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Title:

Heterologous Expression of an Acetoin Reductase leads to D-2,3-butanediol production in *Clostridium acetobutylicum*

Running title:

D-2,3-butanediol production in *C. acetobutylicum*

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ABSTRACT

Acetoin reductase (ACR) catalyzes the conversion of acetoin to 2,3-butanediol. Under certain conditions *Clostridium acetobutylicum* ATCC 824 (and derived strains) generates both D- and L-stereoisomers of acetoin, but due to the lack of an ACR enzyme, does not produce 2,3-butanediol. A gene encoding ACR from *Clostridium beijerinckii* NCIMB 8052 was functionally expressed in *C. acetobutylicum* under control of two strong promoters, the constitutive *thl* promoter and the late exponential *adc* promoter. Both ACR-overproducing strains were grown in batch cultures, during which 89-90% of the natively produced acetoin was converted to 20-22 mM D-2,3-butanediol. Addition of a racemic mixture of acetoin did lead to the production of both, D-2,3-butanediol and *meso*-2,3-butanediol. A metabolic network is proposed that is in agreement with the experimental data. Native 2,3-butanediol production is a first step towards a potential homo-fermentative 2-butanol producing strain of *C. acetobutylicum*.

INTRODUCTION

To meet our future energy needs, it is necessary to develop sustainable and carbon-neutral energy sources. Liquid biofuels are attractive candidates, since little or no change is needed to the current petroleum-based fuel technologies (3). For this purpose, biological production of several alcohols is under investigation, including ethanol and butanol (4, 10).

Several clostridial species are able to ferment carbohydrates to acetone, 1-butanol and ethanol (ABE). Industrial application of this process, also known as the ABE-fermentation, has a long history, but the process economics after 1960 became unfavorable compared to the petrochemical process and its commercial exploitation was gradually abandoned (14). The inefficiency of the fermentation still hampers commercial reintroduction of this renewable butanol production process. Improving the yields and productivities of the solvent products are key to its successful reintroduction.

One of the factors reducing the fermentation efficiency is the toxic effect that 1-butanol has on the culture. Butanol has membrane distorting properties, due to its hydrophobic chain and polar group, which cause severe cell damage (7, 29). Many efforts have been made in the past to obtain clostridial strains with increased 1-butanol tolerance, however with limited success (1, 5, 6, 12, 15, 25).

As an alternative to increasing 1-butanol tolerance, we propose to replace the production of 1-butanol by the production of a compound that has similar physical and chemical properties (heat of combustion, heat of vaporization, and energy density), but which is less toxic to the cell, making higher titers possible. 2-Butanol matches these criteria and has a lower *log* P_{ow} value (octanol:water coefficient) than 1-butanol. The *log* P_{ow} value is a good indicator for the strength of membrane perturbing effects (28).

Generally, the lower the *log* P_{ow} value, the less toxic the compound is to the membrane. However, *C. acetobutylicum* is not known to produce 2-butanol nor its potential precursor 2,3-butanediol (2,3-BD) (9). Nevertheless, it is known to produce acetoin as a minor fermentation product (14).

The 2,3-butanediol biosynthesis route proceeds via pyruvate, acetolactate and acetoin to 2,3-butanediol. Acetolactate is formed *in vivo* by coupling two molecules of pyruvate with the concomitant release of carbon dioxide, catalyzed by acetolactate synthase. Decarboxylation by acetolactate decarboxylase yields acetoin (31), which can be reduced by an acetoin reductase to 2,3-butanediol.

Our aim is to construct a 2,3-BD producing *C. acetobutylicum* strain as a first step towards biological 2-butanol production. In this study, the cloning and functional expression is described of an acetoin reductase (ACR)-encoding gene from *C. beijerinckii* NCIMB 8052 in *C. acetobutylicum*, resulting in the production of D-2,3-butanediol.

MATERIALS AND METHODS

Bacterial strains and plasmids

All bacterial strains and plasmids used during this study are listed in table 1. *E. coli* stocks were stored in 20 % (v/v) glycerol at -80 °C. Stock cultures of *C. acetobutylicum* strains and *C. beijerinckii* NCIMB 8052 were maintained as spore suspensions in 15 % (v/v) glycerol at -20 or -80 ℃. Chemically competent, *E. coli* NEB 5-alpha F' I^q cells were used for cloning and vector maintenance. Electro competent *E. coli* DH10B (pAN1) were used to methylate plasmid DNA before transformation into *C. acetobutylicum* (18).

Media and growth conditions

E. coli strains were cultured in lysogeny broth (LB) medium at 37 ℃, 200 RPM. Sporulation plates were based on media by Nimcevic *et al*. (22) but contained in addition 15 g L^{-1} agar. Prior to inoculation of clostridial pre-cultures, spore suspensions were heat shocked for 10 minutes at 70 or 80 ℃. *C. acetobutylicum* strains were grown in MG medium or modified CGM (mCGM) medium as indicated.

MG medium was based on the semi-synthetic medium described by Nimcevic *et al*. (22), and contained per liter of water: yeast extract, 2.5 g; KH_2PO_4, 1.0 g; K_2HPO_4, 0.76 g; ammonium acetate, 3.0 g; *p*-aminobenzoic acid, 0.10 g; $MgSO_4 \cdot 7\ H_2O$, 1.0 g; and $FeSO_4 \cdot 7\ H_2O$, 0.01 g.

mCGM medium contained per liter of water: yeast extract, 5.0 g; KH_2PO_4, 0.75 g; K_2HPO_4, 0.75 g; $MgSO_4 \cdot 7\ H_2O$, 0.4 g; $MnSO_4 \cdot H_2O$, 0.01 g; $FeSO_4 \cdot 7\ H_2O$, 0.01 g; NaCl, 1.0 g; asparagine, 2.0 g; $(NH_4)_2SO_4$, 2.0 g; cysteine, 0.125 g; and glucose, 12.5 g.

Medium for fermentation was made anaerobic by sparging with nitrogen gas. Serum flasks (250 mL), containing 100 mL MG medium, were inoculated with 2 % (v/v) overnight pre-cultures. Clostridial culture experiments were performed at 37 ℃, without shaking, and anaerobically in (i) an anaerobic chamber; or (ii) in glass serum vials as described previously (16).

Culture media were supplemented with ampicillin (100 μg mL^{-1}), chloramphenicol (30 μg mL^{-1}), erythromycin (40 μg mL^{-1} for liquid cultures and plates; 25 μg mL^{-1} for transformant isolation), kanamycin (50 μg mL^{-1}), IPTG (50 μg mL^{-1}) and X-Gal (40 μg mL^{-1}) when appropriate. For challenge experiments, acetoin or 2,3-BD was added to the medium prior to inoculation with the pre-culture.

The growth of clostridial cultures was monitored spectrophotometrically at 600 nm (Pharmacia Biotech Ultrospec 2000).

DNA isolation, transformation and manipulation

Standard molecular work was performed according to established protocols (24). Genomic DNA from *C. acetobutylicum* or from *C. beijerinckii* was isolated using the GenElute Bacterial Genomic DNA Kit (Sigma-Aldrich). Plasmid DNA from *E. coli* was isolated by the GenElute Plasmid Miniprep Kit (Sigma-Aldrich). PCR amplification of clostridial DNA was done using *Pfu* polymerase (Stratagene), *E. coli* colony PCR reactions were carried out using REDTaq (Sigma-Aldrich).

Methylated plasmids were electroporated into *C. acetobutylicum* according to Oultram *et al*. (23). Correct methylation was checked by restriction analysis using *Fnu4*HI (18).

Cloning of the Cb-acr *gene into* C. acetobutylicum

The clostridial expression plasmids pWUR459 and pWUR460 were constructed as detailed in the supplementary materials. These plasmids, and the control plasmid pMTL500E, were used to transform *C. acetobutylicum*. Each transformation resulted in multiple erythromycin-resistant colonies. After re-streaking, selected colonies were used to prepare spore suspensions for further experiments.

Acetoin reductase enzyme assays

C. acetobutylicum cells were harvested from cultures with an OD of 5 by centrifugation (4816 × *g*, 15 min, 4 ℃) and resuspended in 20 mM Tris-HCl buffer (pH = 7.5) containing 1 mM TCEP as reducing agent, because 2-mercaptoethanol was shown to inhibit enzyme activity. Crude cell extracts were prepared by French press homogenization (two passes at 16 000 psi) and immediately assayed for enzyme activity. Assays were carried out at 37 ℃ in 100 mM phosphate buffer (pH = 6.5) containing 1 mM TCEP, 50 mM D/L-acetoin and 0.28 mM NADPH. The reaction was started by the addition of acetoin. The decrease in absorbance at 340 nm due to NADPH oxidation was followed on a Hitachi U2010 spectrophotometer with correction for background NADPH oxidation. One unit of enzyme activity (U) was defined as the amount of enzyme required for the oxidation of 1 µmol of NADPH per minute. Total protein in crude extracts was determined using Roti-Nanoquant (Carl Roth, Karlsruhe, Germany) with BSA as a standard.

HPLC analysis of glucose and metabolites

Fermentation samples were centrifuged (5 min, 20 800 × *g*) and the supernatants were stored at -20 °C. After thawing, an equivolume amount of internal standard solution (either 100 mM valeric acid (Sigma-Aldrich) in 1 M H_2SO_4, or 30 mM 4-methyl valeric acid (Sigma-Aldrich) in 0.5 M H_2SO_4) was added to the supernatant sample and then filtered (0.2 µm, Whatman). Separation of a 10 µL sample was achieved using a Shodex Ionpack KC-811(RP) column, equipped with a refractive index detector (Waters 2414) and a UV detector (Waters 2487) operating at 210 nm, with 3 mM H_2SO_4 as eluent (flow: 1 mL min^{-1}; column temperature: 85 °C). The order of elution was: glucose, lactic acid, acetic acid, acetoin, *meso*-2,3-BD, D/L-2,3-BD, butyric acid, acetone, ethanol, valeric acid, 4-methyl valeric acid and 1-butanol.

Chiral GC-MS analysis

To determine the enantiomeric distribution of the produced 2,3-BD and acetoin, the fermentation samples were treated like the HPLC samples. However, after thawing, the samples were additionally saturated with sodium chloride and extracted once with an equivolume amount ethyl acetate. To prevent co-extraction of acids, 10 M sodium hydroxide was added, since these compounds interfered with chromatographic analysis. Samples for acetoin analysis were not treated with sodium hydroxide to prevent potential racemization. The extract was then analyzed on a Trace DSQ GC-MS system (Thermo) equipped with a CP-Chirasil-Dex CB (Varian) fused silica capillary column (25 m x 0.25 mm x 0.25 µm) with helium as the carrier gas. Injection port temperature was set at 250 °C, with a split ratio of 1:10. The oven temperature program was as follows: 80 °C (10 min), increased to 120 °C at 10 °C min^{-1} followed by a ramp of 40 °C

1 min^{-1} to 200 ℃ (4 min). Samples (1 or 5 µL) were injected using an autosampler
2 AS3000 (Thermo). The ion source (EI) temperature was set to 200 ℃. Compound
3 identification by column retention time was confirmed by analysis of the mass spectrum.
4 Retention times of chiral standards of D-(-)-(2*R*,3*R*) and L-(+)-(2*S*,3*S*)-2,3-BD
5 (Sigma-Aldrich) were used for peak identification. The elution order of the acetoin
6 enantiomers was inferred from the stereochemistry of the product D-(2*R*,3*R*)-2,3-BD
7 (11). The order of elution was: (3*R*)-acetoin, (3*S*)-acetoin, L-(2*S*,3*S*)-2,3-BD,
8 D-(2*R*,3*R*)-2,3-BD and *meso*-2,3-BD.
9

RESULTS

Characterization of C. acetobutylicum *transformants*

Wild type *C. acetobutylicum* ATCC 824 is known to produce significant levels of acetoin, but no 2,3-butanediol (2,3-BD) (14). Recently, a gene from *C. beijerinckii* NCIMB 8052, *Cbe_1464* annotated as an alcohol dehydrogenase, was functionally expressed in *E. coli* and demonstrated to possess acetoin reductase activity (Siemerink and Kengen, unpublished results). Introduction of this acetoin reductase (ACR)-encoding gene might enable conversion of acetoin to 2,3-BD. Therefore, *C. acetobutylicum* transformants were constructed, containing the *Cb-acr* gene under control of either the *thl* promoter (pWUR459) or the *adc* promoter (pWUR460), and their fermentation pattern was analyzed. Both types of transformants were found to produce D-2,3-BD (Table 2). No D-2,3-BD was produced by the control strain, containing the empty vector. Acetoin was found to accumulate transiently at the end of the exponential growth phase of both transformant strains, with levels reaching 4 ± 1.6, 8 ± 0.7 and 9 ± 0.3 mM for pWUR460 (*thl* promoter), pWUR459 (*adc* promoter) and pMTL500E (empty vector), respectively (Figure 1). These data suggest that the conversion of acetoin to 2,3-BD is limiting, especially in the case of the *thl* promoter construct, which should result in constitutive expression. The final 2,3-BD concentrations and conversion levels for both Cb-ACR strains did not differ significantly from one another: 22 mM and 90% acetoin conversion for the P_{thl}-*Cb-acr* strain, and 20 mM and 89% for the P_{adc}-*Cb-acr* strain. Acetoin levels of the control fermentation reached 19 mM. However, in fermentations of Cb-ACR expressing strains, acetoin was still detected at levels of 2 to 3 mM at the end of the fermentation (Figure 1 and Table 2).

Analysis of medium samples of the control strain harboring pMTL500E (EV), showed a ratio of approximately 12:1 (D:L) in the concentrations of the two acetoin enantiomers (data not shown).

The transformants expressing the *Cb-acr* gene showed similar acid and solvent production patterns as the control strain harboring the empty vector (Table 2). Remarkably, all three transformants produced small but nonetheless detectable amounts of *meso*-2,3-BD (Table 2). To confirm this finding, we also looked at fermentations by our wild type *C. acetobutylicum* strain, in the same (MG) and other media (mCGM and CGM). At the end of all fermentations, small amounts of 1 to 3 mM, *meso*-2,3-BD were found (Figure 2 and data not shown). In all these fermentations, no D- or L-2,3-BD was detected.

Cell homogenates of wild type and transformed *C. acetobutylicum* strains were assayed for acetoin reductase activity. A low, but significant, activity level of 0.042 ± 0.0035 U mg^{-1} and 0.042 ± 0.0044 U mg^{-1} could be detected in cell free extracts for pWUR459 and pWUR460, respectively. Background levels were 0.018 ± 0.0015 U mg^{-1} and 0.024 ± 0.0021 U mg^{-1} for the WUR and pMLT500E vector control strain, respectively. Detection was complicated by the rapid loss of activity also seen with purified enzyme isolated from *E. coli* extracts (Siemerink and Kengen, unpublished results).

Product stereochemistry

There are three stereoisomeric forms of 2,3-BD. The main stereoisomer produced by fermentation in MG medium was identified as D-(2*R*,3*R*)-2,3-BD. However, also low levels of *meso*-2,3-BD were detected in transformant strains, as well as in wild type

fermentations by both HPLC and GC-MS analysis. In all fermentations, L-(2*S*,3*S*)-2,3-BD was below our detection threshold. Figure 2 shows the gas chromatographic analysis of extracts of standard and medium samples of cultures of the various *C. acetobutylicum* strains.

The observation that both the plasmid control strain as well as our wild type strain produced *meso*-2,3-BD is a new observation for *C. acetobutylicum* and prompted us to investigate this further. An independently obtained *C. acetobutylicum* ATCC 824 type strain sample that was grown under identical conditions, did not produce *meso*-2,3-BD. This suggests that our ATCC 824 lab strain has diverged from the type strain. We therefore refer to our lab strain as *C. acetobutylicum* WUR (Table 1).

Acetoin and 2,3-BD challenged batch fermentations

To determine possible inhibitory effects of 2,3-BD on the cultures of *C. acetobutylicum* transformants expressing the *Cb-acr*-gene, fermentations in media supplemented with 20 mM D-(2*R*,3*R*) or 20 mM *meso*-2,3-BD were performed. In cultures challenged with D-2,3-BD, both transformants containing the *Cb-acr* gene produced additional D-2,3-BD in similar amounts (12 mM for strain pWUR459 and 20 mM for strain pWUR460) compared to their non-challenged controls (15 mM and 17 mM respectively). The use of *meso*-2,3-BD resulted in similar behavior (Table 3).

We also supplemented media with racemic acetoin (20 mM) to check if the amount of produced acetoin was limiting for the production of 2,3-BD. Supplemented cultures of ACR expressing strains converted both D- and L-enantiomers into D- and *meso*-2,3-BD (Table 3). This demonstrates that the Cb-ACR enzyme is able to convert both acetoin enantiomers and is therefore not stereoselective for the configuration at the C3 position.

The total amount of both 2,3-BD diastereomers produced in the challenged cultures, 36 mM (pWUR459) and 41 mM (pWUR460), corresponds with the total amount of acetoin consumed.

Interestingly, the plasmid control strain produced more *meso*-2,3-BD in the acetoin challenged cultures. It increased significantly from 2 ± 0.4 mM in the non challenged control to 4 ± 0.2 mM in the racemic acetoin challenged culture. However, still no D- or L-2,3-BD was observed.

DISCUSSION

Acetoin reductase (ACR) is an enzyme that catalyses the reduction of acetoin to 2,3-butanediol. Although *Clostridium beijerinckii* NCIMB 8052 contains a homologue (*Cbe_1464*) of a *Bacillus cereus* 2,3-butanediol dehydrogenase gene in its genome (13), we did not find any report in the scientific literature mentioning the production of either the ACR substrate acetoin or its product, 2,3-butanediol, by *C. beijerinckii*. In this study, we showed that when the *C. beijerinckii acr* gene is expressed in *C. acetobutylicum*, natively produced acetoin is reduced to D-(2*R*,3*R*)-2,3-BD (Table 2). This conversion is in agreement with the proposed acetoin reductase function of the cloned *C. beijerinckii* gene based on functional expression in *E. coli*, and with a recent publication on an acetoin reductase (BdhA) of *Bacillus subtilis* (21). The amino acid sequence of this enzyme is very similar to the *C. beijerinckii* ACR with 51% identical residues and 66% similar residues.

Clostridium acetobutylicum ATCC 824 is known to produce acetoin as a minor fermentation product, but has never been reported to produce *meso*-2,3-BD (8, 30). Our analysis of fermentations of an independently obtained ATCC 824 type strain confirms this. In the course of this study we, however, found that both the wild type and the plasmid control of the *C. acetobutylicum* WUR strain does produce *meso*-2,3-BD. Apparently our laboratory stock, originally acquired as the ATCC 824 type strain, evolved a divergent phenotype. Despite this, we continued to investigate the fermentative behavior of our transformant strains, as they showed interesting properties.

An earlier report describing an attempt to engineer *C. acetobutylicum* to produce 2,3-butanediol by heterologous expression of a *Klebsiella pneumoniae* ACR was unsuccessful (30). Contrary to the reported approach we decided to express the

C. beijerinckii acr gene, which has a GC content (35%) which is comparable to that of the DNA of the *C. acetobutylicum* host (31%), and a similar codon usage (20). We also used a different shuttle vector (pMTL500E) with another origin of replication, in an attempt to increase the gene dosage. The pMLT500E plasmid (pAMβ1 origin) has a higher copy-number in *C. acetobutylicum* (19, 23) than the low-copy-number pSOS84 (pIM13 origin) derived plasmids used by Wardwell *et al*. (30). Combined with strong promoters (either P_{adc} or P_{thl}), our approach resulted in expression levels of the *Cb-acr* gene by *C. acetobutylicum* high enough to lead to 2,3-BD production despite the fact that the detected specific activity is relatively low.

Two different expression constructs, pWUR459 (P_{adc}-*Cb-acr*) and pWUR460 (P_{thl}-*Cb-acr*), were transformed into *C. acetobutylicum* to test the influence of the two different expression profiles of the promoters on the fermentation. Both transformant strains were able to convert approximately 90% of the natively produced acetoin into D-2,3-BD, with values reaching 22-23 mM. Compared to the industrial strain *Klebsiella pneumoniae* SDM*,* which reaches concentrations up to 1664 mM, this is relatively low (17). However, for this species, 2,3-BD is the main fermentation product, whereas for our strain, 2,3-BD is only a side product next to the solvents butanol and acetone whose levels remained unaltered.

During the acidogenic phase, the pWUR460 (*thl*-controlled) fermentation showed a significantly lower acetoin level compared to the pWUR459 (*adc*-controlled) culture or the empty vector control. The level of 2,3-BD was concomitantly higher in the *thl*-controlled culture. This difference in acetoin and 2,3-BD levels between the *adc* and *thl* driven *acr* expression is in good agreement with the differences in promoter activity (2, 26). Later during the fermentation, this difference is leveled out. Despite constitutive

expression of *Cb-acr* by the strain containing pWUR460, accumulation of acetoin in the medium was still observed, which suggests that the acetoin production flux under these conditions is higher than the flux from acetoin to 2,3-BD can accommodate.

Identification of bottlenecks for 2,3-BD production

In our experiments, transformant cultures were challenged by the addition of 20 mM racemic acetoin, resembling the levels observed in the final stages of normal growth. The observation that similar amounts of acetoin were produced by the control strain in both unchallenged and acetoin challenged fermentations (Table 3) indicates that acetoin production is not affected by its extracellular concentration, at the concentrations tested. This is in agreement with the fact that in non-challenged fermentations of *Cb-acr* expressing transformants, the combined levels of acetoin and 2,3-BD do not significantly exceed those of the acetoin levels of the control strain fermentation. In racemic acetoin supplemented fermentations with Cb-ACR expressing strains, all of the additional acetoin was converted into D-2,3-BD and *meso*-2,3-BD. This lack of selectivity for the stereochemistry of the substrate is not uncommon amongst acetoin reductases (27).

When exogenously added D-2,3-BD or *meso*-2,3-BD (20 mM) is present during the fermentation, the amount of converted acetoin is not affected resulting in final D-2,3-BD levels of 40 mM. This shows that at the tested levels, the amount of 2,3-BD is not inhibitory to the reaction or its production. Based on these results, we conclude that the acetoin formation, for the fermentation as a whole, is the limiting factor for the production of 2,3-BD. Even though, initially, acetoin accumulates in the medium (Figure 1).

2,3-BD production model

Based on the combined results of wild type, plasmid control and *Cb-acr* expressing strains, in normal and challenged cultures, we propose the model shown in figure 3 for acetoin and 2,3-BD production in our *C. acetobutylicum* strain. The introduced ACR enzyme can convert both D- and L-acetoin enantiomers into D-2,3-BD and *meso*-2,3-butanediol respectively. The wild type and the plasmid control strain produced small amounts of *meso*-2,3-BD and no detectable levels of D- or L-2,3-BD, as confirmed by GC-MS analysis. In the acetoin challenged cultures the *meso*-2,3-BD formation by the control strain (pMTL500E) doubled from 2 mM to 4 mM. Most likely, one or more of the dehydrogenases that are present in *C. acetobutylicum* WUR do, to some extent, accept acetoin as a substrate. This would suggest that it is D-acetoin that is the source of the endogenous *meso*-2,3-BD production as the native acetoin enantiomer ratio is 12:1 (D:L). Thus, in the acetoin challenge experiment the levels of D-acetoin are increased approximately 1.7 fold (from 14 mM to 24 mM), while the L-acetoin concentration increased more then ten-fold (from 1 mM to 11 mM). If L-acetoin would be the source of the endogenous 2,3-BD production, then a more substantial increase of *meso*-2,3-BD production would be expected.

Future perspective

If a pathway could be established in which 2,3-BD is dehydrated to 2-butanone and then further reduced to 2-butanol, then potentially a redox balanced fermentation of glucose to 2-butanol and carbon dioxide could be established in this organism. The less toxic nature of 2-butanol compared to 1-butanol (28), would make it an alternative approach to circumvent the limited butanol yield of the classic ABE-fermentation.

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1 TABLE 1. Plasmids and bacterial strains

Bacterial strain or plasmid	Relevant genotype	Remarks	Source
Bacterial strains			
C. acetobutylicum WUR	WT	Originally obtained as ATCC 824, but shown to deviate from type strain behavior	Laboratory stock
C. acetobutylicum ATCC 824	WT	Type strain	H. Bahl, Rostock, Germany
C. beijerinckii NCIMB 8052	WT		Laboratory stock
E. coli DH10B (pAN1)	Δ(mrr-hsdRMS-mcrBC)	Methylation strain	Laboratory stock
Plasmids			
pAN1	p15A ori; Cm^r Φ3tI	Expression plasmid for phage Φ3tI methylase gene (18).	Laboratory stock
pMTL500E	ColE1 ori; pAMβ1 ori; MLS^r; Ap^r	Clostridial/*E. coli* shuttle vector (23).	Laboratory stock

pWUR459	ColE1 ori; pAMβ1 ori; MLS^r; Ap^r; P$_{adc}$-*Cb-acr*	Expression plasmid of *Cb-acr* under control of the *C. acetobutylicum* acetoacetate decarboxylase promoter	This study
pWUR460	ColE1 ori; pAMβ1 ori; MLS^r; Ap^r; P$_{thl}$-*Cb-acr*	Expression plasmid of *Cb-acr* under control of the *C. acetobutylicum* thiolase promoter	This study

1

TABLE 2. Concentrations of substrate and products of 100 mL batch fermentations of *C. acetobutylicum* harboring pMTL500E, pWUR459 or pWUR460 on MG medium after 145 hours

	pMTL500E Control	pWUR459 P_{adc}-*Cb-acr*	pWUR460 P_{thl}-*Cb-acr*
Consumed glucose [mM]	318 ± 15	307 ± 29	320 ± 18
Acetoin [mM]	19 ± 2	2 ± 1	3 ± 0.3
meso-2,3-Butanediol [mM]	3 ± 0.1	2 ± 0.1	2 ± 0.3
D-2,3-Butanediol [mM]	0	20 ± 1	22 ± 3
Acetoin + 2,3-butanediol [mM]	22 ± 2	25 ± 2	26 ± 4
Yield of (Acetoin + 2,3-BD) per glucose [mM/mM]	7 ± 0.8 %	8 ± 0.9 %	8 ± 1 %
Other fermentation products			
Initial acetic acid[mM] [a]	37 ± 0.2	38 ± 0.4	36 ± 0.8
Final acetic acid [mM]	14 ± 0.7	20 ± 6	19 ± 2
Butyric acid [mM]	2 ± 1	5 ± 3	6 ± 3
Lactic acid [mM]	3 ± 0.7	6 ± 1	4 ± 0.4
Acetone [mM]	80 ± 9	78 ± 12	86 ± 6
Butanol [mM]	161 ± 6	157 ± 15	166 ± 6
Ethanol [mM]	40 ± 2	34 ± 5	56 ± 14

Data are given as the mean of triplicate fermentations ± standard deviation.

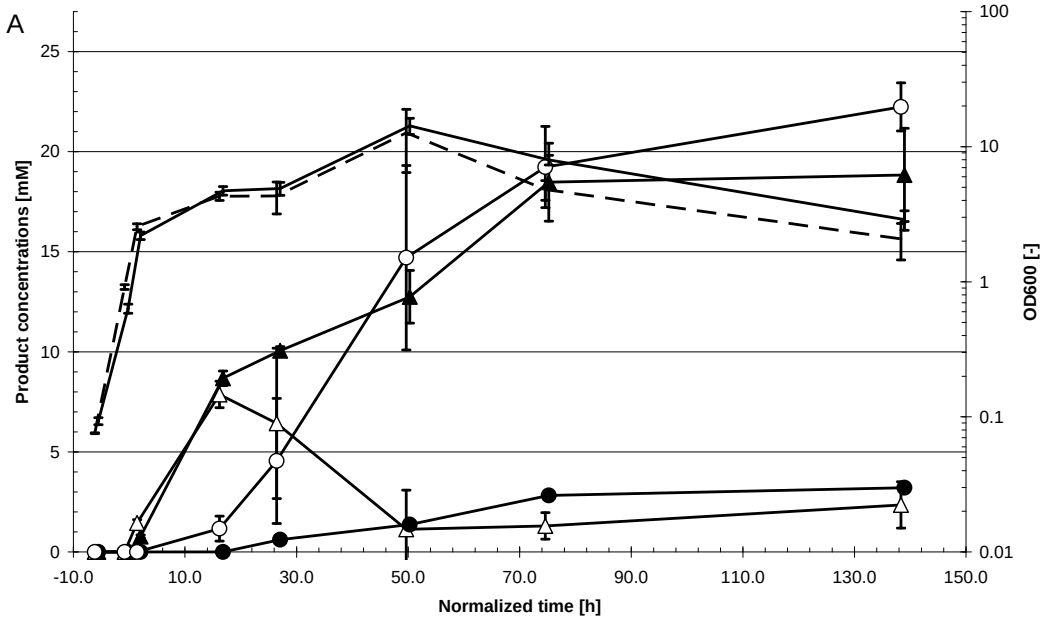
[a] acetate is present at the beginning (t = 0) as a medium component and is consumed during the fermentation.

TABLE 3. Nett change of extra-cellular acetoin and 2,3-butanediol concentrations in challenged batch cultures of *C. acetobutylicum* transformants harboring pMTL500E, pWUR459 or pWUR460, after 72 hours of fermentation, compared to their inoculation levels

Strain	Challenge [a]	Nett change in extra-cellular concentrations [mM] [b]			
		Acetoin	D-2,3-BD [f]	*meso*-2,3-BD	Acetoin + 2,3-BD
pMTL500E (control)	No challenge	15 ± 1	0	2 ± 0.4	17 ± 1
	Acetoin [c]	13 ± 2	0	4 ± 0.2	17 ± 2
	D-2,3-BD [d]	12 ± 2	-0.7 ± 1	1 ± 0.05	13 ± 2
	meso-2,3-BD [e]	15 ± 0.2	0.3 ± 0.6	2 ± 1	18 ± 1
pWUR459 (P_{adc}-*Cb-acr*)	No challenge	0.2 ± 0.4	15 ± 1	1 ± 0.2	16 ± 1
	Acetoin	-20 ± 0.4 [g]	24 ± 1	11 ± 0.5	15 ± 1
	D-2,3-BD	0.1 ± 0.2	12 ± 1	0.9 ± 0.1	13 ± 1
	meso-2,3-BD	0.2 ± 0.4	17 ± 2	-0.5 ± 0.2	17 ± 2
pWUR460 (P_{thl}-*Cb-acr*)	No challenge	0 ± 0.3	17 ± 1	0.8 ± 0.1	18 ± 1
	Acetoin	-21 ± 0.7 [g]	29 ± 4	11 ± 0.2	20 ± 4
	D-2,3-BD	0.2 ± 0.3	20 ± 3	0.9 ± 0.1	21 ± 3
	meso-2,3-BD	0.4 ± 0.4	21 ± 0.5	0.1 ± 1	22 ± 1

[a] Racemic acetoin, D-2,3-BD, or *meso*-2,3-BD were added to the medium before inoculation at concentrations of 20 mM each. [b] Data are given as the mean of triplicate fermentations ± standard deviation and calculated by substracting the concentration

after 72 hours from the concentration at time of inoculation. For example, for the D-2,3-BD challenge of the pWUR460 the initial D-2,3-BD concentration of 20 mM was subtracted from the final concentration of 40 mM resulting in a nett production of 20 mM D-2,3-BD. [c] media supplementation with racemic acetoin; [d] media supplementation with D-(2*R*,3*R*)-2,3-BD; [e] media supplementation with *meso*-2,3-BD, which also contained approximately 10% of racemic D/L-2,3-BD; [f] analysis was done by non-chiral HPLC so no separation of enantiomers was possible, however based on the previous results the D-enantiomer is expected to have been formed; [g] negative values indicate consumption of acetoin relative to inoculation conditions.



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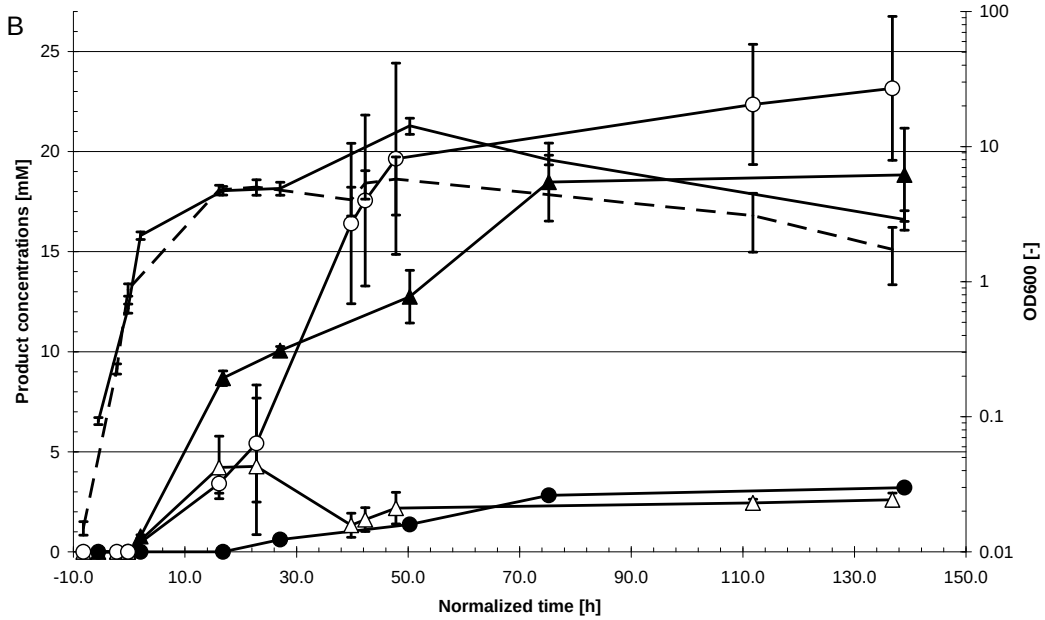

2

3 FIG. 1. Production of acetoin (triangles) and 2,3-BD (circles) during batch fermentations
4 of glucose by transformant strains of *C. acetobutylicum*. Strains codes: EV (panel A and

B, closed symbols), *C. acetobutylicum* (pMTL500E), empty vector control strain; ADC (panel A, open symbols), *C. acetobutylicum* (pWUR459); THL (panel B, open symbols), *C. acetobutylicum* (pWUR460). Optical densities of the cultures are shown by a solid line (EV) and a dashed line (ADC and THL). Normalized time was plotted on the horizontal axis by setting and optical density of 1 at time is zero. The control strain did not produce any D-2,3-BD, only *meso*-2,3-BD. Data represent the mean of triplicate fermentations averaged for each time interval. Error bars indicate standard deviations.

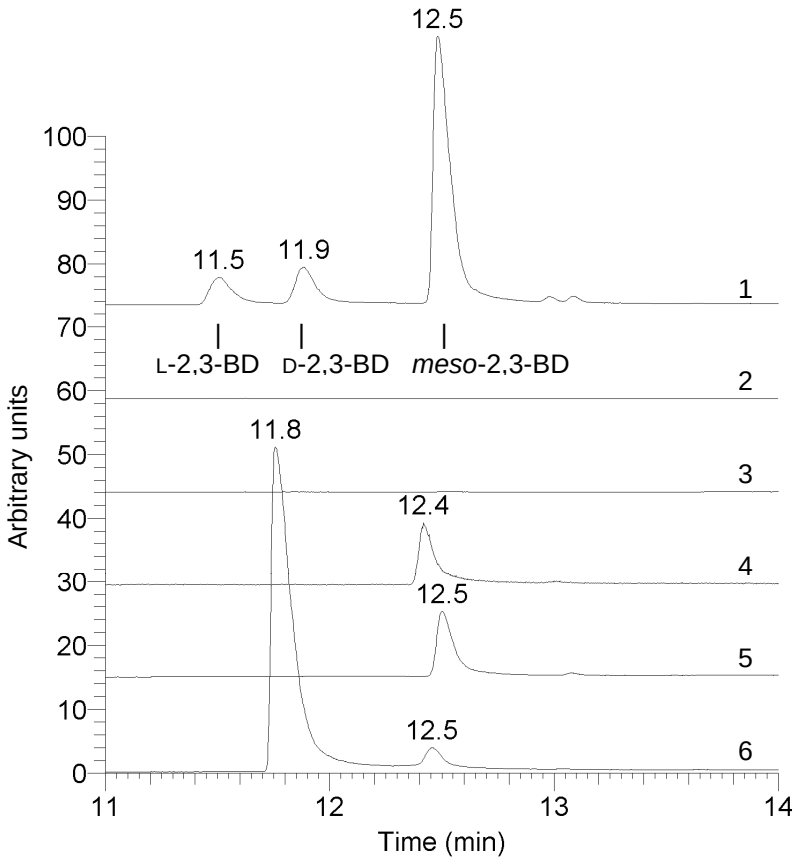

FIG. 2. GC-MS chromatograms (single ion mode, m/z = 45) of extracts of, a standard, GM medium and samples taken at the end of the fermentation of wild-type and transformant cultures. Chromatogram 1, mixture of all three 2,3-BD stereoisomers; Chromatogram 2, GM medium blank; Chromatogram 3, fermentation of *C. acetobutylicum* ATCC 824; Chromatogram 4, fermentation of *C. acetobutylicum* WUR; Chromatogram 5, fermentation of *C. acetobutylicum* WUR harboring the empty vector (EV); Chromatogram 6, fermentation of *C. acetobutylicum* WUR harboring the pWUR460 construct containing the *acr* gene. The retention time of the D-2,3-BD peak in chromatogram 6 is somewhat different due to the high concentration. Spiking experiments confirmed that it is indeed the D-stereoisomer.

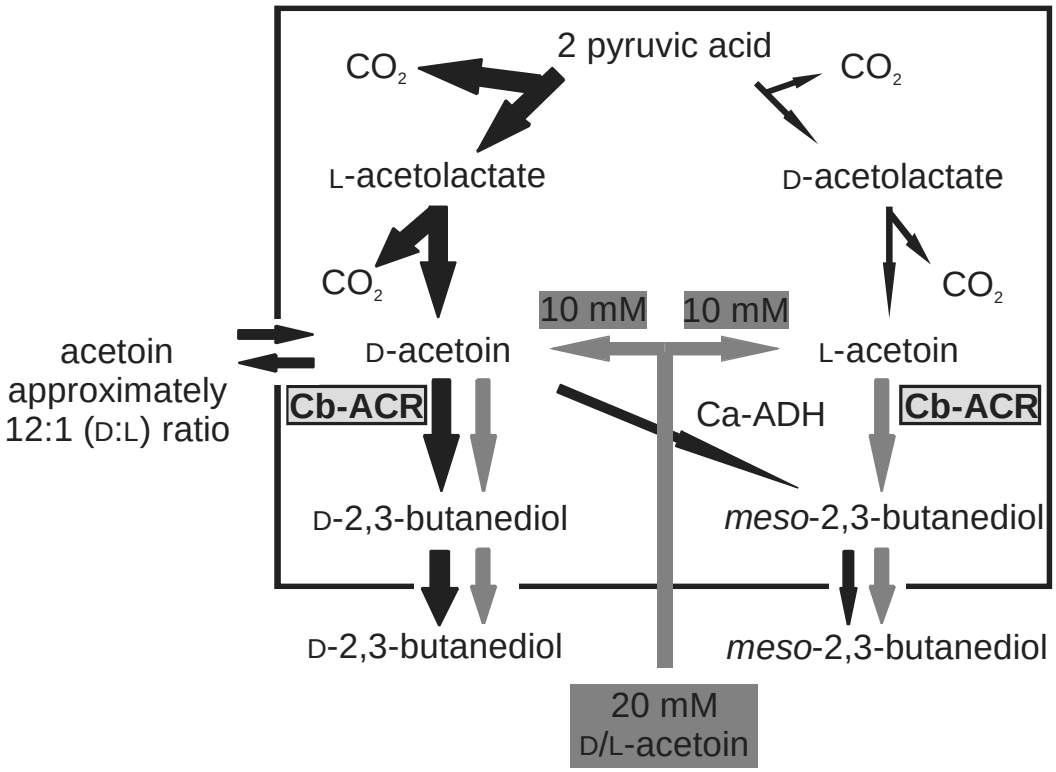

FIG. 3. Proposed 2,3-BD biosynthesis pathway in the ACR expressing *C. acetobutylicum* transformants of this study. The boxed area indicates the intracellular space. The size of the arrows indicates the relative flux towards D- and L-acetoin from pyruvic acid. The gray arrows illustrate the impact of medium supplementation with 20 mM racemic D/L-acetoin. The *C. beijerinckii* acetoin reductase enzyme is indicated in bold on a gray background. The proposed conversion of D-acetoin to *meso*-2,3-BD by an endogenous alcohol dehydrogenase (Ca-ADH) is indicated. In the control strain fermentation the ratio of the two acetoin enantiomers is 12:1 (D:L).