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What Is in a Biocatalyst?

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Abstract:

An area often neglected by synthetic organic chemists beginning studies in biotransformations is the biocatalyst itself. As with homogeneous and heterogeneous metal-catalysed reactions, there is a vast opportunity for elegant process understanding and scale-up with biocatalysis. An understanding of the exact nature of the catalyst and the optimum way in which the catalyst should be presented to the reaction medium are crucial to the successful identification and scale-up of organic reactions catalysed by enzymes in aqueous and, especially, organic solvents.

Introduction

A reasonable number of stable, "easy to use" enzymes can now be purchased and used by synthetic organic chemists who do not have access to a culture collection, microbiology, and fermentation skills, but would like to lever the benefits of biocatalysis in a specific project.[1] These biocatalysts, predominantly hydrolases, are increasingly designed for use in organic synthesis, but many are produced primarily for use in other bulk industries such as food processing, cosmetics, and detergents. Many organic chemists still view these catalysts as "magic powders"—sprinkle some into a reaction, and if they produce the result required, great, if not, back to what we are familiar with! This paper addresses some of the screening and scale-up issues commonly encountered by the synthetic organic chemist when first investigating enzyme-catalysed reactions. An enhanced, more rigorous understanding of the biocatalyst can answer most of the problems encountered.

Discussion

Consider a familiar scenario. A number of synthetic routes to a single-enantiomer target are under consideration. Biocatalysis has been considered as an option, and initial screening reveals an enzyme with a high degree of stereochemical selectivity. This is a common, commercially available enzyme preparation—a lipase. A typical outcome of this is that the reaction is then scaled up, the rate becomes very slow, and the enantiomeric purity of the product changes so that the biocatalytic approach is discounted. The reason for most screening and scale-up failures is, undoubtedly, not considering the nature and type of the biocatalyst during screening and when scaling up the reaction. An acceptable enzyme form for screening may not perform at all well in subsequent scale-up experiments!

Therefore, starting at the screening phase, typically a very dilute solution of substrate (1−20 mg mL^{-1}) will be taken, a large excess of biocatalyst added, and the reaction magnetically stirred or shaken in small vials. Generally at this stage, no attempt will be made to monitor or control the pH or water activity if working in largely organic solvent.[2] Working in dilute solution tends to make the most of any substrate, often a scarce resource at the launch of a project. To a large degree, the dilute substrate/excess enzyme approach is good, since it maximises the chance of getting a reaction hit. A dilute solution will minimise potential problems of substrate/product inhibition and pH change during the reaction, all of which can be addressed later in the development process. At this stage, screening should be for a reaction hit and not an optimised process. If the screening phase presents with a good hit in terms of conversion and ee, the reaction is then scaled-up.

For scale-up, however, a number of factors now need to be considered: much more concentrated solutions will improve space-time yield, mixing will be different (overhead *v*s. magnetic vs shaking), the solvent might be changed to something more amenable to larger scale production, the charge of enzyme reduced to improve process economics, the "same" enzyme might be sourced from a different, cheaper supplier. It is often at this stage in the development process when the screening and initial scale-up results begin to diverge.

Representative Example Using *Candida rugosa* Lipase. The results for a typical screen are shown in Table 1. The reaction was a kinetic resolution via ester hydrolysis; the desired product was the acid, required in high ee and chemical purity—Scheme 1 (full structual details cannot be disclosed at this time). Although the racemic substrate was screened against a large bank of different hydrolytic enzymes, the results in Table 1 refer to only one enzyme, *Candida rugosa* lipase, an enzyme frequently employed in organic synthesis.[3,4] There were 14 different biocatalysts, all obtained commercially either as *C. rugosa* or *C. cylindracia* (older name for *C. rugosa*). The physical form of the biocatalyst varied from simple powders, to CLECs (cross-linked enzyme crystals),[3] CLEAs (cross-linked enzyme aggregates),[5]

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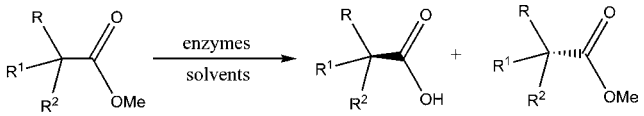
***Table 1. C. rugosa* lipase reaction screen**

biocatalyst number & physical form	comment	% conversion at 48 h in 90% solvent−water (ee) acid product	conversion at 48 h in 90% water−solvent (ee) acid product
1 powder l3	crude lipase	5−10 (n.d.)[a]	55 (50% ee)
2 powder	pure lipase	5−10 (n.d.)	65 (50% ee)
3 CLEC	highly pure crystalline	5−10 (n.d.)	20
4 CLEA	highly pure crystalline	2 (n.d.)	10
5 powder	crude lipase	2 (n.d.)	no reaction
6 powder	crude lipase	50 (95% ee)	90 (n.d)
7 powder	crude lipase	5−10 (n.d.)	65 (n.d.)
8 PCMC	partially purified	1−2 (n.d.)	not tested
9 powder	crude lipase	5−10	90 (n.d.)
10 powder	crude lipase	50 (95% ee)	70 (n.d.)
11 powder	crude lipase	40 (100% ee)	55 (40% ee)
12 macroporus resin	pure lipase	1−2 (n.d.)	no reaction
13 macroporus resin	crude lipase	1−2 (n.d.)	no reaction
14 powder	crude lipase	40 (95% ee)	not tested

[a] (n.d.) = not determined.

Scheme 1



R, R^1, and R^2 are not equal to each other.

PCMCs (protein-coated microcrystals),[6] or protein-supported on macroporus resins (Eupergit C type).[7]

Examination of the results obtained from screening in organic solvent is worthy of discussion. If only biocatalyst numbers **1**−**5**, **7**−**9**, **12**, and **13** had been included in the screening set, it might be reasonable to deduce that *C. rugosa* lipase was inactive, or showed little tendency to hydrolyse the substrate. Catalysts **6**, **10**, **11**, and **14**, however, are all reasonably active and show high enantioselectivity! This behavior cannot be attributed to first set of catalysts being totally inactive or denatured since, prior to screening, activity for all catalysts **1**−**14** was demonstrated by the hydrolysis of a very simple test substrate, *p*-nitrophenyl acetate. It is also clear that catalysts in physical forms that would normally be considered extremely active, such as CLECs or CLEAs or very pure preparations, do not always show high reactivity. Hence, it can be deduced that, for one enzyme, there is great benefit in screening samples from different suppliers and in various physical forms. An interesting comparison also worthy of note is that of results obtained in aqueous medium vs those in organic solvent—Table 1. Most of the catalysts that showed very low activity in organic solvent are now very active in aqueous media, and all those that showed high stereoselectivity now show only moderate stereoselectivity (~95% ee vs 50% ee). If this screen had been run in water alone, it would be reasonable to deduce that the *C. rugosa* lipase was very active, but not really stereoselective! There are several possible reasons for the apparently anomalous stereochemical behavior of enzymes in organic compared to aqueous reaction media. Many lipases are known to be more stereoselective in the presence of certain organic solvents.[8−11] Also, in a mainly organic environment in which lipases are active, the activity of other hydrolytic enzymes present in the biocatalyst (such as esterases or proteases) may be suppressed, but in the aqueous environment these may be active and hydrolyse the substrate with opposite enantiopreference to the lipase, thus changing the overall observed stereoselectivity.[8,12]

Analysis of the Biocatalyst. Most enzyme preparations are sold with very little information by which to characterise the product. A protein assay (mg of protein per mg of solid) might be provided, but it is important to note that not all the protein maybe the desired enzyme! An activity value—for example, units per mg of solid—that refers to the rate of hydrolysis of a standard substrate (usually tributrin in the case of lipases) may be given. For application in a complex organic synthesis, it is much more informative to examine the biocatalyst in more detail.

C. rugosa has genes that encode for five to seven very similar lipases.[13−15] These "isoenzymes" can also undergo post-translational glycosylation to varying degrees. The number of lipases and their distribution is governed by the exact strain of *C. rugosa* and the fermentation conditions (carbon source used, inducers, etc.).[14] The organism also produces other enzymes such as esterases and proteases which could be carried through into the lipase preparation. There appears to be some debate whether the isoenzymes of *C. rugosa* have the same or conflicting enantiopreferences.[14,16−18] A further complicating factor is that *C. rugosa*

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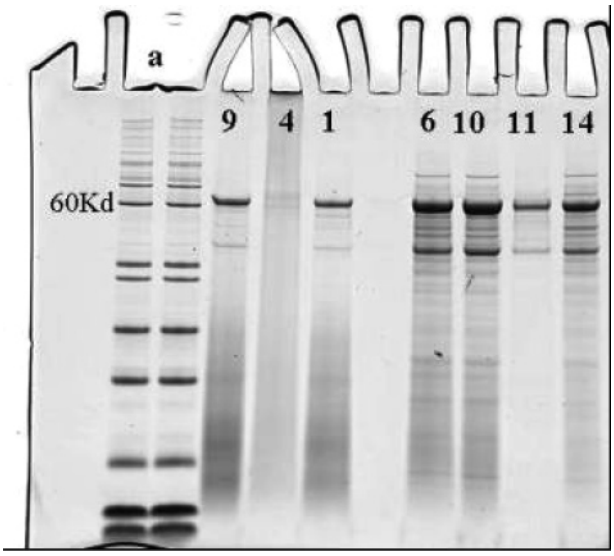

Figure 1. **SDS-PAGE gel of selected *C.rugosa* biocatalyts. Sample a is molecular weight markers.**

is a true lipase. It has a protein fold (or lid) covering the active site.[19] This has to open to allow substrate access, so-called "interfacial activation". Consequently, the lipase can exist in a closed or "low activity", or open, "activated" conformation.[20,21] The distribution between the two forms very much depends on how the lipase is isolated and how reaction conditions are employed (e.g., organic solvents, additives, etc.)[10,11,22−24]

Figure 1 shows a sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE gel) analysis of a selection of the *C. rugosa* biocatalysts listed in Table 1. For all samples, 2 mg of the commercial lipase powder was dissolved in 1 mL of potassium phosphate buffer (50 mM, pH 7.5); 12 μL was loaded to the gel. It can be seen that the major component of ~60 kD molecuar mass is as expected for the isoenzymes of *C. rugosa* lipase,[25] but there is a great deal of variation in band intensity (concentration), and other proteins are present that may be active hydrolases. The very weak response for biocatalyst **4** on the gel is due to this preparation being highly cross-linked hence virtually insoluble in the media used to prepare samples for SDS PAGE gel experiments.

It is important to note here that the composition of each batch of *C. rugosa* lipase can be strictly controlled and manufactured reproducibly—but it should not be expected that identical performance will be obtained with *C. rugosa* lipase from different manufacturers unless identical strains and identical techniques of fermentation and isolation have been used.

Table 2. **Total protein analysis for selected *C. rugosa* catalysts**

lipase sample	% total protein content
1	20
6	32
9	19
10	28
11	14
14	30

Table 3. **Typical analysis[a] of biocatalyst number 6**

components of biocatalyst **6**	average % of total
lipase A	9
lipase B	0.7
lipase C	0.5
other proteins	12
carbohydrates	38
inorganic salts	31
water (LOD)	6

[a] Data supplied by Metio Sangyo.

In addition, many laboratory chemical supply companies will buy and repackage products bought in bulk from primary manufacturers. From our analysis of the soluble catalysts by SDS-PAGE gels, we concluded that several of *C. rugosa* catalysts were virtually identical and probably from the same bulk source; **6**, **10** and **14** look identical and gave very similar performance—see Table 1. Biocatalysts **1**, **9**, and **11** also look very close in composition, but only biocatalyst **11** performed well in the bioresolution.

Total protein analysis was obtained for a subset of the *C. rugosa* biocatalysts, and this is shown in Table 2. It is noteworthy that this is variable, far from 100%, and the analysis technique (Bradford) is not specific for the lipase but is the sum of *all* proteins present. Examination of one particular biocatalyst, **6**, in more detail reveals a complex mixture of three lipases: one major lipase component at 9%, along with other proteins in a matrix of hydrophilic materials such as carbohydrates and inorganic salts—see Table 3. In fact some commercial lipase biocatalysts have as little as 1 wt % of the active desired enzyme. If such a biocatalyst is employed in water, the hydrophilic matrix will dissolve, liberating the enzyme. However, if used in an organic medium, then the biocatalyst is totally insoluble, and the reaction will rely on surface chemistry or complex diffusion of the substrate into, and the product(s) out of, the matrix. The hydophilic components of enzyme powders (carbohydrates, buffer salts) can also cause problems when scaling up reactions in organic media containing small amounts of water. The deliquescent, insoluble powder can rapidly absorb water, thus becoming a thick paste, rendering efficient mixing impossible. However, there are strategies to deal with this

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undesirable behavior.[26] Once all the factors discussed above are considered, it is not really that surprising that scale-related, large variations in rate and so forth can be seen in reactions catalysed by crude enzyme powders.

The enzyme powders may be acceptable for screening, but for scale-up, often a high surface area supported biocatalyst, or a macorporus resin may give better performance and reliability.[7,27] In addition, the preparation of a solid supported biocatalyst often also serves well as an additional purification step, enhancing the concentration of the desired enzyme. This is especially true for *C. rugosa* lipase preparations.[28,29]

Conclusions

For the synthesis described here, the optimum *C. rugosa* biocatalyst identified was number **6**. This is the high activity *C. rugosa* lipase supplied by Metio Sangyo as Lipase OF.[29,30] This biocatalyst has a high concentration of the active lipase and can be manufactured with a high degree of batch-to-batch control.

A key factor to success in identifying and scaling up reactions with biocatalysts is an understanding of the physical nature of the biocatalyst, the number and action of the enzymes present, and how best to present the enzyme to the reaction. Selection of the most appropriate reaction medium and attention to reaction parameters such as pH and water activity are also important. The required thought processes and experiments are not really so different from those needed to optimise homogeneous and heterogeneous metal-catalysed reactions; thus, we should stop thinking about "magic powders" and start thinking about (bio)catalysts! A more in-depth illustration of the importance of correct biocatalyst choice utilising a supported biocatalyst entitled "Selective Lipase-Catalysed Hydrolysis of a 1,2-Diester in the Development of a New Route to AZD2563 DSP" will be published in this issue of *Organic Process Research and Development*'s special section on biocatalysis.

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Note Added after ASAP Publication: In the version published on the Internet April 22, 2006, there was an error in Scheme 1. This has been corrected for the final version published May 19, 2006, and the print version.

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