POLITECNICO
MILANO 1863

SCUOLA DI INGEGNERIA INDUSTRIALE
E DELL'INFORMAZIONE

Upcycling a citrus waste in the chemo-enzymatic synthesis of the muguet fragrance Lilybelle®

Master of Science Thesis in Food Engineering

Gritti, Beatrice, 103219

Advisor:
Prof.ssa Maria Elisabetta Brenna

Co-advisors:
Prof. Fabio Parmeggiani

Academic year:
2022-23

Abstract: This work is aimed at the optimization of the synthesis of the odorous compound Lilybelle® using limonene extracted from citrus waste as starting material and, when possible, introducing biocatalysed steps as an alternative to traditional chemical transformations. Specifically, in the synthetic path devised for the target compound two steps of allylic alcohol oxidation are optimised using alcohol dehydrogenases instead of MnO_2, and the high regioselectivity of ene-reductases in catalysing the reduction of C=C double bonds conjugated to carbonyl groups is exploited. The biocatalysts are employed as crude lysates to avoid cost-effective and time-consuming enzyme purification. The overall procedure is based on well-established, high yield organic transformations and most of the waste is represented by readily biodegradable organic material from cell lysates.

Key-words: biocatalysis, Lilybelle®, waste management, OYE, limonene, fragrances

Table of Contents

1. Introduction

1.1 Fragrances

Fragrances has been part of human history for millennia and still dominate our world today (Figure 1), being high-value chemicals widely used in different fields, for example to enhance flavour and aroma in food and beverages, to provide a fresh scent in cleaning products and to mask unpleasant odours. At this day, the fragrance market size is experiencing significant grow due to the changing in customers preference along with the increasing interest in luxury goods and it is estimated to reach USD 84.02 billion dollars [1] by 2028, subsequently occupying a unique position in terms of annual production volumes.

Figure 1. Fragrances and Perfumes Market: Market Size (%), 2022 [1].

As the interest in perfumes and scented products increases, so does the need for sustainable and scalable production methods in the flavour and fragrance industry, according to the growing demand for sustainable and safe fragrances coupled with the limited availability of natural resources and the urgent need for environmentally friendly production processes [2].

1.2 Sustainability

In 1987 United Nations defined sustainable development as *"meeting the needs of the present without compromising the ability of future generations to meet their own needs."* [3]

In response to the sustainability issue and growing environmental concerns, the 193 member countries of the United Nations elaborated the Agenda 2030, incorporating 17 Sustainable Development Goals (SDGs) (Figure 2) that are a blueprint to achieve a better and sustainable future, addressing global challenges and providing a roadmap to work towards sustainability in order to meet all the 17 goals by not later than 2030. Fragrances and their impact on human health and the environment are relevant to several SDGs, including SDG 3, SDG 14, SDG 15, SDG 11, and SDG 12 [2]. To compel to the Agenda, the *International Fragrance Association* (IFRA) and *the International Organization of the Flavor Industry* (IOFI), constituted by, among others, Symrise, Firmenich, Givaudan and BASF, joined together chaired by the lead R&D of Takasago and launched the IFRA-IOFI Sustainability Charter, aimed at promoting sustainability within the fragrance and flavour industry. In order to achieve these key goals, innovative and sustainable approaches to fragrance production and consumption are needed; this includes the development of alternative methods for the production of chemicals, the use of renewable resources, and the adoption of environmentally benign and ethical sourcing practices [4].

Figure 2. The 17 Sustainable Development Goals (SDGs) of Agenda 2030.

1.2.1 Upcycling and biotechnology

Figure 3. Upcycling process of orange peels [5].

The concept of upcycling has gained importance in the fragrance industry as a sustainable approach to create new scents while reducing waste and complying with SDG 12: Sustainable production and consumption.

Upcycling is a key component of the circular economy model and it consists in the repurposing of waste materials to produce high-value fragrance ingredients (Figure 3) that otherwise would have been sent to landfill; for this aim, companies have started using flowers and fruits that are traditionally considered waste to further processing them and extract essential oils that can be used in fragrance formulations [2].

Upcycled fragrances can also utilise by-products from unrelated industries. For example, wood retrieved from old furniture can be exploited for its resinous, deep notes; coffee grounds, which are commonly disposed of after brewing, can be utilized to produce earthy and bitter notes, transforming useless products or by-products into better quality and high-value products [5].

To fully exploit the potential of this process in the field of fragrances, biotechnology has emerged as a promising tool for upcycling fragrance ingredients and synthetise products at industrial scale with the help of enzymes and microorganisms. The combination of biotechnology and waste valorisation complies with the main principles of Green Chemistry, making the overall process "greener" than conventional one, which typically relies on non-renewable sources. In addition, biotech-derived fragrances can be synthesized at higher concentrations than those naturally occurring in plants, allowing flexibility in the formulation of the fragrances [2].

By sticking to upcycling and alternative pathways like biotechnology, fragrance companies can align with the SDGs and subsequently integrate them into business practices, contributing to global sustainability efforts and demonstrate their commitment to responsible and ethical practices.

To assess and take social responsibility for their action, in the last decade companies began to develop their own procedure to evaluate the sustainability of their solutions; in 2018, Symrise has developed the Product Sustainability Scorecard to evaluate the sustainability of its products. The procedure allows the company to record the environmental impact of raw materials and products in a systematic manner, in order to select the most environmentally friendly options possible when it comes to product development on the basis of ten criteria and enabling the customer to choose the most eco-friendly solution compared to the alternatives [6]. In addition, Symrise identified six SDGs (8, 12, 13, 14, 15, 17) on which the company can strive and have a particularly significant impact through its business activities [7]; moreover, the company always takes further steps into developing and improving biotechnology process, also synthetizing biodegradable molecules. Thanks to these efforts, Symrise earned the "Sustainable Future Award 2023" prize. On the other hand, in 2019, Givaudan has launched the FiveCarbon Path program [8] and touches now upon 10 out of the 17 SDGs, taking action where they can have a great impact [9]. Carbon atoms play a vital role in the fragrance industry and hence is at the centre of the FiveCarbon Path, aiming to use carbon sources wisely. Therefore, in order to develop sustainable molecules and processes, the company focuses on five measurable dimensions: increasing the use of renewable carbon, increasing carbon efficiency in synthesis, maximizing biodegradable carbon, increasing the 'odour per carbon ratio' with high-impact materials, and using upcycled carbon from side streams. Three out of five dimension are linked to the development of efficient processes according to green chemistry principles, however biocatalysis have the potential to positively influence the metrics used to assess each dimension of the FiveCarbon Path [10].

1.2.2 Biocatalysis

Figure 4. The 12 principles of Green chemistry [28].

In the last two decades, the cosmetic industry witnessed dramatic increases in the industrial potential and scope of biocatalysis, completely changing the scenario of this process: many approaches involving biocatalysis have emerged in order to improve quantitively or qualitatively essential oils and natural extracts used in perfumery [8]. Biocatalysis is 'naturally' sustainable, not only because reactions are usually performed in water and under mild conditions (ambient temperature and atmospheric pressure), but is also essentially non-hazardous, non-toxic and biodegradable, with any enzyme discharged into aqueous effluent being rapidly degraded; furthermore, generates less waste than conventional organic syntheses. Consequently, biocatalytic methods have environmental benefits and are more cost-effective, considering that enzymes are produced from readily available and inexpensive renewable resources and the costs of producing them are, therefore, essentially stable and predictable [11]. Producing natural products by biotechnology complies with ten of the twelve principles of green chemistry (Figure 4), and the two remaining principles, the 4th and 10th, are not really relevant in this case since they relate to the product, rather than the process [11]. Enzymes exhibit high chemo-, regio-, and stereoselectivities that are difficult to achieve with standard organic synthesis methods. In particular, the nearly perfect enantioselectivities observed with highly engineered enzymes are practically inimitable [11]. In short, enzyme-based catalysis is safe, selective, sustainable, and good for people, planet, and profit [12]. Enzymes can catalyse reactions with remarkable selectivity, leading to synthetically challenging and often inaccessible by traditional synthetic chemistry compounds, furthermore allowing the production of fragrances with higher concentration and a lower odour threshold, using less material to achieve a similar olfactive effect.

Due to the fact that enzymatic reactions usually involve roughly the same temperature and pressure facilitates the integration of multiple steps into cost-effective and environmentally friendly one-pot processes. One-pot cascades afford several economic and environmental benefits: fewer unit operations, minimized solvent and reactor volume, higher throughputs and space time yields and reduced waste [11].

The growing trend in the use of enzymes has led to the exploitation of the immobilization process. Enzymes are water soluble and their recovery thus is challenging; thanks to immobilization, the catalyst in a heterogeneous phase can be easily recovered by filtration or centrifugation and recycled. This results in process simplification, improved stability and product quality; the drawback could be the loss of activity, but the increase in stability and reusability compensates for this loss [8].

In conclusion, biocatalysis can help reduce waste generation thanks to its high selectivity, the production of biodegradable subproducts, the lower energy consumption using mild process conditions and reduce the use of organic solvents.

1.2.3 Old Yellow Enzymes

The asymmetric hydrogenation of carbon-carbon double bonds, with the potential generation of up to two stereogenic centres, is a crucial transformation in the stereoselective synthesis of enantioenriched molecules, especially in the field of fine chemicals such as pharmaceuticals, agrochemicals and fragrances [13].

The biocatalytic alkene reduction is catalysed by families of enzymes known collectively as ene-reductases (ERs), which gained broad practical use as they accept α,β-unsaturated aldehydes, ketones, carboxylic acids and derivatives [10]. Enzymatic stereoselective carbon-carbon double bonds reductions offer a convenient, versatile and often complementary alternative to their metal catalysed counterpart [13].

The majority of ERs belongs to the well-characterised superfamily of Old Yellow Enzymes (OYEs), first identified in yeasts and, shortly after, discovered also in bacteria, plants, fungi and algae [13]. The name originates from the fact that they contain a flavin mononucleotide (FMN) prosthetic group, which is responsible for catalysis and confers an intense yellow colour to purified samples [13].

The Old Yellow Enzyme family dominate the scene in the enzyme-catalysed reduction of activated carbon-carbon double bonds, thanks to their extremely high enantioselectivity and regioselectivity, which makes them particularly useful for synthetic applications [14]. OYEs can be engineered to preferentially produce one enantiomer over the other, providing a valuable tool for the synthesis of chiral compounds. The mechanism involves a two-step reaction sequence, in which an electron-withdrawing group forms tight H-bond interaction with donor residues. This interaction makes the substrate more electronically reactive to the 1,4-hydride addition at β position. This leads to the hydrogenation of the carbon-carbon double bond with anti-stereospecificity. After the product's release, thanks to a ping-pong bi-bi mechanism (Figure 5), a molecule of nicotinamide cofactor NAD(P)H restores the catalytically active form to restart a new reduction cycle [13]. An industrially attractive technology is achieved by combining the ene-reductase with an NAD(P)H recycling system, such as alcohol dehydrogenase/isopropanol, glucose/glucose dehydrogenase or formate/formate dehydrogenase [11].

In conclusion, ERs applicability at a preparative scale with excellent yields and enantiomeric excess was demonstrated, and their utility in biocatalytic applications is well established [10].

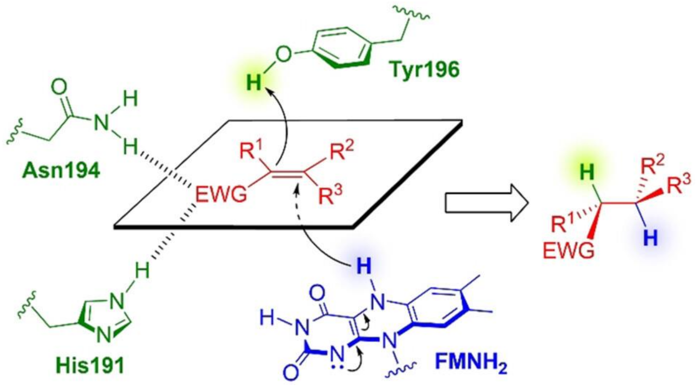


Figure 5. ER-mediated bioreduction of C=C bonds [13].

1.3 Lily-of-the-valley fragrances

Figure 6. Lily-of-the-valley flowers [7].

Lily-of-the-valley (Figure 6), with its characteristic bell-shaped white flower, belongs to the family of the Liliaceae. The aroma of lily-of-the-valley is composed of several volatile organic compounds (VOCs), including linalool, geraniol, and alpha-terpineol [15] which confer to the fragrance its fresh, green, flowery and aldehydic muguet notes.

Unlike many other flowers, it is troublesome to extract essential oil directly from lily-of-the-valley's ones, due to their delicate nature; in addition, the process would be unsustainable because of the intensive harvesting need, which would result in a costly and low yield process either way. As a result, over the last 120 years the interest in the synthesis of compounds which replicates as much as possible lily-of-the-valley scent grew. Nowadays, these odorants account for over 20% of the total perfumery raw materials market, making lily-of-the-valley the most important odour family, not only in terms of value but also in volume [16]; furthermore, it has been used as a keynote in many perfumes of famous companies over the year, such as Dior, Cartier, YSL and the Italian Acqua di Parma.

To address this challenging growing demand, perfumers have resorted to replicate as closely as possible lily-of-the-valley scent; in this perspective, fragrances like Bourgeonal®, Lyral® and Lilial® (Figure 7) were developed.

Over the last ten years, serious concerns were raised over the safety of lily-of-the-valley odorants and many of them were found to be toxic for human use; hence, their use has been restricted or totally prohibited. It is the case of 3-(4-(tert-Butyl)phenyl)-2-methylpropanal, commonly known as Lilial®, patented by Givaudan in 1956. In 2021, the European Chemical Agency added Lilial® to its list of Very High Concerns due to its potential toxicity. Lilial® was definitely banned in all cosmetic products from 1st March 2022.

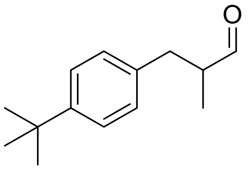
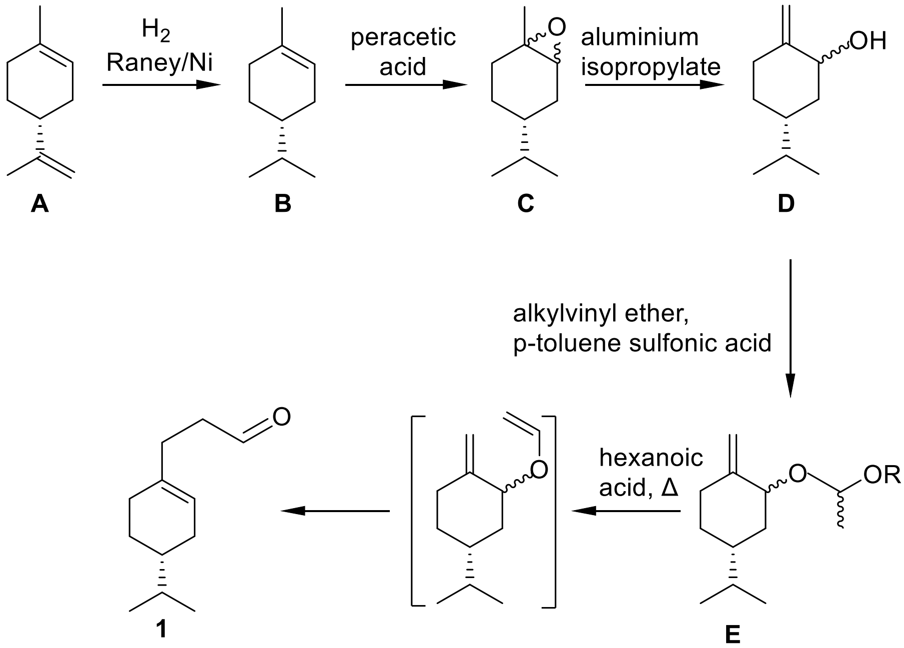


Figure 7. Lilial®.

1.3.1 Lilybelle® synthesis

The challenge of sustainability drove the fragrance company Symrise to develop a new fragrance, 3-[(4*R*)-4-(propan-2-yl)cyclohex-1-en-1-yl]propanal, commercialised under the name of Lilybelle®. Symrise patented this fragrance in 2011, although was not put on the market until 2017 because the five-stage synthesis (Scheme 1) did not perform well at large scale [17].



Scheme 1. Lilybelle® pathway patented by Symrise [17].

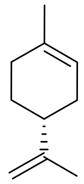
This process is now optimised and uses a starting material from renewable sources, (R)-(+)-limonene contained in by-products from the orange juice industry. Thus, a final product that is 83% derived from renewable raw materials, which respects the 12 principles of Green Chemistry and is also readily biodegradable [18].

In the first stage of the synthesis proposed by Symrise, the isopropenyl moiety of limonene, sustainable starting material accessible from orange peels, is hydrogenated selectively with molecular hydrogen in the presence of Raney-Nichel. After epoxidation, the resulting oxirane is rearranged to an allyl alcohol by refluxing in xylene in the presence of aluminium triisopropoxide. Reaction with butyl vinyl ether and heating to 200°C in a high boiling solvent such as Marlotherm® (dibenzyl toluene) finally provided Lilybelle® via a Claisen rearrangement. The synthetic route stays true to the principles of green chemistry, which includes the use of catalysts at almost every stage of the synthesis, the recycling of solvents, and restrictions of waste production in its manufacturing process [7]. Lilybelle® was developed with the aim of producing a lily-of-the-valley odorant with a reduced environmental impact using the principles of green chemistry and starting from sustainable resources. However, its synthesis process still involves the use of (i) dangerous reagents (peracetic acid is employed at the very beginning of the procedure when a bigger amount of material is manipulated), (ii) scarcely selective reactions (the third and fourth step are rearrangements that, even if characterised by high atom economy, occur with low selectivity affording low purity products, that need extensive purification); (iii) high energy consumption (the third and fourth step require prolonged heating at 140 and 200 °C, respectively). Therefore, finding a more sustainable and eco-friendly synthesis method for Lilybelle® is considered of high interest [19].

1.3.2 Limonene as a sustainable starting material for the production of fragrances

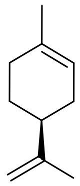
Limonene is a valuable low-cost, low toxicity and biodegradable cyclic monoterpene and due to its numerous applications in the food, cosmetics, and pharmaceutical industries has been extensively investigated in recent years [20].

Limonene is an optically active compound, and exists in two enantiomeric forms: R and S. (R)-limonene (Figure 8), the most abundant enantiomer, it is present in the peels of lemon, orange, and other citrus fruits, while the (S)- limonene (Figure 9) is found in peppermint and in turpentine oil [20].



Fresh citrus, orange-like
Odor Threshold = 200 ppb

Figure 8. (*R*)-*limonene* structure.



Harsh, turpentine-like, lemon note
Odor Threshold = 500 ppb

Figure 9. (*S*)-*limonene* structure.

(*R*)-limonene is a high-value starting material commonly used for the production of synthetic fragrances as it is the most prevalent enantiomer present in citrus peel.

Citrus fruits represent almost 98% of the total industrialized crops, with oranges representing about 82% of the total citrus fruits produced. The global overall citrus fruit production was around 158.49 million metric tons in 2020, with Asia accounting for almost half of total citrus fruit production. Oranges are the majorly processed citrus fruits, followed by tangerines, lemons/limes, and grapefruits [21]. Global citrus fruit processing generates approximately 10 million MT of waste each year; moreover, due to the large quantity and its perishable nature, citrus by-products are also problematic wastes [22]. This waste includes peels, called *flavedo*, which account for the 50–55% of the total fruit mass, seeds, pomace, and wastewater [21].

With citrus waste being a serious ecological issue, there is a need to move toward sustainable processes to manage and upcycling them. The challenge is the transition to a circular economy through the efficient utilization of natural resources and waste management, as it is the main goal of the target 12.5 of SDGs Agenda 2030 [23], through prevention, reduction, recycling and reuse (Figure 10).

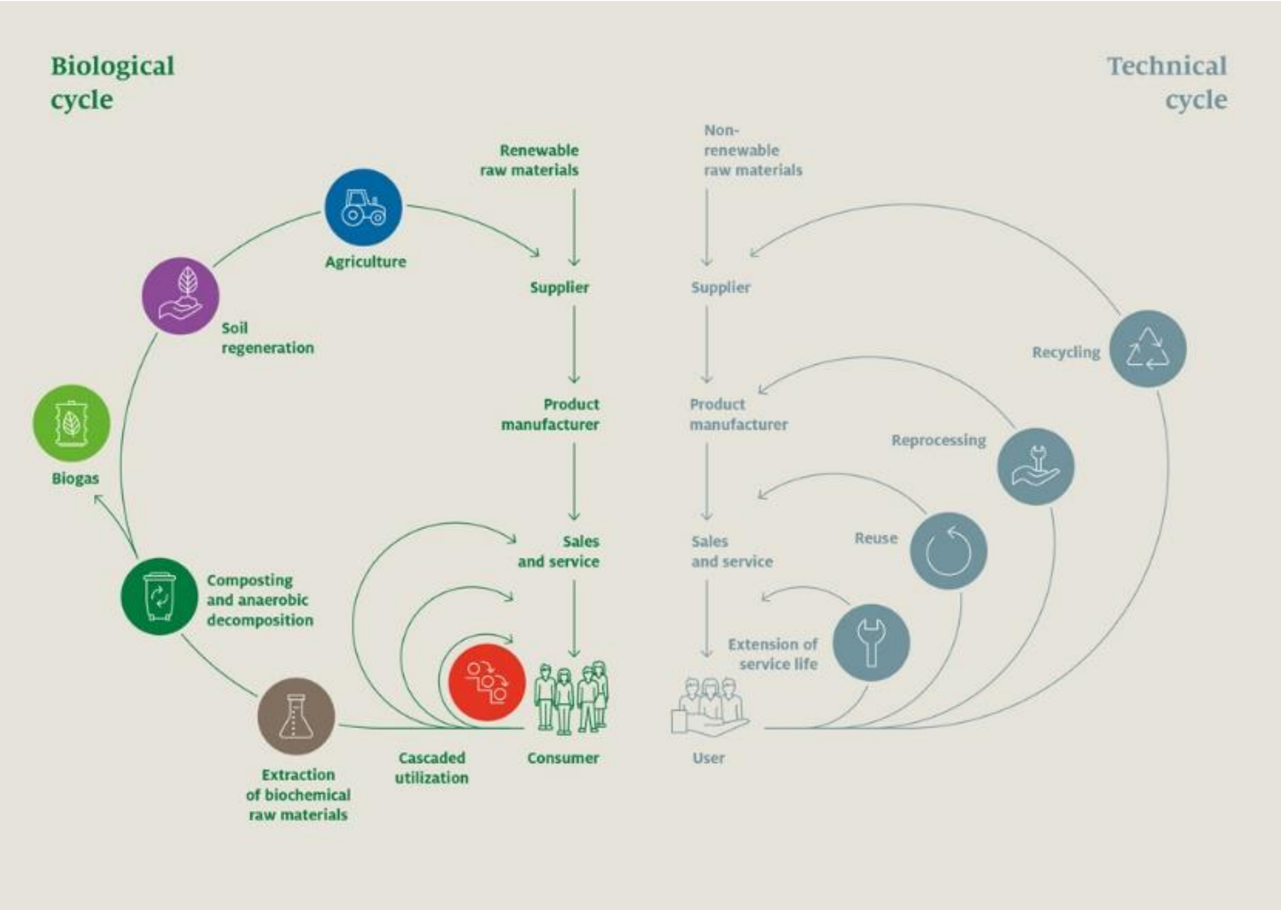


Figure 10. Butterfly circular economy systems diagram, Ellen MacArthur Foundation.

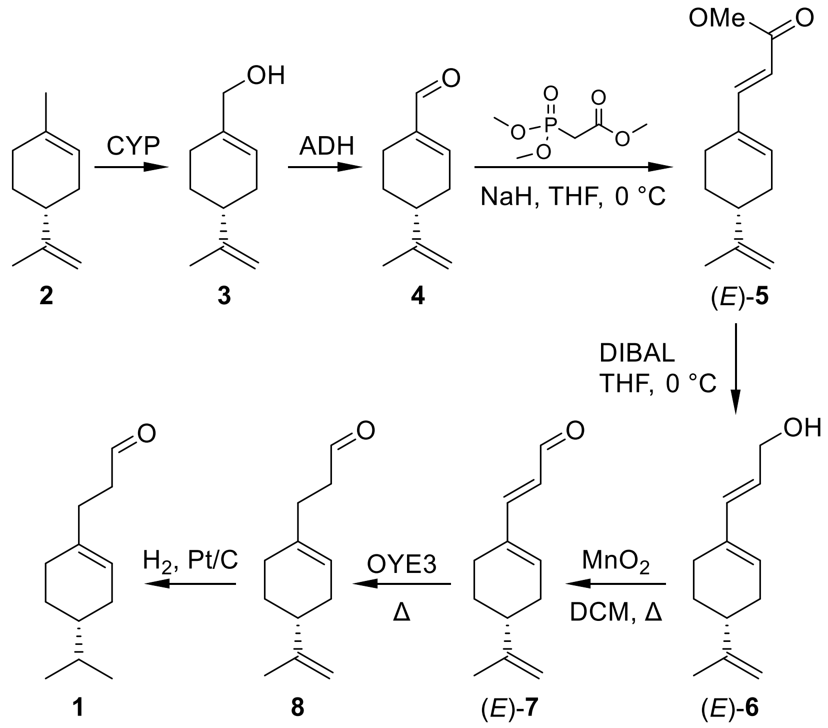
To achieve that, biotechnology can and must be exploited, as it has the potential to transform the chemical and fragrance industries by generating value from waste and low value by-products. The existing large-scale production of limonene presents an opportunity to leverage its availability and affordability in sustainable fragrance production, potentially contributing to the development of eco-friendlier and more economically viable processes [2].

As an example, Symrise developed the Symtrap® technology, a process that extracts much more flavour from fruits than the traditional method. When orange juice is concentrated, a highly aromatic aqueous phase remains; that phase was once disposed or used as water for cleaning the fruit. Symtrap® recovers and treats this aqueous phase in order to capture aroma molecules. The technology can produce one kilogram of the highly concentrated flavour from several hundred kilograms of raw material, which reduces transport weight and thus plays a key role in improving the sustainability profile of the final products [6].

2. Aim of the work

This work aims to implement the overall synthesis path to obtain the fragrance Lilybelle® **1** through the employment of biocatalysis whenever possible. This includes less energy consumption, fewer toxic chemicals, decreased waste generation thanks to the sustainable nature of biocatalysis and the use of highly selective reactions, according to the 12 principles of green chemistry.

To achieve a successful outcome, a key role was played by OYE (Old Yellow Enzyme), a versatile enzyme that can catalyse the reduction of carbon-carbon double bonds activated by an electron withdrawing group, as previously said.



Scheme 2. Synthetic path to Lilybelle® as developed in this work.

The synthetic process features seven steps and was carried out as shown in Scheme 2. The first step consisted in the oxidation of (*R*)-(+)-limonene **2** to alcohol **3**, also known as perillyl alcohol, by means of the cytochrome P450 CYP153A6 from Mycobacterium sp. strain HXN-1500. Subsequently, an alcohol dehydrogenase (ADH) was used to oxidize alcohol **3** to afford perillaldehyde **4**. Next, two carbon atoms were added to perillaldehyde thanks to the olefination of Horner-Wadsworth-Emmons, which was employed to obtain the acrylate **5** that was then reduced to the allylic alcohol **6** using diisobutylaluminium hydride (DIBAL). The resulting alcohol **6** was oxidized to acrylaldehyde **7**, by means of the oxidizing agent MnO_2. After that, OYE3 was employed to hydrogenate the carbon-carbon double bond conjugated to the aldehydic moiety to obtain saturated aldehyde **8** and finally the isopropenyl double bond in **8** was hydrogenated using a Pt/C as a catalyst in a continuous reactor to obtain Lilybelle® **1**.

3. Results and discussion

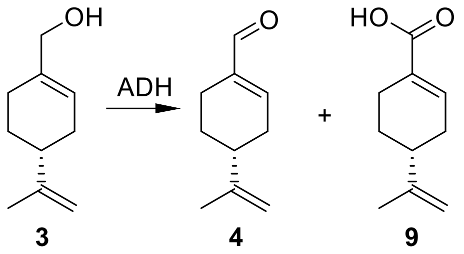
3.1 Oxidation of Limonene

A well-established application of (+)-limonene is its utilization as a very valuable intermediate in organic syntheses for the production of several molecules. One of the main ways to add value to this compound is its conversion to (+)-perillyl alcohol, through epoxidation with metachloroperbenzoic acid, followed by treatment with LDA to isomerize the epoxide to the corresponding allylic alcohol. The aforementioned alcohol is submitted to Claisen rearrangement and subsequently converted into the acetate derivative. Finally, to obtain (+)-perillyl alcohol, a hydrolysis is performed giving the alcohol with a modest overall yield [24].

The research group of Diego Romano (Università degli Studi di Milano) described a more environmentally friendly synthetic path that involves biocatalysis [25]. In particular, they demonstrated that recombinant CYP153A6 - *E. coli* cells were capable of regioselective hydroxylation of monoterpenes like limonene and α-pinene; this could be a powerful tool to make added-value chemicals. Since a collaboration is ongoing between them and the research group in which the thesis has been carried out, we started our experimentation from (+)-perillyl alcohol produced according to their method.

3.2 From (+)-perillyl alcohol to (+)-perillaldehyde via alcohol dehydrogenase – mediated oxidation

A first screening was performed to investigate the oxidation of alcohol **3** to aldehyde **4** by using both commercial ADHs and ADHs recombinantly produced in *E. coli* in the laboratory where this thesis has been carried out and employed as cell free extracts (CFEs). GC-MS analyses were performed to assess conversion and product distribution (Table 1).



Scheme 3. Pathway from **3** to **4**.

Table 1. ADH mediated oxidation from **3** to **4**.

Entry	ADH	Conversion [%]	Product distribution [%]	
			4	**9**
1	Res	49	100	-
2	A	28	100	-
3	hT	100	100	-
4	Sy	8	100	-
5	Evo030	92	100	-
6	Evo010	3	100	-
7	Evo200	23	100	-
8	Evo250	39	64	36
9	Evo270	49	100	-
10	Evo430	33	100	-
11	Evo440	70	79	21
12	Dr	5	100	-
13	Pl	11	100	-

Given the results in Table 1, it has been identified an ADH that converts 100% of **3** in **4**. Further work will be carried out in order to scale up the reaction and optimise the reaction conditions.

3.3 Horner-Wadsworth-Emmons reaction

The third step of the synthetic path involved the application of Horner-Wadsworth-Emmons reaction in order to obtain the ester **5** through the reaction of aldehyde **4** with a stabilised phosphonate carbanion (mechanism showed in Figure 11) following a known procedure [26].

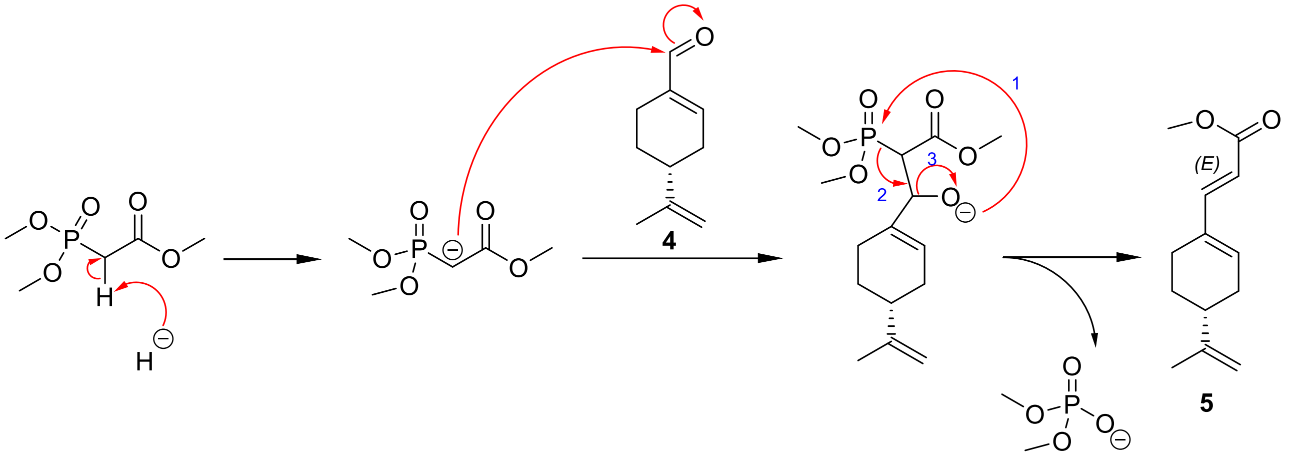


Figure 11. Horner-Wadsworth-Emmons reaction mechanism.

The reaction was conducted at 0 °C in THF, in the presence of NaH and trimethyl phosphonoacetate. Compound **4** was added to the mixture according to the procedure. The conversion of alcohol **4** was almost quantitative, with (*E*)-**5** being the predominant product. The main sub-product is constituted by the isomer with *Z* configuration. The product so synthetized was obtained in 91% isolation yield, with a sufficient purity (92% by GC-MS) to be used for the next step without further purifications.

3.4 Reduction with DIBAL

The synthesis of alcohol **6** was done by reduction of the corresponding α,β-unsaturated ester **5** following a known procedure [26] with DIBAL in THF at 0 °C. The conversion of ester **5** was almost quantitative. The product so synthetized was obtained in 90% isolation yield, with a satisfactory purity (95% by GC-MS) to be used for the next step without further purifications.

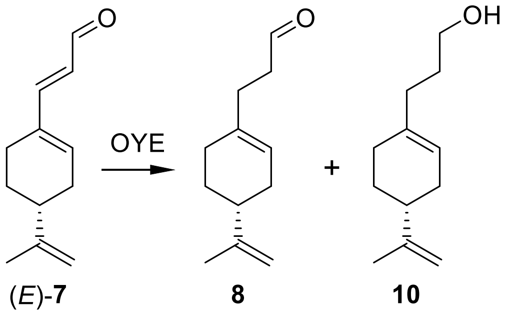
3.5 Oxidation with MnO_2

The fifth step involved the use of MnO_2, a common oxidant used in organic synthesis, in order to oxidise alcohol **6** to the correspondent aldehyde **7** with MnO_2 in CH_2Cl_2 at room temperature. The product so synthetized was obtained with a yield of 64% and a purity of 81% which was submitted to column chromatography to recover pure aldehyde (*E*)-**7**.

3.6 Hydrogenation through OYEs

To perform the selective hydrogenation of (*E*)-**7** to **8,** ene-reductases were employed as catalysts. In particular, a previous screening [19] highlighted that OYE3 was the most suitable enzyme to catalyse the hydrogenation of the conjugated C=C double bond. Hence, starting from the previous results, which shown a higher activity of OYE3 with the substrate **7**, this reduction was carried out with this enzyme, using GDH to recycle the cofactor with glucose as sacrificial substrate. Both OYE3 and GDH were used as cell free extracts to avoid the expensive and time-consuming enzyme purification step.

The first part of the optimization of this reaction involved a screening in homogeneous phase in order to optimize the enzyme concentration and the OYE3/GDH ratio needed to perform the reaction. The first test was carried out on 1.76 mg of substrate **7** in 20 µL of DMSO with OYE3 and GDH in the ratio of 1:1 (100 µL of each one) for a total of 980 µL of aqueous phase. The reaction vial was vertically oriented at 30 °C in a thermoshaker for 24 h. Then, different OYE3/GDH ratios were tested (Table 2). The screening resulted in a conversion >99% of the substrate for all the trials, but GC-MS showed that the cell free extract saturated both the C=C double bond and the carbonyl group, affording the corresponding saturated alcohol **10** (Scheme 4) as the major product. In this case, the alcohol was formed because of the use of a cell free extract, which contains ADHs from *E. coli* that reduce the carbonyl group of the aldehyde to alcohol.



Scheme 4. OYE - mediated reduction of (*E*)-**7** with cell free extracts in homogeneous phase.

Table 2. OYE3 – mediated reductions of (*E*)-**7** in homogeneous phase (20% DMSO) with different OYE3/GDH ratios.

Entry	OYE3/GDH ratio	Conversion [%]	Product distribution [%]	
			8	10
A	1:1	>99	7	93
B	1.5:1	>99	13	87
C	1:1.5	>99	6	94

To minimise the quantity of alcohol produced, a biphasic screening with different solvents was performed on 17.60 mg of substrate **7** with 1:1 OYE3/GDH ratio (200 µL of each one). Toluene, hexane, ethyl acetate and methyl tert-butyl ether (MTBE) were tested. 2000 µL of organic solution (0.05 M in the starting aldehyde **7**) were combined with 2000 µL an aqueous phase (containing 400 µL of a 1M aqueous solution of glucose and phosphate buffer, 1120 µL of phosphate buffer pH 7, 80 µL of NADP$^+$, 200 µL of OYE3 and 200 µL of GDH). With respect to the previous screening the concentration of the substrate in the organic phase was increased five times and the volume of aqueous phase was doubled. No complete conversion of the substrate was achieved and different values of selectivity towards the product **8** were obtained. The highest conversion and selectivity were observed for the reaction using MTBE as organic solvent (entry G, Table 3). Lower solubility of **7** in hexane with respect to MTBE was observed.

Table 3. OYE3 – mediated biphasic reductions of (*E*)-**7** with different solvents.

Entry	**Solvent**	**Conversion [%]**	**Product distribution [%]**		
			8	**10**	**other**
D	Toluene	7	83	17	-
E	Hexane	15	92	8	-
F	Ethyl acetate	3	14	3	83
G	MTBE	28	96	4	-

The next step of the optimization aimed to study the optimal OYE3/GDH ratio in biphasic condition with MTBE as organic solvent; it showed that the appropriate amount of enzymes to use was OYE3 and GDH in a ratio of 1:2 as a selectivity of 85% towards **8** was observed (Table 4).

Table 4. OYE3 – mediated biphasic reductions of (*E*)-**7** with different OYE3/GDH ratios.

Entry	**OYE3/GDH ratio**	**Conversion [%]**	**Product distribution [%]**	
			8	**10**
L	2:1	23	78	22
M	1:2	34	85	15

To avoid the formation of alcohol and favour the conversion of **7** to the needed product **8** it was considered the possibility to entrap the enzymes in a polymeric matrix in order to limit the formation of alcohol **10**. In order to do so, it was used a polyacrylate polymer, commonly known with the commercial name of "magic snow". The peculiarity of this polymer is its capacity to super expand and swell when in contact with the aqueous phase, forming microspheres in which the enzymes can be immobilised. To determine the quantity of polymer to be used, a blank run was performed. The blank run featured various quantities of polymer to be swelled with 5 mL of phosphate buffer and 5 mL of MTBE and left in the thermoshaker at 30 °C in order to study the behaviour of the mixture during time. The runs resulted in the discarding of two out of four trials, being the swelled polymer too dry or too watery (Figure 12). The two remaining samples (and also a new one with an intermediate quantity of polymer), were tested under both mechanic and magnetic stirring, in the presence of MTBE, in order to study the interaction of the swelled polymer with the organic solvent. For all the three cases, it was shown that the macroscopical structure of the swelled polymer did not collapse, retaining the aqueous phase and the separation from the organic layer. After studying the physical performance of the three blank samples, a screening was conducted in the same condition described above (Table 5) in order to verify the effect of the polymer presence on the reaction performance. Moreover, from now on, the reaction vials were horizontally oriented in the thermoshaker at 30 °C, in order to promote a more effective agitation. The adoption of 25 mg/mL of polymer significantly decreased the quantity of alcohol produced.

Table 5. OYE3 – mediated biphasic reductions of (*E*)-**7** with different polymer/aqueous phase ratios.

Entry	**Polymer/aqueous phase ratio [mg/mL]**	**Conversion [%]**	**Product distribution [%]**	
			8	**10**
N	25	62	92	8
O	30	21	81	19
P	40	16	83	17

Figure 12. Trials to assess the most suitable polymer/water ratio.

After determining the optimal quantity of polymer, it was conducted a screening in order to determine the most convenient organic/aqueous phase volume ratio (Table 6). The screening showed that the most convenient organic/aqueous phase ratio was 1:2 and was furtherly adopted for all the following reactions. Moreover, the screening showed only little production of alcohol even after 24 h. After finding the optimum parameters to avoid the production of alcohol the conversion was not satisfactory yet, stopping at almost 50% after 24 hours.

Table 6. OYE3 – mediated biphasic reductions of (*E*)-**7** with different organic/aqueous phase ratios.

Entry	**Organic/aqueous phase ratio**	**Conversion [%]**	**Product distribution [%]**	
			8	**10**
Q	1:1	25	84	16
R	1:2	42	86	14
S	3:2	20	80	20

Following an iterative process, the ratio OYE3/GDH was revised (Table 7) in order to improve the conversion and it was noted that the optimal ratio was 2:1, giving a higher conversion with respect to the previous conditions.

Table 7. OYE3 – mediated biphasic reductions of (*E*)-**7** with different OYE3/GDH ratios.

Entry	OYE3/GDH ratio	Conversion [%]	Product distribution [%]	
			8	**10**
T	1:2	54	83	17
U	2:1	72	90	10
V	1:1	61	80	20

In the attempt to reach complete conversion on a larger scale (660 mg of substrate **7**), it was taken into account the possibility to do consecutive additions of enzymes to the reaction. This practice was beneficial and permitted to reach almost complete conversion in 4 days with a production of alcohol lower than 1% (Table 8), thanks to the possibility to switch from orbital stirring to the mechanical one thanks to a Teflon® stirrer, which guaranteed a homogeneous mixing between organic and aqueous phases.

Moreover, thanks to scaling effect and the consecutive additions of enzymes, it was possible to optimize the consumption of OYE3 and GDH, diminishing the expenditure up to 75% with respect the first optimized reaction.

Table 8. Optimized conditions for the production of **8**.

Solvent	Concentration of 7 in the org. phase [mM]	OYE3/ GDH ratio	Polymer/aq. phase ratio [mg/mL]	Organic/aq. phase initial ratio	Tot OYE3 [mL]	χ [%]	Product distribution [%]	
							8	**10**
MTBE	50	2:1	25	1:2	16	98	99	<1

After the optimization of the conditions to take advantage of the enzyme entrapped in polyacrylate, it was tried to use it in continuous flow, loading the swelled polymer in a column. Being the column packing not optimal, it was decided to investigate other enzyme entrapment methods, such as sodium alginate spheres crosslinked with calcium chloride.

A blank run was made as a first trial. To better investigate the diffusion phenomenon, a food colorant was added to a 1% wt. solution of sodium alginate in water and subsequently spherified (Figure 13a and 13b), with the help of a needleless syringe, in a crosslinking agent such as an aqueous solution 2% wt. of calcium chloride. The dripping of the alginate resulted quite challenging as some air was left entrapped in the spheres due to the syringe nozzle conformation. After the crosslinking, the spheres were recovered from the calcium chloride solution, added to a vial containing MTBE and left in a thermoshaker at 30°C. After 24 hours it was showed that the spheres were still intact but slightly lost their colour (Figure 14).

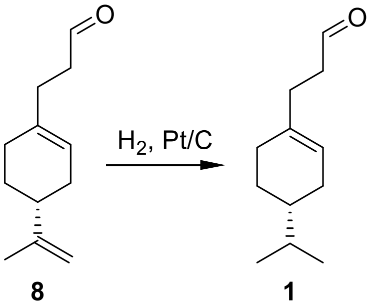
Figure 13a and 13b. Alginate spheres containing food-colouring.

Figure 14. Discoloured sodium alginate spheres in MTBE.

The first test was conducted with a model substrate in which the C=C double bond can be easily reduced, α-methylcinnamaldehyde. The needed amount of aqueous phase, including enzymes, were entrapped in sodium alginate spheres and packed up to six centimetres in the glass column of a continuous flow reactor, which operated at a flow of 100 µL/min. This reaction showed no conversion.

Furthermore, it was performed a trial with mechanical and magnetic stirring. The mechanical stirring showed no conversion, although the spheres were still intact; the reaction with magnetic stirring showed a conversion of 11% after 48 h. To this moment, the entrapment of the enzyme in a natural polymer has not had the desired effect and need further optimization.

3.7 Continuous reactor hydrogenation



Scheme 5. Hydrogenation reaction to obtain Lilybelle®.

After obtaining aldehyde **8**, the last step of selective hydrogenation of the isopropyl C=C double bond was performed in order to achieve Lilybelle® **1** (Scheme 5). In Symrise's patent [17], the hydrogenation of the isopropenyl moiety was performed on starting limonene with 78% yield, probably in order to reduce separation costs. Nevertheless, we decided to postpone the hydrogenation to the last step, in order to reduce the amount of catalyst employed given the lower volume of material to be manipulated. The high selectivity of the Pt/C catalyst employed enabled to achieve satisfactory results in this step.

The hydrogenation was performed using an H-Cube® reactor (Figure 15), which allows to set a wide range of reaction parameters, such as flow rate, pressure, temperature and catalyst used. The catalyst consists into various size cartridges (Figure 16) packed with suitable catalysts, such as Pd/C, Raney Ni and Pt/C. The latter was used in this step. The hydrogen needed in this reaction is provided by a high-efficiency electrolysis cell, making the overall step safer, simpler and relatively environmentally friendly with respect to conventional hydrogenation processes.

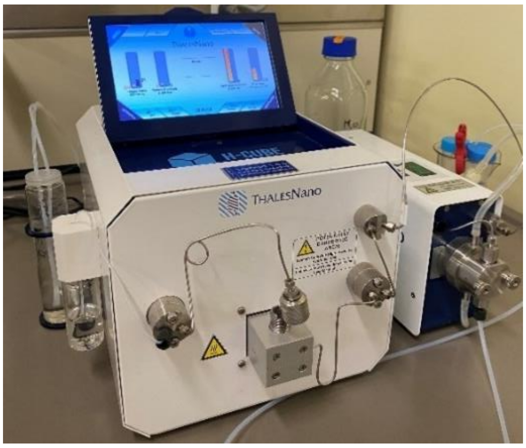
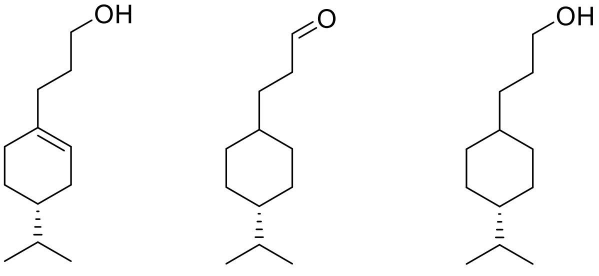


Figure 15. H-Cube® reactor.

Figure 16. ThalesNano cartridge.

The main undesired by-products of the reaction are the alcohol **10** and **12** and the saturated aldehyde **11** represented in figure 17a, 17b and 17c. A large number of tests were conducted in order to optimize the process and minimise the formation of these byproducts. The tests were carried out at ambient temperature. The tested conditions are reported in Table 9.



10 **11** **12**

Figure 17a, 17b and 17c. Undesired subproducts of hydrogenation reaction from **8** to **1**.

Table 9. Parameters of Pt/C catalysed hydrogenation in the continuous-flow reactor.

Entry	Flowrate [mL/min]	Concentration [M]	Pressure [bar]	Conversion [%]	Product distribution [%]			
					11	**1**	**12**	**10**
1	0.3	0.025	10	>99	24	5	64	7
2	0.3	0.025	1	>99	26	40	19	15
3	0.5	0.025	1	>99	14	43	21	22
4	0.7	0.025	1	>99	9	49	14	28
5	2.5	0.025	1	89	7	64	9	20
6	2.5	0.025	5	88	7	69	7	17
7	2.5	0.025	10	90	13	63	13	11

The table shows that entries 5, 6 and 7, despite having the lowest conversion, have the best selectivity. Thus, having the first four entries a complete conversion but a very low selectivity, the trade-off between these two parameters is entry 6 .

3.8 Enzymatic oxidation with ADHs

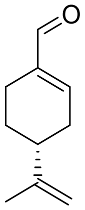
The chemical oxidation involving MnO_2 is par excellence the way to oxidise allylic alcohols to give the correspondent α,β-unsaturated aldehydes. In order to optimize and make the step from **6** to **7** more sustainable and greener, it was developed an enzymatic pathway with the involvement of ADHs to substitute the oxidising agent. A preliminary monophasic screening was conducted with four types of ADH and **6** initially dissolved in dimethyl sulfoxide (DMSO); in a second screening, **6** was dissolved in acetone. The latter screening showed activity for only two ADHs out of four, namely ADH-A and ADH-Res, which were later then utilised in a biphasic screening with various solvents. Out of toluene, hexane, ethyl acetate and methyl tert-butyl ether, only the reaction conducted in hexane showed appreciable conversion. The synthetic pathway from **6** to **8** was then optimized in a cascade reaction. The reaction converting **6** to **7** was performed in hexane and the two aforementioned ADHs. The reaction was the centrifuged and the organic phase collected and used with the polymer, OYE3 and GDH in order to perform the reaction from **7** to **8**. Aldehyde **8** was then extracted, concentrated and used in the last step to obtain Lilybelle® **1**.

4. Materials and methods

4.1 General methods

Chemicals and solvents were purchased from Merck Life Science s.r.l. (Milan, Italy) and used without further purification. TLC analyses were performed on Macherey Nagel pre-coated TLC sheets Polygram® SIL G/UV254 purchased from Chimikart s.r.l (Naples, Italy) and spots were visualized by UV light (254 nm). The continuous flow reactions were performed using an E-Series Integrated Flow Chemistry system from Vapourtec (Alfatech s.p.a., Genoa, Italy) 10 mL standard coiled tubular reactor (ambient to 150°C temperature). H-CUBE MINI PLUS used for hydrogenation reactions was provided by ThalesNano Inc. (Budapest, 7 Zahony Street, Graphisoft ParK). ^1H NMR and ^{13}C NMR spectra were recorded on a 400 MHz spectrometer in $CDCl_3$ solution at r.t. unless otherwise specified; chemical shifts δ are expressed in ppm and coupling constants are in Hz. The chemical shift scale was based on internal tetramethylsilane. GC/MS analyses were performed using an HP5MS column (30 m × 0.25 mm × 0.25 μm, Agilent Technologies Italia s.p.a., Cernusco sul Naviglio, Italy). The following program temperature was used: 60 °C (1 min)/6 °C min^{-1}/150 °C (1 min)/12 °C min^{-1}/280 °C (5 min).

4.2 (*R*)-4-(prop-1-en-2-yl)cyclohex-1-ene-1-carbaldehyde (**4**)



4

To a solution of **3** (500 mM, 10 μL) in DMSO in a 2 mL Eppendorf vial 10 μL of acetone, an aq. solution of NADH (10 mM, 10 μL), an aq. solution of $NADP^+$ (10 mM, 10 μL), a KPi buffer solution (pH 7.0, 50 mM, 870 μL) and ADH (100 μL of a 5 mg/mL of commercial ADH or 100 μL of cell free extract of in-house produced recombinant ADHs) (see table below) were added.

The vials were closed and incubated at 30°C for 24 hours with shaking at 150 rpm. After incubation, 600 μL of ethyl acetate (EtOAc) was added to each vial and the solutions were centrifuged at 11000 rpm for 3 minutes. The organic phases were separated, dried with a small amount of Na_2SO_4, and analyzed by GC-MS.

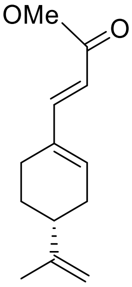
Tables 10a, 10b, 10c. ADHs utilized in the screening.

Evoxx
Evo030
Evo200
Evo270
Evo440
Evo010
Evo020
Evo040
Evo130
Evo140
Evo260
Evo380
Evo420
Evo190
Evo210
Evo250

CFE ADHs
Res from *Rhodococcus erythropolis*
A from *Rhodococcus ruber*
hT from *Bacillus stearothermophilus*
Sy from *Synechocystis*

Sigma Aldrich ADHs
Dr from *Deinococcus radiodurans*
Pl from *Parvibaculum lavamentivorans*

4.3 Methyl(*R*,*E*,-3-(4-(prop-1-en-2- yl)cyclohex-1-en-1-yl)acrylate (**5**)



5

In a three-necked, round-bottomed flask fitted with a dropping funnel and a thermometer was charged with 60 mL of THF and 0.88 g of NaH (60% in mineral oil) (1.10 eq), 3.4 mL of trimethyl phosphonoacetate (1.05 eq) were added drop wise. The suspension was mechanically stirred for 30 minutes with a mechanical stirrer while being cooled with an ice bath. Subsequently, 3.1 mL of (+)-perillaldehyde **4** were added dropwise (1.0 eq). The reaction was followed through TLC (hexane/EtOAc 9:1). The mixture was quenched with HCl 1M (100 mL) and water and extracted with ethyl acetate (3x25 mL). The organic phase was washed with a saturated NaCl solution, dried over anhydrous Na_2SO_4 and concentrated under vacuum to obtain **5.**

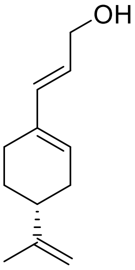
Yield: 91% (3.7 g) as amber oil

^{1}**H-NMR** ($CDCl_3$,400 MHz) δ: 7.31 (d, *J*=15.8 Hz, 1H), 6.18 (m, 1H), 5.77 (d, *J*=15.8 Hz, 1H), 4.76-4.71 (2m, 2H), 3.75 (s, 3H), 2.37-2.09 (m, 5H), 1.92 (m, 1H), 1.75 (s, 3H), 1.53 (m, 1H);

^{13}**C{^{1}H}-NMR** ($CDCl_3$,101 MHz) δ: 168.1, 149.0, 147.7, 138.2, 134.7, 114.7, 109.2, 51.5, 40.8, 31.9, 27.1, 24.6, 20.8;

GC-MS: t_R = 21.32 min, MS (EI) m/z = 206 (69), 191 (13), 175 (16), 163 (18), 147 (33), 79 (100).

4.4 (*R*,*E*,-3-(4-(prop-1-en-2-yl)cyclohex-1-en1-yl)prop-2-en-1-ol (**6**)



6

A three-necked, round-bottomed flask fitted with a dropping funnel and a thermometer was charged with the aldehyde **5** obtained from the previous reaction dissolved in 90 mL of THF. The solution was cooled to 0 °C and DIBAL-H (25% sol. in toluene, 2.2 eq) wasadded dropwise. The reaction was stirred for 30 minutes and followed through TLC (hexane/EtOAc 9:1). The mixture was quenched with HCl 1M (100 mL) and water and extracted with ethyl acetate (3x25 mL). The organic phases were washed with a saturated NaCl solution, dried over anhydrous Na_2SO_4 and concentrated under vacuum to obtain **6**.

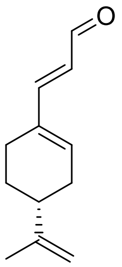
Yield: 90% (2.9 g) as yellow oil.

^{1}**H-NMR** ($CDCl_3$,400 MHz) δ: 6.25 (d, *J*=15.7 Hz, 1H), 5.78 (m, 1H), 5.69 (dt, J=15.7 and 6.1, 1H), 4.77-4.69 (m, 2H), 4.19 (d, *J*=6.1 Hz, 2H), 2.37-2.02 (m, 5H), 1.90 (m, 1H), 1.75 (s, 3H), 1.59-1.44 (m, 1H);

^{13}**C{^{1}H}-NMR** ($CDCl_3$,101 MHz) δ: 149.6, 134.8, 134.7, 129.2, 124.8, 108.8, 63.8, 41.2, 31.3, 27.4, 25.0, 30.8.

GC-MS: t_R = 19.76 min, MS (EI) m/z =178 (13), 160 (15), 145 (25), 134 (34), 105 (44), 79 (100).

4.5 (*R*,*E*,-3-(4-(prop-1-en-2-yl)cyclohex-1-en1-yl)acrylaldehyde (**7**)



7

To a solution of the alcohol **6** (2.9 g) in dichloromethane (30 mL) is added activated MnO_2 (5.0 eq). The reaction is left stirred at room temperature for 24 hours and followed through TLC (hexane/EtOAc 9:1). To increment the conversion, another 5.0 eq of MnO_2 are added and the reaction is heated to 40 °C for 4 hours. The reaction is then filtered over celite and washed with dichloromethane. The filtrate is concentrated under vacuum and purified by silica gel column chromatography with hexane/ethyl acetate (60:40) to obtain **7**.

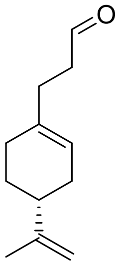
Yield: 64% (1.8 g) as pale-yellow oil.

^{1}**H-NMR** ($CDCl_3$,400 MHz) δ: 9.56 (d, *J*=7.8 Hz, 1H), 7.10 (d, *J*=15.6 Hz, 1H), 6.32 (m, 1H), 6.08 (dd, *J*=15.6 and 7.8 Hz, 1H), 4.81-4.69 (2m, 2H), 2.45-2.14 (m, 5H), 1.95 (m, 1H), 1.76 (s, 3H), 1.56 (m, 1H);

^{13}**C{^{1}H}-NMR** ($CDCl_3$,101 MHz) δ: 194.2, 155.6, 148.6, 140.5, 135.1, 126.1, 109.3, 40.5, 32.0, 26.8, 24.6, 20.7.

GC-MS: t_R = 20.01 min, MS (EI) m/z = 176 (53), 133 (59), 91 (71), 79 (100).

4.6 (*R*)-3-(4-(prop-1-en-2-yl)cyclohex-1-en-1-yl)propanal (**8**)



8

A three-necked, round-bottomed flask was charged with 15 mL of a solution 1M of glucose in water, 84 mL of phosphate buffer pH 7 100 mM, 1000 μL of $NADP^+$, 6000 μL of OYE3 and 3000 μL of GDH. 3750 mg of polymer were then added to the aqueous phase in order to swell the polymer and entrap the enzymes. 660 mg of aldehyde **7** were dissolved in 75 mL of MTBE and added to the flask. The flask was placed in a water bath at the constant temperature of 30 °C and mechanically stirred using a Teflon paddle agitator for 96 hours. The reaction was followed through TLC and GC-MS. After 24 hours, 6000 μL of OYE3 and 3000 μL of GDH were added. 48 hours after the start of the reaction, 3000 μL of OYE3 and 1500 μL of GDH were added to enhance conversion. After 24 hours, another final adding of 1000 μL of OYE3 and 500 μL of GDH was done. Complete conversion is reached after 96 hours from the start and the reaction mixture is centrifuged for 15 minutes at 7000 rpm. A large part of the polymer precipitates to the bottom of bottle, meanwhile the liquid phase is extracted in a funnel in order to recover the organic phase. The reaction is dried over anhydrous Na_2SO_4 and concentrated under vacuum to obtain **8**.

Yield: 85% (560 mg) as pale-yellow oil.

^{1}H-NMR ($CDCl_3$,400 MHz) δ: 9.75 (t, *J*=1.9 Hz, 1H), 5.43 (m, 1H), 4.73-4.66 (m, 2H), 2.56-2.47 (m, 2H), 2.32-2.24 (m, 2H), 2.15-1.88 (m, 5H), 1.81 (m, 1H), 1.72 (s, 3H), 1.46 (m, 1H);

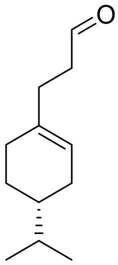
^{13}C{^{1}H}-NMR ($CDCl_3$,101 MHz) δ: 202.7, 150.0, 135.6, 121.6, 108.7, 42.0, 41.1, 30.8, 29.9, 29.0, 27.8, 20.9

GC-MS: t_R = 16.7 min, MS (EI) m/z = 180 (2), 137 (30), 93 (55), 69 (200), 53 (59).

4.6.1 Continuous flow enzymatic reactions

0.147 g of sodium alginate were put in a becker and dissolved in 14.7 mL of aqueous phase containing 1500 μL of a 1M solution of glucose, 8400 μL of phosphate buffer pH 7 100 mM, 300 μL of NAD(P), 3000 mL of OYE3 and 1500 μL of GDH in order to obtain a 1% wt. solution, with the help of a heating and stirring plate. When the sodium alginate solution was homogeneous, it was withdrawn with a needleless syringe and pushed out through the nozzle in a crosslinker solution of 2 g of calcium chloride in 100 ml of water (2%wt.). The results were spheres in which the enzymes were immobilised. The spheres were recovered from the calcium chloride solution and filtered to help eliminate the residual water. The spheres were added to a one- necked balloon and stirred with mechanical and magnetic agitation and sampled every 24 hours through GC-MS.

4.7 (*R*)-3-(4-isopropylcyclohex-1-en-1-yl)propanal (**1**)



1

The H-Cube Mini Plus hydrogen reactor from Thales Nano was used to perform the catalytic reactions. Before each use, mandatory preparatory steps were taken, including filling the water tank reservoir with Milli-Q® water, cleaning all circuits with isopropyl alcohol, and filling the pump cleaning system with a 1:1 mixture of isopropyl alcohol and deionized water. All the solvents and the substrate **8** dissolved in EtOAc were filtered through nylon filters. After multiple cleaning cycles and ensuring no residual material was left in the reactor, which can be seen through the value of the residual pressure in the blank cartridge, a 35 mm catalyst cartridge was inserted into the allotted space. The reactor inlet tube was then inserted into a flask containing the solvent to be used for the reaction, in which the float is immersed in order to withdraw it. After the desired reaction's parameters were set, including pressure, temperature, and flow rate, the hydrogen pressure was set automatically by the system and the reactor initiated a preparatory cycle that ended with the stabilization of the reactor at the chosen conditions. The solvent flask was then quickly replaced with the reaction mixture flask, in order to limit the introduction of air through the float. After the dead volume time, strictly dependent on different flow rates, samples were collected and brought to the GC-MS test. For all the reactions performed, 35 mm Pt/C cartridges were used provided by Thales Nano. All reactions were performed at ambient temperature on 11 ml samples. The various values reaction parameters are already described in results sections.

5. Conclusions

In this work the challenging synthesis of the commercial fragrance Lilybelle® was investigated using as a starting material (+)-(*R*)-limonene, recovered from citrus peels, and introducing biocatalysed steps as more sustainable alternatives to classical chemical transformations. The synthetic procedure to Lilybelle®, planned during this thesis work, includes two allylic alcohol oxidations (compound **3** into **4**, compound **6** into **7**), that were carried out by using suitable ADHs instead of MnO_2. Mn(IV) oxidation of allylic alcohols is a classical high yield chemical reaction, but a large excess of activated MnO_2 over the stoichiometric quantity is required, generating high amount of waste. The ADH-mediated oxidation consumes acetone as a sacrificial substrate in stoichiometric quantity, producing isopropanol as by-product. Another relevant step of the synthetic procedure is represented by the hydrogenation of the unsaturated aldehyde **7** to the corresponding derivative **8**. The use of an ER as a catalyst for this conversion takes advantage of the excellent regioselectivity of this kind of biocatalysed transformation: the mechanism of the reaction is such that only the C=C double conjugated to the carbonyl group is reduced, leaving unaltered the other alkene moieties. Higher selectivity gives higher purity of the products and lower amount of waste generated in the final purification step. Furthermore, the sacrificial substrate of the reaction is glucose producing readily biodegradable gluconolactone as a by-product. Both the ADH oxidations and the ER-reduction occur at atmospheric pressure, at 30°C, with cell free lysates, avoiding expensive and time-consuming enzyme purification. Enzymes are themselves renewable, and the organic biomass remaining after their use can be easily disposed of.

The entrapment of the ER in the polyacrylate matrix enhanced the selectivity of the reaction, avoiding side reactions able to affect the purity of the final compound. Future work will be devoted to use a biodegradable natural polymer for entrapping the ER., and to implement the reaction in a packed – bed reactor for enzyme recovery and re-use.

Hydrogenation with molecular hydrogen in the presence of Pt/C had to be employed for the conversion of the isopropenyl unit into the isopropyl fragment. The reaction was placed at the end of the synthetic sequence, when a lower amount of material is manipulated, for two main reasons: firstly, to reduce the amount of metal catalyst to be used, and secondly, to reduce the waste generated by the incomplete selectivity of the reduction.

A lot of work still needs to be done in order to optimize the cascade reaction from **6** to **8**, in order to lessen the energy and solvents consumption, to optimise the enzymatic reaction from **7** to **8** in continuous flow, and to improve the selectivity of the last step.

Overall, this work demonstrated that is possible to produce useful compounds, supporting circular economy and giving value to cheap subproducts, and support sustainability and waste reduction through environmentally friendly procedures.

Acknowledgements

I would like to extend my heartfelt thanks to Professor Elisabetta Brenna for her endless patience and for providing invaluable knowledge and expertise, Professor Fabio Parmeggiani for his precious help and the whole BiocatLab for any advice given.

References

[1] M. Intelligence. [Online]. Available: https://www.mordorintelligence.com/industry-reports/fragrance-and-perfume-market.

[2] F. Michailidou, “The Scent of Change: Sustainable Fragrances Through Industrial Biotechnology,” *ChemBioChem*, no. 24, pp. 1-16, 2023.

[3] United Nations, “Academic Impact,” [Online]. Available: https://www.un.org/en/academic-impact/sustainability.

[4] IFRA-IOFI, “Sustainability Report,” 2023.

[5] Iberchem, “Upcycled Fragrances: Breathing New Life into Traditional Waste Materials,” Iberchem, [Online]. Available: https://iberchem.com/upcycled-fragrances/.

[6] Symrise. [Online]. Available: https://www.symrise.com/sustainability/innovation/#sustainable-solutions.

[7] Symrise. [Online]. Available: https://www.symrise.com/sustainability/our-approach/#our-contribution-to-the-sdgs.

[8] M. Lecourt and S. Antoniotti, “Chemistry, Sustainability and Naturality of Perfumery Biotech Ingredients,” *ChemSusChem*, no. 13, pp. 5600-5610, 2020.

[9] Givaudan. [Online]. Available: https://www.givaudan.com/sustainability/sustainable-business/sustainable-development-goals.

[10] E. Eichhorn, C. Baumgartner and M. Biermann, "Biotechnology – A Tool to Transform Givaudan's Fragrance Ingredients Palette," *Chimia,* no. 77, pp. 384-389, 2023.

[11] R. Sheldon and D. Brady, "Broadening the Scope of Biocatalysis in Sustainable Organic Synthesis," *ChemSusChem,* vol. 12, pp. 2859-2881, 2019.

[12] J. L. Tucker and M. M. Faul, "Drug companies must adopt green chemistry," *Nature,* vol. 534, pp. 27-29, 2016.

[13] F. Parmeggiani, E. Brenna, D. Colombo, F. Gatti, F. Tentori and D. Tessaro, ""A Study in Yellow": Investigations in the Stereoselectivity of Ene-Reductases," *ChemBioChem,* vol. 23, pp. 1-10, 2022.

[14] Q. e. a. Shi, "Old yellow enzymes: structures and structure-guided engineering for stereocomplementary bioreduction," *Applied Microbiology and Biotechnology,* vol. 104, no. 19, pp. 8155-8170, 2020.

[15] M. Boelens, H. Wobben and J. Heydel, "Muguet in perfumery a review of lily of the valley," Allured publishing, 1980.

[16] R. Ramachanderan and B. Schaefer, "Lily-of-the-valley fragrances," *ChemTexts,* vol. 5, no. 11, pp. 1-34, 2019.

[17] S. AG, "Fragrances with note of lily of the valley". United States Patent US 2013/0090390 A1, 11 April 2013.

[18] "CosmeticOBS-L'Observatoire des Cosmétiques," [Online]. Available: https://cosmeticobs.com/en/articles/news-59/when-symrise-recreates-the-scent-of-the-lily-of-the-valley-3932.

[19] P. Zambelli, "A biocatalytic approach for the synthesis of Lilybelle and other fragrances starting from citrus industry by-products," 2023.

[20] R. Tomar, S. Jain, P. Yadav, T. Bajaj, F. Mohajer and G. Ziarani, "Conversion of Limonene over Heterogeneous Catalysis: An Overview.," *Curr Org Synth,* vol. 19, no. 3, pp. 414-425, 2022.

[21] S. Suri, A. Singh and P. Nema, "Current applications of citrus fruit processing waste: A scientific outlook," *Applied Food Research,* vol. 2, 2022.

[22] D. Zema, P. Calabrò, A. Folino, V. Tamburino, G. Zappia and S. Zimbone, "Valorisation of citrus processing waste: A review," *Waste Management,* vol. 80, pp. 252-273, 2018.

[23] U. Nations, "Sustainable development- Chemicals and waste," [Online]. Available: https://sdgs.un.org/topics/chemicals-and-waste.

[24] K. Geoghegan and P. Evans, “Synthesis of (+)-perillyl alcohol from (+)-limonene,” *Tetrahedron Letters,* vol. 55, no. 8, pp. 1431-1433, 2014.

[25] P. Cannazza, M. Rabuffetti and S. e. a. Donzella, “Whole cells of recombinant CYP153A6-E. coli as biocatalyst for regioselective hydroxylation of monoterpenes.,” *AMB Expr,* vol. 12, no. 48, 2022.

[26] E. Fiocca, “Sintesi e riduzione biocatalitica enantioselettiva di alcheni attivati con sostituenti trifluorometilici,” 2021.

[27] Z. Liu, Y. Gong, S. Byun and R. Bittman, “An improved two-step synthetic route to primary allylic alcohols from aldehydes.,” *New Journal of Chemistry,* vol. 34, no. 3, pp. 470-475, 2010.

[28] [Online]. Available: https://odebohome.in/blogs/news/green-chemistry.