

[Click here to view linked References](#)

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

Revised MICF-S-17-00363

# Engineering *E. coli* for simultaneous glucose-xylose utilization during methyl ketone production

Xi Wang<sup>1,2</sup>, Ee-Been Goh,<sup>1,2</sup> Harry R. Beller\*<sup>1,2,3</sup>

<sup>1</sup> Joint BioEnergy Institute (JBEI), 5885 Hollis St., Emeryville, CA 94608, USA

<sup>2</sup> Biological Systems & Engineering Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

<sup>3</sup> Earth & Environmental Sciences, Lawrence Berkeley National Laboratory, Berkeley, CA 94720, USA

**\*Corresponding author:**

Harry R. Beller  
Joint BioEnergy Institute  
5885 Hollis St.  
Emeryville, CA 94608

E-mail [HRBeller@lbl.gov](mailto:HRBeller@lbl.gov)

Phone (510) 486-7321

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1 **Abstract**

2 **Background:** We previously developed an *E. coli* strain that overproduces medium-chain  
3 methyl ketones for potential use as diesel fuel blending agents or as flavors and fragrances. To  
4 date, the strain's performance has been optimized during growth with glucose. However,  
5 lignocellulosic biomass hydrolysates also contain a substantial portion of hemicellulose-derived  
6 xylose, which is typically the second most abundant sugar after glucose. Commercialization of  
7 the methyl ketone-producing technology would benefit from the increased efficiency resulting  
8 from simultaneous, rather than the native sequential (diauxic), utilization of glucose and xylose.

9 **Results:** In this study, genetic manipulations were performed to alleviate carbon catabolite  
10 repression in our most efficient methyl ketone-producing strain. A strain engineered for  
11 constitutive expression of *xylF* and *xylA* (involved in xylose transport and metabolism) showed  
12 synchronized glucose and xylose consumption rates. However, this newly acquired capability  
13 came at the expense of methyl ketone titer, which decreased 5-fold. Further efforts were made to  
14 improve methyl ketone production in this strain, and we found that two strategies were effective  
15 at enhancing methyl ketone titer: (1) chromosomal deletion of *pgi* (glucose-6-phosphate  
16 isomerase) to increase intracellular NADPH supply and (2) downregulation of CRP (cAMP  
17 receptor protein) expression by replacement of the native RBS with an RBS chosen based upon  
18 mutant library screening results. Combining these strategies resulted in the most favorable  
19 overall phenotypes for simultaneous glucose-xylose consumption without compromising methyl  
20 ketone titer at both 1% and 2% total sugar concentrations in shake flasks.

21 **Conclusions:** This work demonstrated a strategy for engineering simultaneous utilization of C<sub>6</sub>  
22 and C<sub>5</sub> sugars in *E. coli* without sacrificing production of fatty acid-derived compounds.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

24 **Keywords**

25 Carbon catabolite repression; methyl ketones; NADPH; cAMP receptor protein; metabolic  
26 engineering

28 **Background**

29 Medium-chain (e.g., C<sub>11</sub> to C<sub>15</sub>) methyl ketones are among the fatty acid-derived compounds that  
30 have been developed recently for potential application as diesel blending agents [1-5]. A methyl  
31 ketone biosynthetic pathway in *Escherichia coli* that has attained 40% of maximum theoretical  
32 yield with glucose as the sole carbon source includes the following features: (a) overproduction  
33 of  $\beta$ -ketoacyl-coenzyme A (CoA) thioesters achieved by modification of the  $\beta$ -oxidation  
34 pathway (*via* overexpression of native FadB and a heterologous acyl-CoA oxidase from  
35 *Micrococcus luteus*) and (b) overexpression of a native thioesterase (FadM) that is effective at  
36 hydrolyzing  $\beta$ -ketoacyl-CoA thioesters to  $\beta$ -keto acids, which are the immediate precursors of  
37 methyl ketones [2]. Performance of the methyl ketone-producing strain for this technology has  
38 thus far been optimized during growth with glucose [2], which is typically the dominant sugar in  
39 biomass hydrolysates. However, biomass hydrolysates also contain a substantial portion of  
40 hemicellulose-derived xylose (typically, the second most abundant sugar after glucose).  
41 Commercialization of the methyl ketone-producing technology would benefit from the increased  
42 efficiency resulting from simultaneous utilization of glucose and xylose [6].

43 A challenge in cultivating *E. coli* in growth medium containing both glucose and xylose  
44 is diauxic (phased, non-simultaneous) growth, whereby glucose must be depleted before other  
45 sugars, such as xylose, can be metabolized [7]. The underlying mechanism for diauxic growth is  
46 carbon catabolite repression (CCR), which is primarily mediated by components of the

1  
2  
3  
4 47 phosphoenolpyruvate (PEP): carbohydrate phosphotransferase (PTS) system. The glucose-  
5  
6 48 specific EII complex of the PTS system consists of the permease, EIIBC<sup>Glc</sup> (encoded by *ptsG*),  
7  
8  
9 49 and EIIA<sup>Glc</sup> (encoded by *crr*), which has a primary role in modulating carbohydrate metabolism  
10  
11  
12 50 in *E. coli*. During glucose transport, EIIA<sup>Glc</sup> is dephosphorylated, which prevents either the  
13  
14 51 import of non-glucose sugars or their subsequent metabolism, and as a consequence, bacterial  
15  
16 52 cells are devoid of the inducer for the corresponding operons; this is known as inducer exclusion  
17  
18  
19 53 [8]. One consequence of dephosphorylated EIIA<sup>Glc</sup> is a decrease in levels of cyclic AMP  
20  
21 54 (cAMP), which is produced from ATP by adenylate cyclase (activated by phosphorylated  
22  
23 55 EIIA<sup>Glc</sup>). Lower levels of cAMP in turn limit the availability of cAMP–CRP, the complex  
24  
25  
26 56 between cAMP and CRP (cAMP receptor protein). The expression of genes that are involved in  
27  
28  
29 57 the catabolism of sugars other than glucose generally requires the cAMP–CRP complex and,  
30  
31 58 consequently, is repressed in the presence of glucose. In addition, the arabinose transcriptional  
32  
33 59 regulator (AraC) suppresses the xylose-catabolism genes *xylAB* and *xylFGH* by inhibiting the  
34  
35  
36 60 xylose transcriptional activator (XylR), which constitutes the second layer of CCR [9].  
37

38 61 Multiple strategies have been proposed for engineering simultaneous hexose-pentose  
39  
40 62 metabolism in *E. coli* by mitigating CCR, such as inactivation of *ptsG*, mutation of regulatory  
41  
42  
43 63 genes, and constitutive expression of key genes in pentose metabolism [10-16]. However, few  
44  
45  
46 64 studies have applied such strategies for mitigating CCR to production of fatty acid-based  
47  
48 65 biofuels [17]. Challenges can be anticipated in combining metabolic strategies for simultaneous  
49  
50  
51 66 glucose-xylose utilization and methyl ketone overproduction, as changes in central carbon  
52  
53 67 metabolism can substantially alter redox balance, which is needed for efficient conversion of  
54  
55  
56 68 carbon to targeted products [18].  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 69 In this study, we investigated the effects of engineering CCR mitigation into our best  
5  
6 70 methyl ketone-overproducing *E. coli* strain (EGS1895 [2]). We chose to follow the CCR  
7  
8  
9 71 mitigation strategy recently described by Kim and co-workers [12], which was reported to offer  
10  
11 72 advantages over other approaches, most notably, the engineered strains grow well on glucose,  
12  
13  
14 73 unlike some CCR-insensitive mutants defective in the glucose PTS system. Several rounds of  
15  
16 74 engineering were required to optimize both (1) simultaneous glucose-xylose co-utilization and  
17  
18  
19 75 (2) methyl ketone production, as strategies targeting one of these outcomes often adversely  
20  
21 76 affected the other. Ultimately, our results suggest the feasibility of engineering simultaneous  
22  
23 77 utilization of glucose and xylose in *E. coli* along with substantial production of fatty acid-derived  
24  
25  
26 78 biofuels.

## 27 28 79 **Methods**

### 29 30 31 80 **Strains, plasmids and reagents**

32  
33 81 *E. coli* strains and plasmids are listed in Table 1. Strains and plasmids along with their  
34  
35  
36 82 associated information (annotated GenBank-format sequence files) have been deposited in the  
37  
38 83 public version of the JBEI Registry (<https://public-registry.jbei.org>; entries JPUB\_XXX to  
39  
40  
41 84 JPUB\_XXX; Note: these will be added upon publication) and are physically available from the  
42  
43 85 authors and/or addgene (<http://www.addgene.org>) upon request. Our previously developed  
44  
45  
46 86 methyl ketone-overproducing strain EGS1895 [2] was used as the control strain. Q5 High-  
47  
48 87 Fidelity DNA Polymerase was used for all PCR reactions (New England Biolabs, Ipswich, MA).  
49  
50  
51 88 NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA) was used to  
52  
53 89 assemble linear DNA fragments. Plasmid extractions were carried out by using QIAGEN  
54  
55  
56 90 miniprep kits (Valencia, CA). Oligonucleotide primers were synthesized by Integrated DNA  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 91 Technologies, Inc. (San Diego, CA). DNA sequencing was completed by GENEWIZ (South  
5  
6 92 Plainfield, NJ).

### 9 93 **Genetic manipulations and strain development**

10  
11 94 All genome engineering was conducted by using the  $\lambda$ -Red recombination system with  
12  
13  
14 95 vectors pKD13, pKD46, and pCP20 [19, 20]. All primers used in this study are listed in Table  
15  
16 96 S1. CCR mitigation strategies developed by Kim and co-workers [12] (Table 2) were used to  
17  
18  
19 97 engineer the simultaneous utilization of glucose and xylose in strain EGS1895. Specifically, the  
20  
21 98 synthetic constitutive promoters CP6 and CP25 [21] were used to replace native promoters for  
22  
23  
24 99 key genes in pentose transport (*araFGH* and *xyIFGH*) and catabolism (*araBAD* and *xyLAB*),  
25  
26 100 respectively. The arabinose transcription factor *araC* was deleted from the chromosome.  
27  
28  
29 101 Additionally, the arabinose-proton symporter *araE* was inactivated and a point mutation was  
30  
31 102 introduced in the 5'-flanking region of *xylA* (*xylA<sup>up</sup>*). We also deleted *araC* from the methyl  
32  
33  
34 103 ketone-pathway plasmid pEG1675 [2]. By inserting an *araC*-free fragment between SpeI and  
35  
36 104 AgeI restriction sites of pEG1675, a new plasmid, pXW1677, was created. The resultant strain  
37  
38 105 with pXW1677 was named XW1044 (Tables 1 and 2), and the two intermediate strains were  
39  
40  
41 106 named XW1014 and XW1024 (Tables 1 and 2). For constructing the pXW1678 plasmid, the *E.*  
42  
43 107 *coli* DH1 native *maeB* gene was cloned and inserted downstream of *fadM* at an SalI restriction  
44  
45  
46 108 site on pEG1675.

47  
48 109 Studies were conducted to modulate and optimize the expression of the CRP by testing a  
49  
50  
51 110 set of *crp* ribosomal binding sites (RBS). To mutate the RBS for CRP, the RBS Library  
52  
53 111 Calculator [22] was used to design RBS mutant library sequences and  $\lambda$ -Red recombineering  
54  
55  
56 112 was used to integrate the library mutants into the host genome. For RBS calculations, the  $\lambda$ -Red-  
57  
58 113 generated 81-bp scar sequence (5'-

1  
2  
3  
4 114 ATTCCGGGGATCCGTCGACCTGCAGTTCGAAGTTCCTATTCTCTAGAAAGTATAGGA  
5  
6 115 ACTTCGAAGCAGCTCCAGCCTACA-3') was used as the pre-sequence. Based on the native  
7  
8  
9 116 35-bp RBS sequence for *crp* (5'-CTCTGGAGAAAGCTTATAACAGAGGATAACCGCGC-  
10  
11  
12 117 3'), a degenerate RBS sequence (5'-CTHTGGTGAAAGCTTATAACTGAGGMRAACCGCGT-  
13  
14 118 3') was generated with a broad range of predicted translation initiation rates (TIR) for a total 12  
15  
16 119 variant sequences. RBS sequences and their predicted TIR values are listed in Table S2. For  
17  
18  
19 120 reference, the native *crp* RBS sequence in *E. coli* DH1 was predicted to have a TIR value of  
20  
21 121 2441 au based on the RBS Calculator [23, 24].  
22  
23  
24 122  
25  
26 123  
27  
28  
29 124  
30  
31 125  
32  
33 126  
34  
35  
36 127  
37  
38 128  
39  
40  
41 129  
42  
43 130  
44  
45  
46 131  
47  
48 132  
49  
50  
51 133  
52  
53 134  
54  
55 135  
56  
57  
58 136  
59  
60  
61  
62  
63  
64  
65

137 **Table 1** Strains and plasmids used in this study

Strains	Relevant characteristics	Source or Reference
EGS1405	<i>E. coli</i> DH1; $\Delta fadE$ ; $\Delta ackA-pta$ ; $\Delta poxB$	[2]
EGS1895	EGS1405 with pEG1675	[2]
XW1003	<i>E. coli</i> DH1; $\Delta fadE$ ; $\Delta ackA-pta$ ; $\Delta poxB$ ; $\Delta ptsG$	This study
XW1004	XW1003 with pEG1675	This study
XW1013	<i>E. coli</i> DH1; $\Delta fadE$ ; $\Delta ackA-pta$ ; $\Delta poxB$ ; P <sub>CP6-<i>xylF</i></sub> ; P <sub>CP25-<i>xylA</i></sub> ; <i>xylA</i> <sup>up</sup>	This study
XW1014	XW1013 with pEG1675	This study
XW1018	XW1013 with pXW1678	This study
XW1023	<i>E. coli</i> DH1; $\Delta fadE$ ; $\Delta ackA-pta$ ; $\Delta poxB$ ; P <sub>CP6-<i>xylF</i></sub> ; P <sub>CP25-<i>xylA</i></sub> ; <i>xylA</i> <sup>up</sup> ; $\Delta araE$	This study
XW1024	XW1023 with pEG1675	This study
XW1043	<i>E. coli</i> DH1; $\Delta fadE$ ; $\Delta ackA-pta$ ; $\Delta poxB$ ; P <sub>CP6-<i>xylF</i></sub> ; P <sub>CP25-<i>xylA</i></sub> ; <i>xylA</i> <sup>up</sup> ; $\Delta araE$ ; P <sub>CP25-<i>araB</i></sub> ; $\Delta araC$ ; P <sub>CP6-<i>araF</i></sub>	This study
XW1044	XW1043 with pXW1677	This study
XW1053	<i>E. coli</i> DH1; $\Delta fadE$ ; $\Delta ackA-pta$ ; $\Delta poxB$ ; P <sub>CP6-<i>xylF</i></sub> ; P <sub>CP25-<i>xylA</i></sub> ; <i>xylA</i> <sup>up</sup> ; $\Delta pgi$	This study
XW1054	XW1053 with pEG1675	This study
XW1055	XW1053 with pXW1678	This study
XW1063	<i>E. coli</i> DH1; $\Delta fadE$ ; $\Delta ackA-pta$ ; $\Delta poxB$ ; P <sub>CP6-<i>xylF</i></sub> ; P <sub>CP25-<i>xylA</i></sub> ; <i>xylA</i> <sup>up</sup> ; <i>crp</i> -RBS (TIR=13)	This study
XW1064	XW1063 with pEG1675	This study
XW1073	<i>E. coli</i> DH1; $\Delta fadE$ ; $\Delta ackA-pta$ ; $\Delta poxB$ ; P <sub>CP6-<i>xylF</i></sub> ; P <sub>CP25-<i>xylA</i></sub> ; <i>xylA</i> <sup>up</sup> ; <i>crp</i> -RBS (TIR=13); $\Delta pgi$	This study
XW1074	XW1073 with pEG1675	This study
XW1075	XW1073 with pXW1678	This study
Plasmids	Relevant characteristics	Source or Reference
pKD13	$\lambda$ -Red recombineering plasmid	[19]
pKD46	$\lambda$ -Red recombineering plasmid	[19]
pCP20	$\lambda$ -Red recombineering plasmid	[19]
pEG1675	Km <sup>r</sup> ; <i>araC</i> -P <sub>BAD-<i>fadR-co_fadD</i></sub> ; P <sub>trc-<i>fadM</i></sub> ; P <sub>lacUV5-</sub> ' <i>tesA-fadB-co_aco</i>	[2]
pXW1677	pEG1675 with <i>araC</i> deleted	This study
pXW1678	Km <sup>r</sup> ; <i>araC</i> -P <sub>BAD-<i>fadR-co_fadD</i></sub> ; P <sub>trc-<i>fadM-maeB</i></sub> ; P <sub>lacUV5-</sub> ' <i>tesA-fadB-co_aco</i>	This study



1  
2  
3  
4 143 **Table 2** Strategies used for engineering simultaneous glucose-xylose utilization in EGS1895 <sup>a</sup>

No.	Manipulation	Target sequence	XW1014	XW1024	XW1044
1	Replace promoter of <i>xylFGH</i>	CP6 synthetic promoter	+ <sup>b</sup>	+	+
2	Replace promoter of <i>xylAB</i>	CP25 synthetic promoter	+	+	+
3	<i>xylA<sup>up</sup></i> mutation	G (+6) → T of CP25	+	+	+
4	<i>araE</i> inactivation	Truncated <i>araE</i>		+	+
5	Replace promoter of <i>araFGH</i>	CP6 synthetic promoter			+
6	Replace promoter of <i>araBAD</i>	CP25 synthetic promoter			+
7	Chromosomal deletion of <i>araC</i>	$\Delta araC$ (genome)			+
8	<i>araC</i> deletion from pEG1675	$\Delta araC$ (plasmid)			+

22 144 <sup>a</sup> Based on [12].

23 145 <sup>b</sup> + indicates that the specified genetic modification is present in this strain.

24 146  
25  
26 147 **Media and culture conditions**

29 148 Lysogeny broth (LB) was used for routine cell growth and propagation. Kanamycin was  
30  
31 149 added to the growth medium at a final concentration of 50  $\mu\text{g}\cdot\text{mL}^{-1}$ , when required. M9-MOPS  
32  
33 150 minimal medium with 1% total sugars (5  $\text{g}\cdot\text{L}^{-1}$  glucose and 5  $\text{g}\cdot\text{L}^{-1}$  xylose) or 2% total sugars (10  
34  
35 151  $\text{g}\cdot\text{L}^{-1}$  glucose and 10  $\text{g}\cdot\text{L}^{-1}$  xylose) as carbon sources was used for production experiments. The  
36  
37 152 composition of M9-MOPS minimal medium followed the recipe previously described [2]. For  
38  
39 153 production experiments, strains were first adapted in M9-MOPS minimal medium with 1% total  
40  
41 154 sugars for 3 passages before being inoculated into production medium with glucose and xylose.  
42  
43  
44

45 155 **Analysis of cell growth and sugar metabolism**

46 156 Cell growth was monitored by measuring optical density at 600 nm ( $\text{OD}_{600}$ ). Sugars were  
47  
48 157 measured with an Agilent 1100 Series HPLC system, equipped with an Agilent 1200 Series  
49  
50 158 refractive index detector (RID) (Agilent Technologies, CA) and Aminex HPX-87H ion-  
51  
52 159 exclusion column (300-mm length, 7.8-mm internal diameter; Bio-Rad Laboratories, Inc.,  
53  
54 160 Hercules, CA). The column temperature was 50°C, and 4 mM sulfuric acid was used as the  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 161 mobile phase with a flow rate of 0.6 mL·min<sup>-1</sup> for 24 min. The quantification of glucose and  
5  
6 162 xylose was conducted by external standard calibration with authentic standards.  
7  
8

### 9 163 **Production and analysis of methyl ketones**

10  
11 164 For methyl ketone production, strains were inoculated into 50 mL M9-MOPS minimal  
12  
13  
14 165 medium with 1% total sugars, or 25 mL with 2% total sugars, in 250-mL shake flasks and  
15  
16 166 cultured at 37°C with 200-rpm agitation. The starting OD<sub>600</sub> during production was ca. 0.01.  
17  
18  
19 167 Gene expression was induced by adding 0.2 mM IPTG and 1 mM arabinose after 6 h of growth.  
20  
21 168 Five mL of decane (Reagent-Plus ≥ 99% purity, Sigma-Aldrich, St. Louis, MO) amended with  
22  
23  
24 169 perdeuterated tetracosane (C<sub>24</sub>D<sub>50</sub>) and 3-tetradecanone (Sigma-Aldrich, St. Louis, MO) as  
25  
26 170 internal standards was also added to the cultures during induction. The decane overlay was  
27  
28  
29 171 sampled for the measurement of methyl ketones; analysis by electron ionization gas  
30  
31 172 chromatography-mass spectrometry (GC-MS) was conducted as previously described [3].  
32

### 33 173 **Batch fermentation of strains EGS1895 and XW1075 in a 2-L bioreactor for methyl ketone** 34 35 36 174 **production**

37  
38 175 Batch fermentation was carried out in a 2-L bioreactor equipped with a Sartorius  
39  
40  
41 176 BIOSTAT B plus control unit for regulating dissolved oxygen (DO), pH, and temperature.  
42  
43 177 Frozen glycerol stocks of M9-MOPS-adapted cells were used to seed a test tube containing 5 mL  
44  
45  
46 178 of M9-MOPS minimal medium with 1% total sugars (5 g·L<sup>-1</sup> glucose and 5 g·L<sup>-1</sup> xylose) as  
47  
48 179 previously described. After 30 h of growth, the cultures were diluted 1:250 into a 250-mL flat-  
49  
50  
51 180 bottom shake flask containing 50 mL of M9-MOPS minimal medium supplemented with 1%  
52  
53 181 total sugars. This culture was grown for another 30 h as before and was used to inoculate 1.25 L  
54  
55 182 of medium in the bioreactor. The medium was adapted from Korz *et. al.* [25] and was composed  
56  
57  
58 183 of M9 salts (6.8 g·L<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>, 3.0 g·L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 1.0 g·L<sup>-1</sup> NH<sub>4</sub>Cl, 0.5 g·L<sup>-1</sup> NaCl)  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 184 supplemented with 0.5 g·L<sup>-1</sup> of MgSO<sub>4</sub>·7H<sub>2</sub>O, 0.18 g·L<sup>-1</sup> of NH<sub>4</sub>Cl, 1.0 mg·L<sup>-1</sup> thiamine, 10 nM  
5  
6 185 of FeSO<sub>4</sub>·7H<sub>2</sub>O, 100 μM CaCl<sub>2</sub>·2H<sub>2</sub>O, micronutrients as described previously [2], 10 g·L<sup>-1</sup> of  
7  
8  
9 186 glucose, 10 g·L<sup>-1</sup> of xylose, and 50 μg·mL<sup>-1</sup> of kanamycin. The temperature of the bioreactor  
10  
11  
12 187 was maintained at 37°C throughout the fermentation and the culture was maintained at pH 6.5  
13  
14 188 automatically by the addition of a 10 M potassium hydroxide solution. The initial stir rate and  
15  
16 189 airflow were set at 200 rpm and 1 VVM (volume of air per volume of liquid per minute),  
17  
18  
19 190 respectively. Dissolved oxygen was maintained above 40% of saturation *via* cascade control of  
20  
21 191 adjustment of stirrer speed (up to 1600 rpm), followed by air-flow rate (up to 2.0 VVM).  
22  
23  
24 192 Cultures were induced with 1 mM arabinose and 0.5 mM IPTG at 6 h after initiation of batch  
25  
26 193 phase. In addition, 150 mL of dodecane (Sigma-Aldrich, ReagentPlus ≥ 99% purity) amended  
27  
28  
29 194 with 3 mg·mL<sup>-1</sup> of 3-tetradecanone (Sigma-Aldrich) as an internal standard, was added into the  
30  
31 195 bioreactors.

32  
33  
34 196 At selected times, 10- to 15-mL samples were removed from the bioreactors *via* a syringe  
35  
36 197 affixed to the sampling tube while the stirrer was still operating. Approximately 50 μL of cell  
37  
38  
39 198 cultures were filtered through a 0.2-μm syringe membrane filter directly into a vial for HPLC  
40  
41 199 analysis and the rest of the cultures transferred to a 15-mL Falcon tube. After allowing the  
42  
43 200 samples to sit in the 15-mL tube for 1 min, the supernatant dodecane overlay was pipetted out  
44  
45  
46 201 into a 2-mL microcentrifuge tube and centrifuged at 21,130 x *g* for 10 min to obtain a better-  
47  
48 202 resolved aqueous-organic interface. The dodecane overlay was transferred into a glass vial and  
49  
50  
51 203 stored at 4°C until GC-MS analysis.

52  
53 204  
54  
55 205  
56  
57  
58 206  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 **207 Results and discussion**

5  
6 **208 Engineering simultaneous glucose-xylose utilization in methyl ketone-overproducing strain**

7  
8  
9 **209 EGS1895**

10  
11 We engineered several strains by manipulating key genes in pentose metabolism  
12  
13 (XW1014, XW1024, and XW1044; Tables 1 and 2) and evaluated their ability to simultaneously  
14 **211** utilize glucose and xylose (Fig. 1). The control strain, EGS1895, presented a typical diauxic  
15  
16 **212** pattern in which xylose utilization began after glucose was fully depleted. In contrast, newly  
17  
18 **213** engineered strains displayed glucose-xylose co-utilization to varying degrees rather than a strict  
19  
20 **214** diauxic profile. Among these engineered strains, XW1014 (with constitutive expression of *xyIA*  
21  
22 **215** and *xyIF* plus a point mutation in the *xyIA* promoter, *xyIA<sup>up</sup>*) showed the best performance for  
23  
24 **216** simultaneous utilization of glucose and xylose (Fig. 1). This strain had identical consumption  
25  
26 **217** rates for glucose and xylose at 1% sugar conditions, while a slight decrease in xylose  
27  
28 **218** consumption was observed at higher sugar concentration (2%). The inactivation of *araE*  
29  
30 **219** (XW1024; Tables 1 and 2) did not result in better sugar co-utilization than was observed for  
31  
32 **220** strain XW1014, nor did the manipulations made for strain XW1044 (alleviating AraC-mediated  
33  
34 **221** repression through four collective *araC*-related manipulations, including *araC* deletion from  
35  
36 **222** both the genome and plasmid as well as replacement of promoters for *araB* and *araF*). Although  
37  
38 **223** both strains XW1024 and XW1044 showed favorable simultaneous consumption rates of glucose  
39  
40 **224** and xylose at 1% sugar conditions, their xylose consumption dramatically decreased at higher  
41  
42 **225** sugar concentration (2%).  
43  
44 **226**

45  
46  
47  
48  
49  
50  
51  
52  
53 **227** In addition, because *ptsG* deficiency is a well-studied mechanism for mitigating CCR in  
54  
55 **228** *E. coli* [10], the glucose transporter EIIBC<sup>Glc</sup> encoded by *ptsG* was deleted from EGS1895 to  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 229 investigate the effect on sugar co-utilization (strain XW1004; Table 1). Strain XW1004 did not  
5  
6  
7 230 display a better sugar co-utilization profile than strain XW1014 (Fig. 1).

8  
9 231 Methyl ketone production was also investigated among these strains engineered for  
10  
11 232 hexose-pentose co-utilization. Compared with the titer of the control strain EGS1895 (~690  
12  
13  
14 233 mg·L<sup>-1</sup>), methyl ketone production was significantly reduced in all four modified strains (Fig. 2).  
15  
16 234 The best performing strain for sugar co-utilization, XW1014, only produced ~140 mg·L<sup>-1</sup> total  
17  
18  
19 235 methyl ketones (1% total sugars), which is approximately 5-fold lower than for strain EGS1895.  
20  
21 236 Strains with more genetic manipulations produced even lower methyl ketone titers; for example,  
22  
23  
24 237 strains XW1024 and XW1044 produced <60 mg·L<sup>-1</sup> methyl ketones. Although the  $\Delta ptsG$  strain  
25  
26 238 (XW1004) showed the highest methyl ketone titer among these four strains, its diminished  
27  
28  
29 239 glucose utilization was not optimal and it was not pursued further. Despite its relatively low  
30  
31 240 methyl ketone titer, strain XW1014 had the most favorable combination of sugar co-utilization  
32  
33  
34 241 and methyl ketone production of the strains tested.

35  
36 242 **Optimization of methyl ketone production in strain XW1014 by enhancing NADPH**  
37  
38 243 **availability**

39  
40  
41 244 Although strain XW1014 was successfully engineered for simultaneous glucose-xylose  
42  
43 245 consumption, the significantly reduced methyl ketone titer in this strain necessitated further  
44  
45  
46 246 engineering to improve commercial relevance. We hypothesized that enhancing NADPH  
47  
48 247 availability could be a fruitful engineering target because (1) the biosynthesis of fatty acids  
49  
50  
51 248 (methyl ketone precursors) in *E. coli* is an NADPH-demanding process and (2) xylose  
52  
53 249 metabolism, particularly when simultaneous with glucose metabolism, could disrupt NADPH  
54  
55  
56 250 production in a host cell (e.g., strain XW1014) compared to conditions with glucose as a sole  
57  
58 251 carbon source. Fatty acid biosynthesis results in net consumption of NADPH due to demand  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4 252 from two key reductases – FabG ( $\beta$ -ketoacyl-ACP reductase) and potentially, FabI (enoyl-ACP  
5  
6 253 reductase), which can utilize either NADH or NADPH as a cofactor [1, 26]. To illustrate the  
7  
8  
9 254 substantial NADPH demands of fatty acid/methyl ketone biosynthesis, production of 1 mol of a  
10  
11 255 C<sub>13</sub> methyl ketone (2-tridecanone) from glucose using the relevant metabolic pathway [2] would  
12  
13  
14 256 result in net consumption of 6 (or 12) mol of NADPH and net production of 9 (or 15) mol of  
15  
16 257 NADH, depending on FabI cofactor usage.

18  
19 258 By virtue of where xylose enters central carbon metabolism in *E. coli*, xylose metabolism  
20  
21 259 tends to result in less flux than glucose metabolism through the oxidative, NADPH-generating  
22  
23  
24 260 steps of the pentose phosphate pathway (PPP), namely reactions catalyzed by glucose-6-  
25  
26 261 phosphate dehydrogenase (Zwf) and phosphogluconate dehydrogenase (Gnd); however, xylose  
27  
28  
29 262 metabolism can take advantage of other sources of NADPH, such as malic enzyme and  
30  
31 263 transhydrogenase [27]. The situation is likely more complex when considering sugar utilization  
32  
33  
34 264 and NADPH production in strain XW1014 compared to that in control strain EGS1895.  
35  
36 265 Compared with the sequential metabolism from glucose to xylose during diauxic growth (strain  
37  
38 266 EGS1895), simultaneous metabolism of glucose and xylose (strain XW1014) could alter  
39  
40  
41 267 NADPH production by re-distributing flux between glycolysis and the PPP. For example, it is  
42  
43 268 possible that the flux of glucose carbon through the oxidative PPP might be reduced when xylose  
44  
45  
46 269 co-utilization is occurring, because xylose metabolism will satisfy the cell's needs for  
47  
48 270 downstream PPP metabolites required for anabolism, such as erythrose 4-phosphate (needed for  
49  
50  
51 271 aromatic amino acid biosynthesis) and ribose 5-phosphate (needed for nucleic acid biosynthesis).

52  
53 272 We implemented two strategies for increasing NADPH supply in strain XW1014: (1)  
54  
55 273 deleting *pgi* (glucose-6-phosphate isomerase) from the chromosome to divert flux from  
56  
57  
58 274 glycolysis through the oxidative PPP (Fig. 4) and (2) overexpressing *maeB* (malic enzyme),  
59  
60  
61  
62  
63  
64  
65

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

275 which leads to NADPH generation by oxidative decarboxylation of malate to pyruvate (Fig. 4).  
276 <sup>13</sup>C Metabolic flux analysis studies in *E. coli* have shown that *pgi* deletion results in substantial  
277 production of NADPH by diversion of flux from glycolysis through the oxidative PPP, and that  
278 excessive accumulation of NADPH (cofactor imbalance) in  $\Delta pgi$  strains can be at least partially  
279 ameliorated by NADPH consumption through transhydrogenase [28, 29]. In our  $\Delta pgi$  strain  
280 (XW1054; Table 1), it was anticipated that a portion of the NADPH made available by the *pgi*  
281 deletion might facilitate fatty acid/methyl ketone biosynthesis by better satisfying its high  
282 NADPH demands than did central carbon metabolism in strain XW1014.

283 Production results showed that the  $\Delta pgi$  strain (XW1054) had dramatically improved  
284 methyl ketone titer (850 mg·L<sup>-1</sup>) relative to strain XW1014 after 96 h at 1% total sugar  
285 conditions (Fig. 3); this methyl ketone titer was comparable to that of the control strain  
286 (EGS1895). Under 2% total sugar conditions, the methyl ketone titer of strain XW1054 (~1300  
287 mg·L<sup>-1</sup> after 96 h) was also comparable to that of strain EGS1895 (~1600 mg·L<sup>-1</sup>). However,  
288 xylose showed a slower consumption rate than glucose after *pgi* was deleted, and slower cell  
289 growth was also observed during production. In contrast to methyl ketone titer improvement for  
290 strain XW1054, the overexpression of *maeB* with or without *pgi* deletion (strains XW1055 and  
291 XW1018; Table 1) did not result in improvement in methyl ketone production (Fig. S1).

292 Based upon the results for strain XW1054 ( $\Delta pgi$ ), it is possible that NADPH is more limiting  
293 when xylose is used as a carbon source. Indeed, we observed that the control strain (EGS1895)  
294 produced very low methyl ketone titers when xylose was used as the sole carbon source in  
295 minimal medium (Fig. S2).

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

#### **Optimization of methyl ketone production in strain XW1014 by mutating the RBS of *crp***

While enhancing potential NADPH supply (*via pgi* deletion) substantially improved methyl ketone production with mixed glucose-xylose medium, several lines of evidence suggested that the engineered strains were experiencing suboptimal sugar utilization (e.g., strain XW1054 in Fig. 3), and potentially, suboptimal methyl ketone production, that had causes beyond NADPH limitation. For example, NADPH limitation alone does not seem to explain the dramatic reduction in methyl ketone titer in both strain XW1004 ( $\Delta ptsG$ ) and strain XW1014 (introduced constitutive promoters to *xylA* and *xylF*) (Fig. 2), as these genetic modifications are not clearly linked to NADPH supply.

A possible explanation for these results is changes in intracellular distributions of the global regulator CRP. For strain XW1014, promoter replacement for *xylA* and *xylF* resulted in removal of a CRP binding site from the intergenic region between *xylA* and *xylF* [30]. As a global regulator, CRP not only plays an important role in carbon catabolite repression, but also controls the transcription of more than 100 genes in *E. coli*, such as key genes in fatty acid metabolism (e.g., *fadD*, *fadH*) [31] and in central carbon metabolism (e.g., *pgi*, *zwf*, *gnd*) [30, 32]. Thus, the promoter change in strain XW1014 might have altered the level of free CRP and directly and indirectly affected the transcription of many other genes related to fatty acid metabolism. Similarly, changes to intracellular CRP pools might also explain why methyl ketone production was reduced in the  $\Delta ptsG$  strain (XW1004): the absence of PtsG likely increased cAMP availability [33], and in turn, altered the level of free intracellular CRP, which interacts with cAMP to make the cAMP–CRP complex.

Based on this reasoning, one possible strategy for improving methyl ketone production is to optimize the expression level of CRP in strain XW1014. We attempted to modulate CRP



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

availability by replacing the native *crp* RBS with synthetic RBSs of varying strengths. We created a mutant *crp* RBS library with broad range of predicted TIR values (8 to 7291 au, Table S2). A total of 7 RBS variants with different TIRs were identified by sequencing from the mutant library. Screening of this library was conducted with 5-mL cultures in M9-MOPS medium (50-mL test tubes), and one mutant (strain XW1064) was selected that showed significant improvement in methyl ketone production (~900 mg·L<sup>-1</sup> after 96 h with 1% total sugars, Fig. S3). Notably, the predicted TIR of strain XW1064 was 13 au, which is approximately 188-fold lower than the predicted native TIR (2441 au) of *crp*. Scaled up production of strain XW1064 in 250-mL shake flasks resulted in methyl ketone titers up to ~450 mg·L<sup>-1</sup> without compromised cell growth (Fig. 3).

This result supported our hypothesis that optimized expression of CRP is able to improve methyl ketone production in the strains engineered for glucose-xylose co-utilization. However, we also noticed that the consumption rate of xylose in strain XW1064 was slower than that of glucose, especially under 2% total sugar conditions (Fig. 3).

**Seeking the best candidate by combining engineering strategies**

Given the complementary features of the above strategies ( $\Delta pgi$  and CRP downregulation) on cell growth and methyl ketone production, and the fact that they both effectively improved methyl ketone production in strain XW1014, we decided to combine these two strategies to obtain an additive effect. Overall, combining  $\Delta pgi$  and CRP downregulation (strain XW1074; Table 1) created superior phenotypes in cell growth and methyl ketone production compared to use of either strategy alone (Fig. 3). This strain produced up to 570 mg·L<sup>-1</sup> methyl ketones at 1% total sugar conditions, but reached a higher titer at 2% total sugars (~1600 mg·L<sup>-1</sup>) that was comparable to that of the control strain (EGS1895). Glucose and xylose were simultaneously

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

344 consumed by strain XW1074 (Fig. 3) after a lag period, but utilization of xylose was still slower  
345 than that of glucose. Surprisingly, the added *maeB* overexpression (strain XW1075) dramatically  
346 improved sugar co-utilization (albeit with the same lag period, likely caused by *pgi* deletion; [34,  
347 35]). As a result, strain XW1075 achieved synchronized consumption rates for glucose and  
348 xylose at both 1% and 2% total sugar conditions. Methyl ketone titers in strain XW1075 were up  
349 to 700 mg·L<sup>-1</sup> and 1100 mg·L<sup>-1</sup> at 1% and 2% total sugars, respectively. Thus, these two strains  
350 engineered with combined strategies (XW1074 and XW1075) represented a favorable phenotype  
351 displaying simultaneous utilization of glucose and xylose without substantially sacrificing  
352 methyl ketone production relative to the control strain (EGS1895) (Fig. 3, Fig. 5).

### 353 **Strain XW1075 performance during batch fermentation**

354 Strain XW1075 also compared favorably to control strain EGS1895 in batch fermentation  
355 mode. Glucose and xylose were utilized concurrently in strain XW1075 (albeit at unequal rates),  
356 whereas strain EGS1895 displayed a typical diauxic pattern, including sequential sugar  
357 utilization (Fig. 6). Correspondingly, strain XW1075 had a more consistent methyl ketone  
358 production yield (8.7% to 9.8%) than the control strain (6.9% to 10.0%). At 72 h, the methyl  
359 ketone titer of strain XW1075 was 2 g·L<sup>-1</sup>, which was ca. 33% higher than that of strain  
360 EGS1895 (1.5 g·L<sup>-1</sup>).

361 Comparison of the results in Fig. 6 with those of strains XW1075 and EGS1895 grown  
362 with pure glucose or xylose (Fig. S2) reveals that co-utilization of glucose and xylose in strain  
363 XW1075 enabled substantially better methyl ketone production than did utilization of either  
364 sugar alone. In fact, methyl ketone production was negligible for strain XW1075 utilizing either  
365 pure glucose or pure xylose (Fig. S2). Notably, strain EGS1895 also produced negligible methyl  
366 ketones when grown on pure xylose (Fig. S2), but produced substantial methyl ketones while

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

367 metabolizing xylose after diauxic depletion of glucose (Fig. 6). From Fig. 3 and 6, it appears that  
368 glucose metabolism supported both growth and methyl ketone production in strain EGS1895,  
369 whereas xylose metabolism supported methyl ketone production but little or no growth.

### 370 **Conclusions**

371 In this study, genetic manipulations were conducted to alleviate carbon catabolite repression in  
372 our most efficient methyl ketone-producing strain. A strain (XW1014) with constitutively  
373 expressed *xyIA* and *xyIF* plus a *xyIA* promoter mutation showed well-synchronized glucose and  
374 xylose consumption rates. However, this newly acquired capability came at the expense of  
375 methyl ketone titer, which decreased 5-fold. Further efforts were made to optimize methyl  
376 ketone production in this strain, and we found that chromosomal deletion of *pgi* (to enhance  
377 NADPH supply) and CRP downregulation by replacement of the native RBS both effectively  
378 improved methyl ketone production. Combining these strategies resulted in the most favorable  
379 overall phenotypes for simultaneous glucose-xylose consumption without compromising methyl  
380 ketone titer (Fig. 5 and 6).

### 381 **Abbreviations**

382 CCR, carbon catabolite repression; PEP, phosphoenolpyruvate; PTS, phosphotransferase; cAMP,  
383 cyclic AMP; CRP, cAMP receptor protein; PPP, pentose phosphate pathway; RBS, ribosomal  
384 binding site; TIR, translation initiation rate; MK, methyl ketones; CDW, cell dry weight.

### 385 **Declarations**

### 386 **Ethics approval and consent to participate**

387 Not applicable

### 388 **Consent for publication**

389 Not applicable

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

390

**391 Availability of data and materials**

392 All data generated or analyzed during this study are included in this published article and its  
393 supplementary information files.

**394 Competing interests**

395 The authors declare that they have no competing interests

**396 Funding**

397 This work was supported by U.S. Department of Energy (DOE) Phase I Small Business  
398 Technology Transfer (STTR) project DE-SC0015093. The portion of the work conducted at the  
399 Joint BioEnergy Institute (JBEI) was supported by the Office of Science, Office of Biological  
400 and Environmental Research, of the DOE under Contract No. DE-AC02-05CH11231.

**401 Authors' contributions**

402 HB and XW designed the experiments. XW developed strains and performed shake flask  
403 production. EG developed the bioreactor fermentation method and EG and XW performed the  
404 fermentation. XW, HB, EG analyzed the data. XW, HB, and EG wrote the manuscript.

**405 Acknowledgments**

406 We thank Jeremy Minty (Ecovia Renewables, Inc.), Sung Kuk Lee (Ulsan National Institute of  
407 Science and Technology, Korea), and Paul Opgenorth and Joonhoon Kim (Joint BioEnergy  
408 Institute) for valuable discussions.

409

410

411

412

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

**References**

413  
414 1. Beller HR, Lee TS, Katz L. Natural products as biofuels and bio-based chemicals: fatty  
415 acids and isoprenoids. *Nat. Prod. Rep.* 2015;32:1508–1526.

416 2. Goh E-B, Baidoo EEK, Burd H, Lee TS, Keasling JD, Beller HR. Substantial  
417 improvements in methyl ketone production in *E. coli* and insights on the pathway from *in*  
418 *vitro* studies. *Metab. Eng.* 2014;26:67–76.

419 3. Goh E-B, Baidoo EEK, Keasling JD, Beller HR. Engineering of bacterial methyl ketone  
420 synthesis for biofuels. *Appl. Environ. Microbiol.* 2012;78:70–80.

421 4. Lennen RM, Pfleger BF. Microbial production of fatty acid-derived fuels and chemicals.  
422 *Curr. Opin. Biotechnol.* 2013;24:1044–1053.

423 5. Park J, Rodríguez-Moyá M, Li M, Pichersky E, San K-Y, Gonzalez R. Synthesis of  
424 methyl ketones by metabolically engineered *Escherichia coli*. *J. Ind. Microbiol*  
425 *Biotechnol.* 2012;39:1703–1712.

426 6. Kim J-H, Block DE, Mills DA. Simultaneous consumption of pentose and hexose sugars:  
427 an optimal microbial phenotype for efficient fermentation of lignocellulosic biomass.  
428 *Appl. Microbiol. Biotechnol.* 2010;88:1077–1085.

429 7. Deutscher J. The mechanisms of carbon catabolite repression in bacteria. *Curr. Opin.*  
430 *Microbiol.* 2008;11:87–93.

431 8. Deutscher J, Francke C, Postma PW. How phosphotransferase system-related protein  
432 phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol. Mol. Biol.*  
433 *Rev.* 2006;70:939–1031.

434 9. Desai TA, Rao CV. Regulation of arabinose and xylose metabolism in *Escherichia coli*.  
435 *Appl. Environ. Microbiol.* 2010;76:1524–1532.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

436 10. Nichols N, Dien B, Bothast R. Use of catabolite repression mutants for fermentation of  
437 sugar mixtures to ethanol. *Appl. Microbiol. Biotechnol.* 2001;56:120–125.

438 11. Yao R, Hirose Y, Sarkar D, Nakahigashi K, Ye Q, Shimizu K. Catabolic regulation  
439 analysis of *Escherichia coli* and its *crp*, *mlc*, *mgsA*, *pgi* and *ptsG* mutants. *Microb. Cell*  
440 *Fact.* 2011;10:67.

441 12. Kim SM, Choi BY, Ryu YS, Jung SH, Park JM, Kim G-H, Lee SK. Simultaneous  
442 utilization of glucose and xylose *via* novel mechanisms in engineered *Escherichia coli*.  
443 *Metab. Eng.* 2015;30:141–148.

444 13. Xia T, Eiteman MA, Altman E. Simultaneous utilization of glucose, xylose and arabinose  
445 in the presence of acetate by a consortium of *Escherichia coli* strains. *Microb. Cell. Fact.*  
446 2012;11:77.

447 14. Chiang C-J, Lee HM, Guo HJ, Wang ZW, Lin L-J, Chao Y-P. Systematic approach to  
448 engineer *Escherichia coli* pathways for co-utilization of a glucose–xylose mixture. *J.*  
449 *Agric. Food Chem.* 2013;61:7583–7590.

450 15. Groff D, Benke PI, Batth TS, Bokinsky G, Petzold CJ, Adams PD, Keasling JD.  
451 Supplementation of intracellular XylR leads to coutilization of hemicellulose sugars.  
452 *Appl. Environ. Microbiol.* 2012;78:2221–2229.

453 16. Wu Y, Shen X, Yuan Q, Yan Y. Metabolic engineering strategies for co-utilization of  
454 carbon sources in microbes. *Bioeng.* 2016;3:10.

455 17. San K-Y, Li M, Zhang X. Bacteria and method for synthesizing fatty acids. US Patent  
456 2016. US9309543B2.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

457 18. Qiao K, Wasylenko TM, Zhou K, Xu P, Stephanopoulos G. Lipid production in *Yarrowia*  
458 *lipolytica* is maximized by engineering cytosolic redox metabolism. Nat. Biotechnol.  
459 2017;35:173–177.

460 19. Datsenko KA, Wanner BL. One-step inactivation of chromosomal genes in *Escherichia*  
461 *coli* K-12 using PCR products. Proc. Natl. Acad. Sci. USA. 2000;97:6640–6645.

462 20. Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M,  
463 Wanner BL, Mori H. Construction of *Escherichia coli* K-12 in-frame, single-gene  
464 knockout mutants: the Keio collection. Mol. Syst. Biol. 2006;2.

465 21. Jensen PR, Hammer K. The sequence of spacers between the consensus sequences  
466 modulates the strength of prokaryotic promoters. Appl. Environ. Microbiol. 1998;64:82–  
467 87.

468 22. Farasat I, Kushwaha M, Collens J, Easterbrook M, Guido M, Salis HM. Efficient search,  
469 mapping, and optimization of multi-protein genetic systems in diverse bacteria. Mol.  
470 Syst. Biol. 2014;10.

471 23. Espah Borujeni A, Channarasappa AS, Salis HM. Translation rate is controlled by  
472 coupled trade-offs between site accessibility, selective RNA unfolding and sliding at  
473 upstream standby sites. Nucleic Acids Res. 2014;42:2646–2659.

474 24. Salis HM, Mirsky EA, Voigt CA. Automated design of synthetic ribosome binding sites  
475 to control protein expression. Nat. Biotechnol. 2009;27:946–950.

476 25. Korz DJ, Rinas U, Hellmuth K, Sanders EA, Deckwer WD. Simple fed-batch technique  
477 for high cell density cultivation of *Escherichia coli*. J. Biotechnol. 1995;39:59–65.

478 26. Bergler H, Fuchsbichler S, Högenauer G, Turnowsky F. The enoyl-[acyl-carrier-protein]  
479 Reductase (FabI) of *Escherichia coli*, which catalyzes a key regulatory step in fatty acid

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

480 biosynthesis, accepts NADH and NADPH as cofactors and is inhibited by palmitoyl-  
481 CoA. Eur. J. Biochem. 1996;242:689–694.

482 27. Gonzalez JE, Long CP, Antoniewicz MR: Comprehensive analysis of glucose and xylose  
483 metabolism in *Escherichia coli* under aerobic and anaerobic conditions by <sup>13</sup>C metabolic  
484 flux analysis. Metab. Eng. 2017;39:9–18.

485 28. Hua Q, Yang C, Baba T, Mori H, Shimizu K. Responses of the central metabolism in  
486 *Escherichia coli* to phosphoglucose isomerase and glucose-6-phosphate dehydrogenase  
487 knockouts. J. Bacteriol. 2003;185:7053–7067.

488 29. Canonaco F, Hess TA, Heri S, Wang T, Szyperski T, Sauer U. Metabolic flux response to  
489 phosphoglucose isomerase knock-out in *Escherichia coli* and impact of overexpression of  
490 the soluble transhydrogenase UdhA. FEMS Microbiol. Lett. 2001;204:247–252.

491 30. Shimada T, Fujita N, Yamamoto K, Ishihama A. Novel roles of cAMP receptor protein  
492 (CRP) in regulation of transport and metabolism of carbon sources. PLOS ONE.  
493 2011;6:e20081.

494 31. Gama-Castro S, Salgado H, Santos-Zavaleta A, Ledezma-Tejeda D, Muñoz-Rascado L,  
495 García-Sotelo JS, Alquicira-Hernández K, Martínez-Flores I, Pannier L, Castro-  
496 Mondragón JA, et al. RegulonDB version 9.0: high-level integration of gene regulation,  
497 coexpression, motif clustering and beyond. Nucleic Acids Res. 2016;44:D133–D143.

498 32. Zheng D, Constantinidou C, Hobman JL, Minchin SD. Identification of the CRP regulon  
499 using *in vitro* and *in vivo* transcriptional profiling. Nucleic Acids Res. 2004;32:5874–  
500 5893.

501 33. Steinsiek S, Bettenbrock K. Glucose transport in *Escherichia coli* mutant strains with  
502 defects in sugar transport systems. J. Bacteriol. 2012, 194:5897–5908.



1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

503 34. Toya Y, Ishii N, Nakahigashi K, Hirasawa T, Soga T, Tomita M, Shimizu K. <sup>13</sup>C-  
504 metabolic flux analysis for batch culture of *Escherichia coli* and its *pyk* and *pgi* gene  
505 knockout mutants based on mass isotopomer distribution of intracellular metabolites.  
506 Biotechnol. Prog. 2010;26:975–992.

507 35. Kabir MM, Shimizu K. Gene expression patterns for metabolic pathway in *pgi* knockout  
508 *Escherichia coli* with and without *phb* genes based on RT-PCR. J. Biotechnol.  
509 2003;105:11–31.

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

511 **Figure legends**

512 **Fig. 1** Evaluation of glucose-xylose co-utilization in engineered strains (96 h). Symbols: glucose,  
513 blue lines; xylose, green lines; 1% total sugars, dashed lines; 2% total sugars, solid lines. Error  
514 bars indicate one standard deviation ( $n=3$ , except for XW1004, for which  $n=2$ ).

515  
516 **Fig. 2** Methyl ketone production by strains engineered for glucose-xylose co-utilization (1% total  
517 sugars, 96 h). Error bars indicate one standard deviation ( $n=3$ , except for XW1004, for which  
518  $n=2$ ).

519  
520 **Fig. 3** Shake flask production data (growth, methyl ketone production, sugar consumption) for  
521 strains engineered for sugar co-utilization (Table 1) and control strain EGS1895. Symbols:  
522 glucose, blue lines; xylose, green lines; OD<sub>600</sub>, black lines; methyl ketones, red lines; 1% total  
523 sugars, dashed lines; 2% total sugars, solid lines. The starting OD<sub>600</sub> was ca. 0.01. Error bars  
524 indicate one standard deviation ( $n=3$ ).

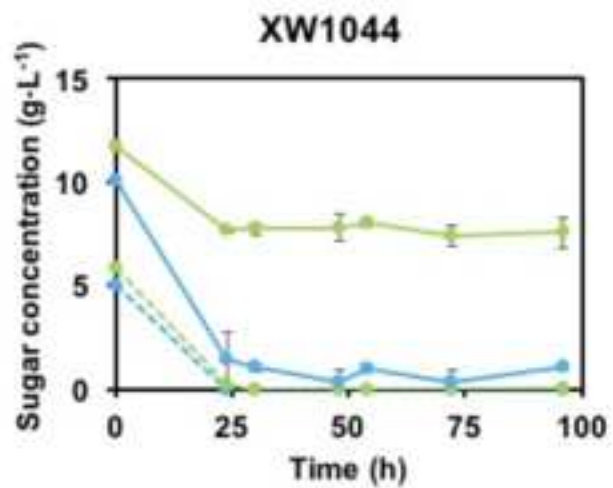
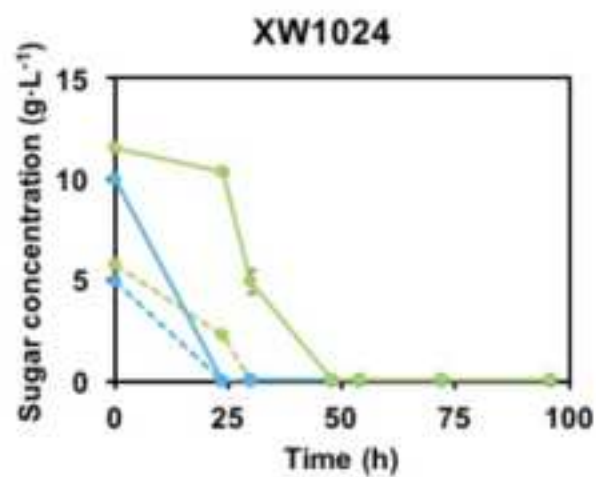
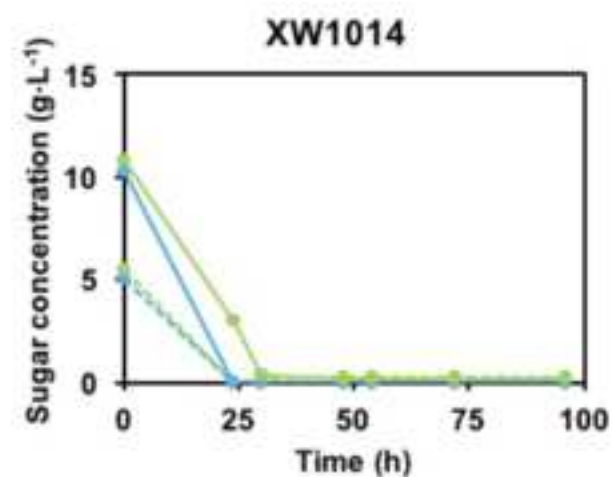
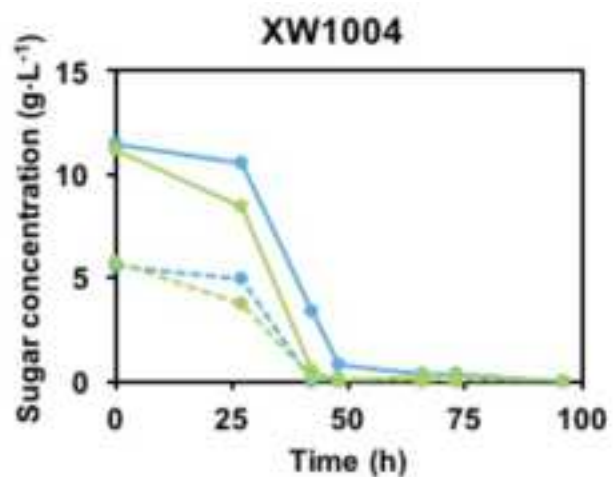
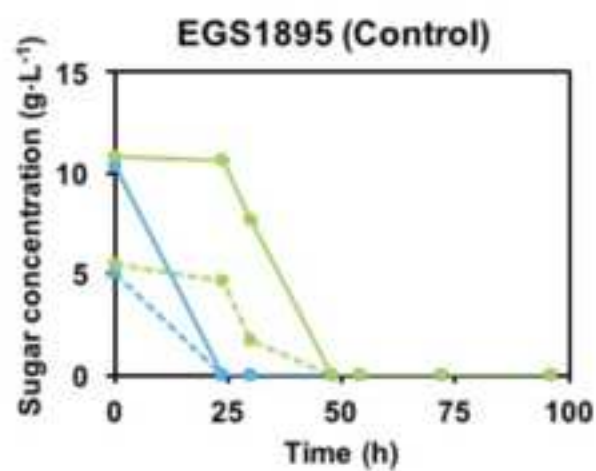
525  
526 **Fig. 4** Overview of central carbon metabolism in *E. coli* highlighting strategies (in red) to  
527 improve NADPH supply in the sugar co-utilizing strain XW1014. G6P, glucose 6-phosphate;  
528 F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; DHAP, dihydroxyacetone  
529 phosphate; GAP, glyceraldehyde 3-phosphate; 13BPG, 1,3-bisphosphoglycerate; 3PG, 3-  
530 phosphoglycerate; 2PG, 2-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; AcCoA,  
531 acetyl-CoA; 2KG: 2-ketoglutaric acid; 6PGL, 6-phosphogluconolactone; 6PG, 6-  
532 phosphogluconate; Ru5P, ribulose 5-phosphate; R5P, ribose 5-phosphate; X5P, xylulose 5-  
533 phosphate; S7P, sedoheptulose 7-phosphate; E4P, erythrose 4-phosphate; *pgi*, glucose 6-

1  
2  
3  
4  
5  
6  
7  
8  
9  
10  
11  
12  
13  
14  
15  
16  
17  
18  
19  
20  
21  
22  
23  
24  
25  
26  
27  
28  
29  
30  
31  
32  
33  
34  
35  
36  
37  
38  
39  
40  
41  
42  
43  
44  
45  
46  
47  
48  
49  
50  
51  
52  
53  
54  
55  
56  
57  
58  
59  
60  
61  
62  
63  
64  
65

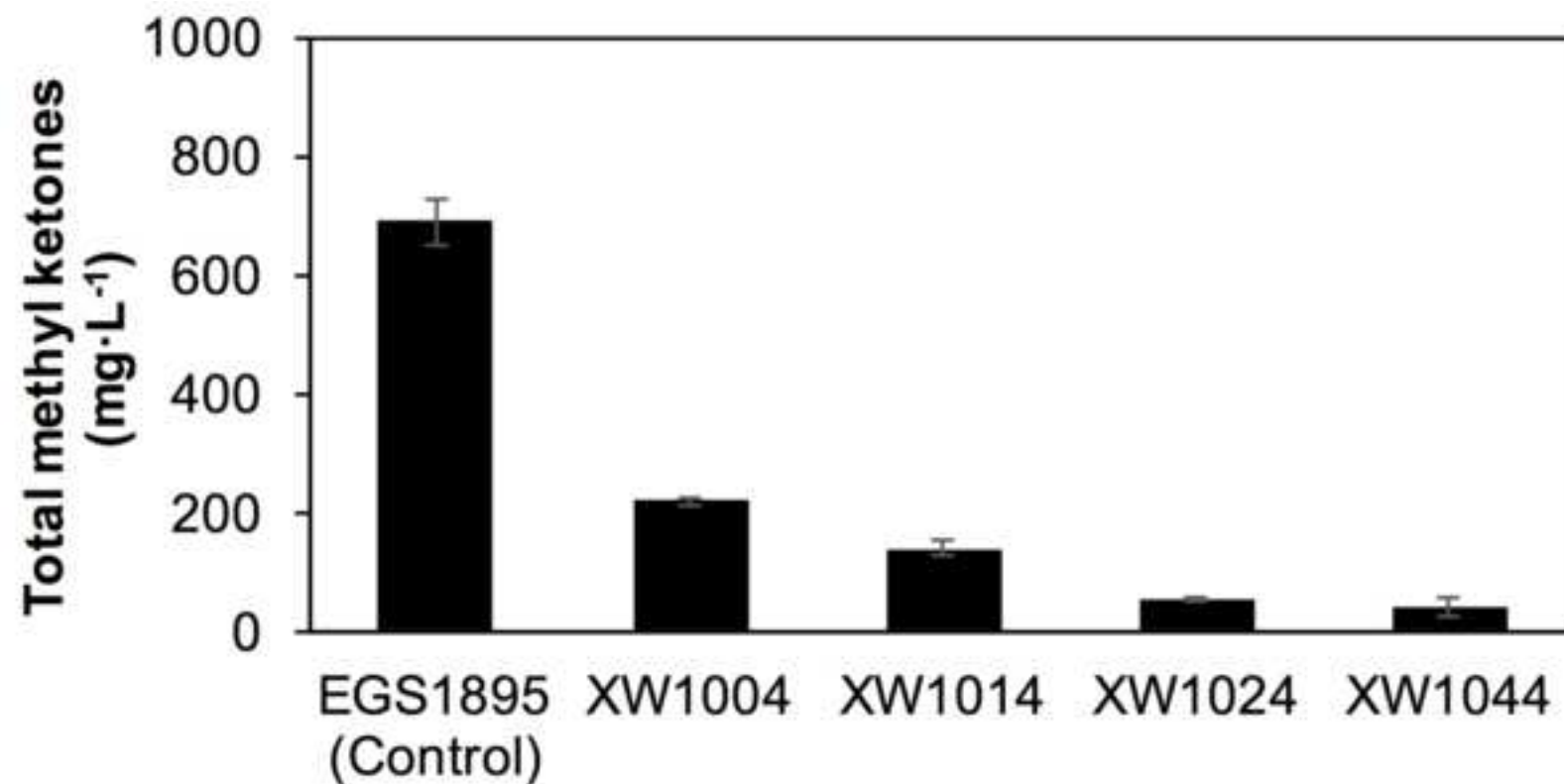
534 phosphate isomerase; *maeB*, malic enzyme; *zwf*, glucose 6-phosphate dehydrogenase; *gnd*,  
535 phosphogluconate dehydrogenase.

536  
**Fig. 5** Summary comparison of methyl ketone production and sugar consumption for engineered  
537 strains. Methyl ketone yield, methyl ketone productivity, and sugar consumption period are each  
538 normalized to the maximum value among the six strains (for cultivation with 2% total sugars).  
539 Blue, methyl ketone yield from glucose + xylose consumed ( $\text{g methyl ketones} \cdot \text{g}^{-1} \text{ total sugars}$ );  
540 red, methyl ketone productivity during the sugar consumption period (from onset of sugar  
541 consumption to >90% total sugar consumption;  $\text{g} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ ); green, the reciprocal of sugar  
542 consumption period (as defined for productivity; the reciprocal was used to make the most  
543 favorable consumption phenotype approach 1 instead of 0 for ease of comparison).

544  
545  
**Fig. 6** Batch fermentation of strains EGS1895 and XW1075 in 2-L bioreactors. Symbols:  
546 glucose, blue line; xylose, green line; cell dry weight (CDW), black line; methyl ketones, red  
547 line; yield, purple line.



Glucose\_1%      Xylose\_1%  
Glucose\_2%      Xylose\_2%



<i>ΔptsG</i>	+			
<i>P<sub>cp6</sub>-xylF</i>		+	+	+
<i>P<sub>cp25</sub>-xylA</i>		+	+	+
<i>xylA<sup>up</sup></i>		+	+	+
<i>ΔaraE</i>			+	+
<i>P<sub>cp6</sub>-araF</i>				+
<i>P<sub>cp25</sub>-araB</i>				+
<i>ΔaraC</i> (genome)				+
<i>ΔaraC</i> (pEG1675)				+

