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1 **Engineering Acetoin and *meso*-2,3-Butanediol Biosynthesis in**
2 ***E. coli***

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Abstract

The functional reconstruction of acetoin and *meso*-2,3-butanediol biosynthetic pathways in *E. coli* have been systematically explored. Pathway construction involved the *in vivo* screening of prospective pathway isozymes of yeast and bacterial origin. After substantial engineering of the host background to increase pyruvate availability, *E. coli* YYC202(DE3) *ldhA*⁻ *ilvC*⁻ expressing *ilvBN* from *E. coli* and *aldB* from *L. lactis* (encoding acetolactate synthase and acetolactate decarboxylase activities, respectively) was able to produce up to 870 mg/L acetoin, with no co-production of diacetyl observed. These strains were also found to produce small quantities of *meso*-2,3-butanediol, suggesting the existence of endogenous 2,3-butanediol dehydrogenase activity that has not before been characterized. Finally, the co-expression of *bdhI* from *S. cerevisiae*, encoding 2,3-butanediol dehydrogenase, in this strain resulted in the production of up to 1120 mg/L *meso*-2,3-butanediol, with glucose a yield of 0.29 g/g. While disruption of the native lactate biosynthesis pathway increased pyruvate precursor availability to this strain, increased availability of NADH for acetoin reduction to *meso*-2,3-butanediol was found to be the most important consequence of *ldhA* deletion.

Introduction

With a carbon chain length of four and two reactive sites, 2,3-butanediol (2,3-BD) is a versatile building block molecule for the synthesis of both fine and commodity chemicals. For example, antiulcer drugs have been produced by diester derivitazation of 2,3-BD [1]. Additionally, fatty acid diester derivatives of 2,3-BD are particularly useful in the production of cosmetics [2]. Via chemocatalytic dehydrogenation, 2,3-BD may be converted to methyl ethyl ketone (MEK), a liquid fuel additive and useful industrial solvent for laquers and resins [3]. Furthermore, technologies for the catalytic conversion of 2,3-BD to 1,3-butadiene have long been established [4, 5]. With an annual global demand of 9 million metric tons in 2005, 1,3-butadiene is a large commodity chemical that is particularly useful as a monomer for the production of a diversity of polymer and co-polymer materials [6]. 2,3-BD has also been used as a coolant for fuel cells [7] and in the manufacturing of plasticizers, inks, fumigants, and explosives [8]. Meanwhile, diacetyl and acetoin (important pathway intermediates, see Figure 1) each have important applications in the food, beverage, and fragrance industries as naturally-derived flavor molecules (butter and cream, respectively) [9].

The natural 2,3-BD biosynthesis pathway is illustrated in Figure 1, beginning with central pathway metabolite pyruvate as the precursor molecule. The first step in the 2,3-BD biosynthetic pathway involves the condensation of two molecules of pyruvate to yield one molecule of acetolactate, and is catalyzed by acetolactate synthase (ALS) [E.C. 2.2.1.6] (A, Figure 1). Such acetohydroxyacid synthases (AHAS) are ubiquitous among living organisms, playing an essential role in the synthesis and regulation of the branched-chain, proteinogenic amino acids L-valine, L-leucine, and L-isoleucine. The

stability of acetolactate is known to be affected by the presence of oxygen, which causes its spontaneous decarboxylation to produce diacetyl. Under anaerobic conditions, however, decarboxylation of acetolactate is catalyzed by acetolactate decarboxylase [E.C. 4.1.1.5] to produce acetoin (B, Figure 1). In the final step (C, Figure 1), acetoin is reduced by a stereospecific, NADH-dependent 2,3-BD dehydrogenase [E.C. 1.1.1.4] to yield 2,3-BD. Since many 2,3-BD dehydrogenase homologs are also known to be capable of catalyzing the reduction of diacetyl to acetoin, in many natural producers 2,3-BD can be synthesized under both aerobic and anaerobic conditions. However the greatest 2,3-BD yields are typically achieved under either anaerobic or microaerophilic conditions, while aerobic conditions predominantly result in the terminal accumulation of a portion of diacetyl as a result of insufficient reducing power [9, 10].

2,3-BD is produced as a natural fermentation product by several bacteria including *Klebsiella pneumoniae*, *Bacillus polymyxa*, *Lactococcus lactis*, *Enterobacter aerogenes*, and *Corynebacterium glutamicum*, as well as by several species of yeast. Product stereospecificity as well as purity, however, is found to vary from organism to organism. For example, *K. pneumoniae* is known to produce a mixture of meso- and (S,S)- stereoisomers [11] whereas *B. polymyxa* produces (R,R)-2,3-BD at optical purities as high as 98% [12, 13]. However, no stereoisomer of 2,3-BD is known to be a natural fermentation product of *Escherichia coli*. In previous studies, *E. coli* has been engineered for the production of (S)-acetoin [14], meso-2,3-BD [15], and (S,S)-2,3-BD [16] by utilizing diacetyl as an exogenously supplied precursor. Production of meso-2,3-BD from glucose was also reported in recombinant *E. coli* harboring a fragment of the *K. pneumoniae* IAM 1063 genome encoding the meso-2,3-BD biosynthetic pathway [15].

Most recently, recombinant *E. coli* strains were engineered to produce (*R,R*)- and *meso*-2,3-BD as enantiopure products from glucose by complementing the BD biosynthetic pathway of *K. pneumoniae* with secondary alcohol dehydrogenases of differing stereoselectivity [17]. By recruiting various bacterial and yeast enzymes as alternative pathway constituents, here we construct and characterize *E. coli* strains for the production of *meso*-2,3-BD biosynthesis, with the objective of complementing and building upon those earlier efforts by other groups. We did so by the following step-wise progression: upon constitution of a functional acetoin pathway, this pathway was then extended by the introduction of an additional enzymatic step to produce *meso*-2,3-BD. In contrast to previous studies, we first chose to explore the use of the native pathway elements of *L. lactis*. Next, we explored the utility of a variety of homologous enzymes from other genetic sources, while also modifying the genetic background of our *E. coli* host in order to enhance the availability of precursors and co-factors.

Materials and methods

Microorganisms

The bacterial strains used in this study are listed in Table 1. Routine cloning and plasmid maintenance was performed using either *E. coli* Electromax DH10B (Invitrogen, Carlsbad, CA) or ElectroTen-Blue (Stratagene, La Jolla, CA). *E. coli* strains MG1655(DE3) and YYC202(DE3) were used as hosts for the expression of genes under the T7lac promoter. *E. coli* MG1655 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). *E. coli* YYC202, a strain previously engineered for the enhanced production of lactate, was generously provided by Prof. John Cronan

(Department of Microbiology, University of Illinois at Urbana-Champaign).
MG1655(DE3) and YYC202(DE3) were then subsequently constructed using the λ DE3
Lysogenization Kit (Novagen, Darmstadt, Germany) for site-specific integration of λ DE3
prophage into each host. *S. cerevisiae* was obtained from the lab of Prof. K. Dane
Wittrup (Department of Chemical Engineering, Massachusetts Institute of Technology).
C. acetobutylicum ATCC824 was purchased from the ATCC (Manassas, VA).

Plasmid Construction

All pathway genes were amplified by polymerase chain reaction (PCR) using genomic
DNA (gDNA) templates. gDNA of *L. lactis* subsp. *lactis* was purchased from the ATCC
(Manassas, VA). *S. typhimurium* LT2 gDNA was provided by Prof. Diane Newman
(Department of Biology, Massachusetts Institute of Technology). All other gDNA
samples were prepared using the Wizard Genomic DNA Purification Kit (Promega,
Madison, WI).

Genes encoding acetolactate synthase were individually cloned into the first
multi-cloning site (MCS-1) of pETDuet-1 (Novagen, Darmstadt, Germany). *ilvBN* from
L. lactis was inserted between *NcoI-Sall* sites of pETDuet-1 to produce pET-ilvBNLI,
whereas *ilvBN* from *E. coli*, *ilvIH* from *E. coli*, and *ilvGM* from *S. typhimurium* were
each PCR amplified with *PciI-EcoRI* sites and inserted between the *NcoI-EcoRI* sites of
pETDuet-1, yielding pET-ilvBNEc, pET-ilvIHEc, and pET-ilvGMSt, respectively (Table
1). The genes encoding acetolactate decarboxylase, including *aldB* and *aldC* from *L.*
lactis and *adc* from *C. acetobutylicum*, were inserted into the second MCS (MCS-2) of
each of the above constructed plasmids. Each gene was PCR amplified with *BglII-AvrII*

sites and inserted between the same. The resultant plasmids are listed in Table 1. This same set of genes was also similarly cloned into the same sites of pETDuet-1 and the resulting plasmids are listed in Table 1. 2,3-butanediol dehydrogenase encoding genes (specifically, *butB* from *L. lactis* and *bdh1* from *S. cerevisiae*) were inserted between the *NcoI-EcoRI* sites of pCDFDuet-1(Novagen, Darmstadt, Germany) after PCR amplification to introduce *PciI-EcoRI* sites, resulting in pCDF-butBL1 and pCDF-bdh1Sc.

In all cases, Phusion High Fidelity DNA Polymerase (Finnzymes, Espoo, Finland) was used for DNA amplification. Custom oligonucleotides (primers) were purchased for all PCR amplifications (Sigma-Genosys, St. Louis, MO). Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs (Ipswich, MA). All positive constructs were identified via restriction digest and nucleotide sequencing. Plasmids constructed in the present work are listed in Table 1.

Strain Development

Serial deletions of *ldhA* and/or *ilvC* in *E. coli* YYC202(DE3) strains were performed according to a previously reported method [18]. All recombinant strains developed in this study are listed in Table 1.

Culture Conditions

For all experiments, seed cultures were prepared by culturing in 5 mL LB with 100 mg/L ampicillin and 50 mg/L streptomycin, as appropriate, in 15-mL culture tubes overnight at 30°C while shaking at 225 rpm. These preinocula were used to seed 25 mL LB medium

supplemented with 10 g/L glucose in 50 mL screw-capped tubes, at an initial optical density at 600 nm (OD_{600}) of ~0.05. Cultures were grown according to a two-stage, aerobic-anaerobic procedure. Growth was first performed at 37°C under aerobic conditions in sealed tubes to promote biomass production. However, with a limited headspace volume, these closed cultures naturally became depleted of oxygen after 3-5 h (as indicated by the addition of 5 mg/L resazurin to the culture medium), at which point OD_{600} had reached ~0.8. 1 mM IPTG was then added to the cultures to induce recombinant protein expression. Following induction, cells were then cultivated at 30°C. In all cases, media was supplemented with 100 mg/L ampicillin and 50 mg/L streptomycin, as appropriate. Cultures were sampled at approximately 24 h intervals for up to 120 h. Culture samples were centrifuged to pellet cells while the aqueous supernatant was collected for HPLC analysis.

Metabolite Analysis

Acetoin, 2,3-BD, diacetyl, and other fermentation products of interest were analyzed via HPLC using an Agilent 1100 series instrument equipped with a refractive index detector (RID). Analyte separation was achieved using an Aminex® HPX-87H anion exchange column (Bio-Rad Laboratories, Hercules, CA) with 5 mM H_2SO_4 as the mobile phase. The mobile phase was pumped at constant gradient between 0.4 and 0.6 mL/min for the first 12 minutes, then held constant at 0.6 mL/min for an additional 12 minutes. The column and detector temperatures were each set to 35°C throughout. This method provided adequate separation of *meso*-2,3-BD from optically active forms, however the

individual (*R,R*) and (*S,S*)-stereoisomers could not be mutually resolved. External standards provided calibration for titer determinations.

Cofactor Determination

The NAD⁺/NADH ratio of each host strain after 72 h culture in the above media was determined by use of the EnzyChrom™ NAD/NADH Assay Kit (BioAssay Systems, Hayward, CA).

Results and discussion

The set of plasmids expressing the individual enzymes of interest to the acetoin biosynthesis pathway (see all pETDuet-1-based plasmids of Table 1) were first individually transformed into *E. coli* MG1655(DE3). However, after 72 h of anaerobic culture in LB with 1% (wt./vol.) glucose and IPTG induction, no acetoin accumulation could be detected from any of these strains. As can be seen in Figure 3, this is likely due to the inability of this host to produce sufficient quantities of pyruvate, the immediate pathway precursor. To promote greater pyruvate biosynthesis we explored modifications to the genetic background of *E. coli*. *E. coli* YYC202 (see Table 1) was previously engineered with mutations to block the respective pathways involved in the conversion of pyruvate to both acetate and acetyl-CoA [19]. The relevant mutations of this strain are summarized in both Table 1 and Figure 2. A previously constructed *ldhA*⁻ mutant of YYC202 has demonstrated the highest pyruvate production potential of any other microorganism [20], being able to achieve pyruvate yields of up to 0.87 g/g on glucose, with titers as high as 110 g/L [21]. This and other mutations were systematically

explored in the *E. coli* YYC202(DE3) background to facilitate acetoin and ultimately *meso*-2,3-BD biosynthesis. For example, strains deficient of acetohydroxy acid isomeroreductase (encoded by *ilvC* and involved in the leucine and valine biosynthesis pathways; Figure 2) were constructed to elevate intracellular acetolactate levels.

Under the conditions of the present study, the engineered strain YYC202(DE3) (as well as its derivatives) was able to produce between 1.5 and 1.9 g/L pyruvate in 72 h (Figure 3), with more being produced in *ldhA*⁻ strains. Although pyruvate is the immediate precursor of the 2,3-BD biosynthesis pathway, acetolactate (the next intermediate molecule in the 2,3-BD biosynthetic pathway; Figures 1 and 2) is also synthesized in *E. coli* by native acetolactate synthases. If the basal activity of these endogenous enzymes was found to be sufficiently high under the conditions examined, then Step A of the proposed pathway (Figure 1) would be rendered superfluous. However, it was found that without ALS over-expression, no acetoin production, and consequently no 2,3-BD production, could be detected in any host (data not shown). Thus, although *E. coli* possesses multiple, functional homologs of ALS (including those encoded by *ilvBN* and *ilvIH*), their basal levels of activity were found to provide insufficient rates of acetolactate synthesis under the studied conditions.

To provide adequate flux towards acetolactate biosynthesis (and ultimately the acetoin and 2,3-BD pathways) the over-expression of additional copies of ALS was required. For such a purpose, the utility of *ilvBN* from *L. lactis* was first examined. Its co-expression with *aldB* together with or without *butB* (both also from *L. lactis*) were intended to constitute the baseline acetoin and 2,3-BD pathways, respectively, for this study. However, it was found that neither acetoin (by strains harboring pET-*ilvBN*LI-

aldBLI) nor *meso*-2,3-BD (by strains harboring pET-ilvBNLI-aldBLI and pCDF-butBLI) could be produced by these initially reconstructed pathways, regardless of the genetic background of the expression host. Speculating that perhaps *ilvBN* from *L. lactis* was not being functionally expressed in *E. coli*, this gene was then replaced with *ilvIH* from *E. coli* (in strains harboring pET-ilvIHEc-aldBLI). In this case, however, acetoin biosynthesis was again not possible (data not shown). As this is an *E. coli* source protein, it seems less likely that poor functional expression was to blame for this less favorable result. However, previous characterizations of *ilvIH* have reported its substrate specificity as strongly preferential for the condensation of pyruvate with 2-ketobutyrate to produce 2-aceto-2-hydroxybutyrate [22]. As can be seen in Figure 3, when finally substituted with either *ilvBN* from *E. coli* (in strains harboring pET-ilvBNec-aldBLI) or *ilvGM* from *S. typhmuri*um (in strains harboring pET-ilvGMSt-aldBLI), elevated ALS activity then resulted in significant acetoin production by all YYC202(DE3)-derived hosts. No diacetyl co-production was detected in any of these cultures. Again, however, no acetoin biosynthesis was detected with MG1655(DE3) as the expression host, despite having identified a set of functional pathway enzymes (data not shown). Since similar acetoin titers could be achieved by strains expressing either *ilvBN* or *ilvGM* (Figure 3), we selected *ilvBN* from *E. coli* as the ALS-encoding gene of preference (by virtue of homologous nature) for the remainder of our study. The utility of *aldC* as an alternative to *aldB* (both from *L. lactis*) within the acetoin pathway was then also examined, however, only lower to equivalent titers were obtained (Figure 3). Therefore, *aldB* was selected as the preferred acetolactate decarboxylase-encoding gene for the remainder of this work.

243 Despite finding that *ldhA* and *ilvC* deletions each rendered a minimal effect on the
244 acetoin titer (Figure 3), the greatest production was still achieved using YYC202(DE3)
245 *ldhA⁻ ilvC⁻* as the host. Accordingly, this strain was selected for use in subsequent 2,3-
246 BD biosynthesis studies. As can be seen in Figure 3, the highest titer observed in this
247 study was 868 ± 5 mg/L, corresponding to a maximum acetoin-glucose yield of 0.22 g/g.
248 Interestingly, it was further observed that small quantities of newly synthesized acetoin
249 were reduced to *meso*-2,3-BD (and none of either optically active stereoisomer) in all
250 *ldhA⁻* strains, albeit in the absence of a heterologously-expressed 2,3-BD dehydrogenase.
251 The highest of such titers, 117 ± 58 mg/L, was found to be produced by YYC202(DE3)
252 *ldhA⁻ ilvC⁻* pET-ilvBNEc-aldCLI, which also produced acetoin to a final titer of 700 ± 40
253 mg/L. As is shown in Figure 4, the measured values of NAD⁺/NADH were 22 to 32%
254 lower in *ldhA⁻* strains of YYC202(DE3) than in their *ldhA⁺* counterparts, indicting an
255 elevated presence of reducing equivalents that may have supported this reduction. A
256 relative reduction of similar magnitude (~33%) has also been previously reported in an
257 *ldhA⁻ pflB⁻* strain of *E. coli* (NZN111) [23]. This observation, however, suggests the
258 possible existence of an endogenous *E. coli* enzyme displaying 2,3-BD dehydrogenase
259 (or, alternatively, acetoin reductase) activity. In such a case, this transformation was
260 possibly co-factor limited in *ldhA⁺* stains, and thus remained unobserved. It should be
261 noted, however, that such a protein has yet to be characterized in *E. coli*. Whereas the
262 ratio of NAD⁺/NADH was measured at a discrete point, it should also be noted that this
263 ratio is known to vary as a function of growth rate [24], and thus would not be expected
264 to remain constant throughout each experiment. Typically reported values of
265 NAD⁺/NADH for anaerobic *E. coli* cultures have been reported to range between 1-2

[25] and 2-6 [24], and can be influenced by the nature of the culture media and selected carbon source [25]. In contrast, the NAD⁺/NADH ratio in exponentially growing aerobic cultures of *E. coli* have been reported to be on the order of 31 [26]. These previously reported values are consistent with the present findings with respect to the magnitude of the NAD⁺/NADH ratio in anaerobically grown *E. coli* YYC202(DE3) and its derivatives. Our measured value of the NAD⁺/NADH ratio in *E. coli* MG1655(DE3) is well above its anticipated level for anaerobic cultures, helps to explain why no desired product formation was observed.

The presence of endogenous 2,3-BD dehydrogenase activity in *E. coli* was then subsequently investigated in a set of complementary experiments in which each relevant host strain was grown anaerobically in LB media with 1% (wt./vol.) glucose and supplemented with 0.2% (wt./vol.) acetoin. In these experiments it was found that all *ldhA*⁻ strains of YYC202(DE3) (specifically YYC202(DE3) *ldhA*⁻ and YYC202(DE3) *ldhA*⁻ *ilvC*⁻) converted approximately 25% of that acetoin to *meso*-2,3-BD. Meanwhile, *ldhA*⁺ strains (specifically MG1655(DE3), YYC202(DE3) and YYC202(DE3) *ilvC*⁻) neither produced *meso*-2,3-BD nor depleted acetoin. These findings further supported our hypothesis that the deletion of *ldhA* provides additional NADH reserves with which acetoin may be reduced to *meso*-2,3-BD via a native enzyme displaying 2,3-BD dehydrogenase activity.

Based upon sequence similarity, it was previously predicted that *idnO* and *yohF* could each encode for diacetyl reductase activity in *E. coli* [27, 28]. It should be noted that many natural 2,3-BD dehydrogenases are also known to dually exhibit diacetyl reductase activity, catalyzing the reversible reduction of diacetyl to acetoin (consuming

289 NADH, or the reverse by reducing NAD⁺). To test whether the converse was true
290 regarding these putative proteins, we constructed both *idnO*⁻ and *yohF*⁻ derivatives of
291 YYC202(DE3) *ldhA*⁻ *ilvC*⁻ to investigate their possible role in the conversion of
292 exogenously supplied acetoin to *meso*-2,3-BD (as above). However, both of these strains
293 could still produce *meso*-2,3-BD from acetoin with equivalent activity and to a similar
294 extent. As an initial step towards identifying a native 2,3-BD dehydrogenase in *E. coli*
295 we then compared the known amino acids sequences of both BUTB from *L. lactis* and
296 BDH1 from *S. cerevisiae* to *E. coli* using BLASTP from NCBI
297 (<http://www.ncbi.nlm.nih.gov/blast/>). For each protein, two major domains were found
298 to be present: an alcohol dehydrogenase catalytic domain which included a zinc-binding
299 site, and a Rossmann-fold NAD⁺ binding domain. This finding came as little surprise as
300 such domains are common among many alcohol dehydrogenases. By phylogenetic
301 analysis, the sequences of both BUTB and BDH1 were also found to share significant
302 homologies with alcohol and threonine dehydrogenases (specifically those containing
303 both zinc and NAD⁺ binding domains) of firmicutes, proteobacteria, actinobacteria, and
304 fungi, as well as significant homology with glycerol and sorbitol dehydrogenases found
305 in yeasts and many eukaryotes (Figure 5). BDH1 and BUTB were both also found to
306 possess the highest similarity with the protein products of *ydjJ* and *ydjL* (about 25% each)
307 of *E. coli*. These genes are each predicted to encode for oxidoreductases that also contain
308 both zinc and NAD⁺ binding domains. Repeated attempts to systematically delete these
309 genes from YYC202(DE3) *ldhA*⁻ *ilvC*⁻ by well-characterized techniques [18], however,
310 remained unsuccessful (despite their success for all other deletions performed in this
311 study). As such, we presently remain unable to comment on the identity and activity of

these specific gene products. It is worth noting that 2,3-BD dehydrogenase activity has previously been demonstrated by glycerol dehydrogenase of the yeast *Hansenula polymorpha* (*Pichia angusta*) [29, 30]. Whereas *E. coli* also possesses a glycerol dehydrogenase (encoded by *gldA*), its substrate specificity with respect to the reduction of acetoin to *meso*-2,3-BD has not yet been examined. Additional studies will be required to improve our understanding of these uncharacterized gene products, as well as their role with respect to *meso*-2,3-BD biosynthesis in engineered *E. coli*.

To produce *meso*-2,3-BD at more appreciable levels the co-expression of known isozymes of 2,3-BD dehydrogenase with the acetoin biosynthesis pathway was then subsequently examined. For this purpose, two previously characterized enzymes were employed, namely those proteins encoded by *butB* of *L. lactis* and *bdh1* of *S. cerevisiae*, which were co-expressed from pCDF-*butB*L1 or pCDF-*bdh1*Sc (Table 1), respectively. In each case, *meso*-2,3-BD was solely produced with no co-production of diacetyl. Figure 6 compares the maximum achievable titers obtained after 5 days of anaerobic culture through co-expression of each of these two isozymes together with pET-*ilvBNec*-*aldB*Ec using YYC202(DE3) *ldhA*-*ilvC*- as host. Compared with the control strain, in which no heterologous 2,3-BD dehydrogenase was expressed, co-expression of *butB* was found to increase the *meso*-2,3-BD titer by nearly 10-fold, up to 400 ± 80 mg/L with a 2,3-BD-glucose yield as high as 0.19 g/g. Among two partially purified 2,3-BD dehydrogenase homologs of *L. lactis*, one was originally identified as being specific for the production of the *meso*- stereoisomer, whereas the other is responsible for production of the (*R,R*)-2,3-BD from (*R*)-acetoin [31]. These two enzymes are encoded by *butB* and *butA* which are co-localized as a single transcriptional unit. The presence of these two,

unique 2,3-BD dehydrogenases results in mixed isomer production of 2,3-BD by *L. lactis*. Although it has not yet been specified as to which of these two stereospecific enzymes is the product of which gene, the confirmed production of *meso*-2,3-BD by pathways expressing *butB* from *L. lactis* in this study suggests that it is *butB* which encodes the *meso*-2,3-BD dehydrogenase. Titters of *meso*-2,3-BD were then further improved by alternatively co-expressing *bdhI*, leading to a maximum *meso*-2,3-BD titer of 1120 ± 180 mg/L, corresponding to a 2,3-BD-glucose yield of up to 0.29 g/g (58% of the theoretical maximum). The co-production of up to 1100 ± 120 mg/L succinate by these strains (Figure 6) represents a significant carbon flux which would preferably be directed towards the desired end product, *meso*-2,3-BD. Succinate production in *E. coli* could be reduced through disruption of the multi-step enzymatic pathway which converts oxaloacetate to succinate. In several previous studies this has been most effectively performed by focusing on the deletion of all or part of the four required subunits (collectively encoded by *frdABCD*) that constitute fumarate reductase [32, 33]. This specific strategy has also been previously demonstrated as effective for increasing pyruvate production (the central metabolic precursor of our constructed pathway) [34].

Although the deletion of *ldhA* was previously found to be of minor importance for increasing the availability of pyruvate as well as the metabolite flux towards the constructed pathway (as discussed above), it is the means by which this deletion made available additional reducing equivalents that was perhaps most impactful on *meso*-2,3-BD production. This hypothesis was supported by an additional subset of experiments which found that, although up to 640 ± 70 mg/L of acetoin could be produced by *ldhA* + strains of YYC202(DE3) harboring pET-ilvBNEc-aldBLI together with pCDF-butBLI or

358 pCDF-bdh1Sc, no *meso*-2,3-BD biosynthesis could be detected by either strain (data not
359 shown). Even with our best *meso*-2,3-BD-producing strain (YYC202(DE3) *ldhA*- *ilvC*-
360 pET-*ilvBNEc*-aldBL1 pCDF-bdh1Sc), up to 330 ± 140 mg/L of residual acetoin remained
361 (Figure 6), suggesting that *meso*-2,3-BD production was not ultimately limited by acetoin
362 availability. Furthermore, we found that pyruvate availability did not limit final product
363 yield since when strains were re-cultured in the presence of exogenously supplied
364 pyruvate (up to 1 g/L), no difference in acetoin or *meso*-2,3-BD biosynthesis was realized
365 (data not shown) by any strains. In such experiments, had we observed elevated acetoin
366 accumulation without further reduction to *meso*-2,3-BD this might suggest that the mode
367 of limitation was associated with insufficient rates of cofactor regeneration. However,
368 since even acetoin biosynthesis became limited in this case, the body of evidence
369 suggests that the activity of our host and its engineered pathway were more broadly
370 disrupted, perhaps by an overall redox imbalance. The combined deletion of pyruvate-
371 formate lyase (*pflB*) and lactate dehydrogenase (*ldhA*) has been shown to additionally
372 inhibit anaerobic growth in glucose minimal medium as a result of redox imbalance [35].
373 This balance can be restored, however, by expression of an appropriate NADH-oxidizing
374 enzyme (thereby recycling NAD⁺), such as pyruvate dehydrogenase (to produce acetyl-
375 CoA and ultimately ethanol) [33], or alcohol dehydrogenase (together with pyruvate
376 decarboxylase to produce ethanol) [36, 37]. In our system, this imbalance could be
377 restored, at least to a certain extent, by the expression of a heterologous 2,3-BD
378 dehydrogenase which reduces acetoin. This is reflected by the fact that greater product
379 yields (up to 0.29 g/g versus 0.22 g/g) and glucose consumption (up to 3.8 g/L versus 2.1
380 g/L) were obtained when the 2,3-BD dehydrogenase was co-expressed (versus the acetoin

pathway alone). However, the realized rate of turnover may have remained inadequate for longer term maintenance of the redox balance, particularly if the activity of one or more of the pathway enzymes was low. Additionally, as a result of deletions to *pflB* and pyruvate dehydrogenase (*aceEF*), *E. coli* YYC202 and its derivatives lack the complete ability to convert pyruvate to acetyl-CoA and acetate. These mutations have been shown to preclude the anaerobic growth of this strain in glucose minimal medium without acetate supplementation [21]. In this study, we have focused solely on the use of complex media (LB) understanding the limitations that our strains will face in glucose minimal media without acetate supplementation. Furthermore, as described above, our culture strategy first involved up to 5 h of growth in the presence of oxygen, allowing for more substantial quantities of biomass to accumulate before cultures naturally became anaerobic. Noting, however, that the overall glucose consumption never exceeded 38% of the 10 g/L originally supplied in the media (data not shown), it appears apparent that our media design and culture protocol were inadequate for maintaining sufficient metabolic activity. These issues will be addressed through more detailed, future investigations focused on improved media design and the use of microaerobic culture conditions.

Whereas there are no other reports of *E. coli* having been engineered to produce acetoin directly from glucose, there currently exists two prior reports regarding the engineering of *E. coli* to produce 2,3-BD stereoisomers. In the first, *meso*-2,3-BD was produced by a strain engineered to express the pathway enzymes of *K. pneumoniae* IAM 1063, including those encoding ALS, ALDC, and *meso*-2,3-BD dehydrogenase [15]. By exclusively exploring the expression of these *K. pneumoniae* –source genes from a

genomic DNA fragment, the authors reported maximum product titers and glucose yields of 17.7 g/L and 0.27 g/g, respectively, using a wild-type host under aerobic conditions. Interestingly, however, in our study no *meso*-2,3-BD (or even acetoin) production was detected in a wild-type host (MG1655(DE3)) under any conditions. Meanwhile, expression of our functional *meso*-2,3-BD biosynthetic pathway in engineered hosts (YYC202(DE3), and its derivatives) under aerobic conditions lead only to trace production of acetoin and diacetyl (data not shown). Note that acetolactate (the precursor to acetoin; Figure 1) is known to be an oxygen sensitive molecule which spontaneously decarboxylates to diacetyl under even microaerophilic conditions [38].

More recently (and during the preparation of this manuscript), functional pathways for the synthesis of both (*R,R*)- and *meso*-2,3-BD were also constructed in *E. coli* through the differential expression of 2,3-BD dehydrogenase (or alternative secondary alcohol dehydrogenases) isozymes with opposing stereospecificities. In that study, acetoin production was catalyzed through the co-expression of *alsS* and *alsD* from *Bacillus subtilis* (encoding ALS and ALDC, respectively). Acetoin was then converted to (*R,R*)-2,3-BD by the enzyme products of *B. subtilis bdhA*, *Clostridium beijerinckii adh*, or *Thermanaerobium Brockii adh*, or alternatively to *meso*-2,3-BD by the enzyme product of *K. pneumoniae budC*. By this approach, 2,3-BD titers were reported as high as 4.5 g/L in wild type hosts, or as high as 6.1 g/L in an engineered background (*adhE*-, *ldhA*-, *frdBC*-, *fnr*-, *pta*-, *pflB*-), corresponding to a 2,3-BD-glucose yield of 0.31 g/g, which is close to our reported value of 0.29 g/g. Whereas a few of these mutations are consistent with those of the strains employed in our study (*ldhA*-, *pflB*-), there also exist notable differences. For example, pyruvate dehydrogenase (encoded by *aceEF*) remained

intact in their production strain. As the transcription of *aceEF* is known to be repressed by both ArcA and Fnr [39], *fnr*- strains may have benefitted by the low level expression of an enzymatically active pathway to acetyl-CoA biosynthesis. This critical feature, which was lacking in our strains, could have resulted in improved host fitness and greater overall production.

As both previous studies have indicated that 2,3-BD could be synthesized in a wild-type *E. coli* host, this suggests that sufficient endogenous precursor (pyruvate) levels should be available. As such, our present inability to produce any 2,3-BD (or even acetoin) in a wild-type *E. coli* host may suggest that the relative activities of our selected pathway enzymes were insufficient to compete with other, endogenous pyruvate-consuming reactions. We observed that the apparent low *in vivo* activity of *ilvBN* of *L. lactis* when expressed in *E. coli* precluded its function in the present pathway. This enzyme has been previously characterized as having a low affinity for pyruvate, with a K_m value of 50 mM [40], compared to reported values as low as 13 mM for a homologous enzyme (encoded by *alsS*) of *Bacillus subtilis* [41]. It has been reported that heterologous expression of this enzyme in *E. coli* successfully resulted in high ALS activity [42]. It is interesting to note that low activity associated with the over-expression of *ilvIH* from *E. coli* was also cited in the same study. Our present ability to achieve similar acetoin titers by using only an engineered host expressing either *ilvBN* of *E. coli* or *ivlGM* of *S. typhmuri* suggests both of these enzymes displayed equivalently inadequate activity, relative to those ALS isozymes employed in previous studies.

Concluding remarks

By following a step-wise approach, we have successfully reconstructed functional acetoin and *meso*-2,3-butanediol biosynthetic pathways in *E. coli*. Engineering of the host background to increased the availability of the pyruvate precursor and, more importantly, reducing equivalents (NADH) was found to be critical in support of the production of both acetoin and *meso*-2,3-butanediol. Overall, however, the productivity of our strains was likely limited by their inability to support a suitable redox balance or sustained metabolic activity and further compounded by a sub-optimal media design. Nevertheless, our 2,3-BD-glucose yields (as high as 0.29 g/g) are consistent with other recent reports using metabolically engineered *E. coli*. As low activity associated with one or more of our composite pathway enzymes may have also attributed to relatively low product titers, additional screening of pathway isozymes from alternative genetic sources should be explored.

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Table 1. *E. coli* strains and plasmids used or constructed in this study.

Name	Relevant Genotype	Reference or Source
<i>E. coli</i> Strains		
Electromax DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 endA1 araD139Δ(<i>ara, leu</i>)7697 <i>galU</i> <i>galK</i> λ <i>rpsL nupG</i></i>	Invitrogen
ElectroTen-Blue	Δ (<i>mcrA</i>)183 Δ (<i>mcrCB-hsdSMR-mrr</i>)173 <i>endA1</i> <i>supE44 thi-1 recA1 gyrA96 relA1 lac</i> Kan ^r [F' <i>proAB</i> <i>lacI</i> ^a Δ M15 Tn10 (Tet ^r)]	Stratagene
MG1655	F ⁻ λ <i>ilvG rfb-50 rph-1</i>	ATCC 700926
MG1655(DE3)	as above, (DE3)	This study
YYC202	Hfr <i>zbi::Tn10 poxB1</i> Δ (<i>aceEF</i>) <i>rpsL pps-4 pfl-1</i>	[19]
YYC202(DE3)	as above, (DE3)	This study
YYC202(DE3) Δ <i>ldhA</i>	as above, Δ <i>ldhA</i>	This study
YYC202(DE3) Δ <i>ilvC</i>	as above, Δ <i>ilvC</i>	This study
YYC202(DE3) Δ <i>ldhA</i> Δ <i>ilvC</i>	as above, Δ <i>ldhA</i> Δ <i>ilvC</i>	This study
Plasmids		
pETDuet-1	ColE1(pBR322) <i>ori, lacI, T7lac, Amp^r</i>	Novagen
pCDFDuet-1	CloDF13 <i>ori, lacI, T7lac, Str^r</i>	Novagen
pET-ilvBNLl ^a	pETDuet-1 harboring <i>ilvBN</i> from <i>L. lactis</i>	This study
pET-ilvBNEc ^a	pETDuet-1 harboring <i>ilvBN</i> from <i>E. coli</i>	This study
pET-ilvIHec ^a	pETDuet-1 harboring <i>ilvIH</i> from <i>E. coli</i>	This study
pET-ilvGMSt ^a	pETDuet-1 harboring <i>ilvGM</i> from <i>S. typhimurium</i>	This study
pET-aldBLl ^a	pETDuet-1 harboring <i>aldB</i> from <i>L. lactis</i>	This study
pET-aldCLl ^a	pETDuet-1 harboring <i>aldC</i> from <i>L. lactis</i>	This study
pET-adcCa ^a	pETDuet-1 harboring <i>adc</i> from <i>C. acetobuylicum</i>	This study
pET-ilvBNLl-aldBLl ^a	pETDuet-1 harboring <i>ilvBN</i> from <i>L. lactis</i> and <i>aldB</i> from <i>L. lactis</i>	This study
pET-ilvBNLl-aldCLl ^a	pETDuet-1 harboring <i>ilvBN</i> from <i>L. lactis</i> and <i>aldC</i> from <i>L. lactis</i>	This study
pET-ilvBNLl-adcCa ^a	pETDuet-1 harboring <i>ilvBN</i> from <i>L. lactis</i> and <i>adc</i> from <i>C. acetobuylicum</i>	This study
pET-ilvBNEc-aldBLl ^a	pETDuet-1 harboring <i>ilvBN</i> from <i>E. coli</i> and <i>aldB</i> from <i>L. lactis</i>	This study
pET-ilvBNEc-aldCLl ^a	pETDuet-1 harboring <i>ilvBN</i> from <i>E. coli</i> and <i>aldC</i> from <i>L. lactis</i>	This study
pET-ilvBNEc-adcCa ^a	pETDuet-1 harboring <i>ilvBN</i> from <i>E. coli</i> and <i>adc</i> from <i>C. acetobuylicum</i>	This study
pET-ilvIHec-aldBLl ^a	pETDuet-1 harboring <i>ilvIH</i> from <i>E. coli</i> and <i>aldB</i> from <i>L. lactis</i>	This study
pET-ilvIHec-aldCLl ^a	pETDuet-1 harboring <i>ilvIH</i> from <i>E. coli</i> and <i>aldC</i> from <i>L. lactis</i>	This study
pET-ilvIHec-adcCa ^a	pETDuet-1 harboring <i>ilvIH</i> from <i>E. coli</i> and <i>adc</i> from <i>C. acetobuylicum</i>	This study
pET-ilvGMSt-aldBLl ^a	pETDuet-1 harboring <i>ilvGM</i> from <i>S. typhimurium</i> and <i>aldB</i> from <i>L. lactis</i>	This study

pET-ilvGMSt-aldC ^a	pETDuet-1 harboring <i>ilvGM</i> from <i>S. typhimurium</i> and <i>aldC</i> from <i>L. lactis</i>	This study
pET-ilvGMSt-adcCa ^a	pETDuet-1 harboring <i>ilvGM</i> from <i>S. typhimurium</i> and <i>adc</i> from <i>C. acetobutylicum</i>	This study
pCDF-butB ^a	pCDFDuet-1 harboring <i>butB</i> from <i>L. lactis</i>	This study
pCDF-bdh1Sc ^a	pCDFDuet-1 harboring <i>bdh1</i> from <i>S. cerevisiae</i>	This study

^a Each gene is under the control of the T7*lac* promoter with a ribosome binding site.

^b Restriction enzyme sites used in the cloning are shown in underlined italics.

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Figure legends

Figure 1. Enzymatic pathway for the biosynthesis of acetoin (Steps A and B) and 2,3-butanediol (Steps A, B, and C) from pyruvate. Note that the stereochemistry of individual metabolites will be dictated by the stereospecificity of the individual enzymes that catalyze each step.

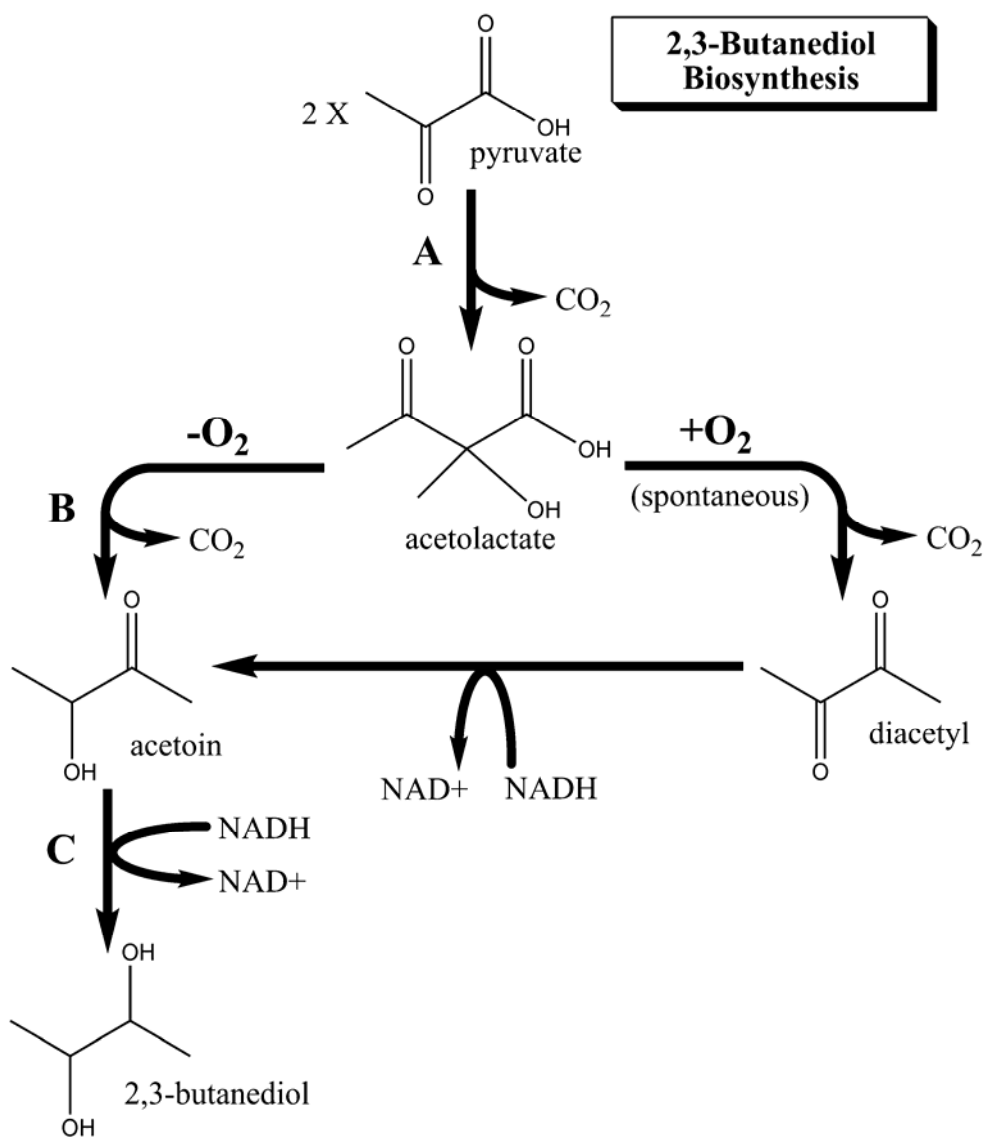
Figure 2. Engineering the mixed acid fermentation pathways of *E. coli* to increase the availability of precursors (pyruvate, and ultimately acetolactate) for the acetoin and 2,3-butanediol pathways. Heterologous pathway elements are indicated by gray arrows. Disrupted pathway steps are indicated by arrow breaks. Over-expressed enzymes are underlined.

Figure 3. Comparison of acetoin titers achieved with different hosts, each expressing the acetoin biosynthesis pathways as constructed with plasmids pET-ilvBNEc-aldCLI (gray), pET-ilvBNEc-aldBLI (white), and pET-ilvGMSt-aldBLI (patterned). Inset data indicates 2,3-BD titers (mg/L) additionally produced by the same cultures. Error bars represent the standard error associated with (at least) triplicate experiments.

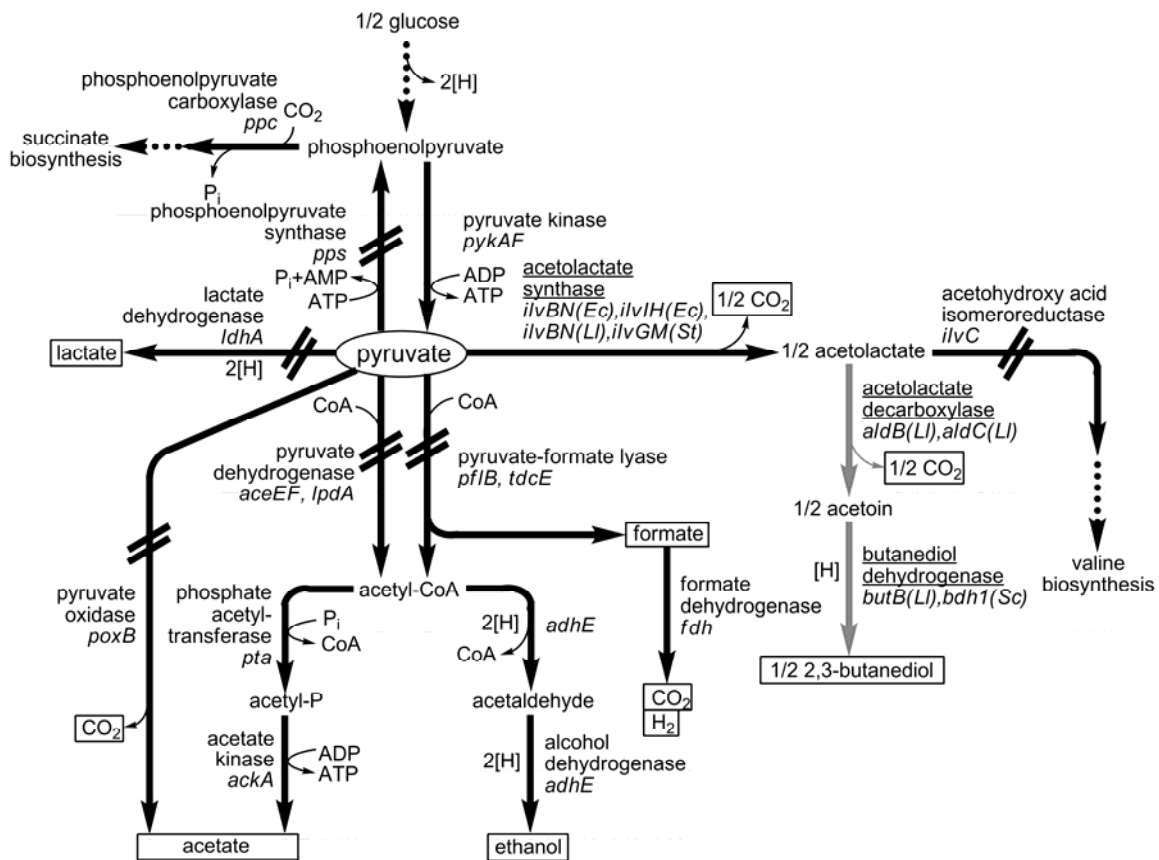
Figure 4. Distribution of fermentation products produced by the hosts used in this study at 72 h of anaerobic culture in LB + 1% (wt./vol.) glucose. Inset data shows experimental NAD⁺/NADH values. Errors are reported at one standard deviation.

Figure 5. Phylogenetic analysis of the butanediol dehydrogenase homologues of interest to this study among a diversity of prokaryotic and eukaryotic organisms. Phylogenetic analysis was performed using homologues of BDH1 of *S. cerevisiae* and BUTB of *L. lactis* (both shown in bold). *E. coli* homologues are also shown in bold.

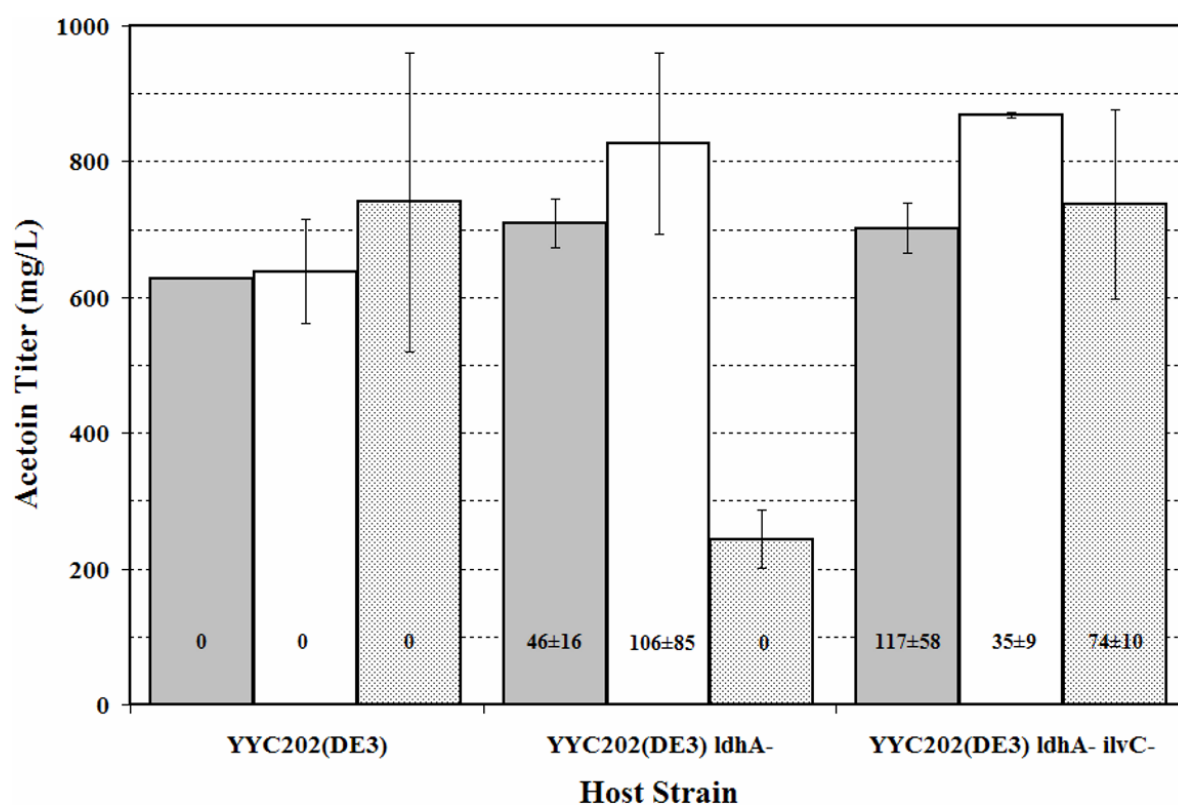
Figure 6. Comparison of the maximum achievable titers of acetoin (white) and 2,3-butanediol (gray) between engineered strains after 5 days of culture. Also shown is the maximum titer of succinate (dotted) concurrently produced as a by-product. Error bars represent the standard error associated with (at least) triplicate experiments.



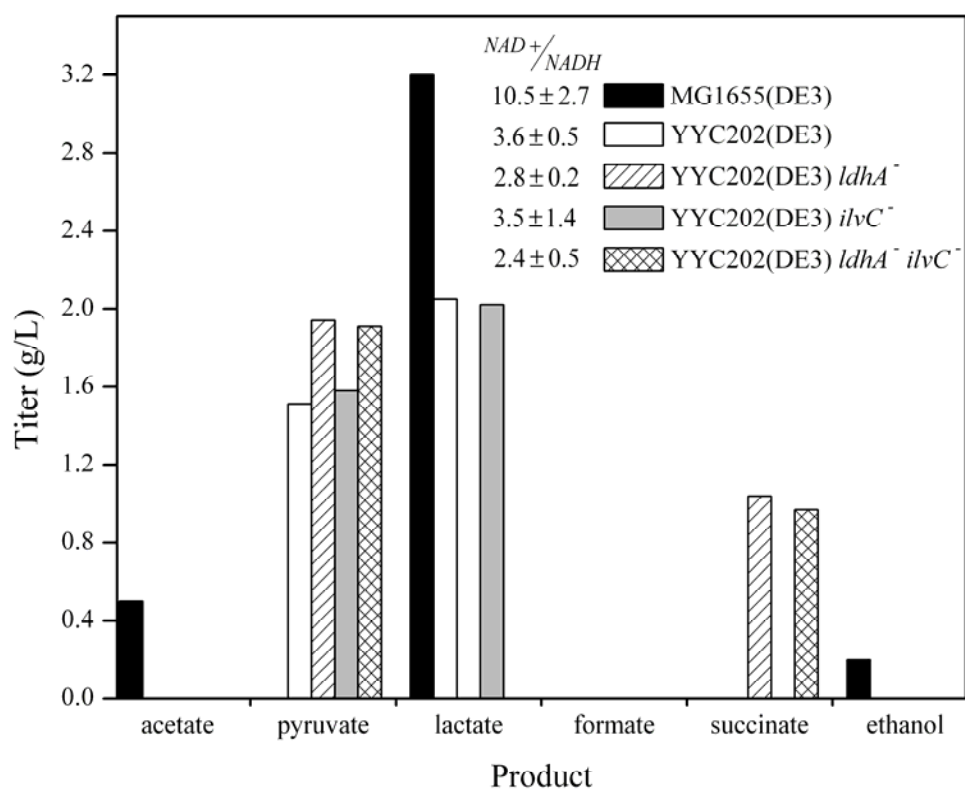
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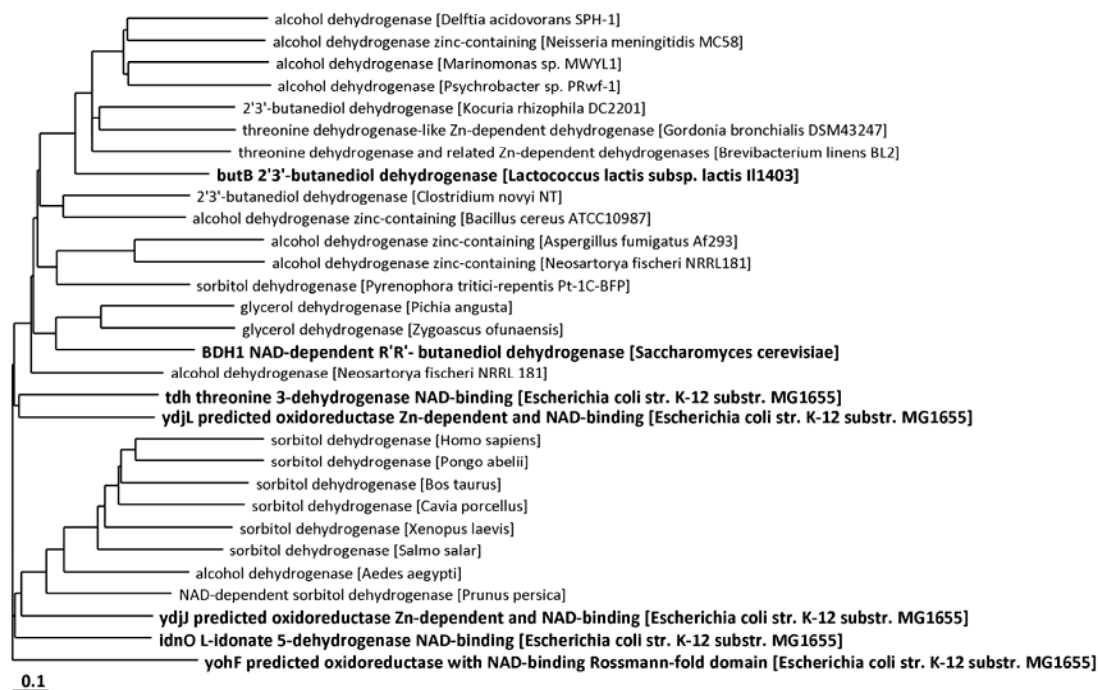
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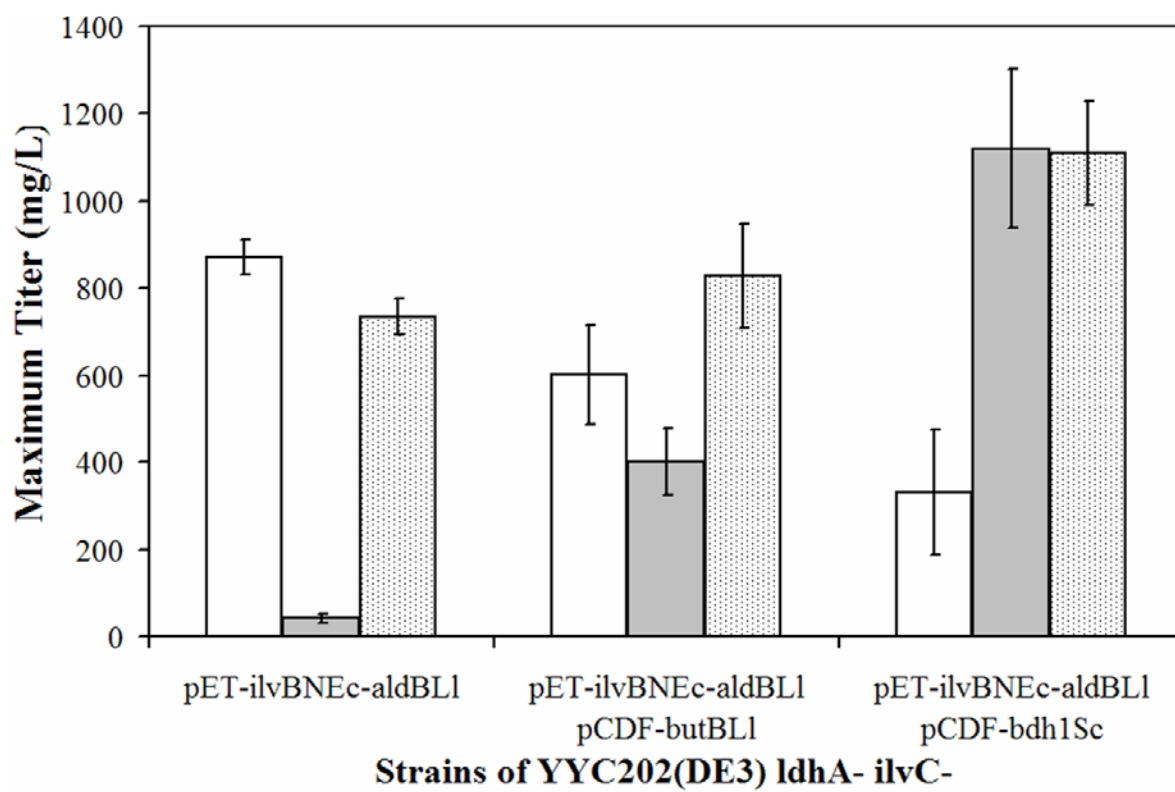


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