Exploiting native endowments by re-factoring, re-programming and implementing novel control loops in Pseudomonas putida for bespoke biocatalysis

**Summary of the context and overall objectives of the project**

Synthetic Biology (SynBio) is bound to be transformative in Industrial and Environmental Biotechnology. The powerful methodologies for high-throughput genome engineering, model-driven design and circuit construction, along with new concepts for bioprocess engineering enable the development of tailored biocatalys-based processes that would be otherwise impossible, unscalable or not economically feasible. SynBio has thus a great potential to foster the transition of a petrochemical to a bio-based economy.

EmPowerPutida aimed at developing a solid, versatile bacterial platform for whole-cell biocatalysis that is bound to take European chemical biotechnology into an unprecedented level of productivity and competitiveness. Accordingly, the project had the overarching goal of re-programming the lifestyle of Pseudomonas putida and designing a modular, streamlined bacterial platform for bespoke biocatalysis. This industrially driven project capitalizes on the outstanding metabolic endowment and stress tolerance capabilities of this versatile bacterium for the production of specialty and bulk chemicals.
To demonstrate this potential, the project dealt with the production of the following selected molecules: isobutanol, butene, tabtoxin and methylmethacrylate were chosen for establishing fermentation conditions for large scale production, with aim to develop a conceptual design for these large-scale production processes.

**Work performed from the beginning of the project to the end of the period covered by the report and main results achieved so far**

We successfully developed and implemented various approaches for streamlining, re-factoring and re-engineering the P. putida chassis to render it more robust to toxic yet valuable products and amenable for process control, plugging in of designed circuits and subsequent bespoke biocatalysis. To this end, the genome of P. putida KT2440 was re-sequenced and fully re-annotated, yielded a number of new insights. A genome-scale metabolic model as a basis for model-driven design of the engineering activities was updated and reconciled with substantial physiological data. High-throughput recombineering tools, MetaBricks, CRISPRi and a MAGE-like system were developed. A basis was set for optimization industrially relevant traits such as rendering P. putida blind and deaf, making the strict-aerobe P. putida able to thrive under anoxic conditions, increase tolerance of P. putida to alcohols and implementing an efficient DNA-repair system. Precise tools were developed to allow the conditional knock down of cellular functions with the aim of disrupting cellular growth while maintaining a state of high metabolic activity, so that production pathways can continue to operate at high productivity. We successfully developed an expression system with a switch-like characteristic to express highly toxic genes, which allows using selective proteases intracellularly without affecting cellular function prematurely. We also identified small RNAs in P. putida that underpin a sRNA knock down method for P. putida.

We developed and implemented novel engineering circuits in P. putida, to enable novel control possibilities, including the build-up of an ATP sensor to sense changing supply of ATP on demand and sRNA-based regulation. In parallel, a detailed dynamic model has been formulated to describe controlled loops of energy supply. The model was used for analysis and design of strain construction and, in conjunction with the genome-scale metabolic model, to assess the interplay between the circuits developed and the chassis.

New synthetic routes to industrially interesting unsaturated products isobutene and butadiene have been established based on renewable resources. A route to crotyl alcohol, a key intermediate, was cloned for use in P. putida. By the implementation of the cascade into P. putida, enzymes degrading crotyl alcohol were identified as targets for potential knock-outs, further widening the application spectrum of the bacterium in future industrial processes. The production of isobutene and butadiene is to date mainly achieved by steam cracking. Our newly developed enzymatic routes play an important role in uncoupling production of these widely used compounds from fossil resources. Applying a dedicated reactor setup, we found that P. putida KT2440 minimizes the large-scale induced stress stimuli to a minimum of transcriptional and metabolic burden on the cell. It does so by rapidly degrading large PHA pools under sudden glucose starvation such that intracellular ATP pools can completely recover. Furthermore, process development for isobutanol production continued identifying intracellular NADPH shortage as a key limiting step hampering further performance improvements. Despite successful enzyme engineering of LinD (linalol dehydratase-isomerase) catalyzing the reaction
of crotyl alcohol to butadiene, producing butadiene from glucose in P. putida KT2440 was not successful yet which prevented any bioprocess development. Also, for isobutene no production strain could be established. For tabtoxin, although its heterologous production could be successfully shown in P. putida, titers were below the threshold that would justify further bioprocess development. The biosynthetic route to methacrylates was established in P. putida KT2440, with the resulting strain being able to produce methacrylates from glucose.

A data management strategy was developed and implemented in the project to ensure findability, accessibility, interoperability and rep

Progress beyond the state of the art and expected potential impact (including the socio-economic impact and the wider societal implications of the project so far)

The game-changing innovations brought in – in particular the uncoupling of ATP-synthesis and production from growth - will provide strong versatility, enhanced efficiency and efficacy to the production processes, thereby overcoming current bottlenecks, matching market needs and fostering high-level research growth and development. The technological and market potential of EmPowerPutida concept is very substantial. The achievement of objectives and exploitation of technologies will not only provide a high impact in the partners of the consortium but also, and more drastically, contribute to boost the global competitiveness of the European industries (reduction of the cost of chemicals and environmental benefits) and to create new economic opportunities. More specifically, the impact on the EU industrial competitiveness can be derived from the added value of the EmPowerPutida to the chemical industry. In the short term period after project completion, EU industry will benefit from the bio-production capacity of the targeted chemicals using novel new-to-nature route starting from renewable glucose sources – overcoming price fluctuation caused by temporal shortages of the established cracker products based on crude oil.