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# Multienzyme-Catalyzed Processes: Next-Generation Biocatalysis

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**Abstract:**

**Biocatalysis has been attracting increasing interest in recent years. Nevertheless, most studies concerning biocatalysis have been carried out using single enzymes (soluble or immobilized). Currently, multiple enzyme mixtures are attractive for the production of many compounds at an industrial level. In this review, a classification of multienzyme-catalyzed processes is proposed. Special emphasis is placed on the description of multienzyme *ex-vivo* systems where several reactions are carried out by a combination of enzymes acting outside the cell. Furthermore, reaction and process considerations for mathematical modeling are discussed for the specific case where the synthetic reactions are carried out in a single reactor, the so-called multienzyme '*in-pot*' process. In addition, options for multienzyme '*in-pot*' process improvements via process engineering and enzyme immobilization technology are described. Finally, enzyme modification via protein engineering is also discussed, such that a better compatibility of the enzymes in the reactor is achieved as a means of assisting the implementation of multienzyme '*in-pot*' processes.**

## Introduction

Most examples of biocatalysis in industrial organic synthetic schemes are found in the pharmaceutical sector.[1] Nevertheless, new opportunities are now arising in the synthesis of lower-value chemicals and biofuels. The reason for such scientific and industrial interest in biocatalysis is due to the exquisite selectivity that can be achieved under mild (and therefore 'green') process conditions.[2,3] Recently, a number of reports have documented the combination of biocatalytic and chemocatalytic methods (heterogeneous and homogeneous) to exploit the advantageous selectivity of biocatalysis alongside the advantageous productivity of chemocatalysis.[4] The resulting processes are frequently more sustainable (being based on either the principles of green chemistry and/or the use of renewable resources).[5,6] Interestingly, the use of renewable feed-stocks results in starting materials that are highly functionalized, and here too, selectivity is an enormous advantage, providing interesting opportunities for biocatalysis.[7] Therefore, provided the cost of the enzyme is low enough, there are today many opportunities for commercial exploitation leading to cost-effective and green processes.[3,8] Existing infrastructure means that it will take time to come up with replacement processes for all but the highest value products. Nevertheless, combined with protein and genetic engineering options for improving the biocatalyst and its production, respectively, we are now at the point that biocatalysis can be considered an established area of catalytic technology.[9−12] However, when implementing a single-step biocatalytic process, it is often seen that the conditions for that single step, while very favorable for that step itself, are frequently different from the others in the synthetic sequence. This raises an obvious question about whether it would be possible to catalyze several, if not all, of the steps using enzymatic methods and thereby minimize changes to conditions through the process. In nature the question has already been answered since 'cell factories' do this with great efficiency. However, for synthetic chemical schemes there are some challenges with implementing such a concept. Several reviews on multienzyme synthetic reactions have been published which start to discuss some of these challenges.[8,13,14] Such reports all contribute to the development of the next generation of biocatalytic applications which include effective cascade reactions, integrated deracemization, and cofactor recycling.[14−17]

In this report a classification of multienzyme-catalyzed processes is proposed. Special emphasis is placed on the description of the multienzyme '*in-pot*' process concept, highlighting the benefits and challenges of this particular application. In order to achieve feasible and optimal implementation of this type of process, mathematical modeling will be required. Indeed, modeling promotes a thorough understanding of what modifications to the conditions for the enzymes are required in order to optimize the process. Considerations

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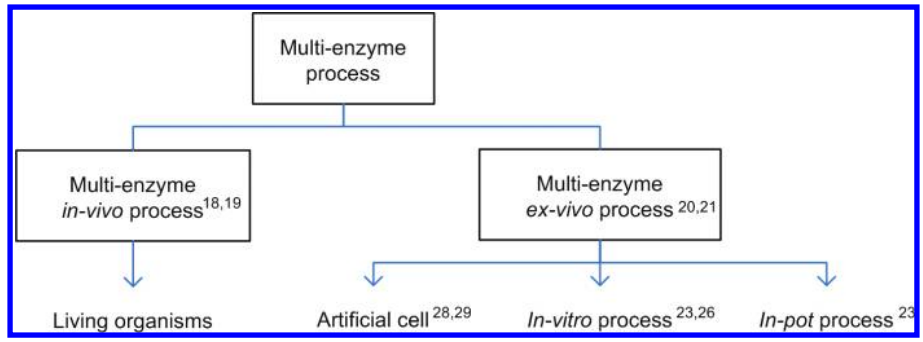


***Figure 1.*** **Classification for the multienzyme processes.**

for modeling are discussed, and finally the implementation of improvement strategies via process engineering, immobilization technology, and protein engineering are suggested as areas that can facilitate or improve the implementation of multienzyme '*in-pot*' processes. For example, improvements in enzyme properties could be achieved via protein engineering,[12,18] or in a similar manner, specific reactor configurations could be realized by new process technology and effective process engineering.[19−21]

## Multienzyme Processes

Multienzyme processes use two or more enzymes to catalyze reactions in a defined pathway via a cascade, a parallel, or a network configuration.[22] Historically, the term 'multienzyme' has most commonly been used to describe the metabolic activity of living microorganisms using established, modified, or *de novo* pathways in the context of biosynthesis.[23,24] However, for organic synthesis, multienzyme processes outside the cell hold particular promise since individual enzyme expression and regulation may be decoupled from the metabolic network. In this way at least two isolated enzymes can be combined in an optimal way, driving the synthesis towards a primary product.

A hierarchical classification of multienzyme processes is shown in Figure 1. In the first division, multienzyme processes are distinguished as multienzyme *in vivo* processes to describe a combination of enzymatic reactions that are carried out inside the cell, and multienzyme *ex-vivo* processes to describe a combination of enzymatic reactions that are carried out outside the cell. A second division is shown for the multienzyme *ex-vivo* processes. These may be classified as artificial cells, multienzyme *in vitro* processes, or multienzyme '*in-pot*' processes. According to the characteristics of each multienzyme process, there are different options available to operate them in single or multiple reactors, as shown in Figure 2. Clearly, the metabolic network of a cell cannot be decoupled into individual reactions, and then it is not feasible to carry out the process in more than one reactor. However, it is also the case that all

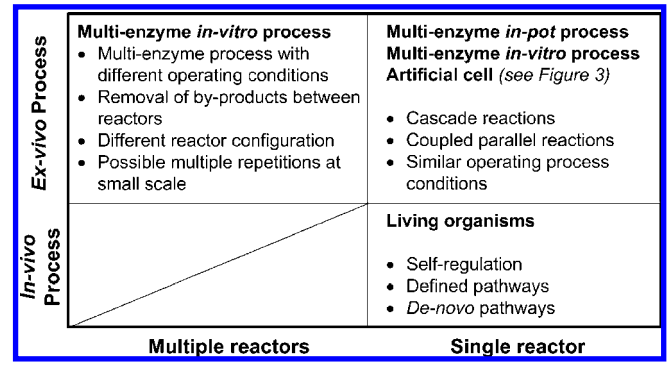


***Figure 2.*** **Opportunities for multienzyme processes performed in a single or multiple reactors.**

multienzyme processes have the potential to be carried out in a single reactor which could bring advantages with respect to the operability of the process. Further discussion about each case is addressed in the following sections.

Characteristics related to the catalyst constraints, process modeling, monitoring, and controllability among others are listed in Table 1. It is clear that several advantages may be realized by working with multienzyme *ex-vivo* processes. For example, modeling of *ex-vivo* processes is simpler than modeling the complex mechanism of the cell, making it more likely to be reliable for the monitoring and control of different variables during the process.

## Multienzyme Ex-Vivo Processes

An isolated enzyme acting out of the cell can be considered as an *ex-vivo* process. Consequently the combination of several isolated enzymes in a single system can also be labelled a multienzyme *ex-vivo* process. For organic synthesis, this approach holds particular promise since individual enzyme expression contributes in the system to drive a given transformation to the subsequent one until the desired product is obtained.

The application of multienzyme *ex-vivo* processes has been explored since the 1970s,[13,14,29] although it was not until 2003 that Bruggink and co-workers published their seminal review.[14]

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***Table 1.*** **Comparison of multienzyme *in-vivo* process and multienzyme *ex-vivo* process in a single reactor**

| characteristic | in-vivo process[25,26] | ex-vivo process[22,27,28] |
|---|---|---|
| Cell/Biocatalyst Constraints | | |
| substrate inhibition | possible | possible |
| product inhibition | possible | possible |
| catalytic stability | low | higher (if immobilized) |
| cost production | low | high |
| Reaction Constraints | | |
| reaction reproducibility | variable | reproducible |
| by-products | possible | unlikely |
| operating conditions | high dependence | high dependence |
| Process Modeling | | |
| process understanding | mechanism not fully understood | possible |
| reaction structure | complex metabolic networks | simpler reactions |
| mathematical model interpretation | difficult | possible |
| Potential Process Controllability | | |
| regulatory control (t, ph, dot) | possible (if online monitoring) | possible |
| supervisory control (concentrations) | difficult (if intermediate products) | possible |
| Process Monitoring | | |
| online measurements | possible | possible |
| intermediate products | unlikely | possible |
| Downstream Processes | | |
| product recovery | possible (if extracellular product) | possible |
| recycling (cell/biocatalysts) | possible | possible (if immobilized) |
| Others | | |
| Green/renewable process | Yes | Yes |
| Current research activity | High | Low |

Interestingly, the application of cascade conversions was the central theme of this paper, and this was supported by a compilation of different cases achieving cascade catalysis via the use of not only multiple enzymes but also multiple chemocatalysts and even combinations of enzymes and chemocatalysts. In a more recent overview, Findrik and Vasic-Racki (2009) again emphasize the importance of developing multienzyme processes. Furthermore, the necessity of using mathematical models and simulation tools was also introduced by these authors as a means to achieve process understanding and optimization.[13] Several processes have now been successfully proven at a laboratory scale for *in situ* cofactor regeneration,[30,31] deracemization,[15,16] and cascade catalysis.[13,14,32] Furthermore, a more limited number of cases have also been reported at pilot and industrial scale.[14] Consequently, although relevant conversions using multiple enzymes have been proven as a valuable concept, only a few scaled examples exist, and therefore, there remains a significant potential for industrial application that has not yet been fully realized.

As previously mentioned, three different types of multienzyme *ex-vivo* processes can be distinguished. Figure 3 shows a scheme for each multienzyme *ex-vivo* concept. They are defined as a multienzyme artificial cell, a multienzyme *in vitro* system, and multienzyme '*in-pot*' system. Historically, each category has contributed to the conceptual evolution of this topic.

**Artificial Cells.** Early work on multienzyme processes was developed in so-called 'artificial cells', which may be described as a solution of multiple enzymes contained in a microcapsule using an ultrathin polymeric membrane of cellular dimensions (about 20 $\mu$m in diameter). In such cases, the diffusion of the substrate, intermediates, and product through the permeable membrane is possible, while remaining impermeable to the larger enzyme molecules.[33] A schematic representation of this type of system is shown in Figure 3a. In an analogous way to enzyme immobilization by encapsulation, the enzymes are kept in solution inside the membrane, and then the free movement of enzymes within the capsule facilitates reaction.[29,34] Such a concept has been used for detoxifiers, immunosorbents, blood substitutes, and drug carriers, among other medical applications.[33,35]

An example of such an application is illustrated by the production of 6-phosphogluconolactone, as shown in Figure 4.

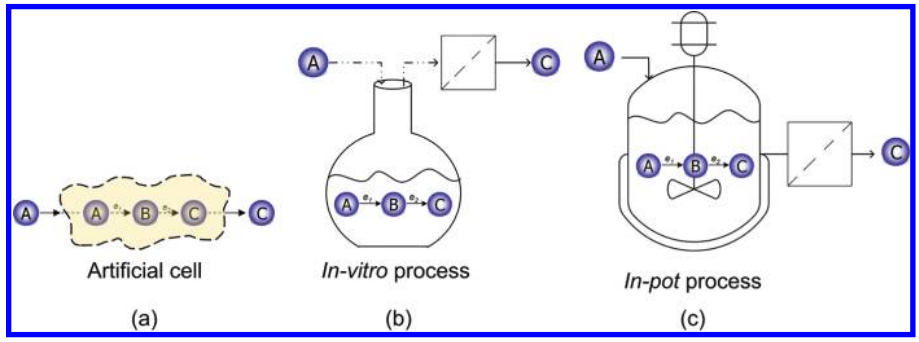


***Figure 3.*** **Types of multienzyme *ex-vivo* processes according to the process scale-up in a single reactor. Dash-dotted lines (—··—··) represent possible supply of substrate and removal of product. A represents substrate; B, intermediate; C, product.**

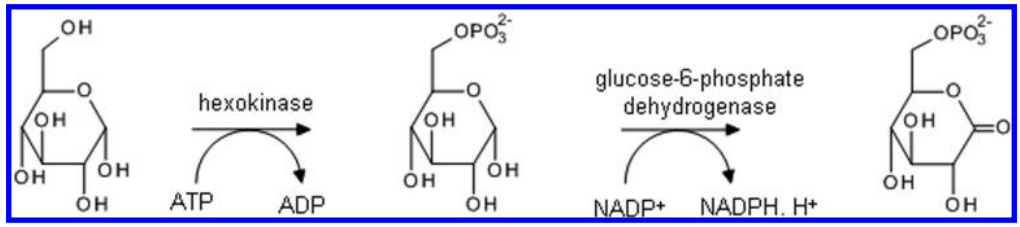


***Figure 4.*** **Bienzymatic (hexokinase and glucose-6-phosphate dehydrogenase) reaction for the production of 6-phosphogluconolactone using glucose as the substrate and glucose-6-phosphate as the intermediate product.**

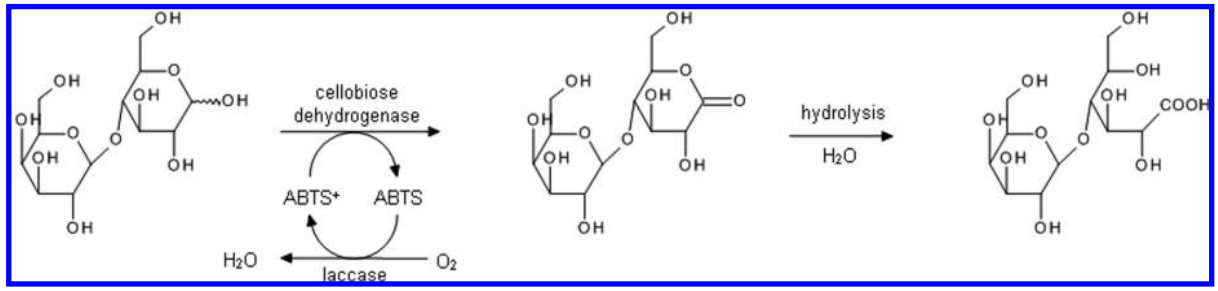


***Figure 5.*** **Reaction scheme for the production of lactobionic acid using lactose and oxygen as substrates, two enzymes (CDH and laccase) and an intermediary redox mediator (ABTS).[31]**

Here, two sequential reactions are carried out using two enzymes, hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (G6PD) (EC 1.1.1.49), respectively. The time dependence of NADPH formation was analyzed in the coupled reaction comparing both the encapsulated format of the enzymes and the free enzymes. Results demonstrated that the encapsulated system showed faster dynamic behavior, reaching steady-state more rapidly than the corresponding soluble system.[36]

Today, the term 'artificial cells' is at the center of a wider scientific discussion, since the concept has changed over the years, together with philosophical considerations of what constitutes a living cell.[37,38] On the other hand, for chemistry the multienzyme concept has evolved into the study of a feasible mixture of enzymes that can perform simpler reactions in a single reactor promoting better possibilities for mathematical modeling and, consequently, process optimization.[13,21,31]

**In-Vitro System.** To date, most of the knowledge on multienzyme processes has been obtained from multienzyme *in vitro* processes.[13,14] Although most of these reactions have been carried out at laboratory scale, the information obtained is actually of considerable value for reaction engineering since laboratory operation has promoted the analysis of individual enzyme and reaction characteristics. As illustrated in Figure 3b, multienzyme *in vitro* systems are mostly operated in batch mode, and the main objective to date has been to analyze optimal conditions and compatibility of the enzymes in the mixture in order to make them work under similar environmental conditions. In this way, the reactions drive the required pathway towards the desired product.

Most applications of multienzyme *in vitro* systems have been developed for *in situ* redox cofactor regeneration and cascade catalysis. Particular attention has been devoted to cofactor regeneration since many biochemical reactions require one or more cofactors to activate the enzymes and thus to carry out conversions effectively.[13,17] For example, in redox biocatalysis, the proper combination of enzymes enables the fast recovery of the cofactor such that it can be reused multiple times. This gives a significant cost reduction, since many cofactors remain expensive. In a similar way, metal or chemical intermediate redox mediators, which are also widely used, can be better exploited since smaller amounts are then required in the system. As a result, cofactors are no longer compounds that limit the desired reaction, and therefore, higher conversions of the main substrates can be achieved.[10] For example, an intermediate redox regeneration has been reported for the bienzymatic production of lactobionic acid.[31] As shown in Figure 5, the first enzyme (flavocytochrome cellobiose dehydrogenase (CDH)(EC 1.1.99.18)) catalyzes the dehydrogenation of lactose to lactobiono-$\delta$-lactone, which is spontaneously hydrolyzed to lactobionic acid, and the second enzyme (laccase (EC 1.10.3.2)) allows the full reduction of the available oxygen to water. Furthermore, the double action of the redox mediator (2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)) is exploited, regenerating the oxidation states of both enzymes.

In this case, a mathematical model was formulated to describe the kinetics of the process. Furthermore, the model allowed successful prediction of the system behavior carried out in a minireactor with integrated, bubbleless oxygenation. Interestingly, the work used a graphical tool to determine the optimal process conditions for the bienzymatic conversion with oxygen as a cosubstrate. In this case it is clear that modeling contributed with a better process understanding.

On the other hand, cascade catalysis is often required in many important synthetic routes,[39−43] for example, the trans-

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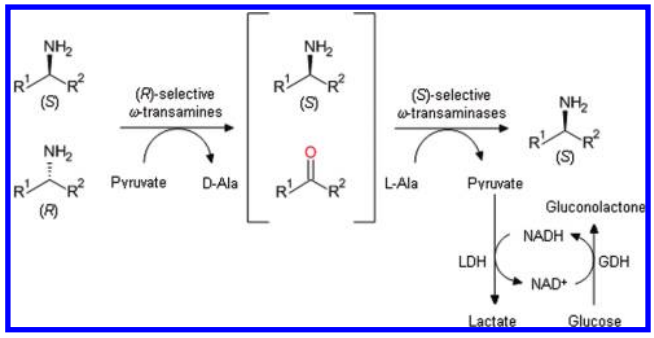


***Figure 6.*** **Reaction scheme for the production of optically pure amines employing two *ω*-transaminases with opposite stereopreference, lactate dehydrogenase (LDH) for pyruvate removal and glucose dehaydrogenase (GDH) for cofactor regeneration.[16]**

formation of D-methionine into L-methionine using four enzymes in a cascade[30] where a mathematical model was also formulated and validated. Likewise, the synthesis of a non-natural carbohydrate (5-deoxy-5-ethyl-D-xylulose) from glycerol using four enzymatic steps was also carried out in *in vitro* using a pH switch method which temporarily reduced the action of one enzyme in the system at a given time.[44]Several reports in the scientific literature offer overviews of specific types of reactions and the relevant laboratory procedures.[8,13,14]

Another illustrative example is the deracemisation of α-chiral primary amines to optically pure amines by the action of specific *ω*-transaminases (EC 2.6.1).[15] Deracemisation is achieved by a one-pot, two-step procedure, as shown in Figure 6. In the first step, kinetic resolution of the chiral racemic amine is carried out by an *ω*-transaminase to yield an intermediate ketone and the residual optically pure amine. In the second step, the ketone intermediate is transformed into the amine by employing alanine as the amine donor and an *ω*-transaminase displaying the opposite stereo preference to that in the first step. In addition in the second step, lactate dehydrogenase (LDH) (EC 1.1.1.27) is used to remove the pyruvate (byproduct) in order to shift the reaction equilibrium to the product side. Here two methodologies were applied. In the first case, the second enzyme was added to the system after the kinetic resolution of the first step was complete. At the end of the conversion, the optical purity of the final product was moderate. In the second case, the conversion was improved by introducing a heat treatment before starting the second step. For such a case, the desired enantiomer was obtained with ee values >99%. From the point of view of multienzyme *ex-vivo* processes, the first case is clearly an interesting study to analyze since high interaction between the enzymes was found, while in the second case both enzymatic reactions were completely decoupled even though the conversions were carried out in a single reactor.

## Multienzyme 'In-Pot' Processes

The so-called multienzyme '*in-pot*' process describes a system to carry out synthetic reactions using two or more enzymes in a single reactor. This option is very well suited to run in an integrated fashion since the conditions in each reaction (i.e., media, concentrations of substrates and products, catalyst pH, catalyst temperature) are typically well matched. When a process operates with this concept, the catalytic activity of all the enzymes working together can be exploited, and thus, a substrate transformed to a first intermediate product can be used by another enzyme and so on, in cascade, parallel, or network reactions. As a further advantage, separation and purification steps of intermediate products are eliminated.[14] Consequently, the multienzyme '*in-pot*' approach potentially leads to considerable process improvements such as a reduction in downstream processing and operating costs. This concept is illustrated in Figure 7. The first part (a) illustrates a process in separate biocatalytic steps, while in the second part (b) the entire sequence of biocatalytic reactions is carried out in a single reactor.

Given a feasible mixture of enzymes, the multienzyme '*in-pot*' process can be analyzed to explore the optimal conditions of the process. This includes the reactor design, operating conditions, and process control.[45] The characteristics of the mixture of enzymes must also be considered. For example, the format of the enzymes (soluble, immobilized, or a mixture of both) as well as the number of phases present in the system needs to be identified. The best selection is achieved by choosing the optimal conditions and compatibility of each reaction in the whole system. Therefore for best operation in the multienzyme '*in-pot*' process it is necessary to focus on process analysis and control such that the process is maintained at the optimal point where productivity can be improved. Most of the reported cases of multienzymatic processes are mainly at laboratory scale,[14] although there are a few exceptions such as the continuous multikilogram-scale production of L-amino acids (e.g., L-methionine, L-norleucine, and L-2-aminobutyric acid).[14]

A number of challenges need to be addressed in order to achieve all the benefits of this approach; one technique to assist in this development is the use of computational tools to simulate the desired process under different conditions. However, as a

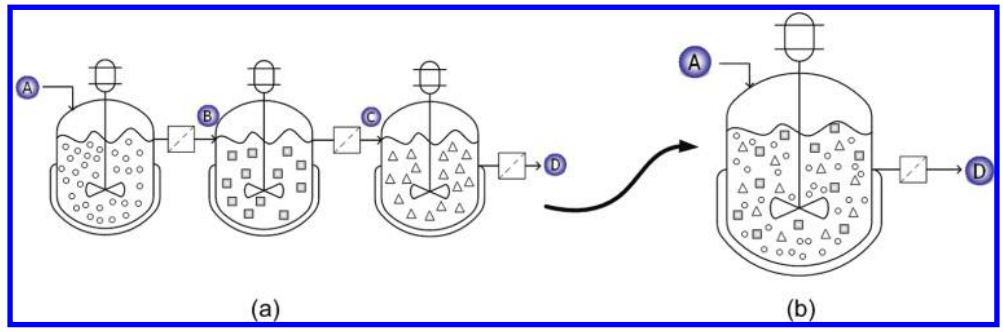


***Figure 7.*** **Three-enzyme process: enzyme 1 (○), enzyme 2 (□), and enzyme 3 (Δ). (a) Process carried out in single biocatalytic steps. (b) Multienzyme '*in-pot*' process to carry out the reactions in a single reactor.**

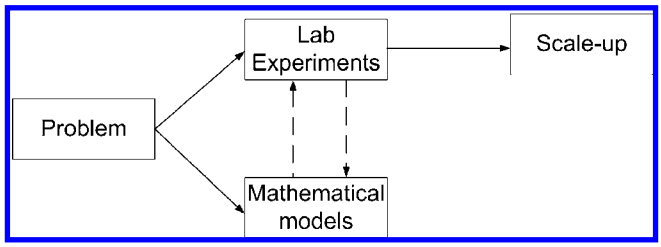


***Figure 8.*** **Basic steps for process developments.**

basis for the simulation, a mathematical model needs to be built and solved, and then the question is whether such models are reliable enough to guide decision making. Applying a systematic framework, the mathematical expressions are formulated, whereas the final model structure will depend on the decisions taken during the analysis of the reaction and process characteristics (as shown in Figure 9) that are related to the real process. Having built a model, the next step is to validate it on the basis of experimental data. Subsequently, a validated model will provide the basis for scale-up. Otherwise, experimental design or model reformulation must be considered again, as shown in Figure 8.

**Process Modeling.** Mathematical process models are tools that describe and contain information about process behavior and the effects of physical conditions.[27,46,47] In the case of a multienzyme '*in-pot*' process, a mathematical model can be the key to exploit the potential of this approach since the individual enzymes may still operate under unfavorable conditions, even though the overall process is optimal. This is well illustrated by the synthesis of non-natural carbohydrates from glycerol which are produced by the use of four enzymes. In this application, the activity of some enzymes was "switched off" by increasing the pH from 4 to 7.5 and "switched on" again by lowering the pH back again to 4.[44] Interestingly, such a procedure enabled the successful production of the desired product, while at the same time maintaining the stability of the enzymes even though some of them were not active at certain times during the overall conversion.

In order to implement a multienzyme '*in-pot*' process, it is advantageous to formulate a mathematical model before proceeding with experimental implementation, because modeling gives an opportunity to evaluate the process feasibility, e.g. analyzing different scenarios such as the operating conditions and alternative reactor configurations. Thus, the effect on the performance can be analyzed in order to explore and clarify advantages as well as limitations of a given process.[20,45] As a result, process feasibility could be proven conceptually ahead of more detailed experimentation, e.g. formulating a reliable experimental design. Frequently, models are formulated for well-known and implemented processes. Some examples, already mentioned before are the production of lactobionic acid[31] and L-methionine,[30] where mathematical models were formulated and successfully validated with experimental data. Nonetheless model simulation could be used as a relatively cheap option to start exploring the realization of new ('*in-pot*') syntheses.[21,27] This also enables the integration of process understanding (e.g., dynamics, productivity, controllability, stability, etc.) and model-based process design as opposed to empirical process design. One example is the bienzyme model formulation and simulation for the production of optically pure lactone. In that case, 'windows of operation' were used as a tool in order to identify feasible economic scenarios.[48] In a similar manner, the bienzyme modeling of aminotriol/aminodiol synthesis was developed. Here, the enzymes transketolase−transaminase (TK−TAm) were used, and the effects of the TAm/TK activity ratio were analyzed by simulation to identify optimal process operation.[49]

An often asked question about modeling is related to the model complexity. Indeed, it is an important issue to address before the model is formulated and implemented. The primary reason is that model complexity is a function of the model purpose.[50] Hence, simple models can be generated for general structural process understanding with limited output capabilities and therefore requiring less accurate input information. In contrast, dynamic models to be used in process optimization, controller performance evaluation and prediction, typically require a more complex model structure. Clearly, good quality input information is required to obtain reliable results. Some of the common modeling objectives could be arranged in increasing order of complexity thusly: structural understanding, exploratory simulation, experimental design, optimization, process control, prediction.[45]

**Modeling Considerations.** In order to define the structure of a mathematical model, a number of different considerations must be carefully discussed. The formulation of any kind of model is obviously built in an iterative manner, because every decision made in one step can affect the decisions in the subsequent steps, and therefore, model reformulations are constantly required.[45] In the case we discuss here, the evaluation is based on the relation between reaction and process characteristics, as shown in Figure 9.[28,51] For a multienzyme process, this evaluation is critically important to achieve a better understanding of the process and to achieve useful modeling and process design.

The following characteristics are listed for the reaction considerations:[20]

• *Knowledge about the compounds involved*: physical properties of the compounds involved in the reaction must be

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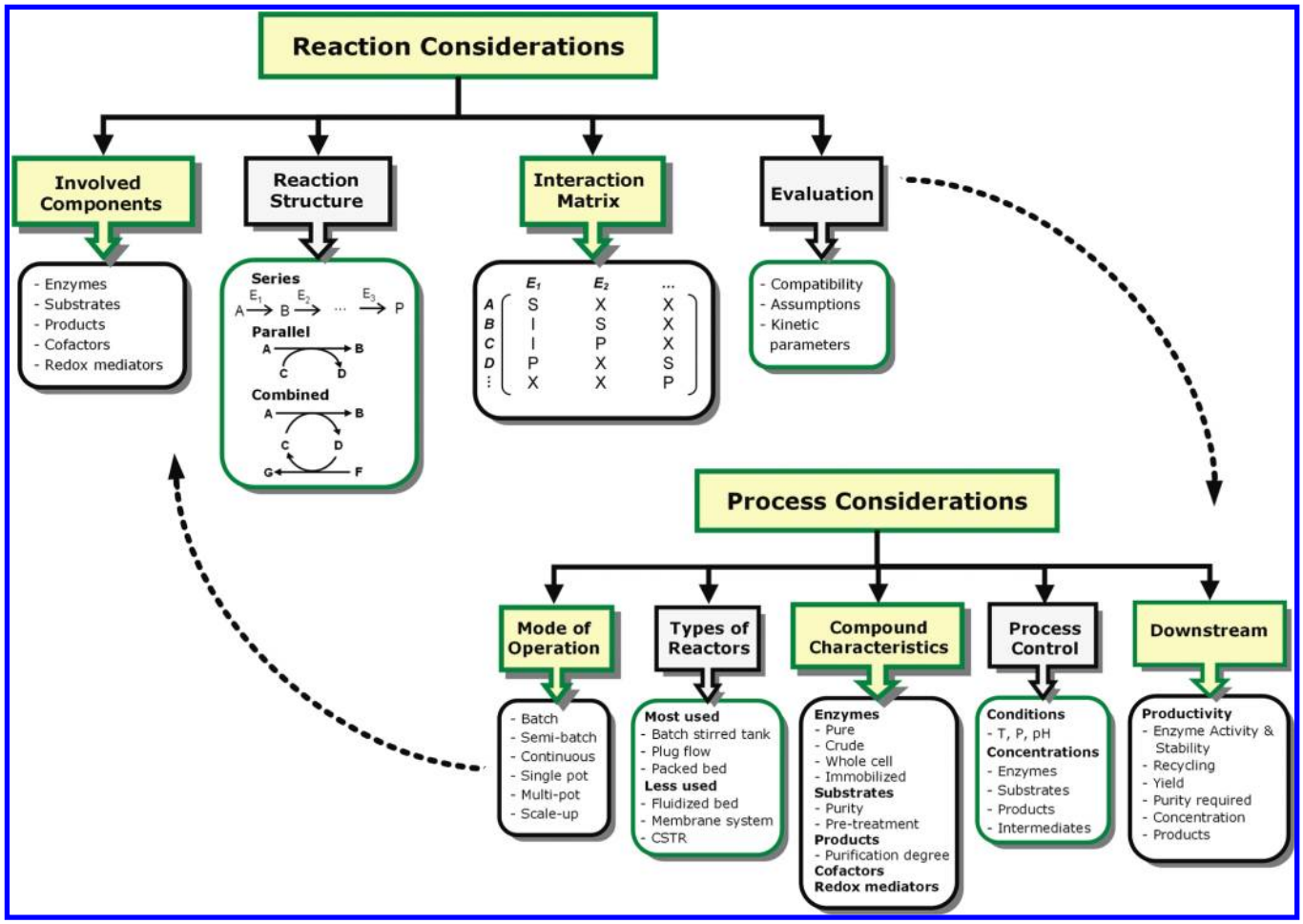


***Figure 9.*** **Reaction and process characteristics to be considered for modeling of multienzyme '*in-pot*' processes.**

known, and for such a task the use of existing databases facilitates the compilation of the available information.[52]

• *Structure of the reaction*: identification of the possible routes to the desired product is the basis for describing the structure of the reactions in the process. The description can be made by the combination of reactions in series or coupled parallel reactions.

• *Interaction matrix*: the compounds involved in the process (i.e., substrates, intermediates, byproduct, products, etc.) are arranged in rows (i.e., A, B, C, ...), and the enzymes ($E_i$) are arranged in columns (for $i = 1, 2, 3, ...$). In this way, the matrix is filled, defining the relationship between each compound and a given enzyme, i.e. for substrate (S), for product (P), for inhibitor (I), or in the case that no interaction between one compound and one enzyme exists (X) (see Figure 9). This compiled information is extremely useful to make decisions for kinetic expression formulations and process design, since relationships unique to a one-pot situation will be identified.

• *Evaluation*: during the modeling procedure, evaluation points are required in order to analyze the feasibility of the selected considerations in the other steps, resulting in model reformulation if necessary.

In the same manner, process considerations are listed as follows:

• *Operating mode*: the selection of the operating mode is related to the liquid exchange characteristics. It is especially relevant when inhibitory effects are present in the system. These relationships can be obtained from the interaction matrix defined for the given reactions.

• *Type of reactor*: the selection of a proper reactor must consider not only technical aspects but also the practical ones. The large number of feasible reactors is reduced by including the physical characteristics and constraints of the system, such as the number of phases involved, media compatibility, enzymatic rates, and product or substrate inhibition. Likewise, the configuration is partly defined by the format of the enzyme(s) (soluble or immobilized) in the process.

• *Component characteristics*: in addition to the physical properties of the components, it is also necessary to provide specifications such as purities of substrates, concentrations and amounts of cofactors, enzyme format (e.g., the whole cell, isolated, immobilized).

• *Process control*: this can be divided in two basic control layers.[53] The regulatory layer manages variables such as pH, temperature, dissolved oxygen (DO); consequently, simple controller designs can be implemented. The supervisory layer manages variables with more impact on the process, such as concentrations of the compounds. In this case more detail in the controller design is required. In general, process control

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***Table 2.*** **Advantages and disadvantages of different reactors for multienzyme '*in-pot*' process**

| characteristic | advantages | disadvantages |
|---|---|---|
| STR | soluble and immobilized enzymes[a]<br>multiphase media<br>good control possible<br>no complex model<br>simplicity of construction<br>easy to clean | possible inactivation of enzymes<br>difficult enzyme recycle |
| PFR | soluble enzymes<br>high conversion | no immobilized enzymes<br>single phase<br>complex model |
| PBR | soluble and immobilized enzymes[a]<br>low enzyme damage<br>high conversion<br>enzyme recycle/separation/exchange | thermal gradient<br>poor control<br>complex model |
| FBR | soluble and immobilized enzymes[a]<br>good enzyme mixing<br>good control possible<br>enzyme recycle/separation/exchange | possible inactivation of enzymes<br>constraint of particle size and density |
| MBR | soluble enzyme<br>low enzyme damage<br>high conversion<br>in-situ separation<br>retention of enzymes/cofactors<br>dosing of a reactant<br>compartmentalization | complex model<br>poor control<br>membrane fouling<br>flow rate restrictions |

[a] Different enzyme configurations are possible by combining soluble and immobilized enzymes in different ways (see Figure 10).

ensures the system maintains optimal operating conditions to achieve the desired process yield and product quality.

• *Downstream processes*: different options can be evaluated according to the required purity of the desired product. The required productivity of the process also plays an important role since it is directly related to the efficiency of the enzymes, yield, recycling, compound purities, etc.

## Reactor Selection

For multienzyme mixtures, different reactor options can be envisaged according to the system characteristics.[28,51] In general, there are two major classes of reactors dependent on the reaction characteristics as well as the model of the process.[19] The first class is formed by the stirred tank reactors (STR) where a homogeneous mixture of all the compounds is assumed; furthermore, the mathematical description of the system in this class is usually achieved by a set of ordinary differential equations (ODEs). The second class contains reactors that are characterized by a concentration gradient through the reactor, e.g. plug flow reactors (PFR), packed bed reactors (PBR) and fluidized bed reactors (FBR). For these reactors, the mathematical description is achieved by means of partial differential equations (PDEs), in the case of a dynamic model.[45] Furthermore, a membrane bioreactor (MBR) can have characteristics of both classes, dependent upon design. The main requirement for a membrane reactor is a semipermeable membrane which allows the free passage of the products while retaining the enzymes and/or cofactors.[54]

The selection of a proper reactor should in principle first consider a large list of existing reactors. However, this list soon narrows down to only a few reactor types when both physical constraints of the desired process and the precise model scope are taken into consideration.[28,55] One important criterion for multienzyme '*in-pot*' reactors is how the combination of enzymes will be arranged in the system, i.e. as soluble or immobilized enzymes or a combination of both.[56] The decision is made according to the characteristics of each enzyme, i.e. kinetics, media compatibility, catalytic stability, operation conditions, and cost. Some advantages and disadvantages of some of the likely reactor configurations are compiled in Table 2. For example, a mixture of soluble enzymes can be carried out in a STR or a PFR in cases where enzymes are not too expensive, negating the need for recycle. The situation is a little different with a PBR, where different configurations of the reactor can be envisaged according to the enzyme format (soluble/immobilized). Figure 10 shows some reactor schemes for different enzyme arrangements. For example, Figure 10d shows one feasible case to operate a PBR; here some enzymes are immobilized and others are soluble in the input flow. One of the advantages described for the MBR is the dosing of a reactant (Table 2). This is well illustrated in the case of the multienzyme production of lactobionic acid.[31] In that case, a membrane bioreactor was used for the biocatalytic reaction and an oxygen-permeable membrane was used for bubble-free

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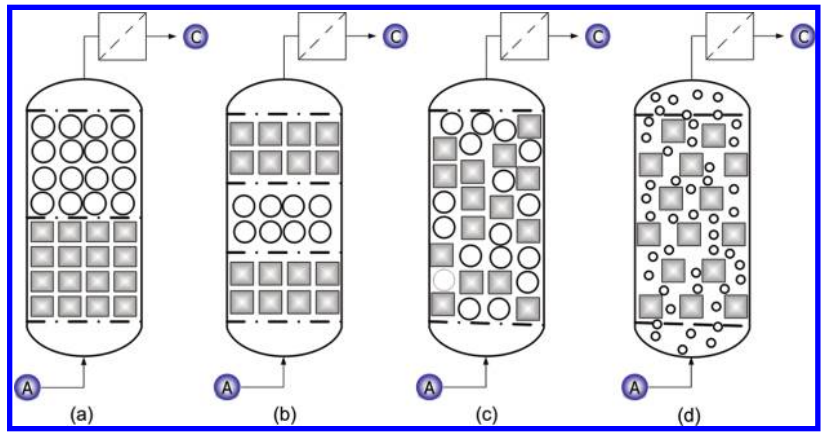


***Figure 10.*** **Immobilized enzyme configurations in a packed bed reactor for multienzyme in-pot processes for two enzymes. (a) Two beds, (b) alternate beds, (c) mixed enzyme bed, and (d) one enzyme bed plus one soluble enzyme. Enzyme 1 (○) and Enzyme 2 (□).**

oxygenation of the reaction system which improved the stability of the enzymes.[57]

## Implementation of Multienzyme 'In-Pot' Processes

In order to implement feasible, scalable, and economic multienzyme '*in-pot*' processes, it is useful to understand which improvements to the catalyst or the reactor or the process are required and how these can be achieved. Process engineering, enzyme immobilization, and protein engineering all have a role to play in improving the process such that it can be implemented effectively. Each of these areas is addressed in the following sections.

**Process Engineering.** Process engineering mainly involves decisions related to reactor design, operating mode, control, and optimization of the process. Currently, all these areas are mostly supported through systematic computer-based methods which facilitate and speed up the implementation of a new process.[58] However, relevant decisions still need to be taken in a realistic manner in order to achieve feasible process implementation. In the case of multienzyme *in-pot* processes, the characteristics of each enzyme and its compatibility in the whole system play an important role in the selection and design of a suitable reactor. As mentioned in the reactor section, standard stirred tank reactors, reactors with concentration gradients, and membrane reactors are commonly used to carry out the reactions using a mixture of enzymes. However, the proper configuration, based on the enzyme format (soluble/immobilized) and operating mode, could open a vast number of possibilities where innovative reactors could enable the combination of more enzymes that can naturally drive the reactions towards the desired product. Some of the relevant process characteristics have been listed in Figure 9. However, even when the reactor has been carefully chosen, together with the operating mode, process control must still be considered. This is necessary to achieve stable real-time operation, consistent product quality, robustness against disturbances, and an optimal operation at predefined set points. For multienzyme '*in-pot*' implementation, process control facilitates stable process operation, especially in cases where limitations of substrate, intermediate product, and product concentrations can result in inhibition or inactivation of the enzymes. Furthermore, a well-designed control system can also handle model uncertainties.[45]

**Enzyme Immobilization Technology.** In a manner similar to that of protein engineering, immobilization of enzymes seeks to achieve better enzyme performance. However, in this case, changes are not made to obtain new enzyme properties. Instead, the mobility of the enzyme is limited by applying a chemical or physical treatment in order to improve the existing characteristics.[59] It is important to orient the enzyme such that steric hindrance is avoided. For multienzyme '*in-pot*' applications, the immobilization of one or more of the enzymes is often essential in order to assist separation and recycle. It can also contribute by improving the productivity of the process, with increased stability and compatibility of the enzymes in the common media.[56,60] Further benefits accrue from the ability to reuse the enzymes, activate or stop the reactions rapidly (by adding or removing the enzymes from the reaction solution, respectively), easier downstream processing, no product contamination with the enzyme, and a considerable process cost reduction.[61] Hence, the issues to be addressed are how should the mixture of enzymes be immobilized? Should it be done individually for each enzyme, or should several enzymes be immobilized on the same support? Which immobilization methods are best to achieve high activity of all the enzymes involved? Material science will also have a role to play via the introduction of novel materials with tailor-made properties that can be used for successful or improved immobilization of enzymes. An interesting example is the covalent immobilization of an enzyme−cofactor−enzyme system. Here, lactate dehydrogenase (LDH) (EC 1.1.1.27), glucose dehydrogenase (GDH) (EC 1.1.99.10), and the cofactor NADH were incorporated into two porous silica glass supports. Effective regeneration cycles of NADH/$NAD^+$ were observed, and enzyme activities were improved when smaller pores were used. Thus, the nanoporous structure of the glass supports could enhance the molecular

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interactions among the immobilized enzymes and cofactor, thus improving the catalytic efficiency of the system.[62,63]

**Protein Engineering.** As the target of multienzyme in pot processes is the combination of different enzymes in one reactor, it is usually necessary to have them work in an efficient and stable manner at the same pH, temperature, and media conditions.[22,64] In reality, the activity of several enzymes do not have a single optimal operating range; i.e. each enzyme acts best around its own optimal pH and temperature values.[65] Furthermore, enzymatic activity is highly sensitive to operating conditions. Consequently, the penalty of enzyme incompatibility can be observed by a lower activity for some enzymes, inhibitory and inactivation effects, deterioration of enzyme structure, and loss of stability. Therefore, in most cases a compromise is required.

Nevertheless, protein engineering can provide an alternative approach since its main scope is based on developing useful and valuable proteins by improving the existing characteristics of a protein or generating new properties.[18,66,67] Consequently, specific changes to the enzyme characteristics can be effected such as adjusting the enzyme activity to be optimal at a given pH or temperature, or making the enzyme reaction rate higher by modifying the kinetic ($K_m$ and $V_{max}$) values. Further characteristics that can also be engineered are the protein folding (structure) that offers thermodynamic stability as well as thermal and environmental stability, and the protein function that includes improvements in binding properties, catalysis performance, and selectivity.[11,12] For example, enzyme improvements were developed for an esterase where the enzyme thermostability was increased without compromising its catalytic activity at lower temperatures.[68]


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## Future Outlook

Biocatalysis today is growing not only in the pharmaceutical sector but also in the production of chemical and bulk products. Consequently, new process demands are revealed, and therefore the next generation of processes will involve new configurations such as chemoenzymatic and multienzymatic systems. In this review it has been discussed that relevant multienzyme *in-pot* processes have a significant potential for industrial application. The current knowledge of these processes supported by mathematical modeling and computational tools is promising for future implementation and process scale-up. It has been discussed that the modeling of these processes brings several benefits such as (i) understanding of the interaction between enzymes, substrate(s), and product(s) in a multienzyme '*in-pot*' process and (ii) the possibility of performing a large number of simulations of the process in order to study different scenarios and conditions of the process. Consequently, a better experimental design can be formulated, saving experimental time and effort.

In the long term it is clear that multienzyme processes will over some decades replace many processes which today are wholly chemically catalyzed processes. Ultimately it is interesting to consider all the enzymes required for an entire process, expressed in a single cell used to effectively produce the desired product. To date several steps have been taken towards this,[37] but in the meantime there are many reactions where combinations of isolated enzymes will be required in multienzyme processes. Here the regulation and control of enzyme activity can be assisted by immobilization on one or more supports and innovative reactor design. Nevertheless several challenges remain for multienzyme processes despite the strong drivers for greener and ever more effective chemical process technology. In this contribution several of the challenges and potential solutions have been discussed along with perspectives on the particular role of mathematical modeling, process technology, and protein engineering. Given the availability of powerful tools to effect new processes, such as those described in this report, it can be expected that in the coming period many more examples of multienzymatic syntheses will make the transition from laboratory curiosity to industrial economic process.


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