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Contents

Introduction

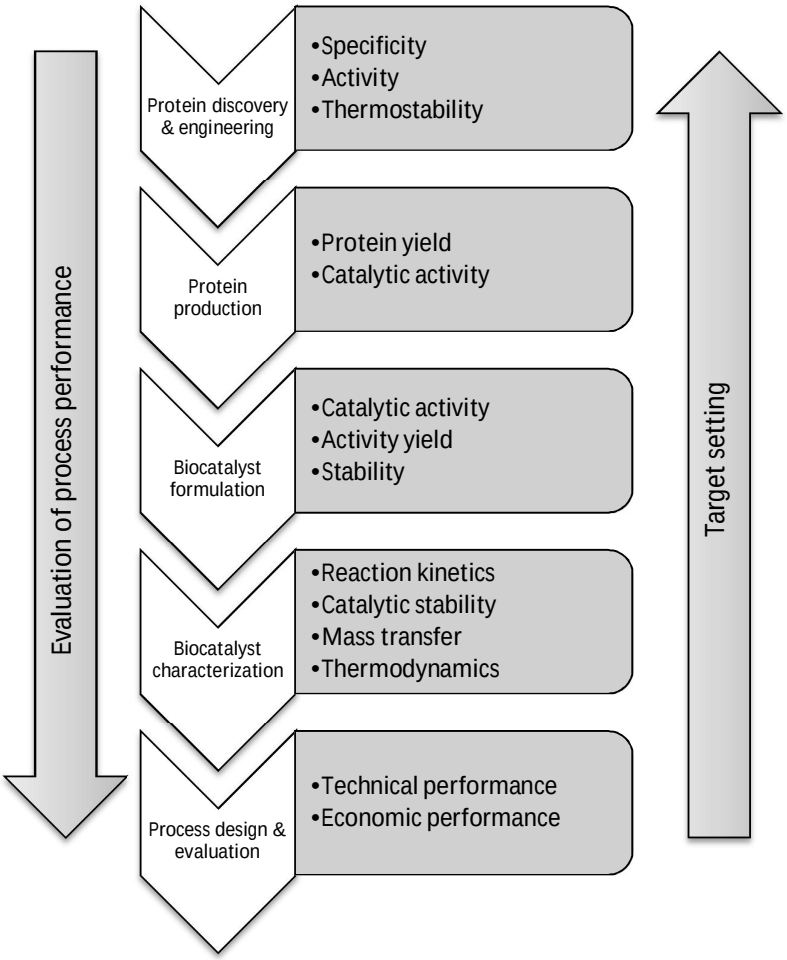
Biocatalytic reactions as an alternative to traditional chemistry for the production of chemicals have at this point been studied for many years (Schmid et al., 2001; Tufvesson et al., 2013). They are typically presented as being more selective, can operate at more benign and less hazardous conditions and, partially as a consequence of these characteristics, are said to have a lower environmental impact than traditional processes (Woodley, 2008). While these are broad statements, it has nevertheless been shown to be the case for many applications, and there are indeed several hundred successful implementations of biocatalytic processes (Blaser and Federsel, 2011; Drauz et al., 2012; Liese et al., 2006; Ramesh et al., 2016). However, in the literature there are also an enormous number of investigated biocatalytic reactions that have not been implemented commercially. Many of these studies are of course aimed at achieving a greater understanding of the reactions and the biocatalysts themselves, and as such benefit other work that is more closely linked to an application. That said, far more studies emphasize the applicability of the work they describe, than is ever actually implemented. Part of the reason for this, in our view, is a lack of understanding of the performance required (for implementation) from a reaction system depending on the intended application. Instead, the tendency is, perhaps understandably, to benchmark performance against other work in one's own field.

The viability of an intended process eventually comes down to its ability to manufacture the intended product at a cost such that it is outweighed by the market volume and the potential selling price of the product, with sufficient margin to cover costs associated with marketing, distribution and required profit. While the exact numbers involved in this will vary between individual cases, general guidelines can in fact be established for a given market value (type of product) and volume. Indeed, establishing such guidelines are a part of the continuing work in ROBOX.

Production processes for chemicals can be broadly grouped in to processes that make low, intermediate and high value products, with production volumes that are correspondingly large, intermediate and small. The distinction between the three groups is fluid, but for the purposes of this document a low value, high volume product is one where the value of the product necessitates a very efficient process that adds little cost to the starting material. At the same time, its production volume is large enough to permit a dedicated production line that can be tailored specifically to the process. At the other end of the scale is the low volume product that is produced in shared equipment, but has a high value which means that the demands on process performance are much more relaxed. Finally, there is an intermediate case where product value is lower but the volume is not sufficient to justify the expense associated with a dedicated process. This intermediate level is in many ways the most challenging, since a lower product value means the process must be highly efficient to keep the processing cost down, while the limitations of shared equipment do not permit the same level of optimization as for the bulk products. At the same time, this intermediate class is highly interesting since a successful implementation of biocatalysis here can provide a platform technology for the production of a large array of intermediate and high value products.

The product types that are the focus of investigation in ROBOX – from larger volume/lower value vitamins over fine chemicals of intermediate volume and value to high value pharmaceutical products and metabolites – include those where the processing cost is critical to the success of the process and where the cost of the catalyst can constitute a significant part of that cost. Such characteristics imply that a process that appears infeasible based on early experiments can potentially be successful by combined efforts in the different levels of development as described below. For the products in ROBOX that fit into

the challenging intermediate product value and volume described above, it turns out that the processes need to be pushed to high product concentrations, relatively high reaction rates and reasonable biocatalyst yields (meaning the amount of product produced for a given amount of catalyst). The phrasing here is intentionally vague as these numbers are still being established, but a rough estimate would be that product concentrations of at least 50 grams per litre need to be achieved in the reaction stage to avoid prohibitive costs for product workup. The product should additionally be generated at an average rate of at least 2 – 10 grams per litre per hour (assuming a maximum reaction time of 24 h).



Scheme 1. Links in the development chain for biocatalytic processes, together with some of the aspects studied at the different levels.

The development of a biocatalytic process can be broken down into key areas, linked to the specialized knowledge and technologies that they rely on. These research areas are shown in Scheme 1 together with some of the aspects that are used to measure performance. As shown in the scheme, the development chain starts with the identification of a catalytic activity of interest. At this level, work is also done to improve the catalyst in different ways by modifying the protein. Once a suitable catalyst has been identified, it must be produced in larger quantities and formulated – for instance as whole cells, as cell free extract or as immobilized catalysts – for further characterization. The catalyst is then characterized with respect to activity and stability, and how these characteristics respond to different properties of the

reaction system such as temperature, pH and substrate and product concentrations. The final steps involve using this information to design a process around the reaction, taking into account the required conditions but also adding on the downstream operations required to remove the catalyst and purify the product. This process also involves designing or choosing the reactor configuration and deciding on batch-wise or continuous operation. At this level, the technical performance of the process can be fully assessed and financial competitiveness can be precisely gauged.

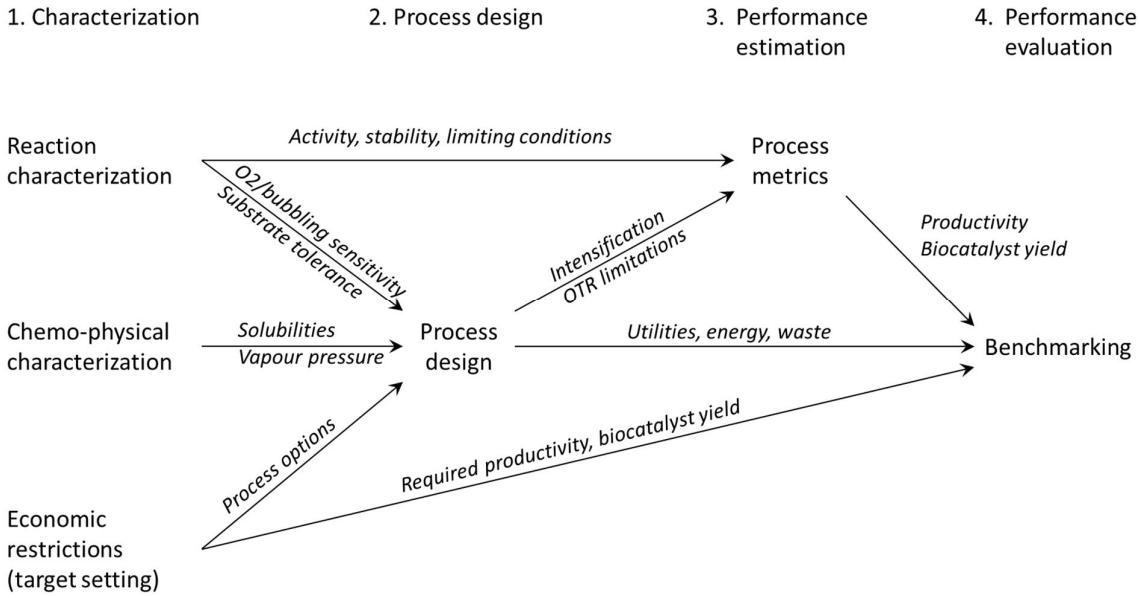
To develop a biocatalytic process in an efficient way, it is important that the performance achieved at each level in the development chain is communicated to the other levels, as well as the performance required from the others – the development benefits from iterative work at each level based on an understanding of what each of the other levels require. However, the work in the different areas, at least in academia, is typically not carried out by the same research group precisely because they involve different areas of expertise. This is in itself a barrier to communication. However, the situation is further complicated due to the differing nature of the respective research fields, since the different partners will typically have different objectives with their research. For example, while the protein chemist has an interest in the effect of protein structure on substrate selectivity, the enzyme technologist is more focused on the kinetics of the biocatalyst. The process engineer, in turn, may be more interested in the performance of product removal methods and mass transfer limitations. It is important to note that while each of these interests are complementary and need to be respected, the value of the individual partner's work for the project can only be fully realized if it is relevant to the other links in the development chain. Specifically, it is essential that if minimum criteria are identified for successful implementation – for example, a minimum final product concentration from the reaction stage – it is important that this is considered by everyone in the chain. It is thus critical that a common language for the development chain is identified; simply put, a common way of measuring performance.

As explained in the preceding paragraphs, there is a need to achieve a certain performance level in the types of process studied in ROBOX. These performance targets can and have to be translated to performance goals at each level of development in the chain. This type of target setting for each of the target molecules is an objective in subsequent work in the project. However, there is also a value in looking in the other direction and estimating the potential performance of a process from the initial performance data that can be collected at each level in the development chain. It is the objective of this report to illustrate the methodology for making such estimates and to provide a protocol to compare them. Making these evaluations at an early stage of development makes it possible to benchmark current (potential) process performance against required performance targets, and can also help in identifying bottlenecks where development efforts should be focused. They are also necessary to allow as much time as possible for molecular biology work to improve the catalyst as needed.

In the following sections, we introduce a protocol detailing the types of information that is required to evaluate the potential of a biocatalytic reaction system at an early stage, as well as the types of experiments that are required to characterize the biocatalyst. This protocol is subsequently implemented to establish conceptual flowsheets for the types of reaction systems studied in ROBOX, to support the estimation of process metrics.

Theory

The introduction section illustrates the different steps and disciplines required for biocatalytic process development. In this section, the type of information that is required from each link to evaluate the feasibility of an intended is presented, together with how that information should be implemented. The evaluation has been broken down into four stages, illustrated by the scheme below.



Scheme 2. The different stages in process evaluation and the types of information transferred.

Characterization involves gathering of experimental and literature data as well as relevant know-how from the involved partners. In the next stage, a process is designed based on the identified characteristics. Once this is done, the potential performance of the process can be estimated based on identified characteristics and the potential improvements and restrictions introduced by the process design. Finally, the results from the performance estimation can be used for benchmarking and analysis of the potential performance relative to identified goals for target performance.

As explained in the introduction, the success of a process is determined by its ability to meet the performance requirements that are defined, or at least linked to, the financial niche of the intended product. Starting from the right in the figure, benchmarking of a process is defined as the comparison of the estimated potential performance with established performance requirements. Beyond giving a pass/fail result, the benchmarking should also include an analysis of any identified shortcomings to identify process bottlenecks and to direct future development efforts. The benchmarking activity is based on identified target performance (which is specific to each case and outside of the scope of this report) and the estimated potential performance, or performance metrics, of the intended process.

The commercial feasibility of a given process is related to its technical performance, which can be measured in many different ways. Here, we have chosen to describe the performance with six key metrics that are in common use (Lima-Ramos et al., 2014; Lundemo and Woodley, 2015; Morris et al., 1996). The first of these, the achievable conversion, is linked to the thermodynamic equilibrium of the reaction and dictates how much of the starting material can be converted in a single reaction stage. For oxidation

reactions, the equilibrium is typically strongly shifted towards product formation; conversion can therefore normally be complete in the absence of other limitations.

The yield of the process describes how much of the starting material is converted into product. This is related to the selectivity of the reaction: for a completely selective conversion, the yield is equal to the conversion. Lower selectivity on the other hand leads to a loss of material to undesired byproducts. This directly translates into a loss for the process (material and processing costs must be carried by a relatively smaller amount of product), but it also complicates and increases the cost for product purification.

Any production process needs to carry a combination of fixed (primarily investments) and operating costs (salaries, utilities) and it is therefore important that the product can be produced at a high rate in the reactor volume available. The metric commonly used to describe this performance is space-time yield, or STY. The STY describes how much product is produced for a given unit of time and volume (*e.g.* g L^{-1} h^{-1}). It is an average measure for the entire reaction and is thus related to catalytic activity present in the reactor; more specifically, the reaction kinetics, catalyst loading and to some extent catalyst stability). For the products intended in ROBOX, the industrial partners have indicated STYs of at least 2-10 g L^{-1} h^{-1} to be desirable.

Another important factor for a production process is the product concentration that can be achieved in the reaction stage. A dilute product stream requires considerably more effort to purify the product, requiring larger equipment/more processing time, larger solvent volumes for extraction, more energy for solvent/water evaporation and so on. Also, separating the product from minor contaminants naturally becomes more complicated when the product itself is present only at similar concentrations. As indicated by the industrial partners, product concentrations of at least 50 and preferably 100 – 250 g L^{-1} is required for the types of products studied in ROBOX.

The final two metrics are the catalyst yield and catalyst loading. The first describes the amount of product produced relative to the amount of catalyst (*e.g.* $g_{product}$ $g_{catalyst}^{-1}$). This is important since the catalyst represents both capital and auxiliary materials that must be carried by the product. The catalyst yield is directly linked to the stability of the catalyst. Biocatalysts invariably (though at different rates) loose activity as they carry out reactions, meaning they can only catalyse the formation of a finite amount of product during a limited time. The lower limit for the catalyst yield from an economic perspective is highly dependent on the catalyst formulation: the cost per enzyme is lower for whole-cell catalysis than for cell free extract, which in turn is lower than for purified enzyme. This is due to the added cost for processing and protein loss in each stage. The cost of immobilized enzymes is even higher, due to the additional material costs introduced (Tufvesson et al., 2011). It should be noted that the required catalyst yield does not necessarily scale directly with the cost of the catalyst, since the formulation of the catalyst influences its performance (both in terms of reaction kinetics and catalyst stability) and also has implications for the configuration of the process. Finally, catalyst reuse can be drastically improve the catalyst yield provided that its stability is sufficient to allow this; however, catalyst reuse also has consequences for process design.

As already mentioned, the space-time yield is an important process metric: there is a lower limit to the overall product formation rate. The last is a function of specific catalytic activity (activity per g catalyst) and catalyst loading ($g_{catalyst}$ L^{-1}). In principle, the space-time yield can thus always be increased by increasing the catalyst loading. There are of course limits to this, the first of which is physical: there is a limit to how much catalyst can fit into the reactor. More importantly, the cost can quickly become limiting. As for the

Table 1. Process metrics

Process metrics	Definition	Unit
Conversion	$\frac{n_{substrate,initial} - n_{substrate}}{n_{substrate,initial}} \times 100$	%
Yield	$\frac{n_{product,} - n_{product,initial}}{n_{substrate,initial}} \times 100$	%
Productivity (STY)	$\frac{m_{product}}{\tau \times V_{reactor}}$	$g\,L^{-1}\,h^{-1}$
Product concentration	$\frac{m_{product}}{V_{reactor}}$	$g\,L^{-1}$
Biocatalyst yield	$\frac{m_{product}}{m_{biocatalyst}}$	$g\,g^{-1}$
Biocatalyst loading	$\frac{m_{product}}{V_{reactor}}$	$g\,L^{-1}$

catalyst yield, the maximum loading depends very much on the catalyst formulation. The restriction on loading can however be lifted if the stability is such that the catalyst can be reused.

The six process metrics described above can be estimated from system characteristics, provided certain assumptions can be made regarding the design of the process. The performance of the oxidation reactions studied in ROBOX largely depend on the catalyst; specifically, its formulation and the reaction kinetics and stability that it can achieve. These aspects are discussed in more detail in the section on 'reaction characterization' below.

The performance of a process is closely linked to its design, which in turn relies on an understanding of the reaction kinetics and catalyst stability, chemo-physical characteristics of the reaction system and the expected production volume. These concepts are discussed in 'process design' below.

Reaction characterization

As explained in the sections above, the overall performance of a process is closely linked to the performance of the reaction, and thus to the achievable performance of the catalyst. This is in turn linked to the enzyme kinetics and achievable activity as well as catalyst stability, all of which depend on the formulation of the catalyst. For the oxidation reactions studied within ROBOX, the supply of oxygen and in relevant cases the additional enzymes that are required for co-factor regeneration, must also be considered.

Reaction kinetics

As mentioned above, reactions of the type studied in ROBOX need to achieve a minimum overall rate (or space-time yield) to be successful. This metric is linked to the activity (and of course amount) of the catalyst, which is in turn determined by the reaction kinetics. These describe the rate of the reaction based on the conditions in the reactor, including parameters such as substrate and product concentrations, pH and temperature. It goes without saying that a complete, detailed characterization of reaction kinetics – such as would be required for mechanistic modelling – is both difficult and time consuming, and

assumptions must typically be made to simplify the work. Generally, a basic characterization of a catalyst will involve a study to identify a pH-profile – how the reaction rate at otherwise fixed conditions varies with pH – and a suitable reaction temperature. Once these are fixed, the activity of the catalyst can be evaluated at different substrate and product concentrations, to assess how the catalyst responds to these (identification of saturation concentrations or K_m values, (Cornish-Bowden, 2004)) and if there is inhibition by either substrate or product that needs to be considered. Ideally, for a system that requires separate addition of co-factor and regenerating enzyme, these should also be characterized separately.

This reduced approach also requires some work, and it can be argued that characterization for the sake of optimizing a reaction is part of the development work that can be addressed by the enzyme technologist. It would be useful if early-stage evaluation of potential process performance could be carried out with less work for characterization. An estimate of catalytic activity can be obtained with less effort, with the provision that the estimate will likely be less accurate: an extrapolation of performance from non-optimal conditions can lead to an underestimation of performance. On the other hand, doing the same without knowledge of possible inhibition phenomena can conversely lead to an overestimation. Ultimately, more understanding is better, but it should also be noted that characterization data for conditions that are similar to those required in a process – especially final product concentrations – are inherently more useful.

Oxygen supply

All of the reactions studied in ROBOX rely on a steady supply of oxygen to allow the reactions to proceed. This is because the solubility of molecular oxygen in an aqueous system is very low; considerably lower than what would be required to fully convert substrates to product at the concentration levels, more than 50 g L^{-1}, mentioned above. The supply of oxygen typically comes in the form of a gas (normally air) that is contacted with the reaction medium. The oxygen supply thus relies on the transfer of oxygen from a gas phase to the liquid reaction phase, at a rate that will be determined by the partial pressure of oxygen in the gas phase and the concentration relative to saturation concentration in the liquid phase (or the relative thermodynamic activity of oxygen in the two phases) and the interfacial area between the phases (Garcia-Ochoa and Gomez, 2009).

The need for a constant supply of oxygen means that the studied reactions can become limited by the oxygen transfer rate (OTR). Just as there is an economic restriction in increasing reaction rates by adding more catalyst, there can thus be a technical restriction to how quickly oxygen can be supplied. The exact limits to OTR will vary with the supply method and the scale of reaction. The established method for reactions at scale in stirred tank reactors is aeration, where gas is bubbled through a vigorously agitated reaction volume (to better disperse the bubbles). The OTR will improve with the rate of aeration, mixing and, as mentioned already, concentration of oxygen in the gas. Further improvement can be achieved by pressurizing the reactor. However, the cost of pushing OTR quickly adds up, and reactors are in most cases run with air for aeration at up to 1 VVM (volume of gas to volume of liquid per min), at ambient pressure with agitation power input around 1 W L^{-1}. Such a setup allows for an OTR on the order of 100 mmol L^{-1} h^{-1} (Tindal et al., 2011). Aeration does come with complications, as will be discussed in the subsection on stability below. In these cases, alternative forms of oxygen supply need to be considered. Such methods are available, but can typically only provide oxygen at a fraction of the rate attainable with aeration (Cote et al., 1989; Van Hecke et al., 2009).

Catalyst stability

Just as important as the rate at which a catalyst can carry out a reaction, is its ability to retain that activity long enough to convert the required amount of material. A loss of activity can to a certain extent be compensated by adding fresh catalyst, but there will be limits with respect to catalyst loading and catalyst yield, as discussed previously.

Loss of activity can be attributed to inhibition or inactivation, or a combination of the two. The distinction is that inhibition is an aspect of enzyme kinetics and is reversible, implying that activity can be regained if conditions become more favourable (for example with substrate inhibition). Inactivation on the other hand is irreversible, meaning that activity is lost permanently (for example through protein denaturation, or through modifications to critical parts of the catalyst). Catalyst stability normally refers to the ability of the catalyst to resist inactivation.

The inactivation of a biocatalyst can be induced in several ways. First, any biocatalyst will spontaneously loose activity over time, including exposure to reaction conditions without ongoing reaction. The rate of inactivation will depend on the exact conditions, and should ideally factor into an optimization of reaction conditions. Second, catalysts can also be inactivated as a consequence of their catalytic cycle. This implies that the enzyme carrying out the reaction can catalyze only a certain number of reactions (on average), and the relative rates of catalysis and catalyst inactivation can be used to calculate a total turnover number (TTN). Third, a catalyst can be inactivated by the presence of compounds that are toxic to it, such as co-solvents. This can in some cases also include the reaction product (and substrate), which means that it can become very difficult to push the product concentration in the reaction medium beyond a certain point.

For oxidation reactions, the method of oxygen supply can potentially also contribute to inactivation. Specifically, bubbling – implying the presence of a gas-liquid interface – can cause protein denaturation. It is important to investigate this aspect of stability at an early stage of process development, as bubble free oxygen supply methods are more complicated and will limit the maximum reaction rate. It should be noted that the dissolved oxygen can also promote inactivation (Cabiscol et al., 2000; Van Hecke et al., 2009; Van Hecke et al., 2011).

A further aspect of stability relevant to some of the oxidation reactions studied in ROBOX concerns the need for co-factors and the required regenerating enzymes. As for the characterization of reaction kinetics, the stability of these catalysts should also be investigated if they are to be added separately to the reaction (Park et al., 2011). Likewise, stability of the co-factor can be an issue that one must be aware of, to avoid that this becomes a limiting factor in the reaction.

Biocatalyst format

There are several ways to implement a biocatalytic reaction with respect to its format, and the choice of format impacts the process (and reaction) in several ways. For the purpose of this discussion, enzyme refers to the protein that catalyzes the intended reaction, whereas biocatalyst will refer to the formulated enzyme(s). The simplest biocatalyst (in terms of how many components it adds to the reaction) is a free, purified enzyme. This is the preferred format for doing mechanistic characterization of the enzyme. However, it is not the most cost-effective choice in that it requires purification from the cell in which it is produced, which involves breaking of the cells and separation from the other components of the cell including other proteins (Tufvesson et al., 2011). The cost associated with isolating an enzyme can potentially be reduced by expressing it in a host that exports the enzyme from the cell.

A further complication of isolated enzymes applies to oxidations that require the use of a cofactor and regenerating enzymes. With isolated enzymes these must be added separately, and the activity and stability of both must be balanced with the enzyme carrying out the intended reaction. With whole-cell biocatalysts, the enzymes are co-expressed and the cell can often, to a degree, maintain the co-factor itself. For this reason, whole-cell catalysis is often recommended for reactions requiring co-factor (Goldberg et al., 2007a; Goldberg et al., 2007b; Knoll et al., 2005; Schmid et al., 2001; Schrewe et al., 2013).

The two previous paragraphs indicate that whole-cell biocatalysis is always the better option. However, sequestering the catalyst in a limited part of the reaction volume (*i.e.* inside a cell or by immobilization, discussed further below) introduces mass transfer limitations for all dissolved reaction components (Nordblad, 2008). For whole cells, this is further exacerbated by the fact that all of these components need to traverse the cell wall. This means that the actual concentration of the substrates (starting material and oxygen) near the catalyst can be considerably lower than in the bulk reaction volume, affecting the overall reaction kinetics. Conversely, the concentration of the product can become relatively higher inside the cell, which poses a challenge for recovery and in cases where the product is inhibitory or inactivating (Ramesh and Woodley, 2014). That said, the stability of the enzymes can also be higher in the native environment of the cell, and whole cells can also be less sensitive to gas-liquid interfaces (*e.g.* from aeration) than the isolated enzymes (Thomas et al., 1979; Thomas and Dunnill, 1979).

Another option for biocatalyst formulation is immobilization, either of enzymes or of whole cells. Performance limitations with respect mass transfer apply depending on the method of immobilization, and the cost of the biocatalyst is increased relative to the active enzyme due to the added efforts and materials involved (Nordblad and Adlercreutz, 2013; Tufvesson et al., 2011). However, immobilization can make the separation of the product from the catalyst much simpler, reducing the processing cost, and also facilitates catalyst reuse. Ultimately, immobilization is best suited for applications where dedicated reactors can be used and where catalyst stability is high enough that stable operation over many reaction cycles can be realized.

Comments on lab conditions vs process limitations

The previous sections illustrate that there are a number of choices to be made related to reaction design for the reactions studied within ROBOX (such as biocatalyst formulation and the method for oxygen supply) and several aspects that need to be characterized once these choices have been made (such as activity and stability). To minimize the required effort and to make the development process as efficient as possible, it is essential that the choices and experiments are made with the intended application in mind. Full characterization of all possibilities can certainly be scientifically valuable, but will not necessarily be relevant to the development effort. It is therefore important to keep the required process performance in mind as the different aspects of a reaction are characterized. For example, the required product concentration for the reactions of interest in ROBOX has been identified as 50 g L^{-1} or ideally higher. This is equivalent, for an assumed product molecular weight of 100 g mol^{-1}, to a product concentration of 0.5 M. This is well in excess of the concentration range typically used to characterize the studied enzymes, partially due to inhibition or toxic effects, and partially because many of the compounds are simply not soluble to this extent.

One way of achieving the required product concentration from the reaction stage is to implement some form of *in situ* product removal (Ramesh et al., 2016). This can be as simple as introducing a solvent to the reaction system (meaning the reaction becomes bi-phasic). Such an approach ideally allows the reaction to

be carried out at concentrations that are suitable for the catalyst, while the product is accumulated in the auxiliary phase. Having the product in separated into a solvent as it leaves the reactor can also greatly simplify subsequent product purification steps, reducing the cost of DSP. In other words, a requirement of high product concentration from the reaction stage does not necessarily preclude biocatalysis at low concentrations, but instead requires a technical solution in the process design. What is critical is that this solution is considered in the characterization of the reaction; in the example, the presence of a solvent must be considered in the characterization of activity and stability at an early stage.

Process design

In addition to the reaction stage required to convert a substrate into an intended product, a production process (as defined for the purposes of this report) also includes units for down-stream processing (DSP) to separate and purify the product from other reaction components. Such separation makes use of differences in chemo-physical properties between the reaction components: size, solubility, hydrophobicity/polarity, vapour pressure etc. There are established unit operations that are based on these principles, from relatively simple – filtering, extraction, evaporation – to more complicated, such as crystallization, distillation and chromatography (Ramesh et al., 2016). Some of these principles can be applied for *in situ* product removal (ISPR) already in the reaction stage, as mentioned previously. ISPR can greatly simplify DSP, but also adds some complexity to the reaction stage (depending on the technology applied).

The design of the reaction stage of a process must accommodate the requirements of the reaction, as outlined in in the previous sections. The choice of unit operations for the subsequent DSP will depend on the chemo-physical characteristics of the components in the reactor output, and also on the required yield and purity of the product. Ultimately, the options available for both DSP and ISPR will be limited if the process cannot be operated with dedicated equipment.

Chemo-physical characterization

There are a number of properties of a reaction system and its components that need to be characterized to support the design of a process suitable for it. This is the case for the choice of separation methods for DSP and ISPR, but the chemo-physical properties also play a role in determining how the reaction can be run. There are too many properties to make an exhaustive list here; instead, an attempt has been made to cover the properties most relevant to the oxidation reactions studied within ROBOX.

Biocatalytic reactions are typically carried out in water, since this is the native environment for the biocatalysts. However, the solubility of many chemicals of interest can be quite low in this solvent. While not altogether a bad thing – for example, a low affinity for the reaction solvent simplifies product separation – this can complicate substrate supply to the catalyst, potentially limiting the rate of reaction. As mentioned before, low solubility is an issue that also affects oxygen. It is worth noting that the limiting concentration for oxygen (and other components) is not fixed but depends on a number of reaction parameters, including temperature and the concentration of other components (including co-solvents).

The solubility of the reaction components in water and other solvents is related to their hydrophilicity/hydrophobicity, presence of any charged groups etc. The relative solubility in water and another solvent (determining the partitioning between the two) is an important factor for the implementation of extraction.

Another important property of substrates and products are their melting points. This is particularly relevant to biocatalysis, since these reactions are typically carried out at much lower temperatures than

with traditional chemistry. Compounds that are solid at the reaction temperature and that are also poorly soluble will therefore be present as solids in the reactor. The solution/dissolution rate from a solid substrate/product is likely to be different to that from a liquid phase (or gaseous, as for oxygen). Additionally, the presence of a solid, or a mixture of solids, in the reactor at the end of reaction is a complication, and something that needs consideration in the design of a process operated at large scale.

At the other end of the spectrum from the melting point of a compound is its volatility, or partial pressure at the reaction conditions (or those in subsequent separation processes). The volatility is particularly relevant in oxidation reactions that rely on aeration. For components with significant vapour pressure, the bubbling of gas through the reactor will over time strip the components form the reaction phase. The vapour pressure of a component is related to its boiling point, but is for dissolved components also determined by its thermodynamic activity in solution (Henry's law). If vapour pressure of a reaction component is an issue for the reaction, it must be addressed in the process design. A volatile substrate needs to be fed to the reaction to keep its concentration low throughout the reaction, whereas it may be necessary to cool the off-gas from the reactor to capture a volatile product to avoid losses (Tufvesson et al., 2014).

A final aspect that needs to be included for especially the reaction product is the stability with respect to degradation. As mentioned previously, the selectivity of the catalyst is critical to achieving a high yield of product. However, it is also important to identify conditions – temperature, pH, concentration of oxidizing components – that cause degradation of the product, so that these can be avoided throughout the process to prevent loss.

Economic restrictions

As explained in the previous sections, the example products studied in ROBOX represent cases that would be profitable to make, provided that the production process performs well enough. However, not all of the example products have the market volume required to justify a dedicated production line. For these products, equipment would typically be shared with the production of other chemicals over time. Even with larger production volumes, it is likely that existing equipment in a plant would be repurposed for the new application.

While the use of standardized equipment is limiting with respect to process design, it also simplifies the early-stage evaluation that is proposed in this work. All operations will likely be (fed-)batch (rather than continuous), and the reactor will likely be a stirred vessel with modifications for aeration and potentially ISPR. The downstream operations will similarly involve units for extraction, liquid-liquid separation, filtration and evaporation. The performance of these are well known and any required additional information specific to the given case (*e.g.* partition coefficients) can typically be determined experimentally at lab scale.

Methodology

This section presents an overview of important experiments for characterizing a biocatalytic reaction. The identified experiments provide much insight into the potential performance of the biocatalyst (as well as its limits). The experiments have been split into two categories: those aimed at characterizing reaction kinetics, and those directed at catalyst stability.

Reaction kinetics

The experiments related to reaction kinetics are summarized in Table 2. The first two of these involve establishing a standard assay for determining activity, preferably based on the intended reaction. 'Standard' in this case means fixing, as far as possible, all reaction parameters such as substrate concentrations, catalyst load, reaction time and pH or temperature; the last two depending on the parameter to be profiled. It should be noted here that a reduction in measured activity, for instance at the high end of the temperature range, can be a result of activity loss during the assay. The exact appearance of pH and temperature profiles thus depend on the chosen assay conditions. The third set of experiments involve initial rate experiments at different substrate concentrations and are directed at identifying standard (apparent) enzyme kinetic parameters such as V_{max} and K_m, as well as inhibition constants, $K_{i,S}$. The fourth set of experiments in Table 2 is similar to the third, but instead focus on product inhibition.

Table 2. Experiments for reaction kinetics

Parameter	Experimental work	Identified characteristic
pH	Initial reaction rate for different pH	pH optimum/profile
Temperature	Initial reaction rate for different temperature	Temperature optimum/profile
Substrate load	Initial reaction rates for different substrate concentrations	V_{max}, K_m, $K_{i,S}$
Product load	Initial reaction rates in the presence of different product concentrations	$K_{i,P}$
Catalyst load	Measure initial reaction rate for the substrate concentration with maximum rate with varying catalyst concentrations	Identify if oxygen is limited and if so at which rate
Catalyst format	Reaction rate in the presence or absence of cell membrane (for whole-cell systems)/ in the absence of immobilization	Presence of mass transfer limitations

The two last sets of experiments relate to mass transfer limitations in the reaction system. Varying the catalyst loading with otherwise fixed conditions can give an indication of limitations with respect to oxygen supply. One might wonder such an experiment is necessary given that the capacity for oxygen transfer in a reaction system is easily established. However, maximum oxygen transfer from the gas phase to the reaction medium occurs when the concentration of oxygen in the liquid is zero. This leaves little oxygen for

diffusion through the liquid and across cell membranes for the enzyme to carry out any reaction. The limiting oxygen supply rate as measured in rate of reaction is therefore unlikely to be as high as the maximum oxygen transfer rate for the system.

The final suggested set of experiments for reaction kinetics relate to reaction systems relate to reaction systems based on whole-cell or immobilized biocatalysts, and involve making experiments with free enzymes (and co-factors and regenerating enzymes as applicable) as a reference. For whole cells, this can be as straight-forward as running an additional set of experiments with permeabilised or homogenized cells. The purpose of such an experiment is to determine if the reaction is limited by mass transfer of substrate (and potentially product) across the cell membrane.

Biocatalyst stability

The other aspect of biocatalyst performance that is important for the design and evaluation of a process is its stability at intended reaction conditions. Table 3 gives an overview of suitable experiments for this characterization. Again, the list is not exhaustive but the data from these experiments can provide a solid basis for process development.

The first experiment in the list is related to turnover stability, meaning the stability of the catalyst as it converts substrate into product. The experiment involves carrying out the same reaction with varying catalyst loads at otherwise equivalent conditions. The data from the reactions (*e.g.* conversion or product concentration) are then plotted against the reaction time multiplied with the catalyst load. In the absence of significant inactivation during the course of the reaction, the plots of the two (or more) reactions should overlap. On the other hand, if the reaction with less catalyst lags behind the other(s), this is an indication of catalyst inactivation as the reaction progresses (Selwyn, 1965).

Table 3. Experiments for catalyst stability

Type of stability	Experimental work
Turnover stability	Full reaction courses with varying biocatalyst loadings. Compare results by plotting reaction data against E*t.
Stability at reaction conditions*	Initial reaction rate after incubating the biocatalyst to reaction pH and temperature for different durations.
Stability towards product	Initial reaction rate after incubating the biocatalyst to different product concentrations for different durations.
Stability towards substrate (Identification of substrate toxicity)*	Initial reaction rate after incubating the biocatalyst at different substrate concentrations in the absence of oxygen.
Stability to oxygen concentration	Initial reaction rate after incubating the biocatalyst at relevant pH and temperature with varying oxygen concentrations.
Stability to bubbling	Expose catalyst at relevant pH and temperature for different duration of aeration.

It should be noted that there is a third possible outcome of the turnover stability experiment, namely that the relative performance with more catalyst is worse than with less catalyst. This would be an indication that something other than the added catalytic activity is limiting the reaction; for these reactions, such a result could for example points to limitations in the oxygen supply.

The next experiment in Table 3 is related to the inherent stability of the catalyst at reaction conditions, and involves incubating the catalyst at the intended conditions (pH, temperature etc.) for a range of relevant durations before adding substrate to measure initial reaction rates as a measure of catalytic activity. The decrease of activity with longer incubation times will give a quick indication of how suitable the chosen reaction condition are for the biocatalyst.

The next two experiments, similarly to the one just described, involve incubating the catalyst in the presence of substrate and products, respectively, at relevant concentrations. It is important, especially for the experiment related to the substrate that the incubation is carried out without supplying oxygen, to avoid conversion during the incubation. The reactions are started after the desired incubation time by adding substrate (for the product stability experiments) and supplying oxygen. The activities (determined through initial rate experiments) are compared to a reference without incubation. High rates of inactivation indicate that the components are toxic to the catalyst, and is something that needs to be considered in the choice of reaction conditions and in the process design.

The last two suggested experiments are related to dissolved oxygen and inactivation caused by gas-liquid interfaces (present when aeration is used for oxygen supply). To separate the two effects, the first experiment involves incubating the catalyst in the presence of different oxygen concentrations. This can be achieved by using different mixtures of oxygen and nitrogen with bubble-free oxygen supply (for instance through headspace or membrane aeration). The last experiment involves bubbling the reaction mixture (including the biocatalyst) with nitrogen for different times, before supplying the system with oxygen to measure remaining activity. Together these two experiments can help in choosing the correct oxygen supply method for the process.

Case studies

The previous sections provide an overview of the information that is needed to make predictions about the potential performance of a process based on an oxidative reaction using biocatalysis. In this section, the evaluation of potential process performance is illustrated through case studies based on the model reaction systems represented within the ROBOX project. The first case is based on a P450 reaction implemented in a demonstration at DSM and has sufficient data to make a full evaluation. This is followed by more brief analyses of three ROBOX cases, focused on Baeyer-Villiger monooxygenase (BVMO), alcohol oxidase (AOX) and alcohol dehydrogenase (ADH). These reactions are in earlier stages of development and their analyses is consequently less comprehensive. Even so, an overview of the currently available information and the consequences for process design (flowsheeting) is provided.

During the preliminary analysis of these cases, it was recognized that for production volumes that do not justify dedicated production lines, processes for the different catalysts appear very similar in the type and order of unit operations. Therefore, it was decided not to draw complete flowsheets for each of the planned ROBOX demonstration cases, but instead exemplify a typical flowsheet with the P450 hydroxylation of diclofenac case presented below.

P450-catalysed oxidation of diclofenac (DSM)

In this case study, cytochrome P450 is used for the selective oxidation of diclofenac into 4'- and 5-OH-diclofenac, both metabolic degradation products of the pharmaceutical. The formation of two regioisomers is not undesirable in this case (reflecting the composition of metabolites when the pharmaceutical is degraded in the body) and both are thus included in the calculation of product yield. The example has been chosen since data is available both for the initial evaluation of the reaction according to the protocol, and for the actual performance of the developed process.

Reaction system characterization

Substrate and product characteristics

Diclofenac is a relatively hydrophobic molecule, and as a consequence its solubility in water is low. Literature data indicates a solubility of 7.6 mM (2.4 g/L) at 25 °C; however, upon investigating DSM found a higher solubility, around 40 mM (12 g/L) at 25 °C and higher at elevated temperatures. The product differs from the substrate through a single hydroxyl group, and is expected to have a somewhat higher, but similar, solubility in water.

The thermodynamics of the reaction are favorable, and full conversion of substrate into product can in principle be achieved as long as the catalytic system is active.

For the type of high-value, low volume product in this example, DSM have judged that substrate and product concentrations in the range of 2-5 g/L are sufficient to support the productivity, space-time yield and biocatalyst yield goals that need to be achieved to make the final process economically feasible. Hence, options for *in-situ* product removal have not been investigated. However, it was found that the substrate is inhibiting to the reaction and it is therefore important that the substrate is dosed over the course of the reaction (fed-batch operation).

Biocatalyst characteristics

The reaction system in the case study employs a whole-cell biocatalyst to oxidize diclofenac into 4´- and 5-OH-diclofenac. The reaction employs oxygen as the oxidizing agent, forming water as a byproduct. The

P450 enzyme is NADPH-dependant, meaning that NADP is formed during the reaction. NADPH is generated in a parallel reaction, where glucose dehydrogenase (GDH) is used to convert glucose into gluconic acid, using NADP as co-factor. As part of the development of the reaction system, DSM have chosen to use whole-cell catalysis with P450 and GDH co-expressed in the cell. Due to the formation of gluconic acid as a by-product, base should be dosed over the course of the reaction to maintain a reaction pH of 7.2.

As already mentioned, initial characterization indicated that the P450 enzyme is sensitive to high substrate concentrations, with inhibition occuring above 6 mM and inactivation in the range of 10-15 mM. This necessitates dosing of the substrate over the course of the reaction. Additionally, it was found that there is an upper limit to the concentration of product, above which conversion of substrate stopped.

Small scale (30 mL) experiments indicate activities up to 0.2 U/mL cell suspension. In fed-batch experiments, this rate is essentially sustained throughout the reaction and conversion of up to 8.3 mM diclofenac is achievable within 8 h, using 10 % catalyst suspension by volume. Conditions in these experiments are summarized in Table 4 below.

Table 4. Experimental conditions in investigation of P450 case

Scale	30 mL	800 mL
Catalyst load (30 % wet cell weight)	10 % (v/v)	20 % (v/v)
Diclofenac	~13 mM	~15 mM
Glucose	0.5 M	0.5 M
NADP	1 mM	0.5 mM
Aeration	0.25 vvm	0.05-0.1 vvm
Cycle time	10 h	4-6 h

For this reaction, DSM have also provided results for experiments in 1 L-scale (800 mL reaction volume). Reaction conditions were similar to those in the smaller scale (see table 2), the main difference being a lower rate of aeration (which was more efficient due to the larger scale) and a doubled catalyst load. As a consequence, maximum conversion was also achieved in approximately half of the time required in the smaller scale. Somewhat higher product concentrations were achieved at this scale: 12 mM product with near-full conversion of substrate.

DSM has further indicated that the stability of the catalyst is not sufficient for reuse, particularly when it is pushed (with high substrate and oxygen concentrations) to high activity and high product concentrations. Predictions of final process performance should thus be based on single use of the catalyst. Further, it has been shown that excessive oxygen supply (high concentrations of dissolved oxygen for much of the reaction time, achieved by sparging with pure oxygen) also has a negative impact on catalyst stability. This illustrates the importance of balancing the oxygen supply with the catalytic activity in a process.

Economic characteristics

DSM are currently working on scaling up the process to 100 L scale, providing the basis for the process design presented in the following section. The exact product value is not available at the time of writing this report. However, the product is a metabolic degradation product of a pharmaceutical, intended for use in clinical studies. As such, it can be classified as a low volume product of high value, and the restrictions with respect to process metrics (space-time yield, product concentration etc.) do not apply fully.

Process design

Details of the reaction conditions developed for the case study are presented in the previous sections. In addition to this, DSM have also developed a procedure to recover the product from the reaction mixture in the small scale experiments. This procedure involves several steps:

- Denaturing of the cells and proteins to facilitate their removal from the reaction. Achieved by addition of 50 % (v/v) methanol.
- Filtering of cell debris (followed by evaporation of methanol).
- Acidification of filtrate (to pH 2.0 using sulfuric acid) to reduce aqueous solubility of the products.
- Extraction of products with 3 volumes of ethyl acetate.
- Evaporation of ethyl acetate, resulting in a concentrated oil with product.
- Column separation of products

The recovery procedure achieves a yield of up to 90 % of the product. To implement a similar procedure at the intended 100 L scale, the procedure is modified slightly and the denaturation of the cells and proteins is achieved by increasing the temperature in the reaction vessel to 70 °C after the reaction is complete and methanol has been added. The methanol is evaporated between the filtration and extraction steps. A suggested process flowsheet for the process is shown in figure 1.

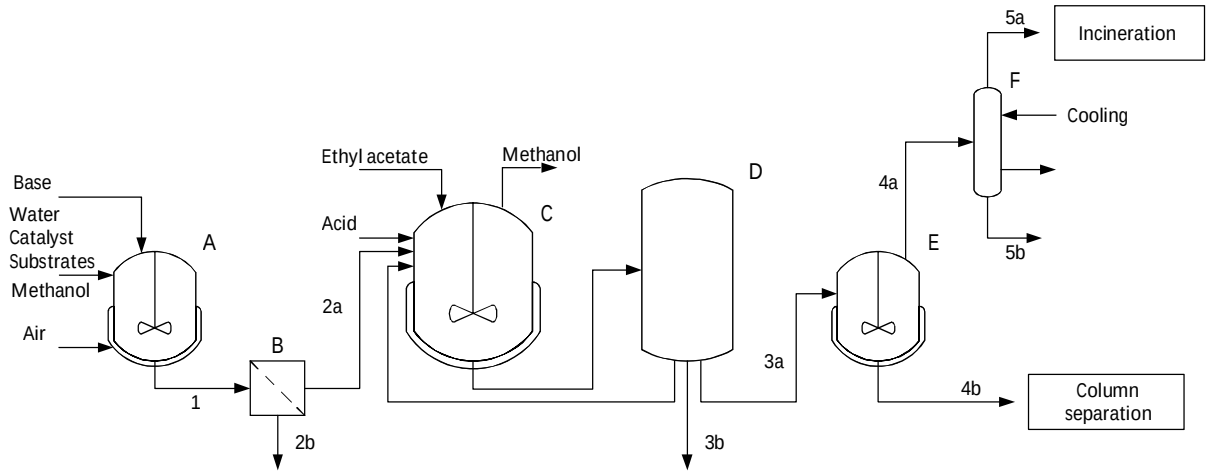


Figure 1. Suggested process flowsheet for the P450 case.

Vessel		Size	Stream	
A	Reactor	150 L	2b	Cell waste
B	Filtration		3b	Reaction waste
C	Extraction	200 L	4b	Product
D	Separation	200 L	5a	Solvent vapors
E	Evaporation	100 L	5b	Solvent waste
F	Condenser			

At this scale, most of the units are operated sequentially in batch mode and the arrows in the flowsheet for these are meant to indicate the direction of the different flows when material is transferred, rather than continuous operation. The process would be operated as follows:

- The reactor (A) is filled and heated to reaction temperature, and feeding of the different substrates started. Total time for filling and reaction is estimated to be 6 h when 20 % (v/v) cell suspension is used.
- Methanol (50 % v/v) is added to the reactor. The reactor is then heated to 70 °C and left for 2 h before cooling; estimated total time 3.5 h.
- The reactor is emptied through a filtration unit (B), with the filtrate directed into the extraction vessel (C). The cell debris (approx. 7 kg) is held back by the filter and goes to waste (stream 2b). Estimated time for filter preparation and filtration 6 h.
- Methanol is evaporated from the filtrate; estimated time 6 h
- Acid is added to the extraction unit, reducing pH to 2.0. Estimated time 0.5 h.
- Extraction is carried out with 3 portions of equal volumes filtrate and ethyl acetate. Between solvent additions, the material is transferred to a separation vessel (D). After settling of the phases, the aqueous phase is returned to the extraction vessel and the solvent phase is transferred to the extraction unit (E). After the last extraction step, the aqueous phase is separated as waste (stream 3b). Each round of extraction and separation is estimated to take 2 h.
- Each portion of ethyl acetate is evaporated at low pressure (40 mbar) in the evaporation unit. The product is collected as a concentrate (stream 4b) with an estimated concentration of 28 % (w/v), based on splitting stream 3a (90 L) into stream 4a (89 L) and stream 4b (1 L). Most of the ethyl acetate is condensed into stream 5b to be discarded as waste, but there will be some solvent vapors in stream 5a that may also have to be collected/treated. It is estimated that each round of evaporation takes 1 h. Since the evaporation is run mostly in parallel with the extraction steps, the evaporation adds only 1 h to the total time of the process.
- Once the process is completed, all equipment must be cleaned. Estimated time 2 h.

Altogether, the total processing time is approx. 32 h.

To support the calculation of process metrics, a mass and energy balance has been established for the case (Table 5). It should be noted some simplifications have been made especially with regard to the energy input required in the different units. A final product concentration of 4.2 g/L (13.5 mM) in the reactor and a product workup yield of 90 % has been used to calculate the material and energy consumption per g product.

Table 5. Mass and energy balance for the suggested P450 process

	Batch	per g product
Materials		
Reaction		
Water (L)	100	0.26
Cells (g wet weight)	6000	15.9
Diclofenac (g)	400	1.06
Glucose (kg)	9	0.02
NADP (g)	37	0.10
NaOH (g)	54	0.14
Denaturation		
Methanol (L)	50	0.13
Extraction		
Sulfuric acid (g)	49	0.13
Ethyl acetate (L)	270	0.71
Utilities		
Heat		
Reactor (MJ)	25.2	0.07
Evaporation (MJ)	125.6	0.33
Total (kWh)	41.9	0.11
Cooling water (L)	357	0.94

Evaluation of process performance

In Table 6, the various performance metrics that are explained in the theory section of the report have been calculated for the presented case study. No attempt has been made to assess the economics of the process at this stage beyond noting that the process has been deemed viable at this scale by DSM. Since the catalyst in this case is representative of one of the enzyme catalyst classes studied in ROBOX, the metrics will be discussed as for a product of intermediate value and product volume in the interests of illustrating the evaluation of process performance.

Table 6. Calculated process metrics for the P450 case

Process metrics	Value	Unit
Reaction conversion	100	%
Reaction yield	100	%
Product concentration	3.8	$g\ L^{-1}$
$STY_{reactor}$	0.76	$g\ L^{-1}\ h^{-1}$
Biocatalyst loading	6	$g_{CWW}\ L^{-1}$
Biocatalyst yield	0.63	$g_{product}\ g_{CWW}^{-1}$
$E\text{-factor}_{solvent}$	750	$g\ g^{-1}$
$E\text{-factor}_{total}$	2000	$g\ g^{-1}$

The product concentration achieved in this case is an order of magnitude lower than what would typically be required for an intermediate-value product. Interestingly, the space-time yield is not far from the minimum level noted for such products in the theory section. However, this has been achieved at the expense of a very high biocatalyst loading that, since the catalyst cannot be reused due to low stability, results in a low biocatalyst yield.

The metric E-factor has been added to the table to give an indication of the amount of waste generated in the process per gram product. The presented values would be very high for an established production process – solvent recovery and reuse could and should for example be implemented at larger scale - but are not surprising given the small scale and early stage of development for this process. It should be noted that the presented values do not take the production of the biocatalyst into consideration.

Due to the small scale, the process is simpler, and hence more wasteful, than it would be for a larger production volume. Implementation of ISPR in the reaction step could potentially increase the biocatalyst yield (gram product per gram catalyst) by increasing the amount of product that could be produced in a batch, without exceeding toxic levels in the reaction phase. An increase in biocatalyst yield could potentially also be achieved by reducing the catalyst load, at the expense of increased reaction time. Furthermore, concentrating the product stream from the filtration unit prior to extraction and optimization of the extraction procedure could more than likely reduce solvent use and waste. However, the required development effort for such improvements must be balanced against the potential gains in terms of future operating costs.

Baeyer-Villiger biooxidation

The target reactions for this case study are shown in Figure 2.

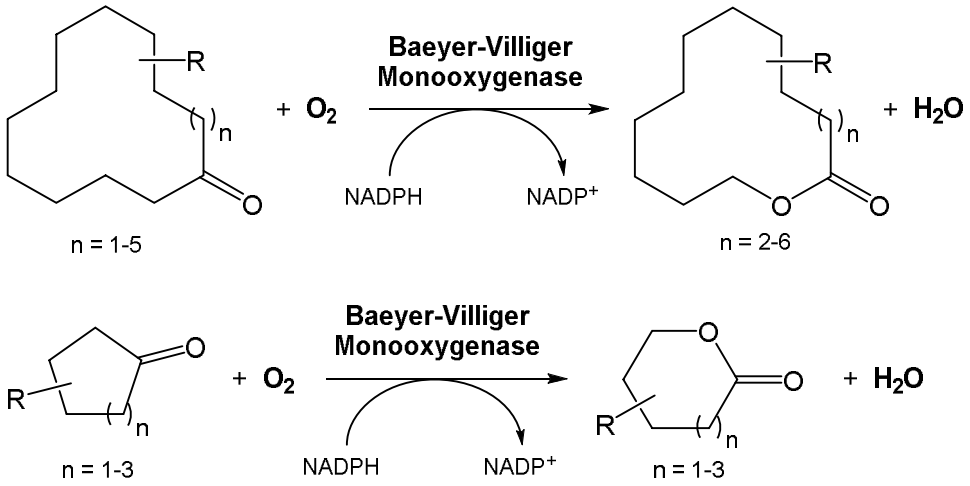


Figure 2. Target reactions for Baeyer-Villiger biooxidation involving the oxidation of macrocyclic and smaller-sized cyclic ketones.

Substrate and product characteristics

The early stage characterization of substrates and products as well as the biocatalyst identifies important challenges for the process development. The substrates and products are poorly soluble in water, and may also cause inhibition of the biocatalyst. This highlights that ISSS and ISPR could be a crucial part of the process implementation. Because of the physiochemical similarity between the substrates and their corresponding products the selectivity of the ISPR method is a challenge. An important consideration to be made is how (if possible) to avoid undesired removal of substrate from the reactor. Potential technologies may be based on liquid-liquid extraction as well as absorption and adsorption. Another consideration concerns the oxygen supply method, which may influence the rate of evaporation of substrates and products as well as any organic solvents/co-solvents present.

Biocatalyst characteristics

The BVMO is expressed in *Escherichia coli*, which are used as biocatalyst in a resting state. The enzyme is co-factor (NADPH) dependent, and the whole-cell catalyst format is therefore preferable since regeneration of the phosphorylated co-factor is challenging using an auxiliary enzyme system. Nevertheless also cell free enzyme formulations will be investigated in free and immobilised form.

Conclusion

Because of the similar basic characteristics to the P450 case described previously (e.g. whole-cell catalyst, poorly soluble substrates and products) it is likely that the overall processes will look very much alike. Details that could differ are e.g. choices of solvent for ISSS and/or ISPR purposes, product isolation (e.g. evaporation or crystallization). Also, since the product categories in this case differ (materials science monomers and fragrances vs. API) the acceptable process cost will inevitably differ, making it more important to optimize e.g. the use of solvents and the overall process time for the lower value product

Alcohol oxidase biooxidation

The alcohol oxidase-catalyzed lactone production is shown in figure 3. In contrast to the case studies for P450 and BVMO biooxidations, the alcohol oxidase catalyzed lactone production (figure 3) differs both in terms of the substrate and product properties, and in that the reaction does not require a co-factor. This simplifies the use of free enzyme.

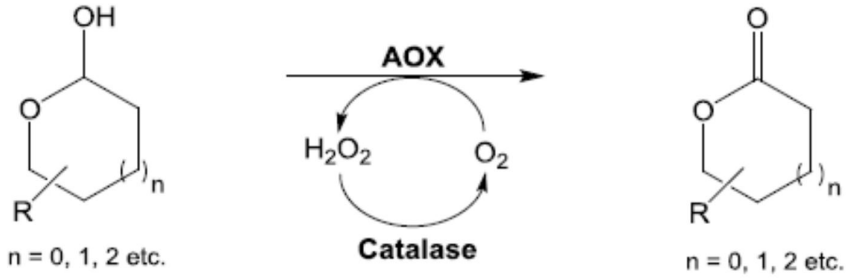


Figure 3. Biooxidation of a lactol using an AOX.

Substrate and product characteristics

In this case, the substrate and product are rather water soluble which lessens problems related to substrate supply. On the other hand, product recovery would be more difficult to achieve through liquid-liquid extraction. The favourable thermodynamics of the reaction allow for high conversion, consequently simplifying the downstream processing since substrate/product separation may not be required. However, the lactone product is sensitive to degradation, and the degradation rate is pH dependent, making the pH monitoring and control throughout the entire process an important issue.

Biocatalyst characteristics

The use of the free enzyme has several implications which differ from the use of whole-cells. Faster mass-transfer rates are expected because there is no barrier (cell membrane) between the substrate/product and the enzyme. The free enzymes, however, may be damaged by e.g. sparging of air or oxygen. The stability towards gas-liquid interfaces is therefore important to investigate, and alternative oxygen supply methods may need to be employed.

Based on the above, a potential flowsheet for the process can be outlined (figure 4).

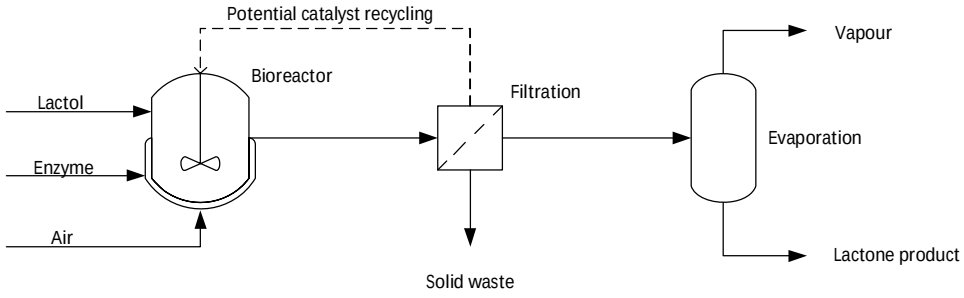


Figure 4. Potential flowsheet for AOX catalyzed lactone production.

Alcohol dehydrogenase biooxidation

The use of alcohol dehydrogenase is an alternative to alcohol oxidase. Thus the reaction schemes and the overall processes look very similar, both consisting of a two-enzyme cascade. However, there is a significant difference in that ADH is a co-factor requiring enzyme, thus regeneration of the oxidized co-factor (NAD^+) is necessary. A particularly interesting way to achieve this is the use of a NAD(P)H oxidase (NOX). This enzyme uses molecular oxygen to oxidize the co-factor, producing only water as the by-product (although some NOXs produce H_2O_2 as a byproduct). The biooxidation using ADH is shown in figure 5.

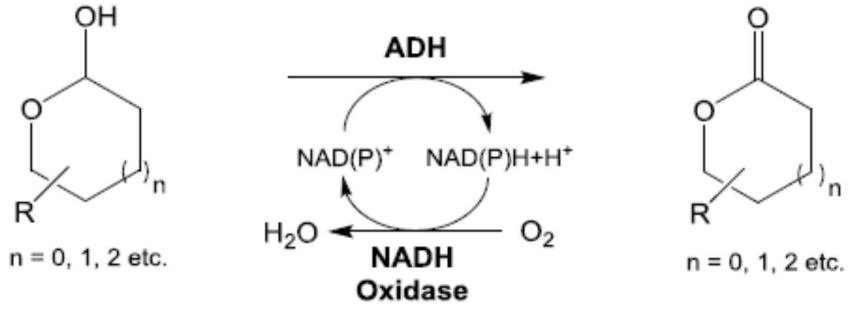


Figure 5. Biooxidation using ADH. Co-factor regeneration achieved using NOX and molecular oxygen.

Naturally, the process design challenges in this case are similar to the AOX case.

Concluding remarks

The presented report illustrates the need for a range of competencies to develop a process based on biocatalysis, and the need for frequent exchange of data between involved parties to make the development efficient. It is shown that it is difficult to design a good process around a biocatalytic reaction without knowing the limitations of that reaction. Conversely, the data generated in the characterization of the reaction (and even in the protein engineering stage) may not be relevant to the development efforts if the economic restrictions of the intended process are not considered. To address this issue, a comprehensive overview of the information that is required to gauge the performance potential of an intended process has been provided. Additionally, a concrete set of experiments have been suggested for characterizing the biocatalyst. Such data must be obtained to evaluate and design the oxidative biocatalytic processes properly and to benchmark them amongst each other and (at a later stage) with the conventional chemical process towards the ROBOX demonstration's target molecules. Finally, the procedure for evaluation has been illustrated through a ROBOX demonstration case study, with suggestions for how the process in the case could be improved.

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