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

Ectoine is an extremolyte and is therefore synthesized whenever a microorganism, which possesses the ectoine pathway genes, has to cope with extreme external conditions. These can for instance be: A high osmolarity of the external medium, extreme dry conditions or extreme temperatures. Under these conditions, protein denaturation usually takes place. Due to the high water coordination ability of Ectoine (seven water molecules per one ectoine molecule), the molecule is able to offer an aqueous environment, in which the proteins are stabilized in their physiological folding. Because of this ability, ectoine or extremolytes in general are also referred to as chemical chaperones.

Ectoine is a small heterocyclic amino acid with a molecular mass of 142.2 g/mol. In its cyclic part it possesses two nitrogen atoms, which are part of a mesomerism-stabilized system. The therefore resulting positive charge and the negative charge from the deprotonated carboxyl group make it into a zwitterion. Ectoine is used in the fields of cosmetics, medicine and biochemistry, in which its water coordination ability is of great use. The worldwide production of ectoine amount to 15.000 tons per year and one kilogram of ectoine is worth 1000 USD which makes it a high value product.

The ectoine pathway branches off from the lysine pathway and consists of three proteins: ectA, ectB and ectC. EctA is an acetyltransferase, ectB a transaminase and ectC the Ectoine synthase. Together they convert the precursor molecule L-Aspartate-semialdehyde into ectoine. The commercial, biotechnological production of ectoine is performed with the halophilic microorganism *Halomonas elongata* in a high salt fermentation process. For that, the cells have to be exposed to a high salinity medium in order to accumulate intracellular ectoine. In a second step, the osmolarity of the medium is drastically decreased, so that the cells secrete the ectoine into the medium to prevent cell rupture by the high water influx into the cells. A high salt fermentation is attended by high operational costs. Therefore, a low salt fermentation process with an engineered *Corynebacterium glutamicum* would be of high economical value.

The starting strain for such an engineered *C. glutamicum* was an optimized lysine producing strain lysC$^{fbr}$. This strain features a single nucleotide exchange (S301Y) in the aspartokinase gene *lysC.* In order to achieve a higher flux to the ectoine pathway, the lysine exporter lysE was deleted in the following strain. Constructive to this strain, the ectoine producing strains were designed.

A library of the ectoine genes together with 19 different promoters and three different bicistronic parts was generated. By doing so, the original polycistronic design of the ectoine pathway gene cluster, which was employed from *Pseudomonas stutzeri,* was changed into a monocistronic design. This allows for a more accurate regulation of the expression and therefore for the production of the three proteins of interest. About 400 strains were selected from this library and further examined by cultivation in miniaturized bioreactors. After comparing the ectoine titer of the strains, it was evident that 28 % of the strains produced more ectoine than the control *ptuf EctABC.* 62 % showed a lower ectoine production and the remaining 10 % produced either no ectoine or too little to be detected.

The expression analysis which was carried out with shotgun proteomics gave insight in the relative expression of the three ectoine genes. These results show, that a high expression of ectB is paramount for a high ectoine production. However, the ratio between ectB and ectA does also play a significant role. Such a high ratio further promotes the production of ectoine. It could also be shown that a lower amount of the sum of ectoine pathway proteins benefits the ectoine production.

The strain with the best ectoine production was the strain P 3.4. It achieved a titer of 65.3 g/L after 56 h, a yield of 0.19 g/g and a volumetric productivity of 1.16 g/L*h. With this, a viable organism for the low salt fermentation process was created.

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