Lonza: 20 Years of Biotransformations

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Abstract: Biotransformations are an important tool in organic synthesis, especially for the production of chiral molecules, and where chemical reactions are not possible or inefficient. Lonza is a major custom manufacturer of intermediates for the life science industries, and uses biocatalysis in many of its processes. A synthetic route may have one or more biotransformation steps. This article gives a number of examples of syntheses that involve biotransformations for the production of both achiral and chiral intermediates, at development and production scales. For each example the reasons and advantages of using a biotransformation are given.

Keywords: biotransformations; hydrolysis; Lonza; multi-enzyme biotransformations; oxidation; reduction

1 Introduction

Biotransformations are enzyme-catalysed conversions of non-natural substrates to products. They are an important tool in organic synthesis, especially for the synthesis of chiral molecules, where the reactions catalysed may be asymmetric syntheses or the resolution of racemates. The main advantages associated with the use of single enantiomer compounds are increased specificity and the avoidance of adverse side effects. Biotransformations are also used for reactions to achiral molecules where a chemical step would not be possible, or where the biotransformation has advantages.

Lonza's biotechnology R&D group was founded in 1983 and consisted of three people. Since then the number of people engaged in biotechnology has risen to over 800. These people are employed in the production of pharmaceutical and agrochemical intermediates by fermentation and biocatalysis, and in the production of biopharmaceuticals with both microbial and mammalian cells. Biotransformations have played a prominent role in the development of biotechnology in Lonza, and processes involving biocatalysis have been developed for both chiral and non-chiral molecules.

The possible synthetic routes for each target molecule are evaluated in order to find the best overall route. The availability of starting materials, the number of steps involved, environmental considerations, scalability, development time, product quality, and down-stream processing are all taken into consideration. The route of choice may be chemical, or a combination of chemistry and biocatalysis. For success, close cooperation between chemistry and biotechnology is essential.

Enzymes for biocatalysis are used in a number of forms. They may be wild-type, or recombinant, or genetically modified to increase their specificity or activity. One or more enzymes that carry out the required steps may be present in whole cells, which may be growing, resting or immobilised. Alternatively, cell-free enzymes may be used in solution, in a membrane reactor, as a suspension, cross-linked, or immobilised. The medium for the reaction may be aqueous, organic or two-phase. For general reviews and

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monographs on biotransformations and their industrial applications the following references are recommended as a starting point.[1–8]

A number of examples are described below for processes that include one or more biotransformation steps. For each example the rationale for using a biotransformation is given.

2 Oxidation

2.1 Oxidation of Alkyl Groups on Aromatic Heterocycles: 5-Methylpyrazine-2-carboxylic Acid and Pyridyl-3-acetic Acid

Chemical oxidation reactions of heteroaromatic compounds with one or more alkyl groups are generally unspecific and lead to the formation of by-products. However, microorganisms grown on xylene or toluene as their sole source of carbon and energy can selectively oxidise single methyl groups on aromatic heterocycles, using the enzymes of the xylene degradative pathway.[9] 5-Methylpyrazine-2-carboxylic acid (MPCA) is an intermediate for the production of Acipimox, which is an antilipolytic drug, and Glipicide, an antidiabetic drug. The oxidation of 2,5-dimethylpyrazine (DMP) to MPCA is carried out with *Pseudomonas putida* (Figure 1). High product concentrations and yields are obtained by carrying out the biotransformation with live cells growing on xylene.[10] DMP is added continuously to the fermentation broth up to 2 g/L. Higher concentrations of DMP are toxic for the biocatalyst. No bacterial degradation products from xylene, which could interfere with product isolation, are detected in the fermentation broth. MPCA is precipitated by acidifying the cell-free fermentation broth. Lonza produces MPCA at the 15 m^3 scale with a product concentration of 24 g/L and an analytical yield of $>95\%$.

In an analogous way, a second group of microorganisms grown on *n*-octane can be used for the terminal oxidation of ethyl groups to the corresponding acetic acid derivatives. For example, *Pseudomonas oleovorans* catalyses the oxidation of 5-ethyl-2-methylpyridine to 6-methylpyridyl-3-acetic acid, and illustrates the selectivity of this reaction: no by-product with an oxidised methyl group in position 2 was detected (Figure 2a). Lonza has used this reaction[9] for the conversion of 3-ethylpyridine to pyridyl-3-acetic acid (Figure 2b), which is an intermediate for the preparation of Risedronate, a drug for the treatment of osteoporosis.

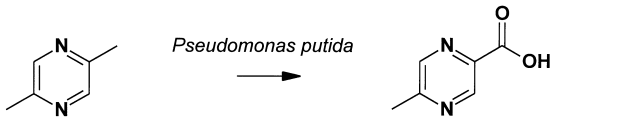

Figure 1. The oxidation of 2,5-dimethylpyrazine to 5-methylpyrazine-2-carboxylic acid by cells of *Pseudomonas putida* growing on xylene.

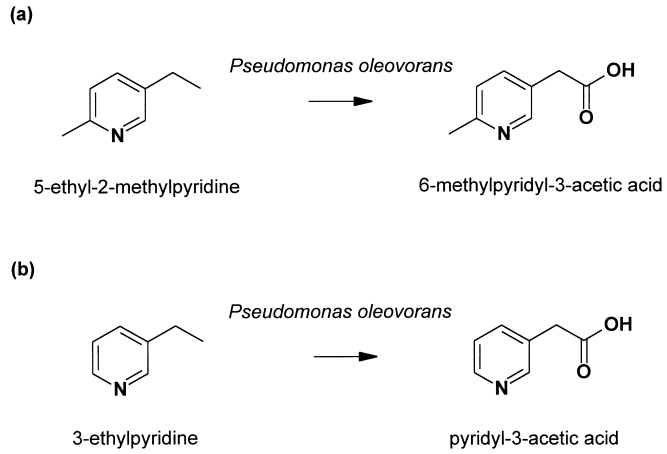

Figure 2. Ethyl group oxidation by cells of *Peudomonas oleovorans.*

2.2 Regiospecific Hydroxylations of Aromatic *N*-Heterocycles: 6-Hydroxynicotinic Acid

The regiospecific hydroxylation of aromatic *N*-heterocycles is difficult to carry out chemically, but progress has been made in this field with biotransformations.[11] Lonza have developed a number of biotransformations for this type of reaction to produce 5-hydroxypyrazinecarboxylic acid, 6-hydroxypicolinic acid and 6-hydroxynicotinic acid.[9,12,13]

The starting product for the biosynthesis of 6-hydroxynicotinic acid is the vitamin nicotinic acid. Nicotinic acid is produced in large quantities by Lonza which ensures both the availability and the competitive pricing of the starting material. 6-Hydroxynicotinic acid is a versatile building block. It is especially useful for the production of insecticides.[13] 6-Hydroxynicotinic acid can be synthesised chemically[14] but the formation of by-products due to non-specific hydroxylation of the pyridine ring and the complex down-stream processing that is required for their separation from the product increases costs, making the chemical process uneconomic. Conversely the biohydroxylation is regiospecific and therefore avoids the above-mentioned problems of the chemical synthesis, making it an attractive alternative.

The enzymatic production of 6-hydroxynicotinic acid occurs in two steps.[15] It is possible to carry out both steps in the same fermenter. Firstly, *Achromobacter xylosoxydans* LK1 (DSM 2783) biomass possessing a highly active nicotinic acid hydroxylase is produced. To achieve a high level of induction of this enzyme nicotinic acid is used as the carbon and nitrogen source as well as the enzyme inducer. In the second step the biomass is used for the hydroxylation of the nicotinic acid. A solution of nicotinic acid that has been neutralised with NaOH is added directly to the reactor containing the *Achromobacter xylosoxydans* LK1 strain. The optimum temperature for the biotransformation is 30 °C and aeration is necessary. The yield after the biotransformation is $>99\%$. The down-stream processing consists of ultrafiltration of the biomass and adjustment of the product solution pH to 1.8 with H_2SO_4. The 6-hydroxynicotinic acid precipitates at this pH and the white crystals are then filtered from the solution. The crystals are washed and then dried under vacuum. The yield of isolated 6-hydroxynicotinic acid is about 93% and the purity $>99\%$.

The degradation of nicotinic acid is described in the literature[16] and is shown in Figure 3. The second enzyme of this pathway, 6-hydroxynicotinic acid hydroxylase is inhibited by $>1\%$ nicotinic acid[13] while the first enzyme, nicotinic acid hydroxylase is unaffected. This phenomenon is exploited in both steps of the process. In the first step where biomass production is critical the nicotinic acid concentration is kept low to ensure that it is completely catabolised and no accumulation of 6-hydroxynicotinic acid occurs. In the second step the reverse situation, accumulation of 6-hydroxynicotinic acid, is desired. In this step the nicotinic acid concentration is kept high ensuring the inhibition of the second enzyme of the pathway which results in the accumulation of 6-hydroxynicotinic acid.

Lonza has successfully produced more than 10 tons of 6-hydroxynicotinic acid with this process.

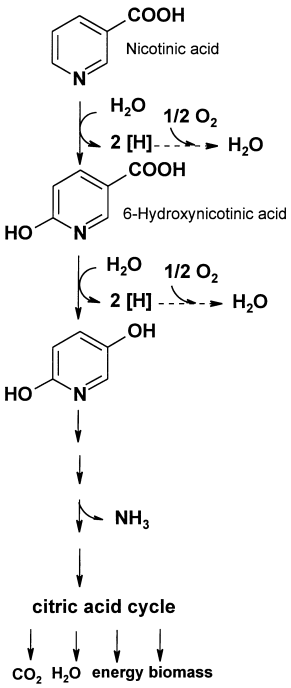

Figure 3. The degradation of nicotinic acid by *Achromobacter xylosoxidans.*

3 Reduction

3.1 (*R*)-Ethyl 4,4,4-Trifluoro-3-hydroxybutanoate

(*R*)-Ethyl 4,4,4-trifluoro-3-hydroxybutanoate is a building block for pharmaceuticals such as Befloxatone, an antidepressant monoamine oxidase-A inhibitor from Synthelabo.[17] The process utilises whole cells of *Escherichia coli* that contain two plasmids.[18,19] One carries an aldehyde reductase gene from the yeast *Sporobolomyces salmonicolor*, which catalyses the reduction of ethyl 4,4,4-trifluoroacetoacetate, and the second carries a glucose dehydrogenase gene from *Bacillus megaterium* to generate NADPH from $NADP^+$ (Figure 4). The strain was originally constructed to catalyse the stereoselective reduction of ethyl 4-chloro-3-oxobutanoate to ethyl 4-chloro-3-hydroxybutanoate, a precursor for L-carnitine synthesis.[20] Productivities of up to 300 g/L, and ee values of up to 92% were reported for the ethyl 4-chloro-3-hydroxybutanoate process.

The Lonza process is carried out in a water/butyl acetate two-phase system to avoid inhibition of the reductase by the substrate and product. An advantage of the two phase system is that the cells are permeabilised, allowing the transfer of $NADP^+$ and NADPH through the cell wall. Cells of *Escherichia coli* JM109 containing the two plasmids are grown at 22 °C to avoid inclusion body formation. The cells are then washed and stored frozen before use in the biotransformation. The product has an ee value of $>99\%$, and the yield is about 50%. Lonza is the world's leading manufacturer of ethyl 4,4,4-trifluoroacetoacetate, so there is optimal backward integration of the process.

$NADP^+$ is both essential and a major cost factor in the biotransformation, so securing a supply at a reasonable price was essential. The concentration of $NADP^+$ required for maximum activity is about 0.5 g/L. Experiments with a number of other esters showed that the isopropyl ester of 4,4,4-trifluoroacetoacetate is at least as good a substrate as the ethyl ester.

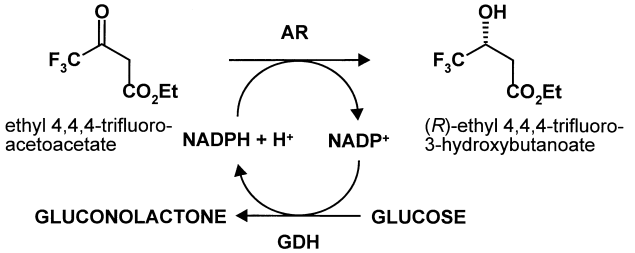

Figure 4. The stereoselective reduction of (*R*)-ethyl 4,4,4,-trifluoro-3-hydroxybutanoate by an aldehyde reductase from S*porobolomyces salmonicolor* in *Escherichia coli*. AR = aldehyde reductase; GDH = glucose dehydrogenase.

4 Hydrolysis

Lonza uses a number of hydrolytic biotransformations to produce both achiral molecules, for example, nicotinamide, and a number of chiral molecules. Enzymes that metabolise nitriles and amides (nitrile hydrolases and amidases) have found many applications.[21] Nitrile hydratases are not usually enantiospecific, whereas amidases can be (Figure 5). Nitrilases, which catalyse the conversion of nitriles directly to the corresponding carboxylic acids, were generally thought not to be enantiospecific, but recently several have been discovered by modern cloning and screening techniques.[22]

4.1 Nicotinamide

Nicotinamide is an essential nutrient in animal and human nutrition. The common source of nicotinamide in human and animal diets is the consumption of vegetables or plant matter. Nicotinamide was shown to cure the skin disease, pellagra[23] hence it is also known as vitamin PP (pellagra preventing). Nicotinamide is a component of vitamin premixes for many animal diets promoting health and growth of the animal. In the area of human nutrition it is used as an additive for refined flour, breakfast cereals and in multi-vitamin preparations.

There are several chemical processes for the production of nicotinamide.[24] One synthesis starts with the reaction of acetaldehyde, formaldehyde and ammonia to produce a mixture of 3-picoline and pyridine. The 3-picoline is converted to 3-cyanopyridine by ammoxidation. The 3-cyanopyridine is then converted to nicotinamide by alkaline hydrolysis. The disadvantages of this process are low yields and the production of a significant amount of nicotinic acid as a side-product which complicates the down-stream processing. Until 1998 Lonza also produced nicotinamide by chemical synthesis. The first step of the Lonza synthesis was the conversion of acetaldehyde and ammonia to 2-methyl-5-ethylpyridine (MEP). MEP was then oxidised with nitric acid to nicotinic acid. A batch amidation of the nicotinic acid and several batch crystallisations produced pure nicotinamide. This Lonza process was no

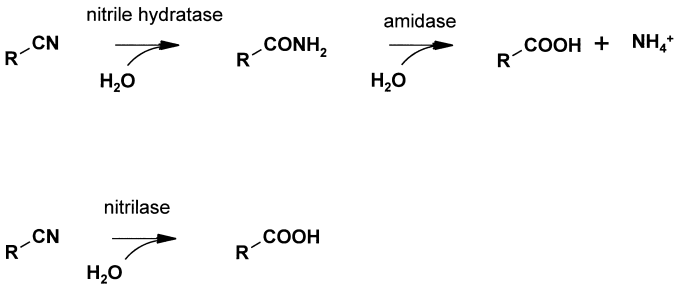

Figure 5. Nitrile- and amide-metabolising enzymes.

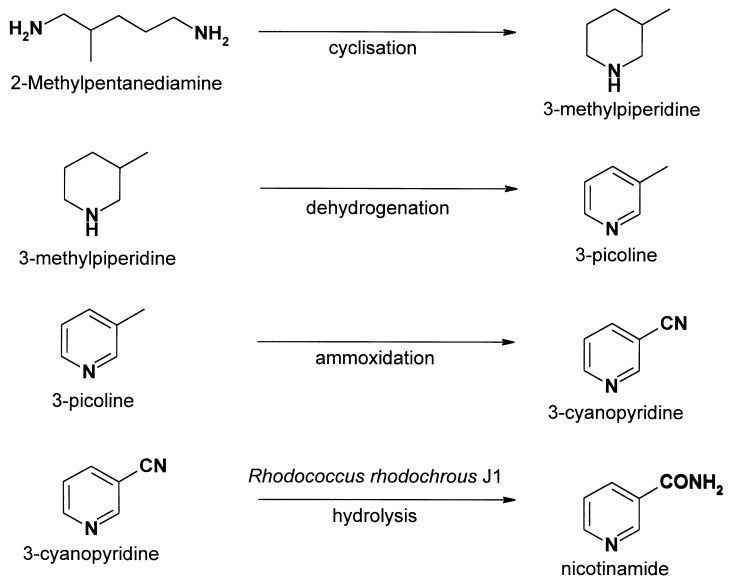

Figure 6. The new Lonza nicotinamide process.

longer competitive and full capacity had been reached. For these reasons Lonza set about developing a new process.

The new Lonza process consists of 4 highly selective, continuous, catalytical reactions (Figure 6). The first three steps are carried out using chemical catalysis. These steps are carried out at temperatures above 300 °C and in the gas phase. The last step, the biohydrolysis using a nitrile hydratase enzyme is the simplest step. It is highly selective (> 99%) and carried out under very mild aqueous conditions. In this step the 3-cyanopyridine is mixed with buffer and continuously dosed into the reactor containing *Rhodococcus rhodochrous* J1 cells immobilised in polyacrylamide. The crude solution of nicotinamide exiting the reactor is decolourised, the bioburden is removed by nanofiltration and the solution is concentrated. In the final step nicotinamide is isolated as a granular, free-flowing, non-caking, white solid. The advantages of this process are high yield, high energy efficiency, only one organic solvent is used and the process water, ammonium and hydrogen are recycled. The process is also environmentally friendly and safe.

The *Rhodococcus rhodochrous* J1 strain was isolated by H. Yamada and T. Nagasawa.[25] This strain was then immobilised in polyacrylamide by Nitto for commercial use. Lonza produces over 3,500 tonnes of nicotinamide per year with this biocatalyst.

4.2 (*S*)-Pipecolic Acid [(*S*)-Piperidine-2-carboxylic Acid]

(*S*)-Pipecolic acid is a building block for a number of pharmaceuticals, such as Incel from Vertex for the treatment of cancer multi-drug resistance[26] and the local anaesthetics Naropin (ropivacaine) from AstraZeneca[27] and Chirocaine (levobupivacaine) from Chiroscience.[28] The Lonza process efficiently combines chemistry and biotechnology to produce (*S*)-pipecolic acid with an ee value of > 99%.[29,30,31]

In the first step whole cells containing a nitrile hydratase convert 2-cyanopyridine to pyridine-2-carboxamide, which is then chemically hydrogenated to (*R*,*S*)-piperidine-2-carboxamide. This compound is the substrate for an amidase-catalysed enantiomer resolution with whole cells of *Pseudomonas fluorescens* DSM9924. The product, (*S*)-pipecolic acid, is isolated by precipitation at acid pH (Figure 7).

The production strain was selected by growth with the racemic substrate as the only source of nitrogen. Strains that hydrolysed about 50% of the substrate, as analysed by thin layer chromatography, were chosen for further investigation to determine the stereospecificity of the amidases.

The pharmaceutical intermediates (*S*)- and (*R*)-piperazine-2-carboxylic acid (Figure 8) are also produced by Lonza using similar processes.[29,30,31]

4.3 CBZ-D-proline [(*R*)-*N*-CBZ-proline]

A further example of a cyclic amino acid that is produced by Lonza for use as a pharmaceutical intermediate is CBZ-D-proline,[30,32] which is used for the synthesis of Eletriptan, a drug from Pfizer for the treatment of migraine.[33]. L-Proline is chemically racemised and derivatised to give racemic *N*-CBZ-proline, which is then stereospecifically hydrolysed by a proline acylase in a strain of *Arthrobacter* sp. (Figure 9). This

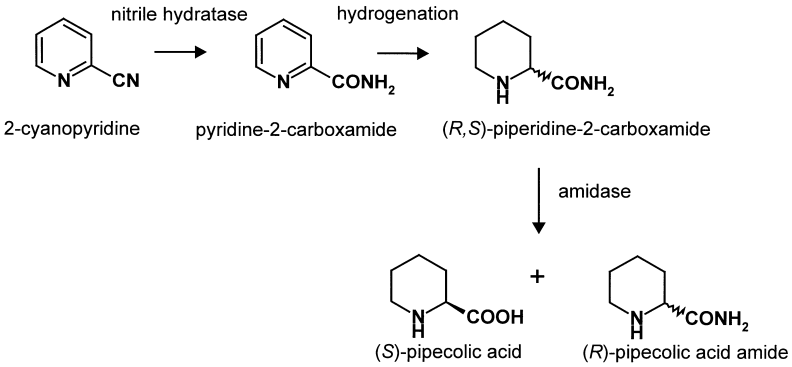

Figure 7. Process scheme for the production of (*S*)-pipecolic acid.

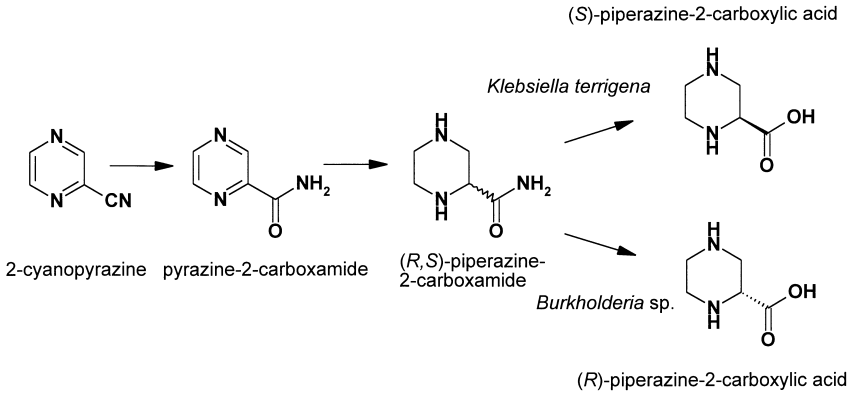

Figure 8. Process scheme for the production of (*S*)- and (*R*)-piperazine-2-carboxylic acid.

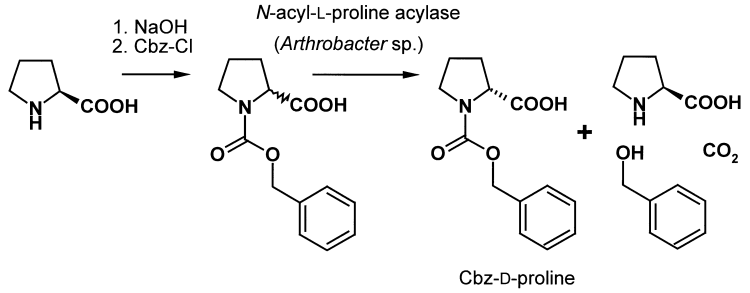

Figure 9. Process scheme for the production of CBZ-D-proline.

strain was newly isolated from soil samples using enrichment methods. (*R*)-*N*-CBZ-proline is obtained with a product concentration of up to 70 g/L, and with an ee value of $>99.5\%$. The process has been scaled up to produce 100 kg amounts. The chemistry for the preparation of the racemic *N*-CBZ-proline is carried out in water to avoid solvent changes, and the final isolation procedure is a simple extraction that yields (*R*)-*N*-CBZ-proline and an aqueous solution of (*S*)-proline (L-proline) that can be recycled as starting material.

4.4 (1*R*,4*S*)-1-Amino-4-hydroxymethylcyclopent-2-ene

(1*R*,4*S*)-1-Amino-4-hydroxymethylcyclopent-2-ene is an intermediate for the Glaxo anti-HIV drug Abacavir.[34] The racemic (*cis*) *N*-acetylamino alcohol was used as the substrate for the selection and screening of microorganisms that could release the acetyl group by hydrolysis and then grow with acetate as the carbon source. In this way a microorganism was isolated that contained a stereospecific amidohydrolase for the hydrolysis of the racemic *N*-acetylamino alcohol (Figure 10). The reaction yields the amino alcohol product [(1*R*,4*S*)-1-amino-4-hydroxymethylcyclopent-2-ene] and the non-hydrolysed (1*S*,4*R*)-*N*-acetylamino alcohol, which it was not possible to recycle.[35]

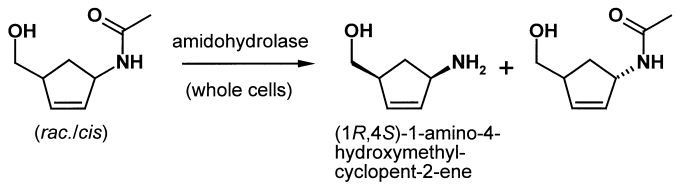

Figure 10. Process scheme for the production of (1*R*,4*S*)-1-amino-4-hydroxymethylcyclopent-2-ene.

Table 1. Cost comparison of stereospecific amidase production with a wild-type or a recombinant strain.

Strain	*C. acidovorans* A:18 (arbitrary units)	*E. coli* DH5/pCAR6 (arbitrary units)
Inducer	17	0
Medium components	7	2
Fermentation	34	3
Total cost	58	5

4.5 (*S*)-2,2-Dimethylcyclopropanecarboxamide

(*S*)-2,2-Dimethylcyclopropanecarboxamide is an intermediate for the production Cilastatin (Merck), which is a renal dehydropeptidase inhibitor that is administered with penem and carbapenem antibiotics to prevent their degradation in the kidney by this enzyme.[36] Chemical and biotechnological processes were developed in parallel, but the bioprocess was simpler, had less unit operations and resulted in higher quality product.

A microbial screening programme resulted in the isolation of several bacterial strains containing amidases that could specifically hydrolyse the (*R*)-amide. One of these strains, *Comomonas acidivorans* A:18 was particularly effective.[37]

The first step in the process is the hydrolysis of the racemic nitrile to the racemic amide (Figure 11) by nitrile hydratase-containing whole cells. This enzymic step resulted in several improvements to the process: (1) a 20% yield improvement compared to the chemical hydrolysis; (2) it reduced the number of unit operations for the synthesis of the starting product; (3) it was carried out by an in-house strain with very high productivity; (4) it made it possible to carry out a one-pot, two-step biotransformation; and (5) the (*R,S*)-amide formed by this biotransformation had better miscibility properties than the chemically synthesised material. For the second step the gene for the amidase was cloned into *Escherichia coli*, resulting in a number of improvements: (1) biomass production was faster than with the *Comamonas* strain; (2) only a minimal growth medium was required; (3) no amide was required in the growth medium to induce the amidase; and (4) the biotransformation was about 20 times faster. These improvements decreased production costs for the stereospecific amidase by about twelve times (Table 1).

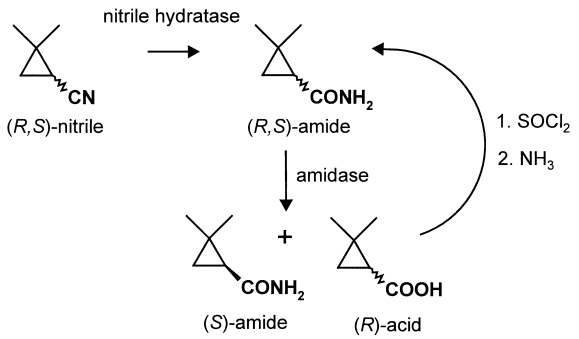

Figure 11. Process scheme for the production of (*S*)-2,2-dimethylcyclopropane carboxamide.

In the first step of the one-pot, two-step biotransformation the (*R,S*)-nitrile is rapidly and quantitatively hydrolysed to the (*R,S*)-amide. It is important that no trace of the racemic nitrile remains in the reactor after this step because the amidase is inhibited by this compound. The amidase-containing biomass is then added so that the (*R*)-amide is specifically hydrolysed to the (*R*)-acid, and the product, the (*S*)-amide accumulates. The down-stream processing steps are ultrafiltration, electrodialysis, ion-exchange chromatography, reverse osmosis, crystallisation, centrifugation and drying. The product has been produced at 15 m^3 scale. The yield was 35% and the product had an ee value of $> 98\%$. The (*R*)-acid can be recycled chemically into the substrate for the amidase which leads to higher yields and minimises waste.

4.6 (*S*)-3,3,3-Trifluoro-2-hydroxy-2-methylpropionic Acid

(*R*)- and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid are intermediates for the synthesis of a number of potential pharmaceuticals, which include ATP sensitive potassium channel openers for the treatment of incontinence,[38] and inhibitors of pyruvate dehydrogenase kinase for the treatment of diabetes.[39]

The aim was to develop efficient syntheses for these intermediates that yielded pure products with high ee values, and that were suitable for large-scale production. Several possible synthetic routes were evaluated. Some were purely chemical and others included biocatalytic steps.

Of the routes tested, the biocatalytic routes were the most promising. For example the resolution of (*R,S*)-ethyl 3,3,3-trifluoro-2-hydroxy-2-methylpropionate with the esterase from *Candida lipolytica* (Figure 12) resulted in the (*S*)-ester with an ee value of 99%.[40]

However, this route was not developed further because of the amount and resulting cost of the enzyme required to complete the reaction in a reasonable time.

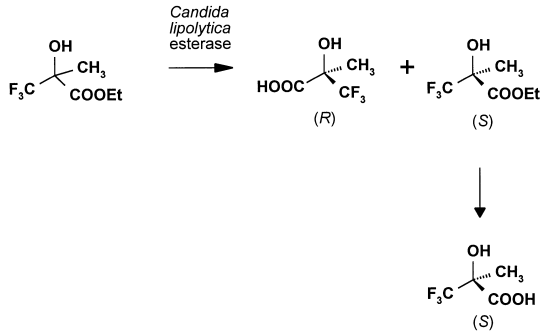

Figure 12. The resolution of (*R,S*)-ethyl 3,3,3-trifluoro-2-hydroxy-2-methylpropionate with the esterase from *Candida lipolytica.*

Other possible routes with one or more biocatalytic steps included those involving an enantioselective oxynitrilase reaction (Figure 13) and various routes starting from the racemic cyanohydrin (Figure 14). Screening for enantioselective oxynitrilases[41] and for enantiospecific nitrilases[42] was started, but discontinued when the amidase route (below) was found to be successful.

For the successful route an enantiospecific amidase from *Klebsiella oxytoca* was isolated, characterised and cloned and used to resolve (*R,S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide, giving (*R*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid and (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide (Figure 15). The (*S*)-amide could then be hydrolysed chemically to (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid.

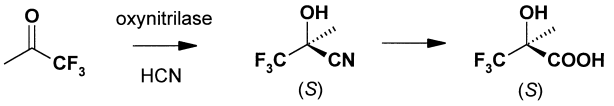

Figure 13. A possible route to 3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid using an oxynitrilase. According to the choice of enzyme, it should be possible to form either the (*R*)- or the (*S*)-enantiomer. The route to the (*S*)-cyanohydrin and (*S*)-acid is shown.

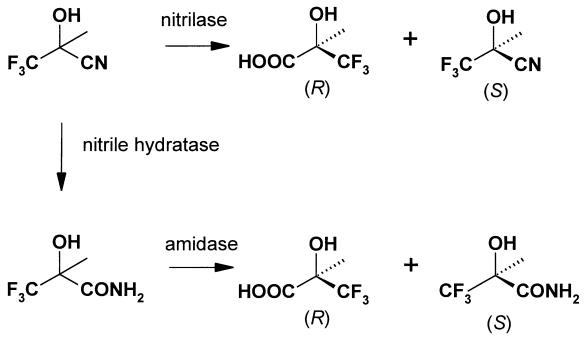

Figure 14. 3,3,3-Trifluoro-2-hydroxy-2-methylpropionic acid: possible routes starting from the racemic cyanohydrin. The routes to the (*R*)-acid are shown. The corresponding routes to the (*S*)-acid should also be possible.

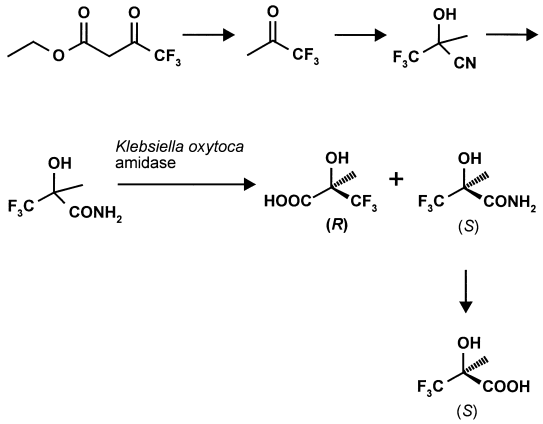

Figure 15. Process scheme for the production of (*R*)- or (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid.

The process can therefore be adapted to produce both *R*- and *S*-enantiomers of 3,3,3-trifluoro-2-hydroxy-2-methylpropionic acid, or (*S*)-3,3,3-trifluoro-2-hydroxy-2-methylpropionamide. The biocatalytic step is part of a combined chemical and biocatalytic route that starts from the Lonza product ethyl 4,4,4-trifluoroacetoacetate, so again backward integration of the process is optimal. The products typically have a purity of greater than 98% and ee values of essentially 100% after isolation. The process has been used to produce 100 g amounts of the (*S*)-acid, and has been successfully scaled up to produce 100 kg amounts of the (*R*)-acid, with the biotransformation carried out at the 1500 L scale.[43,44]

Cloning of the amidase gene into *Escherichia coli* was carried out for a number of reasons: (1) to improve safety; *Klebsiella oxytoca* is a risk class 2 microorganism. Transfer of the amidase gene to a GRAS host such as an *Escherichia coli* K12 derivative facilitated handling and official registration procedures for the production process; (2) the productivity of the biotransformation was improved by cloning the amidase gene under the control of a strong promoter to improve its expression; (3) to avoid the slime-capsule problem encountered when using *Klebsiella oxytoca* as the production strain; (4) to have the possibility to use other microorganisms with, for example, higher substrate or product tolerances, as hosts for the cloned gene.

The amidase from *Klebsiella oxytoca* is robust, stable, and does not require cofactors. It can be heated to 70 °C for 10 min without loss of activity, and therefore, heat treatment of the biomass used in the biotransformation was used to stabilise the enzyme, presumably by inactivating proteases.

For the whole process attention was paid to safety, efficiency and economics. The biotransformation was carried out in aqueous solution (which is often the case with biotransformations). Large volumes of aqueous solution containing relatively low product concentrations are difficult to handle in chemical plants (com-

pared with "normal" processes in organic solutions), so particular attention was paid to the delivery of a biotransformation product solution suitable for downstream processing. It was subjected to ultra-filtration through a 70 kDa membrane to remove proteins that could cause foaming during extraction, and contamination of the product. Concentration by thin-film evaporation then removed as much water as possible before transfer to the chemical plant for product isolation.

5 Multi-Enzyme Biotransformations

5.1 L-Alaninol

L-Alaninol has potential as an intermediate for both pharmaceuticals, such as the quinolone antibacterial Levofloxacin from Daiichi Pharmaceuticals,[45] and agrochemicals, such as the selective herbicide Metolachlor from Novartis.[46] This biotransformation was studied in collaboration with Prof. Thomas Leisinger's group at the ETH in Zürich.[47] Biocatalysis is carried out with whole cells of *Pseudomonas* sp., in which L-alaninol is produced as an intermediate of isopropylamine catabolism. The enzymes involved are a permease, which is involved in transport of the starting material isopropylamine into the cell, a synthetase, a P450 oxygenase and a hydrolase (Figure 16). This product is currently in development.[48]

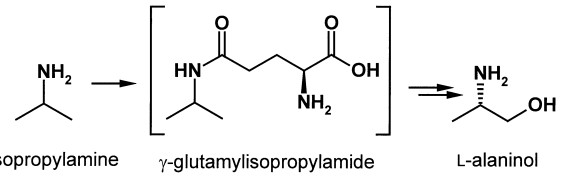

Figure 16. Asymmetric oxidation. Simplified pathway for L-alaninol production by *Pseudomonas* sp.

5.2 L-Carnitine

L-Carnitine or vitamin B_T is an essential growth factor for the meal worm, *Tenebria molitor*. Part of the daily requirements of L-carnitine in humans is met by endogenous synthesis in the liver from lysine and methionine. L-Carnitine functions in the transport of activated fatty acids across the inner mitochondrial membrane. It has pharmaceutical, food and feed applications. Lonza produces L-carnitine in a whole-cell biocatalytic process that utilises a naturally occurring pathway of 4 enzymes (Figure 17). In this process 4-butyrobetaine is converted to L-carnitine, which is excreted into the medium.[13] The biotransformation has a high energy requirement due to the necessity of cofactor regeneration and the transport of the 4-butyrobetaine and L-carnitine across the membrane. For this reason the process must be carried out with growing cells or cells that are in a maintenance state, which has the advantage of low biomass production and high metabolic activity. The pathway is analogous to the β-oxidation of fatty acids and occurs in a new genus of microorganism that is related to *Agrobacterium* and *Rhizobium*.[49] The introduction of the chiral centre is performed by crotonobetainyl-CoA-hydrolase, which belongs to the lyase class of enzymes. The natural degradation of L-carnitine by carnitine dehydrogenase is prevented by a classical mutation in the production strain. L-Carnitine is produced at the 50 m^3 scale with a volumetric yield of greater than 80 g/L, a conversion of substrate to product of 99.5%, and an ee value of 100%.

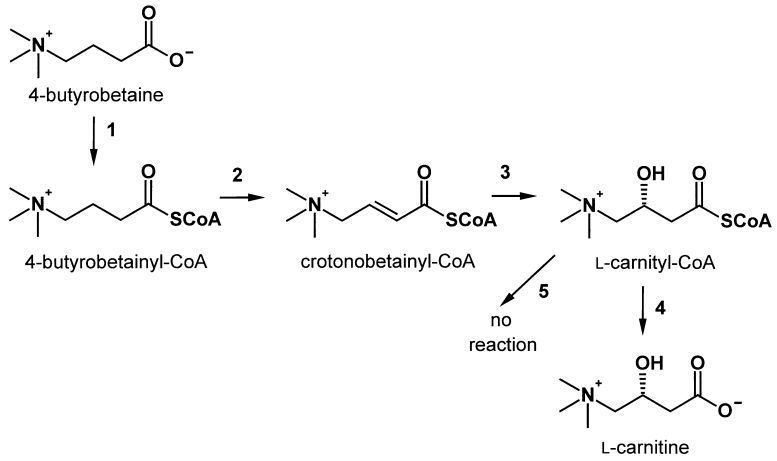

Figure 17. The pathway for L-carnitine production in *Agrobacterium/Rhizobium* HK13. **1** = 4-butyrobetainyl-CoA synthetase, **2** = 4-butyrobetainyl-CoA dehydrogenase, **3** = crotonobetainyl-CoA hydrolase, **4** = thioesterase (?), **5** = carnitine dehydrogenase.

Other possibilities for the biotechnological production of L-carnitine involve either the production from achiral precursors or the resolution of racemic carnitine and its derivatives.[50,51,52] These alternate syntheses have the disadvantages of cofactor requirements, unstable starting product, low productivity, maximum yields of 50%, product ee values of less than 100% or complex down-stream processing steps.

L-Carnitine can also be produced chemically[53,54] starting from epichlorhydrin or diketene. The last step is the racemic resolution of D,L-carnitine using a resolving agent. The yields are low and to reduce costs it is necessary to recover and recycle the resolving agent. A comparison of the waste stream from the bioprocess with that from the Lonza chemical synthesis[55] showed that the biotechnological process was also much more environmentally friendly. The amounts of waste water, total organic carbon, salts, and waste for incineration were all considerably lower for the bioprocess.[56]

6 Conclusion

There are many reasons for the success Lonza has had in the area of biotransformations. In 1983 the emphasis was to create a strong in-house research and development group with specialists in the area of microbiology, biochemistry, molecular biology and fermentation. The fledgling group profited from Lonza's existing infrastructure, especially the analytical capabilities and the synergy with the chemistry research and development groups. Today the pharmaceutical industry is under a lot of price and time pressure due to a number of reasons, for example, patent expiry and a 20 year low in the number of new molecular entities launched onto the market.[57] This has led to a general decrease in the number of products in the development pipelines but it has made the implementation of biosolutions to difficult synthetic problems all the more important. This environment has forced a change in our biotechnological activities. We have diversified so that we can now offer a complete custom manufacturing service in biocatalysis, microbial fermentation and production of biopharmaceuticals with animal and microbial cells. The biotransformation activities are moving to faster and more efficient means of screening for the required enzymic activity. This means implementing higher throughput screening methods, capitalising on our now extensive strain collection, networking and entering into selective collaborations with universities and companies. These collaborations either complement our research efforts, fill deficits in our technology, by-pass patent or licensing problems or gain us access to other extensive strain or enzyme collections.

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