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‘Microreactor Technology for Continuous Enzymatic Reactions Catalyzed By C-C-Bond Forming Enzymes’

EIB.10.012 MicroTechEnz

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Project Coordinator - Zagreb

Project partners

University of Zagreb
Faculty of Chemical Engineering and Technology
Partner 1

Prof. dr. sc. Đurđa Vasić-Rački, project coordinator
Dr. sc. Zvjezdana Findrik Blažević, assoc. prof., project leader

Institute of Bio- and Geosciences
IBG-1: Biotechnology
Forschungszentrum Jülich
Germany
Partner 2

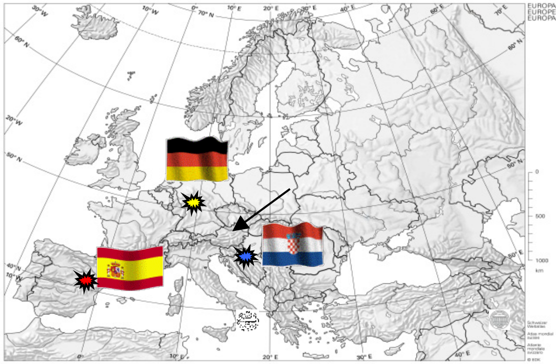
Prof. dr. rer. nat. Martina Pohl

Institute of Advanced Chemistry of Catalonia
Partner 3

Prof. dr. sc. Pere Clapes

Bioglane S.L.N.E.
Partner 4

Mr. Sergi Pumarola



Project objectives

The main goal of the project was the evaluation of the microreactor technology as an appropriate tool for the rapid screening of the enzyme activity and parameter optimization for biotechnological production of important compounds.

Project objectives

–Selection of ThDP-dependent carboligase and D-fructose-6-phosphate aldolases (FSA) for the studied biotransformations.

–Comparison of microreactor technology with classical types of reactors in the reactions catalyzed by ThDP-dependent carboligase and FSA.

–Establishement of microreactor concept using magnetic beads to immobilize benzoylformate decarboxylase (BFD) with a terminal hexahistidine residue (His-tag).

–Preparation and characterization of three FSA variants.

–The development and implementation the cascade reactions to produce valuable compounds in microreactors .

Synthesis of *(S)*-2-hydroxypropiophenone from benzaldehyde and acetaldehyde catalyzed by benzoylformate decarboxylase (BFD) from *Pseudomonas putida*

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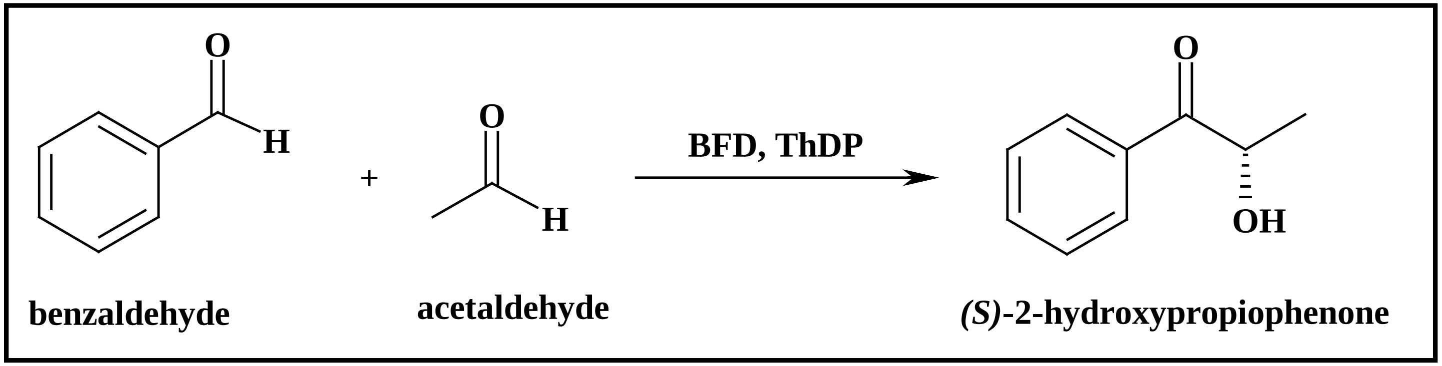


The product of this reaction is the precursor in the enzymatic synthesis of chiral alcohols and pseudo-ephedrine.

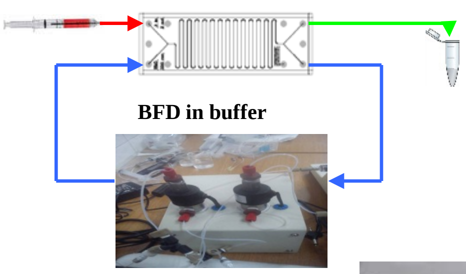
Synthesis of *(S)*-2-hydroxypropiophenone from benzaldehyde and acetaldehyde catalyzed by benzoylformate decarboxylase (BFD) from *Pseudomonas putida*

benzaldehyde + acetaldehyde → *(S)*-2-hydroxypropiophenone

Batch reactor (BR) and repetitive batch reactor (RBR)

Continuously operated ultrafiltration membrane reactor (UFMR)

Microreactor (MR)

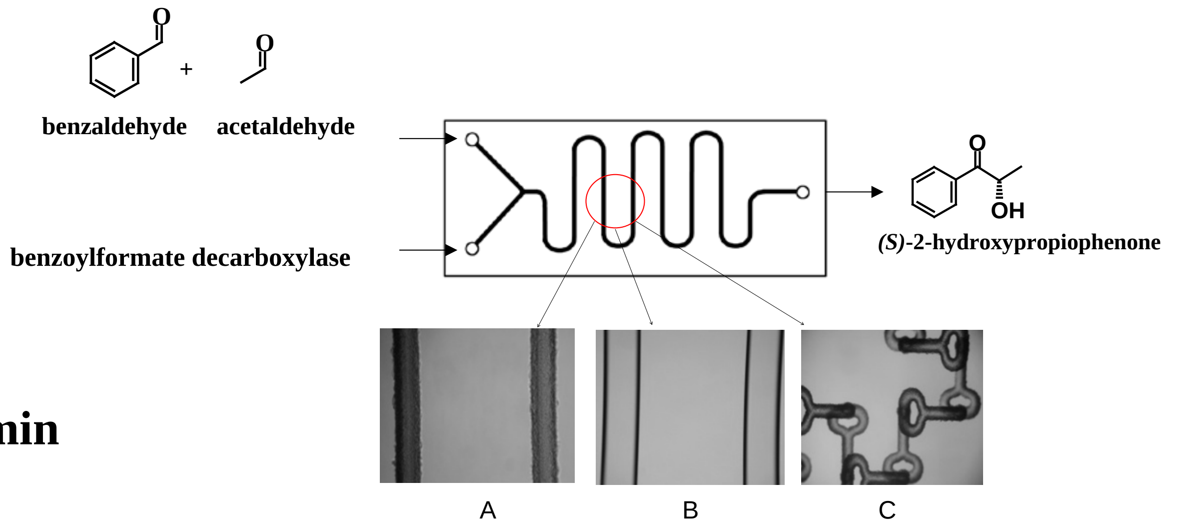


-Soluble enzyme

-Immobilized enzyme

Synthesis of *(S)*-2-hydroxypropiophenone from benzaldehyde and acetaldehyde catalyzed by benzoylformate decarboxylase (BFD) from *Pseudomonas putida*

Microreactors:
-With soluble enzyme



τ = 0.75 min

Q_P=10 mol/(L d) Q_P=9 mol/(L d) Q_P= 14 mol/(L d)

Microreactors have 7-17-fold higher volume productivity than UFMR and 3-5-fold higher volume productivity compared to a batch reactor depending on geometry.

D. Valinger, A. Vrsalovic Presecki, Z. Kurtanjek, M. Pohl, Z. Findrik Blazevic, D. Vasic-Racki (2014) Continuous enzymatic carboligation of benzaldehyde and acetaldehyde in an enzyme ultrafiltration membrane reactor and laminar flow microreactor, J. Mol. Cat. B-Enzym, http//dx.doi.org/10.1016/j.molcatb.2014.02.003.

Synthesis of (*S*)-2-hydroxypropiophenone from benzaldehyde and acetaldehyde catalyzed by benzoylformate decarboxylase (BFD) from *Pseudomonas putida*

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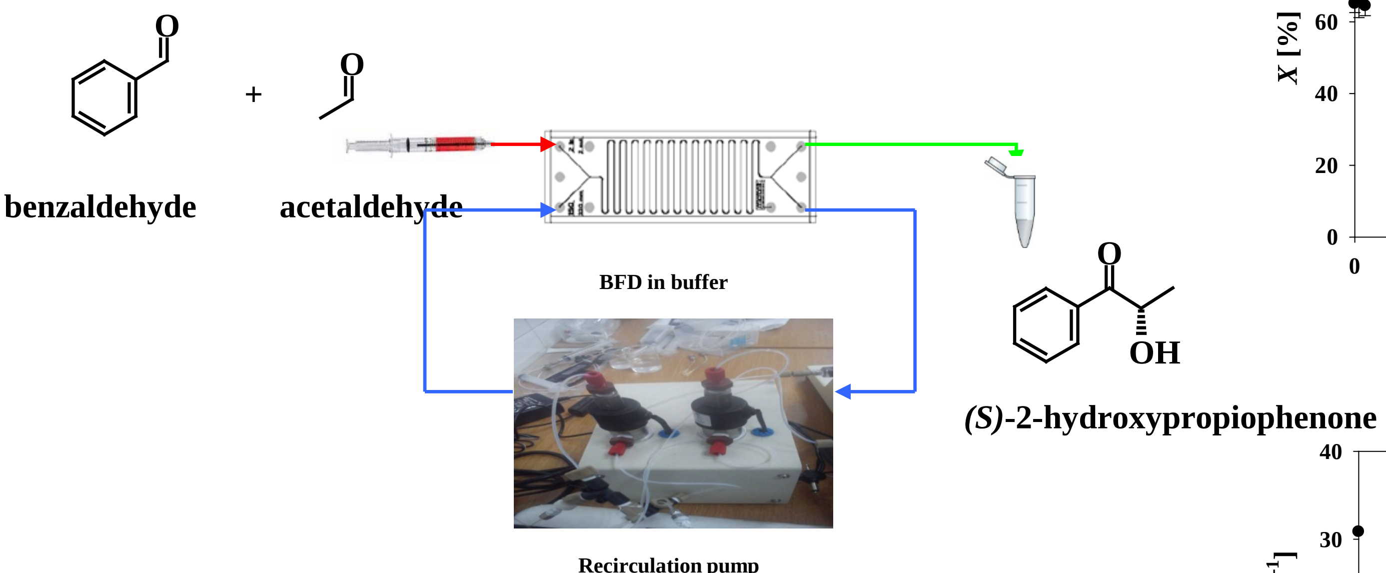
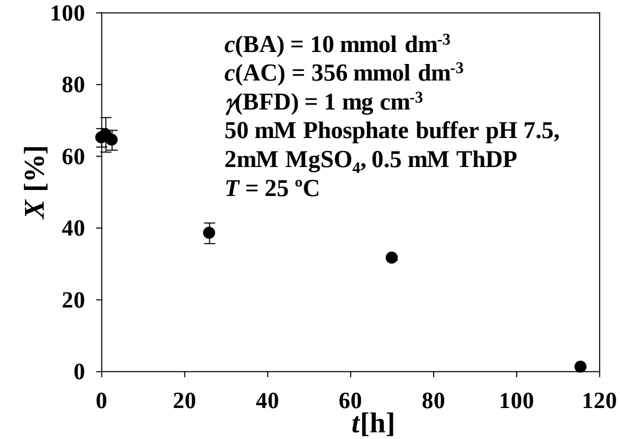
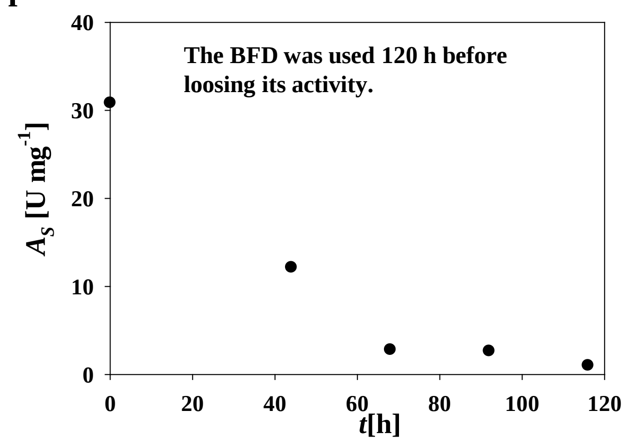
Microreactors:
-With recirculation of soluble enzyme







This approach can be successful in enzyme prolongated use. Steady-state conversion of benzaldehyde was 35 %, because of very high recirculation rate needed to separate enzyme.

Synthesis of *(S)*-2-hydroxypropiophenone from benzaldehyde and acetaldehyde catalyzed by benzoylformate decarboxylase (BFD) from *Pseudomonas putida*

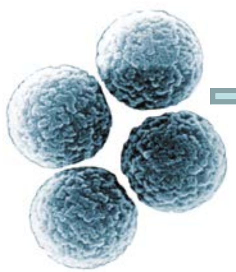
Process	batch	batch	continuous	continuous	continuous
Reactor	Batch Reactor	Repetitive Batch Reactor	Ultrafiltration Membrane Reactor	Microreactor with soluble enzyme*	Microreactor with enzyme recirculation
Volume productivity Q_P [mol /(L d)]	1.5	0.1	0.3	5.1	5.8
Biocatalyst productivity number BPN [g g^{-1}]	1.8	4.7	1.0	1.1	1.2

$$BPN = \frac{C_{A,0} \cdot X_A}{E_0} \quad [\text{g g}^{-1}]$$

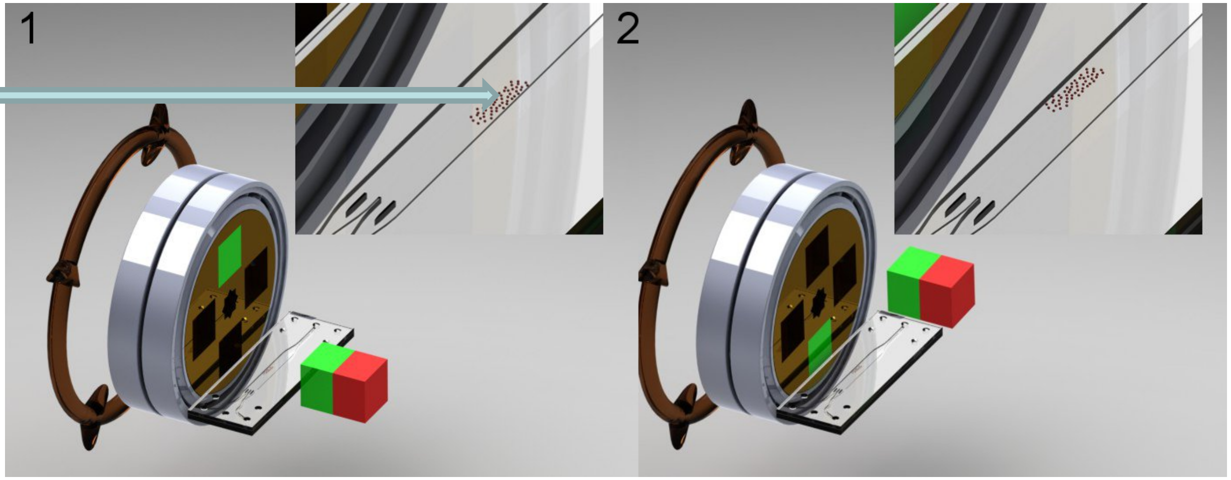
*- microreactor with smooth surface micro-channel walls

Synthesis of *(S)*-2-hydroxypropiophenone from benzaldehyde and acetaldehyde catalyzed by benzoylformate decarboxylase (BFD) from *Pseudomonas putida*

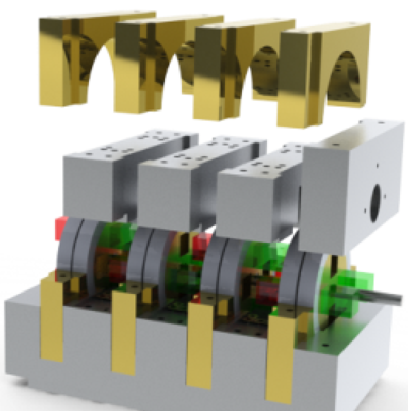
Construction of a new parallelized microfluidic reactor:



enzyme on magnetic beads



permanent magnets in rotating wheels next to the chip retain and mix bead-bound enzyme



final setup with 3 chips

1. D. Jussen and M. Pohl, Mikro-Makroverbindung für mikrofluidische Systeme sowie Verwendung einer elektrischen Leckdetektion, Deutsche Patentanmeldung 10 2013 009 970.3

2. D. Jussen, H. Soltner, M. Pohl, Vorrichtung zum Mischen von Flüssigkeiten in einem Mikrokanal, Deutsche Patentanmeldung 10 2013 006 235.7

Aldol addition of dihydroxyacetone (DHA) to N-Cbz-3-aminopropanal catalysed by D-fructose-6-phosphate aldolase (FSA) variants overexpressed in *Escherichia coli*

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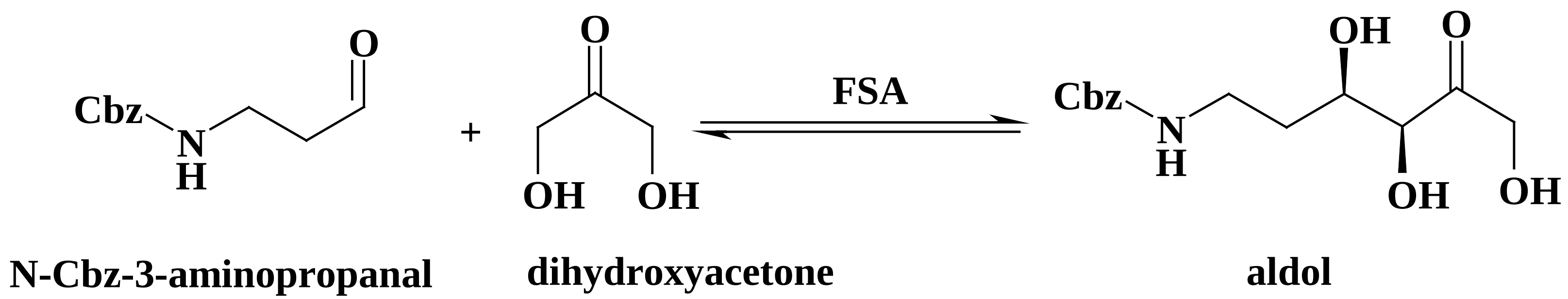

The product of this aldol addition is the precursor of D-fagomine

- Partner 3 from Barcelona prepared 38 different aldolase variants.
- Three aldolase variants were sent to Partner 1 (Zagreb)

Gutiérrez, M.; Parella, T.; Joglar, J.; Bujons, J.; Clapés, P., Structure-guided redesign of D-fructose-6-phosphate aldolase from *E. coli*: remarkable activity and selectivity towards acceptor substrates by two-point mutation. *Chem. Commun.* 2011, *47* (20), 5762-5764.

http://pierre.fkit.hr/miten/

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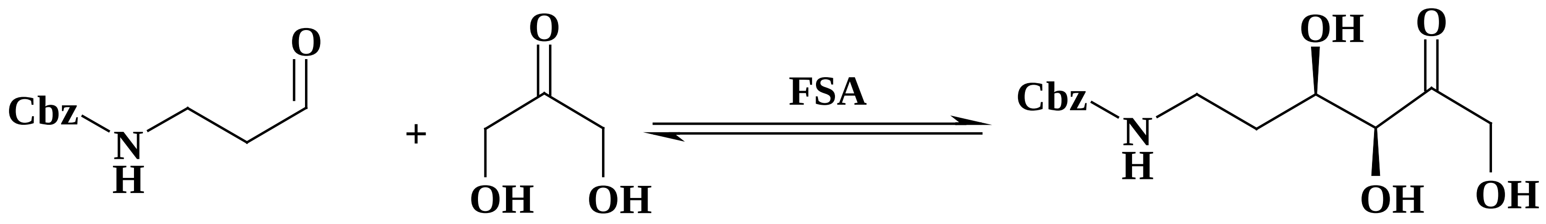
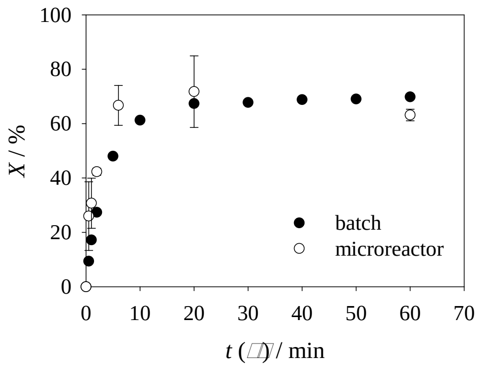


N-Cbz-3-aminopropanal **dihydroxyacetone** **aldol**

Batch reactor (BR)

Continuously operated ultrafiltration membrane reactor (UFMR)

Microreactor (MR)



Aldol addition of dihydroxyacetone (DHA) to N-Cbz-3-aminopropanal catalysed by D-fructose-6-phosphate aldolase (FSA) variants overexpressed in *Escherichia coli*

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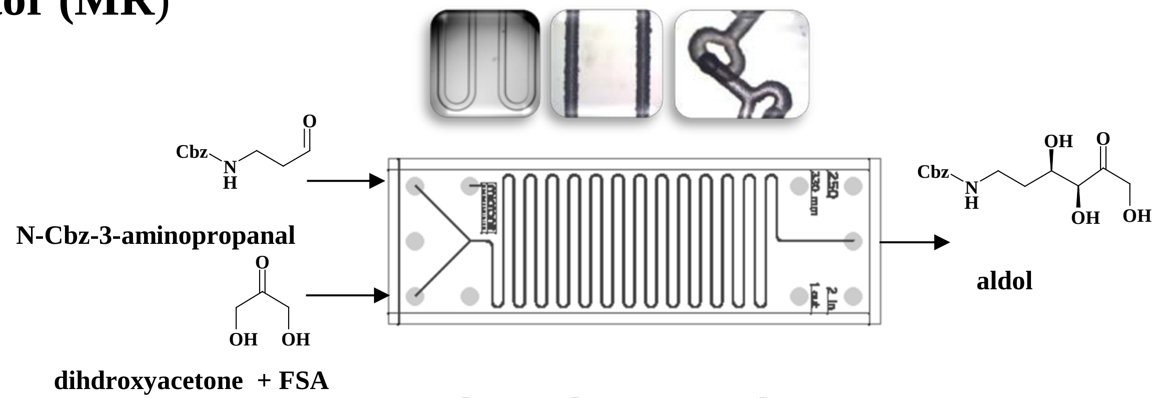
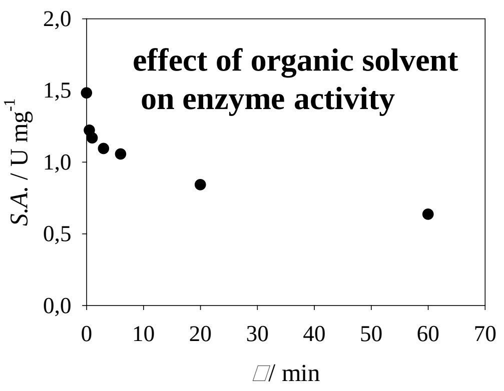
Microreactor (MR)





The volume productivities in microreactors are more than threefold higher than in batch reactor.

The low residence time in microreactor reduces the exposure of enzyme to deteriorating effect of organic solvent and so it is stabilized, because the enzyme is very unstable e.g. in UFMR.

M. Sudar, Z. Findrik, Đ. Vasić-Rački, P. Clapés, C. Lozano, Aldol addition of dihydroxyacetone to N-Cbz-3-aminopropanal catalyzed by two aldolases variants in microreactors. Enzyme Microb. Technol. 2013, 53, 38-45.

M. Sudar, Z. Findrik, Đ. Vasić-Rački, P. Clapés, C. Lozano, Mathematical model for aldol addition catalyzed by two D-fructose-6-phosphate aldolases variants overexpressed in *E. coli*. J. Biotechnol. 2013, 167, 191-200.

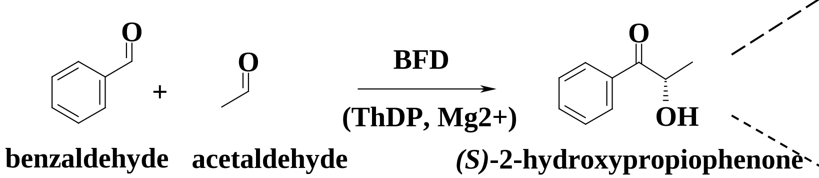
Aldol addition of dihydroxyacetone (DHA) to N-Cbz-3-aminopropanal catalysed by D-fructose-6-phosphate aldolase (FSA) variants overexpressed in *Escherichia coli*

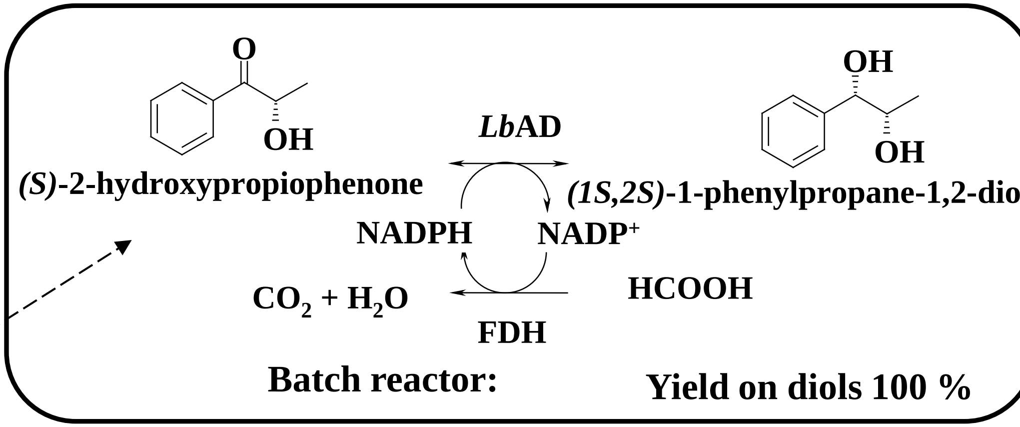
Process	batch	continuous	continuous
Reactor	Batch Reactor	Ultrafiltration Membrane Reactor	Microreactors-soluble enzyme
Volume productivity Q_P [mol /(L d)]	0.3	0.2	0.9
Biocatalyst productivity number *BPN* [g g^{-1}]	0.2	0.2	0.2

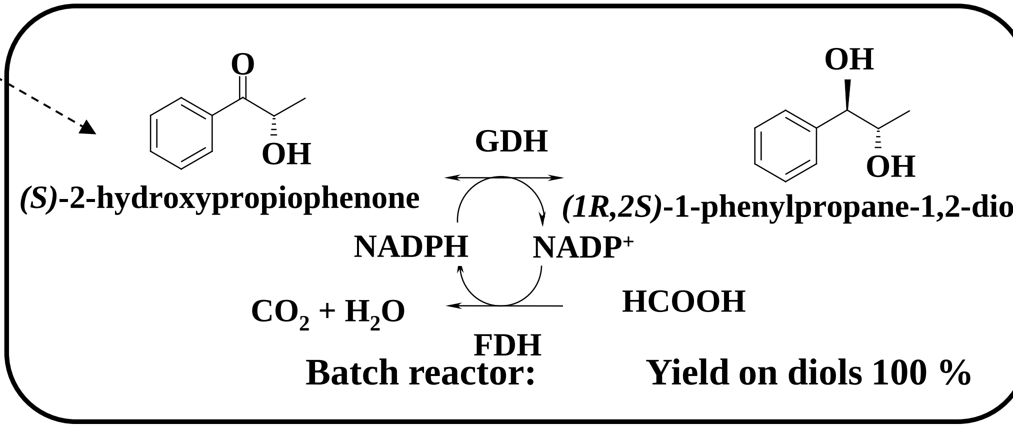
Cascade reactions strategy

One of the goals of project was the integration of carboligation and the oxido-reduction in one pot or as consecutive cascade reaction.

Batch reactors: Integration of carboligation and the oxido-reduction



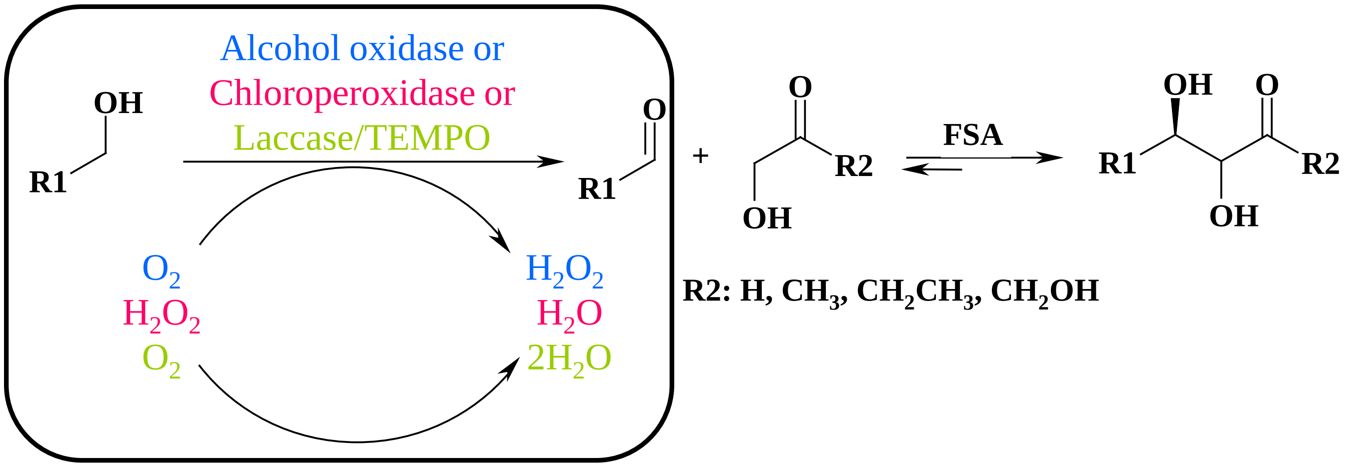




Microreactor: problem coenzyme regeneration; equilibrium conversion in oxido-reduction without regeneration 33-60 %; very high acetaldehyde concentration

Cascade reactions strategy

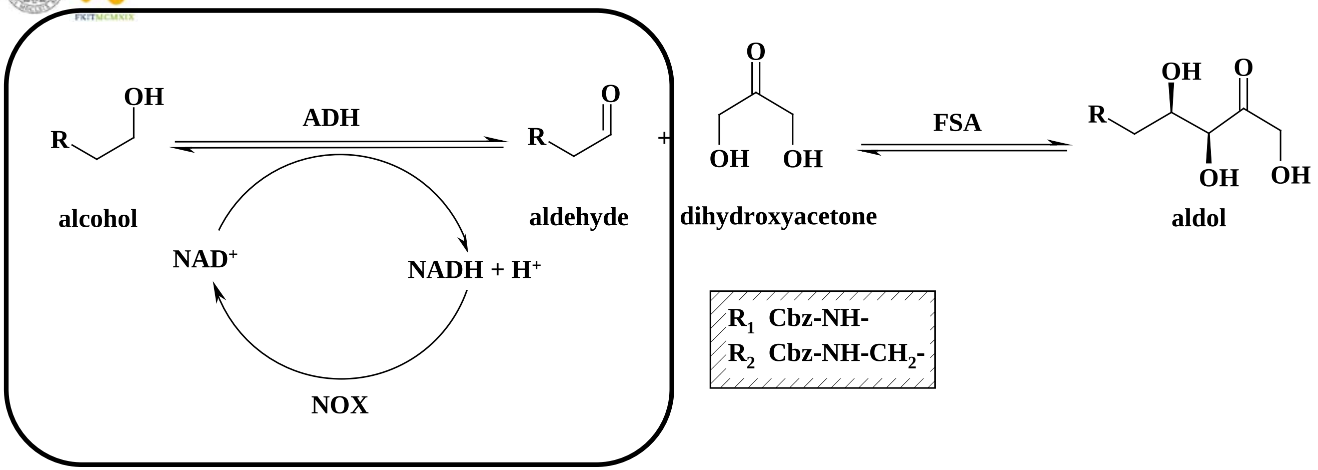
The second goal of project was the integration of the oxidation reaction of the alcohol to aldehyde and subsequent aldol addition in one pot two steps or consecutive cascade reaction.

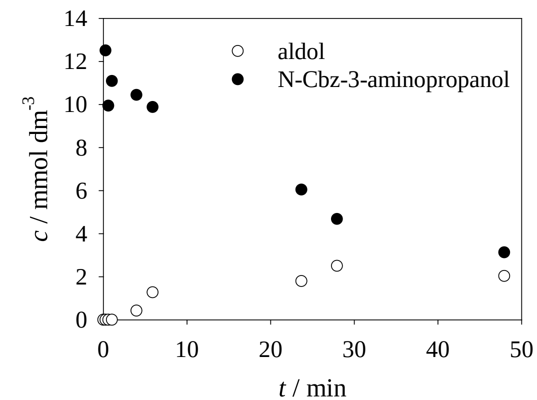


- **Six methods of oxidation reaction were proposed. The best was the laccase/O_2/TEMPO system**

M. Mifsud, A. Szekrényi, J. Joglar, P. Clapés, *In situ* aldehyde generation for aldol addition reactions catalyzed by D-fructose-6-phosphate aldolase. J. Mol. Catal. B: Enzym. 2012, 84, 102-104.

Cascade reactions strategy





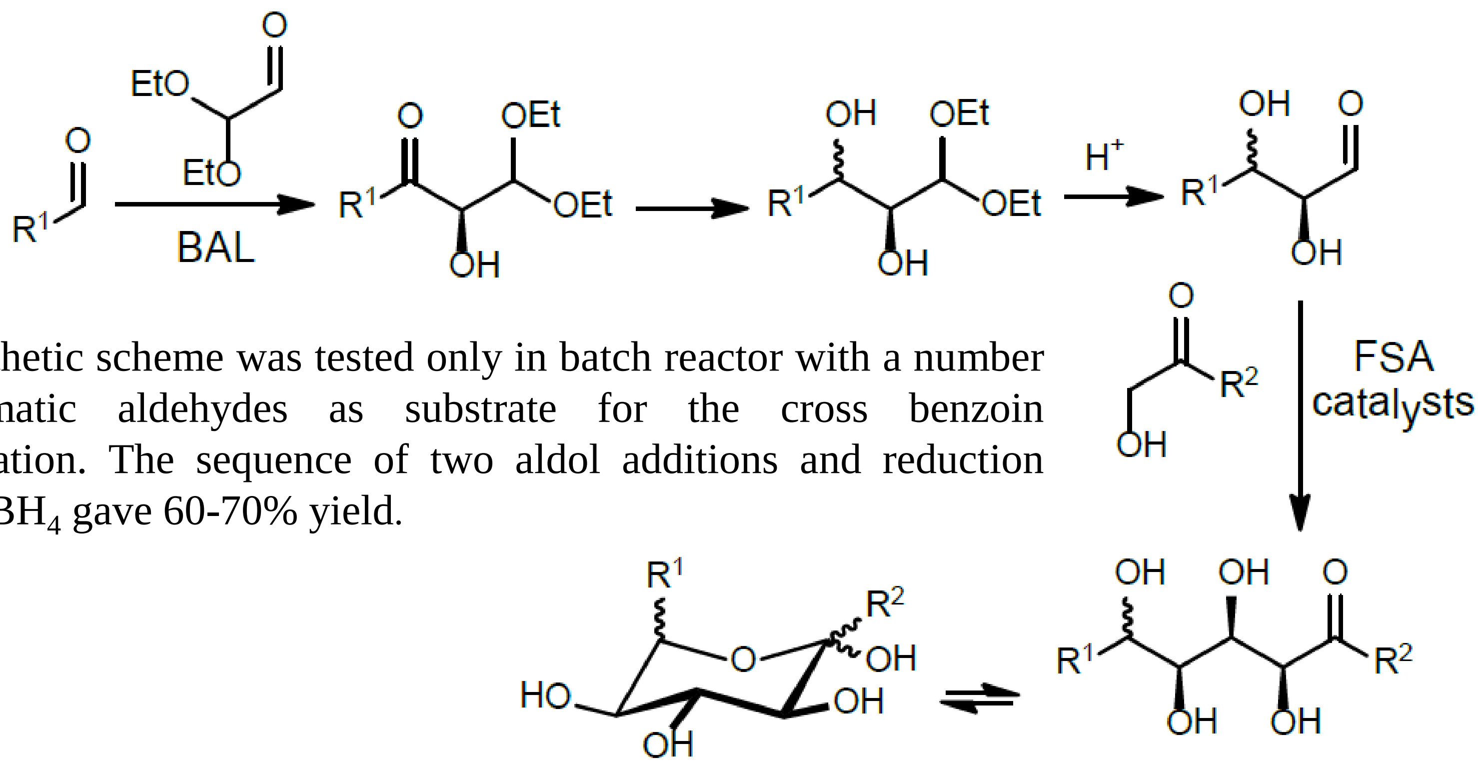
- The new regenerating enzyme NADH oxidase from *Lactococcus lactis* was isolated.

Batch reactor – cascade reaction 20 % yield on aldol

Microreactor – optimization of enzymes concentration needed, currently in progress

M. Sudar, Z. Findrik, M. Vuković Domanovac, Đ. Vasić-Rački, Coenzyme regeneration catalyzed by NADH oxidase from *Lactococcus lactis*, Biochem. Eng. J., submitted

Cascade reactions strategy



The synthetic scheme was tested only in batch reactor with a number or aromatic aldehydes as substrate for the cross benzoin condensation. The sequence of two aldol additions and reduction with $NaBH_4$ gave 60-70% yield.

Scheme: Synthesis of C6 substituted carbohydrate analogues by consecutive catalysis of two carboligases: BAL (benzaldehyde lyase), D-fructose-6-phosphate aldolase (FSA).

Achievement of milestones and objectives

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✓ The evaluation of micro-reactors as an appropriate tool for rapid screening of enzyme activity and production of chiral 2-hydroxy ketones and iminocyclitols was successfully launched.

✓ Biocatalysts which are used in the project: ADH, NADH oxidase from *Lactococus lactis* (new enzyme), FSA variants and BFD are prepared in sufficient quantities and were kinetically characterized.

✓ The method of immobilization of enzyme BFD on magnetic particles was found and the enzyme was immobilized. For the implementation of the reaction with this enzyme in micro-reactor, a completely new magnetic micro-reactor was successfully designed and put into operation.

✓ Characterization of the BFD and FSA reaction systems in laminar flows micro-reactors were carried out.

✓ Aldol addition with FSA variants and carboligation with BFD were performed in micro-reactors and macro-reactors (enzyme membrane reactor and batch reactor).

✓ Investigation and integration of cascade reactions (i.e. oxidation plus aldol addition with FSA mutants) are performed in micro-reactor as well as batch reactor.

Thank you for your attention !

Quality and added value of cooperation between partners

- **Partners exchanged collaborators on the project**
  - Two members of the Zagreb group **Martina Sudar**, PhD student and **Dr Zvjezdana Findrik Blažević** stayed in Barcelona from 1/09/2010 to 30/09/2010 and from 10/10/2011 to 11/11/2011 with a financial support of a COST Action (COST-STSM-CM0701-8306). The purpose was the steady state kinetics characterization for the aldol retroaldol reaction of dihydroxyacetone to N-Cbz-3-aminopropanal catalyzed by the FSA variants A129S and A129S/A165G and their stability under different reaction conditions. Two joint publications were obtained as a result of these investigations**.**
  - **Daniel Jussen** (doctoral student from Jülich) has visited the Lab in Zagreb from April 1- May 31, 2012, were he received valuable support for the setup of the microreactor system. This stay was further used to transfer knowledge on the handling of BFD and instrumental analysis of 2-hydroxypropiophenone from Jülich to Zagreb.
  - **Davor Valinger** (doctoral student from Zagreb) visited the lab in Jülich twice (Oct 10-Oct. 28, 2011, Sept. 1 – Dec 8, 2012. During his first stay Davor learned to purify BFD for his studies and during the second stay he did different measurements concerning the stability and performance of BFD in laminar flow micro reactors.

Quality and added value of cooperation between partners

•**Partners exchanged resources on the project**

- Preparation at medium scale of N-Cbz-2-aminoethanal (Cbz= benzyloxycarbonyl) dimethyl acetal, a stable precursor of the corresponding aldehyde, and the aldol adduct from the addition of dihydroxyacetone to N-Cbz-2-aminoethanal. These products were sent to Partner 1 Faculty of Chemical Engineering and Technology, University of Zagreb. They had a continuous supply of these starting materials.
- The company Bioglane also prepared (Cbz= benzyloxycarbonyl) and the aldol adduct from the addition of dihydroxyacetone to N-Cbz-3-aminopropanal. These products were sent to Partner 1 Faculty of Chemical Engineering and Technology, University of Zagreb. They had a continuous supply of these starting materials.
- The partner from Jülich has provided Partner from Barcelona with benzaldehyde lyase from *Pseudomonas fluorescens* (BAL), a class of enzyme that Jülich has much expertise, which catalyzed the benzoin condensation between two aromatic aldehydes.

Quality and added value of cooperation between partners

- Internal cooperation between partners was excellent. The six consortium meetings were very successful and particularly beneficial for doctoral students. Three doctoral students are at the end of their doctoral thesis. Three joint publications before the end of the project are proof of this good cooperation in a multi-disciplinary team. It should be emphasized that selfless knowledge transfer was present, as well as assistance in the use of equipment. Partners have a good base for future cooperation, because they have gained a completely mutual trust.