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ENZYME REACTION ENGINEERING AS A TOOL TO INVESTIGATE THE POTENTIAL APPLICATION OF ENZYME REACTION SYSTEMS

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It is widely recognized and accepted that although biocatalysis is an exquisite tool to synthesize natural and unnatural compounds under mild process conditions, much can be done to better understand these processes as well as detect resulting bottlenecks and help to resolve them. This is the precise purpose of enzyme reaction engineering, a scientific discipline that focuses on investigating enzyme reactions with the goal of facilitating their implementation on an industrial scale. Even though reaction schemes of enzyme reactions often seem simple, in practice, the interdependence of different variables is unknown, very complex and may prevent further applications. Therefore, in this work, important aspects of the implementation of enzyme reactions are discussed using simple and complex examples, along with principles of mathematical modelling that provide explanations for why some reactions do not proceed as planned.

Keywords: enzyme kinetics, modelling, reaction optimisation

1. Setting up the reaction conditions for an enzyme reaction

In each reaction system, first a proper buffer must be selected and the pH dependence of the enzyme activity determined in order to identify the optimal working conditions [1]. Although the impact of temperature on enzyme activity is also important, it should be remembered that the temperature at which the enzyme exhibits the highest level of activity is not necessarily that at which the enzyme stability is optimal. At higher temperatures, the enzyme activity is often increased but at the cost of progressive and irreversible denaturation due to poor thermal stability [2, 3]. When multiple enzymes are present in the reaction system and are supposed to operate in the same reactor, as is the case in cascade reactions, the optimal conditions can seldom be chosen for all of them. Usually, a compromise must be reached whereby the selection of the reaction conditions depends on the enzyme activity required to catalyse the reaction [4, 5]. After selecting the buffer, temperature and pH for the studied reaction system, it must be analysed in detail, starting from the reaction scheme. Even though the reaction scheme usually clearly depicts the reaction, it should be noted that issues beyond the reaction scheme of the enzymatic reaction need to be discussed and analysed.

In many cases, unwanted but insignificant side reactions may take place that sometimes also have a detrimental effect on the outcome of the reaction. Although this may not be so important on the laboratory scale as far as screening for enzyme activities is concerned, given that the concentrations applied on that scale fall within the range of a few mM, it must be noted that the rate of chemical reactions increases as the concentration of reactants increases, e.g. first- and second-order reactions. Therefore, further analyses to determine the effect of increasing the scale of the reaction by hundreds of mM are required. The same applies to the chemical stability of compounds present in the reactor. In this case, engineering methodology is priceless for the purpose of exploring the possibility of slowly feeding the reactive compound into the reactor. Alternatively, if an intermediate is reactive, the reaction rate in the reactor may be tuned to ensure its concentration is always minimal. For example, in the case of epoxides that are substrates of halohydrin dehalogenases [6], it is known that their stability is poor [7, 8]. As a result, in these reactions, a prochiral substrate is often used to start the reaction [9, 10]. The same is true in this case whereby an epoxide intermediate is formed in situ and immediately spent in the subsequent reaction with the same or a different enzyme such as the one presented in Scheme 1.

Additionally, since both epoxides and their corre-

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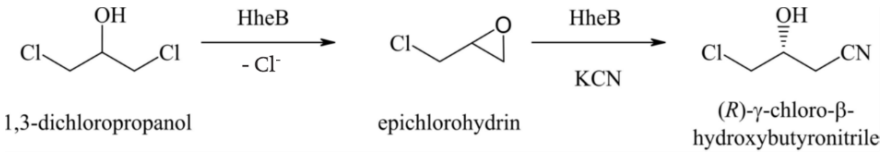
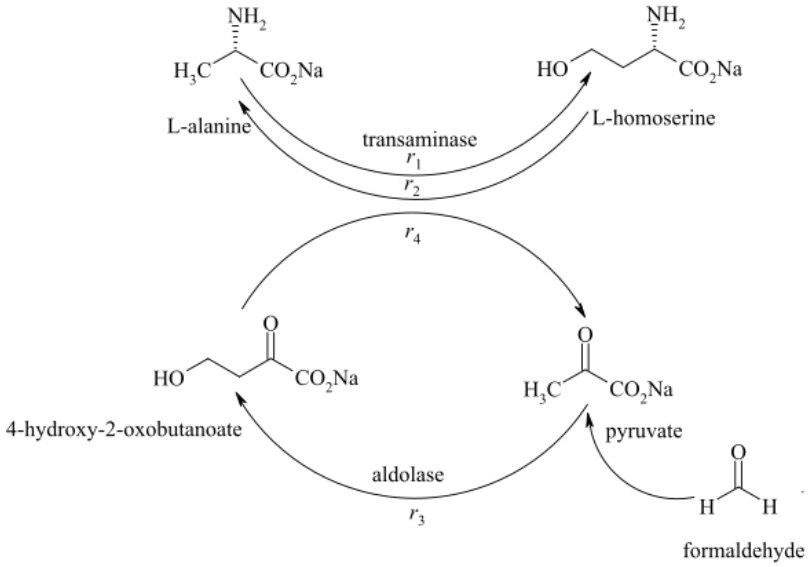


Scheme 1: Synthesis of (*R*)-γ-chloro-β-hydroxybutyronitrile from an achiral substrate



Scheme 2: Synthesis of L-homoserine in a cascade reaction

sponding nucleophiles can inhibit the catalytic activity of an enzyme [11], the selection of their concentrations in the reactor is crucial in facilitating a successful reaction [12]. Clearly, these are very complex reaction systems and the suitable set up of a reactor as well as reaction conditions determined by the model-aided approach can be vital [13].

Multi-step reactions cannot always be performed simultaneously in one pot due to complex relationships between the process variables [14, 18]. In a study of an innovative reaction scheme for the preparation of the atorvastatin side-chain precursor, it was found that the two reaction steps consisting of aldol addition and the oxidation of the corresponding product amino lactol could not be performed simultaneously [14]. This was mostly due to the fact that acetaldehyde as the substrate in the first reaction step interferes with the oxidoreduction and coenzyme regeneration by acting as a substrate for the oxidoreductase or as an inhibitor as well as deactivator of both oxidoreductase and NADH oxidase. It is important to determine if all the reaction steps are compatible with each other before deciding how to develop the reaction. Although this might suggest a significant amount of experimental work, this can be considerably reduced by evaluating the enzyme kinetics [13, 19, 20].

Forming the reaction model enables a vast variable space to be explored in silico. Apart from that, combining the kinetic model with mass balances in different reactors enables different types of reactors to be explored in each system. This was found to be crucial with regard to improving the process metrics in the synthesis of L-homoserine [21], a system governed by the unfavourable equilibrium of the transaminase-catalysed reaction and aided by the pyruvate recycling system catalysed by aldolase (Scheme 2).

The application of model-based optimization techniques led to a doubling of the product concentration (up to 80 gL^{-1}) and an 18% increase in the volumetric productivity (up to 3.2 $gL^{-1}h^{-1}$) in comparison with a previously published work [22]. In this system, it was crucial that both reactions were carried out simultaneously to improve the position of the equilibrium. Formaldehyde was gradually added to the system by using a pump due to its reactivity and inhibiting effect on enzyme activity. Additionally, pyruvate and L-alanine were added sequentially once the pyruvate had been consumed in several doses, which, according to calculations, was found to work in silico experiments (Fig. 1 A-B) and subsequently proved experimentally (Fig. 1 C-D).

2. Side reactions and their effect on the reaction scheme.

When studying a complex reaction system, possible side reactions must be taken into account. These can be caused by the instability of reactants, products or intermediates; by chemical reactions between the compounds present in the reaction mixture; as well as by the side reactions

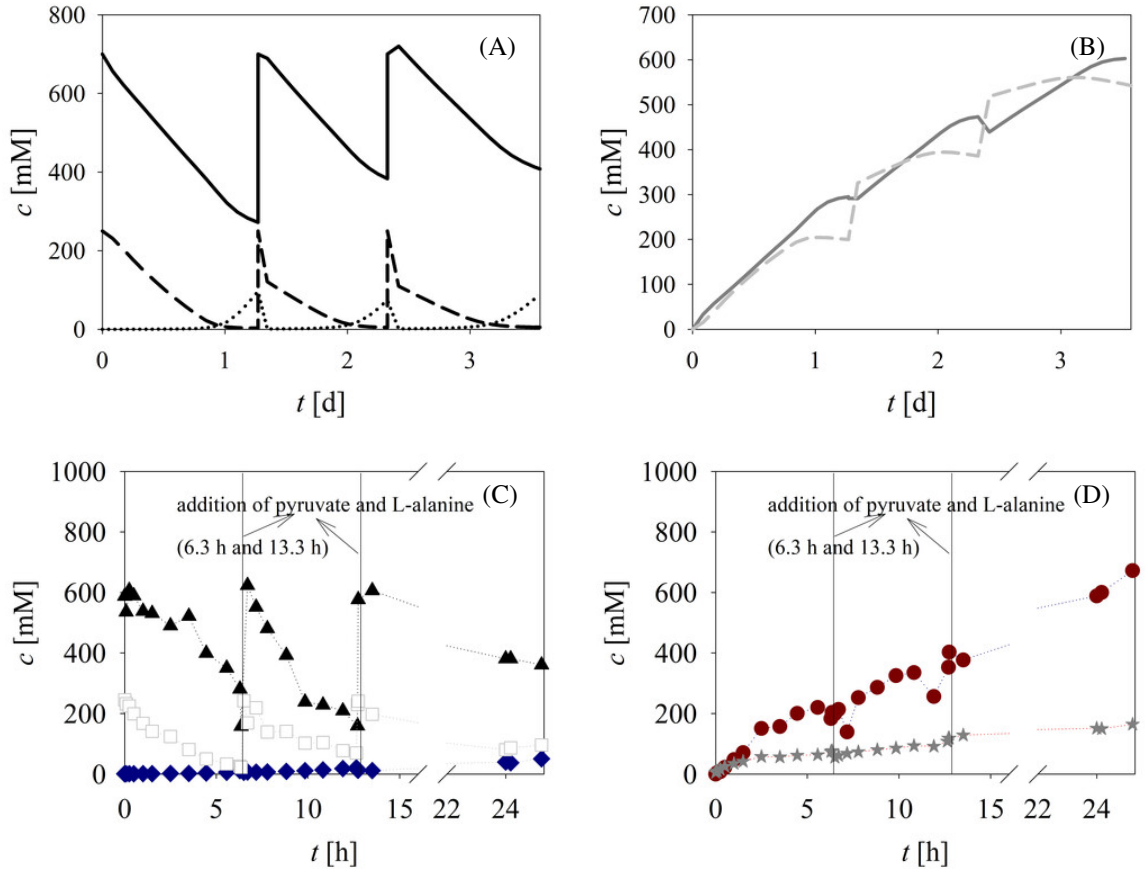
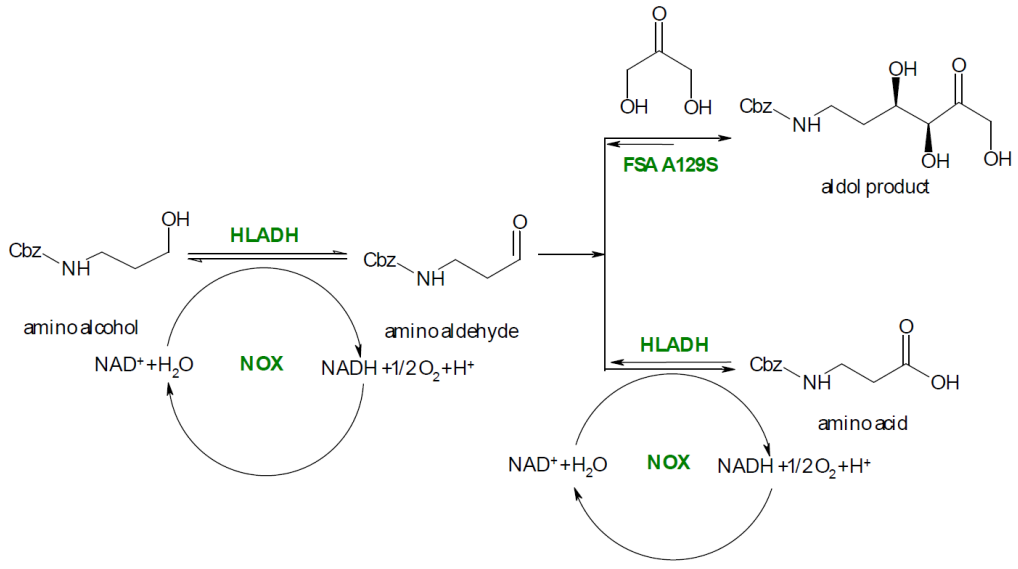


Figure 1: Cascade synthesis of L-homoserine [20] in a fed-batch bioreactor by gradually adding formaldehyde via a pump as well as sequentially adding pyruvate and L-alanine incrementally once the pyruvate had been consumed. (A) (black line – L-alanine, dashed line – pyruvate, dotted line – formaldehyde), (B) (grey line – L-homoserine, grey dashed line – aldol intermediate). In silico experiments, (C) experimental validation (black triangles – L-alanine, white squares – pyruvate, blue diamonds – formaldehyde), and (D) experimental validation (red circles – L-homoserine, grey stars – aldol intermediate).



Scheme 3: Synthesis of the aldol product (3*S*,4*R*)-6-[(benzyloxycarbonyl)amino]-5,6-dideoxyhex-2-ulose in a cascade reaction

caused by the catalytic enzymes due to their low purity or ability to catalyse more than one reaction [8, 23, 24]. Reactions are often carried out with a crude enzyme extract or whole cells in order to reduce the costs of synthesising the protein by avoiding the necessity for purification. Although such systems often offer an additional advantage in terms of enhancing the operational stability of the desired enzyme within the protein mixture or cell compartment, other enzymes in these systems can also catalyse undesirable enzymatic reactions [23].

All of these aforementioned reactions can lower the concentration of the target product as well as decrease the reaction yield, moreover, in some cases, even prevent the formation of the target product. One example of such an event is the oxidation of an alcohol to form an aldehyde catalysed by horse liver alcohol dehydrogenase that reacts further by oxidizing the aldehyde to form the corresponding acid as a side product. In the cascade synthesis of (3*S*,4*R*)-6-[(benzyloxycarbonyl)amino]-5,6-dideoxyhex-2-ulose (Scheme 3), *N*-Cbz-3-aminopropanoic acid was the dominant main product following our first attempt, with only 2% of the target product being formed [25, 26]. Considering the complexity of the system, reaction engineering methodology was applied to determine the reason behind this. A statistical model implied the occurrence of this side reaction [25] which was later confirmed by kinetic studies [26].

Not only did the aforementioned studies reveal the reasons for the poor yield but also determined how to improve it to between 79 and 92%, respectively. In many cases, although commercial compounds that contain small quantities of certain additives are purchased for research purposes, these additives can also frequently act as enzyme inhibitors, such as in the case of 4-methoxyphenol as a stabilizer of acrylonitrile that was used as a substrate in one of the reactions studied by us [27]. In fact, this was one of the crucial reasons why it was not possible to obtain significant amounts of product in any reactor.

3. Investigation of the kinetics of the enzyme-catalysed reaction

To formally identify the system, the effect of all the compounds present in the reaction mixture on the enzyme activity / reaction rate can be evaluated. During these measurements, the effects of all the compounds on the enzyme activity can be measured and, in many cases, substrate, intermediate and product inhibition can be detected, which subsequently help with regard to decision-making and selection of the reactor mode to be used for the reaction. Some examples of reactor designs that can be applied, according to the properties of the studied reaction and desired outcome, are given in Fig. 2. In theory, it is known that the fed-batch bioreactor is a favourable choice for reactions subjected to substrate inhibition to increase the concentration of the obtained product [31].

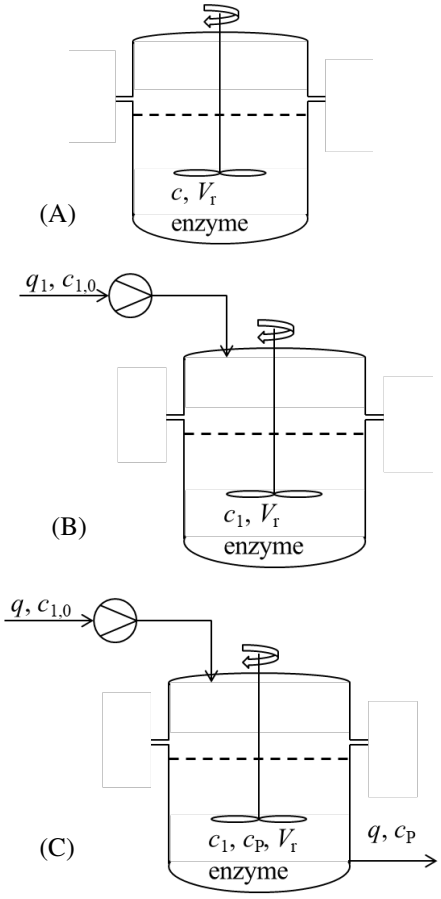


Figure 2: Schemes of different reactors applied in biocatalysis: (A) batch reactor, (B) fed-batch bioreactor, (C) continuous stirred tank reactor

For product-inhibited reactions, a continuous stirred tank reactor operating at the maximum concentration of the product is not recommended and, therefore, the resultant enzyme activity is unsatisfactory [32, 33]. In practice, reactions are rarely inhibited by a single compound, moreover, in many cases, several important inhibitions and/or side reactions take place. Therefore, the reactor mode cannot be easily set by viewing the results of the effect of substrates on the reaction rate. In these cases, kinetic models help simulate different scenarios and enable the best choice for the studied reaction system to be made [13].

The simulations of a relatively simple double-substrate reaction in which the kinetics can be described by double Michaelis-Menten kinetics with both substrate and product inhibition are presented in Fig. 3. The impact of reaction conditions on substrate conversion and volumetric productivity in the batch reactor is presented in Figs. 3A and 3B, while 3C and 3D show the same for the continuous stirred tank reactor (CSTR). Substrate conversion is governed by the enzyme concentration as well as the reaction time and residence time in the batch reactor and CSTR, respectively. The main difference that can be

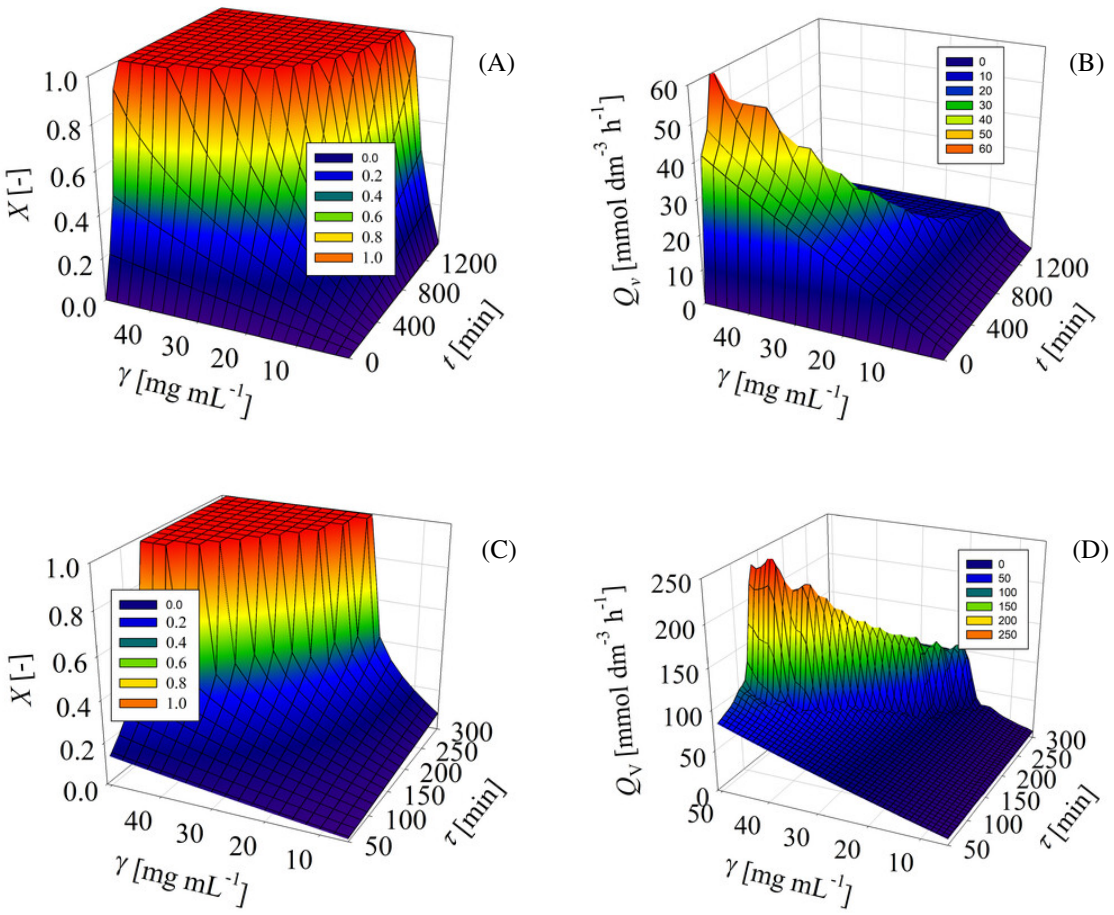


Figure 3: Model simulations demonstrating the impact of the reaction conditions on the conversion and volumetric productivity in the batch and continuous stirred tank reactors

observed is in terms of volumetric productivity, which explains why continuous processes are currently in the spotlight. In this simulation, the maximum volumetric productivity of the CSTR is fourfold greater than that of the batch reactor. Nevertheless, it must be noted that the operational stability of the enzyme is important and that the enzyme activity was assumed to be constant. In practice, since the enzyme activity inevitably drops over time and, therefore, enzymes must be stabilized by a form of immobilization, ensuring the continuous process functions is not a straightforward task.

The first step to investigate enzyme kinetics is to find an appropriate method that will result in the rapid collection of enzyme kinetic data. This can be done by applying a spectrophotometric enzyme assay and microtiter plate reader, however, should these methods be unavailable, this can also be achieved in a traditional manner by determining the initial reaction rates from HPLC or GC data with regard to the concentrations of substrates and products [13]. Given that data collection must be accurate and reliable, analytics is the foundation of the research. Data must be reproducible and trustworthy to be used for modelling. An example of kinetic data is presented in Fig. 4 where the grey line denotes the experiment where the enzyme concentration was too high. Furthermore, even though the linear dependence of absorbance over time is obvious in the initial part of the curve, the error of such measurements can be high and depends on the individual measuring. On the other hand, the black line clearly

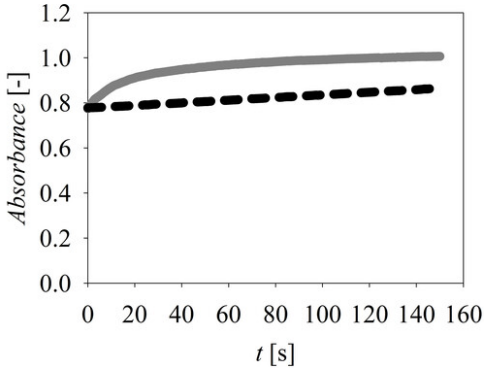


Figure 4: The impact of enzyme concentration on the quality of the experimental data

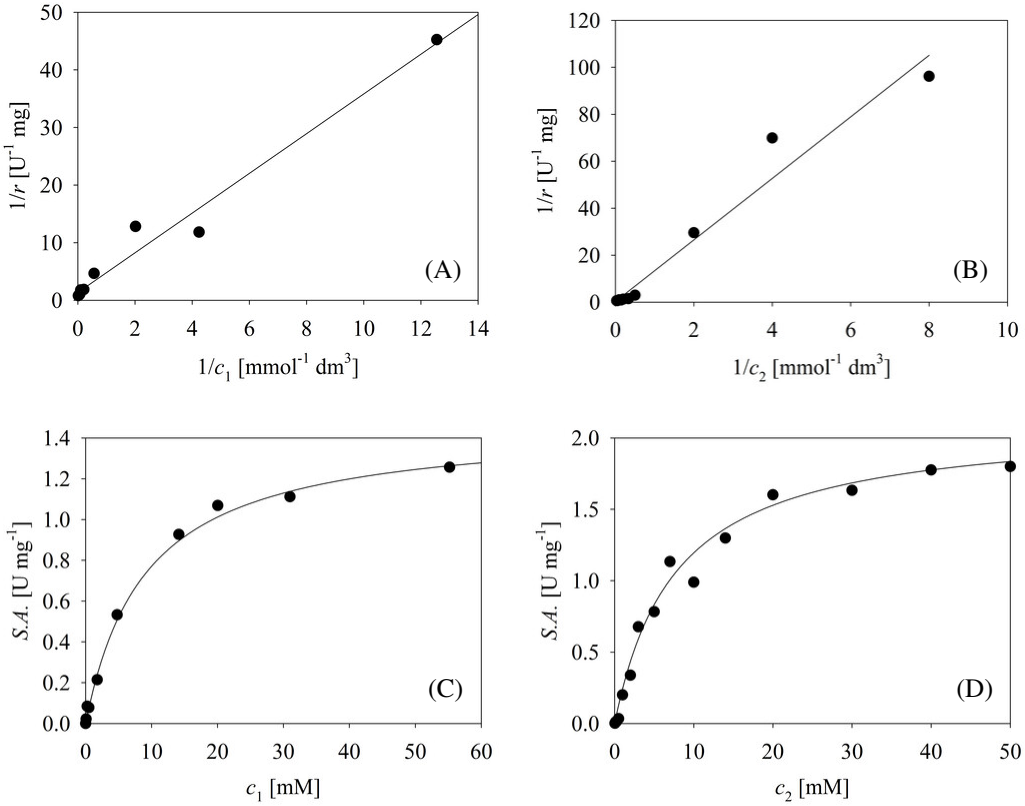


Figure 5: Estimation of kinetic parameters for a two-substrate reaction by applying linear (A and B) and nonlinear regression (C and D) analyses

represents linear data with a relatively small gradient, indicating that the measurements were made properly and the dependence is undoubtedly linear within that range. A series of such experiments performed at different concentrations of substrates, products and intermediates is required to obtain one set of experimental data to be subsequently used for the estimation of kinetic parameters.

Based on the kinetic data, the kinetic parameters can be estimated by using nonlinear regression analysis, which is far better than the still commonly used linear regression analysis [34]. This can be illustrated by the example presented in Fig. 5 whereby a double-substrate reaction was considered and the kinetic data concerning the dependence of the specific enzyme activity on the concentration of the reactants measured. By measuring the initial reaction rate (conversion less than 10%), the effect of product inhibition or enzyme deactivation could be minimized [35]. This example will be used to illustrate the differences between the values of the estimated kinetic parameters when various methods of estimation are applied. In the first case, linear regression analysis was applied by using a Lineweaver-Burk plot (Figs. 5A-5B). The data show discrepancies and, in the case of 5B, two points needed to be removed from the analysis as they were outliers. The estimated kinetic parameters are presented in Table 1. In the second case, the kinetic parameters were estimated by using single-substrate Michaelis-Menten kinetics, which in all likelihood is frequently used in practice. The estimated kinetic parameters presented in Table 1 are very different from the ones obtained following linear regression analysis. Furthermore, since the maximum reaction rates differ for each substrate, the measurements in all probability were not made in the area of substrate saturation. Michaelis constants estimated by using double-substrate Michaelis-Menten kinetics strongly resemble the values estimated by using single-substrate Michaelis-Menten kinetics. However, when the maximum reaction rates are compared, a significant discrepancy between them can still be observed. Estimating the maximum reaction rate by using double-substrate Michaelis-Menten kinetics is the optimum solution offering a unique value of V_m and taking into consideration the case when the non-varying substrate was not saturated. Therefore, in the case when both substrates are saturated, single-substrate Michaelis-Menten kinetics and nonlinear regression analysis offer a suitable solution to estimate the kinetic parameters.

4. Investigation of the operational stability of the enzyme

Enzyme activity inevitably decreases in the reactor over time, which means that the operational stability reduces as well [28–30]. This also needs to be quantified from the

Parameter	Linear regression analysis $\frac{1}{r} = \frac{1}{V_m} + \frac{K_m}{V_m}\frac{1}{c}$	Nonlinear regression analysis – single-substrate kinetics $r = \frac{V_m c}{K_m + c}$	Nonlinear regression analysis – double-substrate kinetics $r = \frac{V_m c_1 c_2}{(K_{m1} + c_1)(K_{m2} + c_2)}$
V_m [U/mg]	0.743 (3.539)	1.448 (2.119)	4.177
K_{m1} [mmol/dm^3]	2.56	8.2	9.09
K_{m2} [mmol/dm^3]	46.366	7.726	9.191

Table 1: Comparison between different methods to estimate the values of kinetic parameters in an enzymatic reaction

experimental data [29] and incorporated into the kinetic model. In many cases, the enzyme activity can be followed by an independent enzyme assay. In other cases, it can be estimated by using the kinetic model and other experiments. The operational stability of enzymes is an important topic not only in terms of research but also with regard to their applications. Understanding and describing quantitatively as well as qualitatively how enzyme function and structure change during conversion in a bioreactor is of crucial importance [28, 36].

In their work, Börner et al. investigated the mechanistic reasons for the poor operational stability of amine transaminases along with the influence of quaternary structure, cofactors and substrates. Through their kinetic and thermodynamic experiments, they were able to identify the structural domain that appears to confer stability. The study revealed that the enzyme is significantly more stable when at rest than in its operational state, moreover, its operational stability was lower and experiments suggested a mechanism that brought about substrate-induced deactivation [28]. In many reports to date, it has been stated that the presence of substrates and their concentrations can have both positive [37] and negative [30] effects on enzyme stability. In a study by Česnik et al. [30], formaldehyde as a substrate was found to have a negative effect on enzyme activity during experiments (Fig. 6A). Subsequently, it was found that this could be correlated with the operational stability of the enzyme (Fig. 6B). Considering the reactivity of formaldehyde and the size of this molecule, chemical damage to the protein may occur in its presence, as reported in other studies. In a study involving the dehalogenation of 1,3-dichloro-2-propanol (1,3-DCP) catalysed by halohydrin dehalogenases (HHDHs), it was found that the substrate 1,3-DCP causes enzyme deactivation during incubation, moreover, as observed in the previously described case, the substrate concentration has a significant effect on enzyme activity (unpublished data, Fig. 6C). Experiments conducted in batch reactors corroborated that the operational stability decay rate constant can be directly correlated to the substrate concentration (unpublished data, Fig. 6D). These are not the only examples of this behaviour. In a study by Vasić-Rački et al., it was also shown that glycolaldehyde caused operational stability decay in the reactor, the rate of which was dependent on its concentration [38]. In all of these cases, the quantification of the operational stability decay rate constant and the modelling approach improved the outcome of the reaction and increased process metrics values.

Another example of the effect of a substrate on enzyme activity can be demonstrated by different oxidases. In one study, the operational stability of D-amino acid oxidase was investigated in the presence and absence of aeration [40]. The enzyme operational stability decay rate of D-amino acid oxidase from porcine kidneys was reduced by increasing the oxygen concentration in the reaction solution and the enzyme activity decreased more rapidly. Similar conclusions were drawn in a later study on glucose oxidase [40]. This can be related to the oxidation of protein residues in the presence of oxygen and requires some sort of quantification to enable development of the reaction by focusing on resolving bottlenecks.

If operational stability is considered in a very simple reaction with only a basic Michaelis-Menten model, its effect during dynamic simulations can be observed (Fig. 7A). When the enzyme activity reduces in the batch reactor, the shape of the curve changes slightly. To the untrained eye, this can also resemble the result of reaching equilibrium or product inhibition. Therefore, if the kinetics of the reaction are completely unknown, it is very difficult to draw the right conclusion. The situation is quite different if the continuous stirred tank reactor is used, since this reactor ideally works at a stationary state and, therefore, no changes in enzyme activity nor in stationary concentrations of reactants and products occur. Hence, enzyme operational stability decay in CSTRs results in the stationary state being lost and the clearly visible shape of the curve caused by the reduction in enzyme activity (Fig. 7B). A third type of reactor often applied in biocatalysis due to substrate inhibition are fed-batch bioreactors. Although enzyme operational stability decay can be observed from the shape of the curve (Fig. 7C), here, like in the case of the batch reactor, it is more difficult to clearly define the reason for this trend. The answer that is suggested here concerns quantification of enzyme activity during the reaction.

5. Choosing the best enzyme variant for the reaction

Techniques for genetically modifying enzymes have advanced greatly over recent years and can be applied to produce industrially suitable catalysts more quickly and

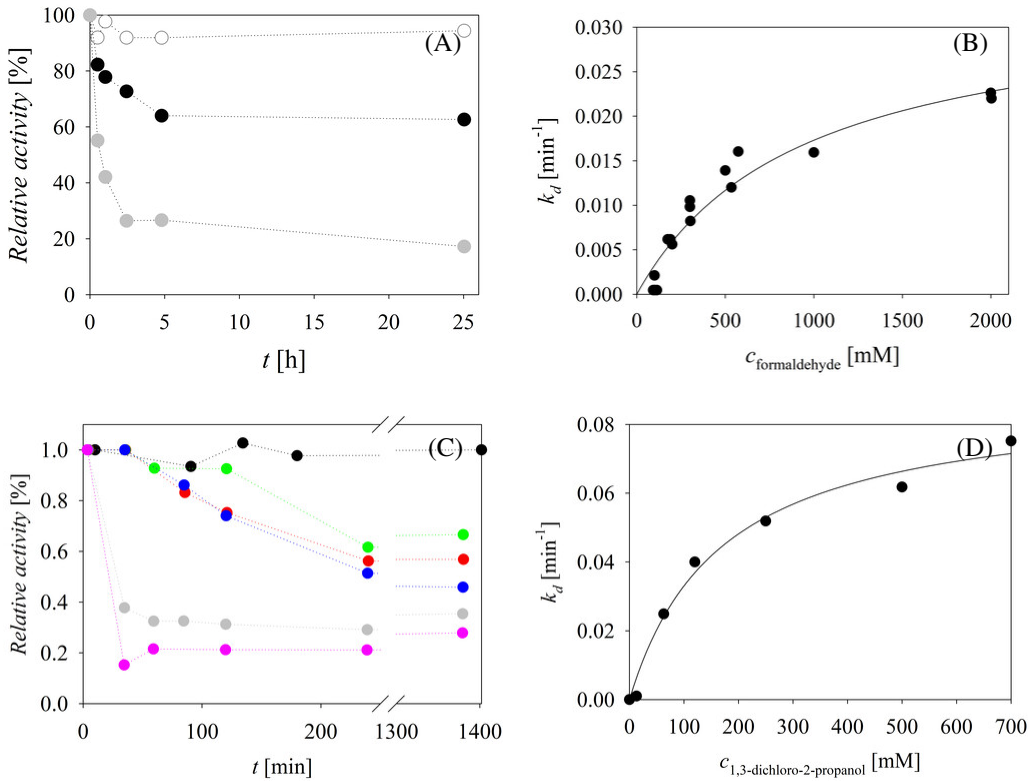


Figure 6: (A)The influence of the initial concentration of formaldehyde on the enzyme activity of FSAD6Q during incubation; (B) Dependence of the operational stability decay rate constants of FSAD6Q on the initial concentration of formaldehyde; (C) The influence of the initial concentration of 1,3-DCP on the enzyme activity of HHDH during incubation; (D) Dependence of the operational stability decay rate constants of HHDH on the initial concentration of 1,3-DCP

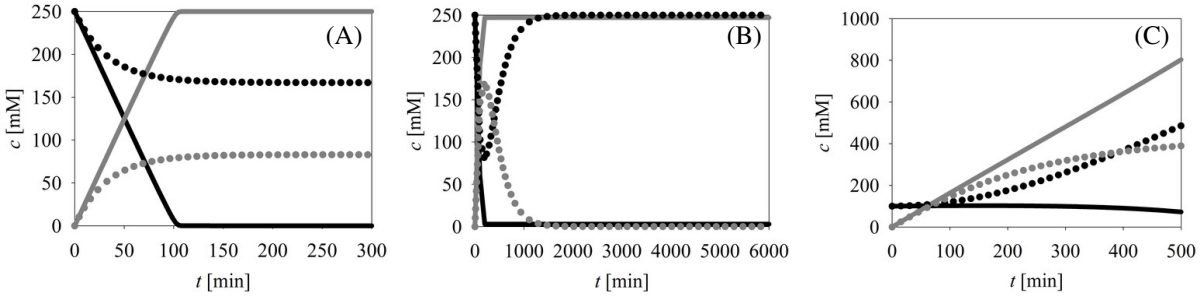


Figure 7: The effect of operational stability decay on model curves in different types of reactors: (A) batch reactor, (B) continuous stirred tank reactor, (C) fed-batch bioreactor

Parameter	Unit	Enzyme 1	Enzyme 2	Enzyme 3
V_{m}	U/mg	3.42 ± 0.05	1.74 ± 0.11	0.74 ± 0.03
K_{m}	mM	102.24 ± 4.01	67.52 ± 7.22	36.33 ± 8.82
K_{i}	mM	679.94 ± 38.81	183.19 ± 29.25	377.28 ± 68.70

Table 2: Estimated kinetic parameters for the three enzyme variants

cost-effectively. However, in order for new biocatalysts to be worthy of industrial large-scale production, reliable and comprehensive methods for the initial kinetic characterization of possible enzyme variants are necessary. In search of an optimal enzyme variant, the enzyme with the highest activity (highest V_m value) or highest affinity for the substrate (lowest K_m value) is often sought [41]. This is only valid when Michaelis-Menten kinetics are applied, however, in practice, the situation is rarely that simple. For example, this is not so in the case

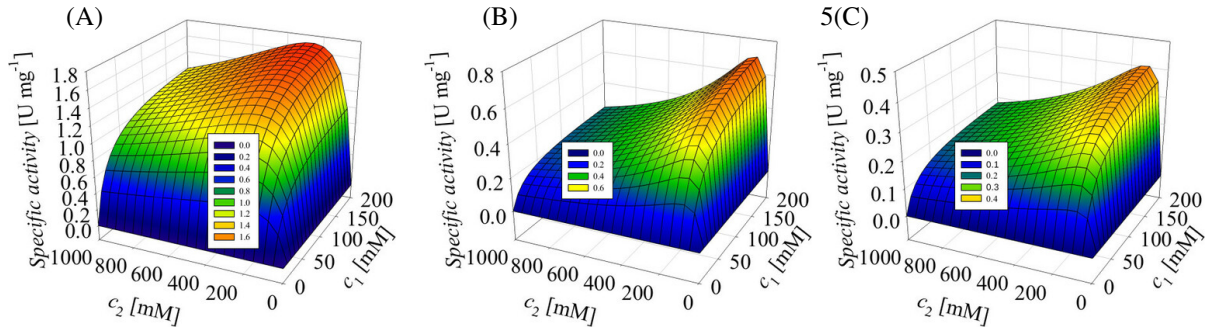


Figure 8: Comparison between the three enzyme variants which exhibit substrate inhibition at different levels

of Michaelis-Menten kinetics with substrate inhibition, when the substrate concentration used during screening is critical. Given that screening seems to only be conducted at one concentration, accurate data of enzyme activity is not provided, considering that the shape of the Michaelis-Menten curve is unknown. Since variants of the same enzyme differ with regard to the estimated values of their kinetic constants, combinations of the relevant kinetic parameters (V_m, K_m, K_i) were obtained for each variant (Table 2). Although it may be assumed that the enzyme with the minimum Michaelis constant and highest activity is most suitable, in practice, this enzyme may exhibit a higher level of substrate inhibition as a result. Three enzyme variants were kinetically characterized and the dependence of their specific enzyme activities on the substrate concentration is presented in Fig. 8, while the kinetic parameters are shown in Table 2. The best applied variant was found to be Enzyme 1, written in bold, in Table 2 because the level of substrate inhibition it is subjected to is by far the least pronounced. In practice, this means a broader substrate concentration area in which the highest enzyme activities can be obtained in the reactor (Fig. 8A) and enhanced stability of the reactor's operating conditions. Simulations presented in Fig. 8 also show that when screening the enzyme variants, it is important to not only evaluate their activities but also estimate all their kinetic parameters.

In further stages of process development, the application of reaction engineering to identify process bottlenecks is required to exploit the full potential of novel enzymes. To develop novel green routes in biocatalysis and scale them up, it is crucial to adopt a multidisciplinary approach by combining the fields of chemistry, biology and chemical engineering.

6. Conclusions

Enzyme reaction engineering can provide explanations for and give answers to different phenomena that occur in bioreactors. This is of particular importance when it comes to multienzyme systems which are very important in terms of sustainable development and green synthesis. Many obstacles to their development must be overcome, for example, adjusting enzyme activities, choosing suitable enzyme variants, selecting the best reactor and determining the optimal reaction conditions while considering the side reactions that may occur. Therefore, a combined effort and multidisciplinary approach are required to prepare complex enzyme reaction systems for industrial applications.

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