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Isolation and Evaluation of *Bacillus* Strains for Industrial Production of 2,3-Butanediol

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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 *Bacillus 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 *B. licheniformis,* which is found widely 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

Owing to its various industrial applications, 2,3-butanediol (2,3-BDO), a chiral bivalent alcohol with three steroisomers ((2R,3S)-, (2S,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]. Moreover, 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 in 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 does the chemical process [2]. It has been reported that 2,3-BDO is produced by a number of microorganisms, such as *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Enterobacter aerogenes*, and *Serratia marcescens* [2, 3, 8–11]. Among them, *Bacillus* species, including *Paenibacillus polymyxa*, *Bacillus licheniformis*, and *Bacillus 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 eight candidate strains shortlisted on the basis of 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* strain GSC3102 showed dramatically enhanced 2,3-BDO production.

Materials and Methods

Isolation and Characterization of *Bacillus* Strains

Soil and plant roots 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 of 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
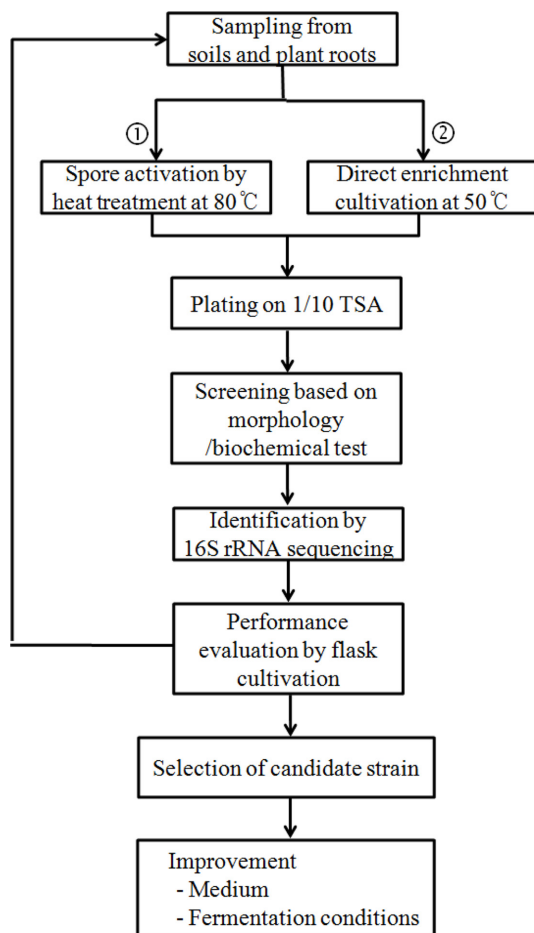

Fig. 1. Schematic of the screening and isolation of 2,3-butanediol-producing *Bacillus* strains.

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 of MRS-VP medium. After 24 h cultivation, culture broth was used for the 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 the reaction was induced by the sequential addition of 60 μl of alpha-naphthol and 20 μl of potassium phosphate. A cherry red color was detected as a positive result if acetoin existed in the culture broth after mixing for 10 min, whereas a yellow-brown color indicated a negative result. The 16S rRNA sequencing was carried out using the following universal bacterial primers: 27F, 5′-GAGAGTTTGATC CTGGCTCAG-3′ and 1492R, 5′-ACGGCTACCTTGTTACGACTT-3′. Multiple alignment and phylogenetic tree construction of the 16S rDNA sequences of the strains were carried out using the PhyML and TreeDyn programs [20, 21]. The 16S rDNA sequence data of the isolated strains were submitted on the PubMed database, and the accession numbers are GSC169 [MG576136]; GSC1836 [MG576137]; GSC2198 [MG576138]; GSC3102 [MG576139]; GSC3107 [MG576140]; GSC3205 [MG576141]; GSC3210 [MG576142]; and GSC3211 [MG576143]. The 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]; and *Bacillus siamensis* N-12 [MG066462.1]. Additional biochemical and physiological characteristics of the 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_2HPO_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 $MgSO_4 \cdot 7H_2O$. 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 realtives of *B. cereus* strains were 37°C, and of *B. subtilis* and *B. 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 disaccharides, including xylose, fructose, and sucrose, were also tested as alternative carbon sources. In a similar way, YE was replaced with corn steep liquor (CSL; Daesang Corporation, 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 a 50 ml conical tube containing 10 ml of TSB, and cultured overnight. Then, 0.5 ml of culture broth was transferred into a 500 ml Erlenmeyer flask containing 300 ml of TSB. When the optical density at 600 nm (OD_{600}) reached around 1.5–2.0, 300 ml of 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 and CelliGen 310 bioreactor (New Brunswick Scientific Co., USA) with 3 L of culture medium. The medium contained 100 g/l glucose, 4.00 g/l K_2HPO_4, 1.00 g/l triammonium citrate, 4.00 g/l sodium acetate, 0.800 g/l $MgSO_4 \cdot 7H_2O$, and 10.0 g/l YE or 40.0 g/l CSL. The pH was controlled at 7.0 using 28% (w/v) NH_4OH and 6 M acetic acid. Foaming was controlled by the addition of Antifoam 289 (Sigma, USA). Fed-batch fermentations were performed by intermittent feeding with 150 g of glucose powder when the residual glucose concentration was 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 the OD_{600} using UV-VIS spectrophotometry (DR5000; Hach Company, USA). 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, Germany). The samples were isocratically eluted on an Aminex HPX-87H column with 0.01 N H_2SO_4 at 80°C and a flow rate of 0.6 ml/min. Stereoisomers of 2,3-BDO were determined by gas chromatography with a flame ionization detector (HP 6890 series; Hewlett Packard, USA) equipped with an HP-chiral 20β column (30 m, 0.32-mm internal diameter, 0.25-μm film thickness; Agilent Technologies) 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: (i) Samples were heat treated at 80°C to activate *Bacillus* spores and kill other bacteria, and (ii) 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 of 4,000 strains were cultured in a 96-deep-well plate to choose fast-growing strains, and the VP test was then conducted to determine potential 2,3-BDO-producing strains. Only VP-positive strains showing a strong cherry red color under the reaction conditions were selected, and those were further subjected to ribotyping. As a result, eight candidate *Bacillus* strains, namely close relatives of *B. cereus* (GSC169, GSC1836, and GSC2198), of *B. subtilis* (GSC3210 and GSC3211), and of *B. licheniformis* (GSC3102, GSC3107, and GSC3205), were identified and their
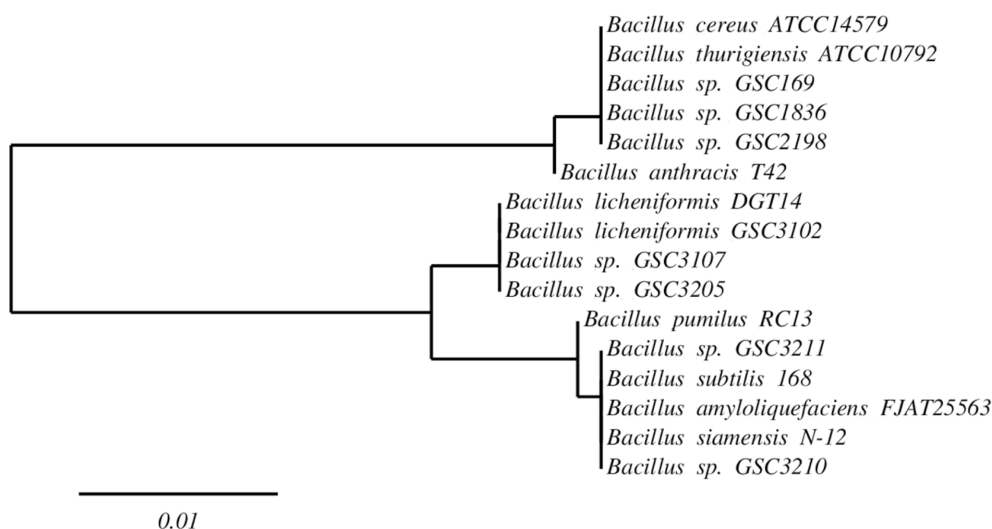

Fig. 2. Phylogenetic tree of isolated *Bacillus* strains and other related species based on 16S rDNA sequences, constructed using the maximum-likelihood principle.
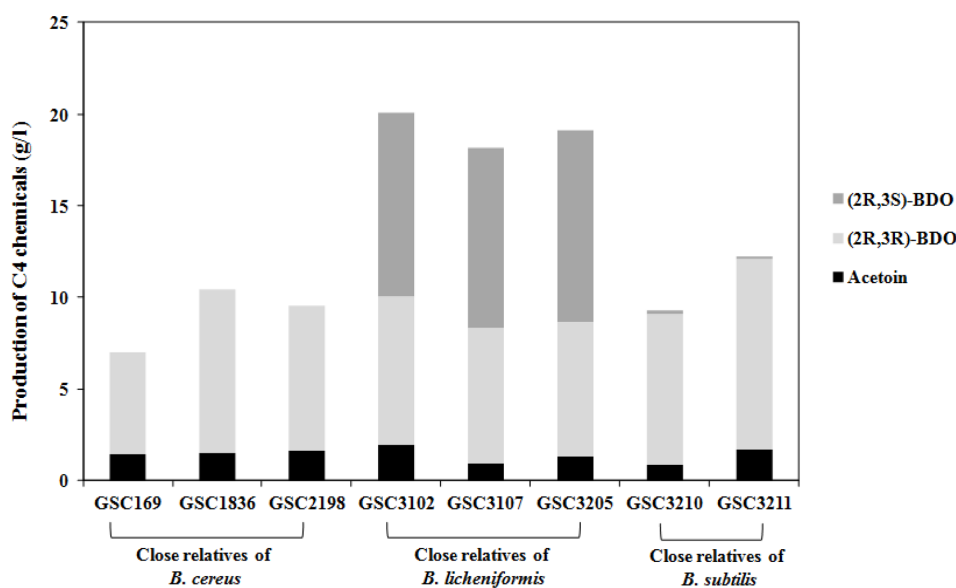The scale bar represents 0.01 substitutions per site.

Fig. 3. Comparison of the total amounts and isomer compositions of C4 chemicals produced by eight isolated candidate strains. Cultivations were performed in a rotary shaker at 180 rpm for 24 h. The temperature was set to 37°C for close relatives of *B. cereus*, and to 50°C for close relatives of *B. subtilis* and *B. licheniformis*. The production medium contained 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, and 0.800 g/l MgSO$_4$·7H$_2$O.

phylogenetic tree based on 16S rDNA sequences was drawn (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 YE and 45.0 g/l glucose. Only eight 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 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 an 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*, strain GSC1836 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*, strain GSC3211 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 strain GSC3102, which produced more than 18.1 g/l of 2,3-BDO. This value was about 102% and 74% higher than those obtained from the GSC1836 and GSC3211 strains, respectively. Although the 2,3-BDO isomer selectivity of GSC3102 was relativity lower than that of the GSC1836 and GSC3211 strains, the selectivity can to be improved through

Table 1. Ratio of 2,3-butanediol (2,3-BDO) stereoisomers produced by *Bacillus* sp. GSC1836, *Bacillus* sp. GSC3211, and *B. licheniformis* GSC3102 in the flask cultivations.

Strain	Acetoin	Total 2,3-BDO (g/l)	Ratio of (2S,3S)-BDO (%)	Ratio of (2R,3R)-BDO (%)	Ratio of (2R,3S)-BDO (%)
Bacilus sp. GSC1836	1.51	8.95	0	100	0
Bacillus sp. GSC3211	1.68	10.6	0	98.4	1.60
B. licheniformis GSC3102	1.98	18.1	0	44.7	55.3

metabolic engineering [24]. Further biochemical analyses of strain GSC3102, such as starch hydrolysis, citrate utilization, and growth at 6.5% NaCl and 55°C, confirmed that the strain was *B. licheniformis* (GSC3102). Apart from issues such as the potential pathogenicity of *B. cereus* [25] and low productivity of isolated close relatives of *B. subtils*, 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 for the economical production of 2,3-BDO was tested in 24 h shake flask cultivation. First, 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
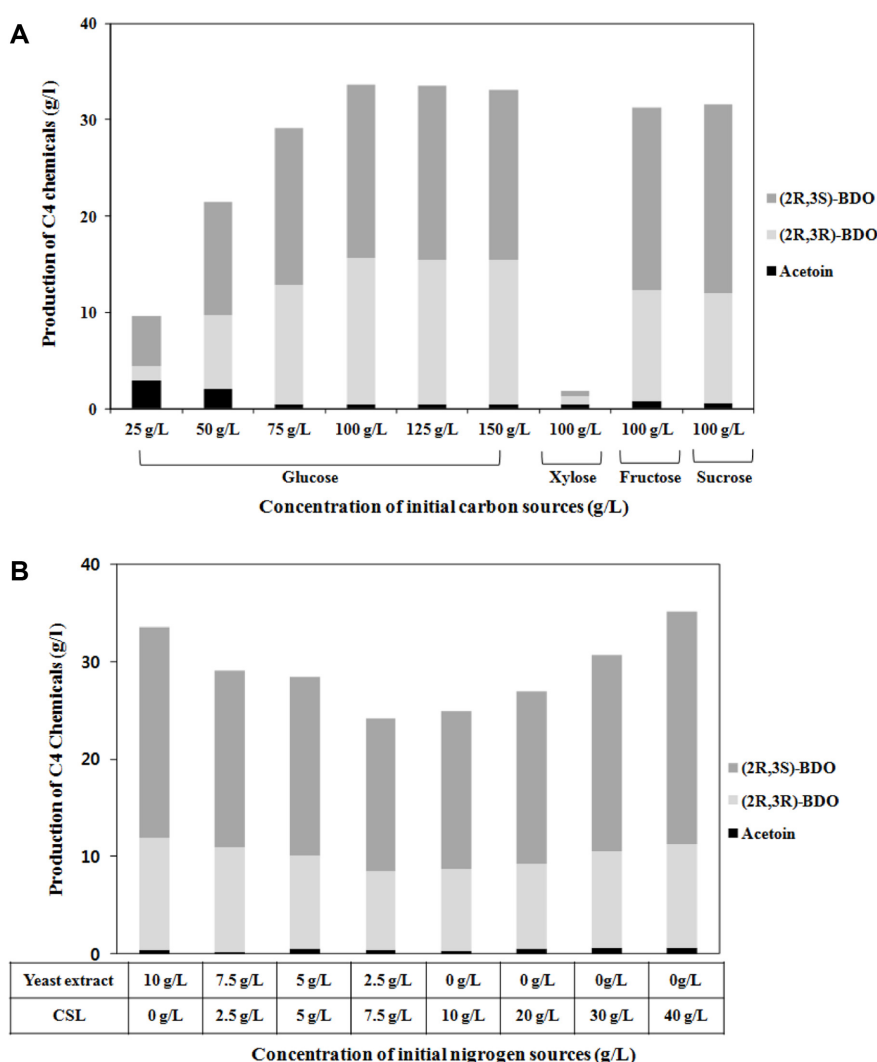

Fig. 4. Comparison of the total amounts and isomer compositions of C4 chemicals produced by *B. licheniformis* strain GSC3102 cultured in a rotary shaker at 180 rpm and 50°C for 24 h.
(**A**) The effects of initial glucose concentrations and other mono- and disaccharides, including xylose, fructose, and sucrose, were tested with medium containing 10.0 g/l yeast extract. (**B**) The effect of nitrogen sources, including yeast extract and corn steep liquor (CSL), was tested with medium containing 100 g/l glucose.

and fructose by *B. licheniformis* GSC3102 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, values that were almost equivalent to that of glucose (Fig. 4A). However, the ability of *B. licheniformis* GSC3102 to utilize xylose, which is a major C5 sugar derived from lignocellulosic feedstock [28], was much 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 ratios of CSL and YE (from 0.0 to 10.0 g/l) were performed using *B. licheniformis* GSC3102. Fig. 4B shows that with increasing CSL content, the 2,3-BDO concentration was gradually decreased from 33.1 g/l, and when YE 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 was slightly higher than that obtained in 10 g/l YE-containing medium. This result indicates that CSL can be used for the industrial fermentative production of 2,3-BDO by *B. licheniformis* strain GSC3102. This could be supported by the previous research that *Clostridium acetobutylicum* produced a higher amount of *n*-butanol in 3−6% CSL-containing medium, instead of YE 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 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 the oxygen transfer rate, oxygen transfer coefficient, and respiratory quotient were utilized as determining factors to find the optimal aerobic condition in conventional studies [32−34]. However, controlling those parameters is not easy in real fermentation circumstances and there was a demand for finding more controllable methods. In this context, two-stage fed-batch fermentation was performed to optmize the condition for the efficient production of 2,3-BDO in this study. The initial agitation speed for 2,3-BDO production was determined on the basis of 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 YE and 40.0 g/l CSL, respectively. 2,3-BDO is synthesized via the dehydration of acetoin, and bacteria also easily take up 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 YE-containing medium, the agitation speed was reduced from 500 to 400 rpm in 40 h fermentation, at which time the concentration of acetoin reached to 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 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 YE-containing medium (17.0 at 20 h and 17.2 at 46 h). Overall, 2,3-BDO was continuously produced during the exponential and stationary growth phases in both conditions. The final titers of 2,3-BDO in YE- 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 YE-containing medium, the differences were not significant (1.50 g/l/h and 0.431 g/g glucose in the YE medium, 1.40 g/l/h and 0.423 g/g glucose in the CSL medium). It can be seen that the production performance was excellent when compared with previous results comprehensively in terms of titer, productivity, and yield (Table 2). In particular, *B. licheniformis* GSC3102 has advantages not only for the performance but also for high temperature (50°C) culturing condition when compared with a similar study carried out by Yang *et al.* [35], who has isolated a *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* strain GSC3102 produced 2,3-BDO with a (2R,3S):(2R,3R) ratio of 1.67:1 in both media. Formate was accumulated as the major byproduct up to 29.1 and 18.9 g/l in the YE- and CSL-containing media, respectively. The accumulation of formate in the culture broth should be avoided, because this byproduct freely penetrates cell membranes, and has a tendency to reduce the intracellular pH, leading to strong cell growth inhibition [31]. In a previous study, we improved the production titer and yield of 2,3-BDO, and eliminated the accumulation of
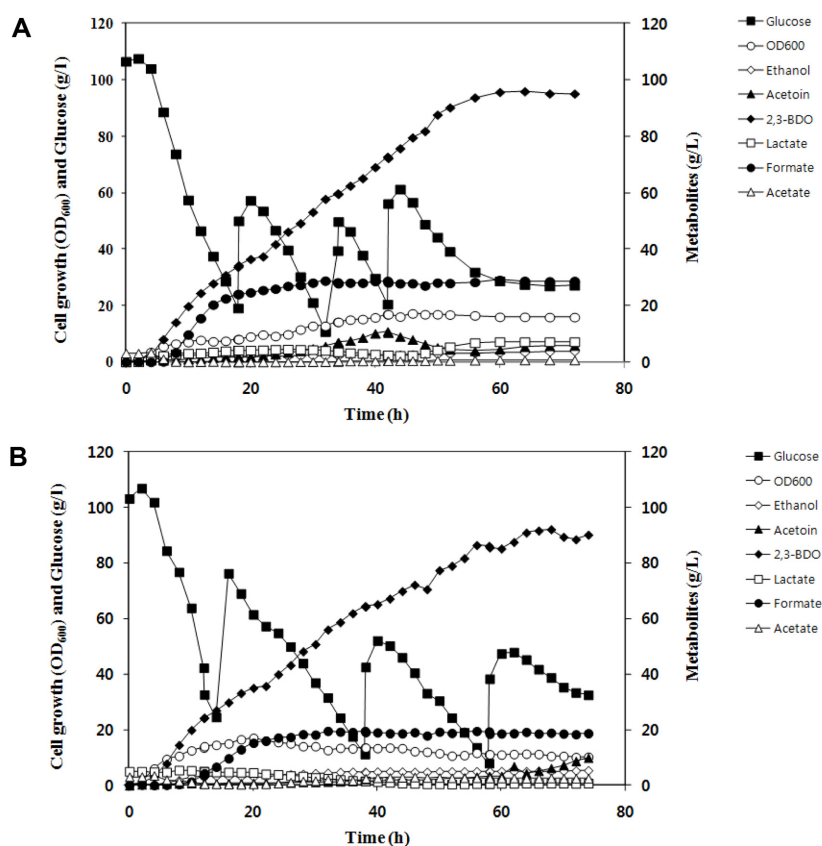
Fig. 5. Two-stage fed-batch fermentation profiles of *B. licheniformis* strain GSC3102 under controlled pH (7.0), temperature (50°C), and aeration rate (1 vvm).

When the acetoin concentration increased above 10 g/l, the agitation speed was reduced from 500 to 400 rpm. Intermittent feeding of 150 g of glucose powder was carried out as the residual glucose decreased to less than 20 g/l. Media containing 10.0 g/l yeast extract (**A**) or 40.0 g/l corn steep liquor (**B**) were used for fed-batch fermentations, respectively.

formate by inactivating the pyruvate formate-lyase activity in *K. oxytoca* [29]. Although a new isolate was employed to determine its natural fermentation capability in this study, it is believed that the performance of the strain could be

Table 2. 2,3-Butanediol (2,3-BDO) production by native *Bacillus* strains.

Strain	Substrate	2,3-BDO isomer	Method (temp.)	Conc. (g/l)	Yield (g/g)	Productivity (g/l/h)	References
Bacillus subtilis AJ1992 (WT)	Glucose	Mixture	Batch (30°C)	6.1	0.340	0.41	[36]
Bacillus vallismortis B-14891 (WT)	Glucose	(2R,3S)-BDO	Batch (39°C)	60.4	0.330	1.10	[15]
Bacillus amyloliquefaciens B10-127 (WT)	Glucose	Mixture	Fed-batch (37°C)	92.3	-	0.96	[35]
Bacillus licheniformis (WT)	Glucose	Mixture	Static flask (37°C)	8.7	0.470	0.12	[37]
Bacillus licheniformis X10 (WT)	Concentrated corn stover hydrolysate	Mixture	Fed-batch (50°C)	74.0	0.470	2.06	[38]
Bacillus licheniformis DSM8785 (WT)	Glucose	Mixture	Fed-batch (30°C)	144.7	0.400	1.14	[39]
Bacillus licheniformis GSC3102	Glucose (with yeast extract)	Mixture	Fed-batch (50°C)	95.9	0.431	1.50	This work
Bacillus licheniformis GSC3102	Glucose (with corn steep liquor)	Mixture	Fed-batch (50°C)	92.0	0.423	1.40	This work

improved by metabolic engineering strategies based on the genetic background in future work.

In conclusion, the newly designed 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 could utilize glucose, fructose, and sucrose as a carbon source with a high initial concentration of up to 100 g/l. Moreover, this strain has the ability to use a cheap and abundant nitrogen source (CSL), instead of the expensive yeast extract. This study demonstrates that *B. licheniformis* strain GSC3102 isolated from the natural environment has great potential for the industrial production of 2,3-BDO. Further studies, including of optimal medium composition, fermentation conditions, and strain improvement, are under way to develop an economically useful 2,3-BDO production system.

Acknowledgments

Conflict of Interest

The authors have no financial conflicts of interest to declare.

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