Efficient Simultaneous Saccharification and Fermentation of Inulin to 2,3-Butanediol by Thermophilic *Bacillus licheniformis* ATCC 14580

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2,3-Butanediol (2,3-BD) is an important starting material for the manufacture of bulk chemicals. For efficient and large-scale production of 2,3-BD through fermentation, low-cost substrates are required. One such substrate, inulin, is a polydisperse fructan found in a wide variety of plants. In this study, a levanase with high inulinase activity and high pH and temperature stability was identified in *Bacillus licheniformis* strain ATCC 14580. *B. licheniformis* strain ATCC 14580 was found to efficiently produce 2,3-BD from fructose at 50°C. Then, the levanase was used for simultaneous saccharification and fermentation (SSF) of inulin to 2,3-BD. A fed-batch SSF yielded 103.0 g/liter 2,3-BD in 30 h, with a high productivity of 3.4 g/liter · h. The results suggest that the SSF process developed with the thermophilic *B. licheniformis* strain used might be a promising alternative for efficient 2,3-BD production from the favorable substrate inulin.

The chemical compound 2,3-Butanediol (2,3-BD) is used as a starting material for the manufacture of bulk chemicals such as methyl ethyl ketone and 1,3-butadiene (1, 2). With a heating value of 27,200 J/g, 2,3-BD compares favorably with ethanol (29,100 J/g) and methanol (22,100 J/g) as a liquid fuel or fuel additive (3). 2,3-BD can be produced by chemical or biotechnological methods (1, 2). Due to the gradual exhaustion of crude oil reserves, interest in the biotechnological production of 2,3-BD has increased greatly in recent years. Currently, *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Serratia marcescens*, and *Bacillus licheniformis* are regarded as the most efficient 2,3-BD producers (4–7). However, in these studies refined glucose and sucrose were used as substrates, which may not be economically feasible. Because the cost of raw materials accounts for more than 30% of the total production costs of fermentation, low-price substrates and efficient strains are required for 2,3-BD production (8). Therefore, from an economic point of view, cheap raw materials, such as lignocellulosic materials, corn cob molasses, corn cob acid hydrolysates, starch hydrolysate, whey permeate, sugarcane molasses, and cassava powder, could be used for 2,3-BD production. However, the productivity and concentration of 2,3-BD obtained by using these cheap substrates are still low (1). Thus, it is desirable to identify cheap, nonfood, renewable sources and develop appropriate fermentation strategies to obtain high 2,3-BD productivity and concentrations.

Fructans are the most abundant nonstructural polysaccharides found in plants after starch, and they are found in wide variety of plants. One of these fructans, inulin, is a polydisperse fructan consisting mainly of β-2,1-α-fructosyl-fructose links terminated by a sucrose residue (9). It serves as a storage polysaccharide in many plants as well as a reserve in sandy soils and tidal areas, where it requires limited human intervention for cultivation, and thus does not compete for arable lands with grain crops (1). Inulin constitutes up to 85% of the dry weight of the Jerusalem artichoke (11) and 16% of the wet weight of chicory root (12). The worldwide production of inulin is currently estimated to be about 350,000 tons (13). Although its total quantity is not comparable to that of lignocellulose biomass, inulin could be easily processed by using currently available technologies, and it may even be easier to process than starch (11). Thus, inulin has recently received much attention as a renewable feedstock for the production of fructose and chemicals, such as ethanol and lactic acid (14–16). However, there have been few studies on 2,3-BD production from inulin as the substrate (17, 18).

Inulin can be hydrolyzed to fructose and glucose by inulinase or levanase. Both inulinase and levanase are fructofuranosyl hydrolases that are produced by a wide variety of organisms, including plants, bacteria, molds, and yeasts (13). Although many inulinas and levanases have been reported, the commercial inulinase, an inulinase from *Aspergillus niger*, is expensive (Novozym 960, $188 for 250 ml [product 16285; Sigma]), and there is no commercial levanase. Dao et al. suggested that a commercial glucoamylase (GA-L New; Genencor) could be used for the hydrolysis of Jerusalem artichoke tubers. However, the optimum pH of glucoamylase for inulin hydrolysis is 4.0, and the stability of this enzyme decreased significantly when the pH was increased to 5.6 (19). These characteristics make GA-L New unsuitable for simultaneous saccharification and fermentation (SSF) of 2,3-BD because the pH range for optimal 2,3-BD production is generally between 5.5 and 7.0 (3). Therefore, identification of an appropri-
ate inulinase or levanase is a key step for industrial-scale production of 2,3-BD from inulin.

Most of the reported inulinases and levanases have optimum temperatures in the range of 45°C to 55°C (13). However, many microbes, including *K. pneumoniae*, *K. oxytoca*, *S. marcescens*, and *Paenibacillus polymyxa*, have the ability to produce 2,3-BD at 28°C to 37°C (1). Utilization of these mesophilic strains for SSF of inulin to 2,3-BD may increase the dosage of inulinase or levanase; therefore, the SSF process with a thermophilic 2,3-BD producer may use a lower dosage of the enzyme. In our previous study, we used the thermophilic bacterium *B. licheniformis* ATCC 14580, which is a potential candidate for the production of 2,3-BD under the 50°C fermentation conditions. In this study, another thermophilic *B. licheniformis* strain, ATCC 14580, was used to produce 2,3-BD from inulin. In addition, a gene in *B. licheniformis* ATCC 14580 strain encoding a levanase was expressed, purified, and partially characterized. Efficiencies of various fermentation strategies, including SSF and separate hydrolysis and fermentation (SHF), were investigated.

**MATERIALS AND METHODS**

**Enzymes and chemicals.** Restriction enzymes were purchased from TaKaRa Bio, Inc. (China). Fast *Pfu* DNA polymerase and T4 DNA ligase were purchased from Transgen Biotech (China) and MBI (USA), respectively. (2R,3R)-2,3-BD (98.0%), (2S,3S)-2,3-BD (99.0%), and meso-2,3-BD (98.0%) were purchased from Acros (Belgium). Isopropyl-ß-D-thiogalactopyranoside (IPTG), diithiothreitol (DTT), and phenylmethylsulfonyl fluoride (PMSF) were obtained from Merck (Germany). Ampicillin was purchased from Amresco (USA). Inulin was purchased from BENEO GmbH (Germany). All other chemicals were of analytical grade and were commercially available.

**Bacterial strains and plasmids.** *Escherichia coli* strains DH5α and BL21(DE3) were used for general cloning and protein expression, respectively. The pEASY-Blunt cloning vector (Transgen) was used as a vector to subclone the sacC gene, and pETDuet-1, which has a T7 promoter, was used for protein expression. *B. licheniformis* strain ATCC 14580 was purchased from ATCC (American Type Culture Collection), and other *B. licheniformis* strains were from our laboratory stocks. Lysogenic broth (LB) medium was used to cultivate *E. coli*. Ampicillin was used at a concentration of 100 µg/ml.

*B. licheniformis* strains were maintained on deMan-Rogosa-Sharpe agar slants. The slants were incubated at 50°C, and fully grown slants were stored at 4°C. The fermentation medium contained the following (g/liter): glucose (50), yeast extract (YE; 5.8), corn steep liquor powder (CSLP; 14.7), sodium acetate (6.5), triammonium citrate (1), K₂HPO₄·3H₂O (2), and MgSO₄·7H₂O (0.25), pH 7.0. The seed culture was prepared as follows: a loop of cells from a fully grown slant was inoculated into 100 ml of the above-described medium in 500-ml Erikmeier flashes and incubated at 50°C for 12 h with agitation. Then, the seed culture was inoculated into Erlenmeyer flasks or bioreactors (5% of the total volume) for 2,3-BD production.

**Strain comparison and fermentation temperature optimization.** The medium used to compare the abilities of various *B. licheniformis* strains to produce 2,3-BD from fructose contained the following (g/liter): fructose (80), YE (5.8), CSLP (14.7), sodium acetate (6.5), triammonium citrate (1), K₂HPO₄·3H₂O (2), and MgSO₄·7H₂O (0.25), pH 7.0. The fermentation was performed in 500-ml Erikmeier flasks containing 100 ml of medium with shaking at 180 rpm on a rotary shaker at 50°C for 10 h.

The optimal fermentation temperature for strain ATCC 14580 was tested using medium containing the following (g/liter): fructose (80), YE (5.8), CSLP (14.7), sodium acetate (6.5), triammonium citrate (1), K₂HPO₄·3H₂O (2), and MgSO₄·7H₂O (0.25), pH 7.0. The fermentation was performed in 500-ml Erikmeier flasks containing 100 ml of medium with shaking at 180 rpm on a rotary shaker at temperatures of 30°C, 37°C, 42°C, 50°C, and 55°C. Samples were collected periodically to determine the concentrations of biomass, fructose, and 2,3-BD.

**Cloning and expression of sacC.** To obtain the sacC gene from strain ATCC 14580 (GenElid 3098029), two primers, P1 and P2, were designed to amplify the sacC sequence by PCR. P1 (5'-GGATCCACTGAAGAAGTATTCCG-3'), which has a BamHI restriction site inserted at the 5’ end, and P2 (5’-AAGCTTTATGTTGCGCATACACAT-3’), which has a HindIII restriction site inserted at the 5’ end were employed to amplify a HindIII restriction site inserted at the 5’ end to employ the sacC gene from the ATCC 14580 genome by using the following program: 30 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s. The resultant PCR product was ligated to the pEASY-Blunt vector. The constructed vector, named pEASY-Blunt-sacC, was confirmed by sequencing (Sangon, Shanghai, China). To construct a sacC expression vector whose expression was under the control of the T7 promoter, pEASY-Blunt-sacC was digested with BamHI and HindIII, and the gel-purified sacC fragment was ligated to pETDuet-1 vector that was ligated with the same restriction enzymes. The resulting plasmid was designated pETDuet-sacC.

**Characterization of the levanase.** Recombinant *E. coli* cells containing pETDuet-sacC were grown at 37°C on a rotary shaker (180 rpm) in LB medium containing ampicillin (100 µg/ml) to an optical density at 620 nm (OD₆₂₀) of 0.6. Expression of sacCin cells was induced by the addition of IPTG to a final concentration of 0.6 mM, and the cells were incubated at 25°C for approximately 12 h. After induction, the cells were harvested by centrifugation at 6,000 × g for 5 min at 4°C and then washed twice with 0.85% NaCl. Harvested cells were resuspended in binding buffer (20 mM potassium phosphate, 500 mM NaCl, 20 mM imidazole, 0.1 mM PMSF, and 1 mM DTT, pH 7.4) and were disrupted by sonication in an ice bath. The homogenate was centrifuged at 15,000 × g for 30 min, and the supernatant (crude extract) was recovered. The crude extract (crude enzyme) was loaded on a 5-ml HisTrap HP column (GE Healthcare, USA) that was equilibrated with 25 mM binding buffer. The column was washed with binding buffer, and proteins were then eluted with 10%, 60%, and 100% elution buffer (20 mM potassium phosphate buffer, 500 mM NaCl, and 500 mM imidazole, pH 7.4). The fraction eluted with 60% elution buffer was collected, concentrated, and desalted in an Amicon Ultra-15 centrifugal filter unit (10-kDa cutoff) with 50 mM Tris-HCl (pH 7.4). Then, the protein was purified by size exclusion chromatography with a Superdex 200 10/300 column equilibrated with two volumes of 50 mM Tris-HCl (pH 7.4) buffer containing 0.15 M KCl. The fraction with inulinase activity was collected and concentrated in an Amicon Ultra-15 centrifugal filter unit (10-kDa cutoff). Expresssed and purified enzymes were analyzed by SDS-PAGE.

A reaction mixture containing 0.1 ml of the enzyme, 0.9 ml of phospho-buffered saline (PBS; 1/15 M, pH 6.5), and 3% inulin was incubated at 50°C for 15 min. Then, the enzyme was inactivated by incubating the reaction mixture at 100°C for 10 min. The reducing sugars in the reaction mixture were measured by using the 3,5-dinitrosalicylic acid method. One unit (U) of levanase was defined as the amount of enzyme that yielded 1 µmol of reducing sugars per min under the assay conditions used in this study. The specific activity of levanase was defined as the units of enzyme (U) divided by the amount of enzyme (mg).

To identify the optimum temperature and pH for levanase, assays were performed at different temperatures (50°C to 70°C) and pH values (5 to 7) in PBS buffer (1/15 M). The thermal stability of the enzyme was determined in the absence of substrate or additives. The enzyme solution was incubated at 40°C, 45°C, 50°C, 55°C, 60°C, and 70°C in PBS buffer (1/15 M, pH 6.5) in a temperature-controlled water bath for 1 h, and the residual activity was measured at regular time intervals. Similarly, the enzyme solution was incubated at different pH values (3 to 11) at 25°C for 1 h to study the effect of pH on enzyme activity.

**SHF and SSF.** To compare 2,3-BD production by SHF and SSF, the fermentation was performed in 500-ml Erikmeier flasks containing 100 ml of medium with shaking at 180 rpm on a rotary shaker. For SHF, the production was tested using medium containing the following (g/liter): fructose (80), YE (5.8), CSLP (14.7), sodium acetate (6.5), triammonium citrate (1), K₂HPO₄·3H₂O (2), and MgSO₄·7H₂O (0.25), pH 7.0. The fermentation was performed in 500-ml Erikmeier flasks containing 100 ml of medium with shaking at 180 rpm on a rotary shaker. For SHF, the
inulin was enzymatically hydrolyzed to reducing sugars; this enzymatic pretreatment was carried out in PBS buffer (pH 6.5, 1/15 M) in a flask at 50°C for 6 h using crude enzyme (10 U/g inulin). The initial concentration of reducing sugars in SHF was approximately 90 g/liter. The seed culture that was prepared as described above was inoculated (5%, vol/vol) into the fermentation medium. For SSF, the initial concentration of inulin was 90 g/liter, and the dosage of levansase was 30 U/g inulin.

The fed-batch SSF was conducted in a 5-liter bioreactor (Biostat B; B. Braun Biotech International GmbH, Germany) with 4 liters of initial medium. The prepared seed culture described above was inoculated (5%, vol/vol) into the fermentation medium. The initial concentration of inulin was 180 g/liter, and the dosage of crude levansase was 30 U/g inulin. The cultivation was carried out at 50°C, with stirring at 400 rpm for the first 10 h, after which time the stirring was decreased to 200 rpm with an airflow rate of 1.0 volume of air/minute of medium/min (vvm) for the fed-batch fermentation. The pH was maintained at 6.5 by automatic addition of 6 M NaOH and acetic acid using a program-controlled peristaltic pump. Samples were collected periodically to determine the biomass and the concentrations of reducing sugars, 2,3-BD, and by-products.

**Analytical methods**. Samples were withdrawn periodically and centrifuged at 12,000 × g for 10 min. The concentration of fructose was measured using the 3,5-dinitrosalicylic acid method after the sample was diluted to an appropriate concentration. The concentration of 2,3-BD was analyzed by gas chromatography (GC) (GC2014c; Shimadzu) using capillary GC columns (AT SE-54; inside diameter, 0.32 mm; length, 30 m [Chromatographic Technology Center, Lanzhou Institute of Chemical Physics, China]). The operating conditions were as follows: nitrogen was used as the carrier gas; the injector temperature and detector temperature were both 280°C; the column oven was maintained at 80°C for 3 min; and the injection volume was 1 μL. The concentrations of by-products, including acetic acid, formic acid, lactic acid, and ethanol, were measured by high-performance liquid chromatography (HPLC) (Agilent 1100 series; Hewlett-Packard) as described by Li et al. (7). The stereoisomeric composition of the 2,3-BD produced by strain ATCC 14580 was analyzed by GC (GC6820; Agilent) as described by Li et al. (7).

**RESULTS**

**B. licheniformis selection for 2,3-BD production.** Kango reported that inulinase and levansase can easily hydrolyze inulin to fructose and some glucose (13). Therefore, with the help of inulinas or levansases that have optimum temperatures in the range of 45°C to 55°C, inulin can be used as a substrate by thermophilic strains in SSF for 2,3-BD production. To select a strain for efficient 2,3-BD production from inulin, we looked for strains that utilize inulin efficiently at a temperature in the range of 45°C to 55°C, inulin can be used as a substrate by thermophilic strains in SSF for 2,3-BD production. To select a strain for efficient 2,3-BD production.

**Fermentation temperature optimization.** In a previous report, the final concentration of 2,3-BD produced by *B. licheniformis* NCIMB 8059 was obviously affected by temperature, and the optimal temperature for 2,3-BD production was 37°C (22). However, our previous study indicated that the optimal fermentation temperature for 2,3-BD production by strain 10-1-A is 50°C (7). Thus, in the current study, the effect of temperature on 2,3-BD production by strain ATCC 14580 was also studied. As shown in Fig. 1. B. licheniformis selection for 2,3-BD production. The fermentation was conducted in 500-ml Erlenmeyer flasks containing 100 ml of medium with shaking at 180 rpm on a rotary shaker for 10 h. The initial concentration of fructose was 80 g/liter.

**Cloning and expression of sacC.** When inulin was directly used as the substrate, approximately 10.0 g/liter 2,3-BD was produced by strain ATCC 14580 in 24 h. This result suggests that there may be an enzyme with inulin hydrolysis activity in the *B. licheniformis* strain used. Based on analysis of the genome sequences of strains ATCC 14580, 5-2-D, and 10-1-A, there are sacC genes in the genomes of the above-mentioned strains that are predicted to encode levansase (20, 23). The amino acid sequence of the levansase in strain ATCC 14580 (GenBank accession number AAU24331.1) shares high identity (79%) with that of *B. subtilis* (GenBank accession number P05656.1) (24). It also shares identity (approximately 50%; amino acids 1 to 500) with other inulinas, based on multiple-sequence alignment (Fig. 2A). Phylogenetic tree analysis of the inulinas and levansases from different strains also indicates that they may have similar functions in inulin hydrolysis (Fig. 2B). Therefore, we speculated that the levansase in strain ATCC 14580 could also hydrolyze inulin. Then, the sacC gene from strain ATCC 14580 was cloned and expressed in *E. coli*. A crude extract of induced *E. coli* BL21(DE3) (pETDuet-sacC) cells showed high inulinase activity (37.9 ± 3.9 U/mg), and no inulinase activity was detected in the control extract. The purified enzyme was detected by SDS-PAGE, as shown in Fig. S2 in the supplemental material. The relative molecular mass of the recombinant enzyme, as estimated by SDS-PAGE, was approximately 66.0 kDa. The specific activity of the purified enzyme was 987.0 U/mg, which was higher than the activities of the levansase in *B. subtilis* (97.0 U/mg) (24), the inulinase in *A. niger* AF10 (156.0 U/mg) (25), and the inulinase in *Candida kluwanensis* sp. nov. KRF1T (188 U/mg) (26). These results suggested that the levansase from *B. licheniformis* ATCC 14580 might be a good candidate for inulin hydrolysis.
Characterization of the ATCC 14580 levanase. Effects of pH on the enzymatic activity and stability of the purified levanase from strain ATCC 14580 are shown in Fig. 3. Maximum inulinase activity was observed at pH 6.5 (Fig. 3A), and considerable activity was observed between pH 5.5 and pH 7.0. In addition, the enzyme was stable at pH values between 5.0 and 11.0, as shown in Fig. 3B. When the purified levanase was incubated at pH values lower than 5.0 for 1 h, inulinase activity decreased rapidly. In contrast, after incubation at pH 11.0 for 1 h, nearly 100.0% of the activity observed at the optimal pH was maintained, indicating strong resistance to alkaline conditions.

Optimum activity of the purified levanase was observed at 60°C (Fig. 3C). At temperatures below 50°C, the levanase showed good thermostability, and the enzyme retained 89% of its original activity after incubation for more than 1 h at 55°C and pH 6.5 (Fig. 3D). After incubation at temperatures above 65°C for 1 h, the enzyme was completely inactivated.

Fermentation of inulin to 2,3-BD by SHF and SSF. As SSF can circumvent the inhibitory effect of a high sugar concentration on cell growth, it usually leads to higher productivity and a higher target product concentration than SHF (14). In this study, we also compared 2,3-BD production from inulin by SSF with that by SHF. An enzyme dosage of 30.0 U/g inulin was chosen to obtain a favorable concentration of reducing sugars in SSF (see Fig. S3 in the supplemental material). As shown in Fig. 4A, in SSF the released reducing sugars were metabolized to 2,3-BD, and almost no reducing sugars were observed in the medium after 12 h. Conversely, in SHF there was greater than 10 g/liter reducing sugar after 12 h. Although the concentrations of 2,3-BD produced in SHF and SSF were nearly the same, the 2,3-BD productivity in SSF
was 1.2 times that in SHF, and the biomass in SSF was also higher than that in SHF (Fig. 4A). The initial reducing sugar concentration in SHF was higher than that in SSF, and the higher sugar concentration might limit cell growth and 2,3-BD productivity in SHF. Thus, compared to SHF, SSF is more suitable for 2,3-BD production from inulin by strain ATCC 14580.

**Fed-batch SSF for production of 2,3-BD.** To achieve higher BD production, a fed-batch SSF was conducted in a 5-liter stirred bioreactor. Inulin (180.0 g/liter) was used as the initial substrate. When the reducing sugar concentration was decreased to approximately 25.0 g/liter, inulin (20.0 g/liter) and levanase (30.0 U/g inulin) were added to the bioreactor. As shown in Fig. 4B, the concentration of reducing sugar increased in the first 4 h and then quickly decreased with the rapid growth of the cells. The highest 2,3-BD concentration obtained was 103.0 g/liter with some acetoin (AC) (2.0 g/liter) after 30 h in the fed-batch SSF, in which 2,3-BD productivity was high (3.4 g/liter · h). Moreover, the stereoisomeric composition of the 2,3-BD produced by strain ATCC 14580 was analyzed by GC. We found that it was a mixture of (2R,3R)-2,3-BD and meso-2,3-BD, at a ratio of nearly 1:1, that was also similar to the 2,3-BD produced by *B. licheniformis* strain 10-1-A (7).

**DISCUSSION**

As substrate materials constitute a large part of the cost for microbial 2,3-BD production, a variety of biomasses, such as molasses, corncob acid hydrolysate, waste starch hydrolysate, glycerol, cassava powder, and corncob molasses, have been tested and show promise to reduce the cost of fermentation (1, 8, 27). Although lignocelluloses are inedible and represent one of the most abundant and inexpensive biomass sources in the world, their utilization is more difficult than storage polysaccharides, such as starch and inulin (1). Recently, Sun et al. showed that a fed-batch SSF is more effective than SHF for the conversion of Jerusalem artichoke powder with inulinase (from Novozymes) to 2,3-BD, and 84.0 g/liter 2,3-BD was obtained by using *Klebsiella pneumoniae* (17). However, this strain is categorized by the World Health Organization as a risk group 2 species (3); therefore, it is not suitable for
Fermentation of Inulin to 2,3-Butanediol

A

![Graph showing concentration of reducing sugars and 2,3-BD in SSF and SHF processes.](http://aem.asm.org/)

B

![Graph showing time course of the fed-batch fermentation.](http://aem.asm.org/)

**TABLE 1** Comparison of 2,3-BD production rates using different low-cost substrates and various bacterial strains

<table>
<thead>
<tr>
<th>Organism</th>
<th>Substrate</th>
<th>Fermentation style</th>
<th>Concentration (g/liter)</th>
<th>Productivity (g/liter·h)</th>
<th>Reference or Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>F. polymyxa</em></td>
<td>Jerusalem artichoke</td>
<td>SSF</td>
<td>44.0</td>
<td>0.8</td>
<td>35</td>
</tr>
<tr>
<td><em>B. licheniformis</em></td>
<td>Inulin extract</td>
<td>SSF</td>
<td>36.9</td>
<td>0.9</td>
<td>18</td>
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<tr>
<td><em>K. pneumoniae</em></td>
<td>Starch</td>
<td>Batch</td>
<td>6.4</td>
<td>0.1</td>
<td>22</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>Inulin hydrolysate</td>
<td>SSF</td>
<td>103.0</td>
<td>3.4</td>
<td>This study</td>
</tr>
<tr>
<td><em>K. pneumoniae</em></td>
<td>Cornstarch</td>
<td>Fed-batch</td>
<td>78.9</td>
<td>1.3</td>
<td>8</td>
</tr>
<tr>
<td><em>E. cloacae</em></td>
<td>Cassava powder</td>
<td>SSF</td>
<td>93.9</td>
<td>2.0</td>
<td>27</td>
</tr>
</tbody>
</table>

*E. cloacae, Enterobacter cloacae.*

Industrial-scale production of 2,3-BD (28). *Paenibacillus polymyxa* has also been used for 2,3-BD production from Jerusalem artichoke, and the highest concentration of 2,3-BD obtained was 44.0 g/liter, with a productivity of 0.79 g/liter·h (35). Although *P. polymyxa* could produce 2,3-BD from inulin without the addition of inulinase, the concentration and productivity are not sufficient for economical production of 2,3-BD. In addition, both of the strains used for 2,3-BD production from inulin have optimum temperatures between 30°C and 37°C. For SSF, it is very important that saccharification and fermentation be carried out under the same conditions, including temperature and pH (29). The conditions for thermophilic fermentation match those of the saccharification of inulin, as most inulinases and levans have an optimum temperature in the range of 45°C to 55°C. In addition, thermophilic fermentation can reduce the risk of bacterial contamination and can even be operated without sterilization, making it more efficient and cost-effective (30). In this study, *B. licheniformis* ATCC 14580, which is widely used for the production of enzymes and antibiotics, was found to produce 103.0 g/liter 2,3-BD from inulin in 30 h, with a productivity of 3.4 g/liter·h at 50°C. The concentration of 2,3-BD and the productivity of the strain are both the highest attained for 2,3-BD production from cheap raw materials (Table 1). Thus, it might be a promising thermophilic strain for the production of 2,3-BD from inulin.

Levan is one of two main types of fructans consisting mainly of β-2,6-α-fructosyl-fructose links in the main chain and β-2,1-α-fructosyl-fructose links in the branch chain (31). Levanase is a fructofuranosyl hydrolase, which hydrolyzes both the β-2,6-α- and β-2,1-α-fructosyl-fructose links in levan. Like inulinase, levanase also hydrolyzes β-2,1-α-fructosyl-fructose links in inulin (24). Generally, the optimum pH values of inulinases from fungi and yeast, such as *Aspergillus awamori* (pH 4.5), *Penicillium janczewskii* (pH 4.8–5.0), *Aspergillus niger* AF10 (pH 4.5), and *Kluyveromyces marxianus* YS-1 (pH 5.5), are in the range of 4 to 5.5 (25, 32–34). However, the optimum pH range for microbial 2,3-BD production is generally between 5.5 and 7.0 (3). In addition, the commercial inulinase (an inulinase from *A. niger*) is expensive (18). These factors make the inulinases from fungi and yeast unsuitable for the production of 2,3-BD from inulin by a SSF process. A commercial glucoamylase, GA-L New (Genencor), could be used for Jerusalem artichoke tuber hydrolysis. However, the optimum pH of this glucoamylase for inulin hydrolysis is 4.0, and the stability of this enzyme decreased significantly when the pH was increased to 5.6 (19). This also makes GA-L New unsuitable for 2,3-BD production via an SSF process. In this study, we found a gene in the genome of strain ATCC 14580 which is predicted to encode a levanase (*sacC*) and exhibits high sequence identity with the levanase in *B. subtilis*. The purified levanase has an optimum pH of 6.5 and an optimum temperature of approximately 60°C (Fig. 3A and C). The specific activity of the purified enzyme was 987.0 U/mg at 50°C, and this was 80% of the maximum at 60°C (18). These factors make the inulinases from fungi and yeast unsuitable for the production of 2,3-BD from inulin by a SSF process. A commercial glucoamylase, GA-L New (Genencor), could be used for Jerusalem artichoke tuber hydrolysis. However, the optimum pH of this glucoamylase for inulin hydrolysis is 4.0, and the stability of this enzyme decreased significantly when the pH was increased to 5.6 (19). This also makes GA-L New unsuitable for 2,3-BD production via an SSF process. In this study, we found a gene in the genome of strain ATCC 14580 which is predicted to encode a levanase (*sacC*) and exhibits high sequence identity with the levanase in *B. subtilis*. The purified levanase has an optimum pH of 6.5 and an optimum temperature of approximately 60°C (Fig. 3A and C). The specific activity of the purified enzyme was 987.0 U/mg at 50°C, and this was 80% of the maximum at 60°C (Fig. 3C). This activity is higher than the specific activities of the levanase from *B. subtilis* and other inulinases, such as those of *A. niger* AF10 and *K. marxianus* YS-1. The levanase from strain ATCC 14580 has significant temperature (Fig. 3D) and pH.
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