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# THESE

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## *Caractérisation des alcools déshydrogénases secondaires microbiennes pour la production d'alcools chiraux et de cétostéroïdes et l'utilisation d'un solvant eutectique profond (DES) dans le milieu réactionnel*

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# Dedication

I dedicate this work to my parents, my siblings, my family, my professors and to all my friends.

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# Résumé

Une partie de cette thèse concerne la caractérisation et la purification d'une alcool déshydrogénase secondaire et d'une cholestérol oxydase à partir de cellules bactériennes du genre *Rhodococcus*. Les souches utilisées étaient GK1: *Rhodococcus equi*, GK3: *Rhodococcus opacus*, GK12: *Rhodococcus erythropolis*. Elles ont été utilisées pour évaluer leur capacité à cataboliser les stérols. Les étapes du catabolisme des stérols comprennent le catabolisme de la chaîne latérale et la dégradation du noyau stéroïdien. Afin d'obtenir des dérivés intéressants, des ions de cobalt et de nickel ont été ajoutés au milieu réactionnel pour inhiber la dégradation du noyau stéroïdien et obtenir des dérivés tels que la 4-androstène-3,17-dione (AD) et le 1,4-androstadiène-3,17-dione (ADD). La deuxième partie de la thèse concerne l'utilisation d'un solvant approfondi (DES) contenant des enzymes ADH de différentes bactéries, du genre *Rhodococcus* et *Ralstonia*, et les enzymes ADH de ces souches bactériennes ont été surexprimées dans les cellules d'*Escherichia coli* (*E. coli*). Lorsque 300 à 400 mM de céto-substrats halogénés ont été utilisés dans les réactions avec DES, d'excellents résultats de conversion et d'excès énantiomérique ont été obtenus. Les meilleurs résultats ont été observés lorsque des ADHs de *Lactobacillus brevis* et de *Rhodococcus ruber* ont été utilisés. Enfin, des réactions en grandes quantités ont été réalisées donnant de bons résultats.

Mots-clefs: ADH, GK1 (*Rhodococcus*), DES, cholesterol oxidase, bioréduction

# Abstract

A part of this thesis is about the characterization and purification of a secondary alcohol dehydrogenase and a cholesterol oxidase from wild type bacterial cells of the genus *Rhodococcus*. The strains that were used from this genus were GK1: *Rhodococcus equi*, GK3: *Rhodococcus opacus*, GK12: *Rhodococcus erythropolis*. They were also utilized to see their ability to catabolize sterols. The steps that are involved in the sterol catabolism include the lateral chain catabolism and the steroid nucleus degradation. In order to obtain valuable derivatives, cobalt and nickel ions were added to the reaction medium to inhibit the degradation of the steroid nucleus and to obtain derivatives such as 4-androstene-3,17-dione (AD), and 1,4-androstadiene-3,17-dione (ADD). The other part of the thesis is about using a Deep Eutectic Solvent (DES) with ADH enzymes from different bacteria ranging from the *Rhodococcus* genus to *Ralstonia*, and the ADH enzymes of those bacterial strains were overexpressed in *Escherichia coli* (*E. coli*) cells. When 300-400 mM of the halogenated keto-substrates were used in the reactions with this novel solvent, excellent conversions and enantiomeric excess results were obtained. The best results were observed when ADHs from *Lactobacillus brevis* and *Rhodococcus ruber* were used. Finally, scale up reactions were performed giving good product yields.

Keywords: ADH, GK1 (*Rhodococcus*), DES, cholesterol oxidase, bioreduction

# Résumé détaillé

Dans cette thèse, différentes alcool-déshydrogénases (ADH) de diverses sources bactériennes ont été caractérisées et analysées. Nous avons testé un nouveau solvant pas cher et qui respecte l'environnement appelé le Deep Eutectic Solvant (DES) sur l'activité de bioréduction des ADH à partir de cellules bactériennes recombinantes d'*E. coli*. Nous avons testé l'effet des: substrats cétoniques, de la température, des solvants, l'ajout des cofacteurs et le recyclage sur les ADH obtenus à partir de différentes sources bactériennes. Le DES a considérablement augmenté l'activité de bioréduction de ces enzymes, conduisant à des alcools énantiopurs, qui sont des précurseurs de certains médicaments pharmaceutiques importants.

Ces solvants sont constitués d'un sel d'ammonium quaternaire (par exemple le chlorure de choline) et d'un donneur de liaison hydrogène (HBD) (glycérol). Dans la réaction, lorsque la quantité de DES augmentait, la stéréosélectivité de l'enzyme s'améliorait. Dans une autre série d'études sur le DES, nous avons utilisé ce solvant qui était composé de ChCl: glycérol (1: 2 mol / mol) avec un tampon Tris-SO4 50 mM pH 7,5 dans une réaction contenant de l'ADH et des cétones halogénées comme substrats.

En outre, nous avons essayé de purifier et de caractériser une alcool déshydrogénase secondaire et une cholestérol oxydase à partir d'échantillons bactériens de type sauvage du genre *Rhodococcus*. Les principales souches de *Rhodococcus* utilisées étaient GK1: *Rhodococcus equi*, GK3: *Rhodococcus opacus*, GK12: *Rhodococcus erythropolis*. Ces souches étaient connues pour leur catabolisme des stérols. Lorsque ces souches ont été utilisées avec un stérol, la chaîne latérale du stérol a été catabolisée et des dérivés précieux

tels que la 4-androstène-3,17-dione (AD), 1,4-androstadiène-3,17-dione (ADD) ont été obtenus par l'ajout de métaux comme le nickel. Ces dérivés pourraient être des précurseurs très importants pour la synthèse de médicaments pharmaceutiques. Il y a trois étapes impliquées dans le catabolisme du cholestérol par le genre *Rhodococcus*: l'initiation de la réaction de cholestérol oxydase, le clivage de la chaîne latérale du stérol et la voie de dégradation du noyau stéroïdien.

Nous avons également étudié la capacité de catalyseur des ADH surexprimés de différents micro-organismes dans les DES. Lorsque 50% v/v de DES a été utilisé dans ces biotransformations, avec 300-400 mM des substrats, d'excellents résultats de conversions (> 90%) et un excès énantiomérique ont été obtenus (*ee*> 99%).

Les meilleurs résultats ont été observés lorsque des ADH de *Lactobacillus brevis* et de *Rhodococcus ruber* ont été utilisés. En raison des résultats prometteurs, des réactions de mise à l'échelle ont été effectuées jusqu'à 250 mg et 1 g.

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## List of Figures and Schemes

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## List of Abbreviations

**AD:** 4-Androsten-3,17-dione
**ADD:** 1,4-Androstadien-3,17-dione
**ADH:** alcohol dehydrogenase
**ADH-A:** alcohol dehydrogenase from *Rhodococcus ruber*
**ADH-T:** alcohol dehydrogenase from *Thermoanaerobacter* sp.
**ABTS:** 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
**ATCC**: American type for culture collection
**BSA:** bovine serum albumin
**CAD:** cinnamyl alcohol dehydrogenase
**CADE:** carbamic acid 1,1 dimethyl ethyl ester
**CBFM:** *o*-chlorobenzoylformate
**Chox:** cholesterol oxidase
**CIP**: Collection Institute of Pasteur
**Cobe:** ethyl 4-chloro-3-oxobutanoate
**Cys:** cysteine
**DEAE:** diethylaminoethyl
**DEA/NO:** diethylamine dinitric oxide
**DES:** deep eutectic solvent
**Dm:** dry matter
**DMSO:** dimethyl sulfoxide
**DSM:** German collection of Microorganisms
***ee*:** enantiomeric excess
**EtOAc:** ethyl acetate
**U:** enzyme unit
**GC:** gas chromatography
**GK:** Germain and Kreit
**GK1:** *Rhodococcus equi*
**GK3:** *Rhodococcus opacus*
**GK12:** *Rhodococcus erythropolis*
**GSH:** glutathione
**GSSG:** glutathione disulfide
**His:** histidine
**HLADH:** horse liver alcohol dehydrogenase
**HpCAD:** *Helicobacter pylori* cinnamyl alcohol dehydrogenase
**HDES**: Hydrophobic Deep Eutectic Solvents

**HPLC:** high performance liquid chromatography
**KDa:** kilodalton
**Km:** Michaelis constant
**LBADH:** *Lactobacillus brevis* ADH
***L. kefir*:** *lactobacillus kefir*
**MBE**: α-methyl-1,3-benzodioxole-5-ethanol
**NAD:** nicotinamide adenine dinucleotide
**NADP:** nicotinamide adenine dinucleotide phosphate
**NMR:** nuclear magnetic resonance
**OD:** optical density
**PAGE:** polyacrylamide gel electrophoresis
**R-CMM:** (*R*)-chloromandelate
**RasADH:** alcohol dehydrogenase from *Ralstonia* sp.
***Re*SADH:** *Rhodococcus erythropolis* ADH
**Rt:** retention time
**SADH:** secondary alcohol dehydrogenase
**SDS:** sodium dodecyl sulfate
**Sp:** species
**SyADH:** alcohol dehydrogenase from *Sphingobium yanoikuyae*
**TbSADH:** ADH from *Thermoanaerobacter brockii*
**TeSADH:** alcohol dehydrogenase from *Thermoanaerobacter ethanolicus*
**TLC:** thin layer chromatography
**Vf:** final volume
**yADH**: yeast ADH
**9α-OH-AD**: 9α-hydroxy-4-androstene-3,17-dione
**9α-OH-ADD**: 9α-hydroxy-1,4-androstadien-3,17-dion

## 1. General introduction

The present thesis can be divided into two parts based on the studies that were conducted. The first part was performed in: (The Biology Laboratory of Human Pathologies at the Faculty of Sciences located in avenue Ibn Battouta, BP. 1014 RP, Rabat-Morocco). We investigated the sterol catabolism by the genus *Rhodococcus*. Two enzymes, cholesterol oxidase and secondary alcohol dehydrogenase are involved in the catabolism of sterols. The second part of the thesis was performed in Spain at the (Organic and Inorganic Chemistry Department, University of Oviedo, Avenida Julián Clavería 8, 33006 Oviedo, Spain) and we tested the effect of a new solvent called the Deep Eutectic Solvent (DES) on the performance of ADHs from different bacterial strains.

Bacterial species from the genus *Rhodococcus* are widely used in research due to many reasons: the ease of utilizing them in the laboratory, genetic manipulation to make a lyophilized recombinant form of that bacterial strain, and their efficacy in catalysis (Busch et al., 2019). The natural habitats of these species are soil and water environments. They are also capable to grow in mesophilic and psychrophilic conditions (Warhurst and Fewson 1994). In addition, these bacteria can metabolize various organic compounds such as acetate, herbicides, hydrocarbons, polychlorinated biphenyls and steroids.

The importance of studying the pathways that this bacterium follows in order to catabolize sterol molecules was to see how we can accumulate steroid derivatives in the medium; because the selective cleavage of the sterol side chain can lead to steroid derivatives that are important in industry.

The species that were studied in this thesis were from the genus *Rhodococcus* and they are described as follows:

*Rhodococcus* sp. CIP 105335 strain GK1, *Rhodococcus* sp. GK12 and *Rhodococcus* sp. GK3 are sterol patent degraders. Originally, these species were isolated from polluted soil (Nancy, France) for their capability to grow on plant sterols (phytosterols) as sole carbon and energy sources. The gene of the 16S rRNA was identified for the strain GK1 (accession number: JQ318031, NCBI Gene Bank) and the strain GK12 (accession number JQ318030, NCBI Gene Bank), and GK3 (accession number NC_012522.1, NCBI Gene Bank).

On the basis of this identification in addition to morphological and physiological observations, the strain GK12 was identified as *R. erythropolis*, and strain GK1 might be a new species of the genus (or *R. equi*), and GK3 could be identified as *R. opacus* (Kreit et al., 2012).

Recently, many research studies have been conducted on alcohol dehydrogenases (ADHs), which catalyze redox reactions. ADHs are important enzymes that are widely used to catalyze reversible redox reactions and produce specific alcohols or ketones (Figure 1). These enzymes are present in many microorganisms such as bacteria and yeast. ADHs are able to catalyze reactions after the redox reaction of the NAD and NADP cofactors (Musa and Phillips, 2011).

The importance of ADHs lay in the fact that they produce pure stereoisomers of chiral alcohols. These pure stereoisomers could be precursors of important drugs, which are highly demanded.

Since the natural environment of enzymes is water, recent advances in biocatalysis have shown the use of water like solvents with these sensitive enzymes, to obtain the desired products while maintaining the enzymes' activity (Musa and Phillips, 2011). From this approach, Deep Eutectic Solvents (DES) have been used in many chemical reactions. DES are composed of a quaternary ammonium salt (like choline chloride) and hydrogen bond donor

molecules (HBDs) like polyols (Müller et al., 2015).

After assessing the use of recombinant whole cells overexpressing oxidoreductases in DES-aqueous mixtures and obtaining good results, we investigated in this study the recycling effect of the biphasic systems, which contain the DES and its impact on the ADHs stability (Müller et al., 2015).

In addition to that, we have examined the effect of several factors like the substrate concentration and the temperature change on the performance of different ADHs and we investigated the effect of cofactors on the biocatalytic activity of different ADHs using DES. The limits in bioconversions at a certain substrate concentration are due to the fact that an enzyme usually reaches a saturation point when all its active sites are occupied with the substrate. For this reason, it is advised to add more enzyme quantity in order to convert the substrate(s) at elevated concentrations.

Due to the promising results that were obtained using DES with ADH mediated systems, higher productivities and selectivities were obtained. We used (choline chloride, glycerol) type of DES at a ratio of 1:2 mol/mol with ADH catalyzed reductions of various halogenated ketones (up to 300-400 mM concentration). ADHs from *Lactobacillus brevis* and *Rhodococcus ruber* were scaled up to 250 mg and 1 g, and this demonstrated that it is possible to use these types of reaction conditions at an industrial level.

### 1.1. Part I: Unconventional Media Used for Enzymatic Reactions

Today, due to the major concerns about the environment, industries and chemical companies are seeking to use a type of solvent that has a green aspect to it. Among the new types of solvents, there is a new type called the deep eutectic solvent (DES). These solvents are composed of two parts: a salt like choline chloride (ChCl) and a hydrogen bond donor (HBD)

like urea.

These solvents have many advantages, such as: they are cheap, eco-friendly, and can enhance the bioreduction activities of many oxidoreductases giving high amounts of products and excellent enantiomeric excess (*ee*) results. They also help in reactions that yield precursors of important pharmaceutical drugs.

Ionic liquids (ILs) are made of cations and anions and have a melting point close to 25 ºC. Walden explained in 1914 the first example of an IL, when he found ethyl ammonium nitrate (Walden 1914). Then the first generation of ILs had emerged. But, this first generation had some drawbacks, like molecular instability and toxicity due to their quick oxidation. Wilkes and Zaworotko established in 1992, the second generation of ILs that were more stable; But had a high cost and toxicity (Wilkes and Zaworotko 1992).

Due to the reasons above, researchers produced easily a new green solvent called the DES, and this solvent had more stable ions, was easily biodegraded, and its components had a cheap price (Abbott et al., 2004). This solvent also enhanced the activities of many enzymes giving good products and enantiomeric excess results (Ibn Majdoub Hassani et al., 2019). It is also very important to note that there is another type of DES called hydrophobic deep eutectic solvents (HDES) in the scientific research field; and this type was synthesized to overcome the limits of hydrophilic DES (like their interaction with polar compounds only). HDES can be used to extract non-polar organic and inorganic compounds from an aqueous medium. HDES can be synthesized by mixing a HBA (*e.g.*, DL-menthol) with a HBD like decanoic acid (DecA) at a molar ratio of 1:1. The solution is heated and stirred (Cao et al., 2017). HDES can be used for various purposes like liquid–liquid extraction of volatile fatty acids (VFAs), liquid-liquid extraction of metal uranyl nitrate from aqueous acid. Moreover, a HDES composed of methyl trioctyl ammonium chloride and various alcohols, could extract

(13-23 mg/g) of polyprenyl acetates from leaves powder of *Ginkgo biloba* (Cao et al., 2017).

**General Objectives**

- To highlight the importance of DES in research as a novel eco-friendly, green medium that can be used in various biotechnological fields.

- To study the activity and selectivity of microbial ADHs in the presence of DES and to synthesize enantiopure halohydrins.

- To deepen and generalize the induction study of the alcohol dehydrogenase and cholesterol oxidase, which is produced by three species of the genus *Rhodococcus*: *R. erythopolis* (strain GK12), *R. opacus* (strain GK3) and *R. equi* (strain GK1).

- To purify the oxidoreductase enzymes using chromatographic techniques (purifying SADH from GK1), and to characterize chox and SADH.

**1.2. The definition of alcohol dehydrogenases (ADHs)**

Alcohol dehydrogenases (ADHs) belong to a large family of oxidoreductases that use the cofactor NAD(P) as an acceptor of electrons. These enzymes catalyze oxidoreduction reactions. ADHs are enzymes that reduce ketones and aldehydes or oxidize alcohols. These enzymes have different protein chain lengths (Figure 1). There are the long chain iron containing ADHs (750 amino acids), the medium chain ADHs (350 amino acids), which are Zn dependent, and the short chain ADHs (250 amino acids which are also Zn dependent) (Persson et al., 1991).

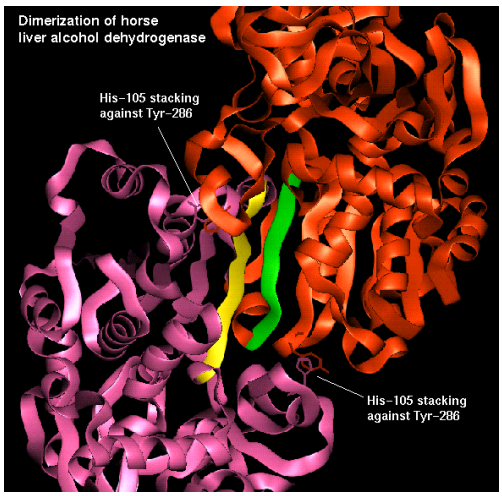



**Figure 1:** the structure of an ADH.

[http://www.cryst.bbk.ac.uk/PPS95/course/9_quaternary/adh_q3sm.gif] Consulted on the 02/05/2018

### 1.3. The ADH system by Hans Jörnvall

In general, the ADH family is composed of four classes, and they can contain Zn. The classes of ADHs have evolved from one another, and the variation in alleles in Class I results in the variety of alcohol metabolism between populations. In humans, there are five classes of ADHs (Table 1).

**Table 1:** the five classes of ADHs in humans (Elalami 2004).

| Classes | Nomenclature of the ADH |
|---|---|
| **1** | (Class I) ADH 1 A, α polypeptide<br>(Class I) ADH 1 B, β polypeptide<br>(Class I) ADH 1 C, γ polypeptide |
| **2** | ADH 4 (class II), π polypeptide |
| **3** | ADH 5 (class III), χ polypeptide |
| **4** | ADH 7 (class IV), μ or σ<br>polypeptide |
| **5** | ADH 6 (class V) |

The major enzyme that metabolizes alcohol in the human body is the ADH, which is present in the liver (Figure 2). The medium chained ADH is the most studied and is present mostly in the liver. ADH is also found in prokaryotes and Drosophila. There are various families of ADHs. The five classes of ADH derive from gene duplication.

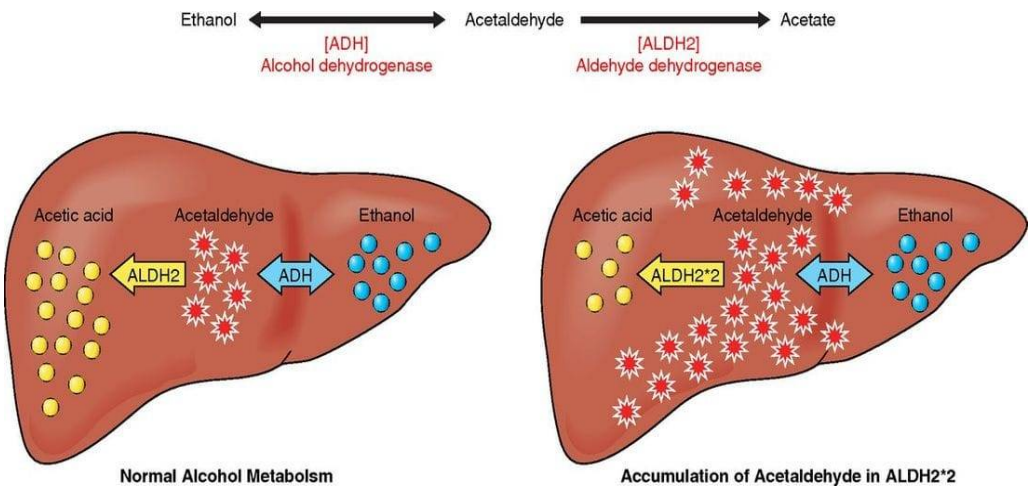



**Figure 2:** ethanol metabolism by an ADH in the liver.

[https://www.thechhe.com/articles/alcohol-intolerance-the-cause-of-your-symptoms] Consulted on the 02/05/2018

Class I is the liver enzyme and it metabolizes ethanol. There are 3 isozymes subunits. Also, the ADH chain length differs from enzyme to enzyme, there are long chains, and short chain ADH and medium chain families. There is also a short chain ADH in Drosophila, and an iron containing ADH. The short chain family of ADH contains 50 different enzymes.

On the other hand, isozymes are enzymes with same function but different amino acid sequence. They are also located in different regions of the body. There are isozymes found in class I of liver ADH. The oldest and ancestral form of ADH is class III and was found in ancient invertebrates. Allelic differences result in alcoholism and diseases related to alcohol.

For this reason, we find the inactivated form of the enzyme in some of the Asian populations and therefore they cannot metabolize alcohol normally.

Also, novel classes of ADHs should be characterized based on their functions and reductase studies should be pushed a little further. Finally, new ADHs families should be investigated in the future (Jörnvall and Höög, 1995).

### 1.4. Groups and types of ADHs

In this section, some types of ADHs will be discussed such as ADHs from **bacteria**, **yeast, plants, mammals, and humans**. ADHs could be divided to different groups based on their catalytic activities. Also, the substrates that ADHs tend to oxidize are primary or secondary alcohols. Other substrates of ADHs include aliphatic, branched, aromatic aldehydes and ketones. The specifities of ADHs in regard to the coenzyme and the substrate depends on the origin of the enzyme. ADHs could be divided into three main groups based on their catalytic activities and their reaction mechanisms (Jörnvall et al., 1987).

The main types of ADHs from some living organisms are summarized in the sections below (Harada et al., 1978).

### 1.5. Human ADHs

There are five classes of ADHs in humans (Table 1) (Sultatos et al., 2004). These enzymes are encoded by different genes, which are located in chromosome 4. The main function of the ADH in the human body is to break down the alcohol and convert it to acetaldehyde, so that it is easily metabolized by the cells of the body (Figure 2). The ADH of Class 1 is a hepatic ADH which consists of α, β, and γ isoenzymes. These ADHs are mostly found in the lining of the liver and the stomach.

ADH1 is a classical ethanol dehydrogenase, which is abundant in the liver of all vertebrates.

The human ADH1 is dimeric and divided to three isoenzymes (ADH1A, ADH1B, ADH1C). Its major function is ethanol metabolism. ADH2 is also found in the liver, the high value of its Km for ethanol plays an important role when ethanol is ingested at high quantities. This enzyme is specific for alcohols and hydrophobic aldehydes. On the other hand, ADH3 is a formaldehyde dehydrogenase which is dependent on glutathione. It catalyzes the oxidation of formaldehyde to formate in the presence of glutathione. This enzyme is considered to be the most ancient form of ADHs, which all other ADHs of vertebrates have evolved from. This enzyme is also active with primary alcohols and aldehydes with a long alkyl chain (Vangnai and Arp, 2001).

### 1.6. ADHs from microorganisms

#### Secondary alcohol dehydrogenases from lactic bacteria

The diacetyl reductase of *Lactobacillus kefir* is known for its capacity to produce acetoin (+). Its purification led to the separation of two isoenzymes E1 and E2 (Hummel et al., 1989). It is a monomeric protein of 66 kDa for E1 (specific activity= 1061 U/mg) and of 74 kDa for E2 (specific activity=366 U/mg). Its optimum pH is estimated to be 4.3 for E1 and 5 for E2. The optimal temperature of the two isoenzymes is 70 ºC. The diacetyl reductase of this bacteria could act on a variety of diketones. The value of its Km constant for diacetyl was estimated at 310 mM in the case of E1 and at 67 mM in the case of E2. These Km values are superior to the ones found in diacetyl reductases of *Kluyveromyces marxianus* (2.5 mM), of *Saccharomyces cerevisiae* (2 to 2.3 mM), of *Bacillus polymyxa* (30 mM), and of *Saccharomyces carlbergensis* (12 mM) (Schwarz and Hang 1994). The enzyme of *L. kefir* could not catalyze the inverse reaction which was the oxidation of acetoin to diacetyl in the presence of NAD. The ions $Cu^{2+}$, $Fe^{2+}$, $Hg^{2+}$ are strong inhibitors of its enzymatic activity. However, $Mn^{2+}$ stabilizes it. Many properties of diacetyl reductase of *L. kefir* are similar to

the ones in lactate dehydrogenases. Its properties include stability of the temperature, the necessity of the $Mn^{2+}$ ions, and an acidic pH.

The species of the genus *Rhodococcus* are known by their power to metabolize a large variety of natural products and of organic toxic molecules. They first oxidize alcohols to aldehydes or ketones. Also, many ADHs were isolated and characterized from this genus, like *R. rhodochrous* PNKb1, *R. erythropolis* DSM 1069, *Rhodococcus* sp. GK1 (Krier et al., 1998; Kreit and Elalami 2002) and *R. ruber* DSM 44541 (Kosjek et al., 2004).

### 1.7. ADH of yeast (*Saccharomyces cerevisiae*)

A research group tried to compare between the ADH of yeast and the ADH of mammals and has found that the molecular weight of the yeast's ADH was twice that of the mammalian and it was 100 times more active (Raj et al., 2014). Another study performed on yeast ADH *Saccharomyces cerevisiae* has proved that the alcohol dehydrogenase I was able to reduce acetaldehyde to ethanol when glucose was fermented. Also, that enzyme was made of 347 amino acid residues; and its structure was determined using X-ray crystallography. ADH I is composed of four subunits A, B, C, D. In addition, that enzyme had a catalytic zinc which was coordinated with Cys-43, Cys-153, and His-66 (Hayes and Velick, 1954).

Another research team led by De Smidt et al. 2008 has analyzed the kinetic characteristics and the structure of ADHs in *S. cerevisiae* and they found that this yeast possessed different isozymes of the ADH. They also determined the primary structure of the ADHs from that yeast and they found that its molecular weight was of 150 kDa, and it also contained four reactive sites. Moreover, they discovered that calcium ions stabilize the ADH from this yeast by inhibiting its unfolding.

That team also found that there was another type of ADH in *S. cerevisiae* entitled ADH4p which was activated by zinc and was structurally like the ADH from *Zymomonas mobilis.*

Similarly, ADH6, which is another type of ADH in *S. cerevisiae*, was found to be NADPH dependent and had a three dimensional crystal structure as it was made of two 40 kDa subunits.

In addition, that research team investigated the active center of ADHs in *S. cerevisiae* and discovered that the monomer components of the tertiary structure of that enzyme can be divided to two domains: the coenzyme binding domain and the catalytic domain. Besides, the active site contains a hydrogen-bonded proton relay system. Generally, that group confirmed that the active sites of *S. cerevisiae* isozymes are the same (De Smidt et al., 2008).

Also, ADHs from this yeast are involved in the redox reactions of either alcohols or ketones, depending on the yeast's growth environment. In ADH1p and ADH2p, the enzyme is responsible to reduce ketones which leads to the corresponding alcohol. These two isoenzymes can also oxidize ethanol. ADH1p shows a high Km value for ethanol (17000-20000 μM). On the other hand, ADH2p tends to produce acetaldehyde and NADH when ethanol levels are low during anaerobic growth (De Smidt et al., 2008).

Finally, the ADHs of this microorganism has a large range of substrate specificity such as: linear, branched alcohols, aldehydes, cinnamyl alcohols, and benzaldehydes (Dickinson and Monger, 1973, Elalami 2004).

**The horse liver alcohol dehydrogenase**

According to kinetic studies, the ADH from horse liver is a dehydrogenase that follows a sequential ordered Bi Bi mechanism (Shanmuganatham et al., 2018). The coenzyme is the first substrate which gets fixed on the free enzyme. This coenzyme fixation induces a conformational change and the enzyme adopts a closed active conformation, limiting the circulation of water inside the protein. The alcohol gets fixed on this active form of the enzyme leading to the formation of a tertiary complex, enzyme-NAD-substrate.

### 1.8. Characteristics of alcohol dehydrogenases from different microbial sources

The species of the genus *Rhodococcus* are known by their power to metabolize a large variety of natural products and organic toxic molecules. The first step in the degradation of alcohols is their oxidation to aldehydes or to the corresponding ketones (Abokitse and Hummel, 2003).

Also, with the new bioengineering techniques, scientists were able to clone the appropriate ADH gene and produce recombinant cells that could transform the organic molecules to the enantiopure product like what was done on the *Rhodococcus erythropolis* DSM 43297 bacteria (Ludwig et al., 1995).

During the work done to elucidate the biodegradation pathway of alkanes and hydrocarbons by this bacteria, a secondary ADH was discovered. It is an NAD-dependent enzyme and is active on a large variety of substrates, especially aliphatic secondary alcohols. The optimal activity is observed with linear secondary alcohols containing eleven carbon atoms. The purified enzyme has shown a high activity for the (*S*) form of 2-octanol.

The physical characterization has shown that the native enzyme is composed of two subunits of 48 kDa each. The biochemical characterization shows that the value of the Km constant decreases when the length of the hydrocarbon chain increases. This shows the importance of

hydrophobic interactions in enzyme-substrate links.

The preference of *R. erythropolis* ADH for secondary alcohols with a medium alkyl chain is explained by the fact that the bacteria grow easily on alkanes containing 12 carbon atoms or more. The strain starts the degradation of alkanes via a subterminal oxidation producing secondary alcohols, which are oxidized by a secondary ADH to the corresponding ketones (Ludwig et al., 1995, Elalami 2004).

### 1.9. *Rhodococcus ruber* DSM 44541

The ADH of this strain of *Rhodococcus* is NAD and zinc dependent. It is a dimeric protein of 38 kDa by subunit. Its maximal activity is obtained at around 40 ºC. Its half-life at 50 ºC is 35 h. Its optimal pH is 6.5 for reduction and 9 for oxidation. The enzymatic catalysis follows a sequential mechanism. Its substrates are secondary alcohols of medium alkyl chain and ketones. The primary alcohols and aldehydes are not accepted. This dehydrogenase can tolerate high concentrations of organic solvents in the reaction media (until 50%, v/v) or 2-propanol (80% v/v), and this is demanded in the NAD cofactor regeneration industry (Kosjek et al., 2004, Elalami 2004).

### 1.10. The alcohol dehydrogenase from *Lactobacillus brevis* (LBADH)

Many research teams tried to characterize the alcohol dehydrogenase from *Lactobacillus brevis* (LBADH), by studying different methods that improved the activity of that enzyme (Leuchs and Greiner 2011). LBADH was found to be an excellent and a widely used enzyme that works perfectly with different factors such as organic solvents, IL, biphasic media, gaseous reaction conditions, and still gives good enantiomeric products. This enzyme can reduce different substrates, among them, substrates with the 2-keto motif and it has a good regio and stereo specificity. In addition, LBADH can be used as a whole cell enzyme overexpressed in *E. coli* and also as an isolated enzyme. This enzyme can also work in soluble

conditions or immobilized ones.

Depending on the products that research teams want to obtain from LBADH a specific ratio method can be chosen while using LBADH/regeneration enzyme systems. This differs from when using whole cells, as intracellular cofactor concentration is enough to get the desired product. Besides, the half-life of LBADH (is usually from a couple of hours to 1000 h), and it depends on several factors like: the presence of the cofactor, organic co-solvents, magnesium, pH, buffer, temperature, etc. (Leuchs and Greiner 2011). Due to the many advantages and promising results that were obtained using the LBADH enzyme from previous research teams, we used it during this thesis in many reactions that contained DES as a solvent.

### 1.11. The alcohol dehydrogenase from *Thermoanerobacter ethanolicus* (TeSADH)

*Thermoanerobacter ethanolicus* (TeSADH) comes from a bacterial strain that usually thrives at high temperatures (70°C) and ferments sugar to ethanol and carbon dioxide. In the following section, some studies will be highlighted to explain the properties and the preferred substrates of this type of ADH (Pham and Phillips 1990).

TeSADH catalyzing performance depends on the type of the substrate, on its structure, and on temperature. A research team led by Bryant et al. (1988), have purified and investigated the properties of a primary and secondary ADH from *Thermoanaerobacter ethanolicus.* They have found that the two enzymes were thermostable and were NADP-dependent; they could also reversibly catalyze ethanol and 1-propanol to aldehydes (Bryant et al., 1988). The substrates of choice for the primary ADH in that strain include several alcohols and aldehydes such as ethanol, propanol, butanol, pentanol, hexanol, heptanol, 2-methyl-1-propanol, acetaldehyde and propionaldehyde. On the other hand, the secondary ADH demonstrated a substrate preference towards ethanol, 1-propanol, 2-propanol, 2-butanol, 2-pentanol, 1,2-propanediol, acetaldehyde, propionaldehyde, acetone, and 2,3-butanedione (Bryant et al.,

1988).

Another study conducted by Pham and Phillips have focused on the effect of substrate structure and temperature on the stereospecifity of a secondary alcohol dehydrogenase from *T. ethanolicus* and the results showed that there was a temperature-dependent reversal of stereospecifity. Based on the $k_{cat}/K_m$ values at reaction at reaction temperatures below 26°C, (*S*)-2-butanol was the perfect substrate. In contrast, above 26°C (*R*)-2-butanol was the substrate of choice. Also, at 60°C (*S*)-2-pentanol was the appropriate substrate; as opposed to (*R*)-2-pentanol, which is well metabolized at temperatures above 70°C (Pham and Phillips, 1990). In addition, (*S*)-2-hexanol is the preferred substrate for that enzyme at 240°C. Also, the enzyme oxidizes more efficiently *S* alcohols with increasing chain length. According to $k_{cat}/K_m$ values, the substrates that were metabolized by TeSADH were: cyclobutanol, cyclopentanol, and cyclohexanol. The *S* enantiomer of 2-butanol, 2-pentanol, and 2-hexanol fit best with the enzyme's active site. There are large and small alkyl-binding pockets in the TeSADH's active site; for the *R* alcohols, it is the small pocket that binds to the methyl and ethyl groups. However, for the *S* alcohols, the large enzyme pocket accepts groups like *n*-butyl (Pham and Phillips, 1990). Finally, these studies demonstrate that TeSADHs are sensitive to temperature and to the substrate's structure.

### 1.12. Group I of ADHs

This group is composed of zinc dependent ADHs which are made of long peptidic chains: 350 to 400 amino acid by subunit. The enzymes of this group are arranged in tetramers, like the ADHs of yeast, or in dimmers like the ADHs of vertebrates. All vertebrate ADHs belong to group I. Some dehydrogenases of this group were isolated from plants, insects and bacteria. Vertebrate ADHs are divided into eight classes which are different by their structure. Group I enzymes are the most studied and are considered to be good models in biochemical research (Harada et al., 1978, Elalami 2004).

### 1.13. Structural particularities

The ADHs of group I exist in the dimeric form, like the ADH of horse liver, or in tetrameric form; like the ADH of yeast. Each subunit is organized in two different domains: the catalytic domain, containing the fixation site of the substrate and the domain of fixation of the coenzyme. The tertiary structure of the horse liver ADH was used as a model for tertiary structures of other ADHs. Even if the sequence homology in amino acids is weak in enzymes of the same family, the conformation of these enzymes is quite similar (Eklund et al., 1974).

The comparison of different primary structures of ADH of group I with the primary structure of horse liver ADH has shown that there is 32% sequence homology. Forty-seven ADHs were studied and have shown the existence of identical sequences in all those dehydrogenases. It had been confirmed that amino acids have an important role at the structural and functional level of the horse liver ADH (Eklund et al., 1974).

### 1.14. Fixation domain of the coenzyme

The coenzyme NAD (Figure 3) is an important factor that activates the ADH. This was confirmed through crystallographic investigations, which were done on horse liver alcohol dehydrogenase (HLADH). Also, change in the enzyme's conformation occur when NAD participates in these redox reactions making the active site dehydrated because NAD become reduced and the substrate gets oxidized by the enzyme leading to the product.

A molecule of NAD is fixed in the fixation site of the enzyme. The NAD usually is fixed at the interface of its domain with the catalytic domain on many amino acid residues and during this process hydrogen bonds and ionic links are formed. The NAD molecule, which is flexible, can adopt different conformations. Its interactions in the fixation site permits to take a different conformation than the one it adopts in its free state (Antoine et al., 1999).

### 1.15. Zn function

All ADHs of group I are Zn dependent. They can possess two atoms of $Zn^{2+}$ by subunit. The zinc has a structural and catalytic role. The four amino acid residues which are implicated in the link with the structural zinc are present in almost all ADHs of group I. The structural zinc is linked to the cysteins 97, 100, 103, and 111. These four residues are at the exterior of the catalytic domain and this pocket has little interaction with the rest of the subunit and protects the Zn atom from water. The catalytic zinc is attached to the cysteins 46 and 174 and to the histidine 67 (Figure 4). This $Zn^{2+}$ is also attached to a molecule of water. When the substrate is linked to $Zn^{2+}$, this molecule will be relocated (Green et al., 1993, Elalami 2004).

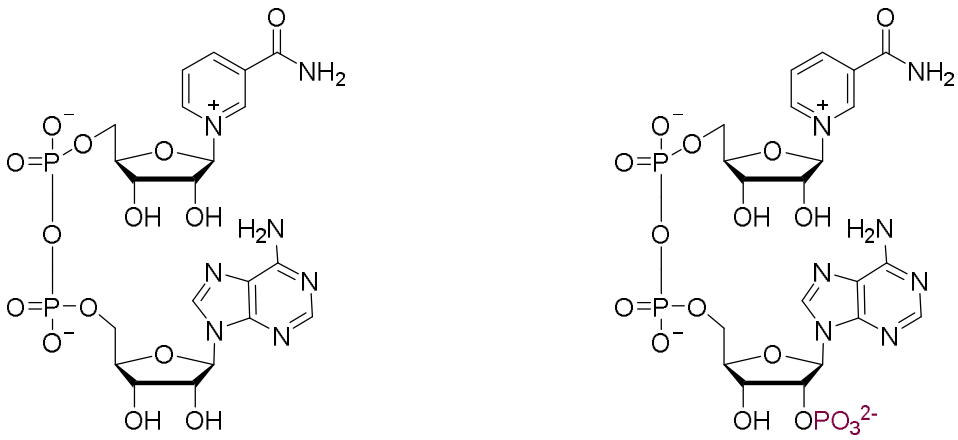



**Figure 3:** the NAD (left) and NADP (right) structures.

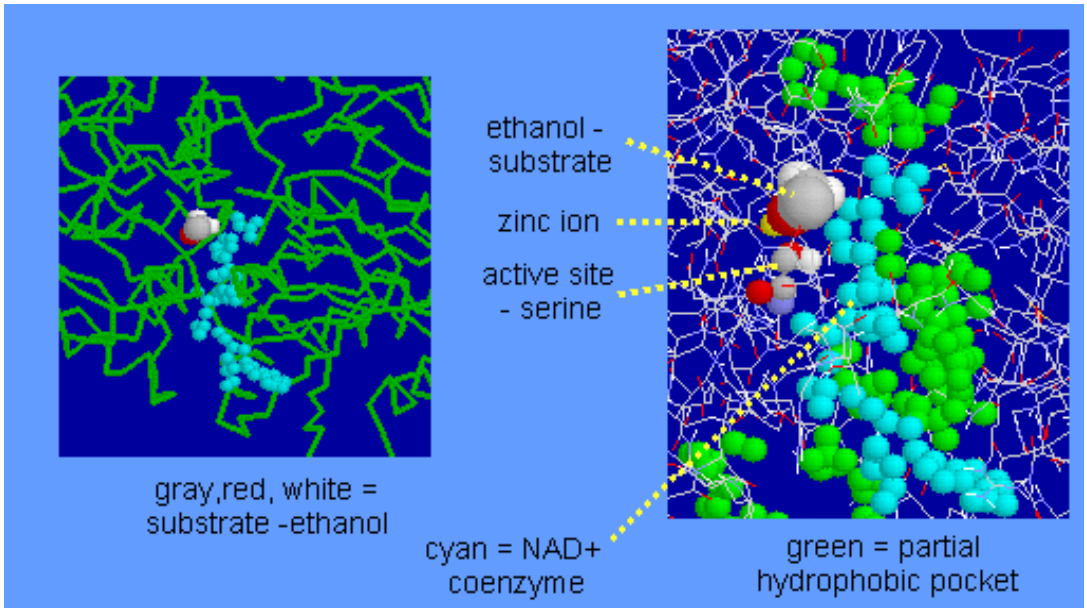



**Figure 4:** an ADH with the $NAD^+$ coenzyme.

[http://chemistry.elmhurst.edu/vchembook/571cofactor.html] Consulted on the 02/05/2018

### 1.16. The fixation site of the substrate

The fixation site of the substrate is a pocket, which does not exist in the apoenzyme, because it is the coenzyme, which by its fixation provokes its formation. The constitutive residues of this pocket are non-polar amino acids, creating a hydrophobic environment (Figure 5). Also, the ADHs specificity to substrates permits to differentiate between two enzymatic types: primary ADHs which oxidize primary alcohols and secondary alcohol dehydrogenases, which oxidize secondary alcohols and reduce ketones to alcohols.

The specificity of ADHs for the substrate is determined by the size and the form of the pocket. The comparison of horse liver ADH and the ADH of the yeast has shown that 12 different residues exist at this site. This amino acid variation explains the specificity of these two enzymes: the ADH of the horse liver is capable of oxidizing alcohols that have a long alkyl chain; on the other hand, the ADH of yeast can only oxidize alcohols with a short chain. It had been shown that the presence of small sized amino acids at the active site of the horse

liver ADH had permitted to this enzyme to oxide alcohols of big sizes. Also, it was suggested that tryptophan forms a perturbance at the entry of the substrate fixation pocket in yeast ADH and this inhibits the entry of alcohols that have a long chain to the active site of the enzyme. In the horse liver ADH, leucine, an amino acid, occupies this position. Also, it was shown that substitutions of amino acids, which enlarge the substrate fixation pocket, permits to enlarge the yeast's ADH specificity so that it can oxide alcohols which have a long alkyl chain (Sofer and Ursprung, 1968, Elalami 2004).

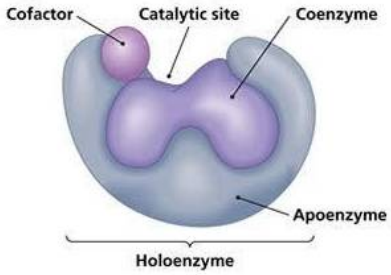



**Figure 5:** the different components of an enzyme.

[https://www.quora.com/What-is-apoenzyme-coenzyme-and-holoenzyme] Consulted on the 02/05/2018

### 1.17. Mechanisms of ADHs

In this section of the thesis, the mechanism of ADHs from different living organisms will be discussed. Another study performed on the liver alcohol dehydrogenase has confirmed that the appropriate substrates for this enzyme were propan-2-ol and butan-2-ol, however that enzyme showed low activity with ketones. The enzyme was able to oxidize alcohols such as propan-1-ol and 2-methylpropan-1-ol. It was also able to reduce propianaldehyde and 2-methylpropionaldehyde. The enzyme was inhibited by primary alcohols because at large concentrations of that substrate, an additional complex of the enzyme was formed and aborted. That complex was composed of two components or pathways; the enzyme NADH complex and the alcohol-NADH complex. Also, this enzymatic reaction followed an ordered sequential mechanism (also known as Theorell-Chance mechanism) when primary alcohols

and aldehydes were used as substrates. In that experiment, they explained how the coenzyme and the substrate were dissociated from the active site of the enzyme (Figure 6) (Kosjek et al., 2004).

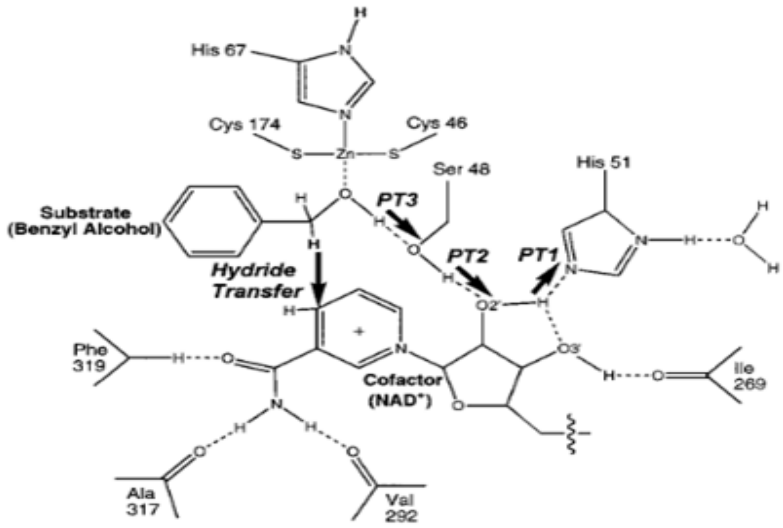



**Figure 6:** the mechanism for the proton and hydride transfer in the liver ADH (Agarwal et al., 2000).

Another research group has studied the oxidation kinetics of some alcohols such as propan-1-ol, ethanol, butanol and propan-2-ol. The enzyme involved was the alcohol dehydrogenase from yeast, and the cofactor for that oxidoreduction reaction was NAD(H). This reaction followed an ordered mechanism and the rate limiting step was the dissociation of the product from the enzyme-$NAD^+$ complex. However, the step that limited the rate in ethanol oxidation was the dissociation of the product enzyme-NADH complex. These studies highlight the importance of understanding the ADH enzyme kinetics as this might lead to other oxidoreductase pathways that can be very important in the pharmaceutical industry (Krier et al., 1998). Also, ADHs are enzymes that use the NADPH cofactor in the oxidoreduction reactions. There is usually a hydride attack that occurs in the *Si*- or *Re*- face of the carbonyl compound. (Explanation: when we have a prochiral $sp^2$ carbon, the *Re* face means that when we look at the face of the substituent it follows a clockwise order-Prelog and the hydrogen

attacks the *Re*-face giving a chiral product and the alcohol in that case is on the dash line and the hydrogen on the bold line. In the *Si* face it is the opposite of the statement above, and it follows a decreasing Cahn-Ingold-Prelog order (counter clockwise) of the substrate). After the bioreduction has occurred, usually an enantiopure (*R*)- or (*S*)-alcohol is produced. Chemists use the Prelog's rule to predict the enantiopure product based on the size of the elements attached to the chiral alcohol. During the bioreduction of alcohols, the NADP cofactor and the alcohol bind in a sequential manner to the active site of the enzyme, here the catalytic mechanism of the ADH will be discussed (Mee et al., 2005).

The ADH from horse liver follows an ordered sequential mechanism (Figures 6 and 7). The coenzyme is the first substrate which gets fixed on the free enzyme. This coenzyme fixation induces a conformational change and the enzyme adopts a closed active conformation, limiting the circulation of water inside the protein (Schneider et al., 1985). The alcohol gets fixed on this active form of the enzyme leading to the formation of a tertiary enzyme-NAD-substrate (Dalziel and Dickinson, 1966).

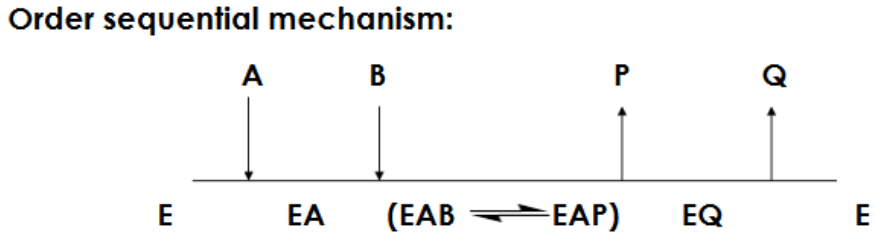



**Figure 7:** the catalytic mechanism of an ADH. **A, B**: substrates. **P, Q**: products. **E**: free enzyme. **EA**: enzyme unstable transitory complex. **EAB**: enzyme central complex. **EAP**: enzyme with the substrate and the formation of the product. **EQ**: enzyme with product.

[http://slideplayer.com/slide/8060700/] Consulted on the 02/05/2018

### 1.18. Group II of ADHs

This group is composed of Zn-independent ADHs with a short peptide chain, which contain 250 amino acids by subunit. These ADHs are found in all biological systems. The

Drosophila's ADH belongs to this group. In a study performed by Sofer and Ursprung, they compared the ADHs from two different strains of *Drosophila melanogaster* and they found that the suitable substrates for that enzyme were secondary alcohols and that it had a molecular weight of 4.4 $x10^4$. Also, that ADH exists in multiple forms and it was first detected using gel electrophoresis of crude fly extracts (Sofer and Ursprung, 1968, Elalami 2004).

**1.19. Group III of ADHs**

This group is composed of ADHs which are activated by iron. Their subunits contain each around 385 amino acids. These ADHs were identified in different bacteria and yeast.

A research team led by Antoine et al. 1999 has purified a NADP-dependent group III alcohol dehydrogenase from *Thermococcus hydrothermalis*. This strain grew at 85 °C and at a pH of 6. The team also cloned the gene that encoded this enzyme and over expressed it in *E. coli*. Then that recombinant enzyme was purified, characterized and compared with the native form of the enzyme. The enzyme structure was determined as a 197-kDa tetramer. The convenient substrate for that enzyme was an aldehyde and it required the NADPH cofactor to function (Figures 3, 4, and 5). That enzyme also oxidized alcohols, methanol, and glycerol (Antoine et al., 1999, Elalami 2004).

**1.20. The alcohol dehydrogenase of plants**

The ADH is a widely distributed enzyme among living organisms as it is also found in plants. In a study conducted on poplar plants, Baucher et al. 1996 have manipulated cinnamyl alcohol dehydrogenase (CAD) in a transgenic poplar plant via genetic methods such as: co-suppression and antisense strategies. CAD is the enzyme that leads to the synthesis of monolignols in plants. Monolignols are involved in the biosynthesis of lignin, which forms cell walls of plants. In that study, the group has manipulated the CAD enzyme in transgenic

poplar plants to see the relation of its activity with xylem tissues. They investigated if these transgenic plants had a reduced activity of the CAD enzyme by examining the xylem of these plants. They found that 3 of the transgenic lines that were modified genetically had a reduced CAD activity by 70% when compared with a control. However, one of the transgenic lines (CAD49) was reduced by 40%. This study was important in the wood industry because lowering the CAD activity in trees could be a good strategy to optimize processes in the pulp and paper industry (Musa and Phillips, 2011).

*ADH in the pea plant*

A research team led by Leblova and Mancal in 1975 have studied and characterized the alcohol dehydrogenase of the pea plant. They isolated, purified, and measured the activity of alcohol dehydrogenase from germinating pea; and they found that the activity of that enzyme was 80000 units per mg protein. They also characterized the ADHs of bean and lentil; and have discovered that the molecular weight of pea and bean ADH was 60000, and for lentil it was 70000. Also, when they compared the three enzymes, they found that they have similar $K_m$ values. The convenient pH for these plant enzymes was (8-9), and these alcohol dehydrogenases were able to oxidize monovalent alcohols. In addition, the enzyme was denatured when the temperature was above 60°C. The inhibitors of plant ADH were pyrazole, imidazole and pyridine. That team also compared these plant ADHs to animal and yeast enzymes and found that they shared similar characteristics (Leblova and Mancal, 1975). The team increased the specific activity of pea ADH 174 times by avoiding the increase of temperature and avoiding ethanol fractionation. Instead, the pea seeds were grown in petri dishes and the ADH enzyme activity was measured at 340 nm in media containing sodium phosphate, mercaptoethanol and 100 mM ethanol and 860 µM NAD at 20°C. After that, the Lowry et al. (1951) method was used for protein determination and the molecular weight of

the ADH from the pea plant was obtained by gel filtration on a column of Sephadex G-200, using 0.01 M tris acetate buffer for elution. When compared to ADH from liver, yeast and tea; the molecular weight of plant ADH (pea, bean, lentil), were lower. Also, plant ADH use NAD as a coenzyme. In addition, plant enzymes oxidize alcohols that do not have a long chain of carbon atoms. The substrate of choice for the three plant enzymes were primary alcohols like mercaptoethanol and methanol for the pea ADH, and 2-propene-1-ol is oxidized by all three ADHs. However, these three enzymes do not oxidize: isooctanol, *n*-dodecan-1-ol, 1,2-propanediol, and 1,3-butanediol. When comparing ADH from plants to the one from horse liver, they found that unlike the latter, plant ADHs do not catalyze cyclohexanol and sec butanol; but they share substrate specificity with animal ADH and with yeast ADH. Also, the Km values for the substrate and the co-enzyme of plant ADH are like those of yeast ADH ($1.7x10^{-2}$ M) for ethanol. Moreover, the team concluded after testing the effect of temperature on the denaturation of the three plant enzymes, that the ADHs lose their activity when they are in temperatures above 60°C. The team also concluded based on the $K_m$ and $K_i$ values that the active centers of plant and animal ADHs are different. In addition, the researchers studied the effect of different inhibitors on the three plant ADHs, and they found that pyrazole was the strongest inhibitor competing with the ethanol substrate (Leblova and Mancal, 1975).

### 1.21. Applications of ADHs: pharmaceutical industry

Today, ADHs from different microorganisms are used to synthesize chiral drugs such as antidepressants, antianxiety drugs, antiasthmatics, anti-cholesterol, antihypertensive, antithrombotic, antiepileptics, antibiotics, anti-inflammatory drugs, anticancer drugs, and anti AIDS drugs (Scheme 1). In the section below, different important precursors of drugs synthesized using ADHs will be discussed (Zheng et al., 2017).

Prochiral ketones: an ADH from *Candida tropicalis* was used to reduce prochiral ketones to (*S*) chiral alcohols. This reaction helped in the synthesis of (*S*)-Duloxetine, an antidepressant

drug. The cofactor used was NADPH and the product yield was of 80% (Scheme 1a, Chen et al., 2016).

Buspirone is a molecule that is used to treat anxiety. *Hansenula polymorpha* dehydrogenase expressed in *Sacharomyces cerevisiae* was able to convert 6-ketobuspirone to *R*-6-hydroxy buspirone with 99% yield and *ee* (Scheme 1b, Goldberg et al., 2006).

Benzoic acid methyl ester (keto ester M) was converted with an ADH from *microbacterium* sp. MB 5614 cells to benzoic acid methyl ester (*S*) hydroxyl ester with more than 95% *ee* (Scheme 1c, Roberge et al., 1996).

Ethyl 4-chloro-3-oxobutanoate (COBE) was converted via an ADH overexpressed in *E. coli* BL 21. Those cells contained a reductase gene that was cloned from *Streptomyces coelicolor*. The cells were able to convert COBE to (*S*)-4-chloro-3-hydroxybutanoate (*S*)-CHBE. This reaction is required to prepare *R*-hydroxynitrile (*R*-HN), which is a precursor of atorvastatin that is used to reduce cholesterol in the body (Scheme 1d, Cai et al., 2012).

Ethyl-2-oxo-4-phenyl butyrate was reduced via an ADH from *Candida boidinii* CIOC21 to ethyl (*R*)-2-hydroxy-4-phenyl butyrate in >99% *ee* was obtained. Ethyl (*R*)-2-hydroxy-4-phenyl butyrate is an intermediate required for the synthesis of antihypertension drugs such as Benazepril (Scheme 1e, D'Arrigo et al., 2010).

In another experiment, *o*-chloro-benzoylformate (CBFM) was reduced via a recombinant *E. coli* to (*R*)-*o*-chloromandelate (*R*-CMM) with 96% *ee*. *R*-CMM is a precursor of *S*-clopidogrel, which is an anticoagulant drug (Scheme 1f, Ema et al., 2008, Ma et al., 2012).

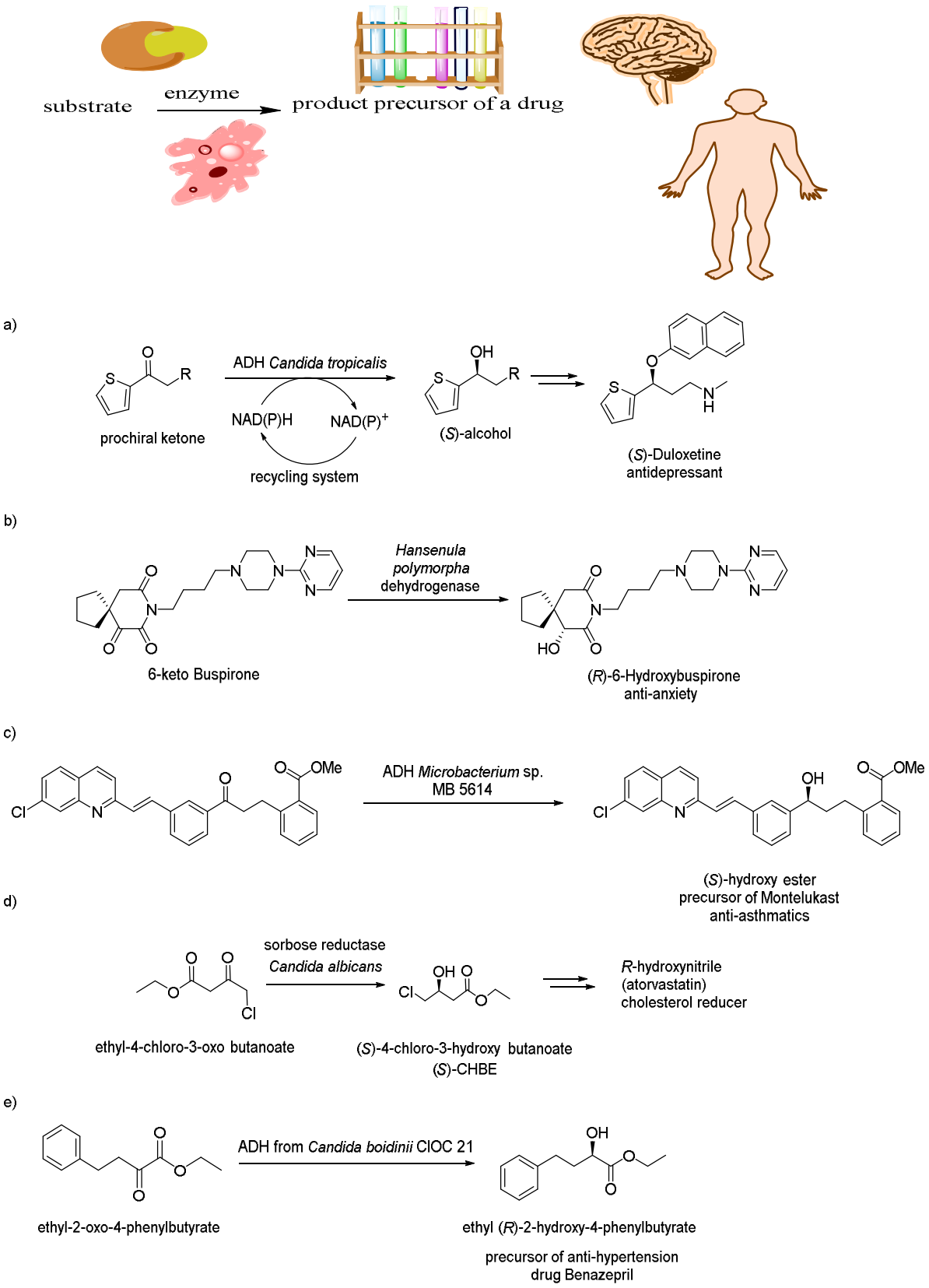
MDA (3,4-methylenedioxyphenylacetone) was reduced to (*S*)-α-methyl-1,3-benzodioxole-5-ethanol (*S*)-MBE, which is a precursor of Talampanel, which is a drug used to treat epilepsy. The reduction occurred via an ADH that was obtained from *Zygosaccharomyces rouxii* and cells from *Debaryomyces hansenii* (Scheme 1g, Erdély et al., 2006).

4-Hydroxybutan-2-one was converted to (*R*)-1,3-butanediol, a precursor of 4-acetoxyazetidinone, a key component for the synthesis of antiobiotics. The conversion was made using an ADH from *Candida parapsilosis* that was expressed in *E. coli* (Scheme 1h, Matsuyama et al., 2001).

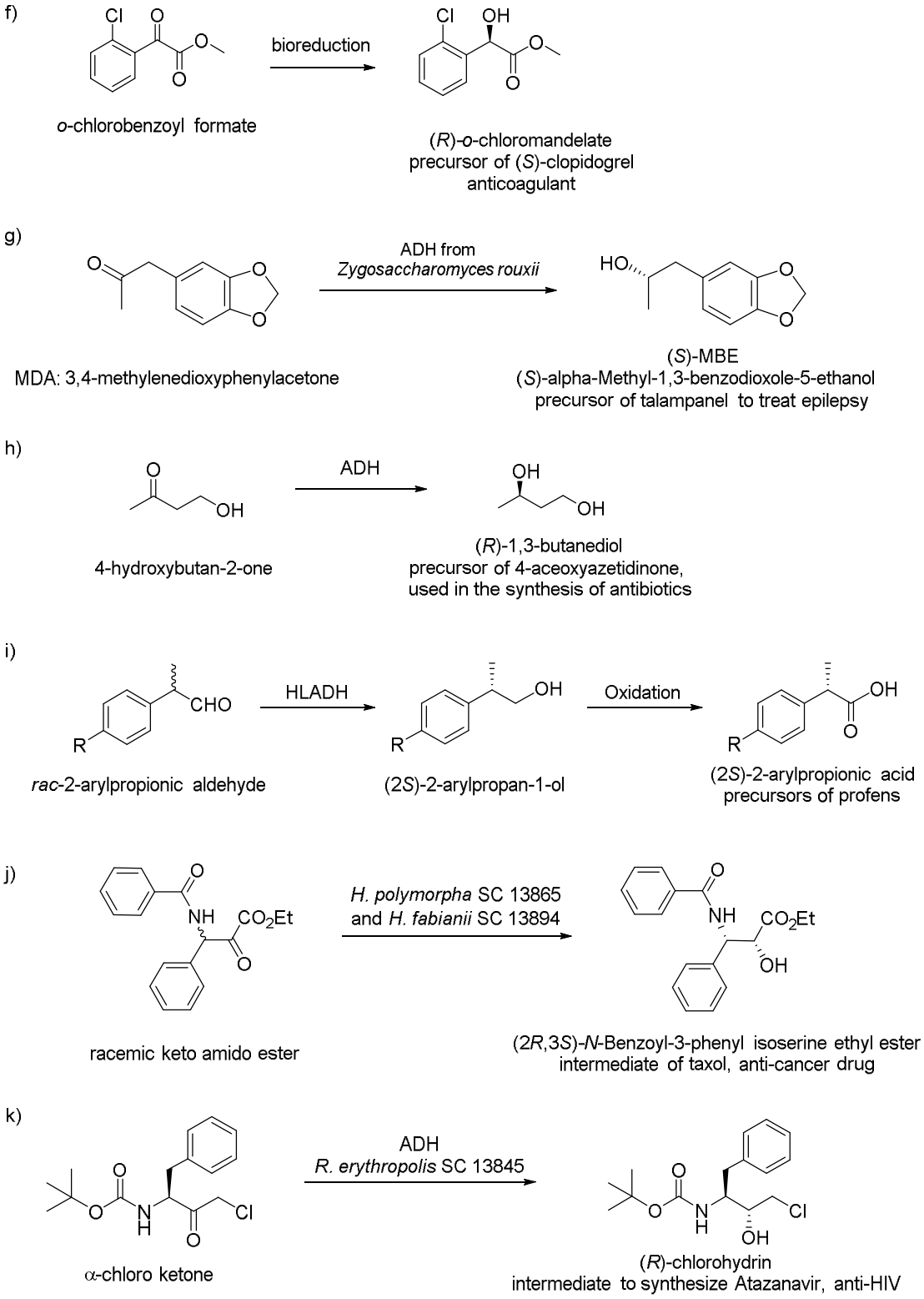
Anti-inflammatory drugs: 2-arylpropionic aldehydes were bioreduced to (2*S*)-2-arylpropanols using HLADH. The oxidation of (2*S*)-2-arylpropanols leads to (2*S*)-2-arylpropionic acids which are precursors of the anti-inflammatory drugs: profens (Scheme 1i, Giacomini et al., 2007).

Anticancer drugs: 2-keto-3-(*N*-benzoylaminophenylpropionic acid ethyl ester) was reduced to its corresponding alcohol(2*R*,3*S*)-*N*-benzoyl-3-phenyl isoserine ethyl ester. This latter compound is an intermediate of taxol C-13 side chain. Taxol is known to be a very important anti-cancer drug. *Hansenula* polymorpha SC 13865 and *Hansenula fabianii* SC 13894 were used to complete this bioreduction, and the obtained results were excellent (>80% product yield, >98% *ee*, Scheme 1j, Patel et al., 1995).

Anti AIDS drugs: (1*S*)-(3-chloro-2-oxo-1-(phenylmethylpropyl)carbamic acid, 1,1-dimethyl-ethyl ester was reduced with an ADH from *R. erythropolis* SC13845 to (1*S*,2*R*)-[3-chloro-2-hydroxy-1-(phenylmethyl)propyl]carbamic acid, 1,1-dimethylethyl ester, (1*S*,2*R*)-CADE) with more than 90% yield and 99% *ee*. This compound is a precursor for the synthesis of Atazanavir, a drug that is used to treat AIDS (Scheme 1k, Patel et al., 2003).




**Scheme 1:** important drugs or drug precursors obtained through ADH-catalyzed biotransformations (Zheng et al., 2017).




**Scheme 1 (Continued):** important drugs or drug precursors obtained through ADH-catalyzed biotransformations (Zheng et al., 2017).

Microorganisms such as mushrooms, yeast, and bacteria can be used for the specific reduction of ketones and diketones to enantiomeric alcohols. This reduction, catalyzed by ADHs from microorganisms, leads to the production of enantiomers, which are used in the chemical industry. The development of this type of biotransformation requires selection of enzymes which have a strong activity for the *S* form or the *R* form of secondary alcohols (Baucher et al., 1996).

Many ADHs, which reduce ketones were isolated from thermophilic microorganisms. The most studied one is of *Thermoanarobacter brokii*, because of its high stability to temperature and to organic solvents. It is a highly demanded enzyme to make chiral synthesis. SADH, which is a NAD dependent enzyme from *Thermoanarobacter ethanolicus* is similar to the one from *T. brokii* (Heiss and Phillips 2000). Its capacity to reduce stereoselectively the ketones depends on three factors: temperature, nature of the cofactor, and the structure of the substrate (Zheng et al., 2017).

**The alcohol dehydrogenases that were used during this thesis**

The oxidoreductases enzymes that were studied in this thesis (Table 2) were from the wild type strains of the genus *Rhodococcus.* Some species of *Rhodococcus* (The Biology Laboratory of Human Pathologies, Faculty of Sciences, Rabat) have been studied for their high capability to catabolize sterols. Among them *Rhodococcus* sp. CIP 105335 (strain GK1), *Rhodococcus* sp. GK12 and *Rhodococcus* sp. GK3 are sterol patent degraders. The gene of the 16S rRNA was identified for the strain GK1 (accession number: JQ318031, NCBI Gene Bank) and the strain GK12 (accession number: JQ318030, NCBI Gene Bank). On the basis of this identification in addition to morphological and physiological observations, the strain GK12 was identified as *R. erythropolis*, and strain GK1 might be a new species of the genus (Kreit et al., 2012). The other enzymes used, were offered by Prof. Wolfgang Kroutil

(University of Graz). The ADHs were from *E. coli* strains that overexpressed the RasADH from *Ralstonia* sp., SyADH from *Sphingobium yanoikuyae*, and TeSADH from *Thermoaerobacter ethanolicus*, ADH-A from *Rhodococcus ruber*, ADH-T from *Thermoanaerobacter* sp., and LBADH from *Lactobacillus brevis.* The lab work with these recombinant bacterial cells was performed at the Faculty of Chemistry, University of Oviedo, Spain.

**Table 2:** characterized ADHs (Stampfer et al., 2003a and 2003b; Kosjek et al., 2004) that were used throughout the experiments of this thesis.

| **ADH** | **Microorganism** | **Cofactor dependency** | **Specificity** |
|---|---|---|---|
| ADH-A | *Rhodococcus ruber* | NADH | (*S*)-specific |
| LBADH | *Lactobacillus brevis* | NADPH | (*R*)-specific |
| RasADH | *Ralstonia* sp. | NADPH | (*S*)-specific |
| SyADH | *Sphingobium yanoikuyae* | NADPH | (*S*)-specific |
| TeSADH | *Thermoanaerobacter ethanolicus* | NADPH | (*S*)-specific |
| ADH-T | *Thermoanaerobacter* sp. | NADPH | (*S*)-specific |
| ADH GK1 | *Rhodococcus equi* | NADH | (*S*)-specific |
| ADH GK3 | *Rhodococcus opacus* | NADH | (*S*)-specific |
| ADH GK12 | *Rhodococcus erythropolis* | NADH | (*S*)-specific |

### 1.22. Systems that include cofactor regeneration (NAD and NADP)

Nicotinamide cofactors such as NAD and NADP are necessary components for enzymes to work properly. Due to the fact that commercial NAD and NADP are expensive (1500 euros/kg for $NAD^+$ and 6000 euros/kg for $NADP^+$), many research teams are testing the oxidoreduction activities of enzymes without the external addition of these cofactors. Usually these nicotinamide cofactors act as either electron acceptor or donor and they exist in oxidized and reduced forms depending on the reaction conditions (Figure 3). An enzyme can either be specific for NADPH or NADH as a hydride source to the substrate.

When the following criteria are met, one can call the system an ideal cofactor regeneration system: (1) when the complementary enzymes used are cheap and stable, (2) when the specific enzymatic activity is high, (3) when the reagents do not interact with the stability of the enzyme, (4) when the turnover number is high, (5) and finally when the chemical equilibrium favors the product formation (Romano and Suzzi, 1996).

For recycling cofactors, a “coupled-enzyme” system can be used. This system uses other dehydrogenases such as glucose dehydrogenase and formate dehydrogenase as a second enzyme and needs a second co-substrate (*e.g.*, glucose or formate, respectively). This system is also known to push the reaction towards the product formation. On the other hand, when the previously mentioned enzymes are unstable, 2-propanol is used as a co-substrate in a “coupled-substrate” system. This method is cheaper and help to shift the equilibrium towards the product formation (Fadda et al., 2002).

### 1.23. Chemical methods to perform ketone reductions (Chemical Industry)

Some enantiopure secondary alcohols are important compounds because they are used in the food and the pharmaceutical industry as well as in the synthesis of perfumes and flavors

(Elalami 2004). These alcohols can be synthesized using a reduction system of their corresponding ketone using chemical protocols. Some of the most important protocols are shown below:

**Hydrogen-transfer (Meerwein-Ponndorf-Verley reduction)**

Meerwein-Ponndorf-Verley reduction is a method in organic chemistry to reduce ketones and aldehydes to alcohols. During this reduction, the aluminum alkoxide acts as a catalyst and a sacrificial alcohol is used.

**Hydrosilylation**

This transformation occurs when a Si-H bond is added to an unsaturated substrate in the presence of a catalyst to give a specific product. For example, iron complexes can be used as catalysts to reduce and make an asymmetric hydrosilylation of ketones. The conditions used in these types of reactions include using a tetrahydrofuran as a solvent, an Fe/N-donor, silane, and a ketone. The temperature is usually set to 65 °C and after 20 h, the ketone gets converted to its corresponding alcohol (~94%) (Morris 2009).

**Metal-catalysed hydrogenation**

In this method hydrogen and a metal catalyst are added to the reaction to reduce a carbonyl compound to the corresponding alcohol. Some metals that catalyze these types of transformations include Al(III), transition metals such as Ir, Fe(II), Ru(II), Rh(III), and Pt group metals (Štefane and Požgan, 2016).

Nowadays, chemists tend to choose biocatalysts instead of chemical catalysts to help in the protection of the environment, and because some of the chemical methods that reduce ketones have several issues. Biocatalysts have many advantages over chemical catalysts: they are cheaper to use, and they require mild conditions and less energy and equipment. They are also more specific into producing enantioselective products with good yields. In the following

section, the problems associated with chemical methods involved in the transformation of ketones will be highlighted:

In order for the reduction to occur in the Meerwein-Ponndorf-Verley method, excess amounts of alcohols are added to the reaction to provide a hydride, the reaction can sometimes occur slowly, undesirable condensation products can appear, and sometimes it is necessary to increase temperature in order to move the reaction equilibrium towards alcohol formation and to remove the side product, acetone (Ooi et al., 1998).

Similarly, among the issues that affect the hydrosilylation reactions are: the mechanical attrition of the catalyst, the weak activity and selectivity of some of the catalysts, their low stability, and the formation of by-products (Nakajima and Shimada, 2015).

Metal catalysts can be very specific when it comes to the choice of substrates resulting in limited conversions of the products when that substrate is not specific to that catalyst; and they selectively convert the substrates to specific products (like in the hydrogenation of alkynes and aromatics). Sometimes hydrogenation is in competition with other reactions; these reactions include dehydrogenation, skeletal rearrangements, cyclization, and hydrogenolysis. Also, the structure of the catalysts' surface is important to determine the speed of the reaction. In addition, one major drawback of this method is the expensive cost of using a metal catalyst. This method is also considered to be complicated because clean metal surfaces give partial dehydrogenation forming new well bonded species (Zaera 2017).

### 1.24. ADHs and Food

An enzyme called diacetyl reductase is used to remove the unwanted taste that diacetyl gives to alcoholic beverages and citric juices. However, diacetyl gives a good taste to fermented dairy products (Fauve and Veschambre, 1990).

Also, *Streptococcus diacetylactis* are producers of this flavor during the transformation of

dairy products. This particular flavor is due to diacetyl and acetoin. During growth, these microorganisms transform citrate to diacetyl and acetoin. The increase in acetoin yield is obtained by the addition of citrate in the reaction medium (Hummel et al., 1989; Heiss and Phillips., 2000).

ADHs can oxidize or reduce compounds based on the type of substrate available. (+)-L-Acetoin is produced in important quantities from diacetyl by the activity of diacetyl reductase a NADP dependent enzyme, which is extracted from pigeon liver (Schwarz and Hang, 1994).

Acetoin is also one of the components which is responsible in the apparition of flavor in alcoholic drinks. It is produced during fermentation by yeast. It is a key product in the synthesis of 2,3-butanediol (Figure 8). Yeast that contribute in the synthesis of wine are divided into three classes based in their ability to produce 2,3-butanediol:
Yeast which have a strong ability to ferment producing only 2,3-butanediol.
Yeast with a medium power to ferment that produce 2,3-butanediol and acetoin.
Yeast with a weak power to ferment, that produce only acetoin.

The tastes of wines differ based on the strain of yeasts that is used to produce them. A strain with a weak power to ferment produces less of 2,3-butanediol and more acetoin, and in that case the taste of wine is strong. However, a strain with a strong power to ferment, like species of *Saccharomyces*, produce less acetoin and more 2,3-butanediol. The taste of wine in that case is weaker. The capacity to produce flavor by yeast is essentially due to the activity of the two enzymes, diacetyl reductase and acetoin reductase (Hummel et al., 1989).

Aliphatic methyl ketones are widely distributed molecules in nature. They are found in plants and dairy products. They are also found in fermented dried sausage, and they also contribute to the salty taste of sausage. Their presence in sausage is associated with the inoculation of

*Staphylococcus carnosus*. To evaluate the ability of these strains to produce methyl ketones, a research team has developed in 2002 an enzymatic method which uses a commercialized secondary ADH, and 2-pentanone like a standard substrate. The assay uses NADP dependent ADH (E.C. 1.1.1.2) of *T. brockii*, known for its large specificity to methyl ketones. This enzyme reduces methyl ketones (like 2-pentanone, 2-octanone) to the corresponding alcohols. The NADPH oxidation velocity is directly linked to the quantity of ketones found in the sample. Compared to GC coupled with mass spectrometry, the enzymatic method offers some advantages like the simplicity, the speed, and the low cost (Bernardo et al., 1984, Elalami 2004).

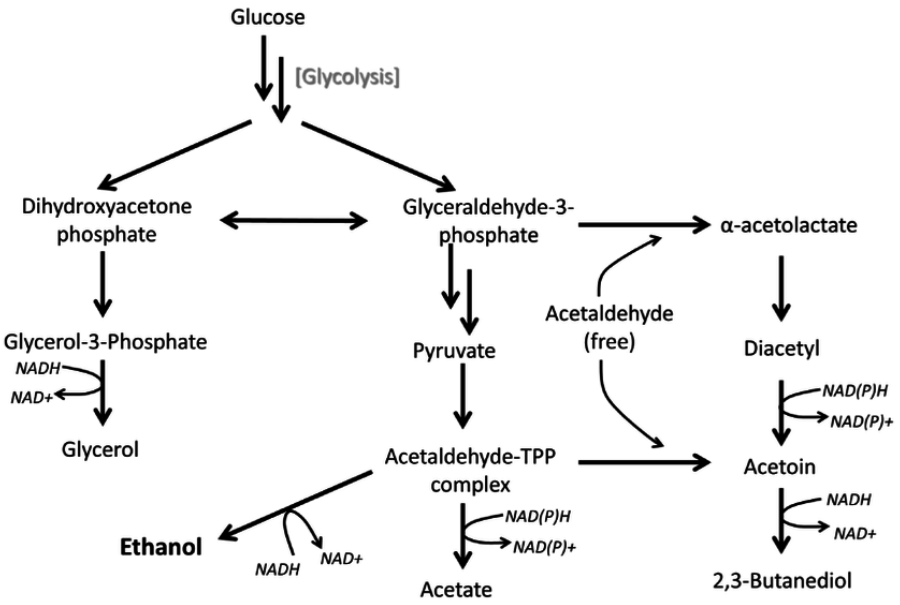



**Figure 8:** the biocatalysis of glucose to ethanol (Macedo and Brigham, 2014).

### 1.25. Recent trends related to ADHs

In the following section of the thesis, recent findings related to the ADH research will be discussed like: the evolution effect on that enzyme and bioinformatics studies. In a recent study about ADHs, an ADH from *Thermoanaerobacter brockii* (TbSADH) was proved to not reduce asymmetrically bulky ketones (Qu et al., 2019). To overcome this challenge Qu et al.

have mutated 2 key residues (A85 and I86) in order to change the shape of the substrate binding pocket of TbSADH; and making the active site more available to bulky ketones. In this study, the team used the mutant (A85G/I86A) as a template and performed a double-code saturation mutagenesis (DCSM) to the selected residues that line the substrate binding pocket with a two-membered reduced amino acid alphabet. As a result, the team created a smart mutant library and the triple-mutant A85G/I86A/Q101A was the best enzyme that gave an *S*-selective product with 99% enantioselectivity and 6555 total turnover number (TTN). Also, this study showed how one can increase the activity of ADH by double-code saturation mutagenesis (DCSM) (Qu et al., 2019).

Today many research groups use bioinformatics in order to analyze genes coding for alcohol dehydrogenases. A team led by Kasirajan et al. in 2019 have used cloning techniques to characterize a cinnamyl alcohol dehydrogenase (CAD) (Kasirajan et al., 2019). This enzyme was used in the phenylpropanoid pathway and is involved in the modification of the biomass in order to enhance saccharification. The length of the CAD gene was of 4 kb and it possessed 4 exons and 3 introns. In addition to that, the region which coded in CAD had 1098 bp open reading frame (ORF) for 365 amino acids (Kasirajan et al., 2019). The researchers also used a protein database entitled PROSITE, and after analysis they discovered that the ADH contained zinc and got attached to NADP (domain motif GLGGLG). This study could be very helpful for lignin engineering in plants and for other biotechnological investigations (Kasirajan et al., 2019).

# ***Part 1:*** *Unconventional Media Used for Enzymatic Reactions*

## 2.1. Introduction

Deep eutectic solvents have gained in the last decade many attentions from the scientific committees and from industries. This is due to their many advantages as they are: eco-friendly and cheap, they are easy to synthesize, and they can be tailored at different molar ratios depending on the purpose of use. Also, they can increase the (bio)catalytic activity of many reactions leading to a high extent of products with excellent enantiomeric excess (*ee*), including important precursors for drugs that are widely used in the pharmaceutical industry (Alonso et al., 2016, Tavares et al., 2013).

In this section of the thesis, different uses of DES in some biocatalytic reactions will be highlighted as DES are very important solvents that increase the product yield and the enantiomeric excess in several important reactions. Nowadays, industries tend to use novel types of solvents that can present advantages over the more typical organic ones due to environmental reasons. Among them, the deep eutectic solvents (DES) have recently appeared, which have a great potential and can have many applications at industrial scale, as they can be beneficial from a green chemistry point of view. DES are mixtures of a salt such as choline chloride (ChCl) and a hydrogen bond donor (HBD) molecule such as urea (Ur).

ILs are salts, consisting of a mixture of cations and anions that do not pack well together, with consequently melting points near room temperature, although they are arbitrarily defined as salts with a melting point below 100 °C (Hallet and Welton 2011). The first example of an IL was described by Walden in 1914 when he obtained ethyl ammonium nitrate (Walden 1914). As a result, the so-called first generation of ILs appeared. However, this first generation suffered from some disadvantages, such as toxicity and molecular instability due to high reactivity with oxygen. In 1992 Wilkes and Zaworotko developed the second generation of ILs that were more stable against water and air (Wilkes and Zaworotko, 1992). The anions were replaced with halides and those ILs had low melting points and were more stable in the

presence of organic solvents and were used, among other applications, in biocatalysis in the 2000s. However, these ILs had again some disadvantages such as high cost and toxicity (Tavares et al., 2013). The reasons above led scientists to synthesize new eco-friendly ILs that could be used in, *e.g.* catalysis, and these types of ILs have been called the DESs. These solvents contain more stable anions and cations which are usually biodegradable. They are easy to prepare and do not require purification. In addition to that, enzymes have shown good activities and stabilities in DES (Tavares et al., 2013). This medium presents many potential advantages in the medical and biotechnological sectors. But before using them, one has to test their safety of use and the extent of their toxicity before implementing them in biotechnological applications, especially the ones involving the utilization of living cells. The toxicity of DESs against different living organisms are summarized in Table 3 (Mbous et al., 2017).

**Table 3:** The effect of deep eutectic solvents (DES) on different organisms.

| Organism | Effect of DES | Reference |
|---|---|---|
| **Bacteria** | DES composed of choline chloride (ChCl) was not toxic | (Smith et al., 2014)<br>(Hayyan et al., 2013a) |
| **Marine organisms** | *Hydra sinensis*: ChCl based DES led to the decomposition of their bodies<br>Brine shrimp: DES was lethal and toxic | (Hayyan et al., 2013a) |
| **Human cells (cancer cells)** | Disrupts the cell membranes and decreases the proliferation of cancer cells | (Hayyan et al., 2013b) |
| **Plants** | Decreases chlorophyll and causes disequilibrium in the homeostasis and cell walls of plant cells | (Gal et al., 2012) |
| **Mice** | Urea type of DES causes death at high levels | (Radošević et al., 2015) |

In general, DES are composed of a hydrogen bond acceptor (HBA) and a HBD. According to Smith et al. (2014) the general formula of DES is made of three components (Hayyan et al., 2015). The first component includes the cations which could be: choline, ammonium, sulfonium, or phosphonium cations. The second component of DES is a halide anion. The cations and halide anions make up the HBA part of the DES. The third component of the DES is a HBD, which includes a range of molecules such as: metal halides, amides, carboxylic

acids, alcohols, urea, acetamide, or polyols. Based on its composition, they have been divided into four different classes. The first type is the one composed by metal halides and imidazolium salts. They are composed by non-hydrated metal halides such as iron chloride. The second type of DES is composed of ChCl and hydrated metal halides such as copper chloride. These solvents are not sensitive to air and moisture. The third type of DES is formed by ChCl as a HBA and an amide, a carboxylic acid, or an alcohol, which act as a HBD. The fourth and last type of DES is made of a transition metal halide like zinc chloride, and with a HBD such as urea, acetamide, ethylene glycol, or hexane-1,6-diol (Hayyan et al., 2015).

Natural deep-eutectic solvents (NADES) can be considered as a type 3 of DES because they are composed of a salt and a HBD molecule. However, one might argue that DES can be considered as green solvents only if they fulfill the standardized safety requirements compared to ILs and organic solvents. For this reason, a standardized protocol to control the quality and greenness of DES must be established in the near future (Mbous et al., 2017).

It is important to mention that the properties of DES can be easily tuned by modifying their structures, by changing both the HBA and the HBD structures, and by adjusting the molar properties of both components. In a study conducted by D'Agostino and co-workers in 2015 to characterize the DES in detail, they examined the composition of different DES and their relation with aqueous mixtures, showing the behavior of DES with water at different proportions (D'Agostino et al., 2015). They also used pulsed field gradient nuclear magnetic resonance (PFG NMR) to determine active species in aqueous mixtures containing DES and to investigate their diffusion behaviors in those mixtures. DES composed of ChCl and glycerol, ethylene glycol, and urea were studied at a 1:2 molar ratio. Authors found that when the water content increased in those three types of DES, the viscosity of those liquids decreased. Therefore, it was concluded that the fluidity of the liquid depended on the hydrogen bonds that were formed between the HBD and the halide anion. This study also

gave new microscopic insights about complex liquid mixtures. NMR was used in this study to detect the mobility of charged and uncharged species.

According to Guajardo et al., DES tend to perform better in reactions that contain a minimum amount of water. Also, the authors highlighted the different applications of DES and showed that they can be used as solvents in different catalytic reactions, such as metal-, organo- or biocatalyzed processes speeding up the reactions and enhancing the isolated product yields (Guajardo et al., 2016). In addition, DES can be used as convenient solvents to separate organic compounds like alcohols and esters from a complex reaction mixture.

Biocatalysis is the field of chemistry that focuses on the use of enzymes from living organisms to catalyze and speed-up the chemical reactions for synthetic purposes (Faber 1992). Regarding the biocatalysis field, Domínguez de María and Hollmann showed that the enzyme activity and selectivity strongly depend on the solvent properties (Domínguez de María and Hollmann 2015). Although biocatalysis is considered a green technology, the authors investigated how in some specific cases biocatalysis can affect negatively the environment. Sometimes, biocatalytic reactions involving water can yield by-products that are toxic to the environment. In these cases, the use of DES can afford a lower impact on the environment by extracting them. The best feature that enables different types of DES to dissolve enzymes and highly polar organic molecules is their capability to form hydrogen bonds.

### 2.1.1. Applications of DES to oxidoreductase-catalyzed reactions

The first report related with the use of DES in the presence of an enzyme was the work of Kazlauskas and co-workers, who confirmed that lipases from different lyophilized bacterial cells had good catalytic activities in DES. It was also shown that other hydrolases had excellent activities in DES (Gorke et al., 2008). That research group conducted several test

reactions to observe the effect of DES on the lipase-catalyzed transesterification of ethyl valerate with butan-1-ol using eight different DES, and they compared their conversions with the ones obtained in toluene. It was found that DES improved significantly the biocatalytic reactions. Also, the initial specific activity of the lipases was higher in these media compared to the usual ILs that were previously used in these types of reactions. DES also enhanced the reaction rates up to 20-fold. The study also confirmed that they were convenient solvents in the case of the epoxide hydrolase-catalyzed transformation of styrene oxide regarding dimethylsulfoxide (DMSO) or acetonitrile.

Oxidoreductases are extremely important enzymes involved in aerobic and anaerobic metabolism in living organisms. As examples, some bioreactions that involve oxidoreductases include: Krebs cycle, glycolysis, amino acid metabolism and oxidative phosphorylation. Nowadays, oxidoreductases have gained attention in the biocatalysis field and have encountered industrial applications due to their ability to synthesize enantiopure compounds under mild conditions (Drauz et al., 2012). In this section, we will discuss the effect of DES on some oxidoreductase enzymes. In order to maintain or improve the activity of the enzyme similar to the one *in vivo*, scientists have used green solvents such as DES to help them transform lipophilic substrates more efficiently (Gotor-Fernández and Paul 2019).

### 2.1.2. Alcohol oxidase and peroxidase-catalyzed transformations

A recent study has shown the use of different enzymatic preparations to selectively oxidize 5-hydroxymethylfurfural (HMF), a natural product, to 2,5-diformylfuran (DFF), 5-hydroxymethyl-2-furancarboxylic acid (HMFCA), 5-formyl-2-furancarboxylic acid (FFCA), and 2,5-furandicarboxylic acid (FDCA) (Qin et al., 2015). These oxidized derivatives are precursors of important compounds. For instance, DFF and FDCA have antifungal and anti-*Pneumocystis carinii* activities. Also, HMFCA is an important monomer for synthesizing

polyesters. Alcohol oxidase (AO) from *Candida boidinii* oxidized HMF and showed a high activity compared to the other alcohol oxidases. AO was able to oxidize HMF to DFF in 41% yield. In addition, galactose oxidase (GO) from *Dactylium dendroides* was also able to perform this transformation in excellent yields (~90%) in the presence of catalase and horseradish peroxidase. Xanthine oxidase (XO) from *Escherichia coli* was able to selectively oxidize the formyl group located in the HMF substrate and gave HMFCA in 94% yield after only seven hours. In addition, this XO-mediated process had many advantages, as it used air instead of the toxic $H_2O_2$ to achieve the oxidation, it did not use harmful organic solvents, it was highly selective, and therefore, no by-products were observed (Qin et al., 2015).

This research team also used three different laccases applied to the oxidation of HMF to FFCA in the presence of the mediator 2,2,6,6-tetramethyl-1-piperidinyloxy radical (TEMPO). At the beginning of the reaction, DFF appeared as an intermediate and was then transformed to FFCA that was accumulated along the time. The laccase from *Panus conchatus* gave the highest yield of FFCA (82%) after 96 h. The laccase from *Trametes versicolor* gave a 68% yield of FFCA after 48 h, and the laccase from *Flammulina velutipes* afforded a 70% yield of FFCA after 72 h. For the synthesis of FDCA, a sequential oxidation protocol was achieved, combining GO and lipase from *Candida antarctica* type B (CAL-B). In a first step, GO oxidized HMF to DFF (75% conversion) as previously described after 48 h. After extraction with ethyl acetate (EtOAc), *t*-butanol, CAL-B and $H_2O_2$ were added to the reaction medium to convert DFF to FDCA. An excellent yield of FDCA in this second step was obtained after 24 h (88%).

In the same study, the successful separation of HMF from DFF with the help of DES was also described after the enzymatic transformations, because DES have affinity towards hydrogen bond-containing molecules, and they enable to dissolve those molecules. The authors were able to separate DFF from HMF using three types of DES. The composition of these solvents

was: choline chloride and glycerol (ChCl:Gly 1:2 mol/mol), choline chloride and urea (ChCl:Ur 1:2 mol/mol), and choline chloride and xylitol (ChCl:Xyl 1:1 mol/mol). They were used to extract HMF from an ethyl acetate solution that contained both DFF and HMF. The researchers discovered that DES composed of ChCl:Gly and ChCl:Ur were the ones that selectively extracted HMF from the mixture. When this mixture was extracted three-times using ChCl:Gly DES, the purity of DFF increased to 97% from the original purity of 76%.

In another investigation conducted by Yang et al., they tested the effect of 24 DES and 21 NADES as co-solvents on the biotransformation of isoeugenol into vanillin using *Lysinibacillus fusiformis* CGMCC1347 whole cells. It was discovered that those eutectic solvents (1% v/v) enhanced the bioconversion because it made the bacterial cell membrane permeable to the lipophilic substrate (Figure 9). Among them, the DES composed of choline acetate (ChAc) were usually better than the ones composed of ChCl in terms of product yields (up to 142%) regarding the control reaction without DES (Yang et al., 2017). The whole cells were immobilized on poly(vinyl alcohol)-alginate beads, and this biotransformation could be repeated up to 13 times in the presence of choline chloride:galactose (ChCl:Gal 5:2 mol/mol, 20% v/v), maintaining their activity to a similar extent, offering this system a promising design for further developments.

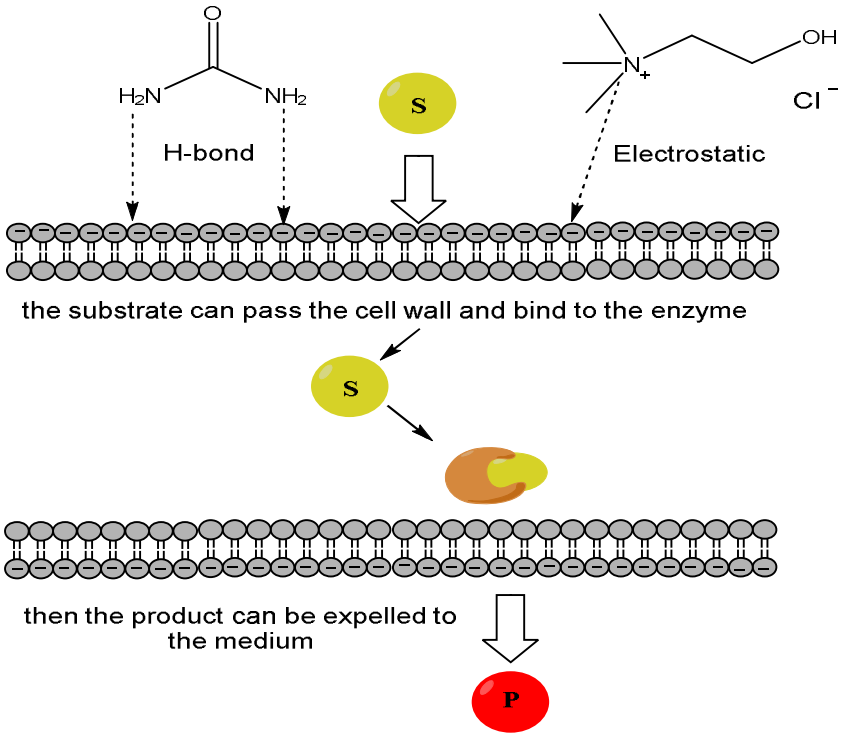



**Figure 9:** the effect of DES on cell membrane permeability. The cations from the choline chloride and urea that form hydrogen bonds can disrupt the cell membrane, allowing the substrate to enter the cells, so then it can bind to the appropriate enzyme. Finally, the product can be released to the medium.

In a study conducted by Wu et al., it was found that DES composed of ChCl and different HBDs (Ur, Gly, acetamide, and EG) at different molar ratios promoted more efficiently the activity of horseradish peroxidase (HRP) compared to DES which was composed of choline acetate. In that study, twenty-four DES were synthesized to see their effect on HRP activity, and they were found to have a stabilizing effect on HRP, especially those when the molar ratio of the salt was higher than that of the HBD. The authors also identified through spectroscopic studies that DES were able to enhance the α-helix conformations thus providing a more relaxed tertiary structure of the enzyme, hence improving its activity. They concluded that DESs can be versatile solvents and found that the hydrogen bonding network that is provided by the DES prevents this solvent from dissociation in aqueous solutions at least at concentrations of 0.5 M (Wu et al., 2014).

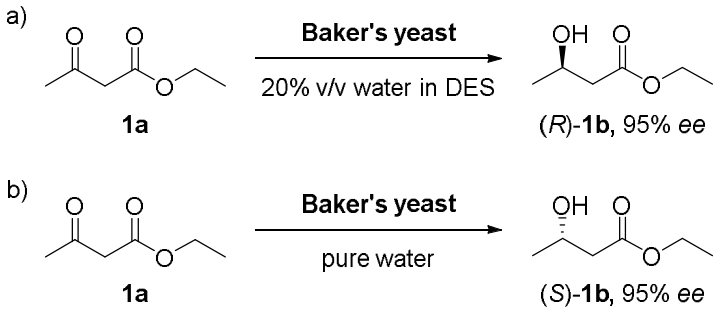
Luna-Bárcenas and co-workers in 2016 have used two types of DES to conduct an enzyme-mediated free radical polymeration of acrylamide. One DES was made of ChCl:Ur and the other was made of ChCl:Gly (1:2 molar ratio). When the DES was used at higher volumes than the phosphate buffer solution, the catalytic activity of the HRP diminished drastically. However, the thermal stability of the enzyme was improved. The enzyme was still able to achieve the initiation of the acrylamide free radical polymerization even at 80% v/v concentrations of DES. Also, the research group was able to synthesize polyacrylamide in ChCl:Gly at 4 °C because it has a low freezing point. The combination of HRP, $H_2O_2$ and pentane-2,4-dione as initiator was responsible to promote the reaction leading to polyacrylamide synthesis in aqueous medium. The free radical polymerization occurred also at room temperature and at 50 °C. HRP was partially denatured in ChCl:Ur because the heme group was extracted into the solution and this caused a decrease in the enzymatic activity at higher DES concentrations. The concentrations of ChCl:Ur and ChCl:Gly were fixed to 80% v/v when used in the reaction media for acrylamide polymerization to protect the hydrogen bonding network in the hydrated medium while decreasing the viscosity of these solvents. Also, a homogenous mixture was obtained throughout the process (Sánchez-Leija et al., 2016).

### 2.1.3. DES and alcohol dehydrogenases (ADHs)

ADHs are probably the most used enzymes corresponding to the family of oxidoreductases for synthetic purposes. They catalyze the reversible transformation between alcohols and carbonyl compounds through redox processes. These reactions are mediated by a nicotinamide cofactor, namely NAD or NADP, which is responsible of the electron transfer from or to the substrate (Kroutil et al., 2004). Apart from using free, isolated enzymes, the setup of whole cell-mediated transformations enables simple and cheaper procedures.

Especially interesting is their use applied to the cofactor-dependent reactions, due to the fact that whole cell microorganisms can provide it on its own and avoid the external addition of these expensive molecules. As eco-friendly and cheap solvents, DES have been selected as good solvent candidates for ADH-mediated protocols. As the subsequent studies will show, DES present many stabilizing effects on biocatalytic reactions involving ADHs from different organisms.

ADHs are extremely important enzymes due to many reasons. For instance, in the human body they are the main enzymes responsible for digesting and degrading the alcohol that is consumed. These enzymes are usually present in the human liver and they detoxify ethanol converting it into acetate that is later used by cells. Moreover, ADHs from yeast are used to convert glucose to ethanol to make alcoholic beverages (Michnick et al., 1997). Also, from a synthetic point of view, ADHs from microorganisms are used to convert ketones into chiral alcohols that can be precursors and building blocks of some important drugs such as cholesterol lowering drugs, anti-arthritic, and antibacterial agents. It is worthy to mention that these enzymes reduce ketones selectively according to the Prelog's rule, which can be used to predict the stereopreference of alcohol dehydrogenase-catalyzed carbonyl reductions. Hence, ADHs usually provide the hydride through the *Re* face of this prochiral moiety. In that case, the ADH follows the Prelog's rule; on the contrary, the biocatalyst will show anti-Prelog selectivity (Prelog 1964).




**Scheme 2:** the stereoselectivity inversion in the formation of alcohol **1b** based on the type of solvent used. (a) Baker's yeast cells showed a high stereoselectivity towards the (*R*) enantiomer when some amount of water was mixed with the DES (ChCl:Gly 1:2 mol/mol). (b) A high (*S*)-enantioselectivity was obtained when pure water was used with that biocatalyst (Maugeri and Domínguez de María 2014).

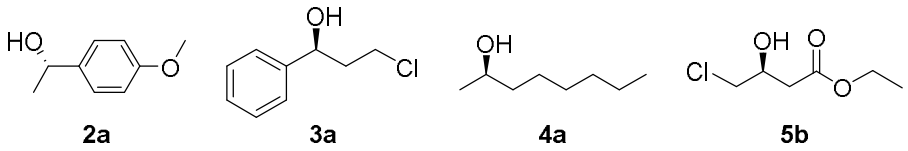
As a first example, Domínguez de María and co-workers demonstrated that DES could be an appropriate media for whole cell catalysis, achieving the bioreduction of ethyl acetoacetate (**1a**) with Baker's yeast (Maugeri and Domínguez de María 2014). This study showed that DES can be efficient at inhibiting oxidoreductases. For instance, alcohol dehydrogenases with (*S*)-stereopreference were inhibited by ChCl:Gly (1:2 mol/mol). When adding different proportions of this DES into water, it was observed the inversion of the stereoselectivity in the bioreduction of **1a** using this biocatalyst. Baker's yeast whole cells showed an excellent stereoselectivity towards the (*S*)-enantiomer of **1b** in pure water (95% *ee*). However, when the reaction was left in a DES medium containing less than 20% volume of water, the product was obtained with high (*R*)-enantioselectivity (95% *ee*, Scheme 2). The reason for that was because Baker's yeast genome contains a mixture of approximately 50 ADHs that can present different selectivities. Therefore, this effect could be explained due to the fact that some (*S*)-selective enzymes were inhibited by DES while (*R*)-selective ones were more active in this medium. In conclusion, DES were proven to be great solvents for whole cell biocatalysis, being able to influence both activity and selectivity.

In 2015 Bubalo et al., tested different DES on yeast mediated reduction of **1a** and found that the DES that were composed of a sugar derivative had the best biocompatible results (Bubalo

et al., 2015). Also, it was found that the amount of water present in the DES influenced the reaction results leading to an increase in the reaction yield when enlarging the percentage of DES. That group tested the effect of different types of DES using the yeast *Saccharomyces cerevisiae* (Baker's yeast). They found that the reaction yield was influenced by the type of HBD (*e.g.*, glucose, fructose, or glycerol) and by the amount of water added to the prepared DES solution. It was concluded that the aqueous solutions of DES (50% v/v of water) gave the highest reaction yield (93%). The viability of yeast cells in DES containing ChCl and Ur was also investigated, and it was found that it decreased in this type of solvent. The increased osmotic pressure on yeast cells grown in the urea type of DES led to a decrease in the viability of the cells. As a result, water diffused out of those cells. However, DES containing a sugar or glycerol demonstrated a yeast viability of 76-99% after 24 h of inoculation of the yeast cells. DES containing a sugar or glycerol provided a pH around 4.5, while the DES containing urea afforded a pH superior to 8. Since the preferred pH range for *S. cerevisiae* growth is from 4 until 6, and as mentioned before the pH for DES containing sugars was 4.5, the best bioreduction yields were observed in these media. These results confirm that pH values and cell viability in bioreduction reactions involving DES can be an important effect, strongly influencing the enzymatic results. Glucose and other sugars such as fructose can also play an important role in the cofactor regeneration necessary for these processes (Bubalo et al., 2015).

In a subsequent study done by (Xu et al., 2015a), the authors used different DES to study the enantioselective oxidation of racemic 1-(4-methoxyphenyl)ethanol (MOPE, **2a**) employing whole cells of *Acetobacter* sp. CCTCC M209061 and acetone to recycle the oxidized nicotinamide cofactor. They found out that the DES composed of ChCl:Gly (1:2 mol/mol) at 10% (v/v) afforded the best results because it improved the permeability of the cells membrane. It also improved the stability of the enzyme(s) involved in the process. Therefore,

it was possible to obtain the remaining (*S*)-**2a** (Figure 10) at 50% conversion in enantiopure form at a substrate concentration of 55 mM, higher than the one used in plain buffer (30 mM). Enantiopure (*S*)-MOPE can be used to make cycloalkyl[*b*]indoles which treat allergic responses.




**2a** **3a** **4a** **5b**

**Figure 10:** examples of chiral alcohols that were obtained in ADH-biocatalyzed processes in the presence of DES.

In another study developed by the same group, it was demonstrated that DES ChCl:Ur (1:2 mol/mol) was able to improve the asymmetric reduction of 3-chloropropiophenone to (*S*)-3-chloro-1-phenylpropan-1-ol (**3a**, Figure 10) catalyzed by *Acetobacter* sp. CCTCC M209061 whole cells immobilized on PVA-sodium sulfate using glucose to recycle the cofactor (Xu et al., 2015b), Among the different DES studied, ChCl:Ur increased the permeability of the bacterial cells as it was confirmed by flow cytometry. By augmenting it, the biocatalyst(s) could bind better to the ketone substrate to convert it into the corresponding enantiopure alcohol. Hence, using 5% v/v of DES at 10 mM substrate concentration, the product yield was 82.3% and the *ee* >99%. Later, utilizing a mixture of this DES with a water-immiscible ionic liquid (1-butyl-3-methylimidazolium hexafluorophosphate) in a biphasic system, it was possible to further increase the productivity of this transformation to 1.87 mmol/L.h.

In 2015, Müller et al. tested the effect of DES on the activity and stereoselectivity of ADHs from *Ralstonia* sp. (RasADH), *Thermoanaerobacter ethanolicus* (TeSADH) and horse liver (HLADH), which were overexpressed on *E. coli*. They used aliphatic and aromatic ketones as substrates and different DES proportions. It was found that these solvents could enhance the selectivity of the ADHs, maintaining a good activity even at high DES concentrations. In addition, the best solvent was the mixture ChCl:Gly (1:2 mol/mol) and buffer at a proportion

of 80:20 v/v. The three ADHs that were used showed excellent activities when different DES-buffer proportions were utilized with the substrates: octan-2-one (for TeSADH), benzaldehyde (for HLADH), and propiophenone (for RasADH). In another set of experiments, the performance of RasADH with propiophenone as a substrate was tested. When the ancillary cosubstrates (ethanol, propan-1-ol, and propan-2-ol) were tested in the DES-aqueous-media mixtures, conversion remained high (>80%) and *ee* towards the (*S*)-alcohol were enhanced up to >95%. For other substrates a similar trend was observed, obtaining high *ee* for some aromatic substrates (Müller et al., 2015).

A research study led by Xu et al. explored different DES compositions and their effects in the reduction of octan-2-one using whole cells of *Acetobacter pasteurianus* GIM1.158 to obtain (*R*)-octan-2-ol (**4a**, Figure 10). They confirmed that DES composed of ChCl:EG (1:2 mol/mol) offered the best results in this transformation. Moreover, when combining this DES with an imidazolium based water-immiscible IL, the productivity of this bioreduction could be highly improved. Thus, using a mixture of ChCl:EG (32% v/v) and 1-butyl-3-methylimidazolium hexafluorophosphate ($C_4MIM•PF_6$, 20% v/v), in the presence of propan-2-ol as a hydrogen donor, 2-octanone (1.5 M) could be reduced at 90% conversion providing the enantiopure (*R*)-alcohol. Authors explained that DES increased the cell membrane permeability and kept the cells stable in the reaction system. In the case of the biphasic system, those solvents improved the substrate consumption by the cells because the second phase acted as a substrate reservoir, diminishing substrate or product inhibition and leading to a good yield of the desired product (*R*)-octan-2-ol (Xu et al., 2016).

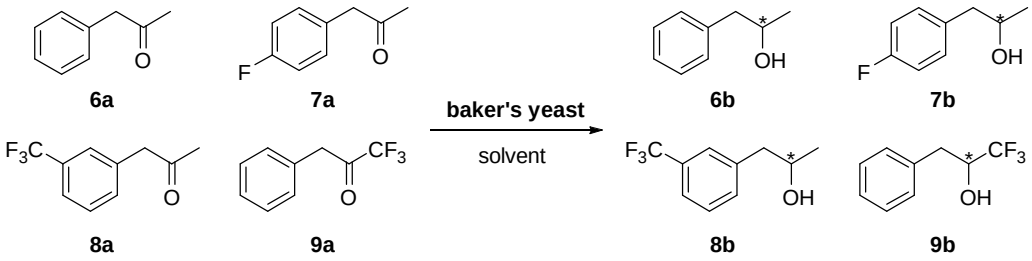
Ethyl (*S*)-4-chloro-3-hydroxybutanoate ((*S*)-CHBE, **5b**, Figure 10) is known to be a precursor for drugs like atorvastatin calcium. In a study conducted by Dai and co-workers, (*S*)-CHBE was produced from the corresponding ketone using recombinant whole cells of *E. coli* CCZU-T15. Thus, when using ChCl:Gly (1:2 mol/mol) at 12.5% v/v in the presence of the surfactant

Tween-80 and L-Glutamine, the substrate could be efficiently transformed (>93%) into the enantiopure alcohol at very high substrate concentrations (>2 M). L-Glutamine participated in the biosynthesis of the nicotinamide cofactors and could promote the biocatalytic activity, so no external addition of NAD was necessary (Dai et al., 2017). Another reason behind the high production of the product (*S*)-**5b**, was that the solvents and the surfactant increased the membrane permeability of the cells helping the substrate to bind to the enzyme(s) involved. This study confirmed that DES containing glycerol were appropriate solvents for the bioconversions that used recombinant bacterial whole cells.

In another investigation performed by Vitale et al., they explored the ability of Baker's yeast to reduce a series of ketones (Table 4) using DES as main solvents. In those experiments, different eutectic mixtures were studied but the best results were obtained with the one composed of ChCl:Gly (1:2 mol/mol) (Vitale et al., 2017). The researchers proved that the amount of water and DES which were added to the reaction mixture was very important even for reversing the selectivity. Hence, the *anti*-Prelog reduction of different ketones could be achieved when ChCl:Gly DES was used with the whole cell biocatalyst due to the inhibition of the (*S*)-oxidoreductases present in it. In the case of phenylacetone (**6a**), the corresponding alcohol was produced with a high stereoselectivity in pure water, giving (*S*)-**6b** in 96% *ee*, while in DES–aqueous mixtures up to 90% w/w of DES, the formation of the *R*-enantiomer was clearly favored (up to 96% *ee*). When water was used at 40% w/w, a racemic mixture of the alcohol was observed and there was no bioconversion when pure DES was employed in the reaction mixture. This team also proved that the outcome of the results depended on the composition of DES. The final stereoselectivity was obtained as a result of an interplay of whole cells "solvation" and the selective "inhibition" of some enzymes based on the nature of DES components. The DES that contained ChCl:glucose (2:1 mol/mol) and ChCl:Ur (1:2 mol/mol) deactivated the biocatalyst when used with 10% w/w water. Also, when water was

replaced by ChCl:D-fructose DES mixture (3:2 w/w), this did not result in the conversion of phenylacetone to 1-phenylpropan-2-ol after five days. However, when this eutectic solvent was used with 40% w/w water, (*S*)-1-phenylpropan-2-ol was obtained with an *ee* value of 78% and the conversion was 31% after five days. This proved that the Baker's yeast cells required a certain amount of water in order to show activity. It must be mentioned that the chiral (*S*)- and (*R*)-1-phenylpropan-2-ol (**6b**) derivatives are used in the preparation of neuroprotective drugs.

**Table 4:** selective reduction of ketones **6-9a** to their corresponding enantioenriched alcohols **6-9b** using baker's yeast in different solvent conditions (Vitale et al., 2017).




| Substrate | Solvent | Conversion (%) | *ee* (%) |
|---|---|---|---|
| **6a**[a] | Water | 88 | 96 (*S*) |
| **6a**[a] | DES A (60% w/w) in water[c] | 31 | 78 (*S*) |
| **6a**[a] | DES B (50% w/w) in water[d] | 88 | 88 (*S*) |
| **6a**[a] | DES B (90% w/w) in water[d] | 36 | 96 (*R*) |
| **7a**[b] | Water | 82 | 94 (*S*) |
| **7a**[b] | DES B (90% w/w) in water[d] | 10 | 90 (*R*) |
| **8a**[b] | Water | 90 | 98 (*S*) |
| **8a**[b] | DES B (90% w/w) in water[d] | 15 | 40 (*R*) |
| **9a**[b] | Water | >95 | 2 (*S*) |
| **9a**[b] | DES B (90% w/w) in water[d] | 12 | 24 (*R*) |

[a] Reaction conditions: ketone **6a** (1.5 mM), baker's yeast (230 mg/mL) at 37°C. [b] Reaction conditions: ketones **7**-**9a** (1.5 mM), baker's yeast (200 mg/mL) at 37°C. [c] DES A: ChCl:D-fructose (3:2 w/w). [d] DES B: ChCl:Gly (1:2 mol/mol).

When the aqueous–ChCl:Gly eutectic mixtures (10:90 w/w) were used with arylpropanone derivatives bearing electron-withdrawing groups (F or $CF_3$), the chiral enantioenriched *R*-configured alcohols were obtained (>40% *ee*) although at low extent. Thus, when 1-(4-fluorophenyl)propan-2-one (**7a**) was incubated at 37°C in water with Baker's yeast, this substrate was reduced to (*S*)-**7b** with 82% conversion in 94% *ee*. However, the (*R*)-

enantiomer was preferentially attained when this substrate was dissolved in a DES–water (80:20 w/w) mixture in 14% conversion and 82% *ee*. When the amount of water was reduced to 10% w/w, the *ee* of (*R*)-**7b** increased to 90% and the conversion was 10%. Also, 1-(4-(trifluoromethyl)phenyl)propan-2-one (**8a**) was reduced to (*S*)-**8b** with very high conversion (90%) and *ee* (98%) in plain water while to (*R*)-**8b** (15% conv. and 40% *ee*) when using a mixture ChCl:Gly/water 90:10 w/w. With 1,1,1-trifluoro-3-phenylpropan-2-one (**9a**), a racemic mixture of the alcohol **9b** was achieved in water and the *ee* increased to 24% to form the (*R*)-enantiomer with a 12% yield utilizing the same DES at 90% w/w.

### 2.1.4. Recent biotechnological applications

In the last years, DES have found potential applications in different biotechnological fields. Herein we just want to briefly mention a few recent interesting uses in this field. In the last decades, biofuels are produced in a less harmful way to the environment, but they are usually obtained at a low purity. In this context, Niawanti and co-workers purified crude biodiesel using DES. As these solvents have high polarities, they were able to separate biodiesel from its impurities like glycerol, water, free fatty acids (FFA), monoglycerides (MG), diglycerides (DG) and triglycerides (TG). This study also confirmed that longer extraction times augmented the purity degree of the biodiesel (Niawanti et al., 2017).

Deep eutectic solvents have been patented several times and have been used to improve activities of enzymes and to reach the desired products. Hertel et al. have patented in 2012 a different type of DES composed of urea as a hydrogen bond donor and betaine monohydrate as a hydrogen bond acceptor. This patent is very important for the cellulose industry and for the properties of the fiber as this type of DES was used to dissolve compounds like starch, cellulose, lignin, and synthetic polymers, which cannot dissolve in other types of media. Also,

when cellulose was treated with Avicel® (purified form of cellulose) and with this type of DES, a 10-15% drop in the crystallinity of cellulose was observed (Hertel et al., 2012).

Capturing post-combustion carbon with aqueous amines is very efficient because of the high absorption capacity of carbon dioxide and its high selectivity for that latter compared to nitrogen. The solubility of carbon dioxide in the presence of primary, secondary, and tertiary amines varies depending on the reaction equilibrium. The research group led by Alnashef et al., have developed a method to solubilize and capture carbon dioxide using three different DES containing amino alcohols (Adeyemi et al., 2017). The first one was composed of ChCl and ethanolamine (ChCl:MEA). The second one was made of ChCl and diethanolamine (ChCl:DEA). The constituents of the third one were ChCl and diethanolmethylamine (ChCl:MDEA). Each solvent was obtained at different molar ratios (1:6, 1:8 and 1:10, respectively). It was found that these amine-based DES had an absorption capacity higher than the ones corresponding to the aqueous amino alcohol solutions and other regular DES. Also, it was discovered that DES composed of ChCl:MEA absorbed the highest quantity of carbon dioxide, and the one that absorbed the least was the one composed of ChCl:MDEA. Hence, the ChCl:MEA mixture was able to solubilize carbon dioxide at 265% compared to the aqueous solution of the amine (at 30% w/v) since it was able to absorb carbon dioxide both physically and chemically. However, the DES composed of ChCl:MDEA absorbed poorly carbon dioxide (16% regarding the aqueous solution of the amine at 30% w/v) (Adeyemi et al., 2017).

Due to the many advantages that DES have, as it was described in the previous section (Unconventional Media Used for Enzymatic Reactions-Introduction); we synthesized a eutectic mixture of choline chloride/glycerol (1:2 mol/mol) in combination of Tris-$SO_4$ 50 mM buffer pH 7.5 (used as a typical reaction medium). This mixture was applied to (ADH)-catalyzed reactions of various α-halogenated ketones at different substrate concentrations. In

the following sections of the thesis, the materials and methods that were used will be discussed (experimental part), and the results will be explained.

## 2.2. Results and Discussion

During our experiments we used lyophilized preparations of *E. coli* overexpressing the (*R*)-selective ADH from *Lactobacillus brevis* (LBADH) and the (*S*)-selective ADHs from *Rhodococcus ruber* (ADH-A), *Thermoanaerobacter ethanolicus* (TeSADH), and *Thermoanaerobacter* sp. (ADH-T), because these enzymes were very stable and easy to handle.

### 2.2.1. The synthesis of the racemic alcohols

In this study, racemic alcohols were synthesized chemically from the ketones that were purchased from Sigma-Aldrich-Fluka. We made those alcohols in order to determine their retention times from the gas chromatography analysis. The ketones that were used to make the alcohols were 2-chloroacetophenone (**10a**), 2,4'-dichloroacetophenone (**11a**), 2,3',4'-trichloroacetophenone (**12a**), 2,2',4'-trichloroacetophenone (**13a**), 2-chloro-4'-fluoroacetophenone (**14a**), 4'-methoxy-2-chloroacetophenone (**15a**), ethyl 4-chloroacetoacetate (**5a**) and ethyl 4,4,4-trifluoro-3-oxobutanoate (**16a**). We chose these substrates for several reasons. Firstly, halogenated ketones were discovered at the end of the eighteenth century and since then, they have showed many advantages in different science fields. Secondly, these compounds are very reactive, and this enables them to be used in different important reactions. They are building blocks for the synthesis of various valuable compounds and this is because they are selectively transformed and can be modified with many reagents. Halogenated ketones can lead to the production of several bio-active heterocyclic compounds. They can also be used in combination with other compounds to design valuable pharmaceuticals (Erian et al., 2003). Lastly, we have selected these substrates

because the halohydrins they produce are precursors and key intermediates of vital drugs that are commercialized nowadays. Some of these drugs are involved in the treatment of many diseases like respiratory disorders (Lu et al., 2011), overactive bladder disease, and hypercholesterolemia (Ibn Majdoub Hassani et al., 2020).

### 2.2.2. Enzymatic bioreduction of halogenated ketones in the presence of DES

We tested the efficiency of DES made of ChCl:glycerol (1:2 mol/mol) at different volume ratios using various ADHs to see their effect on the bioreduction of halogenated ketones at different substrate concentrations. We have selected this type of DES because in a previous study we had investigated the effects of various factors such as: different DESs, different organic solvents, temperature change, and the amount of glycerol on ADH-mediated systems. We also used propiophenone and acetophenone as substrates. The different DESs were composed of choline chloride with either ethylene glycol, urea or glycerol. We found that the DES composed of choline chloride and glycerol gave the best conversions and enantiomeric excess results (Ibn Majdoub Hassani et al., 2016). For this, we performed three types of reactions in different media, one containing plain buffer, the second one in the presence of DES (ChCl:glycerol) at 20% v/v and the third had the DES at 50% v/v. Overall, the substrate **10a** was well metabolized by three ADHs (ADH-A, ADH-T, LBADH); as high conversions and enantioselectivities were obtained (*c* >99%, *ee* >99) at 20 mM substrate concentration in all three conditions mentioned above (Table 5, entries 1-6 and 10-12). However, *E. coli*/TeSADH was not able to convert that substrate in an efficient manner (Table 5, entries 7-9). *E. coli*/ADH-T was able to transform this substrate at 100 mM concentration in the presence of 20% DES in 96% conversion (Table 5, entry 26). *E. coli*/ADH-A was able to reduce effectively this ketone up to 200 mM (>80% conversion, Table 5, entries 31-33) and *E. coli*/LBADH could transform substrate **10a** in >88% conversion at 300 mM (Table 5, entries

37-39). Finally, it is important to mention that excellent stereoselectivities were always observed in all the bioreductions.

**Table 5:** Bioreduction of 2-chloroacetophenone (**10a**) at different substrate concentrations using various proportions of ChCl:glycerol (1:2 mol/mol) DES.

| Entry | ADH | [10a] (mM) | DES (% v/v) | *c* (%)[a] | *ee* (%)[a] |
|---|---|---|---|---|---|
| 1 | *E. coli*/ADH-A | 20 | 0 | >99 | >99 (*R*) |
| 2 | *E. coli*/ADH-A | 20 | 20 | >99 | >99 (*R*) |
| 3 | *E. coli*/ADH-A | 20 | 50 | >99 | >99 (*R*) |
| 4 | *E. coli*/ADH-T | 20 | 0 | >99 | >99 (*R*) |
| 5 | *E. coli*/ADH-T | 20 | 20 | >99 | >99 (*R*) |
| 6 | *E. coli*/ADH-T | 20 | 50 | >99 | >99 (*R*) |
| 7 | *E. coli*/TeSADH | 20 | 0 | 54 | >99 (*R*) |
| 8 | *E. coli*/TeSADH | 20 | 20 | 22 | >99 (*R*) |
| 9 | *E. coli*/TeSADH | 20 | 50 | 3 | n.d. |
| 10 | *E. coli*/LBADH | 20 | 0 | >99 | >99 (*S*) |
| 11 | *E. coli*/LBADH | 20 | 20 | >99 | >99 (*S*) |
| 12 | *E. coli*/LBADH | 20 | 50 | >99 | >99 (*S*) |
| 13 | *E. coli*/ADH-A | 50 | 0 | >99 | >99 (*R*) |
| 14 | *E. coli*/ADH-A | 50 | 20 | >99 | >99 (*R*) |
| 15 | *E. coli*/ADH-A | 50 | 50 | >99 | >99 (*R*) |
| 16 | *E. coli*/ADH-T | 50 | 0 | 73 | >99 (*R*) |
| 17 | *E. coli*/ADH-T | 50 | 20 | >99 | >99 (*R*) |
| 18 | *E. coli*/ADH-T | 50 | 50 | >99 | >99 (*R*) |
| 19 | *E. coli*/LBADH | 50 | 0 | >99 | >99 (*S*) |
| 20 | *E. coli*/LBADH | 50 | 20 | >99 | >99 (*S*) |
| 21 | *E. coli*/LBADH | 50 | 50 | >99 | >99 (*S*) |
| 22 | *E. coli*/ADH-A | 100 | 0 | >99 | >99 (*R*) |
| 23 | *E. coli*/ADH-A | 100 | 20 | >99 | >99 (*R*) |
| 24 | *E. coli*/ADH-A | 100 | 50 | >99 | >99 (*R*) |
| 25 | *E. coli*/ADH-T | 100 | 0 | 75 | >99 (*R*) |
| 26 | *E. coli*/ADH-T | 100 | 20 | 96 | >99 (*R*) |
| 27 | *E. coli*/ADH-T | 100 | 50 | 40 | >99 (*R*) |
| 28 | *E. coli*/LBADH | 100 | 0 | >99 | >99 (*S*) |
| 29 | *E. coli*/LBADH | 100 | 20 | >99 | >99 (*S*) |
| 30 | *E. coli*/LBADH | 100 | 50 | >99 | >99 (*S*) |
| 31 | *E. coli*/ADH-A | 200 | 0 | 97 | >99 (*R*) |
| 32 | *E. coli*/ADH-A | 200 | 20 | 83 | >99 (*R*) |
| 33 | *E. coli*/ADH-A | 200 | 50 | 81 | >99 (*R*) |
| 34 | *E. coli*/LBADH | 200 | 0 | >99 | >99 (*S*) |
| 35 | *E. coli*/LBADH | 200 | 20 | >99 | >99 (*S*) |
| 36 | *E. coli*/LBADH | 200 | 50 | >99 | >99 (*S*) |
| 37 | *E. coli*/LBADH | 300 | 0 | 93 | >99 (*S*) |
| 38 | *E. coli*/LBADH | 300 | 20 | >99 | >99 (*S*) |
| 39 | *E. coli*/LBADH | 300 | 50 | 88 | >99 (*S*) |
| 40 | *E. coli*/LBADH | 400 | 0 | 66 | >99 (*S*) |
| 41 | *E. coli*/LBADH | 400 | 20 | 73 | >99 (*S*) |

[a] Conversions and enantiomeric excess measured by chiral GC. n.d.: not determined.

**Table 6:** Bioreduction of 2,4'-dichloroacetophenone (**11a**) at different substrate concentrations and using various proportions of ChCl:glycerol (1:2 mol/mol) DES.

| Entry | ADH | [11a] (mM) | DES (% v/v) | *c* (%)[a] | *ee* (%)[a] |
|---|---|---|---|---|---|
| 1 | *E. coli*/ADH-A | 20 | 0 | >99 | >99 (*R*) |
| 2 | *E. coli*/ADH-A | 20 | 20 | >99 | >99 (*R*) |
| 3 | *E. coli*/ADH-A | 20 | 50 | >99 | >99 (*R*) |
| 4 | *E. coli*/LBADH | 20 | 0 | >99 | >99 (*S*) |
| 5 | *E. coli*/LBADH | 20 | 20 | >99 | >99 (*S*) |
| 6 | *E. coli*/LBADH | 20 | 50 | >99 | >99 (*S*) |
| 7 | *E. coli*/ADH-A | 50 | 0 | >99 | >99 (*R*) |
| 8 | *E. coli*/ADH-A | 50 | 20 | >99 | >99 (*R*) |
| 9 | *E. coli*/ADH-A | 50 | 50 | >99 | >99 (*R*) |
| 10 | *E. coli*/LBADH | 50 | 0 | >99 | >99 (*S*) |
| 11 | *E. coli*/LBADH | 50 | 20 | >99 | >99 (*S*) |
| 12 | *E. coli*/LBADH | 50 | 50 | >99 | >99 (*S*) |
| 13 | *E. coli*/ADH-A | 100 | 0 | >99 | >99 (*R*) |
| 14 | *E. coli*/ADH-A | 100 | 20 | 98 | >99 (*R*) |
| 15 | *E. coli*/ADH-A | 100 | 50 | >99 | >99 (*R*) |
| 16 | *E. coli*/LBADH | 100 | 0 | >99 | >99 (*S*) |
| 17 | *E. coli*/LBADH | 100 | 20 | >99 | >99 (*S*) |
| 18 | *E. coli*/LBADH | 100 | 50 | >99 | >99 (*S*) |
| 19 | *E. coli*/ADH-A | 200 | 0 | >99 | >99 (*R*) |
| 20 | *E. coli*/ADH-A | 200 | 20 | >99 | >99 (*R*) |
| 21 | *E. coli*/ADH-A | 200 | 50 | 88 | >99 (*R*) |
| 22 | *E. coli*/LBADH | 200 | 0 | >99 | >99 (*S*) |
| 23 | *E. coli*/LBADH | 200 | 20 | >99 | >99 (*S*) |
| 24 | *E. coli*/LBADH | 200 | 50 | 75 | >99 (*S*) |
| 25 | *E. coli*/ADH-A | 300 | 0 | >99 | >99 (*R*) |
| 26 | *E. coli*/ADH-A | 300 | 20 | >99 | >99 (*R*) |
| 27 | *E. coli*/LBADH | 300 | 0 | >99 | 98 (*S*) |
| 28 | *E. coli*/LBADH | 300 | 20 | >99 | >99 (*S*) |
| 29 | *E. coli*/ADH-A | 400 | 0 | >99 | >99 (*R*) |
| 30 | *E. coli*/ADH-A | 400 | 20 | >99 | >99 (*R*) |
| 31 | *E. coli*/ADH-A | 500 | 0 | 97 | >99 (*R*) |
| 32 | *E. coli*/ADH-A | 500 | 20 | 79 | >99 (*R*) |

[a] Conversions and enantiomeric excess measured by chiral GC.

On the other hand, the substrate **11a** (Table 6) was successfully converted at different concentrations (20 mM-500 mM) when *E. coli*/ADH-A and *E. coli*/LBADH were used. When the DES was used at 50% v/v with a substrate concentration of 200 mM for both *E. coli*/ADH-A and *E. coli*/LBADH, a slight drop in the conversion was noticed (Table 6, entries 21 and 24). Moreover, the ability of *E. coli*/ADH-A to convert this substrate decreased as the substrate concentration reached 500 mM (Table 6, entries 31 and 32). However, when DES was used at 20% v/v, excellent conversions were acquired up to 300 mM with *E. coli*/LBADH (entry 28) and up to 400 mM with *E. coli*/ADH-A (entry 30). The amount of the added solvent affects the structure of the enzyme and therefore, its activity. All the factors mentioned above show how sensitive the enzyme is towards many reaction features, confirming that before conducting an ADH reaction at optimal conditions, one has to be very careful towards the choice of the reaction components in order for the bioconversion to occur successfully.

Overall, when the substrate **12a** (Table 7) was used with *E. coli*/ADH-A and *E. coli*/LBADH cells at concentrations varying (from 20 mM to 400 mM), excellent conversions and enantiomeric results were obtained except for some cases (Table 7, entries 28 and 31), and this is due to the increased substrate concentration. Astonishingly, DES at 50% v/v gave good results with this substrate, proving that the conversion results are dependent on several factors like the enzyme, the type of the substrate, the concentration of the substrate, and the percent volume of the DES. For this substrate, *E. coli*/LBADH could reduce it in high conversion and excellent stereoselectivity at 300 mM (entry 29) while *E. coli*/ADH-A afforded very good results even at 400 mM (entries 32 and 33).

**Table 7:** Bioreduction of 2,3',4'-trichloroacetophenone (**12a**) at different substrate concentrations and using various proportions of ChCl:glycerol (1:2 mol/mol) DES.

| Entry | ADH | [12a] (mM) | DES (% v/v) | *c* (%)[a] | *ee* (%)[a] |
|---|---|---|---|---|---|
| 1 | *E. coli*/ADH-A | 20 | 0 | >99 | >99 (*R*) |
| 2 | *E. coli*/ADH-A | 20 | 20 | >99 | >99 (*R*) |
| 3 | *E. coli*/ADH-A | 20 | 50 | >99 | >99 (*R*) |
| 4 | *E. coli*/LBADH | 20 | 0 | >99 | >99 (*S*) |
| 5 | *E. coli*/LBADH | 20 | 20 | >99 | >99 (*S*) |
| 6 | *E. coli*/LBADH | 20 | 50 | >99 | >99 (*S*) |
| 7 | *E. coli*/ADH-A | 50 | 0 | 97 | >99 (*R*) |
| 8 | *E. coli*/ADH-A | 50 | 20 | >99 | >99 (*R*) |
| 9 | *E. coli*/ADH-A | 50 | 50 | >99 | >99 (*R*) |
| 10 | *E. coli*/LBADH | 50 | 0 | 96 | >99 (*S*) |
| 11 | *E. coli*/LBADH | 50 | 20 | >99 | >99 (*S*) |
| 12 | *E. coli*/LBADH | 50 | 50 | 98 | >99 (*S*) |
| 13 | *E. coli*/ADH-A | 100 | 0 | >99 | >99 (*R*) |
| 14 | *E. coli*/ADH-A | 100 | 20 | >99 | >99 (*R*) |
| 15 | *E. coli*/ADH-A | 100 | 50 | >99 | >99 (*R*) |
| 16 | *E. coli*/LBADH | 100 | 0 | 97 | >99 (*S*) |
| 17 | *E. coli*/LBADH | 100 | 20 | 99 | >99 (*S*) |
| 18 | *E. coli*/LBADH | 100 | 50 | 98 | >99 (*S*) |
| 19 | *E. coli*/ADH-A | 200 | 0 | 96 | >99 (*R*) |
| 20 | *E. coli*/ADH-A | 200 | 20 | >99 | >99 (*R*) |
| 21 | *E. coli*/ADH-A | 200 | 50 | 92 | >99 (*R*) |
| 22 | *E. coli*/LBADH | 200 | 0 | 97 | >99 (*S*) |
| 23 | *E. coli*/LBADH | 200 | 20 | 90 | >99 (*S*) |
| 24 | *E. coli*/LBADH | 200 | 50 | 95 | >99 (*S*) |
| 25 | *E. coli*/ADH-A | 300 | 0 | 90 | >99 (*R*) |
| 26 | *E. coli*/ADH-A | 300 | 20 | 91 | >99 (*R*) |
| 27 | *E. coli*/ADH-A | 300 | 50 | 90 | >99 (*R*) |
| 28 | *E. coli*/LBADH | 300 | 0 | 61 | >99 (*S*) |
| 29 | *E. coli*/LBADH | 300 | 20 | 93 | >99 (*S*) |
| 30 | *E. coli*/LBADH | 300 | 50 | 81 | >99 (*S*) |
| 31 | *E. coli*/ADH-A | 400 | 0 | 78 | >99 (*R*) |
| 32 | *E. coli*/ADH-A | 400 | 20 | 95 | >99 (*R*) |
| 33 | *E. coli*/ADH-A | 400 | 50 | 98 | >99 (*R*) |

[a] Conversions and enantiomeric excess measured by chiral GC.

**Table 8:** Bioreduction of 2,2',4'-trichloroacetophenone (**13a**) at different substrate concentrations and using various proportions of ChCl:glycerol (1:2 mol/mol) DES.

| Entry | ADH | [13a] (mM) | DES (% v/v) | *c* (%)[a] | *ee* (%)[a] |
|---|---|---|---|---|---|
| 1 | *E. coli*/ADH-A | 20 | 0 | >99 | >99 (*R*) |
| 2 | *E. coli*/ADH-A | 20 | 20 | >99 | >99 (*R*) |
| 3 | *E. coli*/ADH-A | 20 | 50 | >99 | >99 (*R*) |
| 4 | *E. coli*/LBADH | 20 | 0 | 96 | >99 (*S*) |
| 5 | *E. coli*/LBADH | 20 | 20 | 93 | >99 (*S*) |
| 6 | *E. coli*/LBADH | 20 | 50 | 88 | >99 (*S*) |
| 7 | *E. coli*/ADH-A | 50 | 0 | >99 | >99 (*R*) |
| 8 | *E. coli*/ADH-A | 50 | 20 | >99 | >99 (*R*) |
| 9 | *E. coli*/ADH-A | 50 | 50 | 96 | >99 (*R*) |
| 10 | *E. coli*/LBADH | 50 | 0 | 91 | >99 (*S*) |
| 11 | *E. coli*/LBADH | 50 | 20 | 97 | >99 (*S*) |
| 12 | *E. coli*/LBADH | 50 | 50 | 56 | >99 (*S*) |
| 13 | *E. coli*/ADH-A | 100 | 0 | 99 | >99 (*R*) |
| 14 | *E. coli*/ADH-A | 100 | 20 | 93 | >99 (*R*) |
| 15 | *E. coli*/ADH-A | 100 | 50 | 91 | >99 (*R*) |
| 16 | *E. coli*/LBADH | 100 | 0 | 84 | >99 (*S*) |
| 17 | *E. coli*/LBADH | 100 | 20 | 83 | >99 (*S*) |
| 18 | *E. coli*/ADH-A | 200 | 0 | 92 | >99 (*R*) |
| 19 | *E. coli*/ADH-A | 200 | 20 | 99 | >99 (*R*) |
| 20 | *E. coli*/ADH-A | 200 | 50 | 93 | >99 (*R*) |
| 21 | *E. coli*/LBADH | 200 | 0 | 64 | >99 (*S*) |
| 22 | *E. coli*/LBADH | 200 | 20 | 58 | >99 (*S*) |
| 23 | *E. coli*/ADH-A | 300 | 0 | 98 | >99 (*R*) |
| 24 | *E. coli*/ADH-A | 300 | 20 | 95 | >99 (*R*) |
| 25 | *E. coli*/ADH-A | 300 | 50 | 95 | >99 (*R*) |
| 26 | *E. coli*/LBADH | 300 | 0 | 32 | >99 (*S*) |
| 27 | *E. coli*/LBADH | 300 | 20 | 15 | >99 (*S*) |
| 28 | *E. coli*/ADH-A | 400 | 0 | 99 | >99 (*R*) |
| 29 | *E. coli*/ADH-A | 400 | 20 | 99 | >99 (*R*) |
| 30 | *E. coli*/ADH-A | 400 | 50 | 96 | >99 (*R*) |
| 31 | *E. coli*/ADH-A | 500 | 0 | 51 | >99 (*R*) |
| 32 | *E. coli*/ADH-A | 500 | 20 | 47 | >99 (*R*) |
| 33 | *E. coli*/ADH-A | 500 | 50 | 34 | >99 (*R*) |

[a] Conversions and enantiomeric excess measured by chiral GC.

For the substrate **13a** (Table 8), good conversions and enantioselectivities were noticed when it was used with *E. coli*/ADH-A at various substrate concentrations (20 mM-400 mM), yet there was a decline in the conversions when the substrate concentration reached 500 mM (Table 8, entries 31-33). Usually, slightly better results were achieved at 20% v/v of DES, confirming once again that the best proportion of the DES in these types of reactions seems to be this proportion. However, *E. coli*/LBADH did not show the same satisfying results as we increased the substrate concentration more than 100 mM (Table 8, entries 21-22 and 26-27).

Moreover, for the fluorinated substrate **14a** (Table 9) outstanding conversions and enantioselectivities were perceived for both *E. coli*/ADH-A and *E. coli*/LBADH when that substrate was used at various concentrations (20 mM-400 mM) in DES 20% v/v and DES 50% v/v (Table 9, entries 1-36); however, there was a sharp drop in the conversion of *E. coli*/ADH-A at a substrate concentration of 500 mM (Table 9, entries 37-39). Also, when we used this substrate with the two enzymes at DES 50% v/v, a slight decrease in the conversion rates were noticed compared to the reaction medium at 20% v/v.

**Table 9:** Bioreduction of 2-chloro-4'-fluoroacetophenone (**14a**) at different substrate concentrations and using various proportions of ChCl:glycerol (1:2 mol/mol) DES.

| Entry | ADH | [14a] (mM) | DES (% v/v) | *c* (%)[a] | *ee* (%)[a] |
|---|---|---|---|---|---|
| 1 | *E. coli*/ADH-A | 20 | 0 | >99 | >99 (*R*) |
| 2 | *E. coli*/ADH-A | 20 | 20 | >99 | >99 (*R*) |
| 3 | *E. coli*/ADH-A | 20 | 50 | 96 | >99 (*R*) |
| 4 | *E. coli*/LBADH | 20 | 0 | >99 | >99 (*S*) |
| 5 | *E. coli*/LBADH | 20 | 20 | >99 | >99 (*S*) |
| 6 | *E. coli*/LBADH | 20 | 50 | 95 | >99 (*S*) |
| 7 | *E. coli*/ADH-A | 50 | 0 | >99 | >99 (*R*) |
| 8 | *E. coli*/ADH-A | 50 | 20 | >99 | >99 (*R*) |
| 9 | *E. coli*/ADH-A | 50 | 50 | >99 | >99 (*R*) |
| 10 | *E. coli*/LBADH | 50 | 0 | >99 | >99 (*S*) |
| 11 | *E. coli*/LBADH | 50 | 20 | >99 | >99 (*S*) |
| 12 | *E. coli*/LBADH | 50 | 50 | >99 | >99 (*S*) |
| 13 | *E. coli*/ADH-A | 100 | 0 | >99 | >99 (*R*) |
| 14 | *E. coli*/ADH-A | 100 | 20 | >99 | >99 (*R*) |
| 15 | *E. coli*/ADH-A | 100 | 50 | 98 | >99 (*R*) |
| 16 | *E. coli*/LBADH | 100 | 0 | >99 | >99 (*S*) |
| 17 | *E. coli*/LBADH | 100 | 20 | >99 | >99 (*S*) |
| 18 | *E. coli*/LBADH | 100 | 50 | 95 | >99 (*S*) |
| 19 | *E. coli*/ADH-A | 200 | 0 | >99 | >99 (*R*) |
| 20 | *E. coli*/ADH-A | 200 | 20 | >99 | >99 (*R*) |
| 21 | *E. coli*/ADH-A | 200 | 50 | 96 | >99 (*R*) |
| 22 | *E. coli*/LBADH | 200 | 0 | 97 | >99 (*S*) |
| 23 | *E. coli*/LBADH | 200 | 20 | >99 | >99 (*S*) |
| 24 | *E. coli*/LBADH | 200 | 50 | 92 | >99 (*S*) |
| 25 | *E. coli*/ADH-A | 300 | 0 | 96 | >99 (*R*) |
| 26 | *E. coli*/ADH-A | 300 | 20 | 98 | >99 (*R*) |
| 27 | *E. coli*/ADH-A | 300 | 50 | 99 | >99 (*R*) |
| 28 | *E. coli*/LBADH | 300 | 0 | 98 | >99 (*S*) |
| 29 | *E. coli*/LBADH | 300 | 20 | 99 | >99 (*S*) |
| 30 | *E. coli*/LBADH | 300 | 50 | 99 | >99 (*S*) |
| 31 | *E. coli*/ADH-A | 400 | 0 | 96 | >99 (*R*) |
| 32 | *E. coli*/ADH-A | 400 | 20 | 96 | >99 (*R*) |
| 33 | *E. coli*/ADH-A | 400 | 50 | >99 | >99 (*R*) |
| 34 | *E. coli*/LBADH | 400 | 0 | 88 | >99 (*S*) |
| 35 | *E. coli*/LBADH | 400 | 20 | >99 | >99 (*S*) |
| 36 | *E. coli*/LBADH | 400 | 50 | 98 | >99 (*S*) |
| 37 | *E. coli*/ADH-A | 500 | 0 | 61 | >99 (*R*) |
| 38 | *E. coli*/ADH-A | 500 | 20 | 72 | >99 (*R*) |
| 39 | *E. coli*/ADH-A | 500 | 50 | 63 | >99 (*R*) |

[a] Conversions and enantiomeric excess measured by chiral GC.

**Table 10:** Bioreduction of 2-chloro-4'-methoxyacetophenone (**15a**) at different substrate concentrations and using various proportions of ChCl:glycerol (1:2 mol/mol) DES.

| Entry | ADH | [15a] (mM) | DES (% v/v) | *c* (%)[a] | *ee* (%)[a] |
|---|---|---|---|---|---|
| 1 | *E. coli*/ADH-A | 20 | 0 | >99 | >99 (*R*) |
| 2 | *E. coli*/ADH-A | 20 | 20 | >99 | >99 (*R*) |
| 3 | *E. coli*/ADH-A | 20 | 50 | >99 | >99 (*R*) |
| 4 | *E. coli*/LBADH | 20 | 0 | >99 | >99 (*S*) |
| 5 | *E. coli*/LBADH | 20 | 20 | >99 | >99 (*S*) |
| 6 | *E. coli*/LBADH | 20 | 50 | >99 | >99 (*S*) |
| 7 | *E. coli*/ADH-A | 50 | 0 | >99 | >99 (*R*) |
| 8 | *E. coli*/ADH-A | 50 | 20 | >99 | >99 (*R*) |
| 9 | *E. coli*/ADH-A | 50 | 50 | >99 | >99 (*R*) |
| 10 | *E. coli*/LBADH | 50 | 0 | >99 | >99 (*S*) |
| 11 | *E. coli*/LBADH | 50 | 20 | >99 | >99 (*S*) |
| 12 | *E. coli*/LBADH | 50 | 50 | >99 | >99 (*S*) |
| 13 | *E. coli*/ADH-A | 100 | 0 | >99 | >99 (*R*) |
| 14 | *E. coli*/ADH-A | 100 | 20 | >99 | >99 (*R*) |
| 15 | *E. coli*/ADH-A | 100 | 50 | >99 | >99 (*R*) |
| 16 | *E. coli*/LBADH | 100 | 0 | >99 | >99 (*S*) |
| 17 | *E. coli*/LBADH | 100 | 20 | >99 | >99 (*S*) |
| 18 | *E. coli*/LBADH | 100 | 50 | 99 | >99 (*S*) |
| 19 | *E. coli*/ADH-A | 200 | 0 | 90 | >99 (*R*) |
| 20 | *E. coli*/ADH-A | 200 | 20 | 98 | >99 (*R*) |
| 21 | *E. coli*/ADH-A | 200 | 50 | 98 | >99 (*R*) |
| 22 | *E. coli*/LBADH | 200 | 0 | 96 | >99 (*S*) |
| 23 | *E. coli*/LBADH | 200 | 20 | 98 | >99 (*S*) |
| 24 | *E. coli*/LBADH | 200 | 50 | 98 | >99 (*S*) |
| 25 | *E. coli*/ADH-A | 300 | 0 | 93 | >99 (*R*) |
| 26 | *E. coli*/ADH-A | 300 | 20 | 96 | >99 (*R*) |
| 27 | *E. coli*/ADH-A | 300 | 50 | 87 | >99 (*R*) |
| 28 | *E. coli*/LBADH | 300 | 0 | 93 | >99 (*S*) |
| 29 | *E. coli*/LBADH | 300 | 20 | 93 | >99 (*S*) |
| 30 | *E. coli*/LBADH | 300 | 50 | 95 | >99 (*S*) |
| 31 | *E. coli*/ADH-A | 400 | 0 | 68 | >99 (*R*) |
| 32 | *E. coli*/ADH-A | 400 | 20 | 71 | >99 (*R*) |
| 33 | *E. coli*/ADH-A | 400 | 50 | 76 | >99 (*R*) |
| 34 | *E. coli*/LBADH | 400 | 0 | 91 | >99 (*S*) |
| 35 | *E. coli*/LBADH | 400 | 20 | 94 | >99 (*S*) |
| 36 | *E. coli*/LBADH | 400 | 50 | 71 | >99 (*S*) |

[a] Conversions and enantiomeric excess measured by chiral GC.

Perfect conversions and enantioselectivities were observed with substrate **15a** up to 100 mM, which was also used with *E. coli*/ADH-A and *E. coli*/LBADH at different substrate concentrations (Table 10, entries 1-18). Conversely, there was a decrease in the conversions, as we elevated the substrate concentration until 400 mM (Table 10, entries 19-36). Surprisingly for this substrate, DES at 50% v/v seemed to improve the conversions (Table 10, entries 21, 24, 30, and 33). Moreover, reactions containing DES were improved as opposed to adding only buffer (Table 10, entries 19, 20, 22, 23, 25, 26, 31, 32, 34 and 35).

Likewise, superb conversions and enantioselectivities were perceived with the aliphatic chlorinated substrate **5a**, which was utilized with *E. coli*/ADH-A and *E. coli*/LBADH at various substrate concentrations (Table 11). However, a loss of conversion was obtained when *E. coli*/LBADH was used with a substrate concentration of 200 mM at DES 20% v/v and DES 50% v/v (Table 11, entries 22-24). *E. coli*/ADH-A showed a decrease in the conversion of that substrate when its concentrations reached 400 mM (employing 50% v/v of DES) and 500 mM (under the three studied conditions, Table 11, entries 30-33). These results show that most of the halogenated substrates, at high concentrations, are more efficiently bioreduced by *E. coli*/ADH-A compared to *E. coli*/LBADH.

**Table 11:** Bioreduction of ethyl 4-chloroacetoacetate (**5a**) at different substrate concentrations and using various proportions of ChCl:glycerol (1:2 mol/mol) DES.

| Entry | ADH | [5a] (mM) | DES (% v/v) | *c* (%)[a] | *ee* (%)[b] |
|---|---|---|---|---|---|
| 1 | *E. coli*/ADH-A | 20 | 0 | 97 | >99 (*R*) |
| 2 | *E. coli*/ADH-A | 20 | 20 | >99 | >99 (*R*) |
| 3 | *E. coli*/ADH-A | 20 | 50 | >99 | >99 (*R*) |
| 4 | *E. coli*/LBADH | 20 | 0 | 99 | 95 (*S*) |
| 5 | *E. coli*/LBADH | 20 | 20 | 99 | 92 (*S*) |
| 6 | *E. coli*/LBADH | 20 | 50 | 97 | 97 (*S*) |
| 7 | *E. coli*/ADH-A | 50 | 0 | >99 | >99 (*R*) |
| 8 | *E. coli*/ADH-A | 50 | 20 | >99 | >99 (*R*) |
| 9 | *E. coli*/ADH-A | 50 | 50 | >99 | >99 (*R*) |
| 10 | *E. coli*/LBADH | 50 | 0 | >99 | 94 (*S*) |
| 11 | *E. coli*/LBADH | 50 | 20 | >99 | 96 (*S*) |
| 12 | *E. coli*/LBADH | 50 | 50 | >99 | >99 (*S*) |
| 13 | *E. coli*/ADH-A | 100 | 0 | >99 | >99 (*R*) |
| 14 | *E. coli*/ADH-A | 100 | 20 | >99 | >99 (*R*) |
| 15 | *E. coli*/ADH-A | 100 | 50 | >99 | >99 (*R*) |
| 16 | *E. coli*/LBADH | 100 | 0 | >99 | >99 (*S*) |
| 17 | *E. coli*/LBADH | 100 | 20 | >99 | >99 (*S*) |
| 18 | *E. coli*/LBADH | 100 | 50 | 90 | >99 (*S*) |
| 19 | *E. coli*/ADH-A | 200 | 0 | >99 | >99 (*R*) |
| 20 | *E. coli*/ADH-A | 200 | 20 | >99 | >99 (*R*) |
| 21 | *E. coli*/ADH-A | 200 | 50 | >99 | >99 (*R*) |
| 22 | *E. coli*/LBADH | 200 | 0 | 60 | >99 (*S*) |
| 23 | *E. coli*/LBADH | 200 | 20 | 65 | >99 (*S*) |
| 24 | *E. coli*/LBADH | 200 | 50 | 50 | >99 (*S*) |
| 25 | *E. coli*/ADH-A | 300 | 0 | >99 | >99 (*R*) |
| 26 | *E. coli*/ADH-A | 300 | 20 | >99 | >99 (*R*) |
| 27 | *E. coli*/ADH-A | 300 | 50 | >99 | >99 (*R*) |
| 28 | *E. coli*/ADH-A | 400 | 0 | >99 | >99 (*R*) |
| 29 | *E. coli*/ADH-A | 400 | 20 | >99 | >99 (*R*) |
| 30 | *E. coli*/ADH-A | 400 | 50 | 84 | 97 (*R*) |
| 31 | *E. coli*/ADH-A | 500 | 0 | 84 | 97 (*R*) |
| 32 | *E. coli*/ADH-A | 500 | 20 | 90 | >99 (*R*) |
| 33 | *E. coli*/ADH-A | 500 | 50 | 66 | 88 (*R*) |

[a] Conversions measured by chiral GC. [b] Enantiomeric excess measured by chiral GC after acetylation of the alcohol.

**Table 12:** Bioreduction of ethyl 4,4,4-trifluoroacetoacetate (**16a**) at different substrate concentrations and using various proportions of ChCl:glycerol (1:2 mol/mol) DES.

| Entry | ADH | [16a] (mM) | DES (% v/v) | *c* (%)[a] | *ee* (%)[b] |
|---|---|---|---|---|---|
| 1 | *E. coli*/ADH-A | 20 | 0 | >99 | >99 (*R*) |
| 2 | *E. coli*/ADH-A | 20 | 20 | >99 | >99 (*R*) |
| 3 | *E. coli*/ADH-A | 20 | 50 | >99 | >99 (*R*) |
| 4 | *E. coli*/LBADH | 20 | 0 | 99 | >99 (*S*) |
| 5 | *E. coli*/LBADH | 20 | 20 | >99 | >99 (*S*) |
| 6 | *E. coli*/LBADH | 20 | 50 | >99 | >99 (*S*) |
| 7 | *E. coli*/ADH-A | 50 | 0 | >99 | >99 (*R*) |
| 8 | *E. coli*/ADH-A | 50 | 20 | >99 | >99 (*R*) |
| 9 | *E. coli*/ADH-A | 50 | 50 | >99 | >99 (*R*) |
| 10 | *E. coli*/LBADH | 50 | 0 | 97 | >99 (*S*) |
| 11 | *E. coli*/LBADH | 50 | 20 | >99 | >99 (*S*) |
| 12 | *E. coli*/LBADH | 50 | 50 | >99 | >99 (*S*) |
| 13 | *E. coli*/ADH-A | 100 | 0 | >99 | >99 (*R*) |
| 14 | *E. coli*/ADH-A | 100 | 20 | >99 | >99 (*R*) |
| 15 | *E. coli*/ADH-A | 100 | 50 | >99 | >99 (*R*) |
| 16 | *E. coli*/LBADH | 100 | 0 | >99 | >99 (*S*) |
| 17 | *E. coli*/LBADH | 100 | 20 | 98 | 98 (*S*) |
| 18 | *E. coli*/LBADH | 100 | 50 | 90 | >99 (*S*) |
| 19 | *E. coli*/ADH-A | 200 | 0 | 95 | >99 (*R*) |
| 20 | *E. coli*/ADH-A | 200 | 20 | 99 | >99 (*R*) |
| 21 | *E. coli*/ADH-A | 200 | 50 | 98 | >99 (*R*) |
| 22 | *E. coli*/LBADH | 200 | 0 | 91 | >99 (*S*) |
| 23 | *E. coli*/LBADH | 200 | 20 | 92 | >99 (*S*) |
| 24 | *E. coli*/LBADH | 200 | 50 | 86 | >99 (*S*) |
| 25 | *E. coli*/ADH-A | 300 | 0 | 96 | >99 (*R*) |
| 26 | *E. coli*/ADH-A | 300 | 20 | 94 | >99 (*R*) |
| 27 | *E. coli*/ADH-A | 300 | 50 | 91 | >99 (*R*) |
| 28 | *E. coli*/LBADH | 300 | 0 | 88 | >99 (*S*) |
| 29 | *E. coli*/LBADH | 300 | 20 | 87 | >99 (*S*) |
| 30 | *E. coli*/LBADH | 300 | 50 | 61 | >99 (*S*) |
| 31 | *E. coli*/ADH-A | 400 | 0 | 88 | >99 (*R*) |
| 32 | *E. coli*/ADH-A | 400 | 20 | 91 | >99 (*R*) |
| 33 | *E. coli*/ADH-A | 400 | 50 | 72 | >99 (*R*) |
| 34 | *E. coli*/LBADH | 400 | 0 | 76 | >99 (*S*) |
| 35 | *E. coli*/LBADH | 400 | 20 | 63 | >99 (*S*) |
| 36 | *E. coli*/LBADH | 400 | 50 | 58 | >99 (*S*) |

[a] Conversions measured by chiral GC. [b] Enantiomeric excess measured by chiral GC after acetylation of the alcohol.

Borzęcka et al. had synthesized enantiopure fluorohydrins using ADHs at high substrate concentrations obtaining outstanding results. Also, *E. coli*/ADH-A was utilized without adding external cofactor synthesizing the (*R*)-fluorohydrin derivatives. They also used *E. coli*/LBADH to make the enantiopure (*S*)-fluorohydrins at a 500 mM substrate concentration. This study was important because fluorinated compounds are used in pharmaceuticals and agrochemicals. Fluorohydrins are in fact precursors of steroids and carbohydrates. At the beginning of this study, the researchers used fluorinated α-bromoacetophenones, α-fluoroacetophenone derivatives, 2,2-difluoroacetophenone, and 2,2,2-trifluoroacetophenone as substrates at a small concentration (30 mM) with *E. coli*/ADH-A and *E. coli*/LBADH, and as a result perfect conversions and enantioselectivities were obtained (>99%). In another set of experiments with *E. coli*/LBADH, the substrate concentration could be increased up to 500 mM in the presence of 1 mM of the cofactor while keeping the same amount of the enzyme in the reaction. Hence, fluorinated alcohols were attained with outstanding conversions and enantioselectivities (Borzęcka et al., 2013). In that study, perfect conversion results were obtained although the substrate concentration was high, showing that bioconversions can be affected by the type of substrate.

For the trifluorinated aliphatic ketone that we studied (**16a)**, excellent conversions were observed with *E. coli*/ADH-A and *E. coli*/LBADH when the substrate concentration was 20 mM and 50 mM (Table 12, entries 1-12). However, as we increased the substrate concentration for both enzymes from 100 mM to 400 mM, conversion rates slowly fell down (Table 12, entries 13-36). *E. coli*/LBADH transformed worse this substrate regarding *E. coli*/ADH-A, especially in the case of using DES 50% v/v. In general, biotransformations in the presence of DES 20% v/v presented better conversions than at 50% v/v of DES (Table 12, entries: 17, 18, 23, 24, 29, 30, 32 and 33).

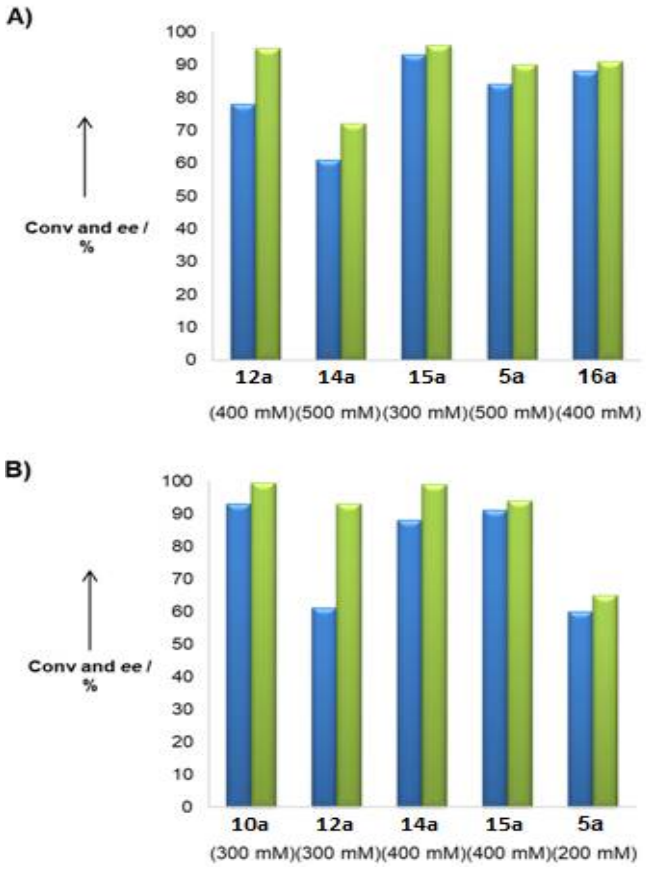



**Figure 11:** comparison between conversions for some selected bioreductions with: A) *E. coli*/ADH-A; and B) *E. coli*/LBADH in plain buffer (blue bars) and in the presence of ChCl:glycerol (1:2 mol/mol) DES at 20% v/v (green bars). In all cases, the obtained *ee* values were >99%.

When comparing the results using plain buffer or DES at 20% v/v for some bioreductions (Figure 11), it was observed that the presence of this eutectic medium gave a beneficial outcome compared to the reactions containing only buffer. This finding demonstrates that the DES could improve the capacity of enzymes to reduce halogenated substrates. This effect can be due to several reasons like: DES helps to solubilize the substrate and make it more available for the enzyme. In addition, glycerol in DES protects the ADH, therefore improving the enzyme capacity to convert the substrate. DES can also improve the permeability of the cell membrane as opposed to only the buffer, enabling the entrance of the substrate inside the cell.

Due to the expensive price of cofactors nowadays, and since we are using lyophilized whole cell systems, we attempted to bioreduce the halogenated substrates (**5a**, **10a**-**16a** at a 200 mM concentration), in the presence of DES at 20% and 50% v/v without adding an extra amount of NADH. Amazingly, we obtained outstanding enantiomeric excess results for all the substrates (*ee* >99%), and excellent conversions for most of the substrates (Table 13, entries 7-8, 11, 13-14, 17, and 19-23).

**Table 13:** bioreduction of the halogenated substrates (200 mM) using *E. coli*/ADH-A without cofactor in the presence of DES at 20% and at 50%.

| Entry | Substrate | DES (% v/v) | Conv (%)[a] | *ee* (%)[a] |
|---|---|---|---|---|
| 1 | **10a** | 0 | 76 | >99 (*R*) |
| 2 | **10a** | 20 | 82 | >99 (*R*) |
| 3 | **10a** | 50 | 70 | >99 (*R*) |
| 4 | **11a** | 0 | 87 | >99 (*R*) |
| 5 | **11a** | 20 | 71 | >99 (*R*) |
| 6 | **11a** | 50 | 46 | >99 (*R*) |
| 7 | **12a** | 0 | >99 | >99 (*R*) |
| 8 | **12a** | 20 | >99 | >99 (*R*) |
| 9 | **12a** | 50 | 81 | >99 (*R*) |
| 10 | **13a** | 0 | 85 | >99 (*R*) |
| 11 | **13a** | 20 | 97 | >99 (*R*) |
| 12 | **13a** | 50 | 85 | >99 (*R*) |
| 13 | **14a** | 0 | >99 | >99 (*R*) |
| 14 | **14a** | 20 | >99 | >99 (*R*) |
| 15 | **14a** | 50 | 92 | >99 (*R*) |
| 16 | **15a** | 0 | 88 | >99 (*R*) |
| 17 | **15a** | 20 | 97 | >99 (*R*) |
| 18 | **15a** | 50 | 91 | >99 (*R*) |
| 19 | **5a** | 0 | >99 | >99 (*R*)[b] |
| 20 | **5a** | 20 | >99 | >99 (*R*)[b] |
| 21 | **5a** | 50 | >99 | >99 (*R*)[b] |
| 22 | **16a** | 0 | 97 | >99 (*R*)[b] |
| 23 | **16a** | 20 | 97 | >99 (*R*)[b] |
| 24 | **16a** | 50 | 91 | >99 (*R*)[b] |

[a] Conversions and enantiomeric excess measured by chiral GC.
[b] Enantiomeric excess measured by chiral GC after acetylation of the alcohol.

The lower conversion rates that were obtained for the substrates **10a** and **11a** at 200 mM, especially when DES was used at 50% v/v could be caused by the substrate concentration. In

fact, when we have set up the reaction with *E. coli*/ADH-A and **10a** as a substrate (20 mM), perfect conversion and stereoselectivities (>99%) were attained in the absence of the NAD cofactor and DES. We conducted the bioreductions without cofactor only with ADH-A because we previously observed poor conversion results with LBADH (data not shown). This can be due to the fact that *E. coli* cells contain more cytosolic concentrations of NADH/$NAD^+$ as opposed to NADPH/$NADP^+$(Ibn Majdoub Hassani et al., 2020). Overall, these good results suggest that we can use ADHs in overexpressed whole-cell preparations with some selected halogenated substrates without having to add the expensive NAD cofactor, and that the DES can play an important role in improving these types of reactions.

### 2.2.3. Scale-up of some selected enzymatic bioreductions in the presence of DES

As a result of the successful bioreduction of the eight halogenated substrates in the presence of DES at 20% v/v and at 50% v/v with ADH-A and LBADH; we scaled-up the reductions of the halogenated ketones to 250 mg and to 1 g in another set of experiments to see if this approach could be applied at a large scale. In these trials, the substrate **13a** was used in two different amounts (250 mg) and (1g).

In most cases, all the results showed excellent conversions (≥96) and perfect enantioselectivities (>99%). In addition, the products were isolated with an average value of 80% yield (Figure 12). Also, **13a** which was scaled up to 1 g with *E. coli*/ADH-A, gave a very good yield of enantiopure halohydrin (*R*)-**13b** (89%). Furthermore, we tested the effect of omitting the cofactor in some of the scale up procedures and have found that when *E. coli*/ADH-A was used with DES 20% v/v using the substrate **12a**, the isolated yield of enantiopure (*R*)-**12b** was 59%.

Therefore, in these scale up reactions we demonstrated that the DESs can also be used with ADH-mediated systems at a larger scale and can still afford high conversions and high yields

of the corresponding alcohols.

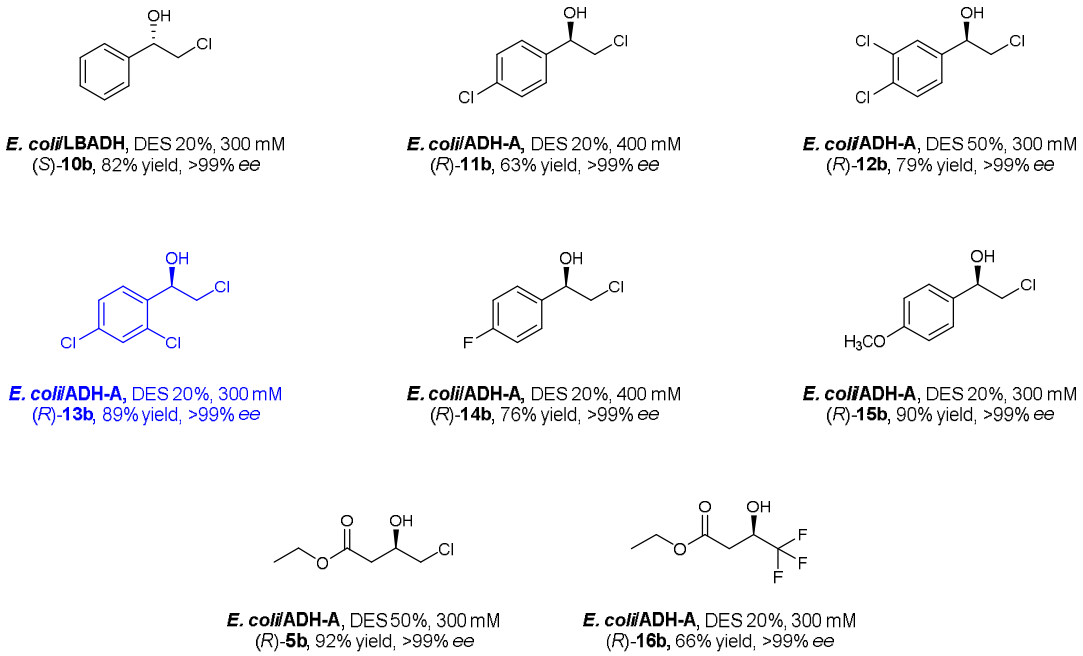



**Figure 12:** preparative transformations at 250 mg (black color) and 1 g-scale (blue color) to synthesize enantiopure halohydrins in the presence of DES.

In order to make a successful scale up reaction, one has to consider the following factors: the choice of the convenient enzyme, a good coenzyme regenerating step, and an appropriate reaction protocol. There are many examples where ADHs have been used at preparative scale reactions. Different NAD(P)H dependent ADHs from various biological sources like horse liver, *Lactobacillus brevis, Rhodococcus erythropolis, Thermobacterium brockii,* and yeast have been utilized in these protocols (Hummel 1999).

Halohydrins are intermediates of several drugs like mirabegron, sertraline, luliconazole, ezetimibe, and empagliflozin (Ibn Majdoub Hassani et al., 2020), and many research teams have synthesized these compounds which are pharmacologically important. For example, in a study conducted by Yamamoto et al., they have produced **5b** by using *E. coli* cells, which expressed a (*S*)-specific secondary alcohol dehydrogenase. The compound **5b** is used to synthesize (*R*)-carnitine, (*R*)-4-amino-3-hydroxybutyric acid and (*R*)-4-hydroxy-2-pyrrolidinone (Yamamoto et al., 2002). Moreover, another research group led by He et al.

have used whole cells of *Saccharomyces uvarum* SW-58 to synthesize **16b**, which is a precursor of the antidepressant befloxatone (He et al., 2007).

## 2.3. Experimental Part

### 2.3.1. General methods

2-Chloroacetophenone (**10a**), 2,4'-dichloroacetophenone (**11a**), 2,3',4'-trichloroacetophenone (**12a**), 2,2',4'-trichloroacetophenone (**13a**), 2-chloro-4'-fluoroacetophenone (**14a**), 4'-methoxy-2-chloroacetophenone (**15a**), ethyl 4-chloroacetoacetate (**5a**) and ethyl 4,4,4-trifluoro-3-oxobutanoate (**16a**), NADPH, NADH, and all the other chemical reagents that were used in this study; were acquired from Sigma-Aldrich-Fluka (Steinheim, Germany).

The enzymes used were obtained from Prof. Wolfgang Kroutil (University of Graz). The ADHs were overexpressed in the *E. coli* strain: ADH-A from *Rhodococcus ruber*, ADH-T from *Thermoanaerobacter* sp., LBADH from *Lactobacillus brevis*, and TeSADH from *Thermoanaerobacter ethanolicus*. Enzymatic activities of the lyophilized whole cells ranged from 0.3-1 U $mg^{-1}$. The DES (ChCl/glycerol 1:2 mol/mol) that was used throughout this study was made of choline chloride (10 g, 71.8 mmol) and glycerol (10.5 mL, 142.6 mmol). For the preparation of this solvent, the constituents were mixed and stirred with a magnetic stirrer at 60 ºC (approximately 2 hours) until a clear solution was observed.

Gas chromatography (GC) analyses were performed on a Hewlett Packard 6890 Series II chromatograph. A ChirasilDex-CB column (25 m x 0.25 mm, 0.25 µm, 12.2 psi $N_2$) was used as a chiral stationary phase. The GC was utilized in order to measure the conversion and enantiomeric excess of the chiral products (Table 14). NMR spectra ($^1$H NMR) were recorded on a Bruker AV300 MHz spectrometer (Figures 13-20).

## 2.3.2. Analytics

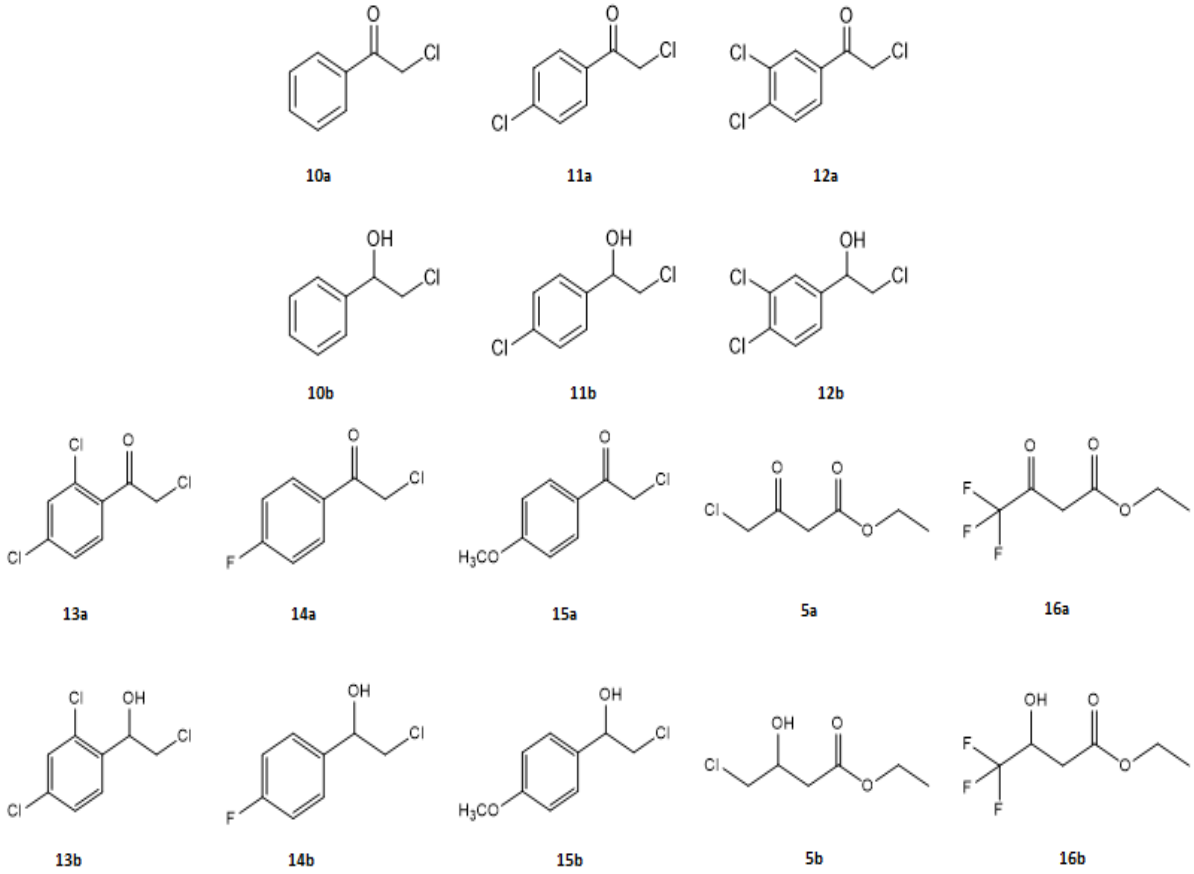



**Table 14:** GC retention times of the ketones and the alcohols that were used in the experiment.

| **Compound** | **Temperature program**[a] | **Time** (min) |
|---|---|---|
| **10a** | 110/0/2.5/120/0/10/200/1 | 6.9 |
| **10b** | 110/0/2.5/120/0/10/200/1 | 8.1 (*S*) and 8.3 (*R*) |
| **11a** | 110/0/2.5/120/0/10/200/1 | 9.1 |
| **11b** | 110/0/2.5/120/0/10/200/1 | 10.5 (*S*) and 10.7 (*R*) |
| **12a** | 110/0/2.5/120/0/10/200/1 | 10.7 |
| **12b** | 110/0/2.5/120/0/10/200/1 | 12.5 (*S*) and 12.7 (*R*) |
| **13a** | 110/0/2.5/120/0/10/200/1 | 11.9 |
| **13b** | 110/0/2.5/120/0/10/200/1 | 14.5 (*S*) and 15.0 (*R*) |
| **14a** | 110/0/2.5/120/0/10/200/1 | 9.0 |
| **14b** | 110/0/2.5/120/0/10/200/1 | 10.7 (*S*) and 11.0 (*R*) |
| **15a** | 110/0/2.5/120/0/10/200/1 | 12.7 |
| **15b** | 110/0/2.5/120/0/10/200/1 | 13.1 (*S*) and 13.3 (*R*) |
| **5a** | 80/0/2.5/120/0/10/200/1 | 9.9 |
| **5b**[b] | 80/0/2.5/120/0/10/200/1 | 14.6 (*R*) and 15.0 (*S*) |
| **16a** | 80/0/2.5/105/0/10/195/1 | 1.5 |
| **16b**[b] | 80/0/2.5/120/0/10/200/1 | 7.7 (*R*) and 8.2 (*S*) |

[a] Initial temperature (ºC)/time (min)/slope (ºC/min)/temperature (ºC)/time (min)/slope (ºC/min)/final temperature (ºC)/time (min). [b] Alcohol acetylated using DMAP and acetic anhydride.

### 2.3.3. Experimental procedures

**General procedure for the synthesis of the racemic alcohols**

To a solution of the corresponding ketone (**5a**, **10a-16a**, 50 mg or 50 μL) in 2 mL of methanol (for **10a-15a**) or ethanol (in case of **5a** or **16a**) at 0 ºC, sodium borohydride (1.2 equiv.) was added. The solution was then stirred at room temperature for three hours and the reaction was monitored by TLC analysis until complete disappearance of the starting ketone. Then, some drops of HCl 1 M and water (20 mL) were added to the solution, which was later extracted with ethyl acetate (EtOAc, 3 x 20 mL) or dichloromethane ($CH_2Cl_2$, 3 x 20 mL). For product **16b**, the solution was extracted using diethyl ether (3 × 20 mL) due to the low boiling point of the alcohol. Then the organic layers were combined and dried over $Na_2SO_4$, filtered and the solvent was evaporated under reduced pressure, obtaining the racemic alcohols in good yields (>80%).

**General procedure for the enzymatic reduction of halogenated ketones in the presence of a deep eutectic solvent**

In a 1.5 mL-Eppendorf vial, the reaction mixture (final volume: 0.6 mL), contains: 10 mg of the corresponding *E. coli*/ADH lyophilized cells, Tris-$SO_4$ buffer 50 mM pH 7.5, $NAD(P)^+$ (1 mM), DES (ChCl: glycerol 1:2 mol/mol, 20% or 50% v/v), propan-2-ol (5-15% v/v) and the halogenated ketone (**5a**, **10a-16a**, Tables 5-12, 20-500 mM), dissolved in DMSO. The final amount of DMSO in the assay was between 2.5-5% v/v. The reduction reactions were carried out during 24 h, at 30 ºC in an orbital shaker at 250 rpm. The crude was extracted with ethyl acetate (2 x 500 μL). The organic layers were collected by centrifugation, combined, and dried over $Na_2SO_4$. Conversion rates and enantiomer excess of the halohydrins were determined by GC on a chiral column (see the Supporting Information). To determine the *ee*

values for halohydrins **5b** and **16b**, an acetylation step was required. 4-Dimethylaminopyridine (DMAP, 1 mg) and acetic anhydride (30 μL) were mixed and then added to a volume of 200 μL of the extracted reaction crude solution. The mixture was shaken for 3 h at 30 ºC, and then quenched with 0.5 mL water for 30 min. The acetylated alcohol was extracted with ethyl acetate (2 x 500 μL), and the combined organic phases were dried over $Na_2SO_4$, and subjected to GC analysis.

**Bioreductions performed without the addition of the nicotinamide cofactor**

Some bioreduction reactions were carried out without adding the cofactors to test their influence on the reactions. To achieve this, the protocols described in the Experimental Section of the manuscript were followed except that $NAD(P)^+$ was not added to the buffer solution.

**Scale-up of the bioreduction of 10a (300 mM) in the presence of DES (20% v/v)**

In an Erlenmeyer flask, 100 mg of *E. coli*/LBADH were introduced. Then, 3.8 mL of Tris-$SO_4$ 50 mM pH 7.5 buffer, 0.54 mL of $NADP^+$ solution (10 mM in Tris-$SO_4$ 50 mM pH 7.5) and 1.1 mL (20% v/v) of DES were added in the Erlenmeyer flask. Then, 300 μL of isopropanol are introduced and 250 mg of 2-chloroacetophenone. The Erlenmeyer flask was closed and introduced in an orbital shaker at 250 rpm for 24 h at 30 ºC. The reaction was transferred to a Falcon tube and it was centrifuged (4,900 rpm, 15 min, 4 ºC). The supernatant was taken from the reaction to an extraction funnel, and the pellet was then washed with EtOAc (10 mL) to extract the product from it by centrifugation (4,900 rpm, 15 min, 4 ºC). This extract was pooled into the extraction funnel. 5 mL of water were added in the extraction funnel. The aqueous and organic phases were separated into two different Erlenmeyer flasks. The aqueous phase was again introduced in the extraction funnel and was extracted again with

pure EtOAc (2 x 20 mL). The organic phase was washed with 10 mL of water (to eliminate the glycerol from the organic phase). Finally, $Na_2SO_4$ was added as a desiccant to the organic phase, the liquid was filtered into a 100-mL round-bottom flask. $Na_2SO_4$ was washed with pure EtOAc (10 mL), and pooled together with the previous filtered organic phase. Then, the organic solvent was evaporated under reduced pressure, affording the alcohol (*S*)-**10b** [(*S*)-2-chloro-1-phenylethan-1-ol, 207 mg, 82% yield] in >99% *ee*.

**Scale-up of the bioreduction of 11a (400 mM) in the presence of DES (20% v/v)**

In an Erlenmeyer flask, 100 mg of *E. coli*/ADH-A were introduced. Then, 2.3 mL of Tris-$SO_4$ 50 mM pH 7.5 buffer, 0.33 mL of $NAD^+$ solution (10 mM in Tris-$SO_4$ or Tris-HCl 50 mM pH 7.5) and 0.66 mL (20% v/v) of DES were added in the Erlenmeyer flask. Then, 300 µL of isopropanol and 250 mg of 2,4'-dichloroacetophenone were introduced. The Erlenmeyer flask was closed and introduced in an orbital shaker at 250 rpm for 24 h at 30 ºC. The reaction was transferred to a Falcon tube and it was centrifuged (4,900 rpm, 15 min, 4 ºC). The supernatant was taken from the reaction to an extraction funnel, and the pellet was then washed with EtOAc (10 mL) to extract the product from it by centrifugation (4,900 rpm, 15 min, 4 ºC). This extract was pooled into the extraction funnel. 5 mL of water were added in the extraction funnel. The aqueous and organic phases were separated into two different Erlenmeyer flasks. The aqueous phase was again introduced in the extraction funnel and was extracted again with pure EtOAc (2 x 20 mL). The organic phase was washed with 10 mL of water (to eliminate the glycerol from the organic phase). Finally, $Na_2SO_4$ was added as desiccant to the organic phase, the liquid was filtered into a 100-mL round-bottom flask. $Na_2SO_4$ was washed with pure EtOAc (10 mL), and pooled together with the previous filtered organic phase. Then, the organic solvent was evaporated under reduced pressure, affording the alcohol (*R*)-**11b** [(*R*)-2-chloro-1-(4-chlorophenyl)ethan-1-ol, 160 mg, 63% yield] in >99% *ee*.

**Scale-up of the bioreduction of 12a (400 mM) in the presence of DES (20% v/v)**

In an Erlenmeyer flask, 100 mg of *E. coli*/LBADH were introduced. Then, 2 mL of Tris-$SO_4$ 50 mM pH 7.5 buffer, 0.28 mL of $NADP^+$ solution (10 mM in Tris-$SO_4$ 50 mM pH 7.5) and 0.56 mL (20% v/v) of DES were added in the Erlenmeyer flask. Then, 300 μL of isopropanol are introduced and 250 mg of 2,3',4'-trichloroacetophenone. The Erlenmeyer flask was closed and introduced in an orbital shaker at 250 rpm for 24 h at 30 ºC. The reaction was transferred to a Falcon tube and it was centrifuged (4,900 rpm, 15 min, 4 ºC). The supernatant was taken from the reaction to an extraction funnel, and the pellet was then washed with EtOAc (10 mL) to extract the product from it by centrifugation (4,900 rpm, 15 min, 4 ºC). This extract was pooled into the extraction funnel. 5 mL of water were added in the extraction funnel. The aqueous and organic phases were separated into two different Erlenmeyer flasks. The aqueous phase was again introduced in the extraction funnel and was extracted again with pure EtOAc (2 x 20 mL). The organic phase was washed with 10 mL of water (to eliminate the glycerol from the organic phase). Finally, $Na_2SO_4$ was added as desiccant to the organic phase, the liquid was filtered into a 100-mL round-bottom flask. $Na_2SO_4$ was washed with pure EtOAc (10 mL), and pooled together with the previous filtered organic phase. Then, the organic solvent was evaporated under reduced pressure, affording the alcohol (*S*)-**12b** [(*S*)-2-chloro-1-(3,4-dichlorophenyl)ethan-1-ol, 176 mg, 70% yield] in >99% *ee*.

**Scale-up of the bioreduction of 12a (300 mM) in the presence of DES (50% v/v)**

In an Erlenmeyer flask, 100 mg of *E. coli*/ADH-A were introduced. Then, 1.52 mL of Tris-$SO_4$ 50 mM pH 7.5 buffer, 0.38 mL of $NAD^+$ solution (10 mM in Tris-$SO_4$ 50 mM pH 7.5) and 1.9 mL (50% v/v) of DES were added in the Erlenmeyer flask. Then, 300 μL of isopropanol and 250 mg of 2,3',4'-trichloroacetophenone were introduced. The Erlenmeyer flask was closed and introduced in an orbital shaker at 250 rpm for 24 h at 30 ºC. The reaction

was transferred to a Falcon tube and it was centrifuged (4,900 rpm, 15 min, 4 ºC). The supernatant was taken from the reaction to an extraction funnel, and the pellet was then washed with EtOAc (10 mL) to extract the product from it by centrifugation (4,900 rpm, 15 min, 4 ºC). This extract was pooled into the extraction funnel. 5 mL of water were added in the extraction funnel. The aqueous and organic phases were separated into two different Erlenmeyer flasks. The aqueous phase was again introduced in the extraction funnel and was extracted again with pure EtOAc (2 x 20 mL). The organic phase was washed with 10 mL of water (to eliminate the glycerol from the organic phase). Finally, $Na_2SO_4$ was added as desiccant to the organic phase, the liquid was filtered into a 100-mL round-bottom flask. $Na_2SO_4$ was washed with pure EtOAc (10 mL), and pooled together with the previous filtered organic phase. Then, the organic solvent was evaporated under reduced pressure, affording the alcohol (*R*)-**12b** [(*R*)-2-chloro-1-(3,4-dichlorophenyl)ethan-1-ol, 198 mg, 79% yield] in >99% *ee*.

**Scale-up of the bioreduction of 13a (250 mg, 300 mM) in the presence of DES (20% v/v)**

In an Erlenmeyer flask, 100 mg of *E. coli*/ADH-A were introduced. Then, 2.65 mL of Tris-$SO_4$ 50 mM pH 7.5 buffer, 0.38 mL of $NAD^+$ solution (10 mM in Tris-$SO_4$ or Tris-HCl 50 mM pH 7.5) and 0.8 mL (20% v/v) of DES were added in the Erlenmeyer flask. Then, 300 μL of isopropanol and 250 mg of 2,2',4'-trichloroacetophenone were introduced. The Erlenmeyer flask was closed and introduced in an orbital shaker at 250 rpm for 24 h at 30 ºC. The reaction was transferred to a Falcon tube and it was centrifuged (4,900 rpm, 15 min, 4 ºC). The supernatant was taken from the reaction to an extraction funnel, and the pellet was then washed with EtOAc (10 mL) to extract the product from it by centrifugation (4,900 rpm, 15 min, 4 ºC). This extract was pooled into the extraction funnel. 5 mL of water were added in the extraction funnel. The aqueous and organic phases were separated into two different Erlenmeyer flasks. The aqueous phase was again introduced in the extraction funnel and was

extracted again with pure EtOAc (2 x 20 mL). The organic phase was washed with 10 mL of water (to eliminate the glycerol from the organic phase). Finally, $Na_2SO_4$ was added as desiccant to the organic phase, the liquid was filtered into a 100-mL round-bottom flask. $Na_2SO_4$ was washed with pure EtOAc (10 mL), and pooled together with the previous filtered organic phase. Then, the organic solvent was evaporated under reduced pressure, affording the alcohol (*R*)-**13b** [(*R*)-2-chloro-1-(2,4-dichlorophenyl)ethan-1-ol, 200 mg, 79% yield] in >99% *ee*.

**Scale-up of the bioreduction of 14a (400 mM) in the presence of DES (20% v/v)**

In an Erlenmeyer flask, 100 mg of *E. coli*/ADH-A were introduced. Then, 2.6 mL of Tris-$SO_4$ 50 mM pH 7.5 buffer, 0.29 mL of $NAD^+$ solution (10 mM in Tris-$SO_4$ or Tris-HCl 50 mM pH 7.5) and 0.72 mL (20% v/v) of DES were added in the Erlenmeyer flask. Then, 300 μL of isopropanol and 250 mg of 2-chloro-4-fluoroacetophenone were introduced. The Erlenmeyer flask was closed and introduced in an orbital shaker at 250 rpm for 24 h at 30 ºC. The reaction was transferred to a Falcon tube and it was centrifuged (4,900 rpm, 15 min, 4 ºC). The supernatant was taken from the reaction to an extraction funnel, and the pellet was then washed with EtOAc (10 mL) to extract the product from it by centrifugation (4,900 rpm, 15 min, 4 ºC). This extract was pooled into the extraction funnel. 5 mL of water were added in the extraction funnel. The aqueous and organic phases were separated into two different Erlenmeyer flasks. The aqueous phase was again introduced in the extraction funnel and was extracted again with pure EtOAc (2 x 20 mL). The organic phase was washed with 10 mL of water (to eliminate the glycerol from the organic phase). Finally, $Na_2SO_4$ was added as desiccant to the organic phase, the liquid was filtered into a 100-mL round-bottom flask. $Na_2SO_4$ was washed with pure EtOAc (10 mL), and pooled together with the previous filtered organic phase. Then, the organic solvent was evaporated under reduced pressure, affording the alcohol (*R*)-**14b** [(*R*)-2-chloro-1-(4-fluorophenyl)ethan-1-ol, 193 mg, 76% yield] in >99%

*ee*.

**Scale-up of the bioreduction of 15a (300 mM) in the presence of DES (20% v/v)**

In an Erlenmeyer flask, 100 mg of *E. coli*/ADH-A were introduced. Then, 3.15 mL of Tris-$SO_4$ 50 mM pH 7.5 buffer, 0.45 mL of $NAD^+$ solution (10 mM in Tris-$SO_4$ 50 mM pH 7.5) and 0.9 mL (20% v/v) of DES were added in the Erlenmeyer flask. Then, 300 μL of isopropanol and 250 mg of 2-chloro-4'-methoxyacetophenone were introduced. The Erlenmeyer flask was closed and introduced in an orbital shaker at 250 rpm for 24 h at 30 ºC. The reaction was transferred to a Falcon tube and it was centrifuged (4,900 rpm, 15 min, 4 ºC). The supernatant was taken from the reaction to an extraction funnel, and the pellet was then washed with EtOAc (10 mL) to extract the product from it by centrifugation (4,900 rpm, 15 min, 4 ºC). This extract was pooled into the extraction funnel. 5 mL of water were added in the extraction funnel. The aqueous and organic phases were separated into two different Erlenmeyer flasks. The aqueous phase was again introduced in the extraction funnel and was extracted again with pure EtOAc (2 x 20 mL). The organic phase was washed with 10 mL of water (to eliminate the glycerol from the organic phase). Finally, $Na_2SO_4$ was added as desiccant to the organic phase, the liquid was filtered into a 100-mL round-bottom flask. $Na_2SO_4$ was washed with pure EtOAc (10 mL), and pooled together with the previous filtered organic phase. Then, the organic solvent was evaporated under reduced pressure, affording the alcohol (*R*)-**15b** [(*R*)-2-chloro-1-(4-methoxyphenyl)ethan-1-ol, 226 mg, 90% yield] in >99% *ee*.

**Scale-up of the bioreduction of 5a (300 mM) in the presence of DES (50% v/v)**

In an Erlenmeyer flask, 100 mg of *E. coli*/ADH-A were introduced. Then, 2.8 mL of Tris-$SO_4$ 50 mM pH 7.5 buffer, 0.31 mL of $NAD^+$ solution (10 mM in Tris-$SO_4$ or Tris-HCl 50 mM pH 7.5) and 3.1 mL (50% v/v) of DES were added in the Erlenmeyer flask. Then, 300 μL of

isopropanol and 250 μL of ethyl-4-chloroacetoacetate were introduced. The Erlenmeyer flask was closed and introduced in an orbital shaker at 250 rpm for 24 h at 30 ºC. The reaction was transferred to a Falcon tube and it was centrifuged (4,900 rpm, 15 min, 4 ºC). The supernatant was taken from the reaction to an extraction funnel, and the pellet was then washed with EtOAc (10 mL) to extract the product from it by centrifugation (4,900 rpm, 15 min, 4 ºC). This extract was pooled into the extraction funnel. 5 mL of water were added in the extraction funnel. The aqueous and organic phases were separated into two different Erlenmeyer flasks. The aqueous phase was again introduced in the extraction funnel and was extracted again with pure EtOAc (2 x 20 mL). The organic phase was washed with 10 mL of water (to eliminate the glycerol from the organic phase). Finally, $Na_2SO_4$ was added as desiccant to the organic phase, the liquid was filtered into a 100-mL round-bottom flask. $Na_2SO_4$ was washed with pure EtOAc (10 mL), and pooled together with the previous filtered organic phase. Then, the organic solvent was evaporated under reduced pressure, affording the alcohol (*R*)-**5b** [(*R*)-ethyl 4-chloro-3-hydroxybutanoate, 284 mg, 92% yield] in >99% *ee*.

**Scale-up of the bioreduction of 5a (400 mM) in the presence of DES (20% v/v)**

In an Erlenmeyer flask, 100 mg of *E. coli*/ADH-A were introduced. Then, 3.3 mL of Tris-$SO_4$ 50 mM pH 7.5 buffer, 0.47 mL of $NAD^+$ solution (10 mM in Tris-$SO_4$ 50 mM pH 7.5) and 0.95 mL (20% v/v) of DES were added in the Erlenmeyer flask. Then, 300 μL of isopropanol and 250 μL of ethyl 4-chloroacetoacetate were introduced. The Erlenmeyer flask was closed and introduced in an orbital shaker at 250 rpm for 24 h at 30 ºC. The reaction was transferred to a Falcon tube and it was centrifuged (4,900 rpm, 15 min, 4 ºC). The supernatant was taken from the reaction to an extraction funnel, and the pellet was then washed with EtOAc (10 mL) to extract the product from it by centrifugation (4,900 rpm, 15 min, 4 ºC). This extract was pooled into the extraction funnel. 5 mL of water were added in the extraction funnel. The aqueous and organic phases were separated into two different Erlenmeyer flasks. The aqueous

phase was again introduced in the extraction funnel and was extracted again with pure EtOAc (2 x 20 mL). The organic phase was washed with 10 mL of water (to eliminate the glycerol from the organic phase). Finally, $Na_2SO_4$ was added as desiccant to the organic phase, the liquid was filtered into a 100-mL round-bottom flask. $Na_2SO_4$ was washed with pure EtOAc (10 mL), and pooled together with the previous filtered organic phase. Then, the organic solvent was evaporated under reduced pressure, affording the alcohol (*R*)-**5b** [(*R*)-ethyl 4-chloro-3-hydroxybutanoate, 268 mg, 87% yield] in >99% *ee*.

**Scale-up of the bioreduction of 16a (300 mM) in the presence of DES (20% v/v)**

In an Erlenmeyer flask, 100 mg of *E. coli*/ADH-A were introduced. Then, 4 mL of Tris-$SO_4$ 50 mM pH 7.5 buffer, 0.57 mL of $NAD^+$ solution (10 mM in Tris-$SO_4$ 50 mM pH 7.5) and 1.15 mL (20% v/v) of DES were added in the Erlenmeyer flask. Then, 300 μL of isopropanol and 250 μL ethyl 4,4,4-trifluoroacetoacetate were introduced. The Erlenmeyer flask was closed and introduced in an orbital shaker at 250 rpm for 24 h at 30 ºC. The reaction was transferred to a Falcon tube and it was centrifuged (4,900 rpm, 15 min, 4 ºC). The supernatant was taken from the reaction to an extraction funnel, and the pellet was then washed with diethyl ether (10 mL) to extract the product from it by centrifugation (4,900 rpm, 15 min, 4 ºC). This extract was pooled into the extraction funnel. 5 mL of water were added in the extraction funnel. The aqueous and organic phases were separated into two different Erlenmeyer flasks. The aqueous phase was again introduced in the extraction funnel and was extracted again with pure diethyl ether (2 x 20 mL). The organic phase was washed with 10 mL of water (to eliminate the glycerol from the organic phase). Finally, $Na_2SO_4$ was added as desiccant to the organic phase, the liquid was filtered into a 100-mL round-bottom flask. $Na_2SO_4$ was washed with pure diethyl ether (10 mL), and pooled together with the previous filtered organic phase. Then, the organic solvent was evaporated under reduced pressure, affording the alcohol (*R*)-**16b** [(*R*)-ethyl 4,4,4-trifluoro-3-hydroxybutanoate, 210 mg, 66%

yield] in >99% *ee*.

**Scale-up of the bioreduction of 13a (1 g, 300 mM) in the presence of DES (20% v/v)**

In an Erlenmeyer flask, 500 mg of *E. coli*/ADH-A were introduced. Then, 10.6 mL of Tris-$SO_4$ 50 mM pH 7.5 buffer, 1.5 mL of the $NAD^+$ solution (10 mM in Tris-$SO_4$ 50 mM pH 7.5) and 3.2 mL (20% v/v) of DES were added in the Erlenmeyer flask. Then, 1.2 mL of propan-2-ol and 1 g of 2,2',4'-trichloroacetophenone were introduced. The flask was closed and the reaction was shaken for 24 h at 30 ºC in an orbital shaker at 250 rpm. The supernatant of the bioreduction mixture was collected by centrifugation and transferred into an extraction funnel. The deposited pellet was washed with ethyl acetate (30 mL) to extract the residual substrate and product in the Falcon tube by centrifugation, and it was pooled into the funnel with the previous organic phase. This organic solvent was washed with water (20 mL). The phases in the funnel were separately collected. The aqueous phase was again extracted with ethyl acetate (50 mL). This organic phase was combined with the previous one to yield a combined extract that was washed with water (10 mL, to eliminate the residual glycerol), collected in an Erlenmeyer flask, dried over $Na_2SO_4$ and filtered into a round-bottom flask. $Na_2SO_4$ was washed with ethyl acetate (10 mL). The solvent was evaporated under reduced pressure, affording the alcohol (*R*)-**13b** (900 mg, 89% yield) in >99% *ee*.

**Scale-up of the bioreduction of 13a (300 mM) in the presence of DES (20% v/v) without cofactor**

In an Erlenmeyer flask, 100 mg of *E. coli*/ADH-A were introduced. Then, 3.05 mL of Tris-$SO_4$ 50 mM pH 7.5 buffer, and 0.8 mL (20% v/v) of DES were added in the Erlenmeyer flask. Then, 300 µL of isopropanol and 250 mg of 2,2',4'-trichloroacetophenone were introduced. The Erlenmeyer flask was closed and introduced in an orbital shaker at 250 rpm for 24 h at 30 ºC. The reaction was transferred to a Falcon tube and it was centrifuged (4,900 rpm, 15 min, 4

ºC). The supernatant was taken from the reaction to an extraction funnel, and the pellet was then washed with EtOAc (10 mL) to extract the product from it by centrifugation (4,900 rpm, 15 min, 4 ºC). This extract was pooled into the extraction funnel. 5 mL of water were added in the extraction funnel. The aqueous and organic phases were separated into two different Erlenmeyer flasks. The aqueous phase was again introduced in the extraction funnel and was extracted again with pure EtOAc (2 x 20 mL). The organic phase was washed with 10 mL of water (to eliminate the glycerol from the organic phase). Finally, $Na_2SO_4$ was added as desiccant to the organic phase, the liquid was filtered into a 100-mL round-bottom flask. $Na_2SO_4$ was washed with pure EtOAc (10 mL), and pooled together with the previous filtered organic phase. Then, the organic solvent was evaporated under reduced pressure, affording the alcohol (*R*)-**13b** [(*R*)-2-chloro-1-(2,4-dichlorophenyl)ethan-1-ol, 176 mg, 70% yield] in >99% *ee*.

### 2.3.4. Absolute configuration assignment

The absolute configuration of the obtained alcohols was assigned based on previous examples described in the bibliography where the same ketones were reduced with LBADH and/or ADH-A:

Ketone **10a** with LBADH (Rodríguez et al., 2014) and with ADH-A (Cuetos et al., 2010), ketones **11a** and **12a** with ADH-A and LBADH (Bisogno et al., 2009), ketone **13a** with ADH-A and LBADH (Mangas-Sánchez et al., 2011), and ketone **5a** with LBADH (Bräutigam et al., 2007).

On the other hand, the absolute configuration for the other alcohols was determined by comparison with previously described GC retention times and optical rotation values: **14b** (Zhu et al., 2009), **15b** (Li et al., 2008) and **16b** (Yadav et al., 2002).

### 2.3.5. $^{1}$H-NMR spectra of the scale-up processes

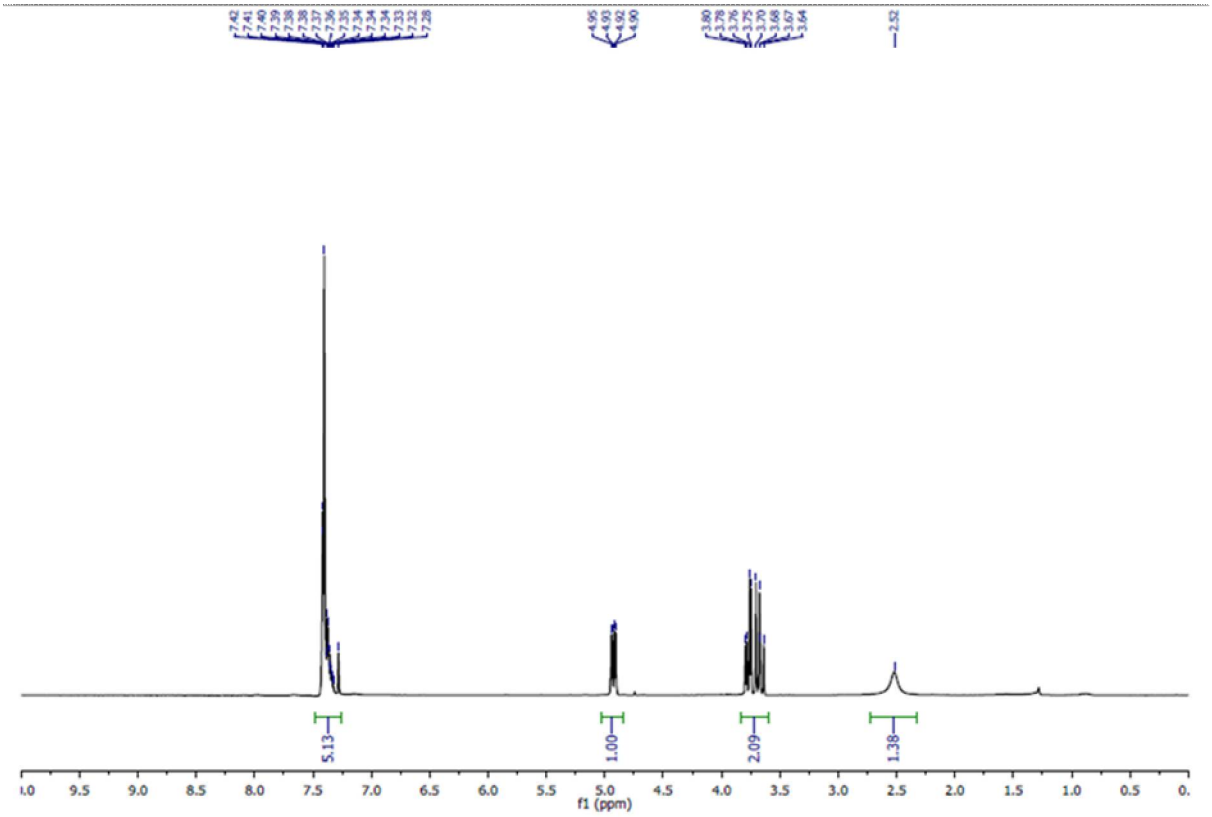



**Figure 13:** $^{1}$H-NMR of alcohol **10b** obtained in the scale-up process ($CDCl_3$).

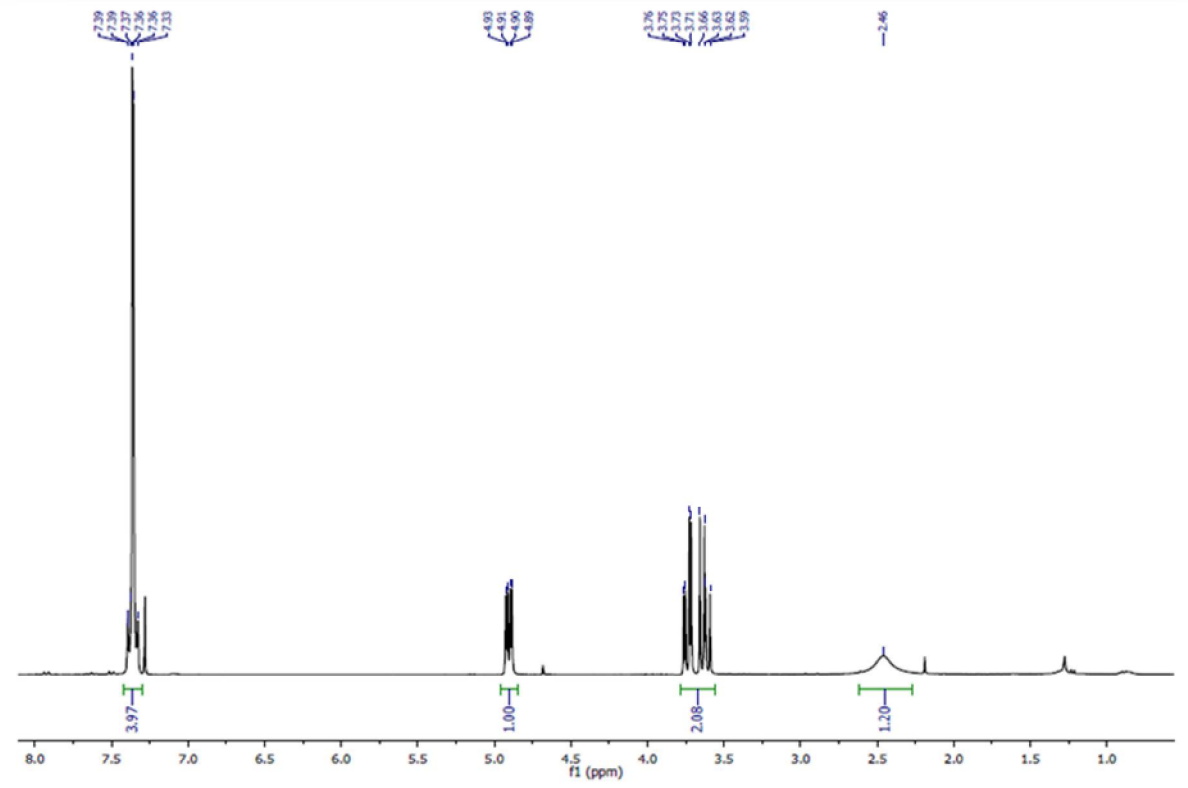



**Figure 14:** $^{1}$H-NMR of alcohol **11b** obtained in the scale-up process ($CDCl_3$).

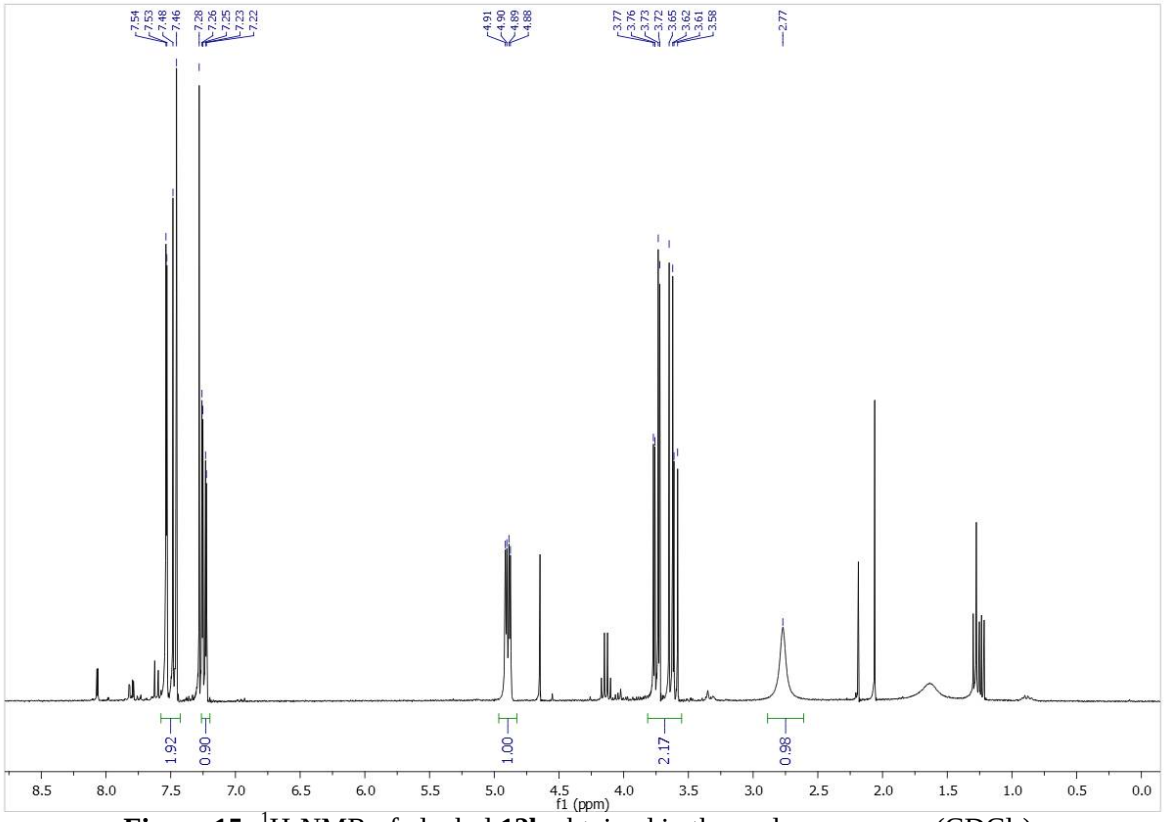



**Figure 15:** $^{1}$H-NMR of alcohol **12b** obtained in the scale-up process ($CDCl_3$).

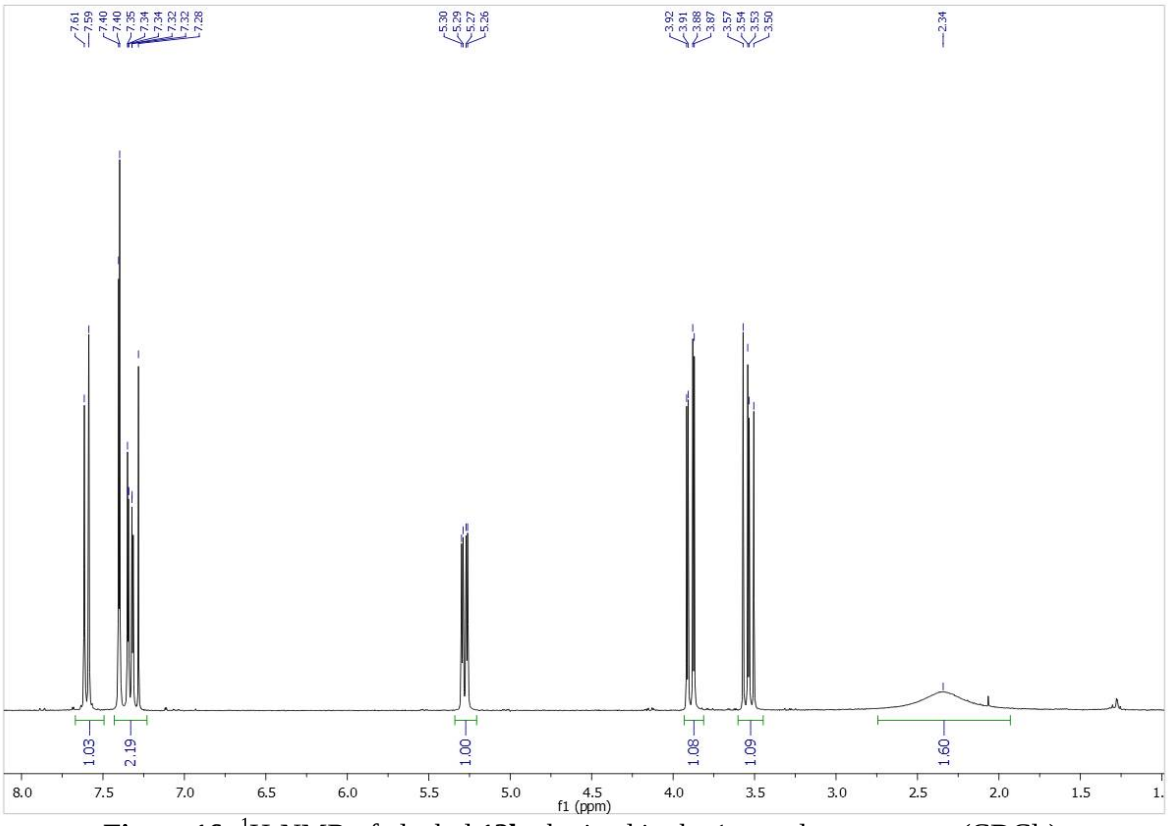



**Figure 16:** $^{1}$H-NMR of alcohol **13b** obtained in the 1-g scale-up process ($CDCl_3$).

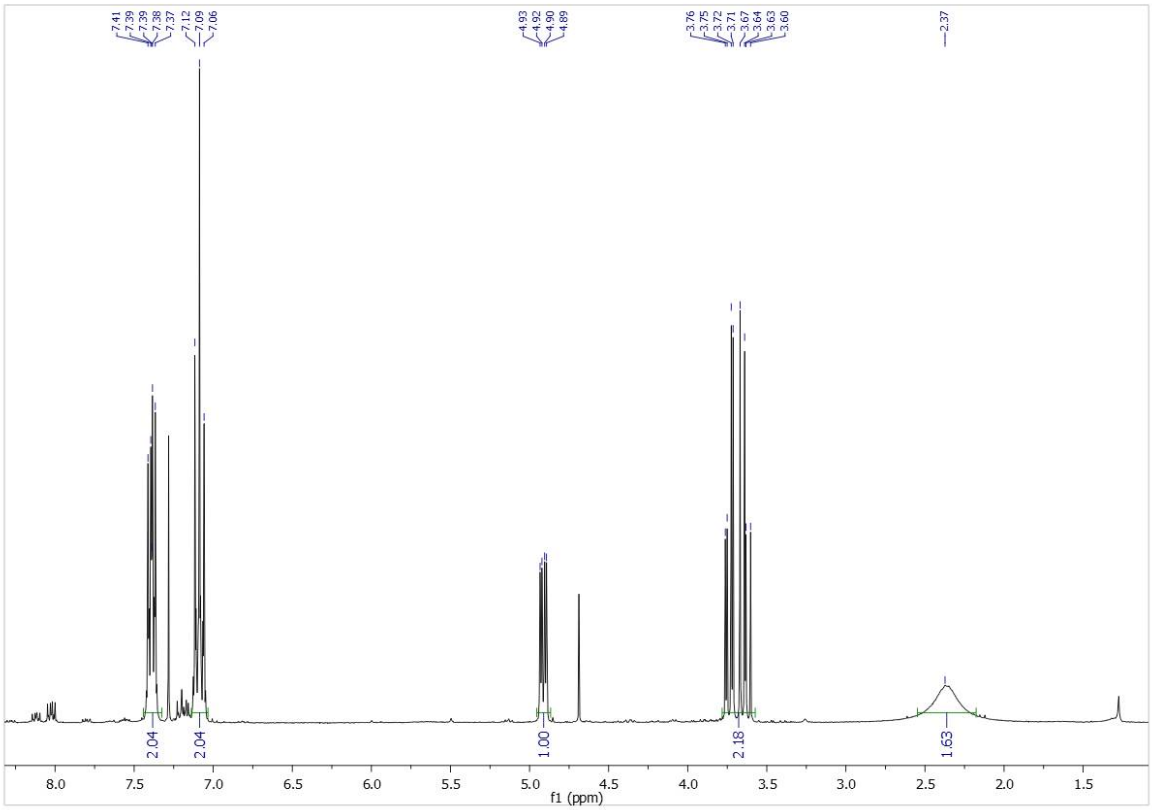



**Figure 17:** $^{1}$H-NMR of alcohol **14b** obtained in the scale-up process ($CDCl_3$).

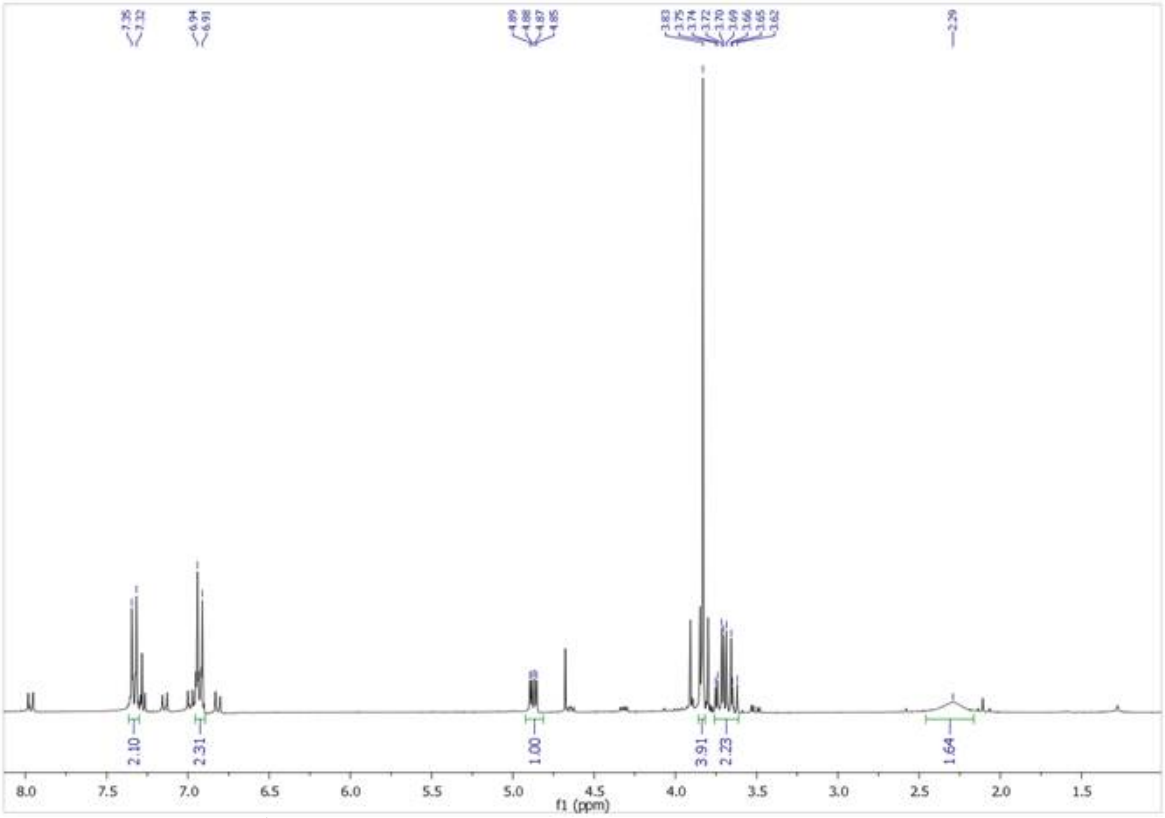



**Figure 18:** $^{1}$H-NMR of alcohol **15b** obtained in the scale-up process ($CDCl_3$).

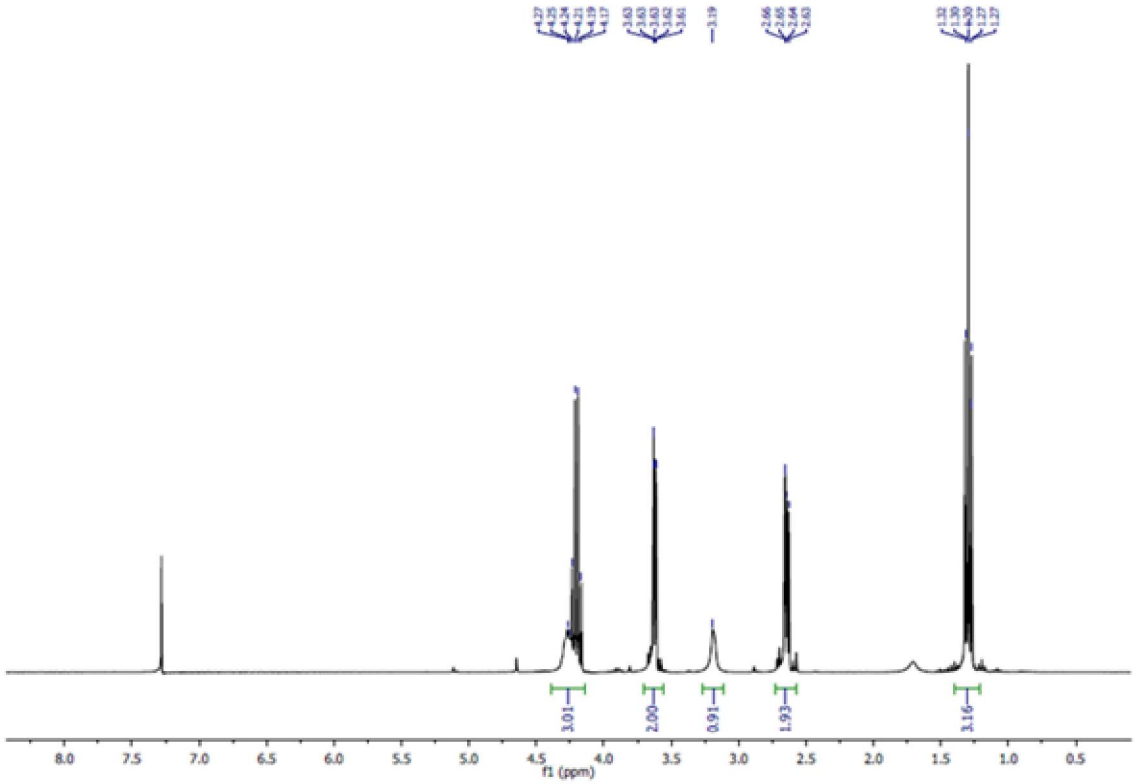



**Figure 19:** $^{1}$H-NMR of alcohol **5b** obtained in the scale-up process ($CDCl_3$).

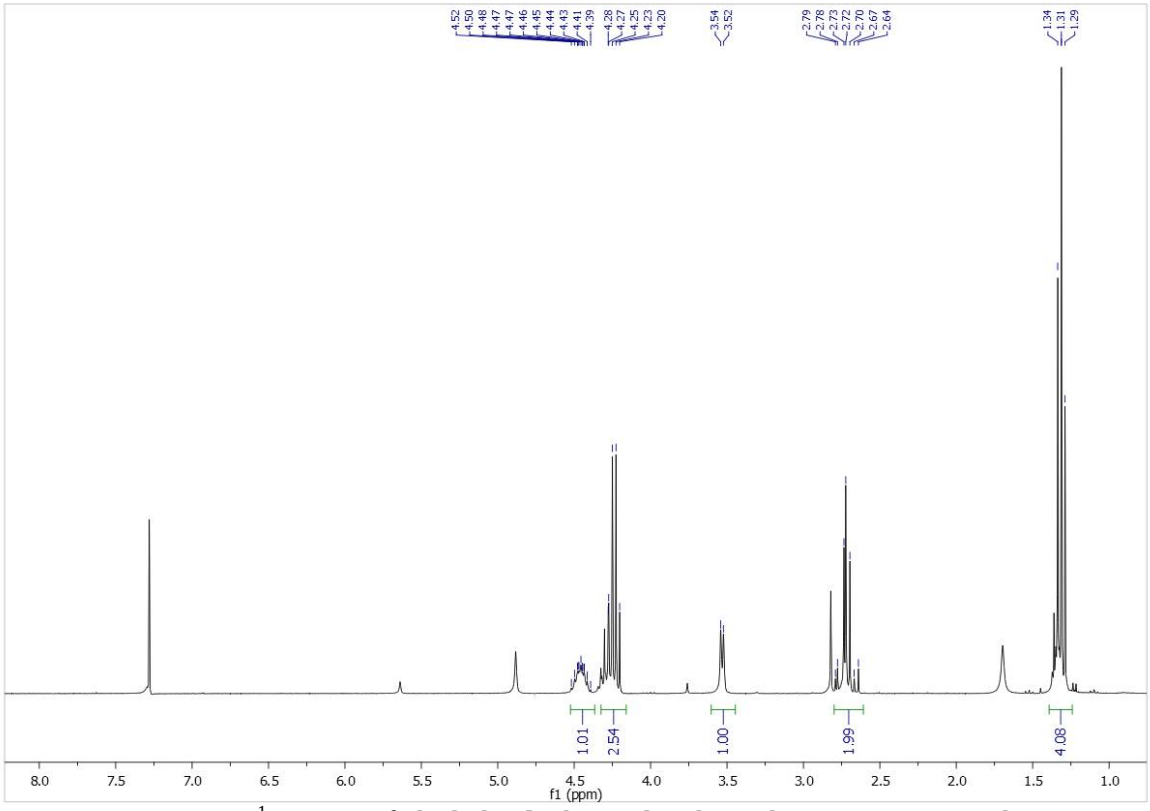



**Figure 20:** $^{1}$H-NMR of alcohol **16b** obtained in the scale-up process ($CDCl_3$).

# *Annex 1*

**Publications derived from Part 1**

F. Z. Ibn Majdoub Hassani, S. Amzazi, I. Lavandera, "The Versatile Applications of DES and their Influence on Oxidoreductase-Mediated Transformations", *Molecules* **2019**, *24*, 2190.

F. Z. Ibn Majdoub Hassani, S. Amzazi, J. Kreit, I. Lavandera, "Deep Eutectic Solvents as Media in Alcohol Dehydrogenase-Catalyzed Reductions of Halogenated Ketones", *ChemCatChem* **2020**, *12*, 832-836.

**Congress and conferences publications derived from Part 1**

F. Z. Ibn Majdoub Hassani, I. Lavandera, J. Kreit, "Stabilizing Effects of Deep Eutectic Solvents on Alcohol Dehydrogenase Mediated Systems". 18$^{th}$ International Conference on Biocatalysis and Applications of Biochemistry (ICBAB 2016), Rome (Italy), 08-09 December 2016, Electronic poster-4.

F. Z. Ibn Majdoub Hassani, I. Lavandera, J. Kreit, S. Amzazi, "The Effect of DES on the Bioreduction Activities of Alcohol Dehydrogenases Using Halogenated Ketones as Substrates". II Jornadas Españolas de Biocatálisis 2018 (JEB 2018), Oviedo (Spain), 25-26 June 2018, Poster P-40.

# ***Part 2:*** *Microbial Sterol Catabolism*

## 4.1. Introduction

Microorganisms use different enzymes to catabolize sterols. In a recent review by Kreit (2019), the major enzymes from microorganisms that are involved in the opening of the 3-ketosteroid nucleus were discussed. The sterol structure is composed of 4 rings, namely, ring A, B, C, and D. Microorganisms degrade aerobically the tetracyclic sterol nucleus starting by the degradation of the A and B rings, then they catabolize the C and D rings. The enzymes that help in the rupture of the B-ring are known as 3-ketosteroid$\Delta^1$-dehydrogenase (KstD) and 3-ketosteroid 9*α*-hydroxylase (KstH). Also, the rupture of ring B takes place at the C9, 10 position and these two enzymes are also involved in the aromatization of C4,5 in ring A leading to the separation of ring B from that latter. The growing bacteria can also have a 5*α*-reductase and as a result the C4,5*α*-hydrogenated 3-ketosteroid can be produced. In this particular situation, besides having (KstD) and (KstH), the microbe produces a 3-ketosteroid$\Delta^4$-(5*α*)-dehydrogenase (Kst4D) and as a result, the A ring becomes an aromatic ring and the B-ring is ruptured from the tetracyclic structure. It is important to note that the enzymes KstD and Kst4D are oxidoreductase enzymes that are FAD-dependent. For the enzyme KstH, it is considered to be both a monooxygenase and a reductase. The catalytic unit of the KstH is the monooxygenase part because it has a Rieske-[2*Fe*-2*S*] center with a non-heme mononuclear iron in the active site of the enzyme. The four main oxidoreductases that are responsible for the catabolism of 3-ketosteroids are KstD, KstH, Kst4D, and Kst5*αR*. In this review, the steps involving the catabolism of 3-ketosteroids using these four enzymes were discussed. In the initiating step, the sterol is oxidized at the C-17 level via the 17β-hydroxysteroid dehydrogenase, then the molecule gets reduced at the C5 positions via the (Kst5*αR*). In addition, the sterol structure goes through a beta oxidation converting the AD via (KstD) and (KstH) to 9α-hydroxy-1,4-androstadien-3,17-dione (9α-OH-ADD) (Kreit 2019). After several reactions, the secophenol derivative ring system opens at the level of C5. As a

result, 2-oxo-4-hexanoic acid, indanedione derivative and central metabolites such as propionate, acetate, succinate, and pyruvate, are observed in the medium (Figure 21). In addition, there is not any particular order in the degradation of the sterol rings and side chains when the microbial (KstD), (KstH), (Kst4D) are used on sterols; as the B-ring can rupture before the degradation of the sterol's side chain (Kreit 2019).

Also, the catabolism of cholesterol can take different pathways, depending on the microorganism that is used in the growth medium. Moreover, the use of genomics and proteomics to study the microbial sterol catabolism can help even more in the understanding of the pathways that are used by microbes to degrade sterols (Kreit 2019).

Cholesterol oxidase is an enzyme produced from microorganisms that transforms the 3β-hydroxy-5-en part of the sterol to the 3-keto-4-en structure, and this triggers the degradation of the sterol. This enzyme belongs to a variety of bacterial genera such as (*Rhodococcus, Streptomyces, Mycobacterium* and *Nocardia*). The enzymes and the cofactors that are used by the species of *Mycobacteria* and *Nocardia* for these types of reactions are dehydrogenases that are NAD(P)-dependent. The starting step or initiating step for the sterol side chain degradation in *Rhodococcus jostii* is when the C26 of the sterol side chain gets oxidized. Then further catabolism occurs at the level of the resulting stenone. This type of catabolism follows two pathways: the cleavage of the sterol side chain and the steroid nucleus degradation.

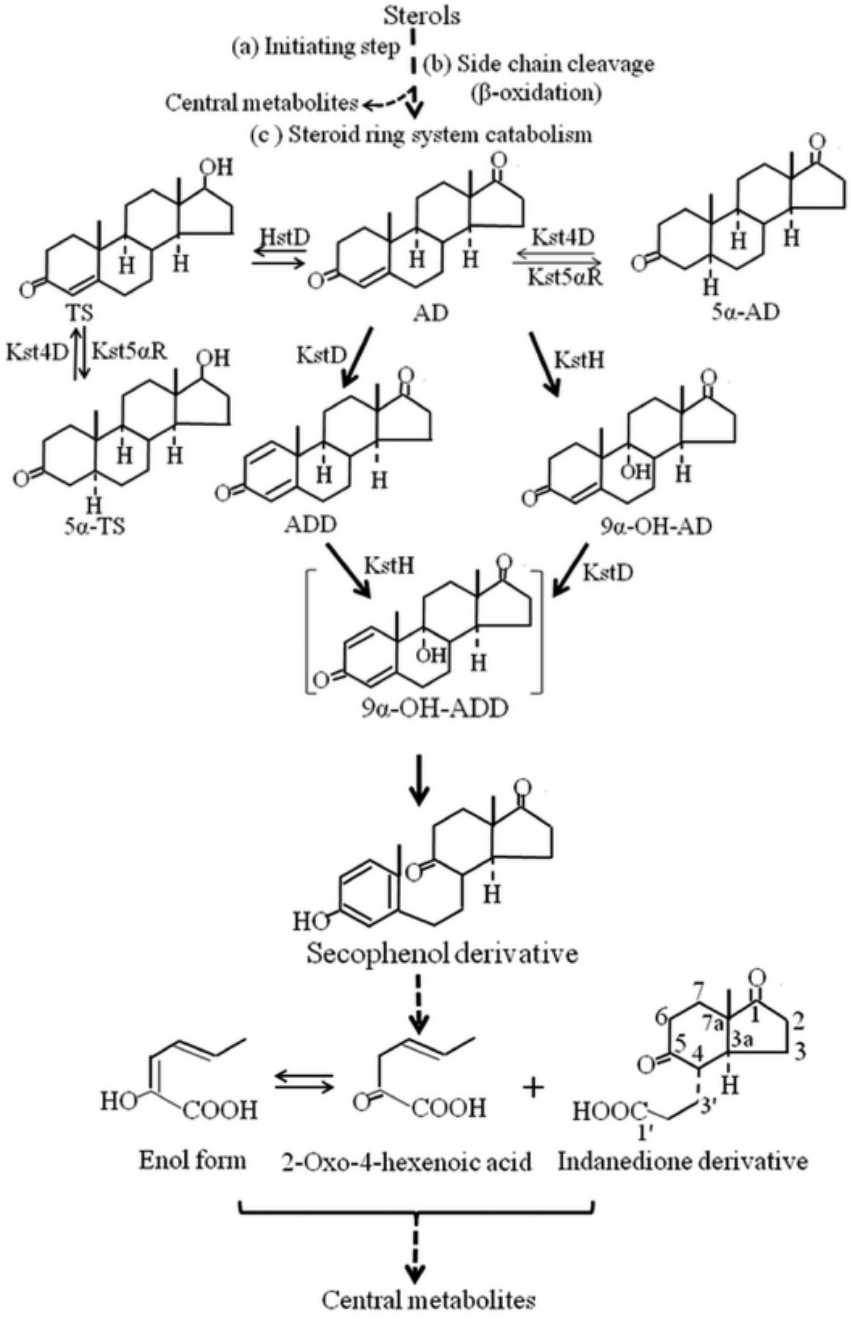



**Figure 21:** aerobic catabolism of sterols in microbes (Kreit 2019).

In actinobacteria, the initiating step in the sterol degradation is the 3β-hydroxy-5-en conversion. The enzyme required for this step is cholesterol oxidase or 3β-hydroxysteroid dehydrogenase (HsD) that is NAD(P)-dependent (Kreit 2017). For the genera of *Mycobacterium* and *Nocaria*, HsD is considered the only enzyme that catabolizes the sterol 3β-ol-5-en to 3-keto-4-en. On the other hand, *Gordonia* and *Rhodococcus* have the cholesterol

oxidase for this step in the reaction. Also, more studies on species taxonomy are needed to better comprehend enzymes that convert 3β-ol-5-en in *Corynebacterineae*. In *rhodococci*, the sterol side chain is important to induce the enzyme; and both the side chain cleavage and the A-B ring degradation make up a subpathway that is controlled by the transcriptional repressor KstR and this helps in the control of cholesterol oxidase in *Rhodococcus* and HsD in *Mycobacteria* and *Nocardia* (Kreit 2017).

The strains from the genus *Rhodococcus* have enzymes that catalyze a broad range of reactions. Some of these reactions catalyzed by these enzymes include redox-reactions, hydrolysis of epoxides or esters, and hydration of fatty acids. Enzymes from *Rhodococcus* are also involved in the aldoxime-nitrile pathway and are used at an industrial scale (Busch et al., 2019).

Also, many genomic studies were performed on *Rhodococcus* strains in order to investigate novel enzymes that have unique properties like large substrate scope, stabilities, and handling severe temperatures of neoteric reaction media (Busch et al., 2019).

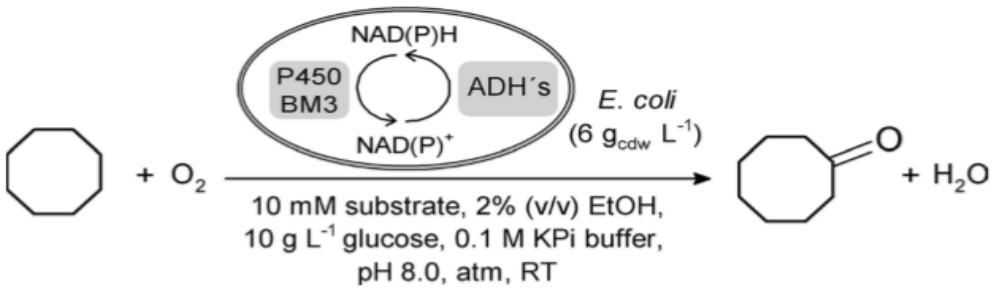
In addition, (*R*)-selective amines can be synthesized by engineering amine dehydrogenase enzymes, and this type of synthesis was difficult in the past. There are two types of bacterial cells from the genus *Rhodococcus* that are used in biotransformations. Some reactions are catalyzed by whole cells of *Rhodococcus* (wild type or mutant). Other reactions occur when enzymes from *Rhodococcus* are expressed in *E. coli* whole cells. In addition, the use of rhodococcal enzymes enable researchers to make new biocascades (Busch et al., 2019).

Also, the use of combination enzymes in bacterial hosts is feasible because these enzymes have common condition ranges (pH, temperature) and many times work on the degradation of the same substrates. Due to the observations above, the genus *Rhodococcus* can be called a

biocatalytic powerhouse due to its strength to catalyze different reactions giving excellent conversions and enantiomeric excess results (Busch et al., 2019). In this contribution it was reported that ADHs from species of *Rhodococcus* gave promising results regarding the production of enantiomeric alcohols. For instance, the (*S*)-enantiomer was obtained successfully when whole cells of *R. ruber* DSM 44541 were used with secondary alcohols as substrates (Stampfer et al., 2002).

Also, when reactions containing *R. erythropolis* cells were used with methyl nonactate, two enantiomers were obtained (+)-and (-)-nonactate. In addition, the stereoselectivity could be switched just by changing the reaction conditions from aerobic to anaerobic (Nikodinovic et al., 2006).

In another study, the researchers showed the large applications of ADHs from *Rhodococcus* in biocascades: three enzymes were combined for the double oxidation of cyclooctane (monooxygenase P450 BM3, *Lactobacillus brevis* ADH (LbADH) and *R. erythropolis* ADH (ReADH)); and all these enzymes were coexpressed in a single host cell (Scheme 3). Usually, the monooxygenase oxyfunctionalizes as a first step the non-activated cyclooctane to cyclooctanol, and then two stereocomplementary ADHs oxidize further the cyclooctanol to form cyclooctanone (Busto et al., 2016).




**Scheme 3:** bioconversion of cyclooctane to cyclooctanone via the single host cell *E. coli* that expressed P450 BM3, *Lb*-ADH, and *Re*-ADH (Müller et al., 2016).

Also, in the same manner, four recombinant enzymes (P450 monooxygenase, *Lb*ADH, *Re*ADH, ω-transaminase (ATA-117)) were co-expressed in *E. coli*, and these cells were able to catalyze the chiral amination of (substituted)-ethylbenzene derivatives in a one-pot three-step process (Figure 22, Both et al., 2016).

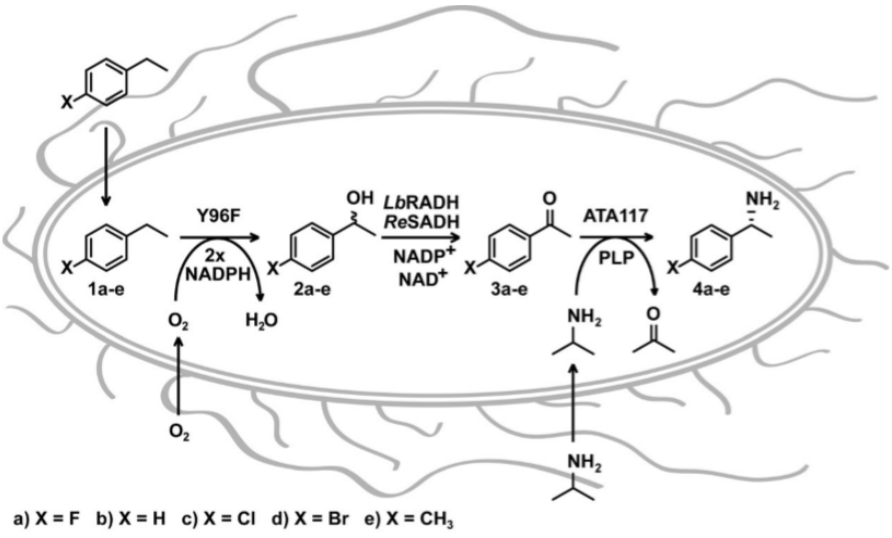
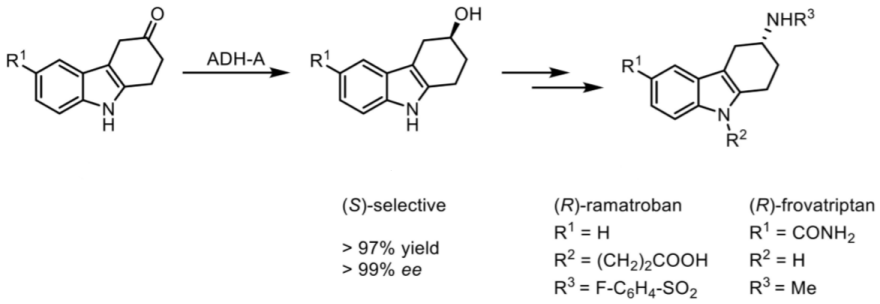



**Figure 22:** *E. coli* BL 21 (DE3) cells that contain four enzymes: a monooxygenase (Y96F), *Lb*RADH and *Re*SADH, and ω-transaminase [ATA117], which convert ethylbenzenes to amines (Both et al., 2016).

KREDs can also catalyze the reduction of ketones in a multistep chemoenzymatic reaction to produce important pharmaceuticals such as (*R*)-ramatroban, which is used to treat coronary artery diseases, and (*R*)-frovatriptan, which is used to treat allergic rhinitis, asthma, or headaches (Scheme 4, Busto et al., 2012, Busto et al., 2013).




**Scheme 4**: a multistep chemoenzymatic reaction via KRED to produce pharmacologically active molecules (Busto et al., 2012, Busto et al., 2013).

In addition, this review also discussed the activities of different enzymes that the *Rhodococcus* genus has and the successful biocatalysis of these enzymes when different substrates are employed.

The use of mutant *Rhodococcus* cells was efficient and helped in the making of intermediates that were used in the dermatological pharmacy field. The enzyme Δ6-desaturase (this is an enzyme that converts between types of fatty acids) from a *Rhodococcus* sp. KSM-B-MT66 mutant cells was utilized in a two-phase system. That system consisted of transforming saturated material into its corresponding product via a *cis*-desaturation. A dehydrogenation occurred at the 9-C-atoms position when substrates such as alkanes, chloroalkanes, and acyl fatty acids were used. This method was used to prepare substituted fatty acids and gave a daily product yield of 16.8 g/L (Takeuchi et al., 1990, Araki et al., 2016, Araki et al., 2004).

*Rhodococcus* cells also contain lactamases which are involved in the hydrolysis reactions (a hydrolysis reaction is the addition of water by the enzyme lactamase to a lactam substrate, and this gives a product containing an amine and a carboxylic acid). Antiviral compounds like carbovir and abacavir are obtained when Vince-lactams are hydrolyzed using lactamases from *Rhodococcus* (Venhoff et al., 2007, Taylor et al., 1990).

When *Rhodococcus* was used for the hydrolysis of (2,2-disubstituted) oxiranes, the epoxide hydrolases (EHs) hydrolyzed the (*S*)-enantiomer giving an (*S*)-diol. For this case, the substrates that were metabolized included: benzyl-substituted epoxides, and linear alkyl-, alkenyl-, alkynyl. In addition, the strains of *Rhodococcus* were able to transform successfully oxiranes that had an alkene or alkyne function. Also, marine sediments containing *Rhodococcus* strains were utilized to hydrolyze styrene oxide and its derivatives (Archelas and Furstoss 2001, Osprian et al., 1997, Wandel et al., 1995, Osprian et al., 2000, Woo et al., 2015).

Finally, the main enzymes in that genus which showed promising results in terms of products and enantioselectivity were: oxidases, oxygenases, amine dehydrogenases, aldoxime dehydratases, nitrile hydratases, amidases, nitrilases, hydrolases, esterases, hydratases, sulfatases, and sulfide monooxygenases (Busch et al., 2019).

### 5.1.1. Convenient solvents and multicatalytic approaches for enzymes from the genus *Rhodococcus*

This section is about the preferred solvents of biocatalytic enzymes from the genus *Rhodococcus*. In a study conducted by de Gonzalo et al. in 2007, it was found that ADH-A from *Rhodococcus ruber* was put into mono and biphasic aqueous-organic solvent systems (50% v $v^{-1}$) and in micro-aqueous organic systems (99% $v.v^{-1}$). It was found that the biocatalytic reaction which involved the reduction of ketones via the enzyme ADH-A occurred successfully. Also, it was found that the more the organic solvent was hydrophobic the higher the enzymatic activity was.

This study confirmed that organic solvents are still important in enzymes such as ADH-A which catalyzes hydrophobic ketones. Also, the study investigated how ADH-A from *Rhodococcus ruber* DSM 44541 overexpressed in *E. coli* catalyzes efficiently the

bioreduction of ketones while recycling its cofactor using 2-propanol. However, it was observed that as they increased the solvent concentration this led to a decrease in the reaction rate while keeping the stereoselectivity and robustness of the enzyme intact.

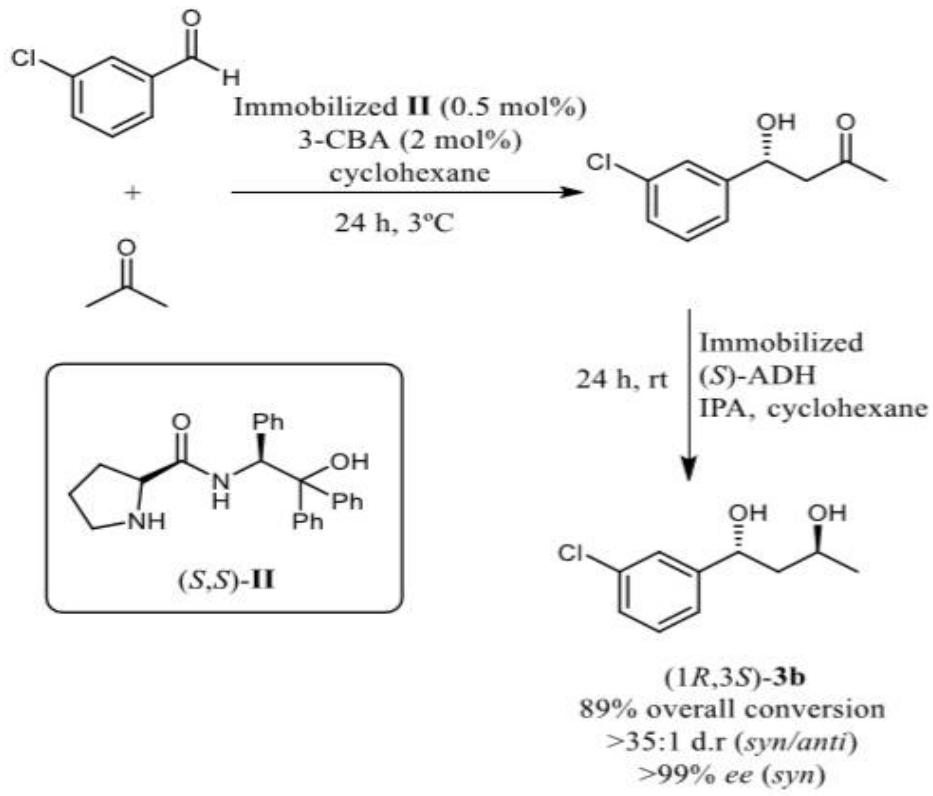
On the other hand, it was observed that when water miscible solvents were used (like ethanol for example), this deactivated both the *E. coli*/ADH-A and the recombinant ADH-A proving that the best co-solvents for ADH-A were, in general, the hydrophobic ones (de Gonzalo et al., 2007).

A review written by Bisogno et al. in 2017 have discussed and explored the benefits of combining biocatalysts with organocatalysts in one-pot procedures. This review is very important because it shows how chemists and researchers can combine different catalysts in order to improve their reactions and products. In the following paragraphs, some of the major findings will be revealed. The authors also highlighted the importance of tailoring the reaction medium of one-pot procedures, which combine both organocatalysts and biocatalysts.

In 2009, a research team made a sequential one-pot synthesis of chiral 1,3-diols. They combined an organocatalytic aldol reaction with an enzymatic ketoreduction reaction in aqueous media (Baer et al., 2009). In addition, they used a proline-based catalyst, *p*-chlorobezaldehyde and 2-propanol as cosubstrate in a substrate-coupled cofactor recycling system, and this gave the four possible isomers of the corresponding 1,3-diols. In that experiment, either (*S*)-ADH from *Rhodococcus* sp. or (*R*)-ADH from *Lactobacillus kefir* were used; and the obtained results were excellent (>95% conversion, >99% *ee*). That team also tested different other substituents at the *para*-position of the aromatic ring, and this time they tried *p*-chlorobenzaldehyde and acetone as substrates. They obtained a β-hydroxy ketone using a proline based catalyst. Then a bioreduction occurred utilizing the (*S*)-ADH and they used 2-propanol 5% $vv^{-1}$ as cosubstrate at room temperature. Finally, after 18 h they obtained

good conversion and *ee* results of the corresponding (1*R*,3*S*)-1-(4-chlorophenyl)butane-1,3-diol (>95% conversion, >99% *ee*) (Baer et al., 2009).

Similarly, the same research group used *meta*-substituted aromatic aldehydes. They applied a one-pot multistep system with *m*-chlorobenzaldehyde and acetone. The team tested different quantities of catalyst loading to reach the desired stereochemical outcome. Furthermore, they also used *m*-chlorobenzaldehyde combined with the organocatalyzed aldol reaction and (*S*)-ADH from *Rhodococcus* sp. or (*R*)-ADH from *Lactobacillus kefir*. The obtained products were 1,3-diols (1*R*,3*S*)-1-(3-chlorophenyl)butane-1,3-diol. In addition, they used acetone in a one-pot procedure in 48 h and achieved excellent results (>95% conversion, >99% *ee*) (Rulli et al., 2011).




**Scheme 5:** one-pot two-step synthesis of (1*R*,3*S*)-1-(3-chlorophenyl)butane-1,3-diol combining organo- and biocatalysis (Heidlindemann et al., 2014).

Another research team prepared differently chiral 1,3-diols using another type of immobilized organocatalyst (*S*,*S*)-**II** in acrylic polymeric beads (Heidlindemann et al., 2014). It was shown that this specific immobilized organocatalyst was involved in the catalysis of an aldol containing aromatic aldehydes and acetone in organic media, and this was like the results that were observed when the free catalyst was used in aqueous media (Kristensen and Hansen 2010, Cozzi 2006). Moreover, (*R*)-4-(3-chlorophenyl)-4-hydroxybutan-2-one, was obtained from *m*-chlorobenzaldehyde with 95% of conversion and *ee* after one day. Then the team recycled the immobilized catalyst by decantation and after evaporation of the organic layer, they performed the bioreduction step. As a result, the chiral (1*R*,3*S*)-1-(3-chlorophenyl)butane-1,3-diol was acquired with excellent conversion (89%) and *ee* (>99%) (Scheme 5). In that case, an immobilized (*S*)-ADH from *Rhodococcus* sp. was used (Heidlindemann et al., 2014).

On the other hand, a study conducted by Cull et al. in 2000 proved that room temperature IL such as 1-butyl-3-methylimidazolium hexafluorophospahate (bmim) $PF_6$ can be used instead of the usual organic solvent when performing a liquid-liquid extraction (technique used to separate compounds based on their solubilities by using 2 immiscible liquids) from the *Rhodococcus* R312 biotransformation of 1,3 dicyanobenzene (Cull et al., 2000).

In a recent study done by Hibino and Ohtake in 2013, they discovered that *R. rhodochrous* NBRC 15564 was able to use some cloned enzyme ADH from *Thermus thermophilus* HB27 ($ADH_{T1}$ and $ADH_{T2}$), to convert 2,2,2-trifluoroacetophenone (TFAP) to alpha-(trifluoromethyl)benzyl alcohol (TFMBA). All the reactions were performed in a solvent free organic medium (400 mg/mL), and they found that the concentration of the TFMBA product was 3.6 M and the overall productivity of that product was of 190 mol TFMBA/kg cells/h) (Hibino and Ohtake 2013).

This confirms that depending on the enzyme's optimal conditions, one can choose to use the rhodococcal enzymes in ionic liquid mediums or in organic ones.

### 4.2. Results and Discussion

Enzymes can be divided into six groups based on the reaction they catalyze. These groups include oxidoreductases, transferases, hydrolases, lyases, isomerases, and ligases. In this section of the thesis, the results that were obtained using microbial oxidoreductases will be discussed. There were two types of enzymes that were used during the experiments: the secondary alcohol dehydrogenases and cholesterol oxidase from wild type bacteria (the genus *Rhodococcus*) and alcohol dehydrogenases from recombinant bacterial cells.

#### *Rhodococcus* identification

*Rhodococcus* sp. GK1 is a bacterium that was isolated from a polluted soil with hydrocarbons from Nancy, France. This species was deposited at the Collection of Pasteur Institute in Paris, France and it was given the number CIP105335. This wild type form of the bacteria was used in this thesis all along with two other strains GK3 (*R. opacus*) and GK12 (*R. erythropolis).* GK1 was the source of two microbial enzymes (the cholesterol oxidase and the alcohol dehydrogenase). The main objective of my experiments was to characterize ADHs from microbial sources and to test a new solvent called DES on them.

#### Catabolism of cholesterol by *Rhodococcus* sp. CIP 105335 (strain GK1) in the presence of 9α-hydroxylase inhibitor

The cholesterol catabolism by the actinomycete genus *Rhodococcus* takes place in three steps (Figure 23): cholesterol oxidase reaction initiating this catabolism, cleavage of the sterol side chain and the steroid's nucleus degradation. Two key enzymes are involved in the opening of the steroid's nucleus: 3-ketosteroid $\Delta^1$-dehydrogenase and 3-ketosteroid 9α-hydroxylase.

Inhibition of one or both of these enzymes leads to the protection of the steroid nucleus from the microbial catabolism, and thus to accumulation of derivatives of interests in the microbial media.

**General scheme of sterol catabolism**

Microbial transformations of steroids, including sterols, are catalyzed by microorganisms belonging to the actinomycetal genera *Arthrobacter, Corynebacterium, Mycobacterium, Nocardia, Rhodococcus* and *Gordonia* (Kreit et al., 2012). Species of *Pseudomonas*, Gram-negative bacteria, are also active in the steroid catabolism. A general scheme encompassing this catabolism is shown in Figure 23. The scheme concerns particularly species of *Rhodococcus* and closely related taxa. The tetracyclic system and the lateral chain of sterols are metabolized independently but simultaneously. Depending on the microorganism, the degradation of the sterol ring system can be more or less rapid than the cleavage of the lateral chain. The first and compulsory step of the sterol catabolism is the conversion of 3β-OH-5-ene to 3-keto-4-ene under the action of cholesterol oxidase. Mycobacteria catalyze this step by a dehydrogenase system, requiring NAD as cofactor (Yang et al., 2007). The catabolism of the steroid nucleus starts when the 1,2-dehydrogenation is followed by the 9α-hydroxylation or vice versa, and this leads to the formation of 9,10-secophenol derivative. The formation of this derivative is via a nonenzymic reverse aldol type reaction. The 9α-hydroxylase, requiring $Fe^{2+}$, can be inhibited by complexion agents of this ferrous ion, or by metals like $Ni^{2+}$ and $Co^{2+}$. In subsequent enzymatic reactions, ring A is degraded to yield the hexahydroindane propionic acid derivative. The side chain is shortened by a mechanism similar to that of the beta oxidation of fatty acids. Following hydroxylation at the C-26, catalyzed by a cytochrome P450 system, and subsequent oxidation of the resulting alcohol group ($-CH_2OH$) into carboxylic group (-COOH), propionate, acetate and propionate are successively removed from the chain of cholesterol. Cleavage of the side chain of beta-sitosterol and campesterol

involves carboxylation at the C28 before the carbonic rupture. As a result, three molecules of propionate and one molecule of acetate are removed from sitosterol, while the side chain of campesterol gives two molecules of each acid (Kreit et al., 2012, Ibn Majdoub Hassani 2013).

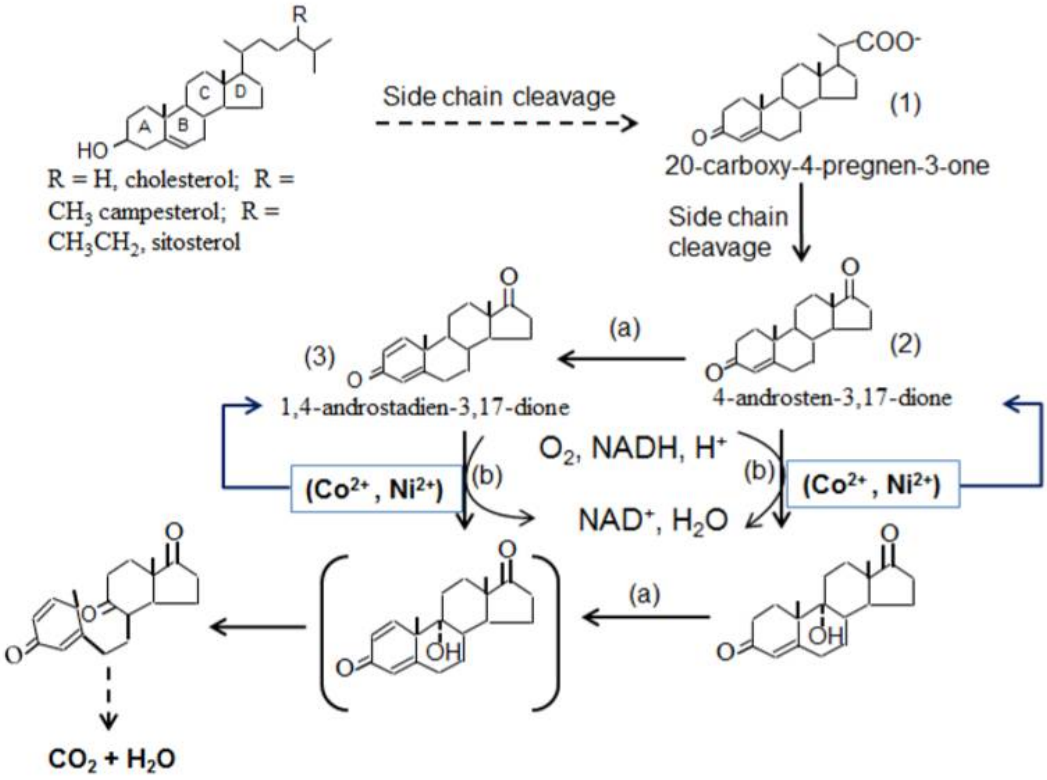



**Figure 23:** cholesterol catabolism by microbes (Capyk et al., 2009, Kreit et al., 2012).

The aim of this experiment was to produce steroid derivatives (AD and ADD) of cholesterol having the tetra cyclic nucleus intact. Culturing the bacterial strain performing the cholesterol oxidase assay, and steroid TLC are according to the procedures described in the experimental part (Figure 24).

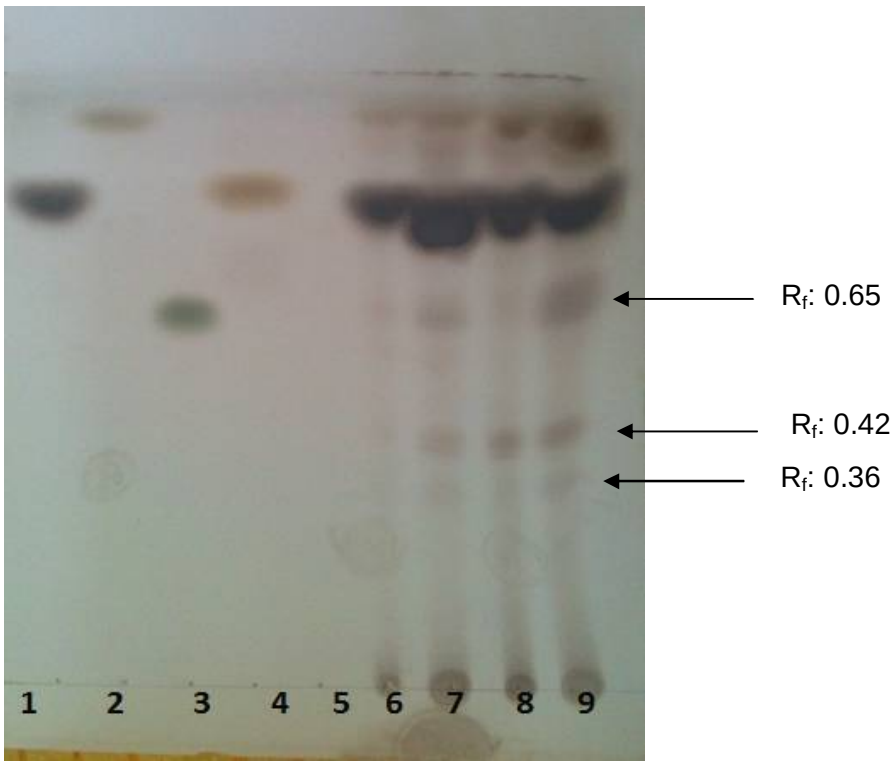



**Figure 24:** TLC analysis of steroids extracted from cultures of *Rhodococcus* sp. Silica gel sheet (13.5 x 10 cm) was used. Resolution solvents: chloroform (14 mL), diethyl ether (10 mL) and ethyl acetate (10 mL). Spot visualization: reagent, 2 g $FeCl_3 \cdot 6H_2O$ in 83 mL isopropanol and 15 mL sulfuric acid; 10 min at 100 °C. References: **1** cholesterol, **2**: cholestenone, **3**: testosterone **4** progesterone, **5-9** cultures with cholesterol; **5** and 7 control cultures; **6** and **8** assay cultures contained 1 mM $Ni^{2+}$. Final concentration of cholesterol was 2 g/L, added as a fine powder (**5** and **7**) or as glycerol suspension (glycerol in the cultures was 1% w/v).

The strain GK1 was identified to belong to the genus *Rhodococcus* according to morphological studies (Figure 25), physiological and cell wall analysis studies. Also, that strain could completely catabolize sterols and the induction of cholesterol catabolic enzymes in cells of GK1 was quantified in term of cholesterol oxidase (chox) specific production. As shown in the Table 15, the chox quantity was insignificant unless cholesterol was added to the strain's growth medium.

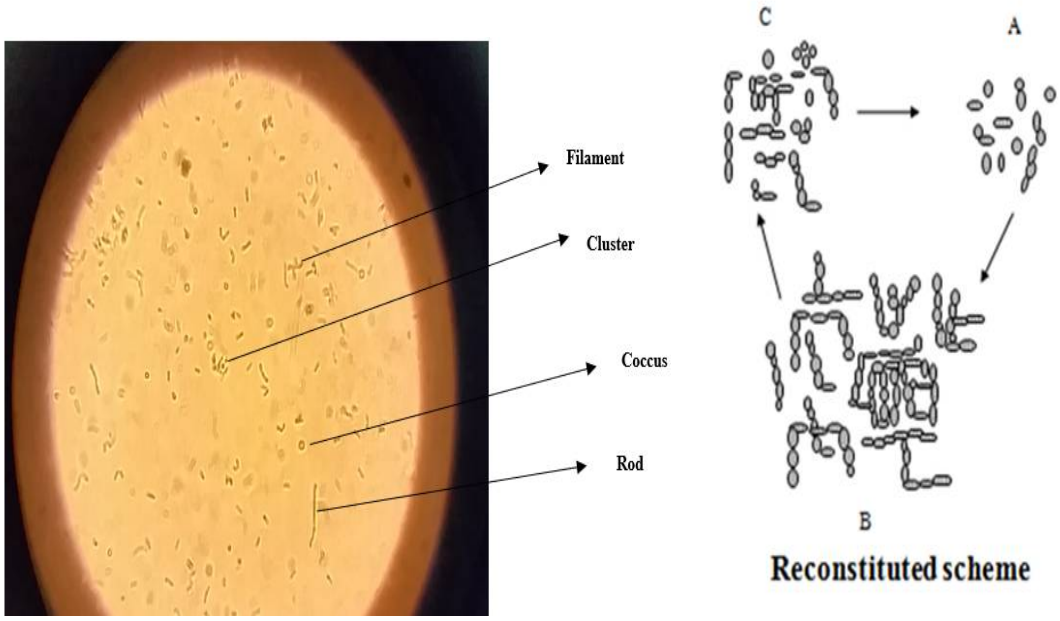
### 4.2.1. Summary about the results obtained from the catabolism of sterols by species of *Rhodococcus*

**Table 15:** growth profile of *Rhodococcus* sp. on acetate and cholesterol. Cholesterol was added as a fine powder (cultures 1, 2) and as glycerol suspension (cultures 3, 4, final [glycerol] = 1% w/v). Cholesterol oxidase (chox) was assayed using a colorimetric assay (U = enzyme units, 1 unit is the quantity of cells catalyzing the conversion of 1 µmol cholesterol/min) (Labuda et al., 2018).

| Cultures | Culture step | db[a] (g/L) | Chox (U/g db[a]) |
|---|---|---|---|
| **1** | - growth | 0.8-1.0 | 2.0-5.0 |
| | - induction | 0.90-1.1 | 10-12 |
| | - derivatives | 0.8-0.9 | 11-12 |
| **2** | - growth | 0.8-1.0 | 2.0-5.0 |
| | - induction | 0.8-1.1 | 10-12 |
| | - derivatives | 0.85-1.0 | 10-11 |
| **3** | - growth | 0.8-1.0 | 2.0-5.0 |
| | - induction | 0.95-1.1 | 14-15 |
| | - derivatives | 1.0-1.1 | 23-25 |
| **4** | - growth | 0.8-1.0 | 2-5 |
| | - induction | 0.95-1.2 | 14-15 |
| | - derivatives | 0.90-1.1 | 10-12 |

[a] db means dry weight or dry biomass.

It seems that $Co^{2+}$ or $Ni^{2+}$ inhibited strain growth and chox induction (see derivatives step). Glycerol could emulsify cholesterol and consequently improves its uptake by growing cells. Glycerol was not used by the strain when used as the sole carbon source.




**(a)** **(b)**

**Figure 25:** (a) cells of *Rhodococcus* sp. GK1 (magnification $10^3$). The strain develops various morphological features during growth. Nucleotide sequence of the rRNA gene of the 16S ribosomal subunit (NCBI, A N: JQ318031) and BLAST showed that GK1 might be the strain of *Rhodococcus equi* or a new species. (b) growth phases of *Rhodococcus* sp. (Kreit et al., 2012).

To extract steroids, the cultures that contained the bacterial strains were acidified with HCl and extracted with chloroform. The solvent phase was evaporated at room temperature and steroid residues were solubilized with appropriate volumes of isopropanol (or methanol). The issued samples were analyzed by UV, TLC and HPLC.

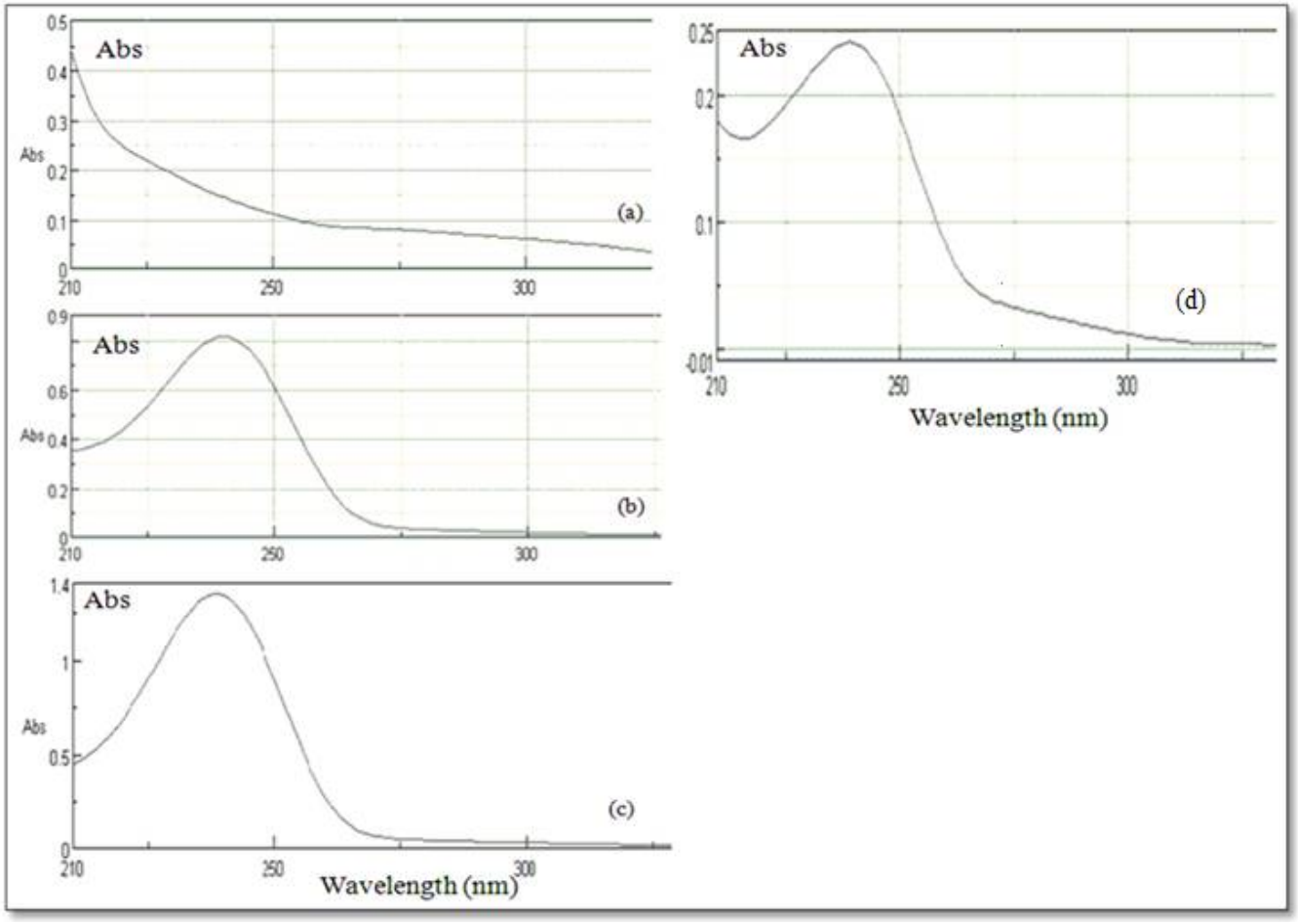



**Figure 26:** UV absorption spectra of steroid solutions in isopropanol. Steroid markers: (a) cholesterol 64 μM, (b) cholestenone 64 μM, (c) 4-androsten-3,17-dione 96 μM; (d) steroids extracted from a culture of the bacterium containing 1 mM $Co^{2+}$.

As shown in (Figure 26), samples of extract steroids absorb in the UV with a maximum at 240 nm which indicated that these extracts contained catabolic derivatives with carbonyl group. In addition to that, those results were also confirmed with TLC analysis and HPLC resolution of steroid extracts (Figures 24 and 27) demonstrated the presence of at least 3 derivatives which might be without a side chain. HPLC resolution of the extracted steroids (Figure 27) showed catabolic derivatives of cholesterol that might be devoid of the cholesterol side chain.

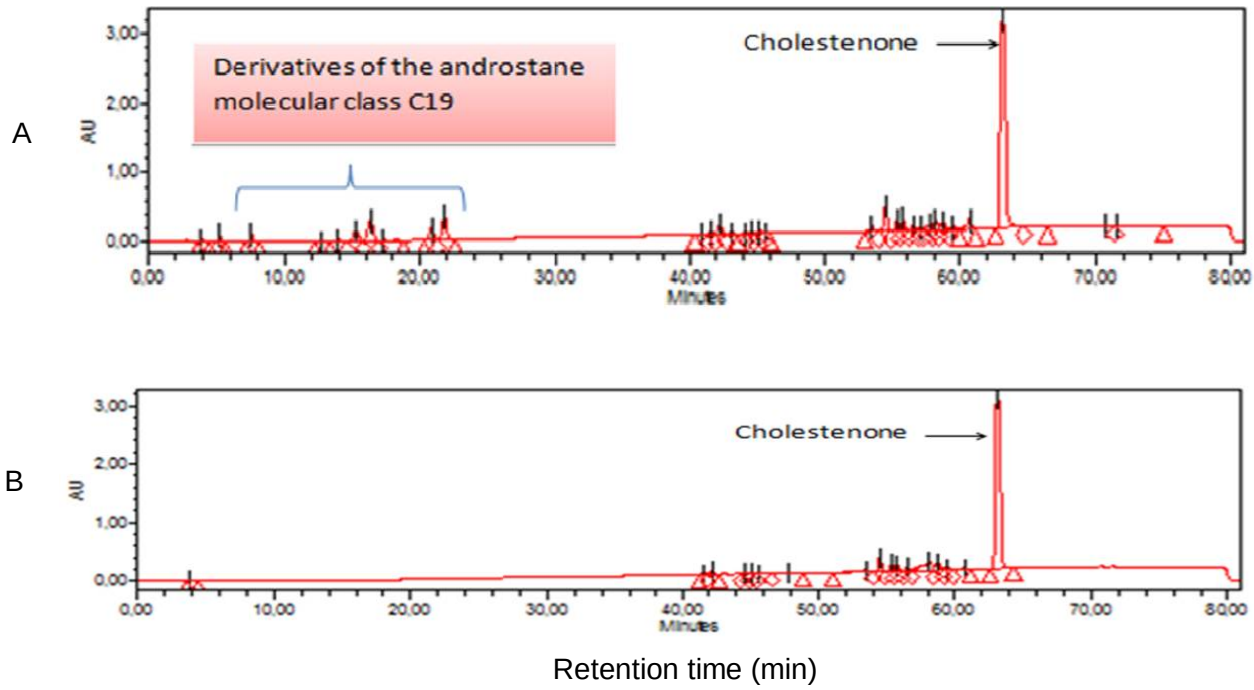



**Figure 27:** HPLC profile of steroids extracted from *Rhodococcus* sp. cultures on acetate-cholesterol.

Extracted steroids (culture of 50 mL) were solubilized with methanol (4 mL). A sample of 10 µL was chromatographed on a C-18 reverse phase column (150 mm x 4.6 mm-particle size 3 µm) using a gradient of mobile phase (methanol-acetic acid (99/1 v/v)/ultrapure water) at 1.2 mL/min. Steroid detection was at 240 nm. A, assay culture (1 mM $Ni^{2+}$), B control culture (Figure 27). The column was standardized with appropriate steroid markers. Based on the results obtained, we concluded that the inhibition of the hydroxylase activity by $Ni^{2+}$ or $Co^{2+}$ appears to be efficient in protection of the steroid nucleus from microbial degradation. The process described here for obtaining catabolic derivatives of interest is worthy of further investigation.

**Purifying the secondary ADH of *Rhodococcus* GK1 and measuring its activity**

We tried to purify the SADH from *Rhodococcus* GK1 using a Sepharose CL-6B column. For the purification of the secondary alcohol dehydrogenases from GK1, the results are depicted in the recovery table (Table 16).

**Table 16:** secondary alcohol dehydrogenase from GK1 recovery table.

| Fraction | Volume (mL) | Enzyme unit | Protein (g/mL) | Specific activity U/mg |
|---|---|---|---|---|
| **Culture** | 20 | 1.67 | 16 | 0.104 |
| **Cell suspension pellet** | 20 | 0.54 | 16 | 0.0419 |
| **Spent medium (supernatant)** | 19 | 4.96 | 8 | 0.011 |
| **Ammonium sulfate fraction** | 15 | 0.259 | 4 | 0.417 |

In order to characterize the secondary ADH from *Rhodococcus* sp. GK1, the following steps were applied: first we grew the bacterial cultures in Erlenmeyer flasks under sterile conditions, each bacterial culture contained a carbon source (cholesterol) and was put in a shaker until a creamy yellowish color was obtained (meaning the bacteria had fully grown). Then we took a volume of the bacterial suspension and measured its OD at 600 nm. Later, sonication and centrifugation were performed on the bacterial solution. In addition to that, several assays were applied to the bacterial suspension (NAD assay and Lowry assay) (Lowry et al., 1951). Then ammonium sulphate fractionation was done. Afterwards, the enzymatic solution was filtered using a CL-6B gel filtration chromatography to determine the active fractions. Unfortunately, we did not obtain a high enzymatic activity from the measured fractions and the activity peaks were very low (below 0.1) for fractions n° 21-22-23-24-25-26-27.

### 4.2.2. Cholesterol oxidase induction in the two strains of *Rhodococcus*: GK1 and GK3

**Induction of cholesterol oxidase in GK1**

We tried to induce cholesterol oxidase from GK1 and measure its enzymatic activity by using with this strain 5 g/L of phytosterols, cholestenone, and 2 g/L of yeast extract. The results are shown in Table 17, at different stages of the bacterial growth. We also confirmed that GK12 can catabolize the lateral chain of the sterol (data not shown).

**Table 17**: activity of cholesterol oxidase from GK1, culture on sterols plus yeast extract kept refrigerated.[a]

| Fraction | Volume (mL) | U total | Specific activity (U/mg) | Protein mg/mL | U/mL | U per L culture |
|---|---|---|---|---|---|---|
| **Culture** | 210 | 89 | 178 | | 0.421 | 421[b] |
| **Cell suspension** | 35 | 49 | | | 1.406 | 234[c] |
| **Spent medium (supernatant)** | 200 | 35 | 0.12 | 1.50 | 0.176 | 167[c] |
| **Triton x-100 (1%, 1 h) extract 1** | 32 | 14 | 0.28 | 1.60 | 0.440 | 67 |
| **Triton extract 2** | 24 | 06 | 0.17 | 1.45 | 0.246 | 28 |
| **Spent medium ($SO_4$) extract** | 20 | 18 | 1.88 | 0.48 | | |
| **Extract $SO_4$** | 10 | 13 | 3.02 | 0.43 | | |

[a] 5 g/L sterols, yeast extract: 2 g/L.

[b] Specific production: 421 U/L, 2.32 g/L= 181 U/g dry biomass.

[c] Issued from a culture of 1 L.

A study conducted by Elalami et al. in 1999, has characterized the secreted cholesterol oxidase from *Rhodococcus* sp. GK1 (CIP 105 335). In that investigation, GK1 cells were grown in a mineral salt medium that contained a mixture of phytosterols (sitosterol, campesterol, and stigmasterol) as the carbon source of the bacteria. These sterols induced the cholesterol oxidase. In addition, the yeast extract was added to the bacterial medium to induce the enzyme's secretion. In that study, the purified cholesterol oxidase was from the supernatant of the culture (Elalami et al., 1999). However, the enzyme activity of the cholesterol oxidase that we worked with was much lower than the activities of the cholesterol oxidase from that study. For example, the specific activity of the pure cholesterol oxidase from their study was 5.5 U/mg as opposed to what we obtained (3.02 U/mg) (Table 17). Furthermore, the specific activity of their supernatant was much higher than ours (they obtained 0.22-0.30 U/mg, and we obtained 0.12 U/mg). In addition, appreciable amounts of cholesterol oxidase were obtained by those researchers (350 to 400 U/L). On the contrary we obtained only 167 U/L. In conclusion, all these results confirm that cholesterol oxidase is a robust enzyme that is very active when the appropriate conditions are available to it.

**Characterization and production of cholesterol oxidase in *Rhodococcus* sp. GK3 cells**

Species of the genus *Rhodococcus* which were isolated from polluted soil could catabolize cholesterol. Those microbes were able to grow successfully in a mineral medium that contains as a source of carbon the following: cholesterol, phytosterols, hexanoate, and acetate. When these sterols were used, cholesterol oxidase was induced. During the microbial catabolism of sterols, cholesterol oxidase (EC 1.1.3.6) is involved in the conversion of $\Delta^5$-3β-alcohol to $\Delta^4$-3-keto derivative and during this reaction, molecular oxygen is converted to hydrogen peroxide. Species of *Rhodococcus* can produce cholesterol oxidase either as a secreted form or/and in a membrane bound form (Aihara et al., 1986; Kreit et al., 1994).

In a study performed by Elalami et al. 2000, the strain GK3 from *Rhodococcus* was utilized to degrade cholesterol. Also, in that same study, the strain was identified and the cholesterol oxidase enzyme was solubilized.

The GK3 strain was grown in a mineral salt medium (Kreit et al., 1992b). The carbon sources for the bacteria were sodium acetate (10 g/L) and cholesterol (2 g/L). Then cholesterol oxidase solutions were prepared to make the enzyme assay and to measure the cholesterol oxidase activity. The GK3 strain was identified as a gram positive, catalase positive, aerobic and coccus-shaped bacteria that did not form endospores. In addition, the strain had a branching-shaped cell growth cycle that started with the assembly of (2 to 4 cells) into more complex structures that split to small assemblies and cocci during the stationary phase. The strain could also grow on media that contained sources like: glucose, saccharose, steroids, phenolic derivatives, acetate, hexanoate, and cholesterol, and it was not able to metabolize cellulose (Lechevalier 1984). Also, GK3 grew very well in the media that contained cholesterol. After 2 to 3 days of culturing, 80% of the initial cholesterol (2 g/L) was transformed; and 2 derivatives were obtained during the exponential growth. One of the derivatives was identified as 4-cholesten-3-one. At the maximal growth, the dry weight yield was 0.88 g per 1 g of consumed cholesterol. This value is more than the dry weight yield that was obtained when grown on acetate media (0.32 g per 1 g of $CH_3COO^-$).

The conversion of cholesterol to 4-cholesten-3-one can be catalyzed by the action of enzymes (NAD-dependent dehydrogenase) and $\Delta^5$-$\Delta^4$ isomerase (Martin 1984). The colorimetric assay enabled the research team to detect the oxidase activity in the whole cells of the microorganism that was grown on cholesterol. In addition, there was no enzyme activity when acetate or hexanoate were used as carbon substrates for GK3. The sterols that triggered the enzyme production in GK3 were cholesterol and soybean sterols. Also, a kinetic study on the

enzyme from GK3 was made, and as the cells were growing, the enzyme amount was increasing reaching the maximum at the stationary phase (54 U/L). Moreover, after 31 h of cultivation, cholestenone was identified as one of the steroids that were extracted from GK3 after 6 hours of growth, a peak of specific enzyme production was obtained (40-45 U/g dry weight). In addition, the produced cholesterol oxidase was a membrane bound enzyme with an active site at the extreme surface of the cell membrane. That enzyme was extracted with Lubrol PX and the extraction yield was more than 70%; and the enzyme extract had a specific activity of 0.53 U/mg, which was much higher than the secreted form (0.014 U/mg).

For GK3, the maximum growth peak was observed after 24 h of growth on cholesterol (2 g/L). The highest enzyme activity peak (specific production) was observed after 9 h of cultivation and after 70 h (for the total enzyme production).

Based on the results obtained above, the *Rhodococcus* genus strains (GK1, GK3, and GK12) still contained some enzymatic activity (chox and SADH). However, due to technical difficulties (contamination and problem with the sonicator), the induced enzymes had a weak activity. These obtained results were in contrast to what was normally obtained when the normal experimental conditions were followed for the induction (Elalami et al., 2004).

Normally, when the reaction conditions are obeyed, the secondary alcohol dehydrogenase from *Rhodococcus*sp. GK1 exhibit an activity when they are cultivated on cholate (182 U/L with diacetyl and 24 U/L with isopropanol). The highest enzymatic production is obtained with phytosterols or cholesterol as a source of carbon and energy (322 U/L with diacetyl and 65 U/L with isopropanol). Throughout the experiments of this thesis, isopropanol was found to be an appropriate inducer of the ADHs from the different bacterial cells.

The secondary alcohol dehydrogenase was partially purified by hydrophobic interaction

chromatography (Krier et al., 1998), or by using ammonium sulfate fractionation followed by filtration on the gel sepharose CL-6B (Kreit and Elalami 2002). The final enzymatic preparation was enriched five times by the first method with a yield of 82%. The second method has permitted a better purification with an activity yield of 66%. The molecular mass of this enzyme was around 65 kDa by filtration on the sephadex gel G-100 (Krier et al., 1998) or on sepharose gel CL-6B (Kreit and Elalami 2002).

This alcohol dehydrogenase is dependent on NAD. The kinetic of the oxidoreduction reaction, which it catalyzes follows the model of Michaelis-Menten for all the substrates used. The $K_m$ constant for the NAD or the NADH with isopropanol and acetone as substrates, follow the order 0.16 mM. Based on the substrate specificity study, which was previously done with the crude extract and the purified enzyme, the following conclusions were made:

- The enzyme was inactive with primary alcohols like ethanol or *n*-propanol.
- The enzyme was active with secondary alcohols like isopropanol, 2-butanol, 2-hexanol, and 2-octanol and with the corresponding ketones like acetone and 2-butanone. The enzyme was also active with diketones like 2,3-butanedione.
- The enzyme was inactive with cyclohexanol, a cyclic alcohol and inactive in the oxidation of the two derivatives of diacetyl, acetoin and 2,3-butanediol.

## 4.3. Experimental part

### Alcohol dehydrogenase and cholesterol oxidase from wild type bacteria

- General information
- Bacterial cultures
- Enzymatic solution preparation
- Filtration on sepharose CL-6B
- Enzymatic activity
- Proteins
- Electrophoresis

### Alcohol dehydrogenase from recombinant bacterial cells

- Solvent preparation: The Deep Eutectic Solvent (DES)
- Procedures for enzymatic bioreduction with DES
- GC analytics

### Chemicals

All the chemical products were of analytical grade, purchased from commercial sources. Molecular biology kits were from specialized commercial companies, as defined below.

### Microorganisms and culturing

Three species of *Rhodococcus* were used in the present study: strain GK12, identified as *R. erythropolis*; strain GK1 (CIP: Pasteur Institute Collection), identified as a new species or a strain of *R. equi* (Kreit et al., 2012); strain GK3, identified as *R. opacus*. These strains were cultured in the media described below (Green, 1933).

### Basic medium (minimal medium)

This is a mineral salt medium that contained per L (Kreit et al., 1992a): 3.00 g $(NH_4)_2SO_4$, 4.16 g $Na_2HPO_4$, 1.20 g $KH_2PO_4$, 0.05-0.10 g yeast extract, 0.8 mg thiamine, and 20 mL of trace element solution. The solution of trace elements was prepared as a stock solution, kept at 4 °C; it contains per L: 12.50 g $MgSO_4.7H_2O$, 1.00 g $CaCl_2.2H_2O$, 0.25 g $FeSO_4.7H_2O$, 0.05 g $MnSO_4.3H_2O$ and 0.05 g $ZnSO_4.7H_2O$.

**Basic medium and carbon source**

The carbon source was variable. The pH of the media was adjusted to around 7.0 with HCl, following the carbon source addition, except for steroid-media and sugar-media. Except where stated otherwise, steroids comprising sterols were added each as a fine powder. Media were sterilized by 20 min autoclaving at 1 bar (120°C).

The following carbohydrates (sugars) were used each as the sole carbon and energy source (molecular weight in g/mol): D-(-)-ribose, 150.13; D-(+)-xylose, 150.13; D-(+)-glucose monohydrate, 198.12; D-(+)-galactose, 180.15; D-(-)-fructose, 180.16; *beta*-lactose, 342.10; maltose monohydrate, 360.32; water soluble starch. Glycerol (mw = 92 g/mol) was also used as a sole carbon and energy source. All these molecules were solubilized with distilled water and each solution was sterilized by filtration throughout a Millipore membrane; then an appropriate volume of the filtrate was added to a defined volume of the basic medium. The media used were labeled according to the used carbon sources.

(a) **Acetate-medium**: 10 g of $CH_3$-COONa.3 $H_2O$ per L, final volume, of the basic medium.

(b) **Acetate-Yeast extract medium**: 4 g $CH_3$-COONa.3 $H_2O$ and 2 g yeast extract per L, final volume, of the basic medium. This medium was used as a control for cholesterol oxidase induction as described in the results section.

(c) **Sterol-medium**: 5 g phytosterols (commercial product from soybean, containing about 60% β-sitosterol, 27% campesterol and 11% stigmasterol) per L of the basic medium. This was a selective medium, used for selectively discard any eventual contaminant that could not be observed under an optic microscope (magnification: 1000 fold). For some cultures, cholesterol (5 g/L) was used instead of the phytosterols.

(d) **Sterol- yeast extract medium**: 2-4 g phytosterols and 1 g yeast extract per L of the basic medium.

(e) **Cholesterol-yeast extract medium**: 2 g cholesterol and 1 g yeast extract per L of the basic medium. $Co^{2+}$ ($CoSO_4.7H_2O$) or $Ni^{2+}$ ($NiCl_2.6H_2O$) was added to this medium as 9α-hydroxylase inhibitor in order to get steroid ring system derivatives from cholesterol, during microbial growth. Cobalt or Nickel solutions were prepared by dissolving the used salts with distilled water. They were sterilized by 30 min boiling at 100°C, and then used for culturing.

**Culturing and growth estimation**

Cultures were carried out in Erlenmeyer flasks under aerobic conditions (medium occupies 1/5 the flask volume) at 30°C ± 2 and mechanical shaking of 240-250 rpm. Unless otherwise specified, culturing was stopped at the maximal growth in the stationary phase; cultures were maintained refrigerated at 5°C. Microbial growth was followed by reading sample OD at 600 nm; OD value was converted to dry biomass using a standard curve. Culture sample containing steroid was diluted with a solution of Triton X-100 (1% w/v)-isopropanol (10% v/v), rigorously agitated on a vortex to dissolve steroids, and then measured for its OD value.

The inoculum was of 4-5% (v/v). The OD of the inoculated biomass at the starting time was around 0.10, except where specified otherwise.

Culture homogeneity was controlled by microscopic observation of untreated cells or Gram-colored cell preparation. When required, cells were separated from the spent media by centrifugation. The collected biomass was washed with distilled water and the cell suspension was centrifuged to recollect the biomass.

**Gram staining**

The applied protocol for the Gram staining method consisted of the following steps:

1. Fix cells on a smear by gentle heating on a Bunsen burner flame. Cover the fixed bacterial smear (frottis) with crystal violet solution (1 g/100 mL distilled water), add 3-5 drops of a bicarbonate solution (5% w/v in distilled water) and let it rest for 2 min.

2. Wash with distilled water, then cover the cells with Lygol solution (0.5 g iodine, 1 g KI, and 100 mL distilled water) for 1 minute.

3. Differentiate the preparation with a mixture of acetone-ethyl ether (75 mL acetone + 25 mL ether) until the liquid drops clear from the smear surface.

4. Wash promptly; recolor with a safranin solution (0.5 g/100 mL distilled water) for 30 seconds.

5. Wash the preparation with distilled water; dry it with water-absorbing paper and observe the smear under a microscope (400 and 1000 magnification).

The Gram coloration reagents can be easily prepared in the laboratory or purchased as a kit from a commercial company.

**Steroids extraction**

Culture sample was acidified with HCl, 10 mL acid solution of 18% for 50 mL culture. Then residual steroids were extracted three times with chloroform (total solvent volume: around 40 mL). The solvent phase (the lower phase) was collected with a Pasteur pipette, dehydrated throughout a filter, composed of cotton-ammonium sulfate on a funnel, and evaporated at room temperature. When required to discard any polar substance from the issued solid fraction of steroids, 15 mL of acidified distilled water, and 15-20 mL of chloroform were added to this fraction. Following rigorous agitation, the solvent layer was up taken and filtered throughout a piece of cotton. The final solid fraction of residual steroids was dissolved with an appropriate volume of 2-propanol. This steroid-solvent fraction was analyzed by thin layer chromatography (TLC), HPLC and spectrometry (Figure 24 and Figure 27).

**Thin layer chromatography**

TLC of steroids was performed on silica gel 60 aluminum sheet (11 x 10 cm) (Merck), using a mixture of chloroform (14 mL), diethyl ether (10 mL) and ethyl acetate (10 mL) at room temperature. Ferric chloride/sulfuric acid was charring reagent, used for spot visualization. The reagent was prepared by dissolving 2.00 g $FeCl_3.6H_2O$ in 83 mL of isopropanol and 15 mL of concentrated sulfuric acid. The TLC sheet was dried from the development solvent mixture in air, immersed in a beaker containing the visualization reagent, and then heated at 90-100°C for about 5 min.

This TLC procedure, established during the present laboratory work, was rapid requiring less than one hour, and reproducible with a good accuracy. The below cited steroids were generally used as molecule markers:

Cholesterol, 4-cholesten-3-one (cholestenone), testosterone, 3β-hydroxy-5-androsten-17-one,

4-androsten-3,17-dione, cholic acid. Each steroid marker was dissolved in 2-propanol at 5 mg/mL.

Three drops and six drops of respectively steroid marker solution and steroid sample solution were deposited with a Pasteur -pipette on the silica sheet for the TLC analysis.

**High performance liquid chromatography**

High-performance liquid chromatography (HPLC) sometimes referred to as high-pressure liquid chromatography, is a chromatographic technique, used in chemical or biochemical analyses to separate sample compounds with the purpose of identifying, quantifying and/or purifying the individual components of the sample. The HPLC of steroids in the present work was carried at the Center of Pharmaceutical Drugs Control, Bab Elirfan, Rabat. The HPLC apparatus used was obtained from Perkin Elmer (USA), Series DAD-200-PE. The apparatus is equipped with one pump, an automatic injector, an ultra-violet light detector, and a system of interface module for computerized peak surface integration and data handling (Total Chrom V 6.3.2). The HPLC column used is a Nucleosil C18 (125 × 3 mm; particle size 5 μm), purchased from Machery Nagel (France).

**Chromatographic procedure**

Samples were analyzed on the system described above by using the gradient mobile phase shown in (Table 18). The injection volume was 10 μL of each sample (blank, crude extract, reference 1, reference 2). Steroid separation was conducted at a flow rate of 1.0 mL/min and steroid detection was achieved by monitoring absorbance at 240 nm.

**Table 18:** HPLC apparatus program and eluent gradient applied for analysis of steroids.

| **Time (min)** | Mobile phase A (% v/v) | Mobile phase B (% v/v) | Curve |
|---|---|---|---|
| **0.5** | 60 | 40 | 0 |
| **5.0** | 60 | 40 | 0 |
| **10.0** | 80 | 20 | 1 |
| **30.0** | 100 | 0 | 1 |
| **10.0** | 100 | 0 | 0 |
| **3.0** | 60 | 40 | 0 |

A: 1% acetic acid in methanol; B: ultrapure water.

**Samples**

- Blank: The blank was 2-propanol.

- Crude extract: it was obtained from culture of cholesterol-growing *Rhodococcus* sp. strain GK1 by extraction with chloroform, solvent evaporation and dissolving solid residues with 2-propanol.

- Reference 1: it consisted of 4-androsten-3,17-dione, dissolved in 2-propanol (approximately 4 mg/mL).

- Reference 2: it consisted of a mixture of 4-cholesten-3-one and testosterone, dissolved with 2-propanol at approximately 1 mg each per mL.

These references were considered for qualitative analysis. Neither cholesterol nor 5-androsten-3*beta*-ol-17-one was integrated in the reference solutions because they do not absorb UV at 240 nm.

**Preparation of enzymatic solutions:**

The supernatant was collected by centrifugations from cultures at 28000 x g during 30 min at 5 ºC. It was used as a crude extract of the extracellular cholesterol oxidase.

The cellular enzyme was extracted using two methods depending on the carbon source used for the microbial growth:

For cells developed on sterols, the cellular suspension (0.5 to 0.8 g humid biomass/10 mL phosphate buffer) is added to the librol PX at 0.5% (w/v) then agitated using a magnetic stirrer for thirty minutes at room temperature (Kreit et al., 1994).

To prepare the enzymatic solution of the alcohol dehydrogenase, the procedure below was followed:

After stopping the growth of the bacteria, the cells were collected by centrifugation during 30 minutes (27000 x g, 5 °C), washed twice with phosphate buffer. Then the cells were mixed as a suspension with the same buffer (0.6 g wet weight/ 10 mL buffer). The cells in suspension were lysed using a sonicator. Then the cellular debris and the cells that were not lysed were eliminated by centrifugation (27000 x g, 5 °C). The clear supernatant is the crude extract of the alcohol dehydrogenase.

For the cells that were developed on sterols and yeast extract or on hexanoate: some part of cellular activity was solubilized by agitation only in buffer. This treatment was repeated two times and the collected extracts were collected and gathered. The residual activity was solubilized by treatment with a detergent.

The enzymatic extracts were collected by centrifugation of cellular suspension at 28000 x g and at 5 °C during 30 min. These enzymatic solutions can be conserved for a week at 4 °C.

For longer periods it was necessary to freeze them at -20°C.

**Enzymatic activity:**

The activity of cholesterol oxidase was measured at a pH of 7 and at 30 °C based on the spectrophotometric methods that were described by Kreit et al., 1992b.

Method 1: the enzymatic activity was measured by estimating the 4-cholestene-3-one formed. The final volume of the assay was 3.2 mL containing 150 µmol of phosphate, 0.16% Triton X-100, 0.98 µmol of cholesterol (in a solution in isopropanol: 0.2 mL added) and an appropriate quantity of the enzyme. The reactional medium was mixed well after adding the enzyme. The evolution of the reaction was monitored continuously by measuring the absorbance of the 4-cholestene-3-one at 240 nm ($\varepsilon = 1.4 \times 10^4\ M^{-1}\ cm^{-1}$) (Figure 29).

Method 2: hydrogen peroxidase which was generated by the enzymatic reaction is determined in the same conditions as the ones mentioned in the first method. In the reaction media, we add 1 µmol of ABTS and 50 µg of peroxidase (Vf=3.2 mL) before the addition cholesterol. The oxidation of ABTS is translated when a green color appears at 600 nm ($\varepsilon = 1.82 \times 10^4\ M^{-1}\ cm^{-1}$).The solutions of Triton X-100 (2% v/v), of ABTS (10 µmol/mL) and of peroxidase (1 mg/mL) are prepared in a phosphate buffer and conserved at 4 ºC.

**Secondary alcohol dehydrogenase**

**Cultures**

The bacterial growth was done in a mineral medium (Kreit et al., 1992a) with only one source of energy. The substrates that were used are: cholic acid (5 g/L), phytosterols (5 g/L) or the cholesterol (5 g/L). The preparation and sterilization conditions are similar to the one used for cholesterol oxidase. The growth was stopped at the beginning of the stationary phase (Figure 28).

**Filtration on sepharose CL-6B**

The enzymatic extract was fractioned using ammonium sulfate with a 60% saturation. The protein precipitation was performed using a magnetic stirrer for 30 min at 0 ºC, followed by a resting state for one hour at the same temperature. The precipitate was collected by centrifugation (27000 x g, 5 ºC) and dissolved in phosphate buffer, then filtered using a column of sepharose CL-6B (108 x 1.5 cm). The column was equilibrated and eluted with the same buffer at 18 mL/h and at 5 °C.

**Enzymatic activity**

The activity of the secondary alcohol dehydrogenase was measured at 30 °C and at a pH of 7, by monitoring the increase in the oxidation of the alcohol or the decrease of the ketone and the absorbance of NADH at 340 nm ($\varepsilon = 5.78 \times 10^3\ M^{-1}\ cm^{-1}$). The final volume of the assay was 3.2 mL containing 150 µmol of phosphate, 6 µmol of NAD or NADH and the substrate at different concentrations and an appropriate quantity of the enzyme.

The enzyme unit (U) is defined as the quantity of the enzyme that catalyzes the conversion of one µmol of substrate per minute according to the specific conditions of the assay method (Labuda et al., 2018). The specific activity is expressed in enzymatic units by mg of proteins

(U/mg).

**Proteins**: the concentration in proteins is determined by the lecture of the absorbance at 220 nm. The absorption coefficient of the proteins at this wavelength (1 mg/mL of solution) is equal to 11.

**Electrophoresis**

Electrophoresis on a polyacrylamide gel was performed using an SDS PAGE protocol and was made for crude extracts or partially purified enzymes. The objective was to investigate whether there was more than a proteic band active in the reaction medium. 14% of Acrylamide and 0.4% of bisacrylamide were mixed with 100 mL distilled water (final volume). From that solution, 12.1 mL were used. Then the following solutions were mixed together (0.375 M Tris HCl (pH 8.8), 0.1% SDS, 0.025% ammonium persulfate, 0.025% by volume of TEMED). Next, this mixture was loaded to the PAGE glasses. After that, the gel was left for 30 minutes in order to solidify. The stacking gel was prepared by mixing the following: (3% acrylamide, Tris buffer 0.125 M Tris HCl pH 6.8, SDS 0.1%, 5 mL of distilled water, 0.5 mL ammonium persulfate, and 0.015 mL TEMED). In addition, the electrode buffer pH 8.3 was also prepared and it contained (0.025 M Tris HCl, 0.192 M glycine, 0.1% SDS) (Laemmli 1970). After the gel had solidified, the protein sample was added to it. Moreover, the sample that contained the protein was prepared using the following method: (0.3 mL of the pellet suspension was mixed with 0.0625 M Tris HCl pH 6.8, and 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.001% bromophenol blue as a dye). Also, 40 µL of the protein sample were loaded into the well. Then the protein was left to migrate overnight (around 8 hours). After that, the gel was stained with coomassie brilliant blue and 50% trichloroacetic acid (TCA). Finally, the gel was washed several times with 7% acetic acid in order to observe the protein band (Elalami et al., 1999).

**The Bioconversion Section**

In order to induce cholesterol oxidase in the GK1 strain, (experiments were conducted at the Faculty of Sciences in Rabat-Morocco from May 2014 until December 2014), the bacterial cells were grown on the acetate yeast extract medium (as it is described in the experimental part, Figure 28). We prepared cultures of 50 mL each in 250 mL Erlenmeyer flasks. Cholesterol and starch were added as a carbon source for the bacteria (25 mg/mL) and propanol was used as a solvent. Then this preparation was left in the shaker for few days at 30°C until a yellowish/beige colour was observed in the flasks. When the nickel solution was prepared (11.88 mg/mL), it was added at the stationary phase of the bacterial growth; and the OD values were estimated after 25 h of incubation. Nickel was added to the medium in order to inhibit further enzymatic degradation and to obtain and accumulate valuable derivatives. Also, the derivatives were visualized by TLC and HPLC (Figures 24 and 27). When isopropanol was added as an emulsifier to the reaction medium, it inhibited the cholesterol oxidase induction and activity. For this reason, we used glycerol instead. We also noticed that the induction step requires 11 hours.

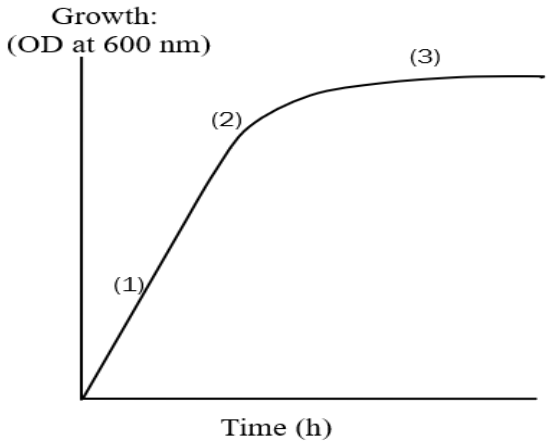



**Figure 28:** growth of *Rhodococcus* sp. for obtaining catabolic derivatives of cholesterol. **(1)** Growth step on acetate (10 g/L $NaCH_3COO.3H_2O$, 3 g/L ammonium sulfate, mineral medium, pH 7.0, 30°C, shaking, 15-20 h. **(2)** Induction step with cholesterol (1 g/L, 15 h). **(3)** Steroid derivatives step (1-2 g cholesterol/L "cholesterol final concentration: 2-3 g/L", 1 mM $Co^{2+}$ or $Ni^{2+}$ "9α-hydroxylase inhibitor", 20-25 h. Cultures were performed in 50 ml medium in Erlenmeyer flasks).

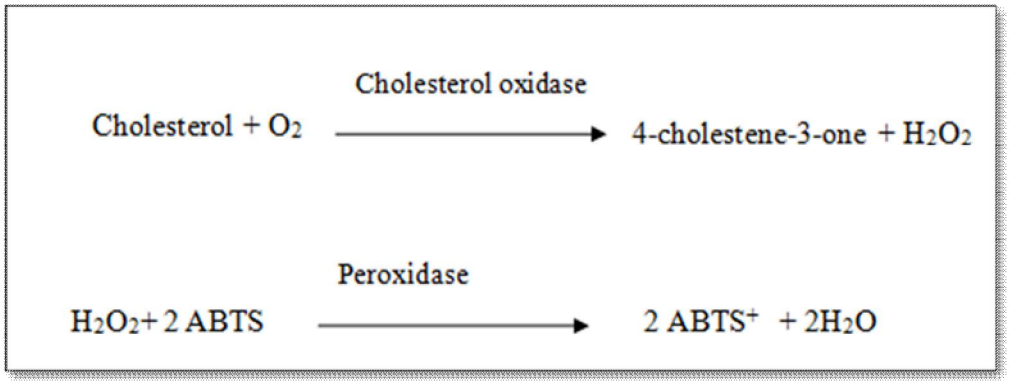



**Figure 29:** the reaction involved to measure the cholesterol oxidase activity.

In the enzymatic purification table (Table 17), the cholesterol oxidase activity is measured at each experimental step; like in the original culture (after removing the bacteria from the shaker and storing them in the fridge), and after each centrifugation. Following the centrifugation step, two phases were obtained: the supernatant and the pellet. The pellet was then mixed with phosphate buffer to obtain an enzymatic suspension. Then for the purpose of purifying the enzyme, we added to that suspension Triton x-100 and we left it for one hour at room temperature to solubilize the cholesterol oxidase. Then we centrifuged and repeated this step another time. Afterwards, we sonicated but we had some difficulties with the sonicator. Then we added to our enzymatic solution ammonium sulphate. At each step we measured the total volume of the solutions and the mass of the pellets after the centrifugations. We also measured the OD at 600 nm. As it can be seen from that table, there was a drop in the volume in each step because after each centrifugation we only kept the pellet containing the enzyme and we dissolved it with the phosphate buffer.

During the month of December 2014, we performed another set of experiments for GK1 and GK3 by adding to the reaction medium (57 mg of cholesterol dissolved in 7.4 mL isopropanol), and we tried to measure the cholesterol oxidase activity (Figure 29). In this experiment, GK1 and GK3 were grown in Tween 80 and cholesterol. Based on the OD and

ABTS results, we concluded that cholesterol oxidase was not present in those species of *Rhodococcus* due to the following reasons:

1- This enzyme was used to convert the 3 beta-ol-5-en moiety to the 3 ketone,
2- This type of cholesterol oxidase can only degrade actively plant sterols,
3- The catalase that was present in the reaction media transformed totally the hydrogen peroxide during the cholesterol/ABTS reaction, before the catalysis of cholesterol oxidase.

Finally, we assumed that there was a weak cholesterol oxidase activity in this experiment, but we were not able to see it in our results due to the catalase activity.

# *Annex 2*

**Congress and conferences publications derived from Part 2**

F. Z. Ibn Majdoub Hassani, M. Talbi, A. Elalami, S. Amzazi, J. Kreit, "Cholesterol: A Molecule of Social and Economic Interest". 12th Edition of the National Science Week-Innovation for Economic and Social Development, Rabat (Morocco), 28th April-2nd May 2014, Poster.

F. Z. Ibn Majdoub Hassani, A. Elalami, S. Amzazi, J. Kreit, "Biocatalysis of Cholesterol Side Chain Cleavage by *Rhodococcus* sp. CIP 105335". 7th International Congress on Biocatalysis, Hamburg (Germany), 31st August-4th September 2014, Conference Paper and Poster.

## 6. General discussion

In this work, we highlighted the importance of DES in research by performing experiments on this new solvent since 2015, and by producing three published articles about it.

In 2016, we used three types of DES; DES 1 was composed of ChCl and ethylene glycol, DES 2 was made of ChCl and glycerol, and the third type of DES was composed of ChCl and urea. From the results, we confirmed that the DES containing glycerol was the best and had stabilizing effects on the performance of *E. coli*/RasADH (Ibn Majdoub Hassani et el., 2016).

We also showed in 2019 in our review, the applications of these solvents and their influence on oxidoreductase mediated transformations (Ibn Majdoub Hassani et al., 2019). DES presented many advantages to different reactions giving excellent conversions, enantiopure products, and very good yields. Also, the DES composed of urea made the cell membrane more permeable enabling the substrate to bind to the enzyme and to release the product. In addition, in 2020 we showed that this neoteric solvent (made of ChCl:glycerol- 1:2 mol/mol) could have an industrial importance because when that solvent was used with interesting halogenated ketones as substrates (at different substrate concentrations), we obtained valuable halohydrins. Moreover, successful scale up reactions were performed using this DES (Ibn Majdoub Hassani et al., 2020).

In the research studies performed in Morocco, we cultured GK1 and we grew it in a basic medium with acetate and cholesterol and we measured its activity and protein quantity at each step of the experiment (by conducting several assays) (Table 15). At the end of the chox induction, we obtained steroids which were then analyzed by TLC and HPLC.

After inducing the SADH in GK1, we tried to purify it and to extract it by centrifugation and by treatment with ammonium sulphate fractionation. Then we purified that enzyme using a

Sepharose CL-6B column. Later, we analyzed the fractions that contained an enzymatic activity. At the end, we concluded that there was a protein quantity and a weak enzymatic activity. Overall, we characterized the chox and the SADH by constructing recovery tables to see the obtained enzymatic activities. As a perspective work in this domain, it would be very important to repeat those experiments according to the protocols described by Kreit et al., 1992b; Kreit and Elalami 2002; and Elalami 2004, but this time, it would be advised to use a DES composed of choline chloride and glycerol with these types of reactions.

**7. General conclusions**

During this first part of the thesis, a green solvent type entitled deep eutectic solvent, was used with alcohol dehydrogenases and with halogenated ketones as substrates. This solvent was composed of choline chloride and glycerol (1:2 mol/mol). In our experiments, we tested the effect of the cofactor addition and the substrate concentration on the oxidoreductase-mediated reactions containing this neoteric solvent. Due to the promising results that were obtained with this solvent, we used it with different ADHs to reduce halogenated ketones at different concentrations. In general, the chlorinated substrates and ethyl 4,4,4-trifluoro-3-oxobutanoate were efficiently bioreduced using this DES by three different alcohol dehydrogenases, namely ADH-A from *Rhodococcus ruber*, ADH-T from *Thermoanaerobacter* sp., and LBADH from *Lactobacillus brevis*. Due to the outstanding results that two of these enzymes demonstrated, we continued working mostly with ADH-A and LBADH. The DES also improved the conversion while keeping the excellent value of enantiomeric excess yields for most of the biotransformations. In most cases, the bioconversion was excellent up to 400 mM or 500 mM. Also, larger scale reactions using these substrates at a quantity of 250 mg and 1 g were carried out.

In general all the results discussed above showed how the DES at 20% v/v can remarkably

improve ADH-mediated reactions with halogenated ketones as substrates at various concentrations using overexpressed ADHs from different bacterial strains in lyophilized cells of *E. coli*.

For all the halogenated substrates that were used in the presence of DES, the ADH-A and LBADH enzymes demonstrated a successful bioreduction at specific substrate concentrations, giving good conversion and *ee* results. This is because DES protects the enzyme's conformation and enhances the solubility of the substrates in these types of reactions. However, TeSADH and ADH-T gave the worst conversion results with DES.

Moreover, two different proportions of DES were studied (20% and 50% v/v), and the best one was usually 20% v/v. All these results might be due to the fact that DES enhances the substrate solubility in the reaction media, allowing a better transport to the enzyme's active site in order to bind and leading to the corresponding alcohol. At a higher proportion of DES the viscosity of the reaction medium increases and maybe this can cause mass transfer limitations.

In addition to that, other reactions were performed with these halogenated ketones but without cofactor to see if the bioreduction occurred successfully in the presence of DES. Overall, excellent conversions and enantioselectivities were obtained for ADH-A catalyzed reactions. Based on these promising results, we performed scale-up reactions involving high substrate concentrations to see if the bioreduction occurred successfully when DES was used at 20% v/v. The substrates that gave the best conversions and *ee* were 2-chloroacetophenone and ethyl 4-chloroacetoacetate. Also, the halogenated alcohols isolated by simple extraction protocols were confirmed by GC and NMR. Overall, these results have confirmed that DESs are appropriate solvents for ADH-mediated systems as they can enhance their ability to reduce halogenated ketones to valuable halohydrins. Summarizing, these solvents can be used

in different reaction conditions and they have the potential to be applied at an industrial scale to make precursors of important drugs. Because the DES has many advantages in the chemical industry, it could even be used as a chemical catalyst. A research team led by Ünlü et al. 2018 have written a mini review about the use of DES as catalysts (Ünlü et al., 2018). In this review, the team discussed that DES can be considered as catalysts depending on their nature. Some of these DES exhibited Lewis acidity and sometimes Brönsted acidity. In addition, DES had an impact on different types of reactions like: esterification, glycolysis, and depolymerization. This confirmed that DES should not be only considered as excellent solvents but also as good catalysts (Ünlü et al., 2018).

Moreover, according to Hooshmand et al. 2020, the DES's characteristics and physicochemical properties enabled them to be used in different biotechnological fields. That study also highlighted their successful use in cross-coupling reactions (using different proportions of their components). In addition, that review article gives a lot of research opportunities for scientists who could use these neoteric solvents with other types of cross-coupling reactions, and it makes it easier for researchers because it showed the results and drawbacks that were obtained from using DES with cross-coupling reactions (Hooshmand et al., 2020).

Based on the previous studies, which used DES with different enzymes, researchers noticed that there were several drawbacks that DES showed with some enzymes, for this reason, it would be interesting as a perspective work to come up with new designed solvents that could solve the issues that were recorded.

The induction and synthesis of cholesterol oxidase is via the addition of steroids that have a lateral chain like cholesterol (8 carbon atoms), beta-sitosterol (10 carbon atoms), or cholic acid (5 carbon atoms, Ibn Majdoub Hassani et al., 2014). The induction mechanism is started

when the steroid has a lateral chain of at least 5 carbon atoms. 4-Cholesten-3-one is produced by the reaction that is catalyzed by cholesterol oxidase. In addition, octanoic acid, hexanoic acid, and Tween 80 stimulated the enzyme synthesis. The induction effect that fatty acids have on this enzyme could be explained by the fact that they possess a lateral chain like the one in steroids.

In a previous study done by Elalami et al. in 2004, it was concluded that three forms of cholesterol oxidase are produced by the GK1 strain. The carbon source that is utilized for the microbial growth influence the level of the cholesterol oxidase production; the cellular form of the enzyme is obtained when the cells are cultivated on sterols. However, when the bacterial cells are cultivated in a medium that do not contain sterols as carbon source, the enzyme in that case is obtained as secreted form. In general, the cholesterol oxidase from GK1 is similar to cholesterol oxidases that are present in species of *Brevibacterium*, *Mycobacterium* and *Rhodococcus*.

For the secondary alcohol dehydrogenase from *Rhodococcus* sp. GK1, it was purified and characterized. The major substrates that it can catabolize are secondary aliphatic alcohols that have between three and eight atoms of carbon. In order to activate the enzyme, a hydrophobic environment is required, and it depends on the NADH cofactor (Elalami 2004).

In this study, taxonomical characteristics of *Rhodococcus* sp. and the sterol catabolism by this genus were discussed. Moreover, we tried to identify further some species of *Rhodococcus*, which degrade sterols like cholesterol; and we attempted to explore the obtained catabolic derivatives. This catabolism consists of two catabolic pathways: the C-17 side chain cleavage and the steroid ring nucleus degradation; these two pathways are independent of each other. The first step in this sterol degradation is catalyzed by cholesterol oxidase to produce a carbonyl group in the steroid nucleus. When the sterol side chain is cleaved selectively, this

enabled us to obtain derivatives with a non-degraded steroid nucleus. This study also confirmed that the strains GK1, GK3, GK12 belong to the genus *Rhodococcus.*

Also, the gene of the 16S rRNA was sequenced for *Rhodococcus* sp. strain GK3. Some parts of the gene were aligned with others from the Gene Bank Data (NCBI), and this confirmed that GK3 was a strain of *Rhodococcus opacus.*

In addition, the GK1 strain of *Rhodococcus* sp. CIP 105335 was employed to selectively cleave the side chain of cholesterol. Cobalt or nickel ions were added to the cultures for this objective. These ions tend to protect the steroid nucleus from opening by a competitive inhibition of 9α-hydroxylase, an enzyme that degrades the steroid nucleus. Three main derivatives were seen on TLC plates and HPLC. These derivatives could be two 3-ketosteroids like 4-androsten-3,17-dione (AD) and 1,4-androstadien-3,17-dione (ADD). Rhodococcal cells have similar cholesterol oxidase induction mechanisms which require a lateral chain, and the sterol structure 3β-ol-5-ene.

It is important to mention that some technical difficulties (such as contamination, and technical difficulties with the sonicator) did not permit us to perform the following experiments such as generalizing the induction study of the alcohol dehydrogenase and cholesterol oxidase (Table 16 and Table 17), which are produced by three species of the genus *Rhodococcus*: *R. erythropolis* (strain GK12), *R. opacus* (strain GK3) and *R. equi* (strain GK1). For this reason, and as a continuation of these experiments, it will be interesting as a perspective work to:

-To purify the oxidoreductase enzymes using chromatographic techniques (purifying SADH from GK1).

-To perform PAGE for cholesterol oxidase and SADH from the genus *Rhodococcus.*

In the future, it will also be very interesting to try different types of DES on the performance of the oxidoreductases enzymes in GK1, GK3, GK12; and to use different DES with new chemical and biochemical reactions.

Our studies about the use of these neoteric solvents is considered to be among the first experiments that use DES to reduce halogenated ketones utilizing overexpressed ADHs to make valuable enantiopure halohydrins. In addition, our findings on DES were beneficial to several other researchers. In this section, an example from other experiments is provided.

In a study conducted by Huang et al. in 2019, they have assessed the behavior of ADHs in DES-water mixtures comparing it to theoretical and classical findings. They have used molecular dynamics (MD) in their studies to know better the relation between enzymes and their media. They have used a HLADH and a DES made of choline chloride and glycerol (glyceline). In addition, they have used MD simulations to analyze quantitively the protein flexibility and the level of hydration. The team also investigated the enzyme activity and stability. They were able to evaluate the HLADH activity studying the initial rate of the reaction and the product formation. HLADH was used in mixtures containing (glyceline/water) and in this case, the water content ranged from 0 to 20 % v/v; and a pure water system was the control. To evaluate the performance of the enzyme, the team used water activity ($a_W$) values and the viscosity of the mixtures containing DES/water. They also shed light on the hydration layer of the enzyme, and its intraprotein hydrogen bonds in different mixtures of the solvent.

This study proved that for the activity of HLADH a value of 0.2 corresponding to the $a_W$, was necessary. Moreover, this type of enzyme did not work well with DES solutions containing a low amount of water. They confirmed that low water contents (<10% v/v in these types of systems) was not enough to hydrate the enzyme, because there was a strong attraction between water and the DES. Finally, to make HLADH more flexible, an increased

water amount was needed (≥10% v/v) (Huang et al., 2019). Finally, all these findings about oxidoreductase enzymes and their appropriate solvents could be developed in the future by using new techniques in order to bring a beneficial effect on different biotechnological fields.

## 8. General references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## *Résumé*

Une partie de cette thèse concerne la caractérisation et la purification d'une alcool déshydrogénase secondaire et d'une cholestérol oxydase à partir de cellules bactériennes du genre *Rhodococcus*. Les souches utilisées étaient GK1 : *Rhodococcus equi*, GK3 : *Rhodococcus opacus*, GK12 : *Rhodococcus erythropolis*. Elles ont été utilisées pour évaluer leur capacité à cataboliser les stérols. Les étapes du catabolisme des stérols comprennent le catabolisme de la chaîne latérale et la dégradation du noyau stéroïdien. Afin d'obtenir des dérivés intéressants, des ions de cobalt et de nickel ont été ajoutés au milieu réactionnel pour inhiber la dégradation du noyau stéroïdien et obtenir des dérivés tels que la 4-androstène-3,17-dione (AD) et le 1,4-androstadiène-3,17-dione (ADD). La deuxième partie de la thèse concerne l'utilisation d'un solvant approfondi (DES) contenant des enzymes ADH de différentes bactéries, du genre *Rhodococcus* et *Ralstonia*, et les enzymes ADH de ces souches bactériennes ont été surexprimées dans les cellules d'*Escherichia coli* (*E. coli*). Lorsque 300 à 400 mM de céto-substrats halogénés ont été utilisés dans les réactions avec DES, d'excellents résultats de conversion et d'excès énantiomérique ont été obtenus. Les meilleurs résultats ont été observés lorsque des ADHs de *Lactobacillus brevis* et de *Rhodococcus ruber* ont été utilisés. Enfin, des réactions en grandes quantités ont été réalisées donnant de bons résultats.

**Mots-clefs :** ADH, GK1 (*Rhodococcus*), DES, cholesterol oxidase, bioréduction

## *Abstract*

A part of this thesis is about the characterization and purification of a secondary alcohol dehydrogenase and a cholesterol oxidase from wild type bacterial cells of the genus *Rhodococcus*. The strains that were used from this genus were GK1 : *Rhodococcus equi*, GK3 : *Rhodococcus opacus*, GK12 : *Rhodococcus erythropolis*. They were also utilized to see their ability to catabolize sterols. The steps that are involved in the sterol catabolism include the lateral chain catabolism and the steroid nucleus degradation. In order to obtain valuable derivatives, cobalt and nickel ions were added to the reaction medium to inhibit the degradation of the steroid nucleus and to obtain derivatives such as 4-androstene-3,17-dione (AD), and 1,4-androstadiene-3,17-dione (ADD). The other part of the thesis is about using a Deep Eutectic Solvent (DES) with ADH enzymes from different bacteria ranging from the *Rhodococcus* genus to *Ralstonia*, and the ADH enzymes of those bacterial strains were overexpressed in *Escherichia coli* (*E. coli*) cells. When 300-400 mM of the halogenated keto-substrates were used in the reactions with this novel solvent, excellent conversions and enantiomeric excess results were obtained. The best results were observed when ADHs from *Lactobacillus brevis* and *Rhodococcus ruber* were used. Finally, scale up reactions were performed giving good product yields.

**Key Words :** ADH, GK1 (*Rhodococcus*), DES, cholesterol oxidase, bioreduction