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Authors: Thu-Ha Nguyen, Barbara Splechna, Montarop Yamabhai, Dietmar Haltrich, Clemens Peterbauer



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1 **Cloning and expression of the**
2 **β -galactosidase genes from *Lactobacillus reuteri***
3 **in *Escherichia coli***

4
5 THU-HA NGUYEN^{1,2}, BARBARA SPLECHTNA^{1,2}, MONTAROP YAMABHAI³,
6 DIETMAR HALTRICH², AND CLEMENS PETERBAUER^{2*}

7
8 ¹*Research Centre Applied Biocatalysis, Petersgasse 14, A-8010 Graz, Austria,*

9 ²*Division of Food-Biotechnology, Department of Food Sciences and Technology,*

10 *University of Natural Resources and Applied Life Sciences Vienna,*

11 *Muthgasse 18, A-1190 Vienna, Austria,*

12 ³*School of Biotechnology, Institute of Agricultural Technology, Suranaree University of*

13 *Technology, 111 University Avenue, Nakhon Ratchasima, 30000, Thailand*

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* Corresponding author (e-mail: clemens.peterbauer@boku.ac.at). Address: Abteilung Lebensmittelbiotechnologie, Department für Lebensmittelwissenschaften und -technologie, Universität für Bodenkultur Wien, Muthgasse 18, A-1190 Vienna, Austria. Phone: 43-1-36006 6274. Fax: 43-1-36006 6251.

1 Abstract

2 Heterodimeric β -galactosidase of *Lactobacillus reuteri* L103 is encoded by two
3 overlapping genes, *lacL* and *lacM*. The *lacL* (1887 bp) and *lacM* (960 bp) genes encode
4 polypeptides with calculated molecular masses of 73,620 Da and 35,682 Da,
5 respectively. The deduced amino acid sequences of *lacL* and *lacM* show significant
6 identity with the sequences of β -galactosidases from other lactobacilli and *Escherichia*
7 *coli*. The coding regions of the *lacLM* genes were cloned and successfully
8 overexpressed in *E. coli* using an expression system based on the T7 RNA polymerase
9 promoter. Expression of *lacL* alone and coexpression of *lacL* and *lacM* as well as
10 activity staining of both native and recombinant β -galactosidases suggested a
11 translational coupling between *lacL* and *lacM*, indicating that the formation of a
12 functional β -galactosidase requires both genes. Recombinant β -galactosidase was
13 purified to apparent homogeneity, characterized and compared with the native β -
14 galactosidase from *L. reuteri* L103.

15

16 1. Introduction

17 β -Galactosidases (β -gal; EC 3.2.1.23) catalyze the hydrolysis and
18 transgalactosylation of β -D-galactopyranosides (such as lactose). β -Galactosidase-
19 catalyzed hydrolysis reactions are widely known for applications in the dairy industries
20 to improve digestibility, solubility and sweetness of lactose, the principle milk
21 carbohydrate (Nakayama and Amachi, 1999). Transgalactosylation reactions catalyzed
22 by β -galactosidases when using lactose or other structurally related galactosides as the
23 substrate yield galacto-oligosaccharides (GOS) (Nakayama and Amachi, 1999; Pivarnik
24 *et al.*, 1995). Galacto-oligosaccharides are classified as prebiotics (Rastall and Maitin,
25 2002) that beneficially affect host health by stimulating selectively the growth of

1 specific species of bacteria such as bifidobacteria and lactobacilli in the gut (Gibson and
2 Roberfroid, 1995; Cummings *et al.*, 2001).

3 *Lactobacillus reuteri* is a dominant strain of the hetero-fermentative lactobacilli in
4 the gastrointestinal tract of human and animals (Benno *et al.*, 1989, Kabuki *et al.*, 1997,
5 Sung *et al.*, 2003). Apart from our recent studies, no information about β -galactosidases
6 from *L. reuteri*, especially regarding their enzymatic and molecular properties, was
7 available. Our previous studies revealed that the intracellular β -galactosidase enzymes
8 from the potentially probiotic isolates of *L. reuteri*, strains L103 and L461, are
9 heterodimers with a molecular mass of 105 kDa, consisting of a 35 kDa and a 72 kDa
10 subunit (Nguyen *et al.*, 2006). Both enzymes were found to be very well suited for the
11 production of galacto-oligosaccharides, components that are of great interest because of
12 their use in functional food (Splechtina *et al.*, 2006).

13 Hence, it was our interest to study the molecular properties of these interesting β -
14 galactosidases from *L. reuteri* in more detail. β -Galactosidase from *L. reuteri* L103 was
15 selected for this study and we identified two partially overlapping genes encoding for
16 this enzyme. In this paper, we describe the cloning of β -gal from *L. reuteri* L103 and its
17 expression in *E. coli*, furthermore, some properties of the recombinant enzyme are also
18 reported.

19

20 **2. Materials and methods**

21 *2.1. Bacterial strains and culture conditions*

22 *Lactobacillus reuteri* strain L103 was obtained from Lactosan (Starterkulturen
23 GmbH & Co KG, Kapfenberg, Austria). The strain was grown anaerobically overnight
24 at 37°C in MRS broth containing peptone 10 g/l, di-potassium hydrogen phosphate 2
25 g/l, meat extract 8 g/l, di-ammonium hydrogen citrate 2 g/l, yeast extract 4 g/l, sodium

1 acetate 5 g/l, magnesium sulfate 0.2 g/l, Tween 80 1 g/l, manganese sulfate 0.04 g/l.
2 Glucose 2% w/v served as the C-source (Lactobacillus broth according to De Man,
3 Rogosa and Sharpe, 1960). *Escherichia coli* TOP10 (Invitrogen Corporation, Carlsbad,
4 CA, USA) was used in the transformation experiments involving the subcloning of the
5 DNA fragments. *Escherichia coli* BL21 Star (DE3) (Invitrogen), which carries the gene
6 for T7 RNA polymerase under control of the *lacZ*-promoter, was used as expression
7 host for the vector carrying the target DNA fragment encoding both large and small
8 subunits (*lacLM*) of β -galactosidase. The *E. coli* strains were grown in Luria broth (LB)
9 containing appropriate antibiotics (100 μ g/ml ampicillin or 50 μ g/ml kanamycin)
10 required for maintaining the plasmids.

11

12 2.2. Chemicals and enzymes

13 All chemicals were purchased from Sigma (St. Louis, MO., USA) unless otherwise
14 stated and were of the highest quality available. MRS broth powder was obtained from
15 Merck (Darmstadt, Germany). All restriction enzymes, *Pfu* DNA polymerase, T4 DNA
16 ligase and shrimp alkaline phosphatase (SAP) were purchased from Fermentas (Vilnius,
17 Lithuania). GoTaq DNA polymerase was from Promega (WI, USA). Isopropyl- β -D-
18 thiogalactopyranoside (IPTG) and agarose were purchased from Roth (Karlsruhe,
19 Germany).

20

21 2.3. DNA preparation

22 Chromosomal DNA was extracted from *L. reuteri* L103 as described by Germond *et*
23 *al.* (2003) with modifications. The strain was grown anaerobically at 37°C in MRS
24 broth to the mid-log phase. Cells were harvested by centrifugation (4500 rpm, 10
25 minutes, 4°), washed twice with 0.8% w/v NaCl and once with 60 mM EDTA. The cells

1 were then subjected to one freeze-and-thaw cycle, resuspended in TE buffer (10 mM
2 Tris-HCl pH 7.5, 1 mM EDTA) containing lysozyme (8 mg/ml) and mutanolysin (40
3 U/ml), and incubated at 37°C for 1 hour. One volume of 0.5% w/v sodium dodecyl
4 sulphate (SDS) was added to lyse the cells, and proteinase K was added to a final
5 concentration of 200 µg/ml. After incubating the mixture at 65 °C for 10 minutes, the
6 DNA was extracted with phenol, precipitated with isopropanol and washed with 70%
7 cold ethanol. The DNA was then dissolved in TE buffer. After DNA was dissolved,
8 RNase A was added to a final concentration of 200 µg/ml and the solution was
9 incubated at 35°C for 30 minutes. The final yield of DNA obtained was approximately
10 0.5 µg/µl.

11 Plasmid DNA from *E. coli* was purified using the Wizard Plus Miniprep DNA
12 Purification System (Promega) and PureLink™ Quick Plasmid Miniprep Kit
13 (Invitrogen).

14

15 2.4. DNA amplification procedure and subcloning of *lacL* (large subunit)

16 The degenerated oligonucleotides β-gal F1 and β-gal R1 (table 1) used for PCR
17 amplification of the *L. reuteri lacL* gene were designed by sequence comparison of β-
18 galactosidase large subunit (*lacL*) gene products from *Lactobacillus spp.*, namely
19 *Lactobacillus acidophilus* (GenBank accession number AB004867), *Lactobacillus*
20 *helveticus* (GenBank accession number AJ512877), and *Lactobacillus sakei* (GenBank
21 accession number X82287). The primers were obtained from VBC-Biotech (Vienna,
22 Austria). The amplifications were performed using a T3 Thermocycler (Biometra;
23 Goettingen, Germany) in a total volume of 50 µl of reaction mixtures containing 0.2
24 mM of each deoxynucleotide triphosphate, 25 pmol of each primer, 5 µl of 10× *Pfu*
25 buffer with MgSO₄ (final concentration of MgSO₄ was 2.5 mM), 1.25 U of *Pfu* DNA

1 polymerase, and 1 μ l of diluted genomic DNA (about 50 ng). The initial denaturation
2 step at 95°C for 2 minutes was followed by 35 cycles of denaturation at 94°C for 40 s,
3 annealing at 42°C for 40 s, and extension at 72°C for 4 minutes. The final cycle was
4 followed by additional 10 minutes elongation at 72°C. The amplified products were
5 visualized by gel electrophoresis at 5 V·cm⁻¹ in a 0.8% agarose gel (containing 1 μ g/ml
6 ethidium bromide) in 1×TBE (Tris-Borate-EDTA) electrophoresis buffer (10.8 g/l Tris
7 base, 5.5 g/l boric acid, 0.9 g/l sodium EDTA; pH 8) and photographed under UV light.
8 The amplified product was purified from the agarose gel using the Wizard[®] SV Gel and
9 PCR Clean-Up System (Promega). The vector pCR-Blunt II-TOPO (Invitrogen) was
10 used for subcloning PCR-amplified product and the resulting plasmid pHA1030 was
11 prepared for DNA sequencing.

12

13 *2.5. Rapid Amplification of Genomic Ends (RAGE) for direct sequencing of lacM (small* 14 *subunit)*

15 RAGE-PCR for direct sequencing of *lacM* was carried out as described in Mizobuchi
16 and Frohman (1993) with modifications. Five μ g each of genomic DNA from L103 and
17 pBluescript II SK (-) plasmid DNA (Stratagene, La Jolla, CA, USA) were digested by
18 single enzyme restriction digests using *Pst*I, *Kpn*I, *Hind*III, *Xho*I, *Xba*I, *Eco*RV at 37°C.
19 After complete digestion, the restriction enzymes were heat inactivated. Digested
20 pBluescript plasmid DNA was then dephosphorylated using shrimp alkaline
21 phosphatase. Digested genomic DNA and pBluescript plasmid DNA were cleaned up
22 using the Wizard[®] SV Gel and PCR Clean-Up System (Promega) and then ligated with
23 T4 DNA ligase at 16°C for 15 h. These ligation mixtures were stored at -20°C until use.

24 The primers β -gal F2, β -gal F3, pBS R1 and pBS R2 (table 1) were designed based
25 on the sequence of *lacL* (large subunit) obtained from previous experimental procedures
26 and the sequence of pBluescript II SK (-) vector (GenBank accession number X52330).

1 The first round of PCR amplification was performed in a total volume of 50 μ l of
2 reaction mixtures containing 1 μ l of the ligation mixture (as template), 0.2 mM of each
3 deoxynucleotide triphosphate, 25 pmol of each primer (β -gal F2 and pBS R1), 10 μ l of
4 5 \times colourless GoTaq reaction buffer, and 2.5 U of GoTaq DNA polymerase (Promega).
5 The initial denaturation step at 95°C for 2 min was followed by 35 cycles of
6 amplification (94°C, 40 s; 53-57°C, 40 s; 72°C, 2 min) and a final elongation at 72°C
7 for 10 min. The second round of PCR amplification was carried out using 1 μ l of the
8 first PCR reaction mix as template and nested primers β -gal F3 and pBS R2 (table 1)
9 under the same conditions described for the first round of PCR amplification. The
10 positive product of RAGE-PCR was cloned into pCR 2.1-TOPO vector (Invitrogen) and
11 the plasmid was prepared for DNA sequencing.

12 The product of first RAGE-PCR did not cover the complete *lacM* sequence. Hence, a
13 second RAGE-PCR was performed using different pairs of primers, β -gal F4 and pBS
14 R1, and nested primers β -gal F5 and pBS R2 (table 1) under the same conditions as
15 described above.

16

17 2.6. DNA amplification procedure and subcloning of β -galactosidase

18 The degenerated oligonucleotides β -gal F6 and β -gal R6 (table 1) used for PCR
19 amplification of *L. reuteri* β -galactosidase genes (*lacLM*) were designed based on the
20 sequences of *lacL* and *lacM* obtained from the experimental procedures described
21 above. The amplification procedure was performed as described above for DNA
22 amplification of *lacL* (large subunit) with some modifications (annealing at 57°C for 40
23 s, and extension at 72°C for 6 min). The vector pCR-Blunt II-TOPO (Invitrogen) was
24 used for subcloning the PCR-amplified product. The resulting plasmid pHA1031
25 contains the complete genes (*lacL* and *lacM*) of β -galactosidase from *L. reuteri*, which
26 was confirmed by sequencing.

1

2 2.7. Nucleotide sequencing and sequence analysis.

3 The nucleotide sequence was determined by VBC-Biotech (Vienna, Austria).
4 Assembly and analysis of DNA sequences were done by using ChromasPro (version
5 1.33) (Technelysium, Australia). The basis local alignment tool (BLAST) from the
6 National Center for Biotechnology Information BLAST website was used for database
7 searches. The comparison of β -galactosidases from *Lactobacillus spp.* with homologous
8 proteins was carried out using the programs ClustalX (version 1.81) (Thompson *et al.*,
9 1997) and GeneDoc (version 2.6.002) (Nicholas *et al.*, 1997).

10

11 2.8. Expression of β -galactosidase

12 Upstream and downstream primers β -gal F7 and β -gal R7 (table 1) were used to
13 amplify the fragment containing β -galactosidase genes from pHA1031. These primers
14 created a restriction site at each end of the gene fragment, *NcoI* and *XhoI*, respectively.
15 The PCR-amplified fragment was digested with *NcoI* and *XhoI* and inserted into the
16 respective sites of the expression vector pET21d (Novagen, Darmstadt, Germany)
17 resulting in the overexpression plasmid pHA1032. The expressed protein carries a C-
18 terminal His-Tag encoded by the vector. *E.coli* BL21 Star (DE3) carrying pHA1032
19 was grown at 37°C in LB medium containing 100 μ g/ml ampicillin until an optical
20 density at 600 nm of 0.6 was reached. Isopropyl- β -D-thiogalactopyranoside (IPTG; 0.1
21 mM) was then added to the culture medium and the cultures were incubated further at
22 25°C for 12 hours. The induced cells were then harvested, washed once with sodium
23 phosphate buffer (50 mM, pH 6.5), and disrupted by using a French press (AMINCO,
24 Maryland, USA). Debris was removed by centrifugation (16,000 rpm, 30 minutes, 4°C)
25 to obtain the crude extract.

26

1 2.9. *Protein purification*

2 The crude extract was loaded on a HisTrap HP column (Ni Sepharose High
3 Performance, 5 ml, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) that was pre-
4 equilibrated with buffer A (20 mM sodium phosphate, 0.5 M NaCl, 20 mM imidazole,
5 pH 6.5). The protein was eluted at a rate of 2.5 ml min⁻¹ with a 75 ml linear gradient
6 from 0 to 100 % buffer B (20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole,
7 pH 6.5). Active fractions were pooled, desalted and concentrated for further analysis.

8

9 2.10. *Protein determination*

10 Protein concentration was determined by the method of Bradford (Bradford, 1976)
11 using bovine serum albumin as standard.

12

13 2.11. *Gel electrophoresis and active staining*

14 Gel electrophoresis and active staining were carried out using 4-methylumbelliferyl
15 β-D-galactoside as the substrate as previously described (Nguyen *et al.* 2006).

16

17 2.12. *Enzyme assays*

18 β-Galactosidase activity was determined using *o*-nitrophenyl-β-D-galactopyranoside
19 (*o*NPG) and lactose as the substrates as described previously (Nguyen *et al.* 2006).
20 When chromogenic *o*NPG was used as the substrate, the reaction was initiated by
21 adding 20 μl of enzyme solution to 480 μl of 22 mM *o*NPG in 50 mM sodium
22 phosphate buffer (pH 6.5) and stopped after 10 min of incubation at 30°C by adding 750
23 μl of 0.4 M Na₂CO₃. The release of *o*-nitrophenol (*o*NP) was measured by determining
24 the absorbance at 420 nm. One unit of *o*NPG activity was defined as the amount of
25 enzyme releasing 1 μmol of *o*NP per minute under the described conditions.

1 When lactose was used as the substrate, 20 μ l of enzyme solution was added to 480
2 μ l of 600 mM lactose solution in 50 mM sodium phosphate buffer, pH 6.5. After 10
3 minutes of incubation at 30°C, the reaction was stopped by heating the reaction mixture
4 at 99°C for 5 minutes. The reaction mixture was cooled to room temperature, and the
5 release of D-glucose was determined colorimetrically using the GOD/POD assay (Kunst
6 *et al.*, 1988). One unit of lactase activity was defined as the amount of enzyme releasing
7 1 μ mol of D-glucose per minute under the given conditions.

8 9 *2.13. Characterization of the recombinant β -galactosidase enzyme*

10 Steady-state kinetic measurements, pH and temperature dependency of activity and
11 stability were obtained following the methods described in our previous report (Nguyen
12 *et al.*, 2006).

13 14 *2.14. Nucleotide sequence accession numbers*

15 The GenBank accession numbers are DQ493596 (nucleotide sequence of both
16 genes), ABF72116 (*lacL*, large subunit) and ABF72117 (*lacM*, small subunit),
17 respectively.

18 19 **3. Results**

20 *3.1. Cloning of β -galactosidase genes from *L. reuteri* and nucleotide sequence analysis*

21 Degenerated oligonucleotides were designed for PCR amplification of the gene
22 encoding the large subunit (*lacL*) of *L. reuteri* L103 β -galactosidase. One complete
23 open reading frame (ORF) of 1887 bp encoding 628 amino acid residues with a
24 calculated molecular mass of 73,620 Da (<http://au.expasy.org/tools/protparam.html>) was
25 obtained. Based on its similarity to the sequences of published *lacL* genes of β -

1 galactosidases from other *Lactobacillus* spp. it was assumed to be the *lacL* gene of *L.*
2 *reuteri* L103 β -galactosidase.

3 To identify the location of the gene encoding the small subunit, RAGE-PCR was
4 carried out for direct sequencing of the flanking region of the 3' end of *lacL*. A second
5 reading frame which partially overlaps the 3' end of *lacL* was obtained. The start codon
6 ATG of this open reading frame starts at position 1870 of *lacL* and its 960 bp encode a
7 protein of 319 amino acids with a calculated molecular mass of 35,682 Da. It was
8 designated as *lacM* based on its similarity to the sequences of published *lacM* genes of
9 β -galactosidases from *Lactobacillus* spp. The gene *lacM* was found downstream of *lacL*
10 and the two genes overlap for 17 base pairs (Figure 1). From our previous study, L103
11 β -galactosidase appeared to be a heterodimer consisting of a 35 kDa and a 72 kDa
12 subunit (Nguyen *et al.*, 2006), hence this was in agreement with the deduced amino acid
13 sequences.

14

15 3.2. Comparison of amino acid sequences

16 The alignments of the deduced amino acid sequences of *lacL* and *lacM* genes of β -
17 galactosidase from *L. reuteri* L103 with other β -galactosidases from *Lactobacillus* spp.
18 and *Leuconostoc lactis* are presented in figure 2 (A,B). The *lacL* and *lacM* genes of β -
19 galactosidase from *L. reuteri* L103 show significant similarity to the reported sequences
20 of β -galactosidases from *L. acidophilus*, 74% and 71%, respectively (accession numbers
21 BAA20536 and BAA20537) (Suzuki *et al.*, 1997); *L. helveticus*, 73% and 70%,
22 respectively (accession numbers CAD55499 and CAD55500) (Fortina *et al.*, 2003); *L.*
23 *johnsonii*, 69% and 65%, respectively (accession numbers AAS08676 and AAS08675)
24 (Pridmore *et al.*, 2004); *L. plantarum*, 64% and 68%, respectively (accession numbers
25 CAD65569 and CAD65570) (Kleerebezem *et al.*, 2003); *Leuconostoc lactis*, 64% and

1 67%, respectively (accession numbers Q02603 and Q02604) (David *et al.*, 1992); and *L.*
2 *sakei*, 58% and 57%, respectively (accession numbers CAA57730 and CAA57731)
3 (Obst *et al.*, 1995). The region showing the lowest conservation in the *lacL* genes of
4 these β -galactosidases is from nucleotide positions 240 to 300, while very high
5 conservation was observed in the region of the 3' end of *lacL* (figure 2A).

6 The amino acid sequence deduced from *L. reuteri* L103 *lacL* shows 33% identity to
7 the *lacZ* gene encoding β -galactosidase from *E. coli* (accession number V00296)
8 (Kalnins *et al.*, 1983). Previous studies suggested that Glu-461 is a general acid-base
9 recognition site (Cupples *et al.*, 1990) and Glu-537 is a nucleophile in the active site of
10 β -galactosidase from *E. coli* (Gebler *et al.*, 1992). Tyr-503 was also reported as a general
11 acid and base catalyst (Ring and Huber, 1990). Glu-416, His-418, Glu-461 appear to
12 coordinate a bound magnesium ion in *E. coli* β -galactosidase (Jacobson *et al.*, 1994).
13 The amino acid sequence alignment of the regions flanking these catalytically important
14 residues in *L. reuteri* L103 *lacL* and *lacZ* from *E. coli* is shown in figure 3. These
15 regions in *lacL* of *L. reuteri* L103 show high similarity to the corresponding regions in
16 *lacZ* from *E. coli*, with the exception of the region flanking a tyrosine residue at position
17 502 with significant lower similarity. The deduced amino acid sequence of *lacM*
18 showed similarity to 180 residues at the 3' end of the *lacZ* gene from *E. coli*. As judged
19 from the sequences of the *lacL* and *lacM* genes from *L. reuteri* L103, they possibly
20 belong to the glycosyl hydrolase family 2 according to the structural classification by
21 Henrissat (Henrissat, 1991; Nakayama and Amachi, 1999).

22

23 3.3. Overexpression of β -galactosidase from *L. reuteri* in *E. coli*

24 To further study the two gene products of *lacLM*, a T7 RNA polymerase expression
25 system was used for the joint overexpression of the *lacL* and *lacM* genes in *E. coli*. The

1 coding region for both genes, *lacL* and *lacM*, was cloned into pET21d resulting in the
2 overexpression plasmid pHA1032. Gene expression was induced by 0.1 mM IPTG with
3 12 h induction at 25°C (figure 4A). β -Galactosidase activity produced by *E. coli* strain
4 BL21 Star (DE3) carrying pHA1032 was determined to be 55 U/mg of protein and
5 approximately 110 kU per liter of fermentation broth. The enzyme was purified with a
6 single-step purification using a HisTrap HP column which gave an overall yield of 75%,
7 and approximately 83 kU of purified recombinant enzyme per liter of fermentation
8 broth with a specific activity of 180 U/mg of protein was obtained.

9 The recombinant β -galactosidase overexpressed in *E. coli* had a molecular mass of
10 approximately 108 kDa and consisted of a ~35 kDa and a ~73 kDa subunit. This is in
11 agreement with the wild-type β -galactosidase from *L. reuteri* L103 (figure 4B). Active
12 staining of both purified wild-type and recombinant β -galactosidases directly on the
13 SDS-PAGE gel after pre-incubating the enzymes with denaturing SDS buffer at 60°C
14 for 5 minutes and using 4-methylumbelliferyl β -D-galactoside as the substrate showed
15 that one band corresponding to a polypeptide of the size of the larger subunit exhibited
16 activity with this substrate. In contrast, the smaller subunit did not show any activity
17 (Figure 4B). As was reported in our previous study, active staining of wild-type β -
18 galactosidase L103 on native PAGE yielded two bands with β -galactosidase activity,
19 one band of approximately 105 kDa, corresponding to the intact heterodimer, and a
20 second band representing a degradation product containing components of both subunits
21 (Nguyen *et al.*, 2006). This observation was also made with recombinant β -
22 galactosidase overexpressed in *E. coli* (data not shown).

23

24 *3.4. Expression of lacL gene product alone did not yield functional β -galactosidase*
25 *activity*

1 Based on these observations with active staining of β -galactosidase L103 using 4-
2 methylumbelliferyl β -D-galactoside as the substrate, it was of interest to determine
3 whether the gene product of *lacL* gene represents an active, monomeric β -galactosidase.
4 To this end, the coding region of only *lacL* was cloned into pET21d resulting in plasmid
5 pHA1033, and this plasmid was introduced into *E. coli*. Gene expression was induced
6 by IPTG, analyzed by SDS-PAGE, and the expressed protein was purified using a
7 HisTrap HP column. A protein with a molecular mass of ~ 73 kDa, which is in
8 agreement with the molecular mass of the large subunit, was obtained, which confirmed
9 that the gene was expressed. However, no detectable β -galactosidase activity was found.
10 This indicates that both genes, *lacL* and *lacM*, are required for the production of active
11 β -galactosidase.

13 3.5. Properties of recombinant β -galactosidase EL103

14 To differentiate from the native β -galactosidase from *L. reuteri* L103, the β -
15 galactosidase overexpressed in *E. coli* was denoted as recombinant β -galactosidase
16 EL103. The steady-state kinetic constants were determined for the hydrolysis of lactose,
17 the natural substrate. Kinetic analysis of recombinant β -galactosidase EL103 with
18 increasing concentrations of lactose as the substrate showed Michaelis-Menten kinetics
19 with the following parameters obtained by nonlinear regression using SigmaPlot (SPSS
20 Inc., Illinois, USA): $V_{\max} = 38$ ($\mu\text{mol D-glucose released/min/mg protein}$) and $K_m = 12 \pm$
21 2 (mM). In case of native β -galactosidase L103, the V_{\max} and K_m values for lactose were
22 34 ($\mu\text{mol D-glucose released/min/mg protein}$), and 13 ± 2 (mM), respectively (Nguyen
23 *et al.*, 2006).

24 The optimum pH of native L103 β -galactosidase is pH 8.0 for both lactose and
25 *o*NPG hydrolysis. The optimum pH of recombinant EL103 β -galactosidase shifts to pH

1 7.0 for *o*NPG and remains at pH 8.0 for lactose hydrolysis (Figure 5A, B). However,
2 both native L103 β -gal and recombinant EL103 β -gal are most stable at pH 6.0,
3 retaining more than 90% and 80% of its activity, respectively, when incubated at pH 6.0
4 and 37°C for 3 hours (Figure 6). EL103 β -gal was slightly less stable than L103 β -gal at
5 pH 6.0, although the difference was not significant. Surprisingly, the recombinant
6 EL103 β -gal showed significant activity at pH 7.0 after 3 hours incubation at 37°C,
7 retaining 26% of its activity, whereas the native L103 β -gal was inactive at this pH.

8 The optimum temperature of both native and recombinant β -galactosidases L103 and
9 EL103 was 50°C when using *o*NPG as the substrate under standard assay conditions
10 (pH 6.5, 10 min). For lactose hydrolysis, the optimum temperatures of L103 and EL103
11 β -galactosidases were found to be in the range of 45-50°C, and 50-60°C, respectively
12 (Figure 5C, D). Both native and recombinant enzymes, L103 β -gal and EL103 β -gal, are
13 very stable at 4°C in the presence of 1 mM 1,4-dithiothreitol (DTT), retaining their full
14 activity after weeks of storage. L103 and EL103 β -galactosidases retained 30% and
15 35% of their activities, respectively, when kept at 37°C for 48 hours. The effect of
16 MgCl₂ on the thermal stability of native L103 β -gal was reported in our previous study
17 (Nguyen *et al.*, 2006) and this observation was also found with recombinant EL103 β -
18 gal. In the presence of 10 mM MgCl₂ both enzymes retained 90% of its activity after 6 h
19 incubation at 42°C, and their half-life time ($t_{1/2}$) of activity at this temperature was
20 increased to approximately 24 h.

21

22 4. Discussion

23 Heterodimeric β -galactosidase from *L. reuteri* is encoded by two overlapping genes,
24 *lacL* and *lacM*. As was reported in our previous study (Nguyen *et al.*, 2006), the larger
25 subunit of *L. reuteri* β -gal showed activity after subunit dissociation and separation by

1 SDS-PAGE while the smaller subunit was inactive. This active staining of the purified
2 β -galactosidase L103 was done directly on the SDS-PAGE gel after pre-incubating the
3 enzymes with denaturing SDS buffer at 60°C for 5 min (a 'milder' form of denaturation
4 resulting predominantly in subunit dissociation but not denaturation) and using 4-
5 methylumbelliferyl β -D-galactoside as the substrate. After pre-incubating the enzymes
6 with denaturing SDS buffer at 99°C (presumably resulting in complete denaturation),
7 both subunits exhibited no activity (data not shown).

8 When both genes *lacLM* were expressed in *E. coli*, functional β -galactosidase was
9 obtained, whereas the expression of *lacL* alone did not yield an active protein. These
10 observations suggest that both *lacL* and *lacM* are required for the synthesis of functional
11 and active β -galactosidase. The precise reason for this is unclear at present, but it can be
12 speculated that the simultaneous presence of polypeptides representing both subunits
13 influences the correct folding of the protein, while a misfolded and therefore inactive
14 protein is formed when only the large subunit is expressed. Translational coupling
15 between *lacL* and *lacM* was also found previously in *Leuconostoc lactis* (David *et al.*,
16 1992).

17 *E. coli* BL21 Star (DE3) was used as the expression host for the expression of β -
18 galactosidase genes. Although this strain has background β -galactosidase activity, the
19 expressed recombinant protein is histidine-tagged and can be easily purified with Ni
20 Sepharose High Performance column and separated from the native β -galactosidase.
21 Heterologous expression of the tagged protein resulted in the production of 110 kU of
22 β -galactosidase activity, which could be efficiently purified by metal affinity
23 chromatography in one single step, and approximately 83 kU of purified recombinant
24 enzyme per liter of fermentation broth with a specific activity of 180 U/mg of protein
25 was obtained. When using the natural source *L. reuteri* L103 for the production of β -

1 galactosidase, 2.5 kU per liter of fermentation broth was formed, of which 175 U (~
2 7%) purified enzyme with a specific activity of 158 U/mg was obtained after a three-
3 step-purification procedure (Nguyen *et al.*, 2006). The β -galactosidase activities
4 described here are given as activity with the chromogenic model substrate *o*NPG, which
5 is approximately 4.6-fold higher than the activity with the natural substrate lactose.

6 Unexpectedly, the optimum pH of recombinant EL103 β -galactosidase shifted 1 pH
7 value for *o*NPG lysis. Recombinant EL103 β -gal also exhibited a slightly broader and
8 higher temperature optimum range for lactose hydrolysis compared to native L103 β -
9 gal. The reason for these changes is not clear, but can probably be attributed to slightly
10 aberrant folding of the polypeptide chains by *E. coli* as compared to *L. reuteri*.
11 However, these changes did not affect the spectrum and the yield of galacto-
12 oligosaccharides (GOS) from lactose using recombinant β -gal EL103. Recombinant β -
13 gal EL103 formed a similar GOS spectrum as native β -gal L103 (data not shown) with
14 the main products, identified being β -D-Galp-(1 \rightarrow 6)-D-Glc (allolactose), β -D-Galp-
15 (1 \rightarrow 6)-D-Gal, β -D-Galp-(1 \rightarrow 3)-D-Gal, β -D-Galp-(1 \rightarrow 3)-D-Glc, β -D-Galp-(1 \rightarrow 6)-Lac,
16 β -D-Galp-(1 \rightarrow 3)-Lac (Splechtna *et al.*, 2006).

17 In conclusion, this work presents the cloning and expression of the genes encoding
18 heterodimeric β -galactosidase from *L. reuteri* in *E. coli*. The cloning of these genes into
19 the expression vector pET21d enables efficient production of the protein of interest in
20 this study in gram scale. The overproduction of this enzyme in yeast or other food-grade
21 expression system, which is ongoing in our project, is also of interest for the
22 applications in food industry.

23 Finally, it should be mentioned that at the time the experimental work of this
24 research was carried out, no sequence of *L. reuteri* β -galactosidases was yet published.

1 Genome shotgun sequence of *L. reuteri* JCM 1112 (GenBank accession number
2 NZ_AAOV01000035) was published during the preparation of this manuscript.

3

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7 (BOKU, Vienna) for the fruitful discussions during the experimental work of this
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1 **FIGURE CAPTIONS**

2 **Fig. 1.** Overlapping coding region of the *lacL* and *lacM* genes of *Lactobacillus reuteri*
3 L103 β -galactosidase.

4 **Fig. 2.** Amino acid sequence alignment of (A) *lacL* and (B) *lacM* genes of the β -
5 galactosidases from *Lactobacillus reuteri* L103 (GenBank accession no. ABF72116 and
6 ABF72117, respectively) and some other *Lactobacillus* spp. and *Leuconostoc lactis*.
7 GenBank accession numbers are indicated.

8 **Fig. 3.** Amino acid alignment of the flanking regions of the presumed acid-base
9 catalyst, nucleophilic recognition site and ligands of the magnesium ion of *lacZ* from *E.*
10 *coli* and *lacL* from *L. reuteri* L103 (GenBank accession no. ABF72116).

11 **Fig. 4.** (A) SDS-PAGE of β -galactosidase (*lacLM*) from *Lactobacillus reuteri* L103
12 overexpressed in *E. coli* BL21 Star (DE3). Lane 1, recombinant molecular weight
13 markers (Biorad); lanes 2 and 3, Coomassie blue staining of whole-cell lysates of *E. coli*
14 containing pHA1032 without induction (lane 2), and with 0.1 mM IPTG induction at
15 25°C for 12 hours (lane 3); lane 4, Coomassie blue staining of purified recombinant β -
16 galactosidase EL103 (B) SDS-PAGE of purified native and recombinant β -
17 galactosidases. Lane 1, recombinant molecular weight markers (Biorad), lanes 2 and 3,
18 Coomassie blue staining of purified native β -galactosidase from *L. reuteri* L103 (lane 2)
19 and recombinant β -galactosidase EL103 (lane 3); lanes 4 and 5, active staining with 4-
20 methylumbelliferyl β -D-galactoside of native β -galactosidase L103 (lane 4) and
21 recombinant β -galactosidase EL103 (lane 5).

22 **Fig. 5.** pH optimum (A, B) and temperature optimum (C, D) of native β -galactosidase
23 from *L. reuteri* L103 and recombinant β -galactosidase EL103 (A, C: *o*NPG as the
24 substrate; B, D: lactose as the substrate). The buffers used as shown in (A,B) were

- 1 sodium citrate (50 mM, pH 4.0-5.5) (circles), sodium phosphate (50 mM, pH 6.0-7.5)
- 2 (triangles) and borate (50 mM, pH 8.0-9.0) (squares).
- 3 **Fig. 6.** pH stability of native L103 and recombinant EL103 β -galactosidases incubated
- 4 at 37°C in sodium citrate buffer (pH 4-5.5), sodium phosphate buffer (pH 6.0 – 7.5) and
- 5 borate buffer (pH 8.0 -9.0) with residual activity measured after 3 h.

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1 TABLES

2

3 **Table 1.** Sequences of the primers used in this study

Primer	Sequence (5'→3')	Location	Reference sequence accession no.
β-gal F1	ATGCAAGCAAAYATMAAWTGG	β-galactosidase (<i>lacL</i>)	DQ493596
β-gal F2	GGTGGTGATTTTCGATGATCGTCAC	β-galactosidase (<i>lacL</i>)	DQ493596
β-gal F3	GGGAGATGGCTTGATGTTTGCTG	β-galactosidase (<i>lacL</i>)	DQ493596
β-gal F4	CCTGGTCTACCACTAGTAGGAATGC	β-galactosidase (<i>lacM</i>)	DQ493596
β-gal F5	CCAACAGTTGCTACCGGCTTTG	β-galactosidase (<i>lacM</i>)	DQ493596
β-gal F6	ATGCAAGCAAATATAAAATGGCTTGATGAACCG	β-galactosidase (<i>lacL</i>)	DQ493596
β-gal F7	TCGCCCCCATGGAAGCAAATATAAAA	(a)	
β-gal R1	TTATTTGTGTAAKCCATARTA	β-galactosidase (<i>lacL</i>)	DQ493596
β-gal R6	TTATTTTGCATTCAATACAAACGAAACTCAAC	β-galactosidase (<i>lacM</i>)	DQ493596
β-gal R7	GGAATTCC <u>TCGAG</u> TGATTTTGCATTCAATAC	(b)	
pBS R1	GTTGTGTGGAATTGTGAGCGG	pBluescript II SK (-)	X52330
pBS R2	ACGCCAAGCGCGCAATTAACC	pBluescript II SK (-)	X52330

4 F: denotes forward primers; R: denotes reverse primers.

5 ^(a) Upstream primer to amplify *lacLM* from pHA1031 with *NcoI* site (underlined)6 ^(b) Downstream primer to amplify *lacLM* from pHA1031 with *XhoI* site (underlined)

1 **FIGURES**

2

ATGCAAGCAAATATAAAATGGCTTGATGAACCGGAAACATTCCGCGTCAATCAATTACCT.....
lacL M Q A N I K W L D E P E T F R V N Q L P

..... AGATACTACTATGGCTTACACAAATAAATTACGCGTGATATATGGTGCAAAATAA
 R Y Y Y G L H K *
lacM M A Y T N K L R V I Y G A K *

3

4 **Fig. 1.**

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1

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*          20          *          40          *          60
L. reuteri-L103       : MQANIKWLDPEE*FRVNLBAHSDHY*YGN*YDE*WRHNN*S*RF*AQNL*DG*QW*QF*NE*AEN*ER*KR* : 60
L. acidophilus_BAA20536 : MQANIKWLDPEE*FRVNLBAHSDH*PF*KNY*RE*QNNH*S*RF*QSL*NG*MW*QF*KE*SK*DQ*SR : 60
L. helveticus_CAD55499 : MQANINWLDNPEE*FRVNLBAHSDH*PF*RDY*RE*QKQ*HS*SY*QSL*NG*KW*RH*FE*SA*NEM*DR : 60
L. johnsonni_AAS08676 : MQANIKWLDPEE*FRVNLBAHSDH*LY*PK*NY*EE*QK*RS*FT*QSL*DG*KW*QF*KE*SNN*Q*TR : 60
L. plantarum_CAD65569 : MQANLQWLDPEE*FRVNLBAHSDH*YH*DTA*EE*KTG*-SR*FL*KSL*NG*AW*RE*NE*AKT*PA*ER : 59
Leu. lactis_Q02603     : MQANLQWLDPEE*FRVNLBAHSDH*YH*DTA*EE*KTG*-SR*FL*KSL*NG*AW*RE*NE*AKT*PA*ER : 59
L. sakei_CAA57730      : MQPNIQWLDPEE*FRVNLBAHSDH*RY*ATL*EA*MA*QQ*SS*FE*QSL*NG*TW*QF*HY*SV*NA*AS*R : 60

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2

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MqaN6 WLD Pe FRVnQ6pAHS*DH 55 E s 5 sL1G W F 5 p R
*          80          *          100         *          120
L. reuteri-L103       : ENDFYKVDYDSS*SE*GTE*VE*PS*EI*EL*NN*MA*QNN*Y*INT*LI*PW*E*GKI*Y*RR*PA*MA*LS*PDD*AQ*EG : 120
L. acidophilus_BAA20536 : EVDFYK*KD*FNT*SS*FT*TP*V*SE*EI*EL*NN*FA*QNY*INT*LI*PW*E*GKI*Y*RR*PA*MA*LN*KSD*AEE*G : 120
L. helveticus_CAD55499 : EODFYQRDFDSS*NED*SH*VP*SE*EI*LS*NY*TQ*NY*INT*LI*PW*E*GKI*Y*RR*PA*MA*LD*PND*HEE*G : 120
L. johnsonni_AAS08676 : ELD*FYK*TN*ED*AS*DF*LD*FP*SE*EI*EL*NN*MA*QNY*INT*LI*PW*E*GKI*Y*RR*PA*MA*SL*GK*TR*- : 118
L. plantarum_CAD65569 : EVDFYQ*PF*DA*DF*DT*QV*PG*HI*EL*AG*Y*QI*Y*INT*LI*PW*E*GKI*Y*RR*PP*Y*T*IN*QD*QL*TP*G : 119
Leu. lactis_Q02603     : EVDFYQ*PF*DA*DF*DT*QV*PG*HI*EL*AG*Y*QI*Y*INT*LI*PW*E*GKI*Y*RR*PP*Y*T*IN*QD*QL*TP*G : 119
L. sakei_CAA57730      : E*KSF*Y*EL*AF*DA*QD*EE*PT*V*Q*HI*EL*AG*Y*EQ*LHY*INT*MI*PW*E*G*HY*Y*RR*PA*ET*SD*DK*QH*LG : 120

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3

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p dFY 51 F I VP IEL 5 Q YInt6 PWEGki5RRP 5 1 g
*          140         *          160         *          180
L. reuteri-L103       : SFSDGDDN*IVG*EYL*KH*FD*LS*PS*LRG*KQ*TR*IR*ED*GER*AMY*V*VL*NG*H*FIG*Y*AED*SFT*PSE*F : 180
L. acidophilus_BAA20536 : SFSEGD*DN*IVG*EYL*KH*FD*LS*PE*LRD*HD*HI*VE*GA*ER*AMY*V*VL*NG*H*FIG*Y*AED*SFT*PSE*F : 180
L. helveticus_CAD55499 : SF*SKG*ADN*IVG*EYL*KH*FD*LS*SAL*IG*KD*VH*IK*EG*VE*Q*AMY*V*VL*NG*H*FIG*Y*AED*SFT*PSE*F : 180
L. johnsonni_AAS08676 : SF*SGG*EDN*IVG*EYL*KH*FD*LS*NS*F*Q*GK*H*VH*VE*GA*ER*AMY*V*VL*NG*H*FIG*Y*GED*SFT*PSE*F : 178
L. plantarum_CAD65569 : LF*S*DA*ADN*IVG*EYL*KH*FD*LS*DA*FK*QR*III*Q*FG*VE*BAL*Y*V*VL*NG*H*FIG*Y*AED*SFT*PSE*F : 179
Leu. lactis_Q02603     : LF*S*DA*ADN*IVG*EYL*KH*FD*LS*DD*V*FK*QR*III*Q*FG*VE*BAL*Y*V*VL*NG*H*FIG*Y*ED*SFT*PSE*F : 179
L. sakei_CAA57730      : MF*SEAD*YN*IVG*EYL*HH*ED*LT*PAL*RN*QR*V*II*RE*EG*VE*Q*AMY*V*VL*NG*H*FIG*Y*AED*SFT*PSE*F : 180

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4

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FS dNtVGsY6k FDL 6 6 F GvE A65VVLNGHf6GY EDSFTPSEF
*          200         *          220         *          240
L. reuteri-L103       : DLT*FY*IQ*DE*GN*LAV*VY*FK*HST*AS*WIED*QD*FR*FG*I*FR*SV*NLL*AG*EL*VH*VED*D*HI*TR*IV : 240
L. acidophilus_BAA20536 : DLT*FY*IK*ED*KN*LAV*VY*FK*HST*AS*WLED*QD*FR*FG*I*FR*SV*BLL*AP*ETH*LV*DD*DL*K*PT*V : 240
L. helveticus_CAD55499 : DLT*FY*IQ*DK*DN*LAV*VY*FK*HST*AS*WLED*QD*FR*FG*I*FR*SV*BLL*GP*ATH*LM*DM*DL*K*BR*V : 240
L. johnsonni_AAS08676 : DLT*FY*LK*EN*NLAV*VY*FK*HST*AS*FLED*QD*FR*FG*I*FR*SV*KLL*AP*ETH*LM*DAL*K*BT*I : 238
L. plantarum_CAD65569 : DLT*FY*IQ*DQ*GN*LAV*VY*FK*HST*AA*FIED*QD*FR*FG*I*FR*DV*NLL*AB*ASH*IT*DD*DI*RB*VP : 239
Leu. lactis_Q02603     : DLT*FY*IQ*DQ*GN*LAV*VY*FK*HST*AA*FIED*QD*FR*FG*I*FR*DV*NLL*AB*ASH*IT*DD*DI*RB*VP : 239
L. sakei_CAA57730      : DLT*FY*LK*ED*TN*CLAV*VY*FK*HSS*AA*FIED*QD*FR*FG*I*FR*DV*KLL*AP*ETH*LM*DAL*K*BT*I : 238

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5

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DLTPy6 N LAV V K S3A 56EDQDMFRFSG6FR V 6La P H6 D6 6 P
*          260         *          280         *          300
L. reuteri-L103       : TDNYQD*GIF*NVD*LQL*HE*EKT*GN*VN*VR*VD*ND*ENT*LV*NETH*PVD*ST*WK*VQ*DQ*FL*ENV*HL*WD : 300
L. acidophilus_BAA20536 : CDNYQD*GIF*NA*EL*KFF*TC*SL*NG*HV*LS*VED*VN*CS*ALL*EQ*DV*PLD*SE*VE*FT*SST*LENI*HL*WD : 300
L. helveticus_CAD55499 : ADNYQD*GIF*NL*KL*HFI*GK*KAG*SF*HL*LK*DI*K*HT*LE*KN*EDI*KEN*VQ*IN*NE*KF*ENV*HL*WN : 300
L. johnsonni_AAS08676 : TDNYHD*GIF*NA*KL*SFT*CT*KD*GY*VR*LK*TD*IN*Q*TL*DE*EH*NLT*ST*T*IEN*KLL*KNI*HL*WD : 298
L. plantarum_CAD65569 : NANLKS*GEL*NI*TK*VT*GE*PAT*LA*-LT*VR*DH*D*ERV*LT*SQ*TQ*TG*SG*ST*FD*TML*FD*QL*HL*WS : 298
Leu. lactis_Q02603     : NANLKS*GEL*NI*TK*VT*GE*PAT*LA*-LT*VR*DH*D*ERV*LT*SQ*TQ*TG*SG*ST*FD*TML*FD*QL*HL*WS : 298
L. sakei_CAA57730      : DVVQQT*G*QV*KL*RL*Q*FS*DEN*R*-VH*LR*IR*DQ*HQ*II*LT*PAD*L*-TSGA*Q*W*ND*LY*KMP*EL*VQ*AMS : 298

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6

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G n G 6 6 D g 6 6 6h1W
*          320         *          340         *          360
L. reuteri-L103       : NHHPYLYQLLIE*IR*DD*EG*NL*VE*LV*YR*FG*FR*RI*E*IN*KH*V*VL*LN*GQ*LI*ING*VNR*HE*WA : 360
L. acidophilus_BAA20536 : NNHPYLYQLLIE*VH*DE*NG*H*VE*LV*YR*FG*FR*RI*E*IN*Q*DR*V*IL*LN*GQ*LI*ING*VNR*HE*WA : 360
L. helveticus_CAD55499 : NHDPYLYQLLIE*VY*DE*Q*Q*NL*LE*LV*YR*FG*FR*RI*E*IS*PE*KV*VL*LN*GQ*KR*LI*ING*VNR*HE*WA : 360
L. johnsonni_AAS08676 : NNHPYLYQLLIE*VH*DS*NK*NL*LE*LD*SY*KE*FR*RI*E*KT*DD*KV*VL*LN*GQ*KR*LI*ING*VNR*HE*WA : 358
L. plantarum_CAD65569 : POTPYLYQLLIE*VY*DAD*RO*LL*EV*YR*FG*FR*TV*ER*DD*KV*Y*V*NN*KR*LV*ING*VNR*HE*WA : 358
Leu. lactis_Q02603     : POTPYLYQLLIE*VY*DAD*HQ*LL*EV*YR*FG*FR*TV*ER*DD*KV*Y*V*NN*KR*LV*ING*VNR*HE*WA : 358
L. sakei_CAA57730      : NQTE*NLY*TL*EL*EV*VD*OAG*ET*IE*IS*QQ*PF*GR*RI*E*I*-K*RV*ML*LN*GQ*KR*LV*ING*VNR*HE*WP : 357

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7

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PyLYqL 6E6 D 16E6 FGFR 6e6 dkV6 6N krL6INGVNRHEW
*          380         *          400         *          420
L. reuteri-L103       : KRGRAT*MTDD*MTSD*IH*FK*BNN*INAV*RT*CHY*PD*QI*HW*YL*CD*DNG*IY*MA*EN*NLES*HAT*W : 420
L. acidophilus_BAA20536 : KTGRS*IT*LN*DM*EK*DI*DF*FK*BNN*INAV*RT*CHY*PN*QI*HW*YL*CD*DNG*IY*MA*EN*NLES*HGT*W : 420
L. helveticus_CAD55499 : KRGRS*IT*MS*DM*TD*IN*FK*BNN*INAV*RT*CHY*PN*QI*HW*YL*CD*DNG*IY*MA*EN*NLES*HGT*W : 420
L. johnsonni_AAS08676 : NSGR*HT*T*KOD*MK*AD*IQ*FK*BNN*INAV*RT*CHY*PN*QI*HW*YL*CD*DNG*IY*MA*EN*NLES*HGT*W : 418
L. plantarum_CAD65569 : HTGRV*IS*MD*MR*AD*IQ*ML*LANN*INAD*RT*CHY*PD*QI*HW*YL*CD*DAG*IY*MA*EN*NLES*HGS*W : 418
Leu. lactis_Q02603     : HTGRV*IS*MD*MR*AD*IQ*ML*LANN*INAD*RT*CHY*PD*QI*HW*YL*CD*DAG*IY*MA*ET*NLES*HGS*W : 418
L. sakei_CAA57730      : ETGR*HT*T*AB*DE*AW*DI*AC*MQR*NH*INAV*RT*SHY*PDR*LS*FY*NG*CD*DAG*IY*MA*ET*NLES*HGS*W : 417

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8

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GR I3 Dm DI t NnINA RTcHYPlq6p5Y LCD GIY6MAE NLESHg3W
*          440         *          460         *          480
L. reuteri-L103       : QKMGATE*PS*YN*VP*GS*VP*Q*MR*DV*VDR*ART*NY*ET*KN*HS*SIL*FWS*LG*NES*YAG*NI*VR*KN*E : 480
L. acidophilus_BAA20536 : QKMG*VE*PS*DN*VP*GS*VE*PE*RE*AV*ID*RARN*NY*ET*KN*HT*SIL*FWS*LG*NES*YAG*NS*IV*AM*NE : 480
L. helveticus_CAD55499 : QKMG*IE*PS*DN*VP*GS*IP*Q*KEA*VID*RAR*IN*Y*ET*KN*HT*SIL*FWS*LG*NES*YAG*DN*II*AM*NE : 480
L. johnsonni_AAS08676 : QKMG*VE*PS*YN*VP*GS*LA*EN*RV*ID*RARS*NY*ET*KN*HT*SIL*FWS*LG*NES*YAG*EN*LI*AM*NQ : 478
L. plantarum_CAD65569 : QKMG*ATE*PS*YN*VP*GD*NPH*ML*AA*VID*RARS*NY*ET*KN*HS*SI*I*FWS*LG*NES*YAG*EN*LI*AM*QA : 478
Leu. lactis_Q02603     : QKMG*ATE*PS*YN*VP*GD*NPH*ML*AA*VID*RARS*NY*ET*KN*HS*SI*I*FWS*LG*NES*YAG*EN*LI*AM*QA : 478
L. sakei_CAA57730      : QKMG*AVE*PS*YN*VP*GS*YDE*MA*AT*LDR*ART*NF*ET*KN*HS*SIL*FWS*LG*NES*YAG*SV*LE*RM*NA : 477

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9

QKMG 6EPS NVPG W v6DRAR N5E fKNH SI6FWSLGNESYAG 6 M

1

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L. reuteri-L103 : FYK*H*DD*SRLVHYEGVCHT*PFYRYRISDVES*SMYL*LPK*EV*EBYLKNN*PK*PF*ME*CEYMHD : 540
L. acidophilus_BAA20536 : FYK*H*DD*SSRLVHYEGV*VHRR*PEL*KDQ*ISD*IES*SHMYL*EPK*EV*ABYL*RNN*PK*PF*ME*CEYMHD : 540
L. helveticus_CAD55499 : FYK*SH*DD*TRLVHYEGV*VHRR*PEL*KDK*ISDVES*SMYL*LPK*EV*EBYL*QND*PK*PF*ME*CEYMHD : 540
L. johnsonni_AA808676 : FYK*SH*DD*TRL*THYEGV*VHT*PD*LK*NI*SD*LES*SMYL*LPK*EA*ERYL*KNN*PK*PF*LE*CEYMHD : 538
L. plantarum_CAD65569 : FYK*SH*DD*SRLVHYEGV*VHT*PEL*KDRI*SDVES*SMYL*EK*EQ*NI*VAYL*EDN*PK*PF*LE*CEYMHD : 538
Leu. lactis_Q02603 : FYK*SH*DD*SRLVHYEGV*FYTP*PEL*KDRI*SDVES*SMYL*EK*EQ*NI*VAYL*EDN*PK*PF*LE*CEYMHD : 538
L. sakei_CAA57730 : Y*YK*Q*DP*TRLVHYEGV*FRAP*PEY*KATI*SDVES*SMYL*AT*PAE*IKAYL*DN*AP*OK*PF*LE*CEYMHD : 537
5YK hD 3RLvHYEGV Pe 4 ISD6ES MY P YL 1 P KPF6 CEYMHD

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2

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L. reuteri-L103 : MGNS*D*GGM*GSYI*SL*LE*KYE*QY*FG*GIW*DFID*QAL*LV*KD*EV*SGQ*EV*MRY*GG*DFD*DR*SDY*E : 600
L. acidophilus_BAA20536 : MGNS*D*GGM*GSYI*KLI*DEY*EQ*YV*GG*FIW*DFID*QAL*LV*DE*IS*KQ*NV*LY*GG*DFD*DR*SDY*E : 600
L. helveticus_CAD55499 : MGNS*D*GGM*GSYI*KLL*DEY*EQ*YV*GG*FIW*DFID*QAL*LV*DE*IS*GHD*V*LY*GG*DFD*DR*SDY*E : 600
L. johnsonni_AA808676 : MGNS*D*GGM*GSYI*KLI*DEY*EQ*YV*GG*FIW*DFID*QAL*LV*DE*IS*GKK*V*LY*GG*DFD*DR*SDY*E : 598
L. plantarum_CAD65569 : MGNS*D*GGM*GSYND*LI*DEY*EQ*YV*GG*FIW*DFID*QAL*LV*DE*IS*T*DQ*DV*LY*GG*DFD*DR*SDY*E : 598
Leu. lactis_Q02603 : MGNS*D*GGM*GSYND*LI*DEY*EQ*YV*GG*FIW*DFID*QAL*LV*DE*IS*T*DQ*DV*LY*GG*DFD*DR*SDY*E : 598
L. sakei_CAA57730 : MGNS*D*GGM*GSYID*LLS*QY*DMY*GG*FIW*DFID*QAL*LV*DE*IS*V*TG*QRE*LY*GG*DFD*DR*SDY*E : 597
MGNS GGM SY L6d Yp Y GGF*IW*DFID*QAL*v Dp63 v6RYGGDFD RhSDYe

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3

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L. reuteri-L103 : FSGDGLMFADRT*E*PK*PAMQ*EV*RY*YGL*HK : 628
L. acidophilus_BAA20536 : FSGDGLMFADRT*E*PK*PAMQ*EV*RY*YGL*HK : 628
L. helveticus_CAD55499 : FSGDGLMFADRT*E*PK*PAMQ*EV*RY*YGL*HK : 628
L. johnsonni_AA808676 : FSGDGLMFADRT*E*PK*PAMQ*EV*KY*YGL*HK : 626
L. plantarum_CAD65569 : FSGDGLMFADRT*E*PK*PAMQ*EV*KY*YGL*HK : 626
Leu. lactis_Q02603 : FSGNGLMFADRT*E*PK*PAMQ*EV*KY*YGL*HK : 626
L. sakei_CAA57730 : FSGDGLVFAT*R*DE*E*PK*PAMQ*EV*RY*YGL*HK : 625
FSG1GL6FAdRt KPAMQEV4YYYGLHK

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4

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Fig. 2 (A)

1

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*           20           *           40           *           60
L.reuteri-L103       : MAYTNKLR-VIYGDAALGLS*DS*HYLPSYERGGLES*LNKKEWLYR*EP*FPWRATTD : 59
L.acidophilus_BAA20537 : MAYTNNL-QIIYGDAALG*TKN*HYLPSYERGGLES*LNKKEWLYR*VP*FPWRATTD : 59
L.helveticus_CAD55500 : MDYTNQLHIIYGDAALGVN*KDE*QYIFSYERGGLES*LVHGKKEWLYR*VP*FPWRATTD : 60
L.johnsonni_AAS08675 : MDYTNK-LHIIYGDAALGVQ*TKK*QYIFSYERGGLES*LNKKEWLYR*IP*FPWRATTD : 59
L.plantarum_CAD65570 : MAYTNNQLHVIYGDGSLGLQ*AN*HYLPSYERGGLES*LVNDKKEWLYR*VP*FPWRATTD : 60
Leu.lactis_Q02604     : MAYTNNQLHVIYGDGSLGLQ*AN*HYLPSYERGGLES*LVNDKKEWLYR*VP*FPWRATTD : 60
L.sakei_CAA57731     : MANTNKR*LAVIFGDV*LG*LP*DE*HYLPSY*CT*GG*ES*LR*IQ*KEWLYR*SP*FPWRATTD : 60
M yTN      6I5GD  LG6 g  F Y6FSY2  GG1ESL 6  KEWLYR P P FWRATTD

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2

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*           80           *           100          *           120
L.reuteri-L103       : NDRGSGFN*RS*QWLAAD*TF*HK*CVG*ID*TV*DN*QHF*AE*LPIA*PI*NN*BS*DP*VSA*EN*V*K*Y* : 119
L.acidophilus_BAA20537 : NDRGSGFN*LK*AS*QWLGAD*MT*TK*CT*KI*EL*KV*DR*QF*DE*LPIA*PI*NN*QF*SN*HE*Y*AD*H*VO*IA*F : 119
L.helveticus_CAD55500 : NDRGSGFN*LK*AA*QWLGAD*MT*TK*CT*DI*EL*KV*DR*HDF*AE*LPIA*PI*NN*RF*SN*HE*Y*AK*S*AB*IS*F : 120
L.johnsonni_AAS08675 : NDRGSGFN*SA*QWLAAD*MT*SQC*SK*HL*TV*DD*QK*ED*ELPIA*PI*NN*QF*SN*HE*Y*AN*LV*K*IS*F : 119
L.plantarum_CAD65570 : NDHGS*GFS*VK*SA*QWYAAD*KR*ST*CD*IE*LT*VDD*QPV*T*ELPIA*PI*NN*KY*T*DE*IE*AT*K*V*S*LAY : 120
Leu.lactis_Q02604     : NDHGS*GFS*VK*SA*QWYAAD*KR*ST*CD*IE*LT*VDD*QPV*T*ELPIA*PI*NN*KY*T*DE*IE*AT*K*V*S*LAY : 120
L.sakei_CAA57731     : NDRGN*QF*PL*SG*M*LAAD*Q*IA*QS*TI*VA*IG*Q*TI*-*ELPIA*PI*NN*RY*SG*RE*TA*Q*EV*TV*TY : 119
ND g gF 64  qW  AD F  C  I 6 6D      LPIAP nN 53  e A  v 6 5

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3

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*           140          *           160          *           180
L.reuteri-L103       : T*ET*LT*VP*AT*QV*TV*TY*EV*N*QGE*K*VT*M*HY*Y*GH*ED*EL*GL*LV*VG*MR*E*IMP*TV*AT*G*FD*Y*Q*GL : 179
L.acidophilus_BAA20537 : W*Y*OT*LT*VP*AT*DV*K*II*N*ID*NT*GC*NI*N*V*M*HY*Y*G*KK*GL*PL*LV*IG*MR*E*IMP*TV*AT*G*FD*Y*E*GL : 179
L.helveticus_CAD55500 : T*Y*OT*LT*VP*AT*NA*K*II*N*ID*DV*GH*K*VT*M*RY*Y*G*KK*GL*PL*LV*IG*MR*E*IMP*TV*AT*G*FD*Y*E*GL : 180
L.johnsonni_AAS08675 : D*Y*KT*T*TP*AT*IC*IT*Y*VID*SS*GH*AT*IK*M*RY*M*CK*E*GL*PL*LV*IG*MR*E*IMP*TV*AT*G*FD*Y*E*GL : 179
L.plantarum_CAD65570 : H*EV*TT*VP*ST*IV*TV*TY*TV*TAD*G*Q*NI*ATH*YS*Q*SD*LP*EL*PA*GL*RE*IMP*TV*AT*G*FD*Y*T*GL : 180
Leu.lactis_Q02604     : H*EV*TT*VP*ST*IV*TV*TY*TV*TAD*G*Q*NI*ATH*YS*Q*SD*LP*EL*PA*GL*RE*IMP*TV*AT*G*FD*Y*T*GL : 180
L.sakei_CAA57731     : T*Y*OT*LT*VP*AT*QV*TV*TY*EV*N*QGE*K*VT*M*HY*Y*GH*ED*EL*GL*LV*VG*MR*E*IMP*TV*AT*G*FD*Y*Q*GL : 179
5 T T P T      6 Y 6      G I 6      Y G      LP LP      G6RF66PT ATgF Y GL

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4

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*           200          *           220          *           240
L.reuteri-L103       : S*GETY*PDR*MAG*ATE*EG*TF*HV*DGL*VP*TK*YL*VP*QEN*GM*HMD*TY*AL*IT*RD*ST*ON*NAD*HS*RE*PF : 239
L.acidophilus_BAA20537 : S*GETY*PDR*MAG*AKE*EG*TF*HV*DGL*VP*TK*YL*VP*QEN*GM*HMD*TY*AL*IT*RD*ST*ON*NAD*Q*SE*-F : 238
L.helveticus_CAD55500 : S*GETY*PDR*MAG*AKE*EG*TF*HV*DGL*VP*TK*YL*VP*QEN*GM*HMD*TY*AL*IT*RD*ST*ON*NAD*RT*NE*KE* : 240
L.johnsonni_AAS08675 : S*GETY*PDR*MAG*AKK*GV*F*HV*RGL*VP*TK*YL*VP*QEN*GM*HMD*TY*AL*IT*RD*ST*ON*NAD*RD*TN*DE : 239
L.plantarum_CAD65570 : S*GETY*PDR*MAG*ATH*GR*F*HV*DGL*VP*TK*YL*VP*QEN*GM*HMD*TY*AL*IT*RD*ST*ON*NAD*HD*NT*PE : 240
Leu.lactis_Q02604     : S*GETY*PDR*MAG*ATH*GR*F*HV*DGL*VP*TK*YL*VP*QEN*GM*HMD*TY*AL*IT*RD*ST*ON*NAD*HD*NT*PE : 240
L.sakei_CAA57731     : S*GETY*PDR*MAG*GIAG*EV*T*GL*VP*TK*YL*VP*QEN*GM*HMD*TY*AL*IT*RD*ST*ON*NAD*CL*HE*VP*ET : 239
SgETY*PDR  AGa  G 5h6  LPVT  YLVPQe  G6HM  T 6 6 R      1N d      f

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5

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*           260          *           280          *           300
L.reuteri-L103       : S*L*IK*Q*DE*Q*FP*AF*S*CL*PY*TA*E*LEN*ATH*IE*EL*PL*AR*TV*LV*IAG*AV*RG*VGG*IDS*WG*AD*VE : 299
L.acidophilus_BAA20537 : S*L*KL*Q*DK*Q*FP*AF*S*CL*PY*TA*E*LEN*ATH*IE*EL*PL*AR*TV*LV*IAG*AV*RG*VGG*IDS*WG*AD*VE : 298
L.helveticus_CAD55500 : S*L*SI*Q*Q*AE*K*EP*AF*S*CL*PY*TA*E*LEN*ATH*IE*EL*PL*VR*TV*LV*IAG*AV*RG*VGG*IDS*WG*AD*VE : 300
L.johnsonni_AAS08675 : Q*LE*INK*TD*REL*N*F*S*CL*PY*TA*E*LEN*ATH*IE*EL*PL*AR*TV*LV*IAG*AV*RG*VGG*IDS*WG*AD*VE : 299
L.plantarum_CAD65570 : S*L*TF*S*Q*AD*APP*AF*S*CL*PY*TA*E*LEN*ATH*IE*EL*PL*AR*TV*LV*IAG*AV*RG*VGG*IDS*WG*AD*VE : 300
Leu.lactis_Q02604     : S*L*TF*S*Q*TD*APP*AF*S*CL*PY*TA*E*LEN*ATH*IE*EL*PL*AR*TV*LV*IAG*AV*RG*VGG*IDS*WG*AD*VE : 300
L.sakei_CAA57731     : G*L*K*FK*M*VD*Q*FP*AF*S*CL*PY*TA*E*LEN*ATH*HS*EL*PA*PH*RT*V*LV*LG*AV*RG*VGG*IDS*WG*AD*VE : 299
L      Pf  FSCLEPYTA  ELENATH  eELPl  rRTVL 6  GaVRGVGGIDSWG  DVE

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6

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*           320
L.reuteri-L103       : EQYH*LPADR*DVE*FS*V*NAK : 319
L.acidophilus_BAA20537 : KQYH*LPED*KDY*FS*ENL*-- : 316
L.helveticus_CAD55500 : SAYH*LPED*HE*FS*ENL*-- : 318
L.johnsonni_AAS08675 : EKYL*DPSS*KNF*FS*PK*LS*-- : 317
L.plantarum_CAD65570 : SPYH*LPAD*OD*ID*FS*ENL*HF*- : 319
Leu.lactis_Q02604     : APYH*LPAN*Q*ID*FS*ENL*HF*- : 319
L.sakei_CAA57731     : VAYQ*LDAT*Q*DR*HE*FS*ENL*HF*- : 318
Y I      1  Fsf 6

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7

8

Fig. 2 (B)

1

Ecoli-lacZ_v00296 : LCDRYGLYVVDEANIETH-----GMVPMNRITD : 428
 L.reuteriL103-lacL : LCDDNGLIYMMABENLESHATWQKMGALIESYNVPG : 434
 LCD G6Y66 E N6E3H 6 P 6

Glu416 ↓ His418 ↓

2

Ecoli-lacZ_v00296 : D-PRMLPAMSERVTRMVDRDRNHPSVILWWSLGNES : 462
 L.reuteriL103-lacL : SVFQMRDVVDRARTNYETFKNHPSILWWSLGNES : 469
 P W 6 R 2 4NHPS66 WSLGNES

Glu415 ↑ His417 ↑

Glu461 ↓

3

Ecoli-lacZ_v00296 : IIC--PMYA-RVDEDQPPAVPKWSIKKNDLSPGE : 529
 L.reuteriL103-lacL : -VCHTEPEYRYRISDVESQMYLEPKVEVEYLK-NNP : 528
 6C P Y R6 2 5 6P 6 5L

Tyr503 ↓

4

Ecoli-lacZ_v00296 : TRPLILCEYAHAMGNSLGGFAKYWQAFROYERLQG : 564
 L.reuteriL103-lacL : DKPFMECEYMRDMGNSDGGMGSYISLLDKYRQYFG : 563
 4P 6 CEY H MGNS GG Y YP G

Tyr502 ↓

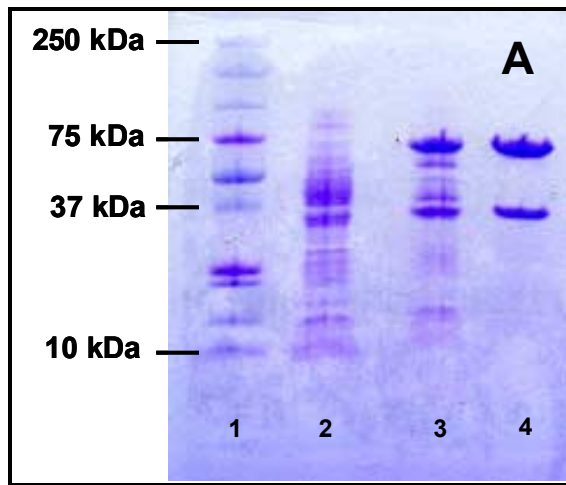
Glu537 ↓

5

Glu536 ↑

6 Fig. 3.

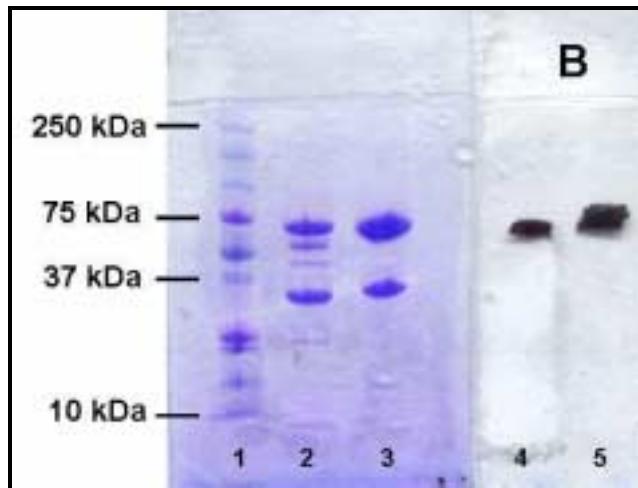
1



2

3

4



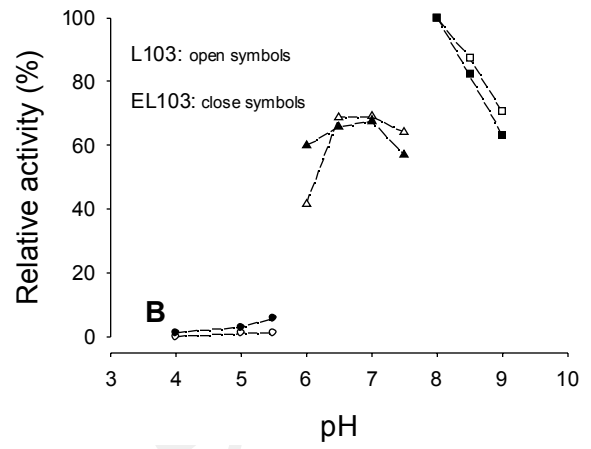
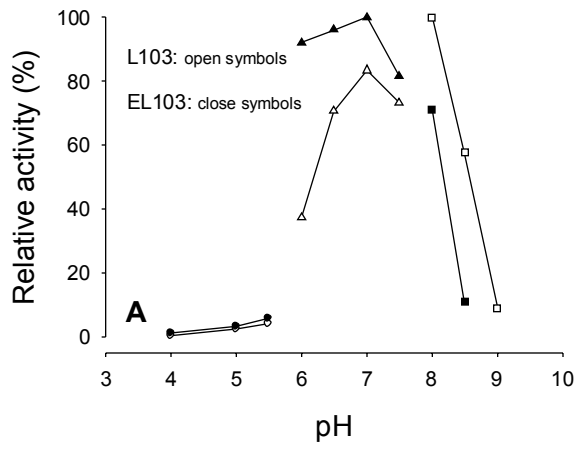
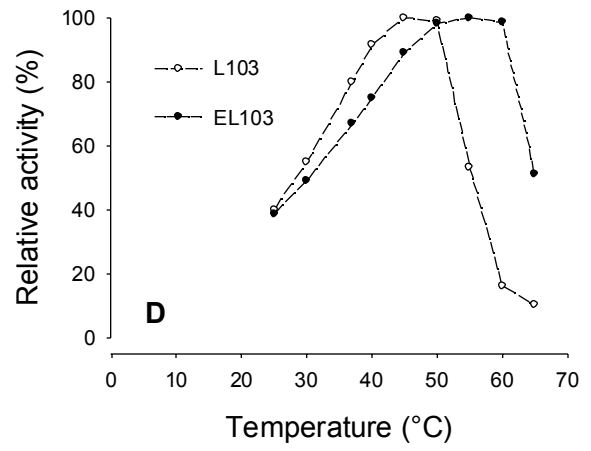
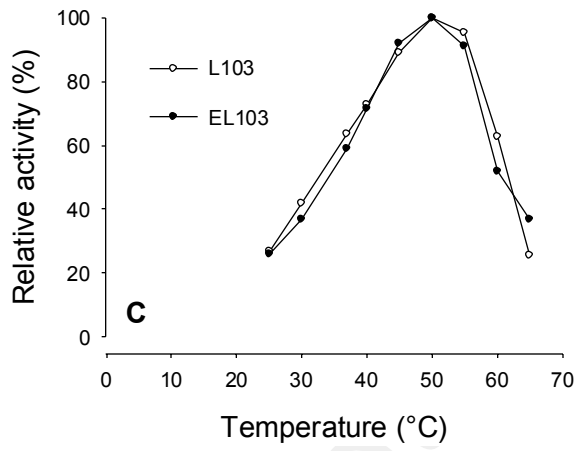
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6

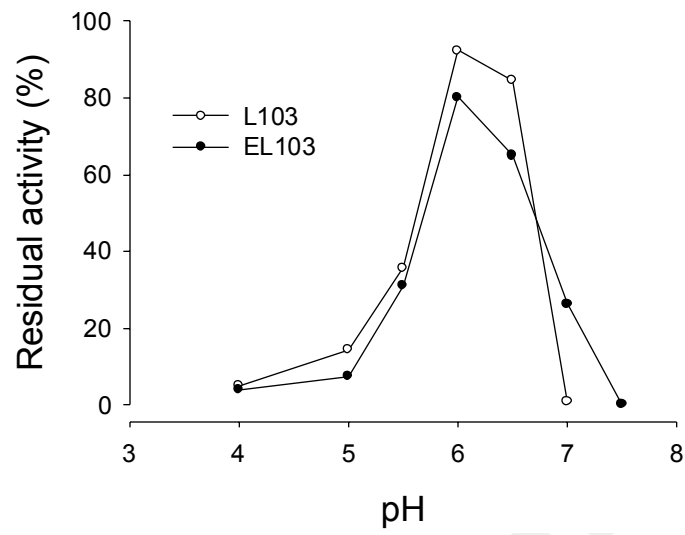
7

Fig. 4.

1

2
34
56 **Fig. 5.**

1



2

3

4 **Fig. 6.**