SUPPLEMENTARY INFORMATION

Boosting the catalytic performance of a marine yeast in a SpinChem reactor for the synthesis of perillyl alcohol

Silvia Donzella,[ab] Concetta Compagno,[a] Francesco Molinari,[a] Francesca Paradisi,[*b] Martina Letizia Contente[*a]

[a.] Department of Chemistry, Biochemistry and Pharmaceutical Sciences, University of Bern, Freiestrasse 3, 3012, Bern, Switzerland
[b.] Department of Food, Environmental and Nutritional Sciences (DeFENS), University of Milan, via Celoria 2, 20133, Milan, Italy
E-mail: martina.contente@unimi.it, francesca.paradisi@unibe.ch

Table of contents

1. Materials

Cell growing and strain maintaining media as well as commercially available reagents were purchased from Thermo Fischer Scientific or Merck (Sigma Aldrich). Organic solvents and chemical standards were bought from Merck (Sigma Aldrich).

2. Yeast strains and screening

All strains used in this work, listed in Table S1, are commercially available. For long-term storage, yeast strains were maintained at −80 ◦C on 15% (v/v) glycerol.
(S)-perillaldehyde was added to growing cells at the concentration of 1 g/L after 24 hours of cultivation in rich YPD medium at 28°C, 110 rpm. The culture broth was then collected after 24 hours and analyzed by TLC and NMR analysis. For the first screening the substrate was added directly to culture broth when the cells reached the concentration of 20-25 OD_{660}.

Table S1: Yeast strains screened for monoterpenes biotransformation. Molar conversion (% of POH) obtained 24h after the addition of 1 g/L of *(S)*-perillaldehyde. UDL: under detection limit.

Species	**Strain**	**Source**	**Molar conversion** (%)
Saccharomyces cerevisiae	CEN-PK	Laboratory strain	22%
Dekkera bruxellensis	Y908	Grape must	UDL
	Y911	Equipment in beer brewery	UDL
	Y871	Sour wine	UDL
	Y870	Sour wine	UDL
	Y906	Tea-beer	UDL
Kluyveromyces lactis	CBS2359	Creamery	UDL
Trichosporon oleaginosus	CCAT2	Dairy plant	31%
Debaryomyces hansenii	Mo40	Deep-sea hydrothermal vents	UDL
Candida viswanathii	Bio1	Deep-sea hydrothermal vents	50%
Lipomyces lipofer	LLDP5	Soil	UDL
Rhodosporidium paludigenum	CBS6566	Juncus roemerianus (marsh)	UDL
Rhodosporidium azoricum	RGRDP3	Soil	UDL
Rhodotorula glutinis	RGNR2	Air	UDL

3. Cell cultivation

For cell flasks cultivation, YPD medium was sterilized in an autoclave at 0.5 atm and at 112 °C for 30 min. Pre-cultures were prepared by inoculating cells from glycerol stocks and cultivating on YPD in bluffed flasks with an air-liquid ratio of 5:1 at 28 °C in a rotary shaker at 150 rpm overnight. Cells were harvested by centrifugation (5000 rpm/2300 rcf, 10 min) and inoculated at OD_{660} 0.1 in bluffed flasks. Cell growth was monitored by measuring the increase of optical density at 660 nm (OD_{660}) using a spectrophotometer (Eppendorf, Milan, Italy).

For the fermentation in bioreactor, yeast cells were initially pre-cultured on YPD medium and were used to inoculate the sterilized bioreactor at initial OD_{660} of 0.2. Fed batch cultivation was performed in a 2L-bioreactor (ez2-Control from Applikon Biotechnology) with a working volume of 1 liter, using pre-treated molasses and yeast extract (1 g/L) dissolved in seawater as culture medium. Before starting of the process, the bioreactor filled up with the medium was sterilized by autoclaving at 112

°C, 0.5 atm for 30 minutes. Temperature was set at 28 °C and pH, measured by AppliSens pH electrode (Applikon Biotechnology), was adjusted and maintained at 6 by automatic addition of 3M KOH or 10% (v/v) solution of H_2SO_4. The dissolved oxygen concentration was measured by an AppliSens oxygen probe (Applikon Biotechnology, Delft, The Netherlands), starting from 100% saturation and controlled by a cascade system that allowed maintaining a constant level (>40%). At specific points, aseptic sampling of 10 mL of culture broth was conducted using a peristaltic pump (Miniplus2 from Gilson, Milan, Italy). Samples were then used to determine cell concentration (OD_{660}), dry weight and sugars concentration. The sugar content was determined by employing commercial enzymatic kits (K-GLUHK and K-SUFRG from Megazyme, Wicklow, Ireland).

4. Molasses pre-treatment

To obtain the waste-derived medium, molasses residues were firstly diluted 1:2 with deionized water or seawater, then the chemical pretreatment was performed by adding H2SO4 95-98% (EMSURE, Germany) at the concentration of 1.5 % (v/v). The obtained solution was then sterilized by autoclave (112 °C, 0.5 atm for 30 minutes) to facilitate the sucrose hydrolysis, resulting in a total sugar content of 335 g/L (170 g/L of glucose and 165 g/L of fructose). To be used as a growth medium, the obtained solution was diluted with deionized water or seawater to reach a concentration of 50 g/L of sugars. All sugar analysis were performed using enzymatic kits from Megazyme, Irland (K-SUFRG, K-GLUHK-220A).

5. Bio1 sequencing

To isolate genomic DNA, pellets corresponding to 30 OD of cells were resuspended in 0.5 ml of 50 mM Tris–HCl, 20 mM M EDTA at pH 7.5. This suspension was transferred to a precooled tube with an equal volume of glass beads (425–600 µm). Mechanical lysis was performed using a TissueLyser LT (Qiagen) alternating 2 min of agitation at 50 Hz with 1 min in ice for four cycles. The supernatant was added with 25 µl of SDS 20% (w/v) and incubated at 65°C for 30 min. Immediately, 0.2 ml of 5 M potassium acetate was added, and the tubes were placed on ice for 30 min. Samples were centrifuged at 13000 rpm for 5 min and supernatants transferred to a fresh microcentrifuge tube. The DNA was precipitated by adding 1 vol of isopropanol. After incubation at room temperature for 5 min, the tubes were centrifuged for 10 min. The DNA was washed with 70% ethanol and dissolved in 50 µl of TE RNAse (10 mM Tris–HCl, 1 mM EDTA, pH 7.5 RNAse 100 µg/ml).

After DNA extraction, 100 µg of DNA were added to a master mix composed of 100 µL of buffer DreamTaq polymerase 10X, 80 µL of dNTPs 2 mM 10X, 50 µL of primer NL1 100 µM, 50 µL of primer 100 µM, 20 µL of MgCl2 50 mM, 5 µL of Phusion TaqPol 5 units/µl. PCR amplification was carried out by denaturing at 94 °C for 2 minutes, followed by 35 cycles of denaturing at 94 °C for 1 minute, annealing at 52 °C for 1 minute, extension at 72 °C for 1 minute, and a final extension at 72 °C for 7 minutes. PCR products were quantified again using spectrophotometer and purified using Isolate II PCR and Gel Kit (Bioline), according to manufacturer's protocol. Sequence analysis were performed by Microsynth AG service, while bioinformatic analysis were performed using NCBI Nucleotide BLAST tool comparing to the reference data available from the database 28S ribosomal RNA sequences (LSU).

Table S2: Primer sequences for strain identification.

Primer	Sequence
NL1 (forward)	5' -GCA TAT CAA TAA GCG GAG GAA AAG-3'

NL4 (reverse)	5' -GGT CCG TGT TTC AAG ACG G-3'

6. Yeast immobilization *via* alginate beads

Bio1 whole cell immobilization has been performed as previusly described by Fernandez-Arrojo *et al.*[1] Briefly, 200 OD_{660} of cells previously grown in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) were mixed 1:1 with a 5% w/v solution of sodium alginate and dropped into a 0.2 M $CaCl_2$ solution through a P200 tip to form beads of 3 mm diameter each. After that the beads were collected and washed twice with distilled water and used for the biotransformation. The alginate beads were stored at 4°C in $CaCl_2$ solution. To check the shelf-life, alginate beads were kept for 1 month at 4°C in $CaCl_2$ solution and then used to perform the reaction under optimized conditions: catalyst concentration 1 g/mL, substrate concentration 4 mM, reaction time 6 hours.

7. Batch small scale biotransformations

1 or 2 mL batch biotransformations have been carried out in glass vials employing physiological solution (NaCl 0.9%, pH 7.0) supplemented with 40 g/L glucose, different catalyst loading (0.1, 0.5, 1.0 g/mL) and substrate concentrations (4.0, 7.0, 14 mM) at 25 °C; the stirring was set up at 150 rpm. The reactions were analyzed *via* TLC and GC (see below) at different times (0, 2, 4, 6, 24 h).

8. Reaction under continuos flow conditions

Flow reactions were performed a R2/R4 Vapourtec flow reactor equipped with an Omnifit glass column (6.6 i.d. x 10 mm). The column (4 mL final volume) was fulfilled with 4 g of alginate beads. Residence time: 15 minutes. Each sample was analyzed by GC analysis (see below).

9. SpinChem reactions

The SpinChem RBR S2 reactor (Nordic ChemQuest AB, Umea, Sweden) was fulfilled using 200 mL physiological solution or 200 mL of filtered and autoclaved seawater, both supplemented with glucose (40 g/L). The internal rotor was packed with 20 g of wet alginates (0.1 g/mL). The speed was set at 300 rpm and every 1-2 h samples were collected using the drain valve to monitor the reaction by GC analysis.

10. Extraction and TLC analysis

After liquid-liquid extraction in ethyl-acetate (1:1), Merck Silica gel 60 F254 (aluminum foil) plates and hexane/EtOAc 7:3 as mobile phase were used for TLC analysis. Detection of TLC analyses have been performed under UV light at 254 and 365 nm or revealed by vanillin staining (15 g vanillin, 250 mL ethanol, 2.5 mL H_2SO_4).

11. GC analysis

GC analysis were performed using an Agilent 8860 GC System 7693A, mobile phase nitrogen, FID detector (275°C), CP-Chirasil-Dex CB column, flow 6.5 mL/min. The analysis was performed in isothermal conditions at 75°C.

(S)-perillyl alcohol and (S)-perillaldehyde concentrations were calculated using a calibration curve constructed using commercially available standards from Merck. (S)-perillyl alcohol and (S)-perillaldehyde retention times were 4.8 and 4.5 minutes, respectively.

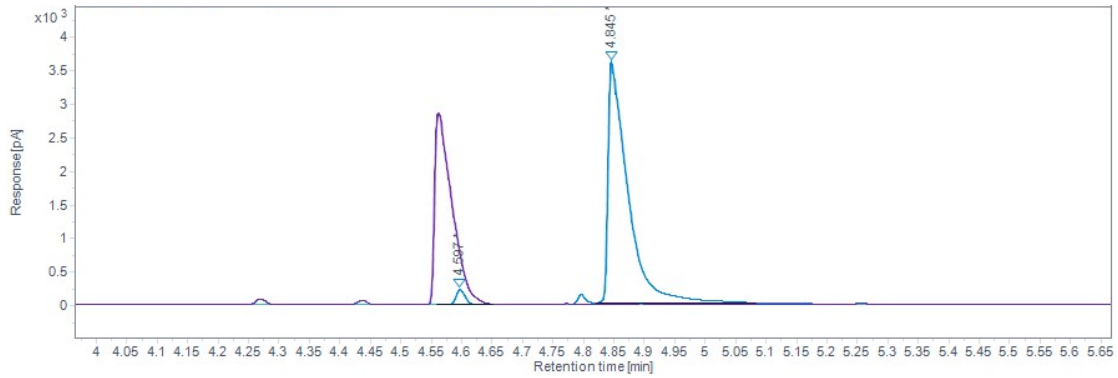

Fig S1: Gas chromatogram (GC) obtained after an 8-hour reaction in the SpinChem reactor using waste-derived Bio1 cells. The purple line represents the chromatogram at t0 (beginning of the reaction), while the blue line represents the chromatogram at 8 hours. 4.5 minutes: Perillaldehyde retention time; 4.8 minutes: Perillyl-alcohol retention time.

12. ^{1}H-NMR spectrum of POH

^{1}H-NMR spectrum of the POH product obtained after an 8-hour reaction in the SpinChem reactor using waste-derived Bio1 cells. After extraction with EtOAc, the organic phase was collected, dried on $Na_2SO_,$ filtered and evaporated under reduced pressure. The crude was purified *via* flash column chromatography was performed on Merck Silica gel (230–400 mesh) (hexane:EtOAc 7:3).
The ^{1}H-NMR spectrum of the product was obtained with a Bruker AV400 (400 MHz) spectrometer. $CDCl_3$ was used as deuterated solvent for the NMR analysis. The spectrum is in agreement with previously reported data.[2]

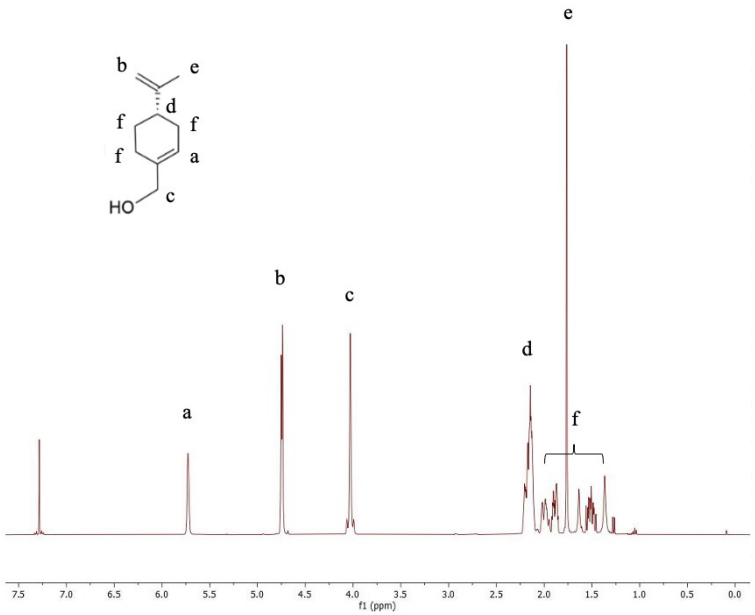

Fig S2: ^{1}H-NMR spectrum of the POH product.

13. References

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2. E. Silva, F. Olivera, J.M. Silva, A. Matias, R.L. Reis, A.R.C. Duarte, *Pharmaceutics*, 2020, **12**, 1121.