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# Microbial Engineering for Production of *N*-Functionalized Amino Acids and Amines

*Melanie Mindt, Tatjana Walter, Pierre Kugler, and Volker F. Wendisch**

***N*-functionalized amines play important roles in nature and occur, for example, in the antibiotic vancomycin, the immunosuppressant cyclosporine, the cytostatic actinomycin, the siderophore aerobactin, the cyanogenic glucoside linamarin, and the polyamine spermidine. In the pharmaceutical and fine-chemical industries *N*-functionalized amines are used as building blocks for the preparation of bioactive molecules. Processes based on fermentation and on enzyme catalysis have been developed to provide sustainable manufacturing routes to *N*-alkylated, *N*-hydroxylated, *N*-acylated, or other *N*-functionalized amines including polyamines. Metabolic engineering for provision of precursor metabolites is combined with heterologous *N*-functionalizing enzymes such as imine or ketimine reductases, opine or amino acid dehydrogenases, *N*-hydroxylases, *N*-acyltransferase, or polyamine synthetases. Recent progress and applications of fermentative processes using metabolically engineered bacteria and yeasts along with the employed enzymes are reviewed and the perspectives on developing new fermentative processes based on insight from enzyme catalysis are discussed.**

## 1. Introduction

Natural products as well as fine and bulk chemicals often contain *N*-specific modifications such as *N*-alkyl-, *N*-hydroxy-, or *N*-acyl-groups. Enzymes for the synthesis of *N*-alkylated compounds such as the *N*-methylated glycopeptide antibiotic vancomycin, *N*-hydroxylated compounds such as *N*-hydroxy-L-pipecolic acid, a plant regulator of systemic acquired resistance to pathogen infection, or *N*-acylated compounds such as the *N*-acetylated hormone melatonin have been identified and characterized. While this review focuses on the microbial engineering of bacteria and yeasts for production of *N*-substituted low-molecular-weight compounds, we also introduce the occurrence, physiological roles, and applications—for example, in the pharmaceutical and fine chemical industries—of these compounds as well as their biosynthetic pathways and enzymes. Where applicable, we describe metabolic engineering strategies for the provision of precursor metabolites and the integration of the *N*-functionalizing enzymes into the metabolism of the microbial production host. Since *Corynebacterium glutamicum* is an ideal host for production of nitrogenous compounds and used in the industrial million-ton-scale amino acid production, many illustrative examples are given for this production host. Performance parameters of illustrative examples are summarized in **Table 1**.

The reader is referred to concise reviews on the chemical synthesis of *N*-functionalized molecules[1,2] or on post-translational modification of proteins at ε-amino groups of lysine residues, for example, in collagen, or at their *N*-termini.[3] We will neither cover *N*-phosphorylated compounds such as *N*-phosphocreatine nor phosphoramidates that contain P—N bonds as in adenosine 5-phosphoramidate[4] nor *N*-nitroso compounds such as streptozotocin.[5]

Technological breakthroughs currently drive new developments in microbial engineering, for example, by CRISPR/Cas genome editing, as well as in enzyme engineering, for example, by directed evolution.[6] This prompted us to propose that combining *N*-functionalizing enzymes with systems metabolic engineering for efficient supply of their substrates may lead to the development of fermentative processes for sustainable production of *N*-alkylated, *N*-hydroxylated, or *N*-acylated bioactive compounds.

## 2. Microbial Production of *N*-Alkylated Amino Acids

*N*-alkylamino acids can be found in plants, mammals, and various microorganisms. They occur in proteins and peptides as well as freely and they extend the chemical repertoire of the 20

Dr. M. Mindt, T. Walter, P. Kugler, Prof. V. F. Wendisch
Genetics of Prokaryotes
Biology and CeBiTec
Bielefeld University
Bielefeld 33615, Germany
E-mail: volker.wendisch@uni-bielefeld.de
Dr. M. Mindt
BU Bioscience
Wageningen University and Research
Wageningen 6708 PB, The Netherlands

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/biot.201900451



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proteinogenic amino acids. *N*-alkylation increases amino acid lipophilicity and renders proteins less prone to proteolysis. Thus, as building blocks of peptide-based drugs they improve certain pharmacokinetic properties of these peptidomimetics such as biological stability, improved bioavailability, or altered conformational stability.[7–9] For example, *N*-methylation of a synthetic integrin ligand improved receptor selectivity and the respective cyclic pentapeptide c(Arg-Gly-Asp-D-Phe-*N*MeVal) was later named Cilengitide. Specifically, docking studies indicated that reduced backbone flexibility of Cilengitide as consequence of *N*-methylation was beneficial for the observed increased receptor selectivity.[10,11]

Amino acid *N*-alkylation reactions occur abundantly for amino acids embedded into peptides, but rarely for free amino acids.[12,13] The methylation of synthetic peptides is performed either at the peptide itself on solid support or by incorporation of *N*-methylated amino acid monomers.[14–16] Approaches for chemical synthesis of free *N*-alkylated amino acids were studied intensively. Synthetic preparation of *N*-alkylated amino acids can be performed i. a. by direct alkylation of protected amino acids, ring opening of 5-oxazolidinones, or by reductive amination.[14,15,17] However, these methods are often limited by low yields, dimethylation, or requirement of heavy metal ions.[17] To overcome these disadvantages, microbial production of *N*-methylated amino acids was developed recently (**Figure 1**). Three independent routes have been engineered depending either on a native pathway for C1-assimilation present in methylotrophic bacteria, reductive alkylamination of 2-oxo acids by the bacterial reductase DpkA, or by *S*-adenosyl-L-methionine (SAM)-dependent methylation of the aromatic compound anthranilate.

**Melanie Mindt** is a postdoctoral researcher at the BU Bioscience at Wageningen University and Research. She studied her Bachelor and Master of Science of Biochemistry at the Heinrich-Heine University, Düsseldorf, Germany. She received her Ph.D. in July 2019 from Bielefeld University for her investigation of the de novo synthesis of *N*-alkylated amino acids using recombinant *Corynebacterium glutamicum* and *Pseudomonas putida* in the Wendisch lab.

**Volker F. Wendisch** is Chair of Genetics of Prokaryotes at the Faculty of Biology at Bielefeld University. Since 2010 he is member of the board of Center for Biotechnology (CeBiTec) and since 2019 its Deputy Scientific Director. He studied biology in Köln University. After having completed his Ph.D. at the Research Center Jülich in 1997, he worked as postdoctoral researcher at the University of California, Berkeley, CA, USA. From 2006–2009 he was Professor for Metabolic Engineering at Münster University. Prof. Wendisch's research interests concern genome-based metabolic engineering of industrially relevant microorganisms, systems, and synthetic microbiology.

### 2.1. *N*-Methylation by Addition of Methylamine to Glutamate Derivatives

Fermentative production of L-theanine and *N*-methylglutamate (Figure 1A) can be realized based on a pathway for methylamine assimilation found in some methylotrophic bacteria like *Methyloversatilis universalis, Methylobacterium extorquens*, and *Methylocella silvestris*.[18–21] The *N*-methylated amino acid *N*-methylglutamate serves as a key intermediate of the eponymous multi-step pathway of methylamine assimilation present in some methylotrophs.[18–21] Expression of the genes coding for *N*-methylglutamate synthase (NMGS) (*mgsABC*) in metabolically engineered *Pseudomonas putida* KT2440 enabled glucose-based production of *N*-methylglutamate by methylamination of the TCA cycle intermediate 2-oxoglutarate. Interestingly, coexpression of *mgsABC* and *gmaS,* which encodes γ-glutamylmethylamide synthetase (GMAS), resulted in higher product titers. Upon derepression of the glycerol catabolism by deletion of regulatory gene *glpR* and integration of a second copy of the endogenous glutamate dehydrogenase gene *gdhA*, *N*-methylglutamate was produced from glycerol and methylamine in a fed-batch bioreactor to a final titer of 18 g $L^{-1}$ at a yield of 0.11 g $g^{-1}$ with a volumetric productivity of 0.13 g $L^{-1}$ $h^{-1}$.[22]

L-theanine, a glutamine derivative ethylated at the $N^5$-position, is a non-proteinogenic amino acid and was first isolated from green tea leaves[23] and was later found also in the mushroom *Xerocomus badius*.[24] Next to its flavor-enhancing ability, L-theanine is thought to have favorable physiological and pharmacological effects.[25–27] In plants, L-theanine is synthesized by addition of ethylamine to L-glutamate in an ATP-dependent manner catalyzed by L-theanine synthetase.[13,14] Carbon labeling experiments revealed that ethylamine derives from L-alanine by decarboxylation.[28,29] Hence, L-theanine can be isolated from tea leaves, but low yields and high costs result in ineffective industrial scale production.[30–32] Due to its low stability, the plant-derived L-theanine synthetase is not used in microbial production. However, side-activity for L-theanine synthesis has been described for L-glutamine synthetase (EC 6.3.1.2[33]), L-glutaminase (EC 3.5.1.2[34]), γ-glutamyltranspeptidase (EC 2.3.2.2[35]), and γ-glutamylmethylamide synthase (GMAS, EC 6.3.4.12[36]). Both L-glutaminase and γ-glutamyltranspeptidase transfer a glutamyl moiety to ethylamine, while L-glutamine synthetase and GMAS catalyze alkylamidation of glutamate in an ATP-dependent manner.[37] Various studies described biological production of the desired compound using free enzymes or whole cell catalysts, either prokaryotic or eukaryotic, but all approaches required external supply of L-glutamate.[37] The first fermentative production of L-theanine starting from glucose has been described recently. Expression of different γ-glutamylmethylamide synthase genes from methylotrophic bacteria in *C. glutamicum* strains enabled a glucose and ethylamine dependent fermentative production of L-theanine.

 

**Table 1.** De novo synthesis of *N*-functionalized amines by (recombinant) microorganisms. Titer, yield, and volumetric productivity values of illustrative production processes for *N*-functionalized amino acid and amines along with proposed production processes are listed.

| Strain | Product | Enzyme(s) | Titer [g $L^{-1}$] | Yield [g $g^{-1}$] | Productivity [g $L^{-1}$ $h^{-1}$] | Fermentation mode | References for established processes |
|---|---|---|---|---|---|---|---|
| *N*-alkylation | | | | | | | |
| *C. glutamicum*[a)] | L-theanine | GMAS | 42.0 | 0.20 | 0.88 | Fed-batch | [39] |
| *Pseudomons putida*[b)] | *N*-methyl-L-glutamate | GMAS, NMGS | 17.9 | 0.11 | 0.13 | Fed-batch | [22] |
| *C. glutamicum* pyruvate producer[c)] [48] | *N*-methyl-L-alanine | DpkA | 31.7 | 0.71 | 0.35 | Fed-batch | [43] |
| *C. glutamicum* glyoxylate producer[d)] [58] | Sarcosine | DpkA | 9.1 | 0.26 | 0.16 | Shake flask | [42] |
| *C. glutamicum* glyoxylate producer[d)] [58] | *N*-ethylglycine | DpkA | 6.1 | 0.17 | 0.11 | Shake flask | [42] |
| 2-Oxoisocaproate producer[e)] [63] | *N*-methyl-L-leucine | DpkA | Hypothetical process | | | | |
| *E. coli*[f)] | *N*-methylanthranilate | | 0.0029 | — | 0.0012 | Shake flask | [70] |
| *E. coli*[f)] | *N*-methyl-*O*-methylanthranilate | | 0.0157 | — | — | Shake flask | [70] |
| *N*-hydroxylation | | | | | | | |
| L-valine producer[g)] [91] | *N*-hydroxy-L-valine | CYP79D1, CYP79D2[88] | Hypothetical process | | | | |
| L-isoleucine producer[h)] [97] | *N*-hydroxy-L-isoleucine | CYP79D1, CYP79D2[88] | Hypothetical process | | | | |
| L-lysine producer[i)] [104] | $N^6$-hydroxy-L-lysine | IucD[101] | Hypothetical process | | | | |
| L-ornithin producer[j)] [109] | $N^5$-hydroxy-L-ornithine | PvdA[108] | Hypothetical process | | | | |
| L-pipecolic acid producer[k)] [116] | *N*-hydroxy-pipecolic acid | FMO01[115] | Hypothetical process | | | | |
| Cadaverine producer[l)] [122] | *N*-hydroxy-cadaverine | AvbB[119] | Hypothetical process | | | | |
| Putrescine producer[m)] [112] | *N*-hydroxy-putrescine | AvbB[119] | Hypothetical process | | | | |
| *N*-acetylation | | | | | | | |
| *Bacillus subtillis*[n)] | *N*-acetylglucosamine | GNA1[146] | 131.6 | 0.38 | 1.37 | Fed-batch | [146] |
| L-glutamate producer[o)] [67] | *N*-acetylglutamate | ArgA[167] | Hypothetical process | | | | |
| L-ornithin producer[j)] [109] | *N*-acetylornithine | ArgJ[170] | Hypothetical process | | | | |
| Putrescine producer[m)] [112] | *N*-acetylputrescine | SnaA[130] | Hypothetical process | | | | |
| Cadaverine producer[l)] [122] | *N*-acetylcadaverin | SnaA[130] | Hypothetical process | | | | |
| Betaines | | | | | | | |
| *Actinopolyspora halophila*[p)] | Glycine betaine | — | 13 | Complex medium | 0.14 | Batch | [200] |
| *Agrobacterium* and *Rhizobium* isolates[q)] | L-carnitine | — | — | 99.5 mol% | 1.21 | Fed-batch | [201] |
| L-lysine producer[i)] [104] | L-carnitine | *N*-lysine methyltransferase[195], carnitine biosynthetic pathway[197] | Hypothetical process | | | | |
| Polyamines | | | | | | | |
| *S. cerevisiae*[r)] | Spermidine | SPE1, SPE2, SPE3 | 0.22 | $2.2\times10^{-3}$ | 0.003 | Fed-batch | [208] |
| *E. coli*[s)] | 1,3-diaminopropane | Dat, Ddc | 13 | 0.1 | 0.19 | Fed-batch | [209] |
| *E. coli*[t)] | Putrescine | SpeC | 42.3 | 0.26 | 1.23 | Fed-batch | [210] |

(*Continued*)

**Table 1.** Continued.

| Strain | Product | Enzyme(s) | Titer [g $L^{-1}$] | Yield [g $g^{-1}$] | Productivity [g $L^{-1}$ $h^{-1}$] | Fermentation mode | References for established processes |
|---|---|---|---|---|---|---|---|
| *C. glutamicum*[u)] | Putrescine | SpeC | 19 | 0.16 | 0.55 | Fed-batch | [211] |
| *C. glutamicum*[v)] | Cadaverine | LdcC | 88 | 0.29 | 2.2 | Fed-batch | [129] |
| *C. glutamicum*[w)] | Cadaverine | LdcC | 103.8 | 0.30 | 1.47 | Fed-batch | [212] |
| Putrescine producer[m)] [112] | *sym*-homospermidine | Homospermidine synthase [225] | Hypothetical process | | | | |
| *S. cerevisiae*[x)] | *N*-methylpyrrolinium | PMT, DAO | 0.017 | — | $0.14\times10^{-3}$ | Shake flask | [226] |

[a)] *C. glutamicum* GKG-047; [b)] *P. putida* KT2440 Δ*upp* Δ*glpR*::P*tac-gdhA* (pEV1-*mgsABC-gmaS*); [c)] *C. glutamicum* ATCC 13032 Δ*aceE* Δ*pqo* Δ*ldhA* ΔC-T *ilvN* Δ*alaT* Δ*avtA*; [d)] *C. glutamicum* ATCC 13032 Δ*aceB icd*$^{GTC}$; [e)] *C. glutamicum* ATCC 13032 Δ*ltbR* Δ*ilvE* Δ*prpC1* Δ*prpC2* PgltA $^{mut\_L1}$ (pBB1leuA $^{EC\text{-}G462D}$); [f)] *E. coli* BL21(DE3) Δ*trpD* Δ*tyrR* Δ*metJ*; [g)] *C. glutamicum* R JCM 18 229 ΔLP_ΔAc+GP_ilvN$^{GE}$C$^{TM}$_ΔAla (pCRB-BN$^{GE}$C$^{TM}$; pCRB-DLD); [h)] *C. glutamicum* IWJ001 (pDXW-8-*cysK*); [i)] *C. glutamicum* ATCC 13020 $P_{sod}$*lysC*$^{T311I}$ $P_{sod}$*dapB* 2x*ddh* 2x*lysA* Δ*pck hom*$^{V59A}$ $P_{sod}$*pycA*$^{P458S}$ *icd*$^{GTC}$ $P_{tuf}$*fbp* $P_{sod}$*tkt*; [j)] *C. glutamicum* ATCC MB001 Δ*argFRG* $P_{gdh4}$-*argB*$^{A49V,M54V}$ *pgi*$^{GTC}$ $P_{tuf}$- *argCJB*$^{A49V,M54V}$*D*; [k)] *C. glutamicum* GRLys1 Δ*sugR* Δ*ldhA* Δ*lysE*$^{2}$ (pVWEx1-*lysDH-proC*); [l)] *B. methanolicus* MGA3 (pBV2mp-*cadA*); [m)] *C. glutamicum* ATCC 13032 Δ*argR* Δ*argF* (pVWEx1-*speC*); [n)] *B. subtilis* Δ*nagP*/ Δ*gamP* Δ*gamA* Δ*ldh* Δ*pta* Δ*glcK* Δ*pckA* Δ*pyk*::*lox72* Δ*nagAB*::$P_{veg}$-*yqaB*, $P_{veg}$-*glmS* Δ*gamR*::*lox72* Δ*amyE*:: $sg_{zwf1}$-$sg_{pfkA2}$-$sg_{glmM2}$ Δ*lacA*::*gamR*-$P_{gamA}$-dCas9 ΔalsSD::lox72 (pSTg-GNA1); [o)] *C. glutamicum* ATCC 13032; triggered with ciprofloxacin; [p)] *Actinopolyspora halophila* MTCC263; [q)] *Rhizobium sp.* HK13 (DSM 2903); [r)] *S. cerevisiae* SR8 Δ*OAZ1* overexpressing *SPE1,2,3* (pTPO1); [s)] *E. coli* W3110 Δ*lacI thrA*$^{C1034T}$ *lysC*$^{C1055T}$ P*ppc*::P*trc* P*aspC*::P*trc* Δ*pfkA* (p15DD$^{opt}$paspC); [t)] *E. coli* W3110 Δ*lacI* Δ*speE* Δ*speG* Δ*argI* Δ*puuPA* P*argECBH*::P*trc* P*speF-potE*::P*trc* P*argD*::P*trc* P*speC*::P*trc* Δ*rpoS* (pKK117SargF); [u)] *C. glutamicum* ORN1 (pVWEx1-*speC-5'*$_{21}$*-argF*); [v)] *C. glutamicum* LYS-12 *bioD*::$P_{tuf}$ldcC$^{opt}$ Δ*NCgl1469* Δ*lysE* $P_{cg2893}$::$P_{sod}$; [w)] *C. glutamicum* PKC *lysE*::$P_{H30}$-ldcC$^{Ec}$; [x)] *S. cerevisiae* BY4742 $P_{TEF1}$-*Ec*ODC-$T_{FBA1}$, $P_{PGK1}$-*At*PMT-$T_{CYC1}$, $P_{TEF1}$-*Aa*DAO3-$T_{PRM9}$, $P_{TCCTDH}$-SAM2-$T_{PGI}$, ΔALD4, ΔALD5, ΔHFD1.

The production titer could be improved by change of the base strain to a glutamate overproducing *C. glutamicum* strain[38] and prevention of glutamate export by deletion of the L-glutamate exporter gene *yggB*. A titer of 42 g $L^{-1}$ was achieved in fed-batch bioreactor cultivation in 5 L scale when *gmaS* from *Methylovorus mays* was expressed.[39] Biochemical characterization of both enzymes GMAS and NMGS elaborated a small substrate spectrum: while GMAS accepts also L-glutamine as substrate,[40] NMGS was described to convert some alkylamines (ethylamine, propylamine, butylamine) with decreasing activity for increasing alkyl moieties.[12] Thus, the access to other *N*-alkylated amino acids produced via the GMAS and NMGS pathway is limited.

### 2.2. Reductive Alkylamination of 2-Oxo Acids Yielding *N*-Alkylated Amino Acids

*N*-alkylamino acid dehydrogenase DpkA from *Pseudomonas* species proved crucial to provide a more versatile route for the production of *N*-alkylated amino acids by microbial fermentation (Figure 1B).[41–43] Natively, DpkA plays a role in degradation of D-lysine and D-proline where it catalyzes imine reduction of the cyclic imines piperidin-2-carboxylate and pyroline-2-carboxylate yielding L-pipecolic acid and L-proline, respectively.[44] In addition, this imine reductase (IRED) catalyzes the reductive alkylamination of 2-oxo acids yielding the respective *N*-alkylamino acids. It has been hypothesized that alkylamines and 2-oxo acids may spontaneously form imines in aqueous solutions that subsequently are reduced by DpkA.[45–47]

Various 2-oxo acids are present in a bacterial cell, for example, as intermediates in glycolysis, the TCA cycle or in amino acid biosynthesis pathways, thus, simple introduction of DpkA into a bacterium is not sufficient for efficient and selective production of a chosen *N*-alkylamino acid. It is pivotal to engineer the bacterial production host for a) high *dpkA* expression and b) for provision of high concentrations of the required 2-oxo acid.[41–43]

Fermentative production of *N*-methyl-L-alanine by *C. glutamicum* involved strain engineering to ensure a) provision of high concentrations of pyruvate from glucose and b) heterologous expression of *dpkA* from *Pseudomonas* species for *N*-methylamination of pyruvate using methylamine.[43] The pyruvate secreting *C. glutamicum* strain ELB-P was chosen as a basis.[48] This strain produced up to 17.6 g $L^{-1}$ pyruvate in shake-flask fermentations due to several modifications of the pyruvate-converting enzymes: accumulation of pyruvate was enabled by deletion of the pyruvate dehydrogenase subunit E1p (*aceE*), the pyruvate-quinone oxidoreductase gene (*pqo*), the C-terminal regulatory domain of the acetohydroxyacid synthase gene (*ilvN*), the lactate dehydrogenase gene (*ldhA*), and both genes for L-alanine synthesizing aminotransferases (*alaT* for alanine aminotransferase and *avtA* for alanine-valine aminotransferase). In an elegant manner, growth and product formation by this recombinant *C. glutamicum* strain are decoupled. This concept, which was transferred to L-lysine, L-valine, isobutanol, and 2-oxoisovalerate production,[49–55] ensures that the strain utilizes carbon sources like sugars for product formation, whereas acetate is required for biomass formation. Balancing the ratios of glucose and acetate as carbon substrates as well as media optimization with respect to ammonium and methylammonium concentrations proved important to obtain *N*-methyl-L-alanine as dominant product in high titers. Finally, transfer to fed-batch bioreactor cultivation allowed for a production of 32 g $L^{-1}$ *N*-methyl-L-alanine at a yield of 0.7 g $g^{-1}$ (glucose) and a volumetric productivity of 0.35 g $L^{-1}$ $h^{-1}$.[43]

Extending this concept to the microbial production of *N*-alkylated glycine derivatives, that show potential as antipsychotic drugs,[56,57] as building blocks for peptidomimetics or detergents, involved a) strain engineering for sufficient supply of the 2-oxo acid glyoxylate and b) engineering of DpkA for better acceptance

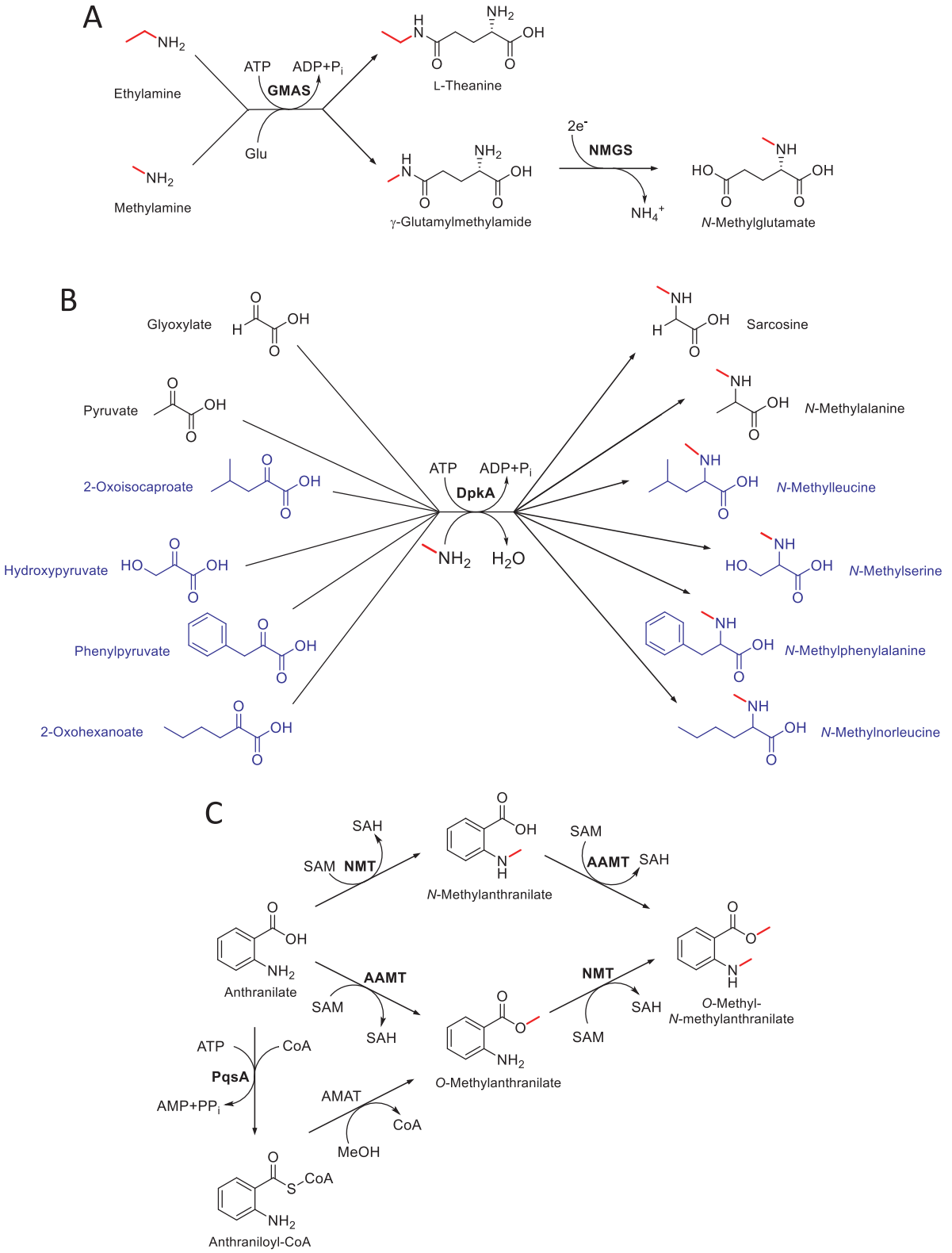


**Figure 1.** Functionalization of amines by *N*-alkylation (depicted in red) can be achieved by different routes depending on the product of interest. The ATP-dependent *N*-alkylation of glutamate is performed by A) γ-glutamylmethylamide syntethase (GMAS) and *N*-methylglutamate synthase (NMGS), B) reductive amination of 2-oxo acids by the *N*-methylamino acid dehydrogenase DpkA, and C) the production of *O*-methyl-*N*-methylanthranilate is catalyzed by SAM-dependent anthranilate *N*-methyltransferase (NMT) and SAM-dependent anthranilate *O*-methyltransferase (AAMT) or by anthranilate CoA ligase and methanol anthraniloyltransferase.

of glyoxylate as substrate for *N*-alkylation.[41,42] A base strain overproducing glyoxylate was derived from a glycolate producing strain which lacks the glyoxylate converting malate synthase and has low isocitrate dehydrogenase activity due to initiation codon exchange from ATG to GTG.[58] Based on the discovery that the imine reductase DpkA does not only accept pyruvate and several 2-oxo acids,[46] but also glyoxylate as substrate with good catalytic activity,[42] DpkA was integrated into the central metabolism of the glyoxylate accumulating *C. glutamicum* base strain and enabled the production of sarcosine (also known as *N*-methylglycine). A final titer of 8.7 g $L^{-1}$ sarcosine and a volumetric productivity of 0.12 g $L^{-1}$ $h^{-1}$ were achieved in a xylose-based process.[42] This process depended on the substrate promiscuity of the IRED DpkA[42] and, thus, rational enzyme engineering and subsequent characterization was employed to develop a more efficient mutant of DpkA.[41] Mutation of a single amino acyl residue in the substrate binding site of DpkA ($DpkA^{F117L}$) increased specific activities for reductive alkylamination of glyoxylate using either methylamine or ethylamine as substrat.[41] Based on $DpkA^{F117L}$, an improved volumetric productivity (0.16 g $L^{-1}$ $h^{-1}$) was achieved for sarcosine production and fermentative production of *N*-ethylglycine to a final titer of 6.1 g $L^{-1}$ could be established.[41] Notably, sustainable production of sarcosine and *N*-ethylglycine from alkylamines and second generation feedstocks such as rice straw hydrolysate could be demonstrated.[41] The DpkA-based approach of *N*-alkylating 2-oxo acids may be diversified further. Regarding provision of the 2-oxo acid precursor, it is conceivable that *E. coli, Clostridium thermocullum, Synechococcus elongates*, and *C. glutamicum* strains developed for biofuel production from 2-oxo acid precursors can be converted to strains overproducing *N*-alkylated amino acids. These strains involve 2-oxo acid decarboxylation using 2-oxoisovalerate decarboxylase KivD from *Lactococcus lactis* and aldehyde reduction by an aldehyde dehydrogenase.[52,59–62] In principle, this would only require a replacement of *kivD* and *adhA* by *dpkA* and feeding alkylamines. Alternatively, strains overproducing a particular 2-oxo acid[38,55,63–69] may be used as basis. However, it must be kept in mind that DpkA may have to be engineered to ensure efficient and selective *N*-alkylamination of the overproduced 2-oxo acid or an alternative IRED has to be used.

### 2.3. SAM-Dependent *N*-Methylation of Amino Acids

SAM-dependent *N*-methyltransferases have been described i.a. for the synthesis of *N*-methylanthranilate[70] and the anthranilate-derived alkaloid 4-hydroxy-1-methyl-2-quinolone.[71] *O*-alkylated *N*-methylanthranilates are known to have flavor and fragrance characteristics as well as antinociceptive and analgesic activities,[72,73] while the group of anthranilate-derived alkaloids include bioactive molecules which show antiseptic, convulsive, or antineoplastic activities. The SAM-dependent *N*-methylation of anthranilate is catalyzed by anthranilate *N*-methyltransferase (Figure 1C). Upon expression of the respective gene from *Ruta graveolens* in an engineered *E. coli* BL21 strain low titers of *N*-methylanthranilate (0.029 g $L^{-1}$) accumulated.[70] This strain was previously optimized by deletion of anthranilate phosphoribosyl transferase gene *trpD*, and the genes *tyrR* and *metJ* encoding negative transcriptional regulators of the aromatic amino acid biosynthesis and SAM biosynthesis, respectively. Similarly, low titers were obtained upon further conversion of *N*-methylanthranilate to either *O*-methyl-*N*-methylanthranilate (0.016 g $L^{-1}$)[70] or 4-hydroxy-1-methyl-2-quinolone (0.018 g $L^{-1}$).[71] By contrast, *O*-methylanthranilate production to high titers (5.74 g $L^{-1}$) has been described as consequence of systems metabolic engineering and the development of fed-batch mode two-phase bioreactor cultivation with tributyrin.[74] It has to be noted that the hydrophobicity and/or volatility of a compound dictates its suitability for two-phase cultivations (e.g., better suited for *O*-methylanthranilate and *O*-methyl-*N*-methylanthranilate than for the charged *N*-methylanthranilate) and that the overlay phase has to be chosen accordingly. However, as upscaling efficiencies of 10 to 70-fold have been observed when moving from two-phase shake flasks to two-phase bioreactors in the case of *O*-methylanthranilate [74] and patchoulol,[75] this approach may prove crucial for commercialization.

## 3. *N*-Hydroxylating Enzymes and Their Application in Microbial Amino Acid and Amine Production

Hydroxylation at different positions of amino acids is well-known in nature.[76–78] While hydroxylation of aliphatic or aromatic carbon atoms, for example, by phenylalanine, tryptophan, or tyrosine hydroxylases, have been studied well due to their physiological roles in mammals as in phenylketonuria or the biosynthesis of catecholamines or serotonin,[77] *N*-hydroxylases that occur in all three domains of life have received less attention. *N*-hydroxylating enzymes are involved in several biosynthesis pathways, for example, in siderophore synthesis in plants and bacteria, but also known for enhancing antibiotic resistance.[79,80]

### 3.1. *N*-Hydroxylation in Nature

Cytochrome P450s (CYPs, EC 1.14) and flavin dependent monooxygenases (FMOs, EC 1.13) are known for their *N*-hydroxylating activities. FMOs, for example, play a role in the adaptation of butterflies to pyrrolizidine-alkaloid-producing plants.[81,82] Higher resistance or nonsusceptibility against the antibiotic rifampicin is observed in many *Nocardia* isolates due to FMO-catalyzed hydroxylation of rifampicin at the $N^2$-position.[79] Aerobactin is an iron chelating siderophore, which helps *E. coli* and related bacteria to survive in iron-poor environments[80] and its synthesis initiates by *N*-hydroxylation of the amino acid L-lysine in an NAPDH dependent manner by the membrane-bound FMO L-lysine $N^6$-monooxygenase. After $N^6$-acetylation of $N^6$-hydroxy-L-lysine (Section 4.1) and subsequent $N^1$-citrylation, aerobactin synthase ligates the resulting $N^1$-citryl-$N^6$-acetyl-$N^6$-hydroxy-L-lysine to $N^6$-acetyl-$N^6$-hydroxy-L-lysine yielding the dihydroxamate siderophore aerobactin.[83–85]

### 3.2. *N*-Hydroxylation for Microbial Production

While C-hydroxylated amino acids have been produced by microbial fermentation, for example, 4-hydroxy-L-isoleucine[86] and 4-hydroxy-L-proline,[87] no fermentative process for *N*-hydroxylated

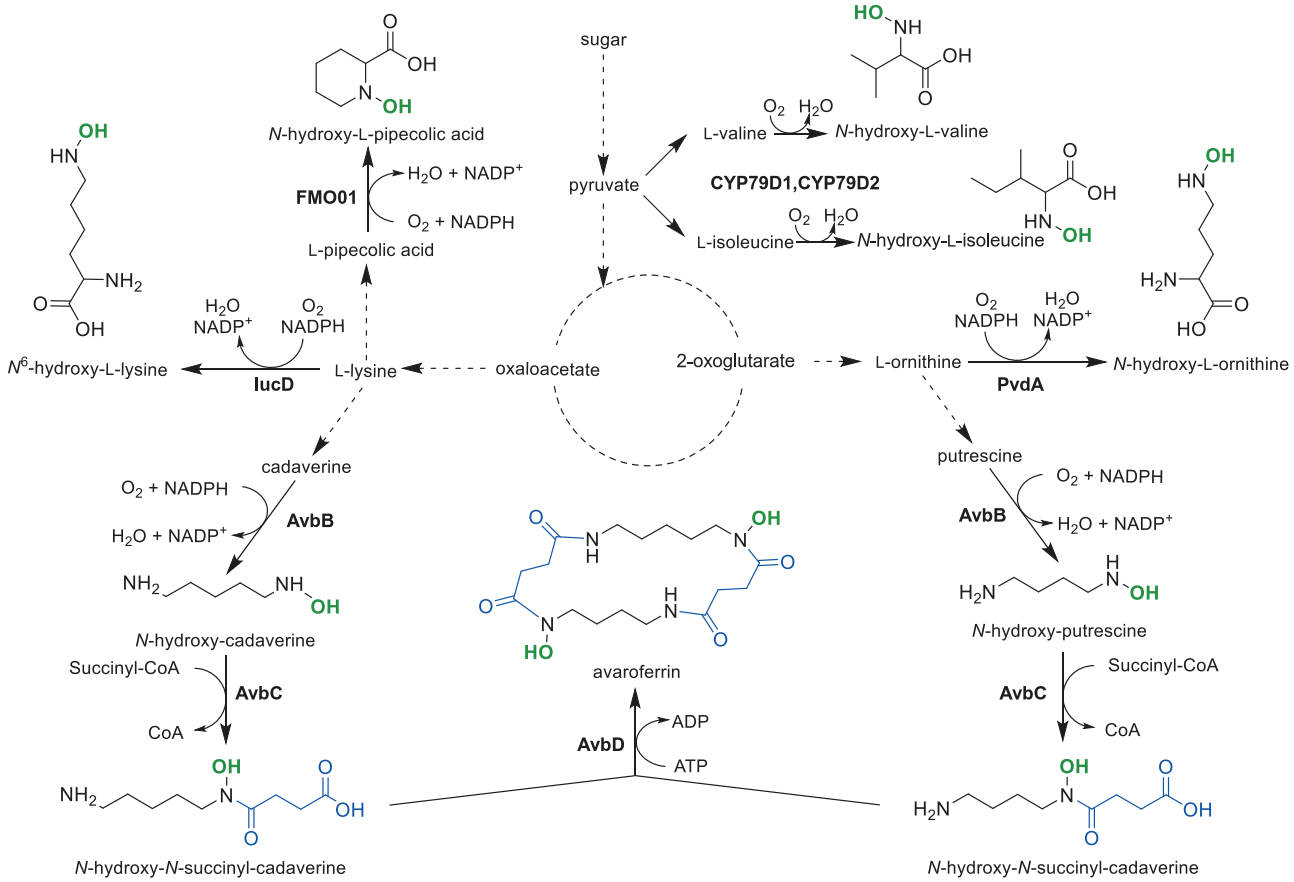


**Figure 2.** Hypothetical processes for production of *N*-acylated (depicted in blue) and *N*-hydroxylated (depicted in green) amino acids and amines. *N*-hydroxy-L-valine and *N*-hydroxy-L-isoleucine formation can be catalyzed by a Cytochrome P450 from cassava, *N*-hydroxy-L-ornithine by L-ornithine $N^5$-hydroxylase PvdA from *P. aeruginosa*. Avaroferrin formation can proceed via *N*-hydroxycadaverine and *N*-hydroxyputrescine formed by *N*-hydroxylase AvbB from *Shewanella algae*, *N*-hydroxy-*N*-succinylcadaverine, and *N*-hydroxy-*N*-succinylputrescine is formed by *N*-succinyl transferase AvbC before condensation by siderophore synthase AvbD. $N^6$-hydroxy-L-lysine formation is catalyzed by $N^6$-L-lysine hydroxylase IucD. *N*-hydroxy-L-pipecolic acid formation can be catalyzed by the flavin dependent monooxygenase FMO01 from *A. thaliana*. Dashed lines refer to multiple reaction steps and continuous lines to one reaction.

amines or amino acids has been published. Potentially, such processes can be envisioned, i.a. based on *N*-hydroxylases active with L-valine or L-isoleucine. For example, the first step in the biosynthesis of the cyanogenic glucosides linamarin and lotaustralin in cassava is catalyzed by *N*-hydroxylating CYPs (CYP79D1, CYP79D2) active with L-valine and L-isoleucine as substrates (**Figure 2**).[88] These CYPs could be functionally expressed in the methylotrophic yeast *Pichia pastoris*, but not in *E. coli*.[88] In principle, microbial production of *N*-hydroxy-L-valine and *N*-hydroxy-L-isoleucine may be possible since strains of *Bacillus licheniformis, C. glutamicum, E. coli*, and *Saccharomyces cerevisiae* have been engineered for high titre L-valine (up to 150 g $L^{-1}$) or L-isoleucine (up to 50 g $L^{-1}$) production.[89–100] Likely, *S. cerevisiae* is the preferred host for expression of plant CYP genes, in particular, since functional expression of cassava CYP79D1 and CYP79D2 failed in *E. coli*.

The proposed concept of expressing an amine *N*-hydroxylase gene in a strain overproducing the respective amine or amino acid to enable fermentative production of the chosen *N*-hydroxylated amine or amino acid may be relevant in several instances, for example, $N^6$-hydroxy-L-lysine, $N^5$-hydroxy-L-ornithine, *N*-hydroxypipecolic acid, *N*-hydroxycadaverine, or *N*-hydroxyputrescine (Figure 2). Fermentative production of $N^6$-hydroxy-L-lysine may be based on expression of *iucD* from *E. coli* encoding L-lysine $N^6$-monooxygenase, which showed a sub-millimolar $K_M$ for L-lysine,[83,101] in an L-lysine overproducing bacterium. The annual fermentative production of L-lysine topped 2.5 million metric tonnes in 2017[102–104] and *C. glutamicum* strains producing L-lysine to titers up to 120 g $L^{-1}$ are known.[104,105] Alternatively, other bacterial hosts such as *E. coli* for glucose-based L-lysine production or *Bacillus methanolicus* for L-lysine production from methanol as alternative carbon source would be suitable.[106,107]

Access to $N^5$-hydroxy-L-ornithine by fermentation may become possible if a gene coding for an FMO with L-ornithine $N^5$-hydroxylating activity is expressed in an L-ornithine overproducing strain.[108] L-Ornithine overproduction has been engineered in *C. glutamicum, E. coli, B. subtilis, Weissella confuse*, and *S. cerevisiae*.[109–114] The FMO PvdA from *Pseudomonas aeruginosa*, which is part of the biosynthetic pathway leading to the siderophore pyoverdin, is a good candidate for fermentative production of $N^5$-hydroxy-L-ornithine due to its relatively low $K_M$

value of 0.58 mM and a high $v_{max}$ of 1.34 $\mu$mol min$^{-1}$ mg$^{-1}$.[108] Production of *N*-hydroxy-L-pipecolic acid (Figure 2) may become possible by overexpression of the L-pipecolate *N*-hydroxylase FMO1 from *Arabidopsis thaliana*[115] in an engineered L-pipecolic acid producer.[116,117]

In principle, both *N*-hydroxycadaverine and *N*-hydroxyputrescine can be accessed via fermentation employing the same FMO, that is, either PubA from *Shewanella putrefaciens*[118] or AvbB from *Shewanella algae*[119] or GorA from *Gordonia rubripertincta*.[120] Expression of one of these enzymes, which show multi-specificity with putrescine, cadaverine, and hexamethylenediamine, in a putrescine overproducing strain will yield *N*-hydroxyputrescine, whereas *N*-hydroxycadaverine is formed upon expression in a cadaverine overproducing strain (Figure 2). Various strains for overproduction of either cadaverine or putrescine are available among the species *C. glutamicum*, *E. coli*, *B. methanolicus*, and *Serratia proteamaculans*.[112,121–128] In *C. glutamicum*, cadaverine production via L-lysine decarboxylase allows for high cadaverine titers without contaminating putrescine formation,[129] and vice versa putrescine production via ornithine decarboxylase enables high putrescine titers without contaminating cadaverine.[130,131] Thus, these *C. glutamicum* strains meet the prerequisite to use a multi-specific *N*-hydroxylase such as GorA for exclusive production of either *N*-hydroxyputrescine or *N*-hydroxycadaverine based on substrate availability for GorA.

## 4. *N*-Acetylation of Amino Acids and Amines for Microbial Production

*N*-acylated and especially *N*-acetylated amines are widespread among bacteria and eukaryotes. *N*-acylation plays a role in melatonin and siderophore biosynthesis, biosynthesis of bacterial and fungal cell walls, and the exoskeleton of crustaceans, post-translational modifications of proteins, or as part of detoxification processes including antibiotic resistance.[132–136]

### 4.1. *N*-Acetylation in Nature

*N*-acetylation requires CoA-activated acyl-groups for transfer to primary or secondary amino groups in proteins or other amines. Depending on their substrate specificity amino acid *N*-acetyltransferases (EC 2.3.1.1), diamine *N*-acetyltransferases (EC 2.3.1.57), and arylamine *N*-acetyltransferases (EC 2.3.1.5) can be distinguished. Co- and post-translational *N*-terminal acylation of proteins occurs in all branches of life and impacts enzymatic activity, stability, or folding patterns of proteins significantly.[136] The proportion of proteins partially or fully acetylated at their *N*-termini is particularly large in eukaryotes: 50–70% in yeast, 72% in *A. thaliana*, and 80–90% in humans.[136,137] Although underestimated for a long time, *N*-terminal acylation of bacterial proteins is of high functional relevance. Bacterial cell walls contain polymeric peptidoglycan comprising *N*-acetyl-D-glucosamine (GlcNAc) and *N*-acetylmuraminic acid (MurNAc) as monomeric constituents.[138] *N*-acetylation of low-molecular-weight amines is important as well, for example, *N*-acetylation of serotonin is a crucial and rate limiting step for the biosynthesis of melatonin, a hormone released by the pineal gland for regulation of the sleep–wake cycle.[132,139] *N*-acetyl or *N*-succinyl transferases also function in siderophore biosynthesis (Figure 2) and often accept $N^6$-hydroxylated substrates that arise from *N*-hydroxylation reactions (Section 3.1). For example, formation of $N^6$-acetyl-$N^6$-hydroxy-L-lysine in *E. coli* aerobactin biosynthesis is catalyzed by $N^6$-hydroxylysine *N*-acetyltransferase IucB.[80,85] In pyoverdine biosynthesis, $N^5$-formyl-$N^5$-hydroxy ornithine and $N^5$-acetyl-$N^5$-hydroxy ornithine are synthesized.[133,140] For avaroferrin synthesis in *S. algae* (**Figure 3**), *N*-hydroxyputrescine and *N*-hydroxycadaverine are succinylated by the *N*-succinyl transferase AvbC.[119] Antibiotic resistance may be due to *N*-acylation. Aminoglycoside acetyltransferases are causal, for example, for resistance of *E. coli* isolates to gentamicin and sisomicin[135] or for ciprofloxacin resistance of clinical isolates of *E. coli*, *Klebsiella pneumoniae*, and *Enterobacter*.[141]

### 4.2. Microbial Production of *N*-Acetylated Compounds

*N*-acetyl-D-glucosamine and its derivative *N*-acetyl-D-neuraminic acid find application in pharmaceutical and health food products.[142] *N*-acetylglucosamine production by *S. cerevisiae* with titers up to 3.0 g L$^{-1}$ in shake flask cultivation were obtained based on systems metabolic engineering.[143] Overexpression of the genes for glutamine-fructose-6-phosphate amidotransferase Gfa1 and the phosphatase YqaB were combined with decreased glucose uptake rate by overexpression of *MTH1*-Δ*T*, which codes for a truncated glucose sensing regulator. In addition, the pyruvate kinase-1 (Pyk1) and phosphofructokinase-1 (PFK-1) genes were repressed by dCas9 and the gene encoding phosphofructokinase 2 (PFK-2) was deleted.[94,134,144] Similarly, *B. subtilis* was engineered to produce 48.9 g L$^{-1}$ GlcNAc at a yield of 0.32 g g$^{-1}$ glucose in fed-batch fermentation.[145] Recently, *B. subtilis* has been engineered for the production of 130 g L$^{-1}$ GlcNAc in 15 L fed-batch cultivation. An autonomous dual-control system was established for balancing the metabolic flux towards the production of GlcNAc by the application of a GlcNAc biosensor in combination with CRISPRi-based NOT gates for self-adjusting gene expression levels.[146] The acetyl transferase GNA1 from *S. cerevisiae* was heterologously overexpressed and high product titers were obtained by the combination of modular engineering of GlcNAc synthesis-related metabolic network, glycolysis, and peptidoglycan synthesis, blocking sporulation, engineering the maintenance metabolism, and blocking acetoin overflow.[142,145,147,148] In *E. coli*, a straightforward strategy employed inactivation of genes involved in glucosamine transport and catabolism, overexpression of the L-glutamine-D-fructose-6-phosphate aminotransferase gene (*glmS*) and one of three different GlcN-6-P *N*-acetyltransferase genes (either from *S. cerevisiae*, *Candida albicans*, or *A. thaliana*). Notably, the best strain produced 110 g L$^{-1}$ GlcNAc with a yield of 0.45 g g$^{-1}$ glucose in two-phase fed batch process.[149] A comparable approach in *E. coli* led to about 73 g L$^{-1}$ GlcNAc.[150,151] The GlcNAc derivative *N*-acetyl-D-neuraminic acid was produced by recombinant *E. coli* to a titer of 13.8 g L$^{-1}$ using glycerol and GlcNAc in a pyruvate-independent biocatalytic process.[152–155]

Long-chain *N*-acylated aromatic amino acids are frequently encountered in antibacterial screens of metagenomic cDNA

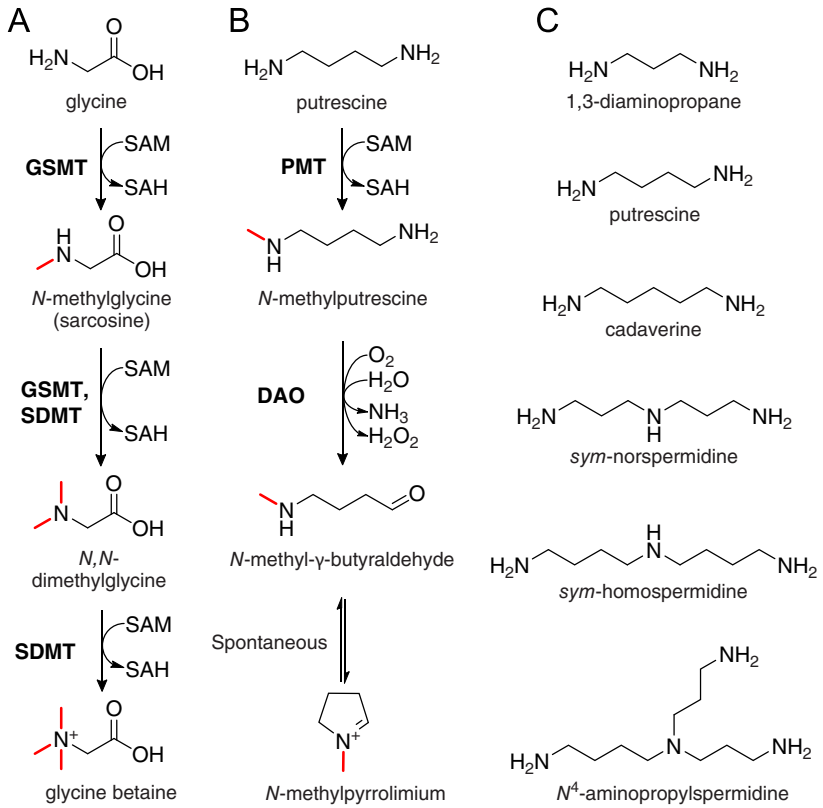


**Figure 3.** Production of betaines and polyamines. A) Glycine betaine formation proceeds via three *N*-methylation (depicted in red) steps by glycine-sarcosine methyltransferase (GSMT) and sarcosine-dimethylglycine methyltransferase (SDMT), which show overlapping substrate specificities. B) Production of *N*-methylpyrrolinium: putrescine is converted to *N*-methylputrescine by putrescine *N*-methyltransferase (PMT) and further oxidized by diamine oxidases (DAO) to yield *N*-methyl-$\gamma$-butyraldehyde, which spontaneously cyclizes to *N*-methylpyrrolinium. C) Overview of the various polyamines discussed in the text.

libraries.[156–158] These long-chain *N*-acylated amino acids arise from acylation of the respective amino acids (L-phenylalanine, L-tyrosine, L-tryptophan, and L-arginine) activated by CoA or acyl carrier proteins. For instance, the *N*-acylating enzyme NasP uses activated fatty acids of typical chain lengths between C12–C20.[157] While various strains for overproduction of the amino acids L-phenylalanine, L-tyrosine, L-tryptophan, and L-arginine are available [159–166] and the genes for *N*-acylating enzymes and acyl activating enzymes are known, a production host has to be chosen to facilitate the accumulation of the very hydrophobic compounds in the cell membranes and to withstand the impact of high concentrations of these antibiotics.

Amino acids such as *N*-acetyl-L-glutamate and *N*-acetyl-L-ornithine occur, for example, as intermediates in amino acid biosynthesis routes. In L-arginine biosynthesis, *N*-acetyl glutamate synthase (EC 2.3.1.1) *N*-acetylates L-glutamate with acetyl-CoA,[167–169] while acetylglutamate-acetylornithine transacetylase (EC 2.3.1.35) catalyzes acetyl transfer between L-ornithine and L-glutamate.[170,171] Production of *N*-acetyl-L-glutamate and *N*-acetyl-L-ornithine has not been followed per se, but has been pivotal during the optimization of strains overproducing L-arginine,[172] L-ornithine,[109] L-proline (via the ornithine cyclodeaminase route),[173] or L-citrulline[174]. Interception of L-arginine biosynthesis in a strain overproducing L-arginine to very high titers may prove suitable, for example, for the overproduction of *N*-acetyl-L-glutamate. Also, in the so-called succinylase and acetylase variants of L-lysine biosynthesis, *N*-succinylated intermediates (e.g., in *C. glutamicum*) or *N*-acetylated intermediates (e.g., in *E. coli*) occur. In *C. glutamicum*, tetrahydrodipicolinate *N*-succinyltransferase DapD forms L-*N*-succinyl-2-amino-6-ketopimelate using succinyl-CoA as a cofactor, while the *E. coli* enzyme generates the *N*-acetylated version of this molecule.[175,176]

Interestingly, *N*-acetylation of the diamines putrescine and cadaverine was very efficient in *C. glutamicum* and the gene for spermi(di)ne *N*-acetyl-transferase *snaA*[130] had to be deleted in putrescine and cadaverine overproducing strains to avoid secretion of these compounds.[131,177–179] Polyamine *N*-acetyltransferases are widespread and found in humans, animals, yeasts, and bacteria.[180–182] Specific processes for production of *N*-acetylated polyamines have not yet been described but appear possible given the ease of biosynthesis and excretion of *N*-acetylputrescine and *N*-acetylcadaverine by recombinant *C. glutamicum* (Section 4.1).

## 5. Production of Betaines and Polyamines

Betaines as well as polyamines are both associated with stress tolerance in nature.[77] Due to their unique chemical properties

they can interact with different cell components and, for example, function as compatible solutes and organic osmolytes. This makes them interesting for applications in the pharmaceutical and cosmetics industry but also for uses as platform chemicals and building blocks in polymer industry.

### 5.1. Occurrence, Functions, and Applications of Betaines

Betaines are low-molecular weight zwitterionic compounds with a negative charge and a quaternary ammonium or phosphonium cation function (Figure 3). The positive charge cannot be balanced by proton migration. The eponymous betaine is also called trimethylglycine or glycine betaine. Betaines may function as compatible solutes or as methyl group donors.[183] Choline is not a betaine sensu stricto, it is used for example as dietary supplement especially for poultry feed[184] or has medical applications as it is closely related to the neurotransmitter acetylcholine.[185] Glycine betaine is used as methyl donor and osmolyte in animal feed and in plants it is used to increase cold, drought, and salt resistance, for example, to prolong storage life of iceberg lettuce. These properties yield to further applications in the pharmaceutical, cosmetics, and fermentation industries.[186] L-Carnitine plays an essential role in the transport of long chain fatty acids and, thus, has many medical applications such as in the treatment of cardiovascular diseases, as dietary supplement, or nutraceutical, where it is being marketed to improve weight management and exercise performance.[187]

### 5.2. Biosynthesis of Betaines

The quaternary ammonium groups of glycine betaine, choline, L-carnitine, and their derivatives arise from three subsequent *N*-methylation reactions catalyzed by SAM-dependent methyltransferases. In the case of choline, the substrate of the SAM-dependent *N*-methyltransferases is ethanolamine or its derivatives.[188–192] Choline can be converted to glycine betaine by two consecutive oxidation reactions with betainealdehyde as intermediate.[191] In an alternative pathway, glycine is *N*-methylated three times by glycine-sarcosine methyltransferase and sarcosine-dimethylglycine methyltransferase, which show overlapping substrate specificity (Figure 3).[183] Biosynthesis of L-carnitine is special as its precursor $N^{\epsilon}$-trimethyllysine (TML) is formed by degradation of proteins such as histones or calmodulin that were post-translationally modified by *N*-methylation of lysyl residues.[193,194] TML is converted to L-carnitine in a four-step pathway. The existence of a fungal *N*-methyltransferase active with free L-lysine remains debated.[195–197] The Rubisco large subunit methyltransferase of pea shows a strong preference for its protein substrate, but some side activity with free L-lysine has been observed.[198]

### 5.3. Fermentative Production of Betaines

Glycine betaine can be fermentatively coproduced with the disaccharide trehalose from acid whey, a waste product of the dairy industry, using the extreme obligate halophile organism *Actinopolyspora halophila* that produces these compounds as compatible solutes.[199] In optimized bioreactor fermentations about 13 g $L^{-1}$ glycine betaine and about 5 g $L^{-1}$ trehalose accumulated.[200]

To date, the lack of an effective lysine *N*-methylase yielding TML hampers development of a fermentative de novo L-carnitine synthesis process. Since biological resolution of racemic D,L-carnitine is not industrially attractive, current processes revolve around biotransformation of precursors crotonobetaine, $\gamma$-butyrobetaine, and 3-dehydrocarnitine to L-carnitine with *E. coli*, *Proteus mirabilis*, or isolates related to *Agrobacterium* and *Rhizobium*.[187] A large-scale fed-batch biotransformation process for the production of L-carnitine from $\gamma$-butyrobetaine was developed by Lonza AG, producing >99.9% ee L-carnitine with a 99.5% yield and a volumetric productivity of 1.2 g $L^{-1}$ $h^{-1}$. This process was chosen over a continuous process making use of cell recycling with a productivity of 5.5 g $L^{-1}$ $h^{-1}$ and 91% yield due to higher downstream processing costs resulting from the lower yield in the continuous process.[201] Biotransformation of crotonobetaine using resting cells of metabolically engineered *E. coli* led to a 95% yield and a maximal productivity of 9.6 g $L^{-1}$ $h^{-1}$ overcoming the need for anaerobic cultivation conditions.[202] A novel non-fermentative approach to carnitine production could make use of engineered halohydrin hydrogen-halide-lyase HheB from *Corynebacterium* sp. N-1074 that catalyzes the conversion of 1,3-dichloro-2-propanol and hydrogen cyanide to *R*-4-chloro-3-hydroxybutyronitrile, a precursor for chemical L-carnitine synthesis.[203]

### 5.4. Occurrence, Functions and Applications of Polyamines

Polyamines are alkaline organic compounds with an aliphatic, saturated carbon backbone, at least two primary amino groups, and a varying number of secondary amino groups. They are applied in a wide variety of commercial applications due to their unique combination of reactivity, basicity, and surface activity. The main commercial interest in biogenic polyamines is their use in polymers. Diamines can be used as monomers for the production of polyamides, whereas higher amines can be applied as curing agents in epoxy-resins.[204] The occurrence, biosynthesis, and applications of diamines have been reviewed elsewhere.[204–206] Here, we give examples for polyamines carrying secondary amine groups (Figure 3), such as the linear triamines and tetramines, or branched polyamines such as $N^4$-aminopropylspermidine, a triamine with a tertiary amine group. These compounds arise from *N*-alkylation of polyamines with primary amine groups or by condensation of several polyamine molecules.

The linear spermidine is synthesized by spermidine synthase that catalyzes the addition of a propylamine moiety to putrescine. Propylamine is derived from decarboxylated *S*-adenosyl-L-methionine (dcSAM) which can be formed by a *S*-adenosylmethionine decarboxylase.[207] Spermidine is present in all living organisms and is closely related to cell growth and the regulation of several other biological processes. Since it was shown to prolong lifespan and reduce inflammation, it has been used for skin anti-ageing, human hair growth stimulation, in the treatment of type 2 diabetes, and in increasing fruit shelf life.[208]

### 5.5. Fermentative Production of Polyamines

A system for spermidine production was constructed in *S. cerevisiae* by overexpressing a polyamine transporter for product secretion and genes of the spermidine biosynthetic pathway in addition to the disruption of a gene responsible for feedback inhibition of the pathway. As an inhibitory effect of glucose on spermidine production was observed, the process was designed to use a mixture of xylose and glucose as carbon source. However, in a fed-batch fermentation only about 0.2 g $L^{-1}$ were produced.[208] This may be due to the fact that SAM or dcSAM have to be regenerated from methylthioadenosine, which arises from dcSAM-dependent *N*-propylamination of putrescine in spermidine biosynthesis. For example, the fermentative 1,3-diaminopropane production by metabolically engineered *E. coli* yielded two orders of magnitude higher titers for 1,3-diaminopropane, that is, 13 g $L^{-1}$.[209] These high titers can also be reached for putrescine production, that is, 42.3 g $L^{-1}$ obtained with *E. coli* in a fed-batch process at a yield of 0.26 g putrescine per g glucose and a volumetric productivity of 1.23 g $L^{-1}$ $h^{-1}$.[210] A *C. glutamicum* fed-batch process led to 19 g $L^{-1}$ putrescine at a yield of 0.16 g putrescine per g glucose and a volumetric productivity of 0.55 g $L^{-1}$ $h^{-1}$.[211]

As *C. glutamicum* is an industrial L-lysine producer it is a suitable host for fermentative cadaverine production as evidenced by cadaverine production from glucose with a titer of 88 g $L^{-1}$ at a yield of 0.29 g cadaverine per g glucose and a productivity of 2.2 g $L^{-1}$ $h^{-1}$.[129] Integration of the *E. coli ldcC* gene driven by the *tuf* promoter into the lysine exporter gene *lysE* on the chromosome of an industrial L-lysine producing *C. glutamicum* strain led to the highest reported cadaverine titer of 103.8 g $L^{-1}$.[212] Cadaverine production with alternative carbon substrates such as xylose,[213] xylooligosaccharides,[214] cellobiose,[215] galactose (by *E. coli*),[216] or methanol (by *B. methanolicus*)[217] has also been described. Cadaverine production has also been shown by two novel approaches: using synthetic mutualistic consortia consisting either of two *E. coli* strains[124] or one *E. coli* and one *C. glutamicum* strain[218] on the one hand and coproduction of cadaverine with succinic acid, the monomeric precursors of polyamide PA5,4 on the other hand.[219] Conversion of L-lysine to cadaverine has been shown by whole-cell-biotransformation[216,220–223] or by biocatalysis using catalytically active inclusion bodies of LdcC from *E. coli*.[224]

Conceptionally, the efficient 1,3-diaminopropane, putrescine, and cadaverine overproducing strains could be used as basis for production of polyamines with secondary amine groups. For example, the bacterial homospermidine synthase found in many proteobacteria such as *Legionella pneumophila* and *P. aeruginosa* condenses two molecules of putrescine to *sym*-homospermidine[225] and might be expressed in a putrescine overproducing strain to enable fermentative production of *sym*-homospermidine. The recent demonstration of production of *N*-methylpyrrolinium (Figure 3), the precursor of alkaloids such as tropane alkaloids, by metabolically engineered *E. coli*, and *S. cerevisiae* strains is important.[226] First, putrescine *N*-methyltransferase from the plant *Anisodus tanguticus* converts putrescine to *N*-methylputrescine, a polyamine with a secondary amine group. Second, diamine oxidases (DAOs) from the related plant *Anisodus acutangulus* oxidize *N*-methylputrescine to *N*-methyl-$\gamma$-butyraldehyde which spontaneously cyclizes to *N*-methylpyrrolinium, a cyclic quaternary imine compound. Titers of 0.002–0.017 g $L^{-1}$ *N*-methylpyrrolinium were obtained.[226]

The discovery of an enzyme from the hyperthermophilic archaeon *Pyrococcus furiosus* catalyzing aminopropylation of the polyamines agmatine, 1,3-diaminopropane, putrescine, cadaverine, and *sym*-norspermidine[227] may open ways to establish aminopropyl derivatives of poylamines such as tridansyl-*N*-(3-aminopropyl)cadaverine.

## 6. Conclusions and Perspectives

The fermentative production of *N*-functionalized compounds has seen first successes, but this field is still in its infancy. For industrial application, key parameters such as titers, yields, and volumetric productivities have to be met and downstream processing requires that the chosen product can easily be separated from structurally similar side-products or formation of these has to be avoided by process operation or by strain engineering.

The employment of *N*-functionalizing enzymes with broad substrate specificity such as DpkA used for alkylamination of 2-oxo acids required metabolic engineering strategies for the selective provision of the required 2-oxo acids (e.g., DpkA in a host engineered for pyruvate overproduction yielded *N*-methyl-L-alanine, while DkpA in a host engineered for glyoxylate production yielded sarcosine). Enzyme engineering and directed evolution have until now played a minor role to direct substrate selectivity towards obtaining a specific *N*-functionalized target product in high purity. However, this dynamically developing research area[6] is expected to have a major impact on realizing biocatalytic and fermentative processes for production of *N*-functionalized fine chemicals.

Embedding the employed *N*-functionalizing enzyme into the metabolism of the production host is critical for production to high titers, yields, and productivities. In this respect, we foresee major improvements of the production hosts through genetic screens facilitated by the new CRISPR/Cas genome editing and CRISPRi gene repression tools that can be multiplexed for higher throughput.[228] Moreover, non-targeted approaches such as adaptive laboratory evolution (ALE) will speed up strain development and in combination with genome re-sequencing the selected genetic changes can be identified. Gene targets identified by CRISPR/Cas and CRISPRi screens as well as genome alterations identified by genome re-sequencing of strains selected in ALE experiments will have to be assessed in follow-up experiments to determine if they are causal for improved performance. This subset of genes can then be used as basis for rational systems metabolic engineering avoiding possible detrimental effects of bystander mutations that may have arisen during ALE experiments.

These technological developments as well as a dramatically growing metagenomics data base provide access to the hitherto unexploited catalyst reservoir present in microorganisms, which cannot be cultivated in the laboratory. They bear the potential to inspire the development of a plethora of efficient fermentative production processes, which would not only lead to *N*-alkylated, *N*-hydroxylated, or *N*-acylated bioactive compounds, betaines, and polyamines, but also to other *N*-functionalized compounds

such as *N*-nitroso or *N*-phosphorylated compounds or phosphoramidates.

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## Conflict of Interest

The authors declare no conflict of interest.

## Keywords

bioactives, fermentation, metabolic engineering, natural products, *N*-functionalization

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[1] Y. Li, C. Wang, F. Zhu, Z. Wang, J. F. Soulé, P. H. Dixneuf, X.-F. Wu, *Chem. Commun.* **2016**, *53*, 142.
[2] K. Natte, H. Neumann, M. Beller, R. V. Jagadeesh, *Angew. Chem., Int. Ed.* **2017**, *56*, 6384.
[3] I. Scott, M. Yamauchi, M. Sricholpech, *Essays Biochem.* **2012**, *52*, 113.
[4] J. J. Petkowski, W. Bains, S. Seager, *Molecules* **2019**, *24*, 866.
[5] T. L. Ng, R. Rohac, A. J. Mitchell, A. K. Boal, E. P. Balskus, *Nature* **2019**, *566*, 94.
[6] S. C. Hammer, A. M. Knight, F. H. Arnold, *Curr. Opin. Green Sustain. Chem.* **2017**, *7*, 23.
[7] J. Chatterjee, F. Rechenmacher, H. Kessler, *Angew. Chem., Int. Ed.* **2013**, *52*, 254.
[8] M. L. di Gioia, A. Leggio, F. Malagrinò, E. Romio, C. Siciliano, A. Liguori, *Mini-Rev. Med. Chem.* **2016**, *16*, 683.
[9] A. Sharma, A. Kumar, S. A. H. A. Monaim, Y. E. Jad, A. El-Faham, B. G. de la Torre, F. Albericio, *Biopolymers* **2018**, *109*, e23110.
[10] J. Chatterjee, O. Ovadia, G. Zahn, L. Marinelli, A. Hoffman, C. Gilon, H. Kessler, *J. Med. Chem.* **2007**, *50*, 5878.
[11] C. Mas-Moruno, F. Rechenmacher, H. Kessler, *Anti-Cancer Agents Med. Chem.* **2010**, *10*, 753.
[12] R. J. Pollock, L. B. Hersh, *J. Biol. Chem.* **1971**, *246*, 4737.
[13] K. Sasaoka, M. Kito, Y. Onishi, *Agric. Biol. Chem.* **1965**, *29*, 984.
[14] L. Gentilucci, R. de Marco, L. Cerisoli, *Curr. Pharm. Des.* **2010**, *16*, 3185.
[15] C. Gilon, M. A. Dechantsreiter, F. Burkhart, A. Friedler, H. Kessler, in *Houben-Weyl Methods of Organic Chemistry* (Eds: M. Goodman, A. Felix, L. Moroder, C. Toniolo), Georg Thieme Verlag, Stuttgart, Germany **2002**, pp. 215–291.
[16] N. Sewald, *Angew. Chem., Int. Ed.* **2002**, *41*, 4661.
[17] L. Aurelio, R. T. C. Brownlee, A. B. Hughes, *Chem. Rev.* **2004**, *104*, 5823.
[18] Y. Chen, J. Scanlan, L. Song, A. Crombie, M. T. Rahman, H. Schafer, J. C. Murrell, *Appl. Environ. Microbiol.* **2010**, *76*, 4530.
[19] C. Gruffaz, E. E. L. Muller, Y. Louhichi-Jelail, Y. R. Nelli, G. Guichard, F. Bringel, *Appl. Environ. Microbiol.* **2014**, *80*, 3541.
[20] E. Latypova, S. Yang, Y.-S. Wang, T. Wang, T. A. Chavkin, M. Hackett, H. Schäfer, M. G. Kalyuzhnaya, *Mol. Microbiol.* **2010**, *75*, 426.
[21] D. D. Nayak, C. J. Marx, *J. Bacteriol.* **2014**, *196*, 4130.
[22] M. Mindt, T. Walter, J. M. Risse, V. F. Wendisch, *Front. Bioeng. Biotechnol.* **2018**, *6*, 159.
[23] Y. Sakato, *J. Agric. Chem. Soc. Jpn.* **1950**, *23*, 262.
[24] J. Casimir, J. Jadot, M. Renard, *Bioch. Biophys. Acta* **1960**, *39*, 462.
[25] M. Nakagawa, *Jpn. Agric. Res. Q.* **1970**, *5*, 43.
[26] M. Saeed, M. Naveed, M. Arif, M. U. Kakar, R. Manzoor, M. E. A. El-Hack, M. Alagawany, R. Tiwari, R. Khandia, A. Munjal, K. Karthik, K. Dhama, H. M. N. Iqbal, M. Dadar, C. Sun, *Biomed. Pharmacother.* **2017**, *95*, 1260.
[27] J. Williams, D. Sergi, A. J. McKune, E. N. Georgousopoulou, D. D. Mellor, N. Naumovski, *Phytother. Res.* **2019**, *33*, 571.
[28] T. Takeo, *Phytochemistry* **1974**, *13*, 1401.
[29] T. Takeo, *Phytochemistry* **1978**, *17*, 313.
[30] A. Ekanayake, J. J. Li (Circulat Biotech Llc, Procter and Gamble Co.), US7303773B2, **2007**.
[31] M. Lachová, J. Lehotay, G. Karasová, I. Skačáni, D. W. Armstrong, *J. Liq. Chromatogr. Relat. Technol.* **2007**, *30*, 2045.
[32] Y. Zhang, B. Chen, Z. Huang, Z. Shi, *J. Liq. Chromatogr. Relat. Technol.* **2004**, *27*, 875.
[33] T. Tachiki, H. Suzuki, S. Wakisaka, T. Yano, T. Tochikura, *J. Gen. Appl. Microbiol.* **1986**, *32*, 545.
[34] T. Takashi, Y. Takeshi, U. Masashi, N. Yasuki, I. Nobuo, H. Yu-ichi, S. Ju-ichi, *Biosci., Biotechnol., Biochem.* **1996**, *60*, 1160.
[35] H. Suzuki, S. Izuka, N. Miyakawa, H. Kumagai, *Enzyme Microb. Technol.* **2002**, *31*, 884.
[36] T. Kimura, I. Sugahara, K. Hanai, Y. Tonomura, *Biosci., Biotechnol., Biochem.* **1992**, *56*, 708.
[37] W. Mu, T. Zhang, B. Jiang, *Biotechnol. Adv.* **2015**, *33*, 335.
[38] Y. Li, L. Sun, J. Feng, R. Wu, Q. Xu, C. Zhang, N. Chen, X. Xie, *Bioprocess Biosyst. Eng.* **2016**, *39*, 967.
[39] H. Ma, X. Fan, N. Cai, D. Zhang, G. Zhao, T. Wang, R. Su, M. Yuan, Q. Ma, C. Zhang, Q. Xu, X. Xie, N. Chen, Y. Li, *Appl. Microbiol. Biotechnol.* **2020**, *104*, 119.
[40] W. V. Shaw, L. Tsai, E. R. Stadtman, *J. Biol. Chem.* **1966**, *241*, 935.
[41] M. Mindt, S. Hannibal, M. Heuser, J. M. Risse, K. Sasikumar, K. M. Nampoothiri, V. F. Wendisch, *Front. Bioeng. Biotechnol.* **2019**, *7*, 232.
[42] M. Mindt, M. Heuser, V. F. Wendisch, *Bioresour. Technol.* **2019**, *281*, 135.
[43] M. Mindt, J. M. Risse, H. Gruß, N. Sewald, B. J. Eikmanns, V. F. Wendisch, *Sci. Rep.* **2018**, *8*, 12895.
[44] H. Muramatsu, H. Mihara, R. Kakutani, M. Yasuda, M. Ueda, T. Kurihara, N. Esaki, *J. Biol. Chem.* **2005**, *280*, 5329.
[45] H.-F. Kung, C. Wagner, *Biochim. Biophys. Acta, Gen. Subj.* **1970**, *201*, 513.
[46] H. Mihara, H. Muramatsu, R. Kakutani, M. Yasuda, M. Ueda, T. Kurihara, N. Esaki, *FEBS J.* **2005**, *272*, 1117.
[47] H. Muramatsu, H. Mihara, R. Kakutani, M. Yasuda, M. Ueda, T. Kurihara, N. Esaki, *Tetrahedron: Asymmetry* **2004**, *15*, 2841.
[48] S. Wieschalka, B. Blombach, B. J. Eikmanns, *Appl. Microbiol. Biotechnol.* **2012**, *94*, 449.
[49] B. Blombach, M. E. Schreiner, M. Moch, M. Oldiges, B. J. Eikmanns, *Appl. Microbiol. Biotechnol.* **2007**, *76*, 615.
[50] B. Blombach, M. E. Schreiner, J. Holatko, T. Bartek, M. Oldiges, B. J. Eikmanns, *Appl. Environ. Microbiol.* **2007**, *73*, 2079.
[51] B. Blombach, A. Arndt, M. Auchter, B. J. Eikmanns, *Appl. Environ. Microbiol.* **2009**, *75*, 1197.

 

[52] B. Blombach, T. Riester, S. Wieschalka, C. Ziert, J.-W. Youn, V. F. Wendisch, B. J. Eikmanns, *Appl. Environ. Microbiol.* **2011**, *77*, 3300.
[53] J. Buchholz, A. Schwentner, B. Brunnenkan, C. Gabris, S. Grimm, R. Gerstmeir, R. Takors, B. J. Eikmanns, B. Blombach, *Appl. Environ. Microbiol.* **2013**, *79*, 5566.
[54] B. J. Eikmanns, B. Blombach, *J. Biotechnol.* **2014**, *192*, 339.
[55] F. S. Krause, B. Blombach, B. J. Eikmanns, *Appl. Environ. Microbiol.* **2010**, *76*, 8053.
[56] R. Werdehausen, D. Kremer, T. Brandenburger, L. Schlösser, J. Jadasz, P. Küry, I. Bauer, C. Aragón, V. Eulenburg, H. Hermanns, *Anesthesiology* **2012**, *116*, 147.
[57] G. Tsai, H.-Y. Lane, P. Yang, M.-Y. Chong, N. Lange, *Biol. Psychiatry* **2004**, *55*, 452.
[58] A. Zahoor, A. Otten, V. F. Wendisch, *J. Biotechnol.* **2014**, *192*, 366.
[59] S. Atsumi, T. Hanai, J. C. Liao, *Nature* **2008**, *451*, 86.
[60] S. Atsumi, W. Higashide, J. C. Liao, *Nat. Biotechnol.* **2009**, *27*, 1177.
[61] J. C. Liao, L. Mi, S. Pontrelli, S. Luo, *Nat. Rev. Microbiol.* **2016**, *14*, 288.
[62] K. M. Smith, K.-M. Cho, J. C. Liao, *Appl. Microbiol. Biotechnol.* **2010**, *87*, 1045.
[63] V. Bückle-Vallant, F. S. Krause, S. Messerschmidt, B. J. Eikmanns, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 297.
[64] H. B. Coban, A. Demirci, P. H. Patterson, R. J. Elias, *Bioprocess Biosyst. Eng.* **2014**, *37*, 2343.
[65] J.-H. Jo, H.-Y. Seol, Y.-B. Lee, M.-H. Kim, H.-H. Hyun, H.-H. Lee, *Can. J. Microbiol.* **2012**, *58*, 278.
[66] N. Kataoka, A. S. Vangnai, T. Pongtharangkul, T. Yakushi, M. Wada, A. Yokota, K. Matsushita, *Biosci., Biotechnol., Biochem.* **2019**, *83*, 372.
[67] D. Lubitz, V. F. Wendisch, *BMC Microbiol.* **2016**, *16*, 235.
[68] Y. Song, J. Li, H. Shin, L. Liu, G. Du, J. Chen, *Bioresour. Technol.* **2016**, *219*, 716.
[69] M. Vogt, S. Haas, T. Polen, J. van Ooyen, M. Bott, *Microb. Biotechnol.* **2015**, *8*, 351.
[70] H. L. Lee, S.-Y. Kim, E. J. Kim, D. Y. Han, B.-G. Kim, J.-H. Ahn, *J. Microbiol. Biotechnol.* **2019**, *29*, 839.
[71] H. J. Choo, J.-H. Ahn, *J. Agric. Food Chem.* **2019**, *67*, 8581.
[72] E. Correa, W. Quiñones, F. Echeverri, *Pharm. Biol.* **2016**, *54*, 569.
[73] N. S. Radulović, A. B. Miltojević, M. McDermott, S. Waldren, J. A. Parnell, M. M. G. Pinheiro, P. D. Fernandes, F. d. S. Menezes, *J. Ethnopharmacol.* **2011**, *135*, 610.
[74] Z. W. Luo, J. S. Cho, S. Y. Lee, *Proc. Natl. Acad. Sci. USA* **2019**, *116*, 10749.
[75] N. Henke, J. Wichmann, T. Baier, J. Frohwitter, K. Lauersen, J. Risse, P. Peters-Wendisch, O. Kruse, V. Wendisch, *Genes* **2018**, *9*, 219.
[76] E. G. Hrycay, S. M. Bandiera, in *Monooxygenase, Peroxidase and Peroxygenase Properties and Mechanisms of Cytochrome P450* (Eds: E. G. Hrycay, S. M. Bandiera), Springer International Publishing, Cham **2015**, pp. 1–61.
[77] P. F. Fitzpatrick, *Biochemistry* **2003**, *42*, 14083.
[78] G. Zurlo, J. Guo, M. Takada, W. Wei, Q. Zhang, *Biochim. Biophys. Acta* **2016**, *1866*, 208.
[79] L.-K. Liu, H. Abdelwahab, J. S. M. D. Campo, R. Mehra-Chaudhary, P. Sobrado, J. J. Tanner, *J. Biol. Chem.* **2016**, *291*, 21553.
[80] J. B. Neilands, *J. Biol. Chem.* **1995**, *270*, 26723.
[81] C. Naumann, T. Hartmann, D. Ober, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 6085.
[82] S. Sehlmeyer, L. Wang, D. Langel, D. G. Heckel, H. Mohagheghi, G. Petschenka, D. Ober, *PLoS One* **2010**, *5*, e10435.
[83] G. L. Challis, *ChemBioChem* **2005**, *6*, 601.
[84] V. de Lorenzo, J. B. Neilands, *J. Bacteriol.* **1986**, *167*, 350.
[85] V. de Lorenzo, A. Bindereif, B. H. Paw, J. B. Neilands, *J. Bacteriol.* **1986**, *165*, 570.
[86] S. V. Smirnov, N. N. Samsonova, A. E. Novikova, N. G. Matrosov, N. Y. Rushkevich, T. Kodera, J. Ogawa, H. Yamanaka, S. Shimizu, *FEMS Microbiol. Lett.* **2007**, *273*, 70.
[87] X.-C. Wang, J. Liu, J. Zhao, X.-M. Ni, P. Zheng, X. Guo, C.-M. Sun, J.-B. Sun, Y.-H. Ma, *J. Biosci. Bioeng.* **2018**, *126*, 470.
[88] M. D. Andersen, P. K. Busk, I. Svendsen, B. L. Møller, *J. Biol. Chem.* **2000**, *275*, 1966.
[89] N. V. Geraskina, E. V. Sycheva, V. V. Samsonov, N. S. Eremina, C. D. Hook, V. A. Serebrianyi, N. V. Stoynova, *PLoS One* **2019**, *14*, e0215777.
[90] G. Han, X. Hu, X. Wang, *Enzyme Microb. Technol.* **2015**, *78*, 27.
[91] S. Hasegawa, M. Suda, K. Uematsu, Y. Natsuma, K. Hiraga, T. Jojima, M. Inui, H. Yukawa, *Appl. Environ. Microbiol.* **2013**, *79*, 1250.
[92] Q.-G. Huang, B.-D. Zeng, L. Liang, S.-G. Wu, J.-Z. Huang, *World J. Microbiol. Biotechnol.* **2018**, *34*, 121.
[93] C. Lange, D. Rittmann, V. F. Wendisch, M. Bott, H. Sahm, *Appl. Environ. Microbiol.* **2003**, *69*, 2521.
[94] K.-M. Lee, S.-K. Kim, Y.-G. Lee, K.-H. Park, J.-H. Seo, *Bioresour. Technol.* **2018**, *268*, 271.
[95] C. Liang, Y. Huo, G. Qi, X. Wei, Q. Wang, S. Chen, *Biotechnol. Lett.* **2015**, *37*, 1243.
[96] E. Lorenz, S. Klatte, V. F. Wendisch, *J. Biotechnol.* **2013**, *168*, 289.
[97] W. Ma, J. Wang, Y. Li, X. Wang, *Biotechnol. Appl. Biochem.* **2019**, *66*, 74.
[98] M. Oldiges, B. J. Eikmanns, B. Blombach, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 5859.
[99] N. Takpho, D. Watanabe, H. Takagi, *Metab. Eng.* **2018**, *46*, 60.
[100] X. Wang, H. Zhang, P. J. Quinn, *Appl. Microbiol. Biotechnol.* **2018**, *102*, 4319.
[101] P. Macheroux, H. J. Plattner, A. Romaguera, H. Diekmann, *Eur. J. Biochem.* **1993**, *213*, 995.
[102] J. H. Lee, V. F. Wendisch, *Biores. Technol.* **2017**, *245*, 1575.
[103] V. F. Wendisch, *Metab. Eng.* **2020**, *58*, 17.
[104] J. Becker, O. Zelder, S. Häfner, H. Schröder, C. Wittmann, *Metab. Eng.* **2011**, *13*, 159.
[105] N. Tatsumi, M. Inui, *Corynebacterium Glutamicum: Biology and Biotechnology*, Springer Science & Business Media, Secaucus, NJ **2012**.
[106] T. Brautaset, Ø. M. Jakobsen, K. F. Degnes, R. Netzer, I. Naerdal, A. Krog, R. Dillingham, M. C. Flickinger, T. E. Ellingsen, *Appl. Microbiol. Biotechnol.* **2010**, *87*, 951.
[107] H. Ying, X. He, Y. Li, K. Chen, P. Ouyang, *Appl. Biochem. Biotechnol.* **2014**, *172*, 3835.
[108] K. M. Meneely, E. W. Barr, J. M. Bollinger, A. L. Lamb, *Biochemistry* **2009**, *48*, 4371.
[109] J. V. K. Jensen, D. Eberhardt, V. F. Wendisch, *J. Biotechnol.* **2015**, *214*, 85.
[110] Y.-J. Lee, J.-Y. Cho, *Biotechnol. Lett.* **2006**, *28*, 1849.
[111] M. Rakhimuzzaman, M. Noda, N. Danshiitsoodol, M. Sugiyama, *Biol. Pharm. Bull.* **2019**, *42*, 1581.
[112] J. Schneider, V. F. Wendisch, *Appl. Microbiol. Biotechnol.* **2010**, *88*, 859.
[113] M. Wang, M. Xu, Z. Rao, T. Yang, X. Zhang, *J. Ind. Microbiol. Biotechnol.* **2015**, *42*, 1427.
[114] B. Zhang, G. Gao, X.-H. Chu, B.-C. Ye, *Bioresour. Technol.* **2019**, *284*, 204.
[115] M. Hartmann, T. Zeier, F. Bernsdorff, V. Reichel-Deland, D. Kim, M. Hohmann, N. Scholten, S. Schuck, A. Bräutigam, T. Hölzel, C. Ganter, J. Zeier, *Cell* **2018**, *173*, 456.e16.
[116] F. Pérez-García, J. M. Risse, K. Friehs, V. F. Wendisch, *Biotechnol. J.* **2017**, *12*, 1600646.
[117] F. Pérez-García, P. Peters-Wendisch, V. F. Wendisch, *Appl. Microbiol. Biotechnol.* **2016**, *100*, 8075.

 

[118] T. Böttcher, J. Clardy, *Angew. Chem., Int. Ed.* **2014**, *53*, 3510.
[119] S. Rütschlin, S. Gunesch, T. Böttcher, O. Enzyme, *Cell Chem. Biol.* **2017**, *24*, 598.e10.
[120] C. O. Esuola, O. O. Babalola, T. Heine, R. Schwabe, M. Schlömann, D. Tischler, *J. Mol. Catal. B: Enzym.* **2016**, *134*, 378.
[121] F. de Filippis, C. Pennacchia, R. di Pasqua, A. Fiore, V. Fogliano, F. Villani, D. Ercolini, *Int. J. Food Microbiol.* **2013**, *165*, 332.
[122] M. Irla, T. M. B. Heggeset, I. Nærdal, L. Paul, T. Haugen, S. B. Le, T. Brautaset, V. F. Wendisch, *Front. Microbiol.* **2019**, *10*, 425.
[123] Y.-M. Moon, S. Y. Yang, T. R. Choi, H.-R. Jung, H.-S. Song, Y. H. Han, H. Y. Park, S. K. Bhatia, R. Gurav, K. Park, J.-S. Kim, Y.-H. Yang, *Enzyme Microb. Technol.* **2019**, *127*, 58.
[124] J. Wang, X. Lu, H. Ying, W. Ma, S. Xu, X. Wang, K. Chen, P. Ouyang, *Front. Microbiol.* **2018**, *9*, 1312.
[125] Z. Bubelová, F. Buňka, M. Tařáková, K. Štajnochová, K. Purevdorj, L. Buňková, *J. Environ. Sci. Health, Part B* **2015**, *50*, 797.
[126] B. del Rio, B. Redruello, V. Ladero, M. Fernandez, M. C. Martin, M. A. Alvarez, *Int. J. Food Microbiol.* **2016**, *232*, 1.
[127] Z. Li, Y.-P. Shen, X.-L. Jiang, L.-S. Feng, J.-Z. Liu, *J. Ind. Microbiol. Biotechnol.* **2018**, *45*, 123.
[128] D. R. Morris, K. L. Koffron, *J. Bacteriol.* **1967**, *94*, 1516.
[129] S. Kind, S. Neubauer, J. Becker, M. Yamamoto, M. Völkert, G. , Abendroth, O. Zelder, C. Wittmann, *Metab. Eng.* **2014**, *25*, 113.
[130] A. Q. D. Nguyen, J. Schneider, V. F. Wendisch, *J. Biotechnol.* **2015**, *201*, 75.
[131] A. Q. D. Nguyen, J. Schneider, G. K. Reddy, V. F. Wendisch, *Metabolites* **2015**, *5*, 211.
[132] J. Jiao, Y. Ma, S. Chen, C. Liu, Y. Song, Y. Qin, C. Yuan, Y. Liu, *Front. Plant Sci.* **2016**, *7*, 1387.
[133] I. L. Lamont, L. W. Martin, T. Sims, A. Scott, M. Wallace, *J. Bacteriol.* **2006**, *188*, 3149.
[134] S.-W. Lee, M.-K. Oh, *Biotechnol. Bioeng.* **2016**, *113*, 2524.
[135] A. M. Ahmed, E. E. A. Younis, S. A. Osman, Y. Ishida, S. A. Elkhodery, T. Shimamoto, *Vet. Microbiol.* **2009**, *136*, 397.
[136] A. Drazic, L. M. Myklebust, R. Ree, T. Arnesen, *Biochim. Biophys. Acta, Proteins Proteomics* **2016**, *1864*, 1372.
[137] K. T. Nguyen, S.-H. Mun, C.-S. Lee, C.-S. Hwang, *Exp. Mol. Med.* **2018**, *50*, 1.
[138] G. Seltmann, O. Holst, *The Bacterial Cell Wall*, 1st ed., Springer-Verlag, Berlin Heidelberg **2002**.
[139] D. Zhao, Y. Yu, Y. Shen, Q. Liu, Z. Zhao, R. Sharma, R. J. Reiter, *Front. Endocrinol.* **2019**, *10*, 249.
[140] M. T. Ringel, T. Brüser, *Microb. Cell* **2018**, *5*, 424.
[141] A. Robicsek, J. Strahilevitz, G. A. Jacoby, M. Macielag, D. Abbanat, C. H. Park, K. Bush, D. C. Hooper, *Nat. Med.* **2006**, *12*, 83.
[142] L. Liu, Y. Liu, H. Shin, R. Chen, J. Li, G. Du, J. Chen, *Appl. Microbiol. Biotechnol.* **2013**, *97*, 6149.
[143] S.-W. Lee, B.-Y. Lee, M.-K. Oh, *J. Agric. Food Chem.* **2018**, *66*, 13191.
[144] S.-W. Lee, M.-K. Oh, *Metab. Eng.* **2015**, *28*, 143.
[145] W. Ma, Y. Liu, H.-D. Shin, J. Li, J. Chen, G. Du, L. Liu, *Bioresour. Technol.* **2018**, *250*, 642.
[146] Y. Wu, T. Chen, Y. Liu, R. Tian, X. Lv, J. Li, G. Du, J. Chen, R. Ledesma-Amaro, L. Liu, *Nucleic Acids Res.* **2020**, *48*, 996.
[147] Y. Liu, Y. Zhu, J. Li, H. Shin, R. R. Chen, G. Du, L. Liu, J. Chen, *Metab. Eng.* **2014**, *23*, 42.
[148] Y. Liu, Y. Zhu, W. Ma, H. Shin, J. Li, L. Liu, G. Du, J. Chen, *Metab. Eng.* **2014**, *24*, 61.
[149] M.-D. Deng, D. K. Severson, A. D. Grund, S. L. Wassink, R. P. Burlingame, A. Berry, J. A. Running, C. A. Kunesh, L. Song, T. A. Jerrell, R. A. Rosson, *Metab. Eng.* **2005**, *7*, 201.
[150] X. Chen, L. Liu, J. Li, G. Du, J. Chen, *Bioresour. Technol.* **2012**, *110*, 534.
[151] X. Chen, L. Liu, J. Li, J. Liu, G. Du, J. Chen, *J. Ind. Microbiol. Biotechnol.* **2012**, *39*, 359.
[152] X. Chen, J. Zhou, L. Zhang, Z. Pu, L. Liu, W. Shen, Y. Fan, *Metab. Eng.* **2018**, *47*, 374.
[153] X. Gao, F. Zhang, M. Wu, Z. Wu, G. Shang, *J. Agric. Food Chem.* **2019**, *67*, 6285.
[154] F. Tao, Y. Zhang, C. Ma, P. Xu, *Appl. Microbiol. Biotechnol.* **2010**, *87*, 1281.
[155] D. Zhu, J. Wu, X. Zhan, L. Zhu, Y. Jiang, *J. Ind. Microbiol. Biotechnol.* **2019**, *46*, 125.
[156] S. F. Brady, J. Clardy, *Org. Lett.* **2005**, *7*, 3613.
[157] J. Clardy, S. F. Brady, *J. Bacteriol.* **2007**, *189*, 6487.
[158] J. W. Craig, F.-Y. Chang, J. H. Kim, S. C. Obiajulu, S. F. Brady, *Appl. Environ. Microbiol.* **2010**, *76*, 1633.
[159] B. Kim, R. Binkley, H. U. Kim, S. Y. Lee, *Biotechnol. Bioeng.* **2018**, *115*, 2554.
[160] Q. Wang, W. Zeng, J. Zhou, *Chin. J. Biotechnol.* **2019**, *35*, 1247.
[161] M. Xu, J. Li, Q. Shu, M. Tang, X. Zhang, T. Yang, Z. Xu, Z. Rao, *J. Ind. Microbiol. Biotechnol.* **2019**, *46*, 1155.
[162] D. Lubitz, J. M. P. Jorge, F. Pérez-García, H. Taniguchi, V. F. Wendisch, *Appl. Microbiol. Biotechnol.* **2016**, *100*, 8465.
[163] C. Zhao, H. Fang, J. Wang, S. Zhang, X. Zhao, Z. Li, C. Lin, Z. Shen, L. Cheng, *Biotechnol. Prog.* **2019**, *5*, e2944.
[164] C. Zang, Z. Zhao, Y. Wang, Y. Zhang, J. Ding, *Chin. J. Biotechnol.* **2014**, *54*, 24.
[165] J. Wu, Y. Liu, S. Zhao, J. Sun, Z. Jin, D. Zhang, *J. Microbiol. Biotechnol.* **2019**, *29*, 923.
[166] C. Zhang, J. Zhang, Z. Kang, G. Du, J. Chen, *J. Ind. Microbiol. Biotechnol.* **2015**, *42*, 787.
[167] D. K. Marvil, T. Leisinger, *J. Biol. Chem.* **1977**, *252*, 3295.
[168] K. Petri, F. Walter, M. Persicke, C. Rückert, J. Kalinowski, *BMC Genomics* **2013**, *14*, 713.
[169] D. Shi, L. Caldovic, Z. Jin, X. Yu, Q. Qu, L. Roth, H. Morizono, Y. Hathout, N. M. Allewell, M. Tuchman, *Acta Crystallogr., Sect. F: Struct. Biol. Cryst. Commun.* **2006**, *62*, 1218.
[170] W. Dou, M. Xu, D. Cai, X. Zhang, Z. Rao, Z. Xu, *Appl. Biochem. Biotechnol.* **2011**, *165*, 845.
[171] A. Iqbal, I. J. Clifton, M. Bagonis, N. J. Kershaw, C. Domene, T. D. W. Claridge, C. W. Wharton, C. J. Schofield, *J. Am. Chem. Soc.* **2009**, *131*, 749.
[172] S. H. Park, H. U. Kim, T. Y. Kim, J. S. Park, S.-S. Kim, S. Y. Lee, *Nat. Commun.* **2014**, *5*, 4618.
[173] J. V. K. Jensen, V. F. Wendisch, *Microb. Cell Fact.* **2013**, *12*, 63.
[174] D. Eberhardt, J. V. K. Jensen, V. F. Wendisch, *AMB Express* **2014**, *4*, 85.
[175] H.-Y. Sagong, K.-J. Kim, *J. Agric. Food Chem.* **2015**, *63*, 10641.
[176] C. Richaud, F. Richaud, C. Martin, C. Haziza, J. C. Patte, *J. Biol. Chem.* **1984**, *259*, 14824.
[177] S. Kind, C. Wittmann, *Appl. Microbiol. Biotechnol.* **2011**, *91*, 1287.
[178] S. Kind, W. K. Jeong, H. Schröder, O. Zelder, C. Wittmann, *Appl. Environ. Microbiol.* **2010**, *76*, 5175.
[179] T. M. Meiswinkel, D. Rittmann, S. N. Lindner, V. F. Wendisch, *Bioresour. Technol.* **2013**, *145*, 254.
[180] B. Liu, A. Sutton, R. Sternglanz, *J. Biol. Chem.* **2005**, *280*, 16659.
[181] D. P. Woolridge, J. D. Martinez, D. E. Stringer, E. W. Gerner, *Biochem. J.* **1999**, *340*, 753.
[182] S. A. Shinsky, D. W. Christianson, *Biochemistry* **2018**, *57*, 3105.
[183] A. Nyyssölä, J. Kerovuo, P. Kaukinen, N. von Weymarn, T. Reinikainen, *J. Biol. Chem.* **2000**, *275*, 22196.
[184] M. Frauenkron, J.-P. Melder, G. Ruider, R. Rossbacher, H. Höke, in *Ullmann's Encyclopedia of Industrial Chemistry*, 6th ed., Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim, Germany **2001**.
[185] C. E. Gidding, in *Kirk-Othmer Encyclopedia of Chemical Technology*, John Wiley & Sons, Inc., Hoboken, NJ **2000**.
[186] P. Mäkelä, *Sugar Tech* **2004**, *6*, 207.

 

[187] V. Bernal, P. Arense, M. Cánovas, in *Industrial Biotechnology of Vitamins, Biopigments, and Antioxidants* (Eds: E. J. Vandamme, J. L. Revuelta), Wiley VCH, Weinheim **2016**, pp. 389–419.
[188] A. H. Datko, S. H. Mudd, *Plant Physiol.* **1988**, *88*, 854.
[189] A. H. Datko, S. H. Mudd, *Plant Physiol.* **1988**, *88*, 1338.
[190] M. L. Nuccio, M. J. Ziemak, S. A. Henry, E. A. Weretilnyk, A. D. Hanson, *J. Biol. Chem.* **2000**, *275*, 14095.
[191] S. D. McNeil, M. L. Nuccio, M. J. Ziemak, A. D. Hanson, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 10001.
[192] M. P. Prud'homme, T. S. Moore, *Plant Physiol.* **1992**, *100*, 1527.
[193] L. Servillo, A. Giovane, D. Cautela, D. Castaldo, M. L. Balestrieri, *PLoS One* **2014**, *9*, e84589.
[194] D. M. Cameron, S. T. Gregory, J. Thompson, M.-J. Suh, P. A. Limbach, A. E. Dahlberg, *J. Bacteriol.* **2004**, *186*, 5819.
[195] P. R. Borum, H. P. Broquist, *J. Biol. Chem.* **1977**, *252*, 5651.
[196] W.-K. Kang, B.-U. Lee, Y. Park, E.-S. Koh, J.-Y. Ju, J. Lee, H. Kim, H. Choi (CJ CheilJedang Corp), *US20080199920A1*, **2008**.
[197] J. Franken, A. Burger, J. H. Swiegers, F. F. Bauer, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 6377.
[198] R. C. Trievel, E. M. Flynn, R. L. Houtz, J. H. Hurley, *Nat. Struct. Mol. Biol.* **2003**, *10*, 545.
[199] J. R. Kar, J. E. Hallsworth, R. S. Singhal, *Environ. Technol. Innov.* **2015**, *3*, 68.
[200] J. R. Kar, R. S. Singhal, *Chem. Eng. Sci.* **2017**, *163*, 83.
[201] H.-P. Meyer, K. T. Robins, *Monatsh. Chem.* **2005**, *136*, 1269.
[202] P. Arense, V. Bernal, D. Charlier, J. Iborra, M. Foulquié-Moreno, M. Cánovas, *Microb. Cell Fact.* **2013**, *12*, 56.
[203] T. Akiyama, F. Watanabe, W. Mizunashi, presented at *Enzyme Engineering XXV*, Whistler, British Columbia, Canada September **2019**.
[204] J. Schneider, V. F. Wendisch, *Appl. Microbiol. Biotechnol.* **2011**, *91*, 17.
[205] V. F. Wendisch, M. Mindt, F. Pérez-García, *Appl. Microbiol. Biotechnol.* **2018**, *102*, 3583.
[206] T. U. Chae, J. H. Ahn, Y.-S. Ko, J. W. Kim, J. A. Lee, E. H. Lee, S. Y. Lee, *Metab. Eng.* **2020**, *58*, 2.
[207] W. H. Bowman, C. W. Tabor, H. Tabor, *J. Biol. Chem.* **1973**, *248*, 2480.
[208] S.-K. Kim, J.-H. Jo, Y.-C. Park, Y.-S. Jin, J.-H. Seo, *Enzyme Microb. Technol.* **2017**, *101*, 30.
[209] T. U. Chae, W. J. Kim, S. Choi, S. J. Park, S. Y. Lee, *Sci. Rep.* **2015**, *5*, 13040.
[210] M. Noh, S. M. Yoo, W. J. Kim, S. Y. Lee, *Cell Syst.* **2017**, *5*, 418.e4.
[211] J. Schneider, D. Eberhardt, V. F. Wendisch, *Appl. Microbiol. Biotechnol.* **2012**, *95*, 169.
[212] H. T. Kim, K.-A. Baritugo, Y. H. Oh, S. M. Hyun, T. U. Khang, K. H. Kang, S. H. Jung, B. K. Song, K. Park, I.-K. Kim, M. O. Lee, Y. Kam, Y. T. Hwang, S. J. Park, J. C. Joo, *ACS Sustainable Chem. Eng.* **2018**, *6*, 5296.
[213] N. Buschke, J. Becker, R. Schäfer, P. Kiefer, R. Biedendieck, C. Wittmann, *Biotechnol. J.* **2013**, *8*, 557.
[214] K. Imao, R. Konishi, M. Kishida, Y. Hirata, S. Segawa, N. Adachi, R. Matsuura, Y. Tsuge, T. Matsumoto, T. Tanaka, A. Kondo, *Bioresour. Technol.* **2017**, *245*, 1684.
[215] R. Matsuura, M. Kishida, R. Konishi, Y. Hirata, N. Adachi, S. Segawa, K. Imao, T. Tanaka, A. Kondo, *Biotechnol. Bioeng.* **2019**, *116*, 2640.
[216] D. H. Kwak, H. G. Lim, J. Yang, S. W. Seo, G. Y. Jung, *Biotechnol. Biofuels* **2017**, *10*, 20.
[217] I. Naerdal, J. Pfeifenschneider, T. Brautaset, V. F. Wendisch, *Microb. Biotechnol.* **2015**, *8*, 342.
[218] E. Sgobba, A. K. Stumpf, M. Vortmann, N. Jagmann, M. Krehenbrink, M. E. Dirks-Hofmeister, B. Moerschbacher, B. Philipp, V. F. Wendisch, *Bioresour. Technol.* **2018**, *260*, 302.
[219] J. Wang, J. Mao, W. Tian, G. Wei, S. Xu, W. Ma, K. Chen, M. Jiang, P. Ouyang, *Green Chem.* **2018**, *20*, 2880.
[220] W. Ma, W. Cao, B. Zhang, K. Chen, Q. Liu, Y. Li, P. Ouyang, *Sci. Rep.* **2015**, *5*, 15630.
[221] J. Shin, J. C. Joo, E. Lee, S. M. Hyun, H. J. Kim, S. J. Park, Y.-H. Yang, K. Park, *Appl. Biochem. Biotechnol.* **2018**, *185*, 909.
[222] W. Ma, W. Cao, H. Zhang, K. Chen, Y. Li, P. Ouyang, *Biotechnol. Lett.* **2015**, *37*, 799.
[223] H. T. Kim, K. A. Baritugo, Y. H. Oh, K. H. Kang, Y. J. Jung, S. Jang, B. K. Song, I. K. Kim, M. O. Lee, Y. T. Hwang, K. Park, S. J. Park, J. C. Joo, *Polymers* **2019**, *11*, 1184.
[224] R. Kloss, M. H. Limberg, U. Mackfeld, D. Hahn, A. Grünberger, V. D. Jäger, U. Krauss, M. Oldiges, M. Pohl, *Sci. Rep.* **2018**, *8*, 5856.
[225] S. Krossa, A. Faust, D. Ober, A. J. Scheidig, *Sci. Rep.* **2016**, *6*, 19501.
[226] Y. Ping, X. Li, B. Xu, W. Wei, W. Wei, G. Kai, Z. Zhou, Y. Xiao, *ACS Synth. Biol.* **2019**, *8*, 257.
[227] G. Cacciapuoti, M. Porcelli, M. A. Moretti, F. Sorrentino, L. Concilio, V. Zappia, Z.-J. Liu, W. Tempel, F. Schubot, J. P. Rose, B.-C. Wang, P. S. Brereton, F. E. Jenney, M. W. W. Adams, *J. Bacteriol.* **2007**, *189*, 6057.
[228] K. Schultenkämper, L. F. Brito, V. F. Wendisch, *Biotechnol. Appl. Biochem.* **2020**, bab.1901.

 