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Sucrose metabolism and exopolysaccharide production by *Lactobacillus sanfranciscensis*

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Contents

1. Introduction

Lactic acid bacteria (LAB) have being used for centuries in production of various fermented foods due to their preservative contribution and metabolic activity, that award the fermented food its characteristic flavour. The most important attribute of LAB is the production of lactic acid, as a result of which the pH is lowered exerting an inhibitory effect on spoilage microorganisms. It is believed that fermentative conservation of food initially happened coincidentally. Nevertheless, the preservative advantages of LAB and the characteristic taste of fermented food have been appreciated by ancient civilisations, which had few possibilities for food preservation. In the last decades the physiology and genetics of LAB were and are still subject of major research efforts. Besides the preservative effect of acid(s) produced, LAB have a diversified metabolic spectrum including the release of flavour precursors and the potential to excrete bacteriocins with sometimes wide inhibitory effect on the accompanying flora. Another interesting property of several LAB is their ability to synthesise exopolysaccharides (EPS), that may improve the texture and "mouthfeel" of food and moreover have health promoting properties.

1.1. Microflora of sourdough

The use and later the cultivation of cereals as a part of human nutrition can be dated to 7000-6000 BC (Lönner and Ahrne, 1995). The addition of water to flour leads to sourdough, this phenomenon has been already observed in ancient times. During excavations in Switzerland a 5500 years old charred wheat sourdough bread was discovered (Währen, 1985). Dumas (1843) attributed the leavening of dough to the alcoholic fermentation of sugars available in flour, and Holliger (1902) described homofermentative lactic acid bacteria as the organisms responsible for the acid production and yeasts for the leavening of sourdough. The first heterofermentative organism from sourdough was identified by Henneberg (1909) as *Bacillus panis fermentati* (syn., *Lactobacillus brevis*, Spicher and Stephan, 1987). Knudsen (1924) isolated the same group of heterofermentative LAB from 300 sourdoughs and described this group as the most important in the sourdough

fermentation. *Lactobacillus plantarum*, *Lactobacillus brevis* ssp. *lindneri* and *Lactobacillus fermentum* were isolated from sourdoughs by Spicher (1958, 1959, 1978). Kline and Sugihara (1971) described a new heterofermentative *Lactobacillus* sp. from San Francisco sourdough and proposed the name *Lactobacillus sanfrancisco*. DNA-DNA-hybridisation confirmed *Lactobacillus sanfrancisco* as new species (Sriranganathan et al. 1973), and taxonomical investigations resulted in its inclusion into the "Approved List of Bacterial Names" (Weiss and Schillinger, 1984). DNA-DNA-homology showed that *Lactobacillus brevis* ssp. *lindneri* and *Lactobacillus sanfrancisco* belong to the same species (Kandler and Weiss, 1986). According to the "International Code of Nomenclature of Bacteria" the specific epithet of *L. sanfrancisco* was changed to *sanfranciscensis* (Trüper and De Clari., 1997). Vogel et al. (1994) applied physiological characteristics, protein patterns and 16S rRNA sequences to identify sourdough lactobacilli. Strains of *Lactobacillus* species accounted for 30 to 80% of microflora of some rye and wheat sourdoughs were isolated. These organisms were differentiated from other sourdough lactobacilli and are closely related to *L. reuteri*. This species was characterised and proposed as *Lactobacillus pontis*.

Böcker et al. (1995) divided sourdoughs into three types based on the fermentation conditions. Type I doughs are continuously (daily) propagated at temperature <26°C. Investigations of the microbiology of type I sourdough revealed that two strains of *L. sanfranciscensis* and one strain of *L. pontis* were present for at least 10 years (Hammes and Gänzle, 1998). Type II doughs with high acid content are fermented for up to 5 days at temperatures of 40°C and usually dominated by acid tolerant lactobacilli e. g. *L. pontis*, *L. reuteri*, *L. panis*, *L. frumenti* and *L. amylovorus*. Type III are dried sourdoughs fermented by dry tolerant lactobacilli e. g *L. plantarum*, *L. brevis* and *Pediococcus pentosaceus* (Böcker et al., 1995). Besides heterofermentative LAB, several yeasts have been isolated from sourdoughs. *Saccharomyces exiguus* and *Candida milleri*, that cannot metabolize maltose unlike *L. sanfranciscensis*, are typical yeasts associated with *L. sanfranciscensis*. Sugihara et al. (1970) attributed this differentiated use of maltose for the lack of competition between *L. sanfranciscensis* and *S. exiguus*. Furthermore, it is proposed that glucose

represses the maltose uptake of the competitors awarding *L. sanfranciscensis* an additional ecological advantage. Another reason for the co-existence between *L. sanfranciscensis* and *Candida milleri* is the tolerance of *Candida milleri* to acetic acid produced by *L. sanfranciscensis* (Hammes and Vogel, 1997). Brandt and Hammes (2001) reported higher acetate production in sourdoughs fermented by *L. sanfranciscensis* and *Candida milleri* compared with doughs fermented only by *L. sanfranciscensis*. It could be shown that *Candida milleri* is able to hydrolyse flour fructosans releasing fructose which used by *L. sanfranciscensis* as electron acceptor.

1.2. Maltose and sucrose metabolism by *L. sanfranciscensis*

Fermentable carbohydrates play an essential role in sourdough fermentation process (Hammes and Vogel, 1997). The utilization of carbohydrates by lactobacilli during sourdough fermentation results in the production of acids, gas and aroma precursors. Maltose is an important carbon source for sourdough lactobacilli. Wheat flour contains 1% maltose and during sourdough fermentation maltose is generated from starch (up to 3.6% dry mass) by amylase activity of flour (Korakli et al. 2001). Virtually all of *L. sanfranciscensis* strains (about 80) isolated from German and Italian wheat and rye sourdoughs can utilize maltose (Hammes and Gänzle, 1998). Maltose is transported into the cell using the proton motive force mediated by maltose/H^+ symport system, and is cleaved by maltose phosphorylase to glucose-1-phosphate and glucose. Glucose-1-phosphate is degraded like other hexoses via pentose-phosphate-pathway, whereas glucose is either phosphorylated or excreted by uniport transport system (Neubauer et al., 1994 and Hammes et al., 1996). The cleavage of maltose by the key enzyme maltose phosphorylase is considered to contribute to the predominance of lactobacilli in sourdough, and enables *L. sanfranciscensis* to save the expenditure of 1 mol ATP per mol of maltose (Stolz et al., 1996). The subsequent metabolism of glucose-1-phosphate results in production of CO_2, lactate and ethanol. In the presence of electron acceptors e. g. fructose, citrate and malate that allow the regeneration of NAD^+ an additional ATP can be gained, and acetate is formed instead of ethanol (Stolz et al. 1996). Acetate affects the flavour of bread, and Salovaara (1987a, b) found a correlation between acetic acid content and the shelf life of the bread. The

combined use of maltose and electron acceptors and the cleavage of maltose via maltose-phosphorylase can explain part of the competitiveness of heterofermentative lactobacilli in sourdoughs (Vogel et al., 1999).

There are various mechanisms for sucrose metabolism by LAB. *Leuconostoc mesenteroides* cleaves sucrose either by the action of dextransucrase (Hestrin et al., 1943) or by sucrose phosphorylase (Kawasaki et al., 1996). Other alternatives for sucrose cleavage are the phosphotransferase system (PTS) found by *Lactococcus lactis* (Thomson and Chassy, 1981) and an invertase activity was described by *Lactobacillus reuteri* (Cuezzo de Gines et al., 2000). In contrast to maltose only a part of *L. sanfranciscensis* strains can metabolize sucrose (Böcker, 1993). The sucrose metabolism by the strain LTH2590 resulted in production of mannitol and exopolyasccharide predominately composed of fructose (Korakli et al., 2000). This EPS was characterised as a levan type polyfructan (Dal Bello et al., 2001). Information about biosynthesis, kinetics of EPS formation and physiological regulation of homopolysaccharides production by lactobacilli are rather scarce. Production of levan type EPS was described by *Lactobacillus reuteri* and a levansucrase was purified (Van Geel-Schutten et al. 1998 and Van Hijum et al. 2001). Levansucrase (E.C. 2.4.1.10) is a fructosyltranferase enzyme that catalyses the following reaction:

$$\text{Sucrose} + (2,6\text{-beta-D-fructosyl})_n \rightarrow \text{glucose} + (2,6\text{-beta-D-fructosyl})_{n+1}$$

Besides the fructosyltransferase activity, levansucrase exhibits ß-D-fructofuranosidase activity (Schomburg and Stephan, 1996). Yanase et al. (1992) reported on the liberation of reducing sugars from substrates having 2-beta-D-fructofuranose residues such as sucrose and raffinose by levansucrase purified from *Zymomonas mobilis*. The energy required for the polymerisation reaction is obtained from the hydrolysis of sucrose. Levan production was observed by numerous bacteria e. g. *Bacillus subtilis* (Chambert et al. 1974), *Streptococcus salivarius* (Song and Jacques, 1999) and is extensively studied by *Zymomonas mobilis* (Yanase et al. 1992). Korakli (1998) suggested levansucrase as the enzyme responsible for sucrose cleavage and EPS synthesis by *L. sanfranciscensis*. The metabolism of sucrose is accompanied with mannitol production indicating

an additional hydrolase activity of levansucrase of *L. sanfranciscensis*. The liberated glucose is used as carbon source and metabolised via pentose-phosphate-shunt.

1.3. Exopolysaccharides

Polysaccharides are a highly diverse group of polymers, of which the functional features are determined by their structural characteristics that may differ in molecular weight, saccharide-linkage type, degree of branching and chemical composition (Kleerebezem et al. 1999). This diversity led to broad applications in industry. Most of the polysaccharides used in the food industry as thickener, stabiliser, texturizing and gelling agents are derived from plant (e. g. starch, pectin, gum arabic) or seaweeds (e. g. alginate, carrageenan). In the last decades several microbial exopolysaccharides (EPS) have been described as alternatives for plant polysaccharides. Microbial polysaccharides have rheological properties that match the technological demands and can be produced in large amounts and high purity. Microbial polysaccharides can be divided based on their location into (i) capsular polysaccharides (CPS) associated with the cell surface, and believed to belong to the virulence characteristics of pathogens (*Streptococcus penumoniae*), and (ii) extracellular polysaccharides (EPS) secreted in the environment of the cell. It is suggested that CPS play a role in protection of microbial cells against desiccation, phagocytosis, phage attack, antibiotics and toxic compounds, and provide the cell with the capability to adhere to solid surfaces (Sutherland, 1977 and Whitfield, 1988). The ability of EPS production is widely spread among bacteria and less among yeasts and fungi (Sutherland, 1998). In the last years several EPS producing lactobacilli were described. Most of these lactobacilli were isolated from dairy products e. g. fermented milk, yoghurts and kefir grains (De Vuyst, 1999). EPS seem not to serve as nutrient reserve, since the most EPS producing bacteria are not able to degrade the EPS they produce (Cerning, 1990). Based on their composition EPS can be classified into homo- and hetero-polysaccharides. Homopolysaccharides consist of one monosaccharide (mostly fructose or glucose),

and are usually produced in large amount from sucrose by the action of glycansucrases (Monsan et al., 2001). In Table 1 are shown some examples of homopolysaccharides.

Table 1. Overview on some homopolysaccharides (modified according to Monsan et al. 2001 and Sutherland 1998).

EPS	trivial name	producing microorganism
ß-glucan	Cellulose	*Acetobacter xylinum*
	Curdlan	*Alcaligenes* ssp., *Rhizobium* ssp.
α-glucan	Dextran	*Leuconostoc mesenteroides*
	Mutan	*Sreptococcus mutans*
	Pullulan	*Aureobasidium pullulans*
Fructan	Fructan	*Lactobacillus sanfranciscensis*
	Levan	*Streptococcus salivarius*
	Inulin	*Streptococcus mutans*
	Fructan and glucan	*Lactobacillus reuteri*
Polygalactan	Polygalactan	*Lactococcus lactis* ssp. *cremoris*

Heteropolysaccharides are mostly composed of identical repeating units consisting of two or more monosaccharides e. g. galactose, glucose, rhamnose and fructose (Tab. 2). Several linkages can occur at the same time in one polysaccharide. Heteropolysaccharides are produced intracellulary as subunits and usually in small amounts up to 1.5 g/l. Sugar nucleotides play an essential role in the synthesis of heteropolysaccharides due to their function in sugar interconversions as well as sugar activation (Cerning, 1990).

Table 2. Overview of some herteropolysaccharides producing LAB.

Strain	composition of EPS	source
Lactobacillus bulgaricus CRL 87	fructose, glucose	Manca et al. 1985
Lb. kefiranofaciens K1	galactose, glucose	Mukai et al. 1990
Lb. casei spp. *casei* NCIB 4114	galactose, glucose	Cerning et al. 1991
Lb. helveticus 776	glucose, galactose	Robjin et al. 1995
Lb. sake 0-1	glucose, rhamnose	Van den Berg, 1995
Lb. acidophilus LMG 9433	glucose, galactose	Robjin et al., 1996
Lb. delbruckii ssp. *bulgaricus* NCFB2772	galctose, glucose	Grobben et al. 1996
Lb. paracasei 34-1	galactose	Robjin et al. 1996
Lb. rhamnosus R	rhamnose, glucose, galactose	Pham et al. 2000
Lactococcus lactis ssp. *lactis*	galactose, glucose	Cerning et al. 1991
Lc. lactis ssp. cremoris B40	glucose, galactose, rhamnose	Van casteren et al. 1998
Streptococcus thermophilus	galactose, glucose	Degeest et al. 1997

Dextran synthesized by *Leu. mesenteroides* was one of the first biopolymers produced at industrial scale in 1948 (Groenwall and Ingelman, 1948) and found several applications in medicine, separation technology and biotechnology (Soetaert, 1995). Xanthan from *Xanthomonas campestris* and gellan (Gelrite®) from *Sphingomonas paucimobilis* are also produced in large amounts and have found various food and non-food applications (Sutherland, 1998). The interest of the food industry in developing "multifunctional additives", that not only provide the desired improvement of the texture but also have additional nutritional properties, led to extensive search for polysaccharides with prebiotic attributes. Prebiotics are non-digestible food ingredients that affect the host beneficially by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon and thus improve host health (Gibson and Roberfroid, 1995). Fructo-oligosaccharides (FOS), xylooligosaccharide and inulin are some prebiotics available for the human consumption (Cummings et al. 2001 and Okazaki et al., 1990). FOS with prebiotic properties (e. g. kestose, nystose) are polymers of D-fructose joined by ß(2→1) linkages and terminated with a D-glucose molecule linked to fructose by an α(1→2) bond as in sucrose (Kosaric et al., 1984). The

degree of polymerization (DP) can vary from 2-35. FOS with DP of 3-5 are called neosugars (Tokunaga et al. 1986) and are enzymatically synthesized from sucrose using fructosyltransferase obtained from *Aspergillus niger* (Hidaka et al., 1988 and 1991). Mckellar and Modler (1989) showed that the maximum activity of ß-fructosidase responsible for the hydrolysis of inulin type polysaccharides by bifidobacteria was observed with neosugars.

1.4. Environmental stress responses

The increasing use of defined starter cultures instead of spontaneous fermentation in the production of fermented foods led to more studies regarding the physiology and genetics of LAB. Most of these studies focused on the response of lactic acid bacteria to environmental stressors, e. g. temperature, pH, osmotic stress and starvation. The response of microorganisms to stress conditions can include gene expression, enhanced/reduced protein synthesis, and metabolic changes. Stress induced gene expression can take place at the level of transcription, translation or mRNA stability. The influence of stress conditions has been studied by analysing their effects on growth and total protein synthesis and by genetic analysis of known stress-related genes. Enhanced and/or reduced protein expression were observed by *Lactococcus lactis* exposed to heat-shock, low temperature, low pH, starvation, salt and UV light (Sanders et al., 1999). Heat-shock proteins were also detected by numerous lactobacilli such as *L. acidophilus*, *L. casei* and *L. helveticus* (Broadbent et al. 1997). On the physiological level low a_w and/or pH by *Lactococcus lactis subsp. cremoris* resulted in decreased growth, lactose utilization and carbon recovery. Lactose fermentation under these conditions was accompanied with galactose accumulation and production of galactose containing saccharide(s) (Liu et al., 1998). The pH tolerance of *L. sanfranciscensis* was increased after sublethal acid pretreatment, and the sensitivity to the low pH was dependent on the acid used. This enhanced acid tolerance was attributed to the induction of stress protein synthesis (De Angelis et al., 2001).

In the last years hydrostatic high pressure was applied in food industry as a non-thermal food processing technique. There are few data about the effect of sublethal high pressure on the metabolism of microorganisms. The effect of high pressure is dependent on the environmental conditions e. g. temperature, pH, concentration of substrate and end products. High pressure treatment (200 MPa) of *L. sanfranciscensis* and subsequent comparison of the protein patterns using 2-D-electrophoresis revealed the detection of some spots, indicating modified protein synthesis under high pressure. Five of these spots could be identified by liquid chromatography-tandem mass spectroscopy, and are homogenic with the cold shock family of *Lactococcus lactis* (Drews et al., 2002). Similar results were also observed by *L. sanfranciscensis* (Scheying, 2002), the spots detected under high pressure conditions showed high homology to glyceraldehyde-3-phosphate-dehydrogenase and alcohol-dehydrogenase. High pressure treatment of microorganisms not only affects the viable cell counts, gene and protein expression but also the metabolic activity. Sublethal high pressure treatment (40-60 MPa) of *Saccharomyces cerevisiae* resulted in reduction of vacuolar-pH and pressure of 25-50 MPa in decreased tryptophan uptake (Abe and Horikoshi, 1998 and 2000).

1.5. Objectives of the thesis

Plant and namely cereal products are the most important staple food throughout the world. The addition of plant polysaccharides are common practice by the preparation of baking mixture, because they improve texture properties, keepability and shelf life of the bread. The use of EPS producing sourdough lactobacilli in sourdough fermentation may allow the replacement of plant polysaccharides and match the demands of consumer for “clean label”. The strain *Lactobacillus sanfranciscensis* LTH2590 was shown to produce EPS from sucrose.

It was the aim of this work to study the metabolism of sucrose by *L. sanfranciscensis* as well as the effect of substrate concentration on sucrose metabolism, EPS production and end products. Furthermore, the sucrose metabolism by *L. sanfranciscensis* in wheat and rye sourdoughs and EPS

formation during dough fermentation should be determined. The ability should be investigated of numerous lactobacilli of sourdough and intestinal origin as well as several bifidobacteria to degrade polysaccharides from wheat and rye and EPS produced by *L. sanfranciscensis*. Finally, the effect should be evaluated of sublethal high pressure on the kinetics of the viable cell counts, substrate consumption and end products formation of maltose or sucrose by *L. sanfranciscensis*.

2. Materials and Methods

2.1. Organisms and culture conditions

2.1.1 Organisms

Lactobacillus sanfranciscensis LTH2590 (TMW1.392) was used as the EPS-producing strain. Other organisms used in this work were:

Lactobacillus sanfranciscensis LTH2581 (TMW1.52)

Lactobacillus sanfranciscensis LTH1729 (TMW1.54)

Lactobacillus animalis TMW1. 972

Lactobacillus brevis DSM5622 (TMW1.220)

Lactobacillus johnsonii TMW1.990

Lactobacillus paracasei subsp. paracasei TMW1.11833

Lactobacillus plantarum TMW1.46

Lactobacillus pontis LTH2587 (TMW1.1191)

Lactobacillus reuteri TMW1.974

Lactobacillus salivarius subsp. salivarius TMW1.992

Bifidobacterium adolescentis DSM20083 (TMW2.454)

Bifidobacterium bifidum DSM20456 (TMW2.455)

Bifidobacterium breve DSM20213 (TMW2.447)

Bifidobacterium infantis TMW2.448

Bifidobacterium lactis DSM10140 (TMW2.462)

Bifidobacterium minimum TMW2.456

2.1.2 Media and culture conditions

L. sanfranciscensis LTH2590 was cultured at 30°C in MRS (De Man et al., 1960) modified to meet the requirements for the EPS production (Su-MRS). Su-MRS contained the following components per litre: peptone from casein, 10g; yeast extract, 5g; meat extract, 5g; $K_2HPO_4.3H_2O$, 2.6g; KH_2PO_4, 4g; cystein-HCl, 0.5g; NH_4Cl, 3g; sucrose, 50g; Tween 80, 1 ml/l. The pH was adjusted to

6.2, and the medium was sterilized at 121°C for 20 min. Stock solutions containing $MgSO_4.3H_2O$ (100 g/l) and $MnSO_4.4H_2O$ (50 g/l) were autoclaved separately, and a vitamin mix containing biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamine, riboflavin, cobalamin and panthothenic acid (0.2 g/l each) was sterilized by filtration (Sartorius AG, Germany, 0.20μm). 1ml/l of each stock solution was added to the sterilized medium. Bifidobacteria were grown at 37°C in DSM 58 medium additionally containing vitamin mix 1ml/l.

To evaluate the effect of sucrose concentration on the sucrose metabolism fermentations at controlled pH were carried out in 350ml jar fermenter (Braun Biotech Int., Germany) at 30°C and 80 rpm without gas inlet. pH was monitored with an online pH sensor and maintained at 5.6 using 4M NaOH. Medium components were dissolved in 60% of the final volume and autoclaved in the fermenter (20 min. at 121°C). Sucrose and fructose were dissolved in the residual volume (40%), sterilized by filtration and added to the autoclaved medium. High pressure experiments were carried out in mMRS4 as described by Stolz et al. (1996) unless otherwise indicated.

2.2. Determination of colony forming units

Cell counts in all experiments were determined on mMRS4 agar as described by Stolz et al (1996). Appropriate dilution was plated using a spiral plater (IUL, Königswinter, Germany) and plates were incubated at 30°C for 48h under controlled atmosphere (76% N_2, 20% CO_2, 4% O_2).

2.3. Determination of the maximum growth rate

The effect of sucrose and fructose concentration on the maximum growth rate were determined in multiple well plates (Sarstedt, USA). Su-MRS with sucrose and fructose concentrations ranging from 0-133 g l^{-1} and 0-53 g l^{-1}, respectively was used. The kinetics of growth and maximum growth rates were determined at 590nm using spectrophotometer (SpectraFlour®, Tecan, Austria). The modeling of the maximum growth rate as a function of sucrose and fructose was performed as described by Gänzle et al. (1998).

2.4. Determination of metabolites

The concentrations of maltose, lactic acid, acetic acid, formic acid and ethanol in the supernatant were determined using polyspher® OA KC column (Merck, Germany); the mobile phase was H_2SO_4 5 mmol/l, flow rate 0.4 ml/min and temperature of the column 70°C. Sucrose, glucose, fructose, kestose, xylose, arabinose and mannitol concentrations were determined using polyspher® CH PB column (Merck, Germany); the mobile phase was deionized H_2O, flow rate 0.4 ml/min and temperature of the column 80°C. For detection a refractive index detector (Gynkotek, Germany) was used. The detection limit was 1 mmol l^{-1} for ethanol, 0.2 mmol l^{-1} for carbohydrates and mannitol, and 0.05 mmol l^{-1} for organic acids.

The retention time of the oligosaccharide produced by *L. sanfranciscensis* LTH2590 was compared with that of kestose using an anion-exchange chromatography with an integrated and pulsed amperometric detector (IPAD) equipped with a gold electrode cell (Dionex, Sunnyvale, USA). The waveform was set as described by Jandik et al. (1999). The column used was AminoPac™ PA10 (Dionex, Sunnyvale, USA) consisting of a guard column and analytical column (2 mm x 250 mm). Three different eluents were used: (A) deionized water; (B) 250mM NaOH and (C) 1M sodium acetate. The water used for the preparation of the eluents had a conductivity of 0.054 μS cm^{-1}. The flow rate was 0.25 ml $min^{-1}$and the temperature of the column 30°C. The following gradient was used to separate sugars and oligosaccharides: 0 min: 80% A, 0% B and 20% C; 16 min: 80% A, 0% B and 20% C; 32 min: 0% A, 80% B and 20% C; 40 min., 0% A, 80% B and 20% C; 40. Helium was used for degassing the eluents and to prevent the formation of carbonate.

In experiments with bifidobacteria concentration of EPS in fermentation broth was calculated as the difference between the fructose concentrations before and after the hydrolysis of EPS.

2.5. EPS Isolation and purification

EPS produced in Su-MRS were isolated after removing the cells by centrifugation and addition of 2 volumes of chilled 95% ethanol to the supernatant. After incubation for 3h at 4°C, the precipitate was collected by centrifugation (8000×g, 10 min). The precipitate was dissolved in deionised water

and solids were removed by centrifugation (8000xg, 10min), the supernatant was precipitated with ethanol again. EPS were dissolved in deionized water, dialyzed (molecular weight cut off 12000-14000, Serva, Germany) against deionized water at 4°C for 24h, and then lyophilized. For experiments with bifidobacteria an EPS stock solution of 50 g l^{-1} was prepared from lyophilised EPS, autoclaved and stored at room temperature. For detection of EPS degradation after heat treatment and the determination of molecular weight, gel permeation chromatography (GPC) was used. The columns Superdex^{TM} 200 HR and Superdex^{TM} peptide (Amersham Pharmacia Biotech, Uppsala, Sweden) were coupled, and samples were eluted with 50 mM NaH_2PO_4 (pH 6.5) at a flow rate of 0.4 ml min^{-1}. EPS in doughs were partially purified from contaminating polysaccharides using gel permeation chromatography (GPC) at room temperature. Samples were injected on Superdex^{TM} 200 HR column (Amersham Pharmacia Biotech, Uppsala, Sweden) and eluted as described above. EPS were detected with a refractive index detector. Purified EPS from Su-MRS were injected on Superdex^{TM} 200 HR column to determine the elution volume of EPS. EPS from *L. sanfranciscensis* eluted at 6 ml, corresponding to an apparent molecular weight of about 2 x 10^6 Dalton. Gel filtration HMW and LMW calibration kits (Amersham Pharmacia, Uppsala, Sweden) were used for calibration of the GPC column. For determination of EPS amount in fermentations carried out in fermenter EPS were isolated from 10ml fermentation broth as described above.

The resistance of EPS to hydrolysis at pH 2 was investigated in HCl-buffer containing the following components per litre: NaCl, 8g, KCl, 0.2g; $Na_2HPO_4.2H_2O$, 8.25g; NaH_2PO_4, 14.35g; $CaCl_2.2H_2O$, 0.1g; $MgCl_2.6H_2O$, 0.18g. The pH 2 was adjusted using HCl 5M.

2.6. Characterization of the oligosaccharide

Cells were removed from fermentation broth by centrifugation and the oligosaccharide was separated from the supernatant using polyspher® CH PB column (Merck, Germany). The molecular weight of the oligosaccharide was determined using gel permeation chromatography (Superdex^{TM} Peptide, Amersham Pharmacia Biotech, Sweden). Bidest. water was used as mobile phase and the determination was carried out at room temperature. Sucrose, raffinose and stachyose were used for

the calibration of the column. For the determination of the composition, the oligosaccharide purified by polyspher® CH PB and Superdex™ Peptide sequential runs was hydrolyzed with 5% perchloric acid as described above by EPS, and the monosaccharide concentrations were determined using HPLC (polyspher® CH PB column). Information about the type of the monosaccharide linkages was obtained by the treatment of the purified oligosaccharide with ß-fructosidase (yeast invertase, r-biopharm, Germany) and subsequent analysis of the monosaccharides liberated by HPLC.

2.7. Isolation of water soluble polysaccharides from wheat and rye flours

One part of each wheat (type 550: flour extraction rate 64-71%) or wohlemeal rye flours was mixed with three parts of water (w/w). Solids were removed by centrifugation for 15 min at 4000xg. Polysaccharides in the supernatant were precipitated in the same way like EPS. Stock solutions of each lyophilised polysaccharide (25 g l^{-1}) were prepared and autoclaved, solid components were removed by centrifugation and the supernatant was used as polysaccharide stock solution. In experiments with bifidobacteria concentrations of polysaccharides from rye- or wheat-WSP were calculated as the difference between the monosaccharides concentration before and after the hydrolysis of WSP.

2.8. Hydrolysis of polysaccharides

For hydrolysis of EPS solution and EPS in supernatant of fermentation broth samples were incubated for 2h at 80°C in the presence of 5% (v/v) perchloric acid (70% w/v). Hydrolysis of dried flour polysaccharides and polysaccharides isolated from fermented doughs were carried out by incubating the lyophilised samples for 2h in 1M H_2SO_4 at 80°C. It was verified by GPC that these hydrolysis conditions quantitatively converted EPS and flour polysaccharides to monosaccharides. Monosaccharides concentration were determined using HPLC as described above.

2.9. Preparation of doughs and bread

Wholemeal rye flour and wheat flour type 550 were used to prepare doughs in yield of 200 (dough yield; [mass of dough/mass of flour]*100). For inoculation of 200 g dough, 10 ml overnight culture of *L. sanfranciscensis* in Su-MRS were harvested by centrifugation at 8000×g for 5 min,

resuspended in 5ml physiological salt solution, and added to the dough. Control doughs were prepared with chloramphenicol (Serva, Germany) and erythromycin (Sigma, USA) to inhibit microbial growth, and pH was adjusted with 90% lactic acid to 4 (Riedel-deHaën, Germany). The dough formulas for wheat and rye doughs are given in table 3. All doughs were incubated at 30°C. Dough samples were taken for Analysis. After addition of 200% (w/w) of water to the dough sample, solid constituents were removed by centrifugation at 8000g for 10 min. Polysaccharides in the supernatant were precipitated with ethanol, dialyzed and lyophilised as described by EPS. The amount of EPS in dough or bread was determined on the basis of fructose released by the acid hydrolysis of polysaccharides extracted from dough or bread. The fructose concentration was compared with that of control doughs (dough without inoculation with lactobacilli and dough inoculated with the non-EPS-producing strain LTH2581).

Table 3. Composition of the wheat and rye doughs (all amounts are in g)

	wheat doughs										rye doughs				
dough	**W I**	**W II**	**W III**	**W IV**	**W V**	**W VI**	**W VII**	**W VIII**	**W IX**	**W X**	**R I**	**R II**	**R III**	**R IV**	**R V**
flour[a)]	100	100	100	100	100	100	100	100	100	100	200	200	200	200	200
water	100	100	100	100	100	100	100	100	100	100	200	200	200	200	200
C_3-sucrose[c)]	0	0	4	8	12	12	0	0	0	0	0	0	0	16	0
C_4-sucrose [c)]	0	0	0	0	0	0	4	8	12	12	0	16	16	0	16
chloramphenicol	0	0.02	0	0	0	0.02	0	0	0	0.02	0	0.04	0.04	0	0
erythromycin	0	0.01	0	0	0	0.01	0	0	0	0.01	0	0.02	0.02	0	0
lactic acid to pH 4	-	+	-	-	-	+	-	-	-	+	-	-	+	-	-
starter culture[b)]	+	-	+	+	+	-	+	+	+	-	+	-	-	+	+

[a)] wheat flour type 550 for wheat doughs and wholemeal rye flour for rye doughs

[b)] *L. sanfranciscensis* LTH2590 washed in tap water and inoculated to a cell count of 1 x 10^7 cfu g^{-1}

[c)] sucrose additions correspont to 20, 38, and 57 g kg^{-1} or53, 106, and 160 mmol kg^{-}

To evaluate the ability of EPS to withstand the baking process control dough, doughs with 20% preferment doughs and dough with EPS addition were prepared. For preparation of sourdoughs the EPS-producing strain *L. sanfranciscensis* LTH2590 und non-EPS-producing strain LTH2581 were used. Preferment doughs composed of 100g wheat flour type 550, 100g water and 10g sucrose and were fermented for 18h with *L. sanfranciscensis* LTH2590 or LTH2581. The formula of the bread doughs are shown in Tab. 4.

Table 4. Composition of bread doughs (all amounts are in grams)

	Yeast dough (I)	Dough with EPS-Addition (II)	Dough with 20% preferment dough LTH2581 (III)	Dough with 20% preferment dough LTH2590 (IV)
Wheat flour 550	500	500	400	400
Tap water 30°C	300	300	200	200
Preferment dough LTH2581	0	0	200	0
Preferment dough LTH2590	0	0	0	200
EPS	0	5	0	0
Bakery yeast	25	25	25	25
Salt	7.5	7.5	7.5	7.5
Sucrose	10	10	0	0

Doughs were incubated for 20 min at 30°C and baked at 200°C for 45-55 min. Breads were dried for 16h at 60°C. For determination of EPS in dough 15 g dough were mixed with 30 g dest. water and centrifuged for 10 min. at 6000 x g. The polysaccharides in the supernatant were precipitated by addition of ethanol (200% v/v), the resulting pellet was dissolved in water und dialysed (molecular weight cut off 12000-14000, Serva, Germany) at 4°C for 24h and subsequently lyophilised. Dried polysaccharides were dissolved in 5 ml dest. water, hydrolysed as described above and analysed using HPLC. For the determination of EPS in bread 70 g dry crumb were homogenized with 210 g

dest. water using Ultra-turax and the suspension was treated with amylase (100units/sample) for 2h at 60°C. After centrifugation (15 min. at 6000 x g) the supernatant was subsequently treated as described for dough samples.

2.10. Degradation of polysaccharides by bifidobacteria and lactobacilli

For the experiments with EPS and polysaccharides from wheat and rye, mMRS and Bifido-medium were prepared without sugars. The media components for 1 litre were dissolved in 800ml deionized water and autoclaved. Various parts (v/v%) of polysaccharide stock-solutions (EPS, wheat- or rye-polysaccharides) were added. During growth of the organisms, samples were taken for determination of the pH, the optical density (measured at 578nm against air), and for subsequent HPLC analysis of metabolites. In pH-regulated fermentations, the pH were kept in the range 5.50-6.50 by the addition of NaOH 5M. Fermentations with dialysed EPS were performed in duplicate with strains *Bifidobacterium breve*, *B. bifidum* and in triplicate with *B. adolescentis* and *B. infantis*. Fermentations with rye-WSP were carried out in duplicate. The coefficient of variation between two fermentations was generally in the range of 5-15%.

2.11. Determination of carbon isotope ratio

The $\delta^{13}C$-value of a compound indicates its content of the ^{13}C isotope relative to the V-PDB standard ($[^{13}C]/[^{12}C]=0.0112372$). Because the ^{12}C isotope is enriched during photosynthesis, carbohydrates have negative $\delta^{13}C$-values. In accordance with the different photosynthetic pathways employed by C_3 and C_4 plants, the $\delta^{13}C$-values of carbohydrates from these plants differ (Schmidt, 1986). The ^{13}C-analysis for EPS and for polysaccharides isolated from dough was performed on CO_2 directly obtained by quantitative combustion of the samples. $\delta^{13}C$-values were then determined using isotope ratio mass spectrometry with double gas inlet system, which measures in alternation the sample and a standard gas (Winkler et al, 1980). The amount of the sample was 2-3 mg. The laboratory working standard was wheat starch ($\delta^{13}C$-value -24.60), which was calibrated with NIST-22 with a $\delta^{13}C$-value of -29.80 (Koziet et al, 1993). To determine the $\delta^{13}C$-values of glucose and fructose in C_3- and C_4-sucrose, a solution (20g/l) of each sucrose was hydrolysed with 1M

H_2SO_4, monosaccharides were obtained after preparative separation with HPLC, lyophilised and measured as described above. Isotope measurements were kindly carried out by Andreas Rossmann, Isolab GmbH, Schweitenkirchen.

2.12. High pressure treatment

An overnight culture of *L. sanfranciscensis* LTH2581 grown on mMRS4 or mMRS4 without fructose was sub-cultured with 1% inoculum in mMRS4 or mMRS4 without fructose, respectively. LTH2590 was grown and sub-cultured with 1% inoculum in Su-MRS4. Late stationary cells were harvested by centrifugation and resuspended in 0.5 volumes of the culturing medium, unless otherwise indicated. This cell suspension was transferred to 2 ml screw reaction tubes, sealed with parafilm avoiding enclosure of air and pressurizing liquid. The high pressure (HP) metabolism and inactivation kinetics of *L. sanfranciscensis* were investigated in HP-autoclaves at 30°C. Compression and decompression rates were 200 MPa min^{-1}. Samples were taken after decompression for determination of viable cell counts, ethanol extraction and the determination of metabolites by HPLC.

3. Results

3.1. Effect of fructose on the utilization of sucrose

The EPS production from sucrose by *L. sanfranciscensis* LTH2590 was previously observed in media containing autoclaved sucrose where a part of sucrose was hydrolysed (Korakli et al. 2000). To evaluate the ability of *L. sanfranciscensis* LTH2590 to metabolise sucrose in the absence of monosaccharide (fructosyltransferase acceptor) maximum growth rates were determined in media containing various contents of sucrose and fructose sterilized by filtration. As shown in Fig. 1, no growth was observed in the absence of fructose at sucrose concentrations ranging from 0 to 133 g l^{-1}. The addition of 4.7 g l^{-1} fructose resulted in maximum growth rate of 0.49 h^{-1} at a sucrose concentration of 2.3 g l^{-1}, indicating that fructose, (and possibly other monosaccharides), are essential for sucrose utilization by *L. sanfranciscensis* LTH2590. The further increase of fructose concentration had no effect on the maximum growth rate.

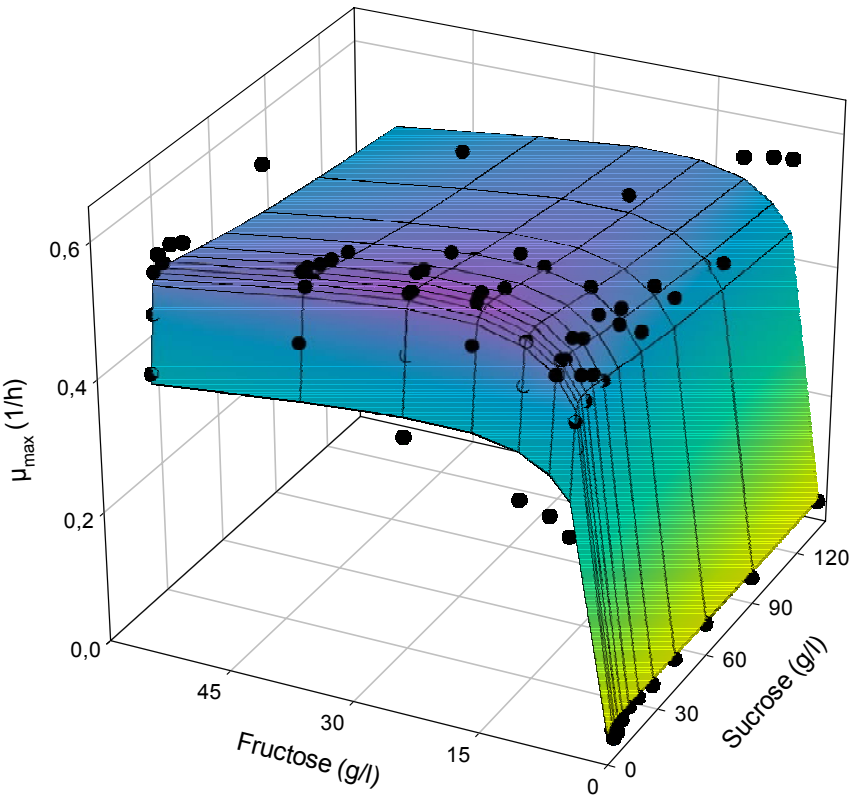

Figure 1. Effect of fructose and sucrose concentration on the growth rate of *L. sanfranciscensis* LTH2590

3.2. Kinetic of sucrose metabolism and EPS production in Su-MRS

The kinetics of sucrose metabolism and EPS production by *L. sanfranciscensis* LTH2590 as well as the end products were determined for all fermentations at sucrose concentrations ranging from 20-160 g l^{-1} and at fructose concentrations of 5 or 15 g l^{-1}. Figure 2 shows as an example the kinetic with 60 g l^{-1} sucrose and 5 g l^{-1} fructose. Cells grew to $1*10^{+9}$ cfu ml^{-1} within 20h. The EPS production was coupled to the growth of the cells and about 10 g l^{-1} EPS were produced. The end products of sucrose metabolism were EPS, mannitol, lactate and acetate or ethanol and the carbon recovery was 93%.

The amount of mannitol produced was higher than the amount of free fructose available at the beginning of the fermentation, demonstrating the ability of *L. sanfranciscensis* LTH2590 to hydrolyse sucrose into glucose and fructose. In figure 3 the kinetics are shown of fructose consumption and mannitol production at two different fructose concentrations. The hydrolysis of sucrose to glucose and fructose by *L. sanfranciscensis* LTH2590 was observed only when the free fructose was almost consumed (Fig. 3). The concentration of free fructose consumed and that one of mannitol produced were identical as long as free fructose was available, indicating that the mannitol produced originates from free fructose only and no fructose is liberated from sucrose. The sum of free fructose and mannitol produced increased only after the free fructose was consumed, indicating that fructose was generated from the sucrose only when free fructose almost completely consumed. The activation of sucrose hydrolysis by *L. sanfranciscensis* LTH2590 upon fructose depletion was observed in fermentations with 5 and 15 g l^{-1} fructose and at all sucrose concentrations tested in this work (data not shown).

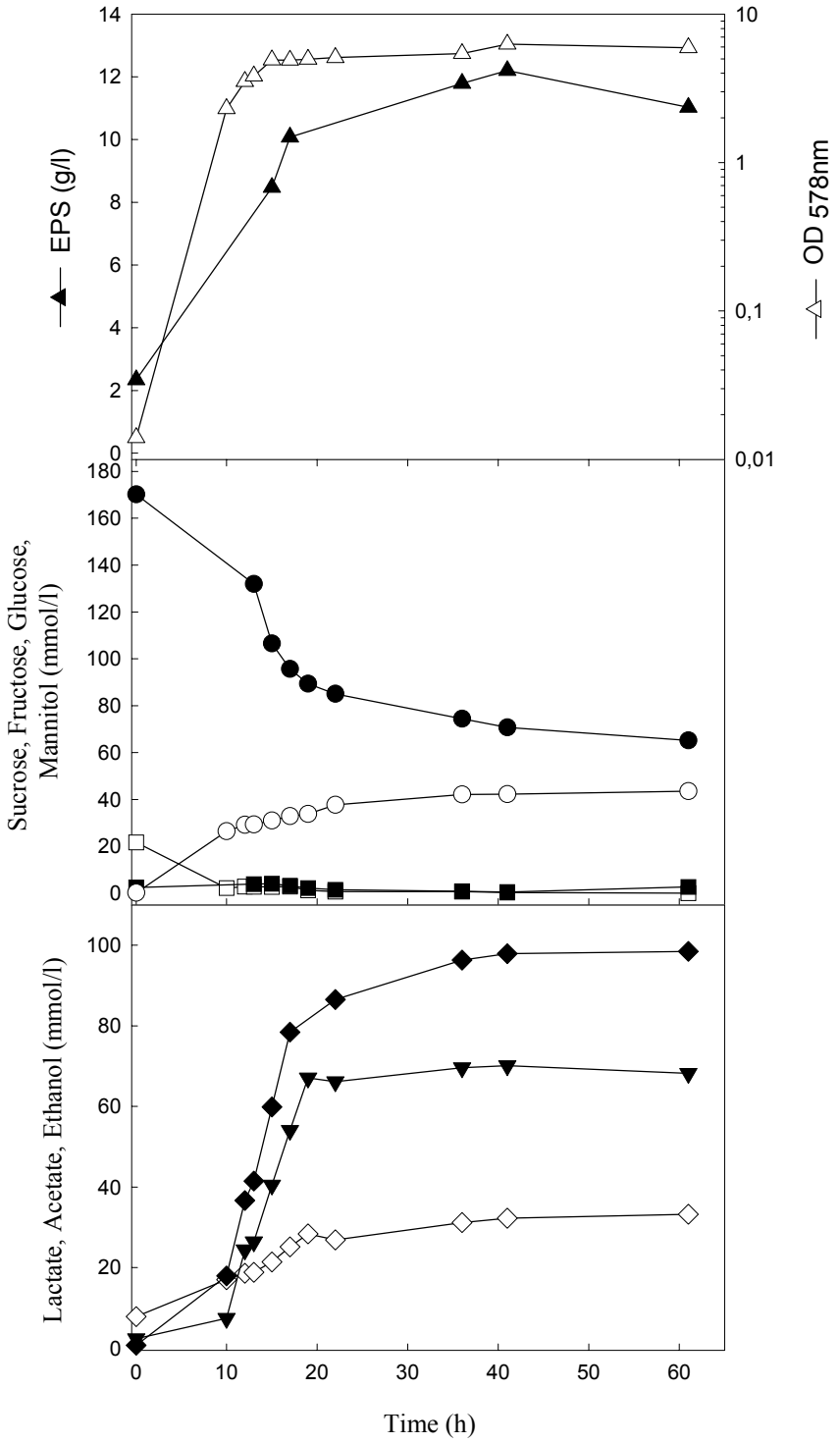

Figure 2. Kinetics of microbial growth, EPS production and fermentation products of *L. sanfranciscensis* LTH2590 on Su-MRS4 containing 60 g l^{-1} sucrose and 5 g l^{-1} fructose at pH 5.6. (Δ) OD_{578nm}, (▲) EPS, (●) sucrose, (❍) mannitol, (□) fructose, (■) glucose, (◆) lactate, (▼) ethanol, (◊) acetate.

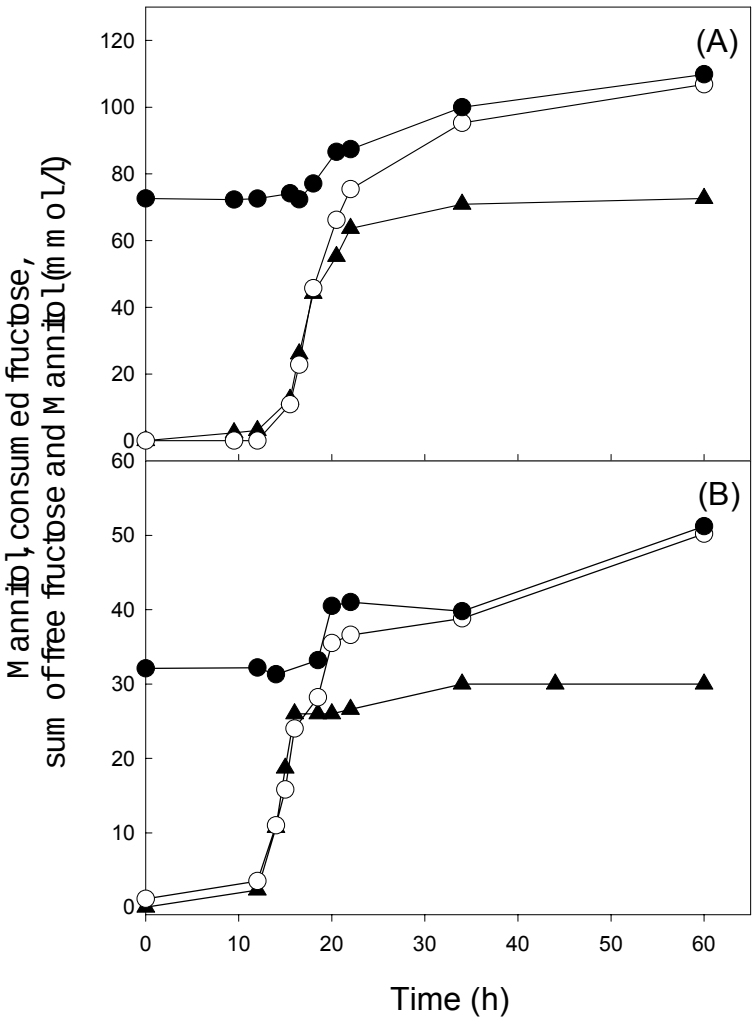

Figure 3. Effect of free fructose amount on the hydrolase activation by *L. sanfranciscensis* LTH2590 in fermentations with (A) 60 g l^{-1} sucrose and 15 g l^{-1} fructose, (B) 90 g l^{-1} sucrose and 5 g l^{-1} fructose. Free fructose consumed (▲), mannitol (❍) and the sum of mannitol and free fructose (●).

3.3. Characterisation and properties of EPS

L. sanfranciscensis LTH2590 produces EPS in media containing sucrose. The composition of dialysed EPS after hydrolysis was 99.30% fructose and 0.70%±0.40 glucose (n=10). After the treatment of pure fructose aqueous solution under the same hydrolysis conditions used for EPS, 0.50% glucose was determined. This result confirms that the EPS produced by *L. sanfranciscensis* LTH2590 is a homopolymer composed of fructose. The molecular weight of dialysed EPS is estimated to be >2x10^6 Dalton (figure 4). Autoclaving of EPS in phosphate-buffer resulted in a partial degradation of the high molecular chains into smaller chains with molecular weight ranging

from 10^4 to 10^6 Dalton. Treatment of aqueous EPS-solution with HCl-buffer pH 2 at 37°C for 1h showed that only 3.30%±2.70 of EPS were hydrolysed.

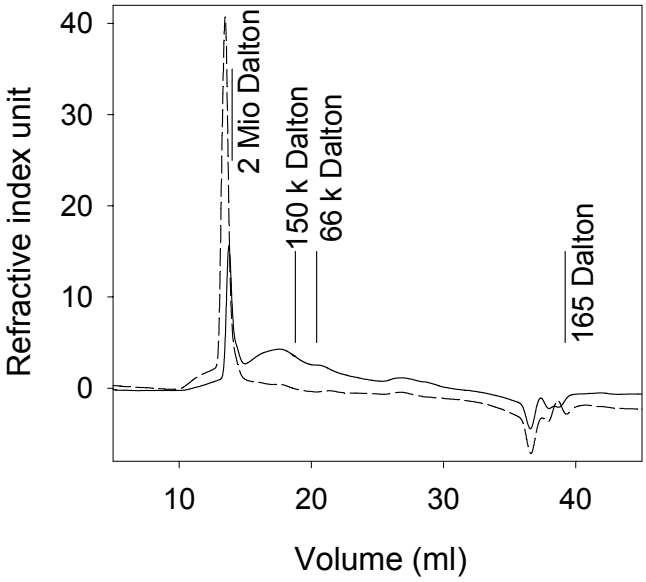

Figure 4. Separation of EPS by GPC. The chromatogram of heat treated EPS (121 °C, 20 min, solid line) is compared to untreated EPS (dotted line).The molecular size of the external calibration standards is indicated.

3.4. Effect of sucrose concentration on the EPS production

To evaluate the effect of initial sucrose and fructose concentration in medium on the yield of EPS and end products formed by *L. sanfranciscensis* LTH2590 fermentations with sucrose concentrations ranging from 20-160 g l^{-1} and at fructose concentration of 5 or 15 g l^{-1} were carried out at pH 5.6. The amount of sucrose consumed increased from 68 mmol l^{-1} at a sucrose concentration of 20 g l^{-1} to 245 mmol l^{-1} at a sucrose concentration of 160 g l^{-1} (Tab. 5). The increase of lactate production was not proportional to the sucrose consumption. The amounts of mannitol produced ranged from 98-111 mmol l^{-1} in fermentation with 15 g l^{-1} fructose and 20-160 g l^{-1} sucrose, and in fermentations with 5 g l^{-1} fructose mannitol concentrations ranged from 38-49 mmol l^{-1}. Acetate production was independent of the sucrose concentration and more acetate was produced in fermentations containing 15 g l^{-1} fructose. Glucose accumulation in the medium was

only observed in fermentations with 15 g l^{-1} fructose and at sucrose concentrations of 60 g l^{-1} or higher (Tab. 5).

Table 5. Concentrations of substrates and end products of fermentations by *L. sanfranciscensis* in media with various sucrose and fructose contents

	15 g/l Fructose						5 g/l Fructose		
Sucrose (g l^{-1})	20[a)]	30	60	70[a)]	120[a)]	160[a)]	20[a)]	60	90[a)]
Measured sucrose at t=0 (mmol l^{-1})	69	92	170	214	356	481	56	170	263
Consumed sucrose at t=end (mmol l^{-1})	-68[b)]	-91	-109	-112	-160	-245	-58	-105	-141
Lactate (mmol l^{-1})	74	86	73	97	103	137	62	98	67
Acetate (mmol l^{-1})	50	55	57	56	52	62	25	25	24
Mannitol (mmol l^{-1})	98	107	111	100	99	104	38	43	49
Fructose (mmol l^{-1})	-75[b)]	-86	-73	-72	-79	-81	-22	-22	-30
Glucose (mmol l^{-1})	0	0	12	6	13	6	0	0	0
Ethanol (mmol l^{-1})	29	25	14	39	40	65	41	66	38
Glucose recovery[c)] (%)	108	95	78	87	73	58	104	93	48

[a)] Values in these fermentations are representative for two independent experiments, the coefficient of variation between two fermentations was generally in the range of 5-12%. [b)] Negative values indicate consumption, positive values indicate production of metabolites. [c)] recovery of the glucose moiety from sucrose consumed in the end products glucose, lactate and acetate or ethanol.

The glucose recoveries (lactate produced/glucose consumed) ranged from 95-108% in media containing up to 30 g l^{-1} sucrose (Tab. 5). Taking into account the experimental error, recoveries in the range of 90-110% can be considered quantitative. In fermentations with sucrose concentrations greater than 60 g l^{-1} the glucose recoveries were lower. In medium containing 160 g l^{-1} sucrose the carbon recovery was only 58%. The glucose recoveries decreased the higher the sucrose concentration, despite the fact that the EPS isolated from fermentation at any sucrose concentrations consisted exclusively of fructose. The low glucose recovery observed at high sucrose concentrations correlated well to the peak area of an unknown metabolite produced during the sucrose fermentation (Fig. 5). The acid hydrolysis of the supernatant upon fermentation resulted in glucose and fructose recoveries of more than 92% (data not shown) and in disappearance of the oligosaccharide peak in HPLC-chromatogram (Fig. 5, A). The retention time of the oligosaccharide peak produced by *L. sanfranciscensis* was the same as that of 1-kestose (15 min.) using polyspher® CH PB, and the treatment of the sample with ß-fructosidase or the acid hydrolysis resulted in complete degradation of the oligosaccharide (Fig. 5, A). The same results could be observed using a second chromatography method, an anion-exchange chromatography with integrated amperometric detection (Fig. 5, B). The molecular weight of the oligosaccharide determined using gel permeation chromatography after separation of the oligosaccharide was 597 ± 27. The hydrolysis of the purified oligosaccharide separated from supernatants (n=3) of different fermentations with various sucrose concentrations revealed a glucose:fructose ratio of 1:2. The molecular weight and glucose:fructose ratio indicate that the oligosaccharide is a trisaccharide. The comparison of the retention time of the oligosaccharide and that of 1-kestose using two different chromatography methods revealed that the oligosaccharide or the major oligosaccharide produced by *L. sanfranciscensis* LTH2590 is 1-kestose.

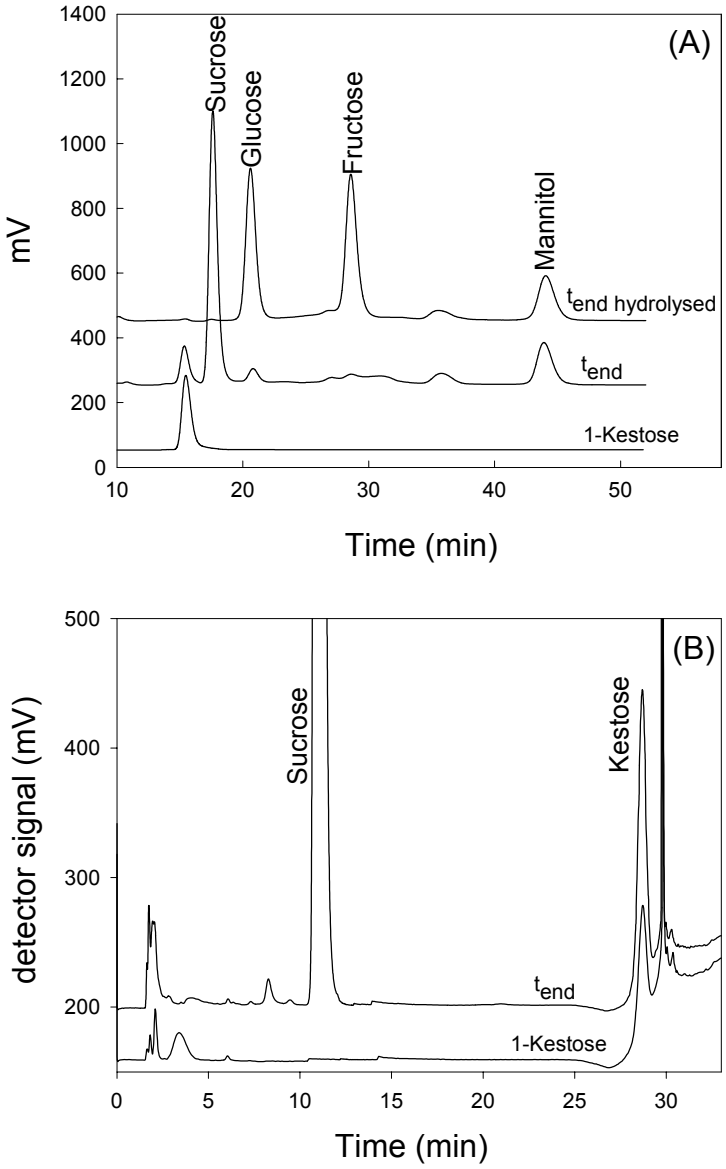

Figure 5. Comparison of HPLC chromatograms using (A) polyspher® CH PB column and (B) anion-exchange chromatography. The chromatograms at the end of a sucrose fermentation and after acid hydrolysis of supernatant compared with that of 1-kestose are shown.

The effect of sucrose concentration on the EPS production, sucrose hydrolysis and kestose formation is shown in Fig. 6. The amount of EPS produced increased the higher the sucrose concentration in medium. The fructose concentration showed no significant effect on the EPS production. About 40 g l^{-1} EPS were produced in medium containing 160 g l^{-1} sucrose (Fig. 6, A). The amount of mannitol produced from sucrose was virtually independent of fructose and sucrose

concentrations in all fermentations (24 ± 6 mmol l^{-1}, n=15), demonstrating that the absolute hydrolase activity is independent of the sucrose concentration (Fig. 6, B). However, the higher the sucrose concentration in medium, the higher the amount of sucrose consumed (table 5), this means that the ratio of the mannitol produced from sucrose to consumed sucrose (relative hydrolase activity) decreased with increasing sucrose concentrations (Fig. 6, B). Almost 40% of the fructose from the sucrose consumed were reduced to mannitol at a sucrose concentration of 20 g l^{-1}, whereas at sucrose concentration of 160 g l^{-1} only 10% of the fructose from the sucrose consumed were reduced to mannitol (Fig. 6, B). The kestose production increased the higher the sucrose concentration in medium and no significant effect of fructose concentration on the production of kestose could be observed (Fig. 6, C).

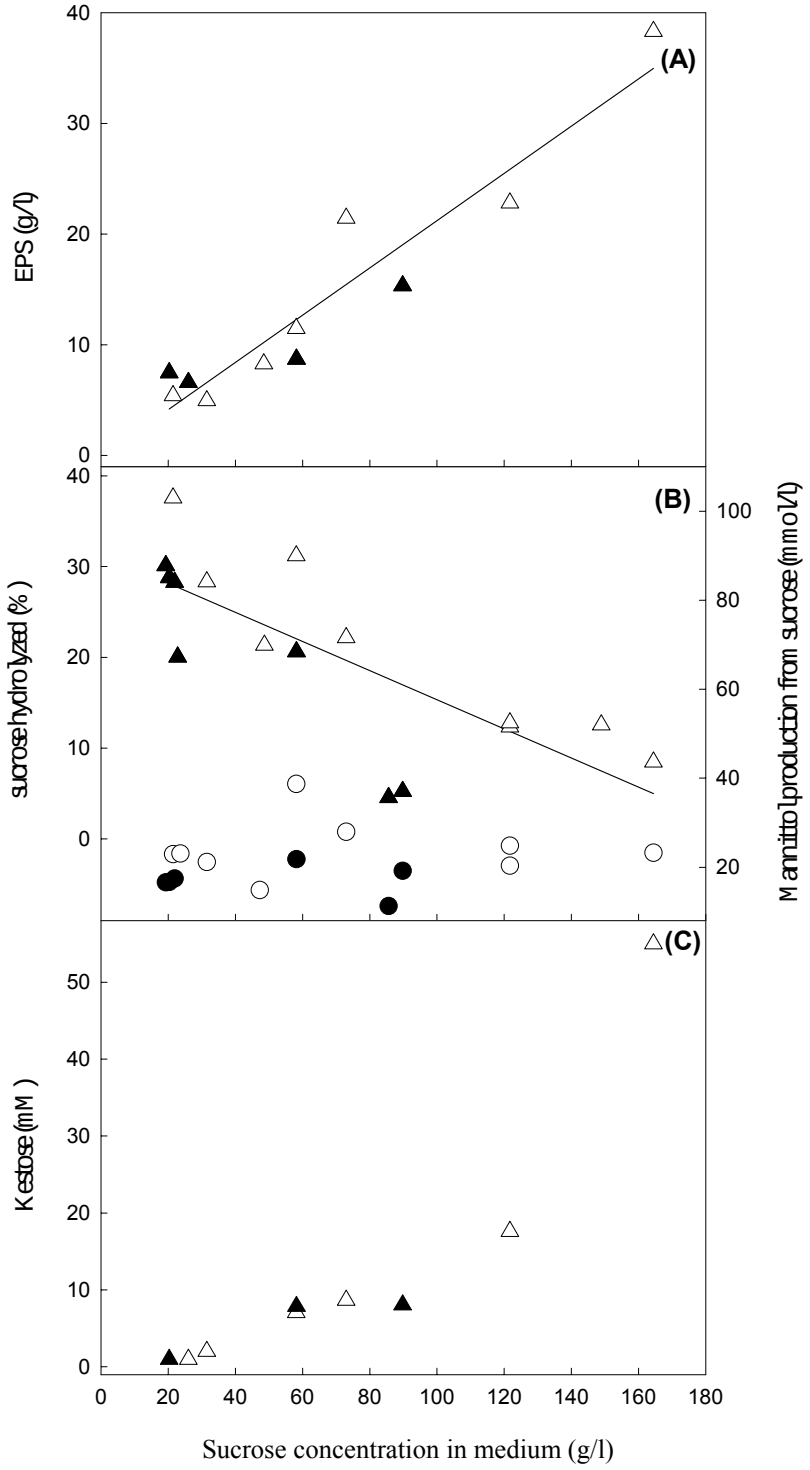

Figure 6. (A). Effect of sucrose concentration on the amount of EPS produced by *L. sanfranciscensis* LTH2590 in Su-MRS containing 5 g l^{-1} (▲) and 15 g l^{-1} (Δ) fructose at pH 5.6. **(B)**. Effect of sucrose concentration on the hydrolysis of sucrose by *L. sanfranciscensis* in Su-MRS containing 5 g l^{-1} (▲) and 15 g l^{-1} (Δ) fructose at pH 5.6 and on mannitol production from sucrose in Su-MRS containing 5 g l^{-1} (●) and 15 g l^{-1} (❍) fructose. **(C)**. Effect of sucrose concentration on the amount of kestose produced by *L. sanfranciscensis* in Su-MRS containing 5 g l^{-1} (▲) and 15 g l^{-1} (Δ) fructose at pH 5.6

3.5. In situ production of EPS during sourdough fermentation

To verify EPS production from sucrose by *L. sanfranciscensis* LTH2590, as well as to demonstrate the changes of δ^{13}C-values through the EPS production in doughs, δ^{13}C-values of C_3- (sugar beet), C_4-Sucrose (sugar cane) and purified EPS were measured. C_3 and C_4 sucrose had δ^{13}C-values of –25.65 and –11.64, respectively. Since the EPS predominately consists of fructose it was important to know whether the δ^{13}C-value of sucrose is the same in its constituents glucose and fructose. Glucose and fructose from C_3 sucrose had δ^{13}C-values of –25.11 and –25.87, respectively, and glucose and fructose from C_4 sucrose had δ^{13}C-values of –12.23 and –11.40. Thus, the differences in δ^{13}C-values between fructose and glucose in either C_3- or in C_4 sucrose are very small. The δ^{13}C-values of EPS produced by *L. sanfranciscensis* during growth in culture media with C_3- and C_4-sucrose followed by dialysis were –26.69 and –17.28. Comparison of these values with the δ^{13}C-values of sucrose shows that the ^{12}C carbon isotope was enriched in the fructose moieties used by *L. sanfranciscensis* for EPS formation, probably because of isotopic partitioning between EPS and other products of sucrose metabolism. During growth of *L. sanfranciscensis* in Su-MRS, the fructose moiety of sucrose is either incorporated into EPS or reduced to mannitol. The glucose moiety is used as carbon source or left unused and accumulated in the medium. The EPS yield from sucrose and the difference between δ^{13}C-values of EPS from C_3- and C_4-sucrose was therefore large enough to expect an impact of EPS production from C_3 or C_4 sucrose in dough on the δ^{13}C-values of overall water soluble polysaccharides.

3.5.1 Carbohydrate Metabolism in wheat and rye sourdoughs

To monitor the microbial metabolism in inoculated and control doughs, substrates and fermentation products were determined (Tab. 6). It was verified by plate counts that the fermentation flora was identical with the starter culture. Contaminants were not detectable and thus accounted for less than 0.1% of the total microflora. In control doughs without inoculum, the total cell counts were less than 10^4 cfu g^{-1} throughout the fermentation, excluding an effect of microbial metabolism on carbohydrates in dough. In control wheat doughs maltose and glucose were accumulated after 24h

in all doughs because of the amylase and α-glucosidase activity of flours. In doughs with sucrose contents of 3.5, 46, 104, and 152 mmol/kg dough, the amount of sucrose metabolised were 2.5, 38, 62, and 77 mmol/kg dough, respectively. The strain LTH2590 is unable to use fructose as carbon source but converts fructose to mannitol with concomitant oxidation of NADH to NAD^+. However, in doughs containing additional sucrose, the amount of sucrose used was greater than the amount of mannitol formed. The accumulated fructose in dough does not account for the molar difference between sucrose metabolised and mannitol produced. Therefore the differences between sucrose consumed and metabolites formed, 2.0, 13, and 27 mmol kg^{-1} fructose in doughs containing 20, 38, and 57 g kg^{-1}sucrose, respectively, indicate EPS production from sucrose. Apparently increased sucrose concentration led to increased EPS production in dough and the proportion of sucrose hydrolysed to the alternative end product fructose was decreased. The lactate and acetate concentrations varied between 58-63 and 19-24 mmol/kg dough respectively, resulting in pH of 3.78-3.85. The metabolic activity was stopped at this pH, although substrates were still available. In dough without added sucrose, the release of fructose from flour oligo- and polysaccharides allowed for the formation of 13 mmol kg^{-1} mannitol. Enzymatic liberation of 8.4 mmol kg^{-1} fructose during the fermentation was observed in the control dough without sucrose addition (dough WII). Reduction of fructose to mannitol allows *L. sanfranciscensis* to produce acetate instead of ethanol in a molar ratio of 2 mol mannitol to 1 mol acetate. Accordingly, increased mannitol concentration in dough with sucrose addition were accompanied by increased acetate and decreased ethanol production, and the molar ratio of mannitol to acetate was approximately 2:1 in all doughs. In doughs containing sucrose, glucose concentrations were substantially higher than in control dough. This indicates that a part of the glucose liberated from sucrose by *L. sanfranciscensis* was not used as carbon source but accumulated in doughs.

Table 6. Concentrations of substrates and products in wheat sourdoughs (mmol kg^{-1})

Dough		pH	Sucrose	Maltose	Glucose	Fructose	Mannitol	Lactate	Acetate	Ethanol
W I	t=0	6.20	3.6	17	1.6	0.2	1.1	0.0	0.0	0.0
	t=24h	3.87	1.2	54	29	0.4	13	72	10	64
W II	t=0	3.96	3.5	16	2.0	1.0	1.4	50	0.0	0.0
	t=24h	4.05	3.3	63	34	8.4	1.5	48	0.0	0.0
W III	t=0	6.31	46	14	0.0	0.8	0.0	0.0	0	0.0
	t=24h	3.81	7.8	54	60	1.1	37	63	19	44
W IV	t=0	6.31	104	15	0.0	0.0	0.0	0.0	0.0	0.0
	t=24h	3.78	42	49	80	6.0	43	60	21	39
W V	t=0	6.27	152	13	0.0	0.0	0.0	0.0	0.0	0.0
	t=24h	3.85	75	50	82	6.6	43	61	24	36
W VI	t=0	3.96	159	13	0.0	0.0	0.0	55	0.0	0.0
	t=24h	3.84	130	39	33	14	0.0	57	0.0	0.0
W VII	t=0	6.19	51	20	1.3	0	0	0	0	0
	t=24h	3.81	6	50.8	64	4.8	36	62.2	18.5	44.9
W VIII	t=0	6.31	103	17.2	0	0	0	0	0	0
	t=24h	3.85	39	50.7	80	11.2	40	59.1	20.7	37.4
W IX	t=0	6.20	166	18.8	0	1.2	0	0	0	0
	t=24h	3.85	67.5	47.3	85	15	43	57.5	21.2	35.3
W X	t=0	3.94	150	18.3	0	0	0	55	0	0
	t=24h	3.93	135	45.5	37	15.5	0	51.4	0	0

Wholemeal rye flour has a higher pentosan and polysaccharide content than wheat flour. To estimate the influence of these polysaccharides on the sourdough fermentation, the kinetics of the main fermentation products in wholemeal rye doughs were determined. The results for doughs R I (no sucrose addition), R III (sterile acidified dough with sucrose addition) and R V (addition of C_4 sucrose) are shown in Fig. 7. The kinetics of substrates and fermentation products in the dough with added C_3-sucrose (R IV) was comparable with that of C_4-sucrose and the differences were within experimental error (data not shown). In the control dough R III acidified to pH 4, glucose accumulation was observed (Fig. 7), whereas nearly no glucose was accumulated in the control dough R II (data not shown). The initial pH of dough R II was 6.36 and the pH fell to 6.13 after 27h of incubation. In fermented doughs, the cell number of *L. sanfranciscensis* reached 10^9 cfu g^{-1} within 8 h independent from sucrose addition. In wohlemeal rye dough without sucrose (dough R I) more mannitol was produced than in the comparable wheat dough, and this is attributed to the higher content of fructans in wholemeal rye compared to in wheat flour type 550. In rye dough with C_4-sucrose addition (dough R V) more mannitol and acetate were produced than in dough R I, where lactate and ethanol were the main end products. In the control dough (R III) glucose accumulation could be observed due to the activity of flour enzymes. The glucose liberated during the fermentation was higher in doughs with sucrose addition (R V).

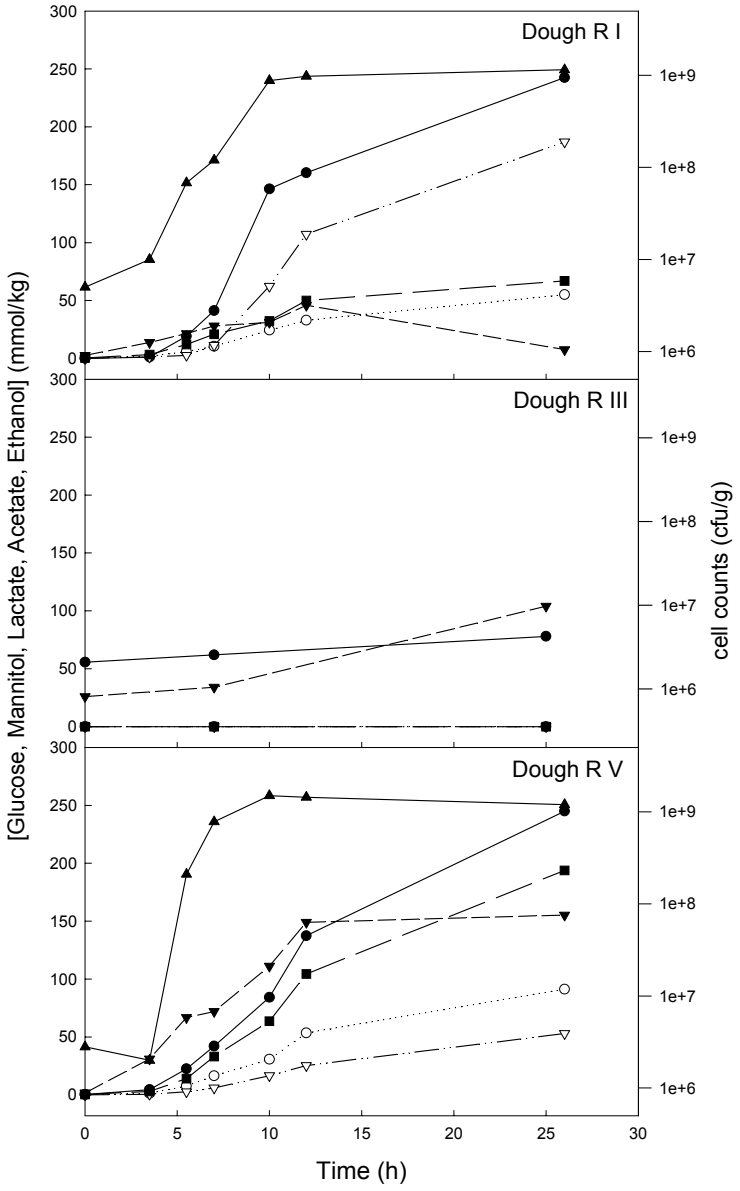

Figure 7. Kinetics of microbial growth and fermentation products in rye sourdoughs. (▼) glucose, (■) mannitol, (●) lactate, (❍) acetate, (▽) ethanol and (▲) CFU.

3.5.2 Composition of water soluble polysaccharides in wheat and rye

The hydrolysis of wheat water soluble polysaccharides following by HPLC analysis demonstrated that these polysaccharides are composed of glucose, arabinose, xylose and fructose. The concentrations of arabinose and xylose were increased during the fermentation in all doughs (Tab. 7). Polysaccharides in flour have a high degree of polymerization (DP), during the incubation the long chains are broken in more water soluble chains. This

degradation of polysaccharides explains the increase of the concentrations of arabinose and xylose during the incubation. This increase was also observed in the control doughs with antibiotics and therefore, this solubilization could be attributed to the activity of flour enzymes. In doughs WV and WIX with sucrose addition (Tab. 7) the concentration of fructose at t=24h in the hydrolysate of water soluble polysaccharides was substantially higher than in control doughs. In doughs WV and WIX, cereal and microbial fructans accounted for up to 30% of water soluble polysaccharides. This increased fructan concentration of water soluble polysaccharides indicated the production of EPS from sucrose.

Table 7. Composition of water soluble polysaccharides in wheat doughs

	(mmol kg^{-1})			
Sample	**glucose**	**fructose**	**xylose**	**arabinose**
0h, all doughs	2.4	2.4	10.8	6.6
24h, W I	3.3	2.0	19.5	16.8
24h, W II	2.0	1.5	21.5	16.3
24h, W V	1.8	14.7	18.6	16.0
24h, W IX	1.9	17.2	20.5	16.0
24h, W X	1.7	2.2	19.2	17.5

Water soluble wholemeal rye polysaccharides have a comparable composition as wheat polysaccharides (Tab. 8). Analog to wheat sourdough fermentation EPS production during rye sourdough fermentation was proven by determination of fructose concentration in hydrolyzed polysaccharides. Polysaccharides from rye doughs were composed of less than 15% fructans. In doughs R IV and R V, the fructan concentrations were higher than in the control doughs, indicating EPS production (Tab. 8). The solubilization of arabinoxylans during rye sourdough fermentations was also observed.

Table 8. Composition of water soluble polysaccharides in rye doughs

	(mmol kg^{-1})			
Sample	**glucose**	**fructose**	**xylose**	**arabinose**
0h, all doughs	2.7	0.0	6.3	2.5
26h, R I	0.4	0.2	30.8	17.7
26h, R II	0.7	0.2	25.0	15.5
26h, R III	0.4	0.6	29.2	15.4
26h, R IV	0.2	6.8	23.4	13.8
26h, R V	0.3	7.5	24.1	13.9

3.5.3 δ ^{13}C values of wheat and rye dough soluble polysaccharides

The δ ^{13}C-values of dialyzed water soluble polysaccharides from wheat sourdoughs are shown in Table 9. Addition of either C_3 or C_4 sucrose to control doughs (W VI and W X) did not affect the δ ^{13}C-values of water soluble polysaccharides as in the fermented doughs with sucrose addition. Therefore, shifts in δ ^{13}C-values of water soluble polysaccharides are attributable to microbial conversion of sucrose to high molecular weight EPS. At t=0 the mean of δ ^{13}C-values of water soluble polysaccharides was -25.37±0.16 in all doughs, as expected for C_3-plants. The difference in δ ^{13}C-value between t=0 and t=24h in the control doughs was –1.12±0.05 (mean of the 3 control doughs). In fermented doughs, decreased δ ^{13}C-values of water soluble polysaccharides were observed if C_3-sucrose was added. The difference was greater in the dough with 57 g kg^{-1} sucrose added (W V) compared to the dough with 20 g kg^{-1} sucrose added (WIII), indicating increasing production of EPS at higher sucrose concentrations. Accordingly, in fermented doughs with C_4-sucrose addition, δ ^{13}C-values of water soluble polysaccharides were higher than those of the control doughs. An enrichment of EPS from the total water soluble dough polysaccharides was achieved by gel permeation chromatography (GPC). Fructan from *L. sanfranciscensis* LTH2590 has a molecular mass of about 2 x 10^6 Dalton and water soluble wheat polysaccharides were

distributed over the range of 10^4 to 5 x 10^6 Dalton (data not shown). The elution volume of 5 to 7 ml, corresponding to a size range of about 5 x 10^5 to 5 x 10^6, was collected for further analysis. After this enrichment of the EPS in the water soluble polysaccharides, the differences in $\delta^{13}C$-values of the fermented doughs with C_3 and C_4 addition relative to the control doughs were more pronounced. The $\delta^{13}C$-value of the dough with C_3-sucrose was more negative than that one without sucrose and had nearly the same $\delta^{13}C$-value as EPS isolated from Su-MRS. Accordingly, the $\delta^{13}C$-value was higher in dough with C_4-sucrose addition. As described above, the $\delta^{13}C$-values of EPS from C_3- and C_4-sucrose were –26.69, -17.28 respectively, and the $\delta^{13}C$-value of the contaminating polysaccharides of wheat –25.84 (Tab. 9). Therefore, when the same amounts of EPS in doughs with C_3- and C_4-sucrose are produced, the change in $\delta^{13}C$-value with C_4-sucrose will be more significant than that one with C_3-sucrose.

Table 9. $\delta^{13}C$-values [‰V-PDB] of water soluble polysaccharides from wheat sourdoughs

	Dough									
	W I	**W II**	**W III**	**W IV**	**W V**	**W VI**	**W VII**	**WVIII**	**W IX**	**W X**
$\delta^{13}C$, 0h	-25.15	-25.29	-25.60	-25.52	-25.28	-25.35	-25.25	-25.44	-25.23	-25.60
$\delta^{13}C$, 24h	-26.59	-26.30	-26.69	-26.81	-27.18	-26.54	-25.77	-25.66	-25.90	-26.75
$\delta^{13}C_{24h}$-$\delta^{13}C_{0h}$	-1.44	-1.01	-1.09	-1.29	-1.90	-1.19	-0.52	-0.22	-0.67	-1.15
	After enrichment of EPS by gel permeation chromatography									
$\delta^{13}C$, 24h	-25.84	ND	ND	ND	-26.44	ND	ND	ND	-21.21	ND

Polysaccharides from rye doughs were composed of less than 15% fructans. In doughs R IV and R V, the fructan concentrations were higher than in the control doughs, indicating EPS

production (Tab. 8). This could also be confirmed by determination of δ ^{13}C-values of water soluble polysaccharides after dialysis (Tab. 10). However, the differences in δ^{13}C-values in dialyzed water soluble polysaccharides did not indicate a clear shift in δ ^{13}C-values with the exception of dough R V, and even after enrichment of EPS with GPC a significant change was only observed in the dough with C_4-sucrose (R V). The evidence for EPS production in rye doughs from C_3- and C_4-sucrose was provided by acid hydrolysis of the water soluble polysaccharides followed by preparative separation of the fructose. The δ ^{13}C-value of fructan from doughs with C_3-sucrose was lower than the corresponding values of the control and the dough without sucrose addition. The difference in δ ^{13}C-value of fructan in dough with C_4-sucrose compared to control dough was more significant.

Table 10. δ ^{13}C-values [‰V-PDB] of water soluble polysaccharides, and fructose in water soluble polysaccharides isolated from wohlemeal rye sourdoughs

	Dough			
	R I	**R III**	**R IV**	**R V**
δ^{13}C, 26h, after dialysis	-24.65	-24.62	-23.84	-23.14
δ^{13}C, 26h, after enrichment with GPC	-23.69	-23.33	-23.33	-20.34
δ^{13}C, 26h, of fructose	-21.44	-20.06	-22.00	-19.14

3.5.4 Detection of EPS in bread

To evaluate the ability of EPS to withstand the baking process experiments were carried out with yeast dough, dough with 20% preferment dough fermented with the non-EPS-producing strain *L. sanfranciscensis* LTH2581, dough with EPS addition and dough with 20% preferment dough fermented with the EPS-producing strain *L. sanfranciscensis* LTH2590. The concentration of fructose was significantly higher in doughs with EPS addition and in doughs with 20% preferment dough fermented with LTH2590 than in control doughs (Tab. 11). After the baking process the retrieval of the fructose in bread was 43% in bread

with EPS addition and 66% in bread with sponge dough fermented by LTH2590. The fructose amounts determined after hydrolysis of EPS extracted from dough or bread represent only the amount of high molecular polysaccharides because of the loss of a part of the polysaccharides with molecular weight < 12000 during the dialysis step carried out prior hydrolysis. Therefore, it is assumed that at least 43% of high molecular polysaccharides withstand the baking process.

Table 11. Concentration of glucose and fructose obtained after hydrolysis of water soluble polysaccharides isolated from various doughs and breads.

	mmol kg^{-1} wheat flour	
	Glucose	Fructose
(I) Yeast dough	6.71	1.38
(II) Dough with EPS-addition	5.75±0.70[a)]	9.65/5.33[b)]
(III) Dough+20% sourdough LTH2581	5.84	1.11
(IV) Dough+20% sourdough LTH2590	5.84±0.84[a)]	3.26/1.18[b)]
After baking process	mmol kg^{-1} dry bread	
Bread I	18.77	0.11
Bread II	17.40±3.40[a)]	3.06/2.84[b)] (43±9%)[c)]
Bread III	8.43	1.61
Bread IV	17.50±4.50[a)]	2.69/0.45[b)] (66±17%)[c)]

[a)] mean and STD of two independent experiments, [b)] Values of two independent experiments, [c)] EPS recovery in bread (%).

3.6. Metabolism of EPS by Bifidobacteria

Based on the composition of EPS produced by *L. sanfranciscensis* LTH2590 and the fact that EPS is produced during sourdough fermentation and withstand the baking process, the ability of several bifidobacteria to metabolise EPS was evaluated. The kinetic of EPS metabolism by *Bifidobacterium breve* is shown in Fig. 8. Only 27% of EPS could be metabolised after 60h. At a pH of 4.3, growth and metabolism stopped. The limitation effect of the pH on the

metabolism could be excluded by regulating the pH in the subsequent fermentations. In Fig. 9 are shown the microbial growth and fermentation kinetics of *B. breve*, *B. bifidum*, *B. infantis* and *B. adolescentis* at controlled pH. The growth of *B. breve* was significantly higher than in the fermentation without pH regulation and EPS was completely consumed. A diauxic growth was observed by all of the bifidobacteria. At the beginning of the fermentation, free fructose was metabolised and only after a second lag-phase, EPS was metabolised.

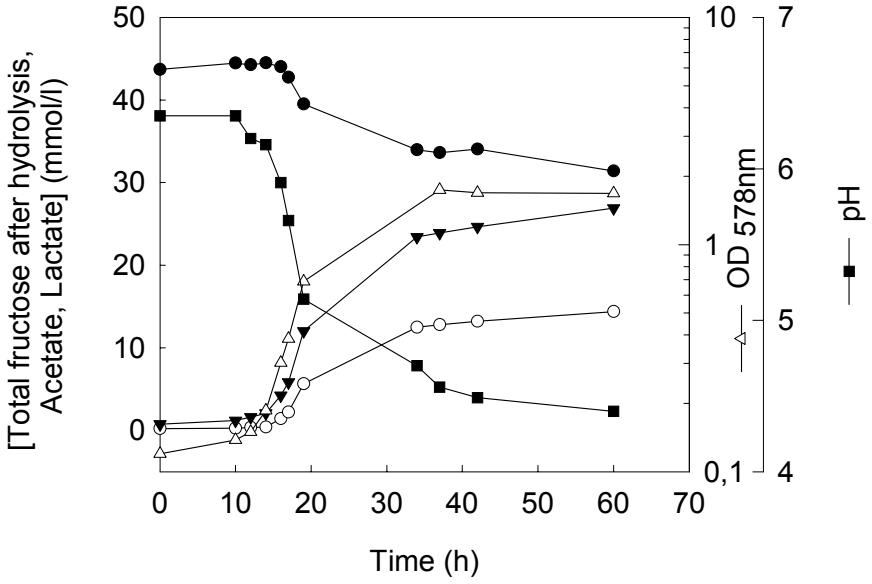

Figure 8. Kinetics of microbial growth and fermentation products of *B. breve* on EPS. (●) total fructose after hydrolysis, (❍) lactate, (▼) acetate, (■) pH, (<) OD.

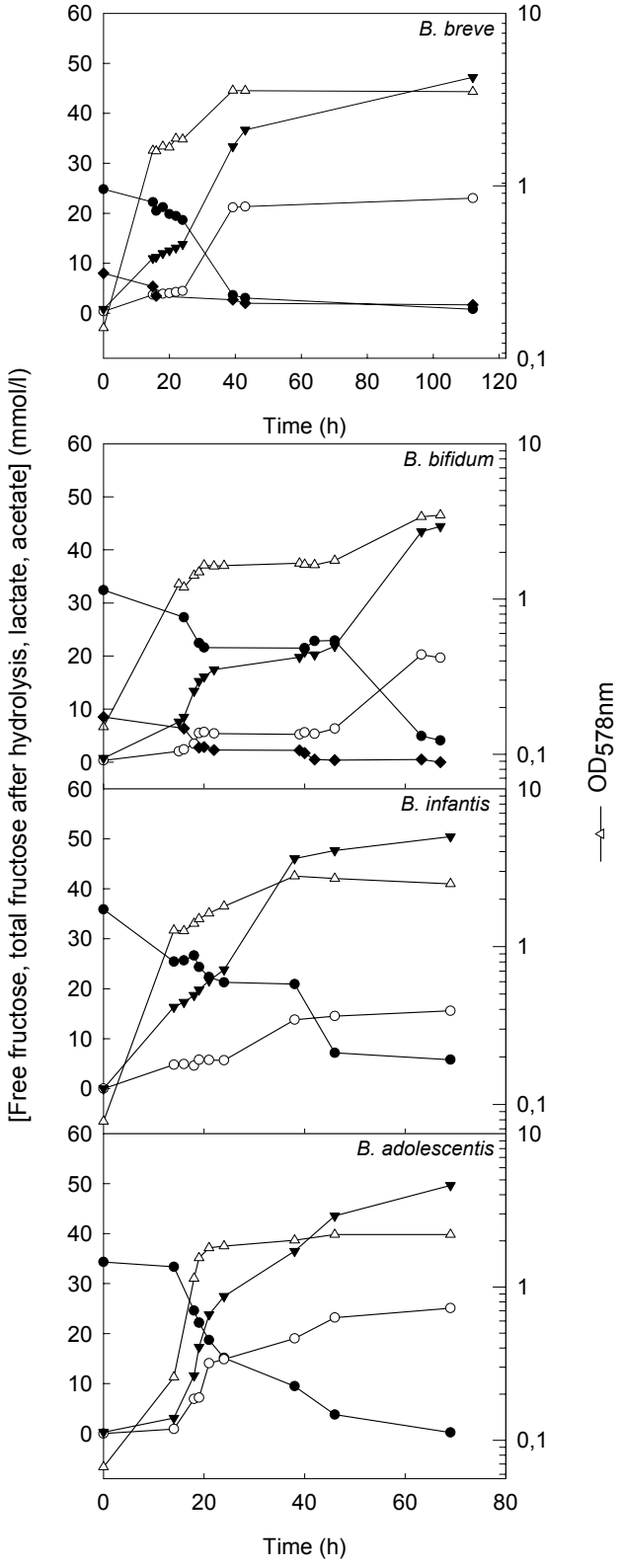

Figure 9. Kinetics of microbial growth and fermentation products of *B. breve* (n=2), *B. bifidum* (n=2), *B. infantis* (n=3) and *B. adolescentis*(n=3) on EPS in pH controlled fermentation. (◆) free fructose, (●) total fructose after hydrolysis, (❍) lactate, (▼) acetate, (<) OD. Results are representative for n independent experiments.

The concentration of the free fructose in the fermentations with *B. infantis* and *B. adolescentis* (Fig. 9) was 11 mmol l^{-1} at the beginning of the fermentation. The acetate/lactate ratio ranged from 1.8 to 2.5 (Table 12).

Table 12. Acetate/lactate ratio of bifidobacteria grown on EPS, fructose or glucose.

	molar ratio of acetate to lactate		
	Glucose-Medium	Fructose-Medium	Dialysed EPS-Medium
B. breve	1.80	1.87	2.00±0.18
B. bifidum	2.95	1.72	2.43±0.25
B. adolescentis	1.45	1.71	1.88±0.01
B. minimum	1.80	2.09	2.26
B. infantis	1.49	2.65	2.52±0.32

Data for fermentations on EPS are means ± standard deviations of two independent experiments

None of the following lactobacilli, *L. sanfranciscensis* LTH2581, *L. sanfranciscensis* LTH1729, *L. pontis* DSM8475, *L. animalis* TMW1.972, *L. brevis* DSM5622, *L. paracasei subsp. paracasei* TMW1.1183, *L. plantarum* TMW1.46, *L. salivarius subsp. salivarius* TMW1.992, *L. reuteri* TMW1.974 and *L. johnsonii* TMW1.990 showed a difference in the optical density when grown on medium containing EPS compared to media without sugar (data not shown). The failure of *L. pontis* and *L. paracasei* to metabolize EPS was confirmed by the determination of the kinetics of microbial growth and by analysis of metabolites produced during fermentations (data not shown).

The ability of different bifidobacteria to metabolise prebiotics was subject of numerous studies in the last years. However, there are few data available about the enzymes involved in the degradation of inulin or fructo-oligosaccharides by bifidobacteria. *Bifidobacterium lactis*

strains are currently used as prebiotic supplements in diverse dairy products (Bonaparte and Reuter, 1996). For better understanding of the bifidogenic nature of prebiotics the gene encoding inulin and FOS hydrolysing ß-fructofuranosidase in *B. lactis* was identified and the enzyme was herterologously expressed in *Escherichia coli* and characterised. The complete work concerning gene identification and enzyme characterisation is presented in the appendix.

3.7. Effect of sublethal high pressure on the metabolism of *L. sanfranciscensis*

The increasing use of defined starter culture instead of spontaneous fermentation in the production of fermented foods led to extensive studies about the physiology and genetics of LAB. Most of these studies focused on the response of lactic acid bacteria to environmental stressors, e. g. temperature, pH, osmotic stress and starvation.

The effect of sublethal high pressure on the metabolism of maltose or sucrose by *L. sanfranciscensis* was investigated. *L. sanfranciscensis* tolerated pressures up to 150 MPa for 3 h without appreciable loss of viability and incubation at 200 MPa decreased viable cell counts by 2 log. Application of 50 MPa did not affect maltose uptake. Incubation at 100, and 150 MPa resulted in a decrease of maltose consumption by 67% and 82%, respectively. The molar ratio of lactate produced to maltose consumed was unchanged by pressures of 100 – 150 MPa. Incubation at 100 – 150 MPa resulted in formation of lactate and acetate only, ethanol formation was not observed. *L. sanfranciscensis* treated at 100 MPa exhibited normal metabolic activity after pressurization but treatment with 150 MPa resulted in an inhibition of maltose metabolism up to 3 h post-treatment. The isotope ratio analysis of carbon in ethanol produced from maltose by *L. sanfranciscensis* LTH2581 revealed that incomplete maltose consumption resulted in an enrichment of ^{12}C in the ethanol due to a kinetic isotope effect. This kinetic isotope effect was enhanced upon metabolism under high pressure conditions with the same maltose turnover levels. Incubation of cells under 100 MPa in fermentations for 24h in medium containing maltose decreased the viable cell counts by less than 2 log,

whereas under 150 MPa the reduction was by 2 log and by more than 4 log in the presence of fructose. Metabolism of maltose or sucrose was inhibited under high pressure conditions. Only 75% and 35% of maltose were consumed after 24h incubation under 100 and 150 MPa, respectively. Maltose metabolism under high pressure was accompanied with glucose accumulation, and the accumulation was under 150 MPa higher than that under 100 MPa. Sucrose consumption rate was 45% and 40% after 24h incubation under 100 and 150 MPa, respectively. Glucose accumulation was also observed during metabolism of sucrose under high pressure. The complete studies of the high pressure effect on the metabolism of *L. sanfranciscensis* are presented in the appendix.

4. Discussion

4.1. Sucrose metabolism by *L. sanfranciscensis*

Lactobacillus sanfranciscensis LTH2590 produces EPS from sucrose (Korakli et al. 2000). Tieking et al. (2002) have recently shown that other species of lactobacilli e. g. *Lactobacillus frumenti*, *Lactobacillus pontis*, *Lactobacillus reuteri*, *Weissella confusa* have the ability to produce EPS of fructan or glucan type indicating, that EPS production from sucrose by lactobacilli seems to be a wide spread phenomenon.

The metabolism of sucrose by *L. sanfranciscensis* LTH2590 was only observed in media containing fructose which apparently is used as acceptor for the fructosyl residues. This activating mechanism of acceptors is common by fructosyltransferases (Schomburg and Stephan, 1996). Korakli (1998) suggested levansucrase as the enzyme responsible for the EPS production by *L. sanfranciscensis*, and Tieking et al. (2002) demonstrated by PCR targeting based on primers derived from amino acid sequence of known levansucrases and sequencing of the resulted PCR-product that the strain *L. sanfranciscensis* LTH2590 carries a levansucrase gene. Levansucrase (E.C. 2.4.1.10) is a fructosyltransferase enzyme that catalyses the following reaction:

$$\text{Sucrose} + (2,6\text{-beta-D-fructosyl})_n \rightarrow \text{glucose} + (2,6\text{-beta-D-fructosyl})_{n+1}$$

Besides the fructosyltransferase activity, levansucrase exhibits ß-D-fructofuranosidase activity (Schomburg and stephan, 1996). Levansucrase has been recently isolated and purified from *Lactobacillus reuteri* that produces polyfructan EPS (Van Hijum et al., 2001 and Van Geel-Schutten et al., 1998). By increasing the initial sucrose concentration in medium more EPS was produced by *L. sanfranciscensis* LTH2590, whereas the amount of mannitol formed from sucrose in fermentation with sucrose content ranging from 20-160 g l^{-1} indicated almost constant hydrolase activity. Because more sucrose was consumed at higher sucrose concentration the relative hydrolase activity decreased by increasing sucrose concentrations. This conforms to the results obtained from *Bacillus subtilis* levansucrase (Euzenat et al.,

1997), where increasing sucrose concentrations resulted in a shift of the enzyme activity from hydrolysis to fructosyltransferase. The highest fructosyltransferase activity by *B. subtilis* levansucrase was 32% at 37°C and 616 g l^{-1} sucrose (Euzenat et al., 1997), whereas more than 90% of the fructose moiety from the sucrose by *L. sanfranciscensis* were transformed to oligo- or polysaccharides at sucrose concentration higher than 120 g l^{-1}.

The glucose recoveries were 95-108% up to sucrose concentration of 30 g l^{-1}. A further increase of the sucrose concentration resulted in a decrease of the glucose recoveries. This was attributed to the production of fructo-oligosaccharide characterized as 1-kestose. The amount of kestose produced was higher the higher the sucrose concentration in medium. Crittenden et al. (1994) reported that the percentage of fructose diverted to oligosaccharide by levansucrase from *Zymomonas mobilis* increased from 18% at sucrose concentration of 92 g l^{-1} to 51% at 280 g l^{-1}, respectively.

The ability of levansucrase isolated from various organisms to produce fructo-oligosaccharides was subject of several works. Table 13 shows an overview of some levansucrases and their end products. Hestrin et al. (1956) reported first the production of fructo-oligosaccharide by *Aerobacter levanicum* levansucrase and Feingold et al. (1956) had characterized this oligosaccharide as 1-kestose. *Bacillus subtilis* levansucrase was also shown to produce fructo-oligosaccharide of 1-kestose type (Euzenat et al., 1997). Structural analysis of the fructo-oligosaccharides produced by *Z. mobilis* levansucrase using ^{13}C-NMR spectroscopy revealed that 98% of the total oligosaccharides were identified as 1-kestose and 1.5% as nystose (Crittenden et al., 1993). The oligosaccharides produced by *Acetobacter diazotrophicus* had a glucose:fructose ratio of 1:2 and 1:3 and were identified as 1-kestose and nystose, respectively (Tambara et al., 1999).

Table 13. Overview of some levansucrases and their end products.

Organism	levan production	sucrose hydrolysis	Oligosaccharide(s)		
			glucose:fructose	type of oligosacch.	literature
Aerobacter levanicum[#]	+	+	1:2	1-kestose, trace of 6-kestose	Hestrin et al.1956 & Feingold et al. 1956
Bacillus subtilis[#]	+	+	ND	1-kestose	Euzenat et al. 1997
Acetobacter diazotrophicus[#]	+	+	1:2 and 1:3	1-kestose, nystose	Tambara et al. 1999
Bacillus macerans[#]	+	+	ND	1-kestose, nystose	Park et al. 2001
Bacillus natto[#]	+	+	ND	1-kestose, 6-kestose	Ben Ammar et al. 2002
Zymomonas mobilis[#]	+	+	1:2	1-kestose, trace of nystose	Yanase et al. 1992 & Crittenden et al. 1993
Lactobacillus reuteri[#]	+	+	ND	ND	Van Geel-Schutten et al. 1999& Van Hijum et al. 2001
Lactobacillus sanfranciscensis[*]	+	+	1:2	1-kestose	present work

[#] Results were obtained by the action of purified levansucrase isolated from the organism.

[*] Results were obtained by analysis of culture broth. ND: no data available

The fructo-oligosaccharides produced by *L. sanfranciscensis* LTH2590 had a glucose:fructose ratio of 1:2. The retention time of the fructo-oligosaccharide from *L. sanfranciscensis* was identical with that one of pure 1-kestose using two different chromatography methods, and the treatment of these purified oligosaccahrides with ß-fructosidase (yeast invertase) led to complete hydrolysis to fructose and glucose (data not shown), indicating that the linkage of the fructose is ß(2→1) and that the oligosaccharide produced is 1-kestose. Low concentration of other oligosaccharides produced by *L. sanfranciscensis* LTH2590 may have escaped our analytical limits. The enzymatic hydrolysis of 1-kestose by the action of ß-fructosidase was also observed by kestose produced by *Aerobacter levanicum* levansucrase (Feingold et al., 1956) and *Z. mobilis* levansucrase (Crittenden et al., 1993). It is surprising that levansucrase which is assumed to catalyze ß(2→6) linkages is also able to catalyze ß(2→1). Euzenat et al. (1997) attributed the production of 1-kestose by levansucrases to the hypothesis that levansucrase catalyses the formation of 1-kestose and can not transfer fructosyl residues onto it leading to its accumulation, while 6-kestose produced by levansucrase is rapidly used as acceptor for the production of levan. Since sucrose is a common part of dough formulas, and the sucrose concentration in the aqueous phase of dough is high enough to expect kestose production, the use of kestose producing lactobacilli in sourdough fermentation is an important step towards developing cereal-based functional foods.

Taking into account the facts known about the metabolism of sucrose by *L. sanfranciscensis*, sucrose is degraded from *L. sanfranciscensis* by the action of the fructosyltransferase enzyme, probably a levansucrase, into glucose, fructo-oligosaccharide and an EPS composed of fructose (Fig. 10). The fructo-oligosaccharide was characterized as 1-kestose. The glucose liberated is used as energy source and degraded via pentose-phosphate-shunt. Sucrose can also be hydrolyzed by *L. sanfranciscensis* into glucose and fructose, the latter is used as electron acceptor and reduced to mannitol.

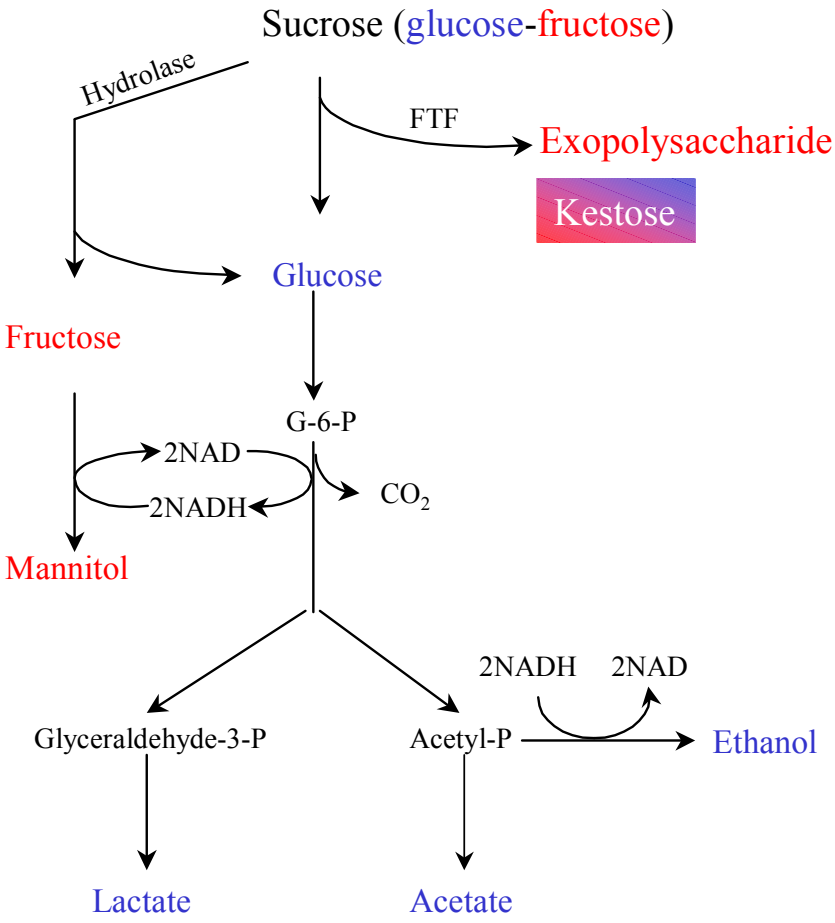

Figure 10. Metabolism of sucrose by *Lactobacillus sanfranciscensis*, FTF: fructosyltransferase.

4.2. Production of EPS during sourdough fermentation

Natural abundance carbon isotope labeling was used as analytical tool to demonstrate in situ EPS production by *L. sanfranciscensis* LTH2590 in a complex food matrix. This technique is a common tool used in ecosystem studies (Henn and Chapela, 2000) and has been applied to detect food adulterations (Schmidt, 1986). The use of natural abundance labeling of carbon isotopes to trace metabolic fluxes in food fermentations is preferable over ^{13}C and ^{14}C carbon isotopes because the substrates are cheap, no safety precautions have to be met, and the products are suitable for sensory evaluation.

Fructan production by lactobacilli may replace the use of plant polysaccharides that are commonly incorporated in baking aids as conditioners, crumb softners and antistaling agents. Furthermore, the use of EPS producing sourdough starters will meet the strict requirements of the modern baking technology for “clean labels”, and consumer demands for a reduced use of additives. The formation of a fructan from sucrose by *L. sanfranciscensis* was previously described (Korakli et al. 2000) but the production of microbial polyfructan from sucrose can not be directly demonstrated in wheat and rye doughs. The high content of polysaccharides including fructans in rye and wheat interferes with the quantification of microbial polysaccharides. Carbon balances of microbial metabolism are difficult to estimate in dough fermentations because maltose, glucose, and other substrates are continuously released during fermentation by cereal enzymes. Qualitatively, the use of natural ^{13}C labelled C_3- and C_4-sucrose followed by the determination of $\delta^{13}C$-values of water soluble polysaccharides of the dough samples demonstrated EPS production from sucrose. Two parameters may be used to estimate the amount of EPS in dough. (i) Sucrose is not formed by cereal enzymes in unstarted doughs and is hydrolysed to a limited extent only. Sucrose metabolism by *L. sanfranciscensis* LTH2590 results in formation of glucose and fructan, or glucose and fructose. The latter is further converted to mannitol. The difference between the amount of sucrose metabolised and the amounts of fructose and mannitol formed, corrected by the mannitol production in doughs without added sucrose, therefore is attributable to EPS and probably kestose formation. (ii) The water soluble polysaccharides in fermented doughs without sucrose addition and in control doughs were predominantly composed of xylose, arabinose and only small amounts of glucose and fructose were detected. An increased proportion of the fructose content in water soluble polysaccharides upon sucrose addition is therefore attributable to fructan synthesis by *L. sanfranciscensis* LTH2590. If the EPS production is calculated based on the carbon balance of sucrose metabolism in dough, it can be estimated that approximately 5 g kg^{-1} EPS, or 1% expressed on flour basis, were produced

in wheat doughs containing 56 g kg^{-1} sucrose. The amount of EPS in the same wheat doughs calculated on the basis of the fructose content of water soluble polysaccharides can be estimated to range between 2.6 and 3.1 g kg^{-1}. The difference between the two methods for calculation relate to the difficulties to establish an exact carbon balance for metabolism of *L. sanfranciscensis* LTH2590 in dough, and possibly to losses of low molecular weight fructan during dialysis. Compared to an EPS yield from sucrose of 57% (fructose in EPS/ fructose in sucrose) during growth of *L. sanfranciscensis* LTH2590 in Su-MRS, the EPS yield from sucrose in wheat doughs was only 15–20%, indicating that under the conditions of dough fermentation an increased proportion of sucrose is hydrolysed to glucose and fructose rather than converted to glucose and fructan. Nevertheless, the fructan content of *L. sanfranciscensis* LTH2590 fermented wheat doughs exceeds that one of hydrocolloids commercially applied as baking aids. The addition of modified cellulose to sourdough bread at a level of 0.3% (flour basis) significantly affects dough rheology as well as bread texture and keepability (Armero and Collar, 1996 and 1998). Although it remains to be established whether or not fructan produced by *L. sanfranciscensis* LTH2590 has a comparable effect as those hydrocolloids commonly used in breadmaking, the amount of fructan produced in dough argues in favour of an effect on textural dough and bread properties.

In wholemeal rye with higher content of polysaccharides the evidence for EPS production through the determination of δ^{13}C-values of water soluble polysaccharides was more difficult than in wheat doughs. Rye flour contains more water soluble polysaccharides interfering with EPS determination. Evidence for EPS production in rye doughs could be provided on the basis of the fructose content of water soluble polysaccharides and by determination of the δ^{13}C-values of fructose released from these polysaccharides by acid hydrolysis. Tieking et al. (2002) have recently demonstrated fructan and glucan production during sourdough fermentation by the hydrolysis of polysaccharides isolated from doughs fermented with various lactobacilli (e. g. *L. frumenti*, *L. pontis*, *L. reuteri*).

In sourdoughs studied in this work, maltose and glucose was formed during the fermentation through starch hydrolysis by cereal amylases and then utilized by the lactobacilli. In doughs with sucrose addition, substantially more glucose was accumulated through the metabolism of sucrose by *L. sanfranciscensis* LTH2590. This accumulation of glucose may affect yeast metabolism in co-cultures of yeast and lactobacilli. High glucose concentrations support the gas production by yeasts and thus contribute to dough leavening, but repress maltose utilisation in bakers yeast.

A correlation between acetic acid content and the shelf-life of the bread was found by Salovaara and Spicher (1987). On the other hand, spontaneous sourdough fermentation reported to improve the volume, crumb grain, staling and shelf-life of bread more than doughs prepared with addition of organic acids (Barber et al. 1992). In rye and wheat sourdough with sucrose addition the concentration of acetate was substantially higher that in doughs without sucrose. Fructose released from sucrose is used as electron acceptor for the regeneration of NADH and results increased acetate formation. In addition to the preservative effect of acetate, it affects the sensorial quality of the resulting bread (Schieberle, 1996).

In the class *Monocotyledoneae* the composition of the hemicelluloses in the endosperm varies greatly, wheat and rye contain mainly arabinoxylans, while ß-glucans predominate in barley and oats (Belitz and Grosch, 1999). These arabinoxylans undergo a degradation process during the fermentation. The long chains of arabinoxylans exhibit less water solubility, and these chains are degraded by cereal enzyms in small more soluble chains during the sourdough fermentation. This solubilisation of arabinoxylans was also reported by Escriva et al. (2000). These higher solubility of arabinoxylans will play an important role by the improvement of the water binding capacity of the dough.

4.3. Metabolism of EPS by Bifidobacteria

The growth promoting effect was shown of EPS from *L. sanfranciscensis* on bifidobacteria using a complex faecal microflora of human origin by DGGE analysis (Dal Bello et al., 2001). To confirm the selective metabolism of EPS by bifidobacteria observed by Dal Bello et al. (2001) and to determine the end products of EPS degradation, the metabolism of EPS by pure cultures of intestinal bifidobacteria was studied. This EPS is stable at pH 2, and heat treatment (121°C, 20 min) of an aqueous EPS-solution resulted in partial degradation only to fragments with 10^4 to 10^6 Da. Oligofructose and inulin are not degraded by pancreatic enzymes and were reported to have an average recovery at the terminal ileum between 85% and 89% (Cummings et al. 2001). EPS of *L. sanfranciscensis* LTH2590 is predominantly composed of fructose and therefore probably not degraded under the conditions in the stomach or small intestine. Thus, EPS meets important requirements for its use as prebiotic (Crociani et al. 1994) as precondition for clinical evaluation of the impact of EPS on the human intestinal microflora or possible effects on human health. Because amounts of up to 5 g EPS kg^{-1} dough are generated by traditional fermentation with appropriate starter cultures, the use of fructan of *L. sanfranciscensis* does not require its declaration as additive. In addition to a possible prebiotic effect of EPS, it is known to improve dough machinability as well as sensory properties of bread (Yasushi and Akifumi, 1995). The EPS recovery in bread from doughs with EPS addition and doughs fermented by *L. sanfranciscensis* LTH2590 was higher than 40%. Besides the promoting effects of prebiotics on the growth of bifidobacteria the end products of carbohydrate metabolism by bifidobacteria (lactate and short chain fatty acids, SCFA) results in a lower luminal pH (Lee, 1999) and inhibits pathogens (Gibson and Roberfroid, 1995). Beneficial effects of SCFA to human health were recently reviewed by Topping and Clifton (2001). Among other factors, the type of substrate, the substrate availability and the pH determine the composition of end products (Marx et al., 2000). Perrin et al. (2001) found that the acetate/lactate ratio was higher in fermentations with fructose in

pH regulated batch compared to non-regulated pH. The metabolites from EPS degraded by bifidobacteria were generally not different from those produced with fructose as carbon source.

5. Summary

Sourdough affects flavour, texture and shelf life of rye and wheat breads, and acidification during dough fermentation is essential in preparation of rye breads. These desired qualities of sourdough are decisively attributed to the metabolic activity of sourdough microflora. In traditionally prepared sourdoughs a stable microflora of herterofermentative lactobacilli and yeasts has been observed. *Lactobacillus sanfranciscensis* is predominant among heterofermentative sourdough lactobacilli. The strain *L. sanfranciscensis* LTH2590 was shown to produce exopolysaccharide (EPS) from sucrose. In this work the sucrose metabolism, EPS production and the end products of sucrose fermentation were studied. Furthermore, the effect of sublethal high pressure on maltose and sucrose metabolism was evaluated.

Sucrose is cleaved by the action of levansucrase in glucose and the fructose moiety is polymerised to high molecular polyfructan. Glucose is used as energy source and degraded via pentose-phosphate-shunt. The end products of sucrose metabolism are EPS, mannitol, lactate, acetate or ethanol. The strain LTH2590 can not utilize fructose but reduces it to mannitol regenerating NAD^+. In all sucrose fermentations mannitol concentration was higher than the free fructose concentration available at the beginning of the fermentation indicating the hydrolysis ability by *L. sanfranciscensis* LTH2590 of sucrose into glucose and fructose. Sucrose was only utilised in the presence of fructose as fructosyl acceptor. The amount of EPS produced increased the higher the initial sucrose concentration in medium. It could be shown that the fructosyltransferase- and the relative hydrolase-activity are dependent on the sucrose concentration. Increasing the sucrose concentration in the medium resulted in a shift of the enzyme activity from hydrolase to fructosyltransferase.

Glucose recovery decreased from 95% at a sucrose concentration of 30g/l to 58% at sucrose concentration of 160g/l due to the synthesis of fructo-oligosaccharide reported for the first time by *Lactobacillus* sp. The fructo-oligosaccharide had a molecular weight of 597±27 and a

glucose:fructose-ratio of 1:2. The comparison of the retention time of the oligosaccharide produced by *L. sanfranciscensis* with standards of 1-kestose and nystose using two different chromatography methods revealed that the fructo-oligosaccharide is 1-kestose.

The use of EPS producing lactobacilli in production of sourdough fermented products might affect the rheology of the dough and the texture and keepability of the bread. Since wheat and rye naturally contain polysaccharides, it is not possible to determine the EPS production during sourdough fermentation directly. In-situ production of EPS during sourdough fermentation with *L. sanfranciscensis* LTH2590 was proven by the extraction of the water soluble polysaccharides at the end of the fermentation followed by dialysis and hydrolysis of these polysaccharides. The fructose concentration in hydrolysate was significantly higher in doughs fermented with the strain LTH2590 than in the control doughs indicating EPS production. The EPS production during dough fermentation with the strain LTH2590 could be confirmed by the use of naturally labelled sucrose of C_3- or C_4-plants as substrate for the EPS production and subsequent isolation and measurement of the δ ^{13}C-values of total polysaccharides. The addition of sucrose to the dough formula resulted in increased mannitol and acetate production and in glucose accumulation. Arabinoxylans were solubilised during sourdough fermentation. Based on the composition of EPS produced by *L. sanfranciscensis* a bifidogenic effect was expected. Fermentations with EPS as the sole energy source revealed that all bifidobacteria tested were able to metabolise EPS. In contrast, none of the tested lactobacilli of human and sourdough origin showed the ability to degrade EPS.

The effect was studied of sublethal high pressure on the viable cell counts, maltose or sucrose metabolism as well as the end products of *L. sanfranciscensis.* In medium with maltose as the sole energy source the reduction of viable cell counts was lower than 2 log at 100 MPa or 150 MPa after 24h of incubation. In the presence of fructose the reduction was at 100 MPa 2 log and at 150 MPa more than 4 log. The reduction of viable cell counts in medium with sucrose was at 100 MPa or 150 MPa 4 log. Maltose or sucrose metabolism was inhibited under high

pressure conditions. 75% or 35% of maltose were metabolised under 100 or 150 MPa, respectively in fermentations after 24h. The inhibition of the metabolism was more pronounced in medium with sucrose, only 40% or 45% of the sucrose were cleaved under 100 or 150 MPa, respectively. The maltose or sucrose metabolism under high pressure conditions was accompanied with glucose accumulation. Glucose accumulation under 150MPa was higher than under 100MPa. The second incubation of cells previously treated with high pressure revealed that the inhibition of metabolism under 100 MPa was reversible und under 150 MPa irreversible. The isotope ratio of the maltose metabolic end product ethanol showed an enrichment of ^{12}C-isotope at an incomplete maltose conversion level under ambient pressure due to a kinetic isotope effect. This kinetic isotope effect was enhanced at the same maltose conversion levels under high pressure conditions.

6. Zusammenfassung

Aus Sauerteig hergestellte Backwaren genießen große Beliebtheit. Neben der Gewährleistung der Backfähigkeit von Roggen dient die Anwendung von Sauerteig auch der Verbesserung von Aroma und Geschmack. Zusätzlich wird dadurch eine verlängerte Frischhaltung und mikrobiologische Stabilität dieser Produkte erzielt. Diese erwünschten Qualitätsverbesserungen sind maßgeblich auf die Stoffwechselaktivität der beteiligten Organismen zurückzuführen. In traditionell, durch kontinuierliches Anfrischen, geführten Sauerteigen entwickelt sich eine stabile Mikroflora, die hauptsächlich aus heterofermentativen Hefen und Lactobazillen besteht, wobei *Lactobacillus sanfranciscensis* einen der wichtigsten Sauerteig-Laktobazillen darstellt. Der Stamm *Lactobacillus sanfranciscensis* LTH2590 zeigte die Fähigkeit, Exopolysaccharid (EPS) aus Saccharose zu synthetisieren. In der vorliegenden Arbeit wurde der Saccharose-Stoffwechsel, die EPS Bildung und die Endprodukte der Saccharose-Fermentation von *Lactobacillus sanfranciscensis* LTH2590 untersucht. Darüber hinaus wurde der Einfluss des subletalen Hochdrucks auf den Maltose- und Saccharose-Stoffwechsel bei *Lactobacillus sanfranciscensis* ermittelt.

Die Saccharose wird durch das Enzym Levansucrase gespalten. Glucose wird als Energiequelle benutzt und über den Pentose-Phosphat-Weg abgebaut, während die Fructose zu einem Exopolysaccharid polymerisiert. Die Endprodukte des Saccharose-Stoffwechsels sind EPS, Mannit, Laktat und Acetat oder Ethanol. Der Stamm LTH2590 kann die Fructose nicht als Energiequelle benutzen. Sie dient als Elektronen-Akzeptor zur Regenerierung von NADH zu NAD^+ und wird somit zu Mannit reduziert. Die Mannit-Konzentration war in allen durchgeführten Fermentationen höher als die Fructose Konzentration, die am Anfang zur Verfügung stand. Dies deutete auf eine Hydrolase Aktivität hin. Saccharose wurde nur in Anwesenheit von Fructose (Fructosyl Akzeptor) verstoffwechselt. Die EPS Bildung stieg mit zunehmender Saccharose-Konzentration an. Es konnte gezeigt werden, dass die Fructosyltransferase- und die relative Hydrolase-Aktivität der Levansucrase stark von der

Saccharose-Konzentration abhängen. Mit zunehmender Saccharose-Konzentration verschob sich das Verhältnis zwischen Hydrolaseaktivität und Fructosyltranferaseaktivität zu Gunsten der fructosyltransferase.

Die Glucose-Bilanz nahm von 95% bei einer Saccharose-Konzentration von 30 g/l auf 58% bei einer Saccharose-Konzentration von 160 g/l ab. Dies konnte auf eine bisher bei Laktobazillen noch nie beschriebene Oligosaccharid-Bildung zurückgeführt werden. Es handelte sich hierbei um ein Fructo-Oligosaccharid, das ein Molekulargewicht von 597±27 und ein Glucose:Fructose-Verhältnis von 1:2 aufweist. Der Vegleich der Retentionszeiten dieses Fructo-Oligosaccharids mit denen von 1-Kestose und Nystose in zwei verschiedenen chromatographischen Trennsystemen ergab, dass es sich bei dem gebildeten Fructo-Oligosaccharid um 1-Kestose handelt.

Der Einsatz von EPS-bildenden Laktobazillen in der Sauerteig Fermentation könnte die Rheologie des Teigs, die Textur sowie die Frischhaltung des Brotes positiv beeinflussen. Da Weizen und Roggen von Natur aus auch Polysaccharide enthalten, konnte der Nachweis der mikrobiellen Polysaccharid-Bildung im Teig nicht direkt, wie im Medium, erbracht werden. Die *in-situ* Produktion von EPS bei *L. sanfranciscensis* LTH2590 im Teig wurde durch Extraktion der wasser-löslichen Polysaccharide, Dialyse und anschließende Hydrolyse nachgewiesen. In Weizen- und Roggen-Teigen mit Saccharose-Zusatz war die Fructose Konzentration im Hydrolysat deutlich höher als in den Teigen ohne Saccharose-Zusatz. Die EPS-Bildung während der Sauerteig-Fermentation konnte durch den Einsatz natürlich markierter Saccharose aus C_3- und C_4-Pflanzen und anschließende Messung der $\delta\ ^{13}C$-Werte der extrahierten Polysaccharide bestätigt werden. Neben der EPS-Bildung verursachte die Zugabe von Saccharose eine erhöhte Mannit- und Acetat-Produktion wie auch die Akkumulation von Glucose. Die Arabinoxylane wurden durch die Sauerteig- Fermentation solubilisiert.

Die Zusammensetzung des von *L. sanfranciscensis* LTH2590 synthetisierten EPS legte die Vermutung nahe, dass dieses von Laktobazillen und Bifidobakterien verwertet werden kann. Fermentationen mit EPS als einziger Energiequelle zeigten, dass EPS von allen getesteten Bifidobakterien vollständig abgebaut wurde. Dahingegen besaß kein einziger der getesteten, aus Menschen und Sauerteigen isolierten Laktobazillen diese Fähigkeit.

Der Einfluss von subletalem Hochdruck auf die Keimzahl von *L. sanfranciscensis*, Maltose oder Saccharose-Verbrauch sowie die Endprodukte war weiterer Gegenstand der Forschungsarbeiten. Im Medium mit Maltose als einziger C-Quelle war die Reduktion der Keimzahl weniger als 2 log unter 100 oder 150 MPa nach 24h Inkubation. Die Reduktion der Keimzahl war im Medium mit Maltose und Fructose nach 24h Inkubation unter 100 MPa höher als 2 log und unter 150MPa höher als 4 log. Die Reduktion der Keimzahl im Medium mit Saccharose unter 100 oder 150 MPa nach 24h Inkubation war 4 log. Die stärkere Abnahme der Keimzahl unter Hochdruck im Medium mit Maltose und Fructose sowie im Medium mit Saccharose könnte auf die Symbiose von Acetat-Bildung und Hochdruck zurückzuführen sein. Sowohl der Maltose- als auch der Saccharose-Stoffwechsel wurde gehemmt. Im Medium mit Maltose wurden 75% bzw. 35% der Maltose unter 100 MPa bzw. 150 MPa verwertet. Mit Saccharose war die Hemmung noch stärker und betrug 45% bzw. 40%. Der Maltose- oder Saccharose-Metabolismus unter Hochdruck ging einher mit der Akkumulation von Glucose, die unter 150MPa stärker als unter 100MPa war. Die zweite Inkubation der für 3h gedrückten Zellen zeigte, dass die Hemmung des Stoffwechsels bei 100 MPa reversibel und bei 150 MPa irreversibel war. Das Isotopen-Verhältnis des Maltose-Stoffwechsel-Produktes Ethanol bei *L. sanfranciscensis* ergab, dass eine Anreicherung des ^{12}C-Isotops im Ethanol bei unvollständigem Maltose-Verbrauch stattfindet. Dieser kinetische Isotopen-Effekt war bei gleichem Maltose-Verbrauch unter Hochdruck ausgeprägter als der unter Normaldruck.

7. References

Abe, F., and K. Horikoshi. 1998. Analysis of interacellular pH in the yeast *Saccharomyces cerevisiae* under elevated hydrostatic pressure: a study in baro-(piezo-)physiology. Extremophiles, **2**: 223-228.

Abe, F., and K. Horikoshi. 2000. Tryptophan permease gene TAT2 confers high-pressure growth in *Saccharomyces cerevisiae*. Mol. Cell. Biol, **20(21)**: 8093-8102.

Armero, E., and C. Collar. 1996. Antistaling additives, flour type and sourdough process effects on functionality of wheat doughs. J. Food Sci. **61(2):**299-303.

Armero, E.; and Collar. C. 1998. Crumb firming kinetics of wheat breads with anti-staling additives. J. Cereal. Sci. **28:**165-174.

Barber, B.; Ortolá, C.; Barber, S.; Fernández, F. 1992. Storage of packaged white bread. III. Effects of sour dough and addition of acids on bread characteristics. Z Lebensm Unters Forsch. **194:** 442-449.

Belitz, H.-D.; Grosch, W. 1999. Food Chemistry; Springer-Verlag: Berlin, Germany.

Ben Ammar, Y., Takayoshi, M., Ito, K., Iizuka, M., and Minamiura, N. 2002. Some properties of levansucrase of Bacillus natto stabilized with periodate oxidized yeast glucomannan. Enz. Microbiol. Technol. **30:**875-882.

Böcker, G. 1993. Ökologische und physiologische Charaktersierung der sauerteigtypischen Stämme *Lactobacillus sanfrancisco* and *Lactobacillus pontis* so. nov.. Dissertation, Universität Hohenheim.

Böcker, G., Stolz, P. and Hammes, W. P. 1995. Neue Erkenntnisse zum Ökosystem Sauerteig und zur Physiologie der Sauerteigtypischen Stämme *Lactobacillus sanfrancisco* and *Lactobacillus pontis*. Getreide Mehl and Brot. **49:**370-374.

Bonaparte, C. and Reuter, G. 1996. Bifidobacteria in commercial dairy products: which species are used? In: Proceeding of the symposium probiotics in man and animal, Berlin, Germany, June 20-22, p33. Deutsche Veterinärmedizinische Gesellschaft, Berlin.

Brandt, M. J. and Hammes, W. P. 2001. Einfluß von fructosanen auf die Sauerteigfermentation. Getreide, Mehl and Brot. **55:** 341-345.

Broadbent, J. R., J. C. Oberg, H. Wang, and L. Wei. 1997. Attributes of the heat shock response in three species of dairy *Lactobacillus*. Syst. Appl. Microbiol. **20:**12-19.

Campbell, J. M., Bauer, L. L., Fahey, G. C., Hogarth, A. J. C. L., Wolf, B. W. and Hunter, D. E. 1997. Selected fructooligosaccharide (1-kestose, nystose, and 1^F-β-fructofuranosylnystose) composition of foods and feeds. J. Agric. Food Chem. **45:** 3076-3082.

Cerning, J. 1990. Exocellular polysaccharides produced by lactic acid bacteria. FEMS Microbiol. Rev. **87:** 113-130.

Cerning, J., C. Bouillanne, M. Landon, and M. Desmazeaud. 1991. Isolation and characterization of exopolysaccharides from slime-forming mesophilic lactic acid bacteria. J. Dairy Sci. **75:**692-699.

Chambert, R., Treboul, G. and Dedonder R. 1974. Kinetic studies of levansucrase of *Bacillus subtilis*. Eur. J. Biochem. **41:**285-300.

Crittenden RG., and HW Doelle. 1993. Structural identification of oligosaccharides produced by *Zymomonas mobilis* levansucrase. Biotechnol. Lett. **15:**1055-1060.

Crittenden RG., and HW Doelle. 1994. Identification and characterisation of the extracellular sucrases of *Zymomonas mobilis* ATCC 39676. Appl. Microbiol. Biotechnol. **41:**302-308.

Crociani, F., Alessandrini, A., Mucci, M.M. and Biavati, B. 1994. Degradation of complex carbohydrates by Bifidobacterium spp. Int. J. Food Microbiol. **24:** 199-210.

Cuezzo de Gines, S., Maldonado, M. C., and Font de Valdez, G. 2000. Purification and characterization of invertase from *Lactobacillus reuteri* CRL1100. Curr. Microbiol. **40:**181-184.

Cummings, J. H., G. T. Macfarlane, and H. N. Englyst. 2001. Prebiotic digestion and fermentation. Amer. J. Clin. Nutr. **73:**415S-420S.

Dal Bello, F., J. Walter, C. Hertel., and W. P. Hammes. 2001. In *vitro* study of prebiotic properties of levan-type exopolysaccharides from lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis. System. Appl. Microbiol. **24(2):**232-237.

De Angelis, M., L. Bini, V. Pallini, P. S. Cocconcelli, and M. Gobbetti. 2001. The acid-stress response in *Lactobacillus sanfranciscensis*. Microbiology. **147:**1863-1873.

De Man, J.C., Rogosa, M., Sharpe, M.E. 1960. A medium for the cultivation of lactobacilli. J. Appl. Bacteriol. **23:** 130-135.

De Vuyst, L., and B. Degeest. 1999. Heteropolysaccahrides from lactic acid bacteria. FEMS Microbiol. Rev. **23:**153-177.

Degeest, B., Van de Ven, S. and De Vuyst, L. 1997. Isolation and production of exopolysaccharides by *Streptococcus thermophilus* and strong evidence for their growth-associated biosynthesis. Proceedings of the 11th Forum for Applied Biotechnology, Gent, Belgium, pp. 1199-1206.

Drews, O., W. Weiss, G. Reil, H. Parlar, R. Wait, and A. Görg. 2002. High pressure effects Step-wise altered protein expression in Lactobacillus sanfranciscensis. Proteomics. **2:**765-774.

Dumas, J. B. 1843. Traité de chimie appliquée aux arts. Paris. **4:**415.

Escrivá, C.; Martínez-Anaya, M. A. 2000. Influence of enzymes on the evolution of fructosans in sourdough wheat process. Eur. Food Res. Technol. **210:** 286-292.

Euzenat, O., A. Guibert, and D. Combes. 1997. Production of fructo-oligosaccharides by levansucrase from *Bacillus subtilis* C4. Proc. Biochem. **32(3):**237-243.

Feingold, D. S., G. Avigad, and S. Hestrin. 1956. The mechanism of polysaccharide production from sucrose. Biochem. J. **64:**351-361.

Gänzle, M. G., M. Ehrmann, and W. P. Hammes. 1998. Modelling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of the sourdough fermentation. Appl. Environ. Microbiol. **64**:2616-2623.

Gibson, G. R., and M. B. Roberfroid. 1995. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J. Nutr. **125:**1401-1412.

Gibson, GR.; Beatty, ER.; Wang, X.; Cummings, JH. 1995. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. Gastroenterol. **108:** 975-982.

Gobetti, M., and A. Corsetti. 1997. *Lactobacillus sanfrancisco* a key sourdough lactic acid bacterium: a review. Food Microbiol. **14:**175-187.

Grobben, G.J., Smith, M.R., Sikkema, J. and De Bont, J.A.M. 1996. In£uence of fructose and glucose on the production of exopolysaccharides and the activities of enzymes involved in the sugar metabolism and the synthesis of sugar nucleotides in *Lactobacillus delbrueckii subsp. bulgaricus* NCFB 2772. Appl. Microbiol. Biotechnol. **46:** 279^284.

Groenwall, A. J., and B. J. A. Ingelman. 1948. Manufacture of infusion and injection fluids. U. S. Patent. **2:**437-518.

Hammes, W. P. and Gänzle, M. G. 1998. sourdough breads and related products, p 199-216 in Wood, B. J.: Microbiology of fermented foods, Chapman&Hall, London.

Hammes, W. P. and Vogel, R. F. 1997. Sauerteig. Die Mikrobiologie der Lebensmittel: Lebensmittel pflanzlicher Herkunft, (Eds., Müller, G., Holzapfel, W., Weber, H.), Behr´s Verlag, Hamburg, 263-285.

Hammes, W. P., P. Stolz, and M. Gänzle. 1996. Metabolism of lactobacilli in traditional sourdoughs. Adv. Food Sci. **18:**176-184.

Henn, M.; and Chapela. H.I. 2000. Differential C isotope discrimination by fungi during decomposition of C_3 and C_4-derived sucrose. Appl. Environ. Microbiol. **66:** 4180-4186.

Henneberg, W. 1909. Gärungsbakteriologisches Praktikum. Betriebsuntersuchungen und Pilzkunde. Paul Parey. Berlin.

Hestirn, S., D. S. Feingold, and G. Avigad. 1956. The mechanism of polysaccharide production from sucrose. Biochem. J. **64:**340-351.

Hestrin, S., S. Averini-Shapiro, and M. Aschner. 1943. The enzymatic production of levan. Biochem. J. **37:**450-456.

Hidaka, H., H. Hirayama, and K. Yamada. 1991. Fructooligosacchrides: Enzymatic preparation and biofunctions. J. carbohydr. Chem. **10:**509-522.

Hidaka, H., H. Hirayama, and N. Sumi. 1988. A fructooligosaccharide producing enzyme from *Aspergillus niger* ATCC 20611. Agric. Biol. Chem. **52:**1181-1187.

Holliger, W. 1902. Bakteriologische Untersuchungen über Mehlteiggärung. Zbl. F. Bakt. II. **9:**305-312.

Hopkins, M.J., Cummings, J.H. and Macfarlane, G.T. 1998. Inter-species differences in maximum specific growth rates and cell yields of bifidobacteria cultured on oligosaccharides and other simple carbohydrate sources. J. Appl. Microbiol. **85:**381-386.

Jandik, P., AP. Clarke, N. Avdalovic, DC. Andersen, and J. Cacia. 1999. Analyzing mixtures of amino acids and carbohydrates using bi-modal integrated amperometric detection. J. Chromatogr B. **732(1):**193-201.

Kandler, O., and N. Weiss. 1986. Regular, Nonsporing Gram-Positive Rods. In: P. H. A. Sneath, N. S. Mair, M. E. Sharpe and J. G. Holt. Bergey`s Manual of Systematic Bacteriology. Vol. 2:1208-1234. Willams and Wilkins, Baltimore.

Kawasaki, H., Nakamura, N. and Ohmori, M. 1996. Screening for bacteria producing sucrose phosphorylase and characterization of the enzymes. Biosci. Biotech. Biochem. 60(2):319-321.

Kleerebezem, M., Van Kranenburg, R. Tuinier, R. Boles, I. C. Zoon, P. Looijesteijn, E. Hugenholz, J. and de Vos, W M. 1999. Exopolysaccharides produced by Lactococcus lactis: from genetic engineering to improved rheological properties?. Antonie van Leeuwenhoek. 76:357-365.

Kline, L., and T. F. Sugihara. 1971. Microorganisms of the San Francisco sour dough Bread process. II. Isolation and characterization of undescribed bacterial species responsible for the souring activity. Appl. Microbiol. **21(3):**459-465.

Knudsen, S. 1924. Über die Milchsäurebakterien des Sauerteiges und ihre Bedeutung für die Sauerteiggärung. Dissertation: Kgl. Veterinaer og Landbrohojsk. Aarskr. 133.

Kontula, P.; Suihko, M.-L.; Suortti, T.; Tenkanen, M.; Mattila-Sandholm, T.;von Wright, A. 2000. The isolation of lactic acid bacteria from human colonic biopsies after enrichment on lactose derivatives and rye arabinoxylo-oligosaccharides. Food Microbiol. **17:**13-22.

Korakli, M. 1998. Die Bildung von Mannit durch *Lactobacillus sanfranciscensis*. Diplomarbeit. Universität Hohenheim.

Korakli, M., A. Rossman, M. G. Gänzle, and R. F. Vogel. 2001. Sucrose metabolism and exopolysaccharide production in wheat and rye sourdoughs by *L. sanfranciscensis*. J. Agri. Food Chem. **49:**5194-5200.

Korakli, M., E. Schwarz, G. Wolf, and W. P. Hammes. 2000. Production of mannitol by *Lactobacillus sanfranciscensis*. Adv. Food Sci. **22:**1-4.

Kosaric, N., GP. Cosentino, and A. Wieczorek. 1984. The Jerusalem artichocke as an agricultured crop. Biomass.**5:**1-36.

Koziet, J.; Rossmann, A; Martin, G.J; Ashurst, P.R. 1993. Determination of carbon-13-content of sugars of fruit and vegetable juices. Anal. Chim. Acta. **271:**31-38.

Lee, Y.K. 1999. Handbook of probiotics. John Wiley & Sons, Inc., New York.

Liu, SQ., Asmundson, RV. Gopal, PK. Holland, R. and Crow, VL. 1998. Influence of reduced water activity on lactose metabolism by *Lactococcus lactis* subsp. *cremoris* at different pH values. Appl. Environ. Microbiol. **64(6):**2111-2116.

Lönner, C., and S. Ahrne. 1995. Lactobacillus (Baking). In Food Biotechnology: Microorganims. pp 797-844. Edited by H. Hui and G. G. Khjachatourians. VCH, Weinheim.

Manca De Nadra, M.C., Strasser De Saad, A.M., Pesce De Ruiz Holgado, A.A. and Oliver, G. 1985. Extracellular polysaccharide production by *Lactobacillus bulgaricus* CRL 420. Milchwissenschaft. **40:** 409-411.

Marx, S. P., Winkler, S. and Hartmeier, W. 2000. Metabolization of ß-(2,6]-linked fructose-oligosaccharides by different bifidobacteria. FEMS Microbiol. Lett. **182:**163-169.

Mckellar, R. C., and H. W. Modler. 1989. Metabolism of fructo-oligosaccharides by *Bifidobacterium* spp. Appl. Microbiol. Biotechnol. **31:**537-541.

Modler, H.W. 1994. Bifidogenic factors-sources, metabolism and applications. Int. Dairy J. 383-407.

Monsan, P.; Bozonnet, S.; Albenne, C.; Joucla, G.; Willemot, R.-M.; Remaud-Siméon, M. 2001. Homopolysaccharides from lactic acid bacteria. Int. Dairy J. **11:**675-685.

Mukai, T., Toba, T. Itoh, T. and Adachi, S. 1990. Structural investigation of the capsular polysaccharide from *Lactobacillus kefiranofaciens* K1. Carb. Res. **204:**227-232.

Neubauer, H., Glaasker, E., Hammes, W. P., Poolman, ?. and W. N. Konings. 1994. Mechanism of maltose uptake and glucose excretion in Lactobacillus sanfranciscensis. J. Becteriol. **176(10):**3007-3012.

Okazaki, M., S. Fujikawa, and N. Matsumoto. 1990. Effects of xylooligosaccharide on growth of bifidobacteria. J. Jap. Soc. Nutr. Food Sci. **43:**395-401.

Park, J. Oh, T. and Yun, J. 2001. Purification and characterization of a novel transfructosylating enzyme from Bacillus macerans EG-6. Proc. Biochem. **37:**471-476.

Perrin, S., Warchol, M., Grill, J.P. and Schneider, F. 2001. Fermentations of fructo-oligosaccharides and their components by *Bifidobacterium infantis* ATCC 15697 on batch culture in semi-synthetic medium. J. Appl. Microbiol. **90:**859-865.

Pham, P. L.; Dupont, I. Roy, D. Lapointe, G. and Cerning, J. 2000. Production of exopolysaccharide by *Lactobacillus rhamnosus R* and analysis of its enzymatic degradation during prolonged fermentation. Appl. Environ. Microbiol. **6:**2302-2310.

Robijn, G., Thomas, J. Haas, H. van den Berg, D. Kamerling, J. and Vliegenthart, J. 1995. The structural of the exopolysaccharide produced by *Lactobacillus helveticus* 766. Carbohydr. Res. **279(1):**137-154.

Robijn, G.W., Gutierrez Gallego, R. van den Berg, D.J.C. Haas, H. Kamerling, J.P. and Vliegenthart, J.F.G. 1996. Structural characterization of the exopolysaccharide produced by *Lactobacillus acidophilus* LMG9433. Carbohydr. Res. **288:** 203-218.

Robijn, G., Wienk, H. van den Berg, D. Haas, H. Kamerling, J. and Vliegenthart, J. 1996. Structural studies of the exopolysaccharide produced by *Lactobacillus paracasei* 34-1. Carbohydr. Res. **285:**129-139.

Salovaara, H; Spicher, G. 1987. Anwendung von Weizensauerteigen zur Verbesserung der Qualität des Weizenbrotes. Getreide Mehl Brot. **41:**116-118.

Salovaara, H; Valjakka T. 1987. The effect of fermentation temperature, flour type, and starter on the properties of sour wheat bread. Int J Food Sci Technol. **22:**591-597.

Sanders, J. W., G. Venema, and J. Kok. 1999. Environmental stress response in *Lactococcus lactis*. FEMS Microbiol. Rev. **23:**483-501.

Scheyhing, C. 2002. Hochdruckinduzierte Genexpression bei Bakterien. Dissertation, TU-München, Wissenschaftszentrum Weihenstephan, *in preparation*.

Schieberle, P. 1996. Intense aroma compounds – useful tools to monitor the influence of processing and storage on bread aroma. Adv. Food Sci. **18:**237-244.

Schmidt, H.-L. 1986. Food quality control and studies on human nutrition by mass spectrometric and nuclear magnetic resonance isotope ratio determination. Fresenius Z Anal Chem. **324:**760-766.

Schomburg, D., and D. Stephan. 1996. Enzyme Handbook, Vol. 12. Springer verlag, Berlin.

Soetaert, W., D. Schwengers, K. Buchholz, and E. J. Vandamme. 1995. A wide range of carbohydrate modifications by a single microorganism: *Leuconostoc mesenteroides.* In: Carbohydrate Bioengineering (Peterson, S. B. Svensson, B. and Pederson, S., Eds.), Vol. 10, pp. 351-358. Elsevier, Amsterdam.

Song, D. D.; Jacques, N. A. 1999. Purification and enzymic properties of the fructosyltransferase of *Streptococcus salivarius* ATCC 25975. Biochem. J. **341:**285-291.

Souci; Fachmann; Kraut. 1987. Die Zusammensetzung der Lebensmittel. Wissenschaftliche Verlagsgesellschaft mbH: Stuttgart, Germany.

Spicher, G. 1958. Die Erreger der Sauerteiggärung. Erste Isolierungsversuche. Brot. Gebäck. **12(3):** 56-63.

Spicher, G. 1959. Die Erreger der Sauerteiggärung. Fortgeführte Isolierungsversuche. Brot. Gebäck. **13(7):** 125-132.

Spicher, G. und H. Stephan. 1999. Handbuch Sauerteig. 5. Auflage. Behr´s Verlag, Hamburg.

Spicher, G. and R. Schröder. 1978. Die Mikroflora des Sauerteiges-IV. Mitteilung: Untersuchungen über die Art der in „Reinzuchtsauer“ anzutreffenden stäbchenförmigen Milchsäurebakterien (Genus Lactobacillus Beijerinck), Z. Lebensm. Unters. Forsch. **167:**342-354.

Sriranganathan, N., R. J. Seidler, W. E. Sandine, and P. R. Elliker. 1973. Cytological and deoxyribonuleic acid-deoxyribonuleic acid hybridization studies on *Lactobacillus* isolates from San Francisco sourdough. Appl. Microbiol. **25:**461-470.

Stolz, P.; Hammes, W.P.; Vogel, R.F. 1996. Maltose-phosphorylase and hexokinase activity in lactobacilli from traditionally prepared sourdoughs. Adv. Food Sci. **18:**1-6.

Sugihara, T. F., L. Kline, and L. B. McCready. 1970. Nature of the San Francisco sourdough French bread process. II. Microbiological aspects. Bakers Dig. **44:**51-56.

Sutherland, I. 1985. Biosynthesis and composition of gram-negative bacterial extracellular wall polysaccharides. Ann. Rev. Microbiol. **39:**243-270.

Sutherland, I. 1990. Food usage of exopolysaccharides, in: Biotechnology of microbial polysaccharides.

Sutherland, I. W. 1997. Biotechnology of microbial polysaccharides. Cambridge University press, Cambridge, United Kingdom.

Sutherland, I.W. 1998. Novel and established applications of microbial polysaccharides. Trends Biotechnol. **16:**41-46.

Tambara, Y., J. V. Hormaza, C. Perez, A. Leon, J. Arrieta, and L. Hernandez. 1999. Structural analysis and optimised production of fructo-oligosaccharides by levansucrase from *Acetobacter diazotrophicus* SRT4. Biotechnol. Lett. **21:**117-121.

Thomson, J. and Chassy, B. 1981. Uptake metabolism of sucrose by *Streptococcus lactis*. J. Bacteriol. **147(2):**543-551.

Tieking, M., **Korakli, M., Ehrmann, M. A., Gänzle, M. G. and Vogel, R .F.** 2002. Production of large amounts of exopolysaccharide by lactobacilli. Submitted.

Tokunaga, T., T. Oku, and N. Hosoya. 1986. Influence of chronic intake of new sweetener fructooligosaccharide (Neosugars) on growth and gastrointestinal function of the rat. J. Nutr. Sci. Vitaminol. **32:**111-121.

Topping, D.L. and Clifton, P.M. 2001. Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiol. Rev. **81(3):**1031-1064.

Trüper, H. G., and L. De Clari. 1997. Taxonomic Note: Necessary correction of specific epithets formed as substantive (nouns) “in Apposition”. Int. J. Syst. Bacteriol. **47:**908-909.

Van casteren, W., Dijkema, C. Schols, H. Beldman, G. and Voragen, A. 1998. Characterization and modification of the exopolysaccharide produced by *Lactococcus lactis* ssp. *cremoris* B40. Carb. Polym. **37(2):**123-130.

Van der Berg, D. 1995. A study to the production of exopolysaccharide by Lactobacillus sake 0-1. Dissertation. University of Utrecht.

Van Geel-Schutten, G. H., E. J. Faber, E. Smit, K. Bonting, M. R. Smith, B. Ten Brink, J. P. Kamerling, J. F. G. Vliegenthart, and L. Dijkhuizen. 1999. Biochemical and structural characterisation of the glucan and fructan exopolysaccharides synthesized by *Lactobacillus reuteri* wild-type strain and by mutant strains. Appl. Environ. Microbiol. **65:**3008-3014.

Van Geel-Schutten, G. H., F. Flesch, B. ten Brink, M.R. Smith, and L. Dijkhuizen. 1998 Screening and characterisation of *Lactobacillus* strains producing large amounts of exopolysaccharides. Appl. Microbiol. Biotechnol. **50:**697-703.

Van Hijum, S. A. F. T., K. Bonting, M. J. E. C. van der Maarel, and L. Dijkhuizen. 2001. Purification of a novel fructosyltransferase from *Lactobacillus reuteri* strain 121 and characterization of the levan produced. FEMS Microbiol. Lett. **205:**323-328.

Vogel, R. F., G. Böcker, P. Stolz, M. Ehrmann, D. Fanta, W. Ludwig, B. Bruno Pot, K. Kersters, K. H. Schleifer and W. P. Hammes. Identification of Lactobacilli from sourdough and description of *Lactobacillus pontis* sp. nov. Int. J. Syst. Bacteriol.**44:**223-229.

Vogel, R. F., R. Knorr, M. R. A. Müller, U. Steudel, M. G. Gänzle, and M. A. Ehrmann. 1999. Non-dairy lactic fermentations: the cereal world. Antonie van Leeuwenhoek. **76:**403-411.

Währen, M. 1985. Die Entwicklungsstationen vom Korn zum Brot im 5. und 4. Jahrtausend. Neuste Untersuchungsergebnisse von Ausgrabungsfunden. Getreide Mehl und Brot. **39(12)**:373-379.

Weiss, N., and U. Schillinger. 1984. *Lactobacillus sanfrancisco* sp. Nov., nom. Rev. System. Appl. Microbiol. **5:**230-232.

Whitfield, C. 1988. Bacterial extracellualr polysaccharides. Can. J. Microbiol. **34:**415-420.

Winkler, J. F.; Schmidt, H.-L. 1980. Einsatzmöglichkeiten der ^{13}C–Isotopen-Massenspektrometrie in der Lebensmitteluntersuchung. Lebensm Unters Forsch. **171:**85-94.

Yanase, H., Iwata, M. Nakahigashi, R. Kita, K. Kato, N. and Tonomura, K. 1992. Purification, crystallization and properties of the extracellular levansucrase from Zymomonas mobilis. Biosci. Biotech. Biochem. **56(8):**1335-1337.

Yasushi, M., Akifumi, Y. 1995. Bread dough improver and production of bread dough. *Japanese Patent Office*. Publication number 07046956A.

8. Appendix

List of the papers that resulted from this Dissertation:

Korakli, M.; Rossmann, A.; Gänzle, M. G.; Vogel, R. F. Sucrose metabolism and exopolysaccharide production in wheat and rye sourdoughs by *Lactobacillus sanfranciscensis*. *Journal of Agriculture and Food Chemistry*. 2001, 49, 5194-5200.

(Die Isotopen-Messungen wurden von A. Rossmann durchgeführt)

Korakli, M.; Gänzle, M. G.; Vogel, R. F. Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. *Journal of Applied Microbiology*. 2002, 92, 958-965.

Korakli, M.; Gänzle, M. G.; Knorr, R.; Frank, M.; Rossmann, A.; Vogel, R. F. Metabolism of *Lactobacillus sanfranciscensis* under high pressure: Investigations with stable isotopes. *Trends in High Pressure Bioscience and Biotechnology*. 2002, 278-294.

(Die Isotopen-Messungen wurden von R. Knorr, M. Frank und A. Rossmann durchgeführt)

Ehrmann, M. A., **Korakli, M**., Vogel, R. F. Identification of the gene for β-fructofuranosidase of *Bifidobacterium lactis* DSM10140^T and characterization of the enzyme expressed in *Escherichia coli*. *Current Microbiology*. 2003. In Press.

(Die Enzym-Charakterisierung wurde von M. Korakli durchgeführt)

Tieking, M., **Korakli, M.** Ehrmann, M. A., Gänzle, M. G., Vogel, R. F. In situ production of exopolysaccharides during sourdough fermentations by cereal and intestinal isolates of lactic acid bacteria. *Applied and Environmental Microbiology*. 2003, 69, In press.

(Der Nachweis von EPS-Bildung im Teig wurde von M. Korakli durchgeführt)

Korakli, M.; Pavlovic, M., Gänzle, M. G.; Vogel, R. F. Exopolysaccharide and kestose production by *Lactobacillus sanfranciscensis* LTH2590. 2002. *Applied and Environmental Microbiology*. 2003, 69, In press.

(Der überwiegende Teil wurde von M. Pavlovic im Rahmen einer von M. Korakli betreuten Diplomarbeit durchgeführt).

Korakli, M.; Pavlovic, M., Rossmann, A., Gänzle, M. G.; Vogel, R. F. Effect of high pressure on the metabolism of maltose and sucrose by *Lactobacillus sanfranciscensis*. In preparation.

(Die Isotopen-Messungen wurden von A. Rossmann und die Saccharose-Fermentationen von M. Pavlovic im Rahmen einer von M. Korakli betreuten Diplomarbeit durchgeführt).

Sucrose Metabolism and Exopolysaccharide Production in Wheat and Rye Sourdoughs by *Lactobacillus sanfranciscensis*

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The exopolysaccharide (EPS) produced from sucrose by *Lactobacillus sanfranciscensis* LTH2590 is predominantly composed of fructose. EPS production during sourdough fermentation has the potential to affect rheological properties of the dough as well as the volume, texture, and keepability of bread. Its in situ production by *L. sanfranciscensis* LTH2590 was demonstrated during sourdough fermentation after the hydrolysis of water soluble polysaccharides. In wheat and rye doughs with sucrose addition the concentration of fructose in the hydrolysate of polysaccharides was significantly higher than that in the hydrolysate of control doughs or doughs without sucrose addition. EPS production by *L. sanfranciscensis* in wheat doughs was confirmed by the determination of δ ^{13}C values of water soluble polysaccharides after the addition of naturally labeled sucrose, originating from C_3- and C_4-plants. In rye doughs, evidence for EPS production with the isotope technique could be demonstrated only by the determination of δ ^{13}C values of fructose from water soluble polysaccharides. In addition to EPS formation from sucrose, sucrose hydrolysis by *L. sanfranciscensis* in wheat and rye sourdoughs resulted in an increase of mannitol and acetate concentrations and in accumulation of glucose. It was furthermore observed that flour arabinoxylans were solublized during the fermentation.

Keywords: *Lactobacillus sanfranciscensis; exopolysaccharide; polyfructan; sourdough; arabinoxylan; carbon isotope ratio*

INTRODUCTION

Polysaccharides from plant source material and their modified forms, for example, starch, carageenans, and arabic gum, as well as microbial polysaccharides are important additives in the food industry (*1*). These polysaccharides improve the textural properties and shelf life of bread and therefore are commonly used as additives for bread production (*2*). Exopolysaccharide (EPS) production by lactic acid bacteria (LAB) during food fermentation allows the replacement of these additives (*3*). EPSs are extracellularly secreted microbial polysaccharides; their amount and chemical structure depend on the microorganism and carbon substrate. They can be classified on the basis of their composition in homo- and hetero-polysaccharides. Homo-polysaccharides consist of only one monosaccharide, such as dextran from *Leuconostoc mesenteroides* and fructan from S*treptococcus salivarius*. Hetero-polysaccharides consist of several different monosaccharides, such as xanthan produced by *Xanthomonas campestris* (*4*, *5*). The properties of EPSs depend on their molecular size, charge, monosaccharide composition, degree of branching, and types of glycosidic linkages. Whereas the application of EPS produced by lactic starter cultures is common practice in the dairy industry, the EPS production in dough in amounts relevant for bread quality has not been described. *Lactobacillus sanfranciscensis* belongs to the microbial flora of traditionally prepared wheat and rye sourdoughs (*6*). The strain *L. sanfranciscensis* LTH2590 was shown to produce EPS from sucrose. This EPS is a high molecular mass fructan homopolymer of the levan type (*7*, *8*). Among the numerous EPS-producing strains of LAB, the sourdough-related species *L. sanfranciscensis* and *Lactobacillus reuteri* contain strains known to produce homo-polysaccharides of the fructan type (*9*). Fructans such as levan, inulin, and the corresponding fructo-oligosaccharides are metabolized by bifidobacteria (*10*). Fructo-oligosaccharides are therefore applied as a prebiotic because they selectively favor the growth of bifidobacteria in the human intestinal tract. It was recently shown that intestinal bifidobacteria are also able to metabolize the high molecular weight fructan produced by *L. sanfranciscensis* LTH2590 (*8*). Analyses of batch cultures inoculated with human feces using denaturing gradient gel electrophoresis and selective culturing techniques demonstrated that fructan from *L. sanfranciscensis* LTH2590 selectively stimulated the growth of bifidobacteria. This finding is corroborated by our finding that this EPS from *L. sanfranciscensis* is degraded by several species of bifidobacteria (unpublished results).

Rye and wheat flours contain 0.8 and 0.6% sucrose, respectively (*11*), and sucrose is a common part of dough formulas. Therefore, the application of EPS-producing sourdough lactobacilli is promising to optimize the technological and nutritional properties of sourdough and bread, respectively. However, it is difficult to estimate the contribution of EPS to the total water

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Table 1. Composition of the Wheat and Rye Doughs (All Amounts Are in Grams)

	wheat doughs										rye doughs				
	W I	W II	W III	W IV	W V	W VI	W VII	W VIII	W IX	W X	R I	R II	R III	R IV	R V
flour[a]	100	100	100	100	100	100	100	100	100	100	200	200	200	200	200
water	100	100	100	100	100	100	100	100	100	100	200	200	200	200	200
C_3-sucrose[c]	0	0	4	8	12	12	0	0	0	0	0	0	0	16	0
C_4-sucrose[c]	0	0	0	0	0	0	4	8	12	12	0	16	16	0	16
chloramphenicol	0	0.02	0	0	0	0.02	0	0	0	0.02	0	0.04	0.04	0	0
erythromycin	0	0.01	0	0	0	0.01	0	0	0	0.01	0	0.02	0.02	0	0
lactic acid to pH 4	−	+	−	−	−	+	−	−	−	+	−	−	+	−	−
starter culture[b]	+	−	+	+	+	−	+	+	+	−	+	−	−	+	+

[a] Wheat flour type 550 for wheat doughs and wholemeal rye flour for rye doughs. [b] *L. sanfranciscensis* LTH2590 washed in tap water and inoculated to a cell count of 1×10^7 CFU g^{-1}. [c] Sucrose additions correspond to 20, 38, and 57 g kg^{-1} or 53, 106, and 160 mmol kg^{-1}.

soluble polysaccharides in dough because rye and wheat flours naturally contain polysaccharides. Consequently, the aim of this study was to determine the sucrose metabolism of *L. sanfranciscensis* in wheat and rye sourdoughs and to provide evidence for in situ EPS production during dough fermentation with this organism. In addition to the determination of substrates and products of microbial metabolism as well as the composition of water soluble polysaccharides, natural abundance stable isotope labeling was used to qualitatively trace sucrose metabolites of *L. sanfranciscensis* in dough.

MATERIALS AND METHODS

Organism and Culture Conditions. *L. sanfranciscensis* LTH2590 was used as the EPS-producing strain (*7*). This strain was isolated from rye sourdough as strain D1 by Böcker GmbH (Minden, Germany) and is deposited as strain LTH2590 and TMW 1.392 in the Lebensmitteltechnologie Hohenheim and Technische Mikrobiologie Weihenstephan strain collections, respectively. The organism was cultured at 30 °C in MRS (*12*) modified to meet the growth requirements of *L. sanfranciscensis* (Su-MRS). Su-MRS contained the following components per liter: peptone from casein, 10 g; yeast extract, 5 g; meat extract, 5 g; $K_2HPO_4 \cdot 3H_2O$, 2.6 g; KH_2PO_4, 4 g; cysteine−HCl, 0.5 g; NH_4Cl, 3 g; sucrose, 50 g; Tween 80, 1 mL/L. The pH was adjusted to 6.2, and the medium was sterilized at 121 °C for 20 min. Stock solutions containing $MgSO_4 \cdot 3H_2O$ (100 g/L) and $MnSO_4 \cdot 4H_2O$ (50 g/L) were autoclaved separately, and a vitamin mix containing biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamin, riboflavin, cobalamin, and panthothenic acid (0.2 g/L each) was sterilized by filtration. Each stock solution was added (1 mL/L) to the sterilized medium.

Preparation of Doughs. Wholemeal rye flour and wheat flour type 550 (ash content = 510−630 mg/100 g of wheat) were used to prepare doughs in a yield of 200 [dough yield; (mass of dough/mass of flour) × 100]. For inoculation of 200 g of dough, 10 mL of overnight culture of *L. sanfranciscensis* in Su-MRS was harvested by centrifugation at 8000*g* for 5 min, resuspended in 5 mL of physiological salt solution, and added to the dough. Control doughs were prepared with chloramphenicol (Serva) and erythromycin (Sigma) to inhibit microbial growth, and the pH was adjusted with 90% lactic acid to 4 (Riedel-deHaën). The dough formulas for wheat and rye doughs are given in Table 1. All doughs were incubated at 30 °C.

Determination of Colony-Forming Units (CFU). Cell counts were determined on mMRS4 agar (*13*). Appropriate dilution was plated using a spiral plater (IUL, Königswinter, Germany), and plates were incubated at 30 °C for 48 h under a controlled atmosphere (76% N_2, 20% CO_2, and 4% O_2).

Determination of Metabolites. Dough samples were taken for HPLC analysis. After the addition of 200% of water to the dough sample (w/w), solid constituents were removed by centrifugation at 8000*g* for 10 min. The concentrations of maltose, lactic acid, acetic acid, and ethanol in the supernatant were determined using Polyspher OA KC column (Merck); the mobile phase was 5 mmol/L H_2SO_4 and the temperature of the column 70 °C. Sucrose, glucose, fructose, xylose, arabinose, and mannitol concentrations were determined using a Polyspher CH PB column (Merck); the mobile phase was deionized H_2O and temperature of the column 80 °C. For detection a refractive index detector (Gynkotek) was used. The detection limits were 1 mmol L^{-1} for ethanol, 0.2 mmol L^{-1} for carbohydrates and mannitol, and 0.05 mmol L^{-1} for organic acids.

EPS Isolation and Purification. EPSs produced in Su-MRS were isolated after cells were removed by centrifugation and the addition of 2 volumes of chilled 95% ethanol to the supernatant. After incubation for 3 h at 4 °C, the precipitate was collected by centrifugation (8000*g*, 10 min). EPSs were dissolved in deionized water, dialyzed (molecular weight cutoff of 12000−14000, Serva) against deionized water at 4 °C for 24 h and then lyophilized. For isolation of water soluble polysaccharides and EPSs from dough, two parts of water were added to one part of dough (w/w) and after centrifugation (8000*g*, 10 min), the resulting supernatant was treated as described above. EPSs in doughs were partially purified from contaminating polysaccharides using gel permeation chromatography (GPC) at room temperature. Samples were injected on a Superdex 200 HR column (Amersham Pharmacia Biotech, Uppsala, Sweden) and eluted at a flow rate of 0.4 mL/min with 50 mM NaH_2PO_4 (pH 6) as a mobile phase. Polysaccharides were detected with a refractive index detector. Purified EPS from Su-MRS was injected on a Superdex 200 HR column to determine its elution volume. EPS from *L. sanfranciscensis* eluted at 6 mL, corresponding to an apparent molecular weight of $\sim 2 \times 10^6$ Da. Gel filtration HMW and LMW calibration kits (Amersham Pharmacia) were used for calibration of the GPC column.

Hydrolysis of Polysaccharides. Hydrolyses of dried EPS and flour polysaccharides were carried out by incubating the samples for 2 h in 1 M H_2SO_4 at 80 °C. It was verified by GPC that these hydrolysis conditions quantitatively converted EPS and flour polysaccharides to monosaccharides. Monosaccharides were determined using HPLC as described above. For determination of δ ^{13}C values of fructose in rye water soluble polysaccharides, fructose eluting from CH PB column was collected and lyophilized.

Determination of Carbon Isotope Ratio. The δ ^{13}C value of a compound indicates its content of the ^{13}C isotope relative to the V-PDB standard ($[^{13}C]/[^{12}C] = 0.0112372$). Because the ^{12}C isotope is enriched during photosynthesis, carbohydrates have negative δ ^{13}C values. In accordance with the different photosynthetic pathways employed by C_3- and C_4-plants, the δ ^{13}C values of carbohydrates from these plants differ (*14*). The ^{13}C analysis was performed on CO_2 directly obtained by quantitative combustion of the samples. δ ^{13}C values were then determined using isotope ratio mass spectrometry with a double gas inlet system, which measures in alternation the sample and a standard gas (*15*). The amount of the sample was 2−3 mg. The laboratory working standard was wheat starch (δ ^{13}C value = −24.60), which was calibrated with NIST-22 with a δ ^{13}C value of −29.80 (*16*). To determine the δ ^{13}C values of glucose and fructose in C_3- and C_4-sucrose, a solution (20 g/L) of each sucrose was hydrolyzed with 1 M H_2SO_4 and

Table 2. Concentrations of Substrates and Products in Wheat Sourdoughs (Millimoles per Kilogram)[a]

cough	time (h)	pH	sucrose	maltose	glucose	fructose	mannitol	lactate	acetate	ethanol
W I	0	6.20	3.6	17	1.6	0.2	1.1	0.0	0.0	0.0
	24	3.87	1.2	54	29	0.4	13	72	10	64
W II	0	3.96	3.5	16	2.0	1.0	1.4	50	0.0	0.0
	24	4.05	3.3	63	34	8.4	1.5	48	0.0	0.0
W III	0	6.31	46	14	0.0	0.8	0.0	0.0	0	0.0
	24	3.81	7.8	54	60	1.1	37	63	19	44
W IV	0	6.31	104	15	0.0	0.0	0.0	0.0	0.0	0.0
	24	3.78	42	49	80	6.0	43	60	21	39
W V	0	6.27	152	13	0.0	0.0	0.0	0.0	0.0	0.0
	24	3.85	75	50	82	6.6	43	61	24	36
W VI	0	3.96	159	13	0.0	0.0	0.0	55	0.0	0.0
	24	3.84	130	39	33	14	0.0	57	0.0	0.0

[a] Shown are data for doughs without sucrose and with addition of C_3-sucrose. Results obtained from doughs to which C_4-sucrose was added (doughs W VII, W VIII, W XI, and W X) correspond within experimental error to the results from doughs W III, W IV, W V, and WVI, respectively.

monosaccharides were obtained after preparative separation with HPLC, lyophilized, and measured as described above.

Statistical Analysis of the Data. The overall experimental error (variation of microbial metabolic activity, flour enzymatic activity, as well as extraction and quantification of substrates and fermentation products) was determined through fermentations under identical conditions,that is, sourdoughs containing equal amounts of C_3- and C_4-sucrose. The coefficient of variation between two fermentations was generally in the range of 5−15%. The extraction of polysaccharides from dough and the determination of δ ^{13}C values were reproducible with a coefficient of variation of 2%.

RESULTS

EPS Production in Su-MRS and δ ^{13}C Values of Sucrose and EPSs Derived Thereof. To verify EPS production from sucrose by *L. sanfranciscensis* LTH2590, as well as to demonstrate the changes of δ ^{13}C values through the EPS production in doughs, δ ^{13}C values of C_3-sucrose (sugar beet), C_4-sucrose (sugar cane), and purified EPS were measured. C_3- and C_4-sucrose had δ ^{13}C values of −25.65 and −11.64, respectively. EPS consists predominantly of fructose, so it was important to know whether the δ ^{13}C value of sucrose is the same in its constituents glucose and fructose. Glucose and fructose from C_3-sucrose had δ ^{13}C values of −25.11 and −25.87, respectively, and glucose and fructose from C_4-sucrose had δ ^{13}C values of −12.23 and −11.40, respectively. Thus, the differences in δ ^{13}C values between fructose and glucose in either C_3- or C_4-sucrose are very small. The δ ^{13}C values of EPS produced by *L. sanfranciscensis* during growth in culture media with C_3- and C_4-sucrose followed by dialysis were −26.69 and −17.28, respectively. Comparison of these values with the δ ^{13}C values of sucrose shows that the ^{12}C carbon isotope was enriched in the fructose moieties used by *L. sanfranciscensis* for EPS formation, probably because of isotopic partitioning between EPS and other products of sucrose metabolism. During growth of *L. sanfranciscensis* in Su-MRS, the fructose moiety of sucrose is either incorporated into EPS or reduced to mannitol. The glucose moiety is used as carbon source or left unused and accumulated in the medium (Korakli et al., 2000, data not shown). The amount of EPS during growth of *L. sanfranciscensis* in Su-MRS was 14 g L^{-1}, corresponding to a yield of 57% based on the fructose content of sucrose. The EPS yield from sucrose and the difference between δ ^{13}C values of EPS from C_3- and C_4-sucrose were therefore large enough to expect an impact of EPS production from C_3- or C_4-sucrose in dough on the δ ^{13}C values of overall water soluble polysaccharides.

Carbohydrate Metabolism in Wheat Sourdoughs. To monitor the microbial metabolism in inoculated and control doughs, substrates and fermentation products were determined (Table 2). The concentration of metabolites in doughs with C_4-sucrose addition (doughs W VII−W X, data not shown) was equivalent to the concentration in the corresponding doughs with C_3-sucrose addition (doughs W III−W VI). It was verified by plate counts that the fermentation flora was identical with that of the starter culture. Contaminants were not detectable and thus accounted for <0.1% of the total microflora. In control doughs without inoculum, the total cell counts were $<10^4$ CFU g^{-1} throughout the fermentation, excluding an effect of microbial metabolism on carbohydrates in dough. In control wheat doughs, maltose and glucose were accumulated after 24 h in all doughs because of the amylase and α-glucosidase activities of flours. In doughs with sucrose contents of 3.5, 46, 104, and 152 mmol/kg of dough, the amounts of sucrose metabolized were 2.5, 38, 62, and 77 mmol/kg of dough, respectively. The strain LTH2590 is unable to use fructose as a carbon source but converts fructose to mannitol with concomitant oxidation of NADH to NAD^+. However, in doughs containing additional sucrose, the amount of sucrose used was greater than the amount of mannitol formed. The accumulated fructose in dough does not account for the molar difference between sucrose metabolized and mannitol produced. Therefore, the differences between sucrose consumed and metabolites formed, 2.0, 13, and 27 mmol kg^{-1} of fructose in doughs containing 20, 38, and 57 g kg^{-1} of sucrose, respectively, indicate EPS production from sucrose. Apparently increased sucrose concentration led to increased EPS production, and the proportion of sucrose hydrolyzed to the alternative end product fructose was decreased. The lactate and acetate concentrations varied between 58 and 63 and between 19 and 24 mmol/kg of dough, respectively, resulting in pH of 3.78−3.85. The metabolic activity was stopped at this pH, although substrates were still available. In dough without added sucrose, the release of fructose from flour oligo- and polysaccharides allowed for the formation of 13 mmol kg^{-1} mannitol. Enzymatic liberation of 8.4 mmol kg^{-1} fructose during the fermentation was observed in the control dough without sucrose addition (dough W II). Reduction of fructose to mannitol allows *L. sanfranciscensis* to produce acetate instead of ethanol in a molar ratio of 2 mol of mannitol to 1 mol of acetate. Accordingly, increased mannitol concentration in dough with sucrose addition was accompanied by increased

Table 3. Composition of Water Soluble Polysaccharides in Wheat Doughs

	mmol kg^{-1}			
sample	glucose	fructose	xylose	arabinose
0 h, all doughs	2.4	2.4	10.8	6.6
24 h, W I	3.3	2.0	19.5	16.8
24 h, W II	2.0	1.5	21.5	16.3
24 h, W V	1.8	14.7	18.6	16.0
24 h, W IX	1.9	17.2	20.5	16.0
24 h, W X	1.7	2.2	19.2	17.5

acetate and decreased ethanol production, and the molar ratio of mannitol to acetate was ~2:1 in all doughs. In doughs containing sucrose, glucose concentrations were substantially higher than in control dough. This indicates that a part of the glucose liberated from sucrose by *L. sanfranciscensis* was not used as carbon source but accumulated in doughs.

Composition of Water Soluble Polysaccharides in Wheat. The hydrolysis of water soluble polysaccharides following HPLC analysis demonstrated that these polysaccharides are composed of glucose, arabinose, xylose, and fructose. The concentrations of arabinose and xylose were increased during the fermentation in all doughs (Table 3). Polysaccharides in flour have a high degree of polymerization (DP); during the incubation the long chains are broken into more water soluble chains. This degradation of polysaccharides explains the increase of the concentrations of arabinose and xylose during the incubation. This increase was also observed in the control doughs with antibiotics and, therefore, this solubilization could be attributed to the activity of flour enzymes. In doughs W V and W IX with sucrose addition (Table 3) the concentration of fructose at $t =$ 24 h in the hydrolysate of water soluble polysaccharides was substantially higher than in control doughs. In doughs W V and W IX, cereal and microbial fructans accounted for up to 30% of water soluble polysaccharides. This increased fructose content of water soluble polysaccharides indicated the production of EPS from sucrose.

***δ* ^{13}C Values of Wheat Dough Soluble Polysaccharides.** The δ ^{13}C values of dialyzed water soluble polysaccharides from wheat sourdoughs are shown in Table 4. Addition of either C_3- or C_4-sucrose to control doughs (W VI and W X) did not affect the δ ^{13}C values of water soluble polysaccharides; therefore, shifts in δ ^{13}C values of water soluble polysaccharides are attributable to microbial conversion of sucrose to high molecular weight EPS. At $t = 0$ the mean of δ ^{13}C values of water soluble polysaccharides was -25.37 ± 0.16 in all doughs, as expected for C_3-plants. The difference in δ ^{13}C values between $t = 0$ and $t = 24$ h in the control doughs was -1.12 ± 0.05 (mean of the three control doughs). In fermented doughs, decreased δ ^{13}C values of water soluble polysaccharides were observed if C_3-sucrose was added. The difference was greater in the dough with 57 g kg^{-1} sucrose added (W V) compared to the dough with 20 g kg^{-1} sucrose added (W III), indicating increasing production of EPS at higher sucrose concentrations. Accordingly, in fermented doughs with C_4-sucrose addition, δ ^{13}C values of water soluble polysaccharides were higher than those of the control doughs. An enrichment of EPS from the total water soluble dough polysaccharides was achieved by GPC. Fructan from *L. sanfranciscensis* LTH2590 has a molecular mass of ~2 $\times$ 10^6 Da, and water soluble wheat polysaccharides were distributed over the range of 10^4–(5×10^6) Da (data not shown). The elution volume of 5–7 mL, corresponding to the elution volume of EPS produced in Su-MRS, was collected for further analysis. After this enrichment of the EPS in the water soluble polysaccharides, the differences in δ ^{13}C values of the fermented doughs with C_3 and C_4 addition relative to the control doughs were more pronounced. The δ ^{13}C value of the dough with C_3-sucrose was more negative than that without sucrose and had nearly the same δ ^{13}C value as EPS isolated from Su-MRS. Accordingly, the δ ^{13}C value was higher in dough with C_4-sucrose addition. As described above, the δ ^{13}C values of EPS from C_3- and C_4-sucrose were -26.69 and -17.28, respectively, and the δ ^{13}C value of the contaminating polysaccharides of wheat was -25.84 (Table 4). Therefore, when the same amounts of EPS in doughs with C_3- and C_4-sucrose are produced, the change in δ ^{13}C value with C_4-sucrose will be more significant than that one with C_3-sucrose.

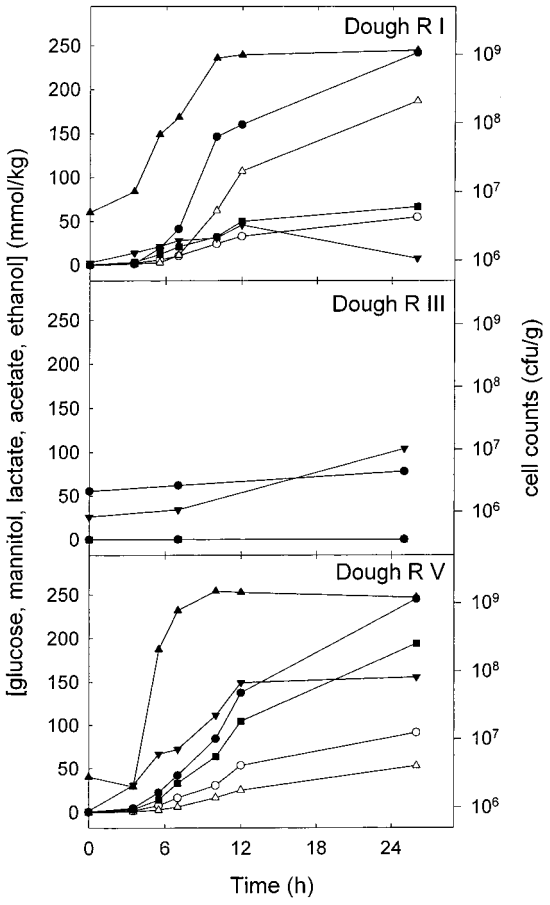

Figure 1. Kinetics of microbial growth and fermentation products in rye sourdoughs: (▼) glucose; (■) mannitol; (●) lactate; (○) acetate; (△) ethanol; (▲) CFU. Dough RI, fermented dough without sucrose addition; dough RIII, control dough with C_4-sucrose added; dough R V, fermented dough with addition of 56 g kg^{-1} C_4-sucrose.

Carbohydrate Metabolism in Rye Sourdoughs. Wholemeal rye flour has a higher pentosan and polysaccharide content than wheat flour. To estimate the influence of these polysaccharides on the sourdough fermentation, the kinetics of the main fermentation products in wholemeal rye doughs were determined. The results for doughs R I (no sucrose addition), R III, and R V (addition of C_4-sucrose) are shown in Figure 1. The kinetics of substrates and fermentation products in the dough with added C_3-sucrose (R IV) was comparable with that of C_4-sucrose, and the differences were within

Table 4. δ ^{13}C Values (‰ V-PDB) of Water Soluble Polysaccharides from Wheat Sourdoughs

	dough									
	W I	W II	W III	W IV	W V	W VI	W VII	WVIII	W IX	W X
δ ^{13}C, 0 h	−25.15	−25.29	−25.60	−25.52	−25.28	−25.35	−25.25	−25.44	−25.23	−25.60
δ ^{13}C, 24 h	−26.59	−26.30	−26.69	−26.81	−27.18	−26.54	−25.77	−25.66	−25.90	−26.75
δ $^{13}C_{24h}$ − δ $^{13}C_{0h}$	−1.44	−1.01	−1.09	−1.29	−1.90	−1.19	−0.52	−0.22	−0.67	−1.15
after enrichment of EPS by GPC										
δ ^{13}C, 24 h	−25.84	ND	ND	ND	−26.44	ND	ND	ND	−21.21	ND

Table 5. Composition of Water Soluble Polysaccharides in Rye Doughs

	mmol kg^{-1}			
sample	glucose	fructose	xylose	arabinose
0 h, all doughs	2.7	0.0	6.3	2.5
26 h, R I	0.4	0.2	30.8	17.7
26 h, R II	0.7	0.2	25.0	15.5
26 h, R III	0.4	0.6	29.2	15.4
26 h, R IV	0.2	6.8	23.4	13.8
26 h, R V	0.3	7.5	24.1	13.9

Table 6. δ ^{13}C Values (‰ V-PDB) of Water Soluble Polysaccharides and of Fructose in Water Soluble Polysaccharides Isolated from Wholemeal Rye Sourdoughs

	dough			
	R I	R III	R IV	R V
δ ^{13}C, 26 h, after dialysis	−24.65	−24.62	−23.84	−23.14
δ ^{13}C, 26 h, after enrichment with GPC	−23.69	−23.33	−23.33	−20.34
δ ^{13}C, 26 h, of fructose	−21.44	−20.06	−22.00	−19.14

experimental error (data not shown). In the control dough R III acidified to pH 4, glucose accumulation was observed (Figure 1), whereas nearly no glucose was accumulated in the control dough R II (data not shown). The initial pH of dough R II was 6.36, which fell to 6.13 after 27 h of incubation. In fermented doughs, the cell number of *L. sanfranciscensis* reached 10^9 CFU g^{-1} within 8 h, independent from sucrose addition. In wholemeal rye dough without sucrose (dough R I) more mannitol was produced than in the comparable wheat dough, and this is attributed to the higher content of fructans in wholemeal rye compared to in wheat flour type 550. In rye dough with C_4-sucrose addition (dough R V) more mannitol and acetate were produced than in dough R I, in which lactate and ethanol were the main end products. In the control dough (R III) glucose accumulation could be observed due to the activity of flour enzymes. The glucose liberated during the fermentation was higher in doughs with sucrose addition (R V).

***δ* ^{13}C Values of Rye Dough Soluble Polysaccharides.** Water soluble wholemeal rye polysaccharides have a composition comparable to that of wheat polysaccharides (Table 5). Polysaccharides from rye doughs were composed of <15% fructans. In doughs R IV and R V, the fructan concentrations were higher than in the control doughs, indicating EPS production. This could also be confirmed by determination of δ ^{13}C values of water soluble polysaccharides after dialysis (Table 6). However, the differences in δ ^{13}C values in dialyzed water soluble polysaccharides did not indicate a clear shift in δ ^{13}C values with the exception of dough R V, and even after enrichment of EPS with GPC, a significant change was observed only in the dough with C_4-sucrose (R V). The evidence for EPS production in rye doughs from C_3- and C_4-sucrose was provided by acid hydrolysis of the water soluble polysaccharides followed by preparative separation of the fructose. The δ ^{13}C value of fructan from doughs with C_3-sucrose was lower than the corresponding values of the control and the dough without sucrose addition. The difference in δ ^{13}C value of fructan in dough with C_4-sucrose compared to control dough was more significant.

DISCUSSION

Natural abundance carbon isotope labeling could be used as an analytical tool to demonstrate in situ EPS production by *L. sanfranciscensis* LTH2590 in a complex food matrix. This technique is a common tool used in ecosystem studies (*17*) and has been applied to detect food adulterations (*14*). The use of natural abundance labeling of carbon isotopes to trace metabolic fluxes in food fermentations is preferable over ^{13}C and ^{14}C carbon isotopes because the substrates are cheap, no safety precautions have to be met, and the products are suitable for sensory evaluation.

Fructan production by lactobacilli may replace the use of plant polysaccharides that are commonly incorporated in baking improvers as conditioners, crumb softeners, and antistaling agents. Furthermore, the use of EPS-producing sourdough starters will meet the strict requirements of the modern baking technology for "clean labels" and consumer demands for a reduced use of additives. The formation of a fructan from sucrose by *L. sanfranciscensis* LTH2590 was previously described (*7*), but the production of microbial polyfructan from sucrose cannot be directly demonstrated in wheat and rye doughs. The high content of polysaccharides, including fructans, in rye and wheat interferes with the quantification of microbial polysaccharides. Carbon balances of microbial metabolism are difficult to estimate in dough fermentations because maltose, glucose, and other substrates are continuously released during fermentation by cereal enzymes. Qualitatively, the use of natural ^{13}C-labeled C_3- and C_4-sucrose followed by the determination of δ ^{13}C values of water soluble polysaccharides of the dough samples demonstrated EPS production from sucrose. Two parameters may be used to estimate the amount of EPS in dough: (i) Sucrose is not formed by cereal enzymes in unstarted doughs and is hydrolyzed to a limited extend only. Sucrose metabolism by *L. sanfranciscensis* LTH2590 results in the formation of glucose and fructan or glucose and fructose. The latter is further converted to mannitol. The difference between the amount of sucrose metabolized and the amounts of fructose and mannitol formed, corrected by the mannitol production in doughs without added sucrose, therefore is attributable to EPS formation. (ii) The water soluble polysaccharides in fermented doughs without sucrose addition and in control doughs were predominantly composed of xylose and arabinose, and only small amounts of glucose and fructose were detected. An increased proportion of the fructose content in water soluble polysaccharides upon sucrose addition

is therefore attributable to fructan synthesis by *L. sanfranciscensis*. If the EPS production is calculated on the basis of the carbon balance of sucrose metabolism in dough, it can be estimated that ~5 g kg^{-1} EPS, or 1% expressed on a flour basis, was produced in wheat doughs containing 56 g kg^{-1} sucrose. The amount of EPS in the same wheat doughs calculated on the basis of the fructose content of water soluble polysaccharides can be estimated to range between 2.6 and 3.1 g kg^{-1}. The differences between the two methods for calculation relate to the difficulties in establishing an exact carbon balance for metabolism of *L. sanfranciscensis* in dough and possibly to losses of low molecular weight fructan during dialysis. Compared to an EPS yield from sucrose of 57% (fructose in EPS/fructose in sucrose) during growth of *L. sanfranciscensis* LTH2590 in Su-MRS, the EPS yield from sucrose in wheat doughs was only 15−20%, indicating that under the conditions of dough fermentation an increased proportion of sucrose is hydrolyzed to glucose and fructose rather than converted to glucose and fructan. Nevertheless, the fructan content of *L. sanfranciscensis* fermented wheat doughs exceeds that of hydrocolloids commercially applied as baking improvers. The addition of modified cellulose to sourdough bread at a level of 0.3% (flour basis) significantly affects dough rheology as well as bread texture and keepability (*2*, *18*). Although it remains to be established whether fructan produced by *L. sanfranciscensis* has an effect comparable to those of hydrocolloids commonly used in bread-making, the amount of fructan produced in dough argues in favor of an effect on textural dough and bread properties.

In wholemeal rye with a higher content of polysaccharides the evidence for EPS production through the determination of δ ^{13}C values of water soluble polysaccharides was more difficult to obtain than in wheat doughs. Rye flour contains more water soluble polysaccharides interfering with EPS determination. Evidence for EPS production in rye doughs could be provided on the basis of the fructose content of water soluble polysaccharides and by determination of the δ ^{13}C values of fructose released from these polysaccharides by acid hydrolysis.

In sourdoughs studied in this work, maltose and glucose were formed during the fermentation through starch hydrolysis by cereal amylases and then utilized by the lactobacilli. In doughs with sucrose addition, substantially more glucose was accumulated through the metabolism of sucrose by *L. sanfranciscensis* LTH2590. This accumulation of glucose may affect yeast metabolism in cocultures of yeast and lactobacilli. High glucose concentrations support the gas production by yeasts and thus contribute to dough leavening but repress maltose utilization in baker's yeast.

A correlation between acetic acid content and the shelf life of the bread was found by Salovaara et al. (*19*, *20*). On the other hand, spontaneous sourdough fermentation reportedly improved the volume, crumb grain, staling, and shelf life of bread more than doughs prepared with addition of organic acids (*21*). In rye and wheat sourdough with sucrose addition the concentration of acetate was substantially higher that in doughs without sucrose. Fructose released from sucrose is used as electron acceptor for the regeneration of NADH and results in increased acetate formation. In addition to the preservative effect of acetate, it affects the sensorial quality of the resulting bread (*22*).

In the class Monocotyledoneae the composition of the hemicelluloses in the endosperm varies greatly: wheat and rye contain mainly arabinoxylans, whereas β-glucans predominate in barley and oats (*1*). These arabinoxylans undergo a degradation process during the fermentation. The long chains of arabinoxylans exhibit less water solubility, and these chains are degraded by cereal enzymes in small more soluble chains during the sourdough fermentation. This solubilization of arabinoxylans was also reported by Escrivá et al. (*23*). These higher solubilities of arabinoxylans will play an important role in the improvement of the water binding capacity of the dough. Arabinoxylans could have furthermore a health-promoting effect according to Kontula et al. (*24*), who found that some lactobacilli isolated from human intestines such as *L. paracasei* could utilize arabinoxylan-oligosaccharides prepared from rye arabinoxylans.

In conclusion, it was shown by analysis of microbial metabolism, composition of water soluble polysaccharides, and an isotope labeling technique that fructan is produced by *L. sanfranciscensis* during sourdough fermentation. On the basis of the amount of EPS produced, 0.5−1% of flour basis, the use of EPS-producing sourdough lactobacilli in order to affect rheological properties of dough as well as the texture and shelf life of bread can be envisaged. Fructan produced during sourdough fermentation might furthermore open new vistas for novel cereal-based functional foods.

ABBREVIATIONS USED

CFU, colony-forming units; DP, degree of polymerization; EPS, exopolysaccharide; GPC, gel permeation chromatography; ND, not done; NIST, National Institute of Standards and Technology; V-PDB, Vienna International Standard Pee-Dee Belemnite.

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LITERATURE CITED

(1) Belitz, H.-D.; Grosch, W. *Food Chemistry*; Springer-Verlag: Berlin, Germany, 1999.
(2) Armero, E.; Collar, C. Antistaling additives, flour type and sourdough process effects on functionality of wheat doughs. *J. Food Sci.* **1996**, *61* (2), 299−303.
(3) De Vuyst, L.; Degeest, B. Heteropolysaccharides from lactic acid bacteria. *FEMS Micrbiol. Rev.* **1999**, *23*, 153−177.
(4) Sutherland, I. W. *Biotechnology of Microbial Polysaccharides*; Cambridge University Press: Cambridge, U.K., 1990.
(5) Sutherland, I. W. Novel and established applications of microbial polysaccharides. *Trends Biotechnol.* **1998**, *16*, 41−46.
(6) Hammes, W. P.; Stolz, P.; Gänzle, M. Metabolism of lactobacilli in traditional sourdoughs. *Adv. Food Sci.* **1996**, *18*, 176−184.
(7) Korakli, M.; Schwarz, E.; Wolf, G.; Hammes, W. P. Production of mannitol by *Lactobacillus sanfranciscensis*. *Adv. Food Sci*. **2000**, *22*, 1−4.
(8) Dal Bello, F.; Walter, J.; Hertel, C.; Hammes, W. P. In vitro study of prebiotic properties of levan-type exopolysaccharides from lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis. *Syst. Appl. Microbiol.* **2001**, *24*, 232−237.

(9) Van Geel-Schutten, G. H.; Faber, E. J.; Smit, E.; Bonting, K.; Smith, M. R.; Ten Brink, B.; Kamerling, J. P.; Vliegenthart, J. F. G.; Dijkhuizen, L. Biochemical and structural characterisation of the glucan and fructan exopolysaccharides synthesized by *Lactobacillus reuteri* wild-type strain and by mutant strains. *Appl. Environ. Microbiol.* **1999**, *65*, 3008−3014.
(10) Gibson, G. R.; Beatty, E. R.; Wang, X.; Cummings, J. H. Selective stimulation of bifidobacteria in the human colon by oligofructose and inulin. *Gastroenterology*. **1995**, *108*, 975−982.
(11) Souci; Fachmann; Kraut. *Die Zusammensetzung der Lebensmittel*; Wissenschaftliche Verlagsgesellschaft: Stuttgart, Germany, 1987.
(12) De Man, J. C.; Rogosa, M.; Sharpe, M. E. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol.* **1960**, *23*, 130−135.
(13) Stolz, P.; Hammes, W. P.; Vogel, R. F. Maltose-phosphorylase and hexokinase activity in lactobacilli from traditionally prepared sourdoughs. *Adv. Food Sci*. **1996**, *18*, 1−6.
(14) Schmidt, H.-L. Food quality control and studies on human nutrition by mass spectrometric and nuclear magnetic resonance isotope ratio determination. *Fresenius' Z. Anal. Chem*. **1986**, *324*, 760−766.
(15) Winkler, J. F.; Schmidt, H.-L. Einsatzmöglichkeiten der ^{13}C-Isotopen-Massenspektrometrie in der Lebensmitteluntersuchung. *Lebensm. Unters. Forsch.* **1980**, *171*, 85−94.
(16) Koziet, J.; Rossmann, A.; Martin, G. J.; Ashurst, P. R. Determination of carbon-13-content of sugars of fruit and vegetable juices. *Anal. Chim. Acta* **1993**, *271*, 31−38.
(17) Henn, M.; Chapela, H. I. Differential C isotope discrimination by fungi during decomposition of C_3- and C_4-derived sucrose. *Appl. Environ. Microbiol.* **2000**, *66*, 4180−4186.
(18) Armero, E.; Collar, C. Crumb firming kinetics of wheat breads with anti-staling additives. *J. Cereal Sci.* **1998**, *28*, 165−174.
(19) Salovaara, H.; Spicher, G. Anwendung von Weizensauerteigen zur Verbesserung der Qualität des Weizenbrotes. *Getreide Mehl Brot*. **1987**, *41*, 116−118.
(20) Salovaara, H.; Valjakka T. The effect of fermentation temperature, flour type, and starter on the properties of sour wheat bread. *Int. J. Food Sci. Technol*. **1987**, *22*, 591−597.
(21) Barber, B.; Ortolá, C.; Barber, S.; Fernández, F. Storage of packaged white bread. III. Effects of sour dough and addition of acids on bread characteristics. *Z. Lebensm. Unters. Forsch*. **1992**, *194*, 442−449.
(22) Schieberle, P. Intense aroma compounds—useful tools to monitor the influence of processing and storage on bread aroma. *Adv. Food Sci*. **1996**, *18*, 237−244.
(23) Escrivá, C.; Martínez-Anaya, M. A. Influence of enzymes on the evolution of fructosans in sourdough wheat process. *Eur. Food Res. Technol.* **2000**, *210*, 286−292.
(24) Kontula, P.; Suihko, M.-L.; Suortti, T.; Tenkanen, M.; Mattila-Sandholm, T.; von Wright, A. The isolation of lactic acid bacteria from human colonic biopsies after enrichment on lactose derivatives and rye arabinoxylo-oligosaccharides. *Food Microbiol*. **2000**, *17*, 13−22.

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Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*

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Aims: The metabolism by bifidobacteria of exopolysaccharide (EPS) produced by *Lactobacillus sanfranciscensis* was investigated. To evaluate the significance of the EPS produced by *Lact. sanfranciscensis* during dough fermentation on the overall prebiotic properties of bread, metabolism by bifidobacteria of water-soluble polysaccharides (WSP) from wheat and rye was investigated.

Methods and Results: Polyglucose and polyfructan contained in WSP from wheat and rye were metabolized by bifidobacteria. In contrast, WSP isolated from fermented doughs were not metabolized by bifidobacteria. The arabioxylan fraction of WSP was metabolized neither by bifidobacteria nor by lactobacilli. All the bifidobacteria tested were able to metabolize fructan from *Lact. sanfranciscensis*. The kinetics of EPS metabolism by various bifidobacteria were characterized by diauxic utilization of fructose and EPS.

Conclusions: Bifidobacteria metabolize fructan from *Lact. sanfranciscensis*. Polyfructan and the starch fractions from wheat and rye, which possess a bifidogenic effect, were degraded by cereal enzymes during dough fermentation, while the EPS were retained.

Significance and Impact of the Study: EPS produced by sourdough lactic acid bacteria will improve the nutritional properties of sourdough fermented products.

INTRODUCTION

Prebiotics are defined as non-digestible food ingredients that affect the host beneficially by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health (Gibson and Roberfroid 1995). The application of prebiotics to the human diet for preferential metabolism by bifidobacteria (bifidogenic factors) results in an increase in both the occurrence and the number of bifidobacteria isolated from faecal material (Modler 1994). The establishment of metabolically-active bifidobacteria is considered to be beneficial for the host; the effects include stabilization of the gut mucosal barrier and prevention of diarrhoea. The end products of carbohydrate metabolism by bifidobacteria (lactate and short-chain fatty acids, SCFA) result in a lower luminal pH (Lee 1999) and inhibit pathogens (Gibson and Roberfroid 1995). Beneficial effects of SCFA to human health were recently reviewed by Topping and Clifton (2001).

Cereal products are the most important staple food throughout the world. Cereal grains are predominantly composed of starch and in addition, non-starch polysaccharides composed of glucose (β-glucan), fructose (polyfructan), xylose and arabinose (arabinoxylan) are present (Belitz and Grosch 1999). Some of these polysaccharides are partially digestible, for example starch, and some of them are believed to serve as dietary fibre, such as arabinoxylan. In addition to polyfructan, wheat and rye flours contain kestose, nystose and other fructooligosaccharides of the inulin type (Campbell *et al.* 1997). Arabinoxylan undergoes degradation by cereal enzymes during the dough resting time and this results in solubilization of arabinoxylan (Korakli *et al.* 2001). Bread

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production traditionally involves sourdough fermentation, and *Lactobacillus sanfranciscensis* belongs to the microflora of traditionally-prepared sourdoughs (Hammes *et al.* 1996). In addition to the polysaccharides from wheat or rye, strains of *Lact. sanfranciscensis* produce exopolysaccharides (EPS) in wheat and rye sourdough (Korakli *et al.* 2000, 2001). The EPS produced by *Lact. sanfranciscensis* TMW1.392 is a high molecular weight fructan of the levan type (Korakli *et al.* 2001; Dal Bello *et al.* 2001). Dal Bello *et al.* (2001) have recently shown a bifidogenic property of fructan produced by two strains of *Lact. sanfranciscensis* using denaturing gradient gel electrophoresis (DGGE). Comparison of an intestinal microflora incubated in media with EPS and media without a carbohydrate source showed that EPS favoured the growth of bifidobacteria by more than 2 log and the growth of clostridia by less than 1 log; stimulation of growth of other bacteria was not observed (Dal Bello *et al.* 2001). Few data are available on the metabolism of polysaccharides isolated from wheat and rye by bifidobacteria and lactobacilli. The aim of this study was to investigate the ability of these bacteria to degrade polysaccharides from wheat and rye, and to determine the effect of cereal enzymes and sourdough fermentation on the degradation of polysaccharides by bifidobacteria. Furthermore, the microbial growth and metabolic kinetics of bifidobacteria grown on fructose or fructan isolated from *Lact. sanfranciscensis* TMW1.392 were determined.

MATERIALS AND METHODS

Organism and culture conditions

The EPS-producing strain *Lactobacillus sanfranciscensis* TMW1.392 (isogenic with *Lact. sanfranciscensis* LTH2590) was cultivated in sucrose-MRS as described by Korakli *et al.* (2001). *Lactobacillus sanfranciscensis* TMW1.52 was used as non-EPS-producing strain. The following bifidobacteria were used: *Bifidobacterium adolescentis* DSM20083, *B. bifidum* DSM20456, *B. breve* DSM20213, *B. infantis* DSM20088 and *B. minimum* TMW2.456. According to the supplier (DSMZ, Braunschweig, Germany), these strains are of human intestinal origin. *Lactobacillus sanfranciscensis* was cultivated in mMRS (Müller *et al.* 2001) at 30°C. Bifidobacteria were grown at 37°C in DSM 58 medium containing a vitamin mix; biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamine, riboflavin, cobalamin and panthothenic acid were dissolved in water to 0·2 g l^{-1}, sterilized by filtration, and 1 ml l^{-1} of this vitamin mix added to the medium. For the experiments on fructose metabolism by bifidobacteria, the same bifidobacteria medium was used but 7 g l^{-1} fructose were added instead of glucose.

Isolation and hydrolysis of EPS

Sucrose MRS medium was inoculated with *Lact. sanfranciscensis* TMW1.392. After 24 h ($O.D._{578nm}$ 4–5), cells were removed by centrifugation (4000 *g*, 15 min) and 2 volumes of chilled 95% ethanol were added to the supernatant fluid. After incubation for 3 h at 4°C, the precipitate was collected by centrifugation (4000 *g*, 15 min). The pellet was dissolved in deionized water and solids were removed by centrifugation (8000 *g*, 10 min). The supernatant fluid was again precipitated with ethanol; EPS were dialysed (molecular weight cut off 12 000–14 000, Serva, Heidelberg, Germany) against deionized water at 4°C for 24 h, and then lyophilized. An EPS stock solution of 50 g l^{-1} was prepared from lyophilized EPS, autoclaved, and stored at room temperature. For detection of EPS degradation after heat treatment and the determination of molecular weight, gel permeation chromatography (GPC) was used. Superdex^{TM} 200 HR and Superdex^{TM} peptide columns (Amersham Pharmacia Biotech, Uppsala, Sweden) were coupled, and samples were eluted with 50 mmol l^{-1} NaH_2PO_4 (pH 6·5) at a flow rate of 0·4 ml min^{-1}. For hydrolysis of EPS, samples were incubated for 2 h at 80°C in the presence of 3·5% (v/v) perchloric acid (Riedel-deHaën).

Isolation and hydrolysis of polysaccharides from wheat and rye flours

One part of each wheat flour (flour extraction rate 64–71%) or wholemeal rye flour was mixed with three parts water (w/w). Solids were removed by centrifugation for 15 min at 4000 *g*. Polysaccharides in the supernatant fluid were precipitated by the addition of two parts (v/v) chilled ethanol to one part supernatant fluid and harvested by centrifugation. Polysaccharides were dissolved in water and again precipitated with ethanol. After centrifugation, the precipitate was dissolved in water, dialysed and lyophilized. Stock solutions of each polysaccharide (25 g l^{-1}) were prepared and autoclaved; solid components were removed by centrifugation and the supernatant fluid was stored and used as a polysaccharide stock solution. For the isolation of water-soluble polysaccharides (WSP) from fermented and control doughs, one part of each flour was mixed with one part water (w/w). Fermentations were carried out for 24 h at 30°C. Doughs were inoculated to a cell count of 5×10^6 cfu g^{-1} with the non-EPS-producing strain *Lact. sanfranciscensis* TMW1.52 harvested from an overnight culture in mMRS and resuspended in phosphate buffer. Control doughs were not inoculated, and 100 mg kg^{-1} chloramphenicol and 50 mg kg^{-1} erythromycin were added. Hydrolysis of WSP was carried out by incubation of the samples for 2 h at 80°C in the presence of 10·5% (v/v) perchloric acid.

Degradation of polysaccharides by bifidobacteria and lactobacilli

For the experiments with EPS and polysaccharides from wheat and rye, mMRS and Bifido-medium were prepared without sugars. The medium components for 1 litre were dissolved in 800 ml deionized water and autoclaved. Various parts (v/v%) of the polysaccharide stock solutions (EPS, wheat or rye polysaccharides) were added. During growth of the organisms, samples were taken for determination of the pH; the optical density (O.D.) was measured at 578 nm against air, and for subsequent HPLC analysis of metabolites. In pH-regulated fermentations, the pH was kept in the range 5·50 to 6·50 by the addition of 5 M NaOH. Fermentations with dialysed EPS were performed in duplicate with *B. breve* and *B. bifidum*, and in triplicate with *B. adolescentis* and *B. infantis*. Fermentations with rye WSP were carried out in duplicate. The coefficient of variation between two fermentations was generally in the range of 5 to 15%. Resistance of EPS to hydrolysis was investigated in HCl buffer containing (l^{-1}): NaCl, 8 g; KCl, 0·2 g; $Na_2HPO_4.2H_2O$, 8·25 g; NaH_2PO_4, 14·35 g; $CaCl_2.2H_2O$, 0·1 g; $MgCl_2.6H_2O$, 0·18 g. The pH was adjusted to 2 using 5 M HCl.

HPLC determination of substrates and metabolites

The concentrations of maltose, lactic acid, acetic acid, formic acid and ethanol were determined as described previously (Müller *et al.* 2001). Sucrose, glucose, fructose, xylose and arabinose concentrations were determined as described by Korakli *et al.* (2001). The concentration of EPS in fermentation broth was calculated as the difference between the fructose concentrations before and after hydrolysis of EPS. The concentrations of polysaccharides from rye or wheat WSP were calculated likewise by comparing monosaccharide concentrations before and after hydrolysis of WSP.

RESULTS

Characterization and properties of EPS

Lactobacillus sanfranciscensis TMW1.392 produces EPS in media containing sucrose. The amount of EPS produced in MRS containing 47 g l^{-1} sucrose and 20 g l^{-1} fructose was 14 g l^{-1} (data not shown). The composition of dialysed EPS after hydrolysis was 99·30% fructose and 0·70 ± 0·40% glucose ($n = 10$). After treatment of pure fructose aqueous solution under the same hydrolysis conditions as those used for EPS, glucose measured 0·50%. This result confirms that the EPS produced by *Lact. sanfranciscensis* TMW1.392 is a homopolymer composed of fructose. The molecular weight of dialysed EPS was estimated to be $> 2 \times 10^6$ Da (Fig. 1). Autoclaving EPS in phosphate buffer resulted in a partial degradation of the high molecular chains into smaller chains, with molecular weights ranging from 10^4 to 10^6 Da. Treatment of aqueous EPS solution with HCl buffer, pH 2, at 37°C for 1 h showed that only 3·30 ± 2·70% of the EPS was hydrolysed.

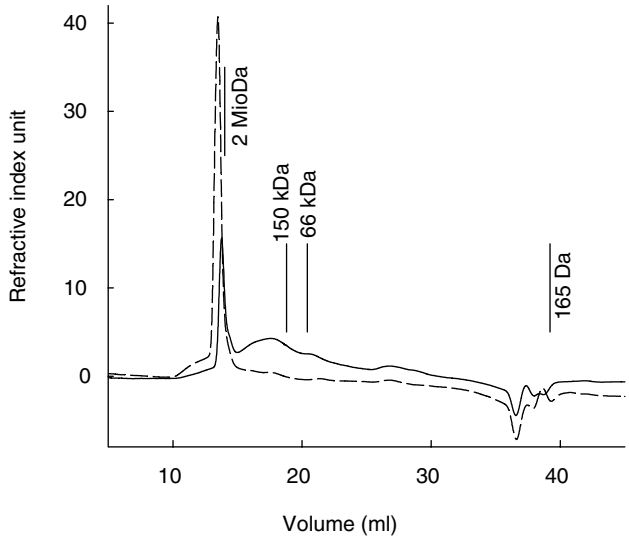


Fig. 1 Separation of EPS by GPC. The chromatogram of heat-treated EPS (121°C, 20 min, solid line) is compared with untreated EPS (dotted line).The molecular sizes of the external calibration standards are indicated

Metabolism of EPS by bifidobacteria

The kinetics of EPS metabolism by *B. breve* are shown in Fig. 2. Only 27% of the EPS had been metabolized after 60 h. At a pH of 4·3, growth and metabolism stopped. The limiting effect of the pH on metabolism could be prevented by regulating the pH in subsequent fermentations. Figure 3 shows the microbial growth and fermentation kinetics of *B. breve*, *B. bifidum*, *B. infantis* and *B. adolescentis* at controlled pH. Growth of *B. breve* was significantly higher than in the fermentation without pH regulation, and EPS was completely consumed. Diauxic growth was seen in all the bifidobacteria. At the beginning of the fermentation, free fructose was metabolized and only after a second lag phase was EPS metabolized. The concentration of free fructose in the fermentations with *B. infantis* and *B. adolescentis* (Fig. 3) was 11 mmol l^{-1} at the beginning of the fermentation. The acetate/lactate ratio ranged from 1·8 to 2·5 (Table 1).

None of the following lactobacilli showed a difference in O.D. when grown on medium containing EPS compared with media without sugar (data not shown): *Lact. sanfranciscensis* TMW1.52, *Lact. sanfranciscensis* TMW1.54, *Lact. pontis* DSM8475, *Lact. animalis* TMW1.972, *Lact. brevis*

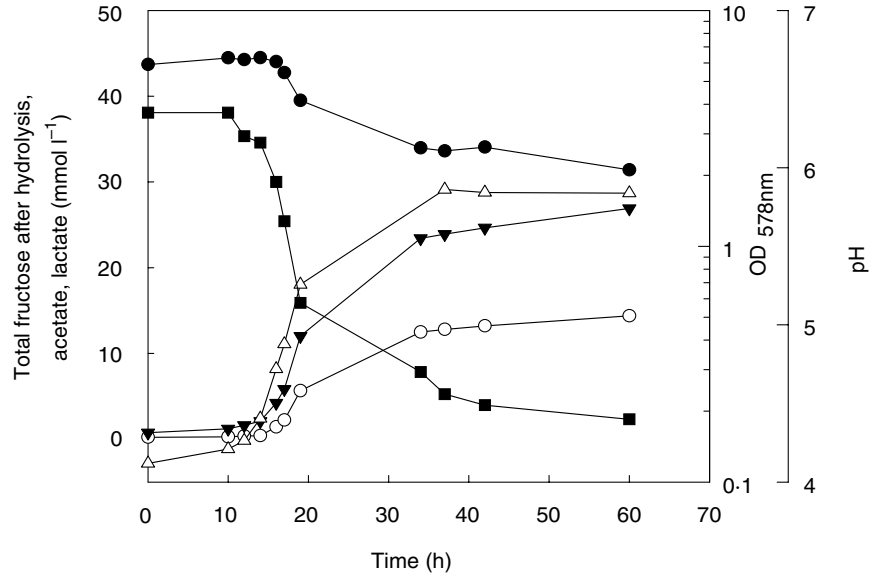


Fig. 2 Kinetics of microbial growth and fermentation products of *Bifidobacterium breve* on EPS. (●) Total fructose after hydrolysis; (○) lactate; (▼) acetate; (■) pH; (△) O.D.

DSM5622, *Lact. paracasei* subsp. *paracasei* TMW1.1183, *Lact. plantarum* TMW1.46, *Lact. salivarius* subsp. *salivarius* TMW1.992, *Lact. reuteri* TMW1.974 and *Lact. johnsonii* TMW1.990. Failure of *Lact. pontis* and *Lact. paracasei* to metabolize EPS was confirmed by determination of the kinetics of microbial growth and by analysis of metabolites produced during fermentation (data not shown).

Utilization of fructose by bifidobacteria

The fermentation of bifidobacteria in media containing EPS was compared with fermentation in media containing fructose as the sole carbon source. As shown in Fig. 4, fructose consumption by all bifidobacteria ranged between 43 and 75% of the initial fructose concentration. *Bifidobacterium adolescentis* and *B. infantis* exhibited the highest fructose consumption (75% and 65%, respectively). At pH values ranging between 4·5 and 4·7, growth and metabolism were inhibited by all bifidobacteria. The acetate/lactate ratio in fermentations with glucose and fructose by the bifidobacteria used in this study varied between 1·45 and 2·95, as shown in Table 1. Generally, the same metabolites were formed from fructose and EPS. However, *B. bifidum* produced more acetate from EPS compared with fermentation with fructose as substrate. In addition to acetate and lactate, small amounts of formate were formed, accounting for about 0·70–2·90% of the carbon substrate. Within experimental error, the substrates consumed were fully recovered as acetate, lactate or formate. In fermentations with EPS or fructose, products accounted for 84–110% of the substrates consumed.

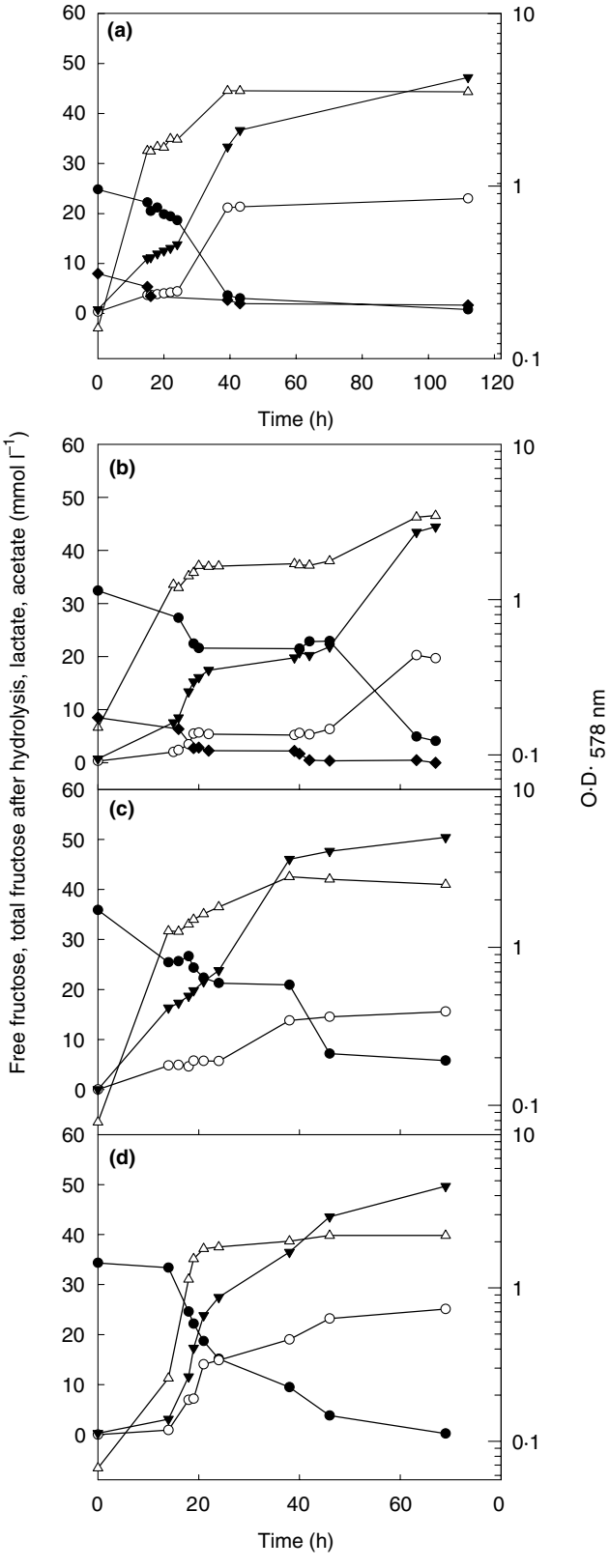
Metabolism of WSP from wheat and rye by bifidobacteria

To determine a possible bifidogenic effect of polysaccharides originating from wheat and rye, fermentations were performed with rye and wheat WSP as the sole source of carbohydrates. Bifidobacteria grown on wheat and rye WSP reached higher cell densities compared with medium

Table 1 Acetate/lactate ratio of bifidobacteria grown on EPS, fructose or glucose

	Molar ratio of acetate to lactate		
	Glucose–Medium	Fructose–Medium	Dialysed EPS–Medium
B. breve	1·80	1·87	2·00 ± 0·18
B. bifidum	2·95	1·72	2·43 ± 0·25
B. adolescentis	1·45	1·71	1·88 ± 0·01
B. minimum	1·80	2·09	2·26
B. infantis	1·49	2·65	2·52 ± 0·32

Data for fermentations on EPS are means ± standard deviations of at least two independent experiments.



without a carbohydrate source (data not shown). Fermentations carried out with WSP from wheat and rye flour and substrates and products of metabolism were determined by HPLC. Figure 5 shows microbial growth and fermentation kinetics of *B. adolescentis* on rye WSP. Xylose and arabinose concentrations remained constant during the fermentation, indicating the inability of *B. adolescentis* to use arabinoxylan as a carbohydrate source. During fermentation, polysaccharides composed of glucose and fructose were degraded. Similar results were also observed with wheat WSP fermented by *B. breve* and *B. infantis* (data not shown).

Effect of sourdough fermentation on the metabolism of WSP

Polysaccharides in doughs undergo enzymatic degradation during dough fermentation. Therefore, it is important to estimate changes in the water-soluble fraction of the polysaccharides, caused by the flour enzymes or by the sourdough starter, on the ability of bifidobacteria to metabolize these polysaccharides. The polysaccharides used for these experiments were isolated from rye and wheat flours after a sourdough fermentation for 24 h with the non-EPS-producing strain, *Lact. sanfranciscensis* TMW1.52, and from a control dough. In the control dough incubated in the presence of antibiotics, viable cell counts were less than 10^4 cfu g^{-1} throughout the fermentation, excluding an influence of microbial metabolism on dough composition. Growth of *B. adolescentis* on medium with polysaccharides isolated from control and fermented rye dough was poor in comparison with WSP isolated from flour. Arabinoxylans were not metabolized, even after their solubilization by flour enzymes. The polysaccharide fraction composed of glucose remained constant, in contrast to the WSP isolated from flours without fermentation. This suggests that *B. adolescentis* was only able to use a certain fraction of glucose polysaccharide in flour, which is probably degraded by flour enzymes during dough fermentation. The absence of fructose in the hydrolysed WSP isolated from fermented and control dough indicates that fructans were also degraded by cereal enzymes. Similar results were also observed during fermentation with *B. infantis*. WSP fractions isolated from fermented and control wheat doughs could not be metabolized by *B. adolescentis* or *B. infantis* (data not shown).

◀

Fig. 3 Kinetics of microbial growth and fermentation products of (a) *Bifidobacterium breve* ($n = 2$), (b) *B. bifidum* ($n = 2$), (c) *B. infantis* ($n = 3$) and (d) *B. adolescentis* ($n = 3$) on EPS in pH-controlled fermentation. (◆) Free fructose; (●) total fructose after hydrolysis; (○) lactate; (▼) acetate; (△) O.D. Results are representative of *n* independent experiments

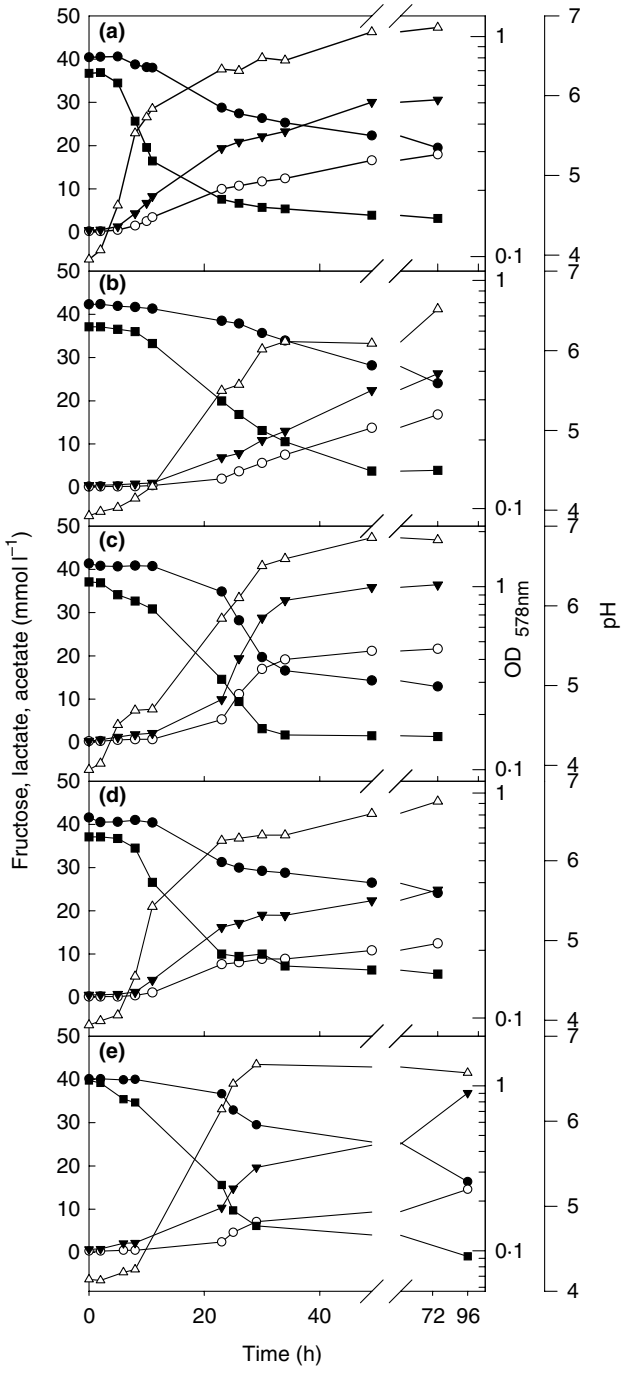


Fig. 4 Microbial growth and metabolization of fructose by (a) *Bifidobacterium breve*, (b) *B. bifidum*, (c) *B. adolescentis*, (d) *B. minimum* and (e) *B. infantis*. (●) Fructose; (○) lactate; (▼) acetate; (■) pH; (△) O.D.

DISCUSSION

Strains of *Lact. sanfranciscensis* produce EPS in wheat and rye sourdough (Korakli *et al.* 2001), and the growth-promoting effect of EPS from *Lact. sanfranciscensis* on bifidobacteria was recently shown by Dal Bello *et al.* (2001) using DGGE analysis. To confirm the selective metabolism of EPS by bifidobacteria observed by Dal Bello *et al.* (2001) using a complex faecal microflora of human origin, the present study describes the metabolism of EPS by pure cultures of intestinal bifidobacteria. This EPS is stable at pH 2, and heat treatment (121°C, 20 min) of an aqueous EPS solution resulted in partial degradation only to fragments of 10^4–10^6 Da. Oligofructose and inulin, which are not degraded by pancreatic enzymes, were reported to have an average recovery at the terminal ileum between 85 and 89% (Cummings *et al.* 2001). EPS of *Lact. sanfranciscensis* TMW1.392 is composed predominantly of fructan and is therefore probably not degraded under the conditions prevailing in the stomach or small intestine. Thus, EPS meets important requirements for its use as a prebiotic (Crociani *et al.* 1994). As amounts of up to 5 g EPS kg^{-1} dough are generated by traditional fermentation with appropriate starter cultures (Korakli *et al.* 2001), the use of *Lact. sanfranciscensis* fructan does not require its application as an additive. In addition to a possible prebiotic effect of fructan in bread, it is known to improve dough machinability as well as the sensory properties of bread (Yasushi and Akifumi 1995).

Bifidobacteria metabolize fructose to form acetate, formate and lactate. Among other factors, the type of substrate, substrate availability and pH determine the composition of the end products (Marx *et al.* 2000). Perrin *et al.* (2001) found that the acetate/lactate ratio was higher in fermentations with fructose in pH-regulated batches compared with those in non-pH-regulated batches. This study revealed that metabolites from EPS were not generally different from those produced with fructose as carbon source.

Wheat and rye contain starch, polyfructan and arabinoxylan. To assess the relevance of fructan produced during sourdough fermentation as prebiotic, fermentations were carried out with WSP from wheat and rye. The bifidobacteria considered in this work degraded polyfructan and a polysaccharide fraction composed of glucose, whereas arabinoxylan could not be degraded. Fermentation of amylose and amylopectin by bifidobacteria was previously described by Crociani *et al.* (1994), but only a few bifidobacteria could ferment xylan. Xylooligosaccharides are poorly utilized by bifidobacteria, but xylobiose was reported to be bifidogenic (Okazaki *et al.* 1990; Hopkins *et al.* 1998). Polysaccharides composed of glucose in WSP isolated from rye sourdough fermented with *Lact. sanfranciscensis*, and from control rye dough, could not be fermented by *B. adolescentis.* This can probably be attributed to the enzymatic degradation of a starch fraction during dough fermentation. Furthermore, polyfructan in flour was completely degraded by cereal enzymes.

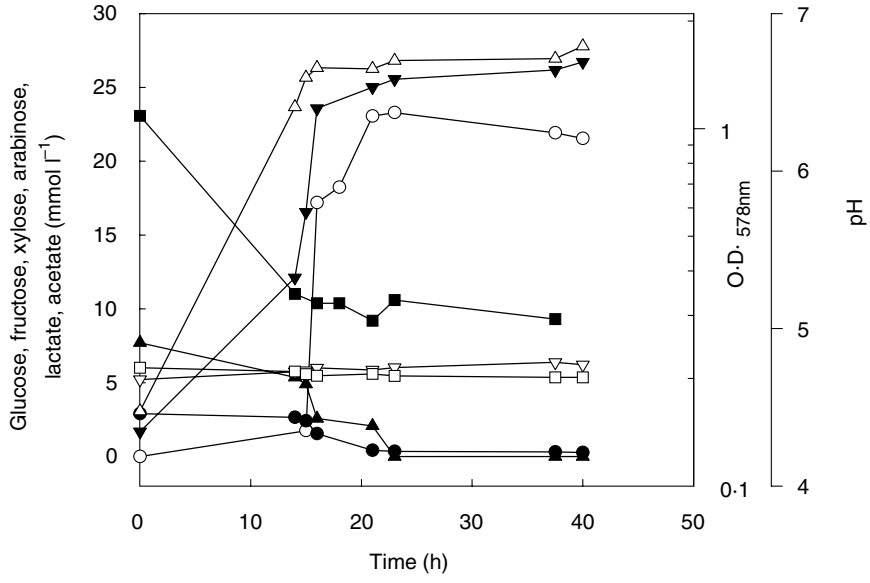


Fig. 5 Kinetics of microbial growth and fermentation products of *Bifidobacterium adolescentis* on rye WSP. (▲) Glucose; (●) fructose; (▽) xylose; (□) arabinose; (○) lactate; (▼) acetate; (△) O.D.; (■) pH. Results are representative of two independent experiments

In conclusion, EPS produced by *Lact. sanfranciscensis* TMW1.392 during sourdough fermentation was metabolized by bifidobacteria. The water-soluble polysaccharides in wheat and rye were also degraded by bifidobacteria. However, polyfructan and the starch fraction, which possess a bifidogenic effect, were degraded by cereal enzymes during dough fermentation while the EPS was retained. The stability of EPS should enable it to withstand the baking process. This suggests that EPS will improve the nutritional properties of sourdough fermented products.

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REFERENCES

Belitz, H.D. and Grosch, W. (1999) *Food Chemistry*. Berlin: Springer-Verlag.

Campbell, J.M., Bauer, L.L., Fahey, G.C., Hogarth, A.J.C.L., Wolf, B.W. and Hunter, D.E. (1997) Selected fructooligosaccharide (1-kestose, nystose, and 1^F-β-fructofuranosylnystose) composition of foods and feeds. *Journal of Agriculture and Food Chemistry* **45,** 3076–3082.

Crociani, F., Alessandrini, A., Mucci, M.M. and Biavati, B. (1994) Degradation of complex carbohydrates by *Bifidobacterium* spp. *International Journal of Food Microbiology*. **24,** 199–210.

Cummings, J.H., Macfarlane, G.T. and Englyst, H.N. (2001) Prebiotic digestion and fermentation. *American Journal of Clinical Nutrition* **73,** 415S–420S.

Dal Bello, F., Walter, J., Hertel, C. and Hammes, W.P. (2001) *In vitro* study of prebiotic properties of levan-type exopolysaccharides from lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis. *Systematic and Applied Microbiology* **24,** 232–237.

Gibson, G.R. and Roberfroid, M.B. (1995) Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. *Journal of Nutrition* **125,** 1401–1412.

Hammes, W.P., Stolz, P. and Gänzle, M. (1996) Metabolism of lactobacilli in traditional sourdoughs. *Advances in Food Sciences* **18,** 176–184.

Hopkins, M.J., Cummings, J.H. and Macfarlane, G.T. (1998) Inter-species differences in maximum specific growth rates and cell yields of bifidobacteria cultured on oligosaccharides and other simple carbohydrate sources. *Journal of Applied Microbiology* **85,** 381–386.

Korakli, M., Rossman, A., Gänzle, M.G. and Vogel, R.F. (2001) Sucrose metabolism and exopolysaccharide production in wheat and rye sourdoughs by *L. sanfranciscensis*. *Journal of Agriculture and Food Chemistry* **49,** 5194–5200.

Korakli, M., Schwarz, E., Wolf, G. and Hammes, W.P. (2000) Production of mannitol by *Lactobacillus sanfranciscensis*. *Advances in Food Sciences* **22,** 1–4.

Lee, Y.K. (1999) *Handbook of Probiotics*. New York: John Wiley & Sons, Inc.

Marx, S.P., Winkler, S. and Hartmeier, W. (2000) Metabolization of β-(2,6)-linked fructose-oligosaccharides by different bifidobacteria. *FEMS Microbiology Letters* **182,** 163–169.

Modler, H.W. (1994) Bifidogenic factors — sources, metabolism and applications. *International Dairy Journal* **4,** 383–407.

Müller, M.R.A., Wolfrum, G., Stolz, P., Ehrmann, M.A. and Vogel. R.F. (2001) Monitoring the growth of *Lactobacillus* species during a rye flour fermentation. *Food Microbiology* **18,** 217–227.

Okazaki, M., Fujikawa, S. and Matsumoto, N. (1990) Effects of xylooligosaccharide on growth of bifidobacteria. *Journal of Japanese Society of Nutrition and Food. Science* **43,** 395–401.

Perrin, S., Warchol, M., Grill, J.P. and Schneider, F. (2001) Fermentations of fructo-oligosaccharides and their components by *Bifidobacterium infantis* ATCC 15697 on batch culture in semi-synthetic medium. *Journal of Applied Microbiology* **90,** 859–865.

Topping, D.L. and Clifton, P.M. (2001) Short-chain fatty acids and human colonic function: roles of resistant starch and non-starch polysaccharides. *Physiological Reviews* **81,** 1031–1064.

Yasushi, M. and Akifumi, Y. (1995) Bread dough improver and production of bread dough. Japanese Patent Office Publication number 07046956A.

Trends in High Pressure Bioscience and Biotechnology
R. Hayashi (editor)

Metabolism of *Lactobacillus sanfranciscensis* under high pressure: investigations using stable carbon isotopes

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1. Abstract

The effect was studied of high pressure on the maltose metabolism of *Lactobacillus sanfranciscensis* in the presence or absence of fructose at pressures ranging from 0.1 to 300 MPa. Substrate consumption and product formation was determined by HPLC. To elucidate mechanisms of pressure induced effects on carbohydrate metabolism, the ratio of ^{12}C / ^{13}C isotopes in maltose and ethanol was measured by IRMS and GC-c-IRMS, respectively.

L. sanfranciscensis tolerated pressures up to 150 MPa for 3 h without appreciable loss of viability and incubation at 200 MPa decreased viable cell counts by 2 log. Application of 50 MPa did not affect maltose uptake. Incubation at 100, and 150 MPa resulted in a decrease of maltose consumption by 67% and 82%, respectively. The molar ratio of lactate produced to maltose consumed was unchanged by pressures of 100 – 150 MPa. Incubation at 100 – 150 MPa resulted in formation of lactate and acetate only, ethanol formation was not observed. These results conform with the preferential production of acetate observed also at comparable maltose turnover levels at ambient pressure. *L. sanfranciscensis* treated at 100 MPa exhibited normal metabolic activity after pressurization but treatment with 150 MPa resulted in an inhibition of maltose metabolism up to 3 h post-treatment.

The isotope ratio analysis of carbon in ethanol produced from maltose by *L. sanfranciscensis* revealed that incomplete maltose consumption resulted in an enrichment of ^{12}C in the ethanol due to a kinetic isotope effect. This kinetic isotope effect was enhanced upon metabolism under high pressure conditions with the same maltose turnover levels.

1. Introduction

Lactic acid bacteria are widely used in food biotechnology for the production of fermented foods and food additives. Studies on the physiology and genetics of these organisms are the basis for their successful use in biotechnological applications. A key element to the application and stable preparation of lactic acid bacteria is their cellular stress response to environmental stressors, e.g. temperature or high salt conditions. Hydrostatic pressure affects the viability, metabolic activity, and gene regulation of biological systems. It was shown that sublethal high pressure results in an acidification of the vacuolar pH in *Saccharomyces cerevisiae* (Abe and Horikoshi, 1998), which was attributed to the ionization of phosphates and carbonates as well as the inactivation of membrane ion pumps. The stress response of *Escherichia coli* to hydrostatic pressure was shown to include the synthesis of pressure inducible proteins (Welch et al., 1993). More recently, differential gene expression as response to elevated hydrostatic pressure was demonstrated for *Escherichia coli* and

Saccharomyces cerevisiae (Abe and Kato, 1999, Iwahashi et al., 2000). High pressure processes have found commercial application in the food industry in the past years, however, few data are available on high pressure effects on metabolism and genetic regulation of lactic acid bacteria. It was therefore the aim of this work to investigate the effects of high pressure on the metabolism of *L. sanfranciscensis*.

L. sanfranciscensis is a heterofermentative lactic acid bacterium with industrial use in sourdough fermentations and has a potential for biotechnological production of food additives (Hammes et al., 1996). *L. sanfranciscensis* degrades hexoses via the pentose-phosphate-shunt; an overview of the metabolic pathways is shown in **Figure 1** (Stolz et al., 1995b, Vogel et al., 1999). Maltose is cleaved by maltose phosphorylase to glucose-1-phosphate and glucose. Glucose is either phosphorylated at the expense of ATP to yield glucose-6-phosphate, or excreted into the medium. Glucose-6-P is converted to xylulose-5-P with concomitant production of CO_2 and reduction of $NADP^+$. Acetyl phosphate represents a major branching point of this metabolism where the carbon flux is directed towards the alternative end products acetate or ethanol. If additional substrates for cofactor regeneration are unavailable, acetyl phosphate is used to regenerate the NADH formed upstream and quantitative conversion of acetyl-phosphate to ethanol is observed. In the presence of electron acceptors, acetate formation is favored over ethanol formation since one additional ATP is gained in the acetate branch of metabolism. Substrates that are used by *L. sanfranciscensis* to regenerate NADH include oxygen, citrate, and fructose (Stolz et al., 1995a).

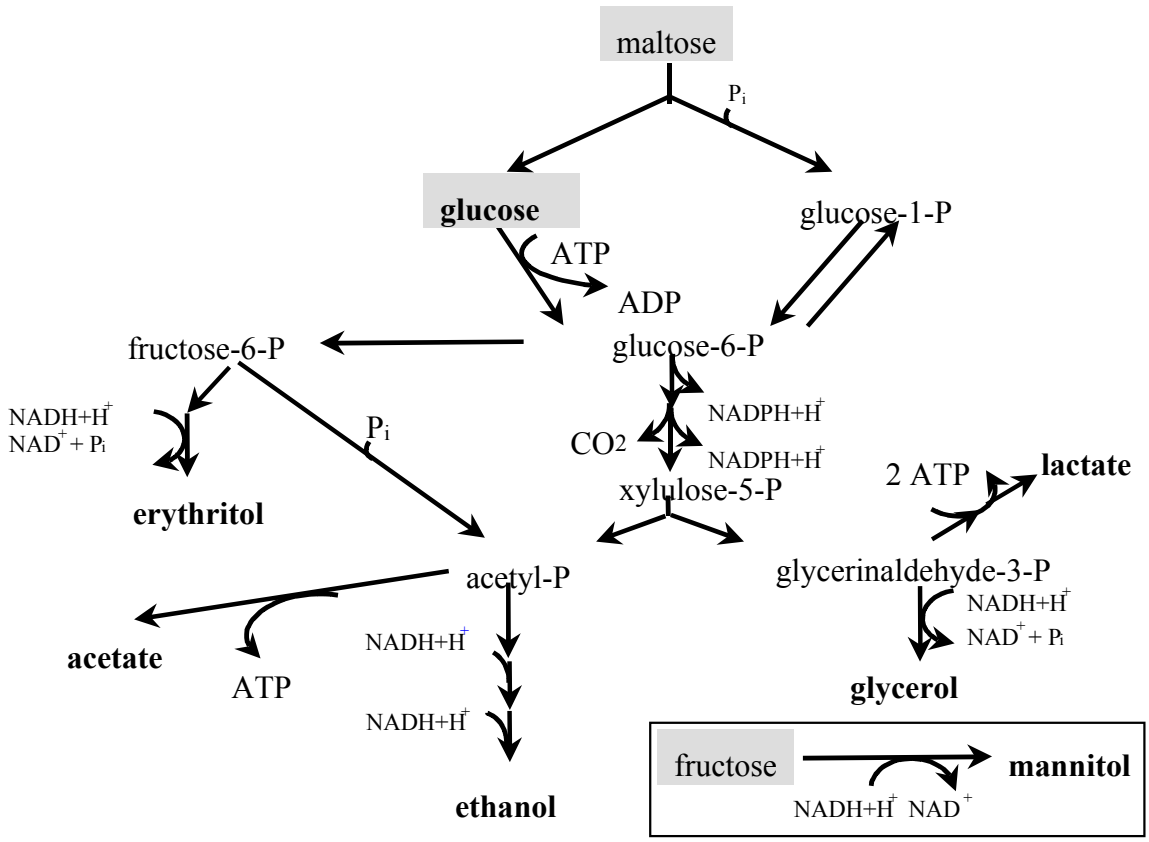

Figure 1. Overview over the metabolic pathways of maltose utilization in *L. sanfranciscensis*. (Stolz et al., 1995, Vogel et al., 1999, modified). Substrates are marked in gray, products are printed in bold letters.

2. Materials and Methods

Organism and culture conditions: *L. sanfranciscensis* LTH2581 and LTH1729 were cultured at 30°C in mMRS4 containing the following components per liter: peptone from casein, 10g; yeast extract, 5g; meat extract, 5g; $K_2HPO_4.3H_2O$, 2.6g; KH_2PO_4, 4g; cystein-HCl, 0.5g; NH_4Cl, 3g; maltose, 10g; fructose, 10g; Tween 80, 1 ml, $MgSO_4$ x H_2O 0.1g, $MnSO_4$ x $4H_2O$, 0.05 g. The pH was adjusted to 6.2, and the medium was sterilized at 121°C for 20 min. Sugars were autoclaved separately. A vitamin mix containing biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamine, riboflavin, cobalamin and panthothenic acid (0.2 g/l each) was sterilized by filtration and 1 ml vitamin mix was added per l mMRS4. For the isotope experiments C_3-MRS4 was used. This medium had the same composition as mMRS4 but did not contain fructose. The maltose used for C_3-MRS4 was derived from a C_3-plant (Sigma, USA) and had a $\delta^{13}C$-value of -25.20‰. Plates were incubated at 30°C under controlled atmosphere (76% N_2, 20% CO_2, 4% O_2).

High pressure treatment. An overnight culture of *L. sanfranciscensis* was sub-cultured with 1% inoculum in mMRS4 or C_3-MRS4. Late stationary cells were harvested by centrifugation and resuspended in an equal volume of mMRS4 or 0.5 volumes of C_3MRS4, respectively. This cell suspension was transferred to 2 ml Eppendorf reaction tubes (ERT), sealed with silicon stoppers avoiding enclosure of air, and pressurized. The high pressure metabolism and inactivation kinetics of *L. sanfranciscensis* were investigated in HP-autoclaves at 30°C for 3 hours. Compression and decompression rates were 200 MPa min^{-1}. Samples were taken after decompression for determination of viable cell counts on mMRS4 agar and the determination of metabolites by HPLC.

Determination of metabolites: Cells from culture samples were separated by centrifugation and the supernatants were analyzed using HPLC. The concentrations of maltose, lactic acid, acetic acid and ethanol in the supernatant were determined using polyspher® OA KC column (Merck, Germany); mobile phase was H_2SO_4 5 mmol/l and temperature of the column 70°C. For detection a refractive index detector (Gynkotek, Germany) was used.

Determination of the ^{12}C / ^{13}C carbon isotope ratio: Ethanol was extracted from medium using liquid-liquid extraction with ethylacetate, adding a mixture of sodiumsulphate / sodiumcarbonate (99:1) for the removal of water. The isotope ratio measurements were performed using a Finnigan MAT δS isotope mass spectrometer (Finnigan MAT, Bremen, Germany) on-line coupled to a Varian 3400 GC via a combustion interface. The GC was equipped with a Poraplot U fused silica capillary column (25m x 0.32 mm; 10 µm film thickness). Helium was used as carrier gas; the sample was applied by 0.3 – 1.0 µl split injection at an injector temperature of 250 °C, the column was held at 140 °C for 3 min followed by a 8°C/min increase to 190 °C. The isotope ratios are expressed as $\delta^{13}C$-values [‰] versus the PDB Standard. The working reference gas was calibrated versus NBS-22 (IAEA, Vienna) using dual inlet IRMS as described previously (Koziet et al. 1993).

3. Results

Inhibition of metabolism by high pressure. The inhibitory effect of high pressure in the range of 0.1 to 250 MPa towards *L. sanfranciscensis* LTH2581 and LTH1729 was evaluated. After 3 h of incubation, cell counts and the maltose consumption were determined (**Fig. 2)**. Cell counts and maltose consumption of *L. sanfranciscensis* LTH2581 remained unaffected by 50 MPa. Incubation at 100 and 150 MPa did not result in appreciable inactivation of the strain, however, the maltose consumption was reduced to 32 and 18 % of the control,

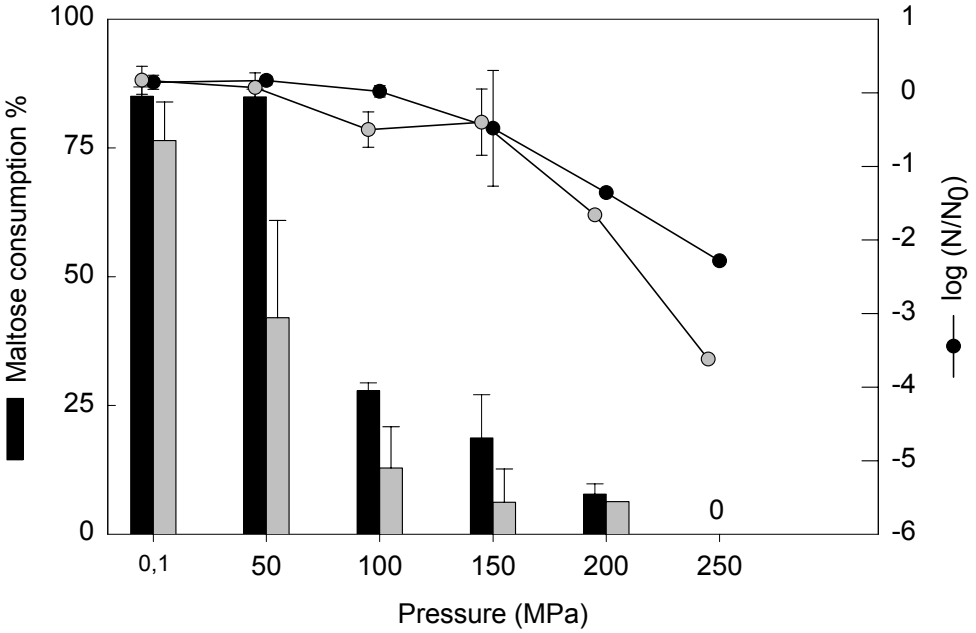

Figure 2. Effect of high pressure on viability and metabolic activity of two strains of *L. sanfranciscensis* in mMRS4 after a 3h incubation period. Shown is the maltose consumption (bar chart) and the viable cell counts (symbols). Black bars and symbols: *L. sanfranciscensis* LTH2581; gray bars and symbols: *L. sanfranciscensis* LTH1729. Results represent means ± standard deviation of two independent experiments

respectively. Pressures of 200 MPa or greater resulted in almost complete inhibition of metabolism, however, this inhibition can largely be attributed to a decrease of cell counts by 90% or greater. The strain *L. sanfranciscensis* LTH1729 was more sensitive to pressure than LTH2581; with the former strain, the application of 50 MPa sufficed to reduce the metabolic activity by more than 50% and a significant reduction of viable cell counts was observed already at 100 MPa.

Composition of metabolites formed during high pressure metabolism. The consumption of substrates and the formation of products during HP metabolism are shown in Table 1. Metabolites other than those shown in Table 1 were not formed during HP metabolism and carbon and electron balances generally accounted for more than 90% of the substrates consumed. At any pressure, maltose was converted to lactate and acetate or ethanol; fructose was not used as carbon source but reduced to mannitol. In accordance with the metabolic pathway shown in Fig. 1, one mole of lactate was formed for each glucose moiety from maltose and reduction of two moles fructose to mannitol resulted in formation of one mole acetate instead of ethanol. High pressure resulted in a decrease of maltose consumption with a concomitant decrease of lactate and ethanol formation. In contrast, the formation of acetate was unaffected by application of 80 MPa. The molar ratio of lactate to acetate was 1.8 after metabolism at ambient pressure but these metabolites were produced in equal amounts during high pressure metabolism. In accordance with the effects of HP on acetate formation, fructose conversion to mannitol was unaffected by 80 MPa and was reduced to 73 and 10 % at 100 and 150 MPa, respectively. The small amounts of glucose present in the medium were utilized during incubation at 0.1, 80 and 100 MPa. However, during incubation at 150 MPa, glucose accumulation equivalent to maltose consumption was observed.

Table 1. Composition of metabolites upon 3 h incubation of *L. sanfranciscensis* LTH2581 in mMRS4 at pressures from 0.1 to 150 MPa.

pressure	**[metabolites] (mmol/l)**						
[MPa]	**maltose**	**glucose**	**fructose**	**mannitol**	**lactate**	**acetate**	**ethanol**
0.1	-16	-1.3	-45	+42	+33	+18	+14
80	-8.8	-1.3	-44	+39	+18	+17	+2.9
100	-5.0	+0.1	-33	+29	+13	+12	+2.5
150	-3.9	+4.1	-6	+5	+2.3	+2.6	+0.6

Values are representative of two independent experiments. Negative values indicate consumption, positive values indicate production of metabolites relative to the medium.

Reversible versus irreversible inhibition of metabolic activity. In order to determine whether HP inactivation of metabolic activity is a reversible or irreversible process, cultures of *L. sanfranciscensis* LTH2581 were incubated for 3 h at 100 and 150 MPa. After this initial incubation time under HP conditions, cells were harvested by centrifugation, resuspended in fresh medium and incubated for further 3 h at ambient pressure. The maltose consumption at the various conditions is shown in Table 2. The maltose consumption at HP conditions were in accordance with the data shown in Fig. 2. During the second incubation step at ambient pressure, the metabolic activity of 100 MPa treated cells was almost fully restored to the activity of untreated cells, indicating that the HP mediated inhibition of metabolic activity is almost fully reversible. However, incubation at 150 MPa resulted in an irreversible inhibition of metabolism since less than 20% maltose consumption was observed during the second incubation at ambient pressure.

High pressure effects on the isotope distribution of metabolic products. The kinetics of maltose consumption was observed and the distribution of the ^{13}C isotope in the metabolic product ethanol was determined. Maltose was the only substrate and lactate and ethanol were the sole products of metabolism. The kinetics of maltose consumption are shown in **Fig. 3**. At ambient pressure, maltose consumption ceased after 4 h. At 100 and 150 MPa, an almost

Table 2. Metabolic activity of *L. sanfranciscensis* during and after a 3 h incubation at high pressure

	Maltose consumption %		
Pressure [MPa]	First incubation at high pressure	harvesting of cells and incubation in fresh media	Second incubation at ambient pressure
0.1	89 ± 4		90 ± 7
100	32 ± 1		70 ± 13
150	22 ± 9		18 ± 11

Results represent means ± standard deviation of two independent experiments

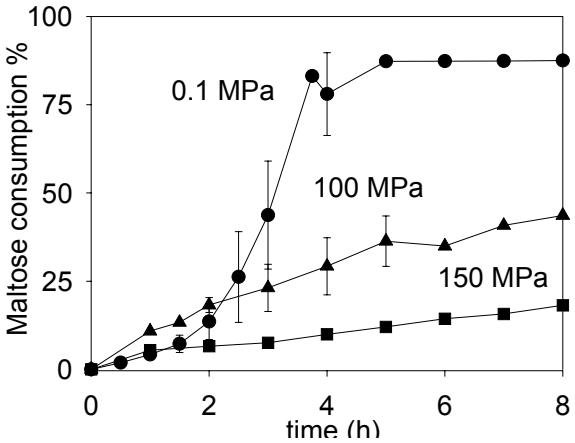

Figure 3. High pressure effects on maltose consumption by *L. sanfranciscensis*. Shown is the maltose consumption of cultures in C_3-MRS during incubation at 0.1, 100, and 150 MPa. Results represent means ± standard deviation of two independent experiments

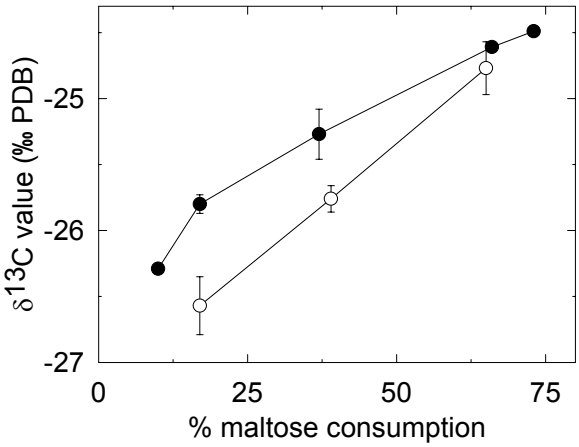

Figure 4. High pressure effects on isotope distribution of ethanol. Shown are the $\delta^{13}C$ values of ethanol from cultures grown in C_3 MRS at 0.1 MPa (●) and 100 MPa (❍).Results represent means ± standard deviation of two independent experiments

linear maltose consumption was observed and after 8 h 40 and 20% of the maltose were utilized, respectively. The $\delta^{13}C$ value of ethanol formed during metabolism at 0.1 and 100 MPa was determined and the values were compared based on equal maltose consumption (Fig 4.). The maltose used in the experiments had a $\delta^{13}C$ value of – 25.2 ‰ and 73% maltose consumption at ambient pressure resulted in an $\delta^{13}C$ value of – 24.8 ‰ in the metabolite ethanol. Because only carbon atoms 2 and 3 from the glucose moiety are recovered in the metabolic product ethanol this discrepancy is attributed to the non-statistical carbon isotope distribution in glucose (Rossmann et al., 1991). At low maltose consumption levels a preference for ^{12}C substrates was apparent. This decreased $\delta^{13}C$ values in the ethanol indicate a kinetic isotope effect. Comparison of the $\delta^{13}C$ values of ethanol formed during metabolism at 0.1 and 100 MPa on the basis of comparable maltose consumption levels demonstrates that this kinetic isotope effect is more pronounced during HP metabolism. For example, at 17% maltose consumption at 0.1 MPa a $\delta^{13}C$ value of – 25.8 ‰ was determined whereas ethanol formed at 100 MPa had a $\delta^{13}C$ value of – 26.6 ‰.

4. Discussion

The response of micro-organisms to increasing hydrostatic pressure includes growth arrest, synthesis of stress proteins, inhibition of metabolism, and cell death. In this communication, we provide a preliminary assessment of the metabolic response of *L. sanfranciscensis* to sublethal high pressure. Whereas up to 50 MPa were tolerated without adverse affects on metabolism, 100 MPa resulted in a reversible inhibition of metabolic activity without adverse effects on cell viability. The inhibitory effect of 150 MPa on metabolism was irreversible up to 3 h post treatment without adverse effects on cell viability. These observations indicate that several targets are involved in inhibition of metabolism. Reversible processes that may account for the decreased metabolic activity include the inhibition of membrane bound transport enzymes due to phase transitions of the cytoplasmic membrane (Ulmer et al., 2000, Wouters et al., 1998), and dissociation of enzymes consisting of several subunits (Deville-Bonne and Else, 1991). High pressure arrest of growth of *Saccharomyces cerevisiae* was

reported to relate to the inhibition of tryptophan transport (Abe and Horikoshi, 2000). Previous work of Wouters et al. (1998) with *L. plantarum* have shown that the glycolytic activity remained unaffected by HP treatment whereas the ability of the organism to transport protons across the membrane was impaired. The irreversible inhibition of metabolic activity in *Lactococcus lactis* and *Lactobacillus plantarum* as determined with a formazan dye occurred concomitant with the loss of viability (Ulmer et al., 2000; Molina-Guttierrez et al., 2000).

High pressure incubation of *L. sanfranciscensis* resulted in a major shift in products formed from maltose and fructose. Whereas the alternative metabolites ethanol and acetate were formed in roughly equal amounts at ambient pressure, only acetate was formed after high pressure metabolism. In the presence of electron acceptors such as fructose, acetate production, associated with the gain of one additional ATP, is favored over ethanol formation. Cofermentation of maltose and fructose results in production of lactate and acetate until the fructose is quantitatively reduced to mannitol, thereafter, lactate and ethanol are produced (Stolz et al., 1993). Therefore, the composition of metabolites observed at high pressure conditions is not markedly different from that observed at comparable maltose consumption levels at ambient pressure. These results imply that the formation of acetate from acetyl-phosphate is not a rate limiting step during high pressure metabolism.

Isotope fractionation during enzymatic reactions reflects kinetic carbon isotope effects because of differences in rate constants k_{12} and k_{13} for ^{12}C and ^{13}C substrates, respectively (O'Leary, 1982). Carbon isotope fractionation effects during photosynthetic CO_2 fixation and decarboxylation reactions are well documented (O'Leary, 1993; Swanson et al., 1998) During ethanol formation from maltose a pronounced kinetic isotope effect was observed resulting in an enrichment of the ^{12}C isotope at incomplete maltose consumption levels. Because the $\delta^{13}C$ values of lactate could not be determined by the analytical setup used in this study, it remains unclear whether this isotope effect is the result of cumulative fractionation over the entire metabolic pathway or may be attributed to a single enzymatic activity. Most remarkably, high pressure metabolism did enhance the kinetic isotope effect. Pressure effects on enzymatic isotope fractionation have so far only been reported for deuterium / hydrogen hydride transfer catalyzed by yeast dehydrogenases (Quirk and Northrop, 2001; Northrop and Cho, 2000). Pressure mediated isotope effects may contain highly specific information about chemical and kinetic mechanisms of enzymatic transformations and may furthermore be relevant for the interpretation of stable isotope data used to trace the carbon flux in deep sea environments (Valentine and Reeburgh, 2000).

5. Literature

Abe, F., and K. Horikoshi. 2000. Tryptophan permease gene TAT2 confers high-pressure growth in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 20:8093-8102.

Abe, F., C. Kato. Barophysiology (Piezophysiology). in: K. Horikoshi, K. Tsujii (eds), Extremophiles in deep-sea environments, pp. 227-248. Springer, 1999.

Deville-Bonne, D., A.J. Else. 1991. Reversible high hydrostatic pressure inactivation of phosphofructokinase from *Escherichia coli*. Eur. J. Biochem. 200:747-750.

Hammes, W.P., P. Stolz, M.G. Gänzle. 1996. Metabolism of lactobacilli in traditional sourdoughs. Adv. Food Sci.. 18:176-184.

Iwahashi, H. H. Shimizu, M. Odani, Y. Komatsu. 2000. Barophysiology of *Saccharomyces cerevisiae* from the aspect of 6000 gene expression levels. HPBB-2000, Kyoto, Japan.

Koziet, J., A. Rossmann, G.J. Martin, P.R. Ashurst. 1993. Determination of carbon-13 content of sugars of fruit and vegetable juices. Anal. Chim. Acta 271:31-38.

Molina-Gutierrez, A., B. Rademacher, M.G. Gänzle, R.F. Vogel. Effect of sucrose and sodium chloride on the survival and metabolic activity of *Lactococcus lactis* under high pressure conditions. Proceedings of the first conference on High Pressure Bioscience and Biotechnology, Kyoto, 26. - 30. November 2000, in press.

Northrop, C.B., Y.-K. Cho. 2000. Effect of pressure on deuterium isotope effects of yeast alcohol dehydrogenase: evidence for mechanical models of catalysis. Biochem. 39:2406-2412

O'Leary, M.H. Biochemical basis of carbon isotope fractionation. in: J.R. Ehleringer, A.E. Hall, J.D. Farquhar (eds), Stable isotopes and plant carbon-water relations, pp. 19-28, 1993 Academic Press.

O'Leary, M.H. Heavy isotope effects on enzyme catalyzed reactions. In: Schmidt, H.L., H. Förstel, and K. Heinzinger (eds), Stable isotopes. pp. 67-75. Analytical Chemistry Symposia Series Vol 11, Elsevier Science 1982.

Quirk, D.J., and D.B. Northrop. 2001. Effect of pressure on deuterium isotope effects of formate dehydrogenase. Biochem. 40:847-851.

Rossmann, A., M. Buthenlechner, and H.-L. Schmidt. 1991. Evidence for a nonstatistical carbon isotope distribution in natural glucose. Plant. Physiol. 96:609-614.

Stolz, P., G. Böcker, W.P. Hammes, R.F. Vogel. 1995a. Utilization of electron acceptors by lactobacilli isolated from sourdough. I. *Lactobacillus sanfrancisco.* Z. Lebensm. Unters. Forsch. 201:91-96.

Stolz, P., G. Böcker, W.P. Hammes, R.F. Vogel. 1995b. Utilization of electron acceptors by lactobacilli isolated from sourdough. II. *Lactobacillus pontis, L. reuteri, L. amylovorus,* and *L. fermentum*. Z. Lebensm. Unters. Forsch. 201:402-410.

Stolz, P., G. Bpöcker, R.F. Vogel, W.P. Hammes. Utilisation of maltose and glucose by lactobacilli isolated from sourdough. FEMS Microbiol. Lett. 109:237-243.

Swanson, T., H.B. Brooks, A.L. Osterman, M.H. O'Leary, M.A. Phillips. 1998. Carbon-13 isotope effect studies of *Trypanosoma brucei* ornithine decarboxylase. Biochem. 37:14943-14947.

Ulmer, H.M., M.G. Gänzle, R.F. Vogel. 2000. Effects of high pressure on survival and metabolic activity of *Lactobacillus plantarum.* Appl. Environ. Microbiol. 66:3966-3973.

Valentine, D.L., W.S. Reeburgh. 2000. New perspectives on anaerobic methane oxidation. Environ. Microbiol. 2:477-484.

Vogel, R.F., R. Knorr, M.R.A. Müller, U.Steudel, M.G. Gänzle, M.A. Ehrmann. 1999. Non-dairy lactic fermentations: The cereal world. Antonie van Leeuwenhoek 76:403-411.

Welch, T.J., A. Farewell, F.C. Neidhardt, D.H. Bartlett. 1993. Stress response of *Escherichia coli* to elevated hydrostatic pressure. J. Bacteriol. 175:7170-7177.

Wouters, P.C., E. Glaasker, and J.P.P.M. Smelt. 1998. Effects of high pressure in inactivation kinetics and events related to proton efflux in *Lactobacillus plantarum*. Appl. Environ. Microbiol. 64:509-514.

Identification of the gene for ß-fructofuranosidase of *Bifidobacterium lactis* DSM10140^T and characterization of the enzyme expressed in *Escherichia coli*

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Running head: The ß-fructofuranosidase from *Bifidobacterium lactis*

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Abstract

Bifidobacterium lactis is a moderately oxygen tolerant, saccharolytic bacterium often used in combination with fructooligosaccharides (FOS) as probiotic supplement in diverse dairy products. This is the first report describing the gene structure and enzymatic properties of a ß-fructofuranosidase [EC 3.2.1.26] from Bifidobacteria. *BfrA* was identified in *Bifidobacterium lactis* DSM 10140^{T} and heterologously expressed in *Escherichia coli*. The G+C content was identical with the G+C content as determined for the total genomic DNA (61.9 mol %). The gene codes for a 532 aa-residue polypeptide of 59.4 kDa. Surprisingly, the deduced aa sequence revealed only minor similarity to other fructofuranosidases (18% to *E. coli cscA*). The enzyme was purified to homogeneity after incorporation of a C-terminal 6xHIS affinity tag. It hydrolased sucrose, 1-kestose, Raftilose®, Actilight®, inulin and raffinose (100%, 91%, 84%, 80%, 37%, 4%). Fructose moieties were released in an exo-type fashion. Substrates with a-glycosidic linkages or residues other than fructose were not attacked.

The kinetic parameters K_m and V_{max} for sucrose hydrolysis were 10.3 mM and 0.031 µM/min (pH 7.6; 37°C). The activity was abolished by Zn^{2+} (1mM) and significantly inhibited by Fe^{2+} and Ni^{2+} (10mM). The enzyme showed its maximal activity at 40°C.

Key words: *Bifidobacterium lactis*, ß-fructofuranosidase [EC 3.2.1.26], fructooligosaccharide

Introduction

Bifidobacteria are considered to be part of the resident microflora of the human large intestine. It is claimed that a high number of bifidobacteria is beneficial for their hosts health [37, 28]. The principal substrates for bacterial growth in the human gut are dietary carbohydrates that have escaped digestion in the upper gastrointestinal tract. The modulation of the gut microflora using bifidogenic carbohydrates is actually a generally accepted strategy [10, 33]. Fructooligosacharides (FOS) are carbohydrates with a proven Bifidobacteria stimulating nature that have already entered commercial marketing as food additives [30]. FOS consist of short linear chains of fructosyl units linked by ß-2? 1 glycosidic bonds carrying a single D-glucosyl unit at the nonreducing end.

The degree of polymerization (DP) varies from 2-60 and strongly depends on way of production [7]. They were either obtained from natural sources (inulin) by partial hydrolyzation (DP 2-30) or synthesized enzymatically from sucrose (DP 2-4). Short chain FOS have been commercially manufactured from sucrose using fungal ß-fructofuranosidase resulting in a mixture of sucrose (GF), 1-kestose (GF_2), nystose (GF_3), 1^F- ß-fructosylfuranosylnystose (GF_4).

Bifidobacterium lactis (synonymous for *B. animalis* [6]) was isolated from yoghurt [25]. Despite the natural host of *B. animalis* is rather animal, it was recently isolated from elderly persons [9]. Various commercial strains (e.g. BB12, DR10, HN019) are currently used as probiotic supplements in different types of yoghurts or infant formulas not seldom in combination with FOS or inulin [3]. Although *B. lactis* is rather linked to lactose derived oligosaccharides as its prebiotic carbohydrate, some strains were shown to markedly utilize FOS [8, 16, 32].

Generally, the bifidogenic character of FOS has been demonstrated many times for *in vitro* fermentations [39, 15] as well as *in vivo* studies [11, 5], but reports about enzymology or genetics of involved enzymes are rare. McKellar et al. screened 43 bifidobacterial strains of human and animal origin for fructofuranosidase activity and growth on fructans[24]. All strains utilized short chain FOS. However, strains of animal origins grow significantly better and showed higher ß-fructofuranosidase activity than strains of human origin. Muramatsu et al. purified a ß-

fructofuranosidase (native M.W. 75.000) from *B. adolescentis* G1 with a unique substrate specificity different from invertase. It prefers GF_n (n=2-8) to inulin and sucrose [27]. Another cell bound inulinase from *B. adolescentis* was reported by Pudjono et al. [29]. From *B. infantis* a cytoplasmatic ß-fructofuranosidase was recovered in the supernatant fraction after disruption of *B. infantis* cells [14]. This enzyme was composed of three identical subunits (M.W. 75000) and catalyzed the hydrolysis of sucrose, 1-kestose, nystose and inulin.

In this study we describe the identification of a gene encoding a FOS hydrolyzing ß-fructofuranosidase in *B. lactis* DSM 10140 as well as in its commercial representative strain BB12. For a better understanding of the bifidogenic nature of FOS metabolism the enzyme was heterologously expressed in *E. coli* and characterized.

Material and Methods

Bacterial strains. *Bifidobacterium lactis* DSM 20451^T was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). *Bifidobacterium lactis* BB12 was isolated from a yoghurt containing this organism as probiotic supplement. *Escherichia coli* BL21 was used as host cell strain for cloning, sequencing, and protein expression.

Growth conditions. *Bifidobacterium lactis* was grown anaerobically at 37°C in *Bifidobacterium* medium as suggested by DSMZ No.58. *E. coli* was grown aerobically at 37°C in LB. Supplements used were 100 mM ampicillin for screening of transformants, and 100mM ampicillin, and 8 or 100 µM isopropyl-ß-D-thiogalactopyranoside (IPTG) for protein expression.

DNA analyses and manipulation of DNA. Chromosomal DNA was isolated according to the procedure described by Marmur [21]. Plasmid DNA was isolated by the use of QIAgen midi-prep columns (QIAgen Ltd., Germany).

PCR was performed in an Omnigene thermocycler (Hybaid, UK) in a total volume of 50 µl containing approximately 200 ng genomic DNA, 10 mM Tris-HCl, pH 8.3, 2.5 mM $MgCl_2$, 50 mM

KCl, 200 µM of each deoxynucleoside triphosphate, 50 pmol of the primers each, and 0.5 U of Taq DNA polymerase (Amersham-Pharmacia, Germany).

PCR conditions were set as follows: one cycle 2 min at 95°C, 35 cycles 30 s at 95°C, 60 s at the respective annealing temperature (see below), 60 s at 72°C, and a final cycle 2 min at 72°C.

Identification of the BfrA encoding gene. Initially, degenerated primers FRU-V (5´-GIYKIITIAAYGAYCCIAAYG) and Fru-R (5´-ACIYIIGGRTCNCKRAART) were used for amplification of the conserved core region within known fructofuranosidase genes (abbreviations according to the IUPAC nucleotide code; I=Inosin). The resulting PCR fragment was cloned in vector pGEM-T (Promega) before sequencing.

DNA sequences were determined by the chain-termination method [35] using ABI PrismTM Dye Terminatorcycle Sequencing Kit (Perkin Elmer, Germany) on an ABI 373 stretch sequencing system.

To gain the total gene sequence, PCR techniques were used to extend the flanking sequences 3´ and 5´ of the initial fragments as follows: 3´-terminus: Single-Specific-Primer PCR (SSP-PCR) with primer FRU4-R (5´- AAGGAGGGCATGATCATCG) was used to amplify an approx. 1.5 kb fragment. 5´-terminus: PCR with Primer FRU5-V (5´-GACGAACTGAAGACCTTCACG) and FRU11-R (5´-GCCCATGCGGTCATGCAGC) after religation of XhoI digested genomic DNA resulted in a fragment of approx. 2.8 kb.

Nucleotide sequence accession number. The complete sequence of the *bfrA* gene from *Bifidobacterium lactis* DSM10140^T has been deposited in the European Molecular Biology Laboratory (EMBL) database under accession number AJ437478.

Heterologous Expression and purification of BfrA. *BfrA* was amplified from genomic DNA with primer FRU8VII (5´- GACAACGCATATGGCAACCCTTCCCAC) and FRU17R (5´-CGTTAGATCTCTCGAGTCCGATGGACTTG) that introduce NdeI and BglII sites, respectively.

The enzyme was expressed in *E. coli* by inserting the DNA into NdeI and BamHI sites of a modified pET3a [34] resulting in plasmid pFRU-his. Positive transformants were identified by PCR assays and correct insertion of the tagging sequence was verified by sequence analysis. The enzyme was purified by means of a C-terminal 6xHIS residue tag (Amersham Biosciences, HIS Trap purification system) and used for enzyme assay as described above.

Protein analysis. Total protein concentration was determined by the method of Bradford with the standard assay kit from BioRad (Germany). Bovine serum albumin was used as standard. Approximately 10 µg of total protein and 0.3 µg of purified protein was used for SDS-PAGE using the buffer of Laemmli[18] and 10% Tris-glycine gels. The gels were stained with Coomassie brilliant blue R (Sigma, Germany).

Enzyme assays. Michaelis-Menten constant (*Km*) and maximum velocity (V_{max}) were determined as a function of the sucrose concentration at 37°C in reaction mixture consisting of 300µl NADP/ATP in triethanolamine buffer (r-Biopharm; Darmstadt, Germany), 6µl hexokinase/glucose-6-phosphate dehydrogenase (r-Biopharm; Darmstadt Germany), 4µl purified enzyme extract (protein concentration 4.7 mg/ml) and 690µl sucrose solution (10mM). The amount of reduced nicotin-amide-adenine dinucleotide phosphate (NADPH) formed is stoichiometric to the amount of glucose and was measured by the increase of the absorbance at 340nm. The slope of the absorbance kinetics after 30min incubation was used for the determination of the relative activity.

The effect of temperature on the enzyme activity was measured in the range of 15-60°C in reaction mixture as described above.

For the determination of pH optimum 900µl phosphate buffer (100mM) with pH ranging 5-8 were mixed with 100µl sucrose solution (200mM) and 5µl purified enzyme extract and incubated at 40°C for 15 min. The reaction was stopped by heating the samples at 80°C for 5 min followed by the measurement of the pH of the buffer solutions. 200µl of each buffer were mixed with 400µl

NADP/ATP solution and 10µl hexokinase/glucose-6-phosphate dehydrogenase and measured at 340nm, the relative activity was plotted against the measured pH of reaction mixtures.

The effects of NaCl, NH_4Cl, $MnCl_2$, $CaCl_2$, $NiCl_2$, $ZnCl_2$, LiCl, $MgCl_2$, $FeCl_2$ and KCl at concentration of 1, 5 and 10mM on the enzyme activity were determined at 37°C in the same reaction mixture (with sucrose) described above. The enzyme activity was expressed as a percentage of the activity without addition of effector (control).

For the investigation of the substrate specificity of the enzyme following reaction mixture was used: 300µl NADP/ATP in triethanolamine buffer, 6µl hexokinase/glucose-6-phosphate dehydrogenase, 6µl phosphoglucose isomerase (r-Biopharm; Darmstadt Germany), 4µl purified enzyme extract (protein concentration 4.7 mg/ml) and 690µl substrate solution. The substrates tested were: sucrose, lactose, maltose, raffinose, melibiose, melezitose, trehalose, palatinose, stachyose, 1-kestose (50mM each), inulin, raftiline®, raftilose®, actilight® (5g/l each). The enzyme activity was expressed as a percentage of the activity determined in sucrose. All results of enzyme assays are means of at least two independent assays. Additionally, carbohydrates were analyzed by thin layer chromatograhy as described by [20].

Results

Identification of the *Bifidobacterium lactis* ß-fructofuranosidase encoding gene (*bfrA*). Primers designed for amplification of an internal region of the gene encoding a fructofuranosidase were designed to target against two highly conserved motifs NDPNG and HFRDP present in most of members of the family-32 hydrolases. A single fragment of 402 bp could be amplified from genomic DNA of *B. lactis* DSM10140 type strain. Overlapping DNA sequences were obtained by the use of single specific primer PCR and ligation anchored PCR-techniques thus gaining the complete open reading frame, that was designated *bfrA*. The ORF consisted of an ATG start codon, a TGA stop codon and 1596 nucleotides which encoded a 532-amino acid polypetide. The molar G+C DNA content (61.9 mol %) corresponded to that as described for the strains total genomic DNA. Eight bases upstream of the initiation codon a putative ribosomal binding site 5´-AAGGA-3´ was present. No classical promotor-like sequence was identified. The calculated mass of the predicted polypeptide was 59.4 kDa.

The alignment of the deduced amino acid sequence of the fructofuranosidase (BfrA) of *B. lactis* against the amino acid sequences of the *E. coli* invertase (CscA EMBL P40714), *Leishmania major* ß-fructofuranosidase (EMBL Q9UOY9), *E. coli* raffinose invertase (RafD, EMBL P16553), *Zymomonas mobil*is invertase (InvA, EMBL P35636), revealed that 16%, 14%, 14%, and 12% of the amino acids were identical (Figure 1).

The hydrophobicity plot did not reveal any regions with highly hydrophobic characters to function in membrane sorting or anchoring (data not shown). The calculated isoelectric point of BfrA is 4.74.

Heterologous expression and purification. The cloned gene product was purified from the soluble fraction of *E. coli* BL21(DE3) harbouring the recombinant plasmid pFRU-his. The plasmid conferred a sucrose-positive phenotype to *E. coli* growing on MacConkey agar when lactose was replaced by sucrose. A one step metal-chelate affinity chromatography was used to purify the his-tagged BfrA. Sucrose hydrolase activity eluted as a single peak with imidazol as competitor. SDS

PAGE analysis of the purified BfrA revealed a single band with an apparent molecular mass of 60 kDa, which agrees well with the size calculated on the basis of the primary structure. No other bands were visible indicating electrophoretical purity (Figure 2).

In gel-permeation chromatography (superdex 200HR, Amersham Pharmacia) the enzyme eluted as a single peak with an estimated mass of 60 kDa, indicative for a monomeric enzyme under native conditions.

Enzymatic characterization. The *B. lactis* ß-fructofuranosidase liberated fructose moieties from linerar ß-(2? 1) linked carbohydrates like sucrose, 1-kestose, Raftilose®, Raftiline®, Actilight®, and inulin with relative activities of 100%, 91%, 84%, 82%, 80% and 37%. Hydrolase of terminal non-reducing ß-D-fructofuranoside residues characterized the enzyme as ß-fructofuranosidase. Even highly polymerized fructans (inulin) were degraded without the appearance of oligomeric intermediates, indicating an exo-type action (thin layer chromatography, data not shown). Only minor activitiy was shown for raffinose, stachyose and palatinose. Saccharides with a-glycosidic bonds and moieties other than fructosyl-residues (maltose, melibiose, lactose, melezitose and trehalose) were not accepted as substrate.

The apparent K_m and V_{max} values of the purified recombinant ß-fructofuranosidase for sucrose were 10.7 mM and 0.032 µM/min, respectively (Figure 3).

The pH dependence of sucrose hydrolysis at 40°C is characterized by an optimum at pH 6.5. More than 50% activity were measured between pH 5.5 and pH 7.5 (Figure 4.). The optimal temperature was found to be 40°C and mirrored the organims optimal growth temperature (Figure 4). The recombinant enzyme remains activ for several month at 4°C without special measures for stabilization.

The addition of 1 mM $ZnCl_2$ and 10mM $FeCl_2$ abolished the activity. Other metal ions (Na^+, NH_4^+, Mn^{2+}, Ca^{2+}, Ni^{2+}, Li^+, Mg^{2+} and K^+) affected the activity moderately (80%) at concentrations of 10mM.

Discussion

The fermentation of fructooligsacharides (FOS) by Bifidobacteria is an often reported fact. Nevertheless, knowledge on enzymes beeing involved is still scarcely. This is the first study reporting the primary structure of a ß-frucofuranosidase of *Bifidobacterium*.

Although the overall amino acid sequence similarity to other known enzymes does not exceed 16%, all conserved regions described to be significant for glycosyl hydrolase enzyme family 32 were present [12, 13]. The motif NDPNG, which was previously shown to contain an active-site aspartate [31], the highly conserved cysteine residue within the triplet sequence ECP also involved in catalysis [22] as well as the consensus FRDP with its typical aspartate-residue were present.

Significant differences to previously published fructofuranosidases of *B. adolescentis* and *B. infantis* are expressed by different molecular weights and substrate specificities (Table 2).

According to Vandamme und Derycke[38] the S/I Index (activity on sucrose/activity on inulin) should be higher than 1600 for invertases *sensu strictu*, but below 50 for ß-fructofuranosidases.

In this regard the described enzyme is a ß-fructofuranosidase despite its prominent invertase activity. Its function *in vivo*, may not be solely the cleavage of sucrose alone but may also be responsible for the growth on FOS. The latter requires either an exoenzyme, a membrane bound enzyme or just the transport of FOS into the cell. In fact, it is described that *B. lactis* cannot ferment inulin [25], while GF_3 is well used as carbon source (this study, data not shown). As neither a secretion signal nor any hydrobhobic membrane anchoring sequences could be identified in the sequence of BfrA, utilization of FOS is probably accomplished by intracellular degradation.

The existence of transport systems specific for tri- or higher polysaccharides in bacteria is not well documented except for a few cases e.g. raffinose uptake by RafY [1], the maltose/maltodextrin system of *Escherichia coli* [4] or the multiple sugar metabolism operon (msm) of *Streptococcus mutans* [36].

From a biotechnological point of view the catalytic activity of the enzyme described here may be of interest for the enzymatical measurement of inulin-type fructans in plant material and food

products. A widely applied method is the hydrolysis of fructans to fructose (and glucose) mainly with laboriously purified endo- and exoinulinases of fungal origins [23]. The food-grade status, the simple purification procedure and the pronounced stability of the bifidobacterial enzyme may be advantageous over hitherto used fructanases.

References

1. Andersen C, Krones D, Ulmke C, Schmid K, Benz R (1998) The porin RafY encoded by the raffinose plasmid pRSD2 of *Escherichia coli* forms a general diffusion pore and not a carbohydrate-specific porin. Eur J Biochem 254:679-84
2. Blatch GL, Woods DR (1993) Molecular charcterization of a fructanase produces by *Bacteroides fragilis* BF-1. J Bacteriol 175:3058-3066
3. Bonaparte C, Reuter G (1996) Bifidobacteria in commercial dairy products: which species are used? In: Proceedings of the symposium probiotics in man and animal, Berlin, Germany, June 20-22, p33. Deutsche Veterinärmedizinische Gesellschaft, Berlin
4. Boos W, Shuman H (1998) Maltose/maltodextrin system of *E.coli*: transport, metabolism, and regulation. Microbiol Mol Biol Rev 62:204-229
5. Bouhnik Y, Flourie B, Riottot M, Bisetti N, Gailing MF, Guibert A, Bornet F, Rambaud JC (1996) Effects of fructo-oligosaccharides ingestion on fecal bifidobacteria and selected metabolic indexes of colon carcinogenesis in healthy humans. Nutr Cancer 26:21-29
6. Cai Y, Matsumoto M, Benno Y (2000) *Bifidobacterium lactis* meile et al. 1997 is a subjective synonym of *Bifidobacterium animalis* (Mitsuoka 1969) scardovi and trovatelli 1974. Microbiol Immun 44:815-820
7. Crittenden RG (1999) Prebiotics,. In: Tannock GW (eds) Probiotics: a critical rewiew. Wymondham, Norfolk: Horizon Scientific Press, pp 141-156
8. Crittenden RG, Morris LF, Harvey ML Tran, LT Mitchell, HL, Playne MJ (2001) Selection of a *Bifidobacterium* strain to complement resistant starch in a synbiotic yoghurt. J Appl Microbiol

90:268-278

9. Gavini F, Cayuela C, Antoine JM, Lecoq C, Lefebvre B, Membré JM, Neut C (2001) Differences in the Distribution of Bifidobacterial and Enterobacterial Species in Human Faecal Microflora of Three Different (Children, Adults, Elderly) Age Groups. Ecol Health Dis 13:40-45

10. Gibson GR. (1999) Dietary modulation of the human gut microflora using the prebiotics oligofructose and inulin. J Nutr 80:1438S-1441S

11. Gill HS, Rutherford KJ, Prasad J, Gopal PK (2000) Enhancement of natural and aquired immunity by *Lactobacillus rhamnosus* (HN001), *Lactobacillus acidophilus* (HN017), and *Bifidobacterium lactis* (HN019). British J Nutr 83:167-176

12. Henrisaat B (1991) A classification of glycosyl hydrolases based on amino acids sequence similarities. Biochem J 280:309-316

13. Henrisaat B, Bairoich A (1993) New families in the classification of glycosyl hydrolases based on amino acid sequence similarities. Biochem J 293:781-788

14. Imamura L, Hisamitsu K, Kobaschi K (1994) Purification and characterization of beta-fructofuranosidase from *Bifidobacterium infantis*. Biol Pharm Bull 17:596-602

15. Kaplan H, Hutkins RW (2000) Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. Appl Env Microbiol 66:2682-2684

16. Kneifel W, Rajal A, Kulbe K D (2000) *In vitro* growth behaviour of probiotic bacteria in culture media with carbohydrates of prebiotic importance. Microb Ecol Health Dis 12**:**27-34

17. Korakli M, Gaenzle MG, Vogel RF (2002) Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye, and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. J Appl Microbiol 92:958-65

18. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685

19. Lampen JO (1971) Yeast and Neurospora invertases In: Boyer PD (eds) The enzymes 3rd ed. Orlando: Academic Press Inc. pp 291-305

20. Liebl W, Brem D, Gotschlich A (1998) Analysis of the gene for beta-fructosidase (invertase, inulinase) of the hyperthermophilic bacterium *Thermotoga maritima*, and characterisation of the enzyme expressed in *Escherichia coli*. Appl Microbiol Biotechnol 50:55-64

21. Marmur J (1961) A procedure for the isolation of deoxyribonucleic acid from microorganisms. J Mol Biol 3:208-218

22. Martin I, Débarbouillé M, Ferrari E, Klier A, Rapoport G (1987) Characterization of the levanase gene of *Bacillus subtilis* which shows homolohy to yeast invertase. Mol Gen Genet 208:177-184

23. McCleary BV, Murphy A, Mugford DC (2000) Measurement of total fructan in foods by enzymatic/spectrophotometric method: collaborative study. J AOAC Int 83:356-64

24. McKellar RC, Modler HW, Mullin J (1993) Characterization of growth and inulinase production by *Bifidobacterium spp*. Bifidobacteria Microflora 12:75-86

25. Meile L, Ludwig W, Rueger U, Gut C, Kaufmann P, Dasen G, Wenger S, Teuber M (1997) *Bifidobacterium lactis* sp. nov., a moderately oxygen tolerant species isolated from fermented milk. System Appl Microbiol 20:57-64

26. Muramatsu K, Onodera S, Kikuchi M, Shiomi N (1992) The production of ß-fructofuranosidase from *Bifidobacterium* spp. Biosci Biotechnol Biochem 56:1451-1454

27. Muramatsu K, Onodera S, Kikuchi M, Shiomi N (1994) Substrate specificity and subsite affinities of ß-fructofuranosidase from *Bifidobacterium adolescentis* G1. Biosci Biotechnol Biochem 58:1642-1645

28. Orrhage K, Nord CE (2000) Bifidobacteria and lactobacilli in human health. Drugs Exp Clin Res 26:95-111

29. Pudjono G, Barwald G, Amanu S (1993) Activity of inulinase of some strains of *Bifidobacterium* and their effects on the consumption of foods containing inulin and other

fructans. In: Fuchs A (eds) Inulin and Inulin-containing crops. Amsterdam: Elsevier, pp. 373-379

30. Rao AV (1998) Dose-response effects of inulin and oligofructose on intestinal bifidogenic effects. J Nutr 80:1442S-1445S

31. Reddy A, Maley F (1990) Identification of an active-site residue of yeast invertase by affinity labeling and site-directed mutagenesis. J Biol Chem 271:13953-13958

32. Roberfroid MB (1998) Prebiotics and synbiotics: concepts and nutritional properties. J Nutr 80:197S-202S

33. Roberfroid MB, Van Loo, JAE, Gibson GR (1998) The bifidogenic nature of chichory inulin and its hydrolysis products. J Nutr 128:11-19

34. Rosenberg AH, Lade BN, Chui DS., Lin SW, Dunn JJ, Studier FW (1987) Vectors for selective expression of cloned DNAs by T7 RNA polymerase. *Gene* 56:125-135

35. Sanger F, Nicklen S, Coulson A (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463-5467

36. Tao L, Sutcliffe IC, Russell RR, Ferretti JJ (1993) Cloning and expression of the multiple sugar metabolism (msm) operon of *Streptococcus mutans* in heterologous streptococcal hosts. Infect Immun 61:1121-5

37. Taper HS, Delzenne NM, Roberfroid MB (1997) Growth inhibition of transplantable mouse tumors by non digestible carbohydrates. Int J Cancer 71:1109-1112

38. Vandamme EJ, Derycke DG (1983) Microbial inulinases: fermentation process, properties, and applications. Adv Appl Microbiol 29:139-176

39. Wang X, Gibson GR (1993) Effects of the *in vitro* fermentation of oligofructose and inulin by bacteria growing in the human large intestine. J Appl Bacteriol 75:373-380

330 **Table 1.** Substrate specificity and relative activities of *B. lactis* ß-fructofuranosidase.

Substrate	Relative activity[b] [%]
Sucrose	100
1-Kestose	91.27±0.40
Raftilose®	84.2±2.80
Raftiline®	82.4±4.60
Actilight® 950P	80.04±0.87
Inulin	37.5±1.40
EPS[a]	6.0±0.10
Raffinose	4.4±1.50
Stachysoe	1.26
Palatinose	0.87
Lactose	-
Maltose	-
Melibiose	-
Melezitose	-
Trehalose	-

331

332 [a] Levan-type EPS (exopolysaccharide) was isolated from *L. sanfranciscensis* as described by

333 Korakli et al., 2002 [17].

334 [b] Relative activity expressed as mean ± SD (n=2).

Table 2. Comparison of enzymatic activities on FOS of microbial ß–fructofuranosidases

	Bif. adoles.[a] extract [26]	*S. cerevisiae*[b] invertase [19]	*P. trzebinskii*[c] inulinase [19]	*B. fragilis*[d] FRUA [2]	*Bif. lactis*[a] BFRA (this study)	*Bif. infantis*[a] BFR [14]
relative activity [%] with:						
Saccharose	100	100	100	100	100	100
1-Kestose (GF2)	353	11.3	89	nd[f]	91	297
GF3	289	6.5	91	nd	80[e]	365
GF4	176	3.3	79	nd	nd	nd
Raftiline	nd	nd	nd	nd	82	nd
Inuline	52	0	42	5.1	37	140
Levan	0	0	0	0.5	6	nd
S/I	1.9	>1600	2.3	19.6	2.7	0.7
Mr [kDa]	74	135	nd	70.2	59.4	3x75

[a] *Bifidobacterium*

[b] *Saccharomyces*

[c] *Penicillium*

[d] *Bacillus*

[e] activity was determined with Actilight® 950P, composed of 35% GF2, 50% GF3 9.5% GF4 and 5% glucose, fructose and sucrose)

[f] nd, not determined..

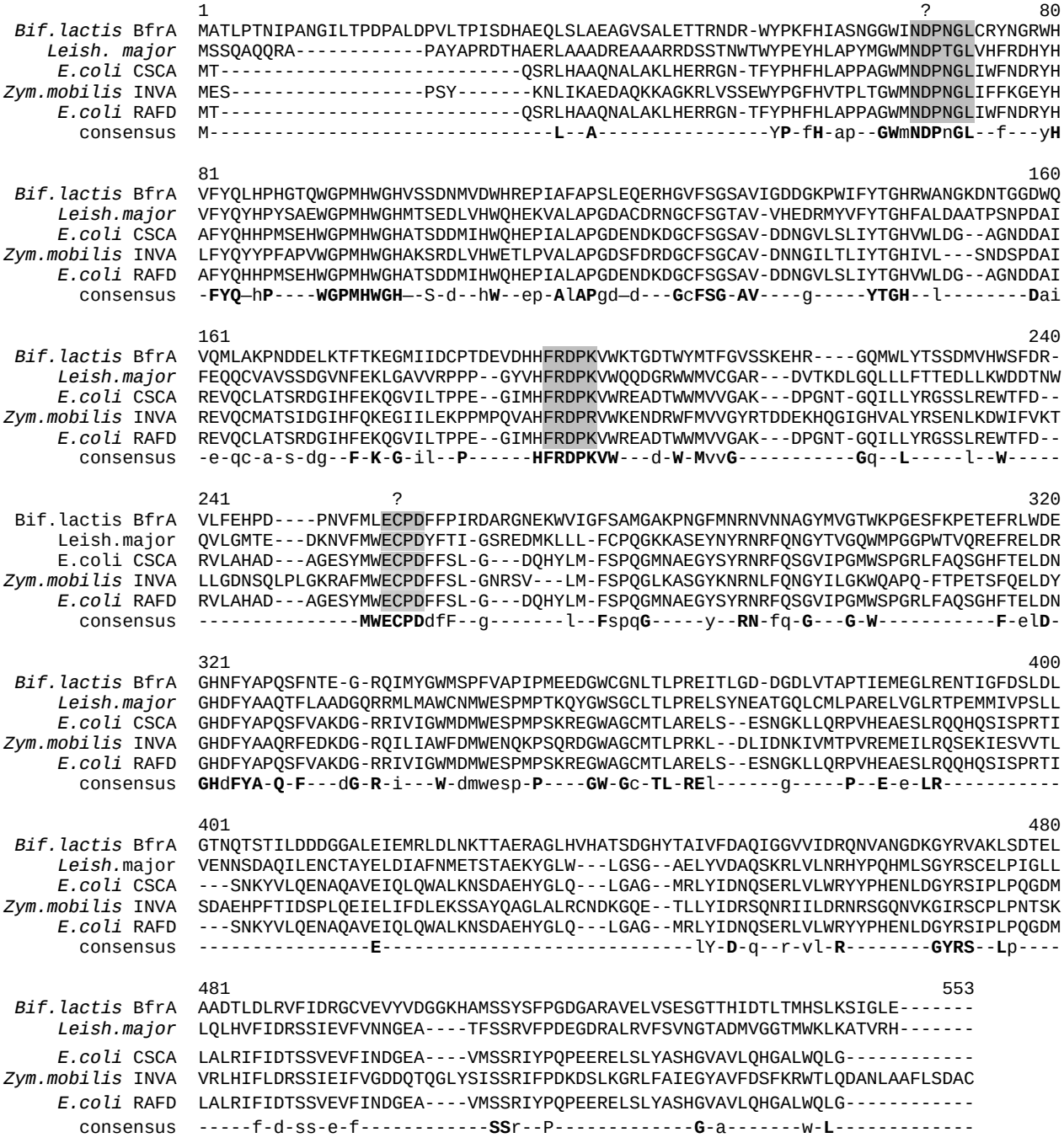
Legends to figures:

Fig. 1. Alignment of amino acid sequences of BfrA from *B. lactis* and most closely related enzymes according to the EMBL Blast search. Accession numbers are: *Leishmania major* EMBL Q9UOY9; *E. coli* CscA EMBL P40714; *Zymomonas mobilis* InvA EMBL P35636; *E. coli* RafD EMBL P16553. Domains that are mostly conserved are shaded. Asterisks indicate an active-site aspartate residue according to [31] and the highly conserved cysteine residue within the triplet sequence ECP that is also involved in catalysis [22].

Fig. 2. SDS PAGE analysis of purified recombinant *B. lactis* ß-fructofuranosidase. Cellular proteins were denatured and separated on 12% polyacrylamide gel and stained with Coomassie Blue. Lane 1 and Lane 4, molecular mass marker; lane 2, purified ß-fructofuranosidase; lane 3, *E. coli* BL21(DE3) cell lysate; the sizes of the marker proteins are indicated.

Fig. 3. Lineweaver-Burk blot of *Bifidobacterium lactis* ß-fructofuranosidase with sucrose as substrate.

Fig. 4. Effect of Temperature and pH on ß-fructofuranosidase activity of *Bifidobacterium lactis* .



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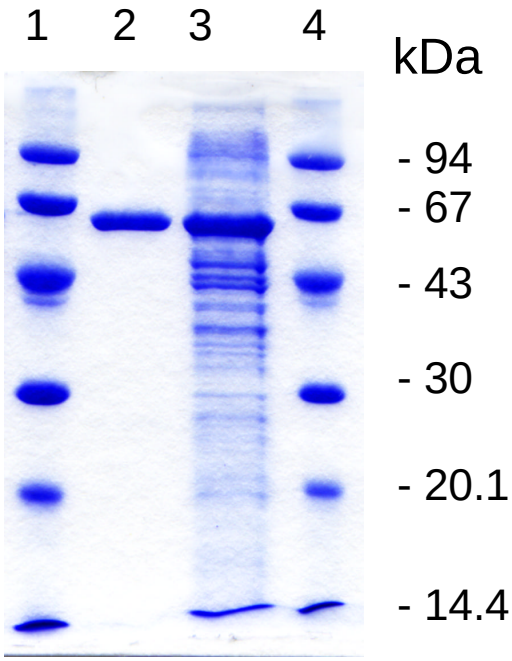

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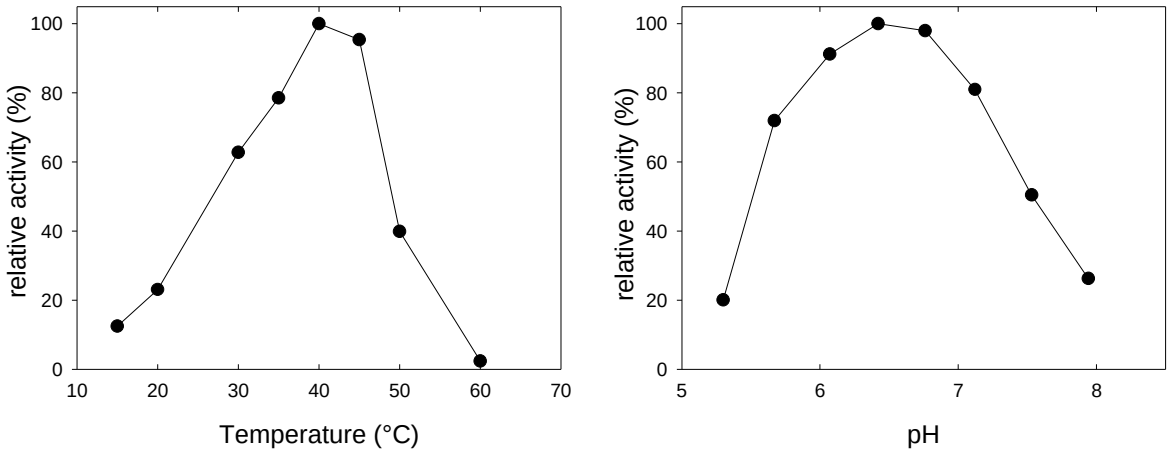
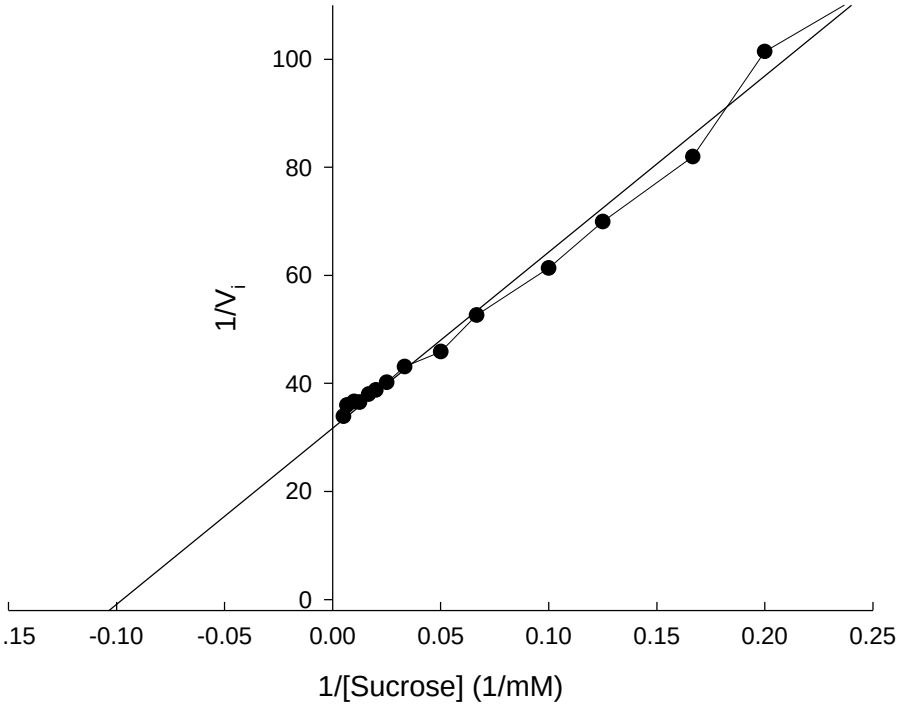


Fig. 4. Effect of Temperatur and pH on ß-fructofuranosidase activity of *Bifidobacterium lactis*

Fig. 3. Lineweaver-Burk plot of *Bifidobacterium lactis* ß-fructofuranosidase with sucrose as substrate.



Exopolysaccharide and kestose production by *Lactobacillus sanfranciscensis* LTH2590

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Abstract

The effect was investigated of sucrose concentration on sucrose metabolism and on the formation of exopolysaccharide (EPS) by *Lactobacillus sanfranciscensis* LTH2590 in pH controlled fermentations with sucrose concentrations ranging from 20-160 g l^{-1}. The EPS production increased and the relative sucrose hydrolysis activity decreased by increasing the sucrose concentration in medium. The carbon recovery decreased from 95% at sucrose concentration of 30 g l^{-1} to 58% at sucrose concentration of 160 g l^{-1}, respectively, because of the production of an unknown metabolite by *L. sanfranciscensis*. This metabolite was characterized as fructo-oligosaccharide. This is the first report describing the ability of a *Lactobacillus* sp. to produce fructo-oligosaccharide. The oligosaccharide produced by *L. sanfranciscensis* was purified and characterized as trisaccharide with a glucose:fructose ratio of 1:2. The comparison of the retention time of this oligosaccharide and that one of pure oligosaccharide standards using two different chromatography methods revealed that the oligosaccharide produced by *L. sanfranciscensis* LTH2590 is 1-kestose. Kestose production increased concomitantly with the initial sucrose concentration in medium.

Introduction

Polysaccharides of plant origin have being used for a long time in the food industry as biothickener, texture stabilizer or gelling agent. In the last decades several microbial exopolysaccharides (EPS) have been described as alternatives for plant polysaccharides. Microbial polysaccharides have rheological properties that match the industrial demands and can be produced in large amounts and high purity. The interest of the food industry in developing "multifunctional additives", that not only provide the desired improvement of the texture but also have additional nutritional properties, led to extensive search for polysaccharides with prebiotic attributes. Prebiotics are non-digestible food ingredients that affect the host beneficially by selectively stimulating the growth and/or activity of specific bacteria in the colon and thus improve host health (12). Fructo-oligosaccharides (FOS), xylo-oligosaccharide and inulin are some of the prebiotics available for the human consumption (5, 24). FOS with prebiotic properties (e. g. kestose, nystose) are polymers of D-fructose joined by ß(2→1) linkages and terminated with a D-glucose molecule linked to fructose by an α(1→2) bond as in sucrose (22). The degree of polymerization (DP) can vary from 2-35. FOS with DP of 3-5 are called neosugars (28) and can be enzymatically synthesized from sucrose using fructosyltransferase from *Aspergillus niger* (16, 17). Mckellar and Modler (23) showed that the maximum activity of ß-fructosidase responsible for the hydrolysis of inulin type polysaccharides by bifidobacteria was observed with neosugars.

Lactic acid bacteria (LAB) are widely used for the production of numerous fermented foods and are generally recognized as safe (GRAS organisms). Several EPS producing LAB e. g. *Lactobacillus delbrueckii* ssp. *bulgaricus*, *Lactococcus lactis*, *Streptococcus thermophilus* have been isolated from fermented dairy products, in particular from yogurt (2, 8). The majority of EPS synthesized by LAB are heteropolysaccharides composed of repeating units consisting of two or more monosaccharides e. g. galactose, glucose, rhamnose and fructose.

Heteropolysaccharides are typically produced in small amounts up to 2 g l^{-1} (7). Furthermore, the ability of LAB to produce homopolysaccharides has been described. *Lactobacillus reuteri* contains strains known to produce homopolysaccharides of fructan type (29, 30). Korakli et al. (19) reported the production of EPS of fructan type by *Lactobacillus sanfranciscensis*, and Tieking et al. (unpublished data) have recently described the ability of several lactobacilli of sourdough and intestinal origin to produce EPS of the fructan and glucan type.

Lactobacillus sanfranciscensis belongs to the microbial flora of traditionally prepared wheat and rye sourdoughs (13). The strain *L. sanfranciscensis* LTH2590 was shown to produce EPS from sucrose (19). This EPS is a high molecular mass fructan homopolysaccharide of the levan type (19, 6). In situ production of EPS by *L. sanfranciscensis* LTH2590 during wheat and rye sourdough fermentation (20), and a bifidogenic activity of this EPS were demonstrated (6, 21). The high potential of sourdough lactobacilli to produce EPS during sourdough fermentation may allow the replacement of plant polysaccharides, whose addition is common practice by the preparation of baking mixture, because they improve textural properties, keepability and shelf life of bread (1).

Information about biosynthesis, kinetics of EPS formation and physiological regulations of homopolysaccharide production by lactobacilli are rather scarce. Hestrin et al. (14) named the enzyme responsible for dextran production by *Leuconostoc mesenteroides* dextransucrase and Van Hijum et al. (31) purified a levansucrase synthesizing EPS of levan type from *Lactobacillus reuteri*. It was the aim of this work to study the metabolism of sucrose as well as the effect of substrate concentration on sucrose metabolism, EPS production and the biochemical nature of the respective end products formed by *L. sanfranciscensis*. The strain *L. sanfranciscensis* LTH2590 was chosen because it produces EPS and can not use fructose as energy source but reduces it to mannitol regenerating NADH to NAD^+ (19).

Materials and Methods

Organism and culture conditions: The EPS-producing strain *Lactobacillus sanfranciscensis* LTH2590 was cultured at 30°C in Sucrose-MRS (Su-MRS) containing the following components per liter: peptone from casein, 10g; yeast extract, 5g; meat extract, 5g; $K_2HPO_4.3H_2O$, 2.6g; KH_2PO_4, 4g; cystein-HCl, 0.5g; NH_4Cl, 3g; Tween 80, 1 ml l^{-1}. Stock solutions containing $MgSO_4.3H_2O$ (100 g l^{-1}) and $MnSO_4.4H_2O$ (50 g l^{-1}) were autoclaved separately, and a vitamin mix containing folic acid, pyridoxal phosphate, thiamine, riboflavin, cobalamin and panthothenic acid (0.2 g l^{-1} each) was sterilized by filtration. 1ml l^{-1} of each stock solution was added to the sterilized medium. Sucrose and fructose were sterilized by filtration, 20 g l^{-1} sucrose and 15g l^{-1} fructose were used unless otherwise indicated.

Fermentations at controlled pH were carried out in 350ml jar fermenter (Braun Biotech Int., Germany) at 30°C and 80 rpm without gas inlet. pH was monitored with an online pH sensor and maintained at 5.6 using 4M NaOH. Medium components were dissolved in 60% of the final volume and autoclaved in the fermenter (20 min. at 121°C). Sucrose and fructose were dissolved in the residual volume (40%), sterilized by filtration and added to the autoclaved medium.

Determination of the maximum growth rate: The effect of sucrose and fructose concentration on the maximum growth rate was determined in multiple well plates (Sarstedt, Germany). Su-MRS with sucrose and fructose concentrations ranging from 0-133 g l^{-1} and 0-53 g l^{-1}, respectively was used. The kinetics of growth and maximum growth rates were determined at 590nm using spectrophotometer (SpectraFlour®, Tecan, Austria). The modeling of the maximum growth rate as a function of sucrose and fructose was performed as described (11), Results are representative for two independent fermentations.

Determination of colony forming units: Cell counts were determined on mMRS4 (26). Appropriate dilution was plated using a spiral plater (IUL, Königswinter, Germany) and

plates were incubated at 30°C for 48h under controlled atmosphere (76% N_2, 20% CO_2, 4% O_2).

Determination of metabolites: During pH-controlled fermentations, samples were taken for determination of viable cell counts, optical density and HPLC analysis. Cells were removed by centrifugation (14000xg for 5min.). The concentrations of lactic acid, acetic acid and ethanol in the supernatant were determined using polyspher® OA KC column (Merck, Germany). The flow rate was 0.4 ml min^{-1}, mobile phase H_2SO_4 5 mmol l^{-1} and temperature of the column 70°C. Sucrose, glucose, fructose, kestose and mannitol concentrations were determined using polyspher® CH PB column (Merck, Germany). The flow rate was 0.4 ml min^{-1}, mobile phase deionized H_2O and the temperature of the column 80°C. For detection a refractive index detector (Gynkotek, Germany) was used.

The retention time of the oligosaccharide produced by *L. sanfranciscensis* LTH2590 was compared with that of kestose using anion-exchange chromatography coupled to an integrated and pulsed amperometric detection (IPAD) equipped with a gold electrode cell (Dionex, Sunnyvale, USA). The waveform was set as described by Jandik et al. (18). The column used was AminoPac™ PA10 (Dionex, Sunnyvale, USA) consisting of a guard column and a analytical column (2 mm x 250 mm). Three different eluents were used: (A) deionized water; (B) 250mM NaOH and (C) 1M sodium acetate. The water used for the preparation of the eluents had a conductivity of 0.054 µS cm^{-1}. The flow rate was 0.25 ml $min^{-1}$and the temperature of the column 30°C. The following gradient was used to separate sugars and oligosaccharides: 0 min: 80% A, 0% B and 20% C; 16 min: 80% A, 0% B and 20% C; 32 min: 0% A, 80% B and 20% C; 40 min., 0% A, 80% B and 20% C. Helium was used for degassing the eluents and to prevent the formation of carbonate.

EPS isolation, purification and hydrolysis: EPS produced was isolated from 10ml fermentation broth after removing of cells by centrifugation and addition of 2 volumes of

chilled 95% ethanol to the supernatant. After incubation for 3h at 4°C, the precipitate was collected by centrifugation (8000×g, 10 min). EPS was dissolved in deionized water, centrifuged again and the supernatant was lyophilized. The lyophilized EPS was weighed and the amount was indicated in g l^{-1}. For hydrolysis of EPS, 5% (v/v) perchloric acid (70% w/v) were added to the samples after removing of cells and incubated at 80°C for 2h.

Characterization of oligosaccharide: Cells were removed from fermentation broth by centrifugation and the oligosaccharide was separated from the supernatant using polyspher® CH PB column (Merck, Germany). The molecular weight of the oligosaccharide was determined using gel permeation chromatography (Superdex™ Peptide, Amersham Pharmacia Biotech, Sweden). Bidest. water was used as mobile phase and the determination was carried out at room temperature. Sucrose, raffinose and stachyose were used for the calibration of the column. For the determination of the composition, the oligosaccharide purified by sequential runs on polyspher® CH PB and Superdex™ Peptide was hydrolyzed with 5% perchloric acid (2h at 80°C). The monosaccharide concentrations were determined using HPLC. Information about the type of the monosaccharide linkages was obtained by the treatment of the purified oligosaccharide with ß-fructosidase (yeast invertase, r-biopharm, Germany) and subsequent analysis of the monosaccharides liberated by HPLC.

Results

Effect of fructose on the utilization of sucrose

The EPS production from sucrose by *L. sanfranciscensis* LTH2590 was previously observed in media containing autoclaved sucrose where a part of sucrose was hydrolyzed (19). To evaluate the ability of *L. sanfranciscensis* LTH2590 to metabolize sucrose in the absence of monosaccharides (fructosyltransferase acceptors) maximum growth rates were determined in media containing various contents of sucrose and fructose sterilized by filtration. As shown in

Fig. 1, no growth was observed in the absence of fructose at sucrose concentrations ranging from 0 to 133 g l^{-1}. The addition of 4.7 g l^{-1} fructose resulted in maximum growth rate of 0.49 h^{-1} at a sucrose concentration of 2.3 g l^{-1}, indicating that fructose, (and possibly other monosaccharides), are essential for sucrose utilization by *L. sanfranciscensis* LTH2590. The further increase of fructose concentration had no effect on the maximum growth rate.

Kinetic of sucrose metabolism and EPS production in Su-MRS

The kinetics of sucrose metabolism and EPS production by *L. sanfranciscensis* LTH2590 as well as the end products were determined for all fermentations at sucrose concentrations ranging from 20-160 g l^{-1} and at fructose concentrations of 5 or 15 g l^{-1}. Figure 2 shows as an example the kinetic with 60 g l^{-1} sucrose and 5 g l^{-1} fructose. Cells grew to $1*10^{+9}$ cfu ml^{-1} within 20h. The EPS production was coupled to the growth of the cells and about 10 g l^{-1} EPS were produced. The end products of sucrose metabolism were EPS, mannitol, lactate and acetate or ethanol and the carbon recovery was 93%.

The amount of mannitol produced was higher than the amount of free fructose available at the beginning of the fermentation, demonstrating the ability of *L. sanfranciscensis* LTH2590 to hydrolyze sucrose into glucose and fructose. In figure 3 the kinetics are shown of fructose consumption and mannitol production at two different fructose concentrations. The hydrolysis of sucrose to glucose and fructose by *L. sanfranciscensis* LTH2590 was observed only when the free fructose was almost consumed (Fig. 3). The concentration of free fructose consumed and that one of mannitol produced were identical as long as free fructose was available, indicating that the mannitol produced originates from free fructose only and no fructose is liberated from sucrose. The sum of free fructose and mannitol produced increased only after the free fructose was consumed, indicating that fructose was generated from the sucrose only when free fructose almost completely consumed. The activation of sucrose hydrolysis by *L.*

sanfranciscensis LTH2590 upon fructose depletion was observed in fermentations with 5 and 15 g l^{-1} fructose and at all sucrose concentrations tested in this work (data not shown).

Effect of sucrose concentration on the EPS production

To evaluate the effect of initial sucrose and fructose concentration in medium on the yield of EPS and end products formed by *L. sanfranciscensis* LTH2590 fermentations with sucrose concentrations ranging from 20-160 g l^{-1} and at fructose concentration of 5 or 15 g l^{-1} were carried out at pH 5.6. The amount of sucrose consumed increased from 68 mmol l^{-1} at a sucrose concentration of 20 g l^{-1} to 245 mmol l^{-1} at a sucrose concentration of 160 g l^{-1} (Tab. 1). The increase of lactate production was not proportional to the sucrose consumption. The amounts of mannitol produced ranged from 98-111 mmol l^{-1} in fermentation with 15 g l^{-1} fructose and 20-160 g l^{-1} sucrose, and in fermentations with 5 g l^{-1} fructose mannitol concentrations ranged from 38-49 mmol l^{-1}. Acetate production was independent of the sucrose concentration and more acetate was produced in fermentations containing 15 g l^{-1} fructose. Glucose accumulation in the medium was only observed in fermentations with 15 g l^{-1} fructose and at sucrose concentrations of 60 g l^{-1} or higher (Tab. 1).

The glucose recoveries (lactate produced/glucose consumed) ranged from 95-108% in media containing up to 30 g l^{-1} sucrose (Tab. 1). Taking into account the experimental error, recoveries in the range of 90-110% can be considered quantitative. In fermentations with sucrose concentrations greater than 60 g l^{-1} the glucose recoveries were lower. In medium containing 160 g l^{-1} sucrose the carbon recovery was only 58%. The glucose recoveries decreased the higher the sucrose concentration, despite the fact that the EPS isolated from fermentation at any sucrose concentrations consisted exclusively of fructose. The low glucose recovery observed at high sucrose concentrations correlated well to the peak area of an unknown metabolite produced during the sucrose fermentation (Fig. 4). The acid hydrolysis of the supernatant upon fermentation resulted in glucose and fructose recoveries of more than

92% (data not shown) and in disappearance of the oligosaccharide peak in HPLC-chromatogram (Fig. 4, A). The retention time of the oligosaccharide peak produced by *L. sanfranciscensis* was the same as that of 1-kestose (15 min.) using polyspher® CH PB, and the treatment of the sample with ß-fructosidase or the acid hydrolysis resulted in complete degradation of the oligosaccharide (Fig. 4, A). The same results could be observed using a second chromatography method, an anion-exchange chromatography with integrated amperometric detection (Fig. 4, B). The molecular weight of the oligosaccharide determined using gel permeation chromatography after separation of the oligosaccharide was 597 ± 27. The hydrolysis of the purified oligosaccharide separated from supernatants (n=3) of different fermentations with various sucrose concentrations revealed a glucose:fructose ratio of 1:2. The molecular weight and glucose:fructose ratio indicate that the oligosaccharide is a trisaccharide. The comparison of the retention time of the oligosaccharide and that of 1-kestose using two different chromatography methods revealed that the oligosaccharide or the major oligosaccharide produced by *L. sanfranciscensis* LTH2590 is 1-kestose.

The effect of sucrose concentration on the EPS production, sucrose hydrolysis and kestose formation is shown in Fig. 5. The amount of EPS produced increased the higher the sucrose concentration in medium. The fructose concentration showed no significant effect on the EPS production. About 40 g l^{-1} EPS were produced in medium containing 160 g l^{-1} sucrose (Fig. 5, A). The amount of mannitol produced from sucrose was virtually independent of fructose and sucrose concentrations in all fermentations (24 ± 6 mmol l^{-1}, n=15), demonstrating that the absolute hydrolase activity is independent of the sucrose concentration (Fig. 5, B). However, the more the sucrose concentration in medium, the higher the amount of sucrose consumed (table 1), this means that the ratio of the mannitol produced from sucrose to consumed sucrose (relative hydrolase activity) decreased with increasing sucrose concentrations (Fig. 5, B). Almost 40% of the fructose from the sucrose consumed were reduced to mannitol at a sucrose

concentration of 20 g l^{-1}, whereas at sucrose concentration of 160 g l^{-1} only 10% of the fructose from the sucrose consumed were reduced to mannitol (Fig. 5, B). The kestose production increased the higher the sucrose concentration in medium and no significant effect of fructose concentration on the production of kestose could be observed (Fig. 5, C).

Discussion

The metabolism of sucrose by *L. sanfranciscensis* LTH2590 was only observed in media containing fructose which was apparently used as acceptor for the fructosyl residues. This activating mechanism of acceptors is commonly found for fructosyltransferases (25). Korakli et al. (19) suggested levansucrase as the enzyme responsible for the EPS production by *L. sanfranciscensis*. We have recently demonstrated by PCR targeting amino acid sequences of known levansucrases, and sequencing of the PCR-product that the strain *L. sanfranciscensis* LTH2590 carries a levansucrase gene (unpublished data). Levansucrase (E.C. 2.4.1.10) is a fructosyltransferase enzyme that catalyses the following reaction:

$$\text{Sucrose} + (2,6\text{-beta-D-fructosyl})_n \rightarrow \text{glucose} + (2,6\text{-beta-D-fructosyl})_{n+1}$$

Besides the fructosyltransferase activity, levansucrase exhibits ß-D-fructofuranosidase activity (25). Levansucrase has been recently isolated and purified from *Lactobacillus reuteri* that produces polyfructan (31, 30). By increasing the initial sucrose concentration in medium more EPS was produced by *L. sanfranciscensis* LTH2590, whereas the amount of mannitol formed from sucrose in fermentation with sucrose content ranging from 20-160 g l^{-1} indicated almost constant hydrolase activity. Thus, the relative hydrolase activity decreased by increasing sucrose concentrations. This conforms to the results obtained from *Bacillus subtilis* levansucrase (9), where increasing sucrose concentrations resulted in a shift of the enzyme activity from hydrolysis to fructosyltransferase. The highest fructosyltransferase activity by *B. subtilis* levansucrase was 32% at 37°C and 616 g l^{-1} sucrose (9), whereas more than 90% of

the fructose moiety from the sucrose by *L. sanfranciscensis* were transformed to oligo- or polysaccharides at sucrose concentration higher than 120 g l^{-1}.

The glucose recoveries were 95-108% up to sucrose concentration of 30 g l^{-1}. A further increase of the sucrose concentration resulted in a decrease of the glucose recoveries. This was attributed to the production of fructo-oligosaccharide characterized as 1-kestose. The amount of kestose produced was higher the higher the sucrose concentration in medium. Crittenden et al. (4) reported that the percentage of fructose diverted to oligosaccharide by levansucrase from *Zymomonas mobilis* increased from 18% at sucrose concentration of 92 g l^{-1} to 51% at 280 g l^{-1}, respectively.

The ability of levansucrase isolated from various organisms to produce fructo-oligosaccharides was subject of several works: Hestrin et al. (15) reported first the production of fructo-oligosaccharide by *Aerobacter levanicum* levansucrase and Feingold et al. (10) have characterized this oligosaccharide as 1-kestose. *Bacillus subtilis* levansucrase was also shown to produce fructo-oligosaccharide of 1-kestose type (9). Structural analysis of the fructo-oligosaccharides produced by *Z. mobilis* levansucrase using ^{13}C-NMR spectroscopy revealed that 98% of the total oligosaccharides were identified as 1-kestose and 1.5% as nystose (3). The oligosaccharides produced by *Acetobacter diazotrophicus* had a glucose:fructose ratio of 1:2 and 1:3 and were identified as 1-kestose and nystose, respectively (27).

These results show similarity to results of this study. The fructo-oligosaccharides produced by *L. sanfranciscensis* LTH2590 had a glucose:fructose ratio of 1:2. The retention time of the fructo-oligosaccharide from *L. sanfranciscensis* was identical with that of pure 1-kestose using two different chromatography methods, and the treatment of these purified oligosaccahrides with ß-fructosidase (yeast invertase) led to completely hydrolysis to fructose and glucose (data not shown), indicating that the linkage of the fructose is ß(2→1) and that the oligosaccharide produced is 1-kestose. Low concentration of other oligosaccharides

produced by *L. sanfranciscensis* LTH2590 may have escaped our analytical set up. The enzymatic hydrolysis of 1-kestose by the action of ß-fructosidase was also observed by kestose produced by *Aerobacter levanicum* levansucrase (10) and *Z. mobilis* levansucrase (3). It is surprising that levansucrase which assumed to catalyze ß(2→6) linkages are also able to catalyze ß(2→1). Euzenat et al. (9) attributed the production of 1-kestose by levansucrases to the hypothesis that levansucrase catalyses the formation of 1-kestose and can not transfer fructosyl residues onto it leading to its accumulation, while 6-kestose produced by levansucrase is rapidly used as acceptor for the production of levan. Since sucrose is a common part of dough formulas, and the sucrose concentration in the aqueous phase of doughs is high enough to expect kestose production, the use of kestose producing lactobacilli in sourdough fermentation is an important step towards development of cereal-based functional foods.

In conclusion, it was shown that the sucrose is degraded by *L. sanfranciscensis* by the action of fructosyltransferase enzyme, probably levansucrase, into glucose, kestose and an EPS composed of fructose (Fig. 6). The glucose liberated is used as energy source and degraded via pentose-phosphate-shunt. Sucrose can also be hydrolyzed by *L. sanfranciscensis* into glucose and fructose, the later is used as electron acceptor and reduced to mannitol. The relative quantity of the alternative end products of sucrose medium are strongly dependant on the sucrose concentration.

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References

1. **Armero, E., and C. Collar.** 1996. Antistaling additives, flour type and sourdough process effects on functionality of wheat doughs. J. Food Sci. **61(2):**299-303.
2. **Cerning, J., C. Bouillanne, M. Landon, and M. Desmazeaud.** 1991. Isolation and characterization of exopolysaccharides from slime-forming mesophilic lactic acid bacteria. J. Dairy Sci. **75:**692-699.
3. **Crittenden RG., and HW Doelle.** 1993. Structural identification of oligosaccharides produced by *Zymomonas mobilis* levansucrase. Biotechnol. Lett. **15:**1055-1060.
4. **Crittenden RG., and HW Doelle.** 1994. Identification and characterisation of the extracellular sucrases of *Zymomonas mobilis* ATCC 39676. Appl. Microbiol. Biotechnol. **41:**302-308.
5. **Cummings, J. H., G. T. Macfarlane, and H. N. Englyst.** 2001. Prebiotic digestion and fermentation. Amer. J. Clin. Nutr. **73:**415S-420S.
6. **Dal Bello, F., J. Walter, C. Hertel, and W. P. Hammes.** 2001. In *vitro* study of prebiotic properties of levan-type exopolysaccharides from lactobacilli and non-digestible carbohydrates using denaturing gradient gel electrophoresis. Sys. Appl. Microbiol. **24(2):**232-237.
7. **De Vuyst, L., and B. Degeest.** 1999. Heteropolysaccahrides from lactic acid bacteria. FEMS Microbiol. Rev. **23:**153-177.
8. **De Vuyst, L., F. De Vin, F. Vaningelgem, and B. Degeest.** 2001. Recent development in the biosynthesis and applications of heteropolysaccharides from lactic acid bacteria. Int. Dairy J. **11:**687-707.
9. **Euzenat, O., A. Guibert, and D. Combes.** 1997. Production of fructo-oligosaccharides by levansucrase from *Bacillus subtilis* C4. Proc. Biochem. **32(3):**237-243.

10. **Feingold, D. S., G. Avigad, and S. Hestrin.** 1956. The mechanism of polysaccharide production from sucrose. Biochem. J. **64:**351-361.
11. **Gänzle, M. G., M. Ehrmann, and W. P. Hammes.** 1998. Modelling of growth of *Lactobacillus sanfranciscensis* and *Candida milleri* in response to process parameters of the sourdough fermentation. Appl. Environ. Microbiol. **64:**2616-2623.
12. **Gibson, G. R., and M. B. Roberfroid.** 1995. Dietary modulation of the human colonic microbiota: Introducing the concept of prebiotics. J. Nutr. **125:**1401-1412.
13. **Hammes, W. P., P. Stolz, and M. Gänzle.** 1996. Metabolism of lactobacilli in traditional sourdoughs. Adv. Food Sci. **18:**176-184.
14. **Hestrin, S., S. Averini-Shapiro, and M. Aschner.** 1943. The enzymatic production of levan. Biochem. J. **37:**450-456.
15. **Hestirn, S., D. S. Feingold, and G. Avigad.** 1956. The mechanism of polysaccharide production from sucrose. Biochem. J. **64:**340-351.
16. **Hidaka, H., H. Hirayama, and K. Yamada.** 1991. Fructooligosacchrides: Enzymatic preparation and biofunctions. J. carbohydr. Chem. **10:**509-522.
17. **Hidaka, H., H. Hirayama, and N. Sumi.** 1988. A fructooligosaccharide producing enzyme from *Aspergillus niger* ATCC 20611. Agric. Biol. Chem. **52:**1181-1187.
18. **Jandik, P., AP. Clarke, N. Avdalovic, DC. Andersen, and J. Cacia.** 1999. Analyzing mixtures of amino acids and carbohydrates using bi-modal integrated amperometric detection. J. Chromatogr B. **732(1):**193-201.
19. **Korakli, M., E. Schwarz, G. Wolf, and W. P. Hammes.** 2000. Production of mannitol by *Lactobacillus sanfranciscensis*. Adv. Food Sci. **22:**1-4.
20. **Korakli, M., A. Rossman, M. G. Gänzle, and R. F. Vogel.** 2001. Sucrose metabolism and exopolysaccharide production in wheat and rye sourdoughs by *L. sanfranciscensis*. J. Agri. Food Chem. **49:**5194-5200.

21. **Korakli, M., M. G. Gänzle., and R. F. Vogel.** 2002. Metabolism by bifidobacteria and lactic acid bacteria of polysaccharides from wheat and rye and exopolysaccharides produced by *Lactobacillus sanfranciscensis*. J. Appl. Microbiol. **92:**958-965.
22. **Kosaric, N., GP. Cosentino, and A. Wieczorek.** 1984. The Jerusalem artichocke as an agricultured crop. Biomass.**5:**1-36.
23. **Mckellar, R. C., and H. W. Modler.** 1989. Metabolism of fructo-oligosaccharides by *Bifidobacterium* spp. Appl. Microbiol. Biotechnol. **31:**537-541.
24. **Okazaki, M., S. Fujikawa, and N. Matsumoto.** 1990. Effects of xylooligosaccharide on growth of bifidobacteria. J. Jap. Soc. Nutr. Food Sci. **43:**395-401.
25. **Schomburg, D., and D. Stephan.** 1996. Enzyme Handbook, Vol. 12.. Springer verlag, Berlin.
26. **Stolz, P., W. P. Hammes, and R. F. Vogel.** 1996. Maltose-phosphorylase and hexokinase activity in lactobacilli from traditionally prepared sourdoughs. Adv. Food Sci. **18:**1-6.
27. **Tambara, Y., J. V. Hormaza, C. Perez, A. Leon, J. Arrieta, and L. Hernandez.** 1999. Structural analysis and optimised production of fructo-oligosaccharides by levansucrase from *Acetobacter diazotrophicus* SRT4. Biotechnol. Lett. **21:**117-121.
28. **Tokunaga, T., T. Oku, and N. Hosoya.** 1986. Influence of chronic intake of new sweetener fructooligosaccharide (Neosugars) on growth and gastrointestinal function of the rat. J. Nutr. Sci. Vitaminol. **32:**111-121.
29. **Van Geel-Schutten, G. H., E. J. Faber, E. Smit, K. Bonting, M. R. Smith, B. Ten Brink, J. P. Kamerling, J. F. G. Vliegenthart, and L. Dijkhuizen.** 1999. Biochemical and structural characterisation of the glucan and fructan

exopolysaccharides synthesized by *Lactobacillus reuteri* wild-type strain and by mutant strains. Appl. Environ. Microbiol. **65:**3008-3014.

30. **Van Geel-Schutten, G. H., F. Flesch, B. ten Brink, M.R. Smith, and L. Dijkhuizen.** 1998. Screening and characterisation of *Lactobacillus* strains producing large amounts of exopolysaccharides. Appl. Microbiol. Biotechnol. **50:**697-703.

31. **Van Hijum, S. A. F. T., K. Bonting, M. J. E. C. van der Maarel, and L. Dijkhuizen.** 2001. Purification of a novel fructosyltransferase from *Lactobacillus reuteri* strain 121 and characterization of the levan produced. FEMS Microbiol. Lett. **205:**323-328.

Figure legends

Figure 1: Effect of fructose and sucrose concentration on the growth rate of *L. sanfranciscensis* LTH2590.

Figure 2: Kinetics of microbial growth, EPS production and fermentation products of *L. sanfranciscensis* LTH2590 in Su-MRS containing 60 g l^{-1} sucrose and 5 g l^{-1} fructose at pH 5.6. (Δ) OD_{578nm}, (▲) EPS, (●) sucrose, (❍) mannitol, (□) fructose, (■) glucose, (◆) lactate, (▼) ethanol, (◊) acetate.

Figure 3: Effect of free fructose amount on the hydrolase activation by *L. sanfranciscensis* LTH2590 in fermentations with (A) 60 g l^{-1} sucrose and 15 g l^{-1} fructose, (B) 90 g l^{-1} sucrose and 5 g l^{-1} fructose. Free fructose consumed (▲), mannitol (❍) and the sum of mannitol and free fructose (●).

Figure 4: Comparison of HPLC chromatograms using (A) polyspher® CH PB column and (B) anion-exchange chromatography. The chromatograms at the end of sucrose fermentation and after acid hydrolysis of supernatant compared with that of 1-kestose are shown.

Figure 5: (A). Effect of sucrose concentration on the amount of EPS produced by *L. sanfranciscensis* in Su-MRS containing 5 g l^{-1} (▲) or 15 g l^{-1} (Δ) fructose at pH 5.6.

Figure 5: (B). Effect of sucrose concentration on the hydrolysis of sucrose by *L. sanfranciscensis* in Su-MRS containing 5 g l^{-1} (▲) or 15 g l^{-1} (Δ) fructose at pH 5.6 and on mannitol production from sucrose in Su-MRS containing 5 g l^{-1} (●) or 15 g l^{-1} (❍) fructose

Figure 5: (C). Effect of sucrose concentration on the amount of kestose produced by *L. sanfranciscensis* in Su-MRS containing 5 g l^{-1} (▲) or 15 g l^{-1} (Δ) fructose at pH 5.6

Figure 6: Sucrose metabolism by *L. sanfranciscensis*, FTF: fructosyltransferase

Table 1. Concentrations of substrates and end products of fermentations by *L. sanfranciscensis* in media with various sucrose and fructose contents

			15 g/l Fructose				**5 g/l Fructose**		
Sucrose (g l^{-1})	20[a)]	30	60	70[a)]	120[a)]	160[a)]	20[a)]	60	90[a)]
Measured sucrose at t=0 (mmol l^{-1})	69	92	170	214	356	481	56	170	263
Consumed sucrose at t=end (mmol l^{-1})	-68[b)]	-91	-109	-112	-160	-245	-58	-105	-141
Lactate (mmol l^{-1})	74	86	73	97	103	137	62	98	67
Acetate (mmol l^{-1})	50	55	57	56	52	62	25	25	24
Mannitol (mmol l^{-1})	98	107	111	100	99	104	38	43	49
Fructose (mmol l^{-1})	-75[b)]	-86	-73	-72	-79	-81	-22	-22	-30
Glucose (mmol l^{-1})	0	0	12	6	13	6	0	0	0
Ethanol (mmol l^{-1})	29	25	14	39	40	65	41	66	38
Glucose recovery[c)] (%)	108	95	78	87	73	58	104	93	48

[a)] Values in these fermentations are representative for two independent experiments, the coefficient of variation between two fermentations was generally in the range of 5-12%. [b)] Negative values indicate consumption, positive values indicate production of metabolites. [c)] recovery of the glucose moiety from sucrose consumed in the end products glucose, lactate and acetate or ethanol.

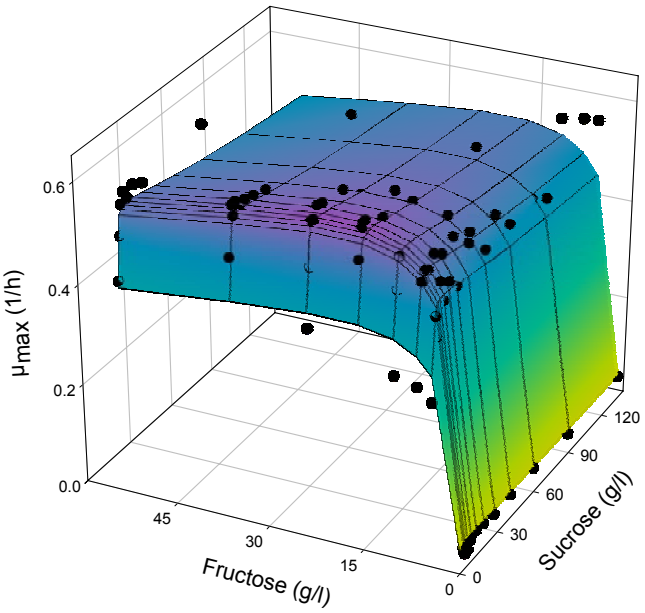

Fig. 1

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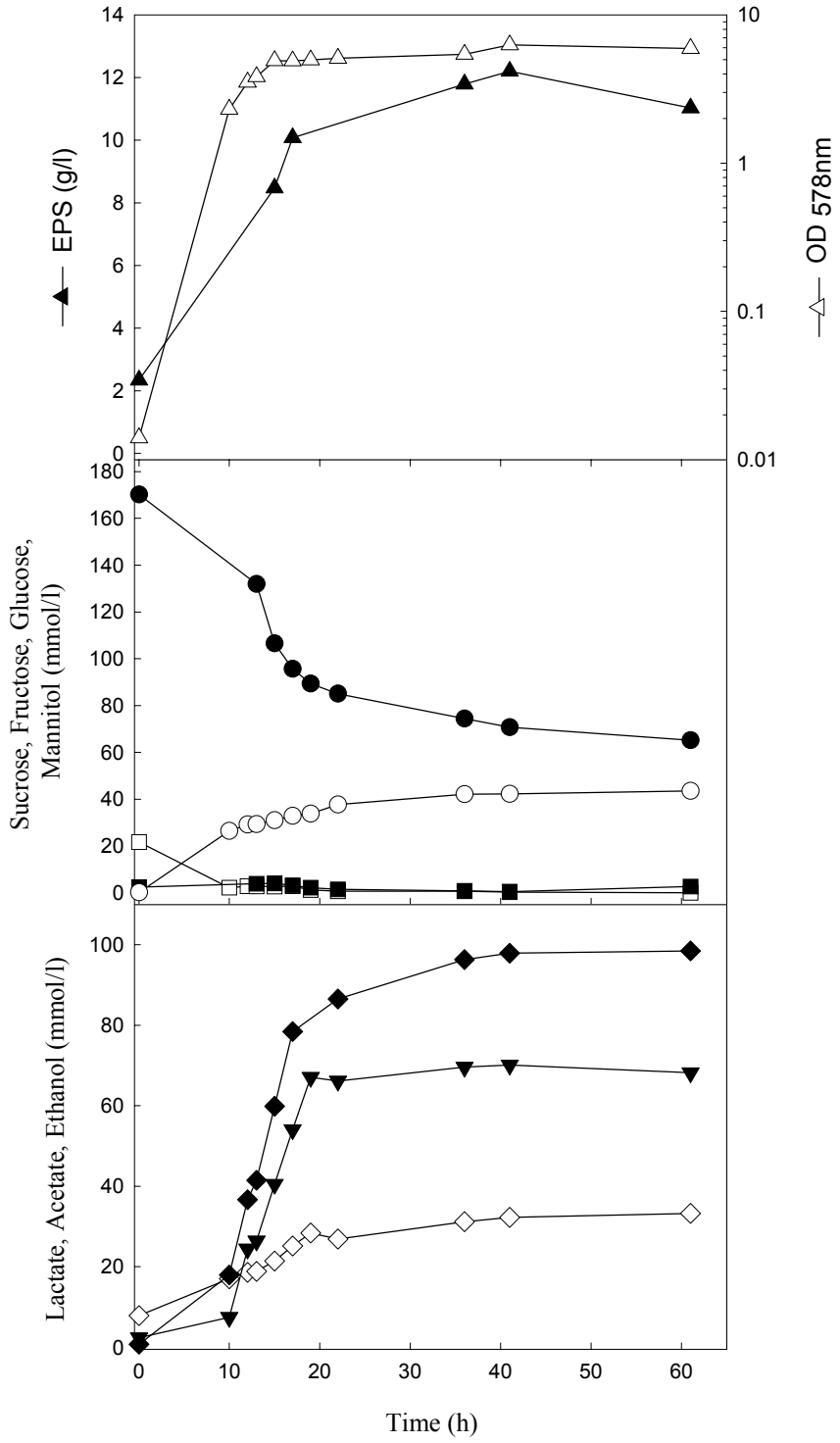

Fig. 2

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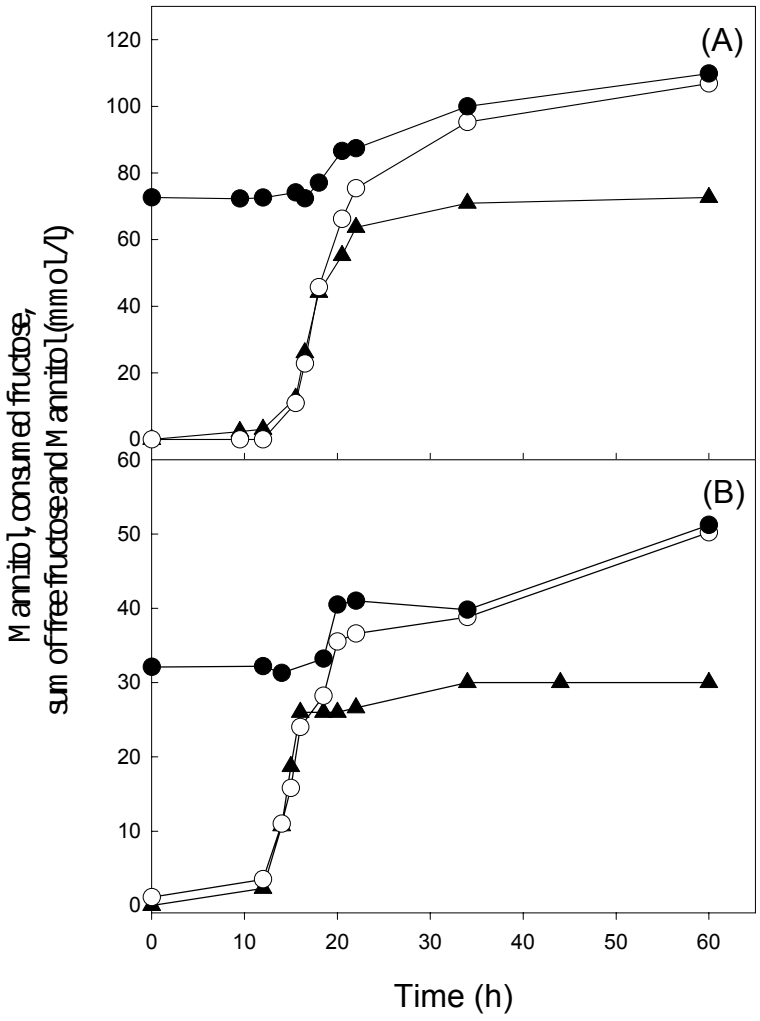

Fig. 3

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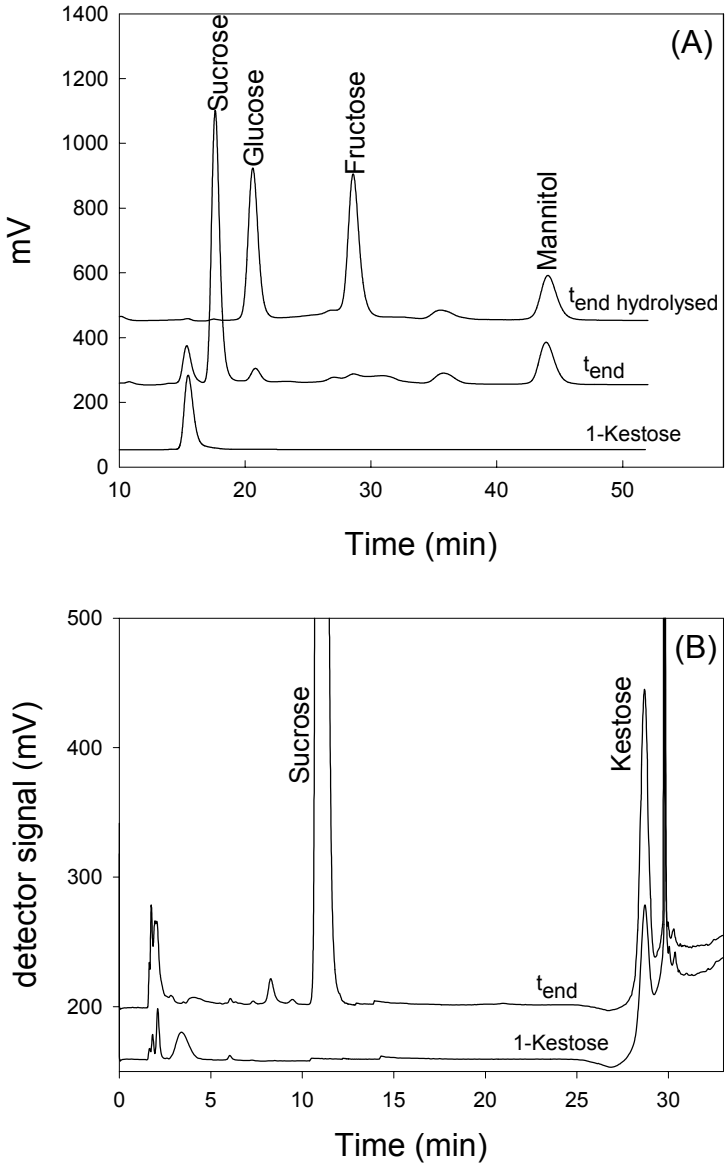

Fig. 4

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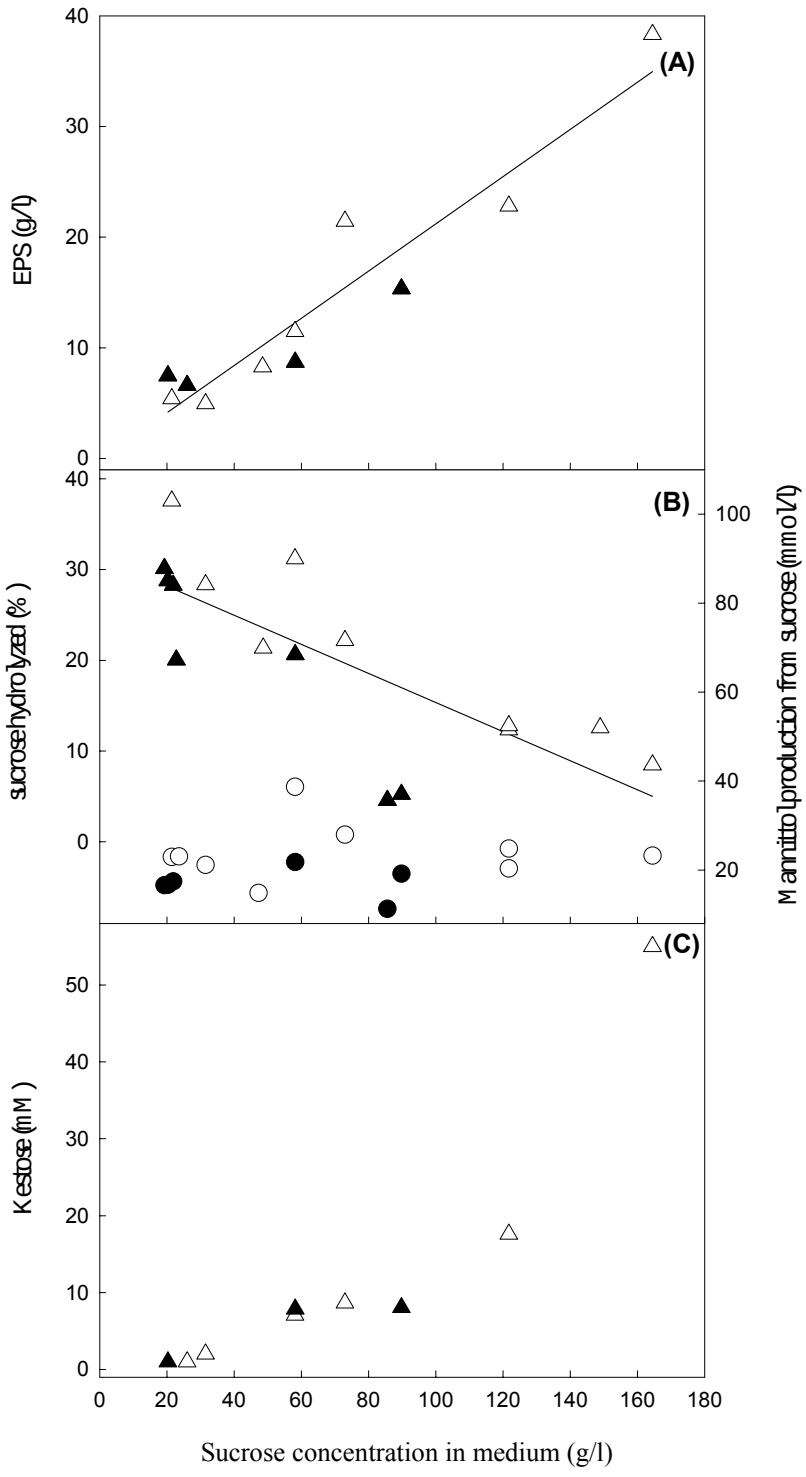

Fig. 5

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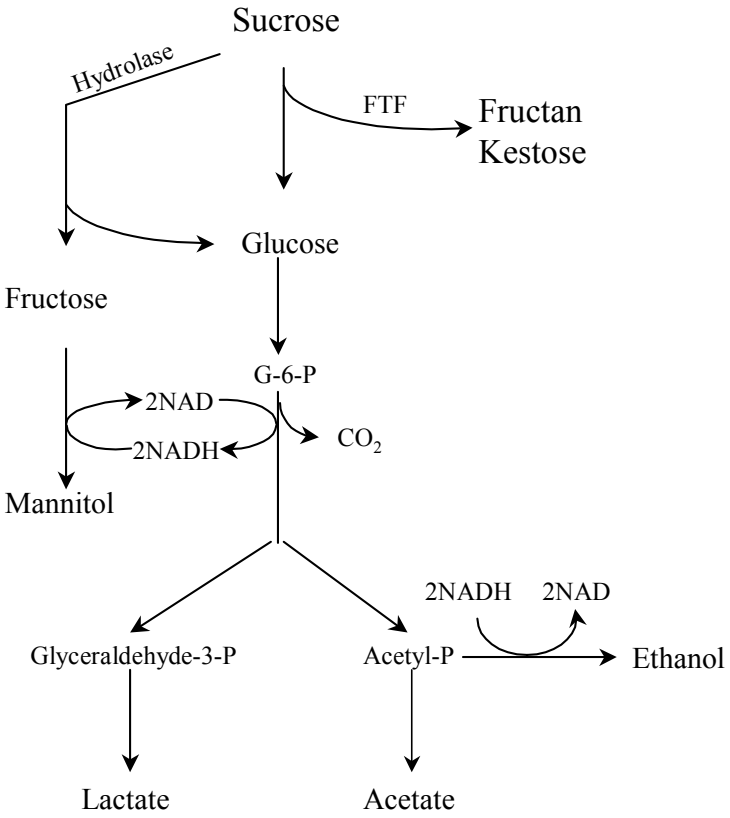

Fig. 6

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Effect of high pressure on the metabolism of maltose and sucrose by *Lactobacillus sanfranciscensis*

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Running title: Metabolism of *Lactobacillus sanfranciscensis* under high pressure

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Aims: The effect was studied of sublethal high pressure on viable cell counts and metabolism of maltose or sucrose by *Lactobacillus sanfranciscensis*.

Methods and Results: Kinetics of viable cell counts, substrate and end products of maltose or sucrose metabolism were determined in fermentations for 24h under ambient pressure, 100 and 150 MPa. Incubation of cells under 100 MPa for 24h in medium containing maltose decreased the viable cell counts by less than 2 log, under 150 MPa the reduction was by 2 log and by more than 4 log in the presence of fructose. Metabolism of maltose by the strain TMW1.52 results in production of lactate, and ethanol or acetate as alternative end products. Metabolism of sucrose by the strain TMW1.392 results in production of lactate, acetate, ethanol, mannitol and exopolysaccharide.

Conclusion: Metabolism of maltose or sucrose was inhibited under high pressure conditions and the inhibition under 150 MPa was higher than under 100 MPa. Maltose or sucrose metabolism under high pressure was accompanied with glucose accumulation.

Significance and impact of the study: Investigation of metabolism under high pressure conditions will contribute to elucidate the mechanisms of high pressure action and the response of lactic acid bacteria to this environmental stressor.

Introduction

Lactic acid bacteria (LAB) have been used for centuries in production of fermented food due to their preservative contribution and metabolic activity, that award fermented foods their characteristic attributes. The increasing use of defined starter culture instead of spontaneous fermentation in the production of fermented foods led to extensive studies on the physiology and genetics of LAB. These studies included the metabolic response of lactic acid bacteria to environmental stressors, e. g. temperature, pH, osmotic stress and starvation. Low a_w and/or pH by *Lactococcus lactis* subsp. *cremoris* resulted in decreased growth, lactose utilization and carbon recovery. Lactose fermentation under these conditions was accompanied with galactose accumulation and production of galactose containing saccharide(s) (Liu et al., 1998).

In the last years hydrostatic high pressure was applied in food industry as a non-thermal food processing technique. There are few data about the effect of sublethal pressure on the metabolism of microorganisms. The effect of high pressure is dependent on the environmental conditions e.g. temperature, pH, concentration of substrate and end products. High pressure treatment of microorganisms affects not only the viable cell counts but also the metabolic activity. Sublethal high pressure treatment (40-60 MPa) of *Saccharomyces cerevisiae* resulted in reduction of vacuolar-pH and pressure ranging from 25-50MPa led to decreased tryptophan uptake (Abe and Horikoshi, 1998 and 2000). The fractionation of stable carbon isotopes by photosynthetic CO_2 fixation and decarboxylation reactions are well documented (O'Leary, 1982). The carbon isotope fractionation observed in enzymatic reactions has been attributed to differences in reaction constants for ^{12}C and ^{13}C substrates (O'Leary, 1982) and forms the basis for the analysis of carbon fluxes through stable isotope analysis. We previously observed pressure effects on the carbon isotope fractionation in maltose metabolism by *L. sanfranciscensis* (Korakli et al., 2002). Pressure mediated carbon isotope effects may be

relevant for the interpretation of stable isotope data used to trace carbon fluxes in deep sea environments (Valentine and Reeburth, 2000).

Lactobacillus sanfranciscensis has frequently been used to determine the stress response to sublethal high pressure. *L. sanfranciscensis* is a heterofermentative lactic acid bacterium that belongs to the microbial flora of traditionally prepared wheat and rye sourdoughs (Hammes et al., 1996). Hexoses are metabolized via pentose-phosphate-shunt, maltose is cleaved by maltose phosphorylase into glucose and glucose-1-phosphate (Stolz, et al. 1996). Sucrose is cleaved by levansucrase into glucose, fructose and an exopolysaccharide (EPS) consisting of fructose (Korakli et al., 2000). We have previously shown that *L. sanfranciscensis* tolerated pressure up to 150 MPa for 3h without appreciable decrease of the viable cell counts, and maltose consumption was reduced under high pressure (Korakli et al. 2002). High pressure treatment (200 MPa) of *L. sanfranciscensis* and subsequent comparison of the protein patterns using 2-D electrophoresis revealed that protein expression is modified under high pressure. Five of the modified spots could be identified by liquid chromatography-tandem mass spectroscopy, and are homologues to cold shock proteins of *Lactococcus lactis* (Drews et al., 2002). It was the aim of this work to evaluate the effect of sublethal high pressure on the kinetics of the viable cell counts, substrate consumption and end products formation of maltose and sucrose in fermentations for 24h by two strains of *L. sanfranciscensis*.

Material and Methods

Media and culture conditions

Lactobacillus sanfranciscensis TMW1.52 (isogenic with LTH2581) was used for the study of maltose metabolism and the strain TMW1.392 (isogenic with LTH2590) for the study of sucrose metabolism. The strain TMW1.52 was cultured at 30°C in Maltose-Fructose-MRS4 (MF-MRS4) or Maltose-MRS4 (M-MRS4). MF-MRS4 contained the following components per litre: peptone from casein, 10g; yeast extract, 5g; meat extract, 5g; $K_2HPO_4x3H_2O$, 2.6g; KH_2PO_4, 4g; cystein-HCl, 0.5g; NH_4Cl, 3g; maltose, 10g; fructose, 10g; Tween 80, 1 ml. The

pH was adjusted to 6.2, and the medium was sterilized at 121°C for 20 min. Sugars were autoclaved separately. Stock solutions containing $MgSO_4x3H_2O$ (100 g l^{-1}) and $MnSO_4x$ $4H_2O$ (50 g l^{-1}) were autoclaved separately, and a vitamin mix containing biotin, folic acid, nicotinic acid, pyridoxal phosphate, thiamine, riboflavin, cobalamin and panthothenic acid (0.2 g/l each) was sterilized by filtration (Sartorius AG, Germany, 0.20 µm).1 ml of each stock solution was added per litre medium. M-MRS4 had the same composition as MF-MRS4 but did not contain fructose. For the isotope investigation in the fermentation end product ethanol maltose derived from C_3-plant (Sigma, USA) was used in M-MRS4 and the $\delta^{13}C$-value of C_3-maltose was -25.20‰. TMW1.392 was cultured on Sucrose-MRS4 (Su-MRS4). Su-MRS4 contained the same components as MF-MRS4 but 50 g l^{-1} sucrose were added instead of maltose and fructose.

Determination of colony forming units

Cell counts in all experiments were determined on MF-MRS4 agar. Appropriate dilutions were plated using a spiral plater (IUL, Königswinter, Germany) and plates were incubated at 30°C for 48h under controlled atmosphere (76% N_2, 20% CO_2, 4% O_2).

Determination of metabolites

For determination of substrates and end products cells were removed from the fermentation broth by centrifugation. The concentrations of maltose, lactic acid, acetic acid and ethanol were determined using a polyspher® OA KC column (Merck, Germany); the mobile phase was 5 mmol l^{-1} H_2SO_4 and temperature of the column 70°C. Sucrose, glucose, fructose, and mannitol concentrations were determined using a polyspher® CH PB column (Merck, Germany); the mobile phase was deionized H_2O and the temperature of the column 80°C. For detection, a refractive index detector (Gynkotek, Germany) was used. The detection limit was 1 mmol l^{-1} for ethanol, 0.2 mmol l^{-1} for carbohydrates and mannitol, and 0.05 mmol l^{-1} for organic acids. For the precipitation of EPS, 200% (v/v) ethanol were added to the culture

supernatant and incubated for at least 3h at 4°C. The pellet was dissolved in dest. water, precipitated again as described above and lyophilised.

High pressure treatment

An overnight culture of *L. sanfranciscensis* TMW1.52 grown on MF-MRS4 or M-MRS4 was sub-cultured with 1% inoculum in MF-MRS4 or M-MRS4, respectively. TMW1.392 was grown and sub-cultured with 1% inoculum in Su-MRS4. Late stationary cells were harvested by centrifugation and resuspended in 0.5 volumes of fresh medium, unless otherwise indicated. This cell suspension was transferred to 2 ml screw reaction tubes, sealed with parafilm avoiding enclosure of air. The high pressure (HP) metabolism and inactivation kinetics of *L. sanfranciscensis* were investigated in HP-autoclaves at 30°C. Compression and decompression rates were 200 MPa min^{-1}. Samples were taken after decompression for determination of viable cell counts and the concentration of metabolites.

Determination of the carbon isotope ratio

Determination of $\delta^{13}C$ values of ethanol produced from C_3-maltose under ambient and high pressure was carried out as described by Korakli et al. (2002).

Results

Effect of high pressure on the metabolism of maltose in M-MRS4

Maltose metabolism by *L. sanfranciscensis* TMW1.52 at pressure of 200 MPa or greater was almost completely inhibited (Korakli et al., 2002). To evaluate the effect of sublethal pressure on the metabolism and cell counts, fermentations in M-MRS4 were carried out at 100 and 150 MPa for 24h. As shown in figure 1 cell counts remained constant ($1\times10^{+9}$ cfu ml^{-1}) at ambient pressure. At 100 MPa the cell counts reduction was about 1 log and even after 24h treatment at 150 MPa the inactivation was less than 2 log. Maltose was almost completely metabolised at ambient pressure within 5h of incubation, whereas at 100MPa only 75% were metabolised within 24h. The inhibition of the metabolism was more significant at 150 MPa, only 35% of the maltose were metabolised within 24h. In the absence of electron acceptors, the

metabolism of maltose by *L. sanfranciscensis* TMW1.52 results in production of lactate and ethanol only (Stolz et al. 1996). The concentrations of substrates and end products after 24h incubation at 0.1, 100 and 150 MPa in M-MRS4 are shown in table 1. Acetate concentration under high pressure conditions was significantly higher than under ambient pressure. Lactate and ethanol concentrations were in accordance with the inhibitory effect of high pressure on maltose metabolisation and the carbon recoveries were 95-105%. The metabolism of maltose under high pressure conditions was accompanied with glucose accumulation throughout the fermentation (Fig. 1). Under ambient pressure, glucose was only accumulated intermediately in the first two hours of the fermentation, whereas the accumulation of glucose was at high pressure conditions proportional to the maltose consumption and at 150 MPa higher than at 100 MPa. To evaluate whether the inhibition of maltose metabolism under high pressure is attributable to pressure effects on the medium pH, maltose consumptions at ambient pressure and 150 MPa in M-MRS4 or M-MRS4 additionally buffered with 50mM imidazol were compared. Maltose was completely consumed in both media after 24h of incubation at ambient pressure. At 150 MPa no significant difference in maltose consumption after 24h incubation in M-MRS4 or M-MRS4 with imidazol (24% and 27% respectively) was observed.

The $\delta^{13}C$ values of ethanol produced from maltose by *L. sanfranciscensis* TMW1.52 during metabolism at ambient pressure and 100 MPa are shown in table 2. At low maltose turnover levels, the end product ethanol was depleted with respect to the ^{13}C isotope, indicating a kinetic isotope effect. At the same maltose consumption levels, the depletion of the ^{13}C isotope was more pronounced under 100 MPa. These results confirm the phenomenon observed in our previous work that the kinetic isotope effect was enhanced upon metabolism under high pressure (Korakli et al., 2002).

Effect of high pressure on the metabolism of maltose in MF-MRS4

In presence of fructose maltose is metabolised to lactate and acetate and the fructose is not used as carbon source but reduced to mannitol (Stolz et al., 1996). The effect of fructose addition on the viable cell counts and metabolic activity during high pressure treatment of *L. sanfranciscensis* TMW1.52 is shown in Fig. 2. The reduction of viable cell counts at the end of the fermentation was similar at ambient pressure and 100 MPa, the reduction of viable cell counts was more than 4 log after 5h of treatment under 150 MPa. Maltose consumption in presence of fructose at ambient and high pressure was comparable to that without fructose. At 150 MPa the metabolism was almost stopped after 6h incubation due to the reduction of the viable cell counts. The metabolism of maltose at ambient pressure and 100 MPa resulted not only in lactate and acetate but also in ethanol production (Tab. 3) due to insufficient fructose amount (54 mmol l^{-1}) needed for the regeneration of NAD^+. Under 150 MPa (metabolic rate 35%) no ethanol was produced and NAD^+ was regenerated only by the reduction of fructose to mannitol. Lactate, acetate and ethanol produced at ambient pressure and 100 MPa accounted for 96-104% of the substrate metabolised and at 150 MPa for 68%. The maltose metabolism in MF-MRS4 under high pressure was also accompanied with glucose accumulation (Fig. 2). However, the glucose amount accumulated in M-MRS4 was higher than that in MF-MRS4.

Effect of high pressure on the metabolism of sucrose

To evaluate whether high pressure effects observed on maltose metabolism are independent of the substrate used, fermentations in Su-MRS4 were carried out with *L. sanfranciscensis* TMW1.392. This strain metabolises sucrose and produces an exopolysaccharide consisting of fructose. Figure 3 showed the viable cell counts, lactate production and sucrose consumption after 3h incubation under pressure ranging from 0.1-400 MPa in Su-MRS4 containing 70 g l^{-1} sucrose and 15 g l^{-1} fructose. The viable cell counts remained unaffected up to 100 MPa and at 400 MPa the reduction of the cells was greater than 6 log. The decrease of lactate

production was more significant than the decrease of sucrose consumption (sucrose consumption at ambient pressure 100%) at high pressure. The sucrose consumptions at 300 and 400 MPa were 40% and 20%, respectively, although only 7% lactate were produced.

The kinetics of sucrose metabolism and viable cell counts at 0.1, 100 and 150 MPa are shown in Fig. 4. The decrease of the viable cell counts after 24h incubation at ambient pressure was about 1 log and at 100 and 150 MPa it was about 4 log. The sucrose consumption kinetics were proportional to the incubation time and surprisingly similar at 100 and 150 MPa (45-50%), although the decrease of viable cell counts was higher than 4 log. This corroborates with the results shown in Fig. 3, that sucrose utilisation seems to be independent of pressure in the range 50-150 MPa. The effect of high pressure on the viable cell counts and sucrose consumption was similar by fermentations carried out with 70 g l^{-1} sucrose (data not shown). The metabolism of sucrose at ambient pressure resulted in production of lactate, acetate, ethanol and EPS, whereas only lactate, acetate and EPS were produced under high pressure (Tab. 4). The ratio of the fructose moiety of sucrose reduced to mannitol to the consumed sucrose ([mannitol produced-fructose consumed]/[sucrose consumed]) was 43%, 46% and 25% at ambient pressure, 100 MPa and 150 MPa, respectively, indicating more EPS production under 150 MPa relative to the sucrose consumption. The sucrose metabolism under high pressure by the strain TMW1.392 was also accompanied with glucose accumulation (Fig. 4), and at 150 MPa more glucose was accumulated than under 100 MPa.

Discussion

In this work the metabolism of maltose and sucrose under sublethal high pressure was studied. In M-MRS4 the reduction of viable cell counts under 100 and 150 MPa after 24h incubation was about 1 log. The reduction of viable cell counts in MF-MRS4 was similar under ambient pressure and 100 MPa after 24h, this reduction is attributed to the combined effect of acetate concentration (28-30 mmol l^{-1}) and the pH. Below pH 4.7, acetate is

undissociated and can penetrate into the cell. Cells were inactivated by 4 log in MF-MRS4 at 150 MPa in the first 6h and the increased inactivation after 6h is probably the result of the synergetic effects of pressure, pH and acetate concentration. The reduction of the viable cell counts of the strain TMW1.392 after 3h or 24h of incubation at 100 or 150 MPa was greater than the reduction of the strain TMW1.52.

The inhibitory effect of high pressure on the metabolism in biological systems are not completely known. However, cell membrane and therewith membrane-bound enzymes represent a major target for pressure effects. Inhibition of Na/K-dependent ATPase involved in the active transport through the membrane was reported by Chong et al. (1985). Marquis and Bender (1987) attributed the inhibition of F_0F_1 ATPase under sublethal HP (50 MPa) to the proton-translocating step and not to the ATP hydrolytic step. Abe and Horikoshi (2000) reported the inhibition of tryptophan uptake into *Saccharomyces cerevisiae* during pressure treatment at 25 MPa. The decreased metabolic activity observed by *Lactobacillus plantarum* was also ascribed to the inhibition of the membrane-bound transport enzymes as a result of the phase transition of the cytoplasmic membrane (Ulmer et al., 2002). The thermodynamic state of the cell membrane during pressure treatment observed in *L. plantarum* could explain the reversibility of the inhibitory effect of high pressure on maltose metabolism observed by *L. sanfranciscensis*. The irreversible inhibition of metabolism was preceded by reversible transition phase, the inhibitory effect was reversible up to 100 MPa and at 150 MPa the inhibition was irreversible (Korakli et al., 2002). The reversible inhibition of metabolism under sublethal high pressure might be also the result of changes in the secondary, tertiary and quaternary structure of proteins, or to changes in hydration, conformation and structure of proteins (Masson, 1992). Deville-Bonne and Else (1991) reported reversible dissociation of the tetrameric phosphofructokinase at 80 MPa.

The effect of high pressure on biological systems depends also on the environmental conditions e. g., temperature, pH and the concentration of substrate. Soluble solids such as

sugars, proteins and lipids exerted a protective action against pectinesterase inactivation by high pressure or heat (Ogawa, 1990). The regulation of the intracellular pH, which is perquisite for the survival of lactic acid bacteria in low pH environment, was also discussed as a possible target of high pressure (Wouters et al. 1998). Molina-Gutierrez et al. (2002) reported the loss of the transmembrane pH gradient of *Lactococcus lactis* and *L. plantarum* during pressure treatment at 200 or 300 MPa. The inhibition of the maltose or sucrose metabolism under high pressure observed in this study cannot be attributed to the loss of the transmembrane pH gradient, since the reduced substrate utilisation was already occurred at the beginning of the fermentation at an almost neutral pH. Furthermore, comparable maltose consumptions were observed at high pressure in media buffered with a different buffering capacity.

Maltose is cleaved by *L. sanfranciscensis* to glucose and glucose-1-phosphate by maltose phosophrylase, this step allows to save ATP compared with maltose cleavage via α-glucosidase. Metabolism of maltose by *L. sanfranciscensis* under high pressure was accompanied with glucose accumulation, indicating that maltose cleavage means maltose phosphorylase is not inhibited even under 150 Mpa, and that the phosphorylation of glucose (with ATP expense) might represent the high pressure sensitive point in the metabolic pathway. The ratio of glucose accumulated to maltose consumed was about 1 at 150 MPa and < 1 at 100 MPa, indicating complete inhibition of glucose phosphorylation at 150 MPa and partial inhibition at 100 MPa. The decrease in sucrose consumption under high pressure was not proportional to the decrease of lactate production, and glucose accumulation under high pressure metabolism of sucrose was also observed. Thus, sucrose cleavage seems not to be a limiting step for the metabolic activity at high pressure.

Stable isotope analysis is a major tool used in ecosystem studies to trace metabolic fluxes (Henn and Chapela, 2000; Korakli et al., 2001; Schmidt et al., 1986). Pressure has been shown to affect deuterium isotope effects (Quirk and Northrop, 2001). In confirmation of a

previous study (Korakli et al., 2002), this is the first report to indicate pressure effects on ^{12}C / ^{13}C isotope fractionation in biochemical reactions. The effect observed here in maltose fermentation of *L. sanfranciscensis* is attributable to enzymatic reactions involving cleavage of carbon-carbon bonds. The specific contribution of the two enzymes of the pentose-phosphate-shunt cleaving C-C-bonds, 6-phospho-gluconate dehydrogenase and phosphoketolase, remains to be determined.

We could demonstrate that sublethal high pressure treatments results in decreasing of the metabolic activity of *L. sanfranciscensis* and increased glucose accumulation. Irreversible inhibition of metabolic activity of lactic acid bacteria by high pressure treatment may be of major interest for the production of fermented food. This will deliver a tool for controlling fermentations without the need to inactivate the cells.

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References

Abe, F. and Horikoshi, K. (1998). Analysis of interacellular pH in the yeast *Saccharomyces cerevisiae* under elevated hydrostatic pressure: a study in baro-(piezo-)physiology. *Extremophiles* 2, 223-228.

Abe, F. and Horikoshi, K. (2000). Tryptophan permease gene TAT2 confers high-pressure growth in *Saccharomyces cerevisiae*. *Molecular and Cell Biology* 20(21)**,** 8093-8102.

Chong, PL., Forted, PA. and Jameson, DM. (1985). Mechanisms of inhibition of Na/K ATPase by hydrostatic pressure studied with fluorescent probes. *Journal of Biological Chemistry* 260, 14484-14490.

Deville-Bonne, D. and Else, A. J. (1991) Reversible high hydrostatic pressure inactivation of phosphofructokinase from *Escherichia coli*. *European Journal of Biochemistry* 200, 747-750.

Drews, O., Weiss, W., Reil, G., Parlar, H., Wait, R. and Görg, A. (2002) High pressure effects Step-wise altered protein expression in *Lactobacillus sanfranciscensis*. *Proteomics* 2, 765-774.

Hammes, W.P., Stolz, P. and Gänzle, M.G. (1996) Metabolism of lactobacilli in traditional sourdoughs. *Advances in Food Sciences* 18, 176-184.

Henn, M.R. and Chapela, I.H. (2000) Differential C isotope discrimination by fungi during decomposition of C_3- and C_4-derived sucrose. *Applied and Environmental Microbiology* 66(10), 4180-4186.

Korakli, M., Gänzle, M.G., Knorr, R., Frank, M., Rossmann, A. and Vogel, R.F. (2002) Metabolism of *Lactobacillus sanfranciscensis* under high pressure: investigation using stable carbon isotopes. In *Trends in high pressure bioscience and biotechnology* ed. Hayashi, R. pp. 287-294. Amesterdam: Elsevier Science.

Korakli, M., Schwarz, E., Wolf, G. and Hammes. W.P. (2000) Production of mannitol by *Lactobacillus sanfranciscensis*. *Advances in Food Sciences* 22, 1-4.

Liu, S.Q., Asmundson, R.V., Gopal, P.K., Holland, R. and Crow, V.L. (1998) Influence of reduced water activity on lactose metabolism by *Lactococcus lactis* subsp. *cremoris* at different pH values. *Applied and Environmental Microbiology* 64(6), 2111-2116.

Marquis, R.E. and Bender, G.R. (1987) Barophysiology of prokaryotes and proton translocating ATPases. In *Current perspective in high pressure biology* ed. Jannasch, H.W., Marquis, R.E. and Zimmermann, A.M. pp. 65-73. London: Academic Press Ltd.

Masson, P. (1992) Pressure denaturation of proteins. In *High pressure and biotechnology* ed Banly, C., Hayashi, R., Heremans, K. and Masson, P. pp. 89, Vol. 224. Montrouge: John Libbey Eurotext.

Molina-Gutierrez, A., Stippl, V., Delgado, A., Gänzle, M.G. and Vogel, R.F. (2002) In situ determination of the intracelluar pH of *Lactococcus lactis* and *Lactobacillus plantarum* during high pressure treatment. *Applied and Environmental Microbiology* 68(9), 4399-4406.

O´Leary, M.H. (1982) Heavy isotope effects on enzyme catalysed reactions. In *Analytica chemistry symposia series* Vol. 11, eds. Schmidt, H.L., Förstel, H. and Heinzinger, K. Stable isotopes pp. 67-75. Amesterdam: Elsevier Science.

Ogawa, H., Fukuhisa, K., Kubo, Y. and Fukumoto, H. (1990) Pressure inactivation of yeasts, molds and pectinesterase in Satsuma mandarin juice: effect of juice concentration, pH, organic acid and comparison with heat sanitation. *Agriculture and Biological Chemistry* 54, 1219-1225.

Quirk, D.J. and Northrop, D.B. (2001) Effect of pressure on deuterium isotope effects of formate dehydrogenase. *Biochemistry* 40, 847-851.

Schmidt, H.L. (1986) Food quality control and studies on human nutrition by mass spectroscopic and nuclear magnetic resonance isotope ratio determination. *Fresenius Journal of Analytical Chemistry* 324, 760-766.

Stolz, P., Hammes, W.P and Vogel, R.F. (1996) Maltose-phosphorylase and hexokinase activity in lactobacilli from traditionally prepared sourdoughs. *Advances in Food Sciences* 18, 1-6.

Ulmer, H.M., Herberhold, H., Fahsel, S., Gänzle, M.G., Winter, R. and Vogel, R.F. (2002) Effects of pressure-induced membrane phase transition on inactivation of HorA, an ATP-dependent multidrug resistance transporter, in *Lactobacillus plantarum*. *Applied and Environmental Microbiology* 68(3), 3966-3973.

Valentine, D.L. and Reeburth, W.S. (2000) New perspectives on anaerobic methane oxidation. *Environmental Microbiology* 2, 477-484.

Wouters, P.C., Glaasker, E. and Smelt, J.P.P.M. (1998) Effects of high pressure on inactivation kinetics and events related to proton efflux in *Lactobacillus plantarum*. *Applied and Environmental Microbiology* 64(2), 509-514.

Figure legends

Figure 1. Effect of high pressure on the viable cell counts, maltose consumption and glucose accumulation in M-MRS4 by *L. sanfranciscensis* TMW1.52, $\log(N_0) = 8.9 \pm 0.0$. Ambient pressure (●), 100 MPa (■) and 150 MPa (▲).

Figure 2. Effect of high pressure on the viable cell counts, maltose consumption and glucose accumulation in MF-MRS4 by *L. sanfranciscensis* TMW1.52, $\log(N_0) = 9.3 \pm 0.1$. Ambient pressure (●), 100 MPa (■) and 150 MPa (▲).

Figure 3. High pressure effect on the viable cell counts and metabolic activity of *L. sanfranciscensis* TMW1.392 in Su-MRS4 containing 70 g l^{-1} sucrose and 15 g l^{-1} fructose after 3h of incubation. Cultured cells were resuspended in an equal volume of culturing medium $\log(N_0) = 8.7 \pm 0.1$. Shown are: $\log(N/N_0)$ (●), sucrose consumption as % of sucrose metabolized after 3h under ambient pressure (black bars) and lactate production as % of lactate produced after 3h under ambient pressure (grey bars).

Figure 4. Effect of high pressure on the viable cell counts, sucrose consumption and glucose accumulation in Su-MRS4 containing 35 g l^{-1} sucrose and 5 g l^{-1} fructose by *L. sanfranciscensis* TMW1.392, $\log(N_0) = 9.1 \pm 0.3$. Ambient pressure (●), 100 MPa (■) and 150 MPa (▲).

Table 1. Concentrations of substrates and end products in M-MRS4 fermented by *L. sanfranciscensis* TMW1.52 at 0.1, 100 and 150 MPa (all concentrations are in mmol l^{-1}).

	pH	Maltose	Glucose	Lactate	Acetate	Ethanol
t=0	5.8±0.0	27.5±0.1	0.3±0.0	0.9±0.1	0.0	1.7±0.0
t=24h, P=0.1 MPa	3.8±0.0	1.3±0.3	0.4±0.2	52.1±0.5	<0.3	49.0±0.9
t=24h, P=100 MPa	4.1±0.3	6.8±4.6	4.7±3.0	35.1±12.8	3.9±0.2	35.8±13.5
t=24h, P=150 MPa	5.0±0.1	17.8±0.7	7.5±1.3	9.7±3.8	4.4±0.4	8.2

Data are means ± standard deviation of two independent fermentations

Table 2. High pressure effect on isotope distribution in ethanol produced by *L. sanfranciscensis* TMW1.52 grown in MF-MRS4.

Maltose consumption (%)	$\delta^{13}C$ value (‰) at 0.1 MPa	$\delta^{13}C$ value (‰) at 100MPa
9.0	-25.6	-26.5
48.2	-24.7	-25.8
90.8	ND	-24.3
96.4	-24.6	ND

Table 3. Concentrations of substrates and end products in mMRS4 fermented by *L. sanfranciscensis* TMW1.52 at 0.1, 100 and 150 MPa (all concentrations are in mmol l^{-1}).

	pH	Maltose	Glucose	Fructose	Mannitol	Lactate	Acetate	Ethanol
t=0	5.7±0.1	27.0±0.1	0.4±0.1	54.2±0.3	0.0	2.8±0.9	5.0±3.8	1.5±0.1
t=24h, P=0.1 MPa	3.8±0.1	1.0±0.1	0.1±0.0	0.0	54.0±1.9	53.0±1.1	28.4±3.6	23.6±0.3
t=24h, P=100 MPa	3.9±0.1	6.2±3.7	1.6±0.7	0.0	53.9±2.9	42.2±7.1	30.1±4.6	12.8±2.8
t=24h, P=150 MPa	4.8±0.0	17.1±0.9	3.6±0.1	42.0	12.0	12.5±1.4	11.4±0.2	<1.0

Data are means ± standard deviation of two independent fermentations

Table 4. Concentrations of substrates and end products in Su-MRS4 fermented by *L. sanfranciscensis* TMW1.392 at 0.1, 100 and 150 MPa (all concentrations are in mmol l^{-1} with exception of EPS).

	pH	Sucrose	Fructose	Glucose	Mannitol	Lactate	Acetate	Ethanol	EPS (g/l)
t=0	5.9±0.1	106.0±1.0	27.6±1.2	1.5±0.4	2.0±1.4	3.4±0.3	9.7±0.8	2.1±0.3	0
t=24h, P=0.1 MPa	3.9±0.1	60.0±3.5	1.0±1.0	2.4±1.0	47.8±4.5	51.1±7.4	32.3±1.0	30.6±2.3	4.8±1.4
t=24h, P=100 MPa	4.4±0.1	79.6	2.6	10.0	37.3	26.8±3.6	30.4±2.4	0	3.2
t=24h, P=150 MPa	4.7	85.6	7.8	11.0	25.7	14.5	24.0	0	2.9

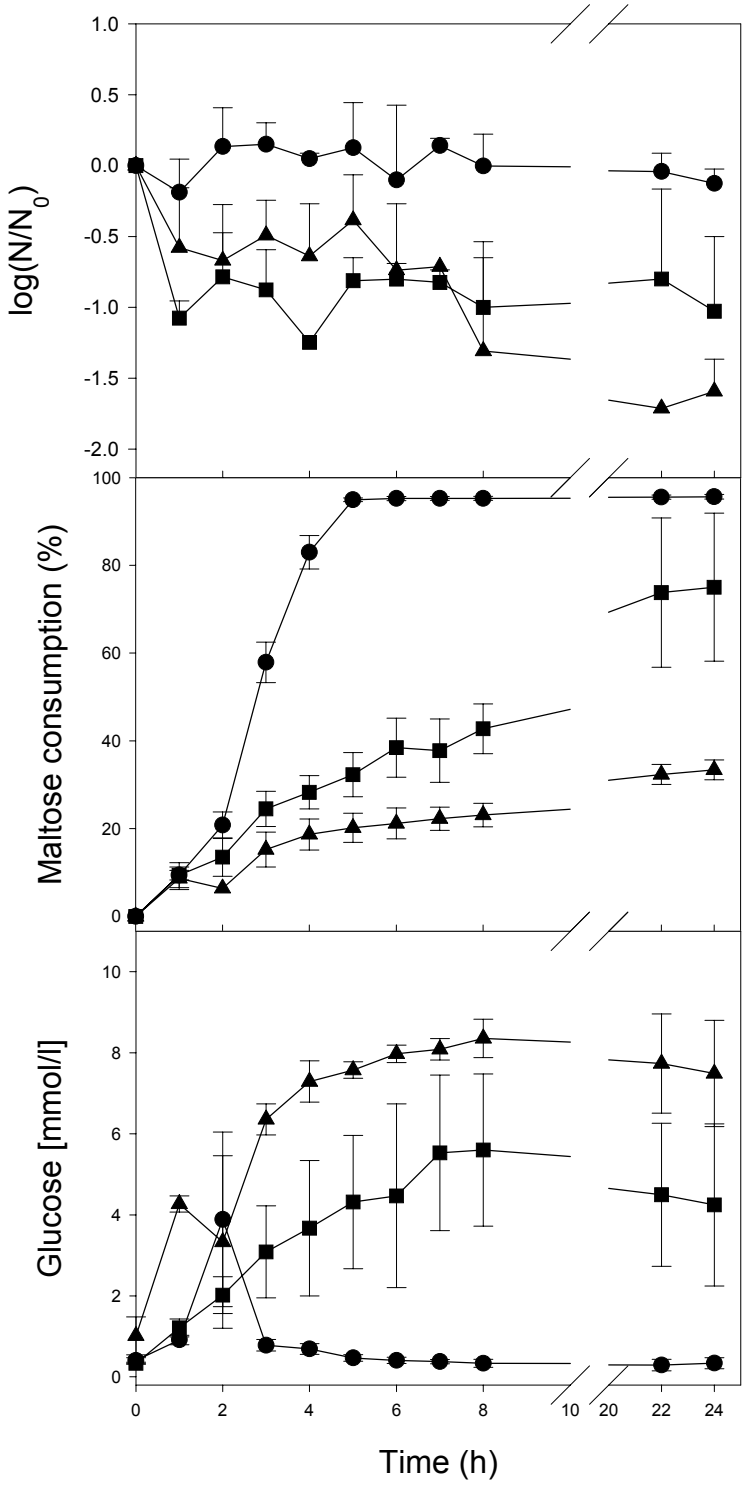

Figure 1
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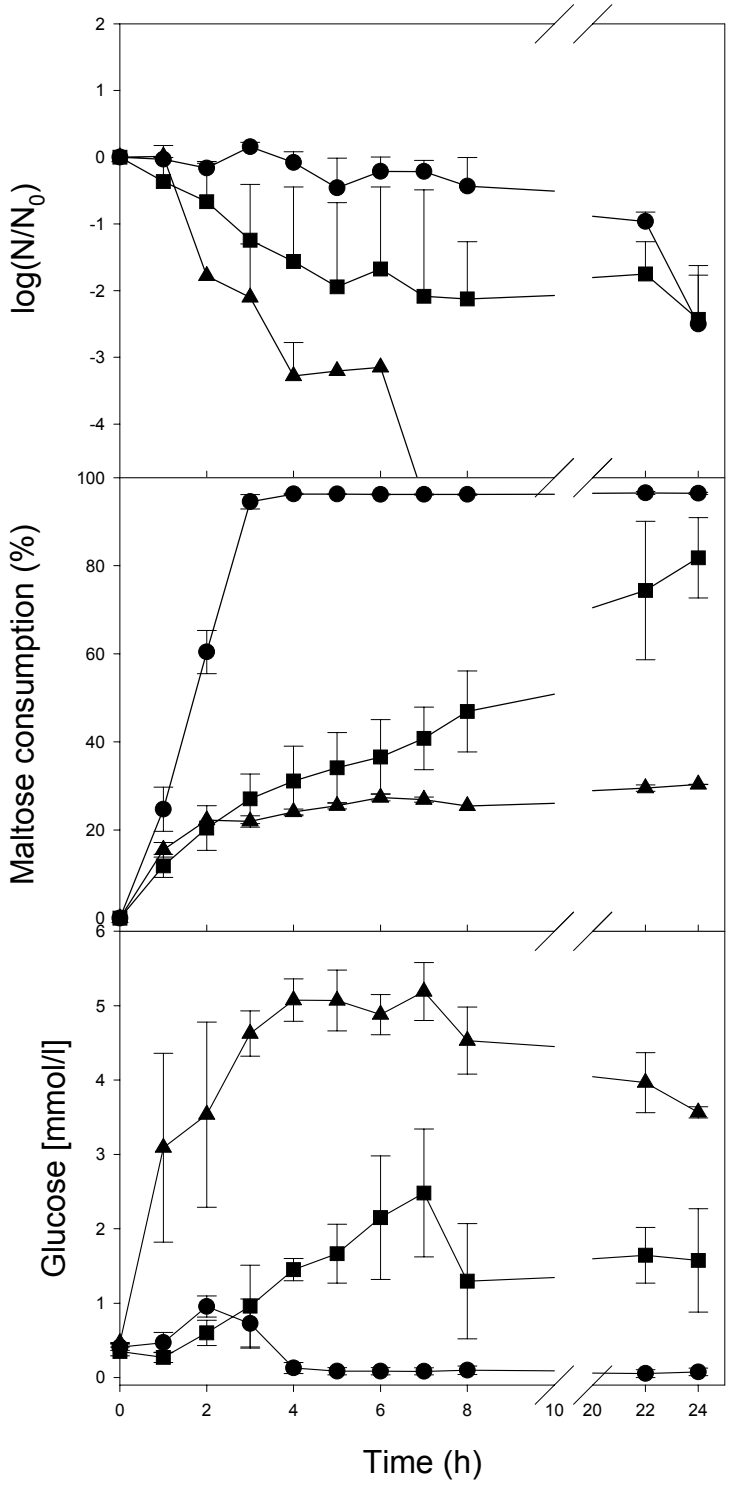

Figure 2
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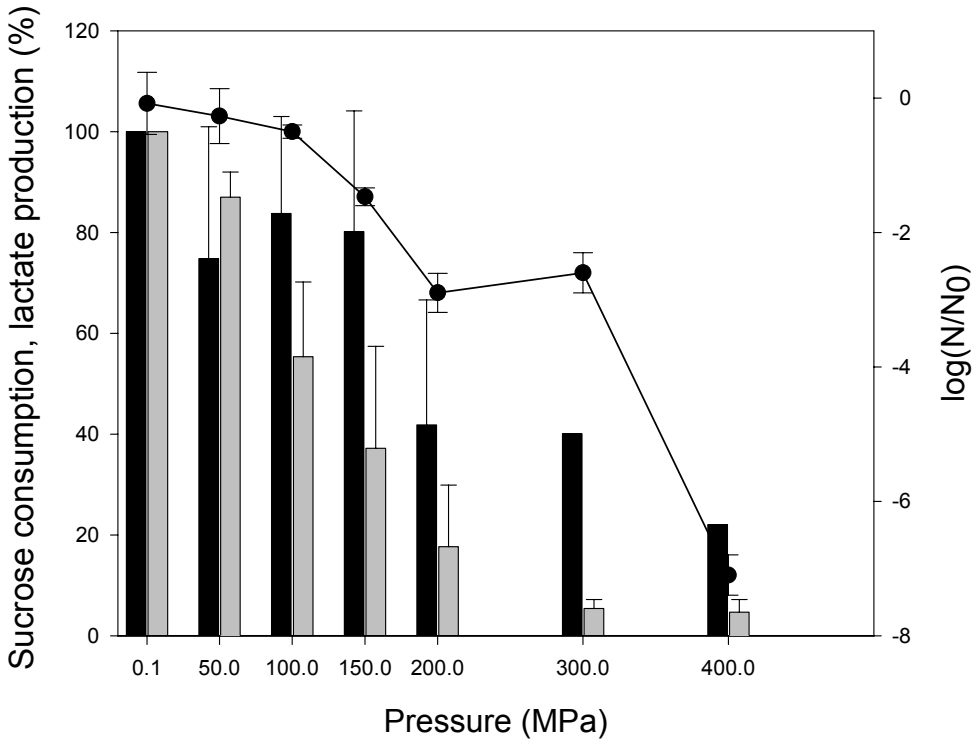

Figure 3

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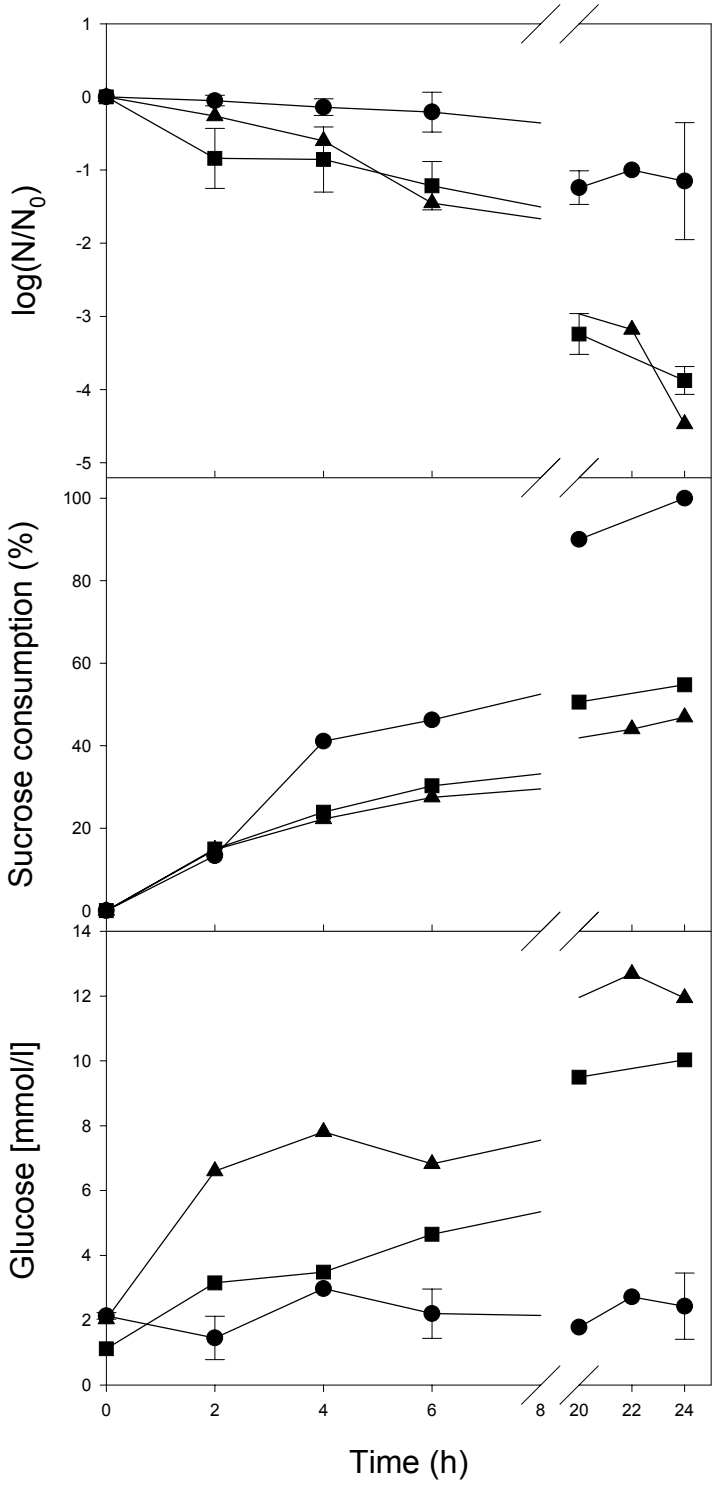

Figure 4
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