DISSERTATIONES BIOLOGICAE UNIVERSITATIS TARTUENSIS
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TRIINU VISNAPUU

Levansucrases encoded in the genome of *Pseudomonas syringae* pv. tomato DC3000: heterologous expression, biochemical characterization, mutational analysis and spectrum of polymerization products

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Department of Genetics, Institute of Molecular and Cell Biology, University of Tartu, Estonia

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Supervisor: Docent Tiina Alamäe, PhD
Institute of Molecular and Cell Biology
University of Tartu
Estonia

Opponent: Professor Maija Tenkanen, PhD
Department of Food and Environmental Sciences
Faculty of Agriculture and Forestry
University of Helsinki
Finland

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LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications which are referred to by Roman numerals in the text:

I. **Visnapuu T**, Mäe A, Alamäe T (2008) *Hansenula polymorpha* maltase gene promoter with sigma 70-like elements is feasible for *Escherichia coli*-based biotechnological applications: Expression of three genomic levansucrase genes of *Pseudomonas syringae* pv. tomato. *Process Biochem* 43: 414–422.

II. **Visnapuu T**, Zamfir AD, Mosoarca C, Stanescu MD, Alamäe T (2009) Fully automated chip-based negative mode nanoelectrospray mass spectrometry of fructooligosaccharides produced by heterologously expressed levansucrase from *Pseudomonas syringae* pv. tomato DC3000. *Rapid Commun Mass Spectrom* 23: 1337–1346.

III. **Visnapuu T***, Mardo K*, Mosoarca C, Zamfir AD, Vigants A, Alamäe T (2011) Levansucrases from *Pseudomonas syringae* pv. tomato and *P. chlororaphis* subsp. *aurantiaca*: Substrate specificity, polymerizing properties and usage of different acceptors for fructosylation. *J Biotechnol* 155: 338–349.

IV. Alamäe T, **Visnapuu T**, Mardo K, Mäe A, Zamfir AD (2012) Levansucrases of *Pseudomonas* bacteria: novel approaches for protein expression, assay of enzymes, fructooligosaccharides and heterooligofructans. In: *Carbohydrate Chemistry*, Vol 38. Rauter AP, Lindhorst TK (Eds.). Specialist Periodical Reports, Royal Society of Chemistry, Cambridge, UK, p 176–191.

* These authors contributed equally to this work.

My contribution to the journal articles referred to in the dissertation is following:

Ref. I – participated in the design of experiments, performed all experimental procedures and analysed the data, participated in writing of the manuscript and preparing the tables and figures, participated in the revision process of the manuscript

Ref. II – participated in the design of experiments, performed the experiments, conducted data analysis and prepared the tables and figures,

contributed to writing of the manuscript, participated in the revision process of the manuscript

Ref. III – participated in the design of experiments, performed experiments concerning wild-type Lsc3 and LscA (except linkage-type assay of the fructan and 3D modelling of enzymes), participated in mutational analysis of Lsc3 (site-directed mutagenesis), conducted data analysis and prepared the tables and figures, contributed to writing of the manuscript, participated in the revision process of the manuscript

Ref. IV – participated in the design of experiments, performed all experiments except 3D modelling of Lsc2, conducted data analysis and prepared the tables and figures, contributed to writing and proofreading of book chapter

ABBREVIATIONS

aa – amino acid
bp – base pair
CAZy – Carbohydrate-Active Enzymes
Da – dalton
DNSA – 3,5-dinitrosalicylic acid
DP – degree of polymerization
ESI – electrospray ionization
FEH – fructan exohydrolase
FFT – fructan:fructan fructosyltransferase
FOS – fructooligosaccharides
FT – fructosyl transferase
GH – glycoside hydrolase
HCT – high-capacity ion trap
HOF – heterooligofructans
HPAEC – high-performance anion-exchange chromatography
HPLC – high-performance liquid chromatography
IPTG – isopropyl β-D-1-thiogalactopyranoside
k_{cat} – catalytic constant (s^{-1}; min^{-1})
k_{cat}/K_m – rate constant of catalytic efficiency (mM^{-1} s^{-1}; M^{-1} min^{-1})
K_i – inhibition constant (mM)
K_m – enzyme's affinity for the substrate (mM)
MS – mass spectrometry
MS/MS or MS^n – tandem mass spectrometry
NMR – nuclear magnetic resonance
PAGE – polyacrylamide gel electrophoresis
PDB – Protein Data Bank
SFT – sucrose:fructan fructosyltransferase
SST – sucrose:sucrose fructosyltransferase
TA – transfructosylation activity
TLC – thin layer chromatography
V_{max} – maximum reaction velocity (U/mg)

INTRODUCTION

Saccharides are extremely abundant substances in nature. They are present in all organisms playing vital roles in their lives. Fructans, polymers of fructose residues, comprise an interesting group of carbohydrates which are synthesized from sucrose and their origin, linkage type, degree of polymerization and branching are diverse. Numerous plant species contain β-2,1-linked inulin-type fructans whereas many bacteria synthesize highly polymeric β-2,6-linked fructan – levan. Aside of functioning as a reserve carbohydrate for the organism, fructans are also implicated in stress tolerance and pathogenesis. Furthermore, fructans are of biotechnological value – they can be used in food technology as prebiotics, emulsifiers, sweeteners and in medicine as therapeutic drugs.

Fructans are synthesized by fructosyl transferases (FTs) which are found in plants, bacteria and fungi. Genes potentially encoding FTs are also present in archaea. FTs are classified to clan J of glycoside hydrolases (GHs) according to Carbohydrate-Active Enzymes (CAZy) database. This clan encompasses GH families 32 and 68 that include hydrolytic enzymes *e.g*. invertases and fructan exohydrolases, but also proteins with transfructosylating activity. The latter group of enzymes includes plant and microbial FTs synthesizing various inulin- or levan-type oligo- and polymers. In plants, at least two distinct proteins are needed to assure production of a polymeric fructan, whereas in bacteria only one enzyme, either levansucrase or inulosucrase is sufficient for the whole process.

Levansucrases are GH68 enzymes present in many bacteria, including numerous strains of plant-pathogenic *Pseudomonas syringae*. Levansucrases produce levan, a fructan with β-2,6 linkages between the fructose residues. These enzymes share a five-bladed β-propeller fold with other GH clan J enzymes. Also, all enzymes of this clan studied so far have two aspartates and one glutamate as key catalytic amino acids (catalytic triad). Although levansucrases of Gram-positive bacteria have been extensively investigated and some of them are crystallized, respective proteins of Gram-negative pseudomonads have been scarcely studied despite of the fact that some of them, for example *P. syringae* strains have multiple levansucrases. Up to now, only one levansucrase from *P. syringae* has been purified and characterized whereas no structure-function study of *P. syringae* has been carried out.

Research presented in this thesis was focused on heterologous expression and characterization of levansucrases of *P. syringae* pv. tomato DC3000. Expression systems were tested and used for heterologous synthesis of *P. syringae* pv. tomato DC3000 levansucrases in *Escherichia coli*. Two of them, the Lsc2 and Lsc3 proteins and their reaction products were characterized. The detailed aims of the study are listed in the beginning of the Results and Discussion section. The most important features of the levansucrases Lsc2 and Lsc3 revealed by us are their high catalytic activity, stability and ability to produce not only polymeric levan, but also fructooligosaccharides and heterooligofructans with potential prebiotic properties. Therefore, these proteins have biotechnological potential.

I OVERVIEW OF LITERATURE

1. Fructosyl transferases

Carbohydrates are extremely widespread compounds in the nature. Primary cell wall of plants contains framework of cellulose microfibrils that is consolidated by xyloglucans and embedded in pectin matrix. All those components have saccharidic origin and they represent bulk of the overall biomass (Carpita and Gibeaut, 1993; Fry, 2001; Cosgrove, 2005). Plant- and algae-derived polysaccharides such as starch, carrageenans, alginates and agar are extracted and extensively used in food manufacture, cosmetics and biotechnology (Donot *et al*., 2012 and references therein).

Saccharides have essential functions in many biological processes like cell adhesion and recognition, signaling, fertilization, immune response, cancer development and metastasis (Dall'Olio and Chiricolo, 2001; Conner *et al*., 2005; Dubber *et al*., 2006; Avci *et al*., 2011; Slawson and Hart, 2011; Taniguchi and Korekane, 2011). Importantly, it has been proposed that more than half of human proteins are glycosylated (Apweiler *et al*., 1999).

Fuctans are composed of fructose residues which are usually originating from sucrose – the main sugar in plant vascular tissues. Besides starch and sucrose, about 15% of flowering plants store their energy and carbon in β-linked fructans of different degree of polymerization (DP) (Hendry, 1993; Ritsema and Smeekens, 2003; Lammens *et al*., 2009). Additionally, fructans have been isolated from several bacteria and fungi that possess fructosyl transferases for their synthesis (Maiorano *et al*., 2008 and references therein).

Fructosyl transferases (FTs) present in many plant, fungal, bacterial and archaeal species synthesize various types of fructans. Fructosyl transferases involved in fructan biosynthesis are classified according to the Carbohydrate-Active Enzymes (CAZy) database to glycoside hydrolase (GH) clan J which encompasses GH families 32 and 68 (http://www.cazy.org; Cantarel *et al*., 2009). This clan also includes a large number of enzymes with hydrolase activity *e.g*. invertases (β-fructofuranosidases), endo- and exo-inulinases and endo-levanases. According to structural and mutational studies, enzymes of families 32 and 68 have highly similar crystal structures and catalytic centre (see Table 1) (Meng and Fütterer, 2003; Martínez-Fleites *et al*., 2005; Verhaest *et al*., 2005; Alberto *et al*., 2006; Verhaest *et al*., 2006; Lammens *et al*., 2008; Alvaro-Benito *et al*., 2010a; Chuankhayan *et al*., 2010; Lammens *et al*., 2012).

Table 1. Enzymes of GH clan J with five-bladed β-propeller fold and crystal structures available in Protein Data Bank (PDB; http://www.rcsb.org). Data were retrieved from CAZy database (http://www.cazy.org). Enzymes belonging to GH family 68 are marked with gray background and enzymes with additional C-terminal domain by an asterisk.

Type of the enzyme	**Designation of the enzyme, organism**	**Catalytic triad**	**PDB code**	**References**
invertase* (EC 3.2.1.26)	AtcwINV1 of *A. thaliana*	Asp23 Asp149 Glu203	2AC1 2OXB 2QQU 2QQV 2QQW 2XQR	Verhaest *et al*., 2006 Lammens *et al*., 2008
invertase* (β-fructosidase) (EC 3.2.1.26)	BfrA of *T. maritima*	Asp17 Asp138 Glu190	1UTW 1UYP 1W2T	Alberto *et al*., 2004 Alberto *et al*., 2006
invertase* (β-fructo-furanosidase) (EC 3.2.1.26)	SoInv of *S. occidentalis*	Asp50 Asp179 Glu230	3KF3 3KF5	Alvaro-Benito *et al*., 2010a
β-fructo-furanosidase* (EC 3.2.1.26)	β-fructofuranosidase of *B. longum* KN29.1	Asp54 Asp181 Glu235	3PIG 3PIJ	Bujacz *et al*., 2011
fructan exohydrolase* (EC 3.2.1.153)	1-FEH IIa of *C. intybus*	Asp22 Asp147 Glu201	1ST8 2ADD 2ADE 2AEY 2AEZ	Verhaest *et al*., 2005 Verhaest *et al*., 2007
exo-inulinase* (EC 3.2.1.80)	Inu1 of *A. awamori*	Asp21 Asp189 Glu241	1Y4W 1Y9G 1Y9M	Nagem *et al*., 2004
fructosyl transferase* (EC 2.4.1.100)	AjFT of *A. japonicus*	Asp60 Asp191 Glu292	3LDK 3LDR 3LEM 3LF7 3LFI 3LIG 3LIH	Chuankhayan *et al*., 2010
sucrose:(sucrose/ fructan) 6-fructo-syltransferase* (EC 2.4.1.-; 2.4.1.10)	Pt6-SST/6-SFT of *P. terminalis*	Asp33 Asp157 Glu211	3UGF 3UGG 3UGH	Lammens *et al*., 2012

Table 1. Continuation.

Type of the enzyme	Designation of the enzyme, organism	Catalytic triad	PDB code	References
inulosucrase* (EC 2.4.1.9)	InuJ of *L. johnsonii*	Asp272 Asp425 Glu524	2YFR 2YFS 2YFT	Pijning *et al*., 2011
levansucrase (EC 2.4.1.10)	SacB of *B. subtilis*	Asp86 Asp247 Glu342	1OYG 1PT2 2VDT 3BYJ 3BYK 3BYL 3BYN	Meng and Fütterer, 2003 Meng and Fütterer, 2008
levansucrase (EC 2.4.1.10)	SacB of *B. megaterium*	Asp95 Asp257 Glu352	3OM2 3OM4 3OM5 3OM7	Strube *et al*., 2011
levansucrase (EC 2.4.1.10)	LsdA of *G. diazotrophicus*	Asp135 Asp309 Glu401	1W18	Martínez-Fleites *et al*., 2005

1.1 Fructosyl transferases of plants and fungi

Fructan-containing plants are distributed over major orders such as *Asterales*, *Poales* and *Liliales*, encompassing many economically important crops and other plants of agricultural significance (Hendry, 1993). So, many edible plants such as onion (*Allium cepa*), chicory (*Cichorium intybus*), rice (*Oryza sativa*), barley (*Hordeum vulgare*) and Jerusalem artichoke (*Helianthus tuberosus*) contain fructans (Van den Ende *et al*., 2009 and references therein). Fructans in plants act mainly as vacuolar storage carbohydrates, whereas additional functions such as protection from drought, cold and even promotion of vacuolar antioxidative activity have been proposed (Hisano *et al*., 2004; Livingston *et al*., 2009; Van den Ende and Valluru, 2009). Plant-protecting effect of fructans might be caused by stabilization of cell membranes and membrane-associated antioxidative proteins (Livingston *et al*., 2009; Van den Ende and Valluru, 2009).

Plant fructans have different chain length and linkage type. Usually plants synthesize low-branched inulin-type fructans consisting of β-2,1-linked fructosyl residues with the chains not exceeding few hundred residues. Shortest **inulin**-type fructan is a trisaccharide 1-kestose (GF_2). Also, many monocot plants contain **levan** – a β-2,6-linked fructan (see Fig. 1) (Vijn and Smeekens, 1999; Ristema and Smeekens, 2003; Livingston *et al*., 2009). In plants, mixture of levan-type fructans with moderate chain length (DP 4-12) is referred as

phlein (Van Riet *et al*., 2006). Interesting groups of fructans present in *Liliaceae* and *Poaceae* are levan- or inulin-type neo-series. They have glucose moiety between the fructosyl chains and they are derived from neokestose (6G-kestotriose) (Fig. 1) (Livingston *et al*., 2009 and references therein).

To synthesize all above-mentioned fructan species, plants use four different types of fructosyl transferases. At least two different enzymes with fructosyl transferase activity are required to produce fructans with higher DP within one plant species (Fig. 1) (Vijn and Smeekens, 1999). All plant-derived enzymes with FT activity are classified within GH family 32 (http://www.cazy.org). Interestingly, it has been suggested that plant FTs originate from vacuolar invertases that have evolved a dual activity on sucrose which acts as a fructosyl donor and also as an acceptor (Lammens *et al*., 2009). The initiation step of fructan biosynthesis in plants takes place in the vacuole where sucrose is converted to 1-kestose and glucose by sucrose:sucrose 1-fructosyltransferase (1-SST) (EC 2.4.1.99), a S-type fructosyl transferase (Fig. 1). 1-SST from onion has been cloned and expressed in protoplasts of the tobacco plant (*Nicotiana plumbaginifolia*) resulting in synthesis of 1-kestose from sucrose (Vijn *et al*., 1998). Depending on the plant, the affinity of 1-SST for sucrose varies to large extent – recorded K_m values for sucrose-splitting reaction by 1-SSTs range from a low millimolar value to 0.5 M (see also The Comprehensive Enzyme Information System BRENDA at http://www.brenda-enzymes.org).

Despite extensive studies concerning sucrose- and fructan-acting enzymes in plants, crystal structure of 1-SST protein has not been solved (http://www.cazy.org). Recently, 3D structure of plant fructosyl transferase from a Japanese spurge (*Pachysandra terminalis*) was published. This enzyme is referred to as sucrose:(sucrose/fructan) 6-fructosyltransferase (6-SST/6-SFT) due to its ability to synthesize not only 6-kestose, but also levan-type fructans with higher DP. Additionally, 1-kestose and highly-branched fructans (graminans) have been disclosed among the reaction products of Pt6-SST/6-SFT (Lammens *et al*., 2012). The overall structure of the enzyme contains N-terminal 5-bladed β-propeller and C-terminal β-sheet sandwich-like folds. The catalytic centre is located in the middle of the β-propeller domain and comprises two aspartates and a glutamate acting as nucleophile, transition-state stabilizer and acid-base catalyst, respectively (Lammens *et al*., 2012). This otherwise quite rare structural fold has been revealed for every crystallized enzyme of the GH32 family including cell-wall invertase 1 from *Arabidopsis thaliana* (AtcwINV1) and fructan 1-exohydrolase IIa (1-FEH IIa) from chicory (Table 1) (Verhaest *et al*., 2005; Verhaest *et al*., 2006).

The other enzymes that are responsible for fructan biosynthesis in plants are (i) fructan:fructan 1-fructosyltransferase (1-FFT) which elongates kestoses (1-kestose and neokestose) and therefore produces mainly inulin-type fructans, (ii) sucrose:fructan 6-fructosyltransferase (6-SFT) which produces β-2,6-liked levans and (iii) fructan:fructan 6G-fructosyltransferase (6G-FFT) that converts 1-kestose to neokestose (Fig. 1) (Livingston *et al*., 2009 and references therein; http://www.brenda-enzymes.org; http://www.cazy.org). Fructans are degraded

by FEHs. Interestingly, it seems that almost all plant species contain fructan hydrolyzing enzymes independently of their ability of fructan synthesis. This could be part of some kind of defensive or adaptational mechanism (Van den Ende *et al*., 2009).

Structural and mutational studies on some GH32 enzymes have been carried out to reveal the catalytic centre and regions required for fructosyl transferase activity. So far, all investigated enzymes from GH clan J harbour two aspartates and one glutamate as a catalytic triad in their catalytic centre. These amino acids are invariant among clan J enzymes regardless of their different (invertase, fructosyl transferase or hydrolase) activities and origin of the protein (Table 1) (http://www.cazy.org).

Multiple alignment of plant GH32 enzyme protein sequences has diclosed several conserved regions. For example AtcwINV1 has eight conserved motifs with three of them situated in close proximity of catalytic residues D23, D149 and E203. Highly conserved motifs in AtcwINV1 are WMN**D**PNG, WGN, WSGSAT, MLYTGI, FR**D**P, W**E**CPD, WGW and GWSG with catalytic triad residues indicated by bold letters (Lammens *et al*., 2008; Van den Ende *et al*., 2009). The main difference between the protein sequences of cell wall invertase from *A. thaliana* and fructan exohydrolase from chicory lays in the hyper-variable loop KISL**D**DTKH close to the acid-base catalyst E203. Analysis of crystal structures and site-directedly mutated enzyme specified Asp239 (indicated in bold) as critical residue for sucrose binding and hydrolysis in AtcwINV1. If this aspartate was mutated to Ala or Phe, invertase activity was lost, but 1-kestose hydrolyzing (FEH) activity was retained (Le Roy *et al*., 2007; Van den Ende *et al*., 2009).

It seems that prevalence of either hydrolytic or transferase activities of GH32 enzymes may be promoted by certain crucial positions in specific regions (loops) that enable hydrogen bonding between different parts of the enzyme or between the enzyme and the substrate. Interestingly, not many alterations are needed to transform a vacuolar invertase to fructosyl transferase or to convert an F-type enzyme to an S-type. For example, a vacuolar invertase from wheat was successfully mutated to act as 1-SST. A double mutant of vacuolar invertase with W23Y and N25S replacements in the **W**MN**N**DPNG motif functioned as a genuine FT producing a significant amount of 1-kestose. It was concluded that switch of the enzyme from invertase to transferase activity was most probably caused by destruction of the hydrogen bond network between W23, N25 and the nucleophile, D26. This specific network is absent in all plant FTs (Schroeven *et al*., 2008; Van den Ende *et al*., 2009).

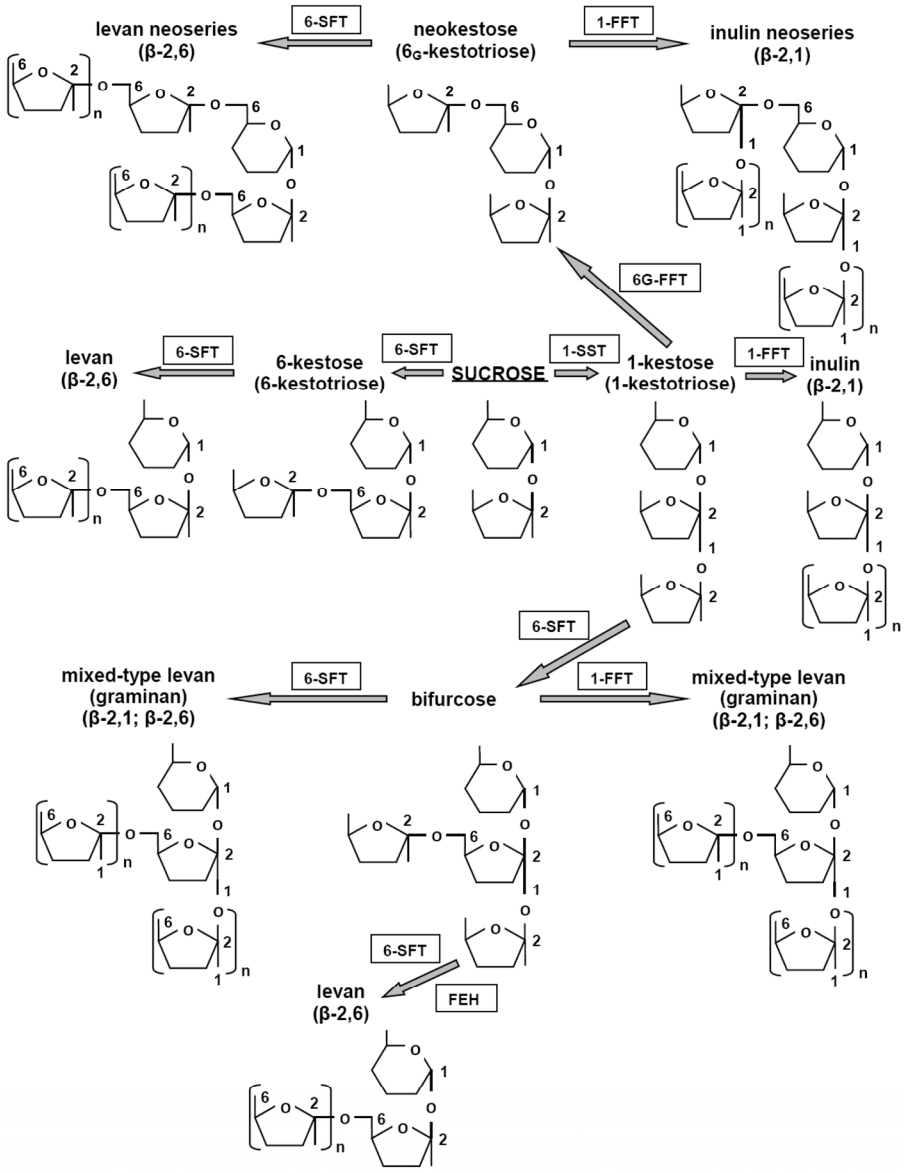

Fig. 1. Schematic representation of fructan synthesis pathways in plants. Substrates, participating enzymes, reaction products and their linkage types are indicated. Sucrose acts as the central substrate for the synthesis of fructans. 1-SST – sucrose:sucrose 1-fructosyltransferase; 6-SFT – sucrose:fructan 6-fructosyltransferase; 1-FFT – fructan: fructan 1-fructosyltransferase; 6G-FFT – fructan:fructan 6G-fructosyltransferase; FEH – fructan exohydrolase. Adapted from Altenbach and Ritsema (2007) and Livingston *et al*. (2009).

In fungi, several FTs and β-fructofuranosidases of GH32 family exist, yet levansucrases have not been detected. Respective enzymes from fungal genera *Aspergillus*, *Penicillum*, *Fusarium* and *Aureobasidium* produce mainly β-2,1-linked short-chain fructooligosaccharides (FOS) such as 1-kestose (DP 3), nystose (DP 4) and 1-fructofuranosylnystose (DP 5) (Rehm *et al*., 1998; Yanai *et al*., 2001; Sangeetha *et al*., 2004; Maiorano *et al*., 2008; Chuankhayan *et al*., 2010; Rodríguez *et al*., 2011). 1-SST gene from *Aspergillus foetidus* was expressed in an invertase-deficient mutant of *S. cerevisiae* and purified. At high sucrose concentration mainly 1-kestose, but also 6-kestose and neokestose were produced (Rehm *et al*., 1998). Interestingly, the FOS spectrum of β-fructofuranosidase from *Schwanniomyces occidentalis* is different – 6-kestose was detected as major product and 1-kestose as a side-product (Alvaro-Benito *et al*., 2010a).

Very high levels of FOS were obtained by using culture fluid or culture broth homogenate from *Aspergillus oryzae* and *Aureobasidium pullulans* as the enzyme source (Sangeetha *et al*., 2004). It has been shown that *Aspergillus niger* ATCC 20611 produces β-fructofuranosidase FopA which synthesizes inulin-type FOS from sucrose (Yanai *et al*., 2001). Industrial production of FOS relies chiefly on fungal proteins and crude enzyme preparation from *A. niger* ATCC 20611 is being used for commercial production of a prebiotic oligofructan mixture under the name of Meioligo (Meiji Seika Kaisha, Tokyo, Japan) (Hidaka *et al*., 1988).

Crystal structures of FT from *Aspergillus japonicus* CB05 and β-fructofuranosidase from *S. occidentalis* have been solved (Alvaro-Benito *et al*., 2010a; Chuankhayan *et al*., 2010). Additionally, the structures of AjFT transition-state stabilizer mutant D191A in complex with substrates sucrose, 1-kestose, nystose and raffinose have been analysed. The overall structures of these enzymes are similar to each other and also to other structures of GH32 proteins consisting of a five-bladed β-propeller domain and a C-terminal β-sandwich-like domain (Table 1) (Alvaro-Benito *et al*., 2010a; Chuankhayan *et al*., 2010).

Very interesting data were obtained for β-fructofuranosidase from *S. occidentalis*. This enzyme was shown to be a homodimeric protein with the active site located in a cleft between the two subunits. Also, for the first time, a potential catalytic role for C-terminal β-sandwich domain as substrate specificity determinant for a GH32 enzyme was suggested (Alvaro-Benito *et al*., 2010a). Based on crystal structure and mutational analysis, three catalytic residues (see Table 1) and positions which are participating in substrate and acceptor binding by *A. japonicus* and *S. occidentalis* enzymes were identified (Alvaro-Benito *et al*., 2010a; Chuankhayan *et al*., 2010). Transferase activity of the *S. occidentalis* enzyme was found to be dependent of residues N52, S196 and P232 (Alvaro-Benito *et al*., 2010b).

In addition to enzymes with fructosyl transferase activity, many fungal species also possess fructan hydrolytic enzymes *i.e*. invertases, endo- and exo-inulinases (Nagem *et al*., 2004; http://www.cazy.org).

1.2 Bacterial fructosyl transferases

1.2.1 Inulosucrases

There are two types of fructosyl transferases in bacteria – **levansucrases** and **inulosucrases**. Inulosucrases (EC 2.1.4.9) produce β-2,1-linked FOS and inulin from sucrose, whereas levansucrases synthesize β-2,6-linked fructans. So far, only lactic acid bacteria *Lactobacillus*, *Streptococcus* and *Leuconostoc* have been shown to possess inulosucrase genes and express the enzyme (Rosell and Birkhed, 1974; Olivares-Illana *et al*., 2003; http://www.cazy.org). Aside from inulin-type oligosaccharides, inulosucrases from *L. johnsonii* and *L. reuteri* also produce high molecular-weight inulin (>10^7 daltons, Da) from sucrose (van Hijum *et al*., 2002; Anwar *et al*., 2008).

Inulo- and levansucrases from lactic acid bacteria are usually large proteins. They consist of three domains: an N-terminal variable domain, an about 500 amino acid (aa) long catalytic domain and a C-terminal domain that may contain a cell wall anchor (van Hijum *et al*., 2006). Analysing the protein sequence of IslA from *Leuconostoc mesenteroides*, Olivares-Illana and colleagues suggested that due to similarity of different domains of IslA to various glycosyl transferases, it is probably a chimeric enzyme resulting from substitution of the catalytic domain of a glycosyl transferase by that of a fructosyl transferase (Olivares-Illana *et al*., 2003). This assumption is plausible because N- and/or C-terminally truncated inulosucrases retain their catalytic activity, but lose their stability (van Hijum *et al*., 2002; Olivares-Illana *et al*., 2003; Anwar *et al*., 2008; del Moral *et al*., 2008). All so far characterized inulosucrases need Ca^{2+}-ions to stabilize their structure and preserve high catalytic activity (van Hijum *et al*., 2003; Anwar *et al*., 2008; del Moral *et al*., 2008).

Catalytic residues of inulosucrase have been determined for *L. johnsonii* InuJ by site-directed mutagenesis and crystal structure analysis (Table 1) (Pijning *et al*., 2011). Active site residues of *L. reuteri* 121 inulosucrase identified by site-directed mutagenesis are D272, D424, E523 corresponding to nucleophile, transition-state stabilizer and acid-base catalyst, respectively, and amino acid positions which have a role in splitting of the substrate and transfructosylation are W271, W340, R423 (Ozimek *et al*., 2004; Ozimek *et al*., 2006). If the latter positions were mutated, total and transfructosylating activity of the inulosucrase was considerably decreased and spectrum of polymerization products was altered (Ozimek *et al*., 2006).

Despite synthesis of products with different bondage type, FTs from lactic acid bacteria share high amino-acid sequence similarity that makes identification of linkage type-specific regions difficult (van Hijum *et al*., 2006). On account of this, crystal structure of *L. johnsonii* NCC533 inulosucrase InuJ active site-containing domain in complex with the synthesis product 1-kestose was solved (see Table 1) (Pijning *et al*., 2011). As the InuJ substrate-binding pocket and binding mode of the substrate occurred almost identical to that of levansucrases, residues farther from the catalytic centre, in particular those of nonconserved 1B-1C loop, were proposed as linkage type determinants of

inulosucrase transfructosylation products. Presence of the C-terminal domain was confirmed for InuJ, but its exact fold still remains unclear (Pijning *et al*., 2011).

1.2.2 Levansucrases

Levansucrases (EC 2.4.1.10) are strictly bacterial enzymes belonging to glycoside hydrolase family GH68 (http://www.cazy.org; Cantarel *et al*., 2009). Levansucrases have been found from various bacterial species *e.g*. bacilli (*Bacillus subtilis*, *B. megaterium*, *B. amyloliquefaciens*, *B. licheniformis*), lactic acid bacteria (*Lactobacillus gasseri*, *L. reuteri*, *L. sanfranciscensis*, *L. panis*, *Leuconostoc mesenteroides*, *Streptococcus mutans*), *Erwinia amylovora*, *Pantoea agglomerans* (previously *E. herbicola*), *Gluconacetobacter* (*Acetobacter*) *diazotrophicus*, *G. xylinus*, *Zymomonas mobilis*, *Rahnella aquatilis*, *Pseudomonas chlororaphis* and *Pseudomonas syringae* pathovars (see http://www.cazy.org and http://www.brenda-enzymes.org for references).

Levansucrases use sucrose as a substrate to synthesize β-2,6-linked fructans: short-chain FOS and also polymeric levan (Lammens *et al*., 2009).

Levansucrases catalyse following reactions:
(i) hydrolysis of the substrate (sucrose): **GF + H_2O → G + F**;
(ii) polymerization of fructose residues: **nGF + acceptor → nG + F_n-acceptor**, with GF corresponding to sucrose, G to glucose and F to fructose.

Levansucrases are considered biotechnologically promising proteins because their polymerization products (levan and FOS) exhibit beneficial properties (see paragraph 2. Applications for fructans). Levansucrases are subject of experimental part of this thesis.

Size, structure and evolutionary origin
Levansucrase proteins of Gram-positive and Gram-negative bacteria differ in size. Those proteins from Gram-positive bacteria are large. For example, levansucrases from *L. sanfranciscensis* and *L. reuteri* are respectively 879 and 804 aa long (van Hijum *et al*., 2004; Tieking *et al*., 2005), whereas those of Gram-negative bacteria are typically 415-431 aa long (see Table 2). Similarily to inulosucrases of Gram-positive bacteria, their levansucrases contain (i) a signal peptide needed for protein secretion, (ii) an N-terminal stretch that varies in length, (iii) a conserved catalytic core of about 500 aa and (iv) a C-terminal region which may contain a cell wall-binding domain (van Hijum *et al*., 2006). In case of *L. reuteri* levansucrase, the N-terminal secretion leader is 36 aa long and in the C-terminus LPXTG cell-wall anchor is present that attaches the enzyme to the peptidoglycan layer (van Hijum *et al*., 2004; van Hijum *et al*., 2006). Secretion signal sequences (29 aa long) are also present in levansucrases of Gram-positive bacteria *B. subtilis* and *B. megaterium*. These proteins,

however, are not as large as levansucrases of lactic acid bacteria (see Table 2). They do not contain a sandwhich-like domain in their C-termini and are thereby structurally similar to levansucrases of Gram-negative bacteria (Meng and Fütterer, 2003; Martínez-Fleites *et al*., 2005; Homann *et al*., 2007). Most levansucrases of Gram-negative bacteria do not have N-terminal signal peptide for secretion and are therefore transported out of the cell in a signal peptide-independent pathway (Kyono *et al*., 1995; Hettwer *et al*., 1998; Song *et al*., 1998; Li *et al*., 2006). LsdA from *G. diazotrophicus* is exceptional – it is synthesized as a precursor with a 30 aa signal peptide that masters the transport of the protein to the periplasm from where it is carried through the outer membrane as a folded protein using type II secretion pathway. So, for the secretion of LsdA, an intricate two-step mechanism is used (Hernández *et al*., 1999a; Arrieta *et al*., 2004).

Even though the first enzyme for which a five-blade β-propeller fold was revealed was arabinanase Arb43A of *Cellvibrio japonicus*, all members of GH families 32 and 68 with their 3D structure available, including levansucrases, share this topology (Nurizzo *et al*., 2002; http://www.cazy.org). First high-resolution crystal structure (1.5 Å) of a levansucrase was published in 2003 (Meng and Fütterer, 2003). By now, two more structures are available (Table 1).

The catalytic centre of levansucrases is located at the bottom of central cavity and catalytic triad amino acids of the levansucrases, two aspartates and a glutamate, are conserved not only in levansucrases, but throughout the GH32 and GH68 family (see Table 1). Interestingly, comparative sequence analysis of glycoside hydrolases has detected regions conserved between the proteins from families GH32, GH68, GH43 (α-L-arabinases) and GH62 (β-xylosidases). Therefore β-fructosidase (furanosidase) superfamily of proteins was proposed and their common evolutionary origin was suggested (Naumoff, 2001).

The origin of bacterial levansucrases is still not clear. It has been proposed that at least some levansucrases have lost the C-terminal β-sandwich domain that is present in all investigated plant and microbial invertases and fructosyl transferases spearing only a robust catalytic core domain (Lammens *et al*., 2009). Summing up, levansucrases seem to be structurally more compact than other glycoside hydrolases with β-propeller fold. Computational analysis of protein sequences of FTs from various organisms suggesed that fungal and bacterial fructosyl transferases have evolved from FTs of dicot plants which in turn originated from an ancestral invertase. Still, bacterial FTs have some different sequence motifs and they cluster separately from fungal enzymes (Alméciga-Díaz *et al*., 2011).

Potential role of levansucrase for bacterial host

A wide variety of bacteria inhabiting different ecological niches possess a levansucrase. Functions for levansucrases and their reaction products have been proposed for a sugar cane symbiont *G. diazotrophicus*, a soil bacterium *B. subtilis*, phytopathogenic *E. amylovora* and *P. syringae* strains, cariogenic *Streptococcus salivarius* and *Actinomyces naeslundii* and a probiotic lactic acid

bacterium *L. reuteri* (Hernandez *et al*., 1995; Hettwer *et al*., 1995; Song and Jacques, 1999; Tambara *et al*., 1999; Bergeron *et al*., 2000; Ozimek *et al*., 2006; Smits *et al*., 2011).

Due to physical properties and chemical composition of polymeric fructans they are probably acting as extracellular energy reserve, attachment mediator and protectant against drought, temperature shift, toxic chemicals, osmotic stress. So, fructans may certainly promote survival and fitness of bacteria in the environment. Fructans have also a role in pathogenesis and symbiotic relationships between the bacterium and its host.

Some examples of physiological functions of levan and levansucrases are listed below.

1) Many bacteria which have a levansucrase also possess a levanase protein. This is the case for *A. naeslundii*, *B. subtilis*, *Geobacillus* (formerly *Bacillus*) *stearothermophilus* and *G. diazotrophicus* (Wanker *et al*., 1991; Li *et al*., 1997; Bergeron and Burne, 2001; Menéndez *et al*., 2002). Sometimes, for example in *G. diazotrophicus* and *G. stearothermophilus*, the two genes are located in one operon (Li *et al*., 1997; Menéndez *et al*., 2002). Having both, a levansucrase and a levanase, under starvation conditions a bacterium can use exogeneous reserve of levan that was produced at sucrose abundance.
2) In oral streptococci, fructans synthesized by FT enhance cariogenicity of the dental plaque. Compared to some other levansucrases, sucrose is more efficient fructosyl donor to FT of *S. salivarius.* This finding emphasizes the importance of FT for bacterial colonization of dental surface and cariogenesis (Song and Jacques, 1999).
3) Exopolysaccharides amylovoran and levan are important in plant pathogenesis by fireblight-causing bacterium *E. amylovora*. Levansucrase-negative mutants of *E. amylovora* exhibited reduced development of necrotic symptoms in pear seedlings (Geier and Geider, 1993). Mutants of *E. amylovora* unable to synthesize amylovoran and levan had severely reduced biofilm formation and pathogenesis. It was concluded that though levan contributes to biofilm formation, the *lsc*-negative mutant was still viable in plant tissues (Koczan *et al*., 2009).
4) 1-kestose produced by LsdA by a sugar cane endosymbiont *G. diazotrophicus* was suggested to serve as substrate in fructan synthesis by the plant, thus evidencing true partnership between the sugar cane and the bacterium (Hernandez *et al*., 1995). As LsdA protein produces from sucrose mostly FOS, and the amount of produced levan is very small, no clogging of plant vessels should occur due to the enzymatic activity (Hernandez *et al*., 1995; Tambara *et al*., 1999).
5) Very recently, importance of levansucrase in abiotic stress tolerance by *G. diazotrophicus* was shown. LsdA-defective mutant was less tolerant to 50–150 mM NaCl, 30% sucrose and desiccation compared to the wild-type. The ability of the mutant to form cell aggregates that is important for biofilm formation was decreased up to 59%. Complementation of the mutant with

functional levansucrase gene caused recovery of the initial phenotype (Velázquez-Hernández *et al*., 2011).

Potential importance of levansucrases for the bacteria is possibly also reflected by duplication of levansucrase gene loci in the genomes of some bacteria. Multiple, up to three copies of levansucrase genes are detected in the genomes of plant pathogenic *P. syringae* strains (for details see Results and Discussion section 2.2 *Pseudomonas syringae* pathovars posess multiple levansucrases in their genomes). On the other hand, loss of levansucrase genes from the genome has also been documented. For example, *E. amylovora* and its hypothetical ancestral strains contain genes for exopolysaccharide (*i.e*. levan) synthesis whereas *E. pyrofoliae* which has much more limited host range than *E. amylovora*, has lost its *lscC* gene (Smits *et al*., 2011).

Reaction mechanism

General reaction mechanism proposed for enzymes of GH families 32 and 68 is double displacement that is also referred to as ping-pong mechanism, acting through enzyme-fructosyl intermediate. The mechanism of polymerization reaction is summarized in Fig. 2.

First, substrate is bound to the active site of the enzyme by hydrogen-bonds, then nucleophilic attack is generated by carboxylate group against the anomeric carbon C2 of the substrate to hydrolyze the glycosidic bond (Fig. 2A). Then nucleophile (Asp) binds covalently the fructosyl residue, acid-base catalyst (Glu) donates the proton to the parting molecule whereas the transition-state stabilizer is additionally needed to stabilize the reaction (Fig. 2B). The next step involves the acceptor binding which can be water (hydrolysis reaction) or some saccharidic molecule *i.e*. sucrose or 1-kestose (transferase reaction) (Fig. 2C). To enable acceptor binding and synthesis of glycosidic bond, acid-base catalyst forms H-bond with neighbouring Arg or His residues and changes its rotameric state which is restored at release of the product (Fig. 2D) (Ozimek *et al*., 2006; Meng and Fütterer, 2008; Lammens *et al*., 2009).

According to the nomenclature of consecutive binding sites of the substrate proposed by Davies *et al*. (1997), hydrolysis of the glycosidic bond takes place between -1 and +1 subsites whereas fructosyl residue locates at -1 subsite. Acceptor binds starting from +1 subsite of the enzyme (Fig. 2) (Ozimek *et al*., 2006; Meng and Fütterer, 2008; Lammens *et al*., 2009). From structural comparison of molecular surfaces of studied enzyme-substrate complexes it was proposed that topology of -1 subsite is conserved between GH32 and GH68 enzymes, but shapes and sizes of following +1, +2, +3 pockets vary to some extent (Chuankhayan *et al*., 2010).

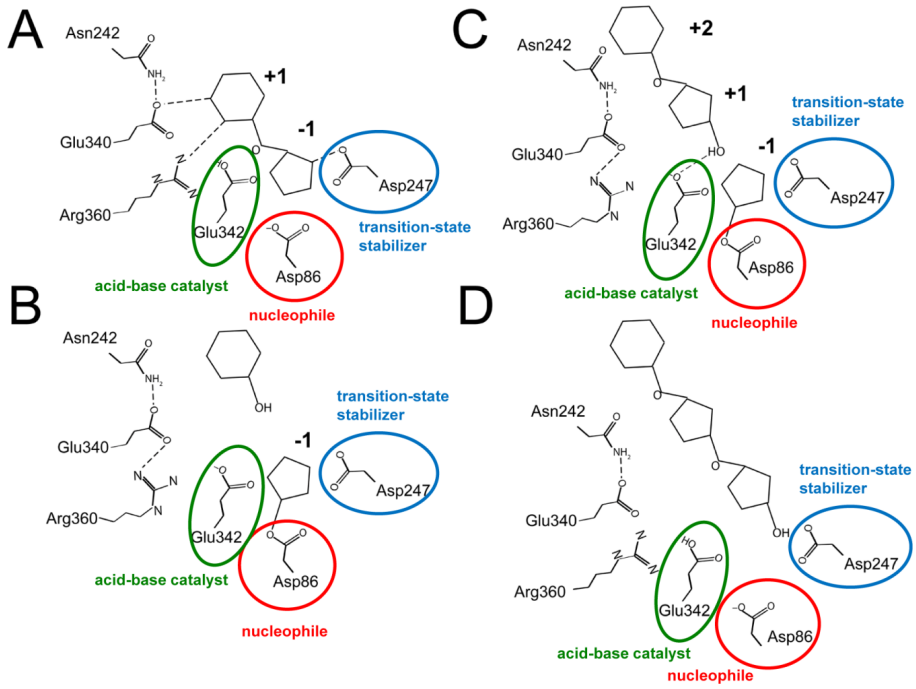

Fig. 2. Schematic representation of reaction mechanism of the levansucrase SacB from *B. subtilis*. The initial complex is formed with the substrate of fructosyl donor, the nucleophile Asp86 is deprotonated while the general acid Glu342 is in protonated state (A). Next, hydrolysis of the glycosidic bond is carried out, the glucose moiety is released and the fructosyl is covalently bound to the nucleophile. Arg360 takes the alternative rotamer state and forms an ionic interaction with Glu340 (B). Binding of the acceptor substrate, in this case second sucrose molecule, is mediated by Arg360 and Asn242. Glu342 is deprotonated and ready to activate the terminal hydroxyl of the acceptor for nucleophilic attack onto enzyme-bound fructosyl (C). The product, 6-kestose is released. Arg360 returns to original conformation (D). Selected non-covalent interactions are indicated by dashed lines. Numbers in bold indicate the substrate-binding subsites of the enzyme. Adapted from Meng and Fütterer (2008).

Biochemical properties

The main substrate and fructosyl donor of levansucrases is sucrose (α-D-Glc*p*-(1→2)β-D-Fru*f*) (Fig. 3). Still, affinities of various enzymes to sucrose differ in quite wide range. The K_m for sucrose of wild-type SacB from *B. megaterium* is as low as 6.6 mM (Homann *et al*., 2007). At the same time, respective values for levansucrases of *Z. mobilis* and *P. syringae* pv. phaseolicola are 125 and 160 mM (Table 2) (Hettwer *et al*., 1995; Yanase *et al*., 2002). Catalytic efficiency (k_{cat}/K_m) which is also dependent on maximum reaction velocity (V_{max}), varies to a great extent between the levansucrases. Higest recorded k_{cat}/K_m value for sucrose, 346 mM^{-1} s^{-1} (2.076 x 10^7 M^{-1} min^{-1}), belongs to SacB from *B. megaterium* (Homann *et al*., 2007). The respective value for SacB of *B. subtilis* is 20.3 mM^{-1} s^{-1} (1.218 x 10^6 M^{-1} min^{-1}) and those for levansucrases from Gram-

negative bacteria are for some reason much lower. So, LevU protein of *Z. mobilis* has k_{cat}/K_m value 0.23 mM^{-1} s^{-1} (1.36 x 10^4 M^{-1} min^{-1}) (Table 2) (Yanase *et al*., 2002; Ortiz-Soto *et al*., 2008). These differences may rely on specific properties of respective enzyme, but also on reaction conditions and purity of the enzyme.

In addition to sucrose, many levansucrases *e.g*. those from *Z. mobilis*, *L. reuteri*, *L. gasseri*, *G. diazotrophicus* and *B. subtilis* can use a trisaccharide raffinose (α-D-Gal*p*-(1→6)α-D-Glc*p*-(1→2)β-D-Fru*f*) (Fig. 3) (Sangiliyandi *et al*., 1999; Yanase *et al*., 2002; Trujillo *et al*., 2004; van Hijum *et al*., 2004; Seibel *et al*., 2006; Anwar *et al*., 2010). The affinities of the enzyme for sucrose and raffinose differ. For example, affinity of *Z. mobilis* levansucrase for raffinose is 84 mM, thus being higher than that for sucrose (Sangiliyandi *et al*., 1999; Yanase *et al*., 2002). Crystal structure analysis of *B. subtilis* levansucrase acid-base catalyst mutant with bound raffinose indicated that galactose residue of raffinose is protruding out from the active site, has minimal contacts with the enzyme and therefore does not interfere with binding of the sucrose moiety of the raffinose (Meng and Fütterer, 2008). As the *Z. mobilis* enzyme has not been crystallized and the binding mode of raffinose in the active site is not known, the reason for high affinity of this enzyme for raffinose remains to be elucidated.

Not much is known on action of levansucrases on a tetrasaccharide stachyose (α-D-Gal*p*-(1→6)-α-D-Gal*p*-(1→6)α-D-Glc*p*-(1→2)β-D-Fru*f*) (Fig. 3). Only couple of reports show that a levansucrase may also act on stachyose (Yanase *et al*., 2002; Teixeira *et al*., 2012). In fact, levansucrase from *Z. mobilis* was shown to produce reducing sugars from stachyose as efficiently as from sucrose and therefore providing the evidence of stachyose as being a suitable substrate for the enzyme (Yanase *et al*., 2002).

Hydrolysis of levansucrase polymerization products *i.e*. oligofructans and levan by the enzymes that produce them has also been detected. Levansucrases of *B. subtilis*, *P. syringae* pv. phaseolicola and *Z. mobilis* hydrolyze polymeric levan (Chambert and Petit-Glatron, 1993; Hettwer *et al*., 1995; Jang *et al*., 2007). Still, the activity on levan is very low compared to sucrose-splitting activity. It is usually less than 1% of sucrose hydrolysis activity (Yanase *et al*., 2002; Jang *et al*., 2007). 1-kestose and nystose can also be hydrolyzed by a levansucrase, but only with very low activity (Yanase *et al*., 2002).

The main biochemical properties of selected bacterial levansucrases *i.e*. K_m for sucrose, catalytic constant (k_{cat}), catalytic efficiency (k_{cat}/K_m), temperature and pH optimums and ability to use raffinose are presented in Table 2.

Levansucrases from Gram-positive bacteria *e.g*. those of bacilli and lactobacilli need Ca^{2+}-ions for activity. The Ca^{2+}-ion is bound to levansucrase protein and most probably acts as enzyme structure stabilizer (Meng and Fütterer, 2003; van Hijum *et al*., 2004; Ozimek *et al*., 2005). It is proved that Ca^{2+} is coordinated by a strictly conserved Asp residue located in **D**(E/Q)(T/I/V)ER (also referred to as DXXER) motif in a close proximity of Glu acting as acid-base catalyst (Meng and Fütterer, 2003). Although this

sequence motif is present in all levansucrases, Ca^{2+}-ions are not needed for catalytic activity of enzymes from Gram-negative bacteria (Martínez-Fleites *et al*., 2005; Ozimek *et al*., 2005). LsdA of *G. diazotrophicus* contains a disulphide bridge between Cys339-Cys395 residues that links the third and fourth blade of the β-propeller structure. The replacement of either Cys339 or Cys395 by a serine reduced the k_{cat} for sucrose hydrolysis approximately 60 times. The disulphide bridge in LsdA is therefore equivalent to Ca^{2+}-binding site of levansucrases from Gram-positive bacteria and probably acts similarly as a fold-stabilizer (Martínez-Fleites *et al*., 2005).

Intriguingly, it was discovered that the levansucrase from *Acetobacter nitrogenifigens* $RG1^T$ is stabilized by Hg^{2+}-ions and exhibits considerably increased hydrolytic and polymerization activity when these ions are present. The authors speculate that due to the presence of mercury ions, the active site of the enzyme is rearranged to a more compact form and thus the enzyme reacts with the substrate and retains its conformation even at high temperatures (Paul *et al*., 2011). However, in case of some other levansucrases such as LsdA from G. *diazotrophicus* and Lev from *L. reuteri*, Hg^{2+}-ions strongly inhibit levansucrase reaction retaining only 4% and 3.4% of respective catalytic activity (Hernandez *et al*., 1995; van Hijum *et al*., 2004).

As levansucrases are extracellular enzymes in their host bacteria, they should be stable and maintain catalytic activity at harsh environmental conditions for a long period of time. Accodingly, resistance against metal ions, detergents, elevated temperature and pH among various levansucrases has been demonstrated (Hernandez *et al*., 1995; Hettwer *et al*., 1995; Sangiliyandi *et al*., 1999; Ben Ammar *et al*., 2002; Rairakhwada *et al*., 2010). Also, levansucrase from *P. syringae* pv. phaseolicola can be stored at 4ºC for 6 months with no loss of activity detectable (Hettwer *et al*., 1995).

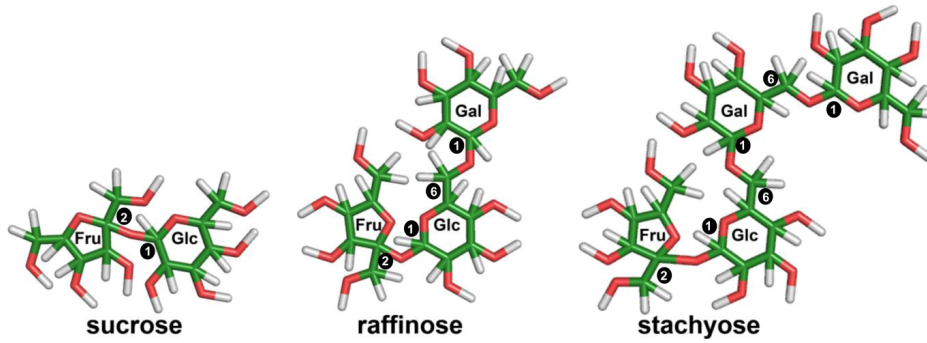

Fig. 3. 2D structures of levansucrase substrates sucrose, raffinose and stachyose. Sugar residues and positions engaged in glycosidic linkage are indicated. Fru corresponds for fructose, Glc to glucose and Gal to galactose. Carbon atoms are marked by green, oxygens by red and hydrogens by gray colour. Structures of the molecules were retrieved from Human Metabolome Database (http://www.hmdb.ca; Wishart *et al*., 2009) and visualized by PyMOL version 0.99 (DeLano, 2002).

Reaction products

Reaction products of levansucrases are β-2,6-linked fructans of various chain length. Major reaction product of *G. diazotrophicus*' levansucrase is a trisaccharide kestose (1-kestose), but most levansucrases, including those of *B. subtilis*, *Z. mobilis*, *L. reuteri* and *R. aquatilis* produce both, FOS and levan (Hernandez *et al*., 1995; Kim *et al*., 1998; Tambara *et al*., 1999; Bekers *et al*., 2002; Ozimek *et al*., 2006). Bacterial levans mostly have a very high DP and molecular mass. For example, β-2,6-linked fructans produced by *L. reuteri* strain 121 can be divided to two fractions of different molecular weigth – 150 kDa and >2 MDa (van Hijum *et al*., 2001). Whereas majority of studied levans are linear low-branched ones, *S. salivarius* produces levan with 30% of β-2,1 side-branches (Newbrun and Baker, 1968; van Hijum *et al*., 2001; Jang *et al*., 2006).

Although the fructosyl donor substrate range is limited to sugars which contain sucrose moiety (see Fig. 3), the spectrum of potential fructosyl acceptors is much wider. If water acts as an acceptor, hydrolysis of sucrose to glucose and fructose proceeds. In case of transfructosylation of sucrose, kestose (GF_2) is produced that can be further elongated in subsequent transfructosylation reactions. In addition to sucrose and fructans, levansucrases can use nonconventional fructosyl acceptors to produce heterooligofructans (HOF). This subject has been thoroughly studied for levansucrase from *B. subtilis*. Using ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy analysis of transfructosylation products, many novel alternative acceptors for SacB were detected: D- and L-galactose, D- and L-xylose, D- and L-fucose, isomaltose, maltose, melibiose, cellobiose, lactose, mannose, 2-deoxy-D-glucose, D-allose, 3-ketoglucose, L-glucose, L-rhamnose. Also, sucrose analogues α-Xyl-1,2-β-Fru and α-Gal-1,2-β-Fru acted as acceptors in transfructosylation reaction (Seibel *et al*., 2005; Seibel *et al*., 2006; Beine *et al*., 2008).

In addition to saccharidic acceptors, some levansucrases were shown to transfructosylate alcohols. By conventional electrospray ionization (ESI) mass spectrometry (MS) it was confirmed that levansucrase of *R. aquatilis* synthesizes methyl-fructoside from sucrose and methanol (Kim *et al*., 2000). Formation of alkyl fructosides by the enzyme was detected if ethanol, ethylene glycol or propylene glycol were serving as acceptors (Kim *et al*., 1998).

Recently it was discovered that the *B. subtilis* levansucrase can also transfructosylate aromatic and aliphatic alcohols such as hydroquinone, 4-hydroxybenzyl alcohol, benzyl alcohol, catechol and resorcinol (Mena-Arizmendi *et al*., 2011). This type of novel glycosidic products could serve as antioxidants or therapeutic agents.

Table 2. Comparison of biochemical properties of selected levansucrases. Enzymes which possess N-terminal secretion signal are marked with an asterisk. Data of protein length were obtained from UniProtKB/Swiss-Prot database (http://www.uniprot.org).

Name of the enzyme, organism	**Protein length (aa)**	**K_m (sucrose; mM)**	**Raffinose hydro-lysis**	**k_{cat} (s^{-1})**	**k_{cat}/K_m (mM^{-1} s^{-1})**	**Optimal pH**	**Optimal temperature (ºC)**		**References**
							Sucrose-splitting	Levan synthesis	
SacB of *B. subtilis*	473*	8	+	164.6	20.3	6	ND	30	Abdel-Fattah *et al*., 2005 Seibel *et al*., 2006 Ortiz-Soto *et al*., 2008
SacB of *B. megaterium*	484*	6.6	ND	2272	346	6.6	45	ND	Homann *et al*., 2007
Lev of *L. reuteri*	804*	9.7	+	147	15.1	4.5-5.5	50	ND	van Hijum *et al*., 2004
LsdA of *G. diazotrophicus*	584*	11.4	+	60	5.3	5	60	ND	Batista *et al*., 1999 Trujillo *et al*., 2004
LevU (or SacB) of *Z. mobilis*	423	125	+	28.3	0.23	5	50	30	Sangiliyandi *et al*., 1999 Yanase *et al*., 2002
Lsc of *P. syringae* pv. phaseolicola	431	160	-	ND	ND	5.8-6.6	60	18	Hettwer *et al*., 1995
LsrA of *R. aquatilis*	415	50	ND	ND	ND	6	50	20	Ohtsuka *et al*., 1992 Song *et al*., 1998

ND, not determined

Mutational analysis and structure-function studies

The active centre of levansucrases resides at the bottom of the funnel that is located in the central part of the β-propeller fold (Meng and Fütterer, 2003; Martínez-Fleites *et al*., 2005; Meng and Fütterer, 2008). Fructose residue of sucrose molecule binds at the bottom of the pocket followed by glucose residue binding on top of it (Fig. 2) (Ozimek *et al*., 2006).

Positions of catalytic triad residues (two aspartates and a glutamate) of LevU from *Z. mobilis*, SacB of *B. megaterium*, SacB of *B. subtilis*, LsdA of *G. diazotrophicus* and Lev of *L. reuteri* have been confirmed experimentally by site-directed mutagenesis (see also Table 1). Most of respective mutants were incapable of levansucrase reaction or showed drastically reduced catalytic activity (Batista *et al*., 1999; Yanase *et al*., 2002; Ozimek *et al*., 2004; Martínez-Fleites *et al*., 2005; Homann *et al*., 2007; Meng and Fütterer, 2008; Strube *et al*., 2011).

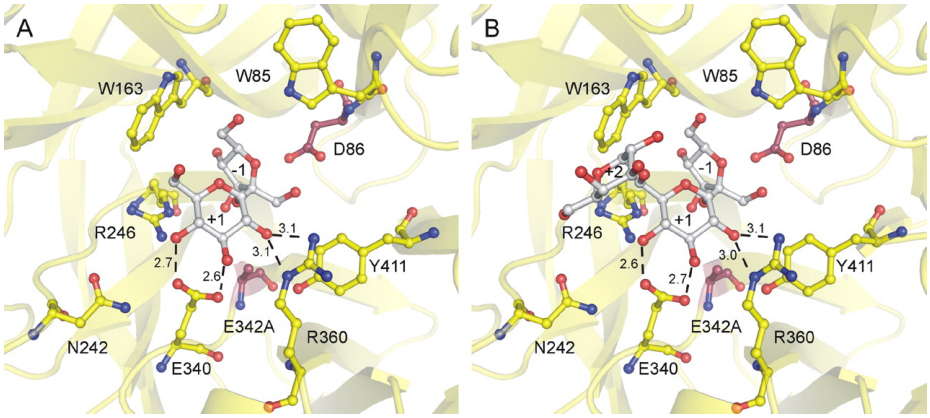

Fig. 4. Active site of *B. subtilis* levansucrase mutant E342A in complex with sucrose (PDB code: 1PT2) (A) and with raffinose (PDB code: 3BYN) (B). Active site residues are coloured in purple. Important amino acid positions for substrate-binding are indicated. Distances are measured in Å. H-bonds are shown as dashed lines. The figure is extracted from Lammens *et al*. (2009).

In *B. subtilis* levansucrase, Glu340 and Glu342 of the conserved D**E**I**E**R motif as well as Arg360 belong to +1 subsite of the substrate-binding pocket. Arg360 and Glu340 form tight hydrogen bonds with hydroxyls of the glucose residue fixing it in a proper orientation that is needed for further reactions (Fig. 4) (Meng and Fütterer, 2008; Lammens *et al*., 2009). Substitution of R360 with H, K, L or S in SacB resulted in significant decrease of catalytic constant and affinity to sucrose. Only oligosaccharides and no polymeric levan were formed as reaction products. Therefore participation of R360 in +1 subsite and a key role in polymerization reaction was confirmed (Chambert and Petit-Glatron, 1991; Ortiz-Soto *et al*., 2008).

2. Applications for fructans

Prebiotics, including fructans of different DP are food ingredients that are potentially beneficial to the health of consumers (Gibson and Roberfroid, 1995). According to Gibson and coworkers (2004), prebiotic is defined as a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confers benefits upon host well-being and health (Roberfroid *et al*., 2010). FOS and dietary fibers *i.e*. inulin are considered as prebiotic agents, because they are selectively metabolized by **probiotic bacteria**, but not by pathogens and are not digested by enzymes of upper digestion tract of humans and animals (Roberfroid *et al*., 1998; Grizard and Barthomeuf, 1999; Bielecka *et al*., 2002; Lomax *et al*., 2012). FOS are so far most thoroughly studied group of prebiotics (Meyer and Stasse-Wolthuis, 2009). A stimulating effect of fructans on beneficial bifidobacteria and lactobacilli has been extensively recorded according to *in vitro* and *in vivo* trials (Tokunaga *et al*., 1993; Gibson and Roberfroid, 1995; Fooks and Gibson, 2002; Roberfroid *et al*., 1998; Biedrzycka and Bieleca, 2004; Roberfroid *et al*., 2010; Van den Abbeele *et al*., 2011; Lomax *et al*., 2012). For example, based on results obtained by quantitative real-time PCR, administration of inulin to healthy volunteers for 16 days increased significantly the levels of *Bifidobacterium adolescentis* and *B. bifidum* in the gut (Ramirez-Farias *et al*., 2009).

Fermentation of fructans by probiotic bacteria in the colon produces a mixture of short-chain fatty acids (acetate, propionate, butyrate and lactate) that have positive effect on gut health. Therefore stimulation of probiotic bacteria may have several implications for human or animal health. Protective effects of lower-DP fructans against colorectal cancer and inflammatory bowel disease, reduction of cholesterol and blood sugar levels, increase of mineral absorption and immune system stimulation of the host have been shown or suggested (Grizard and Barthomeuf, 1999; Bornet *et al*., 2002 and references therein; Lobo *et al*., 2006; Azorín-Ortuño *et al*., 2009).

Inulin and FOS can also have applications in biotechnology and food processing. Inulin is already added to various food products (juices, yoghurt, bread, pet food *etc*.). Also, inulin has been proposed as fat replacer in desserts and sausages (Mendoza *et al*., 2001; Arcia *et al*., 2011). Short-chain FOS can be considered as alternative low-calory sweeteners instead of sucrose because of similar taste profile (Bornet *et al*., 2002).

Fructan-based prebiotics can be obtained in two main ways: (i) by their direct extraction from plants (*e.g*. inulin from chicory root), chemical or enzymatic hydrolysis of plant-derived polysaccharides, (ii) by enzymatic synthesis from sucrose using fructosyl transferases (including levansucrases) from various sources (Roberfroid *et al*., 1998; Grizard and Barthomeuf, 1999; Bornet *et al*., 2002; Lammens *et al*., 2009). In Latvia, a useful system for FOS production by *Z. mobilis* using "levan-levasucrase" sediment has been invented and patented. In this procedure, extracellular levansucrase and levan from

fermentation broth of *Z. mobilis* are co-precipitated by ethanol and used as crude catalyst for FOS production. The resulting fructan syrup contains a high amount of 1-kestose, 6-kestose, neokestose and nystose (Bekers *et al*., 2003).

2.1 Implementation of fructooligosaccharides in technology

Fructans with low DP (2-9) are selective carbon sources for probiotic bifidobacteria. For example, FOS and low-polymerized inulin stimulated growth of *B. longum* and *B. animalis* 2-5 times more compared to lactose. Increase of bifidobacterial numbers was also recorded in case of *in vivo* administration of FOS to rats and humans (Bielecka *et al*., 2002; Ramirez-Farias *et al*., 2009; Lomax *et al*., 2012). Therefore FOS are considered to act as prebiotics and are recommended as supplements in functional food. For example, the BENEO-Orafti company produces several fructose-containing compounds for food industry from inulin extracted from plants. According to the manufacturer, these fructan preparations improve health-related properties of bread, breakfast cereals, candy bars, dairy desserts, cream cheese, pasta and processed meat products (http://www.orafti.com).

We suggest that FOS synthesized by levansucrases may also find application in food technology as prebiotics. According to the investigations, levan-type β-2,6-linked FOS as well as neo-series FOS (neokestose) exert improved prebiotic activity compared to inulin-type β-2,1-linked FOS (Marx *et al*., 2000; Kilian *et al*., 2002).

It has been shown that several fructosyl transferases are capable of producing sucrose analogs and HOF of different DP (Tieking *et al*., 2005; Baciu *et al*., 2005; Seibel *et al*., 2005; Seibel *et al*., 2006; Han *et al*., 2007; Beine *et al*., 2008; Han *et al*., 2009). Those compounds were shown as potential substrates for enzymatic synthesis of novel saccharides, but they may also serve as prebiotics with extended bifidogenic effect. For example, levansucrases synthesize prebiotic lactosucrose (galactosyl-fructoside) and potentially prebiotic xylo-oligofructans (Baciu *et al*., 2005; Han *et al*., 2007; Beine *et al*., 2008; Han *et al*., 2009; Gänzle, 2012).

2.2 Potential biotechnological and medical applications of high-molecular levan

Even though no extensive prebiotic effect for highly polymerized levan has been recorded, different potential applications for levan *e.g*. functioning as industrial gum, blood plasma extender, emulsifier, water-binding agent, stabilizer, thickener, surface-finishing agent, encapsulating material, carrier for flavour and fragrances have been suggested (Bekers *et al*., 2005; Beine *et al*., 2008; Poli *et al*., 2009).

In medicine, levan has been recommended to be used in case of skin disorders as an anti-inflammatory agent to reduce irritation, a moisturizer and a

cell proliferation-promoting substance. All these effects were verified on 3D artificial skin (Kim *et al*., 2005). Additionally, *in vitro* anti-tumor activity of levans has been shown against eight different tumor cell lines, whereas tumor-reducing activity of levans in case of sarcoma and carcinoma was reported in mice (Calazans *et al*., 1997; Calazans *et al*., 2000; Yoo *et al*., 2004). Notably, bacterial levan has no negative effect on normal cells. So, levan produced by *Halomonas* sp. AAD6 did not affect viability and proliferation of osteoblasts and murine macrophages (Poli *et al*., 2009). Interestingly, hypocholesterolemic and hypoglycemic effects of levan in rats have also been observed (Yamamoto *et al*., 1999; Dahech *et al*., 2011).

Levan can also be considered as anti-cytotoxic agent, because low concentrations of levan protected brine shrimp against toxic effects of avarol (Poli *et al*., 2009). Also, levan is protecting organisms from bacterial infection. So, levan synthesized by *B. amyloliquefaciens* prevented *Aeromonas hydrophila* infection of carp and acted as effective immunostimulant (Rairakhwada *et al*., 2007). Interestingly, acetylated, phosphorylated and benzylated levan-type exopolysaccharide from *Paenibacillus polymyxa* EJS-3 was proven *in vitro* as antioxidant and anti-tumor agent of increased efficiency (Liu *et al*., 2012). Therefore, derivatives of levan most probably have promising potential in medical applications whereas the possible toxicity or side-effects of those compounds should be clarified.

Magnetic levan particles with Fe^{2+} and Fe^{3+} have been obtained and succesfully used for trypsin immobilisation (Maciel *et al*., 2012). Therefore levan and its magnetized forms may serve as alternative immobilisation agents for enzymes of interest.

II RESULTS AND DISCUSSION

1. Aims of the study

The main aim of present thesis is to enlighten the entity of levansucrases from *Pseudomonas* bacteria. Levansucrase genes and proteins from these bacteria are rather scarcely studied and not much is known of their biochemical properties, product spectrum and biotechnological applications. Some research has been carried out to reveal the potential role of levansucrases from pseudomonads in plant pathogenesis, to investigate allelic spectrum of levansucrases in *P. syringae* pathovars and expression of these genes in native host (Li and Ullrich, 2001; Laue *et al*., 2006; Li *et al*., 2006). Levansucrase genes of *P. syringae* pv. glycinea and pv. phaseolicola have been cloned and expressed in *Escherichia coli* (Hettwer *et al*., 1998; Li and Ullrich, 2001). Before our work, only one levansucrase protein, that of *P. syringae* pv. phaseolicola was purified and biochemically characterized (Hettwer *et al*., 1995).

My study was focused on heterologous expression and characterization of levansucrases from the bacterium *P. syringae* pv. tomato DC3000 and their reaction products. As a reference, I studied levansucrase from a plant-associated bacterium *P. chlororaphis* subsp. *aurantiaca*. While so far no mutational analysis or structure-function study concerning levansucrases from *Pseudomonas* bacteria has been carried out, mutational approach was implemented to reveal important positions in one of the levansucrases, Lsc3 of *P syringae* pv. tomato DC3000.

The aims of my work can be summarized subsequently:

1. To test if the promoter of the maltase gene from yeast *Hansenula polymorpha* can be applied to produce a foreign protein of interest in a bacterial expression system.
2. To elaborate a serviceable expression system for the synthesis of an adequate amount of recombinant *P. syringae*-derived levansucrase protein in *E. coli*.
3. To clone, express, produce and purify levansucrases from *P. syringae* pv. tomato DC3000 for their further investigation.
4. To biochemically characterize the levansucrases and their products.
5. To initiate structure-function studies of the Lsc3 protein of *P. syringae* pv. tomato.

2. Maltase gene promoter (P_{MAL1}) from *Hansenula polymorpha* is feasible for the production of *Pseudomonas syringae* levansucrases in *Escherichia coli*

Previously our research group has been investigating the *MAL* gene cluster that is responsible for the utilization of disaccharides, maltose and sucrose, in a methylotrophic yeast *Hansenula polymorpha* (synonym *Pichia angusta*). This gene cluster contains maltase, maltose permease and at least one *MAL* activator gene (Alamäe *et al*., 2003; Viigand *et al*., 2005; Viigand and Alamäe, 2007). When cloning the maltase gene (*HpMAL1*; AL432586) of *H. polymorpha* in *E. coli*, a high maltase activity in *HpMAL1*-possessing *E. coli* transformants was observed. It indicated that the promoter (P_{MAL1}) of the maltase gene was functional in a bacterial host (Liiv *et al*., 2001). The feasibility of yeast promoters to operate in a prokaryotic expression system has been barely described, but there are some cases to be marked out. For example, auxotrophic marker genes *LEU2* and *URA3* of *Candida boidinii* have been cloned by complementation of respective *E. coli* mutants and upstream of the genes, sequences homologous to *E. coli* promoter consensus -35 and -10 regions were detected (Sakai *et al*., 1991; Sakai and Tani, 1992; Sakai *et al*., 1992). Intriguingly, genomic DNA sequences of *Saccharomyces cerevisiae* are prone to serve as promoter regions in a bacterial recipient. So, about one half of randomly selected *S. cerevisiae* genomic DNA sequences enabled a considerable expression of a reporter gene in *E. coli*. The existence of potential transcription start sites for bacteria (-10 and -35 consensus for sigma-70 protein) in those sequences was confirmed (Lewin *et al*., 2004).

Several dual expression plasmids and shuttle vectors that function in both, eu- and prokaryotic host have been elaborated. For example, an expression plasmid with a hybrid promoter consisting of the T7 promoter and yeast alcohol oxidase promoter (P_{AOX}) for heterologous gene expression in both, *E. coli* and *Pichia pastoris* has been constructed (Lueking *et al*., 2000). In our previous studies, we have used *H. polymorpha*/*E. coli* shuttle vectors pHIPX8 and pX4-HNBESX (obtained from Dr. J. Kiel, University of Groningen, The Netherlands) which replicate in the bacterium and in the yeast and enable expression of the cloned gene in *H. polymorpha* from P_{TEF2} (promoter of the translation elongation factor 1-alpha gene) or P_{MOX1} (the promoter of the methanol oxidase gene *MOX1*) (Gietl *et al*., 1994), respectively. As in these plasmids the gene to be expressed is under control of a yeast promoter, they should not enable expression of a foreign protein in *E. coli*.

In present study, a new *H. polymorpha*/*E. coli* shuttle vector pHIPMalprom was constructed by replacement of the P_{TEF2} promoter in pHIPX8 with P_{MAL1} in order to investigate functionality and potency of the maltase gene promoter in *E. coli* using levansucrases from *P. syringae* DC3000 as foreign test proteins to be expressed (Ref. I).

2.1 Maltase gene promoter functions in *E. coli* due to σ70-like boxes (Ref. I)

Sigma 70-like hexamers are present in P_{MAL1}: -10 and -35 consensus boxes were detected at positions -310 to -282 (**TTGACA**-N17-**TA**A**AT**T) and -213 to -185 (GGT**ACA**-N17-**TAT**T**AT**) relative to the start codon of the maltase gene (Liiv *et al*., 2001). Nucleotides identical to the *E. coli* σ70 consensus TTGACA-N17-TATAAT (Schumann and Ferreira, 2004) in these boxes are marked by bold font.

To experimentally confirm functional significance of these predicted sigma 70-like boxes, we studied expression from P_{MAL1} and two reference promoters (P_{MOX1} and P_{TEF2}) of *H. polymorpha* in *E. coli*. In P_{MOX1} and P_{TEF2} no σ70-like sequences were detected (Ref. I). In our promoter testing assay we used *H. polymorpha HpMAL1* as a reporter gene since *E. coli* lacks endogenous maltase activity and a simple and cheap chromogenic maltase assay is available (Liiv *et al*., 2001). The reporter plasmids containing the full length (pHIPX8-p51SpeI-SmaI and p51) or truncated (p51MunI and p51HindIII-MunI) variants of P_{MAL1} and unaltered P_{MOX1} and P_{TEF2} were transformed into *E. coli*. Maltase activity was determined and performance of the promoter sequences was evaluated (Table 1 in Ref. I). P_{MOX1} is perhaps the most powerful inducible yeast promoter and it has been widely used for high-level recombinant protein production (van Dijk *et al*., 2000; Dueñas-Sánchez *et al*., 2010). P_{TEF2} is a constitutive and strong promoter in yeasts. In *H. polymorpha*, the strength of P_{TEF2} is similar to P_{HXK1} (promoter of the hexokinase gene of *H. polymorpha*) and sucrose-induced P_{MAL1} is about two times stronger than these two promoters (our unpublished data). It appeared that in contrast to P_{MAL1}, strong yeast promoters P_{TEF2} and P_{MOX1} did not function in *E. coli* (Ref. I). Transformants harboring reporter constructs with full-length P_{MAL1} or its truncated variants with σ70-like sequences still retained, exhibited high maltase activity. Truncated variant of P_{MAL1} with σ70-like sequences excluded could not promote maltase expression in *E. coli* (Ref. I). These results strongly suggest functional significance of predicted σ70 boxes of P_{MAL1} for gene expression in *E. coli*. We have shown earlier that P_{MAL1} functions also in baker's yeast (Alamäe *et al*., 2003). So, we consider that P_{MAL1} can be used for the construction of wide host-range expression vector to select the most suitable host organism for the expression of the protein of interest.

2.2 *P. syringae* pathovars possess multiple levansucrases in their genomes (Ref. I; Ref. IV)

Bacterial genomes usually encode a single levansucrase protein. According to available genomic sequences (http://www.ncbi.nlm.nih.gov/), levansucrase-possessing bacteria such as *G. diazotrophicus*, *B. subtilis* and *Z. mobilis* harbor only one levansucrase gene. Interestingly, plant-associated *P. syringae* pathovars have up to three levansucrase genes in their genomes. Data concerning

distribution of *lsc* alleles in *P. syringae* strains were retrieved from GenBank and are presented in Table 3 (see also Ref. I). Additionally, presence of levansucrase alleles in various *P. syringae* strains has been determined by PCR-screening (Li and Ullrich, 2001).

P. syringae strains are divided to more than 50 pathovars according to their specific host plant. Several of *P. syringae* host plants are important crops (tomato, bean, soybean, cucumber, cauliflower *etc*.) and so *P. syringae* infection may cause severe economical loss (Hwang *et al*., 2005; Cai *et al*., 2011). In the chromosomes of various of *P. syringae* pathovars, 1296 base pair (bp) and 1248 bp variants of levansucrase genes are found, whereas an additional 1296 bp gene copy may reside on a plasmid (Table 3). In case of *P. syringae* pv. tomato, *lsc3* is located on one of the two plasmids present in the strain. Pathovars with no plasmids such as *P. syringae* pv. syringae B728a contain two genes that encode proteins of 431 and 415 aa. Whereas most of characterized *P. syringae* strains contain plasmids (Baltrus *et al*., 2011), the occurrence of additional plasmid-born *lsc* genes is plausible. Most probably the number of *lsc* alleles in *P. syringae* strains (see Table 3) will increase because according to GenBank data several genomes of *P. syringae* pathovars have yet only draft status and in many cases merely partial sequences of *lsc* genes are available (Baltrus *et al*., 2011; http://www.pseudomonas-syringae.org). To date, the importance of multiple levansucrases for a bacterium nor their specific roles in pathogenesis are not clear yet (Srivastava *et al*., 2012).

Table 3. Allelic distribution of levansucrase variants from *P. syringae* species. Fully completed genomes are indicated by an asterisk.

***P. syringae* pathovar and strain**	**Gene name, locus tag**	**Gene length (bp)**	**Genomic location**	**Protein length (aa)**	**References**
pv. tomato DC3000*	*lsc-1*, PSPTO_1453	1296	C	431	Buell *et al*., 2003
	lsc-2, PSPTO_2305	1248	C	415	
	lsc-3, PSPTO_A0032	1296	P (pDC3000 A)	431	
pv. tomato T1	*lsc-1*, PSPTOT1_1070	1296	C	431	Almeida *et al*., 2009
	lsc-2, PSPTOT1_4965	1248	C	415	
	lsc-3, PSPTOT1_4913	1296	ND	431	

Table 3. Continuation.

***P. syringae* pathovar and strain**	**Gene name, locus tag**	**Gene length (bp)**	**Genomic location**	**Protein length (aa)**	**References**
pv. tomato K40	*lsc*, PsyrptK_010100027584	1296	ND	431	Vinatzer *et al*. (unpublished) GenBank
pv. tomato NCPPB 1108	*lsc*, PsyrptN_010100027628	1248	ND	415	Vinatzer *et al*. (unpublished) GenBank
pv. syringae B728a*	*lsc*, Psyr_2103	1248	C	415	Feil *et al*., 2005
	lsc, Psyr_0754	1296	C	431	
pv. phaseolicola 1448A*	*lsc*, PSPPH_2074	1248	C	415	Jordar *et al*., 2005
	lscC, PSPPH_4994	1296	C	431	
	lsc, PSPPH_A0027	1296	P	431	
pv. glycinea race 4	*lsc*, PsgRace4_15609	1248	ND	415	Qi *et al*., 2011
	lscC, PsgRace4_03819	1296	ND	431	
pv. glycinea B076	*lsc*, PsgB076_10300	1248	ND	415	Qi *et al*., 2011
	lscC, PsgB076_00457	1296	ND	431	
pv. glycinea PG4180	lscA	1248	C	415	Hettwer *et al*., 1998 Li and Ullrich, 2001
	lscB	1296	P	431	
	lscC	1296	C	431	
pv. aesculi 2250	*lsc*, Psyrpa2_010100023522	1248	ND	415	Green *et al*., 2010
pv. aesculi NCPPB3681	*lsc*, PsyrpaN_010100019209	1248	ND	415	Green *et al*., 2010
pv. actinidiae M302091	*lscC*, PSYAC_19498	1296	ND	431	Baltrus *et al*., 2011
pv. morsprunorum M302280PT	*lsc*, PSYMP_24576	1296	ND	431	Baltrus *et al*., 2011

Table 3. Continuation.

***P. syringae* pathovar and strain**	**Gene name, locus tag**	**Gene length (bp)**	**Genomic location**	**Protein length (aa)**	**References**
pv. tabaci ATCC 11528	*lsc*, PSYTB_12850	1248	ND	415	Studholme *et al*., 2009 Baltrus *et al*., 2011
	lscC, PSYTB_24342 or PsyrptA_020100005135	1296	ND	431	
pv. lachrymans M301315	*lsc*, PLA107_25445	1248	ND	415	Baltrus *et al*., 2011

C, chromosomal; P, plasmid-born; ND, not determined.

Although the *P. syringae* strains have different host range, their levansucrase sequences are highly identical (see below). Thus, levansucrase proteins are probably not involved in selection of the host and survival in a specific host. *Lsc* alleles of one bacterial strain are also highly identical. Still, some of levansucrase loci may encode proteins with specific functions and novel properties. The main difference between 431 and 415 aa levansucrases of *P. syringae* is in their N-terminal part. Longer protein variants have N-terminal extensions of 16 aa lacking in 415 aa proteins (Ref. I). Role of these N-terminal extensions is not known, but they does not function as a secretion leader (Li and Ullrich, 2001). Very recently it was reported that longer (1296 bp) variants of *P. syringae* levansucrases might have emerged from a shorter (1249 bp) silent horizontally transferred variant through insertion of a prophage-related DNA with promoter elements that probably also added a 16 aa extension to the N-terminus (Srivastava *et al*., 2012).

Longer levansucrase variants of *P. syringae* pv. tomato DC3000, *lsc1* and *lsc3* share nucleotide identity of 94% between them and *lsc2* is 85% identical with those. Analysis of protein sequence using ClustalW program (Thompson *et al*., 1994) showed respectively 96% and 94% of identity. Predictedly, proteins with that high identity level should have highly similar properties. In this thesis, first comparative characterization of two heterologously expressed levansucrases, Lsc2 and Lsc3 from *P. syringae* pv. tomato DC3000 was carried out.

All *lsc* genes present in the genome of a *P. syringae* pathovar might not be expressed. Accordingly, it has been shown that in *P. syringae* pv. glycinea PG4180, LscA was not expressed from its native promoter whereas LscB and LscC both contributed to total levansucrase activity (Li and Ullrich, 2001).

At least one of three *lsc* genes of *P. syringae* DC3000 must be expressed in native host, because if grown on sucrose-containing medium, the bacterium synthesizes levan and has a mucoid phenotype (Ref. IV).

2.3 Expression of levansucrases from *P. syringae* pv. tomato DC3000 in *E. coli* (Ref. I; Ref. III; Ref. IV)

Whereas levansucrases are biotechnologically promising proteins, we decided to clone and heterologously express the three allelic levansucrase genes of *P. syringae* pv. tomato DC3000. This strain had genomic sequence available (Buell *et al*., 2003) and these genes and proteins had not been addressed before. Also, by producing levansucrase proteins in *E. coli* using the *H. polymorpha* maltase gene promoter P_{MAL1} (Liiv *et al*., 2001; Alamäe *et al*., 2003), we intended to evaluate feasibility of this promoter for biotechnological *E. coli*-based applications.

2.3.1 Expression of levansucrases from P_{MAL1}

While yeast-derived P_{MAL1} was shown to function constitutively in *E. coli*, we expressed three *P. syringae* pv. tomato levansucrase genes (*lsc1*, *lsc2* and *lsc3*) from this promoter in the bacterial host. *Lsc* genes were cloned to pHIPMalprom vector with their native Shine-Dalgarno sequences, the resulting plasmids were transformed to *E. coli* strains and expression of recombinant levansucrases was evaluated (Ref. I).

Laboratory strains of *E. coli* do not metabolize sucrose. However, if active levansucrase is synthesized, *E. coli* acquires a sucrose-positive phenotype. This means that it will grow on sucrose as sole carbon source due to glucose that will be liberated from sucrose in levansucrase reaction. Also, it will produce slimy polymeric levan on solid media containing sucrose (Fig. 5B) (Ref. I; Ref. IV). Production of acidic compounds from sucrose-derived glucose can be evaluated on MacConkey agar plates containing sucrose and a pH indicator – neutral red. Dark red zones surrounding *lsc*-expressing colonies indicate production of acid (Fig. 5A).

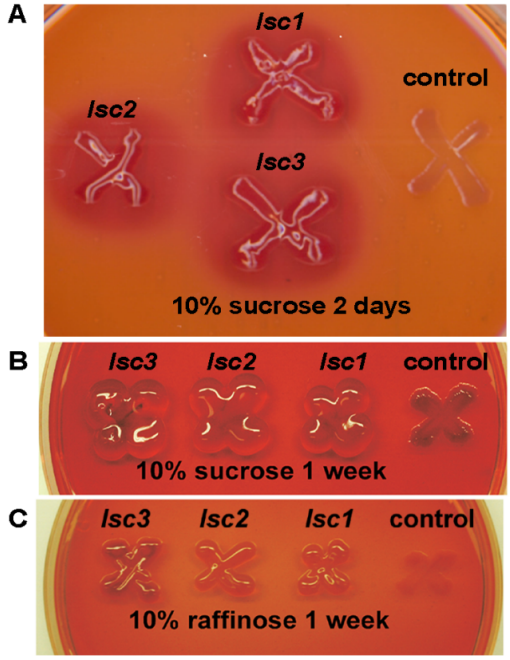

Fig. 5. *E. coli* RA11r transformants harboring pHIPMalprom-lsc1 (*lsc1*), pHIPMalprom-lsc2 (*lsc2*) or pHIP-Malprom-lsc3 (*lsc3*) have a mucoid phenotype due to levan synthesis when grown on MacConkey-sucrose (A, B) and MacConkey-raffinose (C) agar plates. *E. coli* carrying an empty vector pHIPMalprom was analysed as a negative control. At incubation of levansucrase-expressing transformants on MacConkey-sucrose medium at room temperature for 2 days, pH change due to production of acidic compounds from sucrose-derived glucose is clearly evident (A). At extended incubation, levan production from sucrose and raffinose can be observed (B, C).

The fact that none of the three levansucrases of *P. syringae* PG4180 expressed from its own promoter in *E. coli* (Li and Ullrich, 2001), justifies the use of an expression vector-provided promoter for heterologous production of levansucrases. All three levansucrase genes of *P. syringae* pv. tomato expressed from P_{MAL1} in *E. coli* at high level and catalytically active enzymes were produced in each case. The level of expression slightly depended on the host strain and on the gene to be expressed. We recorded the highest levansucrase total activity (70 U/mg) for *lsc3* in *E. coli* HB101 (*lac*+) and the lowest (7 U/mg protein) for *lsc2* in *E. coli RA11r* (Table 2 in Ref. I). When analysed by denaturating polyacrylamide gel electrophoresis (PAGE), Lsc1, Lsc2 and Lsc3 occurred as prominent proteins in cell extracts of recombinant *E. coli*. Lsc3 had the highest expression level compared to the other genes comprising about 20% of total soluble protein. Also, no inclusion body formation was detected (Ref. I). Thus, P_{MAL1} has appropriate strength to produce an adequate amount of soluble catalytically active levansucrase protein in *E. coli*. Exaggeratedly high expression level of protein can lead to aggregation of the proteins and formation of inclusion bodies. For example, this happened when levansucrase from *Z. mobilis* was expressed in fed-batch culture of *E. coli* from a strong constitutive promoter of *R. aquatilis*. Optimization of cultivation conditions, however, enabled production of soluble and active protein (Sunitha *et al*., 2000). It shows that not only the origin of the promoter is vital, but cultivation and induction strategies are important as well. Above-mentioned P_{MAL1}-controlled expression

system was applied for the production of Lsc2 and Lsc3 proteins in *E. coli* HB101 (*lac*+) for their purification and further biochemical characterization (Ref. I-IV).

2.3.2 Expression of levansucrases from P_{T7}

The pURI3 vector (Rivas *et al*., 2007) was used to produce wild-type and mutated Lsc3 proteins of *P. syringae* pv. tomato in *E. coli* BL21(DE3) from the plasmid-based T7 promoter (Ref. III). Cloning of a gene to the pURI family of expression vectors is a restriction- and ligation-free procedure that enables high-level production of recombinant His-tagged fusion proteins in *E. coli* (Curiel *et al*., 2011). This kind of expression system has been succesfully applied to over-express genes for ornithine transcarbamylase from *L. hilgardii* (Rivas *et al*., 2007), p-coumaric acid decarboxylase from *L. plantarum* (Rodríguez *et al*., 2007), phenolic acid decarboxylase from *L. plantarum* (Curiel *et al*., 2011) and many others.

Levansucrase gene *lsc3* was cloned to pURI3 vector and expressed in *E. coli* BL21(DE3). To induce the production of N-terminally His_6-tagged enzyme, 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the transformant culture and temperature was shifted to 22ºC for 20 h. Low temperature during the induction step enabled high yield of catalytically active Lsc3 protein (Ref. III). Extract of *E. coli* BL21(DE3) expressing wild-type His-tagged Lsc3 exhibited total levansucrase activity up to 200 U/mg that is much higher than respective activity achieved when using the pHIPMalprom vector (Ref. I; Ref. III). Implementing the pURI3 vector system, wild-type Lsc3 and its mutant variants were produced for purification by Ni^{2+}-affinity chromatography and further investigation (Ref. III).

3. Purification and characterization of *P. syringae* pv. tomato DC3000 levansucrases Lsc2 and Lsc3

Levansucrases of *P. syringae* have been mostly studied from the aspect of bacterial physiology and plant pathogenesis concentrating on their expression and relevance of the proteins for the native host (Li and Ullrich, 2001; Laue *et al*., 2006; Li *et al*., 2006). When we started our experiments, only one levansucrase protein of this species had been purified and thoroughly characterized (Hettwer *et al*., 1995). Therefore, we certainly contributed to the knowledge on *P. syringae* levansucrases. We consider that biochemical characterization of different levansucrase species should enlighten their specific roles in plant pathogenesis as well as in the life of host bacterium but also suggest some possible new applications of levansucrases.

3.1. Purification strategies for Lsc2 and Lsc3 (Ref. I-IV)

Levansucrases from various bacteria have been purified using cation- or anion-exchange chromatography (Hettwer *et al*., 1995; Ben Ammar *et al*., 2002; Yanase *et al*., 2002; Meng and Fütterer, 2003), reversed-phase high-performance liquid chromatography (HPLC) (Martínez-Fleites *et al*., 2004) or Ni^{+2}-affinity chromatography in case of His-tagged proteins (Tieking *et al*., 2005; Ozimek *et al*., 2006; Li *et al*., 2008; Rairakhwada *et al*., 2010) yielding sufficiently pure and active protein to carry out its characterization.

Lsc2 (UniProtKB/TrEMBL entry Q883P5) and Lsc3 (UniProtKB/TrEMBL entry Q88BN6) were heterologously produced in *E. coli* applying above-mentioned novel expression system (Ref. I). Proteins were purified from extracts of *E. coli* transformants harboring the plasmids pHIPMalprom-lsc2 or pHIP-Malprom-lsc3 using precipitation of proteins with $(NH_4)_2SO_4$ and subsequent size-exclusion chromathography on a Sephacryl S-300 column similarly as in case of *Z. mobilis* levansucrase purification (Vigants *et al*., 2003). Total levansucrase activity of purified Lsc3 preparation measured according to the release of glucose from 100 mM sucrose was ~230 U per mg of protein and V_{max} of the enzyme reached the level of 286 U/mg (Ref. I; Ref. II). Lsc2 preparation showed even higher V_{max} value – 357 U/mg, although its expression level in *E. coli* was about three times lower compared to Lsc3 (see also Table 2 in Ref. I; Ref. IV).

To accelerate and simplify the purification procedure of Lsc3 and its mutants, we cloned variants of the *lsc3* gene into pURI3 vector for over-expression of respective N-terminally His-tagged enzymes in *E. coli*. Ni^{2+}-affinity chromatography on a HisTrap™ FF 1 ml column and eluation with imidazole was used in purification procedure of His-tagged levansucrases (Ref. III).

3.2 Biochemical properties of Lsc2 and Lsc3 (Ref. I-IV)

Purified Lsc2 and Lsc3 proteins were characterized according to their different activities such as substrate specificity and the spectrum of polymerization products. As every splitting event of sucrose by the levansucrase results in release of a glucose molecule, total levansucrase activity is measured by quantification of emitted glucose. We quantified glucose using ready-to-use and sufficiently sensitive Glucose Liquicolor (Human GmbH, Germany) assay that made analysis easy and accurate. Initial reaction rates were measured for calculations and the activity was expressed as μmoles of glucose released from sucrose in 1 min per mg of protein (U/mg) (Ref. I; Ref. III). Based on total activity measurements, K_m for sucrose splitting, maximal reaction velocity (V_{max}) and inhibition constant (K_i) for raffinose on sucrose splitting were determined. Catalytic constants (k_{cat}; min^{-1}) and catalytic efficiencies (k_{cat}/K_m; $min^{-1}M^{-1}$) of the proteins were also calculated (Table 4). The effect of

temperature, pH and metal ions on reaction rate was determined by measuring total levansucrase activity (Ref. I; Ref. III).

To specify the range of substrates reacting with the levansucrase, release of reducing sugars from potential substrates (raffinose, stachyose and levan) was determined using the 3,5-dinitrosalicylic acid (DNSA) method (Ref. I).

Transfructosylation activity (TA) reflects the amount of sucrose-derived fructose that is converted by the levansucrase into polymerization products, levan and FOS. TA is calculated from difference of the amounts between liberated glucose and free (unpolymerized) fructose. The latter measures hydrolytic activity of the levansucrase – transfer of fructosyl units to water. TA is expressed in percentages (Ref. III).

Spectrum of reaction products of levansucrases were determined by thin layer chromatography (TLC) and a novel chip-based mass spectrometry method (see subchapter 3.2.2 Polymerization properties and spectrum of reaction products).

3.2.1 Substrate specificity and kinetic parameters

Levansucrases split their substrate (mainly sucrose) and transfer fructose residues of the substrate to water, another sucrose molecule, FOS or some alternative acceptor. In addition to sucrose, many levansucrases can use a trisaccharide raffinose as a substrate (Yanase *et al*., 2002; van Hijum *et al*., 2004; Seibel *et al*., 2006). Similarly to sucrose, raffinose is a widespread sugar in plants. Notably, levansucrase of *P. syringae* pv. phaseolicola was reported as uncapable of raffinose use (see Table 2) (Hettwer *et al*., 1995).

Nevertheless, our experimental data indicated that Lsc2 and Lsc3 use raffinose as a substrate to produce levan and FOS (Ref. I; Ref. II; Ref. IV). It was first suspected from colony phenotype of levansucrase-expressing *E. coli* RA11r on LB-raffinose plates (see Fig. 5C). Common laboratory strains of *E. coli* are sucrose-negative, but they possess melibiase (α-galactosidase) which is splitting raffinose to galactose and sucrose. We exerted a melibiase-negative RA11r strain (Hanatani *et al*., 1984) for evaluation of levansucrase-mediated raffinose utilization. The RA11r colonies expressing either *lsc1*, *lsc2* or *lsc3* of *P. syringae* pv. tomato produced mucoid colonies on LB agar plates with sucrose or raffinose (Fig. 5B, C). Additionally, native PAGE of *lsc*-expressing *E. coli* extracts and enzymatic activity staining of levansucrases on the gel indicated levan synthesis from both substrates (see Fig. 3 of Ref. I).

Levansucrase assay of bacterial extracts containing Lsc2 or Lsc3 protein proved release of reducing sugars from sucrose, raffinose and also from a tetrasaccharide stachyose (see Fig. 3) showing wider substrate specificity of those proteins that has been reported for any of the *Pseudomonas* levansucrase so far (Ref. III; Ref. IV).

To characterize substrate preference of Lsc3 and Lsc2 proteins, we measured release of reducing sugars from 100 mM sucrose, raffinose and stachyose by

purified enzyme preparations. Velocity of raffinose splitting was 52 ± 5 % (Lsc3) and 48 ± 1 % (Lsc2) from respective values of sucrose splitting. For stachyose, these values were 48 ± 3 % (Lsc3) and 36 ± 4 % (Lsc2). The results indicated that sucrose is the substrate of choice for Lsc2 and Lsc3, whereas raffinose and stachyose were used about twice less efficiently (Ref. III; Ref. IV and our unpublised data).

It has been shown that levansucrase LevU from *Z. mobilis* uses sucrose, raffinose and stachyose as a substrate. In contrast to our results, LevU did not prefer sucrose, but rather raffinose and the rate of raffinose use was 117% of the rate of sucrose use by LevU (Yanase *et al*., 2002). Thus, Lsc3 and Lsc2 are certainly different from LevU with regard to substrate preference.

Kinetic parameters of levansucrases from pseudomonads for the use of different substrates are presented in Table 4. The highest affinity of levansucrases was detected towards sucrose. The K_i of raffinose and stachyose inhibition was about two times higher than the K_m for sucrose confirming that raffinose and stachyose are less preferred substrates. Raffinose and stachyose acted as competitive inhibitors of sucrose-splitting reaction. The inhibition mode of raffinose on sucrose splitting by Lsc2 protein is presented in Fig. 6. Affinity of Lsc3 protein for sucrose was slightly higher than that of Lsc2 (Table 4) (Ref. IV). However, if compared with respective value (K_m=160 mM) of *P. syringae* pv. phaseolicola levansucrase (Hettwer *et al*., 1995), both proteins investigated by us exhibit much higher affinity. Many levansucrases have affinity values for sucrose similar to those of Lsc2 and Lsc3 (see discussion in Ref. I and Table 2). A very high affinity to sucrose, 6.6 mM, has been recorded for SacB of *B. megaterium* (Homann *et al*., 2007).

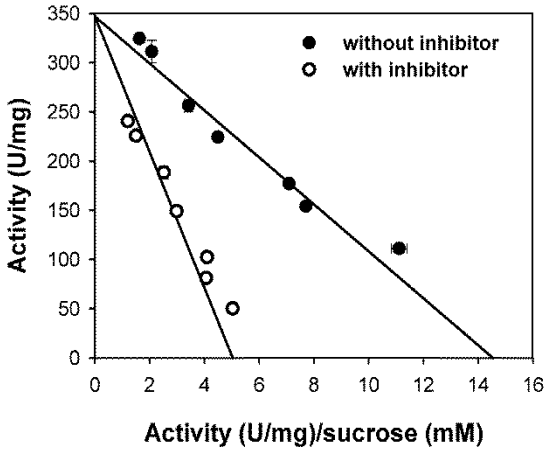

Fig. 6. Kinetics of sucrose splitting by Lsc2 protein of *P. syringae* pv. tomato DC3000. Release of glucose from 10-200 mM sucrose with (○) and without (●) the presence of 100 mM raffinose was assayed. Data were analyzed with Enzyme Kinetics Module 1.1 of the Sigma Plot program and plotted according to Eadie-Hofstee.

Catalytic constants (k_{cat}) and catalytic efficiencies (k_{cat}/K_m) are very high and similar between the two levansucrases of *P. syringae* pv. tomato (Table 4).

Compared to levansucrases from other Gram-negative bacteria such as *G. diazotrophicus* and *Z. mobilis*, catalytic constants of Lsc2 and Lsc3 are 4 to 8 times higher (Yanase *et al*., 2002; Martínez-Fleites *et al*., 2005). It can be partially caused by different temperatures used for activity measurements. We routinely measured levansucrase reaction velocity at 37ºC. In case of *G. diazotrophicus* and *Z. mobilis* levansucrases, catalytic constants were determined at 30ºC. If we conducted our activity measurements at 30ºC, k_{cat} of His-tagged Lsc3 dropped 1.3 times and catalytic efficiency decreased 1.9 times due to some reduction of the affinity (K_m = 27.4 mM) and maximal velocity. Still, k_{cat} and catalytic efficiency of Lsc3 determined at 30ºC were significantly higher than of levansucrases in general and comparable with high values of levansucrases from bacilli (see Table 2 and Table 4) (Homann *et al*., 2007; Ortiz-Soto *et al*., 2008).

Lsc2 and Lsc3 demonstrated some levan-hydrolyzing activity which was less than 1% of respective sucrose-splitting activity with fructose detected as sole end-product (Ref. III; Ref. IV). This coincides well with data of levansucrases from *Z. mobilis* or *R. aquatilis* (Jang *et al*., 2007). No hydrolysis of β-2,1-linked inulin from chicory root was detected (Ref. III; our unpublished data). Thus, levan can be considered as extracellular reserve polymer for *P. syringae* pv. tomato cells contributing to survival of the bacterium in the environment *e.g.* on plant surface.

Table 4. Kinetic parameters of sucrose and raffinose splitting by Lsc2 and Lsc3 from *P. syringae* pv. tomato DC3000 and LscA from *P. chlororaphis* subsp. *aurantiaca*.

Protein	**Molecular weight (kDa)** [b]	**K_m for sucrose (mM)**	**k_{cat} for sucrose (min^{-1})**	**k_{cat}/K_m for sucrose (M^{-1} min^{-1})**	**K_i for raffinose (mM)**	**K_i for stachyose (mM)**
Lsc2	45.9	25.3 ± 1.6 [c]	1.64 x 10^4 [c]	6.47 x 10^5 [c]	53.2 ± 4.3 [c]	53.6 ± 6.4
Lsc3	47.8	20.6 ± 2.1 [a]	1.37 x 10^4 [a]	6.63 x 10^5 [b]	47.6 ± 5.0 [a]	79.1 ± 10.8
Lsc3*	49.6	18.5 ± 2.5 [b]	3.03 x 10^4 [b]	1.64 x 10^6 [b]	39.9 ± 6.1 [c]	ND
LscA	47.0	24.1 ± 1.0 [b]	4.32 x 10^2 [b]	1.79 x 10^4 [b]	80.8 ± 11.8 [b]	ND

* His-tagged protein
ND, not determined
[a] Ref. I
[b] Used in k_{cat} calculations, Ref. III
[c] Ref. IV

3.2.2 Polymerization properties and spectrum of reaction products

Determination of transfructosylating activities

It has been shown that polymerizing activity of levansucrases depends significantly on reaction conditions *e.g.* pH, temperature and substrate concentration (Hettwer *et al*., 1995; Sangiliyandi *et al*., 1999; Ben Ammar *et al*., 2002; van Hijum *et al*., 2004; Goldman *et al*., 2008). Accordingly, we found that polymerizing activities of Lsc3 and Lsc2 were promoted at high substrate concentrations and if incubation was prolonged. When Lsc3 and Lsc2 were incubated with 300 mM sucrose as described for TA assay in Ref. III, by 5 min of reaction only 28% of fructose from reacted sucrose ended up in polymerization products. However, by 20 h of reaction time, respective value increased to 43%. A very high TA was recorded when Lsc3 was reacted with 1200 mM sucrose for 20 h. In that case, 76% of fructose residues from reacted sucrose were incorporated into polymerization products (Ref. III). Under the same conditions, similar level (72%) of polymerization was recorded for Lsc2 (our unpublished data). It seems that both enzymes govern the polymerization process at high sucrose content similarly, so the amount of fructose residues transferred to sucrose or fructan acceptor are comparably high.

Highest polymerization activity of *P. syringae* pv. phaseolicola levansucrase was recorded at 18ºC whereas hydrolysis of sucrose was most effective at 60ºC (Hettwer *et al*., 1995). In case of Lsc2 and Lsc3, we observed similar correlation. At 18ºC, TA was 39% whereas at 60ºC it decreased to about 20%. It should be noted that in these experiments, short reaction time (5 min) and rather low (150 mM) substrate concentration were used (see also Fig. 7). If similar reaction by Lsc3 was conducted for 20 h with 1200 mM sucrose at 20ºC, TA was 80% and respective value at 60ºC was 67% (Ref. III). Although temperature and substrate concentration had a major effect to transfructosylation, the pH of the reaction buffer had no significant effect. The latter characteristic was studied only in case of Lsc3 (Ref. III).

The spectrum of reaction products of Lsc2 and Lsc3

The length of produced fructans largely depends on reaction conditions, but also on intrinsic properties of the levansucrase. So, levansucrases of bacilli synthesize mainly highly-polymerized levan whereas the levansucrase from *G. diazotrophicus* produces mostly FOS (Hernandez *et al*., 1995; Batista *et al*., 1999; Tambara *et al*., 1999; Beine *et al*., 2008). We speculate that the enzyme and its spectrum of transfructosylation products have evolved in accordance with natural environment of the host bacterium (Ref. IV).

If *E. coli* is expressing *lsc* genes of *P. syringae* pv. tomato on a sucrose-containing plates, slimy fructan polymer is synthesized (see Fig. 5) (Ref. IV). According to the literature, LscA of *P. aurantiaca* S-4380 forms fructan polymer that is a typical levan – it has high molecular weight (7×10^5 Da), β-2,6 linkages between fructose residues in the main chain and some branching through β-2,1 linkages (Jang *et al*., 2006). Linkage type in Lsc3-produced

fructan polymer was studied by us using enzymatic method. We incubated the fructan polymer with inulinase from *Aspergillus niger* and analyzed the products using TLC. We concluded that fructan produced by Lsc3 from sucrose is a levan-type fructan (Ref. III). Composition analysis of the levan revealed a 99:1 ratio of fructose to glucose in it that indicates its high DP and molecular weight (Ref. I). As a reference, we analyzed commercial levan preparation of *E. herbicola* (*P. agglomerans*) (provider Sigma-Aldrich) that exhibited similarly high (98%) fructose content.

When we first analysed transfructosylation products of Lsc2 and Lsc3 by TLC, we did not detect FOS due to application of short reaction times (up to 30 min) and relatively low substrate concentrations (up to 100 mM), but levan production from sucrose and raffinose under these conditions was detected (Ref. I). If we extended reaction time up to 20 h and increased substrate concentration till 1200 mM, FOS of different DP were detectable among reaction products of Lsc3 and Lsc2 with sucrose or raffinose (Ref. II; Ref. III; Ref. IV). In the analysis of Lsc3 reaction products from 1200 mM sucrose, faint spots of kestose and nystose were visible on TLC already after a couple of hours of reaction (unpublished data). Lsc3 and Lsc2 both synthesize a considerable amount of FOS, but Lsc2 produces somewhat less levan, otherwise the FOS patterns of the enzymes were highly similar (see Fig. 3 in Ref. IV).

We also quantified and calculated the content of formed levan and FOS in these reaction samples. Lsc3 synthesized 7.2 mg/ml levan and 107.9 mg/ml of total FOS by 20 h of reaction time (Ref. III). Lsc2 produces 100.0 mg/ml FOS under same conditions. Patterns of FOS synthesized from 1200 mM sucrose by Lsc2 and Lsc3 were similar (Ref. II; Ref. III; Ref. IV). These two proteins are therefore clearly different from LsdA of *G. diazotrophicus* which produces 1-kestose as a major transfructosylation product and are more similar at that respect to SacB from Gram-positive *B. subtilis* (Hernandez *et al*., 1995; Tambara *et al*., 1999).

FOS and levan were also produced from sugar beet molasses with product spectrum being similar to that of sucrose (Ref. III).

We evaluated levan production kinetics of purified His-tagged Lsc3 at several sucrose concentrations and one concentration of raffinose. This assay was optimized to be conducted on microtitre plates that enabled to minimize the reaction volume and thereby economy on reagents and proteins (Ref. III). Highest amount of levan was produced from 300 mM sucrose at room temperature (23ºC), whereas very high concentration (1200 mM) of substrate resulted in three times reduced content of levan in the reaction mixture (Fig. 7). At low sucrose concentrations, levan-forming activity was promoted and no short-chain FOS were produced (Ref. II). Some levan (17.8 mg/ml) was also synthesized by Lsc3 from raffinose, but sucrose is certainly more suited substrate for levan synthesis (Fig. 7) (Ref. III).

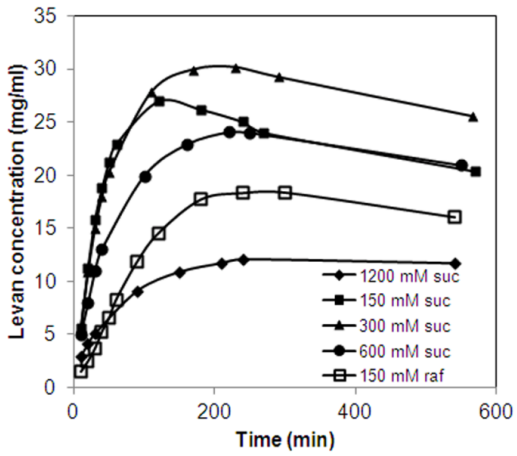

Fig. 7. Time course of levan production by His-tagged Lsc3 protein. Reaction was conducted and levan was quantified as in Ref. III. Average values of four parallel experiments are shown.

Identification of FOS by nanoESI HCT MS

Reaction products of various glycosyl transferases can be analysed by TLC, but also using quantitative and more accurate methods *e.g.* high-performance anion-exchange chromatography (HPAEC), normal phase and reverse phase HPLC. Chemical identities in glycan molecules can be confirmed using ^{1}H and ^{13}C NMR spectroscopy (van Geel-Schutten *et al*., 1999; van Hijum *et al*., 2001; Yanase *et al*., 2002; Ozimek *et al*., 2006; Yamada and Kakehi, 2011).

Mass spectrometrical methods like electrospray ionization (ESI) mass spectrometry (MS) are present-day tools for compositional and structural characterization of oligo- and polysaccharides. ESI MS can provide reliable and sensitive detection of various glycosylated molecules. Quite recently, nanoESI MS for screening of saccharidic molecules and oligosaccharides has been introduced. This method increases even more the capacity and accuracy of the measurements and allows direct sequencing of the ions by tandem mass spectrometry (MS/MS) (Almeida *et al*., 2008; Flangea *et al*., 2011 and references therein). For example, underivatized neutral oligosaccharides present in human milk were analyzed by either positive or negative ion mode nanoESI MS (Pfenninger *et al*., 2002). Therefore, we considered this method for the detection and analysis of fructans synthesized by levansucrases.

Before our research, some reports on application and optimization of conventional ESI MS for the analysis of levansucrase reaction products were available. This method was used to detect methyl-fructoside formation from sucrose and methanol by the *R. aquatilis* levansucrase (Kim *et al*., 2000) and to specify composition of oligosaccharidic reaction products synthesized from sucrose and its analogs by levansucrase variants of *B. subtilis* (Beine *et al*., 2008).

We intended to exert accurate, sensitive and up-to-date MS method to specify the length of the FOS species, presence of which among reaction products

was revealed by us using a traditional TLC method. Experimental conditions for fully automated chip-based nanoESI high-capacity ion trap (HCT) MS were optimized (Ref. II). Both, negative and positive ion modes of the machinery could be used whereas formate or phosphate and sodium additives of FOS were observed, respectively. Those additional groups to the saccharidic ions most probably originated from the reaction buffer and the solution for MS sample preparation. Also, hydrated ions were frequently spotted (Ref. II; Ref. III). Though ionization efficiency of neutral underivatized oligosaccharides is limited and relative abundance of larger ions thereby tends to be reduced, we detected FOS with DP up to 5 synthesized from sucrose or raffinose by Lsc3 (see Table 1 and 2 from Ref. II). To confirm the saccharidic origin of the isolated ions, MS/MS was applied. Depending of the conditions, we obtained MS^n spectra of ions from FOS species up to MS^3 (Ref. II; Ref. III).

The MS method confirmed that at high substrate concentration, FOS synthesis prevailed. According to experimental conditions, the best spectra of FOS were obtained when levansucrase reactions were carried out at high substrate concentration and elevated temperature (Ref. II). This could be explained by synthesis of more FOS and less levan at these conditions that probably enabled better ionization. Interestingly, thermally inactivated protein, unreacted substrate and buffer components of the reaction mixture did not hinder FOS detection. However, longer signal acquisition times were obtained when reaction samples were dialysed or levansucrase reactions were conducted in deionized water (Ref. II; Ref. III). Importantly, when the samples were prepared with Lsc3-containing cell lysates and thereafter analysed without prior purification or by-product removal, the pattern of FOS detected by MS method shared very high similarity with product spectra of purified Lsc3 (unpublished data).

Ref. II is the first report on optimization and introduction of this novel and highly sensitive nanoESI HCT MS method for the analysis of underivatized FOS mixtures formed from sucrose and raffinose by a levansucrase protein. Commercial prebiotic oligofructose preparations (Orafti®P95 and Orafti®Synergy1, BENEO-Orafti, Belgium) were analyzed to validate the MS method. The spectra of commercial FOS were highly similar to those of Lsc3-produced FOS (Ref. II). This suggests potential prebiotic effect of FOS synthesized by Lsc3.

Lsc3 synthesizes heterooligofructans

According to the literature, fructosyl transferases of Gram-positive bacteria can transfructosylate besides sucrose also nonconventional acceptors producing sucrose analogs (Seibel *et al*., 2005; Tieking *et al*., 2005; Seibel *et al*., 2006; Beine *et al*., 2008). Also, brief reports on levansucrases of Gram-negative bacteria *P. aurantiaca* and *Z. mobilis* describing transfructosylation of lactose yielding bifidogenic lactosucrose have been published (Han *et al*., 2007; Han *et al*., 2009).

We used nanoESI HCT MS method to screen possible fructosyl acceptors for Lsc3. D-xylose, D-fucose, L- and D-arabinose, D-ribose, D-sorbitol, xylitol, xylobiose, D-mannitol, D-galacturonic acid, methyl-α-D-glucopyranoside and D-glucosamine were tested. All analyzed acceptor substrates differ according to molecular mass from glucose and fructose that made MS-detection of hetero-oligofructans (HOF) among levansucrase reaction products easy (Ref. III). We showed that all tested nonconventional acceptors, except for D-glucosamine were used by Lsc3 in transfructosylation reaction. The DP of detected HOF were 2-5 in case of Lsc3 (see Supplementary table S1 of Ref. III and Table 3 of Ref. IV).

First we analyzed reaction mixtures without their prior purification. Despite of it, the MS spectra were of sufficient quality to specify HOF. We could detect four different series of sodiated oligosaccharidic ions: 1) HOF with 1-4 fructose residues added to the acceptor residue; 2) hydrated forms of HOF with DP up to 5; 3) conventional FOS with DP up to 5 produced from sucrose as a donor and acceptor; 4) hydrated species of conventional FOS (Ref. III). We conclude that nanoESI HCT MS is a feasible high-throughput method to screen possible acceptor molecules of glycosyl transferases.

Importantly, during this study it was shown for the first time that levansucrases can transfructosylate D-sorbitol, D-galacturonic acid, D-mannitol, xylitol, methyl-α-D-glycopyranoside and disaccharide xylobiose (Ref. III). We consider that use of alternative fructosyl acceptors and synthesis of HOF is probably a common feature of levansucrases. It may be due to relaxed binding properties of the +1 and further subsites of the active centre of the enzyme that enables binding of various acceptor molecules.

According to ^{1}H and ^{13}C NMR spectroscopy data of previously studied HOF, we assume that the linkage-type between a D-isomer of a nonconventional acceptor sugar and an adjacent fructose residue is most probably the same as between D-glucose and fructose in sucrose molecule *i.e.* α-1,2 (Seibel *et al*., 2006; Beine *et al*., 2008) whereas the bond between following fructose residues is most probably β-2,6 as typical for bacterial levans (Beine *et al*., 2008).

3.2.3 Optimal reaction conditions of Lsc3 and Lsc2

Performance of levansucrases depends on several environmental factors such as temperature, pH and substrate concentration whereas optimal reaction conditions vary significantly between the levansucrase proteins of different origin (Table 2). All levansucrases demonstrate two distinct activities, substrate hydrolysis and transfructosylation, and optimal conditions for these two reactions differ. Generally, low temperature enhances TA whereas substrate hydrolysis is promoted at elevated temperature. So, optimum temperature for levan production is usually lower than that for sucrose hydrolysis (see Table 2). Still, for levansucrases of different origin these tempeture optimi may vary. For example, levansucrase from *Bacillus* sp. TH4-2 produces highest amount of

levan at 50ºC which is exceptionally high temperature for efficient polymerization reaction compared to other levansucrases. For *Bacillus* sp. TH4-2 enzyme, the optimum temperature for sucrose hydrolysis was also high – 60ºC (Ben Ammar *et al*., 2002). Therefore, this levansucrase may be considered as a thermoactive and thermostable protein. Some levansucrases *e.g.* those of *Microbacterium laevaniformans* and *L. mesenteroides* also prefer somewhat higher temperature (30ºC) for levan synthesis. Many of studied levansucrases still require rather low temperature for levan synthesis (Kim *et al*., 1998; Park *et al*., 2003; Kang *et al*., 2005). For example, levansucrase of *P. syringae* pv. phaseolicola showed highest levan yield at 18ºC (Hettwer *et al*., 1995) while the optimum temperature for sucrose splitting was 60ºC that is similar to respective value of Lsc3 (see Fig. 8). Even intra- and extracellular levansucrase variants of the same bacterium may vary with regard to optimum temperatures for levan synthesis. So, intracellular levansucrase fraction of *B. amyloliquefaciens* exhibited highest levan synthesis at 25-30ºC, whereas the extracellular one at 40ºC (Tian *et al*., 2011).

We measured total and polymerizing activities of Lsc2 and Lsc3 at various temperatures (Fig. 8). As expected, low temperature increased the ratio between these activities (see also subchapter 3.2.2 Polymerization properties and spectrum of reaction products). Highest sucrose-splitting rates (total activity) were detected at 50ºC and 60ºC for Lsc2 and Lsc3, respectively. Shortly after exceeding this temperature, total activity of the enzymes dropped drastically because of thermal inactivation of the proteins. Polymerizing activity of Lsc2 and Lsc3 stayed almost equal at temperatures between 37-60ºC (Fig. 8) (our unpublished results). In this experiment, polymerizing activity of the enzymes is rather moderate, because short reaction time (5 min) and rather low (150 mM) sucrose concentration were used.

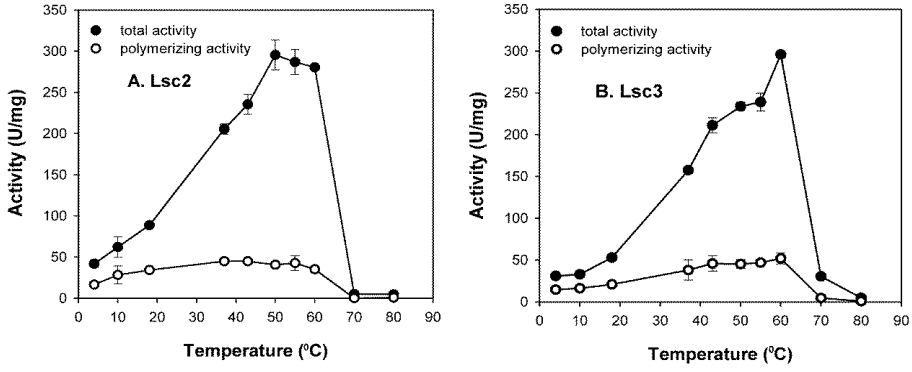

Fig. 8. Effect of temperature on total and polymerizing activity of Lsc2 (A) and Lsc3 (B) of *P. syringae* pv. tomato DC3000. The levansucrase assay with 150 mM sucrose at Na-acetate buffer (pH 5.2) was conducted at different temperatures. Determined total (●) and polymerizing (○) activities are plotted. Mean values and standard deviations of two to three determinations are shown.

We measured total levansucrase activity of Lsc3 and Lsc2 in McIllvaine's buffers with pH ranging from 3.0-8.0 to determine the optimum pH for these levansucrases. For both enzymes, sucrose splitting was highest at pH 6.0 (Ref. III and unpublised results). If pH was decreased below 5.0 or elevated over 7.0, the activity decreased significantly. All levansucrases characterized so far prefer slightly acidic conditions for the catalysis. For instance, levansucrase of *Z. mobilis* has pH optimum at 5.0, *Bacillus* sp. TH4-2 and *B. amyloliquefaciens* perform the best at pH 6.0 (Sangiliyandi *et al*., 1999; Ben Ammar *et al*., 2002; Rairakhwada *et al*., 2010). The optimum pH of *P. syringae* pv. phaseolicola levansucrase was reported to be around 5.8-6.6 (see Table 2) (Hettwer *et al*., 1995). Therefore, Lsc2 and Lsc3 exhibit quite similar properties to other levansucrases in this respect.

According to our data, Lsc2 and Lsc3 of *P. syringae* DC3000 should be considered as proteins with rather wide range of suitable temperature and pH. *P. syringae* is a psychrophilic bacterium with optimum growth at 28ºC and virulence properties being highest at 18ºC (Li *et al*., 2006). Despite of that, we showed that Lsc2 and Lsc3 catalyse well at temperatures higher than 28ºC. As levansucrases are extracellular enzymes in their native host bacteria, they should withstand diverse environmental conditions, including high temperature to survive and perform catalysis.

3.2.4 Stability of the enzymes and resistance to metal ions and detergents

Levansucrases are synthesized inside the native host bacterium to be excreted to the extracellular space. Outside the bacterium, in the environment, they most probably face different kinds of physical and chemical stresses. Our data showed that the Lsc2 and Lsc3 are stable proteins, tolerating prolonged (one-month) maintenance not only at low temperature (4ºC), but also at 37ºC. No loss of activity was detected during storage at 4ºC during six months (Ref. III). The levansucrases also preserve their full activity during repeated freezing-thawing cycles.

Levansucrases, especially Lsc3, also tolerate elevated temperature: the Lsc3 protein maintained ~50% of activity after keeping it during 30 min at 60ºC (Fig. 9). Lsc2 was slightly less thermotolerant – activity was almost lost if incubated at 60ºC, but incubation at 55ºC had only minor effect (our unpublished results). Though total activity of Lsc3 and Lsc2 was highest at 60ºC and 50ºC, respectively (see Fig. 8), they do not withstand long-term incubation at that high temperature.

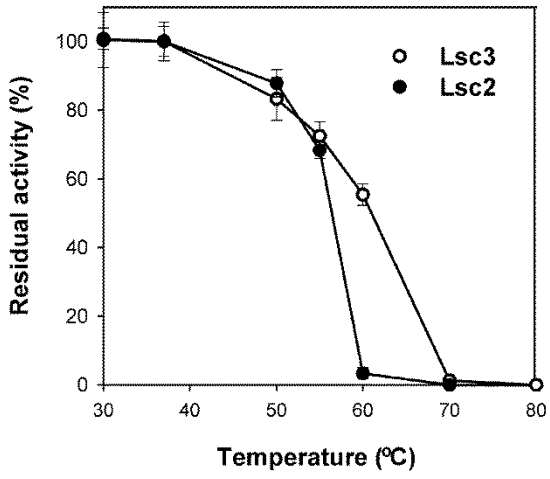

Fig. 9. Thermal stability of *P. syringae* pv. tomato levansucrases. Lsc2 (●) and Lsc3 (○) proteins were preincubated in McIllvaine's buffer (pH 6.0) at different temperatures for 30 min, and thereafter residual levansucrase activity on 100 mM sucrose was measured at 37ºC. Levansucrase activities of untreated preparations taken for 100% were 270.5 ± 12.0 (Lsc2) and 223.9 ± 12.6 U/mg (Lsc3). Average values and standard deviation for four parallel measurements are shown.

To evaluate the effect of metals on levansucrases, the enzymes were preincubated in the presence of 5 mM metal ions for 30 min as in Hettwer *et al*. (1995) and then residual levansucrase activity was determined by measuring the release of glucose from 100 mM sucrose. Non-treated samples were assayed alongside to obtain the reference for 100% activity. The results are gathered to Table 5. Respective literature data on levansucrase of *P. syringae* pv. phaseolicola that was assayed according to the same protocol were included for comparison (Hettwer *et al*., 1995). Hg^{2+} and Ag^{+} caused full inhibition of the levansucrases Lsc2 and Lsc3, whereas Ca^{2+} had no influence. As addressed in subchapter 1.2.2. of Overview of Literature, unlike levansucrases of Gram-positive bacteria, those of Gram-negative bacteria do not need Ca^{2+} ions for activity. Cu^{2+} and Al^{3+} had only minor effects on enzymes from *P. syringae* pv. tomato, whereas they caused 82% and 88% inhibition, respectively, of the pv. phaseolicola enzyme. Unlike Lsc2 and Lsc3, the pv. phaseolicola enzyme was not inhibited by Fe^{2+} and Fe^{3+} ions.

The effect of EDTA, β-mercaptoethanol, Tween 20, urea and SDS on levansucrases of Lsc2 and Lsc3 was also addressed. 1 mM EDTA had only a minor influence, whereas 10 mM β-mercaptoethanol and 1% Tween 20 had no effect on the levansucrases. No effect of β-mercaptoethanol treatment suggested that Lsc2 and Lsc3 proteins are not stabilized by S-S bridges between the cysteins. Although Lsc2 and Lsc3 both encompass four Cys residues (C103, C150, C256, C355 and C119, C166, C272, C371, respectively) no S-S bonds were predicted for these proteins by SoftBerry program CYS_REC (http://linux1.softberry.com/berry.phtml) (our unpublished results).

Treatment with 6 M urea reduced the activity of Lsc2 and Lsc3 to 3% and 4%, respectively. The Lsc2 and Lsc3 proteins were sensitive to SDS – treatment with 1, 0.1 or 0.03% SDS totally abolished the activity. Also, no restoration of

levan-forming activity of the levansucrases was seen after the proteins were separated on SDS gel and the gel was washed to remove the detergent and "revive" the protein (our unpublised results). In contrast to that, activity of levansucrase protein from *G. diazotrophicus* was detected on SDS gel that was washed in distilled water and developed for levan formation by soaking in sucrose solution (Hernández *et al*., 1999b).

Our results presented in chapters 3.2.3 and 3.2.4. confirm that Lsc2 and Lsc3 should be generally considered as robust and stable proteins that is of key importance for their use in desired biotechnological applications.

Table 5. Effect of metal ions on levansucrases of pseudomonads. The values represent residual levansucrase activity retained after the incubation of enzyme preparation with metal ions in percentages from the uninhibited reference. Standard deviation was below 10%.

Metal ion (5 mM)	***P. syringae* pv. tomato Lsc2**	***P. syringae* pv. tomato Lsc3**	***P. syringae* pv. phaseolicola Lsc**[a]
Hg^{2+}	Full inhibition	Full inhibition	Full inhibition
Ag^{+}	Full inhibition	Full inhibition	ND
Ca^{2+}	No effect	No effect	No effect
Cu^{2+}	92	99	18
Al^{3+}	91	75	12
Fe^{2+}	86	87	No effect
Fe^{3+}	90	71	No effect
Zn^{2+}	89	73	ND
Mn^{2+}	93	84	ND

ND, not determined.
[a] Hettwer *et al*., 1995.

4. Characterization of levansucrase LscA from *P. chlororaphis* subsp. *aurantiaca*

There are only scarce data available on levansucrase from *P. chlororaphis* subsp. *aurantiaca* (also *P. aurantiaca*). The *lscA* gene (1275 bp) has been cloned from *P. aurantiaca* S-4380 and expressed in *E. coli* from its own and the *lac* promoter (Jang *et al*., 2002). Feasibility of *P. aurantiaca* levan as starting material for difructose anhydride IV synthesis was also shown (Jang *et al*., 2006). Brief abstracts report on production of FOS and lactosucrose by *P. aurantiaca* levansucrase, respectively (Byun *et al*., 2007; Han *et al*., 2007). In all these studies, sucrose was used as substrate for LscA.

Amino acid sequence identity between Lsc3 and LscA is 73%, whereas the identity of these levansucrases with those from other bacteria is quite low. Therefore, LscA should be considered a close relative of *P. syringae* levansucrases (Ref. III).

As LscA protein was available as a commercial preparation (from Fluka, Sigma-Aldrich), we decided to use this enzyme for comparison of properties of *P. syringae* pv. tomato levansucrases as other similar enzymes from pseudomonads. Substrate specificity, kinetics of sucrose and raffinose usage, polymerization properties and range of products were determined and addressed in the light of respective data for Lsc3 (Ref. III).

4.1 Substrate specificity (Ref. III)

First, we determined substrate specificity of LscA. The enzyme liberated reducing sugars from sucrose, raffinose, stachyose and bacterial levan thereby showing a comparatively wide substrate specificity (Ref. III). Then, we assayed kinetics of glucose release from sucrose as a measure of total activity (see Table 4). K_m of LscA for sucrose splitting was 24.1 mM, which is similar to that of Lsc3. Raffinose inhibited the reaction with K_i of 80.8 mM indicating that raffinose is for LscA even less preferred substrate than for Lsc2 and Lsc3. The V_{max} of the enzyme for sucrose hydrolysis was about 30 times lower than that of Lsc3 evidencing low catalytic efficiency (Ref. III). Still, the catalytic activity of LscA preparation on 100 mM sucrose determined by us agrees with data provided by the manufacturer (Fluka, Sigma-Aldrich). In contradiction to information from the provider, LscA had optimal pH for sucrose splitting at 6.0-6.6, making it more similar to Lsc3 (Ref. III).

So, it seems that in respect of affinity, no big difference is detected between levansucrases from pseudomonads, whereas the turnover rate is very dissimilar. This may be an intrinsic property of the LscA enzyme, but on the other hand, it also may be due to low purity of the preparation or presence of large amount of inactivated enzyme in it.

4.2 Spectrum of reaction products (Ref. III)

According to the literature, *P. aurantiaca* produces low-branced levan as its polymerization product (Jang *et al*., 2006). We confirmed that FOS of various DP were additionally present among the reaction products of LscA. However, when reacted with 1200 mM sucrose, LscA was more prone to synthesis of long-chain FOS compared to Lsc2 and Lsc3 according to TLC analysis (see Fig. 2 in Ref. III). FOS formation by LscA from sucrose and raffinose was also confirmed by nanoESI HCT MS with FOS of DP up to 6 disclosed among reaction products (Ref. III).

Overall polymerization properties of LscA differed to some extent from those of Lsc3. A major difference was detected if TA was assayed from 20 h reaction samples with 300 mM sucrose as substrate at 37ºC. Under these conditions, LscA showed only low transfructosylation activity (15%) compared to Lsc3 (43%). Accordingly, final levan concentration obtained by 20 h of reaction was three times higher for Lsc3. If applying higher substrate concentrations, no significant difference between the transfructosylation values of these two enzymes was detected. Compared to Lsc3, LscA was more prone to FOS synthesis producing 124.9 mg of FOS per ml, with levan synthesis reduced to 4.6 mg/ml (Ref. III).

Both, LscA and Lsc3, favoured lower temperatures for polymerization activity and at higher temperature hydrolytic activity became enhanced. As for Lsc3, no pH dependence for transfructolylation ability was detected for LscA (Ref. III).

Similarly to Lsc3, LscA used 11 out of 12 tested potential fructosyl acceptors. NanoESI HCT MS analysis showed formation of HOF of DP up to 4 (Ref. III). It seems that synthesis of HOF is a widespread property among levansucrases from pseudomonads and possibly among levansucrases in general.

5. Mutational analysis of Lsc3

Crystallization studies have been focusing mostly on levansucrases of Gram-positive bacteria *B. subtilis* and *B. megaterium*. Levansucrases from these bacteria have also been crystallized in complex with the substrate (Meng and Fütterer, 2003; Meng and Fütterer, 2008; Strube *et al*., 2011). Crystal structure of LsdA of *G. diazotrophicus* (PDB code: 1W18) is the single one representing levansucrases of Gram-negative bacteria (see Table 1) (Martínez-Fleites *et al*., 2005). It should be noted that levansucrases of Gram-positive and -negative bacteria are rather diverse according to their amino acid sequences (Ref. III). Similarly, overall sequence identity between LsdA and SacB proteins is low – 26% (Martínez-Fleites *et al*., 2005). Despite of that, LsdA and SacB have similar structure of five-bladed β-propeller with central acidic pocket harboring catalytic triad amino acids (Table 1) (Meng and Fütterer, 2003; Martínez-Fleites *et al*., 2005).

Given that levansucrases from *P. syringae* pv. tomato are distant from other well-studied levansucrases and no mutational analysis and structure-function study has been carried out among levansucrases from pseudomonads, we decided to address this issue. First, we aligned Lsc3 and Lsc2 with other levansucrases and modelled their 3D structure (Supplementary figure S1 of Ref. III; Fig. 2 of Ref. IV). Then we initiated site-directed mutagenesis of Lsc3 to enlighten catalytically essential amino acid positions.

5.1 Prediction of putative catalytic amino acids of Lsc2 and Lsc3 (Ref. III; Ref. IV)

LsdA of *G. diazotrophicus* was found to be closest match for Lsc3 and Lsc2 among crystallized levansucrases. *In silico* 3D models of Lsc3 and Lsc2 were created by Karin Mardo (Ref. III; Ref. IV). According to protein alignment and modeling data, putative active site residues of *Pseudomonas*-originating levansucrases were predicted (see Table 2 of Ref. IV). According to it, the catalytic triad of studied enzymes constitutes two aspartates (Asp) and one glutamate (Glu) that should act as nucleophile, transition-state stabilizer and acid-base catalyst, respectively. The overall fold of Lsc2 and Lsc3 was predicted to be five-bladed β-propeller that is characteristic for levansucrases and for enzymes of GH68 and GH32 families in general (Ref. III; Meng and Fütterer, 2003; Martínez-Fleites *et al*., 2005; Strube *et al*., 2011).

5.2 Site-directed mutagenesis of Lsc3 (Ref. III)

The *lsc3* gene was site-directedly mutated, N-terminally His-tagged Lsc3 mutant variants were produced and purified. Mutations were introduced with mutagenic oligonucleotides using the pURI3 vector system (Ref. III). We substituted histidine (H) at position 321 with arginine (R), lysine (K), leucine

(L) and serine (S). His321 of Lsc3 is equivalent to His296 of *Z. mobilis* LevU and Arg360 of *B. subtilis* SacB. These residues have been shown to act as transfructosylation determinants of these levansucrases (Yanase *et al*., 2002; Li *et al*., 2008; Lammens *et al*., 2009).

Expectedly, substitutions of His321 had serious consequences to Lsc3: the K_m value for sucrose-splitting reaction increased up to 30 times *i.e*. the affinity was drastically reduced, and catalytic efficiency decreased by more than 200 times. Highest negative effect on catalysis was disclosed for H321S mutant. Expectedly, TA of His321 mutants was significantly reduced. Therefore, our results confirm that His321 is certainly of high catalytic importance for Lsc3 protein (Ref. III).

While levan production can easily be monitored in a simple turbidity assay on microplates as in Ref. III (see subchapter 3.2.2 Polymerization properties and spectrum of reaction products), we exerted this method to detect levan production by mutated variants of Lsc3. Our study indicated that synthesis of levan by His321 substitution mutants was strongly retarded. When wild-type His-tagged Lsc3 produced 7.2 mg/ml levan from 1200 mM sucrose by 20 h of reaction, H321R mutant, utmost of H321 variants, synthesized only 4.0 mg/ml of levan under these conditions. The same reaction mixtures were also addressed for FOS content. Total amounts of FOS produced by His321 substitution mutants ranged from 45.3 mg/ml (H321L) to 61.0 mg/ml (H321R). The wild-type Lsc3 protein produced 107.9 mg/ml of total FOS under these conditions. Pattern of FOS produced by the mutants revealed FOS with mobility of kestose as major product for H321R mutant (Ref. III). According to our results, His321 has a definite role in catalytic activity of Lsc3 affecting both, sucrose-splitting and polymerization reactions.

Another important mutation, Thr302Pro, was discovered within the assay of random multiple levansucrase mutants of Lsc3 by Karin Mardo (Ref. III). The mutation of a residue equivalent to Thr302 in Lsc3 has not been described for any of the levansucrase proteins. Thr302 is located in highly conserved region D(E/Q)(**T**/I/V)ERP of levansucrases, it is a neighbour residue of predicted acid-base catalyst Glu303 of Lsc3 and invariant in levansucrases of Gram-negative bacteria. Levansucrases of Gram-positive bacteria have mostly valine (V) or isoleucine (I) at that position (Martínez-Fleites *et al*., 2005). The Lsc3 mutant Thr302Pro was constructed as shown in Ref. III. The T302P mutant had two times reduced affinity for sucrose-splitting and k_{cat} values were reduced by one third if compared to wild-type Lsc3. Our results indicated that replacement of Thr302 with Pro decreased transfructosylation almost two times and especially hindered synthesis of longer-chain FOS (Ref. III).

5.3 Structure-function studies of Lsc3 (Ref. III)

The active centre of levansucrases forms a funnel-shaped pocket in the middle of the β-propeller (Meng and Fütterer, 2003; Martínez-Fleites *et al*., 2005;

Meng and Fütterer, 2008). Fructose residue of sucrose molecule binds at the bottom of the pocket and glucose residue on top of it (Ozimek *et al*., 2006). In *B. subtilis* levansucrase, Glu340 and Glu342 of the D**E**I**E**R motif as well as Arg360 belong to +1 subsite of the pocket. This subsite binds glucose residue of the donor sucrose molecule. Arg360 and Glu340 form tight hydrogen bonds with hydroxyls of the glucose residue fixing it in a proper orientation needed for further reactions (Fig. 4) (Meng and Fütterer, 2008; Lammens *et al*., 2009).

We hypothesize that in Lsc3 protein, His321 and Thr302 belong to +1 subsite of active site pocket. If these residues are mutated, sucrose binding is hindered. That is reflected in increased K_m and reduced catalytic constant values of sucrose-splitting reaction. While +1 subsite is also involved in acceptor binding, His321 and Thr302 substitution mutants of Lsc3 exhibit changed pattern of polymerization products: synthesis of levan and long-chain FOS is reduced whereas short-chain FOS are to some extent still produced (Ref. III).

CONCLUSIONS

Levansucrases are bacterial enzymes belonging to family 68 of glycoside hydrolases (GHs). They catalyze hydrolysis of their substrate but also have prominent fructosyl transferase activity. The main substrate for levansucrases is sucrose which is major disaccharide in plants. Raffinose, likewisely abundant in some plants, is also used as a substrate. Spectrum of reaction products of levansucrases comprises highly polymeric levan and fructooligosaccharides (FOS) of various degree of polymerization (DP). The entity of levansucrase reaction products depends on the enzyme and its origin, but also on reaction conditions enabling manipulation of the product spectrum. Levansucrases share highly similar five-blade β-propeller fold with other GH68 and 32 enzymes including bacterial inulosucrases, plant and microbial invertases, fructan exohydrolases and fructosyl transferases.

In this thesis, levansucrases Lsc2 and Lsc3 from a plant pathogenic bacterium *Pseudomonas syringae* pv. tomato were expressed in a bacterial host *Escherichia coli*, purified and characterized. As a comparison, levansucrase LscA from *P. chlororaphis* subsp. *aurantiaca* was studied.

The main results of this thesis are summarized as follows:

1. Lsc2 and Lsc3 of *P. syringae* pv. tomato were expressed with high yield in a bacterial host *Escherichia coli* exerting two expression systems. The first system relies on maltase gene promoter P_{MAL} from a methylotrophic yeast *Hansenula polymorpha*. We verified that functionality of P_{MAL} in *E. coli* is caused by the presence of σ70-like boxes in the eucaryotic promoter. The P_{MAL} was shown to have suitable strength in *E. coli* providing a sufficient amount of catalytically active protein of interest. Due to its dual activity, it can be used as a promoter shared by yeasts and bacteria in heterologous protein expression trials. A pURI3 vector-based expression system was adjusted to obtain mutant and wild-type N-terminally His-tagged Lsc3 proteins.
2. *P. syringae* pathovars are exceptional among other bacterial species because they possess up to three levansucrase alleles in their genomes. We showed that all three *lsc* genes (*lsc1*, *lsc2*, *lsc3*) of *P. syringae* pv. tomato DC3000 encode functional levansucrase proteins, if expressed from a heterologous promoter in *E. coli*.
3. Enzymology and biochemistry of Lsc2 and Lsc3 was addressed and compared with that of LscA from *P. chlororaphis* subsp. *aurantiaca*. All three proteins were shown to use sucrose, raffinose and stachyose as substrates. Low hydrolytic activity towards levan was also recorded. Affinities for sucrose of Lsc3, Lsc2 and LscA were similar, the K_m values being around 20 mM. The maximum reaction velocity and catalytic efficiency of LscA was much lower than that of Lsc2 and Lsc3 proteins. Polymerization properties of the enzymes differed. At low sucrose concentration, Lsc3 polymerized much more effectively than LscA. At high substrate concentration, the

difference in transfructosylating activity was evened out, but the FOS spectrum was still different – the LscA produced more high-DP FOS than Lsc3 or Lsc2.

4. As a novel feature for levansucrases of pseudomonads, this study shows the ability of Lsc3 and LscA to produce heterooligofructans (HOF) by trans-fructosylating nonconventional fructosyl acceptors. For the first time, levansucrases were shown to transfructosylate D-sorbitol, D-galacturonic acid, D-mannitol, xylitol, methyl-α-D-glycopyranoside and a disaccharide xylobiose. Novel high-throughput nanoESI HCT mass spectrometry method was implemented and optimized to specify the HOF and conventional FOS.
5. Lsc2 and Lsc3 were shown to be stabile and catalytically active proteins that preserved their activity at various pH and temperature values. They also tolerated presence of several metal ions and detergents. Those characteristics are essential for extracellular proteins and they are important for enzymes to be used in industry. As we showed that the levansucrases of *P. syringae* pv. tomato can produce biotechnologically promising products levan, FOS and HOF from a cheap substrate, sucrose, they should certainly be regarded as feasible biocatalysts for technological approaches.
6. Whereas no data on structure-function relationships among levansucrases of *Pseudomonas* bacteria were available, mutational analysis of Lsc3 was initiated. Asp62, Asp219 and Glu303 were predicted as catalytic triad residues of Lsc3. Mutation analysis of Lsc3 specified Thr302 and His321 as residues implicated in substrate binding and transfructosylation reaction possibly belonging to the +1 subsite of the Lsc3 active centre.

REFERENCES

Abdel-Fattah AF, Mahmoud DA, Esawy MA (2005) Production of levansucrase from *Bacillus subtilis* NRC 33a and enzymic synthesis of levan and fructo-oligosaccharides. *Curr Microbiol* **51**: 402–7.

Alamäe T, Pärn P, Viigand K, Karp H (2003) Regulation of the *Hansenula polymorpha* maltase gene promoter in *H. polymorpha* and *Saccharomyces cerevisiae*. *FEMS Yeast Res* **4**: 165–73.

Alberto F, Bignon C, Sulzenbacher G, Henrissat B, Czjzek M (2004) The three-dimensional structure of invertase (beta-fructosidase) from *Thermotoga maritima* reveals a bimodular arrangement and an evolutionary relationship between retaining and inverting glycosidases. *J Biol Chem* **279**: 18903–10.

Alberto F, Jordi E, Henrissat B, Czjzek M (2006) Crystal structure of inactivated *Thermotoga maritima* invertase in complex with the trisaccharide substrate raffinose. *Biochem J* **395**: 457–62.

Alméciga-Díaz CJ, Gutierrez AM, Bahamon I, Rodríguez A, Rodríguez MA, Sánchez OF (2011) Computational analysis of the fructosyltransferase enzymes in plants, fungi and bacteria. *Gene* **484**: 26–34.

Almeida R, Mosoarca C, Chirita M, Udrescu V, Dinca N, Vukelić Z, Allen M, Zamfir AD (2008) Coupling of fully automated chip-based electrospray ionization to high-capacity ion trap mass spectrometer for ganglioside analysis. *Anal Biochem* **378**: 43–52.

Almeida NF, Yan S, Lindeberg M, Studholme DJ, Schneider DJ, Condon B, Liu H, Viana CJ, Warren A, Evans C, Kemen E, Maclean D, Angot A, Martin GB, Jones JD, Collmer A, Setubal JC, Vinatzer BA (2009) A draft genome sequence of *Pseudomonas syringae* pv. tomato T1 reveals a type III effector repertoire significantly divergent from that of *Pseudomonas syringae* pv. tomato DC3000. *Mol Plant Microbe Interact* **22**: 52–62.

Altenbach D, Ritsema T (2007) Structure-function relations and evolution of fructosyltransferases. In: Recent Advances in Fructooligosaccharides Research. Shiomi, N, Benkeblia N, Onodera S (Eds.), Old City Publishing, Philadelphia, Pennsylvania, USA, p 135–157.

Alvaro-Benito M, Polo A, González B, Fernández-Lobato M, Sanz-Aparicio J (2010a) Structural and kinetic analysis of *Schwanniomyces occidentalis* invertase reveals a new oligomerization pattern and the role of its supplementary domain in substrate binding. *J Biol Chem* **285**: 13930–41.

Alvaro-Benito M, de Abreu M, Portillo F, Sanz-Aparicio J, Fernández-Lobato M (2010b) New insights into the fructosyltransferase activity of *Schwanniomyces occidentalis* ß-fructofuranosidase, emerging from nonconventional codon usage and directed mutation. *Appl Environ Microbiol* **76**: 7491–9.

Anwar MA, Kralj S, van der Maarel MJ, Dijkhuizen L (2008) The probiotic *Lactobacillus johnsonii* NCC 533 produces high-molecular-mass inulin from sucrose by using an inulosucrase enzyme. *Appl Environ Microbiol* **74**: 3426–33.

Anwar MA, Kralj S, Pique AV, Leemhuis H, Van der Maarel MJ, Dijkhuizen L (2010) Inulin and levan synthesis by probiotic *Lactobacillus gasseri* strains: characterization of three novel fructansucrase enzymes and their fructan products. *Microbiology* **156**: 1264–74.

Apweiler R, Hermjakob H, Sharon N (1999) On the frequency of protein glycosylation, as deduced from analysis of the SWISS-PROT database. *Biochim Biophys Acta* **1473**: 4–8.

Arcia PL, Costell E, Tárrega A (2011) Inulin blend as prebiotic and fat replacer in dairy desserts: optimization by response surface methodology. *J Dairy Sci* **94**: 2192–200.

Arrieta JG, Sotolongo M, Menéndez C, Alfonso D, Trujillo LE, Soto M, Ramírez R, Hernández L (2004) A type II protein secretory pathway required for levansucrase secretion by *Gluconacetobacter diazotrophicus*. *J Bacteriol* **186**: 5031–9.

Avci FY, Li X, Tsuji M, Kasper DL (2011) A mechanism for glycoconjugate vaccine activation of the adaptive immune system and its implications for vaccine design. *Nat Med* **17**: 1602–9.

Azorín-Ortuño M, Urbán C, Cerón JJ, Tecles F, Allende A, Tomás-Barberán FA, Espín JC (2009) Effect of low inulin doses with different polymerisation degree on lipid metabolism, mineral absorption, and intestinal microbiota in rats with fat-supplemented diet. *Food Chem* **113**: 1058–65.

Baciu IE, Jördening HJ, Seibel J, Buchholz K (2005) Investigations of the transfructosylation reaction by fructosyltransferase from *B. subtilis* NCIMB 11871 for the synthesis of the sucrose analogue galactosyl-fructoside. *J Biotechnol* **116**: 347–57.

Baltrus DA, Nishimura MT, Romanchuk A, Chang JH, Mukhtar MS, Cherkis K, Roach J, Grant SR, Jones CD, Dangl JL (2011) Dynamic evolution of pathogenicity revealed by sequencing and comparative genomics of 19 *Pseudomonas syringae* isolates. *PLoS Pathog* **7**: e1002132.

Batista, FR, Hernandez L, Fernandez JR, Arrieta J, Menendez C, Comez R, Tambara Y, Pons T (1999) Substitution of Asp-309 by Asn in the Arg-Asp-Pro (RDP) motif of *Acetobacter diazotrophicus* levansucrase affects sucrose hydrolysis, but not enzyme specificity. *Biochem J* **337**: 503–6.

Beine R, Moraru R, Nimtz M, Na'amnieh S, Pawlowski A, Buchholz K, Seibel J (2008) Synthesis of novel fructooligosaccharides by substrate and enzyme engineering. *J Biotechnol* **138**: 33–41.

Bekers M, Laukevics J, Upite D, Kaminska E, Vigants A, Viesturs U, Pankova L, Danilevics A (2002) Fructooligosaccharide and levan producing activity of *Zymomonas mobilis* extracellular levansucrase. *Process Biochem* **38**: 701–6.

Bekers M, Upite D, Kaminska E, Laukevics J, Ionina R, Vigants A (2003) Catalytic activity of *Zymomonas mobilis* extracellular ”levan-levansucrase” complex in sucrose medium. *Commun Agric Appl Biol Sci* **68**: 321–4.

Bekers M, Upite D, Kaminska E, Laukevics J, Grube M, Vigants A, Linde R (2005) Stability of levan produced by *Zymomonas mobilis*. *Process Biochem* **40**: 1535–9.

Ben Ammar Y, Matsubara T, Ito K, Iizuka M, Limpaseni T, Pongsawasdi P, Minamiura N (2002) Characterization of a thermostable levansucrase from *Bacillus* sp. TH4-2 capable of producing high molecular weight levan at high temperature. *J Biotechnol* **99**: 111–9.

Bergeron LJ, Burne RA (2001) Roles of fructosyltransferase and levanase-sucrase of *Actinomyces naeslundii* in fructan and sucrose metabolism. *Infect Immun* **69**: 5395–402.

Bergeron LJ, Morou-Bermudez E, Burne RA (2000) Characterization of the fructosyltransferase gene of *Actinomyces naeslundii* WVU45. *J Bacteriol* **182**: 3649–54.

Biedrzycka E, Bielecka M (2004) Prebiotic effectiveness of fructans of different degrees of polymerization. *Trends Food Sci Technol* **15**: 170–5.

Bielecka M, Biedrzycka E, Majkowska A (2002) Selection of probiotics and prebiotics for synbiotics and confirmation of their *in vivo* effectiveness. *Food Res Int* **35**: 125–31.

Bornet FR, Brouns F, Tashiro Y, Duvillier V (2002) Nutritional aspects of short-chain fructooligosaccharides: natural occurrence, chemistry, physiology and health implications. *Dig Liver Dis* **34**: S111–20.

Buell CR, Joardar V, Lindeberg M, Selengut J, Paulsen IT, Gwinn ML, Dodson RJ, Deboy RT, Durkin AS, Kolonay JF, Madupu R, Daugherty S, Brinkac L, Beanan MJ, Haft DH, Nelson WC, Davidsen T, Zafar N, Zhou L, Liu J, Yuan Q, Khouri H, Fedorova N, Tran B, Russell D, Berry K, Utterback T, Van Aken SE, Feldblyum TV, D'Ascenzo M, Deng WL, Ramos AR, Alfano JR, Cartinhour S, Chatterjee AK, Delaney TP, Lazarowitz SG, Martin GB, Schneider DJ, Tang X, Bender CL, White O, Fraser CM, Collmer A (2003) The complete genome sequence of the *Arabidopsis* and tomato pathogen *Pseudomonas syringae* pv. tomato DC3000. *Proc Natl Acad Sci USA* **100**: 10181–6.

Bujacz A, Jedrzejczak-Krzepkowska M, Bielecki S, Redzynia I, Bujacz G (2011) Crystal structures of the apo form of β-fructofuranosidase from *Bifidobacterium longum* and its complex with fructose. *FEBS J* **278**: 1728–44.

Byun SH, Han WC, Kang SA, Kim CH, Jang KH (2007) Production of fructo-oligosaccharides from sucrose by two levansucrases from *Pseudomonas aurantiaca* and *Zymomonas mobilis*. *J Biotechnol* **131**: S112.

Cai R, Yan S, Liu H, Leman S, Vinatzer BA (2011) Reconstructing host range evolution of bacterial plant pathogens using *Pseudomonas syringae* pv. tomato and its close relatives as a model. *Infect Genet Evol* **11**: 1738–51.

Calazans GMT, Lima RC, de França FP, Lopes CE (2000) Molecular weight and antitumour activity of *Zymomonas mobilis* levans. *Int J Biol Macromol* **27**: 245–7.

Calazans GMT, Lopes CE, Lima RMOC, de França FP (1997) Antitumour activities of levans produced by *Zymomonas mobilis* strains. *Biotechnol Lett* **19**: 19–21.

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for glycogenomics. *Nucleic Acids Res* **37**: D233–8.

Carpita NC, Gibeaut DM (1993) Structural models of primary cell walls in flowering plants: consistency of molecular structure with the physical properties of the cell wall during growth. *Plant J* **3**: 1–30.

Chambert R, Petit-Glatron MF (1993) Immobilisation of levansucrase on calcium phosphate gel strongly increases its polymerase activity. *Carbohydr Res* **244**: 129–36.

Chambert R, Petit-Glatron MF (1991) Polymerase and hydrolase activities of *Bacillus subtilis* levansucrase can be separately modulated by site-directed mutagenesis. *Biochem J* **79**: 35–41.

Chuankhayan P, Hsieh CY, Huang YC, Hsieh YY, Guan HH, Hsieh YC, Tien YC, Chen CD, Chiang CM, Chen CJ (2010) Crystal structures of *Aspergillus japonicus* fructosyltransferase complex with donor/acceptor substrates reveal complete subsites in the active site for catalysis. *J Biol Chem* **285**: 23251–64.

Conner SJ, Lefièvre L, Hughes DC, Barratt CL (2005) Cracking the egg: increased complexity in the zona pellucida. *Hum Reprod* **20**: 1148–52.

Cosgrove DJ (2005) Growth of the plant cell wall. *Nat Rev Mol Cell Biol* **6**: 850–61.

Curiel JA, de Las Rivas B, Mancheño JM, Muñoz R (2011) The pURI family of expression vectors: a versatile set of ligation independent cloning plasmids for producing recombinant His-fusion proteins. *Protein Expr Purif* **76**: 44–53.

Dahech I, Belghith KS, Hamden K, Feki A, Belghith H, Mejdoub H (2011) Oral administration of levan polysaccharide reduces the alloxan-induced oxidative stress in rats. *Int J Biol Macromol* **49**: 942–7.

Dall'Olio F, Chiricolo M (2001) Sialyltransferases in cancer. *Glycoconj J* **18**: 841–50.

Davies GJ, Wilson KS, Henrissat B (1997) Nomenclature for sugar-binding subsites in glycosyl hydrolases. *Biochem J* **321**: 557–9.

DeLano WL (2002) The PyMOL Molecular Graphics System DeLano Scientific, San Carlos, CA, USA.

Del Moral S, Olvera C, Rodriguez ME, Munguia AL (2008) Functional role of the additional domains in inulosucrase (IslA) from *Leuconostoc citreum* CW28. *BMC Biochem* **9**: 6.

Donot F, Fontana A, Baccou JC, Schorr-Galindo S (2012) Microbial exopolysaccharides: main examples of synthesis, excretion, genetics and extraction. *Carbohydr Pol* **87**: 951–62.

Dubber M, Sperling O, Lindhorst TK (2006) Oligomannoside mimetics by glycosylation of ‘octopus glycosides’ and their investigation as inhibitors of

type 1 fimbriae-mediated adhesion of *Escherichia coli*. *Org Biomol Chem* **4**: 3901–12.

Dueñas-Sánchez R, Codón AC, Rincón AM, Benítez T (2010) Increased biomass production of industrial bakers' yeasts by overexpression of *Hap4* gene. *Int J Food Microbiol* **143**: 150–60.

Feil H, Feil WS, Chain P, Larimer F, DiBartolo G, Copeland A, Lykidis A, Trong S, Nolan, M, Goltsman E, Thiel J, Malfatti S, Loper JE, Lapidus A, Detter JC, Land M, Richardson, PM, Kyrpides NC, Ivanova N, Lindow SE (2005) Comparison of the complete genome sequences of *Pseudomonas syringae* pv. syringae B728a and pv. tomato DC3000. *Proc Natl Acad Sci USA* **102**: 11064–9.

Flangea C, Serb A, Sisu E, Zamfir AD (2011) Chip-based nanoelectrospray mass spectrometry of brain gangliosides. *Biochim Biophys Acta* **1811**: 897–917.

Fooks LJ, Gibson GR (2002) *In vitro* investigations of the effect of probiotics and prebiotics on selected human intestinal pathogens. *FEMS Microbiol Ecol* **39**: 67–75.

Fry SC (2001) Plant Cell Walls. *eLS*

Geier G, Geider K (1993) Characterization and influence on virulence of the levansucrase gene from the fireblight pathogen *Erwinia amylovora*. *Physiol Mol Plant Pathol* **42**: 387–404.

Gibson GR, Probert HM, Loo JV, Rastall RA, Roberfroid MB (2004) Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr Res Rev* **17**: 259–75.

Gibson GR, Roberfroid MB (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J Nutr* **125**: 1401–12.

Gietl C, Faber KN, van der Klei IJ, Veenhuis M (1994) Mutational analysis of the N-terminal topogenic signal of watermelon glyoxysomal malate dehydrogenase using the heterologous host *Hansenula polymorpha*. *Proc Natl Acad Sci U S A* **91**: 3151–5.

Goldman D, Lavid N, Schwartz A, Shoham G, Danino D, Shoham Y (2008) Two active forms of *Zymomonas mobilis* levansucrase: an ordered microfibril structure of the enzyme promotes levan polymerization. *J Biol Chem* **283**: 32209–17.

Green S, Studholme DJ, Laue BE, Dorati F, Lovell H, Arnold D, Cottrell JE, Bridgett S, Blaxter M, Huitema E, Thwaites R, Sharp PM, Jackson RW, Kamoun S (2010) Comparative genome analysis provides insights into the evolution and adaptation of *Pseudomonas syringae* pv. aesculi on *Aesculus hippocastanum*. *PLoS ONE* **5**: E10224.

Grizard D, Barthomeuf C (1999) Non-digestible oligosaccharides used as prebiotic agents: mode of production and beneficial effects on animal and human health. *Reprod Nutr Dev* **39**: 563–88.

Gänzle MG (2012) Enzymatic synthesis of galacto-oligosaccharides and other lactose derivatives (hetero-oligosaccharides) from lactose. *Int Dairy J* **22**: 116–22.

Han WC, Byun SH, Kim MH, Sohn EH, Lim JD, Um BH, Kim CH, Kang SA, Jang KH (2009) Production of lactosucrose from sucrose and lactose by a levansucrase from *Zymomonas mobilis*. *J Microbiol Biotechnol* **19**: 1153–60.

Han WC, Byun SH, Lee JC, Kim MH, Kang SA, Kim CH, Wha Son E, Jang KH (2007) Synthesis of lactosucrose formed by levansucrase from *Pseudomonas aurantiaca*. *J Biotechnol* **131**: S113.

Hanatani M, Yazyu H, Shiota-Niiya S, Moriyama Y, Kanazawa H, Futai M, Tsuchiya T (1984) Physical and genetic characterization of the melibiose operon and identification of the gene products in *Escherichia coli*. *J Biol Chem* **259**: 1807–12.

Hendry GAF (1993) Evolutionary origins and natural functions of fructans – a climatological, biogeographical and mechanistic appraisal. *New Phytol* **123**: 3–14.

Hernández L, Arrieta J, Betancourt L, Falcón V, Madrazo J, Coego A, Menéndez C (1999a) Levansucrase from *Acetobacter diazotrophicus* SRT4 is secreted via periplasm by a signal-peptide-dependent pathway. *Curr Microbiol* **39**: 146–52.

Hernández L, Ramírez R, Hormaza JV, Madrazo J, Arrieta J (1999b) Increased levansucrase production by a genetically modified *Acetobacter diazotrophicus* strain in shaking batch cultures. *Lett Appl Microbiol* **28**: 41–4.

Hernandez L, Arrieta J, Menendez C, Vazquez R, Coego A, Suarez V, Selman G, Petit-Glatron MF, Chambert R (1995) Isolation and enzymic properties of levansucrase secreted by *Acetobacter diazotrophicus* SRT4, a bacterium associated with sugar cane. *Biochem J* **309**: 113–8.

Hettwer U, Gross M, Rudolph K (1995) Purification and properties of an extracellular levansucrase from *Pseudomonas syringae* pv. phaseolicola. *J Bacteriol* **177**: 2834–9.

Hettwer U, Jaeckel FR, Boch J, Meyer M, Rudolph K, Ullrich MS (1998) Cloning, nucleotic sequence, and expression in *Escherichia coli* of levansucrase genes from the plant phatogens *Pseudomonas syringae* pv. glycinea and *P. syringae* pv. phaseolicola. *Appl Env Microbiol* **64**: 3180–7.

Hidaka H, Hirayama M, Sumi N (1988) A fructooligosaccharides-producing enzyme from *Aspergillus niger* ATCC 20611. *Agric Biol Chem* **52**: 1181–7.

Hisano H, Kanazawa A, Kawakami A, Yoshida M, Shimamoto Y, Yamada T (2004) Transgenic perennial ryegrass plants expressing wheat fructosyltransferase genes accumulate increased amounts of fructan and acquire increased tolerance on a cellular level to freezing. *Plant Sci* **167**: 861–8.

Homann A, Biedendieck R, Götze S, Jahn D, Seibel J (2007) Insights into polymer versus oligosaccharide synthesis: mutagenesis and mechanistic studies of a novel levansucrase from *Bacillus megaterium*. *Biochem J* **407**: 189–98.

Hwang MSH, Morgan RL, Sarkar SF, Wang PW, Guttman DS (2005) Phylogenetic characterization of virulence and resistance phenotypes of *Pseudomonas syringae*. *Appl Environ Microbiol* **71**: 5182–91.

Jang EK, Jang KH, Koh I, Kim IH, Kim SH, Kang SA, Kim CH, Ha SD, Rhee SK (2002) Molecular characterization of the levansucrase gene from *Pseudomonas aurantiaca* S-4380 and its expression in *Escherchia coli*. *J Microbiol Biotechnol* **12**: 603–9.

Jang KH, Kang SA, Kim CH, Lee JC, Kim MH, Son EW, Rhee SK (2007) Characterization of levan hydrolysis activity of levansucrase from *Zymomonas mobilis* ATCC 10988 and *Rahnella aquatilis* ATCC 33071. *Food Sci Biotechnol* **16**: 482–4.

Jang KH, Jang EK, Kim SH, Kim IH, Kang SA, Koh I, Park YI, KimYJ, Ha SD, Chul HK (2006) High-level production of low-branched levan from *Pseudomonas aurantiaca* S-4380 for the production of di-β-D-fructofuranose dianhydride IV. *J Microbiol Biotechnol* **16**, 102–8.

Joardar V, Lindeberg M, Jackson RW, Selengut J, Dodson R, Brinkac LM, Daugherty SC, Deboy R, Durkin AS, Giglio MG, Madupu R, Nelson WC, Rosovitz MJ, Sullivan S, Crabtree J, Creasy T, Davidsen T, Haft DH, Zafar N, Zhou L, Halpin R, Holley T, Khouri H, Feldblyum T, White O, Fraser CM, Chatterjee AK, Cartinhour S, Schneider DJ, Mansfield J, Collmer A, Buell CR (2005) Whole-genome sequence analysis of *Pseudomonas syringae* pv. phaseolicola 1448A reveals divergence among pathovars in genes involved in virulence and transposition. *J Bacteriol* **187**: 6488–98.

Kang HK, Seo MY, Seo ES, Kim D, Chung SY, Kimura A, Day DF, Robyt JF (2005) Cloning and expression of levansucrase from *Leuconostoc mesenteroides* B-512 FMC in *Escherichia coli*. *Biochim Biophys Acta* **1727**: 5–15.

Kilian SG, Kritzinger SM, Rycroft C, du Gibson GR, Preez JC (2002) The effects of the novel bifidogenic trisaccharide, neokestose, on the human colonic microbiota. *World J Microbiol Biotechnol* **18**: 637–44.

Kim KH, Chung CB, Kim YH, Kim KS, Han CS, Kim CH (2005) Cosmeceutical properties of levan produced by *Zymomonas mobilis*. *J Cosmet Sci* **56**: 395–406.

Kim MG, Kim CH, Lee JS, Song KB, Rhee SK (2000) Synthesis of methyl beta-D-fructoside catalyzed by levansucrase from *Rahnella aquatilis*. *Enzyme Microb Technol* **27**: 646–51.

Kim MG, Seo JW, Song KB, Kim CH, Chung BH, Rhee SK (1998) Levan and fructosyl derivates formation by a recombinant levansucrase from *Rahnella aquatilis*. *Biotechnol Lett* **20**: 333–6.

Koczan JM, McGrath MJ, Zhao Y, Sundin GW (2009) Contribution of *Erwinia amylovora* exopolysaccharides amylovoran and levan to biofilm formation: implications in pathogenicity. *Phytopathology* **99**: 1237–44.

Kyono K, Yanase H, Tonomura K, Kawasaki H, Sakai T (1995) Cloning and characterization of *Zymomonas mobilis* genes encoding extracellular levansucrase and invertase. *Biosci Biotechnol Biochem* **59**: 289–93.

Lammens W, Le Roy K, Schroeven L, Van Laere A, Rabijns A, Van den Ende W (2009) Structural insights into glycoside hydrolase family 32 and 68 enzymes: functional implications. *J Exp Bot* **60**: 727–40.

Lammens W, Le Roy K, Van Laere A, Rabijns A, Van den Ende W (2008) Crystal structures of *Arabidopsis thaliana* cell-wall invertase mutants in complex with sucrose. *J Mol Biol* **377**: 378–85.

Lammens W, Le Roy K, Yuan S, Vergauwen R, Rabijns A, Van Laere A, Strelkov SV, Van den Ende W (2012) Crystal structure of 6-SST/6-SFT from *Pachysandra terminalis*, a plant fructan biosynthesizing enzyme in complex with its acceptor substrate 6-kestose. *Plant J* 70: 205–19.

Laue H, Schenk A, Li H, Lambertsen L, Neu TR, Molin S, Ullrich MS (2006) Contribution of alginate and levan production to biofilm formation by *Pseudomonas syringae*. *Microbiology* **152**: 2909–18.

Le Roy K, Lammens W, Verhaest M, De Coninck B, Rabijns A, Van Laere A, Van den Ende W (2007) Unraveling the difference between invertases and fructan exohydrolases: a single amino acid (Asp-239) substitution transforms *Arabidopsis* cell wall invertase 1 into a fructan 1-exohydrolase. *Plant Physiol* **145**: 616–25.

Lewin A, Tran TT, Jacob D, Mayer M, Freytag B, Appel B (2004) Yeast DNA sequences initiating gene expression in *Escherichia coli*. *Microbiol Res* **159**: 19–28.

Li H, Schenk A, Srivastava A, Zhurina D, Ullrich MS (2006) Thermo-responsive expression and differential secretion of the extracellular enzyme levansucrase in the plant pathogenic bacterium *Pseudomonas syringae* pv. glycinea. *FEMS Microbiol Lett* **265**: 178–85.

Li H, Ullrich MS (2001) Characterization and mutational analysis of three allelic *lsc* genes encoding levansucrase in *Pseudomonas syringae*. *J Bacteriol* **183**: 3282–92.

Li SY, Chen M, Li G, Yan YL, Yu HY, Zhan YH, Peng ZX, Wang J, Lin M (2008) Amino acid substitutions of His296 alter the catalytic properties of *Zymomonas mobilis* 10232 levansucrase. *Acta Biochim Pol* **55**: 201–6.

Li Y, Triccas JA, Ferenci T (1997) A novel levansucrase-levanase gene cluster in *Bacillus stearothermophilus* ATCC12980. *Biochim Biophys Acta* **1353**: 203–8.

Liiv L, Pärn P, Alamäe T (2001) Cloning of maltase gene from a methylotrophic yeast, *Hansenula polymorpha*. *Gene* **265**: 77–85.

Liu J, Luo J, Ye H, Zeng X (2012) Preparation, antioxidant and antitumor activities *in vitro* of different derivatives of levan from endophytic bacterium *Paenibacillus polymyxa* EJS-3. *Food Chem Toxicol* **50**: 767–72.

Livingston DP 3rd, Hincha DK, Heyer AG (2009) Fructan and its relationship to abiotic stress tolerance in plants. *Cell Mol Life Sci* **66**: 2007–23.

Lobo AR, Colli C, Filisetti TMCC (2006) Fructooligosaccharides improve bone mass and biomechanical properties in rats. Nutr Res **26**: 413–20.

Lomax AR, Cheung LV, Tuohy KM, Noakes PS, Miles EA, Calder PC (2012) β2-1 Fructans have a bifidogenic effect in healthy middle-aged human subjects but do not alter immune responses examined in the absence of an *in vivo* immune challenge: results from a randomised controlled trial. *Br J Nutr* DOI: http://dx.doi.org/10.1017/S0007114511007276.

Lueking A, Holz C, Gotthold C, Lehrach H, Cahill D (2000) A system for dual protein expression in *Pichia pastoris* and *Escherichia coli*. *Protein Expr Purif* **20**: 372–8.

Maciel JC, Andrad PL, Neri DFM, Carvalho Jr. LB, Cardoso CA, Calazans GMT, Albino Aguiar J, Silva MPC (2012) Preparation and characterization of magnetic levan particles as matrix for trypsin immobilization. *J Magn Magn Mater* **324**: 1312–6.

Maiorano AE, Piccoli RM, da Silva ES, de Andrade Rodrigues MF (2008) Microbial production of fructosyltransferases for synthesis of pre-biotics. *Biotechnol Lett* **30**: 1867–77.

Martínez-Fleites C, Tarbouriech N, Ortiz-Lombardia M, Taylor E, Rodríguez A, Ramírez R, Hernández L, Davies GJ (2004) Crystallization and preliminary X-ray diffraction analysis of levansucrase (LsdA) from *Gluconacetobacter diazotrophicus* SRT4. *Acta Crystallogr D Biol Crystallogr* **60**: 181–3.

Martínez-Fleites C, Ortíz-Lombardía M, Pons T, Tarbouriech N, Taylor EJ, Hernández L, Davies GJ (2005) Crystal structure of levansucrase from the Gram-negative bacterium *Gluconacetobacter diazotrophicus*. *Biochem J* **390**: 19–27.

Marx SP, Winkler S, Hartmeier W (2000) Metabolization of β-(2,6)-linked fructose-oligosaccharides by different bifidobacteria. *FEMS Microbiol Lett* **182**: 163–9.

Mena-Arizmendi A, Alderete J, Águila S, Marty A, Miranda-Molina A, López-Munguía A, Castillo E (2011) Enzymatic fructosylation of aromatic and aliphatic alcohols by *Bacillus subtilis* levansucrase: reactivity of acceptors. *J Mol Catal B: Enzym* **70**: 41–8.

Mendoza E, García ML, Casas C, Selgas MD (2001) Inulin as fat substitute in low fat, dry fermented sausages. *Meat Sci* **57**: 387–93.

Menéndez C, Hernández L, Selman G, Mendoza MF, Hevia P, Sotolongo M, Arrieta JG (2002) Molecular cloning and expression in *Escherichia coli* of an exo-levanase gene from the endophytic bacterium *Gluconacetobacter diazotrophicus* SRT4. *Curr Microbiol* **45**: 5–12.

Meng G, Fütterer K (2003) Structural framework of fructosyl transfer in *Bacillus subtilis* levansucrase. *Nat Struct Biol* **10**: 935–41.

Meng G, Fütterer K (2008) Donor substrate recognition in the raffinose-bound E342A mutant of fructosyltransferase *Bacillus subtilis* levansucrase. *BMC Struct Biol* **8**: 16.

Meyer D, Stasse-Wolthuis M (2009) The bifidogenic effect of inulin and oligo-fructose and its consequences for gut health. *Eur J Clin Nutr* **63**: 1277–89.

Nagem RAP, Rojas AL, Golubev AM, Korneeva OS, Eneyskaya EV, Kulminskaya AA, Neustroev KN, Polikarpov I (2004) Crystal structure of exo-inulinase from *Aspergillus awamori*: the enzyme fold and structural determinants of substrate recognition. *J Mol Biol* **344**: 471–80.

Naumoff DG (2001) Beta-fructosidase superfamily: homology with some alpha-L-arabinases and beta-D-xylosidases. *Proteins* **42**: 66–76.

Newbrun E, Baker S (1968) Physico-chemical characteristics of the levan produced by *Streptococcus salivarius*. *Carbohydr Res* **6**: 165–70.

Nurizzo D, Turkenburg JP, Charnock SJ, Roberts SM, Dodson EJ, McKie VA, Taylor EJ, Gilbert HJ, Davies GJ (2002) *Cellvibrio japonicus* alpha-L-arabinanase 43A has a novel five-blade beta-propeller fold. *Nat Struct Biol* **9**: 665–8.

Ohtsuka K, Hino S, Fukushima T, Ozawa O, Kanematsu T, Uchida T (1992) Characterization of levansucrase from *Rahnella aquatilis* JCM-1683. *Biosci Biotechnol Biochem* **56**: 1371–7.

Olivares-Illana V, López-Munguía A, Olvera C (2003) Molecular characterization of inulosucrase from *Leuconostoc citreum*: a fructosyltransferase within a glucosyltransferase. *J Bacteriol* **185**: 3606–12.

Ortiz-Soto ME, Rivera M, Rudiño-Piñera E, Olvera C, López-Munguía A (2008) Selected mutations in *Bacillus subtilis* levansucrase semi-conserved regions affecting its biochemical properties. *Protein Eng Des Sel* **21**: 589–95.

Ozimek LK, Euverink GJ, van der Maarel MJ, Dijkhuizen L (2005) Mutational analysis of the role of calcium ions in the *Lactobacillus reuteri* strain 121 fructosyltransferase (levansucrase and inulosucrase) enzymes. *FEBS Lett* **579**: 1124–8.

Ozimek LK, Kralj S, van der Maarel MJEC, Dijkhuizen L (2006) The levansucrase and inulosucrase enzymes of *Lactobacillus reuteri* 121 catalyse processive and non-processive transglycosylation reactions. *Microbiology* **152**: 1187–96.

Ozimek LK, van Hijum SA, van Koningsveld GA, van Der Maarel MJ, van Geel-Schutten GH, Dijkhuizen L (2004) Site-directed mutagenesis study of the three catalytic residues of the fructosyltransferases of *Lactobacillus reuteri* 121. *FEBS Lett* **560**: 131–3.

Park HE, Park NH, Kim MJ, Lee TH, Lee HG, Yang JY, Cha J (2003) Enzymatic synthesis of fructosyl oligosaccharides by levansucrase from *Microbacterium laevaniformans* ATCC 15953. *Enz Microb Technol* **32**: 820–7.

Paul A, Samaddar N, Dutta D, Bagchi A, Chakravorty S, Chakraborty W, Gachhui R (2011) Mercuric ion stabilizes levansucrase secreted by *Acetobacter nitrogenifigens* strain RG1^{T}. *Protein J* **30**: 262–72.

Pfenninger A, Karas M, Finke B, Stahl B (2002) Structural analysis of underivatized neutral human milk oligosaccharides in the negative ion mode by nano-electrospray MS(n). *J Am Soc Mass Spectrom* **13**: 1331–48.

Pijning T, Anwar MA, Boger M, Dobruchowska JM, Leemhuis H, Kralj S, Dijkhuizen L, Dijkstra BW (2011) Crystal structure of inulosucrase from *Lactobacillus*: insights into the substrate specificity and product specificity of GH68 fructansucrases. *J Mol Biol* **412**: 80–93.

Poli A, Kazak H, Gürleyendağ B, Tommonaro G, Pieretti G, Öner ET, Nicolaus B (2009) High level synthesis of levan by a novel *Halomonas* species growing on defined media. *Carbohydr Polymer* **78**: 651–7.

Qi M, Wang D, Bradley CA, ZhaoY (2011) Genome sequence analyses of *Pseudomonas savastanoi* pv. glycinea and subtractive hybridization-based comparative genomics with nine pseudomonads. *PLoS ONE* **6**: e16451.

Rairakhwada D, Seo JW, Seo MY, Kwon O, Rhee SK, Kim CH (2010) Gene cloning, characterization and heterologous expression of levansucrase from *Bacillus amyloliquefaciens*. *J Ind Microbiol Biotechnol* **37**: 195–204.

Rairakhwada D, Pal AK, Bhathena ZP, Sahu NP, Jha A, Mukherjee SC (2007) Dietary microbial levan enhances cellular nonspecific immunity and survival of common carp (*Cyprinus carpio*) juveniles. *Fish Shellfish Immunol* **22**: 477–86.

Ramirez-Farias C, Slezak K, Fuller Z, Duncan A, Holtrop G, Louis P (2009) Effect of inulin on the human gut microbiota: stimulation of *Bifidobacterium adolescentis* and *Faecalibacterium prausnitzii*. *Br J Nutr* **101**: 541–50.

Rehm J, Willmitzer L, Heyer AG (1998) Production of 1-kestose in transgenic yeast expressing a fructosyltransferase from *Aspergillus foetidus*. *J Bacteriol* **180**: 1305–10.

Ritsema T, Smeekens S (2003) Fructans: beneficial for plants and humans. *Curr Opin Plant Biol* **6**: 223–30.

Rivas B de las, Curiel JA, Mancheño JM, Muñoz R (2007). Expression vectors for enzyme restriction- and ligation-independent cloning for producing recombinant His-fusion proteins. *Biotechnol Prog* **23**: 680–6.

Roberfroid M, Gibson GR, Hoyles L, McCartney AL, Rastall R, Rowland I, Wolvers D, Watzl B, Szajewska H, Stahl B, Guarner F, Respondek F, Whelan K, Coxam V, Davicco MJ, Léotoing L, Wittrant Y, Delzenne NM, Cani PD, Neyrinck AM, Meheust A (2010) Prebiotic effects: metabolic and health benefits. *Br J Nutr* **104**: S1–63.

Roberfroid MB, Van Loo JA, Gibson GR (1998) The bifidogenic nature of chicory inulin and its hydrolysis products. *J Nutr* **128**: 11–9.

Rodríguez H, de las Rivas B, Muñoz R, Mancheño JM (2007) Overexpression, purification, crystallization and preliminary structural studies of p-coumaric acid decarboxylase from *Lactobacillus plantarum*. *Acta Crystallogr Sect F Struct Biol Cryst Commun* **63**: 300–3.

Rodríguez MA, Sánchez OF, Alméciga-Díaz CJ (2011) Gene cloning and enzyme structure modeling of the *Aspergillus oryzae* N74 fructosyltransferase. *Mol Biol Rep* **38**: 1151–61.

Rosell KG, Birkhed D (1974) An inulin-like fructan produced by *Streptococcus mutans* strain JC2. *Acta Chem Scand B* **28**: 589.

Sakai Y, Kazarimoto T, Tani Y (1991) Transformation system for an asporogenous methylotrophic yeast, *Candida boidinii*: cloning of the orotidine-5'-phosphate decarboxylase gene (*URA3*), isolation of uracil auxotrophic mutants, and use of the mutants for integrative transformation. *J Bacteriol* 173: 7458–63.

Sakai Y, Kazarimoto T, Tani Y (1992) The orotidine-5′-phosphate decarboxylase gene (*URA3*) of a methylotrophic yeast, *Candida boidinii*: nucleotide

sequence and its expression in *Escherichia coli*. *J Ferment Bioeng* **73**: 255–60.

Sakai Y, Tani Y (1992) Directed mutagenesis in an asporogenous methylotrophic yeast: cloning, sequencing, and one-step gene disruption of the 3-isopropylmalate dehydrogenase gene (*LEU2*) of *Candida boidinii* to derive doubly auxotrophic marker strains. *J Bacteriol* **174**: 5988–93.

Sangeetha PT, Ramesh MN, Prapulla SG (2004) Production of fructo-oligosaccharides by fructosyl transferase from *Aspergillus oryzae* CFR 202 and *Aureobasidium pullulans* CFR 77. *Process Biochem* **39**: 753–8.

Sangiliyandi G, Chandra Raj K, Gunasekaran P (1999) Elevated temperature and chemical modification selectively abolishes levan-forming activity of levansucrase of *Zymomonas mobilis*. *Biotechnol Lett* **21**: 179–82.

Schroeven L, Lammens W, Van Laere A, Van den Ende W (2008) Transforming wheat vacuolar invertase into a high affinity sucrose:sucrose 1-fructosyltransferase. *New Phytol* **180**: 822–31.

Schumann W, Ferreira LCS (2004) Production of recombinant proteins in *Escherichia coli*. *Genet Mol Biol* **27**: 422–53.

Seibel J, Moraru R, Götze S (2005) Biocatalytic and chemical investigations in the synthesis of sucrose analogues. *Tetrahedron* **61**: 7081–6.

Seibel J, Moraru R, Götze S, Buchholz K, Na'amnieh S, Pawlowski A, Hecht HJ (2006) Synthesis of sucrose analogues and the mechanism of action of *Bacillus subtilis* fructosyltransferase (levansucrase). *Carbohydr Res* **41**: 2335–49.

Slawson C, Hart GW (2011) O-GlcNAc signalling: implications for cancer cell biology. *Nat Rev Cancer* **11**: 678–84.

Smits TH, Rezzonico F, Duffy B (2011) Evolutionary insights from *Erwinia amylovora* genomics. *J Biotechnol* **155**: 34–9.

Song DD, Jacques NA (1999) Purification and enzymic properties of the fructosyltransferase of *Streptococcus salivarius* ATCC 25975. *Biochem J* **341**: 285–91.

Song KB, Seo JW, Kim MG, Rhee SK (1998) Levansucrase of *Rahnella aquatilis* ATCC33071. Gene cloning, expression, and levan formation. *Ann N Y Acad Sci* **864**: 506–11.

Srivastava A, Al-Karablieh N, Khandekar S, Sharmin A, Weingart H, Ullrich MS (2012) Genomic distribution and divergence of levansucrase-coding genes in *Pseudomonas syringae*. *Genes* **3**: 115–37.

Strube CP, Homann A, Gamer M, Jahn D, Seibel J, Heinz DW (2011) Polysaccharide synthesis of the levansucrase SacB from *Bacillus megaterium* is controlled by distinct surface motifs. *J Biol Chem* **286**: 17593–600.

Studholme DJ, Gimenez Ibanez S, Maclean D, Dangl JL, Chang JH, Rathjen JP(2009) A draft genome sequence and functional screen reveals the repertoire of type III secreted proteins of *Pseudomonas syringae* pathovar tabaci 11528. *BMC Genomics* **10**: 395.

Sunitha K, Chung BH, Jang KH, Song KB, Kim CH, Rhee SK (2000) Refolding and purification of *Zymomonas mobilis* levansucrase produced as

inclusion bodies in fed-batch culture of recombinant *Escherichia coli*. *Protein Expr Purif* **18**: 388–93.

Tambara Y, Hormaza JV, Perez C, Leon A, Arrieta J, Hernandez L (1999) Structural analysis and optimised production of fructo-oligosaccharides by levansucrase from *Acetobacter diazotrophicus* SRT4. *Biotechnol Lett* **21**: 117–21.

Taniguchi N, Korekane H (2011) Branched N-glycans and their implications for cell adhesion, signaling and clinical applications for cancer biomarkers and in therapeutics. *BMB Rep* **44**: 772–81.

Teixeira JS, McNeill V, Gänzle MG (2012) Levansucrase and sucrose phoshorylase contribute to raffinose, stachyose, and verbascose metabolism by lactobacilli. *Food Microbiol* **31**: 278–84.

Thompson JD, Higgins DG, Gibson TJ (1994) Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions specific gap penalties and weight matrix choice. *Nucleic Acids Res* **22**: 4673–80.

Tian F, Inthanavong L, Karboune S (2011) Purification and characterization of levansucrases from *Bacillus amyloliquefaciens* in intra- and extracellular forms useful for the synthesis of levan and fructooligosaccharides. *Biosci Biotechnol Biochem* **75**: 1929–38.

Tieking M, Ehrmann MA, Vogel RF, Gänzle MG (2005) Molecular and functional characterization of a levansucrase from the sourdough isolate *Lactobacillus sanfranciscensis* TMW 1.392. *Appl Microbiol Biotechnol* **66**: 655–63.

Trujillo LE, Gomez R, Banguela A, Soto M, Arrieta JG, Hernández L (2004) Catalytical properties of N-glycosylated *Gluconacetobacter diazotrophicus* levansucrase produced in yeast. *Electron J Biotechnol* **7**: 116–23.

Tokunaga T, Nakada Y, Yasuhito T, Hirayama M, Hidaka H (1993) Effects of fructooligosaccharides intake on the intestinal microflora and defecation in healthy volunteers. *Bifidus* **6**: 143–50.

Van den Abbeele P, Gérard P, Rabot S, Bruneau A, El Aidy S, Derrien M, Kleerebezem M, Zoetendal EG, Smidt H, Verstraete W, Van de Wiele T, Possemiers S (2011) Arabinoxylans and inulin differentially modulate the mucosal and luminal gut microbiota and mucin-degradation in humanized rats. *Environ Microbiol* **13**: 2667–80.

Van den Ende W, Lammens W, Van Laere A, Schroeven L, Le Roy K (2009) Donor and acceptor substrate selectivity among plant glycoside hydrolase family 32 enzymes. *FEBS J* **276**: 5788–98.

Van den Ende W, Valluru R (2009) Sucrose, sucrosyl oligosaccharides and oxidative stress: scavenging and salvaging? *J Exp Bot* **60**: 9–18.

van Dijk R, Faber KN, Kiel JAKW, Veenhuis M, van der Klei I (2000) The methylotrophic yeast *Hansenula polymorpha*: a versatile cell factory. *Enzyme Microb Technol* **26**: 793–800.

Van Geel-Schutten GH, Faber EJ, Smit E, Bonting K, Smith MR, Ten Brink B, Kamerling JP, Vliegenthart JF, Dijkhuizen L (1999) Biochemical and

structural characterization of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains. *Appl Environ Microbiol* **65**: 3008–14.

van Hijum SA, Bonting K, van der Maarel MJ, Dijkhuizen L (2001) Purification of a novel fructosyltransferase from *Lactobacillus reuteri* strain 121 and characterization of the levan produced. *FEMS Microbiol Lett* **205**: 323–8.

van Hijum SA, Kralj S, Ozimek LK, Dijkhuizen L, van Geel-Schutten IG (2006) Structure-function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiol Mol Biol Rev* **70**: 157–76.

van Hijum SA, Szalowska E, van der Maarel MJ, Dijkhuizen L (2004) Biochemical and molecular characterization of a levansucrase from *Lactobacillus reuteri*. *Microbiology* **150**: 621–30.

van Hijum SA, van der Maarel MJ, Dijkhuizen L (2003) Kinetic properties of an inulosucrase from *Lactobacillus reuteri* 121. *FEBS Lett* **534**: 207–10.

van Hijum SA, van Geel-Schutten GH, Rahaoui H, van der Maarel MJ, Dijkhuizen L (2002) Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high-molecular-weight inulin and inulin oligosaccharides. *Appl Environ Microbiol* **68**: 4390–8.

Van Riet L, Nagaraj V, Van den Ende W, Clerens S, Wiemken A, Van Laere A (2006) Purification, cloning and functional characterization of a fructan 6-exohydrolase from wheat (*Triticum aestivum* L.). *J Exp Bot* **57**: 213–23.

Velázquez-Hernández ML, Baizabal-Aguirre VM, Cruz-Vázquez F, Trejo-Contreras MJ, Fuentes-Ramírez LE, Bravo-Patiño A, Cajero-Juárez M, Chávez-Moctezuma MP, Valdez-Alarcón JJ (2011) *Gluconacetobacter diazotrophicus* levansucrase is involved in tolerance to NaCl, sucrose and desiccation, and in biofilm formation. *Arch Microbiol* **193**: 137–49.

Verhaest M, Lammens W, Le Roy K, De Coninck B, De Ranter CJ, Van Laere A, Van den Ende W, Rabijns A (2006) X-ray diffraction structure of a cell-wall invertase from *Arabidopsis thaliana*. *Acta Crystallogr D Biol Crystallogr* **62**: 1555–63.

Verhaest M, Lammens W, Le Roy K, De Ranter CJ, Van Laere A, Rabijns A, Van den Ende W (2007) Insights into the fine architecture of the active site of chicory fructan 1-exohydrolase: 1-kestose as substrate vs sucrose as inhibitor. *New Phytol* **174**: 90–100.

Verhaest M, Van den Ende W, Roy KL, De Ranter CJ, Laere AV, Rabijns A (2005) X-ray diffraction structure of a plant glycosyl hydrolase family 32 protein: fructan 1-exohydrolase IIa of *Cichorium intybus*. *Plant J* **41**: 400–11.

Vigants A, Marx SP, Linde R, Ore S, Bekers M, Vina I, Hicke HG (2003) A novel and simple method for the purification of extracellular levansucrase from *Zymomonas mobilis*. *Curr Microbiol* **47**: 198–202.

Viigand K, Alamäe T (2007) Further study of the *Hansenula polymorpha* MAL locus: characterization of the alpha-glucoside permease encoded by the *HpMAL2* gene. *FEMS Yeast Res* **7**: 1134–44.

Viigand K, Tammus K, Alamäe T (2005) Clustering of MAL genes in *Hansenula polymorpha*: cloning of the maltose permease gene and expression from

the divergent intergenic region between the maltose permease and maltase genes. *FEMS Yeast Res* **5**: 1019–28.
Vijn I, Smeekens S (1999) Fructan: more than a reserve carbohydrate? *Plant Physiol* **120**: 351–60.
Vijn I, van Dijken A, Lüscher M, Bos A, Smeets E, Weisbeek P, Wiemken A, Smeekens S (1998) Cloning of sucrose:sucrose 1-fructosyltransferase from onion and synthesis of structurally defined fructan molecules from sucrose. *Plant Physiol* **117**: 1507–13.
Wanker E, Schörgendorfer K, Schwab H (1991) Expression of the *Bacillus subtilis* levanase gene in *Escherichia coli* and *Saccharomyces cerevisiae*. *J Biotechnol* **18**: 243–54.
Wishart DS, Knox C, Guo AC, Eisner R, Young N, Gautam B, Hau DD, Psychogios N, Dong E, Bouatra S, Mandal R, Sinelnikov I, Xia J, Jia L, Cruz JA, Lim E, Sobsey CA, Shrivastava S, Huang P, Liu P, Fang L, Peng J, Fradette R, Cheng D, Tzur D, Clements M, Lewis A, De Souza A, Zuniga A, Dawe M, Xiong Y, Clive D, Greiner R, Nazyrova A, Shaykhutdinov R, Li L, Vogel HJ, Forsythe I (2009) HMDB: a knowledgebase for the human metabolome. *Nucleic Acids Res* **37**: D603–10.
Yamada K, Kakehi K (2011) Recent advances in the analysis of carbohydrates for biomedical use. *J Pharm Biomed Anal* **55**: 702–27.
Yamamoto Y, Takahashi Y, Kawano M, Iizuka M, Matsumoto T, Saeki S, Yamaguchi HJ (1999) *In vitro* digestibility and fermentability of levan and its hypocholesterolemic effects in rats. *Nutr Biochem* **10**: 13–8.
Yanai K, Nakane A, Kawate A, Hirayama M (2001) Molecular cloning and characterization of the fructooligosaccharide-producing beta-fructofuranosidase gene from *Aspergillus niger* ATCC 20611. *Biosci Biotechnol Biochem* **65**: 766–73.
Yanase H, Maeda M, Hagiwara E, Yagi H, Taniguchi K, Okamato K (2002) Identification of functionally important aminoacid residues in *Zymomonas mobilis* levansucrase. *J Biochem* **132**: 565–72.
Yoo SH, Yoon EJ, Cha J, Lee HG (2004) Antitumor activity of levan polysaccharides from selected microorganisms. *Int J Biol Macromol* **34**: 37–41.

REFERRED WEB PAGES

http://linux1.softberry.com/berry.phtml
http://www.brenda-enzymes.org
http://www.cazy.org
http://www.hmdb.ca
http://www.ncbi.nlm.nih.gov/
http://www.orafti.com
http://www.pseudomonas-syringae.org
http://www.rcsb.org
http://www.uniprot.org

SUMMARY IN ESTONIAN

Pseudomonas syringae pv. tomato DC3000 levaansukraasid: ekspressioon, biokeemiline iseloomustamine, mutatsioonanalüüs ja polümerisatsiooniproduktide spekter

Suhkrud on maakeral äärmiselt levinud molekulid ning neil on väga oluline roll paljudes elusloodus protsessides. Mitmed ensüümid sünteesivad fruktoosijääkidest koosnevad polümeere – fruktaane. Nende sahhariidide polümerisatsiooniaste, sidemetüüp ja hargnemine võib olla sõltuvalt nende päritolust väga erinev. Paljud taimed sisaldavad inuliini, mis on β-2,1 sidemetüübiga fruktaan. Mitmed bakteriliigid sünteesivad polümeerset levaani, milles on fruktoosijääkide vahel β-2,6 side. Fruktaanid toimivad varuainetena, aga neil on leitud ka seoseid organismi stressitaluvuse ja patogeensusega. Lisaks on fruktaane võimalik kasutada biotehnoloogias kapslimaterjalina, toidutehnoloogias prebiootikumide, emulgaatorite või magusainetena ja meditsiinis vereplasma asendajana või immuunsüsteemi stimulaatorina.

Fruktaane sünteesivateks ensüümideks on fruktosüültransferaasid, mida on leitud nii taimedest, seentest kui ka bakteritest. Võimalikke fruktosüültransferaase kodeerivaid geene on ka arhede genoomides. CAZy (*Carbohydrate-Active Enzymes*) andmebaasi järgi kuuluvad fruktosüültransferaasid glükosiidi hüdrolaaside (GH-de) klanni J, mille moodustavad ensüümiperekonnad GH32 ja GH68. Siia kuuluvad taimse ja mikroobse päritoluga suhkruid hüdrolüüsiva aktiivsusega ensüümid (invertaasid, fruktaani eksohüdrolaasid) ja ka fruktosüültransferaasid. Huvitav on see, et kui suure molekulmassiga fruktaani sünteesiks on taimedes tarvis vähemalt kahte ensüümi, siis bakterites toimub kogu protsess üheainsa ensüümi, levaansukraasi või inulosukraasi abil.

Levaansukraasid kuuluvad perekonda GH68 ning neid on leitud mitmetest bakteritest, muuhulgas ka taimepatogeenist *Pseudomonas syringae*. Levaansukraasid lõhustavad substraati, kandes fruktoosijääke aktseptormolekulidele, milleks võib olla vesi (siis toimub hüdrolüüs), aga ka teine substraadi molekul, mille transfruktosüülimisel moodustuvad β-2,6 sidemetüübiga fruktaanid. Levaansukraasid sünteesitavad nii lühikese ahelaga fruktaane ehk fruktooligosahhariide (FOS), aga ka väga pika ahelaga ning suure molekulmassiga levaani. Põhiliseks levaansukraaside substraadiks on taimedes laialt levinud sahharoos, aga mitmetel levaansukraasidel on näidatud ka rafinoosi kasutamist. Hüdrolüütiline aktiivsus ja sünteesitavate fruktaanide spekter sõltub nii levaansukraasi päritolust kui ka reaktsioonitingimustest, mis võimaldavad protsessi mõjutada sobivate saaduste tekke suunas.

Levaansukraasidel on sarnaselt teistele GH klanni J ensüümidele viielabaline β-propellerstruktuur. Katalüütilise kolmiku moodustavad ensüümi aktiivtsentris asuvad kaks aspartaati ja glutamaat. Kuigi grampositiivsetest bakteritest pärinevaid levaansukraase on palju uuritud ja kahel nendest on saadud ka kristallstruktuur, siis gramnegatiivsete pseudomonaadide levaansukraasidest on vähe teada. Kuigi *P. syringae* erinevate patovaride genoomides on kaks või kolm

levaansukraasi geeni, siis kuni käesoleva uurimistööni oli neist ainult üks (bakteri *P. syringae* pv. phaseolicola levaansukraas) puhastatud ja põhjalikumalt kirjeldatud.

Selles doktoritöös on esitatud *P. syringae* pv. tomato DC3000 levaansukraaside ekspresseerimise ja iseloomustamise tulemused. Levaansukraasid Lsc2 ja Lsc3 sünteesiti bakteris *Escherichia coli*, puhastati ning iseloomustati. Võrdlusmaterjalina kasutati bakterist *P. chlororaphis* subsp. *aurantiaca* pärinevat levaansukraasi LscA.

Minu töö põhitulemused võib kokku võtta järgnevalt:

1. *P. syringae* pv. tomato levaansukraase Lsc2 ja Lsc3 ekspresseeriti *E. coli*'s kahe erineva süsteemiga. Esimene ekspressioonisüsteem põhines pärmi *Hansenula polymorpha* maltaasi geeni promootoril (P_{MAL}), mis funktsioneerib tänu σ70-taolistele seondumisjärjestustele ka bakteris. Näitasime, et P_{MAL} on *E. coli*'s sobiva tugevusega, et saada piisav kogus katalüütiliselt aktiivset valku. Tänu eukarüootses ja prokarüootses süsteemis funktsioneerimisele saaks seda promootorit kasutada heteroloogilistes ekspressioonisüsteemides, et valida võõrvalgu tootmiseks välja sobivaim peremeesorganism. Metsiktüüpi ja mutantsete N-terminaalse His-järjestusega valkude saamiseks kasutati pURI3 vektoril põhinevat ekspressioonisüsteemi.
2. *P. syringae* erinevad patovarid on bakterite seas erandlikud, sest nende genoomides on kuni kolm levaansukraasi alleeli. Me näitasime, et *P. syringae* pv. tomato DC3000 kõik kolm *lsc* geeni (*lsc1*, *lsc2*, *lsc3*) kodeerivad *E. coli*'s heteroloogiliselt promootorilt ekspresseerides katalüütiliselt aktiivseid levaansukraase.
3. Lsc2 ja Lsc3 puhastati ning määrati ensüümide biokeemilisi omadusi, mida võrreldi *P. chlororaphis* subsp. *aurantiaca* vastavate näitajatega. Kõik kolm uuritud ensüümi kasutasid substraatidena nii sahharoosi kui ka trisahhariid rafinoosi ja tetrasahhariid stahhüoosi. Väikese aktiivsusega lagundas levaansukraas ka enda sünteesiprodukti – levaani. Lsc3, Lsc2 ja LscA afiinsused sahharoosile olid sarnased, ligikaudu 20 mM. LscA maksimaalne reaktsioonikiirus ja katalüütiline efektiivsus olid tunduvalt madalamad Lsc3 ja Lsc2 vastavatest väärtustest. Näitasime, et madalal sahharoosi kontsentratsioonil polümeriseeris Lsc3 tunduvalt paremini kui LscA. Kõrgel sahharoosi kontsentratsioonil olid transfruktosüüliva aktiivsuse väärtused sarnased, kuid LscA sünteesis rohkem pikemaid oligosahhariide kui Lsc2 ja Lsc3.
4. Leidsime, et Lsc3 ja LscA võivad kanda fruktoosijääke alternatiivsetele aktseptoritele, mille tulemusena moodustuvad heterooligofruktaanid (HOF). Seda pole pseudomonaadidest pärinevate levaansukraaside puhul varem näidatud. Esmakordselt näitasime, et D-sorbitool, D-galakturoonhape, D-mannitool, ksülitool, metüül-α-D-glükopüranosiid ja disahhariid ksülobioos on levaansukraaside aktseptoriteks. Optimiseerisime neutraalsete fruktaanide määramiseks suure läbilaskevõimega uudse nanoESI HCT mass spektromeetria meetodi, mida kasutasime FOS-ide ja HOF-ide analüüsimiseks.

5. Lsc2 ja Lsc3 osutusid stabiilseteks ja katalüütiliselt aktiivseteks valkudeks, mis säilitasid oma katalüüsivõime erinevatel temperatuuridel ja pH väärtustel. Nende aktiivsust ei mõjutanud ka mitmed detergendid ja metalliioonid. Sellised omadused on iseloomulikud rakuvälistele ensüümidele ja väga olulised valkudele, mida soovitakse tööstuses kasutada. Kuna *P. syringae* pv. tomato levaansukraasid toodavad suhteliselt odavast substraadist biotehnoloogiliselt huvipakkuvaid produkte nagu levaan, FOS-id ja HOF-id, siis võiksid need ensüümid tehnoloogias biokatalüsaatoritena rakendust leida.
6. Kuna andmed pseudomonaadidest pärinevate levaansukraaside struktuuri ja funktsiooni seostest puudusid, siis alustasime Lsc3 mutatsioonianalüüsiga. Levaansukraaside valgujärjestuste joonduse ja kirjanduse andmete analüüsi alusel ennustasime, et levaansukraasi Lsc3 katalüütilise kolmiku moodustavad Asp62, Asp219 ja Glu303. Thr302 ja His321 muteerimine Lsc3 valgus vähendas oluliselt substraadi seondumist ja transfruktosüülivat aktiivsust. Arvatavasti kuuluvad Thr302 ja His321 Lsc3 aktiivtsentri +1 alapiirkonda, mis on oluline nii substraadi kui ka aktseptori sidumisel.

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CURRICULUM VITAE

I General data

Name	Triinu Visnapuu
Date of birth	October 31, 1983
Citizenship	Estonian
Contact	University of Tartu, Institute of Molecular and Cell Biology, 23 Riia Str., 51010 Tartu, Estonia +3727375013 visnapuu@ut.ee

Education

Since 2007	PhD student of molecular and cell biology (genetics), in University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology (supervisor Tiina Alamäe, PhD)
2007	University of Tartu, Faculty of Biology and Geography, Institute of Molecular and Cell Biology, Master's degree in gene technology (supervisor Tiina Alamäe, PhD)
2005	University of Tartu, Faculty of Biology and Geography, Bachelor's degree in Gene Technology (supervisor Tiina Alamäe, PhD)
1990–2002	Vinni-Pajusti Gymnasium (graduated with highest honors)

Language skills

Estonian and English, Russian (basic level)

II Scientific activities

Main fields of research

My research is connected to glycobiology, heterologous protein expression in bacterial and yeast systems, enzymology and biotechnology. In my scientific research I have been focusing on bacterial levansucrases – enzymes with biotechnological potential. Levansucrases produce a variety of fructans with different composition and chain lengths. I have been studying levansucrases from *Pseudomonas* bacteria: expressed the respective genes in *Escherichia coli*, purifying the enzymes and investigating their properties. Also, I have been participating in the research of maltase gene promoter from yeast *Hansenula polymorpha* which functions also in prokaryotic host. I have been participating in the grants ETF5676 (2004–2007), GLOMR7528 (2008–2011) and GLOMR9072 (2012–2015) from Estonian Science Foundation.

List of publications

1) Alamäe T, **Visnapuu T**, Mardo K, Mäe A, Zamfir AD (2012) Levansucrases of *Pseudomonas* bacteria: novel approaches for protein expression, assay of enzymes, fructooligosaccharides and heterooligofructans. In: *Carbohydrate Chemistry*, Vol 38. Rauter AP, Lindhorst TK (Eds.). Specialist Periodical Reports, Royal Society of Chemistry, Cambridge, UK, p 176–191. Accepted for publication, expected publication date 31st of August, 2012.
2) **Visnapuu T***, Mardo K*, Mosoarca C, Zamfir AD, Vigants A, Alamäe T (2011) Levansucrases from *Pseudomonas syringae* pv. tomato and *P. chlororaphis* subsp. *aurantiaca*: Substrate specificity, polymerizing properties and usage of different acceptors for fructosylation. *J Biotechnol* **155**: 338–349. * These authors contributed equally to this work.
3) Mardo K, **Visnapuu T**, Alamäe T (2010) Isolation and high-throughput screening methods of levansucrase mutants of a plant pathogen *Pseudomonas syringae* DC3000. In: *Proceedings of the 6th International Conference on Polysaccharides-Glycoscience: 6th International Conference on Polysaccharides-Glycoscience*. Rapkova R, Copikova J, Šarka E (Eds.). Praque, Czech Republic, 2010, p 36–39.
4) **Visnapuu T**, Zamfir AD, Mosoarca C, Stanescu MD, Alamäe T (2009) Fully automated chip-based negative mode nanoelectrospray mass spectrometry of fructooligosaccharides produced by heterologously expressed levansucrase from *Pseudomonas syringae* pv. tomato DC3000. *Rapid Commun Mass Spectrom* **23**: 1337–1346.
5) **Visnapuu T**, Mäe A, Alamäe T (2008) *Hansenula polymorpha* maltase gene promoter with sigma 70-like elements is feasible for *Escherichia coli*-based biotechnological applications: Expression of three genomic levansucrase genes of *Pseudomonas syringae* pv. tomato. *Process Biochem* **43**: 414–422.

Research grants and scholarships

2008–2012	meeting grants for young scientists by FEMS (Federation of European Microbiological Societies) and by EU and DoRa program activity 8
2010	EU and DoRa program activity 6 scholarship for research visit to National Institute for Research and Development in Electrochemistry and Condensed Matter, Timisoara, Romaina
2008	COST928 Short Term Scientific Mission grant to Department of Chemistry and Biology, “Aurel Vlaicu” University of Arad, Arad, Romania
2006	FEMS research grant for young scientists (Research Fellowship) to Institute of Microbiology and Biotechnology, University of Latvia, Riga, Latvia

Special courses

2010	workshop "Structural glycoscience: methods and instrumentation" IBS and ESRF/ILL, Grenoble, France; 29.06–1.07.
2008	10th Summer Course Glycosciences, Wageningen University, Wageningen, The Netherlands; 9-12.06.

Other professional and administrative activities

Since 2011	participation as a supervisor in preparatory course for IBO (International Biology Olympiad)
Since 2010	member of The Euroglycoscience Forum (EGSF)
Since 2010	supervisor of Practical Course of Microbiology and Virology (LOMR.03.021) in University of Tartu, Faculty of Science and Technology, Institute of Molecular and Cell Biology
Since 2005	member of Estonian Society of Microbiologists (member of FEMS)
2010	elaboration, preparation and supervision of practical works in microbiology for Estonian National Biology Olympiad for high-school level
2007–2010	member of COST928 science network "Control and exploitation of enzymes for added-value products"

III Public and social activities

Since 2008	member of Sorority Amicitia

CURRICULUM VITAE

I Üldandmed

Ees- ja perekonnanimi	Triinu Visnapuu
Sünniaeg	31. oktoober, 1983
Kodakondsus	Eesti
Kontaktandmed	Tartu Ülikool, Molekulaar- ja Rakubioloogia Instituut Riia 23, 51010 Tartu, Eesti +3727375013 visnapuu@ut.ee

Hariduskäik

Alates 2007	molekulaar- ja rakubioloogia (geneetika) doktorant Tartu Ülikooli Teadus- ja Tehnoloogiateaduskonnas Molekulaar- ja Rakubioloogia Instituudis (juhendaja Tiina Alamäe, PhD)
2007	geenitehnoloogia magistrikraad Tartu Ülikooli Bioloogia- ja Geograafiateaduskonnas Molekulaar- ja Rakubioloogia Instituudis (juhendaja Tiina Alamäe, PhD)
2005	geenitehnoloogia bakalaureusekraad Tartu Ülikooli Bioloogia- ja Geograafiateaduskonnas Molekulaar- ja Rakubioloogia Instituudis (juhendaja Tiina Alamäe, PhD)
1990–2002	Vinni-Pajusti Gümnaasium (kuldmedal)

Keelteoskus

Eesti ja inglise keel, vene keel algtasemel

II Teaduslik tegevus

Peamised uurimisvaldkonnad

Minu teadustöö on seotud glükobioloogia, heteroloogilise valgueksрessiooniga nii bakteri- kui ka pärmirakkudes, ensümoloogia ja biotehnoloogiaga. Oma uurimistöös keskendusin biotehnoloogilise potentsiaaliga bakteriaalsetele valkudele – levaansukraasidele. Levaansukraaside reaktsiooniproduktideks on erineva ahelapikkuse ja koostisega fruktaanid. Ma uurisin *Pseudomonas*'test pärinevaid levaansukraase: ekspresseerisin vastavaid geene bakteris *Escherichia coli*, puhastasin valgud ning uurisin nende omadusi. Veel olen osalenud pärmist *Hansenula polymorpha* pärineva maltaasi geeni promootori uurimisel. See promootor funktsioneerib nii eukarüootses kui ka prokarüootses peremehes. Olen osalenud järgmiste teadusteemade täitmises: Eesti Teadusfondi grantid ETF5676 (2004–2007), GLOMR7528 (2008–2011) ja GLOMR9072 (2012–2015).

Publikatsioonide loetelu

1) Alamäe T, **Visnapuu T**, Mardo K, Mäe A, Zamfir AD (2012) Levansucrases of *Pseudomonas* bacteria: novel approaches for protein expression, assay of enzymes, fructooligosaccharides and heterooligofructans. Väljaandes: *Carbohydrate Chemistry*, Vol 38. Rauter AP, Lindhorst TK (Eds.). Specialist Periodical Reports, Royal Society of Chemistry, Cambridge, UK, lk 176–191. Publitseerimiseks vastu võetud, ilmumiskuupäevaks on 31. august, 2012.
2) **Visnapuu T***, Mardo K*, Mosoarca C, Zamfir AD, Vigants A, Alamäe T (2011) Levansucrases from *Pseudomonas syringae* pv. tomato and *P. chlororaphis* subsp. *aurantiaca*: Substrate specificity, polymerizing properties and usage of different acceptors for fructosylation. *J Biotechnol* **155**: 338–349. * Jagatud esiautorlus.
3) Mardo K, **Visnapuu T**, Alamäe T (2010) Isolation and high-throughput screening methods of levansucrase mutants of a plant pathogen *Pseudomonas syringae* DC3000. Väljaandes: *Proceedings of the 6th International Conference on Polysaccharides-Glycoscience: 6th International Conference on Polysaccharides-Glycoscience*. Rapkova R, Copikova J, Šarka E (Toim.). Praque, Czech Republic, 2010, lk. 36–39.
4) **Visnapuu T**, Zamfir AD, Mosoarca C, Stanescu MD, Alamäe T (2009) Fully automated chip-based negative mode nanoelectrospray mass spectrometry of fructooligosaccharides produced by heterologously expressed levansucrase from *Pseudomonas syringae* pv. tomato DC3000. *Rapid Commun Mass Spectrom* **23**: 1337–1346.
5) **Visnapuu T**, Mäe A, Alamäe T (2008) *Hansenula polymorpha* maltase gene promoter with sigma 70-like elements is feasible for *Escherichia coli*-based biotechnological applications: Expression of three genomic levansucrase genes of *Pseudomonas syringae* pv. tomato. *Process Biochem* **43**: 414–422.

Saadud uurimistoetused ja stipendiumid

2008–2012	FEMS-i (*Federation of European Microbiological Societies*) ning Euroopa Liidu DoRa programmi tegevus 8 stipendiumid erinevatel konverentsidel osalemiseks
2010	Euroopa Liidu DoRa programmi tegevus 6 stipendium uurimistöö tegemiseks teadusasutuses National Institute for Research and Development in Electrochemistry and Condensed Matter, Timisoaras, Rumeenias
2008	COST928 *Short Term Scientific Mission* stipendium "Aurel Vlaicu" Aradi Ülikooli Keemia ja Bioloogia Osakonda, Aradis, Rumeenias
2006	FEMS-i stipendium uurimistöö tegemiseks (*Research Fellowship*) Mikrobioloogia ja Biotehnoloogia Instituudis, Läti Ülikoolis, Riias, Lätis

Kursused

2010	Seminar "Structural glycoscience: methods and instrumentation" IBS ja ESRF/ILL, Grenoble, Prantsusmaa; 29.06–1.07.
2008	10. Glükoteaduste Suvekool (Summer Course Glycosciences), Wageningeni Ülikool, Wageningen, Holland; 9–12.06.

Muu teaduslik erialane ja organisatsiooniline tegevus

Alates 2011	osalemine rahvusvahelise bioloogiaolümpiaadi (IBO) ettevalmistuskursuse juhendamisel
Alates 2010	The Euroglycoscience Forumi (EGSF) liige
Alates 2010	Mikrobioloogia ja viroloogia praktikumi (LOMR.03.021) juhendaja Tartu Ülikooli Molekulaar- ja Rakubioloogia Instituudis
Alates 2005	Eesti Mikrobioloogide Ühingu liige
2010	Eesti bioloogiaolümpiaadi (EBO) lõppvooru mikrobioloogiateemalise praktilise töö väljatöötamine, ettevalmistamine ja juhendamine
2007–2010	teadlaste võrgustiku COST928 "Control and exploitation of enzymes for added-value products" liige

III Ühiskondlik tegevus

Alates 2008	Üliõpilaskorporatsioon Amicitia liige

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4. **Andres Mäe**. Conjugal mobilization of catabolic plasmids by transposable elements in helper plasmids. Tartu, 1992, 91 p.
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