# A cascade reaction for the synthesis of D-fagomine precursor revisited: kinetic insight and understanding of the system

Martina Sudar[1*], Morana Česnik[1], Pere Clapés[2], Martina Pohl[3], Đurđa Vasić-Rački[1], Zvjezdana Findrik Blažević[1]

[1] University of Zagreb, Faculty of Chemical Engineering and Technology, Savska c. 16, HR-10000 Zagreb, Croatia

[2] Institute of Advanced Chemistry of Catalonia Biotransformation and Bioactive Molecules Group, IQAC-CSIC, Jordi Girona 18-26, 08034 Barcelona, Spain

[3] Forschungszentrum Jülich GmbH, IBG-1: Biotechnology, Wilhelm-Johnen-Straße, 52425 Jülich, Germany

[*]corresponding author, e-mail: msudar@fkit.hr, tel: +385 1 4597 101, fax: +385 1 4597 133

**Abstract**

The synthesis of aldol adduct (3*S*,4*R*)-6-[(benzyloxycarbonyl)amino]-5,6-dideoxyhex-2-ulose, a precursor of an interesting dietary supplement iminosugar D-fagomine, was studied in a cascade reaction with three enzymes starting from Cbz-*N*-3-aminopropanol. This system was studied previously using a statistical optimization method which enabled a 79% yield of the aldol adduct with a 10% yield of the undesired amino acid by-product. Here, a kinetic model of the cascade, including enzyme operational stability decay rate and the undesired overoxidation of the intermediate product, was developed. The validated model was instrumental in the optimization of the cascade reaction in the batch reactor. Simulations were carried out to determine the variables with the most significant impact on substrate conversion and product yield. As a result, process conditions were found that provided the aldol adduct in 92% yield with only 0.7% yield of the amino acid in one-pot one-step reaction. Additionally, compared to previous work, this improved process outcome was achieved at lower concentrations of two enzymes used in the reaction. With this study the advantages are demonstrated of a modelling approach in developing complex biocatalytical processes. Mathematical models enable better understanding of the interactions of variables in the investigated system, reduce cost, experimental efforts in the lab and time necessary to obtain results since the simulations are carried out *in silico*.

**Keywords:** cascade reaction, D-fagomine precursor, mathematical model, reaction optimization

**Symbols and abbreviations**

*c:* molar concentration, mM; *d:* diameter of the cuvette ($d$ = 1 cm); $K_i$: inhibition constant, mmol dm$^{-3}$; $k_d$: operational stability decay rate constant, h$^{-1}$; $K_m$: Michaelis constant, mM;

$r_1$: reaction rate of amino alcohol oxidation, mM $min^{-1}$; $r_2$: reaction rate of amino aldehyde reduction, mM $min^{-1}$; $r_3$: reaction rate of coenzyme regeneration, mM $min^{-1}$; $r_4$: reaction rate of aldol addition, mM $min^{-1}$; $r_5$: reaction rate of retro-aldol reaction, mM $min^{-1}$; $r_6$: reaction rate of amino aldehyde oxidation, mM $min^{-1}$; *S.A.*: specific activity, U $mg^{-1}$; *t*: reaction time, min; *V.A.*: volume activity, U $mL^{-1}$; $V_{enz}$: enzyme volume, mL; $V_m$: maximum reaction rate, U $mg^{-1}$; $V_r$: reactor volume, mL; *X*: substrate conversion, %; *Y*: product yield, %; $\varepsilon_{340}$: extinction coefficient for NADH ($\varepsilon_{340}$ = 6.22 $cm^2$ $\mu mol^{-1}$); $\gamma$: mass concentration, mg $mL^{-1}$;
aldol adduct: (3*S*,4*R*)-6-[(benzyloxycarbonyl)amino]-5,6-dideoxyhex-2-ulose; amino acid: Cbz-*N*-3-aminopropanoate; amino alcohol: Cbz-*N*-3-aminopropanol; amino aldehyde: Cbz-*N*-3-aminopropanal; DHA; dihydroxyacetone: FSA: D-fructose-6-phosphate aldolase;
TEA HCl: triethanolamine hydrochloride; TFA: trifluoroacetic acid

## Introduction

Increasing consideration is being shown to multi-enzyme cascades – processes that resemble those found in living organisms. Cascade reactions, involving several catalysts working simultaneously or stepwise, hold a great potential for the development of industrial processes [1-3]. These systems generate less waste [1], are more cost-effective since all reaction steps are carried out without the separation of intermediate precursors, and improve the atom economy of the transformation [4]. In addition, problems with unstable intermediates can be resolved in a cascade reaction since they are instantly consumed in a subsequent reaction [5]. Cascade reactions are also useful in shifting the reaction equilibrium towards product formation [6]. In the present case, the oxidation of the alcohol to aldehyde is thermodynamically unfavourable and, in addition, the aldehyde is an unstable compound that may undergo side reactions due to its strong electrophilic character. Hence, the synthetic process can be facilitated by coupling the oxidation with the ensuing aldol addition reaction in

which the aldehyde formed is instantly consumed, thus favouring the oxidation step and minimizing the potential side reactions of the aldehyde. Furthermore, for an oxidation catalysed by an oxidoreductase, coenzyme regeneration is of crucial importance because (i) it avoids adding the coenzyme in stoichiometric amounts, which reduces costs, (ii) it shifts the equilibrium of the reaction in the desired direction, and (iii) it avoids high concentrations of the coenzyme in the reaction medium, which can have a negative impact on the activity of the oxidoreductase [7-10].

Even though cascade reactions are considered cost-effective and can enable increased productivity, they must be optimized to fully exploit their advantages. Optimization cannot be achieved by using a simple method of trial and error because typical metrics for a biocatalytic process include product yield, volumetric productivity, specific productivity, product concentration, enzyme yield, etc. [11, 12]. To facilitate the optimization of cascade reactions with complex interdependencies of variables, reaction engineering methods, i.e. mathematical modelling, can be used. A mathematical model of a complex system must be developed by employing a systematic approach. This kind of an approach offers many advantages in studying complex reaction systems [13-15]. As the number of compounds and enzymes in the system increases, the possible interdependencies between variables increase. Such complexity is difficult to understand simply from batch reactor experiments. Thus, with equations that describe these interdependencies, *in silico* evaluation of different process scenarios, i.e. reaction conditions and process outcome, can be used to minimize the cost and time in the wet lab.

A mathematical model of a process consists of kinetic equations describing the rate of each reaction in the process, combined with mass balance equations, in a specific reactor [14, 16].

In addition, the operational stability decay rate of an enzyme can be incorporated into the model [14]. This is a significant property for an industrial biocatalyst, since the loss of enzyme activity during a process can cause a decrease in substrate conversion and product yield. Before the model can be instrumental in process optimization [14, 17, 18], in finding the best reactor mode [19, 20], and in scaling-up [21], it must be validated by comparing the experimental results with simulations under equal conditions. The number of papers that cover modelling of multi-enzymatic cascades has increased constantly over the last decade [22-26], which clearly demonstrates that the value of this methodology for process optimization is widely recognized. Modelling techniques should be applied more often in process development to increase the environmental and cost efficiency of enzyme cascades.

Here, the optimization of a cascade reaction (**Scheme 1**) towards the aldol adduct (3*S*,4*R*)-6-[(benzyloxycarbonyl)amino]-5,6-dideoxyhex-2-ulose (aldol adduct) is described. The cascade comprises (i) oxidation of benzyloxycarbonyl (Cbz)-*N*-3-aminopropanol (amino alcohol) to Cbz-*N*-3-aminopropanal (amino aldehyde) catalyzed by alcohol dehydrogenase (ADH), (ii) regeneration of the coenzyme $NAD^+$ catalyzed by NADH oxidase (NOX), (iii) aldol addition of dihydroxyacetone (DHA) to amino aldehyde catalyzed by D-fructose-6-phosphate aldolase variant A129S ($FSA^{A129S}$) and (iv) oxidation of Cbz-*N*-3-aminopropanal to Cbz-*N*-3-aminopropanoic acid catalyzed by ADH. The product, aldol adduct, is a precursor of the first iminosugar found in plants, D-fagomine [27]. D-Fagomine can lower the blood glucose concentration after food and inhibit the adhesion of pathogenic bacteria to epithelial mucosa, which in turn decreases the risk of insulin resistance (i.e. type 2 diabetes), overweight and obesity, important public health problems in Western society [27-30].

This paper is continuation of a previous study in which statistical optimization of reaction conditions for the abovementioned cascade (**Scheme 1**) was used to improve product yield [5]. Statistical methods can be of great value for process optimization but can also be time

consuming since a large number of experiments are necessary to get results. On the other hand, a validated mathematical model can be used without any additional experimentation to perform simulations of various reaction scenarios to optimize a process. Here, a kinetic mathematical model is developed and validated that allows a complete insight and a better understanding of the reaction system and has been instrumental in improving the efficiency of the process.

## Materials and Methods

*Chemicals*

Acetonitrile, amino alcohol, amino aldehyde, dihydroxyacetone (DHA), triethanolamine hydrochloride (TEA HCl) trifluoroacetic acid (TFA) and bovine serum albumin were obtained from Sigma Aldrich (Munich, Germany). Cbz-*N*-3-aminopropanoate (amino acid), $NAD^+$ and NADH were acquired from Acros Organics (Morris Plains, New Jersey, USA). Aldol adduct and $FSA^{A129S}$ from *E. coli* were produced and characterized at Institute of Advanced Chemistry of Catalonia (Barcelona, Spain). ADH from horse liver was from Evoxx technologies GmbH (Monheim Am Rhein, Germany). NOX from *Lactococcus lactis* was isolated and purified at the Faculty of Chemical Engineering and Technology (Zagreb, Croatia) [7, 31].

*HPLC analysis*

High performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) was used to determine the concentrations of reactants and products using a Lichrospher® column (250x4 mm, 5 μm) and a gradient method (30 to 100% B for 30 minutes, flow rate 1.2 mL $min^{-1}$) at 30 °C and 215 nm [32]. Mobile phase A was water with 0.1% TFA, mobile phase B was 80% acetonitrile, 20% water and 0.095% TFA. The retention times were aldol adduct: 4.6 min,

amino alcohol: 6.8 min, amino acid: 7.4 min, amino aldehyde: 7.7 min. Samples were diluted with methanol to stop the reaction and centrifuged for 2 min at 18659x*g*. The enzymes precipitated and the upper layer was used for the HPLC analysis.

*Protein concentration*

The Bradford method [33] was used to determine protein concentrations in enzyme samples, based on bovine serum albumin as a standard.

*Enzyme kinetics*

The kinetics of each reaction in this cascade system (see **Scheme 1**), i.e. amino alcohol oxidation catalysed by ADH ($r_1$, $r_2$), amino aldehyde oxidation catalysed by ADH ($r_6$), $NAD^+$ regeneration catalysed by NOX ($r_3$), and aldol addition catalysed by $FSA^{A129S}$ ($r_4$, $r_5$), was investigated separately in a batch reactor using the initial reaction rate method, regarded as the most robust method in terms of wide model applicability [16]. The influence of each compound in the reaction mixture on the activity of all enzymes was investigated by varying the concentration of one compound at a time and maintaining the concentration of other compounds constant, and where possible, in saturation. All measurements were made in duplicate.

The kinetics of all reactions was determined in 50 mM TEA HCl buffer, pH 8.0, at 25°C and with 10% v/v of acetonitrile in the reaction mixture. The enzyme concentration was maintained constant in all kinetic experiments at 0.1 mg $mL^{-1}$ for ADH in amino alcohol oxidation and amino aldehyde reduction, 2 mg $mL^{-1}$ for ADH in amino aldehyde oxidation, and 0.84 mg $mL^{-1}$ for $FSA^{A129S}$. For all other experimental conditions see figures and figure legends showing the results of measurements (Suppl. Figures 1-5).

*ADH kinetics*

As ADH catalyses the oxidoreduction ($r_1$, $r_2$, **Scheme 1**), the kinetics for the oxidation of amino alcohol ($r_1$) and the reduction of amino aldehyde ($r_2$) were investigated. The influence

of concentration of amino alcohol, $NAD^+$, amino aldehyde and NADH on the activity of ADH in the oxidation reaction was determined. In the reverse reaction, i.e. reduction, the influence of amino aldehyde, NADH, amino alcohol and $NAD^+$ concentration on the activity of ADH was investigated.

In addition, the kinetics of amino aldehyde oxidation catalysed by ADH ($r_6$, **Scheme 1**), in which amino acid is formed, was investigated. For this reaction, the influence of the concentration of amino aldehyde and $NAD^+$ on the activity of ADH was investigated.

The kinetics of the ADH-catalysed reactions was determined spectrophotometrically by following the absorbance of NADH at 340 nm. Volume and specific activities of the enzyme were calculated from the changes of absorbance over time [34]. One Unit (U) of ADH activity was defined as the amount of ADH necessary to oxidize 1 μmol of amino alcohol per min at 25 °C in 50 mM TEA HCl buffer, pH 8.0, in the presence of 10% v/v acetonitrile.

*NOX kinetics*

The kinetics of NADH oxidation catalysed by NOX ($r_3$, **Scheme 1**) was investigated in detail previously [7]. One U of NOX activity was defined as the amount of enzyme necessary to oxidize 1 μmol of NADH per min at 25 °C in 50 mM TEA HCl buffer, pH 8.0, in the presence of 10% v/v acetonitrile.

*$FSA^{A129S}$ kinetics*

The kinetics of $FSA^{A129S}$ in the aldol addition of DHA to amino aldehyde and in the retro-aldol reaction was investigated in previously [35], but considering the different pH value used in the present study, kinetic parameters were re-evaluated ($r_4$, $r_5$, **Scheme 1**). The dependence of the $FSA^{A129S}$ activity on concentration of amino aldehyde, DHA and the aldol adduct in the aldol reaction was determined ($r_4$, **Scheme 1**). Furthermore, the influence of the concentration of the aldol adduct, amino aldehyde and DHA in the retro-aldol reaction on the activity of $FSA^{A129S}$ was also investigated ($r_5$, **Scheme 1**). The kinetics of $FSA^{A129S}$ in batch experiments

was determined by monitoring the concentration of the aldol adduct by HPLC during the reaction time when substrate conversion was less than 10%. Kinetic experiments were carried out in a 0.5 mL batch reactor placed on a shaker at 1000 rpm. The change in aldol adduct concentration over time was used to calculate the volume and specific activity of the enzyme [35]. One U of aldolase activity was defined as the amount of $FSA^{A129S}$ necessary to produce one μmol of aldol adduct per min at 25 °C in 50 mM TEA HCl buffer, pH 8.0, in the presence of 10% v/v acetonitrile.

*Enzyme operational stability*

Operational stability decay rate constants for NOX and $FSA^{A129S}$ were estimated previously [5]. The constant for ADH used in this work was estimated experimentally from the enzyme activity vs. time data (data not shown) gathered during one of the cascade reactions.

*Batch reactor experiments*

Cascade reactions were carried out in a 1 mL batch reactor on a shaker at 1000 rpm and at 25°C. Different initial concentrations of substrates and enzymes were used to test the applicability of the mathematical model. All experiments were carried out in 50 mM TEA HCl buffer, pH 8.0, in the presence of 10% v/v acetonitrile. For further experimental conditions, see figure legends. The aldol adduct and amino acid yield were calculated as the ratio of aldol adduct and amino acid, respectively, to amino alcohol initial concentration.

*Mathematical model and data handling*

The mathematical model of the cascade reaction (**Scheme 1**) is presented in **Table 1**. The kinetic model was developed based on the kinetic measurements, i.e. estimated kinetic parameters and the reaction scheme (**Scheme 1**).

The reaction rate of amino alcohol oxidation, $r_1$, was described by a double substrate Michaelis-Menten (MM) kinetics with competitive product (amino aldehyde and NADH) inhibition (Equation 1, **Table 1**). The reaction rate of amino aldehyde reduction, $r_2$, was also

described by double substrate MM equation with competitive product (amino alcohol and $NAD^+$) inhibition (Equation 2, **Table 1**). The MM equation with uncompetitive product ($NAD^+$) inhibition [10] (Equation 3, **Table 1**) described the reaction rate of coenzyme regeneration, $r_3$. The double substrate MM kinetics (Equation 4, **Table 1**) described the reaction rate of the aldol addition, $r_4$. The MM equation with competitive amino aldehyde inhibition (Equation 5, Table 1) described the reaction rate of the retro-aldol reaction, $r_5$. The reaction rate of the side-reaction, i.e. amino aldehyde oxidation, $r_6$, was described by double substrate MM equation (Equation 6, **Table 1**). The mass balance equations for amino alcohol, amino aldehyde, $NAD^+$, NADH, DHA, aldol adduct and amino acid in the batch reactor are described by Equations 7-13, respectively (**Table 1**). The operational stability decay rate was described by a first order kinetics (Equation 14, **Table 1**). This equation was used for all three enzymes in the system.

Kinetic parameters ($V_m$, $K_m$, $K_i$) were estimated from the specific activity vs concentration data sets by non-linear regression methods (i.e. simplex and least squares fit) implemented into the SCIENTIST 2.0 software [36]. Experimental kinetic data were obtained as described above (*Enzyme kinetics*). Enzyme operational stability decay rate constants ($k_d$) were estimated from enzyme activity vs time data obtained by monitoring enzyme activity during the reaction in the batch reactor [5]. The statistical analysis for the estimation of kinetic parameters was performed using SCIENTIST 2.0. The result is the estimated parameter with its standard deviation (sd) value with 95% probability. The same software was used for reaction simulations.

## Results and Discussion

The kinetic model of the cascade reaction towards the aldol adduct (**Scheme 1**) was developed here. All experiments were performed in 50 mM TEA HCl buffer, pH 8.0, which

238 represents a compromise for all enzymes, i.e. ADH, NOX and $FSA^{A129S}$, as established
239 previously where experimental optimization using a statistical model was applied [5].
240 *Enzyme kinetics*
241 The influence of reaction compounds on the specific activity of ADH, NOX and $FSA^{A129S}$ is
242 presented in Suppl. Figures 1-5. Kinetic parameters estimated from these data are shown in
243 **Table 2**.
244 The influence of amino alcohol and $NAD^+$ concentration on ADH activity ($r_1$) can be
245 described by the MM kinetics (Suppl. Figure 1A, B). The apparent kinetic constants $V_{m1}$,
246 $K_{m1}^{\text{amino alcohol}}$ and $K_{m1}^{\text{NAD+}}$ were estimated (**Table 2**) from this data. Both amino aldehyde and
247 NADH reaction products inhibited the enzyme in the forward reaction (Suppl. Figure 1C, D).
248 The inhibitions are significant, as shown by the values of the $K_{i1}^{\text{amino aldehyde}}$ and $K_{i1}^{\text{NADH}}$
249 inhibition constants (**Table 2**). As efficient coenzyme regeneration is anticipated in the
250 reaction (**Scheme 1**), inhibition by NADH would not be a problem since regeneration of
251 $NAD^+$ will ensure minimal NADH concentration, i.e. near zero. At the same time, the
252 inhibitory intermediate, amino aldehyde, is concomitantly consumed in the aldol addition,
253 keeping its concentration low. In this way, the effect of amino aldehyde inhibition will be
254 diminished if the process conditions are properly set.
255
256 In the reverse reaction, amino aldehyde reduction ($r_2$), the influence of the concentration of
257 substrates on ADH specific activity (Suppl. Figure 2A, B) was described by MM kinetics.
258 The corresponding apparent kinetic parameters were estimated, i.e. $V_{m2}$, $K_{m2}^{\text{amino aldehyde}}$ and
259 $K_{m2}^{\text{NADH}}$ (**Table 2**). The products, $NAD^+$ and amino alcohol, inhibited ADH and from the
260 values of the estimated kinetic constants ($K_{i2}^{\text{amino alcohol}}$ and $K_{i2}^{\text{NAD+}}$) (**Table 2**) it was
261 concluded that this inhibition is less severe than in the case of the forward reaction. In
262 addition, these inhibitions could bring benefits to the process outcome since they decrease the

reaction rate of the undesired reduction. Once again, an efficient coenzyme regeneration will have an important role in the system, by keeping the concentration of $NAD^+$ practically at the initial value during the reaction, thus inhibiting the reduction reaction. The maximum reaction rate of the amino aldehyde reduction ($V_{m2}$) (**Table 2**) was found to be 2.3-fold higher than that of the amino alcohol oxidation ($V_{m1}$) (**Table 2**). Therefore, it is expected that the reaction equilibrium will be on the side of reduction, as usually is the case in oxidations catalysed by oxidoreductases. The kinetic parameters of the reduction reaction that additionally confirmed this statement are the apparent Michaelis constant values for amino aldehyde ($K_{m2}^{\text{amino aldehyde}}$) and NADH ($K_{m2}^{\text{NADH}}$), which are much lower than those for amino alcohol ($K_{m1}^{\text{amino alcohol}}$) and $NAD^+$ ($K_{m1}^{\text{NAD+}}$) (**Table 2**) in the oxidation reaction. These kinetic data and parameters show that it is necessary to couple the oxidation reaction with coenzyme regeneration and aldol addition to achieve high substrate conversion and product yield.

Even though kinetic parameters for NOX-catalysed NADH oxidation were previously reported [7], they were re-evaluated due to modifications in the isolation procedure; affinity chromatography was carried out as an additional purification step as suggested by [31]. Acetonitrile (10% v/v) was also added to the reaction mixture in these measurements to improve amino alcohol solubility. However, only the value of maximum reaction rate ($V_{m3}$) increased, as expected due to the additional purification step of NOX performed here. The reaction is inhibited by $NAD^+$, which should not present a serious problem if the initial concentration of the coenzyme used in the reaction is kept below 1 mM. NOX showed high affinity towards $O_2$ ($K_m^{O_2}$ = 4.467 μM), which implies maximum enzyme activity even at $O_2$ concentrations as low as 0.02 mM. Therefore, the $O_2$ mass balance and its concentration were not included in the kinetic model of coenzyme regeneration to keep the model as simple as possible (**Table 1**, Equation 3).

As in the case of NOX, the kinetic model for the aldol addition catalysed by $FSA^{A129S}$ was reported earlier [35]. The same equations were used here, but due to the change in pH, the kinetic parameters were re-evaluated using new experimental data. The maximum reaction rate of the retro-aldol reaction ($V_{m5}$) was 3.1-fold higher than that of aldol addition ($V_{m4}$) (**Table 2**). However, this should not pose a problem in the reaction since the enzyme showed higher affinity toward the substrates in the aldol addition ($K_{m4}^{DHA}$ and $K_{m4}^{amino\ aldehyde}$) than toward that in the retro-aldol addition ($K_{m5}^{aldol\ adduct}$) (**Table 2**). Furthermore, the retro-aldol reaction was strongly inhibited by the amino aldehyde, which is evident from the low value of $K_{i5}^{amino\ aldehyde}$ (**Table 2**). To be specific, simulations showed that concentrations above 0.5 mM of amino aldehyde will considerably decrease the reaction rate of the retro-aldol reaction, implying that the reaction equilibrium should be shifted towards the formation of the aldol adduct.

The corresponding amino acid was also formed in this cascade reaction (**Scheme 1**), arising from the oxidation of amino aldehyde catalysed by ADH, as suggested by the literature [37, 38] and a previous statistical model [5]. The kinetics of this reaction was evaluated, and indicated that the maximum reaction rate for the oxidation ($V_{m6}$) was 100-fold lower than that of the aldol addition ($V_{m5}$) (**Table 2**, Suppl. Figure 5). Furthermore, the ADH showed low affinity towards both the amino aldehyde and $NAD^+$ in the oxidation of the amino aldehyde ($K_{m6}^{amino\ aldehyde}$ = 196.313 mM and $K_{m6}^{NAD+}$ = 8.194 mM) indicating that high concentrations of both substrates are required to achieve maximum enzyme activity (Suppl. Figure 5A). These findings imply that the preferred reaction is aldol addition over aldehyde oxidation. Nevertheless, it is expected that the reaction conditions need to be optimized to minimize the impact of this reaction on purity of the final product.

313 *Enzyme operational stability*

314 Operational stability decay rate constants for all three enzymes (**Table 3**) were included in the
315 mathematical model of the cascade reaction. The $k_d$ for ADH from Evoxx technologies GmbH
316 used in this work was very similar to that for ADH from Sigma Aldrich used previously
317 (**Table 3**) [5]. The operational stability decay rates for all three enzymes were described by
318 first order kinetics (**Table 1**, Equation 14).

319 *Batch reactor experiments*

320 To validate the mathematical model, batch reactor experiments of the cascade reaction were
321 carried out at different initial concentration of substrates, amino alcohol, $NAD^+$, DHA and
322 enzymes. The model (**Tables 1-3**) described the experimental data well. Two experiments
323 were performed at similar concentrations of substrates and enzymes, but different $NAD^+$
324 concentrations (**Figure 1**A, B). Even though the amino acid was not formed when the
325 concentration of $NAD^+$ was low (**Figure 1**B), this affected aldol adduct production negatively
326 as well, i.e. 7.4 mM of aldol adduct was obtained with 1 mM of $NAD^+$ (**Figure 1**A) and only
327 3.9 mM at with 0.1 mM of $NAD^+$ (**Figure 1**B). The reason for the poor outcome of the
328 reaction at 0.1 mM of $NAD^+$ could be that the ADH has a relatively high Michaelis constant
329 for $NAD^+$, $K_{m1}^{NAD+}$ = 0.400 mM, in the oxidation of the amino alcohol. Thus, with 0.1 mM of
330 $NAD^+$, the rate of oxidation is low and far from its maximum. In previous work [5] using a
331 statistical method, the influence of the $NAD^+$ concentration on the aldol adduct formation in
332 the cascade reaction was investigated in the range 0.1-1 mM. The results showed that a high
333 coenzyme concentration, i.e. 1 mM, should be used to obtain maximum aldol adduct
334 concentration. These results are the reason for choosing an $NAD^+$ concentration of 1 mM in
335 the cascade reaction. To further corroborate the claims that a higher $NAD^+$ concentration (1
336 mM) should be used in the cascade reaction to ensure high reaction rates, additional

simulations are presented of the cascade reaction in a batch reactor (Suppl. Figure 6) and additional discussion in the Supplementary information.

To improve total turnover number of $NAD^+$, a better oxidoreductase should be found with low $K_m$ values for $NAD^+$ and amino alcohol. Likewise, a NOX to oxidase the NADH efficiently, with an improved operational stability, should also be sought. The model for the cascade reaction is already developed, so that testing different enzymes can be readily performed by simply introducing the corresponding estimated kinetic parameters and running simulations *in silico*. This will speed up the evaluation of new enzymes in this cascade and the process of selecting the most effective.

**Figures 1**A, C show that 74% and 54% yields of aldol adduct can be obtained at initial amino alcohol concentrations of 10 mM (**Figure 1**A) and 50 mM (**Figure 1**C), respectively. Thus, the product yield decreased with an increase of amino alcohol concentration. **Figure 1**D shows an experiment with 100 mM amino alcohol in which only ~8% of aldol adduct yield was achieved. This is mostly due to the low concentration of NOX used in this reaction and will be discussed further below.

To investigate the influence of NOX concentration on reaction outcome, experiments under similar conditions but with different concentrations of NOX were performed (**Figure 2**). They showed that the concentration of NOX is of utmost significance, indicating the importance of efficient coenzyme regeneration. By increasing NOX concentration, it was possible to achieve high aldol adduct concentration, up to 8 mM (approximately 80% yield), as demonstrated experimentally (**Figure 2**, black circles). The simulations showed that it should be possible to achieve 92% aldol adduct yield after 12 hours with a NOX concentration of 5 mg $mL^{-1}$ (**Figure 2**, dashed line). An additional increase of NOX would not further improve the aldol adduct concentration (**Figure 2**, dotted line).

*The influence of variables on process performance for the oxidation of amino alcohol*

Once validated, the model can be used to investigate the influence of all reaction components on process outcome for a better understanding and optimization of the reaction. To confirm the hypothesis that the cascade reaction is a good strategy to obtain high product yield, various simulation experiments were conducted. Simulations of the amino alcohol oxidation reaction, without coenzyme regeneration and aldol addition, indicated that with equimolar concentrations of amino alcohol and $NAD^+$ (**Figure 3**A), maximum amino alcohol conversion of 3% could be achieved with an ADH concentration of 5 mg $mL^{-1}$. When coenzyme regeneration was included into the amino alcohol oxidation, the maximum amino alcohol conversion that can be achieved rose to 30%, at initial amino alcohol concentration of 10 mM, with only 1 mM of $NAD^+$ and 2 mg $mL^{-1}$ of ADH (**Figure 3**B1). Within the concentration range investigated, the increase of ADH and $NAD^+$ caused decreased amino aldehyde yield (**Figure 3**B2), as the formation of the amino acid was enhanced under these conditions (**Figure 3**B3), which was also shown by the previous statistical model [5]. The influence of ADH and NOX concentrations on reaction outcome was further simulated at constant concentrations of amino alcohol (10 mM) and $NAD^+$ (1 mM). The purpose was to test whether high concentrations of the enzymes would benefit substrate conversion and product yield. The simulations (**Figure 3**C) showed that only a narrow operating window of ADH concentration (between 2-5 mg $mL^{-1}$) and high concentrations of NOX (>50 mg $mL^{-1}$) enable a maximum yield of amino aldehyde of ~70% (**Figure 3**C2). This leads to the conclusion that the best strategy to achieve the highest aldol adduct yield is to couple oxidation and coenzyme regeneration with aldol addition, in which the amino aldehyde is constantly consumed by the aldol reaction.

*The influence of variables on process performance for the cascade reaction*

386 The model of the cascade reaction was used to find process conditions at which the yield of
387 aldol adduct can be maximized and formation of amino acid minimized. In the statistical
388 optimization carried out previously [5], three variables were used for optimization:
389 concentration of $NAD^+$ (0.1 to 1 mM), FSA (1 to 3 mg $mL^{-1}$) and ADH (1 to 10 mg $mL^{-1}$).
390 Only three variables were used in order to keep the number of experiments as low as possible.
391 Here, using a validated mathematical model, the range of enzyme concentrations was
392 expanded ($\gamma_{ADH}$ = 1 – 200 mg $mL^{-1}$, $\gamma_{FSA^{A129S}}$ = 1 – 100 mg $mL^{-1}$). Moreover, another variable,
393 the concentration of NOX ($\gamma_{NOX}$ = 0.1 – 100 mg $mL^{-1}$), was included. The influence of these
394 variables on process outcome was investigated without laboratory experimentation. In
395 addition, a shorter time was needed to obtain the results than with the statistical method since
396 the simulations were carried out *in silico*, one of the main benefits of using mathematical
397 modelling.
398
399 In the range investigated of 1-100 mg $mL^{-1}$ of $FSA^{A129S}$, no significant influence on substrate
400 conversion or product yield was observed (data not shown) and thus its concentration was
401 fixed at 50 mg $mL^{-1}$ for further simulations. Regarding the concentrations of ADH and NOX
402 (**Figure 4**), at amino alcohol concentration of 10 mM and $NAD^+$ of 1 mM, the simulations
403 revealed that at 5 mg $mL^{-1}$ NOX and 2 mg $mL^{-1}$ ADH, 92% aldol adduct yield was achieved
404 with only 0.7% of amino acid. Further increase of ADH concentration was detrimental for the
405 aldol adduct yield since amino acid formation increased (**Figure 4**B). The increase of NOX
406 above 5 mg $mL^{-1}$ indicated no significant influence on yield of aldol adduct. Additional
407 simulations (data not shown) showed that the $FSA^{A129S}$ concentration can be reduced from 50
408 mg $mL^{-1}$ to 2 mg $mL^{-1}$ without impairing yield of aldol adduct.
409 To confirm the conclusions from these simulations, an experiment was performed starting
410 from 10 mM amino alcohol and 1 mM $NAD^+$, with 5 mg $mL^{-1}$ NOX, 2 mg $mL^{-1}$ ADH and 2

411 mg $mL^{-1}$ $FSA^{A129S}$. This verified the findings and 92% yield of the aldol adduct was achieved
412 with only 0.7% yield of the amino acid (**Figure 5**), a significant improvement compared to
413 previous work in which this reaction was optimized using a statistical method, where 79%
414 yield of the aldol adduct was achieved with 10% yield of amino acid [5]. Furthermore, the
415 kinetic model made it possible to decrease the concentration of the two enzymes in the
416 reaction, ADH and $FSA^{A129S}$. In previous work, the concentrations of ADH and $FSA^{A129S}$
417 used to achieve the 79% yield of the aldol adduct were 10 and 3 mg $mL^{-1}$, respectively. The
418 simulations here (**Figure 4**), confirmed experimentally (**Figure 5**), showed that product yield
419 was improved with 2 mg $mL^{-1}$ of both ADH and $FSA^{A129S}$. The concentration of the third
420 enzyme, NOX, was slightly increased (5 mg $mL^{-1}$) compared to previous work (3.5 mg $mL^{-1}$).
421
422 Although statistical methods have many advantages for process optimization, in this case the
423 use of a kinetic model enabled a better process outcome. Moreover, it helped to decrease the
424 concentration of two enzymes, out of three used in the reaction. Since the reaction is of great
425 importance for the synthesis of D-fagomine precursor, current efforts are focused on
426 increasing substrate concentration and scaling up the process in order to achieve metrics
427 necessary for industrial application [11]. This will include additional simulations, testing
428 additional enzyme variants to find more active ones and different reactor modes, e.g. fed-
429 batch, which could improve volume productivity for which a simulation has already been
430 performed (Suppl. Figure 7) and showed that fed-batch reactor could indeed improve volume
431 productivity (details and discussion in Supplementary Information), but to scale up this
432 process the effect of $O_2$ will have to be included in the model, implying further investigations
433 regarding volumetric $O_2$ mass transfer coefficients. This is assumed to be the crucial step to
434 increase the rate of oxidation at high concentrations of amino alcohol.
435

The simulations presented here showed that of the three enzymes used in the investigated cascade, only $FSA^{A129S}$ had no significant influence on process outcome when changing its concentration. ADH and NOX had a substantial influence on the yield of both products, aldol adduct and amino acid, which clearly shows the direction in which future investigation of this reaction should be continued.

## Conclusion

A kinetic model of a cascade reaction for the synthesis of the aldol adduct (3*S*,4*R*)-6-[(benzyloxycarbonyl)amino]-5,6-dideoxyhex-2-ulose was developed. The kinetics of all three enzymes in the cascade were determined using the initial reaction rate method. As a side product, the corresponding amino acid was formed arising from the oxidation of the electrophile amino aldehyde. The kinetics of this side reaction was also studied and included in a mathematical model of the entire process. The latter was experimentally validated using different initial conditions and its applicability demonstrated in a wide range of concentrations. The model was instrumental in finding conditions at which the yield of aldol adduct can be maximized and formation of amino acid minimized. Simulations demonstrated that the most appropriate set-up for this enzymatic cascade reaction was in one-pot, where all reactions take place simultaneously. Furthermore, the simulations, as well as experiments, showed that it was possible to achieve 92% yield of the aldol adduct with only 0.7% yield of amino acid side product starting from 10 mM amino alcohol and 1 mM $NAD^+$, with 5 mg $mL^{-1}$ NOX, 2 mg $mL^{-1}$ ADH and 2 mg $mL^{-1}$ $FSA^{A129S}$. This yield of aldol adduct is an improvement compared to previous work where 79% yield was achieved. Additionally, the kinetic model enabled a decreased concentration of the two enzymes in the reaction, ADH and $FSA^{A129S}$, compared to previous work. It should be noted that the system operates at low turnover number for the coenzyme, i.e. 1 mM of $NAD^+$ is needed to convert 10 mM of the

substrate. However, in the reaction investigated, the situation with respect to process efficiency and economics differs relative to other enzymatic products obtained with oxidoreductases, because the coenzyme is usually the most expensive part and not the product. A further advantage here is that the cheapest form of the nicotinamide coenzyme can be used for this process, with the reduced and phosphorylated versions being more expensive. Given the high price of D-fagomine (700-900 $ for 10 mg), which can be obtained from the aldol adduct synthesized here by using selective catalytic reductive amination [39], we believe that the complete kinetic analysis of this reaction is a significant step towards understanding this complex system, and on the way to potential application. Future efforts will be focused on a more detailed investigation into $O_2$ mass transfer related to NOX-catalysed coenzyme regeneration and on increasing the total turnover number of $NAD^+$ coenzyme and substrate concentrations to achieve the process metrics necessary for industrial application. The presented study illustrates the advantage of mathematical modelling in understanding complex relationships between variables in cascade reactions. and can reduce time and cost necessary for the optimization of complex reaction systems. Modelling techniques should therefore be used more widely in process development to increase environmental and cost efficiency of enzyme cascades.

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**Scheme 1** Reaction scheme of the cascade reaction towards aldol adduct (3*S*,4*R*)-6-[(benzyloxycarbonyl)amino]-5,6-dideoxyhex-2-ulose consisting of amino alcohol oxidation to amino aldehyde catalysed by ADH, coenzyme regeneration catalysed by NOX, aldol addition of DHA to amino aldehyde catalysed by $FSA^{A129S}$, and the side reaction oxidation of amino aldehyde to amino acid catalysed by ADH.

**Figure 1** Cascade reaction of amino alcohol oxidation with coenzyme regeneration and aldol addition of DHA to amino aldehyde (25 °C, 50 mM TEA HCl pH 8.0, 10% v/v acetonitrile). **A**. $\gamma_{ADH}$ = 5.00 mg mL$^{-1}$, $\gamma_{NOX}$ = 0.35 mg mL$^{-1}$, $\gamma_{FSA^{A129S}}$ = 2.08 mg mL$^{-1}$, $c_{\text{amino alcohol}}$ = 10.02 mM, $c_{NAD^+}$ = 1.02 mM, $c_{DHA}$ = 25.16 mM, **B.** $\gamma_{ADH}$ = 5.00 mg mL$^{-1}$, $\gamma_{NOX}$ = 0.31 mg mL$^{-1}$, $\gamma_{FSA^{A129S}}$ = 2.10 mg mL$^{-1}$, $c_{\text{amino alcohol}}$ = 9.99 mM, $c_{NAD^+}$ = 0.10 mM, $c_{DHA}$ = 25.00 mM, **C.** $\gamma_{ADH}$ = 5.00 mg mL$^{-1}$, $\gamma_{NOX}$ = 0.35 mg mL$^{-1}$, $\gamma_{FSA^{A129S}}$ = 2.08 mg mL$^{-1}$, $c_{\text{amino alcohol}}$ = 50.15 mM, $c_{NAD^+}$ = 1.02 mM, $c_{DHA}$ = 125.40 mM, **D.** $\gamma_{ADH}$ = 5.00 mg mL$^{-1}$, $\gamma_{NOX}$ = 0.08 mg mL$^{-1}$, $\gamma_{FSA^{A129S}}$ = 2.08 mg mL$^{-1}$, $c_{\text{amino alcohol}}$ = 100.31 mM, $c_{NAD^+}$ = 1.02 mM, $c_{DHA}$ = 250.80 mM. Black circles – aldol adduct, white circles – amino aldehyde, grey circles – amino acid, line – model.

**Figure 2** Cascade reaction of amino alcohol oxidation with coenzyme regeneration and aldol addition of DHA to amino aldehyde (**Scheme 1**) (25 °C, 50 mM TEA HCl pH 8.0, 10% v/v acetonitrile, $\gamma_{ADH}$ = 5.00 mg mL$^{-1}$, $\gamma_{FSA^{A129S}}$ = 2.08 mg mL$^{-1}$, $c_{\text{amino alcohol}}$ = 10.02 mM, $c_{NAD^+}$ = 1.02 mM, $c_{DHA}$ = 25.16 mM). Black circles – $\gamma_{NOX}$ = 0.45 mg mL$^{-1}$, black triangles – $\gamma_{NOX}$ = 0.35 mg mL$^{-1}$, white circles – $\gamma_{NOX}$ = 0.27 mg mL$^{-1}$, dash line – $\gamma_{NOX}$ = 5 mg mL$^{-1}$, dotted line – $\gamma_{NOX}$ = 10 mg mL$^{-1}$, solid line – model.

**Figure 3 A.** Influence of equimolar amino alcohol and $NAD^+$ concentration on amino alcohol (solid line) conversion (*X*), yield (*Y*) of amino aldehyde (short dash line) and amino acid (dotted line) in the reaction of amino alcohol oxidation without coenzyme regeneration ($\gamma_{ADH}$

= 5 mg $mL^{-1}$). Simulations were carried out at $t$ = 24 h. **B.** Influence of ADH and $NAD^+$ concentration on amino alcohol conversion (B1), yield on amino aldehyde (B2) and amino acid (B3) in the reaction of amino alcohol oxidation with coenzyme regeneration ($\gamma_{NOX}$ = 0.4 mg $mL^{-1}$, $c_{amino\ alcohol}$ = 10 mM). Simulations were carried out at $t$ = 24 h. **C.** Influence of ADH and NOX concentration on amino alcohol conversion (C1), yield on amino aldehyde (C2) and amino acid (C3) in the reaction of amino alcohol oxidation with coenzyme regeneration ($c_{amino\ alcohol}$ = 10 mM, $c_{NAD^+}$ = 1 mM). Simulations were carried out at $t$ = 24 h.

**Figure 4** Influence of NOX and ADH concentration on amino alcohol conversion (A), aldol adduct (B) and amino acid (C) yield in the cascade reaction ($c_{amino\ alcohol}$ = 10 mM, $c_{DHA}$ = 25 mM, $c_{NAD^+}$ = 1 mM, $\gamma_{FSA}{}^{A129S}$ = 50 mg $mL^{-1}$). Simulations were carried out at $t$ = 24 h.

**Figure 5** Cascade reaction of amino alcohol oxidation with coenzyme regeneration and aldol addition of dihydroxyacetone to amino aldehyde (25 °C, 50 mM TEA HCl pH 8.0, 10% v/v acetonitrile, $\gamma_{ADH}$ = 2.05 mg $mL^{-1}$, $\gamma_{NOX}$ = 5 mg $mL^{-1}$, $\gamma_{FSA}{}^{A129S}$ = 2 mg $mL^{-1}$, $c_{amino\ alcohol}$ = 12.55 mM, $c_{NAD^+}$ = 1.02 mM, $c_{DHA}$ = 25 mM. Black circles – aldol adduct, white circles – amino aldehyde, grey circles – amino acid, line – model.