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Molecular background, *in situ* production and structure/function relation of bacterial exopolysaccharides in gluten-free dough and bread

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Abbrevations

AF4	asymmetrical flow field flow fractionation
amp	ampicillin
APS	ammonium persulfate
ATP	adenosine triphosphate
bp	base pair
cam	chloramphenicol
CPS	capsular polysaccharide
D_2O	heavy water
DMSO-d_6	deuterated dimethyl sulfoxide
DNA	desoxyribonucleic acid
DTT	dithiothreitol
DY	dough yield
E.	*Escherichia*
EDTA	ethylene diamine tetraacetic acid
EPS	exopolysaccharide
FPLC	free pressure liquid chomatography
Ftf	fructosyltransferase
G6PDH	glucose-6-phosphate dehydrogenase
GLC	Gas-liquid chromatographie
GRAS	Generally Recognized As Safe
Gtf	glucosyltransferase
HePS	heteropolysaccharide
HK	hexokinase
HoPS	homopolysaccharide
HPLC	high performance liquid chromatography
HPMC	hydroxypropylmethylcellulose
kb	kilobase
kDa	kilo dalton
L.	*Lactobacillus*
LAB	lactic acid bacteria
LB	lysogeny broth
Ln.	*Leuconostoc*
MALLS	multiangle laser light scattering
MCS	multiple cloning site

MeI	methyl iodide
Mn	number average molecular weight
MM	Michaelis-Menten
Mw	weight average molecular weight
NADP	nicotineamide adenine dinucleotide phosphate
OD	optical density
ORF	open reading frame
PCR	polymerase chain reaction
PGI	phosphoglucose isomerase
RAPD	randomly amplified polymorphic DNA
rms	root mean square
rpm	rounds per minute
Rw	weight average root mean square
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	tris acetate EDTA
TEMED	tetramethylethylendiamine
TMW	Technische Mikrobiologie Weihenstephan
W.	*Weissella*

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1 Introduction

1.1 Celiac disease and gluten-free baked goods

Celiac disease is an immune-mediated enteropathy leading to intestinal mucosal damage and malabsorption of several important nutrients and is triggered by the ingestion of gluten in genetically susceptible individuals (Catassi and Fasano, 2008). It affects about 1% of the world population (Catassi and Yachha, 2009) and remains undiagnosed in many cases, since celiac disease is often atypical or even silent on clinical grounds. Undiagnosed, there is a risk of it leading to long-term complications, such as osteoporosis, infertility or cancer (Fasano and Catassi, 2001). Celiac disease is detected through serological screening or through biopsy of the luminal surface of the small intestinal mucosa, which will show truncation or loss of villi (Figure 1).

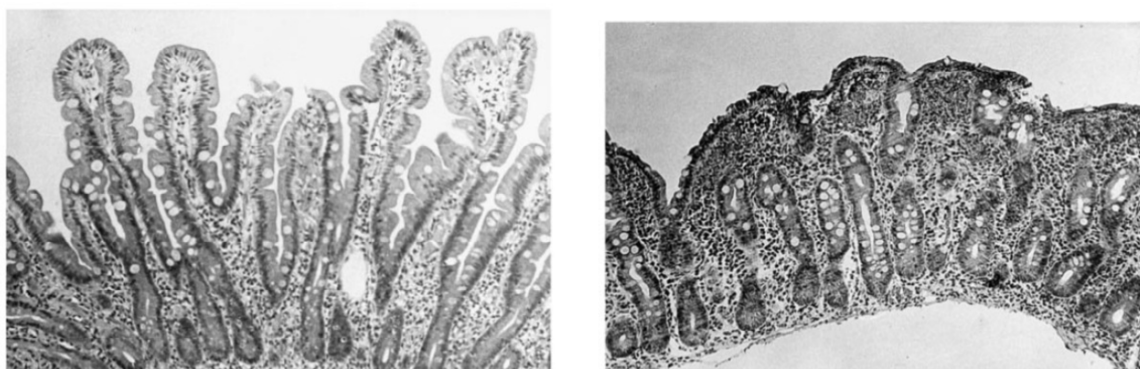


Figure 1: Normal versus celiac disease small-bowel biopsy (Collin et al., 2002).
Left: normal small bowel biopsy with finger-like villi. Right: small bowel biopsy with villous atrophy.

Currently, the only effective treatment for celiac disease is a lifelong gluten-free diet. Gluten-free foods either consist of ingredients which do not contain wheat, kamut, rye, spelt, barley or oats, or, they are made of ingredients from such cereals, which must have been previously processed to remove any gluten (Moroni et al., 2009) in order to comply with the gluten level threshold of 20 mg kg^{-1} (Deutsch et al., 2008).

1.1.1 Composition and quality of gluten-free breads

In recent years there has been an increasing interest in improving gluten-free breads as those commercially available are characterized by poor crumb and crust characteristics as well as poor palate texture and flavor (Gallagher et al., 2003) and because prices of gluten-free foods are often considerably higher than those of conventional products (Hüttner and Arendt, 2010). Furthermore, these commonly rice and starch based products contain very low contents of various nutrients, fiber and vitamins and quickly become stale

(Ahlborn et al., 2005; Hager et al., 2012). Since malabsoption of nutrients is associated with celiac disease, adherence to gluten-free diet worsens an already unbalanced diet (Mariani et al., 1998). In several studies gluten-free nutrient-dense whole grains, such as buckwheat, millet, quinoa, amaranth and brown rice were used to overcome these issues. Gluten-free bread recipes containing flours of these grains have been shown to increase nutritional value with regard to protein, fiber and mineral content (reviewed by Moroni et al., 2009). Notably the pseudocereals amaranth, quinoa and buckwheat were suggested to be a healthy alternative to the frequently used traditional ingredients in gluten-free products (Alvarez-Jubete et al., 2009).

1.1.2 The role of gluten in bread making

Gluten is a protein consisting of glutenin and gliadin. It exhibits cohesive, elastic and viscous properties and is therefore termed the 'structural' protein for bread making. It provides viscoelasticity to the dough, has gas-holding properties and gives a good crumb structure in baked bread (Gallagher et al., 2004). Thus, the development of gluten-free breads is a major technological challenge (Arendt et al., 2008) and possibilities to either improve protein network formation or to increase water absorption by proteins and starch have to be explored. Currently, hydrocolloids and proteins (e.g. dairy powders or eggs) are used to improve gluten-free breads and enzymes (e.g. transglutaminase) are also being investigated intensely (Renzetti et al., 2008). Sourdough fermentation and the application of hydrostatic pressure were also found to be effective in improving the baking performance of gluten-free flours. Of all additives, hydrocolloids in particular were successfully used in several studies to mimic the viscoelastic properties of gluten (Ahlborn et al., 2005; Lazaridou et al., 2007; Moore et al., 2004; Sciarini et al., 2010). The effects of hydrocolloids were dependent on the specific hydrocolloid, as well as its addition level and the type of flour used (Hüttner and Arendt, 2010). Most of the applied hydrocolloids are of seed or root origin (guar gum, locust bean gum), are from microorganisms (xanthan gum, gellan gum) or are cellulose derivates (hydroxypropylmethylcellulose (HPMC)) (Hoefler, 2004).

However, the incorporation of additives in bread formulas has several disadvantages, as these breads are expensive and do not meet consumers' demands for natural products (Moroni et al., 2009).

1.2 Bacterial exopolysaccharide formation

Polysaccharides are widely distributed in nature and are industrially used as stabilisers, thickeners and gelling agents in food products. They originate from different sources, e.g.

bacteria, fungi, algae or plants. Polysaccharides produced by microorganisms can be classified into three groups according to their location in the cell: (i) cytosolic polysaccharides, which are employed as carbon and energy sources, (ii) polysaccharides that make up the cell wall and (iii) polysaccharides that are located outside the cell wall, known as exopolysaccharides (EPSs) (Donot et al., 2012). The latter are divided into capsular polysaccharides (CPSs) that are associated with the cell surface or in polysaccharides that are not attached (Cerning, 1990).

1.2.1 Exopolysaccharides

Based on their composition, EPSs can be differentiated into homo- (HoPS) and heteropolysaccharides (HePS). HoPS are composed of one monosaccharide and are formed from sucrose by glycosyltransferases (Monsan et al., 2001). They contain either glucose or fructose as the sole monosaccharide and are therefore termed glucan and fructan respectively. EPSs are produced by many lactic acid bacteria (LAB) and glucan and fructan production has been reported for members of *Leuconostoc*, *Streptococcus, Weissella* and *Lactobacillus* (Monsan et al., 2001). Among the genera *Lactobacillus*, approximately 30 species of lactobacilli are described as EPS producers (e.g. *L. casei, L. curvatus, L. reuteri, L. animalis, L. sanfranciscensis, L. plantarum*) (Badel et al., 2011). Glucans and fructans are further classified through their main linkage type as pictured in Figure 2. Glucans are divided in α- and β- glucans, whereby β-glucan production by LAB is rare. Up to now four different subtypes of α-glucans are known. The glucan dextran contains α-(1,6) linked glucose and is the most common. Glucans with α-(1,3) linkages are called mutan and glucans with a majority of α-(1,4) linkages reuteran. Alternans are glucans consisting of alternating α-(1,3) and α-(1,6) bonds. Fructans are divided into levan and inulin. Levan contains mainly β-(2,6) linked fructose and inulin mainly β-(2,1) linkages. Further branching in different positions of glucans and fructans is possible. In contrast, HePS consist of different monosaccharides and are formed through various enzymes in more complex biosynthetic pathways. HePS are not examined in this work so the term EPS always refers to HoPS, unless stated otherwise.

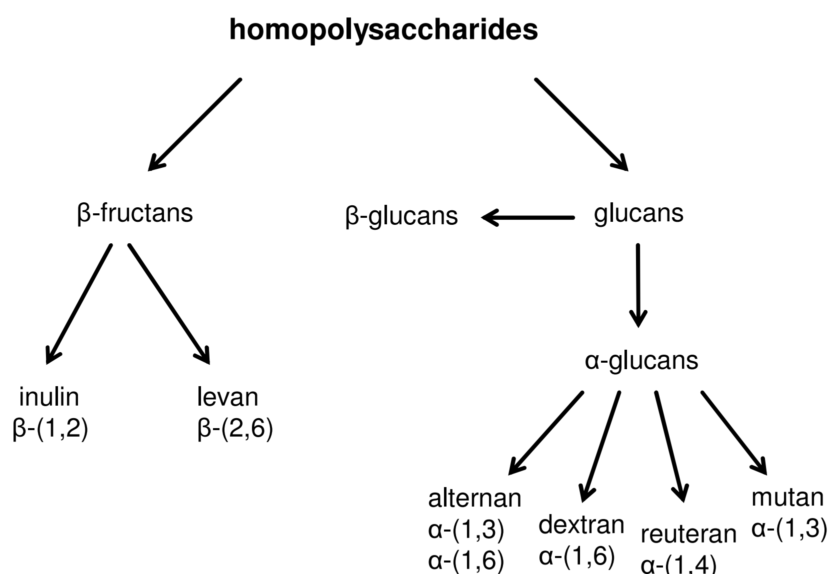


Figure 2: Classification of homopolysaccharides produced by lactobacilli

Different functions are attributed to EPSs but the exact benefit of a cell from EPS formation is often not clear. Since most of the EPS producing bacteria are not able to degrade EPS (lack of respective enzymes), utilization of EPS as a carbon source seems unlikely (Cerning, 1990; Korakli and Vogel, 2006). Rather osmotic and energetic advantages are suggested for EPS forming lactobacilli, as they are able to decrease sucrose concentrations through EPS production (Korakli and Vogel, 2006). Furthermore, EPS is thought to play a role in the protection of cells against desiccation, phagocytosis and phage attack, antibiotics or toxic compounds or in the adhesion to solid surfaces and biofilm formation as seen in dental plaque (Cerning, 1990; De Vuyst and Degeest, 1999).

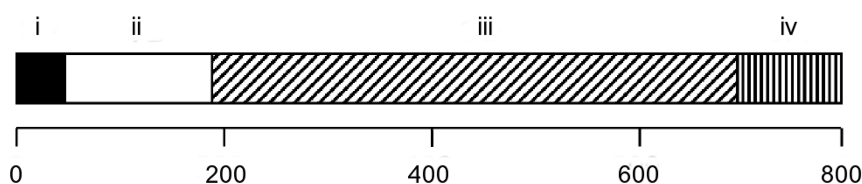
LAB EPSs exhibit a large variety of different linkage types, molecular weight, and branching, resulting in diverse functional properties, which are used by the food industry. EPSs can be used as viscosifying, stabilizing, emulsifying, sweetening, gelling, or water-binding agents (van Hijum et al., 2006). Despite the wide diversity of microbial EPSs, only three bacterial EPSs, namely xanthan gum (HePS), gellan gum (HePS) and curdlan gum (HoPS) and one mold EPS (pullulan, HoPS from *Aureobasidium pullulans*) are authorized as additives in the food industry in the United States and Europe (Badel et al., 2011; Donot et al., 2012). Xanthan is produced by *Xanthomonas campestris* and has a very high molecular weight ranging from 500 to 2000 kilo Dalton (kDa). It consists of D-glucosyl, D-mannosyl and D-glucuronyl acid residues in a molar ratio of 2:2:1 and variable proportions of *O*-acetyl and pyruvyl residues (Rosalam and England, 2006). Due to its superior rheological properties it is used as a rheological control agent in aqueous systems and as stabilizer for emulsions and suspensions. Gellan gum is formed by *Pseudomonas elodea*. The repeating unit of this polysaccharide is composed of β-D-glucose, L-rhamnose and D-

glucuronic acid in a ratio of 3:1:1 (Bajaj et al., 2007). Gellan solutions are stable under autoclaving conditions and the molecular weight of gellan gums ranges between 1000 and 2000 kDa (Hoefler, 2004). Curdlan gum is an insoluble, linear homopolysaccharide without branching, which is produced by *Agrobacterium biobar*. It is composed of 400 to 500 D-glucose residues joined by β-(1,3) linkages (Shih et al., 2009). It improves the textural quality, water holding capacity and thermal stability of various foods (Jezequel, 1998).

1.2.2 Glycosyltransferases

The enzymes responsible for HoPS synthesis from sucrose are called glycosyltransferases. Glycosyltransferases that synthesize glucans are named glucosyltransferases (Gtfs) or glucansucrases. Often they are also named according to their products as dextran-, mutan-, reuteran- and alternansucrase respectively. Glycosyltransferases that form fructans are called fructosyltransferases (Ftfs) or fructansucrases. Levan producing Ftfs are named levansucrases and inulin forming Ftfs are called inulosucrases. These extracellular or cell wall bound enzymes obtain energy from the cleavage of the osidic bond in sucrose (Korakli and Vogel, 2006) and transfer the glucose or fructose moiety to an acceptor molecule, while the other moiety is released into the environment and can be transported into the cell and be metabolized. Depending on the acceptor molecule three glycosyltransferase activities can be distinguished. If water is the acceptor molecule, sucrose is hydrolyzed into glucose and fructose (hydrolysis activity). The second possible reaction is a transfer activity, where the sugar monomer is transferred to another sugar molecule to form oligosaccharides. Moreover, these enzymes show a second transfer activity, where the sugar monomer is transferred to a growing polysaccharide chain. Dominance of these activities can be influenced depending on culture conditions (Korakli et al., 2003; Kralj et al., 2004b).

Gtfs and Ftfs of LAB follow a conserved structure with the (i) N-terminal signal peptide, (ii) variable region, (iii) catalytic domain and (iv) the C-terminal region that is called the glucan binding domain in Gtfs and cell-wall anchor motif in Ftfs (Figure 3).



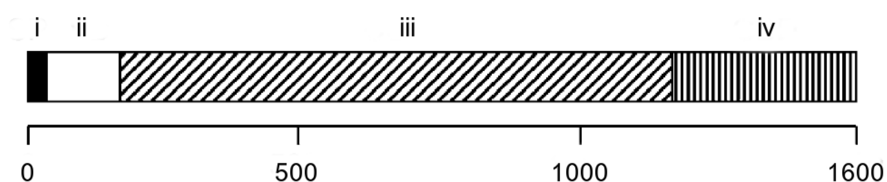


Figure 3: General structure of glycosyltransferases from LAB
(i) N-terminal signal peptide, (ii) variable region, (iii) catalytic domain, (iv) C-terminal region. Scale illustrates length in amino acids (adapted from Korakli and Vogel, 2006).

Gtfs and Ftfs perform similar reactions but do not share a high amino acid sequence similarity and differ strongly in protein structures. In addition they differ in their molecular weight. Gtfs are large proteins with an average amino acid (aa) length of 1600 aa. Ftfs are smaller and have an average length of 800 aa. Though Gtfs can only use sucrose as a substrate, Ftfs are also able to use raffinose (van Geel-Schutten et al., 1999).

1.3 Sourdough and its application in bread

The use of sourdough has a long tradition in the production of wheat and rye breads. Its main functions are leavening, acidification and aroma formation, which is achieved through LAB and yeasts. Incorporated in bread, sourdough improves texture, flavor, volume, shelf life and nutritional value (Arendt et al., 2007). Additionally, special metabolic activities, like proteolysis, EPS formation and synthesis of volatile and antimicrobial compounds can intensify the effects mentioned above (Corsetti and Settanni, 2007; Moroni et al., 2009). Recently, sourdough received increased attention for the production of high quality gluten-free bread, combined with reduction of expensive additives (Moroni et al., 2009), as sourdough was discovered to be crucial for obtaining gluten-free breads with a uniform crumb structure (Schober et al., 2007). Furthermore, sourdough LAB with special peptidase activities can be used to eliminate traces of gluten epitopes caused by cross-contamination of gluten-free products (Hüttner and Arendt, 2010).

However, gluten-free sourdough fermentation can look back on a long tradition. In many Asian and African countries, gluten-free cereal products (e.g. corn, rice and millet), are basic foods, and are often fermented prior consumption (Nout, 2009). Traditionally

fermented gluten-free cereal products are e.g. Ogi, Kenkey, Kisra and Idli. Ogi is a fermented gruel made from corn, millet or sorghum and is common in Africa (Blandino et al., 2003). Kenkey from Ghana is a cooked bread-like corn product and Kisra, which is a typical fermented Sudanese food, is produced from millet or sorghum (Blandino et al., 2003; Nout et al., 1996). Idli is a blend of 80% rice and 20% black gram and is consumed in Southeast Asian countries (Blandino et al., 2003; Nout, 2009).

1.3.1 Ecology of sourdough

The microbial ecology of a sourdough is determined by endogenous factors (e.g. chemical and microbial composition of the dough), exogenous factors (e.g. temperature and redox potential) and by process parameters like dough yield, salt, amount and composition of the starter culture, fermentation time and propagation steps (Arendt et al., 2007). The ecology of wheat and rye sourdoughs has been well explored. Different genus of *Lactobacillus (L.)*, *Saccharomyces* and *Candida* typically occur in these sourdoughs (Gänzle, 2005). Typical LAB detected in these doughs are *L. sanfranciscensis*, *L. plantarum*, *L. pontis*, *L. panis* and *L. spicheri*. In traditional sourdoughs, which are carried out in tropical climates, more thermophilic LAB like *L. fermentum* and *L. reuteri* are commonly present (Moroni et al., 2009). Moroni et al. (2011) found obligately or facultatively heterofermentative LAB in gluten-free sourdoughs, which are commonly associated with wheat or rye sourdoughs. But the spontaneous fermentation resulted also in the selection of atypical species, concluding that appropriate starter cultures and process parameters have to be explored and adapted to different substrates and demands (Moroni et al., 2011).

1.3.2 *In situ* production of EPS through lactobacilli

EPSs produced by LAB are expected to beneficially affect water absorption of the dough, dough rheology and machinability, its stability during frozen storage, loaf volume and bread staling (Tieking and Gänzle, 2005). Several studies have already shown that EPSs are effective in bread making and that EPS producing LAB have been successfully used in wheat sourdough fermentations. Whereas Di Cagno et al. (2006) and Katina et al. (2009) showed that breads created from sourdoughs containing the EPS producers *Weissella* (*W.*) *cibaria*, *W. confusa* or *L. plantarum* had an increased specific volume and a lowered firmness, Kaditzky and Vogel (2008), Korakli et al. (2001, 2003) and Tieking et al. (2003) corroborated *in situ* EPS production in sourdoughs. Associated with EPS formation, LAB can also produce gluco- or fructo-oligosaccharides (Korakli et al., 2003; Tieking and Gänzle, 2005), which improve not only the nutritional value but also the bread

structure; the prebiotic effects of fructo-oligosaccharides being especially well documented (Bierdrzycka and Bielecka, 2004). Both features are extremely promising for gluten-free bread making (Moroni et al., 2009). Though EPS formation in gluten-containing sourdoughs has been investigated quite well, little information is available on EPS forming LAB in gluten-free sourdoughs and their effect on gluten-free breads. Schwab et al. (2008) investigated the formation of EPSs by *L. reuteri* and *W. cibaria* in sorghum sourdoughs and Galle et al. (2010, 2012) studied the suitability of EPS producing strains as starter cultures for sorghum sourdoughs and successfully applied them to improve the bread-making potential of sorghum flour. Peressini et al. (2011), Lazaridou et al. (2007) and Sciarini et al. (2010) explored the effects of commercially available hydrocolloids on gluten-free doughs and breads and improved them to different extents, but the addition of hydrocolloids is in conflict with consumer demand for natural and functional foods. This may be overcome by *in situ* production of EPSs and oligosaccharides from sucrose using LAB as functional starters, as such EPSs do not have to be declared. Up to now insufficient information is available about the effects of EPSs produced from LAB on gluten-free breads. Furthermore, EPSs have to be produced in considerable amounts to replace hydrocolloids. Since the type of EPS, e.g. molecular weight, degree of branching and type of linkages strongly influence its rheological behavior, more research is needed to identify the most suitable EPS (Moroni et al., 2009).

1.4 Motivation and objective

Currently many additives are used to improve the baking performance of gluten-free flours but this does not coincide with consumer interest in natural foods. This converse thinking may be overcome by the use of sourdough, which was found to be prerequisite for the production of high quality gluten-free bread (Schober et al., 2007). Combined with the general positive effects evoked by sourdoughs, special metabolic activities like *in situ* EPS formation may reduce or replace the application of additives, provided that EPSs are produced in adequate amounts. *In situ* EPS formation was already approved in several studies conducted in wheat or rye sourdough fermentations (Kaditzky et al., 2008; Korakli et al., 2001; Tieking et al., 2003). But although EPS formation in gluten-containing sourdoughs has been investigated quite well, only a few attempts have been conducted with gluten-free sourdoughs. And, insufficient information is available concerning the effects of EPSs on gluten-free breads.

Therefore, the objective of this work was to examine the impact of EPSs on the quality of gluten-free bread and to establish a connection between the structure and function of EPSs in gluten-free bread making. Furthermore, the aim was to identify suitable strains for

in situ EPS production and to optimize EPS formation in gluten-free sourdoughs in order to ascertain that *in situ* EPS amounts can reach effectual levels. By analyzing other concomitantly formed compounds resulting from carbohydrate metabolism, which enhance or limit applications and by exploring genes responsible for EPS formation and characterizing resulting proteins to gain more information about EPS forming enzymes, this work should enable to fix parameters for optimal *in situ* EPS production.

2 Materials and methods

Figure 4 gives an overview of the methods performed.

The chemicals used in this thesis were purchased from Sigma-Aldrich, Roche, Merck, Serva, Carl Roth and GERBU Biotechnik GmbH.

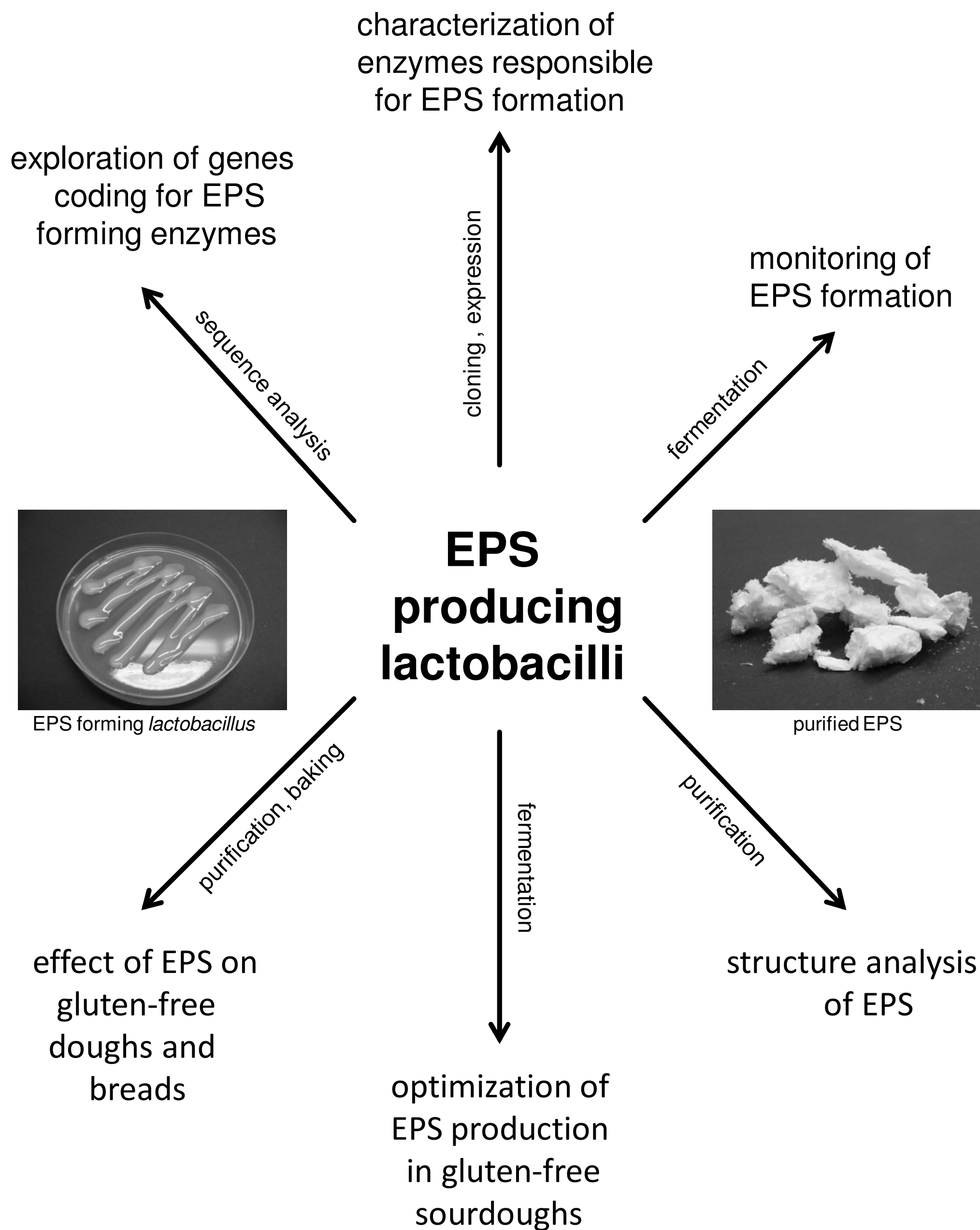


Figure 4: Flowchart of general procedure.

2.1 Strains and culture conditions

All strains used in this work were obtained from the Technische Mikrobiologie Weihenstephan (TMW) culture collection. For subcloning and heterologous expression *Escherichia (E.) coli* TOP10 and Rosetta were utilized. They were cultivated aerobically in lysogeny broth (LB) containing 10 g L^{-1} peptone, 5 g L^{-1} yeast extract and 10 g L^{-1} NaCl (pH 7) at 37 °C unless stated otherwise. To maintain the plasmids 100 µg mL^{-1} ampicillin (amp), and additionally 68 µg mL^{-1} chloramphenicol (cam) for cultivating Rosetta were added respectively.

Table 1: Lactobacilli used in this thesis

Species	Strain TMW no.	Species	Strain TMW no.
L. curvatus	1.7	*L. curvatus*	1.1437
L. curvatus	1.17	*L. curvatus*	1.1438
L. curvatus	1.48	*L. curvatus*	1.1439
L. curvatus	1.49	*L. curvatus*	1.1403
L. curvatus	1.50	*L. curvatus*	1.1447
L. curvatus	1.51	*L. curvatus*	1.1449
L. curvatus	1.167	*L. curvatus*	1.1450
L. curvatus	1.266	***L. reuteri***	**1.106**
L. curvatus	1.407	*L. reuteri*	1.137
L. curvatus	1.421	*L. reuteri*	1.292
L. curvatus	1.439	*L. reuteri*	1.294
L. curvatus	1.440	*L. reuteri*	1.295
L. curvatus	1.591	*L. reuteri*	1.656
L. curvatus	1.593	*L. reuteri*	1.693
L. curvatus	1.594	*L. reuteri*	1.723
L. curvatus	1.595	*L. reuteri*	1.966
L. curvatus	1.596	*L. reuteri*	1.1187
L. curvatus	**1.624**	*L. reuteri*	1.1281
L. curvatus	1.700	*L. sanfranciscensis*	1.52
L. curvatus	1.1291	***L. sanfranciscensis***	**1.392**
L. curvatus	1.1365	*L. sanfranciscensis*	1.450
L. curvatus	1.1381	*L. sanfranciscensis*	1.451
L. curvatus	1.1382	*L. sanfranciscensis*	1.709
L. curvatus	1.1384	*L. sanfranciscensis*	1.710
L. curvatus	1.1389	*L. sanfranciscensis*	1.894
L. curvatus	1.1390	*L. sanfranciscensis*	1.1193
L. curvatus	1.1391	*L. sanfranciscensis*	1.1221
L. curvatus	1.1408	***L. animalis***	**1.971**

bold face: Strains intensively used in this study

LAB were cultivated anaerobically in Homohiochii medium (Kitahara et al., 1957), which is denoted as spicher medium at this institute, containing 7 g L^{-1} glucose, 7 g L^{-1} fructose and 7 g L^{-1} maltose. For EPS production the three sugars were replaced by 80 g L^{-1} sucrose unless stated otherwise. Where appropriate, 15 g L^{-1} agar was added for solid media. Strains of the species *L. reuteri* and *L. animalis* were grown at 37 °C and *L. sanfrancis-censis* and *L. curvatus* at 30 °C. LAB used are listed in Table 1.

2.2 General molecular techniques

2.2.1 Sequence analysis and bioinformatics

Nucleotide and amino acid sequence alignments were performed with the multiple sequence alignment tool ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/).

In order to search for genes, proteins or homologous nucleotide and amino acid sequences the NCBI portal was used (http://www.ncbi.nlm.nih.gov/).

Cloning experiments were designed and documented using Vector NTI Advances ™ 11.0, 2008 Invitrogen Corporation. Furthermore this program was applied for endonuclease restriction site analysis within DNA and plasmid sequences.

Translation of DNA to amino acid sequences was performed by the online translate tool ExPASy (http://web.expasy.org/translate/). The open reading frame (ORF) and the signal peptide of the *gtf* were analyzed with the ORF finder from NCBI (http://www.ncbi.nlm.nih.gov/gorf/gorf.html) and with the SignalP 4.0 Server tool (http://www.cbs.dtu.dk/services/SignalP/), respectively. The graphical codon usage analyzer (http://gcua.schoedl.de/sequential_v2.html) was used to analyze the *gtf* genes for rare *E. coli* codons.

2.2.2 Detection and purification of DNA fragments

Agarose gel electrophoresis

DNA fragments were separated and visualized using agarose gel electrophoresis. Gels were prepared with 1% (w/w) agarose, 1x TAE buffer (stored as 50x TAE buffer: 0.1 M EDTA, 1 M acetic acid, 2 M Tris, pH 8.2) and boiled in a micro wave oven until the agarose was dissolved. After cooling on a stirrer, pouring and hardening, gels were placed in an electrophoresis chamber (Owl B2, Thermo Scientific, Germany) and covered with 1x TAE buffer. Samples to be analyzed were stowed with 6x loading dye (Fermentas GmbH, Germany) and applied to gel. DNA amounts used depended on concentration and kind of analysis. If DNA was purified from gels, more DNA was used. To determine DNA fragment

size the GeneRuler 1 kilobase (kb) plus DNA ladder (Fermentas GmbH, Germany) was applied. Separation was routinely performed at 90 – 120 V (EPS 300, Pharmacia Biotech, England) for 1 - 1.5 h. Electrophoresis of randomly amplified polymorphic DNA (RAPD) patterns was carried out using a 1.3% gel at 80 V for 1.5 - 2 h. Gels were stained with dimidium bromide and documented using UV light (Herolab UVT 28M, Herolab GmbH Laborgeräte, Germany) and a video camera in combination with Intas GDS equipment and software.

Determination of DNA concentrations

Quantity of DNA was determined by analysis with a Nanodrop 1000 device (Peqlab Biotechnologie GmbH, Germany) according to manufacturers' instructions.

Purification of DNA

Isolated DNA fragments from agarose gels were purified using the peqGOLD Gelextraction Kit (Peqlab Biotechnologie GmbH, Germany) following the instructions of the suppliers. To purify polymerase chain reaction (PCR) products the E.Z.N.A Cycle Pure Kit (Omega Bio-Tek Inc., USA) was applied.

2.2.3 Preparation and transformation of chemical competent *E. coli* cells

Preparation of competent cells

To prepare competent cells, 100 mL LB media were inoculated with 1% of an overnight culture of *E. coli* TOP10 or Rosetta. Culture was grown at 37 °C and 180 rounds per minute (rpm) to an optical density of 590 nm (OD_{590}) 0.5 – 0.6. Subsequently cells were harvested by centrifugation for 5 min at 4000 rpm and 4 °C and washed twice with the same volume of ice-cold 0.1 M $CaCl_2$. After centrifugation for a futher 5 min at 4000 rpm and 4 °C the pellet was resuspended in 2 mL ice-cold 0.1 M $CaCl_2$ containing 15% glycerol. Aliquots of 100 µL were initially stored on ice for 5 – 24 h and then directly used for transformation or were stored at -80 °C.

Transformation of *E. coli*

Competent cells were thawed on ice and mixed with the respective amounts of DNA. If a ligation mix was used, then the whole amount was transformed. In the case of pure plasmid DNA, 5 ng were used. Cells were incubated with DNA for 20 min on ice. After heat shocking (42 °C, 50 s), cells were stored again on ice for another 2 min. Afterwards 750 µL LB medium were added and cells were incubated for 60 – 90 min at 37 °C and 180 rpm. After this time had elapsed, 50 µL, 200 µL and 500 µL were plated on LB agar plates supplemented with the respective antibiotic.

2.2.4 Isolation of DNA from lactobacilli and *E. coli*

DNA isolations from lactobacilli

DNA of lactobacilli was isolated using the E.Z.N.A Bacterial DNA Kit (Omega Bio-Tek Inc., USA) according to manufacturers' instructions. The amount of DNA was determined by nanodrop analysis.

Plasmid isolations from *E. coli*

Following to the instructions of the supplier, plasmid DNA from *E. coli* was isolated using a peqGOLD Plasmid Miniprep Kit (Peqlab Biotechnologie GmbH, Germany). Amounts of plasmid were also quantified using nanodrop analysis.

2.2.5 PCR analysis

2.2.5.1 Primer design and general PCR procedure

Primer design

Oligonucleotides used for screening, cloning and sequencing were purchased from Eurofins MWG Operon, Germany. Degenerated primers were designed from the nucleotide sequence alignments of different lactobacilli and specific primers from single nucleotide sequences. Table 2 lists these primers and their uses.

Table 2: Primers used in this dissertation.

Primer	Primer sequence (5' → 3')	Use
M13V	GTTTTCCCAGTCACGAC	RAPD
616V	AGAGTTTGATYMTGGCTCAG	Species identification
609R	ACTACYVGGGTATCTAAKCC	Species identification
Dexf1	THWTRATGACDTGGTGGCC	Gtf screening *L. curvatus* TMW 1.624
Dexr1	GWACCATTRTTTGCWGACC	Gtf screening *L. curvatus* TMW 1.624
Dexf2	ATTCYAAYCCAGTAGTTCAAGC	Gtf screening *L. curvatus* TMW 1.624
Dexr2	TCATYACTDGAACGATATTG	Gtf screening *L. curvatus* TMW 1.624
Dexcf1	TTTGTCCGTGCACACGATAG	Completion of *gtf* from *L. curvatus* TMW 1.624
Dexcr1	GAGAATCACGCCAATTCCTT	Completion of *gtf* from *L. curvatus* TMW 1.624
Dexcf4	CAGCCAGTGTCAGCTGATGT	Completion of *gtf* from *L. curvatus* TMW 1.624
Dexcr4	ATGTTTAGCAGGTCGGCATC	Completion of *gtf* from *L. curvatus* TMW 1.624
Dexcf5	GGGCTTTAATACGCCAACAA	Completion of *gtf* from *L. curvatus* TMW 1.624

Primer	Primer sequence (5' → 3')	Use
Dexcr5	ATCAACGCCCGTATTTGGTA	Completion of *gtf* from *L. curvatus* TMW 1.624
Dexcf6	ACCACGCGTATGTGTTTATT	Completion of *gtf* from *L. curvatus* TMW 1.624
Dexcr6	CACCTGTCACTGAAGGAATCTT	Completion of *gtf* from *L. curvatus* TMW 1.624
Dexcf7	ATAACGGGGCTTTAGCATGA	Completion of *gtf* from *L. curvatus* TMW 1.624
Dexcr7	TCGTCTTTTCGTCAAACTGG	Completion of *gtf* from *L. curvatus* TMW 1.624
dNGTFCurv_XhoI_F	TATACTCGAGGCaAACTATTGCTGGTA AGACGTATTACTTTG	Cloning of *dNgtf* from *L. curvatus* TMW 1.624
GTFCurv_XhoI_F	TATACTCGAGGTTAAGGAATAATTATTT TGGAGAG	Cloning of *gtf* from *L. curvatus* TMW 1.624
GTFCurv _HindIII_R	TATAAAGCTTATCTTTTTtATGCTTTTTA CTTGTAAACAACAGA	Cloning of *dNgtf/gtf* from *L. curvatus* TMW 1.624
dNGTFReu_SacI_F	TATAGAGCTCACAAACTATTAACGGTC AACAATATTATATTG	Cloning of *dNgtf* from *L. reuteri* TMW 1.106
GTFReu _SacI_F	TATAGAGCTCTGATCAACAAGTTCAGT CTTCCACAAC	Cloning of *gtf* from *L. reuteri* TMW 1.106
GTFReu_Bsp119I_R	TATATTCGAAAGTTTTTTCTGATCAGCC AAATTACTAAT	Cloning of *dNgtf/gtf* from *L. reuteri* TMW 1.106
dNGTFAni_XhoI_F	TATACTCGAGTCAAAATATCAAGGGCA ATAACTATTTTG	Cloning of *dNgtf* from *L. animalis* TMW 1.971
GTFAni_ XhoI_F	TATACTCGAGTGATGAGACGAGTAGTA GCAATGAG	Cloning of *gtf* from *L. animalis* TMW 1.971
GTFAni_EcoRI_R	TATAGAATTCGGAATTAAAGTTGTTTTT CCAGTTGTAATG	Cloning of *dNgtf/gtf* from *L. animalis* TMW 1.971
pBAD_F	ATGCCATAGCATTTTTATCC	Control of pBAD/Myc-HisA *gtf* clones
pBAD_R	TCTGATTTAATCTGTATCAGG	Control of pBAD/Myc-HisA *gtf* clones

Recognition sites of restriction enzymes are underlined

General PCR procedure

PCR was performed to discover genes and then to amplify those for further processing like the introduction of restriction sites or as controls. DNA was amplified in an Eppendorf Gradient Cycler (Eppendorf, Germany).

For general amplifications the Taq Core Kit (MP Biomedicals, France) was used following the recommendations of the supplier. Samples contained 1x Taq buffer with $MgCl_2$, 0.2 mM dNTPs, 0.5 µM primers and 0.05 U/µL Taq unless stated otherwise. Amplification conditions with Taq polymerase were carried out under the following temperature profile: Initial denaturation of DNA at 94 °C for 4 min followed by 35 cycles of denaturation (94 °C, 45 s), annealing (melting temperature of primer -2 to 3 °C, 30 s) and elongation (72 °C, 1 min/kb). Final elongation was carried out at 72 °C for 10 min.

For cloning purposes, amplification was performed with the Phusion High-Fidelity DNA Polymerase (Finnzmyes, Finland). This polymerase features 3’ → 5’ exonuclease activity and is capable of amplifying long amplicons, which is advantageous for amplifying the big *gtf* genes. In line with the recommendations of the supplier, a reaction mix was prepared containing 1x Phusion HF buffer, 0.2 mM dNTPs, 0.5 mM primers and 0.02 U/µL Phusion

polymerase. Cycling conditions for amplification with Phusion polymerase were conducted using the following profile: Initial thermal denaturation of DNA (98 °C, 2 min) followed by 30 cycles of denaturation (98 °C, 30 s), annealing (melting temperature ascertained via temperature gradient, 30 s), and elongation (72 °C, 15-30 s/kb). Final extension time was 10 min.

2.2.5.2 Strain verification and strain typing

Strain verification

Prior to starting the molecular biology and sourdough experiments, strain verification of *L. curvatus* TMW 1.624, *L. reuteri* TMW 1.106, *L. animalis* TMW 1.971 and *L. sanfranciscensis* TMW 1.392 was carried out by partial sequencing of the 16S rRNA gene, amplified with primers 616V and 609R (Table 2). Sequencing of PCR products was performed by GATC Biotech GmbH (Konstanz, Germany).

Strain typing

Strain typing was carried out by RAPD-PCR analysis with the Taq polymerase using the primer M13V (Table 2). Reaction mix contained 1x Taq buffer without $MgCl_2$, 5 mM $MgCl_2$, 0.4 mM dNTPs, 1 mM primer M13V and 0.03 U/µL Taq. Either 0.5 µL extracted DNA (50-100 ng) were added to the mixture or PCR was performed as colony RAPD. Therefore colonies from agar plates were dissolved in 5 µL TE buffer (1 mM EDTA, 10 mM Tris, pH 8) and from this 2.5 µL were added to the reaction mix. Cycling conditions comprised 3 cycles consisting of 3 min at 94 °C, 5 min at 40 °C and 5 min at 72 °C, followed by 32 cycles of 1 min at 94 °C, 2 min at 60 °C and 3 min at 72 °C. 8 µL of samples were analyzed on agarose gels as described under section 2.2.2. Strain assertiveness in sourdoughs was evaluated by fragment profile conformity when compared to positive control of DNA or colony amplifications.

2.2.5.3 Exploration of the *gtf* gene from *L. curvatus* TMW 1.624

The objective was to discover the gene coding for the Gtf of *L. curvatus* TMW 1.624. Therefore, degenerated primers were created deduced from the homologous nucleotide sequence alignments of *L. hilgardii* TMW 1.828 (Waldherr et al., 2010), *L. parabuchneri* strain 33 and *L. sakei* Kg15 (Kralj et al., 2004a) (Table 2). Finally, sequence was obtained by primer walking on the basis of the *gtf* gene from *L. sakei* Kg15 (Kralj et al., 2004a). Sequencing of PCR products or plasmids was performed by GATC Biotech GmbH (Konstanz, Germany).

2.2.6 Vector design and inspection

Expression vector

Heterologous expression of different *gtf* genes in *E. coli* was performed with the pBAD/*Myc*-HisA expression vector from Invitrogen (Figure 5). This vector is useful for expression of toxic proteins, since the promoter is tightly regulated by AraC. Furthermore, the C-terminal polyhistidine region enables affinity purification of recombinant fusion proteins on metal-chelating resins.

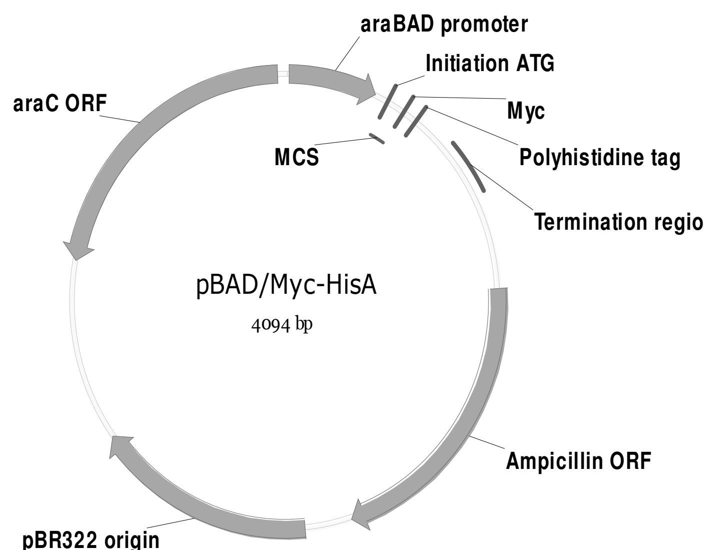


Figure 5: Map of pBAD/Myc-HisA expression vector
araBAD promoter region: 4-276 bp; Initiation ATG: 319-321 bp; Multiple cloning site (MCS): 317-370 bp; Myc epitope: 377-406 bp; Polyhistidine tag: 422-439; rmB transcription termination region: 545-702 bp; Ampicillin ORF: 981-1841 bp; pBR322 origin: 1986-2659 bp; AraC ORF: 4068-3190 bp.

Vector construction and inspection

Restriction enzyme digestions (FastDigest) and ligations with T4-DNA ligase were performed following the recommendations of the supplier (Fermentas GmbH, Germany). Amplifications of *gtf* were carried out with Phusion polymerase as explained in section 2.2.5.1.

The *gtf* genes of *L. curvatus* TMW 1.624 (this work), *L. reuteri* TMW 1.106 (Kaditzky, 2008) and *L. animalis* TMW 1.971 (Tieking, 2005) were completely amplified or without N-terminal region and were termed *gtf* and *dNgtf*, respectively. Primers containing restriction sites were used (Table 2) and PCR products were digested with the appropriate restriction enzyme and ligated into the corresponding site of the expression vector pBAD/*Myc*-HisA. Vector and gene were digested with *XhoI* and *HindIII* for *L. curvatus* TMW 1.624, with *SacI* and *Bsp119I* for *L. reuteri* TMW 1.106 and with *XhoI* and *EcoRI* for *L. animalis* TMW 1.971 *gtf*. The resulting constructs were transformed into the chemical competent *E. coli*

TOP10 or Rosetta cells (see section 2.2.3). Initially clones were analyzed for insertion of the desired genes by PCR with primers targeting the gene (pBAD_F and pBAD_R). Clones showing correct fragment sizes were further investigated using restriction analysis. Thus, plasmids of positive clones were extracted and digested with at least 2 different restriction enzymes. Later a phenotypic fast screening method of clones on agar plates was developed (see section 2.3.1).

2.3 Protein chemical methods

2.3.1 Expression of recombinant proteins in *E. coli*

Expression screening of clones on agar plates

Because of their sheer size sequencing of *gtf* genes is very expensive and controlling clones using the isolation, restriction and gel analysis of plasmids is very time consuming, so a screening method for clones on agar plates was developed. Clones obtained by transformation with the respective constructs, were plated on LB agar supplemented with the respective antibiotics as negative control. For expression, clones were transferred onto plates containing 30 g L^{-1} sucrose and 1 g L^{-1} arabinose as well as the respective antibiotics. Expression plates were incubated at 30 °C over night and were then stored from some hours to several days at 4 °C. Positive clones were conspicuous due to their slimy surface and were reminiscent of the appearance of EPS positive LAB on agar plates supplemented with sucrose.

Expression scale-up and generation of cell free extracts

1 L LB medium, supplemented with 0.3 M sorbitol, 100 µg mL^{-1} and 64 µg mL^{-1} chloramphenicol was inoculated with the cell material of half of the agar plate containing the respective *gtf* Rosetta clone of *L. curvatus*, *L. reuteri* or *L. animalis*. The cells were incubated at 37 °C and 180 rpm to an OD_{590} of 0.6 - 0.7. Then, culture was induced with 100 µM arabinose and cultivated at 16 °C over night (16-18 h). Cells were harvested by centrifugation at 8000 rpm and 4 °C for 30 min. The supernatant was discarded and cells were washed with precooled 50 mM sodium phosphate buffer pH 8, resuspended in 8-10 mL binding buffer A (50 mM NaH_2PO_4, 250 mM NaCl, 10 mM imidazole, pH 8) and broken by sonification (Sonoplus/SH70G Bandelin electronic, Berlin). Sonification was performed on ice (cycle 0.5/ 90 %/ 20 s) with three repeats with one minute breaks in between. Cell debris was separated from crude extract by centrifugation at 14000 rpm and 4 °C for 30 min. The supernatant was pooled, stored on ice and subsequently purified (see section 2.3.2).

2.3.2 Purification of recombinant proteins

Recombinant proteins were purified using free pressure liquid chromatography (FPLC) (Biologic HR Controller, Workstation and Fraction Collector Modell 2128, Bio-Rad Laboratories, Hercules, USA) coupled with a 1 mL HisTrap FF affinity column (GE Healthcare, Germany). All steps during purification were monitored by UV detection and all buffers and solutions were degassed prior to starting. The column was equilibrated with at least 10 column volumes of binding buffer A (50 mM NaH_2PO_4, 250 mM NaCl, 10 mM imidazole, pH 8) with a flow rate of 1 mL min^{-1}. Cell extract was applied manually and washed with buffer A until the absorbance reached a steady state (at least 10 – 15 column volumes). Target protein was released by applying a linear gradient from 100 % buffer A and 0 % elution buffer B to 0 % buffer A to 100 % elution buffer B (50 mM NaH_2PO_4, 250 mM NaCl, 200 mM imidazole, pH 8) and collected in 1 mL fractions. Afterwards column was regenerated by washing with 10 column volumes of buffer B. Finally FPLC system and HisTrap FF column were washed with 20 % ethanol and column was stored at 4 °C.

2.3.3 SDS-PAGE

Protein size and purity were investigated by one dimensional sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970). Generally 12% gels (good separation between 15 and 100 kilo dalton (kd)) were prepared to estimate the purity of proteins and 6% gels (separation between 70-250 kd) were made to estimate protein size of Gtfs (Table 3 and Table 4). Solutions were mixed and cross-linking was started by adding ammonium persulfate (APS) and tetramethylethylendiamine (TEMED). After gelling, protein samples were mixed with Laemmli sample buffer (2x buffer: 1 mL 1 M Tris/HCl, pH 6.8, 0.8 g SDS, 3 g glycerine, 0.37 g dithiothreitol (DTT), 1 mg bromophenol blue, adjusted to 10 mL), and incubated for 10 min at 75 °C. Prior to sample application the gel was put in the pre-assembled Mini Protean III Cell (Bio-Rad Laboratories, USA). For protein size determination 5 µL of PagerRuler Plus Prestained protein ladder (Fermentas GmbH, Germany) was used. After loading samples (5-20 µL) into the gel pockets, proteins were led into the stacking gel at 75 V for 15 minutes. Further separation was performed at 120 V. Electrophoresis was between 5 and 60 min (depending on the desired dissolution) after the bromophenol blue front had left the gel.

Table 3: SDS-PAGE buffer according to Laemmli.

buffer	composition
separating gel	1.5 M Tris/HCl, pH 8.8
stacking gel	0.5 M Tris/HCl, pH 6.8
electrophoresis buffer (5x)	125 mM Tris/HCl, 960 mM glycine, 0.5% SDS, pH 8.3

Table 4: Separating and stacking gel solutions for two SDS gels according to Laemmli.

solution	separating gel (6%)	separating gel (12%)	stacking gel (4%)
separating gel buffer	2.5 mL	2.5 mL	-
stacking gel buffer	-	-	1.25 mL
deionized water	5.35 mL	3.35 mL	3.05 mL
acrylamide (30%)	2 mL	4 mL	665 µL
SDS (25%)	40 µL	40 µL	20 µL
APS (20%)	50 µL	50 µL	25 µL
TEMED	5 µL	5 µL	5 µL

Gels were stained with Colloidal Coomassie Blue using diluted Roti®Blue solution (Carl Roth, Germany) as described in the suppliers manual. Staining of gels was carried out over night without shaking. Destaining of gels took from minutes to hours and was stopped by washing the gel with deionized water. Gels were digitalized by scanning (Epson Expression 1600, Epson, Germany).

2.3.4 Determination of protein concentration

The Bio-Rad Protein Assay, based on the Bradford method was used for determining protein concentrations. The “Standard Procedure for Microtiter Plates” was used following the manufacturers’ instructions.

Dye reagent was prepared by diluting 1 part Dye Reagent Concentrate with 4 parts deionized water and filtrating through a filter to remove particulates. A dilution series of a BSA standard was prepared from 0.05 mg/mL to 0.5 mg/mL (linear range of assay). 10 µl of each standard and sample solution were transferred into separate microtiter plate wells and 200 µl of diluted dye reagent were added. After incubation at room temperature for at least 5 minutes, absorbance was measured at 590 nm using a microtiter plate reader (SpectraFluor, Tecan Deutschland GmbH, Germany) and Magellan V7.0 software (Tecan Deutschland GmbH, Germany).

2.3.5 Characterization of different Gtfs

Enzyme assay

The various Gtf activities were determined enzymatically by measuring the glucose and fructose release from sucrose conversion in duplicate. If not stated otherwise all pipetting steps were performed on ice. In order to start the reaction, 18 µL of assay buffer were added to 2 µL of purified enzyme (Table 5). Then, the mixture was incubated at an appropriate temperature for about 20-60 min and the reaction was stopped by adding 20 µL of 0.5 M NaOH. After storage for 10 min at room temperature, the mixture was neutralized by adding 20 µL of HCl buffer. Released glucose and fructose were quantified at OD_{340} using a plate reader (Tecan SpectraFluor, Tecan Deutschland GmbH, Germany). Chemicals used for this detection were adenosine triphosphate (ATP), which was purchased from Sigma-Aldrich (Germany) and nicotineamide adenine dinucleotide phosphate (NADP), hexokinase (HK)/glucose-6-phosphate dehydrogenase (G6PDH) and phosphoglucose isomerase (PGI) which were all purchased from Roche (Germany). First, 49 µL of determination mix were added without enzymes and measured (blank). Afterwards, 1 µL of a HK/G6PDH solution was added and reaction was monitored until the glucose was completely converted (glucose determination). Subsequently, 1 µL of PGI was added and monitored until the fructose had converted (fructose determination). Each assay was calibrated with glucose (0, 1, 10, 50, and 100 mM) as standard.

Table 5: Buffer and solutions used for enzyme assays

component	concentration
5x assay buffer	
sucrose	1500 mM
buffer (pH 3.2 – 8)	500 mM
$CaCl_2$	5 mM
HCl buffer	
HCl	0.5 M
Tricine/KOH pH 8	0.1 M
Determination mix	
Tricine/KOH pH 8	100 mM
ATP	3 mM
NADP	3 mM
$MgCl_2$	5 mM
HK	2 U mL^{-1}
G6PDH	1 U mL^{-1}
PGI	5 U mL^{-1}

Resulting enzyme activities were calculated as follows: total activity [U mg^{-1} protein] = fructose released [µmol] * [mg protein $min]^{-1}$; hydrolysis activity [U mg^{-1} protein] = glucose released [µmol] * [mg protein $min]^{-1}$; transferase activity [U mg^{-1} protein] = total activity – hydrolysis activity.

Determination of optimum conditions for activity

In order to examine the pH optimum of different Gtf activities, different buffers were used. Sodium acetate buffer (100 mM) was used for measurements in the range of pH 3.2 – 5.2 and sodium phosphate buffer (100 mM) in the range of pH 5.8 – 8.0. Assays with *L. curvatus* Gtf were performed at 30 °C and assays with *L. animalis* and *L. reuteri* Gtf at 40 °C as these are the growth temperatures of the strains.

To determine the optimal temperature, the respective optimal pH of each enzyme was used and reactions were performed in the range of the expected optimal temperature (*L. reuteri* Gtf: 22 – 56 °C; *L. curvatus* Gtf: 15 – 40 °C; *L. animalis* Gtf: 22 – 66 °C).

Determination of influence of ions, EPSs and dough extracts on activity

All following enzyme assays were performed without $CaCl_2$ (Table 5) under the respective optimal conditions.

The influence of different compounds on activity was evaluated by analyzing the following substances at a final concentration of 1 mM: Ethylene diamine tetraacetic acid (EDTA), $CaCl_2$, $MgCl_2$, $ZnCl_2$, KCl, $AlCl_3$, $CuCl_2$, NaCl and $MnCl_2$.

The effect of different EPSs on activity was examined with final concentrations of 0.1 and 0.5 % (w/v). The EPSs were isolated from fermentations with *L. sanfranciscensis* TMW 1.392, *L. reuteri* TMW 1.106, *L. animalis* TMW 1.971 and *L. curvatus* TMW 1.624 as described in section 2.4.

To examine the influence of different dough extracts, doughs with flours of buckwheat core (Schälmühle, Germany), buckwheat wholemeal (Schälmühle, Germany), quinoa (Ziegler & Co. GmbH, Germany), and rice wholemeal (Ziegler & Co. GmbH, Germany) were made. Doughs were prepared with a dough yield (DY) of 250 and centrifuged for 30 min at 9000 rpm. The sticky supernatant was diluted (1:2) with deionized water and incubated for 30 min at 80 °C in order to inactivate flour enzymes. Afterwards the extract was filtered (0.45 µM). Due to flour sugar contents and in order to stay in the valid assay range only 2 µL of these extracts were used for measurements. This final concentration corresponded to one twentieth of the original concentration from the undiluted supernatant.

Since only a small amount of the original dough extract could be used in previous experiments, the aim was to generate an artificial extract of the most important flour minerals. Therefore, rice and buckwheat flour were analyzed for their mineral content according to DIN EN ISO 11885 E22 by the Forschungszentrum for Brau- und Lebensmittelqualität (Freising, Germany). As quinoa and buckwheat core flour were incorporated at a later stage in this project, analyses are missing. Thus no information about mineral content of buckwheat core flour is available. Mineral content of quinoa flour was taken from literature (Hager et al., 2012), who purchased the flour from the same company.

The mineral contents of the three different flours were converted to a content present in doughs with a DY of 250 and were then used for enzyme assays as described above. To investigate the influence of different DY on the activity of the Gtf from *L. curvatus*, mineral solution imitating buckwheat was used for preparing DY of 200, 250, 300 and 350.

Determination of Michaelis-Menten kinetic parameters and EPS production

Kinetik assays were performed using sucrose concentrations ranging from 1 mM to 300 mM and reaction was carried out with 1 mM $CaCl_2$ at optimal pH and temperature conditions for each enzyme. Data obtained were fit to a Michaelis-Menten (MM) kinetic (SigmaPlot 8.0) and K_M and V_{max} were calculated for the overall activity.

Polymer production was performed twice in buffers with optimal pH containing 1 mM $CaCl_2$. The purified enzyme preparations were incubated for 8 days at room temperature with 120 mM sucrose. Afterwards, glucans were isolated by precipitation with two volumes of ethanol, dried, dissolved in deionized water and analyzed for their structure as described in section 2.5.

2.4 Production and purification of EPS from lactobacilli

EPS was produced through bacterial fermentations in Homohiochii medium with *L. curvatus* TMW 1.624, *L. reuteri* TMW 1.106, *L. animalis* TMW 1.971 and *L. sanfranciscensis* TMW 1.392 containing 80 g L^{-1} sucrose, in sourdough fermentations (section 2.7) or through in vitro enzyme reactions (section 2.3.5) and purified according to Korakli et al. (2001). Briefly, EPS was precipitated with two volumes of precooled denatured ethanol, centrifuged (Sigma 6-16 K, Sigma Labortechnik, Germany; Hermle Z 383 K, Hermle Labortechnik, Germany) and dried over night at 40 °C. Afterwards EPS was dissolved and dialyzed (cutoff 12000-14000, Serva Electrophoresis GmbH, Germany) against deionized water for at least 48 h. Finally EPS was freeze dried using a WFK L 05-06 freeze dryer (WFK, Germany). Up to analysis, EPS was stored at room temperature.

2.5 Structural characterization of EPS

Pure EPS of *L. curvatus* TMW 1.624, *L. reuteri* TMW 1.106, *L. sanfranciscensis* TMW 1.392 and *L. animalis* TMW 1.971 were obtained as described in section 2.4 and analyzed via asymmetrical flow field flow fractionation (AF4), multiangle laser light scattering (MALLS), high performance liquid chromatography (HPLC), ^{1}H-NMR, ^{13}C-NMR and methylation analysis.

The influence of different sucrose concentrations (25, 50 and 80 g L^{-1}) and sucrose fed-batch (50 g L^{-1}) on EPS yield and structure were analyzed with *L. curvatus* TMW 1.624. For this, different Homohiochii media were inoculated with the same over night culture and incubated at 30 °C for 48 h. To investigate the effect of sucrose fed-batch, sucrose was added in portions over the first 8 h of fermentation. Metabolites were quantified as described in section 2.6.1 from undiluted supernatant or in the case of EPS after precipitation and purification (see also section 2.4).

Asymmetrical flow field flow fractionation and multiangle laser light scattering

AF4 and MALLS were done in cooperation with H. Rübsam (Lehrstuhl für Brau- und Getränketechnologie, TU München, Germany). Samples of EPSs were separated by size using AF4 (Wyatt Technology, Germany). Weight average molar mass (Mw), number average molar mass (Mn), size distribution, root mean square (rms) radius and weight average root mean square (Rw) were analyzed by MALLS (Dawn, Heleos II, Wyatt Technology, Germany) and a refractive index (RI) as quantitative detector (Agilent Series 1200 G1362A, Agilent Technologies, Germany). The inserted spacer had a height of 350 µm, a width of approximately 21.5 mm at its widest position where the sample is focused before it is eluted, and a long channel of 240 mm. EPS samples of four different fermentations conducted under same conditions were analyzed. About 1 g L^{-1} EPS was dissolved in deionized water and 100 µL were injected. Measurement was carried out at 25 °C. Carrier solvent was composed of 50 mM $NaNO_3$ and 250 ppm NaN_3 in millipore water. Separation was conducted with a 5 kDa membrane (Nadir regenerated cellulose Wyatt Technology, Germany). Inject flow was 0.2 mL min^{-1}. Elution flow was 1 mL min^{-1} and cross flow was decreased from 3 mL min^{-1} to 0.1 mL min^{-1} within 30 min. Then cross flow was held constant for 20 minutes at 0.1 mL min^{-1} and finally reduced to zero within 18 min. The intensity of scattered light was measured simultaneously at 18 different scattering angles ranging from 10° to 160°. A dn/dc of 0.146 mL g^{-1} was used to determine concentration of polysaccharides in aqueous solvents by RI detector. Baselines of RI detector were subtracted from baselines of blank runs, since RI baselines are influenced by different salt and pressure conditions during a cross flow gradient in AF4.

Pullulane standards (1 g L^{-1}) (PSS GmbH, Mainz, Germany) were used for calibration of detectors. Obtained data were processed using ASTRA V 5.3.4.19 software (Wyatt Technology, Germany).

Methylation analysis, ^{1}H and ^{13}C NMR

Methylation analysis was conducted in cooperation with the Institute of Food Chemistry (TU Braunschweig, Germany) in order to determine the branching types of dextrans of *L. reuteri* TMW 1.106 and *L. curvatus* TMW 1.624.

50 mg of the two dextrans were first partially methylated in water with NaOH/methyl iodide (MeI), then twice in dimethyl sulfoxide with pulverized NaOH and MeI according to Ciucanu and Kerek (1984). Products were isolated by dialysis (cut-off 3500) after each methylation step. Yields calculated on the basis of a fully methylated dextran were between 74% and 100% for the individual steps. Attenuated total reflectance infrared spectroscopy of the final products showed near complete disappearance of the OH absorption.

Hydrolysis, reduction and acetylation (acetic anhydride/pyridine) of the permethylated dextran was performed as described by Voiges et al. (2012). Permethylated dextrans were then submitted to methanolysis and trimethylsilylation as described by Vollmer et al. (2009).

Gas-liquid chromatographic (GLC) analysis was carried out with a GLC-FID instrument Shimadzu GC 2010 (ZB5-MS, Phenomenex, Germany, 30 m × 0.32 mm ID, d_f = 0.25 µm; carrier gas: H_2: 40 cm/s, linear velocity mode). Split injection port: 250 °C, split ratio: 0. Temperature programs: PMAA: 60 °C (1 min), with 20 °C/min to 200 °C, 4 °C/min to 250 °C, 20 °C/min to 310 °C (10 min); methyl *O*-Me-*O*-TMS-glucosides: 60 °C (1 min), with 20 °C/min to 130 °C, 4 °C/min to 290 °C (10 min), with 20 °C/min to 310 °C (10 min). For quantitative evaluation, peak areas were corrected according to the effective-carbon-response concept (Addison and Ackman, 1968; Scanlon and Willis, 1985).

A Hewlett Packard 5890A gas chromatograph equipped with a 30 m column (ZB5-MS, Phenomenex, Germany, 30 m × 0.32 mm ID, d_f = 0.25 µm) was used for GCMS analysis. Conditions: Injector 250 °C, temperature program: 100 °C (3 min); 6 °C/min to 310 °C (3 min). The capillary column was directly coupled to a triple quadrupole mass spectrometer Finnigan TSQ 700. Transfer line was set at 250 °C, ion source temperature was set at 150 °C and ionization voltage at 70 eV.

For ^{1}H and ^{13}C NMR analysis about 10 mg of EPSs were dispersed in 0.7 mL of heavy water (D_2O) or deuterated dimethyl sulfoxide (DMSO-d_6) over night. NMR spectra were

recorded with a Bruker DRX-400 and a Bruker A VIII-400 instrument at 400 MHz (^1H, all samples) and 100 MHz (^{13}C, EPS from *L. animalis* Gtf), respectively.

HPLC analysis

In order to analyze monomer composition, a 10 g L^{-1} EPS solution was prepared, hydrolyzed, filtered and analyzed as described in section 2.6.1.

2.6 Quantification and monitoring of metabolite formation

Metabolites were monitored and quantified in order to optimize fermentation conditions or to screen for EPS negative control strains for sourdough fermentations.

2.6.1 Quantification of metabolites

Acid and sugar analysis

For analysis of acids and ethanol, 1 mL supernatant was treated with 50 µL perchloric acid (70%) over night at 4 °C and filtered through 2 µM membrane filters (Phenomenex, Germany). To analyze sugars, 1 mL supernatant was filtered directly. Quantification of acids was carried out by HPLC using a sulfonated styrene-divinylbenzene Rezex ROA column (Phenomenex, Germany) with a flow rate of 0.6 mL min^{-1} (0.005 N H_2SO_4) at 85 °C and an injection volume of 10 µL. Sugars were quantified using a Rezex RPM column (Phenomenex, Germany) with a flow rate of 0.6 mL min^{-1} (deionized water) at 85 °C and an injection volume of 20 µL. Both columns were coupled to a refractive index (RI) detector (Gynkotek, Germany).

EPS analysis

In situ produced EPS was purified according to section 2.4, dissolved in a certain amount of deionized water and hydrolyzed by adding perchloric acid (70%) to 1 mL of sample and incubated at 100 °C for 5 h. Glucans were hydrolyzed with a final concentration of 5% and fructans with 0.5% perchloric acid. Purified and freeze dried EPS from Homohiochii fermentations were used as standard. After filtration, samples were analyzed and quantified by HPLC using a Rezex ROA column as described above.

2.6.2 Monitoring of EPS production

EPS production through *L. curvatus* TMW 1.624, *L. reuteri* TMW 1.106, *L. animalis* TMW 1.971 and *L. sanfranciscensis* TMW 1.392 was monitored over 30 h in Homohiochii medium containing 50 g L^{-1} sucrose. OD_{590} (Novaspellq, Pharmacia Biotech, Germany) and pH (InLab 412 Electrode, Mettler-Toledo, Germany) were monitored and metabolites

were determined from undiluted supernatant or in the case of EPS after precipitation and purification (see sections 2.4 and 2.6.1).

2.6.3 Screening of lactobacilli for their absence of EPS formation

For the purpose of finding suitable EPS negative controls for sourdough fermentations, several strains of the species *L. curvatus*, *L. reuteri* and *L. sanfranciscensis* were screened for EPS production (Table 1). Strains were plated on Homohiochii medium containing maltose, glucose and fructose and on Homohiochii medium containing solely sucrose. EPS formation was judged by colony appearance. Positive strains appeared more slimy and ropy on sucrose agar plates compared to control plates without sucrose. No difference on agar plates was seen for negative colonies. EPS negative strains were then grown for 24 h in Homohiochii medium containing 7 g L^{-1} glucose, fructose, maltose or solely 50 g L^{-1} sucrose or all four sugars. Organic acids were quantified from undiluted supernatant according to section 2.6.1.

2.7 Sourdough fermentations with different lactobacilli

In order to analyze whether effectual amounts of EPS can be produced *in situ*, *L. sanfranciscensis* TMW 1.392, *L. reuteri* TMW 1.106, *L. animalis* TMW 1.971 and *L. curvatus* TMW 1.624 were investigated for their ability to grow and to produce EPS in gluten-free sourdoughs. In addition, fermentations with the EPS negative strains *L. curvatus* TMW 1.1447 and TMW 1.1450 were also conducted.

2.7.1 Dough preparation

Doughs were prepared with gluten-free flour, tap water and sucrose. For inoculation of doughs, cells from an overnight culture were washed and resuspended in peptone water (0.85% NaCl and 0.1% peptone) and added to obtain about 5.0E+07 cells per g dough. Doughs were incubated at optimal growth temperature for each strain. In order to find impact factors on EPS formation different flours were analyzed. Rice, quinoa, buckwheat and buckwheat core flour with a DY of 250 and 7.5% sucrose (flour base) were investigated for each strain. To evaluate the influence of the sucrose concentration on metabolite and EPS formation 3.75%, 7.5%, 11.25% and 15% sucrose (flour base) were tested with previously determined optimal flour for each strain. Furthermore, the influence of inoculated cell count, DY and sucrose fed-batch were analyzed for *L. curvatus* TMW 1.624. So buckwheat doughs (DY 250, 7.5% sucrose) were inoculated with 3.0E+07, 5.0E+07, 7.0E+07 and 1.0E+08 cells per g dough or buckwheat doughs (7.5% sucrose) with DY 200, 250, 300 and 350 were prepared. To investigate the influence of a sucrose

fed-batch on EPS production 7.5% sucrose was added in portions (0 h: 1 g, 4 h: 2 g, 8 h: 3 g) to the dough (DY 250). Control doughs, acidified with lactic acid (90%) or with lactic acid and acetic acid (100%; ratio 4:1) to pH 4 and doughs with EPS negative strains of *L. curvatus* (*L. curvatus* TMW 1.1447 and TMW 1.1450) were prepared to separate the amount of water soluble polysaccharides of flours from *in situ* produced EPS. Samples were taken at the beginning of fermentation and at 6, 24, 30 and 48 h after inoculation. All fermentations were carried out twice.

2.7.2 Determination of cell counts, pH and total titratable acidity

During fermentation, pH and total titratable acidity (TTA) were analyzed as these parameters are an important control for contamination. The pH and the TTA were determined using a glass electrode (InLab 412, pH 0-14, Mettler-Toledo, Germany). TTA is defined as the consumption of 0.1 M NaOH in mL per 10 g sample (DY 200) up to a final pH value of 8.5 ± 0.1. To analyze TTA, 12.5 g dough (DY 250) was titrated with 0.1 M NaOH to pH 8.5 ± 0.1 until the pH was stable for at least one minute. Cell counts, measured as colony forming units (cfu) per g dough, were determined in duplicate from appropriate dilutions of dough in peptone water. Using a spiral plater (Eddy Jet plating machine, IUL Instruments, Germany), dilutions were plated on Homohiochii agar plates and plates were incubated for 1-2 days in a controlled atmosphere (80% N_2 and 20% CO_2) at optimal temperatures for each strain.

2.7.3 Proof of sourdough flora

Beside pH and TTA observations a colony RAPD-PCR was performed to exclude contamination and to verify that the inoculated strains were dominant during fermentation. Colonies of plates from 0 h and 48 h were randomly analyzed by PCR with M13V universal primer (see section 2.2.5.2). DNA and colonies from sequenced stock cultures were used as positive control.

2.7.4 Metabolite analysis of doughs

Metabolites were extracted from dough by dissolving dough samples in deionized water (1:2 w/v) followed by centrifugation at 8000 g, 10 °C for 30 min.

Acid and sugar analysis

For analysis of acids and ethanol, 1 mL extract was treated with 50 µL perchloric acid (70%) over night at 4 °C and filtered through 2 µM membrane filters (Phenomenex, Germany). For sugar analysis, extracts were treated in a different way. 500 µL extract of

samples taken at 0 h and 6 h were treated with 250 µL of $ZnSO_4$ (10% w/v) and 250 µL of 0.5 M NaOH, centrifuged and filtered. The extracts of the other samples (24 h, 30 h and 48 h) were filtered directly. Quantification was carried out as described in section 2.6.1. The relative hydrolase activity was calculated as a ratio of released glucose to consumed sucrose and the fermentation quotient (FQ) of *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392 as a ratio of lactic to acetic acid.

EPS analysis

24 h *in situ* produced EPS and water soluble polysaccharides originating from the flour were purified in triplicate according to section 2.4. Then, EPS was dissolved in deionized water, hydrolyzed with perchloric acid (70%) at 100°C for 5 h and quantified as described in section 2.6.1.

2.8 Investigations with EPS in gluten-free dough and bread

Different EPSs were investigated for their effect on gluten-free bread quality and their properties to act as hydrocolloids. Experiments were conducted in cooperation with Ernst Böcker GmbH & Co. KG, Germany and the Lehrstuhl für Brau- und Getränketechnologie, TU München, Germany. EPS was therefore isolated and purified as mentioned in section 2.4 from *L. sanfranciscensis* TMW 1.971, *L. reuteri* TMW 1.106, *L. animalis* TMW 1.971 and *L. curvatus* TMW 1.624. Afterwards, dried EPS was carefully crushed for no longer than 5 s in a coffee mill (Severin Café KM 3872, Germany).

2.8.1 Determination of EPS water retention in doughs

The water holding capacity of the EPS and HPMC (Mw: 300 kDa – 500 kDa, Methocel K4M food grade, Dow Wolff Cellulosics GmbH, Germany) was determined according to AACC method 56-11 in duplicate. A concentration series of EPS from 0% w/w to 3% w/w in 80 parts rice and 20 parts buckwheat flour was made or 2% w/w of *L. curvatus* TMW 1.624 EPS produced from fermentations with different sucrose concentrations was prepared (section 2.5). The flours were mixed with EPS prior to the addition of water (DY 210) and the mixture was allowed to swell for 20 min in falcon tubes. Doughs were directly transferred to centrifuge for 15 min at 1000 g (Allegra, Beckmann Coulter, Germany). Immediately after, the supernatant was decanted and the tubes were drained at a 45 ° angle for 10 min on a paper towel. The water retention was calculated by reweighing.

2.8.2 Bread formulas and bread preparation

Bread doughs with a DY of 200 were made of 80 parts rice flour, 20 parts buckwheat flour, 3% w/w (flour base) fresh yeast (Uniferm GmbH & Co. KG, Germany), 2% w/w salt and 1% w/w HPMC or EPS, or, without hydrocolloid. All ingredients were blended with a Diosna Dierks & Söhne GmbH (Germany) mixer. Four mixers were used simultaneously (4 min at 30 rpm and 1 min at 55 rpm). Each dough was split up into twelve lots of 400 g and proofed at room temperature (22 °C) for 45 min. Breads were steamed for 5 s and baked in a multi-deck oven (Werner & Pfleiderer Carat Stein Standard, WP Bakery Technologies, Germany) for a total of 30 min (10 min at 230 °C and 20 min at 210 °C). First measurements were carried out at the earliest after 3 h of cooling and breads were wrapped 24 h after baking in PE bags. The breads were stored at room temperature for up to 7 days.

For the purpose of visualizing the effect of EPS on gluten-free breads, bread formulas and bread preparation were modified. Bread doughs were made of rice (54.8%), buckwheat (39.1%), salt (1.6%), dry yeast (1.5%, Fermipan red, Italy) and 1% xanthan or EPS (unground or dissolved). Doughs were acidified with lactate (90%) and acetate (100%) in a ratio of 4:1 to a final pH of 4.8 to ascertain the influence of acids produced by sourdough flora. Doughs were kneaded for 8 min at 25 rpm, rested 20 min at room temperature, divided into 300 g portions and proofed at 30 °C and 80% humidity (KOMA SunRiser, Koeltechnische Industrie B.V., Netherlands) for 45 min. Breads were baked in a multi-deck oven (Werner & Pfleiderer Lebensmitteltechnik, Germany) for a total of 45 min at 220 °C. Resulting breads were judged only optically.

2.8.3 Bread quality evaluation

The specific volume was determined using the rapeseed displacement method. A total of 6 bread loaves from each recipe were analyzed. Crumb firmness was determined according to AACC method 74-09 with a TA-XT2 Texture Analyzer (Stable Micro Systems, UK). A total of 10 slices (1.4 cm thick), 5 from two different loaves (of one bread type) were analyzed using a spherical plunger (SMS P/0.5 S). Each slice was compressed to 40% at 1.7 mm/s speed. Baking loss was calculated through the weight difference of 4 doughs of each recipe and their resulting breads 5 h after cooling. In addition, the weight loss during storage of these 4 breads was also measured. Over several days, moisture was determined according to AACC method 44-15A in duplicate. To do this, about 5 g of the center bread crumb was dried at 130 °C for at least 16 h. In order to measure the porosity (pore area × 100/slice area) 4 slices of each bread were scanned, adapted and analyzed by ImageJ according to Pérez-Nieto et al. (2010) and Sciarini et al. (2010).

Images were saved as bitmap files with an 800 dpi resolution. 1498 × 1050 pixels were selected at the center of the bread slice and converted to grayscale. The threshold was adapted to Huang (Huang and Wang, 1995), outliers were removed and the crumb measurements were achieved.

2.9 Statistical analyses

Statistical analyses were performed using one-way Anova. Where appropriate, Fisher's least significant differences test was used to describe means at 5% significance.

3 Results

The two obligately heterofermentative strains *L. sanfranciscensis* TMW 1.392 and *L. reuteri* TMW 1.106 and the two facultatively heterofermentative strains *L. curvatus* TMW 1.624 and *L. animalis* TMW 1.971 were chosen for this work as the strains and their EPS have already been partially characterized. *L. reuteri* and *L. sanfranciscensis* are key organisms in traditional wheat and rye sourdoughs (Gänzle and Brandt, 2005) and already investigated quite well for *in situ* EPS production (Kaditzky, 2008; Kaditzky et al., 2008; Korakli et al., 2001; Tieking, 2005; Tieking et al., 2003). *L. reuteri* TMW 1.106 was found to produce a branched dextran with 15 to 17% α-(1,4) linkages (Kaditzky, 2008) and *L. sanfranciscensis* TMW 1.392 to form a levan (Tieking, 2005). Beside a glucosyltransferase *L. reuteri* TMW 1.106 also harbors an inulosucrase (Schwab and Gänzle, 2006). The *gtf* gene of this strain was explored and heterologously expressed in studies of Kaditzky (2008). *L. curvatus* TMW 1.624 and *L. animalis* TMW 1.971 are not common in sourdoughs. EPS of *L. curvatus* TMW 1.624 was identified as linear dextran (Grepka, 2003), but branching could not be excluded by the method applied. The *gtf* gene has not been identified and characterized so far (Jänsch, 2004). *L. animalis* TMW 1.971 was found to produce a glucan (Tieking, 2005). The *gtf* gene was identified by Jänsch (2004) and Tieking (2005) but has not yet been heterologously expressed.

3.1 Monitoring of EPS formation

In order to obtain initial information on EPS and metabolite formation, the four chosen strains were investigated in Homohiochii medium containing 50 g L^{-1} sucrose as sole carbon source.

L. sanfranciscensis metabolized the available sucrose already after 22 h. In contrast, sucrose was not depleted in fermentations with *L. animalis, L. curvatus* and *L. reuteri* (Figure 6). Still 79.67 mM, 62.05 mM and 1.83 mM sucrose were found after 30 h fermentation for the strains, respectively. Released glucose was no longer detectable for *L. curvatus* after 7 h and for *L. animalis* after 14 h. The glucose concentration stayed low during the entire fermentation process with *L. reuteri*. The final concentration of glucose amounted to 1.91 mM. In fermentations with *L. sanfranciscensis*, glucose concentration augmented to 38.10 mM after 10 h but decreased shortly after down to 2.42 mM after 30 h. Fructose accumulated in the medium and was not metabolized by *L. curvatus*. After 30 h of fermentation 45.39 mM fructose was still measurable. In fermentations with *L. animalis*, *L. sanfranciscensis* and *L. reuteri*, fructose accumulated temporarily and then metabolized to residual 2.59 mM, 12.44 mM and 41.78 mM after 30 h. Additionally

L. reuteri and *L. sanfranciscensis* converted parts of the fructose to mannitol. Final concentrations were 99.51 mM and 71.28 mM. Mannitol formation and the concomitant fructose consumption and acid production were not completed for fermentations with *L. reuteri* even after 30 h.

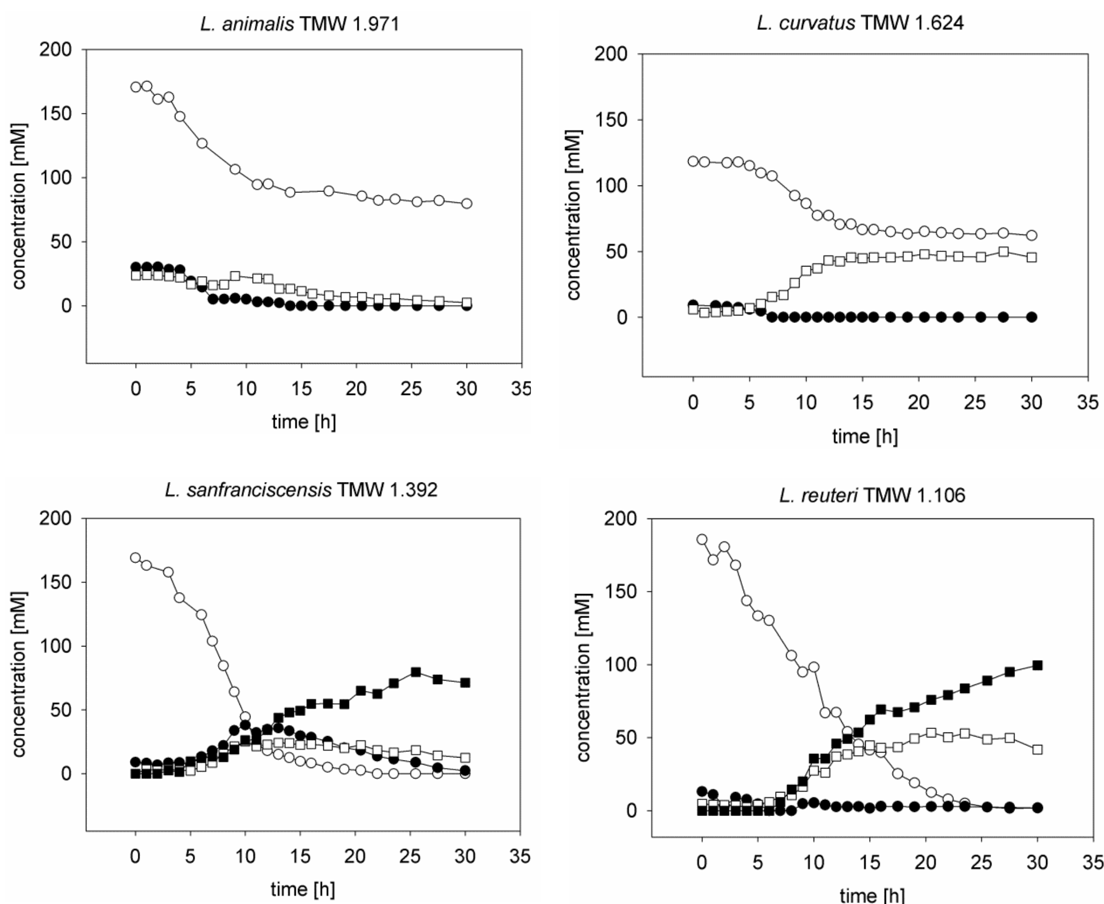


Figure 6: Metabolite formation of *L. animalis*, *L. curvatus*, *L. sanfranciscensis* and *L. reuteri* in Homohiochii medium containing 50 g L^{-1} sucrose. Shown are sucrose (○), glucose (●), fructose (□) and mannitol (■) consumption/formation.

L. animalis and *L. curvatus* only produced lactate, while for the obligately heterofermentative strains *L. sanfranciscensis* and *L. reuteri* acetate and ethanol were also detectable (Table 6). *L. animalis* turned out to be a strong acidifier. The pH decreased quickly to 3.75 and after 30 h fermentation 167.14 mM lactate had been formed. Growth and EPS production had almost stopped after 12 h with a final OD of 5.89 ± 0.24 and a final EPS amount of 3.11 g L^{-1} (Figure 7). *L. curvatus* only produced one third of the lactate of *L. animalis* and the pH value decreased more slowly. Fermentations with *L. curvatus* reached a final pH of 4.50. Growth was finished after 14 h (OD_{590}: 3.99 ± 0.27) and maximal EPS amounts of 10.13 g L^{-1} were found. Compared to *L. reuteri*, *L. sanfranciscensis* grew faster and produced more metabolites. The stationary phase for *L. sanfranciscensis* was already reached after 13 h of fermentation. In contrast, culture of

L. reuteri showed growth till 25.5 h fermentation. Final pH values of cultures were 4.27 for *L. reuteri* and 3.89 for *L. sanfranciscensis*. FQs were 1.41 ± 0.07 and 2.02 ± 0.10, respectively and maximal EPS quantities of 9.24 g L^{-1} and 8.79 g L^{-1} were measurable.

Growth and EPS production correlated strongly for all four strains (*L. animalis*: r = 0.99, *L. curvatus*: r = 0.99, *L. sanfranciscensis*: r = 0.98, *L. reuteri*: r = 0.98). EPS production was initiated at the beginning of the exponential phase. Most EPS was produced in this growth phase. As soon as the strains reached the stationary phase, EPS production decreased and stopped shortly afterwards.

Table 6: Lactate, acetate and ethanol formation of *L. animalis*, *L. curvatus*, *L. sanfranciscensis* and *L. reuteri* after 30 h fermentation in Homohiochii medium containing 50 g L^{-1} sucrose.

strain	lactate [mM]	acetate [mM]	ethanol [mM]
L. animalis TMW 1.971	167.14	-	-
L. curvatus TMW 1.624	54.93	-	-
L. sanfranciscensis TMW 1.392	135.76	64.59	74.22
L. reuteri TMW 1.106	55.95	41.54	6.70

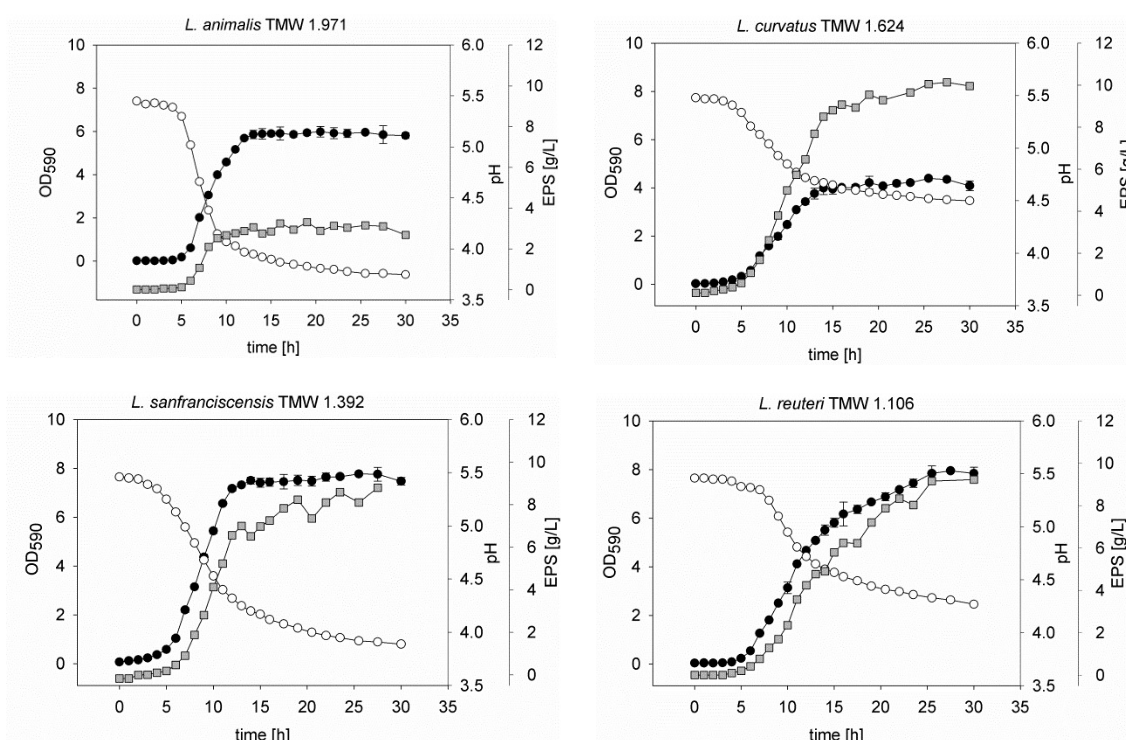


Figure 7: Growth, pH and EPS formation of *L. animalis*, *L. curvatus*, *L. sanfranciscensis* and *L. reuteri* in Homohiochii medium containing 50 g L^{-1} sucrose. Growth (●), pH (○) and EPS formation (■).

3.2 Characterization of EPS

3.2.1 Structure analysis of different EPSs

Analysis of EPS isolated from four different fermentations, conducted under same conditions displayed different Mw (Figure 8).

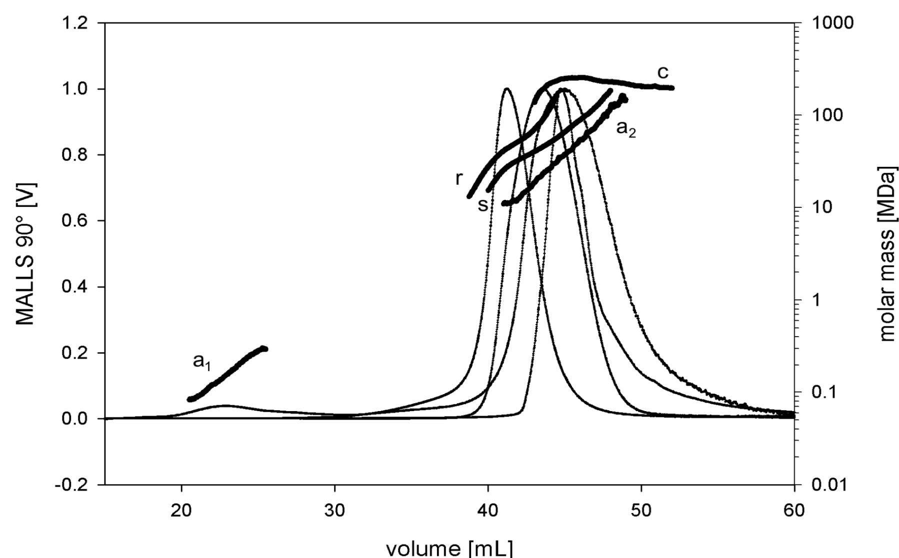


Figure 8: AF4 analysis of EPS from lactobacilli.
Shown are the multiangle laser light scattering signal at 90° angle (thin lines) and molecular weight distribution of EPS (thick lines). *L. curvatus* TMW 1.624 (c), *L. reuteri* TMW 1.106 (r), *L. sanfranciscensis* TMW 1.392 (s), *L. animalis* TMW 1.971 (a_1 and a_2).

Dextran *of L. curvatus* TMW 1.624 showed highest Mw (118 – 242 MDa). Mw of dextran produced by *L. reuteri* TMW 1.106 ranged from 44.3 to 58.7 MDa, and levan from *L. sanfranciscensis* TMW 1.392 from 44.8 – 94.9 MDa. *L. animalis* TMW 1.971 was found to produce two different EPSs with an Mw ranging from 88.4 to 241 kDa (80.1 – 82.4%, a_1), and an Mw ranging from 19.7 to 56 MDa (17.6 – 19.9%, a_2). Analysis of this EPS after hydrolysis with HPLC evidenced glucose (85.96%) and fructose (14.04%) as monomer components. As glycosyltransferases are described as only producing homopoly-saccharides and a similar fraction size was found in the monomer composition when compared to the Mw analysis, it is suggested that the EPS with low Mw is a glucan (a_1) and the other one a fructan (a_2). Figure 9 evidences that dextran of *L. curvatus* TMW 1.624 has a more compact conformation compared to the other EPS since rms radius remains constant, whereas the rms radius of the other EPS increases with higher molecular weight. Rms of glucan of *L. animalis* was not calculable due to its too small size. Rw values were 41.2 – 54.4 nm for *L. reuteri*, 148.6 – 192.2 nm for *L. curvatus* and 56.0 – 76.6 nm for *L. animalis* a_2. The polydispersity index (Mw/Mn) was highest for dextran of *L. reuteri* TMW 1.106 (Mw/Mn = 1.202), for the levan of *L. sanfranciscensis*

TMW 1.392 (Mw/Mn = 1.252) and for the fructan of *L. animalis* TMW 1.971 (Mw/Mn = 2.006), indicating a broader molecular weight and therefore a higher variability of EPS. The glucan of *L. animalis* TMW 1.971 and the dextran of *L. curvatus* TMW 1.624 showed a narrow molecular weight (Mw/Mn = 1.101 and Mw/Mn = 1.036, respectively).

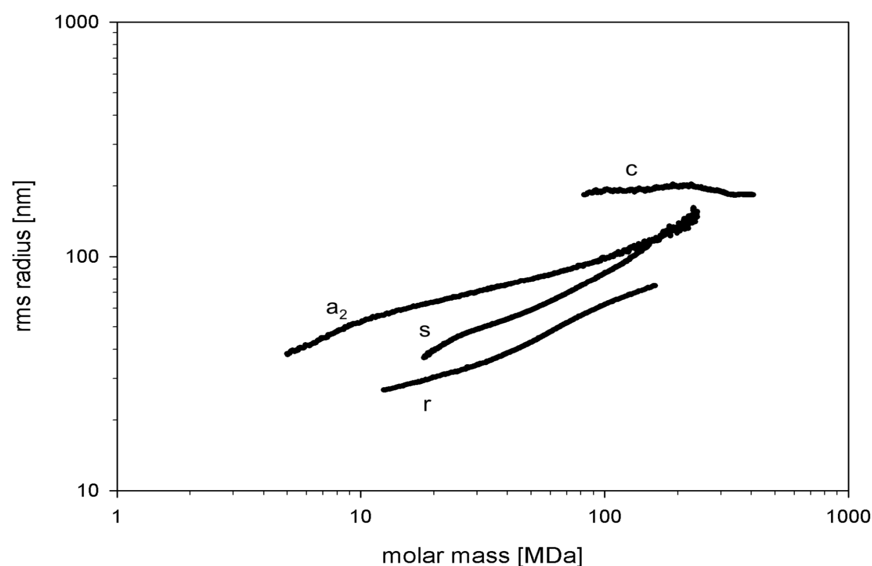


Figure 9: Conformation plot of EPSs from lactobacilli, analyzed by MALLS and refractive index detector. *L. curvatus* TMW 1.624 (c), *L. reuteri* TMW 1.106 (r), *L. sanfranciscensis* TMW 1.392 (s), *L. animalis* TMW 1.971 (a_2), root mean square (rms). Small Mw fraction (a_1) of *L. animalis* is not pictured.

Using ^{1}H-NMR spectroscopy, α-configuration of glucosidic linkages was confirmed. EPS of *L. animalis* consisting of two different EPSs also evidenced α-(1,6) as a main linkage, indicating that glucan (a_1) is a dextran. As the NMR spectrum is overlapped by the fructan (a_2) no exact clarification of structure (e.g. branching) is possible.

Methylation analysis of dextrans of *L. reuteri* TMW 1.106 and *L. curvatus* TMW 1.624 revealed differences in branching. Degree of branching of dextran of *L. curvatus* was determined as 7.8 % (methanolysis) and 9.1 % (hydrolysis) at O-3, respectively. For dextran of *L. reuteri*, values were 17.5 % (methanolysis) and 19.6 % (hydrolysis) branching at O-4.

3.2.2 Influence of sucrose concentration on EPS yield and structure

The influence of different sucrose amounts on EPS yield and structure was investigated in Homohiochii fermentations with *L. curvatus*.

It was found that longer EPS molecules were produced with higher amounts of sucrose (20 g L^{-1}: Mw: 320 MDa, Rw: 197.4 nm, 50 g L^{-1}: Mw: 377 MDa, Rw: 209.6 nm, 80 g L^{-1}: Mw: 552 MDa, Rw: 237.4 nm, Figure 10 A). Also adding sucrose in portions (fed-batch)

influenced the structure. Compared to EPS produced through directly added sucrose, the size of this EPS was increased more (50 g L^{-1} fed-batch: Mw: 511 MDa, Rw: 229.6). Different sucrose amounts showed no influence on the polydispersity index, which ranged between Mw/Mn = 1.000 and 1.007, indicating that all EPS have a close variability. Apparently different sucrose amounts as well as sucrose fed-batch influenced the conformation of the EPS molecule as shown in Figure 10 B. With increasing sucrose concentration the conformation became more linear. ^{1}H-NMR analysis of these EPSs evidenced 5 – 8 % branching, but no rising or falling trend was visible.

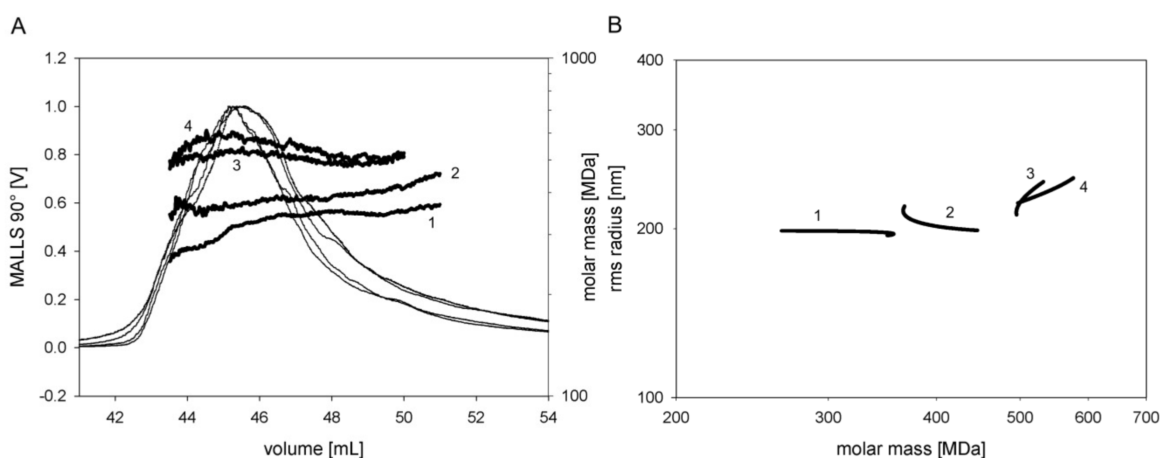


Figure 10: AF4 analysis (A) and conformation plot (B) of EPS of *L. curvatus* produced with different sucrose concentrations. 20 g L^{-1} (1), 50 g L^{-1} (2), 50 g L^{-1} fed-batch (3), 80 g L^{-1} (4) sucrose, root mean square (rms).

Metabolite formation was slightly affected through sucrose (Table 7). Residual sucrose was found in all fermentations except for fermentations with 20 g L^{-1} sucrose, where the sucrose had completely been metabolized. The more sucrose was added, the higher the residual sucrose concentration. Through fed-batch more sucrose was metabolized. Glucose was consumed in all fermentations and no great deviations were detected for final lactate and fructose concentrations.

Table 7: Metabolite formation by *L. curvatus* after 48 h of fermentation with different sucrose amounts.

sucrose [g L^{-1}]	lactate [mM]	sucrose [mM]	glucose [mM]	fructose [mM]
20	82.13	0.00*	0.00	22.44
50	76.48	48.86	0.00	46.98
50 fed-batch	80.20	29.94	0.00	45.50
80	78.42	128.81	0.00	42.71

*zero within the scope of the applied method.

In contrast to other metabolites, EPS yields were strongly influenced (Figure 11). An optimum was found at 50 g L^{-1} sucrose as with higher amounts the EPS yield was reduced. Less sucrose was converted when high concentrations were used. Adding the sucrose via fed-batch did not significantly affect the final EPS amount.

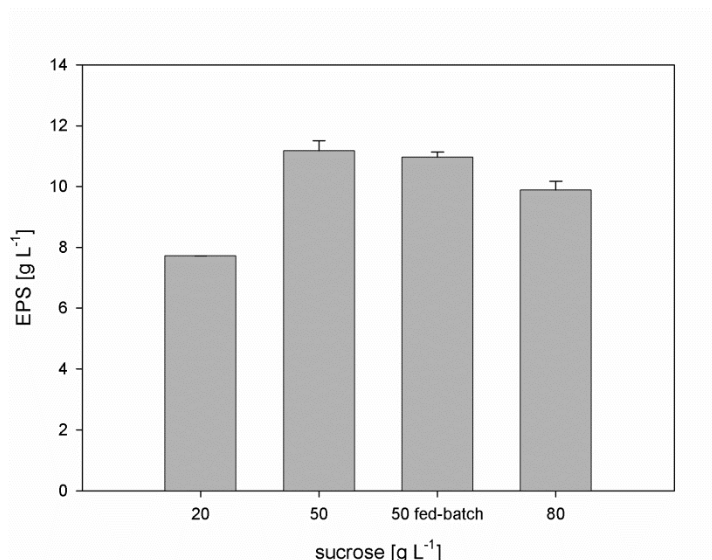


Figure 11: Influence of different sucrose amounts on EPS yields.
Data are given as mean ± SD, n=2 and were subjected to a one-way Anova (P<0.001).

3.3 Effect of EPS on the quality and structure of gluten-free dough and bread

The influence of different EPSs on the quality of gluten-free dough and bread was investigated in order to establish a structure/function relation in gluten-free baked goods. Therefore, EPSs were purified from fermentations conducted under same conditions and used for structural, dough and baking experiments (see also section 3.2).

3.3.1 Water-holding capacity of different types of EPS in doughs

Purified EPSs, which were characterized in section 3.2.1, were used to explore the effect of structurally different EPSs on water retention in doughs. HPMC was used as hydrocolloid control. The flour mixture alone (0% hydrocolloid) assimilated 93.2% of the added water (Figure 12), and of all analyzed EPSs, only the dextran of *L. curvatus* TMW 1.624 was able to retain water. For full water retention, 2% of HPMC and 2.5% of dextran were necessary. For the EPSs of *L. sanfranciscensis* TMW 1.392, *L. animalis* TMW 1.971 and *L. reuteri* TMW 1.106 no effect was visible up to 3%.

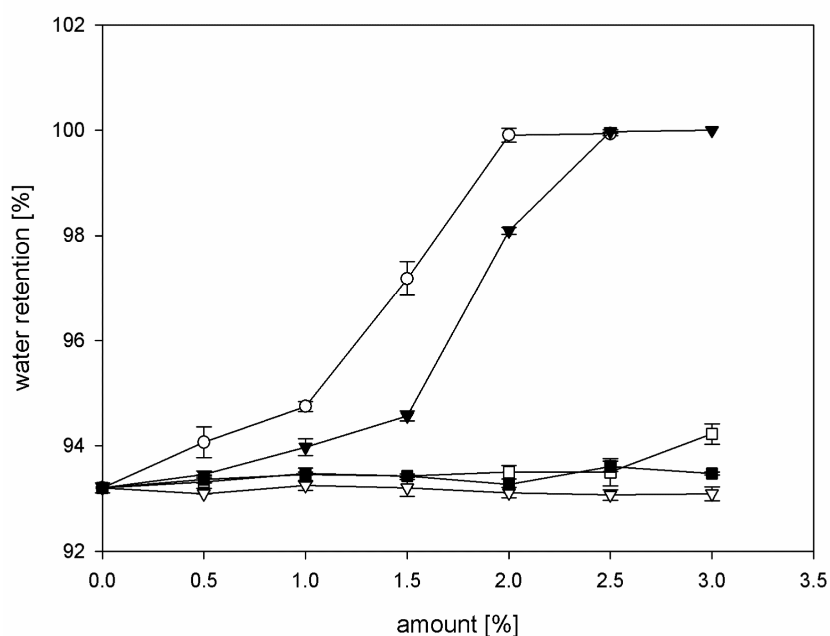


Figure 12: Water retention capacity of HPMC and EPSs from lactobacilli in doughs.
HPMC (○), *L. curvatus* TMW 1.624 (▼), *L. animalis* TMW 1.971 (▽), *L. reuteri* TMW 1.106 (■), *L. sanfranciscensis* TMW 1.392 (□). Data are given as mean ± SD, n=2.

Since the dextran of *L. curvatus* TMW 1.624 was the only EPS that retained water, further water retention experiments were performed with this EPS. The influence of different sucrose amounts on EPS structure and water retention was investigated. Section 3.2.2 already evidenced differences in structure of dextrans produced with different sucrose concentrations. For water retention experiments 2% w/w (flour base) of dextran was used and analyzed in the same way as described previously.

Dextran purified from different fermentations showed different behavior in water retention (Figure 13). The flour mixture absorbed 93.18% water. The more sucrose was used for EPS production, the more the resulting dextrans retained water. Dextran purified from fermentations with 20 g L^{-1} sucrose enhanced the water retention to 93.65% and dextran from fermentations with 80 g L^{-1} had the highest water holding capacity (95.68%). Though sucrose fed-batch resulted in a different dextran structure, no relevant distinction can be seen in the water retention of dextrans resulting from fermentations with 50 g L^{-1} sucrose added directly or via fed-batch.

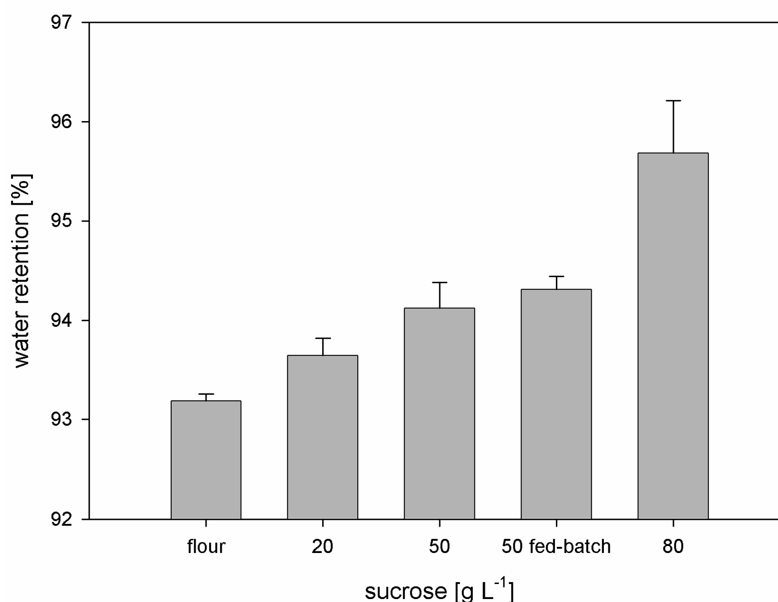


Figure 13: Water retention of 2% w/w flour base dextran from *L. curvatus* TMW 1.624 purified from fermentations with different sucrose concentrations. Sucrose concentrations used for EPS fermentations were 20 g L^{-1}, 50 g L^{-1} (added directly or fed-batch) and 80 g L^{-1}. Data are given as mean ± SD, n=2 and were subjected to a one-way Anova (P<0.001).

3.3.2 Effect of EPS on properties of fresh bread

In view of the fact that it is complex and laborious to produce purified bacterial EPS in amounts to make baking experiments possible, this experiment could only be performed twice. Table 8 comprises the representative results of the first experiment. Results of the second experiment were congruent.

Table 8: Effect of EPS on fresh bread quality parameters.

Formulation	Specific volume [mL g^{-1}]	Baking loss [%]
Without hydrocolloid	1.34 ± 0.03a*	11.95 ± 0.29a
HPMC	1.47 ± 0.04b,d	10.92 ± 0.72b,c
L. animalis TMW 1.971	1.50 ± 0.06c	11.07 ± 0.97c
L. curvatus TMW 1.624	1.48 ± 0.02d	10.54 ± 0.48d
L. reuteri TMW 1.106	1.53 ± 0.02e	11.41 ± 0.55e
L. sanfranciscensis TMW 1.392	1.59 ± 0.04f	11.79 ± 0.65a

*Different letters in the same column indicate statistical significance (P<0.05).

All specific volumes of breads containing additives were significantly different to breads without hydrocolloid (P<0.05). The volume of loaves ranged from 1.34 to 1.59 mL g^{-1}, and

the volume of all breads with EPS and HPMC was higher than that of breads without hydrocolloid. Interestingly, in both experiments, breads with HPMC and dextran of *L. curvatus* TMW 1.624 had similar specific volumes (1.47 and 1.48 mL/g in the first round and 1.53 and 1.53 mL/g in the second round, respectively). The other three EPSs caused higher volumes than HPMC and dextran of *L. curvatus* TMW 1.624 in both rounds.

Baking loss for all breads except for those containing levan of *L. sanfranciscensis* were significantly different from breads without hydrocolloid ($P<0.05$), indicating that all glucans are able to retain water during the baking process. Dextran of *L. curvatus* TMW 1.624 tended to retain most water. The porosity of breads was not significantly different.

3.3.3 Effect of EPSs on properties of stored bread

The course of weight loss in all breads was similar and no significant difference was visible.
Except for breads containing EPS of *L. reuteri*, breads evidenced higher moisture content than the control bread after 48 h. Breads containing dextran of *L. curvatus* TMW 1.624 lost least water after 48 h and 96 h of storage ($P<0.05$) (Table 9). After 96 h of storage, only the water content of breads supplemented with EPS of *L. curvatus* was higher than the control bread without hydrocolloid.

Table 9: Influence of EPS on moisture content of stored bread.

Formulation	Moisture content after 48 h of storage [%]	Moisture content after 96 h of storage [%]
Without hydrocolloid	55.45 ± 0.11a*	54.76 ± 0.24a
HPMC	55.58 ± 0.02b,c,f	54.78 ± 0.27a
L. animalis TMW 1.971	55.53 ± 0.04c,f	54.42 ± 0.35a
L. curvatus TMW 1.624	55.96 ± 0.09d	55.21 ± 0.29b
L. reuteri TMW 1.106	55.30 ± 0.02e	53.90 ± 0.27c
L. sanfranciscensis TMW 1.392	55.61 ± 0.05f	54.65 ± 0.04a

*Different letters in the same column indicate statistical significance ($P<0.05$).

Breads containing EPSs of *L. animalis*, *L. curvatus,* and *L. sanfranciscensis* showed decreased crumb hardening during the whole storage period, while HPMC and EPS of *L. reuteri* evidenced their positive attributes in long-term storage experiments (91 h) ($P<0.05$). Breads supplemented with EPS of *L. curvatus* had the lowest crumb firmness after 4 days of storage (Table 10).

Table 10: Effect of HPMC and EPS on crumb hardness [N] of stored bread.

Formulation	3 h	20 h	91 h
Without hydrocolloid	1.40 ± 0.17a*	1.62 ± 0.19a	2.52 ± 0.27a
HPMC	1.42 ± 0.24a	1.83 ± 0.25b,e	2.30 ± 0.32b,c
L. animalis TMW 1.971	1.22 ± 0.19b,c,e	1.37 ± 0.20c	2.33 ± 0.25c
L. curvatus TMW 1.624	1.24 ± 0.15c,e	1.53 ± 0.15d	2.08 ± 0.14d
L. reuteri TMW 1.106	1.60 ± 0.31d	1.83 ± 0.19e	2.22 ± 0.17e,f
L. sanfranciscensis TMW 1.392	1.25 ± 0.17e	1.48 ± 0.25f	2.21 ± 0.24f

*Different letters in the same column indicate statistical significance ($P<0.05$).

Overall, the quality of stored bread was improved most by dextran of *L. curvatus* TMW 1.624.

3.3.4 Influence of EPS on crumb structure

In order to visualize the effect of EPS on crumb structure, breads were made under different, not optimal conditions compared to previous experiments. Xanthan was used as hydrocolloid control. No further baking experiments were conducted with EPS of *L. animalis* TMW 1.971, since this strain produces low amounts of EPS (~ 3 g L^{-1}) which is, moreover, a mixture of two EPS. First dextran of *L. curvatus* TMW 1.624 was used for experiments as this EPS had the most promising effects on gluten-free breads so far (compare previous sections). Resulting breads are pictured in Figure 14. Obviously breads without hydrocolloid displayed crumbs containing large pores with a tendency to form holes. Pore distribution in breads containing xanthan or dextran of *L. curvatus* TMW 1.624 was consistent. Breads without hydrocolloid and with EPS were split on the surface.

Another baking experiment was done with EPSs of the three strains *L. curvatus* TMW 1.624, *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392 in order to visualize the effect of structurally dissimilar EPSs. As the aim was to produce EPS *in situ*, EPS would already have been dissolved in sourdoughs. Therefore EPS were dissolved and swollen over night prior to their addition to the dough. This experiment was conducted on another day with other charges of flours and yeast and therefore cannot be compared to the previous experiment in this section. All breads were split on the surface. Breads containing levan of *L. sanfranciscensis* TMW 1.392 showed large pores and holes, whereas breads with dextran of *L. reuteri* TMW 1.106 also had holes, but the crumbs showed a better pore distribution (Figure 15). The structure and pores of breads made with dextran of *L. curvatus* TMW 1.624 were most consistent and only one of the three

slices contained holes, while two slices (out of three) taken from loaves containing EPS of the other two strains evidenced holes.

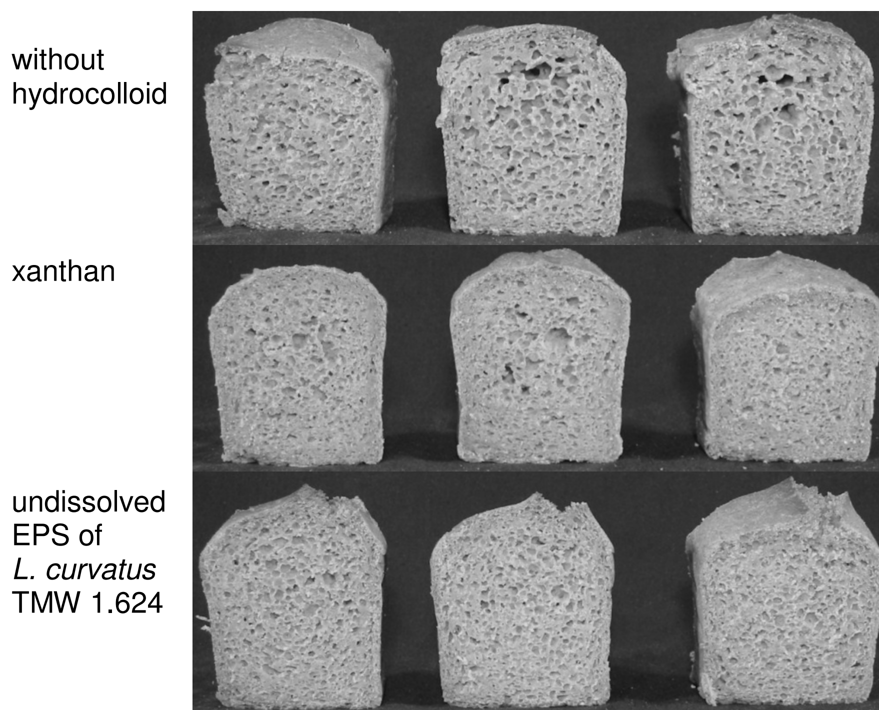


Figure 14: Effect of undissolved EPS on the structure of gluten-free bread.
Shown are breads without hydrocolloid and breads containing 1% w/w flour base xanthan or EPS of *L. curvatus*. Breads are representative for one of two independent experiments.

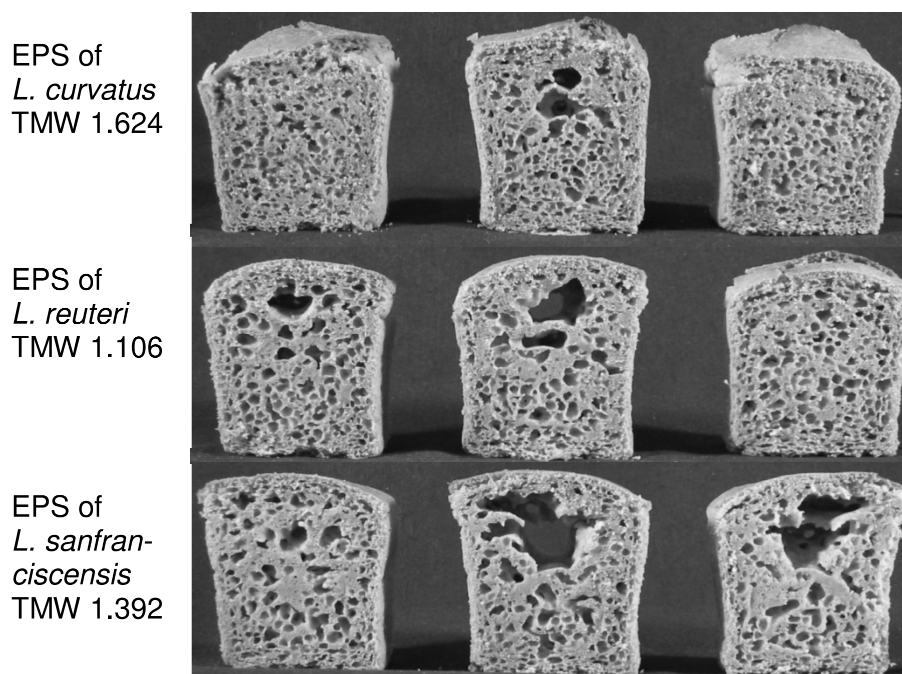


Figure 15: Effect of dissolved EPS on the structure of gluten-free bread.
Shown are breads with 1% w/w flour base EPS of *L. curvatus*, *L. reuteri* and *L. sanfranciscensis*. Breads are representative for one of two independent experiments.

3.4 Screening of lactobacilli for their lack of EPS formation

Different strains of *L. sanfranciscensis*, *L. reuteri* and *L. curvatus* were screened for their ability to produce EPS in order to find suitable non EPS formers as control strains for subsequent sourdough fermentations. This is necessary to separate the amount of water soluble polysaccharides of flours from *in situ* produced EPS. Overall, 35 strains of *L. curvatus*, 11 strains of *L. reuteri* and 9 strains of *L. sanfranciscensis* were screened. EPS formation was assessed visually from the slimy appearance of colonies grown on Homohiochii agar plates containing sucrose compared to those colonies grown on medium without sucrose.

Table 11: Screening of *L. curvatus*, *L. reuteri* and *L. sanfranciscensis* strains for their ability to produce EPS.

Species	Strain TMW no.	Ability to produce EPS	Species	Strain TMW no.	Ability to produce EPS
L. curvatus	1.7	-	*L. curvatus*	1.1437	-
L. curvatus	1.17	-	*L. curvatus*	1.1438	-
L. curvatus	1.48	-	*L. curvatus*	1.1439	-
L. curvatus	1.49	+	*L. curvatus*	1.1403	-
L. curvatus	1.50	+	*L. curvatus*	1.1447	-
L. curvatus	1.51	+	*L. curvatus*	1.1449	-
L. curvatus	1.167	-	*L. curvatus*	1.1450	-
L. curvatus	1.266	-	***L. reuteri***	**1.106**	+
L. curvatus	1.407	+	*L. reuteri*	1.137	-
L. curvatus	1.421	-	*L. reuteri*	1.292	+
L. curvatus	1.439	-	*L. reuteri*	1.294	+
L. curvatus	1.440	+	*L. reuteri*	1.295	+
L. curvatus	1.591	-	*L. reuteri*	1.656	+
L. curvatus	1.593	-	*L. reuteri*	1.693	-
L. curvatus	1.594	+	*L. reuteri*	1.723	+
L. curvatus	1.595	+	*L. reuteri*	1.966	-
L. curvatus	1.596	+	*L. reuteri*	1.1187	-
L. curvatus	**1.624**	+	*L. reuteri*	1.1281	-
L. curvatus	1.700	+	*L. sanfranciscensis*	1.52	-
L. curvatus	1.1291	-	***L. sanfranciscensis***	**1.392**	+
L. curvatus	1.1365	-	*L. sanfranciscensis*	1.450	+
L. curvatus	1.1381	-	*L. sanfranciscensis*	1.451	+
L. curvatus	1.1382	+	*L. sanfranciscensis*	1.709	-
L. curvatus	1.1384	-	*L. sanfranciscensis*	1.710	-
L. curvatus	1.1389	-	*L. sanfranciscensis*	1.894	-
L. curvatus	1.1390	-	*L. sanfranciscensis*	1.1193	-
L. curvatus	1.1391	-	*L. sanfranciscensis*	1.1221	-
L. curvatus	1.1408	-			

bold face: EPS positive strains intensively used in this dissertation

EPS negative strains were then further screened for lactate and acetate formation in Homohiochii medium i) with maltose, glucose and fructose, ii) with sucrose as the sole carbon source and iii) with maltose, glucose, fructose and sucrose. Wherever possible, FQ was calculated and compared to EPS positive strains *L. curvatus* TMW 1.624, *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392.

All EPS negative strains of *L. sanfranciscensis* did not grow on Homohiochii medium, if sucrose was used as the sole carbon source. None of the chosen strains produced comparable amounts of acids to *L. sanfranciscensis* TMW 1.392 in the three media. In case of *L. reuteri* strains, only *L. reuteri* TMW 1.1281 was identified to be sufficiently comparable to *L. reuteri* TMW 1.106. FQs were i) 3.03 vs. 2.94, ii) 1.49 vs. 1.47 and iii) 1.65 vs. 1.56 for *L. reuteri* TMW 1.1281 and 1.106, respectively. *L. curvatus* TMW 1.1447 and 1.1450 came closest to *L. curvatus* 1.624 lactate quantities. Determined lactate amounts were i) 51.55 mM, 61.60 mM vs. 61.66 mM, ii) 18.77 mM, 28.43 mM vs. 27.18 mM and iii) 35.35 mM, 73.82 mM vs. 29.07 mM for *L. curvatus* TMW 1.1447, 1.1450 and 1.624, respectively.

3.5 Sourdough fermentation with EPS negative strains of *L. curvatus*

L. curvatus strains 1.1447 and 1.1450 were chosen for sourdough fermentation experiments in order to ascertain and compare their growth and metabolite formation to *L. curvatus* TMW 1.624. Hence, buckwheat sourdoughs were prepared with and without sucrose. Only the results of sourdoughs containing sucrose are shown, because sourdoughs without sucrose gave similar results except for sugar patterns, and sucrose addition is necessary for EPS production.

The three strains were able to grow in buckwheat sourdoughs. For the EPS negative strains no water-soluble polysaccharides and therefore no EPS were found (Table 12). However, EPS negative strains demonstrated different growth behavior to *L. curvatus* TMW 1.624. The two strains acidified the dough very fast. After 6 h fermentation three to four times more lactate had been produced and pH had decreased faster than pH of *L. curvatus* TMW 1.624. After 24 h fermentation, amounts were closer to the EPS positive strain. Lactate, TTA and pH values were quite similar (Table 12). *L. curvatus* TMW 1.624 metabolized sucrose faster than the other strains and less glucose but more fructose was formed.

Table 12: Metabolite formation and growth behavior of *L. curvatus* TMW 1.1447, 1.1450 and 1.624 in buckwheat flour after 6 h and 24 h fermentation. Data are representative for one of two independent experiments showing the same trends.

L. curvatus **strain**	**lactate***	**sucrose**	**glucose**	**fructose**	**cfu per g dough**	**TTA [mL]**	**pH**	**EPS [g L^{-1}]**
6 h								
TMW 1.1447	171.66	164.98	84.49	-	5.62E+08	14.6	4.5	-
TMW 1.1450	118.60	168.06	89.28	-	1.94E+08	11.95	5.04	-
TMW 1.624	41.58	126.91	61.36	39.74	2.90E+08	7.45	6.09	4.84
24 h								
TMW 1.1447	279.47	48.91	126.17	109.33	6.02E+08	21.2	4.02	-
TMW 1.1450	251.22	50.29	133.18	107.71	4.91E+08	20.75	4.09	-
TMW 1.624	228.35	4.51	121.80	142.53	8.98E+08	18.85	4.15	8.62

*metabolite concentrations are given in mmol kg^{-1} flour.

Since metabolite quantities of EPS negative *L. curvatus* strains differ strongly from fermentations with *L. curvatus* TMW 1.624 and as no suitable strains were available for the other three EPS positive strains *L. reuteri* TMW 1.106, *L. sanfranciscensis* TMW 1.392 and *L. animalis* TMW 1.971, chemically acidified doughs were chosen as the preferred control doughs for subsequent sourdough fermentations.

3.6 Optimization of EPS formation in gluten-free sourdoughs

In section 3.3 it was proved that EPSs positively influenced the quality of gluten-free bread. Because of consumer interest and costs arising from EPS purification, the aim was to produce EPSs *in situ*. Fermentations were conducted to explore the ability of the four chosen lactobacilli strains to grow and to produce EPSs in gluten-free sourdoughs. An optimization was carried out to ascertain whether *in situ* EPS amounts can reach effectual levels to reduce or replace commercially available hydrocolloids.

3.6.1 Impact of different flours on EPS formation

To investigate the influence of different flours on EPS formation, gluten-free sourdoughs with 7.5% sucrose and a DY of 250 were prepared. Doughs were inoculated with *L. animalis* TMW 1.971, *L. reuteri* TMW 1.106, *L. sanfranciscensis* TMW 1.392 and *L. curvatus* TMW 1.624. Results of EPS formation are given in g EPS per kg flour and ANOVA of the data revealed significant differences ($P<0.001$) in EPS amounts for the same strain in different flours. No water soluble polysaccharides were found in chemically acidified control doughs analyzed with this method. A colony RAPD verified that *L. animalis* TMW 1.971, *L. reuteri* TMW 1.106 and *L. curvatus* TMW 1.624 are able to

assert in all gluten-free sourdoughs. *L. sanfranciscensis* TMW 1.392 only grew in buckwheat and buckwheat core doughs.

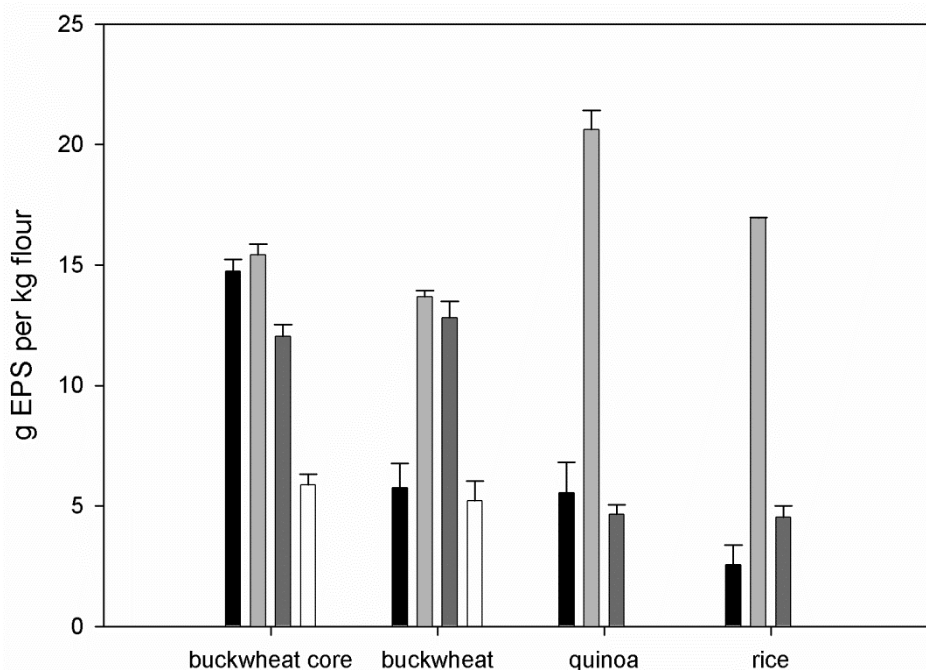


Figure 16: Influence of different flours on EPS production by lactobacilli.
L. animalis TMW 1.971 (black), *L. reuteri* TMW 1.106 (light gray), *L. curvatus* TMW 1.624 (dark gray) and *L. sanfranciscensis* (white). Data are given as mean ± SD, n=3 and were subjected to a one-way ANOVA. Values are significantly different (P<0.001) for each strain in different flours.

Figure 16 shows the respective results of the fermentations and indicates that EPS production strongly depends on type of flour. Each strain demonstrated different patterns for the four flours. *L. animalis* TMW 1.971 produced most EPS on buckwheat core flour (14.74 g kg^{-1}), followed by buckwheat (5.76 g kg^{-1}), quinoa (5.55 g kg^{-1}), and rice flour (2.55 g kg^{-1}). EPS formation of *L. reuteri* TMW 1.106 was best in quinoa sourdoughs. In these fermentations difference in EPS amounts between quinoa (20.63 g kg^{-1}), rice (16.94 g kg^{-1}), buckwheat core (15.43 g kg^{-1}) and buckwheat (14.68 g kg^{-1}) were slight compared to fermentations with *L. animalis* TMW 1.971. *L. curvatus* TMW 1.624 produced most EPS in buckwheat (12.82 g kg^{-1}) and buckwheat core (12.04 g kg^{-1}) sourdoughs. The EPS formation in quinoa (4.65 g kg^{-1}) and rice (4.54 g kg^{-1}) sourdoughs was less, but similar. *L. sanfranciscensis* TMW 1.392 produced 5.22 g kg^{-1} EPS in buckwheat and 5.87 g kg^{-1} EPS in buckwheat core sourdoughs. *L. curvatus* TMW 1.624, *L. sanfranciscensis* TMW 1.392 and *L. reuteri* TMW 1.106 reached their optimal growth after 24 h fermentation, whereas *L. animalis* TMW 1.971 grew very fast and showed highest cell counts after 6 h (Figure 17). A correlation between optimal growth and EPS formation after 24 h was only suggested in fermentations with *L. reuteri* TMW 1.106 (r = 0.84). This strain grew best in quinoa and rice and worse in buckwheat core and

buckwheat. Especially for *L. curvatus* TMW 1.624 no correlation was visible (r = 0.35). Best growth was found on buckwheat core, followed by quinoa, buckwheat and rice (Figure 17). In contrast, most EPS was produced in buckwheat sourdoughs, then in buckwheat core, quinoa and rice (Figure 16). In all fermentations highest TTA were found in buckwheat core and quinoa sourdoughs, indicating a good buffer capacity (Table 13).

L. animalis TMW 1.971 and *L. curvatus* TMW 1.624 accumulated fructose during fermentation, whereas *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392 converted fructose to mannitol. Most lactate, acetate and ethanol were produced in doughs where the highest cfu per g dough were found after 24 h. The FQ decreased in fermentations with *L. reuteri* from rice (2.50) to quinoa (2.30), buckwheat (2.03) and buckwheat core flour (1.82). In fermentations with *L. sanfranciscensis*, FQ of 2.96 (buckwheat) and 2.53 (buckwheat core flour) were measured.

Table 13: Metabolite formation in sourdoughs with different flours (DY 250, 7.5% sucrose) after 24 h fermentation. Data are representative for one of two independent experiments showing the same trends.

flour	lactate[a]	acetate	ethanol	sucrose	glucose	fructose	mannitol	TTA [mL]	pH
L. animalis TMW 1.971									
buckwheat core	545.37	-	-	3.01	19.38	194.94	-	42.00	4.04
buckwheat	376.03	-	-	12.58	76.92	192.66	-	27.70	3.97
quinoa	416.07	-	-	33.55	236.87	186.50	-	35.65	3.88
rice	244.16	-	-	55.20	163.56	187.54	-	18.50	3.78
L. reuteri TMW 1.106									
buckwheat core	257.10	140.80	127.10	-	4.48	-	270.30	34.45	4.41
buckwheat	259.16	126.51	139.72	-	4.36	-	249.98	27.60	4.00
quinoa	386.04	166.78	241.07	-	97.16	-	290.14	44.85	3.91
rice	257.26	103.65	172.56	-	18.03	-	221.39	26.15	3.62
L. curvatus TMW 1.624									
buckwheat core	456.20	-	-	-	124.31	263.15	-	31.30	4.23
buckwheat	296.11	-	-	-	161.43	279.56	-	19.60	4.13
quinoa	382.49	-	-	51.53	468.08	240.99	-	30.30	4.20
rice	141.88	-	-	-	261.98	266.63	-	9.55	4.34
L. sanfranciscensis TMW 1.392									
buckwheat core	283.10	109.69	200.78	-	7.73	-	213.75	34.55	4.30
buckwheat	234.54	77.33	175.70	-	3.80	-	156.10	28.70	3.93
quinoa	-	-	-	-	-	-	-	-	-
rice	-	-	-	-	-	-	-	-	-

[a] metabolite concentrations in mmol kg^{-1} flour

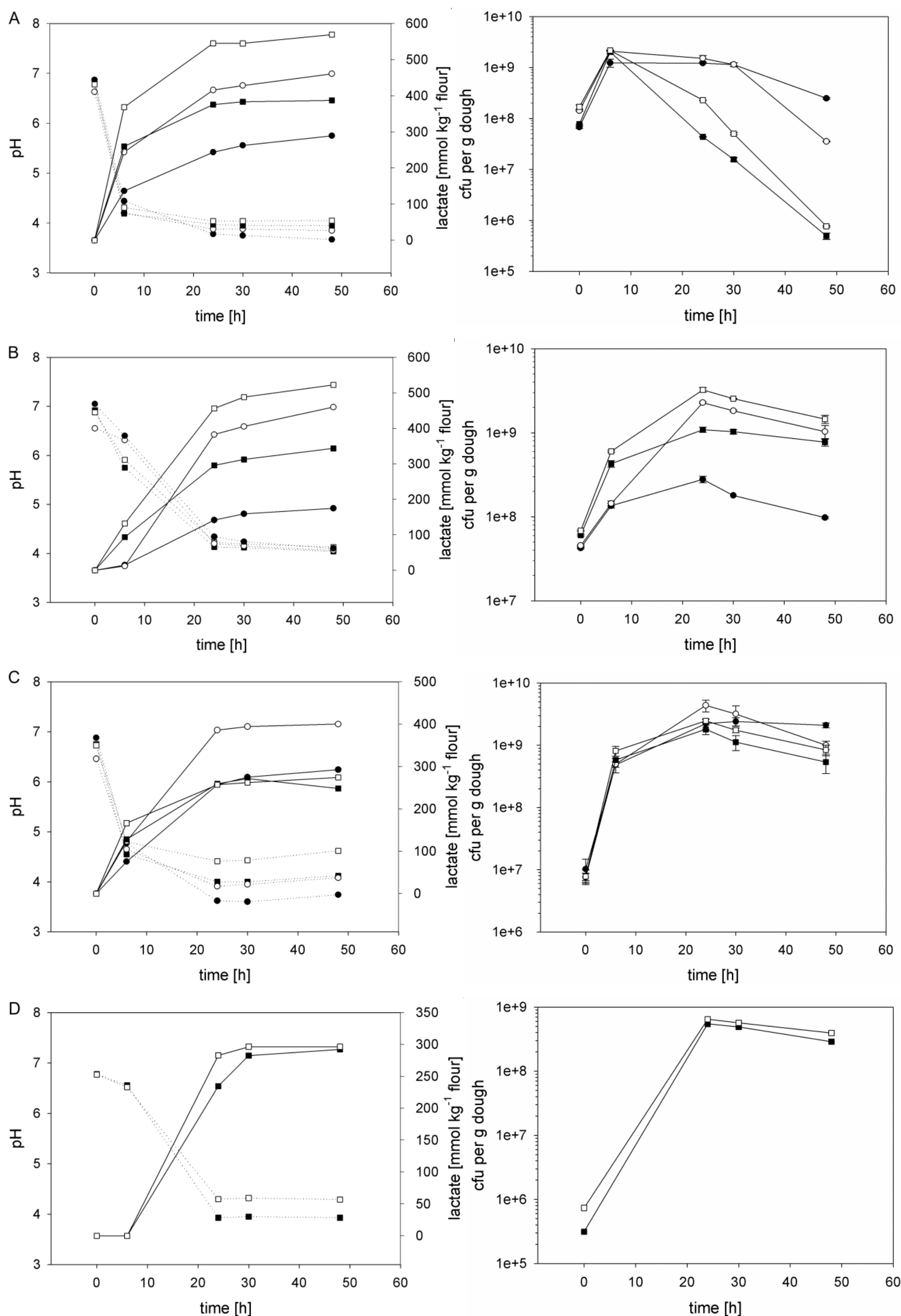


Figure 17: Growth, pH and lactate formation during fermentation in different flours by lactobacilli. Shown are *L. animalis* 1.971 (A), *L. curvatus* TMW 1.624 (B), *L. reuteri* TMW 1.106 (C) and *L. sanfranciscensis* TMW 1.392 (D) in rice (●), quinoa (○), buckwheat (■) and buckwheat core (□) flour.

3.6.2 Influence of sucrose amounts on EPS yields

Dough fermentations with different sucrose concentrations were carried out in different flours. For each strain the optimal flour for EPS production was used. Thus, *L. reuteri* TMW 1.106 was fermented in quinoa, *L. animalis* TMW 1.971 in buckwheat core and *L. curvatus* TMW 1.624 in buckwheat flour. Further optimization steps with *L. sanfranciscensis* TMW 1.392 were not carried out, because gluten-free flours did not seem to be suitable for growth and EPS production of this strain.

ANOVA of the data showed significant differences ($P<0.001$) in EPS yields for each strain in doughs with different sucrose concentrations. The higher the sucrose concentration, the more EPS was produced (Figure 18). The influence on EPS yields was less with higher sucrose amounts. In the case of *L. animalis* TMW 1.971, EPS formation could be increased 2.76 fold by enhancing the sucrose amount from 3.75% to 15%. EPS production in sourdoughs with *L. reuteri* TMW 1.106 could be increased 2.23 fold and in fermentations with *L. curvatus* TMW 1.624 by 2.12 fold.

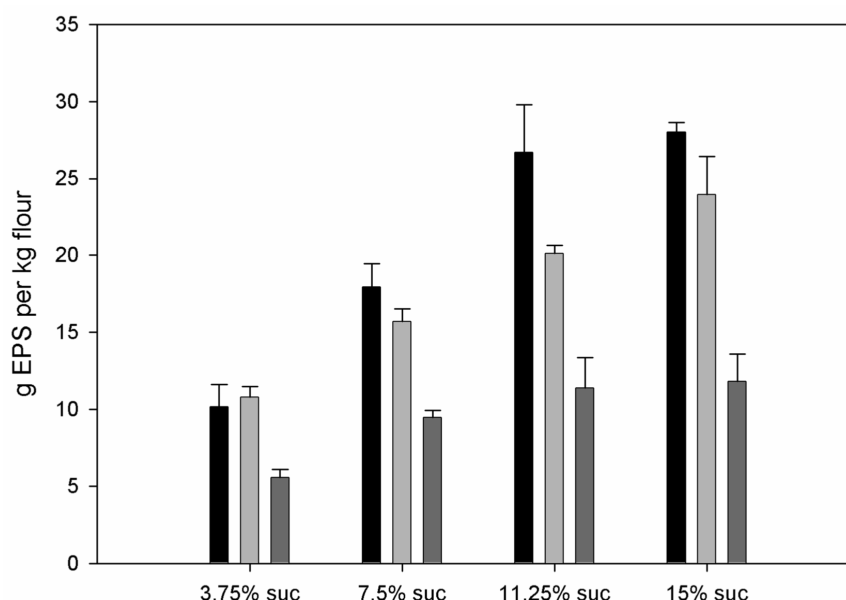


Figure 18: Influence of different sucrose amounts on EPS production by lactobacilli.
L. animalis TMW 1.971 (black), *L. reuteri* TMW 1.106 (light gray) and *L. curvatus* TMW 1.624 (dark gray). Data are given as mean ± SD, n=3 and were subjected to a one-way ANOVA. Values are significantly different ($P<0.001$) for each strain in sourdoughs with different sucrose amounts.

Growth, pH, TTA as well as lactate production of *L. animalis* and *L. curvatus* were not strongly influenced by the sucrose concentration (Table 14 and Figure 19). Only growth and metabolite formation were affected in fermentations with *L. reuteri*. pH was not influenced, but the more sucrose, the more TTA were formed and the FQ decreased from 3.42 (3.75% sucrose) to 1.52 (15% sucrose). Growth decreased as sucrose concentration increased (Figure 19). Furthermore, with increasing sucrose concentration less lactate

and ethanol and more acetate and mannitol were produced. In general, more glucose was released with higher sucrose amounts, but regarding the ratio of released glucose to consumed sucrose (relative hydrolase activity), the hydrolase activity increased in case of *L. animalis* and decreased for *L. reuteri* and *L. curvatus* with increasing sucrose concentration (data not depicted). Residual sucrose could be found in fermentations ≥ 7.5% sucrose with *L. animalis* and *L. curvatus*. In the case of *L. reuteri*, the sucrose was completely metabolized except for doughs containing 15% sucrose (Table 14).

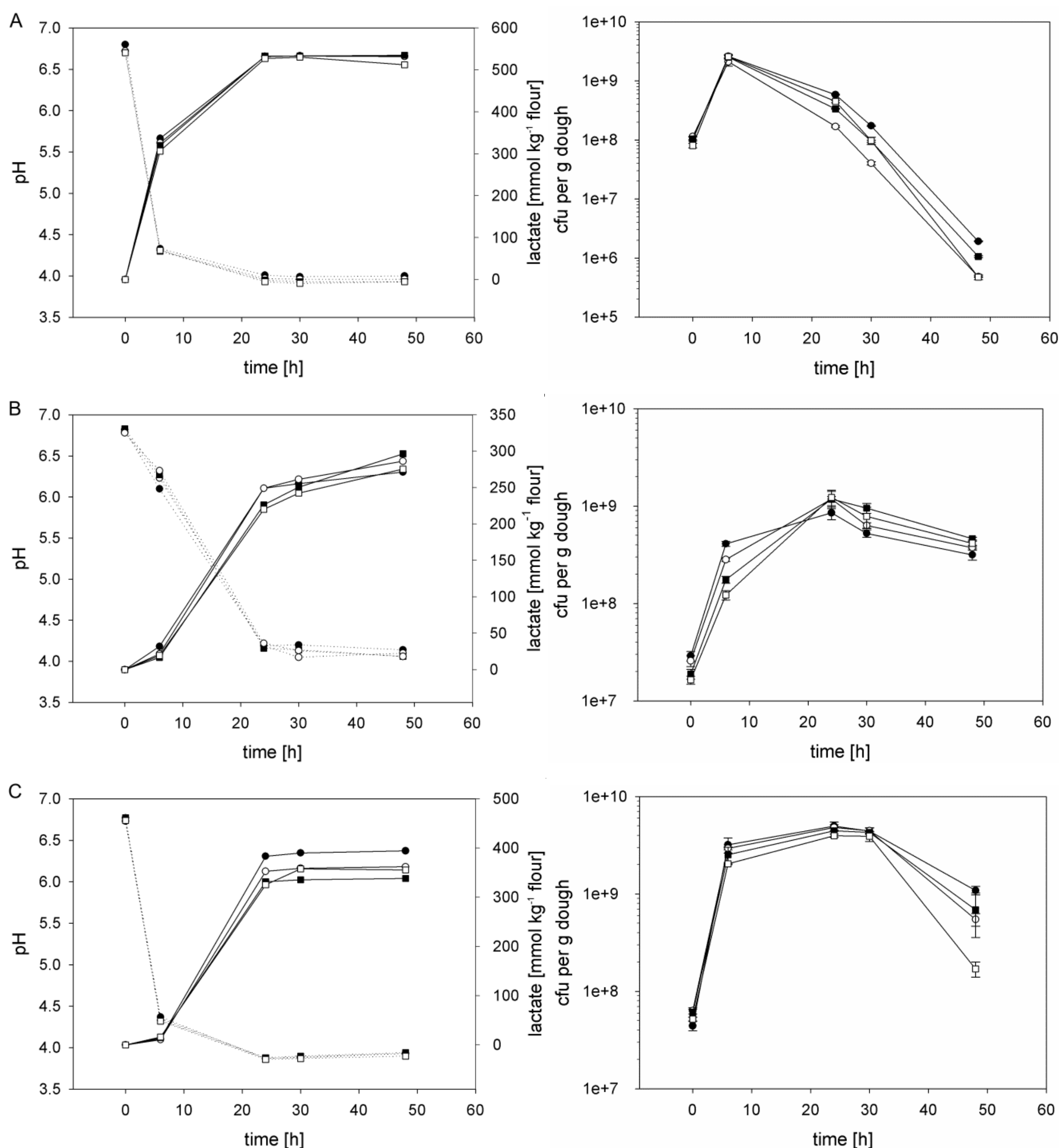


Figure 19: Growth, pH and lactate formation during fermentation with different sucrose concentrations by lactobacilli. Shown are *L. animalis* TMW 1.624 in buckwheat core flour (A), *L. curvatus* TMW 1.624 in buckwheat flour (B) and *L. reuteri* TMW 1.106 in quinoa (C) with 3.75% (●), 7.5% (○), 11.25% (■) and 15% (□) sucrose.

Table 14: Metabolite formation in sourdoughs (DY 250) with different amounts of sucrose after 24 h fermentation. Data are representative for one of two independent experiments showing the same trends.

sucrose [%][a]	lactate[b]	acetate	ethanol	sucrose	glucose	fructose	mannitol	TTA [mL]	pH
L. animalis TMW 1.971[c]									
3.75	531.98	-	-	-	7.75	77.13	-	41.80	4.01
7.50	532.18	-	-	1.49	21.90	167.72	-	42.10	3.97
11.25	532.99	-	-	12.81	51.55	212.50	-	42.45	3.95
15.00	527.25	-	-	36.98	77.69	248.99	-	42.25	3.93
L. reuteri TMW 1.106[d]									
3.75	383.37	110.38	310.51	-	103.99	-	188.27	37.05	3.87
7.50	352.75	149.94	234.58	-	121.11	-	276.53	37.90	3.87
11.25	331.32	190.55	167.19	-	166.04	-	351.33	39.10	3.88
15.00	325.63	210.01	152.50	8.79	174.29	57.80	416.22	40.65	3.86
L. curvatus TMW 1.624[e]									
3.75	249.16	-	-	-	133.51	207.53	-	18.70	4.19
7.50	249.13	-	-	25.89	209.33	304.14	-	18.70	4.19
11.25	226.48	-	-	92.66	219.56	306.36	-	18.60	4.16
15.00	220.23	-	-	175.18	185.85	236.29	-	18.50	4.22

[a] flour base; [b] metabolite concentrations in mmol kg^{-1} flour; fermented in [c] buckwheat core, [d] quinoa, [e] buckwheat

3.6.3 Influence of cell counts on EPS amounts

Further optimization steps were conducted with *L. curvatus* as the EPS of this strain improved the quality of gluten-free bread the most.

The impact of inoculation cell counts on EPS amounts was investigated with *L. curvatus* TMW 1.624 in buckwheat doughs (DY 250, 7.5% sucrose). ANOVA of the data revealed significant differences ($P<0.01$) in the influence of different cell counts of EPS yields. EPS production correlated strongly to cell counts at the beginning of fermentation ($r = 0.98$) but not to the highest cell counts reached after 24 h of fermentation ($r = 0.16$) (Figure 20). Doubling the cell counts enhanced EPS production 1.41 fold. Final pH, TTA and lactate production were not influenced. Just 6 h after inoculation, differences were visible (Figure 21). The higher the inoculated cell counts, the faster the pH decreased and TTA and lactate amounts increased. Sucrose was completely consumed in all doughs after 24 h, in doughs with 7.21E+07 and 1.01E+08 cfu g^{-1} already after 6 h. The more cell counts used, the less glucose, indicating EPS formation, and the more fructose were released (2.89E+07 cfu g^{-1}: 128.96 mmol glucose kg^{-1}, 231.84 mmol fructose kg^{-1}; 5.18E+07 cfu g^{-1}: 108.80 mmol glucose kg^{-1}, 240.10 mmol fructose kg^{-1}; 7.21E+07 cfu g^{-1}: 111.13 mmol

glucose kg^{-1}, 246.39 mmol fructose kg^{-1}; 1.01E+08 cfu g^{-1}: 102.42 mmol glucose kg^{-1}, 261.48 mmol fructose kg^{-1}).

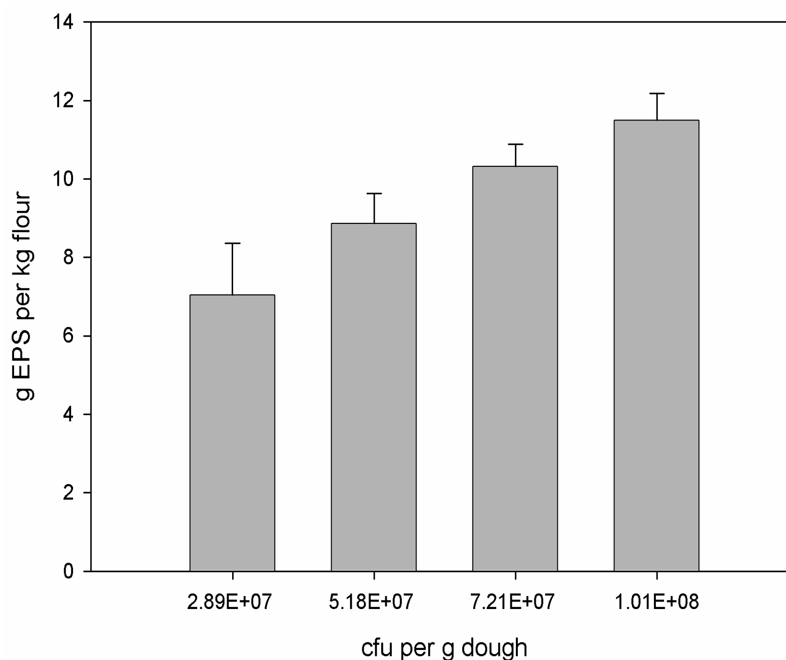


Figure 20: Influence of different inoculation cell counts on EPS production by *L. curvatus* TMW 1.624. Shown are initial cell counts (0 h) and EPS produced after 24 h. Data are given as mean ± SD, n=3 and were subjected to a one-way ANOVA (P<0.001).

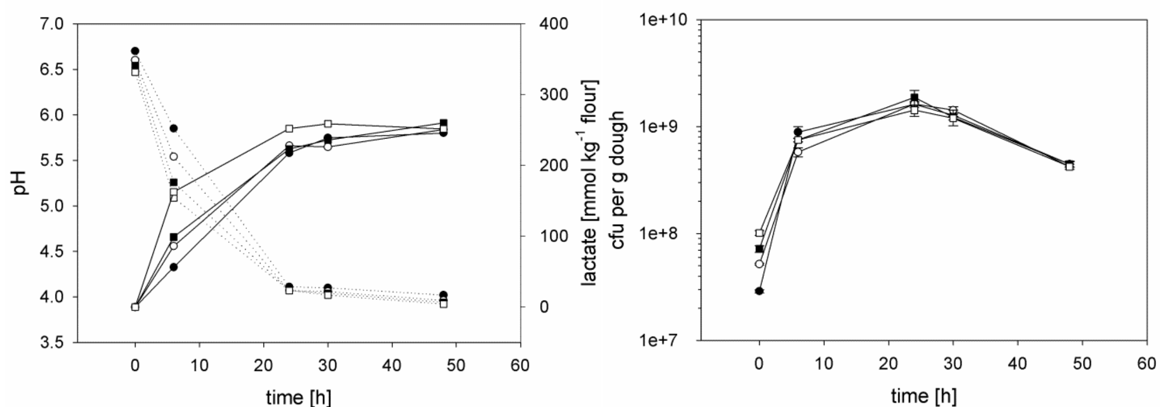


Figure 21: Growth, pH and lactate formation of *L. curvatus* TMW 1.624 in buckwheat doughs inoculated with different cfu. Doughs were inoculated with 2.89E+07 (●), 5.18E+07 (○), 7.21E+07 (■) and 1.01E+08 (□) cfu per g dough.

3.6.4 Impact of dough yield and sucrose fed-batch on EPS formation

Buckwheat doughs were prepared with 7.5% sucrose and different dough yields (DY 200, 250, 300 and 350) and inoculated with *L. curvatus* TMW 1.624. One further fermentation was conducted, where sucrose was given to the dough in portions (DY 250, 7.5% sucrose fed-batch). Figure 22 and Figure 23 present the appropriate results. ANOVA of the data showed that there were significant differences (P<0.001) in the EPS yields produced in

doughs with different DY. With increasing DY less EPS was produced and difference between higher DY became less. By reducing the DY from 250 (previous fermentations) to 200, EPS production could be enhanced 1.28 fold. Final pH, TTA and lactate production were not affected by different DY except for DY 200 (Figure 23). *L. curvatus* TMW 1.624 produced less lactate in this dough during the entire fermentation process. The higher the DY the faster the strain grew. Highest cell counts were therefore found in doughs with DY 350 after 6 h, but in doughs with DY 200 after 24 h. Considering final glucose und fructose concentrations no tendency was visible (data not shown). Sucrose was metabolized after 48 h in all doughs, but after 30 h less sucrose was found in doughs with smaller DY (data not depicted).

It was not possible to enhance the EPS production in the sucrose fed-batch, rather it was influenced negatively (Figure 22). Because of less sucrose at the beginning of fermentation, cells grew and pH fell faster (Figure 23). Residual sucrose after 24 h was highest compared to the other doughs. Especially released glucose and fructose were high in fed-batch fermentations, indicating that the glucose had not been used as much for EPS formation as in direct fermentations (DY 250, 7.5% sucrose direct: 127.77 mmol glucose kg^{-1}, 175 mmol fructose kg^{-1}; DY 250, 7.5% sucrose fed-batch: 157.99 mmol glucose kg^{-1}, 200.68 mmol fructose kg^{-1}).

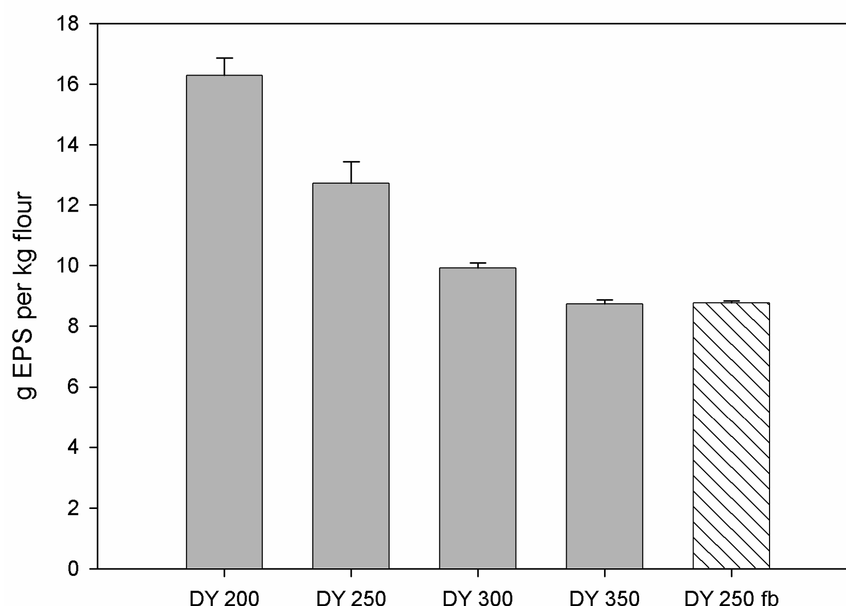


Figure 22: Influence of different dough yields and sucrose fed-batch (fb, hatched) on EPS production by *L. curvatus* TMW 1.624. Shown are EPS yields reached in the appropriate dough after 24 h. Data are given as mean ± SD, n=3 and were subjected to a one-way ANOVA (P<0.001).

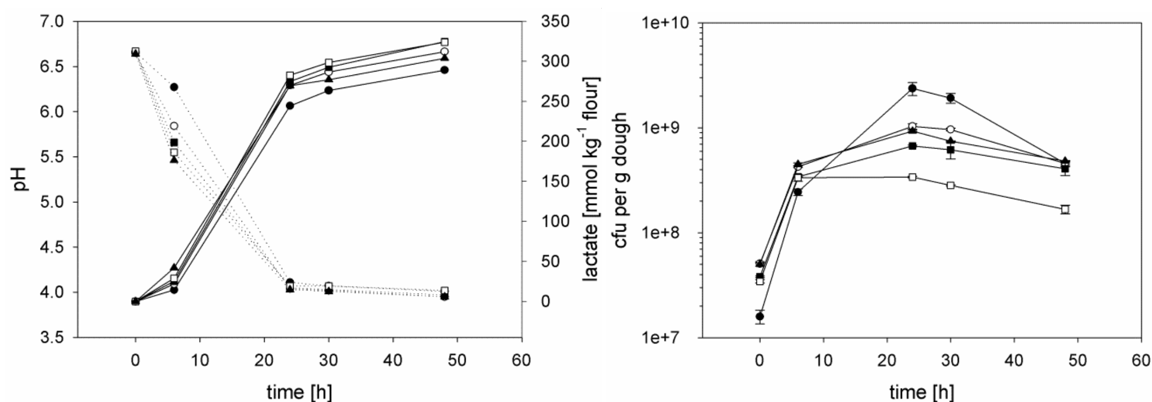


Figure 23: Growth, pH and lactate formation of *L. curvatus* TMW 1.624 in buckwheat doughs with different dough yields and sucrose fed-batch. DY 200 (●), DY 250 (○), DY 300 (■), DY 350 (□) and DY 250 with sucrose fed-batch (▼).

3.7 Exploration and analysis of the *gtf* gene from *L. curvatus* TMW 1.624

In order to gain more knowledge about EPS production in sourdoughs, respective *gtf* genes need to be heterologously expressed and characterized. *Gtf* genes of *L. reuteri* TMW 1.106 and *L. animalis* TMW 1.971 have already been investigated by Kaditzky (2008), Jänsch (2004) and Tieking (2005). No information on *gtf* from *L. curvatus* TMW 1.624 is available and so this was investigated in this dissertation.

Both degenerated primer pairs (Dexf1/Dexr1 and Dexf2/Dexr2) resulted in the expected PCR product sizes of about 2300 base pairs (bp) and 1400 bp, respectively. Sequence BLAST revealed high similarity (99%) to *gtf* of *L. sakei* Kg15 (Kralj et al., 2004a). After several rounds of PCR by primer walking with primers generated from the sequence of *L. sakei* Kg15, a sequence of 5257 bp was obtained (see appendix section 8.1). The open reading frame with 1697 amino acids has a similarity of 93.8% and an identity of 93.6% to *L. sakei* Kg15 (Kralj et al., 2004a). The resulting protein has a calculated molecular weight of 183.63 kDa and is greater than the Gtf of *L. sakei* Kg15, which has a calculated molecular weight of 174.09 kDa. Furthermore, the putative Gtf of *L. curvatus* TMW 1.624 shares the common domain structure of glucansucrases with four distinct structural domains from the N- to the C-terminus (Figure 24). In its variable region, this Gtf contains conserved and less-conserved YG-repeats.

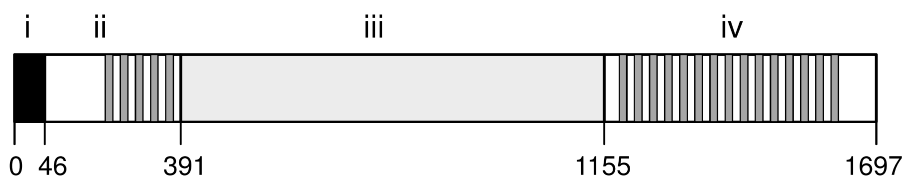


Figure 24: Putative glucansucrase from *L. curvatus* TMW 1.624.
The four regions shown are (i) the N-terminal signal sequence, (ii) the N-terminal variable region, (iii) the catalytic core and (iv) the C-terminal glucan binding domain. Within the regions ii and iv the YG repeats are shaded gray.

The four conserved regions within the catalytic core are completely identical to the conserved regions of Gtf of *L. sakei* Kg15 (Figure 25) (Kralj et al., 2004a). Differences to the protein of *L. sakei* Kg15 can be found in the N- and C-terminal region, which are larger for Gtf of *L. curvatus*. Within the catalytic core region one amino acid is replaced (P786A) and one amino acid occurs which does not exist in Gtf from *L. sakei* Kg15 (-787P, Gtf *L. sakei* Kg15 numbering). Gtfs of *L. sakei* Kg15, *L. fermentum* Kg3 and *L. parabuchneri* 33 were identified to produce dextrans with branching at O-3 (Kralj et al., 2004a) and share several amino acids with *L. curvatus* TMW 1.624 within the conserved regions (Figure 25).

```
                    Region I    Region II     Region III     Region IV
L. reuteri          ADWVPDQ     SIRVDAVDNVD   HLNILEDWSHAD   FIRAHDSNAQDQINN
L. animalis         ADWVPDQ     GIRIDAVDNVD   HISILEDWNDKD   FVRAHDASVQEDILQ
L. curvatus         ADFVPDQ     SVRVDAVDNVD   HLSILEDWGHND   FVRAHDSEVQTVIGD
L. sakei            ADFVPDQ     SVRVDAVDNVD   HLSILEDWGHND   FVRAHDSEVQTVIGD
L. fermentum        ADFVPDQ     AIRIDAVDNVD   HLSILEDWSHND   FVRAHDSEVQTVIAE
L. parabuchneri     DDWVPDQ     GYRVDAVDNVD   HLSILEDWDNND   FIRAHDSEVQTIIAQ
                     *:****     . *:*******   *:.*****.. *   *:****:..*  * :
```

Figure 25: Amino acid sequence alignment of the four conserved regions I, II, III and IV in the catalytic domains of different Gtfs. *L. reuteri* TMW 1.106 (Kaditzky, 2008), *L. animalis* TMW 1.971 (Tieking, 2005), *L. curvatus* TMW 1.624 (this work), *L. sakei* Kg15 (Kralj et al., 2004a), *L. fermentum* Kg3 (Kralj et al., 2004a) and *L. parabuchneri* 33 (Kralj et al., 2004a). The seven fully conserved residues are underlined and catalytic residues are shown in bold type. Amino acids detected in Gtfs as main determinants for α-(1-6) linkages are highlighted gray (Kralj et al., 2005).

3.8 Cloning and heterologous expression of *gtf* genes from *L. curvatus* TMW 1.624, *L. reuteri* TMW 1.106 and *L. animalis* TMW 1.971

The *gtf* genes of *L. curvatus* TMW 1.624, *L. reuteri* TMW 1.106 and *L. animalis* TMW 1.971 were heterologously expressed in *E. coli*. Therefore the genes were amplified with specific primers (Table 2) to obtain PCR products which are listed in Table 15.

Table 15: Rounded sizes of *gtf* PCR products.

strain	*dNgtf* [bp]	*gtf* [bp]
L. curvatus	3950	5110
L. animalis	3120	4660
L. reuteri	3150	5250

The purified PCR products were cloned into a pBAD/*Myc*-HisA expression vector using *XhoI* and *HindIII* (*L. curvatus dNgtf/gtf*) or *XhoI* and *EcoRI* (*L. animalis dNgtf/gtf*) or *SacI* and *Bsp119I* (*L. reuteri dNgtf/gtf*) restriction sites. A His-tag for purification via affinity chromatography was provided by the vector at the C-terminal site of the protein. Correct insertion of *gtf* genes was initially inspected by PCR analysis with pBAD_F and pBAD_R

primers and restriction analysis of isolated vector DNA. Later, a fast phenotypic screening method was developed, which made isolation and sequencing of plasmids unnecessary. Clones obtained by transformation with the respective constructs, were plated on LB agar supplemented with the respective antibiotics as negative control. For expression, clones were transferred onto plates containing sucrose and arabinose as well as the respective antibiotics. First *E. coli* TOP10 was used for screening, but no expression and thus no formation of EPS was visible. A codon usage analysis revealed rare codons for *E. coli* within all three *gtf* genes. Because of this, the strain was changed to Rosetta, which is an *E. coli* strain that contains an additional plasmid with tRNA genes corresponding to rare codons to overcome the codon bias of *E. coli*. After incubation and storage in the fridge, clones producing the active Gtf could be identified due to their slimy surface (Figure 26). Respective Rosetta clones were transferred into the TMW strain collection (TMW 2.1402 – 2.1404).

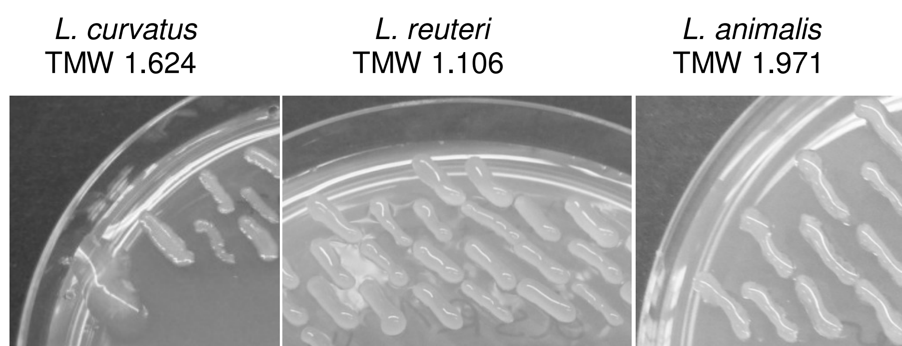


Figure 26: Fast phenotypic screening of Rosetta clones expressing the Gtf.
Shown are Rosetta clones containing expression plasmids with *gtf* genes of *L. curvatus*, *L. reuteri* and *L. animalis*. Beside the positive slimy clone on the agar plate containing *L. curvatus gtf*, negative clones are also apparent above. Rosetta clones containing *L. reuteri* and *L. animalis gtf* are all positive in this picture.

On upscaling to flasks, expression was visible during purification of proteins. When target protein was eluted from the His-Trap column, the UV detector signal peaked. Respective eluted fractions were then analyzed on SDS-PAGE, which revealed significant amounts of nearly pure protein. In addition, the fractions were screened for activity and protein concentration was determined. The fraction with the highest activity was used for subsequent enzyme assays.

3.9 Determination of Gtf activities

Glucosyltransferase activities of purified protein fractions were determined as described in section 2.3.5. The follow-on enzyme assays were performed with dNGtf of *L. reuteri* (TMW 2.1404), Gtf of *L. animalis* (TMW 2.1402) and Gtf of *L. curvatus* (TMW 2.1403) as these enzymes featured the highest activities and as N-terminal domain does not affect

the catalytic properties or the EPS structure (Kralj et al., 2004b, Monchois et al., 1999). The influences of pH, temperature and ions were analyzed with dNGtf and Gtf of *L. animalis*, but no differences were detectable. For simplicity's sake, in the following sections the three enzymes are consistently called Gtf.

3.9.1 Determination of pH and temperature optima

In order to define the best conditions for subsequent studies, the pH and temperature optima of Gtf activities were examined. Total activity was determined by generated fructose and includes hydrolysis and transferase activities.

The pH optima for the *L. reuteri* Gtf as well as the *L. curvatus* Gtf activity were in the range of pH 4.4 – 4.8 (Figure 27). Optimal conditions for Gtf of *L. animalis* were found within a higher pH range (pH 5.8 – 6.0). In contrast to the other enzymes, the activity of *L. animalis* Gtf showed a broad range with activities higher than 60% (pH 4.8 – 7.2). Maximal transferase activities were within the same pH scope and maximal hydrolysis activities showed slightly higher pH optima than the overall activity.

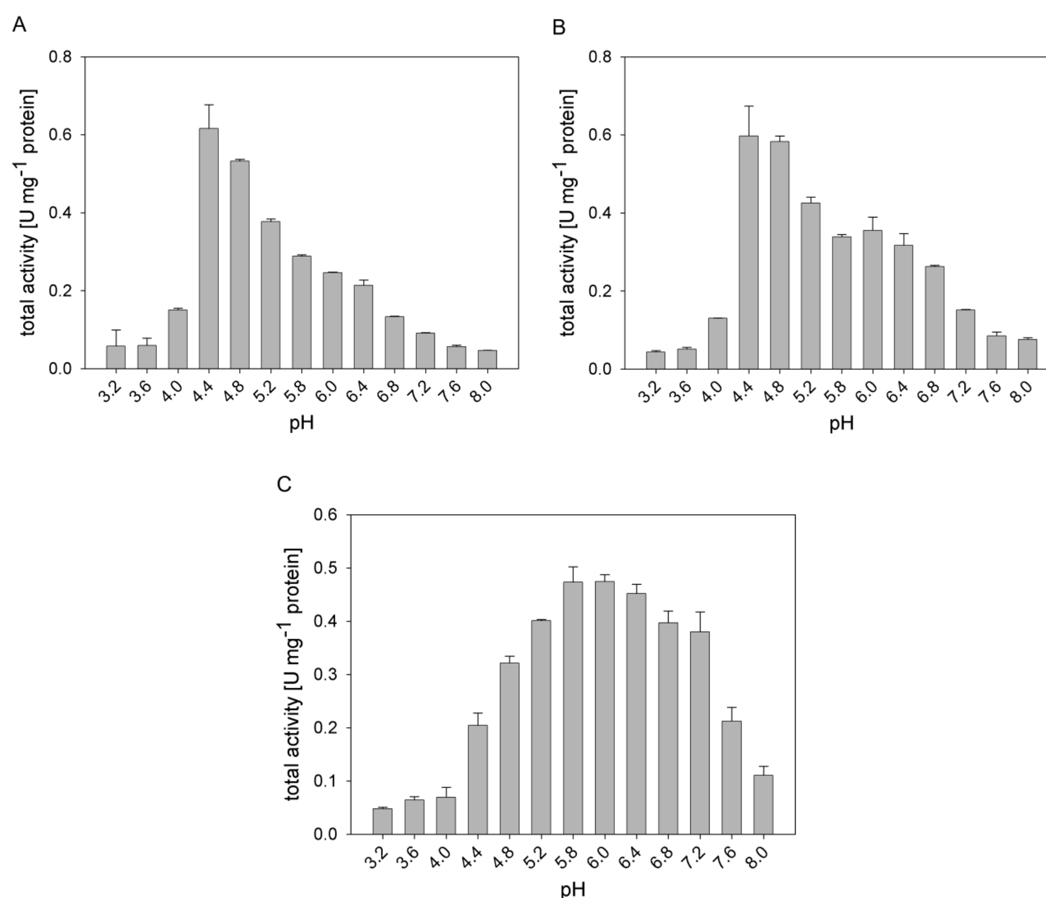


Figure 27: Effect of pH on Gtf activities of *L. reuteri* (A), *L. curvatus* (B) and *L. animalis* (C). The pH optima were analyzed using sodium acetate (pH 3.2 – 5.2) and sodium phosphate (pH 5.8 – 8.0) buffer at the respective growth temperature of the strain (*L. curvatus*: 30 °C; *L. reuteri* and *L. animalis*: 40 °C). Data are given as mean ± SD, n=2.

The optimal temperatures for the three enzymes were close to the optimal growth temperatures (Figure 28). For *L. reuteri* Gtf, the optimal temperature determined for both reactions was 45 °C. Under the selected conditions, transferase activity constituted 85 – 90% of the total activity. The activity distribution remained constant until 48 °C. The increased hydrolysis and decreased transferase activity at high temperatures were due to acid hydrolysis of sucrose, which was verified by control reactions without enzymes.

Optimum temperature for Gtf of *L. curvatus* for both reactions was 31 °C and transferase activity constituted 90 – 99 % of the total activity. At 40 °C no hydrolysis activity could be detected, indicating that hydrolysis activity is more sensitive to higher temperatures.

Gtf of *L. animalis* evidenced two temperature maxima at 42 °C and 53 °C for total activity (Figure 28, C1). The reason for this lies in different temperature maxima for the hydrolysis and transferase activities. Optimal hydrolysis activity was found at 56 °C and the optimum for transferase activity was 42 °C (Figure 28: C1, C2). The hydrolysis activity turned out to be more sensitive to high temperatures shown by the activity distribution at ≥ 63 °C. Overall, *L. animalis* Gtf showed a higher hydrolase activity than the other two enzymes. Up to 42 °C the transferase activity constituted 72 – 75 % and decreased to 59% at 56 °C.

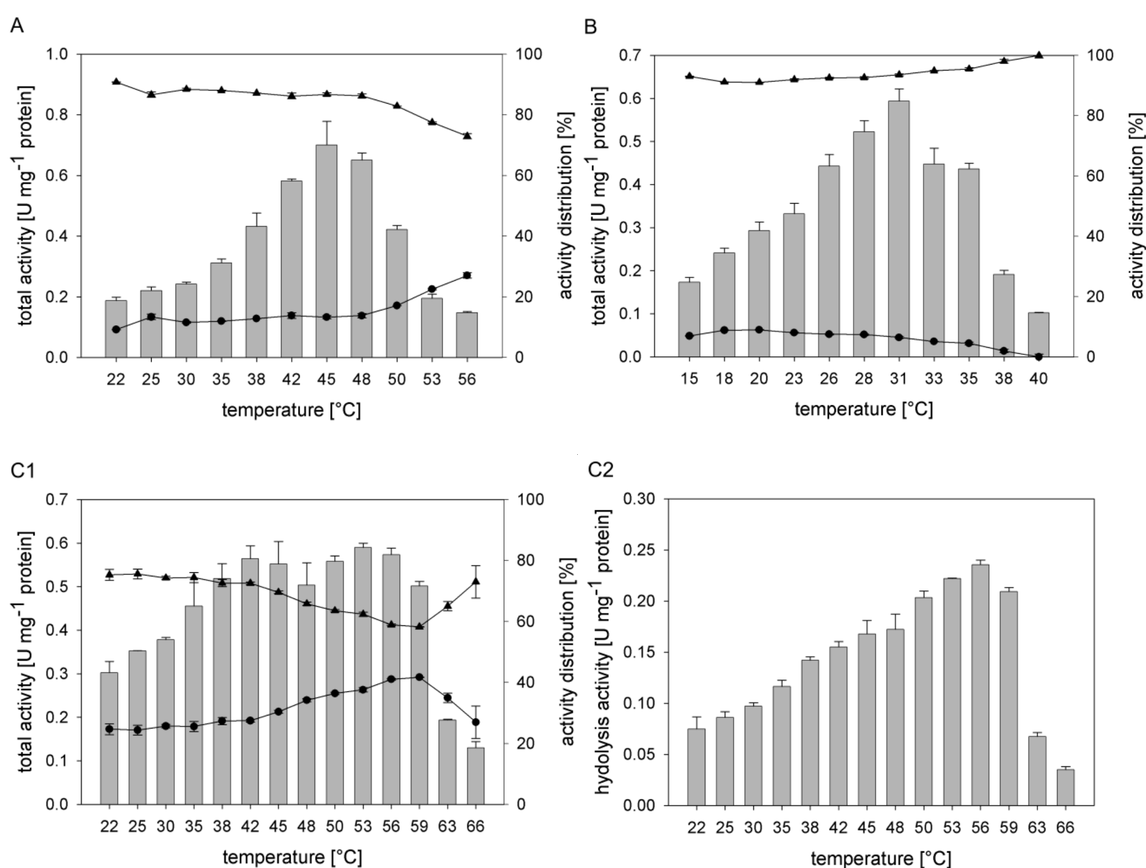


Figure 28: Effect of temperature on Gtf activities of *L. reuteri* (A), *L. curvatus* (B) and *L. animalis* (C). Shown are transferase activity (▼) and hydrolysis activity (●) as activity distribution. To determine the optimal temperature, the respective optimal pH of each enzyme was used (Gtf of *L. reuteri*: pH 4.4; Gtf of *L. curvatus*: pH 4.4; Gtf of *L. animalis*: pH 5.8). Data are given as mean ± SD, n=2.

3.9.2 Influence of different ions and EPS on activity

Different metal ions had varying effects on the overall enzyme activities (Table 16). As differences in transferase and hydrolysis activity were less pronounced, only total activity is depicted and exceptions are mentioned in the text.

L. reuteri Gtf overall activity was significantly inhibited only by Cu^{2+}. The other ions, except for Zn^{2+}, had stimulating effects on enzyme activity. Of these ions, Ca^{2+} had the most stimulating effect. Mn^{2+} played a specific role as it inhibited hydrolysis activity almost entirely, but activated transferase activity, so that the total activity was increased. EDTA had no significant influence on overall enzyme activities.
Total activity of *L. curvatus* Gtf was inhibited by EDTA and Cu^{2+} (Table 16). Hydrolysis activity was significantly inhibited by Zn^{2+} and Mn^{2+}. In contrast, Zn^{2+} had no effect on transferase activity, which was equivalent to more than 90% of the overall activity and therefore had no real influence on overall activity. As for *L. reuteri* Gtf, transferase activity of *L. curvatus* Gtf was activated through Mn^{2+}. K^{+}, Na^{2+} and Ca^{2+} stimulated overall activity while Al^{3+} and Mg^{2+} had no significant influence on activity.
Like the *L. curvatus* Gtf, Gtf of *L. animalis* was inhibited through EDTA and Cu^{2+}. But in contrast to the other two enzymes Mn^{2+} had no influence on hydrolysis and transferase activity and thus no effect on total activity. Al^{3+} and Na^{+} also did not significantly influence overall activity and Mg^{2+}, Zn^{2+}, K^{+} and Ca^{2+} stimulated *L. animalis* Gtf activity.

Overall, Ca^{2+} ions had the most stimulating effect on all three Gtfs.

Table 16: Effect of various compounds on *L. reuteri*, *L. curvatus* and *L. animalis* Gtf-catalyzed activity.

compound [1 mM]	Gtf[a] *L. reuteri*	Gtf *L. curvatus*	Gtf *L. animalis*
none	100.00 ± 6.28a[b]	100.00 ± 2.68a	100.00 ± 8.52a
EDTA	107.50 ± 4.33a,c,e,f	83.84 ± 3.39b,e	67.19 ± 0.26b
$CaCl_2$	162.08 ± 14.18b	176.42 ± 3.72c	146.19 ± 8.04c
$MgCl_2$	112.02 ± 6.73c,d,e,g,h	95.96 ± 5.26a	117.03 ± 11.10d,e,f
$ZnCl_2$	95.49 ± 7.39a	95.95 ± 6.44a	111.90 ± 1.84e,f
KCl	118.09 ± 4.45d,e,g,h	110.15 ± 0.24d,g	117.48 ± 9.22f
$AlCl_3$	111.80 ± 0.28e,g,h	99.85 ± 16.06a	97.06 ± 3.85a,g
$CuCl_2$	35.13 ± 6.60f	79.77 ± 6.52e	88.79 ± 3.82g
NaCl	111.91 ± 8.28g,h	126.59 ± 11.99f	100.85 ± 3.79a
$MnCl_2$	121.76 ± 3.44h	110.71 ± 6.15g	103.63 ± 1.64a

[a] Total enzyme activity [%], [b] Different letters in the same column indicate statistical significance ($P<0.05$). Enzyme activities were determined at optimal conditions for each enzyme (*L. reuteri* Gtf: 45 °C, pH 4.4; *L. curvatus* Gtf: 30 °C, pH 4.4; *L. animalis* Gtf: 42 °C, pH 5.8). Data are given as mean ± SD, n=2.

EPSs from lactobacilli were investigated in this dissertation for their effect on Gtf activities as well. Concentrations of 0.5% w/v and 0.1% w/v, corresponding to 5 and 1 g L^{-1}, were used.

L. reuteri Gtf was stimulated by all EPSs, whereas concentrations of 0.5% caused the strongest effects (Table 17). The ratio between hydrolysis and transferase activity was not influenced through EPS. The transferase activity always varied between 89.8 and 91.6% (without EPS: 90%).

EPSs had a stronger impact on *L. curvatus* Gtf activity. Its activity was stimulated 1.5 to 2 fold through EPSs (Table 17). Concentrations of 0.5% caused the strongest effects here, too. The activity was stimulated mostly by EPS of *L. animalis*. Fructan activated the enzyme as well as glucan. The ratio of hydrolysis to transferase activity was slightly influenced. Samples without EPS had a transferase activity of 98.6% which was reduced on addition of EPS to 95.8-97.0%, indicating that hydrolysis activity was more stimulated than transferase activity.

Table 17: Effect of different EPS on *L. reuteri*, *L. curvatus* and *L. animalis* Gtf-catalyzed activity.

EPS compound	Gtf[e] *L. reuteri*	Gtf *L. curvatus*	Gtf *L. animalis*
none	100.00 ± 3.29a[f]	100.00 ± 0.88a	100.00 ± 3.84a
0.5 % S[a]	129.00 ± 3.00b,c,g,h	161.55 ± 4.36b,d,h	91.84 ± 3.62b
0.1 % S	118.36 ± 2.87c,e,f,g,h	140.59 ± 5.50c,i	101.04 ± 2.93a,h
0.5 % R[b]	129.94 ± 9.37d,g,h	154.66 ± 7.63d,h	127.51 ± 0.22c,g
0.1 % R	111.99 ± 0.72e,f	121.21 ± 6.52e	114.83 ± 1.53d
0.5 % A[c]	116.52 ± 2.76f,g,h	199.95 ± 13.86f	198.69 ± 0.45e
0.1 % A	106.36 ± 7.48a	172.33 ± 5.69g	148.07 ± 3.24f
0.5 % C[d]	123.93 ± 6.93g,h	158.76 ± 1.00h	126.73 ± 3.45g
0.1 % C	122.21 ± 2.09h	133.50 ± 0.87i	106.37 ± 7.45h

[a] Levan of *L. sanfranciscensis* TMW 1.392, [b] Dextran of *L. reuteri* TMW 1.106, [c] EPS of *L. animalis* TMW 1.971, [d] Dextran of *L. curvatus* TMW 1.624. [e] Total enzyme activity [%]. [f] Different letters in the same column indicate statistical significance (P<0.05). Enzyme activities were determined at optimal conditions for each enzyme (*L. reuteri* Gtf: 45 °C, pH 4.4; *L. curvatus* Gtf: 30 °C, pH 4.4; *L. animalis* Gtf: 42 °C, pH 5.8). Data are given as mean ± SD, n=2.

L. animalis Gtf showed a different behavior. This enzyme was most activated by its own EPS (1.5 to 2 fold). The fructan (levan) of *L. sanfranciscensis* had either no effect or an inhibitory one (Table 17). The dextrans of *L. reuteri* and *L. curvatus* stimulated the activity. In contrast to the other two Gtfs, the ratio of hydrolysis and transferase activity of *L. animalis* Gtf was strongly influenced by EPSs. The transferase activity increased from 73.4% (without EPS) to 85.3 and 79.7% when 0.5% or 0.1% EPS from *L. animalis* was added, respectively. EPSs of *L. curvatus* and *L. reuteri* stimulated transferase activity, too

(0.5% C: 78.1% and 0.1% C: 74.4%; 0.5% R: 76.8% and 0.1% R: 73.5%, respectively). Only the fructan decreased the transferase activity from 73.4% to 69.1% (0.5% S) and 71.7% (0.1% S).

3.9.3 Influence of dough and artificial dough extracts on activity

In order to find more keystones to optimize EPS production in sourdoughs, dough extracts were generated and investigated for their influence on Gtf activities. Due to flour sugars and having to stay within the valid range of the assay, only one twentieth of the original concentration present in fermentations (DY 250) could be applied. The results are shown in Table 18.

Nearly all dough extracts inhibited *L. reuteri* Gtf overall activity. Only buckwheat extract slightly stimulated the overall activity and all extracts reduced transferase activity (without extract: 90.5%, quinoa: 81.3%, rice: 84.1%, buckwheat: 81.1% and buckwheat core: 77.9%).

L. curvatus Gtf was stimulated by all extracts, but most strongly by the buckwheat extracts. As for the Gtf of *L. reuteri*, transferase activity of *L. curvatus* Gtf was decreased by the extracts (without extract: 99.8%, quinoa: 84.7%, rice: 93.5%, buckwheat: 92.0% and buckwheat core: 90.7%).

L. animalis Gtf overall activity was also increased through all the dough extracts. The rice extract most strongly activated the enzyme. The transferase activity (without extract: 69.2%) was increased by quinoa (73.7%) and rice (79.0%) and inhibited through buckwheat (67.1%) and buckwheat core (67.7%).

Table 18: Effect of dough extracts on *L. reuteri*, *L. curvatus* and *L. animalis* Gtf-catalyzed activity.

extract compound[a]	Gtf[b] *L. reuteri*	Gtf *L. curvatus*	Gtf *L. animalis*
none	100.00 ± 2.01a[c]	100.00 ± 4.97a	100.00 ± 3.20a
quinoa	72.01 ± 2.71b	140.81 ± 8.07b	157.67 ± 1.64b
rice	92.93 ± 1.67c	129.26 ± 2.37c	219.08 ± 3.91c
buckwheat	106.32 ± 1.00d	163.17 ± 3.57d	146.51 ± 2.31d
buckwheat core	84.20 ± 4.61e	155.11 ± 1.85e	141.00 ± 3.16e

[a] A twentieth of dough extract concentration (DY 250) was used in this assay. [b] Total enzyme activity [%]. [c] Different letters in the same column indicate statistical significance ($P<0.05$). Enzyme activities were determined at optimal conditions for each enzyme (*L. reuteri* Gtf: 45 °C, pH 4.4; *L. curvatus* Gtf: 30 °C, pH 4.4; *L. animalis* Gtf: 42 °C, pH 5.8). Data are given as mean ± SD, n=2.

As only a small amount of the original dough extract could be used in the preceding experiments, the aim was then to generate an artificial extract of the most important flour minerals. Therefore rice and buckwheat flour were analyzed for their minerals according to DIN EN ISO 11885 E22 by the Forschungszentrum for Brau- und Lebensmittelqualität (Freising, Germany). The results are shown in Table 19.

Table 19: Mineral composition of flours.

mineral	rice[a]	buckwheat	quinoa[b]
Calcium	125.0	450.0	497.3
Magnesium	1076.0	1931.0	2299.0
Sodium	11.0	6.1	37.0
Potassium	2646.0	4602.0	5537.7
Copper	1.9	6.6	7.7
Manganese	22.9	19.4	13.5
Zinc	14.4	27.3	32.8

[a] mineral concentration [mg kg^{-1}]. [b] Hager et al., 2012.

Mineral content of the three flours from Table 19 were converted to a content present in doughs with a DY 250 and analyzed for their effect on Gtf activities.

As previously shown with original dough extracts, artificial dough extracts also inhibited *L. reuteri* Gtf overall activity (Table 20). But, in contrast, transferase activity was stimulated (without extract: 90.9%; buckwheat: 95.9%, quinoa: 96.6%, rice: 94.0%).
Overall *L. curvatus* Gtf activity was stimulated by all artificial extracts, with artificial rice extract having the most stimulating effect. Hydrolysis activity was neither detectable for samples without extract nor for artificial extracts.
L. animalis Gtf was also activated through all three artificial extracts. Overall activity was most stimulated through artificial buckwheat extract and transferase activity was stimulated by extracts, too (without extract: 74.1%, buckwheat: 79.5%, quinoa: 76.5%, rice: 74.2%).

Table 20: Effect of artificial dough extracts on *L. reuteri*, *L. curvatus* and *L. animalis* Gtf-catalyzed activity.

artificial extract[a]	Gtf[b] *L. reuteri*	Gtf *L. curvatus*	Gtf *L. animalis*
none	100.00 ± 1.29a[c]	100.00 ± 0.96a	100.00 ± 0.93a
buckwheat	69.18 ± 0.47b	115.61 ± 2.45b	131.57 ± 1.77b
quinoa	71.03 ± 0.27c	109.43 ± 4.17c	116.33 ± 2.20c
rice	84.62 ± 1.37d	127.36 ± 2.77d	108.45 ± 1.52d

[a] Artificial extract corresponds to mineral concentration in doughs with DY 250. [b] Total enzyme activity [%].
[c] Different letters in the same column indicate statistical significance ($P<0.05$). Enzyme activities were determined at optimal conditions for each enzyme (*L. reuteri* Gtf: 45 °C, pH 4.4; *L. curvatus* Gtf: 30 °C, pH 4.4; *L. animalis* Gtf: 42 °C, pH 5.8). Data are given as mean ± SD, n=2.

As different DY had an effect on EPS production through *L. curvatus* in sourdoughs, the mineral solution imitating buckwheat flour was used to investigate the influence of different DY on *L. curvatus* Gtf activity.

Conversely to the sourdough fermentations, higher dough yields stimulated overall Gtf activity more and DY 200 had no effect on activity (Table 21). As before, no hydrolysis activity was detectable.

Table 21: Effect of artificial dough extracts with different dough yield on *L. curvatus* Gtf-catalyzed activity.

artificial extract of buckwheat	Gtf[a] *L. curvatus*
none	100.00 ± 0.98a[b]
DY 200	99.68 ± 1.92a
DY 250	116.41 ± 2.46b
DY 300	133.85 ± 6.03c,d
DY 350	140.08 ± 9.22d

[a] Total enzyme activity [%]. [b] Different letters in the same column indicate statistical significance ($P<0.05$). Enzyme activities were determined at optimal conditions for the enzyme (30 °C, pH 4.4). Data are given as mean ± SD, n=2.

3.9.4 Kinetic studies

Kinetic parameters were investigated in the presence of sucrose and all three enzymes displayed Michaelis-Menten type kinetics for the total enzyme activity (Figure 29). As the kinetic studies were the last enzymatic investigations, the enzymes had already lost part of their activity during storage and V_{max} values differed from those obtained in section 3.9.1.

The Michaelis-Menten kinetic parameters for the release of fructose from sucrose through the enzymes were K_m= 14.91, V_{max}= 0.45 U mg^{-1} (r= 0.993); K_m= 3.29 mM, V_{max}= 0.06 U mg^{-1} (r= 0.992) and K_m= 3.05 mM, V_{max}= 0.3 U mg^{-1} (r= 0.959) for Gtf of *L. reuteri*, *L. curvatus* and *L. animalis*, respectively. The initial transferase rate was 89.2% (2 mM sucrose), which decreased to 88.0% (50 mM sucrose) and 85.7% (300 mM sucrose) for *L. reuteri* Gtf. For *L. curvatus* Gtf, hydrolysis activity was only detectable at sucrose concentrations ≥ 75 mM. The transferase rate decreased from 99.5% (75 mM sucrose) to 91.3% (150 mM) and 85.3% (300 mM sucrose). The initial transferase rate for *L. animalis* Gtf was 10.3% (2 mM sucrose) and increased to 22.9% (15 mM sucrose) and 49.9% (300 mM). These data reveal that Gtf of *L. animalis* favored hydrolysis at low sucrose concentrations and polymerization at high sucrose concentrations. Gtfs of *L. reuteri* and *L. curvatus* showed contradictory behavior favoring polymerization at low sucrose concentrations.

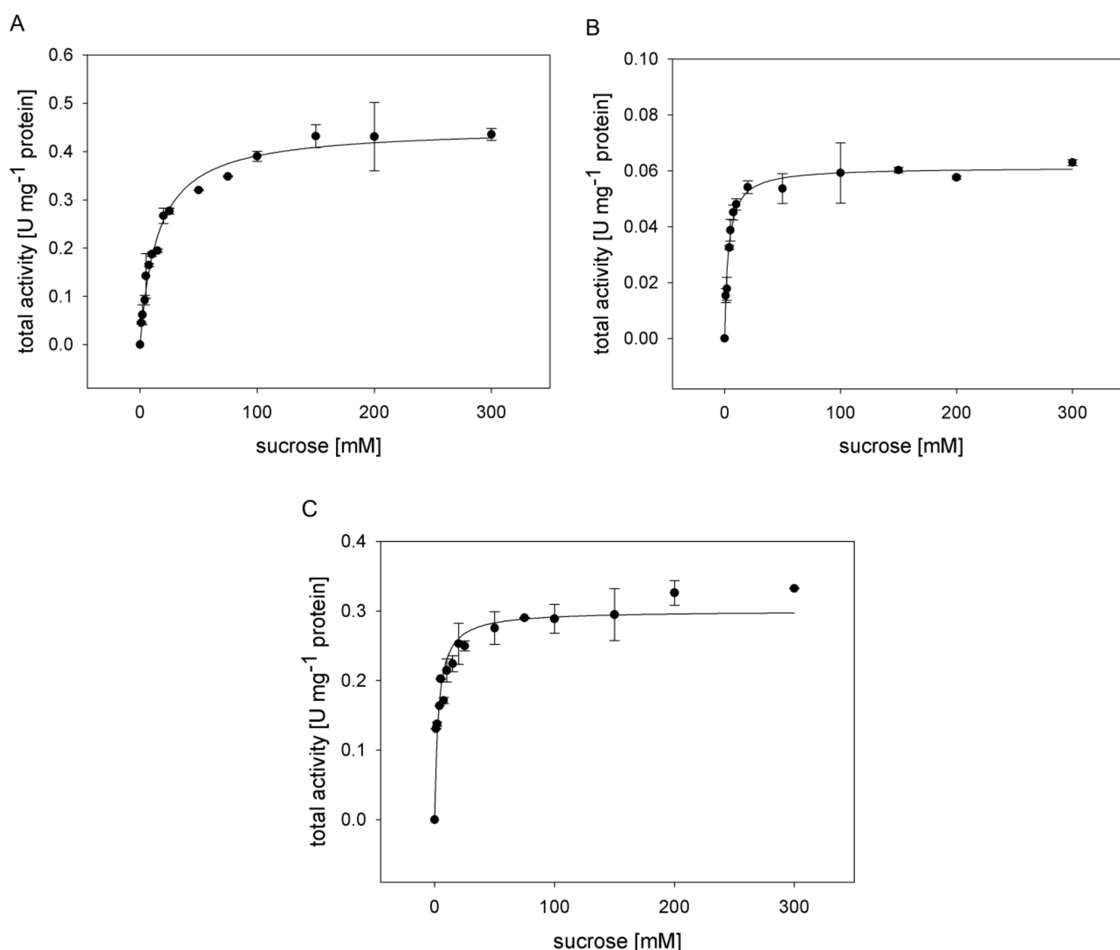


Figure 29: Michaelis-Menten kinetics of Gtfs from *L. reuteri* (A), *L. curvatus* (B) and *L. animalis* (C). Enzyme activities were determined at optimal conditions for each enzyme (*L. reuteri*: 45 °C, pH 4.4; *L. curvatus*: 30 °C, pH 4.4; *L. animalis*: 42 °C, pH 5.8). Data are given as mean ± SD, n=2.

3.10 Analysis of glucans produced through enzymatic reactions

EPSs produced with purified enzymes were analyzed for their structure by AF4, MALLS, ^{13}C-NMR and ^{1}H-NMR and compared to EPSs purified from fermentations. Whereas EPSs of *L. reuteri* and *L. curvatus* Gtfs were completely soluble in water, EPS produced through *L. animalis* Gtf showed a very low tendency to dissolve in water. EPS purified from fermentations with *L. animalis*, however, demonstrated higher but also not full solubility, which indicates a structural change of EPS produced by the enzyme. Nevertheless, the amounts dissolved were just enough for structure analysis.

All glucans formed by enzymes were smaller than the ones from fermentation (compare section 3.2.1). The polydispersity index (Mw/Mn) was slightly higher for *L. reuteri* and *L. curvatus* Gtf formed dextrans, which indicates a higher variability of these dextrans in comparison with dextrans produced during fermentation (Table 22). Glucan from *L. animalis* Gtf had a smaller polydispersity index. The enzyme of *L. animalis* produced

only the small EPS fraction, corroborating the findings from section 3.2.1 that this EPS is a glucan (a_1). Rw was higher for dextran formed through *L. reuteri* Gtf than for dextran purified from fermentations though Mw was smaller. This implies that the dextran from enzymatic reaction had not such a compact conformation. Dextran of *L. curvatus* Gtf demonstrated a smaller Mw and also a smaller Rw.

Table 22: Structural properties of EPSs produced with purified Gtfs.

Enzyme	Mw [MDa]	Mn [MDa]	Mw/Mn	Rw [nm]	branching
L. reuteri	6.53 - 7.02	4.80 - 5.47	1.324	57.7 - 59.7	17% α-(1,4)
L. curvatus	37.50 - 45.67	35.48 - 41.03	1.085	160.0 - 160.2	5% α-(1,3)
L. animalis	0.09 - 0.11	0.08 - 0.09	1.050	n.c.*	n.d.**

*not calculable due to its too small size, **not detected (limit of detection is about 1-2%), Mw: weight average molecular weight, Mn: number average molecular weight, Mw/Mn: polydispersity index, Rw: weight average root mean square

For dextrans from *L. reuteri* and *L. curvatus* Gtf the following chemical shifts (δ) were obtained. *L. reuteri*: ^{1}H-NMR (D_2O, δ [ppm]): 5.36 H-1 *t*-glucose, 5.05 H-1 and α-1,4,6 glucose, 5.02 H-1 α-1,6 glucose, 3.4-4.1 ring protons H-2, H-3, H-4, H-5, H-6a,b. *L. curvatus*: ^{1}H NMR (D_2O, δ [ppm]): 5.36 H-1 *t*-glucose, 5.00 H-1 α-1,6 glucose and α-1,3,6 glucose, 3.5-4.1 ring protons H-2, H-3, H-4, H-5, H-6a,b. Chemical shift of H-1 at 5.00 (*L. curvatus*) and 5.02 ppm (*L. reuteri*) confirms the α-1,6-glucopyranosyl linkages of dextran. Both exopolysaccharides show resonance signals at 5.36, which are referred to terminal glucosyl units from side chains. From integrals, degrees of branching are estimated to be about 5% 1,3- for *L. curvatus*, and about 17% 1,4- for *L. reuteri*, respectively. These branching percentages are slightly lower than estimated for dextrans purified from fermentations. Chemical shifts for EPS from *L. animalis* Gtf revealed that the glucan is an unbranched α-1,6-glucan (dextran): ^{1}H NMR (DMSO-d_6, δ [ppm]): 4.76 H-1, 3.75 H-6a, 3.63 H-5, 3.40-3.50 H-6b, H-3, 3.15-3.22 H-2, H-4; ^{13}C NMR (referred to DMSO at 39.43 ppm): 97.09 C-1, 73.03 C-3, 71.53 C-2; 70.06 C-5, 69.81 C-4, 65.76 C-6. However, branching up to 1-2% cannot be excluded due to the limit of sensitivity of NMR measurements.

As size and structure of the three dextrans produced by enzymes are almost identical to EPSs formed by lactobacilli, results corroborate that respective Gtfs were identified, cloned and investigated.

4 Discussion

The application of EPS forming starter cultures in sourdoughs is a promising approach to improving gluten-free breads, as EPSs are produced *in situ* and can act as hydrocolloids and do not have to be declared. This advantage is combined with the generally positive influence of sourdoughs on breads, like volume, texture, flavor and nutritional value. This work showed that *in situ* EPS formation in gluten-free sourdoughs can be optimized regarding the type of flour, DY, cell count, sucrose amount and sucrose fed-batch to levels sufficient for baking applications. It was further shown that it is necessary to focus on effective EPSs prior to starting the optimization of EPS production in sourdoughs as the specific properties of an EPS are decisive for the effects on bread quality parameters. Besides a high molecular weight, branching types and especially the resulting conformation of the molecule are important for structural influence on gluten-free breads. The dextran of *L. curvatus* TMW 1.624 was the most promising candidate and a structure/function relation is suggested in which high Mw and branching at position 3 of the glucose monomer foster a compact conformation of the molecule, which makes an increased water-binding capacity possible and promotes superior effects in gluten-free breads. Furthermore, optimal conditions for bacterial growth and enzyme activity were identified, which can reduce fermentation time (and cost) and can help to transfer this basic research into industrial applications.

4.1 Comparison of *L. curvatus*, *L. reuteri* and *L. animalis* Gtf

4.1.1 Gene and EPS comparison

Parts of the putative *gtf* gene were isolated from *L. curvatus* TMW 1.624 using degenerated primers. The complete sequence was obtained by primer walking with primers generated from the sequence of *L. sakei* Kg15 (Kralj et al., 2004a). An alignment of the appropriate aa sequence with other glucansucrases revealed clear similarities to Gtfs of LAB (Figure 25). *L. curvatus* Gtf displayed the highest identity and similarity to *L. sakei* Kg15 Gtf. The N-terminal domain of *L. curvatus* Gtf (345 aa) is smaller compared to *L. reuteri* TMW 1.106 (702 aa, Kaditzky, 2008) and *L. animalis* TMW 1.971 (516 aa, Tieking, 2005) Gtf. However, N-terminal region does not influence size and branching as determined for GTFA of *L. reuteri* 121 (Kralj et al., 2004b). The sizes of the catalytic domains are similar for the Gtfs of *L. reuteri* TMW 1.106 (774 aa, Kaditzky, 2008), *L. curvatus* TMW 1.624 (764 aa) and *L. animalis* TMW 1.971 (772 aa, Tieking, 2005) and are within the range of other Gtf enzymes (Kralj et al., 2004a). The conserved amino acids already identified as essential for activity, could be identified in *L. curvatus* Gtf (Figure 25)

(Kralj et al., 2004b, van Hijum et al., 2006, Leemhuis et al., 2012). Recently, Gtf crystal structures with bound maltose revealed that residues interacting with this substrate are from regions II, III and IV but also from other non-conserved regions (Leemhuis et al., 2012). Kralj et al. (2005) identified different aa in GTFA of *L. reuteri* 121 that influenced the main linkage. Introduction of SEV in region IV (Figure 25) converted the enzyme from a mainly reuteran to a mainly dextran synthesizing enzyme. The subsequent introduction of two valins in region II resulted in an even more dextran synthesizing enzyme (Kralj et al., 2005). All sequences of this recombinant mutant enzyme can be found in wildtype *L. curvatus* Gtf, whose EPS is a dextran, and this confirms the findings of Kralj et al. (2005) that these amino acids are determinants for dextran structure. The Gtf of *L. animalis* TMW 1.971 and *L. reuteri* TMW 1.106 does not share the SEV sequence within region IV but contains the two valins in region II. As ^{1}H and ^{13}C NMR analysis of glucan from *L. animalis* Gtf showed that this enzyme also forms a dextran the results corroborate the findings of Kaditzky (2008) who identified these two valins to be the main determinants for α-(1-6) linkage of *L. reuteri* TMW 1.106 glucan. Comparison of the three dextransucrases from *L. reuteri*, *L. curvatus* and *L. animalis* indicates that probably the two valins in region II are more decisive for α-(1-6) linkage than the SEV in region IV. Shimamura et al. (1994) identified the aa residue present at position 567 of GTF-I from *Streptococcus mutans* (equivalent to the 11th residue within region IV of Figure 25) as important for glucan solubility. This was also confirmed by Ito et al. (2011) through the crystal structure of GTF-SI from *Streptococcus mutans*. The presence of an acidic aa resulted in an enzyme synthesizing low levels of soluble glucan (Shimamura et al., 1994). Conversely, the presence of a neutral aa in that position resulted in an enzyme synthesizing primarily soluble glucan. *L. animalis* Gtf harbors an acidic aa at this position, which possibly contributes to the low solubility of the glucan.

In comparison to glucans produced through lactobacilli in this thesis, molecular weights of all three glucans from enzymatic reactions were lower. This is in accordance with the studies of Kaditzky (2008) who also investigated *L. reuteri* TMW 1.106. Recently, Maina (2011) demonstrated that AF4 analyses of dextrans in aqueous solutions had higher Mw than dextrans in DMSO. He proposed that aggregates are formed in water, while values in DMSO originate from individual chains. As these experiments were performed in aqueous solutions, aggregates cannot be excluded. Branching types and percentages of enzymatically formed glucans were the same for *L. curvatus* and *L. reuteri* corroborating the findings of Kralj et al. (2004a) that the N-terminal domain does not affect the EPS structure. This was also recently confirmed through Vujičić-Žagar et al. (2010), who showed with the crystal structure of their Gtf that the N-terminal variable domain is not needed for correct folding of the enzyme. Whereas most of the dextran formed by

L. animalis was soluble in water, dextran synthesized by the enzyme was highly insoluble. A similar observation was made by Funane et al. (2001). In their study recombinant DSRS formed glucan differed from native B-512F dextran as regards the ratio of linkages. They assume that differences in the tertiary structure of recombinant and native enzyme are the reason for this. The difference between the structures of *L. animalis* native and recombinant dextran could not be determined as the strain produces a mixture of a glucan and fructan. No signals for branched units could be assigned in NMR spectra. Thus, within the limit of detection (i.e. < 2% of branching), the glucan from *L. animalis* is considered to be an unbranched α-1,6-glucan.

Nevertheless, the similarity to glucan structures produced in fermentation procedures demonstrates that the investigated *gtf* genes are responsible for EPS production by *L. curvatus* TMW 1.624, *L. reuteri* TMW 1.106 and *L. animalis* TMW 1.971.

4.1.2 Temperature and pH optima

Monchois et al. (1999) and Kralj et al. (2004b) observed that catalytic properties are not influenced if the N-terminal domain is deleted. Similar observations were made with complete and N-terminal shortened Gtf from *L. animalis*, which had nearly identical pH and temperature profiles. Consequently, enzyme assays were performed with enzymes featuring the highest activity.

So far, only two Gtf of *Lactobacillus* strains (*L. reuteri* 121, Kralj et al., 2004b and *L. hilgardii* TMW 1.828, Waldherr et al., 2010) have been biochemically characterized in detail. Most data available are about *Leuconostoc* (*Ln.*) and *Streptococcus* Gtfs. From *L. reuteri* TMW 1.106 Gtf only the pH optimum was determined (Kaditzky, 2008). Therefore, this dissertation is believed to provide the first comparative characterization of three recombinant Gtfs from different *Lactobacillus* species.

The Gtf pH optima, as well as the activity ranges, were comparable to that of other glucansucrases (pH optima: 4.7-7.4, pH activity range: 3.0-8.0, Kralj et al., 2004b and Brenda-Enzymes.org). Maximal activity of *L. reuteri* Gtf was observed at pH 4.4-4.8 and is within the range found by Kralj et al. (2005) and Kaditzky (2008) for *L. reuteri* Gtf.

The optimal temperature of *L. reuteri* 121 Gtf is described as high (50 °C) and this has also been observed for the inulosucrase and levansucrase enzymes of the same strain (Kralj et al., 2004b). These results are in accordance with *L. reuteri* TMW 1.106 Gtf that has an optimum at 45 °C. Temperature optimum of *L. curvatus* Gtf was close to optimal growth temperature (31 °C). Similar optima were found for dextransucrases from *Ln. mesenteroides* (Kim and Day, 2008) and *W. cibaria* (Kang et al., 2009). *L. animalis* Gtf revealed high activity over a broad temperature profile, which has not yet been reported

for other glucansucrases. More than 50% of activity maximum was detected between 22 °C and 59 °C. Noteworthy is that this Gtf was active at temperatures much higher than other previously described enzymes of this class and produced a low-MW glucan. Moreover this enzyme revealed different temperature maxima for hydrolysis and transfer reaction and is, to date, the first demonstration of different temperature profiles for these Gtf-catalysed reactions. So far only different pH profiles have been demonstrated for the two reactions (Kralj et al., 2004b).

4.1.3 Effects of ions

Of all cations tested, Ca^{2+} had the most stimulating effect on all three enzymes (Table 16). The activation of dextransucrases by 1 mM Ca^{2+} has already been reported by many sources, e.g. Miller and Robyt (1986a). EDTA and Cu^{2+} ions significantly inhibited enzyme activity, which has also been reported by Robyt and Walseth (1979) and Kralj et al. (2004b) for *Ln. mesenteroides* and *L. reuteri* Gtf. In previous documentation, Zn^{2+} and Al^{3+} are stated to be an inhibitory compound for *Ln. mesenteroides* Gtf (Miller and Robyt, 1986b; Yalin et al., 2008), but in this study Zn^{2+} and Al^{3+} just activated slightly or did not significantly influence overall activity. Only hydrolysis activity of *L. curvatus* Gtf was significantly inhibited by Zn^{2+}. As reported by Kralj et al. (2004b) for Mg^{2+}, K^{+} and Na^{+} ions and by Yalin et al. (2008) for Mn^{2+}, as well in this study, these ions demonstrated stimulating effects. On the other hand, Mn^{2+} was described as inhibiting Gtf activity by several other sources e.g. Kobayashi and Matsuda (1976) and Robyt and Walseth (1979). In this work Mn^{2+} strongly inhibited hydrolysis activity of *L. reuteri* and *L. curvatus* Gtf but stimulated transferase activity. Since transferase activity constitutes more than 90% of the overall activity of both enzymes, total activity was increased. Ul-Qader et al. (2001) reported that the presence of manganese, magnesium and calcium salts in the medium increased enzyme activity as well as dextran yield, which highlights the importance of medium composition.

4.1.4 Influence of different EPSs

Sucrose hydrolysis of *L. animalis* Gtf was reduced by its own EPS. This stimulation of the transfer reaction has already been described by Mooser et al. (1985) where sucrose hydrolysis rates were reduced using 0.5 mg/mL of dextran. The ratio of hydrolysis to transfer reaction was hardly or not influenced for *L. curvatus* and *L. reuteri* Gtf.

EPSs were found to strongly activate Gtfs of *L. reuteri*, *L. animalis* and *L. curvatus*. Except for Ca^{2+} overall activity was more stimulated by EPSs than by ions. Gtfs were activated by different EPSs and not only by their own particular one (Table 17), corroborating the

findings of Kobayashi and Matsuda (1980) who investigated the influence of different dextrans on *Ln. mesenteroides* Gtf activity. In their study different dextrans stimulated the activity to similar extents. Also other studies demonstrated that Gtfs had much higher catalytic rates upon the addition of acceptor substrates (Leemhuis et al., 2012). The effect of soluble dextran on dextransucrase activities has been widely discussed (e.g. Germaine and Schachtele, 1976; Montville et al., 1977; Robyt and Corrigan, 1977). Robyt and Corrigan (1977) and Robyt et al. (1995 and 2008) proposed an allosteric activation of dextransucrase by exogenous dextran. In this study *L. reuteri* and *L. curvatus* Gtf were also activated through levan, indicating that not only glucans or dextrans are able to stimulate the Gtfs. *L. curvatus* Gtf especially was strongly activated through levan. In contrast, *L. animalis* Gtf was negatively affected. Rozen et al. (2004) found *Streptococcus mutans* Ftf to have a high affinity for glucans. An affinity of Gtf for fructans has not yet been reported. Schwab et al. (2007) and Schroeder et al. (1989) reported that Ftf activity also involved the regulation of the expression of Gtf. Since in fermentations with *L. animalis* a fructan was found, this strain should harbor an Ftf which may possibly also be involved in Gtf regulation through fructan formation or in Gtf expression. However, this assumption still needs to be checked. As up to now no stimulating effects of fructans on Gtfs have been investigated, no overall conclusions can be drawn on these results. Nevertheless, results show that *L. reuteri* and *L. curvatus* may be cultivated simultaneously with levan-forming lactobacilli without negative impact on dextran yields. Rather these strains could be beneficial because of Gtf activation through levan formation and thus enhancing dextran yields of *L. reuteri* and *L. curvatus*.

4.1.5 Effects of dough and artificial dough extracts

Dough and artificial dough extracts of the most important flour minerals were generated and investigated for their influence on Gtf activities. A correlation of high Gtf activity in dough extracts and high EPS yields in sourdough fermentations could only be found for *L. curvatus*. This strain demonstrated highest EPS yields in both buckwheat flours, where also the highest activity for *L. curvatus* Gtf was found. This connection was not visible for *L. reuteri* and *L. animalis*. Interestingly, nearly all extracts negatively influenced *L. reuteri* Gtf, whereas *L. curvatus* and *L. animalis* Gtfs were stimulated by all extracts. Artificial dough extract results differed from original dough extracts. This is most likely due to the lack of influence from other flour components on enzyme activity. Also, the results of different DY differ from fermentation results. Higher DY stimulated overall Gtf activity, whereas EPS yield in sourdough fermentation decreased with increasing DY. These results demonstrate that though Gtfs are extracellular (free or cell bound) enzymes,

results cannot be easily transferred. Also growth of the strain and enzyme expression has to be considered, which play an important role in high Gtf activity and EPS yields (section 3.1 in this thesis and Årsköld et al., 2007).

4.1.6 Influence of substrate concentration

The effect of initial sucrose concentration on the distribution between hydrolysis and polymerization reactions was studied. Kralj et al. (2004b) showed that deletion of the N-terminal domain had a drastic effect on enzyme kinetics regarding the initial transferase activity, which was strongly increased. Also, the site of the his-tag influenced kinetics, whereas the affinity for sucrose remained similar. Kralj et al. (2004b) explain this phenomenon with a steric hindrance caused by the large N-terminal domain. As in this dissertation different deletion mutants were investigated, the initial ratios of hydrolysis to transfer reaction for different sucrose concentrations should, however, be considered with caution. A vector with a C-terminal his-tag was used and this should have had less impact on activity than an N-terminal his-tag which is even closer to the catalytic region, when the N-terminal domain of the enzyme is deleted.

Gtf of *L. animalis* was found to favor hydrolysis at low sucrose concentrations and polymerization at high concentrations as the relation of transferase activity to overall activity increased with increasing sucrose concentration. This has also been observed for the amylosucrase from *Neisseria polysaccharea* (De Montalk et al., 2000) and for the reuteransucrase from *L. reuteri* 121 (Kralj et al., 2004b). For *L. reuteri* and *L. curvatus* Gtfs this phenomenon was the opposite. Both enzymes favored polymerization at low sucrose concentrations. As only a few attempts have been made to investigate the kinetics of Gtfs that distinguish between hydrolysis and transfer reaction, no literature could be found that describes this behavior.

The affinities of *L. curvatus* and *L. animalis* Gtfs for sucrose in the overall reaction were higher than that observed for *L. reuteri* Gtf (section 3.9.4). When compared with literary articles, the affinities of the three analyzed dextransucrases lie in between the affinities observed for GTFA of *L. reuteri* 121 (0.9 mM, Kralj et al., 2004b) and DSRS of *Ln. mesenteroides* NRRL B512F (26 mM, Monchois et al., 1997). V_{max} values are not comparable to literature data as kinetic studies were the last enzymatic investigations and the enzymes had already lost part of their activity during storage.

4.2 EPS formation in laboratory medium

Observation of EPS yields during fermentation clearly showed that EPS production strongly correlated to growth for *L. curvatus* TMW 1.624, *L. animalis* TMW 1.971,

L. sanfranciscensis TMW 1.392 and *L. reuteri* TMW 1.106 and that EPSs are produced during the exponential phase. This is in agreement with Santos et al. (2000) and Ul-Qader et al. (2001) who investigated *Ln. mesenteroides*. For the four strains, only *gtf* and *ftf* expression of *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392 were investigated in earlier studies and were not regulated by sucrose (Tieking 2005, Schwab et al., 2007). As the expression of the enzymes is not influenced by sucrose, the Gtfs are only formed during growth of the producing strain. Thus enzyme and EPS production stopped as soon as cells reached the stationary phase. This is corroborated by Årsköld et al. (2007), who found that expression of *L. reuteri gtf* increased with growth during the exponential phase and reached an optimum at the end of the exponential phase. This gene was also constitutively expressed and not induced by sucrose.

In Homohiochii fermentations, *L. animalis* turned out to be a fast growing and fast acidifying strain producing comparatively low EPS amounts of up to 3 g L^{-1} (Figure 7). The Homohiochii medium provides an initial pH around pH 5.4. *L. animalis* Gtf demonstrated a high pH optimum of 5.8 (Figure 27). Therefore, growth and EPS production began below optimal conditions for the enzyme. As soon as the cells were in the middle of the exponential phase pH had dropped below pH 4.8. As reported by Årsköld et al. (2007) through expression and as demonstrated in this study through EPS yields, most EPS is produced in the exponential phase. From these observations it can therefore be concluded that media with higher initial pH and buffer capacities are much more suitable for EPS production in uncontrolled batch fermentations with this strain. For *L. reuteri* and *L. curvatus* Homohiochii medium is well suited as optimal pH values of enzymes are reached in the late exponential growth phase of these strains.

Further experiments were conducted with *L. curvatus* in order to investigate the influence of different sucrose concentrations on EPS yield and structure. Most EPS was produced with 50 g L^{-1} sucrose and yield decreased with higher sucrose amounts (80 g L^{-1}). With 20 g L^{-1} sucrose, the EPS formation was relatively low because the sucrose had been consumed within the 48 h. Santos et al. (2000), Ul-Qader et al. (2001) and Årsköld et al. (2007) observed that higher concentrations of sucrose lead to higher EPS yields at the end of fermentation, which match the yields obtained within this dissertation except for the reduction in yield at high sucrose concentration. Sarwat et al. (2008) found that dextran yield decreased with higher sucrose concentrations and assumed a substrate inhibition as there was a decrease in sucrose conversion, which affected the yield. In their study as well less sucrose was converted, but a substrate inhibition of the enzyme is unlikely because *L. curvatus* Gtf follows a Michaelis-Menten type kinetic. Nevertheless, 80 g L^{-1} are within the edge region of kinetic measurements done and several studies have shown

that dextransucrases can still be inhibited at excess sucrose concentrations (more than 100 g L^{-1}) (Martinez-Espindola and Lopez-Munguia, 1985, Chellapandian et al., 1998). Thus substrate inhibition cannot be fully ruled out, although it is more likely that growth (not determined in this case) and therefore EPS yields were affected by different sucrose amounts as EPS production strongly correlates to growth (compare section 3.1).

With increasing sucrose concentration, the size of EPSs produced by *L. curvatus* was higher but branching was not influenced. This contradicts the findings of Tsuchiya et al. (1953), Bovey (1959) and Kim et al. (2003) who showed that increasing concentrations of sucrose gave a decreasing amount of high-molecular weight dextran and an increasing degree of branching. In contrast to this study, where the influence on EPS structure was investigated with the strain, they used the pure enzyme. It is known that many other factors (e.g. growth medium and temperature) influence the molecular mass, structure and physical characteristics of the polymer as well and this may explain the contradictory results. Falconer et al. (2011), who also used the pure enzyme, explain their contradictory findings due to Michaelis-Menten kinetics in which the rate of the reaction is proportional to the concentration of the substrate, giving longer and higher molecular weight chains. *L. curvatus* Gtf was also found to follow Michaelis-Menten kinetic, which may additionally explain the higher Mw with increasing sucrose amounts.

4.3 Influence of formulation parameters on *in situ* EPS production

Kaditzky (2008) used the Ftf deletion mutant of *L. sanfranciscensis* TMW 1.392 as sourdough control but she concluded that this mutant is not suitable for direct comparison as different metabolite patterns were observed. Same problems were also demonstrated in this dissertation. Different *L. reuteri*, *L. sanfranciscensis* and *L. curvatus* EPS negative strains were screened for their suitability as control organisms for sourdough fermentations. Though suitable EPS negative strains of *L. curvatus* could be identified in medium fermentations, metabolite patterns (especially sugars) strongly differed from EPS producer *L. curvatus* when grown in buckwheat flour with sucrose. Consequently, for EPS quantification in sourdoughs chemically acidified doughs were preferred as control doughs. For baking experiments these control doughs have to be considered with caution as metabolites directly affect baking results (Barber et al., 1992; Kaditzky, 2008). Other possible variants of controls, beside the ones mentioned above, could be the fermentation with the EPS producing strain without the addition of sucrose or the application of an EPS hydrolyzing enzyme. However, metabolite patterns will also differ from the sample dough and may affect baking performance. Thus finding the optimal control dough is a difficult task and different controls should be examined in order to find the most suitable one.

4.3.1 Impact of different flours and sucrose concentrations

L. animalis TMW 1.971, *L. reuteri* TMW 1.106 and *L. curvatus* TMW 1.624 were all able to grow and to produce EPS in the analyzed flours. They adapted very quickly to the new conditions and were already growing 6 h after inoculation of the flours. *L. sanfranciscensis* TMW 1.392 had some problems in adapting to gluten-free flours and did not grow in rice and quinoa. Cell counts after 6 h of fermentation in buckwheat and buckwheat core are missing because cells died instead of growing (Figure 17). All four gluten-free flours lacked maltose, which is the preferred carbohydrate source for *L. sanfranciscensis*, this being in consequence the most likely cause for the poor growth. This has also already been observed by Galle et al. (2010), who came to the same conclusion. Moreover, *L. sanfranciscensis* is considered as the most adapted species in wheat and rye sourdoughs (Vogel et al., 2011). Genomic analysis revealed several features which may contribute to the competitiveness in these doughs (Vogel et al., 2011). Consequently, the question arises whether *L. sanfranciscensis* is probably too highly specialized on that environment and is hence scarcely able to adapt to gluten-free cereals.

Each strain demonstrated different growth and EPS formation patterns. *L. animalis* TMW 1.971 grew very fast in all sourdoughs and produced high amounts of lactate. Highest final pH and lactate concentrations were found in buckwheat core sourdoughs, an indication of an increased buffer capacity of this flour. As *L. animalis* TMW 1.971 is a strong acidifier and as its Gtf has a comparatively high pH optimum, the buffer capacity could have positively contributed to higher EPS amounts. Also quinoa showed a good buffer capacity in all fermentations. *L. reuteri* TMW 1.106 grew best and produced most metabolites, including EPS, in quinoa sourdoughs. But a good buffer capacity did not necessarily result in high EPS production. EPS amounts generated by *L. reuteri* TMW 1.106 in buckwheat core and by *L. curvatus* TMW 1.624 in quinoa sourdoughs were low, while high amounts of acids were formed. *L. curvatus* TMW 1.624 produced most EPS in both buckwheat flours, suggesting that any ingredient advanced EPS production. A suggested correlation between best growth and highest EPS yields could only be found in fermentations with *L. reuteri* TMW 1.106. This strain grew best in quinoa and rice sourdoughs, where also most EPS was detected. *L. curvatus* TMW 1.624 exhibited a different behavior. Best growth was found in buckwheat core and quinoa, but most EPS was formed in buckwheat sourdoughs, corroborating the fact that any ingredient of buckwheat led to high EPS amounts in fermentations with this strain. Polyphenols are described to protect cell constituents against oxidative damage and to decrease the risk of associated diseases (Alvarez-Jubete et al., 2010). Several extracts of these secondary plant metabolites inhibited strains of *L. hilgardii*, *L. casei*, *L. plantarum*, *Pediococcus pentosaceus* and

Oenococcus oeni to different extents (García-Ruiz et al., 2012). But also growth promoting effects of some polyphenolic extracts on LAB were demonstrated (e.g. *L. acidophilus,* China et al., 2012). Svensson et al. (2010) showed that some LAB can metabolize these compounds and reduce the antimicrobial activity of the flour. Compared to quinoa (0,782 mg g^{-1}) and rice (0,142 mg g^{-1}) the total polyphenol content in buckwheat is high (4,655 mg g^{-1}) (Hager et al., 2012). In this dissertation growth of *L. animalis* was affected in both buckwheat flours. Growth of *L. curvatus* was only impaired in buckwheat flour and growth of *L. reuteri* was only slightly affected. As different studies already evidenced that polyphenols can inhibit LAB it is conceivable that these substances could have affected the growth of the three EPS forming LAB and hence affected EPS formation. Other studies demonstrated that Gtf activities were influenced by various acceptor molecules (reviewed by Leemhuis et al., 2012). Thus, different acceptor substances originating and depending from flour type likely also affected EPS yields in these studies.

The obligately heterofermentative strains *L. reuteri* TMW 1.106 and *L. sanfranciscensis* TMW 1.392 showed a strong mannitol dehydrogenase activity in all flours, since no fructose, but high amounts of mannitol and acetate, were found. The production of acetate is beneficial due to its antifungal properties, but high concentrations may negatively affect taste, dough rheology, bread volume and crumb hardness (Kaditzky et al., 2008). Also a correct ratio between lactic and acetic acid (FQ around four) is important for bread quality and sensory (Barber et al., 1991). In these experiments highest FQ were found in rice and quinoa sourdoughs for *L. reuteri*, which highlights these flours, as also most EPS was produced by this strain in these doughs.

With increasing sucrose concentration EPS amounts produced by the three strains increased. Lactate and acetate may counterbalance the positive effects of EPSs and have to be considered (Kaditzky et al., 2008). Different sucrose amounts only influenced acid production by *L. reuteri* TMW 1.106. The FQ strongly decreased with increasing sucrose concentrations. Due to the formation of metabolites and because of residual sucrose found in sourdoughs, sucrose concentration should not exceed 7.5%. This was also corroborated by the EPS amounts produced, because with higher sucrose concentrations EPS production followed a saturation curve. *L. reuteri* TMW 1.106 was found to produce highest EPS yields in the flour in which the strain grew best. Section 3.1 showed that EPS production strongly correlated to growth in Homohiochii medium. As growth of *L. reuteri* was impaired by high sucrose amounts, EPS yields were probably affected, too, and this may explain the saturation of EPS yields produced with higher sucrose concentrations. Growth of *L. animalis* TMW 1.971 and *L. curvatus* TMW 1.624 were not influenced by sucrose, except at the 6 h time point, suggesting a saturation of EPS forming Gtfs through high sucrose amounts. *L. reuteri*, *L. curvatus* and *L. animalis* Gtfs were found to follow a

Michaelis-Menten kinetic with saturation at high sucrose concentrations. The concentrations of sucrose in sourdoughs correspond to 73 mM (3.75%), 146 mM (7.5%), 219 mM (11.25%) and 293 mM (15%) and are definitely within the saturation-dynamic of the three Gtfs. Actually, theoretically, EPS yields should have increased with prolongation of fermentation time, but it is likely that due to low pH and consequent activity loss, EPS amounts remained constant. After 24 h of fermentation pH values of sourdoughs with *L. reuteri* and *L. animalis* were below 4 and sourdoughs with *L. curvatus* below 4.2. All these pH values are below the pH optima for the three Gtfs (compare Table 14 and Figure 27).

4.3.2 Effect of cell counts, dough yield and sucrose fed-batch

Inoculation cell counts were found to be relevant for high EPS amounts. With increasing cell counts at the beginning of fermentation, EPS production was enhanced. The higher the inoculated cell counts, the faster the pH decreased. For these doughs the optimal *L. curvatus* Gtf pH was reached earlier and thus could have positively contributed to high EPS amounts. The release of glucose and fructose through the hydrolase and transferase activity of the glucansucrase was influenced by the cell counts, too. The more cell counts used, the less glucose and the more fructose were released, indicating an increased EPS formation. Probably, if cell counts are low in the dough, sucrose is used for biomass generation, while there is no need for high cell counts to generate biomass, and sucrose is used for EPS production.

Lactate formation was hardly influenced by different dough yields. Doughs with a DY ≥ 250 showed no influence on lactate formation. A lower lactate content was only measured in doughs with DY 200. This is in accordance with the description of Decock and Capelle (2005) and was observed in the studies of Kaditzky and Vogel (2008), too. Different dough yields were found to affect EPS yields. Increasing dough yields led to lower EPS amounts. In contrast to the study of Kaditzky and Vogel (2008) using *L. reuteri* TMW 1.106, EPS yields decreased with increasing DY for *L. curvatus* TMW 1.624 and yields could not be further increased by prolonging fermentation with *L. curvatus* TMW 1.624. Both strains and their respective Gtf already indicated different behavior in the analyzed flours so it is likely that the DY caused different effects. Kaditzky and Vogel (2008) explain lower EPS yields in firmer doughs with a diminished water activity and therefore a limited diffusion of sucrose and enzyme. EPS is often thought to have a protective function (Cerning, 1990; Badel et al., 2011). Thus, it is possible that the diminished DY is a stress condition for *L. curvatus* TMW 1.624 leading to more EPS. The sucrose concentration and flour ingredients and therefore the osmotic stress is increased due to low DY. Probably EPS is

formed in order to reduce the osmotic stress as already suggested by Korakli and Vogel (2006). This is corroborated by the experiments with different flours. *L. reuteri* TMW 1.106 produced most EPS in flours where also most metabolites were formed (section 3.6.1). In contrast, *L. curvatus* TMW 1.624 produced most EPS in the flour where nearly the least metabolites were generated, indicating that buckwheat is not optimal for growth and metabolic activity and therefore could cause a stress reaction. Furthermore, *L. curvatus* Gtf was found to be stimulated through higher DY, indicating that decreasing EPS yields are probably not caused by an enzyme inhibition. The difference in EPS yields between higher DY became less. The probable reason here is the reduced osmotic stress, whose effect is greater at smaller DY (low water activity) than at higher ones (high water activity).

In contrast to Kaditzky and Vogel (2008) who used wheat doughs, sucrose fed-batch in gluten-free doughs did not enhance EPS production. Rather the fed-batch influenced EPS yield negatively. Released glucose and fructose were higher when compared to doughs with directly added sucrose. Kralj et al. (2004b) found, that the Gtf of *L. reuteri* 121 favors hydrolysis at low and polymerization at high sucrose concentrations. Similar results were reported for a Gtf of *L. sanfranciscensis* (Korakli et al., 2003). Korakli and Vogel (2006) explain this increased polymerization activity behavior of the enzymes at high sucrose concentrations as beneficial, reasoning that sucrose consumption reduces the osmotic stress of organisms. Dols et al. (1997) observed decreased dextran yields for *Ln. mesenteroides* NRRL B-1299 if residual sucrose concentration was low in the medium. Since the sucrose was added in portions in the experiment with *L. curvatus*, the concentration was always low and could have favored hydrolysis. On examining the relative hydrolase activity in doughs with different sucrose concentrations, this observation can be confirmed for *L. reuteri* TMW 1.106 and *L. curvatus* TMW 1.624. In the case of *L. animalis* TMW 1.971 hydrolysis increased with rising sucrose levels. This could be caused by an Ftf, which released glucose while fructan was formed. Cultivation of *L. animalis* in Homohiochii medium demonstrated that this strain also produced a fructan, although in sourdough fermentations no fructan was found. Fructans are very susceptible to acid hydrolysis during heating and released fructose reacts fast to hydroxymethyl furfural (results not shown). In order to measure glucan yields, conditions for hydrolysis are stronger than for fructans and thus released fructose probably reacted further to hydroxymethyl furfural and was not determined by HPLC. Considering the ratio of hydrolysis to transfer activity of Gtf enzymes, all enzymes demonstrated the opposite behavior. However, as already mentioned in section 4.1.6, enzyme kinetic measurements have to be considered with caution, as it has been demonstrated that deletion of the N-terminal domain of the enzyme has a drastic effect on the initial ratio of hydrolysis and

transferase activity (Kralj et al., 2004b). Thus, examined relative hydrolase activities in sourdoughs seem to be more trustworthy than enzymatic measurements.

4.4 Influence of EPS on dough, fresh and stored gluten-free bread quality

4.4.1 Influences of EPS on fresh and stored bread quality

An increase in bread volume was achieved in this study upon addition of EPS and HPMC at 1 % flour base. An explanation for this was given by Rosell et al. (2001). In their opinion, hydrocolloids improve dough development and gas retention by increasing the batter viscosity. In these studies HPMC was used as hydrocolloid control, which has additional properties, such as the formation of gel network structures while heating, for the bread making process. This structure increases the viscosity, which leads to better gas retention and better loaf volume. The analyzed EPSs are not able to form a network when heated (results not shown) and therefore other structural effects must be responsible for the increased volume. Breads supplemented with HPMC and the dextran of *L. curvatus* TMW 1.624 showed similar volumes and the doughs showed similar behavior regarding water retention (Table 8 and Figure 12). The other EPSs did not retain water but led to a higher specific volume.

Baking loss was most influenced by HPMC and dextran of *L. curvatus* TMW 1.624. This is interesting, since only these additives were able to retain water in dough after centrifugation (Figure 12) and it may explain their water retention during baking. Water retention of hydrocolloids most likely depends on their chemical structure and their interactions with the ingredients. However, to date, no clear structure function relations have yet been defined and respective data are unavailable.

Differences in staling are significant for all EPSs, and clear tendencies are visible. Breads with dextran of *L. curvatus* TMW 1.624 underwent staling more slowly than the other breads. Moisture content and its transfer among bread components is believed to be a significant factor contributing to bread staling (Gray and BeMiller, 2003). Several studies, which are listed by Bárcenas et al. (2004), have shown that hydrocolloids improve shelf life by maintaining the moisture content and retarding the staling. Since breads supplemented with dextran of *L. curvatus* TMW 1.624 had the highest moisture content over the experimentation period, their crumb underwent staling more slowly. Rosell et al. (2001) reported an increase of water activity and moisture retention of the crumb due to the higher water holding capacity of the hydrocolloids. Solely dextran of *L. curvatus* TMW 1.624 evidenced water holding capacity beside HPMC, explaining the increased water retention.

In the studies of Mezaize et al. (2009) and Sciarini et al. (2010), softer crumbs were related to higher specific volumes. In these studies, however, specific volume and crumb hardness only correlate, if breads with dextran of *L. curvatus* TMW 1.624 are not considered. Softer crumbing of breads with dextran of *L. curvatus* TMW 1.624 has to be related to a good water retention ability of this EPS.

4.4.2 Structure and function of EPS in gluten-free bread making

Bacterial dextrans have been widely examined in baking applications with wheat and other gluten containing flour, and patents claim the enhancement of such doughs and breads. Studies of Ross et al. (1992) showed the ability of dextrans to influence strength as well as the water-absorption capacity of doughs. Experiments indicate that the effects of levans appeared to be similar (Waldherr et al., 2009). Schwab et al. (2008) analyzed levan and dextran formation in sorghum sourdoughs, but the EPS production had no significant influence on bread volume. Kaditzky et al. (2008) demonstrated that added levan positively influenced water absorption, bread volume and firming of the crumb. However, dextran was reported to be more efficient in influencing the loaf volume of wheat breads than fructans (Tieking and Gänzle, 2005). Results of this thesis demonstrate that such findings cannot be generalized as the size and structure of an EPS, rather than its monomer composition, appear to be decisive for the effects observed in dough and bread. Also, experiments using wheat cannot be transferred to gluten-free bread making. Lacaze et al. (2007) and the patent EP0790003B1 (Vandamme et al., 1997) showed that dextran with a high molecular weight and a linear chain structure is more efficient regarding bread volume than dextran with a high molecular weight and more branching. Specific volumes of breads with dextran of *L. curvatus* were smallest despite the dextrans' highest molecular weight and low branching. Furthermore, specific volumes using dextran of *L. reuteri* were less than with levan of *L. sanfranciscensis*. These findings indicate that the effects of fructan and glucan strongly depend on EPS structure and dough conditions.

The effects of EPSs of *L. animalis* TMW 1.971, *L. sanfranciscensis* TMW 1.392 and *L. reuteri* TMW 1.106 were less distinctive, except for their influence on specific volume, than the effects evoked by dextran of *L. curvatus* TMW 1.624, which can be ascribed to different Mw and conformation. All EPSs, except for dextran of *L. curvatus* revealed Mw below 100 MDa or even less (~100 kDA for biggest fraction of *L. animalis*). Furthermore, AF4 showed that dextran of *L. curvatus* TMW 1.624 has a different, more compact conformation and that its size distribution was narrower than for the other EPSs, resulting in more pronounced impacts. This ramified structure of dextrans has also already been demonstrated by Maina (2011). Just as the dextran of *L. curvatus* the few branched

dextran of *W. confusa* was not linear. Baking loss and moisture content were most influenced by dextran of *L. curvatus* TMW 1.624, and this EPS revealed different behavior to dextran of *L. reuteri* TMW 1.106 in bread volume, water retention and crumb hardness. Volume was less, crumb hardness was more strongly decreased and dextran showed an ability to retain water compared to *L. reuteri* TMW 1.106. Dextran of *L. curvatus* TMW 1.624 was found to be less and differently branched and to have a higher molecular weight than the dextran of *L. reuteri* TMW 1.106, probably causing the resulting improved freshness of the bread through increased water retention. Dextran of *Ln. mesenteroides* LMGP-16878 was identified as being the decisive factor for keeping panettone fresh over months. It consists of long linear chains with a high molecular mass (up to 200 MDa) and has less than 5% α-(1,3) branching (Decock and Cappelle, 2005). Dextran of *L. curvatus* TMW 1.624 has a very similar structure (Mw: 118 MDa– 242 MDa, branching: α-1,3 (8-9%)), but the branching is more distinct. However, the similarity to dextran of *Ln. mesenteroides* corroborates and explains the findings that breads containing dextran of *L. curvatus* TMW 1.624 stay fresh longer during storage. These findings have been further corroborated by rheometer measurements with EPS in watery suspensions (Grepka, 2003). Compared to *L. reuteri* TMW 1.106 dextran and *L. sanfranciscensis* TMW 1.392 levan, *L. curvatus* TMW 1.624 dextran had the highest viscosity and was the only one demonstrating viscoelastic behavior (Grepka, 2003). Also, dextran of *W. cibaria* MG1 with a Mw of 720 MDa showed the best shelf life improvements (Galle et al., 2012), indicating that longer chain lengths are especially effective. Unfortunately, no further information about conformation and degree of branching for this dextran is available.

Additional water retention experiments with EPS from *L. curvatus* with different Mw demonstrated that not just the size of the EPS is important for increased water retention (compare Figure 10 and Figure 13). As same amounts of EPS with a similar variability and branching were used there has to be a connection between conformation and water retention ability. Thus, it is suggested that beside a high molecular weight and type of branching, the resulting conformation of the molecule is especially important for structural influence on gluten-free doughs and breads. Furthermore, short and long chain branching may exert additional, but as yet undefined, effects.

Since two dextrans can display such differences, it can be concluded that only linkage type analysis alone is not sufficient when choosing the right EPS for bread making. More information on molecular weight and degree of branching and conformation is necessary. As the behavior of a hydrocolloid in a food system depends on several factors, hydrocolloids are tested in the application in order to see whether the desired results are achievable (Hoefler 2004). These experiments clarify the importance of analyzing the EPS

in the respective dough system and bread making prior to starting the optimization EPS production in sourdoughs. This ensures that the effective EPS is focused on before any effort is put into *in situ* production, as this is frequently accompanied by many metabolic side effects and requires quite some effort before application level is achieved.

4.5 Perspective of EPS forming cultures in gluten-free baking

Verified through RAPD, *L. animalis* TMW 1.971, *L. reuteri* TMW 1.106 and *L. curvatus* TMW 1.624 demonstrated an ability to assert in gluten-free sourdoughs. Vogelmann et al. (2009) investigated the adaptability of lactobacilli in gluten-free sourdoughs and showed that only a few strains were competitive. Moroni et al. (2011) discovered that an autochthonous strain of *L. sakei* was stable in buckwheat sourdoughs. This strain is closely related to *L. curvatus* and hence evidences its competitiveness. Cell counts and pH values reached in sourdoughs containing *L. reuteri*, *L. curvatus* and *L. animalis* were similar to data for wheat, rye, sorghum and quinoa sourdoughs (Di Cagno et al., 2006; Schwab et al., 2008). This highlights the potential of all three strains as gluten-free sourdough starter cultures. Especially *L. curvatus* seems to be a promising strain for gluten-free bread improvements as its dextran affected gluten-bread quality the most.

Kaditzky and Vogel (2008) fixed 24 h as the optimum fermentation time for *in situ* EPS production toward baking applications. This time period was confirmed in this dissertation, since EPS formation took place within the first 24 h. EPS formation is always associated with metabolite formation. Thus, EPS optimization has a limitation. An upper limit of 7.5% sucrose was found for the three investigated strains in order to avoid high residual sucrose concentrations and to avoid too much acidification. Following this previously mentioned restriction, 17.93 g EPS kg^{-1} flour could be produced with *L. animalis* TMW 1.971 in buckwheat core doughs (DY 250), 15.69 g EPS kg^{-1} with *L. reuteri* TMW 1.106 in quinoa (DY 250) and 16.28 g EPS kg^{-1} flour with *L. curvatus* TMW 1.624 in buckwheat sourdoughs (DY 200). Korakli et al. (2001) obtained 2.6 – 3.1 g EPS kg^{-1} and Tieking et al. (2003) 0.3 – 2 g EPS kg^{-1} flour in their sourdough fermentations. In unoptimized sourdoughs comparable amounts (2.5 – 5 g EPS kg^{-1}) were reached in this thesis. But especially through selecting the most suitable flour, the EPS amounts could be manifold. Converting the values to final EPS concentration in bread doughs considering TTA, EPS amounts ranging from ~3 to ~7 g kg^{-1} flour can be achieved. Hydrocolloids are effective as dough improvers and are typically applied at a 1% level, but smaller amounts were also revealed to be effective (reviewed by Gallagher et al., 2004; Lazaridou et al., 2007). In section 3.3 it was demonstrated that EPSs of these three strains are effective at 1% in gluten-free bread making. Using optimized sourdoughs, 0.3-0.7% EPS can be inserted

into bread dough. These amounts are relevant in gluten-free bread making and show the potential of EPS forming lactobacilli to substitute hydrocolloids.

During sourdough fermentations with *L. reuteri*, *L. animalis* and *L. curvatus* high amounts of glucose, fructose and mannitol were formed from added sucrose. Fructose and mannitol were found to lead to cakes of poor characteristic quality (Psimouli and Oreopoulou, 2011). The effects of high sugar amounts on gluten-free baked goods have not yet been investigated; however, they affect taste, due to their sweetening attribute and may affect color and texture of the finished product. Fructose is a strong sweetener and provides 1.2-1.8 times the sweetness of sucrose (Mariotti and Alamprese, 2012). Glucose and mannitol are 0.5-0.7 fold as sweet as sucrose (Fitch and Keim, 2012). The US Departments of Agriculture and Health and Human Services pattern (2010) for 2,000 kcal, recommends no more than 32 g of added sugars per day as free sugars are digested by humans and have a high glycemic index. Thus, on the one hand these sourdoughs can be used for the purpose of manufacturing sweet bakery products, on the other hand, for other purposes, it is absolutely essential to eliminate the monosaccharides. Therefore, the three strains should be coupled with other sourdough organisms that metabolize the released sugars, especially fructose. A promising candidate could be the yeast *Pichia pastoris* as it is incapable of fermenting sucrose. This yeast has already been successfully used to minimize fructose inhibition of catechin transglucosylation by fructose removal (Meulenbeld et al., 1999). Ávila-Fernández et al. (2011) used this yeast to eliminate the resulting monosaccharides glucose and fructose from hydrolyzed agave fructans to obtain sugar-free prebiotic fructooligosaccharides. Otherwise, if monosaccharides are not removed, addition of EPS containing sourdough results in a drastic increase of gas production by baker's yeast during dough proofing as observed by Galle et al. (2012). Unfortunately, no sensory evaluation of their resulting breads was done to show that remaining sugars do not contribute negatively to bread sweetness.

In conclusion, *L. animalis* TMW 1.971, *L. reuteri* TMW 1.106 and *L. curvatus* TMW 1.624 were found to produce technologically relevant amounts of EPS in sourdoughs after several parameters had been varied. This emphasizes the necessity of undertaking optimization steps in order to realize high EPS yields in sourdoughs. In addition to EPS, further metabolites must also be considered, as they may partly counteract positive EPS functions in dough and bread.

5 Summary

Celiac disease is an immune-mediated enteropathy, which is triggered by gluten in genetically susceptible individuals and leads to intestinal mucosal damage and malabsorption of several nutrients. A lifelong exclusion of gluten-containing cereals from a person's diet is currently the basis for effective treatment of celiac disease. For the baking industry, gluten replacement presents a difficult task, since gluten provides viscoelasticity to the dough, which is crucial for water retention and gas-holding properties. Therefore, additives are used to improve gluten-free breads. Hydrocolloids, in particular, were successfully used to mimic the viscoelastic properties of gluten. Nevertheless, the incorporation of additives in bread formulas does not meet consumers' demands for natural products. This paradox may be overcome by the application of sourdough, which was recently found to be prerequisite for the production of high quality gluten-free bread. Combined with the general positive effects evoked by sourdoughs, special metabolic activities like *in situ* EPS formation may reduce or replace the application of additives.

Therefore, the objective of this work was to examine the potential of different EPS forming *Lactobacillus* species as starter cultures for gluten-free sourdoughs in order to improve gluten-free breads. The strains *Lactobacillus* (*L.*) *animalis* TMW 1.971 (forms a glucan), *L. sanfranciscensis* TMW 1.392 (forms a levan), *L. reuteri* TMW 1.106 (forms a dextran) and *L. curvatus* TMW 1.624 (forms a dextran) were chosen for this work as the strains and their EPSs have already been partially characterized.

Growth experiments in Homohiochii medium showed that EPS formation occurred during the exponential phase and correlated strongly to growth. Further experiments with *L. curvatus* revealed that the sucrose concentration influenced final dextran yields and structure. 50 g L^{-1} were observed to be the optimal concentration. With increasing sucrose amounts less dextran was formed, whereas chain length of the EPS molecules increased. Variability and branching were not affected by different sucrose concentrations.

In baking experiments with 1% flour base EPS it was shown that all four EPSs improved the quality of gluten-free breads to a different extent and that dextran from *L. curvatus* improved bread properties the most. The dextran positively influenced baking loss and moisture content and decreased crumb hardness. Compared to the other EPSs, this dextran resulted in lowest specific bread volumes and was the only one that retained water in dough. A detailed structure analysis of EPS revealed differences. Size of dextran from *L. curvatus* was about 200 MDa containing 8-9% branching at position 3. In contrast, size of dextran from *L. reuteri* was about 50 MDa and with a high degree of branching (18-

19%) in position 4. As well as forming a glucan (~81%, ~100 kDa) *L. animalis* was found to produce a fructan (~19%, ~40 MDa). Through analysis of the EPS mixture and of the glucan produced by the heterologously expressed glucosyltransferase it was shown that the glucan is a linear dextran. Levan of *L. sanfranciscensis* had a molecular weight of about 70 MDa. In addition to the molecular weight and degree and type of branching, the resulting conformation of the molecule was especially crucial for its influence on gluten-free breads. The dextran of *L. curvatus* revealed a more compact structure compared to the other three EPSs and probably caused more pronounced effects in gluten-free doughs and breads. Further water retention experiments with structurally different dextrans from *L. curvatus* corroborated this assumption. Same amounts of dextran with the same variability but demonstrating a different molecular weight and conformation showed different water retention abilities in doughs.

All strains, except *L. sanfranciscensis*, which is a common bacterium for traditional wheat and rye sourdoughs, were able to assert in gluten-free sourdoughs. The investigated gluten-free flours lack of maltose, which is the preferred carbohydrate source for this strain and was most likely the cause for poor growth. For the other strains it was shown that *in situ* EPS formation can be optimized regarding type of flour, dough yield, cell count, sucrose amount and fed-batch, to levels suitable for baking applications. But especially through selecting the most suitable flour, the EPS amounts could be manifold. 17.93 g EPS kg^{-1} flour could be produced with *L. animalis* in buckwheat core doughs and 15.69 g EPS kg^{-1} with *L. reuteri* in quinoa doughs. *L. curvatus*, whose EPS had the most promising impact on gluten-free breads, formed 16.28 g EPS kg^{-1} flour in buckwheat sourdoughs. Through *in situ* optimization with *L. animalis*, *L. reuteri* and *L. curvatus* approximately 0.3% - 0.7% EPS can be achieved in the final bread dough, which equates to generally used amounts of hydrocolloids.

Furthermore, the dextransucrase responsible for dextran formation by *L. curvatus* was elucidated and compared to dextransucrases from *L. reuteri* and *L. animalis*. The three dextransucrases were successfully heterologously expressed in *E. coli* and characterized. Through structural comparison of dextrans formed by enzymes and *lactobacilli* it was shown that the analyzed genes and enzymes are responsible for EPS production by the strains. The dextransucrases followed a Michaelis-Menten kinetic and were stimulated through various ions, dextran, as well as levan. Whereas the activities of *L. animalis* and *L. curvatus* dextransucrase were increased, activity of *L. reuteri* dextransucrase was inhibited through dough extracts. Optimal conditions were pH 4.4 and 45 °C for the dextransucrase of *L. reuteri* and pH 4.4 and 31 °C for *L. curvatus* dextransucrase. The

dextransucrase from *L. animalis* had an optimal pH of 5.8 and revealed more than 50% of activity over a broad temperature profile (22-59 °C). Moreover, this enzyme showed different temperature optima for hydrolysis and transfer reaction, which has, up to date, not yet been reported for other glucosyltransferases.

The results of this dissertation clearly show that gluten-free baked goods can be improved by the application of EPS forming LAB. However, it is necessary to analyze the EPS in the respective dough system and bread making prior to starting optimization of EPS production in sourdoughs. This enables focusing on effective EPSs before any effort is put into *in situ* production, which is frequently accompanied by metabolic side effects, which again may partly counteract positive EPS functions. Both, optimal conditions of organism and of glycosyltransferase have to be considered for process optimization steps in order to ensure high EPS yields.

6 Zusammenfassung

Bei der Zöliakie handelt es sich um eine immunologisch vermittelte Erkrankung des Dünndarmes, die bei genetisch prädisponierten Menschen durch die Aufnahme von Gluten ausgelöst wird. Sie schädigt den Dünndarm und führt zur Malabsorption von Nährstoffen. Eine lebenslange glutenfreie Ernährung ist derzeit die einzige mögliche Therapie, was jedoch eine besondere Herausforderung der Industrie darstellt. Gluten spielt vor allem in der Backindustrie eine große Rolle, da es maßgeblich zur Viskoelastizität von Teigen beiträgt und damit entscheidend für Wasserbindung und Gashaltevermögen ist. Aus diesem Grund werden diverse Zusatzstoffe zur Qualitätsverbesserung glutenfreier Brote eingesetzt. Insbesondere Hydrokolloide konnten erfolgreich Anwendung finden um die viskoelastischen Eigenschaften glutenfreier Teige zu verbessern. Allerdings steht der Einsatz dieser Zusatzstoffe im Konflikt mit der zunehmenden Nachfrage der Konsumenten nach naturbelassenen Lebensmitteln. Dieses Problem kann durch den Einsatz von Sauerteig überwunden werden, der sich kürzlich als unabdingar für die Herstellung qualitativ hochwertiger glutenfreier Brote erwiesen hat. Neben allgemeinen positiven Effekten, können spezielle metabolische Aktivitäten, wie die Bildung von Exopolysacchariden (EPS), dazu genutzt werden den Einsatz von Hydrokolloiden zu reduzieren oder zu ersetzen.

Daher wurde in dieser Arbeit das Potential verschiedener EPS-bildender *Lactobacillus*-Arten als Starter für glutenfreie Sauerteige zur qualitativen Verbesserung glutenfreier Brote untersucht. Aufgrund bereits verfügbarer Informationen über Stamm und EPS, wurden für diese Arbeit *Lactobacillus* (*L.*) *animalis* TMW 1.971 (Glukan-Bildner), *L. sanfranciscensis* TMW 1.392 (Levan-Bildner), *L. reuteri* TMW 1.106 (Dextran-Bildner) und *L. curvatus* TMW 1.624 (Dextran-Bildner) ausgewählt.

Wachstumsversuche in Homohiochii-Medium zeigten, dass die EPS-Bildung aller vier Stämme während der exponentiellen Phase stattfindet und stark mit dem Wachstum korreliert. In weiteren Experimenten mit *L. curvatus* stellte sich zudem heraus, dass die Saccharosekonzentation Einfluss auf die gebildete Dextran-Menge und Struktur hat. Als Optimum für die EPS-Bildung wurde für diesen Stamm eine Konzentration von 50 g L^{-1} Saccharose ermittelt. Bei höherer Konzentration wurde weniger Dextran gebildet, während die Kettenlänge zunahm. Auf den Verzweigungsgrad und die Variabilität hatte die Saccharosekonzentration keinen Einfluss.

In Backversuchen mit 1% EPS (mehlbezogen) zeigte sich, dass die vier EPS die Qualität glutenfreier Brote in unterschiedlichem Maße verbesserten, wobei sich das Dextran von *L. curvatus* auf die Qualität besonders auswirkte. Backverlust und Feuchtegehalt der

Krume glutenfreier Brote wurden positiv durch das Dextran beeinflusst. Darüber hinaus reduzierte es die Krumenhärte und war als einziges der vier EPS in der Lage Wasser im Teig zurückzuhalten. Verglichen mit den anderen EPS-haltigen Broten, zeigten die Brote mit dem Dextran von *L. curvatus* die geringsten spezifischen Volumina. Eine genauere Analyse der EPS offenbarte strukturelle Unterschiede. Das Dextran von *L. curvatus* erwies sich als ein etwa 200 MDa großes Molekül mit 8-9% Verzweigungen in Position 3. Dagegen zeigte das etwa 50 MDa große Dextran von *L. reuteri* mit 18-19% einen sehr hohen Verzweigungsgrad in Position 4. *L. animalis* bildete im Homohiochii Medium neben dem Glukan (~81%, ~100 kDa) noch ein Fruktan (~19%, ~40 MDa). Eine NMR-Analyse des EPS-Gemisches und des Glukans der heterolog exprimierten Glukosyltransferase zeigte, dass es sich bei dem Glukan um ein lineares Dextran handelt. Das Levan von *L. sanfranciscensis* hatte eine Größe von etwa 70 MDa. Neben der Molekülgröße und dem Verzweigungsgrad und –typ, erwies sich besonders die Konformation der Moleküle als entscheidend für den Einfluss auf glutenfreie Teige und Brote. Das Dextran von *L. curvatus* offenbarte eine kompaktere Konformation als die übrigen drei EPS, die wahrscheinlich zu ausgeprägteren Effekten in glutenfreien Teigen und Broten führte. Weitere Wasserrückhalteexperimente mit strukturell unterschiedlichen Dextranen von *L. curvatus* untermauerten diese Vermutung. Gleiche Mengen an Dextran mit gleicher Variabilität und unterschiedlicher Molekülgröße und –konformation führten zu verschieden starker Wasserrückhaltung in Teigen.

In glutenfreien Sauerteigfermentationen konnten sich alle Stämme, ausgenommen von *L. sanfranciscensis*, durchsetzen. Die Ursache für das schlechte bzw. fehlende Wachstum des für Weizen- und Roggensauerteige typischen Milchsäurebakteriums konnte auf einen Maltosemangel glutenfreier Mehle zurückgeführt werden. Für die übrigen Stämme wurde gezeigt, dass die *in situ* EPS-Bildung in glutenfreien Sauerteigen bezüglich Mehl, Teigausbeute, Zellzahl, Saccharose-Menge und Fed-Batch zu backtechnologisch relevanten Mengen optimiert werden kann. Insbesondere durch die Wahl geeigneter Mehle konnten die EPS-Mengen stark gesteigert werden. Durch Optimierung wurden mit *L. animalis* 17.93 g EPS kg^{-1} Mehl in Buchweizenkernmehlsauerteigen erzielt und mit *L. reuteri* 15.69 g kg^{-1} in Quinoateigen. *L. curvatus*, dessen dextran die vielversprechendsten Auswirkungen in Backversuchen zeigte, bildete 16.28 g EPS kg^{-1} Mehl in optimierten Buchweizensauerteigen. Umgerechnet können durch *in situ* Optimierung mit *L. animalis*, *L. reuteri* und *L. curvatus* etwa 0.3 – 0.7% EPS im Brotteig erzielt werden, eine Menge die der typischen Einsatzmenge von kommerziellen Hydrokolloiden entspricht.

Weiterhin wurde im Rahmen dieser Arbeit die für die Dextranbildung von *L. curvatus* verantworliche Dextransucrase aufgeklärt und mit den Dextransucrasen von *L. reuteri* und *L. animalis* verglichen. Die drei Dextransucrasen konnten erfolgreich heterolog in *E. coli* exprimiert und charakterisiert werden. Ein struktureller Vergleich enzymatisch gebildeter Dextrane mit den mikrobiell gebildeten Dextranen zeigte, dass es sich um die für die EPS-Bildung verantwortlichen Enzyme handelt. Die Dextransucrasen folgten einer Michaelis-Menten Kinetik und wurden durch verschiedene Ionen sowie durch Dextran als auch Levan stimuliert. Glutenfreie Teigextrakte steigerten die Aktivität der Dextransucrasen von *L. animalis* und *L. curvatus*, während die Aktivität der Dextransucrase von *L. reuteri* inhibiert wurde. Die optimalen Bedingungen lagen für die Dextransucrase von *L. reuteri* bei pH 4.4 und 45 °C und für die von *L. curvatus* bei pH 4.4 und 31 °C. Die Dextransucrase von *L. animalis* zeigte ein pH-Optimum von 5.8 und erwies sich mit über 50% Aktivität zwischen 22 und 59 °C über einen weiten Temperaturbereich als stabil. Darüber hinaus offenbarte das Enzym zwei unterschiedliche Temperaturoptima für die Hydrolyse- und Transferasereaktion, was bisher noch für keine Glukosyltransferase gezeigt werden konnte.

Die Ergebnisse dieser Arbeit zeigen deutlich, dass glutenfreie Backwaren durch EPS-bildende Milchsäurebakterien qualitativ verbessert werden können. Hierfür besteht jedoch die Notwendigkeit, die Auswirkung verschiedener EPS zunächst im Teig- und Brotsystem zu analysieren. Dadurch können effektive EPS fokussiert werden, bevor eine aufwändige *in situ* Optimierung unternommen wird, welche häufig mit metabolischen Nebeneffekten verbunden ist und der positiven Wirkung der EPS entgegenwirken können. Es müssen, sowohl die optimalen Bedingungen des Mikroorganismus, als auch die der jeweiligen Glykosyltransferase gleichermaßen in die Prozessoptimierung einfließen, um hohe EPS-Ausbeuten gewährleisten zu können.

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8 Appendix

8.1 Sequences of different *gtf* genes

L. curvatus **TMW 1.624** (HE972512)

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atgttaaggaataattattttggagagactaaaacgcattataaattatataaatgcggt
 M  L  R  N  N  Y  F  G  E  T  K  T  H  Y  K  L  Y  K  C  G
aagaactgggctgtcatggggatttcattatttccgctgggattagggatgctagttacc
 K  N  W  A  V  M  G  I  S  L  F  P  L  G  L  G  M  L  V  T
agccagccagtgtcagctgatgtgacagccaccagcacctcaagcagtgcagtgaggacc
 S  Q  P  V  S  A  D  V  T  A  T  S  T  S  S  S  A  V  R  T
gatgcaatcagtgaaagtagtagcagtgcagcaaaggctgaaacgactagtgcaagtagt
 D  A  I  S  E  S  S  S  S  A  A  K  A  E  T  T  S  A  S  S
agcagtgcagtgaaggccgaaacgactagtgcaagtagtagcagtgcagcaaaggctgaa
 S  S  A  V  K  A  E  T  T  S  A  S  S  S  S  A  A  K  A  E
acggctgcgatcactactgcaggtgttgcaaatgctgattcacaaacatcagcagaagta
 T  A  A  I  T  T  A  G  V  A  N  A  D  S  Q  T  S  A  E  V
accgctgactctacttctaccagccaagtggtaactaataattccaataatcaaaataat
 T  A  D  S  T  S  T  S  Q  V  V  T  N  N  S  N  N  Q  N  N
acagcacagccagccggtcaagaagcagccccggtatcagaggacacatcatctgatgat
 T  A  Q  P  A  G  Q  E  A  A  P  V  S  E  D  T  S  S  D  D
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***L. reuteri* TMW 1.106** (Kaditzky, 2008; EF189716.1)

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 E   T   D   M   R   P   I   L   M   V   W   W   P   N   T   L   T   Q   A   Y
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 Y   L   N   Y   M   K   Q   H   G   N   L   L   P   S   A   L   P   F   F   N
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 A   D   A   D   P   A   E   L   N   H   Y   S   E   I   V   Q   Q   N   I   E
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 K   R   I   G   E   T   G   S   T   D   W   L   R   T   L   M   H   D   F   I
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 T   N   N   P   M   W   N   K   D   S   E   N   V   N   F   S   G   I   Q   F
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 Q   G   G   F   L   K   Y   E   N   S   D   L   T   P   Y   A   N   S   D   Y
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 R   L   L   D   R   M   P   I   N   I   D   G   K   G   V   K   G   S   E   F
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 L   Y   Y   L   L   N   F   G   T   I   T   A   N   N   D   Q   A   N   F   D
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 S   I   R   V   D   A   V   D   N   V   D   A   D   L   L   N   I   A   Q   D
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 Y   F   N   A   A   Y   G   M   N   S   D   A   V   S   N   K   H   L   N   I
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 L   E   D   W   S   H   A   D   P   E   Y   I   N   K   I   G   N   P   Q   L
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 T   M   D   D   R   E   K   E   A   L   N   H   G   L   S   D   K   T   N   R
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 W   G   L   D   A   I   V   H   Q   S   L   A   D   R   E   N   D   S   T   E
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 N   I   V   I   P   N   Y   T   F   I   R   A   H   D   S   N   A   Q   D   Q
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 I   N   N   V   I   R   D   L   T   G   K   D   Y   H   T   F   T   F   E   D
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 E   Q   K   G   I   D   T   Y   I   Q   D   Q   N   S   T   V   K   K   Y   N
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 L   Y   N   I   P   S   S   Y   A   I   M   L   T   N   K   D   T   I   P   R
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***L. animalis* TMW 1.971** (Jänsch, 2004; Tieking, 2005; HE972513)

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```

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aatgcaatcattgctcaaaatgttggtctgttcaatagttggggagttactagcttccag
 N  A  I  I  A  Q  N  V  G  L  F  N  S  W  G  V  T  S  F  Q
ttagcaccacaatatgtatctagtcatgacggctcattcttggattcgatcattgataat
 L  A  P  Q  Y  V  S  S  H  D  G  S  F  L  D  S  I  I  D  N
ggttatgccttcactgatcgttatgatcttgcaatgagtaagaataataagtatggttct
 G  Y  A  F  T  D  R  Y  D  L  A  M  S  K  N  N  K  Y  G  S
taccaagatctggtcaatgtattaaaagctctacacgctggtgggatccaagtgatcgct
 Y  Q  D  L  V  N  V  L  K  A  L  H  A  G  G  I  Q  V  I  A
gactgggttccagatcagatctatagcttacctggtaaggaagttgtatcggtcgtacgg
 D  W  V  P  D  Q  I  Y  S  L  P  G  K  E  V  V  S  V  V  R
tctgatgaatttggtaataaagtcgatggtactcagatcgataatactttatatgttgtt
 S  D  E  F  G  N  K  V  D  G  T  Q  I  D  N  T  L  Y  V  V
aatactattggtggtggacaataccaaaaagaatacggtggtcgctatcttgaagaatta
 N  T  I  G  G  G  Q  Y  Q  K  E  Y  G  G  R  Y  L  E  E  L
aaacagaagtatccagaactatttaagacaaagcaaccttcaaccggggtaactatcgat
 K  Q  K  Y  P  E  L  F  K  T  K  Q  P  S  T  G  V  T  I  D
ccaagtgaaaagatcactgaatggtcagctaagtatctaaatgggactaatatcttgcat
 P  S  E  K  I  T  E  W  S  A  K  Y  L  N  G  T  N  I  L  H
cgcggtgcagaatttgtgttacgtgatggtgctacttactttagggtagctgaaacaagt
 R  G  A  E  F  V  L  R  D  G  A  T  Y  F  R  V  A  E  T  S
gaagtattcttgcctagtcaattacgtggtaagatcactaagaatggtttctggaaaaat
 E  V  F  L  P  S  Q  L  R  G  K  I  T  K  N  G  F  W  K  N
gatgctggaaaagttaactattataacagtgaaggcgaaattatgaagaatgcctttgtt
 D  A  G  K  V  N  Y  Y  N  S  E  G  E  I  M  K  N  A  F  V
aaagatggtaaaaacaactggtattatttcgacaacgatggaaatatggtaacaaatact
 K  D  G  K  N  N  W  Y  Y  F  D  N  D  G  N  M  V  T  N  T
gctttaacgatcgatagtgatgctcaagtagcagattactatttcttgagtaatggtatt
 A  L  T  I  D  S  D  A  Q  V  A  D  Y  Y  F  L  S  N  G  I
tctttacgtgatggctttgttcaattagctaatggtgatgtttattactatgatattaat
 S  L  R  D  G  F  V  Q  L  A  N  G  D  V  Y  Y  Y  D  I  N
gggcgtaagttgaagaatggtaaagtaacagtcaataatgtcgaatatactactgacaaa
 G  R  K  L  K  N  G  K  V  T  V  N  N  V  E  Y  T  T  D  K
aatggtaaggtcgtaggcgaaaacattttgaagaagttagatgagatcattacaactgga
 N  G  K  V  V  G  E  N  I  L  K  K  L  D  E  I  I  T  T  G
aaaacaactttaatttaa
 K  T  T  L  I  -
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8.2 Publications

List of publications that resulted from this dissertation

Papers

Rühmkorf, C., Bork, C., Mischnick, P., Rübsam, H., Becker, T., Vogel, R.F., 2012. Identification of *L. curvatus* TMW 1.624 dextransucrase and comparative characterization with *L. reuteri* TMW 1.106 and *L. animalis* TMW 1.971 dextransucrases. Food Microbiol. 34, 52-61.

Rühmkorf, C., Jungkunz, S., Wagner, M., Vogel, R.F., 2012. Optimization of homoexo-polysaccharide formation by lactobacilli in gluten-free sourdoughs. Food Microbiol. 32, 286-294.

Rühmkorf, C., Rübsam, H., Becker, T., Bork, C., Voiges, K., Mischnick, P., Brandt, M.J., Vogel, R.F., 2012. Effect of structurally different microbial homoexopolysaccharides on the quality of gluten-free bread. Eur. Food Res. Technol. 235, 139-146.

Oral presentations

Rühmkorf, C., Kaditzky, S., Brandt, M.J., Bork, C., Mischnick, P., Vogel, R.F., 2011. Auswirkung zweier verschiedener Dextrane auf die Struktur von glutenfreien Broten und *in situ* Bildung der Dextrane in Sauerteigen, Minden, Germany.

Posters

Rühmkorf, C., Rübsam, H., Becker, T., Bork, C., Voiges, K., Mischnick, P., Brandt, M.J., Vogel, R.F., 2012. Knowledge-based bread structure design with exopolysaccharides. 1. Frühjahrstagung des Weihenstephaner Instituts für Getreideforschung, Freising, Germany.

Rühmkorf, C., Kaditzky, S., Vogel, R.F., 2010. Effect of microbial homopolysaccharides on the structure of gluten-free breads. Gluten-free cereal products and beverages, Tampere, Finland.