Biocatalytic modification of monoterpenes using waste-derived yeast cells

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This PhD thesis focused on the development of a sustainable process for the reduction of perillaldehyde to perillyl alcohol (POH) by employing yeast cells immobilized into calcium alginate beads (entrapment technology). After optimizing the reaction conditions in small-scale batch reactions, process was scaled in a rotating bed reactor (SpinChem) reaching 90% of molar conversion. The biomass used as biocatalyst was produced starting from waste material and using seawater instead of the fresh one. By harnessing the potential of immobilized yeast cells in a rotating reactor and employing environmentally friendly resources, this study exemplified a sustainable biocatalytic approach with the potential to be expanded to other natural molecules.

Processo sostenibile per la modifica di terpenoidi utilizzando lieviti immobilizzati

Questa tesi di dottorato si è concentrata sullo sviluppo di un processo sostenibile per la produzione di POH utilizzando cellule intere di lievito immobilizzate via intrappolamento in idrosfere di alginato. Dopo aver ottimizzato le condizioni in batch (1-2 mL), la reazione è stata scalata in un reattore a letto rotante (SpinChem) raggiungendo il 90% di conversione molare. La biomassa utilizzata come biocatalizzatore è stata prodotta partendo da materiale di scarto e utilizzando acqua di mare anziché acqua distillata. Sfruttando il potenziale di cellule immobilizzate in reattore e impiegando materiale eco-sostenibile, questo studio è un esempio di approccio biocatalitico sostenibile con il potenziale di essere esteso a diverse molecole di origine naturale.

**Key words**: Biocatalysis, rotating bed reactor, monoterpenoids, circular economy, enzyme immobilization.

# **1. Introduction**

This oral communication reports the main results of the following four activities directed to:

A1) Screening of yeast collections to identify strains able to modify monoterpenes;

A2) Optimizing the biotransformation conditions in small scale batch reactions;

A3) Scaling-up the process in a rotating bed reactor (SpinChem);

A4) Enhancing the sustainability of the whole process by utilizing of waste materials and seawater.

**2. Whole cells biocatalysis**

Biocatalysis involves the use of biological systems (whole cells or enzymes) to catalyse chemical reactions. This approach has gained attraction in various industries, including pharmaceuticals, chemicals, cosmetics, agriculture, and food companies, due to its potential for sustainable, energy-efficient, and environmentally friendly production processes. Indeed, enzymes operate under mild reaction conditions, such as moderate temperatures, water media (neutral pH), and atmospheric pressure, preserving the biocatalyst activity and avoiding the massive use of solvents. Enzymes can be utilized as isolated proteins, cellular extracts, or within whole cells, either free or immobilized. Isolated enzymes require a long and usually expensive process for their purification, and their recovery and reuse are difficult. On the contrary, whole cells do not require purification steps and can be easily recovered from the culture medium, thus reducing production time and costs (Schrewe *et al*., 2012). In addition, the use of whole cells is usually preferred for reactions involving cofactors as they can synthesize and recycle cofactors themselves (Haque *et al.*, 2019; Filippucci *et al.*, 2020). However, cells present intrinsic metabolic pathways, so competitive secondary reactions due to the presence of other enzymes may occur with the risk of reduced final yields as well as the formation of undesired by-products.

To enhance the usability of whole cells, immobilization techniques can be employed, such as encapsulating or immobilizing them within hydrogel beads that serve as carriers for the biocatalysts. These hydrogel beads provide a protective environment for the cells, allowing them to retain their activity and stability during the reaction process. The immobilization also facilitates the isolation of the product and the separation of the catalyst from the reaction mixture, simplifying downstream processing for industrial scale-up (Rodrigues and de Carvalho, 2022).

Moreover, the use of immobilized catalysts in the SpinChem reactor system, which efficiently facilitates liquid percolation through packed particle bed within the stirring element, presents additional benefits (Mallin *et al*., 2013).

Overall, biocatalysis offers established and significant advantages and continues to be explored and optimized for various applications, driving the shift towards sustainable and environmentally conscious production processes.

# **3. Modification of monoterpenes**

Terpenoids are a diverse group of natural compounds primarily derived from plants, that find applications in various industries such as food, pharmaceuticals, and cosmetic ones. The use of yeast whole cells for the biotransformation of certain monoterpenoids, including carvone, geraniol and limonene, into highly valuable flavoring derivatives has been studied due to their economic potential in the food and beverage, perfume, and soap industry (Goretti *et al*., 2011; van Beilen *et al.*, 2005). Perillaldehyde is a terpenoid found in various plants and essential oils, with perilla herb being the most abundant source. It is commonly used as a food additive for flavoring and in perfumery to provide a spicy aroma. However, in 2015, the European Food Safety Authority (EFSA) conducted an evaluation and determined that perillaldehyde demonstrated genotoxic potential in vivo, raising safety concerns as a flavoring substance (Hobbs *et al.*, 2016; Erhunmwunsee *et al.*, 2021). Subsequently, the European Commission announced its intention to remove perillaldehyde from the EU list of flavorings (http://eur-lex.europa.eu/eli/reg/2015/1760/oj).

Taking into consideration the concerns raised by the use of perillaldehyde as food ingredient, this compound can be used to produce the correspondent natural derivative, perillyl alcohol (POH), which many studies investigated as a possible therapeutic agent in the prevention and treatment of cancer (Shojaei *et al*., 2014). Indeed, POH showed the ability to inhibit different type of tumors at different stages, such as skin, liver, breast, glioma, lung, colon and gastric cancers in animal models (Chen *et al*., 2021). However, this natural compound is present only in low quantities in a few plant oils, so an alternative source of POH is becoming necessary (Chen *et al*., 2021). Although POH can be produced via classical organic synthesis, for example, through the hydrogenation of α,β-unsaturated aldehydes, in recent years there has been a significant preference towards biotransformation processes carried out by using microbial cells as biocatalysts for the production of fine chemicals (Forti *et al.*, 2015). Accordingly, molecules obtained by such bioprocesses can be labeled as ‘natural’ and GRAS (‘Generally Regarded As Safe’), thus increasing their market value (Contente et al., 2020).

# **5. Materials and Methods**

The strain *Candida viswanathii* Bio1 (UBOCC-A-208001) was grown in baffled flasks or in 2L-bioreactor (Applikon), using YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) or molasses (1:2) pre-treated with H2SO4 1.5% (v/v) mixed with filtered and sterilized seawater and yeast extract (1 g/L). The fed-batch process was set as follow: 28 °C, pH maintained at 6, oxygen over 40%. For the biocatalyst immobilization, 200 OD of cells were mixed 1:1 with a 5% w/v solution of sodium alginate and dropped into a 0.2 M CaCl2 solution thought a P200 tip to form beads of size 3 mm each. Biotransformations were performed in 5 mL glass vials, at room temperature (23-25°C), in continuous stirring at 150 rpm. Biotransformation products and substrates, after extraction with EtOAc 1:1, were analyzed through TLC (hexane/EtOAc 7:3, revealed by vanillin staining), 1H-NMR and gas chromatography (GC).

# **5. Results and Discussion**

## **5.1 Screening for monoterpenes reduction**

## A total of fourteen yeast wild-type strains, listed in Table 1, were screened for their ability to modify monoterpenes through biotransformation for the achievement of interesting APIs (Active Pharmaceutical Ingredients). For the first screening, (R)- and (S)-carvone were added at the concentration of 1 g/L after 24 hours of cultivation in rich medium (YPD). The cultural broth was collected after another 24 hours and analyzed by TLC. Interesting results were shown by three yeast strains: CEN-PK and the non-conventional yeast strains Bio1 and CCAT2. These three strains were tested also on other terpenes (Fig. 1): (R)-carvone (a), (S)-carvone (b), (S)-perillaldehyde (c), thymoquinone (d), (R)-pulegone (e), verbenone (f), and β-ionone (g). Notability, Bio1 was the only strain to show positive results for all substrates (except for verbenone and β-ionone).

|  |  |  |
| --- | --- | --- |
| **Species** | **Strain** | **Source** |
| *Saccharomyces cerevisiae* | CEN-PK | Laboratory strain |
| *Dekkera bruxellensis* | Y908 | Grape must |
|  | Y911 | Equipment in beer brewery |
|  | Y871 | Sour wine |
|  | Y870 | Sour wine |
|  | Y906 | Tea-beer |
| *Kluyveromyces lactis* | CBS2359 | Creamery |
| *Trichosporon oleaginosus* | CCAT2 | Dairy plant |
| *Debaryomyces hansenii* | Mo40 | Deep-sea hydrothermal vents |
| *Candida viswanathii* | Bio1 | Deep-sea hydrothermal vents |
| *Lypomyces lipofer* | LLDP5 | Soil |
| *Rhodosporidium paludigenum* | CBS6566 | Juncus roemerianus (marsh) |
| *Rhodosporidium azoricum* | RGRDP3 | Soil |
| *Rhodotorula glutinis* | RGNR2 | Air |

**Table 1** *Yeast strains screened for monoterpenes biotransformation.*

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**Figure 1** *Table of terpenes tested on Bio1 and CCAT2 strains.*

## **5.2 Cell immobilization and optimization of batch biotransformation**

## Based on the screening results, we decided to focus on the reduction of (S)-perillaldehyde into POH performed by Bio1 strain as a model reaction. The putative enzyme involved in this conversion is a carbonyl reductase which catalyzes the reduction of the carbonyl group by using cofactors (Fig. 2). To obtain a more stable and easier to use biocatalyst, Bio1 cells were immobilized into Ca-alginate hydrogel beads. The media for the biotransformation was composed of physiological solution (NaCl 0.9%) supplemented by glucose (40 g/L) to enhance the cofactor recycling system (Filippucci *et al.*, 2020; Goretti *et al.*, 2009). Working on small volumes (1 or 2 mL), different concentrations of alginate beads were tested with increasing concentrations of substrate (Table 2, 3). The best result (89% molar conversion) was archived by using 1 g/mL of immobilized biocatalyst with 4 mM of perillaldehyde in 6-hour reaction time (Table 4).

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**Figure 2** *Reduction of (S)-perillaldehyde into (S)-perillyl-alcohol.*

**Table 2** *Molar conversion obtained using different amount of immobilized biocatalysts (g of alginates/mL).*

|  |  |  |
| --- | --- | --- |
| Immobilized catalyst (g/mL) | POH  (mM) | Molar conversion  (%) |
| 0.1 | 2.83 ± 0.22 | 35 |
| 0.5 | 3.50 ± 0.28 | 44 |
| 1 | 4.18 ± 0.16 | 53 |

## **Table 3** *Molar conversion obtained using 1 g/mL of immobilized biocatalysts with increasing substrate concentrations.*

|  |  |  |
| --- | --- | --- |
| Perillaldehyde  (mM) | POH  (mM) | Molar conversion  (%) |
| 4 | 3.43 ± 0.26 | 90 |
| 7.5 | 4.18 ± 0.22 | 54 |
| 14 | 5.34 ± 0.14 | 37 |

## **Table 4** *Molar conversion obtained using* *1g of alginates and 4 mM of substrate.*

|  |  |
| --- | --- |
| **Time**  (h) | **Molar conversion**  (%) |
| 2 | 76 |
| 4 | 68 |
| 6 | 89 |
| 24 | 85 |

## **5.3 Attempt to scale the process through a flow system**

As a first attempt to scale up the process, we tried to transfer the reaction into a flow system. Specifically, a 12 cm x 0.4 cm glass column (4 mL volume) was packed with 4 g of alginate beads containing Bio1 cells. The stream containing the physiological solution with the substrate was set at 0.3 mL/min (residence time: 15 min). Unfortunately, this system did not work as expected because perillaldehyde adhered to the plastic tubing of the system, resulting in very low conversion as the substrate did not come into contact with the biocatalyst.

## **5.4 Scale-up in a rotating bed reactor (SpinChem reactor)**

The SpinChem reactor is a type of rotating bed reactor (RBR) that exploits centrifugal acceleration to enhance mixing and mass transfer in the system (Fig. 3). Additionally, this reactor facilitates easy scalability, making it suitable for both laboratory-scale and industrial-scale applications. The SpinChem reactor is ideal to be used with cells immobilized in hydrogel beads as catalysts (Fig. 4).

Using this system, more than 90% of molar conversion was reached after 8 hours in a working volume of 200 mL (Table 5). More interestingly, this result was obtained using 10 times less biocatalyst compared to small scale reactions (0.1 g/mL compared to 1 g/mL). The efficient design of the system allowed for effective interaction between the immobilized cells and the reaction medium, ensuring uniform distribution of reactants and enhancing reaction kinetics and overall performance.

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**Figure 3** *SpinChem reactor system.*

## **Figure 4** *The internal cage of a SpinChem fulfilled with the biocatalyst immobilized into Ca-alginate hydrogel beads.*

## **Table 5** **(***S)-perillyl-alcohol production in the SpinChem reactor.*

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Time**  (h) | **(S)-perillaldehyde**  (mM) | **(S)-perillyl-alcohol**  (mM) | **(S)-perillyl-alcohol**  (mg/L) | **Molar conversion**  (%) |
| 0 | 4.16 ± 0.08 | - | - | - |
| 1 | 3.51 ± 0.08 | 0.98 ± 0.05 | 148 | 24 |
| 2 | 3.04 ± 0.07 | 1.85 ± 0.17 | 281 | 45 |
| 3 | 2.65 ± 0.01 | 2.52 ± 0.14 | 384 | 62 |
| 4 | 2.33 ± 0.02 | 3.33 ± 0.35 | 506 | 81 |
| 6 | 1.71 ± 0.01 | 3.07 ± 0.01 | 467 | 75 |
| 7 | 1.36 ± 0.17 | 3.41 ± 0.01 | 518 | 83 |
| 8 | 0.29 ± 0.01 | 4.02 ± 0.03 | 607 | 91 |

## **5.5 Sustainability focus: use of waste materials and seawater**

To adhere to circular economy principles and prioritize sustainable resource management, we evaluated the growth ability of Bio1 strain on a waste-derived medium based on molasses, a by-product of sugar beet refining, instead of the expensive synthetic medium. In addition, considering that Bio1 is a marine yeast strain, we chose to employ seawater instead of distilled water, as it contains essential minerals and reduces the risk of contamination.

Unfortunately, we found that Bio1 strain was not very efficient at metabolizing sucrose, so an acidic pre-treatment of molasses was needed to promote the hydrolysis of sucrose into glucose and fructose. In addition, a small amount of yeast extract (1 g/L, 10 times less than the synthetic medium) was added to stimulate the growth. With this strategy, we were able to produce more than 35 g/L (dry weight) of biocatalyst in 70 hours of process in 2L bioreactor.

To further enhance the sustainability of the overall process, we also decided to replace the physiological solution used as biotransformation medium with seawater, obtaining comparable results in terms of both molar conversion and product titers (data not shown).

# **6. Conclusions and Future Perspectives**

This study serves as a proof of concept to demonstrate the viability and potential applicability of the proposed approach for biocatalytic processes, paving the way for further exploration and utilization in related research areas. The results show the high potential of non-conventional yeasts as biocatalysts for the biotransformation of cheap and largely available monoterpenes (*i.e.,* those present in the screening but not limited to) to valuable derivatives, that have the potential to be explored as potential active pharmaceutical ingredients (APIs) and claimed as 'natural' products, following the European and US regulation. Furthermore, the incorporation of waste materials into the process emphasizes the commitment to sustainability and aligns with the principles of a circular economy. By utilizing molasses and seawater as feedstocks and incorporating them into the production cycle, the overall process becomes more environmentally friendly and resource efficient.

# **7. Nomenclature**

POH, peryllil-alcohol; g/L, Grams per Liter; YPD, Yeast Extract–Peptone–Dextrose broth; APIs, potential active pharmaceutical ingredients; EtOAc, ethyl acetate; TLC, thin layer chromatography; RBR, rotating bed reactor.

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