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ENGINEERING *LACTOCOCCUS LACTIS* FOR STILBENE PRODUCTION

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MAIN CONCLUSION

Heterologous pathway for biosynthesis of the stilbene resveratrol was assembled in *Lactococcus lactis*. The strain was further engineered in order to enhance the production efficiency.

INTRODUCTION

Bacterial hosts for production of bioactive phenolics from berry fruits (BacHBerry) is a 3 year project funded by the Seventh Framework Programme (FP7) of the European Union. The overall aim of the project is to establish a sustainable and economically-feasible pipeline for production of high-value phenolic compounds originating from berries using bacterial platforms. The project covers all levels of the discovery and pre-commercialization process, from berry collection screening and identification of novel bioactive polyphenols to functional characterization of putative biosynthetic pathways and assembly into Gram-positive cell factories, with further optimization of extraction methods and production scale-up by fermentation up to demonstration scale. Within the project DTU fulfills the task of constructing polyphenol-producing bacteria using *Lactococcus lactis* as a host.

L. lactis belongs to the group of Lactic Acid Bacteria (LAB), members of which have a broad range of applications in food industry, including manufacturing of fermented products. The generally-regarded as safe (GRAS) status of this organism, as well as robustness, stress tolerance, and genetic accessibility make this species an attractive candidate for production of food and pharmaceutical ingredients [1].

The polyphenol resveratrol (trans-3,5,4'-hydroxystilbene) is a well-known polyphenol with a variety of health-beneficial properties, including a link to the so-called "French paradox" [2]. In plants the resveratrol biosynthesis occurs through the general phenylpropanoid pathway [2]. The pathway can be shortened by using a tyrosine ammonia-lyase that would produce *p*-coumaric acid directly from tyrosine (Fig. 1A). As this heterologous pathway consists of only three steps, it presents an interesting test case for accessing feasibility of polyphenol production in LAB. Several combinations of TAL, 4CL, and STS enzymes from plant, bacterial, and fungal sources were introduced into *L. lactis* and the outcomes are summarized below.

MATERIALS AND METHODS

Genes encoding for TAL, 4CL, and STS enzymes from plant, bacterial, and fungal sources were cloned into a lactococcal expression vector pNZ8048 under control of the nisin-

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inducible promoter P_{nisA} (Fig. 1B). Cells were grown as static cultures in falcon tubes in chemically-defined medium (CDM) supplemented with 1% glucose, and expression of the pathway was induced with nisin in the early exponential phase. Resveratrol production was assayed by HPLC analysis of culture supernatants. Acetyl-CoA carboxylase (ACC)-encoding genes were integrated into the genome of the production strain using the pSEUDO system.

RESULTS AND DISCUSSION

Analysis of the phenolic content in culture supernatants collected during the stationary phase of growth revealed that the assembled biosynthetic pathway is functional and the best producer strain yielded 3.0 ± 0.7 µM of resveratrol and 0.7 ± 0.1 µM of *p*-coumaric acid (Fig. 1B).

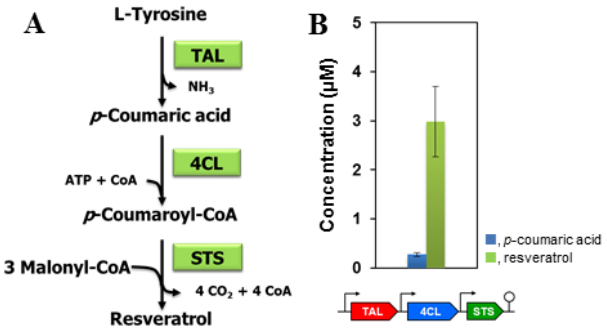

Figure 1. A) Heterologous pathway for resveratrol biosynthesis in *L. lactis*. TAL, tyrosine-ammonia lyase; 4CL, *p*-coumaroyl-CoA ligase; STS, stilbene synthase. B) Resveratrol concentration in the best producer strain and organization of its biosynthetic operon.

The intracellular pool of malonyl-CoA is frequently indicated as the major bottleneck in the bioproduction of polyphenolic compounds [3]. In order to test whether this is also the case for *L. lactis*, the production strain was treated with cerulenin, an inhibitor of fatty acid synthesis that is known to increase malonyl-CoA levels in bacteria. Addition of cerulenin 2 hours post-induction resulted in about 4-fold increase of resveratrol production (data not shown), proving that the malonyl-CoA pool is indeed the limiting factor here. ACC catalyze conversion of acetyl-CoA into malonyl-CoA, thus over-expression of this enzyme should overcome this limitation. Indeed, when ACC genes of plant and fungal origins were integrated into the genome of *L. lactis* and co-expressed together with the resveratrol pathway, production was increased by about three-fold. The above findings demonstrate that *L. lactis* has a potential for being a new type of cell factory for production of polyphenols, especially combined with its capacity to grow on alternative substrates, such as whey.

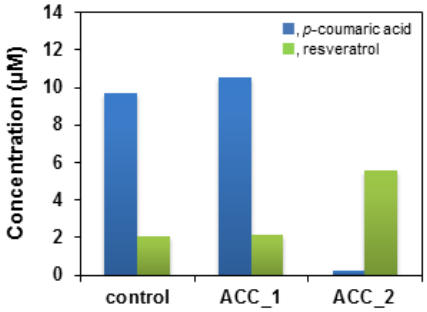

Figure 2. Production of resveratrol in *L lactis* co-expressing different ACCs.

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