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

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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 C₆
22 and C₅ sugars in *E. coli* without sacrificing production of fatty acid-derived compounds.

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24 **Keywords**

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

28 **Background**

29 Medium-chain (e.g., C₁₁ to C₁₅) 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 β -ketoacyl-coenzyme A (CoA) thioesters achieved by modification of the β -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 β -ketoacyl-CoA thioesters to β -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

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4 47 phosphoenolpyruvate (PEP): carbohydrate phosphotransferase (PTS) system. The glucose-
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6 48 specific EII complex of the PTS system consists of the permease, EIIBC^{Glc} (encoded by *ptsG*),
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9 49 and EIIA^{Glc} (encoded by *crr*), which has a primary role in modulating carbohydrate metabolism
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12 50 in *E. coli*. During glucose transport, EIIA^{Glc} is dephosphorylated, which prevents either the
13
14 51 import of non-glucose sugars or their subsequent metabolism, and as a consequence, bacterial
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16 52 cells are devoid of the inducer for the corresponding operons; this is known as inducer exclusion
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19 53 [8]. One consequence of dephosphorylated EIIA^{Glc} is a decrease in levels of cyclic AMP
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21 54 (cAMP), which is produced from ATP by adenylate cyclase (activated by phosphorylated
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23 55 EIIA^{Glc}). Lower levels of cAMP in turn limit the availability of cAMP–CRP, the complex
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26 56 between cAMP and CRP (cAMP receptor protein). The expression of genes that are involved in
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29 57 the catabolism of sugars other than glucose generally requires the cAMP–CRP complex and,
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31 58 consequently, is repressed in the presence of glucose. In addition, the arabinose transcriptional
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33 59 regulator (AraC) suppresses the xylose-catabolism genes *xylAB* and *xylFGH* by inhibiting the
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36 60 xylose transcriptional activator (XylR), which constitutes the second layer of CCR [9].
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38 61 Multiple strategies have been proposed for engineering simultaneous hexose-pentose
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40 62 metabolism in *E. coli* by mitigating CCR, such as inactivation of *ptsG*, mutation of regulatory
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43 63 genes, and constitutive expression of key genes in pentose metabolism [10-16]. However, few
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46 64 studies have applied such strategies for mitigating CCR to production of fatty acid-based
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48 65 biofuels [17]. Challenges can be anticipated in combining metabolic strategies for simultaneous
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51 66 glucose-xylose utilization and methyl ketone overproduction, as changes in central carbon
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53 67 metabolism can substantially alter redox balance, which is needed for efficient conversion of
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56 68 carbon to targeted products [18].
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4 69 In this study, we investigated the effects of engineering CCR mitigation into our best
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6 70 methyl ketone-overproducing *E. coli* strain (EGS1895 [2]). We chose to follow the CCR
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9 71 mitigation strategy recently described by Kim and co-workers [12], which was reported to offer
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11 72 advantages over other approaches, most notably, the engineered strains grow well on glucose,
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14 73 unlike some CCR-insensitive mutants defective in the glucose PTS system. Several rounds of
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16 74 engineering were required to optimize both (1) simultaneous glucose-xylose co-utilization and
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19 75 (2) methyl ketone production, as strategies targeting one of these outcomes often adversely
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21 76 affected the other. Ultimately, our results suggest the feasibility of engineering simultaneous
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23 77 utilization of glucose and xylose in *E. coli* along with substantial production of fatty acid-derived
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26 78 biofuels.

27 28 79 **Methods**

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

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33 81 *E. coli* strains and plasmids are listed in Table 1. Strains and plasmids along with their
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36 82 associated information (annotated GenBank-format sequence files) have been deposited in the
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38 83 public version of the JBEI Registry (<https://public-registry.jbei.org>; entries JPUB_XXX to
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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
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46 86 methyl ketone-overproducing strain EGS1895 [2] was used as the control strain. Q5 High-
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48 87 Fidelity DNA Polymerase was used for all PCR reactions (New England Biolabs, Ipswich, MA).
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51 88 NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs, Ipswich, MA) was used to
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53 89 assemble linear DNA fragments. Plasmid extractions were carried out by using QIAGEN
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56 90 miniprep kits (Valencia, CA). Oligonucleotide primers were synthesized by Integrated DNA
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4 91 Technologies, Inc. (San Diego, CA). DNA sequencing was completed by GENEWIZ (South
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6 92 Plainfield, NJ).

9 93 **Genetic manipulations and strain development**

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

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

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4 114 ATTCCGGGGATCCGTCGACCTGCAGTTCGAAGTTCCTATTCTCTAGAAAGTATAGGA
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6 115 ACTTCGAAGCAGCTCCAGCCTACA-3') was used as the pre-sequence. Based on the native
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9 116 35-bp RBS sequence for *crp* (5'-CTCTGGAGAAAGCTTATAACAGAGGATAACCGCGC-
10
11
12 117 3'), a degenerate RBS sequence (5'-CTHTGGTGAAAGCTTATAACTGAGGMRAACCGCGT-
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14 118 3') was generated with a broad range of predicted translation initiation rates (TIR) for a total 12
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16 119 variant sequences. RBS sequences and their predicted TIR values are listed in Table S2. For
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19 120 reference, the native *crp* RBS sequence in *E. coli* DH1 was predicted to have a TIR value of
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21 121 2441 au based on the RBS Calculator [23, 24].
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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 _{CP6-<i>xylF</i>} ; P _{CP25-<i>xylA</i>} ; <i>xylA</i> ^{up}	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 _{CP6-<i>xylF</i>} ; P _{CP25-<i>xylA</i>} ; <i>xylA</i> ^{up} ; $\Delta araE$	This study
XW1024	XW1023 with pEG1675	This study
XW1043	<i>E. coli</i> DH1; $\Delta fadE$; $\Delta ackA-pta$; $\Delta poxB$; P _{CP6-<i>xylF</i>} ; P _{CP25-<i>xylA</i>} ; <i>xylA</i> ^{up} ; $\Delta araE$; P _{CP25-<i>araB</i>} ; $\Delta araC$; P _{CP6-<i>araF</i>}	This study
XW1044	XW1043 with pXW1677	This study
XW1053	<i>E. coli</i> DH1; $\Delta fadE$; $\Delta ackA-pta$; $\Delta poxB$; P _{CP6-<i>xylF</i>} ; P _{CP25-<i>xylA</i>} ; <i>xylA</i> ^{up} ; Δpgi	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 _{CP6-<i>xylF</i>} ; P _{CP25-<i>xylA</i>} ; <i>xylA</i> ^{up} ; <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 _{CP6-<i>xylF</i>} ; P _{CP25-<i>xylA</i>} ; <i>xylA</i> ^{up} ; <i>crp</i> -RBS (TIR=13); Δpgi	This study
XW1074	XW1073 with pEG1675	This study
XW1075	XW1073 with pXW1678	This study
Plasmids	Relevant characteristics	Source or Reference
pKD13	λ -Red recombineering plasmid	[19]
pKD46	λ -Red recombineering plasmid	[19]
pCP20	λ -Red recombineering plasmid	[19]
pEG1675	Km ^r ; <i>araC</i> -P _{BAD-<i>fadR-co_fadD</i>} ; P _{trc-<i>fadM</i>} ; P _{lacUV5-} ' <i>tesA-fadB-co_aco</i>	[2]
pXW1677	pEG1675 with <i>araC</i> deleted	This study
pXW1678	Km ^r ; <i>araC</i> -P _{BAD-<i>fadR-co_fadD</i>} ; P _{trc-<i>fadM-maeB</i>} ; P _{lacUV5-} ' <i>tesA-fadB-co_aco</i>	This study

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4 143 **Table 2** Strategies used for engineering simultaneous glucose-xylose utilization in EGS1895 ^a

No.	Manipulation	Target sequence	XW1014	XW1024	XW1044
1	Replace promoter of <i>xylFGH</i>	CP6 synthetic promoter	+ ^b	+	+
2	Replace promoter of <i>xylAB</i>	CP25 synthetic promoter	+	+	+
3	<i>xylA^{up}</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>	Δ <i>araC</i> (genome)			+
8	<i>araC</i> deletion from pEG1675	Δ <i>araC</i> (plasmid)			+

22 144 ^a Based on [12].

23 145 ^b + indicates that the specified genetic modification is present in this strain.

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26 147 **Media and culture conditions**

29 148 Lysogeny broth (LB) was used for routine cell growth and propagation. Kanamycin was
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31 149 added to the growth medium at a final concentration of 50 $\mu\text{g}\cdot\text{mL}^{-1}$, when required. M9-MOPS
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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
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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
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37 152 composition of M9-MOPS minimal medium followed the recipe previously described [2]. For
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39 153 production experiments, strains were first adapted in M9-MOPS minimal medium with 1% total
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41 154 sugars for 3 passages before being inoculated into production medium with glucose and xylose.
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45 155 **Analysis of cell growth and sugar metabolism**

46 156 Cell growth was monitored by measuring optical density at 600 nm (OD_{600}). Sugars were
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48 157 measured with an Agilent 1100 Series HPLC system, equipped with an Agilent 1200 Series
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50 158 refractive index detector (RID) (Agilent Technologies, CA) and Aminex HPX-87H ion-
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52 159 exclusion column (300-mm length, 7.8-mm internal diameter; Bio-Rad Laboratories, Inc.,
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54 160 Hercules, CA). The column temperature was 50°C, and 4 mM sulfuric acid was used as the
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4 161 mobile phase with a flow rate of 0.6 mL·min⁻¹ for 24 min. The quantification of glucose and
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6 162 xylose was conducted by external standard calibration with authentic standards.
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9 163 **Production and analysis of methyl ketones**

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11 164 For methyl ketone production, strains were inoculated into 50 mL M9-MOPS minimal
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14 165 medium with 1% total sugars, or 25 mL with 2% total sugars, in 250-mL shake flasks and
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16 166 cultured at 37°C with 200-rpm agitation. The starting OD₆₀₀ during production was ca. 0.01.
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19 167 Gene expression was induced by adding 0.2 mM IPTG and 1 mM arabinose after 6 h of growth.
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21 168 Five mL of decane (Reagent-Plus ≥ 99% purity, Sigma-Aldrich, St. Louis, MO) amended with
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24 169 perdeuterated tetracosane (C₂₄D₅₀) 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
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29 171 sampled for the measurement of methyl ketones; analysis by electron ionization gas
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31 172 chromatography-mass spectrometry (GC-MS) was conducted as previously described [3].
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33 173 **Batch fermentation of strains EGS1895 and XW1075 in a 2-L bioreactor for methyl ketone** 34 35 36 174 **production**

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38 175 Batch fermentation was carried out in a 2-L bioreactor equipped with a Sartorius
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41 176 BIOSTAT B plus control unit for regulating dissolved oxygen (DO), pH, and temperature.
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43 177 Frozen glycerol stocks of M9-MOPS-adapted cells were used to seed a test tube containing 5 mL
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45 178 of M9-MOPS minimal medium with 1% total sugars (5 g·L⁻¹ glucose and 5 g·L⁻¹ xylose) as
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48 179 previously described. After 30 h of growth, the cultures were diluted 1:250 into a 250-mL flat-
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51 180 bottom shake flask containing 50 mL of M9-MOPS minimal medium supplemented with 1%
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53 181 total sugars. This culture was grown for another 30 h as before and was used to inoculate 1.25 L
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55 182 of medium in the bioreactor. The medium was adapted from Korz *et. al.* [25] and was composed
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58 183 of M9 salts (6.8 g·L⁻¹ Na₂HPO₄, 3.0 g·L⁻¹ KH₂PO₄, 1.0 g·L⁻¹ NH₄Cl, 0.5 g·L⁻¹ NaCl)
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4 184 supplemented with 0.5 g·L⁻¹ of MgSO₄·7H₂O, 0.18 g·L⁻¹ of NH₄Cl, 1.0 mg·L⁻¹ thiamine, 10 nM
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6 185 of FeSO₄·7H₂O, 100 μM CaCl₂·2H₂O, micronutrients as described previously [2], 10 g·L⁻¹ of
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9 186 glucose, 10 g·L⁻¹ of xylose, and 50 μg·mL⁻¹ of kanamycin. The temperature of the bioreactor
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12 187 was maintained at 37°C throughout the fermentation and the culture was maintained at pH 6.5
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14 188 automatically by the addition of a 10 M potassium hydroxide solution. The initial stir rate and
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16 189 airflow were set at 200 rpm and 1 VVM (volume of air per volume of liquid per minute),
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19 190 respectively. Dissolved oxygen was maintained above 40% of saturation *via* cascade control of
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21 191 adjustment of stirrer speed (up to 1600 rpm), followed by air-flow rate (up to 2.0 VVM).
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24 192 Cultures were induced with 1 mM arabinose and 0.5 mM IPTG at 6 h after initiation of batch
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26 193 phase. In addition, 150 mL of dodecane (Sigma-Aldrich, ReagentPlus ≥ 99% purity) amended
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29 194 with 3 mg·mL⁻¹ of 3-tetradecanone (Sigma-Aldrich) as an internal standard, was added into the
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31 195 bioreactors.

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

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4 **207 Results and discussion**

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

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9 **209 EGS1895**

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11 We engineered several strains by manipulating key genes in pentose metabolism
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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
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16 **212** pattern in which xylose utilization began after glucose was fully depleted. In contrast, newly
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18 **213** engineered strains displayed glucose-xylose co-utilization to varying degrees rather than a strict
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20 **214** diauxic profile. Among these engineered strains, XW1014 (with constitutive expression of *xyIA*
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22 **215** and *xyIF* plus a point mutation in the *xyIA* promoter, *xyIA^{up}*) showed the best performance for
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24 **216** simultaneous utilization of glucose and xylose (Fig. 1). This strain had identical consumption
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26 **217** rates for glucose and xylose at 1% sugar conditions, while a slight decrease in xylose
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28 **218** consumption was observed at higher sugar concentration (2%). The inactivation of *araE*
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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
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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
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38 **223** both strains XW1024 and XW1044 showed favorable simultaneous consumption rates of glucose
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40 **224** and xylose at 1% sugar conditions, their xylose consumption dramatically decreased at higher
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42 **225** sugar concentration (2%).
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53 **227** In addition, because *ptsG* deficiency is a well-studied mechanism for mitigating CCR in
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55 **228** *E. coli* [10], the glucose transporter EIIBC^{Glc} encoded by *ptsG* was deleted from EGS1895 to
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4 229 investigate the effect on sugar co-utilization (strain XW1004; Table 1). Strain XW1004 did not
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7 230 display a better sugar co-utilization profile than strain XW1014 (Fig. 1).

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

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

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41 244 Although strain XW1014 was successfully engineered for simultaneous glucose-xylose
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43 245 consumption, the significantly reduced methyl ketone titer in this strain necessitated further
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46 246 engineering to improve commercial relevance. We hypothesized that enhancing NADPH
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48 247 availability could be a fruitful engineering target because (1) the biosynthesis of fatty acids
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51 248 (methyl ketone precursors) in *E. coli* is an NADPH-demanding process and (2) xylose
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53 249 metabolism, particularly when simultaneous with glucose metabolism, could disrupt NADPH
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56 250 production in a host cell (e.g., strain XW1014) compared to conditions with glucose as a sole
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58 251 carbon source. Fatty acid biosynthesis results in net consumption of NADPH due to demand
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4 252 from two key reductases – FabG (β -ketoacyl-ACP reductase) and potentially, FabI (enoyl-ACP
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6 253 reductase), which can utilize either NADH or NADPH as a cofactor [1, 26]. To illustrate the
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9 254 substantial NADPH demands of fatty acid/methyl ketone biosynthesis, production of 1 mol of a
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11 255 C₁₃ methyl ketone (2-tridecanone) from glucose using the relevant metabolic pathway [2] would
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14 256 result in net consumption of 6 (or 12) mol of NADPH and net production of 9 (or 15) mol of
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16 257 NADH, depending on FabI cofactor usage.

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

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53 272 We implemented two strategies for increasing NADPH supply in strain XW1014: (1)
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55 273 deleting *pgi* (glucose-6-phosphate isomerase) from the chromosome to divert flux from
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58 274 glycolysis through the oxidative PPP (Fig. 4) and (2) overexpressing *maeB* (malic enzyme),
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275 which leads to NADPH generation by oxidative decarboxylation of malate to pyruvate (Fig. 4).
276 ¹³C 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 Δpgi strains can be at least partially
279 ameliorated by NADPH consumption through transhydrogenase [28, 29]. In our Δpgi 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 Δpgi strain (XW1054) had dramatically improved
284 methyl ketone titer (850 mg·L⁻¹) 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⁻¹ after 96 h) was also comparable to that of strain EGS1895 (~1600 mg·L⁻¹). 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 (Δpgi), 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).

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

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

307 A possible explanation for these results is changes in intracellular distributions of the
308 global regulator CRP. For strain XW1014, promoter replacement for *xylA* and *xylF* resulted in
309 removal of a CRP binding site from the intergenic region between *xylA* and *xylF* [30]. As a
310 global regulator, CRP not only plays an important role in carbon catabolite repression, but also
311 controls the transcription of more than 100 genes in *E. coli*, such as key genes in fatty acid
312 metabolism (e.g., *fadD*, *fadH*) [31] and in central carbon metabolism (e.g., *pgi*, *zwf*, *gnd*) [30,
313 32]. Thus, the promoter change in strain XW1014 might have altered the level of free CRP and
314 directly and indirectly affected the transcription of many other genes related to fatty acid
315 metabolism. Similarly, changes to intracellular CRP pools might also explain why methyl ketone
316 production was reduced in the $\Delta ptsG$ strain (XW1004): the absence of PtsG likely increased
317 cAMP availability [33], and in turn, altered the level of free intracellular CRP, which interacts
318 with cAMP to make the cAMP–CRP complex.

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

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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⁻¹ 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⁻¹ 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 (Δpgi 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 Δpgi 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⁻¹ methyl ketones at 1% total sugar conditions, but reached a higher titer at 2% total sugars (~1600 mg·L⁻¹) that was comparable to that of the control strain (EGS1895). Glucose and xylose were simultaneously

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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⁻¹ and 1100 mg·L⁻¹ 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⁻¹, which was ca. 33% higher than that of strain
360 EGS1895 (1.5 g·L⁻¹).

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

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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

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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

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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.

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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; OD₆₀₀, black lines; methyl ketones, red lines; 1% total
523 sugars, dashed lines; 2% total sugars, solid lines. The starting OD₆₀₀ was ca. 0.01. Error bars
524 indicate one standard deviation ($n=3$).

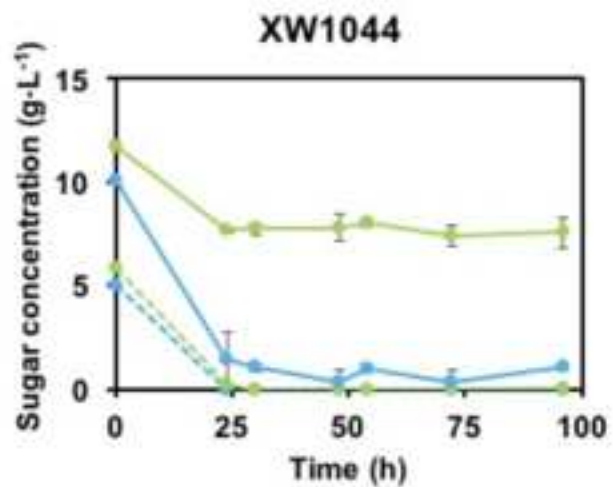
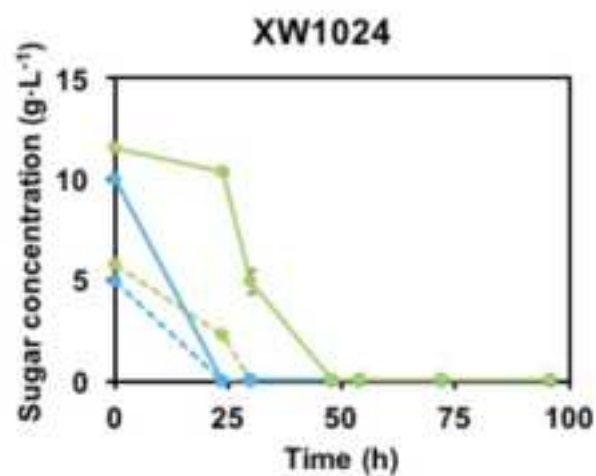
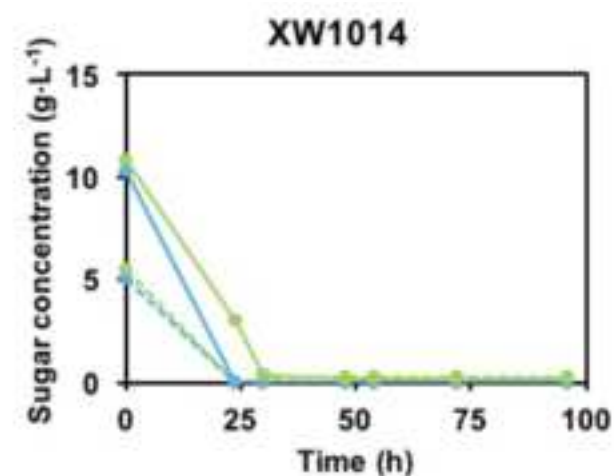
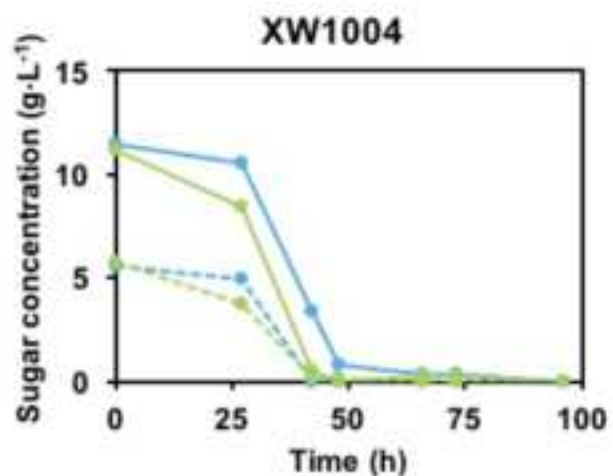
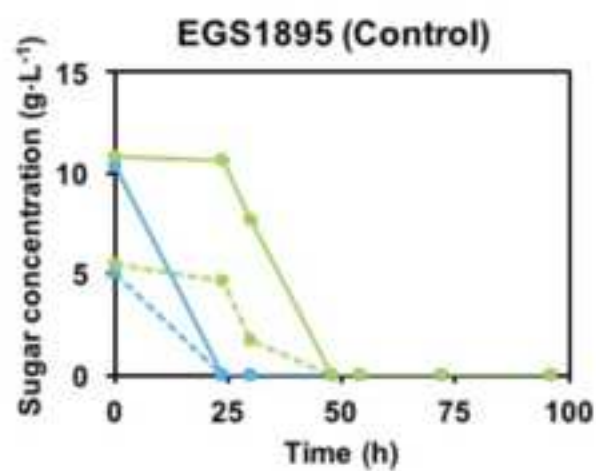
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526 **Fig. 4** Overview of central carbon metabolism in *E. coli* highlighting strategies (in red) to
527 improve NADPH supply in the sugar co-utilizing strain XW1014. G6P, glucose 6-phosphate;
528 F6P, fructose 6-phosphate; FBP, fructose 1,6-bisphosphate; 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-

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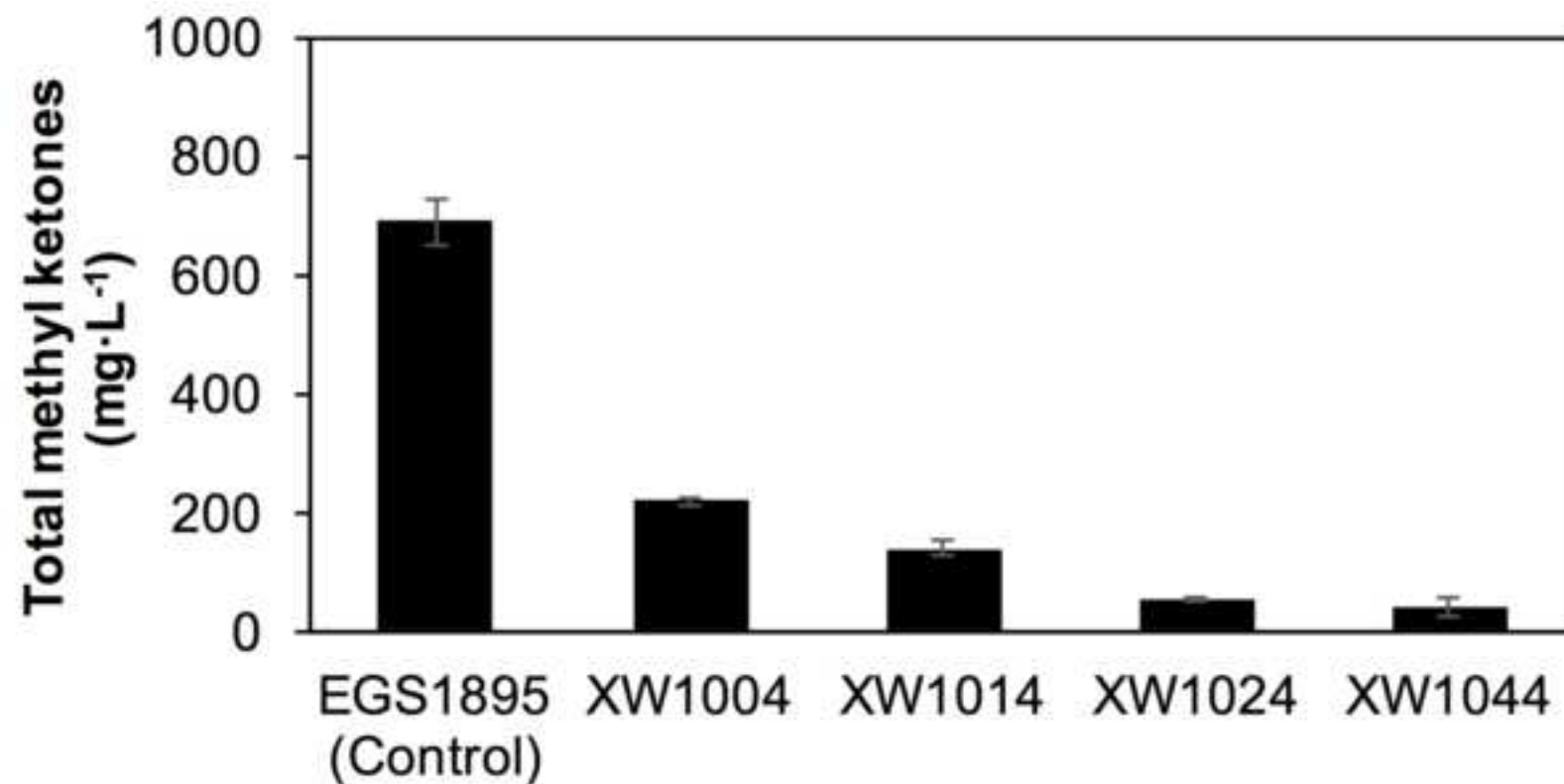
534 phosphate isomerase; *maeB*, malic enzyme; *zwf*, glucose 6-phosphate dehydrogenase; *gnd*,
535 phosphogluconate dehydrogenase.

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_{cp6}-xylF</i>		+	+	+
<i>P_{cp25}-xylA</i>		+	+	+
<i>xylA^{up}</i>		+	+	+
<i>ΔaraE</i>			+	+
<i>P_{cp6}-araF</i>				+
<i>P_{cp25}-araB</i>				+
<i>ΔaraC</i> (genome)				+
<i>ΔaraC</i> (pEG1675)				+

