Engineering *Pseudomonas putida* KT2440 for the production of isobutanol

Robert Nitschel\(^1\) | Andreas Ankenbauer\(^1\) | Ilona Welsch\(^1\) | Nicolas T. Wirth\(^1\) | Christoph Massner\(^1\) | Naveed Ahmad\(^2\) | Stephen McColm\(^2\) | Frédéric Borges\(^3\) | Ian Fotheringham\(^2\) | Ralf Takors\(^1\) | Bastian Blombach\(^1,4\)

\(^1\)Institute of Biochemical Engineering, University of Stuttgart, Stuttgart, Germany
\(^2\)Ingenza Ltd., Roslin Innovation Centre, Charnock Bradley Building, Easter Bush Campus, Roslin, UK
\(^3\)Laboratoire d’Ingénierie des Biomolécules (LJBi), Université de Lorraine, Nancy, France
\(^4\)Microbial Biotechnology, Campus Straubing for Biotechnology and Sustainability, Technical University of Munich, Straubing, Germany

**Correspondence**

Bastian Blombach, Microbial Biotechnology, Technical University of Munich, Schulgasse 22, 94315 Straubing, Germany
Email: bastian.blombach@tum.de

**Abstract**

We engineered *P. putida* for the production of isobutanol from glucose by preventing product and precursor degradation, inactivation of the soluble transhydrogenase SthA, overexpression of the native *ilvC* and *ilvD* genes, and implementation of the feedback-resistant acetolactate synthase AlsS from *Bacillus subtilis*, ketoacid decarboxylase KivD from *Lactococcus lactis*, and aldehyde dehydrogenase YqhD from *Escherichia coli*. The resulting strain *P. putida* Iso2 produced isobutanol with a substrate specific product yield (\(Y_{\text{IsoS}}\)) of 22 ± 2 mg per gram of glucose under aerobic conditions. Furthermore, we identified the ketoacid decarboxylase from *Carnobacterium maltaromaticum* to be a suitable alternative for isobutanol production, since replacement of *kivD* from *L. lactis* in *P. putida* Iso2 by the variant from *C. maltaromaticum* yielded an identical \(Y_{\text{IsoS}}\). Although *P. putida* is regarded as obligate aerobic, we show that under oxygen deprivation conditions this bacterium does not grow, remains metabolically active, and that engineered producer strains secreted isobutanol also under the non-growing conditions.

**KEYWORDS**

isobutanol, ketoacid decarboxylase, metabolic engineering, microaerobic, *Pseudomonas putida*

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**1 | INTRODUCTION**

Biofuel production from renewable feed stocks is of special importance because of the finite nature of the currently used crude oil derivatives and growing concerns about climate change [1]. Isobutanol is an attractive alternative to the employed fossil fuels. It has several advantages such as a higher energy density, compatibility with existing engines, lower vapor pressure and volatility, as well as a lower corrosivity compared to bio-ethanol [2,3]. Furthermore, isobutanol is used in the chemical industry and can be used to produce the gaseous alkene precursor isobutene [4].

Isobutanol can be synthesized via the branched-chain amino acid biosynthesis and the so-called Ehrlich pathway to convert pyruvate to isobutanol (Figure 1). The first step in this route is the conversion of two pyruvate molecules to...
2-acetolactate catalyzed by the acetolactate synthase (AlsS), which is usually feedback inhibited by the branched-chain amino acids L-valine, L-leucine, and L-isoleucine. However, AlsS from Bacillus subtilis has been shown to be feedback-resistant and therefore has been applied for isobutanol production in several studies [5,6]. Then, 2-acetolactate is reduced to 2,3-dihydroxyisovalerate and subsequently converted to 2-ketoisovalerate (2-KIV) by the ketoacid reductoisomerase IivC and dihydroxyacid dehydratase IivD, respectively. Finally, isobutanol is synthesized from 2-KIV in two more reaction steps of the Ehrlich pathway. The decarboxylation of 2-KIV to isobutyraldehyde is catalyzed by ketoacid decarboxylases (KDCs) that are not widespread in nature. Especially KivD from Lactococcus lactis has been proved as an efficient variant in, e.g. E. coli and C. glutamicum [5,7]. The last step from isobutyraldehyde to isobutanol requires an aldehyde reductase or alcohol dehydrogenase. A number of NADH and NADPH dependent enzymes are available that catalyze this reaction [8].

Several microorganisms have been engineered for isobutanol production such as E. coli, C. glutamicum, B. subtilis, and yeast such as Saccharomyces cerevisiae [5,7,9,10]. Although highly efficient E. coli and C. glutamicum strains have been constructed [6,7], the relatively low tolerance of most microbial systems against isobutanol hampers commercialization of isobutanol production processes. In contrast, pseudomonads have an intrinsic tolerance against organic compounds and solvents [11,12] making them promising candidates for isobutanol production.

Among them, Pseudomonas putida is a Gram-negative, saprophytic soil bacterium with a genome size of 6.18 Mbp [13]. It has been reported to promote plant growth, prevent plant diseases, and can efficiently remove organic soil pollutants and environmental contaminants [14]. P. putida features a versatile metabolism using the Entner–Doudoroff pathway for glucose catabolism, shows resistance against oxidative stress conditions, and genetic engineering tools are readily available [15–17]. The carbohydrate substrate spectrum is limited and confined to hexoses [18], however, P. putida has been recently engineered to concomitantly consume xylose, cellobiose, and glucose, which are the basic building blocks of the abundant polysaccharides cellulose and hemicellulose [19]. As a result of these achievements, P. putida has emerged as a promising candidate for industrial biotechnology [20,21]. Recent works have engineered this bacterium for the production of polyhydroxyalkanoates, the nylon precursor cis,cis-muconic acid [22] and aromatic compounds like p-coumaric acid or trans-cinnamate [23,24]. P. taiwanensis VLB120 has been applied for the production of phenol [25,26].

In this study, we engineered P. putida for the production of isobutanol from glucose by preventing product and precursor degradation and increasing the flux from pyruvate towards isobutanol. We identified KivD from Carnobacterium maltaromaticum as a suitable alternative to KivD from L. lactis to drive the decarboxylation of 2-ketoisovalerate and finally we showed that isobutanol production can also be achieved under oxygen deprivation conditions with this obligate aerobic bacterium.

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and plasmids

Bacterial strains, their respective genotype, plasmids, and oligonucleotides used in this study are listed in Table 1.

2.2 | Media and culture conditions

E. coli DH5α was grown aerobically in Lysogeny broth (LB) complex medium containing 10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl [34] at 37°C as 5 mL cultures in glass test tubes on a rotary shaker at 120 rpm (Infors AG, Bottmingen, Switzerland). C. maltaromaticum and L. lactis were grown in brain–heart infusion (BHI) broth (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) at 30°C on a rotary shaker at 120 rpm. For longtime storage, P. putida was kept as 30% (w/v) glycerol stock at ~70°C and was streaked out for cultivation on LB solid medium with 15 g/L agar. The first preculture of P. putida was prepared by inoculation of 5 mL LB medium in a test tube with a single colony. The culture was cultivated at 30°C on a rotary shaker (Edmund Bühler GmbH, Bodelshausen, Germany) at 175 rpm overnight and used to inoculate, a second overnight preculture to an optical density of 600 nm (OD₆₀₀) of 0.01–0.02 in 50 mL DeBont minimal medium (pH 7) [35], which was supplemented with...
FIGURE 1 The central metabolism of *P. putida* KT2440 with the Ehrlich pathway. Abbreviations (coding genes are given in brackets): G6P: glucose-6-phosphate, 2-KG: 2-ketoglutarate, 2-K6PG: 2-keto-6-phosphogluconate, 6-PG: 6-phosphogluconate, 2-KDPG: 2-keto-3-deoxy-6-phosphogluconate, G3P: glyceraldehyde-3-phosphate, 1,3-bPG: 1,3-bisphosphoglycerate, 3-PG: 3-phosphoglycerate, 2-PG: 2-phosphoglycerate, PEP: phosphoenolpyruvate, DHAP: dihydroxyacetone-phosphate, F-1,6-bP: fructose-1,6-bisphosphate, F6P: fructose-6-phosphate, CoA: co-enzyme A, Gcd: glucose dehydrogenase (*gcd*), Gad: gluconate 2-dehydrogenase (*gad*), PQQ: pyrroloquinoline quinone, Glk: glucokinase (*glk*), Zwf: glucose-6-phosphate 1-dehydrogenase (*zwf*), GnuK: gluconate kinase (*gnuK*), KguD: 2-6-phosphoketogluconate reductase (*kguD*), KguK: 2-ketogluconate kinase (*kguK*), Edd: 6-phosphogluconate dehydratase (*edd*), Eda: 2-keto-3-deoxy-6-phosphogluconate aldolase (*eda*), IlvHI/AlsS: acetolactate synthase (*ilvHI/alsS*), IlvC: ketolactate reducto-isomerase (*ilvC*), IlvD: dihydroxyacid dehydratase (*ilvD*), KivD: ketoacid decarboxylase (*kivD*), Bkd: branched-chain ketoacid dehydrogenase complex (*bkd*), YqhD: aldehyde reductase (*yqhD*), AldH: aldehyde dehydrogenases, PntAB: pyridine nucleotide transhydrogenase (membrane bound) (*pntAB*), SthA: pyridine nucleotide transhydrogenase (soluble) (*sthA*).

5.4 g/L glucose and 0.5 g/L yeast extract. Cells from the second preculture were harvested by centrifugation (4500 × g, 15 min, 4°C), resuspended in DeBont medium, and used to inoculate 50 mL DeBont medium, to an OD<sub>600</sub> of about 0.1–0.2. The main culture was supplemented with 5.4 g/L glucose, 0.5 g/L isobutanol, or 2.9 g/L 2-ketoisovalerate, respectively. The second pre- and main cultures were performed in 500 mL baffled Erlenmeyer flasks filled with 50 mL medium on a rotary shaker at 175 rpm at 30°C. Micro-aerobic shaking flask cultivations were carried out in sealed 100 mL Müller-Krempel bottles as 25 mL cultures that were inoculated to an OD<sub>600</sub> of 15–20. To obtain sufficient biomass, the second preculture was performed in 100 mL LB medium in a 500 mL baffled Erlenmeyer flask that was cultivated on a rotary shaker (175 rpm) overnight at 30°C. Cells from the second preculture were harvested by centrifugation (4500 × g, 15 min, 4°C) and resuspended in 25 mL DeBont minimal medium (pH 7) supplemented with 5.4 g/L glucose and 15 g/L 3-morpholino-propanesulfonic acid. To induce plasmid-based gene expression, 0.2% (w/v) L-arabinose was supplemented. For plasmid-bearing strains, 50 μg/mL kanamycin or 50 μg/mL apramycin were added to the medium.
### Table 1: Overview of strains, plasmids and oligonucleotides used in this study

| Strain, plasmid or oligonucleotide | Relevant characteristic(s) or sequence (5′ → 3′) | Source, reference or purpose |
|-----------------------------------|-------------------------------------------------|-----------------------------|
| **Strains**                       |                                                 |                             |
| *Pseudomonas putida* KT2440       | Wild type strain, DSM-6125, ATCC47054            | [27]                        |
| *Carnobacterium maltaromaticum* LMA28 |                                               | [28]                        |
| *Lactococcus lactis* subsp. cremoris MG1363 |                     | [29]                        |
| *Corynebacterium glutamicum*      | Wild type strain ATCC13032                      | American type culture collection |
| *P. putida* GN346                 |                                                  | This work                   |
| *P. putida* EP1                   |                                                  | This work                   |
| *P. putida* EP2                   |                                                  | This work                   |
| *P. putida* Iso1                  |                                                  | This work                   |
| *P. putida* Iso2                  |                                                  | This work                   |
| *P. putida* Iso3                  |                                                  | This work                   |
| *P. putida* Iso5                  |                                                  | This work                   |
| *P. putida* Iso6                  |                                                  | This work                   |
| **Plasmids**                      |                                                 |                             |
| pBB1                              | pACYC184/pBL1 derivative, chloramphenicol resistance, P_<sub>lac</sub> promoter and trpA terminator | [31]                        |
| pSA55                             | Expression plasmid for *adh2* of *S. cerevisae* and *kivD* of *L. lactis* | [5]                         |
| pBB1 yqhD                         | pBB1 P_<sub>lac</sub> yqhD                      | This work                   |
| pBB1 kivD yqhD                    | pBB1 P_<sub>lac</sub> kivD yqhD                 | This work                   |
| pNG413.1                          | pBRR1MCS2 derivative, apramycin resistance, *araC*, *P_BAD*, *lacZ* | [32]                        |
| pSEVA231                          | pBBR1 derivative, kanamycin resistance, mobilizable (oriT) | [33]                        |
| pIP01                             | pSEVA231P_<sub>lac</sub> kivD yqhD              | This work                   |
| pIP02                             | pNG413 araC P_<sub>BAD</sub> kivD yqhD alsI ivlC ivlD | This work                   |
| pIP03                             | pIP02, yqhD was changed for *adhA* from *L. lactis* | This work                   |
| pIP04                             | pIP02, yqhD was changed for *adhA* from *C. glutamicum* | This work                   |
| pIP05                             | pIP02, kivD was changed for *kdcA* from *C. maltaromaticum* | This work                   |
| pEMP04                            | pSEVA231 P_<sub>lac</sub> kivD yqhD alsI ivlC ivlD | Ingenza Ltd.               |
| pEMP012                           | pEMP04, yqhD was changed for *adhA* from *L. lactis* | This work                   |
| pEMP013                           | pEMP04, yqhD was changed for *adhA* from *C. glutamicum* | This work                   |
| pEMP014                           | pEMP04, kivD was changed for *kdcA* from *C. maltaromaticum* | This work                   |
| **Oligonucleotide**               |                                                 |                             |
| yqhhd1                            | AACTGCAGAACCACATGCATTTGGAGAGACACACACACACACACACACCCCGCTTGGAAAGTTTCTGGGCGGCTTGTGATATACG | Construction of pBB1yqhhd, PstI site underlined |
| yqhd2                             | CCGCTGGAAGAGCCTTTAGCGGCGGCTTCTGTATACG            | Construction of pBB1yqhhd, Xhol site underlined |
| kivd1                             | TCCCCCCGGAGGAGACACACACATGTATACGAGTAGGAGATCCCTAT | Construction of pBB1 kivd yqhhd, Xmal site underlined |
| kivd2                             | CCAATGCATTGGGTTCAGACACAAAATGATATTAGGAGATCCCTAT | Construction of pBB1 kivd yqhhd, PstI site underlined |
| bkdaa1                            | CTGGATCCCATTCCAGACCTCTCATGACC                    | Deletion of *bkdaA*         |
| bkdaa2                            | CGGCCTTCCAGACGTCACTGACATGATGAACGCGCAACACCTCTAG  | Deletion of *bkdaA*         |
| bkdaa3                            | TGTTGTGGTCATCATGCTGAGCTCTGAGCGCC                | Deletion of *bkdaA*         |

(Continues)
| Strain, plasmid or oligonucleotide | Relevant characteristic(s) or sequence (5′ → 3′) | Source, reference or purpose |
|------------------------------------|-------------------------------------------------|-----------------------------|
| bkdaa4                             | GCTTGTCGACCCGTCGTCACTGCCGTAG                    | Deletion of bkdAA           |
| bkdaagc1                           | GTACCGACGATGCGCT                              | Verification of bkdAA deletion |
| bkdaagc2                           | GCCGTGCACTAAGTGTAG                             | Verification of bkdAA deletion |
| stha1                              | GCCGCTTGGTGGCCGATCCACGACATCGTACGTGTCGCC        | Deletion of sthA             |
| stha2                              | GTGAAATCGGTCAGGTGACGAGAGACGCCACAGTAATCACC     | Deletion of sthA             |
| stha3                              | GAAATGTCGGCGCGGTTCAGATGCGAAGAGACCGATTTCACAC   | Deletion of sthA             |
| stha4                              | TTGCAATGCGCTGCAAGTCGAGTTGGGGCAAAACCTGCT       | Deletion of sthA             |
| sthagc1                            | ATGGGCTATTCCGACGCTGTTACGTG                    | Verification of sthA deletion |
| sthagc2                            | ACTATGCGTCAACGTCTGCTG                         | Verification of sthA deletion |
| gcd1                               | GCCGCTTGGTGGCCGATCCACGACATCGTACGTGTAATCATTAGGC | Deletion of gcd             |
| gcd2                               | GACCTGGGGAGAAGCTACTAGATAGCGGCAGTGAACTACGAC    | Deletion of gcd             |
| gcd3                               | GTGTCGGTACTCGGGTTCATTAGGTGGTTCTGGTCAAGGTAC   | Deletion of gcd             |
| gcd4                               | TTGCAATGCGCTGCAAGTCGAGCGACAACATCGCAAGCACC    | Deletion of gcd             |
| gcdgc1                             | GGGATGGGTGTTTCAAATGTTACGT                     | Verification of gcd deletion |
| gcdgc2                             | GCCACAAGATGGTCTCAAGAG                      | Verification of gcd deletion |
| png1                               | AGCTCTAGAAGGTGTTATATAAAACATATGCTAGTGTTACATTACC | Construction of pIP02, pIP03, pIP04 and pIP05 |
| png2                               | GAGAATAGGAACTCGAAGGTGACGTCGACAGGGGCTCGAGC    | Construction of pIP02, pIP03 and pIP04 |
| png3                               | AGCTCTAGAAGGTGTTATATAAAACATATGCTAGTGTTACATTACC | Construction of pIP05 |
| pbb1                               | TCGGGAGCTCGCCGAATGTCAAGCGTAACTCCTGCGGAGC     | Construction of pIP01, SacI site underlined |
| pbb2                               | ATCGGATCTCTATGGCGCGGCGGCTCGTAT                | Construction of pIP01, BamHI site underlined |
| kdca1                              | TTGCTAAACAAAAATTCATAAAACATGCGAGAATCGAACCAGTC | Amplification of kdca gene   |
| kdca2                              | AATGCATTGCTGTGCAGTTTGTTAATGGAATTTGTTTACGCA   | Amplification of kdca gene   |
| p41                                | TTGCTAAACAAAAATTCATAAAACATGCGAGAATCGAACCAGTC | Construction of pEMP014      |
| p42                                | TAATTTCACAGTGACATGTGGTTTTCAAATTGCGGCGG         | Construction of pEMP014      |
| p43                                | TAATTTCACAGTGACATGTGGTTTTCAAATTGCGGCGG         | Construction of pEMP014      |
| p44                                | CGTACTACTGCTGGCTCTGTGGTTTTCAAATTGCGGCGG       | Construction of pEMP014      |
| p45                                | GTGTCGGGATTCGTTAAGCGAGGAGAGAGCTGACG          | Construction of pEMP013      |
| p46                                | TGGGGTGGCGACGATGCGTCTGCTGCTCGGTGTTGTTTACGCA   | Construction of pEMP013      |
| adha1                              | GCATGGGAGGAGACACAACATGAAAGAAGCAGCTGTAGCAGA   | Amplification of adhaA gene from L. lactis |
| adha2                              | GTCATAGCTGTCTCCTGCTGCTTATTAGTAGAAATCAAGACATCC | Amplification of adhaA gene from L. lactis glutamicum |
| adha3                              | TGCATAGGAGGAGACACAACATGACAGCAGCTGACG         | Amplification of adhaA gene from C. glutamicum |
| adha4                              | GTCATAGCTGTCTCCTGCTTATTAGAAAGAAGCAGCTGACG   | Amplification of adhaA gene from C. glutamicum |

(Continued)
| Strain, plasmid or oligonucleotide | Relevant characteristic(s) or sequence (5′ → 3′) | Source, reference or purpose |
|-----------------------------------|-----------------------------------------------|-----------------------------|
| alss1                             | GAGGAAAGCGGCCGCGTCTTCGGGGCGAGCTTGTTG          | Construction of pEMP04, NotI site underlined |
| alss2                             | TTAGATCTGAGGTCTTCCGGCTATAGAGCTTTCG TTTTCA    | Construction of pEMP04, XhoI site underlined |
| ilvc1                             | GAGGAAAGCGGCCGCGTCTTCGAAGAAAGTCGCCATCATC     | Construction of pEMP04, NotI site underlined |
| ilvc2                             | TTAGATCTGAGGTCTTCCGGCTATAGCTTTCG TTTTCAG    | Construction of pEMP04, XhoI site underlined |
| ilvd1                             | GAGGAAAGCGGCCGCGTCTTCGGGGCGGCCGCGTGG          | Construction of pEMP04, NotI site underlined |
| ilvd2                             | TTAGATCTGAGGTCTTCCGGCTTCAGAGCGCTTCG          | Construction of pEMP04, XhoI site underlined |
| pip011                            | GAGGAAAGCGGCCGCGTCTTCGGCTTCAGAGCGCTTCG      | Construction of pEMP04, NotI site underlined |
| pip012                            | TTAGATCTGAGGTCTTCCGGCTTCAGAGCGCTTCG          | Construction of pEMP04, XhoI site underlined |

2.3 | Recombinant DNA work

Standardized cloning procedures such as PCR and DNA restrictions were carried out according to Sambrook and Russell, 2001. Plasmids were isolated from 5 mL liquid cultures using the E.Z.N.A.® Plasmid Mini Kit (Omega Bio-tek, Inc., Norcross, USA) following manufacturer’s instructions. PCR fragments were purified with the NucleoSpin® Gel and PCR Clean-up Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) according to the manufacturer’s instructions. Chromosomal DNA of E. coli MG1655, P. putida, C. maltaromaticum, and L. lactis was isolated using the Nucleospin® Microbial DNA Kit (Macherey-Nagel GmbH & Co. KG, Düren, Germany) following the protocol of the manufacturer. Electrocompetent cells were prepared for E. coli and P. putida as described previously [53,54]. E. coli DH5α and P. putida strains were electroporated with an Eporator (Eppendorf AG, Hamburg, Germany) at 2.5 kV with 600 Ω resistance. All enzymes for recombinant DNA work were obtained from Thermo Fisher Scientific Inc. (Darmstadt, Germany) and oligonucleotides were synthesized by biomers.net GmbH (Ulm, Germany, listed in Table 3).

2.4 | Plasmid construction

yqhD was amplified from genomic DNA of E. coli MG1655 using the primers yqhd1/yqhd2, digested with PstI/Xhol and ligated into PstI/Xhol-digested pBB1 yielding pBB1 yqhD. kivD was subsequently added before yqhD, amplified from pSA55 with the primer pair kivd1/kivd2, digested with PstI/XmaI, and ligated into PstI/XmaI-digested pBB1 yqhd creating plasmid pBB1 kivD yqhD. P_tac, kivD, and yqhD were amplified from plasmid pBB1 kivD yqhD using the primers pbb1/pbb2. The resulting PCR fragment was digested with BamHI/SacI and subsequently ligated into BamHI/SacI-digested pSEVA231 to create plasmid pIP01. Plasmid pEMP04 was constructed using the inABLE DNA assembly method from Ingenza Ltd. The B. subtilis alsS and P. putida ilvC and ilvD genes were amplified using primer pairs alss1/als2, ilvc1/ilvc2, and ilvd1/ilvd2, respectively. Additionally, a 5′ truncated version of pIP01 was amplified using primer pair pip011/pip012. The PCR products were digested using SapI and annealed oligonucleotides were ligated at each terminus. Ligation of the oligonucleotides results in the generation of 5′ and 3′ 16 nt single stranded overhangs that are complementary between fragments resulting in the DNA assembling in the predefined order. The genes of pEMP04 were amplified using the primers png1/png2 and cloned by Gibson Assembly with a PCR fragment from pEMP04 that was amplified with the primers p41/p42, p43/p44, or p45/p46 to construct plasmid pEMP014, pEMP012, and pEMP013. To exchange P_tac with araC P_BAD the genes of pEMP012, pEMP013 and pEMP014 were amplified using the primers png1/png2 for pEMP012/013 and png3/png4 for pEMP014 and cloned by Gibson Assembly into NdeI/SalI-digested pNG413.1, constructing the plasmids pIP03, pIP04, and pIP05.
2.5 | Determination of $\mu$ and $Y_{X/S}$

Growth rates were determined by linear regression of ln($\text{OD}_{600}$) plotted against time (in hours) during the exponential growth phase. Biomass yields $Y_{X/S}$ (g/g) were calculated by linear regression of the biomass concentration $c_x$ (g/L) plotted against the respective glucose concentration (g/L) during the exponential growth phase.

2.6 | Construction of P. putida deletion mutants

Chromosomal deletions in P. putida were carried out using the 5-fluorouracil (5-FU)/upp counterselection system [37]. Deletions of the bkdAA gene (encoding the α-subunit of the ketoacid dehydrogenase complex), the sthA gene (encoding soluble transhydrogenase) and the gcd gene (encoding glucose dehydrogenase) were performed using the integration vector pJOE6261.2. The flanking regions (about 500 bp) of each gene were amplified by PCR from chromosomal DNA of P. putida using the primer pairs bkdAA1/bkdAA2 and bkdAA3/bkdAA4, sthA1/sthA2, and sthA3/sthA4, gcd1/gcd2 and gcd3/gcd4. The two respective PCR fragments were purified and cloned into SalI/BamHI-restricted pJOE6261.2 by Gibson Assembly. Finally, the assembly mix was used to transform P. putida by electroporation. The first selection was carried out on LB agar with 50 μg/L kanamycin and a kanamycin-resistant clone was afterward grown in liquid LB medium for 24 h. The second recombination event was induced by plating cells on LB agar with 50 μg/L 5-FU. Deletion mutants were identified by colony PCR using the primer pairs bkdAA1/bkdAA2, sthA1/sthA2, and sthA3/sthA4, gcd1/gcd2 and gcd3/gcd4, respectively.

2.7 | Analytics

Biomass formation was measured by determination of the OD$_{600}$ (Ultraspec 10, GE Healthcare, USA) at specific time points. The cell dry weight (g$_{CDW}$/L) was correlated to the OD$_{600}$ in several independent cultivations with a correlation factor of 0.346 g$_{CDW}$/L per OD (data not shown). Shaking flasks were sampled directly in the incubator using an injection syringe (100 Sterican®, 0.80 × 120 mm, B.Braun, Melsungen, Germany). For the determination of isobutanol, 2-KIV, and 2-ketogluconate (2-KG), and glucose concentrations, 2 mL of the main culture was harvested by centrifugation (12 100 × g, 5 min, room temperature (RT)) and the supernatant was analyzed via HPLC. Glucose concentrations were measured enzymatically with a test kit from r-biopharm (r-biopharm AG, Darmstadt, Germany).

2.8 | HPLC metabolite quantification

Isobutanol, 2-KIV and 2-KG were measured with a Agilent 1200 series HPLC system equipped with a Rezex ROA organic acid H (8%) column (300 by 7.8 mm, 8 μm; Phenomenex) protected by a Phenomenex guard column carbo-H (4 by 3.0 mm inside diameter) [38]. Samples and standards were treated with a phosphate precipitation protocol before HPLC measurements. More precisely, 500 μL of sample volume was mixed with 45 μL 4 M NH$_3$ and 50 μL 1.2 M MgSO$_4$ followed by 5 min incubation at RT and centrifugation for 5 min at 7000 × g. Pellets were discarded and the supernatant was mixed with 500 μL 0.1 M H$_2$SO$_4$, incubated for 15 min at RT, and centrifuged for 15 min at 7000 × g. The resulting supernatant was used for HPLC injection with an injection volume of 10 μL. Separation was carried out under isocratic conditions at 50°C column temperature for 60 min with 5 mM H$_2$SO$_4$ as the mobile phase at a constant flow rate of 0.4 mL/min. Detection of isobutanol, 2-KIV, and 2-KG was achieved with a refractive index detector at 32°C. Quantification of all analytes was done with a 7-point calibration curve for each component as an external reference standard.

3 | RESULTS

3.1 | Preventing product and precursor degradation

Pseudomonads are well-known for their ability to degrade a variety of organic substances to utilize them as carbon and energy sources [21]. Since the genomic repertoire provides annotated routes for the degradation of isobutanol and 2-ketoisovalerate (Figure 1), we initially characterized growth on both compounds (Figure 2). P. putida showed exponential growth on isobutanol with a $\mu$ of 0.27 ± 0.01 h$^{-1}$ as well as on 2-KIV with a $\mu$ of 0.33 ± 0.01 h$^{-1}$ that is 52% of the growth rate on glucose (Figure 2A,B). Recently, several enzymes involved in n-butanol degradation were identified [39] and Simon et al. [30] constructed P. putida Δupp ΔpedE ΔpedI ΔpedH ΔaldB-I (P. putida GN346) to inactivate two alcohol dehydrogenases (PedE, PedH) and two aldehyde dehydrogenases (PedI, AldB-I) and showed that the introduced deletions prevented n-butanol consumption. Accordingly, P. putida GN346 was unable to utilize isobutanol as sole carbon and energy source (Figure 2A).

P. putida possesses a branched chain ketoacid dehydrogenase (BCKDH) complex that converts 2-ketoacids to the respective decarboxylated CoA-derivatives [40,41] which are, after further conversion steps, funneled into the TCA cycle. To prevent the consumption of the precursor 2-KIV, we inactivated the α-subunit of the BCKDH by deletion of the bkdAA gene in P. putida GN346. In contrast to the wild-type, the resulting strain P. putida EP1 was unable to grow on 2-KIV as carbon source (Fig. 2B), and therefore was used as basis for further strain engineering.
3.2 | Engineering *P. putida* for isobutanol production

To drain the carbon from pyruvate to 2-KIV, we constructed a plasmid harboring the *alsS* gene encoding the acetolactate synthase from *Bacillus subtilis*, which is not feedback inhibited by branched chain amino acids, and the native ilvCD genes encoding the ketolacid reductoisomerase and dihydroxyacid dehydratase (Figure 1). For the conversion of 2-KIV to isobutanol, we additionally cloned *kivD* encoding the KDC from *Lactococcus lactis* and *yqhD* encoding an aldehyde reductase from *E. coli* (Figure 1). *AlsS*, *KivD*, and *YqhD* were previously applied for isobutanol production in other hosts such as *C. glutamicum* and *E. coli* [5,7]. The resulting plasmid pIP02 expresses all cloned genes under control of the L-arabinose inducible P<sub>BAD</sub> promoter and was used to transform *P. putida* EP1 yielding *P. putida* Iso1. In minimal medium with glucose, *P. putida* Iso1 showed a $\mu = 0.56 \pm 0.02$. Although the $Y_{X/S}$ was reduced by 25% compared to the wild-type, no isobutanol was produced during the cultivation (Table 2).

The synthesis of isobutanol from glucose requires 2 mol NAD(P)H per mol isobutanol. The reduction of acetolactate is catalyzed by NADPH-dependent ketolacid reductoisomerase (IvC), while the conversion of isobutyraldehyde to isobutanol can be catalyzed by NAD(P)H-dependent aldehyde/alcohol dehydrogenases such as *YqhD* (Figure 1). Since *YqhD* is NADPH-dependent, the engineered isobutanol pathway should consume 2 mol NADPH per mol isobutanol. *P. putida* possesses a membrane-bound and a soluble transhydrogenase. The latter is encoded by the *sthA* gene [42] and has in *E. coli* been reported to favor the re-oxidation of NADPH to NADP<sup>+</sup> under reduction of NAD<sup>+</sup> to NADH [43,44].

To test whether the inactivation of the soluble transhydrogenase is beneficial for isobutanol production, we deleted the *sthA* gene in *P. putida* EP1, yielding *P. putida* EP2 which was transformed with the plasmid pIP02. The resulting strain *P. putida* Iso2 showed in minimal medium containing 5.4 g/L glucose, a growth rate of $0.26 \pm 0.01$ h<sup>-1</sup>, a $Y_{X/S}$ of of $0.13 \pm 0.01$ g/g, and produced $438 \pm 20$ mg/g<sub>GLC</sub> 2-KG and for the first time isobutanol with a $Y_{Iso/S}$ of $22 \pm 2$ mg/g<sub>GLC</sub> (Table 2, Figure 3). We also replaced the aldehyde reductase gene *yqhD* on the overexpression plasmid pIP02 with the *adhA* genes encoding NADH-dependent alcohol dehydrogenase variants from *Lactococcus lactis* and *Corynebacterium glutamicum*, respectively. The plasmids pIP03 and pIP04 were used to transform *P. putida* EP2, yielding *P. putida* Iso3 and Iso4.

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**TABLE 2** Overview of growth, 2-ketogluconate (2-KG) and isobutanol production of *P. putida* and its engineered derivatives

| Strain | $\mu$ [h<sup>-1</sup>] | $Y_{X/S}$ [g/g] | $Y_{2-KG/S}$ [mg/g<sub>GLC</sub>] | $Y_{Iso/S}$ [mg/g<sub>GLC</sub>] |
|--------|-----------------|----------------|-------------------------------|-------------------------------|
| KT2440 | $0.62 \pm 0.01$ | $0.40 \pm 0.01$ | 0                             | 0                             |
| GN346  | $0.59 \pm 0.01$ | $0.39 \pm 0.01$ | 0                             | 0                             |
| Iso1   | $0.56 \pm 0.02$ | $0.30 \pm 0.01$ | 0                             | 0                             |
| Iso2   | $0.25 \pm 0.01$ | $0.13 \pm 0.01$ | $438 \pm 20$                  | $22 \pm 2$                   |
| Iso3   | $0.14 \pm 0.01$ | $0.06 \pm 0.01$ | $833 \pm 100$                 | $13 \pm 1$                   |
| Iso4   | $0.19 \pm 0.01$ | $0.09 \pm 0.02$ | $771 \pm 15$                  | $14 \pm 0.0$                 |
| Iso5   | $0.18 \pm 0.02$ | $0.28 \pm 0.01$ | 0                             | 0                             |
| Iso6   | $0.28 \pm 0.01$ | $0.10 \pm 0.01$ | $633 \pm 39$                  | $21 \pm 1$                   |

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**FIGURE 2** (A) Growth of *P. putida* KT2440 and *P. putida* GN346 in DeBont minimal medium containing 0.5 g/L isobutanol (filled symbols) or 5.4 g/L glucose (open symbols). (B) Growth of *P. putida* and *P. putida* EP1 in DeBont minimal medium containing 2.9 g/L 2-ketoisovalerate. Experiments were performed in triplicates and error bars represent the corresponding standard deviation.
which were characterized in minimal medium with glucose (Table 2). Both strains showed reduced growth rates and about 40% lower product yields compared to P. putida Iso2. All engineered strains with deletion of sthA converted 40% to 83% of the available glucose into 2-KG that was secreted into the culture broth (Table 2). To avoid 2-KG secretion and to improve isobutanol production, we constructed P. putida EP3 by deletion of the gcd gene encoding periplasmatic glucose dehydrogenase in P. putida EP2. To construct P. putida Iso5, P. putida EP3 was transformed with the plasmid pIP02. In fact, P. putida Iso5 did not secrete any 2-KG, however, inactivation of GCD also abolished isobutanol production completely (Table 2).

### 3.3 Ketoacid decarboxylase from Carnobacterium maltaromaticum is suitable for isobutanol production

The key enzyme for isobutanol production via the Ehrlich pathway is ketoacid decarboxylase (KDC) converting 2-KIV to isobutyraldehyde (Figure 1). So far, only KDC from L. lactis has been proven as suitable variant that efficiently catalyzes this reaction [2,5]. Recently, the genome sequence of Carnobacterium maltaromaticum LMA28 [28] was published that harbors a gene encoding a putative KDC. KDC from C. maltaromaticum shows 54% identity to the KDC enzyme from L. lactis. To test the suitability of KDC from C. maltaromaticum for isobutanol production, we replaced the kivD gene on plasmid pIP02 with the respective gene from C. maltaromaticum. Plasmid pIP05 was transformed into P. putida EP2 and the resulting strain P. putida Iso6 was characterized. P. putida Iso6 showed a growth rate of 0.28 ± 0.01 h⁻¹, a YX/S of 0.10 ± 0.01 g/g, and produced 633 ± 39 mg/gGLC 2-KG. Furthermore, P. putida Iso6 secreted as much isobutanol as P. putida Iso2 with a Yx/s of 21 ± 1 mg/gGLC (Table 2) showing that KDC from C. maltaromaticum LMA28 is a useful alternative to KDC from L. lactis.

### 3.4 Microaerobic isobutanol production in P. putida

P. putida is regarded as an obligate aerobic bacterium [18]. However, since the implementation of the synthetic isobutanol pathway theoretically enables a closed redox balance, we tested the capabilities of our engineered P. putida strains to produce isobutanol from glucose in a zero-growth bioprocess under oxygen deprivation conditions [45]. Therefore, we inoculated P. putida WT and Iso2–6 to an OD₆₀₀ of 15–20 in closed bottles filled with minimal medium containing 5.4 g/L glucose and characterized substrate consumption and (by-) product formation (Table 3). In the micro-aerobic environment P. putida WT showed no growth, but remained metabolically active and consumed the glucose that was converted to 2-KG. With the exception of P. putida Iso5, all other engineered strains consumed glucose and produced isobutanol. P. putida Iso6 showed the best performance under oxygen deprivation conditions. Compared to the WT the qs was reduced by 37% and P. putida Iso6 produced about 10% less isobutanol compared to the aerobic shaking flask experiments (Table 3).

### Table 3 Overview of engineered P. putida strains cultivated under oxygen deprivation conditions

| Strain | Yx/S [mg/gGLC] | qs [g g⁻¹ h⁻¹] | Y2-KG/S [mg/gGLC] |
|--------|----------------|----------------|-------------------|
| KT2440 | 0              | 0.11 ± 0.01    | 42 ± 32           |
| Iso2   | 9 ± 1          | 0.14 ± 0.01    | 120 ± 9           |
| Iso3   | 5 ± 2          | 0.12 ± 0.01    | 183 ± 8           |
| Iso4   | 4 ± 1          | 0.13 ± 0.01    | 193 ± 12          |
| Iso5   | 0              | 0.01 ± 0.00    | 0                 |
| Iso6   | 19 ± 2         | 0.07 ± 0.01    | 397 ± 14          |

### 4 DISCUSSION

P. putida is an emerging host for industrial biotechnology [46–48]. However, this bacterium is also known to efficiently metabolize a broad range of substrates including amino and organic acids and alcohols [21]. As shown here, P. putida grows rapidly on isobutanol as well as on its precursor 2-KIV. Although P. putida KT2440 possesses four aldehyde dehydrogenases and about 10 alcohol dehydrogenases, Simon et al. [30] showed that deletion of the two alcohol dehydrogenase genes pedE and pedH and the two aldehyde dehydrogenase genes pedI and aldB-I is sufficient to prevent n-butanol degradation. Accordingly, we found that this strain background also prevents growth on
the branched-chain alcohol isobutanol. P. putida possesses a branched chain ketoacid dehydrogenase complex that converts 2-ketoacids to the respective decarboxylated CoA-derivatives [40,41]. As expected and also observed for P. taiwanensis VLB120 [41], inactivation of the BKDH abolished growth on 2-ketoisovalerate. To avoid auxotrophies, we relinquished the inactivation of the L-valine forming transaminase IlvE, the 2-isopropylmalate synthase LeuA and the 2-ketoisovalerole hydroxymethyltransferase PanB as has been applied to improve isobutyric acid production with P. taiwanensis strain VLB120 [41].

Since AHAIR is usually NADPH-dependent, the synthesis of one molecule of isobutanol either requires two molecules of NADPH or one NADH plus one NADPH molecule depending on the applied alcohol/aldheyde dehydrogenase variant for the reduction of isobutyraldehyde to isobutanol. Optimization of NAD(P)H availability has already been shown to be a crucial factor for isobutanol production with other hosts such as E. coli and C. glutamicum [6,49]. Recently, Nikel et al. [16] showed that P. putida cells growing on glucose exhibit a slight catabolic overproduction of reducing power and run a biochemical cycle that favors NADPH formation. Therefore, we applied in our experiments the broad-substrate range NADPH-dependent aldehyde reductase YqhD [50], which has also been successfully applied for isobutanol production with E. coli [8]. However, expression of the synthetic pathway in P. putida Iso1 to channel pyruvate toward isobutanol did not result in isobutanol production from glucose. Similar to E. coli, P. putida possesses a membrane bound (PntAB) and a soluble transhydrogenase (SthA) to balance the overall redox state of the cell (Figure 1). SthA has in E. coli been reported to favor the oxidation of NADPH to NADP⁺, accompanied with the reduction of NAD⁺ to NADH [43,44]. To improve NADPH availability, we inactivated SthA that resulted in isobutanol formation in P. putida Iso2 under aerobic conditions. Accordingly, expression of two adhA genes encoding NADH-dependent alcohol dehydrogenases from L. lactis and C. glutamicum, which have previously been shown to be suitable for isobutanol production [7,8], instead of YqhD, led to significantly reduced isobutanol yields in the ΔsthA background (Table 2).

Inactivation of SthA resulted in isobutanol production, however, also in the secretion of significant amounts of 2-KG. In P. putida a majority of the glucose is converted in the periplasm by glucose dehydrogenase (Gcd) to gluconate, which is transported to the cytoplasm and activated by the gluconate kinase to feed the Entner–Doudoroff pathway with 6-phosphogluconate. Usually, only a small fraction of gluconate is converted in the periplasm by gluconate dehydrogenase to 2-KG, which is subsequently transported into the cytoplasm to finally form 6-phosphogluconate via 2-KG kinase and 2-ketogluconate-6-P reductase [16]. Since deletion of gcd abolished 2-KG production completely, the synthesis of this molecule occurs solely in the periplasm via the described route. The accumulation of 2-KG in the culture broth indicates a transport inhibition of gluconate and/or 2-KG from the periplasm to the cytoplasm by an unknown mechanism and/or an inhibition or limitation of the ATP-dependent conversion to the phosphorylated derivatives. The latter might result as consequence of a perturbed redox state due to the inactivated transhydrogenase SthA.

P. putida is an obligate aerobic bacterium, however, in a bioelectrochemical system P. putida was metabolically active under anoxic conditions when an electron mediator was applied for redox balancing in a high-yield 2-KG production system [51,52]. Since isobutanol synthesis enables regeneration of NAD(P)⁺, we cultivated P. putida WT and the engineered derivatives under microaerobic conditions. All strains showed no growth (data not shown) but with the exception of P. putida Iso5, remained metabolically active and P. putida Iso2-4 and 6 also secreted 2-KG, isobutanol, and further unidentified products. However, according to the zero-growth the qS values are low compared to aerobic conditions (e.g. for P. putida WT 0.11 vs. 1.55 g g⁻¹ h⁻¹). The capability of P. putida to remain metabolically active opens the possibility to develop dual-phase production processes that comprise an aerobic growth phase for rapid biomass formation and a micro-aerobic or anaerobic production phase [45].

This study paths the way to construct more efficient P. putida strains for isobutanol production in future studies. The overall isobutanol yield is significantly higher compared to other engineered P. putida strains [41], however, rather low compared to tailored E. coli and C. glutamicum strains [49]. Product and precursor degradation can be prevented by the presented deletions in this study, however, improving NAD(P)H and pyruvate availability [49] will be crucial to achieve high-yield isobutanol production strains.

ACKNOWLEDGEMENTS

We thank Mira Lenfers-Lück and Sven Göbel (Institute of Biochemical Engineering, University of Stuttgart, Germany) for assistance during HPLC analysis and experimental procedures. This work was funded by the European Commission H2020 project Empowerputida under the grant agreement No. 635536. This work was supported by the German Research Foundation (DFG) and the Technical University of Munich (TUM) in the framework of the Open Access Publishing Program.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.
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How to cite this article: Nitschel R, Ankenbauer A, Welsch I, et al. Engineering *Pseudomonas putida* KT2440 for the production of isobutanol. *Eng Life Sci.* 2020;20:148–159. https://doi.org/10.1002/elsc.201900151