Optimization of the expression of levansucrase L17 in recombinant E. coli

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ABSTRACT
Levansucrases synthesize levans and fructooligosaccharides, which are of interest in the food and pharmaceutical industry. Leuconostoc mesenteroides Lm 17 produces levansucrase of about 120 kDa. The encoding gene from this strain was cloned and expressed in Escherichia coli BL21 (DE3). The cloned gene encodes a 1022 amino acids long levansucrase LevS from L. mesenteroides NRRL B-512F strain. The induction and expression of the levansucrase gene were performed at temperatures between 15 °C and 37 °C, and concentration of the inducer from 0.1 to 2.0 (mmol/L)^{-1}. We report for the first time recombinant expression of a levansucrase gene at a low temperature of induction, after cell biomass accumulation at 37 °C. The highest enzyme activity of 1.90 (U/mg)^{-1} was measured in TB medium at 18 °C temperature of induction, and concentration of the inducer from 0.1 to 1.0 (mmol/L)^{-1}. The in situ analysis of the purified enzyme showed an active band of about 120 kDa, similar to the one produced by the native strain. The purified enzyme has a temperature optimum at 35 °C, pH optimum at 5.5, and K_{m} = 64 (mmol/L)^{-1} of sucrose.

Introduction
In our previous studies, we showed that Leuconostoc mesenteroides Lm 17 strain produces several types of glycosyltransferases enzymes [1,2]. We identified one of them as a fructosyltransferase (FTF) synthesizing a levan type fructose polysaccharide. According to the literature, many lactic acid bacteria (LAB) belonging to genera Leuconostoc, Lactobacillus and Weissella produce FTFs [3–5]. These enzymes are grouped into glycosyde hydrolase family 68 (GH68) [6]. FTFs synthesizing polysaccharide composed of β-(2→6)-linked fructose units from sucrose are called levansucrases (EC 2.4.1.10), and the corresponding fructans – levans [7]. The levansucrase enzymes produced by bacteria have molecular masses ranging from 46 to 120 kDa [8–11]. Alternatively, the levansucrases can catalyze the transfer of fructose units from both sucrose and raffinose to various other acceptor fructosaccharides and glucosaccharides. Thus, the transferase reaction catalysed by the levansucrase enzymes leads to the synthesis of low-molecular weight fructooligosaccharides (FOSs) [12]. The effectiveness of FOSs synthesis by levansucrases strongly depends on the kinetic properties of the particular enzyme, the concentration of sucrose, the type and concentration of the acceptors, the temperature and pH of the reaction [9,13,14].

Synthesis of FOSs from cloned levansucrases has been reported for Lactobacillus sanfranciscensis TMW 1.392 [15] and L. mesenteroides S12FMC [8]. Levans and FOSs synthesized by levansucrases are important for human health because of their prebiotic properties and their use as immunomodulators, anti-tumour agents and inhibitors of dental caries [11,16–18]. Some L. mesenteroides and Lactobacillus strains produce a mixture of both glycosyltransferases (GTFs) and FTFs [19]. This makes the biochemical characterization of these enzymes difficult as well as the study of the structure and therapeutic potential of the synthesized products. The isolation, molecular and biochemical characterization of levansucrases, followed by optimization of their recombinant expression are needed as a first step towards their industrial-scale application for the production of levans and novel functional oligosaccharides [20–22].

The aim of the present work was to optimize the heterologous expression of L. mesenteroides Lm 17 levansucrase in E. coli. This goal is achieved by the cultivation of the recombinant strain in different media, at different temperatures of expression and concentrations of the
Materials and methods

**Bacterial strains and culture media**

*L. mesenteroides* Lm 17 strain isolated from fermented cabbage was obtained from the bacterial culture collection of the Department of General and Industrial Microbiology, Sofia University (Bulgaria). The strain is also deposited in the National Bank for Industrial Microorganisms and Cell Cultures (Bulgaria) under number NBIMCC 8953. *L. mesenteroides* LBAE-G15 strain isolated from sourdough was obtained from the LBAE collection (culture collection WDCM 1016 Université Paul Sabatier, Auch, France). The strains were routinely grown overnight in De Man, Rogosa and Sharpe (MRS) medium (Biokar Diagnostic) at 30 °C. *Escherichia coli* 10 G cells (Lucigen Corporation, Middleton, USA) were used for the construction of clones in the pETite vectors were grown in Luria–Bertani medium (LB) (Sigma) containing 30 μg/mL kanamycin at 37 °C, 250 rpm. For inducible expression, the cloned FTF genes were expressed in *E. coli* BL21 (DE3) (Lucigen Corporation, Middleton, USA). The transformants were grown in LB medium and Terrific Broth medium (TB) (12.0 g/L tryptone, 24.0 g/L yeast extract, 9.4 g/L K₂HPO₄, 2.2 g/L KH₂PO₄) supplemented with 30 μg/L kanamycin on a rotary shaker (250 rpm) at 37 °C.

**Cloning of the levansucrase-encoding gene**

*L. mesenteroides* total DNA was extracted using Blood and Tissue kit DNeasy (Qiagen). The primers for the isolation of levansucrase genes were designed after alignment of the sequences of *LevS* from *L. mesenteroides* NRRL B512F (AAAY19523.1), *LevC* (ABJ62503.1) and *Levl* (ABJ62504.4) from *L. mesenteroides* ATCC8293, and flanking sequences (in bold) according to the manufacturer's instruction for cloning into the pETite N-His SUMO vector (Lucigen Corporation, Middleton, WI, USA). The designed primers were: *LevCSUMOFordeg* 5’-GGCGAACAGATT-GAGGTTAGAAWAAARYTATAARKCWGGG-3’ where W = T or A, R = G or A, Y = T or C, K = T or G; *LevCSUMOrev* 5’-GTG GCGGCCGCTCTATTAACGTAAAGTATATGTGC-3’ (Eurogentec, Belgium). The amplification by polymerase chain reaction (PCR) was performed using a Gradient Master Thermocycler (Eppendorf) and appropriate concentrations of Expand Long Template PCR System containing reaction buffer with 17.5 mmol/L MgCl₂, Taq DNA polymerase (Roche), 20 mmol/L deoxyribonucleoside triphosphates and 0.4 μmol/L of the designed primers (Eurogentec). The cloning procedure was performed by Expresso™ T7 SUMO Cloning and Expression kit (Lucigen Corporation, Middleton, WI, USA). The amplicon containing the full-size gene was mixed with pETite N-His SUMO vector and transformed directly into *E. coli* 10 G chemically competent cells for construction of clones. The obtained colonies grown on LB medium containing 30 μg/mL kanamycin were screened for inserts by performing colony PCR using the first SUMO Forward primer (5’-ATCCAAGCTGATCAGACCCCTGA-3’) and pETite™ Reverse primer (5’-CTCAAGACCGGTTTAGAGGC-3’) (Lucigen Corporation, Middleton, WI, USA), according to the instruction provided by the supplier. Plasmid DNA from the positive transformants was isolated and purified using a QiAprep Spin Miniprep kit (Qiagen). The positive clones in the pETite™ vectors were transformed into *E. coli* BL21(DE3) cells for the purpose of expression.

**DNA and protein sequences analysis**

DNA sequencing was carried out by Millegen (Toulouse, France). Accession numbers have been assigned by GenBank to levansucrase genes from strains Lm 17 (L17 - KR052819) and LBAE-G15 (KR059028). DNA sequence information was analyzed by ORF FINDER (http://www.ncbi.nlm.nih.gov/projects/orf/) and BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) from the National Center for Biotechnology Information (NCBI) and transeq (http://www.ebi.ac.uk/Tools/st/emboss_transeq/) from EMBL-EBI.

Protein sequences were analyzed by Interpro software from EMBL-EBI (http://www.ebi.ac.uk/interpro/) in order to identify functional domains and were aligned together with the pairwise alignment tool EMBOSs Needle (http://www.ebi.ac.uk/Tools/psa/emboss_needle/) and Clustal Omega from EMBL-EBI (http://www.ebi.ac.uk/Tools/msa/clustalo/), in order to determine the identity between sequences. Repeated elements in the sequences were analyzed using RADAR (http://www.ebi.ac.uk/Tools/pfa/radar/) from EMBL-EBI. MAFFT version 7, Archaeopteryx (v0.9901), and the neighbour-joining method (http://mafft.cbrc.jp/alignment/server/) was used to get phylogenetic information [23].

**Optimization of the conditions for expression of levansucrase L17 gene in recombinant *E. coli* BL21**

Recombinant *E. coli* BL21(DE3) containing the pETite N-His vector with levansucrase gene from *L. mesenteroides* Lm 17 was grown in two different media: LB broth and TB broth, supplemented with 30 μg/mL kanamycin on a rotary shaker (250 rpm) at 37 °C. When the optical density at 600 nm (OD₆₀₀) reached 0.6, isopropyl-β-D-
thiogalactopyranoside (IPTG) was added as an inducer to a final concentration of 1.0 mmol/L. Then the protein expression was studied by growing the recombinant cells at five different temperatures: 15 °C, 18 °C, 20 °C, 25 °C and 37 °C. The enzyme activity was measured at different hours during the cultivation. Additionally, we optimized the expression of the studied levansucrase in the presence of inducer from 0.1 to 2.0 mmol/L concentration.

**Preparation of a cell-free extract and purification of levansucrase L17**

*E. coli* BL21(DE3) cells were collected by centrifugation (8500 g, 15 min, 4 °C) and washed two times with 20 mmol/L sodium acetate buffer (pH 7.5). The cells were resuspended in a cold sonication buffer containing 20 mmol/L sodium acetate buffer (pH 7.5), 300 mmol/L NaCl, 5 mmol/L imidazole and 5 mmol/L β-mercaptoethanol and then were sonicated (Hielscher Ultrasound Technology UP50H, Germany). The cell debris was separated by centrifugation (8500 g 15 min, 4 °C) and the supernatant was used as a cell-free extract. Levansucrase L17 was purified from the extract by affinity chromatography using His-Trap FF 5 mL Ni Sepharose Fast Flow column (GE Healthcare). The column was equilibrated with binding buffer (20 mmol/L sodium acetate buffer, pH 7.5; 500 mmol/L NaCl, 30 mmol/L imidazole) and then the cell-free extract was added to the column. To remove the unbound proteins, the column was washed with three volumes of the same buffer. Then the bound His-tagged protein was eluted from the Ni Sepharose column by elution buffer (binding buffer with 500 mmol/L imidazole). The eluted fraction was concentrated 10-fold using 10 000 MW cut off concentrators (Sartorius) and dialyzed against 20 mmol/L sodium acetate buffer (pH 5.5).

**Levansucrase enzyme activity assays and protein determination**

One unit of levansucrase activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol glucose per minute at 30 °C in 20 mmol/L sodium acetate buffer (pH 5.5); 0.05 g/L CaCl₂ and 100 g/L of sucrose. Levansucrase enzyme activity was determined by measuring the amount of reducing sugars derived from sucrose by the 3,5-dinitrosalicylic acid method (DNS) [24]. Additionally, the released D-glucose and D-fructose during the enzyme reactions were measured enzymatically (K-FRUGL kit from Megazyme, International Ireland Ltd, Wicklow, Ireland). For determination of the specific enzyme activity, the protein content was assayed by the method of Bradford [25], using bovine serum albumin as a standard.

All measurements were performed at least in triplicate with standard deviation (±SD).

**Kinetic studies of levansucrase L17**

The influence of temperature on enzyme activity during the reaction was studied in the standard reaction mixture described above, at 20–60 °C. The effect of pH was studied by dissolving the substrate in buffers with different pH and subsequent determination of enzyme activity. The used buffers were: 20 mmol/L sodium citrate (pH 3.0–4.0); 20 mmol/L sodium acetate (pH 4.5–5.5); potassium phosphate (pH 6.0–9.0).

The substrate-dependent kinetic parameters of the enzyme were determined at optimum temperature and pH in the presence of 0.05 g/L CaCl₂ and sucrose concentrations from 20 to 1000 mmol/L. $K_m$ and $V_{max}$ were determined by the Michaelis–Menten equation and non-linear regression approach.

All measurements were performed in triplicate and the received data were processed using SigmaPlot 12.0 (Systat Software, Inc., Chicago, USA).

**Electrophoresis analysis of levansucrase L17**

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE; 70 × 80 mm slab gels, 5% acrylamide) was performed according to the method of Laemmli [26]. The proteins were stained with Coomassie Brilliant Blue R 250 (Sigma) and also according to the silver nitrate staining protocol [27]. Levansucrase activity was detected by incubation of the gels in 10% sucrose or 5% raffinose overnight, followed by staining of polysaccharides according to a periodic acid-Schiff’s procedure (PAS) [28]. The High Molecular Weigh Calibration Kit for SDS Electrophoresis (Amersham, UK) was used as a protein standard.

**Polymer synthesis and analysis**

Polymers were synthesized by incubation with 0.5 U/mL of levansucrase L17 in 20 mmol/L sodium acetate buffer (pH 5.5) containing 100 g/L of sucrose, 0.05 g/L of CaCl₂ and 1 g/L of NaN₃ at 35 °C for 48 h. The synthesized polymers were precipitated twice with ethanol at a 75% (v/v) final concentration, washed twice with ultrapure water and freeze-dried.

The molecular weight of the purified polymer was determined by high-performance size exclusion chromatography (HPSEC) as described previously [29].
For nuclear magnetic resonance (NMR) analysis, freeze-dried polymer samples were suspended in deuterated water at a final concentration of 20 mg/mL. Nuclear magnetic resonance (NMR) spectra were recorded on an Advance 500 MHz spectrometer (Bruker, Billerica, Massachusetts, USA) operating at 500 MHz for $^1$H NMR and 125 MHz for $^{13}$C using a 5 mm z-gradient TBI probe. The data were processed using TopSpin 3 software. 1D $^1$H NMR spectra were acquired by using a zgpr pulse sequence (with water suppression). All the measurements were performed at 298 K and the chemical shifts were referenced to an internal reference sodium $2,2,3,3$-tetradeutero-3-trimethylsilylpropanoate ($^1$H = 0 ppm) and acetone ($^{13}$C = 31.08 ppm).

Results and discussion

Cloning and sequence analysis

Levansucrase genes from L. mesenteroides Lm 17 and LBAE-G15 strains were cloned by PCR amplification. Designed primers enabled synthesis of amplicons of about 3.7 kb which were cloned into the SUMO vector system from Lucigen. This system utilizes an N-terminal small ubiquitin modifier to enhance the solubility of the protein and a histidine-tag to enable easy purification of the protein.

Cloned sequences from L. mesenteroides Lm 17 and LBAE-G15 strains encode levansucrases of 1022 aa and 1034 aa, respectively. They are highly homologous to each other (97% identity) and to levansucrase LevS from L. mesenteroides NRRL B-512F strain (96% identity) [30], and they have similar predicted pi of 4.6 and theoretical molecular weight of 113 kDa. Interpro analysis showed that these proteins have a K$\times$Y$\times$G$\times$K$\times$W motif, which is specific for signal peptides of secretory proteins as defined by Bensing [31]. Eight cell-wall anchoring motifs LPTXG (CW) at the C-terminus part of the two levansucrases from Lm 17 and LBAE-G15 strains are also available. Four 33-aa long weak repeats are present in the variable N-terminus region before the catalytic domain specific for levansucrase enzymes. The sequences are also homologous to other levansucrases from other L. mesenteroides strains: 97% to hypothetical levansucrase MI1—06210 from J18; 82%, 77% and 68% to hypothetical levansucrases LEUM—1410 (LevC), LEUM—1409 and LEUM—1411 (LevL) from ATCC8293 [32,33]. In contrast, Lm 17 and LBAE-G15 levansucrases share only 12% identity with M1FT levansucrase from L. mesenteroides B-512FMC [8].

The catalytic domains of levansucrases from Lm 17 and LBAE-G15 strains are 451-aa long and have 99% identity to each other. A phylogenetic analysis of the catalytic domains from Lm 17, LBAE-G15 and several other FTFs was carried out (Figure 1). The catalytic domains of all Leuconostoc spp. levansucrases, except M1FT, were clustered together with those of LevSMI1—06 210 and LevC being positioned at a closer distance to the Lm17

![Figure 1. Phylogenetic analysis of levansucrase catalytic core.](image)

Note: The analysis was performed with MAFFT by the neighbour-joining method [23]. Bar: substitutions/nucleotide position. Numbers indicate bootstrap percentages per 1000 repeats.
NRRL B-512F and ATCC8293 strains [30,33]. The protein (Figure 2 (a)). Similar results have been reported for several activity bands ranging from 116 to 86 kDa expected molecular weight of about 120 kDa, and also nose and PAS staining, showed an activity band with an L. mesenteroides recombinant expression of FTFs from product of 120 kDa levansucrase [2].

Obtained by the native strain and could be a degradation with molecular weight of 86 kDa is similar to the one case with M1FT.

In situ analysis of the induced cells by the zymogram test, i.e. incubation of the gels in 10% sucrose or 5% raf and concentration (Silver staining); 2 – after dialysis (Silver staining); 3 – after elution (supernatant after lysis); 4 – after dialysis (incubation on 10% sucrose and PAS staining); 5 – after diafiltration (incubation on 5% raffinose and PAS staining).

Figure 2. In situ analysis of the crude (a) and purified (b) levansucrase L17 expressed in E. coli BL21.

Note: M: protein molecular-weight size marker (Amersham, UK); (a) PAS staining after incubation in 10% sucrose: 1 – supernatant after lysis; 2 – cells before lysis. Silver staining: 3 – supernatant after lysis; 4 – cells before lysis. PAS staining after incubation in 5% raffinose: 5 – supernatant after lysis; 6 – cells before lysis. (b) In situ analysis of the purified levansucrase: 1 – after elution and concentration (Silver staining); 2 – after dialysis (Silver staining); 3 – after dialysis (incubation on 10% sucrose and PAS staining); 4 – after dialysis (incubation on 5% raffinose and PAS staining).

and LBAE-G15 catalytic domains. This cluster differs significantly from the one formed by the levansucrases from Gram-negative bacteria Pseudomonas chlororaphis subsp. aurantiaca (LscA), Erwinia amylovora (Lsc), Glucobacter oxidans 621H (GOX0873) and L. mesenteroides B-512FMC (M1FT) (Figure 1). This correlates well with the phylogenetic analysis performed by Velazquez-Hernandez et al. [11], according to which some levansucrases by Gram-positive bacteria are a result of horizontal gene transfer from Gram-negative bacteria as seems to be the case with M1FT.

In situ analysis of the induced cells by the zymogram test, i.e. incubation of the gels in 10% sucrose or 5% raffinose and PAS staining, showed an activity band with an expected molecular weight of about 120 kDa, and also several activity bands ranging from 116 to 86 kDa (Figure 2(a)). Similar results have been reported for recombinant expression of FTFs from L. mesenteroides NRRL B-512F and ATCC8293 strains [30,33]. The protein with molecular weight of 86 kDa is similar to the one obtained by the native strain and could be a degradation product of 120 kDa levansucrase [2].

Optimization of the expression temperature of levansucrase L17 gene in recombinant E. coli BL21

In order to optimize the production of levansucrase L17, we studied the expression levels at various temperatures by growing the recombinant strain in two different media (LB and TB) and 1 mmol/L IPTG as an inducer. Levansucrase activity was detected at 15, 18 and 20 °C temperature of expression on both used media. At higher temperatures of expression (25 and 37 °C), enzyme activity was not detected, contrary to what has been reported for the expression of levansucrase M1FT [8]. The profiles of enzyme activity for both used media are shown in Figures 3 and 4. The highest enzyme activity was detected at the 16th hour at 18 °C in TB medium (1.90 U/mg) (Figure 4(b)). In LB medium, the highest activity of 1.60 U/mg was measured at the 12th hour from the induction at 18 °C (Figure 3(b)). After expression at 15 °C in LB and TB media, the enzyme activity was 13% and 9% lower compared to the values obtained at 18 °C (Figures 3(a) and 4(a)). When the expression was performed at 20 °C, the highest enzyme activity was detected at the 9th and the 12th hour from the induction in LB and TB media: 0.36 and 0.57 U/mg, respectively (Figures 3(c) and 4(c)). The results showed more than 70% reduction in the levansucrase activity in both media. The recombinant production of the studied levansucrase at 15 and 18 °C was more effective than the expression at temperature conditions nearer to the cultivation optimum of E. coli. It appears that, at temperatures higher than 18 °C, the synthesis potential of the cell is directed towards the reproduction of its own essential structures including proteins and nucleic acids, and the batch of the shared building blocks (nucleotides, amino acids, co-factors) and machinery are insufficient to maintain the recombinant expression. In contrast, the optimal temperature for expression of recombinant levansucrases M1FT and LevC have been detected at 28 and 37 °C, respectively [2,8]. Yi et al. [34] reported the expression of dextranucrase gene in E. coli at 15 °C in LB medium achieving 330-fold higher production than at 37 °C. Cote and Skory [35] also reported the expression of dextranucrase gene at 15 °C in LB medium as an optimum for production of the enzyme. In all the cases, higher levansucrase activity was measured during the cultivation and expression in TB medium, which could be explained by the higher concentration of yeast extract and phosphate salts facilitating the cellular stress response, preventing pH changes that can affect the normal metabolic systems, and contributing to the higher cell growth and production of recombinant proteins [36,37]. The decrease in enzyme activity observed after the 16th hour in TB medium is most likely due to an increase in the pH value above 7.0, and probably production of proteases [37].

Optimization of IPTG concentration for the expression of levansucrase L17 gene in recombinant E. coli BL21

The influence of several concentrations of the inducer on the expression levels of levansucrase L17 was studied at optimum expression conditions (TB medium, induction
Figure 3. Profile of levansucrase activity and cell growth during the recombinant expression in strain *E. coli* BL21 in LB medium at 15 °C (a), 18 °C (b) and 20 °C (c) after induction with IPTG. Note: All values are means (±SD) from triplicate measurements from two independent cultures.

Figure 4. Profile of levansucrase activity and cell growth during the recombinant expression in strain *E. coli* BL21 in TB medium at 15 °C (a), 18 °C (b) and 20 °C (c) after induction with IPTG. Note: All values are means (±SD) from triplicate measurements from two independent cultures.
Effect of temperature and pH on the activity of levansucrase L17

In order to determine the optimum reaction conditions of the purified levansucrase, we investigated the influences of temperature and pH on the enzyme activity. The highest activity (10.26 U/mg) was measured at 35 °C in 20 mmol/L sodium acetate buffer, pH 5.5 (Figure 6(a,b)). At temperatures of 40 and 45 °C, the residual enzyme activity decreased with 62% and 96%, respectively (Figure 6(a)). The enzyme activity decreased with more than 50% at pH < 5.0 and pH > 6.0 (Figure 6(b)).

Similar optimum temperatures have been reported for other Leuconostoc levansucrases: LevC [33], LevS [30] and M1FT [8]. This is in contrast to enzymes produced by

at 18 °C, 16 h of expression) (Figure 5). At IPTG concentrations from 0.1 to 1.0 mmol/L, there was no significant difference in levansucrase production. At 2.0 mmol/L IPTG, the enzyme activity decreased by about 30%. Similar effects are observed during the recombinant expression of other proteins in E. coli BL21, and could be attributed to the toxicity of IPTG when added at concentrations higher than 1 mmol/L [38]. On the other hand, achieving the same level of expression at 10-times lower concentration of the inducer represents a significant interest for industrial production of proteins using recombinant strains of E. coli [39]. Based on the obtained results for the influence of the IPTG concentration on the expression of the studied levansucrase, the most effective and economically advantageous concentration of the inducer was 0.1 mmol/L.

Purification and characterization of levansucrase L17

The activity of the crude enzyme extract was 3.32 U/mg of proteins. The levansucrase was purified three times using Ni-Sepharose chromatography, and the activity in the purified fraction was 10.0 U/mg (Table 1). In situ analysis of the purified levansucrase by zymogram showed a single activity band of about 120 kDa (Figure 2(b)).

Table 1. Purification of levansucrase L17.

| Step              | Total activity (U) | Protein (mg) | Activity (U/mg) | Purification (fold) | Yield (%) |
|-------------------|--------------------|--------------|-----------------|--------------------|-----------|
| Crude extract     | 42.92 ± 1.85       | 12.92 ± 0.50 | 3.32 ± 0.12     | 1.00               | 100       |
| Purified protein  | 15.00 ± 0.60       | 1.50 ± 0.06  | 10.00 ± 0.4     | 3.00               | 35.0      |

Note: All analyses of enzyme activity were performed in triplicate from two independent purification experiments and average values with standard deviation (± SD) are presented.
Lactobacillus spp., which have a higher temperature optimum: 35–45 °C for levansucrases from *Lb. sanfranciscensis* TMW 1.392 [32] and 50 °C for the enzyme from *Lb. reuteri* 121 [36]. Levansucrase L17 showed an optimum pH similar to that of some enzymes produced by *Lactobacillus* spp.: pH 4.5–5.5 from *Lb. reuteri* 121 [10] and pH 5.4 for a levansucrase from *Lb. sanfranciscensis* TMW 1.392 [15]. The pH optimum for the studied enzyme was lower than those of others produced by *L. mesenteroides* strains – optimum pH ≥ 6.0 [8,30,33].

**Levansucrase L17 kinetic parameters**

The $K_m$ and $V_{max}$ values were determined at concentrations of sucrose ranging from 20 to 1000 mmol/L and calculated by the Michaelis–Menten equation using the nonlinear regression approach. The $K_m$ of levansucrase L17 was 63.48 ± 6.39 mmol/L sucrose, and the $V_{max}$ value was 12.24 ± 0.29 U/mg. The value of $K_m$ was higher than that of other levansucrases expressed in *E. coli*: $K_m = 36.7 ± 5.4$ mmol/L for LevS [30]; $K_m = 27.3$ mmol/L for LevC [33]; $K_m = 26.6$ mmol/L for levansucrase M1FT [8] and $K_m = 14.5 ± 4.2$ mmol/L for levansucrase from *Lb. sanfranciscensis* TMW 1.392 [15].

**Polymer characterization**

The polysaccharide synthesized by levansucrase L17 was analyzed by both $^{13}$C and $^1$H NMR analysis (Figure 7). The produced polymer is a $\beta-2\rightarrow 6$ levan, similar to the one produced by the native strain. $^1$H NMR analysis of the polymer produced from the cloned levansucrase L17 gene showed chemical shifts of 4.10 and 4.19 ppm, in the bulk region (3.3–4.3 ppm). $^{13}$C NMR spectroscopy analysis showed anomeric C2 resonance at 105.04 ppm characteristic for levan, which correlates well with our previous studies [2].

The analysis of the molecular weight distribution of the synthesized levan showed presence of a single high molecular fraction of about $4 \times 10^6$ Da. Such a monomodal production is documented also by Olvera et al. [33] for levansucrase LeVC, which synthesizes single fraction of levan with molecular weight of $3 \times 10^6$ Da. In contrast, the levansucrase from *Lb. reuteri* 121 produces two levan fractions with molecular weights of $2 \times 10^4$ (96%) and $3 \times 10^6$ Da (3%), respectively [10]. Overall, the characteristics of the levansucrase reported here suggest that it would be of interest for production of levan-type polysaccharide, and also for synthesis of prebiotic FOSs like 1-kestose and nystose [9]. In addition, the enzyme could be tested for its ability to transfer fructose units to acceptors other than carbohydrates – fructosylation of alkaloids, flavonoids and aromatic alcohols, and thus potentially improving their physical-chemical properties and bioactivity [40].

**Conclusions**

In this study, we reported for the first time a two-step process for optimization of the recombinant production of levansucrase in *E. coli*: 1) cultivation of the recombinant strain at 37 °C for cell biomass accumulation and 2) low-temperature induction and expression of the gene encoding levansucrase L17 on two different media, LB and TB. From both media used for cultivation and production of the studied enzyme, TB medium supplemented with 0.1–1.0 mmol/L IPTG, at 18 °C temperature of induction gave the highest yield of levansucrase. The purified enzyme has a molecular mass of about 120 kDa, temperature optimum at 35 °C, pH optimum at 5.5, and $K_m = 64$ mmol/L of sucrose. The optimum pH value for
the action of the studied enzyme is lower, and the $K_m$ value is higher than those reported for levansucrases from other *L. mesenteroides* strains. This investigation of the production and purification of levansucrase reveals the potential industrial use of this enzyme for levan and fructooligosaccharide synthesis. Further studies of levansucrase L17 shall be directed towards the synthesis of different oligosaccharides and evaluation of their prebiotic potential.

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**Disclosure statement**

The authors have no conflicts of interest to disclose.

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