Increased Cellulose Production from Sucrose with Reduced Levan Accumulation by an Acetobacter Strain Harboring a Recombinant Plasmid

Naoto Tonouchi,† Hideshi Yanase,* Yukiko Kojima, Takayasu Tsuchida, Fumihiro Yoshinaga, and Sueharu Horinouchi**

Bio-Polymer Research Co., Ltd., 3-2-1 Sakato, Takatsu-ku, Kawasaki, Kanagawa 213-0012, Japan
*Department of Biotechnology, Faculty of Engineering, Tottori University, Tottori 680-0945, Japan
**Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan

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Cellulose production from sucrose by Acetobacter strains is accompanied by the accumulation of a water-soluble polysaccharide, called levan. To improve cellulose productivity, a levan-sucrase-deficient mutant, LD-2, was derived from Acetobacter strain 757 and used as a host for the construction of recombinant strains. An LD-2 mutant harboring a plasmid containing the sucrase gene, sucZ3, from Zymomonas mobilis together with zilS, a gene that encodes a secretion-activating factor under the control of the Escherichia coli lac promoter, had sucrase activity and produced much cellulose and little levan in a medium containing sucrose. In addition, a mutant levan-sucrase gene, mutant sacB, from Bacillus subtilis, which encodes a protein with little levan-forming activity, was generated by site-directed mutagenesis and introduced into the LD-2 mutant. This introduction also resulted in the higher cellulose productivity and little levan.

Key words: Acetobacter; cellulose; sucrose; levan; levansucrase

Some Acetobacter strains produce and accumulate cellulose in the culture. This bacterial cellulose has various useful properties and is expected to be a novel industrial material. For efficient industrial production, strain improvement is needed. There have been few attempts at strain improvement by standard or recombinant DNA techniques.

We have isolated several Acetobacter strains that produce cellulose efficiently from sucrose, the most suitable carbon source for the economic production. However, these strains have a draw-back in that a large amount of a water-soluble polysaccharide accumulates in the culture broth when grown with sucrose. This polysaccharide appeared to be levan, because Acetobacter diazotrophicus produces a polysaccharide composed of fructose from sucrose by the action of levansucrase.

A wide range of microorganisms produce levan from sucrose. Accumulation of the polysaccharide decreases the yield of cellulose yield and hampers its purification from the culture broth.

To overcome this problem, we first derived a levansucrase-deficient mutant from Acetobacter strain 757 by mutagenesis. We then introduced foreign genes encoding enzymes degrading sucrose into the mutant cells. The genes we tested were sucZ3, which encodes an extracellular sucrase from Zymomonas mobilis, and a mutant sacB gene, which encodes a levansucrase from Bacillus subtilis. As expected, the mutant Acetobacter strain harboring the plasmid containing the sucrase genes produced cellulose from sucrose with high yields and without producing levan.

Materials and Methods

Acetobacter strain producing cellulose and isolation of levansucrase-deficient mutants. Acetobacter sp. strain 757, which produces cellulose efficiently from sucrose, was used. For isolation of levansucrase-deficient mutants, cells of strain 757 were treated with N-methyl-N'-nitro-N-nitrosoguanidine (NTG) to give 1.0% survival, as described previously, and plated onto a minimal medium containing sucrose. Several small nonmucous colonies were selected as possible levansucrase-deficient mutants.

Purification and identification of composition of the water-soluble polysaccharide. The polysaccharide was isolated by ethanol precipitation from the supernatant from a culture of strain 757 culture, and purified by removal of protein by chloroform extraction, dialysis, and freeze-drying. The sugar composition was examined after the purified polysaccharide was hydrolyzed for 6 h at 100°C in 2 N trifluoroacetic acid. For detection of fructose, hydrolysis was done under mild conditions (30 min at 60°C in 0.02 N HCl), because fructose is known to be degraded by hydrolysis under standard conditions. Composite sugars were detected by post-column fluorometric HPLC with arginine.

Cellulose production and analytical methods. A CSL-Suc medium, that contained 4% sucrose and 4% corn steep liquor (CSL), was used for cellulose production. Suc-F+C medium, that contained 2% fructose and 2% glucose as carbon sources was used as a control. When cells contained plasmids, 100 μg/ml ampicillin was added to the culture medium.
Cellulose production in a jar fermentor and estimation of cellulose yield were done as described previously. The amount of levan was estimated by measuring the total sugar content of the ethanol precipitate of the culture broth after cellulose removal by the phenol-sulfate method.

Recombinant DNA studies. Construction of the plasmids is shown in Fig. 1. The extracellular saccharase gene, sucZ3, from Z. mobilis has been described previously. From the EcoRI-HindIII fragment of plasmid pKZE3d containing sucZ3, a BamHI-PstI fragment was obtained and used. Z. mobilis extracellular saccharase, SucZ3, has no signal sequence, and for secretion of SucZ3 by E. coli, another gene, zisS, is needed. The zisS gene was amplified by PCR with a previously described plasmid, pZS1, and the synthetic DNA primers ATCAGAATTCCGTCAATAAATC and TTAGGATTTGATCTGGAAG. The fragments were ligated into an Acetobacter-Escherichia coli shuttle vector, pSA19, at the BamHI-PstI or EcoRI-PstI site, and cloning of the fragment was confirmed by sequencing of the nucleotide.

The levansucrase gene, sacB, was prepared by PCR with chromosomal DNA of B. subtilis Marburg 168 as the template and two primers, GAAGGATCCGCTAACACAGTACATA and CCCGCTGAGTTCATATGGATTCACCT, of which the design was based on the nucleotide sequence of the gene.

A mutated sacB gene encoding a protein that produced little levan was generated by two-step PCR. For base substitution at Arg331, a set of primers (a sequence shown in Fig. 2 and its complement sequence) were used. The genes were ligated into vector pSA19 at the BamHI and PstI sites, giving the plasmid pSAR331H. The fragments cloned were confirmed by nucleotide sequencing.

These plasmids were introduced into Acetobacter cells by electroporation, and cells harboring the plasmids were selected as ampicillin-resistant colonies.

Enzyme activity. The cells were collected and incubated with 20 mM phosphate buffer (pH 6.5) at 30°C for 20 min. Extracellular enzymes are released from the cell surface by this procedure, so the cell suspension was centrifuged and the supernatant was used as the crude enzyme fraction. Sucrase activity was measured as described previously. One unit was defined as the amount of enzyme that produces reducing sugars equivalent to 1 µmol of glucose per minute. For characterization of the enzyme, the amounts of glucose and fructose liberated from sucrose were measured separately by HPLC.

Results

Composition of the polysaccharide produced by strain 757

To determine the sugar composition of the water-soluble polysaccharide produced by strain 757, the polysaccharide was extracted from the culture broth, hydrolyzed under standard conditions, and analyzed by HPLC as described in Materials and Methods. HPLC showed a small amount of fructose in the polysaccharide hydrolyzed under ordinary conditions, but no amount of fructose was detected by HPLC. The amount of fructose recovered by this hydrolysis procedure was 82.5% of the polysaccharide applied to the column. Since no peaks corresponding to other sugars were detected, the water-soluble polysaccharide was found to be consisted only of fructose.

Isolation of levansucrase-deficient mutant strain

Starting with strain 757, we isolated mutants deficient in levansucrase, the enzyme responsible for the production of the fructose polysaccharide. From some 3000

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sequence</th>
<th>Amino Acid</th>
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<tbody>
<tr>
<td>327</td>
<td>TTC ACT GAC TCC CGC GGA TCA AAA...</td>
<td>Phe Thr Asp Ser Arg Gly Ser Lys</td>
</tr>
<tr>
<td>328</td>
<td>TTC ACT GAC TCC CGC GGA TCA AAA...</td>
<td>Phe Thr Asp Ser Arg Gly Ser Lys</td>
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<td>TTC ACT GAC TCC CGC GGA TCA AAA...</td>
<td>Phe Thr Asp Ser Arg Gly Ser Lys</td>
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<td>330</td>
<td>TTC ACT GAC TCC CGC GGA TCA AAA...</td>
<td>Phe Thr Asp Ser Arg Gly Ser Lys</td>
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<td>TTC ACT GAC TCC CGC GGA TCA AAA...</td>
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<td>TTC ACT GAC TCC CGC GGA TCA AAA...</td>
<td>Phe Thr Asp Ser Arg Gly Ser Lys</td>
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<tr>
<td>R331H</td>
<td>TTC ACT GAC TCC CGC GGA TCA AAA...</td>
<td>Phe Thr Asp Ser His Gly Ser Lys</td>
</tr>
</tbody>
</table>

*Fig. 2.* Primers for Introduction of the Mutation into the sacB Gene.

The mutant gene was generated by two-step PCR using synthetic DNA with the sequence indicated and its complementary sequence, in addition to the primers for cloning of the sacB gene. The numbers of the amino acid residues of the SacB protein are also indicated.
NTG-treated cells grown on a medium containing sucrose, we selected several small nonmucous colonies. Some strains produced only 0.5–1.5 g/l of cellulose in a medium containing glucose, and might have had mutations in the genes involved in cellulose synthesis. On the other hand, several strains, including LD-1 and LD-2, produced cellulose efficiently from glucose (4.2–4.5 g/l). The amounts of cellulose and glucose by LD-1 and LD-2 are shown in Table I. The two strains produced significantly less cellulose from sucrose than from glucose, but their cellulose production from glucose was almost the same as that of the parent 757 strain. These strains probably had a defecting levansucrase gene, and indeed, no levansucrase activity was detected in LD-1 or LD-2 cultures. Therefore, we selected strain LD-2 as the host for the subsequent recombinant DNA studies.

**Cellulose production by recombinant Acetobacter strain containing Z. mobilis genes for extracellular sucrase and secretion-activating factor**

The sucZ3E3 gene was inserted into the Acetobacter-E. coli shuttle vector pSA19 under the control of the E. coli lac promoter and introduced into LD-2. The lac promoter functions efficiently in *Acetobacter* strains. The LD-2 strain harboring this recombinant plasmid pSAZE3 was cultured and its sucrose activity was measured. No sucrase activity was detected in the extracellular fraction (Table II). This was consistent with our observation that the sucrase enzyme is secreted from *E. coli* cells only when the zII5S gene is present together with sucZ3E3. We then inserted both sucZ3E3 and zII5S into pSA19 and introduced the recombinant plasmid pSAZE3zII5S into the LD-2 mutant. As expected, significant sucrase hydrolyzing activity (0.26 unit/mg protein) was detected in the extracellular fraction (Table II). This observation suggests that ZII5S serves as a secretion-activating factor for sucrase in *Acetobacter* species as well as in *Z. mobilis* and *E. coli*.

Cellulose and levan production by this recombinant strain is shown in Fig. 3. The amount of cellulose produced by LD-2 cells harboring pSAZE3zII5S was greater than that produced by LD-2 or by 757 cells. That was the same as that produced by 757 cells when fructose and glucose were the carbon sources (Fig. 3(a)). Strain 757 produced much levan, but the LD-2 (pSAZE3zII5S) strain produced little (Fig. 3(b)).

**Cellulose production by an Acetobacter strain harboring a recombinant plasmid containing the B. subtilis mutansucrase gene**

The sacB gene was cloned from *B. subtilis* Marburg 168, and was introduced into the LD-2 mutant with the pSA19 vector. Levansucrase activity was detected in the extracellular fraction (Table II). We also constructed a mutant sacB gene, sacB (R331H), that resulted in little levan formation, but sucrase hydrolyzing activity was detected in the extracellular fraction. The properties of the enzyme are shown in Table II. Frc/Glc indicates the ratio of the amount of fructose to the amount of glucose generated from sucrose by the enzyme. The mutant enzyme SacB(R331H) produced little levan from sucrose, because it generated similar amounts of glucose and fructose from sucrose. In contrast, little fructose (Frc/Glc = 0.61) was generated by native levansucrase.

The strain harboring the pSAR331H plasmid also produced little levan and an increased amount of cellulose (Fig. 3). These results are compatible with the enzyme properties shown in Table II. Therefore, cellulose production by the strain harboring either pSAR331H or pSAZE3zII5S was greater than that of the host strain, LD-2; more cellulose was produced than the amount produced by strain 757.

**Discussion**

For prevention of levan accumulation during the production of bacterial cellulose from sucrose, two genes encoding sucrase-hydrolyzing enzymes were in-

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**Table I. Cellulose Production from Sucrose and Glucose by Levansucrase-Deficient Strains**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Produced with sucrose:</th>
<th>Produced with glucose:</th>
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<tbody>
<tr>
<td></td>
<td>Cellulose, g/l</td>
<td>Levan, g/l</td>
</tr>
<tr>
<td>LD-1</td>
<td>1.4</td>
<td>0.0</td>
</tr>
<tr>
<td>LD-2</td>
<td>1.5</td>
<td>0.0</td>
</tr>
<tr>
<td>757</td>
<td>6.1</td>
<td>5.0</td>
</tr>
</tbody>
</table>

The culture medium contained 4% sucrose or 4% glucose. The produced cellulose and levan were measured after 4 days culture.

**Table II. Extracellular Sucrase Activity of LD-2 Strains with Newly Introduced Genes**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Activity (U/mg)</th>
<th>Frc/Glc</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD-2</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>LD-2 (SACE3)</td>
<td>ND</td>
<td>—</td>
</tr>
<tr>
<td>LD-2 (pSAZE3zII5S)</td>
<td>0.26</td>
<td>0.94</td>
</tr>
<tr>
<td>LD-2 (pASA&amp;3B)</td>
<td>0.47</td>
<td>0.61</td>
</tr>
<tr>
<td>LD-2 (pSAR331H)</td>
<td>0.79</td>
<td>0.95</td>
</tr>
<tr>
<td>757</td>
<td>0.40</td>
<td>0.78</td>
</tr>
</tbody>
</table>

ND, not detected. —, not applicable. Frc/Glc, ratio of the amount of fructose produced to the amount of glucose produced by this sucrase.

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**Fig. 3. Cellulose and Levan Production in a Jar Fermentor from Sucrose by LD-2 Cells Harboring Sucrase Genes.**

A medium containing sucrose was used for all cultures except that specified below. (a) Cellulose production. (b) Levan production. Crosses, LD-2; open circles, 757; closed circles, 757 grown with fructose and glucose; squares, LD-2 (pSAR331H); triangles, LD-2 (pSAZE3zII5S).
introduced into a levansucrase-deficient mutant. As expected, efficient cellulose production without levan formation was possible, because strain 757 produces a polysaccharide composed only of fructose, unlike other cellulose-producing *Acetobacter* strains that produce a heteropolysaccharide, called both AM-2 \(^{25}\) and acetan \(^{26}\) composed of glucose, mannose, rhamnose, and glucuronate. \(^{25,26}\)

*Z. mobilis* extracellular sucrase, SucZ3, is not secreted by *E. coli*. \(^{19}\) For secretion of SucZ3 by *E. coli*, another gene, *zis*, is needed. \(^{17}\) The function of the *zis* gene product is not clear, but it may be a secretion-activating protein. \(^{18}\) Consistently with this suggestion, no sucrose activity was detected in the extracellular fraction from the LD-2 mutant containing only sucZ3, but when *zis* was introduced as well, the enzyme was detected in the extracellular fraction. Therefore, *zis* seems to function as a secretion-activating factor in *Acetobacter* strains producing cellulose, as well as in *Z. mobilis* and *E. coli*.

**B. subtilis** SacB accumulates in the intracellular fraction of *E. coli* rather than being secreted, even though a signal sequence has been identified. \(^{27}\) In addition, the growth of *E. coli* cells containing *sacB* is inhibited in a medium containing sucrose, \(^{28}\) probably because the levan produced by the action of *sacB* accumulates in the cell membranes. In contrast, in the *Acetobacter* strain with *sacB*, levansucrase activity was detected in the extracellular fraction, and growth inhibition was not observed in a medium containing sucrose. Therefore, the signal sequence of the *B. subtilis* *sacB* gene may be recognized by the secretion machinery of *Acetobacter*. This possibility is consistent with our previous observation that an endoglucanase from *B. subtilis* is secreted by an *Acetobacter* strain. \(^{29}\)

More cellulose was produced by recombinant strains in the medium containing sucrose than by strain 757 in the same medium, and the amount produced was the same as that produced by strain 757 in the medium containing fructose and glucose. These findings suggest that cellulose production increased because by levan formation decreased.

In conclusion, cellulose production from sucrose by the recombinant strains described in this study was efficient and levan was not formed. The approach used (the introduction of foreign genes) is a useful way to achieve economical cellulose production by *Acetobacter*.

References