Cloning, expression, purification and characterization of β-galactosidases from probiotic strains of *Lactobacillus* spp. in *Lactobacillus plantarum* WCFS1 and *Lactobacillus sakei* Lb790

Diplomarbeit

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Elisabeth Halbmayr

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Abstract

The work of this diploma thesis presents the cloning and expression of the genes encoding heterodimeric β-galactosidases from *Lactobacillus reuteri* L103, *Lactobacillus acidophilus* R22, *Lactobacillus plantarum* WCFS1 and *Lactobacillus sakei* Lb790. These enzymes consist of two subunits of 73 kDa and 35 kDa, which are encoded by two overlapping genes, *lacL* and *lacM*, respectively. The cloning of these genes into the expression vectors pSIP403 and pSIP409 and expressing them in the host strains *Lactobacillus plantarum* WCFS1 and *Lactobacillus sakei* Lb790 enables over production of β-galactosidase in a food-grade expression system which is of high interest for the applications in food industry.

Recombinant β-galactosidase from *L. reuteri* L103 expressed in *L. plantarum* WCFS1 was purified by affinity chromatography to apparent homogeneity and characterized. K_m-value, V_{max}, pH optimum, pH stability, temperature optimum and temperature stability were determined and compared with the native β-galactosidase from *L. reuteri* L103 and recombinant β-galactosidase from *L. reuteri* L103 expressed in *Escherichia coli*. Recombinant β-galactosidases from probiotic lactobacilli can be used for synthesis of prebiotic galacto-oligosaccharides. To investigate the spectrum of galacto-oligosaccharides produced from transgalactosylation of lactose catalyzed by recombinant β-galactosidase from *L. reuteri* L103 expressed in *L. plantarum* WCFS1, lactose conversion experiment with the purified enzyme and the crude enzyme extract were carried out.

Zusammenfassung

In dieser Diplomarbeit wurden die Gene für β-Galaktosidasen aus *Lactobacillus reuteri* L103, *Lactobacillus acidophilus* R22, *Lactobacillus plantarum* WCFS1 and *Lactobacillus sakei* Lb790 kloniert und exprimiert. Diese Enzyme bestehen aus zwei Untereinheiten mit der Größe von 73 kDa und 35 kDa. Beide Untereinheiten werden von zwei überlappenden Genen, *lacL* and *lacM*, codiert. Diese Gene wurden in die Expressionsvektoren pSIP403 und pSIP409 kloniert und anschließend in *Lactobacillus plantarum* WCFS1 and *Lactobacillus sakei* Lb790 exprimiert. Somit ist die Überproduktion von β-Galaktosidase in einem lebensmitteltauglichen Expressionssystem möglich, was für die Anwendung dieses Enzyms in der Lebensmittelindustrie von großem Interesse ist.

Rekombinante β-Galaktosidase aus *L. reuteri* L103 exprimiert in *L. plantarum* WCFS1 wurde mittels Affinitätschromatographie gereinigt und charakterisiert. Es wurden der K_m-Wert, V_{max}, das pH-Optimum, die pH-Stabilität, das Temperaturoptimum und die Temperaturstabilität ermittelt. Die Ergebnisse wurden mit denen der nativen β-Galaktosidase aus *L. reuteri* L103 und der rekombinanten β-Galaktosidase aus *L. reuteri* L103 exprimiert in *Escherichia coli* verglichen.

Rekombinante β-Galaktosidase aus probiotischen Lactobacillen kann für die Synthese von prebiotischen Galakto-Oligosacchariden verwendet werden. Um das Spektrum der Galakto-Oligosaccharide, die durch Transgalaktosylierung von Laktose durch die rekombinante β-Galaktosidase aus *L. reuteri* L103 exprimiert in *L. plantarum* WCFS1 erzeugt wurden, aufzuzeigen, wurde eine Laktosekonversion mit dem gereinigten Enzym und dem Rohextrakt durchgeführt.

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High-level expression of recombinant β-galactosidases in *Lactobacillus plantarum* and *Lactobacillus sakei* using a sakacin P-based expression system

Elisabeth Halbmayr[1], Geir Mathiesen[2], Thu-Ha Nguyen[1, 3], Clemens Peterbauer[1], Vincent Eijsink[2] and Dietmar Haltrich[1,*]

[1] Division of Food Biotechnology, Department of Food Sciences and Technology, BOKU - University of Natural Resources and Applied Life Sciences Vienna, Muthgasse 18, A-1190 Vienna, Austria

[2] Department of Chemistry, Biotechnology and Food Science, Agricultural University of Norway, N-1432 Ås, Norway

[3] Research Centre Applied Biocatalysis, Petersgasse 14, A-8010 Graz, Austria

[*] corresponding author

Dietmar Haltrich, Department für Lebensmittelwissenschaften und –technologie, Universität für Bodenkultur Wien

phone: +43-1-36006 6275, fax: +43-1-36006 6251, e-mail: dietmar.haltrich@boku.ac.at

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Abstract

This work presents the cloning and expression of the genes encoding heterodimeric β-galactosidases from *Lactobacillus reuteri* L103, *Lactobacillus acidophilus* R22, *Lactobacillus plantarum* WCFS1 and *Lactobacillus sakei* Lb790. These enzymes consist of two subunits of 73 kDa and 35 kDa, which are encoded by two overlapping genes, *lacL* and *lacM*, respectively. The cloning of these genes into the expression vectors pSIP403 and pSIP409 and expressing them in the host strains *Lactobacillus plantarum* WCFS1 and *Lactobacillus sakei* Lb790 facilitates over production of β-galactosidase in a food-grade expression system which is of high interest for applications in food industry. Recombinant β-galactosidase from *L. reuteri* L103 expressed in *L. plantarum* WCFS1 was purified by affinity chromatography to apparent homogeneity characterized and compared with the native β-galactosidase from *L. reuteri* L103 and recombinant β-galactosidase from *L. reuteri* L103 expressed in *Escherichia coli*. Recombinant β-galactosidases from probiotic lactobacilli can be used for synthesis of prebiotic galacto-oligosaccharides.

Keywords: inducible expression of proteins, bacteriocins, sakacin, Lactobacillus, lactic acid bacteria

Introduction

At present, most host strains for the production of recombinant enzymes for food applications have been derived from a relatively small number of bacterial and fungal species, primarily *Bacillus subtilis, B. licheniformis, Aspergillus niger* or *A. oryzae*. These microorganisms have a long history of use as safe sources of their native enzymes in food biotechnology and ample documentation of efficient growth under industrial production conditions. In addition, they are amenable to genetic manipulations and the necessary techniques and tools have been developed (*1*). Similarly, *Lactobacillus* spp. or other lactic acid bacteria (LAB) have a great potential as food-grade cell factories, e.g., for the production of food-related enzymes (*2, 3*). Many *Lactobacillus* spp. are traditionally used in food and feed industries for a wide variety of different important processes and products (*4*), and many of them have the generally regarded as safe (GRAS) status. A number of lactobacilli have furthermore been proposed and are applied as probiotic strains, i.e., live organisms that are used as food supplement in order to benefit health (*5-7*). Proven and proposed beneficial effects of probiotics are manifold and quite diverse, ranging from the production of important digestive enzymes and alleviation of lactose intolerance to a non-specific stimulation of the immune system (*8, 9*), to name a few. Probiotics also show a normalizing effect on the intestinal microbiota by inhibiting growth or adhesion of pathogenic microorganisms. This is achieved not merely by forming lactic acid and this lowering the pH value but also by the secretion of bacteriocins, peptides with antimicrobial activity. Production of these peptide bacteriocins, such as most of the class II bacteriocins, is often regulated via quorum-sensing mechanisms based on secreted peptide pheromones with no or little bacteriocin activity (*10, 11*). This pheromone then activates a two-component regulatory system of a histidine kinase that senses the pheromone, and a response regulator protein that, when activated by the histidine kinase, induces the promoters of the operons involved in bacteriocin production. Using these regulatory mechanisms of

bacteriocin formation, several inducible gene expression systems have been developed for efficient, regulated overproduction of heterologous proteins in LAB, using for example *Lactococcus lactis* (*12*), *Streptococcus thermophilus* (*13*), or *Lactobacillus sakei* and *L. plantarum* (*2, 14-16*) as expression hosts. The expression systems for the latter lactobacilli use vectors, the so-called pSIP expression vectors, based on promoters and regulatory genes involved in the production of the class II bacteriocins sakacin A [*sap* gene cluster; (*17*)] or sakacin P [*spp* gene cluster (*18, 19*)] with an engineered *Nco*I site for translational fusion cloning (*16*). The operational parts of the versatile expression vectors consist of the histidine kinase (HK) gene, the response regulator (RR) gene and a cognate bacteriocin promoter (Fig. 1). The vectors are inducible with pheromone and can be used as models for food-grade and `self-cloning´ systems (*16*). The applicability and usefulness of these new expression systems was shown in previous studies using β-glucuronidase from *Escherichia coli* (*gusA*) and aminopeptidase N from *Lactococcus lactis* (*pepN*) as reporter genes (*15, 20*).

The goal of the present study was to further develop and compare these different expression systems, using two different promoters and two different host strains, for the overproduction of the industrially relevant enzyme β-galactosidase from *Lactobacillus spp.*, namely *L. acidophilus, L. plantarum, L. reuteri* and *L. sakei*, which are probably among the best known probiotic lactobacilli. The β-galactosidases from *L. acidophilus*, *L. reuteri*, *L. plantarum* and *L. sakei* are heterodimeric proteins consisting of two subunits of approximately 35 and 73 kDa, which are encoded by two overlapping genes, *lacL* and *lacM* (*21-25*).

β-Galactosidases (β-gal, EC 3.2.1.23) catalyze the hydrolysis and transgalactosylation of β-D-galactopyranosides such as lactose. β-Galactosidase-catalyzed hydrolysis reactions are widely known for applications in the dairy industries to improve digestibility, solubility and sweetness of lactose, the principle milk carbohydrate (*26*). Transgalactosylation reactions catalyzed by β-galactosidases when using lactose or other structurally related galactosides as

the substrate yield galacto-oligosaccharides (*26, 27*). These attract an increasing amount of attention for both their prebiotic effects and tailor-made functions (*28-30*).

Here we describe the cloning of β-galactosidases from four different *Lactobacillus* species, *L. acidophilus* R22, *L. reuteri* L103, *L. plantarum* WCFS1 and *L. sakei* Lb790, and the expression of the genes (*lacL* and *lacM*) encoding these enzymes in *Lactobacillus* expression systems. It was our interest to increase the production of these attractive and important enzymes to high levels in expression systems that can be easily adapted to the needs of food industry for the production of food-grade enzymes. Furthermore, properties of the recombinant β-galactosidase from *L. reuteri* L103 expressed in *L. plantarum* WCFS1 are reported.

Materials and methods

Chemicals and enzymes

All chemicals were purchased from Sigma (St. Louis, MO) unless otherwise stated, and were of the highest quality available. MRS broth powder (*Lactobacillus* broth according to De Man, Rogosa and Sharpe (*31*) was obtained from Oxoid (Hampshire, UK) and Merck (Darmstadt, Germany). BHI (Brain Heart Infusion) broth was purchased from Oxoid, 1,4-Dithiothreitol (DTT) was from Roth (Karlsruhe, Germany), and glucose oxidase (GOD) from *Aspergillus niger* (lyophilized, 205 U/mg enzyme preparation) was from Fluka (Buchs, Switzerland). Horseradish peroxidase (POD) (lyophilized, 210 U/mg) and the test kit for the determination of D-glucose were from Boehringer (Mannheim, Germany). All restriction enzymes and Quick T4 DNA ligase were purchased from New England Biolabs (Beverly, MA, USA), while Phusion DNA Polymerase was from Finnzymes OY (Espoo, Finland).

Bacterial strains and culture conditions

L. reuteri strain L103, isolated from calf, was obtained from Lactosan (Starterkulturen GmbH & Co. KG, Kapfenberg, Austria). *L. acidophilus* R22, originating from probiotic yoghurt, was obtained from the culture collection of the Division of Food Microbiology, BOKU University of Natural Resources and Applied Life Sciences, Vienna. *L. plantarum* WCFS1, isolated from human saliva as described in Kleerebezem *et al.* (*24*), and *L. sakei* Lb790, isolated from meat as described in Schillinger and Lücke (*32*), were from the culture collection of the Norwegian University of Life Sciences and used as host strains. *E. coli* One Shot TOP10 chemical competent cells used for chemical transformation and electroporation were purchased from Invitrogen (Carlsbad, CA, USA). S.O.C. medium, containing bacto-tryptone 20 g/l, bacto yeast extract 5 g/l, sodium chloride 0.57 g/l, potassium chloride 0.19 g/l, magnesium sulphate 2.47 g/l and glucose 3.6 g/l, was used for transforming *E. coli* One Shot TOP10 competent cells. *E. coli* was grown in BHI medium at 37°C with shaking. *Lactobacillus* strains were grown in MRS medium at 30°C in tightly capped flasks and without shaking. Agar plates were made using BHI medium for *E. coli* and MRS medium for *Lactobacillus* strains. When appropriate, antibiotics were added as follows: 100 µg/ml kanamycin or 200 µg/ml erythromycin for *E. coli,* and 5 µg/ml erythromycin for lactobacilli.

DNA preparation

The preparations of the plasmids pHA1031 and pHAR22 containing the complete genes (*lacL* and *lacM*) of β-galactosidases from *L. reuteri* L103 and *L. acidophilus* R22 were described previously in Nguyen *et al.* (*21, 23*), respectively. Chromosomal DNA from *L. plantarum* WCFS1 and *L. sakei* Lb790 was extracted using the E.Z.N.A. Bacterial DNA kit (Omega Bio-Tek, Doraville, GA). Expression vectors pSIP403, pSIP409 and pSIP405 were prepared as previously described in Sørvig *et al.* (*2, 16*) and Mathiesen *et al.* (*33*). Plasmid DNA was purified using the E.Z.N.A. Plasmid Miniprep Kit I (Omega Bio-Tek).

Cloning of *lacLM* genes

The oligonucleotides LacReuF and LacReuR, LacAciF and LacAciR, LacLMPlaF and LacLMPlaR, Lb790LacF and Lb790LacR (Table 1) used for PCR amplification of the *L. reuteri lacLM*, *L. acidophilus lacLM*, *L. plantarum lacLM* and *L. sakei lacLM* genes, respectively, were designed based on the sequences of β-galactosidases from *L. reuteri* L103 (GenBank accession number DQ493596), *L. acidophilus* R22 (GenBank accession number EF053367), *L. plantarum* WCFS1 (GenBank accession number AL935262) and *L. sakei* subsp. *sakei* (GenBank accession number X82287). *Bsa*I restriction sites were added to the 5´ends of the forward primers, and *Xho*I restriction sites were added at the 5´end of reverse primers, except for LacAciR, which contains an added *EcoR*I site. These primers were obtained from Operon Biotechnologies GmbH (Cologne, Germany). The amplification was performed with Phusion High-Fidelity DNA Polymerase using an Eppendorf Mastercycler (Eppendorf; Hamburg, Germany) in a total volume of 50 µl of reaction mixtures containing 0.2 mM of each deoxynucleotide triphosphate, 0.5 µM of each primer, 10 µl of 5× Phusion HF buffer (final concentration of $MgCl_2$ was 1.5 mM), 1 U of Phusion DNA Polymerase, and approximately 50 ng genomic DNA. The initial denaturation step at 98°C for 30 s was followed by 35 cycles of denaturation at 98°C for 5 s, annealing at 49/53°C for 10 s, and extension at 72°C for 1 min. The final cycle was followed by an additional 10-min elongation step at 72°C. The amplified products were visualized by gel electrophoresis at 7 V/cm in a 1.2% agarose gel (containing ethidium bromide at 0.2 µg/ml) in 1× TAE (Tris-Acetate) electrophoresis buffer (Tris base 4.8 g/l, acetic acid 1.2 g/l, 1 mM EDTA pH 8.0) and photographed under UV light. The amplified products were purified from the agarose gel using the NucleoSpin Extract II (Macherey-Nagel; Bethlehem, PA), and ligated into the vector pCRII-Blunt-TOPO (Invitrogen; Carlsbad, CA) according to the manufacturers instructions, resulting in the plasmids pTReu, pTAci, pTLacLMPlant, pTLacLMSak

containing the complete genes (*lacL* and *lacM*) of β-galactosidases from *L. reuteri*, *L. acidophilus*, *L. plantarum* and *L. sakei*, respectively.

Sequencing of *lacLM* genes and sequence analysis

The nucleotide sequences were determined by the Department of Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, by primer walking using the sequencing primers listed in Table 1. Assembly and analysis of DNA sequences were done using BioEdit Sequence Alignment Editor version 7.0.4.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). The basic local alignment search tool (BLAST) from the National Centre for Biotechnology Information BLAST website was used for database searches.

Construction of *lacLM* expression vectors

The four genes encoding for the complete heterodimeric β-galactosidase (*lacLM*) were cut out from plasmids pTReu, pTAci, pTLacLMPlant and pTLacLMSak using the restriction enzymes *Bsa*I and *Xho*I (*EcoR*I instead of *Xho*I for pTAci). The resulting fragments were visualized by agarose gel electrophoresis and purified from the agarose gel using the NucleoSpin Extract II (Macherey-Nagel). The β-glucuronidase reporter gene was cut out from the pSIP403 and pSIP409 expression vectors (*2*) using restriction enzymes *Nco*I and *Xho*I (or *EcoR*I for the vectors designated for *lacLM* from *L. acidophilus*), and empty vector fragments were purified from the agarose gel using the NucleoSpin Extract II (Macherey-Nagel). Expression is controlled by the P_{sspA} promoter in pSIP403, while pSIP409 contains the P_{sspQ} (formerly P_{orfX}) promoter (*2, 34*). The Quick Ligation Kit (New England BioLabs; Beverly, MA) was used for ligating the empty pSIP403/pSIP409 vectors with the *lacLM* genes resulting in 8 plasmids pSIP403LacLM-Reu, pSIP409LacLM-Reu, pSIP403LacLM-Aci, pSIP409LacLM-Aci, pSIP403LacLM-Plant, pSIP409LacLM-Plant, pSIP403LacLM-Sak and pSIP409LacLM-Sak. Transformation of the newly constructed plasmids into *E. coli* was

performed using *E. coli* One Shot TOP10 chemical competent cells (Invitrogen) following the manufacturers protocol.

Expression of β-galactosidases

To generate 16 expression strains carrying the 8 newly constructed plasmids pSIP403LacLM (Reu, Aci, Plant, Sak) and pSIP409LacLM (Reu, Aci, Plant, Sak) electroporation of electro-competent *L. plantarum* WCFS1 and *L. sakei* Lb790 cells was conducted according to the protocol by Aukrust and Blom (*35*), and transformant colonies were selected on MRS agar containing 5 μg/ml erythromycin. Single colonies were inoculated into 50 ml of MRS medium containing 5 μg/ml erythromycin and grown at 30°C. When an optical density at 600 nm of 0.3 was reached the cultures were induced with 25 ng/ml peptide pheromone IP-673 (Molecular Biology Unit, University of Newcastle-upon-Tyne, UK). Cells were harvested at an optical density at 600 nm of 1.8 by centrifugation at 5200 rpm for 5 min at 4°C, washed once with 0.9% sodium chloride solution and disrupted using a sonicator (Sonics vibra-cell; Sonics and Materials, Newtown, CT). Debris was removed by centrifugation (13200 rpm, 5 min, 4°C) to obtain the crude enzyme extract. The same growth conditions and procedures were followed with cultures that were not induced with pheromone. Background expression of native β-galactosidases was checked with *L. plantarum* pSIP405 and *L. sakei* pSIP405, which carry a *cat* (chloramphenicol) gene instead of the *lacLM* genes, under similar growth conditions as those for the strains carrying constructs with *lacLM*.

Fermentation of the selected *Lactobacillus* strain

L. plantarum WCFS1 carrying pSIP403LacLM-Reu, the plasmid containing the complete genes (*lacL* and *lacM*) of β-galactosidase from *L. reuteri* L103, was selected for further studies. This strain was grown in MRS broth containing 5 μg/ml erythromycin in a 1-L glass bottle capped tightly at 30°C. Precultures were grown overnight in 5 ml MRS broth medium

containing 5 μg/ml erythromycin and inoculated into 1000 ml fresh medium to an optical density at 600 nm of 0.1. When optical density at 600 nm reached 0.3, pheromone (25 ng/l) was added to the culture medium and the cultures were incubated further for 20 h until an optical density at 600 nm of approximately 6.0 was reached. The induced cells were harvested, washed once with and then resuspended in 50 mM sodium phosphate buffer (pH 6.0). Cell disruption was performed using a French press (Aminco; Jessup, MD), and debris was removed by centrifugation at 13000 rpm for 45 min at 4°C to obtain the crude extract.

Protein purification

The crude extract was loaded onto an affinity column (10 ml of *p*-aminobenzyl 1-thio-*β*-D-galactopyranoside immobilized on crosslinked 4% beaded agarose; Sigma) that was pre-equilibrated with 50 mM sodium phosphate buffer pH 6.0 (buffer A). The enzyme was eluted at a rate of 0.6 ml/min by using a linear gradient of 0 to 2 M sodium chloride in buffer A (15 column volumes). The active fractions were pooled, desalted, and concentrated. The purified enzyme was stored in 50 mM sodium phosphate buffer (pH 6.0) containing 1 mM DTT at 4°C.

Protein determination

Protein concentration was determined by the method of Bradford (*36*) using bovine serum albumin as standard.

Gel electrophoresis and active staining

To compare the expression of the different β-galactosidases in *L. plantarum* WCFS1 and *L. sakei* Lb790 denaturing sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using precast polyacrylamide gels (NuPAGE Novex Bis-Tris Gels 10%, Invitrogen). The sample preparation, running buffer and running conditions were as

described in the NuPAGE manual from Invitrogen with slight modifications (samples were heated at 100°C for 5 min). Coomassie blue staining was performed for visualization of the protein bands. Furthermore, gel electrophoresis and active staining of homogenous β-galactosidase from *L. reuteri* overexpressed in *L. plantarum* WCFS1 were carried out using 4-methylumbelliferyl β-D-galactoside as the substrate as previously described (*22*).

Enzyme assays

β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside (*o*NPG) and lactose as the substrates as described previously (*22*). When chromogenic *o*NPG was used as the substrate, the reaction was initiated by adding 20 µl of enzyme solution to 480 µl of 22 mM *o*NPG in 50 mM sodium phosphate buffer (pH 6.5) and stopped after 10 min of incubation at 30°C and shaking at 600 rpm by adding 750 µl of 0.4 M Na_2CO_3. The release of *o*-nitrophenol (*o*NP) was measured by determining the absorbance at 420 nm. One unit of *o*NPG activity was defined as the amount of enzyme releasing 1 µmol of *o*NP per minute under the described conditions.

When lactose was used as the substrate, 20 µl of enzyme solution was added to 480 µl of 600 mM lactose solution in 50 mM sodium phosphate buffer, pH 6.5. After 10 min of incubation at 30°C, the reaction was stopped by heating the reaction mixture at 99°C for 5 min. The reaction mixture was cooled to room temperature, and the release of D-glucose was determined colormetrically using the GOD/POD assay (*37*). One unit of lactase activity was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions.

Characterization of the recombinant β-galactosidase enzyme

Steady-state kinetic data for the substrate lactose were obtained following the methods described in our previous report (*22*). The pH dependence of the enzymatic release of *o*-

nitrophenol (*o*NP) from *o*NPG and D-glucose from lactose was measured between pH 4 and 9 using Britton-Robinson buffer (containing 20 mM each of phosphoric, acetic and boric acid adjusted to the required pH with NaOH). The activity was determined at different pH values under otherwise standard assay conditions. To determine the pH stability of the recombinant β-galactosidase, the enzyme samples were incubated at various pH values using Britton-Robinson buffer as mentioned above at 37°C for up to 24 h, and the remaining enzyme activity was measured using *o*NPG as the substrate under standard assay conditions.

The temperature dependence of enzyme activity (both *o*NPG and lactose activity) was measured by assaying the enzyme samples over the range of 25–70°C for 10 min. The temperature stability of both enzymes was studied by incubating the enzyme samples in 50 mM sodium phosphate buffer of pH 6.0 at various temperatures (4, 25, 30, 37 and 42 °C). At certain time intervals, samples were withdrawn and the residual activity was measured with *o*NPG as the substrate under standard assay conditions.

Results

Cloning and expression of β-galactosidase genes from *L. reuteri*, *L. acidophilus*, *L. plantarum* and *L. sakei* in *Lactobacillus* expression systems

Degenerated oligonucleotides were designed for PCR amplification of the entire *lacLM* gene encoding both the large and the small subunits of β-galactosidase from *L. reuteri* L103, *L. acidophilus* R22, *L. plantarum* WCFS1 and *L. sakei* Lb790, and the genes were amplified and cloned into standard plasmid vectors as well as – using restriction sites that were introduced with the primers – expression vectors based on the pSIP403 and pSIP409 systems (*16*). Table

2 shows the size of the respective genes as well as the calculated molecular masses for the large and the small subunits of the β-galactosidases. The overlapping coding regions for both genes, *lacL* and *lacM*, were cloned into pSIP403 and pSIP409 vectors, containing either the P_{sppA} or P_{sppQ} promoter, resulting in eight expression plasmids pSIP403LacLM-Reu, pSIP409LacLM-Reu, pSIP403LacLM-Aci, pSIP409LacLM-Aci, pSIP403LacLM-Plan, pSIP409LacLM-Plan, pSIP403LacLM-Sak and pSIP409LacLM-Sak. These plasmids were transformed into *L. plantarum* and *L. sakei*, and expression of the introduced β-galactosidase genes was studied. Gene expression was induced by 25 ng/ml peptide pheromone, which was found to be optima for the expression in these systems (*15*), and cultivation was continued at 30°C until the optical density at 600 nm reached 1.8, then the harvested cells were homogenized, and the extracts analyzed for the presence of the gene products. SDS-PAGE of cell-free extracts showed two additional bands of approx. 75 and 35 kDa, corresponding to the gene products of *lacL* and *lacM,* respectively (Fig. 2). β-Galactosidase activities produced by *L. plantarum* WCFS1 and *L. sakei* Lb790 carrying the plasmids above mentioned are compared in Fig 3. As can be seen from both of these figures, expression levels of the introduced β-galactosidase genes vary significantly both between the expression hosts, where *L. plantarum* gives higher activity levels of the *L. reuteri* and the *L. plantarum* genes for both promoters used as well as with pSIP403-LacLMSak, and also among the different genes. The *lacLM* genes from *L. sakei* and *L. acidophilus* formed significantly less enzyme activity upon expression with both promoters and in both expression hosts. Exchange of the P_{sspA} promoter with the P_{sppQ} promoter did not significantly affect the levels of β-galactosidase activity formed in both host strains.

L. plantarum WCFS1 and *L. sakei* Lb790 carrying the expression plasmids were also grown the same way as described above but without the addition of the inducing pheromone to check for leakage of the two promoters. The basal activities formed under non-induced conditions together with the induction factors for the different constructs, based on the formation of β-

galactosidase activity, are given in Table 3. With only few exceptions, these basal activities obtained under non-induced conditions were not significantly higher than the background β-galactosidase activities derived from the native genomic β-galactosidase-encoding genes of *L. plantarum* WCFS1 pSIP405 and *L. sakei* Lb790 pSIP405 when grown on glucose, which were approximately 0.07 U/mg. Generally, the *L. plantarum* variants showed higher basal activities with most of the expression plasmids than *L. sakei.* Furthermore, consistently lower basal activities were obtained for constructs based on the expression plasmid pSIP409 containing the P_{sppQ} promoter, which results in higher induction factors for *L. sakei* as expression host and constructs based on pSIP409.

In addition, the levels of β-galactosidase activity produced by the wild-type strains *L. reuteri, L. acidophilus and L. plantarum* were tested. To this end, these strains were cultivated under identical conditions in MRS medium that contained lactose instead of glucose. Cells were harvested when the optical density reached approximately 2, and the intracellular β-galactosidase activity was determined after cell rupture. These activities were 7.90 ± 0.78, 4.00 ± 0.56, and 2.22 ± 0.14 for *L. reuteri, L. acidophilus* and *L. plantarum*, respectively. When compared to the data obtained for the heterologous or homologous expression of the β-galactosidases, this again shows the efficiency of the expression systems studied especially for the two β-galactosidases from *L. reuteri* and *L. plantarum.* The highest expression levels of the enzyme of interest were found for the plasmid pSIP403LacLM-Reu (carrying the *lacLM* gene from *L. reuteri*) when expressed in *L. plantarum* WCFS1. Thus this strain and its recombinant β-galactosidase were selected for further study.

Overexpression of β-galactosidase genes from *L. reuteri* in *L. plantarum*

β-Galactosidase activity produced by *L. plantarum* WCFS1 carrying pSIP403LacLM-Reu was determined to be 136 U/mg of protein, and approximately 23 kU per liter of fermentation broth was obtained. β-Galactosidase constituted approximately 55% of the total amount of intracellular soluble proteins. The enzyme was purified in one single step to apparent homogeneity using an affinity column (*p*-aminobenzyl 1-thio-*β*-D-galactopyranoside immobilized in crosslinked 4% beaded agarose), which gave an overall yield of 12% and a specific activity of 245 U/mg of protein. The recombinant β-galactosidase overexpressed in *L. plantarum* had a molecular mass of approximately 108 kDa and consisted of a 73 and a 35 kDa subunit as was shown by SDS-PAGE (Fig. 4A). This compares well to the calculated molecular masses of the two subunits of 73,492 Da and 35,683 Da as based on the sequence of the *lacLM* gene. Active staining of purified recombinant β-galactosidases expressed in *L. plantarum* directly on SDS-PAGE gel after preincubating the enzyme with denaturing SDS buffer at 60°C for 5 min and using 4-methylumbelliferyl β-D-galactoside as the substrate showed that one band corresponding to a polypeptide of the size of the larger subunit exhibited activity with this substrate. In contrast, the smaller subunit did not show any activity (Fig. 4A); this is in agreement with previous reports (*22, 23*). Active staining of wild-type β-galactosidase L103 and recombinant β-galactosidase EL103 expressed in *E. coli* on native PAGE yielded two bands with β-galactosidase activity, one band of approximately 105 kDa, corresponding to the intact heterodimer, and a second band representing a degradation product containing components of both subunits (*22*). This observation was also made with recombinant β-galactosidase overexpressed in *L. plantarum* (fig. 4B).

Properties of recombinant β-galactosidase LpL103

To differentiate between the native β-galactosidase from *L. reuteri* L103 (*22*) and the recombinant β-galactosidase overexpressed in *E. coli* EL103 (*23*), the β-galactosidase overexpressed in *L. plantarum* was denoted as recombinant β-galactosidase LpL103.

The steady-state kinetic constants were determined for the hydrolysis of lactose, the natural substrate of this enzyme. Kinetic analysis of recombinant β-galactosidase LpL103 with increasing concentrations of lactose (9.6–576 mM) as the substrate showed Michaelis-Menten kinetics with the following parameters obtained by nonlinear regression using SigmaPlot (SPSS Inc.; Chicago, IL): v_{max} = 43 (μmol D-glucose released per min and per mg protein), and K_m = 12 ± 2 mM. In cases of native β-galactosidase L103 and the recombinant β-galactosidase EL103, the v_{max} and K_m values for lactose were 34 and 38 (μmol D-glucose released per min and per mg protein), and 13 ± 2 and 12 ± 2 mM, respectively (*22, 23*).

The optimum pH of recombinant LpL103 β-galactosidase was found to be pH 7.0 for *o*NPG hydrolysis and pH 6.5–7.5 for lactose hydrolysis. Again, this compares very well to the data obtained for both the native L103 β-galactosidase and the recombinant EL103 β-galactosidase expressed in *E. coli* (*22, 23*). Recombinant LpL103 is most stable at the rather narrow pH range from 5.5 to 6.5, retaining 80% of its activity at this pH range when incubated at 37°C for 3 h (fig. 5), and half-life times of activity ($t_{½}$) ranging from approx. 16 h at pH 5.5 to 26 h at pH 6.5. Even small changes of the pH reduce stability of the enzyme significantly, and the $t_{½}$ values for pH 5.0 and 7.0 were found to be below 2 h.

The optimum temperature of recombinant β-galactosidase LpL103 was 50°C when using *o*NPG as the substrate under standard assay conditions (pH 6.5 and 10 min) as was also found for both native and recombinant β-galactosidase L103 and EL103 (*22, 23*). For lactose hydrolysis, the optimum temperature was found to be 55°C. Figure 6 shows the effect of temperature on the stability of native L103 and recombinant EL103 and LpL103 β-

galactosidase after incubation for 48 h at different temperatures. All three enzymes are very stable at 4°C in the presence of 1 mM 1,4-dithiothreitol (DTT), retaining full activity after several weeks of storage. LpL103, EL103 and L103 β-galactosidases retained 70%, 95% and 60% of their activities, respectively, when incubated at pH 6.0 and 30°C for 48 h (Figure 6). The effect of $MgCl_2$ on the thermal stability of native L103 and recombinant EL103 was reported previously (*22, 23*), with $MgCl_2$ concentrations of 1 and 10 mM increasing the thermal stability to approximately the same extent. In the presence of 1 mM $MgCl_2$ LpL103 retained 85% of its activity after 4.5 h incubation at 42°C and the half-life time ($t_{½}$) of activity at this temperature was increased to approximately 20 h. Again, this compares very well to the data obtained for both enzymes L103 and EL103 at 42°C in the presence of 10 mM $MgCl_2$, for which a $t_{½}$ value of approximately 24 h was determined (*22, 23*).

Discussion

At present, *Escherichia coli* is the preferred bacterium for cloning genes and recombinant protein production. It is well characterized, many of its biological processes are understood, and a number of different genetic tools are available for molecular biology work. Yet, one single organism is unlikely to fulfill every need and to be suitable for every application (*38*). For food-related applications and the production of food-relevant enzymes, *E. coli* is probably not the first choice, and expression systems based on food-grade microorganisms, which have the 'generally regarded as safe' status, are of great interest for food applications (*39*). Lactic acid bacteria (LAB) are widely used in industrial fermentations, and therefore a wealth of information is available about their growth conditions and nutrient requirements. Furthermore, significant progress has been made in the genetic engineering tools and molecular characterization of these organisms, and therefore LAB can be highly attractive cell-factories for the production of recombinant enzymes but also as delivery vehicles for

proteins such as antibodies or antigens (*40*). Several inducible and controlled expressions systems based on LAB as host organisms have been developed, with the nisin-controlled gene expression system (NICE) in *Lactococcus lactis* being probably the best known (*12, 41*). In this paper we studied a related bacteriocin-based expression system for lactobacilli (*2, 15, 16*) using *L. plantarum* WCFS1 and *L. sakei* Lb790 as expression hosts. The background β-galactosidase activities of the chromosomal β-galactosidases of these two strains when grown on glucose were found to be negligible, hence the activities reported in this paper can be considered as originating exclusively from homologous or heterologous expression of the introduced β-galactosidase genes. These β-galactosidase genes originated from four *Lactobacillus* species, *L. reuteri* L103, *L. acidophilus* R22, *L. plantarum* WCFS1 and *L. sakei* Lb790. These were introduced into two different expression vectors, pSIP403 and pSIP409, differing in the promoters P_{sppA} and P_{sppQ} (formerly P_{orfX}). No significant differences were found when comparing the levels of β-galactosidase activity obtained with these two promoters (Fig. 3). This is in clear contrast to recent results, where the P_{sppQ} (P_{orfX}) promoter resulted in considerably higher expression levels of β-glucuronidase from *E. coli* (GusA) and aminopeptidase from *L. lactis* (PepN). Apparently, the performance of this expression system depends on the combination of the gene of interest, the promoter, the replicon, and the host strain used (*2*).

Protein expression was induced by the addition of 25 ng/ml of the peptide pheromone. This concentration was previously found to result in maximum production of PepN when the corresponding gene was under control of the P_{sppA} promoter (*15*). Addition of the inducing pheromone was clearly necessary for the efficient high-level production of the recombinant β-galactosidases, as the basal activities found under non-induced conditions are significantly lower (Table 3). These basal activities were always somewhat higher in *L. plantarum* than in *L. sakei* when compared for identical expression plasmids, and as a consequence, the *L. sakei* variants showed higher induction factors ranging from 37 to 240, compared to values of 13 to

183 obtained for the *L. plantarum* variants. Similarly, the P_{sppQ} promoter seemed to be more tightly regulated than the P_{sppA} promoter as it consistently gave higher induction factors (Table 3) for both expression hosts carrying pSIP409-based constructs. These high induction factors indicate that β-galactosidase expression is tightly controlled by the two promoters used. Both of these promoters precede 'product' genes, i.e., sakacin structural genes, in the sakacin P gene clusters of *L. sakei* (*34*), and these were found to be most tightly regulated (*10*).

Significant differences between the expression levels of the four β-galactosidase genes were, however, observed. These genes are almost identical in size (Table 1), vary in GC content from 36.8 to 47.2%, and show high identity on a nucleotide basis. Activities of the recombinant enzymes that are encoded by these genes and produced in the expression systems studied were significantly lower for the two *lacLM* genes from *L. sakei* and *L. acidophilus* and for both promoters used, even when the *L. sakei lacLM* gene was homologously overexpressed in *L. sakei*. Activities of the products from the β-galactosidase genes from *L. plantarum* and *L. reuteri* were considerably higher, again essentially independent of the two promoters with slightly higher activities obtained in *L. plantarum*. The four heterodimeric β-galactosidases considered in this study share 58–74% identity on an amino acid basis and are very similar with respect to their activity (*21, 22*). Hence the differences found pertaining to the β-galactosidase activities formed can be attributed to varying levels of protein synthesized for the four different *lacLM* genes studied. Since similar results — significantly higher activities for the expression of the *L. reuteri* and *L. plantarum lacLM* genes — were obtained for both expression hosts and both expression plasmids, the difference in the expression levels must be caused by the structural genes themselves. Important factors for efficient expression of heterologous genes are codon usage and GC content of the genes of interest, amongst others. As the *lacLM* genes are derived from closely related lactobacilli, codon usage should obviously not play a major role. Also, no correlation could be found between the GC content

of the genes studied and their expression efficiency in the two hosts *L. plantarum* and *L. sakei*. The *lacLM* genes of *L. reuteri* (accession number DQ493596) and *L. plantarum* (accession number AL935262), which both express well, have a GC content of 42.0 and 47.2%, respectively, while that of the *lacLM* genes of *L. acidophilus* (accession number EF053367) and *L. sakei* (accession number X82287) are 36.8 and 44.3%, respectively. Again, the performance of the expression systems studied seems to depend strongly on the combination of the gene of interest, the promoter, the replicon, and the host strain used.

The highest expression levels were found for the expression host *L. plantarum* WCFS1 and the plasmid pSIP403 containing the *lacLM* gene from *L. reuteri*, and therefore this system was studied in more detail. A simple cultivation in a 1-liter bottle yielded approximately 23 kU of β-galactosidase activity per liter of cultivation. The high-level expression is further evident from the fact that this constitutes 55% of the total intracellular protein of *L. plantarum*. This compares favorably with the highest levels of protein production of to 50% reported for the related and much better studied NICE expression system (*12*). The highest yields of 23 kU per l are furthermore considerably higher than the maximal value of 2.5 kU per l of fermentation broth reported for the wild-type *L. reuteri* L103 strain (*22*). This represents a nine-fold increase in the production of recombinant β-galactosidase activity.

Purification of the recombinant β-galactosidase was straightforward. Because of the high specific activity of the crude recombinant enzyme of 136 U/mg, one single step of purification using affinity chromatography on *p*-aminobenzyl 1-thio-*β*-D-galactopyranoside agarose was sufficient for the purification of the protein to apparent homogeneity. This single purification step of recombinant β-galactosidase LpL103 is a clear advantage compared to the three-step-purification protocol based on ammonium sulphate precipitation, hydrophobic interaction chromatography, and affinity chromatography used for the wild-type β-galactosidase from *L. reuteri* L103, which was obtained with a significantly lower specific activity of 9.6 U/mg (*22*).

Properties of the recombinant β-galactosidase produced in *L. plantarum* were found to be essentially the same as those of the wild-type enzyme. Recently, we also reported the application of β-galactosidase from *L. reuteri* for the production of galacto-oligosaccharides GOS (*29, 42*). The spectrum and the yield of GOS produced from lactose using recombinant β-galactosidase LpL103 are similar to GOS formed by native β-galactosidase L103 (data not shown). The main products formed are β-D-Gal*p*-(1→6)-D-Glc (allolactose), β-D-Gal*p*-(1→6)-D-Gal, β-D-Gal*p*-(1→3)-D-Gal, β-D-Gal*p*-(1→3)-D-Glc, β-D-Gal*p*-(1→6)-Lac, β-D-Gal*p*-(1→3)-Lac (*29*).

In conclusion, we showed the efficient and inducible expression of recombinant β-galactosidases in *L. plantarum* and *L. sakei* using expression vectors based on bacteriocin operons found in *L. sakei* strains. These vectors are simple to use and can easily be adapted for a particular purpose, such as production of food-grade enzymes, by exchanging one or more of the 'cassettes' in the vector, e.g., the antibiotic markers used for selection. As lactobacilli are widely used in food industry, these efficient and tightly regulated expression systems for the overproduction of enzymes and proteins can be of great interest.

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Figure captions

Figure 1

Schematic overview of the pSIP-vector used in this study.

Light-grey regions, replication determinants; dark-grey regions, erythromycin resistance marker; vertically hatched regions, histidine kinase (*sppK*) and response regulator (*sppR*) genes; horizontally hatched regions, structural gene (*lacLM*); dotted region, inducible bacteriocin promoters; white region, inducible P_{sppA} promoter for pSIP403 and P_{sppQ} (formerly P_{orfX}) promoter for pSIP409; lollypop structures, transcriptional terminator; black box, multicloning site (Sørvig *et al.*, 2005).

Figure 2

SDS-PAGE of whole-cell lysates of *L. plantarum* WCFS1 (panel A) and *L. sakei* Lb790 (panel B) carrying the plasmids containing the complete β-galactosidase genes (*lacL* and *lacM*) from *L. reuteri* L103, *L. acidophilus* R22, *L. plantarum* WCFS1 and *L. sakei* Lb790. Recombinant β-galactosidase formation was induced by the addition of 25 ng/ml pheromone peptide and cells were harvested at OD_{600} of 1.8. Gels were stained by using Coomassie blue. Panel A: Lane 1, Benchmark protein ladder (Invitrogen); *L. plantarum* carrying pSIP403LacLM-Reu (lane 2), pSIP409LacLM-Reu (lane 3), pSIP403LacLM-Aci (lane 4), pSIP409LacLM-Aci (lane 5), pSIP403LacLM-Plant (lane 6), pSIP409LacLM-Plant (lane 8), pSIP403LacLM-Sak (lane 9), pSIP409LacLM-Sak (lane 10); and pSIP409LacLM-Aci without induction (lane 7). (Reu: *L. reuteri*, Aci: *L. acidophilus*, Plant: *L. plantarum*, Sak: *L. sakei*)

Panel B: *L. sakei* carrying pSIP403LacLM-Reu (lane 1), pSIP409LacLM-Reu (lane 3), pSIP403LacLM-Aci (lane 4), pSIP409LacLM-Aci (lane 6), pSIP403LacLM-Plant (lane 7), pSIP409LacLM-Plant (lane 8), pSIP403LacLM-Sak (lane 9), pSIP409LacLM-Sak (lane 10) with 25 ng/ml pheromone induction and cells were harvested at OD_{600} of 1.8; and pSIP409LacLM-Aci without induction (lane 2); lane 5, Precision Plus Protein (BioRad). (Reu: *L. reuteri*, Aci: *L. acidophilus*, Plant: *L. plantarum*, Sak: *L. sakei*)

Figure 3

Specific activities of β-galactosidases from *L. reuteri* L103, *L. acidophilus* R22, *L. plantarum* WCFS1, *L. sakei* Lb790 overexpressed in *L. sakei* Lb790 and *L. plantarum* WCFS1. The cultures were induced with 25 ng/ml pheromone and the cells were harvested when the OD_{600} of the cultures reached 1.8. All data are the mean of three independent experiments; the error bars indicate the standard deviations. Dark bars indicate *L. sakei* Lb790 as expression host, while lighter bars show data obtained when the enzymes were expressed in *L. plantarum* WCFS1.

Figure 4

4 A)

SDS-PAGE of β-galactosidase (*lacLM*) from *L. reuteri* L103 overexpressed in *L. plantarum* WCFS1 and *E. coli* BL21 Star (DE3).

Lane 1, recombinant molecular weight markers, (dual colour, Biorad); lane 2, Coomassie blue staining of purified LpL103 β-galactosidase overexpressed in *L. plantarum*; lane 3, Coomassie blue staining of purified EL103 β-galactosidase overexpressed in *E. coli*; lane 4, recombinant molecular weight markers (unstained, Biorad); active staining with 4-methylumbelliferyl β-D-galactoside of recombinant EL103(lane 5), and LpL103 (lane 6) β-galactosidases.

4 B)

Native PAGE of purified β-galactosidase (*lacLM*) from *L. reuteri* L103 overexpressed in *L. plantarum* WCFS1 and *E. coli* BL21 Star (DE3).

Lane 1, high molecular weight marker (Amersham); lanes 2 and 3, Coomassie blue staining of purified recombinant LpL103 (lane 2) β-galactosidases and EL103 (lane 3); lanes 4 and 5, active staining with 4-methylumbelliferyl β-D-galactoside of recombinant LpL103 (lane 4) and EL103 (lane 5) β-galactosidases.

Figure 5

pH stability of native L103 and recombinant EL103, LpL103 β-galactosidases. Residual activity after an incubation for 3 h at 37°C in buffers with different pH.

Figure 6

Temperature stability of native L103 and recombinant EL103, LpL103 β-galactosidases. Residual activity after an incubation for 48 h at different temperatures in sodium phosphate buffer at pH 6.0.

Tables

Table 1 Sequences of the primers used in this study

Primer	Sequence (5´→ 3´)	Location	Reference sequence accession no.
LacReuF	**GGTCTCAC**ATGCAAGCAAATATAAAAT	a	DQ493596
LacReuR	**CTCGAG**TTATTTTGCATTCAATACAAAC	b	DQ493596
SeqReuF	CCCTCTGAATTTGACCTGACT	β-gal	DQ493596
SeqReu2F	CCGTCACGAATGGAATGCT	β-gal	DQ493596
SeqReu3F	GAGGAGTACTTGAAGAACAATC	β-gal	DQ493596
SeqReu4F	CCGAGGAAGTGGCTTTAAT	β-gal	DQ493596
LacAciF	**GGTCTCTC**ATGCAAGCAAACATAAAAT	c	EF053367
LacAciR	GG**GAATTC**TTAATTAAGATTGAAAGAAAATTCGT	d	EF053367
SeqAciF	CCGTCGTCCTGCTTACGC	β-gal	EF053367
SeqAci2F	GGCTTGAAGATCAAGACATGT	β-gal	EF053367
SeqAci3F	CGCCATGAATGGAATGCCAAAAC	β-gal	EF053367
SeqAci4F	GAGTGTGAATACATGCATGAC	β-gal	EF053367
SeqAci5F	GCACTAAAATCGAATTAAAGGTAGAT	β-gal	EF053367
LacLMPlaF	**GGTCTCTC**ATGCAAGCTAATCTTCAAT	e	AL935262
LacLMPlaR	**CTCGAG**TTAGAAATGAATATTAAAGCT	f	AL935262
SeqPlanF	GGCCTATTCAGCGACGCT	β-gal	AL935262
SeqPlan2F	GGAGCCTGCCAGCCATATT	β-gal	AL935262
SeqPlan3F	GCGATGACAAAGTCATTTACGT	β-gal	AL935262
SeqPlan4F	GCTTACTTGGAAGATAACCC	β-gal	AL935262
SeqPlan5F	GGCGACAACCGATAATGAT	β-gal	AL935262
SeqPlan6F	GCATGCACATGCAAACTGAAC	β-gal	AL935262
Lb790LacF	**GGTCTCTC**ATGCAACCTAATATTCAAT	g	X82287
Lb790LacR	**CTCGAG**TTAAAACGAAATTTCAAATTCAAAAT	h	X82287
SeqSakF	GCGTCCCGCTTTTTCAACGT	β-gal	X82287
SeqSak2F	GCAGTAGTGCGGCTTTCAT	β-gal	X82287
SeqSak3F	GGCGTTAATCGGCATGAAT	β-gal	X82287
SeqSak4F	GCCCCACAAAAACCATTTATT	β-gal	X82287
SeqSak5F	GCGGCAACCAATTCCCATT	β-gal	X82287
SeqSak6F	GCTACCTGTGACGCCTTAT	β-gal	X82287
pSekF	GGCTTTTATAATATGAGATAATGCCGAC	pSIPvector	Sørvig et al., 2003
pSIPseqR	TCTATTTAGGGTATTCCCGCC	pSIPvector	Sørvig et al., 2003

F: denotes forward primers; R: denotes reverse primers

[a] Upstream primer to amplify *lacLM* from pHA1031 with *Bsa*I site (bold, underlined)

[b] Downstream primer to amplify *lacLM* from pHA1031 with *Xho*I site (bold, underlined)

[c] Upstream primer to amplify *lacLM* from pHAR22 with *Bsa*I site (bold, underlined)

[d] Downstream primer to amplify *lacLM* from pHAR22 with *EcoR*I site (bold, underlined)

[e] Upstream primer to amplify *lacLM* from genomic DNA *L. plantarum* WCFS1 with *Bsa*I site (bold, underlined)

[f] Downstream primer to amplify *lacLM* from genomic DNA *L. plantarum* WCFS1 with *Xho*I site (bold, underlined)

[g] Upstream primer to amplify *lacLM* from genomic DNA *L. sakei* Lb790 with *Bsa*I site (bold, underlined)

[h] Downstream primer to amplify *lacLM* from genomic DNA *L. sakei* Lb790 with *Xho*I site (bold, underlined)

Table 2 Number of base pairs and calculated molecular masses for the large (lacL) and the small (lacM) subunits of β-galactosidases from different lactobacilli

Source of β-galactosidase	lacL		lacM		Accession number
	Base pairs	Molecular mass [Da]	Base pairs	Molecular mass [Da]	
L. reuteri L103	1,887	73,492	960	35,683	DQ493596
L. acidophilus R22	1,887	73,254	951	35,590	EF053367
L. plantarum WCFS1	1,881	72,180	960	35,223	AL935262
L. sakei Lb790	1,878	72,457	993	36,742	X82287

Table 3. Specific β-galactosidase activity obtained in non-induced and induced (25 ng inducing peptide per ml) cultures of *L. plantarum* WCFS1 and *L. sakei* Lb790 containing various expression plasmids. Data given are the average and the standard deviation of three independent experiments. The induction factor was calculated from the maximum β-galactosidase activity obtained under induced conditions divided by the maximum β-galactosidase activity under non-induced conditions.

Strain	Specific activity [U/mg]		Induction factor
	without induction	with induction	
WCFS1 (p403Reu)	1.610 ± 0.195	61.1 ± 7.2	38.0
Lb790 (p403Reu)	0.213 ± 0.061	22.3 ± 6.2	104
WCFS1 (p409Reu)	0.290 ± 0.031	54.5 ± 7.0	183
Lb790 (p409Reu)	0.123 ± 0.006	29.5 ± 4.0	240
WCFS1 (p403Aci)	0.273 ± 0.015	4.21 ± 0.47	15.4
Lb790 (p403Aci)	0.130 ± 0.000	7.42 ± 1.06	57.1
WCFS1 (p409Aci)	0.057 ± 0.006	2.96 ± 1.22	51.9
Lb790 (p409Aci)	0.043 ± 0.006	4.32 ± 0.81	100
WCFS1 (p403Pla)	1.203 ± 0.049	49.1 ± 10.9	40.8
Lb790 (p403Pla)	0.267 ± 0.021	10.7 ± 4.4	40.1
WCFS1 (p409Pla)	0.360 ± 0.026	44.8 ± 1.3	124
Lb790 (p409Pla)	0.230 ± 0.017	18.5 ± 2.9	80.4
WCFS1 (p403Sak)	0.376 ± 0.065	4.87 ± 0.48	13.0
Lb790 (p403Sak)	0.060 ± 0.010	2.23 ± 0.22	37.2
WCFS1 (p409Sak)	0.320 ± 0.010	5.23 ± 0.39	16.3
Lb790 (p409Sak)	0.106 ± 0.006	6.91 ± 0.63	65.2

Figures

Figure 1

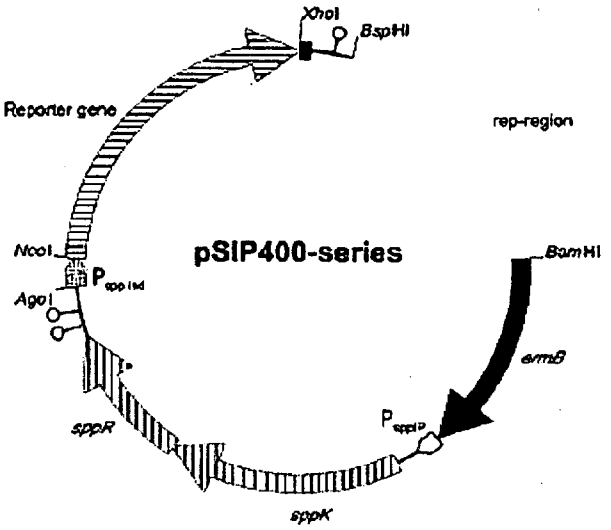
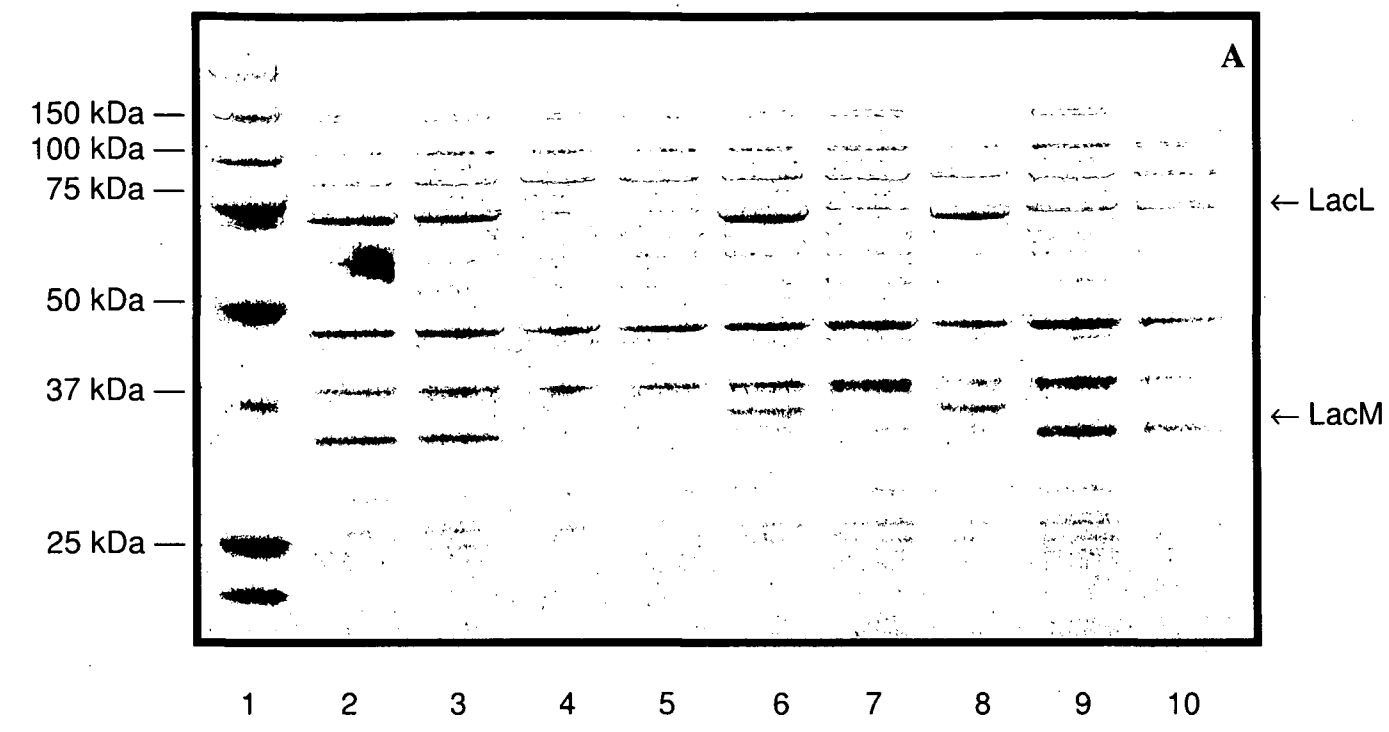


Figure 2


A
150 kDa —
100 kDa —
75 kDa —
50 kDa —
37 kDa —
25 kDa —
← LacL
← LacM
1 2 3 4 5 6 7 8 9 10

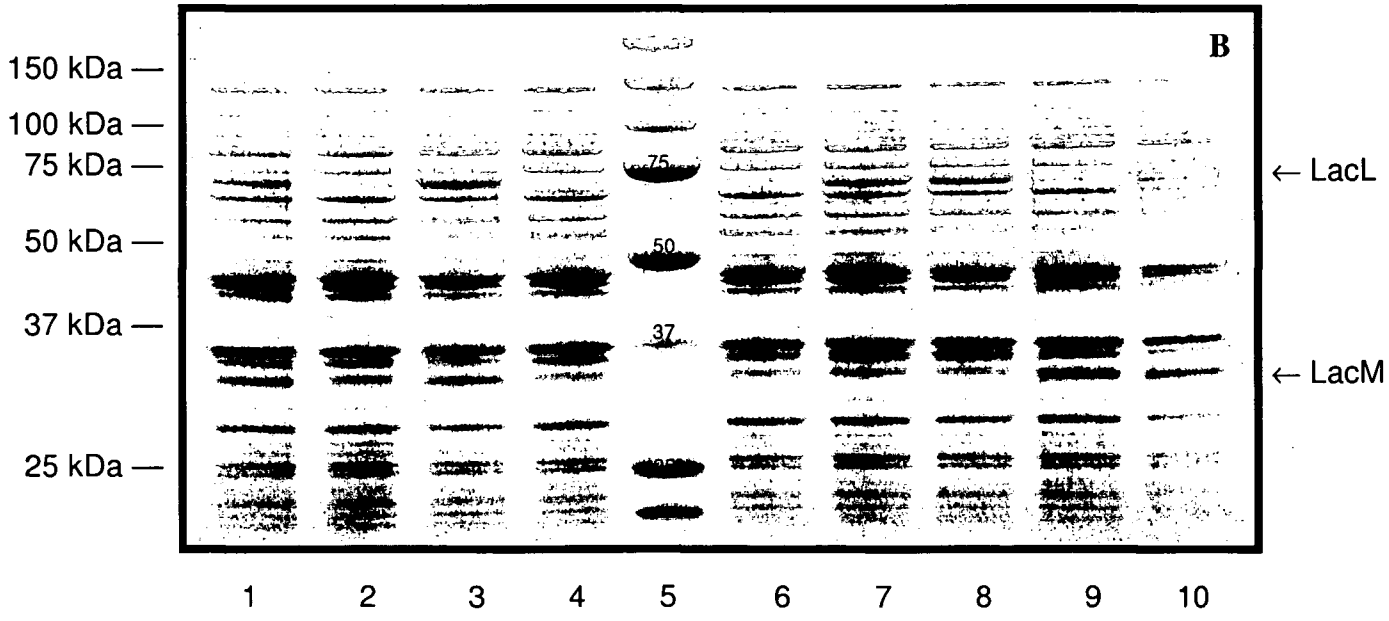

B
150 kDa —
100 kDa —
75 kDa —
50 kDa —
37 kDa —
25 kDa —
75
50
37
← LacL
← LacM
1 2 3 4 5 6 7 8 9 10

Figure 3

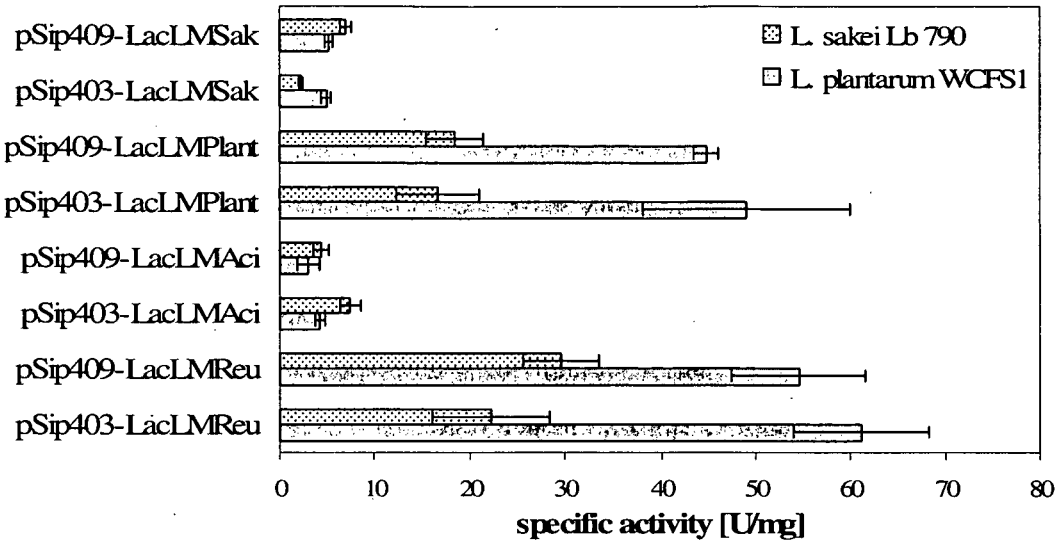

pSip409-LacLMSak
pSip403-LacLMSak
pSip409-LacLMPlant
pSip403-LacLMPlant
pSip409-LacLMAci
pSip403-LacLMAci
pSip409-LacLMReu
pSip403-LacLMReu
L. sakei Lb 790
L. plantarum WCFS1
0
10
20
30
40
50
60
70
80
specific activity [U/mg]

Figure 4 A

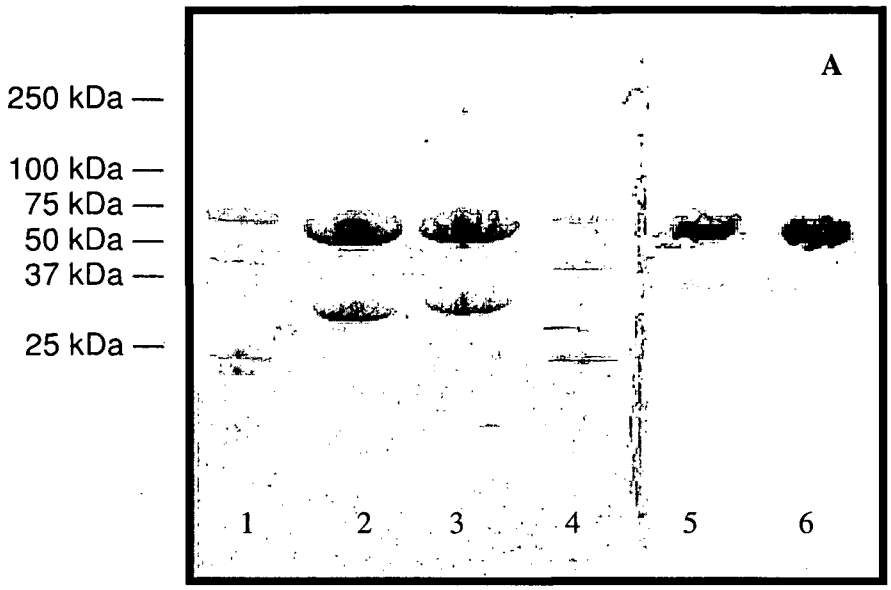


Figure 4 B

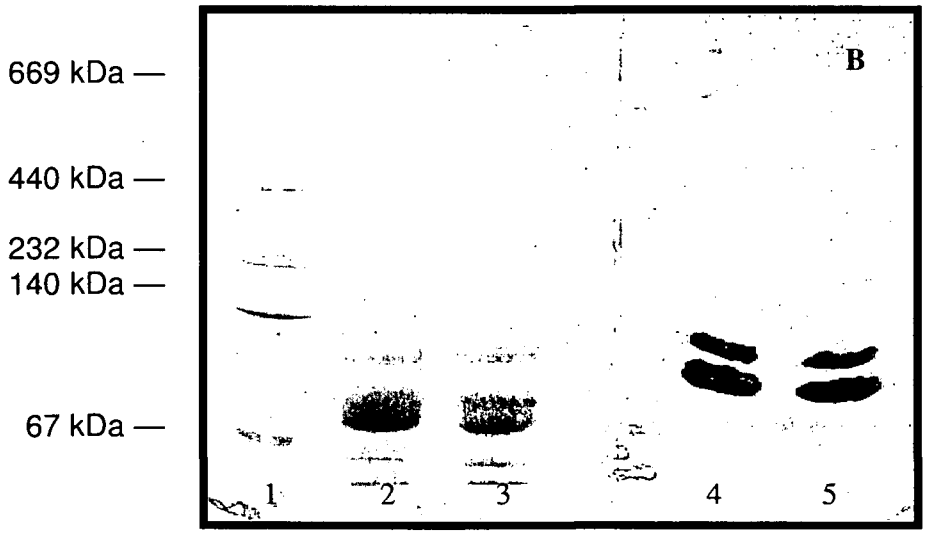


Figure 5

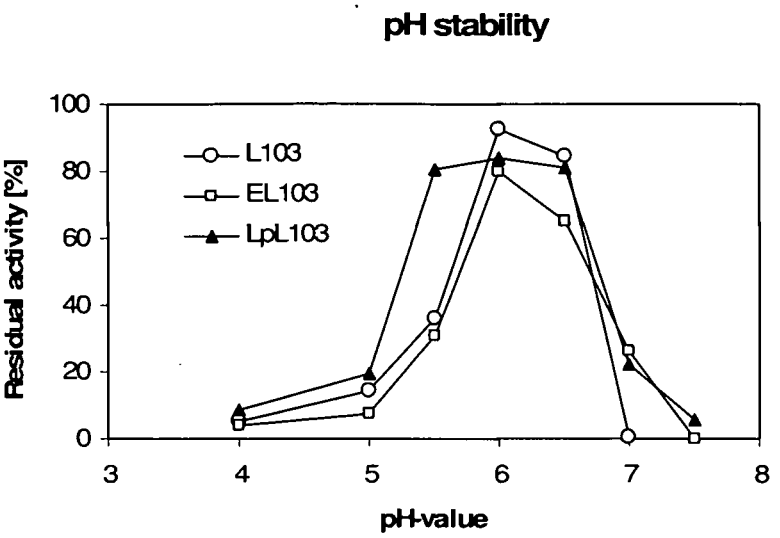
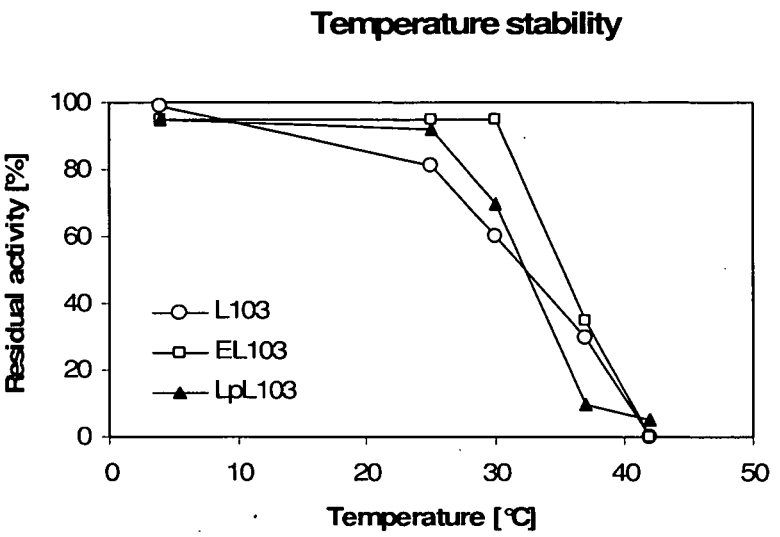

pH stability
Residual activity [%]
100
80
60
40
20
0
3
4
5
6
7
8
pH-value
L103
EL103
LpL103

Figure 6


Temperature stability
Residual activity [%]
100
80
60
40
20
0
0
10
20
30
40
50
Temperature [°C]
L103
EL103
LpL103

A1 The vector system

A1.1 Schematic overview of the pSIP-vector system

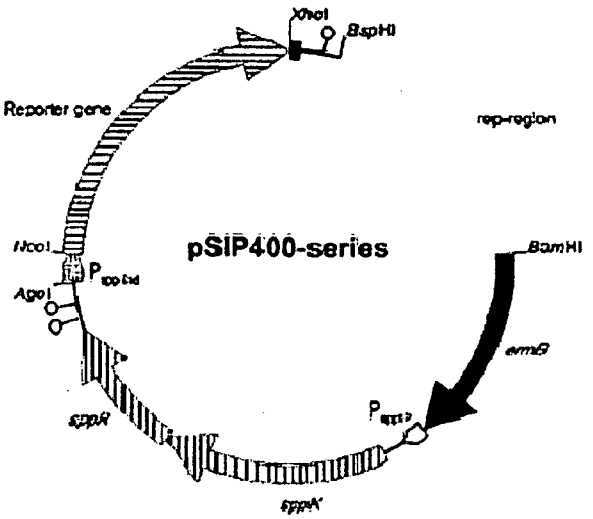


Figure A 1: pSIP-vectors used in this study

Light-grey regions, replication determinants; dark-grey regions, erythromycin resistance marker; vertically hatched regions, histidine protein kinase (*sppK*) and response regulator (*sppR*) genes; horizontally hatched regions, structural (reporter) gene (*lacLM*); dotted region, inducible bacteriocin promoters; white region, inducible P_{sppA} promoter for pSIP403 and P_{orfX} promoter for pSIP409; lollypop structures, transcriptional terminator; black box, multicloning site (Sørvig *et al.*, 2005)

A1.2 Induction of the pSIP-vector

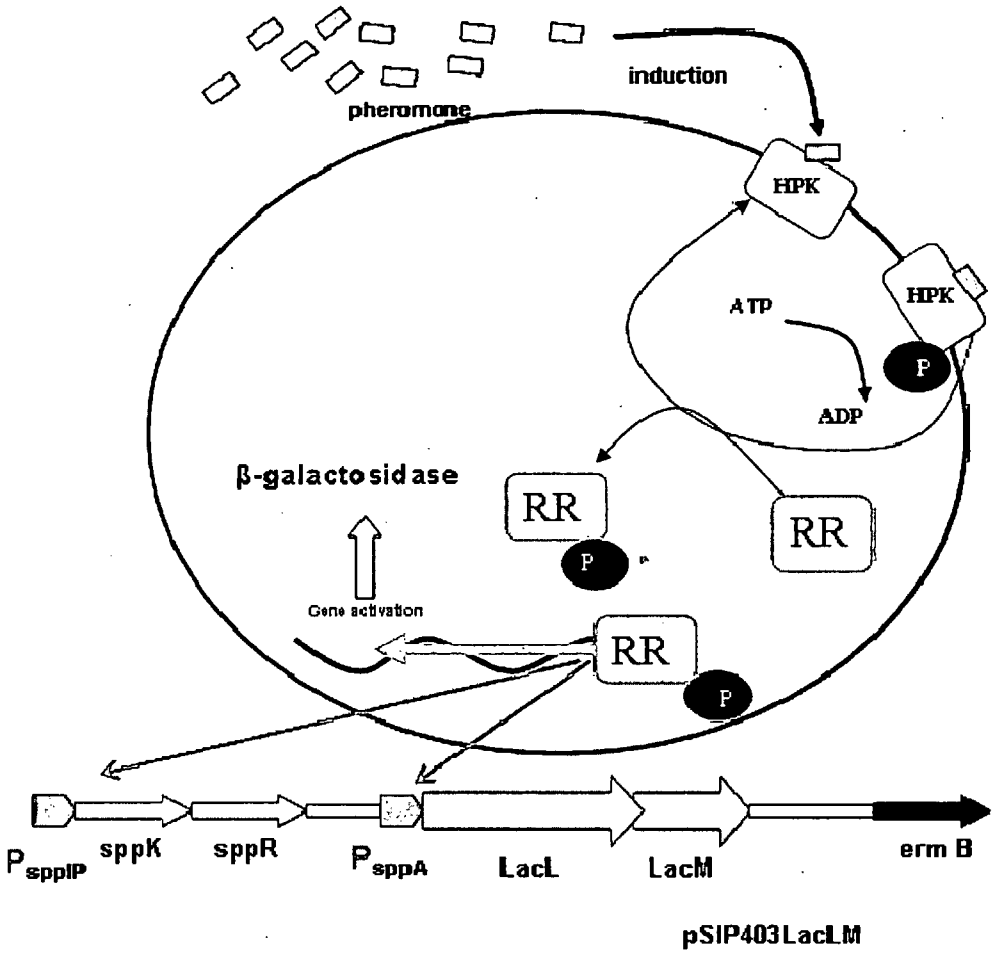


Figure A 2: Schematic overview of the induction

RR	Response Regulator (*sppR*)
HPK	Histidin Kinase *(sppK)*
	Pheromone
P	Phosphate
P_{sppIP}	inducible promotor
P_{sppA}	inducible promotor
LacLM	genes for β-galactosidase
erm B	erythromycin resistance gene

For the induction pheromone is used. When pheromone is added to the media, it binds to the receptor histidine-kinase (*sppK*) and that gets phosphorylated. Thus the response regulator (*sppR*) is phosphorylated and can bind to the DNA. The promotors are activated and the gene of interest can be transcribed, in this case β-galactosidase is produced.

A2 Materials and methods

A2.1 Buffers

Sodium phosphat buffer (NaPP):

50 mM sodium phosphate ($NaH_2PO_4.2H_2O$) buffer, pH 6.5

"Sonication buffer":

50 mM sodium phosphate buffer, pH 6.5

20% glycerol

1 mM DTT

TEN-buffer:

10mM Tris-HCl, pH8

1 mM EDTA, pH 8

100 mM NaCl

GTE-buffer:

50 mM glucose

25 mM Tris-HCl, pH 8

10 mM EDTA, pH 8

A2.2 Media

S.O.C. medium: (was used for transforming *E. coli* One Shot®TOP10 competent cells)

bacto-tryptone 20 g/L

bacto yeast extract 5 g/L

sodium chloride 0.57 g/L

potassium chloride 0.19 g/L

magnesium sulphate 2.47 g/L

glucose 3.6 g/L

MRSSM: (was used for transforming *L. plantarum* WCFS1 and *L. sakei* Lb790)

MRS broth powder 52 g/L

0.5 M sucrose

$MgCl_2x6H_2O$ 20 g/L

A2.3 Cultivation of the strains

Overnight cultures:

10 mL MRS + erythromycin (final concentration of 5 µg/mL)

in 15 mL plastic tubes capped tightly and without shaking

incubation temperature 30°C/37°C

Cultivation conditions:

Prewarmed (30°C) MRS broth containing 5 µg/mL erythromycin was inoculated with the overnight culture. 50 mL plastic tubes capped tightly were used. Optimum growing conditions for *L. plantarum* and *L. sakei* are 30 °C and no shaking.

Induction:

Cells were induced at OD_{600nm} of 0.3 with pheromone solution (final concentration of 25 ng/mL). After adding the pheromone solution the tubes were inverted once for mixing.

A2.4 Cell harvesting

Procedure:

After OD_{600nm} reached required value (1.8) the tubes were put on ice and centrifuged 5 min at 5200 rpm and 4°C followed by a washing step with 10 mL NaCl (0.9%) 5 min at 5200 rpm and 4°C. The cell pellet was resuspended in 1 mL buffer (sonication buffer) and transferred to a 2 mL eppi.

Sonication:

Sonicator:

Sonics vibra-cell

Sonics and Materials Inc, USA

Modell VCX500

Power 500 W

Frequence 20 kHz

Fuse size: 8AMPS SLO-BLO

Set up:

Amplitude 25%

Pulse 5 sec on 5 sec off

Eppis on ice during whole sonication procedure

Probe 0.5 cm under surface of sample

A2.5 Assays

A2.5.1 Protein determination – Method of Bradford

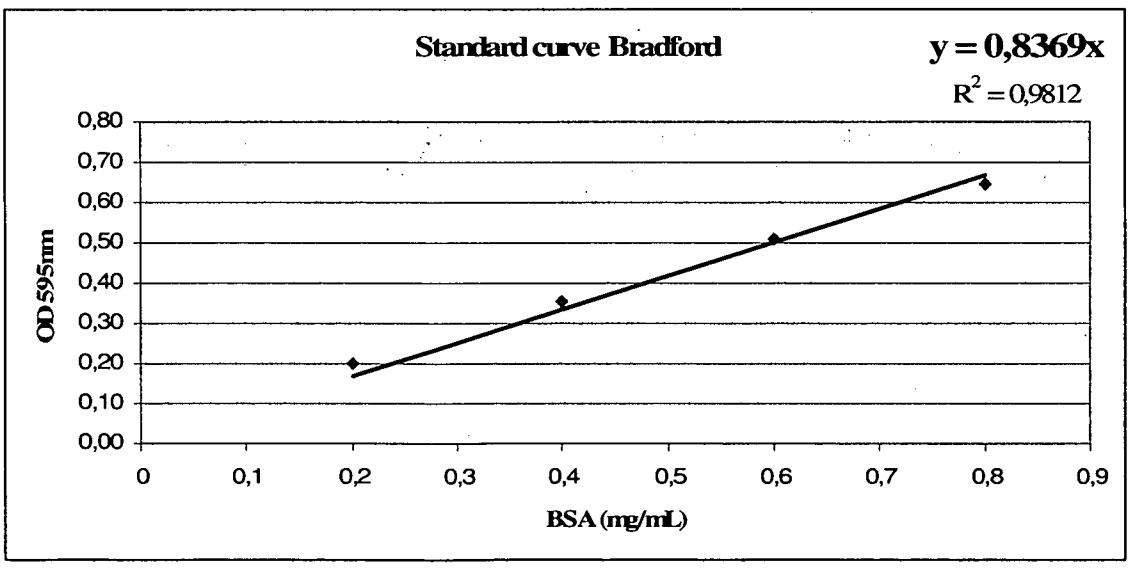


Figure A 3: Standard curve method of Bradford

A2.5.2 β-Galactosidase assay with *o*NPG as substrate

Materials:

1) 50 mM sodium phosphate buffer, pH 6.5

$NaH_2PO_4x2H_2O$ (MW=156.01 g/mol)

1000 mL.....7.8005 g of $NaH_2PO_4x2H_2O$ is required

Correct pH with 4M NaOH

2) 22 mM *o*NPG (in 50 mM sodium phosphate buffer, pH 6.5)

o-Nitrophenyl- β-D-galactopyranosides $C_{12}H_{15}NO_8$ (MW=301.3 g/mol)

25 mL buffer.....0.16571 g of $C_{12}H_{15}NO_8$ is required

Solution is very unstable and light sensitive, store (only a few days) in dark bottle at 4°C, if the solution gets yellow, prepare a new one!

3) 0.4 M Na_2CO_3 (MW= 105.99 g/mol)

1000 mL.....42.396 g of M Na_2CO_3 is required

4) *o*NP-standard solution (in 50 mM sodium phosphate buffer, pH 6.5)

o-Nitrophenol $C_6H_5NO_3$ (MW=139.1 g/mol)

2 mM *o*NP stock solution

50 mL buffer.....0.01391 g

Hard to dissolve (ultrasonic bath!)

Standard curve:

standard solution	1	2	3	4	5	6
stock solution *o*NP [mL]	0	1	2	3	4	5
sodium phosphate-buffer [mL]	5	4	3	2	1	0
concentration [mM]	0	0.4	0.8	1.2	1.6	2

- mix 0.5 mL standard solution and 0.75 mL Na_2CO_3
- measure absorption at wavelength of 420 nm, blank with distilled water or sodium phosphate buffer

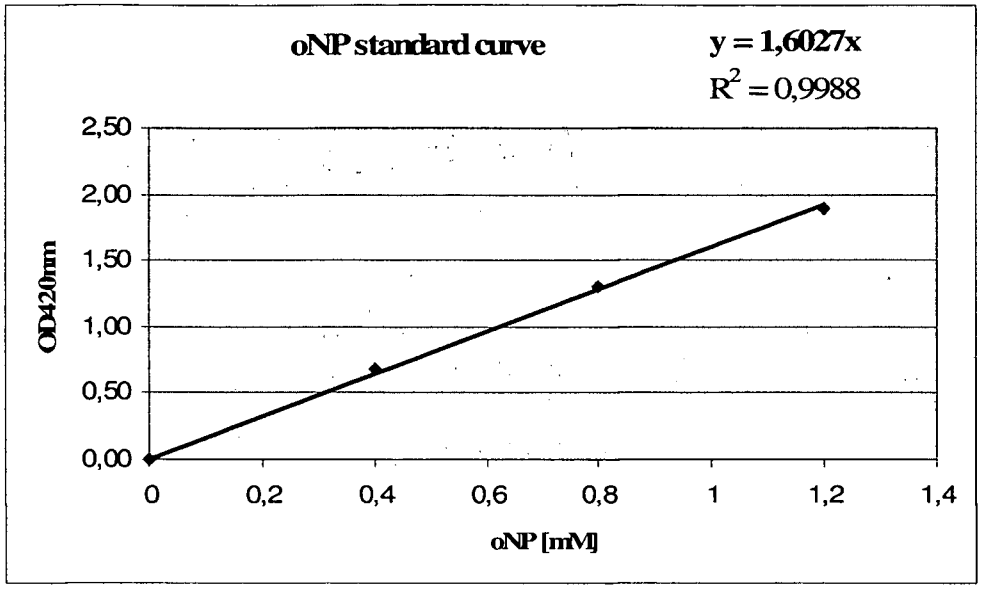


Figure A 4: Standard curve *o*NPG assay

β-Galactosidase activities from chapter A5 and following chapters were calculated with a different standard curve:

y= 1.1828x + 0.019 R^2= 0.9993

Enzyme assay:

β-Galactosidase splits *o*NPG in *o*NP and galactose, free *o*NP is measured at 420nm at alkaline conditions

Preparation:

Bring 480µL of 22 mM *o*NPG solution to 30°C (not for too long, otherwise it gets yellow)
Add 20µL of enzyme sample (reaction is started) - vortex - and incubate on the thermo mixer (30°C and 600 rpm) for exact 10 minutes
(In Norway a water bath at 30 °C and vortexed before and after the water bath - instead of the thermo mixer was used)
Stop reaction by adding 750 µL of Na_2CO_3 - vortex
Measure absorbance at 420 nm

Calculation:

$U/mL = OD_{420}/k \times 1/t \times[(V_{oNPG}+V_{enzyme})/V_{enzyme}] \times Dil$

U/mL	activity of β-galactosidase
OD_{420}	measured absorption at 420 nm
k	slope of the standard curve
t	reaction time (10 min)
V_{oNPG}	volume of oNPG (480µL)
V_{enzyme}	volume of enzyme sample (20 µL)
Dil	dilution factor

A2.5.3 β-Galactosidase assay with lactose as substrate

Materials:

1) 600 mM lactose (in 50 mM NaPP, pH 6.5)

2) GOD

2.77 g KH_2PO_4

32.1 mg 4-amino-anti-pyrine

mixed in 160 mL dH_2O and pH was adjusted to 7.0

20 mg glucoseoxidase (from *A. niger*)

1.25 mg horseradish peroxidase

Filled up to 200 mL

Stored in a dark bottle at 4°C

3) Phenol (560 mmol/L)

4) Glucose assay solution: 50 mL GOD + 1 mL phenol

Preparation:

When lactose was used as the substrate, 20 µL of enzyme solution was added to 480 µL of 600 mM lactose solution in 50 mM sodium phosphate buffer, pH 6.5. After 10 min of incubation at 30°C, the reaction was stopped by heating the reaction mixture at 99°C for 5 min. The reaction mixture was cooled to room temperature, and the release of D-glucose was determined colormetrically using the GOD/POD assay (Kunst *et al.*, 1988).
One unit of lactase activity was defined as the amount of enzyme releasing 1 µmol of D-glucose per minute under the given conditions.

For the glucose assay (GOD/POD assay) 600 µL assay solution and 60 µL sample were incubated in the dark at room temperature for 40 min, then OD_{546nm} was measured. Measured absorption should be in the range of 0.3-0.7.

Standard curve:

$y = 0.4514\ x$

Calculation:

$$U/mL = OD_{546}/k \times 1/t \times [(V_{lactose}+V_{enzyme})/V_{enzyme}] \times Dil$$

U/mL	activity of β-galactosidase
OD_{546}	measured absorption at 546 nm
k	slope of the standard curve
t	reaction time (10 min)
$V_{lactose}$	volume of 600 mM lactose (480µL)
V_{enzyme}	volume of enzyme sample (20 µL)
Dil	dilution factor

A3 Preliminary studies

When I started with the lab work for my diploma thesis I already had *L. plantarum* WCFS1 and *L. sakei* Lb790 carrying the *lacLM* genes from *L. reuteri* L103 in the pSIP403 vectors. My Norwegian supervisor Geir Mathiesen cloned these genes into these hosts.

Before I could start with more cloning work I had to answer some questions:

- What method of cell disruption should I use to be reproducible? Glass bead method or sonication? What is the best set-up for sonication?

- What is the best optical density at 600 nm to harvest the cells? What compromise between yield of protein and time for growing the cells should I accept?

- Is there active β-galactosidase expressed from the *lacLM* genes of the bacterial chromosome? Is there chromosomal background?

- Is β-galactosidase expressed from the vector when the system is not induced? Is there leakage of the induction system?

At the lab in Norway a bead mill was used for disrupting the cells. At BOKU sonication or French press is used. First I had to find out what conditions for sonication in Norway are the best to be reproducible, to get enough protein out of the cells and to save time.

A3.1 Test 1: Optimization of cell disruption conditions for highest protein concentrations

L. plantarum WCFS1 pSIP403LacLM-Reu and *L. sakei* Lb790 pSIP403LacLM-Reu were grown and harvested in 5 parallel samples.

The 5 pellets for each strain were treated differently:

Table A 1: Treatment of the cell pellets

Pellet	Buffer (1 mL)	Pre-treatment*	Cell disruption
1	NaPP	no	sonication
2	NaPP	yes	sonication
3	Sonication buf.	no	sonication
4	Sonication buf.	yes	sonication
5	NaPP	no	Glass bead mill

***Pre-treatment**:

Mutanolysin (100 U/mL) and lysozyme (6 mg/mL) were added to the resuspended pellet and incubated at 37°C in a water bath for 45 min.

Glass bead mill:

Fast Prep FP 120

Bio 101 Sorvant

Bead mill set up:

45 sec programme 6 (shaking)

For the bead mill procedure, the samples were transferred to a special eppi and 0.5 cm glass beads were added before the treatment in the bead mill.

For the samples that were treated with the sonicator, samples were taken after every 2 min (1, 3,.....19 min) of sonication.

All samples were centrifuged at 13200 rpm for 5 min at 4°C using a table centrifuge. Each supernatant (which is the crude extract of proteins) was filled in a new eppi.

Determination of protein concentration with method of Bradford was performed with all samples.

Results of test 1:

Table A 2: Results of test 1 – sonication *L. plantarum* WCFS1 pSIP403LacL-Reu

OD 600nm	Pellet 1			Pellet 2		
	1.65			1.7		
sonication time [min]	OD 595nm	1 protein conc. [mg/mL]	1 protein conc. /OD600	OD 595nm	2 protein conc. [mg/mL]	2 protein conc. /OD600
1	0.582	0.695	0.421	2.53	3.023	1.778
3	1.75	2.091	1.267	2.66	3.178	1.870
5	2.65	3.166	1.919	2.71	3.238	1.905
7	4.53	5.413	3.281	3.19	3.812	2.242
9	4.37	5.222	3.165	2.91	3.477	2.045
11	5.41	6.464	3.918	3.75	4.481	2.636
13	6.01	7.181	4.352	3.66	4.373	2.573
15	6.,07	7.253	4.396	3.47	4.146	2.439
17	5.85	6.990	4.236	3.63	4.337	2.551
19	6.86	8.197	4.968	3.67	4.385	2.580
OD 600nm	Pellet 3			Pellet 4		
	1.6			1.65		
sonication time [min]	OD 595nm	3 protein conc. [mg/mL]	3 protein conc. /OD600	OD 595nm	4 protein conc. [mg/mL]	4 protein conc. /OD600
1	0.87	1.040	0.650	2.47	2.951	1.789
3	2.5	2.987	1.867	2.94	3.513	2.129
5	4.11	4.911	3.069	3.13	3.740	2.267
7	4.87	5.819	3.637	3.48	4.158	2.520
9	5.47	6.536	4.085	3.66	4.373	2.650
11	6.2	7.408	4.630	4.67	5.580	3.382
13	6.72	8.030	5.019	4.73	5.652	3.425
15	6.09	7.277	4.548	4.5	5.377	3.259
17	6.48	7.743	4.839	4.22	5.042	3.056
19	7.23	8.639	5.399	5.01	5.986	3.628

Table A 3: Results of test 1 - bead mill *L. plantarum* WCFS1 pSIP403LacLM-Reu

OD 600nm	Pellet 5		
	1.8		
	OD 595nm	5 protein conc. [mg/mL]	5 protein conc./OD600
	3.87	4.624	2.569

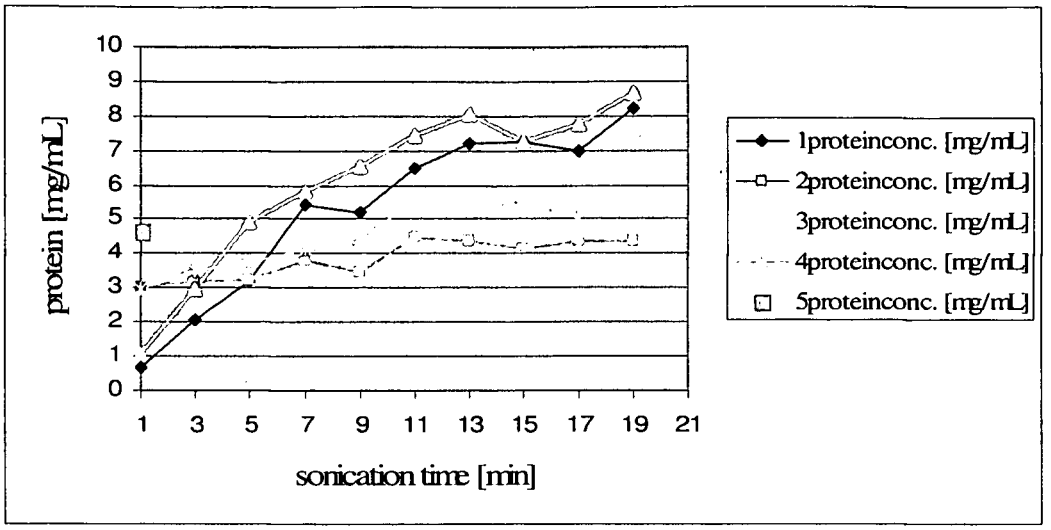


Figure A 5: Results of test 1 for *L. plantarum* WCFS1 pSIP403LacLM-Reu in term of protein concentration [mg/mL]

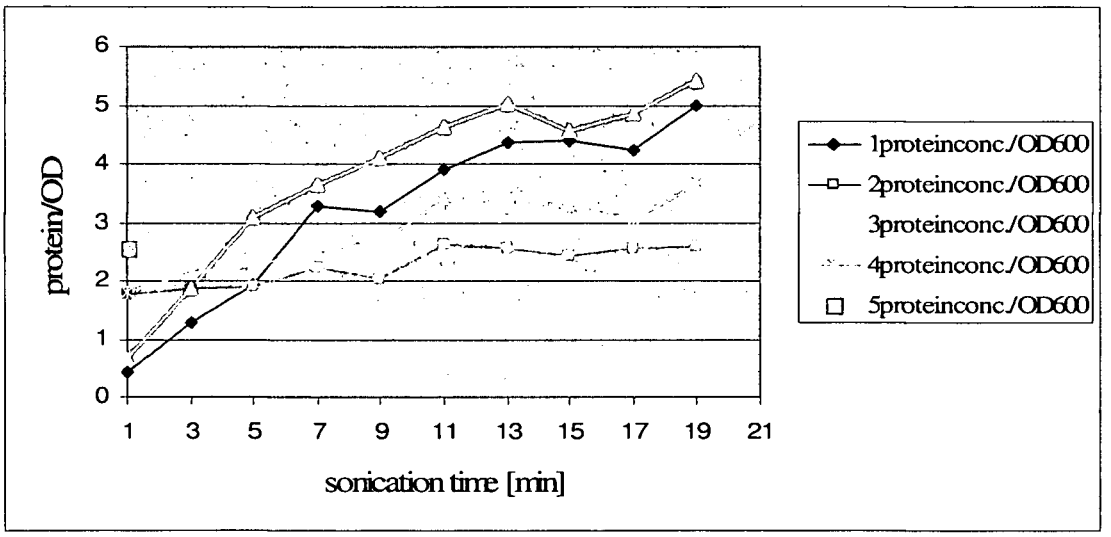


Figure A 6: Results of test 1 for *L. plantarum* WCFS1 pSIP403LacLM-Reu in term of protein concentration divided by OD600nm

Table A 4: Results of test 1 - sonication *L. sakei* Lb790 pSIPLacLM-Reu

OD 600nm	**Pellet 1**			**Pellet 2**		
	1.3			1.25		
sonication time [min]	**OD 595nm**	**1 protein conc. [mg/mL]**	**1 protein conc. /OD600**	**OD 595nm**	**2 protein conc. [mg/mL]**	**2 protein conc. /OD600**
1	0.676	0.808	0.621	2.59	3.095	2.476
3	1.81	2.163	1.664	2.67	3.190	2.552
5	2.51	2.999	2.307	3.16	3.776	3.021
7	2.92	3.489	2.684	2.74	3.274	2.619
9	4.28	5.114	3.934	2.58	3.083	2.466
11	4.39	5.246	4.035	4.25	5.078	4.063
13	4.52	5.401	4.155	4.22	5.042	4.034
15	4.11	4.911	3.778	3.2	3.824	3.059
17	4.58	5.473	4.210	3.52	4.206	3.365
19	5.01	5.986	4.605	3.83	4.576	3.661
OD 600nm	**Pellet 3**			**Pellet 4**		
	1.15			1.3		
sonication time [min]	**OD 595nm**	**3 protein conc. [mg/mL]**	**3 protein conc. /OD600**	**OD 595nm**	**4 protein conc. [mg/mL]**	**4 protein conc. /OD600**
1	1.62	1.936	1.683	3.52	4.206	3.235
3	2.15	2.569	2.234	3.54	4.230	3.254
5	3.31	3.955	3.439	3.7	4.421	3.401
7	3.49	4.170	3.626	3.47	4.146	3.189
9	3.71	4.433	3.855	3.55	4.242	3.263
11	4.01	4.791	4.167	3.61	4.314	3.318
13	4.27	5.102	4.437	3.6	4.302	3.309
15	4.83	5.771	5.019	3.97	4.744	3.649
17	4.85	5.795	5.039	3.78	4.517	3.474
19	5.77	6.894	5.995	4.64	5.544	4.265

Table A 5: Results of test 1 – bead mill *L. sakei* Lb790 pSIPLacLM-Reu

OD600nm	**Pellet 5**	
	1.65	
OD 595nm	**5 protein conc. [mg/mL]**	**5 protein conc./OD600**
1.42	1.697	1.028

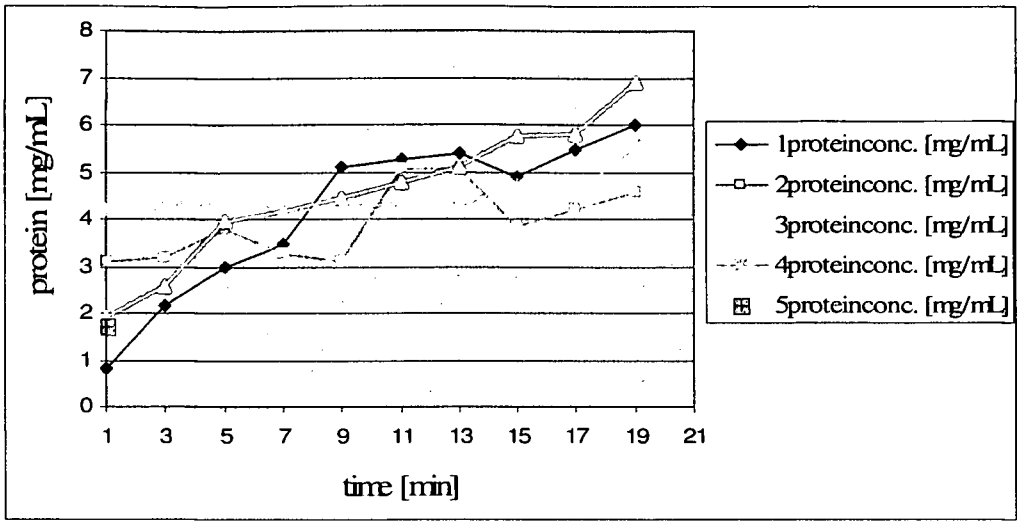


Figure A 7: Results of test 1 for *L. sakei* Lb790 pSIP403LacLM-Reu in term of protein concentration [mg/mL]

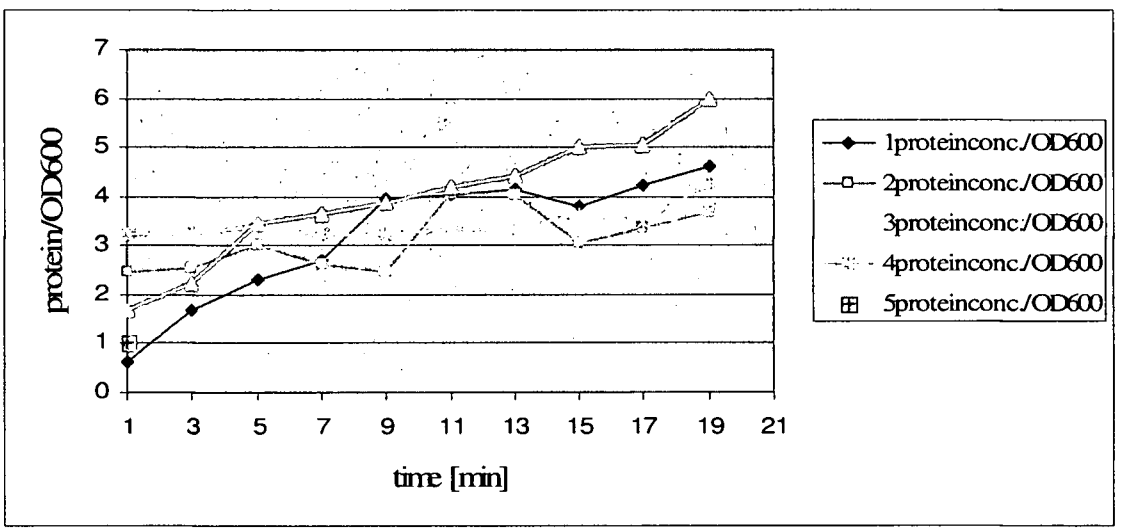


Figure A 8: Results of test 1 for *L. sakei* Lb790 pSIP403LacLM-Reu in term of protein concentration divided by OD600nm

The results of the test showed a clear trend: without pre-treatment there was low protein concentration at the beginning but after longer sonication time high protein concentration was obtained. Sonication buffer gives higher protein concentration than NaPP. With pre-treatment there is high protein concentration after 3 min of sonication (green curves).

A3.2 Test 2: Optimization of cell disruption conditions for optimal protein concentration and enzyme activity

L. plantarum WCFS1 pSIP403LacLM-Reu and *L. sakei* Lb790 pSIP403LacLM-Reu were grown in 3 parallel samples. All cultures were induced and harvested at OD_{600nm} of 2.3. The pellets were resuspended in sonication buffer and pre-treated (mentioned above).Cell disruption was performed with the sonicator. During sonication samples were taken at 0, 1, 3, 5, 7 and 9 min. All samples were centrifuged at 13200 rpm for 5 min at 4°C using a table centrifuge. Each supernatant was filled in a new eppi.

Protein concentration and β-galactosidase activity of the crude extract were determined using Bradford and *o*NPG assays, respectively.

Results of test 2:

Table A 6: Results of test 2 for *L. plantarum* WCFS1 pSIP403LacLM-Reu

sonication time [min]	average activity [U/mL]	average protein conc. [mg/mL]	specific activity [U/mg]
0	95.16	2.3	41.92
1	140.4	2.6	53.41
3	266.24	3.2	82.52
5	263.64	4.2	62.45
7	356.72	5.1	69.97
9	439.14	6.5	68.06

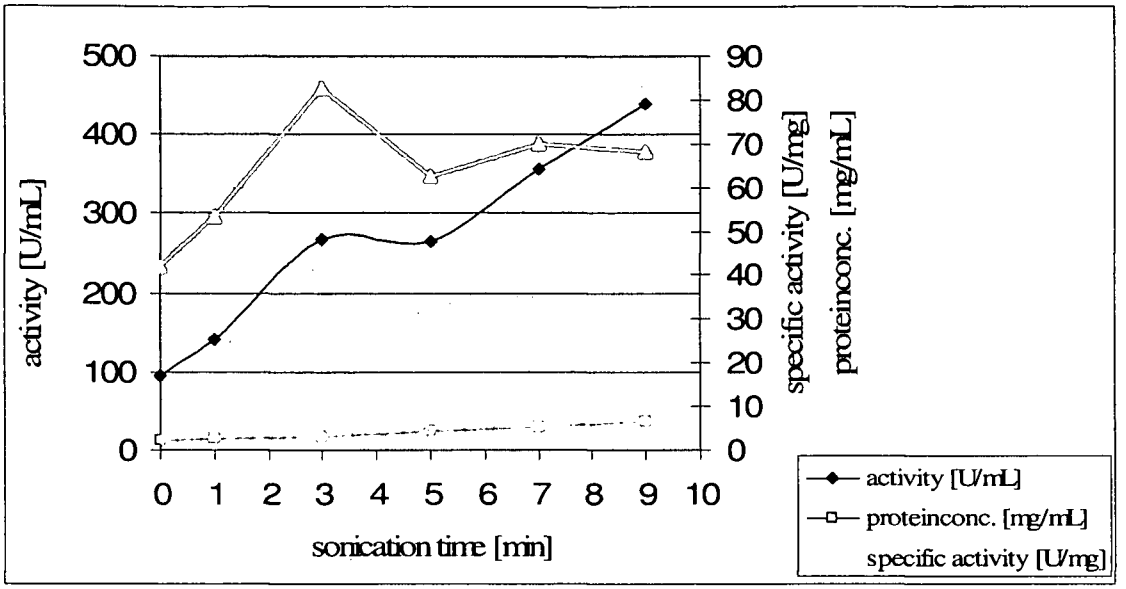


Figure A 9: Results of test 2 for *L. plantarum* WCFS1 pSIP403LacLM-Reu in terms of protein concentration and enzyme activity

Table A 7: Results of test 2 for *L. sakei* Lb790 pSIP403LacLM-Reu

sonication time [min]	average activity [U/mL]	average protein conc [mg/mL]	specific activity [U/mg]
0	185.64	4.3	42.76
1	179.4	5.6	32.17
3	184.6	5.9	31.11
5	187.72	6.3	30.02
7	179.92	6.3	28.77
9	191.36	7.0	27.14

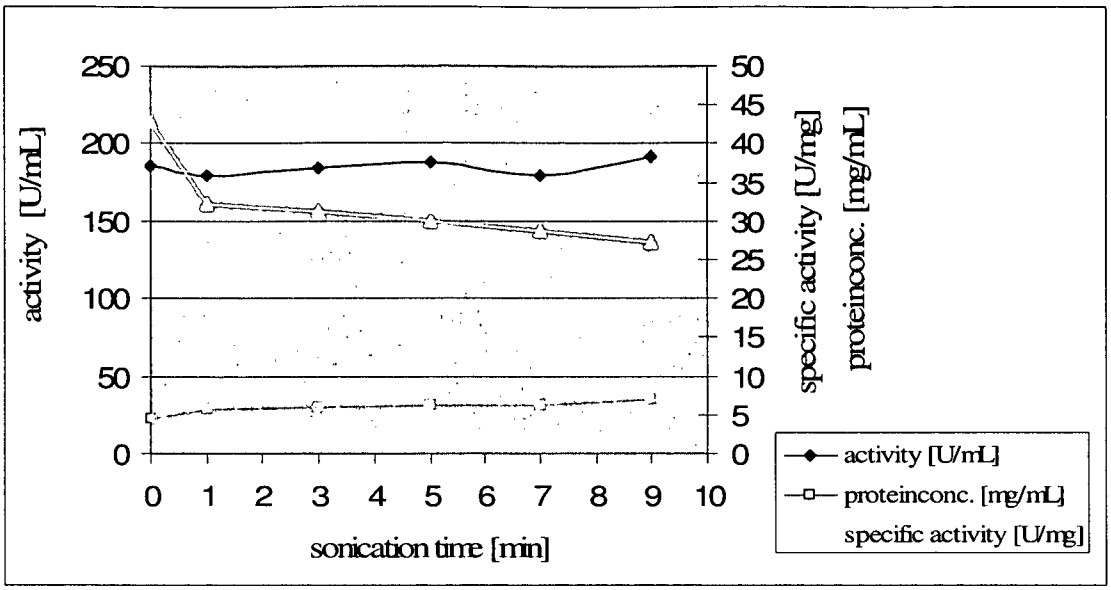


Figure A 10: Results of test 2 for *L. sakei* Lb790 pSIP403LacLM-Reu in terms of protein concentration and enzyme activity

A3.3 Test 3: Optimization of cell disruption conditions with and without pre-treatment for optimal protein concentration and enzyme activity

L. plantarum WCFS1 pSIP403LacLM-Reu and *L. sakei* Lb790 pSIP403LacLM-Reu were grown in 4 parallel samples. All cultures were induced and harvested at OD_{600nm} of 2.3. All pellets were resuspended in 1 mL sonication buffer and 2 pellets of each strain were pre-treated with mutanolysin (200 U/mL) and lysozyme (12 mg/mL) for 5min in a water bath at 37°C.

Cell disruption was performed with the sonicator. During sonication samples were taken at 0, 1, 3, 5, 7 and 9 min. All samples were centrifuged at 13200 rpm for 5 min at 4°C in a table centrifuge. Each supernatant was filled in a new eppi.

Protein concentration and β-galactosidase activity of the crude extract were determined using Bradford and *o*NPG assays, respectively.

Results of test 3:

Table A 8: Results of test 3 for *L. plantarum*WCFS1 pSIP403LacLM-Reu

No pre-treatment			
sonication time [min]	average activity [U/mL]	average protein conc. [mg/mL]	specific activity [U/mg]
0	0.15	0	0
1	29.42	0.41	71.99
3	109.93	1.63	67.51
5	182.57	2.60	70.15
7	199.18	3.40	58.52
9	272.20	4.79	56.85
Pre-treatment			
sonication time [min]	average activity [U/mL]	average protein conc. [mg/mL]	specific activity [U/mg]
0	0.03	2.27	0.01
1	0.21	1.96	0.10
3	0.48	1.45	0.33
5	2.01	0.92	2.20
7	17.07	1.07	15.90
9	95.45	1.71	55.66

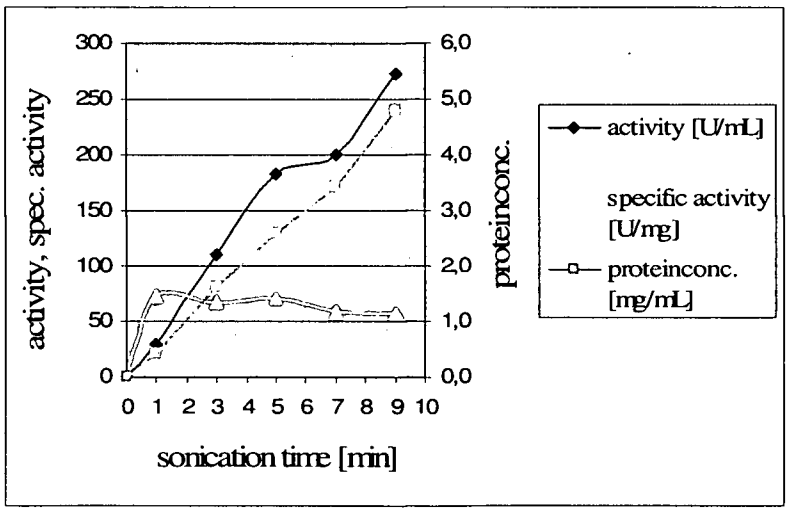


Figure A 11: Results of test 3 for *L. plantarum* WCFS1 pSIP403LacLM-Reu in terms of protein concentration and enzyme activity – without pre-treatment with mutanolysin and lysozyme

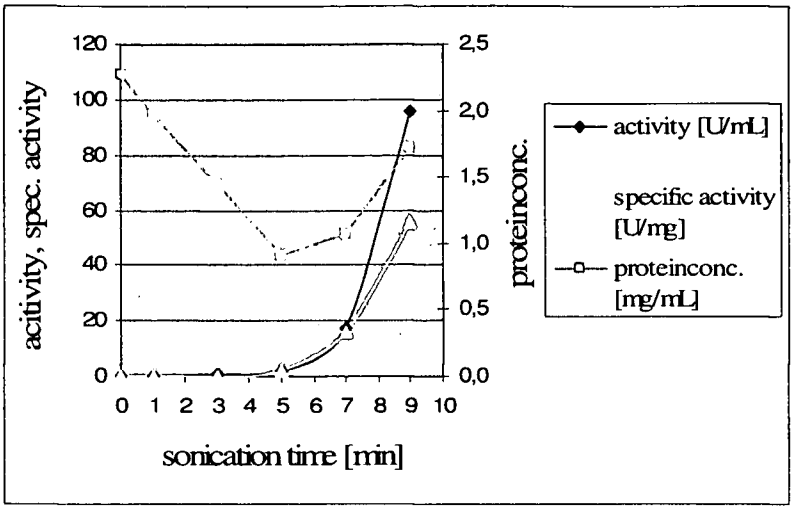


Figure A 12: Results of test 3 for *L. plantarum* WCFS1 pSIP403LacLM-Reu in terms of protein concentration and enzyme activity – with pre-treatment with mutanolysin and lysozyme

The results of test 3 for *L. plantarum* with pre-treatment do not fit with the other results from this test. Probably something went wrong there with the pre-treatment or the sonication.

Table A 9: Results of test 3 for *L. sakei* Lb790 pSIP403LacLM-Reu

No pre-treatment			
sonication time [min]	average activity [U/mL]	average protein conc. [mg/mL]	specific activity [U/mg]
0	2.14	0.08	25.64
1	15.90	0.71	22.40
3	39.33	1.63	24.06
5	59.17	2.45	24.16
7	76.87	2.99	25.73
9	91.35	4.02	22.75
Pre-treatment			
sonication time [min]	activity [U/mL]	protein conc. [mg/mL]	specific activity [U/mg]
0	22.55	1.04	21.79
1	42.35	1.67	25.41
3	59.21	2.20	26.93
5	66.66	2.71	24.55
7	66.95	3.25	20.62
9	75.78	3.70	20.47

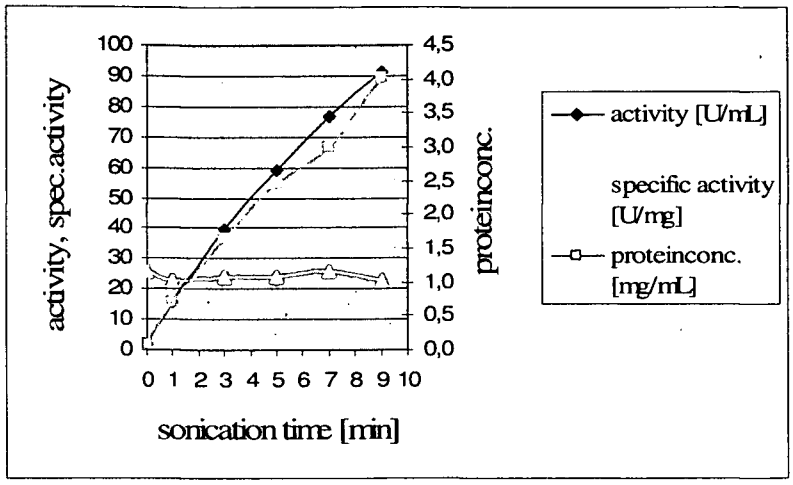


Figure A 13: Results of test 3 for *L. sakei* Lb790 pSIP403LacLM-Reu in terms of protein concentration and enzyme activity – without pre-treatment with mutanolysin and lysozyme

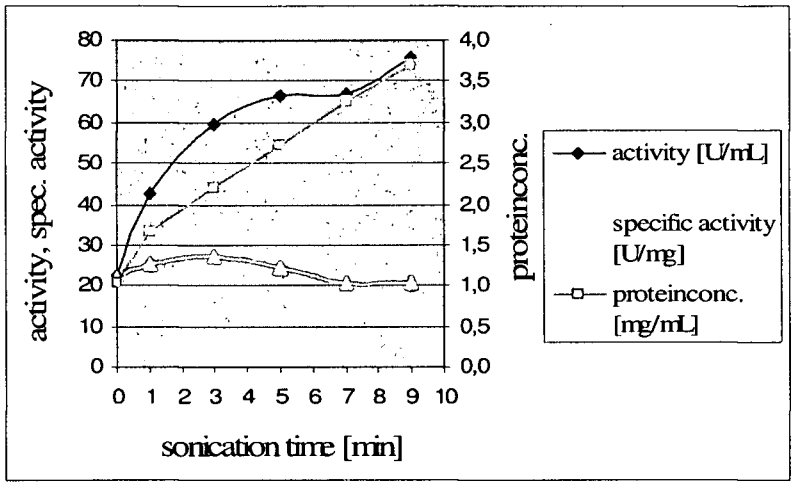


Figure A 14: Results of test 3 for *L. sakei* Lb790 pSIP403LacLM-Reu in terms of protein concentration and enzyme activity – with pre-treatment with mutanolysin and lysozyme

Specific activities were the same for different time duration of sonication. It is easier to use no pre-treatment, because pre-treatment requires time.

A3.4 Conclusion from tests 1-3

The best buffer is the sonication buffer. The compromise between time and yield of protein is to use no pre-treatment and to sonicate for 3 min.

A3.5 Test 4: Correlation between OD_{600nm} and expressed active β-galactosidase

The next step was to check if there was β-galactosidase activity derived from the chromosomal *lacLM* genes and to see the correlation between OD_{600nm} and expressed active β-galactosidase.

Chromosomal β-galactosidase activity was tested with *L. plantarum* WCFS1 pSIP405(*cat*) and *L. sakei* Lb790 pSIP405(*cat*). These strains have a pSIP vector with the reporter gene *cat* (which encodes chloramphenicol) instead of the *lacLM* genes.

Overnight cultures were set up for:
- *L. plantarum* WCFS1 pSIP403LacLM-Reu
- *L. plantarum* WCFS1 pSIP405(*cat*)
- *L. sakei* Lb790 pSIP403-LacLM-Reu
- *L. sakei* Lb790 pSIP405(*cat*)

For each strain 13 tubes were prepared. Cultivation was started with OD_{600nm} of 0.1. Cultivation and induction were performed. Cells were harvested at different OD_{600nm} (0.3, 0.8, 1.3,....) and the pellets were resuspended in 1 mL sonication buffer. Cell disruption was performed with the sonicator.

Protein concentration and β-galactosidase activity in the crude extract were determined using method of Bradford and *o*NPG assays, respectively.

Results of test 4:

Table A 10: Results of test 4 for *L. sakei* Lb790 pSIP405

incubation time [h]	OD 600nm	protein conc. [mg/mL]	activity [U/mL]	specific activity [U/mg]
2	0.33	0.78	0.15	0.19
3.5	0.79	2.26	0.04	0.02
4.5	1.32	2.63	0.05	0.02
5.5	1.7	2.69	0.04	0.02
7	2.2	3.38	0.05	0.01
8.5	2.7	2.95	0.47	0.16
11.5	3.3	2.07	0.19	0.09
12	3.5	1.90	0.04	0.02
27	3.9	0.57	0.04	0.07

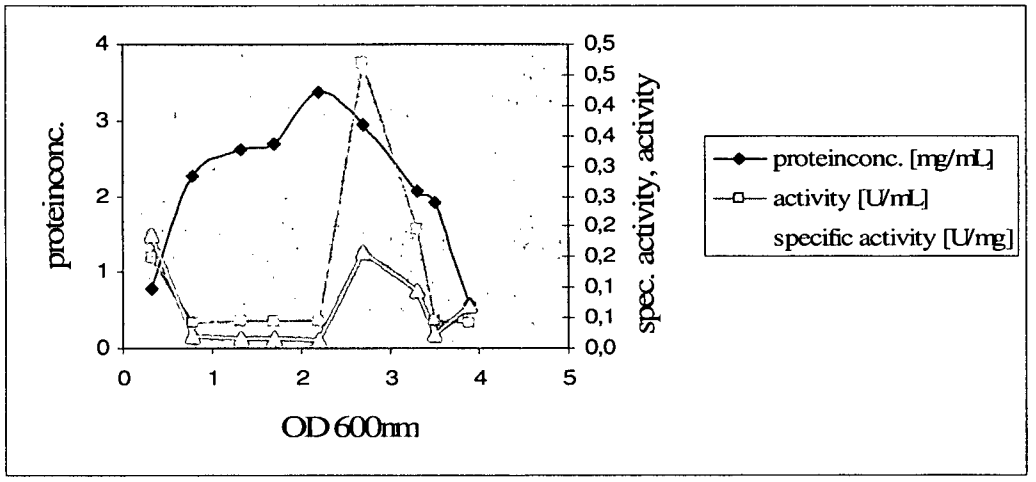


Figure A 15: Results of test 4 for *L. sakei* Lb790 pSIP405 in terms of protein concentration and enzyme activity

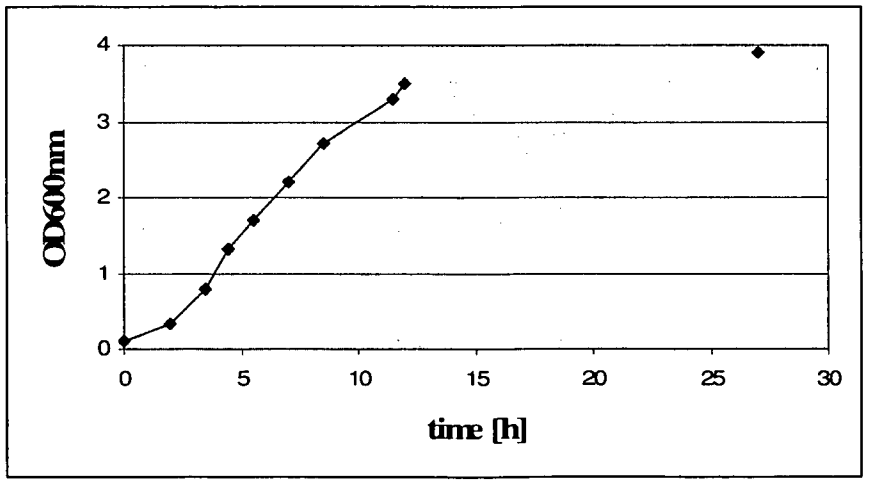


Figure A 16: Growth curve for *L. sakei* Lb790 pSIP405

Table A 11: Results of test 4 for *L. sakei* Lb790 pSIP403LacLM-Reu

incubation time [h]	OD 600nm	protein conc. [mg/mL]	activity [U/mL]	specific activity [U/mg]
3	0.33	0.81	0.07	0.08
4.5	0.78	2.44	36.04	14.78
5.5	1.26	3.70	76.60	20.68
6.5	1.95	3.86	75.97	19.68
7	2.2	3.42	61.00	17.85
9	2.7	2.81	52.26	18.61
12	3.2	2.07	39.31	19.02
27	3.8	0.72	14.87	20.74

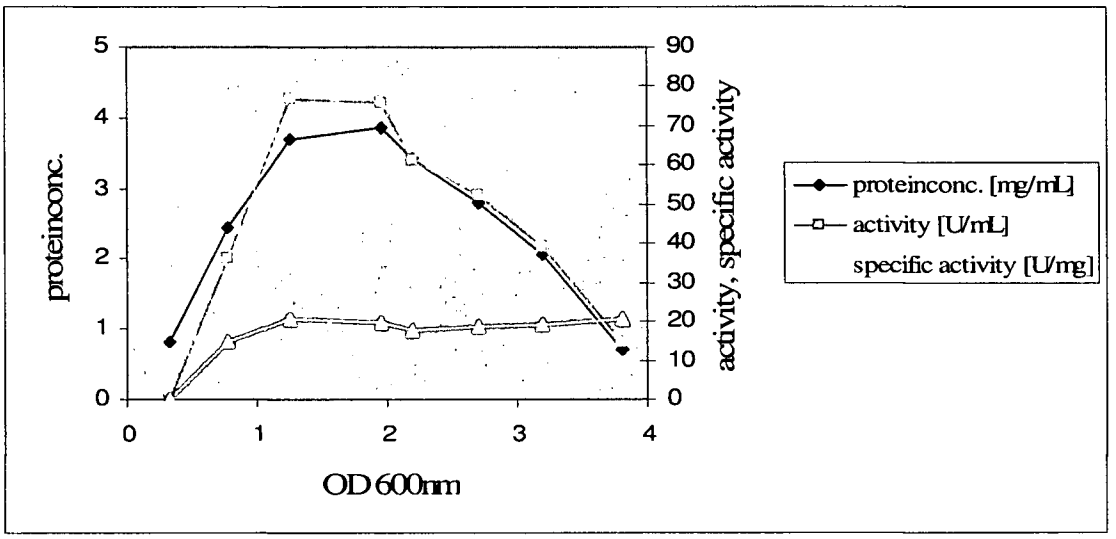


Figure A 17: Results of test 4 for *L. sakei* Lb790 pSIP403LacLM-Reu in terms of protein concentration and enzyme activity

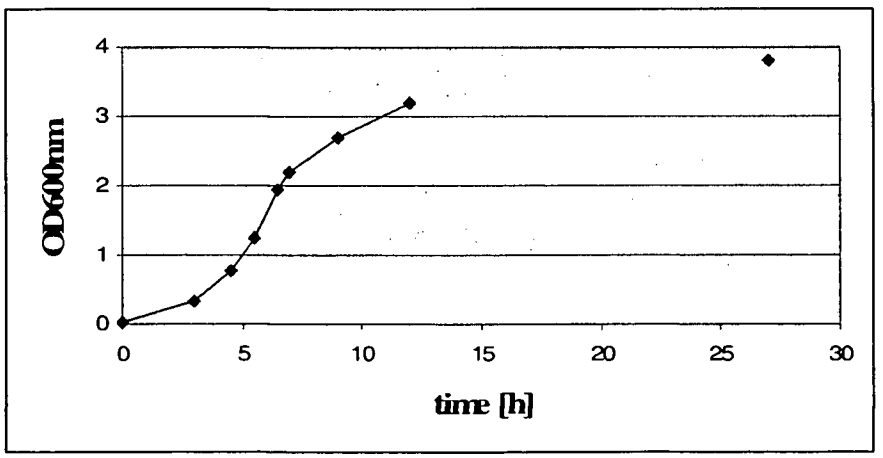


Figure A 18: Growth curve for *L. sakei* Lb790 pSIP403LacLM-Reu

Table A 12: Results of test 4 for *L. plantarum* WCFS1 pSIP405

incubation time [h]	OD 600nm	protein conc. [mg/mL]	activity [U/mL]	specific activity [U/mg]
2	0.31	0.63	0.07	0.11
3.5	0.76	0.98	0.04	0.04
5	1.22	1.26	0.05	0.04
5.5	1.8	1.13	0.11	0.10
7	2.4	1.13	0.06	0.05
7.5	2.65	0.98	0.05	0.06
8.5	3.25	0.90	0.17	0.19
9	3.7	0.78	0.06	0.08
10.5	4.2	0.68	0.05	0.07
11.5	4.7	0.47	0.15	0.31
27	10.6	0.32	0.05	0.14

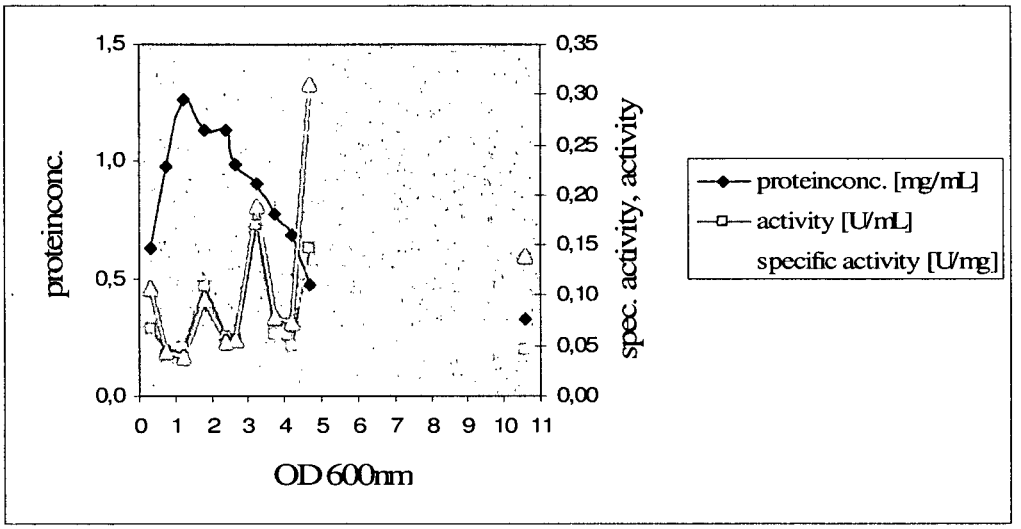


Figure A 19: Results of test 4 for *L. plantarum* WCFS1 pSIP405

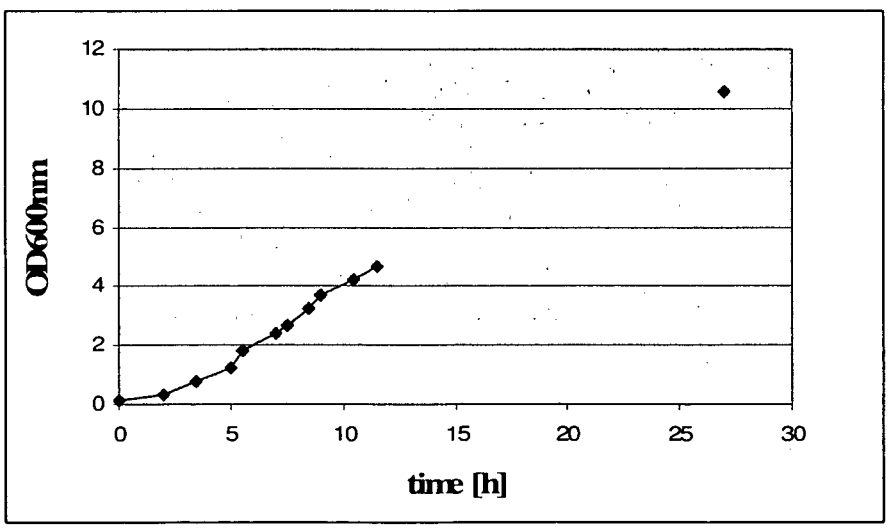


Figure A 20: Growth curve for *L. plantarum* WCFS1 pSIP405

Table A 13: Results of test 4 for *L. plantarum* WCFS1 pSIP403LacLM-Reu

incubation time [h]	OD 600nm	protein conc. [mg/mL]	activity [U/mL]	specific activity [U/mg]
2	0.32	0.79	0.94	1.20
4	0.82	1.68	59.59	35.37
5	1.24	2.76	110.29	39.96
5.5	1.8	3.61	204.36	56.63
6.5	2.55	2.20	149.29	67.90
7	3	2.13	128.08	60.22
7.5	3.6	1.70	151.79	89.46
8	3.8	1.48	129.64	87.49
9	4.6	1.48	135.88	91.71
9.5	4.9	1.32	143.21	108.46
26.5	10.6	0.26	12.11	46.05

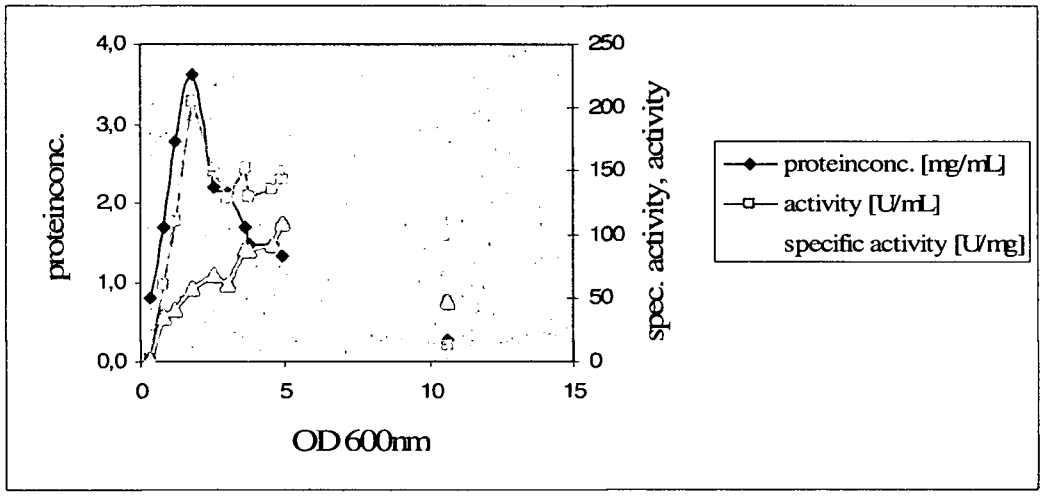


Figure A 21: Results of test 4 for *L. plantarum* WCFS1 pSIP403LacLM-Reu

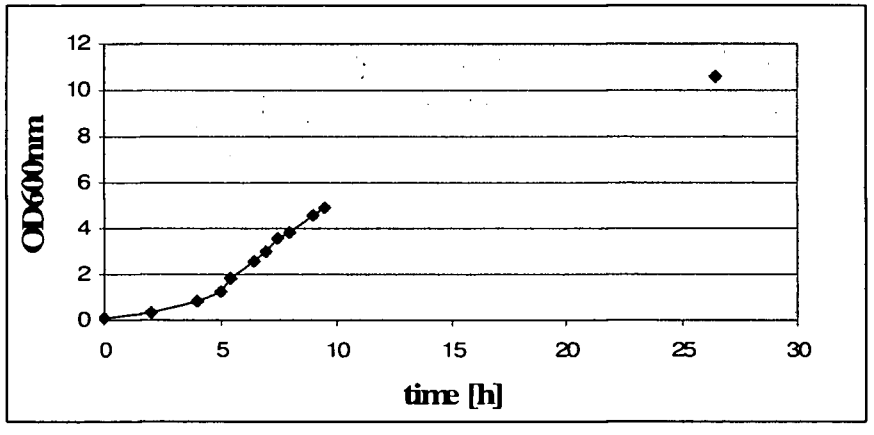


Figure A 22: Growth curve for *L. plantarum* WCFS1 pSIP403LacLM-Reu

L. sakei strains grew very slowly and stopped when OD_{600nm} reached 3.5. Most specific activity in the crude extract of *L. sakei* was found at OD_{600nm} above 1.2. For *L. plantarum* most specific activity in the crude extract was found between OD_{600nm} of 4 and 10. To get reproducible results and for better handling at the lab it was decided to harvest the cells at OD_{600nm} of 1.8.

The comparison of specific β-galactosidase activity expressed from *L. sakei* Lb790 pSIP403LacLM-Reu, *L. plantarum* WCFS1 pSIP403LacLM-Reu and *L. sakei* Lb790 pSIP405, *L. plantarum* WCFS1 pSIP405 did not show any significant specific β-galactosidase activity expressed from *L. sakei* Lb790 pSIP405 and *L. plantarum* WCFS1 pSIP405. In fact there is no significant β-galactosidase activity derived from the chromosomal genes when these strains are grown on glucose (blue lines in the following two figures).

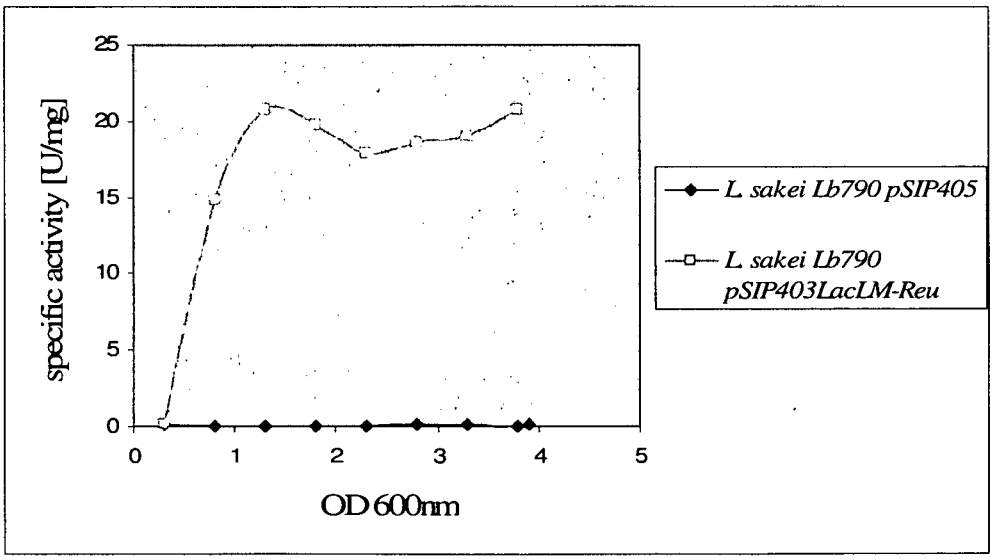


Figure A 23: Comparison of specific activity from *L. sakei* Lb790 pSIP405 and *L. sakei* Lb790 pSIP403LacLM-Reu

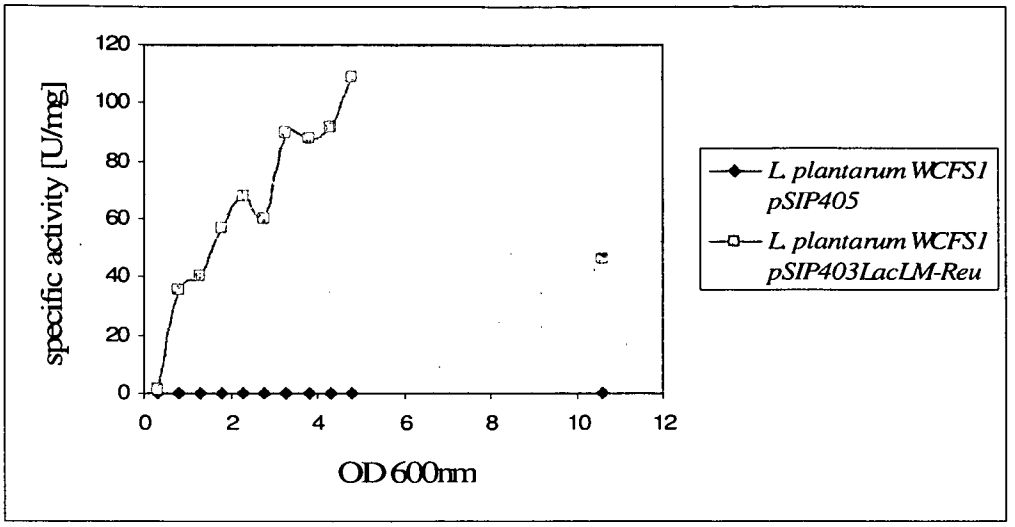


Figure A 24: Comparison of specific activity from *L. plantarum* WCFS1 pSIP405 and *L. plantarum* WCFS1 pSIP403LacLM-Reu

A4 Cloning and expression of β-galactosidase genes from *L. reuteri* L103, *L. acidophilus* R22, *L. plantarum* WCFS1 and *L. sakei* Lb790 in *L. plantarum* WCFS1 and *L. sakei* Lb790

A4.1 Cloning strategy

- Isolation of bacterial DNA
- PCR → incorporation of restriction sites
- TOPO-Cloning
- Digestion
- Ligation with pSIP-vectors and transformation to *E. coli*
- Sequencing
- Transformation to *Lactobacillus*

A4.2 The vectors

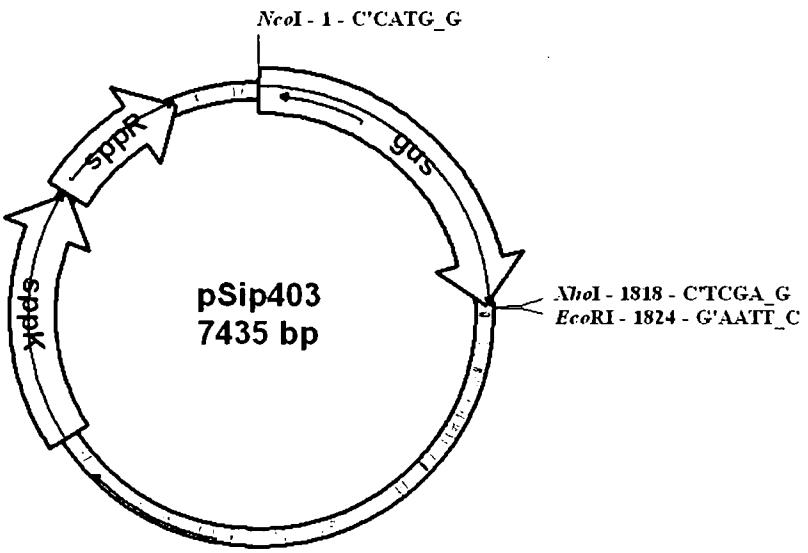


Figure A 25: pSIP403 vector

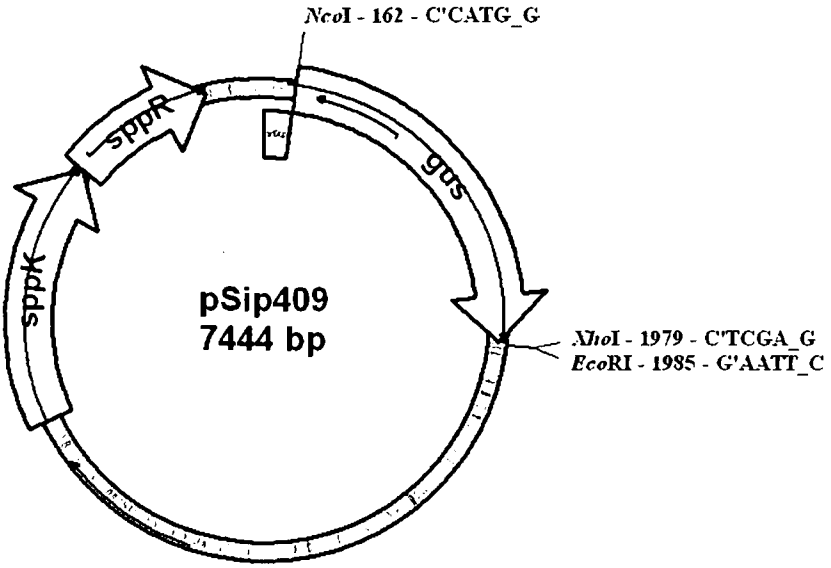


Figure A 26: pSIP409 vector

A4.3 The lacLM genes

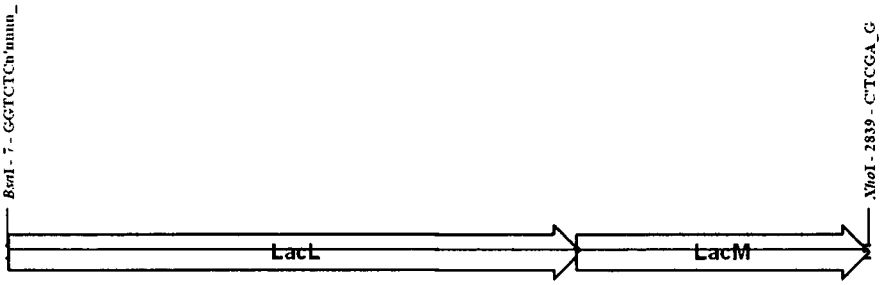


Figure A 27: lacLM genes from *L. reuteri* L103

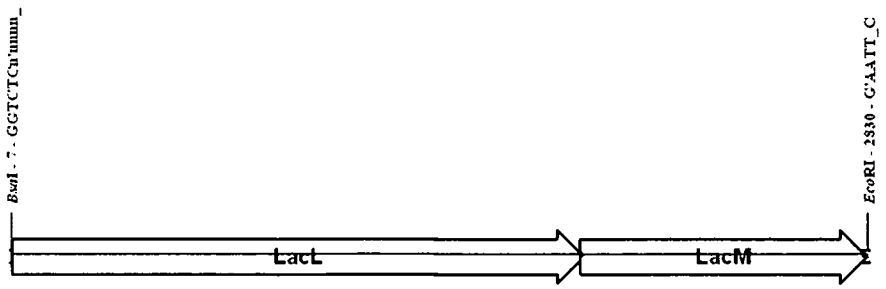


Figure A 28: lacLM genes from *L. acidophilus* R22

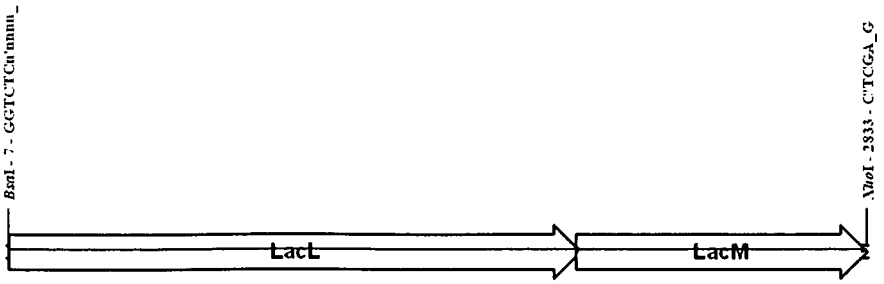


Figure A 29: lacLM genes from *L. plantarum* WCFS1

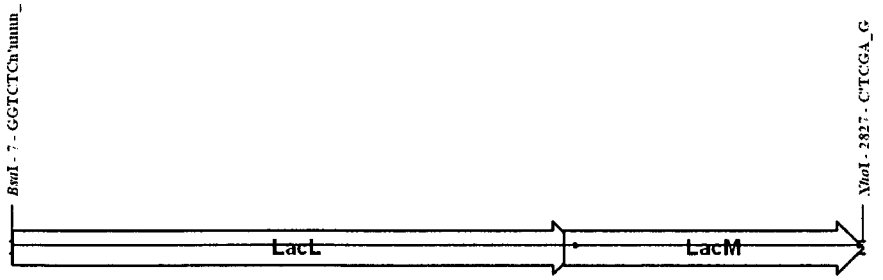


Figure A 30: lacLM genes from *L. sakei* Lb790

A4.4 Restriction sites

- *Bsa*I was inserted at the 5´end of the *lacL* genes from all 4 lactobacilli
- *Nco*I is in the multicloning site at the 3´end of the vectors
- *Xho*I was inserted at the 3´end of the *lacM* genes of *L. reuteri*, *L. plantarum*, *L. sakei* and is in the multicloning site at the 5´ end of the vectors
- *EcoR*I was inserted at the 3´end of the *lacM* gene of *L. acidophilus*
- *Bsa*I and *Nco*I are compatible sites

A4.5 Plasmid purification and digestion of the pSIP vectors

Purification of the plasmids from the strains *L. plantarum* WCFS1 pSIP403(*gus*), *L. sakei* Lb790 pSIP409(*gus*) was performed using the E.Z.N.A.™ Plasmid Miniprep Kit I (Omega Bio-Tek. Inc. Doraville, GA).

The β-glucuronidase gene (*gus*) had to be cut out from the pSIP403 and pSIP409 expression vectors using restriction enzymes *Nco*I and *Xho*I (or *EcoR*I for the vectors that will be ligated with *lacLM* from *L. acidophilus*) to obtain the empty vectors.

Table A 14: Digestion of the pSIP vectors

	volumes
pSIP403 or pSIP409 (1 µg)	15 µL
Buffer Neb 4	5 µL
BSA 10x	5 µL
*Nco*I (25 U)	2,5 µL
*Xho*I (50 U))	2,5 µL
dH_2O	20 µL
37°C water bath for 3 h	

The resulted fragments were visualized by gel electrophoresis at 7 Vcm^{-1} in a 1.2% agarose gel (containing ethidium bromide 0.2 µg/ml) in 1x TAE (Tris-Acetat) electrophoresis buffer (Tris base 4.8 g/l, acetic acid 1.2 g/l, 1 mM EDTA pH 8.0) and photographed under UV light.

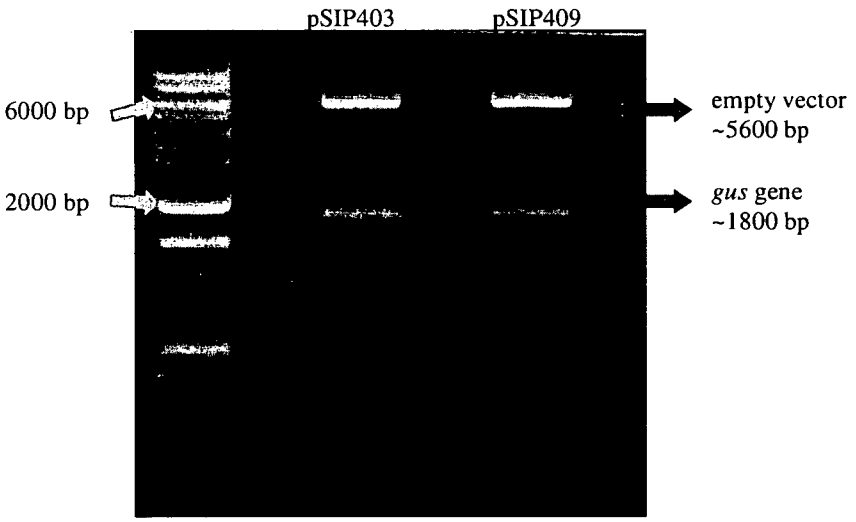


Figure A 31: 1.2 % agarose gel - digested vectors

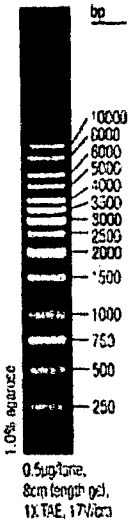


Figure A 32: GeneRuler™ 1 kb ladder

The empty vectors with the size of ~5600 bp were purified from the agarose gel using the NucleoSpin Extract II (Macherey-Nagel Inc., Bethlehem, USA). Vectors were frozen at -20°C for later use.

A4.6 DNA preparation

The preparations of the plasmids pHA1031 and pHAR22 containing the complete genes (*lacL* and *lacM*) of β-galactosidases from *L. reuteri* L103 and *L. acidophilus* R22 were described previously in Nguyen *et al.* (2007a; 2007b), respectively.
Chromosomal DNA from *L. plantarum* WCFS1 and *L. sakei* Lb790 was extracted using the E.Z.N.A.™ Bacterial DNA kit (Omega Bio-Tek. Inc. Doraville, GA).

A4.7 DNA amplification and incorporation of restriction sites

The oligonucleotides LacReuF and LacReuR, LacAciF and LacAciR, LacLMPlaF and LacLMPlaR, Lb790LacF and Lb790LacR (Table A 16) used for PCR amplification of *L. reuteri lacLM*, *L. acidophilus lacLM*, *L. plantarum lacLM* and *L. sakei lacLM* genes, respectively, were designed based on the sequences of β-galactosidases from *L. reuteri* L103 (GenBank accession number DQ493596), *L. acidophilus* R22 (GenBank accession number

EF053367), *L. plantarum* WCFS1 (GenBank accession number AL935262) and *L. sakei* subsp. *sakei* (GenBank accession number X82287). The amplification was performed with Phusion™ High-Fidelity DNA Polymerase from Finnzymes OY (Espoo, Finland) using an Eppendorf Mastercycler® (Eppendorf AG, Hamburg, Germany) in a total volume of 50 µL of reaction mixtures containing 0.2 mM of each deoxynucleotide triphosphate, 0.5 µM of each primer, 10 µl of 5xPhusion HF buffer (final concentration of $MgCl_2$ was 1.5 mM), 1 U of Phusion DNA Polymerase, and ~50 ng genomic DNA.

Table A 15: Set-up for PCR reaction

	volumes
5xPhusion HF buffer	10 µL
10 mM dNTPs	1 µL
Primer F *	1 µL
Primer R *	1 µL
Template DNA **(50 ng)	1 µL
Polymerase (Phusion)	0.5 µL
dH_2O	35.5 µL

* primer concentration was 50 pmol/mL

** extracted chromosomal DNA was diluted 1:5 before used in the PCR reaction

The initial denaturation step at 98°C for 30 s was follow by 35 cycles of denaturation at 98°C for 5 s, annealing at 49/53 °C for 10 s, and extension at 72°C for 1 min. The final cycle was followed by additional 10 min elongation at 72°C.

Table A 16: Sequences of the primers used for incorporation of the restriction sites

Primer	Sequence (5´→ 3´)	notes	Reference sequence accession no.
LacReuF	**GGTCTCA**CATGCAAGCAAATATAAAAT	a)	DQ493596
LacReuR	**CTCGAG**TTATTTTGCATTCAATACAAAC	b)	DQ493596
LacAciF	**GGTCTCT**CATGCAAGCAAACATAAAAT	c)	EF053367
LacAciR	GG**GAATTC**TTAATTAAGATTGAAAGAAAATTCGT	d)	EF053367
LacLMPlaF	**GGTCTCT**CATGCAAGCTAATCTTCAAT	e)	AL935262
LacLMPlaR	**CTCGAG**TTAGAAATGAATATTAAAGCT	f)	AL935262
Lb790LacF	**GGTCTCT**CATGCAACCTAATATTCAAT	g)	X82287
Lb790LacR	**CTCGAG**TTAAAACGAAATTTCAAATTCAAAAT	h)	X82287

F: denotes forward primers; R: denotes reverse primers

a) Upstream primer to amplify *lacLM* from pHA1031 with *Bsa*I site (bold, underlined)

b) Downstream primer to amplify *lacLM* from pHA1031 with *Xho*I site (bold, underlined)

c) Upstream primer to amplify *lacLM* from pHAR22 with *Bsa*I site (bold, underlined)

d) Downstream primer to amplify *lacLM* from pHAR22 with *EcoR*I site (bold, underlined)

e) Upstream primer to amplify *lacLM* from genomic DNA *L. plantarum* WCFS1 with *Bsa*I site (bold, underlined)

f) Downstream primer to amplify *lacLM* form genomic DNA *L. plantarum* WCFS1 with *Xho*I site (bold, underlined)

g) Upstream primer to amplify *lacLM* from genomic DNA *L. sakei* Lb790 with *Bsa*I site (bold, underlined)

h) Downstream primer to amplify *lacLM* form genomic DNA *L. sakei* Lb790 with *Xho*I site (bold, underlined)

The amplified products were visualized by gel electrophoresis at 7 Vcm^{-1} in a 1.2% agarose gel (containing ethidium bromide 0.2 µg/ml) in 1x TAE (Tris-Acetat) electrophoresis buffer (Tris base 4.8 g/l, acetic acid 1.2 g/l, 1 mM EDTA pH 8.0) and photographed under UV light.

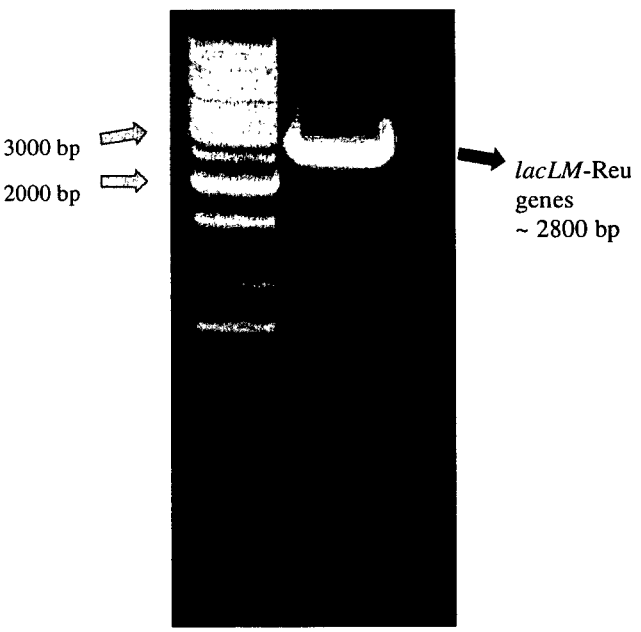


Figure A 33: 1.2% agarose gel – amplified lacLM genes from *L. reuteri* L103

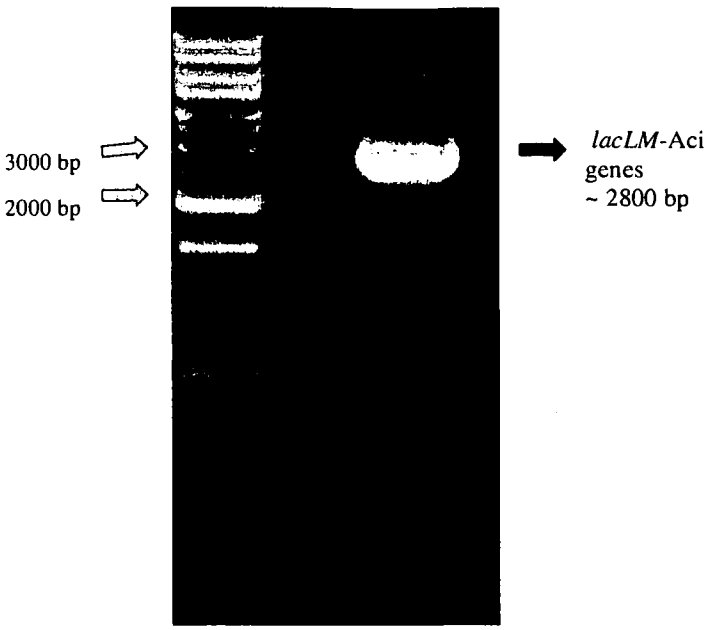


Figure A 34: 1.2% agarose gel – amplified lacLM genes from *L. acidophilus* R22

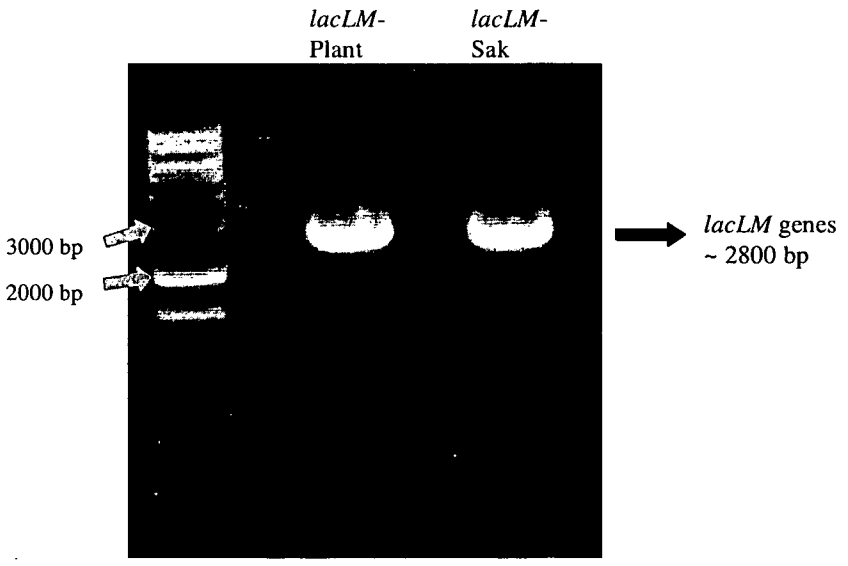


Figure A 35: 1.2% agarose gel – amplified lacLM genes from *L. plantarum* WCFS1 and *L. sakei* Lb790

The amplified products were purified from the agarose gel using the NucleoSpin Extract II (Macherey-Nagel Inc., Bethlehem, USA).

A4.8 Subcloning of lacLM into TOPO® vectors

The Zero Blunt® TOPO® PCR Cloning Kit with vector pCR®II-Blunt-TOPO® (Invitrogen, Carlsbad, CA) was used for subcloning PCR-amplified products.

Table A 17: Set-up for TOPO® cloning

	volumes
PCR product	4 µL
Salt solution	1 µL
pCR®II-Blunt-TOPO®	1 µL

The tubes were mixed gently, incubated for 30 min at room temperature and placed on ice. For transformation to *E. coli* One Shot®TOP10 chemical competent cells the manual of the kit was followed.

The plasmids pTReu, pTAci, pTLacLMPlant, pTLacLMSak containing the complete genes (*lacL* and *lacM*) of β-galactosidases from *L. reuteri*, *L. acidophilus*, *L. plantarum* and *L. sakei*, respectively, were generated. Restriction sites *Bsa*I were inserted at the 5´end of the *lacL* genes and *Xho*I were inserted at the 3´end of the *lacM* genes, except *lacM* from *L. acidophilus*. *Eco*RI was inserted at the 3´end of the *lacM* gene from *L. acidophilus*.

A4.9 Digestion of TOPO® plasmids

The *lacLM* genes with the designated restriction sites were cut out from the pTReu, pTAci, pTLacLMPlant and pTLacLMSak plasmids by digestion with the restriction enzymes *Bsa*I and *Xho*I (*Eco*RI instead of *Xho*I for pTAci). Because of the similar size of the *lacLM* genes (2800 bp) and the empty TOPO® vectors (3000 bp) there would be a problem to separate them on the agarose gel. Therefore the vectors were digested with a third restriction enzyme to obtain smaller fragments for a better separation.

A4.9.1 Digestion of pTReu

pTReu was digested in two steps with *Bsa*I, *Nco*I and *Xho*I:

Table A 18: Digestion of pTReu - first step

pTReu	20 µL
Buffer 3 Neb	4 µL
*Bsa*I (20 U)	2 µL
dH_2O	14 µL
50°C water bath for 2.5 h 37°C water bath for 5 min	

Table A 19: Digestion of pTReu - second step

*Nco*I (20 U)	2 µL
*Xho*I (40 U)	2 µL
BSA 10x	6 µL
Buffer 3 Neb	2 µL
dH_2O	8 µL
37°C water bath for 2.5 h	

The resulted fragments were visualized by agarose gel electrophoresis at 7 Vcm^{-1} on a 1.2% agarose gel and photographed under UV light.

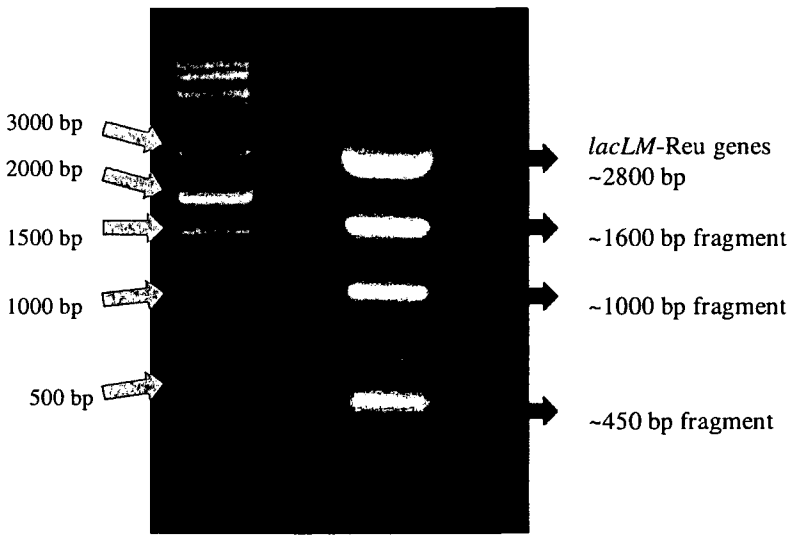


Figure A 36: Digested pTReu on 1.2 % agarose gel

A4.9.2 Digestion of pTAci

pTAci was digested in two steps with *Bsa*I, *Eco*RI and *Sph*I:

Table A 20: Digestion of pTAci - first step

pTAci	20 µL
Buffer 4 Neb	4 µL
*Bsa*I (20 U)	2 µL
dH_2O	14 µL
50°C water bath for 2.5 h, 37°C water bath for 5 min	

Table A 21: Digestion of pTAci - second step

*Eco*RI (40 U)	2 µL
*Sph*I (20 U)	2 µL
Buffer 4 Neb	2 µL
dH_2O	14 µL
37°C water bath for 2.5 h	

The resulted fragments were visualized by agarose gel electrophoresis at 7 Vcm^{-1} on a 1.2% agarose gel and photographed under UV light.

Figure A 37: Digested pTAci on 1.2% agarose gel

A4.9.3 Digestion of pTLacLM-Plant

pTLacLM-Plant was digested in two steps with *Bsa*I and *Xho*I:

Table A 22: Digestion of pTLacLM-Plant - first step

pTLacLM-Plant	10 µL
Buffer 4 Neb	4 µL
*Bsa*I (20 U)	2 µL
dH_2O	24 µL
50°C water bath for 2 h, 37°C water bath for 5 min	

Table A 23: Digestion of pTLacLM-Plant - second step

Buffer 4 Neb	2 µL
*Xho*I2 (40 U)	2 µL
BSA 10x	6 µL
dH_2O	10 µL
37°C water bath for 2 h	

A third restriction enzyme could not be found to digest the TOPO® vector in smaller fragments therefore the resulted fragments were visualized by agarose gel electrophoresis on a 2.0% agarose gel.

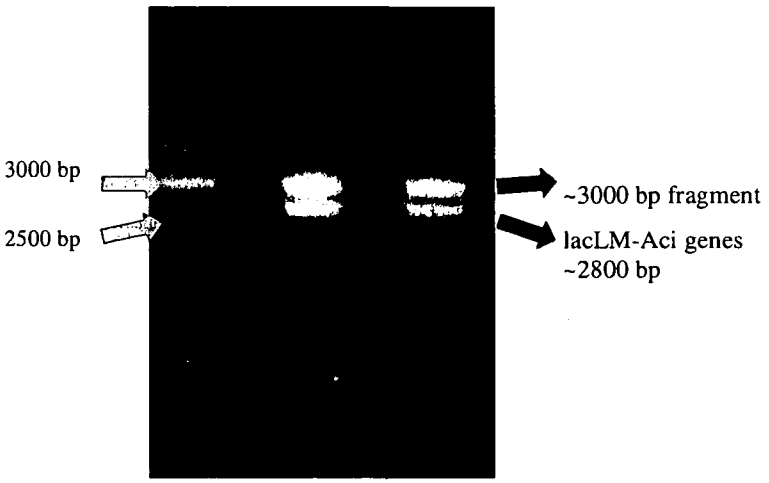


Figure A 38: Digested pTLacLM-Plant on 2 % agarose gel

A4.9.4 Digestion of pTLacLM-Sak

pTLacLM-Sak was digested in two steps with *Bsa*I, *Xho*I and *Nco*I:

Table A 24: Digestion of pTLacLM-Sak - first step

pTLacLM-Sak	20 µL
Buffer 4 Neb	4 µL
*Bsa*I (20 U)	2 µL
dH_2O	14 µL
50°C water bath for 2.5 h, 37°C water bath for 5 min	

Table A 25: Digestion of pTLacLM-Sak - second step

*Xho*I (40 U)	2 µL
*Nco*I (20 U)	2 µL
Buffer 4 Neb	2 µL
BSA 10x	6 µL
dH_2O	14 µL
37°C water bath for 2.5 h	

Resulted fragments were visualized by gel electrophoresis at 7 Vcm^{-1} in a 1.2% agarose gel and photographed under UV light

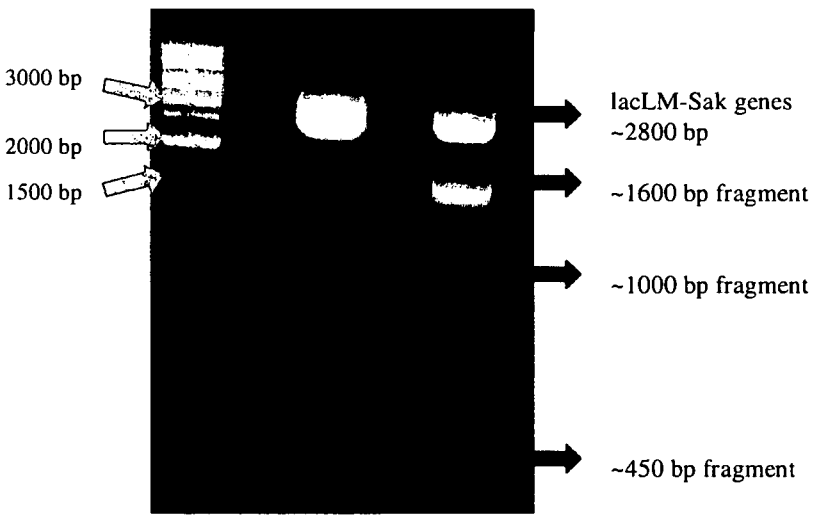


Figure A 39: Digested pTLacLM-Sak on 1.2% agarose gel

All fragments containing *lacLM* genes were purified from the agarose gel using the NucleoSpin Extract II (Macherey-Nagel).

A4.10 Cloning of lacLM into pSIP vectors

The Quick Ligation™ Kit (New England BioLabs, Beverly, MA, USA) was used for ligating the empty pSIP403/pSIP409 vectors with the *lacLM* genes.

Table A 26: Ligation set-up

	ligation	control	
empty pSIP409	1 µL	2 µL	
lacLM-Reu	9 µL		
dH_2O		8 µL	
	ligation	control	
empty pSIP403	5 µL	5 µL	
lacLM-Aci	5 µL		
dH_2O		5 µL	
	Ligation*	control	
empty pSIP409	1 µL	1 µL	
lacLM-Aci	9 µL		
dH_2O		9 µL	
	Ligation*		control
empty pSIP403	1 µL	3 µL	3 µL
lacLM-Plant	9 µL	7 µL	
dH_2O			7 µL
	Ligation*		control
empty pSIP409	1 µL	3 µL	3 µL
lacLM-Plant	9 µL	7 µL	
dH_2O			7 µL
	ligation	control	
empty pSIP403	3 µL	3 µL	
lacLM-Sak	6 µL		
dH_2O	1 µL	7 µL	
	ligation	control	
empty pSIP409	3 µL	3 µL	
lacLM-Sak	6 µL		
dH_2O	1 µL	7 µL	

* 3 min at 68 °C water bath, then on ice

Transformation of the newly created plasmids to *E. coli* was performed using *E. coli* One Shot®TOP10 chemical competent cells following the One Shot® Chemical Transformation from Zero Blunt® TOPO® PCR Cloning Kit protocol (Invitrogen).
Each transformation was spread out on BHI agar plates containing erythromycin (200µg/mL) and incubated at 37°C over night.

No or just one or two colonies were grown on the control plates. Four of each positive transformants were picked and grown over-night in MRS containing 5 µg/mL erythromycin.

After purification of the plasmid [using the E.Z.N.A.™ Plasmid Miniprep Kit I (Omega Bio-Tek. Inc. Doraville, GA)] the plasmids were digested with specific restriction enzymes to check if the *lacLM* genes were inserted into the vectors.

A4.10.1 Digestion of pSIP409LacLM-Reu

pSIP409LacLM-Reu was digested with *EcoR*I:

Table A 27: Digestion of pSIP409LacLM-Reu

pSIP409LacLM-Reu, pSIP409(*gus*)	15 µL
Buffer 3 Neb	5 µL
*EcoR*I (50 U)	2.5 µL
dH_2O	27.5 µL
37°C water bath for 2 h	

The pSIP409LacLM-Reu plasmid has three *EcoR*I sites. The resulted fragments of 7060 bp, 1039 bp and 360 bp were visualized by gel electrophoresis at 7 Vcm^{-1} on a 1.2% agarose gel and photographed under UV light. Additionally, a pSIP409(gus) control [C] was digested with *EcoR*I and run on the gel. The pSIP409(gus) plasmid has just one *EcoR*I site resulting in one fragment with the size of 7444 bp.

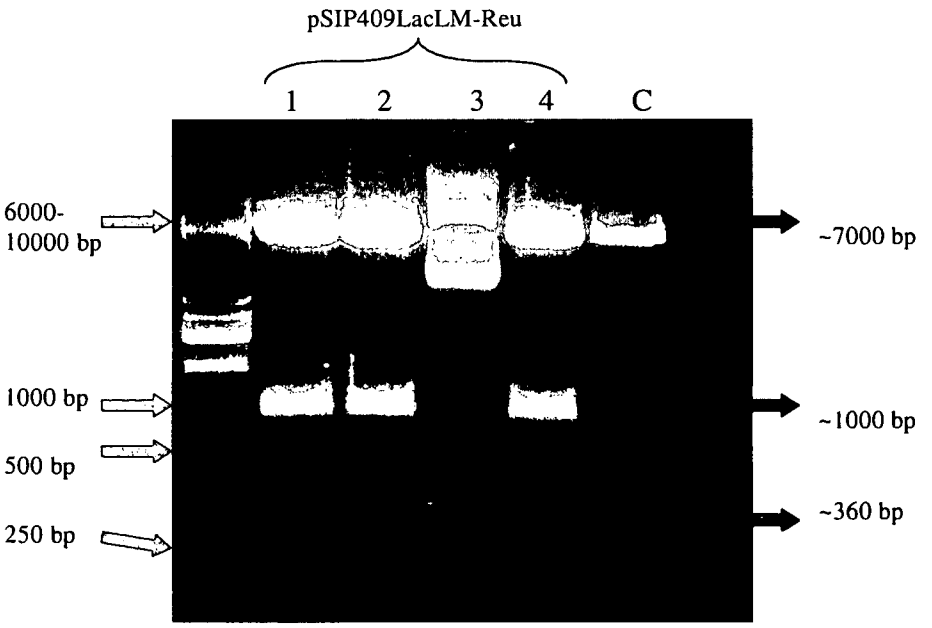


Figure A 40: Vector pSIP409LacLM-Reu digested with *EcoR*I on 1.2% agarose gel

Plasmids numbered 1, 2 and 4 showed correct fragments, thus their *lacLM* genes could be sequenced.

A4.10.2 Digestion of pSIP403LacLM-Aci and pSIP409LacLM-Aci

pSIP403LacLM-Aci and pSIP409LacLM-Aci was digested with *Kpn*I:

Table A 28: Digestion of pSIP403LacLM-Aci and pSIP409LacLM-Aci

pSIP403/409LacLM-Aci, pSIP403/409(gus)	10 µL
Buffer 1 Neb	3 µL
*Kpn*I (20 U)	2 µL
BSA 10x	3 µL
dH_2O	12 µL
37°C water bath for 2 h	

The pSIP403LacLM-Aci and pSIP409LacLM-Aci plasmids have two *Kpn*I sites. The resulting fragments of ~7000 bp and ~1600 bp were visualized by gel electrophoresis at 7 Vcm^{-1} on a 1.2% agarose gel and photographed under UV light. Additionally, a pSIP403(gus) [C1] and pSIP409(gus) control [C2] was digested with *Kpn*I and run on the gel. The pSIP403(gus) and the pSIP409(gus) plasmids have just one *Kpn*I site resulting in one fragment with the size of ~7444 bp.

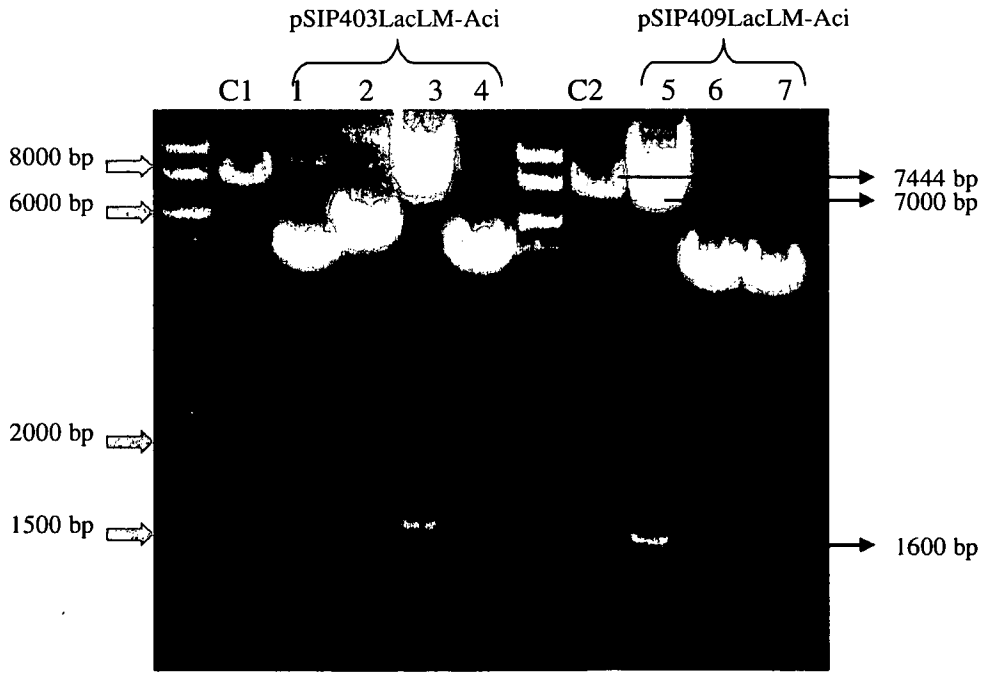


Figure A 41: Vectors pSIP403LacLM-Aci and pSIP409LacLM-Aci digested with *Kpn*I on 1.2% agarose gel

Plasmids numbered 3 and number 5 showed correct fragments, thus their *lacLM* genes could be sequenced.

A4.10.3 Digestion of pSIP403LacLM-Plant and pSIP409LacLM-Plant

pSIP403LacLM-Plant and pSIP409LacLM-Plant was digested with *EcoR*I:

Table A 29: Digestion of pSIP403LacLM-Plant and pSIP409LacLM-Plant

pSIP403/409LacLM-Plant, pSIP403/409(*gus*)	10 µL
Buffer 1 Neb	5 µL
*EcoR*I (50 U)	2.5 µL
dH_2O	32.5 µL
37°C water bath for 2.5 h	

The pSIP403LacLM-Plant and pSIP409LacLM-Plant plasmids have four *EcoR*I sites. The resulting fragments of ~5500 bp, ~1600, ~1000 bp and ~170 bp were visualized by gel electrophoresis at 7 Vcm^{-1} on a 1.2% agarose gel and photographed under UV light. Additionally, a pSIP403(*gus*) [C1] and pSIP409(*gus*) control [C2] was digested with *EcoR*I and run on the gel. The pSIP403(*gus*) and the pSIP409(*gus*) plasmids have just one *EcoR*I site resulting in one fragment of ~7444 bp.

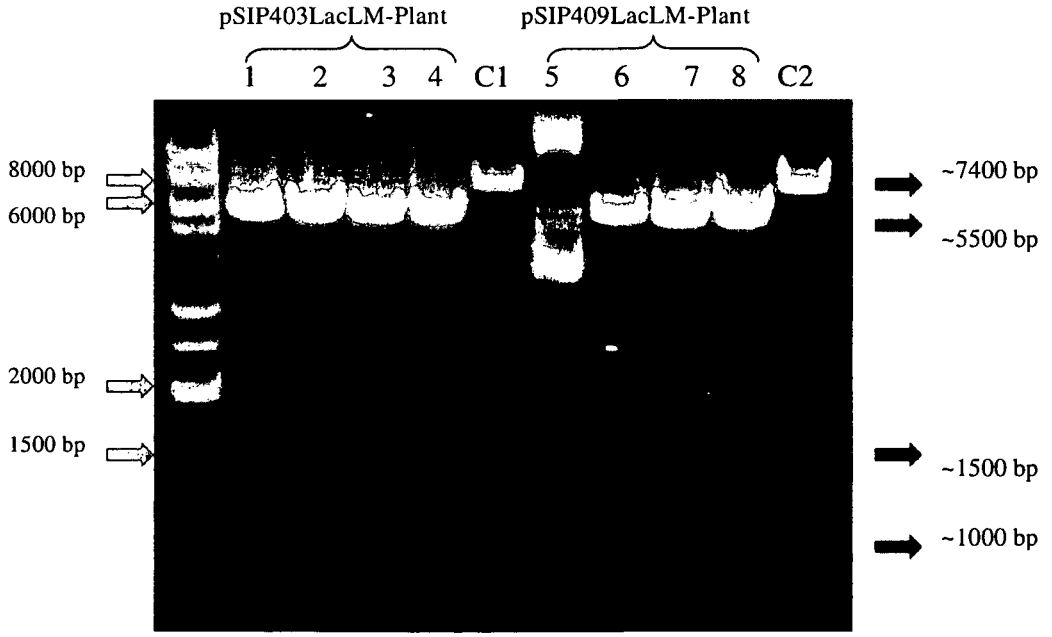


Figure A 42: Vectors pSIP403LacLM-Plant and pSIP409LacLM-Plant digested with *EcoR*I on 1.2%agarose gel

Plasmids numbered 1-4 and 6-8 showed correct fragments, thus their *lacLM* genes could be sequenced.

A4.10.4 Digestion of pSIP403LacLM-Sak and pSIP409LacLM-Sak

pSIP403LacLM-Sak and pSIP409LacLM-Sak was digested with *EcoR*I:

Table A 30: Digestion of pSIP403LacLM-Sak and pSIP409LacLM-Sak

pSIP403/409LacLM-Sak, pSIP403/409(*gus*)	15 µL
Buffer 1 Neb	5 µL
*EcoR*I (50 U)	2.5 µL
dH_2O	27.5 µL
37°C water bath for 2 h	

The pSIP403LacLM-Sak and pSIP409LacLM-Sak plasmids have two *EcoR*I sites. The resulting fragments of ~7200 bp and ~1200 bp were visualized by gel electrophoresis at 7 Vcm^{-1} in a 1.2% agarose gel and photographed under UV light. Additionally, a pSIP403(*gus*) [C1] and pSIP409(*gus*) control [C2] was digested with *EcoR*I and run on the gel. The pSIP403(*gus*) and the pSIP409(*gus*) plasmids have just one *EcoR*I site resulting in one fragment with the size of ~7444 bp.

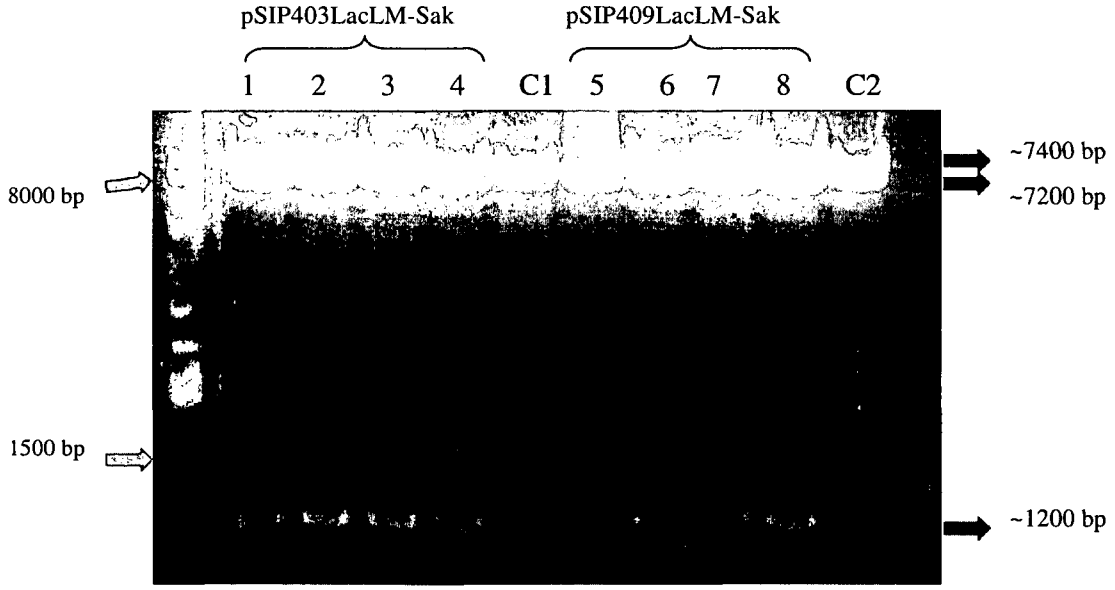


Figure A 43: Vectors pSIP403LacLM-Sak and pSIP409LacLM-Sak digested with *EcoR*I on 1.2% agarose gel

Plasmids number 1-4 and 6-8 showed correct fragments, thus their *lacLM* genes could be sequenced.

A4.11 Sequencing of lacLM genes

The nucleotide sequences were determined by the department of Biotechnology and Food Science, Norwegian University of Life Sciences, Ås. Assembly and analysis of DNA sequences were done using BioEdit Sequence Alignment Editor version 7.0.4.1. The basic local alignment tool (BLAST) from the National Centre for Biotechnology Information BLAST website was used for database searches.

The sequences of *lacLM* from *L. reuteri* L103, *L. acidophilus* R22 and *L. plantarum* WCFS1 in the plasmids pSIP403LacLM-Reu, pSIP409LacLM-Reu, pSIP403LacLM-Aci, pSIP409LacLM-Aci, pSIP403LacLM-Plant and pSIP409LacLM-Plant show 100 % identity with the sequences from the Gen Bank.
The sequence of *lacLM* from *L. sakei* Lb790 is not published yet thus the sequence of the inserted *lacLM* genes could not be compared.

Table A 31: Primers used for sequencing of the *lacLM* genes

Primer	Sequence (5´→ 3´)	Location	Reference sequence accession no.
SeqReuF	CCCTCTGAATTTGACCTGACT	β-gal	DQ493596
SeqReu2F	CCGTCACGAATGGAATGCT	β-gal	DQ493596
SeqReu3F	GAGGAGTACTTGAAGAACAATC	β-gal	DQ493596
SeqReu4F	CCGAGGAAGTGGCTTTAAT	β-gal	DQ493596
SeqAciF	CCGTCGTCCTGCTTACGC	β-gal	EF053367
SeqAci2F	GGCTTGAAGATCAAGACATGT	β-gal	EF053367
SeqAci3F	CGCCATGAATGGAATGCCAAAAC	β-gal	EF053367
SeqAci4F	GAGTGTGAATACATGCATGAC	β-gal	EF053367
SeqAci5F	GCACTAAAATCGAATTAAAGGTAGAT	β-gal	EF053367
SeqPlanF	GGCCTATTCAGCGACGCT	β-gal	AL935262
SeqPlan2F	GGAGCCTGCCAGCCATATT	β-gal	AL935262
SeqPlan3F	GCGATGACAAAGTCATTTACGT	β-gal	AL935262
SeqPlan4F	GCTTACTTGGAAGATAACCC	β-gal	AL935262
SeqPlan5F	GGCGACAACCGATAATGAT	β-gal	AL935262
SeqPlan6F	GCATGCACATGCAAACTGAAC	β-gal	AL935262
SeqSakF	GCGTCCCGCTTTTTCAACGT	β-gal	X82287
SeqSak2F	GCAGTAGTGCGGCTTTCAT	β-gal	X82287
SeqSak3F	GGCGTTAATCGGCATGAAT	β-gal	X82287
SeqSak4F	GCCCCACAAAAACCATTTATT	β-gal	X82287
SeqSak5F	GCGGCAACCAATTCCCATT	β-gal	X82287
SeqSak6F	GCTACCTGTGACGCCTTAT	β-gal	X82287
pSekF	GGCTTTTATAATATGAGATAATGCCGAC	pSIPvector	Sørvig *et al.*, 2003
pSIPseqR	TCTATTTAGGGTATTCCCGCC	pSIPvector	Sørvig *et al.*, 2003

A4.12 Transformation of the plasmids to Lactobacillus

To generate 16 strains carrying the plasmids pSIP403LacLM- (Reu, Aci, Plant, Sak) and pSIP409LacLM- (Reu, Aci, Plant, Sak) electroporation of electro-competent *L. plantarum* WCFS1 and *L. sakei* Lb790 cells was performed according to the protocol by Aukrust and Blom (1992).

To check if the transformation was correct the plasmids were isolated from *L. plantarum* WCFS1 and *L. sakei* Lb790 carrying the pSIP403LacLM and pSIP409LacLM plasmids.

Plasmids were isolated from 8 mL overnight cultures. The cells were harvested by centrifugation for 5 min at 5200 rpm. The supernatant was discarded and the pellet was

washed with 0.5 mL TEN-buffer. Cells were resuspended in 125 µL GTE-buffer with 100 µL lysozyme (40 mg/mL), 3 µL mutanolysin (5000 U/mL in 0.1 M K-phosphate buffer pH 6.2) and 25 µL RNAse (10 mg/mL). The solution was incubated in a 37 °C water bath for 1 h for *L. plantarum* and 30 min for *L. sakei*. Further isolation procedure was done according to E.Z.N.A.™ Plasmid Miniprep Kit I (Omega Bio-Tek. Inc. Doraville, GA) and in the final elution step only 30 µL of buffer was added.

A4.13 Expression of β-galactosidases

A4.13.1 Induced experiments

The strains:

- *L. plantarum* WCFS1 pSIP403LacLM-Reu
- *L. sakei* Lb790 pSIP403LacLM-Reu
- *L. plantarum* WCFS1 pSIP409LacLM-Reu
- *L. sakei* Lb790 pSIP409LacLM-Reu
- *L. plantarum* WCFS1 pSIP403LacLM-Aci
- *L. sakei* Lb790 pSIP403LacLM-Aci
- *L. plantarum* WCFS1 pSIP409LacLM-Aci
- *L. sakei* Lb790 pSIP409LacLM-Aci
- *L. plantarum* WCFS1 pSIP403LacLM-Plant
- *L. sakei* Lb790 pSIP403LacLM-Plant
- *L. plantarum* WCFS1 pSIP409LacLM-Plant
- *L. sakei* Lb790 pSIP409LacLM-Plant
- *L. plantarum* WCFS1 pSIP403LacLM-Sak
- *L. sakei* Lb790 pSIP403LacLM-Sak
- *L. plantarum* WCFS1 pSIP409LacLM-Sak
- *L. sakei* Lb790 pSIP409LacLM-Sak
- *L. plantarum* WCFS1 pSIP405
- *L. sakei* Lb790 pSIP405

were grown at 30°C in MRS medium (50mL-tubes) containing 5 µg/ml erythromycin. When an optical density at 600 nm of 0.3 was reached the cultures were induced with 25 ng/ml peptide pheromone. Cells were harvested at an optical density at 600 nm of 1.8 by

centrifugation at 5200 rpm for 5 min at 4°C and washed once with 0.9 % sodium chloride solution. The pellet was resuspended in 1 mL sonication buffer and cells were disrupted using a sonicator (Sonics vibra-cell, Sonics and Materials Inc, USA). Sonication was performed 3 min with amplitude of 25%, pulse 5 sec on 5 sec off, eppis were placed on ice the whole sonication procedure. Debris was removed by centrifugation (13200 rpm, 5 min, 4°C) to obtain the crude enzyme extract.

The background expression from the chromosomes of *L. plantarum* pSIP405 and *L. sakei* pSIP405, which carry a *cat* (chloramphenicol) gene instead of the *lacLM* gene on their plasmids, were tested with same growth conditions as the strains carrying the *lacLM* genes on their plasmids.

All growth experiments were done in three independent tests.

Protein concentration and β-galactosidase activity in the crude extract were determined using method of Bradford and *o*NPG assays, respectively.

Table A 32: First harvesting with induction

Lactobacillus	plasmid	OD600nm	activity [U/mL]	protein conc. [mg/mL]	specific activity [U/mg]	U/L
plantarum	403-LacLMReu	2.05	224.6	3.62	62.05	4493
sakei	403-LacLMReu	1.7	97.3	3.35	29.10	1947
plantarum	409-LacLMReu	1.8	157.6	2.52	62.49	3151
sakei	409-LacLMReu	1.85	107.6	3.17	33.99	2153
plantarum	403-LacLMAci	2	10.61	2.26	4.70	212
sakei	403-LacLMAci	1.8	27.9	3.25	8.59	558
plantarum	409-LacLMAci	2	8.52	1.95	4.36	170
sakei	409-LacLMAci	1.8	15.1	3.51	4.31	303
plantarum	403-LacLMPlant	2	171.6	2.78	61.64	3432
sakei	403-LacLMPlant	1.75	39.3	2.82	13.94	786
plantarum	409-LacLMPlant	2.1	117.2	2.70	43.38	2343
sakei	409-LacLMPlant	2.05	76.4	3.81	20.05	1529
plantarum	403-LacLMSak	2.25	12.9	2.44	5.29	258
sakei	403-LacLMSak	1.7	4.7	2.29	2.04	94
plantarum	409-LacLMSak	1.9	9.1	1.61	5.68	182
sakei	409-LacLMSak	1.7	11.2	1.69	6.62	224
plantarum	405	1.8	0.1	0.96	0.06	2
sakei	405	1.75	0.2	2.03	0.10	4

Table A 33: Second harvesting with induction

Lactobacillus	plasmid	OD600nm	activity [U/mL]	protein conc. [mg/mL]	specific activity [U/mg]	U/L
plantarum	403-LacLMReu	2.15	183.92	2.71	67.81	3678
sakei	403-LacLMReu	1.75	73.63	3.54	20.82	1473
plantarum	409-LacLMReu	1.75	158.50	3.07	51.61	3170
sakei	409-LacLMReu	2.1	64.43	2.45	26.30	1289
plantarum	403-LacLMAci	1.75	9.45	2.51	3.77	189
sakei	403-LacLMAci	1.95	29.72	4.15	7.17	594
plantarum	409-LacLMAci	1.9	3.63	1.70	2.13	73
sakei	409-LacLMAci	1.85	14.73	4.18	3.52	295
plantarum	403-LacLMPlant	1.85	93.44	2.19	42.73	1869
sakei	403-LacLMPlant	2.25	49.06	3.42	14.36	981
plantarum	409-LacLMPlant	2.15	123.08	2.74	44.98	2462
sakei	409-LacLMPlant	1.75	44.62	2.95	15.12	892
plantarum	403-LacLMSak	2.05	11.28	2.59	4.35	226
sakei	403-LacLMSak	1.75	7.30	3.33	2.19	146
plantarum	409-LacLMSak	1.85	7.41	1.46	5.08	148
sakei	409-LacLMSak	1	11.20	1.73	6.49	224
plantarum	405	1.85	0.08	1.17	0.07	2
sakei	405	2	0.05	2.26	0.02	1

Table A 34: Third harvesting with induction

Lactobacillus	Plasmid	OD600nm	activity [U/mL]	protein conc. [mg/mL]	specific. activity [U/mg]	U/L
plantarum	403-LacLMReu	1.8	152.10	2.84	53.48	3042
sakei	403-LacLMReu	1.8	54.44	3.19	17.07	1089
plantarum	409-LacLMReu	1.85	111.70	2.26	49.46	2234
sakei	409-LacLMReu	1.9	97.03	3.43	28.29	1941
plantarum	403-LacLMAci	1.85	8.53	2.04	4.18	171
sakei	403-LacLMAci	1.95	28.39	4.36	6.51	568
plantarum	409-LacLMAci	1.75	5.15	2.15	2.39	103
sakei	409-LacLMAci	1.85	19.81	3.86	5.13	396
plantarum	403-LacLMPlant	1.8	116.53	2.72	42.77	2331
sakei	403-LacLMPlant	1.8	74.57	3.43	21.74	1491
plantarum	409-LacLMPlant	1.75	128.08	2.78	46.00	2562
sakei	409-LacLMPlant	1.9	74.57	3.69	20.20	1491
plantarum	403-LacLMSak	1.85	11.20	2.26	4.96	224
sakei	403-LacLMSak	1.75	9.69	3.93	2.,46	194
plantarum	409-LacLMSak	1.75	5.79	1.17	4.94	116
sakei	409-LacLMSak	1.7	14.15	1.85	7.64	283
plantarum	405	1.85	0.07	0.95	0.08	1
sakei	405	1.85	0.05	2.49	0.02	1

Table A 35: Conclusion of the harvestings with induction

Lactobacillus	Plasmid	specific activity [U/mg]	specific activity [U/mg]	specific. activity [U/mg]	average [U/mg]	standard deviation [U/mg]
plantarum	403-LacLMReu	62.05	67.81	53.48	61.11	7.21
sakei	403-LacLMReu	29.10	20.82	17.,07	22.33	6.16
plantarum	409-LacLMReu	62.49	51.61	49.46	54.52	6.99
sakei	409-LacLMReu	33.99	26.30	28.29	29.53	3.99
plantarum	403-LacLMAci	4.70	3.77	4.18	4.21	0.47
sakei	403-LacLMAci	8.59	7.17	6.51	7.42	1.06
plantarum	409-LacLMAci	4.36	2.13	2.39	2.96	1.22
sakei	409-LacLMAci	4.31	3.52	5.13	4.32	0.81
plantarum	403-LacLMPlant	61.64	42.73	42.77	49.05	10.90
sakei	403-LacLMPlant	13.94	14.36	21.74	16.68	4.39
plantarum	409-LacLMPlant	43.38	44.98	46.00	44.79	1.32
sakei	409-LacLMPlant	20.05	15.12	20.20	18.46	2.89
plantarum	403-LacLMSak	5.29	4.35	4.96	4.87	0.48
sakei	403-LacLMSak	2.04	2.19	2.46	2.23	0.22
plantarum	409-LacLMSak	5.68	5.08	4.94	5.23	0.39
sakei	409-LacLMSak	6.62	6.49	7.64	6.91	0.63
plantarum	405	0.06	0.07	0.08	0.07	0.01
sakei	405	0.10	0.02	0.02	0.05	0.04

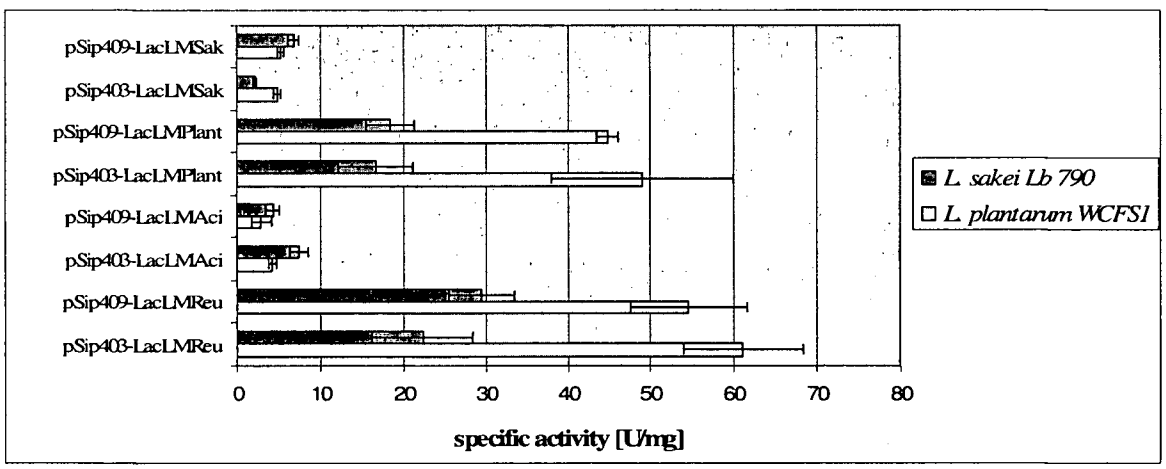


Figure A 44: Expression of β-galactosidase in *L. plantarum* WCFS1 and *L. sakei* Lb790

Background β-galactosidase activities derived from the genome of *L. plantarum* WCFS1 and *L. sakei* Lb790 when grown on glucose were not significant (see data of *L. plantarum* WCFS1 pSIP405 and *L. sakei* Lb790 pSIP405).

L. plantarum WCFS1 is the better host for expression of β-galactosidase from *L. reuteri*, *L. plantarum* cloned in both vectors and from *L. sakei* cloned in vector pSIP403. *L. sakei* is the better host for expression of β-galactosidase from *L. acidophilus* cloned in both vectors and *L. sakei* cloned in vector pSIP409.

It was found that *L. plantarum* WCFS1 carrying the plasmid pSIP403LacLM-Reu, which contains the complete β-galactosidase genes from *L. reuteri*, gave the highest expression level of the enzyme of interest in this work. Thus this strain and its recombinant β-galactosidase were selected for further studies.

A4.13.2 Non-induced experiments

To check if there was a leakage of expression, *L. plantarum* WCFS1 and *L. sakei* Lb790 carrying the overexpression plasmids were grown the same way as mentioned above but without induction.

Table A 36: First harvesting without induction

Lactobacillus	Plasmid	OD600nm	activity [U/mL]	protein conc. [mg/mL]	specific activity [U/mg]
L. plantarum	pSip403-LacLMReu	1.8	4.94	2.70	1.83
L. sakei	pSip403-LacLMReu	2.1	0.70	3.48	0.20
L. plantarum	pSip409-LacLMReu	1.95	0.86	2.96	0.29
L. sakei	pSip409-LacLMReu	1.85	0.45	3.67	0.12
L. plantarum	pSip403-LacLMAci	2.1	0.70	2.64	0.27
L. sakei	pSip403-LacLMAci	2.05	0.57	4.27	0.13
L. plantarum	pSip409-LacLMAci	1.85	0.15	2.83	0.05
L. sakei	pSip409-LacLMAci	2.1	0.18	3.88	0.05
L. plantarum	pSip403-LacLMPlant	2.1	3.35	2.65	1.26
L. sakei	pSip403-LacLMPlant	1.95	0.93	3.58	0.26
L. plantarum	pSip409-LacLMPlant	1.8	0.90	2.70	0,33
L. sakei	pSip409-LacLMPlant	1.9	0.73	3.47	0.21
L. plantarum	pSip403-LacLMSak	1.75	1.28	2.89	0.44
L. sakei	pSip403-LacLMSak	1.7	0.17	3.58	0.05
L. plantarum	pSip409-LacLMSak	1.8	0.93	2.99	0.31
L. sakei	pSip409-LacLMSak	1.85	0.43	3.75	0.11

Table A 37: Second harvesting without induction

Lactobacillus	Plasmid	OD600nm	activity [U/mL]	protein conc. [mg/mL]	specific activity [U/mg]
L. plantarum	pSip403-LacLMReu	1.95	5.30	3.44	1.54
L. sakei	pSip403-LacLMReu	3.1	1.02	4.37	0.23
L. plantarum	pSip409-LacLMReu	2.35	0.79	2.57	0.31
L. sakei	pSip409-LacLMReu	1,65	0.33	2.86	0.12
L. plantarum	pSip403-LacLMAci	2	0.73	2.86	0.26
L. sakei	pSip403-LacLMAci	1.75	0.40	3.11	0,13
L. plantarum	pSip409-LacLMAci	2.15	0.17	2.80	0.06
L. sakei	pSip409-LacLMAci	1.8	0.12	2.98	0.04
L. plantarum	pSip403-LacLMPlant	2.1	3.27	2.80	1.17
L. sakei	pSip403-LacLMPlant	1.9	0.89	3.50	0.25
L. plantarum	pSip409-LacLMPlant	2.05	0.84	2.27	0.37
L. sakei	pSip409-LacLMPlant	1.75	0.70	2.96	0.24
L. plantarum	pSip403-LacLMSak	1.95	0.90	2.88	0.31
L. sakei	pSip403-LacLMSak	1.7	0.16	2.75	0.06
L. plantarum	pSip409-LacLMSak	2.15	0.85	2.66	0.32
L. sakei	pSip409-LacLMSak	1.75	0.35	3.38	0.10

Table A 38: Third harvesting without induction

Lactobacillus	Plasmid	OD600nm	activity [U/mL]	Protein conc. [mg/mL]	specific activity [U/mg]
L. plantarum	pSip403-LacLMReu	1.7	4.69	3.20	1.46
L. sakei	pSip403-LacLMReu	1.9	0.53	3.35	0.16
L. plantarum	pSip409-LacLMReu	2.05	0.76	2.81	0.27
L. sakei	pSip409-LacLMReu	1.7	0.39	3.09	0.13
L. plantarum	pSip403-LacLMAci	2	0.94	3.18	0.29
L. sakei	pSip403-LacLMAci	1.75	0.37	2.95	0.13
L. plantarum	pSip409-LacLMAci	1.75	0.19	3.04	0.06
L. sakei	pSip409-LacLMAci	1.9	0.13	3.18	0.04
L. plantarum	pSip403-LacLMPlant	1.95	3.55	3.00	1.18
L. sakei	pSip403-LacLMPlant	1.7	0.85	2.87	0.29
L. plantarum	pSip409-LacLMPlant	2.15	0.92	2.43	0.38
L. sakei	pSip409-LacLMPlant	2.05	0.82	3.43	0.24
L. plantarum	pSip403-LacLMSak	1.9	1.16	3.08	0.38
L. sakei	pSip403-LacLMSak	1.8	0.18	2.77	0.07
L. plantarum	pSip409-LacLMSak	1.8	0.79	2.40	0.33
L. sakei	pSip409-LacLMSak	1.7	0.34	3.14	0.11
L. sakei	pSip403-LacLMReu	2.1	0.70	3.48	0.20
L. plantarum	pSip409-LacLMReu	1.95	0.74	2.28	0.33

Table A 39: Conclusion of the harvestings without induction

Lactobacillus	Plasmid	specific activity [U/mg]	specific activity [U/mg]	specific activity [U/mg]	average [U/mg]	standard deviation [U/mg]
L. plantarum	pSip403-LacLMReu	1.83	1.54	1.46	1.61	0.19
L. sakei	pSip403-LacLMReu	0.28	0.20	0.16	0.21	0.06
L. plantarum	pSip409-LacLMReu	0.29	0.33	0.27	0.30	0.03
L. sakei	pSip409-LacLMReu	0.12	0.12	0.13	0.12	0.01
L. plantarum	pSip403-LacLMAci	0.27	0.26	0.29	0.27	0.02
L. sakei	pSip403-LacLMAci	0.13	0.13	0.13	0.13	0.00
L. plantarum	pSip409-LacLMAci	0.05	0.06	0.06	0.06	0.00
L. sakei	pSip409-LacLMAci	0.05	0.04	0.04	0.04	0.00
L. plantarum	pSip403-LacLMPlant	1.26	1.17	1.18	1.21	0.05
L. sakei	pSip403-LacLMPlant	0.26	0.25	0.29	0.27	0.02
L. plantarum	pSip409-LacLMPlant	0.33	0.37	0.38	0.36	0.03
L. sakei	pSip409-LacLMPlant	0.21	0.24	0.24	0.23	0.01
L. plantarum	pSip403-LacLMSak	0.44	0.31	0.38	0.38	0.06
L. sakei	pSip403-LacLMSak	0.05	0.06	0.07	0.06	0.01
L. plantarum	pSip409-LacLMSak	0.31	0.32	0.33	0.32	0.01
L. sakei	pSip409-LacLMSak	0.11	0.10	0.11	0.11	0.01

No significant β-galactosidase activities were found in the uninduced cultures. Thus there is no leakage of the vector system.

A4.13.3 Wild types grown on lactose

To compare the overexpression of recombinant β-galactosidase with the activity of native β-galactosidase from the *Lactobacillus* wild types they were grown on MRS with lactose instead of glucose.

- *L. reuteri* L103
- *L. acidophilus* R22
- *L. plantarum* WCFS1
- *L. sakei* Lb790

were grown at 37°C in "home-made" MRS medium with lactose instead of glucose (50mL-tubes). Cells were harvested at an optical density at 600 nm of ~2.0 by centrifugation at 5200 rpm for 5 min at 4°C and washed once with 0.9 % sodium chloride solution. The pellet was resuspended in 1 mL sonication buffer and cells were disrupted by using a sonicator (Sonics

vibra-cell, Sonics and Materials Inc, USA). Sonication was performed 3 min with amplitude of 25%, pulse 5 sec on 5 sec off, eppis were placed on ice during the whole sonication procedure. Debris was removed by centrifugation (13200 rpm, 5 min, 4°C) to obtain the crude enzyme extract.

Protein concentration and β-galactosidase activity in the crude extract were determined using method of Bradford and *o*NPG assays, respectively.

Table A 40: Wild types grown on lactose

37°C	Media	OD600nm	activity [U/mL]	protein conc. [mg/mL]	specific activity [U/mg]	average [U/mg]	standard deviation [U/mg]
L. reuteri	MRS-lactose*	1.9	33.07	4.71	7.02		
L. reuteri	MRS-lactose*	2	31.20	3.82	8.16	7.90	0.78
L. reuteri	MRS-lactose*	2.05	32.29	3.79	8.53		
L. acidophilus	MRS-lactose*	1.95	19.73	4.25	4.64		
L. acidophilus	MRS-lactose*	2.05	19.94	5.33	3.74	4.00	0.56
L. acidophilus	MRS-lactose*	1.75	18.16	5.02	3.62		
L. plantarum	MRS-lactose*	2.1	3.67	1.62	2.26		
L. plantarum	MRS-lactose*	2	3.92	1.68	2.33	2.22	0.14
L. plantarum	MRS-lactose*	2.32	3.27	1.59	2.06		
L. sakei	MRS-lactose*	0.6	0.05	0.70	0.08		
L. sakei	MRS-lactose*	0.6	0.05	0.79	0.06	0.07	0.01
L. sakei	MRS-lactose*	0.5	0.05	0.83	0.06		

* "home-made" MRS medium with lactose

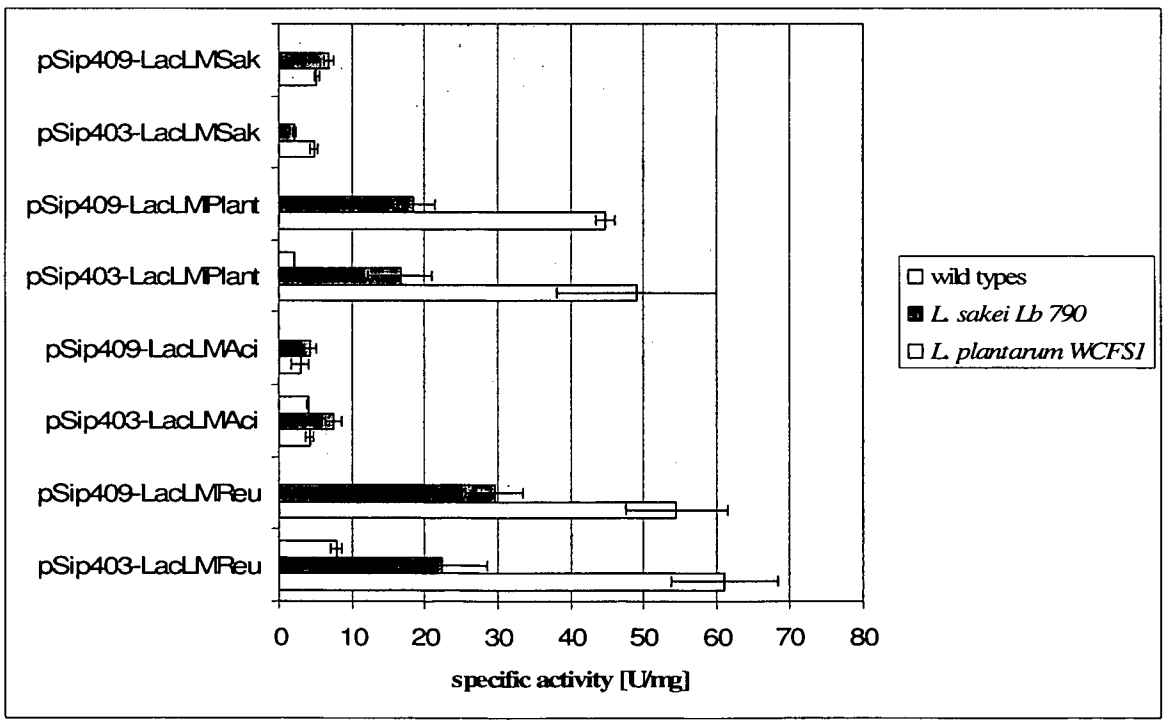


Figure A 45: Comparison of expression levels of native and recombinant β-galactosidases

Expression levels of recombinant β-galactosidases are much higher than that of the native β-galactosidases. Results from *L. sakei* Lb790 can not be included because this strain is very sensitive to temperature and did not grow properly at 37°C and the growth was completely stopped at OD_{600nm} of 0.6. Its optimum growing temperature is at 30°C.

To see if there is a difference in the expression level at 30°C and 37°C some recombinant strains were grown and induced at 37°C. To compare the "home-made" MRS media with the commercial one a "home-made" MRS with glucose was also prepared.

Table A 41: Comparison growth medium (MRS) and temperature

37°C	media	OD600nm	activity [U/mL]	protein conc. [mg/mL]	specif. activity [U/mg]	specif. activity at 30°C [U/mg]
L. plantarum 403Reu	MRS-glucose*	1.95	147.26	1.91	77.27	61.1±7.2
L. plantarum 409Reu	MRS-glucose*	1.8	124.33	2.10	59.29	54.5±7.0
L. plantarum 403Reu	MRS**	1.95	106.55	1.65	64.62	61.1±7.2
L. plantarum 409Reu	MRS**	1.95	63.34	1.40	45.11	54.5±7.0

* "home-made" MRS medium with glucose

** commercial MRS

There is no significant difference between the expression levels at 30°C and 37°C. The "home-made" MRS seems to give a little bit higher expression rates than the commercial one. This result could be based on the fact that the sugars in the "home-made" one were autoclaved separately from other ingredients of the media thus no maillard reaction took place and more sugar is available for the bacteria. This experiment was carried out without independent parallels thus a statistical statement is not possible.

A4.14 SDS-PAGE of crude enzyme extract from 16 strains

See figure 2 in the manuscript.

A5 Protein purification and characterization

A5.1 Preliminary tests

To find out at which OD_{600nm} it is the best for harvesting the cells to get high specific activity of the enzyme a preliminary test was carried out in which the cells were harvested at different OD_{600nm}.

L. plantarum WCFS1 carrying pSip403LacLM-Reu, the plasmid containing the complete genes (*lacL* and *lacM*) of β-galactosidase from *L. reuteri* L103 was grown in MRS broth (containing 5 µg/ml erythromycin) in a 1 L glass bottle capped tightly at 30°C. Precultures were grown overnight in MRS broth medium containing 5 µg/ml erythromycin, reactivated three times and inoculated into fresh media to an optical density at 600 nm of 0.1. When optical density at 600 nm reached 0.3, pheromone (25 ng/l) was then added to the culture medium and the cultures were incubated further for 30 h. When an optical density at 600 nm of 5.2, 5.5, 7.6 and 8.5 was reached 100 mL sample was taken and cells were harvested, washed once with 50 mM sodium phosphate buffer (pH 6.0), and resuspended in 3 mL of the same phosphate buffer as used for washing the cells. Cell disruption was performed using a French press (AMINCO, Maryland, USA) and debris was removed by centrifugation at 13000 rpm for 15 min at 4°C to obtain the crude extract. Protein concentration and β-galactosidase activity of the crude extract were determined.

Table A 42: Results of the preliminary tests

Sample	Incubation time [h]	OD600nm	Protein conc. [mg/mL]	Activity [U/mL]	Specific activity [U/mg]	U/L fermentation broth
A	15	5.2	6.15	690	112	17250
B	16	5.5	8.18	915	110	22870
C	27	7.6	4.31	390	90	9750
D	33	8.5	5.58	373	70	9325

The best optical density of the cells is determined to be between 5 and 6 in terms of enzyme activity and yield.

A5.2 Fermentation of *L. plantarum* WCFS1 pSIP403LacLM-Reu

See Fermentation of the selected *Lactobacillus* strain in the manuscript.

A5.3 Protein purification

Buffer A:

50 mM sodium phosphate, pH 6.0

Buffer B:

50 mM sodium phosphate, pH 6.0

2 M NaCl

Affinity chromatography

3 mL crude extract was loaded onto the affinity column (10 ml of *p*-aminobenzyl 1-thio-β-D-galactopyranoside immobilized on crosslinked 4% beaded agarose; Sigma) that was pre-equilibrated with 50 mM sodium phosphate buffer pH 6.0 (buffer A). The enzyme was eluted at a rate of 0.6 ml/min by using a linear 0 to 2 M sodium chloride gradient in 15 column volumes. The active fractions were pooled, desalted, and concentrated. The purified enzyme was stored in 50 mM sodium phosphate buffer (pH 6.0) containing 1 mM DTT at 4°C.

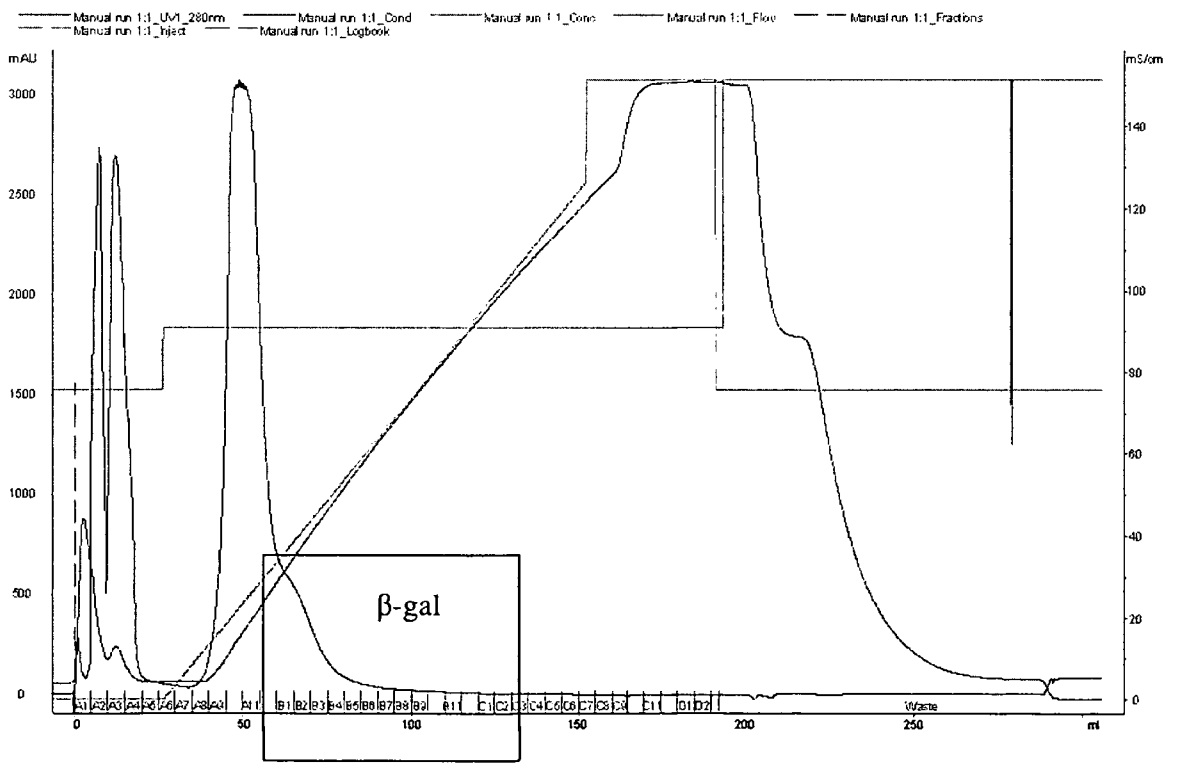


Figure A 46: Chromatogram - purification of recombinant β-galactosidase from *L. reuteri* L103 expressed in *L. plantarum* WCFS1

Table A 43: Pooled active fractions

Pool	fractions	Volume [µL] after concentration	Protein conc.[mg/mL]	Activity [U/mL]	Specific activity [U/mL]
1	A_{12}-B_1	450	9.4	620	66
2	B_2-B_5	350	12.2	3000	245
3	B_6-B_{10}	550	1.73	250	145
4	B_{11}-C_3	400	0.48	45	95

Table A 44: Yield of pooled active fractions

	Total activity [U]	Total protein [mg]	Specific activity [U/mg]	Purification factor	Yield [%]
Crude extract	8670	63.6	136	1	100
Pool 1	280	4.23	66	0.49	0.03
Pool 2	1050	4.27	245	1.8	12
Pool 3	138	0.95	145	1.07	1.6
Pool 4	18	0.192	95	0.7	0.2

A5.4 Gel electrophoresis and active staining

See also Gel electrophoresis and active staining in the manuscript.

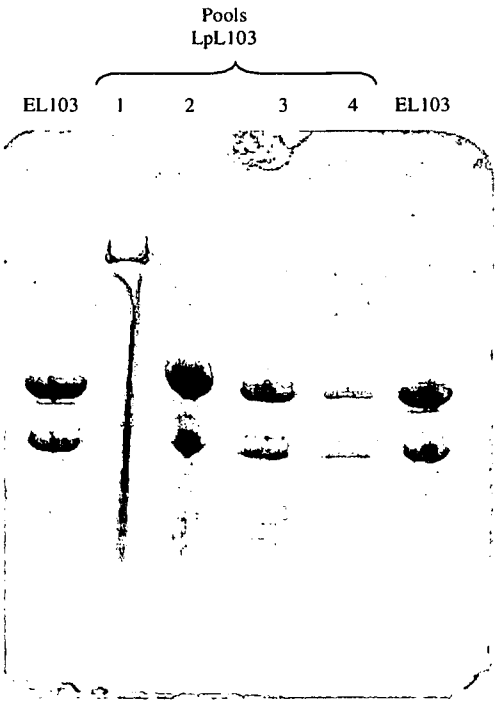


Figure A 47: Pooled active fractions of LpL103 on SDS-PAGE

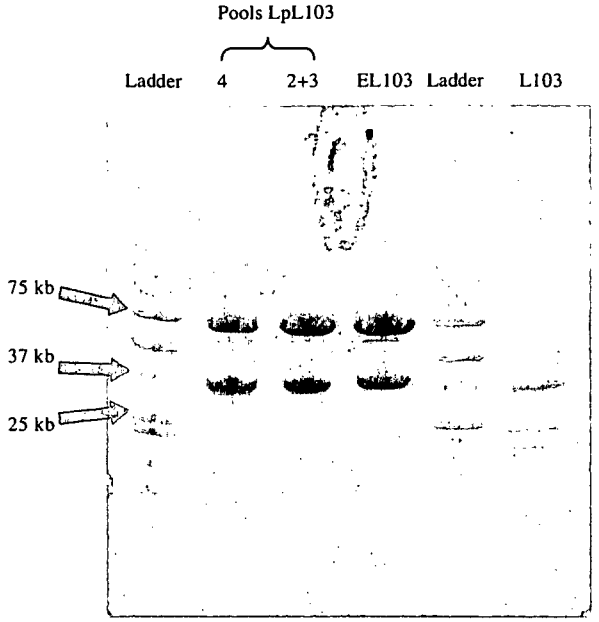


Figure A 48: Pooled active fractions of LpL103, EL103 and L103 on SDS-PAGE

A5.5 Characterization of the recombinant β-galactosidase enzyme LpL103

A5.5.1 *Km* and *Vmax* with lactose as substrate

Solutions of 0, 10, 20, 50, 75, 100, 150, 300 and 600 mM lactose (in 50 mM NaPP pH 6.5) were prepared.

β-Galactosidase activity was determined with lactose as substrate. For the assay 480 µL of the different lactose solutions (0-600 mM) were used.

Three assays were run with each solution (two parallels of the enzyme sample and one blank test with dH_2O instead of the sample).

Table A 45: Measurements for Km and Vmax

Lactose [mM]	Dilution	Blank OD546	OD546	OD546	average-blank	activity [U/mL]	specific activity [U/mg]
600	100	0.1003	0.5139	0.5054	0.40935	226.71	48.24
300	100	0.0796	0.4747	0.4615	0.3885	215.16	45.78
150	100	0.0691	0.4236	0.4359	0.36065	199.74	42.50
100	100	0.0686	0.4067	0.4072	0.33835	187.39	39.87
75	100	0.0699	0.3842	0.3663	0.30535	169.11	35.98
50	100	0.0688	0.382	0.355	0.2997	165.98	35.32
20	100	0.0699	0.3102	0.3085	0.23945	132.62	28.22
10	100	0.0661	0.2741	0.2753	0.2086	115.53	24.58
0	100	0.0633	0.0601	0.0619	0	0	0

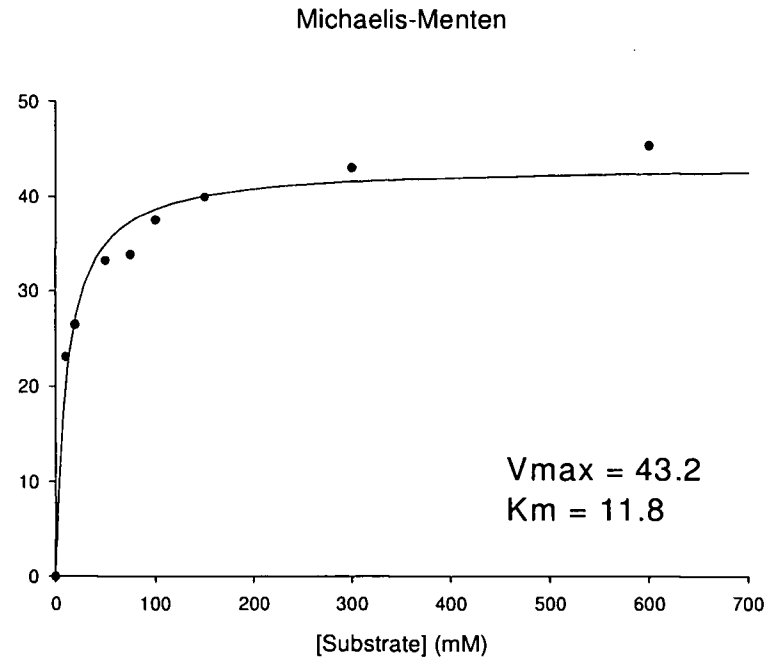


Figure A 49: Michaelis-Menten

A5.5.2 pH optimum

The pH dependence of the enzymatic release of *o*-nitrophenol (*o*NP) from *o*NPG and D-glucose from lactose was measured between pH 4 and 9 using Britton-Robinson buffer (containing equimolar amounts, 20 mM, of phosphoric, acetic and boric acids adjusted to the required pH with NaOH). The activity was determined at different pH values under standard assay conditions.

Table A 46: pH optimum of LpL103with *o*NPG as the substrate

pH	Dilution	BlankOD420	OD420	OD420	average-Blank	activity [U/mL]	rel. activity [%]
4	2	0.0841	0.3863	0.3794	0.29875	1.26	0.10
5	200	0.0796	0.4221	0.4322	0.34755	146.92	11.97
5.51	1000	0.082	0.3827	0.3943	0.3065	647.83	52.79
6.06	1500	0.082	0.3602	0.3635	0.27985	887.25	72.30
6.49	1500	0.08	0.4022	0.3903	0.31625	1002.65	81.70
7.04	2000	0.0804	0.3671	0.3743	0.2903	1227.17	100.00
7.52	2000	0.0809	0.3321	0.3305	0.2504	1058.51	86.26
8.05	2000	0.0864	0.2833	0.2776	0.19405	820.30	66.84
8.49	1000	0.0819	0.3108	0.3159	0.23145	489.20	39.86
9.01	200	0.0809	0.2529	0.2461	0.1686	71.27	5.81

Table A 47: pH optimum of LpL103with lactose as the substrate

pH	Dilution	BlankOD546	OD546	OD546	average-Blank	activity [U/mL]	Rel. activity [%]
4	1	0.0894	0.1373	0.1356	0.04705	0.26	0.13
5	10	0.0895	0.5574	0.561	0.4697	26.01	13.13
5.51	50	0.0911	0.3752	0.3888	0.2909	80.54	40.66
6.06	100	0.0839	0.3989	0.3847	0.3079	170.49	86.08
6.49	100	0.0937	0.4367	0.4519	0.3506	194.13	98.02
7.04	100	0.122	0.4729	0.4694	0.34915	193.33	97.61
7.52	100	0.1163	0.4766	0.4714	0.3577	198.06	100.00
8.05	100	0.1136	0.4257	0.4286	0.31355	173.62	87.66
8.49	100	0.108	0.4194	0.4078	0.3056	169.21	85.43
9.01	100	0.1072	0.3476	0.3485	0.24085	133.36	67.33

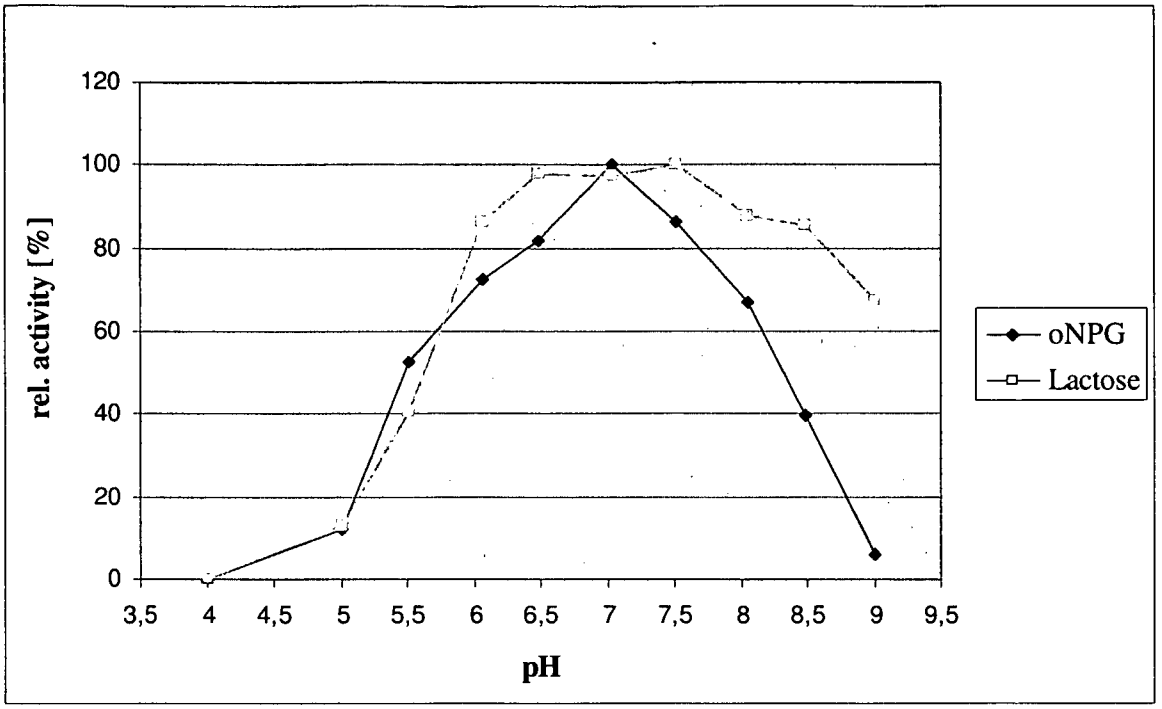


Figure A 50: pH optimum LpL103

Table A 48: pH optimum of EL103 with *o*NPG as the substrate

pH	Dilution	BlankOD420	OD420	OD420	average-Blank	activity [U/mL]	Rel. Activity [%]
4	50	0.0843	0.4946	0.4848	0.4054	42.84	2.27
5.02	500	0.0833	0.3985	0.4003	0.3161	334.06	17.67
5.6	1000	0.082	0.4156	0.412	0.3318	701.30	37.10
6.01	2000	0.0831	0.4309	0.4281	0.3464	1464.32	77.46
6.48	2000	0.0792	0.4975	0.5065	0.4228	1787.28	94.55
7.04	2500	0.0817	0.4449	0.434	0.35775	1890.38	100.00
7.5	2500	0.0798	0.3947	0.3965	0.3158	1668.71	88.27
8	2000	0.0792	0.4013	0.3966	0.31975	1351.67	71.50
8.49	1000	0.0808	0.5626	0.5545	0.47775	1009.79	53.42
9.02	200	0.0791	0.5477	0.5365	0.463	195.72	10.35

Table A 49: pH optimum of EL103 with lactose as the substrate

pH	Dilution	BlankOD546	OD546	OD546	average-Blank	activity [U/mL]	Rel. Activity [%]
4	5	0.0823	0.3308	0.3252	0.2457	6.80	2.77
5.02	20	0.0736	0.4991	0.4958	0.42385	46.94	19.14
5.6	50	0.0738	0.4322	0.4287	0.35665	98.74	40.27
6.01	200	0.0754	0.2467	0.2379	0.1669	184.83	75.38
6.48	200	0.0755	0.2733	0.2773	0.1998	221.26	90.24
7.04	200	0.0794	0.296	0.3056	0.2214	245.18	100.00
7.5	200	0.0839	0.2761	0.2851	0.1967	217.83	88.84
8	200	0.0834	0.2645	0.2652	0.18145	200.94	81.96
8.49	200	0.0844	0.2464	0.2449	0.16125	178.57	72.83
9.02	100	0.083	0.3673	0.3608	0,28105	155.62	63.47

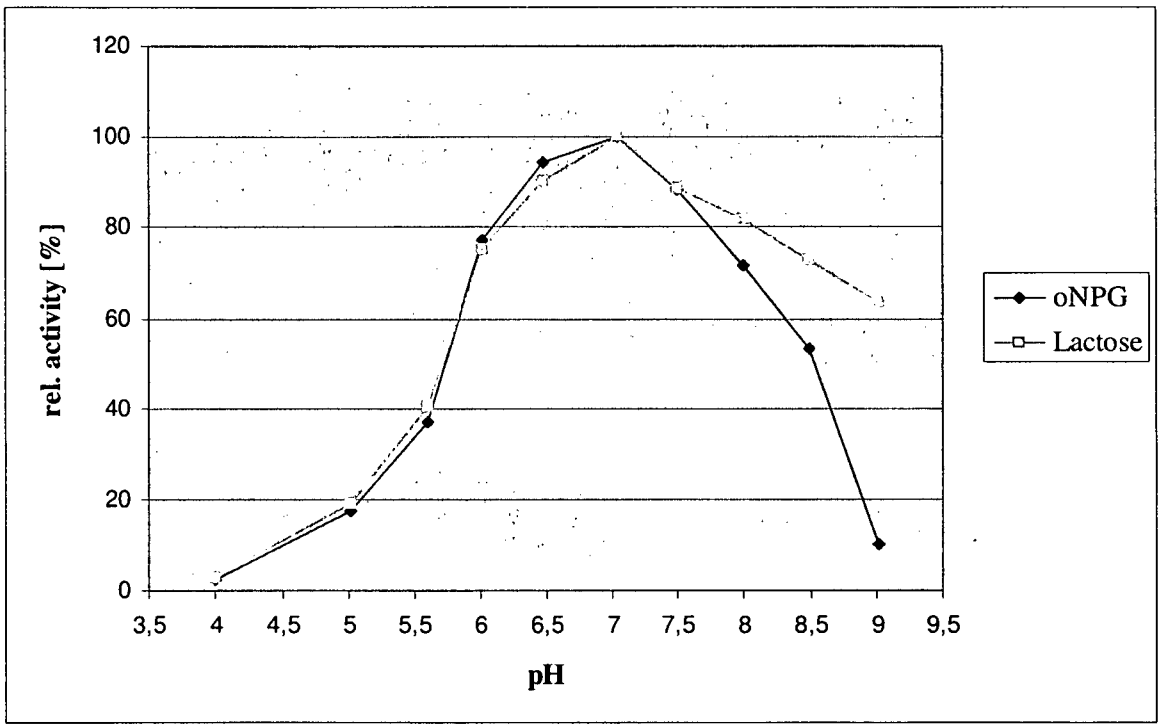


Figure A 51: pH optimum EL103

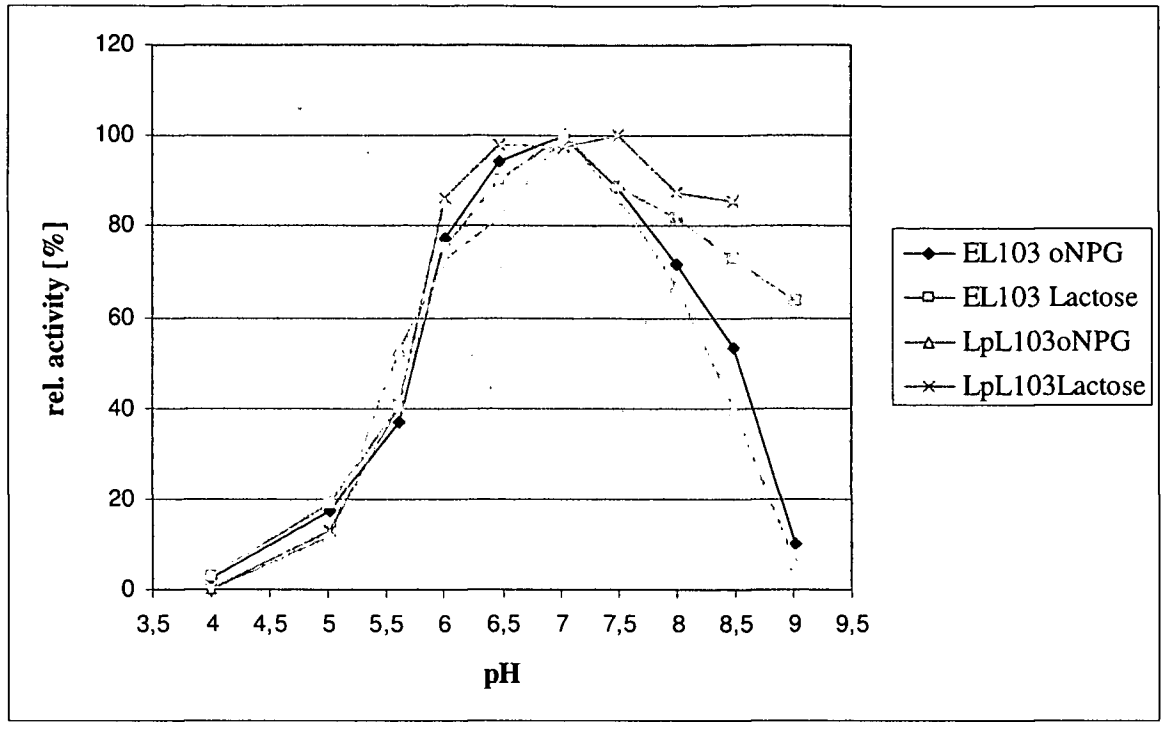


Figure A 52: Comparison of pH optimum between LpL103 and EL103

A5.5.3 pH stability

To determine the pH stability of the recombinant β-gal, the enzyme samples were incubated at various pH using Britton-Robinson buffer as mentioned above at 37°C for up to 24 h, and the residual enzyme activity was measured at time intervals using *o*NPG as the substrate under standard assay conditions.

Table A 50: Stability of LpL103 at pH 4.0

time [h]	Dil	Blank	OD420	OD420	Average-Blank	activity [U/mL]	rel. activity [%]
0	1500	0.0829	0.254	0.2513	0.16975	538.18	100.00
0.5	500	0.081	0.2436	0.2309	0.15625	165.13	30.68
1	100	0.0785	0.4259	0.4386	0.35375	74.77	13.89
2	100	0.075	0.3583	0.3671	0.2877	60.81	11.30
4	100	0.075	0.2931	0.3028	0.22295	47.12	8.76
6	100	0.075	0.2186	0.217	0.1428	30.18	5.61
24.5	100	0.0984	0.2322	0.2449	0.14015	29.62	5.50
50.5	100	0.0984	0.2181	0.1986	0.10995	23.24	4.32

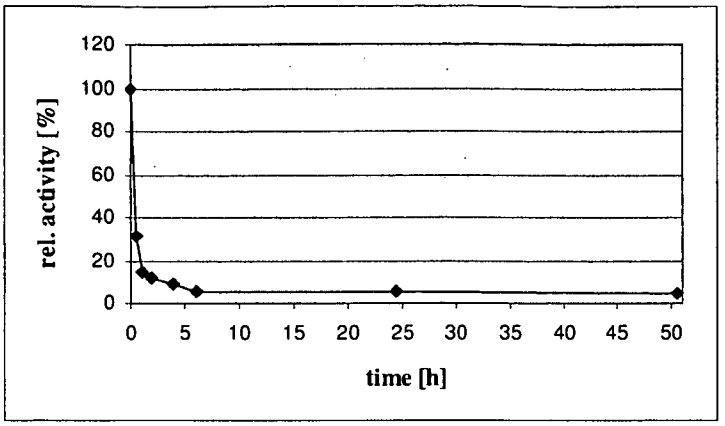


Figure A 53: Stability of LpL103 at pH 4.0

Table A 51: Stability of LpL103 at pH 5.0

time [h]	Dil	Blank	OD420	OD420	Average-Blank	activity [U/mL]	rel. activity [%]
0	1500	0.0829	0.254	0.2513	0.16975	538.18	100.00
0.5	1000	0.081	0.3118	0.2993	0.22455	474.62	88.19
1	500	0.0785	0.3366	0.3139	0.24675	260.77	48.45
2	200	0.075	0.6125	0.5879	0.5252	222.02	41.25
4	200	0.075	0.3268	0.3207	0.24875	105.15	19.54
6	100	0.075	0.618	0.6166	0.5423	114.62	21.30
24.5	100	0.0984	0.1981	0.2353	0.1183	25.00	4.65
50.5	100	0.0984	0.1862	0.1907	0.09005	19.03	3.54

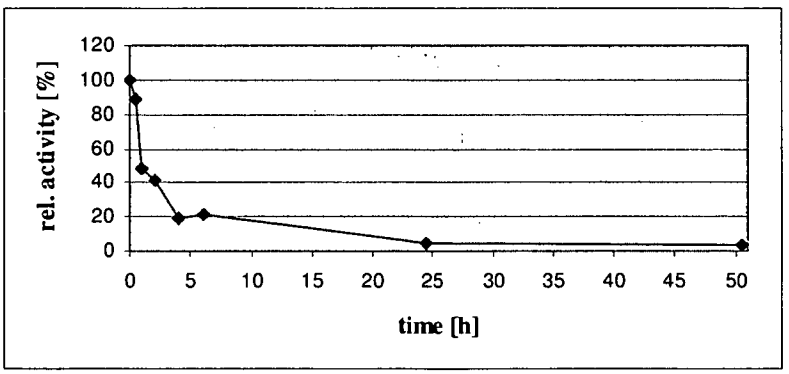


Figure A 54: Stability of LpL103 at pH 5.0

Table A 52: Stability of LpL103 at pH 5.5

time [h]	Dil	Blank	OD420	OD420	Average-Blank	activity [U/mL]	rel. activity [%]
0	1500	0.0829	0.254	0.2513	0.16975	538.18	100.00
0.5	1000	0.081	0.3908	0.4176	0.3232	683.12	126.93
1	1000	0.0785	0.3277	0.2936	0.23215	490.68	91.17
2	500	0.075	0.6347	0.6349	0.5598	591.60	109.93
4	500	0.075	0.5948	0.5948	0.5198	549.33	102.07
6	500	0.075	0.668	0.6715	0.59475	628.54	116.79
24.5	500	0.0984	0.4	0.389	0.2961	312.92	58.14
50.5	300	0.0984	0.2169	0.2111	0.1156	73.30	13.62

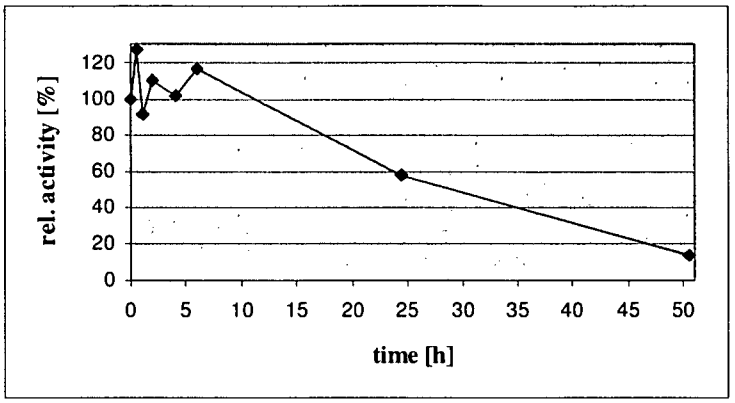


Figure A 55: Stability of LpL103 at pH 5.5

Table A 53: Stability of LpL103 at pH 6.0

time [h]	Dil	Blank	OD420	OD420	Average-Blank	activity [U/mL]	rel. activity [%]
0	1500	0.0829	0.254	0.2513	0.16975	538.18	100.00
0.5	1000	0.081	0.4401	0.4339	0.356	752.45	139.81
1	1000	0.0785	0.3841	0.3895	0.3083	651.63	121.08
2	1000	0.075	0.4271	0.4207	0.3489	737.45	137.03
4	1000	0.075	0.3785	0.3704	0.29945	632.93	117.60
6	1000	0.075	0.4016	0.39	0.3208	678.05	125.99
24.5	500	0.0984	0.1864	0.1784	0.084	88.77	16.49
50.5	200	0	0	0	0	0	0

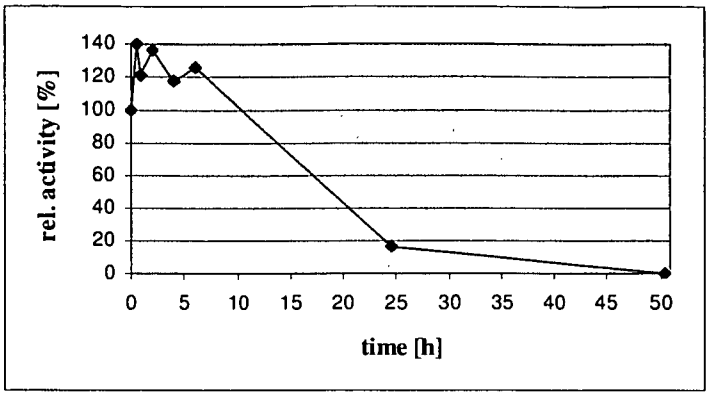


Figure A 56: Stability of LpL103 at pH 6.0

Table A 54: Stability of LpL103 at pH 6.5

time [h]	Dil	Blank	OD420	OD420	Average-Blank	activity [U/mL]	rel. activity [%]
0	1500	0.0829	0.254	0.2513	0.16975	538.18	100.00
0.5	1000	0.081	0.4709	0.3994	0.35415	748.54	139.09
1	1000	0.0785	0.3606	0.3654	0.2845	601.33	111.73
2	1000	0.075	0.3613	0.3629	0.2871	606.82	112.75
4	1000	0.075	0.3595	0.3649	0.2872	607.03	112.79
6	1000	0.075	0.3367	0.3572	0.27195	574.80	106.80
24.5	500	0.0984	0.166	0.1625	0.06585	69.59	12.93
50.5	200	0	0	0	0	0	0

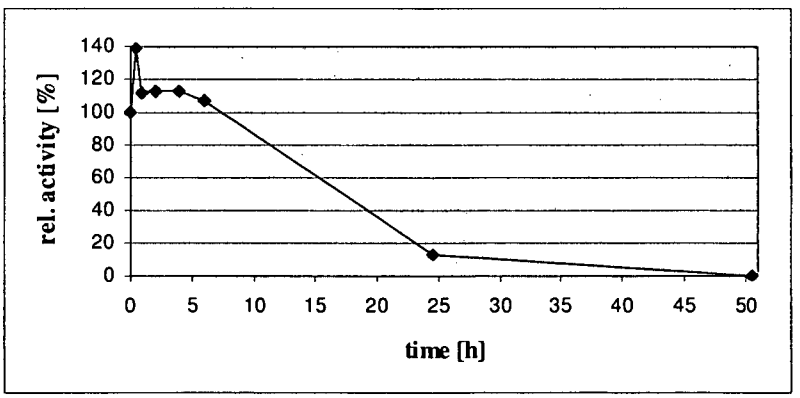


Figure A 57: Stability of LpL103 at pH 6.5

Table A 55: Stability of LpL103 at pH 7.0

time [h]	Dil	Blank	OD420	OD420	Average-Blank	activity [U/mL]	rel. activity [%]
0	1500	0.0829	0.254	0.2513	0.16975	538.18	100.00
0.5	1000	0.081	0.3785	0.3542	0.28535	603.12	112.07
1	1000	0.0785	0.3013	0.2986	0.22145	468.06	86.97
2	500	0.075	0.3534	0.3306	0.267	282.17	52.43
4	200	0.075	0.3944	0.388	0.3162	133.67	24.84
6	200	0.075	0.2641	0.2669	0.1905	80.53	14.96
24.5	0	0	0	0	0	0	0

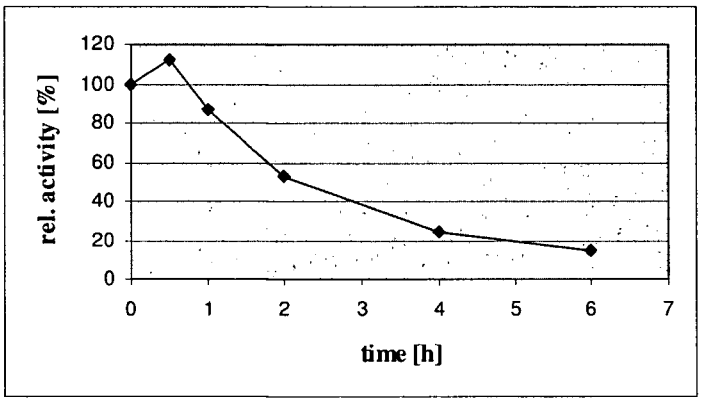


Figure A 58: Stability of LpL103 at pH 7.0

Table A 56: Stability of LpL103 at pH 7.5

time [h]	Dil	Blank	OD420	OD420	Average-Blank	activity [U/mL]	rel. activity [%]
0	1500	0.0829	0.254	0.2513	0.16975	538.18	100.00
0.5	500	0.081	0.5222	0.5175	0.43885	463.78	86.18
1	500	0.0785	0.1893	0.2024	0.11735	124.02	23.04
2	200	0.075	0.2714	0.2693	0.19535	82.58	15.34
4	100	0.075	0.2183	0.2215	0.1449	30.63	5.69
6	0	0	0	0	0	0	0

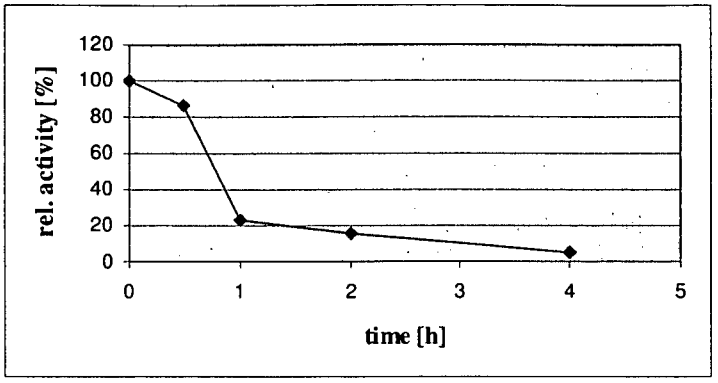


Figure A 59: Stability of LpL103 at pH 7.5

Table A 57: Stability of LpL103 at pH 8.0

time [h]	Dil	Blank	OD420	OD420	Average-Blank	activity [U/mL]	rel. activity [%]
0	1500	0.0829	0.254	0.2513	0.16975	538.18	100.00
0.5	200	0.0785	0.3848	0.38	0.3039	128.47	23.87
1	100	0.0785	0.2784	0.2771	0.1986	41.98	7.80
2	0	0	0	0	0	0	0

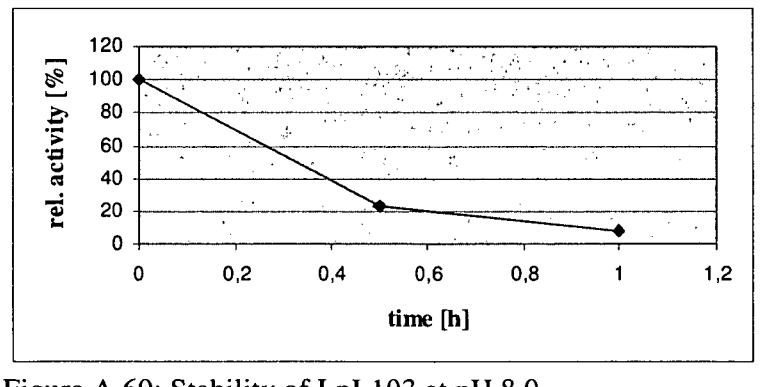


Figure A 60: Stability of LpL103 at pH 8.0

Table A 58: Stability of LpL103 at pH 8.5

time [h]	Dil	Blank	OD420	OD420	Average-Blank	activity [U/mL]	rel. activity [%]
0	1500	0.0829	0.254	0.2513	0.16975	538.18	100.00
0.5	0	0	0	0	0	0	0

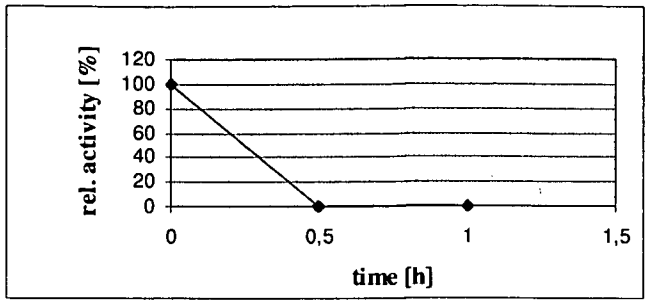


Figure A 61: Stability of LpL103 at pH 8.5

Table A 59: Stability of LpL103 at pH 9.0

time [h]	Dil	Blank	OD420	OD420	Average-Blank	activity [U/mL]	rel. activity [%]
0	1500	0.0829	0.254	0.2513	0.16975	538.18	100.00
0.5	0	0	0	0	0	0	0

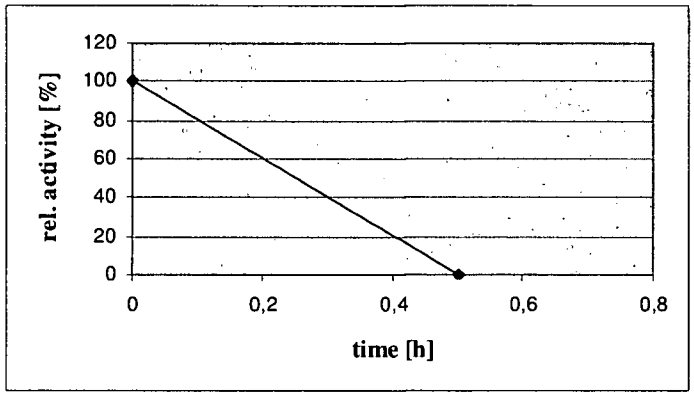


Figure A 62: Stability of LpL103 at pH 9.0

A5.5.4 Temperature optimum

The temperature dependence of enzyme activity (both with *o*NPG and lactose as substrate) was measured by assaying the enzyme samples over the range of 25-70°C for 10 min.

Table A 60: Temperature optimum with lactose as substrate

°C	Dilution	Blank OD546	OD546	OD546	average	activity [U/mL]	Rel. activity [%]
25	100	0.0902	0.4133	0.3971	0.4052	174.46	39.87
30	100	0.093	0.4648	0.4726	0.4687	208.07	47.55
37	100	0.1016	0.5785	0.5921	0.5853	267.89	61.22
40	150	0.0927	0.4762	0.4813	0.47875	320.71	73.29
45	200	0.0985	0.4299	0.416	0.42295	359.38	82.13
50	200	0.0945	0.4488	0.4643	0.45655	401.03	91.65
55	200	0.0896	0.4826	0.4867	0.48465	437.58	100.00
60	200	0.0947	0.4423	0.4145	0.4284	369.63	84.47
65	100	0.0929	0.5153	0.5191	0.5172	234.99	53.70
70	100	0.0943	0.2246	0.2141	0.21935	69.26	15.83

Table A 61: Temperature optimum with *o*NPG as substrate

°C	Dilution	Blank OD420	OD420	OD420	average	Activity [U/mL]	Rel. activity [%]
25	1000	0.0759	0.3575	0.3491	0.3533	586.32	21.00
30	1000	0.0751	0.5212	0.4964	0.5088	916.68	32.83
37	1500	0.0855	0.4705	0.4508	0.46065	1189.39	42.60
40	2000	0.0805	0.4194	0.4156	0.4175	1424.59	51.02
45	2000	0.084	0.5026	0.4813	0.49195	1724.51	61.76
50	2500	0.0862	0.6239	0.6053	0.6146	2792.10	100.00
55	2500	0.1041	0.3926	0.3772	0.3849	1483.77	53.14
60	2500	0.0968	0.2844	0.2862	0.2853	996.05	35.67
65	1000	0.1234	0.5084	0.4701	0.48925	773.27	27.69
70	1000	0.1223	0.3016	0.2915	0.29655	368.30	13.19

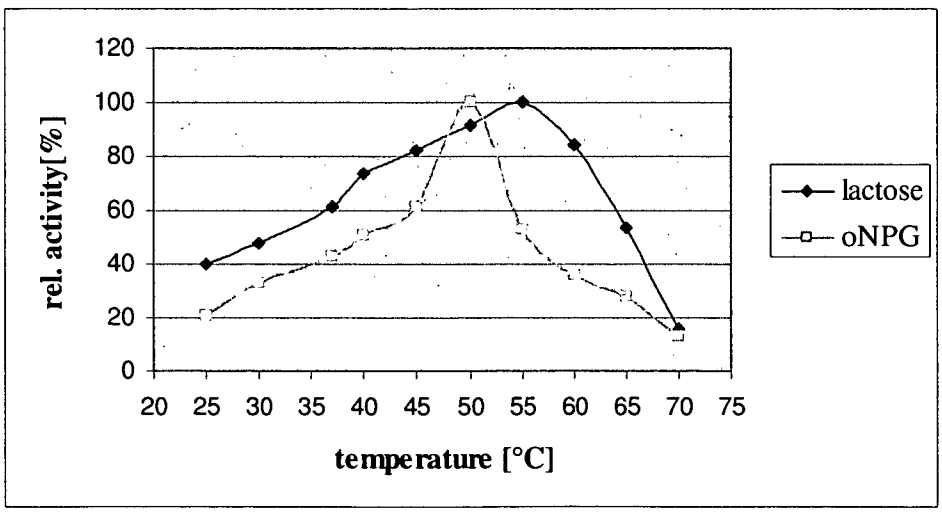


Figure A 63: Temperature optimum LpL103

A5.5.5 Temperature stability

The temperature stability of LpL103 was studied by incubating the enzyme samples in 50 mM sodium phosphate buffer at pH 6.0 at various temperatures (4, 25, 30, 37 and 42 °C). At certain time intervals, samples were withdrawn and the residual activity was measured with *o*NPG as the substrate under standard assay conditions.

Table A 62: Stability of LpL103 at 4°C

time [d]	Dilution	Blank OD420	OD420nm	OD420nm	average	activity [U/mL]	rel. activity [%]
0	1500	0.0762	0.6398	0.6286	0.6342	1180.67	100.0
1	1000	0.0756	0.6398	0.6286	0.6342	1180.67	100.0
2	1000	0.0756	0.50469	0.48317	0.49393	884.19	74.9
3	1000	0.0903	0.5793	0.5721	0.5757	1025.96	86.9
4	1000	0.0807	0.5012	0.491	0.4961	878.00	74.4
5	1000	0.0761	0.5646	0.5364	0.5505	1002.71	84.9
17	1500	0.08	0.4022	0.3903	0.39625	1002.65	84.9
27	1000	0.0766	0.4967	0.5033	0.5	894.91	75.8

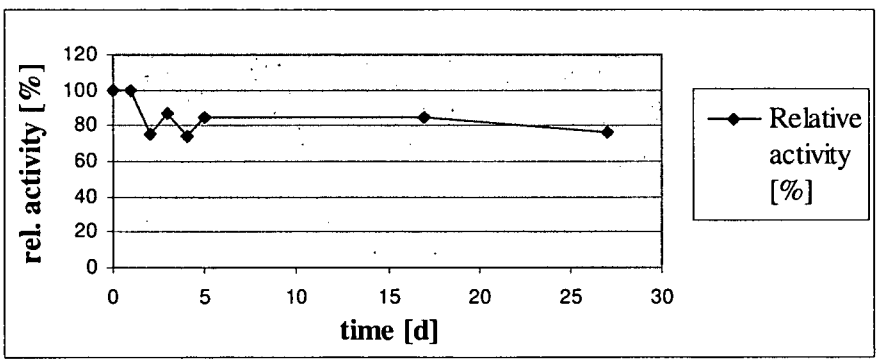


Figure A 64: Stability of LpL103 at 4°C

Table A 63: Stability of LpL103 at room temperature

time [h]	Dilution	Blank OD420	OD420nm	OD420nm	average	activity [U/mL]	rel. activity [%]
0						1064.00	100.0
19	1000	0.0756	0.594	0.564	0.579	1064.00	100.0
46	1000	0.0756	0.54354	0.53553	0.539535	980.59	92.2
72	1000	0.0903	0.5786	0.5669	0.57275	1019.72	95.8
94	1000	0.0807	0.4979	0.519	0.50845	904.10	85.0
143	1000	0.0787	0.3674	0.3738	0.3706	616.97	58.0
432	500	0.0769	0.272	0.2836	0.2778	212.31	20.0

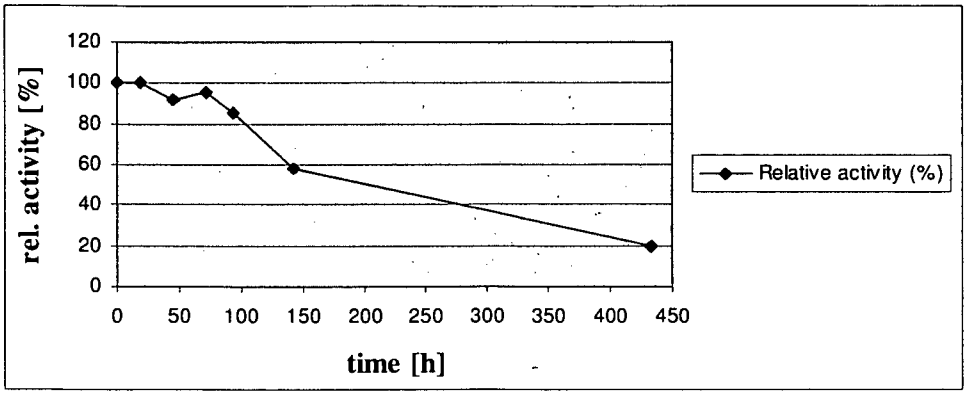


Figure A 65: Stability of LpL103 at room temperature

Table A 64: Stability of LpL103 at 30°C

time [h]	Dilution	Blank OD420	OD420nm	OD420nm	average	activity [U/mL]	rel. activity [%]
0	1500	0.0762	0.3361	0.3193	0.3277	797.37	100
19	1000	0.0756	0.5758	0.5599	0.56785	1040.43	100.0
46	1000	0.0756	0.42669	0.40492	0.415805	719.07	69.1
72	1000	0.0903	0.33	0.3235	0.32675	499.77	48.0
94	500	0.0807	0.4151	0.421	0.41805	356.52	34.3
120	500	0.0761	0.3297	0.3155	0.3226	260.50	25.0
143	250	0.0787	0.3	0.3315	0.31575	125.26	12.0

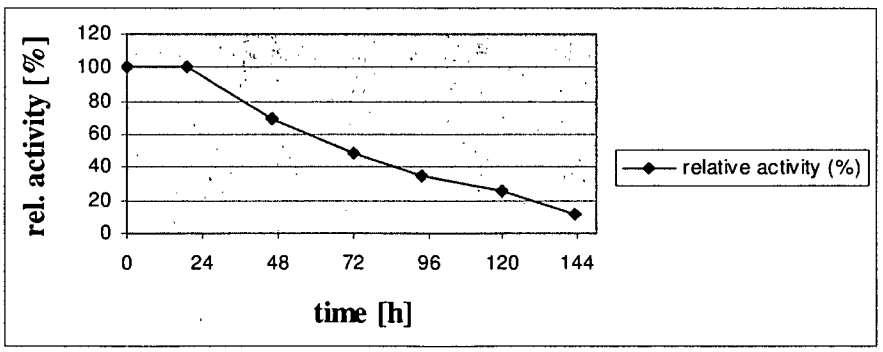


Figure A 66: Stability of LpL103 at 30°C

Table A 65: Stability of LpL103 at 37°C

time [h]	Dilution	Blank OD420	OD420nm	OD420nm	average	activity [U/mL]	rel. activity [%]
0	1500	0.0762	0.3361	0.3193	0.3277	797.37	100.0
19	1000	0.0756	0.3229	0.3139	0.3184	513.19	64.4
46	500	0.0756	0.16086	0.130445	0.1456525	74.03	9.3
72	100	0.0903	0.2937	0.2816	0.28765	41.71	5.2
94	50	0.0807	0.349	0.3152	0.3321	26.57	3.3
120	50	0.0761	0.1443	0.1521	0.1482	7.62	1.0

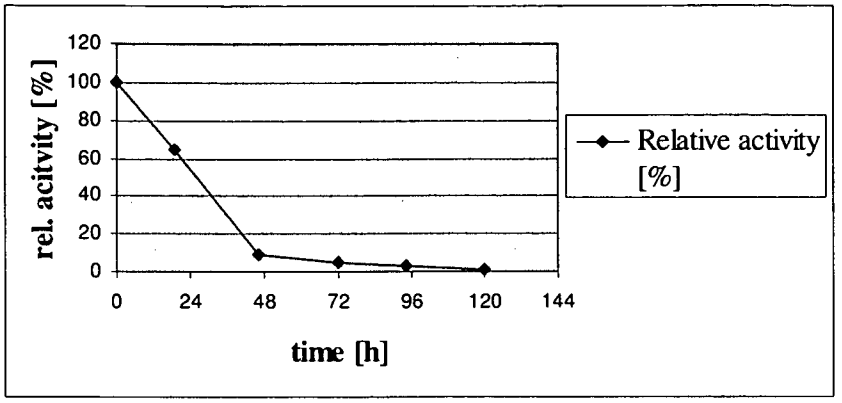


Figure A 67: Stability of LpL103 at 37°C

Table A 66: Stability of LpL103 with 1mM MgCl2 at 37°C

time [h]	Dilution	Blank OD420	OD420	OD420	average	activity [U/mL]	rel. activity [%]
0	1250	0.0769	0.377	0.3733	0.37515	787.99	100.0
20	1000	0.0769	0.335	0.342	0.3385	552.93	70.2
47	500	0.0769	0.656	0.6434	0.6497	605.34	76.8
70	500	0.0769	0.4983	0.4965	0.4974	444.39	56.4
96	500	0.0769	0.4005	0.3922	0.39635	337.60	42.8

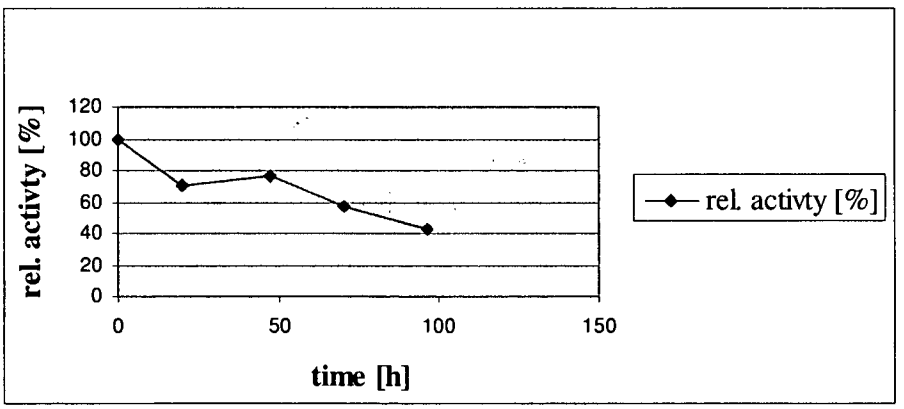


Figure A 68: Stability of LpL103 with 1mM $MgCl_2$ at 37°C

Table A 67: Stability of LpL103 with 1mM MgCl2 at 42°C

time [h]	Dilution	Blank OD420	OD420	OD420	average	activity [U/mL]	rel. activity [%]
0	1250	0.0769	0.377	0.3733	0.37515	787.99	100.0
0.5	1250	0.0769	0.4583	0.4615	0.4599	1011.90	128.4
1.5	1250	0.0769	0.398	0.375	0.3865	817.97	103.8
3	1250	0.0769	0.2876	0.2964	0.292	568.30	72.1
4.5	1000	0.0769	0.3762	0.4227	0.39945	681.75	86.5
20	500	0.0769	0.315	0.3035	0.30925	245.55	31.2
27.5	250	0.0769	0.5029	0.5016	0.50225	224.76	28.5
47	100	0.0769	0.2675	0.2669	0.2672	40.22	5.1
52	1	0.0769	0.4571	0.4578	0.45745	0.80	0.1
70	1	0.0769	0.1892	0.1924	0.1908	0.24	0.0

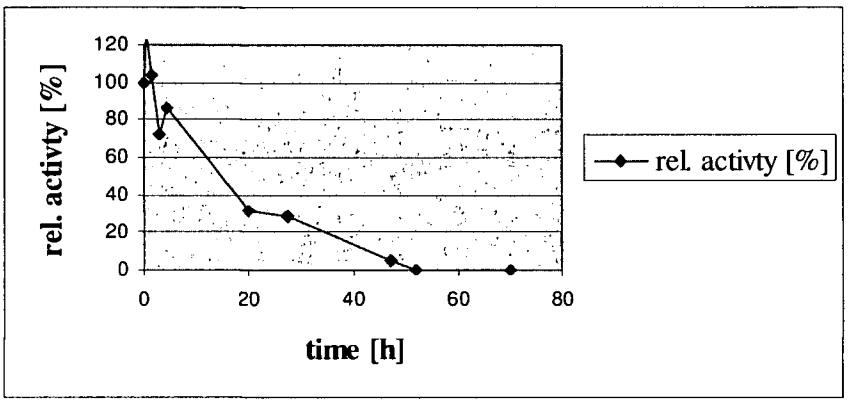


Figure A 69: Stability of LpL103 with 1mM $MgCl_2$ at 42°C

A6 Lactose conversion

A6.1 Conversion reaction

To investigate the spectrum of galacto-oligosaccharides produced from transgalactosylation of lactose catalyzed by recombinant β-galactosidase from *L. reuteri* L103 expressed in *L. plantarum* WCFS1, lactose conversion experiment with the purified enzyme and the crude enzyme extract were carried out.

Table A 68: Reaction mixture 1

LpL103	1:2 diluted	24 µL
1 M $MgCl_2$		2 µL
600 mM lactose	in NaPP pH 6	1974 µL

Table A 69: Reaction mixture 2

Crude extract LpL103*	1:5 diluted	25 µL
1 M $MgCl_2$		2 µL
600 mM lactose	in NaPP pH 6	1973 µL

* whole-cell lysates of *L. plantarum* pSIP403LacLM-Reu

There are 10.7 units of β-galactosidase in each reaction mixture.

Incubation was performed on a thermo mixer at 37 °C and 600 rpm. 50 µL and 150 µL samples were taken after 0, 0.5, 1, 2, 4, 6, 8, 20 h of conversion. 50 µL-samples were used to perform an *o*NPG assay and 150 µL-samples were heated at 99°C for 5 min and used for sugar analysis.

The *o*NPG assay showed constant activity of the β-galactosidase in the reaction mixture.

For sugar analysis the concentrations of glucose and galactose in the samples were determined enzymatically. Furthermore analysis using thin-layer chromatography, HPLC and CE were performed.

A6.2 Glucose determination

The concentration of free D-glucose was determined colormetrically using the GOD/POD assay (Kunst *et al.*, 1988) described earlier.

Table A 70: Results of glucose determination in samples from reaction mixture 1 (purified LpL103)

time [h]	Dilution	BlankOD546	OD546	OD546	average-Blank	glucose [mM]	glucose [g/L]
0	10	0.0659	0.368	0.3712	0.3037	6.73	1.21
0.5	100	0.0659	0.2196	0.2236	0.1557	34.49	6.21
1	100	0.0659	0.3748	0.3726	0.3078	68.19	12.28
2	100	0.0659	0.5992	0.5972	0.5323	117.92	21.24
4	400	0.0659	0.3514	0.3407	0.28015	248.25	44.71
6	400	0.0659	0.4329	0.4306	0.36585	324.19	58.39
8	400	0.0659	0.5647	0.5609	0.4969	440.32	79.30
24	400	0.0659	0.6814	0.7073	0.62845	556.89	100.30

Table A 71: Results of glucose determination in samples from reaction mixture 2 (crude enzyme extract LpL103)

time [h]	Dilution	BlankOD546	OD546	OD546	average-Blank	glucose. [mM]	glucose [g/L]
0	10	0.0659	0.4011	0.4128	0.34105	7.56	1.36
0.5	100	0.0659	0.202	0.2034	0.1368	30.31	5.46
1	100	0.0659	0.3471	0.3392	0.27725	61.42	11.06
2	100	0.0659	0.5054	0.4904	0.432	95.70	17.24
4	400	0.0659	0.2707	0.2907	0.2148	190.34	34.28
6	400	0.0659	0.3546	0.3594	0.2911	257.95	46.46
8	400	0.0659	0.4363	0.4319	0.3682	326.27	58.76
24	400	0.0659	0.6616	0.6766	0.6032	534.51	96.27

A6.3 Galactose determination

For galactose determination a test kit from Boehringer Mannheim/ R-Biopharm was used.

Materials and preparation:

Table A 72: Materials and preparation of galactose assay

	blank	samples
	Volume [µL]	Volume [µL]
Solution 1	40	40
Sample solution		20
Solution 3	200	200
dH2O	410	390
Suspension 4	10	10

It was started with pipeting water, following other ingredients, mixing and reading absorbance of the solution (A_1) after approximately 3 min. Then the reaction was started by adding suspension 4, mixing and waiting until the reaction stopped (10-15 min). Now the absorbance was read (A_2).

The absorbances of A_1 should be between 0.14-0.16 and A_2 should not be over 0.6 otherwise the samples have to be diluted.

Calculation:

$\Delta A = (A_2\text{-}A_1)\text{sample} - (A_2\text{-}A_1)\text{blank}$

$c = [V \times Mr/\varepsilon \times d \times v \times 1000] \times \Delta A \times Dil$

c	galactose concentration [g/L]
A_1	absorbance after 3 min incubation
A_2	absorbance 15 min after suspension 4 was added
V	final volume [mL].....0.66
Mr	molecular weight of galactose
ε	extinction coefficient of NADH at 340nm....6.3
d	light path [cm].....1
v	sample volume [mL]
Dil	dilution factor

Table A 73: Results of galactose determination in samples from reaction mixture 1 (purified LpL103)

Time [h]	Dil	A1 Blank	A1 Blank	A2 Blank	A2 Blank	Blank A2-A1	A1	A1	A2	A2	ΔA	**galactose [g/L]**
0	10	0.1409	0.1437	0.1454	0.1508	0.0058	0.1555	0.1426	0.234	0.2314	0.07785	**0.73**
0.5	50	0.1409	0.1437	0.1454	0.1508	0.0058	0.1419	0.1479	0.2349	0.2407	0.0871	**4.11**
1	100	0.1409	0.1437	0.1454	0.1508	0.0058	0.1532	0.1398	0.2217	0.2307	0.0739	**6.97**
2	100	0.1409	0.1437	0.1454	0.1508	0.0058	0.1437	0.1427	0.2677	0.267	0.11835	**11.17**
4	100	0.1409	0.1437	0.1454	0.1508	0.0058	0.1471	0.1402	0.3946	0.4006	0.24815	**23.42**
6	100	0.1409	0.1437	0.1454	0.1508	0.0058	0.1408	0.1412	0.5185	0.5014	0.36315	**34.27**
8	100	0.1409	0.1437	0.1454	0.1508	0.0058	0.1455	0.1402	0.6491	0.6474	0.4996	**47.15**
24	200	0.1409	0.1437	0.1454	0.1508	0.0058	0.1396	0.1399	0.5855	0.5876	0.441	**83.23**

Table A 74: Results of galactose determination in samples from reaction mixture 2 (crude enzyme extract LpL103)

Time [h]	Dil	A1 Blank	A1 Blank	A2 Blank	A2 Blank	Blank A2-A1	A1	A1	A2	A2	ΔA	**galactose [g/L]**
0	10	0.1409	0.1437	0.1454	0.1508	0.0058	0.1437	0.1399	0.2356	0.2354	0.0879	**0.83**
0.5	50	0.1409	0.1437	0.1454	0.1508	0.0058	0.1373	0.1409	0.2063	0.2108	0.06365	**3.00**
1	100	0.1409	0.1437	0.1454	0.1508	0.0058	0.1414	0.1444	0.2234	0.2074	0.0667	**6.29**
2	100	0.1409	0.1437	0.1454	0.1508	0.0058	0.1394	0.148	0.2426	0.241	0.0923	**8.71**
4	100	0.1409	0.1437	0.1454	0.1508	0.0058	0.1534	0.1402	0.3418	0.3541	0.19535	**18.44**
6	100	0.1409	0.1437	0.1454	0.1508	0.0058	0.1532	0.1439	0.4166	0.3999	0.2539	**23.96**
8	100	0.1409	0.1437	0.1454	0.1508	0.0058	0.139	0.1395	0.4847	0.4847	0.33965	**32.05**
24	200	0.1409	0.1437	0.1454	0.1508	0.0058	0.1415	0.141	0.5316	0.5304	0.38395	**72.47**

A6.4 Thin-layer chromatography

Running solution for thin-layer chromatography:

Butanol, propanol, ethanol and dH_2O

2:3:3:2

Staining solution for thin-layer chromatography:

0.5 g thymol

95 mL ethanol

5 mL conc. H_2SO_4

Standards:

Glucose	20 g/L
Galactose	20 g/L
Lactose	20 g/L
Elixor	20 g/L

The thin-layer plate was activated for 10 min at 130°C. The samples were diluted 1:10 with dH_2O and 2 µL were loaded on the plate. The plate was run two times in solvent and dried in between. The plate was dipped quickly into the staining solution and dried for 5 min at 130 °C until pink spots were visible.

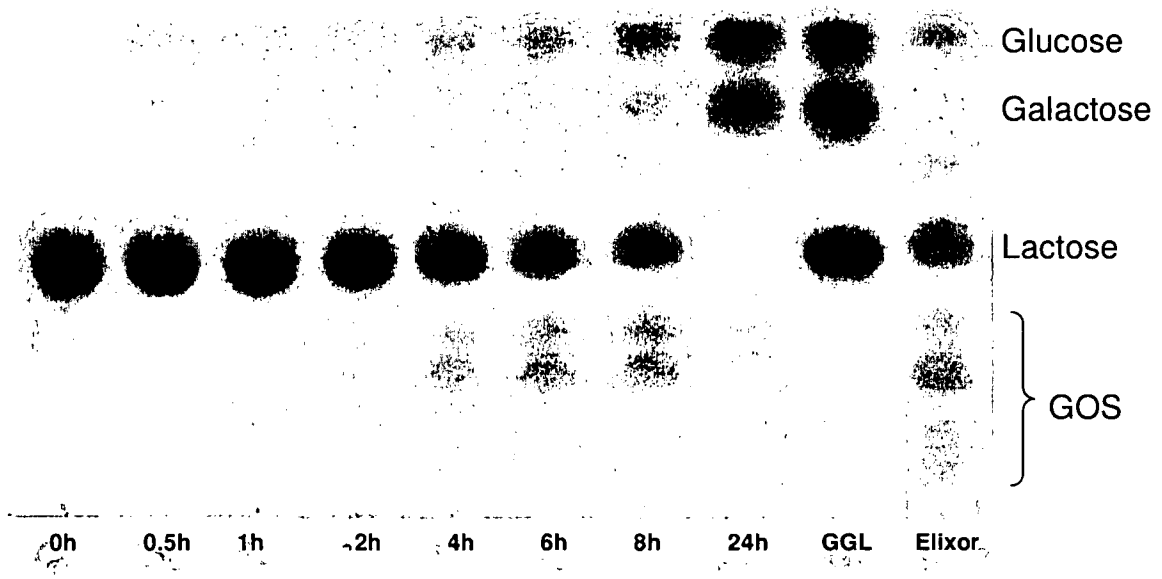


Figure A 70: Thin-layer chromatography of samples from lactose conversion reaction mixture 1 (purified LpL103)

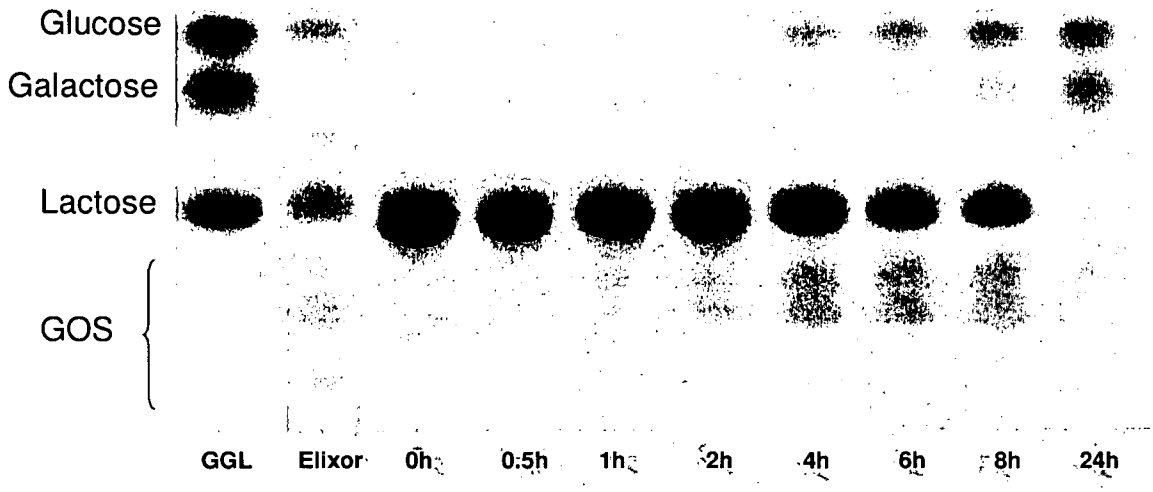


Figure A 71: Thin-layer chromatography of samples from lactose conversion reaction mixture 2 (crude enzyme extract LpL103)

A6.5 HPLC

All samples were diluted 1:20 for running with gradient for all components analysis and 1:600 for running with gradient for monosaccharides, lactose and allolactose separation.

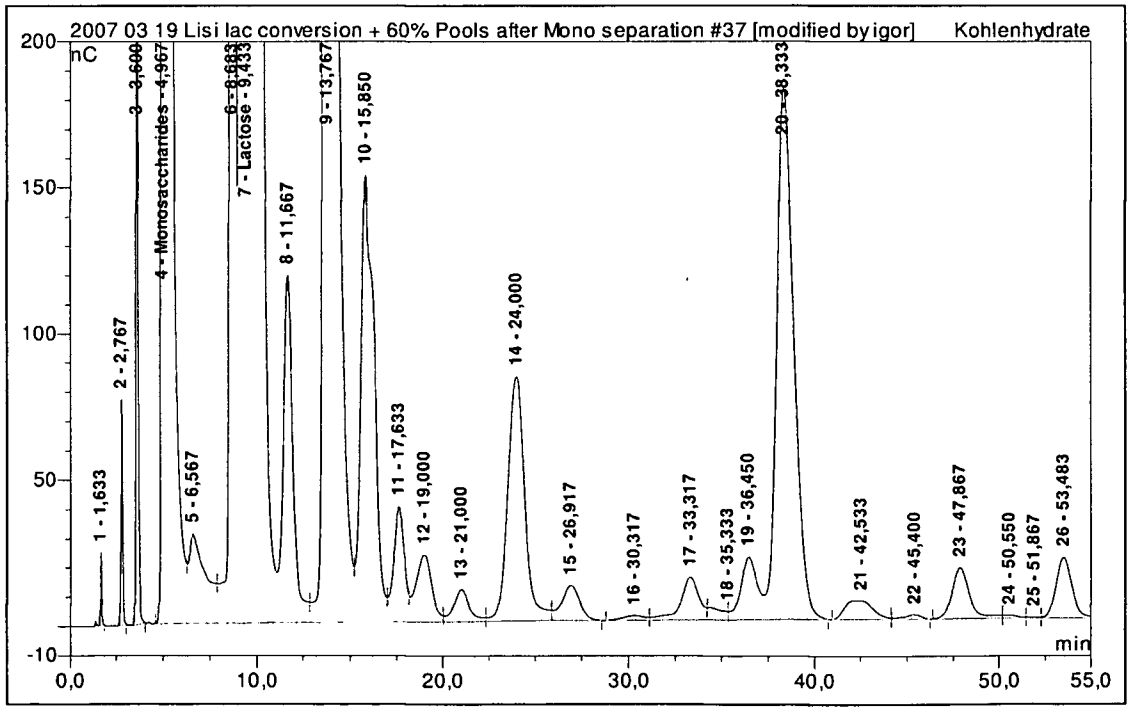


Figure A 72: HPLC – chromatogram (gradient "4 O" for all components analysis) of sample after 8 h lactose conversion with the crude enzyme extract of LpL103

A6.6 Capillary electrophoresis

Standards

Glucose	5 g/L
Galactose	5 g/L
Lactose	5 g/L
Elixor	30 g/L and 40 g/L

AP (aminopyridine):

600 µL methanol

1 g aminopyridine

in 470 µL acetic acid

borohydride:

59 mg NaCNBH3

1 mL 30 % acetic acid

Sample preparation for capillary electrophoresis

10 µL of the samples (1:2 dilution and 1:10 dilution) and standards were dried in the speed-vac for 2 h. For derivatization 20 µL AP were added and the samples were incubated for 15 min at 90 °C without shaking. Then the samples were dried for 2 h at 60 °C in the speed-vac. 25 µL borohydride were added and the samples were incubated for 30 min at 90°C and dried in the speed-vac at 60°C for 3 h. The dried samples were then resuspended in 200 µL ddH_2O for analysis.

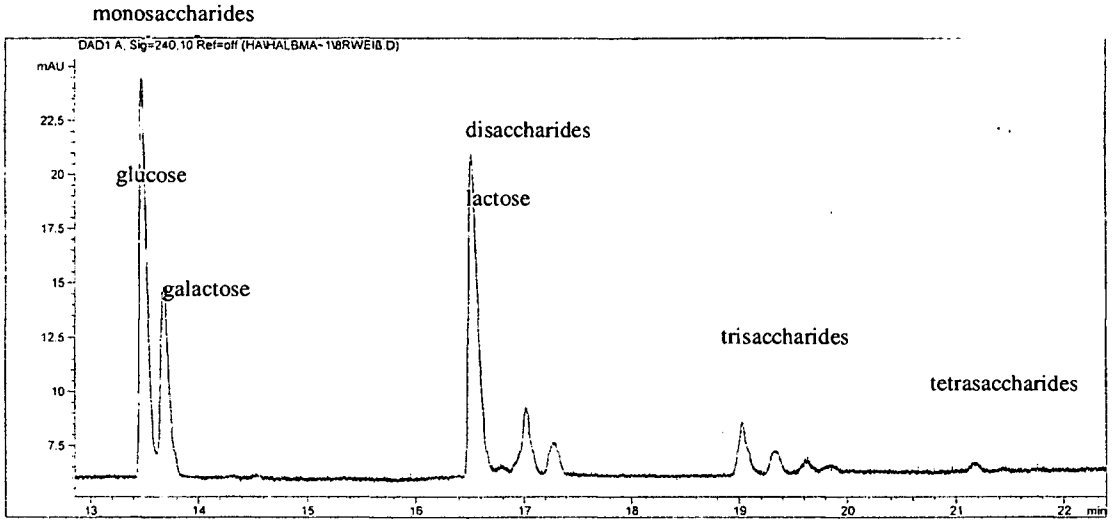


Figure A 73: Capillary electrophoresis - chromatogram of sample (1:10 diluted) after 8 h lactose conversion with the crude enzyme extract of LpL103