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Enhancing the productivity of the bi-enzymatic convergent cascade for ε-caprolactone synthesis through design of experiments and a biphasic system

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ABSTRACT

A two-step Design of Experiments (DoE) strategy followed by a two-liquid-phase system (2LPS) was applied to enhance the ε-caprolactone yield in the cyclohexanone monooxygenase (CHMO)-alcohol dehydrogenase (ADH) convergent cascade system. The key reaction parameters were identified and optimized for the determination of an optimal operational window for the aqueous media. In the 2LPS system, high partitioning of the lactone product was observed in 2-MeTHF and in toluene; however, these solvents led to drastically reduced enzymatic activity. Dodecane was chosen as the non-miscible organic phase owing to the enzymes' high residual activity, despite the low partitioning of the lactone. Cyclohexanone concentrations up to 75 mM were applied in the aqueous media. The turnover numbers for the nicotinamide cofactor and for the ADH reached up to 980 and 392,000, respectively whereas a turnover number value of 5600 was achieved for the CHMO. By employing a 2LPS, whereby 91 mM of cyclohexanone was applied in the second phase, turnover numbers were slightly increased.

1. Introduction

Nowadays, the use of biocatalysts has been widely recognized as a resource efficient, energy saving, safe, economical and an environmentally benign way for the synthesis of high value-added products in chemical transformations.[1] A technically relevant example where the above-given advantages play a major role can be found in the synthesis of bulk lactones.[2] Bulk lactones have a broad range of industrial applications and are of huge commercial importance. In particular, ε-caprolactone (ECL), with a global annual production of multi-kilotons, is a key precursor in the production of biodegradable, thermoplastic, and elastomeric polymers.[3]

A fully enzymatic approach for the synthesis of lactones in a linear cascade fashion (i.e., single substrate—one intermediate—single product) was first reported in the early 90s.[4] Nowadays, this cascade approach is used for ECL synthesis, whereby oxidation of cyclohexanol (CHL) by an alcohol dehydrogenase (ADH) (or a designed polyol dehydrogenase) is coupled with the further oxidation of cyclohexanone (CHO) to ECL by a cyclohexanone monooxygenase (CHMO).[5] Recently, we reported a new, fully enzymatic system for the synthesis of ECL running in a convergent cascade fashion (i.e., bi-substrate—no intermediate—single product).[6] The bi-enzymatic bi-substrate convergent system consists of a CHMO for oxidation of CHO and an ADH for two-step oxidation of a so-called 'double-smart cosubstrate' 1,6-hexanediol (1,6-HD) for the in situ regeneration of the nicotinamide cofactor. The enzymes employed in this reaction cascade were wild-type CHMO from *Acinetobacter* sp. NCIMB 9871[7] and ADH from *Thermoanaerobacter ethanolicus* (TeSADH)[8] (Scheme 1), as their coupling showed the highest product yield in our proof-of-concept study. Our previous study revealed the formation of CHL from the ADH-catalyzed reduction of CHO, which however gradually converted to ECL during the course of reactions.

The characterization of the bi-enzymatic cascade system described above and the identification of its optimal operational window necessitate a detailed analysis of interactions between the various reaction parameters. To achieve this, design of experiments (DoE),[9] an organized and systematic approach for studying multi-parameter systems, is of a substantial usefulness. Herein, a suitable DoE model to obtain a maximum amount of information from a minimum number of experiments is used to map the reaction

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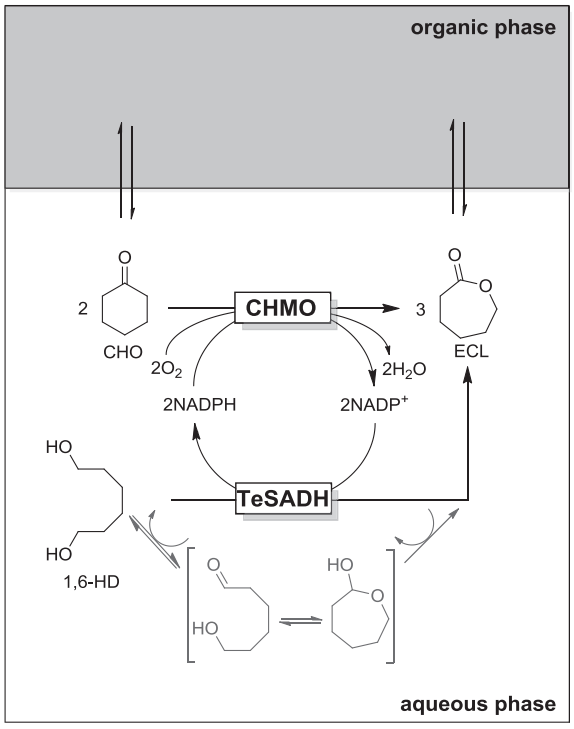

Scheme 1. The two-liquid-phase system (2LPS) applied for the removal of ε-caprolactone (ECL) synthesized in a CHMO-TeSADH convergent cascade coupled for the oxidation of cyclohexanone (CHO) and for the two-step oxidation of 1,6-hexanediol (1,6-HD) for simultaneous regeneration of NADPH.

system in terms of different responses, with respect to different parameters and their interactions. The DoE data are used for statistical modeling and analysis of the reaction to understand the parameters' impacts on target responses e.g., substrate conversion, by-product formation, product yield, turnover number (TON) for the cofactor and for the enzymes etc. The bi-enzymatic convergent system described herein is influenced by several parameters such as (1) temperature, (2) pH, (3) available O_2 amount, (4) substrates concentrations, (5) cofactor concentration and (6) the enzymes amounts. Each of these parameters can directly and interactively influence the reaction profile and the product (analytical) yield.

For optimization purposes, the so-called two-liquid-phase system (2LPS) is an established method to overcome a range of challenges encountered especially in oxidoreductase-catalyzed reactions.[10] Substrate toxicity and product inhibition issues can be overcome through the use of an organic phase as a substrate reservoir and a product sink. By doing so, high concentrations of hydrophobic substrates can be applied to achieve high space-time yields. Furthermore, the reaction can be shifted to the product side by selectively removing the (co)product. In order to alleviate these limitations mentioned above other strategies, for example, fed-batch synthesis[11] and the use of resins for substrate feeding and product removal (SFPR)[12] have been documented in the literature. In addition to those, in order to overcome the production inhibition due to ECL, the use of CAL-A for in situ oligomerization has been shown to be useful.[5d]

Based on our previous experience with the recently developed CHMO-ADH convergent cascade, we became interested in enhancing its productivity. Therefore, the aim of the present study was to first identify an optimal operational window for the CHMO-ADH convergent cascade through a two-step DoE approach. Thereafter, in order to further enhance the productivity, we established a two-liquid-phase (2LPS) system (Scheme 1) for the selective extraction of the lactone product.

2. Results and discussion

2.1. DoE for screening of reaction parameters

A two-step DoE approach was used to screen and optimize the CHMO-ADH convergent cascade with several key factors involved. For screening purposes, eight reaction parameters (i.e., temperature, O_2, pH, c(CHO), c(CHMO), c(TeSADH), c($NADP^+$), and time) were evaluated for their impacts on four target responses chosen (i.e., conversion of CHO, c(ECL), c(CHL), TON for $NADP^+$). The concentration of 1,6-HD was always kept at a half molar equivalent of CHO, hence it was not further included in the reaction parameters. It is worth mentioning here that the kinetic analysis of TeSADH for CHO reduction and for 1,6-HD oxidation revealed that the ADH exhibits 19-fold higher V_{max} for the reduction compared to the oxidation, while similar K_M values for CHO and 1,6-HD were detected.[6] With respect to the cofactor recycling, total turnover numbers (TTNs) at least 100,000 are aimed for the technical scale applications, whereas TTN values of 1000–10,000 are sufficient for the laboratory scale.[13]

A total of 19 experiments (16 individual experiments and three replicate center points, Table SI1 and SI2) based on a 'fractional factorial design' were evaluated, instead of all 256 potential experiments. A partial least squares (PLS) model was well fitted (R^2 in the range of 0.9–1.0) to the data for the aforementioned four target response values shown in Table SI3 by the DoE software (MODDE 8.0, Umetrics, Umeå, Sweden). The summary of the PLS model gave high predictabilities (Q^2>0.8) for c(ECL) and TON ($NADP^+$), whereas moderate predictabilities (Q^2≥0.5) were obtained for the conversion of CHO and for the formation of CHL (Table SI4). Moreover, the summary of the PLS model showed very high reproducibility values (>0.99) (Table SI4).

According to the variable importance plot (VIP, Fig. SI1) representing the normalized values corresponded to the different model parameters (the VIP of 1.0 represents an average importance), four of the parameters, namely: pH, temperature, O_2, and time, were found to have lower impacts on the CHMO-ADH convergent cascade, compared to the other parameters. Through understanding some of the influences of these four parameters (i.e., pH, temperature, O_2 and time) on the responses (Fig. SI2), the values for these factors were fixed in such a way as to improve the reactions the most. Therefore, a slightly alkaline pH value of 8.0, the highest O_2 amount by having the maximal headspace ratio $\left(\frac{Headspace\ volume(mL)}{Volume\ of\ the\ reaction(mL)}\right)$, and room temperature (~20 °C), were chosen for the optimization step, in order to obtain high product (analytical) yields within 24–48 h.

2.2. DoE for optimization of the reaction parameters identified in the screening step

Based on the results of the screening study, we focused our attention on four reaction parameters, i.e., c(CHO), c(CHMO), c(TeSADH), and c($NADP^+$) in the optimization step. Based on a 'central composite face-centered design', in total 27 reactions (24 individual experiments and three replicate center points) were run (Table SI5). Herein, we considered it worthwhile to change the target responses for optimization purposes. The responses for the screening study were (1) conversion of CHO, (2) c(ECL), (3) c(CHL), and (4) TON ($NADP^+$), whereas for the optimization step we chose the responses as (1) analytical ECL yield, (2) TON ($NADP^+$), (3) TON (CHMO), and (4) TON (ADH). These target responses were chosen mainly because the monitoring of ECL formation represents the performance of the convergent cascade better compared to the conversion of CHO, as the consumed amount of CHO cannot be