

## What makes a good biocatalyst?

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### Abstract

A biocatalyst is an enzyme or cell with both the required activity in high enough levels to make its use cost-effective, and also the other characteristics necessary for its successful and cost-effective use on an industrial scale. The biocatalyst is usually the lynchpin of a process, without which the product could not be made, despite the biocatalyst being only a relatively small element of total production costs for most of the processes that have achieved commercial success. This paper attempts to identify some of the factors necessary for success and to give illustrative examples. These include improved process integration and downstream processing with a proper appreciation that the patentability and cost of the product are of vital importance. Screening is highlighted as very important in achieving successful processes, including the consideration of plant sources of enzymes as worthwhile. Because biocatalyst success is closely linked with how high a concentration of product can be made, the importance of finding or designing biocatalysts with increased resistance to product inhibition should be strongly encouraged especially as so little research effort has been carried out in this area so far. © 1998 Elsevier Science B.V. All rights reserved.

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### 1. Introduction

To achieve success using a biocatalyst it is very necessary to clearly define both the market need, in a properly focused and quantified form: and the performance criteria required of the biocatalyst necessary to meet the market need. Therefore it is important to consider ‘What makes a good biocatalyst’ from the points of view of all con-

cerned, including the inventing technologists, the biocatalyst producer and user, and the benefits that can be provided to the end consumer, and taking into account that cost is a key part of the product!

So as to help to answer the question ‘What makes a good biocatalyst?’ it is useful to restate it as ‘What is needed to make good products using biocatalysts?’ and also to consider the following key points: a wide range of different activities are provided by biocatalysts, which include fermenta-

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tions in which de novo synthesis takes place, so-called precursor fermentations, single or multi-step bioconversions, enzyme processes using single enzymes, and of course, various forms of immobilised enzyme and cells (Table 1). This broad capability has yielded some good examples of successful biocatalyst-based manufacturing processes. These give good indications of the high productivities required to achieve sustained industrial success. Successful industrial biocatalysts include nitrile hydratase (Nitto Chemicals) which has a productivity 50 g acrylamide  $l^{-1} h^{-1}$ . Penicillin G amidase (SmithKline Beecham and others) which has a productivity of 1–2 tonnes 6-APA per kg of immobilised enzyme; and glucose isomerase (Novo Nordisk, etc.) which has a productivity of 20 tonnes HFCS per kg of immobilised enzyme (Cheetham, 1994a).

## 2. Examples

However, having first shown the difficulty of developing biocatalysis processes, it is equally easy to demonstrate how much success is actually being achieved, both in terms of specific new products, such as L-carnitine, which is gaining markets as a health supplement (Fig. 1). More generally, the success of biocatalysts is demonstrated by the rapidly expanding sales of industrial enzymes. These sales figures show not just that all segments of the industrial enzyme market are growing, but that some, such as starch enzymes, and enzymes for non-dairy food uses are growing far faster than others, which is substantially due to quite new uses being developed, as well as increased demands from pre-existing processes. Indeed enzyme sales values are actually an underestimate of the true economic value of biocatalysts, as they not only exclude the use of whole cell biocatalysts, but also do not record the much greater aggregated sales of all the products made using the biocatalysts. This effect is best illustrated by a typical supply chain, in which although the product is absolutely dependant on a biocatalysts to make it, the real economic value is from the sales of branded products containing the active ingredient made using the biocatalysts.

Table 1

The various bio-routes for the manufacture of food ingredient chemicals

Method	Complexity	Examples
'De novo' production by fermentation <sup>a</sup>	Multistep	Citric acid, MSG
Precursor fermentation	Multistep	Gamma and delta-decalactones
Bioconversion by nongrowing cells	Single step	Sorbitol to L-sorbitose (ascorbic acid)
Isolated 'sacrificial' enzymes	Multistep	Methylketones
	Single step	Lipases, proteases phosphodiesterases <sup>b</sup>
	Multistep	$\alpha$ -Amylase/glucosylase
Immobilised enzymes	Combined use	Lipases and esterases for EMCs
	Single step	Glucose isomerase

<sup>a</sup> Also used for making whole foods, e.g. beer, wine, cheese and yoghurt etc.

<sup>b</sup> Most microbial enzymes are now cloned products.

Thus the cost of enzyme is usually less than 10% of the overall processing costs, and total process costs can be only 10–20% of final product costs once other costs such as formulation, costs of sales etc. are taken into account. Thus, given that the volume of high fructose corn syrups sold is some  $10 \times 10^7$  million tonnes per annum, then, glucose isomerase has created a very big market, but with the value of the actual glucose isomerase sold being far smaller. One reason for this success is the big improvement in the productivity of the new forms of glucose isomerase product that have now been adopted by corn starch refiners (Table 2; Poulsen personal communication). This illus-

### L-Carnitine production

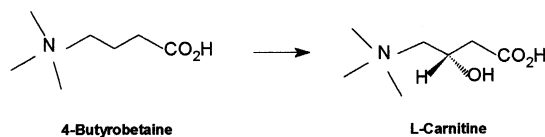


Fig. 1. Bioconversion process to make L-carnitine from 4-butyrobetaine as an example of a new nutraceutical food ingredient.

Table 2

The market constantly seeks improved products and so 'step-jump' improvements in biocatalysts must be made

	'Old' GI introduced in 1974	'New' from <i>S. murinus</i>	Improvement
Productivity (tonnes 42% HFCS per kg GI)	0.25–0.3	15–20	× 60
HFCS cost (\$ per tonne)	5	0.15	× 30

HFCS, high fructose corn syrup; GI, glucose isomerase.

trates that it is not just new biocatalysts that have good economic prospects, but also improved versions of existing biocatalysts. However, in order to achieve such a market dominance these new versions of existing biocatalysts have to achieve a big step-jump improvement in performance, rather than just a more marginal improvement.

Despite the promise of newer techniques such as site-directed mutagenesis and catalytic antibodies the major source of new biocatalysts continues to be from nature, particularly using selective screening methods and/or high throughput screening, coupled with sampling from novel and/or extreme environmental niches. Once a strain with the required enzyme activities has been isolated a sufficiently active biocatalyst can be developed using a range of techniques including mutagenesis, fermentation optimisation, gene cloning; or even a second stage screen targeted at the isolation of a more active and/or higher yielding strain.

Using this approach significant new biocatalysts are being used. Efficient and cost-effective integration of the biocatalysis step into the manufacturing process is critical. Examples include hydroxylating biocatalysts such as for the formation of L-carnitine (Hoeks, 1990), the formation of  $\delta$ -decalactone by mid-chain hydroxylation of linoleic acid formation of a hydroperoxide derivative by lipoxygenase, followed by reduction using strains of *Cladosporium suaveolens* (Gatfield et al., 1993). Also there is the bioconversion of the terpene valencene into nootkatone, which is an important grapefruit flavour chemical, which involves first a site-specific hydroxylation and then a second oxidation step to form the ketone. In addition to its flavour nootkatone is also of interest as it has cytochrome P450 inhibiting properties that may allow its use to reduce the in vivo

metabolism of some drugs, thereby enhancing their effects (Sime et al., 1996). Another product of a new biotransformation process has a similar mode of action. This is cilastatin, which retards the metabolism of certain antibiotics in the kidney and is made on a 15 m<sup>3</sup> scale process that uses chemical steps, and also two biosteps, involving nitrile hydratase and stereoselective amidase reactions (Robins and Gilligan, 1992).

The success of microbial screening as a source of new useful biocatalysts is remarkable considering that it is estimated that less than 1% of the worlds microorganism have been tested, and that a host of strains remain unknown because of the difficulties of growing them in vitro. For these strains new screening methods based on sampling soil DNA may prove more successful. By contrast it is estimated that of the 300000–500000 known plant species some 3000 have been brought into agricultural cultivation.

Interim criteria for the ideal biocatalyst can be drawn-up. Criteria for the ideal biocatalyst include: sufficiently high volumetric activity (g l<sup>-1</sup> day<sup>-1</sup>), required selectivity, broad substrate specificity, rapid process definition, ease of process integration and DSP, cheap, especially in terms of capital costs and DSP operations, easy scale-up, validation and commissioning, reproducible and reliable, patentable and makes a product for which there is a very good market need and value (Hoeks et al., 1995). In particular, it is important to stress the wide range of biocatalyst productivities that have proved sufficiently effective to achieve the status of successful manufacturing processes (Table 3) but with success also depending of course on raw materials costs, capital equipment requirements, product market sizes and profit margins etc. Again, L-carnitine provides a good example (Fig. 2) which shows how

Table 3  
The range of bioprocess productivities

	Concentration of product in reactor ( $\text{g l}^{-1}$ )	Scale of production (tonnes per annum)
Citric acid	120–150	500 000
Glutamic acid	80	300 000
Methylketone (2-heptanone)	75	
L-Phenylalanine	25	
Fructose in high fructose corn syrup	17	8 000 000
Penicillin	10–15	5000
Gamma decalactone	5	
Lactic acid	2.8	20 000
Vitamin B <sub>12</sub>	0.06	6

long it took to develop a sufficiently productive process to achieve a cost-effective product, even when a microbial strain with the required activity had already been found by screening. Biological limits to how productive processes can be achieved are important, with a major determinant being the normal rates of biosynthesis of the different types of cell constituents (Table 4), which substantially limit the rates of production and concentrations of product that it is possible to achieve.

Biocatalysts performance has been improved by a range of strategies. These include the use of two microbial strains carrying out different reactions, but at the same pH, so that they can be used more efficiently in a single reactor to carry out the two-step production of L-lysine (Fukamura, 1976, 1977) (Fig. 3). Then there is the selection of a raw

material that also gives a by-product of significant value, such as the hydrolysis of chlorogenic acid into caffeic and quinic acids, both of which are useful synthons for making functional end-use molecules (Myers et al., 1996) (Fig. 4). In many cases the development of such effective biocatalysts depends on the isolation by screening of novel microbial strains that have the required enzyme activities. Screening enables challenging problems to be overcome, scientifically novel biocatalysts can be discovered, but the main importance of screening is the new and useful properties of the biocatalysts discovered. But these new strains must also have desirable growth properties so as to facilitate their easy and cost-effective large scale use. Some desirable features of indus-

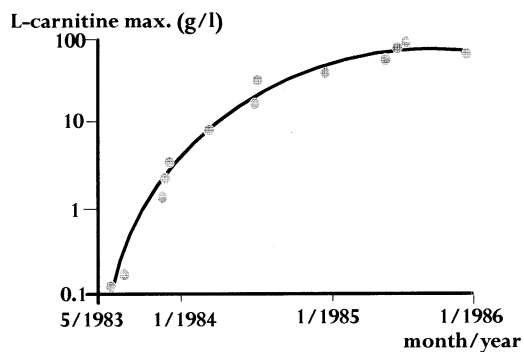


Fig. 2. An indication of the time required for research and development to optimise the concentration of a bioconversion product to a commercially viable level. Data for Lonza's R&D to optimise its L-carnitine process (Kulla, 1991).

Table 4  
The limits to microbial productivity

Substrate family	Reaction type	Turnover rate ( $\text{mmol g}^{-1} \text{h}^{-1}$ )
Carbon source	Uptake, oxidation	10
Monomers/polymers	Synthesis, polymerisation	1
Amino acids	Uptake, synthesis, incorporation in protein	0.1–0.5
Bases, nucleotides	Uptake, synthesis, incorporation in RNA	0.15
	Incorporation in DNA	0.01
Vitamins, coenzymes	Uptake, synthesis	0.001–0.005

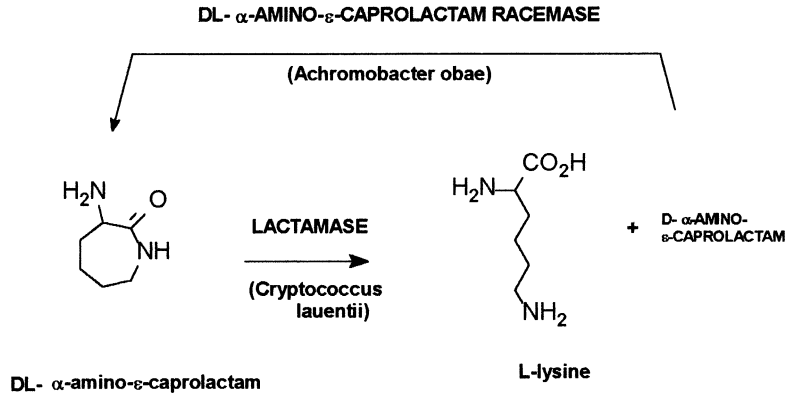


Fig. 3. Bioconversion process for the production of L-lysine from DL- $\alpha$ -aminocaprolactam with both the lactamase and racemase used together in the same reactor as both enzymes have been selected to be active at the same pH (pH 8–9).

trial microorganisms include genetic stability, ease of strain improvement by mutation and rDNA, rapid growth rates, non-fastidious growth requirements, absence of toxin production, no processing problem, e.g. foam production, not susceptible to contamination, metabolic versatility, easy substrate uptake and product export.

The full range of sources of biocatalysts should be examined to obtain a good enough process. These should include plant enzymes as well as microbial enzyme sources, simply because the di-

versity of enzymes made by plants is greater. A good example is the use of plant seed  $\alpha$ -galactosidase to convert guar gum into a polysaccharide with a structure that is essentially the same as that of the more expensive locust bean gum (McCleary et al., 1984; Cheetham and Underwood, 1993). This process which is now carried out on a manufacturing scale. It involved cloning of the gene for the enzyme into yeast for ease of production, and also allowed enzyme reaction on dough-like partially hydrated guar flavour so as to maximise process intensity. Another important but rather less well recognised example is bread making. This involves the ascorbic acid oxidase and glutathione reductase enzymes that are naturally present in wheat flour. By this process the degree of disulphide cross-linking of the bread gluten and gliadin protein molecules, can be controlled, which determine bread structure and quality (Fig. 5). A more recent example of such clever use of enzymes is in the enzyme peeling of citrus fruit so as to save on expensive labour or equipment. This process depends on the use of pectinase enzymes that have a high polygalacturonidase activity, which hydrolyse the fibrous material between the fruit segments and the waxy rind, facilitating the subsequent mechanical removal of the peel (Berry et al., 1988). Enzyme is applied under vacuum and allows easier peel removal, a reduction in adhering albedo, easier separation of fruit sections and good flavour and colour.

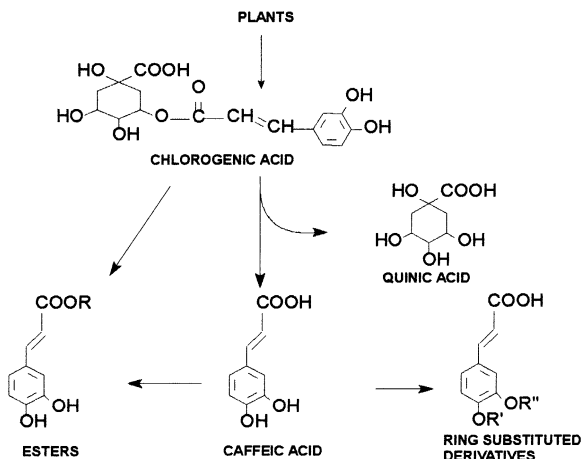


Fig. 4. Formation of caffeic acid useful as an intermediate for the synthesis of its esters, and other derivatives, by enzymic hydrolysis of chlorogenic acid or by transesterification to make the esters directly, and with quinic acid formed as the by-product.

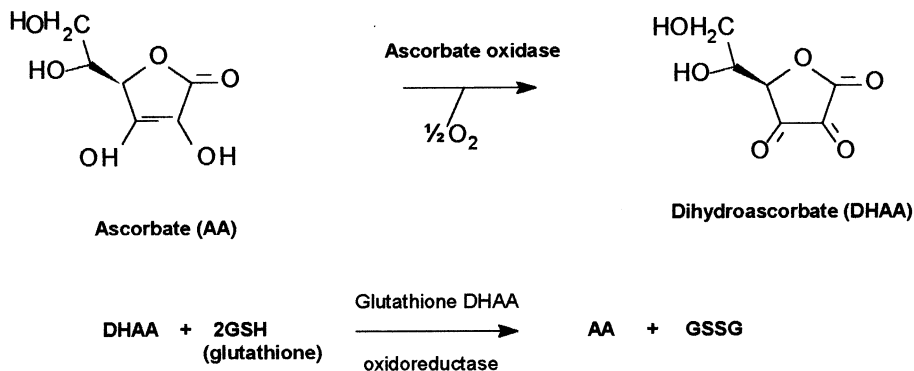


Fig. 5. The role of the endogenous ascorbate oxidase and glutathione–DHAA oxidoreductase enzymes present in wheat flour to control the level of oxidised and reduced glutathiones present, and thus the degree of disulphide bond mediated cross-linking in bread flour proteins.

The growing impact of biocatalysis is also shown by the establishment of a second generation bioprocesses for drugs, such as the semi-synthetic antibiotic cephalexin. Recent advances now make possible a new much simpler process. In the old process, DL-phenylglycine is resolved chemically, then a salt of the D(–) phenylglycine is formed, which is next converted into a mixed anhydride for chemical coupling with protected ADCA to form cephalexin. Instead in the new much simpler process DL-phenylglycinamide or its ester can be resolved and the D(–) isomer coupled enzymatically with unprotected ADCA (Bruggink, 1996). Two stage bioprocessing is also well established in proven manufacturing processes such as for *p*-hydroxyphenylglycine, a key intermediate for semisynthetic antibiotics such as amoxicillin. This is carried out by first a D-selective hydantoinase reaction to form the D-*N*-carbamoyl amino acid; and then a treatment with carbamoylase (amidohydrolase) to produce the D-hydroxyphenylglycine. The yield of the second reaction is enhanced by the rapid racemisation of the unreacted L-hydantoin from the first reaction, which makes quantitative conversion through to *p*-hydroxyphenylglycine more possible (Fig. 6). In addition, some microbial strains have been found that contain both hydantoinase and amido hydrolase activities and so allow possible on step reaction from racemic hydantoin to D-hydroxyphenylglycine.

More recent developments include the use of a *Pseudomonas putida* aminopeptidase to make chiral L- $\alpha$ -amino acids as pharmaceutical and agrochemical intermediates. This has been augmented by different aminopeptidases from *Mycobacterium neoaurum* specific for L- $\alpha$ -alkylamino acids, and most recently from an *Ochrobactrum anthropi* strain obtained by selective enrichment culture that has improved specific activity over a broader pH range and to a greater range of substrates ranging from aliphatic to aromatic, polyaromatic and heterocyclic molecules (Van den Tweel et al., 1993). In all these cases these processes have resulted only after each biocatalyst strain has been developed following screening studies, fermentation to optimise their production, and the bioprocess development work to optimise their uses in the process.

However, it must be admitted that despite all these successes in some cases biocatalysts have not proved successful so far. General ‘misses’ include biocatalysts capable of removing hydroxyl groups, or in carrying out the Diels-Alder reaction. More specifically an enzyme capable of the deformylation of protected aspartame has never been found and an enzyme capable of deacetylating cephalosporins has proved very much more difficult to find and commercialise than the corresponding acylase (penicillin amidase). One general problem that seems to have been little investigated is the effect of production inhibition. This can be either competitive, uncompetitive or non-competi-

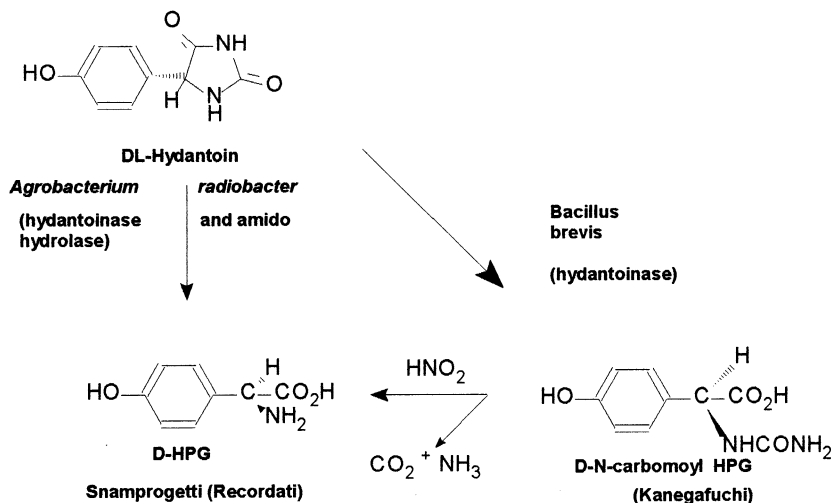


Fig. 6. Routes for the synthesis of D-hydroxyphenylglycine by either D-specific hydantoinase bioconversion followed by either treatment with nitrous acid, or by a carbamoylase (amido-hydrolyase) bioconversion.

tive inhibition by the product of the reaction, slowing down the reaction and preventing full reaction of the substrate into product. In turn this makes difficult both the efficient utilisation of costly raw materials and also the easy recovery of product because of the need to purify it free of remaining raw material(s).

So the important question of ‘What makes a good biocatalysts’ requires both scientific and business answers. These include genuinely meeting market needs, especially product criteria such as cost, quality, functionality, and safety; and also, cost-effective manufacturing process and proprietary technology. The biocatalyst must be cost-effective, and both safe to use, and free to use as regards competitors patents. Also success for a

biocatalysts is much more likely if there is no obvious and direct competition from an existing chemical or agricultural process although in many cases biocatalysis complements chemical or agricultural approaches, for instance the chemical process to make ascorbic acid is completely dependant on the key microbial conversion step of sorbitol into L-sorbose. So it is absolutely necessary to achieve good results not just for the technical performance of the process, but also to answer financial questions on the profitability of the product, and in addition the many technico-commercial requirements, such as safety and regulatory approval, and patenting requirements that must be satisfied. In particular, Table 5 shows some of the problems peculiar to the patenting of inventions for biocata-

Table 5

Problems in patenting microbial inventions

- 1 Functionally identical biological materials (e.g. enzymes) can have significant different structures and amino acid sequences
- 2 Functionally identical biological materials can be obtained from very different biological sources, e.g. enzymes with the same substrate specificity from different microbial sources
- 3 The requirement to deposit samples of microorganism(s) is a substantial overdisclosure
- 4 Biological materials, especially living organisms are too complex to be described in sufficient detail and with enough precision to easily satisfy the requirement of patent law. For instance, existing microbial classification systems are not always directly applicable to newly isolated microorganisms
- 5 Varying criteria and definitions are used to distinguish biocatalysts, e.g. substrate and inhibitor specificity and immuno-chemical cross-reactivity in the case of enzymes, and nutrient requirements and fatty acid composition, etc. in the case of microorganisms

Table 6  
Requirements to create a successful biocatalyst

Discovery scientists	Novel science
↓	
Development	Reliable, reproducible and easy process integration
↓	
Production	Minimal commissioning problems, speed to first production
↓	
Company	Good returns on investments, low capital costs
↓	
Consumer	New or better quality products and services at more affordable prices
↓	
Society	Job creation, export creation
↓	
The planet	Use of renewable resources, environmental benefits

lyst processes and products that must be surmounted in order to not only avoid infringing competitors patents but also to gain patent protection oneself (Cheetham, 1994b).

### 3. Conclusions

So as to increase the number of novel and valuable biotechnology products, two major challenges must be met. Firstly, it is absolutely essential to discover new biocatalysts with novel and improved activities. This is because our current range of biocatalysts is still quite limited both in terms of the number and range of bioreactions it is possible to properly carry out. The other big advance required is to be able to scale-up bioprocesses, including both raw material work-up and DSP operations on a systematic and reproducible basis, rather than the present individual basis, that is rather expensive, time consuming and risky.

In conclusion, it should also be remembered that people with many different types of skills are needed to create a successful biocatalysts, and with the contribution of each specialism absolutely vital for success (Table 6). It is to these *Homo sapiens* 'biocatalysts', as well as to the enzymes and microbial strains they use, that we

look to for future benefits, which are not just economic, as biocatalysis also has the potential to provide a truly eco-friendly technology.

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