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Secretory Production of *Rahnella aquatilis* ATCC 33071 Levansucrase Expressed in *Escherichia coli*

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Abstract To investigate the production and characteristics of thermostable levansucrase from *Rahnella aquatilis* ATCC 33071, the levansucrase gene from *R. aquatilis* was cloned and expressed in *Escherichia coli* without induction system. Expression of levansucrase gene in *E. coli* had no notable or detrimental effect on the growth of host strain, and the recombinant levansucrase exhibited levan synthesis activity. Levansucrase was secreted to the periplasm in *E. coli*, and addition of 0.5% glycine yielded further secretion of levansucrase to the growth medium and resulted in an increase of total levansucrase activity. Furthermore, the cellular levansucrase was evaluated for the production of levan by using toluene-permeabilized whole-cells. The levansucrase was thermostable at 37°C. The molecular size of levan was 1×10^6 Da, as determined by HPLC, and the degree of polymerization of levan varied with incubation temperatures: Low incubation temperature was preferable for the production of high-molecular size levan. The present study demonstrated that the mass production of levan and levan oligosaccharides can be achieved by glycine supplementation to the growth medium or by toluene-permeabilized whole-cells.

Key words: Levansucrase, functional food, *Rahnella aquatilis*, levan, secretion

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Fructan, inulin, and levan are homopolysaccharides composed of D-fructofuranosyl residues joined by β-(2,6) and β-(2,1) linkages. Chemically, levan consists of β-D-fructofuranosyl residues linked predominantly through β-(2,6) as 6-kestose of the basic trisaccharide, with extensive branching through β-(2,1) linkages. In contrast, inulin is composed of β-D-fructofuranose attached by β-(2,1) linkages [8]. Application of levan as an emulsifier, formulation aid, stabilizer, thickener, surface-finishing agent, encapsulating agent, and carrier of flavors and fragrances have been suggested [25]. Levans produced by *Aerobacter levanicum* [18] and *Zymomonas mobilis* [3] have been reported to have antitumor and immunomodulatory activities. These authors also suggested that the antitumor activities of levan depend on the molecular weight of polysaccharide, and that levan with a specific range of molecular weight is effective for such activity.

Although levan is found in plants and in byproducts of microorganisms, only a few are available in sufficient quantities to be useful for industry [8, 9, 21]. Therefore, it appears to be advantageous to produce levan from microbes. Microbial levans are produced by levansucrase (sucrose 6-fructosyltransferase, EC 2.4.1.10) from a wide range of taxa such as bacteria, yeasts, and fungi [8, 19]. Amongst them, levansucrase of *Z. mobilis* has attracted special attention, because it has the capacity of producing levan at very low temperature; 4°C [20, 31]. In contrast, the optimal temperature for levansucrase of *Rahnella aquatilis* is relatively high; 30–40°C [21, 25]. Recently, the enzymatic process for the efficient production of levan has been developed by using levansucrase originated from *Z. mobilis* and expressed in *E. coli* [31]. However, one of the problems with the above system was the instability of the enzyme activity during extended fermentation at 37°C, due to formation of insoluble inclusion body [31]. In contrast, our preliminary investigation showed that recombinant *R. aquatilis* levansucrase produced in *E. coli* was stable in the extended fermentation.

Although *E. coli* is widely used as hosts for recombinant protein production, unfortunately, with an exception of a few classes of proteins such as toxins [1] and hemolysins [6], *E. coli* does not normally secrete the foreign proteins extracellularly. Traditional methods to recover the recombinant intracellular proteins expressed in *E. coli* require cell disruption by either mechanical, physical, or chemical means. The main drawbacks of these methods are insufficient product release and potential degradation of enzymes, due to shear, heat, or chemical-mediated inactivation. Considering the tedious enzyme purification procedures [23], secretory enzyme production is an advantageous way for the industrial application of enzyme. Fortunately, levansucrase can easily be recovered from the culture fluids, where the number of cellular products are less than cells.

In the present study, two approaches were taken to explore the mass production systems for levan and levan oligosaccharides; glycine supplementation to the growth media for the secretory production of *R. aquatilis* levansucrase expressed in *E. coli* into culture fluids, and preparation of permeabilized *E. coli* cells by toluene treatment, followed by washing step [30].

MATERIALS AND METHODS

Materials

Restriction enzymes, calf intestinal alkaline phosphatase, Klenow fragment, T4 polynucleotide kinase, T4 DNA ligase, and dNTP mix were from Boehringer Mannheim or Takara. Unless otherwise specified, chemicals were purchased from Sigma.

Bacterial Strains, Plasmids, and Growth Conditions

E. coli DH5α [*supE*44 Δ*lac*U169(p80*lacZ*ΔM15) *hsdR*17 *recA*1 *endA*1 *gyrA*96 *thi*-1 *relA*1] was employed as a host strain for the cloning and expression of levansucrase gene (*lsr*A) of *R. aquatilis* ATCC 33071 (GenBank Accession No. U91484). The plasmid used was pRL1CP, a pBluescript II KS(+) (Stratagene, U.S.A.) derivative carrying the promoter region and structural gene of levansucrase from *R. aquatilis* ATCC 33071 [28]. *R. aquatilis* ATCC 33071 and *E. coli* were grown aerobically in Luria-Bertani medium [LB; 1% (w/v) Bacto-trypton, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl] at 30°C. When necessary, ampicillin was added to a final concentration of 100 mg/ml. To test the effect of glycine on the growth rate and the enzyme activities, cells were inoculated into 100 ml of LB in 250-ml culture flasks supplemented with glycine (0–2%, w/v), with an initial optical density of 0.1–0.2 at 600 nm. Chromosomal DNA isolation, restriction enzyme digestion, agarose gel electrophoresis, ligation, transformation, restriction endonuclease mapping, and PCR were all performed as described elsewhere [11, 27, 28].

Cell Fractionation

The cell fractionation method was described by Osborn *et al*. [22] and modified in the present work as follows. After cultivation of *E. coli* cells for 10 h in 100 ml of LB medium, cells were harvested, washed with 0.05 M sodium phosphate buffer (pH 6.0) (buffer A), and then centrifuged. The supernatant was combined and used as a extracellular fraction. The washed cells were resuspended in 10 ml of 0.05 M Tris·Cl (pH 8.0) containing 25% sucrose and 200 mg of lysozyme, and the suspension was incubated at 25°C for 30 min and centrifuged. The supernatant was used as a periplasmic-located enzyme source. The remaining cell precipitate was resuspended in 10 ml of buffer A and disrupted by ultrasonication with a SONIFER (Branson, U.S.A.) at 4°C for 3 min, which was used as a cytoplasmic-located enzyme source. The remaining precipitate was suspended in 10 ml of buffer A and used as the source of mixture of outmembrane- and cytoplasmic membrane-bound enzyme.

Reproducibility of analyses of levansucrase localization in *E. coli* cells was verified as follows. Two different *E. coli* cells, *E. coli* DH5a/pBluescript II KS(+) and *E. coli* DH5α/pRL1CP, were used. Each experiment was performed in replicates, and several independent experiments were performed for each set of conditions. Results for each type of experiment were consistent between replicates, but some quantitative variations between separate analyses were observed. The trends of relative proportions of enzyme activities and protein concentrations for any particular strain or set of experimental conditions were identical in every analysis; hence, mean of percentage composition between experiments is shown in Results and Discussion, and results are expressed as mean±SE.

Preparation of Toluene-Permeabilized Whole-Cells

For preparation of toluene-permeabilized whole-cells, 100 ml of cultures were harvested and resuspended in 5 ml of 50 mM phosphate buffer (pH 7.0). Permeabilized whole-cell preparations were used to measure NADH oxidase (a cytoplasmic-membrane protein in *E. coli*) and β-lactamase (a periplasmic protein in *E. coli*) activities in the supernatant after centrifugation. In order to prepare the permeabilized whole-cells, toluene was added to the whole-cells at 1:10 (v/v) and vortexed for 5 min at room temperature. The mixture was centrifuged at 5,000 ×*g* and 4°C for 1 min and the permeabilized whole-cells was collected. After washing the cells with buffer A, the washed cells were used as the source of toluene-permeabilized whole-cells.

Enzyme Assay

One unit of levansucrase activity was defined as the amount of enzyme to release one micromole of glucose per minute. Levansucrase activity in the toluene-permeabilized whole-cells was calculated by dividing the enzyme activity in the intact whole-cells by the total enzyme activity obtained in the cells disrupted by ultrasonication. Assay for NADH oxidase was performed as described by Osborn *et al.* [22]: The reaction mixture (1.0 ml) contained 0.05 M Tris·Cl (pH 7.5), 0.28 mM NADH, 0.2 mM dithiothreitol, and the enzyme source, and the rate of absorbance decrease at 340 nm was measured at 25°C. β-Lactamase activity (a periplasmic protein in *E. coli*) was determined by measuring the rate of degrading penicillin-G per minute at 37°C, as described by Chalmers *et al.* [4].

HPLC Analysis

Quantitative determination of glucose, fructose, oligosaccharides, and levan was conducted by HPLC equipped with a refractive index detector and gel filtration column (Shodex Ionpack KS-802, 300×8 mm, Japan). Deionized water was used as a mobile phase at 0.4 ml/min. The degree of polymerization of levan was also determined by HPLC equipped with two successive columns at 30°C [GPC 4,000-GPC 1,000 (Polymer Laboratories, U.S.A.)] and a refractive index detector. Deionized water was used as a mobile phase at 0.4 ml/min. Polyethylene oxide (8.0×10^6), dextran standards (1.8×10^6, 7.5×10^5, 1.7×10^5, 4.0×10^4) and sucrose were used as standard compounds.

Analytical Methods

During batch cultivation, cell growth was monitored by measuring optical density at 600 nm. Protein samples were analyzed by electrophoresis on SDS-PAGE gels, containing 10% polyacrylamide, as described by Laemmli [17]. The gels were stained with Coomassie brilliant blue R-250 (Bio-Rad). The amount of protein was determined with a protein assay kit (Bio-Rad, U.S.A.) by using bovine serum albumin as the standard [2].

RESULTS AND DISCUSSION

Expression of Levansucrase Gene from *R. aquatilis* in *E. coli*

Expression of the *B. subtilis* levansucrase gene (*sac*B) in *E. coli* confers a lethal effect on the host in the presence

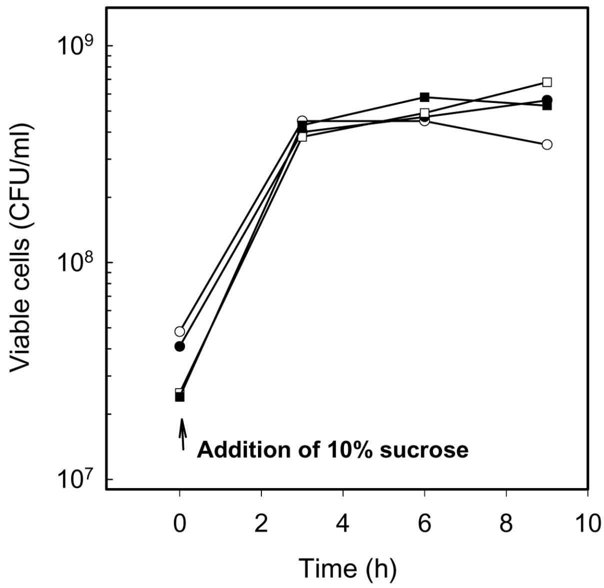

Fig. 1. Effect of *lsr*A expression in *E. coli* on the viability of cells. Ten % (w/v) sucrose was added to the cultures at exponential phase of growth (indicated by arrow sign).
Symbols: circle, *E. coli*/pBluescript II KS(+); square, *E. coli*/pRL1CP; open symbols, no sucrose addition; closed symbols, sucrose addition.

of 10% sucrose [24]. Therefore, we became interested in whether the expression of *lsr*A gene originated from a Gram-negative bacterium *R. aquatilis* is also toxic to the *E. coli*. However, as shown in Fig. 1, the viability of *E. coli*/pRL1CP was not affected by the expression of the *lsr*A gene, indicating that the expression of the *lsr*A gene was not toxic to the *E. coli*.

Localization of Levansucrase Expressed in *E. coli*

The localization of levansucrase in wild strain and whole-cells of *E. coli*/pRL1CP was examined. First of all, NADH oxidase activity was not detected in the culture fluids, indicating that cells were not lysed under the cell growth and analytical conditions used, and 35% of the total activities were found in the whole-cells of *E. coli*/pRL1CP. To investigate subcellular localization of the levansucrase, *E. coli*/pRL1CP was first subfractionated into extracellular, periplasmic, and a mixture of outmembrane- and cytoplasmic membrane fractions, and marker enzymes which are known to be specific for each of the subcellular fractions were assayed along with levansucrase (Table 1). *E. coli* DH5α/pBluescript II KS(+) was used as control. Levansucrase activity was not detected in *E. coli* DH5α/pBluescript II KS(+). More than 95% of the levansucrase activity were found in the intracellular fractions of *E. coli*/pRL1CP; the levansucrase activities in the periplasmic and cytoplasmic fractions were 47.7±3.7 and 48.6±6.6%, respectively (Table 1). However, although no signal sequence was found in the deduced amino acid sequence of *R. aquatilis*

Table 1. Localization of three enzymes expressed in *E. coli* DH5α/pRL1CP and *E. coli* DH5α/pBluescript II KS(+)[a].

	% Protein concentration	% Levansucrase activity	% β-Lactamase activity	% NADH oxidase activity
E. coli DH5α/pRL1CP				
CB	7.1±0.8	2.0±1.4	19.8±0.8	0
Periplasm	32.2±2.1	47.7±3.7	65.6±3.5	0.6±0.3
Cytoplasm	50.6±2.7	48.6±6.6	11.0±10.2	27.0±14.5
CM/OM	9.0±2.4	6.9±6.6	2.9±1.1	72.5±14.7
E. coli DH5α/pBluescript II KS(+)				
CB	8.6±1.9	0	9.1±4.0	0
Periplasm	9.9±0.5	0	90.1±3.1	0
Cytoplasm	72.9±0.5	0	0.3±0.3	7.9±2.6
CM/OM	9.0±1.2	0	0.3±0.3	92.1±2.6

[a]Numbers given in Table represent relative percentage values of each sample and are mean±SE.
Abbreviations: CB, culture broth; CM/OM, mixture of outmembrane- and cytoplasmic membrane-bound fractions.

levansucrase [28], the localization experiment indicated that a certain amount of levansucrase expressed in *E. coli* might be associated with the outer membrane of cells or be located in the periplasmic space. This is in support of an earlier observation [14]: The volume of periplasmic space in *E. coli* was estimated to be 20–40% of the cell volume [14].

SDS-PAGE profiles of proteins from the periplasmic space and cytoplasm fractions of *E. coli*/pRL1CP showed an identical band, corresponding to levansucrase (Fig. 2). This indicates that the secretion of levansucrase into the periplasmic space does not require the signal peptide, as reported in a number of proteins; hemolysin of *E. coli*, proteases of *Erwinia chrysanthemi*, and alkaline protease of *Pseudomonas aeruginosa* [26]. It is of interest to note that Kim *et al.* [15] recently reported that the levansucrase gene of *R. aquatilis* ATCC 15552 was expressed in *E. coli* BL21(DE3) by the IPTG induction, and that the protein was found in the cytoplasm (93.5%) and periplasm (6.2%). Previously, we reported that the levansucrase gene (*lsrA*) was expressed well in *E. coli*/pRL1CP from its natural promoter upstream of the gene [29]. Expression of the *lsrA* gene was tightly regulated by the growth phase of the host cell; low-level expression was observed in the early phase of cell growth, but the expression was stimulated in the late phase. Therefore, we assume that the expression vector system might be important for the localization of *R. aquatilis* levansucrase in *E. coli*. In preliminary experiments, serial deletion mutants of the C-terminal domain of the *Z. mobilis* levansucrase gene were constructed, and the results showed that the C-terminal region modulated the initial velocity of levan synthesis, but localization of *Z. mobilis* levansucrase in *E. coli* DH5α was unaffected by the deletions. The C-terminal domain seemed to play no role in the secretion of levansucrase. Similar results were also observed with N-terminal deletion mutants. We are currently further pursuing the signal-independent secretion motif in bacterial levansucrase to sufficiently understand the nature of the genetic composition of levansucrase.

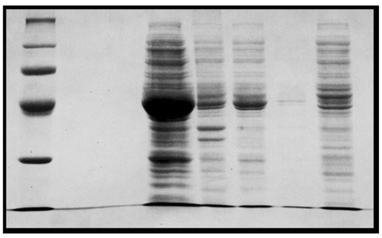

Fig. 2. SDS-PAGE analysis of levansucrase produced in *E. coli*/pRL1CP. Protein standards are indicated on the left.
Lane 2, extracellular culture fluids; lane 3, extracellular culture fluids after washing with buffer A and following centrifugation; lane 4, total cell fraction from *E. coli*/pRL1CP; lane 5, periplasm fraction; lane 6, cytoplasm fraction; lane 7, cytoplasmic membrane-bound fraction; lane 8, total cell fraction from *E. coli*/pBluescript II KS(+).

Extracellular Discharge of Levansucrase

Glycine supplement to the growth medium is a simple method to secrete the intracellular proteins into the culture fluids [10, 12]. This technique is based on the hypothesis that glycine substitutes for alanine residue in the peptidoglycan, thus impairing cross-linking and weakening the peptidoglycan structure of bacteria [7]. The effect of various glycine concentrations, ranging from 0 to 2% (w/v), on the growth of cells and the production of β-lactamase and levansucrase was investigated (Table 2, Table 3, and Table 4). Glycine

Table 2. Effect of glycine supplement on cell growth of *E. coli*/pRL1CP[a].

% of glycine	Optical density at 600 nm			
	6 h	9 h	12 h	24 h
0	2.0	2.9	4.0	5.1
0.5	1.9	2.8	3.8	4.5
1.0	1.2	2.3	2.4	2.4
2.0	0.2	0.2	0.2	0.5

[a]Numbers given in Table represent optical density of each sample at 600 nm in incubation.

Table 3. Effect of glycine supplement on β-lactamase production and secretion in *E. coli*/pRL1CP.

% of glycine	Total β-lactamase (U/ml of culture broth)[a]				Extracellular β-lactamase (U/ml of culture broth)			
	6 h	9 h	12 h	24 h	6 h	9 h	12 h	24 h
0	2.3	8.1	14.2	14.4	0.7	0.6	2.5	0.8
0.5	2.0	5.2	11.5	14.2	N.D.[b]	1.5	4.9	8.0
1.0	1.3	4.8	11.3	12.8	N.D.	3.3	9.9	10.7
2.0	2.9	4.3	8.8	5.2	N.D.	0.8	0.5	0.7

[a]Total: sum of extra- and intracellular β-lactamase activities.
[b]N.D.: not detected.

concentration at 0.5% did not appear to have a significant effect on the growth of *E. coli*/pRL1CP cells. Interestingly, however, the production of levansucrase in *E. coli*/pRL1CP at 24 h of incubation was increased by 50% by the addition of 0.5% glycine, compared to that of cells grown in LB. However, further increase in the glycine concentration inhibited the cell growth, and the cell growth in the presence of 2% glycine was markedly decreased by 90%, compared to that of cells grown in LB. The production of levansucrase in *E. coli*/pRL1CP grown in LB progressively increased as the cultivation time increased, consequently reaching to 74.5 U/ml of culture broth after 12 h of cultivation, and then slightly decreasing to 66.6 U/ml of culture broth after 24 h of cultivation (Table 4). It should be noted that the formation of insoluble levansucrase in the present work was much lower than that from *Z. mobilis* [12, 31]: The activity of *Z. mobilis* levansucrase in *E. coli* DH5α/pRZ4 grown in LB progressively increased with the increasing cultivation time, consequently reaching 9.1 U/ml of culture broth after 12 h of cultivation, but began thereafter to decrease, showing only 1.8 U/ml of culture broth after 24 h of cultivation [12]. This might probably be due to the thermostable nature of *R. aquatilis* levansucrase. The cultivation of *E. coli*/pRL1CP in the LB medium without glycine did not induce any significant amount of extracellular discharge of the levansucrase and β-lactamase (Tables 3 and 4). However, the presence of glycine (0.5%) caused the increase of levansucrase and β-lactamase secretion to the culture broth. Further increase of glycine supplement up to 1% elevated the secretion of β-lactamase into the medium of 24 h incubation. SDS-PAGE analysis of the culture broth obtained from 24 h incubation medium in the presence of 2% glycine showed a few differences in protein patterns between extracellular and intracellular fractions. Based on the results in the current study, it is postulated that 0.5% glycine might be enough to impair the peptidoglycan structure of *E. coli*/pRL1CP, so that the levansucrase located in the periplasmic space of *E. coli*/pRL1CP was released to the culture broth. At 0.5% glycine concentration, the highest levansucrase activity (115.3 U/ml of culture broth) was obtained. In the cases of *Z. mobilis* [12] and *Pseudomonas aurantiaca* [16], the highest levansucrase activity was 24 and 145 U/ml of culture broth, respectively. Recently, we found that the addition of 0.3– 0.5 M glycine to insoluble form of levansucrase from *Z. mobilis* resulted in 20% increase of refolding yield [32]. Although it is not yet known what action glycine plays in the refolding process of levansucrase, it has been suggested that glycine increases the refolding efficiency by either increasing the solubility of insoluble levansucrase [32] or by decreasing the cell growth, consequently slowing down the production of levansucrase and resulting in more correct folding of levansucrases.

Table 4. Effect of glycine supplement on levansucrase production and secretion in *E. coli*/pRL1CP.

% of glycine	Total levansucrase (U/ml of culture broth)[a]				Extracellular levansucrase (U/ml of culture broth)			
	6 h	9 h	12 h	24 h	6 h	9 h	12 h	24 h
0	9.3	40.5	74.5	66.6	0.8	2.5	1.8	1.1
0.5	7.3	38.8	68.4	115.3	N.D.[b]	0.3	1.7	21.3
1.0	11.7	45.8	89.7	62.0	0.8	2.7	1.8	3.0
2.0	7.6	9.3	11.2	25.1	2.0	2.6	4.1	10.6

[a]Total: sum of extra- and intracellular levansucrase activities.
[b]N.D.: not detected.

Properties of Toluene-Permeabilized Whole-Cells

To further increase the enzyme activity in the whole-cells, cells were mixed with 10% (v/v) toluene, as reported previously [13]. It has been suggested that toluene treatment confers the cell better permeability to the substrate, thereby

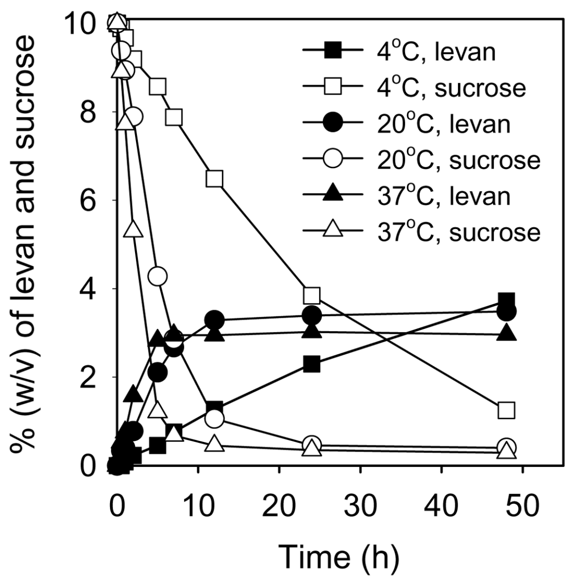

Fig. 3. Temporal profiles of sugar comsumption of toluene-permeabilized whole-cells of *E. coli*/pRL1CP in a batch fermentation at various temperatures.

For the reaction, 100 ml of permeabilized cells were added to 1 ml of 50 mM phosphate buffer (pH 6) containing 10% sucrose.

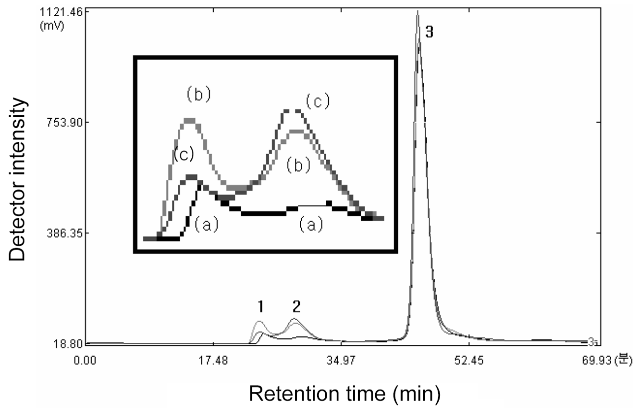

Fig. 4. Typical HPLC chromatograms of enzymatic products by using toluene-permeabilized whole-cells incubated with 10% sucrose in 1 ml of 50 mM phosphate buffer (pH 6), at 4°C (a), 20°C (b), 37°C (c).

HPLC analyses were performed by using two successive GPC columns with water as a mobile phase at 0.4 ml/min. 1, High-molecular size levan; 2, low-molecular size levan; 3, mixture of sucrose, glucose, and fructose.

resulting in improved productivity [5]. In order to study the effects of toluene on cell permeability, different amounts of toluene were added to the cell, and investigated at 5 min of a batch permeation. Of the varying concentrations of toluene employed, 10% (v/v) toluene increased the conversion efficiency of sucrose to levan by more than 35% during 48 h of operation [13]. Using the toluene-permeabilized whole-cells, the enzyme reaction was carried out under standard conditions with substrate (100 g sucrose/l) for 48 h at pH 6 and various temperatures (4, 20, and 37°C). As shown in Fig. 3, sucrose was consumed, and levan, oligosaccharides, and glucose were produced. The velocities of levan formation from sucrose by the toluene-permeabilized whole-cells were dissimilar: At low temperature (4°C), low velocity of levan formation was observed, and maximum velocity of levan formation was observed at 37°C. The polymerization degree of levan formed by the toluene-permeabilized whole-cells was greatly affected by the reaction temperature, as reported by other workers using purified levansucrase [33, 34]. The estimated molecular sizes of levan formed at 4, 20, and 37°C were similar (1×10^7 Da) (Fig. 4). However, the polymerization degree and yield of levan at 20°C and 37°C were higher than that at 4°C: At lower temperature such as 4°C and 20°C, high-molecular size levan was preferentially produced, whereas productions of low-molecular size levan or oligosaccharides were preferred at higher temperature such as 37°C (Fig. 4).

In conclusion, we have developed a culture method for *E. coli*/pRL1CP. The high levansucrase activity (115.3 U/ml of culture broth) obtained in this study demonstrates the possibility of mass production of levan by levansucrase from *E. coli*/pRL1CP. Furthermore, the current study showed that the production of levan by using *E. coli*/pRL1CP can be achieved without enzyme purification. Optimization of the operating parameters in a fermentor system deserves further investigation.

Acknowledgments

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REFERENCES

1. Blight, M. A., C. Chervaux, and I. B. Holland. 1994. Protein secretion pathways in *Escherichia coli. Curr. Opin. Biotech.* **5:** 468–474.
2. Bradford, M. M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein of protein-dye binding. *Anal. Biochem.* **72:** 248–254.
3. Calazans, G. M. T., R. C. Lima, F. P. Francisca, and C. E. Lopes. 2000. Molecular weight and antitumor activity of *Zymomonas mobilis*. *Int. J. Biol. Macromol*. **27:** 245–247.
4. Chalmers, J. J., E. Kim, J. N. Telford, E. Y. Wong, W. C. Tacon, M. L. Shuler, and D. B. Wilson. 1990. Effects of temperature on *Escherichia coli* overproducing β-lactamase or human epidermal growth factor. *Appl. Environ. Microbiol.* **56:** 104–111.
5. Chun U. H. and P. L. Rogers. 1988. The simultaneous production of sorbitol from fructose and gluconic acid from glucose using an oxidoreductase of *Zymomonas mobilis*. *App. Microbiol. Biotechnol*. **29:** 19–24.
6. Goebel, W. and J. Hedgpeth. 1982. Cloning and functional characterization of the plasmid-encoded hemolysin determinant of *Escherichia coli. J. Bacteriol.* **151:** 290–298.
7. Hammes, W., K. H. Scheifer, and O. Kandler. 1973. Mode of action of glycine on the biosynthesis of peptidoglycan. *J. Bacteriol*. **116:** 1029–1053.
8. Han Y. W. 1990. Microbial levan. *Adv. Appl. Microbiol.* **35:** 171–194.
9. Hendry, G. A. F. and R. K. Wallace. 1993. The origin, distribution, and evolutionary significance of fructans. *In* M. Suzuki and N. J. Chatterton (eds.), *Science and Technology of Fructans.* CRC Press.
10. Ikura, Y. 1986. Effect of glycine and its derivatives on production and release of beta-galactosidase by *Escherichia coli. Agric. Biol. Chem.* **50:** 2747–2753.
11. Jang, E. K., K. H. Jang, I. Koh, I. H. Kim, S. H. Kim, S. A. Kang, C. H. Kim, S. D. Ha, and S. K. Rhee. 2002. Molecular characterization of the levansucrase gene from *Pseudomonas aurantiaca* S-4380 and its expression in *Escherchia coli. J. Microbiol. Biotechnol*. **12:** 603–609.

12. Jang, K. H., J. W. Seo, K. B. Song, C. H. Kim, and S. K. Rhee. 1999. Extracellular secretion of levansucrase from *Zymomonas mobilis* in *Escherichia coli*. *Bioprocess Eng.* **21:** 453–458.
13. Jang, K. H., K. B. Song, C. H. Kim, B. H. Chung, S. A. Kang, U. H. Chun, R. W. Choue, and S. K. Rhee. 2001. Comparison of characteristics of levan produced by different preparations of levansucrase from *Zymomonas mobilis. Biotechnol. Lett.* **23:** 339–344.
14. John E. Van W. and A. D. Johannis. 1990. How big is the periplasmic space? *Trends Biochem. Sci.* **15:** 136–137.
15. Kim, H. J., H. E. Park, M. J. Kim, H. G. Lee, J. Y. Yang, and J. H. Cha. 2003. Enzymatic characterization of a recombinant levansucrase from *Rahnella aquatilis* ATCC 15552. *J. Microbiol. Biotechnol.* **13:** 230–235.
16. Kim, S. H., E. K. Jang, I. H. Kim, K. H. Jang, S. A. Kang, and B. I. Chang. 2003. Effect of glycine supplement on extracellular secretion of levansucrase from *Pseudomonas aurantiaca* S-4380 in recombinant *Escherichia coli*. *Korean J. Biotechnol. Bioeng.* **18:** 312–317.
17. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227:** 680–685.
18. Leibovici J., S. Kopel, A. Siegel, and O. Gal-Mor. 1986. Effect of tumor inhibitory and stimulatory doses of levan, alone and in combination with cyclophosphamide, on spleen and lymph nodes. *Int. J. Immunopharmacol.* **8:** 391–403.
19. Loo, J. V., J. Cummings, N. Delzenne, H. Englyst, A. Frank, M. Hopkins, N. Kok, G. Macfalane, D. Newton, M. Quigley, M. Roberfroid, T. van Vliet, and E. van den Heuvel. 1999. Functional food properties of non-digestible oligosaccharides: A consensus report from the ENDO project (DGXII AIRII-CT94-1095). *Br. J. Nutr.* **81:** 121–132.
20. Lyness, E. W. and H. W. Doelle. 1983. Levansucrase from *Zymomonas mobilis. Biotechnol. Lett.* **5:** 345–350.
21. Ohtsuka, K., S. Hino, T. Fukushima, O. Ozawa, T. Kanematsu, and T. Uchida. 1992. Characterization of levansucrase from *Rahnella aquatilis* JCM-1683. *Biosci. Biotech. Biochem.* **56:** 1373–1377.
22. Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson. 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*; isolation and characterization of cytoplasmic and outer membranes. *J. Biol. Chem.* **247:** 3962–3972.
23. Patil, N. K., U. Sharanagouda, J. H. Niazi, C. K. Kim, and T. B. Karegoudar. 2003. Degradation of salicylic acid by free and immobilized cells of *Pseudomonas* sp. strain NGK1. *J. Microbiol. Biotechnol.* **13:** 29–34.
24. Pelicic, V., J. M. Reyrat, and B. Gicquel. 1996. Expression of the *Bacillus subtilis* sacB gene confers sucrose sensitivity on mycobacteria. *J. Bacteriol.* **178:** 1197–1199.
25. Rhee, S. K., K. B. Song, C. H. Kim, B. S. Park, E. K. Jang, and K. H. Jang. 2002. Levan, pp 351–377. *In* S. Alexander, E. Vandamme, and S. De. Baets (eds.), *Biopolymers*, vol. **5**. Wiley-VCH, Weinheim, Germany.
26. Salmond, G. P. C. and P. J. Reeves. 1993. Membrane traffic wardens and protein secretion in Gram-negative bacteria. *Trends Biochem. Sci.* **18:** 7–12.
27. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, U.S.A.
28. Seo, J. W., K. B. Song, K. H. Jang, C. H. Kim, B. H. Jung, and S. K. Rhee. 2000. Molecular cloning of a gene encoding the thermoactive levansucrase from *Rahnella aquatilis* and its growth phase-dependent expression in *Escherichia coli*. *J. Biotechnol*. **81:** 63–72.
29. Seo, J. W., K. H. Jang, S. A. Kang, K. B. Song, E. K. Jang, B. S. Park, C. H. Kim, and S. K. Rhee. 2002. Molecular cloning of the growth phase-dependent expression of the *lsrA* gene, encoding levansucrase of *Rahnella aquatilis. J. Bacteriol*. **184:** 5862–5870.
30. Singh, Y. 2003. Photosynthetic activity, and lipid and hydrocarbon production by alginate immobilized cells of *Botrycoccus* in relation to growth phase. *J. Microbiol. Biotechnol*. **13:** 687–691.
31. Song, K. B., H. Belghith, and S. K. Rhee. 1996. Production of levan, a fructose polymer, using an overexpressed recombinant levansucrase. *Ann. N. Y. Acad. Sci.* **799:** 601–607.
32. Sunita, K., B. H. Chung, K. H. Jang, K. B. Song, C. H. Kim, and S. K. Rhee. 2000. Refolding and purification of *Zymomonas mobilis* levansucrase produced as inclusion bodies in fed-batch culture of recombinant *Escherichia coli*. *Protein Expres. Purif.* **18:** 388–393.
33. Tanaka, T., S. Oi, and T. Yamamoto. 1979. Synthesis of levan by levansucrase. *J. Biochem.* **85:** 287–293.
34. Yanase, H., M. Iwata, R. Nakahigashi, K. Kita, N. Kato, and K. Tonomura. 1992. Purification, crystallization and properties of the extracellular levansucrase from *Zymomonas mobilis*. *Biosci. Biotech. Biochem*. **56:** 1335–1337.