

# Pilot-scale Operation of a Multi-Enzymatic Cascade Reaction in a Multiphase System

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This work aims to emphasize the importance of scale-up in biotechnological research, in order to introduce more environmentally friendly bio-catalyzed processes to the industry. Here, a complex biotechnological reference reaction system is investigated, and, for the first time, a successful synthesis of cinnamyl cinnamate from cinnamyl aldehyde in a multiphase system is established. A multi-enzymatic cascade reaction sequence with integrated co factor regeneration and *in situ* intermediate separation is applied. This highly integrated reaction system is successfully implemented in a pilot-scale reactor plant. Experimental results show complex relationships between physically separated unit operations.

## 1. Introduction

Industrial biotechnology in engineering is widely and controversially discussed in literature as a high-potential area to replace current chemical production processes through novel, sustainable processes (Vivien *et al.*, 2019, Kiss *et al.*, 2014). In particular, the production of specialty and fine chemicals is a strongly growing field in the chemical industry, with a high potential for greener processes (Wohlgemuth, 2010, Johannsen *et al.*, 2019). This also applies to flavors and fragrances (Johannsen *et al.*, 2020), which show annual growth rates of up to 5 % in a 30 billion US-\$ market volume (IAL Consultants, 2018). Hereby, the production of natural flavors is of growing interest especially in Europe and North America.

While this field is the focus of important academic research, the phases of development, ranging from generating a new idea to its final industrial application, remain mostly in laboratory scale. The present work aims to draw attention to the importance of taking the next step in process development, and investigates the challenges that these biotechnological ideas face when implemented on a larger scale.

Enzymatic reactions allow for high selectivity and purity under mild reaction conditions, with less by-products within an overall sustainable production process (Mata *et al.*, 2017, Caetano *et al.*, 2012). Hereby, multi-enzymatic cascade reactions in particular show a high potential for specialty and fine chemicals as products, like flavors and fragrances (Ricca *et al.*, 2011, Xue and Woodley, 2012). Bi-enzymatic cascade reactions primarily use the possibility of regenerating co factors (Findrik and Vasic-Racki, 2009), but cascades up to four enzymes are also described in literature (Ricca *et al.*, 2011). However, such cascades are limited to the application in aqueous phases, due to enzyme stability. The use of an enzyme cascade in aqueous and organic media has been investigated in very few examples only (Bruggink *et al.* 2003). Usually, the extractive character is used in order to advance the reaction turnover, but no reaction takes place subsequently in the organic phase. Some examples to advance the reaction in an aqueous phase with phase-interfacial active enzymes by an extraction can be found. These processes are implemented as one-pot processes (Gargouri and Legoy, 1997).

In general, complex biotechnological processes are difficult to model, an essential step for scale-up (Kuhn *et al.* 2010). In this study, we introduce a complex multi-enzymatic cascade reaction sequence within a two-phase system in a pilot-scale reaction plant. Due to the dimensions of these plants, these processes face more challenges in contrast to the usual biotechnological reaction systems, implemented on laboratory scale

only (Reismann, 1993, Formenti *et al.*, 2014). The complexity of having various reactions with individual optima, and additionally intermediate phase transition, requires the process windows to be characterized carefully. Moreover, the residence times in the individual unit operations influences the overall productivity of the process.

## 2. Process description

A novel *in vitro* multi-enzymatic cascade reaction sequence for the sustainable production of cinnamyl cinnamate from cinnamyl aldehyde in a multiphase system is introduced, as shown in figure 1. Cinnamyl cinnamate is a natural component of storax and of Balsam of Peru, from which it is extracted, and it is used as flavor in perfumes and cosmetic products (Burdock and Fenaroli, 2010). Chemically, cinnamyl cinnamate is directly produced by the synthesis of cinnamic aldehyde in absolute ether with aluminum ethylate (Burdock and Fenaroli, 2010), or by oxidative carbonylation of styrene with carbon monoxide, oxygen and aliphatic alcohols in the presence of palladium and sodium propionate, the selectivity being about 95 % (Hsu, 1986). In this work, cinnamyl cinnamate is synthesized in a three-enzyme cascade reaction sequence. Two co factor coupled dehydrogenases are used in a water-based buffer system, thus ensuring co factor regeneration within the reaction pathway. An alcohol dehydrogenase (ADH) converts the educt, cinnamyl aldehyde, to cinnamyl alcohol. The required co factor  $\text{NADH}+\text{H}^+$  is regenerated in a parallel reaction step by a formate dehydrogenase (FDH), which converts  $\text{NAD}^+$  and sodium formate to  $\text{NADH}+\text{H}^+$  and  $\text{CO}_2$ . This has been successfully established in laboratory-scale (Engelmann *et al.*, 2020). In a subsequent step, the intermediate, cinnamyl alcohol, is extracted *in situ* by an organic solvent. For this, eight different organic solvents were tested, considering extraction performance, enzyme deactivation, and safety as crucial determinants. As a result, xylene was chosen as the most suitable solvent. The intermediate cinnamyl alcohol and added cinnamyl acid are the educts of the final step of this multi-enzymatic cascade reaction. A lipase-catalyzed esterification reaction results in the final product cinnamyl cinnamate, and the by-product water. Here, we present, for the first time, the experimental results of a pilot-scale multi-enzymatic cascade reaction for the production of cinnamyl cinnamate in a multiphase system.

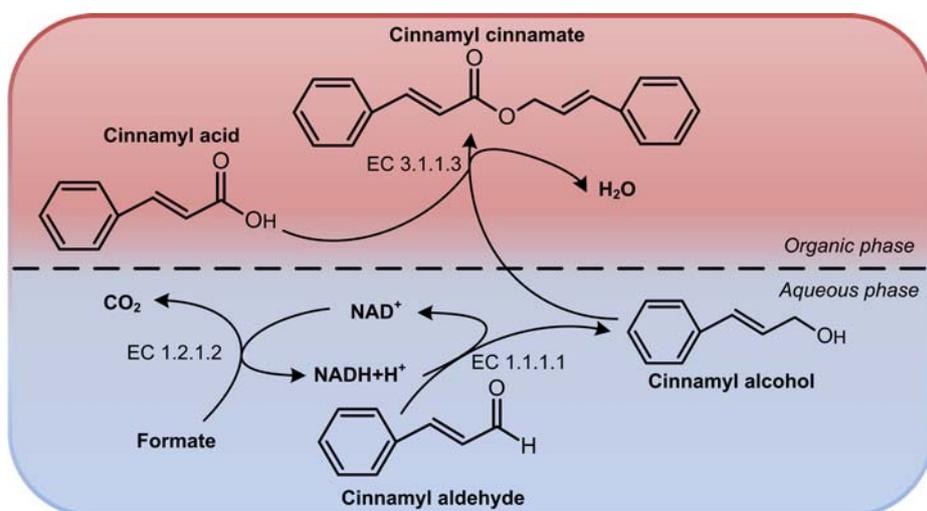


Figure 1: Multi-enzymatic cascade reaction sequence in a two-phase system with integrated co factor regeneration and *in situ* intermediate extraction producing cinnamyl cinnamate from cinnamyl aldehyde.

## 3. Pilot-scale reactor plant

A pilot-scale reactor plant was constructed in order to perform the reaction cascade from figure 1 on a larger scale. Figure 2 shows the flow chart of the reactor plant, consisting of two cycles (aqueous and organic) that are connected to each other for the extraction step. A continuously stirred tank reactor containing the aqueous phase (0.1 M potassium buffer at pH 8.0) is used for the co factor coupled red-ox reactions, catalyzed by ADH and FDH (figure 2, A). The organic phase cycle consists of three segments. A buffer tank is used for the organic phase and for the feed of the cinnamyl acid (figure 2, B). An extractive centrifuge (CINC CS 50) connects the two cycles, simultaneously mixes the organic and the aqueous phase to extract the intermediate product, and subsequently separates both phases within one apparatus (figure 2, C). A fixed bed reactor containing 21 g of immobilized lipase (Novozym<sup>®</sup> 435) is used for the esterification reaction (D).

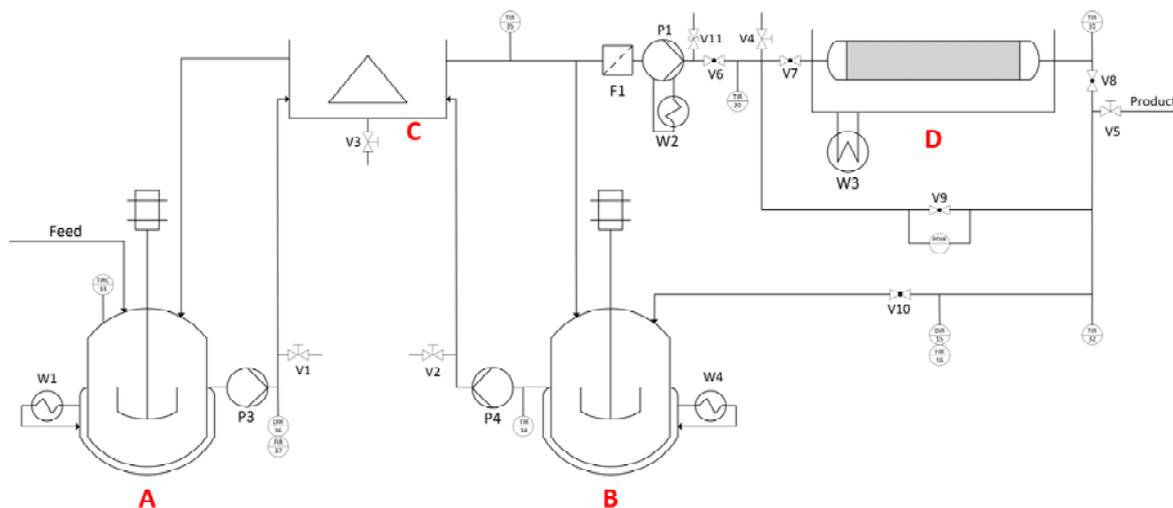


Figure 2: Flow chart of the pilot-scale reactor plant consisting of a continuously stirred tank reactor (CSTR) (A), a buffer tank (B), an extractive centrifuge (C) and a fixed bed reactor (D).

In most cases, academic research processes with enzymes such as dehydrogenases are carried out in the milliliter range. The total volume of the reactor plant is 5 l, which increases the scale by a factor of  $10^3$ . The reactor plant is equipped with 7 temperature sensors, 4 heating baths, 2 mass flow meters, 3 pumps, 4 pressure sensors, and 1 difference pressure sensor. The data obtained from the sensors are fed into a LabVIEW<sup>®</sup>-based process monitoring system, allowing for on-line process monitoring and control. With the reactor plant shown in figure 2, the multi-enzymatic cascade reaction can be performed on pilot scale. The dehydrogenases are immobilized on silica particles (Engelmann, 2020), and placed in a SpinChem<sup>®</sup> RBR S2 rotating bed reactor. 23.3 mM cinnamyl aldehyde, 0.1 mM NADH+H<sup>+</sup>, and 64 mM cinnamyl acid are used as starting concentrations in 800 ml aqueous buffer phase (figure 2, A) and 500 ml organic phase (figure 2, B). Due to the low reaction rates of the immobilized dehydrogenases, the aqueous phase reaction (figure 2, A) was started 49 h prior to the esterification reaction, in order to obtain a suitable starting concentration of the intermediate for the esterification reaction. After 49 h the organic cycle was connected through the extractive centrifuge (figure 2, B). Figure 3 shows the concentrations of all four cinnamyl components over the reaction time in the organic phase. The samples were taken from the buffer tank of the organic phase (figure 2, B) and analyzed using a Clarus 500 gas chromatograph from Perkin Elmer, equipped with a SLB SUPELCO 30 m column with a diameter of 0.25 mm x 0.25  $\mu$ m.

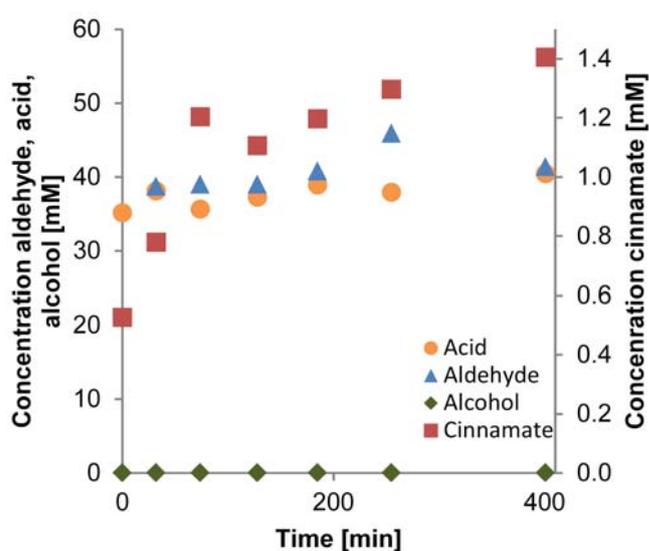


Figure 3: Concentration of all cinnamyl components in the xylene buffer tank (figure 3, B) as function of time, according to the experimental setup described in chapter 3.

As shown in figure 3, the concentration of the final product cinnamyl cinnamate continuously increases. The cinnamyl alcohol concentration is constantly below the detection limit, as the produced intermediate in the aqueous phase is immediately converted in the esterification reaction. Due to the low reaction rates, the concentrations of cinnamyl aldehyde and cinnamyl acid also remain nearly constant over the course of the experiment. 1.4 mM of product was produced after 400 min reaction time. These results prove the feasibility of the multi-enzymatic cascade reaction sequence in the pilot-scale reactor plant.

#### 4. Process analysis and improvement

The phase interface plays a crucial role in the production process, in that it connects the two separate reaction systems to each other and can greatly influence the available concentration of each component for the enzyme reaction. Therefore, it is important to determine the two most influential parameters of the phase interface: The partition coefficients of each component and the water saturation concentration in xylene. The latter parameter is especially crucial, since water is a product of the final step of this reaction (esterification) and can potentially hinder product formation. Albeit neglected in a continuous process due to constant product removal, the theoretical maximum conversion in the thermodynamic equilibrium is also decreased. The partition coefficient defines the concentration change in each phase at the beginning of and during an experiment, and it is defined according to equation (1).

$$P_{\text{component } i} = \frac{[\text{component } i]_{\text{organic phase}}}{[\text{component } i]_{\text{aqueous phase}}} \quad (1)$$

##### 4.1 Partition coefficients

Partition coefficients were determined by adding different concentrations of the four cinnamyl components, both separately and together, to the two-phase system of aqueous buffer phase and xylene. The organic phase was analyzed using the Clarus 500 gas chromatograph from Perkin Elmer described in chapter 3. The experiments were carried out at temperatures varying from 20 °C to 70 °C. No influence of the temperature on the partition coefficients of the components was observed. Table 1 shows the partition coefficient for each of the four cinnamyl components for the aqueous buffer phase and for the organic xylene phase according to equation (1).

Table 1: Partition coefficients of the cinnamyl components according to equation (1).

Component	P <sub>i</sub> [-]
Cinnamyl alcohol	33.0 ± 19.8
Cinnamyl aldehyde	15.2 ± 8.6
Cinnamyl acid	5.1 ± 3.7
Cinnamyl cinnamate	4.5 ± 2.0

As expected, the data in table 1 show that the solubility of cinnamyl alcohol is much greater in xylene than it is in water. This benefits the intermediate extraction and enhances the aqueous phase reactions. The educt cinnamyl aldehyde is also preferably soluble in the organic phase. Since this component has to be made available for the ADH, it seems as if the partition coefficient is hindering the reaction. However, since the reaction rates are much lower than the extraction rates, the concentration of cinnamyl aldehyde can actually be controlled with the help of the organic phase and the known partition coefficient. In continuous operation, together with the feed stream, the aldehyde concentration can be set to a suitable range for the enzymes. Of course, this requires a precise, validated model to find optimal operating points. Due to their higher polarity, cinnamyl acid and cinnamyl cinnamate have a lower partition coefficient than alcohol and aldehyde. Nevertheless, the final product is more soluble in xylene, which is highly beneficial for product separation.

##### 4.2 Water saturation in xylene

The saturation concentration of water in xylene during the extraction step is of interest, because water is a product of the subsequent esterification reaction. The water saturation concentration was determined by mixing water and xylene at 1300 rpm. Samples from the organic phase were analyzed in a C30-All-round Coulometer from Mettler Toledo. The experiments were performed between 10 °C and 60 °C, in order to describe the water content in the organic phase in dependency of the temperature (figure 4).

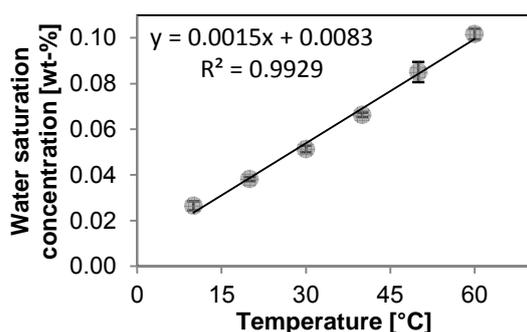


Figure 4: Temperature dependency of the saturation concentration of water in xylene at atmospheric pressure.

The temperature dependency of water saturation concentration in xylene is linear with a coefficient of determination of 0.99, as shown in figure 4. These results fall in line with comparable literature values (Hai, 2015), allowing for a precise determination of the water concentration in the organic phase, and consequently for an exact determination of the reaction rates inside the fixed bed reactor.

#### 4.3 Influence of the extraction temperature

As shown in chapter 4.2, the temperature has a great influence on water concentration in the organic phase. Therefore, it was expected, that the temperature in the extraction step (figure 2, C) strongly affects the subsequent esterification reaction (figure 2, D). Figure 5 shows the relative lipase productivity in dependency of the temperature in the extractive centrifuge. All experiments shown in figure 5 were conducted with the same set of parameters, including the starting concentration of each component (45 and 60 mM of alcohol and acid respectively), the volume flow rates (100 ml/min, figure 2, P1; 300 ml/min, figure 2, P3; 350 ml/min, figure 2, P4), and 50 % centrifuge power. The esterification reaction was carried out at 60 °C, according to the provider's information.

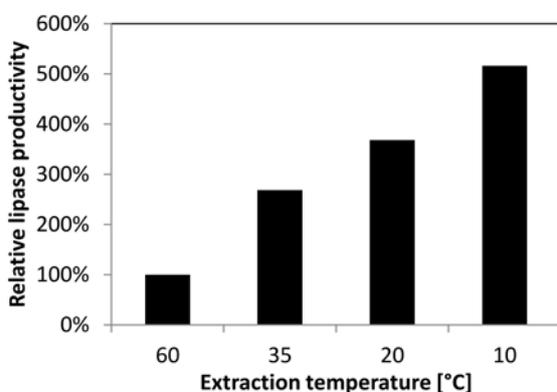


Figure 5: Influence of the temperature in the extractive centrifuge (figure 2, C) on lipase productivity.

Figure 5 shows that, by decreasing the temperature in the extractive centrifuge from 60 °C to 10 °C, the lipase productivity in the fixed bed reactor (figure 2, D) can be increased by over 500 %, starting from a rate of 4  $\mu\text{M}/\text{min}$  as 100 % at 60 °C extraction temperature for a production time of 4 hours. This is possible due to the temperature dependency of the water saturation concentration, as described in chapter 4.2. A lower water saturation concentration in the organic phase leads to less by-product of the esterification reaction (water) entering the fixed bed reactor. These results show that further drying of the organic phase is desirable.

## 5. Conclusion and Outlook

For the first time, the successful production of cinnamyl cinnamate from cinnamyl aldehyde with a multi-enzymatic cascade reaction sequence across a phase interface with integrated co factor regeneration and *in situ* intermediate separation was presented, and implemented on a pilot-scale reactor plant. The crucial process step of intermediate extraction was investigated in detail, showing a strong influence of the

temperature in the extractive centrifuge on lipase productivity in the final reaction step: decreasing the extraction temperature from 60 °C to 10 °C leads to an over 500 % increase in lipase productivity. Further process improvement aims to decrease water concentration in the organic phase to enhance lipase productivity. Hereby, promising results were achieved using an additional integrated adsorption step. For a continuous production process, *in situ* product separation has to be investigated. Due to the high boiling points of the components and the rather low final product concentrations, thermal separation methods seem unfit. However, promising results were achieved using a simulated moving bed separation sequence, which takes advantage of the differences in molecular size between the final product and the intermediates (size exclusion chromatography).

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