such as maltose in the wheat bran.

Chapter 8 The gluco-oligosaccharides from *Weissella confusa* Cab3 were produced by acceptor reaction of dextransucrase using sucrose and maltose. In the presence of sucrose and maltose, dextransucrase catalyzed the synthesis of gluco-oligosaccharides along with the synthesis of dextran. The gluco-oligosaccharides were separated from dextran by ethanol precipitation. The gluco-oligosaccharides were structurally characterized by FT-IR, NMR, HPAEC-PAD and ESI-TOF-MS analyses. HPAEC-PAD analysis showed that the acceptor reaction products viz. panose (6²-O- α -D-glucopyranosyl-maltose) and a homologous series of 6'-isomaltodextrinosyl maltose were produced. The FT-IR spectrum of the gluco-oligosaccharides was used to establish the conformational properties such as α -dominating configuration and the carbohydrate nature of the acceptor reaction products. ¹H and ¹³C NMR spectroscopic analysis showed that the dextransucrase synthesized oligosaccharides that contained both α -(1→6) and α -(1→4) linkages. The detail structural elucidation of the gluco-oligosaccharides was carried out using various hydrolyzing enzymes viz. endodextranase from *Chaetomium erraticum*, α -glucosidase from *Aspergillus niger* and α -glucosidase from *Bacillus stearothermophilus*. The enzymatic action of

endodextranase from *Chaetomium erraticum* on gluco-oligosaccharides resulted in the conversion of gluco-oligosaccharides into isomaltose, isomaltotriose and panose. α -Glucosidase from *Bacillus stearothermophilus* could not hydrolyze the panose series of oligosaccharides as they contained α -(1 $\rightarrow$ 4) linked glucosyl residue at reducing end. However, due to the transglycosilation reaction of the α -glucosidase from *Bacillus stearothermophilus*, isomaltose and isomaltotriose were produced using the gluco-oligosaccharides as substrate. Therefore, NMR spectroscopic analysis and the incomplete hydrolysis of the gluco-oligosaccharides by α -glucosidase from *Bacillus stearothermophilus* showed that that gluco-oligosaccharides contained maltose at their reducing end. The gluco-oligosaccharides were fractionated by gel permeation chromatography by Biogel P2 column. The HPAEC-PAD analysis of different fractions from Biogel P2 column confirmed the separation of oligosaccharides. The eluted fractions of gluco-oligosaccharides were analyzed by ESI-TOF-MS. The ESI-TOF-MS analysis of the acceptor reaction products (mixture of gluco-oligosaccharides) and the fractions, confirmed the separation of the gluco-oligosaccharides of different degree of polymerization. ESI-TOF-MS analysis of acceptor reaction products revealed the presence of the gluco-oligosaccharides corresponding to a homologous series of panose ranging from DP-3 to DP-8.

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Chapter 1

General Introduction

1.1 Introduction

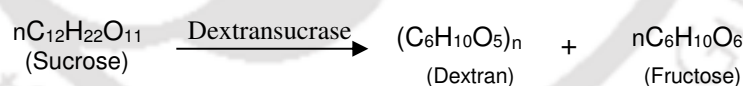
Lactic acid bacteria (LAB) consist of a number of bacterial genera within the phylum Firmicutes. The genera *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Lactosphaera*, *Leuconostoc*, *Melissococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* are recognized as LAB (Stiles and Holzapfel, 1997; Ercolini *et al.*, 2001; Holzapfel *et al.*, 2001). LAB are generally regarded as safe microorganism (GRAS) and have wide application in food fermentation where they contribute in acid production, proteolytic activity and aroma formation. Lactic acid bacteria (LAB) are gram positive (Fooks *et al.*, 1999), non motile and non-sporulating bacteria. LAB ferment carbohydrates and forms lactic acid as a primary metabolite (Nair and Surendran, 2005). LAB are either homofermentative or heterofermentative based on the organism's metabolic pathway. Homofermentative bacteria such as *Lactococcus* sp and *Streptococcus* sp yield two moles of lactic acid from one mole of glucose molecule, whereas the heterofermentative bacteria such as *Leuconostoc* sp and *Weissella* sp transform a glucose molecule into lactate, ethanol and carbon dioxide (Caplice and Fitzgerald, 1999; Kuipers *et al.*, 2000). In addition, LAB produce small organic compounds that

give the aroma and flavor to the fermented product (Caplice and Fitzgerald, 1999). LAB also produce acetaldehyde, hydrogen peroxide, diacetyl, carbon dioxide, polysaccharides and bacteriocins (Caplice and Fitzgerald, 1999; de Vuyst and Degeest, 1999; Rodríguez *et al.*, 2003). Some LAB strains synthesize ribosomally encoded antimicrobial peptides, or bacteriocins, targeted to inhibit other Gram-positive bacteria (Barefoot and Nettles, 1993; Abee, 1995; Caplice and Fitzgerald, 1999; O'Sullivan *et al.*, 2002). LAB are regarded as a major group of probiotic bacteria (Collins *et al.*, 1998; Tannock, 1998; Schrezenmeir and de Vrese, 2001). The probiotic concept was defined by Fuller (1989) that means “a live microbial feed supplement which beneficially affects the host animal by improving its intestinal microbial balance”. LAB along with other gut microbiota ferment various substrates like lactose, biogenic amines and allergenic compounds into short-chain fatty acids and other organic acids and gases (Gorbach, 1990; Gibson and Fuller, 2000). LAB synthesizes enzymes, vitamins, antioxidants and bacteriocins (Fernandes *et al.*, 1987; Knorr, 1998). Probiotics may regulate local and systemic immunity, thereby reducing allergic disease severity and susceptibilities of infants and children to allergies and atopic diseases (Hsieh and Versalovic 2008). Lactic acid bacteria produce wide range of exopolysaccharides. Exopolysaccharide producing LAB finds their application in dairy industries in the production of yoghurt cheese and fermented vegetables and beverages. Lactic acid bacteria produce extracellular enzyme glucansucrase. Glucansucrase utilizes sucrose as substrate. *Leuconostoc* and *Lactobacillus* produce inducible glucansucrase which produce glucansucrase only in the presence of sucrose while *Streptococcus* sp are constitutive for this enzyme. Additionally LAB play an essential role in producing fermented foods, as a component for probiotic-foods, and as bio-preservatives (Zambonelli *et al.*, 2002). The antimicrobial effect of LAB may

be due to the production of organic acids (lactic and acetic acids), hydrogen peroxide and bacteriocins or antimicrobial peptides (Daeschel, 1989). Bacteriocins are ribosomally encoded peptides which inhibit genetically related organisms. They have applications in food additives as a substitute to chemical additives in order to inhibit growth of undesirable microorganisms (Barefoot and Nettles, 1993).

1.2 Glucansucrase

Glucansucrases (GSs) are classified as glycoside hydrolase family 70 (GH70) enzymes based on amino acid sequence similarity (<http://www.cazy.org/>). The GSs (GH70) are mechanistically, structurally and evolutionary closely related to the GH13 and GH77 family enzymes (Stam *et al.*, 2006). They catalyze the synthesis of high molecular weight D-glucose polymers named glucans from sucrose by polymerization reaction, yielding dextran, mutan, alternan or reuteran. Dextransucrase (1,6- α -D-glucan-6- α -glucosyltransferase, EC 2.4.1.5) catalyses the formation of the soluble dextran by a transfer of the D-glucosyl moieties of sucrose to a growing polymer chain.



1.2.1 Molecular architecture of dextransucrase

The dextransucrase is a large protein consisting of approximately acid sequence (Monchois *et al.*, 1999). The schematic structure of dextransucrase is shown in Fig. 1.1. Dextransucrases encoding gene have been cloned which suggested that it consists of A, signal peptide; B, variable region; C, N-terminal catalytic domain; D, C-terminal glucan binding domain (Russell, 1990; Giffard *et al.*, 1993; Simpson *et al.*,

1995; Vickermann *et al.*, 1997). N-terminal part contains typical signal peptides of Gram-positive bacteria (Monchois *et al.*, 1998a). The main characteristic of the structure of glucansucrase signal peptides is that it is well conserved (Monchois *et al.*, 1998a). The N-terminal signal peptide of approximately 32-34 amino acid residues aids in the translocation of dextransucrase across bacterial membrane. The signal peptide region is followed by a stretch of 121-129 amino acids which is highly variable. The non-conserved region located just downstream of the signal peptide tends to have no important role in the enzyme mechanism. Its deletion does not affect the enzyme activity (Abo *et al.*, 1991). As shown by protein sequence alignments of different dextransucrases, the N-terminal domain is highly conserved catalytic domain of about 1000 amino acids (Ferretti *et al.*, 1987). The catalytic domain is the glucan binding domain, capable of binding and cleaving sucrose. The C-terminal end of glucansucrases covering about 500 amino acids is a functional glucan binding domain which is composed of a series of tandem repeats (Monchois *et al.*, 1999).

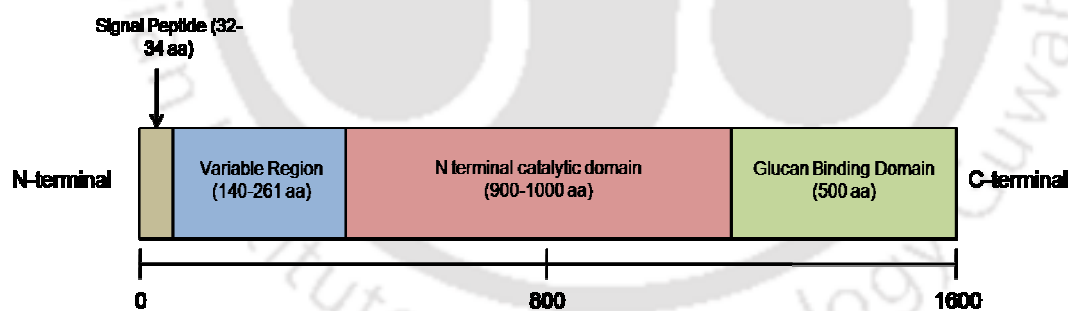


Fig. 1.1 General schematic diagram of dextransucrase.

1.2.2 Three dimensional (3D) structure of dextransucrase

Recently the first three-dimensional structures of GH70 GSs obtained with truncated forms of the enzyme have become available (Vujicic- Zagar *et al.*, 2010; Ito *et al.*, 2011; Brison *et al.*, 2012). Crystal structures of complete GS enzymes have not yet been reported. The crystal structure of a fully active, 1,031-residue fragment

encompassing the catalytic and C-terminal domains of GTF180 from *Lactobacillus reuteri* 180 is shown in Fig. 1.2 as described earlier by Vujicic- Zagar *et al.*, 2010. The catalytic core of the GSs consists of three core domains, which resemble the A, B and C domains which are also found in glycoside hydrolase family 13 (GH13) enzymes. There are two extra domains, called IV and V (Fig. 1.2A), attached to the core domains (Vujicic- Zagar *et al.*, 2010; Leemhuis *et al.*, 2012b).

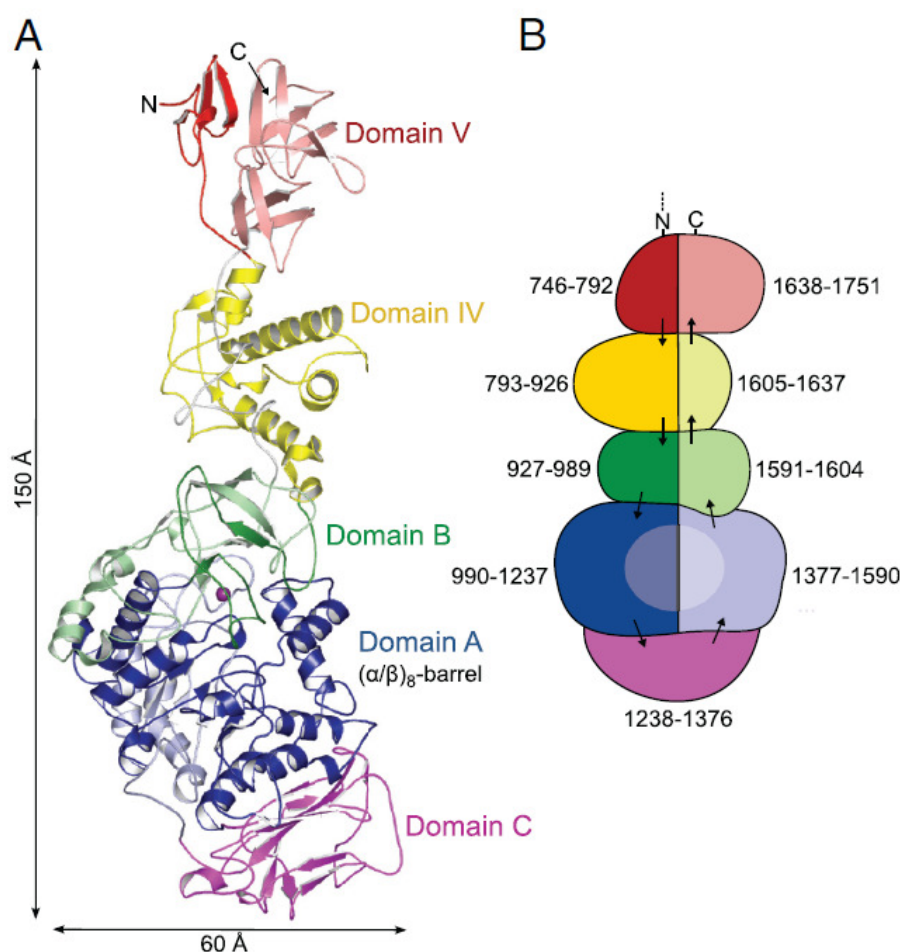


Fig. 1.2 Overall structure of *Lactobacillus reuteri* 180 GTF180-ΔN as reported by Vujicic- Zagar *et al.*, 2010. (A) Crystal structure of GTF180-ΔN, the N- and the C-terminal ends of the polypeptide chain are indicated; (B) Schematic presentation of the “U-shaped” course of the polypeptide chain. Domains A, B, C, IV, and V are coloured in blue, green, magenta, yellow, and red, respectively, with dark and light colors for the N- and C-terminal stretches of the peptide chain.

Starting from residue 746 in, the A, B, IV and V domains in glucansucrase from *Lactobacillus reuteri* 180 (GTF180-ΔN) follows a U-shaped course which is arranged in two discontinuous segments as shown in Fig. 1.2B (Vujicic-Zagar *et al.*, 2010). On the other hand, the C domain consists of only one contiguous polypeptide. Therefore, from N- to C-terminus the polypeptide chain follows” V, IV, B, A, C, A, B, IV, and V domains (Leemhuis *et al.*, 2012b). The domain A comprises a $(\beta/\alpha)_8$ barrel which contain three proposed catalytic residues (the nucleophilic aspartate, the acid/base glutamate and the transition state stabilizing aspartate) at the bottom of a deep pocket (Vujicic-Zagar *et al.*, 2010). Domain A comprises four homology region viz. I, II, III and IV. Homology regions II, III and IV are located in the N-terminal part of domain A whereas, homology region I is located at the C terminal part of the domain A. Domain B forms a highly twisted antiparallel five or six-stranded β -sheet and lies next to the catalytic domain. Domain B contribute to shaping the groove near the catalytic site and the residues at the interface of domain B with domain A and forms a calcium binding site at about 10°A from the nucleophilic aspartate. Domain C is well conserved in all GS structures as well as in most GH13 enzymes, and is located next to the catalytic domain and ‘opposite’ of the domain B. The function of Domain C in glucansucrase has not yet been understood. The structure of domain IV revealed a novel fold with no similarity to any other known protein structure. Domain IV helps in connecting domains B and V. Ito *et al.*, 2011 propose that domain IV may act as a ‘hinge’ that brings domain V bound with glucan near or away from the catalytic site. Domain V, located next to domain IV, is either built up of both N- and C-terminal segments or of an N-terminal segment alone.

Glucan binding domains of GSs facilitate the transfer of synthesized products from the catalytic site, but the detail mechanism is still unknown. However, it has

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

1.2.3 Production, purification and biochemical characterization of dextransucrase

Dextransucrase are produced by various *Leuconostoc* (Kim and Robyt, 1995a; Padmanabhan and Kim, 2002), *Pediococcus* (Patel *et al.*, 2011a), *Lactobacillus* (Hammond, 1969), *Weissella* (Maina *et al.*, 2008; Shukla and Goyal, 2011) and *Streptococcus* sp (Chludzinski *et al.*, 1974). Dextransucrase is an inducible enzyme requiring sucrose in the medium for the induction with the exception of recently isolated constitutive mutant strains of *Leuconostoc mesenteroides* viz. B-512 FMC (Kitaoka and Robyt, 1998), B-742 (Kim and Robyt, 1995a), B-1299 (Kim and Robyt, 1995b) and B-1355 (Cote *et al.*, 1999). Dextransucrase production is affected by temperature, aeration and medium components (Cortezi *et al.*, 2005). Tsuchiya *et al.*,

1952 studied the effect of sucrose, corn steep liquor and phosphate on dextransucrase production from *Leuconostoc mesenteroides* strain NRRL B-512. Goyal and Katiyar, 1997 used low yeast extract and high K_2HPO_4 concentration for enhanced dextransucrase production from *Leuconostoc mesenteroides* NRRL B-512F. However, dextransucrase production by wild-type *Leuconostoc mesenteroides* grown on glucose or maltose instead of sucrose also has been reported (Smith and Zahnley, 1999). Behravan *et al.* 2003 used sugar-beet molasses as a sucrose source and wheat bran as substitute for yeast extract. The *Leuconostoc mesenteroides* NRRL B-512F displayed increased production of biomass and enzyme with the increase in K_2HPO_4 concentrations (Rodrigues *et al.*, 2003). The aeration conditions were optimized for production and scale up of dextransucrase from *Leuconostoc mesenteroides* B/110-1-1 strain in a bioreactor (Michelena *et al.*, 2003). Purama and Goyal 2008a studied dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 in a bioreactor at different aeration rates and reported that the enzyme activity was 36% higher at 1.5 vvm aeration than that at 0 vvm.

Various purification methods such as salt, alcohol precipitation, fractionation by polyethylene glycol, ultra-filtration, chromatography and phase-partitioning have been successfully used for purification of dextransucrase from different strains of lactic acid bacteria (Majumder *et al.*, 2007). Among all the reported purification methods, fractionation by polyethylene glycol (PEG) is an effective, rapid and single step purification method for dextransucrase from *Leuconostoc mesenteroides* (Purama and Goyal, 2008b). Polyethylene glycol (PEG) is a non-ionic hydrophilic polymer which selectively precipitates high molecular weight or aggregated form of proteins (Goyal and Katiyar, 1994). The dextransucrase from *L. mesenteroides* NRRL B-512F (Goyal and Katiyar, 1994), *Leuconostoc mesenteroides* NRRL B-640 (Purama and

Goyal 2008b), *Leuconostoc dextranicum* NRRL B-1146 (Majumder *et al.*, 2008) and *Pediococcus pentosaceus* (Patel *et al.*, 2011a) have been purified by fractionation with PEG of different molecular weights. PEG fractionation method does not remove the associated dextran from the dextransucrase. This is achieved by a combination of dextranase treatment, ion-exchange and affinity chromatography after PEG precipitation (Majumder *et al.*, 2007). *Leuconostoc mesenteroides* NRRL B-512F was mutated by N-nitrosoguanidine (designated as B-512FM) which resulted in 300 times increase in the activity (Fu and Robyt, 1990). The mutated dextransucrase was purified by treatment with crude dextranase, followed by column chromatography using Bio-Gel A-5m (Fu and Robyt, 1990a). Kobayashi *et al.* (1986) reported the efficacy of DEAE-cellulose and Sephadex G-100 column chromatography for dextransucrase from the same strain with a specific activity 26 U/mg and 679-fold purification.

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

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

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

Fine-tuning of nutrient concentrations is essential to regulate the microbial metabolism and associated metabolic product production. For optimization of nutrient's concentration, various methods are reported in the literature such as Box Behnken method (Navaneeth *et al.*, 2009), central composite design (CCD) (Patel *et al.*, 2011b), neural networking (Singh *et al.*, 2008) and Taguchi's orthogonal array based methodology (Umesaki *et al.*, 1977). Purama and Goyal, 2008a employed response surface methodology for maximizing dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 in a bioreactor. Singh *et al.*, 2008 used artificial intelligence based optimization method for enhancement of dextransucrase production from *Leuconostoc dextranicum* NRRL B-1146. Patel *et al.*, 2011b optimized a medium for enhanced dextransucrase production from an isolate *Pediococcus pentosaceus* (SPAm) using response surface methodology.

1.3 Dextran from lactic acid bacteria

Lactic acid bacteria synthesize various homopolysaccharides by glucansucrase enzyme. Glucansucrase are produced by lactic acid bacteria belonging to the genera: *Leuconostoc*, *Lactobacillus*, *Pediococcus* and *Weissella*. These glucansucrase produce four types of glucans based on the types of the linkages present. They are dextran, mutan, alternan and reuteran. The dextran is composed of mainly α -(1 $\rightarrow$ 6) linkage

and occasionally α -(1 $\rightarrow$ 2), α -(1 $\rightarrow$ 3) and α -(1 $\rightarrow$ 4) linkage as branching points (Sidebotham, 1974; Monchois *et al.*, 1999). The degree of branching involving α -(1 $\rightarrow$ 2), α -(1 $\rightarrow$ 3) and α -(1 $\rightarrow$ 4) linkages in dextrans vary according to the origin of dextransucrase (Seymour and Knapp, 1980). Mutan is a type of insoluble glucan having more than 50% α -(1 $\rightarrow$ 3) linkages (Sidebotham, 1974; Mooser, 1992). Alternansucrase synthesizes the glucan, alternan, which contains alternating α -(1 $\rightarrow$ 6) and α -(1 $\rightarrow$ 3) glucosidic linkages, with some degree of α -(1 $\rightarrow$ 3) branchings (Jeanes *et al.*, 1954; Seymour and Knapp, 1980; Cote and Robyt, 1982). Dextran from *Leuconostoc mesenteroides* NRRL B-512F consists of 95% of α -(1 $\rightarrow$ 6) linkages and 5% of α -(1 $\rightarrow$ 3) branched linkages (Bertrand *et al.*, 2006). *L. mesenteroides* NRRL B-1299 produces a highly branched dextran containing between 27-35% of α -(1 $\rightarrow$ 2) branched linkages (Dols *et al.*, 1997). Molecular weight of dextran play key role in determining their application. High molecular weight dextran with linear structure finds their application in food industries as hydrocolloids. Whereas, low molecular weight dextrans are important in medical field. Kim *et al.*, 2003 studied the effect of sucrose concentration, pH and temperature on size and branching of dextran from *Leuconostoc mesenteroides* NRRL B-512F. Dextran plays various physiological function in bacteria such as providing protection from adverse conditions such as desiccation (Looijestejn *et al.*, 2001), adhesion of the bacteria to solid surfaces (Marshall, 1992) and biofilm formation to aggregate bacteria. In some bacteria, dextran can be used as reserve energy source (Cerning, 1990; Colby and Russel, 1997).

1.3.1 Mechanism of dextran biosynthesis

The mechanism of dextran biosynthesis involving a “two-site insertion mechanism” was first proposed for the synthesis of dextran by dextransucrase by

Ebert and Schenk, 1968. Experimental evidence and further elaboration of the two catalytic site and insertion mechanism was provided by Robyt *et al.* 1974 and Robyt and Walseth, 1978. Robyt *et al.* 1974 by pulse-chase experiments demonstrated that glucan and glucosyl residues coming from sucrose cleavage were both covalently linked with enzyme through their reducing end. Robyt *et al.* 1974 proposed that the two identical nucleophilic sites are involved for glucan biosynthesis without requiring the presence of exogenous primer at the beginning of the reaction. Moulis *et al.*, 2006 recently rejected the two-site insertion mechanism for B-512F dextranase. Moulis *et al.* (2006) concluded that the synthesis is a semi-processive mechanism, involves a single active site, and that the glucosyl residues are added to the non-reducing end of a growing dextranyl chain. According to Moulis *et al.* 2006, dextranase first hydrolyzes sucrose into glucose and fructose as a minor reaction. Then dextranase uses both glucose and sucrose, as initiator primers for the elongation of dextran, which continues by the addition of glucose to the non-reducing end of the isomaltodextrin chain. Robyt *et al.*, 2008 reinvestigated the dextranase reaction mechanism using pulse and chase experiments with soluble B-512FMC dextranase, as a conformational evidence. Robyt *et al.*, 2008 experimentally proved that the high molecular weight dextran does not require a primer and also that it does not follow a non-reducing-end synthesis mechanism. Their experiments showed that glucose, sucrose or isomaltodextrins are not initiator primers for the synthesis of dextran by dextranase (Robyt *et al.*, 2008). In “two site insertion mechanism” D-glucose and the growing dextran chain are covalently attached to the active site of the dextranase (Robyt *et al.*, 2008). The D-glucose is transferred to the reducing end of the growing dextran chain.

However, studies on a 117 kDa crystal structure of a glucansucrase fragment (GTF180- Δ N) from *Lactobacillus reuteri* 180 have supported the non-reducing end growth mechanism (Vujicic-Zagar *et al.*, 2010). The crystal structure of the glucansucrase fragment (GTF180- Δ N) from *Lactobacillus reuteri* 180 has confirmed that there was only one active site with no space for another covalently bound glucosyl residue or dextranyl chain (Vujicic-Zagar *et al.* 2010).

1.3.2 Mechanism for branching in dextran

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

1.3.3 Purification and characterization of dextran

Extracellular dextran extraction from culture medium usually involves cell removal, dextran precipitation and drying of the precipitated dextran (Ruas-Madiedo and de los Reyes-Gavilan, 2005). Membrane-filtration, anion-exchange and gel permeation chromatography (Sanz and Martine, 2007) have been successfully employed to purify dextran. Precipitation of proteins with trichloroacetate and hydrolysis of proteins with proteases can be done to remove protein contamination from dextran. High-performance size exclusion chromatography coupled with refractive index detection (HPSEC-RI) and with multi-angle laser light scattering (HPSEC-MALLS) (Picton *et al.*, 2000) field flow fractionation (FFF) and hydrodynamic chromatography (HDC) (Isenberg *et al.*, 2010; Cave *et al.*, 2009), are the techniques for the determination of the molecular weight (MW) of dextran. The monomer composition of dextran can be determined by acid hydrolysis followed by monomer detection using high-performance anion-exchange chromatography (HPAEC) with pulse amperometric detection (PAD) (Cataldi *et al.*, 2000). Structural analysis of the dextran have been studied by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy which provides information about the type of constituent monosaccharides, including ring size and anomeric configuration, and the position of glycosidic linkages. The FT-IR, NMR, scanning electron microscopy and rheological studies on dextrans of *Weissella ciberia* have been carried out (Jagan and Goyal, 2012; Ahmed *et al.*, 2012). The dextran from *Weissella confusa* E392 containing α -(1 $\rightarrow$ 6) and α -(1 $\rightarrow$ 3) linked branches was characterized using NMR spectroscopic analysis (Maina *et al.*, 2008). *Leuconostoc mesenteroides* NRRL B-640 synthesizes a α -(1 $\rightarrow$ 6) linked linear dextran (Purama *et al.*, 2009) and *Leuconostoc dextranicum* NRRL B-1146 synthesizes a α -(1 $\rightarrow$ 6) linked dextran with α -(1 $\rightarrow$ 4) branching

(Majumder and Goyal, 2009). Shukla *et al.* (2011) studied the structure of insoluble dextran from *Leuconostoc mesenteroides* NRRL B-1149, which revealed the branched structure containing α -(1 $\rightarrow$ 6) and α -(1 $\rightarrow$ 3) glucosidic linkages.

1.3.4 Applications of dextran

Low molecular weight dextran (Dextran 70 and dextran 40) are used as blood plasma substitutes (Harrison, 1954). Dextran has been used as adjuvant, emulsifier, carrier and stabilizer in food and pharmaceutical industries (Janciauskaite *et al.*, 2008, Hernandez *et al.*, 2011; Patel *et al.*, 2012). Dextran improve rheology, texture, stability and mouth feel of fermented milk products (Patel *et al.*, 2012). Dextran are also used as matrix of chromatography column, anticoagulant, metal-plating processing, for enhanced oil recovery, biomaterials. Dextran has been reported to be suitable for cell-resistant coatings on biomaterial surfaces (Massia *et al.*, 2000). *In-situ* production of dextran during sourdough fermentation improves the volume, moist feel in the mouth and softness of rye breads and rye mixed breads (Di Cagno *et al.*, 2006; Lacaze *et al.*, 2007). Important features of dextran for its use in sourdough bread are its high yield, low degree of branching and high molecular weight. The physical properties such as surface morphology, gelling nature, structure and rheology are crucial in determining the potential of dextrans (Stoodley *et al.*, 1999). Dextrans have many industrial applications due to their non-ionic character and good stability under normal operating conditions. They have been also used for preparing blood plasma substitutes, plasminogen activators and antithrombogenic agents (Soetaert *et al.*, 1995, Purama and Goyal, 2005). Dextran with high molecular weight can be used as osmotic agents and they are being used to treat hypovolemia (Alpar and Killampalli, 2004). Iron dextran is used to treat iron deficiency anaemia (Thayu and

Mamula, 2005). In clinical settings, dextran is used as effective volume replacement and infusion of dextrans provides thromboembolic prophylaxis (Koekenberg, 1962). Water uptake, erosion and dissolution profile studies for dextran tablets established that glucose polymer with molecular weight $M_w \geq 2 \times 10^6$ is suitable for the development of soluble drugs with controlled drug release (Gil *et al.*, 2008). 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). Dextran hydrogels have received an increased attention for their variety of biotechnological and biomedical applications. Due to their good tissue biocompatibility and the possibility of to transport specific drugs, they appear to be a viable alternative to the existing drug carriers (Hennink *et al.*, 1996; van Dijk *et al.*, 1997; Chen *et al.*, 2013). Researchers have realized and studied a variety of different dextran hydrogels as transdermal delivery systems (Moriyama *et al.*, 1999; Van Tomme and Hennink, 2007). Dextran hydrogels can be used for the removal of unwanted water or some chemical substances in environment and controlled release of fertilizer and pesticide in agricultural area (Demirbilek and Dinc, 2012). Dinu *et al.*, 2011 established the potential of dextran hydrogel in controlled drug-delivery systems. In a recent study, vitamin E was loaded into a dextran hydrogel containing ferulic moieties, covalently linked, to improve its topical delivery, and also to increase its relative poor stability, which is due to direct exposure to UV light (Cassano *et al.*, 2009). Dextran has been used as cryoprotectant in ice cream preparation (McCurdy *et al.*, 1994). Recently, Maillard reaction between proteins and polysaccharides has been investigated to improve functional properties, especially solubility and surface properties of proteins (Dickinson, 2008; Sun *et al.*, 2011; Zhang *et al.*, 2012). The functional properties of peanut protein isolate (PPI)

were remarkably improved by conjugation with dextran (Liu *et al.*, 2012). In comparison with PPI, mixture and conjugates had more compacted tertiary conformation but exhibited better thermal stability, protein solubility, emulsifying and foaming properties (Liu *et al.*, 2012). Dextran based nanoparticles find application in specific drug targeting where dextran is used as polymer coating, which can be functionalized (McBain *et al.*, 2007). Biosensor analysis based on carboxymethylated dextran (CM-dextran) surfaces has provided reliable platform for the rapid determination of a wide range of compounds relevant to food safety and quality (Situa *et al.*, 2008). Dextran is used as a stabilizing coating for protecting metal nanoparticles against oxidation (Bautista *et al.*, 2005). Dextran coating on biomaterials are being done to prevent undesirable protein absorption to improve their biocompatibility (Sengupta *et al.*, 2006). Dextran is used in nanotechnology as tool for antigen delivery in vaccination (Sahoo *et al.*, 2007).

1.4 Oligosaccharides production by acceptor reaction

Dextranase has been known for years to catalyze secondary, transglycosylation reactions in which glucose moiety of sucrose is transferred primarily to the C6-OH at the nonreducing-ends of low molecular weight carbohydrates such as mono-, di-, and tri-saccharides present in reaction mixture (Robyt and Walseth, 1978; Robyt and Eklund, 1983). As the result of transglycosylation reaction, a series of glucooligosaccharides are produced. However, dextranase from *Leuconostoc mesenteroides* NRRL B-512F can transfer glucosyl residue either to nonreducing end or to reducing end of maltodextrin acceptor by synthesis of α -(1 $\rightarrow$ 6) linkages (Fu and Robyt, 1990b). Maltose, isomaltose, and glucose and many other carbohydrates are known acceptor molecules which can

accept glucose at the active-site of glucansucrases to give a series of isomaltodextrin “acceptor products” presenting an isomaltosyl residue at their non-reducing end (Robyt and Walseth, 1978). Synthesis progresses by successive transfers of glucosyl units to oligosaccharides, which are alternately product and substrate. These acceptor reaction products are synthesized in decreasing amounts, as the size of the products increase (Robyt and Eklund, 1983; Fu and Robyt, 1990b; Su and Robyt, 1993). The major product is the first oligosaccharide homologue, e.g. isomaltose from glucose, isomaltotriose from isomaltose and panose from maltose. Fructose and cellobiose are the exceptional acceptor molecules which give only one product. Fructose gives the disaccharide leucrose, and lactose gives a trisaccharide, with glucose attached to the C2-OH of the reducing-end, glucose moiety (Robyt and Eklund, 1983). Cellobiose, an analogue of lactose, gave a series of isomaltodextrins attached to the C2-OH of the reducing-end glucose moiety (Robyt and Eklund, 1983).

1.4.1 Acceptor molecules

The acceptor efficiency of various sugar molecules and their corresponding acceptor reaction products have been studied (Robyt and Walseth, 1978; Robyt and Eklund, 1983). The efficiency of the acceptor molecules vary depending upon their ability to compete with glucan synthesis or their effect on the reaction velocity (Sidebotham, 1974). Acceptors such as maltose and isomaltose are classified as strong acceptors (Koepsell *et al.*, 1952; Robyt and Walseth, 1978; Robyt and Eklund, 1983). They have activator effect on the reaction velocity and strongly inhibit the yield of glucan synthesis (Robyt and Elkund, 1983). On the other hand weak acceptors like fructose or melibiose, have an inhibitory effect on the reaction (Koepsell *et al.*, 1953) therefore the yield of oligosaccharides is low (Robyt and Elkund, 1983). The yield,

branching and length of the acceptor reaction products can be controlled by changing the acceptor molecule concentration in the reaction mixture of dextran sucrose along with sucrose. The yield of oligosaccharides decreases with increasing the ratio of sucrose concentration to maltose concentration (Monchois *et al.*, 1998b). However, by increasing the sucrose/maltose ratio (S/M), it is possible to catalyze the synthesis of oligosaccharides of increasing degree of polymerization (Vasileva *et al.*, 2010). For an S/M ratio of 7, both linear oligosaccharides (only composed of α -(1 $\rightarrow$ 6) linkages and a maltose residue at the reducing end) and branched oligosaccharides were produced (Vasileva *et al.*, 2010).

1.4.2 Kinetics and mechanism of acceptor reaction

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

mechanistic model. Heincke *et al.*, 1999 also proposed the existence of a separate acceptor binding site between the two active sites for dextran biosynthesis. It is assumed that occupation of the acceptor site by sucrose or an acceptor molecule causes hindrance to the dextran formation reaction. Both sucrose and acceptor molecule compete for the acceptor binding site (Heincke *et al.*, 1999). Therefore, increasing acceptor concentration with respect to sucrose enhances oligosaccharide production (Heincke *et al.*, 1999).

1.4.3 Prebiotic potential of gluco-oligosaccharides

Non digestible oligosaccharides (NDOs) are oligomeric carbohydrates, the osidic bond of which is in a spatial configuration that allows resistance to hydrolytic activities of intestinal digestive enzymes (Roberfroid, 2000). These NDOs possess physiological and physiochemical properties and they functions as dietary fibers and as a prebiotics (Grootaert *et al.*, 2007). The ability of a probiotic lactic acid bacterium to survive in the gastrointestinal tract was promoted by oligosaccharides facilitating the metabolism and growth of LAB (Salminen *et al.*, 1998). The dietary fiber, mainly oligosaccharides and polysaccharides in the colon may act as prebiotics (Ziemer and Gibson, 1998; Fooks *et al.*, 1999). The maltose acceptor reaction has been used to produce these non-digestible glucooligosaccharides (Remaud-Simeon *et al.*, 1994). These glucooligosaccharides are presently marketed for human nutrition and dermocosmetic applications. The production of glucooligosaccharides having α -(1 $\rightarrow$ 3) branched linkages has been done using glucosyltransferases from *Leuconostoc mesenteroides* NRRL B-1355 and NRRL B-742 (Remaud-Smeon *et al.*, 1999; Cote and Sheng, 2006). Kim *et al.*, 2009 studied the production of linear isomalto-oligosaccharides (IMO) with DP2 - DP10 using engineered fusion enzyme (DXSR) of

endo-dextranase and only α -(1 $\rightarrow$ 6) glucan synthesizing dextransucrase using sucrose as sole substrate. *Leuconostoc mesenteroides* NRRL B-1299 dextransucrase synthesizes specifically α -(1 $\rightarrow$ 2) linear and branched glucooligosaccharides which are highly resistant to glycolytic digestive enzymes and can be fermented by beneficial species of the intestinal microflora (Ramaud-Simeon *et al.*, 1994; Dols *et al.*, 1998). Branched oligosaccharides produced by GTFs coming from *Leuconostoc mesenteroides* M2860 were readily catabolized by *Lactobacilli* but not by *Escherichia coli* and *Listeria innocua* strains, pointing toward their application in intestinal microflora modification. Thermo acid-stable oligosaccharides (TASO) were produced from *Leuconostoc mesenteroides* B-512 FCM (Seo *et al.*, 2007). These resistant oligosaccharides inhibited the growth of *Streptococcus sobrinus*. However, it stimulated the growth of probiotic organisms such as *Bifidobacterium* sp (Seo *et al.*, 2007). Gluco-oligosaccharides are known to selectively stimulate the growth of bifidobacteria. In particular, long-chain glucooligosaccharides with a degree of polymerization of 3 or higher are preferred to short-chain oligosaccharides because of the longer persistence in the colon (Chung and Day, 2002). These probiotic strains get advantage over other harmful bacteria in the sense that they can ferment these oligosaccharides. Short chain fatty acids like propionic acids are formed as the results of the fermentation of these oligosaccharides which play decisive role in the prevention of colon cancer (Cummings, 1981).

1.5 Sourdough and lactic acid bacteria

Sourdough is ancient way to improve flavor, texture and shelf life of bread and is widely utilized in whole grain bread baking (Lorenz and Brummer, 2003). Sourdough is a process in which flour and water (and other ingredients) are mixed

together and the fermentation takes place by microbes from preceding sourdough, commercial starter culture, bakery equipment or from flour. The most important quality characteristics for wheat breads are high volume, soft and elastic crumb structure, good shelf-life and microbiological safety of the product (Cauvain, 2003). Sourdough fermentation is based on lactic acid and alcoholic fermentation depending on the composition of microflora and fermentation conditions. The dominating microbes in spontaneously fermented dough are homofermentative lactobacilli and *Pediococci*. Typical homofermentative lactic acid bacteria in spontaneous sourdoughs are *Lactobacillus casei*, *Lb. delbrueckii*, *Lb. farciminis*, *Lb. plantar* and *Pediococcus pentosaceus*. Among heterofermentative LAB are *Lactobacillus brevis*, *Lb. buchneri*, and *Lb. fermentum* (Stolz, 2003). Various yeast strains have also been isolated from spontaneous fermentations of sourdough such as *Saccharomyces cerevisiae* and *Pichia satoi* (Beech and Davenport, 1971). Homofermentative lactic acid bacteria ferment hexose sugars to produce mainly lactic acid. Many heterofermentative strains can also ferment pentosans to produce lactic acid, acetic acid and ethanol. The formation of end product is dependent on the processing conditions of sourdough and type of heterofermentative strain used (Röcken *et al.*, 1992). The most common lactic acid bacteria identified in sourdoughs are capable of fermenting pentoses, hexoses, sucrose and maltose, although some species such as *Lb. sanfransiscensis* are specific to maltose.

In wheat products, textural characteristics are mainly based on the formation of gluten network during bread baking, which has the ability to extend and keep the gas from yeast fermentation and makes a direct contribution to the formation of a cellular crumb structure. In order to improve particularly texture and shelf-life of the breads on the market, several food additives such as emulsifiers and enzymes have

been introduced in bread baking. However, most of these additives are either E-numbered additives or produced with genetically modified organisms (GMOs). Nowadays these additives do not fulfill the current consumer trends which prefer GMO-free, high-quality products baked without chemical additives. Therefore, Sourdough baking is an alternative to the use of additives. Sourdough fermentation is known to improve the nutritional value of cereal products (Liukkonen *et al.* 2003, Kariluoto *et al.* 2004).

The majority of gluten-free breads available are of poor sensory and textural quality. Bran supplementation usually weakens the structure and baking quality of wheat dough and decreases bread volume and the elasticity of the crumb. Recently, it has been reported that certain lactic acid bacteria are able to produce exopolysaccharides, which might have a positive effect on bread volume and shelf-life (Korakli *et al.*, 2001, Tieking *et al.*, 2003). In gluten-free bread, dextran from *Weissella cibaria* improved nutritional and organoleptic properties of gluten-free bread (Schwab *et al.*, 2008). Dextran enriched *Weissella cibaria* MG1 sourdough increased loaf volume and improved crumb softness and provided mildly acidic bread with an improved shelf life of gluten-free sorghum sourdough bread (Galley *et al.*, 2012). In addition to dextran, *Weissella cibaria* MG1 also produced oligosaccharides during sorghum sourdough fermentation contributing to the nutritional benefits of gluten-free sorghum bread (Galley *et al.*, 2012). Bread prepared by adding 50% sourdough produced with the starter cultures of *Leuconostoc citreum* HO12 and *Weissella koreensis* HO20, exhibited consistent ability to retard the growth of bread spoilage fungi (*Penicillium roqueforti* and *Aspergillus niger*) and rope-forming bacterium (*Bacillus subtilis*) (Choi *et al.*, 2012).

Hydrocolloids have been reported to improve bread quality (Rosell *et al.*, 2001). The exopolysaccharides influence the product texture, mainly due to their ability to influence viscosity. *Weissella* sp. has emerged as potential *in-situ* dextran producer in sourdough. Katina *et al.*, 2009 established the potential of *Weissella confusa* VTT E-90392 in sourdough fermentation. *Weissella confusa* VTT E-90392 produced significant amounts of polymeric dextran and isomaltooligosaccharides in wheat sourdough without strong acidification (Katina *et al.*, 2009).

The endogenous cereal proteases of flours have been shown to degrade cereal prolamins under acidic conditions (Kawamura and Yonezawa, 1982, Brijs *et al.*, 1999). Increased proteolysis during sourdough fermentation leads to the liberation of amino acids in wheat and rye dough (Spicher and Nierle, 1988, Collar *et al.*, 1991, Gobbetti *et al.*, 1994). Furthermore, sourdough fermentation results in a solubilization and depolymerization of the gluten macropolymer (Thiele *et al.*, 2004). Lactic acid bacteria also produce volatile compounds which is strain-specific. Homofermentative Lactobacilli are characterized by the high production of diacetyl, acetaldehyde, hexanal and heterofermentative strains are characterized by the production of ethyl acetate, alcohols and aldehydes. Isoalcohols (2-methyl-1-propanol, 2,3-methyl-1-butanol), with their respective aldehydes and ethylacetate are characteristic volatile compounds of yeast fermentation (Damiani *et al.*, 1996).

1.6 *Weissella confusa*

The *Weissella* genus, first described by Collins *et al.*, 1993, includes Gram-positive, obligate heterofermentative, catalase negative *coccobacilli*, producing D- or DL-isomers of lactic acid as main end products of fermentation (Collins *et al.*, 1993). *Weissella confusa* has been isolated from a variety of sources such as sugar cane,

carrot juice, milk, fermented foods and beverages and human and animal samples (Bjorkroth *et al.*, 2002; Dworkin *et al.*, 2006, Fusco *et al.*, 2011; Kumar *et al.*, 2011).

It is more commonly found in many fermented cereals and vegetables (Bjorkroth *et al.*, 2002; Dworkin *et al.*, 2006, Fusco *et al.*, 2011).

1.6.1 Phylogentic relationship of *Weissella confusa*

The phylogeny of the bacteria classified currently in the genus *Weissella* was clarified in 1990. The genus *Weissella* is phylogenetically related to *Leuconostoc* and *Oenococcus* and arose from the reclassification of *Leuconostoc paramesenteroides* and some related atypical heterofermentative Lactobacilli (Björkroth and Holzapfel, 2006). In a study of *Leuconostoc*-like organisms originating from fermented sausages (Collins *et al.*, 1993) the taxonomy of these species was further assessed. Thus there are currently eight species in the genus *Weissella*, *Weissella confusa*, *Weissella halotolerans*, *Weissella hellenica*, *Weissella kandleri*, *Weissella minor*, *Weissella paramesenteroides*, *Weissella thailandensis* and *Weissella viridescens*. *Weissella confusa* was formerly known as *Lactobacillus confuses*. *Weissella confusa* possessed the highest 16S rDNA sequence similarity to *Weissella cibaria*, but the DNA-DNA reassociation experiment showed hybridization levels below 49% between the strains studied (Björkroth *et al.*, 2002).

1.6.2 Identification of *Weissella confusa*

The identification of *Weissella confusa* and the other *Weissella* species traditionally relies on their biochemical and physiological features. These biochemical and physiological features include the production of gas from carbohydrates, the presence within the cell wall of lysine and alanine joined with an intrapeptide bond,

the hydrolysis of arginine, the formation of D, L-lactate and the ability to ferment different sugars (Björkroth *et al.*, 2002; Collins *et al.*, 1993; De Bruyne *et al.*, 2008; Koort *et al.*, 2006). Given its widespread use for biotechnological applications, Fusco *et al.*, 2011 developed rapid and reliable method involving polymerase chain reaction (PCR) using Amplified Fragment Length Polymorphism (AFLP) derived primers for the identification of *Weissella* sp. Recently, the first genome sequence for the *Weissella confusa* LBAE C39-2 species has become available (Amari *et al.*, 2012). A total of 86,967 filtered reads corresponded to 33 Mb and 14-fold coverage of in the genome sequence of *Weissella confusa* LBAE C39-2 were obtained (Amari *et al.*, 2012). The phylogenetic analysis of the genome sequence of *Weissella confusa* LBAE C39-2 showed that less than 30% contig sequences matched with *Weissella ciberia* KACC11862 (Amari *et al.*, 2012).

1.6.3 Metabolic traits of *Weissella confusa*

Weissella confusa shows multiple metabolic traits which can be exploited for biotechnological applications. *Weissella confusa* has been successfully employed in a multi-species semi-liquid ready-to-use sourdough starter (Gaggiano *et al.*, 2007). Di Cagno *et al.*, 2009 used strains of *Weissella confusa* as autochthonous starters to ferment tomato juice as well as red and yellow peppers. Some metabolic traits other than lactic acid fermentation, such as exopolysaccharide production from sucrose (Maina *et al.*, 2008; Shukla and Goyal, 2011) and antifungal activity (Ndagano *et al.*, 2011; Beak *et al.*, 2012), have been reported, highlighting that *W. confusa* could be attractive for diverse biotechnological applications. *W. confusa* is also present in the normal microflora of human intestines (Mitsuoka, 1992) and has been described as a potential probiotic species (Lee, 2005). Three *Weissella confusa* and five *Weissella*

cibaria strains were isolated from human faeces and their potential as probiotics was examined by Lee *et al.*, 2012. *W. confusa* is a suitable alternative to the widely used *Leuconostoc mesenteroides* NRRL B-512F in the production of high amounts of linear dextran (Maina *et al.*, 2008). *Weissella confusa* has been successfully employed for *in-situ* production of dextrans and isomaltooligosaccharides in wheat sourdoughs without strong acidification (Katina *et al.*, 2009).

1.6.4 Dextranucrase, dextran and oligosaccharide production from *Weissella confusa*

There are very few reports where the dextran production capacity of *Weissella confusa* has been explored. While dextranucrase from *Leuconostoc* are inducible by sucrose, it has been reported that dextranucrase from several *Weissella sp.* are constitutive in nature (Bounaix *et al.* 2010). Dextranucrase of 180 kDa size from *Weissella cibaria* and *Weissella confusa* (LBAE-C39-2 and DSM 20196) have been reported in medium containing sucrose or glucose as the carbon source (Bounaix *et al.*, 2010). Bounaix *et al.*, 2010 also reported an additional sucrose inducible dextranucrase of approximately, 300 kDa for *W. confusa* DSM 20196 which did not form in the medium containing glucose. Malik *et al.*, 2009 characterized three glucantransferase genes (*gtf*) and one fructantransferase gene (*ftf*) from *Weissella confusa* strains MBF8-1 and MBF8-2. Amari *et al.*, 2012 reported the first complete gene sequence encoding dextranucrase from a *W. confusa* strain (LBAE C39-2) along with the one from a *W. cibaria* strain (LBAE K39). A recombinant dextranucrase (rDSRC39-2) of approximately, 180 kDa from *Weissella confusa* isolated from sourdough has been characterized (Amari *et al.*, 2012). The dextran produced by *Weissella confusa* has gained importance recently due to its linear structure (Maina *et al.*, 2008). Dextran produced by *Weissella sp.* is more linear in

nature (Maina *et al.*, 2008) as compared to 95% linearity in glucan formed from *Leuconostoc mesenteroides* NRRL B-512F (Seymour and Knap, 1980). Bounaix *et al.*, 2010 also reported that *Weissella* strains isolated from sourdoughs which produce linear dextran containing α -(1 $\rightarrow$ 6) glucose residues with few α -(1 $\rightarrow$ 3) linkages from sucrose produced by a constitutive dextransucrase of 180 kDa size. The production of exopolysaccharide by *Weissella* strains, isolated from a traditional Thai food (plasom) was investigated using sugarcane molasses and white sugar from sugarcane as substrates in comparison to analytical grade sucrose (Tayuan *et al.*, 2011). The dextran (rDSRC39-2 dextran) produced by a recombinant dextransucrase from *Weissella confusa* C39-2 contained consecutive α -(1 $\rightarrow$ 6)-linked D-glucopyranosyl units with few α -(1 $\rightarrow$ 3)-linked branches (Amari *et al.*, 2012). Several reports suggest that *Weissella* sp are very promising candidates for sourdough fermentation due to the production of significantly high dextran (Katina *et al.*, 2009; Galle *et al.*, 2010). Galle *et al.*, 2010 reported *in-situ* production of panose and glucosylated panose with a degree of polymerization of up to 14 from *Weissella ciberia* MG1. Katina *et al.*, 2009 also reported similar observation of oligosaccharide production in wheat sourdough.

1.7 Objectives of the present study

The screening of industrially important lactic acid bacteria from the untapped Indo-Burma biodiversity hot spot, the Northeast India, deserves special attention. The North-Eastern region is a genetic treasure house of plant, animal and microbial resources. The north eastern Indian region has been in focus for its high biodiversity and has been a priority for leading conservation agencies of the world. The hot and humid tropical climate of Assam is conducive for isolation of microbes from the soil producing rare and novel industrial enzymes and bioactive exopolysaccharides. This North-Eastern India region is a promising genetic hub due to its vast untrapped resources. The aim of the current study is to explore the natural biodiversity to isolate novel hyper dextranucrase and dextran producing strains of lactic acid bacteria. On the basis of higher enzyme activity, the bacterial colonies will be selected. The physiological and biochemical characterization such as Gram staining, catalase test, carbohydrate utilization pattern and antibiotic susceptibility experiments will be carried out to distinguish the isolate from other closely related lactic acid bacteria. The isolated bacterium will be identified at species and genus level based on 16S rRNA gene sequencing. The culture conditions for maximum enzyme production will be optimized. The growth parameters *viz.* the cell optical density, pH, enzyme activity and protein concentration of the selected isolate will be studied. The purification of dextranucrase from the isolate will be carried out using the polyethylene glycol fractionation and gel filtration. The purified dextranucrase will be characterized by non-denaturing gel and zymogram analysis. The reaction conditions for enzyme such as sucrose concentration, temperature, pH and ionic strength of the buffer will be optimized. The effects of different salts, temperature and additives on the dextranucrase activity of the isolate will be studied.

The purified dextransucrase will be used to synthesize dextran. The crude dextran will be purified by alcohol precipitation and subjected to lyophilization. The dextran structure will be analyzed by optical rotation, FT-IR, NMR and SEM studies. HPSEC analysis will be carried out to determine the M_w of dextran from the isolate. The dextran will be studied for the rheological properties. Dynamic light scattering (DLS) analysis of dextran will be studied to understand the molecular weight distribution pattern of dextran. Cytotoxicity test of the dextran will be performed to evaluate its biomedical potential. Statistical methods will be employed to enhance the dextransucrase and dextran production from the natural isolate of lactic acid bacterium. The natural isolate will be explored for its potential *in situ* dextran production in sourdough fermentation using wheat bran. The purified dextransucrase from the isolate will be used for the production of gluco-oligosaccharides using maltose as the acceptor molecule. The gluco-oligosaccharides produced will be purified and characterized using various techniques such as HPAEC-PAD, FT-IR and NMR spectroscopy, gel permeation chromatography and ESI-TOF-MS analysis.

1.8 Specific objectives of present study

1. Isolation, characterization and identification of dextran producing isolate of lactic acid bacteria from sauerkraut.
2. Production, purification and characterization of dextransucrase from the isolate.
3. Production, purification and characterization of dextran from isolate.
4. Medium optimization for dextransucrase and dextran production from the isolate by statistical methods.
5. Sourdough fermentation involving *in situ* dextran production by isolate for improving textural property of wheat bran.
6. Production of gluco-oligosaccharides by acceptor reaction of dextransucrase and their purification and characterization.

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Chapter 2

Identification and characterization of a dextran hyper-producing *Weissella confusa* Cab3 from sauerkraut

2.1 Introduction

Members of the genus *Weissella* have been isolated from a variety of sources, such as fresh vegetables, fermented silage, meat or meat products. The *Weissella* species are Gram-positive, non-spore-forming, heterofermentative, nonmotile, irregular or coccoid rod-shaped organisms. Several lactic acid bacteria belonging to the genera *Weissella* have been introduced to wheat sourdough baking for *in situ* production of exopolysaccharides (Katina *et al.*, 2009). *Weissella kimchii* PL9023 was selected as a probiotic as it produced most hydrogen peroxide that inhibited the growth and adherence of vaginal isolates of *Candida albicans*, *Escherichia coli*, *Staphylococcus aureus* and *Streptococcus agalactiae* (Lee, 2005). Lactic acid bacteria (LAB) that produce dextrans include various *Leuconostoc*, *Streptococcus* and *Lactobacillus* species (Sidebotham, 1974). Smitinont *et al.*, 1999 had emphasized on dextran synthesizing ability of the *Pediococcus* genus. Patel *et al.*, 2010 reported the dextran production ability of *Pediococcus pentosaceus* for the first time. Additionally, dextran production has typically served as a phenotypic test in the identification of bacteria classified in the genus *Weissella* (Collins *et al.*, 1993). Maina *et al.*, 2008

reported industrially useful dextran producing *Weissella confusa* E392 which could be suitable alternative to the widely used *L. mesenteroides* B512F in the production of linear dextran.

First proposed by Collins *et al.*, 1993 on the basis of the results of 16S rDNA phylogenetic analyses, the genus *Weissella* encompasses a phylogenetically coherent group of lactic acid bacteria and includes twelve validated *Leuconostoc*-like species currently including *Weissella confusa* (formerly *Lactobacillus confusus*), *W. minor* (formerly *Lactobacillus minor*), *W. kandleri* (formerly *Lactobacillus kandleri*), *W. halotolerans* (formerly *Lactobacillus halotolerans*), *W. viridescens* (formerly *Lactobacillus viridescens*), *W. paramesenteroides* (formerly *Leuconostoc paramesenteroides*), *W. hellenica*, *W. thailandensis*, *W. cibaria*, *W. kimchii*, *W. soil* and *W. koreensis*. Alternative molecular biological approaches have been developed in place of classical phenotypic methods for the identification of *Weissella* species. Such methods include soluble whole cell protein pattern analyses (Villani *et al.*, 1997), restriction pattern of ribosomal DNA (Villani *et al.*, 1997), fatty acid analyses (Samelis *et al.*, 1998), random amplified polymorphic DNA-PCR (RAPD-PCR) (Kurzak *et al.*, 1998), denaturing gradient gel electrophoresis (DGGE) (Ampe *et al.*, 1999) and PCR targeting 16S/23S rRNA gene spacer region (Kurzak *et al.*, 1998). In the present study, a novel *Weissella* species isolated from sauerkraut was characterized up to genus and species level using 16S rDNA based sequence analyses.

2.2 Material and Methods

2.2.1 Chemicals, reagents and kits

The antibiotic tests were performed using commercially available antibiotic octodiscs from Hi-media Pvt. Ltd. India. Ingredients required for the maintenance and enzyme production media were from Hi-Media Pvt. Ltd., India. All the chemicals required for reducing sugar estimation and buffer preparation were of highest purity grade available commercially. *Taq* DNA polymerase was supplied by Bangalore Genei Ltd., Bangalore, India. The dNTPs and $MgCl_2$ were acquired from Bioline, USA. PCR tubes (0.2 ml) used were from Axygen, Germany. RNase solution (20 mg/ml), glacial acetic acid (99.9 % pure) Trizma base (Tris free base), ethidium bromide and DNase-RNase free water (pH 8.0). The miniprep plasmid isolation kit was purchased from Sigma-Aldrich Pvt. Ltd., India. The gel extraction kit was purchased from Qiagen, Germany. The DNA marker *viz* Hyperladder I was purchased from Bioline, UK. Private Limited.

2.2.2 Fermentation of cabbage for sauerkraut production

The fresh cabbage sample was collected from the market of Indian Institute of Technology Guwahati (Assam). 130 g of cabbage was chopped into small pieces without peeling and soaked in 100 ml of NaCl (2.5%) solution in a 500 ml glass bottle. The bottle was tightly closed so that the NaCl solution shall not come in contact with outside air and kept for incubation at 30°C for 5 days.

2.2.3 Media used for the isolation and propagation of lactic acid bacteria

The MRS agar medium (DeMan *et al.*, 1960) contained (% w/v): glucose, 2; yeast extract powder, 0.5; beef extract, 1 and peptone, 1; dipotassium hydrogen

orthophosphate, 0.2; tri-ammonium citrate, 0.2; sodium acetate, 0.5; Tween 80, 0.1 (v/v); $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.02 and agar, 1.5. The pH of the medium was adjusted to 6.4 by of concentrated acetic acid. The MRS medium was sterilized by autoclaving at a steam pressure of 10.3 kPa (15 lb/in²) and a temperature of 121°C for 20 min.

2.2.4 Medium For enzyme production

The enzyme production medium described by Tsuchiya *et al.* (1952) contained (g/l) sucrose, 20; yeast extract, 20; K_2HPO_4 , 20; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.2; $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.01; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.01; NaCl 0.01; 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/in²) and a temperature of 121°C for 20 min.

2.2.5 Isolation of bacterial strain

One gram of fermented cabbage (sauerkraut) was ground to paste and mixed in 10 ml of saline (0.9% w/v) homogeneously in test tubes. The serial dilutions were made till the dilution factor 10^{-5} . One hundred microliter from all the dilutions of sauerkraut from 10^0 to 10^{-5} dilution was taken and spread plated on MRS agar plates with glucose as carbon source (DeMan *et al.*, 1960) as described earlier in Section 2.2.3. The petriplates were incubated at 28°C for 24 h. A total of 25 distinct and larger colonies were picked by sterile inoculation loop and streaked further on MRS agar plates (DeMan *et al.*, 1960) as described earlier in Section 2.2.3 and incubated at 28°C for 24 h. A loopful of cultures were grown in 5 ml enzyme production medium as described by Tsuchiya *et al.*, 1952 (described earlier in Section 2.2.4) for 12 h at 28°C and 180 rpm. 1% of these 12 h grown culture was again inoculated in 5 ml sterile

medium of Tsuchiya *et al.*, 1952 (described earlier in Section 2.2.4) in test tubes and grown for 12-14 h at 28°C and 180 rpm for enzyme assays. The enzyme activities of all the natural isolates were determined and the isolate with highest dextransucrase activity was selected. The protocol for assay of dextransucrase activity is given later in this Chapter in Section 2.2.10.2. The isolated bacterium was propagated as stab in MRS agar supplemented with sucrose as carbon source (Goyal and Katiyar, 1996) and subcultured every 2 weeks at 28°C and stored at 4°C. For long term preservation, the isolate was kept in 20% (v/v) glycerol at –80°C.

2.2.6 Morphological and biochemical characterization of isolate Cab3

The colony morphology of isolate grown on MRS medium (described earlier in Section 2.2.3) for overnight at 30°C was observed directly and by light microscopy. The Gram reaction was performed using the KOH method of Gregesen, 1978. The cell morphology of the selected isolate was examined by scanning electron microscopy (SEM) (Leo1430 VP, Leo Electron Microscopy Ltd., Cambridge, UK) operated at 10.0 kV. The cell sample for SEM was prepared by centrifuging 1 ml of 12 h grown culture at 5,000 rpm at 4°C for 10 min. The pellet was washed twice with 1 ml of saline solution (0.85%, w/v). The sample was fixed with glutaraldehyde (2.5%, v/v) for 4 h. The sample was dehydrated using different percent of alcohol 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. The catalase activity of the isolate was determined by transferring fresh colonies from MRS agar (described earlier in section 2.2.3) to a drop of 5% (v/v) H₂O₂ on a glass slide (Kannan, 2002). The *E. coli* culture was treated with H₂O₂ under

identical conditions as positive control for catalase activity. For the temperature tolerance of the microorganism, the growth of selected isolate was carried out using MRS medium at different temperatures *viz.* 4°C, 10°C, 15°C, 20°C, 30°C, 37°C, and 42°C for 2 d. For salt tolerance of the isolate the growth was observed using MRS medium in the presence of 4.0 and 6.5% (w/v) of NaCl incubated at 25°C under shaking at 180 rpm for 2 d. The production of dextran (slimy layer) by isolate Cab3, from sucrose was observed on MRS agar in which glucose was replaced by sucrose (Goyal and Katiyar, 1996).

2.2.7 Antibiotic sensitivity profile of isolate Cab3

The isolate Cab3 was tested for its susceptibility to 29 antibiotics using agar disc diffusion test (Barry and Thornsberry, 1980). The antibiotic test was performed using commercially available antibiotic octodiscs impregnated with Amoxyclav, Cephalexin, Ciproflaxacin, Clindamycin, Claxacillin, Erythromycin, Tetracyclin, Ampicillin, Carbenicillin, 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 petri-plates were first laid with MRS medium containing (as described in Section 2.2.3) 1.8% (w/v) agar. The isolate Cab3 was grown in MRS liquid medium, mixed in MRS-soft agar (0.8%, w/v agar) and poured over the MRS medium containing 1.8% (w/v) agar. 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 overnight at 30°C and were observed next day for zone of inhibition around the discs (Purama *et al.*, 2008).

2.2.8 Carbohydrate fermentation profile of isolate Cab3

The isolate Cab3 was tested for its ability to ferment various carbohydrates using the method of Kandler and Weiss (1986). From the overnight grown MRS broth (described earlier in section 2.2.3) containing 2% (w/v) glucose as carbohydrate source, 50 µl was inoculated in 5.0 ml liquid MRS medium lacking glucose but containing phenol red and other test carbohydrates to give a final inoculum to medium ratio of 1% (v/v). The test media were incubated for 2 d at 30°C without shaking. The acid production was recorded between 24-48 h. The acid production was indicated by a change in color of the phenol red indicator dye from red to yellow.

2.2.9 16S rRNA based identification of isolate Cab3

2.2.9.1 Extraction of genomic DNA

The genomic DNA was extracted by HiPurA Bacterial and Yeast Genomic DNA Purification Spin Kit (Himedia India Pvt. Ltd.) as described below:

1. The bacterial culture of Cab3 (1% inoculum) was grown in MRS medium (as described in Section 2.2.3) at 28°C and 180 rpm for 14-16 h. The 5 ml of broth was centrifuged at 12000g for 2 min at 4°C. The supernatant was completely discarded.
2. The cell pellet was resuspended in 200 µl lysozyme solution (2.0×10^5 U/ml, 2 mg/ml) and incubated for 30 min at 37°C.
3. 20 µl of the RNase A solution (20 mg/ml) (DS0003) was added to the above solution, mixed and incubated for 2 min at room temperature.
4. 20 µl of the proteinase K solution (20 mg/ml) (RM2957) was added to the above solution and vortexed thoroughly for 15 sec and incubated at 55°C for 10 min.

5. DNA was then precipitated by adding 200 μ l of absolute alcohol to the lysate and mixed thoroughly by vortexing for 15 min.
6. The lysate was passed through HiElute miniprep spin column (DBCA02) provided with the kit by centrifugation at 12000g for 10 min. The flow through liquid was discarded and the spin column was placed in a new 2.0 ml collection tube.
7. 500 μ l of prewash (PWB: DS0012) solution was added to the column and centrifuged for 3 min at maximum speed (16,000g) to dry the column. The column should be free of ethanol before eluting the DNA.
8. 50 μ l of the elution buffer (ET: DS0040) was added directly to the column without spilling to the sides and incubated for 1 min at room temperature. The column was then centrifuged at 8000g to elute the DNA.

The extracted DNA was purified using GenElute Gel Extraction Kit (Sigma, St. Louis, USA) as described later in Section 2.2.9.5. DNA concentrations were determined in duplicate using a UV-Visible spectrophotometer (Varian, Carry 100). The quality of purified DNA was evaluated on 0.8% agarose gel (as described later in Section 2.2.9.3).

2.2.9.2 Amplification of 16S rRNA gene by Polymerase Chain Reaction

The genomic DNA of isolate Cab3 was used for amplification of 16S rRNA gene. The universal 16S rDNA primers, forward primer 8F and reverse primer 1492R (Table 2.2.1) were used for the polymerase chain reaction (PCR) using *Taq* DNA polymerase (Bangalore Genei Ltd., Bangalore, India). The details of 50 μ l PCR reaction mixture and amplification conditions or PCR cycles are mentioned below (Table 2.2.2 and 2.2.3). The PCR amplifications were performed using a thermal

cycler (Applied Biosystems, GeneAmp® PCR System 9700). The amplified fragment was detected on 0.8 (% w/v) agarose gel as mentioned in Section 2.2.9.3.

Table 2.2.1 Universal primers used for amplification of 16S rRNA gene from isolate Cab3.

Primer	Primer sequence
8F	5'-AGTTGATCCTGGCTCAG-3'
1492R	5'-ACCTTGTTACGACTT-3'

Table 2.2.2 PCR mixture for amplification of 16S rRNA gene from isolate Cab3.

PCR components	Volume (µl)	Final concentration
10x reaction buffer	5.0	1x
dNTP mix (100 mM)	1.0	2.5 mM
Forward primer (15 µM)	1.7	0.5 mM
Reverse primer (15 µM)	1.7	0.5 mM
Genomic DNA (10 ng/µl)	1.0	10 ng
<i>Taq</i> DNA polymerase (5 U/µl)	1.0	5 U
MgCl ₂ (1 M)	2.5	50 mM
DNase free water, pH 8.0 (Sigma Aldrich)	36.1	---
Total	50.0	

Table 2.2.3 PCR cycles for amplification 16S rRNA gene from isolate Cab3.

Steps	Time (min)
I. Denaturation at 94 °C	4.0
II. 30 cycles of	i) Denaturation at 94 °C
	ii) Annealing at 55 °C
	iii) Extension at 72 °C
III. Final extension at 72 °C	10.0

2.2.9.3 Agarose gel electrophoresis of PCR amplified and other DNA

Fifteen µl of PCR amplified product was run on 0.8% agarose gel prepared in 1x TAE buffer. A stock solution of 10x TAE (400 mM Tris-acetate, 10 mM EDTA pH 8.0) buffer was prepared according to method reported by Sambrook and Russell (2001). The gel was prepared by dissolving 400 mg of agarose in 50 ml of 1x TAE

buffer (for 0.8% gel) by heating in a microwave oven for few min to get a clear solution. Then 5.0 μ l of ethidium bromide (5.0 mg/ml) was added when the solution temperature was around 50°C. The solution was mixed and poured on the casting apparatus, combs were placed and the gel was allowed to set for 30 min. For the separation of PCR amplified DNA, 1x TAE (Tris-acetate-EDTA) buffer was used for preparation of agarose gels and as an electrophoresis running buffer (Sambrook and Russel, 2001). The DNA sample and 5x DNA sample loading buffer (as described in Section 2.2.9.4) were mixed in 4:1 ratio and the gel was run at 60 Volts for 1h. The bands were then visualized under UV illumination in a gel documentation system (Bio-RAD).

2.2.9.4 DNA sample loading buffer

The DNA sample loading buffer was prepared by mixing the components mentioned below in Table 2.2.4. A 5x stock solution of DNA loading buffer was prepared and was mixed with 4 volumes of DNA to make it to 1x before loading on to the agarose gel. The final pH of the DNA loading buffer adjusted to pH 8.0.

Table 2.2.4 Composition of 5x DNA loading buffer.

Components	Final concentration (5x)
Tris-HCl	50 mM
Glycerol	25% (w/v)
EDTA	5.0 mM
Bromophenol blue	0.2% (w/v)
Xylene cyanol	0.2% (w/v)

2.2.9.5 Extraction of DNA from agarose gel

The PCR amplified DNA or other plasmid DNA was purified from agarose gel using a kit (Qiagen, QIAquick® Gel Extraction Kit), following the protocol provided by the manufacturer as described below:

DNA gel extraction protocol

1. Weights of 1.5 ml sterile, empty microcentrifuge tubes were noted.
2. The extracted genomic DNA or PCR-amplified DNA were excised from gel using sharp sterile scalpel and transferred to empty microcentrifuge tubes. The tubes were weighed again and the weight of excised gel was determined by subtracting the empty tube weight (noted above).
3. Now, 3 volumes of buffer QG were added to every 1 volume of gel (100 mg ~ 100 µl).
4. The microcentrifuge tubes containing excised gel were incubated at 50°C for 10 min (or until the gel slice has completely dissolved). When the gel slice dissolved completely, the color of the solution became yellow (similar to Buffer QG without dissolved agarose).
5. 1 gel volume of isopropanol was added to above yellow colored solution in case of PCR amplified DNA (For higher yield of DNA fragments <500 bp and >4 kb).
6. QIAquick spin column (DNA binding column) was placed in 2 ml collection tube provided with the kit. The above solution containing extracted genomic DNA or PCR-amplified DNA (750 µl) were added to DNA binding columns and centrifuged at 17,900g for 1 min at room temperature, and the flow

- through was discarded. If the volume was more than 750 μ l, the remaining solution was centrifuged similarly and again the flow through was discarded.
7. 500 μ l of buffer QG was added to each QIAquick spin column and the mixtures were centrifuged again at 17,900g for 1 min at room temperature, and the flow through was discarded.
 8. Now, 750 μ l of buffer PE was added to each column containing extracted genomic DNA or PCR-amplified DNA (750 μ l) and the mixture was centrifuged at 17,900g for 1 min at room temperature. The flow through was discarded and the column was given an additional spin of 1 min at 17,900g, to completely remove the residual ethanol.
 9. Now the column containing bound DNA was placed in a fresh 1.5 ml sterile microcentrifuge tube. 50 μ l of DNase free water (Sigma-Aldrich Pvt. Ltd., USA) or elution buffer (10 mM Tris-Cl, pH 8.5) was added at the centre of the column. The column was incubated for 2 min at room temperature and centrifuged at 17,900g for 1 min.
 10. The extracted genomic DNA or PCR-amplified DNA were eluted from QIAquick spin columns got collected in 1.5 ml sterile microcentrifuge tube. The extracted genomic DNA or PCR-amplified DNA (50 μ l) were stored at -20°C for further use.

2.2.9.6 Sequencing of 16S rRNA gene

The sequence of 16S rRNA gene was obtained from Xcelris Labs Limited, Ahmedabad, India. Forward and reverse DNA sequencing of PCR amplicon was carried out with 8F and 1492R primers using BDT v 3.1 Cycle sequencing kit on ABI

3730xl Genetic Analyzer. Consensus sequence of 1288 bp of 16S rDNA gene was generated from forward and reverse sequence data using aligner software.

2.2.9.7 Sequence alignment of 16S rRNA gene

The 16S rDNA gene sequence of the isolate Cab3 was used to carry out BLAST with the nrdatabase of NCBI genbank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4 (Tamura *et al.*, 2007).

2.2.10 Production of dextransucrase under different culture condition

2.2.10.1 Preparation of reagents for reducing sugar estimation

The reagents for estimation of reducing sugar were prepared by the method of Nelson, 1944 and Somogyi, 1945.

Reagent A: Sodium carbonate anhydrous (25 g), sodium potassium tartarate (25 g), sodium bicarbonate (20 g) and sodium sulfate anhydrous (200 g) dissolved in water and the volume made upto 1 l. 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 water and mixed and placed in an incubator at 37°C for 24 h.

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

2.2.10.2 Enzyme activity 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 was added for reducing sugar estimation. The solution was mixed and heated for 20 min in boiling water bath. It was cooled to the room temperature and 100 µl of reagent C was then added. The color developed rapidly and simultaneously with evolution of carbondioxide. The mixture was diluted by adding 700 µl distilled water. The absorbance of color developed was measured at 500 nm on a UV-visible spectrophotometer (Varian, Cary 100). Fructose was used to plot the standard graph.

2.2.10.3 Calculation of enzyme activity

One unit (U) of dextransucrase activity is defined as the amount of enzyme that liberates 1 µmole of reducing sugar per min. 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

2.2.10.4 Effect of temperature on enzyme production

The dextranucrase was produced in the enzyme production medium as described by Tsuchiya *et al.* (1952) as described earlier in Section 2.2.4. Fermentation of *Weissella confusa* Cab3 was carried out in triplicate sets of 60 ml enzyme-production medium in a 250 ml Erlenmeyer flask incubated at various temperatures 22°C, 25°C, 28°C, 30°C, 32°C, 37°C and 40°C at 180 rpm in an orbital shaking incubator to determine the optimum incubation temperature for maximum enzyme production. The culture (1.0 ml) were withdrawn at the indicated time intervals and centrifuged at 8000g at 4°C for 10 min to pellet the cells. The cell free supernatant was analyzed for enzyme activity as described in Section 2.2.10.2.

2.2.10.5 Effect of shake flask culture on enzyme production

The production of dextranucrase was compared under shaken flask condition with the static flask culture at 25°C in triplicate sets of 60 ml enzyme production medium in 250 ml Erlenmeyer flasks. The shaking was carried out in an orbital shaking incubator at 180 rpm. The samples (1.0 ml) were withdrawn at indicated time intervals and centrifuged at 8000g for 10 min at 4°C to pellet out the cells. The cell free extract was analyzed for enzyme activity as described earlier in section 2.2.10.2.

2.2.11 Sucrose estimation

The sucrose concentration was determined by estimating the reducing sugars by the method of Sumner and Sisler (1944) as described in Chapter 3, Section 3.2.3.

2.3 Results and Discussion

2.3.1 Selection of the isolate Cab3

After 24 h incubation of the isolates on MRS agar medium (described earlier in Section 2.2.3) and observation under the colony counter, a total of 25 colonies were picked up for their enzyme assay. Among them, six colonies showing relatively higher enzyme activity were selected for further study. They were named as Cab1, Cab3, Cab4, Cab7, Cab9 and Cab14 respectively after the source and colony numbers. The enzyme activities of the isolates are listed in Table. 2.3.1.

Table 2.3.1 Enzyme activity of the six selected isolates.

S. No.	Isolates	Enzyme activity (U/ml)
1	Cab1	1.9
2	Cab3	5.3
3	Cab4	2.3
4	Cab7	2.1
5	Cab9	3.1
6	Cab14	2.9

Among the six isolates, Cab3 showed significantly higher, enzyme activity (5.3 U/ml) at 28°C and 180 rpm. The production of dextransucrase can further be optimized to increase dextransucrase activity from the natural isolate Cab3. This isolate Cab3 was used for further characterization and study.

2.3.2 Morphological and biochemical characterization of isolate Cab3

Based on higher dextransucrase activity, a microbe Cab3 isolated from the sauerkraut was further biochemically and physiologically characterized (Shukla and Goyal, 2011). The isolated culture was identified according to their morphological,

cultural, physiological and biochemical characteristics (Green *et al.*, 1991; Patel *et al.*, 1994). The tests used were: Gram reaction, production of catalase and growth at different temperatures and salt concentrations, acid production from carbohydrates (2%, w/v). *Weissella confusa* can be identified and differentiated from other species such as *Enterococcus* spp., *Streptococcus* sp., *Lactococcus* spp., and *Leuconostoc* spp. by its biochemical and physiological properties like arginine deamination, esculin hydrolysis, growth at 42°C, and acidification of certain carbohydrates (Olano *et al.*, 2001). The purple colored bacterial cells of Cab3 after following Gram staining protocol showed its Gram-positive nature (Fig. 2.3.1).

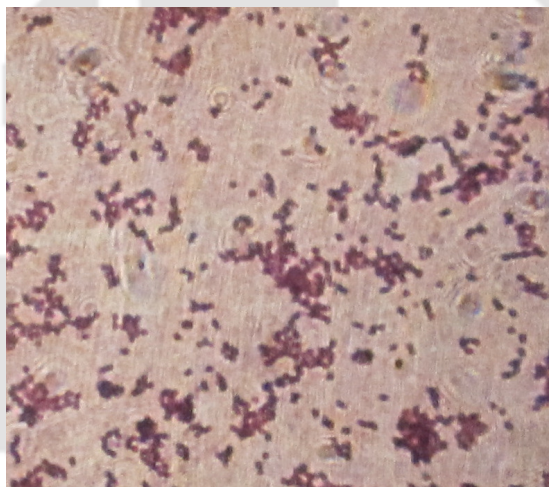


Fig. 2.3.1 Gram staining of the isolate Cab3 showing purple colour, indicating its Gram positive nature.

Scanning electron microscopy of the isolate Cab3 showed phenotypically homogeneous with irregular short rod cells in pairs or chains with a width and length of 0.5 -0.6 μm and 1.2-1.4 μm , respectively (Fig. 2.3.2).

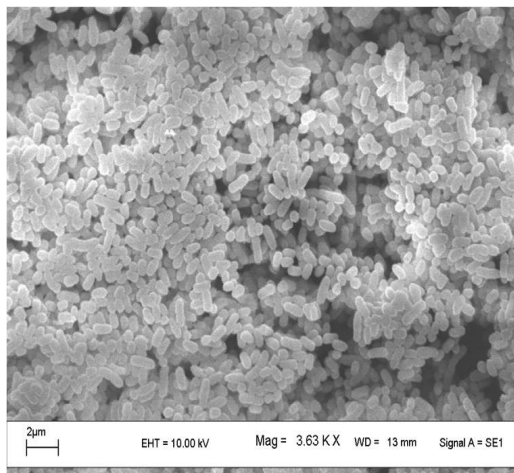


Fig. 2.3.2 Scanning Electron Microscopy (SEM) of the isolate Cab3.

The colonies on MRS agar medium (described earlier in section 2.2.3) were opaque, circular and slightly convex (Fig. 2.3.3 A). The colony produced slimy layer when grown on modified MRS agar plate supplemented with sucrose (Fig. 2.3.3 B). The slime production indicated the production of extracellular polysaccharide.

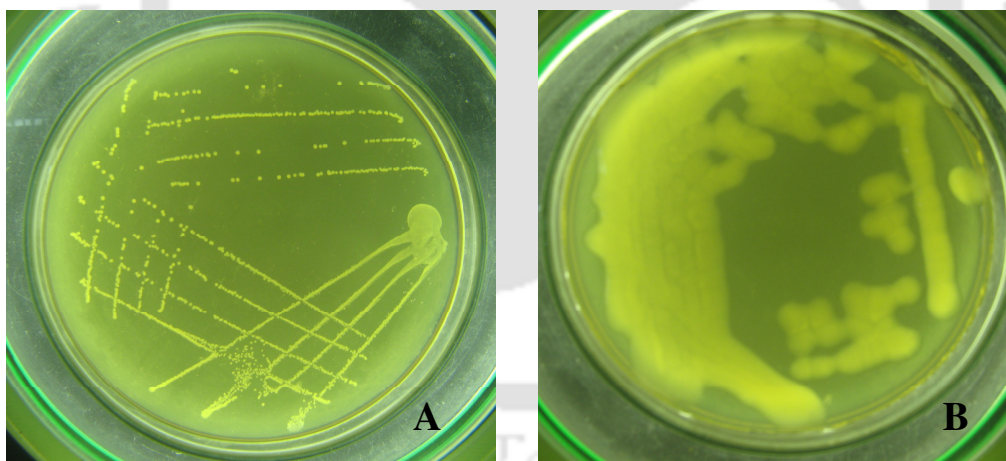


Fig. 2.3.3 The colony morphology of the isolate Cab3 on (A) MRS agar medium, (B) modified MRS agar plate showing slime formation.

The isolate Cab3 showed growth between 15-42°C. The isolate Cab3 could grow in presence of 4.0, 5.0, 6.0, and 6.5% (w/v) NaCl, but not beyond 8.0% NaCl.

The isolate Cab3 gave negative catalase test (Fig. 2.3.4A). The negative result was due

to inability of the isolate Cab3 to hydrolyse H_2O_2 . However, *Escherichia coli* strain was used as positive control for catalase test and this culture showed vigorous bubbling immediately after addition of 5% H_2O_2 solution to the 18-20 h old *Escherichia coli* culture (Fig. 2.3.4B).



Fig. 2.3.4 Catalase activity test of (A) No bubble formation in culture of isolate Cab3 showing its catalase negative nature (B) Bubble formation in culture of *Escherichia coli* showing its catalase positive nature.

2.3.3 Antibiotic susceptibility

The susceptibility of isolate Cab3 towards different antibiotics was checked by standardized filter-paper disc-agar diffusion assay (Fig. 2.3.5). If the test microorganism is sensitive towards a particular antibiotic a clear ring appears around the disc in 24/48 h. This ring is called the zone of inhibition. The larger the zone of inhibition, the more effective that antibiotic is against the bacterium. The effect of antibiotics on the isolate Cab3 were defined by measuring the diameter of the zone of inhibition and based on this the test organism was classified to be resistant, moderate or susceptible to a particular antibiotic. The effects of antibiotics on the isolate Cab3 are listed in Table 2.3.2. The isolate Cab3 was sensitive towards chloramphenicol, erythromycin, tetracycline, moderately resistant towards Gentamicin (M), Kanamycin (M) and showed resistant features towards Norfloxacin (R) and Vancomycin (R) as listed in Table 2.3.2. These are the typical characteristic features of *Weissella confusa*

as reported by Liu *et al.*, 2009. The lactic acid bacteria are intrinsically resistant to glycopeptide antibiotic vancomycin (Barton *et al.*, 2001).

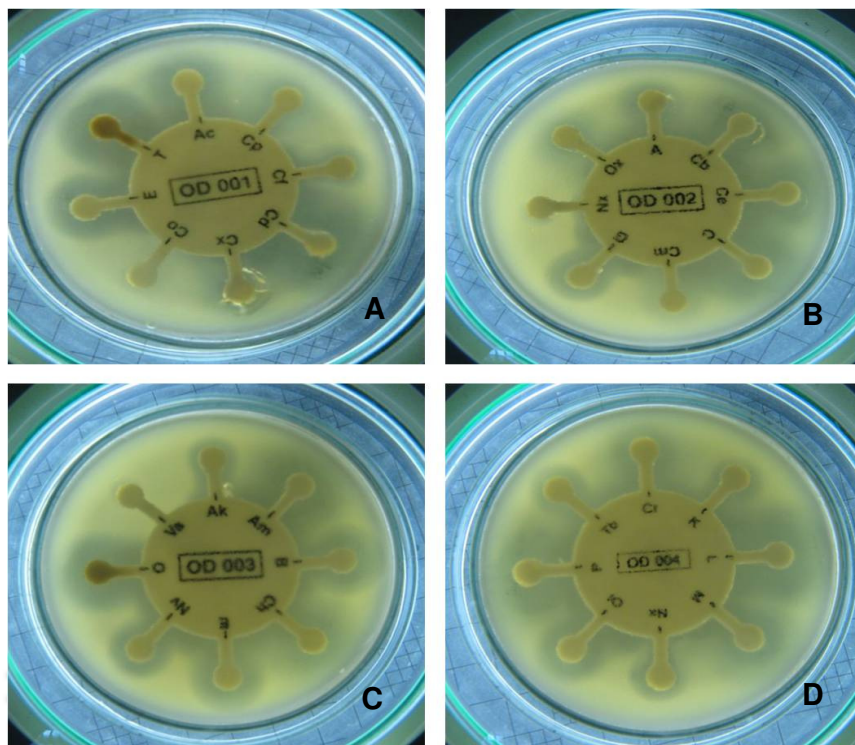


Fig. 2.3.5 Antibiogram of the isolate Cab3 using antibiotic octodiscs (A-D) on MRS agar.

Table 2.3.2 Effect of antibiotics on the isolate Cab3.

S. No	Antibiotic	Concentration (mg)	Isolate Cab3
1	Amikacin	10	M
2	Amoxycillin	10	S
3	Amoxyclav	10	S
4	Ampicilin	10	M
5	Bacitracin	10 Units	S
6	Carbenicillin	100	S
7	Cephalexin	10	S
8	Cephalexidine	30	S
9	Cephalothin	30	S
10	Cephatoxamine	30	S
11	Chloramphenicol	30	S
12	Ciproflaxacin	10	M
13	Claxaciln	1	M
14	Clindamycin	2	S
15	Co-Trimazine	25	M
16	Erythromycin	15	S
17	Gentamicin	10	M
18	Kanamycin	30	M
19	Linomycin	2	S
20	Methicillin	5	S
21	Norfloxacin	10	R
22	Novobiocin	30	S
23	Oleandomycin	15	S
24	Oxacillin	5	S
25	Oxytetracyclin	30	S
26	Penicillin-G	10 Units	S
27	Tetracyclin	30	S
28	Tobramycin	10	M
29	Vancomycin	30	R

R-Resistant- (0-2 mm^a); M- Moderate (3-6 mm^a); S- Sensitive- (7-13 mm^a)

^aValues in millimetres are the distance of zone of inhibition of growth of isolate Cab3.

2.3.4 Carbohydrate fermentation pattern

The ability of the isolate Cab3 to degrade and ferment carbohydrates with the production of acid was tested (Fig. 2.3.6). The strain very efficiently utilised cellobiose, galactose, maltose, sucrose, xylose but poorly fermented mellibiose, raffinose and trehalose (Table 2.3.3) which was in accordance with fermentation pattern of *Weissella confusa* reported earlier by Liu *et al.*, 2009. The Cab3 did not utilize rhamnose which is also characteristic of *Weissella* sp. (Olano *et al.*, 2001). The results of both physiological and biochemical tests are shown in Table 2.3.4. The microorganism (Table 2.3.4, lane 1) was identified by comparing the results with those from the literature (Table 2.3.4). The results of comparison showed that the isolate Cab3 has same characteristics as *Weissella confusa* (Table 2.3.4, lane 4).

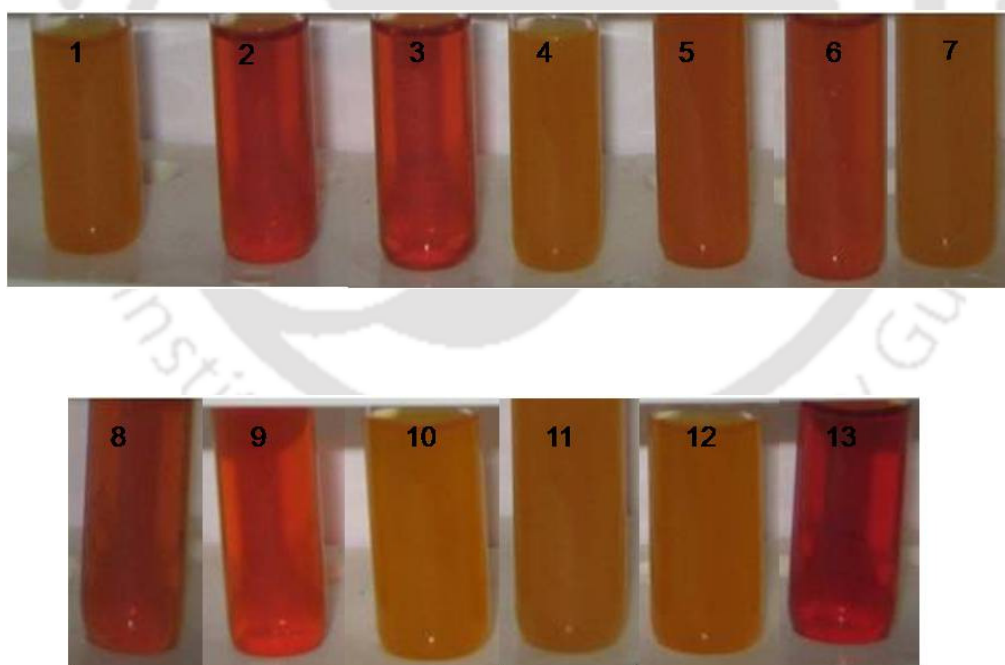


Fig. 2.3.6 The Carbohydrate fermentation pattern of the isolate Cab3 using 13 sugars, 1:Fructose, 2:Raffinose, 3:Trehalose, 4:Sucrose, 5:Galactose, 6:Lactose, 7:Maltose, 8:Mellibiose, 9:Mannitol, 10:Dextrose, 11:Xylose, 12:Cellobiose, 13: Rhamnose.

Table 2.3.3 Carbohydrate fermentation of the isolate Cab3 after 24 h incubation.

S. No.	Carbohydrates	Fermentation response
1	Cellobiose	+++
2	Dextrose	+++
3	Fructose	+++
4	Galactose	++
5	Lactose	+
6	Maltose	+++
7	Mannitol	+
8	Melibiose	+
9	Raffinose	+
10	Rhamnose	-
11	Sucrose	+++
12	Trehalose	+
13	Xylose	+++

(+++) strongly positive; (++) fairly positive; (+) weakly positive; (-) negative

Table 2.3.4 Characteristics of *Weissella confusa* and other lactic acid bacteria.

1, <i>Weissella confusa</i> Cab3; 2, <i>Weissella soli</i> ; 3, <i>Weissella kandleri</i> ; 4, <i>Weissella confusa</i> ; 5, <i>Weissella hellenica</i> ; 6, <i>Weissella kimchii</i> ; 7, <i>Pediococcus pentosaceus</i> ; 8, <i>Leuconostoc mesenteroides</i> B-512F; 9, <i>Leuconostoc mesenteroides</i> B-640.									
Characteristics	1 ^a	2 ^b	3 ^c	4 ^c	5 ^d	6 ^e	7 ^f	8 ^g	9 ^g
Acid production from:									
Ribose	+	+	+	+	-	-	NT	NT	NT
D- Xylose	+	+	-	+	-	+	+	+	+
Galactose	+	-	+	+	-	+	+	+	+
D- Fructose	+	-	+	+	+	NT	+	+	+
Cellobiose	+	-	-	+	-	+	+	-	+
Melibiose	-	+	-	-	-	+	+	+	+
Sucrose	+	+	-	+	+	+	+	+	+
Trehalose	-	+	-	-	+	-	+	+	+
D- Raffinose	-	+	-	-	-	-	+	+	-
Maltose	+	-	-	+	+	+	-	+	+
Dextran Formation	+	-	+	+	-	+	+	+	+
Cell Morphology	Short Rods	Short Rods, often thickened to one end	Irregular rods	Short Rods, thickened at one end	Large spherical or lenticular cells	NT	Cocci shaped and random arrangement in groups or chains	NT	NT

Symbols: +, 90% or more strains were positive; -, 90% or more strains were negative; NT, not tested.

a. Shukla and Goyal, 2011

b. Magnusson *et al.*, 2002

c. Kandler and Weiss, 1986

d. Collins *et al.*, 1993

e. Choi *et al.*, 2002

f. Patel and Goyal, 2010

g. Purama *et al.*, 2008

2.3.5 16S rRNA gene sequence analysis

The genomic DNA of isolate Cab3 was extracted by HiPurA Bacterial and Yeast Genomic DNA Purification Spin Kit (Himedia India Pvt. Ltd.) (Described earlier in Section 2.2.9.1) and purified using Qiagen, QIAquick® Gel Extraction Kit (as described earlier in Section 2.2.9.5). A single band of high molecular-weight DNA of about 19.7 kb from isolate Cab3 was observed on 0.8 % Agarose gel (Fig. 2.3.7). The genomic DNA along with universal primers was used for PCR amplification of 16S rRNA gene as described earlier in Section 2.2.9.6. The amplified product of 16S rRNA gene showed a single band of 1,500 bp (Fig. 2.3.8). The analysis of 16S rRNA gene sequence allowed the identification of the isolate Cab3 at both genus and species levels.

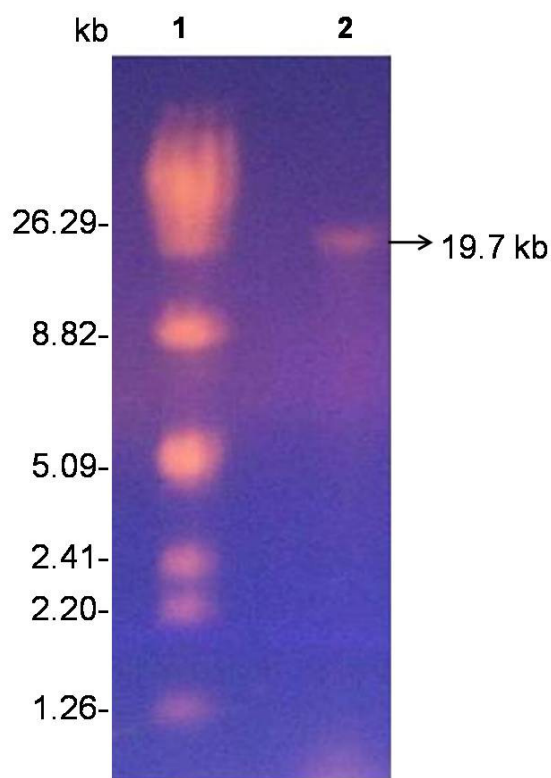


Fig. 2.3.7 Agarose gel (0.8%) showing genomic DNA of the isolate Cab3 extracted using HiPurA bacterial and yeast genomic DNA purification Spin Kit. Lane 1: DNA ladder; Lane 2: Genomic DNA of Cab3.

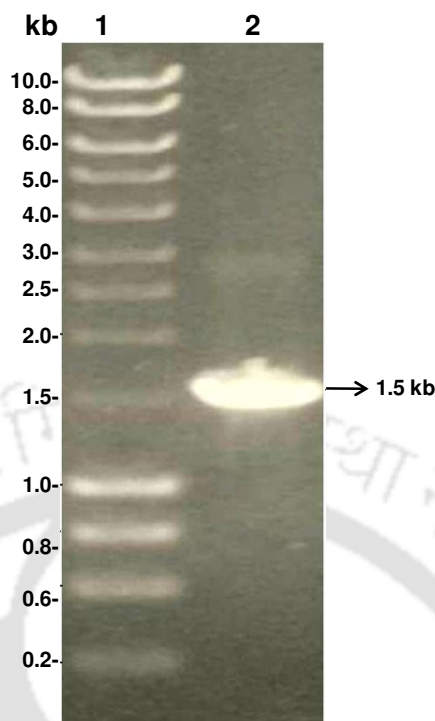


Fig. 2.3.8 Agarose gel (0.8%) showing full length 16S rRNA gene (1.5 kb) of the isolate Cab3 amplified with universal primers. Lane 1: DNA ladder; Lane 2: Amplified product of full length 16S rRNA gene.

The DNA sequence of the PCR amplified 16S rRNA gene was obtained from Xcelris Labs Limited, Ahmedabad, India. Forward and reverse DNA sequencing of PCR amplicon was carried out with 8F and 1492R primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyzer. The consensus sequence of 1288 bp of 16S rDNA gene was generated from forward and reverse sequence data using aligner software (Fig. 2.3.9).

ATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTGGGAAACCTACCTCTTAGCAG
 GGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATGACAACCGCATGGTTGTTATTTAAAAGA
 TGGTCTGCTATCACTAAGAGATGGTCCCGCGGTGATTAGCTAGTTGGTAAGGTAATGGCTTACCAAG
 GCGATGATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTA
 CGGGAGGCAGCAGTAGGGAAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATG
 AAGGGTTTCGGCTCGTAAAACTGTTGTAAGAGAAGAATGACATTGAGAGTAAGTGTTCATGTGTGA
 CGGTATCTTACCAGAAAGGAACGGCTAAATACGTGCCAGCAGCCGCGGTAATACGTATGTTCCAAGCGT
 TATCCGGATTTATTGGGCGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAAGCCCTCAGCTC
 AACTGAGGAATTGCTTTGGAACTGGATGACTTGAGTGCAGTAGAGGAAAGTGGAAGTCCATGTGTAGC
 GGTGAAATGCGTAGATATATGGAAGAACACCAAGTGGCGAAGGCGGCTTTCTGGACTGTAAGTACGTTG
 AGGCTCGAAAGTGTGGGTAGCAAACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGAGTGT
 AGGTGTTTGAGGGTTTCCGCCCTTAAGTGCCGAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGA
 CCGCAAGGTTGAACTCAAAGGAATTGACGGGGACCGGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
 AGCAACGCGAAGAACCTTACCAGGTCTTGACATCCCTTGACAACCTCCAGAGATGGAGCGTTCCCTTCGG
 GGACAAGGTGACAGGTGGTGCATGGTTGTCTGCTCAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAA
 CGAGCGCAACCCCTTATTACTAGTTGCCAGCATTCAAGTGGGCACTCTAGTGAGACTGCCGGTGACAAAC
 CGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCTTATGACCTGGGCTACACACGTGCTACAATG
 GCGTATACAACGAGTTGCCAACC CGCAGGGTGAGCTAATCTCTTAAAGTACGTCTCAGTTCCGATTGT
 AGGCTGCAACTCGCCTACATGAAGTCGGAATCGTAGTAATCGCGG

Fig. 2.3.9 16S rRNA gene sequence (1288 bp full length) of the isolate Cab3.

The 16S rRNA sequence was compared with reference bacteria from National Centre for Biotechnological Information (NCBI) Genbank (<http://www.ncbi.nlm.nih.gov>) and Ribosomal Database Project (RDP) (Table 2.3.5 and 2.3.6). Similarity searches were carried out using the BLAST algorithms available at (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple sequence alignment was performed using ClustalW (<http://www.ebi.ac.uk/clustalw/>) (Casanellas and Fernandez-Sanchez, 2008).

Table 2.3.5 Alignment results using combination of NCBI GenBank and RDP database.

Accession	Description	Max score	Total Score	Query coverage	E value	Max identity
GU369773.1	<i>Weissella confusa</i> strain JS-9-2	2379	2379	100%	0.0	100%
GU138614.1	<i>Weissella confusa</i> IMAU:10286	2379	2379	100%	0.0	100%
GU138608.1	<i>Weissella confusa</i> IMAU:10280	2379	2379	100%	0.0	100%
GU138599.1	<i>Weissella confusa</i> IMAU:10271	2379	2379	100%	0.0	100%
GU138596.1	<i>Weissella confusa</i> IMAU:10268	2379	2379	100%	0.0	100%
GU138592.1	<i>Weissella confusa</i> IMAU:10264	2379	2379	100%	0.0	100%
GU138573.1	<i>Weissella confusa</i> IMAU:10245	2379	2379	100%	0.0	100%
GU138518.1	<i>Weissella confusa</i> IMAU:10190	2379	2379	100%	0.0	100%
FJ429978.1	<i>Weissella confusa</i> strain C5-7	2379	2379	100%	0.0	100%
FJ429975.1	<i>Weissella confusa</i> strain C4-17	2379	2379	100%	0.0	100%

Table 2.3.6 Distance Matrix based on Nucleotide Sequence Homology (Using Kimura-2 parameter).

Sample	1		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GU369773.1	2	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GU138614.1	3	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
GU138608.1	4	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000	0.000
GU138599.1	5	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000	0.000
GU138596.1	6	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000	0.000
GU138592.1	7	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000	0.000
GU138573.1	8	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000	0.000
GU138518.1	9	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000	0.000
FJ429978.1	10	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000		0.000
FJ429975.1	11	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	

The alignment was checked visually and corrected manually using the sequence editor. The homology in sequences identified by above methods was used to find out common ancestry. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The bootstrap consensus tree inferred from 500 replicates was taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Kimura 2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+ Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1288 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007). The isolate Cab3 and the strain *Weissella confusa* IMAU: 10190 (Genbank Accession Number GU GU138518.1) clustered together (Fig. 2.3.10) which confirmed the isolate to be

Weissella confusa. The 16s RRNA gene sequence of *Weissella confusa* Cab3 was submitted to NCBI GenBank data base and assigned the Genbank Accession Number JX649223.

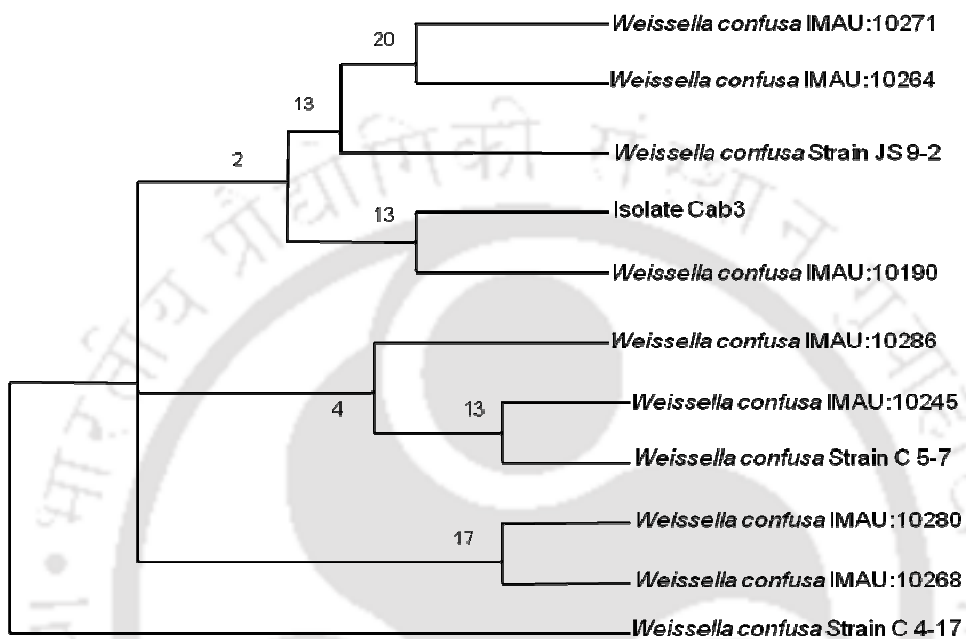


Fig. 2.3.10 Phylogenetic tree generated in MEGA 3.1 software using Neighbour joining method. Reference strains from NCBI database found as nearest homologs are indicated on the right side of the figure.

A bioinformatic tool, GeneDoc software, version 2.7.1 was used for more 16S rRNA gene sequence investigation of the other closely related *Weissella* sp. The partial sequence (1288 nucleotides) of 16S rDNA of *Weissella confusa* Cab3 was 100% similar to 16S rRNA genes from other related strains of *Weissella* sp. in NCBI (Fig. 2.3.11A, 2.3.11B and 2.3.11C).

```

      *      20      *      40      *      60      *
Wc1 : ---GAACGCTGGCGGCGTGCCCTAATACATACAAGTCTGAACGCTTTGTGGTTCAACTGATTTGAAGAGCTTGC : 69
Wc3 : ----CGCTGGCGGCGTGCCCTAATACATGCAAGTCTGAACGCTTTGTGGTTCAACTGATTTGAAGAGCTTGC : 66
Cab3 : -----
Wc2 : -----GTCTGAACGCTTTGTGGTTCAACTGATTTGAAGAGCTTGC : 39
Wc4 : TGTAACGCTTGGCGGCGTGCCCTAATACATGCAAGTCTGAACGCTTTGTGGTTCAACTGATTTGAAGAGCTTGC : 72
      gtcgaacgctttgtggttcaactgattgaagagcttgc

      80      *      100      *      120      *      140
Wc1 : TCAGATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTTGGGAAACCTACCTCTTAGCA : 141
Wc3 : TCAGATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTTGGGAAACCTACCTCTTAGCA : 138
Cab3 : ----ATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTTGGGAAACCTACCTCTTAGCA : 68
Wc2 : TCAGATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTTGGGAAACCTACCTCTTAGCA : 111
Wc4 : TCAGATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTTGGGAAACCTACCTCTTAGCA : 144
      tcagATATGACGATGGACATTGCAAAGAGTGGCGAACGGGTGAGTAACACGTTGGGAAACCTACCTCTTAGCA

      *      160      *      180      *      200      *
Wc1 : GGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATGACAACCGCATGGTTGTTATTTAAAAGATG : 213
Wc3 : GGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATGACAACCGCATGGTTGTTATTTAAAAGATG : 210
Cab3 : GGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATGACAACCGCATGGTTGTTATTTAAAAGATG : 140
Wc2 : GGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATGACAACCGCATGGTTGTTATTTAAAAGATG : 183
Wc4 : GGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATGACAACCGCATGGTTGTTATTTAAAAGATG : 216
      GGGGATAACATTTGGAAACAGATGCTAATACCGTATAACAATGACAACCGCATGGTTGTTATTTAAAAGATG

      220      *      240      *      260      *      280
Wc1 : GTTCTGCTATCACTAAGAGATGGTCCCGCGGTGCATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGAT : 285
Wc3 : GTTCTGCTATCACTAAGAGATGGTCCCGCGGTGCATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGAT : 282
Cab3 : GTTCTGCTATCACTAAGAGATGGTCCCGCGGTGCATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGAT : 212
Wc2 : GTTCTGCTATCACTAAGAGATGGTCCCGCGGTGCATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGAT : 255
Wc4 : GTTCTGCTATCACTAAGAGATGGTCCCGCGGTGCATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGAT : 288
      GTTCTGCTATCACTAAGAGATGGTCCCGCGGTGCATTAGCTAGTTGGTAAGGTAATGGCTTACCAAGGCGAT

      *      300      *      320      *      340      *      360
Wc1 : GATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGC : 357
Wc3 : GATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGC : 354
Cab3 : GATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGC : 284
Wc2 : GATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGC : 327
Wc4 : GATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGC : 360
      GATGCATAGCCGAGTTGAGAGACTGATCGGCCACAATGGGACTGAGACACGGCCCATACTCCTACGGGAGGC

      *      380      *      400      *      420      *
Wc1 : AGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGG : 429
Wc3 : AGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGG : 426
Cab3 : AGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGG : 356
Wc2 : AGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGG : 399
Wc4 : AGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGG : 432
      AGCAGTAGGGAATCTTCCACAATGGGCGAAAGCCTGATGGAGCAACGCCGCGTGTGTGATGAAGGGTTTCGG

      440      *      460      *      480      *      500
Wc1 : CTCGTAAAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAAGTTCATGTGTGACGGTATCTTACCAG : 501
Wc3 : CTCGTAAAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAAGTTCATGTGTGACGGTATCTTACCAG : 498
Cab3 : CTCGTAAAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAAGTTCATGTGTGACGGTATCTTACCAG : 428
Wc2 : CTCGTAAAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAAGTTCATGTGTGACGGTATCTTACCAG : 471
Wc4 : CTCGTAAAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAAGTTCATGTGTGACGGTATCTTACCAG : 504
      CTCGTAAAACACTGTTGTAAGAGAAGAATGACATTGAGAGTAAGTTCATGTGTGACGGTATCTTACCAG

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Fig. 2.3.11A 16S rRNA gene sequence (1-504 nucleotides) investigation of *Weissella confusa* Cab3) by bioinformatics tool, GeneDoc software version 2.7.1. Wc1: WcI10190, Wc2: W.c strain_JS-9-2, Wc3: W.c_IMAU:10264, Wc4: W.c_IMAU:10271, Cab3: isolated strain.


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      *      520      *      540      *      560      *
Wc1 : AAAGGAACGGCTAAATACGTGCCAGCAGCCGCGTAAATACGTATGTTCCAAGCGTTATCCGGATTATTGGG : 573
Wc3 : AAAGGAACGGCTAAATACGTGCCAGCAGCCGCGTAAATACGTATGTTCCAAGCGTTATCCGGATTATTGGG : 570
Cab3 : AAAGGAACGGCTAAATACGTGCCAGCAGCCGCGTAAATACGTATGTTCCAAGCGTTATCCGGATTATTGGG : 500
Wc2 : AAAGGAACGGCTAAATACGTGCCAGCAGCCGCGTAAATACGTATGTTCCAAGCGTTATCCGGATTATTGGG : 543
Wc4 : AAAGGAACGGCTAAATACGTGCCAGCAGCCGCGTAAATACGTATGTTCCAAGCGTTATCCGGATTATTGGG : 576
      AAAGGAACGGCTAAATACGTGCCAGCAGCCGCGTAAATACGTATGTTCCAAGCGTTATCCGGATTATTGGG

      580      *      600      *      620      *      640
Wc1 : CGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAGGCCCTCAGCTCAACTGAGGAATTGCTTTGGA : 645
Wc3 : CGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAGGCCCTCAGCTCAACTGAGGAATTGCTTTGGA : 642
Cab3 : CGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAGGCCCTCAGCTCAACTGAGGAATTGCTTTGGA : 572
Wc2 : CGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAGGCCCTCAGCTCAACTGAGGAATTGCTTTGGA : 615
Wc4 : CGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAGGCCCTCAGCTCAACTGAGGAATTGCTTTGGA : 648
      CGTAAAGCGAGCGCAGACGGTTATTTAAGTCTGAAGTGAAGGCCCTCAGCTCAACTGAGGAATTGCTTTGGA

      *      660      *      680      *      700      *      720
Wc1 : AACTGGATGACTTGAGTGCAGTAGAGGAAAGTGAAGTCCATGTGTAGCGGTGAAATGCGTAGATATATGGA : 717
Wc3 : AACTGGATGACTTGAGTGCAGTAGAGGAAAGTGAAGTCCATGTGTAGCGGTGAAATGCGTAGATATATGGA : 714
Cab3 : AACTGGATGACTTGAGTGCAGTAGAGGAAAGTGAAGTCCATGTGTAGCGGTGAAATGCGTAGATATATGGA : 644
Wc2 : AACTGGATGACTTGAGTGCAGTAGAGGAAAGTGAAGTCCATGTGTAGCGGTGAAATGCGTAGATATATGGA : 687
Wc4 : AACTGGATGACTTGAGTGCAGTAGAGGAAAGTGAAGTCCATGTGTAGCGGTGAAATGCGTAGATATATGGA : 720
      AACTGGATGACTTGAGTGCAGTAGAGGAAAGTGAAGTCCATGTGTAGCGGTGAAATGCGTAGATATATGGA

      *      740      *      760      *      780      *
Wc1 : AGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAAC TGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACA : 789
Wc3 : AGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAAC TGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACA : 786
Cab3 : AGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAAC TGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACA : 716
Wc2 : AGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAAC TGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACA : 759
Wc4 : AGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAAC TGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACA : 792
      AGAACACCAGTGGCGAAGGCGGCTTTCTGGACTGTAAC TGACGTTGAGGCTCGAAAGTGTGGGTAGCAAACA

      800      *      820      *      840      *      860
Wc1 : GGATTAGATACCCCTGGTAGTCCACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTG : 861
Wc3 : GGATTAGATACCCCTGGTAGTCCACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTG : 858
Cab3 : GGATTAGATACCCCTGGTAGTCCACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTG : 788
Wc2 : GGATTAGATACCCCTGGTAGTCCACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTG : 831
Wc4 : GGATTAGATACCCCTGGTAGTCCACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTG : 864
      GGATTAGATACCCCTGGTAGTCCACACCGTAAACGATGAGTGCTAGGTGTTTGAGGGTTTCCGCCCTTAAGTG

      *      880      *      900      *      920      *
Wc1 : CCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGG : 933
Wc3 : CCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGG : 930
Cab3 : CCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGG : 860
Wc2 : CCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGG : 903
Wc4 : CCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGG : 936
      CCGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGG

      940      *      960      *      980      *      1000
Wc1 : GACCCGCACAAGCGGTGGAGCATGTGGTTTAATTGGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC : 1005
Wc3 : GACCCGCACAAGCGGTGGAGCATGTGGTTTAATTGGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC : 1002
Cab3 : GACCCGCACAAGCGGTGGAGCATGTGGTTTAATTGGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC : 932
Wc2 : GACCCGCACAAGCGGTGGAGCATGTGGTTTAATTGGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC : 975
Wc4 : GACCCGCACAAGCGGTGGAGCATGTGGTTTAATTGGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC : 1008
      GACCCGCACAAGCGGTGGAGCATGTGGTTTAATTGGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCC

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Fig. 2.3.11B 16S rRNA gene sequence (505-1008 nucleotides) investigation of *Weissella confusa* Cab3 by bioinformatics tool, GeneDoc software version 2.7.1. Wc1: WcI10190, Wc2: Wc strain_JS-9-2, Wc3: Wc_IMAU:10264, Wc4: Wc_IMAU:10271, Cab3: isolated strain.


```

      *      1020      *      1040      *      1060      *      1080
Wc1 : CTTGACAACCTCCAGAGATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTC : 1077
Wc3 : CTTGACAACCTCCAGAGATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTC : 1074
Cab3 : CTTGACAACCTCCAGAGATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTC : 1004
Wc2 : CTTGACAACCTCCAGAGATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTC : 1047
Wc4 : CTTGACAACCTCCAGAGATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTC : 1080
      CTTGACAACCTCCAGAGATGGAGCGTTCCCTTCGGGGACAAGGTGACAGGTGGTGCATGGTTGTCGTCAGCTC

      *      1100      *      1120      *      1140      *
Wc1 : GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTTATTACTAGTTGCCAGCATTTCAGTTGGG : 1149
Wc3 : GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTTATTACTAGTTGCCAGCATTTCAGTTGGG : 1146
Cab3 : GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTTATTACTAGTTGCCAGCATTTCAGTTGGG : 1076
Wc2 : GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTTATTACTAGTTGCCAGCATTTCAGTTGGG : 1119
Wc4 : GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTTATTACTAGTTGCCAGCATTTCAGTTGGG : 1152
      GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTTATTACTAGTTGCCAGCATTTCAGTTGGG

      1160      *      1180      *      1200      *      1220
Wc1 : CACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCCTTATGA : 1221
Wc3 : CACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCCTTATGA : 1218
Cab3 : CACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCCTTATGA : 1148
Wc2 : CACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCCTTATGA : 1191
Wc4 : CACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCCTTATGA : 1224
      CACTCTAGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCCTTATGA

      *      1240      *      1260      *      1280      *
Wc1 : CCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTTAA : 1293
Wc3 : CCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTTAA : 1290
Cab3 : CCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTTAA : 1220
Wc2 : CCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTTAA : 1263
Wc4 : CCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTTAA : 1296
      CCTGGGCTACACACGTGCTACAATGGCGTATACAACGAGTTGCCAACCCGCGAGGGTGAGCTAATCTCTTAA

      1300      *      1320      *      1340      *      1360
Wc1 : AGTACGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCA : 1365
Wc3 : AGTACGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCA : 1362
Cab3 : AGTACGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGG---- : 1288
Wc2 : AGTACGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCA : 1335
Wc4 : AGTACGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGATCA : 1368
      AGTACGTCTCAGTTCGGATTGTAGGCTGCAACTCGCCTACATGAAGTCGGAATCGCTAGTAATCGCGGatca

      *      1380      *      1400      *      1420      *      1440
Wc1 : GCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAAACACCCA : 1437
Wc3 : GCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAAACACCCA : 1434
Cab3 : ----- : -
Wc2 : GCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAAACACCCA : 1407
Wc4 : GCACGCCGCGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGTCACACCATGAGAGTTTGTAAACACCCA : 1440
      gcacgccgcggtgaatacgttcccggtcttgtacacacgcccgtcacaccatgagagtttgtaacacca

      *      1460      *      1480      *
Wc1 : AAGCCGCTGGGGTAACCTTCGGGAGCCAGCCGTCTAAGGTGGGACAGATGATTAGGGTG : 1496
Wc3 : AAGCCGCTGGGGTAACCTTCGGGAGCCAGCCGTCTAAGGTGGGACAGAT----- : 1483
Cab3 : ----- : -
Wc2 : AAGCCGCTGGGGTAACCTTCGGGAGCCAG----- : 1436
Wc4 : AAGCCGCTGGGGTAACCTTCGGGAGCCAGCCGTCTAAGGTGGGACAGATGATTAGGGTG : 1499
      aagccgctggggtaaccttcgggagccag

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Fig. 2.3.11C 16S rRNA gene sequence (1009-1288 nucleotides) investigation of *Weissella confusa* Cab3 by bioinformatics tool, GeneDoc software version 2.7.1. Wc1: WcI10190, Wc2: W.c strain_JS-9-2, Wc3: W.c_IMAU:10264, Wc4: W.c_IMAU:10271, Cab3: isolated strain.

2.3.6 Production of dextransucrase under different culture conditions

2.3.6.1 Effect of temperature

Temperatures ranging from 22°C to 40°C were studied for production of enzyme under shaking at 180 rpm (Fig. 2.3.12). At 25°C the activity achieved was maximum (6.2 U/ml), at lower temperature than 25°C, the cell growth was slow which might be the cause for lower enzyme activity as shown in Fig. 2.3.12. At higher temperature the enzyme activity decreased which might be due to the deactivation of the enzyme at higher temperatures (Shukla and Goyal, 2011).

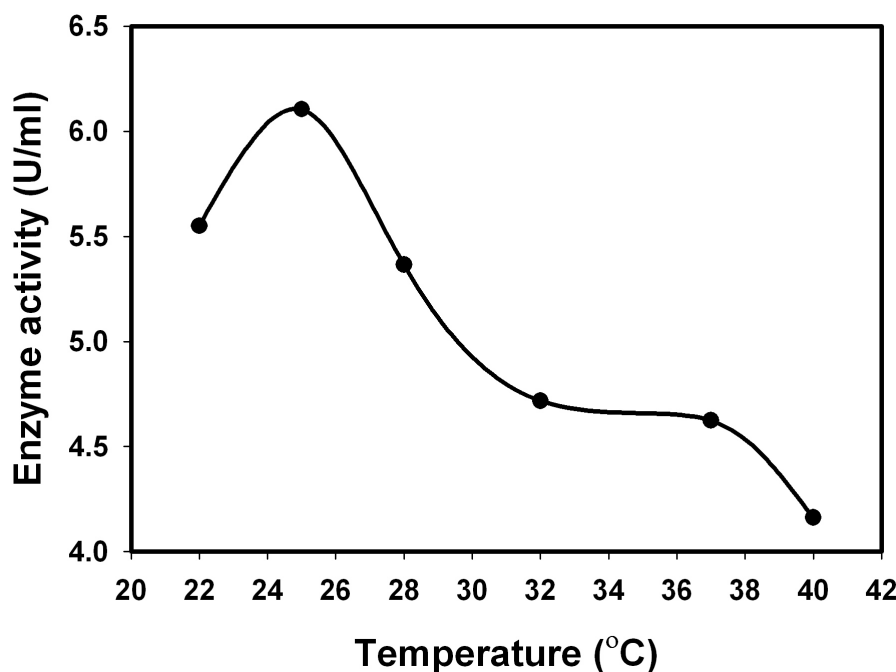


Fig. 2.3.12 Effect of temperature on dextransucrase production from *Weissella confusa* Cab3. The maximum enzyme activity obtained at each temperature was plotted.

2.3.6.2 Effect of shaken flask culture

Shaking condition significantly favored the enzyme production giving 6.1 U/ml enzyme activity which was 1.5 times higher than the enzyme activity observed

under static condition (4.1 U/ml) as shown in Fig. 2.3.13. The maximum enzyme activity was achieved 4 h later in case of static flask culture.

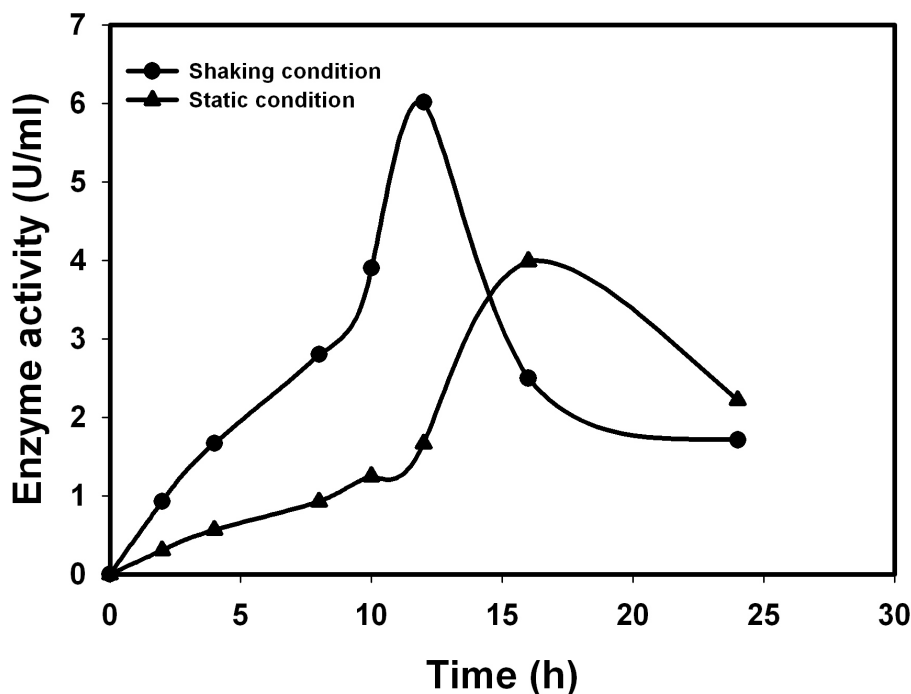


Fig. 2.3.13 Effect of shaken and static flask culture on dextranucrase production.

Fig. 2.3.14 shows the fermentation profile of the new strain of *Weissella confusa* Cab3. The cell OD₆₀₀, pH, enzyme activity (described in Section 2.2.10.2) and sucrose concentration (as described in Chapter 3, Section 3.2.3) were analyzed at regular intervals. The enzyme production was cell growth associated. The sucrose concentration decreased sharply between 10-12th h of fermentation of *Weissella confusa* Cab3 (Fig. 2.2.14). The fermentation profile of the new strain of *Weissella confusa* Cab3 was compared with other lactic acid bacterial strains using the same medium composition and the parameters compared are listed in Table 2.3.7. It was reported that 23°C temperature under static flask culture condition was optimum for the production of dextranucrase from *Leuconostoc mesenteroides* NRRL B-512F (Goyal *et al.*, 1997) and 25°C temperature under shaking condition was found to

optimum for *Leuconostoc mesenteroides* NRRL B-640, whereas, for *Weissella confusa* Cab3 the temperature, 25°C and shaking conditions were optimum. From the results it was clear that the enzyme activity (6.1 U/ml) from *Weissella confusa* Cab3 was 50%, 27% and 65% higher than the enzyme activity from *L. mesenteroides* NRRL B-512F (4.1 U/ml), *L. mesenteroides* NRRL B-640 (4.8 U/ml), and *P. pentosaceus*, respectively, as shown in Table 2.3.7. The cell growth obtained in all strains after 12 h was similar. There was a sharp decrease in pH of the broth with *Weissella confusa* Cab3 after 12 h (Fig. 2.3.14), when the maximum activity was achieved, whereas the pH drop was much lower from the point where the maximum enzyme activity was observed for *L. mesenteroides* NRRL B-512F. The pH range for maximum enzyme production for *Weissella confusa* Cab3 was narrow 5.2-5.6, whereas for *L. mesenteroides* NRRL B-512F, *L. mesenteroides* NRRL B-640 (Purama and Goyal, 2009), and *P. pentosaceus* (Patel and Goyal, 2010) it was 6.0-6.5, 4.4-5.9, 4.0-5.0, respectively.

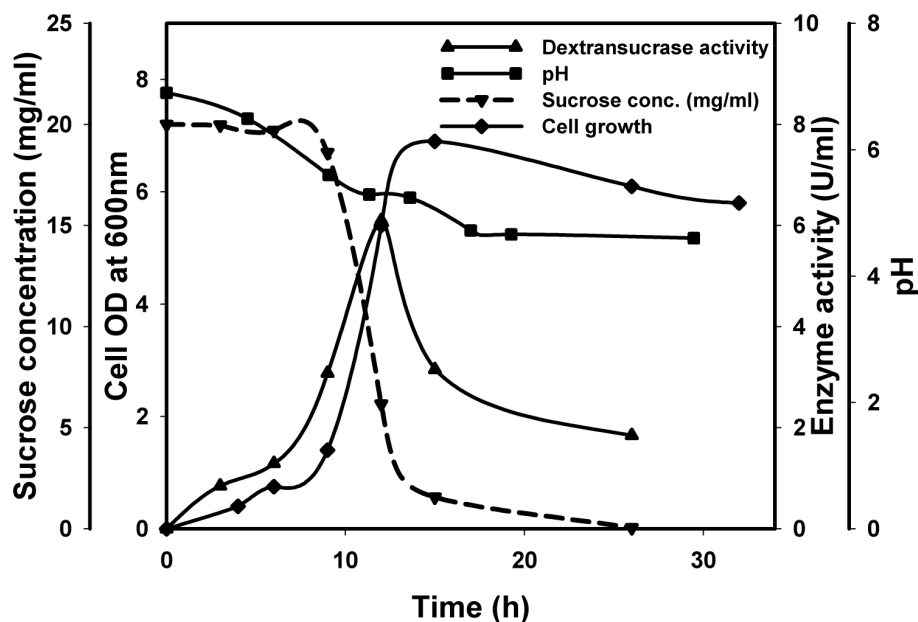


Fig. 2.3.14 Fermentation profile of *Weissella confusa* Cab3. The enzyme activity, cell growth, pH and sucrose concentration changes with time are shown.

Table 2.3.7 Comparison of *Weissella confusa* Cab3 for fermentation characteristics with other lactic acid bacteria.

Fermentation parameters	<i>Weissella Confusa</i> Cab3	<i>Leuconostoc mesenteroides</i> NRRL B-512F	<i>Leuconostoc mesenteroides</i> NRRL B-640	<i>Pediococcus pentosaceus</i> (SPA)
Duration (h)	12	12 ^a	14 ^b	16 ^c
Maximum enzyme activity (U/ml)	6.1	4.1	4.8 ^b	3.4 ^c
pH range for maximum enzyme production	5.2-5.6	6.0 – 6.5 ^a	4.4-5.9 ^b	4-5 ^c
Temperature (°C)	25 °C	23 °C ^a	25 °C ^b	25 °C ^c
Cell density (OD _{600 nm})	5.4	5.8	5.0	8.1

a. Goyal *et al.*, 1997

b. Purama and Goyal., 2009

c. Patel and Goyal, 2010



2.4 Conclusions

The physiological and biochemical characterization were carried out to distinguish the isolate from other closely related lactic acid bacteria. Based on biochemical and physiological studies the strain showed its relation with other *Weissella* sp. The Cab3 was Gram's positive short rod shaped. The cell morphology of Cab3 was studied by scanning electron microscopic study which revealed it to be short rod shaped. Being a lactic acid bacterial strain Cab3 was catalase negative. The Cab3 produced slimy colony when streaked on modified MRS medium which showed the production of polysaccharide. The antibiogram and carbohydrate fermentation profile study enabled the identification of isolated bacterium as it exhibited the typical characteristic features of *Weissella* sp. *Weissella confusa* Cab3 was sensitive towards chloramphenicol, erythromycin, tetracycline, a common trend in *Weissella* sp. The strain was resistant to gentamicin, kanamycin, norfloxacin and vancomycin. The isolate *Weissella confusa* Cab3 could not ferment mellibiose, raffinose and trehalose, a common trait of *Weissella confusa* Cab3. The isolate Cab3 fermented cellobiose, galactose, maltose, ribose, sucrose, xylose but poorly utilized mellibiose, raffinose and trehalose. The carbohydrate fermentation profile of the isolate also supported its similarity with *Weissella* sp. The isolated bacterium Cab3 was identified at species and genus level based on 16S rDNA sequencing to be *Weissella confusa* and assigned the Genbank Accession Number JX649223. The culture conditions for maximum enzyme production were optimized. The 25°C temperature and shaking condition were optimum for enzyme production. At optimum culture conditions *Weissella confusa* Cab3 produced 6.1 U/ml enzyme activity. The enzyme activity of the isolate *Weissella confusa* Cab3 was quite high as compared to other lactic acid bacterial strains. The production of dextransucrase could be increased further by optimizing the

fermentation process. Till date not much attention has been paid to the dextran production capacity of *Weissella confusa* which has recently drawn attention for its very high dextran yield and more linearity in linkage pattern, which makes it very potent candidate for dextran production at industrial level. Wide applications of dextran in food, pharmaceutical and cosmetics industries emphasize the importance of exploration of the new strains and characterization of their traits. This study reveals a novel high dextran producing bacterial isolate which could be a good alternative for dextran production in the future.



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Chapter 3

Optimization of dextransucrase production from *Weissella confusa* Cab3

3.1 Introduction

The enzyme synthesizing dextran from sucrose is known as dextransucrase (1,6- α -D-glucan-6- α -glucosyltransferase, EC 2.4.1.5). It catalyzes the transfer of glucosyl residues from sucrose to dextran polymer and liberates fructose (Hehre, 1951). Dextransucrase are produced by various *Leuconostoc*, *Pediococcus*, *Lactobacillus*, *Weissella* and *Streptococcus* species (Monchois *et al.*, 1999; Monsan *et al.*, 2001, Patel *et al.*, 2010, Shukla and Goyal, 2011a) and by the mold *Rhizopus* spp. (Sankpal *et al.*, 2001). Dextransucrase is an inducible enzyme requiring sucrose in the medium for the induction with the exception of recently isolated constitutive mutant strains of *Leuconostoc mesenteroides* viz B-512 FMC (Kitaoka and Robyt, 1998), B-742 (Kim and Robyt, 1995a), B-1299 (Kim and Robyt, 1995b) and B-1355 (Cote *et al.*, 1999). Dextran is composed of a linear chain of glucosyl residues linked through $\alpha(1\rightarrow6)$ glucosidic bonds and several $\alpha(1\rightarrow2)$, $\alpha(1\rightarrow3)$ or $\alpha(1\rightarrow4)$ branched linkages (Robyt, 1995). Dextran is used in various industries because of their inertness, porous structure and gelling properties (Patel *et al.*, 2012). These are used as food syrup stabilizers, matrix of chromatography columns, blood plasma substitutes,

antithrombogenic agents, treatment for iron deficiency anaemia and drug carriers (Purama and Goyal, 2005; Patel *et al.*, 2012).

Microorganisms utilize various substrates as nutrient source for their growth and metabolic activities. There are several reports on sucrose effect on dextransucrase production by various strains of *Leuconostoc* spp. Tsuchiya *et al.* (1952) studied the effect of sucrose, corn steep liquor and phosphate on dextransucrase production from *Leuconostoc mesenteroides* strain NRRL B-512. Goyal and Katiyar (1997) used low yeast extract and high K_2HPO_4 concentration for enhanced dextransucrase production from *Leuconostoc mesenteroides* NRRL B-512F. However, dextransucrase production by wild-type *Leuconostoc mesenteroides* grown on glucose or maltose instead of sucrose also has been reported (Smith and Zahnley, 1999). Behravan *et al.* (2003) used sugar-beet molasses as a sucrose source and wheat bran as substitute for yeast extract. The *Leuconostoc mesenteroides* NRRL B-512F displayed increased production of biomass and enzyme with the increase in K_2HPO_4 concentrations (Rodrigues *et al.*, 2003). The aeration conditions were optimized for production and scale up of dextransucrase from *Leuconostoc mesenteroides* B/110-1-1 strain in a bioreactor (Michelena *et al.*, 2003). Fine-tuning of nutrient concentrations is essential to regulate the microbial metabolism and associated metabolic product production. For optimization of nutrient's concentration, various methods are reported in the literature such as Box Behnken method (Navaneeth *et al.*, 2009), central composite design (CCD) (Patel *et al.*, 2011), neural networking (Singh *et al.*, 2008) and Taguchi's orthogonal array based methodology (Umesaki *et al.*, 1977). Purama and Goyal (2008b) employed response surface methodology for maximizing dextransucrase production from *Leuconostoc mesenteroides* NRRL B-640 in a bioreactor. Singh *et al.*, 2008 used artificial intelligence based optimization method

for enhancement of dextransucrase production from *Leuconostoc dextranicum* NRRL B-1146. Patel *et al.*, 2011 optimized a medium for enhanced dextransucrase production from an isolate *Pediococcus pentosaceus* (SPAm) using response surface methodology.

Weissella confusa is lactic acid bacterium which produces dextransucrase. There are very few reports where the dextran production capacity of this strain has been explored. In our earlier report, the dextransucrase and dextran production by *Weissella confusa* Cab3 isolated from sauerkraut were studied (Shukla and Goyal, 2011a). *Weissella confusa* Cab3 produced dextransucrase (6.0 U/ml) in the medium as described by Tsuchiya *et al.* (1952). The effects of various carbon sources, nitrogen sources and buffering agents were investigated for their effects on dextransucrase production from *Weissella confusa* Cab3 (Shukla and Goyal, 2011b). Taguchi orthogonal array design is widely used statistical technique for screening and optimization of medium components at shake-flasks level (Bajaj *et al.*, 2006). The basic principle of this method serves as screening filters, which examine the effects of many process variables and identify those factors which have major effects on process using a few experiments (Dasu *et al.*, 2003). In this method the best levels of the factors maximizes the Signal-to-Noise ratios which are log functions of desired output characteristics (Attar *et al.*, 2011). In the present study the medium components were optimized for enhanced dextransucrase production by *Weissella confusa* Cab3 using Taguchi's orthogonal method. Subsequently, the dextransucrase production by this strain was scaled up to lab scale bioreactor using the statistically designed medium.

3.2 Material and Methods

3.2.1 Microorganism, maintenance and preparation of inoculum

The bacterial strain *Weissella confusa* Cab3 (Genbank Accession Number JX649223) isolated from sauerkraut (Shukla and Goyal, 2011a) was used for optimization of medium composition for dextransucrase production. The organism was maintained in MRS medium (DeMan *et al.*, 1960) slants incubated at 25°C, stored at 4°C and subcultured every two weeks. The MRS medium composition has been described in Chapter 2, Section 2.2.3. Fermentation experiments were carried out using *Weissella confusa* Cab3, subcultured and grown (12-14 h old) in medium as described by Tsuchiya *et al.* (1952) (as described in Chapter 2, Section 2.2.4) at optimized culture conditions of 25°C and 180 rpm as described in Chapter 2, Section 2.3.6.

3.2.2 Production of dextransucrase

The production of dextransucrase was carried out in 250 ml Erlenmeyer flasks containing 100 ml medium as described by Tsuchiya *et al.*, 1952 (as described in Chapter 2, Section 2.2.4) or in the medium as per the statistical design and inoculated with 1% culture inoculum. The inoculated flasks were incubated under orbital shaking at 180 rpm and 25°C for 12-15 h. The samples (1 ml) were withdrawn at regular time intervals and centrifuged at 8,000g and 4°C for 10 min to separate the cells. The cell free supernatant was analyzed for enzyme activity as described in Chapter 2, Section 2.2.10.2.

3.2.3 Sucrose estimation

3.2.3.1 Preparation of DNS reagent for reducing sugar estimation

3,5-Dinitrosalicylic acid (DNS) reagent was prepared as per the method as described by Sumner and Sisler, 1944. Sodium potassium tartarate (Rochelle salt, 183 g) was dissolved in 500 ml of hot water and mixed with 262 ml of 2 N NaOH. DNS (6.3 g) was slowly added to above mixture and stirred continuously. Redistilled phenol (5 g) and anhydrous sodium metabisulphite (5 g) were added to the above reagent mixture and cooled to room temperature. The volume was made upto 1 l and stored in an amber color bottle.

3.2.3.2 Estimation of sucrose

To the 0.5 ml of cell free supernatant, 1 ml 1N HCl was added and heated in a boiling water bath for 10 min. After cooling to room temperature, 1 ml of 1N NaOH and 3 ml of DNS reagent were added. The mixture was diluted by adding 9.5 ml distilled water. The absorbance was taken at 540 nm using UV-Visible spectrophotometer (Varian, Cary 100). The fructose in the range of 0.1 to 1 mg/ml was used to plot standard.

3.2.4 Effect of sucrose on dextransucrase production

The effect of sucrose on dextransucrase production was studied by varying its concentration from 1 to 6% (w/v) in the enzyme production medium as described earlier in this Chapter, Section 3.2.2. The concentrations of other components were kept constant. The enzyme production medium as described by Tsuchiya *et al.*, 1952 (as described in Section 2.2.4) containing 2 (% w/v) sucrose was considered as control.

3.2.5 Effect of nitrogen sources on dextransucrase production

Three different nitrogen sources *viz.* yeast extract, beef extract and peptone were investigated for their effects on dextransucrase production. For this yeast extract, peptone and beef extract were used as sole nitrogen sources in the medium as described by Tsuchiya *et al.*, 1952 (as described in Chapter 2, Section 2.2.4) by replacing 2%, w/v yeast extract with the different concentrations of nitrogen sources. The concentrations of nitrogen sources were varied from 1% to 4% (w/v). The concentrations of other components were kept constant.

3.2.6 Effect of sodium acetate and K₂HPO₄ on dextransucrase production

Sodium acetate concentration was varied from 6.0 mM to 36 mM to observe its effect on enzyme production. 1 to 3% (w/v) K₂HPO₄ was used to study its effect on enzyme production. Enzyme production medium as described by Tsuchiya *et al.*, 1952 (as described in Chapter 2, Section 2.2.4) was considered as control in each case (Sodium acetate and K₂HPO₄).

3.2.7 Effect of Tween 80 on dextransucrase production

The effect of Tween 80 on enzyme production was studied by varying its concentration from 0.02 to 0.3% (v/v) in the medium. Enzyme production medium as described by Tsuchiya *et al.*, 1952 (as described in Chapter 2, Section 2.2.4) was considered as control.

3.2.8 Optimization of dextransucrase production from *Weissella confusa* Cab3 by Taguchi's orthogonal array methodology

An L16 orthogonal array in four levels was used consisting of 16 different experimental trials for the medium optimization for dextransucrase production by

Weissella confusa Cab3. The design for the L16 OA was developed and analyzed using “MINITAB 15” software. All four selected factors, their assigned levels and the experimental design along with dextransucrase production data are listed in Tables 3.3.2 and 3.3.3, respectively. To achieve the maximum dextransucrase production by *Weissella confusa* Cab3, sucrose (% w/v), Yeast extract (% w/v), di-potassium hydrogen orthophosphate (K_2HPO_4 , % w/v) and Tween 80 (% v/v) were selected for medium optimization for enhanced dextransucrase production because they had significant impact on dextransucrase production as screened in our earlier findings (Shukla and Goyal, 2011b). Sucrose was the most effective medium component for dextransucrase production. However yeast extract, K_2HPO_4 , and Tween 80, displayed moderate effect on dextransucrase production from *Weissella confusa* Cab3. Experimental results were fitted in Taguchi software to analyze further for predicted values, individual and interactive influences, ANOVA, optimum conditions and to know the contribution of each selected fermentation factor in the production of dextransucrase by this bacterial strain. Validation experiments were performed using optimized parameters of fermentation medium components and levels by software. The dextransucrase production medium was validated at shake flask level. The optimized medium for dextransucrase production from *Weissella confusa* Cab3 were inoculated with 1% of fresh seed culture of *Weissella confusa* Cab3 and various fermentation parameters like optical density of cell (OD_{600}), enzyme activity (Chapter 2, Section 2.2.10.2) and sucrose concentration (as described earlier in Section 3.2.3) were determined. All the experiments were carried out in triplicate and the average value was taken.

3.2.9 Scale up of dextransucrase production at bioreactor level using statistically designed medium

The dextransucrase production using optimized medium was scaled up in 1 l volume of culture medium in a 3 l bioreactor (Applikon, model Bio Console ADI 1025). The bioreactor is equipped with pH probe, oxygen probe, foam sensor, and stirrer of two–six bladed Rushton turbines. For controlled pH cultivations, the pH was maintained at 7.0 by addition of 2 M NaOH and 2 M HCl solution. During the experiment, temperature and aeration rate were controlled at 25°C and 2 vvm, respectively. The Dissolved Oxygen (DO) was calibrated to 100% before inoculation. The initial agitation speed was set to 200 rpm and it was changed accordingly to maintain the DO above 30%. 1% inoculum from 12 h grown culture was added to 1 l medium in the bioreactor. Sterile Soybean oil was simultaneously employed as anti-foam agent. The parameters like dextransucrase activity, sucrose concentration, cell optical density and dry cell weight were analyzed at regular interval. The dextransucrase activity was analyzed as described in Chapter 2, Section 2.2.10.2. The cell optical density was measured at 600 nm. The sucrose concentration was determined by estimating the reducing sugars by the method of Sumner and Sisler, 1944 as described earlier in this Chapter, Section 3.2.3. All the experiments were carried out in triplicate and the average value was taken.

3.3 Results and Discussion

3.3.1 Effect of sucrose on dextransucrase production

Among the nutrients, carbon source sucrose was chosen as it induces the dextransucrase production and is also, a substrate for dextran production from *Weissella confusa* Cab3. The increase in dextransucrase activity was 3-fold, from, 6.0 U/ml to 18.2 U/ml (Fig. 3.3.1, Table 3.3.1) with an increase in sucrose concentration from 2% (control) to 5%, in the medium.

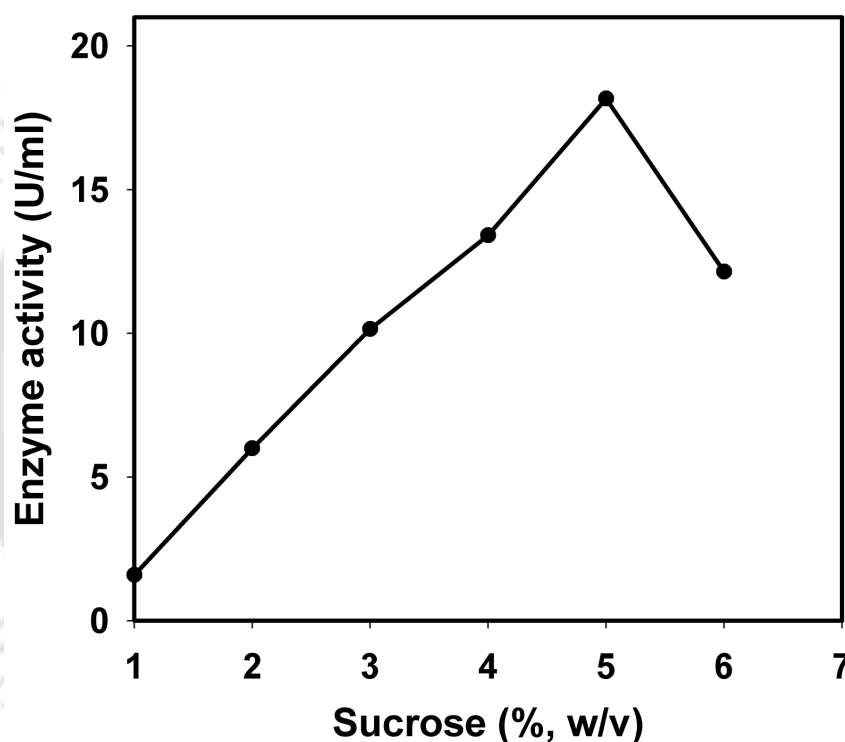


Fig. 3.3.1 Effect of sucrose on dextransucrase production.

As the sucrose concentration increased there was an increase in viscosity of the broth due to the subsequent formation of exopolysaccharide from the available sucrose by the released enzyme, in the medium. Above 5% sucrose concentration the enzyme activity started decreasing, which could be due to some inhibitory effect of substrate on enzyme production. Increment in sucrose concentration is also known to enhance the enzyme activity in other lactic acid strains viz. *Leuconostoc*

mesenteroides NRRL B-512F, where, increase in sucrose concentration from 2% to 4%, resulted in the increase in the dextranucrase activity from 1.4 /ml to 2.45 U/ml (Goyal and Katiyar, 1997). *Leuconostoc mesenteroides* NRRL-B640 also showed 3 fold increase in the dextranucrase activity from 4.8 to 15.0 U/ml, with an increase in sucrose concentration from 2 to 7% (Purama and Goyal, 2008a).

Table 3.3.1 Effect of nutrients on enzyme activity.

Medium component	Concentration (% w/v)	Enzyme activity (U/ml)	Relative activity (%)
Control medium*		6.0	100
Sucrose	5.0	18.2	303
Yeast extract	1.5	6.4	107
Peptone†	2.0	6.2	103
Beef extract†	3.0	4.8	80
Sodium acetate	0.05	6.0	100
Tween 80 (% v/v)	0.1	7.0	117
K ₂ HPO ₄	1.5	6.4	107

* Tsuchiya *et al.* (1952) as described in Chapter 2, Section 2.2.4.

† Medium did not contain yeast extract, whereas control medium contained 2% (w/v) yeast extract

3.3.2 Effect of nitrogen sources on dextranucrase production

The effects of various nitrogen sources like yeast extract, peptone and beef extract on dextranucrase production by *Weissella confusa* Cab3 were studied. Yeast extract was most effective nitrogen source for dextranucrase production. At 1.5% (w/v) yeast extract concentration, 6.4 U/ml enzyme activity was obtained (Fig. 3.3.2, Table 3.3.1) which was 7% more than that observed in control medium containing 2% yeast extract. There was no change in the enzyme activity beyond 2% (w/v) yeast extract concentration.

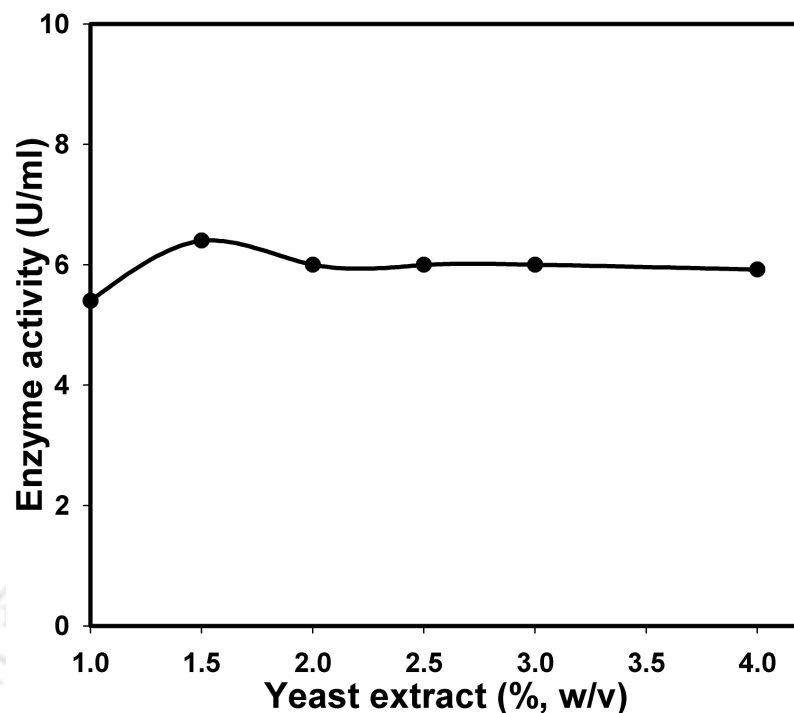


Fig. 3.3.2 Effect of yeast extract on dextranucrase production.

Effect of peptone and beef extract as nitrogen source on dextranucrase production from *Weissella confusa* Cab3 was studied by replacing yeast extract from the medium as described by Tsuchiya *et al.*, 1952 (Chapter 2, Section 2.2.4) with different concentrations of peptone and beef extract ranging from 1-4 (% w/v). Peptone significantly increased dextranucrase activity from 2.3 U/ml to 6.2 U/ml, as the concentration of peptone was increased from 1 to 2%, w/v. Beyond 2%, w/v peptone concentration there was no further increase in the dextranucrase production rather a saturation effect was observed. Beef extract did not support dextranucrase activity. Increasing beef extract concentration from 1-3%, increased dextranucrase production from 2.5 to 4.8 U/ml (Fig. 3.3.3).

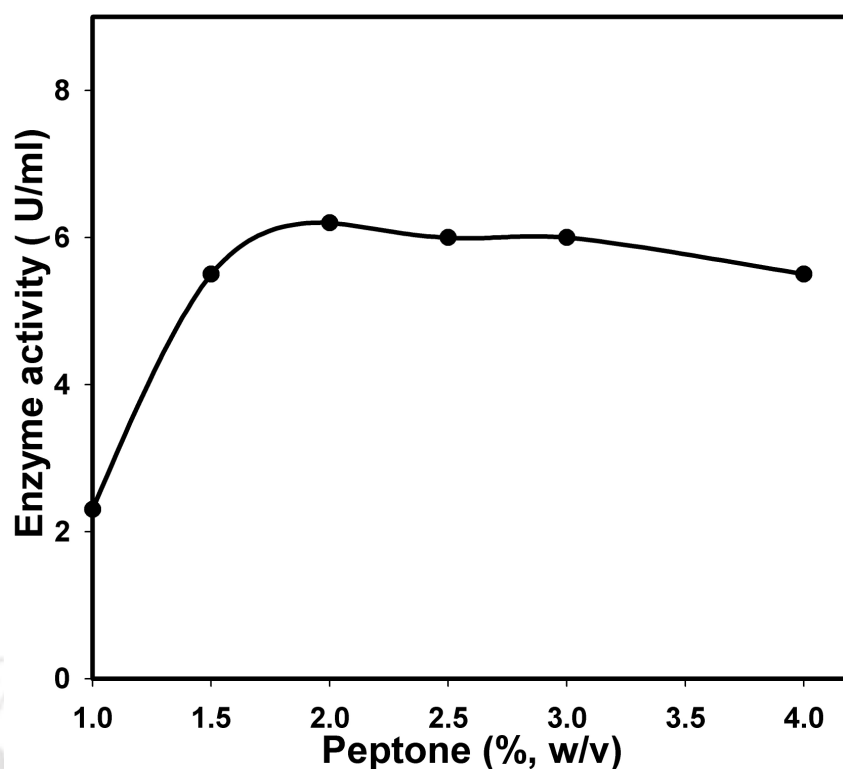


Fig. 3.3.3 Effect of Peptone on dextranucrase production.

The maximum enzyme activity (4.8 U/ml) obtained in the medium containing 3 (% w/v) beef extract as sole nitrogen source (Fig. 3.3.4) was much lower as compared to 6.4 U/ml and 6.2 U/ml enzyme activity obtained in the medium containing 1.5 (% w/v) yeast extract and 2 (% w/v) peptone, respectively as sole nitrogen sources. From the results it was concluded that higher concentrations of nitrogen sources inhibited dextranucrase which could be due to their complex nature.

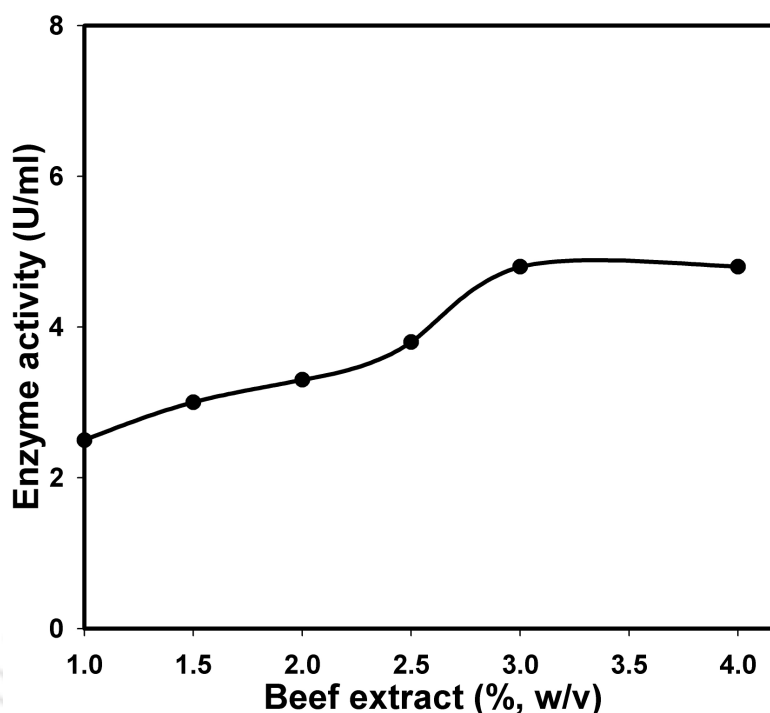


Fig. 3.3.4 Effect of beef extract on dextranucrase production.

3.3.3 Effect of sodium acetate and K_2HPO_4 on dextranucrase production

Sodium acetate acts as a buffering agent and helps to maintain the pH in the fermentation process (Kim *et al.*, 2003). Sodium acetate was used in the medium to study its effect on dextranucrase production. At lower concentration upto 12 mM it did not have any effect on dextranucrase production (Fig. 3.3.5). However, beyond 12 mM, sodium acetate negatively affected enzyme production. Whereas, Sawale and Lele (2009) and Sawale and Lele (2010) reported positive effect of sodium acetate on dextranucrase (1.51% w/v, 0.18 M) and dextran (1.69% w/v, 0.20 M) production, respectively by a *Leuconostoc mesenteroides* strain isolated from fermented idli batter.

Increasing the K_2HPO_4 concentration up to 1.5% (w/v), dextranucrase production increased. 1.5% (w/v) K_2HPO_4 concentration was optimum for dextranucrase production resulting in the 7% (6.4 U/ml) increase in enzyme activity as compared with the control medium (Fig. 3.3.6, Table 3.3.1). The medium as described by Tsuchiya *et al.*, 1952 (as described in Chapter 2, Section 2.2.4) which

contained 2%, w/v K_2HPO_4 was considered as control. Beyond 1.5% (w/v) K_2HPO_4 , dextransucrase production decreased.

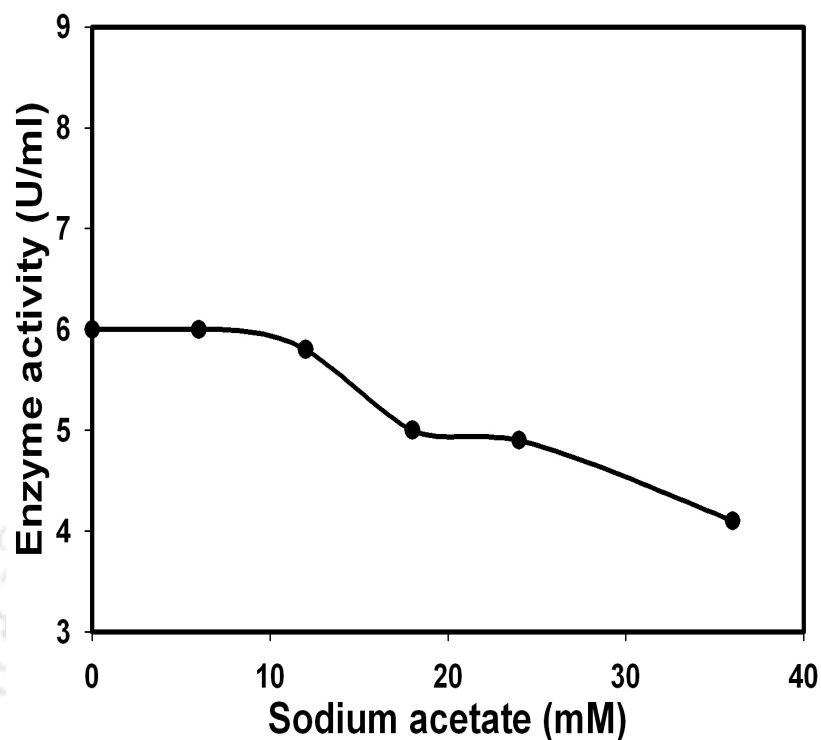


Fig. 3.3.5 Effect of sodium acetate on dextransucrase production.

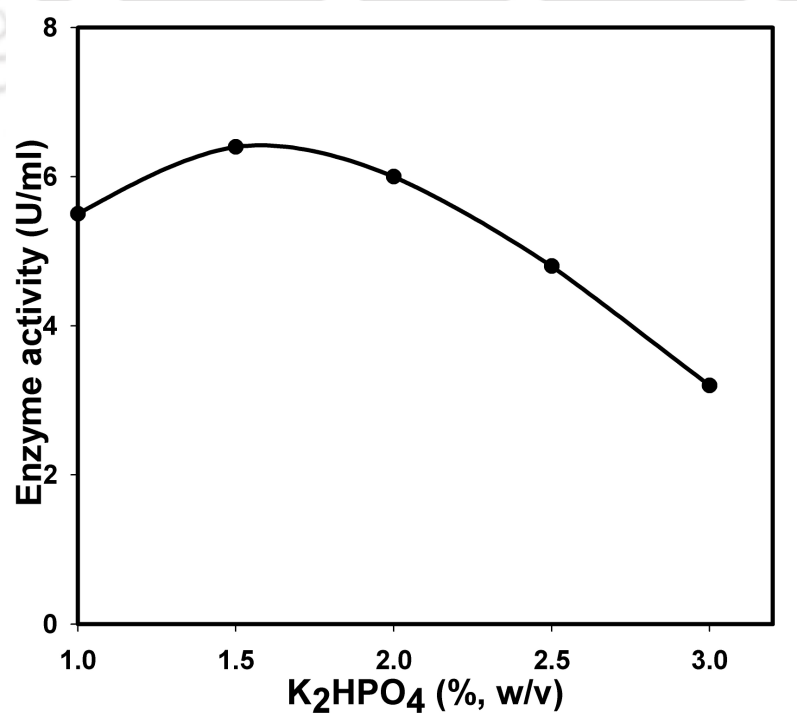


Fig. 3.3.6 Effect of K_2HPO_4 on dextransucrase production.

3.3.4 Effect of Tween 80 on dextransucrase production

The production of dextransucrase increased with increase in concentration of Tween 80 upto 0.1% (v/v) (Fig. 3.3.7, Table 3.3.1) resulting in 17% increase in the enzyme activity as compared with control. Further increase in the Tween 80 concentration showed saturation in dextransucrase production. This result is similar to the earlier reports where they showed that addition of Tween 80 to enzyme production medium altered the fatty acid composition of the membrane thus enhancing the secretion of the dextransucrase and its activity (Umesaki *et al.*, 1977; Sato *et al.*, 1989; Goyal and Katiyar, 1997; Purama and Goyal, 2008a).

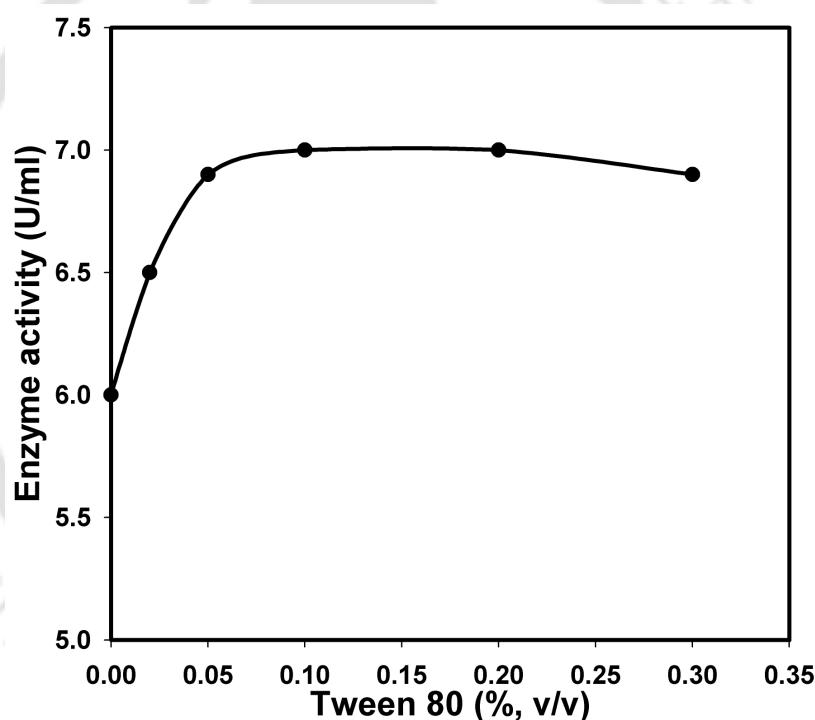


Fig. 3.3.7 Effect of Tween 80 on dextransucrase production.

3.3.5 Medium optimization for enhanced dextransucrase production by Taguchi's orthogonal array method

The optimum temperature, pH and shaking condition were 25°C, 7.0 and 180 rpm, respectively for dextransucrase production from *Weissella confusa* (Shukla and Goyal, 2011a). *Weissella confusa* Cab3 grew well within the range of pH 5.0 to 7.0,

and temperature 20°C to 45°C however *Weissella confusa* Cab3 produced maximum dextransucrase (6.0 U/ml) at pH 7.0 and 25°C in the enzyme production medium (Shukla and Goyal, 2011a). As described in previous section, the effects of several medium components were investigated and sucrose, Tween 80, yeast extract and K₂HPO₄ were effective nutrients which displayed higher dextransucrase production (Shukla and Goyal, 2011b). Based on the earlier results, L-16 Taguchi's orthogonal array method was designed, where the factors were varied in four levels for determining optimal medium components for dextransucrase production from *Weissella confusa* Cab3. Level 1 for each factor was fixed at negative side, considering the factors role in dextransucrase production whereas, level 2 and 3 were considered as intermediate level for the production of dextransucrase. Level 4 of each factor was selected at relatively higher concentration range. Table 3.3.2 indicates the selected fermentation factors and their levels for optimization of dextransucrase production by this bacterial strain.

Table 3.3.2 Selected factors and their assigned levels for dextransucrase production by *Weissella confusa* Cab3.

Factor	Level 1	Level 2	Level 3	Level 4
Sucrose (% w/v)	2.0	3.0	4.0	5.0
Yeast extract (% w/v)	0.1	0.5	1.0	2.0
K ₂ HPO ₄ (% w/v)	0.1	0.5	1.0	2.0
Tween 80 (% v/v)	0.01	0.1	0.5	1.0

The design matrix and dextransucrase production data are represented in Table

3.3.3.

Table 3.3.3 Fractional factorial design of L-16 orthogonal array used for optimization of dextransucrase production optimization by *Weissella confusa* Cab3.

S. No.	Sucrose	Yeast extract	K ₂ HPO ₄	Tween 80	Dextransucrase activity (U/ml)	
					Experimental	Predicted
1	2.0	0.1	0.1	0.01	1.60	1.19
2	2.0	0.5	0.5	0.1	1.67	0.85
3	2.0	1.0	1.0	0.5	2.87	3.37
4	2.0	2.0	2.0	1.0	4.00	4.73
5	3.0	0.1	0.5	0.5	5.80	6.53
6	3.0	0.5	0.1	1.0	3.99	4.49
7	3.0	1.0	2.0	0.01	4.60	3.78
8	3.0	2.0	1.0	0.1	6.68	6.26
9	4.0	0.1	1.0	1.0	12.00	11.18
10	4.0	0.5	2.0	0.5	10.42	10.01
11	4.0	1.0	0.1	0.1	6.40	7.13
12	4.0	2.0	0.5	0.01	11.29	11.79
13	5.0	0.1	2.0	0.1	11.85	12.35
14	5.0	0.5	1.0	0.01	12.87	13.61
15	5.0	1.0	0.5	1.0	14.96	14.55
16	5.0	2.0	0.1	0.5	17.00	16.18

A minor variation was observed between software-predicted and experimental values in dextransucrase production. The variation in predicted dextransucrase activity by the software MiniTab version 15 is shown in Fig. 3.3.8 and Table 3.3.3. The experimental values of dextransucrase production by *Weissella confusa* Cab3 widely varied between 1.6-17.0 U/ml (Table 3.3.3). From Fig. 3.3.8 it was clear that sucrose, 5 (% w/v); yeast extract, 2 (% w/v); K₂HPO₄, 1 (% w/v) and Tween 80, 0.5 (% w/v) maximized the dextransucrase production. These concentrations of the four nutrients were fitted in the software (MiniTab version 15) to obtain the predicted maximized dextransucrase activity (17.54 U/ml). The predicted dextransucrase activity using the optimized medium was validated experimentally and described later in this Chapter, Section 3.3.5.

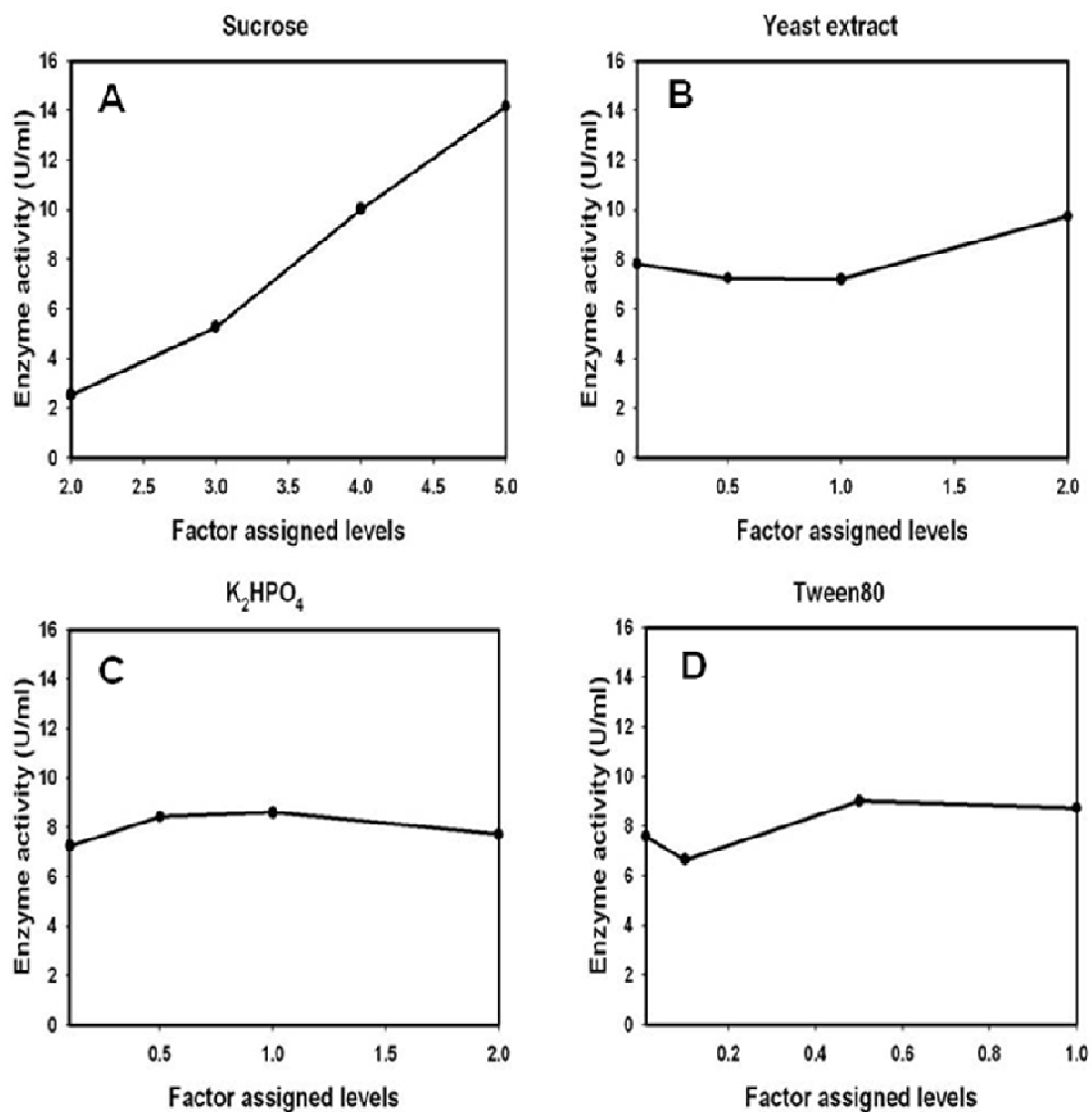


Fig. 3.3.8 Multiple graphs of predicted main effects of (A) Sucrose, (B) Yeast extract, (C) K_2HPO_4 and (D) Tween 80 on dextranucrase production by *Weissella confusa* Cab3 using Design Expert software version 8.

The difference between average value of each factor at higher level and lower level indicated the relative influence of the effect at their individual capacities. The order in which the individual components selected in the present study affected the dextransucrase production can be ranked as sucrose > yeast extract > Tween 80 > K_2HPO_4 , suggesting that sucrose, yeast extract and Tween 80 had substantial effect and K_2HPO_4 had least effect on dextransucrase production by *Weissella confusa* (Table 3.3.4). Highest delta value of sucrose (11.635) reflected it to be most significant factor for the dextransucrase production. As the concentration was increased to 5% (level 4), maximum dextransucrase production occurred. Yeast extract and Tween 80 were the next important nutrients for dextransucrase production, 2% (w/v) and 0.5% (v/v) being optimum for dextransucrase production.

Table 3.3.4 Impact of fermentation factors and their assigned levels on dextransucrase production by *Weissella confusa* Cab3.

Factor	Level 1	Level 2	Level 3	Level 4	Delta	Rank
Sucrose	2.536	5.267	10.028	14.172	11.635	1
Yeast extract	7.814	7.239	7.208	9.742	2.534	2
K_2HPO_4	7.248	8.431	8.605	7.718	1.358	4
Tween 80	7.592	6.649	9.024	8.738	2.375	3

The significance of each factor on dextransucrase production can be seen from the corresponding t and P values listed in Table 3.3.5. The ANOVA table demonstrates that the sucrose was the significant factor for the dextransucrase production, as is evident from the Fisher's F-test with a very low probability value ($p > F$) = 0.005 (Table 3.3.5).

Table 3.3.5 Analysis of variance of experimental data on dextransucrase production by *Weissella confusa* Cab3.

Factors	DF	SS	MS	F- value	Prob. (P)>F
Sucrose	3	318.095	106.032	48.88	0.005
Yeast extract	3	17.107	5.702	2.63	0.224
K ₂ HPO ₄	3	4.790	1.597	0.74	0.596
Tween 80	3	14.338	4.779	2.20	0.267
Residual error	3	6.507	2.169		
Others	15	360.837			

$R^2 = 99.1\%$; Adj $R^2 = 99.6\%$.

DF: degrees of freedom, SS: sum of squares, MS: mean square

Table 3.3.6 represents the optimum conditions required for the production of maximum dextransucrase production. The experimental data revealed that selected level 4 value of sucrose and yeast extract of the medium were observed to be optimum for dextransucrase production, whereas for K₂HPO₄ and Tween 80, selected level 3 values, were observed to be good for optimal enzyme production. The model was highly significant considering to its 99.1% R^2 value.

Table 3.3.6 Optimum conditions and levels of factors.

Factor	Optimum Concentration	Level
Sucrose (% w/v)	5.0	4
Yeast extract (% w/v)	2.0	4
K ₂ HPO ₄ (% w/v)	1.0	3
Tween 80 (% v/v)	0.5	3

3.3.6 Validation of medium optimization for enhanced dextransucrase production by Taguchi's orthogonal array method and scale up at bioreactor level

Taguchi's orthogonal methodology predicted the maximum dextransucrase production of 17.54 U/ml, in a medium containing (g/l) sucrose, 50.0; K₂HPO₄, 10; yeast extract, 2.0; Tween 80, 5 (ml/l); MgSO₄·7H₂O, 0.2; MnSO₄·4H₂O, 0.01; FeSO₄·7H₂O, 0.01; CaCl₂·2H₂O, 0.01 and NaCl 0.01. The dextransucrase production using statistically optimized medium was validated at shake flask level and scaled up in a 3 l lab scale bioreactor using 1 l of statistically designed medium. The fermentation profiles of dextransucrase production from *Weissella confusa* Cab3 at shake flask and bioreactor level are shown in Fig. 3.3.9A and B, respectively. Table 3.3.7 shows comparison of maximum attained fermentation parameters using unoptimised medium and optimized medium. For the bioreactor the online data such as dissolved oxygen, pH, temperature and agitation were monitored and the offline data like enzyme activity (as described earlier Chapter 2, Section 2.2.10.2), sucrose concentration (as described earlier in this Chapter, Section 3.2.3) and cell optical density were plotted with time (Fig. 3.9B). The enzyme activity and the cell optical density reached maximum at 10-12 h of fermentation at both shake flask and bioreactor level. In both the cases (shake flask and bioreactor level) sucrose concentration profiles showed maximum consumption of sucrose during first 10-15 h, with the maximum production of dextransucrase using the same optimized medium.

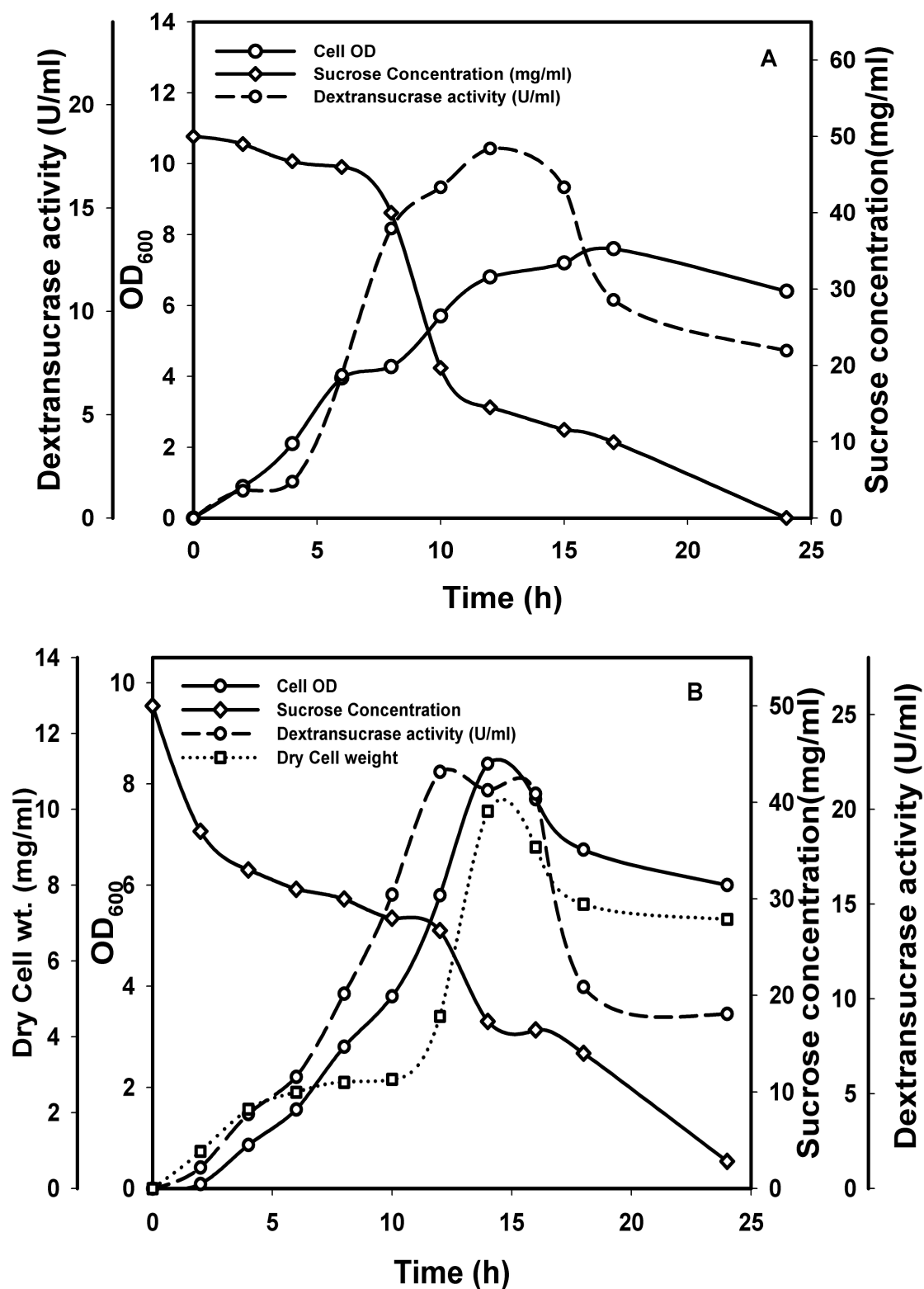


Fig. 3.3.9 Fermentation profile of *Weissella confusa* Cab3 using statistically designed medium for dextranucrase production at (A) Shake flask culture, 100 ml and (B) Bioreactor 1000 ml level.

The experimentally calculated maximum dextransucrase activity at shake flask level was 17.9 U/ml which was in agreement with the predicted value. The increase in dextransucrase activity of the *Weissella confusa* Cab3 after medium optimization (17.9 U/ml) was about 3.0 fold higher as compared with unoptimized medium (6.0 U/ml). The dextransucrase activity at bioreactor level after 10-12 h was 22.0 U/ml which was 17% more than that observed at shake flask level using the optimized medium (Table 3.3.7). Oxygen is known to have positive effects on the growth of certain strains of *Leuconostoc mesenteroides* (Veljkovic *et al.*, 1992). Thus the higher production of dextransucrase in bioreactor as compared to flask culture is possibly be due to the effect of oxygen mass transfer rates on biosynthesis of dextransucrase.

Table 3.3.7 Comparison of fermentation parameters for dextransucrase production from *Weissella confusa* Cab3 using unoptimized and optimized medium.

Tsuchiya medium*				
Levels	Enzyme activity (U/ml)	Specific activity (U/mg)	Cell OD ₆₀₀	Dry cell wt (mg/ml)
Shake Flask (100 ml)	6.0	1.0	5.4	4.1
Optimized medium				
Shake Flask (100 ml)	17.9	3.0	7.6	7.1
Bioreactor (1000 ml)	22.0	3.5	8.4	9.9

* Tsuchiya *et al.*, 1952 as described in Chapter 2, Section 2.2.4.

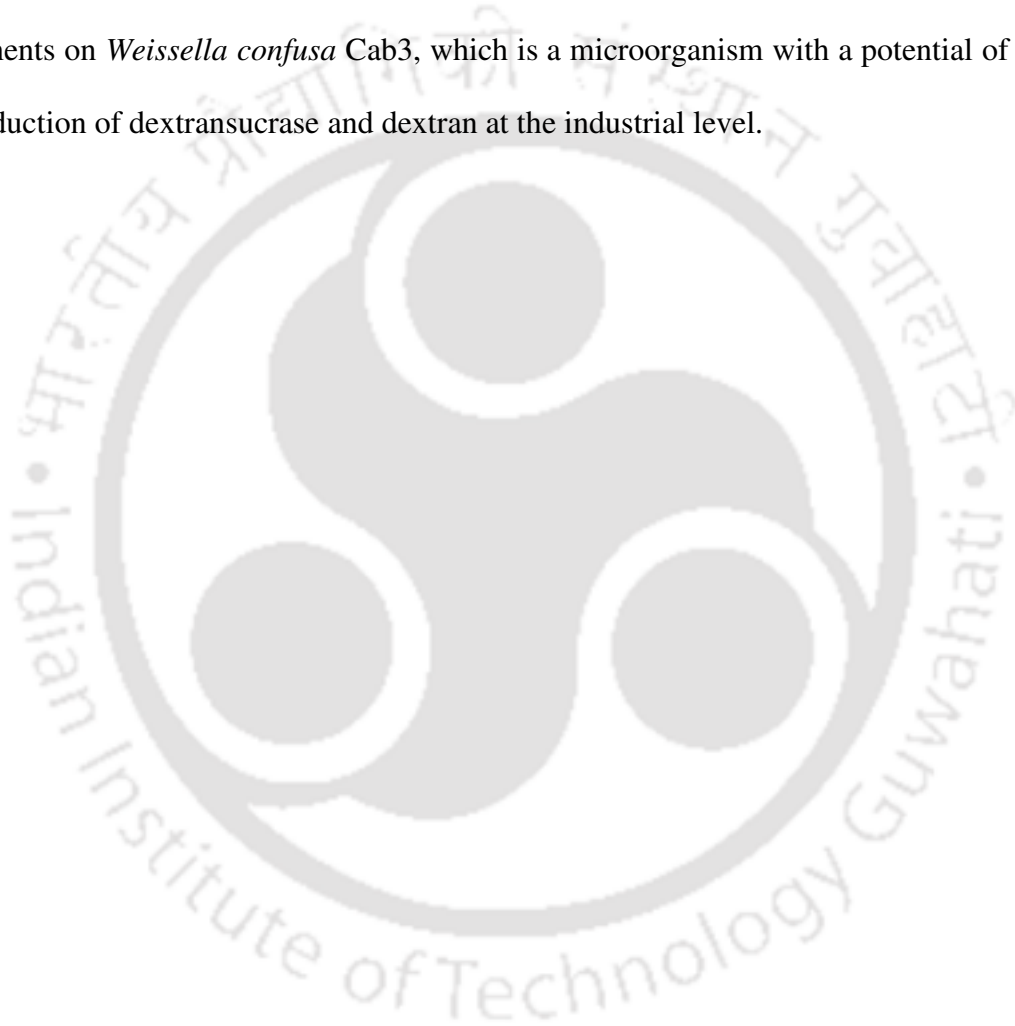
The dextransucrase production (22.0 U/ml) using statistically optimized medium by *Weissella confusa* Cab3 at lab scale bioreactor level is higher than that observed with other lactic acid strains in their respective optimized medium. There is no literature available about the optimization of medium composition for dextransucrase production from *Weissella confusa*. *Leuconostoc mesenteroides* NRRL B-640 produced 10.7 U/ml dextransucrase activity in the optimised medium (Purama and Goyal, 2008b). However, according to a recent study by Patel *et al.*, 2011 a mutant *Pediococcus pentosaceus* (SPAm) showed substantially higher dextransucrase activity (15.6 U/ml). A *Leuconostoc mesenteroides* strain isolated from idli batter, an Indian fermented food was used for the optimisation for enhanced dextransucrase yield using response surface methodology (Sawale and Lele, 2009). They reported 489.12 DSU/ml (23.8 U/ml) activity after optimization which was 5.5 fold higher than that of basic medium, however, a very high concentration of sucrose (13.75 %, w/v) was used. In contrast to their findings, in present study lesser concentration of sucrose (5.0 %, w/v) resulted in significantly high dextransucrase production.

3.4 Conclusions

The influence of medium ingredients on extracellular dextransucrase production by a new bacterial strain *Weissella confusa* Cab3 (Genbank Accession Number JX649223) was evaluated. *Weissella confusa* Cab3 produced substantially high dextransucrase in basic unoptimized enzyme production medium. The effects of various macronutrients such as sucrose, yeast extract, beef extract, peptone, sodium acetate, K_2HPO_4 and Tween 80 were studied on dextransucrase production from *Weissella confusa* Cab3 using one factor at a time approach (OVAT). The medium used as control gave 6.0 U/ml enzyme activity. Sucrose (5%), Tween 80 (0.1%), yeast extract (1.5%), peptone (1.5%) and K_2HPO_4 (1.5%) were effective nutrients displaying higher dextransucrase production giving 18.2, 7.0, 6.4, 6.2 and 6.4 U/ml enzyme activity, respectively. Sodium acetate and beef extract were not effective for enzyme production.

The effect of medium ingredients on dextransucrase production was evaluated using fractional factorial design of Taguchi's orthogonal array. Metabolism influencing four factors viz. sucrose, yeast extract, K_2HPO_4 and Tween 80 were selected to optimize dextransucrase production by *Weissella confusa* Cab3 using fractional factorial design of Taguchi method. Based on the influence of interaction components of fermentation, least significant factors of individual level have higher interaction severity index and *vice versa* for enzyme production from *Weissella confusa* Cab3. Sucrose and yeast extract were most significant factors which positively influenced the dextransucrase production. The optimized medium composition consisted of sucrose 5% (w/v); yeast extract 2% (w/v); K_2HPO_4 1.0% (w/v); Tween 80 0.5% (v/v), based on Taguchi orthogonal array method. The optimized composition gave an experimental value of dextransucrase activity of 17.9

U/ml at shake flask level which corresponded well with the predicted value of 17.54 U/ml by the model. After scaling up the dextransucrase production in a lab scale bioreactor 22.0 U/ml enzyme activity was obtained. The optimized medium gave 3.0 and 3.7 fold higher dextransucrase production at shake flask and bioreactor level, respectively from *Weissella confusa* Cab3 as compared with unoptimized medium. The result displayed successful effort in understanding the effects of various medium components on *Weissella confusa* Cab3, which is a microorganism with a potential of the production of dextransucrase and dextran at the industrial level.



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Chapter 4

Production, purification and characterization of dextransucrase from *Weissella confusa* Cab3

3.1 Introduction

Dextransucrase is an extracellular enzyme of high molecular weights typically in the range of 120-200 kDa belonging to Glycoside hydrolase family 70 (<http://www.cazy.org/>). Dextransucrase are mechanistically, structurally and evolutionary closely related to the GH13 and GH77 enzymes (Stam *et al.*, 2006). Recently, the detail 3 dimensional crystal structure of these enzymes has become available (Vujicic-Zagar *et al.*, 2010; Ito *et al.*, 2011; Brison *et al.*, 2012). Dextransucrase has been reported among genera of *Lactobacillus* (Hammond, 1969), *Leuconostoc* (Kim and Robyt, 1995; Padmanabhan and Kim, 2002), *Streptococcus* (Sidebotham *et al.*, 1971) and *Weissella* (Maina *et al.*, 2008; Shukla and Goyal, 2011). However, Smitinont *et al.* 1999 and Patel *et al.* (2011) reported the production of dextransucrase from *Pediococcus pentosaceus* strain. However, not much work has been done on the dextransucrase production of *Weissella confusa*. This enzyme is of high industrial importance due to its dextran synthesizing capacity. This enzyme utilises sucrose as substrate and cleaves it to release free glucose and fructose. The released glucosyl units are polymerized to form the polymer dextran. The dextran has

numerous industrial and food applications (Neubauer *et al.*, 2003). In addition, dextransucrase can transfer glucose residues from sucrose onto sugars or hydroxylated exogenous acceptor molecules, resulting in oligosaccharides or glucoconjugates synthesis which results in increase solubility and/or biological activity (Monsan *et al.* 2010).

Dextransucrases are large proteins existing in multiple forms ranging from molecular mass of 64-245 kDa (Willemot *et al.*, 1988). This variation is associated with the presence of dextran in the purified preparations, with the disassociation of subunits from a high molecular mass multimeric complex (Kobayashi and Matsuda 1980; Kobayashi and Matsuda, 1986). The multiple molecular forms of dextransucrase could also result as the action of proteases (Miller and Robyt, 1986; Argüello-Morales *et al.*, 2005). Sanchez-Gonzalez *et al.*, 1999 showed that the presence of different molecular mass forms of dextransucrase from *L. mesenteroides* strains B-512F and B-512FMC are the result of proteolytic processing. The PEG-400 purified dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 also showed multiple protein bands on SDS-PAGE with a prominent band corresponding to the size 180 kDa (Purama and Goyal, 2008) and a single band on non-denaturing native-PAGE analysis (Purama and Goyal, 2009a). While dextransucrase from *Leuconostoc* are inducible by sucrose, it has been reported that dextransucrase from several *Weissella* sp. are constitutive in nature (Bounaix *et al.*, 2010). Constitutive dextransucrase of 180 kDa size from various *Weissella* sp (Five strains of *W. cibaria* (LBAE-C36-1, D38, D39, H25 and K39) and two strain of *W. confusa* (LBAE-C39-2 and DSM 20196) have been reported in medium containing sucrose or glucose as the carbon source (Bounaix *et al.*, 2010). Bounaix *et al.*, 2010 also reported an additional

sucrose inducible dextransucrase of approximately, 300 kDa, for *W. confusa* DSM 20196 which did not form in medium containing glucose.

Various purification methods such as salt, alcohol precipitation, fractionation by polyethylene glycol, ultra-filtration, chromatography and phase-partitioning have been successfully used for purification of dextransucrase from different strains of lactic acid bacteria (Majumder *et al.*, 2007). Among all the reported purification methods, polyethylene glycol (PEG) is an effective, rapid and single step purification method for dextransucrase from *Leuconostoc mesenteroides* (Purama and Goyal, 2008). Polyethylene glycol (PEG) is a non-ionic hydrophilic polymer which selectively precipitates high molecular weight or aggregated form of proteins (Goyal and Katiyar, 1994). The dextransucrase from *L. mesenteroides* NRRL B-512F (Goyal and Katiyar, 1994), *Leuconostoc mesenteroides* NRRL B-640 (Purama and Goyal 2008), *Leuconostoc dextranicum* NRRL B-1146 (Majumder *et al.*, 2008) and *Pediococcus pentosaceus* (Patel *et al.*, 2011) have been purified by fractionation with PEG of different molecular weights. *Leuconostoc mesenteroides* NRRL B-512F was mutated by N-nitrosoguanidine (designated as B-512FM) which resulted in 300 times increase in the activity (Fu and Robyt, 1990). The mutated dextransucrase was purified by treatment with crude dextranase, followed by column chromatography using Bio-Gel A-5m (Fu and Robyt, 1990).

In the present study, purification of dextransucrase from the isolate *Weissella confusa* Cab3 was carried out by polyethylene glycol fractionation followed by gel filtration. The enzyme was identified and confirmed by Periodic Acid Schiff's (PAS) staining and the approximate molecular weight was determined. The reaction conditions such as temperature, pH, ionic strength and sucrose concentration for maximum enzyme activity were optimized. The effects of various metal ions,

chaotropic agents, additives and storage temperatures on the dextranucrase activity were studied.



4.2 Materials and methods

4.2.1 Chemicals and Reagents

The ingredients required for the preparation of MRS medium and enzyme production medium (Tsuchiya *et al.*, 1952) such as sucrose, yeast extract, beef extract, peptone, tri-ammonium citrate and dipotassium hydrogen orthophosphate were purchased from Hi-Media Pvt. Ltd., India. Sodium acetate, Tween 80, magnesium sulphate heptahydrate, manganese sulphate tetrahydrate, ferrous sulphate heptahydrate and calcium chloride dihydrate were purchased from Fisher Scientific Pvt. Ltd., India. The chemicals required for reducing sugar estimation (sodium carbonate anhydrous, sodium potassium tartrate, sodium bicarbonate, sodium sulphate anhydrous, cupric sulphate, ammonium molybdate and sodium arsenate), protein estimation (sodium carbonate, sodium hydroxide, sodium potassium tartrate, cupric sulphate and phenol reagent) and buffer preparation were of highest purity grade. PEG-400 from Qualigens Pvt. Ltd., India and PEG-1500 from BDH, U.K. were used for fractionation of glycosyltransferases. The chemicals required for Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS-PAGE) were purchased from Sigma Aldrich Pvt. Ltd., USA. The protein ladder (10-200 kDa) for SDS-PAGE was purchased from Fermentas, Germany.

4.2.2 Maintenance of the isolate *Weissella confusa* Cab3

Weissella confusa Cab3 (GenBank Accession Number: JX649223) was isolated from fermented cabbage as described in Chapter 2, Section 2.2.2. This isolate was maintained as stab in modified MRS agar (as described in Chapter 2, section 2.2.3) at 4°C and sub-cultured every two weeks.

4.2.3 Production of dextransucrase from *Weissella confusa* Cab3

For the development of inoculum, a loopful of culture from modified MRS agar stab (as described in Chapter 2, Section 2.2.3) was transferred to 5 ml of medium described by Tsuchiya *et al.* 1952 (as described in Chapter 2, Section 2.2.4). The isolate *Weissella confusa* Cab3 was incubated at 25°C at 180 rpm for 12 h. 1% of the culture was again inoculated in 100 ml enzyme production medium (as described in Chapter 2, Section 2.2.4) in a 250 ml Erlenmeyer flask and incubated for 12 h at 25°C under shaking conditions at 180 rpm. The culture broth was centrifuged at 8000g at 4°C for 10 min and the cell free supernatant was used for enzyme assay and protein concentration determination and enzyme purification as described in next Sections 4.2.4, 4.2.5 and 4.2.7, respectively.

4.2.4 Enzyme activity assay

The assay of dextransucrase was conducted in 1 ml of a reaction mixture containing 20 mM sodium acetate buffer (pH 5.4), 5% sucrose and 20 µl cell free supernatant containing enzyme. The reaction mixture was incubated at 35°C for 15 min. The enzyme activity was analyzed by estimating the reducing sugars by the Nelson-Somogyi protocol (Nelson, 1944; Somogyi, 1945) as described in Chapter 2, Section 2.2.10.2. Aliquots (0.1 ml) from the reaction mixture were analyzed for reducing sugar. The absorbance was recorded at 500 nm in a UV-visible spectrometer (Varian, model Cary 100) against a blank. D-fructose was used as a standard.

4.2.5 Protein determination

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 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's 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.

4.2.6 Estimation of protein

To 0.2 ml of sample containing protein or BSA, 1 ml of reagent C was added. After 10 min, 0.1 ml of phenol 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:

$$\text{Protein Concentration} = \frac{\Delta A_{660} \times C \times 10^{-3} \times V}{v} \text{ (mg/ml)}$$

where,

A_{660} = Optical density of protein sample at 660 nm.

C = Amount (µg/ml) of BSA at OD=1 from standard plot.

V = Volume (ml) of reaction mixture.

v = Volume (ml) of the protein sample.

4.2.7 Purification of dextransucrase by fractionation using polyethylene glycol

Among all the reported purification methods, polyethylene glycol (PEG) is an effective, rapid and single step purification method for dextransucrase (Purama and Goyal, 2008). The dextransucrase could be purified using different molecular weights of PEG. For purification of dextransucrase, the cell free supernatant (as described in Section 4.2.3) was subjected to fractionation by different molecular weight of polyethylene glycol according to Purama and Goyal, 2008. The ice cold polyethylene glycols of different molecular weights (PEG-400 and PEG-1500) were added to 30 ml cell free supernatant of *Weissella confusa* Cab3. The PEG-400 concentration varied from 10-50 (% v/v) in cell free supernatant. 40% (w/v) of PEG-1500 solution was prepared in distilled water and added to the 30 ml cell free supernatant to get final concentrations between 5-25% (v/v). The mixtures were incubated at 4°C for 12 h to allow the dextransucrase to fractionate. The mixture was centrifuged at 12,000g for 30 min at 4°C to separate the fractionated dextransucrase. The enzyme pellet was separated and dissolved in 20 mM sodium acetate buffer (pH 5.4). The resuspended enzyme samples were subjected to dialysis using 5 kDa cut off membrane with four changes of same buffer. The dextransucrase samples were analyzed for enzyme activity (as described in Chapter 2, Section 2.2.10.2) and protein concentration (as described earlier in Section 4.2.6).

4.2.8 Purification of dextransucrase by gel filtration

The dextransucrase (10.5 U/mg, 0.84 mg/ml) purified by PEG-400 (30%) fractionation (as described in Section 4.2.7) was further purified by gel filtration. 2.0 ml of the dialyzed enzyme (10.5 U/mg, 0.84 mg/ml) was applied to a glass column (30 cm x 1.5 cm) containing Sephacryl S-200HR with a bed volume of 40 ml

previously equilibrated with 20 mM sodium acetate buffer (pH 5.4). The column was run on chromatography system with UV detector (Biologic LP, BioRad Labs. India) equipped with a fraction collector (Bio RAD, Model 2110 fraction Collector). The enzyme was eluted at a flow rate of 0.2 ml/min using the same buffer and total of 40 fractions (2 ml each) were collected and analyzed for enzyme activity (as described in Chapter 2, Section 2.2.10.2) and protein concentration (as described earlier in 4.2.6). Fractions containing the highest specific activity were pooled. The pooled enzyme fraction was used for the estimation of enzyme activity, protein concentration and was lyophilized for further analysis by SDS-PAGE.

4.2.9 SDS-PAGE analysis of purified enzyme

4.2.9.1 Stock solutions preparation

(A) Acrylamide/ Bis acrylamide solution (29.2%, w/v acrylamide and 0.8% w/v Bisacrylamide)

29.2 g acrylamide and 0.8 g N, N'- methylene-bis acrylamide to 100 ml distilled water. The solution was filtered through Whatmann no. 1 and stored at 4°C in dark.

(B) Tris-HCl (1.5 M, pH 8.8)

54.45 g Tris base (121.14 g/mol) was first dissolved in 150.0 ml of distilled water. The pH of solution was adjusted to 8.8. Finally the volume of the solution was made up to 300 ml and stored at 4°C.

(C) Tris-HCl (0.5 M , pH 6.8)

6.0 g Tris base (121.14 g/mol) was first dissolved in 60.0 ml of distilled water. The pH of solution was adjusted to 6.8. Finally the volume of the solution was made

up to 100 ml and stored at 4°C.

(D) SDS (10%, w/v)

10.0 g SDS was first dissolved in 60.0 ml of distilled water with gentle stirring. Finally the volume of the solution was made up to 100 ml.

(E) Ammonium persulphate (10%, w/v)

100.0 mg ammonium persulphate (APS) was dissolved in 1.0 ml of distilled water.

(F) Sample buffer

The protein sample buffer was prepared by mixing the components mentioned below in Table 4.2.1. A 5x stock solution of sample buffer was prepared and was mixed with 4 volumes of protein sample to make it to 1x before loading on to the gel.

Table 4.2.1 Composition of 5x sample buffer

Sample buffer Component	Amount (ml)	Final Concentration
0.5 M Tris HCl (pH 6.8)	1.0	62.5 mM
Glycerol	1.6	20% (v/v)
10 (% w/v) SDS	1.6	2% (v/v)
0.5 (% w/v) Bromo Phenol Blue in water	0.4	0.025% (w/v)
Distilled water	3.4	
Total volume	8.0	

(E) 5x Running buffer

45.0 g Tris base, 216.0 g glycine and 15.0 g SDS was dissolved dissolved in 200.0 ml of distilled water. Finally the volume of the solution was made up to 300 ml.

4.2.9.2 Electrophoretic analysis of purified dextranucrase

SDS-polyacrylamide gel electrophoresis was performed using a vertical slab mini gel unit (BioRad) using 1.5 mm thick gels, following the method of Laemmli (1970). 7.0% (w/v) acrylamide for resolving gel and 4% (w/v) for stacking gel were prepared as mentioned in Table 4.2.2 and Table 4.2.3, respectively. For running the enzyme samples under non denaturing conditions the sample buffer (5x) used did not contain β -mercaptoethanol (as described in Table 4.2.1) and the enzyme sample mixed with sample buffer was not subjected to heat denaturation. The samples from all the purified fractions with different molecular weights were loaded on 7.0% acrylamide gel under SDS non-denaturing conditions (without β -mercaptoethanol and heat treatment). Each protein samples to be analyzed were loaded in duplicate on the 7.0% polyacrylamide gel. Electrophoresis was carried out with a current of 2 mA per lane using 1x running buffer (as described in Section 4.2.9.1). Following the run, the gel was cut in two parts. One part of the gel was subjected to silver staining as described in Section 4.2.10 and the other part of the gel was analyzed for *in-situ* activity detection by Periodic acid Schiff (PAS) staining protocol as described in Section 4.2.11. High range molecular mass marker proteins (from 10 kDa to 200 kDa) purchased from Fermentas, India, was used as standard for SDS-PAGE.

Table 4.2.2 Components for the preparation of 7% resolving gel

Component	Volume (ml)
Acrylamide-bisacrylamide solution (30%, w/v)	2.30
SDS solution (10%, w/v)	1.00
Glycerol (50%, v/v)	1.00
1.5 M Tris (pH 8.8)	3.30
APS solution (10%, w/v)	0.10
TEMED	0.01
Deionized water	2.30
Total volume	10.00

Table 4.2.3 Components for the preparation of 4% stacking gel

Component	Volume (ml)
Acrylamide-bisacrylamide solution (30%, w/v)	0.70
SDS solution (10%, w/v)	0.50
1.0 M Tris (pH 6.8)	1.00
APS solution (10%, w/v)	0.05
TEMED	0.01
Deionized water	2.74
Total volume (ml)	5.00

4.2.10 Silver staining protocol

4.2.10.1 Preparation of buffers for silver staining

Fixing solution	: 40 (% v/v) ethanol, 10 (% v/v) acetic acid in water
Sensitizing solution	: 0.2 (% w/v) $\text{Na}_2\text{S}_2\text{O}_3$, 6.8 (% w/v) Sodium acetate, 30 (% v/v) ethanol and 0.025 (% v/v) glutaraldehyde
Silver nitrate	: 0.1 (% w/v) AgNO_3 , 0.076 (% w/v) formalin
Developer	: 2.5 (% w/v) Na_2CO_3 , 0.05 (% w/v) formaldehyde
Stop solution	: 1.46 (% w/v) EDTA

4.2.10.2 Silver staining procedure

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

4.2.11 Identification of dextransucrase by activity staining using Periodic Acid Schiff's (PAS) protocol

In situ activity of dextransucrase was detected on a 7.0% acrylamide gels run under SDS non denaturing conditions (as described in Section (4.2.8) using the protocol described by Holt *et al.*, 2001. The samples from all fractions were loaded on two identical, 7.0% acrylamide gels and were under SDS-non denaturing condition as described in Section 4.2.8. After run the gel was treated thrice with a solution containing 20 mM sodium acetate buffer (pH 5.4), 0.1% (w/v) Triton-X-100 and 0.005% (w/v) calcium chloride for 20 min to remove SDS. Then the gel was incubated with 5% (w/v) sucrose/raffinose solution in 20 mM sodium acetate buffer, pH 5.4 for 10-12 h at 30°C. Following incubation the gel was washed twice with 75% (v/v) ethanol for 20 min and incubated in solution with 0.7% (w/v) periodic acid in 5% (v/v) acetic acid for 20 min at room temperature. The gel was then washed thrice with 0.2% (w/v) sodium meta bisulfate in 5% (v/v) acetic acid solution and finally stained with Schiff's reagent (0.5%, w/v basic fuschin, 1%, w/v sodium bisulfite and 0.1N HCl) until the discrete magenta bands appeared within the gel. The other gel was incubated in 5 (% , w/v) raffinose, which is a specific substrate for fructosyltransferases (inulansucrase and levansucrase), in order to exclude the presence of fructosyltransferases.

4.2.12 Optimization of reaction conditions for dextransucrase activity

4.2.12.1 Effect of temperature on dextransucrase activity

To study the effect of temperature on the activity of purified dextransucrase (10.5 U/mg, 0.84 mg protein/ml), the temperature range 20-45°C was used for enzyme assay. The enzyme reaction was carried out in 1 ml reaction mixture in 20 mM sodium acetate buffer (pH 5.4) containing 146 mM (5%) final concentration of

sucrose, 20 μ l of enzyme (0.84 mg protein/ml) for 15 min. The assay mixtures were incubated at different temperatures for 15 min. Aliquots (0.1 ml) of reaction mixture were taken for reducing sugar analysis as described earlier in Chapter 2, Section 2.2.10.2.

4.2.12.2 Effect of ionic strength of buffer on dextranucrase activity

To study the effect of different ionic strength of buffer on the enzyme activity of purified dextranucrase (10.5 U/mg, 0.84 mg protein/ml), the enzyme assay was conducted at different ionic strength of sodium acetate buffer (pH 5.4) ranging from 10-400 mM. The reaction was carried out in 1 ml mixture in sodium acetate buffer of pH 5.4 containing 146 mM (5%, w/v) final concentration of sucrose, 20 μ l of enzyme (0.84 mg protein/ml) and varying ionic strength of sodium acetate buffer (pH 5.4). The mixture was incubated at 35°C in water bath for 15 min and the enzyme activity was determined by estimating the released reducing sugar, as described earlier in Chapter 2, Section 2.2.10.2.

4.2.12.3 Effect of pH of buffer on dextranucrase activity

To study the effect of different pH of buffer on the enzyme activity of purified dextranucrase (10.5 U/mg, 0.84 mg protein/ml), the enzyme assay was conducted at different buffer pH ranging from 4.0-7.0. The reaction was carried out in 1 ml mixture in 20 mM sodium acetate buffer of varying pH containing 146 mM (5%) final concentration of sucrose and 20 μ l of enzyme (0.84 mg protein/ml). The mixture was incubated at 35°C in water bath for 15 min and the enzyme activity was determined by estimating the released reducing sugar, as described earlier in Chapter 2, Section 2.2.10.2.

4.2.12.4 Effect of sucrose concentration on dextransucrase activity

To study the effect of sucrose concentration on the enzyme activity of the purified dextransucrase (specific activity 10.5 U/mg, 0.84 mg/ml), the enzyme assay was conducted at different sucrose concentration ranging from 0.1-8 (% w/v). The reaction was carried out in 1 ml mixture in 20 mM sodium acetate buffer pH 5.4 containing 20 µl of enzyme (0.84 mg protein/ml) and varying concentration of sucrose. The mixture was incubated at 35°C in water bath for 15 min. The enzyme activity was determined by taking 0.1 ml of reaction mixture and estimating the released reducing sugar, as described earlier in Chapter 2, Section 2.2.10.2.

4.2.13 Effect of salts and EDTA on the activity of dextransucrase

The effects of MgCl₂, CaCl₂, CoCl₂, NiCl₂, ZnCl₂ and MnCl₂ between 0-10 mM and EDTA between 0-7 mM concentrations on dextransucrase activity were studied. The reaction was carried out in 1 ml reaction mixture of sucrose solution (5%, w/v) in 20 mM sodium acetate buffer pH 5.4 containing 20 µl of enzyme (0.84 mg protein/ml) at 35°C and varying concentrations of salts or EDTA. The enzyme activity was determined as described earlier in Chapter 2, Section 2.2.10.2.

4.2.14 Thermostability studies of dextransucrase

For determining the thermostability, the enzyme (10.5 U/mg, 0.84 mg protein/ml) was incubated at different temperatures (10-60°C) for 1 h. The aliquots (10-20 µl) were assayed for residual enzyme activity in 1 ml reaction mixture containing 5% sucrose in 20 mM sodium acetate buffer, pH 5.4 by incubating at 35°C following the method as described earlier in Chapter 2, Section 2.2.10.2.

4.2.15 Effects of storage temperatures and additives on dextransucrase activity

The effects of various additives on stability of dextransucrase at 30°C were determined. Aqueous solutions of dextran (500 kDa), PEG-8000, glutaraldehyde, glycerol and Tween 80 were added to dextransucrase solution (10.5 U/mg, 0.84 mg protein/ml) in sodium acetate buffer (pH 5.4) to obtain the final concentrations of 2 µg/ml (w/v) dextran (500 kDa), 10 µg/ml (w/v) PEG-8000, 0.1% (v/v) glutaraldehyde, 0.5% (v/v) glycerol and 0.1% (v/v) Tween 80, respectively, in a final volume of 1.0 ml. Storage stability was studied by incubating the dextransucrase at different temperatures (0°C, 4°C and -20°C). The aliquots (20 µl) were taken periodically for residual enzyme activity in 1 ml reaction mixture containing 5% sucrose in 20 mM sodium acetate buffer (pH 5.4) by incubating at 35°C. The half life ($t_{1/2}$) of additives treated dextransucrase was determined only at 30°C. The residual activity and half life ($t_{1/2}$) of dextransucrase were measured at different temperatures with respect to time without any additives by assuming that the decay followed first order kinetics. All the calculations were done according to Naidu *et al.* (2003). It was assumed that the active enzyme state E directly converts to inactive state E_d without providing any significant amount of intermediates according to single step two-stage theory (Sadana, 1995). The two-state mechanism is as follows:



The first-order deactivation can be represented as:

$$dE/dt = -k_d[E] \quad \dots(2)$$

Integration of Eq. (2) leads to:

$$\alpha = \exp(-k_d t) \quad \dots(3)$$

where, $\alpha = E_d/E$.

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

$$t_{1/2} = \ln 2 / k_d \quad \dots(4)$$

where,

$t_{1/2}$ = half-life of enzyme (time).

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



4.3 Results and Discussion

4.3.1 Purification of dextransucrase from *Weissella confusa* Cab3 by fractionation

The cell free supernatant containing crude dextransucrase (6.1 U/ml, 6.0 mg protein/ml) from *Weissella confusa* Cab3 was purified by polyethylene glycols of different molecular weights. The partially purified dextransucrase samples were analyzed for enzyme activity and protein content and compared with that of crude dextransucrase. The purification of the crude enzyme by polyethylene glycols increased the dextransucrase activity significantly. The enzyme activity profiles with PEG-400 and PEG-1500 are shown in Fig. 4.3.1. A concentration of 30% (v/v) PEG-400 and 15% (w/v) PEG-1500 gave maximum enzyme activity (Fig. 4.3.1). Table 4.3.1 shows the maximum specific activity, fold purification and % yield of dextransucrase with corresponding concentrations of PEG-400 and PEG-1500. PEG-400 concentration range between 20%, v/v to 45%, v/v was used to study its effect on dextransucrase purification. The concentration of 30% (v/v) PEG-400 gave the highest specific activity of 10.5 U/mg with 10 fold purification and 26.4% overall yield (Fig. 4.3.1 and Table 4.3.1). The higher or lower concentrations than 30% (v/v) of PEG-400 resulted in decrease of specific activity of dextransucrase (Fig. 4.3.1). 15% (v/v) concentration of PEG-1500 resulted in slightly higher specific activity of 10.9 U/mg with 11 fold purification with 24% overall yield of dextransucrase (Fig. 4.3.1 and Table 4.3.1). The dextransucrase (10.5 U/mg, 0.84 mg/ml) purified by fractionation with 30% (v/v) PEG-400 (PEG-400 being economical) was selected for further experiments.

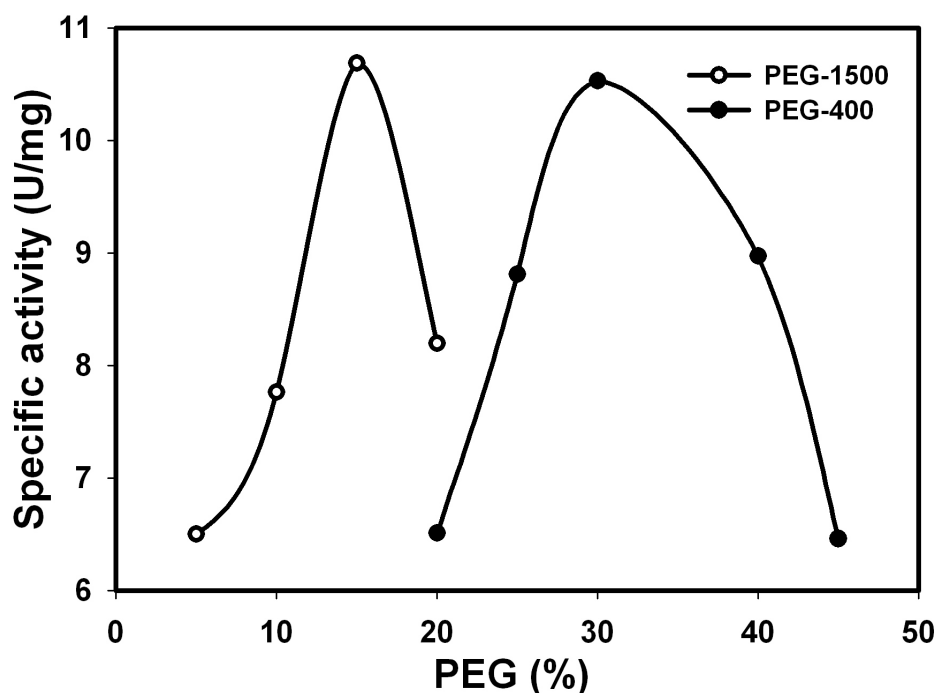


Fig. 4.3.1 Purification of dextransucrase from *Weissella confusa* Cab3 by PEG-400 (% v/v) and PEG-1500 (% w/v). The specific activities of purified dextransucrase by different final concentrations of PEGs are plotted.

Table 4.3.1 Purification of dextransucrase of *Weissella confusa* Cab3.

Sample	Enzyme activity (U/ml)	Vol. (ml)	Total Units	Over all % Yield	Protein mg/ml	Specific activity U/mg	Fold purification
By PEG fractionation							
Crude	6.01	30.0	180.4	-	6.0	1.0	1.0
PEG-400 (30%)	8.53	5.4	46.1	25.5	0.84	10.2	10
PEG-1500 (15%)	4.90	9.0	44.1	24.4	0.45	10.9	11
By gel filtration							
Sephacryl S-200 HR	0.66	8	5.3	25.0	0.02	33.0	33.0

4.3.2 Non-denaturing SDS-PAGE analysis and identification by activity staining of purified enzyme by PEG fractionation

The dextransucrase samples obtained after purification with 30 (% v/v) PEG-400 and 15 (% w/v) PEG-1500 were run on one SDS-PAGE gel in triplicate and run under non denaturing conditions (Fig. 4.3.2). The dextransucrase purified by PEG fractionation by 30% (v/v) PEG-400 and 15% (w/v) PEG-1500 showed single band of approximately, 180 kDa molecular size on the non-denaturing gel stained with silver

staining (Fig. 4.3.2, lane 1 and 2, respectively). The second part of the gel was subjected to activity staining for *in-situ* activity detection. The presence of magenta color bands (Fig. 4.3.2B, lane 3, 4) after PAS staining using sucrose showed the active form of dextranucrase which corresponded well with 180 kDa size of dextranucrase that was obtained with silver staining method (Fig. 4.3.2A, lane 1, 2, respectively). No activity band after PAS staining appeared after the incubation of the third part of non-denaturing SDS-PAGE gel in raffinose (Fig. 4.3.2C; lane 5 and 6), thus excluding the presence of fructosyltransferase.

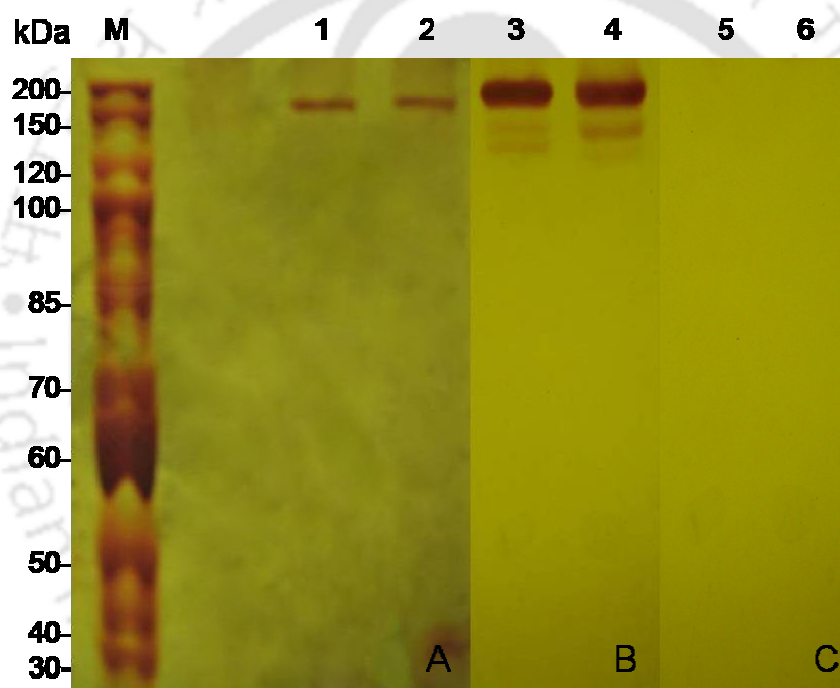


Fig. 4.3.2 Non denaturing SDS-PAGE analysis of dextranucrase by 7% acrylamide gel after PEG fractionation. **(A)** Silver staining of dextranucrase, Lanes: M- Protein molecular weight marker (10-200 kDa), 1- PEG-400 (30%, v/v) fraction; 2- PEG-1500 (15%, w/v) fraction. **(B)** Identification of purified dextranucrase by staining of dextran formed using sucrose; Lanes: 3- PEG-400 (30%, v/v) fraction; 4- PEG-1500 (15%, w/v) fraction and **(C)** Raffinose; Lane 5- PEG-400 (30%, v/v) fraction; 6- PEG-1500 (15%, w/v) fraction.

4.3.3 Purification of dextransucrase by gel filtration

The dextransucrase (10.5 U/mg, 0.84 mg/ml) purified by PEG-400 (30%) fractionation was further purified by gel filtration. The Sephacryl S-200HR column (30 cm x 1.5 cm) was pre equilibrated with 20 mM sodium acetate buffer (pH 5.4) containing 0.1 M NaCl with a flow rate of 1 ml/min. 2 ml of enzyme (10.5 U/mg, 0.84 mg/ml) was loaded on to the Sephacryl S-200HR column. The fractions (2.0 ml) were eluted with the same buffer at a flow rate of 0.2 ml/min. The elution profiles of dextransucrase with the specific activity and protein are shown in Fig. 4.3.3. The dextransucrase activity was found in fraction numbers 3 to 8 as shown in Fig. 4.3.3. The fraction number 3, 4, 5 and 6 were pooled. The pooled enzyme was used for the estimation of enzyme activity and protein concentration. A specific activity of 33 U/mg with 33 fold purification was obtained by gel-filtration (Table 4.3.1).

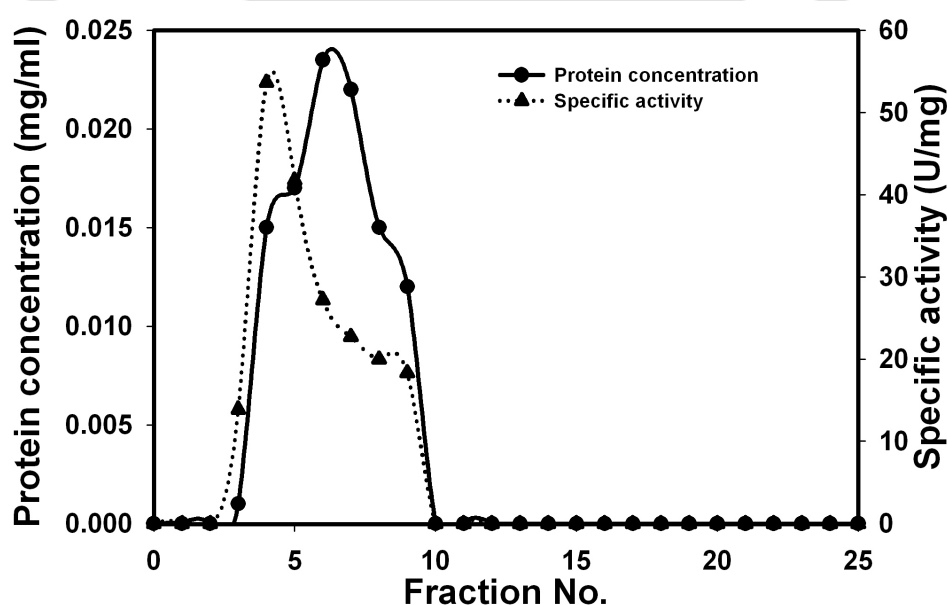


Fig. 4.3.3 Purification of dextransucrase by gel filtration. Elution pattern of dextransucrase on Sephacryl S-200 HR. Protein concentration (mg/ml) and specific activity (U/mg) are shown.

4.3.4 Non-denaturing SDS-PAGE analysis of purified dextransucrase by gel filtration

The dextransucrase samples obtained after purification by gel filtration was run on one SDS-PAGE gels in duplicate and run under non denaturing conditions (Fig. 4.3.4). The dextransucrase purified by gel filtration showed single band of approximately 180 kDa molecular size on the non-denaturing gel stained with silver staining (Fig. 4.3.4, lane 1). The other part of the gel was subjected to activity staining for *in-situ* activity detection. The presence of magenta color band (Fig. 4.3.4B, lane 2) after PAS staining using sucrose showed the active form of dextransucrase which corresponded well with 180 kDa size of dextransucrase that was obtained with silver staining method (Fig. 4.3.4A, lane 1).

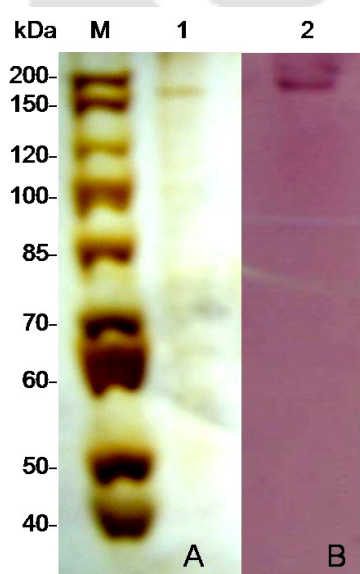


Fig. 4.3.4 Non denaturing SDS-PAGE analysis of dextransucrase by 7% acrylamide gel after gel filtration. (A) Silver staining of dextransucrase, Lanes: M- Protein molecular weight marker (10-200 kDa), 1- Sephacryl S-200 HR pooled fraction. (B) Identification of purified dextransucrase by staining of dextran formed using sucrose; Lane 2- Sephacryl S-200 HR pooled fraction.

4.3.5 Optimization of reaction conditions for maximum dextransucrase activity

4.3.5.1 Effect of temperature on dextransucrase activity

Temperatures ranging from 22°C to 45°C were studied for activity of dextransucrase (Fig. 4.3.5). The purified dextransucrase (specific activity 10.5 U/mg) was used for this study. The results showed that the temperature of 35°C was the optimum for dextransucrase activity (Fig. 4.3.5). The enzyme activity of dextransucrase from *Weissella confusa* Cab3 gradually decreased after 35°C. At 40°C and 45°C the dextransucrase activity decreased by 15% and 28%, respectively as compared to that observed at 35°C. Dextransucrase from *Leuconostoc mesenteroides* NRRL B-640 showed maximum activity in the temperature range of 30°C to 35°C (Purama and Goyal, 2009b).

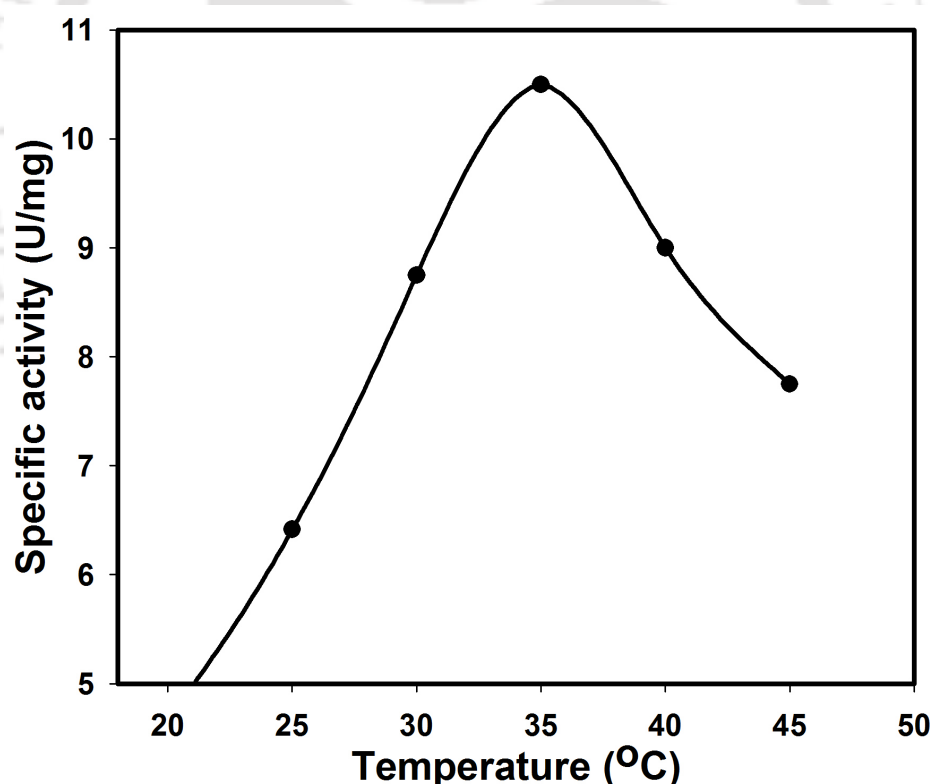


Fig. 4.3.5 Effect of temperature on dextransucrase activity from *Weissella confusa* Cab3. The enzyme activity was determined by carrying out the assay in 20 mM sodium acetate buffer (pH 5.4) containing 5%, w/v sucrose.

4.3.5.2 Effect of ionic strength of buffer on dextransucrase activity

The purified dextransucrase (specific activity 10.5 U/mg) was used to study the effect of different ionic strength of buffer on dextransucrase activity by varying the molar concentrations between 10-400 mM. The dextransucrase from *Weissella confusa* Cab3 showed maximum activity in the ionic strength range of 10-20 mM (Fig. 4.3.6). As the ionic strength of the buffer was increased to 50 mM, the enzyme lost its 41% activity. Beyond 50 mM the decrease in the enzyme activity was not significant with the increase of ionic strength.

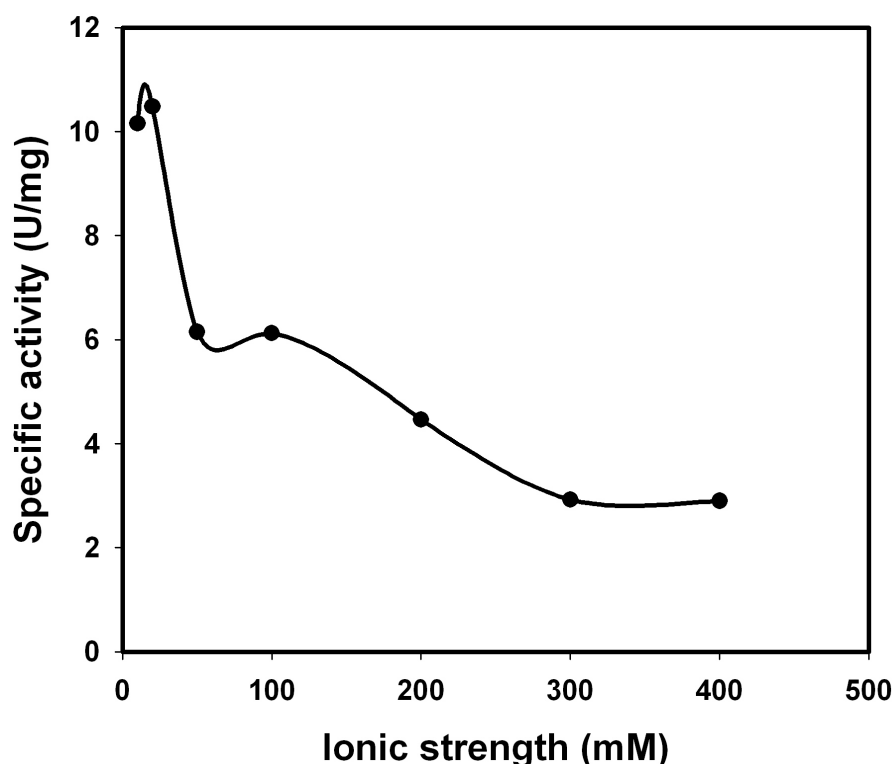


Fig. 4.3.6 Effect of ionic strength on dextransucrase activity from *Weissella confusa* Cab3. The enzyme activity was determined by carrying out the assay at 30°C, in sodium acetate buffer of different ionic strengths (pH 5.4) containing 5% w/v sucrose.

4.3.5.3 Effect of buffer pH on dextransucrase activity

The purified dextransucrase (specific activity 10.5 U/mg) was used for studying the effect of pH of buffer on dextransucrase activity by varying the pH between 4.0- 7.0. The maximum enzyme activity (10.5 U/mg) was observed at pH 5.4 (Fig. 4.3.7). At pH below and above than 5.4, a sharp decrease in enzyme activity was observed. At pH 5.6, 31% decrease in the enzyme activity was seen as compared to enzyme activity observed at pH 5.4. However, 38% loss in the enzyme activity was observed at pH 5.0. The optimum pH 5.2-5.4 of dextransucrase was also observed in the strains, *Leuconostoc mesenteroides* NRRL B-512F (Goyal *et al.*, 1995), NRRL B-640 (Purama and Goyal, 2009b), *Leuconostoc dextranicum* NRRL B-1146 (Majumder *et al.*, 2008) and *Pediococcus pentosaceus* (Patel *et al.*, 2011).

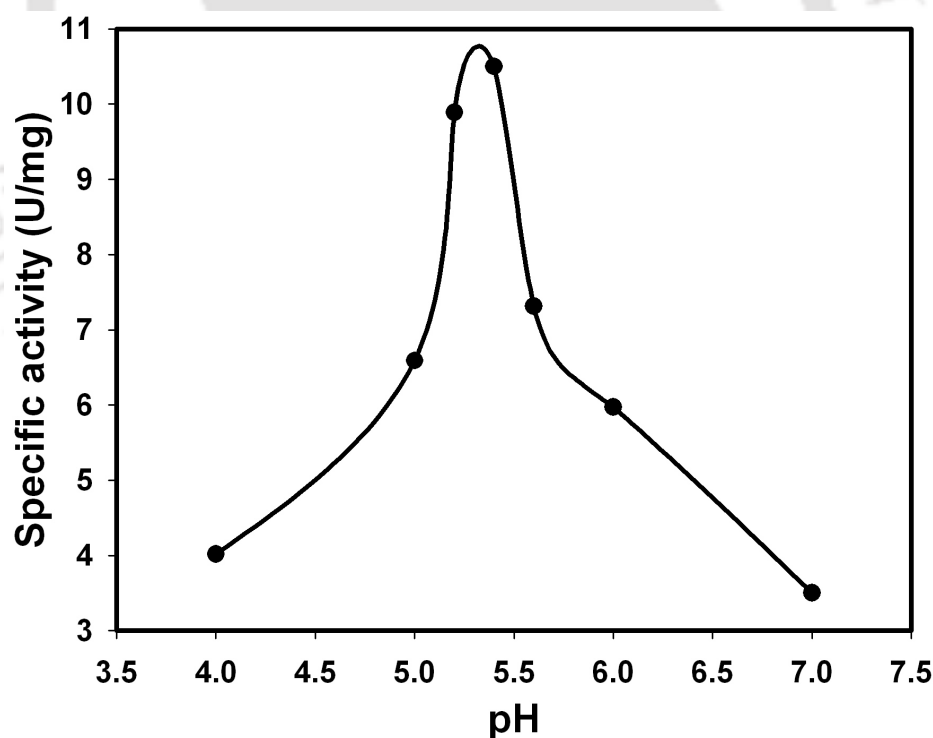


Fig. 4.3.7 Effect of pH on dextransucrase activity from *Weissella confusa* Cab3. The enzyme activity was determined by carrying out the assay at 30°C in 20 mM sodium acetate buffer containing 5% w/v sucrose.

4.3.5.4 Effect of sucrose concentration on dextransucrase activity

The purified dextransucrase (specific activity 10.5 U/mg) was used for studying the effect of sucrose concentration on dextransucrase activity by varying sucrose concentration between 0.1-10% final concentrations in the assay mixture. 5% (w/v) final sucrose concentration was optimum which gave maximum enzyme activity (10.5 U/mg). Beyond 5% (w/v) sucrose concentration saturation effect was observed in enzyme activity (Fig. 4.3.8).

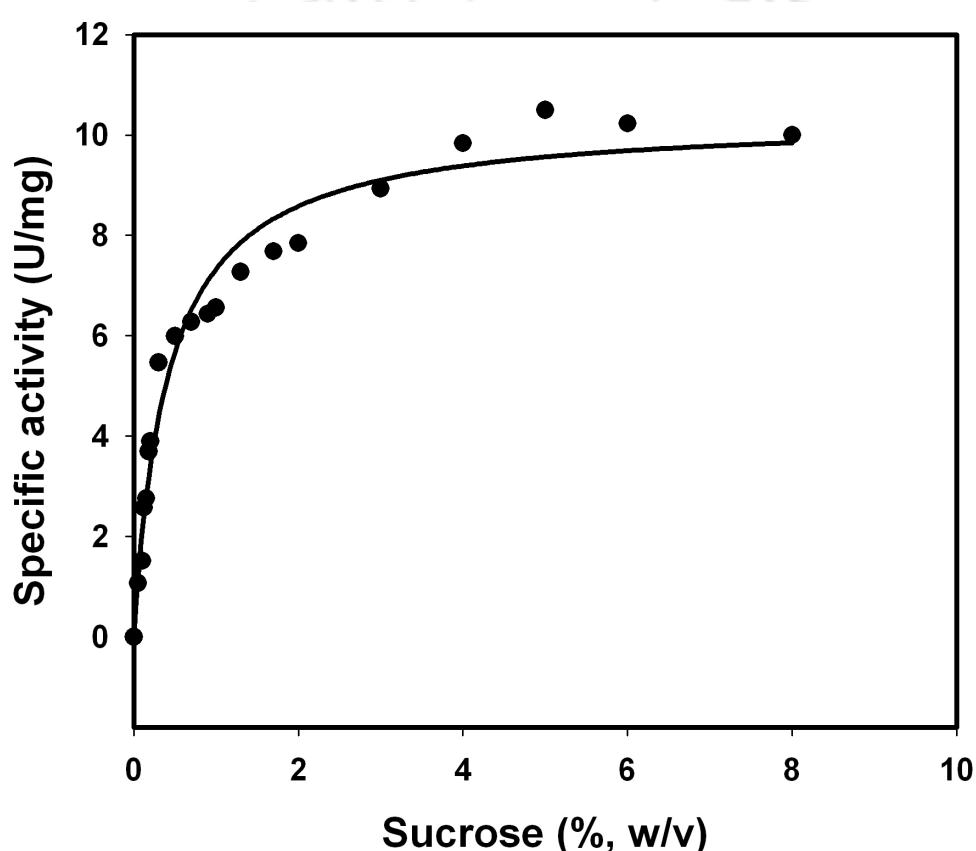


Fig. 4.3.8 Effect of sucrose concentration on dextransucrase activity from *Weissella confusa* Cab3 assayed at 35°C in 20 mM sodium acetate buffer, pH 5.4. The enzyme activity was determined by carrying out the assay at 30°C in 20 mM sodium acetate buffer (pH 5.4) containing varying concentrations of sucrose.

The reaction kinetics fitted a Michaelis Menten model, with a K_m of 13.2 mM, and V_m of 11.2 $\mu\text{mol/mg/min}$. The recombinant dextransucrase rDSRC39-2 from *W. confusa* C39-2 catalyzed dextran synthesis from sucrose with a K_m of 8.6 mM and a

V_m of 20 $\mu\text{mol}/\text{mg}/\text{min}$ (Amari *et al.*, 2012). This result was similar with the finding of Purama and Goyal, 2009b and Patel *et al.*, 2011 where 5% sucrose was optimum concentration for dextransucrase activity of *Leuconostoc mesenteroides* NRRL B-640 and *Pediococcus pentosaceus*, respectively. However, the optimum sucrose concentration for dextransucrases enzyme assays of *Leuconostoc mesenteroides* NRRL B-512F (Goyal *et al.*, 1995) and *Leuconostoc dextranicum* NRRL B-1146 (Majumder *et al.*, 2008) was 10% (w/v).

4.3.6 Effect of salts and EDTA on dextransucrase activity

Certain metal ions show positive effects on activity of enzymes. These solutes stabilize the catalytic activity of enzymes by stabilizing the three-dimensional protein structure. Salts are believed to affect the water structure of enzymes thus affecting the solubility and activity (Forman and Kennedy, 1977). The effects of certain metal ions were studied on dextransucrase activity from *Weissella confusa* Cab3. The enzyme activity increased by 8% at 2 mM MgCl_2 (Fig. 4.3.9A and Table 4.3.2). However, beyond 2 mM MgCl_2 , the enzyme activity remained unchanged (as compared to control dextransucrase activity) up to 5 mM.

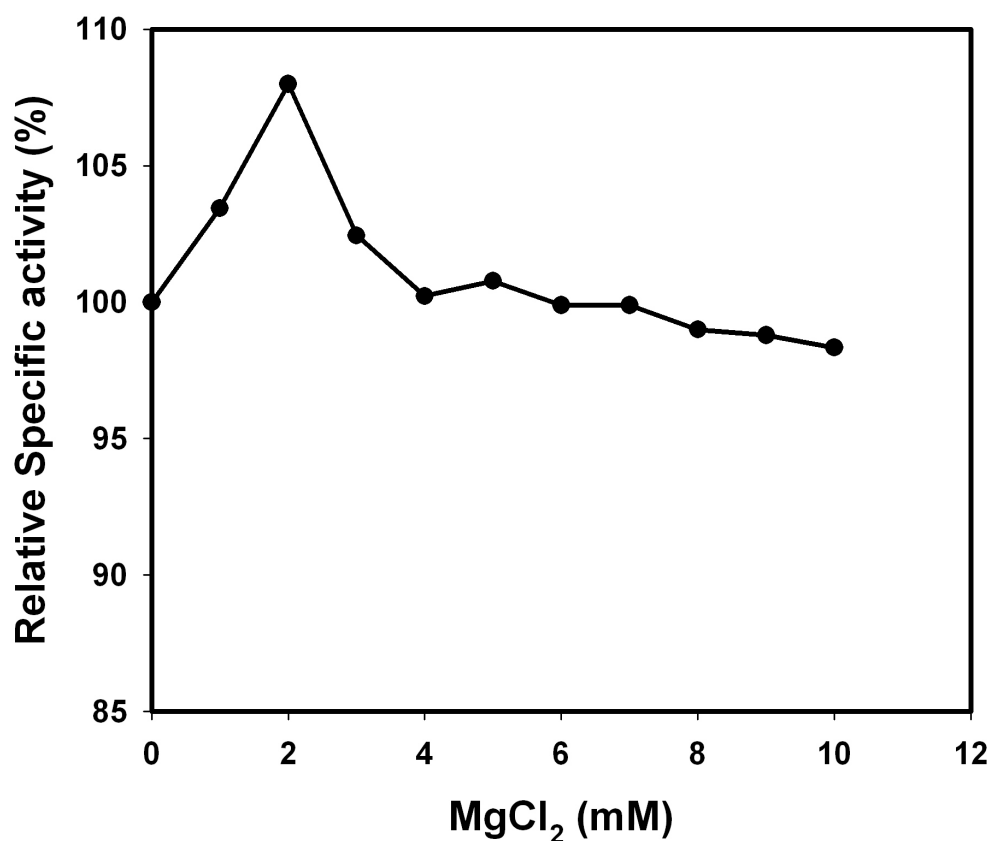


Fig. 4.3.9A Effect of MgCl_2 on the activity of purified dextransucrase of *Weissella confusa* Cab3.

Table 4.3.2. Effects of salts on purified dextransucrase from *Weissella confusa* Cab3.

Effect of metal ions	
Dextransucrase + Metal ions	Relative enzyme activity (%)
Control	100
CoCl_2 (1 mM)	121
CaCl_2 (1 mM)	120
ZnCl_2 (1 mM)	109
MgCl_2 (2 mM)	108
NiSO_4 (2 mM)	107
MnSO_4 (10 mM)	58
EDTA (5 mM)	02

The Co^{2+} and Ca^{2+} ions were the most effective for increasing the enzyme activity of dextransucrase. The enzyme activity increased by 21% and 20% at 1 mM CoCl_2 and 1 mM CaCl_2 , respectively (Fig. 4.3.9B, Fig. 4.3.9C and Table 4.3.2). The Ca^{2+} ion has been reported to be associated with the catalytic sites of dextransucrases

(Miller and Robyt, 1986). CaCl_2 (6 mM) increased the dextransucrase activity by 150% from *Pediococcus pentosaceus* (Patel *et al.*, 2011) and 4 mM CaCl_2 increased by 108% from *Leuconostoc mesenteroides* NRRL B-640 (Purama *et al.*, 2010).

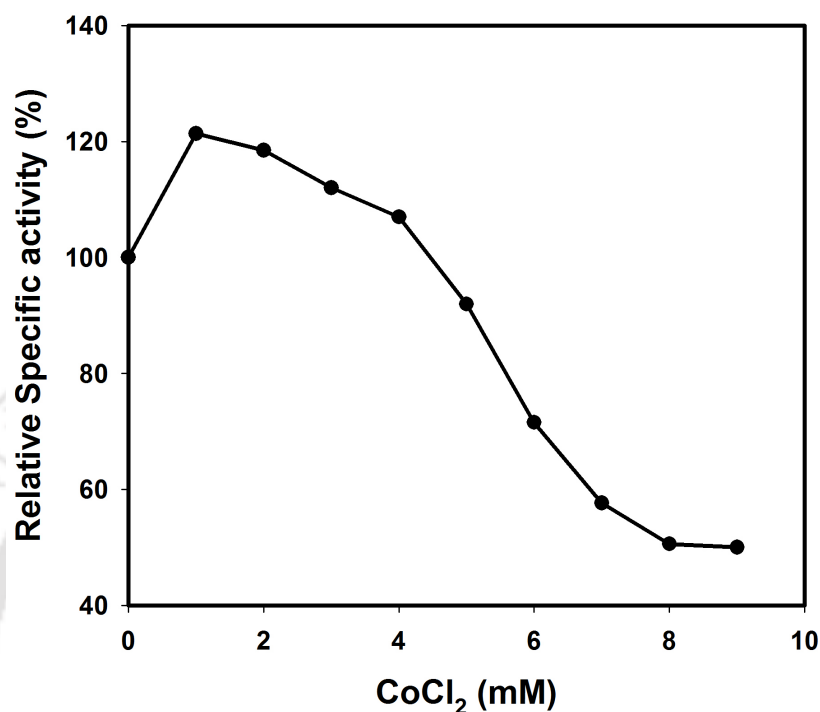


Fig. 4.3.9B Effect of CoCl_2 on the activity of purified dextransucrase of *Weissella confusa* Cab3.

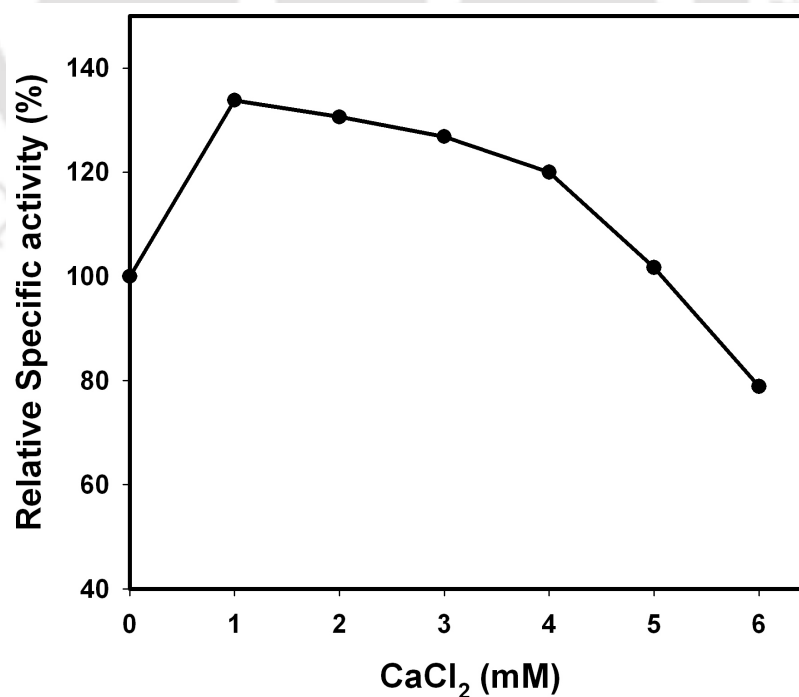


Fig. 4.3.9C Effect of CaCl_2 on the activity of purified dextransucrase of *Weissella confusa* Cab3.

The addition of ZnCl_2 (1 mM) and NiCl_2 (2 mM) to the enzyme reaction resulted in 9% and 7%, respectively increase in the enzyme activity (Fig. 4.3.9D, Fig. 4.3.9E and Table 4.3.2). Beyond 1 mM ZnCl_2 and 2 mM NiCl_2 concentration, these metal ions did not have any effect on enzyme activity. However, increasing the concentration of ZnCl_2 to 2 mM drastically reduced the enzyme activity to 77% residual activity. MnCl_2 negatively affected the enzyme activity. At lower concentration MnCl_2 (1 mM) decreased the enzyme activity by 4% (Fig. 4.3.9F and Table 4.3.2). At 10 mM MnCl_2 , the enzyme activity lost by 42% of its initial value. 5 mM EDTA displayed 2% residual activity of dextranucrase from *Weissella confusa* Cab3 (Fig. 4.3.9G and Table 4.3.2).

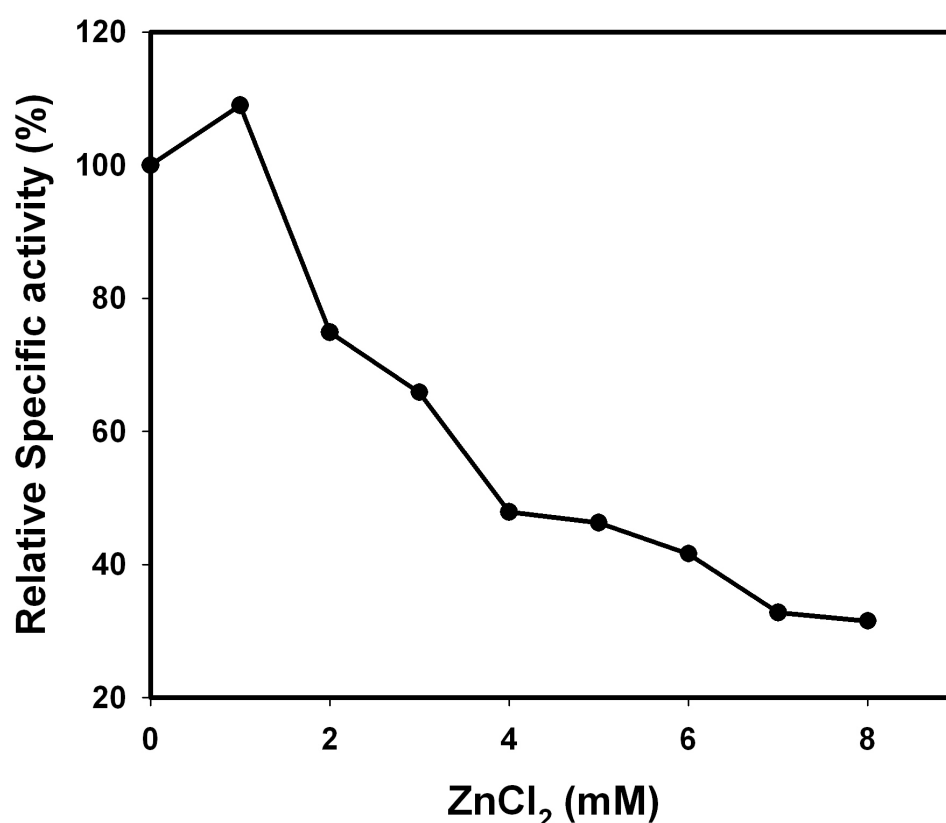


Fig. 4.3.9D Effect of ZnCl_2 on the activity of purified dextranucrase of *Weissella confusa* Cab3.

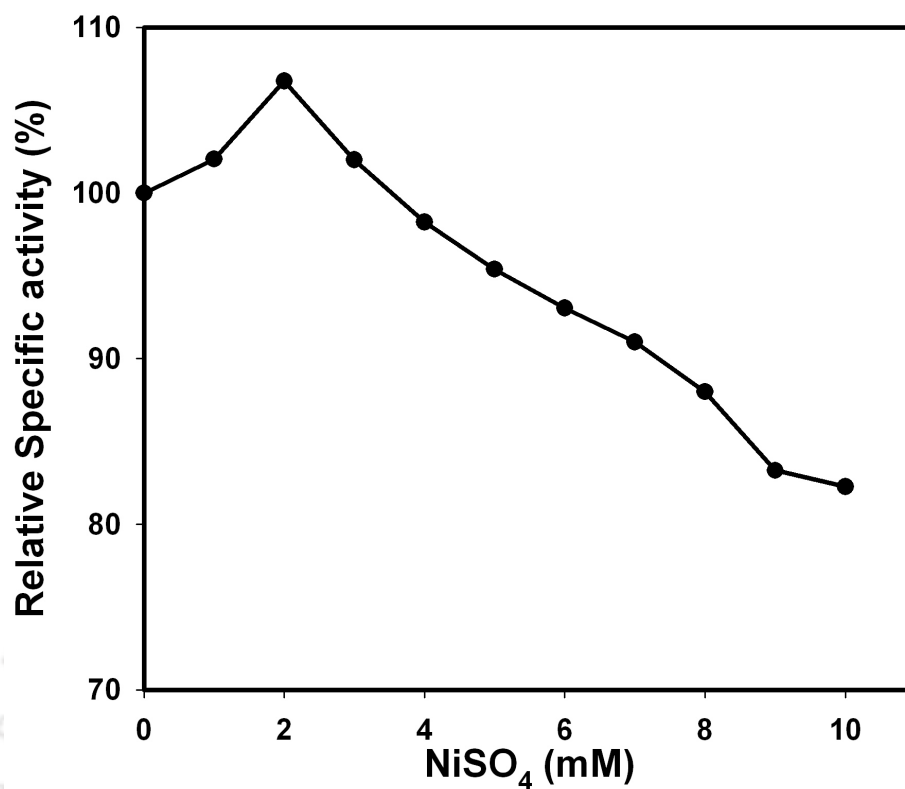


Fig. 4.3.9E Effect of NiSO_4 on the activity of purified dextranase of *Weissella confusa* Cab3.

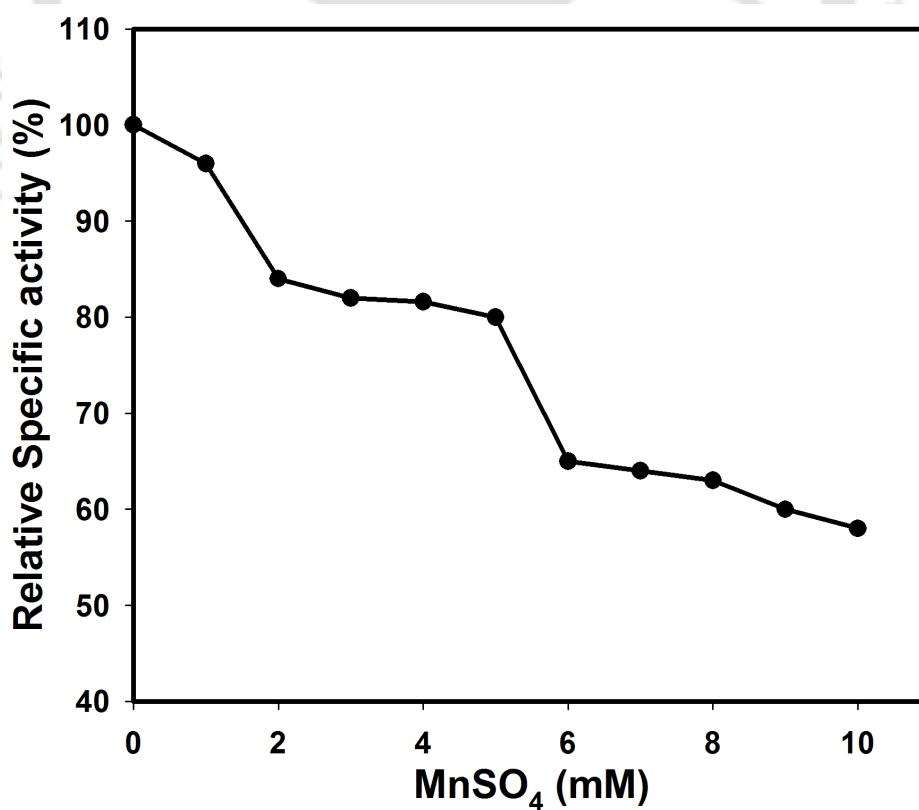


Fig. 4.3.9F Effect of MnSO_4 on the activity of purified dextranase of *Weissella confusa* Cab3.

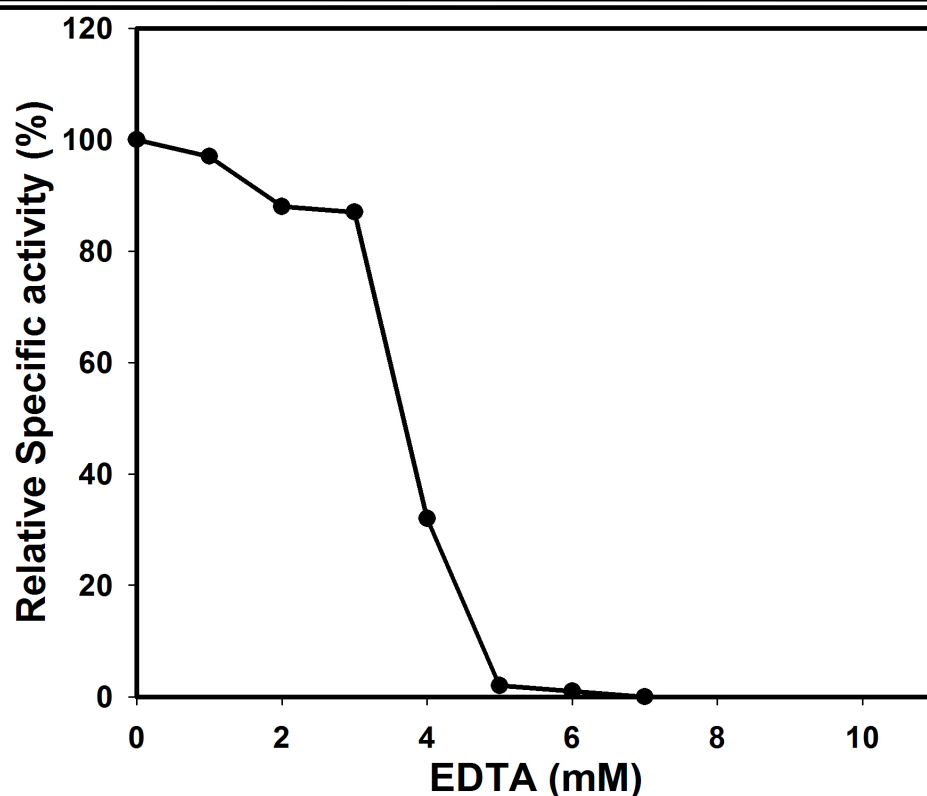


Fig. 4.3.9G Effect of EDTA on dextranase activity of *Weissella confusa* Cab3.

4.3.7 Thermostability study of dextranase *Weissella confusa* Cab3

Dextranase exhibited a mesophilic nature and showed stability up to 40°C, above which it rapidly lost the activity upon incubation for 1 h (Fig. 4.3.10). The optimum temperature range for the assay of dextranase from *Weissella confusa* Cab3 was also in the range of 30-35°C (Shukla and Goyal, 2011). The enzyme showed relatively thermostable nature as compared to other dextranase enzyme from *Leuconostoc mesenteroides* NRRL B-640 which rapidly lost its activity above 30°C after 10 min of incubation (Purama *et al.*, 2010).

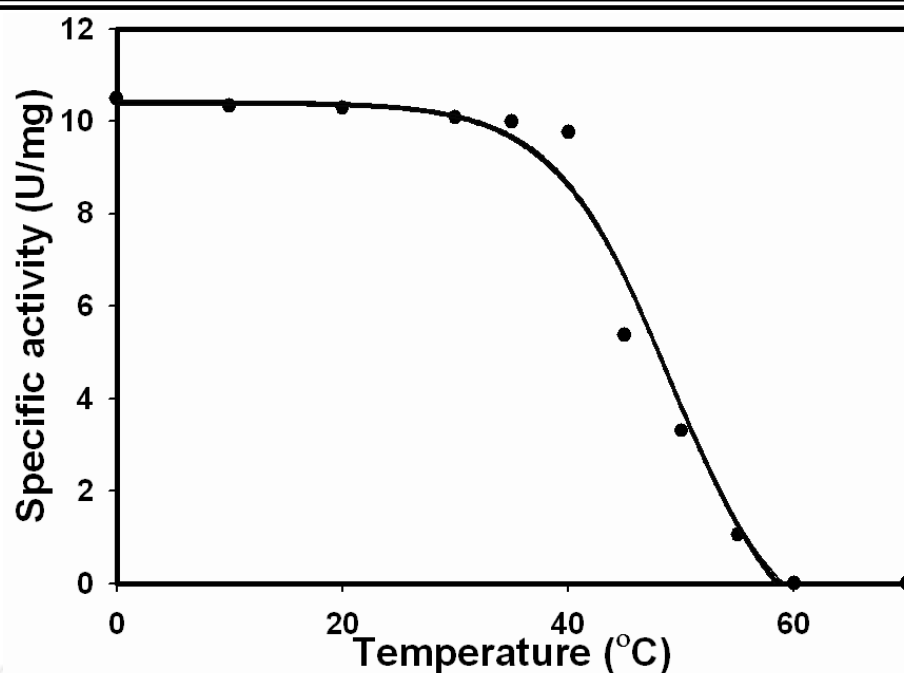
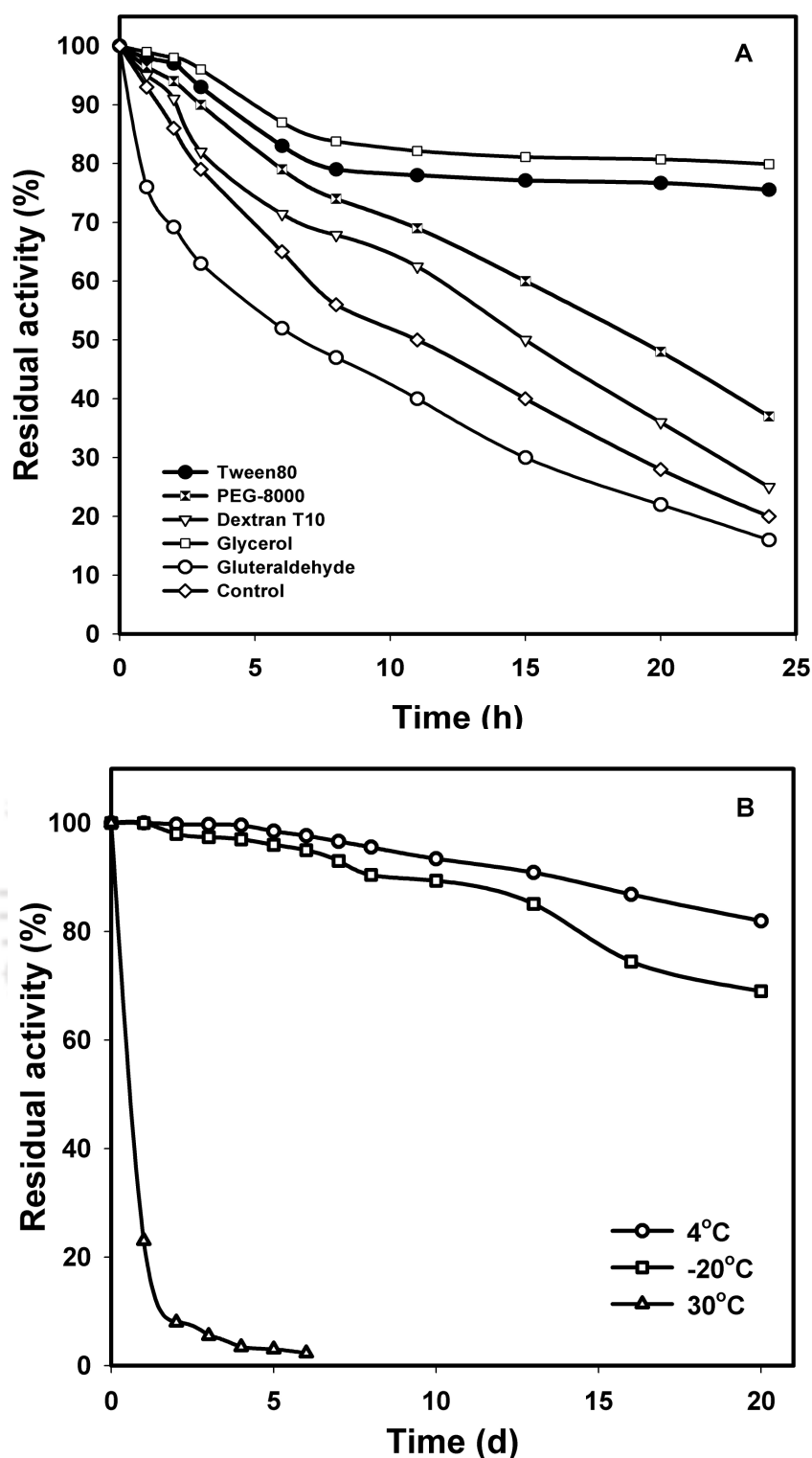


Fig. 4.3.10 Effect of temperature on stability of dextranase. Thermostability of dextranase was determined at different temperatures by pre-incubating for 1 h and assaying at 35°C in a 1 ml reaction mixture as described in methods.

4.3.8 Effect of storage temperature and additives on stability of dextranase

The effects of different additives on stability of dextranase at 30°C were studied. The residual activity of dextranase at 30°C after 24 h with glycerol, Tween 80 and PEG 8000 were 80%, 76% and 37.0%, respectively (Fig. 4.3.11A). The dextran (500 kDa) and glutaraldehyde did not stabilize the enzyme. The residual activity of dextranase with dextran (500 kDa) and glutaraldehyde at 30°C after 24 h was only 25% and 16%, respectively (Fig. 4.3.11A). The residual activity of dextranase with glutaraldehyde at 30°C after 24 h was lower than the residual activity of dextranase without any additive (20%). Thus glutaraldehyde negatively affected dextranase. The residual enzyme activity profile of storage stability at -20°C, 4°C and 30°C of purified enzyme was studied. Dextranase lost only 1.5% and 4% enzyme activity after 5 days of incubation at 4°C and -20°C temperature, respectively (Fig. 4.3.11B).



4.3.11 Effect of storage temperatures and various stabilizers on dextransucrase activity at 35°C. **(A)** Effects of various stabilizers on dextransucrase activity at 30°C. Dextransucrase (10.5 U/mg, 0.84 mg/ml) in 20 mM sodium acetate buffer (pH 5.4) was pre-incubated with additives at 30°C; **(B)** Effect of temperatures at 30°C, 4°C and -20°C on storage stability of dextransucrase. Dextransucrase (10.5 U/mg, 0.84 mg/ml) in 20 mM sodium acetate buffer (pH 5.4) was pre-incubated at 30°C, 4°C and -20°C and aliquots were assayed for dextransucrase activity at 35°C at 1 day intervals.

The half-life time ($t_{1/2}$) of dextransucrase and additives treated dextransucrase was calculated by assuming that the decay followed first order kinetics and were listed in Table 4.3.3.

Table 4.3.3 Effects of additives on purified dextransucrase from *Weissella confusa* Cab3.

Dextransucrase+ additive	30 °C	4 °C	-20 °C
Dextransucrase	10.33 h	69.4 d	37.9 d
Dextransucrase + Glycerol (0.5%)	74.12 h	nd	nd
Dextransucrase + Tween 80 (0.1%)	59.26 h	nd	nd
Dextransucrase + PEG-8000 (10 µg/ml)	16.72 h	nd	nd
Dextransucrase + Dextran T-500 (2 µg/ml)	12.0 h	nd	nd
Dextransucrase + Glutaraldehyde (0.1%)	9.07 h	nd	nd

nd: not determined

Amongst all the additives used glycerol and Tween 80 were the most efficient stabilizers giving stabilization of dextransucrase with $t_{1/2}$ of 74.12 h and 59.26 h at 30°C, respectively (Table 4.3.3). A small enhancement in the $t_{1/2}$ (16.72 h) was observed using PEG 8000 at 30°C as compared to control (10.33 h). Not much difference in the $t_{1/2}$ of dextransucrase was observed with Dextran-500 (12 h) at 30°C. The addition of glutaraldehyde negatively affected dextransucrase stability (9.07 h) when compared to dextransucrase with no additive ($t_{1/2}$ =10.33 d) at 30°C. The half life ($t_{1/2}$) of enzyme increased from 10.33 h to 69.4 d with decreasing the storage temperature from 30°C to 4°C. Thus the storage temperature 4°C for dextransucrase was selected.

4.4 Conclusions

Crude dextransucrase (6.1 U/mg and 6.0 mg/ml) from *Weissella confusa* Cab3 (GenBank Accession Number: JX649223) was purified by PEG fractionation. The concentration of 30% (v/v) PEG-400 gave the highest specific activity of 10.5 U/mg with 10 fold purification and 25% overall yield. The partially purified dextransucrase was subjected to gel filtration which resulted in the specific activity of 33 U/mg with 33 fold purification. The purified dextransucrase showed a single band of approximately 180 kDa molecular size on the non-denaturing gel with silver staining. The dextransucrase was identified by Zymogram analysis using activity staining protocol. The partially purified dextransucrase was used for further biochemical characterization. 35°C temperature, pH 5.4 and 20 mM ionic strength were optimum for the dextransucrase assay. The reaction kinetics of dextransucrase fitted a Michaelis Menten model, with a K_m of 13.2 mM and V_m of 11.2 $\mu\text{mol/mg/min}$. The dextransucrase activity was enhanced by Co^{2+} , Ca^{2+} , Zn^{2+} Mg^{2+} and Ni^{4+} ions by 21%, 20%, 9%, 8% and 7%, respectively. EDTA inhibited activity of dextransucrase which showed the requirement of metal ion for the catalytic efficiency of dextransucrase from *Weissella confusa* Cab3. The thermo-stability results showed that dextransucrase was stable at lower temperatures (10-40°C) and loses rapidly the enzyme activity at temperatures higher than 40°C. Glycerol and Tween 80 significantly enhanced the stability of dextransucrase at 30°C increasing half life ($t_{1/2}$) to 74.12 h and 59.26 h, respectively as compared with half life ($t_{1/2}$) of dextransucrase without any additive (10.33 h). The storage stability of purified enzyme at -20°C, 4°C and 30°C was studied. The half life ($t_{1/2}$) of enzyme increased from 10.33 h to 69.4 d with decreasing the storage temperature from 30°C to 4°C. Thus 4°C was selected for storage of dextransucrase.

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Chapter 5

Kinetics of dextran production and statistical optimization of medium for maximizing dextran production from *Weissella confusa* Cab3

5.1 Introduction

Dextran is composed of chains of α -D glucose units of varying length having linear α -(1 $\rightarrow$ 6) linked glycosidic linkages with varying percentage of α -(1 $\rightarrow$ 4), α -(1 $\rightarrow$ 3) and α -(1 $\rightarrow$ 2) branched linkages. The type of dextran production depends on the type of the bacterial strain (Kim *et al.*, 2003). Dextransucrase produced by lactic acid bacteria catalyses the formation of dextran when abundant sucrose is present in their surroundings. They hydrolyze the glycosidic bond of the sucrose moiety. The released glucose is used for the synthesis of polymer dextran and the fructose moiety is used for metabolic purpose by the microorganism (Lawford *et al.*, 1979). Dextrans have immense applications in various food, pharmaceutical and fine chemical industries (Janciauskaite *et al.*, 2008, Gloria Hernández *et al.*, 2011). It is used medicinally as an antithrombotic agent to reduce blood viscosity and as a blood volume expander (Purama and Goyal, 2005; Naessens *et al.*, 2005). Dextran based nanoparticles find application in specific drug targeting where dextran is used as polymer coating, which can be functionalized (McBain *et al.*, 2008). Biosensor analysis based on carboxymethylated dextran (CM-dextran) surfaces has provided reliable platform for the rapid determination of a wide range of compounds relevant to

food safety and quality (Situa *et al.*, 2008). Dextran is used as a stabilising coating for protecting metal nanoparticles against oxidation (Bautista *et al.*, 2005). Dextran coating on biomaterials are being done to prevent undesirable protein absorption to improve their biocompatibility (Sengupta *et al.*, 2006). Other than these applications several lactic acid bacteria belonging to the genera *Leuconostoc*, *Lactobacillus* and *Weissella* have been introduced to wheat sourdough baking by *in situ* production of exopolysaccharides (Katina *et al.*, 2009).

Dextran is produced by lactic acid bacteria such as *Leuconostoc mesenteroides* (Majumder and Goyal, 2008) *Pediococcus pentosaceus* (Smitinont *et al.* 1999), *Bacillus subtilis* (Sidebotham, 1973) and *Weissella confusa* (Maina *et al.*, 2008; Amari *et al.*, 2012). The dextran produced from *Leuconostoc mesenteroides* NRRL B-512F is used at industrial scale (Alsop, 1983). However, dextran produced by *Weissella confusa* has gained importance recently due to its linear structure (Maina *et al.*, 2008). A hyper dextran producer *Weissella confusa* Cab3 was isolated from fermented cabbage (Shukla and Goyal, 2011a). This bacterial strain produced 9.0 mg/ml dextran in the medium as described by Tsuchiya *et al.*, 1952 under optimized culture conditions. The production of dextran is influenced by medium components such as carbon sources, nitrogen sources and inorganic salts and buffering agents. Thus, the effects of various medium components were studied on dextran production by *Weissella confusa* Cab3 using one factor at a time approach (Shukla and Goyal, 2011b). However, one variable at a time approach does not depict the interaction among nutrients, making it necessary to fine tune the medium components using statistical approach for better and enhanced dextran production by *Weissella confusa* Cab3. Box Behnken method, central composite design (CCD), neural networking and Taguchi's orthogonal array based methodology are among most used statistical

methods for the optimization of medium components. Shukla and Goyal (2012) employed Taguchi's orthogonal array methodology for enhanced glucansucrase and glucan production. Patel *et al.*, 2011 employed response surface methodology for maximizing dextran production from *Pediococcus pentosaceus* SPAm in a bioreactor.

In the present study a sequential statistical approach was applied for fine tuning and optimization of the medium components. First Plackett–Burman design was used to find out the significant components addressing high impact on dextran production by *Weissella confusa* Cab3. In second stage a central composite design (CCD) was used to investigate optimal medium composition for maximizing dextran production.

5.2 Materials and Methods

5.2.1 Chemicals

Ingredients required for the maintenance and enzyme production media were from Hi-Media Pvt. Ltd., India. All the chemicals required for reducing sugar estimation and buffer preparation were of highest purity grade available commercially.

5.2.2 Microorganisms

Weissella confusa Cab3 (Genbank Accession Number JX649223) a potent dextran producer was isolated from fermented cabbage as described earlier in Chapter 2, Section 2.3.1 (Shukla and Goyal, 2011a). The cultures were maintained as stab in modified MRS agar (Goyal and Katiyar, 1996) as described in Chapter 2, Section 2.2.3 at 4°C and sub-cultured every 2 weeks. The strain *Leuconostoc mesenteriodes* NRRL B-512F was procured from Agricultural Research Service (ARS-Culture collection), USDA, Peoria, USA.

5.2.3 Cultivation and seed culture

From the culture maintained as MRS agar stab at 4°C, 1 loopful was inoculated in the 5 ml enzyme production medium as described by Tsuchiya *et al.* 1952 (Chapter 2, Section 2.2.4) and grown at 25°C at 180 rpm for 18-20 h. It was then inoculated either into a 250 ml flask containing 100 ml enzyme production medium (as described in Chapter 2, Section 2.2.4) or statistically designed medium. One percent inoculum was used and culture flask was incubated at 25°C and 180 rpm for 12 h, which are the optimum conditions for dextransucrase production from *Weissella confusa* Cab3 (Shukla and Goyal, 2011a). *Leuconostoc mesenteriodes* NRRL B-512F was grown at 23°C and 180 rpm for 18 h (Goyal *et al.*, 1995).

5.2.4 Dextransucrase activity assay

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

5.2.5 Analysis of dextran content of the *Weissella confusa* Cab3

5.2.5.1 By phenol sulphuric acid method

The dextran concentration of the *Weissella confusa* Cab3 was determined by phenol-sulphuric acid method (Dubois *et al.*, 1956) in a micro-titre plate (Fox and Robyt, 1991). *Weissella confusa* Cab3 was grown in 60 ml liquid medium as described by Tsuchiya *et al.*, 1952 (as described earlier in Chapter 2, Section 2.2.4) at 25°C and 180 rpm upto 32 h. The samples (1 ml) were withdrawn at regular intervals. To 200 µl of the culture supernatant three volume of the pre-chilled ethanol was added and centrifuged at 12,000g. The supernatant was discarded and the precipitate was resuspended in 200 µl distilled water. The process was repeated two more times. The final precipitate was air dried and dissolved in 200 µl distilled water. To 25 µl of sample containing dextran in a microtitre plate, 25 µl of 5% (v/v) phenol was added. The mixture was mixed by shaking the plate on a vortex mixer for 30s. Then the plate was placed on an ice bath and 125 µl of concentrated sulphuric acid was added to each well containing the mixture. The plate was again shaken for 30s to ensure proper

mixing of the contents of the wells. Then the plate was wrapped in cling film and incubated in water bath at 80°C for 30 min. After cooling to room temperature, the absorbance was determined at 490 nm on a multimode microplate reader (Tecan, model Infinite™ 200). A standard graph was plotted using dextran T40 (Sigma Aldrich, USA) in the concentration range 0.1-1.0 mg/ml.

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

Where,

$$A_{490} = \text{Optical Density (OD) change at 490 nm}$$

$$C = \text{1 OD equivalent concentration (mg/ml) from standard plot}$$

5.2.5.2 By estimating dry weight of dextran

The dextran production from *Weissella confusa* Cab3 was also analyzed in terms of dry weight of dextran and compared with *Leuconostoc mesenteroides* NRRL B-512F. For the production of dextran, the *Weissella confusa* Cab3 and *Leuconostoc mesenteroides* NRRL B-512F were grown in 100 ml of enzyme production medium (as described in Chapter 2, Section 2.2.4) and grown under their respective optimum culture conditions. The cells were separated by centrifugation at 8000g at 4°C for 10 min. To 30 ml of the cell free supernatant, three volume of the pre-chilled ethanol (90%) were added and centrifuged at 12,000g. The supernatant was discarded and the precipitate was resuspended in 30 ml distilled water. The process was repeated two more times. The precipitate was dissolved in 30 ml of distilled water with slight heating of tubes in hot water and dried by lyophilization to obtain dry powder of dextran. The lyophilized dextran samples were weighed to determine the amount of dextran produced from respective strains.

5.2.6 Estimation of sucrose

The sucrose concentration of the cell free supernatants was analyzed by the method given by Sumner and Sisler (1944). The details of protocol for the determination of the sucrose concentration have been as described earlier in Chapter 3, Section 3.2.3.

5.2.7 Cell growth and dry cell weight measurement

Cell growth of the broth was determined by measuring optical density at 600 nm with required dilutions against a blank of the initial sterile fermentation medium. The optical density was measured using UV-visible spectrophotometer (Varian, model Cary-100 Bio). The dry cell weight was determined in triplicate and the average value was taken. For the dry weight analysis, 1 ml of fermentation broth was withdrawn in pre-weighed eppendorf tubes and centrifuged at 8,000g at 4°C for 10 min. The cell free supernatants were discarded and the cell pellets were allowed to dry in an oven at 50°C. The difference in the weight of eppendorf tubes were considered as the dry cell weights of respective cultures (g/l).

5.2.8 Determination of kinetic parameters for dextran production from *Weissella confusa* Cab3

The stoichiometric parameters were studied to understand the kinetics of dextran production from *Weissella confusa* Cab3. The biomass yield $Y_{x/s}$ (gg⁻¹), Dextran yield $Y_{P/s}$ (gg⁻¹), specific dextran production q_P (gg⁻¹h⁻¹) and specific substrate uptake rate q_S (gg⁻¹h⁻¹) were calculated from the fermentation profiles of *Weissella confusa* Cab3 as a function of time. The stoichiometric parameters were calculated at different time intervals (t= 6, 9, 12, 15, 18 and 21 h) according to the following equations as reported earlier (Shuler and Kargi, 2002):

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

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

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

Where,

$Y_{X/S}$ = Biomass yield (gg^{-1})

ΔX = variation in the biomass ($X_t - X_0$)

ΔS = Sucrose consumption ($S_0 - S_t$)

$t = 0, 3, 6, 9, \dots, 21$ h.

5.2.8.2 Dextran yield ($Y_{Dex/Suc}$)

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

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad \dots (2)$$

Where,

$Y_{P/S}$ = Dextran yield (gg^{-1})

ΔP = variation in the dextran concentration ($P_t - P_0$) in time t ($t=t_i - t_0$)

ΔS = Sucrose consumption ($S_0 - S_t$) in time t ($t=t_i - t_0$)

$t = 0, 3, 6, 9, \dots, 21$ h.

5.2.8.3 Specific dextran production rate (q_{Dex})

Dextran production occurred simultaneously with the growth of *Weissella confusa* Cab3. The specific dextran production rate is proportional to the growth rate of *Weissella confusa* Cab3. The dextran production rate was calculated according to the equation (3):

$$q_{Dex} = \frac{\Delta P}{\Delta t} \frac{1}{\Delta X} \quad \dots (3)$$

Where,

q_{Dex} = Specific dextran production rate ($\text{gg}^{-1}\text{h}^{-1}$)

ΔP = Change in dextran concentration ($P_t - P_0$)

ΔX = Change in biomass ($X_t - X_0$)

dt = change in time t ($t=t_i - t_0$)

$t = 0, 3, 6, 9, \dots, 21$ h.

5.2.8.4 Specific sucrose uptake rate (q_{suc})

Sucrose consumption led to the cell growth and dextran production from *Weissella confusa* Cab3. The sucrose consumption rate was determined with respect

to the change in the biomass. The specific sucrose consumption rate was calculated according to the equation (4):

$$q_{Suc} = \frac{\Delta S}{\Delta t} \frac{1}{\Delta X} \quad \dots(4)$$

q_{Suc} = Specific sucrose uptake rate ($g g^{-1} h^{-1}$)

ΔS = Sucrose consumption ($S_0 - S_t$) in time ($t=t_i - t_0$)

ΔX = Change in biomass ($X_t - X_0$)

dt = Change in time ($t=t_i - t_0$)

$t = 0, 3, 6, 9, \dots, 21$ h.

5.2.9 Screening of nutrients on dextran production from *Weissella confusa* Cab3 by One Variable at a Time Approach (OVAT)

5.2.9.1 Effect of sucrose on dextran production

The effect of sucrose concentration on dextran production from *Weissella confusa* Cab3 was studied by varying its concentration from 1 to 6% (w/v) in the enzyme production medium by keeping the concentration of other components constant. *Weissella confusa* Cab3 was grown in 100 ml medium (as described in Chapter 2, Section 2.2.4) containing different sucrose concentration at 25°C and 180 rpm. The enzyme production medium (as described in Chapter 2, Section 2.2.4) containing the 2% (w/v) sucrose was considered as control.

5.2.9.2 Effect of nitrogen sources on dextran production

The effects of three different nitrogen sources viz. yeast extract, beef extract and peptone on dextran production were investigated. For this yeast extract, peptone and beef extract were used as sole nitrogen sources in the medium as described by

Tsuchiya *et al.*, 1952 (as described in Chapter 2, Section 2.2.4) by replacing 2%, w/v yeast extract with the different concentrations of different nitrogen sources. The concentrations of nitrogen sources were varied from 1% to 4% (w/v). The concentrations of other components were kept constant. Enzyme production medium as described by Tsuchiya *et al.*, 1952 (as described in Chapter 2, Section 2.2.4) was considered as control.

5.2.9.3 Effect of sodium acetate, Tween 80 and K_2HPO_4 on dextran production

Sodium acetate concentration was varied from 6.0 mM to 36 mM to observe its effect on dextran production. The effect of Tween 80 on dextran production was studied by varying its concentration from 0.02 to 0.3% (v/v) in the medium as described by Tsuchiya *et al.*, 1952 (as described in Chapter 2, Section 2.2.3). The medium without sodium acetate and Tween 80 served as a control for studying the effect of sodium acetate and Tween 80. 1 to 3% (w/v) K_2HPO_4 was used to study its effect on dextran production. The enzyme production medium (Tsuchiya *et al.*, 1952) containing 2%, w/v K_2HPO_4 was considered as control as described in Chapter 2, Section 2.2.4.

5.2.10 Experimental design for statistical optimization procedure and data analysis

After screening the medium nutrients by one variable at a time approach (OVAT) the medium was optimized for enhanced dextran production from *Weissella confusa* Cab3. The statistical design was applied in two steps. The first step was applied to identify the nutrients having significant effect on dextran production using Plackett-Burman Design (Plackett and Burman, 1946) and the second step was applied for the fine tuning of nutrient's concentration using Central Composite Design

(CCD). The experimental design and statistical analysis of the data were done by Minitab statistical software (Version 15).

5.2.10.1 Plackett-Burman Design

Plackett-Burman (PB) design a very useful tool, was used to screen 'n' variables in just 'n+1' number of experiments (Plackett and Burman, 1946). In this part, the PB design was used to evaluate the relative significance of six nutrients *i.e.* sucrose, yeast extract, K_2HPO_4 , Tween 80, $CaCl_2$ and initial pH of medium for dextran production in batch fermentation. Among the nutrients, sucrose was selected as the carbon source as it is the inducer and substrate of dextransucrase which is responsible for dextran production (Tsuchiya *et al.*, 1952). Yeast extract was chosen as the nitrogen source because it displayed significant effect on dextran production (Shukla and Goyal, 2011b). K_2HPO_4 was chosen as it acts as a buffering agent in the fermentation medium and maintain its pH for a longer duration as reported earlier (Goyal and Katiyar, 1997). It has been reported that increasing the K_2HPO_4 concentration in the medium enhances the dextransucrase production which in turn increases dextran production (Tsuchiya *et al.*, 1952). The surfactant Tween 80 was selected as it changes the membrane permeability and enhances the release of the extracellular dextransucrase, which in turn increases dextran biosynthesis (Yamashita and Takehara, 1989; Goyal and Katiyar, 1997). Tween 80 is also a known stabilizer of dextransucrase (Purama *et al.*, 2010). Therefore, Tween 80 stimulates dextran production by stimulating and stabilizing dextransucrase. $CaCl_2$ was considered because of its stimulatory effect on dextransucrase production and dextran polymerization (Qader *et al.*, 2008). Trace elements such as $MgSO_4 \cdot 7H_2O$, $MnSO_4 \cdot 4H_2O$, $FeSO_4 \cdot 7H_2O$, $CaCl_2$, and NaCl were added in each medium according

to their level in the medium as described by Tsuchiya *et al.*, 1952 (as also described in Chapter 2, Section 2.2.4). This design does not consider the interaction effects among the variables and is used to screen the important variables affecting the dextran production. The experiments were carried out in triplicate and the averages of the dextran concentrations were taken as response. The experimental design for screening of medium components is shown in 5.3.4 and 5.3.5. Each variable was set at two levels, that is, high level (+1) and low level (-1) (Plackett and Burman, 1946). The high level of each variable was set far enough from the low level to identify which ingredients of the medium have significant influence on the dextran production. Plackett-Burman experimental design is based on the first order polynomial model:

$$Y = \beta_o + \sum \beta_i x_i \quad \dots (5)$$

Where, Y is the response (dextran concentration), β_o is the model intercept and β_i is the linear coefficient, and x_i is the level of the independent variable. This model is useful for screening and evaluation of the significant factors that influence the response. From the regression analysis the variables, which were significant at 90% level ($P < 0.1$) were considered to have greater impact on dextran production and were further optimized by a central composite design. The experimental design and statistical analysis of the data were done by Minitab statistical software package (Version15).

5.2.10.2 Central Composite Design

The significant components affecting the dextran yield were identified by Plackett-Burman design. A 2^3 full-factorial central composite design (CCD) with three identified significant medium constituents, *i.e.* sucrose, K_2HPO_4 and Tween 80 was generated by Minitab statistical software. The factors and their coded values are

listed in Table 5.3.7. In this study, the experimental plan consisted of 20 run orders (Table 5.3.8). The experiments were carried out in triplicates and the dextran production (Y , mg/ml) was used as the output variable. For statistical calculations the variables X_i were coded as x_i according to Eq. (6)

$$x_i = \frac{(X_i - X_0)}{\Delta X_i} \quad \dots (6)$$

where, x_i is the dimensionless coded value of the variable X_i , X_0 is the value of X_i at the center point, and ΔX_i is the step change. The relationships among the variables were determined by fitting the following second-order polynomial equation to the data obtained from 20 experiments

$$Y = \beta_o + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_i \sum_j \beta_{ij} X_i X_j \quad \dots (7)$$

Where, Y is the predicted response, k is the number of factor variables, β_o is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, β_{ij} is the interaction coefficient. X_i and X_j denote the factor variable in its coded form. F-test was used to evaluate the statistical significance of the quadratic polynomial. The multiple coefficients of correlation R and the determination coefficient of correlation R^2 were calculated to evaluate the performance of the regression equation. P-values below 0.1 were regarded as statistically significant. The optimum levels of the selected variables were obtained by solving the regression equation using a multi-stage Monte-Carlo optimization (Conley, 1984) program and also by analyzing the response-surface plots (Khuri and Cornell, 1987).

5.2.10.3 Validation of model at shake flask and scale up to bioreactor level

The confirmation and validation of the medium composition optimized using a central composite design was done at shake flask and bioreactor level. The experiment at shake flask level was performed in 250 ml flask containing 100 ml optimized culture medium and grown at 25°C at 180 rpm. The parameters like cell optical density, pH, enzyme activity (as described in Chapter 2, Section 2.2.10.2), dextran concentration (as described in Section 5.2.5.1) and sucrose concentration (as described in Chapter 3, Section 3.2.3) were analyzed at regular interval. The dextran production using RSM optimized medium was scaled up in 1 l volume of culture medium contained in a 3l bioreactor (Applikon, model Bio Console ADI 1025). For controlled pH cultivation, the pH was maintained at 7.0 by addition of 2M NaOH and 2M HCl solution. During the experiment, temperature and aeration rate were controlled at 25°C and 2 vvm, respectively. The dissolved oxygen (DO) was adjusted to 100% before inoculation. The agitation was set at 200 rpm at the beginning of the run but changed accordingly to keep the DO above 30%. The formation of foam was prevented by adding sterile soyabean oil. The parameters like dextran concentration, enzyme activity, sucrose concentration, cell optical density and dry cell weight were analyzed.

5.3 Results and Discussion

5.3.1 Determination of stoichiometric parameters for kinetics of dextran production of *Weissella confusa* Cab3

Weissella confusa Cab3 was grown at 25°C and 180 rpm in a 250 ml Erlenmeyer flask containing 100 ml medium (pH 7) as described by Tsuchiya *et al.*, 1952 (Chapter 2, Section 2.2.4). An initial sucrose concentration of 20 g/l was used to understand the kinetics of dextran production by *Weissella confusa* Cab3. The changes in the cell optical density, cell dry weight, sucrose consumption and dextran production profiles of *Weissella confusa* Cab3 are shown in Fig. 5.3.1A. Sucrose is essentially required for the induction and production of dextransucrase enzyme which in turn produces dextran. Sucrose metabolism by *Weissella confusa* Cab3 led to its multiplication and a maximum cell OD₆₀₀ of 6.9 at 15 h and dry cell weight of 5.0 g/l) was obtained (Fig. 5.3.1A). The maximum dextran production of 9.0 mg/ml at 12 h of fermentation was observed (Fig. 5.3.1A).

The production of dextran induced the visible change in the texture of the medium. The medium became viscous due to the accumulation of the dextran. As shown in Fig. 5.3.1A the dextran production occurred during growth phase and sucrose consumption phase and ceased when almost all sucrose got consumed by the bacterium. At the end of the fermentation whole available sucrose (20 g/l) was consumed by *Weissella confusa* Cab3 (Fig. 5.3.1A). The variation in biomass yield ($Y_{X/Suc}$) and dextran yield ($Y_{Dex/Suc}$) are illustrated in Fig. 5.3.1B. The stoichiometric parameters of production kinetic of dextran are shown in Table 5.3.2.

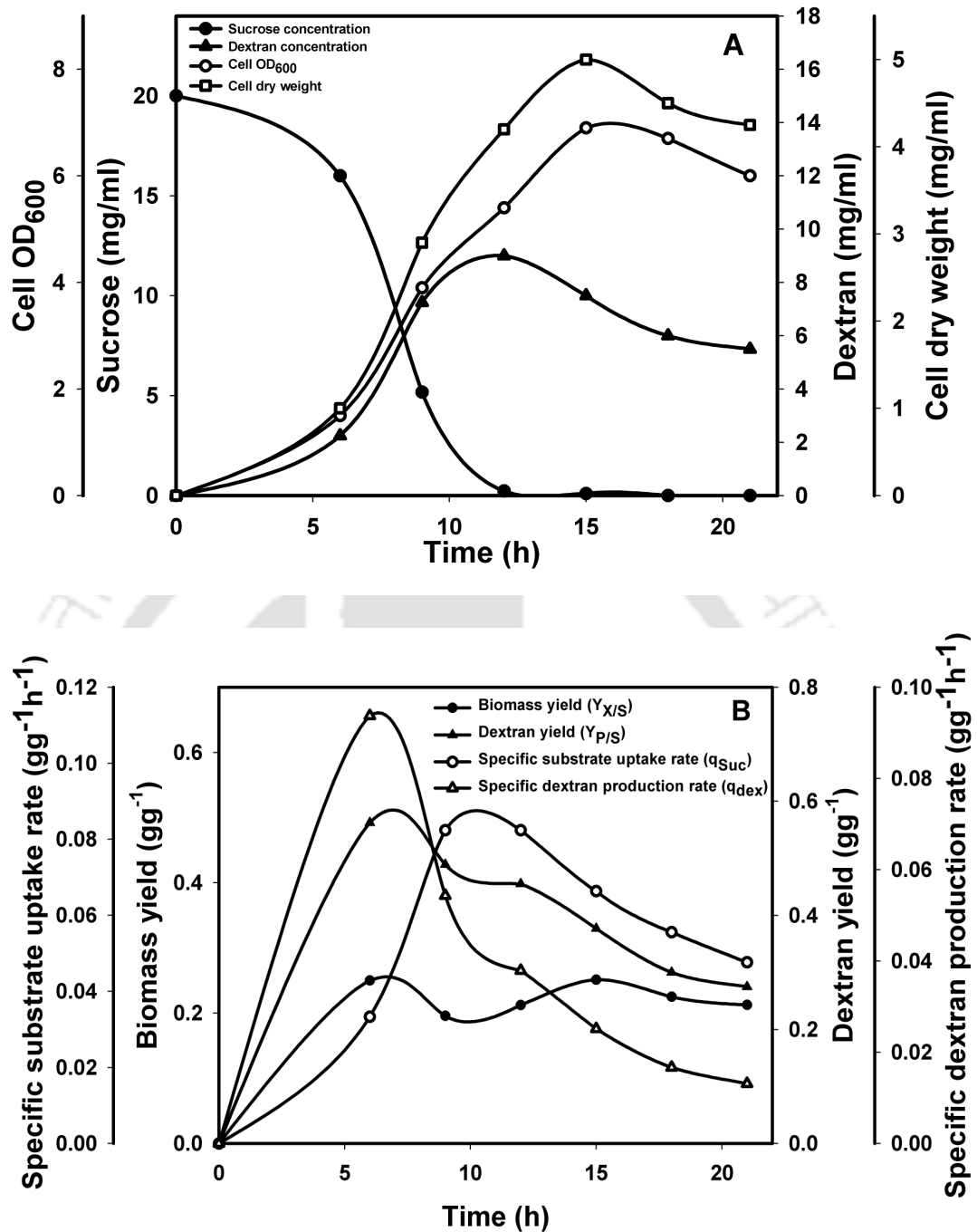


Fig. 5.3.1 Variation in (A) Cell OD₆₀₀, cell dry weight, sucrose concentration and dextran production and (B) Specific substrate uptake rate, specific dextran production rate, biomass yield and dextran yield in *Weissella confusa* Cab3. *Weissella confusa* Cab3 was grown at 25°C and 180 rpm in the 100 ml medium as described by Tsuchiya *et al.*, 1952.

The maximum biomass yield ($Y_{X/Suc}$) obtained from *Weissella confusa* Cab3 was very low *i.e.* 0.27 g g^{-1} (Fig. 5.3.1B and Table 5.3.1). The low biomass yield of *Weissella confusa* Cab3 obtained could be due to efficient utilization of sucrose by the dextransucrase enzyme resulting in substantial high dextran yield ($Y_{Dex/Suc}$) of 0.49 g g^{-1} (Fig. 5.3.1B and Table 5.3.1), at early stage of fermentation (9^{th} h). From the Fig. 5.3.1B, it was clear that the specific substrate (sucrose) uptake rate curve and specific dextran production rate profile were similar. By the time the sucrose uptake rate (q_{suc}) started decreasing, the dextran production rate (q_{Dex}) also ceased.

Table 5.3.1 Kinetic parameters for dextran production from *Weissella confusa* Cab3.

Strain	$Y_{X/suc}$ (g g^{-1})	$Y_{Dex/suc}$ (g g^{-1})	P (g l^{-1})	q_{suc} ($\text{g g}^{-1} \text{ h}^{-1}$)	q_{dex} ($\text{g g}^{-1} \text{ h}^{-1}$)
<i>Weissella confusa</i> Cab3	0.24	0.49	9.0	0.08	0.09

The dextran production from *Weissella confusa* Cab3 was also analyzed on the basis of dry weight of dextran and compared with *Leuconostoc mesenteroides* NRRL B-512F which is being used at industrial scale for dextran production. The soluble dextran was extracted from 30 ml of bacterial culture as described earlier in Section 5.2.5.2. As the three volume of ethanol (90%, v/v) was added to the cell free supernatant, white color precipitate was observed as shown in Fig. 5.3.2 which was then lyophilized. The dried lyophilized dextran was weighed to determine the dextran production by the respective bacterial strains (Table 5.3.2). *Weissella confusa* Cab3 and *Leuconostoc mesenteroides* NRRL B-512F produced 255 mg and 120 mg, respectively dextran from 30 ml of crude cell free supernatants. To determine the dextran concentration from respective bacterial strains, the dextrans (mg) obtained from 30 ml cell free supernatant (mg) as shown in Table 5.3.2 were divided by 30 ml. Table 5.3.3 shows the comparison of dextran production from *Weissella confusa* Cab3

and *Leuconostoc mesenteroides* NRRL B-512F. *Weissella confusa* Cab3 produced significantly higher dextran (8.5 mg/ml) as compared to *Leuconostoc mesenteroides* NRRL B-512F which produced 4.0 mg/ml dextran using same medium as described by Tsuchiya *et al.*, 1952 (as described in Chapter 2, Section 2.2.4) under their optimized culture conditions. The dextran concentration determined by phenol sulphuric acid method (as described in Section 5.2.5.1) of *Weissella confusa* Cab3 and *Leuconostoc mesenteroides* NRRL B-512F was 9.0 and 4.5 mg/ml, respectively which were in good agreement with the dextran concentration determined by dry weight method (as described in Section 5.2.5.2). The dextran concentration of *Weissella confusa* Cab3 was 2.1 fold higher than that of *Leuconostoc mesenteroides* NRRL B-512F. Therefore, *Weissella confusa* Cab3 can be used as a potent alternative of *Leuconostoc mesenteroides* NRRL B-512F for dextran production at industrial scale. However, Santos *et al.*, 2000 reported 6.9 mg/ml dextran concentration from *Leuconostoc mesenteroides* NRRL B-512F under controlled aeration condition in a 2 l bioreactor scale.

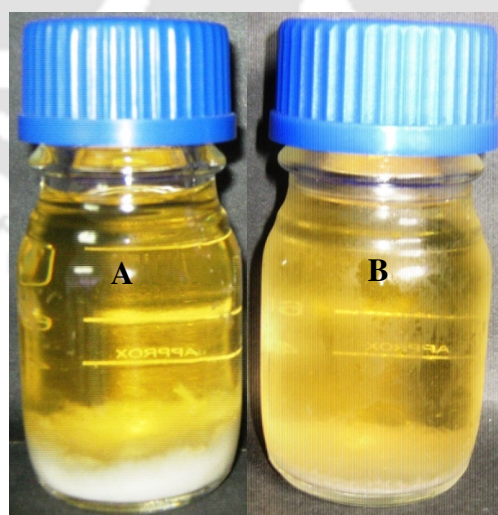


Fig. 5.3.2 Crude dextran (white precipitate) at the bottom of each bottle after 1st extraction from (A) *Weissella confusa* Cab3 (B) *Leuconostoc mesenteroides* NRRL B-512F.

Table 5.3.2 Dextran production by lactic acid bacteria on the basis of dry weight analysis and phenol sulphuric acid method from 30 ml cell free supernatant.

Lactic acid bacterial strain	Dextran (mg/ml)	
	Dry weight method ^a	Phenol sulphuric acid method ^b
<i>Weissella confusa</i> Cab3	8.5	9.0
<i>Leuconostoc mesenteroides</i> NRRL B512-F	4.0	4.5

^a as described in Section 5.2.5.2. Dextran (mg) obtained after lyophilisation was divided by 30 ml to determine concentration.

^b as described in Section 5.2.5.1.

5.3.2 Screening of nutrients on dextran production from *Weissella confusa* Cab3 by One Variable at a Time Approach (OVAT)

5.3.2.1 Effect of sucrose on dextran production

Among the nutrients, carbon source sucrose was chosen as it induces the dextransucrase production and is also, a substrate for dextran production from *Weissella confusa* Cab3. Increased sucrose concentration (upto 6%, w/v) was seen to have positive effect on dextran production by *Weissella confusa* Cab3. The increase in dextran was 5 fold, from 4.5 mg/ml to 22.4 mg/ml with an increase in sucrose concentration from 1% to 5%, in the medium (Fig. 5.3.3 and Table 5.3.3). Due to the high dextran production, the texture of the culture medium became thicker and viscous. Above 6% sucrose concentration the dextran production started decreasing which could be due to the inhibitory effect of higher sucrose concentration on the dextransucrase.

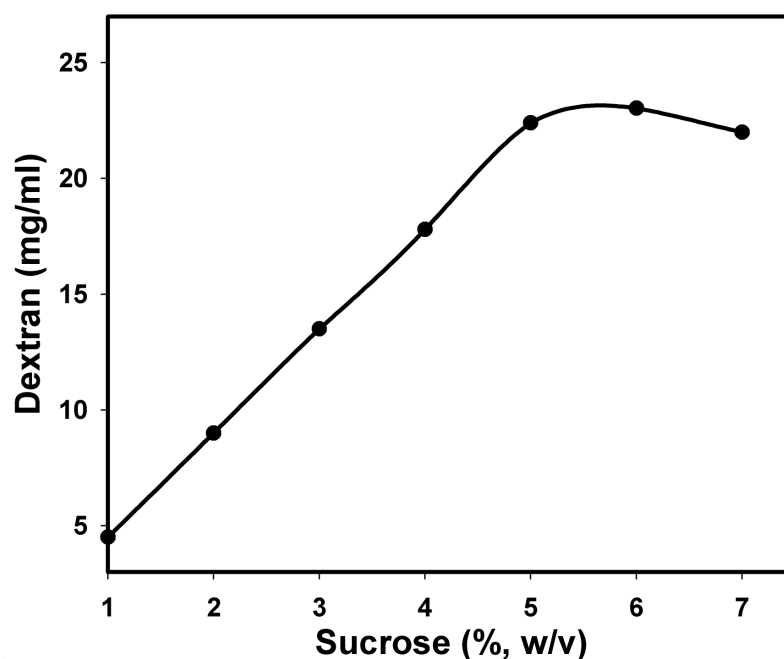


Fig. 5.3.3 Effect of sucrose on dextran production from *Weissella confusa* Cab3.

Table 5.3.3 Effect of nutrients on dextran production from *Weissella confusa* Cab3.

Medium component	Concentration (% w/v)	Dextran (mg/ml)	Relative dextran production (%)
Control medium ^a	-	9.00	100
Sucrose	5.0	22.4	249
Yeast extract	1.5	10.0	111
Peptone ^b	2.0	9.9	110
Beef extract ^b	3.0	7.4	82
Sodium acetate (mM)	6.0	9.0	100
Tween 80 (% v/v)	0.1	10.0	111
K ₂ HPO ₄	1.5	9.8	109

^a Tsuchiya *et al.*, 1952 as described in Chapter 2, Section 2.2.4.

^b Medium did not contain yeast extract, whereas control medium contained 2% (w/v) yeast extract.

5.3.2.2 Effect of nitrogen sources on dextran production

Effect of various nitrogen sources like yeast extract, peptone and beef extract on dextran production by *Weissella confusa* Cab3 were studied. Yeast extract was most effective nitrogen source for dextran production. At 1.5% (w/v) concentration, 10.0 mg/ml dextran was obtained (Fig. 5.3.4A and Table 5.3.3), which was 11% more than that observed in control medium containing 2% yeast extract (Table 5.3.3). Higher concentration of yeast extract beyond 1.5%, w/v started inhibiting dextran production (Fig. 5.3.4A). To study the effect of the peptone and beef extract as a sole nitrogen source the yeast extract was replaced from the medium. The medium used was as described by Tsuchiya *et al.*, 1952 (Chapter 2, Section 2.2.4) with different concentrations of peptone and beef extract ranging from 1-4 (% w/v).

Peptone significantly increased dextran production from 3.4 mg/ml to 9.9 mg/ml, as the concentration of peptone was increased from 1 to 2%, w/v. Beyond 2% peptone, the dextran production decreased (Fig. 5.3.4B and Table 5.3.3) as observed with yeast extract.

Beef extract was not an effective nitrogen source for dextran production from *Weissella confusa* Cab3 as compared with yeast extract and peptone. Increasing beef extract concentration from 1-3%, increased dextran production from 3.8 to 7.35 mg/ml (Fig. 5.3.4C). However 1.5%, w/v yeast extract and 2%, w/v peptone produced much higher dextran (10.0 mg/ml and 9.9 mg/ml, respectively) as compared with 3%, w/v (7.35 mg/ml) beef extract as the sole nitrogen source in the medium. From the results it was concluded that higher concentrations of nitrogen sources inhibited dextran which could be due to their complex nature.

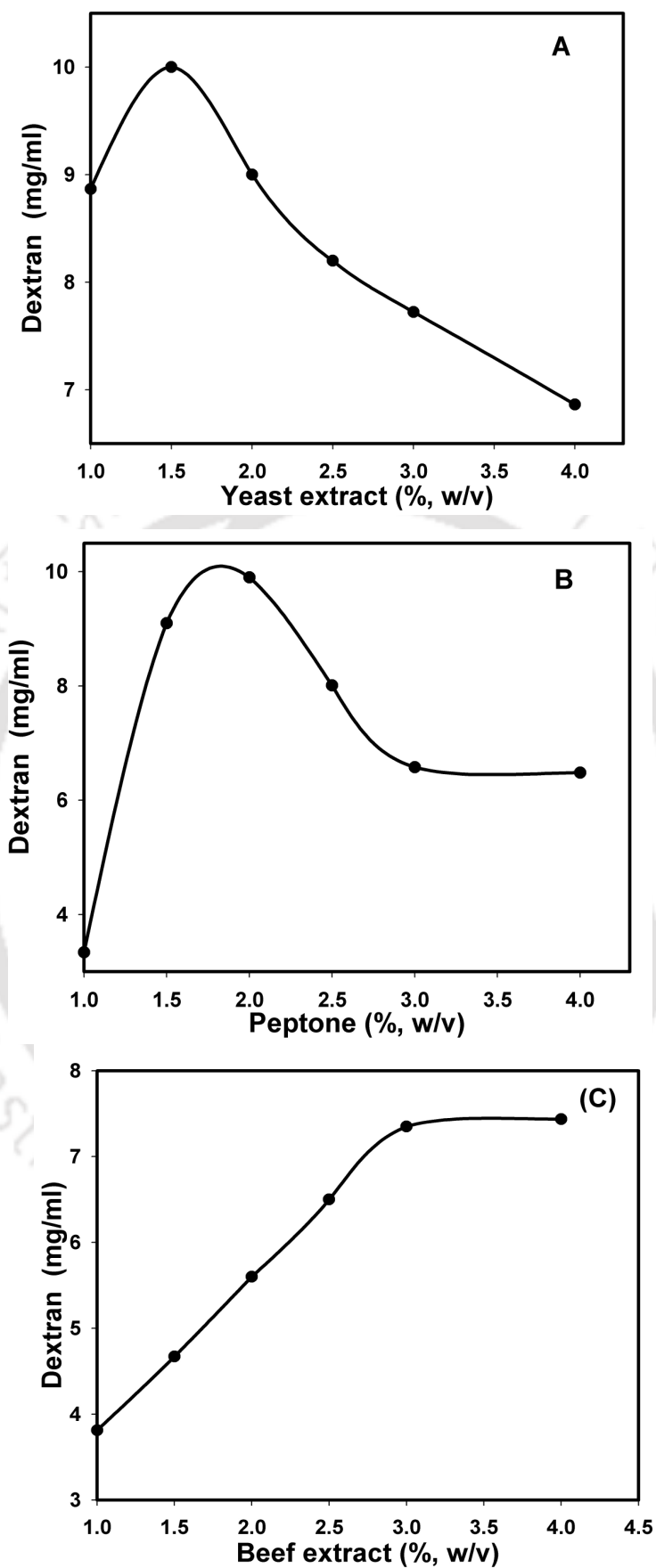


Fig. 5.3.4A Effect of (A) Yeast extract; (B) Peptone and (C) Beef extract on dextran production from *Weissella confusa* Cab3.

5.3.2.3 Effect of sodium acetate, Tween 80 and K_2HPO_4 on dextran production

Sodium acetate acts as a buffering agent and helps to maintain the pH in the fermentation process (Kim *et al.*, 2003). Sodium acetate was used in the medium to study its effect on dextran production. At lower concentration (6 mM) it did not have any effect on dextran production and it was similar to that of control (Fig. 5.3.5A). Increasing the concentration of sodium acetate beyond 6 mM, adverse effect on dextran production was observed (Fig. 5.3.5A). However, Sawale and Lele (2010) reported positive effect of sodium acetate (1.69% w/v, 0.20 M) on dextran production by a *Leuconostoc mesenteroides* strain isolated from fermented idli batter.

The production of dextran increased to 10 mg/ml with increase in concentration of Tween 80 upto 0.1% (v/v) (Fig. 5.3.5B) resulting in 11% as compared with control which produced 9.0 mg/ml dextran (Table 5.3.3). Further increase in the Tween 80 concentration did not enhance the dextran production. Rather at 0.3%, v/v Tween 80, a slight decrease in the dextran production was observed but still dextran concentration was higher (110 %) than the control. This result is similar to the earlier reports where they showed that addition of Tween 80 to enzyme production medium altered the fatty acid composition of the membrane thus enhancing the secretion of dextransucrase and its activity (Umesaki *et al.*, 1977; Sato *et al.*, 1989; Goyal and Katiyar, 1997; Purama and Goyal, 2008). 1.5% (w/v) K_2HPO_4 concentration was optimum for dextran production which gave 9.8 mg/ml dextran concentration showing a 9% increase as compared with control (Fig. 5.3.5C and Table 5.3.3). Beyond 1.5% (w/v) K_2HPO_4 , dextran production decreased. The medium as described by Tsuchiya *et al.* (1952) containing 2% K_2HPO_4 (as described in Chapter 2, Section 2.2.4) was considered as control.

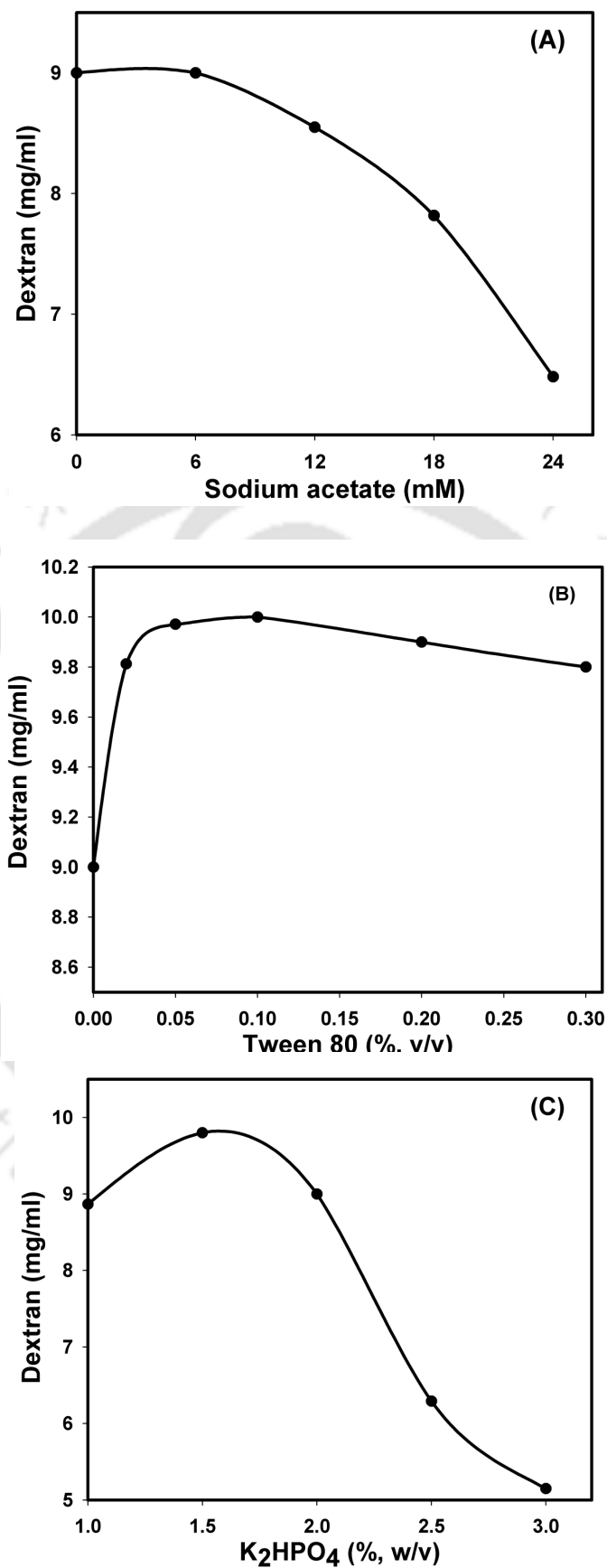


Fig. 5.3.5 Effect of (A) sodium acetate; (B) Tween 80 and (C) K_2HPO_4 on dextran production from *Weissella confusa* Cab3.

5.3.3 Experimental design for statistical optimization procedure and data analysis

5.3.3.1 Screening of significant factors for dextran production by Plackett-Burman Design

A 12 run Plackett Burman design consisting of 12 run was developed to identify the significant factors for dextran production by *Weissella confusa* Cab3. The assigned concentrations of variables at different levels in Plackett-Burman design for dextran production are listed in Table 5.3.4.

Table 5.3.4 Assigned concentrations of variables at different levels in Plackett–Burman design for dextran production.

S. No.	Variables	Concentration (% w/v)	
		Lower Level (-1)	Higher Level (+1)
1	Sucrose	2.00	6.0
2	Yeast extract	0.20	2.0
3	K ₂ HPO ₄	0.20	2.0
4	Tween 80 (% v/v)	0.10	1.0
5	CaCl ₂	0.01	0.1
6	pH	6.00	8.0

The experimental runs were executed to determine the dextran concentration in 12 different medium combinations (generated by Plackett Burman design) consisting of the six variables as listed in Table 5.3.4. The experimental and predicted dextran concentration in each of 12 experimental run is given in Table 5.3.5. The data listed in Table 5.3.5 showed a wide variation in dextran production from 4.62 mg/ml to 22.82 mg/ml in the twelve trials. This variation in dextran production with varying medium components showed the importance of medium optimization for enhanced dextran production.

Table 5.3.5 Plackett-Burman design for six variables with coded values with observed results for dextran production.

Run Order	Sucrose (A)	Yeast extract (B)	K ₂ HPO ₄ (C)	Tween 80 (D)	pH (E)	CaCl ₂ (F)	Dextran (mg/ml)	
							Experimental	Predicted
1	-1	1	1	-1	1	-1	6.48	2.74
2	1	1	-1	1	-1	-1	22.82	21.73
3	-1	-1	-1	1	1	1	14.20	12.88
4	1	-1	1	1	-1	1	21.05	20.05
5	-1	-1	1	1	1	-1	4.62	7.03
6	-1	1	-1	-1	-1	1	7.34	8.18
7	1	-1	-1	-1	1	1	22.79	21.38
8	1	1	-1	1	1	-1	21.05	22.14
9	1	1	1	-1	1	1	13.19	16.17
10	1	-1	1	-1	-1	-1	15.67	15.11
11	-1	-1	-1	-1	-1	-1	5.63	7.53
12	-1	1	1	1	-1	1	7.34	7.26

According to the resulting effects of these six variables on dextran production, the associated significant levels are presented in Table 5.3.6. Sucrose and Tween 80 having coefficient 5.913 and 1.666, respectively showed positive effect and its presence at higher concentrations in the medium enhanced the dextran production. The positive effect of sucrose was due to the direct correlation of dextransucrase and dextran production with sucrose concentration. K₂HPO₄ having coefficient of -2.123 was found negatively significant and its presence in the medium in lower concentration stimulated dextran production. The variables with confidence levels greater than 90% were considered as significant. With the help of relative ranking and from the regression analysis (Table 5.3.6) the variables sucrose, K₂HPO₄ and Tween 80 which were significant at 99.9%, 95.1% and 90.2% levels, respectively were considered to have greater impact on dextran production and were further optimized by a central composite design. Excluding insignificant variables the model equation

for dextran concentration after screening by Plackett-Burman Design could be written as

$$Y = 13.515 + 5.913A - 2.123C + 1.666D \quad \dots(8)$$

Where, Y = Dextran concentration, A = Sucrose, C = K_2HPO_4 and D =Tween 80

Table 5.3.6 Statistical analysis of Plackett-Burman Design showing coefficient, t and P values for each variable.

Variable	Coefficient	t- value	P-value	Rank
Intercept	13.515	16.47	0.000	-
Sucrose (A)	5.913	7.21	0.001	1
Yeast extract(B)	-0.478	-0.58	0.585	5
K_2HPO_4 (C)	-2.123	-2.59	0.049	2
Tween 80 (D)	1.666	2.30	0.098	3
pH (E)	0.206	0.25	0.811	6
$CaCl_2$ (F)	0.805	0.98	0.372	4

5.3.3.2 Optimization of medium composition for dextran production by Response Surface Methodology

The relationships, interrelationships and the optimal level of medium constituents of the variables were determined by fitting the second order polynomial equation to data obtained from 20 experiments. Based on the Plackett-Burman design, where sucrose, K_2HPO_4 and Tween 80 were selected for their significant effects on the dextran production, a 2^3 central composite design was used for further optimization. The effects of the selected significant variables were studied in five levels which are listed in Table 5.3.7.

Table 5.3.7 Coded values of variables used in central composite design (CCD).

Coded value X_i	Independent variables X_i		
	Sucrose (X_1)	K ₂ HPO ₄ (X_2)	Tween 80 (X_3)
-1.633	2.05	0.025	0.01
-1	3.00	0.50	0.20
0	4.50	1.25	0.50
1	6.00	2.00	0.80
1.633	6.94	2.47	0.98

Table 5.3.8 2³ full factorial central composite design matrix of two variables in uncoded units and experimental response.

Run order	Sucrose (%, w/v) X_1	K ₂ HPO ₄ (%, w/v) X_2	Tween 80 (%, v/v) X_3	Dextran (mg/ml)	
				Experimental	Predicted
1	0	0	-1.633	22.58	21.49
2	0	0	0	26.26	25.51
3	0	0	+1.633	15.55	17.03
4	-1.633	0	0	10.66	9.44
5	0	+1.633	0	21.67	21.65
6	0	-1.633	0	13.71	14.12
7	+1.633	0	0	24.42	26.04
8	0	0	0	25.95	25.51
9	0	0	0	26.26	26.31
10	1	+1	-1	28.70	28.71
11	1	-1	1	18.61	17.39
12	0	0	0	26.26	26.31
13	-1	-1	-1	10.96	11.94
14	-1	+1	1	14.63	14.74
15	0	0	0	25.95	26.42
16	1	+1	1	25.34	24.09
17	1	-1	-1	23.50	23.13
18	-1	+1	-1	13.71	14.67
19	0	0	0	25.95	26.42
20	0	-1	1	11.57	11.30

Other nutrient's concentrations were set at their middle levels. Table 5.3.8 gives the design and results of experiments carried out by the CCD design. The closer value of R^2 to 1 of the model explains better for the variability of experimental values with the predicted values (Liu *et al.*, 2007). The coefficients of regression were calculated and the following regression equation was obtained,

$$Y = 26.0811 + 5.0818X_1 + 2.3055X_2 - 1.3669X_3 + 0.7650 X_1X_2 - 1.2240 X_1X_3 + 0.2295 X_2X_3 - 2.9158X_1^2 - 2.8603X_2^2 - 2.3440X_3^2 \quad \dots\dots (9)$$

Where, Y is the response value, that is, the dextran production, and X_1 , X_2 and X_3 are the coded levels of sucrose, K_2HPO_4 and Tween 80, respectively. The statistical significance of above equation was checked by F-test, the results of ANOVA for dextran production are shown in Table 5.3.9. The results demonstrated that the model is highly significant, and is evident from Fischer's, F test with a very low probability value ($P_{\text{model}} > F = 0.001$) (Table 5.3.9). The model's goodness of fit was checked by determination coefficient (R^2). In this case, the value of the determination coefficient ($R^2=0.9808$) indicated that only 1.92% of the total variations were not explained by the model. The value of the adjusted determination coefficient [$\text{Adj } (R^2) = 0.9545$] was also very high thus supported the high significance of the model.

Table 5.3.9 ANOVA of Central Composite Design for dextran concentration.

Source	DF	Sum of squares	Mean Square	F-value	Prob. P > F
Model	9	714.018	79.335	45.27	0.000
Residual (error)	8	14.020	1.752		
Lack of fit	5	13.973	2.795	179.07	0.001
Pure error	3	0.047	0.016		
Total	19	731.562			

$R^2 = 0.9808$, $\text{Adj } R^2 = 0.9545$, DF: degrees of freedom.

Model coefficients estimated by regression analysis for each variable representing their effect on dextran concentration is shown in Table 5.3.10. The significance of each coefficient was determined by t-values and P-values which are listed in Table 5.3.10.

Table 5.3.10 Model coefficient of dextran estimated by multiple linear regression

Model Term	Coefficient	Standard error of Coefficient	t-value	P-value
Intercept	26.0811	0.5399	48.310	0.000
X_1	5.0818	0.3625	14.017	0.000
X_2	2.3055	0.3625	6.359	0.000
X_3	-1.3669	0.3625	-3.770	0.005
X_1^2	-2.9158	0.3643	-8.004	0.000
X_2^2	-2.8603	0.3643	-7.852	0.000
X_3^2	-2.3440	0.3643	-6.435	0.000
X_1X_2	0.7650	0.4680	1.634	0.141
X_1X_3	-1.2240	0.4680	-2.615	0.031
X_2X_3	0.2295	0.4680	0.490	0.637

The results showed that among the independent variables, X_1 (sucrose) and X_2 (K_2HPO_4) have strong positive linear effect on the dextran production with $P = 0.000$ and $P = 0.000$, respectively [Table 5.3.10, Eq. (5)]. The increase in their concentration can increase the product yield. The negative coefficient (-1.3669) observed for the X_3 (Tween 80) indicated that decrease in its concentration can increase the dextran production. Among the interactions X_1X_2 ($P < 0.7650$), X_2X_3 ($P < 0.2295$) have positive coefficients, while X_1X_3 ($P < 1.2240$) had negative coefficients. By analyzing 3D response surface and 2D contour plots *i.e.* the graphical representations of regression equations (Zhong and Wang, 2010) the relationship and interaction between responses and variable were studied.

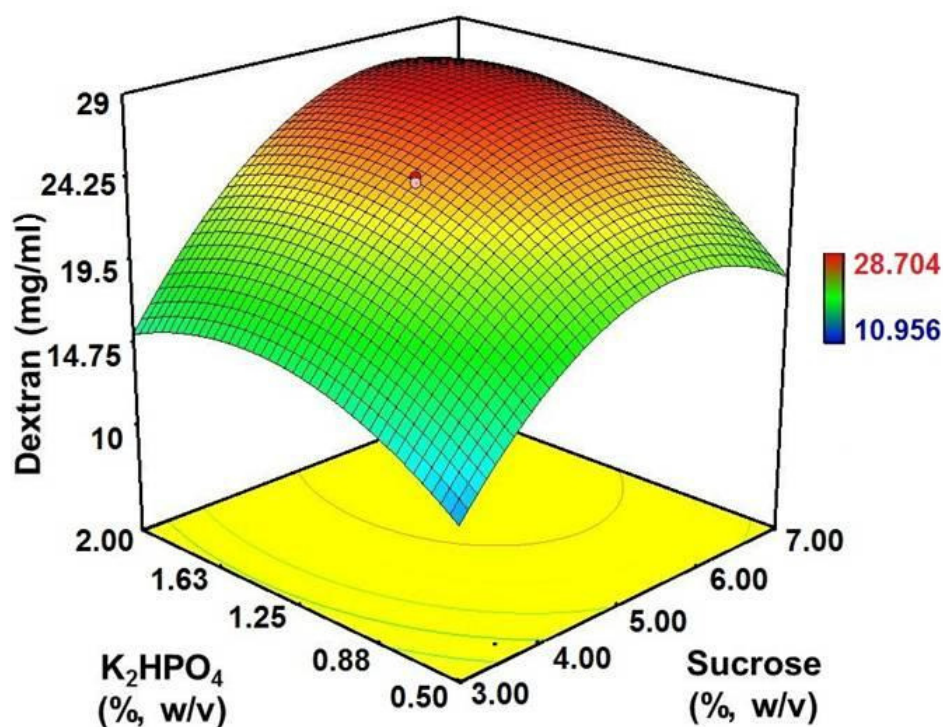
For the determination of the optimal levels of the variables for maximum dextran production, three dimensional response surface plots (Fig. 5.3.6) were constructed by plotting the response against any two of the three independent variables and by maintaining the other variable at their middle (zero) levels. The 3D plot illustrated that there was an increase in dextran production when high concentrations of sucrose (4.5-6.0% w/v), high K_2HPO_4 (1-2% w/v) and low

concentration of Tween 80 (0.2-0.4 %, v/v) were used (Fig. 5.3.6). Fig. 5.3.6A represents the effects of sucrose and K_2HPO_4 concentrations on dextran production from *Weissella confusa* Cab3. The figure demonstrated that, the dextran concentration increases with the increase in the concentrations of both variables viz. sucrose and K_2HPO_4 . Beyond the K_2HPO_4 concentration of 1.63 (% w/v) and sucrose concentration of 6.0 (% w/v) a decline in the dextran production was observed. Fig. 5.3.6B shows the interaction effect between sucrose and Tween 80 on dextran production at constant K_2HPO_4 concentration (1.25%, w/v). The dextran production increased sharply with increasing sucrose concentration upto about 6.0%, w/v and beyond this concentration saturation in the dextran production was achieved (Fig. 5.3.6B). Increase in the Tween 80 concentration upto 0.4 (% v/v) enhanced the dextran production from *Weissella confusa* Cab3 however, beyond this concentration dextran production decreased revealing a negative interaction effect between these factors at their higher level (Fig. 5.3.6B). Fig. 5.3.7C displayed the effects of Tween 80 and K_2HPO_4 on dextran production at constant sucrose concentration (4.5%, w/v). The surface plot revealed that no significant change in dextran production occurred with change in the Tween 80 or K_2HPO_4 concentration. The optimum was observed near the central values of Tween 80 and relatively higher side of K_2HPO_4 (1.63%, w/v).

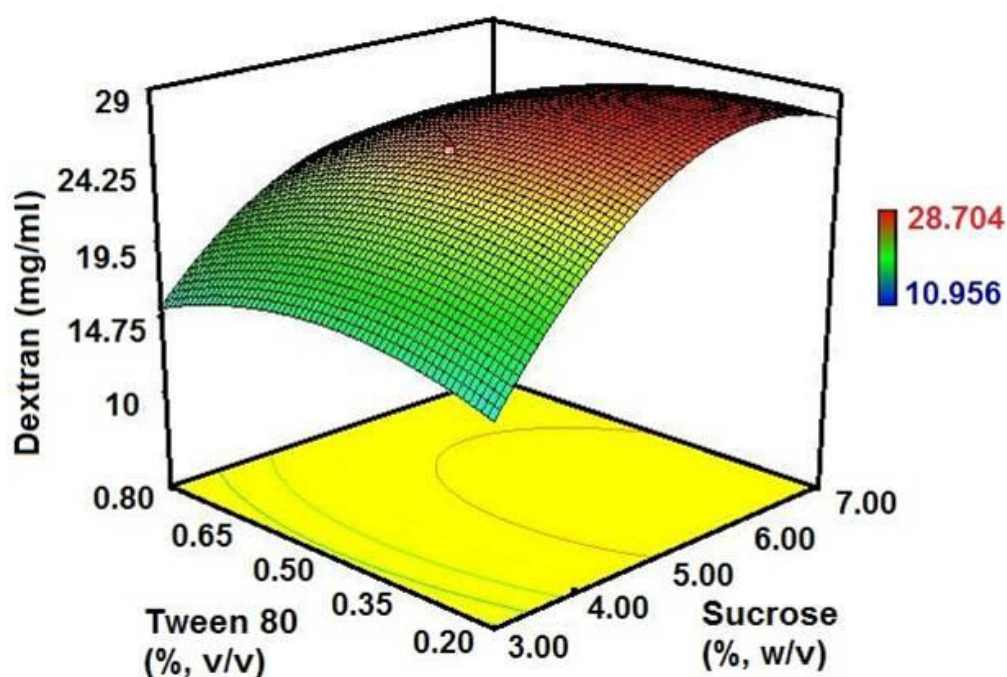
The shapes of the contour plots indicate the significance of mutual interactions between the variables. Circular contour plot reflects the negligible interactions between the corresponding variables, whereas elliptical contour plot indicates the significant interactions between the corresponding variables (Zhong and Wang, 2010). The contours plots are helpful proper identification of the type of interactions between test variables. The surface confined in the smallest curve of the contour diagram can

be used to predict optimum response of the system. To illustrate the above mentioned interaction effect between the variables, typical contour plots between a pair of variable are depicted at the bottom of the corresponding response surface plots of Fig. 5.3.6. The corresponding coordinates in the region of the contour diagram (Fig. 5.3.6) gave the optimum values of the respective factors. The response surface contour plots of mutual interaction between the variables, viz., sucrose and Tween 80 (Fig. 5.3.6B) was elliptical, indicating significant interaction between them. Besides the contour plot showing significant interaction between sucrose and Tween 80, response surface contours drawn between sucrose and K_2HPO_4 (Fig. 5.3.6A) and Tween 80 and K_2HPO_4 (Fig. 5.3.6C) were circular indicating non-significant nature of their interactions. The predicted optimum levels of the tested variables were obtained by applying regression analysis on Eq. (9) using Minitab software version 15.

(A)



(B)



(C)

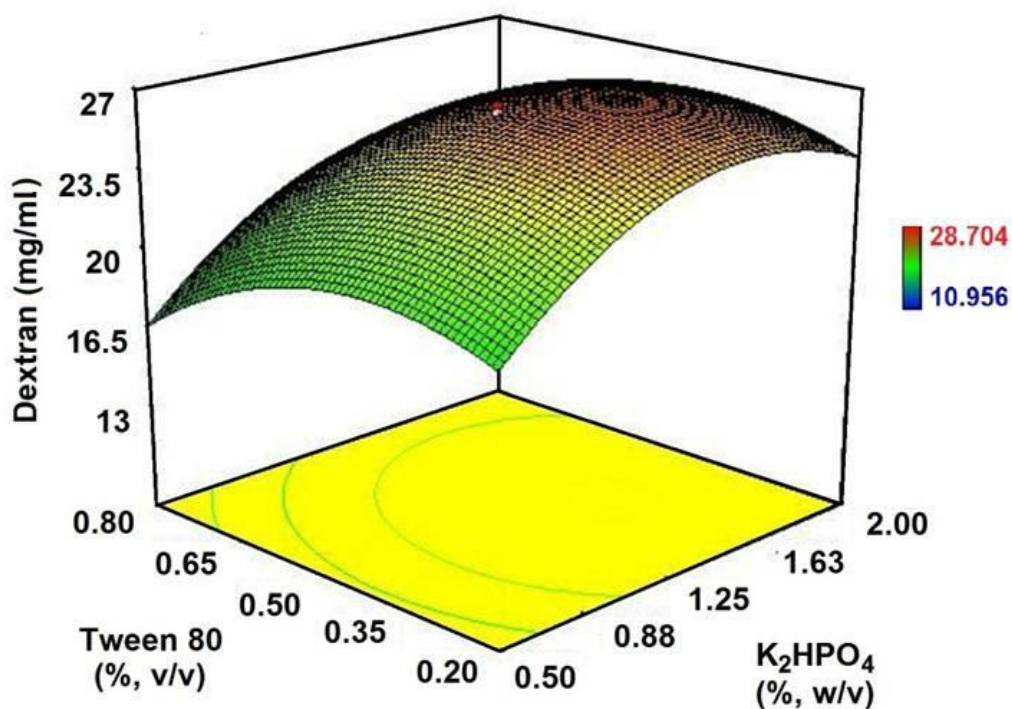


Fig. 5.3.6 Surface and contour plot of the combined effects of the significant factors on dextran sucrose activity of *Weissella confusa* Cab3. (A) Sucrose and K_2HPO_4 (Fixed level: Tween 80=0.5) (B) Sucrose and Tween 80 (Fixed level: K_2HPO_4 =1.25) (C) K_2HPO_4 and Tween 80 (Fixed level: Sucrose=4.5).

The optimization by response surface methodology predicted the maximum dextran production of 29.7 mg/ml with desirability 0.989, at sucrose concentration 6.06 %, w/v, K₂HPO₄ concentration 1.634 %, w/v and Tween 80, 0.34 % v/v (Fig. 5.3.7).

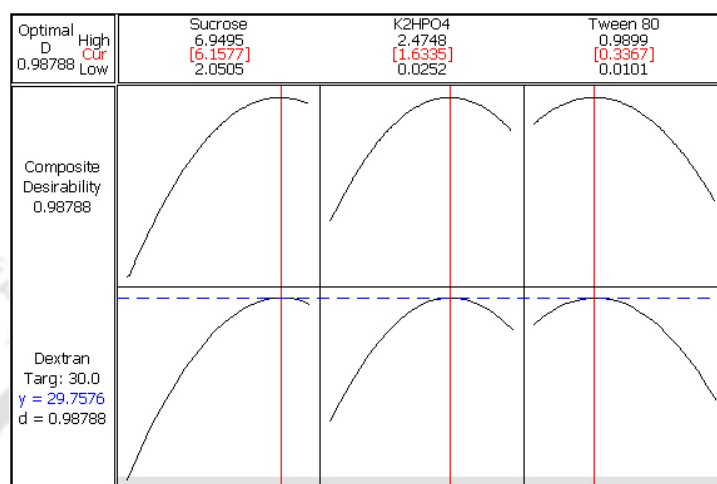


Fig. 5.3.7 Optimization graph for maximization of dextran production showing the optimum concentrations of sucrose, K₂HPO₄ and Tween 80 on dextran concentration from *Weissella confusa* Cab3.

5.3.3.3 Validation and Scale up of dextran production using statistically optimized medium

The optimum levels of the variables were obtained by solving the regression equation and by analyzing the response surface contour plots. The model predicted a maximum dextran production of 29.7 mg/ml in the medium containing sucrose, 6.06% (w/v); K₂HPO₄, 1.63% (w/v); Tween 80, 0.34% (v/v) by keeping the other components according to their level in the medium as described by Tsuchiya *et al.*, 1952. To validate the predicted model, triplicate set of experiment was run at shake flask level with 100 ml of optimized medium at 25°C and 180 rpm. *Weissella confusa* Cab3 produced 28.0 mg/ml dextran concentration at shake flask level using statistically optimized medium which was in good agreement with the predicted model. The excellent correlation of dextran concentration between predicted and measured values of these experiments justifies the validity of the response model and

the existence of an optimum point. The dextran production from *Weissella confusa* Cab3 using statistically designed medium was scaled up to 3 l lab scale bioreactor level with 1 l working volume. The fermentation profile of dextran production at shake flask level and bioreactor level using statistically optimized medium is shown in Fig. 5.3.9A and B. The enzyme activity and the cell optical density reached maximum at 12-15 h of fermentation at both shake flask and bioreactor level. In both the cases (shake flask and bioreactor level) sucrose concentration profiles showed maximum consumption of sucrose during first 10-15 h, with the maximum production of dextran using the same optimized medium. At shake flask level, a maximum of 28 mg/ml dextran production occurred, along with the maximum cell OD₆₀₀, maximum enzyme activity and specific activity of 9.6, 20.0 U/ml and 3.2 U/mg, respectively after 12 h of fermentation (Fig. 5.3.9A).

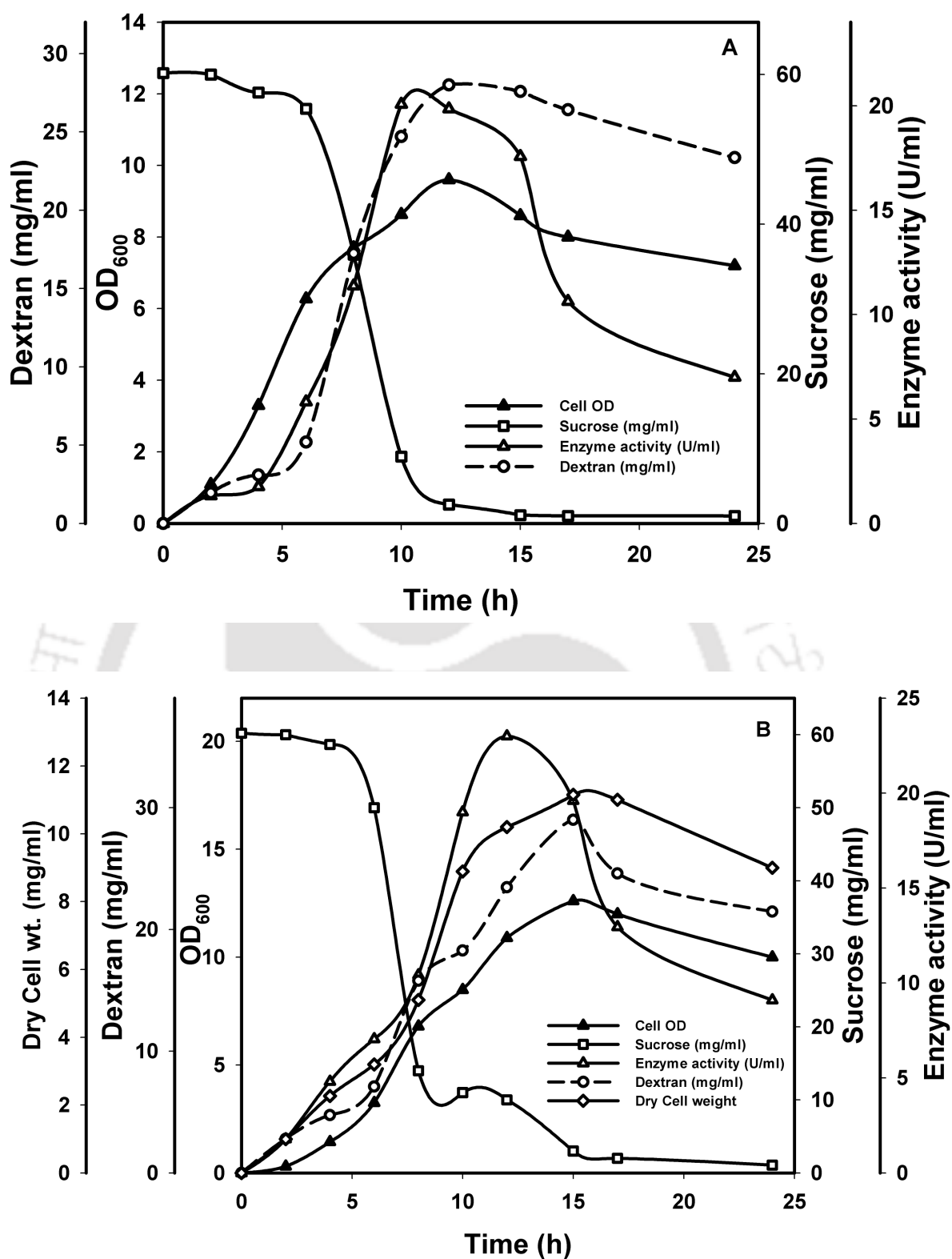


Fig. 5.3.9 Fermentation profile of *Weissella confusa* Cab3 using statistically designed medium for dextran production at (A) Shake flask and (B) Lab scale bioreactor level.

The scale up of batch cultivation from the flask culture containing 100 ml medium to a bioreactor containing 1000 ml of the same optimized medium at 25°C, and aeration rate of 2.0 vvm resulted in a dextran concentration of 29.0 mg/ml (Fig. 5.3.9B). At bioreactor level, the maximum cell OD₆₀₀, maximum enzyme activity and specific activity of 12.6, 23.0 U/ml and 3.6 U/mg, respectively after 15 h of fermentation (Fig. 5.3.9B). Table 5.3.11 shows comparison of maximum attained fermentation parameters in unoptimised medium and optimized medium from *Weissella confusa* Cab3 using statistically optimized medium for dextran production.

Table 5.3.11 Comparison of fermentation parameters for dextran production in different medium.

Unoptimized medium*					
Level	Enzyme activity (U/ml)	Specific activity (U/mg)	Cell OD ₆₀₀	Dry cell wt. (mg/ml)	Dextran mg/ml
Shake Flask	6.0	1.0	5.4	4.1	9.0
Optimized medium					
Shake Flask	20.0	3.20	9.6	8.9	28.0
Bioreactor	23.0	3.60	12.6	11.2	29.0

*Enzyme production medium as described by Tsuchiya *et. al.*, 1952 (as described in Chapter 2, Section 2.2.4).

The cell OD₆₀₀ and dry cell weight (mg/ml) at bioreactor level were higher as compared with those obtained at shake flask level using the same optimized medium (Table 5.3.11). The increase in the cell OD₆₀₀ and dry cell weight at bioreactor level might be the outcome of controlled aeration and pH which supported cell growth. The viscosity of the culture increased synchronously with the production of dextran in the fermentation broth. The growth of *Weissella confusa* Cab3 in the statistically optimized medium under controlled pH adjustment resulted in enhanced dextran

production (29.0 mg/ml) as compared to 28.0 mg/ml dextran production at shake flask level without pH control as listed in Table 5.3.11. Several other studies have also shown that growth in medium with pH adjustment resulted in enhanced polysaccharide production (Gamer *et al.*, 1997; Degeest *et al.*, 2001). Thus increased production of dextran (29.0 mg/ml) at bioreactor level might be the result of pH maintenance and aeration. The kinetics of dextran production by *Weissella confusa* Cab3 showed that the dextran production occurred mainly in exponential phase of growth curve. Upon prolonged fermentation, a decrease in the dextran production was observed after 15 h of fermentation. Several other studies also showed a decrease in the exopolysaccharide concentration upon prolonged fermentation (De Vuyst *et al.*, 1998; Pham *et al.*, 2000).

The maximum enzyme activity observed at shake flask and bioreactor level was 20.0 and 23.0 U/ml, respectively at 12 h of the fermentation (Fig. 5.3.9A and Table 5.3.11). The specific activities for dextransucrase obtained at flask culture (3.20 U/mg) and bioreactor (3.6 U/mg) level using statistically designed medium were 3.2 and 3.6 fold higher than that observed in unoptimized medium (Table 5.3.11). Thus along with the enhancement in dextran production, an increment in the dextransucrase production was also observed which shows the linked production of the dextransucrase and the dextran.

The statistical methods proved to be a powerful tool for maximizing dextran production from *Weissella confusa* Cab3. Majumder and Goyal, 2008 reported 1.01 mg/ml glucan production from *Leuconostoc dextranicum* NRRL B-1146, using statistically optimized medium. Sawale and Lele, 2010 reported production of 60.3 mg/ml dextran from *Leuconostoc* sp, using statistically optimized medium for dextran production. However, they used very high sucrose concentration (220 mg/ml).

5.4. Conclusions

Weissella confusa Cab3 (Genbank Accession Number JX649223), isolated from fermented cabbage was used for the dextran production. The stoichiometric parameters were determined to study the kinetics of dextran production. The dextran concentration (**P**), dextran yield (**Y_{Dex/suc}**) and specific dextran production rate (**q_{dextran}**) from *Weissella confusa* Cab3 were 9.0 g/l 0.49 gg⁻¹ and 0.09 gg⁻¹h⁻¹, respectively. The dextran concentration from *Weissella confusa* Cab3 was compared with the lactic acid bacterial strain *Leuconostoc mesenteroides* NRRL B-512F which has been used for dextran production at used at industrial scale. *Weissella confusa* Cab3 produced significantly higher dextran 9.0 mg/ml than *Leuconostoc mesenteroides* NRRL B-512F (4.5 mg/ml) using the same medium.

First step towards medium optimization for enhanced dextran production by *Weissella confusa* Cab3 was to screen the medium components by one variable at a time approach. The effects of various macronutrients such as sucrose yeast extract, beef extract, peptone, sodium acetate, K₂HPO₄ and Tween 80 were studied on dextran production from *Weissella confusa* Cab3. The medium used as control gave 9.0 mg/ml dextran concentration. Sucrose (5%, w/v), Tween 80 (0.1%, v/v), yeast extract (1.5%, w/v), peptone (1.5%, w/v) and K₂HPO₄ (1.5%, w/v) were effective nutrients displaying higher dextran production with 22.4, 10.0, 10.0, 9.9 and 9.8 mg/ml dextran concentration, respectively. Sodium acetate and beef extract were not effective for dextran production.

The statistical method was applied to optimize fermentation medium for dextran production by *Weissella confusa* Cab3. Six nutrients were tested using Placket-Burman Design for their significance. Based on the results of the Placket-Burman Design three nutrients viz. sucrose, K₂HPO₄ and Tween 80 showing significant effect

on dextran production were selected for further optimization in order to increase dextran production of *Weissella confusa* Cab3 by response surface methodology. A quadratic polynomial equation obtained by the CCD with $R^2=0.9808$ was used for the determination of the optimal concentrations of constituents having significant effects on dextran production. Under the optimal condition, 28.7 mg/ml dextran production was predicted by the software which upon validation at shake flask resulted in 28.0 mg/ml dextran production. In an attempt to approximate industrial conditions for dextran production, scale-up was carried out in a lab scale bioreactor by using optimized medium. At lab scale 3 liter bioreactor level containing 1 l optimized medium, 29.0 mg/ml dextran was obtained. The dextran concentration of *Weissella confusa* Cab3 was enhanced up to 3.2 fold in the optimized medium as compared with the unoptimized medium. These results are encouraging for pilot scale or industrial scale production of dextran from hyper producing *Weissella confusa* Cab3.

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Chapter 6

Production, purification and characterization of dextran from *Weissella confusa* Cab3

6.1 Introduction

Dextran is a class of homopolysaccharides made up of repeating units of D-glucose. Dextran is catalyzed by dextranase, an extracellular enzyme synthesized by lactic acid bacteria viz., *Leuconostoc*, *Lactobacillus* and *Streptococcus* spp. (Majumder and Goyal, 2008). Smitinont *et al.* 1999 reported the production of dextran from *Pediococcus pentosaceus* strain. Recently *Weissella confusa* has been focused for the efficient dextran production (Maina *et al.*, 2009; Shukla and Goyal, 2011). Dextran is produced by the catalytic cleavage of the osidic bond of the sucrose by dextranase enzyme (Purama and Goyal, 2008). When some acceptor carbohydrate molecules are present in the reaction, some of the glucosyl unit of sucrose is transferred onto the acceptor molecule and produce oligosaccharides (Robyt and Eklund, 1983). Maltose and isomaltose are the most efficient acceptors, leading to the production of homologous series of oligosaccharides as the acceptor reaction product (Robyt and Walseth, 1978).

Dextran has been classified according to their structural and functional properties (Jeanes *et al.*, 1954). Dextran are briefly divided in to three groups viz.

class 1, class 2 and class 3 depending on the types and percentages of the various linkages (Maina *et al.*, 2008). The physical and chemical properties of the dextran vary according to its type and percentage of linkage (Majumder *et al.*, 2009). Dextran improve rheology, texture, stability and mouth feel of fermented milk products (Patel *et al.*, 2012). Dextran has been used as adjuvant, emulsifier, carrier and stabilizer in food and pharmaceutical industries (Patel *et al.*, 2012). Low molecular weight dextran (Dextran 70 and dextran 40) are used as blood plasma substitute (Harrison, 1954). Dextran are also used as matrix of chromatography column, anticoagulant, metal-plating processing, for enhanced oil recovery, biomaterials. Dextran has been investigated suitable for cell-resistant coatings on biomaterial surfaces (Massia *et al.*, 2000). *In situ* production of dextran during sourdough fermentation improves the volume, moist feel in the mouth and softness of rye breads and rye mixed breads (Di Cagno *et al.*, 2006; Lacaze *et al.*, 2007). Important features of dextran for its use in sourdough bread are its high yield, low degree of branching and high molecular weight. The physical properties such as surface morphology, gelling nature, structure and rheology are crucial in determining the applications potential of dextrans (Stoodley *et al.*, 1999).

The NMR spectroscopic techniques have been used to analyze the structures of dextran from various lactic acid bacterial strains. The NMR analysis revealed α -(1 $\rightarrow$ 2) linkages in the dextran of *Leuconostoc citreum* E497 (Maina *et al.*, 2008). However, dextran from *Weissella confusa* E392 was highly linear containing only 3% α -(1 $\rightarrow$ 3) linkages (Maina *et al.*, 2008). The structural characterization of purified EPS by FTIR, ^1H and ^{13}C NMR spectroscopy demonstrated that *W. cibaria* CMGDEX3 synthesized a linear dextran that predominately had α -(1 $\rightarrow$ 6) glycosidic linkages with only a few (3.4%) (1 $\rightarrow$ 3) linked branches (Ahmad *et al.*, 2012).

In the present study, a dextran producing bacterial isolate *Weissella confusa* Cab3 was isolated from fermented cabbage (Shukla and Goyal, 2011). Considering the great industrial and pharmaceutical applications, the dextran was produced by enzymatic synthesis and purified by the successive steps using ethanol precipitation and gel filtration. The purified dextran was physically and structurally characterized using FT-IR, NMR, scanning electron microscopy and rheological studies. To study the biocompatibility of the dextran from the isolate *Weissella confusa* Cab3, the cytotoxicity test was conducted using human cervical cancer cell line (HeLa) cell line.



6.2 Material and Methods

6.2.1 Chemicals and reagents

All the chemicals required for reducing sugar estimation, total sugar estimation, protein estimation and buffer preparation were from Hi-Media Pvt. Ltd., India and Fisher Scientific Pvt. Ltd., India. Sucrose used for the dextran biosynthesis was purchased from Hi-media Pvt. Ltd., India. The cervical cancer (HeLa) cell line, Dulbecco's Modified Eagle's Medium (DMEM) containing 4.0 mM L-glutamine and 110 mg/l sodium pyruvate, foetal bovine serum (FBS) and 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from National Centre for Cell Science (NCCS), Pune, India. W/5000 units Penicillin and Streptomycin (5 mg/ml in 0.9% normal saline), dimethyl sulfoxide (DMSO) and phosphate buffer saline (PBS), DMSO (cell culture grade) were purchased from Himedia India Pvt. Ltd., India. 1x trypsin-EDTA solution (T001-100ML, 0.25% trypsin and 0.2% EDTA in Dulbecco's Phosphate Buffered saline) and phosphate buffer saline (PBS, pH 7.4) were purchased from Himedia India Pvt.

6.2.2 Microorganism and culture condition

The bacterial strain *Weissella confusa* Cab3 (Genbank Accession Number JX649223) isolated from fermented cabbage (Shukla and Goyal, 2011) was used for the production of dextran. The organism was maintained in MRS medium (DeMan *et al.*, 1960) slabs incubated at 25°C, stored at 4°C and subcultured every two weeks. The MRS medium composition has been described in Chapter 2, Section 2.2.3.

6.2.3 Production of dextransucrase

Weissella confusa Cab3 was grown at 25°C in enzyme production medium as described by Tsuchiya *et al.*, 1952 (as described in Chapter 2, Section 2.2.4). The culture supernatant was used for enzyme purification. Partially purified dextransucrase (10.5 U/mg, 0.84 mg protein/ml) was obtained by fractionation using 30% PEG-400 (as described earlier in Chapter 4, Section 4.2.7). The dextransucrase (10.5 U/mg, 0.84 mg/ml) was used for the production of dextran as described later in Section 6.2.4.

6.2.4 Production of dextran using partial purified dextransucrase from *Weissella confusa* Cab3

For dextran synthesis, 24.0 ml of enzyme (10.5 U/mg, 0.84 mg protein/ml) was incubated in 200 ml of 5% sucrose as substrate in 20 mM sodium acetate (pH 5.4) containing 0.3 mM CaCl₂ and 15 mM sodium azide. The reaction mixture was incubated at 35°C for 48 h. The jelly like viscous solution was produced at the end of the incubation indicating the production of dextran which was further subjected to purification as described in Section 6.2.6.

6.2.5 Purification of dextran by ethanol precipitation

The viscous solution (200 ml) containing dextran synthesized by the enzyme was treated with three volume of ethanol (90%, v/v) to precipitate the dextran. The solution was centrifuged at 12,000g at room temperature for 20 min and the pellet was resuspended in 500 ml of deionized water. The same process was repeated on the solution (500 ml) two more times to ensure removal of any remaining sucrose. Finally the precipitated dextran was dissolved in 500 ml of deionized water. The solution was then freeze dried using a lyophilizer (Christ GmbH, model ALPHA 1-4 LD) at -51°C

at a vacuum pressure of 35×10^{-3} mbar for 24 h for further analysis. The sample was stored at room temperature for further characterization. The purified dextran was analyzed for protein content by the method of Lowry *et al.*, 1951 using bovine serum albumin as the standard (as described in Chapter 4, Section 4.2.5) and total carbohydrate content (as described in Chapter 5, Section 5.2.5.1).

6.2.6 Estimation of dextran

The total carbohydrate content of the test sample (dextran) was determined by phenol-sulphuric acid method (Dubois *et al.*, 1956) in a micro-titre plate (Fox and Robyt, 1991) as described in Chapter 5, Section 5.2.5.1).

6.2.7 Physical properties of dextran

6.2.7.1 Rheological properties of dextran

The steady shear viscosity (η) of semi-dilute dextran (0.5%) purified by ethanol precipitation at 25°C was measured using a rheometer (Haake RheoStress RS1, Thermo Electron, Karlsruhe, Germany) interfaced with a, HAAKE RheoWin 323 software. The shear rate was in the range of 0.1–350 s⁻¹.

6.2.7.2 Scanning Electron Microscopic analysis of dextran

Lyophilized sample of purified dextran (as described in Section 6.2.6.1) from *Weissella confusa* cab3 was attached to the SEM stub with double-sided tape and coated with 10 nm Au in a sputter coater (SC 7640, Leo Electron Microscopy Ltd., Cambridge, UK). The surface of the sample was analyzed in Scanning Electron Microscope operated at 10.0 kV.

6.2.7.3 High performance size exclusion chromatography (HPSEC) of dextran from *Weissella confusa* Cab3

Dextran (purified by ethanol precipitation as described in Section 6.2.6.1) was dissolved in DMSO to a final concentration of 2.0 mg/ml. HPSEC analysis were accomplished using DMSO based (DMSO + 100 mM LiBr) eluent. The HPSEC equipment comprised an integrated autosampler and pump module (GPCmax, Viscotek Corp., Houston, TX, USA), a combined light scattering and viscometric detector (270 Dual Detector, Viscotek Corp.), a refractive index (RI) detector (VE 3580, Viscotek Corp.) and the light scattering detector angles: 7° and 90°.

IF-804 Column (300 x 8 mm, exclusion limit 2×10^6 , Showa Denko, Tokyo, Japan) with LF-G (10 x 4.6 mm) guard columns was used. The flow rate was 1 ml/min and injection volume was 10 μ l. The HPSEC data (molar mass (n) and R_{η} = viscometric radius), were processed with the Omni SEC 4.5 software (Viscotek Corp.). The d_{η}/d_c value of 0.1435 ml/g for dextran in DMSO-based eluent (Basedow *et al.*, 1978) was used.

6.2.7.4 Dynamic light scattering (DLS) of dextran from *Weissella confusa* Cab3

Dynamic light scattering (also known as photon correlation spectroscopy or quasi-elastic light scattering) can be used to determine the size distribution profile of small particles in suspension or polymers in solution (Schaetzel, 1991). In particle size analysis DLS has been established over several years for the characterization of sub-micron particles and macromolecules (Schaetzel, 1991, Urban and Schurtenberger, 1998). Dynamic light scattering (DLS) measurement of the dextran from *Weissella confusa* Cab3 was performed at 25°C using a Photol dynamic laser scattering spectrometer Zetasizer NanoZS (Malvern Instruments, UK). The ethanol purified dextran solution (7 mg/ml) was diluted to a final concentration of 0.35 mg/ml

(20x) in MilliQ water and filtered through 0.2 µm filter. The sample volume of 500 µl of diluted dextran sample was used for the DLS analysis. The particle size distribution from DLS measurement was derived by deconvolution of the measured intensity autocorrelation function of the dextran sample. Deconvolution was accomplished using a non-negatively constrained least squares (NNLS) fitting algorithm, common examples being CONTIN, Regularization, and the General Purpose and Multiple Narrow Mode algorithms included in the Zetasizer Nano software.

The polydispersity (Pd) was evaluated according to Eq. (1), where SD is the standard deviation of the distribution and D(H) is the mean hydrodynamic diameter from DLS measurement:

$$\text{Polydispersity (PD)} = \frac{\text{SD}}{\text{D (H)}} \times 100 \quad (\%) \quad \text{..... (1)}$$

SD = standard deviation of the hydrodynamic diameter

D (H) = mean hydrodynamic diameter

6.2.8 Structural characterization of dextran from *Weissella confusa* Cab3

6.2.8.1 Optical rotation

The optical rotation property of ethanol purified dextran (as described in Section 6.2.6.1) from isolate *Weissella confusa* Cab3 was recorded using a polarimeter (Perkin-Elmer Instruments, model 343 Polarimeter) using a sodium D-line (589 nm) at 25°C.

6.2.8.2 Monosaccharide analysis of dextran from *Weissella confusa* Cab3

The monosaccharide analysis of dextran (purified by ethanol precipitation as described in Section 6.2.6.1) from *Weissella confusa* Cab3 was carried out by High

Performance Anion Exchange Chromatography (HPAEC). 50 mg of purified dextran sample was hydrolyzed by 1 ml of 0.05 M sulphuric acid at 100°C for 6 h. The hydrolyzed dextran was neutralized by adding 2M Na₂CO₃ (~100 µl) solution and filtered with 0.2 µm membrane. The sample was analyzed by HPAEC using ion chrome system (ICS-3000 system, Dionex Corporation, USA) using CarboPac PA-20 analytical column (150 x 3 mm) and CarboPac PA-20 guard column (30 x 3 mm). The temperature of the column was adjusted to 30°C and the injection volume was 25 µl. The eluent used was 250 mM NaOH at 0.2 ml/min. Glucose and fructose (10 µg/ml, filtered through 0.2 µm membrane) was used as standard monosaccharides for comparison.

6.2.8.3 Fourier-Transform Infrared Spectroscopic analysis

The dextran (purified by ethanol precipitation as described in Section 6.2.6.1) was characterized using a Fourier Transform Infra-Red (FT-IR) Spectrophotometer (Perkin-Elmer Instruments, Spectrum One FT-IR Spectrometer). The dried polymer was grinded with Potassium bromide (KBr) powder and pressed into pellet for FT-IR spectral measurement in the frequency range of 4000–400 cm⁻¹.

6.2.8.4 ¹H and ¹³C Nuclear Magnetic Resonance spectral analysis of dextran

¹H and ¹³C Nuclear Magnetic Resonance (NMR) spectra for dextran (purified by ethanol precipitation as described in Section 6.2.6.1) were recorded at 50°C with a 600 MHz NMR spectrometer (Bruker Avance III NMR spectrometer). Dextran sample (10 mg/ml) was exchanged three times with D₂O and then placed in NMR tubes (Wilmad NMR tubes, 5 mm, ultra-imperial grade, from Aldrich chemical

company, Milwaukee, WI, USA). The chemical shifts were referenced to acetone (^1H = 2.225 ppm and ^{13}C = 31.55 ppm).

6.2.9 Cytotoxicity test of dextran

6.2.9.1 Culturing and maintenance of Human cervical cancer cell line

Human cervical cancer (HeLa) cells were maintained in the Dulbecco's Minimal Essential Medium (DMEM) containing 0.1 mM nonessential amino acids, 18.0 mM sodium bicarbonate, 2 mM L-glutamine and 1.0 mM sodium pyruvate (National Centre for Cell Science) that was supplemented with 10% heat inactivated foetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (1000 U/ml penicillin G, 10 mg/ml streptomycin sulfate, 5 mg/ml gentamycin and 25 $\mu\text{g}/\text{ml}$ amphotericin B). The cells were cultured in T-25 tissue culture flask (Corning®) at 37°C in a humidified Incubator (Heal Force, HF 160 W, China) supplemented with 5% CO_2 . At 80-90% confluence, the medium was carefully aspirated without disturbing the surface and washed with PBS twice to remove dead cells in suspension. 1x trypsin-EDTA (0.25% trypsin and 0.2% EDTA in Dulbecco's Phosphate Buffered saline) solution was slowly added sufficient to cover the monolayer of cells and incubated at 37°C in 5% CO_2 atmosphere for 4-5 min. The cell count was done using a haemocytometer before proceeding for experiments.

6.2.9.2 Indirect contact based in vitro cytotoxicity assay

The cytotoxicity test of dextran (purified by ethanol precipitation as described in Section 6.2.6.1) from *Weissella confusa* Cab3 on human cervical cancer (HeLa) cell line was performed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described by Mosmann, 1983. To prepare a stock solution,

10 mg of lyophilized dextran powder was dissolved in 1 ml of sterile serum free DMEM. For MTT assay, the cell count was recorded using a haemocytometer and cells were seeded at a concentration of 1×10^4 /well in a 96-well plate (Cell Bind, Corning). After 24 h of growth, the medium was replaced with the serum free medium (DMEM medium) that contained varying concentrations (100 μ g-1500 μ g) of dextran and incubated for 24 and 48 h. The serum free medium without dextran was used as negative control. After incubation period, medium was removed and cells were gently washed with phosphate-buffered saline (PBS, pH 7). The contents of the each well were replaced with 100 μ l of MTT solution (0.5 mg/ml in serum free DMEM) and incubated for 4 h at 37°C. After 4 h incubation the medium was removed and 100 μ l of dimethyl sulphoxide (DMSO) was added to each well to solubilise the formazan crystals. The yellow color MTT is reduced to an insoluble purple formazan complex by mitochondrial reductases that are active only in the mitochondria of living cells. Thus, the formation of color complex can be directly correlated to the number of viable cells. The concentration of formazan was determined by measuring its absorbance at 570 nm using a multiwell plate reader (Tecan, model Infinite™ 200). The cell viability was calculated with the following equation.

$$\text{Cell viability (\%)} = (A_{\text{treated}} / A_{\text{control}}) \times 100$$

Where, A_{treated} and A_{control} are the absorbances of the treated and untreated cells, respectively. All experiments were done in five independent runs and the data was expressed as Mean $\pm$ Standard deviation (n=5). To minimize the experimental errors, exponentially growing cells of the same passage were used for each of the experiment.

6.3 Results and Discussion

6.3.1 Production and purification of dextran

The *in vitro* dextran synthesized by dextransucrase from *Weissella confusa* Cab3 was purified by ethanol precipitation in 3 successive steps as described earlier in Section 6.2.6.1. The ethanol purified dextran (500 ml) was analyzed for its purity in terms of protein contamination analyzed by the method of Lowry *et al.*, 1951 (as described in Chapter 4, Section 4.2.5). The protein content of the ethanol purified dextran was 0.13 mg/ml which was less than 2%. The total carbohydrate content (as described in Section 6.2.5.1) of the purified dextran sample was 7.5 mg/ml. The purified dextran was freeze-dried at -20°C and lyophilized for further structural and functional characterization.

6.3.2 Physical properties of dextran from *Weissella confusa* Cab3

6.3.2.1 Rheological properties of dextran from *Weissella confusa* Cab3

Rheological study of dextran from *Weissella confusa* Cab3 showed that the viscosity is inversely proportional to the shear rate and the semi liquid dextran exhibited a typical non-Newtonian pseudoplastic behavior, as expected in polymer solutions of high molecular weight (Fig. 6.3.1). The characteristic of pseudoplastic fluid is a shear thinning behavior. The polymers displaying non-Newtonian characteristics are employed in the food industry as gelling, stabilizers or thickening agents (Feddersen and Thorp, 1993). Rheology has emerged as a crucial quality control tool for the biopolymers because recently biopolymers have emerged as promising food additives (Zakaria and Rahman, 1996). Several biopolymers like alginates, carrageenans, agar, guar gum, arabic gum, methylcellulose and carboxymethylcellulose are hydrocolloids are being used in numerous food

formulations like sauces, ice creams etc. (Williams and Phillips, 2000). Exopolysaccharides are considered as biothickeners or hydrocolloids and represent a good alternative to additives obtained from plant (e.g. starch), animal (e.g. caseinate and gelatin), and seaweed (e.g. carrageen), which are used in the baked goods industry (Palomba *et al.*, 2012). Dextran affect the viscoelastic properties and stabilize the rheological properties of dough during freezing storage. The final product has better texture and flavor and an extended shelf-life (Tieking and Ganzle, 2005). The dextran from isolate *Weissella confusa* Cab3 showed similar rheological property thus it could be potentially used in food industries.

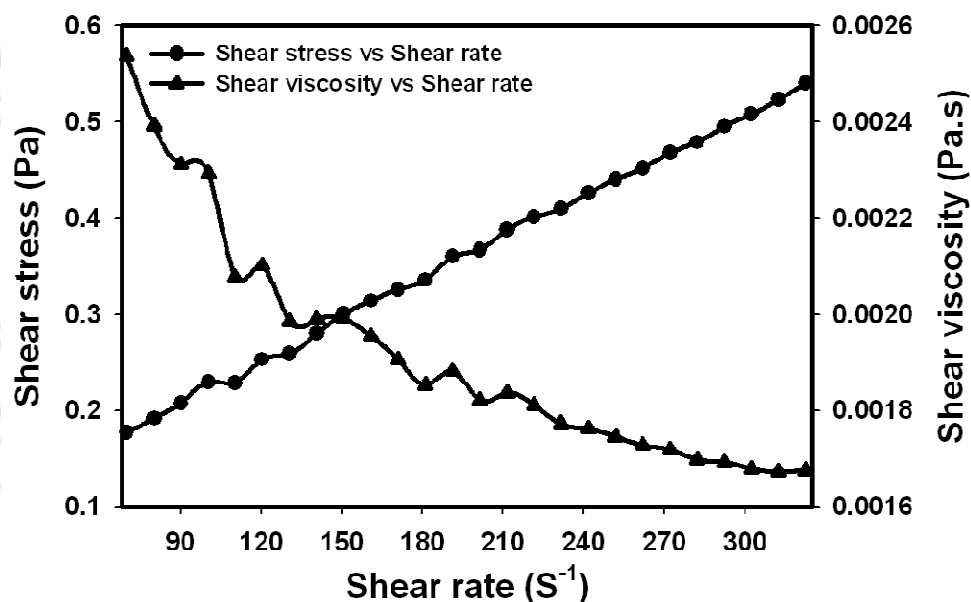


Fig. 6.3.1 Correlation of shear viscosity and shear stress of dextran of *Weissella confusa* Cab3 with respect to shear rate.

6.3.2.2 Scanning Electron Microscopic analysis of dextran

The surface morphology of dextran from the isolate *Weissella confusa* Cab3 was studied by scanning electron micrograph. The objectives of this study were to examine the surface morphology of the dextran. Numerous pores were observed throughout the surface of dextran (Fig. 6.3.2). Owing to its small pore size

distribution, the polymer can hold water and can be used as a texturing agent in food industry as additive. Several reports are available for the application of dextran in the production of biocompatible hydrogels (Sun *et al.*, 2011; Brunsen *et al.*, 2012)

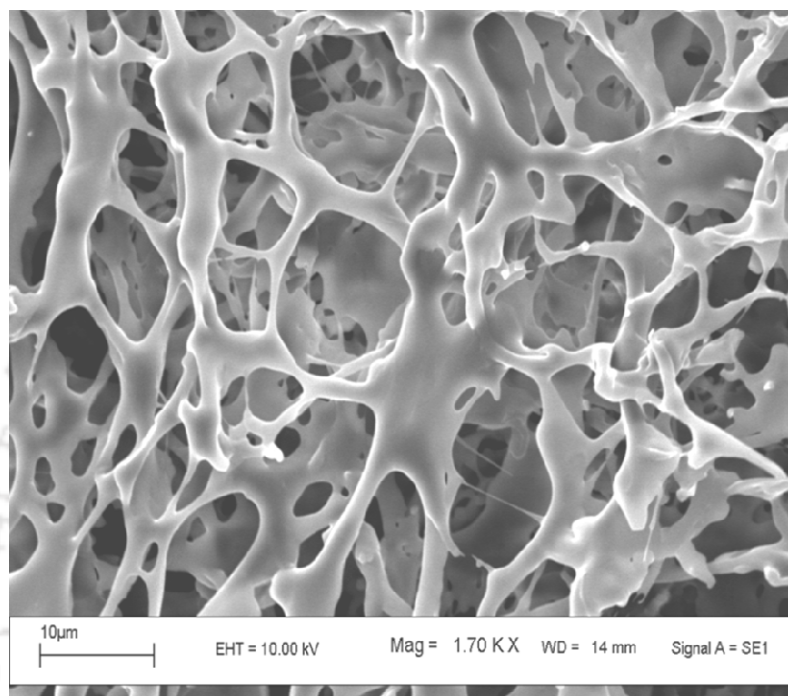


Fig. 6.3.2 Scanning Electron Micrograph of dextran from the isolate *Weissella confusa* Cab3 showing the porous reticular surface morphology.

6.3.2.3 High performance size exclusion chromatography (HPSEC) of dextran from *Weissella confusa* Cab3

The HPSEC chromatograms (aqueous solution) of dextran from *Weissella confusa* Cab3 is shown in Fig. 6.3.3. The HPSEC chromatogram of *W. confusa* Cab3 dextran shows only one major peak for both RI and LS signals. The macromolecular parameters of the samples based on these analyses are shown in Table 6.3.1. HPSEC in DMSO gave M_w values of 1.77×10^7 g/mol for *W. confusa* Cab3 dextran.

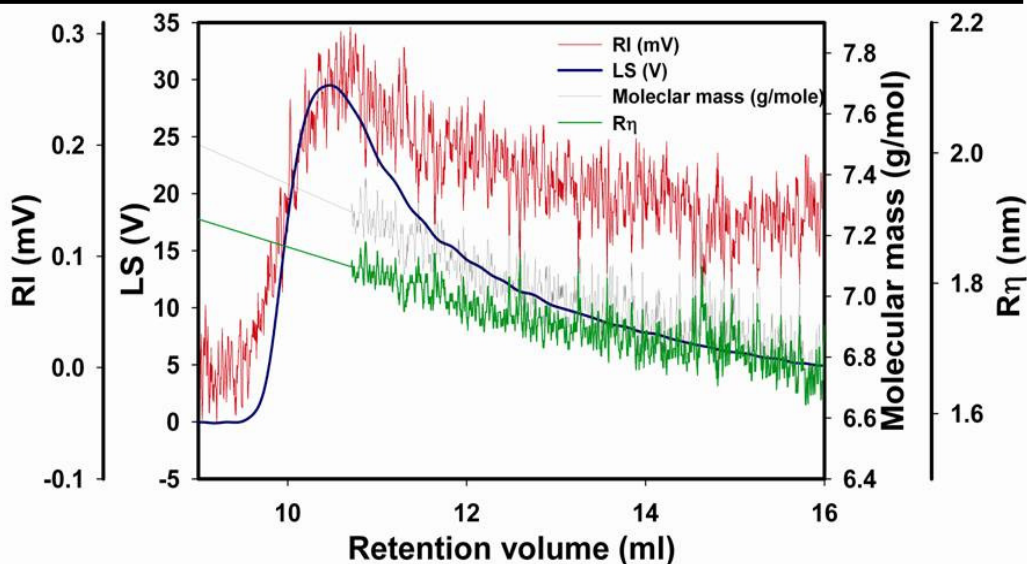


Fig. 6.3.3 Refractive index (RI, black line) and light scattering at 90° (LS 90°, red line) profiles, from HPSEC analysis of *W. confusa* Cab3 dextran. The molar mass distribution and viscometric radius (R_{η}) are shown.

The peak eluting between 17 and 19 minutes is for low molar mass materials according to the HPSEC elution theory (Maina, 2012). The dextran from *W. confusa* Cab3 peaked between 10 to 12 min showing very high molecular weight. Dextran from *W. confusa* E392 has been reported to be of molecular weight 1.473×10^6 g/mol (Maina, 2012). An average molecular weight of dextran from *Weissella cibaria* CMGDEX3 was found to be $>2,000,000$ Da (Ahmad *et al.*, 2012). Use of high molecular weight microbial dextran with low degree of branching is preferable in sourdough baking (Maina *et al.*, 2008). The average molecular weight of dextran produced by *Leuconostoc mesenteroides* NRRL B512F was determined in the range of 9×10^6 to 500×10^6 Da (Antonini *et al.*, 1964). The molecular size of the EPS from *Weissella confusa* Cab3 can be controlled by changing the culture parameters. There are several reports for the controlled biosynthesis of dextran (Remaud-Simeon *et al.*, 2000; Brison *et al.*, 2010). The clinical grade dextran can be produced from high molecular weight dextran from *Weissella confusa* Cab3 by controlled acid hydrolysis.

Robyt and Taniguchi, 1976 produced low molecular acceptor dextran by controlled acid hydrolysis method.

Table 6.3.1. Macromolecular properties of dextran from *Weissella confusa* Cab3 determined with HPSEC.

Sample	$M_w \times 10^6$ (g/mol)	M_w/M_n	R_h (nm)	R_g (nm)
<i>Weissella confusa</i> Cab3	17.72	1.31	63.65	90.8
<i>Weissella confusa</i> E392*	1.473	1.08	24.0	51.0
<i>Leuconostoc citreum</i> E497*	1.849	1.05	29.0	78.0

*Maina, 2012

To assess the variation in dextran length from *Weissella confusa* Cab3 polydispersity index was determined. The width of a polymer's molecular weight distribution is estimated by calculating its polydispersity index (M_w/M_n). The closer value of polydispersity index to 1 suggests that the molecular weight distribution of the polymer is narrow. If all of the polymer molecules in a polymer sample are of the same molecular size, then all of the molecular weight averages would be identical. However, this is the ideal hypothetical condition which can not be achieved in practice generally. The molecular population having $M_w/M_n < 1.5$ are considered to be distributed over relatively narrow molecular weight distribution (Matyjaszewski, 1998). Polydispersity (M_w/M_n) of naturally occurring polysaccharides vary from 1.1 to 35 (Chuang, 1990; Williams *et al.*, 1994). The polydispersity index (M_w/M_n) of dextran from *Weissella confusa* Cab3 was calculated to be 1.31 by HPSEC analysis. This observation suggests that the molecular distribution of dextran molecules from *Weissella confusa* Cab3 was relatively narrower. However, the polydispersity index (M_w/M_n) of dextran from *Weissella confusa* Cab3 (1.31) was relatively higher than that observed in dextrans molecules from *Weissella confusa* E392 and *Leuconostoc citreum* E497 which yielded the polydispersity index (M_w/M_n) of 1.08 and 1.05,

respectively. The higher polydispersity index (M_w/M_n) of dextran *Weissella confusa* Cab3 may be contributed to its very high molecular weight (1.77×10^7 g/mol) in respect to the molecular weights of the dextran from *Weissella confusa* E392 (1.47×10^6) and *Leuconostoc citreum* E497 (1.85×10^6). Grun *et al.* (2005) demonstrated that the α -glucan of strain FH023 was distributed in a relatively narrow molecular mass distribution having a polydispersity index of 2.41.

The molecular dimensions of dextran are dependent on solvent system, concentration and molecular weight (M_w). Dextran behave as flexible, slightly branched random coils in dilute solutions (Nordmeier, 1993). Dextran coils are rather compact in poor solvents, but they expand considerably in a good solvent such as water, methyl sulphoxide or formamide (Basedow and Ebert, 1979; Snabre *et al.*, 1985). Above M_w 2000 kDa, dextran behaves as an expandable coil in solution (Granath, 1958). The hydrodynamic radius (R_h) and radius of gyration (R_g) of dextran molecule from *Weissella confusa* Cab3 in DMSO based solvent were 63.65 nm and 90.8 nm, respectively as calculated by HPSEC. Hydrodynamic radius (R_h) and radius of gyration (R_g) of dextran from *Weissella confusa* Cab3 were very high as compared to the dextran molecules from other lactic acid bacteria viz. *Weissella confusa* E392 and *Leuconostoc citreum* E497 as listed in Table 6.3.1. Higher R_h and R_g values also reflected the high molecular weight of dextran from *Weissella confusa* Cab3.

6.3.2.4 Dynamic light scattering (DLS) of dextran from *Weissella confusa* Cab3

Dynamic light scattering (DLS) analysis of dextran is shown in Fig. 6.3.4. The dextran eluted as a single peak showing that the dextran molecules were not distributed over different molecular mass range. The hydrodynamic radius (R_h) of dextran molecule in water based system was 121.4 ± 25 nm. The hydrodynamic radius

(121.4±25 nm) observed in water based system as determined by dynamic light scattering (DLS) was higher than that obtained in DMSO based solvent (63 nm). This variation in the hydrodynamic radius (R_h) may be attributed to the different solvent system used. It has been reported that the aggregates of polysaccharides can be broken into a single one in a powerful solvent such as DMSO, leading to a significant difference in the molecular mass hence in the hydrodynamic radius from that in water (Wang *et al.*, 2009). Polydispersity (%) was calculated as 21% which also showed that the molecular mass of dextran from *Weissella confusa* Cab3 are distributed over relatively narrow molecular distribution range. Therefore, the dextran from *Weissella confusa* Cab3 was considered to be in a monodispersed form.

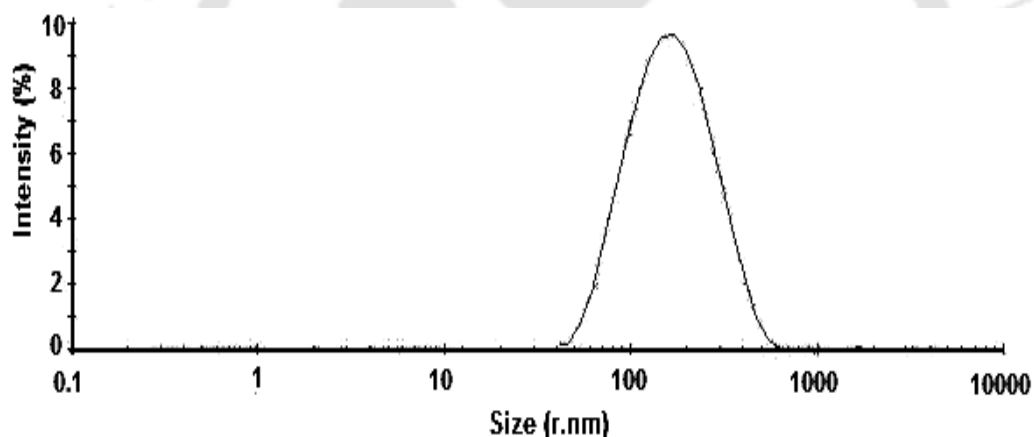


Fig. 6.3.4 Dynamic light scattering (DLS) analysis of dextran from *Weissella confusa* Cab3.

6.3.3 Structural characterization of dextran from *Weissella confusa* Cab3

6.3.3.1. Optical rotation

The dextran synthesized by the isolate *Weissella confusa* Cab3 showed an optical rotation $[\alpha]_D$ of +165° at $C^{0.2}$. It confirmed the *dextro* rotatory property of the dextran.

6.3.3.2 Monosaccharide analysis of dextran

The chromatogram of purified dextran (as described in Section 6.2.6.1) sample hydrolyzed with 0.05 M H₂SO₄ from *Weissella confusa* Cab3 is shown in Fig. 6.3.5.

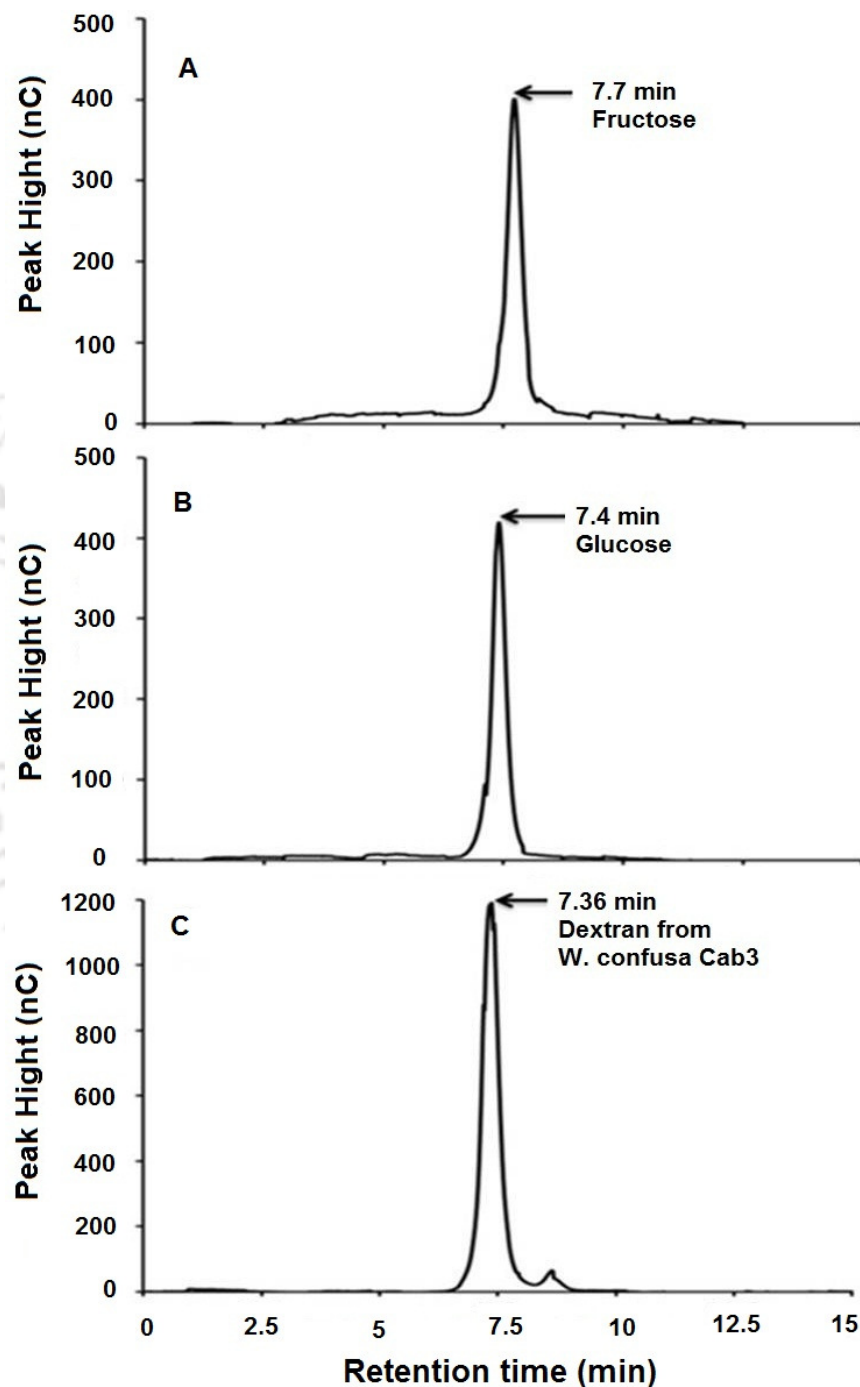


Fig. 6.3.5 Chromatogram of different sugar samples with 250 mM NaOH (A) fructose standard; (B) glucose standard; (C) Hydrolyzed sample of dextran from *Weissella confusa* Cab3.

The chromatogram of fructose and glucose standard (Fig. 6.3.5 A and B) was compared with the hydrolyzed dextran sample (Fig. 6.3.5 C). The retention time of hydrolyzed dextran sample was 7.36 min which is comparable with the retention time of glucose (7.4 min). This study enabled us to establish the fact that the dextran produced was made up of glucose units, hence it could be glucan.

6.3.3.3 Fourier-transform infrared spectrometric (FT-IR) analysis

The FT-IR spectra were used to investigate the functional groups, monomeric units and linkages present in commercial dextrans and pullulans (Shingel, 2002). FT-IR spectrum of the purified dextran from *Weissella confusa* Cab3 is presented in Fig. 6.3.6. It showed bands in the region $950\text{--}1200\text{ cm}^{-1}$. This region is a typical fingerprinting region for all polysaccharides (Cerna *et al.*, 2003). The band at 3394 cm^{-1} represents hydroxyl stretching vibration of the polysaccharide. The band at 2930 cm^{-1} is due to C–H stretching vibration and the band in the region of 1639 cm^{-1} is for presence of bound water. Similar results have been reported earlier (Seymour *et al.*, 1980; Shingel, 2002; Cao *et al.*, 2006; Liu *et al.*, 2007). The transmission peak at 908 cm^{-1} is a characteristic peak for α -glycosidic bond. The band at about 1150 cm^{-1} was assigned to the valent vibrations of the C–O–C bond and glycosidic bridge (Majumder *et al.*, 2009). The peak at 1101 cm^{-1} represents vibration of C–O bond at C-4 position of D-glucose. A similar band was observed at 1103 cm^{-1} in previous reports (Shingel, 2002). The peak at 1022 cm^{-1} indicates great chain flexibility around the α -(1 $\rightarrow$ 6) glycosidic bonds in the FTIR spectrum of dextran from *Weissella confusa* Cab3. Similar finding was observed by Shingel, 2002. The dextran from *Weissella confusa* Cab3 showed band at 1418 cm^{-1} . Similar band at 1416 cm^{-1} is commonly observed in most of the polymers representing the symmetric stretching of --COO (Pongjanyakul

and Puttipipatkachorn, 2007). Evidence supporting absence of β -anomeric carbons in the dextran from *Weissella confusa* Cab3 was confirmed by FTIR spectroscopy (Fig. 6.3.6). The peaks at 890 cm^{-1} and 1370 cm^{-1} are typical of β -(1 $\rightarrow$ 3) glucans (Scweiger-Hufnagel *et al.*, 2000) and β -glucans (Gutierrez *et al.*, 1996), respectively. Therefore, the absence of these peaks showed the absence of β -conformation in dextran from *Weissella confusa* Cab3. Therefore, the absence of these peaks showed the absence of β -conformation in dextran from *Weissella confusa* Cab3. Therefore, the dextran from *Weissella confusa* Cab3 was characterized to be made up of α -(1 $\rightarrow$ 6) glycosidic linkages.

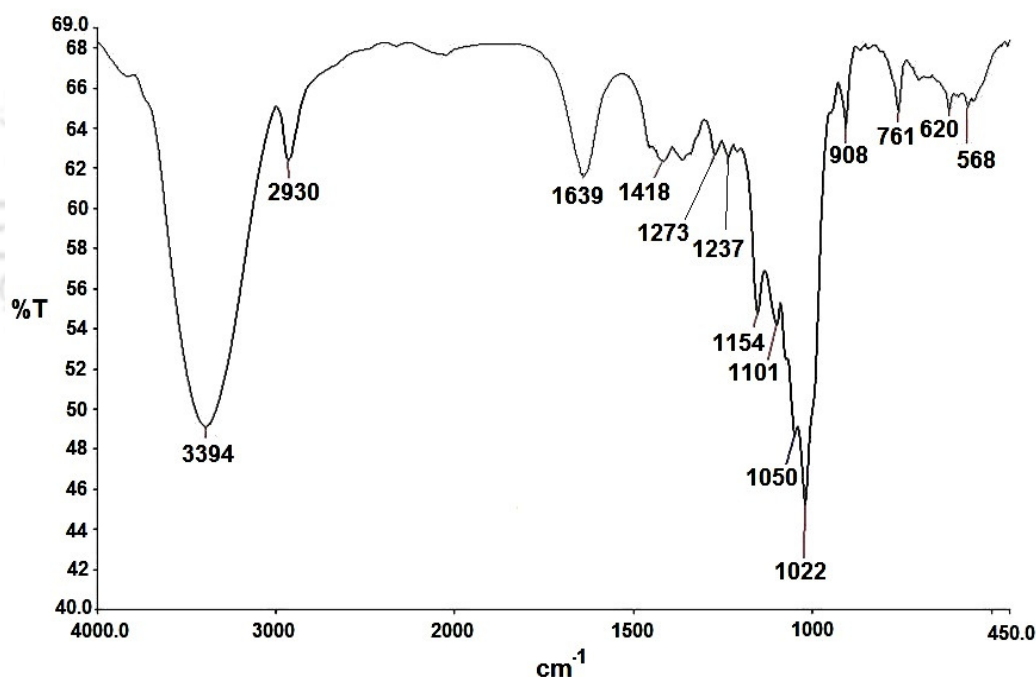


Fig. 6.3.6 FT-IR spectrum of dextran produced from the purified dextranase of isolate *Weissella confusa* Cab3.

6.3.3.4 ^1H nuclear magnetic resonance (NMR) spectral analysis of dextran

The anomeric proton resonances for the dextran produced by the isolate *Weissella confusa* Cab3 is shown in Fig. 6.3.7. The 1D ^1H NMR analysis of the isolate *Weissella confusa* Cab3 showed five spectral resonances at 3.58, 3.73, 3.51,

3.91 and 3.97 ppm corresponded to C-2, C-3, C-4, C-5 and C-6, respectively and an anomeric C-1 signal at 4.97 ppm. These results are in accordance with the earlier findings (Sidebotham, 1974; Seymour, 1979). Various dextran have ^1H NMR spectral resonances (C-2, C-3, C-4, C-5 and C-6) in 3 to 4 ppm region and the hemiacetal C-1 resonance in 4-6 ppm region (Sidebotham, 1974). The ^1H spectrum of dextran from *Weissella confusa* Cab3 showed a typical α -(1 $\rightarrow$ 6) chain-extending anomeric signal centered at 4.97 ppm. An additional low intensity anomeric proton signal at 5.32 ppm (Fig. 6.3.7 and Table 6.3.2) was also observed in ^1H spectrum of dextran from *Weissella confusa* Cab3 which was due to α -(1 $\rightarrow$ 3) linkages. The relative intensities (%) of two peaks labeled as spin system A and B were 96.8% and 3.2%, respectively which reflects that the dextran from *Weissella confusa* Cab3 was comprised of 96.8% linear α -(1 $\rightarrow$ 6) linkages with 3.2% α -(1 $\rightarrow$ 3) linkages. The two anomeric signal at 4.97 and 5.32 named A and B, respectively in the ^1H NMR spectrum of dextran from *Weissella confusa* Cab3 were comparable with the anomeric protons signal at 4.98 and 5.32 ppm of dextran from *Leuconostoc citreum* E497 (Maina *et al.*, 2008). However, ^1H spectrum of dextran from *Leuconostoc citreum* E497 contained four anomeric signals at 4.98, 5.11, 5.18 ppm and a low intensity anomeric signal at 5.32 ppm (Maina *et al.*, 2008). The signals at 5.11 and 5.32 ppm were attributed to α -(1 $\rightarrow$ 2) and α -(1 $\rightarrow$ 3) linked branches, respectively (Maina *et al.*, 2008). In the spectrum of dextran from *Weissella confusa* E392, one additional low intensity branched anomeric signal was observed at 5.32 ppm (2.7%) along with one main anomeric signal at 4.98 ppm (97.3%) (Maina *et al.*, 2008). The dextran from *Weissella cibaria* CMGDEX3 also showed an additional low intensity anomeric signal at 5.32 ppm which was attributed to the presence of α -(1 $\rightarrow$ 3) linked branches (Ahmed *et al.*, 2012). The dextran from *Weissella confusa* Cab3 was analyzed to be

linear dextran made up of 96.8% linear chain of α -(1 $\rightarrow$ 6) linked glucose residues with low degree (3.2%) of α -(1 $\rightarrow$ 3) branching based on ^1H NMR spectroscopy.

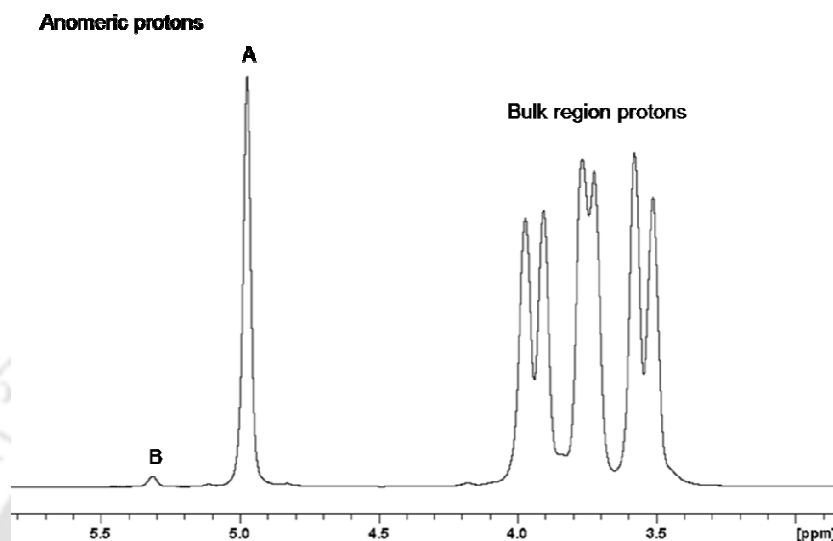


Fig. 6.3.7 The 1D ^1H spectrum of EPS from *Weissella confusa* Cab3 recorded at 600 MHz in D_2O at 50°C . Anomeric protons are labeled A and B according to the increasing chemical shifts. The peaks are referenced to internal acetone ($^1\text{H} = 2.225$ ppm).

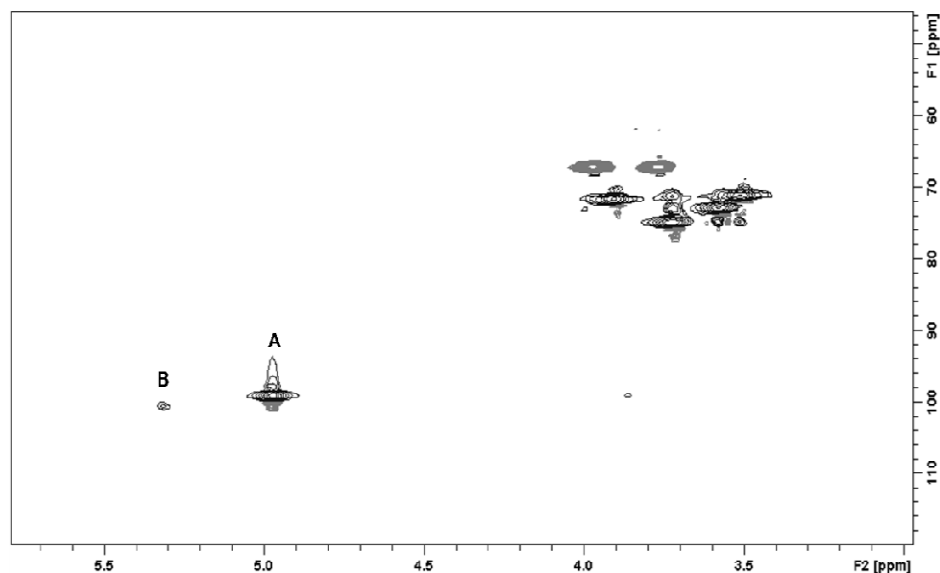
Table 6.3.2 Assignments of ^1H and ^{13}C chemical shifts (ppm) in EPS from *Weissella confusa* Cab3 determined by 1D and 2D NMR experiments recorded at 600 MHz in D_2O at 50°C .

	H-1	H-2	H-3	H-4	H-5	H-6	H-6'
Atoms	4.97	3.58	3.73	3.51	3.91	3.97	3.97
	C-1	C-2	C-3	C-4	C-5	C-6	C-6'
	99.1	72.8	74.7	71.0	71.5	67.1	67.1

6.3.3.5 ^{13}C nuclear magnetic resonance (NMR) spectral analysis of dextran

^{13}C NMR spectroscopy has been used to study the structure of a series of dextran. Carbon chemical shifts of dextran from *Weissella confusa* Cab3 were determined by 2D heteronuclear single quantum correlation spectroscopy (HSQC).

The HSQC spectrum of dextran from *Weissella confusa* Cab3 (Fig. 6.3.8) showed two anomeric ^{13}C chemical shifts at 99.1 ppm and 100.5 ppm corresponding to ^1H anomeric signals at 4.97 ppm (Spin system A), and 5.32 ppm (Spin system B), respectively (Fig. 6.3.8). The anomeric ^{13}C chemical shift at 100.5 ppm was due to the presence α -(1 $\rightarrow$ 3) linkages. Apart from the anomeric signal dextran from the isolate *Weissella confusa* Cab3 showed five ^{13}C NMR resonances at 100 MHz: 72.8, 74.7, 71.0, 71.5 and 67.1 ppm (Fig. 6.3.8 and Table 6.3.2) characteristic of linear dextran. Dextran from *Weissella cibaria* CMGDEX3 showed ^{13}C NMR and ^1H NMR peak at 76.0 ppm and 5.32 ppm, respectively which indicated a branch linkage at C-3 (Ahmed *et al.*, 2012). Dextran have typical ^{13}C anomeric signals (C-1) downfield at about 90 ppm which is characteristic of anomeric carbon C-1 involved in glycosidic bond linked carbohydrates. The chemical shifts for C-2, C-3, C-4 and C-5 appear in the 70-75 ppm region, and C-6 is normally up field at about 60 ppm (Seymour *et al.*, 1976). In present study the major resonance in the anomeric regions at 99.1 ppm rather than at about 90 ppm indicated that the C-1 was linked and the signal at 67.1 ppm rather than at 60 ppm showed that the C-6 was also linked. The absence of chemical shifts downfield of 102.2 ppm, suggested that the biopolymer from *Weissella confusa* Cab3 does not have β -glycosidic linkages. This result was supported by Barbosa *et al.* (2003).



6.3.8 HSQC spectrum of dextran from *Weissella confusa* Cab3 recorded at 600 MHz in D₂O at 50°C. Anomeric correlation peaks are labeled A–B according to the increasing proton chemical shifts. Peaks are referenced to internal acetone (^1H = 2.225 ppm and ^{13}C = 31.55 ppm).

6.3.4 Cytotoxicity and cellular compatibility test of dextran

The cell viability was estimated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described earlier in Section 6.2.4.4. After post treatment, there was no effect of dextran synthesized by *Weissella confusa* Cab3 on the viability of HeLa cells when observed for 48 h even at high concentration of 1500 μg (Fig. 6.3.9). The viability of the all treated cells showed alike results as the control. The lack of any noticeable toxicity of the dextran proved to be highly biocompatible.

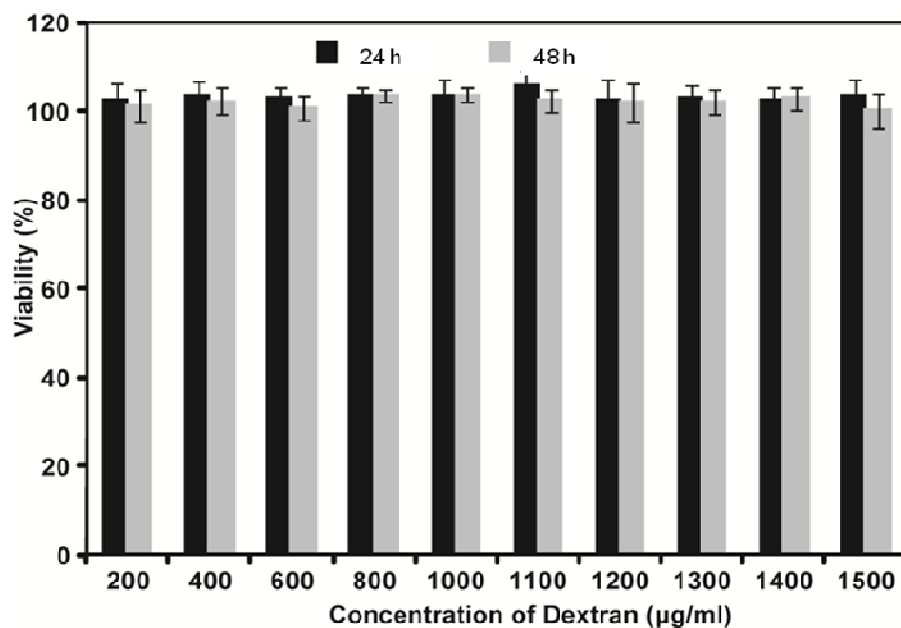


Fig. 6.3.9 The indirect contact based *in vitro* cytotoxicity assay showing the cell viability was unaltered after treatment with various concentrations of dextran (100 µg/ml to 1500 µg/ml) from the natural isolate *Weissella confusa* Cab3 over a period of 24 h to 48 h incubation.

Dextran is known to have wide applications in contact lenses, cell encapsulation, drug delivery and tissue engineering. Also, it can be potentially used as viscosifying and texturizing or gelling agents (Patel *et al.*, 2012). Thus, the cytotoxicity test revealed that the dextran synthesized from *Weissella confusa* Cab3 is biocompatible, non-toxic and is safe for use as a biomaterial for biomedical applications.

6.4 Conclusions

Weissella species are gram-positive, catalase-negative, short rods or coccobacilli, obligately heterofermentative lactic acid bacteria (LAB). Recently *Weissella* sp. has drawn attention for its high dextran production capacity and linearity in its dextran. The dextran from *Weissella confusa* Cab3 was produced by enzyme synthesis method and was purified by ethanol precipitation. The purified dextran contained less than 2% protein. The dextran was physically and structurally characterized. The *dextro* rotatory property of the biopolymer from *Weissella confusa* Cab3 was established by polarimetry. From the rheological study it was observed that dextran at low concentration followed a typical non Newtonian pseudoplastic behavior. Such fluids can be employed in the food industry as gelling, stabilizers or thickening agents. The scanning electron microscopic study revealed the highly porous structure of the dextran which aid in the water holding capacity of dextran. Therefore, dextran can be used as potent hydrocolloid in sourdough and food industries as texturizing and viscosifying agent. Dextran supplementation improves dough stability and gas retention through a structure build up of the dextran and interaction with gluten network. HPSEC analysis of dextran from *W. confusa* Cab3 in DMSO based system gave M_w values of 1.77×10^7 g/mol. The hydrodynamic radius (R_h) and radius of gyration (R_g) of dextran molecule from *Weissella confusa* Cab3 in DMSO based solvent as determined by HPSEC were 63.65 nm and 90.8 nm, respectively. Dynamic light scattering (DLS) analysis of dextran in water based system showed that the dextran was monodispersed with hydrodynamic radius (R_h) 121.4 ± 25 nm. The monosaccharide analysis of the dextran from *Weissella confusa* Cab3 showed only glucose moiety as monomer suggested that the biopolymer produced was glucan. FT-IR analysis helped to identify the functional groups present

in the dextran. The ^1H and ^{13}C NMR spectral analysis of dextran from *Weissella confusa* Cab3 confirmed the presence of dextran with 96.8% linear α -(1 $\rightarrow$ 6) linkages with 3.2% α -(1 $\rightarrow$ 3) branching. The dextran from the isolate *Weissella confusa* Cab3 was non-toxic and biocompatible as evident from the *in vitro* contact based cytotoxicity assay. Therefore, the non-toxic and biocompatible dextran from *Weissella confusa* Cab3 can be used as a biomaterial for drug delivery, tissue engineering and various other biomedical, pharmaceutical and biotechnological applications. The properties like high yield, linear chain with low degree of branching, high-molecular-weight, monodispersity, pseudoplastic behavior and biocompatibility of dextran from *Weissella confusa* Cab3 can be efficiently used to improved rheological (gelling, thickening) or physico-chemical (emulsion stabilisation, particle suspension etc.) properties which are important for food product formulation such as sourdough bread.

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Chapter 7

Sourdough fermentation of wheat bran involving *in situ* dextran Production by *Weissella confusa* Cab3

7.1 Introduction

Sourdough is ancient way to improve flavor, texture and microbiological shelf life of bread and is widely utilized in whole grain rye baking (Lorenz and Brummer, 2003). Cereal fermentation by yeast and lactic acid bacteria (LAB) is commonly referred to as sourdough or yeasted preferment. Cereal fermentations also show significant potential in improvement and design of the nutritional quality and health effects of foods and ingredients (Poutanen *et al.*, 2009).

Several lactic acid bacteria belonging to the genera *Leuconostoc* (Bounaix *et al.*, 2010; Palomba *et al.*, 2012), *Lactobacillus* (Corsetti and Settanni, 2007; Palomba *et al.*, 2012) and *Weissella* (Katina *et al.*, 2009) have been introduced to wheat sourdough baking for *in situ* production of dextran. The addition of sourdough fermented with lactic acid bacteria that synthesize organic acids, oligo and exopolysaccharides (EPS) from sucrose, enhances the texture, nutritional value, and machinability of wheat through a structure build up, rye and gluten-free bread (Galle *et al.*, 2010). The majority of gluten-free breads available are of poor sensory and textural quality. *Weissella* sp. has emerged as potential *in situ* dextran producer in sourdough. Katina *et al.*, 2009 established the potential of *Weissella confusa* VTT E-

90392 in sourdough fermentation. *Weissella confusa* VTT E-90392 produced significant amounts of polymeric dextran and isomaltooligosaccharides in wheat sourdough without strong acidification (Katina *et al.*, 2009). Dextran enriched sourdoughs increased the viscosity that improved the bread quality. Increased release of glucose and fructose from sucrose during fermentation can enhance CO₂ production by yeast. Dextran formed during sourdough fermentation can mask the effect of the organic acids leading to a softer crumb in the fresh and stored bread. In addition to this dextran production can improve dough stability interacting with the dough gluten network (Waldherr and Vogel, 2009).

Celiac disease is a chronic inflammatory disorder which is characterized by damage of the small intestinal mucosa. This disease is caused by the gliadin fraction (protein) of wheat gluten and similar alcohol-soluble proteins (prolamins) of barley and rye (Maki and Collin, 1997; Fasano and Catassi, 2001). This disease can be controlled by maintaining a strictly gluten-free diet. Therefore, gluten free bread production with good texture and softness is becoming a challenge now a days. The *in situ* dextran production in sourdough can overcome the requirement of gluten in bread in order to maintain the same texture and sensory qualities of bread. Bran sourdough enriched with dextran can effectively compensate the negative effect of added wheat bran on bread volume and shelf life in high-fibre baking (Katina, 2005). Sourdough has been used as nutritionally superior raw materials for all cereal foods (Katina, 2005). The sourdough fermentation improves the nutritional value of the cereal products such as rye, oat and wheat products (Liukkonen *et al.* 2003, Kariluoto *et al.*, 2004).

In the present study, the impact of *in situ* dextran production from *Weissella confusa* Cab3 on sourdough fermentation of wheat bran was studied. In Chapter 6, the

physiological, rheological and biochemical properties of dextran from *Weissella confusa* Cab3 were studied. The properties like high yield, linear chain with low degree of branching, high-molecular-weight, monodispersity, pseudoplastic behavior and biocompatibility of dextran from *Weissella confusa* Cab3 seemed very promising for its application in sourdough fermentation as reported in Chapter 6. Therefore, *Weissella confusa* Cab3 was used for sourdough fermentation of wheat bran for *in situ* dextran production. For sourdough preparation *Weissella confusa* Cab3 was added to wheat bran. *Weissella confusa* Cab3 produced significant amount of dextran in sourdough which improved the texture of the wheat bran. Therefore, *in situ* dextran from *Weissella confusa* Cab3 could be used as a potent replacer of hydrocolloids in bread baking.

7.2 Materials and Methods

7.2.1 Chemicals and reagents

All the chemicals required for buffer preparation were of high purity grade available commercially. The enzymes endodextranase from *Chaetomium erraticum* (Sigma-Aldrich, Germany) and α -glucosidase from *Bacillus stearothermophilus* (Megazyme, Ireland) were used. Reagents required for maintenance medium were of high purity grade (as described in Chapter 2, Section 2.2.3).

7.2.2 Microorganisms and culturing conditions

The bacterial strain *Weissella confusa* Cab3 (Genbank Accession Number JX649223) isolated from fermented cabbage (Shukla and Goyal, 2011) was used for sourdough experiment. The organism was maintained in MRS medium (DeMan *et al.*, 1960) stabs incubated at 25°C, stored at 4°C and subcultured every two weeks. The MRS medium composition has been described in Chapter 2, Section 2.2.3.

7.2.3 Bran fermentation and texture analysis

Weissella confusa Cab3 was cultivated twice in succession in MRS broth (Oxoid) with 2% (w/v) sucrose and grown at 25°C for 20 h for sourdough fermentations. The cells were harvested from the liquid cultures by centrifugation (8000g, 15 min, 4°C), washed once with sterile saline water and re-suspended in sterile tap water for a cell density of 5×10^8 - 1×10^9 cfu per ml. The sourdough was prepared by mixing 480 g of tap water, 1200 g of wheat bran, 120 g of sucrose (10%, w/w of wheat bran) and a cell suspension (target count 10^6 - 10^7 cfu/g dough) of *Weissella confusa* Cab3 in a large beaker (5000 ml) and covered with aluminium foil. Bran was kept for fermentation at 25°C for 20 h.

7.2.4 Microbial growth and pH in sourdoughs

Samples (10 g) were taken before and after fermentation of wheat bran and analyzed for viable cell counts of *Weissella confusa* Cab3 as described by Katina *et al.*, 2007. Dextran producing *Weissella confusa* Cab3 were enumerated by counting the number of dextran forming colonies grown on MRS agar with 2% (w/v) sucrose. Sourdough samples were analyzed for pH before and after fermentation according to Katina *et al.* (2006). The pH value was measured from an aliquot of 50 g of sourdough directly by pH meter.

7.2.5 Texture analysis of wheat bran sourdough

Sourdough is traditionally one of the key methods for enhancement of the flavor (Katina *et al.*, 2006). The texture change in wheat bran before and after 20 h fermentation by *Weissella confusa* Cab3 was visually examined by noticing the slime production and increase in viscosity of sourdough.

7.2.6 Preparation of sourdough samples for *in situ* dextran and free sugars analyses

50 g samples from sourdough (wheat bran) before and after fermentation were stored at -20°C in a deep freezer. For the analyses, the sourdough samples were lyophilized (Christ, Alpha 2-4 LD plus) and further homogenized. These powdered sourdough samples were processed for *in situ* dextran and sugar analyses.

7.2.7 Enzyme-assisted method for dextran analysis of sourdough samples

The amount of dextran produced during sourdough fermentation was quantified by an enzyme-aided assay method developed by Maina *et al.*, 2008. The principle of enzyme aided methodology was to specifically hydrolyze dextran

produced *in situ* during sourdough fermentation, by involving two dextran hydrolyzing enzymes *viz.* endodextranase and α -glucosidase. The details of the dextran analysis by enzyme-aided assay method are given in the following sections:

7.2.7.1 Dextran hydrolyzing enzymes

Endodextranase from *Chaetomium erraticum* and α -glucosidase from *Aspergillus niger* were used in a synergistic manner to hydrolyze *in situ* dextran produced in sourdough samples. The enzymatic activity of the endodextranase was determined by applying a similar method to the one used for β -glucanase activity (Zurbriggen *et al.*, 1990). In the endodextranase assay, a 1% solution of commercial dextran in sodium citrate buffer (pH 5.5) was used as a substrate. The measured activity of the *C. erraticum* endodextranase was 1,95,808 nkat/ml. No activity of the *C. erraticum* endodextranase could be detected in control sample (wheat bran) towards barley β -glucan or starch, thus the endodextranase was specific to dextran hydrolysis. The activity of the α -glucosidase (16,670 nkat/ml) was provided by the manufacturer. According to the manufacturer, α -glucosidase from *A. niger* is able to hydrolyse α -(1 $\rightarrow$ 2), α -(1 $\rightarrow$ 3), α -(1 $\rightarrow$ 4) and α -(1 $\rightarrow$ 6) glucosidic linkages in the corresponding disaccharides.

7.2.7.2 Quantification of *in situ* dextran production in sourdough

1. 100 mg lyophilized homogenized flour (as described in section 7.2.6) were mixed in duplicate with 6 ml of 50%, v/v ethanol (to remove free sugars and short oligosaccharides, which could interfere with the assay by increasing the level of background glucose) vortexed and placed in a boiling water bath for 5 min.

2. The samples were centrifuged at 8000g at 25°C for 10 min to separate the free sugars already present in the sample.
3. Supernatants containing free sugars were discarded and the pellets containing dextran and wheat bran were resuspended in 4.5 ml sodium citrate buffer (50 mM, pH 5.5).
4. For the enzymatic hydrolysis of dextran to release free sugars, endodextranase from *Chaetomium erraticum* (10,000 nkat/g) and α -glucosidase from *Aspergillus niger* (1000 nkat/g) were added to solution (4.5 ml) containing dextran.
5. In another set of solution (4.5 ml), only α -glucosidase from *Aspergillus niger* was added to a final concentration of 1000 nkat/g. This was considered as control sample of the dextran-containing sourdough.
6. The solutions (4.5 ml) containing enzymes were incubated in a waterbath with mixing at 30°C for 48 h.
7. After incubation, the solutions (4.5 ml) were kept in a boiling waterbath to inactivate the hydrolytic enzymes.
8. The solutions (4.5 ml) were centrifuged at 8000g at 25°C for 10 min. The supernatant contained the total released glucose by the action of endodextranase and α -glucosidase. Whereas, the pellet containing wheat bran material were discarded.
9. The total glucose concentration released from *in situ* dextran in the supernatant was determined by HPAEC-PAD analysis (as described in Section 7.2.7.3).

7.2.7.3 HPAEC-PAD analysis of free sugars

The HPAEC-PAD equipment consisted of an autosampler (Waters 2707, Milford, MA, USA), three HPLC pumps (Waters 515, Milford, MA, USA), an analytical CarboPac PA-1 column (250 x 4 mm, i.d, Dionex, Sunnyvale, CA, USA) and a pulsed amperometric detector (PAD) (Waters 2465, Milford, MA, USA). The analyses of sugar containing samples were carried using a 200 mM NaOH milli-Q-water gradient at a flow rate of 1 ml/min as described by Rantanen *et al.*, 2007. The samples were filtered using a 0.45 mm nylon membrane and the injection volume was 10 µl. D-glucose, D-fructose, sucrose, maltose in five concentrations (for each sugar) were used as standards (Table 7.2.1) for identification of respective compounds in the samples.

Table 7.2.1 Concentration range of standard sugars for HPAEC-PAD analysis

Standard sugar	Point 1	Ponit 2	Point 3	Point 4	Point 5
Sucrose (µg/ml)	5.0	25.1	100.2	250.5	450.9
Glucose (µg/ml)	3.0	15.1	50.3	100.6	176.1
Fructose (µg/ml)	5.0	25.0	75.0	175.0	300.0

7.2.7.4 Calculation of dextran concentration in sourdough

Dextran concentration (% w/w) in the sourdough samples were calculated according to the following equation:

$$\text{Dextran concentration} = \frac{(\text{GlcA}-\text{GlcB}) \text{ mg/ml} \times 4.5 \text{ ml} \times 0.9 \times 2}{100 \text{ mg}} \times 100$$

Where,

GlcA = Glucose concentration in sourdough sample treated with both enzymes (endodextranase and α-glucosidase).

GlcB = Glucose concentration in control sourdough sample treated with α glucosidase to correct background sugars.

In the dextran concentration calculation, 0.9 is a correlation factor for anhydro-glucose and 2 is a correction factor determined from the recovery of dextran added to model doughs (Katina *et al.*, 2009).

7.2.8 Analysis of free sugars in sourdough samples

The free sugars (sucrose, glucose and fructose) in the sourdough samples (as mentioned in Section 7.2.6) were determined according to Katina *et al.*, 2009. The steps followed for the free sugar analysis are as follows:

1. 100 mg wheat bran from each sample was weighed in an eppendorf tube. 1350 μ l of distilled water was added to the tube and the tube was vortexed properly to mix the content.
2. Each sample was boiled for 10 min and again vortexed properly.
3. After boiling, all samples were centrifuged at 12000g at 25°C for 10 min.
4. Viscous supernatants containing free sugars as well as dextran produced during sourdough fermentation were filtered using 0.5 ml centrifugal filters (Amicon Ultra). Each sample was diluted (25x) and analyzed for sugar contents by HPAEC-PAD analysis (as described in Section 7.2.7.3).

7.2.9 Analysis of oligosaccharides in sourdough samples

Presence of oligosaccharides in sourdough sample after 20 h of fermentation by *Weissella confusa* Cab3 was carried out. The sourdough samples were prepared in a similar manner as followed for free sugar analysis in Section 7.2.8. However, the sourdough samples were analyzed for the presence of oligosaccharides by HPAEC-PAD analysis using a carboPak PA-100 column as described in Chapter 8, Section 8.2.6.

Results and Discussion

7.3.1 Growth of *Weissella confusa* Cab3 and pH in sourdough

The comparison of cell count and pH before and after fermentation of wheat bran by *Weissella confusa* Cab3 are listed in Table 3.3.1. The initial count of *Weissella confusa* Cab3 in wheat bran was in the range 1.8×10^6 cfu/g. After 20 h fermentation *Weissella confusa* Cab3 multiplied efficiently and the cell count increased to 2.1×10^8 (Table 7.3.1). Lactic acid bacteria produce organic acid (acetic acid, propionic acid and lactic acid) which aid in the decrease of pH during wheat bran fermentation. The pH values of sourdough after 20 h of fermentation was pH 5.43 (Table 7.3.1). Mildly acidic (pH 4.9–5.1) dextran enriched sourdough can be used at high addition level (43%, w/w) in wheat baking without negative impact in flavor and texture of bread (Katinal *et al.*, 2009). Therefore, the mild acidic (pH 5.43) sourdough fermented by *Weissella confusa* Cab3 can be efficiently be used at high addition level in bread making.

Table 7.3.1 Bacterial cell counts and pH before and after sourdough fermentation.

Growth parameters	Before fermentation	After fermentation
Bacterial cell count (cfu/g)	1.8×10^6	2.1×10^8
pH	6.65	5.43

7.3.2 Fermentation induced texture changes in wheat bran sourdough

Weissella confusa Cab3 induced significantly change in the texture of the wheat bran after 20 h of fermentation at 25°C. After fermentation, the texture of the sourdough became very smooth, viscous and homogenous (Fig. 7.3.1). As shown in Fig. 7.3.1B, the slime formation occurred due to subsequent dextran production. It has been reported that in bread baking, the removal of wheat gluten in order to maintain

good bread texture and for retention of softness during storage is a challenge. The sourdough enriched with exopolysaccharide, has been used in baking of gluten-free bread for improving its texture and for delayed staling (Moore *et al.*, 2007; Moore *et al.*, 2008). The use of exopolysaccharide producing bacterial strains has been one way of improving properties in sorghum sourdoughs (Schwab *et al.*, 2008). Therefore, wheat bran enriched with dextran from *Weissella confusa* Cab3 can be used to produce gluten-free bread.

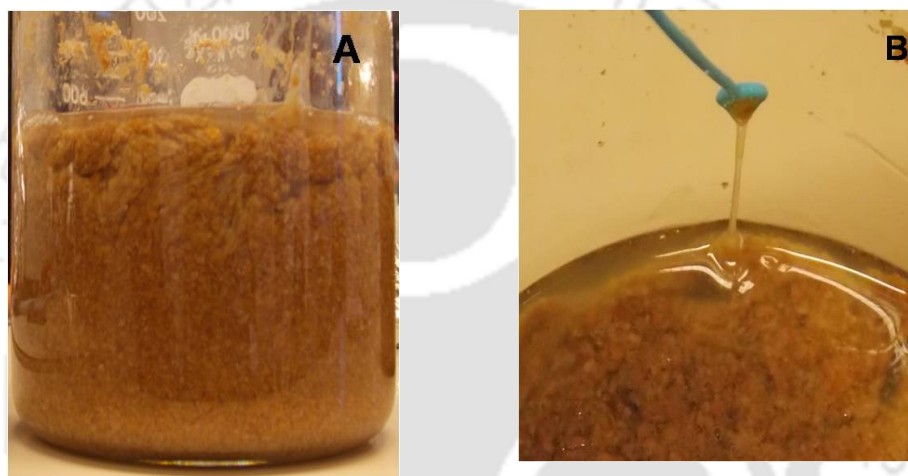


Fig. 7.3.1 Texture change during sourdough fermentation at 25°C for 20 h, involving *Weissella confusa* Cab3. Slime production due to *in situ* dextran production from *Weissella confusa* Cab3 is shown. (A) Texture of wheat bran sourdough; (B) Slime formation due to the production of dextran.

7.3.3 Enzyme-assisted dextran analysis of sourdough samples

The slime production in sucrose supplemented wheat bran sourdough after 20 h of fermentation was due to the production of *in situ* dextran from *Weissella confusa* Cab3. 10% w/w sucrose was added in wheat bran for *in situ* dextran production by *Weissella confusa* Cab3. Fig. 7.3.2A shows that there was no free glucose before sourdough fermentation by *Weissella confusa* Cab3. However, some trace amount of sucrose was present in the sourdough sample before fermentation. After 20 h of

fermentation *Weissella confusa* Cab3 utilized sucrose which was present in the sourdough sample and produced *in situ* dextran and released fructose (Fig. 7.3.2B). The *in situ* dextran production from *Weissella confusa* Cab3 in wheat bran sourdough was determined by the enzyme-aided hydrolysis assay method developed by Maina *et al.*, 2008 (as described in Section 7.2.7). For the dextran analysis, 100 mg sourdough samples were treated with hydrolyzing enzymes viz. endodextranase and α -glucosidase to release the free glucose from dextran as described in Section 7.2.7.2. Dextran with low degree of branching can be hydrolyzed by endodextranase, but highly branched dextran is resistant to endodextranase hydrolysis. Endodextranase hydrolyzes polymeric dextran to release glucose, isomaltose, isomaltotriose and larger isomalto-oligosaccharides. However, some of the oligosaccharides produced by the action of endodextranase which contain α -(1 $\rightarrow$ 2), α -(1 $\rightarrow$ 3), α -(1 $\rightarrow$ 4) glucosidic linkages other than α -(1 $\rightarrow$ 6) linkages are resistant to further hydrolysis by endodextranase (Taylot *et al.*, 1985; Khalikova *et al.*, 2005). Endodextranase can further hydrolyze isomalto-oligosaccharides to release glucose. α -Glucosidase synergistically act along with endodextranase on isomaltose, isomaltotriose and larger isomalto-oligosaccharides to release free glucose. α -Glucosidase can also hydrolyze oligosaccharides which are resistant to hydrolysis by endodextranase action due to presence of α -glucosidic linkages other than α -(1 $\rightarrow$ 6). Therefore, the monomeric glucose was released by the hydrolysis of dextran produced in wheat bran sourdough sample using the synergistic action of endodextranase and α -glucosidase enzyme (Fig. 7.3.2). The free glucose concentration (released from dextran) was determined by HPAEC-PAD analysis (as described in Section 7.2.7.3). The *in situ* dextran content in sourdough was 3.7%, w/w (Table 7.3.2) using the formula as described in Section 7.2.7.4.

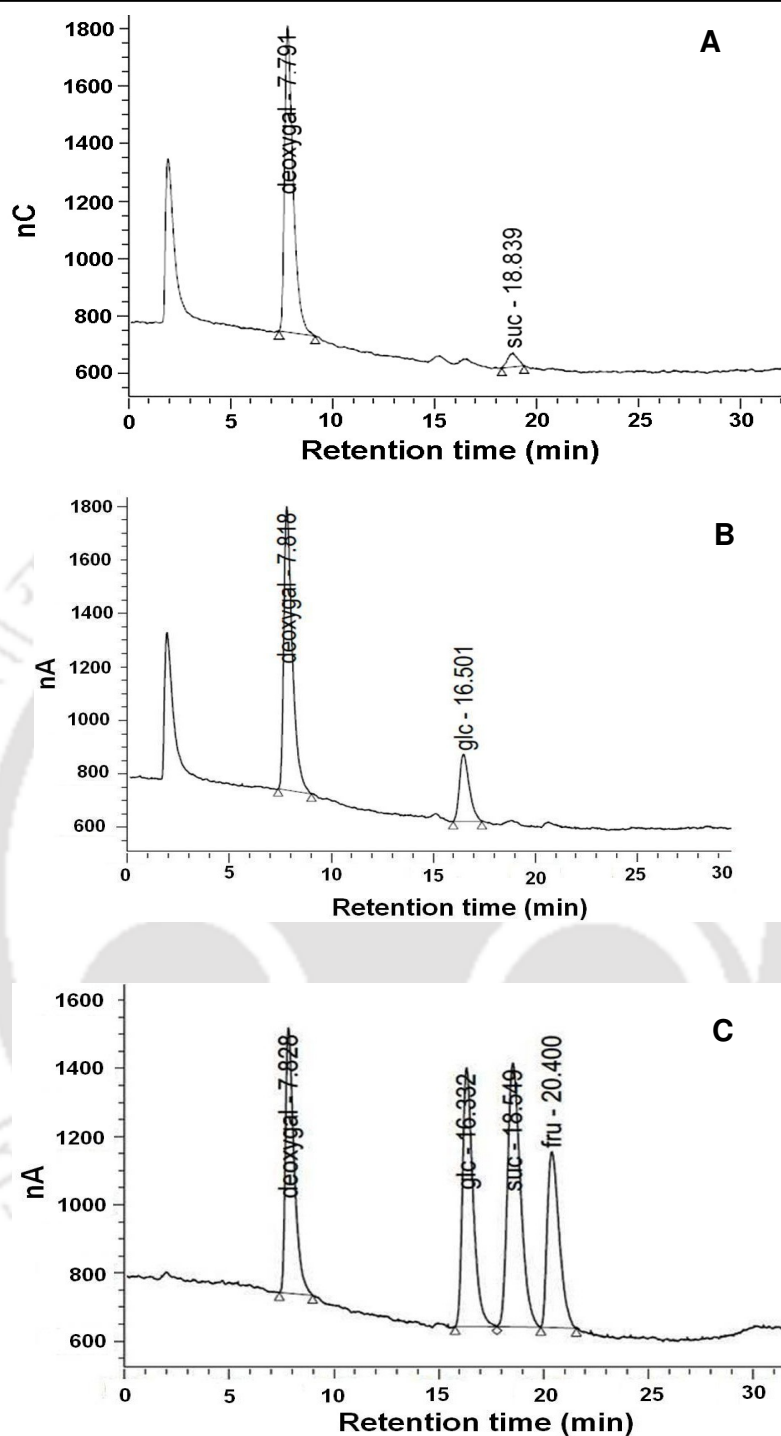


Fig. 7.3.2 Determination of *in situ* dextran in sourdough sample by enzyme assisted method (A) Sourdough sample before fermentation showing added sucrose in wheat bran; (B) Sourdough sample after fermentation showing total released glucose by synergistic action of hydrolyzing enzymes (endodextranase and α -glucosidase) from *in situ* dextran and (C) Standard sugars. (deoxygal: deoxygalactose; glc: glucose; suc: sucrose; fru: fructose).

7.3.4 Analysis of free sugars in sourdough samples

The sucrose consumption during sourdough fermentation by *Weissella confusa* Cab3 was verified by analyzing the free sugars as described in Section 7.2.8. The amount of free sucrose and fructose were analyzed by HPAEC-PAD analysis (Fig. 7.3.3) as described in Section 7.2.7.3. For *in situ* dextran production from *Weissella confusa* Cab3, 10% w/w sucrose was added to wheat bran prior to the fermentation. However, wheat bran also contains considerable amount of sucrose (Saunders *et al.*, 1969). Therefore, total available sucrose in wheat bran was more than 10%, w/w. Analysis of the sourdoughs samples before fermentation showed that there was no anhydro-glucose which might come from the complex nature of the wheat bran. Therefore, it was assumed that all the glucose residues were coming from the enzymatic treatment of dextran. The release of glucose and fructose and consequently dextran production was observed by the enzymatic action of dextransucrase from *Weissella confusa* Cab3. *Weissella confusa* Cab3 efficiently utilized sucrose and consequently produced dextran and released fructose. It was clear from the Fig. 7.3.3A that, before fermentation there was no free mono sugars present in the sourdough but only sucrose was present at higher concentration (11.2%, w/w). During fermentation, *Weissella confusa* Cab3 grew well (as described in section 7.3.1) and produced 3.7% (w/w) dextran (Table 7.3.2). The fructose was released in the sourdough after 20 h of fermentation as shown in Fig. 7.3.3B. The concentration of released fructose in sourdough sample after 20 h of fermentation by *Weissella confusa* Cab3 was 3.8%, w/w (Table 7.3.2).

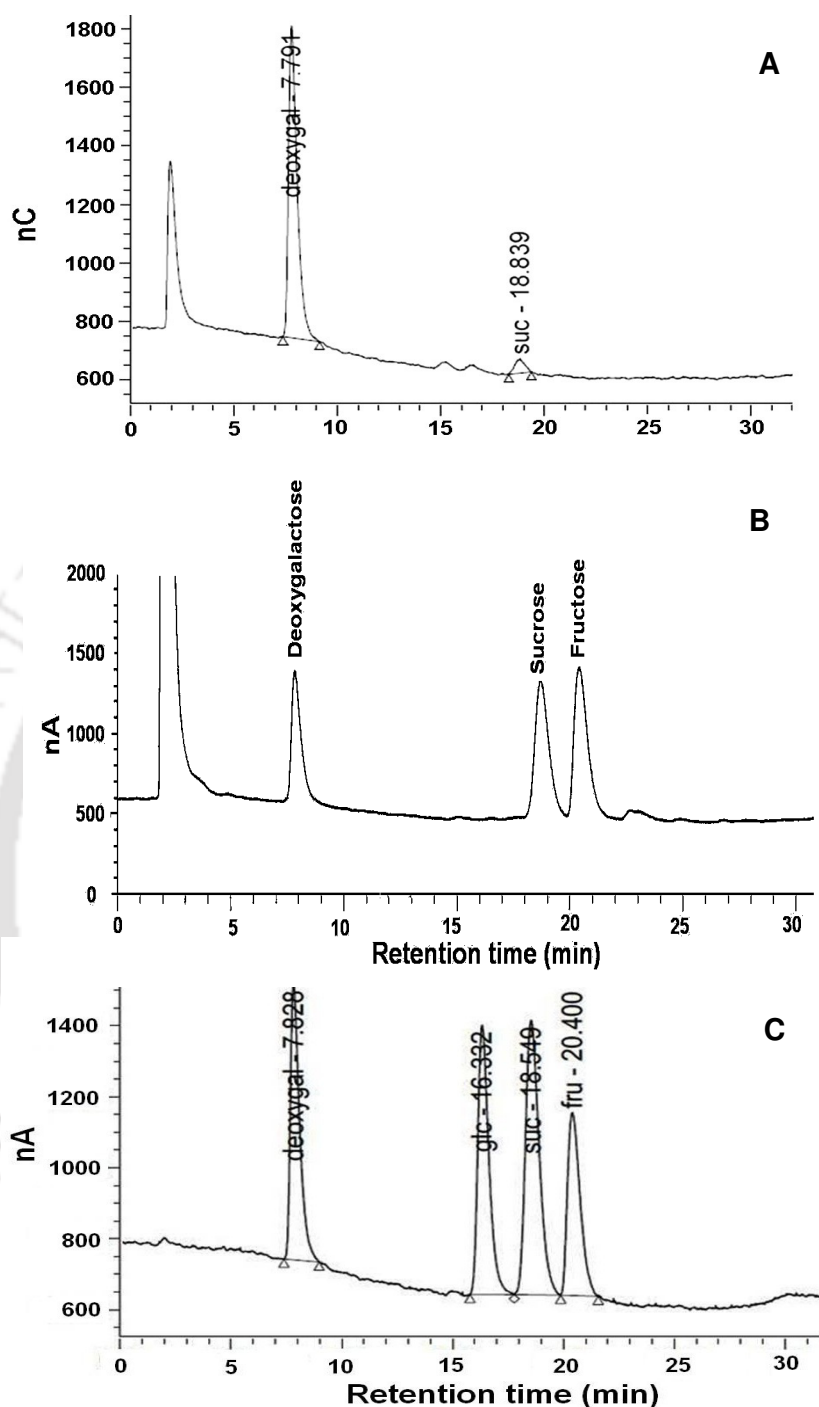


Fig. 7.3.3 Determination of sugar analysis in sourdough sample (A) Sourdough sample before fermentation showing added sucrose in wheat bran; (B) Sourdough sample after fermentation showing released fructose (C) Standard sugars (deoxygal: deoxygalactose; glc: glucose; suc: sucrose; fru: fructose).

Table 7.3.2 Dextran concentration and sugar analysis in sourdough.

Fermentation time (h)	Sucrose (% w/w)	Sucrose consumption (% w/w)	Fructose (% w/w)	Dextran (% w/w)
0	11.2	-	-	-
20	3.5	7.7	3.8	3.7

Weissella confusa E392 is reported to be an efficient producer of dextran (1.8%, w/w) in wheat sourdough *in situ* dextran in sourdough (Katina *et al.*, 2009). *Weissella ciberia* WC 4 is reported to produce 2.5%, w/w dextran in wheat sourdough (Di Cagno *et al.*, 2006). Therefore, *Weissella confusa* Cab3 which produced 3.7%, w/w *in situ* dextran can be considered as a potent *in situ* dextran producer in wheat sourdough.

7.3.5 Analysis of oligosaccharide production in sourdough samples

The amount of maltose in wheat bran was also assayed by HPLC-PAD analysis (Fig. 7.3.4). The absence of maltose peak in sourdough samples before fermentation by *Weissella confusa* Cab3 clearly showed that there was no free maltose present in wheat bran (Fig. 7.3.4A). Therefore, during sourdough fermentation, no oligosaccharide production took place by acceptor reaction of dextransucrase. As shown in Fig. 7.3.4B, no peak for oligosaccharide production could be detected after 20 h fermentation of sourdough by *Weissella confusa* Cab3. However, Katina *et al.*, 2009 showed that in wheat flour, maltose was present in considerable amount, which resulted in the production of oligosaccharides along with dextran during sourdough fermentation by *Weissella confusa* E392 (Katina *et al.*, 2009).

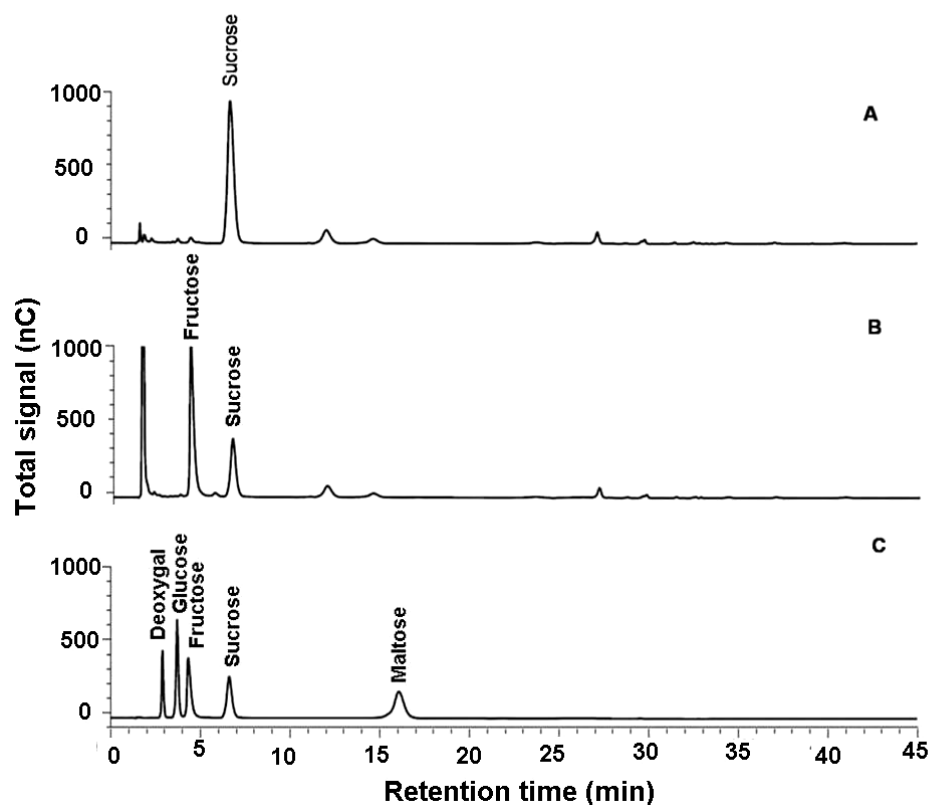


Fig. 7.3.4 HPAEC-PAD profiles of free sugars (Sucrose, glucose and fructose) during sourdough fermentation by *Weissella confusa* Cab3. (A) Before sourdough fermentation; (B) After 20 h of sourdough fermentation; (C) Sugar Standards, from left to right, Dexoygalactose; Glucose; Fructose; Sucrose and Maltose.

7.4 Conclusions

The impact of *in situ* dextran production from *Weissella confusa* Cab3 on wheat bran sourdough fermentation was studied. This study established the potential of *Weissella confusa* Cab3 to produce significant amounts of *in situ* dextran in wheat bran without strong acidification. *Weissella confusa* Cab3 grew well in wheat bran and the pH of the wheat bran sourdough decreased showing the production of organic acids in sourdough. After fermentation of the wheat bran sourdough, the texture of the sourdough was improved. The *in situ* dextran produced in sourdough sample during fermentation by *Weissella confusa* Cab3 was estimated by enzyme assisted hydrolysis method using endodextranase and α -glucosidase. These enzymes hydrolyzed the *in situ* dextran into free glucose residues. The *in situ* dextran content in sourdough sample after fermentation was 3.7%, w/w as determined by enzyme assisted hydrolysis method. *Weissella confusa* Cab3 efficiently utilized sucrose (7.7 %, w/w) and consequently produced significantly higher dextran (3.7 g/g) and released fructose (3.8%, w/w) after fermentation. *Weissella confusa* Cab3 did not produce gluco-oligosaccharides in wheat bran because of the absence of any acceptor molecule such as maltose in the wheat bran.

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Chapter 8

Production of gluco-oligosaccharides by dextransucrase from *Weissella confusa* Cab3 and their purification and characterization

8.1 Introduction

Dextransucrase is a glucosyltransferase (EC 2.4.1.5) which catalyses the synthesis of dextran from sucrose (Hehre, 1955). The mechanism of dextran synthesis by dextransucrase involves a glucosyl-enzyme intermediate (Mooser and Iwaoka, 1989) and polymerization of dextran occurs by an insertion mechanism at the reducing end of the molecule (Robyt *et al.*, 1974; Ditson and Mayer, 1984). In the presence of an auxiliary sugar, called an acceptor, the enzyme not only synthesizes dextran but also catalyses the formation of oligosaccharides by transferring glucosyl residues to the non-reducing end of acceptors (Koepsell *et al.*, 1953; Robyt and Walseth, 1978). Many sugars such as monosaccharide, disaccharide or trisaccharide can act as acceptor molecules. The efficiency of these acceptor molecules varies (Robyt and Eklund 1983; Fu and Robyt, 1990). For example, D-Fructose act as a weak acceptor giving only a single acceptor product, leucrose (Stodola *et al.*, 1956). On the other hand, efficient acceptors such as maltose and isomaltose give a series of homologous oligosaccharides (Robyt and Walseth, 1978).

The average degree of polymerization of the gluco-oligosaccharides (GOS) produced, as well as the amount of dextran formed, can be increased by increasing the sucrose/maltose ratio (Paul *et al.*, 1986; Remaud *et al.*, 1992). Remund *et al.*, 1992 demonstrated that by increasing the sucrose/maltose (S/M) ratio, the synthesis of oligosaccharides occurred with increase in the degree of polymerization. They also showed that by increasing the S/M ratio to 7 branched oligosaccharides were also produced along with the linear oligosaccharides (Remund *et al.*, 1992). There are many reports which show that the GOS obtained from the acceptor reaction of dextransucrase from *Leuconostoc mesenteroides* NRRL B512-F using maltose as acceptor molecule in the presence of sucrose are composed of a maltose residue located at the reducing end and additional glucosyl residues linked by α -(1 $\rightarrow$ 6) linkages (Robyt and Walseth, 1978; Paul *et al.*, 1986). As the result of acceptor reaction of maltose a homologous series of isomalto-oligosaccharides are produced. Panose is the first major component in this series which subsequently act as next acceptor molecule to give next member of the homologous series (Fu and Robyt, 1990). Panose is a trisaccharide containing three glucose moieties with α -(1 $\rightarrow$ 4) and α -(1 $\rightarrow$ 6) linkages (α -D-Glc-(1 $\rightarrow$ 6)- α -D-Glc-(1 $\rightarrow$ 4)-D-Glc), and it is classified as an isomaltooligosaccharide (IMO) (Kohmoto *et al.* 1988). The glucooligosaccharide with degree of polymerisation 3 were produced by microwave assisted hydrolysis of the glucan from *Leuconostoc mesenteroides* NRRL B742 (Majumder *et al.*, 2009). Various oligosaccharides including isomalto-oligosaccharides are widely used for various foods and as feed additive because of their functional properties such as bifidus factor activity and low cariogenic properties. Panose composed of both α -(1 $\rightarrow$ 6) and α -(1 $\rightarrow$ 4) linkages is known to prevent dental caries (Hamada *et al.*, 1984). Morales *et al.*, 2001 studied the prebiotic oligosaccharides produced by the acceptor

reaction of alternansucrase from *Leuconostoc mesenteroides* NRRL B-23192 using cellobiose as an acceptor molecule.

Isomalto-oligosaccharides are also known as prebiotic (Fernandez-Arrojo *et al.*, 2007). The two requirements of a prebiotic are that it is not hydrolyzed in the small intestine and that it is selectively fermented in the colon by certain beneficial members of the colonic microbiota (*Bifidobacterium* and *Lactobacillus* genera). Kohmoto *et al.* 1988 reported that panose was well utilized by *Bifidobacterium adolescentis*, *Bifidobacterium longum*, *Bifidobacterium breve* and *Bifidobacterium infantis*. The physico-chemical properties and the functionality of oligosaccharides depend on their structure. Structural analysis requires determination of monosaccharides, their sequence, type of linkages, branching and anomeric configuration (Patel and Goyal, 2011).

In the present study, the dextransucrase from *Weissella confusa* Cab3 was used for the production of gluco-oligosaccharides using maltose as the acceptor molecule. The gluco-oligosaccharides produced were purified and characterized using various techniques such as Thin layer chromatography (TLC), Fourier transform infrared spectroscopy (FT-IR) High Performance Anion Exchange Chromatography with Pulsed Amperometric Detector (HPAEC-PAD), electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI) and nuclear magnetic resonance (NMR) spectroscopy.

8.2 Material and Methods

8.2.1 Chemicals and reagents

All the chemicals required for buffer preparation were of highest purity grade available commercially. Sucrose and maltose used for the oligosaccharides biosynthesis and standards were purchased from Sigma-Aldrich, USA. The Bio-gel P-2, isomaltose, isomaltotriose and panose were purchased from TCI Europe, Swijdrecht, Belgium. The other standards such as D-glucose and D-fructose were purchased from Merck, Germany. The enzymes used such as *Chaetomium erraticum* dextranase from Sigma-Aldrich (Germany), α -glucosidase from *Aspergillus niger* (Megazyme, Ireland) and α -glucosidase from *Bacillus stearothermophilus* (Megazyme, Ireland) were used.

8.2.2 Microorganism and culture condition

The bacterial strain *Weissella confusa* Cab3 (Genbank Accession Number JX649223) isolated from fermented cabbage (Shukla and Goyal, 2011) was used for the production of gluco-oligosaccharides. The microorganism was maintained in MRS medium (DeMan *et al.*, 1960) slabs incubated at 25°C, stored at 4°C and subcultured every two weeks. The MRS medium composition has been described in Chapter 2, Section 2.2.3.

8.2.3 Enzyme

The 12 h old culture of *Weissella confusa* Cab3 from enzyme production medium as described by Tsuchiya *et al.*, 1952 (as described in Chapter 2, Section 2.2.4) grown at 25°C was used for enzyme purification. Partially purified dextransucrase (10.5 U/mg, 0.84 mg/ml) was obtained by PEG fractionation using

30% PEG-400 (as described earlier in Chapter 4, Section 4.2.7). The dextransucrase (10.5 U/mg) was used for the production of oligosaccharides as described later in Section 8.2.4.

8.2.4 Production of oligosaccharides by acceptor-reaction of dextransucrase

Oligosaccharides were produced by reaction of dextransucrase with sucrose using maltose as acceptor molecule following method of Dols *et al.*, 1998. The reaction mixture (100 ml) contained dextransucrase 12.0 ml (10.5 U/mg, 0.84 mg protein/ml) final 292 mM sucrose and 146 mM maltose in 20 mM sodium-acetate buffer (pH 5.4). The reaction was allowed to proceed at 35°C for 24 h. The reaction was stopped by keeping the reaction mixture in a boiling waterbath for 10 min.

8.2.5 Purification of gluco-oligosaccharides by acceptor-reaction of dextransucrase

The reaction mixture (containing gluco-oligosaccharides) was centrifuged at 8000g at 25°C for 10 min. The absence of pellet indicated the absence of insoluble dextran. The supernatant was subjected to ethanol precipitation with three volume of the pre-chilled ethanol and centrifuged at 12,000g at room temperature for 10 min. The supernatant part (400 ml) contained reducing sugar, unutilized sucrose and acceptor reaction products (gluco-oligosaccharides) whereas the pellet contained soluble dextran. The supernatant containing gluco-oligosaccharides (400 ml) was concentrated to a final volume of 22 ml by rotary vacuum evaporator (Heidolph, laboratory digital 4001). The total carbohydrate content of the concentrated purified gluco-oligosaccharides (22 ml) was determined by phenol sulphuric acid method (Dubois *et al.*, 1956) in a micro-titre plate (Fox and Robyt, 1991) as described in Chapter 5, Section 5.2.5.1.

8.2.6 Analysis of gluco-oligosaccharides by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD)

The gluco-oligosaccharides (18 mg/ml) were analyzed by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD). The analyses were carried out using the oligosaccharide method with a 100 mM NaOH-1M NaOAc (sodium acetate) gradient at a flow rate of 1 ml/min as described by Rantanen *et al.* 2007. The high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) equipment consisted of a autosampler (Waters 717), two pumps (Waters 515 HPLC), an analytical CarboPac PA-100 column (250 x 4 mm, i.d, Dionex, Sunnyvale, CA, USA) and a Decade detector with a gold electrode (Antec Leyden, Zoeterwoude, The Netherlands). The gluco-oligosaccharide mixture was filtered using a 0.45 μm nylon membrane and the injection volume was 10 μl . D-glucose, D-fructose, sucrose, maltose and panose (10 $\mu\text{g/ml}$) were used as standards for identification of respective compounds in the samples.

8.3.7 FT-IR spectroscopic analysis of gluco-oligosaccharides

The FT-IR spectrum of the gluco-oligosaccharides was recorded on a FT-IR spectrometer (Perkin Elmer, Spectrum 2) in transmittance mode over a wavelength range of 4,000 and 400 cm^{-1} . The gluco-oligosaccharides were incorporated with KBr powder and then pressed into pellets.

8.2.8 Oligosaccharide analysis by NMR spectroscopic analysis

The gluco-oligosaccharides solution (as described in Section 8.2.5) was lyophilized for NMR spectroscopic analysis. The lyophilized sample of oligosaccharide was dissolved in D_2O (99.96%) at concentrations of 10 mg/ml. ^1H

and ^{13}C NMR spectra for gluco-oligosaccharide were recorded at 30°C by a 400 MHz NMR spectrometer (Varian, AS400) equipped with VnmrX for Sun Microsystems, California, SA, Ver. 6.1 software (Operating frequencies 400 MHz for ^1H and 100 MHz for ^{13}C NMR). ^1H chemical shifts were referenced to D_2O (4.8 ppm at 30°C).

8.2.9 Enzyme-assisted structural elucidation of gluco-oligosaccharide

To establish the nature of oligosaccharide produced, gluco-oligosaccharides were hydrolyzed with dextranase and two types of α -glucosidase. The enzymatic activity of the dextranase (195808 nkat/ml) was determined using a similar method to the one used for β -glucanase activity (Zurbriggen *et al.* 1990). The activity of the α -glucosidase (16,670 nkat/ml) was provided by the manufacturer. The oligosaccharide solutions (18 mg/ml) in 0.05 mM sodium citrate buffer pH 5.5 were prepared and separately hydrolyzed with dextranase (1380 nkat/g), *A. niger* α -glucosidase (130 nkat/g) and *B. stearothermophilus* α -glucosidase (66 U/g) at 30°C for 48 h. The reaction was terminated by putting the samples in a boiling water bath for 10 min, after which the degradation profile of oligosaccharides in the hydrolysate were determined by high-performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD).

8.2.10 Purification and separation of oligosaccharides by gel permeation chromatography

Oligosaccharides produced by the acceptor reaction of dextranase from *Weissella confusa* Cab3 were purified by gel permeation chromatography (GPC) with biogel P2 column (95 x 5 cm; Biorad, Hercules, CA, USA) using water as eluent. The gluco-oligosaccharides were first filtered with 0.45 μm membrane filters (Acrodisc 13, Pall corporation, Ann Arbor, Michigan, USA) and 3.0 ml of sample (60 mg/ml)

injected into the column. The flow rate was first kept 1.0 ml/min to elute void volume (600 ml) and then adjusted to 0.3 ml/min for collection of 4 ml fractions. The fractions from Biogel P2 column were analyzed for oligosaccharides by HPAEC-PAD as described in Section 8.2.6.

8.2.11 Oligosaccharide analysis by mass spectrometry

Oligosaccharides were analyzed by mass spectrometry using an ESI-TOF-MS Premier mass spectrometer (Waters, Q-TOF Micromass, HAB 273). The mass spectrometer was operated in positive ion mode and prior to analysis was calibrated over the range 100–1400 m/z. Mass spectra were acquired manually over the m/z range 100–1400. ESI/MS data were acquired with the collision energy adjusted in such a way that the majority of the precursor ion was attenuated. Collision energies used were typically between 50 eV and 100 eV.

8.3 Results and Discussion

8.3.1 Production and purification of oligosaccharides

The gluco-oligosaccharides (GOS) by acceptor reaction of dextransucrase were separated from dextran by precipitation using three volumes of 90%, v/v ethanol. The GOS were concentrated to a final volume of 22 ml using a rotary vacuum evaporator and was used further for the structural characterization. The total carbohydrate content of the concentrated GOS in 22 was 600 mg/ml as determined by phenol sulphuric acid method (as described in Chapter 5, Section 5.3.5.1).

8.3.2 Analysis of gluco-oligosaccharides by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD)

Dextransucrase catalyzed the secondary acceptor reaction in presence of maltose along with sucrose. The concentrated mixture containing GOS produced by the acceptor reaction of dextransucrase from *Weissella confusa* Cab3 was analyzed by high performance anion exchange chromatography with pulse amperometric detection (HPAEC-PAD) using CarboPac PA-100 column (as described in Section 8.2.6). The acceptor products of maltose with dextransucrase were identified as panose (6²-O- α -D-glucopyranosyl-maltose) and a homologous series of 6'-isomaltodextrinosyl maltose by HPAEC-PAD analysis. The first molecule of the homologous series of gluco-oligosaccharides produced by the acceptor reaction of dextransucrase from *Weissella confusa* Cab3 was identified as a trisaccharide panose as the first acceptor molecule (based on its retention time) by transferring a glycosyl residue from sucrose to non reducing end of maltose (Fig. 8.3.1A). Panose acted as an acceptor to give 6²-O- α -isomaltosyl-maltose as the second acceptor product, which was subsequently utilized as next acceptor molecule to give the third acceptor product. Thus, a

homologous panose series of oligosaccharides was formed, in which isomaltodextrins of varying lengths are attached to the nonreducing glucosyl residue of maltose.

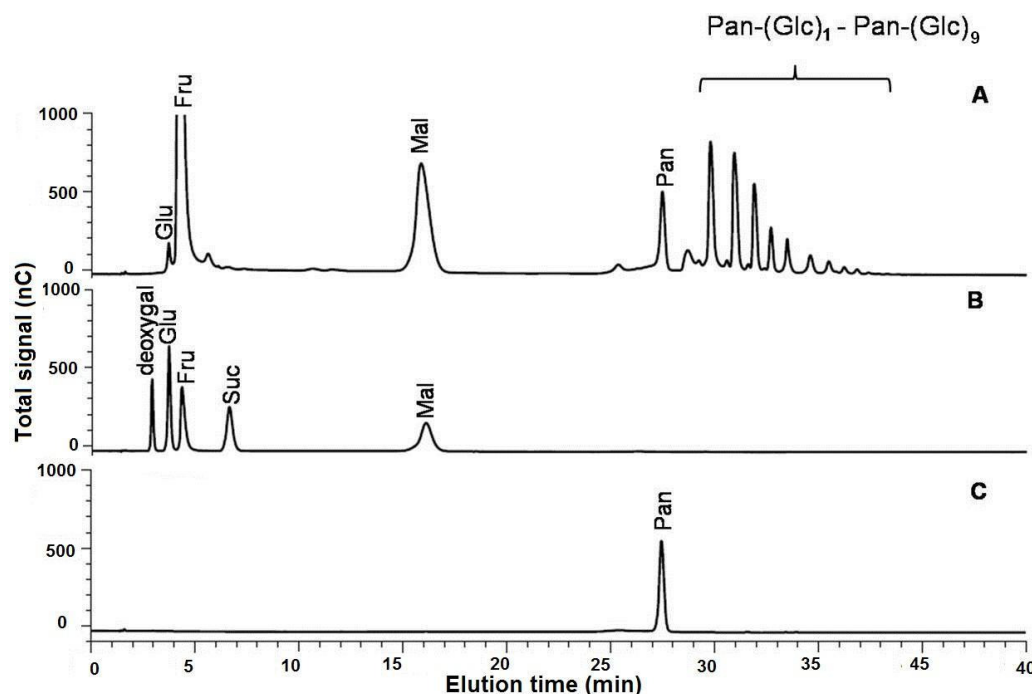


Fig. 8.3.1 HPAEC-PAD chromatograms showing (A) the profile of gluco-oligosaccharide formed by the acceptor reaction of dextran sucrose from *Weissella confusa* Cab3 in presence of 5%, w/v maltose (as acceptor molecule) along with 10%, w/v sucrose, showing Glucose, Fructose, Maltose and the acceptor reaction products (Panose and the homologous series of panose: Pan-(Glc)₁ to Pan-(Glc)₉ ranging from DP-3 to DP-12; (B) Sugar Standards, from left to right deoxygalactose (internal reference), Glucose, Fructose, Sucrose and Maltose, respectively; (C) Panose standard (Glu: glucose; Fru: fructose; Mal: maltose; Pan: panose).

Similar results have been reported by Robyt *et al.*, 1974. From the HPAEC-PAD analysis it was clear that the oligosaccharides (homologous series of panose) ranging from degree of polymerization DP-3 to DP-10 were produced (Fig. 8.3.1). Very small amount of DP-11 and DP-12 were also observed in acceptor reaction of dextran sucrose from *Weissella confusa* Cab3. Sucrose was absent in the chromatogram of acceptor reaction products as observed by comparing with the

sucrose standard. Thus it was concluded that sucrose was completely consumed by dextranucrase however, some maltose was left unutilized as the peak for maltose residue was present in the acceptor reaction products (Fig. 8.3.1A).

8.3.3 FT-IR spectroscopic analysis of gluco-oligosaccharides

FT-IR spectroscopy was used to evaluate conformational properties of the gluco-oligosaccharides produced by the acceptor reaction of dextranucrase from *Weissella confusa* Cab3. The FT-IR spectrum of the gluco-oligosaccharides is shown in Fig. 5. The band in the region of 3380 cm^{-1} was due to the hydroxyl-stretching vibration of the gluco-oligosaccharides. The bands in the region of 2932.5 cm^{-1} was due to C–H stretching vibration, and the bands in the region of 1639 cm^{-1} was due to the associated water.

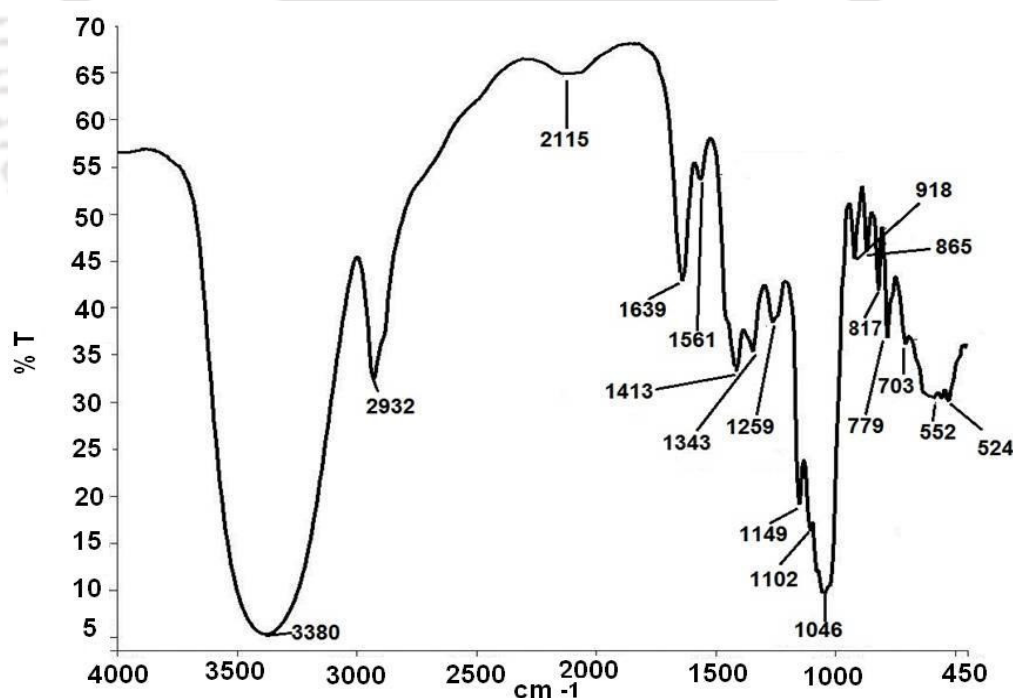


Fig. 8.3.2 FT-IR analysis of gluco-oligosaccharides produced by the acceptor reaction of dextranucrase from *Weissella confusa* Cab3 using maltose as acceptor molecule along with sucrose.

Peak at 1102, 1149, 1343, 1413 cm^{-1} were attributed to the C-O-H bending, C-O, C-C stretching, C-O-H bending and CH_2 twisting, C-O, C-C stretching and CH_2 bending and C-O-O stretching, respectively. Similar bands were observed at 1094, 1163, 1344 and 1415 cm^{-1} , respectively in the FT-IR spectra of the amylose (Cael *et al.*, 1975). The peak at 1259 cm^{-1} was due to the CH_2OH (side chain) related mode as also reported earlier by Cael *et al.*, 1975. The band at 1046 cm^{-1} was mainly due to the vibrational modes of $-\text{CH}_2\text{OH}$ groups and the C-O stretching coupled with C-O bending of the C-OH groups of carbohydrates. Parker (1971) also reported similar observations. The absorptions at 779 and 918 cm^{-1} were typical for the ring vibrations of D-glucose in pyranose form. Similar peaks at 770 and 918 cm^{-1} have been reported earlier (Coral, 1991). Moreover, the characteristic band at 817 cm^{-1} suggested the α -dominating configuration of the gluco-oligosaccharides. FT-IR analysis established the α -dominating configuration and the carbohydrate nature of the acceptor reaction products (gluco-oligosaccharides).

8.3.4 ^1H NMR spectroscopic analysis of gluco-oligosaccharides

The structure of the gluco-oligosaccharides was investigated by ^1H NMR spectroscopic analysis. ^1H NMR spectra of the acceptor reaction products showed anomeric protons in the region 4 ppm to 6 ppm and the bulk region protons were centered in 3-4 ppm which reflected their carbohydrate nature (Fig. 8.3.3). It has been observed that the distribution of ^1H NMR spectral resonances range between 3 and 6 ppm for different oligosaccharides (Seymour, 1979). Goffin *et al.*, 2009 showed that the anomeric signals of various oligosaccharides are found between 4 ppm – 6 ppm and the bulk region protons are located in 3-4 ppm region in ^1H NMR spectra. In the present study, the peaks centered at 4.859 and 5.38 ppm, corresponded to α -(1 $\rightarrow$ 6)

and α -(1 $\rightarrow$ 4) linked glucose units, respectively. This indicated the presence of a maltose residue which contains α -(1 $\rightarrow$ 4) linked glucosyl residues. Similar peaks at 4.93 ppm and 5.38 ppm were also reported in the ^1H NMR spectra of panose (Goffin *et al.*, 2009). Signal at 4.859 ppm, 3.538 ppm, 3.72 ppm, 3.415 ppm, 3.687 ppm and 3.816 ppm, 3.758 ppm corresponded to H-1, H-2, H-3, H-4, H-5, H-6 and H-6', respectively of the non reducing end residue in α -anomeric form. Signal at 5.38 ppm, 3.578 ppm, 3.661 ppm, 3.492 ppm, 3.92 ppm, 3.987 ppm, 3.72 ppm were assigned to H-1, H-2, H-3, H-4, H-5, H-6 and H-6', respectively the central residue in α -anomeric form in a oligosaccharide, connected to the reducing end residue, which may exist in α or β anomeric forms. Similar results have been observed for panose spectra by Goffin *et al.*, 2009. ^1H NMR spectrum showed the gluco-oligosaccharides contained α -(1 $\rightarrow$ 6) and α -(1 $\rightarrow$ 4) linked glucose units.

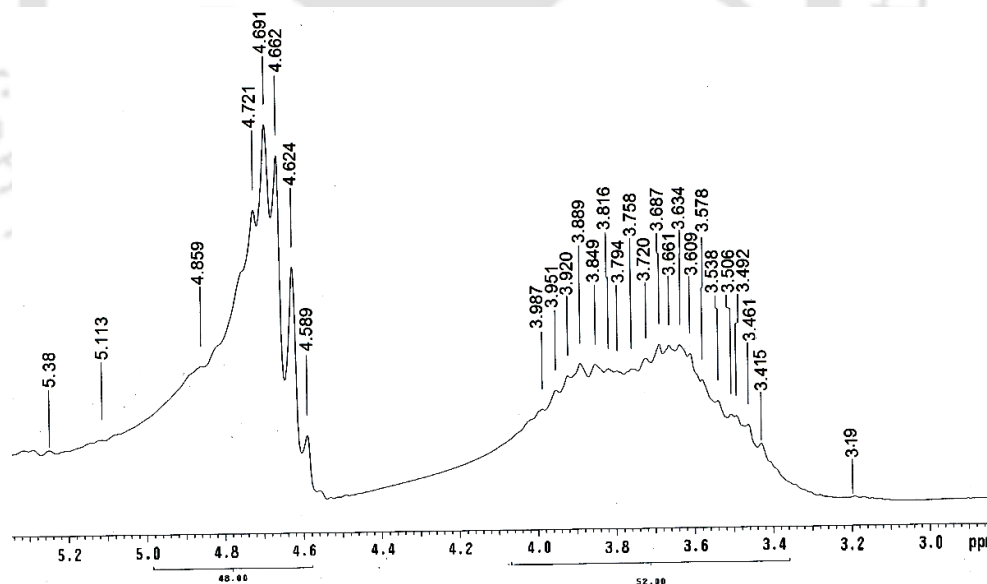


Fig. 8.3.3 ^1H NMR spectrum of the acceptor reaction products (gluco-oligosaccharides) of dextranucrase from *Weissella confusa* Cab3 using maltose as acceptor molecule along with sucrose, recorded at 400 MHz in D_2O at 30°C.

8.3.5 ^{13}C NMR spectroscopic analysis of gluco-oligosaccharides

Structural analysis of gluco-oligosaccharides by ^{13}C NMR showed that they are linked mainly by α -(1 $\rightarrow$ 4) and α -(1 $\rightarrow$ 6) linkages (Fig. 8.3.4). Various signals in the ^{13}C NMR spectra of gluco-oligosaccharides were assigned as described by Seymour *et al.* 1976 and Remaud *et al.* 1992. Peaks in the region 85-105 ppm, are assigned for anomeric region. However, in the present ^{13}C NMR spectra of gluco-oligosaccharides this anomeric region was located between 95-102 ppm because of presence of only trace amount of reducing sugar such as glucose and fructose (Fig. 8.3.4). Two closely separated peaks at 101.68 ppm showed presence of α -(1 $\rightarrow$ 4) linkages in gluco-oligosaccharides produced by the acceptor reaction of dextranase from *Weissella confusa* Cab3 in presence of maltose and sucrose. This peak showed that the α -(1 $\rightarrow$ 4) linkage is located at the reducing end of isomaltosyl residues containing α -(1 $\rightarrow$ 6) linkages. Similar results have been reported earlier (Remaud *et al.*, 1992; Chung, 2002). Goffin *et al.*, 2009 also demonstrated the similar peak at 101.10 ppm in ^{13}C NMR spectrum of panose. The resonance peaks around 99 ppm (99.883 ppm and 98.221 ppm) corresponded to α -(1 $\rightarrow$ 6) linked glucosyl residues. Remaud *et al.*, 1992 also reported that the peaks at 99.1, 98.9, 98.87 and 98.65 ppm in the ^{13}C NMR spectrum of oligosaccharides corresponded to various degrees of polymerization ranging from DP-4 to DP-6. Signal at 95.956 ppm and 92.52 ppm, are the resonance peaks corresponding to the reducing end residue. These chemical shifts are similar to the chemical shift observed in the spectrum of maltose corresponding to the C-1 β and C-1 α . Similar peaks at 96.7 ppm and 92.8 ppm have been reported in branched gluco-oligosaccharides which were corresponding to the C-1 β and C-1 α of the panose molecule from *Leuconostoc mesenteroides* NRRL B-742 (Remaud *et al.*, 1992). The resonance peaks due to bonded C-2, C-3 and C-4 of the glucose residue

and the peaks for C-2, C-3 and C-5 of the β form of the reducing end glucose lie in the range 75 ppm - 85 ppm. Chemical shifts at 75.654, 74.731 and 76.34 correspond to the free carbon C-2 β , C-3 β and C-5 β of glucose residue (β anomer) located at the reducing end. Multiple split signals in the region 60 ppm to 61 ppm correspond with the multiple free C-6 located at the reducing end of the gluco-oligosaccharides. Based on these results it was confirmed that a panose series of gluco-oligosaccharides was produced as the result of acceptor reaction of dextranase from *Weissella confusa* Cab3 in the presence of maltose.

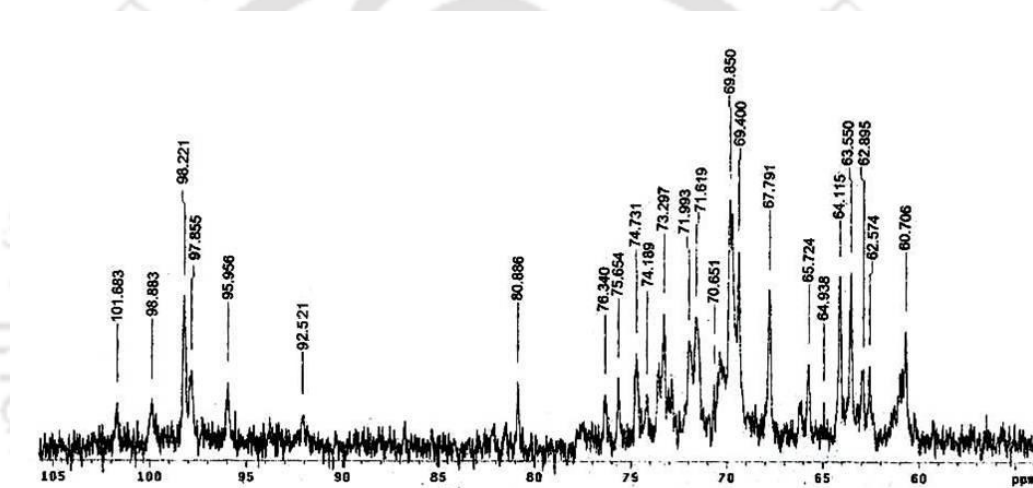


Fig. 8.3.4 ^{13}C NMR spectrum of the acceptor reaction products (gluco-oligosaccharides) of dextranase from *Weissella confusa* Cab3 using maltose as acceptor molecule along with sucrose recorded at 400 MHz in D_2O at 30°C .

8.3.6 Enzyme-assisted structural elucidation of gluco-oligosaccharide

The types of linkage present in gluco-oligosaccharides produced by acceptor reaction product of dextranase from *Weissella confusa* Cab3 were established by enzymatic action of three specific linkage cleaving enzymes. The chromatographic profiles of gluco-oligosaccharides (produced by the acceptor reaction of dextranase from *Weissella confusa* Cab3 in presence of maltose) hydrolyzed by endodextranase from *Chaetomium erraticum*, α -glucosidase from *Aspergillus niger*

and α -glucosidase from *Bacillus stearothermophilus* are shown in Fig. 8.3.5. HPAEC-PAD analysis of oligosaccharides without any enzyme treatment revealed the presence of fructose, maltose and a panose series of oligosaccharides ranging from DP-3 to DP-12 (Fig. 8.3.5A).

Endodextranase hydrolyzes the α -(1 $\rightarrow$ 6) glycosidic linkage in dextran chain. The enzyme cleaves the linkages of the dextran molecule and releases shorter isomaltosaccharides. Endodextranase can also hydrolyze isomaltooligosaccharides from the reducing end to release isomaltotriose (IM₃), isomaltose (IM₂) and glucose. Therefore, gluco-oligosaccharides hydrolyzed with endodextranase from *Chaetomium erraticum* showed glucose, isomaltose (IM₂) and isomaltotriose (IM₃) as main product (Fig. 8.3.5 B). Endodextranase from *Chaetomium erraticum* catalyzed the hydrolysis of linear α -(1 $\rightarrow$ 6) linkages of oligosaccharides from nonreducing end and also from random sites. Therefore, the action of endodextranase resulted in the conversion of gluco-oligosaccharides into IM₂, IM₃ and panose (Fig. 8.3.5B). Hydrolysis of isomaltose is slightly difficult because of initial condensation to isomaltotetraose followed by hydrolysis to glucose and isomaltotriose (Khalikova *et al.*, 2005). However, it could not completely hydrolyze DP-3 and DP-4 which might be due to the presence of α -(1 $\rightarrow$ 4) linkages in the vicinity of α -(1 $\rightarrow$ 6) or due to inadequate enzyme concentration. It has been reported that dextran and oligosaccharides containing non- α -(1 $\rightarrow$ 6) linkages becomes resistant to endodextranase action (Taylor *et al.*, 1985). Maltose remained unhydrolyzed by the action of endodextranase as it contained α -(1 $\rightarrow$ 4) linkage.

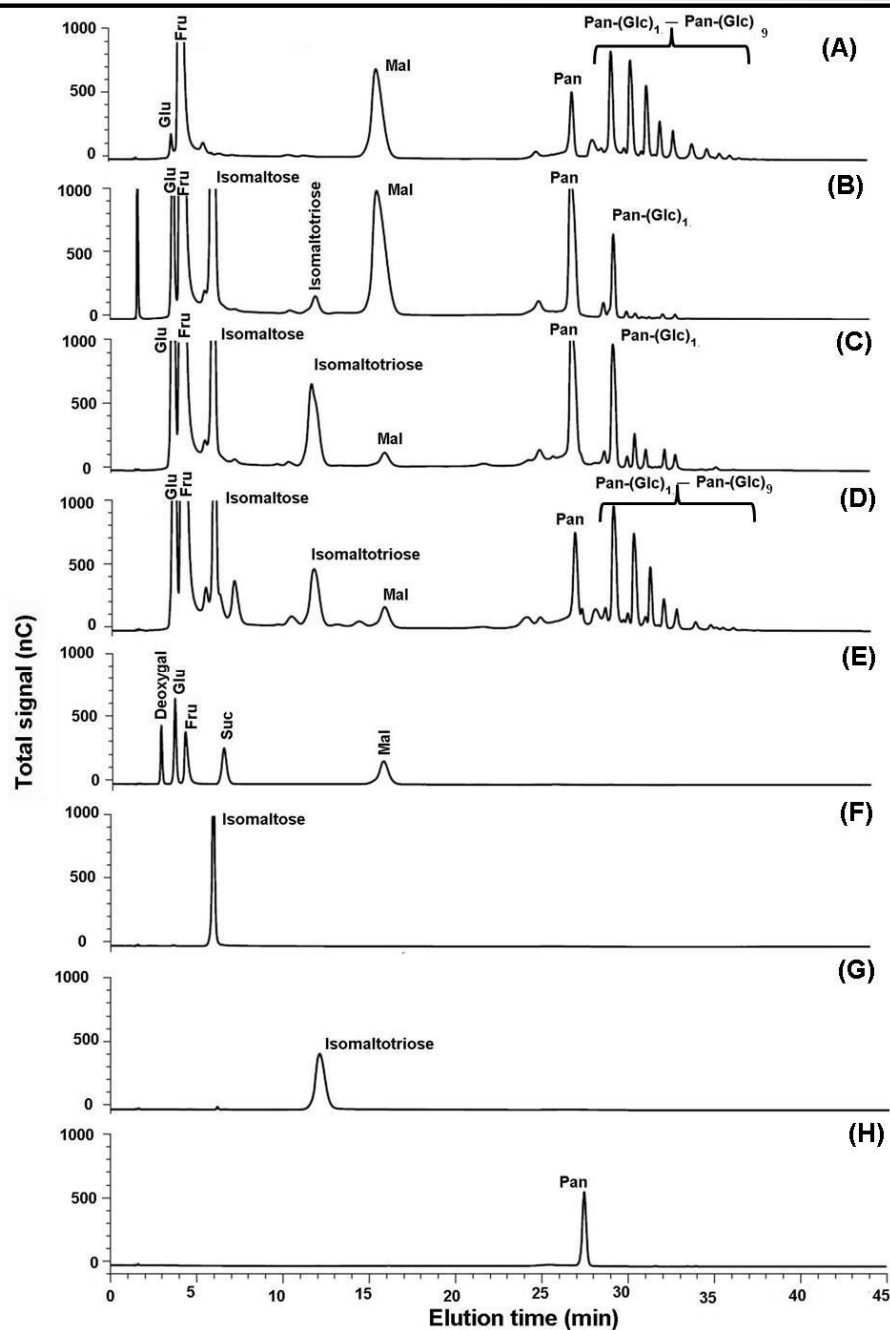


Fig. 8.3.5 HPAEC-PAD chromatograms of (A) Mixture of gluco-oligosaccharides formed by acceptor reaction of dextranase from *Weissella Confusa* Cab3. Hydrolysis products of gluco-oligosaccharides formed by acceptor reaction of dextranase from *W. confusa* Cab3 by (B) Dextranase from *Chaetomium erraticum*; (C) α -glucosidase from *Aspergillus niger* and (D) α -glucosidase from *Bacillus stearothermophilus* (E) Sugar standards from left to right deoxygalactose (internal reference), Glucose, Fructose, Sucrose and Maltose, respectively; (F) Isomaltose standard; (G) Isomaltotriose standard and (H) Panose standard (Glu: glucose; Fru: fructose; Mal: maltose; Pan: panose).

α -Glucosidase from *Aspergillus niger* hydrolyzes terminal α -(1 $\rightarrow$ 6) and α -(1 $\rightarrow$ 4) linked glucosyl residue located towards non reducing end of the carbohydrate. Therefore, its action on gluco-oligosaccharides from *Weissella confusa* Cab3 hydrolyzed both maltose and panose series of oligosaccharides, liberating glucose, IM2 and IM3 (Fig. 8.3.5C). On the other hand, α -glucosidase from *Bacillus stearothermophilus* has only α -(1 $\rightarrow$ 4) activity towards non reducing end of the carbohydrate. So, it can only hydrolyze remaining maltose residues from the acceptor reaction products into free glucose. α -Glucosidase from *Bacillus stearothermophilus* can not hydrolyze the panose series of oligosaccharides because they contain α -(1 $\rightarrow$ 4) linked glucosyl residue at reducing end. It was clear that panose series of oligosaccharide remained unchanged and maltose was cleaved into free glucosyl residues after the enzyme hydrolysis by α -glucosidase from *Bacillus stearothermophilus* (Fig. 8.3.5D). The hydrolysis profile of gluco-oligosaccharides treated with α -glucosidase from *Bacillus stearothermophilus* also showed the presence of IM2 and IM3 (Fig. 8.3.5D) which can not be obtained by the hydrolysis of higher oligosaccharides because this α -glucosidase can not hydrolyze α -(1 $\rightarrow$ 6) linked glucose residues. Therefore, these IM2 and IM3 are produced by the transglycosylation reaction of α -glucosidase. It has been reported that when maltose and oligosaccharides are present in higher concentration, α -glucosidase can also catalyze transglycosilation reaction (Fernandez-Arrojo *et al.*, 2007). Maltose and water residue compete for the active site. Thus, the hydrolysis of maltose and polymerization of IM2 and IM3 remains in equilibrium. The hydrolysis pattern of gluco-oligosaccharides by endodextranase from *Chaetomium erraticum*, α -glucosidase from *Aspergillus niger* and α -glucosidase from *Bacillus*

stearothermophilus showed that the gluco-oligosaccharides contained both α -(1 $\rightarrow$ 6) and α -(1 $\rightarrow$ 4) linkages and maltose residue located towards their reducing end.

8.3.7 Purification and separation of gluco-oligosaccharides by gel permeation chromatography

Dextranucrase catalyzed acceptor reaction appeared as an efficient method for production of oligosaccharides from DP-3 to DP-12. However, the mixture that resulted from the reaction of dextranucrase contained high levels of fructose and maltose which have to be eliminated. Thus the gluco-oligosaccharides were purified by gel permeation chromatography (GPC) using Biogel P2 column. Gluco-oligosaccharides fractionated according to their degree of polymerization (DP), largest DP sugar eluting first and smallest DP sugar at the last from the Biogel P2 column (Fig. 8.3.6). The HPAEC-PAD analysis of different fractions confirmed the separation of oligosaccharides as shown in Fig. 8.3.6. The gluco-oligosaccharide eluted in the fraction number 195 was identified as panose based on the similar retention time of panose standard. Subsequent fraction numbers 175, 150, 135, 115, 100, 90 and 80 were identified as homologous series of panose with higher degree of polymerization ranging from DP4 (Pan-(Glc)₁) to DP10 (Pan-(Glc)₇). These fractions were further identified according to their molecular weights as described in Section 8.3.8.

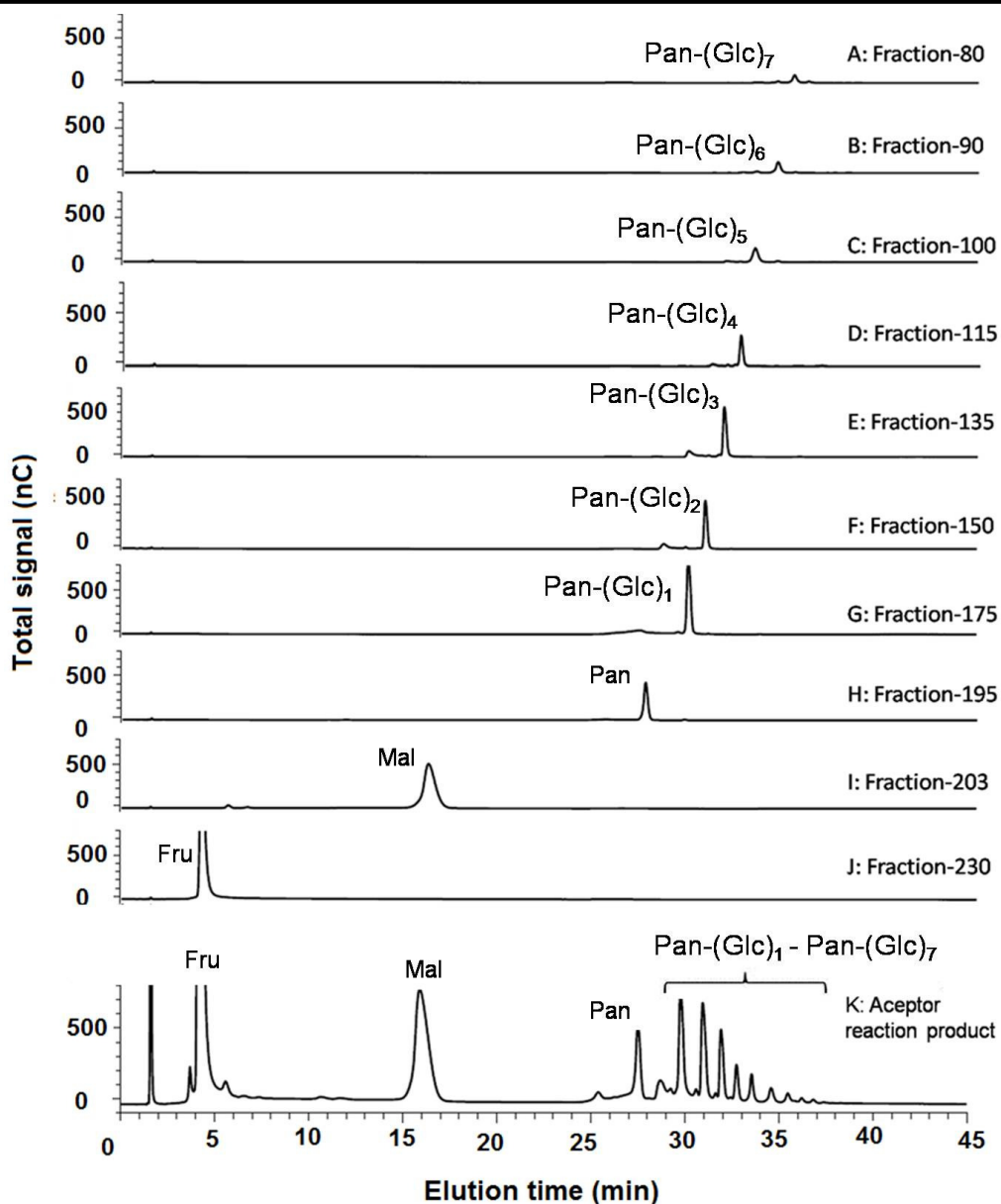
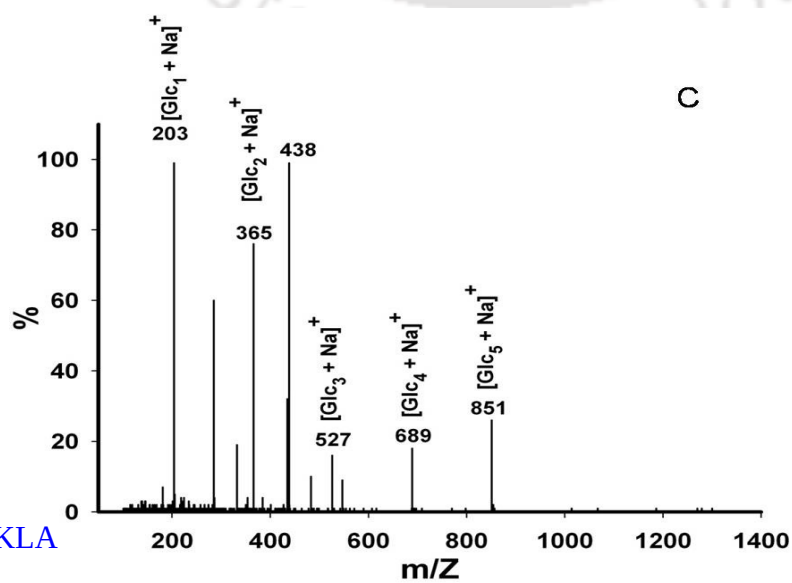
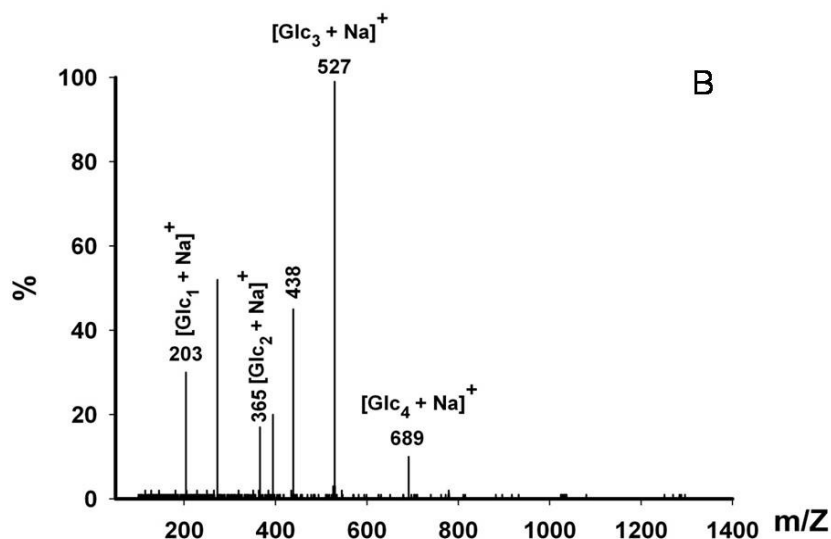
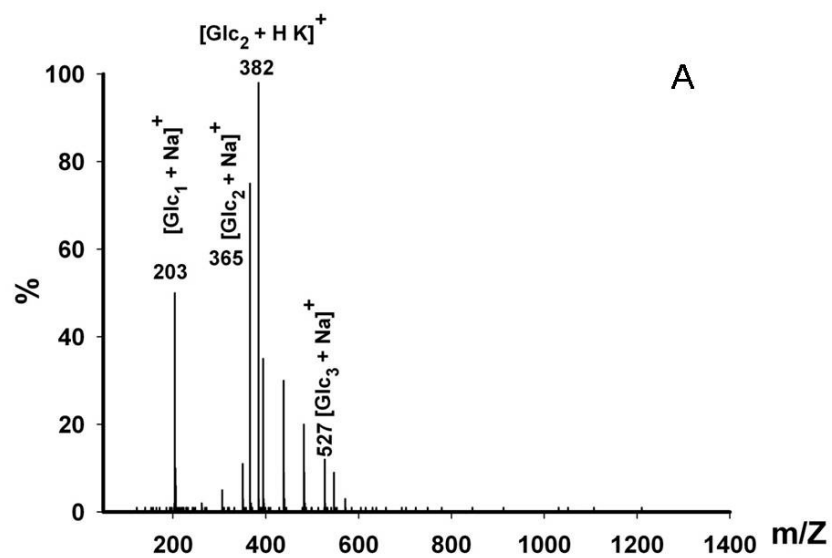


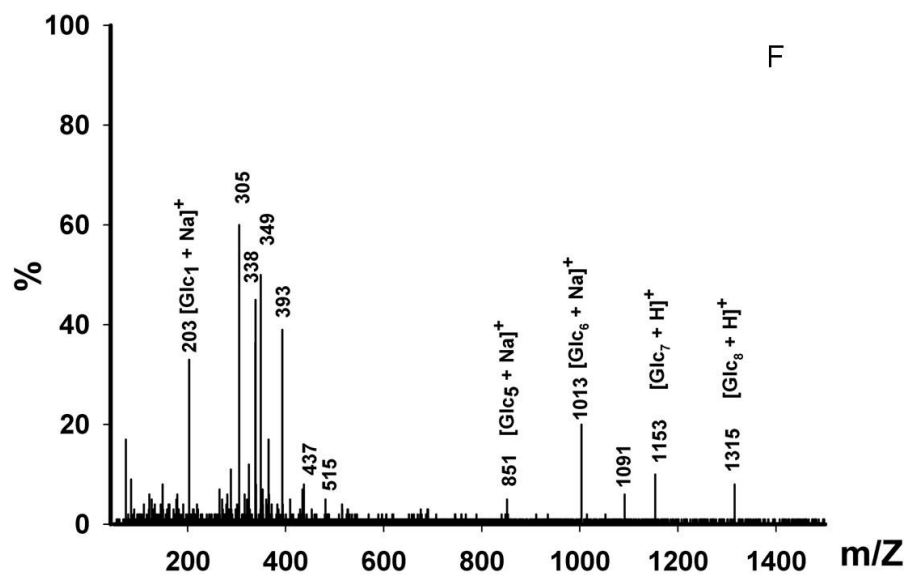
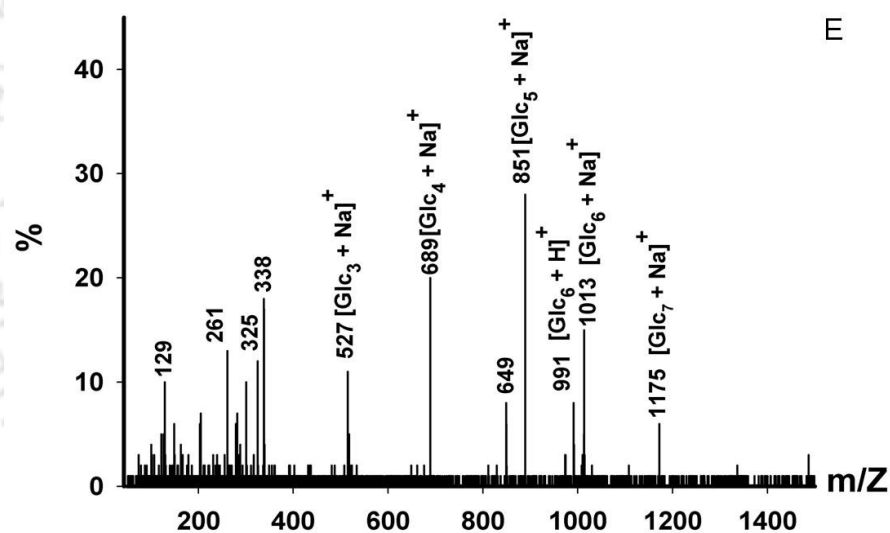
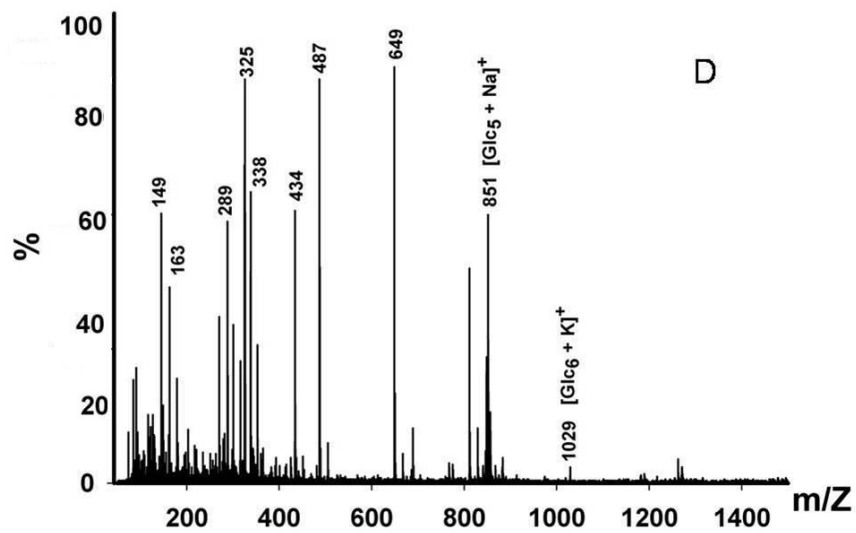
Fig. 8.3.6 HPAEC-PAD chromatograms of some of the fractions from gel permeation chromatography using Biogel P2 column showing gluco-oligosaccharide of different degree of polymerization (DP). From A to J: Fractions 80th, 90th, 100th, 115th, 135th, 150th, 175th, 195th, 203th and 230th fraction; K: Mixture of gluco-oligosaccharides formed by acceptor reaction using maltose and sucrose with dextransucrase from *Weissella Confusa* Cab3. (Glu: glucose; Fru: fructose; Mal: maltose; Pan: panose; Pan(Glc)₁: DP-4; Pan(Glc)₂: DP-5; Pan(Glc)₃: DP-6; Pan(Glc)₄: DP-7; Pan(Glc)₅: DP-8; Pan(Glc)₆: DP-9; Pan(Glc)₇: DP-10).

8.3.8 Gluco-oligosaccharide analysis by mass spectroscopy

The gluco-oligosaccharides produced by the acceptor reaction of dextranucrase were separated using a GPC using a biogel P2 column as described in Section 8.3.7. The oligosaccharides that eluted in different fraction numbers 195, 175 and 150 along with the mixture of the glucooligosaccharides produced by acceptor reaction, were characterized by ESI-TOF-MS as shown in Fig. 8.3.7. Fig. 8.3.7A, B, C, D, E and F shows the ESI-TOF-MS spectra of different gluco-oligosaccharide eluted in the fraction numbers 195, 175 and 150, 135, 115 and 100 respectively. However, the ESI-TOF-MS spectra of the mixture of gluco-oligosaccharides produced by the acceptor reaction of dextranucrase are shown in Fig. 8.3.7G.

The gluco-oligosaccharide eluted in the fraction no. 195 was identified as panose as described in Section 8.3.6. Further confirmation of fraction no. 195 was done by analyzing m/z value using ESI-TOF-MS. The ESI-TOF-MS of the fraction no 195, 175 and 150 (as described in Section 8.3.6) revealed $[M+Na]^+$ pseudomolecular ions at m/z 203, 365 and 527. These molecular weight of these peaks corresponded to the $[Glc_1+Na]^+$, $[Glc_2+Na]^+$ and $[Glc_3+Na]^+$ suggesting the monomer, dimer and trimer of glucose residue, respectively. Additional peak at m/z 689 in fraction no 175 and at 851 in fraction no 150 was observed in the ESI-TOF-MS spectra corresponding to the $[Glc_4+Na]^+$ and $[Glc_5+Na]^+$, respectively suggesting the tetramer and pentamer of the glucose residue. Peak at m/z 438 present in the ESI-TOF-MS spectra of fractions 175 and 150 were attributed to the glycosylium ions obtained by interglycoside cleavages.





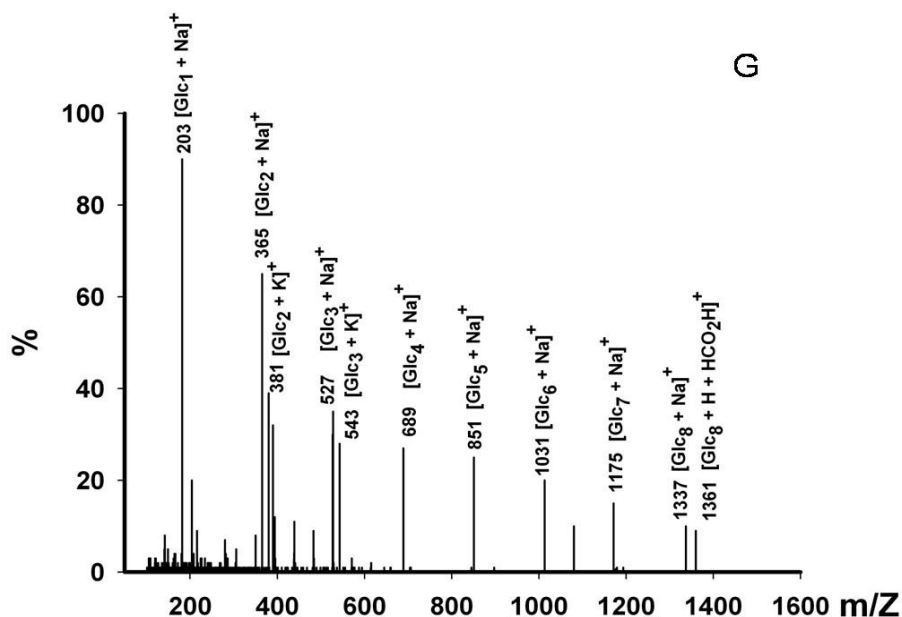


Fig. 8.3.7 ESI-TOF-MS analysis of the (A) Fraction 195; (B) Fraction 175; (C) Fraction 150; (D) Fraction 135 eluted; (E) Fraction 115; (F) Fraction 100 from biogel P2 column and (G) mixture of gluco-oligosaccharides produced from produced by the acceptor reaction of dextransucrase from *Weissella confusa* Cab3 using maltose as acceptor molecule along with sucrose.

The ESI-TOF-MS spectra of fraction 135 revealed m/z at 325, 487, 649, 851 respectively (Fig. 8.3.7D). The ions at m/z 325, 487 and 649 were generated due to the ring cleavage of the glycosidic rang at various place. These results were in accordance with the fragmentation pattern of malto-oligosaccharides of the Bosso *et al.*, 1991. Peak at m/z 851 and 1029 in the ESI-TOF-MS spectrum of the fraction 135, corresponded with the $[\text{Glc}_5 + \text{Na}]^+$ and $[\text{Glc}_6 + \text{K}]^+$, respectively (Fig. 8.3.7D).

The ESI-TOF-MS spectrum of the fraction 115 is depicted in Fig. 8.3.6E. The peak at m/z 1175 was due to the $[\text{Glc}_7 + \text{Na}]^+$ adduct showing that the degree of polymerization of the gluco-oligosaccharide eluted in fraction number 115 from

biogel P2 column was a heptamer (Pan-(Glc)₄). By the loss of one, two, and three glucose units, ions at m/z 1013, 851, 689 and 527, were formed, corresponding to the $[\text{Glc}_6 + \text{Na}]^+$, $[\text{Glc}_5 + \text{Na}]^+$, $[\text{Glc}_4 + \text{Na}]^+$ and $[\text{Glc}_3 + \text{Na}]^+$, respectively (Fig. 8.3.6E). The signal at m/z 991 was due to the $[\text{Glc}_6 + \text{H}]^+$ adduct (Fig. 8.3.6E) which might have produced due to loss of one glucose residue from the heptasaccharide. The data confirmed that the compound was a linear heptamer of glucose units. Peak at m/z 261 (Fig. 8.3.6E) was originating from non reducing end cross-ring cleavages which was in accordance with the Lee *et al.*, 2012. The peak at m/z 325 and 649 were generated due to the ring cleavage of the glycosidic ring at various place (Fig. 8.3.6E). The ESI-TOF-MS spectrum of the fraction 100 is depicted in Fig. 8.3.6F. With respect to the glucose octasaccharide eluted in fraction number 100, the fragmentation of its $[\text{Glc}_8 + \text{H}]^+$ ion at m/z 1315 is observed in Fig. 8.3.6F. By the loss of one, two, and three glucose units, ions at m/z 1153 $[\text{Glc}_7 + \text{H}]^+$, 1013 $[\text{Glc}_6 + \text{Na}]^+$, 851 $[\text{Glc}_5 + \text{Na}]^+$, formed (Fig. 8.3.6F). Peak at m/z 305 corresponded to the ring cleavage of the glycosidic bond and the peak at m/z 203 corresponded to the $[\text{Glc}_1 + \text{Na}]^+$ ions produced due to the fragmentation of the higher gluco-oligosaccharides (Fig. 8.3.6F).

The ESI-TOF-MS of acceptor reaction products (as described in Section 8.3.6) revealed $[\text{M} + \text{Na}]^+$ pseudomolecular ions at m/z 203, 365, 527, 689, 851, 1031, 1175 and 1337 corresponding to the $[\text{Glc}_1 + \text{Na}]^+$, $[\text{Glc}_2 + \text{Na}]^+$ and $[\text{Glc}_3 + \text{Na}]^+$, $[\text{Glc}_4 + \text{Na}]^+$ and $[\text{Glc}_5 + \text{Na}]^+$, $[\text{Glc}_6 + \text{Na}]^+$, $[\text{Glc}_7 + \text{Na}]^+$ and $[\text{Glc}_8 + \text{Na}]^+$ respectively (Fig. 8.3.7G). Gluco-oligosaccharides beyond DP8 could not be detected using ESI-TOF-MS, which might be due to the fragmentation of the higher gluco-oligosaccharides. Some $[\text{M} + \text{K}]^+$ ions were also observed in the ESI-TOF-MS spectra of the acceptor reaction product at m/z 381 and 543 corresponding to the $[\text{Glc}_2 + \text{K}]^+$ and $[\text{Glc}_3 + \text{K}]^+$ ions (Fig.

8.3.7G). Peak at m/z 1361 was due to the formation of $[\text{Glc}_8+\text{H}+\text{HCO}_2\text{H}]^+$ adduct (Fig. 8.3.7G).

This analysis showed that the fractionation of the acceptor reaction products using a biogel P2 column resulted in the separation of the gluco-oligosaccharides of different degree of polymerization. The ESI-TOF-MS spectra of the acceptor reaction product showed a mixture of the gluco-oligosaccharides ranging from the DP-3 to DP8 (Fig. 8.3.7G). On the other hand, the fractions 195, 175, 150, 135, 115 and 100 showed the presence of DP-3, DP-4, DP-5, DP-6, DP-7 and DP-8 corresponding to the molecular weight of 504, 666, 828, 990, 1152 and 1314 Da, respectively.

8.4 Conclusions

The gluco-oligosaccharides from *Weissella confusa* Cab3 were produced by acceptor reaction of dextransucrase using sucrose and maltose. In the presence of sucrose and maltose, dextransucrase catalyzed the synthesis of gluco-oligosaccharides along with the synthesis of dextran. The gluco-oligosaccharides were separated from dextran by ethanol precipitation. The gluco-oligosaccharides were structurally characterized by FT-IR, NMR, HPAEC-PAD and ESI-TOF-MS analyses. HPAEC-PAD analysis showed that the acceptor reaction products *viz.* panose (6²-O- α -D-glucopyranosyl-maltose) and a homologous series of 6'-isomaltodextrinosyl maltose were produced. The FT-IR spectrum of the gluco-oligosaccharides was used to establish the conformational properties such as α -dominating configuration and the carbohydrate nature of the acceptor reaction products. ¹H and ¹³C NMR spectroscopic analysis showed that the dextransucrase synthesized oligosaccharides that contained both α -(1 $\rightarrow$ 6) and α -(1 $\rightarrow$ 4) linkages. The structural elucidation of the gluco-oligosaccharides was carried out using various hydrolyzing enzymes *viz.* endodextranase from *Chaetomium erraticum*, α -glucosidase from *Aspergillus niger* and α -glucosidase from *Bacillus stearothermophilus*. The enzymatic action of endodextranase from *Chaetomium erraticum* on gluco-oligosaccharides resulted in the conversion of gluco-oligosaccharides into isomaltose, isomaltotriose and panose. α -Glucosidase from *Bacillus stearothermophilus* could not hydrolyze the panose series of oligosaccharides as they contained α -(1 $\rightarrow$ 4) linked glucosyl residue at reducing end. However, due to the transglycosilation reaction of the α -glucosidase from *Bacillus stearothermophilus*, isomaltose and isomaltotriose were produced using the gluco-oligosaccharides as substrate. Therefore, NMR spectroscopic analysis and the incomplete hydrolysis of the gluco-oligosaccharides by α -glucosidase from *Bacillus*

stearothermophilus showed that that gluco-oligosaccharides contained maltose at their reducing end. The gluco-oligosaccharides were separated by gel permeation chromatography by Biogel P2 column. The HPAEC-PAD analysis of different fractions from Biogel P2 column confirmed the separation of oligosaccharides. The eluted fractions of gluco-oligosaccharides were analyzed by ESI-TOF-MS. The ESI-TOF-MS analysis of the acceptor reaction products (mixture of gluco-oligosaccharides) and the fractions confirmed the separation of the gluco-oligosaccharides of different degree of polymerization. ESI-TOF-MS analysis of acceptor reaction products revealed the presence of the gluco-oligosaccharides corresponding to a homologous series of panose ranging from DP-3 to DP-8.

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List of publications

1. **Shraddha Shukla**, Qiao Shi, Ndegwa Maina, Minna Juvonen, Maija Tenkanen and Arun Goyal (2014) *Weissella confusa* Cab3 Dextranase: Properties and in Vitro Synthesis of Dextran and Glucooligosaccharides, *Carbohydrate Polymers*, 101, 554-564.
2. **Shraddha Shukla** and Arun Goyal (2013) Medium optimization of fermentation for enhanced dextran production from *Weissella confusa* Cab3 by statistical methods, *Current Biotechnology*, 2, 39-46.
3. **Shraddha Shukla** and Arun Goyal (2012) Development of efficient fermentation process at bioreactor level by Taguchi's orthogonal Array methodology for enhanced dextranase production from *Weissella confusa* Cab, *Advances in Microbiology*, 2, 277-283.
4. **Shraddha Shukla** and Arun Goyal (2011) 16S rRNA based identification of a glucan hyper-producing *Weissella confusa*. *Enzyme Research*, 2011, ID 250842, 10 pages, doi:10.4061/2011/250842.
5. **Shraddha Shukla** and Arun Goyal (2011) Optimization of fermentation medium for enhanced glucanase and glucan production from *Weissella confusa*. *Brazilian Archives of Biology and Technology*, 54 (6), 1117-1124.
6. Rishikesh Shukla, **Shraddha Shukla** and Arun Goyal (2011) Structural characterization of insoluble dextran produced by *Leuconostoc mesenteroides* NRRL B-1149 in the presence of maltose. *Food Technology and Biotechnology*, 49 (3), 291-296.

Conferences/Symposia/Meetings

1. Qiao Shi, **Shraddha Shukla**, Ndegwa Maina, Minna Juvonen, Arun Goyal and Maija Tenkanen (2013) Characterization of dextran and glucooligosaccharides produced by *Weissella confusa* Cab3 dextranase 10th Carbohydrate Bioengineering Meeting, April 21-24, 2013, Institute of Microbiology, Academy of Sciences of the Czech Republic, Prague, Czech Republic.
2. **Shraddha Shukla** and Arun Goyal (2012) Potentials of dextran hyper-producing *Weissella confusa* Cab3 isolated from fermented cabbage for sourdough. V International Symposium on Sourdough - Cereal Fermentation for Future Foods, Oct 10-12, 2012, Helsinki, Finland.
3. **Shraddha Shukla** and Arun Goyal (2012) Enhancement and scale up of dextran production by *Weissella confusa* using Response surface methodology. 18th International Conference on Perspective and Challenges in chemicals and Biological Sciences, Jan 28-30, 2012, Institute of Advanced Study in Science & Technology, Guwahati, India.
4. **Shraddha Shukla** and Arun Goyal (2011) Production, purification and characterization of glucan and gluco-oligosaccharides from *Weissella confusa*.

- Symposium on Carbohydrates at the Interface of Chemistry and Biology, Nov. 23-25, 2011 Indian Institute of Chemical Biology, Kolkata, India.
5. **Shraddha Shukla** and Arun Goyal (2011) Purification and characterization of dextranase from a dextran hyper-producing *Weissella confusa*. International Conference on Genomic Sciences, Nov 3-6, 2011 Panjab University, Chandigarh, India.
 6. **Shraddha Shukla** and Arun Goyal (2011) Enhancement of dextranase production by *Weissella confusa* using Taguchi's orthogonal array method. International Conference on Genomic Sciences, Nov 3-6, 2011, Panjab University, Chandigarh, India.
 7. **Shraddha Shukla** and Arun Goyal (2011) 16S rRNA based identification of a glucan hyper-producing *Weissella confusa*. 9th Carbohydrate Bioengineering Meeting, May 15-18, 2011 Lisboa, Portugal.
 8. **Shraddha Shukla** and Arun Goyal (2010) Production and characterization of glucan from a new strain of "*Weissella confusa*" isolated from fermented cabbage. 7th BRSI Convention and International Conference on Genomic Sciences, Nov 12-14, 2010, Madurai Kamraj University, Madurai, India.
 9. **Shraddha Shukla**, T. Jagan Mohan Rao and Arun Goyal (2010) Optimisation of culture conditions for production and assay conditions of glucanase 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.
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 11. Seema Patel, **Shraddha Shukla** and Arun Goyal (2009) Production, purification and characterization of dextranases from two natural isolates of lactic acid bacteria, SPO and SPA. 6th BRSI Convention and International Conference on Emerging Trends in Biotechnology, Dec 4-6, 2009, Banaras Hindu University, Varanasi, India.
 12. **Shraddha Shukla**, Rwivoo Baruah and Arun Goyal (2013) Biochemical characterization of dextranase from *Weissella confusa* Cab3. National Seminar on Micro- and Macro- resources in Biomolecular Technology. February 25-26, 2013, North Bengal University, Siliguri, West Bengal, India.

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