Production of diacetyl by metabolically engineered Enterobacter cloacae

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Diacetyl, a high value product that can be extensively used as a food ingredient, could be produced from the non-enzymatic oxidative decarboxylation of α-acetolactate during 2,3-butandiol fermentation. In this study, the 2,3-butandiol biosynthetic pathway in Enterobacter cloacae subsp. dissolvens strain SDM, a good candidate for microbial 2,3-butandiol production, was reconstructed for diacetyl production. To enhance the accumulation of the precursor of diacetyl, the α-acetolactate decarboxylase encoding gene (budA) was knocked out in strain SDM. Subsequently, the two diacetyl reductases DR-I (gdh) and DR-II (budC) encoding genes were inactivated in strain SDM individually or in combination to decrease the reduction of diacetyl. Although the engineered strain E. cloacae SDM (AbudAAbudC) was found to have a good ability for diacetyl production, more α-acetolactate than diacetyl was produced simultaneously. In order to enhance the nonenzymatic oxidative decarboxylation of α-acetolactate to diacetyl, 20 mM Fe(III) was added to the fermentation broth at the optimal time. In the end, by using the metabolically engineered strain E. cloacae SDM (AbudAAbudC), diacetyl at a concentration of 1.45 g/L was obtained with a high productivity (0.13 g/(L·h)). The method developed here may be a promising process for biotechnological production of diacetyl.
diacetyl through metabolic engineering. In the present work, the ALDC encoding gene budA has been knocked out and the DRs encoding genes were also inactivated to construct a diacetyl producer (Figure 1). Fe⁺³ was added to the medium to improve the nonenzymatic oxidative decarboxylation of α-acetolactate to produce diacetyl. Through the metabolic engineering approach described, 1.45 g/L diacetyl was synthesized within 11.3 h with a high yield of 0.21 mol/mol using glucose as substrate.

**Results**

**Potential for diacetyl production by E. cloacae SDM.** 2,3-BD exists in three stereoisomeric forms: (2R,3R)-2,3-BD, (2S,3S)-2,3-BD, and meso-2,3-BD. Recently, the mechanism of 2,3-BD stereoisomer formation was identified in 2,3-BD producing strains including K. pneumonia. (2R,3R)-2,3-BD and meso-2,3-BD are mainly produced from the (3R)-AC by the reactions catalyzed by (2R,3R)-2,3-BDH and meso-2,3-BDH, respectively41. (2S,3S)-2,3-BD could only be produced by the meso-2,3-BDH catalyzed reduction of (3S)-AC, which is not an enzymatic decarboxylation product of α-acetolactate but a reduction product of diacetyl41. As shown in Figure 2 I-B, the main metabolic products of E. cloacae SDM were meso-2,3-BD and (2S,3S)-2,3-BD when glucose was used as the carbon source. Low concentrations of (3R)-AC and (3S)-AC were also produced under aerobic conditions. (2S,3S)-2,3-BD could only be produced via the meso-2,3-BDH catalyzed two step reduction of diacetyl, the nonenzymatic oxidative decarboxylation product of α-acetolactate. Thus, diacetyl was produced as an intermediate of 2,3-BD biosynthesis in E. cloacae SDM. Redirecting more carbon flux toward the 2,3-BD into diacetyl through metabolic engineering of E. cloacae SDM might result in an efficient strain for the production of diacetyl.

**Metabolic characteristics of the ALDC mutant of E. cloacae SDM.** Although there was nonenzymatic oxidative decarboxylation of α-acetolactate in E. cloacae SDM, little diacetyl accumulated in the 2,3-BD fermentation process (Figure 2 I-A Figure 2 I-C and Table 1). Since α-acetolactate is mainly subjected to enzymatic conversion to (3R)-AC catalyzed by ALDC besides nonenzymatic oxidative conversion to diacetyl, lack of diacetyl accumulation might be due to the high degradation rate of α-acetolactate. Since the enzymatic irreversible reaction drains the available pool of α-acetolactate for diacetyl formation, knockout of the ALDC might be an effective method for the enhancement of diacetyl production in the E. cloacae strain SDM.

In this study, E. cloacae SDM (ΔbudA) was constructed by knock-out of the budA gene (Gene bank: 13167655) through allele exchange (Figure 2 II-A). The effects of budA gene deletion on the ALDC activity and diacetyl formation of strain SDM are shown in Table 1 and Figure 2 II-C, respectively. In the native strain, the ALDC activity towards α-acetolactate was 3.81 ± 0.16 U/mg while little ALDC activity (0.04 ± 0.00 U/mg) was detected in E. cloacae SDM (AbudA). After 36 h fermentation, the concentration of diacetyl produced by E. cloacae SDM (AbudA) was 59.7 mg/L while only 2.85 mg/L diacetyl was obtained by the native strain SDM (Table 1). Besides diacetyl, (3R)-AC, (3S)-AC, (2R,3R)-2,3-BD, (2S,3S)-2,3-BD, and meso-2,3-BD were also detected in the medium (Figure 2 II-B). These results indicate that the diacetyl would also be converted into those compounds in E. cloacae SDM (AbudA).

**Inactivation of DR-I in the ALDC mutant of E. cloacae SDM.** Glycerol dehydrogenase (GDH) belongs to the medium-chain dehydrogenase family and accepts a broad range of substrates. Diacetyl could be reduced to (3R)-AC and (2R,3R)-2,3-BD by the GDH in K. pneumonia. A gdh gene (Gene bank: 13166340), which exhibits 59% sequence identity with that of K. pneumonia, was identified in the genome sequence of E. cloacae SDM. In this study, the protein encoded by gdh gene was renamed as DR-I due to its diacetyl reduction activity. As shown in Table 1, inactivation of DR-I would result in a lower DR activity of E. cloacae SDM (ΔbudAAgdh) than that of the strain E. cloacae SDM and the mutant strain E. cloacae SDM (AbudA). However, the concentration of diacetyl increased modestly to only 326.7 mg/L (Table 1). (3R)-AC, (3S)-AC, (2R,3R)-2,3-BD, (2S,3S)-2,3-BD, and meso-2,3-BD would still accumulate during the fermentation (Figure 2 III-B).

**Inactivation of DR-II in the ALDC mutant of E. cloacae SDM.** The genes that encode ALDC, ALS, and meso-2,3-BDH are sequentially clustered in one operon in E. cloacae SDM (Figure S1). Our previously studied enzymatic reactions showed that meso-2,3-BDH can catalyze the conversion of diacetyl to (3S)-AC and further to (2S,3S)-2,3-BD as well as (3R)-AC to meso-2,3-BD. In this study, the meso-2,3-BDH (renamed as DR-II) encoding gene budC (Gene bank: 13167657) was knocked out through the allele exchange in E. cloacae SDM (AbudA) (Figure 2 IV-A).

As shown in Table 1, inactivation of DR-II would result in a sharp decrease of DR activity in E. cloacae SDM (AbudAΔbudC). The concentration of diacetyl increased to 416.1 mg/L after 36 h fermentation (Figure 2 IV-C, Table 1). The budC mutant lost the ability to produce (2S,3S)-2,3-BD and meso-2,3-BD (Figure 2 IV-B). This
phenotype indicates that the formation of both \((2S,3S)-2,3\)-BD and \(\text{meso}-2,3\)-BD depends on the activity of DR-II.

Then, DR-I and DR-II were both inactivated in the ALDC mutant of \(E.\ cloacae\) SDM (Figure S2). As shown in Table 1, the DR activity would further decrease in the DR-I and DR-II double mutant. However, the glucose consumed, biomass, and concentration of diacetyl would also decrease in the mutant of \(E.\ cloacae\) SDM (\(D\)\(budA\)\(D\)\(budC\)\(D\)\(gdh\)). Since the concentration (416.10 mg/L) of diacetyl obtained by \(E.\ cloacae\) SDM (\(D\)\(budA\)\(D\)\(budC\)) was higher than that of other strains, \(E.\ cloacae\) SDM (\(D\)\(budA\)\(D\)\(budC\)) was chosen for further investigation.

Diacetyl production by \(E.\ cloacae\) SDM (\(D\)\(budA\)\(D\)\(budC\)). Diacetyl production using \(E.\ cloacae\) SDM (\(D\)\(budA\)\(D\)\(budC\)) was conducted at 37 °C in 300-mL shake flasks containing 50 mL medium. The medium was M9 medium supplemented with 18 g/L glucose and 5 g/L yeast extract\(^2\). The initial pH was 7.4. As shown in Figure 3, 59.8 mg/L diacetyl was obtained from 15 g/L glucose after 12 h of bioconversion. The yield of diacetyl was only at 0.83% of the theoretical value.

The concentration of \(\alpha\)-acetolactate produced by \(E.\ cloacae\) SDM (\(D\)\(budA\)\(D\)\(budC\)) was also analyzed during the 12 h of bioconversion. \(\alpha\)-Acetolactate of 2.94 g/L was produced. This indicated that the strain \(E.\ cloacae\) SDM (\(D\)\(budA\)\(D\)\(budC\)) showed an almost 325 (mol/mol) co-production of \(\alpha\)-acetolactate and diacetyl. Thus, diacetyl production could be further enhanced by the transformation of \(\alpha\)-acetolactate accumulated in medium.

### Table 1 | Enzyme activities, glucose consumption and diacetyl production of \(E.\ cloacae\) SDM and its derivatives

| Strain                  | DR (U/mg) ± SD | ALDC (U/mg) ± SD | Glucose (g/L) ± SD | Diacetyl (mg/L) ± SD |
|-------------------------|----------------|------------------|--------------------|----------------------|
| SDM                     | 14.20 ± 1.11   | 3.81 ± 0.16      | 35                 | 2.85 ± 1.57          |
| \(D\)\(budA\)          | 5.72 ± 0.23    | 0.04 ± 0.00      | 19.5               | 59.70 ± 11.24        |
| \(D\)\(budA\)\(gdh\)  | 5.29 ± 0.13    | 0.003 ± 0.00     | 17                 | 326.66 ± 7.54        |
| \(D\)\(budA\)\(budC\) | 0.53 ± 0.09    | 0.004 ± 0.00     | 17                 | 416.10 ± 13.66       |
| \(D\)\(budA\)\(budC\)\(gdh\) | 0.47 ± 0.04 | 0.003 ± 0.00 | 14.5               | 318.31 ± 33.08       |

DR: diacetyl reductase; ALDC: \(\alpha\)-acetolactate decarboxylase.

*Data are the means ± standard deviations (SDs) from three parallel experiments.
of diacetyl was 0.21 mol/mol glucose. During the two-step bioconversion process, diacetyl was produced with a high productivity of 0.13 g/(L·h).

**Discussion**

Diacetyl has a strong buttery flavor and is mainly existed at low concentration in many dairy products, such as butter, beer, and fresh cheeses. Its formation in dairy products mainly results from the catabolism of α-acetolactate during 2,3-BD fermentation by certain species of lactic acid bacteria. Due to the excellent performance of *E. cloacae* SDM as an efficient 2,3-BD producing strain, developing a metabolically engineered strain based on *E. cloacae* SDM through redirecting carbon flux toward the 2,3-BD pathways for the production of diacetyl is quite attractive and promising.

In the present study, the diacetyl production from glucose by *E. cloacae* SDM was firstly conducted through two genetic strategies: (i) inactivation of the ALDC gene (*budA*) to avoid enzymatic conversion of the diacetyl precursor α-acetolactate to (3R)-AC as described previously, and (ii) inactivation of the DR gene to avoid enzymatic reduction of diacetyl. Two DRs encoding genes (*gdh* and *budC*) were identified in the genome sequence of *E. cloacae* SDM. *E. cloacae* SDM (*AbudAAbudCgdh*) produced diacetyl at a concentration (318.31 mg/L) lower than that of *E. cloacae* SDM (*AbudAAbudC*) (416.10 mg/L). This result indicates that DR might be important to strain SDM for glucose utilization and cell growth. On the other hand, when DR-I and DR-II were both inactivated in the ALDC mutant, (3R)-AC, (3S)-AC, and (2R,3R)-2,3-BD could still be detected (Figure S2), indicating the presence of the third DR (DR-III) responsible for these chemical production in *E. cloacae* strain SDM (Figure 1).

Although 2.94 g/L α-acetolactate was produced from 15 g/L glucose after 12 h of bioconversion, only 59.8 mg/L diacetyl was obtained and the final molar ratio of α-acetolactate and diacetyl was 32:1 (Figure 3), implying an inefficient NOD of α-acetolactate to diacetyl. Thus, besides redirecting carbon flux toward production of α-acetolactate through genetic methods, more efficient chemical conversion of α-acetolactate into diacetyl should also be developed for optimal production of diacetyl. In the study by Gao et al., an efficient chemical conversion of α-acetolactate to diacetyl could be achieved by addition of Fe³⁺. However, it was indicated that Fe³⁺ would also influence the glucose consumption (Figure 4B) and hence might decrease the diacetyl production during the fermentation process. Thus, the addition time of 20 mM Fe³⁺ was also optimized in the present study. As shown in Figure 4A, when added at 10 h,
20 mM Fe$^{3+}$ could accelerate the NOD of $\alpha$-acetolactate, and accumulate the highest concentration of diacetyl.

Several biotechnological routes have been used to produce diacetyl (Table 2). Among all of the reported biotechnological processes, the group of Liu obtained the highest diacetyl concentration of 4.7 g/L with a metabolically engineered C. glabrata$^{25}$. Efforts have been tried in order to increase the yield of diacetyl through inactivation of ALDC and overexpression of NADH oxidase in L. lactis. Using 5 g/L glucose as the substrate, the recombinant L. lactis produced 0.38 g/L diacetyl at a high yield of 0.16 mol/mol glucose$^{1}$. In this study, metabolic engineering based on 2,3-BD pathway was used to reconstruct E. cloacae SDM as a novel biocatalyst for diacetyl production. Under optimal conditions, the recombinant E. cloacae SDM ($\Delta$budA$\Delta$budC) could produce diacetyl with rather high concentration (1.45 g/L), productivity (0.13 g/(L h)) and yield (0.21 mol/mol). Both the productivity and yield of diacetyl produced by the recombinant E. cloacae were new records for diacetyl production (Table 2). The carbon flux channeled into the diacetyl biosynthetic might be further enhanced since there were still (3R)-AC, (3S)-AC, and (2R,3R)-2,3-BD accumulated during the fermentation (Figure 2 IV-C Figure 2 IV-B). This may be accomplished by searching the undiscovered diacetyl reductase, or overexpressing NADH oxidase, which could lead to prevention of NADH dependent reduction of diacetyl.

**Methods**

Chemicals and biochemicals. (2R,3R)-2,3-BD (98.0%), (2S,3S)-2,3-BD (99.0%), and meso-2,3-BD (98.0%) were purchased from ACROS (The Kingdom of Belgium). Racemic AC, ethyl 2-acetoxy-2-methyl-acetoacetate, and diacetyl were purchased from Sigma. NADH was purchased from Amresco. Restriction enzymes were purchased from TaKaRa Bio Inc. (China). PCR primers were prepared by Sangon (Shanghai, China). FastPfu DNA polymerase and T4 DNA ligase were purchased from Transgen Biotech (China) and MBI (USA), respectively. All other chemicals were of analytical grade and commercially available.

**Bacterial strains and plasmids.** All the strains and plasmids used in this study are listed in Table 3. E. coli DH5$\alpha$ was used for general cloning procedures. The pKR6K was used for gene knock-out in E. cloacae strain SDM$^{15}$. E. coli S17-1, which is able to host pKR6K and its derivatives, was used for conjugation with E. cloacae SDM. Lysogenic broth (LB) medium was used for the culture of E. coli and E. cloacae SDM. The selection medium in the conjugation experiments was M9 minimal medium supplemented with 1% sodium citrate as the carbon source and 0.05% ammonium chloride as the nitrogen source. Solid LB medium with 10% sucrose was used to select plasmid excision from the chromosome through the gene allelic exchange experiments. Kanamycin was used at a concentration of 50 $\mu$g/mL.

**Knock out of the genes in E. cloacae SDM.** Primers used in this study are listed in Table S1. Isolation of vectors, restriction enzyme digestion, agarose gel electrophoresis, and other DNA manipulations are carried out by standard protocols$^{22}$. Mutants of E. cloacae strain SDM were generated by allele exchange using the suicide plasmid pKR6K$^{23}$. The left and right flanking sequences were amplified from E. cloacae SDM and then ligated through PCR to get $\Delta$budA fragment using primer pairs PAbudA.f (EcoRI)/PAbudA.r (overlap), PAbudA.r (over)/PAbudA.t (BamHI). The gel-purified $\Delta$budA fragments were ligated to the pKR6K vector digested with the EcoRI and BamHI.

**Table 2 | Comparison of diacetyl production by different microorganisms**

| Strain | Engineering strategy | Diacetyl (g/L) | Yield (mol/mol) | Productivity (g/(L h)) | Reference |
|--------|----------------------|---------------|-----------------|------------------------|-----------|
| L. lactis | Inactivation of ALDC, overexpression of NADH oxidase | 0.38 | 0.16 | 0.03 | 14 |
| L. lactis | Random mutagenesis | 0.52 | – | 0.02 | 17 |
| L. rhamnosus | WT | 0.2 | 0.2 | 0.06 | 19 |
| E. aerogenes | UV mutation and medium optimization | 1.35 | 0.03 | – | 16 |
| L. lactis | Inactivation of ALDC, overexpression of NADH oxidase | 0.36 | 0.12 | 0.03 | 18 |
| C. glabrata | Overexpression of ALS, inactivation of ALDC and DR, medium optimization | 4.70 | 0.10 | 0.07 | 12 |
| E. cloacae | Inactivation of ALDC and DR, Fe$^{3+}$ addition | 1.45 | 0.21 | 0.13 | This study |

$^a$Not mentioned in the reference.
Batch fermentation. The batch fermentation was conducted in 300-mL shake flasks containing 50 mL medium. The medium consisted of M9 medium supplemented with 18 g/L glucose and 2 g/L casamino acids. The cultures were grown for 8 h, then centrifuged at 13,000 g for 15 min. The cell debris was removed through centrifugation at 13,000 g for 15 min. Enzyme activity was assayed in the resulting supernatant.

The activity of ALDC was assayed by detecting the production of AC from L-α-αacetolactate. α-Aacetolactate was prepared immediately before use from ethyl 2-acetoxy-2-methyl-acetacetate according to the protocol supplied by the manufacturer. One unit of ALDC activity was defined as the amount of protein that produced 1 µmol of AC per min.

The activity of DR was assayed spectrophotometrically by measuring the change in absorbance at 340 nm corresponding to the oxidation of NADH (εmax = 6220 M⁻¹ cm⁻¹) at 30°C using a UV/visible spectrophotometer (UltraSpec 2100 pro, Amersham Biosciences, USA)26. The reaction solution for DR assay contained 5 mM of diacetyl and 0.2 mM of NADH in 67 mM phosphate buffer (pH 7.4). One unit of activity was defined as the amount of enzyme that consumed 1 µmol of NADH per min. The protein concentration was measured by the Lowry method, with bovine serum albumin as the standard27.

Analytical methods. Samples were withdrawn periodically and centrifuged at 12,000 × g for 10 min. The cell density was determined by monitoring the absorbance at 600 nm using a spectrophotometer (LENGUANG-721, China) after an appropriate dilution. The concentration of glucose was measured enzymatically by a bio-analyzer (SBA-40D, Shandong Academy of Sciences, China) after diluting to an appropriate concentration. The concentrations of 2,3-βĐ and AC were analyzed by GC as described in Ma et al.28 The concentrations of α-acetolactate and diacytale were determined by the methods described in the previous reports25,26.

Table 3. Bacterial strain and plasmid used in this study.

| Strain or plasmid | Characteristics(s) | Source or reference |
|-------------------|---------------------|---------------------|
| E. coli DH5α | F-, ∆80 lacZΔ15, lacY-argF-U169, recA1, endA1, hsdR17, phoA, supE44, thi-1, gyrA96, relA1 | Novagen |
| E. coli S17-1 | recA, pro, thi, conjugative strain able to host λ-pir-dependent plasmids | 31 |
| E. cloacae SDM | Wild-type | 5 |
| SDM (∆budA) | E. cloacae SDM budA disruption mutant strain | This study |
| SDM (∆budA budC) | E. cloacae SDM budA and budC disruption mutant strain | This study |
| SDM (∆budA budC) | E. cloacae SDM budA and gdh disruption mutant strain | This study |
| SDM (∆budA budC CsgA) | E. cloacae SDM budA budC and gdh disruption mutant strain | This study |
| pEASYBlunt | Ap+, cloning vector | Transgen |
| pKR6K | Km+, gene replacement vector derived from plasmid pK18mobSacB, R6K origin, Mob+ SacB | 24 |
| pKDbudA | Km+, pKR6K derivative, carries a 587 bp deletion of budA | This study |
| pKDbudC | Km+, pKR6K derivative, carries a 639 bp deletion of budC | This study |
| pKDgdh | Km+, pKR6K derivative, carries a 302 bp deletion of gdh | This study |

*Ap*, ampicillin resistance; *Km*, kanamycin resistance.
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Author contributions
C.G. and C.M. participated in the design of the study. L.Z., Y.Z., Q.L. and L.M. executed the experimental work. L.Z., M.H., K.L. and M.L. analyzed the data. C.G., C.M. and P.X. contributed reagents and materials. L.Z., C.G., C.M. and P.X. wrote and revised the manuscript. All authors read and approved the final manuscript.

Additional information
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