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ORIGINAL PAPER

# Gene cloning, characterization, and heterologous expression of levansucrase from *Bacillus amyloliquefaciens*

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**Abstract** Although levan produced by *Bacillus amyloliquefaciens* is known to have efficient immunostimulant property which gives 100% survival of common carp when infected with *Aeromonas hydrophila*, no detailed reports are available describing kinetic studies of D-glucose production and levan formation. In this study, we cloned and characterized the enzymatic kinetics using levansucrase expressed in *Escherichia coli*. Optimum pH for D-glucose production and levan formation was 6.0 and 8.0, respectively, whereas optimum temperature was 30°C and 4°C, respectively. The $K_m$ and $V_{max}$ values for levansucrase were calculated to be 47.81 mM sucrose and 57.47 μmole/min mg protein, respectively. Prominent expression of levansucrase was obtained through xylose induction in *Bacillus megaterium*, where most of the $His_6$-tagged protein was secreted into the culture broth, giving levansucrase activity of 12,906 U/l. Response-surface methodology (RSM) was further employed to optimize the fermentation conditions and improve the level of levansucrase production. Maximum levansucrase activity of 20,251 U/l was obtained in 12 h of fermentation carried out at 28°C, starting induction with 0.735% xylose when $A_{600}$ was 1.2, which was 1.6- and 62-fold higher than those obtained in the nonoptimized conditions for the recombinant strain and the native strain, respectively.

**Keywords** *Bacillus amyloliquefaciens* type 1 · Levansucrase · *Escherichia coli* · *Bacillus megaterium* · Gene expression · Response-surface methodology

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## Introduction

Levan, a polymer of fructose synthesized by levansucrase, is employed in food and nonfood industries as a viscosifier, stabilizer, emulsifier, gelling or water-binding agent. Levan has been reported to have a number of biological functions such as the promotion of infection and necrosis, tumor inhibition, tumor stimulation, and increasing the permeability of cells to cytotoxic agents [5]. Yano et al. [28] studied the effect of levan and other polysaccharides for their abilities to enhance the protection of common carp, *Cyprinus carpio*, against bacterial infection. However, no protection against *Aeromonas hydrophila* and *Edwardsiella tarda* was conferred by levan. Irrespective of the study carried out by Yano et al. [28], levan obtained from wild-type *Bacillus amyloliquefaciens* type 1 at concentration of 0.5% conferred protection against *A. hydrophila*, thus acting as an effective immunostimulant for common carp in aquaculture [18].

The production of levan using the wild-type strain is not cost-effective as levan is produced in the culture supernatant. Harvesting of levan from the culture supernatant requires an initial precipitation with ethanol, which leads to the precipitation of media components and other extracellular proteins that might add to the impurities in levan. Hence, an additional purification step, ultrafiltration, is required to obtain levan that is sufficiently purified for use as an immunostimulant.

Considering the exceptional properties of levan produced by *B. amyloliquefaciens* type 1, in this study we cloned the levansucrase gene from *B. amyloliquefaciens* type 1, expressed the gene in *Escherichia coli*, and determined its reaction kinetics in order to obtain the maximum yield.

Moreover, considering the commercial importance of the enzyme levansucrase, an attempt was also made to express the protein in *B. megaterium* MS941, which is a defined protease-negative mutant [26]. The capacity of selected *Bacillus* strains to produce and secrete large quantities (20–25 g/l) of extracellular enzymes has placed them among the most important industrial enzyme producers, producing about 60% of the commercially available enzymes [19]. Compared with *B. subtilis*, the most studied model Gram-positive bacterium, *B. megaterium*, appears to be an attractive alternative host for recombinant protein production due to its intrinsic lack of alkaline proteases as well as the high stabilities in replication and maintenance of recombinant plasmids it hosts [24].

The conventional experimental approach used for media optimization and fermentation conditions employs a method based on changing one factor at a time. This method is time consuming, may lead to misinterpretation of results when interactions between different components are present, and requires a large number of experiments to study the effect of individual factors. Unfortunately, this approach also frequently fails to locate the region of optimum response, thus an efficient approach is required for optimization. A combination of factors generating a certain optimum response can be identified through factorial design and the use of response-surface methodology (RSM) [2, 4]. RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effect of factors, and searching for optimum conditions of factors for desirable responses [14]. It combines fractional factorial design and a second-degree polynomial model to investigate complex processes, and it has been widely used in different fields. It is an efficient statistical technique for the optimization of multiple variables with the minimum number of experiments [25].

In the present study, we cloned and expressed the levansucrase gene of *B. amyloliquefaciens* type 1 in *E. coli* and *B. megaterium* and enhanced the levansucrase production in the recombinant *B. megaterium* through optimization of fermentation conditions using RSM.

## Materials and methods

### Strains, plasmids, and media

*Escherichia coli* DH5α was used as a host for cloning, whereas *E. coli* BL21 (DE3) pLysS and *B. megaterium* MS941 were used as the host strain for expression of levansucrase gene from *B. amyloliquefaciens* type 1. *B. amyloliquefaciens* type 1 was grown in Luria–Bertani (LB) (10 g/l NaCl, 5 g/l yeast extract, 10 g/l tryptone) media at 30°C, whereas *E. coli* and *B. megaterium* MS941 were grown in LB medium and LB with 2.5 mM $CaCl_2$, respectively, at 37°C. The plasmid pGEM-T Easy was used for cloning; pET28a and pMM1525 were used for the expression of the levansucrase gene in *E. coli* and *B. megaterium*, respectively. The expression of levansucrase in *E. coli* and the preliminary expression studies of levansucrase in *B. megaterium* were carried out using 50 ml of respective media as mentioned above in 250-ml baffled flask.

For optimization of fermentation conditions through RSM for recombinant levansucrase in *B. megaterium* the seed cell were prepared in 250-ml flask containing 50 ml LB medium. The flasks were incubated at 37°C for 12 h and were subsequently inoculated into the fermenter at 2.5% (v/v) concentration. The cultivations were carried out in 5-l stirred vessel fermenter (Kobiotech Co., Ltd., Korea) containing 2 l fermentation media (LB containing 2.5 mM of $CaCl_2$). pH was controlled by automatic addition of 2 M HCL and $NH_4OH$. The agitation rate was adjusted to 200 rpm. Antibiotics were added to the growth media at the following final concentrations: ampicillin at 50 μg/ml, kanamycin at 20 μg/ml, and tetracycline at 10 μg/ml in *E. coli* and 50 μg/ml in *B. megaterium.*

### Cloning and construction of plasmid for *B. amyloliquefaciens sacB* expression in *E. coli* BL21 (DE3) pLysS

The DNA fragment encoding the open reading frame (ORF) of levansucrase from *B. amyloliquefaciens* type 1 was obtained by polymerase chain reaction (PCR) amplification using the primers based on the *sacB* of *B. subtilis*, P1 (5′-ATGAACATCAAAAA-3′) and P2 (5′-TTATTT GTTAACTGTTAATTG-3′). The resulting PCR products were ligated into pGEM-T Easy (Promega). The DNA sequence of the entire *B. amyloliquefaciens* type 1 levansucrase ORF was determined. The ORF encoding the active SacB protein lacking the secretion signal peptide region was further amplified using two different primer sets, one set for the expression of *sacB*, P3 (5′-CCATGG AAGAAAATACCCAAA-3′; the underlined letters indicate a *Nco*I site) and P4 (5′-CTCGAGTTAGTTGTT AACCGT-3′; the underlined letters indicate a *Xho*I site), and one set for the expression of *sacB* fused to an amino-terminal $His_6$ tag, P5 (5′-CATATGAAAGAAAATA CCCAA-3′; the underlined letters indicate a *Nde*I site) and P4, generating SacB-Nc-X and SacB-Nd-X, respectively. The PCR products digested with *Nco*I and *Xho*I or *Nde*I

and *Xho*I were ligated into the corresponding restriction sites of pET28a, resulting in pET-sacB-NcX and pET-sacB-NdX.

### Construction of plasmid for *B. amyloliquefaciens sacB* expression in *B. megaterium* MS941

For the expression of *B. amyloliquefaciens sacB* in *B. megaterium* the ORF encoding the active region of SacB gene was amplified using two different primers, one for the expression of *sacB*, P6 (5′-GGATCCCT*GGCGCC*AAA GAAAATACCCAA-3′; the underlined letters indicate a *Bam*HI and *Sfo*I site, respectively) and P7 (5′-GCATGC TTAGTTGTTAACCGT-3′; the underlined letters indicate a *Sph*I site) and one set for the expression of *sacB* fused to an carboxy-terminal $His_6$ tag, P6 and P8 (5′-GCATGC TTAGTGATGGTGATGGTGATGGTTGTTAAC-3′; the underlined letters indicate a *Sph*I site). The PCR products were digested with *Bam*HI and *Sph*I and ligated with the corresponding restriction sites of pMM1525, resulting in pMM-sacB and pMM-sacB-His.

### Heterologous expression and purification of *B. amyloliquefaciens* SacB in *E. coli* through IPTG induction

*Escherichia coli* BL21 (DE3) pLysS clone harboring pET-sacB-Nc-X or pET28-sacB-Nd-X was grown to mid-exponential phase at 37°C with aeration until $A_{600}$ reached 0.6. Expression of *sacB* was induced by adding isopropylthio-*β*-D-galactoside (IPTG) at concentration of 0.5 mM and then incubating for 24 h at 20°C. *E. coli* clone harboring pET28a was used as a control. The protocol described by Kang et al. [11] was used to obtain the soluble and the insoluble fractions, and the soluble fractions were purified using $Ni^{2+}$-nitrilotriacetic acid (NTA) affinity chromatography.

### Levansucrase assay

Levansucrase assays were performed on all the fractions, followed by the estimation of glucose in the reaction mixture using the glucose oxidase/peroxidase kit (Sigma). One unit (U) of enzyme activity was defined as the amount of enzyme needed for the release of 1 μmole glucose per min. A reaction mixture containing 100 μl metabolic filtrate and 100 μl 200 mM sucrose prepared in sodium acetate buffer (pH 6.0) was incubated at 30°C for 30 min.

### Optimization of levansucrase activity and levan formation for recombinant levansucrase in *E. coli*

The effect of pH ranging from 4.0 to 9.0, effect of temperature (4°C, 10°C, 20°C, 30°C, 40°C, 50°C, and 60°C), and the effect of sucrose concentration within a range of 100–500 mM on D-glucose production activity and levan formation was also estimated. A time course of levan formation was conducted using 1 U enzyme and 100 mM sucrose prepared in sodium phosphate buffer (pH 8.0) and incubating the reaction mixture at 4°C for varying incubation periods. Following incubation, levan was harvested and its weight was estimated as described below.

### Analysis of levan produced by *B. amyloliquefaciens* SacB

Purified levansucrase (1 U) was added to 100 mM sucrose prepared in 50 mM phosphate buffer (pH 8.0). Following incubation at 4°C for 24 h, three volumes of chilled ethanol was added and levan was allowed to precipitate at 4°C for overnight. The precipitated mixture was then centrifuged at 13,000*g* for 30 min at 4°C, the supernatant was discarded, and levan was dried at 60°C to constant weight.

The polymer was analyzed by thin-layer chromatography (TLC) as described by Park et al. [16]. $^{13}$C nuclear magnetic resonance (NMR) was used to determine the structure and the linkages of the polysaccharide and Fourier-transform infrared (FTIR) analysis was performed for the determination of the functional groups in the polysaccharide.

### Heterologous expression of *B. amyloliquefaciens sacB* in *B. megaterium* MS941

Plasmids were transformed into *B. megaterium* strain as described before [26]. The expression of *B. amyloliquefaciens sacB* in *B. megaterium* MS941 was carried out at 37°C in LB medium containing 2.5 mM $CaCl_2$. *B. megaterium* harboring pMM-sacB or pMM-sacB-His was grown at 37°C at 200 rpm until $A_{600}$ reached 0.4. Expression of *sacB* was induced by adding D-xylose at concentration of 0.5% and then incubated further at 37°C. Aliquots were collected 3, 6, and 9 h after induction. *B. megaterium* clone harboring pMM1525 was used as a control. After respective incubation periods, the cells were harvested by centrifugation at 5,000*g* for 20 mins at 4°C and the supernatant was used for the levansucrase assay.

### Response-surface methodology

RSM was used to optimize the fermentation conditions for extracellular production of levansucrase by the recombinant *B. megaterium* (pMM-SacB-His). A central composite design was used to investigate the effects of four independent variables: xylose concentration ($X_1$), inoculum density to start induction ($A_{600}$) ($X_2$), time of incubation ($X_3$), and temperature ($X_4$) on the levansucrase activity ($Y$).

**Table 1** Levels of the factors used in the experimental design

| Independent variables | Symbol code | Coded levels | | | | |
|---|---|---|---|---|---|---|
| | | −2 | −1 | 0 | 1 | 2 |
| Xylose concentration (%) | $X_1$ | 0.25 | 0.5 | 0.75 | 1.0 | 1.25 |
| Inoculum density ($A_{600}$) | $X_2$ | 0.0 | 0.4 | 0.8 | 1.2 | 1.6 |
| Time (h) | $X_3$ | 3 | 6 | 9 | 12 | 15 |
| Temperature (°C) | $X_4$ | 25 | 28 | 31 | 34 | 37 |

The independent variables were coded at five levels (−2, −1, 0, 1, and 2), and the complete design consisted of 30 experimental points including six replications of the center points (all variables were coded as zero) (Tables 1, 2). The coded values were calculated according to the following equation:

$$\text{Coded value} = \frac{\text{Actual level} - (\text{high level} + \text{low level})/2}{(\text{high level} - \text{low level})/2}. \quad (1)$$

Levansucrase activity ($Y$) was analyzed by using a second-order polynomial equation and the data was fit to the equation by a multiple regression procedure. The model equation for analysis is given below:

$$\begin{aligned} Y = {} & \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{12} X_1 X_2 \\ & + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 \\ & + \beta_{34} X_3 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2, \end{aligned} \quad (2)$$

where $X_1$, $X_2$, $X_3$, and $X_4$ represent the coded level of the independent variable, and $\beta_0$, $\beta_1$ … $\beta_{34}$ represent coefficient estimates, with $\beta_0$ having the role of a scaling constant. Design-Expert (version 7.0.0, 2005; Stat-Ease, Inc., Minneapolis, MN) was used to design the experimental plan and conduct the analysis of the results.

Nucleotide sequence accession number

The nucleotide sequence of the *sacB* gene from *B. amyloliquefaciens* type 1 was deposited in the GenBank database under accession no. EU668142.

## Results and discussion

Cloning and sequence analysis of *sacB* from *Bacillus amyloliquefaciens* type 1

Levan obtained from *B. amyloliquefaciens* type 1 has the unique property of being an efficient immunostimulant for common carp, giving 100% survival against *Aeromonas hydrophila* when used at concentration of 0.5% in fish feed. Obtaining higher yield of levan in the purified form using the wild-type strain is not cost-effective; hence gene cloning and characterization of *B. amyloliquefaciens* type 1 levansucrase and heterologous production of the protein using a safe host such as *B. megaterium* is important for the production of higher yield of highly purified levan.

**Table 2** Central composite design and levansucrase activity obtained from the fermentation trails

| Run | $X_1$ | $X_2$ | $X_3$ | $X_4$ | Response [levansucrase activity (U/l)] |
|---|---|---|---|---|---|
| 1 | −1.00 | −1.00 | −1.00 | −1.00 | 9615.38 |
| 2 | 1.00 | −1.00 | −1.00 | −1.00 | 9615.38 |
| 3 | −1.00 | 1.00 | −1.00 | −1.00 | 8903.13 |
| 4 | 1.00 | 1.00 | −1.00 | −1.00 | 13746.40 |
| 5 | −1.00 | −1.00 | 1.00 | −1.00 | 22151.00 |
| 6 | 1.00 | −1.00 | 1.00 | −1.00 | 16453.00 |
| 7 | −1.00 | 1.00 | 1.00 | −1.00 | 20156.70 |
| 8 | 1.00 | 1.00 | 1.00 | −1.00 | 18304.80 |
| 9 | −1.00 | −1.00 | −1.00 | 1.00 | 7051.28 |
| 10 | 1.00 | −1.00 | −1.00 | 1.00 | 7478.63 |
| 11 | −1.00 | 1.00 | −1.00 | 1.00 | 7621.08 |
| 12 | 1.00 | 1.00 | −1.00 | 1.00 | 5911.68 |
| 13 | −1.00 | −1.00 | 1.00 | 1.00 | 8190.88 |
| 14 | 1.00 | −1.00 | 1.00 | 1.00 | 5769.23 |
| 15 | −1.00 | 1.00 | 1.00 | 1.00 | 5626.78 |
| 16 | 1.00 | 1.00 | 1.00 | 1.00 | 5626.78 |
| 17 | −2.00 | 0.00 | 0.00 | 0.00 | 11609.70 |
| 18 | 2.00 | 0.00 | 0.00 | 0.00 | 16880.30 |
| 19 | 0.00 | −2.00 | 0.00 | 0.00 | 12891.70 |
| 20 | 0.00 | 2.00 | 0.00 | 0.00 | 20548.00 |
| 21 | 0.00 | 0.00 | −2.00 | 0.00 | 8903.13 |
| 22 | 0.00 | 0.00 | 2.00 | 0.00 | 12891.70 |
| 23 | 0.00 | 0.00 | 0.00 | −2.00 | 14316.20 |
| 24 | 0.00 | 0.00 | 0.00 | 2.00 | 6054.13 |
| 25 | 0.00 | 0.00 | 0.00 | 0.00 | 15598.30 |
| 26 | 0.00 | 0.00 | 0.00 | 0.00 | 15883.20 |
| 27 | 0.00 | 0.00 | 0.00 | 0.00 | 15740.70 |
| 28 | 0.00 | 0.00 | 0.00 | 0.00 | 16310.50 |
| 29 | 0.00 | 0.00 | 0.00 | 0.00 | 16025.60 |
| 30 | 0.00 | 0.00 | 0.00 | 0.00 | 16168.10 |

The open reading frame (ORF) of *B. amyloliquefaciens sacB* was cloned and nucleotide sequencing determined the complete 1,419-bp ORF of SacB, which coded for 473 amino acids (aa) including 29 aa of the expected signal peptide. BLAST searches of the SacB protein sequence showed 99% identity at the amino acid sequence level with SacB from *B. amyloliquefaciens* FZB 4 [23]. There were two amino acid residues that varied between these sequences, Pro-41 and Lys-156, which were replaced by Gln-41 and Thr-156 in the *B. amyloliquefaciens* FZB42

sequence. The deduced amino acid sequence of the *B. amyloliquefaciens* type 1 *sacB* was compared with those of other bacterial levansucrases. The SacB protein exhibited good identity with levansucrase originated from Gram-positive bacteria originated from *B. subtilis* (80%) and *B. licheniformis* (58%). The SacB sequence, however, showed low amino acid identity to SacB from Gram-negative bacteria such as *Zymomonas mobilis* (12%) and *Pseudomonas syringae* pv. *glycinea* (9%). In the multiple alignments of deduced amino acid sequences, the *B. amyloliquefaciens* type 1 levansucrase also shared several conserved regions of all bacterial levansucrase [20].

## Expression and purification of *B. amyloliquefaciens* SacB in *E. coli*

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) analysis (Fig. 1) of the cytoplasmic fraction of recombinant *E. coli* harboring pET-sacB-NcX or pET-sacB-NdX confirmed prominent expression of the levansucrase protein and showed a band of approximately 49 kDa, which is the expected molecular weight of the *B. amyloliquefaciens* type 1 levansucrase protein. No such band was found in the cytoplasmic fraction of the control cells (pET28a). Levansucrase assays were carried out as described in the “Material and methods” section on total cell lysate and insoluble and cytoplasmic fractions of the cells, and the reaction mixtures were analyzed by thin-layer chromatography (TLC). Figure 2a shows the formation of levan as a dark spot, and released glucose is also clearly visible. The sucrose in the control group remained intact, as no levansucrase gene was present to hydrolyze sucrose and form the polymer. The $His_6$-tagged recombinant levansucrase from the cytoplasmic fraction of *E. coli* harboring pET-sacB-NdX was purified by Ni–NTA affinity column chromatography. The crude enzyme from the cytoplasmic fraction yielded D-glucose production activity of 8.67 μmole glucose released/min mg protein while the D-glucose production activity for Ni–NTA-purified cytoplasmic fraction was 37.32 μmole of glucose released/min mg protein, thus purification resulted in a fourfold increase in D-glucose production activity.

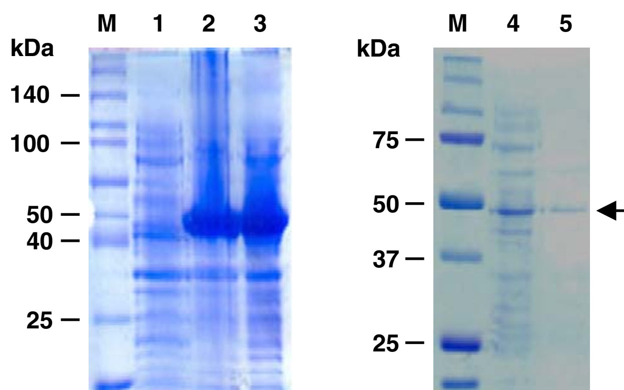


**Fig. 1** SDS–PAGE analysis of the cytoplasmic fraction of *E. coli* clones expressing *B. amyloliquefaciens sacB*. *Lane* 1, *E. coli* harboring pET28a; *lane* 2, *E. coli* harboring pET-sacB-NdX ($His_6$-tagged SacB); *lane* 3, *E. coli* harboring pET-sacB-NcX (untagged SacB); *lane* 4, SacB purified by Ni–NTA affinity chromatography (*arrow*)

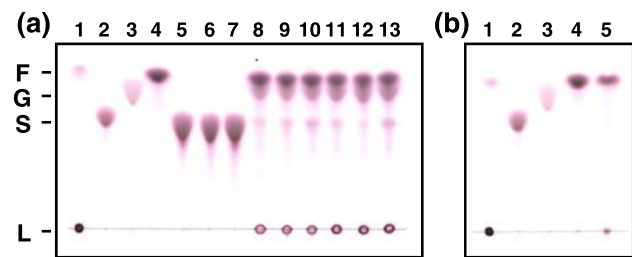


**Fig. 2** (**a**) Standard levan (*lane* 1), sucrose (*lane* 2), glucose (*lane* 3), and fructose (*lane* 4). Thin-layer chromatography (TLC) analysis of the enzyme reaction mixture of total cell lysate (*lanes* 5, 8, and 11) and soluble (*lanes* 6, 9, and 12) and insoluble (*lanes* 7, 10, and 13) fractions of *E. coli* clones harboring pET28a (control), pET-sacB-NdX ($His_6$-tagged SacB), and pET-sacB-NcX (untagged SacB), respectively. (**b**) Standard levan (*lane* 1), sucrose (*lane* 2), glucose (*lane* 3), and fructose (*lane* 4). The acid hydrolysis product of the polymer (*lane* 5). L, levan; S, sucrose; G, glucose; F, fructose

## Characterization of *B. amyloliquefaciens* SacB produced in *E. coli*

The effects of initial pH, temperature, substrate concentration, and time were investigated to determine the optimum conditions. As shown in Fig. 3a, the optimum pH for D-glucose production and levan formation was 6.0 and 8.0, respectively. The pH activity profile indicates that the catalysis involves two ionic groups with $pK_a$ values of 4.5–5.0 and 7.0–7.5. The lower $pK_a$ is ascribed to acidic residues based on its $pK_a$ values of the solution. The acidic residue (aspartate or glutamate) in the active site has been shown to be responsible for catalysis in most glycosyl hydrolases [6]. This residue is conserved in the fructofuranosidase family including fructosyltransferases, invertases, levanases, inulinases, and sucrose-6-phosphatase. The optimum pH (8.0) for levan formation was slightly higher than those reported for *B. subtilis* (pH 6.0) [5] and *Z. mobilis* (pH 4.0–6.0) [1]. The optimum temperatures for D-glucose production and levan formation were 30°C and 4°C, respectively (Fig. 3b). A vast difference in the optimum temperatures for D-glucose production and levan formation was found. Such variation of the optimum temperatures for these two activities has been reported for levansucrase from *R. aquatilis* [12] and *Z. mobilis* [21].

The effect of substrate concentration on D-glucose production activity (at pH 6.0 and 30°C) and levan formation (at pH 8.0 and 4°C) was investigated. As shown in Fig. 3c,

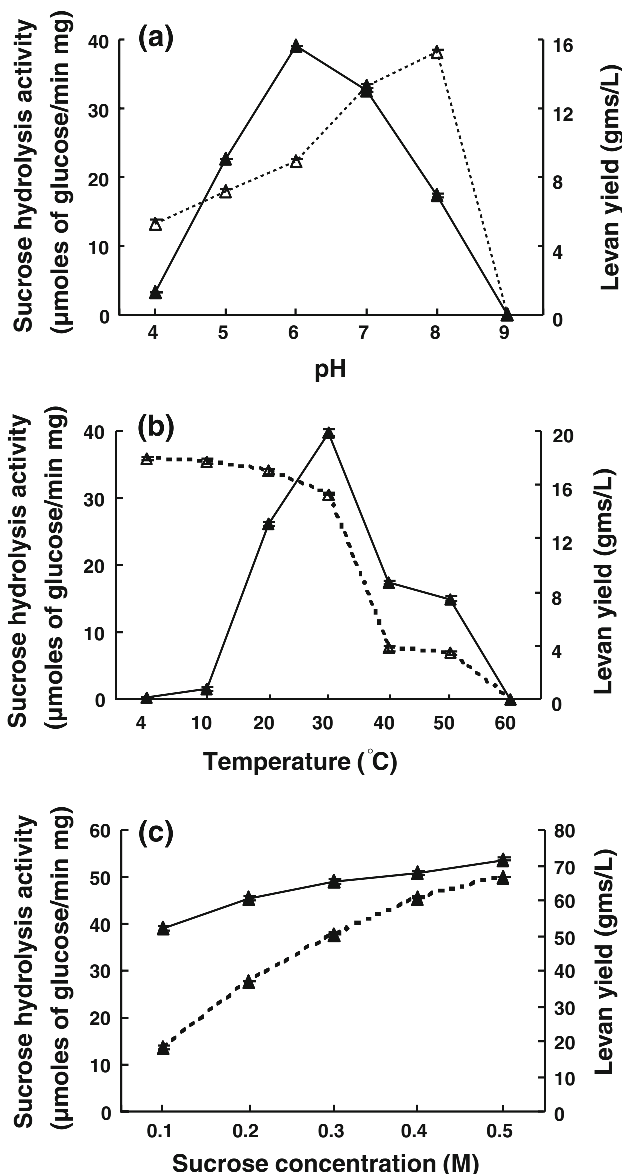


**Fig. 3** Effect of pH (**a**), temperature (**b**), and substrate concentration (**c**) on D-glucose production activity and levan formation. The effect of substrate concentration on D-glucose production activity and levan formation were determined at conditions of pH 6.0 and 30°C and pH 8.0 and 4°C, respectively. Sold line indicates D-glucose production; dashed line indicates levan yield

the D-glucose production activity was found to increase with increasing concentration of sucrose, and even levan formation was found to increase with increasing concentration of sucrose. The $K_m$ of levansucrase was 47.81 mM sucrose and the $V_{max}$ was 57.47 µmole/min mg protein. The $K_m$ (47.81 mM) of this enzyme was similar to the $K_m$ of levansucrase from *R. aquatilis* (50 mM) [15]. However, levansucrases from *Pseudomonas syringae* [9], *L. mesenteroides* B-512 FMC [11], and *B. megaterium* [10] were reported to have varying $K_m$ values of 122, 26.6, and 6.6 mM respectively.

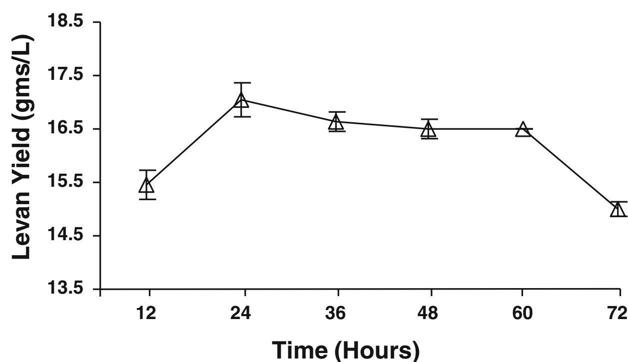


**Fig. 4** Effect of reaction time on levan formation (at pH 8.0, 4°C, 100 mM sucrose)

The effect of enzyme reaction time on levan formation was studied at pH 8.0 and 4°C using 100 mM sucrose. As shown in Fig. 4, a maximum amount of levan was synthesized in 24 h; prolonged incubation led to a decrease in levan yield due to the hydrolysis activity possessed by levansucrase. After saturation of the levan formation reaction, the formed levan was reversibly hydrolyzed. Similar results were obtained for levansucrase from *R. aquatilis* wherein prolonged incubation reduced the levan yield, consequently increasing the amount of oligosaccharides and free fructose in the reaction mixture [22].

## Analysis of the levan produced by *B. amyloliquefaciens* SacB

The monomer component of the levan produced by the recombinant $His_6$-tagged levansucrase was identified by TLC of the acid hydrolysate as shown in Fig. 2b. Fructose was detected as the sole sugar component of the polymer, indicating that it was a fructose polymer. $^{13}$C NMR spectra for the polysaccharide obtained from the recombinant levansucrase of *B. amyloliquefaciens* type 1 was determined (data not shown). The chemical shifts were similar to the chemical shifts observed for levan obtained from *Bacillus polymyxa* (chemical shifts: C1 to C6—60.7, 104.2, 77.0, 75.7, 80.5, 63.6 ppm) [8], which characterizes the fructan as levan. The FTIR spectra of levan showed an overlapping broad band with maximum at ~1,030 1/cm and stronger absorption at ~940 1/cm, which is in agreement with the IR spectra for levan as described by Grube et al. [7].

## Expression of *B. amyloliquefaciens* SacB in *B. megaterium*

Expression of levansucrase in *B. megaterium* was carried out considering the commercial importance of levansucrase and the generally recognized as safe (GRAS) status of *B. megaterium*. The Gram-positive bacterium *B. megaterium* MS941 was employed for expression of levansucrase

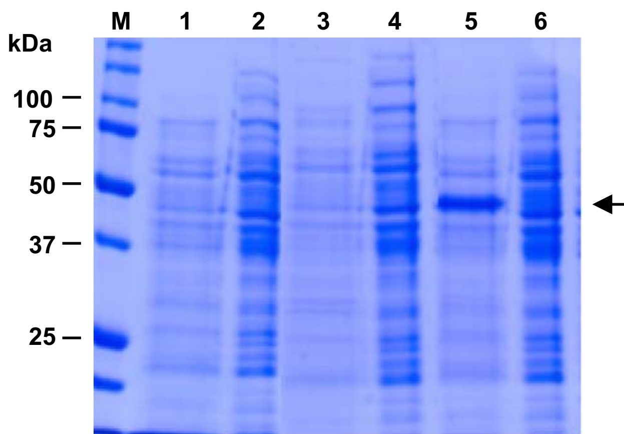


**Fig. 5** SDS–PAGE analysis of the cell free growth medium (*lanes* 1, 3, and 5; pMM, pMM-sacB, and pMM-sacB-His, respectively) and total cell fraction (*lanes* 2, 4, and 6; pMM, pMM-sacB, and pMM-sacB-His, respectively) of *B. megaterium* clones expressing *B. amyloliquefaciens sacB*

from *B. amyloliquefaciens* type 1. Expression was induced with 0.5% (w/v) xylose at $A_{600} = 0.4$. The protein from the cell free growth medium was concentrated by precipitation with ammonium sulfate at 70% saturation. The concentrated protein was used for SDS–PAGE analysis, which revealed prominent expression of His$_6$-tagged levansucrase at molecular weight of 49 kDa (Fig. 5); no protein could be viewed in SDS–PAGE for the cell free growth medium of the untagged levansucrase and in the control group containing no target protein. This was contradictory to the results obtained by Biedendieck et al. [3] wherein slightly less secretion of the protein fused with different tags was observed as compared with the untagged protein. However, the enhanced secretion of His$_6$-tagged protein could be an advantage for purification via the C-terminal His$_6$ tag, which would lead to the purification of intact levansucrase. As shown in Table 3 the recombinant protein with His$_6$ tag obtained 3 h after induction showed the maximum activity of 12,906 U/l, which was around 21-fold higher than the activity obtained by the untagged protein. The total activity obtained for the recombinant levansucrase from *B. amyloliquefaciens* from the cell free growth media was 30-fold higher than the total activity obtained for the recombinant levansucrase from *Lactobacillus reuterii* in *B. megaterium* [3]. Recombinant His$_6$-tagged levansucrase was secreted up to 3 h after induction, showing maximum enzyme activity of 12,906 U/l, and further incubation up to 6 and 9 h led to degraded levansucrase showing 10- and 31-fold lower enzyme activity (1,210 and 410 U/l, respectively). This was in contradiction to the earlier report, where a minimum of incubation time of 12 and 22 h was found to be optimum for expression of cobaltochelatase gene in *B. megaterium* [13]. The decline in activity beyond 3 h incubation could be due to nutritional limitations leading to decline in growth of the recombinant strain, thus responsible for the decline in levansucrase activity.

In comparison with the total enzyme activity of the protein from the cell free growth medium of the wild-type strain *B. amyloliquefaciens* type 1 and of the purified protein in *E. coli*, the total activity for the unconcentrated protein from the cell free growth medium for recombinant protein from *B. megaterium* was 40- and 47-fold higher, respectively. Further optimization of levansucrase activity was carried out through RSM, considering temperature as one of the parameters not yet considered while carrying out the optimization of protein production in *B. megaterium*.

## Optimization of fermentation conditions for levansucrase production in *B. megaterium*

Various combinations of genetic and process strategies such as the selection of the efficient signal peptide, media optimization, fermentation condition optimization, etc. have been studied to improve the production and secretion of recombinant proteins in *B. megaterium* [17, 27]. RSM has also been used as a stratagem by Radha and Ganasekaran

**Table 3** Levansucrase activity in recombinant *B. megaterium* strains and *B. amyloliquefaciens* type 1

| Strain | Total cell lysate | | | Culture supernatant | | |
|---|---|---|---|---|---|---|
| | Protein (mg/l) | Specific activity (U/mg) | Total activity (U/l) | Protein (mg/l) | Specific activity (U/mg) | Total activity (U/l) |
| *B. amyloliquefaciens* type 1 | 783.0 | 0.16 | 125.3 | 30.0 | 10.80 | 325.2 |
| *B. megaterium*/pMM1525 | 429.0 | 0.04 | 15.0 | 20.0 | 25.71 | 514.2 |
| *B. megaterium*/pMM-SacB | 397.0 | 0.18 | 69.5 | 16.0 | 38.69 | 619.0 |
| *B. megaterium*/pMM-SacB-His | 464.5 | 1.33 | 617.0 | 28.0 | 461.0 | 12,906.0 |
| *B. megaterium*/pMM-SacB-His (RSM optimized condition) | 430.3 | 1.29 | 555.0 | 30.2 | 669.6 | 20,251.0 |

Comparison of the total levansucrase activity from the cell free growth medium and total cell lysate of *B. megaterium* containing pMM1525 (no target gene), pMM-SacB (untagged protein), pMM-SacB-His (His$_6$-tagged protein), and the wild-type strain *B. amyloliquefaciens* type 1 incubated for 3 h after induction for the recombinant *B. megaterium* strains and 6 h of total incubation for *B. amyloliquefaciens* type 1

[17] for improved production of keratinase by recombinant *B. megaterium*, obtaining threefold increases in keratinase production over the wild-type strain. Media components, signal peptide, and effect of affinity tags for levansucrase production by recombinant *B. megaterium* were optimized, which resulted in maximum levansucrase activity of 417 U/l [21].

Inducer concentration (xylose), inoculum density to start induction, period of incubation, and temperature had a significant effect on levansucrase activity. Hence, to examine the combined effect of these independent variables on levansucrase production, a central composite factorial design (CCD) was performed with fixed middle points of 0.75% xylose (w/v), $A_{600}$ 0.8 inoculum density to start induction, 9 h incubation period, and temperature of 31°C. The experimental range and the levels of variables used in the design are presented in Table 1. The levels of these factors used in the optimization studies by RSM were determined by preliminary experiments. The effect of the four variables, each at five levels, and their interactions on levansucrase activity were determined by carrying out 30 experiments given by the model (Table 2). The mathematical model, which represents a second-order polynomial, is given by Eq. 3, where the variables are expressed as their coded values.

$$\begin{aligned} Y = {} & 15954.42 + 172.13X_1 + 623.22X_2 + 1679.63X_3 \\ & - 3424.74X_4 + 560.90X_1X_2 - 845.80X_1X_3 \\ & - 62.32X_1X_4 - 329.42X_2X_3 - 436.25X_2X_4 \\ & - 2377.14X_3X_4 - 802.77X_{12} - 179.55X_{22} \\ & - 1639.66X_{32} - 1817.72X_{42}. \end{aligned} \quad (3)$$

$Y$ represents the response, which is a dependent variable, levansucrase activity (U/l), and $X_1$, $X_2$, $X_3$, and $X_4$ represent coded values of independent variables. Statistical significance of the second-order model equation was checked by analysis of variance (ANOVA) (Table 4). The fit of the model was also expressed as the coefficient of determination, $R^2$, which was found to be 0.8537 (the closer the $R^2$ value is to 1, the better the model fits the experimental data), indicating that 85.37% of the variability in the response can be explained by the model. The coefficient of variation (CV) indicates the degree of precision with which the treatments are compared. The lower the value of the CV (21.48%), the greater the reliability of the experiments. The model $F$-value of 6.25 implied that the model is significant, wherein there is only a 0.05% chance that a model $F$-value this large could occur due to noise. The lack-of-fit $F$-value of 149.40 implies that the lack of fit is significant, wherein there is only a 0.01% chance that a lack-of-fit $F$-value this large could occur due to noise.

**Table 4** Analysis of variance for the quadratic model

| Source | SS | DF | MS | $F$-value | $P > F$ |
|---|---|---|---|---|---|
| Model | 6.213E + 008 | 14 | 4.438E + 007 | 6.25 | 0.0005 |
| Residual | 1.065E + 008 | 15 | 7.098E + 006 | | |
| Lack of fit | 1.061E + 008 | 10 | 1.061E + 007 | 149.40 | <0.0001 |
| Pure error | 3.551E + 005 | 5 | 71022.15 | | |
| Total | 7.278E + 008 | 29 | | | |

$R^2 = 0.8537$; $R^2(\text{adj}) = 0.7172$; CV = 21.48%

*SS* sum of squares, *DF* degree of freedom, *MS* mean of squares

**Table 5** The least-square fit and parameter estimates (significance of regression coefficient)

| Factor | Coefficient | Standard error | $F$-value | $P$-value |
|---|---|---|---|---|
| Intercept | 15954.42 | 1087.63 | | |
| $X_1$ | 172.13 | 543.81 | 0.10 | 0.7560 |
| $X_2$ | 623.22 | 543.81 | 1.31 | 0.2697 |
| $X_3$ | 1679.73 | 543.81 | 9.54 | 0.0075 |
| $X_4$ | −3423.74 | 543.81 | 39.66 | <0.0001 |
| $X_1X_2$ | 560.90 | 666.03 | 0.71 | 0.4129 |
| $X_1X_3$ | −845.80 | 666.03 | 1.61 | 0.2235 |
| $X_1X_4$ | −62.32 | 666.03 | 8.765E-003 | 0.9267 |
| $X_2X_3$ | −329.42 | 666.03 | 0.24 | 0.6281 |
| $X_2X_4$ | −436.25 | 666.03 | 0.43 | 0.5224 |
| $X_3X_4$ | −2377.14 | 666.03 | 12.74 | 0.0028 |
| $X_1^2$ | −802.77 | 508.69 | 2.49 | 0.1354 |
| $X_2^2$ | −179.55 | 508.69 | 0.12 | 0.7290 |
| $X_3^2$ | −1639.66 | 508.69 | 10.39 | 0.0057 |
| $X_4^2$ | −1817.72 | 508.69 | 12.77 | 0.0028 |

The $P$ value serves as a tool for checking the significance of each of each of the coefficients. Values of "$Prob > F$" less than 0.05 indicate that the model terms are significant. In this case, the responses taken from Table 5 reveal that the quadratic coefficients of $X_3$, $X_4$, $X_3X_4$, $X_3^2$, and $X_4^2$ have remarkable effect on the level of levansucrase activity. Values greater than 0.1000 indicate the model terms are not significant. "Adeq precision" measures the signal-to-noise ratio. A ratio greater than 4 is desirable; the ratio of 9.525 indicates an adequate signal.

The three-dimensional response surface and the two-dimensional contour plots are graphical representations for the regression equation. Figure 6 shows the response-surface curves and also contour plots for variations in levansucrase activity as a function of two variables with the order variable maintained at its respective zero level. By using the response-surface plot, the interaction between two variables and their optimum levels can be easily understood and located. Plots showing the interaction between xylose and inoculum density, xylose and incubation time, xylose and temperature, inoculum density and incubation time, inoculum density and temperature, and time and temperature are depicted in Fig. 6.

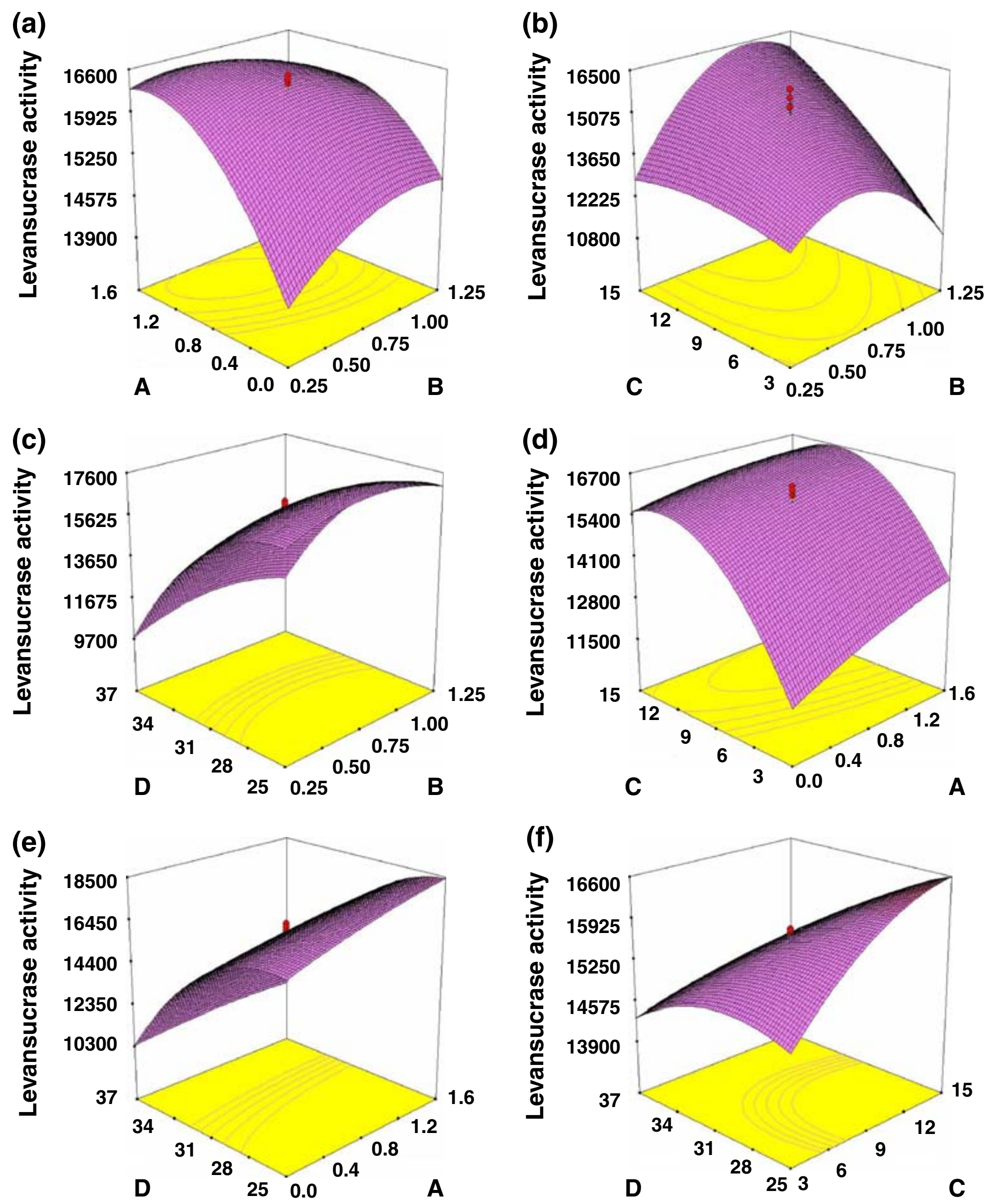


**Fig. 6** Response-surface plot showing the effect of each independent variable and also their mutual effect on the levansucrase activity (**a**–**f**). A, inoculum density ($A_{600}$); B, xylose concentration (%); C, induction time (h); D, temperature (°C); levansucrase activity (U/l)

The results predicted maximum levansucrase activity of 20,528 U/l under the experimental conditions of 0.735% (w/v) xylose concentration, inoculum density to start induction of 1.2 optical density (OD), incubation time of 12 h, and temperature of 28°C. An experiment was conducted to validate the model under optimum condition in 5-l stirred vessel fermenter (Kobiotech Co., Ltd., Korea) containing 2 l fermentation medium. The maximum levansucrase activity obtained experimentally under the optimal conditions was found to be 20,251 U/l, which was in close agreement with the model prediction.

Our study on the optimization of fermentation condition for levansucrase production by recombinant strain suggested that the model accurately represents the data in the experimental design. After optimization with RSM, *B. megaterium* (pMM-SacB-His) showed 1.6-fold increased levansucrase activity under optimal conditions (20,251 U/l) [xylose 0.735%, $A_{600}$ to start induction of 0.8, period of incubation after induction of 9 h, and temperature of 28°C] in comparison with the unoptimized conditions (12,906 U/l) [xylose 0.5%, $A_{600}$ to start induction of 0.4, period of incubation after induction of 3 h, and temperature of 37°C]. In comparison with the wild-type strain *B. amyloliquefaciens* type 1, there was a 62-fold increase in levansucrase activity. Hence, temperature plays a very significant role in improved secretion of levansucrase secretion in the *B. megaterium* expression system. Considering this as one of the important factors, the secretion of various proteins can be improved when using the *B. megaterium* expression system.

## Conclusions

In the present study we obtained successful expression of levansucrase in *E.coli* and *B. megaterium* using T7 promoter and xylose inducible promoter, respectively. Using RSM, it was possible to model the individual and interactive effects of media components on production of levansucrase. The validity of the model was confirmed by close agreement between experimental and predicted values. Medium optimization by RSM effectively enhanced levansucrase production by 62-fold in reference to the wild-type strain. The optimum conditions for the improved level of levansucrase production are as follows: 0.735% xylose, $A_{600}$ of 1.2, 12 h of incubation, and temperature of 28°C. The design proved to be crucial for achieving a remarkable increase in levansucrase activity.

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