Title: Isolation and evaluation of Bacillus strains for industrial production of 2,3-butanediol

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Isolation and evaluation of *Bacillus* strains for industrial production of 2,3-butanediol

Chan Woo Song, Chelladurai Rathnasingh, Jong Myoung Park, Julia Lee, Hyohak Song*

Research and Development Center, GS Caltex Corporation, Yuseong-gu, Daejeon 34122, Republic of Korea

* Corresponding author: Hyohak Song

Research and Development Center, GS Caltex Corporation, Yuseong-gu, Daejeon 34122, Republic of Korea

Tel: +82-42-866-1613

Fax: +82-42-866-1769

E-mail address: hyohaks@gscaltex.com

Running title: Isolation of *Bacillus* strains for 2,3-butanediol production
Abstract

Biologically produced 2,3-butanediol (2,3-BDO) has diverse industrial applications. In this study, schematic isolation and screening procedures were designed to obtain generally regarded as safe (GRAS) and efficient 2,3-BDO producers. Over 4,000 candidate strains were isolated by pretreatment and enrichment, and the isolated Bacillus strains were further screened by morphological, biochemical, and genomic analyses. The screened strains were then used to test the utilization of the most common carbon (glucose, xylose, fructose, sucrose) and nitrogen (yeast extract, corn steep liquor) sources for the economical production of 2,3-BDO. Two-stage fed-batch fermentation was finally carried out to enhance 2,3-BDO production. In consequence, a newly isolated B. licheniformis GSC3102 strain produced 92.0 g/L of total 2,3-BDO with an overall productivity and yield of 1.40 g/L/h and 0.423 g/g glucose, respectively, using a cheap and abundant nitrogen source. These results strongly suggest that Bacillus licheniformis, which is widely found in nature, can be used as a host strain for the industrial fermentative production of 2,3-BDO.

Keywords: 2,3-Butanediol • Bacillus licheniformis • Isolation • Screening • Fermentation
Introduction

Due to its various industrial applications, 2,3-Butanediol (2,3-BDO), a chiral bivalent alcohol with 3 stereoisomers ((2R,3S)-, (2R,3R)-, and (2S,3S)-BDO) [1], has been regarded as a promising chemical. Dehydration of 2,3-BDO leads to bulk chemicals, which include methyl ethyl ketone, an important solvent, and 1,3-butadiene, a building block of synthetic rubber [2, 3]. Also, owing to its high heating value (27,200 J/g) and low freezing point, it can be used as a liquid fuel additive and antifreeze agent [2]. Furthermore, its usage has been suggested in many industrial areas, such as food, cosmetics, medicine, and agriculture [4, 5]. The range is still expanding and the market size of 2,3-BDO is expected to reach 74 kilo tons by 2018 [6]. Despite the aforementioned industrial potential of 2,3-BDO, its usage has still been quite limited, mainly because of its high production cost [7]. 2,3-BDO is currently manufactured by hydrolysis of 2,3-butene oxide at high temperature (160–220°C) and pressure (50 bar), via a number of catalytic reactions [2].

There is growing interest in the fermentative production of 2,3-BDO, because this process is environmentally friendly (using renewable biomass) and can be operated under mild conditions (37°C, normal pressure). In particular, the fermentative 2,3-BDO production process enables the economical production of each stereoisomer with much higher optical purity than the chemical process [2]. It has been reported that 2,3-BDO is produced by a number of microorganisms, such as Klebsiella pneumoniae, K. oxytoca, Enterobacter aerogenes, and Serratia marcescens [2, 3, 8–11]. Among them, Bacillus species, including Paenibacillus polymyxa, Bacillus licheniformis, and B. amyloliquefaciens, have been recognized as generally regarded as safe (GRAS) and efficient 2,3-BDO producers [12–15].

In this study, we conducted the isolation and screening of Bacillus strains from soil and plant roots to obtain a robust 2,3-BDO producer classified as a GRAS microorganism.
We obtained more than 4,000 colonies by applying newly designed schematic isolation and screening procedures. The shortlisted 8 candidate strains based on biochemical and 16S rRNA sequencing tests were further evaluated in terms of their 2,3-BDO production ability. The finally selected GSC3102 strain was identified as *B. licheniformis*, and showed the best 2,3-BDO production performance. Further fed-batch fermentation studies using *B. licheniformis* GSC3102 strain showed dramatically enhanced 2,3-BDO production.

**Materials and Methods**

**Isolation and characterization of Bacillus strains**

Soil and plant root samples were collected from several sampling sites around Mt. Gyeryongsan (GPS coordinate: N36°20’33”E127°12’20.999”) and Mt. Geumseongsan (GPS coordinate: N35°2’53.502”E126°42’13.625”) of South Korea, as many *Bacillus* species are found in food, soil, and plant growing areas [16–18]. Samples were heat treated at 80°C for 30 min after suspension in 50 mL 0.1 M MgSO$_4$ solution for spore activation, or directly enriched in MRS medium at 50°C (Fig. 1). Small aliquots of those heat-treated spores or cultivated cells were spread on 1/10 diluted tryptic soy agar (TSA) medium containing 3 g/L tryptic soy broth (TSB) and 10 g/L glucose. Grown colonies were inoculated into 96-deep well plates filled with 800 μL MRS-VP medium. After 24 h cultivation, culture broth was used for Voges-Proskauer (VP) test to distinguish acetoin, 2,3-BDO precursor, producing cells from background [19]. A small aliquot (100 μL) of culture broth was added into each 96-well plate, and a reaction was induced by the sequential addition of 60 μL alpha-naphthol and 20 μL potassium phosphate. A cherry red color was detected as a positive result if acetoin existed in the culture broth after mixing for 10 min, while a yellow-brown color indicated a negative result. The 16S rRNA sequencing was carried out using the universal bacterial
primers as follows: 27F, 5’-GAGAGTTTGATCCTGGCTCAG-3’ and 1492R, 5’-ACGGCTACCTTGTACGACTT-3’. Multiple alignment and phylogenetic tree construction of the 16S rDNA sequences of strains were carried out using PhyML and TreeDyn programs [20, 21]. The 16S rDNA sequence data of isolated strains were submitted on the PubMed database and accession numbers are, GSC169 [MG576136]; GSC1836 [MG576137]; GSC2198 [MG576138]; GSC3102 [MG576139]; GSC3107 [MG576140]; GSC3205 [MG576141]; GSC3210 [MG576142]; GSC3211 [MG576143]. Accession numbers for related species are, Bacillus cereus ATCC14579 [NR_074540.1]; Bacillus thurigiensis ATCC10792 [CP020754.1]; Bacillus subtilis 168 [CP019662.1]; Bacillus licheniformis DGT14 [KX768314.1]; Bacillus amyloliquefaciens FJAT25563 [KY038794.1]; Bacillus anthracis T42 [KM019703.1]; Bacillus pumilus RC13 [KM459021.1]; Bacillus siamensis N-12 [MG066462.1]. Additional biochemical and physiological characteristics of GSC3102 strain, including starch hydrolysis, growth in 6.5% NaCl at 55°C, and citrate utilization were determined according to Bergey’s manual of Determinative Bacteriology [22]. The newly isolated B. licheniformis GSC3102 strain was deposited in the Korean Collection for Type Cultures (KCTC), and the accession number given by the international depositary authority is KCTC13282BP.

**Medium composition and flask cultivation**

Isolated cells were cultivated in the production medium containing: 45.0 g/L glucose, 4.00 g/L K\( _2 \)HPO\(_4 \), 1.00 g/L triammonium citrate, 4.00 g/L sodium acetate, 10.0 g/L yeast extract (YE), and 0.800 g/L MgSO\(_4\)·7H\(_2\)O. Cultivation was conducted in 100 mL Erlenmeyer flasks containing 20 mL of the medium in a rotary shaker at 180 rpm. The cultivation temperatures for close relatives of Bacillus cereus strains were 37°C, and of Bacillus subtilis and
Licheniformis strains were 50°C [11]. The effect of initial glucose concentrations on 2,3-BDO production was investigated for the values of (25, 50, 75, 100, 125, and 150) g/L. Other mono- and di-saccharides, including xylose, fructose, and sucrose, were also tested as alternative carbon sources. In a similar way, yeast extract was replaced with corn steep liquor (CSL, Daesang Corporation, Gunsan, Korea) as a cheap nitrogen source. The mixing compositions of nitrogen sources used in this study were as follows: 10 g/L YE, 7.5 g/L YE + 2.5 g/L CSL, 5 g/L YE + 5 g/L CSL, 2.5 g/L YE + 7.5 g/L CSL, 10 g/L CSL, 20 g/L CSL, 30 g/L CSL, and 40 g/L CSL.

**Two-stage fed-batch fermentation**

Single colonies from 1/10 diluted TSA medium plates were inoculated into 50 mL of conical tube containing 10 mL of TSB, and cultured overnight. 0.5 mL culture broth was transferred into a 500 mL Erlenmeyer flask containing 300 mL TSB. When the optical density at 600 nm (OD$_{600}$) reached around 1.5–2.0, 300 mL culture broth was inoculated into a 5 L bioreactor. The tube and flask cultivations were carried out in a rotary shaker at 180 rpm and 50°C. Fed-batch fermentations were carried out in a 5 L BIOFLO&CELLIGEN 310 bioreactor (New Brunswick Scientific Co., Edison, NJ) with 3 L of culture medium. The medium contained 100 g/L glucose, 4.00 g/L K$_2$HPO$_4$, 1.00 g/L triammonium citrate, 4.00 g/L sodium acetate, 0.800 g/L MgSO$_4$·7H$_2$O, and 10.0 g/L yeast extract or 40.0 g/L CSL. The pH was controlled at 7.0 using 28% (w/v) NH$_4$OH and 6 M acetic acid. Foaming was controlled by the addition of Antifoam 289 (Sigma, St. Louis, MO). Fed-batch fermentations were performed by intermittent feeding of 150 g glucose powder when the residual glucose concentration is around 20 g/L. Two-stage fermentation was performed by changing the agitation speed. Samples were periodically taken for the analysis of cell growth (OD$_{600}$) and metabolites.
Analytical procedures

Cell growth was monitored by measuring OD$_{600}$ using UV-VIS spectrophotometry (DR5000, Hach Company, CO). Concentrations of glucose and metabolites, including 2,3-BDO, formate, ethanol, acetate, lactate, succinate, and acetoin, were determined by HPLC equipped with UV-VIS and RI detectors (Agilent 1260 series, Agilent Technologies, Waldbronn, Germany). The samples were isocratically eluted by Aminex HPX-87H column with 0.01 N H$_2$SO$_4$ at 80°C and a flow rate of 0.6 mL/min. Stereoisomers of 2,3-BDO were determined by gas chromatograph with flame ionization detector (GC-FID; HP 6890 series, Hewlett Packard, Palo Alto, CA, USA) equipped with HP-chiral 20ß column (30 m, 0.32-mm internal diameter, 0.25-μm film thickness; Agilent Technologies, Waldbronn, Germany) based on a previous method [23].

Results and Discussion

Isolation of 2,3-BDO producers from soil and plant root samples

Schematic isolation and screening procedures were designed in this study to find 2,3-BDO producing Bacillus strains (Fig. 1). We could obtain diverse types of Bacillus strains by employing two independent isolation methods: 1) Samples were heat treated at 80°C to activate Bacillus spores and kill other bacteria, and 2) Samples were directly enriched at 50°C to obtain thermophilic Bacillus species. Heat-treated or enriched cells were plated on 1/10 diluted TSA to isolate a single cell from the mixture of diverse cells.

Morphologically large colonies were selected and a total 4,000 strains were cultured in a 96 deep-well plate to choose strains which are fast-growing, and sequentially VP-test was conducted to determine potential 2,3-BDO producing strains. Only strains displayed VP
positive, showing strong cherry red color under reaction condition were selected and those were further subjected for ribotyping. As a result, 8 candidate Bacillus strains, namely close relatives of *B. cereus* (GSC169, GSC1836, and GSC2198), close relatives of *B. subtilis* (GSC3210 and GSC3211), and close relatives of *B. licheniformis* (GSC3102, GSC3107, and GSC3205), were identified, and their phylogenetic tree was drawn based on 16S rDNA sequence (Fig. 2). The close relatives of *B. cereus* were only isolated from the heat treatment method, but close relatives of *B. subtilis* and *B. licheniformis* were isolated by the direct enrichment method.

**Performance evaluation of isolated strains for 2,3-BDO production**

The isolated 2,3-BDO producing *Bacillus* strains were further studied in a shake flask cultivation for 24 h, in which the medium contained 10.0 g/L yeast extract and 45.0 g/L glucose. Only 8 candidate strains from the screening library yielded more than 5 g/L of 2,3-BDO (Fig. 3). Close relatives of *B. cereus* (GSC169, GSC1836, and GSC2198) and close relatives of *B. subtilis* (GSC3210 and GSC3211) mainly produced (2R,3R)-BDO with isomer selectivity of more than 97.0%. On the other hand, close relatives of *B. licheniformis* (GSC3102, GSC3107, and GSC3205) simultaneously produced (2R,3R)- and (2R,3S)-BDO with isomer ratio of 2:3. Lactate and acetate were observed as major byproducts, and their production profiles were similar in all the candidate strains.

Among close relatives of *B. cereus*, GSC1836 strain produced the highest amount of (2R,3R)-BDO (8.95 g/L), with a selectivity of almost 100% (Table 1). In the case of close relatives of *B. subtilis*, GSC3211 strain showed the maximum (2R,3R)-BDO production ability (10.4 g/L), with a selectivity of 98.4%. The best performance on 2,3-BDO production among all the isolated strains was observed with GSC3102 strain, which produced more than
18.1 g/L of 2,3-BDO. This value is about 102 and 74\% higher than those obtained from GSC1836 and GSC3211 strains, respectively. Although the 2,3-BDO isomer selectivity of GSC3102 is relatively lower than those of GSC1836 and GSC3211 strains, the selectivity is to be improved through metabolic engineering [24]. Further biochemical analyses of GSC3102 strain such as starch hydrolysis, citrate utilization, and growth at 6.5% NaCl and 55°C, confirmed that the strain is characterized as *B. licheniformis* (GSC3102). Apart from issues such as the potential pathogenicity of *B. cereus* [25] and low productivity of isolated close relatives of *B. subtilis*, the thermophilic characteristics of *B. licheniformis* are advantageous in industrial fermentation, in terms of reducing contamination risk, cooling energy, and increasing the simultaneous saccharification and fermentation performance [26]. Consequently, we chose *B. licheniformis* GSC3102 as the host strain for further studies.

**Feasibility test on the economical utilization of carbon and nitrogen sources**

The utilization of a variety of carbon and nitrogen sources by *B. licheniformis* GSC3102 strain for the economical production of 2,3-BDO was tested in 24 h shake flask cultivation. Firstly, the effect of substrate inhibition on 2,3-BDO production was investigated in various initial glucose concentrations ranging from 25 to 150 g/L [27]. As a result, as the initial glucose concentration increased to 100 g/L, the concentration of 2,3-BDO gradually increased to 33.2 g/L (Fig. 4A). However, further increase in the initial glucose concentration led to a drop in 2,3-BDO production. The utilization of sucrose and fructose by *B. licheniformis* GSC3102 strain was tested, because sugarcane containing sucrose, fructose, and glucose is a dominant fermentation feedstock. The strain produced 31.0 and 30.4 g/L of 2,3-BDO using sucrose and fructose as the sole carbon source, respectively, which values are almost equivalent to that of glucose (Fig. 4A). However, the ability of *B. licheniformis*
GSC3102 strain to utilize xylose, which is a major C5 sugar derived from lignocellulosic feedstock [28], is quite lower than that of the other carbon sources. This means that the development of *B. licheniformis* strains through metabolic engineering or adaptive evolution is required to use the most abundant lignocellulosic biomass.

In order to evaluate the industrial application of CSL as a complex nitrogen source for 2,3-BDO production, batch fermentation in media containing different ratio of CSL and yeast extract (from 0.0 to 10.0 g/L) were performed using *B. licheniformis* GSC3102 strain. Fig. 4B shows that with increasing CSL content, the 2,3-BDO concentration was gradually decreased from 33.1 g/L, and when yeast extract was fully replaced with CSL, dropped to 24.6 g/L. However, the final concentration of 2,3-BDO in 40.0 g/L CSL-containing medium was 34.6 g/L, which is slightly higher than that obtained in 10 g/L yeast extract containing medium.

This result indicates that CSL can be used for the industrial fermentative production of 2,3-BDO by *B. licheniformis* GSC3102 strain. This could be supported by the previous research that *Clostridium acetobutylicum* produced a higher amount of n-butanol in 3 to 6% CSL-containing medium, instead of yeast extract and L-asparagine as a nitrogen source [24].

**Two-stage fed-batch fermentation strategy for the enhanced production of 2,3-BDO**

The dissolved oxygen (DO) level has been known as a key parameter in the effective production of 2,3-BDO by fermentation [29]. To find a proper oxygen supply condition, several parameters such as oxygen transfer rate (OTR), oxygen transfer coefficient (KLa), and respiratory quotient (RQ) were utilized as determining factors to find optimal aerobic condition in conventional studies [32-34]. However, controlling those parameters were not easy in a real fermentation circumstances and there was a demand for finding more controllable methods. In this context, two-stage fed-batch fermentation was performed to
optimize the condition for the efficient production of 2,3-BD in this study. The initial agitation speed for 2,3-BDO production was determined based on previous studies [29–31]. The aeration rate was fixed at 1 vvm, and the initial agitation speed was set to 500 rpm. The fermentation was carried out in medium containing 10.0 g/L yeast extract and 40.0 g/L CSL, respectively. 2,3-BDO is to be synthesized via the dehydration of acetoin, and the bacteria also easily uptake 2,3-BDO to produce acetoin under high oxygen conditions. Furthermore, acetoin has been known to strongly inhibit the growth of 2,3-BDO producing bacteria [31]. Therefore, in 10.0 g/L yeast extract-containing medium, the agitation speed was reduced from 500 to 400 rpm in 40 h fermentation, at which time the concentration of acetoin reached 10.2 g/L, and gradually decreased to 3.93 g/L in 16 h (which is 56 h fermentation) with the increment of 2,3-BDO from 69.0 g/L to 93.7 g/L (Fig. 5A). On the other hand, at the maximum production of 2,3-BDO in the CSL-containing medium, the concentration of acetoin was 6.15 g/L, and therefore the agitation speed was maintained at 500 rpm during the whole period of fermentation (Fig. 5B). The maximum OD$_{600}$ values were almost similar in both cases, but it was observed that CSL-containing medium reached the maximum OD$_{600}$ value faster than yeast extract-containing medium (17.0 at 20 h and 17.2 at 46 h). Overall, 2,3-BDO was continuously produced during exponential and stationary growth phases in both conditions. The final titers of 2,3-BDO in yeast extract- and CSL-containing media were not much different (95.9 and 92.0 g/L). Although the overall productivity and yield on glucose were slightly lower in 40.0 g/L CSL-containing medium than in 10.0 g/L yeast extract-containing medium, the differences were not significant (1.50 g/L/h and 0.431 g/g glucose in the yeast extract medium, 1.40 g/L/h and 0.423 g/g glucose in the CSL medium). It can be seen that the production performance is excellent when compared with previous results comprehensively in terms of titer, productivity and yield (Table 2). In particular, B.
**licheniformis** GSC3102 strain has advantages not only for the performance but also for high temperature (50°C) culturing condition when compared with similar study carried out by Yang et al, who has isolated *B. amyloliquefaciens* strain producing 92.3 g/L of 2,3-BDO with a productivity of 0.96 g/L/h at 37°C based on optimization experiments in terms of initial glucose concentration, temperature, pH and agitation on 2,3-BDO production [35]. The nitrogen source also did not affect the ratio of 2,3-BDO isomers. *B. licheniformis* GSC3102 strain produced 2,3-BDO with (2R,3S) : (2R,3R) ratio of 1.67:1 in both the media. Formate was accumulated as the major byproduct up to (29.1 and 18.9) g/L in the yeast extract- and CSL-containing media, respectively. The accumulation of formate in culture broth should be avoided, because this byproduct freely penetrates cell membranes, and has a tendency to reduce intracellular pH, leading to strong cell growth inhibition [31]. In the previous study, we improved production titer and yield of 2,3-BDO, and eliminated accumulation of formate by inactivating the pyruvate formate-lyase activity in *K. oxytoca* [29]. Although new isolate was employed to see natural fermentation capability in this study, it is believed that the performance of the strain could be improved by metabolic engineering strategies based on genetic background in the future works.

In conclusion, newly designed schematic isolation and screening procedures were well validated for the efficient isolation of 2,3-BDO producers in this study. The selected GSC3102 strain was identified as *B. licheniformis*, and it produced a high amount of 2,3-BDO at 50°C. *B. licheniformis* GSC3102 strain could utilize glucose, fructose, and sucrose as a carbon source with a high initial concentration of up to 100 g/L. Also, *B. licheniformis* GSC3102 strain has the ability to use a cheap and abundant nitrogen source (CSL), instead of an expensive yeast extract. This study demonstrates that *B. licheniformis* GSC3102 strain isolated from the natural environment has great potential for the industrial production of 2,3-
BDO. Further studies, including optimal medium composition, fermentation conditions, and strain improvement, are underway to develop an economically useful 2,3-BDO production system.
Acknowledgement

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Conflict of interest

All authors declare that they have no conflict of interest.

Figure legend

Fig. 1. A schematic of the screening and isolation of 2,3-BDO producing Bacillus strains.

Fig. 2. Phylogenetic tree of isolated Bacillus strains and other related species were constructed based on 16S rDNA sequences using the maximum-likelihood principle. The scale bar represents 0.01 substitutions per site.

Fig. 3. Comparison of the total amounts and isomer compositions of C4 chemicals produced by 8 isolated candidate strains. Cultivations were performed in a rotary shaker at 180 rpm for 24 h. Temperature was set to 37°C for close relatives of B. cereus, and to 50°C for close relatives of B. subtilis and licheniformis. Production medium contains 45.0 g/L glucose, 4.00 g/L K$_2$HPO$_4$, 1.00 g/L triammonium citrate, 4.00 g/L sodium acetate, 10.0 g/L yeast extract (YE), and 0.800 g/L MgSO$_4$·7H$_2$O.

Fig. 4. Comparison of the total amounts and isomer compositions of C4 chemicals produced by B. licheniformis GSC3102 strain cultured in a rotary shaker at 180 rpm and 50°C for 24 h. The effect of initial glucose concentrations and other mono and di-saccharides, including xylose, fructose, and sucrose, was tested with medium containing 10.0 g/L yeast extract (A). Also, the effect of nitrogen sources, including yeast extract and CSL, was tested with medium containing 100 g/L glucose (B).
Fig. 5. Two-stage fed-batch fermentation profiles of *B. licheniformis* GSC3102 strain under controlled pH (7.0), temperature (50°C), and aeration rate (1 vvm). When the acetoin concentration increased above 10 g/L, the agitation speed was reduced from 500 to 400 rpm. Intermittent feeding of 150 g glucose powder was carried out as the residual glucose decreased to less than 20 g/L. The media containing 10.0 g/L yeast extract (A) or 40.0 g/L CSL (B) were used for fed-batch fermentations, respectively.
Table 1. Ratio of 2,3-BDO stereoisomers produced by *Bacillus* sp. GSC1836, *Bacillus* sp. GSC3211, and *B. licheniformis* GSC3102 in the flask cultivations.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Acetoin</th>
<th>Total 2,3-BDO (g/L)</th>
<th>Ratio of (2S,3S)-BDO (%)</th>
<th>Ratio of (2R,3R)-BDO (%)</th>
<th>Ratio of (2R,3S)-BDO (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus</em> sp. GSC1836</td>
<td>1.51</td>
<td>8.95</td>
<td>0</td>
<td>100</td>
<td>0</td>
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<tr>
<td><em>Bacillus</em> sp. GSC3211</td>
<td>1.68</td>
<td>10.6</td>
<td>0</td>
<td>98.4</td>
<td>1.60</td>
</tr>
<tr>
<td><em>B. licheniformis</em> GSC3102</td>
<td>1.98</td>
<td>18.1</td>
<td>0</td>
<td>44.7</td>
<td>55.3</td>
</tr>
</tbody>
</table>
Table 2. 2,3-Butanediol (2,3-BDO) production by native *Bacillus* strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Substrate</th>
<th>2,3-BDO isomer</th>
<th>Method (temp.)</th>
<th>Conc. (g/L)</th>
<th>Yield (g/g)</th>
<th>Productivity (g/L/h)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em> AJ1992 (WT)</td>
<td>Glucose</td>
<td>Mixture</td>
<td>Batch (30°C)</td>
<td>6.1</td>
<td>0.340</td>
<td>0.41</td>
<td>[36]</td>
</tr>
<tr>
<td><em>Bacillus vallismortis</em> B-14891 (WT)</td>
<td>Glucose</td>
<td>(2R,3S)-BDO</td>
<td>Batch (39°C)</td>
<td>60.4</td>
<td>0.330</td>
<td>1.10</td>
<td>[15]</td>
</tr>
<tr>
<td><em>Bacillus amyloliquefaciens</em> B10-127 (WT)</td>
<td>Glucose</td>
<td>Mixture</td>
<td>Fed-batch (37°C)</td>
<td>92.3</td>
<td>-</td>
<td>0.96</td>
<td>[35]</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em> (WT)</td>
<td>Glucose</td>
<td>Mixture</td>
<td>Static flask (37°C)</td>
<td>8.7</td>
<td>0.470</td>
<td>0.12</td>
<td>[37]</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em> X10 (WT)</td>
<td>Concentrated corn stover hydrolysate</td>
<td>Mixture</td>
<td>Fed-batch (50°C)</td>
<td>74.0</td>
<td>0.470</td>
<td>2.06</td>
<td>[38]</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em> DSM8785 (WT)</td>
<td>Glucose</td>
<td>Mixture</td>
<td>Fed-batch (30°C)</td>
<td>144.7</td>
<td>0.400</td>
<td>1.14</td>
<td>[39]</td>
</tr>
<tr>
<td><em>Bacillus licheniformis</em> GSC3102</td>
<td>Glucose (with Yeast extract)</td>
<td>Mixture</td>
<td>Fed-batch (50°C)</td>
<td>95.9</td>
<td>0.431</td>
<td>1.50 This work</td>
<td></td>
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<tr>
<td><em>Bacillus licheniformis</em> GSC3102</td>
<td>Glucose (with CSL)</td>
<td>Mixture</td>
<td>Fed-batch (50°C)</td>
<td>92.0</td>
<td>0.423</td>
<td>1.40 This work</td>
<td></td>
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</table>
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Fig. 2. Phylogenetic tree of isolated *Bacillus* strains and other related species were constructed based on 16S rDNA sequences using the maximum-likelihood principle. The scale bar represents 0.01 substitutions per site.
Fig. 3. Comparison of the total amounts and isomer compositions of C4 chemicals produced by 8 isolated candidate strains. Cultivations were performed in a rotary shaker at 180 rpm for 24 h. Temperature was set to 37°C for close relatives of *B. cereus*, and to 50°C for close relatives of *B. subtilis* and *licheniformis*. Production medium contains 45.0 g/L glucose, 4.00 g/L K$_2$HPO$_4$, 1.00 g/L triammonium citrate, 4.00 g/L sodium acetate, 10.0 g/L yeast extract (YE), and 0.800 g/L MgSO$_4$·7H$_2$O.
Fig. 4. Comparison of the total amounts and isomer compositions of C4 chemicals produced by *B. licheniformis* GSC3102 strain cultured in a rotary shaker at 180 rpm and 50°C for 24 h. The effect of initial glucose concentrations and other mono and di-saccharides, including xylose, fructose, and sucrose, was tested with medium containing 10.0 g/L yeast extract (A). Also, the effect of nitrogen sources, including yeast extract and CSL, was tested with medium containing 100 g/L glucose (B).
Fig. 5. Two-stage fed-batch fermentation profiles of *B. licheniformis* GSC3102 strain under controlled pH (7.0), temperature (50°C), and aeration rate (1 vvm). When the acetoin concentration increased above 10 g/L, the agitation speed was reduced from 500 to 400 rpm. Intermittent feeding of 150 g glucose powder was carried out as the residual glucose decreased to less than 20 g/L. The media containing 10.0 g/L yeast extract (A) or 40.0 g/L CSL (B) were used for fed-batch fermentations, respectively.