JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# Glucan and Fructan Production by Sourdough *Weissella cibaria* and *Lactobacillus plantarum*

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After a large screening on sourdough lactic acid bacteria, exopolysaccharide (EPS)-forming strains of *Weissella cibaria*, *Lactobacillus plantarum*, and *Pediococcus pentosaceus* were selected. After 6 days of incubation at 30 °C, the synthesis of EPS in MRS-based broth ranged from 5.54 to 7.88 mg $mL^{-1}$. EPS had an apparent molecular mass of ca. $10^4$ Da. As shown by carbohydrate consumption, the synthesis of EPS was found from sucrose only. Two types of homopolysaccharides were synthesized: glucans simultaneously with growth and fructans after 1 day of incubation. Two protein bands of ca. 180−200 kDa were in situ detected on SDS-PAGE gels incubated with sucrose. PCR products of ca. 220 bp were found for *L. plantarum* PL9 (100% of identity to putative priming glycosyltransferase of *L. plantarum* WCFS1) and *W. cibaria* WC4 (80% of identity to putative glycosyltransferase, epsD, of *Bacillus cereus* G9241) by using hybrid primers for the priming *gtf* genes. Degenerated primers DexreuR and DexreuV showed a unique PCR product, and the predicted amino acid sequences were identical for *W. cibaria* WC4 and *L. plantarum* PL9. The sequence had similarity with polysaccharide biosynthesis glycosyltransferases. *W. cibaria* WC4 or *L. plantarum* LP9 synthesized ca. 2.5 g $kg^{-1}$ EPS during sourdough fermentation with sucrose added. Compared to the sourdough started with an EPS-negative strain, the sourdough started with *W. cibaria* WC4 or *L. plantarum* LP9 increased the viscosity, and the resulting bread had higher specific volume and lower firmness. The synthesis of EPS by selected sourdough lactic acid bacteria could be considered as a useful tool to replace the additives for improving the textural properties of baked goods.

**KEYWORDS: Glucans; fructans; sourdough; *Weissella cibaria*; *Lactobacillus plantarum*; lactic acid bacteria**

## INTRODUCTION

Microbial exopolysaccharides (EPS) are extracellular polysaccharides associated with the cell surface in the form of capsules or secreted into the extracellular environment in the form of slime. In their natural environment, EPS protect the microbial cell against desiccation, phagocytosis, phage attack, and antibiotics and favor adhesion to solid surfaces and the formation of biofilms. Several food grade microorganisms synthesize EPS also, for example, lactic acid bacteria, propionibacteria, and bifidobacteria. EPS synthesized by lactic acid bacteria differ for chemical composition, size, structure, molecular organization, and type of genes involved in the biosynthesis. Basically, they are distinguished in (i) extracellularly synthesized homopolysaccharides, which contain only one type of monosaccharide (e.g., glucans and fructans made of glucose and fructose, respectively); and (ii) heteropolysaccharides with (ir)regularly repeating units (e.g., glucose, galactose, fructose, and rhamnose) that are synthesized from intracellular sugar nucleotide precursors (*1*). Transglycosylases or glycosyl-transferase (GTF) enzymes (EC 2.4.1.) (e.g., glucosyl-transferase/glucansucrase and fructosyl-transferase/levansucrase for glucans and fructans, respectively) are involved in the synthesis of EPS.

Currently, hydrocolloids from plant source material and their modified forms, for example, starch, carageenans, methylcellulose, hydroxypropylmethylcellulose (HPMC), and arabic gum, as well as microbial EPS are important additives in bakery

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10.1021/jf061393+ CCC: $33.50 
Published on Web 12/01/2006

products (*2*−*6*). These polysaccharides improve the rheological properties of the dough and the physical (moisture, hardness, volume, crumb color), and sensory properties and shelf life of bread and therefore are commonly used as food additives for bread production (*2*, *5*, *6*). Some examples are xanthan, acetan, and gellan, produced by Gram-negative bacteria, or dextran, produced by *Leuconostoc mesenteroides* (*7*). Xanthan induced a desiderable increase in dough resistance to extention, bread volume, and moisture (*2*, *8*). Several patents claim that fructan or FOS improve the rheological proprieties of wheat doughs and bread quality (*9*). In addition, certain EPS are thought to have beneficial effects on human health such as cholesterol-lowering ability (*10*), immunomodulating, and antitumoral activities (*11*, *12*) and probiotic effects (*6*, *13*, *14*). The synthesis of EPS by lactic acid bacteria has gained remarkable interest to replace the use of additives from plant (starch, pectin, and cellulose) and seaweed (alginate and carageenans) sources for improving the textural properties of fermented foods. Whereas the use of EPS synthesized by starter cultures, mainly heteropolysaccharides, is a common practice in the dairy industry, EPS production during sourdough fermentation has been reported only recently (*15*−*18*). Sourdough, a cocktail of lactic acid bacteria and yeasts originating from wheat or rye flour, has traditionally been used as the leavening agent in the manufacture of baked goods. Currently, sourdough fermentation has an importance in baking biotechnology because of the improved aroma, texture, shelf life, and nutritional and health properties of sourdough wheat and rye baked goods (*19*). Sourdough lactic acid bacteria seem to synthesize homopolysaccharides mainly (for a review, see ref *6*). Fructans are produced by *Lactobacillus sanfranciscensis* LTH2590 during wheat and rye sourdough fermentations (*16*), and fructans and levans are in situ synthesized by cereal and intestinal isolates of lactic acid bacteria (*Lactobacillus reuteri*, *Lactobacillus pontis*, and *Lactobacillus frumenti*) (*17*). The synthesis of glucans and fructans has also been shown in strains belonging to species sometimes isolated from sourdoughs such as *Lactobacillus reuteri* (*20*), *Leuconostoc* spp. (*21*), *Lactobacillus fermentum* (*22*), and the fructosyl-transferase and glucosyl-transferase of *L. reuteri* have been subjected to biochemical and molecular characterizations (*23*−*25*).

The use of EPS-producing sourdough starters meets the strict requirements of the modern baking biotechnology for clean labels and consumer demands for a reduced use of additives. Nevertheless, the few studies dealing with EPS produced by sourdough lactic acid bacteria (*16*−*18*) showed their in situ synthesis but did not demonstrate the effective improvement of the started baked goods. The texturizing and antistaling properties of EPS markedly depend on their molecular size, charge, monosaccharide composition, degree of branching, and types of glycosidic linkages (*26*). Furthermore, the capacity to synthesize EPS is often an unstable characteristic at the genetic level along with the instability of the EPS texture itself (*1*).

After an initial large screening on sourdough lactic acid bacteria, this paper reports (i) the synthesis of glucans and fructans by not previously reported sourdough species such *Weissella cibaria*, *Lactobacillus plantarum*, and *Pediococcus pentosaceus* strains; (ii) the partial sequencing of the GTF genes of *W. cibaria* and *L. plantarum*; and (iii) the in situ production of EPS, which was shown to improve the textural properties of wheat bread.

## MATERIALS AND METHODS

**Strains, Media, and Growth Conditions.** Two-hundred and thirty-five strains of lactic acid bacteria, isolated from Italian sourdoughs and belonging to the Culture Collection of the Dipartimento di Protezione delle Piante e Microbiologia Applicata, Università degli Studi di Bari, Italy, were screened for exopolysaccharides (EPS) formation. Previously, isolates were identified by partial sequencing of the rRNA 16S gene. The following species were used: *Lactobacillus rossiae* (30 strains), *Lactobacillus sanfranciscensis* (*66*), *Weissella cibaria* (*29*), *Lactobacillus plantarum* (*41*), *Lactobacillus pentosus* (*10*), *Lactobacillus brevis* (*2*), *Lactobacillus paralimentarius* (*5*), *Lactobacillus farciminis* (*21*), *Lactobacillus fermentum* (*12*), and *Pediococcus pentosaceus* (*19*). Strains were routinely cultivated at 30 °C for 24 h in modified MRS (de man Rogosa and Sharpe, mMRS) broth (Oxoid, Basingstoke, Hampshire, U.K.) (*27*) with the addition of fresh yeast extract (5%, v/v) and 28 mM maltose at a final pH of 5.6.

**Screening for EPS Synthesis.** Preliminarily, the synthesis of EPS was determined by growing cell colonies in agar plates. Colonies from cell suspensions of each strain precultivated in mMRS broth were allowed to grow in mMRS agar with the addition of 292 mM sucrose, 146 mM glucose, or 146 mM fructose. After incubation at 30 °C for 6 days, the synthesis of EPS was observed by visual appearance of the mucoid colonies. The screening was carried out in three separate experiments.

**Synthesis of EPS from Different Carbon Sources and Metabolism of Carbohydrates.** Twenty-four-hour-old cells of EPS-forming strains were inoculated at 4% (v/v) in a laboratory-made MRS broth (pH 5.6) (*27*) with the addition of fresh yeast extract (5%, v/v) and only one carbon source: (i) 146 mM glucose (MRS-G); (ii) 146 mM fructose (MRS-F); (iii) 292 mM with sucrose (MRS-S); (iv) 146 mM glucose and 292 mM sucrose (MRS-G-S); or (v) 146 mM glucose and 146 mM fructose (MRS-G-F). MRS without carbohydrates was used as the control. After incubation at 30 °C for 1 or 6 days, the concentration of EPS and the consumption of carbohydrates were determined according to the phenol−sulfuric method of Dubois et al. (*28*) and by enzymatic assays (Diffchamb, Lyon, France) (*29*, *30*), respectively. Quantification of EPS in culture media and during sourdough fermentation was also carried out by high-performance gel permeation chromatography (HP-GPC) analysis (see below) (*17*).

**Purification, Molecular Mass Determination, and Monosaccharide Composition of EPS.** EPS were purified according to the method described by Cerning et al. (*31*). After incubation at 30 °C for 1 and 6 days on MRS-S, cultures were heated at 100 °C for 15 min to inactivate the enzymes potentially capable of polymer degradation, and cells were removed by centrifugation (9000*g*, 10 min, 4 °C). EPS were precipitated with 3 volumes of chilled 95% (v/v) ethanol. After standing overnight at 4 °C, the resultant precipitate was collected by centrifugation (11325*g*, 20 min). The EPS were dissolved in distilled water, dialyzed against distilled water at 4 °C for 24 h, and lyophilized. The lyophilized powder was dissolved in 10% trichloroacetic acid to remove proteins. After centrifugation (11325*g*, 20 min), the supernatant was dialyzed at 4 °C against distilled water for 5 days and lyophilized. Freeze-dried EPS were dissolved in potassium phosphate buffer (50 mM, pH 7.0) and filtered through a 0.45-$\mu$m-pore-size filter (Millex-HA, Millipore S.A., Saint Quentin, France).

For molecular mass determination, purified EPS were subjected to gel filtration chromatography (LC-GPC) and determined by a refractive index detector (Gynkotek, Germering, Germany). A Superose 12 HR 10/30 (Amersham Biosciences AB, Uppsala, Sweeden) was used, applying 50 mM potassium phosphate buffer (pH 7.0) as the eluent at a flow rate of 0.6 mL $min^{-1}$. High molecular weight and low molecular weight gel filtration calibration kits (Amersham Biosciences) were used for calibration of the column.

Fractions containing EPS collected from gel filtration chromatography were dialyzed, freeze-dried, and further purified using HPLC-GPC analysis. The purification was carried out at room temperature by using a Progel TSK guard column, followed by a Progel TSK G6000 PW column, and a refractive index detector as described by Van Geel-Schutten et al. (*20*). Each fraction containing EPS was dialyzed, freeze-dried, and dissolved in 800 $\mu$L of distilled water. To hydrolyze the EPS, 15% (v/v) perchloric acid (70%) was added, and the samples were heated at 80 °C for 1 h. To precipitate perchlorate, 250 $\mu$L of 5 M KOH was added. Precipitated potassium perchlorate was removed by centrifugation (12000*g*, 5 min, 4 °C), and the supernatant was used for

**Table 1.** Nucleotide Sequences, Microbial Species, and Targeted Gene of the Primers Used for PCR Amplifications

| primer | specificity/gene target | oligonucleotide sequence (5′ → 3′) | reference/NCBI GenBank accession no. (of reference sequence) |
|---|---|---|---|
| LevV/LevR | degenerated primers targeting levansucrase [conserved sequences DVWDSWP, DEV(I,L)ER] | GAYGTITGGGAYWSITGGC<br>TCITYYTCRTCISWIRMCAT | Tieking et al. (*17*)<br>P05655, P94468, P21130, P11701, Q55242 |
| DexwobV/DexwobR | conserved sequences DAVDNV and EGFSNF in bacterial glucosyltransferase | GAYGCIGTIGAYAAYGTI<br>YTGRAARTTISWRAAICC | Tieking et al. (*18*) |
| DexreuV/DexreuR | *gtfA*-gene of *Lactobacillus reuteri* 121 | GTGAAGGTAACTATGTTG<br>ATCCGCATTAAAGAATGG | Tieking et al. (*18*); Kralj et al. (*22*) |
| Inu2F/Inu2R | *inu* gene of *Lactobacillus reuteri* 121 | CTATAATTCTACCGCGGTTTC<br>GTATAACTCTGCCACCTTAG | Van Hijum et al. (*23*) |
| FtfV/FtfR | *ftfA* gene *Lactobacillus reuteri* LTH5448 | GAATGGCTATCAACTTCTG<br>CTTCTACTTGCGGGTTC | Schawab and Gänzle (*43*) |
| 28IS/29IS | inulosucrase | GATGTKTGGGATAGYTGGGCKTTRCAAGAT<br>ATCRACAAAYTGMGCMCCMGCTTCACCATATGG | AY191311 |
| Deg1/Deg2i | degenerated primers targeting levansucrase of *Lactobacillus reuteri* 121 | AAYTATAAYGGYGTTGCNGAAGT<br>TTCAACTTCATCNSWNGCCAT | Van Hijum et al. (*34*) |
| 5FTF/6FTF | degenerated primers targeting fructosyltransferase from *Bacillus amyloliquefaciens* SacB (X52988), *Bacillus subtilis* SacB (X02730), *Streptococcus mutans* FTF (M18954), and *Streptococcus salivarius* FTF (L08445) | GAYGTNTGGGAYWSNTGGGCC<br>GTNGCNSWNCCNSWCCAYTSYTG | Van Hijum et al. (*23*) |
| GTF1/GTF1R | glycosyltransferase | TCATTTTCGTAAAACCTCAATTGAYGARYTNCC<br>AATATTATTACGACCTSWNAYYTGCCA | Provencher et al. (*56*) |

the analysis of monosaccharides. The monosaccharide composition was analyzed by HPLC using a Spherisorb 5 $\mu$L $NH_2$ column (Waters Co., Milford, MA) and a refractive index detector (Gynkotek, Germering, Germany). The mobile phase consisted of acetonitrile containing distilled water (10%) and HCl (0.01%), and samples were eluted at a flow rate of 1.0 mL $min^{-1}$. For peak identification, an external standard containing arabinose, fructose, glucose, xylose, and rhamnose was used. Determination of the monosaccharide composition was done in three independent experiments (*1*, *17*).

**Activity Staining of EPS-Synthesizing Enzymes.** In situ detection of EPS-synthesizing enzymes was carried out by staining with Schiff's reagent (*32*) of a sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE). Whole cells of EPS-forming strains grown in MRS-S were resuspended in sterile potassium phosphate buffer (50 mM, pH 7.0), mixed with an equal volume of sample buffer, and incubated at 37 °C for 2 h to denature the enzymes reversibly. After incubation, samples were centrifuged (10000*g*; 1 min) to remove cell debris and loaded (25 $\mu$L) into stacking-gel sample wells. SDS-PAGE was performed with a vertical slab gel unit (Hoefer Pharmacia Biotech Inc., San Francisco, CA) according to the method of Laemmli (*33*). Electrophoresis was performed in a 12.5% resolving gel at 12 °C and at a current of 30 mA/gel. Following SDS-PAGE, the gel was washed three times in 20 mM sodium acetate buffer (pH 5.4) containing 0.3 mM $CaCl_2$ and 0.1% (v/v) Tween 80, at 4 °C for a total time of 30 min. The gel was then incubated for 72 h at 30 °C in sodium acetate buffer supplemented with 10% of sucrose or glucose to allow the in situ polymer formation. After incubation, the gel was washed once with a solution of methanol/acetic acid (50:10) for 30 min and then with water for 30 min and incubated at room temperature in a periodic acid solution (1% periodic acid and 3% acetic acid) for 45 min. After the periodic acid treatment, the gel was washed with water for 2 h. The gel was then stained with 7 mL of Schiff reagent (1% pararosanilina−HCl w/v, 4% sodium bisulphite, and 0.25 M HCl) for 8 min and finally washed with 0.5% sodium bisulphite for 30 min. EPS-synthesizing enzymes appeared as discrete magenta bands within the gel matrix (*34*).

**DNA Isolation, PCR Conditions, and Sequencing of Amplification Products.** Total DNAs were obtained according to the method of De Los Reyes-Gavilàn et al. (*35*) from 2 mL samples of overnight cultures grown in mMRS at 30 °C. The final concentration of lysozyme used for cell lysis was 2 mg $mL^{-1}$ (w/v). The concentration and purity of DNA were assessed by determining the optical densities at 260 and 280 nm, as described by Sambrook et al. (*36*). The set of specific primers used to amplify EPS-synthesizing enzymes genes is reported in **Table 1**. Fifty microliters of each PCR mixture contained 200 $\mu$M of each 2′-deoxynucleotide 5′-triphosphate (dNTPs), 1 $\mu$M of both primers, 2 $\mu$M $MgCl_2$, 2 U of *Taq* DNA polymerase (Invitrogen Life Technologies, Carlsbad, CA), in the supplied buffer, and ca. 50 ng of DNA. Polymerase Chain Reaction (PCR) amplification was performed using the GeneAmp PCR System 9700 thermal cycler (Applied Biosystems). Amplification conditions were changed according to the primer used. PCR products were separated by electrophoresis on 1.5% (wt $vol^{-1}$) agarose gel (Gibco BRL) and stained with ethidium bromide (0.5 $\mu$g $mL^{-1}$).

The amplicons obtained were eluted from gel and purified by the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). DNA sequencing reactions were performed by PRIMM srl (San Raffaele Biomedical Science Park, Milan, Italy). Sequence comparison was performed by using the Basic BLAST database. Translations of nucleotide sequences analyses were performed by using OMIGA software (Oxford Molecular, Madison, WI) or the ExPASy translation routine at the ExPASy Moleculary Biology Server of the Swiss Institute of Bioinformatics (http://ca.expasy.org/). Similar studies with the advanced BLAST algoritm str available at the National Center for Biotechnology Information site (htpp://www.ncbi.nlm.nih.gov/). Sequences alignments were conducted with the ClustalW algorithm at the ClustalW server at the European Bioinformatics Institute (http://www.ebi.ac.uk/clustalw/index.html).

**Preparation of Doughs.** The wheat flour (*Triticum durum* var. Svevo) used had the following characteristics: moisture, 12.8%; protein (N × 5.70), 10.7% of dry matter (dm); fat, 1.8% of dm; and ash, 0.6% of dm. *W. cibaria* WC4, *L. plantarum* PL9, and one EPS-negative strain (*Lactobacillus sanfranciscensis* SF17) were used for dough fermentations. Twenty-four-hour-old cells of each strain cultivated in MRS-S were harvested by centrifugation (9000*g*, 10 min, 4 °C), washed twice with 50 mM sterile potassium phosphate buffer (pH 7.0), and resuspended in sterile distilled water to a final optical density at 620 nm ($OD_{620}$) of 2.5 [$10^9$ colony-forming units (CFU) $mL^{-1}$]. Three-hundred and twelve grams of wheat flour, 137.5 mL of sterilized distilled water, 50 g of sucrose, and 50 mL of a cellular suspension containing $10^9$ CFU of WC4 or PL9 or SF17 strains per milliliter (final cell number of ca. $5 \times 10^8$ CFU $g^{-1}$ of dough) were used to produce 500 g of dough (dough yield, dough weight × 100/flour weight, 160) with a continuous high-speed mixer (60*g*; dough mixing time, 5 min) (Chopin & Co., Boulogne, Seine, France). A control dough without microbial inoculum was prepared also. It was chemically acidified to pH 3.9 with a mixture of lactic and acetic acids at a molar ratio 4:1,

**Table 2.** EPS Production[a] by Sourdough Strains after Growth in MRS-S and MRS-G-S at 30 °C for 1 and 6 Days

| | EPS (mg/mL) | | | |
|---|---|---|---|---|
| | 1day | | 6 days | |
| EPS-forming strain | MRS-S[b] | MRS-G-S[c] | MRS-S | MRS-G-S |
| *W. cibaria* WC4 | 3.88 ± 0.07 | 6.05 ± 0.20 | 7.88 ± 0.13 | 8.50 ± 0.09 |
| *W. cibaria* WC3 | 2.65 ± 0.16 | 4.95 ± 0.13 | 6.67 ± 0.23 | 6.75 ± 0.20 |
| *W. cibaria* WC9 | 1.53 ± 0.21 | 3.05 ± 0.21 | 5.54 ± 0.15 | 5.65 ± 0.14 |
| *L. plantarum* PL9 | 3.14 ± 0.20 | 5.95 ± 0.18 | 7.39 ± 0.20 | 7.55 ± 0.22 |
| *P. pentosaceus* PP1 | 2.65 ± 0.10 | 4.87 ± 0.22 | 6.85 ± 0.10 | 6.95 ± 0.25 |

[a] Data are means ± standard deviations of three independent experiments. [b] 292 mM sucrose as sole carbon source (MRS-S). [c] 146 mM glucose and 292 mM sucrose as sole carbon sources (MRS-G-S).

which corresponds to that usually found after sourdough fermentation (*37*). Another two doughs were produced under the same conditions, except for the addition of lyophilized EPS (5 g $kg^{-1}$ of dough) from *W. cibaria* WC4 or xanthan gum (2.5 g $kg^{-1}$ of dough, E415, Chimab S.p.a., Milan, Italy). All of the doughs were incubated at 30 °C for 24 h.

**Determination of Cell Numbers and pH in Sourdoughs.** Cell numbers were determined on mMRS agar, PCA agar (Oxoid), and malt agar (Oxoid) for lactic acid bacteria, total mesophilic aerobic bacteria, and yeasts and molds, respectively. The values of pH were determined by a Foodtrode electrode (Hamilton, Bonaduz, Switzerland).

**EPS Isolation and Determination from Doughs.** The isolation of water-soluble polysaccharides originating from the flour and microbial EPS during sourdough fermentation was carried out as described by Tieking et al. (*17*) with a few modifications. Nine parts of distilled water was added to one part of dough (w/w). Solids were removed by centrifugation (8000*g*, 10 min), and the resulting supernatant was precipitated with ethanol, dialyzed against distilled water ($M_r$ cutoff, ca. $10^4$), and lyophilized. The concentration of EPS was determined according to the method of Dubois et al. (*28*) and by HP-GPC analysis (*17*, *26*).

**Rheological Measurement of Doughs and Breads.** Rheological measurement of doughs was carried out by using a Viscotester VT 500 (Haake, U.K.) kept at a constant temperature of 30 °C. The readings of the apparent viscosity ($\eta$) were taken at rotational speeds from 2 to 50 rpm, to obtain increasing shear rates ($D$), and then drawing a curve that described the behavior of the sample. An empirical power-law equation was used to calculate the apparent viscosity and the consistency coefficient index ($k$) (*38*)

$$\eta = k\gamma^{(n-1)}$$

where $n$ is the flow behavior index, which is <1 for pseudoplastic behavior.

Three types of sourdough breads were manufactured by using EPS-forming or -negative (*L. sanfranciscensis* SF17) strains. Sourdoughs were fermented at 30 °C for 24 h according to the protocol described above. After sourdough fermentation, 250 g of wheat flour, 150 mL of distilled water, 10 g of baker's yeast, and 100 g of sourdough were mixed. Doughs were fermented at 30 °C for 2 h and baked at 220 °C for 50 min. The determinations of bread mass, specific volume, and firmness were carried out according to AACC 10-10 and AACC 74-09 official methods.

**Statistical Analysis.** Experimental data were subjected to analysis of variance (ANOVA), and pair comparison of treatment means was achieved using Tukey's procedure at $P < 0.05$ using the statistical software Statistica for Windows (Statistica 6.0 for Windows 1998).

## RESULTS

**Screening for EPS Synthesis.** Fourteen of the 235 sourdough strains had the capacity to synthesize EPS during growth at 30 °C for 6 days in mMRS agar containing 292 mM sucrose. Seven strains (WC1, WC2, WC3, WC4, WC9, WC12, and WC15) of *W. cibaria*, 4 (PL9, PL12, PL15, and PL16) of *L. plantarum*, and 3 (PP1, PP5, and PP8) of *P. pentosaceus* were selected. No synthesis of EPS was found when 146 mM glucose or fructose was added to mMRS agar medium.

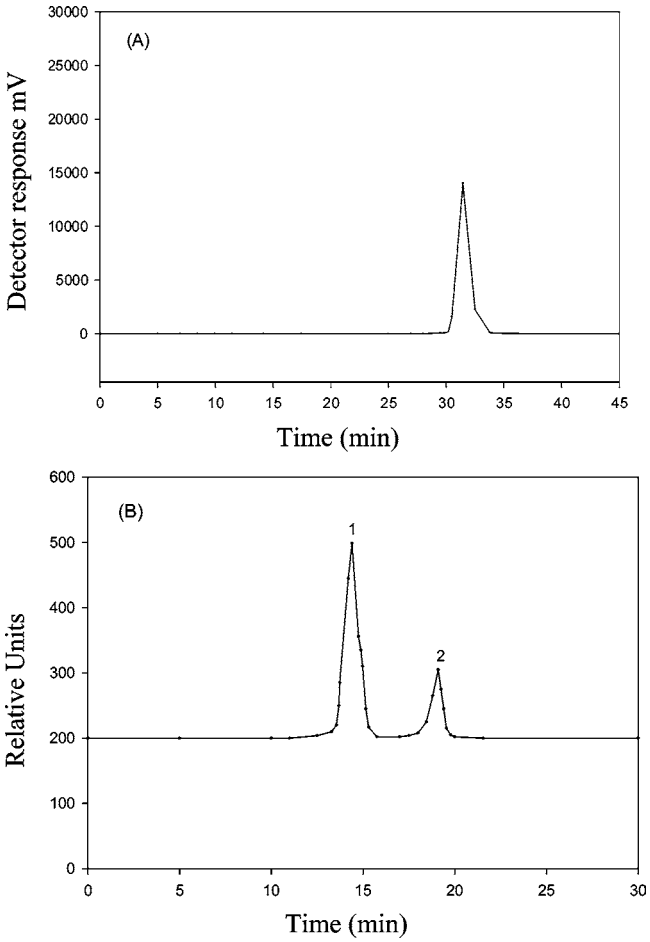


**Figure 1.** Representative gel filtration chromatography (**A**) and high-performance gel permeation chromatography (HP-GPC) (**B**) profiles of EPS isolated and purified after growth of *W. cibaria* WC4 at 30 °C for 6 days on MRS with 292 mM sucrose as sole carbon source (MRS-S).

**Synthesis of EPS from Different Carbon Sources, Molecular Mass Determination, and Carbohydrate Composition.** Selected strains were cultivated in a laboratory-made MRS broth containing different carbon sources. The best EPS-forming strains cultivated in MRS-S showed a production that ranged from 1.53 to 3.88 mg $mL^{-1}$ and from 5.54 to 7.88 mg $mL^{-1}$ after 1 and 6 days, respectively (**Table 2**). *W. cibaria* WC4 synthesized the highest amount of EPS. After 6 days, the other nine selected strains not included in **Table 2** gave concentrations of EPS lower than 3 mg $mL^{-1}$ and were excluded from further characterization. The synthesis of EPS was also found during growth in MRS-G-S. After 1 day, it was about twice (3.05−6.05 mg $mL^{-1}$) that found in MRS-S. According to the preliminary screening on agar plate, no EPS synthesis was found after growth in MRS-G, MRS-F, or MRS-G-F.

EPS synthesized by selected strains in MRS-S were purified and subjected to LC-GPC (**Figure 1A**). The EPS synthesized by *W. cibaria* WC3, WC4, and WC9, *L. plantarum* PL9, and *P. pentosaceus* PP1 had similar retention times (31−32 min), showing an apparent molecular mass of ca. (1.1−1.3) × $10^4$ Da. Fractions containing EPS collected from LC-GPC were further purified using HPLC-GPC analysis (**Figure 1B**). Two peaks (1 and 2) were separated for all of the sourdough strains

**Table 3.** Consumption[a] of Carbohydrates (Millimolar) and Residual Sucrose (Millimolar) by *W. cibaria* WC4 after Growth in MRS Broth with Different Carbon Sources at 30 °C for 1 and 6 Days

| | 1 day | | | 6 days | | |
|---|---|---|---|---|---|---|
| carbon source | glucose | fructose | residual sucrose | glucose | fructose | residual sucrose |
| MRS-G[b] | 63.9 ± 3.0 | nd[g] | nd | 74.7 ± 1.5 | nd | nd |
| MRS-F[c] | nd | 56.7 ± 1.0 | nd | nd | 79.7 ± 1.0 | nd |
| MRS-S[d] | 166.7 ± 2.0 | 0 | 108.0 ± 1.3 | 193.5 ± 4.0 | 122.7 ± 2.5 | 58.5 ± 1.0 |
| MRS-G-S[e] | 227.0 ± 4.5 | 9.7 ± 2.5 | 89.1 ± 0.9 | 265.3 ± 3.5 | 71.8 ± 3.0 | 59.4 ± 0.7 |
| MRS-G-F[f] | 61.6 ± 3.0 | 0 | nd | 72.1 ± 3.0 | 10.2 ± 0.8 | nd |

[a] Data are means ± standard deviations of three independent experiments. [b] 146 mM glucose (MRS-G). [c] 146 mM fructose (MRS-F). [d] 292 mM with sucrose (MRS-S). [e] 146 mM glucose and 292 mM sucrose (MRS-G-S). [f] 146 mM glucose and 146 mM fructose (MRS-G-F). [g] Not determined.

which eluted in the range of 15−20 min. Fractions containing EPS synthesized after 6 days of growth in MRS-S were collected from HP-GPC analysis, freeze-dried, and subjected to acid hydrolysis. Glucose and fructose were the hydrolysis end-products for peaks 1 and 2 (**Figure 1B**), respectively. HPLC-GPC analysis of EPS synthesized after 1 day of growth in MRS-S showed only one peak with the same retention time as peak 1 found after 6 days of incubation. Glucose was the only hydrolysis end-product (data not shown).

**Metabolism of Carbohydrates. Table 3** shows the consumption of carbohydrates by *W. cibaria* WC4 during growth in MRS broth containing different carbon sources. After 1 day, the cell number was in the range of $(5-7) \times 10^9$ CFU $mL^{-1}$ for all of the conditions assayed and did not increase during prolonged incubation. The consumption of glucose was ca. 63 mM either in MRS-G or in MRS-G-F. *W. cibaria* WC4 used preferentially glucose with respect to fructose for growth. Cultivation in MRS-F showed a consumption of ca. 57 mM fructose. During growth in MRS-S, 3.88 mg $mL^{-1}$ of EPS was synthesized (**Table 2**) and residual sucrose amounted to ca. 108 mM (**Table 3**). Only the glucose moiety of the hydrolyzed sucrose (ca. 184 mM) was almost totally consumed (ca. 167 mM), whereas fructose was used neither for growth nor for EPS synthesis. During growth in MRS-G-S, the concentration of EPS increased to 6.05 mg $mL^{-1}$ (**Table 2**). Compared to growth in MRS-S, an increase (ca. 60 mM) of the consumption of glucose (total consumption of ca. 227 mM) was found, which approximately corresponded to the amount consumed for growth alone. The residual sucrose slightly decreased (ca. 89 vs 108 mM), and only ca. 10 mM fructose was consumed (**Table 3**). As a consequence of the above results, it was confirmed that EPS synthesized after 1 day of growth were made of glucose alone. During prolonged incubation (6 days), the carbohydrate consumption in MRS-G, MRS-F, and MRS-G-F increased only slightly. On the contrary, in both MRS-S and MRS-G-S the concentration of EPS increased to 7.88 and 8.50 mg $mL^{-1}$, respectively (**Table 2**). This was in agreement with an increased consumption of glucose (ca. 193 and 265 mM, respectively) and, especially, fructose (ca. 122 and 71 mM, respectively), which reflected the decreased concentration of residual sucrose (ca. 58.5 mM) (**Table 3**). Because the growth stopped after 1 day of incubation, fructose was used for the synthesis of another homopolysaccharide during prolonged incubation. *W. cibaria* WC3 and WC9, *L. plantarum* PL9, and *P. pentosaceus* PP1 showed almost similar kinetics of carbohydrate consumption (data not shown).

**Activity Staining of EPS-Synthesizing Enzymes.** EPS-synthesizing enzymes of *W. cibaria* WC4 were detected in situ on SDS-PAGE gels incubated with sucrose and stained with Schiff's reagent (**Figure 2**). Two protein bands of ca. 180−200 kDa were found. No protein bands were detected after incubation with glucose (data not shown). *W. cibaria* WC3 and WC9, *L. plantarum* PL9, and *P. pentosaceus* PP1 also exhibited two similar protein bands (data not shown).

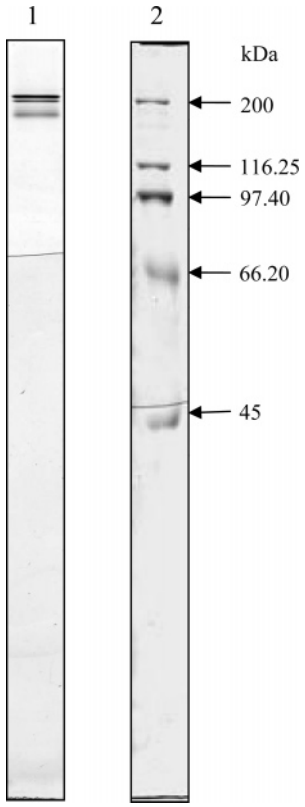


**Figure 2.** In situ detection of EPS-synthesizing enzymes by *W. cibaria* WC4 as detected in SDS-PAGE gels stained with Schiff's reagent (see Materials and Methods): lane 1, whole cells; lane 2, high molecular weight standards.

**Molecular Characterization of EPS Genes.** *W. cibaria* WC4 and *L. plantarum* PL9, the two best EPS-forming strains, were screened for *gtf* and *ftf* genes. Hybrid primers for the priming *gtf* genes gave a PCR product of ca. 220 bp for both strains. The predicted amino acid sequences showed for *L. plantarum* PL9 100% of identity to putative priming glycosyltransferase (GTF) of *L. plantarum* WCFS1 (A.N. NP_784894.1) and for *W. cibaria* WC4 80% of identity to putative GTF (epsD) of *B. cereus* G9241 (A.N. ZP_00236343.1). Alignment of the internal *gtf* fragment of *W. cibaria* WC4 and *L. plantarum* PL9 with the four most similar sequences from putative priming GTF is shown in **Figure 3**. Degenerated primers DexreuR and DexreuV showed a unique PCR product, and the predicted amino acid sequences were identical for *L. plantarum* and *W. cibaria*. The sequence showed similarity with polysaccharide biosynthesis protein GTF family 2 of (i) *L. plantarum* WCFS1 (A.N. NP_786160.1) (100% of identity), (ii) *B. licheniformis* ATCC

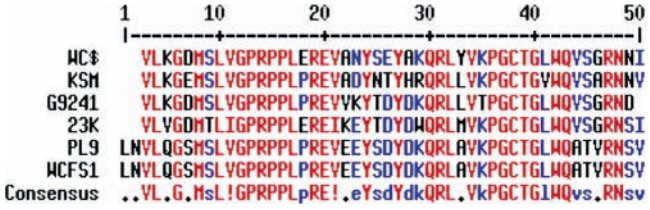


**Figure 3.** Alignment of the internal *gtf* fragment of *W. cibaria* WC4 and *L. plantarum* PL9 with the four most similar sequences from putative priming glycosyltransferase of *L. plantarum* WCFS1 (A.N. NP_784894.1), putative glycosyltransferase of *B. cereus* G9241 (A.N. ZP_00236343.1), sugar transferase of *B. clausii* KSM-K16 (A.N. YP_177191.1), and putative priming glycosyltransferase of *L. sakei* 23K (A.N. CAI55817.1). Regions with strong homologies between the sequences are red.

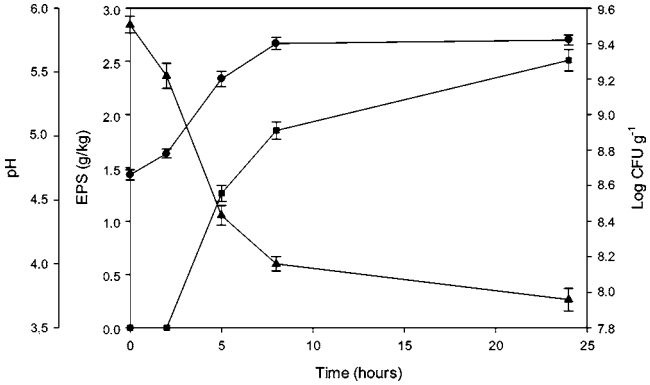


**Figure 4.** pH kinetics (▼), EPS production (g kg$^{-1}$) (■), and cell number (CFU g$^{-1}$) (●) of sourdough started with *W. cibaria* WC4 at 30 °C for 24 h.

14580 (A.N. AAU25137.1) (35% of identity), (iii) *L. reuteri* (A.N. ABD13910.1) (31% of identity), and (iv) *Lactobacillus acidophilus* NCFM (A.N. YP_193055.1) (31% of identity). PCR products without any identity to *gtf* were found by using other primers constructed targeting regions of known bacterial glucosyl-transferase and fructosyl-transferases (**Table 1**).

**In Situ EPS Production and Rheological Characterization of Doughs and Breads.** Sourdoughs were started at 30 °C for 24 h with *W. cibaria* WC4, *L. plantarum* PL9, or *L. sanfranciscensis* SF17 (EPS-negative strain). After 24 h of fermentation, *W. cibaria* WC4 reached the cell number of ca. $4.0 \times 10^9$ CFU g$^{-1}$ and caused an acidification to pH 3.8 (**Figure 4**). Almost the same values of pH and cell number were found in the sourdough started with the EPS-negative strain. Cell numbers of mesophilic aerobic bacteria in control dough without microbial inoculum were $<5 \times 10^3$ CFU g$^{-1}$ throughout fermentation, excluding an effect of other microorganisms on the EPS formation. No contamination by yeasts and molds was detected. In situ production of EPS by *W. cibaria* WC4 started after ca. 7 h of fermentation and reached the final concentration of 2.5 g kg$^{-1}$. The EPS synthesized were made of glucose alone. Similar results were obtained for the sourdough started with *L. plantarum* PL9 (data not shown). When compared to control acidified dough, no EPS were determined in the sourdough started with *L. sanfranciscensis* SF17, and this dough was taken as negative control for further analyses. After fermentation, the sourdoughs were subjected to viscosity measurement and compared to the dough with added 5 g kg$^{-1}$ of lyophilized EPS synthesized by *W. cibaria* WC4 or 2.5 g kg$^{-1}$ of xanthan gum (**Figure 5**). All of the doughs showed a pseudo-plastic behavior. The consistency index ($k$) was 80.3 mPa s$^{-1}$ for the sourdough

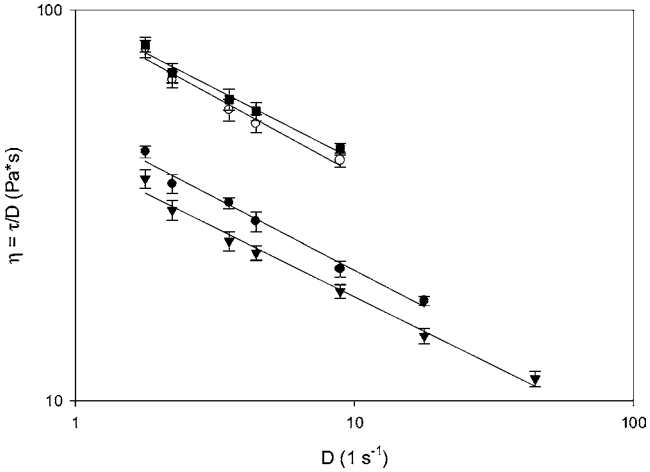


**Figure 5.** Apparent viscosity of sourdoughs started at 30 °C for 24 h in relation to shear rate (log–log). Sourdoughs were started with *W. cibaria* WC4 (●) or *L. sanfranciscensis* SF17 (EPS-negative strain) (▼). Aseptic dough was amended with lyophilized EPS (5 g kg$^{-1}$ of dough) produced by *W. cibaria* WC4 (○) or xanthan gum (2.5 g kg$^{-1}$ of dough) (■).

started with *W. cibaria* WC4 and 55.8 mPa s$^{-1}$ for the sourdough fermented with *L. sanfranciscensis* SF17. The values of $k$ for the doughs with added lyophilized EPS or xanthan gum were 123.5 and 132.1 mPa s$^{-1}$, respectively. Results similar to those for *W. cibaria* WC4 were obtained for the sourdough started with *L. plantarum* PL9 (data not shown). After fermentation, the sourdoughs started with *W. cibaria* WC4, *L. plantarum* PL9, and *L. sanfranciscensis* SF17 were used as starters (20% of the total dough weight) for breadmaking. Breads were characterized in terms of specific volume and firmness (**Table 4**). The breads containing sourdough fermented for 24 h with the EPS-forming strains showed higher volume (ca. 1015 mL) when compared to the EPS-negative strain (854 mL). According to EPS concentration in the doughs, no significant increase of the bread volume was obtained after 6 or 8 h of fermentation. An improvement in bread volume was obtained also using 0.5% of lyophilized EPS produced by *W. cibaria* WC4 or 0.25% of xanthan gum. The presence of EPS-forming strains after 24 h or the addition of lyophilized EPS or xanthan also augmented significantly ($P < 0.05\%$) the mass of bread. However, the bread containing EPS from lactobacilli or xanthan showed an increase in specific volume in the range from 116.7 to 129.2% compared to the negative control. The crumb firmness analysis displayed the opposite results according to the substances tested. EPS from lactobacilli reduce the crumb firmness, giving a softer crumb than the bread obtained with sourdough started with the EPS-negative strain. Conversely, the addition of xanthan increased the firmness of the bread crumb. The results obtained with xanthan supplementation agree with previous findings of Guarda et al. (*2*) and Rosell et al. (*39*), who observed the increase in volume and firmness of the bread.

## DISCUSSION

Fourteen of the 235 sourdough strains (ca. 6%) subjected to the preliminary screening were found to produce EPS from sucrose. A larger proportion of EPS-forming strains was found in another study dealing with cereal and intestinal isolates (*17*), and other studies reported the production of EPS by *L. sanfranciscensis* strains (*16*, *18*), which was found to be EPS-negative under our conditions. Selected sourdough *W. cibaria*, *L. plantarum*, and *P. pentosaceus* strains seemed to synthesize two types of homopolysaccharides, glucans and fructans, from

**Table 4.** Effect of the Sourdough Produced by EPS-Forming Strains on Volume, Mass, and Firmness of Wheat Bread[a]

| | volume (mL) | | | bread mass (g) | | | specific volume index (%) | | | firmness (N) | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| EPS-forming strain | 6 h[b] | 8 h[b] | 24 h[b] | 6 h[b] | 8 h[b] | 24 h[b] | 6 h[b] | 8 h[b] | 24 h[b] | 6 h[b] | 8 h[b] | 24 h[b] |
| *W. cibaria* WC4 | 850a | 852a | 1025b | 460.1a | 460.9a | 465.7b | 100.0a | 100.0a | 118.9b | 27.9b | 26.7b | 11.9a |
| *L. plantarum* PL9 | 851a | 850a | 1005b | 460.3a | 460.2a | 464.8b | 100.0a | 100.0a | 116.7b | 28.6b | 27.4b | 12.1a |
| EPS-WC4[c] | 1120c | 1108c | 1119c | 468.2b | 467.5b | 467.8b | 129.3c | 128.1c | 129.2c | 10.3a | 10.2a | 10.3a |
| xanthan gum[d] | 1020b | 1036b | 1024b | 466.8b | 466.3b | 466.4b | 117.8b | 120.0b | 118.6b | 35.9c | 34.3c | 35.0c |
| *L. sanfranciscensis* SF17[e] | 852a | 850a | 854a | 460.0a | 460.1a | 460.0a | 100.0a | 100.0a | 100.0a | 27.6b | 27.2b | 27.2b |

[a] Details about bread production are described under Materials and Methods. Data are the mean of three independent fermentations, and values in the same column with different letters differ significantly ($P < 0.05$). [b] Sourdough fermentation time. [c] Chemically acidified dough with added lyophilized EPS (5 g kg$^{-1}$ of dough) produced by *W. cibaria* WC4. [d] Chemically acidified dough with added lyophilized xanthan gum (2.5 g kg$^{-1}$). [e] Sourdough started with *L. sanfranciscensis* (EPS-negative strain).

sucrose only. The synthesis of EPS from sucrose only was also reported for other lactic acid bacteria isolated from Thai fermented foods (*40*). The production of EPS by *L. plantarum* was reported previously (*41*) but only for heteropolysaccharides, which contained glucose, galactose, and *N*-acetylgalactosamine (*41*). Overall, the concomitant synthesis of glucans and fructans was described for *L. reuteri*, *L. pontis*, and *L. frumenti* (*17*, *20*). Outside the genus *Lactobacillus*, glucans and fructans have been found in *Leuconostoc* spp. (*42*). As shown by the metabolism of carbohydrates during growth in MRS-S, the synthesis of fructans by *W. cibaria*, *L. plantarum*, and *P. pentosaceus* seemed to be delayed (after 1 day of incubation) with respect to glucans, which are produced almost simultaneously with growth. These results may suggest a major specificity of GTF enzymes for glucose rather than for fructose. Nevertheless, the synthesis of fructans during prolonged incubation has also been found in *L. reuteri*, probably as a response to stress conditions (*43*). The apparent molecular masses of glucans and fructans synthesized by sourdough lactic acid bacteria were ca. $10^4$ Da, which corresponds to the lowest limit of the homopolysaccharides characterized from lactic acid bacteria (*17*, *20*). The TCA treatment before gel filtration chromatography might cause a partial hydrolysis of the polymer, thus slightly affecting the estimation of the apparent molecular mass (data not shown). The molecular mass is considered to be one of the factors determining the functional properties of EPS (*1*, *44*, *45*).

Gel staining of the whole cell incubated with sucrose as substrate revealed the presence of two GTF enzymes of ca. 180−200 kDa. GTFs are key carbohydrate-interacting enzymes involved in the synthesis of complex carbohydrate structures which have high region stereospecificities and the potential availability for many different glycosidic linkages. One of the major limitations is the low number of enzymes that have been characterized (*46*). Gel staining of sucrose culture supernatants incubated with sucrose revealed the presence of a single glucansucrase of 146 kDa in *L. reuteri* LB 121 (*20*) and, generally, glucansucrases are large, extracellular proteins with average molecular masses of 160 kDa (*47*). Overall, glucansucrases are mostly produced by lactic acid bacteria belonging to the genera *Leuconostoc*, *Streptococcus*, and *Lactobacillus* (*15*, *26*). Levansucrase was produced in cultures of *Leuconostoc mesenteroides* NRRL B-512F in addition to dextransucrase. Levansucrase from *L. sanfranciscensis* LTH2592 was responsible for 100% of the reducing sugars produced when grown in the presence of sucrose (*18*).

The genetics of EPS have been elucidated in streptococci, lactococci, and lactobacilli. The chromosomally located *eps* gene cluster of *Streptococcus thermophilus* Sfi6 contains at least 14.52 kb of DNA encoding 13 genes from *eps*A to *eps*M (*48*). In *Lactococcus lactis* subsp. *cremoris* NIZO B40 the 14 *eps* genes (12 kb region) are located on the 40 kb pNZ4000 plasmid (*49*). *Eps* clusters containing *wzd*, *rmlA*, *welE*, *wzr*, and *wzb* genes and *epsE* to *epsO* genes have been identified in *Lactobacillus rhamnosus* and *Lactobacillus helveticus*, respectively (*46*, *50*). According to homology searches, the genes seem to be organized in four functional regions: the first contains regulatory genes; the second codes for proteins proposed to be involved in determining polymer chain length; the third contains genes showing homology with GTFs specifically required for the biosynthesis of the EPS-repeating unit; and the fourth encompasses genes involved in transport and polymerization (*1*, *48*, *49*). *Eps* genes involved in (i) the first step of the biosynthesis of EPS-repeating unit (priming GTF) and (ii) the transfer of sugar from UDP-glucose to growing EPS chain (GTF family 2) were detected in *L. plantarum* PL9 and *W. cibaria* WC4. Overall, the GTF involved in linking the first sugar of the repeating unit to the lipid carrier, referred to as the priming GTF, plays a key role in EPS biosynthesis and shows high homology among Gram-positive bacteria. The function of priming GTF was elucidated by disrupting the *epsD* gene in *Lc. lactis* subsp. *cremoris* SMQ-461 (*51*) and NIZO B40 (*52*), thus generating non-EPS-producing reversible mutants. In the present study, no *gtf* genes were detected by using the primers targeting regions of known bacterial glucosyl-transferase and fructosyl-transferases (*18*, *53*). Overall, most of the GTF genes needed for the synthesis of repeating units are often unique or have little similarity to each other (*49*, *54*). Tieking et al. (*18*) showed that no *gtf*-PCR product was found with the chromosomal DNA of the glucan-positive *L. reuteri* strains, neither with dexwobV/R nor with primers targeting the *gtf*A gene. Fructosyl-transferase genes were present in only 6 of the 15 fructan-forming strains and in none of the fructan-forming *L. reuteri* strains. Eight additional strains of *L. sanfranciscensis* produced fructans not characterized on the structural level but that were Lev-PCR negative (*17*). The results of this study could be useful for further biochemical and molecular characterization of *L. plantarum* and *W. cibaria* GTF enzymes (*34*, *52*).

Rye and wheat flours contain ca. 0.8 and 0.6% sucrose, respectively, and sucrose is a common part of dough formulas. This study showed the synthesis of ca. 2.5 g kg$^{-1}$ EPS by *W. cibaria* WC4 or *L. plantarum* LP9 during sourdough fermentation with sucrose added. During the 24 h of fermentation, only glucans were synthesized. The amount of EPS synthesized is almost in compliance with the concentration of EPS (2.6−3.1 g kg$^{-1}$) found in the sourdough started by *L. sanfranciscensis* LTH2590 (*16*). First, this study showed that the production of EPS by sourdough lactic acid bacteria under industrial conditions (20% refreshment) was texturizing relevant. Compared to a sourdough bread started with an EPS-negative strain, the sourdough started with *W. cibaria* WC4 or *L. plantarum* LP9 showed an increase of the viscosity, and the resulting bread increased the specific volume and decreased the firmness. The concentration of EPS found in the dough fermented by *W. cibaria* WC4 or *L. plantarum* LP9 exceeded that of hydrocol-

loids commercially applied as baking improvers (e.g., 0.3% on flour basis), which significantly affected dough rheology as well as bread texture and shelf life (*55*).

The results of this study show the synthesis of homopolysaccharides by probably different enzymes from those currently reported for sourdough lactic acid bacteria and demonstrate that some new EPS-forming species (e.g., *W. cibaria* and *L. plantarum*) can be deliberately used in sourdough breadmaking as a useful tool to replace the texturizing effect of additives from plant and seaweed sources.

## LITERATURE CITED


(1) De Vuyst, L.; Degeest, B. Heteropolysaccharides from lactic acid bacteria. *FEMS Microbiol. Rev.* **1999**, *23*, 153−177.

(2) Guarda, A.; Rosell, C. M.; Benedito, C.; Galotto, M. J. Different hydrocolloides as bread improvers and antistaling agents. *Food Hydrocolloids* **2004**, *18*, 241−247.

(3) Bárcenas, M.; Rosel, C. M. Different approaches for increasing the shelf life of partially baked bread: Low temperatures and hydrocolloid addition. *Food Chem*. **2007**, *100*, 1597−1601.

(4) Bárcenas, M.; Rosel, C. M. Effect of HPMC addition on the microstructure, quality and aging of wheat bread. *Food Hydrocolloids* **2005**, *19*, 1037−1043.

(5) Azizi, M. H.; Rao, G. V. Effect of surfactant gel and gum combinations on dough rheological characteristics and quality of bread. *J. Food Qual*. **2004**, *27*, 320−336.

(6) Tieking, M.; Gänzle, M. G. Exopolysaccharides from cereal-associated lactobacilli. *Trends Food Sci. Technol*. **2005**, *16*, 79−84.

(7) Ruas-Madiedo, P.; de los Reyes-Gavilán, C. G. Methods for screening, isolation, and characterization of exopolysaccharides produced by lactic acid bacteria. *J. Dairy Sci*. **2005**, *88*, 843−856.

(8) Collar, C.; Andreu, P.; Martìnez, J. C.; Armero, E. Optimization of hydrocolloid addition to improve wheat bread dough functionality: a response surface methodology study. *Food Hydrocolloids* **1999**, *13*, 467−475.

(9) Takehiro, U.; Teruo, N.; Akemi, W.; Terumi, U. Modifier for frozen bread dough and production of frozen bread dough. Jpn. Patent 08009892 A, 1994.

(10) Pigeon, R. M.; Cuesta, E. P.; Gilliland, S. E. Binding of free bile acids by cells of yogurt starter culture bacteria. *J. Dairy Sci*. **2002**, *85*, 2705−2710.

(11) Kitazawa, H.; Harata, T.; Uemura, J.; Saito, T.; Kaneko, T.; Itoh, T. Phosphate group requirement for mitogenic activation of lymphocytes by an extracellular phosphopolysaccharide from *Lactobacillus delbrueckii* spp. *bulgaricus*. *Int. J. Food Microbiol*. **1998**, *40*, 169−175.

(12) Chabot, S.; Yu, H. L.; De Léséleuc, L.; Cloutier, D.; van Calsteren, M. R.; Lessard, M.; Roy, D.; Lacroix, M.; Oth, D. Exopolysaccharide from *Lactobacillus rhamnosus* RW-9595 stimulate TNF, IL-6 and IL-12 in human and mouse cultured inmunocompetent cells, and IFN-g in mouse splenocytes. *Lait* **2001**, *81*, 683−697.

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

(14) 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*. *J. Appl. Microbiol*. **2002**, *92*, 958−965.

(15) Van Hijum, S. F. T.; Kralj, S.; Ozimek, L. K.; Dijkhuizen, L.; van Geel-Schutten, I. G. H. Structure−function relationships of glucansucrase and fructansucrase enzymes from lactic acid bacteria. *Microbiol. Mol. Biol. Rev*. **2006**, *70*, 157−176.

(16) Korakli, M.; Rossman, A.; Gänzle, M. G.; Vogel, R. F. Sucrose metabolism and exopolysaccharide production in wheat and rye sourdoughs by *Lactobacillus sanfranciscensis*. *J. Agric. Food Chem*. **2001**, *49*, 5194−5200.

(17) Tieking, M.; Korakli, M.; Ehrmann, M. A.; Gänzle, M. G.; Vogel, R. F. In situ production of exopolysaccharides during sourdough fermentation by cereal and intestinal isolates of lactic acid bacteria. *Appl. Environ. Microbiol*. **2003**, *69*, 945−952.

(18) Tieking, M.; Kühnl, W.; Gänzle, M. G. Evidence for formation of heterooligosaccharides by *Lactobacillus sanfranciscensis* during growth in wheat sourdough. *J. Agric. Food Chem*. **2005**, *53*, 2456−2461.

(19) Gobbetti, M.; De Angelis, M.; Corsetti, A.; Di Cagno, R. Biochemistry and physiology of sourdough lactic acid bacteria. *Trends Food Sci. Technol*. **2005**, *16*, 57−69.

(20) 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 characterization of the glucan and fructan exopolysaccharides synthesized by the *Lactobacillus reuteri* wild-type strain and by mutant strains. *Appl. Environ. Microbiol*. **1999**, *65*, 3008−3014.

(21) Holt, S. M.; Al-Sheikt, H.; Shin, K. J. Characterization of dextran-producing *Leuconostoc* strains. *Lett. Appl. Microbiol*. **2001**, *32*, 185−189.

(22) Kralj, S.; van Geel-Schutten, G. H.; Dondorff, M. M. G.; Kirsanovs, S.; van der Maarl, M. J. E. C.; Dijkhuizen, L. Glucan synthesis in the genus *Lactobacillus*: isolation and characterization of glucansucrase genes, enzymes and glucan products from six different strains. *Microbiology* **2004** *150*, 3681−3690.

(23) Van Hijum, S. A. F. T.; van Geel-Schutten, G. H.; Rahaoui, H.; van der Maarel, M. J. E. C.; Dijkhuizen, L. Characterization of a novel fructosyltransferase from *Lactobacillus reuteri* that synthesizes high-molecular-weight inulin and inulin oligosaccharides. *Appl. Environ. Microbiol*. **2002**, *68*, 4390−4398.

(24) Kralj, S.; van Geel-Schutten, G. H.; Rahaoui, H.; Leer, R. J; Faber, E. J.; van der Maarel, M. J. E. C.; Dijkhuizen, L. Molecular characterization of a novel glucosyltransferase from *Lactobacillus reuteri* strain 121 synthesizing a unique, highly branched glucan with α-(1→4) and α-(1→6) glucosidic bonds. *Appl. Environ. Microbiol*. **2002**, *68*, 4283−4291.

(25) Jolly, L.; Stingele, F. Molecular, organization and functionality of exopolysaccharide gene cluster in lactic acid bacteria. *Int. Dairy J*. **2001**, *11*, 733−745.

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

(27) De Man, J. C.; Rogosa, M.; Sharpe, M. E. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol*. **1960**, *23*, 130−135.

(28) Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A.; Smith, F. Colorimetric, method for determination of sugars and related substances. *Anal. Chem*. **1956**, *28*, 350−356.

(29) Bergmeyer, H. U.; Bernt, E. In *Methods of Enzymatic Analysis*; Bergmeyer, H. U., Ed.; Verlag Chemie Weinheim/Academic Press: New York, 1974; Vol. 3, pp 1176−1179.

(30) Schmidt, F. H. Die enzymatische Bestimmung von Glucose und Fructose Nebeinander. *Klinische. Wochenschrift*. **1961**, *39*, 1244−1247.

(31) Cerning, J.; Renard, C. M. G. C.; Thibault, J. F.; Bouillanne, C.; Landon, M.; Desmazeaud, M.; Topisirovic, L. Carbon source requirements for exopolysaccharide production by *Lactobacillus casei* CG11 and partial structure analysis of the polymer. *Appl. Environ. Microbiol*. **1994**, *60*, 3914−3919.

(32) Schiff, H. Eine neve Reihe organischer Diamine. *Justus Liebigs Ann. Chem*. **1866**, *140*, 92−137.

(33) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriofage T4. *Nature* **1970**, *227*, 680−685.

(34) Van Hijum, S. A. F. T.; Szalowska, E.; van der Maarel, M. J. E. C.; Dijkhuizen, L. Biochemical and molecular characterization of a levansucrase from *Lactobacillus reuteri*. *Microbiology* **2004**, *150*, 621−630.
(35) De Los Reyes-Gavilàn, C. G.; Limsowtin, G. K. Y.; Tailliez, P.; Sechaud, L.; Accolas, J. P. A *Lactobacillus helveticus*-specific DNA probe detects restriction fragment length polymorphism in this species. *Appl. Environ. Microbiol.* **1992**, *58*, 3429−3432.
(36) Sambrook, E. F.; Fritsch, J.; Maniatis, M. *Molecular Cloning. A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1989.
(37) Gobbetti, M. Interactions between lactic acid bacteria and yeasts in soudoughs. *Trends Food Sci. Technol.* **1998**, *9*, 9267−274.
(38) Heikal, Y. A.; Chhinnan, M. S. Rheological characterization of tomato puree at different temperatures using two types of viscometers. In *Engineering and Food*; Spies, W. E. L., Shubert, H., Eds.; Elsevier: London, U.K., 1990; Vol. 1, pp 151−158.
(39) Rosell, C. M.; Rojas, J. A.; Benedito de Barber, C. Influence of hydrocolloids on dough rheology and bread quality. *Food Hydrocolloids* **2001**, *15*, 75−81.
(40) Smitinont, T.; Tansakul, C.; Tanasupawat, S.; Keeratipibul, S.; Navarini, L.; Bosco, M.; Cescutti, P. Exopolysaccharide-producing lactic acid bacteria strains from traditional thai fermented foods: isolation, identification and exopolysaccharide characterization. *Int. J. Food Microbiol.* **1999**, *51*, 105−111.
(41) Tallon, R.; Bressollier, P.; Urdaci, M. C. Isolation and characterization of two exopolysaccharides produced by *Lactobacillus plantarum* EP56. *Res. Microbiol.* **2003**, *154*, 705−712.
(42) Robyt, J. F.; Walseth, T. Production, purification, and properties of dextransucrases from *Leuconostoc mesenteroides* NRRL B-512F. *Carbohydr. Res.* **1979**, *68*, 95−111.
(43) Schwab, C.; Gänzle, M. Effect of membrane lateral pressure on the expression of fructosyltransferases in *Lactobacillus reuteri*. *Syst. Appl. Microbiol.* **2006**, *29*, 89−99.
(44) Funane, K.; Yamada, M.; Shiraiwa, M.; Takahara, H.; Yamamoto, N.; Ichishima, E.; Kobayashi, M. Aggregated form of dextransucrases from *Leuconostoc mesenteroides* NRRL B-512F and its constitutive mutant. *Biosci., Biotechnol., Biochem.* **1995**, *59*, 776−780.
(45) Giffard, P. M.; Simpson, C. L.; Milward, C. P.; Jacques, N. J. Molecular characterization of a cluster of at least two glucosyltransferase genes in *Streptococcus salivarius* ATCC 25975. *J. Gen. Microbiol.* **1991**, *137*, 2577−2593.
(46) Jolly, L.; Newell, J.; Porcelli, I.; Vincent, S. J. F.; Stingele, F. *Lactobacillus helveticus* glycosyltransferases: from genes to carbohydrate synthesis. *Glycobiology* **2002**, *12*, 319−327.
(47) Remaund-Simeon, M.; Willemot, R. M.; Sarcabal, P.; Potocki de Montalk, G.; Monsan, P. Glucansucrases: molecular engineering and oligosaccharides synthesis. *J. Mol. Catal.* **2000**, *10*, 117−128.
(48) Stingele, F.; Neeser, J. R.; Mollet, B. Identification and characterization of the *eps* (exopolysaccharide) gene cluster from *Streptococcus thermophilus* Sfi6. *J. Bacteriol.* **1996**, *178*, 1680−1690.
(49) Van Kranenburg, R.; Marugg, J. D.; van Swam, I. I.; Willem, N. J.; de Vos, W. M. Molecular characterization of the plasmid-encoded *eps* gene cluster essential for exopolysaccharide biosynthesis in *Lactococcus lactis*. *Mol. Microbiol.* **1997**, *24*, 387−397.
(50) Péant, B.; LaPointe, G.; Gilbert, C.; Atlan, D.; Ward, P. Roy, D. Comparative analysis of the exopolysaccharide biosynthesis gene clusters from four strains of *Lactobacillus rhamnosus*. *Microbiology* **2005**, *51*, 1839−1851.
(51) Dabour, N.; LaPointe, G. Identification and molecular characterization of the chromosomal exopolysaccharide biosynthesis gene cluster from *Lactococcus lactis* subsp. *cremoris* SMQ-461. *Appl. Environ. Microbiol.* **2005**, *71*, 7414−7425.
(52) Van Kranenburg, R.; Vos, H. R.; van Swam, I. I.; Kleerebezem, M.; de Vos, W. Functional analysis of glycosyltransferase genes from *Lactococcus lactis* and other gram-positive cocci: complementation, expression, and diversity. *J. Bacteriol.* **1999**, *181*, 6347−6353.
(53) Monchois, V.; Remand-Simèon, M.; Monsan, P.; Willemot, R. M. Cloning and sequencing of an extracellular dextransucrase (DSR-B) from *Leuconostoc mesenteroides* NRRL B1299-synthesizing only α(1−6)glucan. *FEMS Microbiol. Lett.* **1998**, *159*, 307−315.
(54) Kolkmann, M. A. B.; Walkarchuk, W.; Nuijten, P. J. M.; van der Zeijst, B. A. M. Capsular polysaccharide synthesis in *Streptococcus pneumoniae* serotype 14: molecular analysis of the complete *eps* gene locus and identification of genes encoding glycosyltransferases required for the biosynthesis of the tetrasaccharide subunit. *Mol. Microbiol.* **1997**, *26*, 197−208.
(55) Armero, E.; Collar, C. Crumb firming kinetics of wheat breads with anti-staling additives. *J. Cereal Sci.* **1998,** *28*, 165−174.
(56) Provencher, C.; LaPointe, G.; Siros, S.; van Calsteren, M. R.; Roy, D. Consensus degenerate hybrid oligonucleotide primers for amplification of priming glycosyltransferase genes of the exopolysaccharide locus in strains of the *Lactobacillus casei* group. *Appl. Environ. Microbiol.* **2003**, *69*, 3299−3307.

**Received for review May 17, 2006. Revised manuscript received August 28, 2006. Accepted September 6, 2006.**

JF061393+