Metabolic engineering of *Corynebacterium glutamicum* for *de novo* production of 3-hydroxycadaverine

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**ABSTRACT**

Functionalization of amino acids and their derivatives opens up the possibility to produce novel compounds with additional functional groups, which can expand their application spectra. Hydroxylation of polyamide building blocks might allow crosslinking between the molecular chains by esterification. Consequently, this can alter the functional properties of the resulting polymers. *C. glutamicum* represents a well-known industrial workhorse and has been used extensively to produce lysine and lysine derivatives. These are used as building blocks for chemical and pharmaceutical applications. In this study, it was shown for the first time that C3-hydroxylated cadaverine can be produced *de novo* by a lysine overproducing *C. glutamicum* strain. The lysine hydroxylase from *Flavobacterium johnsoniae* is highly specific for its natural substrate lysine and, therefore, hydroxylation of lysine precedes decarboxylation of 4-hydroxylsine (4-HL) to 3-hydroxycadaverine (3-HC). For optimal precursor supply, various cultivation parameters were investigated identifying the iron concentration and pH as major effectors on 4-HL production, whereas the supply with the cosubstrate 2-oxoglutarate (2-OG) was sufficient. Deletion of the gene coding for the lysine exporter LysE suggested that the exporter may also be involved in the export of the structurally similar 4-HL. With the optimised setting for 4-HL production, the pathway was extended towards 3-HC by decarboxylation. Three different genes coding for lysine/4-HL decarboxylases, LdcC and CadA from *E. coli* and DC, from *F. johnsoniae*, were expressed in the 4-HL producing strain and compared regarding 3-HC production. It was shown in a semi-preparative biocatalysis that all three decarboxylases can accept 4-HL as substrate with varying efficiencies. In vivo, LdcC supported 3-HC production best with a final titer of 11 mM. To improve titers a fed-batch cultivation in 1 L bioreactor scale was performed and the plasmid-based overexpression of *LdcC* was induced after 24 h resulting in the highest titer of 8.6 g L⁻¹ (74 mM) of 3-hydroxycadaverine reported up to now.
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Introduction

Microbial enzymes are exceptionally attractive to be incorporated in chemical processes as they are sustainable and environmentally friendly (Sheldon and Woodley, 2018). One major advantage of these biocatalysts is their ability to catalyse interconversions of many functional groups with well-defined selectivity (chemo-, regio-, stereo-) ensuing reduced waste during synthesis and high atom economy. The recent developments in bioinformatics, synthetic biology, and computational biology enabled a deeper understanding of the enzymatic reactions and the establishment of effective biotransformation systems for industrial use (Bornscheuer et al., 2012; Sun et al., 2018). It is important to discover novel enzymes or unknown side activities of known enzymes to access new products, e.g. for the functionalization of non-activated C-H bonds, which are difficult for organic chemists (Bastard et al., 2018). One group of enzymes, which catalyse the formation of various C-heteroatom bonds, are the iron (II)/α-ketoglutarate dependent dioxygenases (KDO) (Hausinger, 2004; Wan et al., 2017), that e.g., hydroxylate C-H bonds (Dunham et al., 2018; Hausinger, 2004). These enzymes require the cofactor iron (II) and three substrates: molecular oxygen, 2-OG and a primary substrate. One oxygen atom is transferred to the primary substrate to yield the hydroxylated product, and the second oxygen is used for oxidation of 2-OG to succinate and carbon dioxide (Bastard et al., 2018; Martínez and Hausinger, 2015; Mitchell et al., 2017). Typical substrates for these metalloenzymes are amino acids, which are hydroxylated. KDOs are mainly involved in the biosynthesis of secondary metabolites and can be found in bacteria, where they hydroxylate the side chains of free amino acid (derivatives) or tether peptides in non-ribosomal peptide biosynthesis (Wu et al., 2016). The hydroxylated amino acids are highly attractive intermediates in pharmaceutical industries and find application as valuable chiral building blocks for fine chemistry (Jing et al., 2021; Ren and Fasan, 2021). KDOs that are active towards amino acids and their derivatives belong to the Clavamine Synthase Like (CSL) family. They are highly substrate specific and exhibit high regio- and stereoselectivities (Bastard et al., 2018; Hara et al., 2017). Among the KDOs which act specifically on L-lysine, the KDOs from Catenulidpora apicillum (Baud et al., 2014; Peters and Buller, 2019) and Kineococcus radiotolerans SRS30216 (Hara et al., 2017) yield L-3-hydroxylysine, while the KDOs from Flavobacterium johnsoniae UW101 (Bastard et al., 2018; Baud et al., 2014) or Niasella korensis (Wang et al., 2020) show different regioelectivity and form D-4-hydroxylysine.

As the physiological function of hydroxylysine depends on the location of the hydroxyl group, the regio- and stereoselective synthesis is highly important. In nature, the most abundant isomer of hydroxylated lysine is D-5-hydroxylysine and it is found in a particular type of collagen peptide (Peters and Buller, 2019). It frequently occurs in the extracellular matrix of animal cells, where it stabilizes the collagen scaffold by subsequent O-glycosylation. Moreover, L-lysine residues present in proteins can be hydroxylated as a posttranslational modification by lysyl hydroxylase (EC 1.14.11.4) (Turpeenniemi-Hujanen and Myllylä, 1984). 5-hydroxylysine (5-HL) can also be found in bacteria, like Staphylococcus aureus, where it is used as a cell-wall precursor instead of lysine (Smith et al., 1965). Moreover, regio- and stereoisomers are highly demanded as synths for pharmaceutical agents. For example, C3-hydroxylation of L-lysine provides the precursor for synthesis of the antibiotic tambroline (X. Zhang et al., 2018), a precursor of the antibiotic tambromycin (Goering et al., 2016). In addition, L-3-hydroxylysine (3-HL) is an intermediate in the synthesis of (-)-balanol in Verticillium balanoides, which is a potent protein kinase C inhibitor (Lampe et al., 1996). L- and D-4-Hydroxylysine (4-HL) are also promising precursors for pharmaceutical agents, like functionalized piperidine-2-ones, which are highly versatile building blocks for the synthesis of many bioactive substances (Herbert et al., 2012). They can also be used for the synthesis of 4-hydroxypipeolic acid, a constituent of certain cyclic peptide antibiotics; palinavir, a potent HIV protease inhibitor (Lamarre et al., 1997), or for the anti-cancer agent Glidobactin A (Amatuni and Renata, 2019).

Like lysine, one of the most industrially relevant amino acids, hydroxylysine could be used as a precursor for chemical synthesis. By decarboxylation of lysine, the C2-diamine polymer building block cadaverine, also known as 1,5-diaminopentane, can be obtained (Cheng et al., 2018). Using hydroxylysine as substrate would yield hydroxylated cadaverine, which could be incorporated as novel building block for polyamides with new properties. Additional hydroxyl groups are attractive in polymers, as they can undergo various reactions, e.g., esterification. Moreover, they are hydrophilic and can act as initiation sites for ring-opening polymerization of cyclic esters, thus enabling easy access to complex polymers (Gómez and Varela, 2007; Kakwere and Perrier, 2011). Orgueira et al. (2001) showed the production of a hydroxylated polymer derived from the building blocks cadaverine and δ-2-hydroxyglutarate (Orgueira et al., 2001). Biomaterials, which are derived from polysaccharides showed promising applications in biomedical realms because of their good biocompatibility on the hydroxyl-enriched material surfaces (Yang and Yang, 2014). For the production of cadaverine several approaches have been established, including efficient whole-cell biocatalysis in E. coli (Kim et al., 2019). Furthermore, C. glutamicum has been engineered for in vivo production of cadaverine by overexpression of the genes coding for different pyridoxal phosphate (PLP) dependent decarboxylases from E. coli: CadA (Mimitsuka et al., 2007) and LdcC (Kind et al., 2011). The deletion of snaA coding for spermi(di)ne-N-
acetyltransferase increased product titers by avoiding N-acetylation of cadaverine (Kind et al., 2010; Nguyen et al., 2015b). Further optimisation strategies included the application of synthetic promoter-based expression cassettes and integration of different ldcC variants in the genome yielding up to 125 g L\(^{-1}\) cadaverine (Kind et al., 2010; Nguyen et al., 2015b). Further optimisation strategies included the application of synthetic promoter-based expression cassettes and integration of different ldcC variants in the genome yielding up to 125 g L\(^{-1}\) cadaverine (Kim et al., 2018; Oh et al., 2015). C. glutamicum was further engineered to exploit starch, wheat sidestream concentrate hydrolysate (WSCH) and methanol as carbon sources for the production of cadaverine (Burgardt et al., 2021; Leßmeier et al., 2015; Tateno et al., 2009).

In this study, a lysine overproducing C. glutamicum strain was chosen to extend the lysine biosynthesis pathway to hydroxylated lysine by overexpression of codon-optimised genes coding for KDOs with different regiospecificity to yield either 3-HL (KDO\(_{Kr}\) encoded by Krad_3958 from K. radiotolerans) or 4-HL (KDO\(_{Fj}\) encoded by Fjoh_3169 from F. johnsoniae). To improve production, the effects of cofactor and cosubstrate supply as well as substrate/product export and pH alterations were tested. Since hydroxylated lysines can be decarboxylated in vitro to hydroxylated cadaverines by different decarboxylases as demonstrated by Bastard et al. (2018), the substrate specificity of the lysine decarboxylases LdcC (Yamamoto et al., 1997) and CadA (Sabo et al., 1974) from E. coli and the predicted 4-hydroxylysine decarboxylase DC\(_{Fj}\) (Fjoh_3171) (Bastard et al., 2018) were investigated with regard to produce 3-hydroxycadaverine (3-HC) from 4-HL (Fig. 1). Additionally, the inverse route was explored with decarboxylation of lysine to cadaverine prior to hydroxylation of cadaverine to yield 3-hydroxyxycadaverine.

### Material and methods

#### Microorganisms and cultivation conditions

*C. glutamicum* WT strains were cultivated in lysogeny broth (LB) (Bertani, 1951) supplemented with 25 μg mL\(^{-1}\) kanamycin. *C. glutamicum* GRLys1 derived strains were cultivated in brain heart infusion with 0.5 M sorbitol (BHIS), supplemented with 25 μg mL\(^{-1}\) kanamycin, 100 μg mL\(^{-1}\) spectinomycin or 5 μg mL\(^{-1}\) tetracycline, when appropriate. All bacterial strains and plasmids are listed in Tables 1 and 2. Growth experiments with *C. glutamicum* were performed in CGXII minimal medium at pH 7.0 (Eggeling and Bott, 2004) supplemented with 40 g L\(^{-1}\) glucose as sole carbon source and induced with 1 mM IPTG if necessary. The amount of iron and the pH were adjusted to 1.04 mM and 6.5, respectively, as indicated in the result section. Overnight cultures were harvested and washed twice in TN buffer (50 mM Tris-HCl, 50 mM NaCl, pH 6.3) before inoculation to an initial OD\(_{600}\) of 1. The cultivations in the BioLector microcultivation system (m2p-labs, Baesweiler, Germany) were performed in 3.2 mL Flower-Plates at 30 °C, 1100 rpm and a filling volume of 1000 μL.

#### Molecular biology methods

Isolation of genomic DNA and classical methods which include plasmid isolation, molecular cloning and heat-shock transformation of *E. coli* and electroporation of *C. glutamicum* were performed as described previously (Eikmanns et al., 1994; Simon et al., 1983).

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**Fig. 1.** Schematic overview of the predicted route towards 3-hydroxycadaverine from lysine. Enzymes are depicted next to their reaction. Heterologous proteins are boxed. Deletion of coding gene is marked by a red cross. DC: Decarboxylase from different organisms (lysine decarboxylase from *E. coli* MG1655 (LdcC, CadA), PLP-dependent decarboxylase from *Flavobacterium johnsoniae* UW101 (DC\(_{Fj}\)) ; KDO\(_{Fj}\): α-ketoglutarate dependent dioxygenase/lysine 4-hydroxylase from *Flavobacterium johnsoniae* UW101; SnaA: spermi(di)ne-N-acetyltransferase from *C. glutamicum* WT; LysE: lysine/4-HL exporter; CgmA: cadaverine/3-HC exporter, PTS: phosphotransferase system). Question marks display transport systems that may be involved for export of 4-HL and 3-HC, respectively, based on genetic evidence, but in the absence of biochemical evidence. Dashed lines represent multiple reactions. 2-OG: 2-oxoglutarate; SA: succinic acid; TCA: tricarboxylic acid cycle; AR: anaplerotic reaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
The oligonucleotides which were used as primers in this study are to amplify DNA sequences with plasmid or genomic DNA as template. ALLin HiFi DNA Polymerase (HighQu, Kraichtal, Germany) was used.

**Table 1**

| Strain          | Relevant Characteristics                  | Reference/source |
|-----------------|-------------------------------------------|------------------|
| E. coli DH5α    | ΔlacU169 (q80lacZ ΔM15), aspE44,             | Hanahan, 1985    |
|                 | hisD17, recA1, endA1, gyrA96, thr-1,        |                  |
|                 | relA1                                      |                  |
| C. glutamicum   | ATCC 13002 (DM1933ΔGFP123)                 |                  |
| GRLys1          | ATCC C. glutamicum with modifications: Δpsk, |                  |
|                 | psk<sup>exo</sup>, hom<sup>DNA</sup> – 2 copies |                  |
|                 | of lys<sup>G</sup>Δ111, 2 copies of asd,    |                  |
|                 | 2 copies of dapA, 2 copies of dapB, 2       |                  |
|                 | copies of lysA, 2 copies of lysF, in-      |                  |
|                 | frame deletion of prophages CGP1           |                  |
|                 | (cg1507-cg1524), CGP2 (cg1746-cg1752) and  |                  |
|                 | CGP3 (cg1899-cg2071).                      |                  |

Table 2

| Strain          | Relevant Characteristics                  | Reference     |
|-----------------|-------------------------------------------|---------------|
| pECX99A-        | Tet<sup>+</sup>, C. glutamicum/E. coli     | Henke et al.,|
| P<sub>amp</sub>-| shuttle vector (P<sub>amp</sub>)          | 2022          |
| pECX99A-        | Flavobacterium johnsoniae UW101           |               |
| KDO<sub>2</sub>|                            |               |
| pEKEx3         | Spe<sup>-</sup>, C. glutamicum/E. coli     | Stansen et al.,|
|                | shuttle vector (P<sub>amp</sub> lac<sup>+</sup> | 2005          |
|                | pBluI, ori<sub>V</sub>C)                  |               |
| pEKEx3-         | pEKEx3, expressing adh (cg1630)         | This study    |
| odhT<sup>14A</sup> | carrying SNP T14A from C.              |               |
| glutamicum WT   |                                            |               |
|                  | pEKEx3-lysF                              | Lubitz et al.,|
|                  | pEKEx3, expressing lysF (cg1424)         | 2016          |
|                  | from C. glutamicum WT                    |               |
| pVWE1x          | Kan<sup>+</sup>, C. glutamicum/E. coli     | Peters-        |
|                | shuttle vector (P<sub>amp</sub> lac<sup>+</sup>) | 2001          |
| pVWE1x          | pVWE1x, expressing lclC (CX41_00970)     | Jorge et al., |
|                | from E. coli MG1655                      | 2017          |
| pVWE1x          | pVWE1x, expressing cadA (CX41_22535)     |               |
|                | from E. coli MG1655                      |               |
| pVWE1x          | pVWE1x, expressing Fjoh_3171 from        |               |
|                | Flavobacterium johnsoniae UW101          |               |
| pVWE4x          | Kan<sup>+</sup>, C. glutamicum/E. coli     | Henke et al.,|
|                | shuttle vector (P<sub>amp</sub> lac<sup>+</sup>) | 2022          |
| pVWE4x          | pVWE4x expressing KDO<sub>2</sub> (Krd_3958) |               |
| pVWE4x          | pVWE4x expressing KDO<sub>2</sub> (Fjoh_3169) |               |
| pVWE4x          | Flavobacterium johnsoniae UW101           |               |

Unthan et al., 2015

GSL
GRLys1 with in-frame deletions: modi
(cg2115), idaA (cg3219) from
Pérez-García et al., 2018

GSLA
GSL with in-frame deletion: znuA
(cg1722) from
Pérez-García et al., 2018

GSL2
GSL with deletion of the two copies of
lysF (cg1424) from
Pérez-García et al., 2017

GSLA2
GSL with in-frame deletion: cgmA
(cg2893) from
Pérez-García et al., 2018

Lys1
GSL (pVWE4) from
This study

Lys2
GSL2 (pVWE4) (pEKEx3) from
This study

Lys3
GSL2 (pVWE4) (pEKEx3-lysF) from
This study

Lys4
GSL (pVWE4) (pEKEx3) from
This study

Lys5
GSL (pVWE4) (pEKEx3-odh<sup>T14A</sup>) from
This study

Lys6
GSLA (pECX99A-P<sub>amp</sub>) (pVWEEx1) from
This study

HLys1
GSL (pVWEEx4-KDO<sub>2</sub>) from
This study

HLys2
GSL2 (pVWEEx4-KDO<sub>2</sub>) from
This study

HLys3
GSL2 (pVWEEx4-KDO<sub>2</sub>) from
This study

HLys4
GSL (pVWEEx4-KDO<sub>2</sub>) from
This study

HLys5
GSL (pVWEEx4-KDO<sub>2</sub>) from
This study

HLys6
GSLA (pECX99A-P<sub>amp</sub>-KDO<sub>2</sub>) from
This study

HLys6ΔcgmA
GSL2A (pECX99A-P<sub>amp</sub>-KDO<sub>2</sub>) from
This study

pVWEEx1
GSLA (pECX99A-P<sub>amp</sub>-KDO<sub>2</sub>) from
This study

Table 2

| Strain          | Relevant Characteristics                  | Reference     |
|-----------------|-------------------------------------------|---------------|
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| P<sub>amp</sub>-| shuttle vector (P<sub>amp</sub>)          | 2022          |
| pECX99A-        | Flavobacterium johnsoniae UW101           |               |
| KDO<sub>2</sub>|                            |               |
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|                | shuttle vector (P<sub>amp</sub> lac<sup>+</sup> | 2005          |
|                | pBluI, ori<sub>V</sub>C)                  |               |
| pEKEx3-         | pEKEx3, expressing adh (cg1630)         | This study    |
| odhT<sup>14A</sup> | carrying SNP T14A from C.              |               |
| glutamicum WT   |                                            |               |
|                  | pEKEx3-lysF                              | Lubitz et al.,|
|                  | pEKEx3, expressing lysF (cg1424)         | 2016          |
|                  | from C. glutamicum WT                    |               |
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|                | from E. coli MG1655                      | 2017          |
| pVWE1x          | pVWE1x, expressing cadA (CX41_22535)     |               |
|                | from E. coli MG1655                      |               |
| pVWE1x          | pVWE1x, expressing Fjoh_3171 from        |               |
|                | Flavobacterium johnsoniae UW101          |               |
| pVWE4x          | Kan<sup>+</sup>, C. glutamicum/E. coli     | Henke et al.,|
|                | shuttle vector (P<sub>amp</sub> lac<sup>+</sup>) | 2022          |
| pVWE4x          | pVWE4x expressing KDO<sub>2</sub> (Krd_3958) |               |
| pVWE4x          | pVWE4x expressing KDO<sub>2</sub> (Fjoh_3169) |               |
| pVWE4x          | Flavobacterium johnsoniae UW101           |               |

Quantification of amino acids, carbohydrates and organic acids by HPLC

The quantification of extracellular amino acids, their derivatives and carbohydrates in the cultivation medium was performed with a high-performance liquid chromatography system (1200 series, Agilent Technologies Deutschland GmbH, Böblingen, Germany). After centrifugation of 1 ml of cell cultures at 20238 g for 10 min the supernatant was stored at −20 °C prior to analysis. Analysis of amino acids, diamines and ω-amino acids was performed by an automatic pre-column derivatization with ortho-phthalaldehyde (OPA) and separated on a reversed phase HPLC using a a pre-column (LiChropher 100 RP18 EC-5 μ (40 × 4 mm), CS Chromatographic Service GmbH, Langerwehe, Germany) and a main column (LiChropher 100 RP18 EC-5 μ (125 × 4 mm), CS Chromatographic Service GmbH). A mobile phase of buffer A (0.25% (v/v) sodium acetate, pH 6.0) and buffer B (methanol) was used. The following gradient was applied: 0–1 min 20 % B (0.7 ml min<sup>−1</sup>), 1–11 min a linear gradient of B from 20% to 85% (1.2 ml min<sup>−1</sup>), 12–13 min a linear gradient of B from 85% to 20% (1.2 ml min<sup>−1</sup>). To quantify amino acids, diamines and ω-amino acids, the standards of ornithine, lysine, 5-HL, putrescine, cadaverine, GABA and 5AVA were analysed in a range from 50 to 800 μM. The peak area was normalised to the internal standard of 100 μM asparagine (Schneider and Wendisch, 2010) by division of the respective standard peak area by the area of the internal standard.
The area quotient was plotted against the concentration of the respective standard. The slope of the calibration curve was used to quantify the area quotient. The slope of the calibration curve was used to quantify the biocatalytic activity of the hydroxylase. To identify the substrate specificity of the different decarboxylases, the following assay was performed: 1.5 mL reaction mix contained 50 mM HEPES buffer (pH 7.5), 1 mM PLP, 1 mM DTT and 10 mM of the respective substrate (lysine, 5-HL, 4-HL from fermentation broth) (Baud et al., 2017). The reaction was started by addition of 1 mg mL⁻¹ protein. Incubation of the reaction mix, sampling, sample treatment and analysis were performed as described for the KDO assay.

**Fermentative production of 3-NC**

A baffled bioreactor with a total volume of 3.6 L was used for scale-up experiments (KLF, Bioengineering AG, Switzerland). Two six-bladed Rushton turbines were placed on the stirrer axis with a distance from the bottom of the reactor of 6 and 12 cm. The stirrer to reactor diameter ratio was 0.39. The dissolved oxygen concentration in the batch phase was kept at a minimum of 30% by stepwise increases of the stirrer rate. A constant airflow of 0.75 L min⁻¹ was maintained from the bottom through a sparger, corresponding to an aeration rate of 0.75 vvm. The pH was kept constant at 6.50 ± 0.05 by automatic addition of phosphoric acid (10% (v/v)) and ammonia (25% (w/v)). The temperature was maintained at 30 °C. To prevent foaming 0.6 mL L⁻¹ of the anti-foam agent AF204 (Sigma Aldrich, Darmstadt, Germany) was added, and a mechanical foam breaker was present to serve as an additional foam control. The fermentation was performed with a head space overpressure of 0.5 bar. The initial working volume of 1 L was inoculated to an O₂ saturation of 1.5 from a shake flask pre-culture in CGXII minimal medium (pH 6.5) supplemented with 40 g L⁻¹ glucose and 1.5 mM FeSO₄. Samples were collected by an autosampler and cooled down to 4 °C until further use. The feed consisted of 400 g L⁻¹ glucose, 0.75 g L⁻¹ MgSO₄·7H₂O and 1.5 mM FeSO₄ (ρ = 1.15 kg m⁻³). The feed was applied at 1.2 mL min⁻¹ as long as the pO₂ surpassed 60%. If the feed-pump was constantly active for more than 1 min, the feed was halted to prevent overheating. The plasmid-based overexpression of ldcC was induced with 1 mM IPTG after 24 h.

**Table 3**

| Primer | Sequence (5’→3’) | Construct |
|--------|------------------|-----------|
| PC48   | CAGCTTGAGCTTGGTAGTTTCTTCGTCCTGAGCCGAACGCGGCGCTTTTTAAAGGAGGTTTTTATGCTCTCCGTTCC | pVWEx4- KDO₂₇ |
| PC49   | GAATTCGAGCTTGGTACCCGGGGATCTTTGCTAAG | pVWEx4- KDO₂₇ |
| PC52   | CAGCTTGAGCTTGGTAGTTTCTTCGTCCTGAGCCGAACGCGGCGCTTTTTAAAGGAGGTTTTTATGCTCTCCGTTCC | pVWEx4- KDO₂₇ |
| PC53   | GAATTCGAGCTTGGTACCCGGGGATCTTTGCTAAG | pVWEx4- KDO₂₇ |
| CA34   | GCCCTGAGGCTTGGACTCTAGAGAAAGGAGGCGCCTTGAGTTGCGACAAACCGGC | pEKEx3-odB²⁷ |
| CA35   | AAATGAGCTGCTGATCCGGCGCCTTTCACTACGAGCCGGCCTGC | pEKEx3-odB²⁷ |
| CA36   | CAGCTTGAGCTTGGTACCCGGGGATCTTTGCTAAG | pEKEx3-odB²⁷ |
| CA26   | CAGCTTGAGCTTGGTACCCGGGGATCTTTGCTAAG | pEKEx3-odB²⁷ |
| CA27   | CAGCTTGAGCTTGGTACCCGGGGATCTTTGCTAAG | pEKEx3-odB²⁷ |
| CA28   | CAGCTTGAGCTTGGTACCCGGGGATCTTTGCTAAG | pEKEx3-odB²⁷ |
| CA29   | CAGCTTGAGCTTGGTACCCGGGGATCTTTGCTAAG | pEKEx3-odB²⁷ |
| CA30   | CAGCTTGAGCTTGGTACCCGGGGATCTTTGCTAAG | pEKEx3-odB²⁷ |
| CA31   | CAGCTTGAGCTTGGTACCCGGGGATCTTTGCTAAG | pEKEx3-odB²⁷ |
| CA32   | CAGCTTGAGCTTGGTACCCGGGGATCTTTGCTAAG | pEKEx3-odB²⁷ |

The areal quotient was plotted against the concentration of the respective standard. The slope of the calibration curve was used to quantify the area quotient. The slope of the calibration curve was used to quantify the biocatalytic activity of the hydroxylase. To identify the substrate specificity of the different decarboxylases, the following assay was performed: 1.5 mL reaction mix contained 50 mM HEPES buffer (pH 7.5), 1 mM PLP, 1 mM DTT and 10 mM of the respective substrate (lysine, 5-HL, 4-HL from fermentation broth) (Baud et al., 2017). The reaction was started by addition of 1 mg mL⁻¹ protein. Incubation of the reaction mix, sampling, sample treatment and analysis were performed as described for the KDO assay.

**Fermentative production of 3-NC**

A baffled bioreactor with a total volume of 3.6 L was used for scale-up experiments (KLF, Bioengineering AG, Switzerland). Two six-bladed Rushton turbines were placed on the stirrer axis with a distance from the bottom of the reactor of 6 and 12 cm. The stirrer to reactor diameter ratio was 0.39. The dissolved oxygen concentration in the batch phase was kept at a minimum of 30% by stepwise increases of the stirrer rate. A constant airflow of 0.75 L min⁻¹ was maintained from the bottom through a sparger, corresponding to an aeration rate of 0.75 vvm. The pH was kept constant at 6.50 ± 0.05 by automatic addition of phosphoric acid (10% (v/v)) and ammonia (25% (w/v)). The temperature was maintained at 30 °C. To prevent foaming 0.6 mL L⁻¹ of the anti-foam agent AF204 (Sigma Aldrich, Darmstadt, Germany) was added, and a mechanical foam breaker was present to serve as an additional foam control. The fermentation was performed with a head space overpressure of 0.5 bar. The initial working volume of 1 L was inoculated to an O₂ saturation of 1.5 from a shake flask pre-culture in CGXII minimal medium (pH 6.5) supplemented with 40 g L⁻¹ glucose and 1.5 mM FeSO₄. Samples were collected by an autosampler and cooled down to 4 °C until further use. The feed consisted of 400 g L⁻¹ glucose, 0.75 g L⁻¹ MgSO₄·7H₂O and 1.5 mM FeSO₄ (ρ = 1.15 kg m⁻³). The feed was applied at 1.2 mL min⁻¹ as long as the pO₂ surpassed 60%. If the feed-pump was constantly active for more than 1 min, the feed was halted to prevent overheating. The plasmid-based overexpression of ldcC was induced with 1 mM IPTG after 24 h.

**Results**

**Determination of the substrate spectra of the lysine-4-hydroxylase KDO₂₇**

Hydroxylases are known for their high substrate specificity. Therefore, it is essential to test if non-natural substrates like cadaverine can be accepted. It was shown before that the lysine-4-hydroxylase KDO₂₇ can accept 5-HL and convert it to 4,5-dihydroxylysine (4,5-DHL) with a low efficiency of 15% conversion next to its natural substrate lysine (65% conversion) (Baud et al., 2014), but not ornithine. In this study, the diamine cadaverine and putrescine as well as the ω-amino acids ω-
aminobutyrate (GABA) and 5-aminovalerate (5AVA) were tested as well. Indeed, it could be confirmed that 79% of the added lysine was converted to 4-hydroxylysine using crude extract of C. glutamicum WT (pVWEx4-KDO<sub>2</sub>) within 24 h (Table 4). Moreover, 4,5-DHL could be produced from 5-HL. However, none of the other substrates was hydroxylated by crude extract of C. glutamicum WT (pVWEx4-KDO<sub>2</sub>) within 24 h (n.c. = not converted). Thus, as cadaverine was not hydroxylated by KDO<sub>2</sub>, conversion of lysine to 3-CHC had to occur in a reaction sequence with lysine hydroxylation occurring before 4-HL decarboxylation.

### Determination of the substrate specificity of different lysine decarboxylases

The capability of different PLP-dependent decarboxylases to decarboxylate lysine and hydroxylated lysines was tested. It was described before that the decarboxylase from Flavobacterium johnsoniae D<sub>Fj</sub> decarboxylates 4-HL with high efficiency, but not 5-HL (Baud et al., 2017). Crude extract of C. glutamicum WT (pVWEx1-D<sub>Fj</sub>) converted 4-HL (18% of 10 mM), whereas neither 5-HL nor lysine were decarboxylated within 24 h (Table 5, Figure S2, Table S1). By contrast, CadA and LdcC from E. coli fully converted lysine and 4-HL within 24 h (Table 5) yielding cadaverine and 3-CHC, respectively. Only about 50% of 10 mM 5-CHC was converted to 2-hydroxycadaverine by LdcC and CadA (Table 5).

### Fermentative production of hydroxylated lysines via regiospecific C-H-hydroxylation using different KDOs

Lysine biosynthesis in C. glutamicum was extended by regiospecific hydroxylases (KDOs) to produce hydroxylated lysines fermentatively. C. glutamicum strain GSL was chosen as base strain, as this lysine overproducer has been successfully engineered to produce lysine-derived compounds (Pérez-García et al., 2018, 2017; Prell et al., 2021). By addition to growth medium, 5-CHC was shown neither to be toxic to C. glutamicum GSL nor to be catabolized (data not shown). Genes encoding two KDOs showing different regioselectivity were tested. To obtain 3-CHC a codon-optimised version of Krad_3958 (KDO<sub>2</sub>) from K. radiotolerans was heterologously overexpressed in GSL, whereas for 4-CHC production a codon-optimised version of Fjoh_3169 (KDO<sub>1</sub>) was used. In shake flask cultivation, 3 ± 1 mM 3-CHC was accumulated by GSL (pVWEx4-KDO<sub>2</sub>) with concomitant production of 35 ± 3 mM lysine (Fig. 2B). In contrast, overexpression of KDO<sub>1</sub> led to a higher 4-CHC concentration (23 ± 1 mM) and a lower lysine (15 ± 2 mM) concentration (Fig. 2B). Biomass formation of both producers was comparable to the empty vector carrying strain GSL (pVWEx4), whereas the growth rate was significantly reduced in the 4-CHC producer (μ = 0.2 ± 0.00 h<sup>−1</sup>) compared to the control strain (μ = 0.25 ± 0.02 h<sup>−1</sup>) and the 3-CHC producer (Fig. 2A). Since 4-CHC production led to higher titers than 3-CHC production, production of 4-CHC was further optimised and the strains GSL (pVWEx4) and GSL (pVWEx4-KDO<sub>2</sub>) were named Lys1 and HLlys1, respectively.

### Table 5

| Enzyme | Substrate [10 mM] | Conversion [%] |
|--------|------------------|----------------|
| Lysine-4-hydroxylase | Lysine | 79 |
| | 5-Hydroxylysine | 14 |
| | Ornithine | n.c. |
| | Putrescine | n.c. |
| | Cadaverine | n.c. |
| | γ-Aminobutyrate | n.c. |
| | 5-Aminovalerate | n.c. |

### Table 4

Substrate specificity of lysine-4-hydroxylase KDO<sub>2</sub> in 2 mg mL<sup>−1</sup> crude extract after 24 h (n.c. = not converted).

| Enzyme | Substrate [10 mM] | Conversion [%] |
|--------|------------------|----------------|
| Lysine-4-hydroxylase | Lysine | 98 |
| | 5-Hydroxylysine | 48 |
| | 4-Hydroxylysine | 100 |
| CadA | Lysine | 98 |
| | 5-Hydroxylysine | 50 |
| | 4-Hydroxylysine | 100 |
| DC<sub>Fj</sub> | Lysine | n.c. |
| | 5-Hydroxylysine | n.c. |
| | 4-Hydroxylysine | 18 |

### Role of LysE for 4-hydroxylysine production

Transport engineering is a promising approach for optimising producer strains (Krämer, 2002; Pérez-García and Wendisch, 2018). For example, overexpression of the gene coding for the lysine exporter LysE accelerated lysine production (Gunji and Yasueda, 2006), while its deletion improved production of the lysine-derived α-picolinic acid by minimizing loss of lysine as precursor of α-picolinic acid (Pérez-García et al., 2017). Strains unable to export lysine while overexpressing it suffer from growth perturbation (Pérez-García et al., 2017) as up to 1 M lysine may accumulate intracellularly as consequence of such metabolic imbalance (Vršič et al., 1996). Growth perturbation may be overcome by conversion of lysine to another compound that is exported independently of LysE, e.g., conversion of lysine to α-picolinic acid (Pérez-García et al., 2017). To test if the deletion of lysE might affect 4-CHC production, strain GSL2 which lacks lysE was used. As expected, the absence of LysE severely perturbed growth of the lysine producer GSLE2 (pVWEx4) (pEKE3) (=Lys2) (Table 6). Growth of the 4-CHC producer GSLE2 (pVWEx4-KDO<sub>2</sub>) (pEKE3) (=HLlys2) was severely perturbed under the tested conditions as well (Table 6). When both strains were complemented by plasmid-based overexpression of lysE, (strains named Lys3 and HLlys3) growth, accumulation of lysine (16 ± 1 mM) as well as of 4-CHC (2 ± 0 mM) in the supernatant were restored (Table 6). Thus, unlike conversion of lysine to α-picolinic acid in the absence of LysE (Pérez-García et al., 2017), conversion of lysine to 4-CHC did not restore growth in the absence of LysE (Table 6). One may speculate that LysE is not only exporting lysine, arginine and citrulline out of the C. glutamicum cell (Lubitz et al., 2016), but may also be involved in export of 4-CHC.

### Effect of increased supply of 2-oxoglutarate as cosubstrate on 4-CHC production

It was demonstrated before that sufficient supply of the cosubstrate 2-OG is crucial to facilitate hydroxylation of amino acids catalysed by KDOs. By dynamic modulation of the 2-oxoglutarate dehydrogenase complex (ODHC) in C. glutamicum production of 4-hydroxyisoleucine (C. Zhang et al., 2018) and trans-4-hydroxyproline (Long et al., 2020) were improved. In this study, the impact of extracellularly added 2-OG on 4-CHC production in HLlys1 was investigated first. Increased biomass formation and a decreased growth rate were only observed at 60 mM 2-OG (Fig. 3A). Notably, 2-OG concentrations remained stable when 30 mM or less 2-OG were added, but 2-OG appeared to be catabolised at 45 mM and 60 mM (Fig. 3A). The addition of 30 mM 2-OG or more only resulted in a minor increase from 20 ± 0 mM up to 22 ± 0 mM 4-CHC. Interestingly, the lysine concentrations gradually decreased from 14 ± 0 mM to 7 ± 1 mM with increasing 2-OG concentrations up to 60 mM. Therefore, addition of 2-OG hardly affected 4-CHC titers but was beneficial as it decreased production of lysine as major by-product (Fig. 3B).

As a next step, supply of 2-OG was adjusted by overexpression of the gene adhF<sup>T14A</sup> coding for the inhibitor of the ODHC with a T14A
were grown in CGXII minimal medium with 40 g L⁻¹ glucose supplemented with 1 mM IPTG in 500 mL baffled shake flasks. The supernatants were analysed after 72 h. Values and error bars represent means and standard deviations (n = 3 cultivations). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 6
Production and biomass formation by ᵃ₃₅E deficient strains Lys2 and HLys2 in comparison to the complemented strains Lys3 and HLys3 (n.d. = not detectable).

| Strain | ΔOD₆⁰₀₀ [-] | Lysine [mM] | 4-HL [mM] |
|--------|-------------|-------------|----------|
| Lys2   | not grown   | n.d.        | n.d.     |
| Lys3   | 23 ± 1      | 16 ± 1      | n.d.     |
| HLys2  | 28 ± 2      | 14 ± 0      | 2 ± 0    |
| HLys3  | 29 ± 1      | n.d.        | 2 ± 0    |

amino acid exchange (Nguyen et al., 2015a). The amino acid exchange abolishes phosphorylation of one of two phosphorylation sites, thus, increasing inhibition of ODHC which requires unphosphorylated OdhI. Indeed, the overexpression of odhI⁻¹⁴₄ resulted in a 4.4-fold increase of 2-OG accumulation in the culture broth (31 ± 1 mM) by C. glutamicum Lys5 in comparison to the control strain Lys4 with 7 ± 0 mM (Fig. 3D). Concomitantly, the glutamate titers were increased (23 ± 1 mM compared to 10 ± 0 mM), while lysine production (60 ± 1 mM compared to 37 ± 2 mM) (Fig. 3D) and the growth rate (0.18 ± 0.00 h⁻¹ compared to 0.14 ± 0.00 h⁻¹ decreased (Fig. 3C). Overexpressing odhI⁻¹⁴₄ also affected growth by the 4-HL producer HLys5 (Fig. 3C). While the glutamate concentrations accumulated by both strains were similar (7 ± 0 mM by HLys4 and 6 ± 1 mM by HLys5), about 2-fold more 2-OG accumulated in odhI⁻¹⁴₄ overexpressing 4-HL producing strain HLys5 (Fig. 3D). However, about 2- to 3-fold less 4-HL were produced, but more about 2-fold more lysine accumulated. Thus, modulating ODHC activity by overexpressing odhI⁻¹⁴₄ did not improve 4-HL production.

**Effect of increased supply of iron as cofactor on 4-HL production**

The influence of the KDO cofactor iron (II) was investigated since it was shown before that elevated concentrations of iron (II) in the cultivation medium improved production of l-2-hydroxyglutarate by C. glutamicum significantly which involved hydroxylation of glutarate (Prell et al., 2021). Therefore, up to 2.5 mM iron sulfate were added to the CGXII minimal medium, which already contained 37 μM Fe²⁺. Addition of iron sulfate to the growth medium slowed growth of strains Lys1 and HLys1 (Fig. 4A; Kᵢ = 1.9 mM for Lys1 and Kᵢ = 1.3 mM for HLys1). Lysine production by Lys1 was increased by addition of 0.5 mM or more iron sulfate (Fig. 4B). Of note, gradually increasing the addition of iron sulfate to the culture broth of HLys1 reduced lysine accumulation and increased 4-HL production proportionally (Fig. 4D). When 1.0 mM Fe²⁺ were added, HLys1 produced 29 ± 1 mM 4-HL, but only 6 ± 0 mM lysine in comparison to standard cultivation conditions (20 ± 0 mM 4-HL, 14 ± 0 mM lysine) (Fig. 4D). Since at 2.5 mM Fe²⁺ only slightly more 4-HL titer (32 ± 1 mM) were produced, but the growth rate dropped to 0.07 ± 0.00 h⁻¹, addition of 1.0 mM Fe²⁺ was chosen for the following experiments since the growth rate was still 0.11 ± 0.00 h⁻¹.

**Adaption of extracellular pH for improved precursor supply and 4-HL production**

For KDO₉ various pH values ranging from 6.0 (Hara et al., 2017) to 7.5 (Baud et al., 2014) were described as optimal condition for the enzymatic reaction of the purified protein. Although pH homeostasis of C. glutamicum is effective in this pH range (Jakob et al., 2007), the influence of the external pH on lysine and 4-HL production was investigated. Growth of HLys1 was affected stronger at pH 6.5 than that of Lys1 (Fig. 5A, C). Lysine production by Lys1 was decreased from pH 6.5 (65 ± 5 mM) to pH 7.5 (47 ± 3 mM), while lysine production by HLys1 was affected less by the medium pH (Fig. 5B, D). Notably, 4-HL production was maximal at pH 6.5 with a titer of 54 ± 4 mM and only 9 ± 1 mM lysine accumulating as by-product (Fig. 5D). Although slow growth was observed at pH 6.5, this medium pH supported the highest 4-HL titers with the least accumulation of lysine as by-product. Thus, pH 6.5 was chosen as standard for the following experiments.

**Extension of the pathway from 4-HL to 3-HC**

Cadaverine and potentially 3-HC are N-acetylated by spermi(di)ne-N-acetyltransferase (Nguyen et al., 2015b). Therefore, the respective
gene snzA was deleted and the strain carrying the empty vectors pECXT-Psyn and pVWEx1 was called Lys6. Strain HLys6 expressed the KDO\textsubscript{Fj} gene under a strong synthetic promoter (Psyn) on plasmid pECXT-Psyn. The genes coding for the different decarboxylases LdcC, CadA and DC\textsubscript{Fj} were expressed under the control of an IPTG-inducible promoter on plasmid pVWEx1 either in Lys6 (yielding strains Cad1-3) or in HLys6 yielding strains HCad1-3). Lys6 produced 89 ± 4 mM lysine, whereas overexpression of the KDO\textsubscript{Fj} in HLys6 resulted in 36 ± 0 mM 4-HL and 40 ± 9 mM lysine as by-product (Fig. 6). Plasmid-based overexpression of ldcC (Cad1) resulted in 100 ± 5 mM cadaverine with only 1 ± 0 mM lysine as by-product (Fig. 6). Upon overexpression of cadA (Cad2) 39 ± 2 mM cadaverine and 53 ± 4 mM lysine accumulated. As expected, no cadaverine, but 73 ± 1 mM lysine was produced by Cad3 as DC\textsubscript{Fj} cannot accept lysine as substrate.

Strains HCad1-3 that expressed the lysine-4-hydroxylase gene in combination with one of the genes coding for the different decarboxylases (HCad1: ldcC; HCad2: cadA; HCad3: DC\textsubscript{Fj}) produced 3-HC to varying degrees (Fig. 6A). HCad2 produced 1 ± 0 mM 3-HC, 30 ± 3 mM cadaverine, 15 ± 1 mM 4-HL and 36 ± 2 mM lysine (Fig. 6A). As expected, HCad3 produced no cadaverine since decarboxylase DC\textsubscript{Fj} is highly specific for 4-HL, but 2 ± 0 mM 3-HC, 16 ± 0 mM 4-HL and 56 ± 3 mM lysine accumulated. The highest titer of 11 ± 0 mM 3-HC was obtained with HCad1 (Fig. 6). While lysine and 4-HL were no significant by-products, but 72 ± 2 mM cadaverine accumulated. Taken together, 3-HC production by strain HCad1 was investigated further.

Hydroxylation of lysine to 4-HL and subsequent decarboxylation of 3-HC competes with lysine decarboxylation and export of cadaverine as by-product. Thus, cgmA coding for the cadaverine exporter was
deleted. As consequence, export of cadaverine was almost completely abolished. Moreover, 3-HC was no longer exported in HCad1ΔcgmA and only lysine (46 ± 2 mM) and 4-HL (17 ± 2 mM) were detected in the supernatant (Fig. 6B). These results indicated that CgmA might also be the exporter for 3-HC. Thus, deletion of cgmA could not be used to avoid accumulation of cadaverine during 3-HC production.

Fermentative production of 3-HC in reactor scale

A 1 L bioreactor cultivation was performed to test if 3-HC production is stable and if operation in the fed-batch mode provides a means to reduce cadaverine formation as by-product. During the batch phase, the aeration rate was kept at 0.75vvm since this supported 4-HL production using KDOFj best (data not shown). After 21.5 h the feed started and 1 mM IPTG was added to induce the expression of ldcC after 24 h. The cells grew in the batch phase with a growth rate of 0.13 h⁻¹ to an OD₆₀₀ of 29 (Fig. 7). In the feed phase, a maximum OD₆₀₀ of 114 was reached after 42 h. After that, the rDOS steadily rose up to 85%, and no more feed was applied. After 44.5 h nitrogen was added manually using the base pump. The decrease in the rDOS led to the addition of more feed solution indicating that nitrogen might be the limiting factor. Even though more feed was applied, no cell growth and further product accumulation could be observed. After 42 h, 74 mM 3-HC (8.8 g L⁻¹, corresponding to 11.4 g L⁻¹ when normalized to the initial volume of 1 L) was produced with a volumetric productivity of 1.55 g L⁻¹ h⁻¹ (corresponding to 2.0 g L⁻¹ h⁻¹ when normalized to the initial volume of 1 L) and a product yield on biomass of 0.31 g per g CDW (Fig. 7). The product yield on substrate was low (0.07 g g⁻¹) as cadaverine accumulated as main by-product (390 mM; 39.8 g L⁻¹) besides 4-HL (25 mM; 4.1 g L⁻¹). Taken together, compared to the microscale cultivation (11 mM; 1.3 g L⁻¹, 0.44 g L⁻¹ h⁻¹, 0.03 g g⁻¹, Fig. 6), the bioreactor fed-batch cultivation enabled a 7-
fold higher 3-HC titer, a 3.5-fold higher volumetric productivity, and more than 2-fold higher product yield on substrate.

Discussion

In this study, C. glutamicum was engineered to produce 4-HL and 3-HC by fermentation. Lysine biosynthesis was extended first by lysine-4-hydroxylase to yield 4-HL and second by a decarboxylase (E. coli LdcC > E. coli CadA > DCFj from F. johnsoniae) for 3-HC production. To reduce formation of cadaverine as by-product, two-phasic fed-batch cultivation in which KDOFj was expressed constitutively, whereas expression of ldcC was induced when the feed was started, resulted in production of 8.8 g L⁻¹ 3-HC (Fig. 7).

The observation that decarboxylation of cadaverine and 4-HL are comparable in vitro (Table 5), while more cadaverine, but much less 3-HC accumulated in vivo (Fig. 6) points to the conclusion that lysine hydroxylation is the bottleneck of 3-HC production. This may be explained by cadaverine being derived from lysine by direct decarboxylation, whereas 3-HC formation depends on two consecutive reactions: lysine hydroxylation followed by decarboxylase. Thus, there is a need to identify more efficient lysine-4-hydroxylases. The lysine-4-hydroxylase KDOFj accepts lysine and with lower affinities 3-HL and 5-HL with low efficiency as substrates in vitro, but not ornithine (Baud et al., 2014; Hara et al., 2017). Hydroxylation of simple (di)amines such as cadaverine is not possible, likely because a conserved arginine residue interacts with the carboxylate group of lysine via a salt bridge (Baud et al., 2014). The α-amino group of lysine is directly involved in the lid closure via an H-bond with Asn232, which may be relevant for substrate binding (Strieker et al., 2007), i.e. the lid is in its opened-form in the absence of substrates, while it is in closed form when lysine is bound (Bastard et al., 2018). Our finding that
cadaverine is a major by-product of 3-HC production and is commensurate with the inability of KDO enzymes to hydroxylate cadaverine to 3-HC. Identification and use of KDO enzymes hydroxylating also cadaverine may help increase 3-HC production.

Hydroxylation by KDOs is associated with loss of carbon as carbon dioxide, which negatively impacts the carbon efficiency of the process in addition to the loss of carbon dioxide by decarboxylation of 4-HL.

The use of enzymes, which are capable to perform C-H hydroxylation without using 2-OG as cosubstrate is desirable, such as monooxygenases (EC 1.14.16), which use pteridines as cosubstrates to regiospecifically hydroxylate aromatic amino acids (Pey et al., 2006; Zhang et al., 2006, 2006, p. 2). Comparable to the KDOs, they are highly specific for their natural and closely related substrates. The tyrosine 3-monoxygenase from Homo sapiens can accept next to tyrosine with lower efficiency structurally related amino acids like phenylalanine and tryptophan (Roberts and Fitzpatrick, 2013). Alternatively, NAD(P)H/FADH2-dependent monooxygenases (EC 1.14.13.) use reduction equivalents as cofactors, and form the N-hydroxylated product and water. One example is the L-lysine N6-monooxygenase, which converts lysine to N6-hydroxy-L-lysine. (Dick et al., 2002).

Fig. 6. Comparison of different decarboxylases for production of 3-hydroxycadaverine (A) and impact of deleting the gene coding for the export system CgmA (B). The strains were cultivated in the Biolector microcultivation system in CGXII minimal medium (pH 6.5) supplemented with 40 g L⁻¹ glucose, 1 mM IPTG and 1.04 mM FeSO₄. Supernatants were analysed after 72 h (A) and 48 h (B). Values and error bars represent means and standard deviations (n = 3 cultivations).

Fig. 7. 3-HC production by C. glutamicum HCad1 operated in fed-batch mode. HCad1 was cultivated in 1 L CGXII minimal medium in fed-batch mode over 48 h, containing 40 g L⁻¹ glucose from the batch medium and 95 g L⁻¹ glucose from the feeding solution. (A) OD₆₀₀ is shown in black squares, glucose concentration (g L⁻¹) is plotted as pink empty triangles, feed solution (mL) is depicted as pink line, the relative dissolved oxygen saturation (rDOS, %) is indicated in light blue and the stirrer frequency (rpm) is shown as black line. (B) 3-HC production is indicated in light blue diamonds (mM), OD₆₀₀ is shown in black squares, lysine in dark blue circles (mM), 4-HL in turquoise squares (mM), and cadaverine concentration (mM) in grey triangles. Cultivation was performed at 30 °C, 0.75vvm and a constant pH 6.5 regulated with 10 % (v/v) H₃PO₄ and 25% (w/v) ammonia. The pO₂ was kept above 30% by a stepwise increase in stirrer rate. An overpressure of 0.5 bar was applied. 0.6 mL L⁻¹ of antifoam agent AF204 (Sigma Aldrich, Taufkirchen, Germany) was added to the medium manually before inoculation. Plasmid-based overexpression of ldcC was induced after 24 h with 1 mM IPTG. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
KDO enzymes require iron and increasing the iron concentration in the growth medium improved production of hydroxylated glutarate involving CsiD from P. putida (Prell et al., 2021) and, as described here, of hydroxylated lysine involving KDO enzymatic activity (et al., 2002), while overexpression of cgmA for improved cadaverine production (Kind et al., 2011; Nguyen et al., 2015). It was demonstrated that deletion of cgmA abolished 3-OG accumulation (Fig. 6B). CgmA likely functions as export system for lysE (Table 6). Overexpression of lysE increased arginine production in Corynebacterium crenatum (Xu et al., 2013). It is known that CgmA is involved in cadaverine export and cgmA can be overexpressed for improved cadaverine production (Kind et al., 2011; Nguyen et al., 2015a). As it was demonstrated that deletion of cgmA abolished 3-OG accumulation (Fig. 6B), CgmA likely functions as export system for 3-OG. In the case of 4-HP and lysine, indirect evidence may indicate that LysE is involved in 4-HP export. In the absence of LysE, high intracellular lysine concentrations (e.g. due to dipeptide feeding or lysine overproduction) drastically perturb growth of C. glutamicum (Vrijic et al., 1996), which can be avoided by conversion of lysine, e.g., to pipercolic acid, which is exported independently of LysE (Pérez-Garcia et al., 2017). Conversion of lysine to 4-HP did not abolish the growth perturbation observed in the 4-HP producer HLYs2 that lacks LysE (Table 6). Overexpression of lysE may lead to loss of lysine as precursor of 4-HP, thus, decreasing 4-HP production. As alternative, lysine re-uptake into the cell by overexpression of the gene coding for the lysine importer LysE (Seep-Feldhaus et al., 1991) may improve 4-HP production under the hitherto unknown condition that LysE does not accept 4-HP.

Different metabolic engineering strategies were applied to increase provision of 2-OG as cosubstrate of KDOs (Jing et al., 2021). Smirnov et al. (2010) blocked the TCA cycle in E. coli by several deletions to avoid conversion from 2-OG to succinate and to couple KDO activity, which provides succinate from 2-OG besides hydroxylation of the primary substrate (in this case isoleucine dioxygenase was used) (Smirnov et al., 2010). In a comparable approach, a tunable circuit for dynamic attenuation of ODHC activity was adopted to enhance the flux towards 2-OG and consequently trans-4-hydroxypyrroline production (Long et al., 2020). In this study, addition of extracellular 2-OG decreased the lysine titer but did not increase the 4-HP titer. Increasing repression of ODHC by OdhT led to 4.4-fold more accumulation of 2-OG in Lys5, but at the expense of lysine production as its biosynthesis is derived from oxalacetate, an intermediate of the TCA cycle (Georgi et al., 2005; Schrumpf et al., 1991). Moreover, glutamate accumulated, while 4-HP was not improved. Possibly, withdrawal of oxaloacetate for lysine production is more important than provision of 2-OG for KDOs.

Activities of KDOs respond to the intracellular pH. Lowering the external pH of the cultivation media to 6.5 resulted in increased production of lysine and 4-HP, while perturbing growth. C. glutamicum maintains pH homeostasis in a medium pH range from 6.0 to 9.0 (Tüttüber et al., 2021). Pleiotropic effects of changing the medium pH (i.a., iron starvation, protein folding, and stabilization) (Martín-Galliano et al., 2005) preclude interpretation of these effects of 4-HP production.

The inducible and constitutive lysine decarboxylases, CaDA (Kanjeet al., 2011; Sabo et al., 1974) and LdcC (Yamamoto et al., 1997) from E. coli were tested with regard to decarboxylation of 4- and 5-hydroxylysine as alternative substrates. CaDA is highly efficient, but the enzyme has its optimum at acidic pH (pH 5.0–6.0) and is rapidly inactivated at higher pH (>8.0) (Lemonnier and Lane, 1998) and inhibited at high concentrations of lysine (Kim et al., 2015) or cadaverine (Sabo et al., 1974). Therefore, in vivo production LdcC proved to be superior as it exhibits a broader pH range (Kind et al., 2010) and is hardly inhibited by its substrate (Shin et al., 2018). The third decarboxylase DC tested in this study was investigated before as the genes coding for the lysine-4-hydroxylase KDO enzymatic activity (Prell et al., 2021). It remains to be studied if 3-OG can be separated from cadaverine efficiently in downstream processing. This and other challenges remain to be solved before the proof-of-concept of 3-OG production can be transferred to industrial application.

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CRediT authorship contribution statement

Carina Prell: Conceptualization, Methodology, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. Sophie-Ann Wunderbank: Investigation. Florian Meyer: Methodology, Investigation, Writing – review & editing. Fernando Pérez-García: Conceptualization, Writing – review & editing. Volker F. Wendisch: Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix

Figure S1: Overlayed HPLC chromatograms of enzymatic KDO assays with crude extracts from C. glutamicum. Figure S1: Overlayed HPLC chromatograms of enzymatic DC assays with crude extracts from C. glutamicum. Table S1. Retention times and structures for different substrates and products derived from the enzymatic assays.

Ethical approval

This article does not contain any studies with human participants or animal experiments performed by any of the authors.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crbiot.2021.12.004.

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