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Co-Enzymes with Dissimilar Stabilities: A Discussion of the Likely Biocatalyst Performance Problems and Some Potential Solutions

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Abstract: Enzymes have several excellent catalytic features, and the last few years have seen a revolution in biocatalysis, which has grown from using one enzyme to using multiple enzymes in cascade reactions, where the product of one enzyme reaction is the substrate for the subsequent one. However, enzyme stability remains an issue despite the many benefits of using enzymes in a catalytic system. When enzymes are exposed to harsh process conditions, deactivation occurs, which changes the activity of the enzyme, leading to an increase in reaction time to achieve a given conversion. Immobilization is a well-known strategy to improve many enzyme properties, if the immobilization is properly designed and controlled. Enzyme co-immobilization is a further step in the complexity of preparing a biocatalyst, whereby two or more enzymes are immobilized on the same particle or support. One crucial problem when designing and using co-immobilized enzymes is the possibility of using enzymes with very different stabilities. This paper discusses different scenarios using two co-immobilized enzymes of the same or differing stability. The effect on operational performance is shown via simple simulations using Michaelis–Menten equations to describe kinetics integrated with a deactivation term. Finally, some strategies for overcoming some of these problems are discussed.

Keywords: co-immobilization; cascade reaction; enzyme stability; enzyme kinetics

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1. Introduction

Today, enzymes have proved themselves as potential biocatalysts in many industrial applications [1–8] because of their exquisite specificity and selectivity in aqueous media at ambient temperature and atmospheric pressure. Additionally, enzymatic selectivity overcomes the need for protection (and de-protection) steps when converting highly functionalized molecules [9–12]. Thus, implementing enzymes into chemical synthetic schemes is often preferred from the view of sustainable and green chemistry principles [7,10,13–15].

However, enzymes in nature need to fulfil the physiological requirements of a given organism and, in this way, usually possess features that do not fit the requirement of an industrial catalyst [16]. For example, enzymes are water-soluble molecules and often unstable over prolonged periods of operation. Their excellent catalytic features have evolved to convert a physiological substrate under physiological conditions, not for the conversion of synthetic compounds in an industrial reactor. Fortunately, today, there are many tools to solve these enzyme shortcomings, such as the remarkable developments in metagenomics [17,18], enzyme modelling and site-directed mutagenesis [19,20], directed

evolution [21–23], as well as chemical modification of proteins [24–27]. With the advent of such powerful tools, the complexity of the processes in which enzymes are used is growing. For example, mimicking nature, many enzymes are now used in cascade reactions, where the product of one enzyme reaction is the substrate for the subsequent one [28–30]. Likewise, researchers have generated an enzyme bearing several active centres [31]. In an elegant example, an enzyme bearing an artificial "biological" active centre and an organometallic catalyst (plurizyme) (by using an irreversible inhibitor bound to the native active centre) was created and employed in a cascade [19]. Later, this was extended to add an active biological centre bearing different activity (creating a transaminase/hydrolase plurizyme) to catalyze other cascade reactions [20]. Despite the many benefits, a significant drawback for many enzymes as industrial biocatalysts remains their poor stability.

Several strategies are known to improve the stability of enzymes, including protein engineering aiming to increase the melting temperature and other properties [23,32,33]. Additionally, recent findings have shown that deep eutectic solvents (DES) can improve the stability of coenzymes in aqueous solutions [34]. Finally, enzyme immobilization was developed to create heterogeneous enzyme biocatalysts. This may be achieved by creating a 'solid' involving the enzyme [35] (e.g., cross-linked enzyme aggregates [36–39], cross-linked enzyme crystals [40–43], microcrystals coated with proteins [44,45], copolymers [46–48], nanoflowers [49]) or by binding the enzyme to a pre-existing solid (non-porous or porous particles) [50–54].

Currently, enzyme immobilization has been proven to improve many enzyme properties [55,56], such as stability [57–59], activity (mainly under harsh conditions) [57,60], selectivity or specificity [61,62], inhibition [60], and even to be integrated with enzyme purification [63]. However, these benefits are only obtained if the immobilization is properly designed and controlled. Moreover, an inadequate immobilization protocol can even worsen some enzyme features [64]. In this way, enzyme immobilization becomes an essential step in preparing an industrial biocatalyst, not merely as means to make it easily recoverable and reusable.

Enzyme co-immobilization is a further step in the complexity of preparing a biocatalyst [65–67]. Co-immobilization consists of the immobilization of more than one enzyme in the same particle and has received particular attention in multi-enzymatic systems used to perform cascade reactions, due to the kinetic advantages that co-immobilized enzymes present in such systems [68]. For example, when using enzymes that have been co-immobilized in porous supports, the second enzyme in the cascade may be exposed from the first moment of the reaction to a high concentration of its substrate (the product of the first enzyme), eliminating the usual lag time observed in cascades [29,55]. This effect is mainly relevant in the first moments of the reaction and determines the initial reaction rate. This is because, after some reaction time, it may be expected that the concentration of the product of the first enzyme increases in the bulk and the second enzyme may become exposed to a saturating concentration of this substrate after some reaction time even if they are not co-immobilized [55]. That way, the impact of enzyme co-immobilization on the global reaction cycle will depend on many factors related to the kinetic features of the individual enzymes, the used substrate concentrations, etc. [29,69–83]. In some instances, e.g., when the product of the first enzyme is unstable [55,84,85], co-immobilization is the only way to obtain a high yield of the desired product since even a short time might permit its modification of another undesired compound. Several cases of this kind of cascade have also been reported in the scientific literature [86–91].

The simultaneous use of multiple enzymes is not restricted to cascade reactions. For example, one possible application of enzyme co-immobilization is using an enzyme (e.g., lysozyme) to prevent the microbial contamination of an immobilized biocatalyst composed of other enzymes [92,93]. In these cases, co-immobilization of both (lytic and target) enzymes seems to be the only way to entirely prevent the growth of microorganisms inside the biocatalyst particles [92,93]. Moreover, using several enzymes may be of interest for modifying multifunctional substrates (e.g., oils) or when the reaction conditions change

through the course of the reaction (e.g., pH, concentrations of substrates/products) [94–100]. It may also be necessary if some synergy between the involved enzymes exists and needs to be co-localized. For example, the co-immobilization of enzymes and cofactors permits the continuous reuse of the cofactors but also necessitates the co-immobilization of the cofactor regeneration enzyme and target enzyme with cofactors to be useful [101–103]. Enzymes have also been co-immobilized to prepare multipurpose biocatalysts [104–106], i.e., enzymes able to catalyze unrelated reactions. However, the interest in this combi-biocatalyst is unclear since it will never surpass the complete ultimate multipurpose biocatalyst: the whole cell [85]. Thus, there are examples where co-immobilization is a strict necessity, other instances where co-immobilization may increase the initial rate but perhaps insufficiently justified for implementation, and others where the co-immobilization advantages are not always obvious.

Some problems related to co-immobilization were recently reviewed [85]. First, the relationship between the activities of the involved enzymes must be carefully optimized to give the maximum volumetric productivity of the biocatalyst [85,107,108]. Additionally, the location of the enzymes inside the porous particle (co-localized or forming concentric enzyme crowns that may be ordered in different fashions) may also be critical to achieving the desired results [65,67,72,79,109]. Moreover, exposing the different enzymes to the same immobilization conditions, even if for a short time, restricts the range of conditions that can be used in the immobilization [55,60]. Additionally, the protocol for the immobilization strategy should be considered, as using the same protocol might not be the optimal strategy for all involved enzymes [52].

One crucial problem in the design and use of co-immobilized enzymes is the possibility of using enzymes bearing very different stabilities [85,110–115]. This problem is not usually considered in combi-biocatalyst design but may have some important practical implications. For example, when using standard immobilization protocols, one may discard the whole combi-biocatalyst even though some co-immobilized enzymes still retain a high percentage of initial activity [85,110–115]. There are some recent reports in the scientific literature showing the fundamental importance of this problem (i.e., co-immobilized enzymes where one of the enzymes is almost entirely inactivated while the other enzyme retains its activity) and suggesting some protocols that permit the reuse of the most stable enzyme to build new co-immobilized biocatalysts [85,110–115]. These strategies are based on the use of a reversible immobilization concept to immobilize the least stable enzyme: the least stable enzyme is released from the combi-biocatalyst after inactivation, and a new batch of the soluble enzyme can subsequently be immobilized, regenerating a combi-biocatalyst with similar properties to the initial one, and reusing the most stable immobilized enzyme [85,110–113,115].

Another problem promoted by the different stabilities of the co-immobilized enzymes, which has been even less considered in the scientific literature, is how these different enzyme stabilities may affect the long-term operating performance of the combi-biocatalyst. The effects on the process performance may also depend on the rationale for co-immobilization (if it is just a kinetic advantage or if it is critical because the product of the first enzyme needs to be rapidly modified). These problems are the focus of the current paper.

2. Long-Term Performance of a Combi-Biocatalyst using Enzymes with Dissimilar Stabilities

As stated in the introduction, one of the key points in designing a co-immobilized enzyme preparation is the relationship between the activities of the involved enzymes [85,107,108]. This activity ratio is vital in optimizing a combi-biocatalyst volumetric activity and requires significant effort. Therefore, the use of an excess of one of the enzymes may be considered wasteful, as the global initial volumetric activity of the combi-biocatalyst will be lower than that obtained using the correct ratio between all the involved enzymes and the excess of the over-represented enzyme will be wasted, at least at first glance.

However, such optimization efforts are usually performed using soluble enzymes in a single reaction cycle. To the best of our knowledge, no studies have been undertaken regarding the influence of the dissimilar inactivation rate of enzymes during operation, especially if the enzyme stabilities are very different.

In this paper, to avoid excessive complexity and clarify this phenomenon's relevance, we will focus on the simplest case, where only two enzymes are co-immobilized. We will show the effects of dissimilar enzyme inactivation simulating the use of mixtures of the soluble enzymes or immobilized enzymes where there are no diffusional considerations that may alter the enzyme features, discussing later that this becomes far more complex when utilizing co-immobilized enzymes. The dynamic of the enzyme rate expressions will be illustrated, assuming no measurement errors or noise. To further simplify the situation, the only considered effect of enzyme deactivation is the loss in activity over time, causing a decrease in the overall reaction rate. Thus, the maximum reaction rate (Vmax) is the only affected kinetic parameter. Hence, enzyme features, such as specificity and selectivity, will not be considered. The deactivation of the least stable enzyme activity is assumed to have no significant effect on the remaining active enzyme's performance in the process (except if co-immobilized). Additionally, we will assume that the enzymes are not being substrate-inhibited.

Furthermore, using co-immobilized enzymes, we did not consider the diffusional effect of the initial substrate. At the same time, accumulating the intermediate product will be an advantage for co-immobilization. In this way, the rapid diffusion of this intermediate is vital in this instance. The support particle porosity will define this tortuosity of the pore channels, pore diameter and the particle size of the biocatalytic support, together with the activity of the different enzymes and diffusional constants of the target compounds under the reaction conditions [116,117]. The optimal loading of the different enzymes is assumed to be 50% of each enzyme (optimized with respect to the volumetric activity of the biocatalysts for the production of the second product). We also define the target as using the biocatalyst for 50 cycles. These 50 cycles are the half-life of the least stable enzyme. In contrast, the most stable remains fully active, and the enzyme recovery of the soluble, immobilized and co-immobilized enzyme is 100%. Of course, this simplified situation is not the actual situation in all aspects but is selected here for clarity [118,119].

We can consider a situation where two enzymes deactivate at a different rates, where the intermediate remains stable. The simultaneous use of both enzymes only affects the reaction kinetics, enabling a more rapid action of the second enzyme in the cascade and giving some kinetic advantages, resulting in a prolongation of the reaction time but not affecting the yield. However, as shown in this first approximation, even in this oversimplified example for co-immobilized enzymes, the operational performance is more complex than individually immobilized enzymes.

Another case is when the rapid and efficient action of the second enzyme is critical to prevent the modification of the product. This is the case of an intrinsically unstable intermediate product or the production of a reactive by-product (e.g., hydrogen peroxide) able to destroy the target product [86–91]. In this case, the inactivation of the first enzyme may have a similar effect to the above case. However, if the second enzyme is the least stable, the repercussions may be much higher. This situation will be discussed in future works.

2.1. Enzyme Deactivation and Kinetics

We will show the results using a mixture of soluble enzymes or individually immobilized enzymes where there are no diffusion constraints. This simplification enables us to generate simple models of the effect of enzyme inactivation on the biocatalyst performance and the reaction cycle. Furthermore, it is assumed that both enzymes are acting in a cascade reaction, where the first enzyme (E_1) converts a substrate (S) to an intermediate (I) which is the substrate for the second enzyme (E_2) that is converted to the target product (P). Thus, the reaction scheme is as follows:

$$S \xrightarrow{E_1} I \xrightarrow{E_2} P$$

The kinetics for the two reactions can then be described by the well-known Michaelis–Menten equation, assuming no product inhibition. The change in concentration over time is then described as:

$$v = \frac{dS}{dt} = -\frac{V_{max1} \cdot [S]}{K_{M,S} + [S]} \quad (1)$$

$$v = \frac{dI}{dt} = \frac{V_{max1} \cdot [S]}{K_{M,S} + [S]} - \frac{V_{max2} \cdot [I]}{K_{M,I} + [I]} \quad (2)$$

$$v = \frac{dP}{dt} = \frac{V_{max2} \cdot [I]}{K_{M,I} + [I]} \quad (3)$$

Additionally, the maximum velocity (V_{max}) can be expressed as:

$$V_{max} = k_{cat} \cdot \mathrm{E}_0 \quad (4)$$

The reaction rate is compromised in the presence of a typical enzyme deactivation. Thus, the maximum reaction rate (V_{max}) will decrease, as illustrated in Figure 1 (assuming that the other enzyme features are intact).

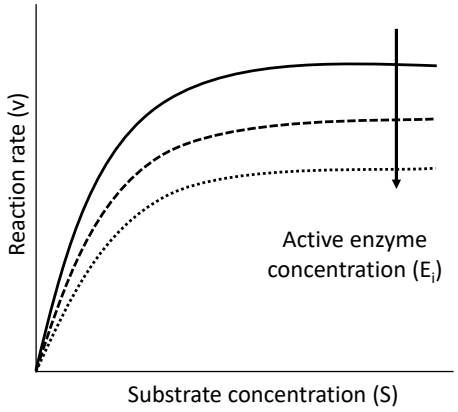


Figure 1. Effect of enzyme deactivation on the reaction rate (v = a). The arrow illustrates the decrease in the active enzyme concentration. The full line is without deactivation, and the dotted lines show the reaction rate when a loss of activity occurs.

The enzyme inactivation is assumed to follow a first-order decay [100,101]

$$\ln\left(\frac{a}{a_0}\right) = -k_d \cdot t \quad (5)$$

where a is the initial measured enzyme activity, a_0 is the activity at a given time, and k_d is the deactivation constant. A deactivation term will be implemented in the rate expressions (Equations (1)–(3)) [120,121]:

$$\frac{dS}{dt} = -\frac{V_{max1} \cdot e^{-k_{d1} \cdot t} \cdot [S]}{K_{M,S} + S} \quad (6)$$

$$\frac{dI}{dt} = \frac{V_{max1} \cdot e^{-k_{d1} \cdot t} \cdot [S]}{K_{M,S} + [S]} - \frac{V_{max2} \cdot e^{-k_{d2} \cdot t} \cdot [I]}{K_{M,I} + [I]} \quad (7)$$

$$\frac{dP}{dt} = \frac{V_{max2} \cdot e^{-k_{d2} \cdot t} \cdot [I]}{K_{M,I} + [I]} = a \quad (8)$$

2.2. Description of the Case Study

In this theoretical paper, enzyme deactivation is investigated over 50 reaction cycles, where each reaction cycle is assumed to take 8 h. Kinetic parameters are required to show the dynamics of the deactivation model. These are estimated based on a total substrate conversion to the product after 8 h and are listed in Table 1. Figure 2 shows a non-dimensional simulation of the time course of the substrate, intermediate, and product concentration, with an initial substrate concentration at 100 mM equivalent to 100%.

Table 1. Assumed kinetic parameters for enzymes 1 and 2.

Parameter	Enzyme 1	Enzyme 2
V_{max}	4 μM/s	6 μM/s
K_m	2 mM	4 mM
k_{cat}	$1{\cdot}10^{-3}$ s^{-1}	$2{\cdot}10^{-3}$ s^{-1}
$[E_i]$	4 mM	6 mM

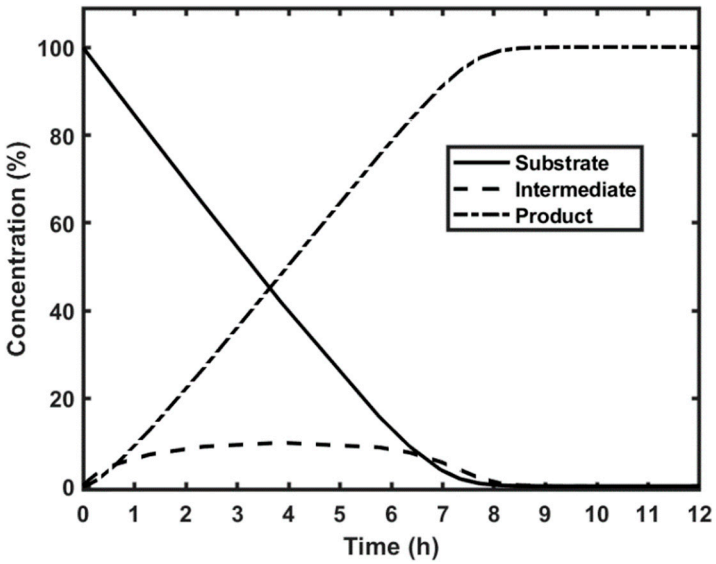


Figure 2. Reaction course in the first cycle of the cascade reaction catalyzed by E_1 and E_2 using the kinetic parameters and initial enzyme concentrations listed in Table 1.

Implementing the assumption that the half-life of the enzyme (residual activity at half the initial activity) is reached after 50 reaction time cycles and that a reaction time where a cycle is 8 h, the deactivation constant may be calculated using Equation (5) [32]:

$$\ln(0.5) = -k_d \cdot (50 \text{ cycles} \cdot 8 \text{ h}) \Rightarrow k_d = 0.002 \text{ h}^{-1}$$

Both enzymes are assumed to act together without compromising activity and operational stability.

3. Effect of Enzyme Inactivation when the Combined Use of Enzymes Only Increases the Volumetric Activity of the Combi-Biocatalysts

This section will discuss three different situations regarding enzyme stability:

(1) Both enzymes deactivate at a similar rate.
(2) The first enzyme in the cascade deactivates, while the second fully retains its activity.
(3) The second enzyme in the cascade deactivates, while the first fully retains its activity.

To simplify the modelling of the effect on the biocatalyst's performance, we will consider the case where both enzymes can be fully recovered and reused efficiently for the different reaction cycles without any modification on other features.

3.1. Effect of Enzyme Deactivation on Biocatalyst Performance

3.1.1. Both Enzymes Deactivate at a Similar Rate

The most straightforward situation to discuss is when both enzymes deactivate at a similar rate. Then, both enzymes lose activity at a similar rate. The rate expressions are described by Equations (6)–(8), with a deactivation constant equal to 0.002 h^{-1}, corresponding to a half-life of 50 reaction cycles, as calculated in Section 2.2. The first-order enzyme inactivation for both enzymes with that deactivation constant is illustrated in Figure 3A. When the enzymes deactivate, their catalytic activity will be compromised, causing the reaction rate to decrease and prolong the reaction cycle.

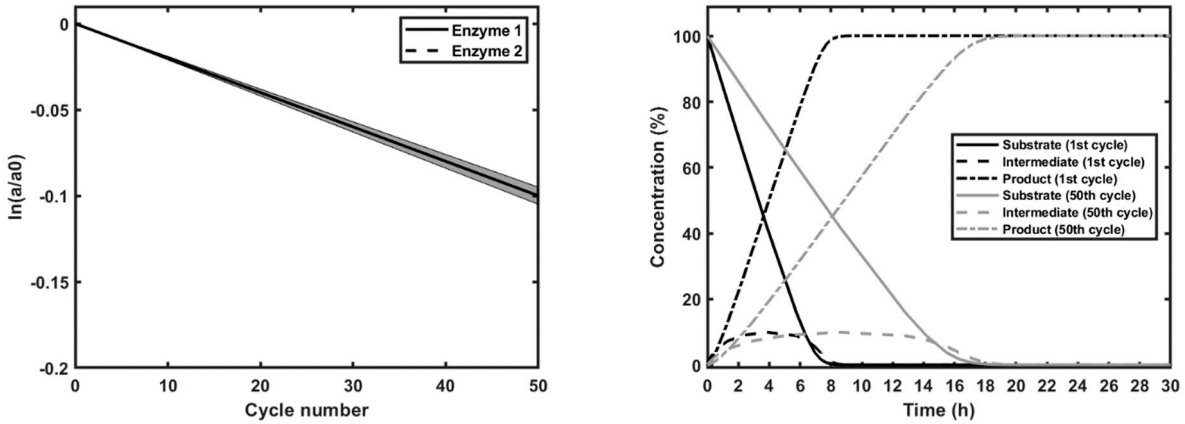


Figure 3. Effect on reaction course when both enzymes deactivate at a similar rate. (**A**) Deactivation course of enzyme 1 and enzyme 2. The deactivation is assumed to be first-order, with a deactivation constant of 0.002 h^{-1} for both enzymes. The shaded area indicates an error of ±5%. (**B**) Simulated time course of the concentration profile. The black line shows the initial reaction cycles, and the grey lines illustrate the time course of the 50th reaction cycle in the presence of deactivation, with a deactivation constant at 0.002 h^{-1}. Kinetic parameters and initial enzyme concentrations are listed in Table 1.

Figure 3B illustrates the dynamics of the rate expressions when enzyme deactivation occurs, as described by Equations (6)–(8). Assuming the enzymes have similar stability parameters, the prolongation of both reaction steps will be similar. The main effect when both enzymes deactivate is prolonging each reaction cycle. Thus, the intermediate product concentration will follow a similar pattern to the initial course during all cycles. Thus, the same intermediate amount accumulates, but the conversion from the substrate to the product takes longer.

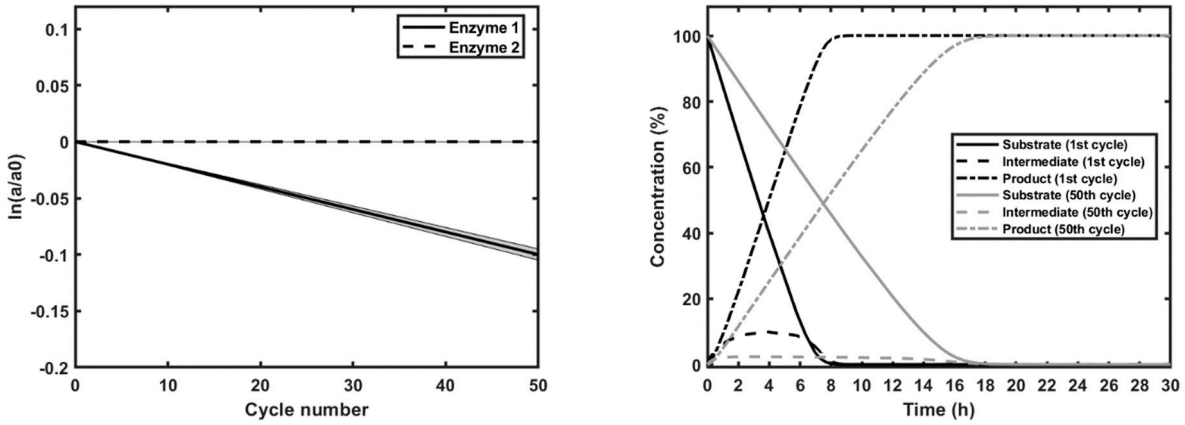
3.1.2. Enzyme 1 Deactivates while Enzyme 2 Remains Fully Active

Another possibility is that the first enzyme in the cascade deactivates while the second remains fully active (Figure 4A). This situation has been reported in different instances [85,110–115,122]. Again, the inactivation kinetics of the least stable enzyme is assumed to follow first-order decay, with the half-life reached after 50 reaction cycles. In this scenario, only the rate expression related to the substrate modification will include a deactivation term, as enzyme 1 (E_1) catalyzes the conversion of the substrate to the intermediate. The rate expressions to this scenario appear in Equations (9)–(11). The loss of activity is illustrated in Figure 4A. Only the rate expressions for the substrate and the intermediate will be changed in this case. The simulated results of the concentration profiles in the initial reaction compared to the concentration profiles in the presence of deactivation are illustrated in Figure 4B.

$$\frac{dS}{dt} = -\frac{V_{max1} \cdot e^{-k_d 1 \cdot t} \cdot [S]}{K_{M,S} + S} \quad (9)$$

$$\frac{dI}{dt} = \frac{V_{max1} \cdot e^{-k_{d1} \cdot t} \cdot [S]}{K_{M,S} + [S]} - \frac{V_{max2} \cdot [I]}{K_{M,I} + [I]} \quad (10)$$

$$\frac{dP}{dt} = \frac{V_{max2} \cdot [I]}{K_{M,I} + [I]} \quad (11)$$



(**A**) (**B**)

Figure 4. Enzyme 1 deactivates, while enzyme 2 remains fully active. (**A**) The deactivation is assumed to be first-order decay, with a deactivation constant of 0.002 h^{-1} for both enzymes. The shaded area indicates an error of ±5%. (**B**) Simulated time course of the concentration profile. The black line shows the initial reaction cycles, and the grey lines illustrate the time course of the 50th reaction cycle in the presence of deactivation, with a deactivation constant at 0.002 h^{-1}. Kinetic parameters and initial enzyme concentrations are listed in Table 1.

Superficially, Figures 3B and 4B have similarities, but if enzyme 1 deactivates, the amount of accumulated intermediate will be lowered, as illustrated by Figure 3B. The second enzyme will use the intermediate faster, relative to its production rate, than in the initial situation, as it is assumed that E_2 remains fully active. The product formation will be faster than when both enzymes lose activity, as the second enzyme in the cascade quickly uses the intermediate. Still, the decrease in the intermediate production will prolong the reaction cycles, assuming there is an excess of K_M (Figure 4B).

3.1.3. Enzyme 2 Deactivates while Enzyme 1 Remains Fully Active

The final situation we present is when the second enzyme in the cascade deactivates while the first enzyme remains fully active (Figure 5A). In this case, only the rate expressions for the intermediate modification and the product production changed:

$$\frac{dS}{dt} = -\frac{V_{max1} \cdot [S]}{K_{M,S} + S} \quad (12)$$

$$\frac{dI}{dt} = \frac{V_{max1} \cdot [S]}{K_{M,S} + [S]} - \frac{V_{max2} \cdot e^{-k_{d2} \cdot t} \cdot [I]}{K_{M,I} + [I]} \quad (13)$$

$$\frac{dP}{dt} = \frac{V_{max2} \cdot e^{-k_{d2} \cdot t} \cdot [I]}{K_{M,I} + [I]} \quad (14)$$

The first reaction rate will be the same in the initial and further reaction cycles, and the substrate consumption will be similar during all cycles. However, there will be an excess of accumulated intermediate, where enzyme 2 takes longer to convert, as illustrated in Figure 5B. Thus, the activity of enzyme 1 is in excess after some cycles in this situation, and the time to complete production of the target product will be prolonged.

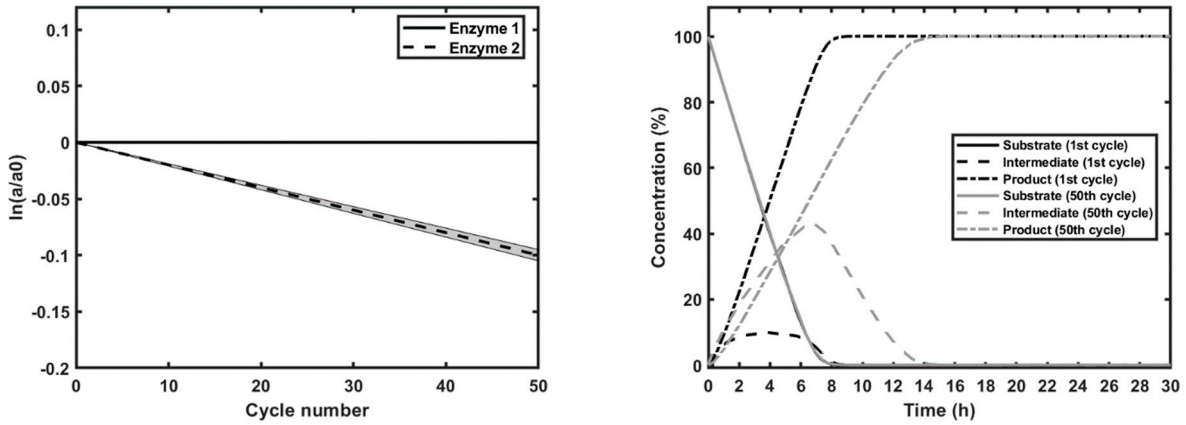


Figure 5. Enzyme 2 deactivates, while enzyme 1 remains fully active. (**A**) The deactivation is assumed to be first-order decay, with a deactivation constant of 0.002 h^{-1}. The shaded area indicates an error of ±5%. (**B**) Simulated time course of the concentration profile. The black line shows the initial reaction cycles, and the grey lines illustrate the time course of the 50th reaction cycle in the presence of deactivation, with a deactivation constant at 0.002 h^{-1}. Kinetic parameters and initial enzyme concentrations are listed in Table 1.

3.2. Strategies to Maintain a Similar Reaction Course during the 50 Cycles

Using soluble enzymes or individually immobilized enzymes, the strategy to regain the initial reaction course and recover the lost activity is straightforward: to add the least stable enzyme in the percentage it inactivated in the previous cycle (or cycles) before starting the subsequent cycle. Depending on the reactor and production plant requirements, this can be performed each cycle (around 1% of the least stable enzyme should be added before starting a new cycle) or when the inactivation of the least stable enzyme reaches a pre-determined value (e.g., a 5 or a 10%), leaving the reaction course to prolong in this extension. That way, the situation will be similar to the case where a single enzyme is used in a one-step reaction.

3.3. Use of Co-Immobilized Enzymes

Using co-immobilized enzymes, the situation generated by the enzyme inactivation becomes more complex. It is assumed that co-immobilized enzymes are used because this has some advantages. That way, if we add only make-up enzymes, in an individually immobilized form, we will need more enzyme than the percentage of the enzyme that has been inactivated to maintain the cycle time. This will depend on the advantages that the co-immobilization present, which in terms depends on the kinetic constants of the involved enzymes, and the diffusional problems of the substrate (that will occur in many processes, although we are going to discard then to simplify the discussion) and, most importantly, the diffusional problems of I, as the accumulation of this compound inside the particle is the origin of the advantages of enzyme co-immobilization [85]. Thus, adding only the make-up enzyme will require an excess of individually immobilized enzymes (including enzyme and support). This will decrease the interest in co-immobilized enzymes, as, ultimately, we will use each enzyme immobilized in an individual form.

Even in the simplest situation, when both enzymes are inactivated at the same rate, and we add fresh combi-biocatalyst to compensate for the activity drop, the effects on the reaction course may not be identical to the case of the soluble enzymes or individually immobilized enzymes. Using co-immobilized enzymes, the decrease in the combi-biocatalyst activity will be produced for two reasons. First, is the obvious decrease of enzyme activity, but second, is if the first enzyme is inactivated, because the accumulation of the intermediate inside the biocatalyst particle is lower than using the initial combi-biocatalyst. That way, it may not only be less active by its inactivation. Still, it may also not be exposed to

saturation concentrations of its substrate, which can further decrease the activity of the second enzyme during operation. This will depend not only on the kinetics of the enzymes but also on all the parameters that determine the diffusion of the substrate and intermediate, related to the medium and the particle properties. That way, we cannot discard that, even if we can maintain the overall reaction time, the intermediate accumulation may differ after the inactivation of the first enzyme.

Simplifying the problem to cases where the concentration of the intermediate inside the biocatalyst particle still is enough to maintain the second enzyme under saturation conditions, adding fresh co-immobilized enzymes to compensate for the drop in the enzyme activity may be a way to maintain the cycle time. For example, for every 10% decrease in the reactor (in the eleventh cycle), add 10% of fresh co-immobilized enzymes. However, even in this ideal situation, we will be wasting the excess of the active enzyme. It is also possible that this excess of the second enzyme can make the second step faster, making necessary a careful analysis to maintain the reaction cycle time within the desired ranges.

However, in cases where the effect in E_2 activity is higher because the enzyme is not under saturation concentrations of the substrate on the partially inactivated combi-biocatalyst, focusing on the decrease of P production rate may be a better solution than focusing on the E_1 activity decrease, adding the corresponding amount of fresh combi-biocatalyst when the P production rate has decreased in a 10%. In this case, the concentration profile of I during the reaction course may change in the different reaction cycles, which may become a problem in certain cases for the automation of the process.

That way, even under an oversimplified situation where the co-immobilization has just an effect on the reaction rate, the use of co-immobilized enzymes poses new problems in maintaining the reaction cycle time, mainly when the first enzyme is inactivated. The simplest solution is to use an excess of E_2, increasing the operating cost of the process.

Other more sophisticated solutions are also possible to save the excess of immobilized stable enzyme used above; the process scientist should consider the suitability of each of them for their specific requirements.

One possibility is to use an excess of the least stable enzyme in the initial combi-biocatalyst. Suppose the biocatalyst initially is not utilized at full load, and it is possible to increase the amount of the least stable enzyme without decreasing the amount of the other enzyme. In that case, this solution may provide good results. If E_1 is the least stable enzyme, an excess of E_1 can be employed. We can assume that the concentration of intermediate in the bulk will increase. However, E_2 will remain under saturation conditions, as it is in the optimal combi-biocatalyst as now there are double of E_1 producing the substrate of this second enzyme, which may reach a double concentration inside the particle. It may be expected that the excess of E_1 mainly produces a more rapid conversion of substrate to intermediate. Still, the conversion of the intermediate to the product may be in a similar fashion, giving reaction times similar to that of using the optimal biocatalyst (apparently wasting the excess of E_1 in the initial cycles). When E_1 activity decreases to 50% after 50 cycles, the activities of both enzymes will be similar to the one designed for the optimal biocatalyst. That way, E_2 remains fully active and saturated, and the reaction cycle time will be as designed using the optimal biocatalyst. However, if the "optimal" biocatalyst is fully loaded with both enzymes, to increase the amount of E_1, the amount of E_2 must be decreased. To have a reaction time cycle similar to that using the "optimal" biocatalyst, the amount of combi-biocatalyst should also be increased (that is, the cost of the support increased, we only save the most stable enzyme costs). The amount of extra-biocatalyst utilized to maintain the reaction cycle time to the desired one will depend on the specific kinetics of the enzymes involved in the reaction and the diffusional features of the support regarding the substrates and products (the activities ratio will not be optimal, that way one can be sure only that more biocatalyst than the "optimal" will be necessary). Again, E_2 should be saturated at the desired level in cycle 1 (in fact, more E_1 is presented in the biocatalyst). Still, we can assume that when the activity of E_1 decreases in the successive cycles, the activity of E_1 in the biocatalyst used by several cycles may become under the

value that it presented in the "optimal biocatalyst", and perhaps E_2 may be no longer under the desired saturation conditions. This possible solution will require more profound studies to be implemented.

If E_2 is the least stable enzyme, the use of an excess of E_2 should also be properly analyzed. Suppose it is possible to immobilize this excess of E_2 without affecting the loading of E_1 (that is, the support was not fully loaded in the optimal biocatalyst). In that case, intermediate accumulation will decrease, as it will be consumed faster. When E_2 is inactivated after 50 cycles to 50%, the biocatalyst performance should be similar to the "optimal" one, with the cost of an initial excess of E_2, but not support. If the optimal biocatalyst is designed using fully loaded biocatalyst, the decrease in E_1 necessary to increase E_2 can drive a sub-optimal concentration of intermediate inside the biocatalyst particle, making the optimization and control of the process complex. This will not be compensated by using an excess combi-biocatalyst to maintain the amount of E_1 in the reactor. The secondary problem is that E_2 is not under the target saturation conditions in the optimization. That way, the initial activity of the biocatalyst may further decrease, making it necessary to add more combi-biocatalyst to maintain the reaction cycle time. Hence, this solution may not be easy to implement and will require deep analysis before being implemented.

Suppose the least stable enzyme is reversibly immobilized, and the support remains active. In that case, there is another possibility to keep the duration of the reaction cycles when one of the enzymes is inactivated for a long time. However, this strategy is only valid if, in optimizing the optimal biocatalyst support, the support is not fully loaded with enzymes 1 and 2, leaving some free surface to immobilize more of the least stable enzyme. Or, if a multi-layer immobilization strategy has been developed and the immobilization of more enzyme molecules over the previous enzyme layer did not affect the most stable enzyme activity, always the least stable enzyme is on the upper layer [110,115,123,124]. When the least stable enzyme is inactivated by 10%, we can immobilize an additional 10% of this enzyme in the combi-biocatalyst to compensate for this activity loss, recovering a biocatalyst with a performance almost identical to the initial one.

If the optimal biocatalyst is fully loaded and it is impossible to add more enzymes when required, this strategy requires decreasing the load of immobilized E_1 or E_2. This decrease in the enzyme loading may have the unwanted effects discussed above. Furthermore, it can make it necessary to re-optimize the biocatalyst with a lower loading to obtain similar performance to the optimal fully loaded combi-biocatalyst (very likely using more of this lower loaded biocatalyst than that necessary to replace the amount of enzyme compared to the optimal combi-biocatalyst and altering the E_1/E_2 rate ratio). On the other hand, the simplicity of this strategy to maintain the reaction time course may compensate for using more combi-biocatalyst at a lower loading and efforts to re-optimize the combi-biocatalyst.

The best strategy to be used in each case will depend on many factors, including company facilities, the cost of support, the cost of the involved enzymes, and the immobilization cost.

In this way, using co-immobilized enzymes if the enzyme activity decreases along the reaction cycles, and more if this occurs in a very different fashion, it may be expected that to maintain the reaction course and the cycle time, even in the simplest situation, may not be a simple goal. This means using independently immobilized enzymes may be preferred. However, we can regain an identical reaction course by adding the specifically inactivated enzyme. That way, the kinetic advantages of co-immobilization should be analyzed in light of the difficulties of maintaining advantage along the entire reaction lifetime before discarding the use of individually immobilized enzymes. Moreover, the problems of the co-immobilized enzyme caused by the enzyme inactivation will increase when their kinetic advantages increase.

4. Design of Combi-Biocatalysts: Strategies to Improve Biocatalyst Performance and Reuse the More Stable Enzyme

Finally, we would like to link these operational performance problems of combi-biocatalysts to the current strategies that have been developed to solve the discarding of the whole combi-biocatalyst after the deactivation of the least stable enzyme while the more stable retains activity. These strategies enable reusing the more stable immobilized enzyme [66]. The few strategies proposed for recovery and reuse of the more stable co-immobilized enzyme involve reversibly immobilizing the least-stable enzyme on the combi-biocatalyst [66].

As discussed below, these strategies are compatible with the most sophisticated strategy proposed in this paper to solve the problems generated by co-immobilized enzyme inactivation, the progressive immobilization of the least stable enzyme when its activity has decreased significantly.

For example, one of the strategies is coating the most stable immobilized enzyme with an ionic polymer and immobilizing the least stable enzyme on top of that [94,96,125] (Figure 6).

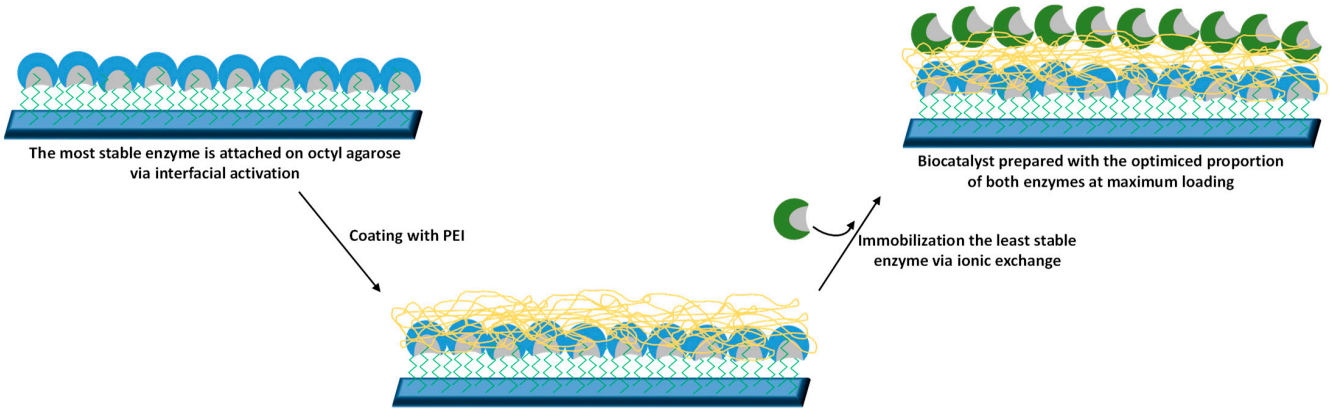


Figure 6. Preparation of a combi-biocatalyst where the most stable enzyme may be reused. First, the most stable enzyme is immobilized on the support. Then, it is coated with PEI, and finally, the least stable enzyme is immobilized on the PEI layer. The least stable enzyme may be selectively released by using high ionic strength.

It should be considered that the polymers may coat the whole surface of the support, even if not all of the support surface is occupied by the most stable immobilized enzyme, enabling the strategy of not saturating the support with the enzymes and immobilizing more of the fresh least-stable enzyme when its activity decreases (Figure 7). When the support surface is saturated with the least-stable enzyme, if the more stable co-immobilized one is still with high activity, it may be recovered and used to produce a new combi-biocatalyst after releasing the inactivated least-stable enzyme [94,96,125] (Figure 7).

The immobilization of the least-stable enzyme in multi-layers built using ionic polymers when the initial layer of the least stable enzyme has been inactivated could also be an interesting solution for this problem [102–104,114] (Figure 8). Still, it should be considered that diffusional problems might occur for the more stable enzyme (that is immobilized on the surface of the support, below the least stable enzyme and polymer layers) when the second layer of the least-stable enzyme is produced due to the increase of the tortuosity of the path the substrate must follow to reach the enzyme immobilized on the support surface [105–108] (Figure 9). This can reduce the overall activity of the biocatalyst, even while maintaining the ratio of active enzyme molecules, but may be adequate in some instances. In this way, a more specific investigation is required.

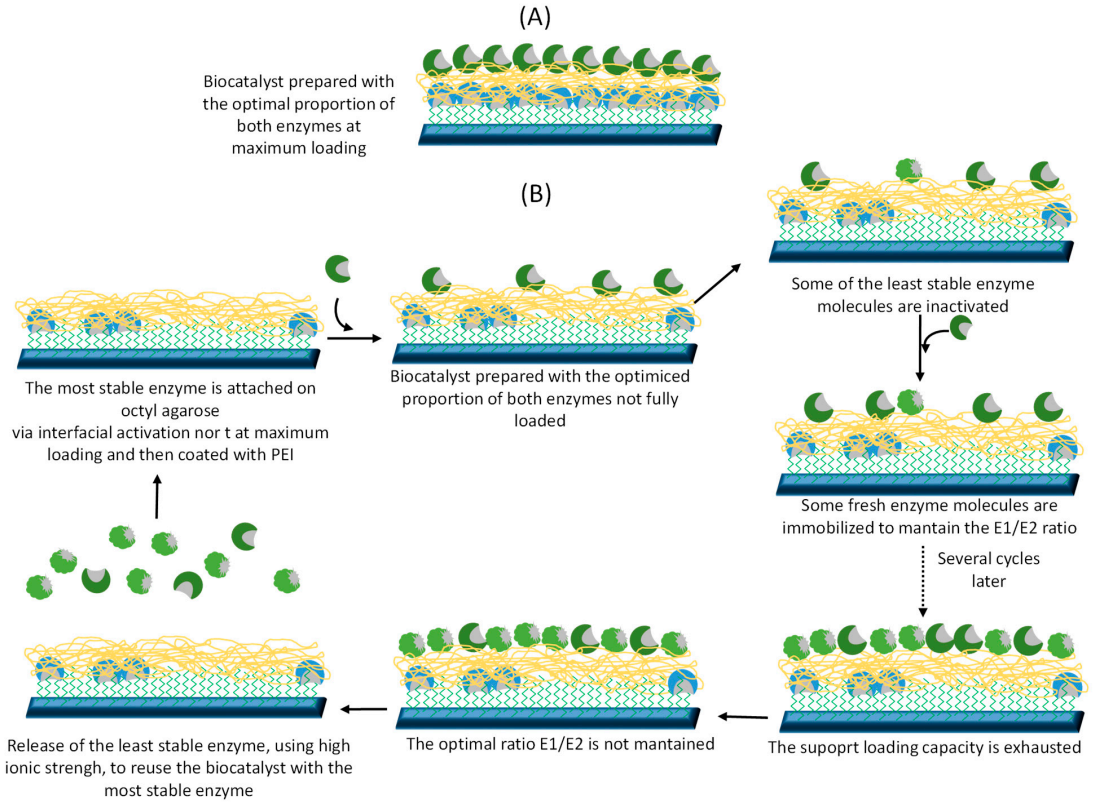


Figure 7. Compatibility of the combi-biocatalysts prepared following Figure 6 with the strategy of using an enzyme loading under the maximum loading and adding more of the least stable enzyme when required, but with the possibility of reusing the most stable enzyme to prepare more combi-biocatalyst. (**A**) Biocatalyst prepared under the optimal initial conditions at the maximum loading. (**B**) Biocatalyst was prepared considering the inactivation of the least stable enzyme to keep the necessary E_1/E_2 ratio.

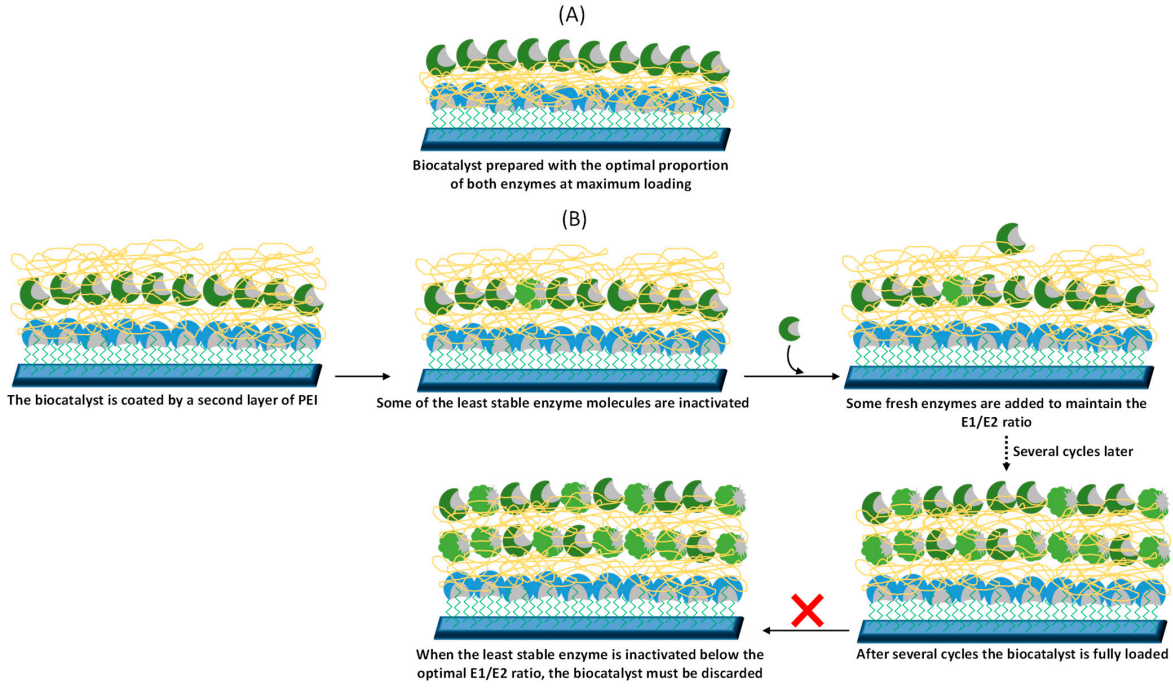


Figure 8. Use of multi-layer combi-biocatalyst to permit the maintenance of the enzyme activity ratio. (**A**) Biocatalyst prepared under the optimal initial conditions at the maximum loading. (**B**) Biocatalyst prepared to permit further immobilization of the least stable enzyme to maintain the E_1/E_2 rate ratio.

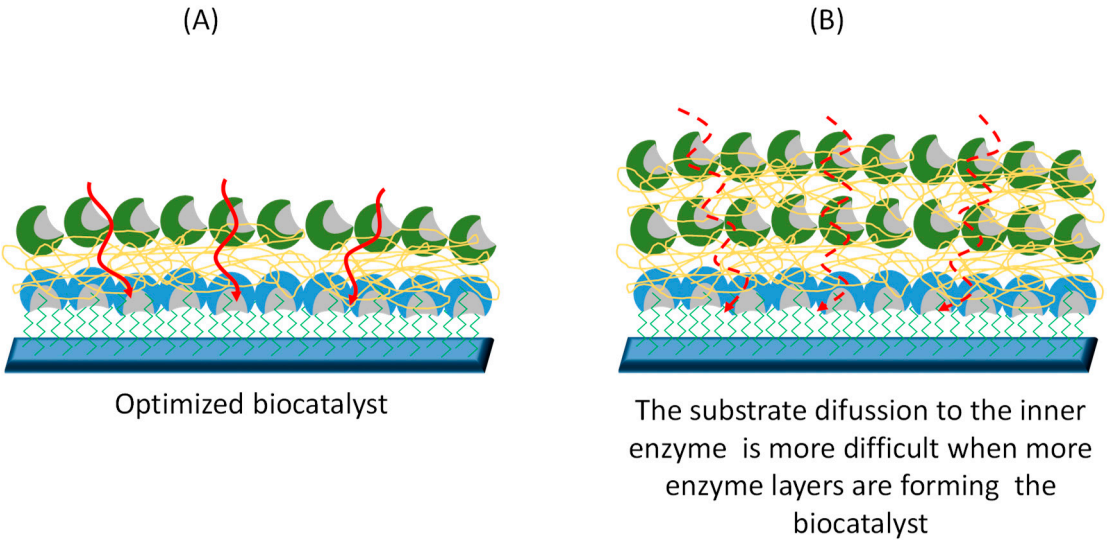


Figure 9. Increased tortuosity results in a different lifetime for the substrate to the enzyme immobilized on the support surface when using a multi-layer biocatalyst. (**A**) In a biocatalyst with one or two layer or enzyme the substrate diffuses with relative ease through the layers. (**B**) When more layers are added the substrate may experiment diffusional problems when reaching the deepest layers.

Similarly, the enzyme-by-enzyme co-immobilization of lipases using octyl-glyoxyl-supports (Figure 10) is compatible with this strategy of adding the least stable enzyme when its activity decreases by leaving a percentage of the support surface available to immobilize it in the initial combi-biocatalyst [95,126] (Figure 10).

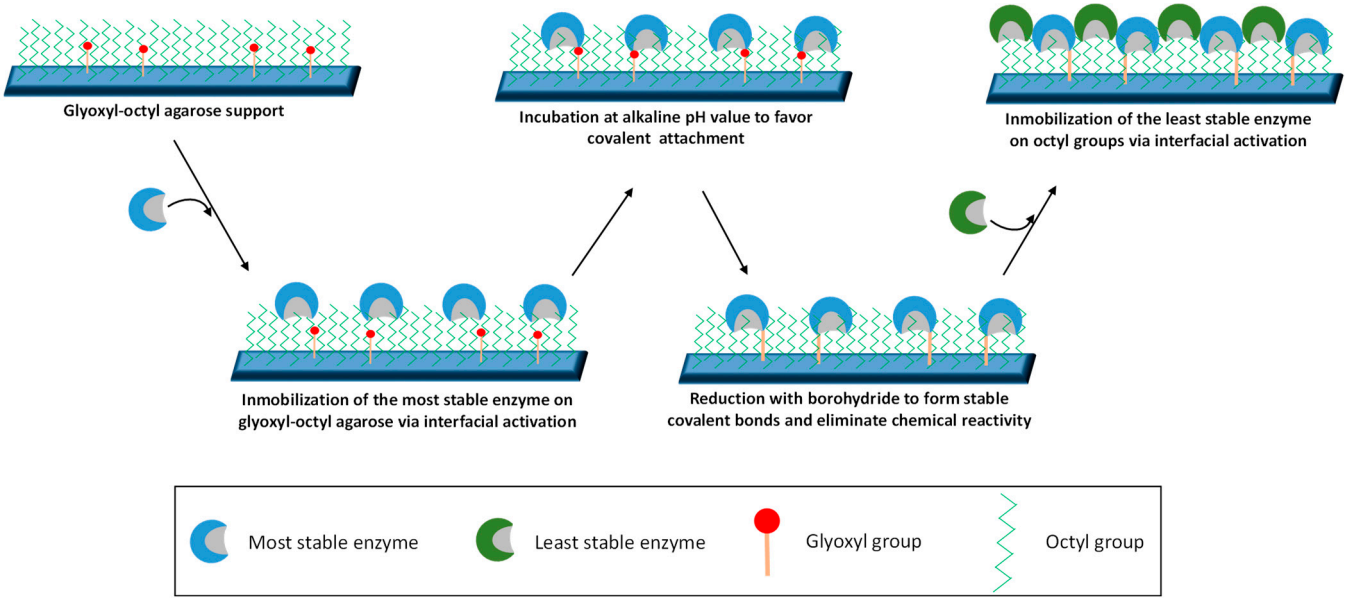


Figure 10. Using octyl-glyoxyl supports to prepare a combi-biocatalyst by lipase immobilization enables the reuse of the most stable enzyme to prepare a new combi-biocatalyst after the least stable enzyme inactivation. The most stable enzyme is covalently immobilized, the support is reduced to eliminate the chemical reactivity, and then the least stable enzyme is just physically immobilized via interfacial activation. The least stable enzyme may be selectively released by using detergents.

After saturating the support surface with the least stable enzyme, it is possible to release it from the support and recover the most stable immobilized one to produce a new combi-biocatalyst similar to that used in cycle 1 [95,126] (Figure 11). The same may be

said of using the strategy based on the step-by-step co-immobilization of enzymes using supports activated with divinyl sulfone [109,127] (Figure 12) that are fully compatible with this strategy (Figure 13).

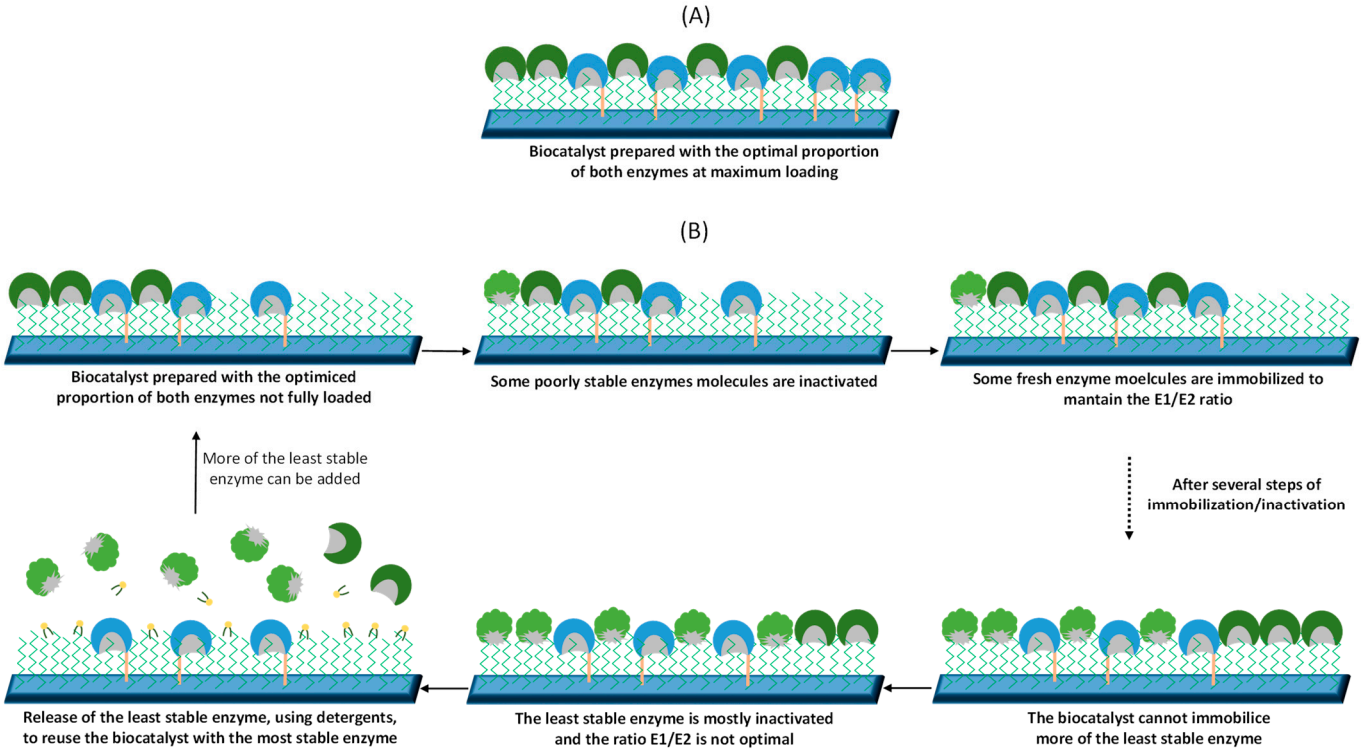


Figure 11. Compatibility of the combi-biocatalysts prepared following Figure 10 with the strategy of using an enzyme under the maximum loading and adding more of the least stable enzyme when required, but with the possibility of reusing the most stable enzyme to prepare more combi-biocatalyst. (**A**) Biocatalyst prepared under the optimal initial conditions at the maximum loading. (**B**) Biocatalyst was prepared considering the inactivation of the least enzyme to keep the optimal E_1/E_2 rate ratio.

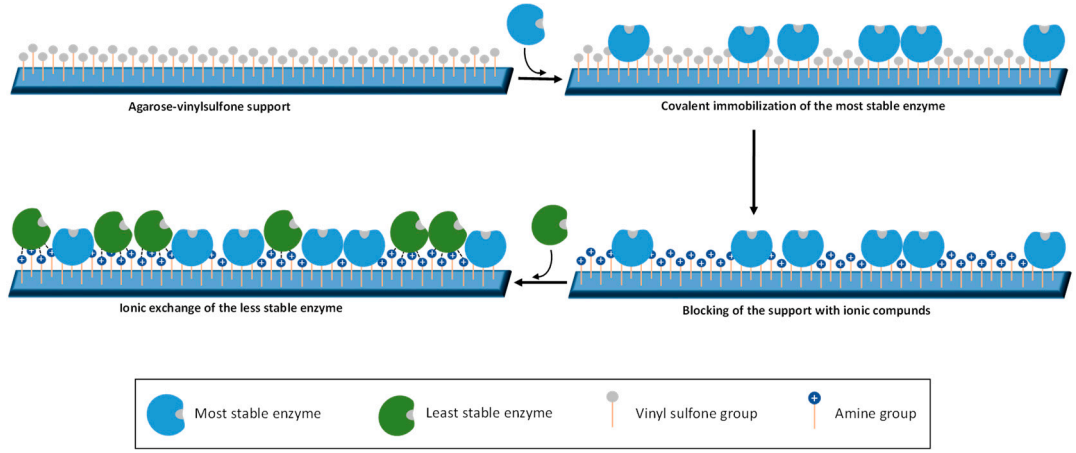


Figure 12. Use of vinyl-sulfone activated support to prepare combi-biocatalysts that enable the reuse of the most stable enzyme after the least stable enzyme inactivation. The most stable enzyme is covalently immobilized in the vinyl-sulfone groups. The support is blocked with ionic compounds, and the least stable enzyme is immobilized solely via ionic exchange. After inactivating the least stable enzyme, it may be released by incubation at high ion strength. The more stable enzyme may be reused to build a new combi-biocatalyst.

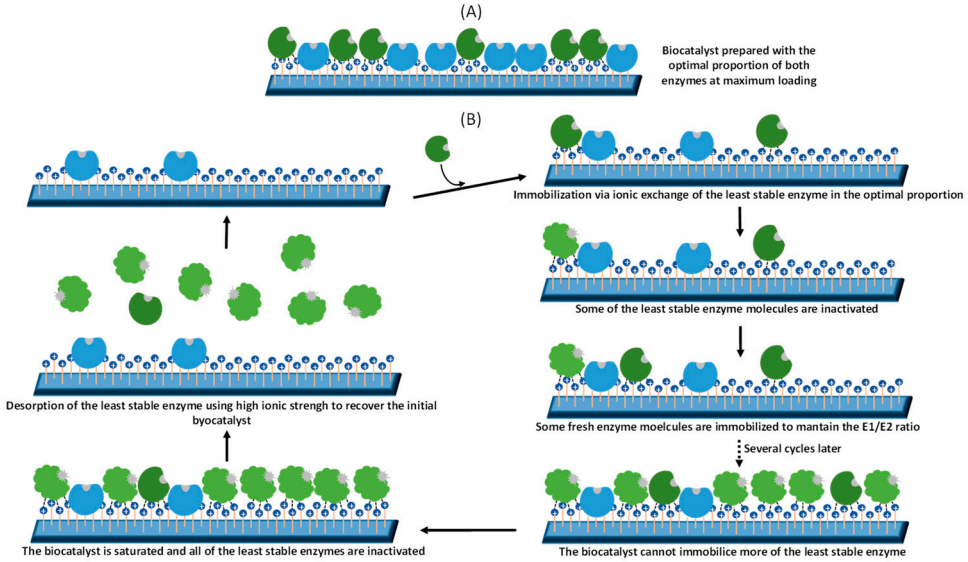


Figure 13. Compatibility of the combi-biocatalysts prepared following Figure 3 with the strategy of using an enzyme loading under the maximum and adding more of the least stable enzyme when required, but with the possibility of reusing the most stable enzyme to prepare more combi-biocatalyst. (**A**) Biocatalyst prepared under the optimal initial conditions at the maximum loading. (**B**) Biocatalyst prepared considering the inactivation of the least stable enzyme to keep the optimal E_1/E_2 rate ratio.

These strategies are also compatible with some of the simpler solutions offered in the previous section. For example, adding fresh combi-biocatalyst when the initial combi-biocatalyst is partially inactivated to maintain the least stable enzyme activity in cases where this is possible (e.g., if the intermediate is stable). After inactivating the least stable enzyme to a defined level, we can add more combi-biocatalyst, although one of the enzymes remains fully active. When we decide that the amount of biocatalyst in the reactor has reached the maximum, we can release the least stable enzyme, recover the more stable one and still, with a high activity level, use it to build a new combi-biocatalyst. On the other hand, if the least stable enzyme is not stable even under storage conditions, then the only problem is that we will have an excess of the most stable immobilized enzyme that should be stored. Therefore, the least stable enzyme should be immobilized when the fresh combi-biocatalyst is added to the reactor.

This way, some solutions may be applied to solve operational performance and reuse the more stable enzyme to build a fresh combi-biocatalyst.

5. Concluding Remarks

From the problems presented in this paper, it seems evident that when designing a combi-biocatalyst, even under simplified conditions (e.g., not considering differing kinetic features of the involved enzymes nor the existence of diffusional matters in the initial reaction), new problems and perspectives from the point of view of biochemical engineering and design of the biocatalyst are opened.

One point to be considered in the design of combi-biocatalysts is that the researcher should focus on improving the stability of the least-stable enzyme. This will mean submitting this enzyme to more exhaustive pre-immobilization procedures, such as site-directed mutagenesis, directed evolution, use of biodiversity to identify alternative enzymes, etc., immobilization (selecting the optimal protocol for this weak point of the chain), or even post-immobilization (submitting the immobilized enzyme to further chemical or physical modifications) stabilization strategies. In this way, the impact of dissimilar activities on the performance of the biocatalyst can be minimized.

The simplifications adopted in this initial approach to the problem to clearly explain the problems raised by the dissimilarity of enzyme stabilities may not be realistic in many

cases to give solutions to the different situations raised in the paper. Furthermore, in many cases, changes in the kinetic properties of the remaining least-stable enzyme will also need to be considered [97,98]. This means performing careful studies of the biocatalysts under different operational inactivation levels (inactivation under stress conditions may not be fully equivalent). Moreover, the first reaction may present some diffusional limitations (substrate diffusion limitations, pH gradients). In these instances, the decrease of enzyme 1 activity will reduce them. This may affect the calculations to recover the initial combi-biocatalyst performance and the activity of the second enzyme (mainly if pH gradients exist). In this way, again, deep characterization of the performance of the enzymes at different levels of inactivation needs to be performed. A handful of possible solutions may be the easy way to find one which works in a given case.

In general, if a combi-biocatalyst is considered just as a unique entity (i.e., if we can discard any significant change of the enzyme properties during inactivation (except activity), the addition of fresh combi-biocatalyst may be a straightforward way to keep the reactor performance during operation, even if this means over-spending on the most stable immobilized enzyme. We have proposed more sophisticated strategies to solve this problem, trying to reduce costs; the optimal option will depend on the cost of materials and operation in each case.

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