**An Enzymatic Cascade with Integrated Process Intensification for Synthesis of Natural Flavors.**

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**Highlights**

* Establishment of a 3-enzyme cascade in a two phase system
* Integrated *in situ* cofactor regeneration and *in situ* product extraction
* Process optimization by enzyme immobilization

**1. Introduction**

Biotechnical processes are a promising alternative to conventional chemical reactions, if they fulfill certain requirements like less byproduct formation, less intermediate separation, environmentally friendly and sustainable reactions. With different methods to improve the productivity, like efficient cofactor regeneration and simplified downstream processing, these enzymatic reactions are of increasing importance for industry.[1,2] In this study a three enzyme cascade in two phases is evaluated, as shown in figure 1. In the aqueous phase an alcohol dehydrogenase (ADH) is reducing the substrate cinnamal to the corresponding alcohol. Since this enzyme is NADH cofactor dependent an *in situ* regeneration is established by formate dehydrogenate (FDH). The product is extracted *in situ* by an organic solvent to realize the esterification by a lipase to the target product cinnamyl cinnamate.



**Figure 1.** Reaction scheme of the enzyme cascade.

**2. Methods**

Three different enzymes are investigated, whereby the enzymes used in the aqueous phase are immobilized with different techniques. Characterization of free and immobilized enzymes is performed via activity and kinetic, as well as stability measurements. The whole cascade is carried out in a reactor concept with an integrated extraction centrifuge and a fixed-bed reactor for the esterification.

**3. Results and discussion**

A suitable organic solvent has to be identified for the enzymes in the aqueous phase, as well as for the esterification. Buffer saturated with different solvents are screened regarding ADH activity, whereby compounds with high logP values showed the lowest influence on enzyme activity. Xylene is chosen as second phase, due to its high logP value, dissolution of the substrates and good properties regarding process safety. Based on this, the individual enzymes are characterized in xylene saturated buffer and their respective kinetic parameters (Michaelis-Menten) were determined. In addition, the esterification in xylene by an immobilized lipase was carried out and high yields of over 95% for the two reaction steps show the feasibility of this cascade.

To improve the enzyme stability and simplify the downstream processing, immobilization of the biocatalysts in the aqueous phase is investigated in detail, shown in figure 2. Several techniques are screened, whereby cross-linked enzyme crystals (CLEAs) and covalent immobilization on hydrophilic amino-activated supports showed good immobilization yields and the highest residual activities.

 

**Figure 2.** Results of the screened immobilization methods.

**4. Conclusions**

A multi-enzyme cascade in a two phase system was successfully established and evaluated in detail. Xylene was chosen as suitable organic solvent for the second phase and the enzymes were characterized regarding these reaction conditions. Different immobilization techniques were studied, whereby covalent immobilization on hydrophilic supports showed the most promising results for the ADH. The established system will be further investigated in a larger reactor set-up.

**References**

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