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Considerations for the Application of Process Technologies in Laboratory- and Pilot-Scale Biocatalysis for Chemical Synthesis

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1.1 Introduction

The development and implementation of an efficient new biocatalytic process relies upon successful communication between the scientists establishing the chemical reaction (organic chemists, process chemists, analysts, etc.), those developing the biocatalyst (microbiologists, biochemists and molecular biologists, analysts, etc.), and those scaling up the process (process, biochemical, and chemical engineers). The working relationship between the first two groups has strengthened enormously in recent years, but nevertheless successful scale-up also requires process engineering involvement from an early stage. In the pharmaceutical industry, it is easy to argue that the rate of attrition of new target molecules is such that any consideration for scale-up should be delayed for as long as possible. However, the reality is that to address the process aspects too late is equally problematic. The problem is exacerbated by many of the chemical reactions of greatest commercial interest in transforming non-natural substrates. In some cases, the selectivity of an enzyme is not compromised, but its activity is nearly always found to be lower than on a

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comparable natural substrate. Additionally, the conditions under which these enzymes are expected to operate in industry are also very often far from those found in nature, further affecting their activity and stability. Therefore, this necessitates improvements not only to the biocatalyst, but also to the reactor and process, such that a suitable system can be designed and implemented for scale-up. This demands effective communication and dialog between the various scientists at an early stage of process development.

This chapter is written with the intent of giving chemists, biologists, and engineers a basic idea of the concepts involved in implementing a biocatalytic reaction for development, scale-up, and, ultimately, production of a target product. Frequently, information relevant to these fields is scattered, and therefore a deliberate attempt has been made here to bring it together into a single compilation to help in disseminating the available knowledge to those working in all aspects of biological chemical conversions.

It is hoped that this will give a better understanding to scientists working in unifying these fields for efficient process development. For example, a biologist should be able to use this chapter to help understand the importance of setting commercial targets to measure the success of the biocatalysts they have developed. Likewise, process chemists can appreciate the key differences between the application of chemical catalysis and biological catalysis. Additionally, this chapter aims to guide chemists and biologists in designing experiments to obtain relevant data that might help in a smooth transition from a laboratory proof-of-concept to a scalable chemical synthesis with product isolation. Engineers will also abstract the differences between biochemical and conventional chemical transformations.

The aim of this chapter is to provide readers with an understanding of the important tools and technologies available for use in biocatalysis. Specifically, the technologies that can be implemented at laboratory and pilot scale will be addressed. Quantitative information will be provided when possible for application of these technologies, which will hopefully guide the reader to make educated decisions on how to efficiently operate their processes. The purpose, therefore, is not to answer all questions, but to give a quick overview of the different characteristics and considerations for the said technologies.

Finally, it is of vital importance to acknowledge that this text is based on the contributions and experiences of many scientists and engineers (both in academia and in industry) from different spheres involved in the establishment of fundamental and applied research of the discussed technologies.

1.2 Process Intensification and Proposed Scale-Up Concept

The arguments for the application of biocatalysis as a catalytic tool in organic synthesis and production are numerous, but are perhaps most usually focused on the exquisite selectivity that biocatalysts offer [1]. Clearly, the rationale for implementation depends upon the industrial sector and the value of the product to be produced.

One of the central challenges in the development and implementation of new enzymatic processes in industry is translating an established laboratory-scale reaction into a commercial process. The first step in that journey should be to establish suitable conditions for the reaction, in particular the required selectivity and product purity. This is mainly the work of organic and process chemists. The enhancement of enzyme properties is also a major

preoccupation at this point, carried out by molecular biologists. In order to make a process that can meet the demands of industry, scale-up also needs to be considered. As we have suggested in several recent publications, it is best to address this in two steps: first, by improving the process via enzyme modification and process intensification, and second by considering scale-up by volume increase [2]. This two-part philosophy builds confidence at an early stage that the process is indeed scalable and helps to test the limits of the process technology, both at laboratory and at pilot scale.

Biological conversions using enzyme(s) or enzymes in cells (in a non-fermentative state) are also known as "biocatalytic reactions," and are typically carried out in aqueous media. Biocatalysts have evolved to work on particularly low concentrations of natural substrates, so as to make them highly efficient in nature. However, in industrial applications, biocatalysts are often subject to non-natural environments, such as reaction solutions with high concentrations of substrate or product. Both the activity (reaction rate) and stability (maintenance of reaction rate over time) of the biocatalyst are affected by this. However, both parameters are also critical for the performance of the biocatalytic process. The rationale behind operating the reaction at high concentrations of substrate and product is the need to meet the minimal process metrics (in order to fulfill the required commercial targets). Reaction (and process) yield, as well as space–time yield, are determined by the commercial targets of a particular product. In other words, a certain supply rate of product will be required for economic feasibility (either in an existing facility or in a dedicated facility). These commercial targets will, in turn, also set the process metrics. For a biological conversion with isolated (immobilized) enzymes or resting whole cells, the process metrics can be defined by the product concentration and the biocatalyst yield (linked to the allowable cost for the biocatalyst). For biological conversions with growing cells (fermentations), space–time yield replaces the biocatalyst yield metric (since the time required for cell growth limits the process). The various commercial targets and process metrics are defined as follows:

Reaction yield (in engineering terms – same as "conversion" in laboratory terms) is a measure of the mass of product formed per mass of substrate consumed (usually expressed in units such as $g.g^{-1}$). The reaction yield may also be expressed on a molar basis. Process yield (rather than reaction yield or conversion) can be used to take into account losses in the downstream recovery of the product following the reaction. It is important to consider process yield in order to ensure sufficient product is made to achieve the necessary commercial metrics. Together with the difference in value between the reaction substrate and product, the reaction (and process) yield will determine the value added to the substrate as a result of the reaction. It is therefore the paramount commercial metric.

$$\textit{Reaction yield} = \textit{mass of product formed}/\textit{mass of substrate consumed}$$

Biocatalyst yield is the mass of product formed per mass of biocatalyst provided (expressed in units such as $g.g^{-1}$). In many cases, biocatalysts are recovered and then recycled following a reaction. Hence, the biocatalyst yield should reflect the cumulative mass of product formed over all the batches in which the biocatalyst is used. Together with the absolute cost of the biocatalyst (determined by the supplier or the fermentation conditions), the biocatalyst yield will determine the cost contribution of the biocatalyst to the final operating cost. In many cases, this is essential to ensuring the cost of the

manufacturing process is sufficiently low for the product value to meet market expectations.

$$Biocatalyst\ yield = mass\ of\ products\ formed / mass\ of\ biocatalyst\ provided$$

Product concentration is the mass of product formed per reaction volume (usually expressed in units such as $g.L^{-1}$). The product concentration defines the scale of the downstream product recovery process and will therefore determine both the operating cost and the capital cost for dedicated plants.

$$Product\ concentration = mass\ of\ product\ formed / volume\ of\ reactor$$

Space–time yield is the mass of product formed per reaction volume per time (usually expressed in units such as $g.L^{-1}.h^{-1}$) and is a measure of the capacity of a process. For a given production rate, the space–time yield thus defines the scale of the reactor to achieve a given commercial target. For a process based on growing cells (fermentation), the space–time yield is largely governed by the time required for the growth of the cells, which thus determines the cost contribution of the fermentation to the overall conversion process.

$$Space - time\ yield = mass\ of\ product\ formed / volume\ of\ reactor / time$$

There are as yet no accepted guidelines for the minimum process metrics, and in any case they are, of course, in large part dependent on the potential production volume (market size), required purity, and value of the product (e.g., bulk chemical, speciality chemical, or small-molecule pharmaceutical). Nevertheless, some example values are given in Table 1.1 as a first guide. The required biocatalyst yield is closely linked to the allowable cost for the biocatalyst. The guidelines in Table 1.1 are for immobilized enzymes and the required yields are consequently high (see Section 1.3.1).

In order to achieve such process metrics, it is clear that in almost all cases a strategy of biocatalyst improvement (via targeted protein engineering, biocatalyst modification, and improved biocatalyst production) should be complemented by the implementation of process intensification options into the reaction (and process) of interest. The purpose of the following sections is to introduce the reader to the available process-intensification options. With that in mind, the sections have been formulated by first describing the

Table 1.1 *Example guidelines for minimum process metrics (biocatalyst yield and product concentration) for immobilized enzyme-catalyzed reactions in different industrial sectors.*

Product market	Product value ($.kg^{-1})	Biocatalyst yield ($kg.kg^{-1})	Product concentration ($kg.m^{-3})
High value (e.g., pharmaceuticals)	500	50	50
Medium value (e.g., flavors)	100	500	150
Low value (e.g., bulk chemicals)	1	5000	300

technologies available and then outlining some of the considerations required for the implementation of each. The purpose is not to give answers to the question of how a process should be operated, but rather to provide a guide for selection of the most suitable options in which to invest time, effort, and money in further research in a given case. Necessarily, the chapter does not aim to be comprehensive.

1.3 Enabling Technologies

Enabling technologies are those technologies that allow the process to be implemented. While biocatalyst immobilization (Section 1.3.1) is not essential to implementation, consideration of the options is nearly always required. On the other hand, all reactions will need to be operated in an available reactor, or, more rarely, a dedicated reactor (Section 1.3.2). Understanding the implications of using a given option is of great importance to achieving the required process metrics for given commercial targets.

1.3.1 Biocatalyst Immobilization

Immobilization is the process of attaching soluble enzyme, or alternatively whole cells, on to or into larger particles of inert support materials, with the primary aim of facilitating an easy separation so as to remove (and frequently recover and recycle) the biocatalyst from the product stream. A secondary objective is to improve the operational stability of the biocatalyst. Biocatalyst immobilization is a process of significant importance and plays a central role in the operational performance of a biocatalyst. Immobilization can be applied to both enzymes and whole cells. Very many (in fact, several hundred) techniques, which can broadly be classified into carrier immobilization, carrier-free immobilization, and entrapment, are available for this purpose [3,4]. Carrier-immobilization and carrier-free-immobilization methods will be outlined here.

Attaching the biocatalyst to a support material brings the advantage of separation by simple filtration (e.g., using microfiltration, or even sieves – so-called "Johnson" screens – for larger particles). Such separation facilitates the first step in the downstream process, enabling complete removal of the biocatalyst (which is especially required in pharmaceutical manufacture). Provided the biocatalyst has sufficient stability (this is often improved on immobilization), it can potentially be recovered for subsequent recycle, enabling an increase in biocatalyst yield. The insoluble format of the immobilized enzyme makes the use of continuous-packed-bed and fluidized-bed reactors possible.

Another aspect of immobilization is that it allows for specific control of the biocatalyst's microenvironment. For example, the hydrophilic/hydrophobic balance of the immobilized biocatalyst can be changed by altering the support material. Finally, multi-enzyme and chemoenzymatic cascades can be made possible using such technology to co-immobilize them on to a single support as a multifunctional catalyst, when both (bio)catalysts have similar stability (provided that the activities are balanced). When this is not the case, immobilization can allow the separation of (bio)catalysts inside a reactor (by compartmentalization).

The stated advantages of separating the biocatalyst from the bulk reaction media are applicable to the majority of biocatalytic reactions, most of which are carried out in aqueous media. For those biocatalytic reactions that are performed in organic or biphasic media, the

formulation of an immobilized biocatalyst is essential, since the biocatalysts cannot generally be dissolved in such systems.

1.3.1.1 General Considerations for Implementation

The first and most important consideration is that the process of immobilization itself may lead to a loss of enzyme activity. The conditions for immobilization are often harsh, and potentially up to 50% of the activity can be lost in the preparation step [5]. Furthermore, the carrier must be added, and this, combined with operational costs for immobilization, adds considerably to the final cost of the biocatalyst [6]. Hence, biocatalyst recovery and recycle, while clearly advantageous, may in many cases also be a necessity, in order to recover the losses caused by the immobilization procedure, as well as the cost of the support material in the formulation. In some rare cases, recycle of the carrier (support) material may also be possible, even if the enzyme is not stable enough to be recycled.

A further complication with immobilized biocatalysts is the potential for substrate mass transfer limitations. During operation, the substrate(s) needs access to the enzyme, which is rarely on the surface of the support alone, but usually in pores throughout the carrier particle. In general, a large surface area, typically greater than $100\,m^2.g^{-1}$, is required for immobilization [7,8]. Consequently, very small particles (with an average diameter less than 50 μm) would be required were surface immobilization used alone, in order to provide sufficient activity per volume, and this would make filtration problematic, defeating the primary purpose of the immobilization. For this reason, most particles are larger (with an average diameter of 150–500 μm), implying that the rate of substrate transport into the support (and the rate of product out of the support) can potentially be rate-limiting. In fast reactions, with a low-porosity structure (sometimes used to give the required strength to the support), diffusional limitations can therefore be expected [9]. The measured reaction rate in such cases may be lower than the equivalent rate measured with soluble enzyme, depending upon the K_m of the enzyme. Mass transfer limitations can also be detrimental to the selectivity of the biocatalyst.

1.3.1.2 Carrier-Bound Supported Enzymes

1.3.1.2.1 Adsorption. Adsorption refers to the binding of the enzyme (or, potentially, whole-cell biocatalysts) on to a porous support (or carrier) via physical interactions such as hydrogen bonds, van der Waals forces, and hydrophobic interactions [10]. The maximum adsorption of a protein on a hydrophobic carrier usually occurs around its isoelectric pH (pI value). A schematic representation of the adsorption process is shown in Figure 1.1.

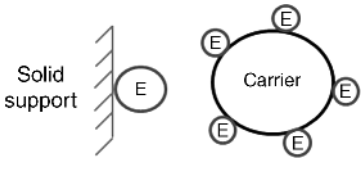


Figure 1.1 *Schematic representation of enzyme immobilization by adsorption (E, enzyme).*

In such cases, the activity loss is minimal provided conformational changes are avoided. The method is also simple and versatile [11]. The amount of protein that can be loaded is somewhere between 2 and 50 mg of protein per gram of support. The support is usually between 100 and 200 μm in diameter. A classical industrial example of immobilization by adsorption is the immobilization of *Candida antarctica* B-lipase on a polymeric carrier (Novozym 435).

Considerations for Implementation. With high protein loading, some steric hindrance may lead to lower measured reaction rates compared to the equivalent measurements using soluble enzyme. Perhaps more serious is that desorption (sometimes referred to as "leaching") of protein is possible: this means not only that the enzymatic activity will be reduced, but also that some protein can pass downstream. For pharmaceutical applications, this is a serious limitation, and usually necessitates an extra ultrafiltration step immediately downstream of the reactor, prior to the other product recovery operations. However, potential desorption is very dependent upon operating media. For example, in organic media, adsorption works particularly well, since the protein does not leach from the surface.

1.3.1.2.2 Covalent Binding. A second group of immobilization methods can be classified as those based on covalent binding of the enzyme to the support material. The carrier is bound to the enzyme by means of functional groups (i.e., the amino acid residues) of the protein. Care should be taken that use of these residues does not interfere with the active site or the substrate binding sites of the enzyme. For glycosylated enzymes, there is also the option of coupling using their carbohydrate moiety. The most common modes of covalent binding are diazo-coupling, peptide bond formation, and alkylation or arylation. Other, less common methods include Schiff's base formation and amidation. A schematic representation of enzyme immobilization by covalent binding is shown in Figure 1.2.

Clearly, covalent immobilization establishes a permanent bond between the enzyme and the support, meaning that the enzyme is usually stabilized by maintaining its tertiary structure intact and the potential for desorption of the protein is eliminated. There are many support materials that can be used, provided the material can be activated so as to form a covalent bond with the enzyme. Eupergit C is an excellent example of enzyme immobilization by covalent bonding [12]. An application of this type has been demonstrated in the production of N-acetylneuraminic acid using N-acetylneuraminic acid aldolase

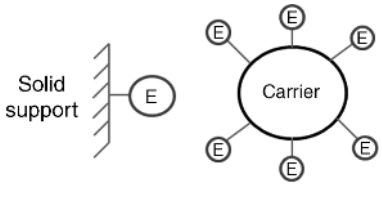
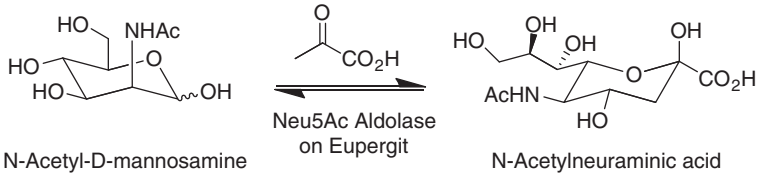


Figure 1.2 *Schematic representation of enzyme immobilization by covalent binding (E, enzyme).*



Scheme 1.1 *Synthesis of N-acetylneuraminic acid using supported enzyme.*

immobilized in Eupergit C. The immobilized enzyme was reused for at least nine cycles without significant loss in activity (Scheme 1.1) [13].

Considerations for Implementation. Potentially, the enzyme can change conformation during covalent bond formation, leading to a significant loss in activity and/or selectivity. It is important to consider that the carrier needs to be chemically activated prior to formation of the covalent bond. In some cases, the coupling agent can deactivate the enzyme of interest, lowering the applicability.

1.3.1.2.3 Ionic Binding. Ionic binding is a method of immobilization that exploits the ionic interaction between the carrier and the support to facilitate binding. Both cationic and anionic exchangers can be used as the support. A schematic diagram of such enzyme immobilization is depicted in Figure 1.3.

Clearly, one of the major advantages of using ionic interactions to attach the enzyme to the support is that there are already many cheap and readily available ion-exchange resins on the market that can potentially be used. While the binding forces are stronger than those of physical adsorption, they are not as strong as covalent binding.

Considerations for Implementation. The binding stability (balance of adsorption and desorption) is affected by the pH and the ionic strength of the reaction medium, which must be well understood and controlled. Reactions in which the ionic strength of the medium changes (e.g., reactions where an acid or base is added to maintain constant pH changes on account of the reaction) need to be examined with particular care. Likewise, for whole-cell immobilization, cell age, pH, ionic strength, and surface charges can all affect the performance of the immobilization procedure.

1.3.1.3 Carrier-Free Immobilization

Carrier-free immobilization is the preparation of insoluble enzymes (without the use of carriers), which can be useful for industrial biological conversions. Several methods exist,

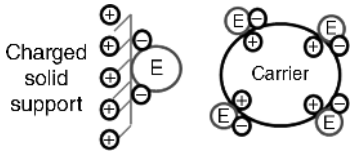


Figure 1.3 *Schematic representation of enzyme immobilization by ionic binding (E, enzyme).*

including cross-linked enzyme aggregates (CLEAs), cross-linked enzyme crystals (CLECs), flocculation, and aggregation [14].

1.3.1.3.1 Cross-Linked Enzyme Aggregates (CLEAs^{TM}). An alternative and potentially easier approach to conventional immobilization of a carrier support material is to aggregate the proteins. Proteins usually aggregate in the presence of salts, non-ionic polymers, or water-miscible organic solvents. Subsequently, the aggregates can be cross-linked to render them permanently insoluble. Such cross-linked insoluble aggregates are termed "CLEAs" [15,16].

Protein aggregation is a simple immobilization technique amenable to rapid optimization. There are few process steps for catalyst formulation, and it can potentially be used to combine purification and immobilization in a single unit operation [17]. It is a relatively low-cost method, since it avoids the use of expensive carrier supports and leads to a short development time. Additionally, high catalyst activity can be achieved, since the enzyme activity is concentrated as an insoluble aggregate with very high protein content. Likewise, catalyst stability can be very high. Furthermore, the technique can stabilize quaternary structures of multi-meric enzymes [18].

Recently, it has been shown that simple multi-enzyme immobilization is also possible. This results in combinations of enzymes that can be immobilized together and have potential for use in cascade reactions (combi-CLEAs) [19]. Inevitably, typical biocatalyst aggregate sizes are quite small – usually with diameters between 5 and 50 μm – but larger sizes are also available [20]. Nevertheless, biocatalyst recycle is relatively easy using either filtration or the magnetic attraction of iron trapped in the aggregates [21].

Considerations for Implementation. Enzymes that have a low number of surface-reactive amino groups lead to the formation of mechanically unstable CLEAs due to poor cross-linking, and this can also lead to leaching of the enzyme during operation. Additionally, a new immobilization protocol has to be developed for the aggregation and cross-linking of each enzyme. In some cases, this may require some purification of the crude protein prior to CLEA formation. The glutaraldehyde linker in high concentrations tends to cause a loss of enzyme activity, due either to chemical modification of the functional groups or to denaturation induced by derivatization [15]. As with other immobilizations, the optimal size of the CLEA particles is a trade-off between their being small enough to minimize substrate mass transfer limitations and their being large enough to facilitate process handling. Although crucial for enzyme activity, control of CLEA size is difficult. Also, the CLEA particles may not always be able to maintain their mechanical stability under agitated conditions.

1.3.2 Reactor Options

As with conventional chemical synthesis, several reactor options are at the disposal of a process engineer wishing to implement and develop a biological conversion for larger-scale application. Both whole cells and enzymes (free and immobilized) have been used in several reactor configurations. The characteristics of these reactor designs will be discussed in this section. In an industrial setting (especially in the pharmaceutical industry), the reactor is often already defined, and the reactions need to be fitted to the equipment. In such cases, it is also useful to understand the characteristics of each reactor type, in order to understand

compromises and trade-offs that have to be considered. Finally, from the perspective of the process chemist and biologist, the reaction and biocatalyst characteristics have a profound influence on the choice of equipment to be used, or, alternatively, on the degree of compromise required to fit into existing equipment, in any given case.

1.3.2.1 Ideal Reactors

Classical chemical reaction engineering classifies reactors into groups dependent upon hydrodynamic properties and mode of operation. Three "ideal" reactor configurations are defined based on the concept of "ideal" hydrodynamics (well-mixed or plug-flow) and mode of operation (batch or continuous). At large scale, such "ideality" does not exist, but nevertheless the classification provides a useful basis for reactor characterization. In a reactor with well-mixed hydrodynamics, the reaction mixture is agitated so that the concentration, temperature, and pH are identical throughout the vessel [22–24]. For a continuous system, this also implies the leaving concentrations are the same as those in the tank, which has important implications for achievable yield and kinetics. For ideal plug-flow hydrodynamics, there is no mixing in the direction of flow through the reactor, and substrate and product concentration are a function of distance traveled (also expressed as "residence time"). Hence, three types of ideal reactor can be distinguished: batch stirred-tank reactor (BSTR), continuous stirred-tank reactor (CSTR), and continuous plug-flow reactor (CPFR). Each comes with a number of advantages and disadvantages related to, for example, volumetric efficiency, space–time yield, and achievable conversion, which will be discussed in the following subsections.

1.3.2.2 Modes of Operation

Most chemical processes can be characterized as either batch or continuous processes. The two clearly differ in that the former outputs product in discreet volumes – batches – and the latter delivers product continuously. However, the two modes of operation are also different in that the continuous process normally operates at steady state (reaction composition at a given point in the process does not change with time), whereas a batch process is dynamic. Both modes of operation come with several advantages and disadvantages. The initial investigation and reaction development for biocatalytic processes is almost exclusively conducted in batch or fed-batch mode.

There is often an interest in modifying processes to make them suitable for continuous operation as they are implemented for full-scale production. Among the clear advantages of continuous operation are the simplified process control for a system that operates at steady state and the potential for improved productivity, since the downtime for filling and emptying vessels (which can represent a significant part of the overall processing time in large-scale processes) is removed. However, a continuous process also comes with a number of challenges. Truly continuous operation requires dedicated equipment and either a high operational catalyst stability or a method for continuously resupplying the process with fresh catalytic activity. Starting up and balancing multiple process steps can also be a complicated task. Finally, continuous operation inherently makes it more difficult to separate product into distinct groups, or batches. As a consequence, errors or contamination in the process become more difficult to trace than in a batch process, and such problems can therefore potentially be more costly in a continuous system.

1.3.2.3 Well-Mixed Reactor Hydrodynamics

Regardless of the mode of operation (batch, fed-batch, or continuous) in well-mixed reactors, the composition of the tank contents is homogenous [25]. This has an important implication: the concentration of any given species is the same at all points in the reactor, meaning there is no concentration gradient across any part of the reactor.

1.3.2.3.1 Stirred Tanks. By far the most common type of well-mixed reactor is the stirred tank. Such reactors are very flexible but also have some important features to be borne in mind if they are to be used for biocatalytic reactions. For example, a maximum loading of immobilized biocatalyst of approximately 10% by volume can be tolerated in a conventional stirred-tank reactor. Higher biocatalyst loadings lead to a higher rate of particle attrition (due to a higher frequency of collision), which, aside from affecting both the activity and the stability of the biocatalyst, can also create a significant problem for downstream biocatalyst filtration for removal, or for recovery and subsequent reuse [26,27]. The aspect ratio of the reactor (ratio of tank height to tank diameter) also affects bulk mixing. High aspect ratios result in poorer overall mixing, so a ratio of unity is normally applied [24]. However, in cases where mass transfer of poorly water-soluble gases (such as oxygen) into the reaction is required, the aspect ratio is increased to as high as 3 in order to increase the residence time of the gas phase in the reactor, and consequently the uptake of gaseous solutes. Typically, reactors are baffled in order to prevent vortex formation and improve the stirring efficiency of the power input. Four baffles are normally used, with a dimension of around 1/10 the diameter of the vessel [24]. When solids are present in the medium (or an immobilized biocatalyst/CLEA is used), the agitator speed must be sufficient to allow good suspension of the particles. The minimum agitation speed required to keep all the immobilized particle in suspension is termed just off-speed limit [28]. Reactors may use more than one impeller (dependent on the scale), with a typical spacing of between 2/3 T and T, and where $D/T \approx 0.4$ (D and T are the impeller and tank diameters, as illustrated in Figure 1.5). Power input for stirring should be around 1–1.5 $W.L^{-1}$. Indeed, for reactors up to a volume of around 500 L, a power input of 1–1.5 $W.L^{-1}$ gives a well-mixed reaction volume [24]. Nevertheless, as scale increases, mixing time (inversely proportional to the agitator speed) will also increase for a given power input. Stirred-tank reactors can be operated in batch, fed-batch, or continuous mode of operation. The advantages and disadvantages of these modes are discussed later in this section.

Considerations for Implementation

- **Mixing type:** Axial mixing is typically used for reactors handling solids, while radial mixing is extensively used for two-liquid phase systems [29]. A schematic representation of the types of mixing can be found in Figure 1.4.
- **Impeller position:** When handling multiphasic systems, the impeller must be placed in the phase that is to be under continuous operation conditions.
- **Motor type:** In order to give some flexibility, the motor used will typically be a variable speed motor.
- **Aspect ratio:** Mixing time increases with the height-to-width ratio of the tank, hence it is always beneficial to keep this ratio close to unity. However, in some systems (where gas is required, for example) it may be necessary to use higher ratios [23]. In such cases, it is common practice to place multiple impellers on the shaft.

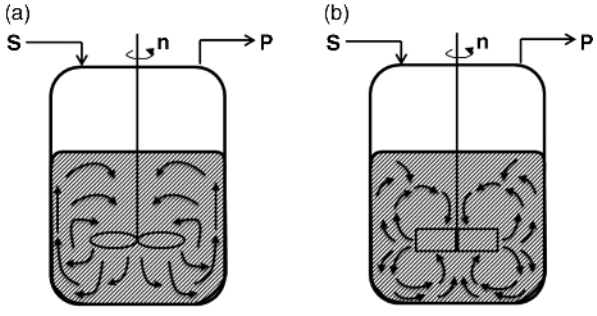


Figure 1.4 *Stirred reactors with (a) axial (down) flow and (b) radial flow (S, substrate; P, product; n, number of rotations per unit time).*

- **Mechanical stress on biocatalyst particles:** Mechanical stress is usually high on immobilized enzymes, due to agitation, especially at higher biocatalyst loadings [30–32]. This stress can be limited by reducing the mixing speed/power input. However, this will result in poorer overall mixing.
- **Heat transfer:** Heat transfer in the reactor is usually achieved through a jacket fitted around the reactor. Although maintenance of the correct temperature is important, control is rarely an issue in biocatalytic reactions, with the exception of enzyme-catalyzed polymerization (which can be exothermic).

1.3.2.3.2 Batch Stirred-Tank Reactors (BSTRs). BSTRs are stirred-tank reactors that are operated in batch mode. All the reactants (substrates) are charged into the reactor at the start of the operation and allowed to react. Upon completion, the product is recovered (Figure 1.5). The conversion achieved is a function of the batch operation time, and in principle complete conversion is possible.

This type of reactor can be used for kinetically slow reactions. Many enzymatic reactions typically fall into this category. The reactor may also be operated in fed-batch mode, meaning that some (or all) of the substrates are fed to the reactor in a controlled manner, at

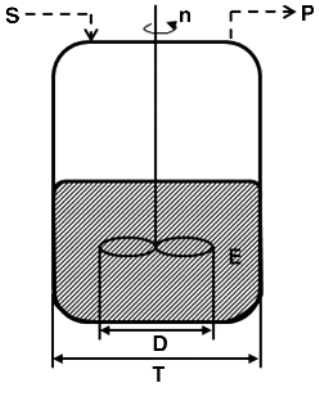


Figure 1.5 *Schematic diagram of a batch stirred-tank reactor (BSTR) (E, immobilized enzyme; S, substrate; P, product; D, diameter of the stirrer; T, diameter of the tank reactor; n, number of rotations per unit time).*

constant or variable feed rate [33]. This is particularly useful for substrates that are inhibitory or toxic to the biocatalyst. Likewise, the mixing means that this configuration offers ease of handling for multiphasic (gas–liquid, solid–liquid, or liquid–liquid) reaction mixtures. The reactor type can accommodate all types of biocatalyst and is relatively easy to scale up. Finally, the BSTR is relatively straightforward to adapt at industrial scale, as it is commonly available and many industrial processing sites have suitable vessels already available for use with minimal capital investment.

Considerations for Implementation

- **Batch shift:** Sometimes it is desirable for the batch reaction to fit within a regular 6- or 8-hour working shift. This dictates the target space–time yield for the process and must be considered.
- **Catalyst deactivation:** It is often desirable to reuse a biocatalyst in multiple batches, especially for immobilized formulations (due to the added cost of the immobilization procedure). However, the performance of the catalyst will typically vary from batch to batch, due to a gradual loss of activity over time. This activity loss can be compensated for in several ways. One method is to add fresh catalyst to each batch; this approach is limited by the amount of fresh biocatalyst that can be added to the reactor (without exceeding loading limitations). Alternatively, the reactor can be operated for longer time periods, to account for lower activity as a function of time. In order to manage biocatalyst deactivation, it may also be possible to increase the operating temperature in the reactor, but this is limited by the thermal stability (or otherwise) of the biocatalyst and reaction components [34]. Loss of a batch of immobilized biocatalyst can have serious economic implications, since it prevents reuse, significantly increasing the cost contribution of the biocatalyst.

1.3.2.3.3 Continuous Stirred-Tank Reactors (CSTRs). CSTRs use the same type of reaction vessel as BSTRs but operate with a continuous reactant feed and product removal stream (Figure 1.6). For an ideal reactor, this implies operation at the exit substrate concentration. A direct consequence of this is that reaching complete conversion is not

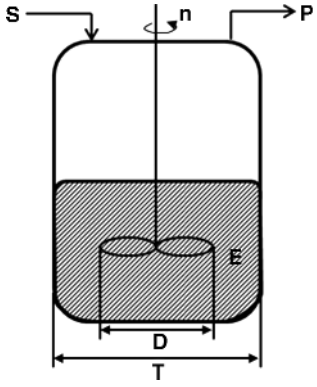


Figure 1.6 *Schematic diagram of a continuous stirred-tank reactor (CSTR) (E, immobilized enzyme; S, substrate; P, product; D, diameter of the stirrer; T, diameter of the tank reactor; n, number of rotations per unit time).*

possible (since some substrate must be present for the reaction to take place). It also indicates that there is a trade-off between achievable conversion and reaction rate (dependent upon the value of the K_m of the enzyme) (see, for example, [35]).

As explained earlier, compared to the equivalent BSTR, the CSTR is usually larger (dependent upon the K_M value of the enzyme of interest), since the kinetics are not as favorable. Nevertheless, the saving on downtime (filling, emptying, and cleaning) can be significant. This is emphasized in batch reactors with a relatively short operating time (e.g., those limited by product inhibition). In cases where the kinetics are poor in a CSTR (i.e., the exit concentration of substrate is at a value that is just a fraction of the enzyme K_M), the reaction may be improved by simulating plug-flow operation, achieved by operating several CSTRs in series (although, for economies of scale, it is unlikely more than three will be used). Interestingly, retrofit of existing batch reactors into CSTRs should be fairly straightforward, provided pumps are available. Such systems can easily cope with multiphasic media, although care should be taken about feed and draw streams in relation to the phase ratio [32]. The CSTR is also highly efficient in terms of man power and control, since the system is in steady state and typically exhibits a first-order response to any perturbations.

Considerations for Implementation. The main disadvantages of a CSTR are that it generally operates at a lower average reaction rate than a BSTR and that it by definition cannot achieve equilibrium conversion. Another aspect that should be considered early in process development is the method of catalyst recycling and reuse, if this is required for the economic feasibility of the process. When a non-immobilized biocatalyst is employed, it must be separated from the product stream and recycled to the reactor in a way that does not inactivate the biocatalyst. Even so, both immobilized and non-immobilized biocatalysts will gradually lose activity over time. In order to manage biocatalyst deactivation, one of the following two methods should be adopted [36]:

1. **Add fresh biocatalyst to the system:** The design of the CSTR must then take into account that fresh biocatalyst will be added. Clearly, there will be a volumetric limit to how much fresh biocatalyst can be added to the system.
2. **Lowering the flow rate:** Depending on the productivity required from the plant, the flow rate can be reduced to some extent to cope with the loss of biocatalytic activity. This means that identical conversion can be maintained, thereby simplifying the downstream process. Ideally, an average productivity value for the plant over the whole of the usable lifetime of the biocatalyst will be used for plant sizing calculations, such that reduced throughput toward the end of a cycle is taken into account.

1.3.2.3.4 Alternative Well-Mixed Reactors

Continuous Fluidized-Bed Reactors (CFBRs). In a continuous fluidized-bed reactor (CFBR), mixing of solid material can be achieved by driving a gas (or, in principle, liquid) phase through solid or porous particles at such a speed that the particles become "fluidized." The particles are therefore held in suspension within the reactor by means of the fluid passing through the system (Figure 1.7) [37]. Operated in continuous mode, such reactors provide good opportunity for multiphasic systems.

Such reactors have the advantage of high biocatalyst loading, without the pressure-drop problems of packed-bed reactors. However, the scientific literature reports only a few cases

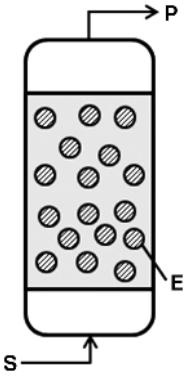


Figure 1.7 *Schematic diagram of a fluidized-bed reactor (FBR) (E, immobilized enzyme; S, substrate; P, product).*

of biocatalytic fluidized beds [38–40]. Since the immobilized enzymes are suspended in liquid, the system should be treated with care to prevent the enzyme being washed away. Conventional fluidized beds use solids suspended in gases, where the density difference between phases is much greater. Hence, while small particles of biocatalyst can be used (which would give a high-pressure drop in a packed-bed reactor), there is always the risk of wash-out where biocatalyst is lost from the reactor. Small particles have the benefit of having no diffusional limitations but are hard to retain in the reactor. Extremely small immobilized enzyme particles (around 10 μm) may be used with a fluidization aid. On the other hand, large biocatalyst particles, which would not be suited to use in a CSTR, due to attrition, may be suitable in a CFBR. Due to the "fluid-like" behavior, the handling of solids is easy, so these reactors are also well suited to reactions with solid substrates or products [34,41]. Likewise, viscous substrates and products can be used.

Considerations for Implementation. A CFBR is a well-mixed reactor, implying kinetics similar to those of a CSTR. In principle, if rated correctly, the biocatalyst will be retained in the reactor without the need for a sieve. Nevertheless, care should be taken, since small immobilized biocatalyst particles can easily be removed in the effluent stream, necessitating further ultrafiltration downstream. While some immobilized biocatalyst particle attrition can be expected, far higher loadings of immobilized biocatalyst can be used than in the equivalent stirred-tank reactor. Reactors are usually fitted with a perforated plate at the bottom to facilitate distribution of fluids. In general, operating such reactors requires extensive knowledge and experience, which also makes scale-up potentially problematic.

Continuous Packed-Bed Reactors (CPBRs). Continuous packed-bed reactors (CPBRs) are tubular reactors filled with (bio)catalyst particles, which are retained by means of a filter [42]. The feed is often pumped through the bottom of the reactor to enable any intrinsic gas bubbles to escape from the column (Figure 1.8). The material flows through the column at the same velocity, and parallel to the axis of the column, without back-mixing (hence, plug flow). Since the biocatalyst is fixed inside the column, the residence time is a function of the position of the material in the column: the longer the column, the higher the conversion. However, the pressure drop across the column also increases in proportion with column length, limiting the

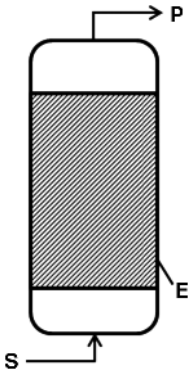


Figure 1.8 *Schematic diagram of a continuous packed-bed reactor (CPBR) (E, immobilized enzyme; S, substrate; P, product).*

maximum length of the column [20]. Hence, for scale-up, the width of the column can be increased in order to increase throughput without having to use a large number of columns. Care must be taken to increase the width in such a way that plug flow is maintained. The implication of having plug flow is that there is no concentration gradient across the width of the column. One example of the use of such a reactor is presented by Marrazzo and co-workers [43], and there are numerous others in the scientific literature.

The space–time yield is high, as there is a significant amount of biocatalyst in the reactor [33]. Space–time yields up to six times greater than those found in a stirred-tank reactor can be achieved. Likewise, the kinetic profile is favorable, and therefore shorter residence times are required than in the equivalent CSTR. The kinetic profile is identical to that in a BSTR, replacing the reaction time dimension with reactor length. The high concentration of biocatalyst means that reactors are smaller for a given conversion, and hence investment is lower (in terms of both capital and operating costs) than in the CSTR option. Additionally, no stirring is required, although pumps are necessary to pump reactants through the reactor. Immobilized biocatalyst particles will be exposed to less mechanical stress, since there is no stirring and the particles are stationary in the column. On the other hand, the particles must instead be able to withstand a (high) pressure drop, meaning they must be rigid and maintain their structure under pressure.

One successful commercial implementation of a biocatalytic PBR system is the trans-esterification process developed by Novozymes. The process uses a lipase immobilized on silica particles to exchange fatty acids between the triglycerides of different oils and fats, providing a superior product to that made in the alternative chemical processes.

Considerations for Implementation. Mass transfer limitations are prominent due to the absence of mixing, and it therefore appears attractive to use small particles. On the other hand, small particles result in a high pressure drop. A compromise must therefore be reached. Given the high space–time yield, this is usually achievable. Without a good distribution system at the inlet of the reactor, there is a risk of so-called "channeling" or "tunneling" of material through the column, which will lead to deviations from plug flow

and a reduction in the kinetic performance of the reactor. As stated previously, dependent upon the size and porosity of the particles, a high pressure drop across the column is possible. The ideal particle size for the immobilized particles used in packed-bed reactors (to ensure low back pressure) ranges from 200 to 400 μm [44]. Because the CPBR is a closed system, it is difficult to add or remove material as the reaction medium travels through the column. This is typically handled by splitting the process into multiple CPBRs in series. Coupling with *in situ* product removal (ISPR) is possible via an external loop, since the biocatalyst is retained in the reactor. However, control of pH (and temperature) is more difficult, as there will be a gradient in the column for reactions undergoing a pH change. Nevertheless, such a system can be operated with an external loop fitted to a small stirred tank, where acid or base can be added to neutralize changes in the column. Clearly, the pH change over the column must be sufficiently small to be effectively managed by the biocatalyst. Byproduct accumulation in the column is a common problem, and can be particularly significant when the byproduct causes inhibition of the biocatalyst. CSTRs are not good for multiphasic systems, since distribution leads to varying phase ratios, channeling, and even blockage of the column. When filling the reactor with biocatalyst, allowances should be made for biocatalyst swelling. The calculated 60% volumetric occupation by immobilized biocatalyst assumes no swelling. Swelling can also cause an increase in pressure drop over the column.

Continuous Expanded-Bed Reactors (CEBRs). Continuous expanded-bed reactors (CEBRs) are tubular reactors that are operated as fluidized beds, except at lower velocities. In order to maintain plug flow. the immobilized biocatalyst particles are usually of a variety of sizes and/or densities, so that they each find their correct suspension position in the reactor. Hence, the biocatalysts, based on their sizes and densities, align themselves in the reactor in such a way that there is a gradient of particle size and density along the length of the column: the larger particles are at the bottom of the column, the smaller particles are at the top (Figure 1.9). The flow of the fluid in the column then follows plug flow.

Interestingly, since there is voidage in the column, solid particles can be used in the feed; these would block a CPBR. Hence, a prefiltration step is not required when using a CEBR,

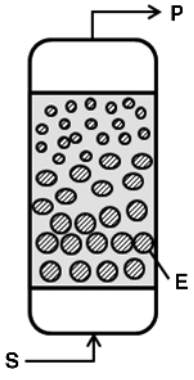


Figure 1.9 *Schematic diagram of a continuous expanded-bed reactor (CEBR) (E, immobilized enzyme; S, substrate; P, product).*

unlike a CPBR. In principle, particles of similar sizes and densities can be used, held in place via a magnetic field if they are constructed around iron particles, although there are only rather limited reports of such systems in the scientific literature. Integration with ISPR and other product recovery operations is also possible using adsorbent or absorbent resins in the bed, together with immobilized particles. Particle sizes are between 50 and 400 μm and densities between 1.1 and 1.3 $g.mL^{-1}$ [45].

Considerations for Implementation. CEBRs are sensitive to operating conditions. While a CEBR has significant benefits over a CFBR, such as reduced particle attrition, it is still essential to use care and carry out small-scale tests to ensure plug-flow distribution in the reactor.

1.3.2.3.5 Membrane Bioreactors (MBRs). Membrane bioreactors (MBRs) have found applications in a myriad of fields, including the petrochemical, water-treatment, food, and pharmaceutical industries. Such reactors have also been applied in bioprocesses [46–48]. The use of these reactors exploits the fact that reaction and separation processes can be combined. Hence, the motivation for their development is the cost saving derived from the reduced number of processing stages. They can be used in two main applications: (i) where the membrane acts as a support upon which the enzyme is immobilized (Figure 1.10) and (ii) where the membrane is used for separation of the product integrated with the reaction (Figure 1.11). Several configurations of MBR are available for use, although their details are not discussed here.

There are many advantages of such a reactor type, dependent upon configuration, but primarily it is a simple system to operate. Normal operation is in continuous mode. High

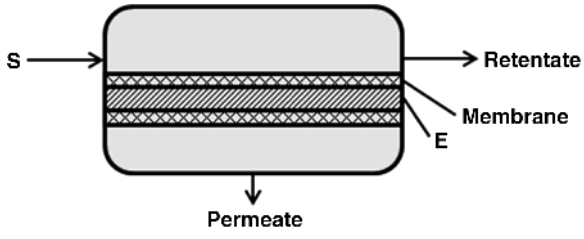


Figure 1.10 *Schematic diagram of a membrane bed reactor containing immobilized catalyst (E, immobilized enzyme; S, substrate).*

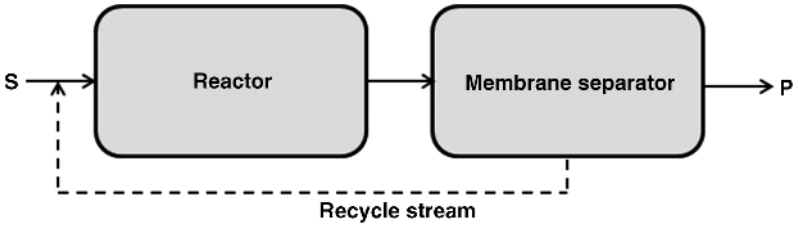
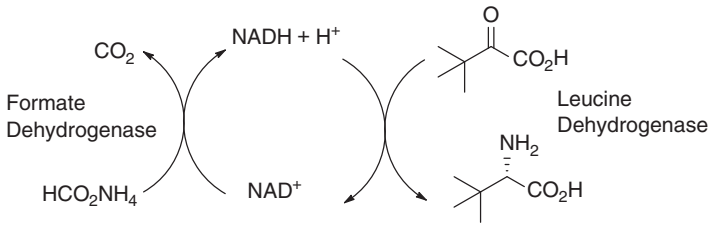


Figure 1.11 *Schematic diagram of a membrane bioreactor (MBR), used for separation integrated with reaction (E, immobilized enzyme; P, product).*



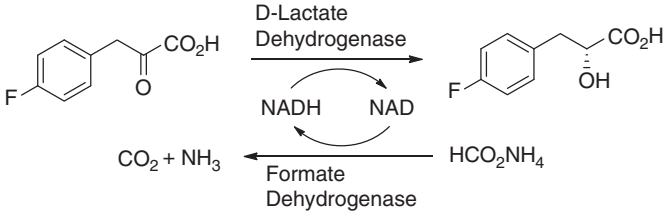
Scheme 1.2 *Synthesis of* tert*-leucine with membrane separation.*

yields (and potentially high selectivity) can be expected, since the basic concept allows reaction and separation steps to be integrated. Likewise, the system can be integrated with an ISPR [49]. Potentially, biocatalyst yield can be enhanced by maintaining high reactor productivity and increasing the usable biocatalyst lifetime by eliminating direct contact between the biocatalyst and inhibitory compounds. The biocatalyst can be separated to allow reuse and recycle, improving biocatalyst yield [50,51]. In a similar manner, expensive co-factors can also be retained for regeneration and recycle. Enzymes grafted on to the membrane surfaces can sometimes be more stable and resistant toward organic solvents than their soluble counterparts. Operation is usually via ultrafiltration membranes, with an average pore size around 0.1 to several micrometers. In such a system, the enzymes can be retained. In order to scale up, it is desirable to keep the membrane surface area-to-reaction volume ratio constant.

Degussa's dehydrogenase technology for the continuous production of (*S*)-*tert*-leucine is a classic example of a membrane reactor in which the membrane is used to retain the biocatalyst in the reactor (Scheme 1.2) [52].

Another example of a continuous process for the synthesis of (*R*)-3-(4-fluorophenyl)-2-hydroxy propionic acid was demonstrated by Pfizer: a multi-kilogram-scale synthesis with a space–time yield of 560 g.L.day^{-1} (Scheme 1.3) [53].

Considerations for Implementation. Membrane fouling and concentration polarization mean that flux (area-based flow rate) is subject to reduction as a function of time (lowering of flux is seen in [48]). Care should be taken to design (and rate) the system with this in mind. The molecular size of all components in the system (substrate(s), product(s), enzyme(s), and co-factor(s)) compared to the pore size of the membrane should be taken into account, since the performance of the reactor is in large part determined by the mass transfer across the membrane (or retention by the membrane) of these components. Such a reactor will therefore



Scheme 1.3 *Synthesis of* (R)*-3-(4-fluorophenyl)-2-hydroxy propionic acid.*

require appropriate fluid-dynamic conditions and reactor design for application. However, when membranes are used in a reaction vessel to execute separation, there is a certain loss in flexibility of the operation, as the conditions that are optimal for the reaction will not necessarily be optimal for the separation process. The cost of such a system can be high, due to cost of membrane replacement. The reactor performance is also affected by electrostatic and hydrophobic interactions between the biological molecules and the membrane. Flow of the substrate can be either axial (dead-end filtration) or tangential (cross-flow). Cross-flow is more suitable for large-scale applications. Membrane fouling is a common problem in operating such reactors, and consequently membrane cleaning and sterilizing techniques between consecutive operations are critical to the success of these reactors.

1.4 Enhancing Technologies

1.4.1 *In Situ* Product Removal (ISPR)

In order to meet the required productivities for successful application of an industrial biocatalytic process, reactions need to be operated at high substrate and therefore high product concentration. However, this situation is often unsuitable for the biocatalyst, and a lower product concentration needs to be maintained in its vicinity in order to overcome toxic and inhibitory effects. In principle, an increase in productivity and yield can also be achieved by shifting the equilibrium of thermodynamically unfavored reactions, although this is far from straightforward [54,55]. Another, relatively common situation is that the reaction product(s) are unstable under the operating conditions, necessitating immediate removal to avoid yield loss. Many ISPR techniques are available for the removal of products from the site of the reaction. This section will briefly discuss the different possibilities (for more extensive reviews, see [56–58]).

1.4.1.1 Considerations for Implementation

The application of ISPR is not widespread, and the choice of method will depend on the combination of product and substrate/impurity properties that gives the largest driving force for separation. Hence, application is complicated when substrates and products have very similar properties, which is often the case in biocatalysis. For thermodynamically unfavorable reactions, it will be essential to remove products more effectively than substrates in order to shift the equilibrium, and hence selectivity is of utmost importance. One successful example of the shift of thermodynamic equilibrium using selective removal is described by Stevenson and co-workers [59]. It may also depend on the relative concentrations of the components and requires careful laboratory testing prior to implementation. For ISPR technologies using an external loop, it is important to account for the volume in the loop in volumetric productivity calculations.

1.4.1.2 ISPR by Adsorption on Resins

ISPR by binding of the product on to polymeric resins by adsorption is a common technique – perhaps the most studied to date (Figure 1.12). The interaction between the product and the resin characterizes the success of this technique.

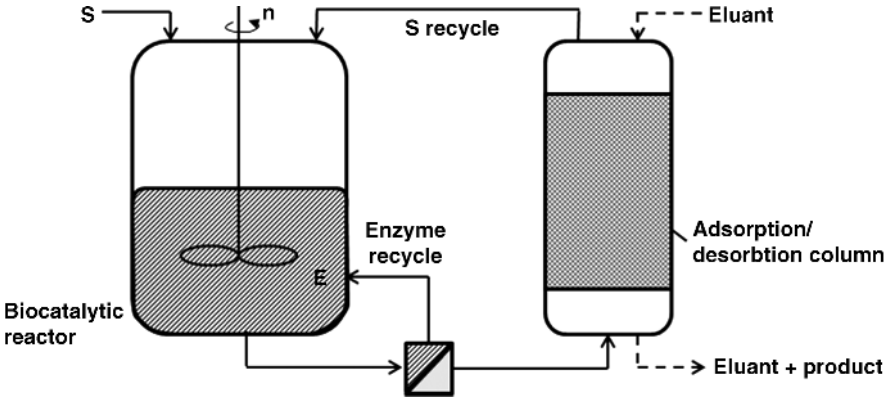
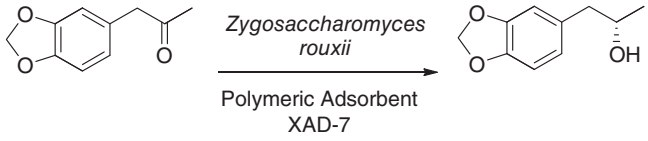


Figure 1.12 *Schematic diagram of a scheme using ISPR with adsorbent resins in an external loop (E, immobilized enzyme; S, substrate; n, number of rotations per unit time).*

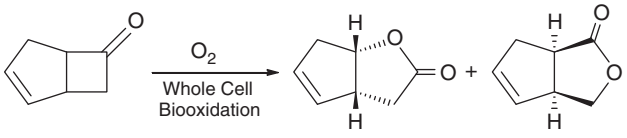
Such a system is in principle highly selective, with high mechanical (when operated as an external loop) and chemical stability, rapid adsorption kinetics, sterilizability (provided that the resin can withstand sterilization conditions), and easy regeneration and recycle. Adsorption usually follows the Langmuir adsorption isotherm. Both fluidized and expanded-bed reactors can be used. However, capacity is limited (typically $\sim$40 mg.g^{-1} of resin), which is a significant limitation in the production of small molecules.

Eli Lilly has adopted the use of resins (XAD-7) for substrate supply and product removal strategies for the production of (3,4-methylenedioxyphenyl)-2-propanol using whole cells expressing alcohol dehydrogenase. The process was run at 300 L scale, where it achieved 96% yield, >99.9% ee, and a space–time yield of 75 g.L.day^{-1} (Scheme 1.4) [60].

A further example of the use of resins for substrate supply was adopted by Sigma-Aldrich for the production of lactone using whole-cell Baeyer-Villiger monooxygenase, Over 200 g of the combined lactone was produced (Scheme 1.5) [61].



Scheme 1.4 *Synthesis of (S)-3,4-methylene-dioxypheyl isopropanol.*



Scheme 1.5 *Biooxidation of bicyclo[3.2.0]hept-2-en-6-one.*

1.4.1.2.1 Considerations for Implementation. It is desirable for the adsorbent to have the following characteristics: high capacity for the target molecule, a favorable adsorption isotherm, low non-specific binding, mechanical and chemical stability, biocompatibility, sterilizability, and low cost (or, at least, the capability for being regenerated and recycled). It is not uncommon that the substrate and product have similar characteristics, so thorough research into the adsorption characteristics is necessary for the successful implementation of the technology [62]. Adsorption is affected by the pH of the media, so the pH effects must also be evaluated, although it is not necessary that the pH optimum for adsorption is the same as the optimum pH for the biological conversion. The amount of adsorbent material that can be employed in a reaction is limited by the type of reactor. Molecular imprinting of polymeric resin or directed evolution of enzyme to operate at the conditions favorable for adsorption can also be adopted to increase the process efficiency.

1.4.1.3 ISPR Using Expanded-Bed Adsorption (EBA)

Expanded-bed adsorption (EBA) uses an expanded-bed reactor and combines solid–liquid separation with adsorptive purification. The reactor design is similar to that of a CEBR, except that the immobilized enzymes are replaced with adsorbent resins, on to which the product will bind (Figure 1.13) [63,64].

This technique combines separation and purification, decreasing the number of downstream processing steps and thereby increasing the potential yield of the process.

1.4.1.3.1 Considerations for Implementation. Reduction in adsorbent binding capacity due to binding of other impurities (cell debris, etc.) can be a problem, but, in principle, "dirty" feed streams can be used. The inlet may include perforated plates of metal mesh to produce back pressure in the system, which might clog with solids. For high flow rates or a higher viscosity of the feed solution, an increase in adsorbent size or density is desirable. It will probably be necessary to evaluate the effects of impurities on the adsorption of the resins prior to their use. The choice of appropriate adsorbent may be difficult. Laboratory experimental measurements are essential prior to scale-up for the pilot plant. Elution of the EBA is usually

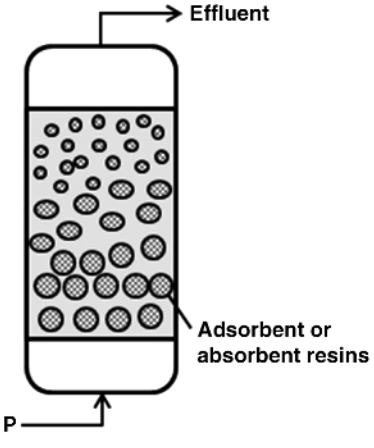


Figure 1.13 *Schematic diagram of expanded-bed adsorption (EBA) for ISPR (P, product).*

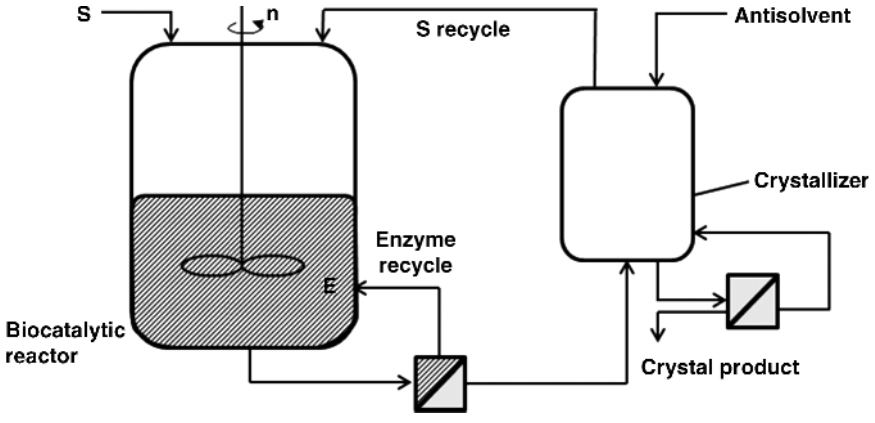


Figure 1.14 *Schematic diagram of an ISPR scheme using crystallization (E, immobilized enzyme; S, substrate; n, number of rotations per unit time).*

done in packed-bed mode to reduce the amount of eluent required. Equipment and adsorbents that can handle a large amount of feedstock have to be developed.

1.4.1.4 ISPR by Crystallization

Crystallization of the product can be coupled with the reaction in order to precipitate the product when the concentration reaches above the saturation limit (Figure 1.14).

In cases where super-saturation is reached, product crystallization occurs, decreasing the product concentration and thereby increasing the productivity and yield (this is especially useful for unstable products or in cases of product inhibition) [65]. Crystallization can provide a valuable tool in industrial biocatalysis by simplifying product recovery. A successful example of crystallization as an ISPR technology has been established by DSM in a multi-thousand-ton production of aspartame by thermoase/thermolysin [66,67].

1.4.1.4.1 Considerations for Implementation. When organic solvents are used for precipitation prior to crystallization, the number of processing steps increases, which decreases the yield of the product. This also leads to high waste generation and hence higher production costs. When product inhibition occurs below the solubility limit, *in situ* crystallization may not be viable and the use of an external loop should be considered. The rate of crystallization must be able to keep up with the production rate in order for the process to be efficient. Accumulation of byproducts can interfere with crystallization. In cases where a co-solute is used, the effect of the solute on the product purity must be evaluated.

1.4.2 Substrate Feeding Strategies

Biocatalysts are often required to work at high substrate concentrations, which seldom correspond to their natural working environment. This can lead to reduced activity or performance. Additionally, they may be inhibited at high substrate concentrations. One solution to this problem is to supply the substrates such that their concentration is kept below the inhibitory or toxic concentration. It is therefore of vital importance to select and adopt substrate feeding strategies for different bioprocesses, as appropriate. Many strategies for

substrate supply are available [68–70]. The principles behind some of them are very similar to the concepts discussed in Section 1.4.1.

1.4.2.1 Fed-Batch Operation

One of the most common supply methods is fed-batch operation, where the substrate is fed at a high concentration continuously into the reactor, in order to keep its concentration below inhibitory or toxic concentration [71,72]. The feed concentration is set by its solubility, which can limit such a method when a high concentration of product is required. Substrate concentration in the feed stream is typically kept high, in order to avoid a significant increase in volume corresponding to the dosing. The feed rate is set by the rate of dissolution of the substrate into the solution and should of course match the usage rate by the biocatalyst, so as to avoid too low or too high a concentration in the reactor itself. A fed-batch system can also be used to feed gaseous substrates [60].

Fed-batch processes are widely implemented in the industry. When the solubility of the substrate is low, solid feeding can be adopted. Lonza has adopted feeding of solids for the conversion of nicotinic acid to 6-hydroxynicotinate, catalyzed by whole cells expressing nicotinic acid hydroxylase. By adopting this feeding strategy, Lonza achieved a product concentration of 75 $g.L^{-1}$ and a reaction yield of more than 90% [73].

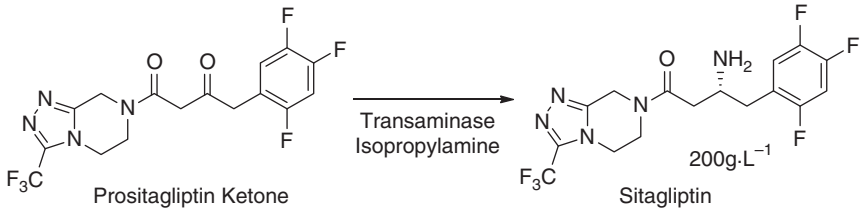
1.4.2.1.1 Considerations for Implementation. The dosing profile needs to be adjusted and altered according to the intended use of the fed-batch operation. For instance, if the solubility profile changes with the composition of the reaction mixture, this should be designed into the dosing profile.

1.4.3 Non-Conventional Media

The natural environment for the great majority of biocatalysts is an aqueous solution. Unfortunately, many of the substrates and products that are of interest for industrial biocatalysis have low solubility in water, or can be inhibitory or indeed toxic to the biocatalyst at the concentrations required for a commercially viable bioprocess. One method of addressing these problems is to use a non-aqueous solvent, either in a single liquid reaction phase with neat solvent or water and a co-solvent, or in a two-liquid phase system [74,75].

1.4.3.1 Single Non-Conventional Liquid Phase Systems

Replacing water with another reaction solvent offers a number of potential advantages in a biocatalytic reaction. Using a water-soluble co-solvent can greatly improve substrate solubility, but it also stresses the stability of the catalyst, as exemplified by the Sitagliptin process developed by Codexis and Merck (Scheme 1.6) [76].



Scheme 1.6 *Synthesis of sitagliptin-6 g.L^{-1} enzyme in 50% DMSO with 92% yield.*

Another option is to replace water completely as a solvent. In many cases, this can drastically change the thermodynamic reaction equilibrium. This has allowed the use of hydrolytic enzymes in synthetic chemistry through catalysis of the reverse reaction [77].

Biocatalytic processes in non-conventional media typically involve the use of highly polar (water-miscible) or non-polar (only sparingly soluble in water) organic solvents. Another option is supercritical CO_2, which circumvents some of the hazards of solvent use.

1.4.3.1.1 Considerations for Implementation. Selection of organic solvents can prove a major challenge. Proteins are typically very difficult to dissolve in non-aqueous solvent without loss of their structural functionality. Because of this, successful applications tend to use water and highly polar co-solvents (such as DMSO or DMF) or, alternatively, neat hydrophobic solvents that interact little with the protein (log P typically higher than 2). It is important to note that, since free enzyme formulations (or cells, for that matter) will not dissolve in neat solvents, they need to be formulated as immobilized catalysts to be efficient in such systems [78,79].

Many of the organic solvents that are useful for biocatalytic conversions are flammable, and thus introduce a complication in the process. Additionally, they can be hazardous to both health and the environment, and care must therefore be taken to limit both waste and emissions. These challenges are avoided in a process based on supercritical CO_2, but the construction of such a process is more complex (and costly) due to the high pressures involved.

1.4.3.2 Aqueous–Organic Two-Liquid Phase Systems

Aqueous–organic two-liquid phase systems may be used for *in situ* substrate supply where the water-solubility of the substrate is so low as to preclude fed-batch operation. In principle, this approach can simultaneously be used for product recovery via extractive ISPR [61].

Substrate supply is driven by mass transfer from the organic to the aqueous phase. In principle, neat (poorly water-soluble) liquid substrates can be used as the second liquid phase. However, in many cases (e.g., when the substrate toxicity limit is lower than the solubility limit or where the substrate is solid), it is useful to use an organic solvent in which to dissolve the substrate (and from which to extract the product).

1.4.3.2.1 Considerations for Implementation. The considerations for single non-conventional liquid phase systems also apply here. Additionally, when using two-liquid phase systems, emulsions may form, which can prove hard to break downstream, making product recovery and/or biocatalyst recovery and recycle difficult. Dissolved levels of solvent can affect the biocatalyst (i.e., when the solvent has low log P values), but interfacial effects have also been found to be important.

1.4.3.3 Aqueous–Ionic Liquid Two-Liquid Phase Systems

Biocatalysis often takes place in aqueous media, but some of the organic components (usually the substrate and products) exhibit low solubility levels in water. The solubility of such reactants needs to be improved, often by using water-immiscible organic solvents (see Section 1.4.3.1). Ionic liquids (ILs) can also be used in place of the organic solvent, again forming a two-liquid phase system. ILs are essentially organic salts that are liquid at (or near to) room temperature. The use of ILs has attracted significant interest in recent years [80,81].

This system is considered a potential "green" alternative to organic solvents, dependent on the type of IL. Likewise, many ILs have been found to be biocompatible. The synthesis of ILs is such that tailoring an IL toward the need of the process by choosing an appropriate cation and anion becomes a real possibility.

1.4.3.3.1 Considerations for Implementation. It is important to consider that the availability and cost of ILs today mean that recycle is essential. Furthermore, the anion of an IL may cause conformational change of the enzyme and lead to a loss in activity. Likewise, the interaction between water and IL can cause complications in the intended reaction system and make separation of IL from the product difficult.

1.4.4 Oxygen Supply Strategies

Biocatalytic oxidation has been gaining importance in synthetic chemistry owing to its high selectivity compared to its chemical counterparts. Supply of molecular oxygen is a key part of biocatalytic oxidation reactions, and although oxygen is a substrate for such biocatalysts (e.g., oxidases, monooxygenases, dioxygenases), it is a special substrate and is hence dealt with separately here. The major advantage of supplying gas to the liquid is the high mass transfer to the liquid via the interface between the gas and liquid phase. On the other hand, oxygen compromises the stability of many biocatalysts, either through interfacial effects or through chemical modification (e.g., oxidation of amino acid residues). It should be noted that oxygen can be introduced into a reactor either as pure gas or in the form of air to drive the reaction. This choice is more of a strategic decision for each reaction, and therefore will not be separately discussed. Several reactor configurations have been used for oxygen supply, and some of them will be discussed in this section. It should be noted that the oxygen supply strategies and the available literature catering to biocatalysis and bioprocesses are scarce. However, it is rather easier to get information on fermentation, and therefore, in some of these cases, the reactors were used for fermentation or growth of cells and are discussed here to give an idea of the potential alternatives available for bioprocess engineers.

1.4.4.1 Surface Aeration

Surface aeration is a method by which oxygen/air is transferred into the bulk liquid phase through the gas–liquid interface at the top of the reactor. Therefore, there is no bulk gas transfer through the bulk liquid. This kind of transfer is always present in reactors unless special modifications have been made to the reactor design to avoid it. It is commonly used in laboratories when biocatalysis requiring oxygen is carried out using shake-flasks or vials. Figure 1.15 represents surface aeration in a batch reactor with agitation.

The efficiency of this method of aeration depends on the mass transfer limitations of the gas and the surface area: mass transfer occurs through the surface of the reactor. It is more useful for small-scale reactors – the reactor size limitation will depend on the oxygen requirement for the system, but it is clearly very limited.

1.4.4.1.1 Considerations for Implementation. Such a method is obviously unsuitable for anything other than the laboratory. It is nevertheless widely used, and experimentalists should be careful about oxygen limitation leading to low observed reaction rates.

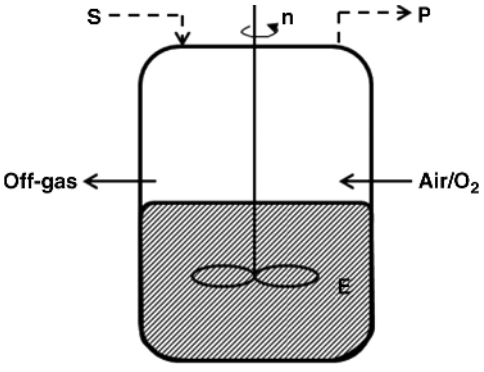


Figure 1.15 *Surface aeration with agitation in a batch reactor (S, substrate; P, product; n, number of rotations per unit time).*

1.4.4.2 Sparged Aeration

Sparged aeration is a method by which oxygen/air is introduced at the bottom of the reactor either through distribution plates or using a sparger. A sparger is more commonly used in stirred-tank reactors, while distribution plates are commonly found in bubble-column reactors.

Sparged aeration is a highly convenient method of oxygen supply for reactors above 1 L working volume. In a stirred system, the stirrer will break the air bubbles so that they are small, and thereby create a large interfacial area for mass transfer. The driving force will be determined in large part by the concentration of oxygen in the liquid in equilibrium with the gas. Although in such systems the liquid is well mixed, it can often be the case that the gas phase flows through the reactor in plug-flow mode. This has a significant effect on the oxygen transfer that is possible.

1.4.4.2.1 Considerations for Implementation. Correlations for oxygen mass transfer potential versus gas flow rate and mixing are rather poor in general, but as a guide, reactors should operate with a gas input of around 0.5–1.0 vvm (volume/volume/minute) and a stirrer power input of about 1.0–1.5 $W.L^{-1}$. Maximum oxygen transfer rates of around 100 $mmol.L^{-1}.h^{-1}$ can be expected when air is supplied [82].

1.4.4.3 Bubble-Column Reactors

Bubble-column reactors are simple reactors in which the gas is sparged through the bottom. The motion of the bubble from the source (usually distribution plates) through the reactor causes the mixing, avoiding any need for stirring (see also [83,84]).

Mass transfer in such a system depends on the superficial gas velocity and the initial bubble size, both of which are affected by the diameter of the orifice (of the sparger). Industrial bubble-column bioreactors have capacities up to 400 m^3.

The flow direction of the liquid has little effect on the gas hold-up, although a downflow bubble column is preferred when longer gas phase residence times are desired. Bubble-column reactors offer reasonable biocatalyst stability, since there is no stirrer. Due to the lack of moving parts, maintenance costs are low.

1.4.4.3.1 Considerations for Implementation. Often, high sparging rates are required to achieve turbulent flow inside the bubble column. Indeed, the superficial gas velocity and the reactor diameter affect the flow regime of the bubbles inside the reactor. A further consideration is coalescence of bubbles, since increased coalescence will decrease the aeration efficient. Additionally, the viscosity of the medium is frequently important, since it has a significant effect on the gas–liquid mass transfer.

1.5 Conclusion

The technologies highlighted in this chapter have been chosen for practical reasons to illustrate the integration of process technology with early-stage considerations for biocatalytic process development. For more detailed descriptions of particular technologies, the reader is advised to consult the references herein.

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