Multienzymatic processes involving Baeyer-Villiger monoxygenases

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Abstract: Baeyer-Villiger monoxygenases (BVMOs) are flavin-dependant oxidative enzymes capable to catalyse the insertion of an oxygen atom between a carbonylic Csp2 and the Csp3 at the alpha position, therefore transforming linear and cyclic ketones into esters and lactones. These enzymes are dependent on nicotinamides (NAD(P)H) for the flavin reduction and subsequent reaction with molecular oxygen to furnish peroxyflavin, the ultimate responsible for the substrate oxidation. BVMOs can be included in cascade reactions, coupled to other redox enzymes such as alcohol dehydrogenases (ADHs) or ene-reductases (EREDs), so that the direct conversion of alcohols or α,β-unsaturated carbonylic compounds to the corresponding esters can be achieved. This way, it is possible to develop smart synthetic strategies with a convenient cofactor recycling, both using whole cells (native or genetically engineered) as well as isolated enzymes, via multi-steps reaction through sequential or parallel methodologies. Some examples will be commented dealing with these biotransformations, highlighting the advantages of the coupling of enzymatic steps.

Keywords: biocatalysis; whole cells; cascade reactions; redox enzymes; monoxygenases; Baeyer-Villiger alcohol dehydrogenases; ene-reductases.

1. Introduction

The elucidation and rational understanding of the internal organization of the different biocatalytic reactions occurring inside biological cells, in which several enzymatic reactions proceed in a concatenated manner, is one of the basis of Systems Biology [1]. The development of this discipline has fostered the design of coupled systems of biocatalytic reactions, and the number of publications dealing with this topic has increased considerably during the last years [2-11]. In this area, oxidoreductases are enzymes frequently used in cascade reactions, as usually a pair of these enzymes are simultaneously applied for the required in situ recycling of the cofactors [12-15]. Inside this type of enzymes, Baeyer-Villiger monoxygenases (BVMOs) are undoubtedly one of the most attractive members of this family; these flavin-dependant oxidative enzymes [16-20] are capable to catalyse the insertion of an oxygen atom between a carbonylic Csp2 and the Csp3 at the alpha position, therefore transforming linear and cyclic ketones into esters and lactones [20-27], as schematized in Scheme 1.

![Scheme 1: Baeyer-Villiger monoxygenase reaction](image-url)
BVMOs require nicotinamides (NADPH) for the flavin reduction to FADH₂, which reacts with molecular oxygen to form the reactive peroxyflavin responsible for the substrate oxidation. As NADPH is an expensive compound and its presence at high concentrations can inhibit the biocatalyst, it has to be recycled to obtain feasible biocatalytic procedures. Most of the BVMOs can be included in the type B subclass of flavin dependant monooxigenases [28], according to their protein sequence motifs, electron donor and type of oxygenation reaction. Thus, they are single-component enzymes possessing two α/β Rossmann-like domains for the FAD and NAD(P)H binding, respectively, and they keep this last one bound during catalysis, while the substrate binds after the flavin-peroxide is formed [17]. Baeyer-Villiger monooxygenases were first discovered in the 60s of the last century, but until thirty years later, they have not been widely applied with biocatalytic purposes for the preparation of high valuable compounds as esters, lactones and sulfoxides, among others. Advances in genome mining have allowed to discover several BVMOs active on different types of compounds, but still nowadays, cyclohexanone monooxygenase (CHMO) from Acinetobacter calcoaceticus NCIMB 9871, discovered more than 40 years ago [29], is the BVMO with the highest applicability due to its large substrate profile and excellent selectivity. Nevertheless, CHMO presents some drawbacks as its thermal instability and its low stability to organic cosolvents [30]. Remarkably, most BVMOs display an excellent behaviour when employed in multi-step cascade reactions coupled to other enzymes. Thus, in this review we will comment different reported cases in which BVMOs have been applied to this purpose. For practical reasons, we have classified the reported examples in two main categories, cascades including BVMOs catalysed by whole cells or by isolated enzymes.

2. Muti-step reactions including BVMO activity catalysed by whole cells.

Generally speaking, the use of whole cells makes the cascade easier compared to those employing isolated biocatalysts, as the recycling of the required cofactors is produced inside the cell metabolic machinery, so that it is not mandatory to implement an external recycling methodology. The most attractive methodology using whole cells implies the use of a la carte engineered cells, inside which different enzymatic activities are overexpressed in order to generate the desired multi-step procedure [31]. This methodology, which has been sometimes termed as Systems Biocatalysis [32], presents several advantages [31,33-36]: i) as already mentioned, the intra-cellular medium provides the natural enzymatic environment and the cofactors regeneration machinery; ii) it is relatively easy and economical to have available cells by cultivation without any additional downstream process; iii) enzymes inside cell walls and membranes are somehow protected from extreme reaction conditions, and iv) the different enzymes involved in the cascade are co-localized inside cells, so that their local concentration is increased therefore reducing the diffusion of intermediates.

2.1. Muti-step reactions including ADH/BVMO activity catalysed by whole cells.

Most of the examples of biocatalytic cascades employing BVMOs have been developed together with alcohol dehydrogenases (ADHs, EC 1.1.1.x), also called ketoreductases (KREDS). These enzymes are able to catalyse the reversible reduction of carbonyl compounds into the corresponding alcohols (Scheme 2), in general with high selectivity [37-40]. ADHs require the presence of nicotinamide cofactors for performing their activity, being required to employ effective regeneration systems if these enzymes are employed as isolated biocatalysts.
Scheme 2. Alcohol dehydrogenases catalysed reversible reduction of carbonyl compounds and oxidation of alcohols.

Initial studies on linear cascades combining ADHs and BVMOs were performed at the beginning of the 90s, using whole cells systems for the direct biooxidation of alcohols to the corresponding lactones. Thus, the starting alcohol was oxidized to the corresponding ketones by the ADH at the expense of NAD(P)$^+$, whereas the obtained ketone was further oxidized in presence of the BVMO to the desired lactone, employing NAD(P)H which was converted again into NAD(P)$^+$. A pioneer example is the oxidation of alcohol 1, shown in Scheme 3, employing fractured cells of *Acinetobacter calcoaceticus* NCIMB to the corresponding lactone 3, described in 1991 [41]. At short reaction times, 1 was converted into ketone 2 in a great extent (80% after 6 hours). Longer reaction time led to an increase in the lactone production, reaching a maximum value of 40% after 48 hours, whereas concentration of 2 started to decrease after 10 hours. A similar pattern was observed in the biooxidation of both *endo-* and *exo-*bicyclo[2.2.1]heptan-2-ols. Alcohol concentration decreased as the ketone became formed and lactone concentration started to increase during the course of the biocatalytic process. Fractured cells of *A. calcoaceticus* were also employed in the biooxidation of 6-*endo-*bicyclo[3.2.0]hept-2-en-6-ol 4 to obtain regioisomeric lactones 5 and 6 with complete conversion after 1 hour, as shown in Scheme 3. A 15% of a by-product, which authors assumed that was *exo-*4, was observed in the crude mixture.

Scheme 3. *Acinetobacter calcoaceticus* cells catalyzed biotransformations of alcohols into lactones.

The combination of BVMOs and ADHs has been further developed for the preparation of *ε*-caprolactone (ECL, 9, Scheme 4). This compound is a valuable material with several applications in the field of colorants, adhesives and coating materials, as ECL can be easily polymerized yielding biodegradable thermoplastics and elastomeric polymers [42,43]. By these reasons, several approaches have been studied for its preparation under sustainable reaction conditions. BVMOs and ADHs have been employed as whole cells or as isolated enzymes for the synthesis of ECL starting from cyclohexanol 7, in a process in which this alcohol is oxidized by the ADH to cyclohexanone 8, which is converted into ECL in presence of the BVMO, with an *in situ* NAD(P)H cofactor regeneration. When employing this approach, the stability of the BVMO is critical for the process, as this biocatalyst is very sensitive to the cyclohexanol and ECL concentration in the reaction medium. By this reason, several attempts have made in order to overcome this bottleneck.
Thus, the bienzymatic system employing whole cells of ADH-BVMO for the preparation of ECL can be coupled with the lipase-catalysed hydrolysis of this compound in order to obtain oligo-caprolactone. This approach was performed for the first time in 2015 [44]. In order to overcome the BVMO deactivation caused by the presence of ECL in the reaction medium, this product subjected to \textit{in situ} ring-opening oligomerization catalysed by CALA (lipase from \textit{Candida antarctica}, Scheme 4). The formed oligo-ECLs were easily removed from the reaction by extraction or precipitation. CALA is able to catalyse the formation of the polymers even in presence of high amounts of water, not showing any hydrolytic activity on ECL at high substrate concentrations (1.0 M). After a few hours in presence of CALA, the lactone was converted into oligomers with a maximum molecular weight of 1200 g/mol, which can be transformed into high-molecular weight polymer. Recombinant \textit{E. coli} cells of \textit{Lactobacillus brevis} ADH (LbADH) and the stable mutant C376L/M400I of \textit{Acinetobacter calcoaceticus} cyclohexanone monoxygenase (AcCHMO) were combined with lipase CALA in a one-pot process at different concentrations of cyclohexanol. A decrease in the ECL concentration in these reactions was detected when compared to those in absence of the lipase, thus indicating the beneficiary effect of the conversion of the ECL into oligo-ECL. When both the ADH and the BVO were employed as separate cells, results were better than expressing both biocatalysts into the same cell-system. LbADH/AcCHMO ratio was optimized 1:10 in order to achieve the highest conversion. Addition of acetone and glucose as co-substrates was also positive for a faster regeneration of the endogenous NADPH in the cell systems. The optimized cascade was performed at preparative scale and, after 48 hours, a complete conversion was achieved when starting from 200 mM of 7, yielding a 75% of oligo-ECL and a 25% of ECL. Higher substrate concentrations, 300 mM and 500 mM, afforded lower conversions, 74% and 43% respectively.

In a further development of this trienzymatic system, the same authors described the preparation of methyl-substituted ECL derivatives in a one-pot two-step process [45], as shown in Scheme 5.

A diastereomeric mixture of 4-methylcyclohexanol (10) was employed as starting substrate; under those conditions, AcCHMO was able to convert 4-methylcyclohexanone 11 into the enantiopure lactone (S)-12 with high yield and complete selectivity. Initial experiments were performed with \textit{E. coli} cells expressing \textit{Lactobacillus kefir} ADH (LkADH)
and wild type AcCHMO in 1:10 ratio, but the conversions at all concentrations tested (5-
20 mM) were low, achieving only a 34% of the enantiopure lactone as highest value when
working at 5 mM. Poor conversion values were caused by the low conversion in the ADH
catalysed oxidation, due to the inhibition of this biocatalyst by the ketone 11 formed. In
order to improve this process, a faster second oxidation process, with the aim of the fast
removing of 11 from the reaction medium was envisioned. Thus, a more stable mutant of
AcCHMO was employed (C376L/M400I/T415C/A463C, the so-called AcCHMO QM) and
the ratio of the E. coli cells for both biocatalysts was optimized to 1:1. The supply of pure
oxygen to the reaction also resulted in a higher conversion, close to 90% after 24 hours at
25°C. The addition of CALA to the enzymatic system led to a further decrease in (S)-12
concentration, as it was being hydrolysed into the oligo-(S)-lactone.

The production of ECL starting from cyclohexanol employing E. coli cells of ADH
and AcCHMO has been studied using a computational approach in 2017 [46], applying a
kinetic model for the study this cascade reaction in both batch and fed-batch synthesis. To
this purpose, a fed-batch synthesis was developed in order to circumvent the CHMO in-
hibition caused by cyclohexanol, which hampered the use of this substrate at concentra-
tions higher than 60 mM when employed in batch. Thus, 7 was added to the reaction me-
dium at different ratios from 14.2 to 19.0 μmol/min; feed rates higher than 17.5 μmol/min
led to accumulation of 7, therefore reducing the rate formation of ECL due to CHMO in-
hibition. The amount of ECL in the reaction medium was reduced by hydrolysing this
compound to 6-hydroxyhexanoic acid (13, Scheme 6) in a process catalysed by Candida
antarctica lipase B (CALB). After 6 hours, 162 mM of 13 was obtained with a feed rate of
19 μmol/min. The addition of CALB maintained a low ECL concentration in the reaction
medium (lower than 10 mM).

Scheme 6. Preparation of ECL 9 and its oligomers through a biocatalytic procedure employing
whole cells of Lactobacillus brevis ADH and Acinetobacter calcoaceticus CHMO M15.

The production of ECL has been optimized by performing the co-expression of the
BVMO and the ADH in E. coli employing a Duet™ vector [47], in order to obtain a higher
efficiency for the enzymatic cascade. The pRSFDuet plasmid form Novagen was selected
as dual expression vector, cloning the AcCHMO QM gene into the first multiple cloning
site and the LkADH gene into the second multiple cloning site. With the aim of balancing
the expression levels of the cascade enzymes, the ADH gene was subjected to engineering
of the ribosome binding site (RBS), employing the native RBS sequence as well as two
point mutations. In order to compare the results, both type of enzymes were expressed
separately into E. coli cells, performing the biotransformations with an optimized ratio of
CHMO:ADH cells 5:1 and in presence of glucose and acetone and co-substrates, as higher
conversions were obtained than in absence of these compounds. This result can be ex-
plained by the difficult diffusion of the NADPH formed in the E. coli cells of the LkADH
and the AcCHMO QM cells. After 16 hours, it was possible to achieve a 90% conversion
from 20 mM of cyclohexanol employing wild type RBS, while much lower conversions
were measured with the other two RBS mutations, indicating that a decreased LkADH
expression is required to obtain a similar enzyme ratio for optimal conversion. When the
same process was carried out with the E. coli cells containing the double gene, a complete
conversion was obtained after 16 hours for all the three RBS preparations in the absence
of co-substrates. In presence of glucose and acetone, complete conversion was also ob-
served for the CHMO co-expressed with one of the RBS mutations of the ADH after 16
hours, whereas after 2 hours, a 54% conversion was reached. This system was the most efficient for the co-expression of both biocatalysts, suggesting that the ratios of expressed AcCHMO QM and LkADH were similar in this system. When higher substrate concentrations were employed, lower conversion values were achieved, mainly debt to the inhibitory effect of both 7 and 9 on AcCHMO.

Very recently, the conversion of cyclohexanol to ECL by combining LkADH with two stable mutants of CHMO has been studied in order to obtain a suitable process at 200 mM scale. Different parameters that affect the process were analysed and optimized in order to achieve the most productive process for the preparation of the final lactone [48]. The reaction was carried out in a stirred-tank reactor with maintained temperature and pH, bubbling a mixture of oxygen with synthetic air and pumping cyclohexanol at a constant rate proportional to the whole cell cascade activity. As both 7 and 8 present a high vapor pressure, they can be stripped out of the system by the off gas. The key component of the complete system, as previously stated, is CHMO, due to its low stability and the requirement of a proper oxygen supply. This last parameter was optimized by performing a bubble aeration technique. BVMO stability was improved by assessing two CHMO mutants; thus, AcCHMO QM showed a 40% higher long-term stability, whereas AcCHMO M15, containing eight mutations, has a higher oxidative stability combined with an improved thermostability. These two variants were compared employing E. coli cells co-expressing ADH and the CHMO at pH 7.5 and 30°C, with 200 mM cyclohexanol fed at 4.5 mM/h. When using AcCHMO QM, the formation of ECL achieved a value of 45 mM after 13 hours, but at longer reaction times this concentration decreased to 39 mM, probably debt to the ECL autohydrolysis to yield 13. The same experiment was performed with AcCHMO M15, reaching a maximum concentration of ECL after 18 hours (79 mM). Longer reactions times resulted in substrate accumulation in the reactor with no further formation of the desired product. AcCHMO M15 showed longer activity, but it was not possible to achieve complete conversion. The stability of both CHMO mutants under the process conditions was analysed; when employing AcCHMO QM, the biocatalyst retained its complete activity for one hour and it decreased to 85% after 8 hours. After this reaction time, there was an important drop in the biocatalyst activity. On the other hand, AcCHMO M15 maintained its activity (98% of the initial one) even after 18 hours. Thus, when working with AcCHMO QM, fresh cells were added after 8 hours, whereas no extra addition was required for AcCHMO M15. Further experiments showed that an increase in the cells loading in the reactor led to a decrease in the volumetric mass transfer, thus resulting in a lower efficiency. The complete optimization of the biocatalytic process allowed to achieve conversions higher than 98% for both CHMO mutants, being possible to obtain a higher final product concentration (21.1 g/L), space time yield (1.1 g/ L h) and isolated ECL amount (9.1 g) employing AcCHMO M15, due to its higher operational stability. These values represent a 100% increase regarding the non-optimized processes, indicating the development of a more efficient process for the preparation of ECL.

Fusion proteins have been initially employed to enhance the soluble expression of proteins or to improve the enzyme purification, but in the last years some examples have appeared in which these systems have been used with synthetic purposes [49]. In 2015, the preparation of an enzyme fusion formed by the ADH from Micrococcus luteus NCTC2665 and the BVMO from Pseudomonas putida KT2440, able to catalyse the double oxidation of long-chain unsaturated secondary alcohols (14a-c) to the corresponding esters (16a-c) through the corresponding ketones 15a-c, was reported [50], as shown in Scheme 7. These products can be hydrolysed into valuable ω-hydroxycarboxylic acids and n-alkanoic acids, valuable compounds. The design of the fusion enzyme showed the highest expression level when using a glycine-rich linker, formed by 12 aminoacids, between the two biocatalysts. When the biooxidations were carried out with the fused biocatalyst expressed in E. coli, higher conversions were achieved for all the substrates when compared with the independent ADH and BVMO expressed in E. coli. Thus, esters 16a-c can be recovered with conversions higher than 75%, with activities higher than 22 µmol/ g dry cells min. This positive effect of the fusion enzyme in the cascade activity can be debt to a
higher functional expression of the BVMO and/or to a better mass transport efficiency, due to the presence of both active centres at close positions.

Scheme 7. Preparation of ECL 9 and its oligomers through a biocatalytic procedure employing whole cells of Lactobacillus brevis ADH and Acinetobacter calcoaceticus CHMO M15.

2.2. Multi-step reactions including ERED/BVMO activity catalysed by whole cells.

Ene-reductases (EREDs) from the old yellow enzyme (OYE) family are flavin-dependent enzymes that catalyse the chemo- and stereo-selective asymmetric reduction of electronically activated carbon–carbon double bonds [25,27,51-53], as depicted in Scheme 8.

Scheme 8. Ene-reductases for the reduction of C=C.

Several examples can be found in literature illustrating the use of whole cells-catalysed cascades coupling BVMO and ERED activities. For instance, Silva et al. [54] reported the preparation of ECL 9 starting from either cyclohexenone 17, cyclohexanone 8 or cyclohexanol 7 using whole cells of Brazilian Geotrichum candidum CCT 1205 (Scheme 9).

When using cyclohexanone 8 as substrate, these authors reported quantitative yields of the desired ε-caprolactone 9 after only 5 hours, using a higher amount (3 g) of whole cells (otherwise, the cascade accumulated 7 and 8). Using 7 or 8 as substrates, the reaction was even faster (4 hours) using only 10 g of cells. These results are better than those previously reported by Mihovilovic et al. [55], leading to 52% of 9 in 48 h using BVMO expressed in E.coli. Recently, Silva et al. [56] have shown that it is possible to immobilize the whole cells from Geotrichum candidum CCT 1205 in modified silica (n SiO–Cl, SiO–NH2 and SiO–SH supports), without altering their catalytic performance and also allowing the stabilization and reuse of the cells.

Although the previous example illustrates the use of non-engineered cells, it is becoming more usual the use of genetically modified cells. Hence, Liu and Li described an enantioselective reduction–oxidation–hydrolysis cascade catalysed by engineered whole cells for the synthesis of (R)-2-alkyl-β-lactones 22–23 (useful flavour and fragrance materials) starting from the corresponding 2-alkylidene cyclopentanones 18–19 (Scheme 10)[57].

Scheme 10. Preparation of enantiopure (R)-2-alkyl-β-lactones by using a whole cell-catalysed cascade involving BVMO activity.

In this example, the cascade catalysis was started with 50 mL of cell suspension of Acinetobacter sp. RS1 in Tris buffer (12 g cdw/L) containing 40 mg of alkylidene ketones 18 or 19 and 20 mg/mL glucose. After 3 hours of reaction to allow the ERED-mediated accumulation of alkyl ketones 20 and 21 (R-configuration), 150 mL of Tris buffer containing...
engineered cells of *E. coli* (CHMO-GDH) (10 g cdw/L) was added to start the Baeyer-Villiger oxidation. After simultaneous oxidation and hydrolysis for 1.5 hour, extraction with ethyl acetate and purification by flash chromatography, compounds (R)-22 (56% yield, 98% ee) or (R)-23 (41% yield, 97% ee) could be obtained. The presence of a hydrolytic activity inside *Acinetobacter* sp. RS1 cells was responsible for the hydrolysis of the intermediate (S)-22 or (S)-23 leading to δ-hydroxyacids 24 or 25, obtained with low enantioselectivity (E = 8-11).

The different enantiomers of carvolactones (7-methyl-4-(prop-1-en-2-yl)oxepan-2-ones) are gaining importance as monomers for the preparation of polymeric thermoplastic elastomers (shape-memory polymers) and pressure-sensitive adhesive components [58-60]. These carvolactones can be obtained starting from biogenic carvones after a C=C reduction and a Baeyer-Villiger oxidation. To this purpose, Iqbal et al. [61] have reported the concurrent redox cascade to transform (-) carvone and (+)-carvone [(R) and (S)-26] into the correspondent lactones, as depicted in Scheme 11.

![Scheme 11. Preparation of carvolactone stereoisomers 28 and 29 by a concurrent redox cascade.](image)

Crude cell extracts from *E. coli* BL21 (DE3) containing the corresponding EREDs or BVMOs were used. Initially, each biotransformation step was carried out independently, starting with bioreductions of enantiomerically pure carvones (R) or (S)-26 and subsequent biooxidation with different BVMOs to furnish different stereoisomers of carvolactones 28 and 29. Finally, the reduction-oxidation steps were performed in a concurrent one-pot cascade. Both for the individual steps and the cascade, NADP⁺, glucose -6-phosphate and glucose -6-phosphate dehydrogenase were used for cofactor recycling. The EREDs expressed *E. coli* BL21 (DE3) were: SYE-4 (from *Shewanella oneidensis* [62]), OPRI and OPRI3 (12-oxophytodienoate reductase from *Lycopersicon esculentum*, tomato [63]), YqIM (from *Bacillus subtilis* [64]) and the variant W116I (replacement of tryptophan-116 by isoleucine in the OYE from *Saccharomyces pastorianus* [65]). On the other hand, the BVMOs were either cyclohexanone monoxygenases (CHMOs: AcCHMO [66], CHMO_{Brevi1} from *Brevibacterium sp.* [67], or cyclopentanone monoxygenases (CPMOs-type enzymes: CHMO_{Brevi2} from *Brevibacterium sp.* [67] and CPMO_{Coma} from *Comamonas sp.* [68]). Regarding the reaction stereochemistry of the bioreduction, as depicted in Scheme 5,
when starting from (−)-carvone (R)-26 all the EREDs catalysed the trans C=C bioreduction to afford (−)-trans-dihydrocarvone (2R,5R)-27. Opposite, if (−)-carvone (S)-26 was the initial substrate, most of the EREDs catalyzed the cis C=C bioreduction to afford (−)-cis-dihydrocarvone (2R,5S)-27, while the trans bioreduction, leading to (−)-trans-dihydrocarvone (2S,5S)-27, was observed only when employing W116l. Analysing the regioselectivity of the BVMOs, depending on the absolute configuration of 27, some of the enzymes were leading to the “normal” lactones 28 (classical migration of the Criegge intermediate leading to the oxygen insertion in the most substituted alpha carbon [69]) or the “abnormal” ones 29, in good yields and enantiopurity.

In another example, Oberleitner et al. [70] reported the preparation of carvolactone (4S,7R)-28 starting from (R)-limonene ((R)-30) directly extracted from orange peel by means of a biocatalytic cascade shown in Scheme 12.

Scheme 12. Preparation of carvolactone (4S,7R)-28 using a whole cells-catalyzed cascade involving a BVMO.

To this purpose, two different types of cells were used: i) an engineered *Pseudomonas putida* S12 expressing cumene dioxygenase (CumDO) and, ii) *E. coli* BL21 (DE3) cells possessing different oxidative activities. These last ones had been previously reported as capable to catalyse the conversion from carveol (1R,5S)-31 into (4S,7R)-28 [71], as they contain the expression of an alcohol dehydrogenase (RR-ADH from *Rhodococcus ruber*), an ERED (XenB from *Pseudomonas* sp.) and a BVMO (AcCHMO). Through the mixed culture approach, combining the two bacterial strains in one pot, 47% of (4S,7R)-28 was produced after 20 hours. By using a sequential approach, where hydroxylation of (R)-30 by CumDO was performed first, and *E. coli* BL21 (DE3) resting cells were added to the reaction vessel after 10 h, observing full conversion to (4S,7R)-28 in 20 hours. Then, these authors explored the direct use of waste product orange peel (biomass loading of about 3% (w/v)), obtaining 3.2 mg of (4S,7R)-28 per g orange peel. Anyhow, by lowering the orange peel amount to 1.5% (w/v) and using the mixed-culture sequential combination, it was possible to furnish 6.3 mg of carvolactone per g orange peel (29% of (4S,7R)-28 from (R)-30 over four biocatalytic steps, 73% per step), only relying on orange peel as the substrate reservoir in aqueous buffer without any additives.

Recently, Avalos et al. [72] have described a similar process, using engineered *E. coli* cells, already designed to follow the *Mentha spicata* route leading to (−)-carvone (R)-26, in which the addition of a ERED and a BVMO activity finally furnished (4R,7R)-28, also known as (−)-dihydrocarvide (−)-DHCD, as shown in Scheme 13.

Scheme 13. Preparation of (−)-DHCD using and engineered whole cells-catalyzed cascade.
The initial step into the *M. spicata* biosynthesis of (-)-carvone (R)-26 is the hydroxylation of (S)-limonene (S)-30 to furnish carveol (1S,5R)-31 catalysed by limonene-6-hydroxylase (L6H, a P450 enzyme) coupled to SmCPR, a cytochrome P450 reductase from *Salvia miltiorrhiza*. Based on this system, authors constructed the rest of the enzymatic system by adding genes from ADH (RR-ADH was the best option), ERED (PETNR, pentaerythritol tetranitrate reductase from *Enterobacter cloacae* PB2 [73,74]) and BVMO (CHMO3M, a triple mutant from *Rhodococcus* sp. Phi1 CHMO [75]). Under optimal conditions, the complete biosynthesis of (+)-DHCD from glucose in *E. coli* was reported at 6.6 mg/L.

3. Muti-step reactions including BVMO activity catalyzed by isolated enzymes.

3.1. Linear ADHs/BVMOs cascades.

The initial experiments in which multienzymatic processes involving ADHs and BVMOs were described [41] were also carried out using isolated enzymes, combining purified *Thermoanaerobium brockii* ADH (TBADH) with purified CHMO form *Acinetobacter* sp. NCIMB 9871 in presence of a catalytic amount of NADPH, which was recycled by both enzymes. As depicted in Scheme 14, oxidation of endo-32 afforded a regioisomeric mixture of lactones 33 and 34 with 79% conversion (67% yield) after 5.5 hours. Lactone 33 was the major product in the mixture, being recovered with low optical purity (>95% purity, 11% ee). Same conditions were applied to the biotransformation of endo-bicyclo[3.2.0]hept-2-en-6-ol 4 leading to a 41% of a mixture of lactones 5:6 in 2:1 ratio after 8.5 hours. (1S,5R)-5 was recovered with 86% ee. The same approach was performed three years later in the oxidation of racemic norbornenol 32 to the corresponding lactone 33 [76] as solely product. Starting alcohol was non-selectively oxidized to norbornanone by TBADH, which was oxidized to 33 in presence of *Acinetobacter calcoaceticus* NCIMB 9871 with a 67% global yield and 11% ee. Only 3.5 mol% of NADPH cofactor was required to ensure the complete conversion of the starting alcohol. The same authors described the formation of two regioisomeric lactones (5 and 6) in the biooxidation of alcohol endo-4 in presence of horse liver alcohol dehydrogenase (HLADH) and *Pseudomonas putida* monooxygenase MO1. HLADH catalyzed the non-selective oxidation of the alcohol to the ketone, which was selectively oxidized to a mixture of (3aR,6aS)-5 (58% yield, 79% ee) and (3aS,6aR)-6 (42% yield, 97% ee) by MO1 using in this system NADH as coenzyme.

![Scheme 14](image)

Scheme 14. Biocatalyzed cascades employing isolated ADHs and BVMOs for the synthesis of bicyclic lactones.

The preparation of ECL combining isolated ADHs and BVMOs have been also widely studied. One of the first examples used a chimeric polyol dehydrogenase (PDH), formed by introducing a substrate recognition loop from the *Rhodobacter sphaeroides* PDH into the thermostable PDH from *Deinococcus goethermalis* [77]. When the resulting PDH
was tested together with AcCHMO, conversions around 80% were obtained when working at cyclohexanol concentrations of 5-10 mM. As previously shown, higher substrate concentrations led to lower BVMO activities, resulting this enzyme critical for the development of a valuable procedure. When the process was carried out at preparative scale, a 55% yield of ECL with complete purity was obtained after 2 hours. Authors sustained that this low yield was caused by the volatility of cyclohexanol. In order to increase the BVMO stability, both CHMO and PDH were co-immobilized onto Relizyme™ HA403 using glutaraldehyde as linker. A CHMO:PDH ratio 10:1 led to the highest activity of the biocatalytic system, but still CHMO showed a low stability in this preparation. Only a 34% conversion was achieved after 5.5 hours. The immobilized biocatalyst was reused for several cycles, being measured a residual activity of 10% after the first cycle. This loss was debt to the instability of the CHMO, as the addition of fresh BVMO after each cycle reduced the loss in activity observed.

In 2013, Gröger et al. also described a linear cascade for the synthesis of ECL starting from cyclohexanol [78]. For the oxidation of cyclohexanol to cyclohexanone, a crude extract of LkADH was employed, whereas a commercial preparation of AcCHMO (Enzymicals AG) catalysed the ketone oxidation. When starting from 20 mM cyclohexanol, it was possible to obtain ECL with an excellent conversion (97%) after 24 hours. Increasing substrate concentration up to 60 mM also led to excellent conversion values, whereas higher values led to high drop for product obtained. Further studies on these results showed a significant inhibition on the BVMO at high ECL concentrations, as well as a negative impact on this enzyme of both cyclohexanol and cyclohexanone.

In 2017, the two redox enzymes involved in the biotransformation of cyclohexanol to ECL were fused into a self-sufficient bifunctional catalyst [79]. The BVMO that was used was the cyclohexanone monooxygenase form Thermocrispum municipale (TmCHMO) [80], whereas three NADPH-dependent alcohol dehydrogenases were tested: TBADH, ADH from Pyrococcus furiosus and the ADHMi from Mesotoga infera. Fusion catalysts were prepared in both orientations, with the TmCHMO either at the C- or at the N-terminus of the ADH. From the six fusion catalysts prepared, Tb-Tm, which contains TBADH at the N-terminus and TmCHMO at the C-terminus, showed the highest thermostability and was tested with synthetic purposes. When starting from 10 mM of cyclohexanol, a complete conversion was observed after 24 hours at 37ºC. The use of cyclohexanol concentrations higher than 100 mM did not afford any conversion, due to the inhibition of TmCHMO by this substrate. In order to overcome this drawback, the starting alcohol was pumped into the reaction medium, achieving a 64% conversion after 24 hours. Subsequently, with the aim of analysing the effect of ECL inhibition, CALA (Candida antarctica lipase A) was added in the reaction medium to hydrolyze the lactone product into oligo-caprolactone (see Scheme 4). In these conditions, conversion was increased up to 81%. During this reaction, a pH decrease was observed due to the hydrolytic process, and by this reason, authors employed a phosphate buffer pH 8.0 of high concentration. After all these modifications, conversion higher than 99% was obtained when starting from 200 mM of cyclohexanol, in a process with a turnover number (TON) of 13,333. Further experiments showed that TmCHMO was the slowest enzyme of the fusion protein, being the oxidation of the cyclohexanone to ECL the rate-limiting step. The fusion protein seemed to have a better performance than both individual biocatalysts. This result can be attributed to several factors, as the lower stability of the non-fused TmCHMO, higher activity of the BVMO or the localization of both enzymes at closer positions in the fused biocatalyst.

In order to optimize the preparation of ECL through the cascade involving ADHs and BVMOs, a solvent engineering study has been developed by carrying out the oxidation of cyclohexanol to ECL combining isolated Lactobacillus kefir ADH (LkADH) and AcCHMO [81]. Different hydrophobic organic solvents, including ethyl acetate, methyl tert-butyl ether, toluene, methylecyclohexane, n-heptane and isooctane were tested in this process at 10% v/v. With most of the solvents, lower conversions were obtained, but in presence of isooctane, complete conversion was achieved after 23 hours, the same result to the one obtained in buffer alone. The time course of the reaction when employing 40 mM of
starting material revealed that the process was faster in presence of 10% v/v of the organic cosolvent, as higher conversions were measured after 1, 2, 3 and 4 hours, a result that is maintained at higher substrate concentration (80 mM). The physical state of the BVMO was also tested in the reactions with 10% v/v of isooctane. When employing cell free extracts of AcChMO, a slightly lower reaction course was obtained when compared with the purified enzyme, whereas the use of the whole cells of the BVMO only led to a 7% conversion after 23 hours.

The preparation of lactones through a multicatalytic process in which a BVMO is involved has been developed starting from cycloalkanes [82]. In a three-step procedure, shown in Scheme 15, the alkane was oxidized to the cyclic alcohol in a reaction catalysed by a cytochrome P450 monoxygenase (CYP450). These biocatalysts are able to perform a wide set of oxyfunctionalizations, in general with high regio- and enantioselectivity, which makes them useful enzymes in organic synthesis [83,84]. In the third step, the alcohol led to the corresponding lactone by described bienzymatic system ADH-BVMO. As this process is not redox balanced, the biocatalysts were employed as cell-free extracts, which contains endogenous NAD(P)H required for cofactor regeneration, together with glycerol and glucose and co-substrates.

Scheme 15. Enzymatic preparation of lactones in a three-step biocatalytic cascade combining a CYP450, an ADH and a BVMO.

Initial experiments were carried out with cycloheptane 35 at a high concentration (165 mM), in order to have a second phase at the reaction medium. The best conditions were found employing the CYP102A1 mutant of cytochrome P450 BM3, a NADH-dependent CYP450 able to convert 35 into cycloheptanol 36, together with Thermoaerobacter ethanolicus ADH (TeSADH) which converts 36 into ketone 37 and AcCHMO, NADPH-dependent enzymes, recovering 0.41 g/L of final lactone 38. The NADH required for CYP102A1 activity was recycled by the E. coli extract, whereas the ADH and the BVMO were the responsible for the NADPH regeneration. In order to prepare a redox balance cascade, formate dehydrogenase (FDH), a NADH dependent enzyme, was added to the reaction system, allowing an increase in the production of oxocan-2-one 38 (1.4 g/L). Addition of external NADH and NADPH also led to a higher formation of 38. Finally, the use of higher biocatalyst concentration by employing highly concentrated cells suspensions in the formation of the cell free extracts allowed a high productivity at reaction times of 12 hours (2.9 g/L), whereas longer times led to a deactivation of both CYP450 and CHMO. The optimized cascade was also applied to the formation of ECL (n=1) and caprylolactone (oxonan-2-one, n=3), with productivities lower for these compounds (around 0.6 g/L).
A multienzymatic system for the conversion of cyclohexanol into aminohexanoic acid (42, Scheme 16), the monomer of nylon-6, was developed by combining one mutant of AcCHMO with different biocatalysts [85].

![Scheme 16. Multienzymatic system for the preparation of 6-aminohexanoic acid (42).](image)

The complete system was based in two different modules. In the first one, well described in the present paper (see Schemes 4, 6 and 8), ADH from *Lactobacillus brevis* (LbADH) was combined with the C376L M400I mutant of AcCHMO with recycling of NADPH in order to generate ECL with excellent conversion (98%), even at high cyclohexanol concentration (96% conversion at 200 mM). When this process was carried out at preparative scale, a 75% yield was achieved. The second module comprises the transformation of ECL into 6-aminohexanoic acid and initially was studied as a combination of a lactonase (from *Rhodococcus* sp.), to open the ECL into compound 13, an ADH from *E. coli* for the oxidation of 13, an alanine dehydrogenase (AlaDH) from *Bacillus subtilis* and different transaminases for the amination reaction to obtain 42. When this system was applied, the formation of compound 13 occurred with excellent conversion, but a very low amount of final amine was recovered. This result was caused by the carboxylic acid moiety of 6-hydroxyhexanoic acid, which inhibited the ADH-catalysed oxidation. In order to overcome this drawback, a novel synthetic route was analyzed, thus converting ECL into a methyl ester 39 employing methanol as nucleophile for the opening of the lactone. In this way, the carboxylic acid 13 was not presented in the reaction medium, allowing the reaction to occur with higher conversions. Different esterases were tested for this purpose, obtaining the best results with the horse liver esterase (HLE). When the treatment of ECL 9 was carried out in buffer containing 10% v/v of methanol as cosolvent in presence of this biocatalyst, it was observed the formation of double amount of the methyl ester 39 until all the ECL was consumed, before starting with a slow hydrolysis of the methyl ester to the carboxylic acid. Once obtained, this compound was oxidized by an ADH (prim-ADH from *Bacillus stearothermophilus*) to aldehyde 40, which was subsequently aminated by a ω-transaminase (ω-TA) in presence of an alanine dehydrogenase (AlaDH) into compound 41. This reaction requires L-alanine as amine donor, which generates pyruvate, that is recycled by AlaDH, consuming ammonia and the NADH generated in the ADH oxidation, thus regenerating this cofactor. The final step is the esterase-catalysed hydrolysis of the aminoester 41 to furnish 6-aminohexanoic acid 42. When the complete system was tested,
it was observed that the presence of methanol in the medium was a problem for the ACCHMO stability. Thus, this solvent concentration was lowered down to 2% v/v, being possible to obtain after 24 hours a 24% of 6-aminohexanoic acid, with a 35% of starting cyclohexanol, 30% 6-hydroxyhexanoic acid and a 10% of cyclohexanone.

3.2. Parallel ADHs/BVMOs cascades

Apart from linear cascades, in which one reaction is conducted before the start of the subsequent reaction, parallel or tandem cascades, with both reactions occurring simultaneously, have been developed combining ADHs and BVMOs.

One of the first examples of parallel cascades with BVMOs was shown in 2010. These isolated catalysts and ADHs have been employed in a parallel fashion for the simultaneous preparation of optically active compounds [86]. This methodology, called Parallel Interconnected Kinetic Asymmetric Transformations (PIKAT), allowed minimizing the quantity of reagents, thus maximizing the redox economy of the system. The PIKAT was performed by coupling two different asymmetric transformations: (a) via two kinetic resolutions (Scheme 17) or (b) via a kinetic resolution and a desymmetrisation reaction (Scheme 18).

![Scheme 17](image)

**Scheme 17.** Parallel interconnected kinetic asymmetric transformation for the concurrent synthesis of optically active alcohols, ketones and esters combining ADHs and BVMOs in a double kinetic resolution.

By the proper selection of both catalysts (an ADH and a BVMO) and using a catalytic amount of NADPH, which acts as the connector between the two transformations, all possible enantiomers can be obtained in a one-pot process. Phenylacetone monoxygenase (PAMO) from *Thermobifida fusca* [87] and 4-hydroxyacetophenone monoxygenase (HAPMO) from *Pseudomonas fluorescens* ACB [88] were selected as BVMOs, due to their excellent behaviour in the kinetic resolution of racemic ketones [89], whereas two NADPH-dependent ADHs with opposite selectivity were employed: LbADH and ADH from *Thermoanaerobacter sp.* (ADH-T). Initial experiments were carried out in the interconnected kinetic resolution of racemic 2-phenylpentan-3-one (43a) and 2-octanol (45a) with BVMOs and ADHs in presence of a catalytic amount of NADPH (0.2 mM). When using HAPMO and ADH-T at 20ºC and pH 7.5, it was possible to obtain (R)-43a and (S)-44a in a process with excellent enantioselectivity (E > 200). ADH-T was able to oxidized (S)-45a to ketone 46a, remaining (R)-45a with high enantiomeric excess. When LbADH was used instead ADH-T, (S)-45a was recovered in an excellent double kinetic resolution when the PIKAT was carried out at pH 8.5. PAMO was also tested in this process. When combined with ADH-T, (R)-43a, (S)-44a and (R)-45a were obtained with conversions around 50% and excellent enantioselectivities. The pH and the temperature effect was studied in the coupled system PAMO/LbADH, being observed that the system can work even at 60ºC,
but the best results were achieved at pH 8.5 and 30°C. A small amount of hexyl acetate (47a) was recovered in the reaction medium. This compound was obtained by the PAMO-catalyzed oxidation of 2-octanone 46a in a secondary reaction. The parallel resolution was extended to other secondary alcohols such as undecanol (45b) or sulcatol (45c) in combination with the BVMO-catalysed resolution of rac-43a. For all the BVMOs and ADHs, excellent results were obtained in the resolution of racemic 45b and 43a, being observed that PAMO led to better conversions and enantioselectivities than with HAPMO. (R)-sulcatol was obtained with low optical purities in the ADH-T-biocatalysed reactions, as this enzyme was not able to perform a selective biooxidation of the racemic alcohol. When the reactions were carried out with LbADH, excellent resolutions were achieved. 4-Phenylhexan-3-one (rac-43b) was resolved in presence of 45a, with excellent selectivity and high optical purities for all the biocatalysts combinations, thus recovering (R)-43b, (S)-44b and (R)- or (S)-45a depending on the enzymes employed. In a further development of the PIKAT system, the kinetic resolution of racemic alcohols catalysed by isolated ADHs was coupled with the desymmetrisation of different prochiral sulphides employing BVMOs [90], as shown in Scheme 17.

Scheme 18. Combination of ADHs and BVMOs for the kinetic resolution of racemic 2-octanol and the enantioselective sulfoxidation of prochiral sulphides in a concurrent fashion.

Thus, racemic 2-octanol was selectively oxidized by both LBADH and ADH-T in a process coupled with the enantioselective oxidation of sulphides 48a-f catalysed by PAMO, its M446G mutant [91] or HAPMO. In order to ensure the best performance for these biocatalysts, PAMO and its mutant were used at 30°C whereas HAPMO was employed at 20°C. Aromatic sulfoxides (49a-d) were obtained with moderate to good conversions and high optical purity, whereas the oxidation of rac-45a led to the (R)- or (S)-alcohol with high enantiomeric excess and conversions around 50%. Some amount of ester 44a is formed, due to the BVMO oxidation of ketone 43a. The system was also applied to the sulphoxidation of a heteroaryl alkyl sulphide (45e), being possible to obtain both enantiomers of the sulphoxide with high selectivity, whereas (S)-cyclohexyl methyl sulfoxide (49f) was obtained with excellent optical purity and high conversion in the system catalysed by HAPMO and both of the ADHs. The concurrent synthesis of (S)-methyl phenyl sulfoxide (49a; R = Ph, n = 0) catalysed by HAPMO was coupled with the kinetic resolution of several secondary alcohols in presence of LBADH, achieving excellent results in the oxidation of 2-octanol, 2-undecanol and sulcatol to yield the (S)-enantiomers. When the hydroxyl moiety was in position 3 of the starting alcohol (3-octanol) or diols were employed, lower optical purities were measured. Finally, the NADPH cofactor in the reaction medium was optimized, in order to ensure an effective large-scale application. Studies revealed that when the cofactor concentration was 5 µM the system performance was optimal in the concurrent sulphoxidation of thioanisole (48a) and the kinetic resolution of 2-octanol combining HAPMO and LBADH. Lower concentrations (1 µM) can be used in the
double kinetic resolution of rac-43b and rac-45a catalysed by PAMO and LBADH, respectively, as this ketone is a very good substrate for the BVMO.

The production of ε-caprolactone combining isolated BVMOs and ADHs has been also described through a convergent cascade [92], depicted in Scheme 19. In this approach, two different substrates were employed, which leads to the final product without the formation of any intermediate.

Scheme 19. Convergent cascade for the synthesis of ECL starting from cyclohexanone and 1,6-hexanediol employing BVMOs (AcCHMO) and ADHs (TeSADH).

Thus, two equivalents of cyclohexanone 8 were oxidized by a BVMO to caprolactone 9, whereas the ADH-catalysed oxidation of one equivalent of 1,6-hexanediol 50 afforded the same lactone, with simultaneous regeneration of the NAD(P)H cofactor, as shown in Scheme 18. Biocatalysts were employed as crude extracts free of cells. The initial study for finding the best ADH in this oxidation pointed towards to Thermus sp. ATN1 ADH (TADH) as a promising candidate for this reaction. Unfortunately, this biocatalyst is NADH dependent and no suitable BVMOs with preference for this cofactor were found for the cascade proposed. Then, different bienzymatic systems were tested, achieving the highest concentration of final lactone when combining AcCHMO with TeSADH, a NADPH-dependent biocatalyst. Thus, 10 mM of ECL were formed when starting from 20 mM of cyclohexanone and 10 mM of 1,6-hexanediol. Reactions in absence of one or two of the catalytic members (cofactor, CHMO and ADH) were carried out. ECL was obtained in 6 mM concentration after 72 hours in absence of TeSADH, indicating that some endogenous ADH presented in the crude CHMO preparation can present some activity on 50. A very low concentration of ECL was recovered (2 mM) in absence of NADPH, maybe owing to the cofactor present in the extracts. No lactone formation was observed in absence of CHMO. In addition, some ECL hydrolysis was observed in the reaction conditions. The CHMO-TeSADH system was optimized by increasing the concentration of substrates and biocatalysts. Addition of extra CHMO after 48 hours allowed obtaining complete conversion after 72 hours, whereas doubling the starting amount of enzymes led to the same conversion after 48 hours, but with a lower turnover frequency (2.0 vs 4.8 h⁻¹). Increasing cyclohexanone concentration to 100 mM led to the same TOF (1.8 h⁻¹) with only 32% conversion after 72 hours. The study of the reaction showed that cyclohexanol was formed during the process as a by-product, achieving a maximum value after 24 hours, and then decreasing its concentration until its disappearance at 72 hours. The convergent cascade was run at 50 mL scale, starting with 98 mg of ketone (20 mM) and 59 mg of diol (10 mM). After 18 hours, complete cyclohexanone conversion was observed, with 19.7 mM of ECL. This result can be debt to a better oxygenation in the multimilligram scale. After 24 hours, lactone concentration was 19 mM and 3.7 mM of diol were measured. Reaction work up afforded 150 mg of a mixture containing ECL, poly-ECL and diol at 1:1.5:0.6 ratio.

3.3. Sequential EREDs/BVMOs cascades

Issa et al. [93] have recently studied the enzymatic bioreduction of synthetic analogies of (-)-carvone (R)-13 (Scheme 5) decorated with different substituents at position C3.
and/or C6, and their subsequent transformation into the corresponding carvactones by means of BVMOs, as shown in Scheme 20.

EREDs tested were OYE2 and OYE3 [94] from S. cerevisiae and PETNR [73,74], while CHMO_Ph1 [75] was used for the Baeyer-Villiger oxidation. Data provided by these authors [93] showed that the configuration of the extra methyl (R') at C6 did not affect the binding and/or orientation of the substrate in the bioreduction compared to the parent compound (R)-13. Thus, (6S) stereochemistry of 51a was always leading to (2S) stereoisomers of 52a, and (6R) isomers of 51a were furnishing (2R) stereoisomers of 51a. Notably, the stereo-configuration of the methyl at C6 does significantly impact the rate of conversion; in fact, (6S)-51a was converted more slowly and to a lower yield (15% with OYE2 in 24 h, 17% with PETNR in 24 h) compared to (6R)-51a (90% with OYE2 in 2 hours, 88% with PETNR at the same time). This reduction in conversion upon transforming the pure diastereomers (5R,6S)-51a and (5R,6R)-51a was also observed using a mixture of them, where a more rapid depletion of (5R,6R)-51a and a quick formation of (2R,3R,6R)-52a was reported. Remarkably, a computational model to understand this stereochemical pattern was proposed [93].

In a second step, the effect of heteroatom substitution at C6 was explored, using 6-OH substitution instead of 6-Me (substrates 51b). In this case, (5S,6S)-51b and (5S,6R)-51b were respectively converted to the corresponding 6-hydroxidihydrocarvones (2S,3S,6R)-52b and (2R,3S,6R)-52b, by OYE2 with moderate yields (40% and 30% after 24 hours) but with very high diastereomeric excesses (≥99% de) in all cases. On the other hand, PETNR performed better, affording 66 and 80% yields of (2S,3S,6R)-52b and (2R,3S,6R)-52b, respectively, after 2 h, also with ≥99% de. As with the 6-methyl derivatives, these were poorer substrates for OYE3 under the same conditions. The corresponding 6-hydroxyxycarvone diastereoisomers derived from (+)-carvone (5)-13, (5R,6S)-51b and (5R,6R)-51b, were also poor substrates for OYE2, affording <10% yields at 2–24 h. A similar behaviour was reported with PETNR on the (5R,6S)-51b (5–10% maximum yield), although this enzyme allowed yields (85–95%) of (2R,3R,6R)-52b when starting from (5R,6R)-51b, but with much reduced de (<30 %). Finally, synthetic C3-Me-(−)-carvone analogue 51c is a regioisomer substrate of 51a, but introducing a methyl at the site of enzymatic conjugate reductive attack. Bioreductions catalysed by OYE2 and PETNR proceeded with low yields (≤ 10%), although with high diastereoselectivity (de≥99) for compound (2S,3R,6R)-52c.

After this exhaustive stereochemical study of the EREDs performance, Issa et al [93] assessed the BV oxidation with the different diastereomers of 52a and 52b. The (2S,3R,6R)-52a isomer was completely converted to lactone (3R,6S,7R)-53a (oxygen insertion between C1-C2), leading to with apparently complete regioselectivity (≥99%), with no traces of the lactone coming from the oxygen insertion between C1-C6. Remarkably, there was no reaction at all when starting with (2S,3R,6R)-52a; this indicates a remarkable diastereoisomer-selectivity, as an inversion of 6-methyl absolute configuration avoids the enzymic
transformation. Thus, a cascade reaction was carried out at 50 mg scale, as shown in Scheme 21.

![Scheme 21](image)

**Scheme 21.** Enzymatic sequential conversion of (5R,6R)-51a into dihydrocarvone (2R,3R,6R)-52a and carvolactone (3R,6S,7R)-53a.

Regarding 3-methyl modified dihydrocarvones 52c, they were chemically synthesized starting from (R)-13, as shown in Scheme 22. This chemical reduction with CuBr and trimethylaluminium was carried out because of the poor ERED outcome using OYE2 and PETNR on 51c, so that a mixture of four isomers with a diastereomeric ratio of 1.5:1:1:0.1 was obtained.

![Scheme 22](image)

**Scheme 22.** Chemical synthesis of dihydrocarvones 49c and subsequent enzymatic Baeyer-Villiger oxidation to carvolactones 50c.

The two 3R diastereomers ((2S,3R,5R)-52c and (2R,3R,5R)-52c, about 70–75% of total) were separated from the two (3S)-isomers, so that it was possible to evaluate valuation of all four isomers, and of the separate pairs of C3 diastereomers with CHMO_Phi1. Bioreductions were run at 25°C for 24 hours, with the system NADP⁺/GDH employed as the hydride donor, leading to a complete conversion of (2S,3R,5R)-52c and (2R,3R,5R)-52c to their corresponding enantiopure lactones (3S,4R,6S)-53c and (3R,4R,6S)-53c in 98% yield (Scheme 21). This process was scaled up to 50 mg. However, no lactone formation was observed from the biooxidation of (2R,3S,5R)-52c and (2S,3S,5R)-52c diastereomers.


In the last few years, the development of multienzymatic processes has been largely spread, being possible to design novel biotransformations avoiding the isolation of reaction intermediates and reducing the consumption of solvents and energy, thus maximizing the atom economy of the reactions. By this reason, several processes in which two or more biocatalysts are employed in a sequential or concurrent fashion have been designed. Among these biocatalyst, Baeyer-Villiger monooxygenases have demonstrated to be useful biocatalysts for the synthesis of valuable compounds employing mild and environmentally friendly conditions. Thus, these enzymes have been employed in multienzymatic reactions in combination with other oxidoreductases, mainly coupled to enolate reductases or alcohol dehydrogenases, being described some examples in which cytochrome P450 are also involved. EREDs have been generally employed for the hydrogenation of α,β-unsaturated cycloketones to the corresponding cyclic ketones, which are then oxidized to valuable lactones.

When combined with alcohol dehydrogenases, most of the synthetic procedures described balance redox systems in which the nicotinamide cofactor required by both biocatalysts is recycled through an initial ADH-catalysed oxidation of an alcohol to a ketone.
with a subsequent BVMO-catalysed formation of ester or lactone. Most of these examples involved the preparation of ε-caprolactone or some of its analogues from cyclohexanols in a biocatalytic transformation combining the ADH-catalysed oxidation of the alcohol with the BVMO-catalysed oxidation of the ketone with in situ cofactor regeneration. This model system has been studied employing both whole cell and cell free systems, but in all the cases, a critical parameter is the low operational stability of the Baeyer-Villiger monooxygenase, very sensitive to the substrate and product concentration or to the reaction conditions. Different approaches have been made to overcome this drawback, including the application of more stable biocatalysts able to tolerate higher concentrations of substrates and products, the use of organic cosolvents or the inclusion of a further biocatalytic step with the aim of diminishing the ECL concentration in the reaction medium, as the lipase-catalysed conversion of ECL to oligo-ECL or 6-hydroxyhexanoic acid. After all these improvements, the results obtained indicate the requirement of further developments are still required to ensure the productive biocatalytic synthesis of ECL starting from cyclohexanol.

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