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"Giulio Natta"

INVESTIGATION OF REDOX REACTIONS BY WHOLE CELL MICROORGANISMS

Relatore: Prof.ssa Maria Elisabetta Brenna

Tesi di Laurea di:

Flavia CANNAVALE

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Contents

Abstract

Redox reactions are central transformations in organic chemistry. Starting off with stoichiometric toxic reagents, it is coming out a new age of catalytic procedures which use environmentally benign oxidants and reducing agents. Biotransformations using microorganisms are a useful and complementary tool for preparative organic chemistry. In the course of a biotransformation the chemo-, regio- and stereoselective transformation of a substrate often takes place under mild and ecologically-compatible conditions. Furthermore for numerous asymmetric reactions starting from prochiral compounds the desired products are formed in excellent enantiomeric excess of >99%. This is the reason why biocatalysed processes are particularly useful for the synthesis of the single enantiomers of chiral molecules and represent attractive alternatives to "traditional" chemical procedures.

Today it is possible to perform organic synthetic processes with enzymes as catalysts. Enzymes are more often specific for selected reactions and their use may require only small-sized equipment and simple workup, but they are generally more expensive than a chemocatalist, and addition or recycling of enzyme cofactors might be necessary. Whole cells instead contain a range of enzymes which are able to accept a wider selection of non-natural substrates and possess all the required cofactors and cofactor-recycling pathways within their natural cellular environment.

In this search for alternatives to the classical chemical routes, we try to develop a biocatalytic method both for primary alcohols/meso diols oxidation and esters reduction which hold a great potential with respect to environmental compatibility and catalytic efficiency.

In Chapter 3 all biotranformations employ whole cells of *Acetobacter aceti*. Some strains of acetic acid bacteria can been employed for their capability to oxidize primary alcohols to the corresponding carboxylic acids. These observations, accompanied by the demand for greener, more atom efficient oxidation methods, led us to investigate the synthetic significance of the kinetic resolution of racemic primary alcohols to the corresponding acids, and the desymmetrisation of meso diols to the corresponding hydroxymethyl acids. We have finally found an economic biooxidation process to obtain chiral building blocks and to simplify multi-step synthetic routes with a single enzyme-catalysed transformation.

In Chapter 4 we report the first results of the bioreduction of carboxylic acids and esters conducted in a very mild, efficient and chemoselective way by using whole cells of a zygomycete fungus from Mycotheca Universitatis Taurinensis. The results show a particular carboxylic acid reductase behavior which could be widely used in the chemical industry.

Estratto

Le reazioni di ossidoriduzione sono trasformazioni di centrale importanza in chimica organica. Anziché utilizzare reagenti tossici in quantità stechiometrica, si sta sviluppando una nuova era di processi catalitici che usano ossidanti e riducenti "ecocompatibili". A tal proposito, le biotrasformazioni con microrganismi rappresentano una risorsa utile e uno strumento complementare per la chimica organica preparativa. Nel corso di un bioprocesso, la trasformazione del substrato in termini di chemo-, regio- e stereoselettività, avviene in condizioni blande ed ecologicamente compatibili. Inoltre, in numerose reazioni asimmetriche, partendo da composti prochirali si ottengono i prodotti desiderati con elevati eccessi enantiomerici >99%. È questa la ragione per cui, ad oggi, sempre più processi biocatalitici sono utilizzati per la sintesi di molecole chirali e rappresentano una valida alternativa ai processi delle chimica tradizionale.

Oggi è possibile condurre processi di sintesi organica utilizzando enzimi come catalizzatori. Gli enzimi sono molto specifici e il loro utilizzo richiede apparecchiature di piccole dimensioni e un workup semplice. Da un altro punto di vista sono però più costosi di un catalizzatore chimico, e richiedono l'aggiunta e/o il riciclo di cofattori. Gli enzimi possono essere utilizzati sia in forma isolata che come cellule intere. Queste ultime hanno il vantaggio di contenere già all'interno del loro ambiente cellulare naturale una serie di enzimi e cofattori in grado di accettare una selezione più ampia di substrati.

In questa ricerca di alternative ai percorsi chimici classici, abbiamo sviluppato due processi biocatalitici: un'ossidazione di alcoli primari e meso dioli, e una riduzione di acidi carbossilici ed esteri. Entrambe le vie sintetiche possiedono un grande potenziale per quanto riguarda compatibilità ambientale ed efficienza catalitica.

Nel Capitolo 3 tutte le biotrasformazioni impiegano cellule intere di *Acetobacter aceti*. Alcuni ceppi di batteri acetici, infatti, possono essere impiegati per la loro capacità di ossidare alcoli primari ai corrispondenti acidi carbossilici. Tale osservazione, accompagnata dalla crescente domanda di metodi di ossidazione più "verdi", ci ha portato a indagare il significato sintetico della risoluzione cinetica di alcoli primari racemi ai corrispondenti acidi, e la dissimmetrizzazione di meso dioli ai corrispondenti acidi idrossimetilici. Abbiamo quindi sviluppato un processo di biossidazione economico per ottenere composti chirali in grado di condensare le tradizionali vie multi-step in una singola trasformazione catalizzata da enzimi.

Nel Capitolo 4 si riportano i primi risultati di bioriduzioni di esteri carbossilici condotti in modo molto ecologico, efficiente e chemoselettivo usando cellule intere di uno zigomicete dalla Mycotheca

Universitatis Taurinensis. I risultati mostrano un particolare comportamento di tipo carbossile reduttasi che può rappresentare una grande innovazione nell'industria chimica.

CHAPTER 1

1.1 White Biotechnology

In recent years biology wants to take revenge on synthetic, petroleum-based consumer goods. Stricter environmental regulations and the growing mass of non-degradable synthetics in landfills have made biodegradable products appealing again[1].

Growing concerns about the dependence on imported oil, particularly in the USA, and the awareness that the world's oil supplies are not limitless are additional factors prompting the chemical and biotechnology industries to explore nature's richness in search of methods to replace petroleum-based synthetics.

An entire branch of biotechnology, known as "white biotechnology", is devoted to this. It uses living cells - from yeast, moulds, **bacteria** and plants - and enzymes to synthesize products that are easily degradable according to synthetic procedures that require less energy and create less waste during their production.

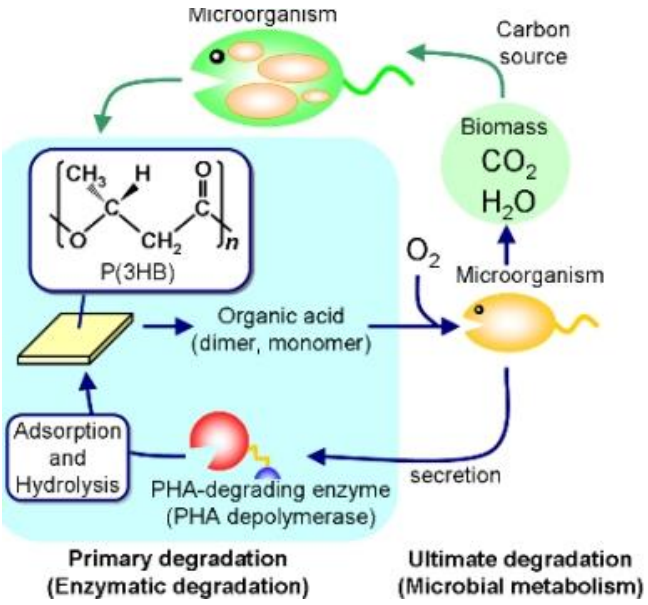
This is not a recent development: in fact, biotechnology has been contributing to industrial processes for some time. For decades, bacterial enzymes have been used widely in food manufacturing (e.g. yeast fermentation of sugar to produce the carbon dioxide that raises the dough) and as active ingredients in washing powders to reduce the amount of artificial surfactants.

The benefits of exploiting natural processes and products are manifold: they do not rely on fossil resources, are more energy efficient and their substrates and waste are biologically degradable, which all helps to decrease their environmental impact.

White biotechnology is already bringing many innovations to the chemical, textile, food, packaging and health care industries. It is no surprise then that academics, industry and policy makers are increasingly interested in this new technology, its economy and its contributions for a sustainable development.

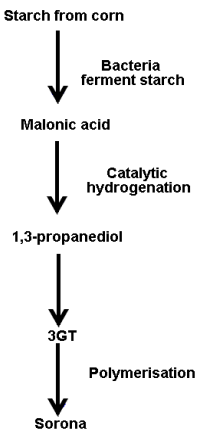
One of the first goals of white biotechnology has been the production of **biodegradable plastics**. Polymers are traditionally produced from limited fossil resources such as oil and natural gas[2]. Bio-based polymers use renewable resources like sugars or corn as raw material.

Over the past 20 years, these efforts have concentrated mainly on polyesters of 3-hydroxyacids (PHAs), which are naturally synthesized by bacterial fermentation of sugar or lipids as an energy reserve and carbon source[3]. These compounds have properties similar to synthetic thermoplastics and elastomers, but are completely and rapidly degraded by bacteria in soil or water. The most abundant PHA is poly-R-(3-hydroxybutyrate) (PHB), which bacteria synthesize from acetyl-CoA. Growing on glucose, the bacterium *Ralstonia eutropha* can amass up to 85% of its dry weight in PHB, which makes this microorganism a sort of "biopolymer".



A major limitation of the commercialization of such bacterial plastics has always been their cost, as they are 5-10 times more expensive to produce than petroleum-based polymers.

Besides this new wave of polymers has enormous potential but the timing of its evolution is uncertain. All this translates into heavy investments in new infrastructures and processing systems and into a considerable amount of time.



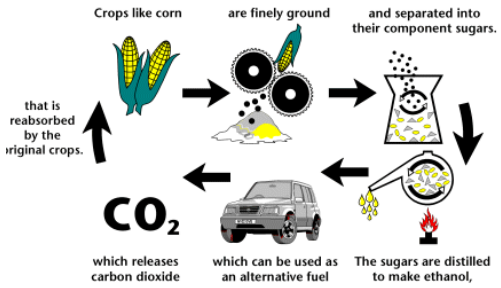
DuPont has for many years been developing a polymer based on 1,3-propanediol (PDO), Sorona®, with new levels of performance, resilience and softness. It was decided to use a bio-route to the alcohol that is thereafter polymerized with terephthalic acid. Conventional polyester is made using 1,2 propane diol as the alcohol, and this is easily obtained from propene by oxidation, followed by hydrolysis of the oxide. But this chemical route is too expensive and requires the use of harsh chemicals that present significant health hazards. Now DuPont uses a fermentation process to manufacture 1,3 propane diol from corn sugar.

Cargill Dow has developed an innovative biopolymer, NatureWorks®, which can be used to manufacture items such as clothing, packaging and office furnishings. The polymer is derived from lactic acid, which is obtained from the fermentation of corn sugar, and requires 25 to 55 % less fossil resources.

Another sector that could benefit greatly from innovative biotechnology is **paper industry**. Much of the cost and considerable pollution involved in the paper-making process is caused by "krafting", a method for removing lignin from the wood substrate to obtain almost pure cellulose fibers. It requires treatment of wood chips with a mixture of sodium hydroxide and sodium sulfide that breaks the bonds that link lignin to the cellulose. On the contrary, the bio-treatment is based on the use of lignin-degrading enzymes which remove lignin to soften paper.

The company Novozymes provides enzymes for the scouring process in the **textile industry**. Scouring is the removal of the brown, non-cellulose parts of cotton, which in the traditional process involves the use of relatively harsh chemical solution. The application of enzymes in this sector can lead to fewer emissions into the water and reduced primary energy demand at 20 % lower cost.

White biotechnology also concentrates on the production of **energy** from renewable resources and biomasses. Starch from corn, potatoes, sugar cane and wheat is already used to produce ethanol as a substitute for gasoline. Today, some motor fuel sold in Brazil is pure ethanol derived from sugar cane, and the rest has a 20% ethanol content. In the USA, 10% of all motor fuel sold is a mixture of 90% petrol and 10% ethanol.

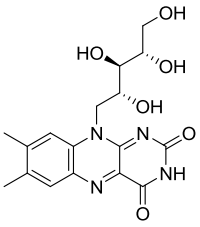


But turning starch into ethanol is neither the most environmentally nor economically efficient method, as growing plants for ethanol production involves the use of herbicides, pesticides, fertilizers, irrigation and machinery.

Companies such as Novozymes, Genencor and Maxygen are therefore exploring avenues to derive ethanol specifically from celluloid material in wood, grasses and, more attractively, agricultural waste. Much of their effort is concentrated on developing more effective bacterial cellulases - produced by fungi, bacteria, and protozoans - that can break down agricultural waste into simple sugars in order to create a more plentiful and cheaper raw substrate for the production of ethanol. Novozymes Cellic® CTec3 is a state-of-the-art cellulase and hemicellulase complex that allows for the most cost-efficient conversion of pretreated lignocellulosic materials to fermentable sugars for cellulosic ethanol production.

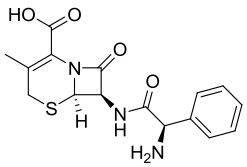
Making biomass an effective feedstock is not a cheap process: to get the production of biofuel up and running on a commercial basis, a different pricing of biofuel will be required. The price structure for fossil fuel is fixed in the market by regulatory frameworks. If the biofuel production is to be successful, it will be necessary to enforce policies that introduce subsidies to bioethanol production, for instance, or put taxes on fossil fuel production.

White biotechnology may also benefit **medicine**. For example, vitamin B2 (riboflavin) is widely used in animal feed, human food and cosmetics and has traditionally been manufactured in a six-step chemical process. BASF's new biotech process reduces production to one-step process. This single step is fermentation whereby the raw material is fed to a mould (*Ashbya gossypii)* that transforms it into the finished product.



riboflavin

Similarly, cephalexin, an antibiotic that is active against Gram-negative bacteria and is normally produced in a lengthy ten-step chemical synthesis, is now produced in a shorter fermentation-based process at DSM. However other vitamins and drugs are still cheaper to produce with classic organic chemistry than by innovative white biotechnology.



cephalexin

Nevertheless, the potential environmental benefits of shifting to biofeedstocks and bioprocesses are substantial. The new bioprocesses will substitute complex chemistry reactions. This corresponds to significant energy and water savings and it also benefits the atmosphere. The carbon needed to make bioethanol from biomass was sequestered by plants from the atmosphere, so putting it back by burning ethanol does not add to global warming.

White biotechnology has potentially large benefits, both economically and environmentally, for a wide range of applications. The way for its development is being paved, but it remains a relatively young technology that has to compete with a mature oil-based chemical industry that has had nearly a century to optimize its methods and production processes. Nevertheless, the growing concerns about the environment and the possibility of cheaper oil in the future make white biotechnology a serious contender.

1.2 Differences in US and EU approaches[4]

Huge differences exist in the ways white biotechnology is managed in Europe and the USA. First of all, the overall sum invested in the US in the white biotechnology business is $250 million, a sum which by far exceeds the total European investment.

Probably driven by a stronger geopolitical will of becoming independent from fossil fuel import, the US has shown a clearer propensity in the development of such technologies. Europe, on the other hand, is culturally more cautious and less adventurous in accepting innovative methodologies.

By taking advantage of technological breakthroughs in biotechnology (Figure1), governments, industries and nongovernmental organizations (NGOs) are developing strategies and action plans to prevent social and economic crises.

Metagenomics - techniques to genetically exploit the previously inaccessible majority of non-cultivable microorganisms and their complement of genes, enzymes and bioactive molecules[5].
In Vitro Evolution - test-tube-format technologies to randomly mutate and functionally select genes and their encoded enzymes in an iterative manner, to improve properties and performance[6].
Functional genomics, expression and flux analyses - high-throughput sequencing, mapping, manipulation and monitoring of genomes and genes in a functional context, in combination with *in situ* analysis of metabolites, enabling "metabolic engineering" and the creation of designer bugs as microbial factories[7].
Biocatalysis, process design - innovative methods of *in situ* cofactor regeneration[8] and whole-cell biotransformation of toxic or water-insoluble substrates[9].

Figure1 Technological breakthroughs facilitating white biotechnology.

The steps necessary for a biobased revolution are problematic and require immediate and coordinated strategies and commitments through legislation, policy, education and research. Importantly, the EU and the USA view the subject from different angles and the outcomes might differ as a consequence (Figure2).

US
1. Centrally coordinated **top-down approach** (initiated by government and/or administration): bio-based economy declared national security issue (potential-focused view).
2. Multi-agency effort (US Department of Energy, US Department of Agriculture) involving academia and industry.
3. Guiding national procurement policy ("buy bio") and massive public research funding.
4. **Agro-based** and **energy-focused approach**. Vast productive agricultural hinterland and nationally accepted GM plant technologies available.
5. Early start with a potential to outcompete foreign economies in IP on key technologies.
6. Milestone achievements and bulk products already launched: massive engagement in bio-ethanol technology based on starch and cellulose; bio-polymer technical platforms launched.
EU
1. **Bottom-up approach** (initiated by chemical industry and industrial stakeholders) driven by concerns on global competitiveness.
2. Comparatively larger impact of environmental protection issues (e.g. Kyoto Protocol compliance) and consumer safety concerns: strong environmentalist movements and little societal acceptance of GMO release (risk-focused view).
3. Vast agricultural hinterlands, especially in new eastern member states and idle fertile lands provide potential to grow renewable biomass.
4. Highly regulated agricultural sector in the EC domestic market: high-price policy with no access to cheap world market sugar.
5. Centralized (EU) efforts in progress to coordinate a fragmented white biotechnology scene with parallel national programs and competencies.
6. **Strong chemical industry** and large academic and industrial expertise in biocatalysis, fine chemistry, fermentation (DSM, Degussa, BASF) and enzyme technologies (Novozymes, Danisco-Genencor).

Figure2 Industrial biotechnology in the USA versus the EU.

Unsurprisingly, US and EU attitudes appear to differ significantly on several aspects.

The US initiatives are focused on biomass-based energy supply and the production of bulk products. Individual mobility is largely dependent on imported fossil fuels making the manufacture of domestic biofuels a strategic imperative. The presence of a large productive agricultural sector and experience in the large-scale deployment of GMO crops (such as soy or maize) will provide ample biomass as feedstock for the industry. Furthermore, central administration enthusiasm and the injection of massive amounts of research funding give the US a considerable advantage.

The EU initiatives, however, started primarily by globally acting industry leaders, such as DSM, Degussa or BASF and a few active national NGOs; although with the support of the EU commission, a pan-European consortium of stakeholders is currently developing. The initial focus of the EU is the manufacture of novel, high-margin products and a reduction in environmental footprints, while individual mobility is addressed as a lower priority.

The main goal is the optimization of biocatalysed manufacturing processes which could represent valid alternatives to the existing procedures based on classical organic synthesis. Research is mainly devoted to find new enzymes or evolving the existing ones to develop sustainable variants of most organic reactions.

The future of white biotechnology will depend on commercial success (i.e. competitiveness and market penetration of its products) and this, particularly in the case of energy and bulk chemicals, largely depends on fossil oil and gas prices, which are regulated by global supply and demand.

CHAPTER 2

2.1 Enzyme Catalysis[10]

Enzymes are large biological molecules responsible for the thousands of chemical processes that sustain life. They are highly selective catalysts and work by lowering the activation energy for a reaction, increasing the rate of the reaction. As a result, products are formed faster and reactions reach their equilibrium state more rapidly.

Enzyme catalysis in organic synthesis is used in the preparation of a wide range of chemical compounds. Notably, this is true not only for academic syntheses but also for industrial-scale applications[11]. For numerous molecules the synthetic routes based on enzyme catalysis have turned out to be competitive and often superior compared with classic chemical as well as chemocatalytic synthetic approaches. Thus, enzymatic catalysis is increasingly recognized by organic chemists in both academia and industry as an attractive synthetic tool besides the traditional organic disciplines such as “classic” synthesis, metal catalysis, and organocatalysis.

By means of enzymes a broad range of transformations relevant in organic chemistry can be catalyzed, including, for example, redox reactions, carbon-carbon bond forming reactions, and hydrolytic reactions. Nonetheless, for a long time enzyme catalysis was not realized as a first choice option in organic synthesis. In fact organic chemists did not use enzymes as catalysts because of observed disadvantages such as restrict substrate range, limited stability of enzymes under organic reaction conditions, low efficiency when using wild-type strains, and diluted substrate and product solutions, thus leading to non-satisfactory volumetric productivities. However, due to tremendous progress in enzyme discovery, enzyme engineering, and process engineering, in recent years numerous examples of organic syntheses with enzymes have been developed that avoid these disadvantages.

A large number of enzymes show excellent performance and today are typically prepared in a highly attractive economic manner by fermentation for example. This economically attractive access to highly efficient bio-catalysts enables an excellent opportunity to realize the development of organic synthetic processes with enzymes as catalysts.

Organic chemists starts to apply these biocatalysts very successfully in a broad range of organic syntheses and many of those synthetic examples have been found to be suitable even for industrial-scale productions instead of “classic” chemical or chemocatalytic approaches.

2.2 Biocatalysts General Properties

The unique functions of enzymes as catalysts are a result of their complex three-dimensional structures and the active site integrated therein[12]. This enables a highly specific recognition of specific substrates, leading to excellent selectivities.

The stereoselectivity of enzymes is in general high to excellent and, furthermore, this is typically true for regio-, diastereo-, as well as enantioselectivity. The unique properties of enzymes to stereoselectively recognize a substrate is the key factor of their practical use.

Also reaction conditions play a very important role in enzyme catalysis. It is difficult to define properties under which in general enzymes are able to operate as a catalyst. At the same time, however, it is evident that enzyme catalysis requires specific suitable reaction conditions such as pH, temperature, and solvent, which have to be considered in bioprocess development. For enzymes, pH and temperature are certainly highly important reaction parameters in terms of both activity and stability. Typically, enzymes operate in a more or less neutral or weakly basic/acidic pH range, usually between pH 5 and 10, although exceptions are known. The natural reaction environment for enzymes is water but is not necessarily required and many enzymatic transformations (including industrial processes) are run in organic reaction medium.

2.3 Enzyme Classes

Enzymes are typically classified according to the types of reactions they catalyze. In the Enzyme Nomenclature Classification[13] they are subdivided and categorized into six main enzyme classes corresponding to the type of reactions such enzymes catalyze.

Figure3 gives an overview of this categorization, in particular the main enzyme classes.

Enzyme class	EC	Selected reactions
Oxidoreductases	1	Reduction of C=O and C=C; reductive amination of C=O; oxidation of C-H, C=C, C-N, and C-O; cofactor reduction/oxidation
Transferases	2	Transfer of functional groups such as amino, acyl, phosphoryl, methyl, glycosyl, nitro, and sulfur-containing groups
Hydrolases	3	Hydrolysis of esters, amides, lactones, lactams, epoxides, nitriles, and so on, as well as the reverse reactions to form such functionalities
Lyases (synthases)	4	Addition of small molecules to double bonds such as C=C, C=N, and C=O
Isomerases	5	Transformation of isomers (isomerizations) such as racemizations, epimerizations, and rearrangement reactions
Ligases (synthetases)	6	Formation of complex compounds (in analogy to lyases) but enzymatically active only when combined with ATP cleavage

Figure3 Enzyme Nomenclature Classification.

With respect to applications of enzymes in organic synthesis, enzymes from nearly all enzyme classes play an important synthetic role in organic chemistry. As an exception, at least in part, one might

regard enzymes from enzyme class 6 (ligases). Since *in situ* regeneration of the cofactor ATP is still a challenge, ligases have found limited use as catalysts for in vitro applications in organic syntheses. In contrast, enzymes from enzyme classes EC 1–5 turned out to be highly efficient catalysts for a broad range of organic synthetic transformations that, in part, are also suitable for technical-scale applications.

With oxidoreductases (EC 1) many successful reduction and oxidation processes have been realized. Figure4 summarizes selected oxidoreductase-catalyzed reaction types that have gained broad interest in organic chemistry.

With respect to (asymmetric) reductions as a synthetically important reaction in organic chemistry, the reduction of a carbonyl moiety to an alcohol (when using, for example, alcohol dehydrogenases or α-hydroxy acid dehydrogenases as catalysts) or amino functionality (when using α-amino acid dehydrogenases in reductive aminations) has already found a wide application range in organic chemistry as well as industrial applications.

A more recent trend is the increasing tendency to apply enzymes also for C=C double bond reductions with the so-called “old yellow enzymes”.

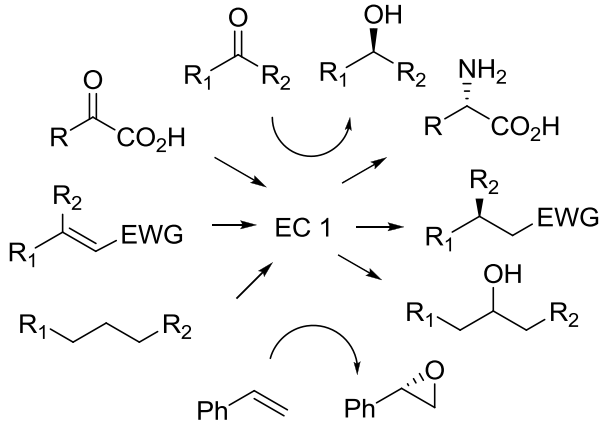


Figure4 Selected reactions catalyzed by enzymes from EC 1

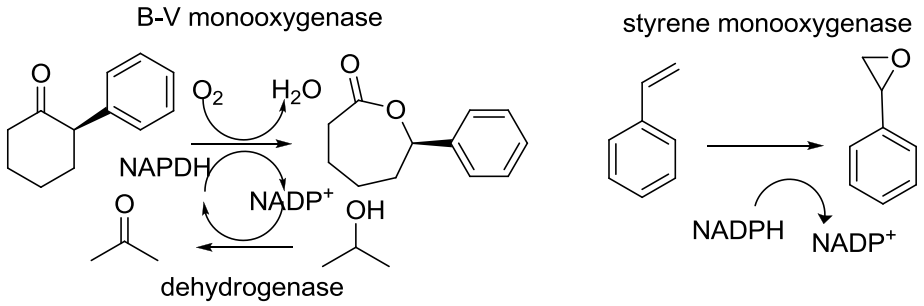
A further recent and current “hot topic” is the field of oxidation reactions using suitable oxidoreductases. Key advantages of using enzymes as catalysts in redox processes are:

- the excellent selectivity even when using nonfunctionalized compounds;
- the use of molecular oxygen as a cheap and sustainable oxidizing agent.

There is a high demand for this reaction type, also from an industrial perspective. Given the excellent stereoselectivity of enzymes, it is no surprise that today hydroxylation of steroids is industrially carried out by means of biocatalytic hydroxylation instead of chemical methods[14].

As well as hydroxylation other oxidative processes with enzymes are also of interest in organic syntheses, such as, for example, reactions with Baeyer-Villiger monooxygenases (for Baeyer–Villiger

oxidations leading to lactones from ketones) and styrene monooxygenases (for epoxidation of styrenes).



Accordingly to the overview of Liese and coworkers[15] bioredox catalysis accounts for one third of all commercialized enzymatic processes. From these, about half comprise oxidation process. Interestingly, simpler alcohol and amine oxidations are only a minor fraction (22%) compared to enantiospecific oxyfunctionalisations such as hydroxylations, dihydroxylations, epoxidations and Bayer-Villiger oxidations (Figure5).

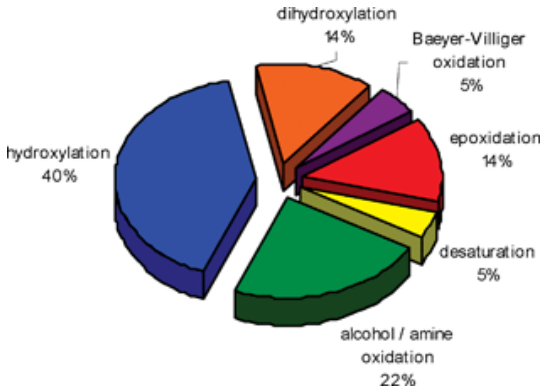


Figure5 Distribution of reaction types amongst the industrial biocatalytic oxidation reactions.

One explanation of this discrepancy might be due to the existing optimized toolbox for the oxidation of alcohols which makes the transition to biocatalysis less attractive. Another interesting feature that becomes clear from the review is that all industrial oxidation processes are based on whole, metabolic active cells because of the higher price and the required regeneration approach of isolated enzymes.

2.4 Cofactors

Cofactors are non-proteinogenic compounds that are required for the catalytic activity of enzymes and which can bind to the enzyme either in a covalent or non-covalent mode[16]. A broad variety of cofactors are known (e.g. ATP, FAD, NADH, NADPH etc.), consisting of organic molecules and inorganic ions. In the covalent mode, when the cofactor is permanently bound to the enzyme, the cofactor is called a prosthetic group. In case of a non-covalent binding of the cofactor to the enzyme it is called a coenzyme. Since the coenzyme is modified during the catalytic process (by transferring

electrons or chemical groups to the substrate), its regeneration in a subsequent reaction is a key issue in order to use the cofactor in catalytic amounts. Thus, the cosubstrate required for the cofactor's regeneration is needed in stoichiometric amounts.

With exception of hydrolases (EC 3), members of all other enzyme classes show a cofactor dependency, although in some cases cofactors are not necessarily involved in the catalytic process. For most enzymes belonging to enzyme classes EC 1-5, however, cofactors are involved in the catalytic process. Furthermore, the availability of an opportunity to regenerate such cofactors efficiently under the chosen organic reaction conditions often decides whether this method can be developed towards an attractive synthetic process.

In the following this shall be exemplified for the regeneration of a cofactor in its reduced and oxidized form, namely, NADPH and $NADP^+$, which are used in enzymatic redox processes. Since the reducing agent for the oxidoreductase in a reduction process is NADPH, in total a stoichiometric amount of such a reducing agent is needed (as in "classic" organic chemistry a stoichiometric amount of molecular hydrogen or borane or sodium borohydride is used). Taking into account both the very high molecular weight and price of cofactors such as NADPH, the use of a stoichiometric amount of such molecules would not enable any synthetically useful process. Thus, *in situ* cofactor regeneration of such cofactors, enabling their use in catalytic amounts, is a prerequisite to conduct such biocatalytic redox processes in a synthetically useful and attractive fashion. Such *in situ* cofactor regeneration can be achieved through combination with a second enzymatic transformation, which regenerates the cofactor (Figure5). To make the cofactor regeneration economically attractive it is important that the substrate consumed in this second enzymatic process is cheap and readily available, since this substrate (the so-called cosubstrate) is required in stoichiometric amount.

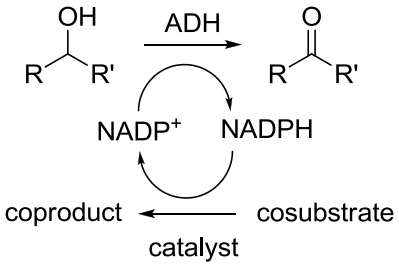


Figure5 Example of ADH regeneration system.

In conclusion, redox cofactors like NADH, NADPH and flavines are prohibitively expensive if used in stoichiometric amounts and need to be recycled using additional enzymes and/or specific auxiliary substrates. When whole cells are employed, the recycle of cofactors does not constitute a problem because cofactor regeneration is carried out within the metabolism in the cell.

2.5 Factors Affecting Enzymatic Reactions

As with chemocatalysts, enzymes also have a typical application range with respect to reaction parameters, which have to be considered in those transformations. These “typical” reaction parameters are in general related to physiological conditions under which the corresponding enzymes work. In particular, the pH and temperature profile of enzymes should be determined prior to use in organic synthesis. For most enzymes a pH in the range 6-10 and temperatures of 20-50°C are preferred although many exceptions are known. Notably, enzymes suitable for catalyzing the reactions at very low or high pH and also at elevated temperature exceeding 80°C have been found. A typical natural source of such types of enzymes is the group of so-called extremophilic microorganisms. For example, thermophilic microbial strains from hot springs are an interesting source of enzymes that can be impressively active at high temperatures.

A further important criteria when setting up biotranformation is the choice of reaction medium. The preferred reaction media for enzymes - when taking into account their natural function - are aqueous (buffered) solutions. However, notably, many enzymes are highly tolerant towards the presence of organic solvents. This has been demonstrated in particular for lipases as catalysts. The reaction medium of choice for most of enzymes is nevertheless water (or related buffer solutions). Since organic substrates are often hydrophobic, water-miscible and water-immiscible organic solvents have been added in biotransformations to ensure sufficient solubility of the substrate.

2.6 Advantages and Drawbacks

Criteria for choosing a specific synthetic route for biotransformations are high conversion, enantioselectivity, and an ideally quantitative conversion which has not only the advantage of consuming the maximal amount of substrate (thus contributing to a decrease in substrate costs) but also simplifies downstream-processing.

With respect to enantioselectivity, typically a high enantiomeric excess of >99% (as required from the FDA for chiral drugs) for the resulting product is desirable. Besides conversion and selectivity issues, substrate and product concentrations as well as volumetric productivities are further important criteria for biotransformations in organic chemistry.

Among further important criteria for realizing an efficient synthetic process are an attractive access to the biocatalyst component and the technical feasibility of the process.

A major advantage of enzymes as catalysts in organic synthesis, which is often regarded as the major advantage of biocatalysts, are the excellent selectivities enzymatic reactions typically show. For numerous asymmetric reactions starting from prochiral compounds the desired products are formed in excellent enantiomeric excess of >99%. This is the reason why biocatalysed processes are particularly favourable for the synthesis of the single enantiomers of chiral molecules for pharmaceutical applications. Some well-known examples are reported in Figure6.

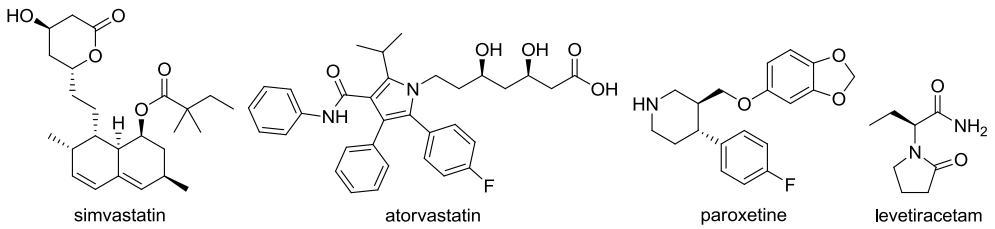


Figure6 Examples of chiral active principles used in medicine.

Whereas chirality and a defined absolute configuration is also an important criterion in nature, high volumetric productivities and a substrate input of, for example,>100 g/l is a desirable feature for organic syntheses, but not for processes in living organisms. Thus, unsurprisingly, for many enzymes the development of synthetic processes running at a high substrate input and leading to a high space-time yield turned out to be a challenging task (but at the same time it should be added that many enzymes turned out to be able to do so efficiently!).

Besides the specific activity, a further key feature for attractive biocatalysts is an economic production method for their preparation. Certainly, this criterion has long been a limiting factor and drawback in enzymatic chemistry when regarding the decade long biotransformations with wild-type organisms. The use of wild-type organisms has major drawbacks, for example, because the expression of the desired protein is very low, thus requiring a large amount of biomass for the biotransformation. Often substrate loading is below 1 g/l, accompanied by a high biomass loading of >25 g/l in such biotransformations (as it can be seen, for example, in many reactions with baker's yeast as a biocatalyst). A further consequence of low protein expression of the desired enzyme in wild-type microorganisms is the significant impact of side reactions.

Owing to impressive advances in molecular biology related to protein engineering[17] today enzymes can be mostly made available in recombinant form.

Overexpression in host organisms such as, for example, Escherichia coli often exceeds 20%, which not only contributes to a high biocatalyst amount in the recombinant whole-cell but also to suppressing side reactions due to the favored ratio of desired enzyme over other enzymes catalyzing for competing side-reactions.

A further major advantage of biocatalysis over chemocatalysis is the possibility to simplify multi-step synthetic routes with a single enzyme-catalysed transformation or with the combination of several enzymes in one-pot procedures.

Biocatalysis offers many unique advantages over chemical alternatives, thus representing an exciting complementary alternative to the pool of "classic" chemical and chemocatalytic synthetic methods available to the organic chemist today.

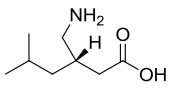
The historical development of biotransformations in organic synthesis has been mainly driven from two perspectives. The first is based on earlier available fermentation methods applied in food industry and this experience was then used for the production of chemicals. The second is based on

an increasing understanding of the occurrence and function of enzymes as molecular catalysts which led to the development of biotransformations using isolated enzymes.

However, compared to microbial systems, access to isolated enzymes is connected with additional purification efforts that represent a cost factor. Whereas fermentation of biomass as a catalyst is a cheap process, purification of enzymes (dependent on the purification degree) makes the enzyme component increasingly expensive. Owing to such higher costs for manufacturing isolated (purified) proteins over microbial whole-cells obtained as biomass directly from the fermentation, during process development there has been great interest in immobilization and recycling of isolated (purified) enzymes and reaction engineering issues to attain an economically attractive biocatalyst and economically favorable data for the biotransformation on a large scale.

2.7 New Production Pathways in Industry

A major application area of enzyme catalysis is the synthesis of pharmaceuticals and intermediates thereof, since such molecules often are chiral and, at the same time, enzymes show excellent stereoselectivities. Despite this "ideal fit" of synthetic requirement and enzyme stereoselectivity properties, the number of enzyme-catalyzed processes in organic syntheses has been rather limited for a long time compared with classic chemical or chemocatalytic syntheses. One reason that might represent in part an explanation for this is the surprising observation that retrosynthetic strategies in natural product or drug synthesis have often been designed mainly based on classic organic synthetic routes without consideration of biocatalytic key steps. This might be because biocatalysis still needs to be more implemented as a "standard synthetic tool" in teaching courses on organic chemistry. Another reason for the limited use of enzymatic steps in natural product and drugs synthesis is that for a long time there was a lack of process efficiency of enzymes despite the excellent stereoselectivities (such as, for example, low volumetric productivities). Although highly efficient processes have long been reported mainly when using hydrolases as catalysts (and in part oxidoreductases), other enzyme classes have been used to a lesser extent with respect to the development of highly efficient production processes.



pregabalin

However, in the last two decades tremendous progress has been made to overcome these two above-mentioned limitations. In particular for pharmaceuticals, exciting retrosynthetic approaches have been designed that are now based on enzymatic key. For example, Tao and coworkers develop an alternative pathways towards pregabalin as the active pharmaceutical ingredient of Pfizer's neuropathic pain drug Lyrica®. The original synthetic route starts from a β-cyano-malonate, which is then transformed by classic chemical synthetic steps into the desired racemic γ-amino acid. The final step is a "classic" resolution based on diastereoselective salt formation with a chiral acid. By means of this method pregabalin

was obtained in an overall yield of 20%. Its main disadvantage is the lack of a suitable racemization for the unwanted enantiomer, since the β-amino acid is difficult to racemize due to the lack of a C-H-acidic functionality at the β-position. This has been addressed in a "second-generation" route (Figure7) as an improved alternative, which is based on a regio- and enantioselective hydrolysis of 2-isobutylsuccinonitrile as the starting material in the presence of a nitrilase as a biocatalyst, leading to the required intermediate in 45% yield and with an enantiomeric excess >97%.

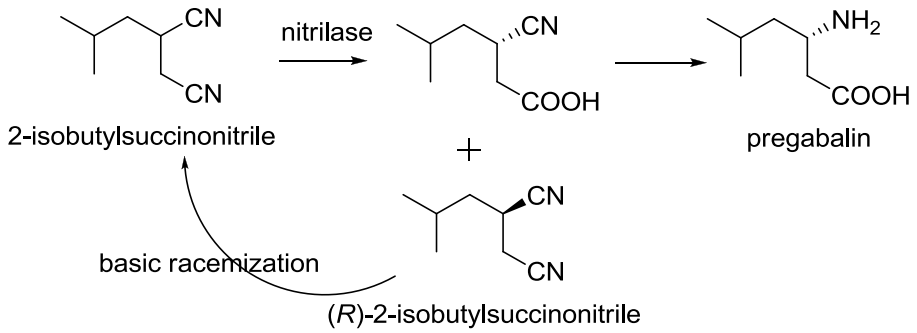
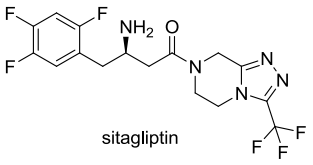


Figure7 Second generation synthetic route to pregabalin based on enzymatic resolution.

This strategy turned out to be superior to the first route since the resolution process now leads to an unwanted enantiomer that can be easily racemized under basic conditions due to the presence of an α-C-H-acidic functionality at the stereogenic center. Thus, this chemoenzymatic multistep synthesis of pregabalin is a very elegant approach, combining an efficient enzymatic resolution with the idea of implementing the resolution at an early stage, thus enabling racemization of the unwanted enantiomer.



sitagliptin

The next example addresses the tremendous progress that has been made to make enzymes suitable for the highly competitive industrial synthesis of complex, pharmaceutically relevant molecules. Without doubt, one of the highlights in recent years has been the development of a chemoenzymatic production process for the antidiabetic drug sitagliptin phosphate (named Januvia®) by Merck and Codexis researchers. Notably, this enzymatic process, based on a transaminase as a biocatalyst, has turned out to be advantageous over the previously developed and also industrially established chemocatalytic alternative. In the chemical synthesis, using an asymmetric metal-catalyzed hydrogenation as a key step, first the ketone used as a starting material is transformed into an enamine, which is subsequently hydrogenated enantioselectively. The final step consists of salt formation of the drug sitagliptin phosphate. Despite an excellent hydrogenation process, the whole synthetic route possesses two drawbacks. First, direct transformation of the ketone into the amine instead of a two-step process with an enamine intermediate formation would be more desirable. Even more important, however, is having a heavy-metal catalyzed process nearly at the end of the multistep synthesis; the need to remove metal traces from a drug intermediate at a late stage is tedious and disadvantageous. These drawbacks have been solved by applying a direct

enzymatic transformation with a transaminase, allowing direct conversion of the ketone substrate into the desired amine. Furthermore, heavy metals (required as catalyst component in the chemocatalytic asymmetric hydrogenation step) are no longer involved. A major challenge, however, had to be solved to realize this process, namely, the development of a transaminase showing sufficient activity for the sterically demanding ketone substrate. The enzyme optimization carried out also underlined today's tremendous opportunities in protein engineering. Starting from a wild-type enzyme showing negligible activity for the ketone substrate, eleven mutation rounds led to a highly efficient mutant (Figure8).

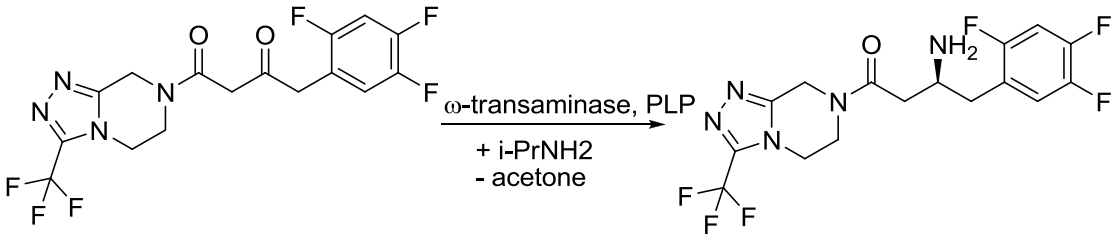


Figure8 Employing a modified ω-transaminase the asymmetric amination of a sterically hindered ketone was achieved.

In the presence of this optimized enzyme, a process has been realized that runs at impressive substrate input and leads to the desired product with the excellent enantioselectivity of 99.95%. These two recent examples in the field of chemoenzymatic multistep drug's synthesis underline the tremendous potential of enzyme-catalyzed processes for the multistep synthesis of complex molecules such as drugs and natural products.

Thus, in future an increasing tendency to integrate biocatalytic key steps into multistep routes to such molecules can be expected, thus contributing to the development of both economically attractive and sustainable production processes.

2.8 Use of enzymes in the chemical industry in Europe[18]

The chemical industry represents one of the biggest economic sectors worldwide and European companies play a dominant role. The European chemical industry is highly developed, but introduction of new technologies continues, including biocatalysis.

Enzyme applications, which are in different development stages, are found mainly in the organics, drugs, and cleaners segments. Today, the market for basic, intermediate, fine and specialty chemicals and polymers made by bioprocesses accounts for only 2% of the chemical market or $25 billions in revenue, excluding revenues from chemicals like ethanol made by traditional fermentation[19].

The sustainability of a process relates to energy and raw material use, waste production, process stability/safety, and product quality. Process improvements such as increase in yield and reductions

in raw material demand, emissions, and waste result in process cost saving can give bioprocesses advantages over traditional chemic al routes. This was shown by Ciba (UK) for the synthesis of acrylic acid on pilot-scale using nitrilase (Figure9).

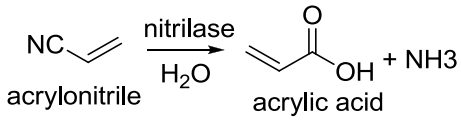
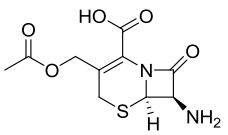


Figure9 Ciba's bio-route to acrylic acid.

Similarly, Budel Zink (NL) are using sulfate reducing bacteria to treat 30 m^3 of wash tower acid per hour and recover 8.5 tons/day of ZnS, an impressive application in the industry sector of inorganics. Today, bioprocesses based on genetically modified organisms (GMOs) are economically feasible, for example is the new plant for the enzymatic production of 7-aminocephalosporanic acid, the core chemical structure for the synthesis of cephalosporin antibiotics and intermediates, by Biochemie (Germany).



7-ACA

Nowadays, key reactions in bioprocesses running on production scale are mostly hydrolytic or the processes are based on fermentations of cheap starting materials like glucose (Figure10).

Company	Strategy	Product(s)	Catalyst(s)
Avecia	Kinetic resolution	(*S*)-2-chloropropionic acid	Whole cells, (*S*)-specific dehalogenase
BASF	Fermentation	L-lysine (*R*)-isobutyl lactate	*Corynebacterium glutamicum* microorganism
	Kinetic resolution	Enantiopure alcohols	Lipases
		Chiral amines	Lipases
	Dynamic resolution	(*R*)-mandelic acid	Nitrilase
	Oxidation	(*R*)-2-(4-hydroxyphenoxy)propionic acid	Whole cells oxidase
Chirotech	Kinetic resolution	Various α-amino acids	Lactamases
		Various D-amino acids	D-aminoacylase
		Various L-amino acids	N-acetyl-L-amino acid amidohydrolase
	Dynamic resolution	Various S-ester amides	Traiacylglycerol lipase
Degussa	Fermentation	L-threonine	Whole cells
	Dynamic resolution	Enantipure L-amino acids	L-acylases
		Enantipure D-amino acids	Hydantoinases
	Enantioselective production	L-tert-Leucine	Leucine dehydrogenase
DSM	Kinetic resolution	(2*R*,3*S*)-3-(p-methoxyphenyl)glycidyl methylester	Lipase
		Enantipure L-amino acids	Amidases, esterases, proteates
	Dynamic resolution	Enantipure D-amino acids	Hydantoinase, decarbamylase
	Enantioselective production	L-aspartic acid	Ammonia lyase
		Aspartame	Thermolysine
		Enantiopure cyanohydrins	Hydroxynitrile lyase
	Enzymatic hydrolysis	6-APA, 7ADCA	Penicillin acylase
Lonza	Hydrolysis	Niacinamide	Immobilized whole cells, nitrile hydratase
	Hydrolysis	2-pyridinamide	Whole cells, nitrile hydratase
	Kinetic resolution	(*S*)-pipecolic acid	Whole cells, (*S*)-amidases
		(*S*)-2-phenylpropionic acid	Whole cells, nitrile hydratase
	Oxidation	L-carnitine	Whole cells, desaturase and hydratase
		Ring-hydroxylated pyridine derivatives	Whole cells, nitrilase and dehydrogenase
		5-methylpyrazine-2-carboxylic acid	Whole cells, xylene degradation pathway
Novartis	Amino oxidation and hydrolysis	7-ACA	D-amino acid oxidase and glutaryl amidase
Roche	Fermentation	Riboflavin	Genetically engineered *B. subtilis*

Figure10 Commercialized bioprocesses.

Mechanistic exceptions are the hydroxylations of trimethylaminobutyrate and heteroaromatic compounds by Lonza, using oxygen derived from water[20]. DSM is using ammonia lyase for the production of L-aspartic acid and is implementing a process for the enantioselective production of cyanohydrins[21].

A frequently underestimated point is the importance of integrated process development as a main factor for success of industrial biocatalysis. The key determining factor of a new bio-route is not the identification of an appropriate enzyme but the speed of scale up. Furthermore a close collaboration with academic institutitions has great importance. Thanks to that Avecia successfully develops a process for the production of (S)-chloropropionic acid (2000 tons/year).

New reactions evaluated in industry now include oxidations[22] and carbon-carbon bond formations[23], processes often limited by product inhibition. Redox reactions includes for example regiospecific oxidation mediated by *Escherichia coli* and the use of wild-type fungus *Merulius tremellosus* to produce various enantiopure benzylic alcohols from prochiral ketones[24].

Nature, but also strain collections, harbour a large pool of still unused but industrially interesting enzymes. The diversity of natural compounds suggests the existence of a, yet undiscovered, potential for functional group biocatalysis. The characterization and application of new enzymes to catalyze reactions with commercial potential will significantly enlarge the spectrum of industrial biocatalysis.

CHAPTER 3

3.1 Acetic acid bacteria

Acetobacter aceti is a Gram negative aerobic bacterium that belongs to the family *Acetobacteraceae.* This big family includes at least 25 genera including *Acetobacter*, *Acidomonas*, *Asaia*, *Gluconacetobacter*, *Gluconobacter*, *Granulibacter*, *Kozakia*, *Neoasaia*, *Saccharibacter* or *Swaminathania*. Other genera have emerged that usually include one species and display common characteristics for the entire family (*Belnapia*, *Leahibacter*, *Oleomonas*, *Rhodopila* or *Rubritepida*).

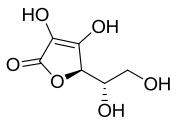
Acetic acid bacteria occur mainly in sugary, acidic and alcoholic habitats and have been studied extensively, since they can play a positive, neutral or detrimental role in foodstuff and beverages. Multiple species of acetic acid bacteria are capable of incomplete oxidation of carbohydrates and alcohols to aldehydes, ketones and organic acids[25]. Oxidation products are secreted outside cells, owing to which they may be isolated directly from a culture medium.

The use of acetic acid bacteria dates back to distant years of our civilization. Probably, as early as in the Babylonian times use was made, though unconsciously, of their capability for the preservation of food. Some notes on the consumption of vinegar have been encountered in the Old Testament.

Contemporary knowledge on biochemical, physiological and genetic characteristics of acetic acid bacteria enables their well-thought-out and targeted application in a number of industry branches.

A typical trait of acetic acid bacteria is the production of acetic acid from ethanol. This capability is employed in the food industry for the production of **vinegar**.

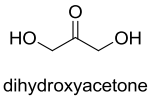
Apart from the production of vinegar, an increasing interest has been observed in the application of those bacteria for the production of **vitamin C** (L-ascorbic acid). Nowadays, vitamin C is synthesized in a seven-step Reichstein's cycle, with D-glucose used as a substrate. That processes covers six chemical syntheses and one reaction of microbiological oxidation of D-sorbitol to L-sorbose. The proper course of that transformation requires high pressure and temperature so, in recent years, research have focused on the elaboration of alternative methods for the production of ascorbic acid, especially the biotechnological ones. In order to minimize production costs a study was undertaken to modify methods applied so far with the use of industrial strains of acetic acid bacteria. It was proved that the



L-ascorbic acid

application of enzymes originating from *Gluconobacter oxydans* enabled facilitating that process and additionally minimized production costs[26].

Selected strains of acetic acid bacteria have been shown to be capable of cellulose biosynthesis (e.g. *Acetobacter xylinum)*, which affords new possibilities of that biopolymer's production. Paper produced from such a cellulose is highly elastic, resistant and, more importantly, completely biodegradable[27]. Apart from the paper industry, **bacterial cellulose** may also be applied for the electrophoretic separation of DNA. Yet the greatest hopes are fostered by the possibility of applying that easily-available bacterial biopolymer in medicine. Attempts are underway to apply this bio-cellulose for reconstruction of destroyed or damaged organs, fragments of skin, for healing wounds and for the synthesis of artificial tissues[28]. Out of multiple compounds produced by acetic acid bacteria, worthy of special notice is **dihydroxyacetone**. It is produced as a result of glycerol oxidation catalyzed by glycerol dehydrogenase. Dihydroxyacetone is an important component of self-tanning creams and a secondary metabolite on the pathway of obtaining other chemical compounds[29].



dihydroxyacetone

Some strains of acetic acid bacteria can been employed for their capability to partially oxidize primary alcohols to the corresponding carboxylic acids. Those observations, accompanied by the demand for greener, more atom efficient oxidation methods, led us to investigate the synthetic significance of the kinetic resolution of racemic primary alcohols to the corresponding acids, and the desymmetrisation of meso diols to the corresponding hydroxymethyl acids by means of acetic acid bacteria.

3.2 Oxidations of primary alcohols and meso diols via bacteria

Direct catalytic oxidation of primary alcohols to carboxylic acids or their salts has been rarely accomplished[30]. Acids are produced so far from primary alcohols in one step by the use of toxic strong oxidants such as iodate or chlorite with catalytic ruthenium and chromium oxides, resulting in great amount of waste[31]. Recent literature examples of primary alcohol oxidation to acids still involve expensive and non-atom-economic stoichiometric reagents[32]. Besides these environmental and technical limitations, standard chemical oxidations of alcohols are often poorly stereoselective.

Biocatalysis is now emerging as an effective tool for the optimization of sustainable catalytic oxidation procedures[33], especially for the exquisite chemo-, regio- and stereoselectivity, the mild conditions of temperature, pressure and pH, the use of non-problematic solvents, and the high rates of turnover. The oxidation of primary alcohols to carboxylic acids is mainly performed by means of whole cells biotransformations, in particular by using acetic acid bacteria. The latter have an incomplete oxidative metabolism, thus they can efficiently convert primary alcohols into the corresponding carboxylic acids, while aldehydes are not normally isolated.

This kind of biocatalysed chemical transformation can be employed to prepare single enantiomers of chiral compounds, according to two well defined synthetic strategies: the kinetic resolution of racemic alcohols and the desymmetrisation of meso diols. Only a few examples are reported in literature describing the use of acetic acid bacteria in this type of optical activation procedure[34].

Production of (*R*)-2-hydroxy-propionic acid showing an enantiomeric excess (ee) value of 99%, in nearly theoretical yields (48%) was carried out through microbial oxidation of racemic 1,2-propanediol by *G. oxydans*[35], when the substrate concentration was lower than 20 g/l.

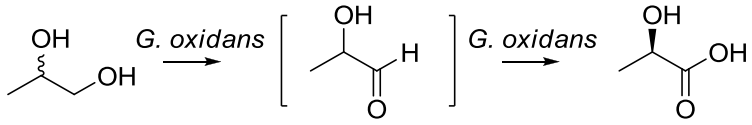


Figure10 Biosynthesis of (*R*)-2-hydroxypropionic acid.

G. oxydans was also successfully applied to the bioconversion of 2-methylbutanol to enantiopure (*S*)-2-methylbutanoic acid[36].

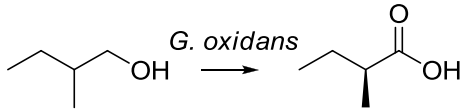
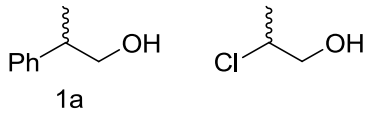


Figure11 Biosynthesis of (*S*)-2-methylbutanoic acid.

The oxidations of 2-phenyl-1-propanol (**1a**)[37] [38] and 2-chloro-1-propanol were investigated with several acetic acid bacteria.



In the case of 2-phenylpropanol the best results were achieved with *A. aceti* DSM 3508, affording the corresponding (*R*)-carboxylic acid (**2a**) with ee of 95% and 40% conversion (c) yields. The oxidation of the racemic mixture of 2-chloropropanol occurred with much lower ee values not higher than 50%. *Acetobacter pasteurianus* was described to oxidize glycidol to (*S*)-glycidic acid with high activity, comparable to the oxidation of ethanol[39].

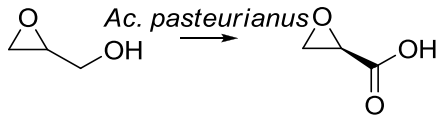
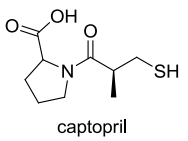


Figure11 Biosynthesis of (*S*)-glycidic acid.

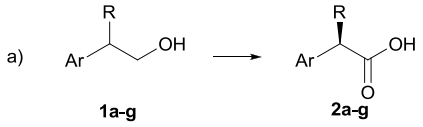
As for the desymmetrisation of meso 2-substituted-1,3-diol, the only successful reactions were reported for 2-methyl and 2-butyl-1,3-diol (**3a** and **3d**). In 2003 it was described the microbial oxidation of compound **3a** into (*R*)-3-hydroxy-2-methyl propionic acid (**4a**), and important building block for the synthesis of Captopril, with 97% enantiomeric excess and 100% molar conversion of 5 g/L within 2 hours by using



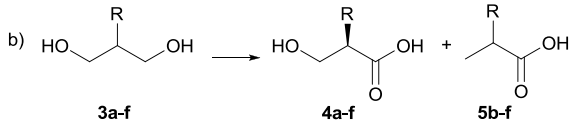
captopril

Acetobacter pasteurianus DSM 8937[40]. *Acetobacter pasteurianus* IAM 12073 was found to produce the (*S*)-enantiomer of the corresponding hydroxymethyl carboxylic acid **4d** with 89% enantiomeric excess after 24 hours of incubation.

The few available data on the synthesis of chiral carboxylic acids by means of microbial oxidations and the necessity of developing efficient sustainable methods for a mild oxidation of primary alcohols under high stereochemical control prompted us to investigate the synthetic significance of the kinetic resolution of racemic alcohols **1a-g** to acids **2a-g**, and the desymmetrisation of meso diols **3a-f** to hydroxymethyl acids **4a-f** by means of acetic acid bacteria (Scheme1).



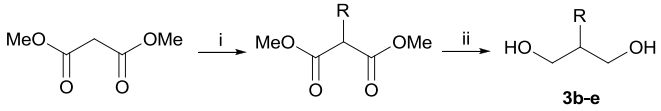
	Ar	R
a	Ph	Me
b	Ph	Et
c	PhO	Me
d	*o*-ClC_6H_4O	Me
e	*m*-ClC_6H_5O	Me
f	*p*-ClC_6H_4O	Me
g	*p-iso*-BuC_6H_4	Me



	R
a	Me
b	Et
c	*n*-Pr
d	*n*-Bu
e	Ph
f	CH_2Ph

Scheme1: a) Microbial kinetic resolution of primary alcohols; b) microbial desymmetrisation of meso diols.

Compounds **1a**, **1b** and **3a, e, f** were commercially available. Substrates **1c**-**f** were obtained by $LiAlH_4$ reduction of the corresponding methyl esters prepared from commercial carboxylic acids. Diols **3b**-**f** were prepared by $LiAlH_4$ treatment of the corresponding 2-substituted malonates, obtained by monoalkylation of dimethyl malonate with the suitable alkyl bromide according to Scheme2.



Scheme2: i) NaH, THF, R-Br; ii) $LiAlH_4$, THF, reflux.

Preliminary investigations were performed with three different aced acid bacteria, namely *Acetobacter aceti* MIM 2000/28*, Asaia sp.,* and *Gluconobacter oxydans* DSM 2343. Oxidation was obtained only with *A. aceti,* whereas the other two bacteria show either scarce or no activity towards substrates **1a**-**g** and **3a**-**f**.

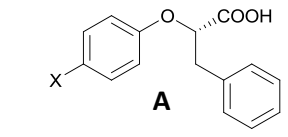
3.3 Kinetic resolution of primary alcohols

The conversion, enantiomeric excess values and absolute configurations of the *A. aceti* mediated oxidations of **1a**-**g** are reported in Table1**.**

Substrate	c[a] (%)	ee[b] (%)
1a (Ph, Me)	35	95 (*R*)[c]
1b (Ph, Et)	13	80 (*R*)
1c (C_6H_5O, Me)	19	90 (*S*)
1d (*o*-Cl-C_6H_4O, Me)	1	-
1e (*m*-Cl-C_6H_4O, Me)	37	98 (*S*)
1f (*p*-Cl-C_6H_4O, Me)	18	69 (*S*)
1g (*p-iso*Bu-C_6H_4)	33	60 (*R*)

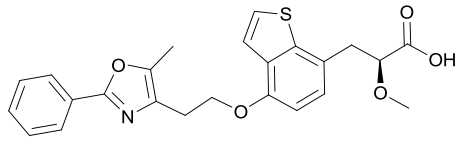
Table1 Results of the biocatalysed oxidations of substrates 1a-g to afford carboxylic acids 2a-g.

α-Substituted alkanoic acids, such as derivatives of type **2,** are important chiral building blocks for the preparation of active pharmaceutical ingredients and agrochemicals. For example, α-aryloxy-hydrocynnamic acids of type **A** and α-alkoxy derivatives, such as Aleglitazar and Saroglitazar, are considered very attractive because they can be used as dual-acting PPARα/γ agonists in the treatment of dyslipidemic type 2 diabetes[41].

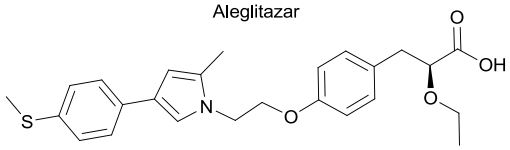


A

X = Cl, CF_3, CH_3O, CH_3CO, Ph, CH_2OH



Aleglitazar



Saroglitazar

Enantiomerically enriched carboxylic acids are usually prepared by fractional crystallisation of their diastereoisomeric salts with chiral amines[42]. The need for trial-and-error optimisation of conditions, the employment of stoichiometric amounts of chiral resolving agents, the necessity of repeated crystallisations, and the requirement of large volumes of solvents are often detrimental for the

[a] Conversions calculated by GC analysis of the crude mixture after 72 h reaction time after treatment with diazomethane.
[b] Enantiomeric excesses calculated by GC analysis on a chiral stationary phase after treatment with diazomethane.
[c] Absolute configurations determined by correlation with α_D reported in literature.

efficiency and throughput of the whole synthetic process in which the resolution procedure is involved. One of the alternatives is kinetic resolution, which has been obtained up to now by enantioselective alcoholysis of activated carboxylic acid derivatives in the presence of chiral chemocatalysts[43] or by biocatalysed hydrolysis of esters, amides and nitriles[44].

The oxidation of racemic **1a**, **1c** and **1e** afforded carboxylic acids with high enantiomeric excess (90-98%); the best conversion yields were obtained with substrates **1a** and **1e**, whereas compound **1d** was left unreacted. In the case of compound **1e** the unreacted alcohol enriched in the (*R*)-enantiomer (ee = 38%), recovered from a first oxidation reaction, was submitted again to *A. acetic* oxidation to afford another sample of (*R*)-**2e** (c = 40%) with ee = 73% and the primary alcohol (*R*)-**1e** with ee = 99%.

The reaction was characterised by the same stereochemical course, independently from the structure of the substrate: the formation of the enantiomers showing the spatial distribution of substituents around the stereogenic carbon atom shown in Scheme1 was obtained. This is the stereochemistry required for the above mentioned active pharmaceutical ingredients. The change in the descriptor from substrates **2a**,**b** to **2c**,**f** is due to a switch of the priority order of substituents occurring when an oxygen atom is inserted between the aryl group and the stereogenic centre.

An example of kinetic resolution for compound **1a** is reported in Figure12.

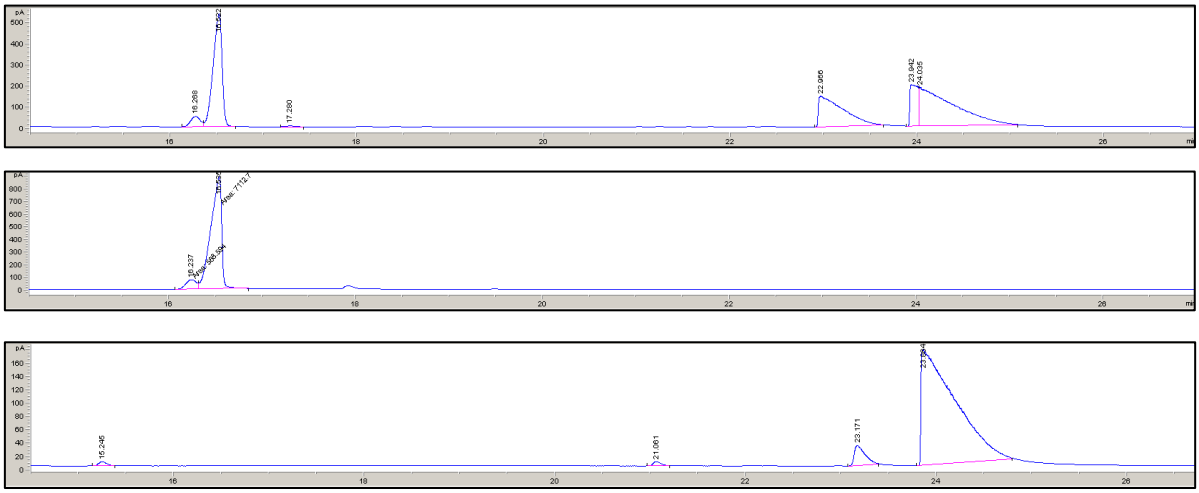


Figure12 Example of kinetic resolution: we can see the formation of the enantiomer of the acidic product while the not involved alcohol's enantiomer is left unreacted.

To improve the efficiency of the kinetic resolution and avoid the waste of half of the starting materials, an all-enzymatic procedure for the recovery and racemisation of the unwanted enantiomer should be performed.

3.4 Desymmetrisation of meso diols

The results of the *A.aceti* mediated oxidations of compounds **3a**-**f** are reported in Table2 and Table3.

Substrate	Hydroxy Acid (4)	Deoxygenated Product (5)	Unreacted Diol (3)
3b	69	31	-
3c	28	72	-
	30	70	-
3d	21	43	36
	76	7	17
	59	4	37
3e	28	14	58
3f	33	25	41
	14	30	54
	34	21	45

Table2 Conversions[a] of the biocatalysed desymmetrisation of diols 3b-f to afford hydroxymethyl acids 4b-f with *A. aceti* **in the presence of 1% DMSO as cosolvent**.

Substrate	Hydroxy acid (4)		Deoxygenated product (5)	
	c[a] (%)	**ee[b] (%)**	**c[a] (%)**	**ee[b] (%)**
3a (Me)			n.d.	
3b (Et)	20	50	80	75
3c (*n*-Pr)	80	36 (*S*)	20	
3d (*n*-Bu)	87	80 (*S*)	12	
3e (Ph)	56	rac	44	n.d.
3f ($PhCH_2$)	70	92 (*R*)	30	58

Table3 Results of the biocatalysed desymmetrisation of diols 3a-f to afford hydroxymethyl acids 4a-f in with *A. aceti* **without cosolvent**.

Only few examples of the enantioselective synthesis of derivatives of type **4** are present in patent literature and most often are described in patents by pharmaceutical companies. Methyl esters of 3-hydroxypropyl alkanoic acids of type **4** were obtained in enantiomerically enriched form by enzyme-mediated reductions of the corresponding 2-formylderivatives by Kaneka corporation[45]. The procedure of Evan's oxazolidines was employed by Vernalis[46], Smithkline Beecham Corporation[47], and Therevance[48] for the synthesis of derivatives **4** to be used in the preparation of antibacterial agents. Recently, the interest in these hydroxymethyl acids has increased because they can be easily converted into β-lactones (Figure13), which are currently studied as caseinolytic protease (ClpP) inhibitors.

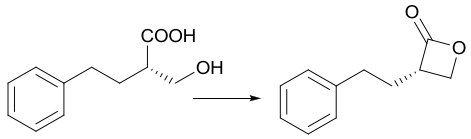
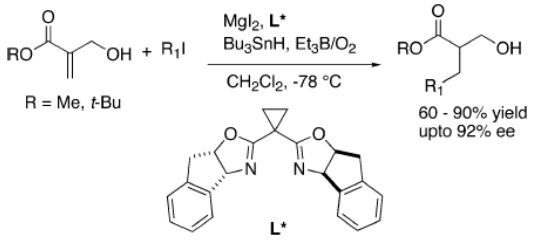


Figure13 Example of hydroxymethyl acid easily converted into β-lactone.

The necessary (*S*) configuration of the suitable hydromethyl acid derivative was obtained with the use of Evans' chiral oxazolidinone auxiliary.

A different synthetic approach was reported in by Sibi *et al.* in 2004[49]: radical alkylation of hydroxyacrylates, obtained by Baylis-Hillman reaction, with alkyl iodides in the presence of chiral Lewis acids as catalysts gave hydromethyl compounds of type **4** with ee values up to 92%.



Thus, we investigated the selective oxidation of only one of the two primary alcohol moieties of meso 2-substituted-1,3-diols by means of acetic acid bacteria, in order to optimise another synthetic procedure to this kind of chiral compounds.

Diols **3a-f** were submitted to *A. aceti* treatment in buffer (pH = 7.2, 32°C) in the presence of 1% DMSO as a cosolvent with a substrate concentration of about 1mg/ml, depending on the molecular weight of the compound. During the oxidation reactions of diols **3b–f** the formation of a deoxygenated product of type **5** was observed (Scheme3).

The same side-products were obtained also when the reactions were performed without the use of a cosolvent. The data of conversions for the reactions are reported in Table3.

In the case of diol **3d** and **3e** the reactions performed without cosolvent were characterised by a complete conversion of the starting diol into a mixture of hydroxyacid **4** and 2-methylalkanoate **5**. On the contrary the use of DMSO afforded a final reaction mixture still containing the unreacted starting diol (Figure14, from data in Table2). The oxidation of diols **3b** and **3c** left no unreacted diol in both the two ways in which the oxidation was performed.

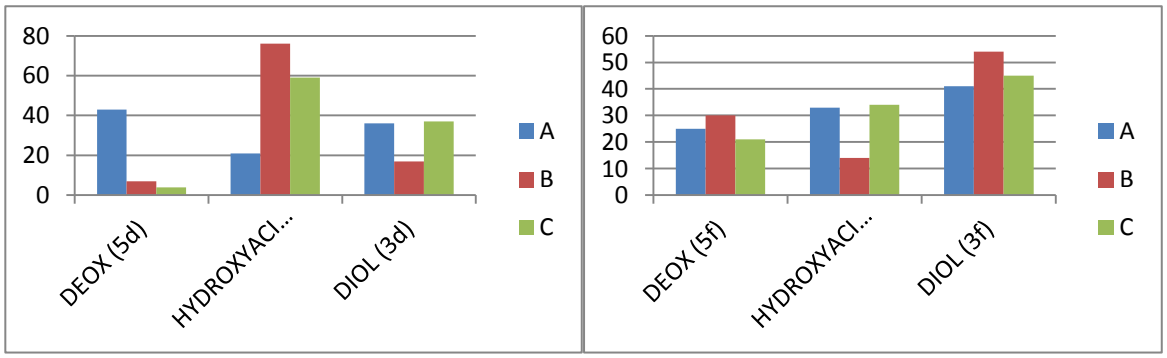
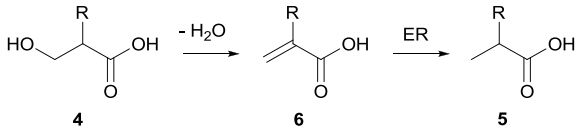


Figure14 Results of the biocatalysed desymmetrisation of diols 3d and 3f with *A. aceti*, in the presence of 1% DMSO as a cosolvent (comparison of three experiments).

The formation of hydroxyacids **4b-f** and of the deoxygenated products **5b-f** was monitored as a function of reaction time.

All the deoxygenated products were found to be enantiomerically enriched (see Table2 for the corresponding ee values), thus their formation was supposed to involve an enzyme-mediated transformation. A dehydration of the hydroxymethyl acid to give an α-methylenic carboxylic acid of structure **6**, followed by the reduction of the C=C double bond by means of an ene-reductase, could be hypothesized (Scheme3).



Scheme3 Tentative explanation of the formation of deoxygenated products 5

The ee values of the desired hydroxymethyl derivatives **4** were found rather satisfactory, especially when they were compared to the values reported in the literature for the above-mentioned procedure: *e.g.*in the Kaneka patent (*R*)-**4f** was obtained with ee values in the range 72-93%, and (*S*)-**4d** only with ee = 15%. The procedure employing chiral Lewis acids afforded both the enantiomers of **4d** with ee values from 40 to 75%.

3.5 Conclusions and future work

As regards results obtained in this chapter, the maximum yield of kinetic resolutions in only 50%, which is economically and ecologically unattractive unless recycling of the undesired enantiomer is easily achievable. Future research would be based on the development of a method which includes *in situ* racemization of the enantiopure alcohol not involved in the reaction. Combining several enzymes it is possible to perform the quantitative conversion of the racemic substrate into a single enantiomer in an efficient one-pot procedure (Figure15)

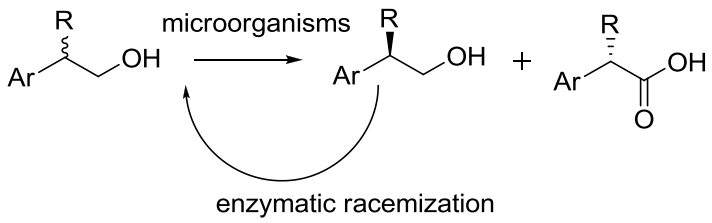


Figure15 Possible one-pot procedure to increase yield and conversion of an enzymatic reaction: oxidation to aldehyde followed by racemization and reduction.

Furthermore the study should be extended to other substrates useful for optically pure enantiomers as building blocks for pharmaceuticals and agrochemicals.

CHAPTER 4

4.1 Reductions of aldehydes and ketones

Biocatalytic reduction of carbonyl group such as aldheydes and ketones are common processes for preparative applications using deydrogenases (DHs). Approximately 300 different DH subclasses with individual members from thousands of organisms are registered giving access to an enormous range of potential substrate and products[50]. Classical chemical routes can easily be substituted using DHs thanks to the inherent specificity of these enzymes resulting in levels of regiospecificity unachievable for a "chemical" catalyst. Especially enantiospecificity in reduction of prochiral ketones is widely appreciated.

Compared to the huge number of biocatalytic reduction of ketones, aldehyde reduction plays only a minor role[51]. There are however a couple of interesting applications such as the reduction of sugars to sugar alcohols (xylitol, mannitol, sorbitol) instead to the established Ni-catalysed hydrogenation or the synthesis of green note compounds (C6-C9 alcohols) for the flavor and fragrance industry. Figure11 and Figure12 show two ways to obtain xylitol.

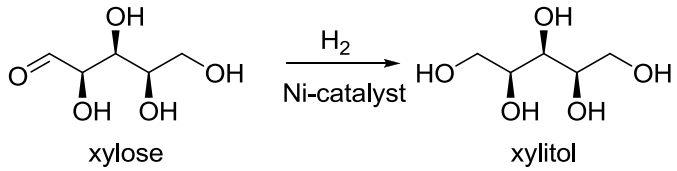


Figure11 Traditional route to xylitol by hydrogenation of xylose, which converts the sugar (an aldehyde) into a primary alcohol

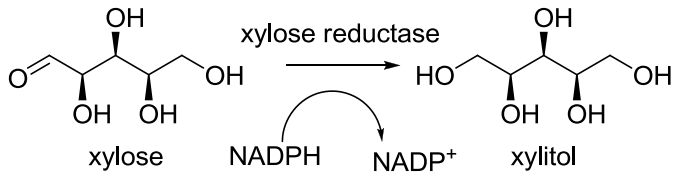


Figure12 Bio-route to xylitol by engineered microbial strains

Nevertheless DH-catalysed reductions require stoichiometric amounts of the reducing equivalents which are provided in form of the reduced nicotinamide cofactors. So these catalysts need an efficient cofactor regeneration system to enable their catalytic use.

Reduction of aldehydes does not directly lead to the formation of a chiral center but DHs exceed enantio-discrimination to positions along the chain and allow kinetic resolutions. The intrinsic drawback of a kinetic resolution of theoretically 50% yield can be circumvented under certain

circumstances. If activated, C-H acidic aldehydes (α-aryl propionals) are used, *in situ* racemization of the starting materials leads to a dynamic kinetic resolution with theoretically full conversion of the starting material into enantiopure product.

The biocatalytic reduction of ketones to corresponding enantiopure alcohols, either using whole cells or isolated enzymes, is widely used in a variety of industrial-scale reduction processes focused on chiral intermediates for agro-, pharma-, and fine chemical applications. The scientific literature contains a vast number of examples for biocatalytic reduction of ketones and a complete view of these topics is practically impossible. Some examples of chiral alcohols derived from DH-catalysed reduction of corresponding ketones is reported in Figure13.

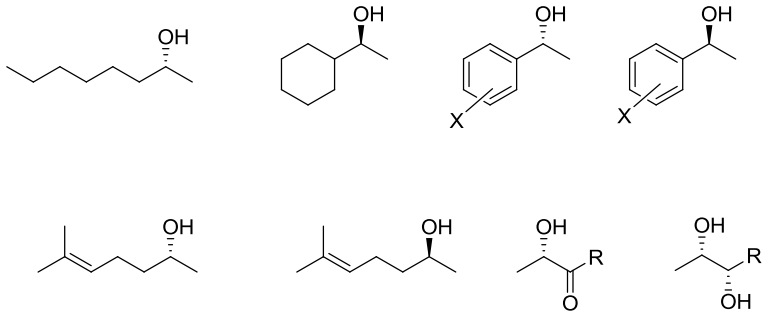


Figure13 Examples of chiral alcohols derived from DH-catalysed reduction of corresponding ketones.

4.2 Reductions of acids and esters via fungi

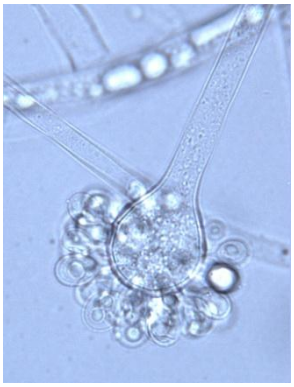
The reduction of esters and carboxylic acids to primary alcohols is a key reaction of organic chemistry[52], which has widespread application in the manufacturing processes of fine and bulk chemicals. This transformation still relies on the stoichiometric use of metal hydride reagents and their derivatives, often with the requirement of extensive heating, and troublesome work up. Recently, transition metal catalysed hydrogenation procedures have been investigated to address the waste challenge connected with this type of reactions, by using molecular hydrogen as a reducing agent. However, only a limited number of them work at room temperature and low hydrogen pressure. Other chemocatalytic methods, such as the homogeneous catalysed hydrosilylation of esters, have received considerable interest as a tool for carboxylic group reduction to primary alcohols.

As for biocatalysed acid and ester reductions, this is still a challenging task, because it is characterised by a very low redox potential, and it has been described up to now only for a limited number of microorganisms and cultured plant cells[53].

The resting cells of *Clostridium thermoaceticum* and *Clostridium formicoaceticum* were described to reduce carboxylic acids or carboxylates to alcohols at the expense of carbon monoxide or, less efficaciously, in the presence of formate[54]. Sodium carboxylates C_6-C_{10} were transformed into the corresponding alcohols by growing cultures of *Colletotrichum gloeosporoides*[55]. Cultures of *Nocardia*

species were found to reduce carboxylic acids first to aldehydes, then to primary alcohols, subsequently converted into acetyl esters, thanks to the action of at least three enzymes[56]. In 1999, it was first reported that the hyperthermophilic organism *Pyrococcus furiosus* reduced carboxylic acids with starch as carbon and energy source at 90°C[57]. Recently, the use of this microorganism has been reconsidered, and it has been employed for the reductions of carboxylic acids at 40°C under 5 bar molecular hydrogen pressure[58]. Modest yields were obtained for the reduction of a few acids with nine plant cell cultures.

In all these reports the intermediate reduction of the carboxylic acid moiety to aldehydes was described.



We now wish to report on the possibility of reducing carboxylic acid and esters in a very mild, efficient and chemoselective way by using the whole cell a zygomycete fungus. This new enzymatic activity was discovered within a project, in which the research group of the Department of Chemistry, Materials and Chemical Engineering tutoring me for my thesis work was involved. The project is aimed to the development of microbial whole cell- and enzyme-catalysed processes as sustainable synthetic tools to be used by organic chemists for the production of fine chemicals, *i.e.* active pharmaceutical ingredients (APIs), pesticides, herbicides, flavours and fragrances. In particular, it is devoted to the optimisation of bio-catalysed variants of classical organic reactions and to the search of new and improved enzymatic activities by screening one of the three Italian Biological Resource Centres (BRCs): the Mycotheca Universitatis Taurinensis of the University of Torino (MUT) specialised in filamentous fungi.

4.3 Biological Resource centres

Biological resource centres are an essential part of the infrastructure underpinning life sciences and biotechnology. They consist of service providers and repositories of the living cells, genomes of organism, and information relating to heredity and the functions of biological systems. BRCs contain collections of culturable organisms (e.g. micro-organisms, plant, animal and human cells), replicable parts of these (e.g. genomes, plasmids, viruses, cDNAs), viable but not yet culturable organisms, cells and tissues, as well as databases containing molecular, physiological and structural information relevant to these collections and related bioinformatics.

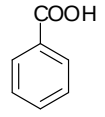
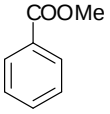
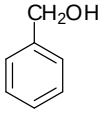
BRCs must meet the high standards of quality and expertise demanded by the international community of scientists and industry for the delivery of biological information and materials. They must provide access to biological resources on which R&D in the life sciences and the advancement of biotechnology depends. Their many crucial roles include:

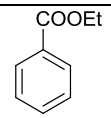
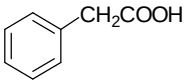
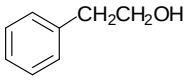
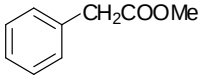
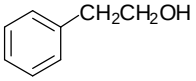
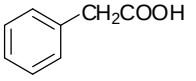
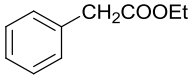
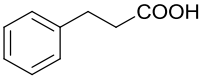
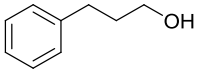
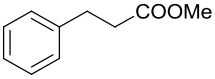
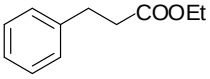
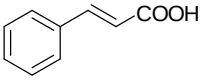
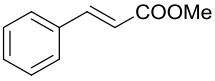
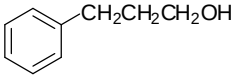
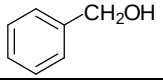
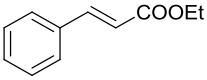
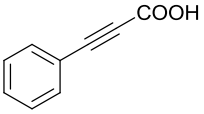
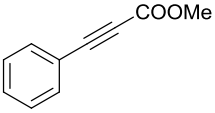
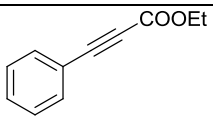
1. preservation and provision of biological resources for scientific, industrial, agricultural, environmental and medical R&D and applications;
2. performance of R&D on these biological resources;
3. conservation of biodiversity;
4. repositories of biological resources for protection of intellectual property;
5. resources for public information and policy formulation.

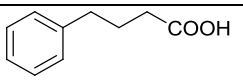
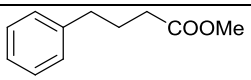
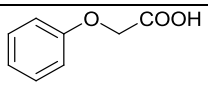
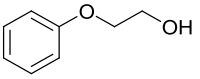
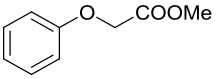
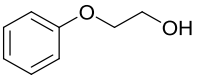
Biological resources, such as microorganisms and their derivatives, are the essential raw material for the advancement of biotechnology, human health and research and development in the Life Sciences. The Mycotheca Universitatis Taurinensis (MUT) collection is one of the largest banks of microorganisms in Italy and, based on the definition of Biological Resource Centre (BRC) given by the OECD, one of the three BRCs existing in Italy at present. Since many years, the MUT is affiliated to the European Culture Collection Organization (ECCO) and to the World Federation of Culture Collections (WFCC) and currently preserves about 5000 strains belonging to almost all classes of filamentous fungi. These fungi have been isolated from very different habitats and substrates in arctic, temperate, tropical and subtropical zones. Many fungi have been isolated from soil, herbaceous and woody plants, lichens, food, and air compost, marine and polluted environments. They include plant growth promoters, symbionts of plants and lichens, biocontrol and bioindicator agents as well as enzyme producers to be used in industrial and bioremediation applications. Hence, MUT holds living microbiological material that can be exploited for global research, cross cutting the agricultural, food, healthcare and biotechnological sectors providing a basis for a bioeconomy.

4.4 Results and discussion

We investigate the reducing capabilities of a particular zygomycete selected from the MUT collection. A preliminary screening was performed by using unsubstituted carboxylic acids and esters to define the structural basic scaffold of the substrate that could be accepted and transformed by the fungus. The results are reported in Table4.

Substrate	Products	Conversions (%)	
		1	2
 **101**	-	n.r	n.r
 **102**		87 (VII)*	84 (VII)

 **103**	-	n.r	n.r
 **104**		44 (VII)	28 (VII)
 **105**	  altri	27 34 12 (VII)	32 37 19 (VII)
 **106**	-	n.r	n.r
 **107**		73 (VII)	40 (VII)
 **108**	-	n.r	n.r
 **109**	-	n.r	n.r
 **110**	-	n.r.	n.r.
 **111**	 	60 40 (VII)	47 53 (VII)
 **112**	-	n.r.	n.r.
 **113**	-	n.r	n.r
 **114**	-	n.r.	n.r.
 **115**		n.r.	n.r.

 **116**	-	n.r.	n.r.
 **117**	-	n.r.	n.r.
 **118**		100 (VII)	100 (VII)
 **119**		100 (VII)	100 (VII)

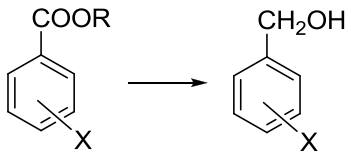
*The distribution of products obtained by GC/MS analysis after seven days is given.

Table4 Data of Preliminary Screening.

Methyl benzoate (**101**) could be reduced nearly completely to benzylic alcohol, whereas benzoic acid and ethyl benzoate could not be transformed. Phenylacetic acid (**104**) was partially converted into phenylmethanol; its methyl esters gave a mixture of alcohol, of carboxylic acid from hydrolysis reaction and of other side products. The ethyl ester was left unreacted. Phenyl propanoic acid (**107**) was reduced, its methyl and ethyl esters remained untouched. In the cinnamic series the methyl ester **111** afforded a mixture of phenylpropanol and benzyl alcohol: the first was obtained by reduction of the carboxylic moiety and of the C=C double bond, and the latter was obtained by a process of degradation with loss of one carbon atom. The three compounds **113**-**115** showing a triple bond and the two phenyl butanoic acid derivatives **116** and **117** were not reduced.

A fast and complete transformation of phenoxy acetic acid **118** as well as of its methyl ester **119** afforded the corresponding alcohol.

In a second phase of the project, the effect of substituents present on the basic molecular scaffold on the bioreduction was investigated. As for substituted carboxylic acids and esters (Table5), the reduction was favoured by the presence of a strong electron withdrawing group such the -OCH_3 moiety in *o*- and *m*-position of the aromatic ring. The activation towards reduction due to these groups was more effective on the carboxylic acids rather than on the methyl or ethyl esters.



Substrate	**Conversions (%)**	
R, X	**1**	**2**
201 H, *o*-Me	n.r.	n.r.
202	n.r.	n.r.

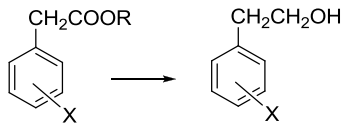
Me, *o*-Me		
203 H, *m*-Me	n.r.	n.r.
204 Me, *m*-Me	n.r.	n.r.
205 H, *p*-Me	n.r.	n.r.
206 Me, *p*-Me	n.r.	n.r.
207 H, *o*-Cl	10 (VII)	n.r.
208 Me, *o*-Cl	n.r.	n.r.
209 H, *m*-Cl	n.r.	n.r.
210 Me, *m*-Cl	n.r.	n.r.
211 H, *p*-Cl	85 (VII)	n.r.
212 Me, *p*-Cl	n.r.	n.r.
213 H, *o*-NO_2	n.r.	n.r.
214 Me, *o*-NO_2	n.r.	n.r.
215 H, *m*-NO_2	n.r.	n.r.
216 Me, *m*-NO_2	n.r.	n.r.
217 H, *p*-NO_2	n.r.	n.r.
218 Me, *p*-NO_2	n.r.	n.r.
219 H, *o*-OMe	89 (VII)	85 (VII)
220 Me, *o*-OMe	42 (VII)	24 (VII)
221 H, *m*-OMe	21 (VII)	n.r.
222 Me, *m*-OMe	n.r.	6 (VII)
223 H, *p*-OMe	69 (VII)	n.r
224 Me, *p*-OMe	n.r.	n.r.
225 H, *p*-Br	5 (VII)	15 (VII)
226 Me, *p*-Br	90 (VII)	90 (VII)
227 Et, *p*-Br	50 (VII)	31 (VII)

228 H, *o*-NH_2	n.r.	n.r.
229 Me, *o*-NH_2	n.r.	n.r.
230 H, *m*-NH_2	n.r.	n.r.
231 Me, *m*-NH_2	n.r.	n.r.
232 H, *p*-NH_2	n.r.	n.r.
233 Me, *p*-NH_2	n.r.	n.r.
234 Et, *p*-NH_2	n.r.	n.r.
235 H, (3,4,5-triOMe)		67 (VII)
236 Me, (3,4,5-triOMe)	n.r.	n.r.
237 2-furoic acid	100 (VII)	100 (VII)
238 methyl furoate		8 (VII)
239 nicotinic acid		
240 methyl nicotinate		

Table5 Data of Substituted benzoic acids and esters.

The presence of a bromine atom in *p*-position promoted the reduction of the corresponding methyl esters in better conversions yields than the corresponding ethyl ester or carboxylic acid. Three methoxy groups and the electron rich furan ring in compounds **235** and **237** favoured the reduction of the carboxylic acids to primary alcohol.

The data reported in Table6 show that either a methoxy or a nitro group were beneficial for the reduction of phenyl acetic acid derivatives, especially in *o*- and *m*-position. The methyl ester of the thiophene derivative **334** was completely reduced.



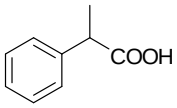
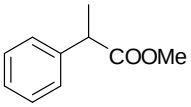
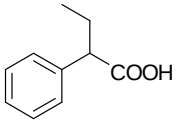
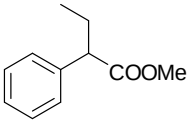
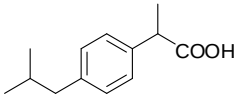
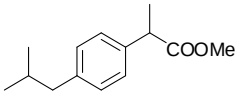
Substrate **R, X**	**Conversions (%)**	
	1	**2**
301 H, *o*-Me	52 (VII)	38 (VII)
302 Me, *o*-Me	n.r	n.r.

303 H, *m*-Me	n.r.	11 (VII)
304 Me, *m*-Me	n.r.	n.r.
305 H, *p*-Me	n.r.	n.r.
306 Me, *p*-Me	n.r.	n.r.
307 H, *o*-Cl	n.r.	n.r.
308 Me, *o*-Cl	n.r.	n.r.
309 H, *m*-Cl	n.r.	n.r.
310 Me, *m*-Cl	n.r.	n.r.
311 H, *p*-Cl	n.r.	n.r.
312 Me, *p*-Cl	n.r.	n.r.
313 H, *o*-OMe	96 (VII)	87 (VII)
314 Me, *o*-OMe	n.r.	n.r.
315 H, *m*-OMe	100 (VII)	100 (VII)
316 Me, *m*-OMe	100 (VII)	5 (VII)
317 H, *p*-OMe	94 (VII)	n.r.
318 Me, *p*-OMe	n.r.	n.r.
319 H, (3,4-di-OMe)	n.r.	n.r.
320 Me, (3,4-di-OMe)	n.r.	n.r.
321 H, *o*-Br	n.r.	n.r.
322 Me, *o*-Br	n.r.	n.r.
323 H, *m*-CF_3	n.r.	n.r.
324 Me, *m*-CF_3	n.r.	n.r.
325 H, *o*-NO_2	100 (VII)	
326 Me, *o*-NO_2		
327 H, *m*-NO_2		
328		

Me, *m*-NO_2		
329 H, *p*-NO_2	38 (VII)	48 (VII)
330 Me, *p*-NO_2	100 (VII)	100 (VII)
331 1-naphthyl acetic acid	n.r.	n.r.
332 methyl 1-naphthyl acetate	n.r.	n.r.
333 2-thiophene acetic acid	n.r.	n.r.
334 methyl 2-thiophene acetate	100 (VII)	100 (VII)
335 1-tetrazolyl acetic acid		
336 1-tetrazolyl acetate		

Table6 Data of Substituted phenyl acetic acids and esters.

The data of Table7 show that the presence of an alkyl substituent or of an amino group in the benzylic position of the arylalkanoic acids and esters inhibited the reduction.

Substrate	**Conversions (%)**	
	1	**2**
 **401**	n.r.	n.r.
 **402**	n.r.	n.r.
 **403**	n.r.	n.r.
 **404**	n.r.	n.r.
 **405**	unknown compounds	unknown compounds
 **406**	unknown compounds	unknown compounds

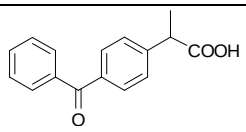
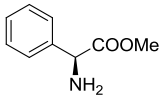
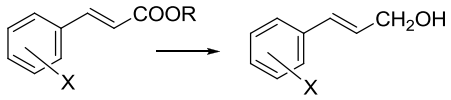
 **407**	n.r.	n.r.
 **408**	n.r.	n.r.

Table7 Data of α-Substituted phenyl acetic acids and esters.

As for substituted cinnamic acids and esters of Table8, in the case of the *p*-methyl derivatives the saturated alcohol was obtained either from the acid and the methyl ester.



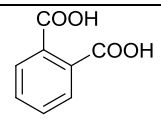
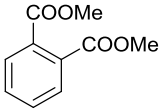
Substrate **R, X**	**Conversions (%)**		
	1	**2**	**3**
501 (H, *p*-Me)	87 +7** (VII)	87 +11** (VII)	84 +16** (VII)
502 (Me, *p*-Me)	88 +12**(VII)	92 +8** (VII)	89 +11** (VII)
503 (H, *m*-NO_2)	Complex mixture of products		
504 (Me, *m*- NO_2)	Complex mixture of products		
505 methyl β-methylcinnamate,	100 (VII) product of MM198		

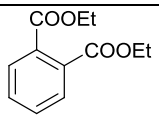
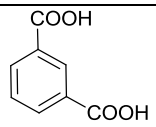
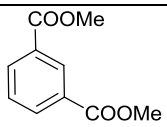
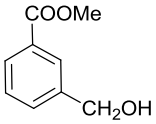
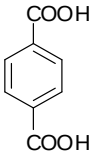
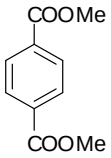
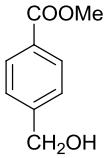
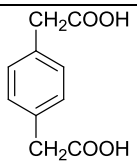
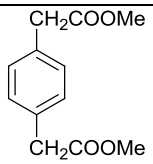
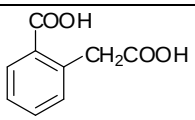
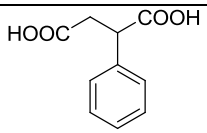
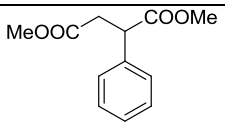
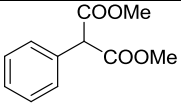
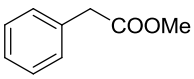
**% saturated alcohol.

Table8 Data of Substituted cinnamic acids and esters

In the series of dicarboxylic acids (Table9), the bioreduction of a single carboxylic group was observed for methyl isophthalate and terephthalate. Malonate derivatives **613** and **614** underwent decarboxylation and the dimethyl succinate **612** derivative was left unreacted.

As for aliphatic esters and acids (Table10), only the methyl ester of 2-hexenoic acid (**704**) was reduced to the corresponding unsaturated primary alcohol.

Substrate	**Products**	**Conversions (%)**	
 **601**	Complex mixture of products	-	-
 **602**	phthalic anhydride		

 **603**	phthalic anhydride		
 **604**	Complex mixture of products	-	-
 **605**		100 (VII)	100 (VII)
 **606**	Complex mixture of products	n.r.	n.r.
 **607**		49 (VII)	64 (VII)
 **608**	Complex mixture of products		
 **609**	Hydrolysis of one COOMe group		
 **610**	Complex mixture of products	n.r.	n.r.
 **611**			
 **612**	.	n.r.	n.r.
 **613**		100 (VII)	100 (VII)

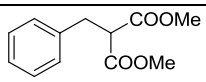
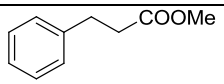
 **614**		100 (VII)	100 (VII)

Table9 Data of Dicarboxylic acids and esters.

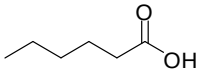
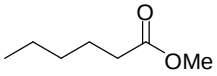
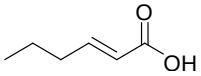
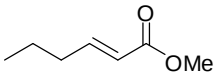
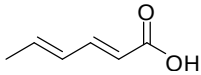
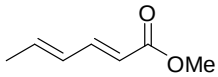
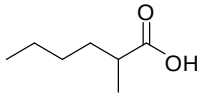
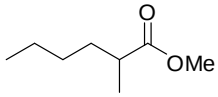
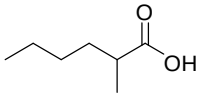
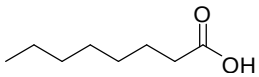
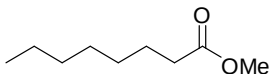
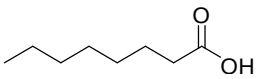
Substrate	Products	Conversions(%)	
		1	2
 **701**	-	n.r.	n.r.
 **702**	Complex mixture of products		
 **703**	-	n.r.	n.r.
 **704**			
 **705**	Complex mixture of products		
 **706**	-	n.r.	n.r.
 **707**	-	n.r.	n.r.
 **708**	 Hydrolysis product	84	
 **709**	Complex mixture of products		
 **710**	 Hydrolysis product	58	

Table10 Data of Aliphatic acids and esters.

4.5 Conclusions and future work

As regards results obtained in this chapter, the zygomycete fungus has shown a particular behavior towards reduction of esters. A more detailed screening and focused studies have to be made.

Future investigations will be devoted to establish first whether this peculiar reducing activity is strain dependent and, then, to isolate the enzyme responsible for this reduction. Once the enzyme will be isolated and characterised work will be done to optimize the reaction conditions, and increase the yields of the reduction.

CHAPTER 5

5.1 General remarks

^{1}H and ^{13}C NMR spectra were recorded on a Bruker ARS 400 spectrometer (400 MHz) in CDCl3 or DMSO-d_6 solution at r.t. using TMS or DMSO-d_6 as internal standard for ^{1}H and $CDCl_3$ or DMSO-d_6 for ^{13}C; chemical shifts are expressed in ppm relative to TMS and J values in Hz.

GC-MS analyses were performed on an Agilent HP 6890 gas-chromatograph equipped with a 5973 mass detector and an Agilent HP-5 (30 m × 0.25 mm × 0.25 μm) column. Temperature program: 60 °C (1 min) / 6 °C min-1 / 150 °C (1 min) / 12 °C min-1 / 280 °C (5 min).

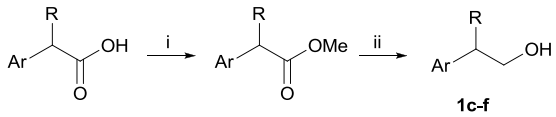
Optical rotations were determined on a Dr. Kernchen Propol digital automatic polarimeter. TLC analyses were performed on Merck Kieselgel 60 F254 plates. All the chromatographic separations were carried out on silica gel columns.

All reagents and solvents were purchased from Aldrich or Carlo Erba Reagenti and used without any further purification.

The enantiomeric excess values were determined by chiral GC analysis performed on a DANI HT 6.10 gas chromatograph equipped with a DAcTBSil.BetaCDX column (25 m × 0.25 mm × 0.25 μm).

5.2 Enzyme mediated oxidations

Preparation of racemic alchols 1c-f



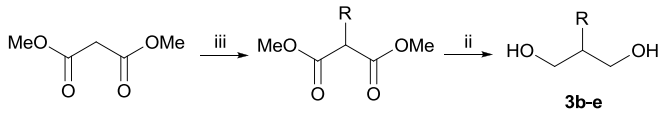
1c-f

i) A solution of the suitable acid and H_2SO_4 in methanol was heated to reflux (64.7°C) for 1-3 hours till consumption of the starting materials (GC/MS). The reaction mixture was poured into brine and then extracted with AcOEt. The organic layer dried over anhydrous Na_2SO_4 and concentrated under reduced pressure.

ii) To a mechanically stirred solution of LiAlH4 and THF a mix of the corresponding methyl ester dissolved in THF was added portionwise. Then the solution was heated to reflux (66°C) till

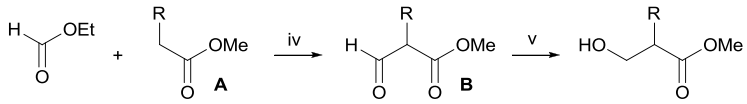
consumption of the starting materials (GC/MS). After 24 h, at room temperature, the solution was poured into acidic water and the organic phase was extracted with Et2O. The mixture was dried over Na_2SO_4 and concentrated under reduced pressure.

Preparation of diols 3b-f



iii) Dimethyl malonate in THF was added to a stirred suspension of NaH (60% in oil) in THF at 0°C. After warming to r.t. and stirring, a solution of the corresponding alkyl bromide in THF was added. The reaction was stirred and then quenched with saturated NH_4Cl. The organic layer was separated and the aqueous phase was extracted with EtOAc. The combining organic layers were dried and the solvent was removed in vacuo.

Preparation of racemic hydroxymethyl acids 4b-f



iv) NaH was suspended in THF and compound of type **A** was added dropwise to the suspension solution thus prepared at room temperature. After the suspension solution was raised in temperature to 40°C and stirred for 15 minutes, ethyl formate was added portionwise for 30 minutes. After the solvent was distilled off, the reaction was cooled in an ice bath and washed with water. The organic layer was separated and the aqueous phase was extracted with EtOAc. The combining organic layers were dried and the solvent was removed under reduced pressure to obtain compounds **B**.

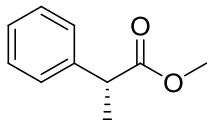
v) Compounds **B** were submitted to a reduction with $NaBH_4$ as reducing agent at 0°C.

Microorganism, growth and biotransformation conditions

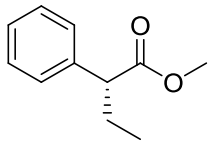
A. *aceti* MIM 2000/28 from the collection MIM (Microbiologia Industriale Milano) was routinely maintained on GYC slants (glucose 50 g l-1, yeast extract 10 g l-1, CaCO3 30 g l-1, agar 15 g l-1, pH 6.3) at 28°C. The strain, grown on GYC slants for 24 h at 28°C, was inoculated into 100 ml Erlenmeyer flasks containing 10 ml of the liquid medium GLY (glycerol 25 g l-1, yeast extract 10 g l-1, pH 5, distilled water) and incubated on a reciprocal shaker (100 rpm); the flask liquid cultures of *A*. *aceti* MIM 2000/28 were used for inoculating a 1 L reactor with 200 mL working volume, agitation speed 250 rpm, air flow rate 1 vvm. The dry weight was determined after centrifugation of 100 ml of cultures, cells were washed with distilled water and dried at 110°C for 24 h.

Compounds **1** and **3** (5mM) dissolved in DMSO (1%v/v) were added to the aqueous bacterial colture (100mL) directly inside the reaction vessel. The flasks were shaken at 150 rpm at 30°C. After 72 hours the solutions were extracted with Et_2O and the combined organic phases dried with anhydrous Na_2SO_4. The residue was chromatographed on a silica gel column, eluting with hexane and increasing amount of ethyl acetate to obtain products **2** and **4**.

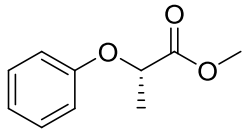
(*R*)-methyl 2-phenylpropanoate, (R)-2a



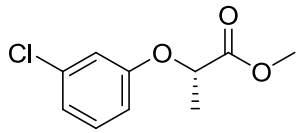
(*R*)-methyl 2-phenylbutanoate (2b)



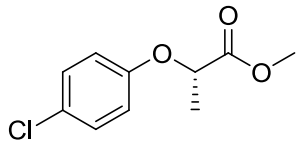
(*S*)-methyl 2-phenoxypropanoate (2c)



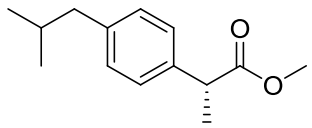
(*S*)-methyl 2-(3-chlorophenoxy)propanoate (2e)



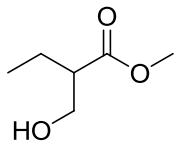
(*S*)-methyl 2-(4-chlorophenoxy)propanoate (2f)



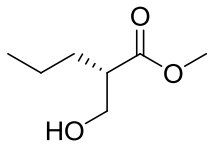
(*R*)-methyl 2-(4-isobutylphenyl)propanoate (2g)



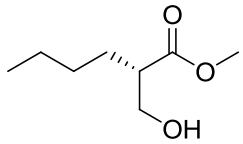
methyl 2-(hydroxymethyl)butanoate (4b)



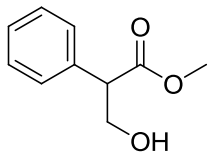
(*S*)-methyl 2-(hydroxymethyl)pentanoate (4c)



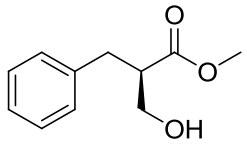
(*S*)-methyl 2-(hydroxymethyl)hexanoate (4d)



methyl 3-hydroxy-2-phenylpropanoate (4e)



(*R*)-methyl 2-benzyl-3-hydroxypropanoate (4f)



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