

Chiral technology: industrial biocatalysis with standard hydrolytic bulk enzymes

Explained is why enzymatic resolution and enzyme immobilisation are key components in the chiral toolbox of the process chemist.

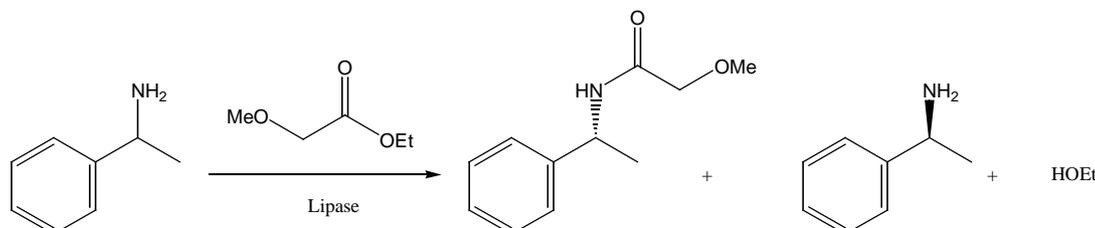
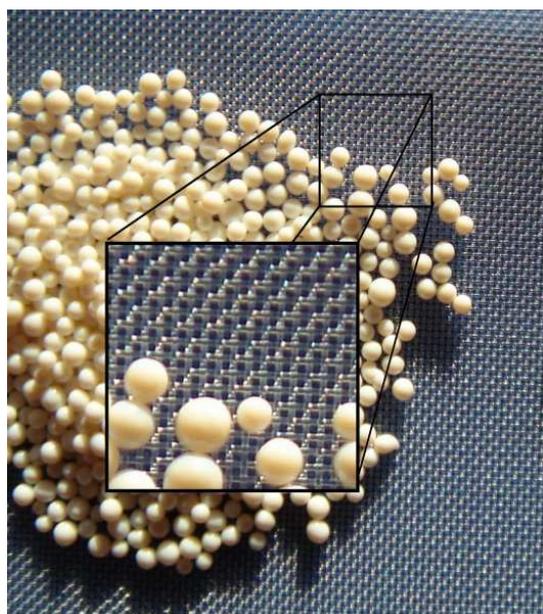


Fig 2. Lipase catalysed kinetic resolution by BASF

Despite the impressive progress in asymmetric synthesis, the dominant production method to obtain a single enantiomer in industrial synthesis consists of the resolution of racemates. The resolution of enantiomers can be divided into several groups consisting of direct crystallization, crystallization of diastereomeric salts, chromatography and kinetic resolution (either by esterification / acylation or hydrolysis). For kinetic resolutions enzymes are found to be most versatile. Besides lipases the group of proteases are used since in many cases opposite chiral selectivity is found; the substrate range for proteases tends to be a bit smaller, however. Many fine chemicals producers also employ esterases, acylases and amidases.



ChiralVision's Immozyme: polymeric beads (150 – 300 µm in size) with covalently bound enzyme filters very easy on a 50 micrometer sieve.

The success of a kinetic resolution is dependent on the fact that the two enantiomers react at different rates with a chiral entity. In contrast to an asymmetric synthesis, a kinetic resolution yields at maximum 50% of the desired enantiomer. To achieve higher yields, the undesired enantiomer can be separated and racemised.

Alternatively, racemisation can be achieved *in-situ* by a dynamic kinetic resolution (DKR) by using metal catalyst or racemases. The current acceptable enantiomeric excess is normally 99%, although the future target that companies have set for themselves is 100% yield and 100% e.e.. There seems to be no limit in the range of compounds accessible. Examples can be found for many chiral compounds comprising alcohols and diols, amines, amides, α -amino acids, β -amino acids, amino alcohols, carboxylic acids and esters.

Smart throughput screening

Since the list of most attractive commercially available (bulk) hydrolytic enzymes is about 100, one can ask what chance there is for a successful process. The answer is simple: the enzyme is but one of the variables in the equation. The combination with clever substrate derivatisation (yielding the same end product) and medium optimisation (including solvent system, temperature, pH, buffer salts, etc.) yields a choice of at least 10,000 – 100,000 distinctively different process conditions with varying yield and enantioselectivity. Detailed knowledge of the behaviour of various enzymes under diverse conditions is needed to be successful in designing such a process. Successful it is though: by using only commercially available bulk enzymes a success rate of over 90% is feasible is learned in several years of enzymatic process development. Large chemical producers now have their own

biocatalysis groups (e.g. BASF, Dowpharma, Degussa, DSM) to have this expertise in-house. They have a strong product based focal point. The early integration of biocatalysis with chemistry is essential. It enables the integration of catalyst improvement, substrate modification and optimisation of reaction conditions in a coordinated approach leading to rapid development. Many companies are using this approach, although discipline-specific thinking is difficult to avoid but must be avoided for successful application. Although these companies also have the capability to develop new enzymes, many commercial processes are using "standard" enzymes. The available literature on enzymatic resolution has steadily increased over the last two decades (Fig 1)

reflecting the growing need for chiral compounds. The amount of patents claiming enzymatic resolution technology has remain fairly constant, however. Many smaller companies (e.g. ChiralVision, Biocatalytics) offer their knowledge and skills in enzyme screening and process optimization to the chiral market thus making this technology general accessible for the industry and thus contributing in a broad sense to white biotechnology's growth. More recently, directed evolution has been applied in making "standard" mutants of the most successful lipases used in resolutions. This will further boost the amount of future applications in which resolution can be useful for the synthesis of enantiomerically pure compounds.

Table 1. Some examples of enzymatic resolution used in production

Manufacturer	compound	enzyme	scale (tons / y)
BASF	chiral amines	<i>C. antarctica</i> lipase B*	1000
BASF	(R)-mandelic acid	(S)- or (R)-nitrilases	multi ton
DSM	L- or D-amino acids	amidase	multi ton
DSM	chiral alcohol	lipase*	multi ton
DSM Chemie Linz	2-halopropionic acids	porcine lipase	0.1
DSM-Andeno	(R)-glycidyl ester	lipase	multi ton
Dowpharma	β -phenylalanines	lipase	0.1-0.5
Dowpharma	carbocyclic nucleosides intermediate	γ -lactamase	multi ton
Kaneka	D-amino acids	hydantoinase* / carbamoylase*	1-5000
Schering	protein farnesyl transferase inhibitor	lipase LIP-300	0.1
various	L-methionine	L-aminoacylase	1-5000
various	6-aminopenicillanic acid	penicillin G acylase*	20,000

* immobilised

Enzymatic resolution on industrial scale

Chiral intermediates and fine chemicals are in high demand from the pharmaceutical and agrochemical industries for the preparation of bulk drug substances and agricultural products.

A number of multi-ton industrial processes use enzymatic resolution, often with lipases that tolerate different substrates (Table 1). BASF, for example, makes a range of chiral amines by acylating racemic amines with proprietary esters. Only one enantiomer is acylated to an amide, which can be readily separated from the unreacted amine, the unreacted amine can be racemised off-line and fed back into the process to increase the final yield (Fig 2). Other resolutions use nitrilases where only one isomer is converted in the corresponding acid. DSM uses enzymatic resolution for racemic 2-pentanol and 2-heptanol with lipase B from *Candida antarctica*. S-(+)-2-pentanol is a key chiral intermediate required for synthesis of anti-Alzheimer's drugs. The resolution of 2-halopropionic acids, a starting materials for the

synthesis of phenoxypropionate herbicides is being carried out on a 100-kg scale by Chemie Linz Co. (Austria) under a license from the Massachusetts Institute of Technology. The process is based on the selective esterification of (S)-isomers with butanol catalysed by porcine pancreatic lipase in anhydrous hexane. Typically, >99% enantiomeric excess (ee) is obtained at 38% yield and the resolution is complete in several hours.

Another commercial application of lipases in the resolution of racemic mixtures is the hydrolysis of epoxy alcohol esters. The highly enantioselective hydrolysis of (R,S)-glycidyl butyrate has been developed by DSM-Andeno (the Netherlands). The reaction products (R)- glycidyl esters and (R)-glycidol, are readily converted to (R)- and (S)-glycidyltosylates, which are very attractive intermediates for the preparation of optically active beta-blockers and a wide range of other products. Dowpharma is also using hydrolases and prepares enantiomerically pure α - and β -amino acids like e.g. 99% enantiopure β -phenylalanines on a scale

of hundreds of kilograms. Kaneka uses immobilised recombinant hydantoinase and carbamoylase enzymes to make several thousand metric tons per year of D-amino acids. Immobilised penicillin G acylase has been used for decades already to produce 6-aminopenicillanic acid by several manufacturers.

Many patents are filed (Table 2) claiming new applications for hydrolytic enzymes. It shows that the industry is still recognising the power of enzymatic resolution. Interestingly, in virtual all patents the use of an immobilised version of the selected enzyme is also claimed.

Table 2. Recent patents (>2003) claiming enzymatic resolution technology

Manufacturer	compound	enzyme
Aventis Pharma	amino acids	Glutaryl 7-ACA acylase
Hoechst	L-PTC (herbicidal)	acylase / protease / esterase
Pfizer	selective estrogen receptor modulators	(immobilised) lipase / esterase
Bristol Myers Squibb	T-butyl taxanes (antitumor)	esterase, lipase, amidase or acylase
Merck	indole ester	<i>Pseudomonas fluorescens</i> lipase
Dow	Glycol ether acetates	lipase
Degussa	β -amino acids	Lipase PS

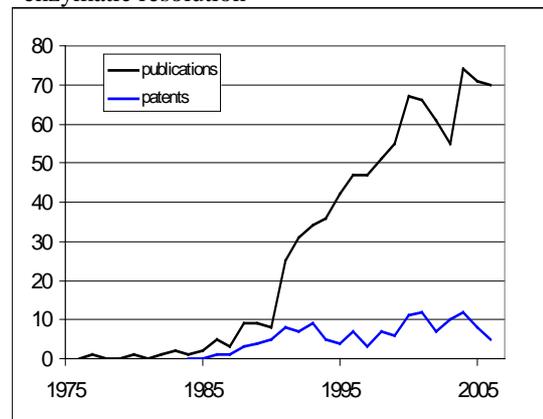
Enzyme immobilisation

One of the most important decisions when designing an enzymatic manufacturing process is whether the conversion is carried out using a whole-cell (or extract) or an isolated enzyme. The development process and the final manufacturing process may differ a lot. For the production of the first kilogram quantities often an isolated enzyme is used; for low upstream costs cells are often used for the final process. Whole-cells can provide an efficient way to regenerate cofactors in situ and contain multiple enzymes enabling multi-step chemistry. However, they generally will not tolerate high concentrations of substrate or product, have limited use with organic solvents and can produce unwanted side products as a result of the presence of multiple enzymes. Isolated enzyme processes are simple to implement because they are more suited for short-time process development. The price to be paid for these benefits is higher upstream costs and enzyme reuse is often essential to keep these costs manageable.

Immobilization of enzymes offers easy separation and reuse (Fig 3), thereby making production processes more cost effective. Additionally process conditions can be chosen with increased flexibility. In a hydrolytic two-phase reaction emulsion formation is prevented and in a water-free reaction immobilisation normally increases enzyme activity 1 or 2 orders compared to using pure enzyme powders. The aim is further to minimize the enzyme purification and to use the crude form of the enzyme in the process. In this way, enzyme immobilisation serves as a purification step in which the enzyme is bound and various unspecified components from the fermentation are washed away. Another source of trouble is additives or stabilisers in formulated enzymes: especially

alcohols (like glycerol) can lead to contaminating esters as side products; in freeze-dried enzymes salts and other additives may hamper activity in low water or water free conditions.

Fig 1. Articles and patents (annually) on "enzymatic resolution"



Since only a dozen immobilised enzymes are used as catalysts in divers industrial processes, only a few enzymes are commercially available in immobilised form. The most well-known one used in chemical applications is NZ 435 produced by Novozymes which is *Candida antarctica* lipase B absorbed on macroporous beads. This product has become an industrial standard over the years. It is presumably used by BASF in their 1000 ton p/a chiral amine process. For the production of antibiotics covalent immobilised penicillin G acylase has been used for over decades. Few others are available, most of them are exclusively used in food processing like immobilised glucose isomerase for the continuous production of fructose syrup. This has forced some chemicals producing



companies to immobilize enzymes themselves: although both the enzyme and the carrier are available on the market, no immobilised enzyme “to-go” can be obtained. More companies including enzyme producers are now offering an increasing range of immobilised enzymes with a wide choice of enzymes and different carriers suiting both batch and continuous processes. Most of the enzyme carriers used are readily available on the market and guarantee minimal costs and bulk availability. Enzyme immobilization services provides this specific knowledge to the market making the technology also accessible for custom enzymes.

The discovery of German chemists Eduard Buchner and Hans Buchner in 1897 that a cell-free extract of yeast could cause alcoholic fermentation and the discovery in 1916 by Nelson and Griffin that invertase “exhibited the same activity when absorbed on a solid (charcoal or aluminium hydroxide) at the bottom of the reaction vessel as when uniformly distributed throughout the solution”, initiated a technological development that came to full application more than 100 years later. Novel catalyst formulation based on

immobilisation technology has resulted in improved types of highly active and stable biological catalysts that are being commercialised. As a result of these developments, industrial biocatalysis is steadily growing and the ongoing progress will ensure continued success in meeting new industrial challenges. Clearly, enzymatic resolution and enzyme immobilisation have become an indispensable tool in the chemist’s chiral toolbox.

Further information

ChiralVision
Wassenaarseweg 72
2333 AL Leiden
The Netherlands
Tel: +31 71 332 2124
Fax: +31 71 332 2125

Internet links: info@chiralvision.com
www.chiralvision.com