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Advances in Biological Conversion Technologies: New Opportunities for Reaction Engineering

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Reaction engineering needs to embrace biological conversion technologies, on the road to identify more sustainable routes for chemical manufacture. There are new options in biological conversion including modifying the properties of an enzyme by protein engineering, a pathway by metabolic engineering or a microbial host cell. However, here also lies the challenge of a multitude of technologies and modes of operation, which the reaction engineer must navigate successfully in order to implement a new process. This perspective summarizes some of the recent developments in the field and the implications for reaction engineering, with a focus on sustainable chemical production.

Introduction

Reaction engineering is one of the most fundamental and unifying fields within the twin disciplines of chemical engineering and process chemistry. It provides the necessary intellectual framework for the design and optimization of all chemical conversion processes, and likewise has application in thermal and physical conversion processes. The framework includes a description of the kinetics of the conversion (with a rate law) and the establishment of the thermodynamic limits to conversion as a basis for screening the best reaction conditions and catalysts, with the ultimate aim of designing a suitable reactor where the conversion can occur in an optimal scenario. The development of the field over the past century has informed the design of much of the modern chemical industry, the products of which we enjoy in our daily lives. Nevertheless, the modern chemical industry is based on converting petroleum-based feed-stocks into useful products and this is not sustainable for the future. Of the various alternatives put forward, one of the most interesting concepts now being developed is the use of bio-based renewable feed-stocks (such a glucose or lignin). Such feed-stocks may come from a variety of sources, including waste biomass (lignocellulose), where for example sub- or supercritical thermal processes can enable the release of useful substrates (such a glucose and xylose) from the cellulose and hemicellulose fractions.¹ Of even greater interest, is that such substrates in combination with biological conversion

technologies (such as fermentation or biocatalysis) can be used to produce higher-value chemicals and products.²⁻⁶ Interest in such an approach has grown enormously in the last decade and one of the major challenges is that there now exist many formats for the biological catalyst (including growing microbial cells and isolated enzymes) and operational modes (including continuous biocatalysis and two-stage fermentation), as well as a plethora of tools for modifying the properties of the biocatalyst itself (including protein engineering, metabolic pathway engineering and newer synthetic biology tools such as CRISPR). This suite of biological conversion technologies (see Table 1) presents the reaction engineer with extra degrees of freedom for design as well as extra decisions, which need to be taken during reaction, reactor and process development.

In this brief perspective, advances in biological conversion technologies will be outlined in the context of reaction engineering for the synthesis and production of valuable chemicals, primarily as a means to sustainable production technologies for the future.

Biological conversion technologies

The biological synthesis of molecules using growing fermentative microbial cells, has been very well established over many decades. Indeed, for complex molecules such as proteins, fermentation is today the established route to manufacture. Today the production of proteins is routinely carried out using fermentation, to produce both high-priced products (e.g. therapeutic proteins) as well as bulk products (e.g. enzymes). Both prokaryotic and eukaryotic systems can be used to produce protein either intracellularly or preferentially extracellularly, dependent upon the host organism chosen. Although cell-free systems are being developed today⁷, the synthesis of proteins is a complex task, and therefore non-biological alternatives do not exist. Such processes have been well optimized for the effective production of low-priced enzymes in bulk and high-priced therapeutic proteins in much smaller quantities. The need for glycosylated proteins and very specific post-translational modifications drives the search for new hosts.

For less complex molecules, such as chemical intermediates and products, fermentation can also be used, starting from renewable feed-stocks such as xylose, glucose and other sugars. Examples of products include antibiotics and other natural products, of particular value to the pharmaceutical industry. While a few large-volume chemicals such as lactic acid and citric acid have been established fermentative products for many years, more recently focus has been placed on several other large-volume and low-priced chemicals driven by the desire to replace petroleum-based feed-stocks by renewable feed-stocks (e.g. glucose) and in the longer-term by sustainable feed-stocks (e.g. glucose derived from waste lignocellulosic

material). To this end, fermentation scientists and metabolic engineers have made significant progress⁸⁻¹² which has led to some well-publicized successes in (bulk) chemical production (e.g. industrial scale production of 1,3-propane diol¹³, succinic acid¹⁴ and 1,4-butane diol¹⁵). Indeed, opportunities for still lower-priced chemicals such as biofuels^{16,17} might even be within reach in the not too distant future. Nevertheless, it takes considerable molecular biology to optimize such systems and despite the effort some remain limited just beneath the required process performance metrics of yield, productivity and product concentration for economically viable industrial implementation (e.g. itaconic acid¹⁸). Recently, the current status of many chemicals which could potentially be produced by microbial fermentation was reviewed in an excellent and comprehensive paper.¹⁹

In reality, there are several modes of operation, and although conventional fermentation is the most established of these, other technologies exist as illustrated in Figure 1. Microbial fermentation is characterized by growing cells and in the usual growth-associated product formation, the cells are grown and simultaneously product is produced. This means that a significant amount of substrate can end up being diverted from product to cell biomass and energy as well as for cellular maintenance. Progress in metabolic engineering allows substrate to be directed towards desired products, using genetic modification techniques. Other tricks can also be used to redesign metabolism via so-called cell factory engineering, helping to eliminate non-productive pathways and channel the required redox and energy.^{20,21} This is what was achieved and enabled the industrial implementation of the second generation of chemical products such as 1,3-propane diol as previously mentioned. However, in all cases it is essential to balance redox and energy in the cell,²² which sets the maximum possible (theoretical) yield of product on substrate, excluding cell growth. Unlike conventional chemical reactions where the stoichiometry is fixed, in biological systems it is dependent upon cellular conditions. Similarly, the rate of product formation is determined by the rate of cell growth. These complications provide a limit to what can be achieved in growing cells (termed fermentation), no matter how effective the metabolic engineering. Recognizing that cells contain pathways of great potential to convert cheap (and importantly, impure) renewable feed-stocks to more valuable chemicals, an alternative approach has been to use non-growing cells, in which the necessary enzymes are merely contained within the cell (like a kind of bag). In such cases, the cells are non-viable and non-growing and are frequently termed 'whole-cell' conversions. A third option is to use so-called 'resting cells' where the cells are not growing, but remain viable. Here, the associated network for cofactor regeneration and energy supply is also present, and may also require some additional carbon. In such a system the growth (first stage) and conversion (second stage) are separated (as occurs naturally with secondary metabolites such as antibiotics). A recent review explains the significant benefits which can accrue even from simple two-stage operation.²³ Recently it was

reported that balancing ATP in such systems is not only necessary but can in some cases become limiting.²⁴ Nevertheless, the real benefit of 'two-stage' operation becomes clear through modifications to the process configuration such as (1) recycling of resting cells after the second stage (thereby obtaining greater product from the original cell biomass), and (2) dewatering between the first (growth) and second (conversion) stage (so as to enable higher resting cell concentrations to be used in the second stage). The former allows higher overall yields to be achieved and the latter, higher productivities. In an improved system, as described here, a new limitation may be the product concentration and in this case *in situ* product removal (ISPR) can be implemented.²⁵ A summary of these potential options is given in Table 2. It is interesting to note that the majority of solutions today use biological, rather than process, strategies to solve existing limitations.

An alternative to using growing cells (or using enzymes within a non-growing cell (whole cell or resting cell)) for the production of chemicals is to isolate the relevant enzymes (see Figure 2). This type of biological conversion using isolated enzymes is commonly termed biocatalysis. In some cases, the cells may secrete the relevant enzyme(s) extracellularly making biocatalyst production much easier (and therefore cheaper). In other cases, the cells must be broken open to release the desired enzyme. For many single-step reactions, it makes sense to isolate the enzyme from the cell, so that the exquisite selectivity of a single enzyme-catalyzed reaction is not disturbed by other enzymes present within the cell. In other cases, especially where biocatalyst cost is dominant over purity considerations, then whole-cells will prove the better choice. The final selection of biocatalyst format needs assessment on a case-by-case basis, dependent upon the economic drivers.

Enzyme-catalyzed reactions have found widespread use in synthetic organic chemistry, especially where high selectivity under mild conditions is needed.²⁶ Unsurprisingly therefore most applications using enzymatic biocatalysis can be found in the pharmaceutical industry,²⁷⁻²⁹ and to a lesser extent in the agrochemical industry and as well as the flavors and fragrances sector. As more enzymes become commercially available, the field continues to expand.³⁰ Likewise, recent advances in bioprocess technology have led to further reductions in the cost of enzyme production through better expression and simpler recovery techniques.³¹

Nevertheless, for many applications significant investment is still required, in particular in enzyme improvement. For example, considerable effort is required to understand the optimal way to use enzymes in an industrial environment, which is frequently very different to that found in nature. For example, in an industrial reaction much higher concentration of substrates and products will be present than is possible in nature, affecting enzyme kinetics.

Likewise, gas-liquid and liquid-liquid interface may be present in industrial reactors and can adversely affect enzyme stability. Those responsible for establishing the right chemistry (synthesizing industrially valuable and relevant target products) are quite correctly preoccupied with the problem of how to modify enzymes to accept non-natural substrates and enable new-to-nature (NTN) reactions to occur. Their objective therefore is to broaden the substrate scope of a given enzyme, or at least know how to change the scope when required. Whether expanding, or changing, the substrate scope scientists have built on the natural promiscuity of enzymes, which is an interesting evolutionary feature of enzymes.³² Such an approach uses protein engineering tools such as directed evolution to screen for improved enzymes in successive rounds of mutation (swapping amino acids and recombining variants).³³ The latest approaches incorporate machine learning algorithms³⁴ to accelerate the procedure, as demanded by the pharmaceutical sector.³⁵ Once significant enzyme activity has been built, the focus can be shifted to expression of the enzyme in a suitable host cell. The enzyme can then be used either within the cell, or else as a lysate providing a crude and cheap form of the enzyme. In a natural progression, the field now moves towards cascades of enzymes. Some excellent examples have been reported in a number of recent reviews.³⁶⁻⁴⁰ The control of such cascades will be a major area of research in the future, and a recent paper reports the latest developments including stimulus-response techniques using stimuli such as changes in light, temperature, magnetic field and pH.⁴¹ The next developments will be towards lower-priced chemical products where aside from some well-established processes, such as glucose isomerase catalyzed isomerization of glucose to fructose, rather few examples currently exist. A recent report on the status of the biocatalytic synthesis of bulk amines⁴² emphasizes the importance of achieving sufficiently high product concentration, as well as other process performance metrics. Hence, the importance of reaction engineering in this field will continue to grow.

Most recently, gene-editing technologies using CRISPR now allow the expression of non-natural enzyme cascades in cells (termed synthetic biology). Individual enzymes can be further improved, in terms of specific activity (to reduce loading in the host cell), by protein engineering. The development of synthetic biology allows the editing of genes to enabling the construction of entirely new pathways to valuable products. Indeed reliance on the natural pathway, or modifications of it via metabolic engineering, is no longer a restriction. This brings the option of cell factories, which can be designed rather than modified based on an existing template (metabolic engineering). A recent example concerns the production of 1,4-butane diol using CRISPR and CRISPRi systems.⁴³ While synthetic organic chemists, in concert with biologists, make significant progress in the field, several challenges still exist, especially concerning the burden placed on the cell and the need for robust circuits.⁴⁴ Indeed, other non-conventional microbial platforms may ultimately prove more useful.⁴⁵ Potentially, this

represents the future of metabolic engineering using sophisticated modelling and genetic editing tools.⁴⁶

Ultimately, the goal is to use the best catalyst (in whatever format) for a given reaction. For example, catalytic hydrogenation is a particularly attractive non-biological reaction with low cost reductants and 100% atom efficiency.^{47,48} Likewise, provided glucose and xylose are pure enough (and that is an important requirement), catalytic routes to particular products, such as furans and lactones by dehydration, or hydrocarbons by reduction, would also seem promising.^{49,50} This also means that alongside the biological catalyst options it is also possible to consider using and integrating heterogeneous catalysis in new schemes from a given sustainable feedstock to a potentially valuable product. Several recent publications have emphasized the importance of such an approach to capitalize upon the best properties of both catalyst types.⁵¹⁻⁵⁵ In the broadest sense this may involve the integration of all catalyst types (in compartmentalized packages to minimize changes of conditions along the way) on the route from sustainable feedstock to final product. Indeed compartmentalization is used in nature, where different part of cells operate under different condition. Likewise, proximity of enzymes to each other can assist catalysis of cascades via co-localization, such that the product of one reaction is suitably placed at the active site for the next.⁵⁶⁻⁵⁸

Reaction Engineering Concepts

Required conditions for industrial operation

For low-priced products, reaction engineering is of particular importance because reaction economics dominate the possibilities for industrial exploitation. For the sustainable biological production of chemicals therefore this places particular emphasis on the required conditions for industrial operation, which are invariably far from those where the particular biocatalyst of interest (whether cell, pathway or enzyme) was originally obtained.

Indeed, this difference between laboratory and process conditions is what limits the application of such systems today for the production of bulk chemicals. As argued in a previous publication⁵⁹ the cost of enzymes means that purification can rarely be justified in an industrial setting (in particular for lower-priced chemical products). However, when using crude (impure) enzymes any interfering activities from other enzymes also need consideration. Indeed, in principle the rate law should be assessed with the same purity of enzyme that will be used in the potential industrial operation. This necessitates establishing rates based on mass, rather than molarity. In a recent publication, we have provided

estimates for the allowable amount of enzyme which can be used to make a given quantity of product dependent upon product value (so called biocatalyst yield, frequently expressed as g product/g biocatalyst⁶⁰). The enzyme concentration needed is determined by the productivity requirements (mass product required per year) for a given combination of reactor size (reaction volume), the maximum rate of reaction (maximum enzyme activity), the efficiency of enzyme use (observed enzyme activity/maximum enzyme activity) and the enzyme stability (enzyme activity with respect to time). Figure 2 represents this schematically where the linear relationship between enzyme concentration needed and the required productivity is shown. The gradient of this line is determined by enzyme efficiency and stability, while the upper limit is determined by the enzyme specific activity and again emphasizes the importance of adequate activity (and effective operation). If the enzyme is stable enough it can be recycled multiple times. Immobilizing the enzyme on (or within) an insoluble support material (immobilization) can help with downstream filtration and recycle, and may even confer added operational stability, but needs to account for the cost of immobilizations. In the case of immobilization on a support, this can be a key factor, but if a cross-linked enzyme aggregate (CLEA) is used, then multifunctional reagents may prove the dominant cost.⁶¹ Thus, when immobilizing and enzyme supports and reagents can be costly and need to be evaluated. Assessment of immobilized enzyme performance at a laboratory scale (with recycle if appropriate) is complicated, but remains important in many cases to establish if the process will be economically viable or otherwise.^{62,63} Minimizing the amount of enzyme used to make a given quantity of product is an important objective in the development of many biological conversion processes.

Whilst enzymes in nature have been evolved to operate at low concentrations, the use of such concentrations in an industrial reactor results in a dilute feed to the downstream process. This not only means the downstream equipment will be very large (and therefore costly), but additionally the driving force for separation is low and therefore the separation is difficult and may even compromise selectivity. Finally, significant progress is made towards the use of enzyme catalysis in alternative media (including suitable organic solvents, ionic liquids and deep eutectic solvents)⁶⁴ to enable higher concentration of substrates in the reactor, either via a second phase or in the catalytic solution itself. In some cases, the stability of enzymes can even be enhanced.⁶⁵ Nevertheless, in many cases the product recovery is still from water (since enzymes are most usually used in an aqueous environment). This further complicates separation because evaporation costs become significant at scale.

Towards a systematic approach to reaction engineering

In conventional chemical reaction engineering the provision of the catalyst provides the first step prior to screening of suitable reaction conditions such as pH, temperature, pressure, reactant(s) concentration and catalyst concentration. Benchmarks for suitable process performance have been established,⁶⁶ and today such an approach can even be automated, dependent upon the complexity.⁶⁷ For biological conversions the situation is a little different since it is rarely necessary to screen against pH, temperature and pressure, except over a relatively limited range. Most biological catalysts operate under relatively benign conditions, but even for those enzymes from so-called extremophiles (whose cells preferentially grow under marginally more extreme conditions), activity will be found within a relatively limited window. Although this makes screening for conditions much easier, an added complexity is the need for cofactors (which may or may not be tightly bound^{68,69}), and other activation agents. In a recent example an in depth (and time-consuming) analysis to establish the correct values of activating agents for an oxidase proved essential prior to more conventional reaction engineering studies.⁷⁰ A further complication when using enzymes is the rate law (which follows the so called Michaelis-Menten kinetics for a single reactant to single product reaction). While in principle this could follow the established procedures of kinetic analysis,⁷¹ at the reactant concentrations required to deliver a suitable product concentration, inhibitory effects on the enzyme are frequently observed (altering the rate law). Clearly, establishing the rate law is an important first step in all reaction engineering studies, although in biocatalysis today, much of the emphasis remains on a limited number of activity measurements, which frequently hide the effects of changes in reaction rate with reactant (and product) concentration. Additionally, while the simple Michaelis-Menten rate law describes a single-reactant to a single-product reaction, in reality the most useful reactions usually use a second reactant (co-substrate) (and may often also make a co-product). This complicates the rate law and also makes the collection of data more difficult. For example, it is necessary to saturate the enzyme with each reactant in turn in order to obtain an estimate of both Michaelis constants for each reactant. Likewise, inhibition constants should be obtained. Furthermore, for conversion of solid substrates (either those above saturation concentration, where high concentrations are required or those, which at the conversion temperature, have a low solubility in the reaction medium (for example cellulose in water)) the rate law needs adjusting to account for surface catalysis.⁷² In all cases, the sensitivity of the observed reaction rate to the concentration of all reactants and products emphasizes the need to obtain the rate law, with the estimated kinetic constants. Several procedures are now in existence for the collection of such data, both at a more global level to establish rate limiting effects,⁷³ as well as to obtain kinetic constants.⁷⁴⁻⁷⁶ Particularly important in this procedure is to check the sensitivity to measurement errors and to provide upper and lower bounds for the kinetic constants. Since enzymes in nature work at relatively low concentrations of reactants, it is also important to ensure that the analytical methods are adequate (especially since the

aqueous environment can swamp many more sensitive methods). Recent advances in NMR may provide a suitable generic method.^{77,78} Such work also needs reporting in databases to build biocatalytic synthesis-based characterization profiles for interesting enzymes in order to complement enzymology-based characterization.⁷⁹

Bioprocess engineering tools

Bioprocess engineering will prove an essential part of the implementation of all these processes with a variety of different catalysts. Several reviews have recently emphasized the importance of bioprocess engineering in the context of sustainable industrial production of chemicals.⁸⁰⁻⁸² Enzymes rarely work in isolation in cells and operate in a network rather than solely in linear sequences. This allows the removal of inhibitory products and shifting of unfavorable equilibria, but can also be used to regenerate cofactors and supply substrates, via coupled reactions. These complexities also need studying to understand when isolated enzymes or when whole-cell processes should be used. As mentioned previously, a key restriction with cellular systems is that energy and redox need to be balanced. A second issue concerns the use of single or multiple reactors. Although the idea of a single 'one-pot' synthesis appears attractive since it potentially overcomes intermediate isolation (and purification) steps, for many reactions temporal or spatial separation (termed compartmentalization) of parts of an enzyme cascade may prove not only beneficial but are essential to avoid cross-reactivity.

Such schemes as well as being complex demand quantitative analysis to understand the feeds required, the need for intermediate isolation of compounds as well as the relative amount of enzymes to add. *In silico* modelling and *in vitro* testing are both routes towards designing the optimal *in vivo* systems. Such approaches are being used already to assess limitations in enzyme cascades.⁸³

pH control is an important feature of many biological conversions since the majority of reactions occur in aqueous solution and many reactions consume or produce acidic or basic species. Most enzymes operate within a narrow pH window and therefore control is of utmost importance. Whole-cells and soluble enzyme processes are therefore best operated in stirred tank reactors to allow for pH control. Operation in a stirred tank is also favored for those reactions requiring substrate feeding (due to enzyme inhibition at higher concentrations). Likewise, many enzymes are limited in performance by product inhibition where *in situ* product removal^{25,84} or liquid-liquid extraction of poorly water-soluble products, may prove beneficial to allow reaction times to higher product concentrations to be increased (thereby

making better use of the reactor). Substrates which do not prove inhibitory, but are nevertheless poorly water-soluble, can be added 'neat' to the reactor with minimum water to allow dissolution of water-soluble components and adequate operation of the biocatalyst. In recent years' some of these operations have been tested and validated, although a full analysis has not yet been made. Likewise new possibilities using flow biocatalysis, capitalizing upon the benefits of plug-flow reactor operation are forthcoming.^{85,86}

Finally, in order to build on these options it will also be necessary to guide route selection driven by the sustainability of the particular route (including starting feedstock), thermodynamic considerations, as well as the availability of enzymes and heterogeneous catalysts. Retrosynthetic approaches are based on analysis of a product molecule to establish potential precursors that when connected form the product. The repeated application of this algorithm allows an entire pathway to be constructed. A complementary approach is function-oriented synthesis where simpler scaffolds can provide suitable starting points, rather than natural materials⁸⁷. The objective in both these approaches is to ensure the substrate/feedstock molecules are used effectively in making the product (so called atom economy) and reduced number of reaction steps (so called step economy). Such approaches which have been used for several decades in organic synthetic chemistry,⁸⁸ now include biological catalysts. Identified bonds which can be formed through biocatalytic reactions have now been added into the methodology of bond disconnections. This will be an important tool to help solve evaluate alternate synthesis routes and several recent reports outline the significant progress that has been made in this area in biocatalysis,⁸⁹⁻⁹¹ and also in biosynthesis for the design of *de novo* synthetic pathways.⁹² Nevertheless, inevitably, the total number of disconnections available through biocatalysis is still limited compared to conventional approaches.

Future Perspectives

The introduction of a suite of biological catalysts into organic synthesis and potentially industrial production enables new reactions and new routes to products, based on alternative starting materials, including sustainable feedstocks.⁹³ The challenges are significant since the number of options for operating processes has expanded enormously and rules are required to guide selection of the optimum path. Figure 3 schematically illustrates the selection, which includes (a) the route, (b) the biocatalyst and (c) the process. In (a) the route is identified against specific targets such as feedstock sustainability, atom economy and step economy. In (b) the biocatalyst format is selected for the given enzymes found in (a). Economic analysis reveals the cost/benefit of host cell options and immobilization. In (c) the process is selected for the given route and biocatalyst from (a) and (b). Here operational considerations

determine the reactor choice as well as feeding and product removal strategies. Ultimately, in further rounds of integration, this should be integrated with biocatalyst engineering to capitalize upon the ability to tune enzyme properties not just for specific chemistry but also for process-specific traits.^{94,95} More work is also required on scale-up. For example, although the performance metrics of an enzyme in the laboratory may be enhanced many fold through techniques such as protein engineering, still adequate productivity in the process setting is the ultimate test for development of a commercially viable and scalable process. This requires far greater emphasis on stability studies in an industrial environment, in order to ensure that the excellent activities so carefully engineered in the enzyme are maintained, in the harsh conditions of the industrial reactor environment. Whilst enormous progress has been made on the biological aspects of expressing and improving the enzymes required for catalysis of a given reaction, as well as the surrounding network of reactions in the case of cascades (and metabolic engineering and synthetic biology of known and new pathways), much greater emphasis is still required on the development of a suitable methodology to translate laboratory reaction(s) into industrial processes. Indeed bioprocess engineers are still building the necessary reaction engineering framework to systematically evaluate all the options of biocatalyst format and operational mode.⁹⁶ Nevertheless, these challenges also mean that reaction engineers of all types will have an increasingly important role to play in translating such research into industrially viable processes for a sustainable future.

Conflicts of Interest

There are no conflicts of interests to declare.

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Table 1. Microbial bioconversion technologies, including biological engineering options at the level of host (H), pathway (P), enzyme (E) and gene (G), using ^agenetic engineering, ^bmetabolic engineering, ^cprotein engineering and ^dCRISPR, respectively.

Conversion type		Fermentation				Two-stage fermentation				Microbial biocatalysis			
		Fermentative bioconversion				Resting cell bioconversion				Whole-cell bioconversion			
Process options	Viability	Viable				Viable				Non-viable			
	Growth	Growing				Non-growing				Non-growing			
	Cell recycle	Unusual				Possible				Possible			
	Immobilization	Unusual				Possible				Possible			
Biological engineering options		H ^a	P ^b	E ^c	G ^d	H ^a	P ^b	E ^c	G ^d	H ^a	P ^b	E ^c	G ^d

Table 2. Potential solutions to common limitations in microbial fermentation. *The most commonly used strategies today.

Limitation	Improvement Strategy	Common solutions	Methodology
Yield too low	*Biological	Divert substrate to product rather than cell growth and maintenance	Metabolic engineering
	Process	Cell recycle	Two-stage fermentation
Productivity too low	*Biological	Increased enzyme load in cell	Expression of selected genes
		Higher specific activity	Protein engineering
	Process	Higher cell concentration	Two-stage fermentation with dewatering between stages
Process concentration too low	*Biological	Increase cell tolerance to higher product concentrations	Host engineering
	Process	Removal product throughout the fermentation	<i>In situ</i> product removal

Figure 1. Biological conversion technologies for the industrial production of chemicals. (a) Growing microbial cells (growth-associated product formation); (b) microbial biocatalysis (growth or cells independent from product formation); (c) enzymatic biocatalysis (pathway or cascade of enzymes independent of cellular constraints). In the figure, S represents substrate, P product, C cells and E enzyme(s). The small red arrows represent an enzyme pathway (in a microbial cell (orange)). The large red arrows indicate points of entry and exit from the cell, across the cell wall.

Figure 2. Biocatalytic reactor design diagram, where required productivity (mass of product/time) determines the enzyme concentration needed in the reactor. Enzyme efficiency is a function of stability as well and effective use of the maximum enzyme rate possible (i.e. the extent to which operation is in excess of the Michaelis constant for the limiting substrate). *Maximum efficiency may be limited by the required conversion if the Michaelis constant is above the allowable final residual substrate concentration. **The enzyme concentration may reach an upper limit in the reactor for practical purposes.

Figure 3. Process options for biological conversions, including route, biocatalyst as well as reactor and separation options. In each case, selection of options starts with the inner core and as each part is defined moves in stages to the outer options.

(a) Route options

(b) Biocatalyst options

(c) Reactor and separation options