Projektleitung: **Dr. Florian Rudroff** Projektnummer: *P24483_B20*

Projekttitel**: A 'retrosynthetic' approach for the design and application of non-native enzyme cascades in living cell factories**

III. KURZBERICHT ÜBER DEN PROJEKTFORTSCHRITT im vorangegangenen Kalenderjahr (Deutsch oder Englisch)

>Short report on the project's progress for the last calendar year (German or English)<

Kurzbericht zum Kalenderjahr___2016____
(Short report for the calendar year)

The current progress of the project is summaritzed in 3 accepted publications. Another manuscript is currently submitted and in preparation. We were able to demonstrate the power of mathematical modelling to describe a synthetic cascade in silico and identify putative bottlenecks (paper #1). Furhtermore we were able to show the applicability of our synthetic cascade, composed of an alcohol dehydrogenase and an aldolase for the production of chiral polyhydroxylated compounds. We could optimized this cascade on the genetic as well as on the down stream processing level (paper #2). Finally we could publish a novel strategy for the persistence of reactive aldehyde species in living cells by applying a so called hidden resevoir concept (paper #3).

Paper #1:

Abstract: This paper describes the development of a kinetic model for the simulation and optimization of an *in vivo* redox cascade in *E. coli,* using a combination of an alcohol dehydrogenase, an enoate reductase, and a Baeyer-Villiger monooxygenase for the synthesis of lactones. The model was used to estimate the concentrations of active enzyme in the sequential biotransformations to identify bottlenecks together with their reasons and how to overcome them. We estimated adapted Michaelis-Menten parameters from *in vitro* experiments with isolated enzymes, and used these values to simulate the change in concentrations of intermediates and products during the *in vivo* cascade reactions. Remarkably, the model indicated the fastest enzyme to be rate-determining due to the unexpectedly low concentration of the active form, opening up reversible reaction channels towards side products. We also provide substantial experimental evidence, that a low intracellular concentration of flavin and nicotinamide cofactors drastically throttled the performance of the *in vivo* cascade.

Paper #2:

Abstract: In this study, we investigated an artificial "mini" pathway for the transformation of primary alcohols into polyhydroxylated compounds in *Eschericia coli* (*E. coli*) by the *in situ* preparation of cytotoxic aldehyde intermediates and subsquent aldolase mediated C–C bond formation. For this reaction, an enzymatic toolbox consisting of the alcohol dehydrogenase (ADH) AlkJ from *Pseudomonas putida* (*P. putida*) and the dihydroxyacetone/hydroxyacetone (DHA/HA) accepting aldolase variant Fsa1-A129S were expressed in *E. coli*. To improve the overall performance, different strategies including genetic modifications and the optimization of process

parameters were applied. Three different arrangements of the *alkJ* and the *fsa1-A129S* genes in operon, monocistronic, and pseudo-operon configuration were tested. The latter proved to be most beneficial regarding bacterial growth and protein expression levels. The optimizied whole cell catalyst combined with a refined and easy to apply solid phase extraction (SPE) downstream purification protocol provides diastereomerically pure carbohydrate derivatives under mild reaction conditions in up to 91% isolated yield over two reaction steps.

Paper #3:

Abstract: Synthetic enzyme cascades in living cells often lack efficiency due to formation of by-products by endogenous enzymes, or toxicity of cascade intermediates. Highly reactive aldehyde species can trigger a metabolic stress response, leading to undesired side reactions and decrease yields. Due to the metabolic background of Escherichia coli (E. coli), aldehydes may be irreversibly oxidized to carboxylic acids or reduced to the corresponding alcohols. Herein, we applied an approach to equilibrate the aldehyde concentration in vivo. We oxidized primary alcohols to the corresponding aldehydes by AlkJ, an alcohol dehydrogenase (ADH) from Pseudomonas putida (P. putida). Introduction of a carboxylic acid reductase from Norcadia iowensis (CARNi), allowed retrieving the target compound from the carboxylate sink. Further reduction of aldehydes to the alcohols by endogenous E. coli enzymes completes the equilibration between alcohols, aldehydes, and carboxylic acids.Thus, aldehyde concentrations remained below non-viable concentrations. We demonstrated the concept on several primary alcohols, which reached the redox equilibrium within 6h and persisted up to 24 h. Subsequent combination with a dihydroxyacetone (DHA) dependent aldolase (Fsa1-A129S, E coli) demonstrated, that the reactive aldehyde species are freely available and resulted in the formation of aldol product (3S,4R)-1,3,4-trihydroxy-5-phenylpentan-2-one in 70% isolated yield within short reaction times.

Conference talks:

1. Rudroff, F. **From waste to value - Direct utilization of limonene from orange peel in a biocatalytic cascade reaction towards chiral carvolactone**, European summit of biotechnology (ESIB) **2016**, Graz, Austria

2. Rudroff, F. **Convergent carbon flux *via* substrate funneling for the *in vivo* production of reactive aldehyde species**, 8th International Congress on Biocatalysis (Biocat), August **2016** Hamburg, Germany.

3. Rudroff, F. **Stabilization of Flavin monooxygenases 50 to 100-fold under reaction conditions**, Oxizymes 2016, July **2016**, Wageningen, Netherlands.

4. Rudroff, F. **Inexpensive additives stabilize notoriously fragile Flavin monooxygenases 50 to 100-fold under reaction conditions**, Gordon Research Seminar on Biocatalysis (GRS), July **2016**, Biddeford, ME, USA.

Posters:

1. T. Bayer, S. Milker, T. Wiesinger, M.D. Mihovilovic, **F. Rudroff* 'Substrate/redox funneling' as a novel flux optimization tool for synthetic enzyme cascades in vivo** 5th International Conference on Novel Enzymes, October **2016**, Groeningen, Netherlands.

2. Wiesinger, T.; Bayer, T.; Milker, S.; Mihovilovic, M.D.; **Rudroff, F.* Fine-tuning of an artificial "mini" - pathway for the synthesis of polyhydroxylated compounds** 5th International Conference on Novel Enzymes, October **2016**, Groeningen, Netherlands.

3. T. Bayer, S. Milker, T. Wiesinger, M.D. Mihovilovic, **F. Rudroff* 'Substrate/redox funneling' as a novel flux optimization tool for synthetic enzyme cascades in vivo** Gordon Research Conference on Biocatalysis (GRC), July **2016**, Biddeford, ME, USA.

4. S. Milker, T. Bayer, M.D. Mihovilovic, **F. Rudroff*** Influence of DHA kinase on intracellular fluxes of E. coli COST training school: "Systems Biocatalysis" April **2016**, Siena, Italy.