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Design of enzymatic cascade processes for the production of low-priced chemicals

<https://doi.org/10.1515/znc-2018-0190>

Received November 22, 2018; revised January 4, 2019; accepted January 7, 2019

Abstract: While the application of enzymes to synthetic and industrial problems continues to grow, the major development today is focused on multi-enzymatic cascades. Such systems are particularly attractive, because many commercially available enzymes operate under relatively similar operating conditions. This opens the possibility of one-pot operation with multiple enzymes in a single reactor. In this paper the concept of modules is introduced whereby groups of enzymes are combined in modules, each operating in a single reactor, but with the option of various operating strategies to avoid any complications of non-productive interactions between the enzymes, substrates or products in a given reactor. In this paper the selection of modules is illustrated using the synthesis of the bulk chemical, gluconic acid, from lignocellulosic waste.

Keywords: biocatalyst; biocatalytic reactions; multi-enzymatic cascade; reaction modules.

1 Introduction

Today, the use of enzymes to effect single synthetic steps has become well established in the chemical industry. Numerous examples can be found in the synthesis of high-priced pharmaceutical products and even some low-priced bulk chemicals [1–4]. More recently, the use of cascades of enzymes (operating sequentially) has been used to mimic what happens in Nature, where metabolic pathways in microorganisms produce primary, as well

as secondary, metabolites [5]. Today, with the advent of numerous genetic techniques, entirely new non-natural pathways can be built de novo. Indeed, combinations of enzymes can be applied not only linearly but also orthogonally as well as in parallel, to enable redox-neutral catalysis, employing auxiliary enzymes [6]. The advantages of such an approach are mainly related to the possibility of overcoming the problems of inhibitory (or toxic) cascade intermediates, to shift unfavorable reaction equilibria, and to decrease the number of reaction steps and unit operations, as recovery of cascade intermediates can largely be avoided [7–9]. In this way, far higher yields can be achieved and thereby lower costs and waste compared with classical step-by-step synthesis [8]. Likewise, possibilities for chemo-enzymatic cascades can also be of great interest [10]. However, for biocatalytic cascades, a particular feature of many commercial enzymes is that they operate under relatively similar environmental conditions (e.g. pH and temperature), meaning that the potential integration of such cascade systems into a single reactor becomes possible. For high-priced pharmaceutical products, the key drivers for implementation will be speed of process development and adhering to the strict regulatory requirements. However, for lower-priced products, the cost of production will be critical to the final decision concerning implementation. So, from the perspective of scale-up and industrial implementation of cascades for the production of low-priced chemical products, operation in a single reactor (thereby saving capital) looks very attractive [11–14]. Nevertheless, evaluation needs to be made in order to establish whether this is always the case. To date, this design task has not been addressed, but it is now timely as many cascades of interest are being operated in vitro, with the advantage of avoiding cellular constraints and time-consuming metabolic engineering. In particular, many enzymatic cascades reported in the literature are of interest en route to lower-priced products, starting from sustainable feedstocks. In this paper, a systematic methodology for designing an enzymatic cascade will be discussed and illustrated using the multi-enzymatic synthesis of the bulk chemical, gluconic acid. This example illustrates well the type of conceptual considerations required at an early stage, prior to more detailed simulations being carried out. The aim here is therefore to focus the options for more detailed design work.

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2 Gluconic acid and its production

Gluconic acid and its derivatives (i.e. sodium gluconate, calcium gluconate and D-lactone) have applications in the chemical, food and pharmaceutical sectors. Conventionally, gluconic acid was produced by fermentation, using either *Aspergillus niger* or *Gluconobacter suboxydans*. However, the growing demand for gluconic acid, with an annual growth rate of around 9% [15], has encouraged the study of alternative synthetic routes. Current production costs are reported around 1.2–8.5 USD/kg [15], where the fermentation substrate and downstream processing cost contribute to the majority of the cost [16]. In reality, around the 60% of the operational cost is defined by the substrate [17] and 30–40% by the downstream product recovery [18]. Alternative processes for the production of gluconic acid include chemical, electrochemical, catalytic and enzymatic oxidation. Many reports claim particular advantages for such methods [19–22], and these are summarized in Table 1. Of particular note is the commercial gold-catalyzed oxidation of glucose, where high productivities have been achieved of greater than 200 g/L h [23].

3 Proposed multi-enzymatic route for gluconic acid production

The potential industrial process for gluconic acid production conceived in our work starts from the perspective that large-scale production must use a sustainable feedstock, meaning one that is not only renewable but also of low cost and readily available. For fermentation, the most commonly used substrate is hydrolyzed corn starch [16],

which is supplemented with additional nutrients such as nitrogen and phosphorus [24]. In reality, both the carbon and the supplementary nutrients have an impact on the substrate cost [17]. Hence, by operating a nonfermentative system, the nutrient cost contribution can be much reduced. Furthermore, using hydrolyzed starch, whether produced from corn, wheat or potatoes, comes with risks as the use of these renewable raw material sources may unfortunately potentially threaten food supply.

Taking into account the cost of substrate on gluconic acid production, we argued that an alternative process using an enzymatic cascade, based on cellulose as the carbon source, would be interesting to consider. This has the potential advantage of the ability to use impure feedstocks, at high concentrations in a flexible process. This also suits potential application in a biorefinery context. Enzymes are highly selective catalysts, which do not require supplementary nutrients, and the requirements for aseptic conditions (while still present) are less rigorous than for a fermentation process. Furthermore, cellulose is a relatively cheap and available, renewable feedstock. The proposed enzymatic cascade uses a combination of cellulases, β -glucosidase, glucose oxidase (GOx) and catalase (CAT) to produce gluconic acid from waste cellulosic material.

The proposed reaction cascade is shown in Scheme 1 and starts with the production of cellobiose from cellulose by the action of cellulases. Afterwards, β -glucanase catalyzes the cellobiose hydrolysis, and glucose is generated. In the presence of GOx, glucose is further oxidized to D-glucono- δ -lactone, and finally, the gluconic acid is produced from this lactone via spontaneous non-enzymatic hydrolysis.

A particular feature of GOx is that it is a flavoprotein, which therefore requires an end-electron acceptor to oxidize the co-factor (FAD), previously reduced during

Table 1: Description and features of technologies used for gluconic acid production.

Production technology	Description	Features
Fermentation	Glucose fermentation by <i>Aspergillus niger</i> or <i>Gluconobacter suboxydans</i>	<ul style="list-style-type: none"> – Product yield: 80–85% on glucose – Substrate renewable but not sustainable – Rigorous aseptic procedures require
Chemical	One-step process, using H_2O_2 , O_2 and O_3 as oxidants	<ul style="list-style-type: none"> – Product yield: 60–80% on glucose – High byproduct formation (poor selectivity)
Electrochemical	Electrolysis of an acid solution in the presence of a halogen, such as bromide	<ul style="list-style-type: none"> – Product yield: 80–97% on glucose – Energetically demanding – Poor environmental impact
Catalytic	Catalytic oxidation using Pt, Pd or Au	<ul style="list-style-type: none"> – Product yield: 98% on glucose – High operation cost – Poor catalyst stability
Enzymatic	Glucose oxidation using glucose oxidase	<ul style="list-style-type: none"> – Product yield: almost 100% on glucose – High selectivity – Low byproduct formation (high selectivity)

Scheme 1: Proposed cascade for the synthesis of gluconic acid from cellulose.

glucose oxidation (Scheme 2). The established way to do this is to carry out the oxidation in the presence of molecular oxygen (acting as the end-electron acceptor). Molecular oxygen is also a particularly attractive oxidant on an industrial scale. A second feature of this reaction is the formation of the byproduct H_2O_2 from the reduction of the oxygen. As H_2O_2 inhibits GOx activity at relatively low concentrations, it needs to be removed upon formation, which can easily be achieved using another enzyme, CAT. CAT converts one mole of H_2O_2 into a mole of water, with the concomitant release of half a mole of oxygen. This means that in the net reaction (glucose to D-glucono- δ -lactone), only a half molecule of oxygen is required.

4 Module design

Potentially, all the enzymes in the proposed synthetic route could be used together in a one-pot system, as they

share a common operating range of pH and temperature (Figure 1). The use of such data is to establish at a very early stage whether operation in a single reactor is possible or some separation is necessary, either in time or in space (more than one reactor).

Nevertheless, simply because the operating windows of the various cascade enzymes overlap does not, in itself, justify the operation of the cascade solely in one reactor. Indeed, it may prove more efficient to operate that reactor with changes in conditions or enzymes over time. Operating in a single reactor with such changes we define as a reaction module. In other words, intermediate separation, and potentially catalyst recovery, only occurs between modules. Hence, the key question to address is whether operation should be separated in space or time. A further related question concerns the reactor choice and its operating mode. Options include stirred tank reactors (all the contents well-mixed) or plug-flow reactors (contents only mixed orthogonal to the direction of flow). The enzymes could be in solution or immobilized on a solid support.

Scheme 2: Enzymatic glucose oxidation for gluconic acid production.

Figure 1: Possible reaction condition operating window for cellulases and β -glucosidase (C- β g), glucose oxidase (GOx) and catalase (CAT).

Operation could be in batch, fed-batch or continuous mode. A broad discussion of characteristics, advantages, drawbacks and selection criteria of these topics can be found in the scientific literature [25, 26].

Analysis of the gluconic acid scheme discussed here identifies three potential process configurations. In all cases the use of batch stirred tank reactors and soluble enzymes were chosen as the process needs to be flexible

and operated at low cost. Indeed, the ultimate concept could be to install the first module near to the feedstock source, thereby saving on the transport costs of very dilute raw material. The three possible options are described beneath and illustrated in Figure 2:

- **Two module process, two reactors each at different conditions and with different enzyme combinations:** The first process (Figure 2A) consists of two modules using two bioreactors. In the first bioreactor, glucose is produced from cellulose using cellulase and β -glucanase. The operating conditions in this step will be pH 5.0, 50 °C, which are the best conditions for these enzymes. Both enzymes have a lower activity compared with GOx and CAT, so the concept we propose is to favor the operating conditions of cellulases and β -glucanase in order to obtain a higher reaction rate. Avoiding a compromise in conditions is only possible by separating the operation of the first two enzymes, from the last two, into two separate modules. When the process in the first bioreactor has finished, substrate is added to the second bioreactor where the production of gluconic acid takes place in the presence of GOx and CAT. In this step, the operational conditions will be pH 6.0, 40 °C, with a constant flowrate of oxygen at around 1 volume per volume per minute (VVM), to favor GOx activity. Although the operating window in Figure 1 shows that

Figure 2: Enzymatic cascade processes for production of gluconic acid. — — — — — ➔ Material added/removed at the beginning/end of the reaction. - - - - - ➔ Material added in the beginning or in the course of the reaction. (A) represents a two-module process, while (B) and (C) represent single-module alternatives.

GOx and CAT also have similar activity at the same pH and temperature as the first bioreactor, with those conditions the experiments show that GOx and CAT lost around half of their initial activity within 24 and 1 h, respectively [22]. In contrast, at pH 6.0, 40 °C, the same loss of activity took place after 100 h [22]. This also emphasizes that the screening done in Figure 1 is a necessary, but nevertheless insufficient, requirement for operation in a single reactor or module, as an evaluation of enzyme stability is also required.

- **Single-module process, single reactor with change in types of enzyme over time:** The second process (Figure 2B) is a one-pot system where cellulase and β -glucanase start the cellulose hydrolysis and GOx and CAT are added at different times during the process. The operational conditions will be pH 6.0, 40 °C, and air will be supplied at a constant rate of 1 VVM. Temperature and pH conditions were chosen to ensure adequate enzyme stability of GOx and CAT, as they will remain for long periods of time in the bioreactor, limited by the reaction rate of the cellulase and β -glucanase. These conditions could also be beneficial for cellulase and β -glucanase in the presence of lignin material in the substrate, because the use of a lower process temperature reduces the adsorption of these enzymes onto lignin [27]. Hence, the loss of enzyme activity can be reduced.
- **Single-module process, single reactor with change in operating conditions over time:** The third process (Figure 2C) is similar to the process presented in Figure 2B, with the difference now that the air flow rate will change over time. In our enzymatic cascade, the oxygen demand changes through the conversion. At the start of the reaction, the oxygen demand is highest but will then decrease. Bubbling air (or oxygen) is an expensive requirement and so should be optimized as much as possible. Therefore, using a specific feeding policy could help use the oxygen in the most efficient way possible and thereby reduce production costs.

In addition to the conditions that have been mentioned for the three process options outlined here, there are other decisions that need to be made to carry out each process. For example, selection of initial enzyme concentration, enzyme feeding policies, oxygen feeding policies and initial cellulose concentration are all essential for process optimization. It is not easy to make decisions to implement a process, when multiple configurations are possible. This is a major motivation for the development of modeling tools, whereby the process (described by kinetics and mass balances) can be evaluated in terms

of process performance metrics under alternative operating strategies. The use of modeling and simulation is particularly helpful because it allows a fast evaluation of many alternatives. Likewise, model implementation provides system understanding to improve the experimental design, because it is possible to identify bottlenecks and system constraints. Once an approximate process behavior is known, it is also easier to move on to the experimental part and with greater confidence. In the final stage of process development, these targeted processes will be simulated. Although beyond the scope of this paper, the conceptual framework for such a simulation environment is presented in the following section.

5 Evaluation of processes using simulation

Once an enzymatic cascade has been selected for the production of a desirable product, different operating conditions can be evaluated. In principle, very many different enzymatic cascade processes that can process alternatives (with different configurations and operating policies) can be analyzed, in the search for the optimal solution. The conventional methodology for process development uses experimental platforms to search for the best operating conditions [28]. However, this is time consuming, and it is always possible that the results do not show the best solution, especially in cases where there are very many possibilities. Conversely, there are approaches where all the possible process options are evaluated simultaneously using mathematical models [29]. Nevertheless, for this to be effective, a significant amount of information is required beforehand. Indeed, in biological processes in particular, the main limitation of this approach is the lack of information. To overcome this, hybrid methods lying somewhere between experimental and simulation methods have also been proposed. Their aim is to develop process models in a systematic and iterative way, where the process is adjusted until it meets established targets [30–32]. This iterative system allows a quick identification to focus on viable options, while eliminating non-viable options.

The approach that we propose therefore is for the design of an enzymatic cascade process to be based initially on a systematic and iterative methodology [31]. Figure 3 shows a schematic representation of a methodology, which is divided into three parts, termed A, B and C. Part A is the most time demanding, where the main sections (shown in gray) have an interaction in both

Figure 3: Proposed systematic method to evaluate promising conceptual processes.

directions, indicating that they work together to build the alternative processes that will be evaluated. The other sections (white) are related with decisions that need to be identified from the start, on the basis of that information. Assigning an implementation order to the various sections would be ideal, but in reality, this is unlikely to happen in the development of a conceptual design. Part B is the evaluation of the alternative processes, which are compared with the targets, in order to identify if the process is worthy of further investigation (in part C), or not.

6 Data requirements

Although it is not easy to define the step sequence in the construction of a conceptual process design, it is clear that the starting point is the information review and compilation related with the process. There are data that are required from the start, such as market size, alternative production processes, product price, physicochemical properties of the reaction system and information about the biocatalyst(s). Conversely, there is additional information that should be searched, dependent on the requirements of the experimental, modeling and simulation parts. Therefore, the information section has different roles during the conceptual process design. In general, the first stage should cover the justification of the study and the selection of a synthesis route, process performance indices and targets.

From an early stage of a process design, it is recommended that a process cost analysis should be made, because it can be used as a decision-making tool [33]. However, a cost analysis requires more time than can be justified at an early stage of design. A simpler alternative, in order to compare processes at an early stage, is the use of process performance indices, calculated from the operational profile and the final concentrations [34]. It is well known that volumetric productivity (g/L h) is a relevant economic index to compare processes. Nevertheless, in the case of biocatalytic processes, the biocatalyst cost also has a strong influence on the total production costs. The rate of reaction can always be improved by adding more enzyme (this means an improvement in the productivity), but increasing the concentration of enzyme involves increasing its cost contribution [14]. Therefore, a second economic index to be analyzed is the specific enzyme productivity, which is in terms of gram of product per gram of enzyme per hour ($\text{g}_p/\text{g}_e \text{ h}$). Related specifically to the gluconic acid example here, another aspect to take into account in the selection of performance indices is the high dependence on oxygen by GOx, related to the cost of bubbling oxygen in a bioreactor. This is an important economic aspect to be evaluated, as oxygen supply could be at different flow rates dependent on the reaction progress. Therefore, the last performance index to evaluate is the specific oxygen requirement calculated as gram of product per gram of oxygen per hour ($\text{g}_p/\text{g}_{\text{O}_2} \text{ h}$).

7 Modeling and simulation

This section of the methodology is required to define the models and simulation that will be implemented, taking into account the information that is available, the performance indices selected and the information that it is possible to access with the experimental platforms available. The most relevant activities to be developed in this section are shown as follows:

- To define the type of models to use,
- To decide the kind of bioreactor and process operation (continuous, batch, fed-batch), and
- To decide the process configuration (one module or more).

The first step in the model selection is to identify the main objective of the modeling. Although validated models with highly detailed phenomena descriptions can be used for process optimization, controller performance evaluation and prediction [35], in some studies this complexity level

is not required, and a model with less phenomenological detail will prove sufficient. Modeling strategies can be divided into white box, black box and gray box. The first uses an in-depth phenomenological knowledge of the process and is therefore highly predictive. Nonetheless, the development time for white-box models is significant. Conversely, black-box modeling often does not have reliable extrapolation properties, because it is developed mainly from experimental data in a defined operating window. Gray-box modeling is a combination of white box and black box, where the resulting models have good interpolation and extrapolation properties, without rigorous modeling or having to make so many experiments [36]. The modeling strategy that best fits the purpose here is most likely the gray box, using first principles models and experimental data for parameter estimation.

8 Conclusions

Enzymatic cascade systems provide promising approaches to more flexible routes to lower-priced chemicals. The flexibility arises not only from the perspective of using alternative feedstocks but also through the option of distributed manufacturing where individual biocatalytic modules can be physically separated. In the context of the illustrative example used here, this means that, for example, gluconic acid could be partially produced on a farm with excess bagasse, which might otherwise be burnt. However, the implementation of these systems to industrial applications can be a slow task, because of their complexity. Especially for low-priced products, each biocatalytic system will demand a significant work among different disciplines, where all share a common objective of optimization. For higher-priced products, this may not prove critical, but as lower-priced products come into focus, this will become increasingly important. The systematic methodology proposed here divides the design task into two. The first part is concerned with module identification and process synthesis. The second task is to simulate each process in such a way that it can be optimized against given constraints for a particular objective function. In this brief article the first of these steps was illustrated for gluconic acid production, where three alternative processes were identified.

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