Large-scale integrating project targeted to SMEs

co-funded within the FP7

Knowledge Based Bio-Economy (KBBE) Work Programme

Grant agreement N. 613868

OPTIBIOCAT

Optimized esterase biocatalysts for cost-effective industrial production

Project type:	Large-scale integrating project targeted to SMEs		
Start date of project:	1st December 2013	Duration:	48 months

Second midterm report

This project has received funding from the European Union's Seventh Programme for Research, Technological Development and Demonstration under Grant Agreement N. 613868

1. Publishable summary

Summary description of project context and objectives

The main goal of the project is to develop competitive and eco-friendly bioconversions based on esterification reactions catalyzed by FAEs and GEs, for production of molecules with antioxidant activity belonging to the classes of:

• phenolic fatty esters obtained by esterification of hydroxycinnamic (ferulic, p-coumaric, caffeic, sinapinic) acids having antioxidant properties with aliphatic alcohols, to make them more lipophilic
and improve their application in oil-based processes

• sugar esters, resulting from the esterification of sugars with antioxidants such as cinnamyl and benzyl alcohols or hydroxyphenyl alcohols in non conventional reaction media giving these molecules more hydrophilic compared to the starting antioxidant compounds.

The main targeted compounds are prenyl ferulate, prenyl caffeate, 5-O-(trans-feruloyl)-arabinofuranose, glyceryl ferulate, benzyl D-glucuronate and prenyl-D-glucuronate. Moreover, a library of 60 novel compounds belonging to the classes of phenolic fatty esters and phenolic sugar esters will be generated and fully characterized for their antioxidant activity.

OPTIBIOCAT will develop biocatalysts based on FAEs and GEs for the synthesis of these compounds optimizing cost-efficiency and environmental impact of the whole process of their production and use.

The specific objectives of OPTIBIOCAT are:

• An inventory of novel fully characterized recombinant FAEs and GEs:
- 50 novel esterases from fungi
- 500 novel esterases from bacteria
- 25 rationally designed mutants
- 20 best directed evolved mutants

• Optimized biocatalysts based on FAEs and GEs for production of the aforementioned compounds
in the rigors of the industrial environment, exhibiting:

- ✓ higher operational stability: recyclability for at least ten cycles
- ✓ at least 3-fold higher thermo-resistance and resistance to solvents
- ✓ higher yield: up to the theoretical yield of 100% for phenolic fatty esters and 80% for phenolic sugar esters
- ✓ higher productivity: up to 1 g/l/h and 0.5 g/l/h productivity for the synthesis of alkyl hydroxycinnamates and sugar hydroxycinnamates, respectively

• The six main targeted biological active compounds -prenyl ferulate, prenyl caffeate, 5-O-(trans-feruloyl)-arabinofuranose, glyceryl ferulate, benzyl D-glucuronate and prenyl-Dglucuronate-fully characterized for their antioxidant

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activity and exhibiting an increase of 1.5-2 fold of the antioxidant activity and an improvement of hydrophilicity/hydrophobicity
• A library of 60 novel compounds belonging to the classes of phenolic fatty esters and phenolic sugar esters fully characterized for their antioxidant activity
• Schemes of reactions for biotechnological production of these compounds based on FAEs and GEs, characterized by
- ✓ lower temperature than that of the chemical process
- ✓ fewer steps (one step) than the chemical process
- ✓ no production of unwanted side reactions causing darkening and formation of odors produced in the chemical process.
- ✓ synthesis of one product instead of a mixture of esters
- ✓ no need of removal of by-product and catalyst residues in order to produce clean and high quality substances with the potential use in the cosmetics or pharmaceutical industry

• Scale-up of production of at least four FAEs- and GEs- biocatalysts beyond 1L to 20 L with an
expression level in the g/L scale
• Four new chemical entities (leads) for the cosmetic industry
• Techno-economic viability of the developed OPTIBIOCAT processes, within their supply/value chain and applying life cycle thinking (LCA), with demonstration of a significant improvement of the economic efficiency and environmental performance of existing and future biorefineries:
- more cost-effective production due to at least 40% reduction of investment cost (CAPEX) and operating cost (OPEX);
- at least 40% reduction of primary energy consumption and greenhouse gas emissions;
- zero-waste production

• Enhanced awareness about OPTIBIOCAT biocatalysts, bioconversions and products of among the Stakeholders

Description of work performed and main results

WP2

- Annotation of four FAE encoding genes of DNL's proprietary fungus Myceliophthora thermophila C1. Mining of other three proprietary fungal genomes of DNL and cloning of the coding sequences of three esterases.
- Homology modelling, active site determination and modelling of surface charge for two FAEs and one GE selected for mutagenesis.
- Sequencing of DNAs and RNAs from six selected fungi. Annotation of FAE and GE coding genes.

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- Finalization of the database, its upload on the project website and periodic updating.

WP3

- Characterization of all previously cloned fungal FAEs and GEs
- Production of five site-directed mutants of two FAEs and one GE in P. pastoris and characterization of some of them
- Recombinant production of 500 putative prokaryotic esterases (480 FAEs and 20 GEs) and characterization of 480 recombinant FAEs
- Nucleophilic elbows of Sorangium cellulosum FAE have been mutated and an additional double mutant has been cloned for production in E. coli
- Design of site-directed mutants for FoFaeC and StGe2
- Construction of 30,000 mutants of FoFaeC by directed evolution in Yarrowia lipolytica, screening for improved activity towards pNC-Fe and selection of mutants with improved features
- Two rounds of evolution for AnFaeA in Y. lipolytica, screening of the mutants for better thermoresistance and solvent resistance and selection of mutants with improved features
- Development of a strategy for construction and screening of a directed evolution mutant library of MtFAE1a in S. cerevisiae

WP4

- Improved production for C1 esterases by strain improvement and fermentation optimization.
- Improvement of protein production in Pichia by multi-copy strains harboring expression vectors with RVE proprietary elements and fermentation optimization
- Production of nine new enzymes in Pichia for WP5 tests.
- Synthesis of new immobilization materials for enzymes with low loading on SBA-15.
- Immobilization of four C1 FAEs on six mesoporous materials, a C1 FAE as a mCLEA,
- three FAEs on hydrophobic beads and process upscale for use in ester synthesis.
- Immobilization of Fae799 on beads but resulted unsuccessful, probably due to heavy glycosylation of the enzyme.
- Process scheme of the overall bioprocess chain prepared and optimized.
- Protein production data for both C1 and Pichia systems exploited for mass and energy balances and LCA models.

WP5

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- Development of 4NTC-linker-Fe for FAE activity and chromogenic and fluorogenic substrates for GE activity
- Synthesis of library based on ferulate derivatives
- Development of a glucuronyl donor for transfer reactions with GEs and successful test with several alcohols
- Synthesis of a new donor of ferulic acid as a possible substitute for vinyl ferulate in preparation for large scale bioconversions.
- Synthesis of 24 vinyl esters at g scale for library generation
- Evaluation of 285 FAE transfer reactions
- Parallel synthesis of 190 library compounds with FAEs by combination of hydroxycinnamic and benzoic acids with sets of structurally diverse alcohols at 5-30 mg
- Evaluation of the cytotoxicity and antioxidant activity of 47 compounds
- Development of protocol and evaluation of 22 FAEs for the synthesis of 4 targeted compounds
- Optimization of reaction conditions using 5 FAEs from M. thermophila C1
- Synthesis using free and immobilized FAEs from M. thermophila C1
- Optimized immobilization of C1FaeB1 and re-usability over 9 reaction cycles
- Development of the generic process chain of bioconversion and design of environmental impact

WP6

- Fermentations for the two optimized strains of P. pastoris expressing the same model enzyme
- Fermentation of the optimized and not optimized strain of P. pastoris expressing a new enzyme
- Vinyl ferulate successfully used as donor substrate in all small scale reactions in WP5

WP7

- Organisation of two OPTIBIOCAT workshops
- Dissemination of the second OPTIBIOCAT newsletter
- Updates of the communication materials and news on the project's website
- Dissemination of OPTIBIOCAT project results

Expected final results and their potential impacts and use (includind socio-economic impact and the wider societal implications of the project so far)

Expected final results

OPTIBIOCAT biocatalysts: Improving synthetic capabilities of FAEs and GEs

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OPTIBIOCAT bioconversions: -Overcoming limitations of the current production of antioxidants substituting industrial chemical synthesis with enzymatic synthesis; - Increasing yield and productivity of FAEs and GEs reactions

OPTIBIOCAT products: -Improving properties of ferulic and caffeic acids for their applications in cosmetic industry by producing as the main targeted products prenyl ferulate, prenyl caffeate, 5-O-(trans-feruloyl)-arabinofuranose, glyceryl ferulate, benzyl D-glucuronate and prenyl-D-glucuronate and a library of 60 novel compounds belonging to the classes of phenolic fatty esters and phenolic sugar esters fully characterized for their antioxidant activity.

OPTIBIOCAT process: achieving process optimization

ECONOMICAL IMPACT

Enhancement of competitiveness and sustainability of the European Biotech

This project is targeted to the needs of SMEs regarding the development of cost-effective processes for biocatalysts production and recovery and for their application in the synthesis of antioxidants.

These achievements will bridge the gap between laboratory and industrial scale meeting the EU

Strategy for KET and Lead Market Initiative on Bio-based products

Regarding the market sectors within the scope of this project – enzymes and antioxidants for cosmetic industry - this project will provide new products for a market in expansion with environmental and economic advantages in relation to the currently existent products.

- Industrial Enzymes. The global market for industrial enzymes is expected to reach $4.4 billion by 2015, a compound annual growth rate (CAGR) of 6% over the 5-year forecast period. Europe represents the largest market for industrial enzymes. There is a huge opportunity for enzyme producing SMEs like Dyadic and NZYT by entering in such rapidly growing market segments.
- Antioxidants. A global sale of around $34 billion has been estimated for the most produced antioxidants for the year 2010, with Japan, USA and China being the top producers. Europe represents the largest regional market for antioxidants with strong growth in UK and Ireland (marketresearchreport.com). Antioxidants are being used in an increasing number of applications and their market is growing at a considerable rate.
- Cosmetic sector. The global market for natural and organic cosmetics has been estimated at 6.5 billion Euros in 2010, where Europe and USA are the main stakeholders. Within Europe, Germany is the biggest natural cosmetics market (marketresearchreport.biz).

ENVIRONMENTAL IMPACT

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- Reduction of the environmental impact of processes for production of compounds with applications in the cosmetic field by substituting the chemical processes with biotechnological ones providing product life cycles with neutral greenhouse gas emissions minimizing the use of toxic reagents or solvents and just requiring ambient temperature.

SOCIAL IMPACT

- Production of natural ingredients not requiring tests on animals. OPTIBIOCAT will produce antioxidants which will not need tests on animals. This will support European Directive 2010/63/EU establishing the replacement and reduction of the use of animals for scientific purposes and the Protocol on the Protection and Welfare of Animals.
- Improvement of life quality of citizens. The main contributions of OPTIBIOCAT are its eco-friendly processes which will positively affect the life quality of the Europe citizens. The project will also particularly boost innovation of European health related industries, with development and validation of new sustainable and efficient healthcare products.
- More jobs. This project will increase competitiveness and innovation, creating quality jobs and looking for new tools for social, economic, environmental and technological developments. This would improve the conditions for conducting research and ultimately improve Europe's potential in creating jobs and improving social wealth

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