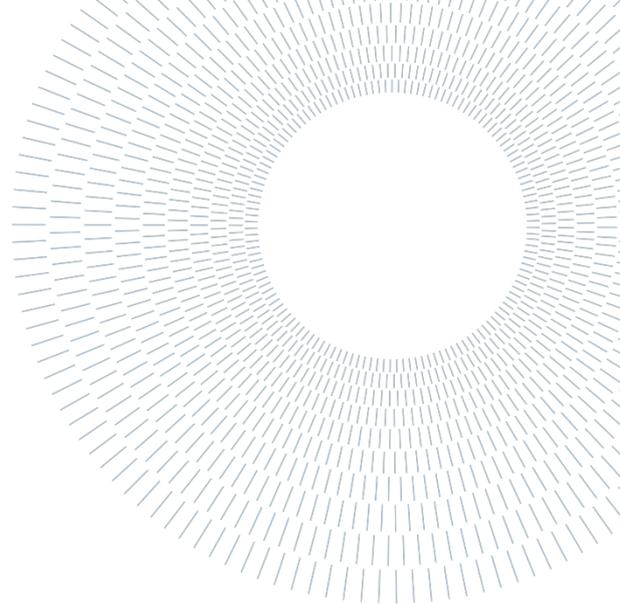




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EXECUTIVE SUMMARY OF THE THESIS

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

TESI MAGISTRALE IN FOOD ENGINEERING – INGEGNERIA ALIMENTARE

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1. Introduction

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.

1.1 Upcycling and biotechnology

Upcycling is a key component of the circular economy model and it consists in the repurposing of waste materials in order to produce high-value

fragrance ingredients 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 process them and extract essential oils that can be used in fragrance formulations [1]. 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 synthesise products at an 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.

1.2 Biocatalysis

Producing natural products by biotechnology complies with the main principles of green chemistry. Enzymes exhibit high chemo-, regio-, and stereoselectivities that are difficult to achieve with standard organic synthesis methods. In short, enzyme-based catalysis is safe, selective and sustainable. Enzymes can catalyze 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.

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.

1.3 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 [2].

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.

The vast 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.

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.

1.5 Lily-of-the-valley fragrances

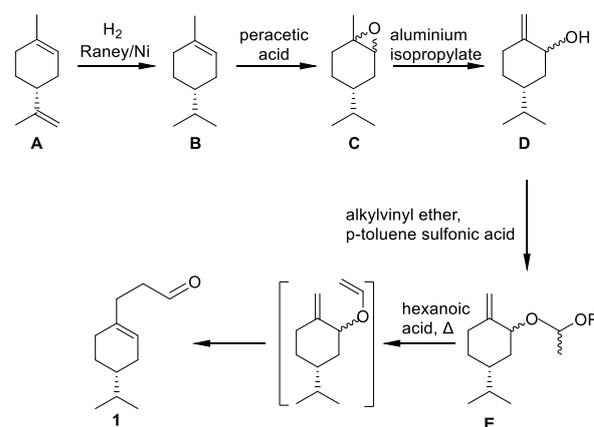
The aroma of lily-of-the-valley is composed of several volatile organic compounds (VOCs), including linalool, geraniol, and alpha-terpineol which confer to the fragrance its fresh, green, flowery and aldehydic muguet notes [3].

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. To address this challenging growing

demand, perfumers have resorted to replicating as closely as possible lily-of-the-valley scent.

1.4 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 [4].



Scheme 1. Lilybelle® pathway patented by Symrise [4].

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 [3].

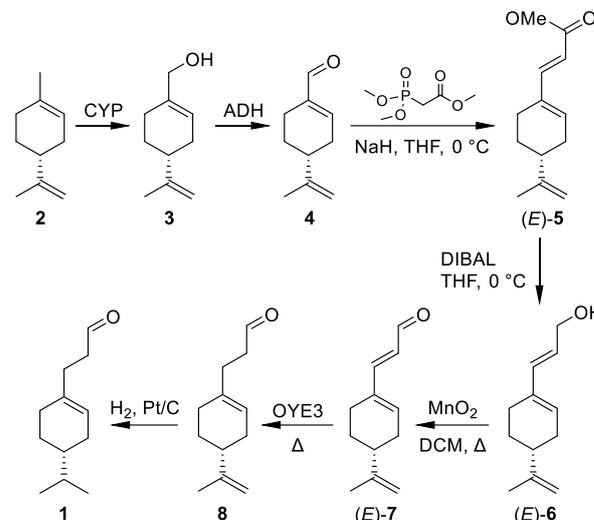
In the first stage of the synthesis proposed by Symrise, the isopropenyl moiety of limonene, sustainable starting material accessible from orange peels, is hydrogenated selectively with molecular hydrogen in the presence of Raney-Nichel. After epoxidation, the resulting oxirane is rearranged to an allyl 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. 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 characterized 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.

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. 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 MnO₂. 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**.



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

3. Results and discussion

3.1 Preparation of α,β -unsaturated aldehyde **7**

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 [5]. The research group of Diego Romano (Università degli Studi di Milano) described a synthetic path that involves biocatalysis; 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. The aforementioned alcohol was submitted to a first screening 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). The screening resulted in the identification of an ADH that converts 100% of **3** in **4**.

The next 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 stabilized phosphonate carbanion; the corresponding

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. To determine the quantity of polymer to be used, a screening was conducted in order to verify the effect of the polymer presence on the reaction performance. The adoption of 25 mg/mL of polymer significantly decreased the quantity of alcohol produced (Table 3).

Table 3. Result of the screening with various polymer/aqueous phase ratios.

Polymer/aq. phase ratio [mg/mL]	Conversion [%]	Product distribution [%]	
		4	10
25	62	92	8

3.2.5 organic/aqueous phase volume 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. The screening showed that the most convenient organic/aqueous phase ratio was 1:2 (Table 4) and was further adopted for all the following reactions.

Table 4. with different organic/aqueous phase ratios.

Organic/aq. phase ratio	Conversion [%]	Product distribution [%]	
		4	10
1:2	42	86	14

3.2.6 Optimization of OYE3/GDH ratio

Following an iterative process, the ratio OYE3/GDH was revised (Table 5) 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 5. Result of the screening with different OYE3/GDH ratios.

OYE3/GDH ratio	Conversion [%]	Product distribution [%]	
		4	10
2:1	72	90	10

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

In the attempt to reach complete conversion (Table 6) on a larger scale, 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%. 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 to the first optimized reaction.

Table 6. Optimized conditions for the production of **8**.

Conditions	
Solvent	MTBE
Molarity of 7 in the org. phase [mM]	50
OYE3/GDH ratio	2:1
Polymer/aq. phase ratio [mg/mL]	25
Organic/aq. phase initial ratio	1:2
Conversion [%]	98

3.3 Continuous flow hydrogenation

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**. In Symrise’s patent [4], the hydrogenation of the isopropenyl moiety was performed on starting limonene; 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 us to achieve satisfactory results in this step.

Table 7. Parameters of Pt/C catalysed hydrogenation in the continuous-flow reactor.

Conditions	
Flowrate [mL/min]	2.5
Concentration [M]	0.025
Pressure [bar]	5
Conversion [%]	88
Product distribution [%]	69 of 1 31 of others

4. 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 MnO₂. Mn(IV) oxidation of allylic alcohols is a classical high yield chemical reaction, but a large excess of activated MnO₂ 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 using 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.

Bibliography

- [1] F. Michailidou, "The Scent of Change: Sustainable Fragrances Through Industrial Biotechnology," *ChemBioChem*, no. 24, pp. 1-16, 2023.
- [2] 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.
- [3] R. Ramachandran and B. Schaefer, "Lily-of-the-valley fragrances," *ChemTexts*, vol. 5, no. 11, pp. 1-34, 2019.
- [4] Symrise, "Fragrances with note of lily of the valley". United States Patent US 2013/0090390 A1, 11 April 2013.
- [5] K. Geoghegan and P. Evans, "Synthesis of (+)-perillyl alcohol from (+)-limonene," *Tetrahedron Letters*, vol. 55, no. 8, pp. 1431-1433, 2014.

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