Enzyme production and immobilization in mesoporous materials

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The aim of this study was to understand the micro-environment enzymes experience inside the pores of mesoporous materials as well as to understand the immobilization process as such. Feruloyl esterases (FAE) were used as the model enzymes.

Background

Mesoporous silica (MPS) materials possess properties such as large surface area, defined pores geometry, mechanical and thermal stability and they are tunable.

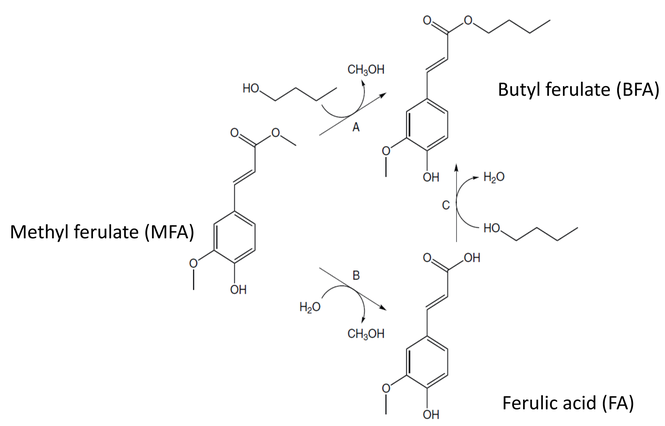
FAEs are of utmost interest in degrading lignocellulosic biomass: they catalyze the hydrolysis of ester linkages releasing ferulic acid and other hydroxycinnamic acids from hemicellulose. They can also catalyze the reverse reaction: transesterification.

Esterified hydroxycinnamic acids are bioactive compounds recognized for their antioxidant, tumor suppressing and antibacterial properties[1].

Previous results have demonstrated:

- ✓ Operational stability and reusability of an FAE cocktail (Depol740L) immobilized on mesoporous silica material [1]
- ✓ Following immobilization of Depol740L, transesterification is increased and hydrolysis decreased [1]
- ✓ Immobilization yield and enzymatic reactions are pH dependent [2]
- ✓ The pH during the immobilization affects the specific activity of the enzyme [2]
- ✓ Methods to study pH inside the pores and to follow immobilization [3] [4].

Some reactions catalyzed by FAEs

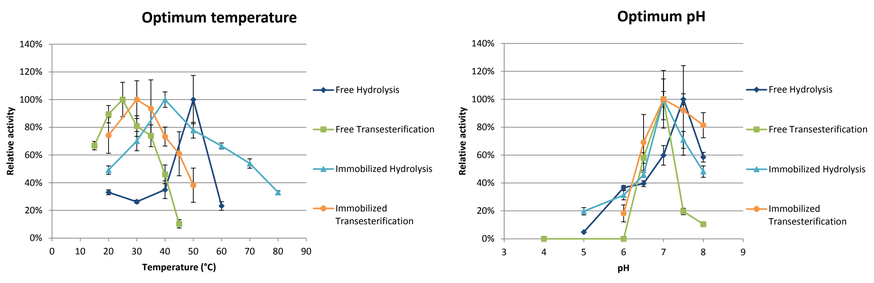


FAEs can catalyse different reactions: (A) Transesterification of MFA with 1-butanol generating BFA and methanol. (B) Hydrolysis of MFA generating ferulic acid and methanol (natural reaction at high water contents). (C) Esterification of FA with 1-butanol generating BFA and water.[1]

Work on a commercial enzyme

E-FAERU is a commercially available FAE (Megazyme) coming from a rumen microorganism.

E-FAERU was characterized in terms of pH and T optimums in four different conditions: free/immobilized and in hydrolysis/transesterification.



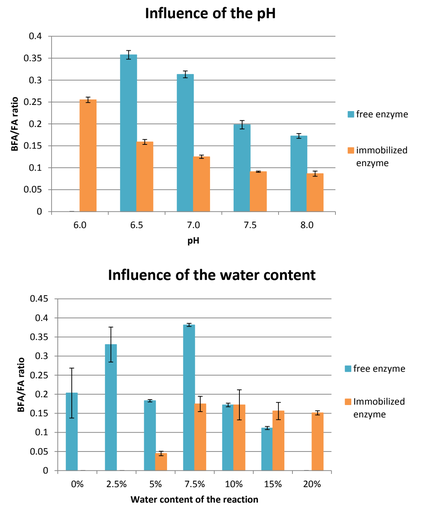
Both for the free and immobilized enzyme, a decrease in the Topt was observed for the transesterification. As expected immobilization seemed to increase the thermo-stability of the enzyme. Regarding the pH no significant differences were observed.

The kinetic parameters were then studied at the defined optimum conditions.

	Km (mM)	kcat (s^{-1})	kcat/Km ($s^{-1}M^{-1}$)
free enzyme - hydrolysis	4,31E-01	1,13E+05	2,63E+02
free enzyme - transesterification	3,60E+01	4,92E+02	1,37E-02
immobilized enzyme - hydrolysis	4,31E-01	2,33E+04	5,40E+01
immobilized enzyme - transesterification	3,09E+01	5,20E+01	1,68E-03

We observed a 100-fold increase in the affinity of the enzyme between hydrolysis and transesterification. Interestingly the affinity was not affected upon immobilization. However the turnover number was reduced upon immobilization in both cases resulting in a reduced catalytic efficiency.

pH and water content (in the solvent/buffer reaction mixture) effect on the transesterification vs hydrolysis ratio were then studied.



The same trend was observed for the free and the immobilized enzyme with a decreasing BFA/FA ratio with increasing pH.

For the free enzyme there was an optimum water content at 7,5%. Interestingly even at very low water content it was still active. For the immobilized enzyme at least 5% of water were needed to have activity.

Our results demonstrate that immobilization changes properties of the enzyme and is influenced by a set of parameters. Results obtained here will serve as a comparison point for the newly produced enzymes.

From selection to purification of enzymes

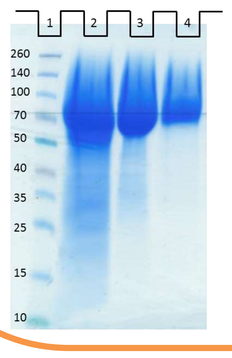
From the genomes of *Aspergillus glaucus* and *Aspergillus zonatus*, five enzymes were selected to be studied.

The selected enzymes were putative FAEs or Tannases and were quite distant in a phylogenetic tree containing *A.oryzae* and *A.glaucus* enzymes.

The five genes were cloned into *Pichia pastoris* SMD1168H using pPiczα as a vector. The enzymes were then expressed in fermenters in a fed batch process.

The produced enzymes were then purified by IMAC or ion exchange chromatography.

1: MW
2: Crude extract 70058
3: cross flow filtrated 70058
4: ion exchange purified 70058



70058

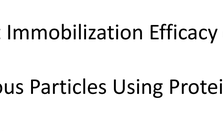
Sample	Activity (mM/min)	Concentration (mg/mL)	Total activity (mM/min)	Specific activity (mM/min/mg)	Purification fold	Yield (%)
Crude extract	0,019	4	3330	0,9	1,0	100%
Cross Flow filtered	0,027	4,5	3288	1,2	1,3	99%
Q purified enzyme	0,026	3	3072	1,7	1,8	92%

The data suggested that most of the enzyme was recovered (92%) and of high purity (single band on the SDS-PAGE gel).

The five enzymes were successfully produced, some of them need purification optimization.

Enzyme activity and type determination

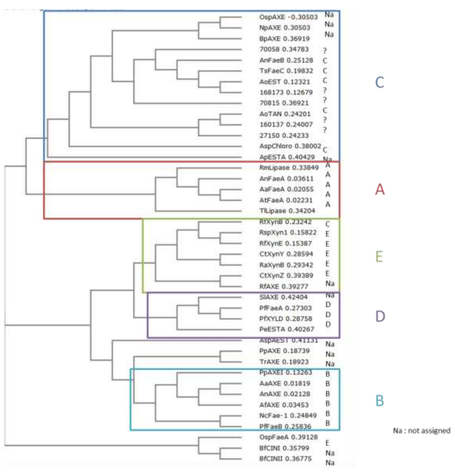
Different activities were screened to determine the enzymes activities.



	168173	70815	27150	70058	160137
FAE (MFA)	yes	no	no	no	no
FAE (MCA)	yes	yes	no	no	no
FAE (MSA)	yes	yes	no	no	no
FAE (MpCA)	yes	no	no	no	no
Tannase (methyl gallate)	no	no	yes	yes	no
Aryl esterase (phenyl acetate)	no	no	no	no	no
Acetyl esterase (pNP-Acetate)	yes	no	yes	yes	yes
Paraoxonase (paraoxon)	no	no	no	no	no
PHB depolymerase (PHB)	no	no	no	no	no
Carboxyl esterase (pNP-palmitate)	no	no	no	no	no

FAE, Tannase or Esterase activity was found in the crude extract of all five expressed enzymes. Allowing further processing of the proteins.

Using proteins sequences of the FAE family and relatives a cladogram was constructed.



Based on the classification from Crepin *et al* [5], all five enzymes belong to the Type C FAE family.

Different activities were found for the five expressed enzymes.
They all belong to the Type C feruloyl esterase family.

[1] Thörn, Christian, Hanna Gustafsson, and Lisbeth Olsson. "Immobilization of Feruloyl Esterases in Mesoporous Materials Leads to Improved Transesterification Yield." *Journal of Molecular Catalysis B: Enzymatic* 72, no. 1–2 (October 2011): 57–64. doi:10.1016/j.molcatb.2011.05.002.
[2] Thörn, Christian, D.B.R.K. Gupta Udatha, Hao Zhou, Paul Christakopoulos, Evangelos Topakas, and Lisbeth Olsson. "Understanding the pH-dependent Immobilization Efficacy of Feruloyl esterase-C on Mesoporous Silica and Its Structure–activity Changes." *Journal of Molecular Catalysis B: Enzymatic* 93 (September 2013): 65–72. doi:10.1016/j.molcatb.2013.04.011.
[3] Thörn, Christian, Nils Carlsson, Hanna Gustafsson, Krister Holmberg, Björn Åkerman, and Lisbeth Olsson. "A Method to Measure pH Inside Mesoporous Particles Using Protein-bound SNARF1 Fluorescent Probe." *Microporous and Mesoporous Materials* 165 (January 1, 2013): 240–246. doi:10.1016/j.micromeso.2012.08.028.
[4] Thörn, Christian, Hanna Gustafsson, and Lisbeth Olsson. "QCM-D as a Method for Monitoring Enzyme Immobilization in Mesoporous Silica Particles." *Microporous and Mesoporous Materials* 176 (2013): 71–77. doi:10.1016/j.micromeso.2013.04.001.
[5] Crepin, V.F., Faulds, C.B., Connerton, I.F., "Functional classification of the microbial feruloyl esterases." *Appl Microbiol Biotechnol* (2004) 63: 647–652