Metabolic Engineering of *Bacillus licheniformis* for Production of Acetoin

Chuanjuan Lü¹, Yongsheng Ge¹, Menghao Cao¹, Xiaoting Guo¹, Peihai Liu¹, Chao Gao¹, Ping Xu² and Cuiqing Ma¹*

¹ State Key Laboratory of Microbial Technology, Shandong University, Jinan, China, ² State Key Laboratory of Microbial Metabolism, Joint International Research Laboratory of Metabolic and Developmental Sciences, School of Life Sciences and Biotechnology, Shanghai-Jiao Tong University, Shanghai, China

Acetoin is a potential platform compound for a variety of chemicals. *Bacillus licheniformis* MW3, a thermophilic and generally regarded as safe (GRAS) microorganism, can produce 2,3-butanediol with a high concentration, yield, and productivity. In this study, *B. licheniformis* MW3 was metabolic engineered for acetoin production. After deleting two 2,3-butanediol dehydrogenases encoding genes *budC* and *gdh*, an engineered strain *B. licheniformis* MW3 (Δ*budCΔgdh*) was constructed. Using fed-batch fermentation of *B. licheniformis* MW3 (Δ*budCΔgdh*), 64.2 g/L acetoin was produced at a productivity of 2.378 g/[L h] and a yield of 0.412 g/g from 156 g/L glucose in 27 h. The fermentation process exhibited rather high productivity and yield of acetoin, indicating that *B. licheniformis* MW3 (Δ*budCΔgdh*) might be a promising acetoin producer.

Keywords: acetoin, *Bacillus licheniformis*, metabolic engineering, 2,3-butanediol dehydrogenase, 2,3-butanediol

INTRODUCTION

Acetoin (3-hydroxy-2-butanone) is widely used as a common additive in food industry and a building block for various chemicals (Liu et al., 2011; Xiao and Lu, 2014; Yang et al., 2017). It was listed as one of 30 platforms compound whose development and utilization was given priority by the US Department of Energy in 2004 (Xiao and Lu, 2014). Currently, commercially available acetoin is primarily produced from fossil feedstocks by chemical methods involving radical reactions and severe environmental pollution. With increasing concerns about production cost and environment protection, microbial fermentation production of acetoin has attracted extensive attention in recent years (Xiao and Lu, 2014; Yang et al., 2017).

Many native or recombinant microorganisms, including the genera *Serratia*, *Klebsiella*, *Saccharomyces*, *Glucosobacter*, and *Bacillus*, have abilities to produce acetoin (Sun et al., 2012; Wang et al., 2013, 2017; Bae et al., 2016; Jia et al., 2017). Among these species, *Bacillus* species which can produce various industrial products have become the research hotspot for acetoin production (Sun et al., 2012; Wang et al., 2013, 2017; Bae et al., 2016; Jia et al., 2017). Among these species, *Bacillus* species production of acetoin has been successfully improved acetoin production by *B. subtilis* through disruption of
acetoacetyl-CoA thiolase and then cleaved into acetoin and diacetyl. Subsequently, acetoin is reduced to 2,3-butanediol, which is a water-soluble compound.

**RESULTS**

The recombinant strain B. licheniformis MW3 (ΔbudCΔgdh) was constructed with a yield of 2.378 g/L and a productivity of 0.412 g/L. Considering its desirable characteristics, B. licheniformis MW3 (ΔbudCΔgdh) may be a promising alternative for fermentation production of acetoacetate.

**MATERIALS AND METHODS**

**Enzymes and Chemicals**

FastPfu DNA polymerase and T4 DNA ligase were purchased from TransGen Biotech (China) and Thermo Fisher (United States), respectively. Restriction enzymes were purchased from Thermo Fisher (United States). Polymerase chain reaction (PCR) primers were provided by Sangon (Shanghai, China). Racemic acetoin, diacetyl, and meso-2,3-butanediol were purchased from Sigma (United States). 2-3-butanediol and glucose were purchased from Thermo Fisher (United States). Yeast extract was purchased from Roche (United States). Yeast extract was defined as the amount of enzyme that consumed or formed NADH or reduction of NAD+ in 67 mM phosphate buffer (pH 7.4).

**Enzyme Activity Assays**

To measure 2,3-butanediol dehydrogenase activities in B. licheniformis MW3 and B. licheniformis MW3 (ΔbudCΔgdh), the cells of the strains were grown for 12 h, harvested, washed twice and resuspended in 67 mM phosphate buffer (pH 7.4). One unit of activity was defined as the amount of enzyme that consumed or formed 1 µmol of NADH per minute. The protein concentration in crude extract was measured by the Lowry method, with bovine serum albumin as the standard (Hartree, 1972).

**Bacterial Strains, Media, and Plasmids**

All the strains and plasmids used in this study are listed in Supplementary Table S1. The plasmids pKVM1-ΔbudC and pKVM1-Δgdh based on the temperature sensitive plasmid pKVM1 were used for gene knockout in B. licheniformis MW3 (Wachskau et al., 2008; Rachinger et al., 2013; Ge et al., 2016). Lysogenic broth (LB) medium was used for cultivation of Escherichia coli and B. licheniformis. NB medium (g/L Nutrient Broth, Difco) supplemented with 50 mM glucose was used for selection of B. licheniformis containing pKVM1. Ampicillin was used at a concentration of 100 µg/mL for the selection of E. coli. Erythromycin (5 µg/mL) and polymyxin B (40 µg/mL) were used for the selection of B. licheniformis. X-Gal was added at a concentration of 40 µg/mL for blue-white screening.

**Gene Knockout in B. licheniformis MW3**

E. coli S17-1 λpir was used as donor strain to allow the conjugal transfer of plasmids pKVM1-ΔbudC and pKVM1-Δgdh into B. licheniformis MW3. After growth in liquid NB/glucose medium with erythromycin at 30°C, serial dilutions and spreading on NB/glucose plates with erythromycin and X-gal and incubation at 42°C, one of blue colonies of B. licheniformis MW3 with plasmid integration by homologous recombination was picked, inoculated in NB/glucose medium without antibiotic, and cultivated at 30°C for at least two passages. After dilutions and spreading on NB/glucose with X-gal, white colonies in which plasmids used to knockout were cured resulting from a second recombination event were picked. PCR was used to verify the disruption event of gene budC and gdh using primer pairs ΔbudC-t/ΔbudC-r and Δgdh-t/Δgdh-r (Supplementary Table S2), respectively.

**Batch and Fed-Batch Fermentations**

B. licheniformis MW3 was maintained on LB agar slants at 4°C. A loop of cells from the fully grown slant was inoculated into 100 mL of LB in 500 mL Erlenmeyer flasks and incubated at 50°C on a rotary shaker at 180 r/min for 12 h to prepare the seed culture. Then, the seed culture was inoculated (5%, v/v) into the bioreactors for acetoin production.

Batch and fed-batch fermentations were conducted in a 1-L bioreactor (Infor AG, Bottmingen, Switzerland) with 0.8 L initial medium and a 5-L bioreactor (BIOSTAT B, B. Braun Biotech International GmbH, Germany) with 4 L initial medium, respectively. The seed culture was inoculated into the fermentation medium containing about 70 g/L glucose; 1 g/L triammonium citrate; 12 g/L yeast extract; 2 g/L K2HPO4·3H2O; 6.5 g/L sodium acetate; 0.25 g/L MgSO4·7H2O; 0.0225 g/L FeSO4·7H2O; 0.0075 g/L ZnSO4·7H2O; and 0.0038 g/L MnSO4·H2O. The cultivation was carried out at 50°C, stirring at 500 r/min, and airflow at 1.0 vvm (volume air per volume broth per minute). The pH was maintained at 7.0 by automatic addition of 6 M acetic acid and 6 M NaOH using a
program-controlled peristaltic pump. Samples were collected periodically to determine the biomass, concentrations of glucose, 2,3-butanediol, and acetoin.

**Analytical Methods**

Samples were centrifuged at 12,000 g for 10 min and the concentration of glucose was measured enzymatically using a bioanalyzer (SBA-40D, Shandong Academy of Sciences, China) after diluting to an appropriate concentration. The concentrations of 2,3-butanediol and acetoin were analyzed by gas chromatography (GC; GC2014c, Shimadzu) with capillary GC columns (AT-SE-54, inside diameter, 0.32 mm; length, 30 m; Chromatographic Technology Center, Lanzhou Institute of Chemical Physics, China) as described previously (Zhang L. et al., 2016).

**RESULTS AND DISCUSSION**

**Construction of the Strain**

*B. licheniformis* MW3 (∆budC∆gdh)

*B. licheniformis* MW3 is a GRAS and thermophilic strain which can efficiently produce *meso*-2,3-butanediol and (2R,3R)-2,3-butanediol with a simple stereoisomer formation mechanism (Ge et al., 2016). As shown in Figure 1A, glucose is converted to pyruvate by glycolytic pathway, then α-acetolactate synthase (ALS) condenses two molecules of pyruvate to generate one molecule of α-acetolactate, and then α-acetolactate is transformed into one molecule of acetoin by α-acetolactate decarboxylase (ALDC). Two stereospecific *2,3*-butanediol dehydrogenases, (2R,3R)-2,3-butanediol dehydrogenase (encoded by *gdh*) and *meso*-2,3-butanediol dehydrogenase (encoded by *budC*), are responsible for the production of (2R,3R)-2,3-butanediol and *meso*-2,3-butanediol, respectively (Ge et al., 2016). To construct an acetoin producing strain based on *B. licheniformis* MW3, both (2R,3R)-2,3-butanediol dehydrogenase and *meso*-2,3-butanediol dehydrogenase encoding genes should be deleted. The temperature sensitive plasmid pKM1 was utilized as described previously. The mutant strain *B. licheniformis* MW3 (∆budC∆gdh) was selected and the result in Figure 1B shows that the PCR using the primer pairs generated products of the expected sizes.

**Activities of 2,3-Butanediol Dehydrogenases in B. licheniformis* MW3 (∆budC∆gdh)**

The activities of (2R,3R)-2,3-butanediol dehydrogenase and *meso*-2,3-butanediol dehydrogenase in *B. licheniformis* MW3 (∆budC∆gdh) were assayed. As shown in Supplementary Table S3, (2S,3S)-2,3-butanediol oxidation activity was rather low in both *B. licheniformis* MW3 and *B. licheniformis* MW3 (∆budC∆gdh). Both (2R,3R)-2,3-butanediol and *meso*-2,3-butanediol oxidation activities decreased in *B. licheniformis* MW3 (∆budC∆gdh). Especially, decrease of acetoin reduction activity occurred after deletion of (2R,3R)-2,3-butanediol dehydrogenase and *meso*-2,3-butanediol dehydrogenase encoding genes. Taking advantage of its low acetoin reduction activity, acetoin produced by ALDC might not be reduced to 2,3-butanediol and the strain *B. licheniformis* MW3 (∆budC∆gdh) was used for acetoin production in the subsequent experiments.

**Batch Fermentation of Acetoin by**

*B. licheniformis* MW3 (∆budC∆gdh)

Batch fermentation was conducted in a 5-L bioreactor with 4 L initial medium to analyze acetoin production by *B. licheniformis* MW3 (∆budC∆gdh). As shown in Figure 2, inactivation of both *budC* and *gdh* led to a high acetoin accumulation in *B. licheniformis* MW3 (∆budC∆gdh). This strain consumed 63 ± 1 g/L glucose in 14 h and produced 27.4 ± 1.45 g/L acetoin, with a yield of 0.435 ± 0.023 g/g (Figure 2). Although the growth of *B. licheniformis* MW3 (∆budC∆gdh) was slightly lower than that of *B. licheniformis* MW3, this strain can produce acetoin instead of 2,3-butanediol as its major product (Supplementary Table S4).

**Fed-Batch Fermentation of Acetoin by**

*B. licheniformis* MW3 (∆budC∆gdh)

To achieve higher acetoin concentrations, fed-batch fermentation was carried out with an initial glucose concentration of about 70 g/L using *B. licheniformis* MW3 (∆budC∆gdh). As shown in Figure 3, 64.2 g/L acetoin from 156 g/L glucose was obtained in 27 h by *B. licheniformis* MW3 (∆budC∆gdh). The yield of acetoin was 84.1% of the theoretical value and the average productivity was 2.378 g/[L h]. The carbon flux channeled into the acetoin biosynthetic might be further enhanced since there were still 2,3-butanediol accumulated during the fermentation (Figure 3 and Supplementary Figure S1). Toxicity of acetoin can hinder its higher production by various microorganisms. It was reported that an approximate 30% cell growth of *B. licheniformis* will be inhibited with 40 g/L exogenous acetoin (Yuan et al., 2019). Thus, production of 2,3-butanediol may be due to induction of an unidentified acetoin reductase or *meso*-2,3-butanediol dehydrogenase to resist the toxicity of acetoin at high concentrations. Higher production of acetoin by *B. licheniformis* may be accomplished by searching the undiscovered acetoin reductase or overexpressing NADH oxidase, which could lead to prevention of NADH-dependent reduction of acetoin.

Several recombinant *Bacillus* strains have been used to produce acetoin (Table 1). Based on a two-stage pH control strategy, the group of Li obtained a high acetoin concentration of 73.6 g/L with a productivity of 0.77 g/[L h] using metabolically engineered *B. subtilis* overexpressing 2,3-butanediol dehydrogenase (Zhang et al., 2014a). Fan et al. successfully improved the coproduction of uridine and acetoin by modification of acetoin metabolism in engineered *B. subtilis*. The recombinant strain can produce 60.48 g/L acetoin and 40.62 g/L uridine after 48 h of fed-batch fermentation (Fan et al., 2018). The group of Chen isolated a *B. licheniformis* strain WX-02 which can produce γ-poly-glutamic acid accompanied with 2,3-butanediol (Li X. et al., 2014). *B. licheniformis* WX-02 ∆budC∆acoR was constructed through deleting *budC* and acetoin degradation transcriptional activator encoding gene *acoR*. Fed-batch fermentation through a three-stage agitation...
strategy during which 2,3-butanediol was first accumulated and then converted to acetoin using *B. licheniformis* WX-02ΔbudCΔacoR can produce 78.79 g/L acetoin with a yield of 0.31 g/g and a productivity of 0.58 g/[L h] at 37°C (Li et al., 2017). *B. licheniformis* MW3 is a thermophilic strain which can produce 2,3-butanediol as its major fermentation product with higher yield and productivity than that of *B. licheniformis* WX-02 at 50°C, implying a latent capacity of strain MW3 for efficient acetoin production (Ge et al., 2016; Qiu et al., 2016). Thus, *B. licheniformis* MW3 (ΔbudCΔgdh) was constructed and the recombinant strain was able to produce 64.2 g/L acetoin with a relatively high productivity (2.378 g/[L h]) through a simple fermentation process. The values of productivity and yield of acetoin produced using *B. licheniformis* MW3 (ΔbudCΔgdh) were the highest ever obtained in acetoin production by recombinant *Bacillus* species. *B. licheniformis* MW3 (ΔbudCΔgdh) might be useful for the production of acetoin on a commercial scale.
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FIGURE 3 | Time-course of fed-batch fermentation by B. licheniformis MW3 (ΔbudCGdh). •, Glucose; ■, OD620nm; †, 2,3-butanediol; ▼, acetoin. The experiment was conducted in a 5-L bioreactor (BIOSTAT B, B. Braun Biotech International GmbH, Germany) with 4 L initial medium with an initial glucose concentration of 70 g/L approximately. Cultivation was carried out at 50°C and an initial pH of 7.0. The pH was maintained at 7.0 by automatic addition of 6 M acetic acid and 6 M NaOH. The agitation speed was 500 r/min and the aeration rate was 1 vvm. The fed-batch fermentation was conducted by feeding glucose solution when the residual glucose in the fermentation was lower than 10 g/L. Data were from a representative fed-batch fermentation repeated for three times.

TABLE 1 | Fermentation production of acetoin by recombinant Bacillus species.

| Strain               | Engineering strategy                                                                 | Concentration (g/L) | Yield (g/g) | Productivity (g/[L h]) | References          |
|----------------------|----------------------------------------------------------------------------------------|---------------------|------------|------------------------|---------------------|
| B. subtilis F126-2   | Disrupting 2,3-butanediol dehydrogenase gene bdhA and overexpression of alsSD operon based on a uridine producing strain | 60.48               | /          | 1.26                   | Fan et al., 2018    |
| B. subtilis BSA      | Over-expression of 2,3-butanediol dehydrogenase in B. subtilis JNA 3-10, applying a two-stage pH control strategy | 73.6                | 0.408      | 0.77                   | Zhang et al., 2014a |
| B. subtilis BMN       | Moderate-expression of the water-forming NADH oxidase C in bdhA disrupted B. subtilis JNA 3-10 | 56.7                | 0.675      | 0.639                  | Zhang et al., 2014b |
| B. subtilis 168/pMAS-zwf | Over-expression of glucose-6-phosphate dehydrogenase G6PDH in B. subtilis 168 | 43.3                | 0.33       | 0.36                   | Bao et al., 2015    |
| B. subtilis BS168D    | Blocking of 2,3-butanediol dehydrogenase gene bdhA in B. subtilis 168                 | 24.6                | 0.246      | 0.342                  | Zhang et al., 2017  |
| B. subtilis ZB02      | Over-expression of xylose transport protein AraE, xylose isomerase XylA, and xylulokinase XylB in B. subtilis 168ARSRCpΔacoAΔbdhA, using glucose, xylose, and arabinose as substrates | 62.2                | NR         | 0.864                  | Zhang B. et al., 2016 |
| B. subtilis BSK814A4  | Deleting araR, bdhA, acoA and inserting a native xyl operon into genome-reduced B. subtilis strain BSK814 | 23.3                | 0.46       | 0.194                  | Yan et al., 2018    |
| B. subtilis BSUW06    | Overexpressing alsSD operon and deleting bdhA, acoA, and pta in B. subtilis 168      | 19.8                | NR         | 0.566                  | Wang et al., 2012   |
| B. subtilis PAR       | Moderate enhancement of ALsR expression using promoter PbdhA in B. subtilis 168       | 41.5                | 0.35       | 0.43                   | Zhang X. et al., 2013 |
| B. licheniformis WX-02ΔbudCGdh | Deleting budC and acetoin degradation transcriptional activator encoding gene acoR in B. licheniformis WX-02 | 78.79               | 0.31       | 0.58                   | Li et al., 2017     |
| B. licheniformis MW3 (ΔbudCGdh) | Deleting budC and gdh in B. licheniformis MW3 | 64.4                | 0.412      | 2.378                  | This work           |

NR, not reported.

CONCLUSION

We constructed a recombinant strain B. licheniformis MW3 (ΔbudCGdh) for acetoin production. Acetoin at a high concentration (64.2 g/L) was produced with a productivity of 2.378 g/[L h] and a yield of 0.412 g/g through fed-batch fermentation by B. licheniformis MW3 (ΔbudCGdh). Because of its GRAS characteristic and ability to produce acetoin with
high concentration, productivity, and yield, this strain should be a promising alternative for the practical production of acetoin.

**DATA AVAILABILITY STATEMENT**

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

**AUTHOR CONTRIBUTIONS**

CL, YG, and MC performed experiments. CL, CG, and CM wrote the manuscript and conceived the study. CL, YG, MC, XG, and PL were involved in analysis and interpretation of experimental data. CM and PX coordinated the project.

**FUNDING**

This work was supported by the National Natural Science Foundation of China (31670041), the grants of National Key R&D Program of China (2019YFA0904900 and 2019YFA0904803), Shandong Provincial Funds for Distinguished Young Scientists (JQ 201806), Natural Science Foundation of Shandong Provincial (ZR2018PC008), Key R&D Program of Shandong Provincial (2019GSF107034 and 2019GSF107039), and Qilu Young Scholar of Shandong University. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

**ACKNOWLEDGMENTS**

The authors greatly thank Prof. Dr. Friedhelm Meinhardt for kindly providing *B. licheniformis* MW3 and Prof. Dr. Armin Ehrenreich for plasmid pKVM1. The authors also thank Chengjia Zhang and Nannan Dong from Core Facilities for Life and Environmental Sciences (State Key Laboratory of Microbial Technology, Shandong University) for assistance in microbial fermentation.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fbioe.2020.00125/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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