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Bioactive Oligosugar Producing Lactic Acid Bacteria *Lactobacillus plantaram* LAB 16 from Fermented Maize (*Zea mays* L)

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The production of fructo oligo sugars (FOS) and fructosyl transferases (FTase) has gained tremendous commercial importance worldwide. It is worthwhile to find out microbes with the ability to synthesize these products for therapeutic purposes. In the present study, 25 strains of lactic acid bacteria (LAB) were isolated from fermented maize product and screened for transfructosylation activity. Among the six positive isolates, LAB16 exhibited the highest enzyme activity (210.89 U.ml^{-1}) and identified as *Lactobacillus plantaram*. In order to improve the production, different acceptor molecules were tested. Among them, the highest enzyme production (308.02 U.ml^{-1}) was observed with glucose grown cultures followed by sucrose (253.09 U.ml^{-1}). The maximum growth and glycansucrase activity of *L. plantaram* LAB 16 was attained after 36h of growth, both in glucose and sucrose MRS broth. With varying level of sucrose concentration, the higher one inhibited the microbial activity. The entire protein fraction prepared from the ammonium sulphate method showed FTase activity, but the highest enzyme activity was observed with 50-60 % of ammonium sulphate saturation level. *In vitro* FTase activity in SDS-PAGE showed two isoforms of Fructosyl transferase (FTase) at 25 KDa and 38 KDa, respectively. Thin layer chromatography analysis of crude enzyme extract of *L. plantaram* LAB 16 revealed the presence of bioactive oligosugars such as kestose and nystose.

Key words: Oligosugars, Lactic acid bacteria, Fructosyl transferase, Kestose, Nystose,

The human colon is one of the body's most metabolically active organ because of its dweller microbiota. The use of diet to fortify certain gut flora components is a popular current aspect of functional food sciences and prebiotics have a significant role (Wang, 2009). Prebiotics are short chain carbohydrates that are non-digestible by digestive enzymes in humans and selectively fermented ingredients that allow specific changes, both in the composition and / or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health. Prebiotics also render many other health benefits in the large intestine such as reduction of cancer risk and increase calcium and magnesium absorption (Quigley *et al,* 1999; Al-Sheraji *et al.,* 2013).

Presently, the demand for functional foods has been on the rise due to consumer interest in the link between diet and health. Other factors fueling the increase in demand for functional foods include the growth in the cost of health-care, individual interest in living a healthy life, and the aging population interested in finding a diet that will help them retain optimal health longer. Credible evidence from scientific research has revealed many potential benefits that can be gained from functional foods (Licht *et al*., 2013; Valcheva and Dieleman, 2016)

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Glycan-oligosaccharides have received particular attention recently, because of their excellent biological and functional properties, mainly for prebiotic compounds (Urgell and Orleans, 2001; Patel and Goyal, 2011). Cereals and legumes are effective substrates for the production of probiotic-incorporated functional food, as they can be used as sources of non-digestible carbohydrates, which stimulate the growth of *Lactobacilli* and *Bifidobacteria*. They are very rich sources of water soluble fibers like ß-glucan, galactooligosaccharides and fructo-oligosaccharides, that act as prebiotics digested by selective groups of lactic acid bacteria (Swennen *et al.,* 2006). These cereal associated lactic acid bacteria can produce a variety of exopolysaccharides (EPS) and oligosaccharides from a variety of carbon substrates through the activity of glycosyltransferases (Tieking *et al.,* 2005).

Microbial production of fructo-oligosaccharides by the action of fructosyltransferase (FTase) is more feasible at industrial level and it provides a cost effective and convenient alternative to chemical synthesis. In view of the great demand of glycanoligosaccharides as food ingredients, scope exists for screening and identification of newer strains capable of producing glycansucrases. The present study aimed to isolate and screen the lactic acid bacteria from fermented cereal for glycansucrase activity and prebiotic oligosugars production.

Material and Methods

Maize fermentation

Maize (*Zea mays*) grains were purchased from a local market in Coimbatore, Tamil Nadu, India and thoroughly washed with clean water and soaked for 24 h. Then grains were wet milled and allowed to undergo fermentation naturally for a period of 48 h.

Isolation and characterization of lactic acid bacteria (LAB)

Fermented maize samples were homogenized in phosphate buffer (0.1M, pH 7.2), serially diluted and pour plated on De Man, Rogosa and Sharpe agar (MRS agar, Hi media, Bangalore) and anaerobically incubated at 37°C for 24 to 48 h. Colonies which were different from each other in their morphology and phenotypic appearance were picked and purified by streak plate method and then stored at -4°C on MRS agar slants for further studies.

Screening of LAB Isolates for fructosyltransferase (FTase) activity

FTase activity was determined in cell-free supernatants and in harvested cell fractions by measuring the release of reducing sugars using the DNS technique (Sumner and Howell, 1935) in the presence of 60% (w/v) sucrose in 0.1Mcitrate buffer, pH 5.4. The reaction was carried out at 55±1ºC for 1 h using a water bath under the assay reaction conditions (Morales-Arrieta *et al.*, 2006). One unit of FTase activity was defined as the amount of enzyme required to release 1 µmol of glucose. mL^{-1} or mg. $min.^{-1}$ under the above mentioned reaction conditions (Park *et al.,* 2001 and Sangeetha *et al.* 2005).

Selection of acceptor molecule for high transfructosylation activity

Different acceptor molecules *viz.*, xylose, glucose, fructose, lactose, maltose and sucrose were tested for higher transfructosylation activity by *L. plantaram* LAB 16 by DNS technique.

Kinetics of Lactobacillus plantaram LAB 16

Flask cultures were performed in 50 mL of MRS broth at 30°C and 150 rpm to define optimum growth conditions for *L. plantaram* LAB16 and to achieve adequate enzyme activities. Microbial growth followed by the OD600 measurements and the enzyme activity were monitored at 4 h interval.

Extraction of crude glycansucrase of L. plantaram LAB 16

The culture was grown in 1L of MRS broth for 36 h at 37°C and the supernatant was collected by centrifugation at 12, 000x g for 20 min at 40C (Kubota, model 6800, Japan). The centrifuged supernatant was gradually added with ammonium sulphate to achieve 20% saturation in order to precipitate the enzyme (Wingfield, 2001) by constant stirring and the mixture was kept in a refrigerator at 4°C for overnight. Later, it was centrifuged at 12,000x g for 20 min and the precipitate was collected and dissolved in sodium phosphate buffer 0.05 M (pH 7.0). The supernatant was subsequently adjusted to 40, 50, 60 and 70% saturation levels by further addition of solid ammonium sulphate. The precipitated material, in each time was dissolved in sodium phosphate buffer. A saturated ammonium sulfate solution (750) was added to the supernatant (470 ml) @ 50 $mL.min^{-1}$ under continuous stirring. The resulting 60% ammonium sulfate solution was centrifuged once again for 15 min at 10000x g. The precipitate was resuspended in 10 ml sodium phosphate buffer (10 mM, pH 6.0) and dialyzed overnight against 1L of sodium phosphate buffer (10 mM, pH 6.0). The dialyzed sample (10 ml) was loaded on a hydroxyapatite column (Bio-Rad; 10U6 clm;flow rate 1 ml min^{-1}) equilibrated with sodium phosphate buffer (10 mM, pH 6.0; A). The column was eluted with sodium phosphate buffer (200 mM, pH 6.0; B; flow rate 1 ml. min^{-1}) and fractions collected from 0% B to 40% B were screened for FTase activity by DNS method.

***In situ* FTase activity by SDS PAGE**

In order to measure enzyme activity in SDS-PAGE gels, periodic acid-Schiff reagent staining (PAS) (Kapitany and Zebrowski, 1973) was done. Protein (approximately 1g) was run on SDS-PAGE gels, followed by washing in a pre-incubation buffer (25 mMNaAc [pH 5.4] 1 mM CaCl2, 0.5% Triton X-100) and overnight incubation in pre-incubation buffer with 50 mM sucrose. The gels were washed for 30 min. in a 12.5% trifluoroacetic acid solution in demineralized water and incubated for 50 min. in a 1% periodic acid–3% hydrogen acetate buffer. The periodic acid was washed away carefully with demineralized water, after which the gels were stained with Schiff reagent (Sigma-Aldrich, St. Louis, Mo.), yielding purple spots, where fructan polymer was produced.

Kinetics of FTase Enzyme

The specific activity of FTase enzyme was recorded at different sucrose concentration 28mM to 176mM. The kinetic plot was drawn and K_m and V_{max} value were calculated from Lineweaver-burk plot.

Detection of oligosugars by TLC

For the detection of oligosaccharides produced by *L. plantaram* LAB16, the end products of enzyme reaction (1µl) were spotted on a thin layer chromatography (TLC) ready foil. The TLC foils were run in choloroform:aceticacid:water (6:7:1) and the sugars were specifically stained with diphenyl amine (1.8% w/v) and aniline in phosphoric acid. Sigma grade 1- kestose (GF_2), 1-nystose (GF_3), glucose and sucrose were also used as reference sugars.

Results and Discussion

New sources of nutrients should be more exploited for varying the human diet and also to benefit from new functional ingredients and natural food components. Today, lactic acid bacteria are focus of intensive international research for their essential role in most fermented food and also for

their ability to produce various compounds promoting probiotic properties (Temmerman et al., 2002). In the present study, the benefits of fermented cereals were utilized for isolation of potent functional lactic acid bacteria for prebiotic compound production. Totally 25 colonies were isolated from fermented maize, but only 6 isolates showed potent fructosyltransferase activity. Morphologically, the cells of lactic acid bacterial isolates were of coccus to rod type and arranged either in pairs or chain. Their colonies on MRS agar were circular, low convex with entire margin and cream or white colored (Table 1). Among the LAB isolates, LAB 16 showed higher transfructosylation activity (210.89 µ mole of glucose released mL^{-1}).

Table 1. Characteristics and fructosyltransferase activity of LAB isolates from fermented maize EU-µ mol of glucose released /ml

Isolate	Colony morphology	Fructosyltransferase activity (EU*)
LAB2	Gram negative, small, circular shaped, white and chain of cocci	15.67
LAB7	Gram negative, no definite shaped, cream colour and chain of cocci	167.31
LAB11	Gram negative, small, circular shaped, cream colour and chain of small rods	205.78
LAB12	Gram negative, small, circular shaped, white and clump of cocci	195.09
LAB14	Gram negative, small, circular shaped, white and chain of cocci	200.12
LAB16	Gram negative, small, circular shaped, white and chain of cocci	210.89

Table 2. FTase activity of *L. plantaram* (LAB16) strainU- Amount of enzyme required to liberate 1 µmole of glucose

Isolate	Fructosyltransferase enzyme activity (U*)				
	Ammonium sulphate saturation level (%)				
	20	40	50	60	70
LAB 14	26.37	40.81	41.93	47.85	27.63

Table 3.Kinetic behavior of fructosyltransferase from *L. plantarum* (LAB16) strain

Sucrose conc.	Specific activity(U.ml^{-1})
28 (10)	56
43 (15)	61.82
58 (20)	62.9
73 (25)	65.48
88 (30)	66.9
(103) 35	66
116 (40)	65.6
(131) 45	61.5
146 (50)	59
161 (55)	54
176 (60)	51.2

U- Amount of enzyme required to liberate 1 µmole of glucose

Table 4. Detection of sugars formed by fructosyl transferase enzyme activity of *L.plantarum* LAB16 by TLC

R_f value of standard	R_f value of LAB 16 isolate	sugar identity
0.541	0.541	Glucose
0.424	0.428	Sucrose
0.250	0.251	kystose

The carbohydrate source is an essential constituent in the cultivation media, being important for formation of cell constituents. FTase production is significantly influenced by acceptor / donor molecule. Maximum enzyme activity by *L. plantarum* LAB 16 from fermented maize on various sugars was determined after 48 h incubation. Among the different acceptor molecule tested, the highest enzyme activity (308.02 U.mL^{-1}) was observed in glucose grown cultures followed by sucrose (253.09 U.mL^{-1}). Growth on maltose, lactose and xylose yielded enzyme activities of 77.78, 169.14 and 170.99U. mL^{-1}, respectively (Fig.1). This study was supported by the report of van Hijum *et al*. (2006) who indicated that, the carbohydrates vary in their efficiency to act as acceptor to fructansucrases. The effect of different carbon sources was also studied by Yun *et al*. (1997) for fructosyltransferase production by *Aspergillus pullulans* where, sucrose was also found to be the preferred carbon source. It is frequently reported that the sucrose is the best inducer for F-Tase production (Hayashi *et al.*, 1992; Wang and Zhou, 2006). In this present study also, higher enzyme activity (256.54 EU) and growth (OD600) was exhibited, when the

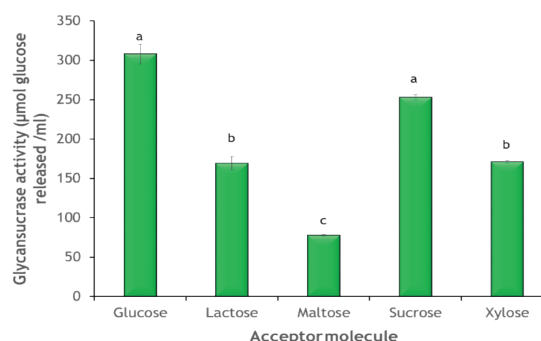

Fig.1. Effect of different acceptor molecule on glycansucrase activity of *L. plantarum* LAB 16 isolate from fermented maize

cultures grown on glucose medium than sucrose (Fig. 2A and 2B) but, comparing the cost of glucose to sucrose, sucrose is the cheapest substrate preferred for oligosugars production (Hector *et al*., 2015). In sucrose broth, the maximum FTase activity was recorded at 36 h.

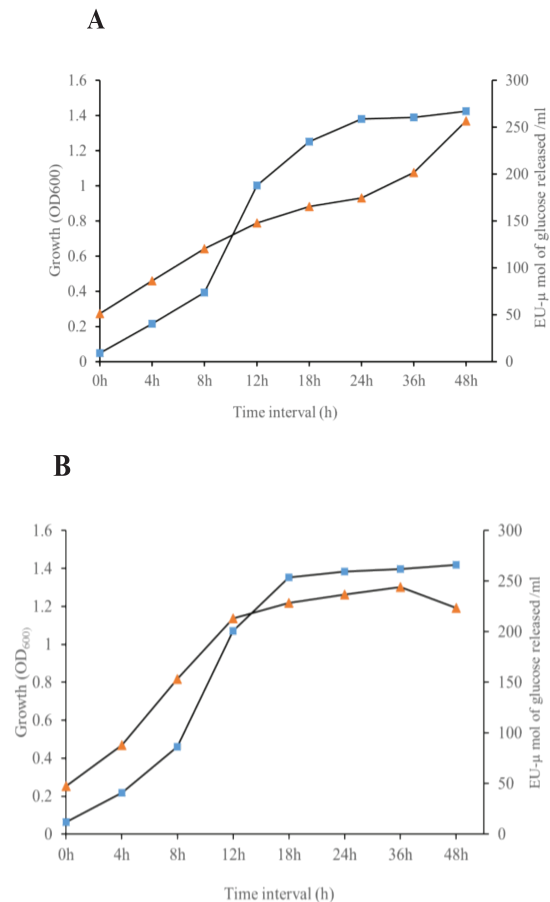

Fig. 2. Correlation of growth and glycansucrase activity of isolate *L. plantarum* LAB 16 in MRS medium with glucose (A) or sucrose (B) as an acceptor molecule at different time intervalA) Glucose-MRS medium B. Sucrose-MRS medium.

The FTase was pre-purified by ammonium sulphate precipitation. Different saturation levels of ammonium sulphate such as 20%, 40%, 50%, 60% and 70% were done to precipitate the FTase. The FTase activity was observed at all the fraction, but the highest activity was observed at 50-60 saturation (Table 2). At this step, up to two-fold increase in specific activity was recorded. The specific activity was recorded in different sucrose concentrations (Table 3). The inhibition of high substrate concentration above 116 mM (40 % w/v) of sucrose level on enzyme activity was found from kinetic plot. The kinetic constants Vmaxand Km (Michaellis–Menton constant) values calculated were 88 mM and 66.9 U. ml^{-1}, respectively.

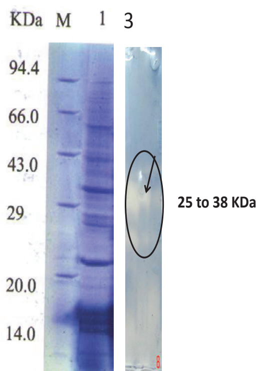

Fig.3. SDS-PAGE and *in situ* gel activity showing fructosyltransferase activity

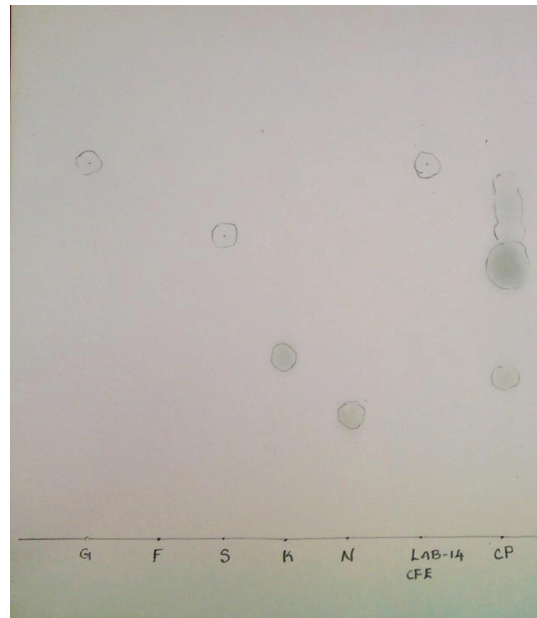

Fig. 4. Identification of fructooligosugars produced by L. *plantarum* LAB16 by TLC. G- Glucose; F - Fructose; S - Sucrose; K - Kestose; N - Nystose, CFE - LAB 14 Cell free extract, CP -LAB 14 Concentrated product

The *in vitro* FTase activity in sodium dodecyl sulphate-polyacrylamide (SDS-PAGE) revealed well clear zone in the range of 25 to 38 KDa size (Figure 3). Levan polymers are either linear or branched (to varying degrees) at the C-1 position, with molecular masses varying between 20 kDa and several MDa. The molecular mass of FTase enzymes from LAB origin (Streptococci only) as reported by Song and Jacques (1999) is typically around 90 to 100 KDa, whereas FTase enzymes of Bacillus sp. (Chambert *et al*. 1974) or Gram-negative bacteria (Hernandez,1995) have molecular masses of 50 to 60 KDa. It will be interesting to study the *in situ* functional properties of *L. reuteri* strain 121 and the levan produced; their possible roles in the probiotic properties contributed to *L. reuteri* strains (De Roos and Katan, 2000). In *L. paracasei* P 4134 (Muller and Seyfarth, 1997), the extracellular preparation showed a single protein band in SDS-PAGE gel with a mobility corresponding to molecular weight of 42kDa. But in *L. plantarum* LAB 16, it was concluded that two molecular weight of fructan-degrading enzyme (25 KDa and 38 KDa) had existed.

Microbial fructansucrase enzyme belongs to the glycosidic hydrolase family, catalase trans fructosylation reactions with sucrose resulting in the synthesis of fructo-oligosaccharide and / or a fructan polymer (Ozimek *et al.,* 2006). The fructan transferring reaction of FTase enables production of oligosugars maintaining a similar sweetness level as well as preventing unfavorable polymer synthesis. In the present study, TLC analysis of crude and concentrated enzyme extract of *L. plantarum* LAB16 showed that the accumulation of short chain oligosugars or fructooligosugars (Kestose and Nystose) at the application site (Fig.4) as the Rf values of these sugars corresponded with the authenticated standards in the chromatogram of the isolate *L. plantarum* LAB 16 (Table 4).

Conclusion

In the present study, bioactive sugar (FOS) producing lactic acid bacteria were isolated from fermented maize. This oligosugar producing isolate has been anticipated to have enormous potential in food biotechnology for *in situ* prebiotic production through fermented cereal foods.

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References

Al-Sheraji S.H.,A.I.M Yazid Manap.,S. Mustafa.,R., M. Yusof and F.A.Hassan. 2013. Prebiotics as functional foods: *A review Journal of Functional Foods*, **5 (4)**:1542-1553.

Arruda, H.S., G.A. Pereira and M.E.F. Alfieda.2013. Frontiers in Natural Product Chemistry (ed.,Atta-ur-Rahman). *Current perspective and future challenge in prebiotic research* **3:** 237

Chambert, R., G. Treboul and R. Dedonder. 1974. Kinetic studies of levansucrase of *Bacillus subtilis*. *European Journal of Biochemistry*, **41**:285-300.

De Roos, N.M. and M.B. Katan. 2000. Effects of probiotic bacteria on diarrhea, lipihd metabolism and carcinogenesis: a review of papers published between 1988 and 1998. *Am. J. Clin. Nutr.,***71**: 405-411.

Hayashi, S., K., Matsuzaki Takasaki, Y.Ueno and H. K. Imada.1992. Production of fructofuranosidase by *Aspergillus japonicus*. *World J Microbiol Biotechnol*., **8**:155–159

Hector, S. K., Willard, R., Bauer I.,Mulako., E.Slabbert., J..Kossmann and G M George. 2015. Bacteria isolated from Milled Sugarcane: Implications for Cane Spoilage and Sucrose Yield. *Plos one*, 1-10.

Hernandez, L., J.Arrieta, C. Menendez, R. Vazquez, A. Coego, V. Suarez, G. Selman, M.F. Petit-Glatron and R. Chambert. 1995. Isolation and enzymic properties of levansucrase secreted by *Acetobacterdiazotrophicus* SRT4, a bacterium associated with sugar cane. *Biochem.J.*, **309**: 113-118.

Kapitany, R. A., and E. J. Zebrowski.1973. A high resolution PAS stain for polyacrylamide gel electrophoresis. *Anal. Biochem.,***56**:361–369.

Morales-Arrieta, S, M.E. Rodríguez, L. Segovia, A.López-Munguía and C.Olvera-Carranza. 2006. Identification and functional characterization of levS, a gene encoding for a levansucrase from *Leuconostocmesenteroides* NRRL B-512. *Gene,***376**:59–67.

Ozimek, L., K. Kralj, S.Kaper, T. van der Maarel and L. Dijkhuizen. 2006. Single amino acid residue changes in subsite-1 of inulosucrase from *Lactobacillus reuteri* 121 strongly influence the size of products synthesized. *FEBS Journal,***273**: 4104-4113.

Park, Y.S, S.W. Kang, J.S. Lee, S.I. Hong and S.W Kim. 2002. Xylanase production in solid state fermertation by *Aspergillus niger* mutant using statistical experimental designs. *Appl. Microbiol. Biotechnol.*,***58(6):***761-766.

Patel, S. and A.Goyal. 2011. Functional oligosaccharides: production, properties and applications. *World Journal of Microbiol Biotechnol***.**, **27**:1119–1128.

Quigley, M. E., , G. J. Hudson and H. N. Englyst, (1999). Determination of resistant short-chain carbohydrates (non-digestible oligosaccharides) using gas-liquid chromatography. *Food Chemistry***,** **65**: 381–390.

Sangeetha, P.T., M.N. Ramesh and S.G.Prapulla. 2004. Production of fructo-oligosaccharides by fructosyltransferase from *Aspergillusoryzae* CFR 202 and *Aureobasidiumpullulans* CFR 77. *Process Biochem.*,**39**:753–758

Song, D.D. and N.A. Jacques, 1999.Purification and enzymic propertiesof the fructosyltransferase of *Streptococcus salivarius* ATCC25975. *Biochem. J.,***341**: 285-291.

Sumner, J.B. and S.F. Howell. 1935. A method for determination of saccharase activity. *J Biol Chem*., **108**:51–54.

Tambekar, D.H. and S.A.Bhutada. 2010. An evaluation of probiotic potential of lactobacillus sp. From Milk of domestic animals and commercial available probiotic Preparations in prevention of enteric bacterial infections.*J .Microbiol.,***8**:1-6.

Tieking, M., M.G.Ganzle, M.Tieking and M.G.Ganzle. 2005. Exopolysaccharides from cereal-associated lactobacilli.*Food sci. technol*., **16**: 79-84.

Urgell, M. and A. Orleans. 2001.Oligosaccharides: Application in infant food.*Early Hum Dev.,***65**:43-44

van Hijum, S.A.F.T., S. Kralj, L.K. Ozimek, L. Dijkhuizen and I.G.H. van Geel-Schutten. 2006. Structure–function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiol.Molec. Biol. Rev.,* **70***:*157–176.

Wang, L.M. and H.M. Zhou. 2006. Isolation and identification of a novel *Aspergillusjaponicus* JN19 producing b-fructofuranosidase and characterization of the enzyme. *J Food Biochem*.,**30:**641–658

Wingfield, P.T. 2001. Protein Precipitation Using Ammonium Sulfate HHS Public Access Published in final edited form as: *Curr Protoc Protein Sci*.,**1:**1-12.

Yun, J.W. 1996. Fructooligosaccharides: Occurrence, preparation, and application. *Enzyme and Microbial Technology*, **19:** 107–117.

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