

Project **BIOCAT**  
**Modular biocatalyst platform for chiral synthesis of chemical compounds by  
structure-based directed evolution**  
**(the 2009 annual report)**  
Academy of Finland, project no. 117874

Funding period: 01.01.2007 - 31.12.2010  
Site of research: University of Oulu

Project webpage: <http://www oulu fi/bioprocess/biocatalysis.pdf>

Partners:

Professor Dr. Peter Neubauer, From 1.9.2008 Professor at the Technical University of Berlin, project coordinator <http://www oulu fi/bioprocess/personne3.htm#Peter>

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Scientists working in the project:

Post-Docs: Dr. Mari Ylianttila (BPEL, BIOCATKAL)  
Dr. Vanja Kapetanidou (Biochemistry, Finnish Cultural Foundation)

PhD-students: M.Sc. Mikko Salin (Biochemistry)  
M.Sc. Marco Casteleijn (BPEL)  
M.Sc. Matti Vaismaa (Chemistry)  
M.Sc. Nanna Alho. (Chemistry)  
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Pro-Gradu student: Kathleen Szeker (BPEL)

Technical staff: Ville Ratas (Biochemistry)

Collaborator: M.Sc. Mirja Krause (Technical University, Berlin)

**Project abstract and objectives:**

- BIOCAT is a new approach for the creation of tailor-made engineered biocatalysts to be used for the efficient, environmentally sound, biotransformation of chemical building blocks into highly pure enantiomers. It uses as a scaffold a monomeric form of

triosephosphate isomerase (TIM). Wild type TIM is a dimeric C3-sugarphosphate isomerase, which can only interconvert the C3-sugarphosphates dihydroxyacetone-phosphate and D-glyceraldehyde-3-phosphate. The BIOCAT project studies the properties of A-TIM, which is a monomeric form of TIM, in which the substrate binding pocket has been made more extended as compared to the wild type TIM.

- The interdisciplinary consortium consisting of five research groups from three faculties of the University of Oulu, was established during an earlier Academy-of-Finland project where a new approach was developed to create a platform of non-natural enzyme catalysts. This concept was developed using as an example the well-known TIM-barrel framework of TIM which was successfully engineered to accept a wide range of chemical ligands. Now, the extended consortium aims to develop a series of efficient biocatalysts for the cofactor-free synthesis of chirally pure chemical compounds. Therefore emerging techniques such as structure-based modeling and directed evolution of the biocatalyst in connection to chemical synthesis of a library of new compounds are jointly applied. The multidisciplinary approach facilitates stimulating discussions at the regular project meetings.
- BIOCAT clearly addresses to strengthen basic research in protein engineering and chemistry with a clearly applied relevance, namely strengthening scientific expertise and further developing research environments in biocatalysis, as a key area supporting sustainable production and products. Despite a solid past in industrial enzymes and chemistry, these areas have been separated in Finland. The project consortium creates a multidisciplinary team intertwining chemistry, biochemistry, molecular biology, structural biology and bioprocessing. By the use of enzyme catalysts, working in aqueous solutions at ambient temperatures and pressure, BIOCAT is directly targeting to new eco-efficient production concepts of chemical products. The project is well integrated into national and international networks which are used to further strengthen this research area and to disseminate research results. The project is closely connected to the interest in Finland for building up competence in industrial biotechnology and green chemistry.

## Results of the research (publications, posters, patents etc)

### Publications:

- Vaismaa, M.J.P , Leskinen, M.V., Lajunen, M.K. Microwave-assisted one carbon chain extension in the preparation of terminal  $\alpha$ -hydroxy ketones, *Synth. Commun.* 39, **2009**, 2042-2052.
- Vaismaa M, J.P., Yau, S.C., Tomkinson, N.C.O. Organocatalytic  $\alpha$ -oxybenzoylation of aldehydes, *Tetrahedron Lett.* 50, **2009**, 3625-3627.

### Diploma and PhD theses:

- Szekér, K. High throughput methods for the expression of Phosphorylase libraries. February 2009 (Diploma thesis of Technical University of Berlin in cooperation with the University of Oulu, where practical work was done)
- Vaismaa, M.J.P. Development of benign synthesis of some terminal  $\alpha$ -hydroxy ketones and aldehydes. August 2009 (PhD thesis)

### Lectures at scientific meetings:

- Casteleijn, M.G. Parallel High Throughput Expression of a Thermostable Phosphorylase. PEGS2009, 6-10 April, 2009, Boston, USA (presentation, hosting round table discussion).
- Ylianttila M.S. Creating new enzymes: High-throughput Protein Expression optimization of monomeric TIM libraries using EnBase® Technology. PEGS Europe-Protein Engineering Summit, Next Generation Technologies for Protein Science, 6.-8.10.2009, Hannover, Germany.
- Neubauer, P. "Currently available A-TIM libraries and *E.coli* knock-out strains". Scientific Workshop, 26.8.-27.8.2009, Berlin, Germany.
- Ylianttila M.S. Directed evolution of TIM: Towards D-xylose isomerase and L-arabinose isomerase activities. Structure based evolution of new protein activities, activities, Scientific Workshop, 26.8.-27.8.2009, Berlin, Germany.
- Kapetaniou, E. Protein crystallographic binding studies with A-TIM. Scientific Workshop, 26.8.-27.8.2009, Berlin, Germany.
- Wierenga, R.K. Scientific questions connected to the A-TIM directed evolution approaches. Scientific Workshop, 26.8.-27.8.2009, Berlin, Germany.
- Krause, M. "Directed evolution experiments towards DR5PI activity, current status and future plans" Scientific Workshop, 26.8.-27.8.2009, Berlin, Germany.
- Vaismaa, M. Green preparation methods of  $\alpha$ -hydroxy and aldehydes, 31.8.2009, Scientific Workshop: Chemistry Days, Oulu, Finland
- Wierenga R.K. Structural enzymological studies of triosephosphate isomerases: a highly evolved biocatalyst. (Oct 9, 2009) University of Copenhagen (Host: Prof. Dr. S. Larsen)

#### Posters:

- Salin, M., Kapetaniou, V., Krause, M., Ylianttila, M., Pääkkonen, S., Casteleijn, M., Pursiainen, J., Neubauer, P., and Wierenga, R.K. "Engineering new enzymatic activities on the TIM-barrel framework". Biocenter Oulu Discovery-of-the-year seminar, 17.12.2009. The poster was chosen as Best poster.
- Széker, K., Pelttari, S., Neubauer, P., Mattila, S., Ylianttila, M., Casteleijn, M.G., Parallel High Throughput Expression of a Thermostable Phosphorylase. PEGS2009, 6-10 April, 2009, Boston, USA.
- Hylkinen, L., Pelttari, S., Hiltunen, L., Pikkuhookana, H., Rautio, A., Alho, N., Vaismaa, M., Casteleijn, M., Lajjunen, M., Wierenga, R., Neubauer, P., Mattila, S. "Altered Affinity from TIM Derived Biocatalysts: Protein Ligand Interactions Studied by NMR", XXXI National NMR Symposium, 10-12 June, 2009, Ruka, Kuusamo.
- Casteleijn, M., Pelttari, S., Ratas, V., Alho, N., Wierenga, R., Neubauer, P., and Mattila, S. "High Yield Protein Expression of Isotope Labeled Monomeric Triosephosphate Isomerase". XXXI National NMR Symposium, 10-12 June, 2009, Ruka, Kuusamo.

### **Impact of the research**

- Generally the BIOCAT research work in 2009 has enhanced our expertise in this field, highlighting the crucial interplay of genetic methods, protein characterization, X-ray, NMR and MassSpec. The progress of the characterisation of the crystal structures (X-ray) and the solution structures (NMR) will allow for an interesting comparison of these properties.
- This project forms an excellent starting point for implementing directed evolution methods. In various project meetings we have discussed how to optimally implement these ongoing directed evolution approaches, using the selection method with well-defined *E.coli* knock-out strains and a range of gene libraries.

### **The progress of the research versus the original plan**

- The crystallographic binding studies have shown that 3PGA (3-phosphoglycerate) and 4PEH (4-phospho-D-erythronohydroxamic acid) do bind to the active site of A-TIM. These molecules are analogues of C4 and C5 sugar phosphates, respectively. A manuscript on these studies will be submitted soon. Two PhD-theses on the characterization of A-TIM will be completed in 2010 (Marco Casteleijn, February 2010; Mikko Salin, March 2010).
- Methodology for the production of  $^{15}\text{N}$  stable isotope labeled TIM samples for NMR studies has been finalized (manuscript under preparation).

- In regards to applications, where A-TIM variants could act as enzymes for the creation of precursors of modified nucleosides, a structural model of a thermostable Uridine Phosphorylase(UP) has been created. In the scheme: (A-TIM variants, Ribose kinase) + (UP variants) followed by Purine Nucleoside Phosphorylase, the focus of UP has been on special properties of the active site (manuscript under preparation). Thermostable UP has an industrial benefit in regards to the biocatalytic process. This includes the methodology to measure UP activity in cell lysate by means of NMR (in collaboration with Dr. Sampo Mattila; NMR), the creation of protein-fusion expression libraries and subsequent purification protocols.
- The ribose isomerase (Rpi) assay, needed to evaluate hits in the selection-screening of A-TIM variants with Rpi activity, is currently under active development.
- New *E.coli* minus strains are being generated currently in the lab by ourselves. In this way the minus strains will be precisely defined. It concerns *E.coli* knock-out strains in which respectively the genes for D-ribosephosphate isomerase, D-xylose isomerase, and L-arabinose isomerase will be removed. This work is done in close collaboration with Mirja Krause and Peter Neubauer at the Technical University of Berlin, Germany
- The methodology for reliable and fast determination of dissociation constants and general protein ligand binding by NMR and MS solution methods were further studied. One PhD thesis manuscript (Nanna Alho) is in preparation, defense is scheduled to occur 2010 and three manuscripts of the recent results are in preparation. The manuscripts, when published, would constitute majority of the PhD thesis of Silja Pelttari (although the actual defense would probably be in the beginning of 2011).
- The synthetic work related to the sulphonyl  $\alpha$ -hydroxy ketone-based substrate candidates and transition state analogues for the mutated TIM was successfully finished. A new method for an asymmetric organocatalytic  $\alpha$ -oxybenzoylation reaction of aldehydes was developed. The design and synthetic work were carried out in accordance to principles of green chemistry. Results of the work were presented in two publications and in the public defense of the PhD thesis (Matti Vaismaa) in August 2009.
- The synthesis of suicide inhibitors that are analogues of C4 and C5 sugars and sugar-phosphates has been initiated. Such molecules, being either epoxides or bromo-ketone analogues of these sugars are difficult to make, but we think that such molecules are important probes of the properties of A-TIM and its new variants. These molecules can also be tested on other sugar or sugar-phosphate isomerases.

## Concluding remarks

In the last year of the BIOCAT-project we will focus on implementing the directed evolution technology as well as on completing the solution binding studies with NMR and mass spec approaches. Also, we will continue to work on the NMR solution structure of A-TIM.

The directed evolution technology is seen as very important for any new enzyme discovery project. In the context of the ongoing A-TIM studies it will be fascinating to see if active variants can be obtained using this directed evolution technology and subsequently to understand the rationale of the mutations in these new A-TIM variants.

It will also be interesting to see the results of the NMR and mass spec solution studies and compare these results with the information obtained from the available crystal structures. Of key interest will also be to get information on the flexibility of the A-TIM molecule in solution, which, possibly, can be obtained through the NMR approaches.