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DESIGNING BIOCATALYSTS BASED ON ENZYMES CO-IMMOBILIZATION WITH APPLICATION FOR CASCADE REACTIONS

Experimental report (I)

MASTER: CHEMISTRY OF ADVANCED MATERIALS

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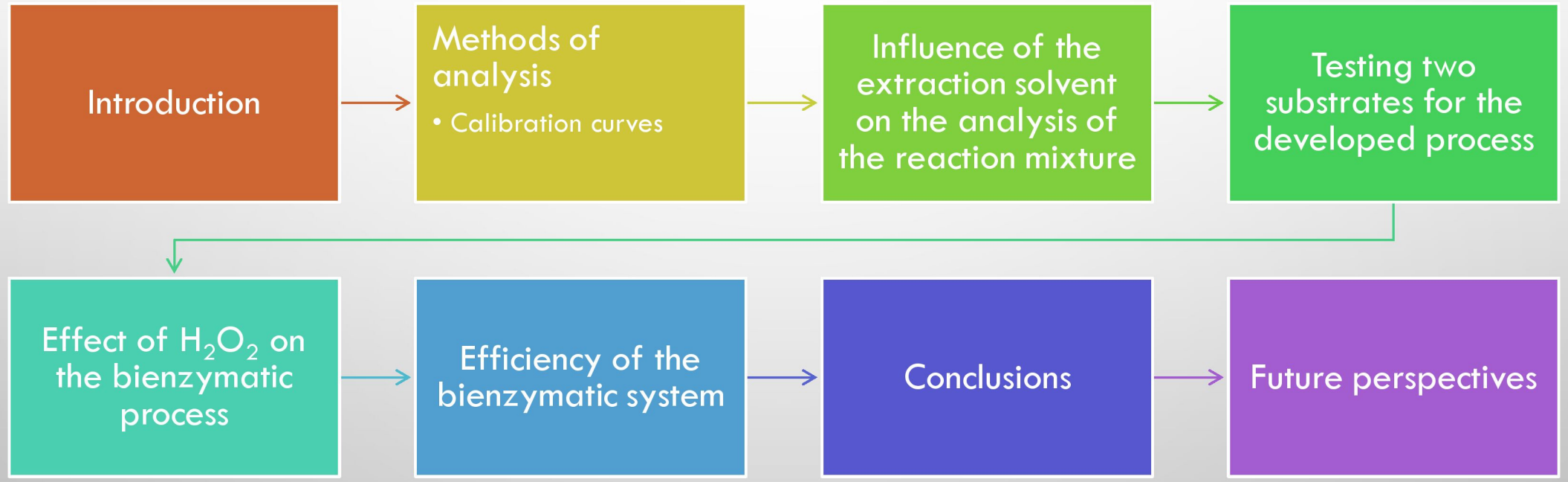
September 2020

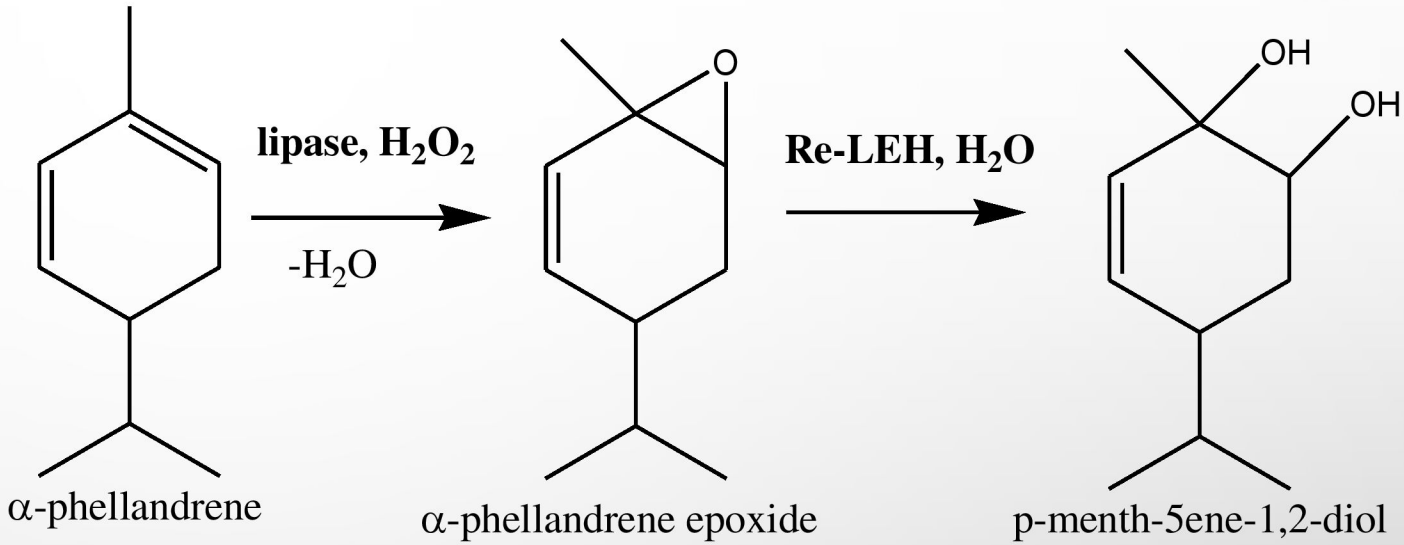
OUTLINE


Introduction
Methods of analysis
• Calibration curves
Influence of the extraction solvent on the analysis of the reaction mixture
Testing two substrates for the developed process
Effect of H_2O_2 on the bienzymatic process
Efficiency of the bienzymatic system
Conclusions
Future perspectives

Introduction

We propose a bienzymatic cascade system based on:

- Monoterpenoid epoxidation indirectly biocatalyzed by lipase from *Aspergillus niger*
- Epoxide hydrolysis directly biocatalyzed by hydrolase Re-LEH



In this experimental report, we present our results for:

- Analysis of reaction mixture
- Testing two substrates for the developed process
- Optimization of H_2O_2 concentration

Methods of analysis

A sample contains:
- 1.6 M octanoic acid
- 1.6 M substrate
- 0.1 M PBS (pH 8)
- 4.39% v/v lipase from Aspergillus niger
- 2.81% v/v hydrolase Re-LEH
- > 0.44 mM H_2O_2

- contains: NaCl, KCl, Na_2HPO_3, K_2HPO_3 in water - pH adjusted with NaOH
- is used to maintain a constant volume among all samples

24 h
25°C
1000 rpm
(Thermoshaker)

Extraction:
- 1:1 volume ratio in ethyl acetate or n-pentane

Agitation at room temperature (30 min)

Acetylation:
- 100 µL sample
- 150 µL pyridine
- 66 µL acetic anhydride

24 h
25°C
1000 rpm
(Thermoshaker)

Sample analysis:
- Gas chromatography

GC-FID (flame ionization):
- Requsted the acetylation step for analysis
- Enantioseparation

Process enantioselectivity

GC-MS (mass spectrometer):
- Does not need the acetylation step
- Reverse phase separation

Substrate conversion

Masks unreacted substrate

Pre-treatment	Unreacted α-phellandrene (mols/L)
Extraction	19.00×10^{-2}
Extraction +acetylation	6.54×10^{-2}

Values obtained from interpolation on calibration curve

Methods of analysis

Calibration curves:

- For a proper determination of substrate and interested product in the reacted mixture

- 8 samples
- Starting with around 3.3 M the concentrations are halved till 0.05 M
- The final sample has 0 M
- Solvents : n-pentane and ethyl acetate

Acetylation:

- 100 µL sample
- 150 µL pyridine
- 66 µL acetic anhydride

24 h
25°C
1000 rpm
(Thermoshaker)

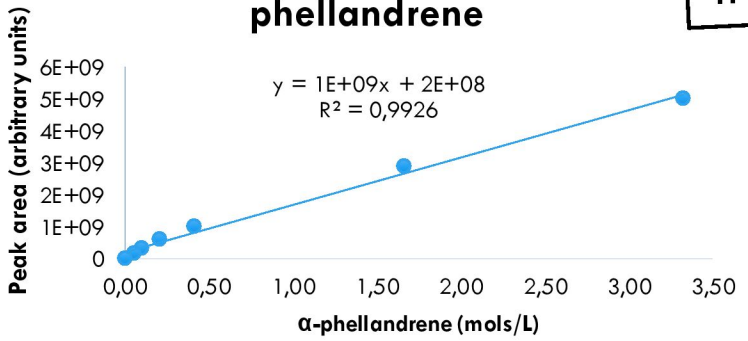
GC-MS:

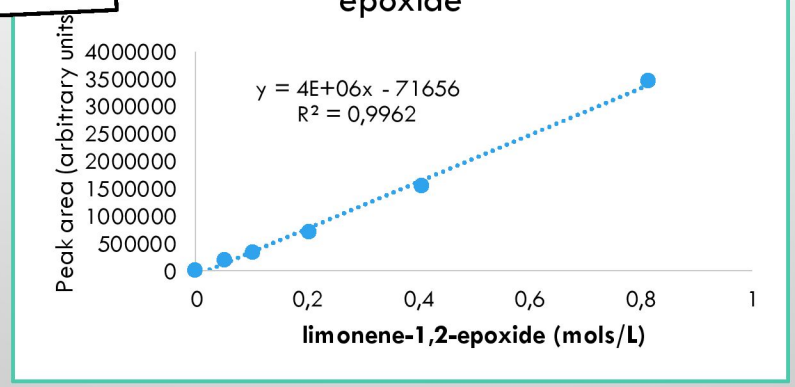
- More sensible in changes in substrate concentration

GC-FID:

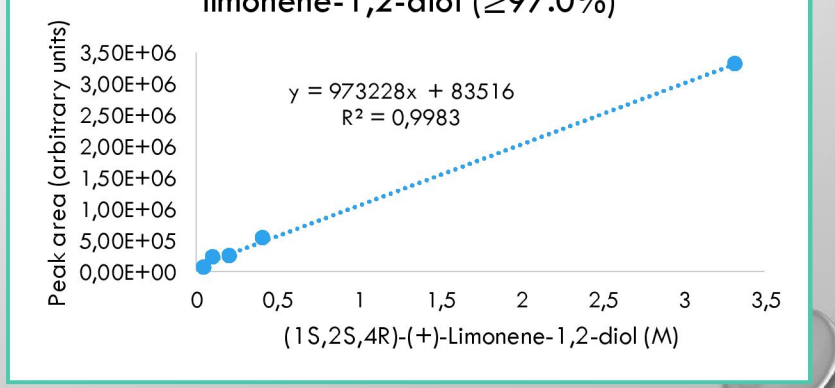
- Can distinguish between R and S isomers of resulting diols

n-pentane





ethyl acetate



All 3 calibration curves have a correlation coefficient close to 1 → **Good linearity**

Influence of the extraction solvent

Sample:
- 1.60 M octanoic acid
- 1.6 M α-phellandrene
- PBS pH 8
- 4.39% (v/v) lipase Aspergillus niger
- 2.81% (v/v) hydrolase Re-LEH
- 0.44 mM H_2O_2

24 h
25°C
1000 rpm
(Thermoshaker)

Extraction:
- 1:1 volume ratio in ethyl acetate or n-pentane

✓ Low polarity
✓ Less volatile

✓ Non-polar → better for extracting the non-polar substrate
X Highly volatile

Agitation at room temperature (30 min)

GC-MS

Extraction solvent	Unreacted α-phellandrene (mols/L)	Conversion (%)
Ethyl acetate	1.25×10^{-1}	89
n-pentane	1.67×10^{-1}	90

- The difference between the determined concentrations is low
- The conversion is not affected substantially

Ethyl acetate

Testing two substrates for the developed process

2 samples:

- 1.60 M octanoic acid
- 1.6 M α-phellandrene or 1.59 M (+)-limonene
- PBS pH 8
- 4.39% (v/v) lipase Aspergillus niger
- 2.81% (v/v) hydrolase Re-LEH
- 0.44 mM H_2O_2

- Extraction (ethyl acetate)
- Acetylation
- GC-FID

The conversion of α-phellandrene is influenced by the reaction environment

The difference in selectivity is not reflected in the amount obtained

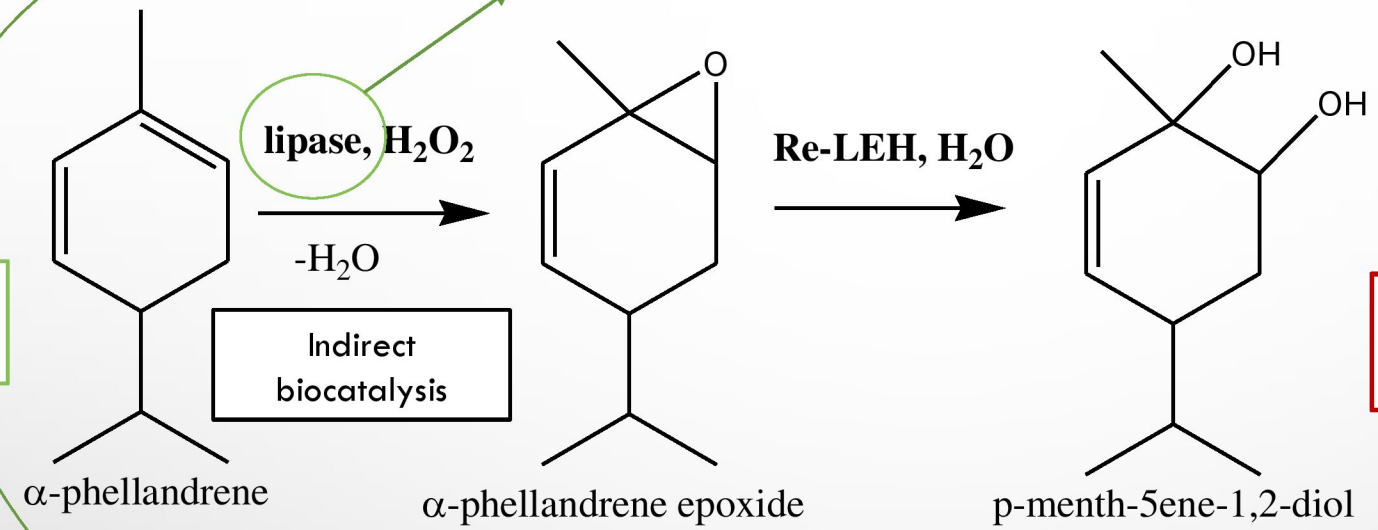
Process performance	α-phellandrene	(+)-limonene
Conversion (%)	87	62
Selectivity for epoxide (%)	0.1	2.0
Epoxide (mols/L)	1.82×10^{-2}	2.37×10^{-2}
Selectivity for p-cymene (%)	17	27
Selectivity for diols (%)	83	71
Diols (mols/L)	1.25	0.75
Enantiomeric excess (%)	91	87

p-cymene is detected for both cases

Enantiomeric excess around 90 % in both cases

Effect of H_2O_2 on the bienzymatic process

Catalyzes the oxidation of octanoic acid



Indirect biocatalysis

Oxidation agent: H_2O_2

Octanoic peracid

H_2O_2 30%

1. 0.44 mM H_2O_2
2. 0.23 mM H_2O_2
3. 0.12 mM H_2O_2
4. 0.02 mM H_2O_2
5. 0.01 mM H_2O_2

H_2O_2 30% 1:10 in water

- 1.60 M octanoic acid
- 1.6 M α-phellandrene
- PBS pH 8
- 4.39% (v/v) lipase
- 2.81% (v/v) hydrolase
- H_2O_2

24 h
25°C
1000 rpm
(Thermoshaker)

Extraction:
- 1:1 volume ratio in ethyl acetate

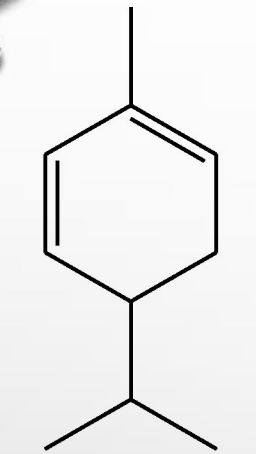
Agitation at room temperature (30 min)

Acetylation:
- 100 µL sample
- 150 µL pyridine
- 66 µL acetic anhydride

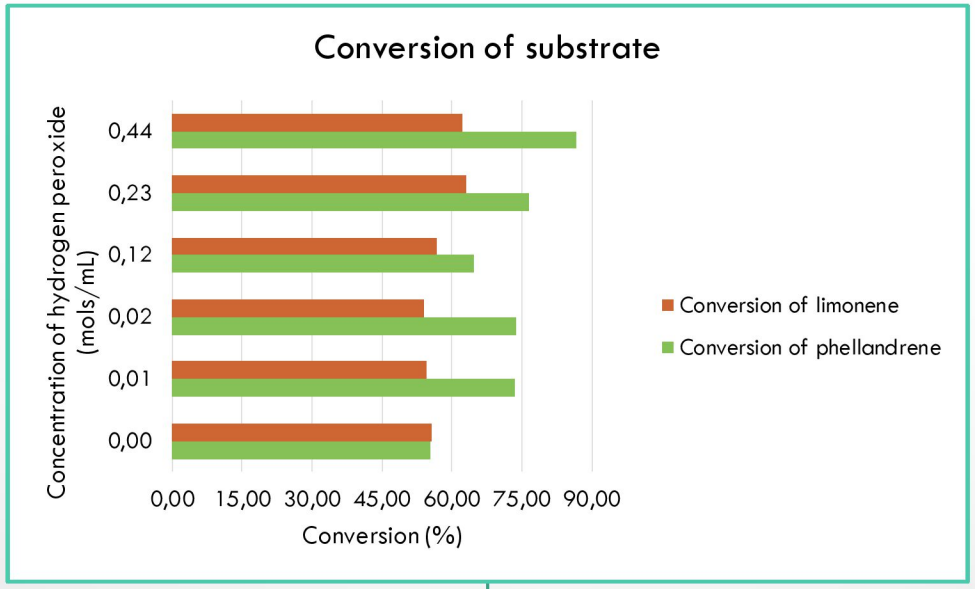
24 h
25°C
1000 rpm
(Thermoshaker)

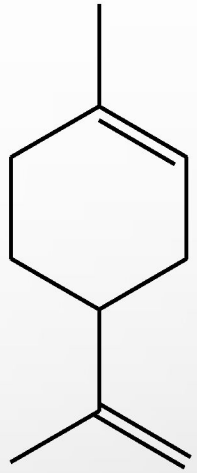
GC-FID

Effect of H_2O_2 on the bienzymatic process



α-phellandrene





limonene

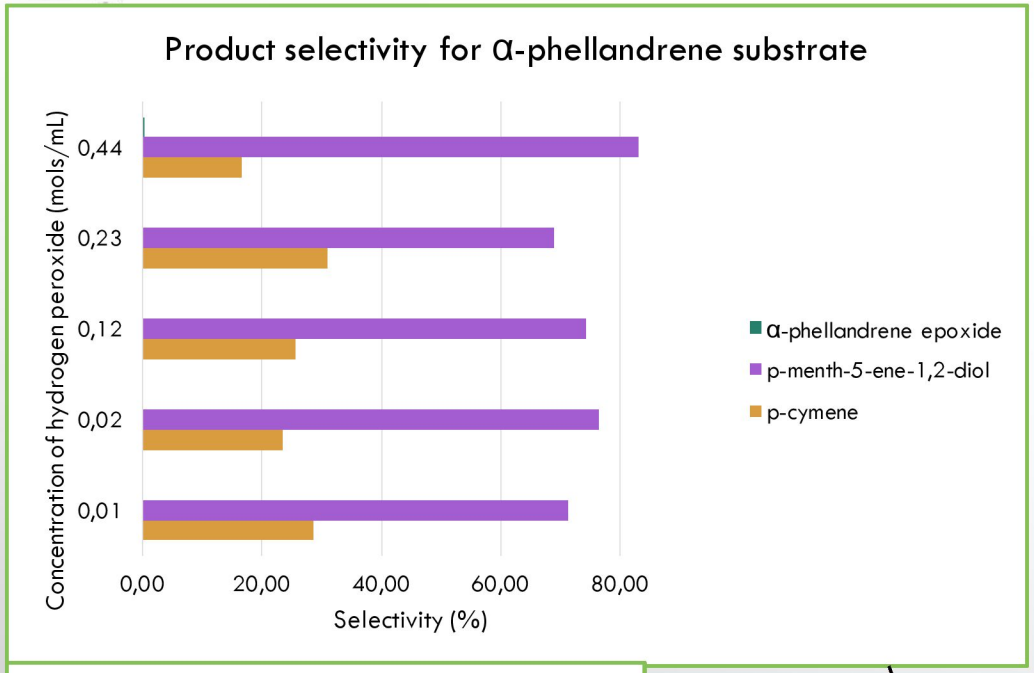
- **Maximum conversion:** 87% for 0.44 mM H_2O_2

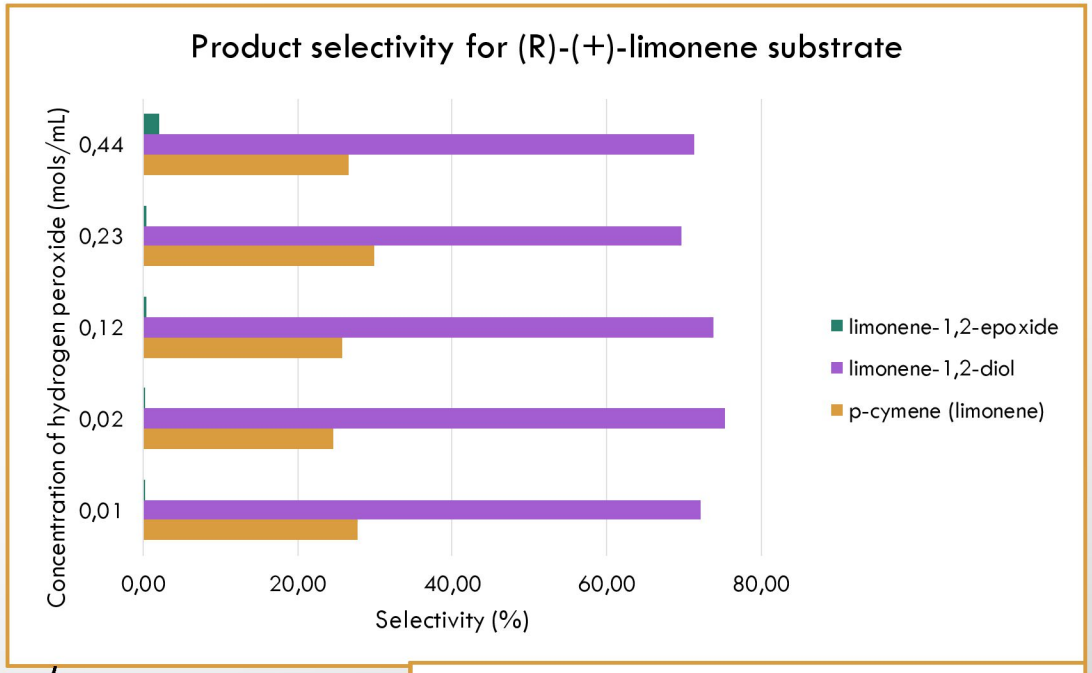
- The conversion increases together with the concentration of hydrogen peroxide

- **Maximum conversion:** 63% for 0.23 mM H_2O_2

Better transformation of phellandrene compared with limonene!

Effect of H_2O_2 on the bienzymatic process





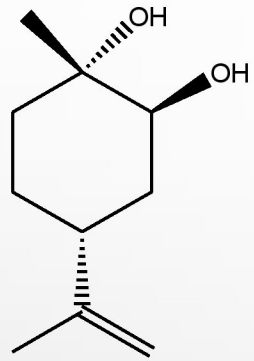
0.44 mM H_2O_2

- Presence of the epoxide
- the highest diol selectivity (83%)
- the lowest p-cymene selectivity (17%)

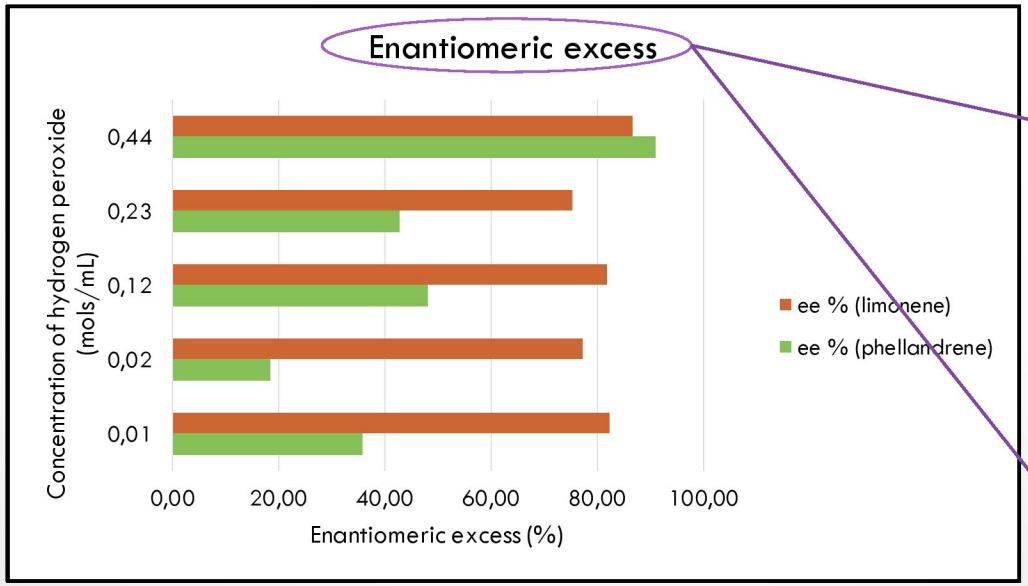
- Low selectivity for p-cymene and epoxide intermediates
- Above 70% selectivity for diols

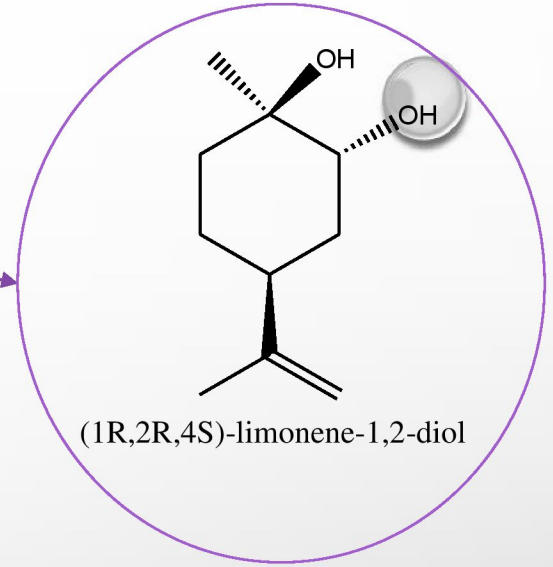
- Epoxide selectivity decreases together with the concentration of H_2O_2
- Selectivity for p-cymene between 25 – 30%
- Diol selectivity around 75%

Effect of H_2O_2 on the bienzymatic process

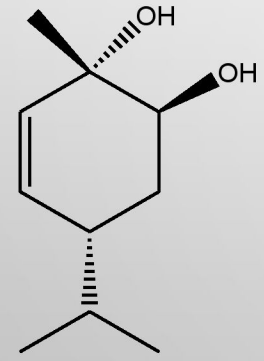


(1S,2S,4R)-limonene-1,2-diol



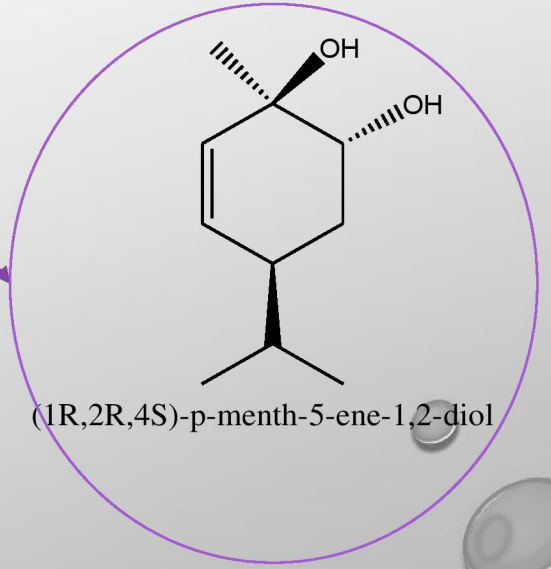


(1R,2R,4S)-limonene-1,2-diol



(1S,2S,4R)-p-menth-5-ene-1,2-diol

$$ee\% = \frac{R - S}{R + S} * 100$$



(1R,2R,4S)-p-menth-5-ene-1,2-diol

- Highest ee% for 0.44 mM H_2O_2 (87% for limonene and 91% for phellandrene)
- ee% decreases at lower H_2O_2 concentrations
- ee% for α-phellandrene is more affected by H_2O_2 concentration than limonene

Efficiency of the bienzymatic system

Sample 1(bienzymatic system):
- 1.60 M octanoic acid
- 1.6 M α-phellandrene
- PBS pH 8
- 4.39% (v/v) lipase Aspergillus niger
- 2.81% (v/v) hydrolase Re-LEH
- 0.44 mM H_2O_2

Sample 2 (mono-enzymatic system):
- 1.60 M octanoic acid
- 1.6 M α-phellandrene
- PBS pH 8
- 4.39% (v/v) lipase Aspergillus niger
- 0.44 mM H_2O_2

Sample 3 (without biocatalyst):
- 1.60 M octanoic acid
- 1.6 M α-phellandrene
- PBS pH 8
- 0.44 mM H_2O_2

24 h
25°C
1000 rpm
(Thermoshaker)

Extraction:
- 1:1 volume ratio in ethyl acetate

Agitation at room temperature (30 min)

Acetylation:
- 100 µL sample
- 150 µL pyridine
- 66 µL acetic anhydride

24 h
25°C
1000 rpm
(Thermoshaker)

GC-FID

Sample:	Bienzymatic system	Mono-enzymatic system	Without biocatalyst
p-menth-5ene-1,2-diol (mols/L)	1.25	1.41	0.88

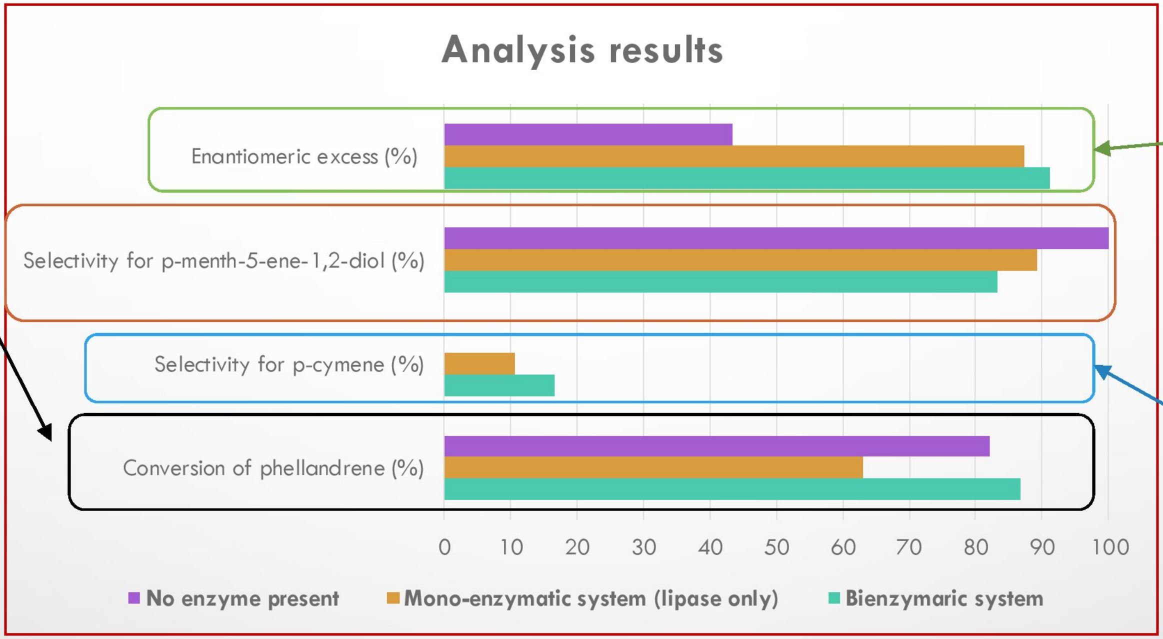
Lowest value

The presence of at leas one enzyme results in higher diol amount

Efficiency of the bienzymatic system

Conversion:
- *Highest:* bienzymatic system (87%)
- *Lowest:* mono-enzymatic system (63%)

At this moment we do not have an explanation for the high conversion degree obtained in the system without biocatalysts, because we were unable to identify all products.



ee%:
- *Highest:* bienzymatic system (91%)
- *Lowest:* no enzyme system (44%)

p-cymene selectivity:
- *Highest:* bienzymatic system (17%)
- *Lowest:* no enzyme system (0%)

Sample 1(bienzymatic system)

Sam 2 ono-e ymatic sys)

S mpl (with t iocata t)

CONCLUSIONS

- We propose two methods of analysis for our samples, the GC-FID method that can be used for the determination of the enantioselectivity, and GC-MS method for substrate conversion.

- Ethyl acetate is more efficient compared with n-pentan as extraction solvent.

- The bienzymatic system is highly effective in converting both monoterpenoids (phellandrene and limonene) with promising enantioselectivity of the resulted products.

- Hydrogen peroxide concentration has a high influence on the conversion of phellandrene, compared to limonene. However, the highest concentration of hydrogen peroxide offered the best performance of the process.

Optimum reaction mixture:

- 1.6 M octanoic acid
- 1.6 M substrate
- 0.1 M PBS (pH 8)
- 4.39% v/v lipase from Aspergillus niger
- 2.81% v/v hydrolase Re-LEH
- 0.44 mM H_2O_2

Future perspectives

1

We propose a more detailed study on the effect of substrate concentration on the conversion, selectivity, and enantiomeric excess.

because

The behavior of the two monoterpenoids used in our study is quite different.

e.g. phellandrene was greatly affected by the concentration of H_2O_2, but limonene substrate is less influenced by it

therefore

A study for the substrate influence on the bienzymatic system is requested.

2

We propose a biocatalyst designed by immobilization approach of lipase from *Aspergillus niger* and hydrolase Re-LEH.

Why?

Enzyme immobilization is an important step for our design because it provides **higher stability of the biocatalyst,** and the biocatalyst can be **recovered from the reaction phase,** which provides great future perspectives for reusing the biocatalyst.

How?

We propose **cross-linking** and **entrapment.**

Why?

Minimum effect on the catalytic activity of the enzymes

Thank you for your attention!