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ARTICLE

BIOTECHNOLOGY and BIOENGINEERING

Co-Production of Acetone and Ethanol With Molar Ratio Control Enables Production of Improved Gasoline or Jet Fuel Blends

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ABSTRACT: The fermentation of simple sugars to ethanol has been the most successful biofuel process to displace fossil fuel consumption worldwide thus far. However, the physical properties of ethanol and automotive components limit its application in most cases to 10–15 vol% blends with conventional gasoline. Fermentative co-production of ethanol and acetone coupled with a catalytic alkylation reaction could enable the production of gasoline blendstocks enriched in higher-chain oxygenates. Here we demonstrate a synthetic pathway for the production of acetone through the mevalonate precursor hydroxymethylglutaryl-CoA. Expression of this pathway in various strains of *Escherichia coli* resulted in the co-production of acetone and ethanol. Metabolic engineering and control of the environmental conditions for microbial growth resulted in controllable acetone and ethanol production with ethanol:acetone molar ratios ranging from 0.7:1 to 10.0:1. Specifically, use of gluconic acid as a substrate increased production of acetone and balanced the redox state of the system, predictively reducing the molar ethanol:acetone ratio. Increases in ethanol production and the molar ethanol:acetone ratio were achieved by co-expression of the aldehyde/alcohol dehydrogenase (AdhE) from *E. coli* MG1655 and by co-expression of pyruvate decarboxylase (Pdc) and alcohol dehydrogenase (AdhB) from *Z. mobilis*. Controlling the fermentation aeration rate and pH in a bioreactor raised the acetone titer to 5.1 g L^{-1}, similar to that obtained with wild-type *Clostridium acetobutylicum*. Optimizing the metabolic pathway, the selection of host strain, and the physiological conditions employed for host growth together improved acetone titers over 35-fold (0.14–5.1 g/L). Finally, chemical catalysis was used to upgrade the co-produced ethanol and acetone at both low and high molar ratios to higher-chain oxygenates for gasoline and jet fuel applications.

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KEYWORDS: metabolic engineering; fermentation; biofuels; catalytic alkylation

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Introduction

The increasing demand for petroleum-based fuels, coupled with the deleterious effects of increasing carbon dioxide emissions, has driven efforts to develop approaches for the production of transportation fuels from renewable resources. The fermentation of sugars to ethanol by yeast or bacteria has been exploited by society for thousands of years (Cavalieri et al., 2003). Currently, a significant proportion of the ethanol produced through this fermentative process is blended with gasoline as an anti-knock, octane-enhancing additive. However, ethanol production for fuel use in 2010 was 23.0 billion gallons, accounting for only 6.8% of the world's gasoline consumption (EIA data). The United States leads all nations in ethanol production at 13.3 billion gallons per year but has seen significant resistance recently to increase production above the 10 vol% blend-wall (Strogen et al., 2012). The production of longer-chain biofuels (Atsumi et al., 2009; Jang et al., 2012; Machado et al., 2012) can address the blend-wall problem. However, several of these longer-chain biofuel molecules are highly toxic to the producing organism, severely limiting the final titers achieved during fermentation (Lehmann and Lütke-Eversloh, 2011).

Acetone, also known as dimethyl ketone, is a colorless liquid used primarily as a solvent in the synthesis of polymers. Acetone is

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not well-suited as a fuel molecule because of its high volatility and polymer swelling susceptibility but is only mildly toxic to most organisms, with tolerances in excess of 60 g L^{-1} reported (Jones and Woods, 1986). The α-carbons on acetone can be rendered nucleophilic and were previously shown to form carbon-carbon bonds with primary alcohols via an alkylation reaction under basic conditions with an appropriate transition-metal catalyst (Anbarasan et al., 2012). Therefore, the co-production of acetone and ethanol, combined with a chemical catalysis alkylation reaction, enables the production of C_5 and C_7 mono-ketones as precursors for gasoline blendstocks. Bormann et al. (2014) demonstrated that tuning the ratio of fermentation products for catalytic upgrade has a major impact on the distribution of long-chain fuel precursors.

Figure 1 (Scenario 1) describes how a high ethanol:acetone ratio (in excess of 5:1) would produce an improved gasoline blendstock enriched in C_5 and C_7 oxygenates. Alternatively, controlling the ethanol:acetone ratio to a level of 3:1 or below, as depicted in Scenario 2 of Figure 1, enables nearly complete conversions of both fermentation products to mono-ketones of C_5–C_7. Balakrishnan et al. (2015) and Sacia et al. (2015) recently demonstrated that the mono-ketones produced from this initial alkylation reaction could be upgraded further to a highly branched C_{10} and C_{14} or to cyclic C_{15} mono-ketones via self-condensation reactions to dimers and trimers (Fig. 1, Scenario 2). The high degree of branching and longer-chain length of these C_{10}–C_{15} products makes them well suited as jet fuel.

Biological production of acetone was demonstrated on the industrial scale with *Clostridium acetobutylicum* from the 1920s to

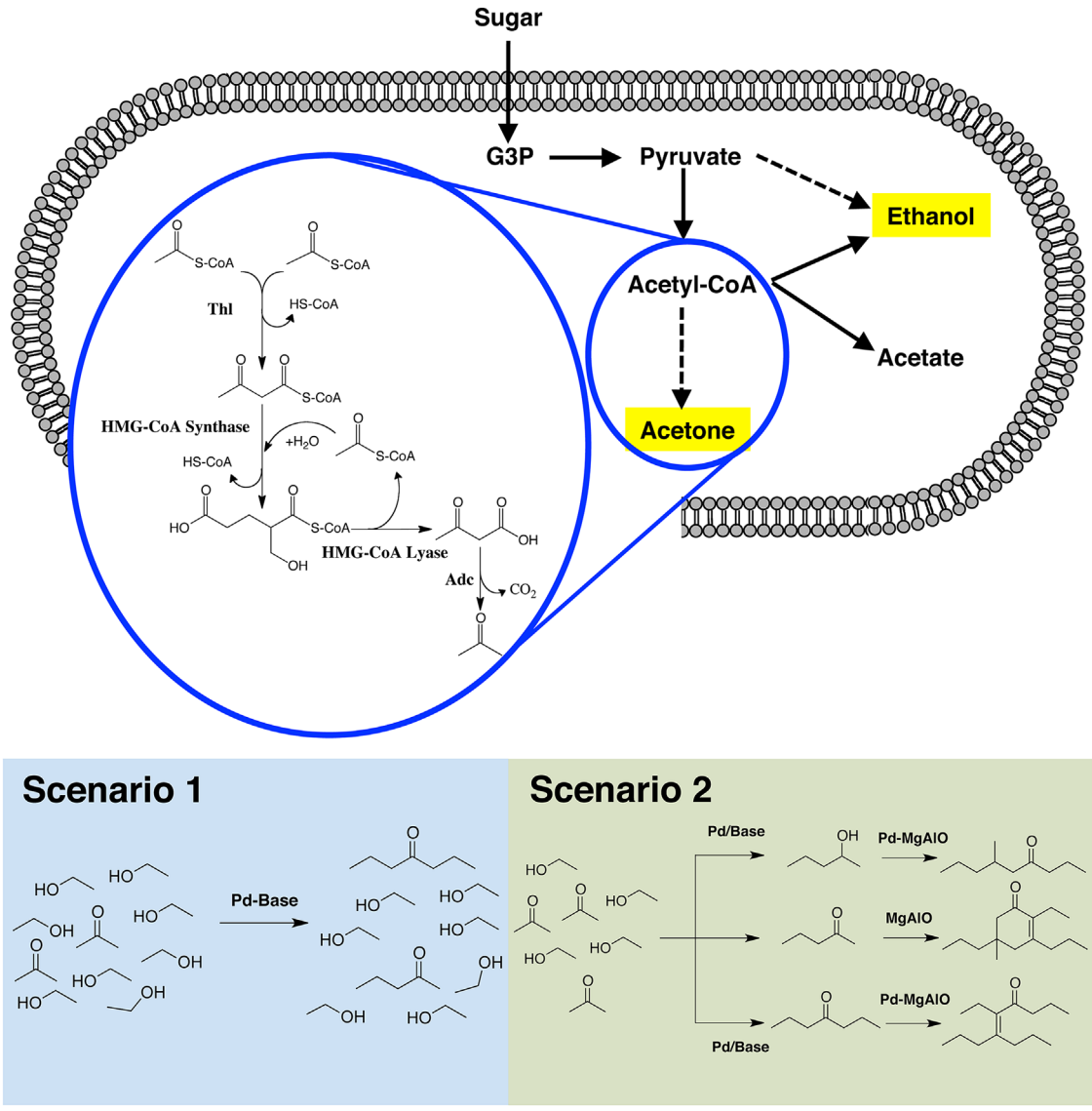

Figure 1. Summary of proposed metabolic pathway for co-production of acetone and ethanol in *E. coli* (dashed lines indicate recombinant pathways, solid lines native pathways) coupled with proposed chemical catalysis to enriched gasoline or jet blendstocks. Acetone and ethanol from the fermentation can be upgraded to either an enriched gasoline blendstock with an ethanol:acetone ratio of at least 5:1 (Scenario 1) or highly branched jet fuel blendstock using an ethanol:acetone ratio of at most 3:1 (Scenario 2).

1950s (Tracy et al., 2012). The metabolic pathway for producing acetone in *C. acetobutylicum* was successfully transferred to *Escherichia coli* (Bermejo et al., 1998) and resulted in titers comparable to those of wild-type *C. acetobutylicum*. The pathway for acetone production in *C. acetobutylicum* requires the presence of acetate or butyrate in their un-dissociated forms. A CoA-transferase (CtfA or CtfB) transfers a Co-A moiety from acetoacetyl-CoA to the un-dissociated acid, producing acetyl- or butyryl-CoA and acetoacetate. Wiesenborn et al. (1989) reported very high acetate and butyrate Km values of 1,000 and 660 mM, respectively, for the purified *C. acetobutylicum* Co-A-transferase. Acetoacetate is then decarboxylated to acetone by acetoacetate decarboxylase (Adc). May et al. (2013) described a modified acetone production pathway using a thioesterase from *Haemophilus influenzae* (*ybgC*) that did not require acetate or butyrate reassimilation and increased the acetone titer to 7.1 g L^{-1}. However, the thioesterase activity toward acetyl-CoA, coupled with the aerobic environment of the fermentation, limited ethanol production to at most 10 mM or 0.5 g/L. This low ethanol:acetone ratio is incompatible with the chemical catalysis upgrading, which requires a minimum molar ratio of 1:1 for complete conversion of the acetone. To address this challenge, we identified an alternative acetone production pathway not requiring acid reassimilation that allowed for sufficient ethanol co-production by expressing a hydroxymethylglutaryl-CoA (HMG-CoA) synthase and HMG-CoA lyase in *E. coli*, as depicted in Figure 1.

HMG-CoA synthases are responsible for mevalonate production and have been investigated extensively for the microbial synthesis of isoprenoids (Peralta-Yahya et al., 2011; Ro et al., 2006; Tsuruta et al., 2009; Wang et al., 2011), whereas HMG-CoA lyases found in higher eukaryotes are responsible for the final enzymatic step of ketoneogenesis and leucine catabolism in the liver. Isolates from prokaryotes are implicated in mevalonate catabolism and maintain activity under oxidative conditions by lacking residue Cys^{323} (Pié et al., 2007). The metabolism of glucose to acetone in most microorganisms is not balanced with respect to reducing equivalents; therefore, fermentations occur under aerobic conditions. For this reason, this study only considered HMG-CoA lyase isolates from previously described prokaryotes.

In this work, we describe the use of an alternative acid-independent acetone production pathway expressed in *E. coli* using both an HMG-CoA synthase and an HMG-CoA lyase in a single inducible-operon with the *C. acetobutylicum* genes *thl* and *adc*. Several sources of the synthase and lyase enzymes are tested, and suitable candidates are combined with a second inducible operon containing either the *E. coli* MG1655 alcohol/aldehyde dehydroganse (AdhE) or both the *Z. mobilis* pyruvate decarboxylase (Pdc) and alcohol dehydrogense (AdhB) to improve the co-production of ethanol. Optimization of the fermentation conditions and sugar substrate affords significant improvements in overall solvent production and control of the ethanol:acetone ratio. This control over the solvent ratio enables catalytic upgrading to either gasoline or jet fuel blendstocks.

Material and Methods

Chemicals, Microorganisms, and Plasmids

All chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO) and Spectrum Chemicals. Enzymes were purchased from New England Biolabs (Ipswich, MA) and used according to the manufacturer's instructions. Strains used in this work and their sources are described in Table I. Primers used and plasmids created in this work are described in Supplemental Table SI and Table II, respectively.

Strain Maintenance

All *E. coli* strains were first streaked onto Luria Broth (LB) agar plates supplemented with the appropriate antibiotic (60 μg mL^{-1} kanamycin and/or 100 μg mL^{-1} carbenicillin) if necessary. Single colonies were picked and inoculated into liquid LB media overnight at 37°C before sub-culturing into fresh liquid LB media. Strains were stored in 15 wt% glycerol at −80°C.

Assembly of Expression Plasmids

All plasmid used in this study were constructed using isothermal DNA assembly as previously described (Gibson et al., 2009). The genes *C. acetobutylicum thl*, *adc*, and *E. coli* strain MG1655 *adhe* were amplified from genomic DNA. Pyruvate decarboxylase (*pdc*) and alcohol dehydrogenase (*adhB*) from *Z. mobilis* were amplified from the pLOI297 vector. HMG-CoA synthase and lyase genes from *E. faecalis EF1363*, *S. aureous mvaS*, *L. acidophilis LAC30SC_03150*, *P. mevalonii mvaB*, and *B. subtilis yngG* were synthesized with codon optimization for both *E. coli* and *C. acetobutylicum* through geneArt (see sequences in Supplementary Materials). The HMG-CoA lyase gene from *A. baumannii AB57_1597* was also synthesized by

Table I. Strains used in this study.

Strain	Description	Source
E. coli BL21 (DE3)	F− ompT hsdSB(rB− mB−) gal dcm (λDE3)	Invitrogen
E. coli MG1655 (DE3)	K-12;λ−; F− (λDE3)	Trinh et al. (2011)
E. coli XL1B	F′ proAB lacIqZΔM15 Tn10 (Tetr) K-12; λ−; F− lacIq rrnBT14 ΔlacZWJ16	Stratagene
E. coli BW25113	hsdR514 ΔaraBADAH33 ΔrhaBADLD78	Trinh et al. (2011)
E. coli BFA7 (DE3)	BW25113, λDE3 Δzwf Δmdh ΔfrdA Δndh Δpta ΔpoxB ΔldhA::Kan	Trinh et al. (2011)
E. coli ATCC 11303	Harboring pLOI297	ATCC
C. acetobutylicum ATCC 824	Wild-type	ATCC

Table II. Plasmids used in this study.

Name	Description	Source
pET 21b	f1 *ori*, Amp^r	Novagen
pET 24b	f1 *ori*, Kan^r	Novagen
p7EFPM	pET21b, pT7 *E. faecalis HMG-CoA SYN* + *P. mevalonii HMG-CoA LYS* + *C. acetobutylicum ADC* + *C. acetobutylicum THL tT7*	This study
p7LAPM	pET21b, pT7 L. *acidophilus HMG-CoA SYN* + *P. mevalonii HMG-CoA LYS* + *C. acetobutylicum ADC* + *C. acetobutylicum THL tT7*	This study
p7SAPM	pET21b, pT7 *S. aureus HMG-CoA SYN* + *P. mevalonii HMG-CoA LYS* + *C. acetobutylicum ADC* + *C. acetobutylicum THL tT7*	This study
p7EFAB	pET21b, pT7 *E. faecalis HMG-CoA SYN* + *A. baumanii HMG-CoA LYS* + *C. acetobutylicum ADC* + *C. acetobutylicum THL tT7*	This study
p7LAAB	pET21b, pT7 *L. acidophilus HMG-CoA SYN* + *A. baumanii HMG-CoA LYS* + *C. acetobutylicum ADC* + *C. acetobutylicum THL tT7*	This study
p7SABS	pET21b, pT7 *S. aureus HMG-CoA SYN* + *B. subtilis HMG-CoA LYS* + *C. acetobutylicum ADC* + *C. acetobutylicum THL tT7*	This study
pK7AT	pET24b, pT7 *E. coli strain MG1655 ADHE* + *C. acetobutylicum THL tT7*	This study
p7negSYN	pET21b, pT7 *P. mevalonii HMG-CoA LYS* + *C. acetobutylicum ADC* + *C. acetobutylicum THL tT7*	This study
p7negLYS	pET21b, pT7 *E. faecalis HMG-CoA SYN* + *C. acetobutylicum ADC* + *C. acetobutylicum THL tT7*	This study
p7negADC	pET21b, pT7 *E. faecalis HMG-CoA SYN* + *P. mevalonii HMG-CoA LYS* + *C. acetobutylicum THL tT7*	This study
p7negTHL	pET21b, pT7 *E. faecalis HMG-CoA SYN* + *P. mevalonii HMG-CoA LYS* + *C. acetobutylicum ADC tT7*	This study
pK7PA	pET24b, pT7 *Z. mobilis PDC* + *Z. mobilis ADHB tT7*	This study

geneArt but codon optimization could not be performed. All four of the genes for the recombinant acetone pathway were cloned into a pET21b vector as a single operon (HMG-CoA synthase-HMG-CoA lyase-*adc*-thl). Plasmids harboring only three of the recombinant acetone pathway genes (p7ΔSYN, p7ΔLYS, p7ΔADC, p7ΔTHL) were constructed from the parent plasmid p7EFPM (see Supplementary Materials). Ethanol production plasmids (pK7AT and pK7PA) were constructed from a pET24b vector. A detailed description of plasmid assembly can be found in the supplementary information. All PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, CA) prior to the isothermal DNA assembly reaction.

DNA Isolation and Manipulation

All plasmids were propagated in *E. coli* XL1B (Stratagene, Carlsbad, CA) and purified using the QIAprep Spin Miniprep Kit (Qiagen). *E. coli* XL1B and BL21 (DE3) (Invitrogen, Carlsbad, CA) were transformed using the standard heat shock protocol. Transformations in *E. coli* BFA7 and MG1655 were performed by electroporation as previously described (Trinh et al., 2011).

Fermentation

All fermentation experiments were carried out at 37°C. Precultures were grown to an OD_{600} of 1.0 before inoculating in Terrific broth (24 g L^{-1} yeast extract, 12 g L^{-1} tryptone, 4 mL L^{-1} glycerol, 17 mM KH_2PO_4, and 72 mM K_2HPO_4) at a 1:20 dilution. Initial 5-mL fermentation experiments were carried out with 14-mL round bottom tubes. Oxygen was provided to the culture by shaking at 250 RPM. After the OD_{600} reached 0.6–1.0, IPTG was added to the culture to a final concentration of 1 mM. Feeding of concentrated sugar or concentrated sugar acid was initiated 1 h after induction. The culture pH was stabilized using 1 M NH_4OH for all sugar acid fermentations.

Fermentations were scaled-up in a pH- and oxygen-controlled 750-mL DasGIP bioreactor (Eppendorff, Hamburg, Germany) operated in fed-batch mode. Seed cultures (30 mL Terrific broth, 100 μg mL^{-1} carbenicillin and 60 μg mL^{-1} kanamycin) were inoculated with 2 mL of preculture and grown to an OD_{600} of 2.0. A 30 mL aliquot of the seed culture was inoculated into 720 mL of Terrific broth supplemented with 100 μg mL^{-1} carbenicillin and 60 μg mL^{-1} kanamycin. Oxygen was supplied via aeration at a constant rate of 10 or 0.5 L h^{-1} (STP), for aerobic or microaerobic conditions, respectively, at a constant agitation rate of 500 rpm. The bioreactor pH was controlled at pH 6.0 with a 28% NH_4OH solution. After the culture OD_{600} reached 1.0, IPTG was added to the fermenter to a final concentration of 1 mM and used to induce expression of the acetone pathway. Concentrated feeding (400 g L^{-1} glucose or gluconic acid) was initiated after IPTG induction.

Alkylation Reaction of Acetone and Ethanol Mixtures

A 12 mL Q-tube reactor was loaded with a small stir bar, 12 mg of 5% Pd-Carbon and 300 mg K3PO. The Q-tube was then covered with a veined butyl rubber septa and sealed. Dodecane, ethanol, and acetone were added to the Q-tube via syringe while weighing on a balance until desired mass was achieved. The reactor was then cooled to −10°C for 20 min in a freezer. The butyl rubber septum was removed and the tube was covered with a rigid PTFE coated disk. The Q-tube was then placed in the nylon sleeve and the aluminum cover screwed down firmly to make a tight seal between the glass tube and PTFE disk. The reaction mixture was then placed in an aluminum block, which had been preheated to 120°C, and allowed to react for 18 h.

The Qtube was then removed from heat, cooled to room temperature and frozen at −80°C in a dry ice-acetone bath for 30 min. While still in the dry ice bath, 5.5 mL of toluene was added to the reaction mixture via syringe and allowed to warm back to room temperature. The Q-tube was shaken vigorously to remove solids from the tube wall and centrifuged for 5 min at 3500 rpm to remove all suspended particles. A 200 μL aliquot was withdrawn from the reactor via syringe, injected into a sealed GC vial through the septum and diluted with 1.2 mL of toluene. The sample was then analyzed via GC-FID, and acetone, ethanol, 2-pentanone, and 4-heptanone were quantified.

Analytical Methods

Major metabolite concentrations for glucose, gluconic acid, acetate, succinate, formate, pyruvate, ethanol, acetone, and lactate were determined using a Shimadzu Prominence HPLC System

(Shimadzu, Kyoto, Japan) equipped with both UV/Vis and refractive index detectors. Samples were separated with a Bio-Rad (Hercules, CA) Aminex HPX-87H ion exchange column and a Cation H guard column with 5 mM sulfuric acid as the mobile phase and a flow rate of 0.7 mL min^{-1} at 35°C.

Results and Discussion

Acetone Production in *E. Coli* Strains Via the Alternative Pathway

To evaluate whether the synthetic acetone pathway would express in *E. coli*, plasmids (p7EFPM and p7LAPM) containing the HMG-CoA synthase from either *E. faecalis* or *L. acidophilis*, the HMG-CoA lyase from *P. mevalonii*, and the *thl* and *adc* genes from *C. acetobuytlicum* were transformed into *E. coli* strain BL21 (DE3). Expression of the pathway was induced by 1 mM IPTG and analyzed by SDS–PAGE 18 h after induction; see Supplemental Figure S1. Expression of the HMG-CoA synthase, HMG-CoA lyase, and thiolase genes could be clearly detected compared to the negative control, while expression of the acetoacetate decarboxylase could not. However, 5-mL shake flask fermentations of BL21 p7EFPM and BL21 p7LAPM produced 190 and 120 mg L^{-1} of acetone, respectively, from 6 g L^{-1} glucose (Table III), while no acetone production was detected in uninduced cultures (Supplemental Table SII).

Plasmids p7EFPM and p7LAPM were transformed into *E. coli* strains MG1655 (DE3) and BFA7 (DE3). Fermentation of the p7EFPM and p7LAPM BFA7 strains showed similar acetone titers to those of the previously tested BL21 strains; more specifically, BFA7 p7EFPM and p7LAPM both produced 140 mg L^{-1} of acetone. However, both variants expressed in strain MG1655 produced only 30 mg L^{-1} of acetone, with most of the carbon flux going to acetate production (Table III). May et al. (2013) described similar strain variability with respect to acetone production, citing genetic background of the strain as a crucial factor for acetone production. Since the p7EFPM variant achieved the highest acetone titer, further characterization of the alternative acetone pathway was performed using this variant.

Table III. Comparison of acetone production for variants p7EFPM and p7LAPM in various *E. coli* strains. Fermentation results from 5-mL shake flask experiments with 1 mM IPTG induction and addition of 6 g L^{-1} glucose.

Variant	*E. coli* strain	Acetate (mg L^{-1})	Ethanol (mg L^{-1})	Acetone (mg L^{-1})
p7EFPM	BL21	80	510	190
	BF7	70	620	140
	MG1655	2540	310	30
p7LAPM	BL21	120	570	120
	BF7	220	570	140
	MG1655	2470	300	30

Verifying the Essential Role of Each Gene in the Pathway by Single Gene Deletions

To verify that the observed variability in acetone production was not a function of a native enzyme and that all enzymes in the pathway are necessary for acetone production, plasmids with single gene deletions were constructed from p7EFPM (Table II). These single gene deletion plasmids were transformed into strain BL21 and evaluated for acetone production in 5-mL shake flask fermentations. No acetone production was detected in the thiolase (p7ΔTHL), HMG-CoA synthase (p7ΔSYN), and HMG-CoA lyase (p7ΔLYS) deletion variants (Fig. 2). However, acetone production was detected in the acetoacetate decarboxylase (p7ΔADC) deletion variant. Interestingly, when the p7ΔADC variant was transformed into the BFA7 strain, no acetone production was detected (Fig. 2). No enzymes could be identified from a homology blast search of the *C. acetobutylicum* acetoacetate decarboxylase gene in strain BL21 to account for this result. It is known that acetoacetate is naturally unstable under acidic conditions and readily degrades to acetone. Therefore, this effect could be explained by differences in intracellular pH between the two strains.

Fed-batch fermentations in 5-mL shake flasks were carried out to determine whether the deletion of the *adc* gene in the strain BL21 had any effect on final acetone titers. Figure 3A shows that there is no statistical difference in acetone production when grown on glucose between variants p7EFPM (0.85 g L^{-1}) and p7ΔADC (0.96 g L^{-1}) expressed in strain BL21, with *P*-value = 0.52. Strain

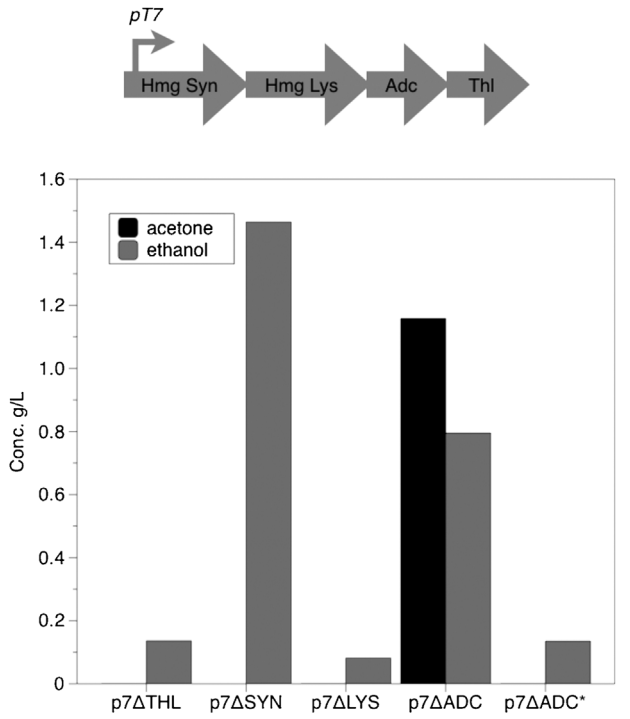

Figure 2. Single gene deletions of the alternative acetone pathway in *E. coli* BL21 (p7ΔTHL, p7ΔSYN, p7ΔLYS, p7ΔADC) and *E. coli* BFA7 (p7ΔADC*). Solvent concentrations for the single gene deletion variants fermented on 6 g L^{-1} glucose. Samples taken at least 24 h after 1 mM IPTG induction.

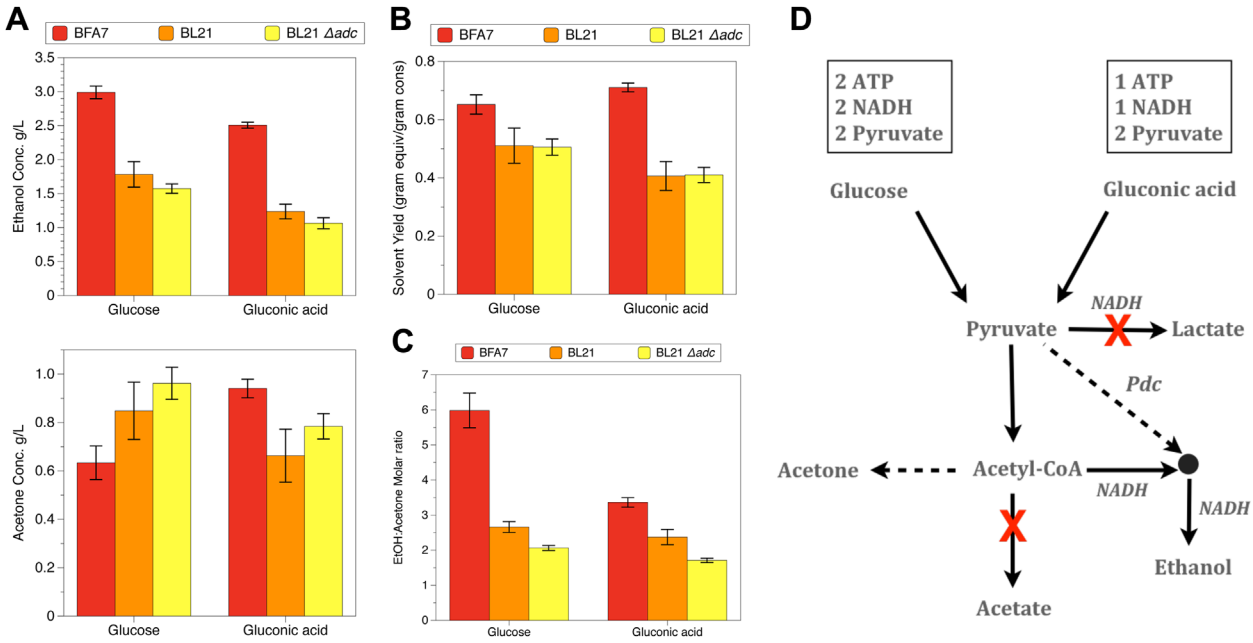

Figure 3. Fermentation results with glucose and gluconic acid as the major carbon source. For each condition, bars correspond to BFA7 (p7EFPM), BL21 (p7EFPM), BL21 (p7 ΔADC), from left to right. (**A**) Final solvent titers, (**B**) solvent yields with respect to theoretical sugar consumption, (**C**) ethanol to acetone molar ratios. Values are the average of biological triplicates. (**D**) *E. coli* metabolic pathways for glucose and gluconic acid catabolism. Dashed lines represent non-native pathways in *E. coli*. Red "X" denotes knockouts found in *E. coli* strain BFA7. NADH requirements for all of the metabolic steps are depicted.

BFA7 expressing variant p7EFPM had a lower acetone titer of 0.63 g L^{-1} and higher ethanol titer of 3.0 g L^{-1} compared to the BL21 variants. The BFA7 strain has knockouts for several of the competing metabolic pathways to ethanol and acetone production (e.g., acetate and lactate). A carbon balance analysis of the two strains of *E. coli* demonstrates that these knockouts in BFA7 enable more efficient production of the desired solvents with solvent yields (defined by moles of glucose equivalent converted to acetone and ethanol/moles of glucose consumed) of 0.65 for the BFA7 strain as opposed to 0.51 in both variants of the BL21 strain; see Figure 3B.

Controlling the Ethanol:Acetone Ratio

The ideal molar ratios of ethanol:acetone for the catalytic production of an enhanced gasoline blend and a jet fuel blendstock are quite different. To align with these desired ratios, we envisioned engineering both the fermentation conditions and central metabolism of *E. coli*. By changing the substrate from glucose to the oxidized sugar acid gluconic acid, acetone production in strain BFA7 p7EFPM was increased by almost 50% to 0.94 g L^{-1} while ethanol production decreased to 2.5 g L^{-1}. This effect is explained by the difference in reducing equivalent requirements for the production of acetone and ethanol; see Figure 3D. The catabolism of gluconic acid generates 2 moles of pyruvate but only 1 mole of NADH, as opposed to the 2 moles generated from glucose. Additionally, each mole of pyruvate converted to ethanol requires either 1 or 2 moles of NADH, depending on the metabolic pathway used, while acetone requires no NADH and consumes 2 moles of pyruvate. Therefore, in the more metabolically constrained BFA7 strain, the cell must decrease the ratio of ethanol to acetone produced when catabolizing gluconic acid. This effect is highlighted in Figure 3C, which demonstrates that changing the carbon source from glucose to gluconic acid reduced the ethanol:acetone ratio of BFA7 p7EFPM from 6.0 to 3.4. Switching from glucose to gluconic acid in the BL21 strains had no effect on the ethanol:acetone ratio. This result is explained by the functional acetate production pathway in BL21 strains, which, like the acetone pathway, does not consume NADH. Therefore, BL21 strains can increase production of either acetone or acetate in response to switching the substrate from glucose to gluconic acid. Supplemental Figure S2 shows that acetate production in the BL21 strain expressing variants p7EFPM and p7ΔADC increased by at least 70% when the carbon source was changed from glucose to gluconic acid.

Evaluating Different HMGS and HMGL Variants

Initial strain and fermentation optimization for variant p7EFPM increased acetone titer fivefold but it still remained below 1 g L^{-1}. To address this issue, we evaluated acetone production with several additional HMG-CoA synthases and lysases (p7EFAB, p7LAAB, p7SAPM, p7SABS) in strains BL21 and BFA7; see Table IV. In all variants tested, strain BL21 produced higher acetone titers. Of the new variants tested, p7EFAB and p7LAAB produced the highest acetone titers, 0.54 and 0.77 g L^{-1}, respectively.

To improve the ethanol production in BL21 strains, the alcohol/aldehyde dehydrogenase (*adhE*) gene from *E. coli* MG1655 was cloned into a pET24b plasmid (pK7AT) and co-transformed with the best acetone pathway variants (p7EFPM, p7EFAB, and

Table IV. Comparison of acetate and solvent production titers for new HMGS and HMGL variants in 5 mL shake flask fermentations. Values are the average of biological triplicates.

Variant	*E. coli* strain	Acetate ($g\,L^{-1}$)	Ethanol ($g\,L^{-1}$)	Acetone ($g\,L^{-1}$)
p7EFAB	BL21	5.37 ± 0.30	1.7 ± 0.05	0.54 ± 0.07
	BFA7	0.73 ± 0.01	2.11 ± 0.02	0.08 ± 0.0
p7LAAB	BL21	2.94 ± 0.04	1.77 ± 0.04	0.77 ± 0.03
	BFA7	0.617 ± 0.03	1.91 ± 0.06	0.14 ± 0.0
p7SAPM	BL21	5.687 ± 0.73	1.76 ± 0.04	0.41 ± 0.03
	BFA7	1.45 ± 0.03	3.15 ± 0.08	0.07 ± 0.02
p7SABS	BL21	8.52 ± 0.47	1.60 ± 0.06	0.27 ± 0.01
	BFA7	0.64 ± 0.01	2.01 ± 0.03	0.06 ± 0.001
p7EFPM	BL21	1.38 ± 0.13	1.78 ± 0.19	0.85 ± 0.12
	BFA7	4.34 ± 1.85	2.99 ± 0.09	0.63 ± 0.07

p7LAAB). Fed-batch 5-mL shake flask fermentations of these BL21 variants were conducted with glucose or gluconic acid as the carbon source; see Table V. Ethanol production on glucose increased by 126%, 167%, and 111% for variants p7EFPM/pK7AT, p7EFAB/pK7AT, and p7LAAB/pK7AT, compared to the strains not harboring the alcohol/aldehyde dehydrogenase plasmid. Ethanol production was further increased fourfold to $15.1\,g\,L^{-1}$ when plasmid pK7PA harboring the pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis* was co-expressed with p7LAAB; see Table V. Acetone production in this strain was moderate at $1.82\,g\,L^{-1}$, yielding an ethanol:acetone molar ratio of 10:1. Variant p7LAAB/pK7AT produced the highest acetone titer, $3.05\,g\,L^{-1}$, when fermented on glucose, which is over a threefold improvement from variant p7EFPM. Combining plasmids pK7AT or pK7PA with plasmids harboring the acetone production pathway, we demonstrated that *E. coli* can be engineered to produce a wide variety of ethanol:acetone substrate ratios ranging from 10:1 to 1.5:1. To further demonstrate that these differences in substrate ratio are required for downstream synthesis of different types of fuel blends, catalytic upgrading of low and high ratio solvent mixtures was investigated.

Catalytic Upgrading of Low and High Acetone:Ethanol Ratio Blends

Molar ratios of ethanol:acetone of 3:1 and 6:1 were selected to represent low and high fermentation solvent ratios and were reacted over a palladium catalyst on carbon support (Pd/C) with molar quantities of solid tribasic potassium phosphate (K_3PO_4). The yield and selectivity for 2-pentanone (2-C_5) and 4-heptanone (4-C_7) from these reactions are listed in Table VI. Overall yields of C_5+ ketones were greater than 40% for both molar ratios of ethanol:acetone tested with no side products observed on the GC trace; see Supplemental Figure S3A and B. As expected, decreasing the molar ratio of ethanol:acetone from 6:1 to 3:1 favored the production of the mono-alkylated 2-C_5 product, increasing selectivity from 17% to 45% and reducing the production of di-alkylated 4-C_7. Additionally, reducing the ethanol:acetone molar ratio increased the final ketone weight fraction of the corresponding fuel blend to 27%. This enriched ketone fuel blend can be further reacted over either hydrotalcite (MgAlO), TiO_2 or niobic acid (Nb_2O_5) to form cyclic and branched oxygenates of carbon length C_{10}–C_{15} (Sacia et al., 2015), ideal for jet fuel applications. Operating the catalysis reaction at the higher ethanol:acetone ratio of 6:1 still enabled the production of a gasoline-based fuel blend that was enriched with 20 wt% of higher molecular weight ketones, predominately composed of 4-C_7 with a ketone selectivity of 83%. Combining the 6:1 ratio catalysis reaction with the fermentation of variant p7EFAB/pk7AT (ethanol:acetone ratio of 5.5:1) enables the production of 920 mg/L of 4-heptanone and 1.06 g/L of C_{5+} ketones starting from glucose. To our knowledge, this is the highest reported titer of 4-heptanone from a biomass-derived substrate, and it highlights the potential of integrated biological and chemocatalytic processes. Further increases to the concentration of acetone without eliminating ethanol production in the fermenter would enable even higher titers of C_{5+} oxygenate.

Optimizing Fermentation Conditions for Acetone and Ethanol Co-Production

To further improve acetone titer in the p7LAAB/pK7AT strain and enable higher long-chain oxygenate production, culture conditions were optimized in 750-mL DasGIP (Eppendorff) bioreactors. Initial fermentation studies performed on BL21 p7EFPM showed that acetone production in *E. coli* could not be achieved under strictly anaerobic conditions (Supplemental Figure S4A). Similar results were previously demonstrated in Bermejo et al. (1998) and May et al. (2013) when expressing the native *C. acetobutylicum* and synthetic acetone pathways in *E. coli*. When the air sparge-rate in the bioreactor was set to $10\,sL\,h^{-1}$, making the culture fully aerobic, acetone was the only solvent produced (Supplemental Figure S4B). Decreasing the sparge-rate to $1\,L\,h^{-1}$ (STP) kept the fermentation

Table V. Product concentrations for improved ethanol variants in *E. coli* strain BL21. Fed-batch 5-mL shake flask fermentations with glucose or gluconic acid as the major carbon source. Values are averages of biological triplicates.

Variant	Carbon source	Acetate ($g\,L^{-1}$)	Ethanol ($g\,L^{-1}$)	Acetone ($g\,L^{-1}$)
p7EFAB/pK7AT	Glucose	3.02 ± 1.100	4.54 ± 0.42	1.06 ± 0.27
	Gluconic acid	6.75 ± 0.64	2.39 ± 0.11	0.78 ± 0.20
p7LAAB/pK7AT	Glucose	3.32 ± 0.01	3.74 ± 0.33	3.05 ± 0.32
	Gluconic acid	4.69 ± 1.43	2.53 ± 0.18	2.03 ± 0.27
p7EFPM/pK7AT	Glucose	2.55 ± 0.50	4.03 ± 0.23	0.87 ± 0.13
	Gluconic acid	3.13 ± 0.11	1.89 ± 0.02	0.49 ± 0.01
p7LAPM/pK7AT	Glucose	4.68 ± 0.08	3.76 ± 0.22	0.58 ± 0.07
	Gluconic acid	4.79 ± 0.23	2.00 ± 0.04	0.38 ± 0.01
P7LAAB/pK7PA	Glucose	2.81 ± 0.78	15.1 ± 0.41	1.82 ± 0.10

Table VI. Alkylation reaction of high and low ethanol/acetone molar ratio blends.

Reactant loading (mg)			Yield[a]			Ketone selectivity		Fuel Blend
Acetone	EtOH	EtOH: Acetone	2-C_5 (%)	4-C_7 (%)	C_{5+} Ketones (%)	2-C_5 (%)	4-C_7 (%)	Ketone wt%
140	640	6:1	9 ± 3	44 ± 7	53 ± 10	17 ± 6	83 ± 4	20
230	550	3:1	19 ± 2	24 ± 2	43 ± 0	45 ± 4	55 ± 4	27

[a]Yields based on limiting reagent acetone. Ketone selectivity based on mole % of 2-pentanone (2-C_5) and 4-heptanone (4-C_7) in product mixture. Ketone wt% is defined as $\frac{\text{mass ketone products}}{\text{mass of reactants}}$ and includes un-reacted acetone in the ketone product mass ($\leq$3% total mass).

microaerobic, enabling co-production of both acetone and ethanol (Supplemental Figure S4C). Controlling the sparge-rate to ensure microaerobic conditions in the bioreactor, a fed-batch fermentation of BL21 p7LAAB/pK7AT on glucose produced 3.90 g L^{-1} acetone and 4.50 g L^{-1} ethanol (Fig. 4A). Switching the carbon source to gluconic acid resulted in 5.1 g L^{-1} acetone and 2.76 g L^{-1} ethanol production, reducing the ethanol to acetone molar ratio to 0.7:1 (Fig. 4B). These results demonstrate that overall acetone titers can be improved by operating the fermentation in an aerated bioreactor

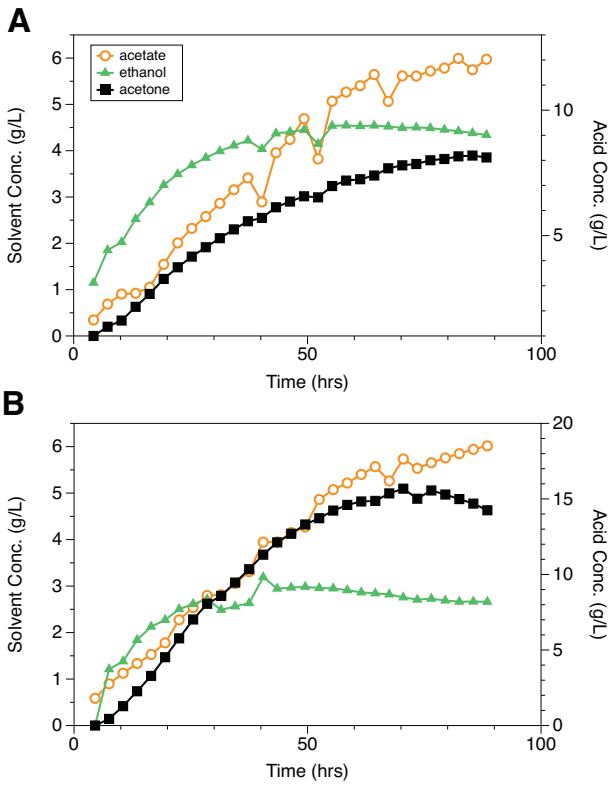

Figure 4. Time course of major metabolites in 750-mL pH controlled fed-batch fermentations of *E. coli* BL21 variant p7LAAB/pK7AT. Cultures fed with concentrated (40 wt%) glucose **(A)** and gluconic acid **(B)**. Aeration rate controlled at 1–2 sL h^{-1} and pH controlled $\geq$ 6.0 with 28 wt% ammonium hydroxide.

compared to oxygen-limited shake flask experiments. Moreover, adjustment of oxygen supply by changing the aeration rate enables control over the acetone:ethanol ratio and titers.

Discussion

The co-production of acetone and ethanol with subsequent catalytic upgrading to C_5 to C_{15} oxygenates has major implications for the ethanol industry to address gasoline and jet blend limitations. Here, we demonstrate in *E. coli* an acid-independent pathway for the production of acetone through the mevalonate precursor HMG-CoA. Acetone production was detected in all strains of *E. coli* tested; however, strain background contributed significantly to final titers and acetoacetate decarboxylation activity.

Acetone production was predictably increased with respect to ethanol production when the primary carbon source was changed from glucose to gluconic acid in the metabolically constrained BFA7 strain. This enabled control of the ethanol:acetone ratio, which is critical to align with the chemical catalysis operation (Bormann et al., 2014). The BL21 strain responded to the gluconic acid feeding by increasing acetate production, highlighting the importance of metabolic push in this *E. coli* system. Though gluconic acid is not found at appreciable concentrations in any of the conventional biofuel feedstocks, it can be readily produced from glucose either enzymatically with glucose oxidase or through fermentation with *Aspergillus niger* (Ramachandran et al., 2006). Alternatively, bacterial metabolism of galacturonic acid or glucuronic acid produces the same amount of NADH, ATP, and pyruvate as gluconic acid. Galacturonic acid is a major component of pectin and has previously been studied as a feedstock for biofuel production (Benz et al., 2014).

To improve acetone titers several variants of the HMG-CoA synthase and HMG-CoA lyase were tested for acetone production in strains BL21 and BFA7. A majority of these variants produced higher acetone titers in the BL21 strain. This could be attributed to improved protein expression of the heterologous pathway in the BL21 strains compared to BFA7 strains. The combination of HMGS from *L. acidophilus* and HMGL from *A. baumanii* produced the highest acetone titer. Ethanol titers and ethanol:acetone ratios could be increased by the co-expression of either the aldehyde/alcohol dehydrogenase from *E. coli* MG1655 or pyruvate decarboxylase and alcohol dehydrogenase from *Z. mobilis*. Varying the ratio of ethanol:acetone has a major impact on the ketone selectivity and serves as a tool to influence the downstream catalysis to produce drop-in fuels for either gasoline or jet applications. It was demonstrated that catalytic upgrading of improved acetone strain fermentations enables production of advanced biofuel oxygenates greater than 1 g/L.

Following metabolic engineering and substrate investigations, optimization of air sparge rates in pH-controlled fed-batch fermentations improved acetone titers to *C. acetobutylicum* wild-type levels. May et al. (2013) described a synthetic acetone pathway capable of producing the highest acetone titer reported to date of 122 mM or 7.08 g/L. However, the synthetic pathway and fermentation conditions employed in their study prevented any appreciable ethanol co-production. It is critical for advanced biofuel applications that ethanol be co-produced to convert all of the

acetone into higher molecular oxygenates through the downstream catalysis step.

Metabolic engineering and control of the environmental conditions for microbial growth increased acetone titers over 35-fold to 5.1 g/L and ethanol:acetone molar ratios ranging from 0.7:1 to 10.0:1. Further optimization of the strain background and fermentation conditions for efficient acetone production, improved solvent yields, and catalyst screening are necessary for commercialization. Though currently no commercial *E. coli* ethanol process exists, the pathway described in this work should be transferable to other industrial ethanologens. Specifically, with recent advances in S. cerevisiae cytosolic acetyl-coA production (Kozak et al., 2014) and the development of industrial isoprenoid yeast strains, transfer of this combined acetone/ethanol process to yeast is a promising route to commercialize advanced biofuels with the flexibility to address both gasoline and jet fuel applications.

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