



Metabolic Engineering of *Bacillus licheniformis* for Production of Acetoin

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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 ($\Delta budC \Delta gdh$) was constructed. Using fed-batch fermentation of *B. licheniformis* MW3 ($\Delta budC \Delta gdh$), 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 ($\Delta budC \Delta gdh$) 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*, *Gluconobacter*, 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 (Zhang Y. et al., 2013; Fan et al., 2018; Liu et al., 2019). Acetoin is an intermediate of 2,3-butanediol fermentation-pathway (Ji et al., 2011; Yang and Zhang, 2019). Except a few native acetoin producers, most of the *Bacillus* strains produce 2,3-butanediol but not acetoin as their major product (Fu et al., 2016; Dai et al., 2019). Thus, different metabolic engineering strategies were proposed to redistribute the carbon flux to acetoin in various *Bacillus* strains. For example, Zhang et al. successfully improved acetoin production by *Bacillus subtilis* through disruption of

acetoin reductase (also called as 2,3-butanediol dehydrogenase) and expression of a water-forming NADH oxidase. Acetoin at a concentration of 56.7 g/L was produced with a yield of 0.378 g/g and a productivity of 0.639 g/[L h] (Zhang et al., 2014b).

Bacillus licheniformis, a generally regarded as safe (GRAS) strain with the characteristics of strong sugar consumption and fast growth rate, is a promising chassis strain for acetoin production. Natural *B. licheniformis* strains usually produce a mixture of (2*R*,3*R*)-2,3-butanediol and *meso*-2,3-butanediol isomers (Li L. et al., 2014; Qi et al., 2014; Qiu et al., 2016). In this study, two 2,3-butanediol dehydrogenases encoding genes in *B. licheniformis* MW3 were deleted and an engineered strain *B. licheniformis* MW3 ($\Delta budC\Delta gdh$) was constructed. The recombinant strain *B. licheniformis* MW3 ($\Delta budC\Delta gdh$) can produce 64.2 g/L acetoin within 27 h with a productivity of 2.378 g/[L h] and a yield of 0.412 g/g. Considering its desirable characteristics, *B. licheniformis* MW3 ($\Delta budC\Delta gdh$) may be a promising alternative for fermentative production of acetoin.

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 2,3-butanediol were purchased from Sigma. (2*R*,3*R*)-2,3-butanediol (98.0%), (2*S*,3*S*)-2,3-butanediol (99.0%), and *meso*-2,3-butanediol (98.0%) were purchased from ACROS (The Kingdom of Belgium). NADH was purchased from Roche (United States). Yeast extract (FM902) was a kind gift from Angel Yeast Co., Ltd. (Yichang, Hubei, China). All other chemicals were of analytical grade and commercially available.

Bacterial Strains, Media, and Plasmids

All the strains and plasmids used in this study are listed in **Supplementary Table S1**. The plasmids pKVM1- $\Delta budC$ and pKVM1- Δgdh based on the temperature sensitive plasmid pKVM1 were used for gene knockout in *B. licheniformis* MW3 (Waschkau 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 (8 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 μ g/mL for the selection of *E. coli*. Erythromycin (5 μ g/mL) and polymyxin B (40 μ g/mL) were used for the selection of *B. licheniformis*. X-Gal was added at a concentration of 40 μ g/mL for blue-white screening.

Gene Knockout in *B. licheniformis* MW3

E. coli S17-1 λ pir was used as donor strain to allow the conjugal transfer of plasmids pKVM1- $\Delta budC$ 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 $\Delta budC$ -f/ $\Delta budC$ -r and Δgdh -f/ Δgdh -r (**Supplementary Table S2**), respectively.

Enzyme Activity Assays

To measure 2,3-butanediol dehydrogenase activities in *B. licheniformis* MW3 and *B. licheniformis* MW3 ($\Delta budC\Delta gdh$), the cells of the strains were grown for 12 h, harvested, washed twice and resuspended in 67 mM phosphate buffer (pH 7.4), and disrupted by sonication with a Sonics sonicator (500 W; 20 kHz). Cell debris was removed through centrifugation (12,000 g, 15 min) and the resulting supernatants were used as the crude extracts for enzyme activity assays.

Activities of 2,3-butanediol dehydrogenase were spectrophotometrically assayed by measuring the change in absorbance at 340 nm corresponding to the oxidation of NADH or reduction of NAD⁺ at 30°C using a UV/visible spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences, United States). For the reduction reaction, the reaction solution contains 0.2 mM of NADH and 5 mM of acetoin or diacetyl in 67 mM phosphate buffer (pH 7.4). For oxidation reactions, the reaction solution contains 10 mM (2*R*,3*R*)-2,3-butanediol, (2*S*,3*S*)-2,3-butanediol, and *meso*-2,3-butanediol and 1 mM NAD⁺ in 67 mM phosphate buffer (pH 7.4). One unit of activity was defined as the amount of enzyme that consumed or formed 1 μ mol of NADH per min. The protein concentration in crude extract was measured by the Lowry method, with bovine serum albumin as the standard (Hartree, 1972).

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 (Infors 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 bio-analyzer (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 ($\Delta budC\Delta gdh$)

B. licheniformis MW3 is a GRAS and thermophilic strain which can efficiently produce *meso*-2,3-butanediol and (2*R*,3*R*)-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, (2*R*,3*R*)-2,3-butanediol dehydrogenase (encoded by *gdh*) and *meso*-2,3-butanediol dehydrogenase (encoded by *budC*), are responsible for the production of (2*R*,3*R*)-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 (2*R*,3*R*)-2,3-butanediol dehydrogenase and *meso*-2,3-butanediol dehydrogenase encoding genes should be deleted. The temperature sensitive plasmid pKVM1 was utilized as described previously. The mutant strain *B. licheniformis* MW3 ($\Delta budC\Delta gdh$) 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 ($\Delta budC\Delta gdh$)

The activities of (2*R*,3*R*)-2,3-butanediol dehydrogenase and *meso*-2,3-butanediol dehydrogenase in *B. licheniformis* MW3 ($\Delta budC\Delta gdh$) were assayed. As shown in **Supplementary Table S3**, (2*S*,3*S*)-2,3-butanediol oxidation activity was rather low in both *B. licheniformis* MW3 and *B. licheniformis* MW3 ($\Delta budC\Delta gdh$). Both (2*R*,3*R*)-2,3-butanediol and *meso*-2,3-butanediol oxidation activities decreased in *B. licheniformis* MW3 ($\Delta budC\Delta gdh$). Especially, decrease of acetoin reduction activity occurred after deletion of (2*R*,3*R*)-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 ($\Delta budC\Delta gdh$) was used for acetoin production in the subsequent experiments.

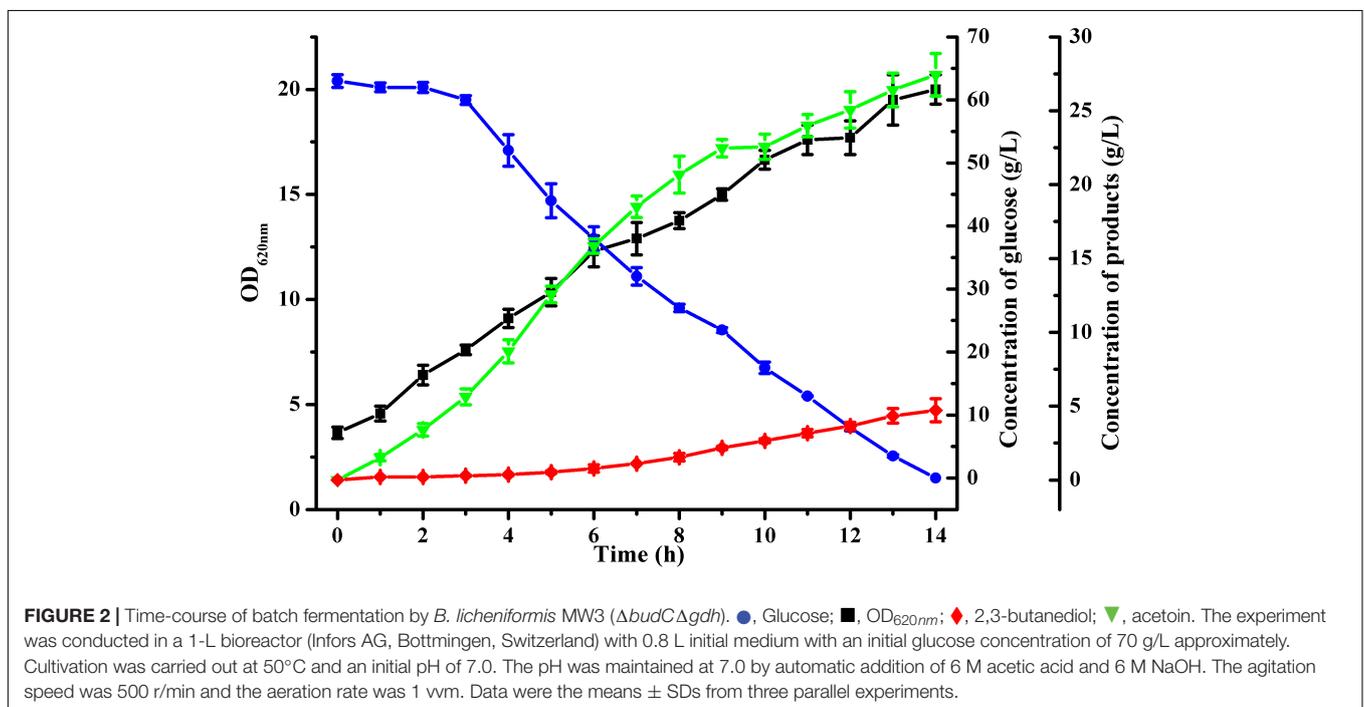
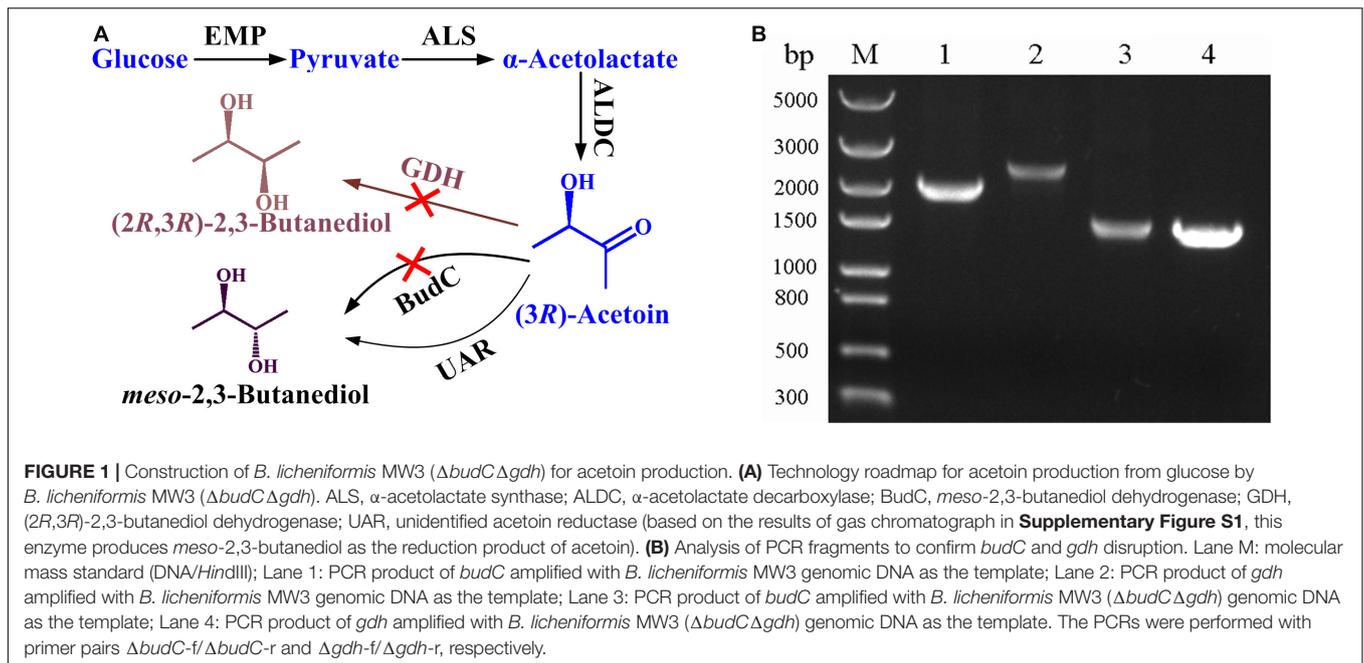
Batch Fermentation of Acetoin by *B. licheniformis* MW3 ($\Delta budC\Delta gdh$)

Batch fermentation was conducted in a 5-L bioreactor with 4 L initial medium to analyze acetoin production by *B. licheniformis* MW3 ($\Delta budC\Delta gdh$). As shown in **Figure 2**, inactivation of both *budC* and *gdh* led to a high acetoin accumulation in *B. licheniformis* MW3 ($\Delta budC\Delta gdh$). 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 ($\Delta budC\Delta gdh$) 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 ($\Delta budC\Delta gdh$)

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 ($\Delta budC\Delta gdh$). As shown in **Figure 3**, 64.2 g/L acetoin from 156 g/L glucose was obtained in 27 h by *B. licheniformis* MW3 ($\Delta budC\Delta gdh$). 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 $\Delta budC\Delta acor$ 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-02 $\Delta budC\Delta acoR$ 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 ($\Delta budC\Delta gdh$) 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 ($\Delta budC\Delta gdh$) were the highest ever obtained in acetoin production by recombinant *Bacillus* species. *B. licheniformis* MW3 ($\Delta budC\Delta gdh$) might be useful for the production of acetoin on a commercial scale.

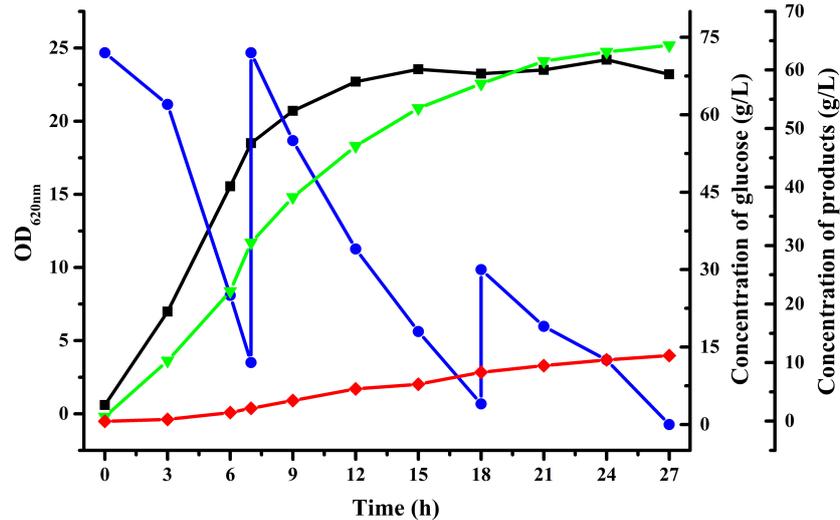


FIGURE 3 | Time-course of fed-batch fermentation by *B. licheniformis* MW3 ($\Delta budC \Delta gdh$). ●, Glucose; ■, OD_{620nm}; ♦, 2,3-butanediol; ▼, acetoin. The experiment was conducted in a 5-L bioreactor (BIOSTAT B, B. Braun Biotech International GmbH, Germany) with 4 L initial medium with an initial 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
<i>B. subtilis</i> F126-2	Disrupting 2,3-butanediol dehydrogenase gene <i>bdhA</i> and overexpression of <i>alsSD</i> operon based on a uridine producing strain	60.48	/	1.26	Fan et al., 2018
<i>B. subtilis</i> BSA	Over-expression of 2,3-butanediol dehydrogenase in <i>B. subtilis</i> JNA 3-10, applying a two-stage pH control strategy	73.6	0.408	0.77	Zhang et al., 2014a
<i>B. subtilis</i> BMN	Moderate-expression of the water-forming NADH oxidase C in <i>bdhA</i> disrupted <i>B. subtilis</i> JNA 3-10	56.7	0.675	0.639	Zhang et al., 2014b
<i>B. subtilis</i> 168/pMA5-zwf	Over-expression of glucose-6-phosphate dehydrogenase G6PDH in <i>B. subtilis</i> 168	43.3	0.33	0.36	Bao et al., 2015
<i>B. subtilis</i> BS168D	Blocking of 2,3-butanediol dehydrogenase gene <i>bdhA</i> in <i>B. subtilis</i> 168	24.6	0.246	0.342	Zhang et al., 2017
<i>B. subtilis</i> ZB02	Over-expression of xylose transport protein AraE, xylose isomerase XylA, and xylulokinase XylB in <i>B. subtilis</i> 168ARSRCP $\Delta acoA \Delta bdhA$, using glucose, xylose, and arabinose as substrates	62.2	NR ^a	0.864	Zhang B. et al., 2016
<i>B. subtilis</i> BSK814A4	Deleting <i>araR</i> , <i>bdhA</i> , <i>acoA</i> and inserting a native <i>xyl</i> operon into genome-reduced <i>B. subtilis</i> strain BSK814	23.3	0.46	0.194	Yan et al., 2018
<i>B. subtilis</i> BSUW06	Overexpressing <i>alsSD</i> operon and deleting <i>bdhA</i> , <i>acoA</i> , and <i>pta</i> in <i>B. subtilis</i> 168	19.8	NR	0.566	Wang et al., 2012
<i>B. subtilis</i> PAR	Moderate enhancement of ALsR expression using promoter P_{bdhA} in <i>B. subtilis</i> 168	41.5	0.35	0.43	Zhang X. et al., 2013
<i>B. licheniformis</i> WX-02 $\Delta budC \Delta acoR$	Deleting <i>budC</i> and acetoin degradation transcriptional activator encoding gene <i>acoR</i> in <i>B. licheniformis</i> WX-02	78.79	0.31	0.58	Li et al., 2017
<i>B. licheniformis</i> MW3 ($\Delta budC \Delta gdh$)	Deleting <i>budC</i> and <i>gdh</i> in <i>B. licheniformis</i> MW3	64.4	0.412	2.378	This work

^aNR, not reported.

CONCLUSION

We constructed a recombinant strain *B. licheniformis* MW3 ($\Delta budC \Delta gdh$) 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 ($\Delta budC \Delta gdh$). 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.

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REFERENCES

- Bae, S. J., Kim, S., and Hahn, J. S. (2016). Efficient production of acetoin in *Saccharomyces cerevisiae* by disruption of 2,3-butanediol dehydrogenase and expression of NADH oxidase. *Sci. Rep.* 6:27667. doi: 10.1038/srep27667
- Bao, T., Zhang, X., Zhao, X., Rao, Z., Yang, T., and Yang, S. (2015). Regulation of the NADH pool and NADH/NADPH ratio redistributes acetoin and 2,3-butanediol proportion in *Bacillus subtilis*. *Biotechnol. J.* 10, 1298–1306. doi: 10.1002/biot.201400577
- Dai, J., Wang, Z., and Xiu, Z. (2019). High production of optically pure (3R)-acetoin by a newly isolated marine strain of *Bacillus subtilis* CGMCC 13141. *Bioprocess Biosyst. Eng.* 42, 475–483. doi: 10.1007/s00449-018-2051-8
- Fan, X., Wu, H., Jia, Z., Li, G., Li, Q., Chen, N., et al. (2018). Metabolic engineering of *Bacillus subtilis* for the co-production of uridine and acetoin. *Appl. Microbiol. Biotechnol.* 102, 8753–8762. doi: 10.1007/s00253-018-9316-7
- Fu, J., Huo, G., Feng, L., Mao, Y., Wang, Z., Ma, H., et al. (2016). Metabolic engineering of *Bacillus subtilis* for chiral pure meso-2,3-butanediol production. *Biotechnol. Biofuels.* 9:90. doi: 10.1186/s13068-016-0502-5
- Ge, Y., Li, K., Li, L., Gao, C., Zhang, L., Ma, C., et al. (2016). Contracted but effective: production of enantiopure 2,3-butanediol by thermophilic and GRAS *Bacillus licheniformis*. *Green Chem.* 18, 4693–4703. doi: 10.1039/C6GC01023G
- Hartree, E. F. (1972). Determination of protein: a modification of the Lowry method that gives a linear photometric response. *Anal. Biochem.* 48, 422–427. doi: 10.1016/0003-2697(72)90094-2
- Ji, X., Huang, H., and Ouyang, P. (2011). Microbial 2,3-butanediol production: a state-of-the-art review. *Biotechnol. Adv.* 29, 351–364. doi: 10.1016/j.biotechadv.2011.01.007
- Jia, X., Peng, X., Liu, Y., and Han, Y. (2017). Conversion of cellulose and hemicellulose of biomass simultaneously to acetoin by thermophilic simultaneous saccharification and fermentation. *Biotechnol. Biofuels.* 10:232. doi: 10.1186/s13068-017-0924-8
- Li, L., Li, K., Wang, K., Chen, C., Gao, C., Ma, C., et al. (2014). Efficient production of 2,3-butanediol from corn stover hydrolysate by using a thermophilic *Bacillus licheniformis* strain. *Bioresour. Technol.* 170, 256–261. doi: 10.1016/j.biortech.2014.07.101

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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>

- Li, L., Wei, X., Yu, W., Wen, Z., and Chen, S. (2017). Enhancement of acetoin production from *Bacillus licheniformis* by 2,3-butanediol conversion strategy: metabolic engineering and fermentation control. *Process Biochem.* 57, 35–42. doi: 10.1016/j.procbio.2017.03.027
- Li, X., Gou, X., Long, D., Ji, Z., Hu, L., Xu, D., et al. (2014). Physiological and metabolic analysis of nitrate reduction on poly-gamma-glutamic acid synthesis in *Bacillus licheniformis* WX-02. *Arch. Microbiol.* 196, 791–799. doi: 10.1007/s00203-014-1014-y
- Liu, Y., Liu, L., Li, J., Du, G., and Chen, J. (2019). Synthetic biology toolbox and chassis development in *Bacillus subtilis*. *Trends Biotechnol.* 37, 548–562. doi: 10.1016/j.tibtech.2018.10.005
- Liu, Z., Qin, J., Gao, C., Hua, D., Ma, C., Li, L., et al. (2011). Production of (2S,3S)-2,3-butanediol and (3S)-acetoin from glucose using resting cells of *Klebsiella pneumoniae* and *Bacillus subtilis*. *Bioresour. Technol.* 102, 10741–10744. doi: 10.1016/j.biortech.2011.08.110
- Qi, G., Kang, Y., Li, L., Xiao, A., Zhang, S., Wen, Z., et al. (2014). Deletion of meso-2,3-butanediol dehydrogenase gene *budC* for enhanced D-2,3-butanediol production *Bacillus licheniformis*. *Biotechnol. Biofuels.* 7:16. doi: 10.1186/1754-6834-7-16
- Qiu, Y., Zhang, J., Li, L., Wen, Z., Nomura, C. T., Wu, S., et al. (2016). Engineering *Bacillus licheniformis* for the production of meso-2,3-butanediol. *Biotechnol. Biofuels.* 9:117. doi: 10.1186/s13068-016-0522-1
- Rachinger, M., Bauch, M., Strittmatter, A., Bongaerts, J., Evers, S., Maurer, K. H., et al. (2013). Size unlimited markerless deletions by a transconjugative plasmid-system in *Bacillus licheniformis*. *J. Biotechnol.* 167, 365–369. doi: 10.1016/j.jbiotec.2013.07.026
- Sun, J., Zhang, L., Rao, B., Shen, Y., and Wei, D. (2012). Enhanced acetoin production by *Serratia marcescens* H32 with expression of a water-forming NADH oxidase. *Bioresour. Technol.* 119, 94–98. doi: 10.1016/j.biortech.2012.05.108
- Wang, M., Fu, J., Zhang, X., and Chen, T. (2012). Metabolic engineering of *Bacillus subtilis* for enhanced production of acetoin. *Biotechnol. Lett.* 34, 1877–1885. doi: 10.1007/s10529-012-0981-9
- Wang, X., Lv, M., Zhang, L., Li, K., Gao, C., Ma, C., et al. (2013). Efficient bioconversion of 2,3-butanediol into acetoin using *Gluconobacter oxydans* DSM 2003. *Biotechnol. Biofuels* 6:155. doi: 10.1186/1754-6834-6-155

- Wang, Y., Tao, F., Xin, B., Liu, H., Gao, Y., Zhou, N., et al. (2017). Switch of metabolic status: redirecting metabolic flux for acetoin production from glycerol by activating a silent glycerol catabolism pathway. *Metab. Eng.* 39, 90–101. doi: 10.1016/j.ymben.2016.10.020
- Waschkau, B., Waldeck, J., Wieland, S., Eichstadt, R., and Meinhardt, F. (2008). Generation of readily transformable *Bacillus licheniformis* mutants. *Appl. Microbiol. Biotechnol.* 78, 181–188. doi: 10.1007/s00253-007-1278-0
- Xiao, Z., and Lu, J. (2014). Strategies for enhancing fermentative production of acetoin: a review. *Biotechnol. Adv.* 32, 492–503. doi: 10.1016/j.biotechadv.2014.01.002
- Yan, P., Wu, Y., Yang, L., Wang, Z., and Chen, T. (2018). Engineering genome-reduced *Bacillus subtilis* for acetoin production from xylose. *Biotechnol. Lett.* 40, 393–398. doi: 10.1007/s10529-017-2481-4
- Yang, T., Rao, Z., Zhang, X., Xu, M., Xu, Z., and Yang, S. (2017). Metabolic engineering strategies for acetoin and 2,3-butanediol production: advances and prospects. *Crit. Rev. Biotechnol.* 37, 990–1005. doi: 10.1080/07388551.2017.1299680
- Yang, Z., and Zhang, Z. (2019). Recent advances on production of 2, 3-butanediol using engineered microbes. *Biotechnol. Adv.* 37, 569–578. doi: 10.1016/j.biotechadv.2018.03.019
- Yuan, H., Xu, Y., Chen, Y., Zhan, Y., Wei, X., Li, L., et al. (2019). Metabolomics analysis reveals global acetoin stress response of *Bacillus licheniformis*. *Metabolomics* 15:25. doi: 10.1007/s11306-019-1492-7
- Zhang, B., Li, X., Fu, J., Li, N., Wang, Z., Tang, Y., et al. (2016). Production of acetoin through simultaneous utilization of glucose, xylose, and arabinose by engineered *Bacillus subtilis*. *PLoS One* 11:e159298. doi: 10.1371/journal.pone.0159298
- Zhang, J., Zhao, X., Zhang, J., Zhao, C., Liu, J., Tian, Y., et al. (2017). Effect of deletion of 2,3-butanediol dehydrogenase gene (*bdhA*) on acetoin production of *Bacillus subtilis*. *Prep. Biochem. Biotechnol.* 47, 761–767. doi: 10.1080/10826068.2017.1320293
- Zhang, L., Liu, Q., Ge, Y., Li, L., Gao, C., Xu, P., et al. (2016). Biotechnological production of acetoin, a bio-based platform chemical, from a lignocellulosic resource by metabolically engineered *Enterobacter cloacae*. *Green Chem.* 18, 1560–1570. doi: 10.1039/C5GC01638J
- Zhang, X., Bao, T., Rao, Z., Yang, T., Xu, Z., Yang, S., et al. (2014a). Two-Stage pH control strategy based on the pH preference of acetoin reductase regulates acetoin and 2,3-butanediol distribution in *Bacillus subtilis*. *PLoS One* 9:e91187. doi: 10.1371/journal.pone.0091187
- Zhang, X., Zhang, R., Bao, T., Rao, Z., Yang, T., Xu, M., et al. (2014b). The rebalanced pathway significantly enhances acetoin production by disruption of acetoin reductase gene and moderate-expression of a new water-forming NADH oxidase in *Bacillus subtilis*. *Metab. Eng.* 23, 34–41. doi: 10.1016/j.ymben.2014.02.002
- Zhang, X., Zhang, R., Bao, T., Yang, T., Xu, M., Li, H., et al. (2013). Moderate expression of the transcriptional regulator AlsR enhances acetoin production by *Bacillus subtilis*. *J. Ind. Microbiol. Biotechnol.* 40, 1067–1076. doi: 10.1007/s10295-013-1303-5
- Zhang, Y., Li, S., Liu, L., and Wu, J. (2013). Acetoin production enhanced by manipulating carbon flux in a newly isolated *Bacillus amyloliquefaciens*. *Bioresour. Technol.* 130, 256–260. doi: 10.1016/j.biortech.2012.10.036

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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