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Metabolic Engineering of *Corynebacterium glutamicum* for High-Level Ectoine Production: Design, Combinatorial Assembly, and Implementation of a Transcriptionally Balanced Heterologous Ectoine Pathway

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Ectoine is formed in various bacteria as cell protectant against all kinds of stress. Its preservative and protective effects have enabled various applications in medicine, cosmetics, and biotechnology, and ectoine therefore has high commercial value. Industrially, ectoine is produced in a complex high-salt process, which imposes constraints on the costs, design, and durability of the fermentation system. Here, *Corynebacterium glutamicum* is upgraded for the heterologous production of ectoine from sugar and molasses. To overcome previous limitations, the ectoine pathway taken from *Pseudomonas stutzeri* is engineered using transcriptional balancing. An expression library with 185,193 variants is created, randomly combining 19 synthetic promoters and three linker elements. Strain screening discovers several high-titer mutants with an improvement of almost fivefold over the initial strain. High production thereby particularly relies on a specifically balanced ectoine pathway. In an optimized fermentation process, the new top producer *C. glutamicum ectABC*^{opt} achieves an ectoine titer of 65 g L^{-1} and a specific productivity of 120 mg g^{-1} h^{-1}. This process is the first reported example of a simple fermentation process under low-salt conditions using well-established feedstocks to produce ectoine with industrial efficiency. There is a compelling case for more intensive implementation of transcriptional balancing in future metabolic engineering of *C. glutamicum*.

1. Introduction

Various microbes accumulate ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine-carboxylic acid) as a protective extremolyte under conditions of environmental stress, e.g., high osmolarity, dryness, and extreme temperature.[1,2] The chemical is of recognized commercial value because of its remarkable ability to protect macromolecules, cells, and tissues.[3,4] Its current sales price is approximately US$1000 kg^{-1}, making ectoine a high-priced product.[5] The applications of ectoine are manifold and include skin preservation, skin protection against cell damage and aging,[6] and the treatment of diseases such as atopic dermatitis,[7] lung inflammation,[8] allergic rhinitis,[9] and Alzheimer's disease.[10] Furthermore, ectoine is used as a medium ingredient to enhance biotechnology processes.[11]

Commercially, ectoine production is realized by fermentation of the halophilic bacterium *Halomonas elongata*.[12] The microbe naturally synthesizes ectoine

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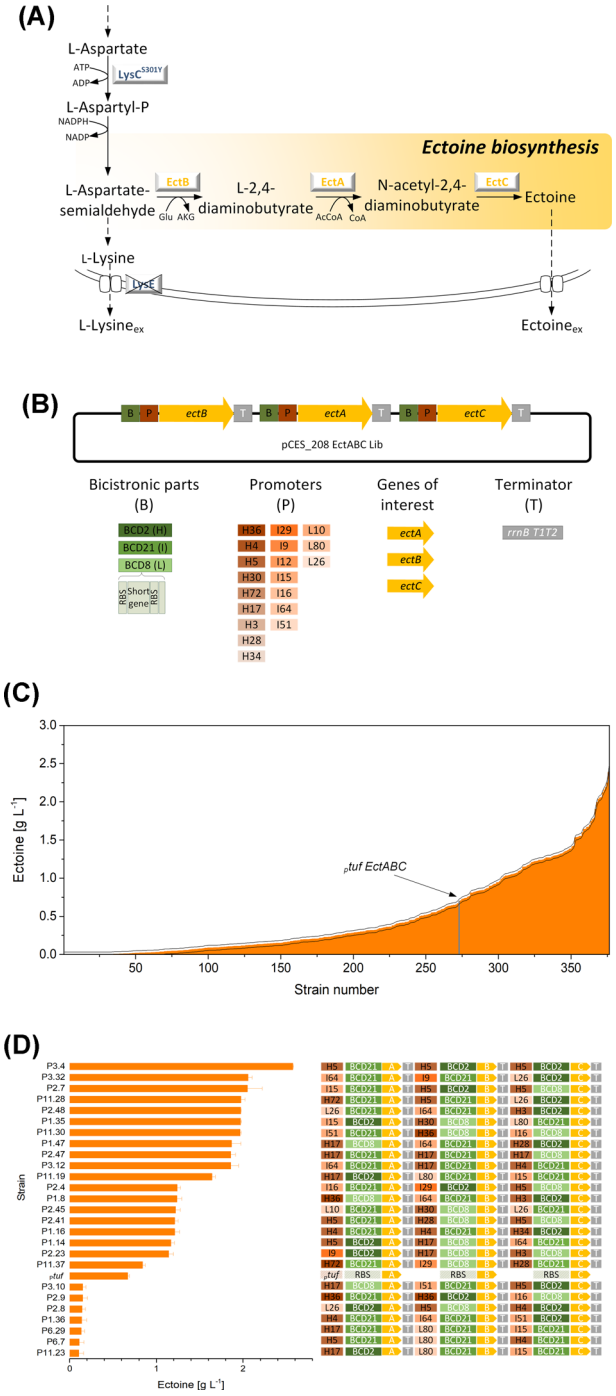

Figure 1. Ectoine production in metabolically engineered *C. glutamicum*. A) Metabolic pathway and B) design of a monocistronic ectoine library for transcriptionally balanced expression of the individual ectoine genes. The library comprised three expression modules for the ectoine genes *ectA*, *ectB*, and *ectC* (yellow). Each gene was randomly linked to one out of 19 promoters (orange), one out of three BCDs (green), and one transcriptional terminator (gray), and subsequently assembled into an expression vector. The genetic elements differed in expression strength, as visualized by the color. C) Characterization of the mutant library for ectoine overproduction. The strains were grown in a miniaturized cultivation system. Strains are sorted according to the observed ectoine titer. The initial producer *lysC*^{fbr} Δ*lysE ectABC*^{basic} is shown for comparison. Twenty strains with different production levels were grown in triplicate to determine an average experimental error of 5% for the ectoine titer. This error range is indicated in the figure. D) Comparison of phenotype and genotype for different ectoine-producing strains. The initial producer *lysC*^{fbr} Δ*lysE ectABC*^{basic} is shown for comparison. ADP, adenosine diphosphate; ATP, adenosine triphosphate; NADP, nicotinamide adenine dinucleotide phosphate; RBS, ribosome-binding site.

from the precursor L-aspartate-β-semialdehyde (ASA), a central hub in microbial amino acid production. The biosynthesis comprises a cascade of three biochemical conversions catalyzed by L-2,4-diaminobutyrate transaminase (EctB), L-2,4-diaminobutyrate acetyltransferase (EctA), and ectoine synthase (EctC) (**Figure 1**A).[1] The industrial fermentation setup for ectoine production, introduced approximately 20 years ago, is somewhat complex: the cells are first grown at a high-salt level, accumulating ectoine intracellularly, and then subjected to an osmotic downshock that causes the release of ectoine through the transient opening of mechanosensitive channels.[12] The cells can be regenerated and reused for additional cycles of intracellular ectoine formation and secretion, and the popular term "bacterial milking" has been coined for this process concept.[12]

Throughout the years, concerted efforts have enhanced the efficiency of ectoine production in *H. elongata*.[13,14] In addition, alternative microbial production strains,[15–21] more sustainable raw materials,[22] and modifications of the fermentation and milking procedures[23,24] have been considered.

In a pioneering study, low-salt ectoine production was realized in recombinant *Escherichia coli*, which expressed the *ectABC* operon from the halophilic bacterium *Marinococcus halophilus*.[25] In addition, the industrial amino acid producer *C. glutamicum* was metabolically engineered to produce 4.5 g L^{-1} ectoine at low-salt levels.[18] Subsequently, an enhanced supply of ASA[26] and systematic metabolic engineering further increased ectoine production in strains of *E. coli*[27] and *C. glutamicum*.[28] However, the implemented changes resulted in poor growth and the accumulation of high levels of L-lysine as an undesired by-product, indicating metabolic imbalances in the obtained production strains.[28]

Here, we describe a novel strain of *C. glutamicum* that expresses an optimized heterologous ectoine pathway with a fine-tuned expression of the individual genes. The engineered mutant, designated *C. glutamicum ectABC*^{opt}, produces 65 g L^{-1} ectoine within 56 h.

2. Experimental Section

2.1. Strains, Plasmids, and Recombinant DNA Work

Strains, primers, and plasmids were constructed in this work or obtained from previous work (Table S1, Supporting Information).[29–33] Details are given elsewhere[18,34–36] and in the Supporting Information.

2.2. Construction and Expression of an Ectoine Library

The plasmid library was constructed from 19 promoters,[33] three bicistronic designed elements (BCDs),[37] and the transcriptional terminator *rrnBT1T2*[38] (Figure S1, Supporting Information). Details are given in the Supporting Information.

2.3. Media, Cultivation, and Screening of Transformants

Shake flask cultivations were conducted as previously described.[39,40] For screening purpose, the Biolector (m2p-labs; Baesweiler, Germany) was used.[18] Details are given in the Supporting Information.

2.4. Fed-Batch Process

The fed-batch production of ectoine was conducted in stirred bioreactors (DASGIP AG, Jülich, Germany).[18,39] Details are given in the Supporting Information.

2.5. Substrate and Product Quantification

If not specified elsewhere in the Supporting Information, products and substrates were quantified as previously described.[34,41,42]

2.6. Proteome Analysis

Proteins were quantified by shotgun proteomics.[43–45] Details are given in the Supporting Information.

3. Results

3.1. Dysregulation of L-Lysine Biosynthesis and Elimination of L-Lysine Export in *C. glutamicum* Provides a Useful Chassis for Ectoine Production

The L-lysine producer *C. glutamicum lysC*^{fbr} served as a starting point for strain engineering. To prevent L-lysine secretion into the broth in later ectoine producers, this export was eliminated by the deletion of the L-lysine exporter *lysE*. Positive clones were identified using PCR (Figure S2, Supporting Information). One of the clones was designated *C. glutamicum lysC*^{fbr} *ΔlysE* and was analyzed further. As expected, it no longer secreted L-lysine, as its ancestor *C. glutamicum lysC*^{fbr} did (**Table 1**). Instead, small amounts of L-glycine and L-glutamate were observed as by-products (data not shown). In the next step, *C. glutamicum lysC*^{fbr} *ΔlysE* was refurbished to produce ectoine. For this purpose, it was transformed with an episomal plasmid to express the genes *ectABC* under control of the *tuf* promoter. The resulting mutant *C. glutamicum lysC*^{fbr} *ΔlysE ectABC*^{basic} accumulated ectoine as the main product (Table 1). However, the introduced ectoine pathway could not take in all the carbon potentially available at the aspartate-semialdehyde node. The yield for ectoine was only 0.03 mol mol^{-1}, threefold lower than that for L-lysine in the parent strain.

3.2. Modulated Expression of the Ectoine Genes Is Achieved by Synthetic Clusters

To investigate the impact of different expression levels of the ectoine genes, we designed a synthetic cluster with a modular monocistronic design (Figure 1B). The cluster comprises the codon-optimized genes (*ectA, ectB, ectC*) in a conserved order identical to that in the native operon. Each gene was randomly linked upstream to one out of 19 different synthetic promoters (Table S3, Supporting Information) and one out of three bicistronic linkers (Table S4, Supporting Information). Furthermore, each construct was flanked downstream by the *rrnB*T1T2 terminator. In total, this design provided 185,193 theoretical variants to express the ectoine pathway. The created library (p*EctABC*-Lib) was first transformed into *E. coli* XL1-Blue, and a total of 13.2×10^6 colonies were obtained, a number much higher than the theoretical library size (185,193). From this library, 20 clones were randomly selected for sequencing and it was confirmed that the modules were assembled in the correct order and contained different combinations of synthetic promoters and BCDs, which indicated that the constructed library was fully randomized (data not shown).

The *ectABC* library was then transformed into the chassis strain *C. glutamicum lysC*^{fbr} *ΔlysE*. Positive clones were selected on kanamycin agar plates. For initial validation, one clone was randomly picked and grown in the glucose minimal medium. After 24 h of incubation, the culture supernatant of this mutant

Table 1. Kinetics and stoichiometry of L-lysine- and ectoine-producing strains of *C. glutamicum*.

Strain	*lysC*^{fbr}	*lysC*^{fbr} *ΔlysE*	*lysC*^{fbr} *ΔlysE ectABC*^{basic}	P3.4	P3.32	P11.28	P11.37
μ [h^{-1}]	0.35 ± 0.00	0.34 ± 0.00	0.33 ± 0.00	0.16 ± 0.01	0.26 ± 0.00	0.29 ± 0.02	0.34 ± 0.03
q_S [mmol g^{-1} h^{-1}]	4.38 ± 0.14	4.36 ± 0.10	4.71 ± 0.12	3.24 ± 0.11	3.54 ± 0.10	3.45 ± 0.19	4.19 ± 0.10
$q_{Ectoine}$ [mmol g^{-1} h^{-1}]	—	—	0.32 ± 0.01	0.85 ± 0.03	0.71 ± 0.03	0.57 ± 0.02	0.33 ± 0.01
$Y_{X/S}$ [g mmol^{-1}]	0.08 ± 0.00	0.08 ± 0.00	0.07 ± 0.00	0.05 ± 0.00	0.07 ± 0.00	0.09 ± 0.00	0.08 ± 0.00
$Y_{Ectoine/S}$ [mol mol^{-1}]	n.d.	n.d.	0.03 ± 0.00	0.26 ± 0.01	0.20 ± 0.00	0.16 ± 0.00	0.08 ± 0.00
$Y_{L\text{-}Lysine/S}$ [mol mol^{-1}]	0.09 ± 0.00	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

The data comprise the specific growth rate (μ), substrate uptake (q_S) and ectoine production rate ($q_{Ectoine}$), and the yields of biomass ($Y_{X/S}$) and secreted products ($Y_{Product/S}$); $n = 3$.
n.d., not detected.

contained 0.1 g L^{-1} of ectoine. The formation of ectoine served as an important proof of the general functionality of the synthetic design. Additional studies revealed that ectoine was formed selectively. Organic acids or amino acids, which are often associated with the growth of *C. glutamicum*, were not excreted in significant amounts. The almost exclusive formation of ectoine enabled a fast analytical protocol for its quantification for screening purposes. Using high-performance liquid chromatography for minimal chromatographic separation efforts, ectoine was precisely quantified within only 3 min of total analysis time per sample, enabling high analytical throughput (Figure S3, Supporting Information).

3.3. Screening of the Synthetic Pathway Library Reveals Great Diversity in Ectoine Production Efficiency

For high-throughput analysis of the synthetic mutants, the cultivation process was scaled down to 500 µL using a miniaturized cultivation system. Initial tests confirmed that the basic producer *C. glutamicum* $lysC^{fbr}$ *ΔlysE ectABC*^{basic} grew well in this system. Similar to cultures in shaken flasks, the cells grew exponentially, the level of dissolved oxygen remained above 30%, and the pH and temperature were stable (data not shown). Under these conditions, *C. glutamicum* $lysC^{fbr}$ *ΔlysE ectABC*^{basic} formed approximately 0.5 ± 0.03 g L^{-1} of ectoine. The high experimental reproducibility was taken as a reasonable indication that the observed production differences throughout the later screening could be attributed to clonal variations rather than to experimental noise.

Approximately 400 synthetic mutants were then screened, which revealed strong differences in ectoine production efficiency (Figure 1C). Approximately 28% of the mutants accumulated more ectoine than the basic strain, but only a small subset of 6% appeared as high producers. The best synthetic mutants accumulated up to fivefold more ectoine than the basic strain. Most of the obtained strains (62%) formed less product. In addition, 10% did not form ectoine at all or below the detection limit. This observation revealed the great impact of the ectoine pathway design on the flux. It was interesting to note that the production level increased gradually across the library, rather than clustering in specific groups. Moreover, the tested strains covered the full range of production efficiency. There was no gap in production performance, indicating that the number of tested mutants was sufficient to provide a representative overview of the library.

3.4. Optimum Ectoine Flux Is Enabled by a Specific Combination of Genetic Elements in the Encoding Operon

To study the link between genotype and phenotype, the ectoine cluster was isolated from different strains and sequenced (Figure 1D). The results revealed a certain complexity. First, the fine-tuned expression of each ectoine gene appeared crucial to improve production performance. Second, the better producers contained relatively strong promoters and binding sites but not the strongest ones. Third, strong expression of *ectB* seemed particularly important for high ectoine formation, whereas the expression of *ectA* and *ectC* had a less pronounced effect. High product levels were also achieved when these two genes were equipped with weaker control elements.

Several strains were selected for more detailed kinetic and stoichiometric characterization, including the best performer from the prescreening (P3.4), and gradually weaker producers with different promoter–BCD combinations (P3.32, P11.28, P11.37) (Figure 1D). None of the low-efficiency producers was further analyzed because these strains appeared to be of limited value for achieving high production performance. The studied strains scaled well in production performance (Table 1; Figure S4, Supporting Information). The strain P11.37 accumulated ectoine at a yield of 0.08 mol mol^{-1}, which was similar to the amount of L-lysine formed by the parent $lysC^{fbr}$ strain (Table 1). In comparison to P11.37, the strain P3.4 revealed an almost threefold increase in the product yield and specific productivity (Table 1). The fact that the ectoine yield was much higher than the original yield for L-lysine in the parent $lysC^{fbr}$ strain indicated that the ectoine pathway actively pulled carbon out of the central metabolism. As already suggested by the screening, *C. glutamicum* P3.4 emerged as the most productive strain in terms of titer, yield, and productivity. It was designated *C. glutamicum ectABC*^{opt}.

3.5. High Expression of EctB, a Low EctA:EctB Ratio, and a Low Total Amount of Ectoine Pathway Enzymes Are Key for Efficient Ectoine Production

It was now interesting to see how the different promoter–BCD combinations affected the protein levels of EctA, EctB, and EctC in the investigated strains. Quantitative shotgun proteomics enabled precise quantification of the proteins of interest (**Figure 2**). The studied strains revealed great variation in the protein levels of EctA (0.7–3.0) and EctC (0.7–3.0) (Figure 2A). In contrast, differences in EctB levels (1.1–2.3) were less pronounced. Strikingly, EctB had the strongest impact on production performance. Its relevance became immediately obvious when the correlation of production efficiency with the EctB level was examined (Figure 2B): ectoine production gradually decreased along with EctB. This finding matched the observed high frequency of strong promoters and BCDs as crucial control elements for *ectB* expression among the high producers (Figure 2D). Interestingly, not only the absolute EctB level but also the ratio between EctB and EctA played an important role in production efficiency (Figure 2C). Third, an increase in the total protein level of the ectoine pathway, i.e., the sum of the levels of EctA, EctB, and EctC was not beneficial for ectoine production, as one might have expected (Figure 2D).

3.6. The Cell Factory *C. glutamicum ectABC*^{opt} Performs Well under Fed-Batch Conditions and Accumulates More Than 65 g L^{-1} of Ectoine within 56 h

To assess performance under industrially relevant conditions, we benchmarked the *C. glutamicum ectABC*^{opt} strain in a fed-batch process on a glucose–molasses medium (**Figure 3**). During the batch phase, the strain grew exponentially (Figure 3A) and accumulated ectoine with a yield of 0.13 mol mol^{-1} (Figure 3B).

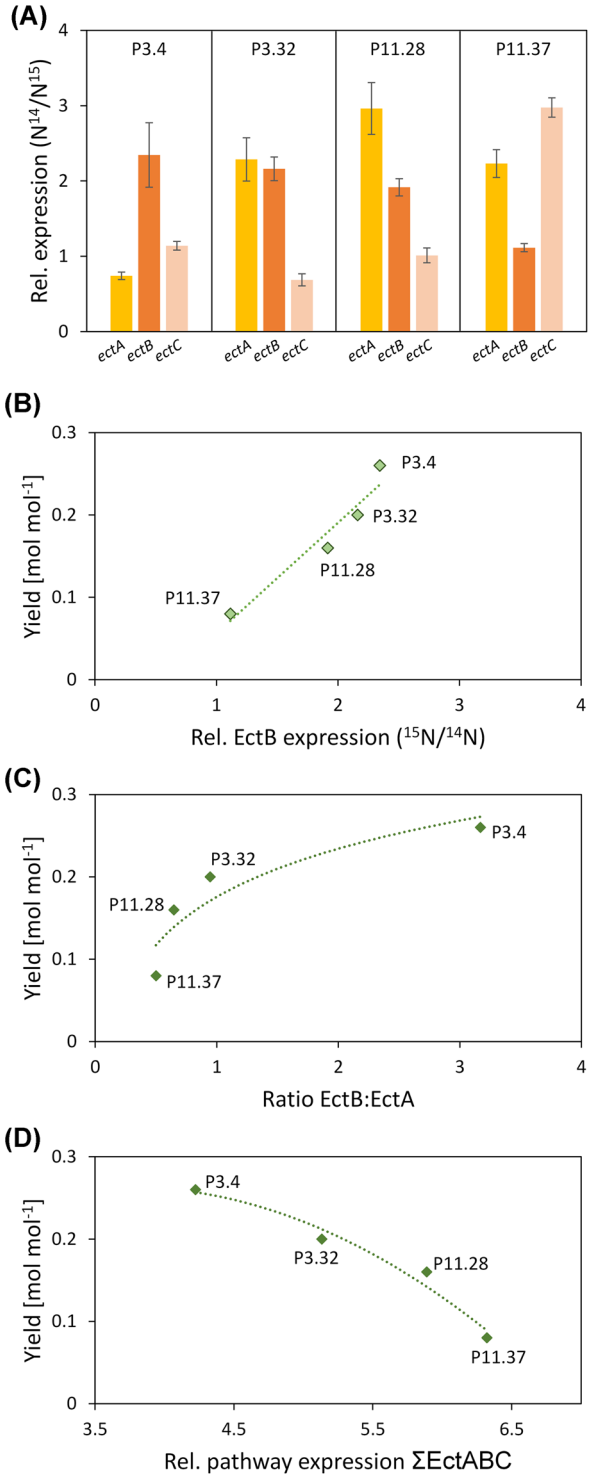

Figure 2. Proteome analysis of different ectoine-producing strains of *C. glutamicum*. A) The protein levels of EctA, EctB, and EctC are given as relative levels based on the quantification of peak areas of ^{15}N peptide ions (standard) and ^{14}N peptide ions (sample). $n = 3$. B) Impact of the relative EctB level on the ectoine yield. C) Impact of the relative expression ratio of EctB:EctA on the ectoine yield. D) Impact of the overall protein level of the ectoine pathway (given as the sum of the relative protein levels of EctA, EctB, and EctC) on the ectoine yield.

After 15 h, the initially supplied sugar was depleted, and the feed phase was started (Figure 3A). Sucrose and fructose, contained at a lower level as molasses-based sugars (data not shown), were efficiently consumed during the first hours and later remained below the detection limit. The concentrated feed was added pulsewise afterwards. This type of feeding is typically used during the industrial fermentation of *C. glutamicum*[46] and has proven most valuable to achieve high production of, e.g., L-lysine,[47] aminovalerate,[34] and glutarate.[39] During the feed phase, the ectoine level continuously increased from 20 g L^{-1} at the end of the batch phase to a final titer of more than 65 g L^{-1} after 56 h. The cells produced ectoine almost exclusively. Only 3 g L^{-1} trehalose accumulated as the only by-product. The molar ectoine yield increased during the feed phase (Figure 3B). The space–time yield for ectoine was maximal at the beginning of the feed phase (2.3 g L^{-1} h^{-1}). Averaged over the full process, production occurred at more than the half-maximum rate (1.16 g L^{-1} h^{-1}).

4. Discussion

4.1. *C. glutamicum ectABC*^{opt} Has Set a Benchmark in Ectoine Production

In this study, *C. glutamicum*, a well-established industrial microbe,[47–49] was upgraded for the production of ectoine from sugar and molasses. From an industrial perspective, ectoine is a high-priced molecule (€900 kg^{-1}) with a rather small market volume.[3,50] This market situation prioritizes the key indicators for economic performance in the order titer > productivity > yield. Therefore, we primarily aimed to enhance titer and productivity. To overcome previous limitations, the flux through the three-step ectoine pathway was engineered. Using libraries of synthetic promoters and BCDs, expressional balancing of the pathway flux was achieved. The implemented genetic changes did not cause any observable growth defects. In fact, the producing strain exhibited high vitality, which resulted in high ectoine productivity. Screening of approximately 0.2% of the total initial library led to the discovery of several high-titer mutants, which revealed an improvement of almost fivefold over the initial strain. The engineered strain *C. glutamicum ectABC*^{opt} achieved an ectoine titer of 65 g L^{-1}, which surpasses previously reported values for recombinant *C. glutamicum* by approximately threefold and, furthermore, exceeds the maximum titers observed to date for ectoine production in general (**Table 2**). With regard to specific productivity, *C. glutamicum ectABC*^{opt} outperforms strains reported to date by sevenfold (Table 2 and **Figure 4**). Driven by the high-flux synthetic pathway, the producer formed ectoine at a specific rate of 120 mg g^{-1} h^{-1}, whereas previously derived native and heterologous producers achieved far lower values: 17 mg g^{-1} h^{-1} (*C. glutamicum* ECT-2), 15 mg g^{-1} h^{-1} (*C. glutamicum* Ecto5), 7 mg g^{-1} h^{-1} (*H. elongata*),[12] and 2 mg g^{-1} h^{-1} (*E. coli* pASK-*ectABC*).[52] Likewise, the volumetric productivity achieved is among the top values reported (Table 2). The conversion yield (although we achieved one of the highest values observed so far) still leaves space for improvement. In comparison to titer and productivity, it does not have a great impact on ectoine process economics, in contrast to bulk chemicals: the raw material costs for production at a yield of 0.19 g g^{-1} represent only 0.2% of the ectoine price, considering the EU reference price

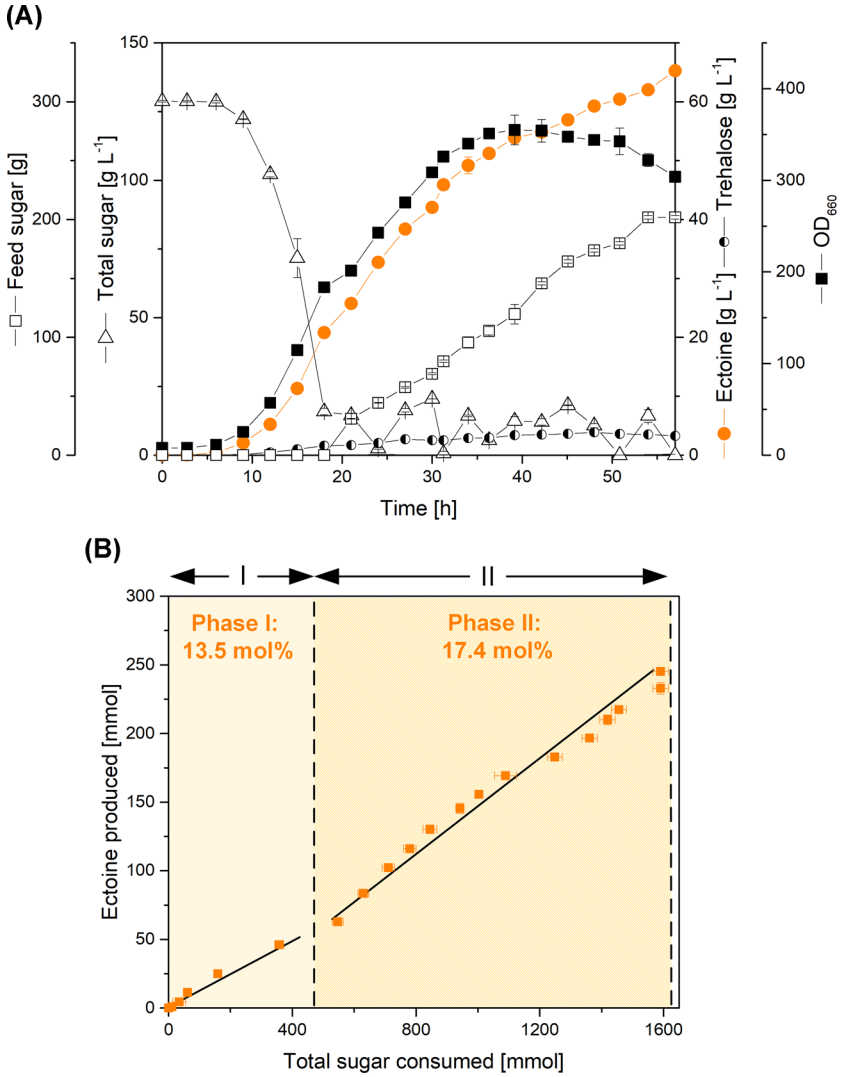

Figure 3. Fed-batch production of ectoine by *C. glutamicum ectABC*^{opt}. The substrate is given as total sugar, i.e., the lumped concentrations of glucose, sucrose, and fructose, added either as pure glucose or as molasses-based sugar. A) After depletion of the initial sugar, pulses of feed were added manually. B) The molar amount of sugar reflects hexose units; $n = 2$.

for sugar over the past ten years (€0.40 kg^{-1}). However, higher yields would still be beneficial and could be more specifically addressed in future studies. For example, our previously developed strain *C. glutamicum* ECT-2 secretes ectoine together with hydroxyectoine.[18] The summed yield of both products together slightly exceeded the value observed here. This observation could potentially indicate a remaining bottleneck at the level of ectoine export,[36] which remains only partially understood in *C. glutamicum*.

4.2. *C. glutamicum ectABC*^{opt} Opens the Door to Industrial Ectoine Production Using Standard Fermentation Processes at Low-Salt Levels

Natural producers such as *H. elongata*[12] and *Chromohalobacter salexigens* (*C. salexigens*)[51] form ectoine inside the cell as a protectant under stress conditions. When industrially producing ectoine, the specific lifestyle of these bacteria requires fermentation conditions that impose stress on the cells to drive transcription of the ectoine genes and trigger production.[4,53] Using such setups, extremophilic microbes have achieved impressive performance and form the economic basis of industrial ectoine production to date.[1,12,14,24] Unfortunately, the need for high-salinity media in the fermentation process imposes notable constraints on the investment and operation costs, design, and durability of the reactor systems.[18] Moreover, high-salinity processes require complex reactor cascades with cell recycling and sequential changes in the salt level and temperature to drive intracellular ectoine accumulation and ectoine excretion into the broth for harvesting.[51] Such setups are difficult if not even infeasible to operate in standard biotechnology production plants, which has stimulated the search for efficient low-salt alternatives. However, low-salt

Table 2. Biobased production of ectoine using natural and engineered micro-organisms.

Strain	Titer [g L^{-1}]	Yield [g g^{-1}]	Volumetric productivity [g L^{-1} h^{-1}]	Reference
C. glutamicum ectABC^{opt}	65.3	0.19	1.16	This work
C. glutamicum Ect-2	4.5	0.23	0.22	[18]
C. glutamicum Ecto5	22.0	0.16	0.32	[28]
E. coli ECT05	25.1	0.11	0.84	[27]
C. salexigens DSM3043[a)]	32.9	—	1.35	[51]

[a)]Two continuously operated reactors with cell retention and sequential cycles of salt level and temperature, yielding ectoine/hydroxyectoine mixtures.

processes in heterologous hosts have previously failed to achieve the performance of industrially used extremophiles (Table 2).[16,18,25,27,28] Our process enabled production with low salt. The medium contained low levels of mineral salts and glucose, and the addition of salt (NaCl) from feeding molasses (0.13 g L^{-1}) was negligible.

Moreover, the production process established here was highly selective and provided ectoine almost without by-products. This result is a great advance over previous developments, which suffer from significant extra costs in downstream purification due to the formation of ectoine in a mixture with its derivative hydroxyectoine[13,18,51] and other by-products.[28] In addition, the product was secreted into the broth without any external trigger, further facilitating downstream processing. Altogether, the novel strain developed in this work enables highly selective ectoine production at industrial efficiency in a simple fed-batch process on well-established feedstocks and without the need for extra salt. Thus, it represents a milestone in industrial ectoine fermentation.

4.3. The High-Level Ectoine Pathway Drains Extra Carbon from the Central Metabolism and Removes Metabolic Imbalances from Previous Producer Strains

From a metabolic perspective, ectoine is closely linked to the biosynthesis of various amino acids, which all compete for the same precursor L-aspartate-semialdehyde (Figure 1A). It is interesting to note that the expression of the native polycistronic ectoine cluster *ectABC* in *C. glutamicum* fails to efficiently drain carbon into the ectoine pathway, redirecting only 30% of the potentially available carbon from L-lysine (Table 1). Similar limitations have also been observed in other studies. As an example, previously developed *C. glutamicum* strains secreted significant amounts of L-lysine as a competing by-product.[18,28] Attempts to delete the L-lysine exporter and redirect extra carbon towards ectoine were only partially successful because the carbon made available by the modification was not fully utilized to form ectoine and the resulting mutants suffered from poor growth and low vitality.[18,28] A common trait of all previous studies was the use of a polycistronic ectoine gene cluster. In all cases, the expression control for the ectoine genes relied on a single, constitutive or inducible, promoter placed upstream of *ectA*. However, as shown here and in previous studies, this cluster organization seems not to be well suited for high-level ectoine production in a heterologous host.

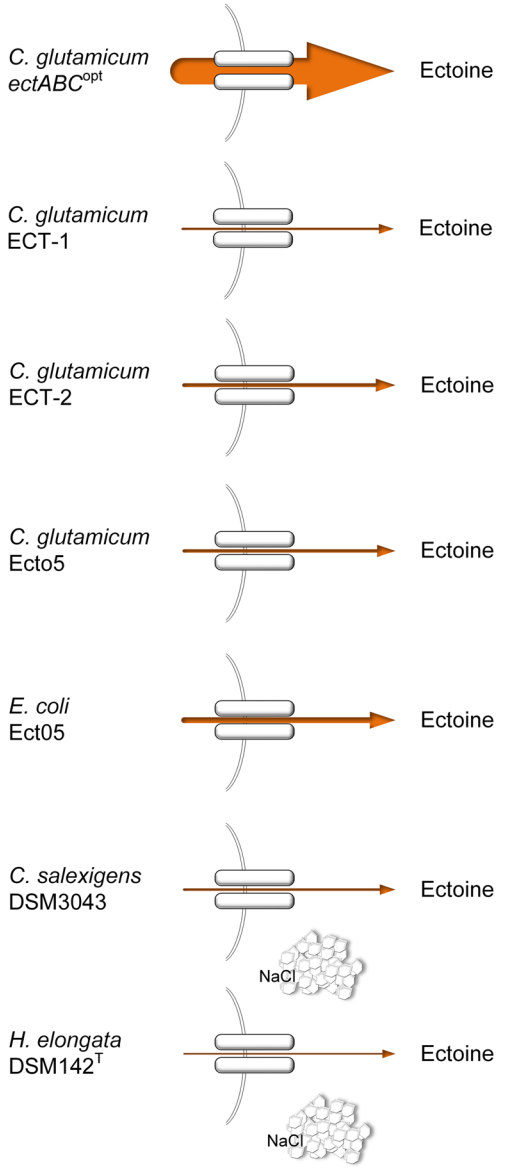

Figure 4. Ectoine production performance in various microbial producers. The arrow width represents the specific ectoine production rate for *C. glutamicum ectABC*^{opt} (120 mg g^{-1} h^{-1}) (this work), *C. glutamicum* ECT-1 (10 mg g^{-1} h^{-1}) and ECT-2 (17 mg g^{-1} h^{-1}),[18] *C. glutamicum* ecto5 (15 mg g^{-1} h^{-1}),[28] *E. coli* Ect05 (26 mg g^{-1} h^{-1}),[27] *C. salexigens* DSM3043 (11 mg g^{-1} h^{-1}),[51] and *H. elongata* DSM 142 (7 mg g^{-1} h^{-1}).[12] The data are related to the cell dry mass. The value for *C. salexigens* DSM3043 is estimated from the previous work, using data on biomass-specific intracellular accumulation and biomass production.[51] *C. glutamicum* and *E. coli* secrete the product continuously. The specific production rate for these microbes equals the specific secretion rate. In contrast, *C. salexigens* and *H. elongata* require cycles of osmotic up- and downshock for ectoine accumulation and secretion, visualized by the salt crystals.

In contrast to previous approaches, the monocistronic design of this work allows individual control of gene expression and, for the first time, yielded an optimal balance in enzymatic capacity for the different steps involved.

Proteome analysis of the studied mutants revealed three key features. The optimum ectoine pathway flux was driven by high activity of EctB (Figure 2B), catalyzing the entry step of the ectoine pathway, and a well-balanced expression ratio between EctB and the enzyme of the subsequent step, EctA (Figure 2C). In addition, the fine-tuned expression was far better than the overall high-level expression of the entire pathway (Figure 2D). As demonstrated, this new feature had a tremendous impact on the pathway flux. The ectoine yield of the advanced producers was even higher than the L-lysine yield in the parent strain, which indicated that the ectoine pathway actively drained the extra carbon from the central metabolism. The high-flux ectoine pathway avoided the metabolic imbalances observed in previous producers. By-products were hardly formed by the optimized cell factory and the vitality of the strain was excellent (Figure 3; Figure S4, Supporting Information).

4.4. Transcriptional Balancing of the Ectoine Genes Is Crucial for Optimal Pathway Flux

Our results highlight the potential effect of transcriptional balancing on the flux through extrinsic pathways and nicely complement similar findings for violacein production in *E. coli*.[54,55] We sequenced strains with different production efficiencies (Figure 1D) and correlated the identified promoters to their previously measured expression strengths[33] to further investigate the cause of the variations in ectoine production and to better understand why some mutants exhibited particular high production. The strongest promoter (H36) occurred at a frequency of only 4% in the ten best producers, whereas medium and weak promoters were much more prominent (Figure 1D). In line with this finding, we found that high-level expression of the entire ectoine pathway (Figure 2D) did not favor ectoine production, as one might have expected. High-titer production was achieved using an average promoter strength of only 50%, normalized to the strongest promoter H36 at 100% (Figure 1D). Furthermore, all low-titer producers contained at least one, in most cases even two strong promoters. These findings provide another compelling case for the use of reduced strength promoters for metabolic engineering applications involving the expression of heterologous pathways.[54]

Interestingly, weak (49%) and medium clusters (53%) had the same average promotor strength as the strong ones (50%): product formation obviously did not correlate to a generally higher or lower expression level, as one might have guessed. This general independence could explain why the previously used polycistronic design is not well suited to produce ectoine. In fact, pronounced differences in the expression of individual genes were crucial to obtain high production. A closer look reveals that the expression ratio between *ectA* and *ectB* is a key feature that determines pathway flux. High-level producers had a stronger promoter for *ectB* than for *ectA*, while the opposite was observed for the low-level producers, and the medium producers had an intermediate balance of promoter strength (Figure S5, Supporting Information). Interestingly, the same trend was observed for the BCD elements, although the differences were less pronounced and were directly observed in the proteome data (Figure 2C). On average, the expressional control elements were slightly stronger for *ectB* (35%) than for *ectA* (29%) in high-level producers and the opposite situation, i.e., *ectB* (32%) and *ectA* (43%), was found for low-producing mutants. Based on the predicted expression strength, an optimal EctA:EctB ratio of 1:1.4 for high producers was deduced. In medium (1:0.9) and low producers (1:0.7), the ratio shifts to favor EctA. Interestingly, this trend was also reflected by the proteome data: the best producer, P3.4, exhibited an EctA:EctB ratio of 1:3.1 and, similar to the prediction, this ratio gradually shifted towards EctA with decreased ectoine production (Figure 2C). The observed importance of a high level of *ectB* expression for a high-flux pathway is consistent with previous studies that identified EctB as the rate-limiting enzyme.[19,56–58] Although this picture seems to be quite clear, other factors, such as plasmid copy number and application-specific parameters, must also be considered before more general conclusions can be drawn.

5. Conclusions

As shown, transcriptional balancing of the ectoine pathway in *C. glutamicum* provides the high-value product at industrial efficiency, using the engineered cell factory in a simple, low-salt fermentation process on well-established feedstocks. In this regard, the created ectoine producer follows *C. glutamicum* cell factories previously described for the production of amino acids,[47,59,60] amino acid-related compounds,[31,34,61] organic acids,[39,48,62,63] and alcohols[64,65] and continues the success story of the microbe as an industrial production host. Thus, transcriptional balancing boosted the flux through the ectoine pathway. This strategy holds promise for application to other *C. glutamicum*-based strains in the future.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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The contributions of the authors to the work were as follows. E.J.J. and S.S.Y. designed and constructed the plasmid library. G.G. constructed the chassis strain. G.G. and D.D. conducted transformation, screening of the strains, and shake flask cultivation. M.K., G.G., and D.D. carried out fed-batch fermentation. L.J. conducted growth experiments and sample preparation for proteome analysis. F.S. and M.S. carried out protein precipitation, digestion, and LC–MS/MS measurements for proteome analysis. D.Z. and T.M. evaluated the MS data from proteome measurements. J.B. and C.W. conceived and structured the work. J.B., G.G., K.J.J., and C.W. assessed the data and wrote the manuscript. All authors read and approved the final manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

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[1] K. Schwibbert, A. Marin-Sanguino, I. Bagyan, G. Heidrich, G. Lentzen, H. Seitz, M. Rampp, S. C. Schuster, H. P. Klenk, F. Pfeiffer, D. Oesterhelt, H. J. Kunte, *Environ. Microbiol.* **2011**, *13*, 1973.
[2] J. M. Pastor, M. Salvador, M. Argandoña, V. Bernal, M. Reina-Bueno, L. N. Csonka, J. L. Iborra, C. Vargas, J. J. Nieto, M. Cánovas, *Biotechnol. Adv.* **2010**, *28*, 782.
[3] L. Czech, L. Hermann, N. Stöveken, A. Richter, A. Höppner, S. Smits, J. Heider, E. Bremer, *Genes* **2018**, *9*, 177.
[4] H. Kunte, G. Lentzen, E. Galinski, *Curr. Biotechnol.* **2014**, *3*, 10.
[5] L. Czech, L. Hermann, N. Stoveken, A. A. Richter, A. Hoppner, S. H. J. Smits, J. Heider, E. Bremer, *Genes* **2018**, *9*, 177.
[6] A. Mamalis, D. H. Nguyen, N. Brody, J. Jagdeo, *J. Drugs Dermatol.* **2013**, *12*, 780.
[7] A. Marini, K. Reinelt, J. Krutmann, A. Bilstein, *Skin Pharmacol. Physiol.* **2014**, *27*, 57.
[8] K. Unfried, U. Krämer, U. Sydlik, A. Autengruber, A. Bilstein, S. Stolz, A. Marini, T. Schikowski, S. Keymel, J. Krutmann, *Int. J. Chronic Obstruct. Pulm. Dis.* **2016**, *11*, 2573.
[9] N. Werkhäuser, A. Bilstein, U. Sonnemann, *J. Allergy* **2014**, *2014*, 176597.
[10] M. Kanapathipillai, S. H. Ku, K. Girigoswami, C. B. Park, *Biochem. Biophys. Res. Commun.* **2008**, *365*, 808.
[11] L. Zhang, Y. Lang, C. Wang, S. Nagata, *Process Biochem.* **2008**, *43*, 642.
[12] T. Sauer, E. A. Galinski, *Biotechnol. Bioeng.* **1998**, *57*, 306.
[13] K. Grammann, A. Volke, H. J. Kunte, *J. Bacteriol.* **2002**, *184*, 3078.
[14] K. Tanimura, T. Matsumoto, H. Nakayama, T. Tanaka, A. Kondo, *Enzyme Microb. Technol.* **2016**, *89*, 63.
[15] S. Nagata, Y. B. Wang, *J. Biosci. Bioeng.* **2001**, *91*, 288.
[16] A. U. Kuhlmann, E. Bremer, *Appl. Environ. Microbiol.* **2002**, *68*, 772.
[17] N. Stoveken, M. Pittelkow, T. Sinner, R. A. Jensen, J. Heider, E. Bremer, *J. Bacteriol.* **2011**, *193*, 4456.
[18] J. Becker, R. Schäfer, M. Kohlstedt, B. J. Harder, N. S. Borchert, N. Stöveken, E. Bremer, C. Wittmann, *Microb. Cell Fact.* **2013**, *12*, 110.
[19] Y.-Z. He, J. Gong, H.-Y. Yu, Y. Tao, S. Zhang, Z.-Y. Dong, *Microb. Cell Fact.* **2015**, *14*.
[20] W. C. Chen, C. C. Hsu, J. C. W. Lan, Y. K. Chang, L. F. Wang, Y. H. Wei, *J. Biosci. Bioeng.* **2018**, *125*, 578.
[21] Y. H. Wei, F. W. Yuan, W. C. Chen, S. Y. Chen, *J. Biosci. Bioeng.* **2011**, *111*, 336.
[22] K. Tanimura, H. Nakayama, T. Tanaka, A. Kondo, *Bioresour. Technol.* **2013**, *142*, 523.
[23] Y. Lang, L. Bai, Y. Ren, L. Zhang, S. Nagata, *Extremophiles* **2011**, *15*, 303.
[24] R. Chen, L. Zhu, L. Lv, S. Yao, B. Li, J. Qian, *World J. Microbiol. Biotechnol.* **2017**, *33*, 116.
[25] P. Louis, E. A. Galinski, *Microbiology* **1997**, *143*, 1141.
[26] T. Bestvater, P. Louis, E. A. Galinski, *Saline Syst.* **2008**, *4*, 12.
[27] Y. Ning, X. Wu, C. Zhang, Q. Xu, N. Chen, X. Xie, *Metab. Eng.* **2016**, *36*, 10.
[28] F. Pérez-García, C. Ziert, J. M. Risse, V. F. Wendisch, *J. Biotechnol.* **2017**, *258*, 59.
[29] H.-M. Kim, E. Heinzle, C. Wittmann, *J. Microbiol. Biotechnol.* **2006**, *16*, 1174.
[30] W. Jäger, A. Schäfer, A. Pühler, G. Labes, W. Wohlleben, *J. Bacteriol.* **1992**, *174*, 5462.
[31] S. Kind, S. Neubauer, J. Becker, M. Yamamoto, M. Völkert, G. Abendroth, O. Zelder, C. Wittmann, *Metab. Eng.* **2014**, *25*, 113.
[32] N. Buschke, J. Becker, R. Schäfer, P. Kiefer, R. Biedendieck, C. Wittmann, *Biotechnol. J.* **2013**, *8*, 557.
[33] S. S. Yim, S. J. An, M. Kang, J. Lee, K. J. Jeong, *Biotechnol. Bioeng.* **2013**, *110*, 2959.
[34] C. M. Rohles, G. Gießelmann, M. Kohlstedt, C. Wittmann, J. Becker, *Microb. Cell Fact.* **2016**, *15*, 154.
[35] S. Kind, J. Becker, C. Wittmann, *Metab. Eng.* **2013**, *15*, 184.
[36] S. Kind, S. Kreye, C. Wittmann, *Metab. Eng.* **2011**, *13*, 617.
[37] S. S. Yim, J. W. Choi, R. J. Lee, Y. J. Lee, S. H. Lee, S. Y. Kim, K. J. Jeong, *Biotechnol. Bioeng.* **2016**, *113*, 163.
[38] V. K. Mutalik, J. C. Guimaraes, G. Cambray, C. Lam, M. J. Christoffersen, Q. A. Mai, A. B. Tran, M. Paull, J. D. Keasling, A. P. Arkin, D. Endy, *Nat. Methods* **2013**, *10*, 354.
[39] C. M. Rohles, L. Gläser, M. Kohlstedt, G. Gießelmann, S. Pearson, A. del Campo, J. Becker, C. Wittmann, *Green Chem.* **2018**, *20*, 4662.
[40] N. Buschke, H. Schröder, C. Wittmann, *Biotechnol. J.* **2011**, *6*, 306.
[41] A. Lange, J. Becker, D. Schulze, E. Cahoreau, J. C. Portais, S. Haefner, H. Schröder, J. Krawczyk, O. Zelder, C. Wittmann, *Metab. Eng.* **2017**, *44*, 198.
[42] J. Becker, C. Klopprogge, C. Wittmann, *Microb. Cell Fact.* **2008**, *7*, 8.
[43] T. Mühlhaus, J. Weiss, D. Hemme, F. Sommer, M. Schroda, *Mol. Cell. Proteomics* **2011**, *10*, M110.004739.
[44] A. Hammel, D. Zimmer, F. Sommer, T. Mühlhaus, M. Schroda, *Front. Plant Sci.* **2018**, *9*, 1265.
[45] J. E. Schaff, F. Mbeunkui, K. Blackburn, D. M. Bird, M. B. Goshe, *Plant J. Cell Mol. Biol.* **2008**, *56*, 840.
[46] R. Kelle, T. Hermann, B. Bathe, in *Handbook of Corynebacterium glutamicum* (Eds: L. Eggeling, M. Bott), CRC Press, Boca Raton, FL 2005.
[47] J. Becker, O. Zelder, S. Häfner, H. Schröder, C. Wittmann, *Metab. Eng.* **2011**, *13*, 159.
[48] J. Becker, M. Kuhl, M. Kohlstedt, S. Starck, C. Wittmann, *Microb. Cell Fact.* **2018**, *17*, 115.
[49] J. Becker, C. M. Rohles, C. Wittmann, *Metab. Eng.* **2018**, *50*, 122.
[50] S. Cantera, R. Lebrero, S. Rodríguez, P. A. García-Encina, R. Muñoz, *Chem. Eng. J.* **2017**, *328*, 44.
[51] C. Fallet, P. Rohe, E. Franco-Lara, *Biotechnol. Bioeng.* **2010**, *107*, 124.
[52] T. Schubert, T. Maskow, D. Benndorf, H. Harms, U. Breuer, *Appl. Environ. Microbiol.* **2007**, *73*, 3343.
[53] C. Vargas, M. Jebbar, R. Carrasco, C. Blanco, M. I. Calderon, F. Iglesias-Guerra, J. J. Nieto, *J. Appl. Microbiol.* **2006**, *100*, 98.
[54] J. A. Jones, V. R. Vernacchio, D. M. Lachance, M. Lebovich, L. Fu, A. N. Shirke, V. L. Schultz, B. Cress, R. J. Linhardt, M. A. G. Koffas, *Sci. Rep.* **2015**, *5*, 11301.
[55] M. Jeschek, D. Gerngross, S. Panke, *Nat. Commun.* **2016**, *7*, 11163.
[56] T. Li, X. Chen, M. T. Chaudhry, B. Zhang, C. Y. Jiang, S. J. Liu, *J. Biotechnol.* **2014**, *192*, 355.
[57] X. H. Shen, Y. Huang, S. J. Liu, *Microb. Environ.* **2005**, *20*, 160.
[58] S. Y. Lee, Y. H. Kim, J. Min, *Appl. Microbiol. Biotechnol.* **2010**, *85*, 713.
[59] T. Jojima, M. Fujii, E. Mori, M. Inui, H. Yukawa, *Appl. Microbiol. Biotechnol.* **2010**, *87*, 159.
[60] T. Yang, Z. Rao, B. G. Kimani, M. Xu, X. Zhang, S. T. Yang, *J. Ind. Microbiol. Biotechnol.* **2015**, *42*, 1157.
[61] 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.

[62] Y. Tsuge, S. Yamamoto, N. Kato, M. Suda, A. A. Vertès, H. Yukawa, M. Inui, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 4679.
[63] S. C. Chung, J. S. Park, J. Yun, J. H. Park, *Metab. Eng.* **2017**, *40*, 157.
[64] S. Yamamoto, M. Suda, S. Niimi, M. Inui, H. Yukawa, *Biotechnol. Bioeng.* **2013**, *110*, 2938.
[65] T. Jojima, R. Noburyu, M. Sasaki, T. Tajima, M. Suda, H. Yukawa, M. Inui, *Appl. Microbiol. Biotechnol.* **2015**, *99*, 1165.

