Metabolic Engineering of 2-Pentanone Synthesis in *Escherichia coli*

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Expanding the chemical diversity of microbial fermentation products enables green production of fuel, chemicals, and pharmaceuticals. In recent years, coenzyme A (CoA) dependent chain elongation, resembling the reversed β-oxidation pathway, has attracted interest for its use in producing higher alcohols, fatty acids, and polyhydroxyalkanoate. To expand the chemical diversity of this pathway, we metabolically engineered Escherichia coli to produce 2-pentanone, which is not a natural fermentation product of E. coli. We describe the first demonstration of 2-pentanone synthesis in E. coli by coupling the CoA-dependent chain elongation with the acetone production pathway. By bioprospecting for enzymes capable of efficient hydrolysis of 3-keto-hexanoyl-CoA, production of 2-pentanone increased 20 fold, reaching a titer of 240 mg/L. © 2013 American Institute of Chemical Engineers *AIChE J*, 59: 3167-3175, 2013
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Introduction

In the past century, the chemical industry is relying almost exclusively on fossil resources including petroleum, coal, and natural gas. The use of such nonrenewable raw materials presents sustainability and environmental challenges. Biological processes allow the use of renewable resources such as sugars,[1] lignocellulose,[2] waste proteins,[3] or even CO_2[4–6] as raw materials, and represent an important approach to address the energy and environmental problems.[7] To achieve green manufacturing and to replace fossil raw materials in the chemical industry, expanding the chemical repertoire that a living organism can produce is essential. To meet this goal, microorganisms have been engineered to produce a wide array of products.[8–12]

Specifically, ketones are an important class of solvents produced in large quantities. The simplest form, acetone, has been produced by microbial processes using *Clostridium* in a process known as acetone, butanol, ethanol fermentation.[13] To expand the chemical diversity of microbial synthesis, here we aim to produce a higher chain methyl ketone, 2-pentanone. Methyl ketones are used as solvents, and have been found in small quantities in microbial fermentation products.[14] Methyl ketones are also used as food additives for providing scent and flavoring.[15] In particular, 2-pentanone is a phytochemical naturally produced in banana and carrot, and has been demonstrated to inhibit cyclooxygenase 2 (COX-2),[16] which is involved in inflammatory processes and potentially associated with colon cancer. Recently, *E. coli* has been engineered to produce long chain methyl ketones with 11–15 carbons by reassimilating fatty acids into fatty acyl-coenzyme A (CoA).[17] Fatty acyl-CoA is then partially metabolized by β-oxidation to β-ketoacyl-CoA, which is then hydrolyzed and decarboxylated to methyl ketone. Here, we describe the engineering of *E. coli* to synthesize 2-pentanone without requiring fatty acid production.

To produce 2-pentanone, we extend the CoA-dependent pathway (Figure 1) that leads to acetone and *n*-butanol production[18,19] for one more round of carbon chain elongation

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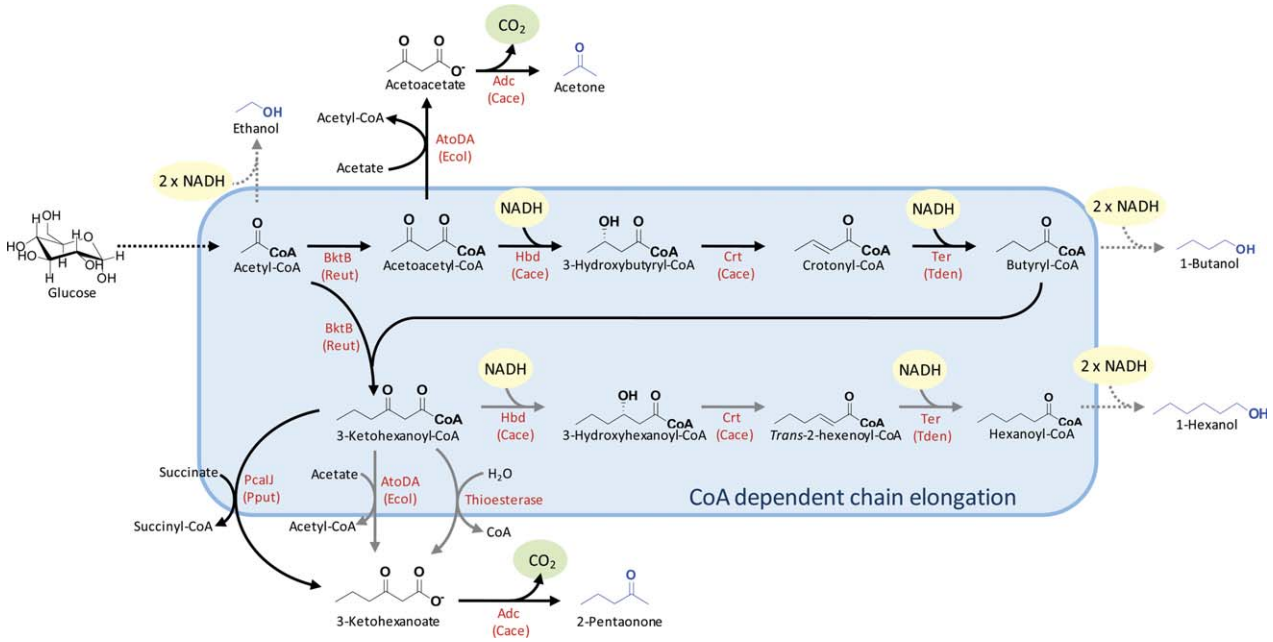

Figure 1. Schematic of 2-pentanone synthesis.

Other products made from the CoA-dependent pathway are also shown as references. BktB, β-keto-thiolase; Hbd, hydroxybutyryl-CoA dehydrogenase; Crt, crotonase; Ter, *trans*−2-enoyl-CoA reductase; PcaIJ, 3-oxoadipate CoA-succinyl transferase; AtoDA, acetate CoA-transferase. Reut, *Ralstonia eutropha*; Cace, *Clostridium acetobutylicum*; Tden, *Treponema denticola*; Pput, *Pseudomonas putida*; Ecol, *Escherichia coli*. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

by applying the principle described for keto-acid chain elongation.[20,21] Note that the CoA-dependent pathway resembles the reaction sequence of the β-oxidation in reverse.[22] The CoA-dependent pathway is of interest for its ability to produce higher alcohols for biofuels and polyhydroxyalkanoate for biodegradable plastics. The CoA-dependent pathway (Figure 1) starts from acetyl-CoA, an essential metabolite in all organisms. The carbon number in acetyl-CoA can be increased to make acetoacetyl CoA, which after hydrolysis and decarboxylation produces acetone. If the chain length of acetoacetyl-CoA is further increased to become 3-ketohexanoyl-CoA (Figure 1), similar biochemical transformations can be performed on this compound to produce 2-pentanone. To achieve this goal, efficient expression of the CoA-dependent chain elongation pathway is essential.

Many groups have transferred the *Clostridium* *n*-butanol pathway into heterologous organisms as an alternative platform for *n*-butanol production.[23–27] However, one of the enzymes, butyryl-CoA dehydrogenase electron transferring flavoprotein complex (Bcd/Etf), in the *Clostridium* CoA-dependent pathway was not expressed well in heterologous organisms and resulted in low titers of butanol produced. This difficulty prevented further engineering of the CoA-dependent pathway. To solve this problem, Bcd/Etf was replaced by *trans*−2-enoyl-CoA reductase (Ter). This replacement partially overcomes the difficulty of heterologous *n*-butanol production.[1,28] Further engineering of increasing acetyl-CoA and NADH concentration as driving forces proved to be most effective to push the flux against the thermodynamic gradient of the condensation of two acetyl-CoA, a reaction with $\Delta G^{\circ\prime}$ of 28.5 kJ/mol. Together, higher acetyl-CoA and NADH concentrations lower the energy barrier of the pathway and produce high titer and yield of *n*-butanol.[1] These results paved the way for further engineering of the CoA-dependent pathway.

The native CoA-dependent pathway extends the carbon chain length from two (acetyl-CoA) to four (butyryl-CoA). This chain length extension enables the production of *n*-butanol, rather than ethanol. The same reaction sequence could be repeated to increase the carbon chain length by using enzymes that are active toward various chain lengths. For example, expression of a promiscuous thiolase, which catalyzes the first step of the chain elongation by condensation of acyl-CoA's, enables successive rounds of carbon chain elongation to produce 1-hexanol and 1-octanol.[29,30]

Here, we constructed the CoA-dependent chain elongation pathway for two rounds of acyl-CoA condensation and demonstrated for the first time the production of 2-pentanone in an engineered organism. We identified the acyl-CoA hydrolysis or thiol group transfer as a limiting step for 2-pentanone synthesis. After improving this step, the best strain produces 2-pentanone to 240 mg/L, representing an increase of 20 fold.

Materials and Methods

Chemicals and reagents

All chemicals were purchased from Fisher Scientifics (Pittsburgh, PA) or Sigma-Aldrich (St. Louis, MO). KOD (*Thermococcus kodakaraensis* KOD1) hot start and KOD-extreme hot start DNA polymerases were purchased from EMD Biosciences (San Diego, CA). Phusion DNA polymerase and ligases were purchased from New England Biolabs (Ipswich, MA). T5-Exonuclease was purchased from Epicentre Biotechnologies (Madison, WI). Oligonucleotides were obtained from IDT (San Diego, CA).

Plasmid constructions

Plasmids used in this work are listed in Table 1. All plasmids were constructed by the isothermal DNA assembly method.[31] Plasmids were propagated and stored in *E. coli* strain XL-1 blue. Promoter and enzyme coding regions of all plasmids were verified by DNA sequencing performed by Genewiz (San Diego, CA).

Table 1. Strain and Plasmid List

Strain	Relevant Genotypes	Reference
XL1-Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F′ *proAB lacI*^{q}*ZΔM15 Tn10* (Tet^R)]	Stratagene
BW25113	*rrnB*$_{T14}$ Δ*lacZ*$_{WJ16}$ *hsdR*514 Δ*araBAD*$_{AH33}$ Δ*rhaBAD*$_{LD78}$	1
JCL16	BW25113 / F′ [*traD36, proAB+, lacI*^{q} *ZΔM15* (Tet^R)]	1
JCL166	JCL16 Δ*ldhA* Δ*adhE* Δ*frdBC*	1
JCL299	JCL16 Δ*ldhA* Δ*adhE* Δ*frdBC* Δ*pta*	1
Plasmid	Genotypes	Reference
pTA30	$P_L$lacO$_1$::*atoB atoDA adc;* ColE1; Amp^R	25
pIM8	$P_L$lacO$_1$::*ter;* ColA; Kan^R	1
pDK69	$P_L$lacO$_1$::*ter crt hbd;* ColA; Kan^R	This work
pDK73	$P_L$lacO$_1$::*bktB atoDA adc;* ColE1; Amp^R	This work
pEL137	$P_L$lacO$_1$::*bktB ctfAB(C.ace) adc;* ColE1; Amp^R	This work
pEL138	$P_L$lacO$_1$::*bktB Cbei_3833 3834 adc;* ColE1; Amp^R	This work
pEL139	$P_L$lacO$_1$::*bktB Cbei_2654 2653 adc;* ColE1; Amp^R	This work
pEL140	$P_L$lacO$_1$::*bktB ctfAB(C.dif) adc;* ColE1; Amp^R	This work
pEL142	$P_L$lacO$_1$::*bktB pcaIJ(P.put) adc;* ColE1; Amp^R	This work
pEL143	$P_L$lacO$_1$::*bktB scoAB(H.pyl) adc;* ColE1; Amp^R	This work
pEL144	$P_L$lacO$_1$::*bktB IpsIJ(X.cam) adc;* ColE1; Amp^R	This work
pEL145	$P_L$lacO$_1$::*bktB R.eut_1331 1332 adc;* ColE1; Amp^R	This work
pEL146	$P_L$lacO$_1$::*bktB scoAB(B.sub) adc;* ColE1; Amp^R	This work
pDC13	$P_L$lacO$_1$::*bktB cat1(C.klu) adc;* ColE1; Amp^R	This work
pDC14	$P_L$lacO$_1$::*bktB cat2(C.klu) adc;* ColE1; Amp^R	This work
pDC15	$P_L$lacO$_1$::*bktB cat3(C.klu) adc;* ColE1; Amp^R	This work
pDC16	$P_L$lacO$_1$::*bktB Cbei_2103 adc;* ColE1; Amp^R	This work
pDC17	$P_L$lacO$_1$::*bktB tesB adc;* ColE1; Amp^R	This work
pDC18	$P_L$lacO$_1$::*bktB fadM adc;* ColE1; Amp^R	This work
pDC20	$P_L$lacO$_1$::*bktB paaI adc;* ColE1; Amp^R	This work
pDC21	$P_L$lacO$_1$::*bktB ybgC adc;* ColE1; Amp^R	This work

Kan^R, kanamycin resistance; Amp^R, ampicillin resistance.
atoB (*E. coli*), thiolase; *bktB* (*R. eutropha*), β-keto-thiolase; *hbd* (*C. acetobutylicum*), hydroxybutyryl-CoA dehydrogenase; *crt* (*C. acetobutylicum*), crotonase; *ter* (*T. denticola*), *trans*−2-enoyl-CoA reductase; *pcaIJ* (*P. putida*), 3-oxoadipate CoA-succinyl transferase; *atoDA* (*E. coli*), acetate CoA-transferase; R. eut, *Ralstonia eutropha*; C. ace, *Clostridium acetobutylicum*; T. den, *Treponema denticola*; P. put, *Pseudomonas putida*; E. col, *Escherichia coli*; C. bei, *Clostridium beijerinckii*; C. dif, *Clostridium difficile*; H. pyl, *Helicobacter pylori*; X. cam, *Xanthomonas campestris*; C. klu, *Clostridium kluyveri*.

Plasmids pEL134, pEL135, pEL136, pEL137, pEL138, pEL139, pEL140, pEL142, pEL143, pEL144, pEL145, pEL146, pDC13, pDC14, pDC15, pDC16, pDC17, pDC18, pDC20, and pDC21 were constructed by replacing the *atoDA* gene (Figure 2A) of pDK73 with individual genes listed in Table 1. These plasmids were constructed by DNA assembly of a linear DNA fragment containing *adc*, ColE1 origin, ampicillin resistance, $P_L$lacO$_1$ promoter, and *bktB* with individual genes listed in Table 1 for each plasmid. Primers used in this work are listed in Table 2.

Culture condition for 2-pentanone production

Production of 2-pentanone was either carried out in LB (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter of water) or in Terrific Broth (TB) (12 g tryptone, 24 g yeast extract, 2.31 g KH_2PO_4, 12.54 g K_2HPO_4, 4 mL glycerol per liter of water) supplemented with glucose (1% for LB and 4% for TB). Precultures were grown in LB overnight in 37°C. 300 μL of the precultures were then used to inoculate 3 mL of fresh medium in 15-mL BD vacutainer. Microaerobic condition was achieved by capping the BD vacutainer without anaerobic purging. The cultures were grown to OD630 of 0.4–0.6, which was then induced with 0.1 mM IPTG (Isopropyl β-D-1-thiogalactopyranoside). Induced cultures were then incubated in 37°C shaker (250 rpm; New Brunswick Scientific, Enfield, CT) until sampling. For the bioprospecting enzymes capable of hydrolyzing CoA from 3-ketohexanoyl-CoA experiments, LB with 1% (w/v) glucose was used as the culture medium. For time-course experiments, TB with 4% (w/v) glucose was used.

Toxicity test for 2-pentanone and acetone

3 mL of fresh TB supplemented with 4% glucose and varying concentration of 2-pentanone or acetone in BD

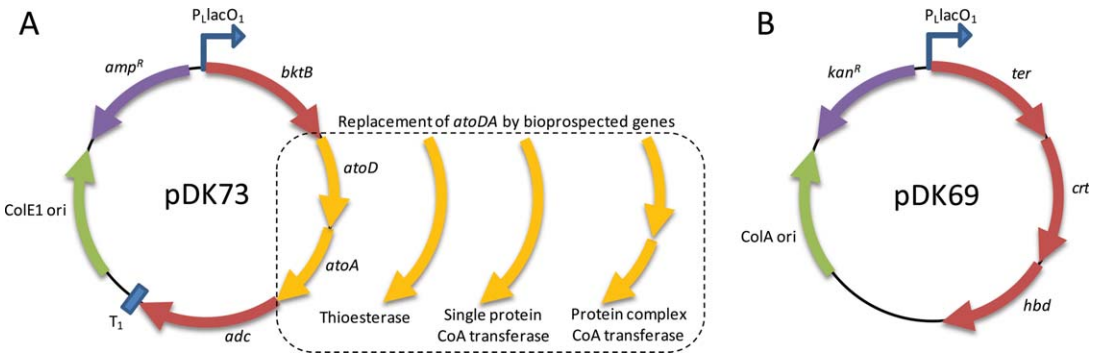

Figure 2. Plasmid map of (A) pDK73 and its derivatives, (B) pDK69. Coexpression of these two plasmids enabled synthesis of 2-pentanone.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 2. Primer Sequences

Primers	Sequence (5′ -> 3′)	Used for Plasmid
rEL-556	TAATGATCTAGAAGGAGATATACCATGTTAAAGGA	pEL137, pEL138, pEL139 pEL140, pEL142, pEL143, pEL144, pEL145, pEL146, pDC13, pDC14, pDC15, pDC16, pDC17, pDC18, pDC20, pDC21
rEL-557	CATGGTACCTTTCTCCTGCATGCTTAGATAC	pEL137, pEL138, pEL139 pEL140, pEL142, pEL143, pEL144, pEL145, pEL146, pDC13, pDC14, pDC15, pDC16, pDC17, pDC18, pDC20, pDC21
rEL-558	TCTAAGCATGCAGGAGAAAGGTACCATGAACTCTAAAATAATTAGATTTGAAAATTTAAG	pEL137
rEL-559	TATCATTAATCATGGTATATCTCCTTTATGCAGGCTCCTTTACTATATAATTTATAAG	pEL137
rEL-560	CTGCATAAAGGAGATATACCATGATTAATGATAAAAACCTAGCGAAAGAAATAAT	pEL137
rEL-561	GTATATCTCCTTCTAGATCATTAAACAGCCATGGGTCTAAGTTCATTG	pEL137
rEL-562	TCTAAGCATGCAGGAGAAAGGTACCATGAATAAATTAGTAAAATTAACAGATTTAAAGCG	pEL138
rEL-563	AAGTAATGTTCATGGTATATCTCCTTTAAGCCGCCTCCTTAACGATATAATC	pEL138
rEL-564	AGGCGGCTTAAAGGAGATATACCATGAACATTACTTTTGAATCAGAAAATGGC	pEL138
rEL-565	GTATATCTCCTTCTAGATCATTATATATCCATAATCTTTAAGTTATCTGGAATAA	pEL138
rEL-566	TCTAAGCATGCAGGAGAAAGGTACCATGACAAAGATTAAGACAGTACAGGAAGCAG	pEL139
rEL-567	CATTTTCATGGTATATCTCCTTTATCTCATTTTATTAGTTCTTATCCATAAATCTT	pEL139
rEL-568	TGAGATAAAGGAGATATACCATGAAAATGATGAATGGTAAAGAGATAATTGC	pEL139
rEL-569	GTATATCTCCTTCTAGATCATTATATTTCCATTTCCTTAACATTATCAGCTATTA	pEL139
rEL-570	TCTAAGCATGCAGGAGAAAGGTACCATGAATAAAATAGTTAGCATTGATGAAGCTCTA	pEL140
rEL-571	ATTTATCCATGGTATATCTCCTTTATAAATTACCTCCTTCAACGATGTAATTC	pEL140
rEL-572	AATTTATAAAGGAGATATACCATGGATAAATTAGAAATGCAAGAATATATTGC	pEL140
rEL-573	GTATATCTCCTTCTAGATCATTAAACAACAGATATTTTTCCATTTAATGAATC	pEL140
rEL-578	TCTAAGCATGCAGGAGAAAGGTACCATGATCAATAAAACGTATGAGTCCATCG	pEL142
rEL-579	TGGTAATGGTCATGGTATATCTCCTTTAGACAGCGTTTGCGATCGAAGAA	pEL142
rEL-580	AAACGCTGTCTAAAGGAGATATACCATGACCATTACCACAAAACTTTCCC	pEL142
rEL-581	GTATATCTCCTTCTAGATCATTACTTGATCAGCGGCACACCACTG	pEL142
rEL-582	TCTAAGCATGCAGGAGAAAGGTACCATGAACAAGGTTATAACCGATTTAGACAAA	pEL143
rEL-583	CCTCTCTCATGGTATATCTCCTTTATTTCGCACTCCTTGTGGTGGTTT	pEL143
rEL-584	GTGCGAAATAAAGGAGATATACCATGAGAGAGGCTATCATTAAACGAGCG	pEL143
rEL-585	GTATATCTCCTTCTAGATCATTATAGATGCACTTCAAATTCAGCTTCTGT	pEL143
rEL-586	TCTAAGCATGCAGGAGAAAGGTACCATGGACAAGGTGGTCGCCACAGC	pEL144
rEL-587	GTGTCCAGGCCATGGTATATCTCCTTTAGCCGCTCACCGTCCCGGTT	pEL144
rEL-588	ACGGTGAGCGGCTAAAGGAGATATACCATGGCCTGGACACGCGAGGAGA	pEL144
rEL-589	GTATATCTCCTTCTAGATCATTACGAGCGGATCTCCTCCGGG	pEL144
rEL-590	TCTAAGCATGCAGGAGAAAGGTACCATGAACCAAGTTGCAAGGGG	pEL145
rEL-591	GGGTCCAGGCCATGGTATATCTCCTTTACTTGTCTCCCTGGCGCACGG	pEL145
rEL-592	GAGACAAGTAAAGGAGATATACCATGGCCTGGACCCGCGATCAGA	pEL145
rEL-593	GTATATCTCCTTCTAGATCATTAGCCTCTTGTTTGTACAACGAAC	pEL145
rEL-594	TCTAAGCATGCAGGAGAAAGGTACCATGAACAAGGTCTACGCCAGCGC	pEL146
rEL-595	CGTGTCCATGCCATGGTATATCTCCTTTAGCTGGCCGCGCGCACGGTG	pEL146
rEL-596	GCGGCCAGCTAAAGGAGATATACCATGGCATGGACACGTGACGAAATGG	pEL146
rEL-597	GTATATCTCCTTCTAGATCATTACAGCAGCGGAGCGCCAGTC	pEL146
rEL-598	TCTAAGCATGCAGGAGAAAGGTACCATGGGAAAAGTGCTGTCATCAAGC	pEL147
rEL-599	TCGCTTCCTTCATGGTATATCTCCTTTACTTGGCCTCACCCTTTCCC	pEL147
rEL-600	TGAGGCCAAGTAAAGGAGATATACCATGAAGGAAGCGAGAAAACGAATGG	pEL147
rEL-601	GTATATCTCCTTCTAGATCATTAAGAATTGAGTACAGACTGGCTTACAGC	pEL147
DC23-cat1-F	GAGCGTATCTAAGCATGCAGGAGAAAGGTACCATGAGTAAAGGGATAAGAATTC	pDC13
DC24-cat1-R	TAACATGGTATATCTCCTTCTAGATCATTATTTCATACTACCAGTTTTTATAA	pDC13
DC25-cat2-F	TTCGAGCGTATCTAAGCATGCAGGAGAAAGGTACCATGGAGTGGGAAGAGATATAT	pDC14
DC26-cat2-R	CTTTAACATGGTATATCTCCTTCTAGATCATTAAAATCTCTTTTTAAATTCATTCATT	pDC14
DC27-cat3-F	GCGTATCTAAGCATGCAGGAGAAAGGTACCATGGTTTTTAAAAATTGGCAGGATCT	pDC15
DC28-cat3-R	CATCCTTTAACATGGTATATCTCCTTCTAGATCATTAAAGCTTACAACTGAATCTT	pDC15

TABLE 2. *Continued*

Primers	Sequence (5′ -> 3′)	Used for Plasmid
DC29–2103-F	AGCATGCAGGAGAAAGGTACCGTGTCTAAGATTAGCTGGAAAGATTTATACAAGAGTAA	pDC16
DC30–2103-R	CATGGTATATCTCCTTCTAGATCATTAAAATTTCACTTTAAACCTCTTTTCCCACTCTT	pDC16
DC31-tesB-F	GAGCGTATCTAAGCATGCAGGAGAAAGGTACCATGAGTCAGGCGCTAAAAAATTTAC	pDC17
DC32-tesB-R	CCTTTAACATGGTATATCTCCTTCTAGATCATTAATTGTGATTACGCATCACCCCT	pDC17
DC33-fadM-F	GAGCGTATCTAAGCATGCAGGAGAAAGGTACCATGCAAACACAAATCAAAGTTC	pDC18
DC34-fadM-R	ATGGTATATCTCCTTCTAGATCATTACTTAACCATCTGCTCCAGCTTTTCGCGC	pDC18
DC-37-paal-F	CGTATCTAAGCATGCAGGAGAAAGGTACCATGAGTCATAAGGCCTGGCAAAAT	pDC20
DC-38-paal-R	TCATCCTTTAACATGGTATATCTCCTTCTAGATCATTAGGCTTCTCCTGTAATGGTG	pDC20
DC-39-ybgC-F	CGAGCGTATCTAAGCATGCAGGAGAAAGGTACCGTGAATACAACGCTGTTTCGATGGC	pDC21
DC-40-ybgC-F	TCCTTTAACATGGTATATCTCCTTCTAGATCATTACTGCTTAAACTCCGCGACAAT	pDC21
pDK024 BB F2	CGACGGTATCGATAAGCTTGATATCGAATTCCTG	pDK69
pDK037 BB R1	GGTATATCTCCTTCTAGACTAAATCCTGTCGAACCTTTC	pDK69
pDK069 crt-hbd F1	GGATTTAGTCTAGAAGGAGATATACCATGGAACTAAACAATG	pDK69
pDK069 crt-hbd R1	CGATATCAAGCTTATCGATACCGTCGATTATTTTGAATAATCGTAGAAACC	pDK69
pDK062 BB F1	CTAGAGGCATCAAATAAAACGAAAGGC	pDK73
pDK006 BB R1	CCTCTTTAATGAATTCGGTCAGTGCGTCC	pDK73
pDK006 bktB F1	CGCACTGACCGAATTCATTAAAGAGGAGAAAGGTACCATGACGCGTGAAGTGGTAGTGG	pDK73
pDK049 fars BB R1	CTCCTGCATGCTTAGATACGCTCGAAG	pDK73
pDK072 atoDA F1	CGAGCGTATCTAAGCATGCAGGAGAAAGGTACCATGAAAACAAAATTGATGACATTAC	pDK73
pDK072 atoDA R1	CATCCTTTAACATGGTATATCTCCTTCTAGATCATAAATCACCCCGTTGCGTATTTC	pDK73
pDK068 adc F1	GGAGATATACCATGTTAAAGGATGAAGTAATTAAAC	pDK73
pDK071 adc R1	GCCTTTCGTTTTATTTGATGCCTCTAGATTACTTAAGATAATCATATATAAC	pDK73

vacutainer was inoculated at 0.1% (3 μL cell per 3 mL medium) with strain JCL299 overnight preculture. Cultures were then incubated in 37°C shaker at 250 rpm for 6 h. After 6 h of incubation, the cells were taken out for optical density measurement using Beckman Coulter DU 800.

Quantification of 2-pentanone

Culture samples were prepared by centrifuging (21,000 × g) the production cultures to separate the cell and supernatant. 200 μL of the supernatant was then mixed with 800 μL of 0.1% (v/v) 2-methyl-1-pentanol as the internal standard. The sample mixtures were then analyzed by gas chromatography equipped with flame ionization detector (Model 6850, Agilent Technologies, Santa Clara, CA). The separation of products was carried out with a DB-FFAP capillary column (Agilent Technologies, 30 m; 0.32 mm inner diameter; 0.25 μm film thickness). The GC result was analyzed by Agilent software Chem Station (Rev.B.04.01 SP1). The amount of 2-pentanone in the sample was then calculated based on the ratio of its integrated area and that of the 2-pentanone standard.

Helium was used as the carrier gas with 9.52 psi inlet pressure. The injector and detector temperatures were maintained at 225°C. Injection volume was 1 μL. Column flow rate was 1.7 mL/min. The oven program was as follows: 60°C for 2 min, ramp to 85°C at 45°C/min, 85°C for 2 min, ramp to 235°C at 45°C/min, 235°C for 1 min.

GC–MS analysis

To analyze the supernatant of the production culture, 2-pentanone was extracted with *n*-hexane. 500 μL of supernatant was mixed with 200 μL of hexane. The organic layer was then analyzed by GC–MS system (model 6890N GC/5973N MSD, Agilent Technologies) equipped with a HP-5MS capillary column (Agilent Technologies, 30 m; 0.25 mm inner diameter; 0.25 μm film thickness). Helium (constant flow 1 mL/min) was used as a carrier gas. The temperature of the injector was 250°C. The oven program was as follows: 50°C for 3 min, ramp to 100°C at 5°C/min, 100°C for 0 min, ramp to 250°C at 50°C/min, 250°C for 1 min.

Results

Constructing the 2-pentanone production pathway

Previously, a modified CoA-dependent chain elongation pathway[29] was constructed in *E. coli* by overexpression of a promiscuous β-keto-thiolase (BktB) from *Ralstonia eutropha* with rest of the CoA-dependent pathway enzymes, thiolase (AtoB), 3-hydroxy butyryl-CoA dehydrogenase (Hbd), crotonase (Crt), and Ter. This synthetic pathway enabled the production of six carbon CoA intermediates as demonstrated by the synthesis of 1-hexanol. As BktB also catalyzes the condensation of two acetyl-CoA into butyryl-CoA, AtoB was removed from the pathway in this work, reducing the enzymes required for CoA-dependent chain elongation pathway to BktB, Crt, Hbd, and Ter.

Methyl ketones are produced from β-keto acids derived from β-ketoacyl-CoA. Acetone, the simplest ketone, is naturally produced by *Clostridia*. The *Clostridium* acetone production pathway branches out from the CoA-dependent pathway from the acetoacetyl-CoA node. Acetoacetyl-CoA goes through a transthiolation by reacting with acetate to form acetoacetate and acetyl-CoA using acetoacetyl-CoA

transferase from *Clostridium acetobutylicum* (CtfAB) or from *E. coli* (AtoDA).[32,33] Acetoacetate is then decarboxylated into acetone by acetoacetate decarboxylase (Adc). By coexpressing the CoA-dependent chain elongation (BktB, Crt, Hbd, and Ter) with AtoDA and Adc, we expected to synthesize 2-pentanone from 3-ketohexanoyl-CoA (Figure 1).

To simultaneously express AtoDA, Adc, and the CoA-dependent chain elongation pathway, we constructed two plasmids (Figure 2) with different origins of replication. Plasmid pDK73 (Figure 2A) harbored genes *bktB*, *atoDA*, and *adc* under an IPTG inducible promoter $P_L$lacO1. Plasmid pDK69 (Figure 2B) harbored *ter*, *crt*, and *hbd,* which were also transcribed by promoter $P_L$lacO1. To minimize the formation of side products, mixed acid fermentation pathways were knocked out in the host strain JCL166 (Δ*ldh*, Δ*frdB*, Δ*adhE*). As shown in Figure 3A, strain JCL166 expressing plasmid pTA30 (*atoB*, *atoDA*, *adc*) produced only acetone, and 2-pentanone was undetectable as expected. On the other hand, 6 mg/L of 2-pentanone (Figure 3C) was produced by strain JCL166 expressing plasmid pDK73 (*bktB*, *atoDA*, *adc*) and pDK69 (*ter*, *crt*, *hbd*). Interestingly, 2 mg/L of 2-pentanone (Figure 3B) was also produced by strain JCL166 expressing only plasmid pDK73, indicating that some native *E. coli* enzymes had the catalytic properties of the CoA-dependent chain elongation. The identity of 2-pentanone produced was verified by GC–MS. The fragmentation pattern of the product (Figure 3D) matched that of the 2-pentanone standard (Figure 3E), confirming the compound produced by JCL166/pDK73/pDK69 was 2-pentanone. In all cases, the major product was acetone, indicating that either AtoDA and Adc were highly selective for four carbon substrates or they outcompeted chain elongation enzymes (BktB, Hbd, Crt, and Ter) in diverting carbon flux to acetone.

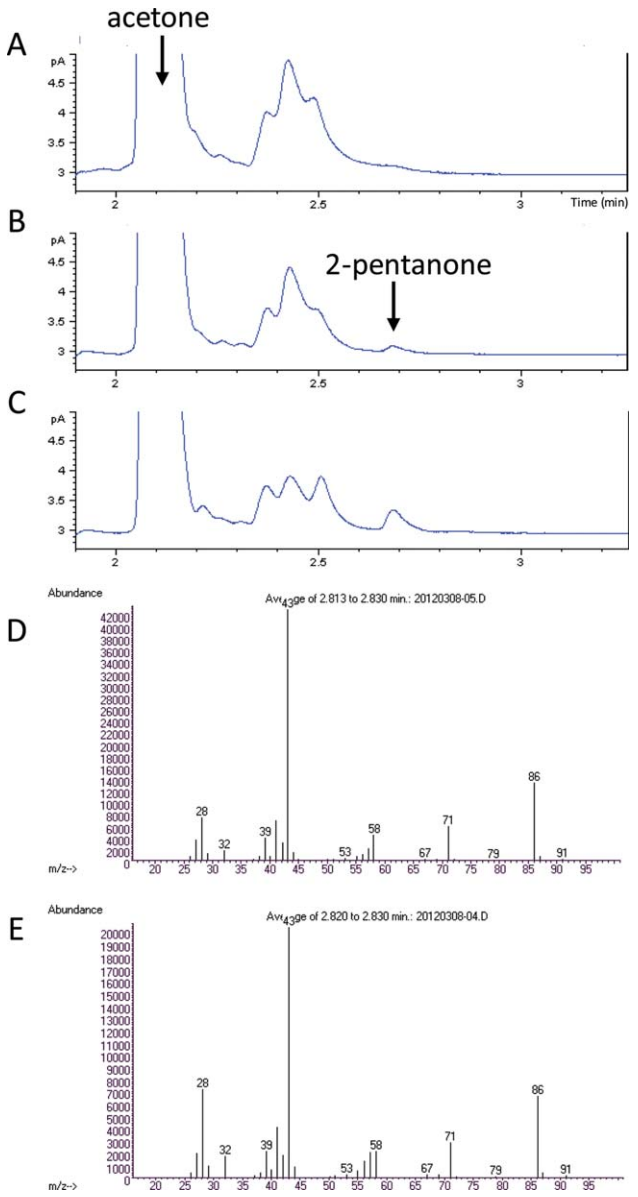

Figure 3. Gas chromatogram of the 2-pentanone production.

(A) JCL166/pTA30, (B) JCL166/pDK073 and (C) JCL166/pDK073/pDK069. Mass spectrum of (D) 2-pentanone produced by JCL166/pDK073/pDK069 and (E) 2-pentanone standard. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

CoA transferase enables production

To determine the limiting step for 2-pentanone synthesis, we first compared 2-pentanone production pathway with that of the 1-hexanol production. The two pathways share the common intermediate 3-ketohexanoyl-CoA as the result of second round of carbon chain elongation. We previously demonstrated the production of 1-hexanol up to 500 mg/L,[30] which is at least two orders of magnitude higher than the 2-pentanone produced. Therefore, the formation of 3-ketohexanoyl-CoA catalyzed by BktB is less likely to be the limiting step. Next, we rule out Adc as potential limiting step. Decarboxylation of β-keto acids to methyl-ketone is likely to occur spontaneously[17] and enables production of long chain methyl ketone up to 200 mg/L. Therefore, we reasoned that the potential limiting step for 2-pentanone synthesis was AtoDA.

To search for a CoA transferase more suitable than AtoDA for 2-pentanone synthesis, we used the protein sequence of AtoD and BLAST to identify potential CoA transferases that are capable of removing CoA from 3-ketohexanoyl-CoA. We cloned CoA transferases from organisms including *Clostridium acetobutylicum*, *Clostridium beijerinckii*, *Clostridium difficile*, *Pseudomonas putida*, *Helicobacter pylori*, *Xanthomonas campetris*, *Ralstonia eutropha*, and *Bacillus subtilis*. The identities of these homologues to AtoD range from 40 to 54%. CtfAB from *C. acetobutylicum*[34] and *C. difficile* have been demonstrated and annotated, respectively, to catalyze the CoA transfer between acetate and acetoacetyl-CoA as well as between butyrate and acetoacetyl-CoA. On the other hand, the other transferases were annotated for catalyzing the CoA transfer between 3-keto acid and succinyl-CoA. To broaden our search for an enzyme efficient in converting 3-ketohexanoyl-CoA to 3-ketohexanoate, we cloned the single protein CoA transferase Cbei_2103 from *C. beijerinckii* and Cat1, Cat2, and Cat3 from *Clostridium kluyveri*. Additionally, we cloned thioesterases TesB, FadM, PaaI, and YbgC from *E. coli* to directly hydrolyze 3-ketohexanoyl-CoA.

The genes encoding for these CoA transferases and thioesterases were individually cloned to replace *atoDA* in plasmid pDK73 (Figure 2A). These plasmids were transformed into *E. coli* with pDK69 to complete the pathway for 2-pentanone synthesis. The transformants vary greatly in colony size,

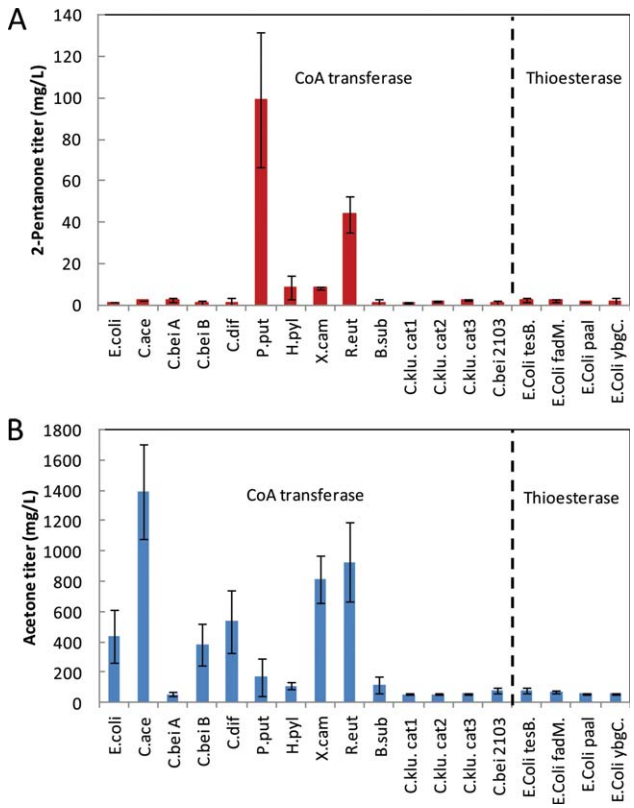

Figure 4. Production of 2-pentanone from expression CoA-dependent chain elongation and bioprospected enzymes for converting 3-ketohexanoyl-CoA to 3-ketohexanoate.

Host strain used was JCL166 (Δ*adhE*, Δ*ldh*, Δ*frdB*). Production was carried out in LB 1% glucose for 20 h. Cbei A, 3833, 3834; Cbei B, 2654, 2653; gene names for other enzymes are listed in Table 1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

indicating potential metabolic stress. Therefore, as a standard practice, we chose the smaller colonies to continue production assay. As shown in Figure 4A, CoA transferase from *P. putida*, *H. pylori*, *X. campetris*, and *R. eutropha* increased the production of 2-pentanone as compared to AtoDA. In particular, PcaIJ of *P. putida* and Reut_1331_1332 of *R. eutropha* achieved the highest increase of 2-pentanone production to 99 ± 33 and 44 ± 9 mg/L, respectively. The CoA transferases from *E. coli*, *C. acetobutylicum*, *C. beijerinckii* (*Cbei_2654_2653*), and *C. difficile* are selective for acetoacetyl-CoA as demonstrated by high acetone production (Figure 4B) and minimal production of 2-pentanone. With the exception of PcaIJ from *P. putida,*[35] the other enzymes demonstrated to aid 2-pentanone synthesis are uncharacterized proteins. Furthermore, enzymatic activities toward 3-ketohexanoyl-CoA of all enzymes tested were previously unknown. Therefore, the findings of this study may bring insights for the substrate specificity of these enzymes.

Time course of 2-pentanone production

The precursors for CoA-dependent chain elongation and 2-pentanone production are acetyl-CoA and NADH. For each mole of 2-pentanone produced, three moles of acetyl-CoA and two moles of NADH are required. Glycolysis provides two acetyl-CoA and four NADH per mole of glucose consumed. Therefore, two moles of glucose can produce three moles of 2-pentanone, giving it a maximum theoretical yield of 67% molar conversion. However, for each mole of 2-pentanone produced, four moles of NADH are produced in excess. Therefore, microaerobic environment was necessary for 2-pentanone production to avoid accumulation of NADH in strain JCL166. To increase the driving force for 2-pentanone production, we used *E. coli* strain JCL299 which is strain JCL166 with *pta* deleted to increase intracellular acetyl-CoA concentration. Accumulation of acetyl-CoA overcomes the large thermodynamic barrier[1] of the condensation reactions catalyzed by BktB.

To compare the effectiveness of different enzymes (PcaIJ vs. *Reut_1331_1332*) and acetyl-CoA driving force (with or without pta deletion), plasmids pEL142 (harboring *bktB*, *pcaIJ*, and *adc*) and pEL145 (harboring *bktB*, *Reut_1331_1332*, *adc*) were individually transformed into JCL299 (Δ*ldhA* Δ*adhE* Δ*frdBC* Δ*pta*) and JCL166 (Δ*ldhA* Δ*adhE* Δ*frdBC*) with plasmid pDK69 (harboring *ter*, *crt*, and *hbd*). Time courses of the 2-pentanone and acetone productions in these strains are shown in Figure 5. As expected, increasing intracellular acetyl-CoA by *pta* deletion (in JCL299) increased the production of 2-pentanone. 2-Pentanone production (Figure 5A) by JCL299/pEL142/pDK69 (overexpressing PcaIJ) reached the highest titer of 240 mg/L. Strain JCL299/pEL145/pDK69 (overexpressing *Reut_1331_1332)* produced less 2-pentanone, reaching final titer of 110 mg/L. On the other hand, acetone production (Figure 5B) from strain JCL299/pEL145/pDK69 exceeded that from strain JCL299/pEL142/pDK69 by around fourfold, indicating that *Reut_1331_1332* is more selective for acetoacetyl-CoA than PcaIJ.

To test if product toxicity inhibited production, we inoculated strain JCL299 into the TB medium supplemented with various concentrations of 2-pentanone and acetone. As shown in Figure 6, 600 mg/L of 2-pentanone inhibits 50% of the cell growth. At 5 g/L of 2-pentanone, growth was inhibited completely. On the other hand, acetone is much less toxic as 50% growth inhibition occurs at 12 g/L of acetone. In our best producing strain JCL299/ pEL142/pDK69, the production of both 2-pentanone and acetone are below toxicity levels, indicating that the cease of production is unlikely due to toxicity, and further improvement is possible.

Discussion

Natural organisms use a finite set of pathways and chemistry to synthesize metabolites required for growth and survival. Some of these metabolites may serve as fuels, chemicals, and pharmaceuticals. To expand the chemical space available from microbes, synthetic biology and metabolic engineering methods are used for designing new metabolic pathways. By hybridizing different pathway features, new chemicals are produced by recombinant microbes. These synthetic pathways can then be integrated into various microorganisms capable of utilizing a variety of resources such as CO_2,[36,37] syngas,[38] and waste proteins,[3] thus broadening the choice of green production strategies.

Here, we engineered a strain of *E. coli* to produce 2-pentanone at 240 mg/L in 3 days by constructing a synthetic 2-pentanone production pathway based on CoA-dependent chain elongation. CoA transferase step was identified as the potential limiting step for 2-pentanone synthesis as demonstrated by enhanced production upon expression of PcaIJ from *P. putida*. PcaIJ has been identified as *β*-

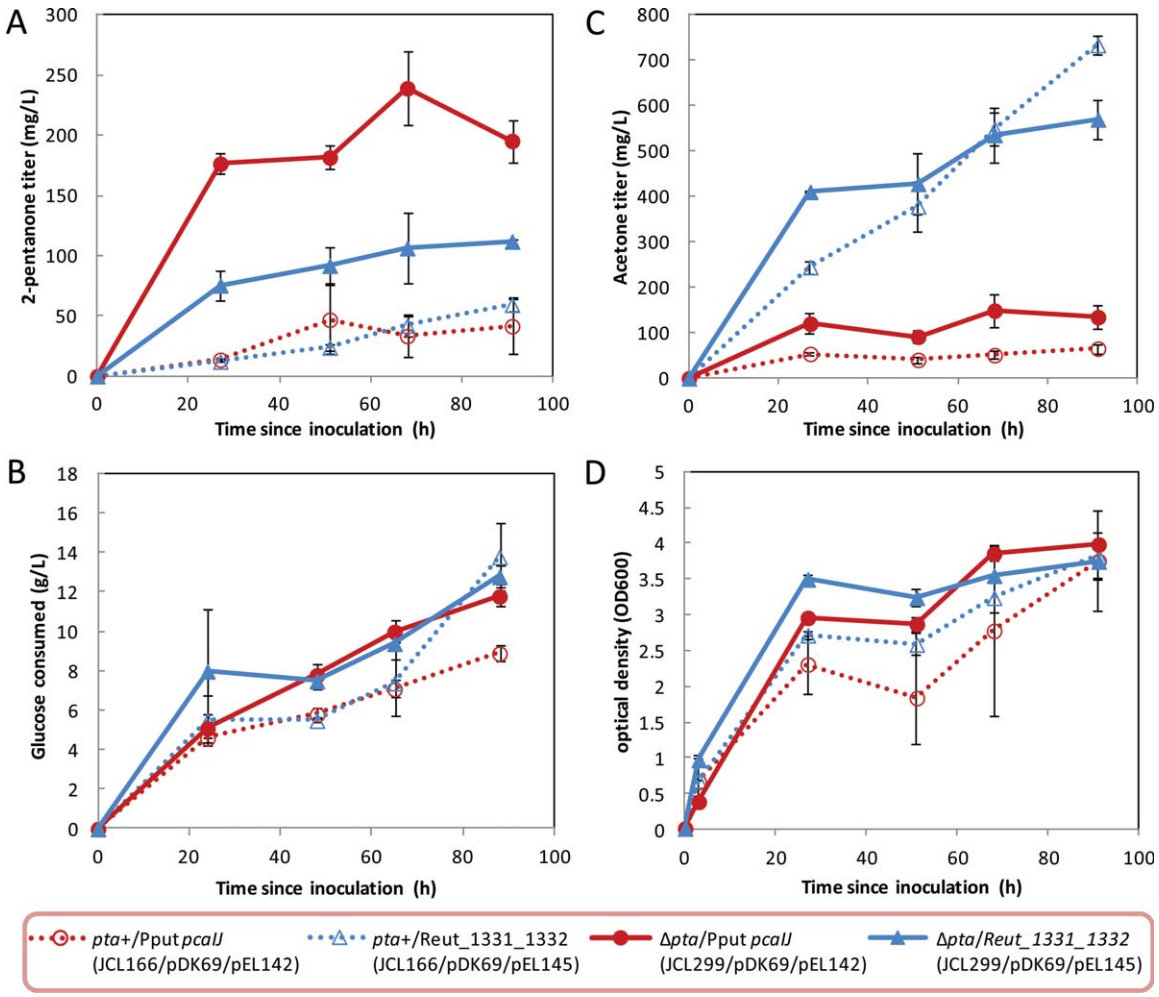

Figure 5. Time course for production of (A) 2-pentanone and (B) acetone, (C) glucose consumed, (D) cell density. *pta*+ and Δ*pta* represent the presence and absence of pta on the chromosome, respectively.

[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

ketoadipate:succinyl-CoA transferase[35] involved in the degradation of benzoate and 4-hydroxybenzoate. It is likely that PcaIJ outperformed the other CoA transferases and thioesterases for 2-pentanone synthesis because of the similarity between its natural substrate 3-ketoadipate and 3-ketohexanoate, the precursor for 2-pentanone (Figure 1). It is also possible that a competition for carbon flux exists between carbon chain elongation and the synthesis of acetone. A less efficient enzyme for freeing the CoA from acetoacetyl-CoA may facilitate chain elongation, preferentially enabling the synthesis of 2-pentanone.

When compared to the fatty acid-dependent synthesis of methyl ketones, the ATP requirement of the 2-pentanone production pathway presented here is lower. The CoA-dependent chain elongation is more efficient in ATP conservation than fatty acid synthesis because it directly utilizes acetyl-CoA as carbon addition unit instead of having to activate acetyl-CoA into malonyl-CoA with ATP. Furthermore, CoA transferase conserves the chemical energy stored in thioester bond whereas the hydrolysis catalyzed by thioesterase does not.

With some notable exceptions,[5,39] minimizing ATP expenditure has been an important strategy for metabolic engineering, as increased ATP consumption from heterologous pathways may lead to adverse effects in the cell and reduce biomass formation. Thus, the pathway presented here may be particularly suitable for organisms where conserving ATP is beneficial and manipulating acetyl-coA driving force is possible.

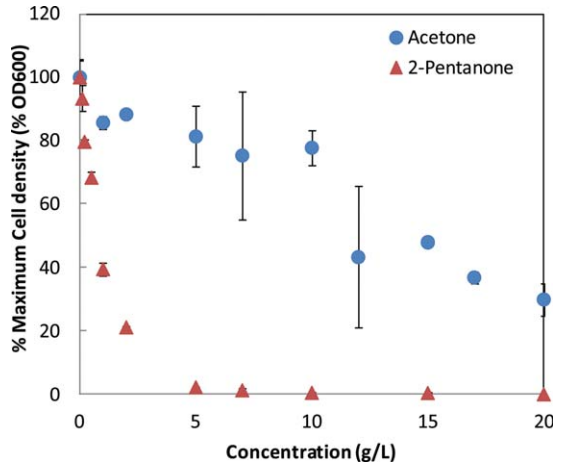

Figure 6. Toxicity level of 2-pentanone and acetone.

Cell tolerance for 2-pentanone is significantly lower than that of acetone. 600 mg/L of 2-pentanone inhibited 50% of growth. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Acknowledgments

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