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

Experimental report (II)

MASTER: CHEMISTRY OF ADVANCED MATERIALS

Student: MARIA CRISTINA GHEȚU

Coordinator: Assoc Prof. MĂDĂLINA SĂNDULESCU

February 2021

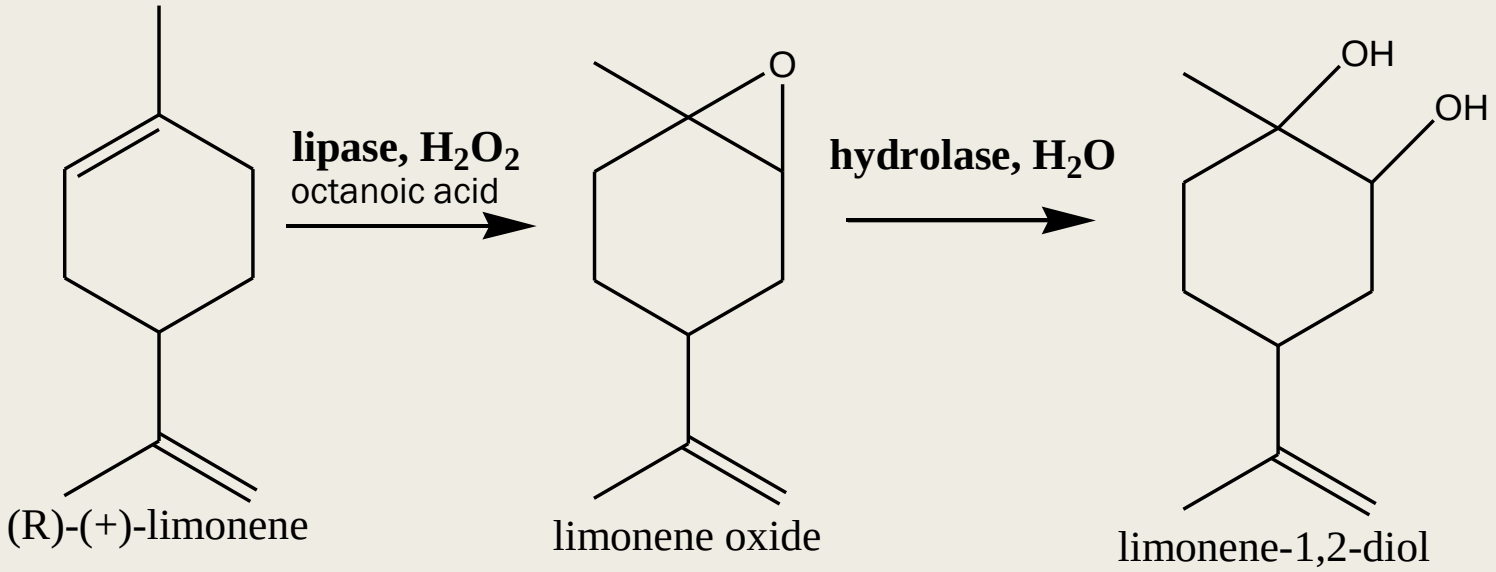
Disclaimer: This was realised with the EEA Financial Mechanism 2014-2021 financial support. Its content (text, photos, videos) does not reflect the official opinion of the Programme Operator, the National Contact Point and the Financial Mechanism Office. Responsibility for the information and views expressed therein lies entirely with the author(s).

OUTLINE

- Introduction
- Sample preparation
- Methods of analysis
- Free enzyme vs Immobilized enzyme
- Effect of reaction temperature on enzymatic process
- Possible bienzymatic systems
- Conclusions
- Future perspectives

Introduction

- We propose a bienzymatic cascade process for converting monoterpenoids into flavor and fragrance products



1. The lipase indirectly catalyzes the substrate epoxidation by transforming the fatty acid present in the system into the corresponding peracid using hydrogen peroxide

2. The hydrolase directs the transformation of limonene oxide toward obtaining preferentially (1R,2R,4S)-limonene-1,2-diol

Sample preparation

A sample contains:
- 1.6 M substrate
- 1.6 M octanoic acid
- 0.44 mM H_2O_2
- 4.39% v/v lipase
- 2.81% v/v hydrolase*
- 0.1 M PBS pH 8

*some studies were realized on a monoenzymatic system

Procedure:

1. **Reaction:** 24 h, 25 – 50 °C, 1000 rpm (thermoshaker)
2. **Pretreatment after reaction:**

- extraction - 1:1 = sample : ethyl acetate → 30 min mixing at room temperature;
- derivatization** - 100 µL extract, 150 µL pyridine, 66 µL acetic anhydride → 24 h, 25 °C, 1000 rpm (thermoshaker)

3. **Analysis:** GC-MS, GC-FID, GC*-FID

**acetylation step is used only for bienzymatic samples because it increases the resolution for diol isomers for GC*-FID method

Methods of analysis

GC-MS

Uses:
- Structural elucidation of sample components
- Samples are analyzed after extraction

GC-FID

- Once the peaks are attributed, this is the preferred method for samples analysis

- **Stationary phase of GC column**
  (5%-phenyl)-methylpolysiloxane

✓ (R)-(+)-limonene conversion
✓ limonene oxide yield

GC*-FID

Uses:
- Determination of enantiomeric excess of S/R limonene-1,2-diol
- Only acetylated samples

- **Stationary phase of GC column**
  β-cyclodextrin modified with S-hydroxypropyl

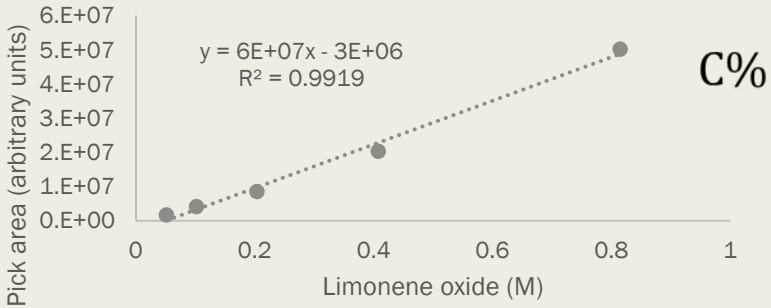
✓ Limonene-1,2-diol yield
✓ Enantiomeric excess (e.e.)

Methods of analysis

• Calibration curves for the evaluation of limonene biotransformation

GC-FID

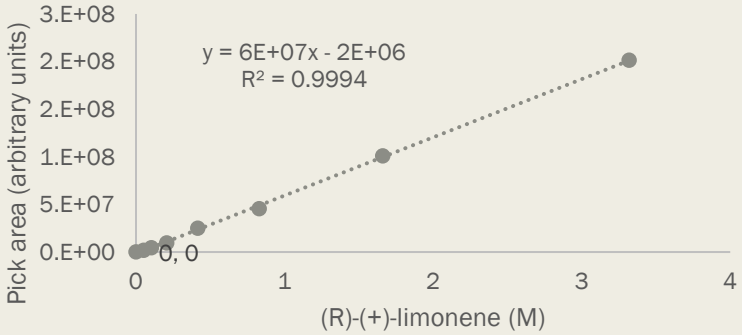
Calibration curve for limonene oxide (GC-FID)



Limonene conversion (%)

$$C\% = \frac{c_i\ \text{limonene} - c_{\text{limonene}}\text{curve}}{c_i\ \text{limonene}} * 100$$

Calibration curve for (R)-(+)-limonene (GC-FID)



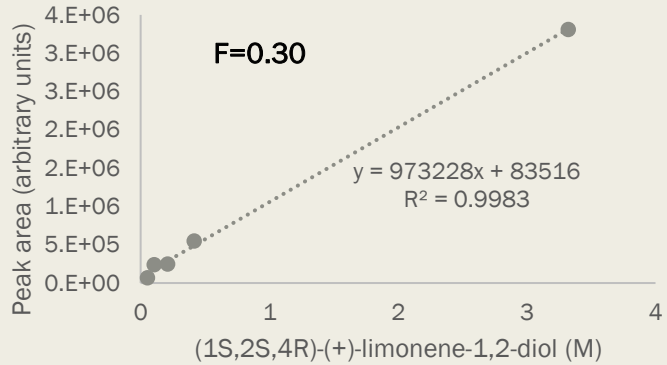
Epoxide yield (%)

$$\text{yield}_{\text{epoxide}}\% = \frac{c_{\text{epoxide}}\text{curve}}{c_i\ \text{limonene}} * 100$$

$$ee\% = \frac{\text{peak area S} - \text{peak are R}}{\text{peak area S} + \text{peak are R}} * 100$$

GC*-FID

Calibration curve for (1S,2S,4R)-(+)-limonene-1,2-diol (≥97.0%)



Diol yield (%)

$$\text{yield}_{\text{diol}}\% = \frac{c_{\text{diol}}\text{curve} * F}{c_i\ \text{limonene}} * 100$$

- The enantiomeric excess is obtained from the chromatogram

➢ This calibration curve has a correction factor (the substance is not commercially available anymore)

Free enzyme vs Immobilized enzyme

Samples:
- 1.6 M substrate
- 1.6 M octanoic acid
- 0.44 mM H_2O_2
- 4.39% v/v lipase
- 0.1 M PBS pH 8

1. **Reaction:** 24 h, 25 – 50 °C, 1000 rpm
2. **Pretreatment after reaction:**

- extraction - 1:1 = sample : ethyl acetate

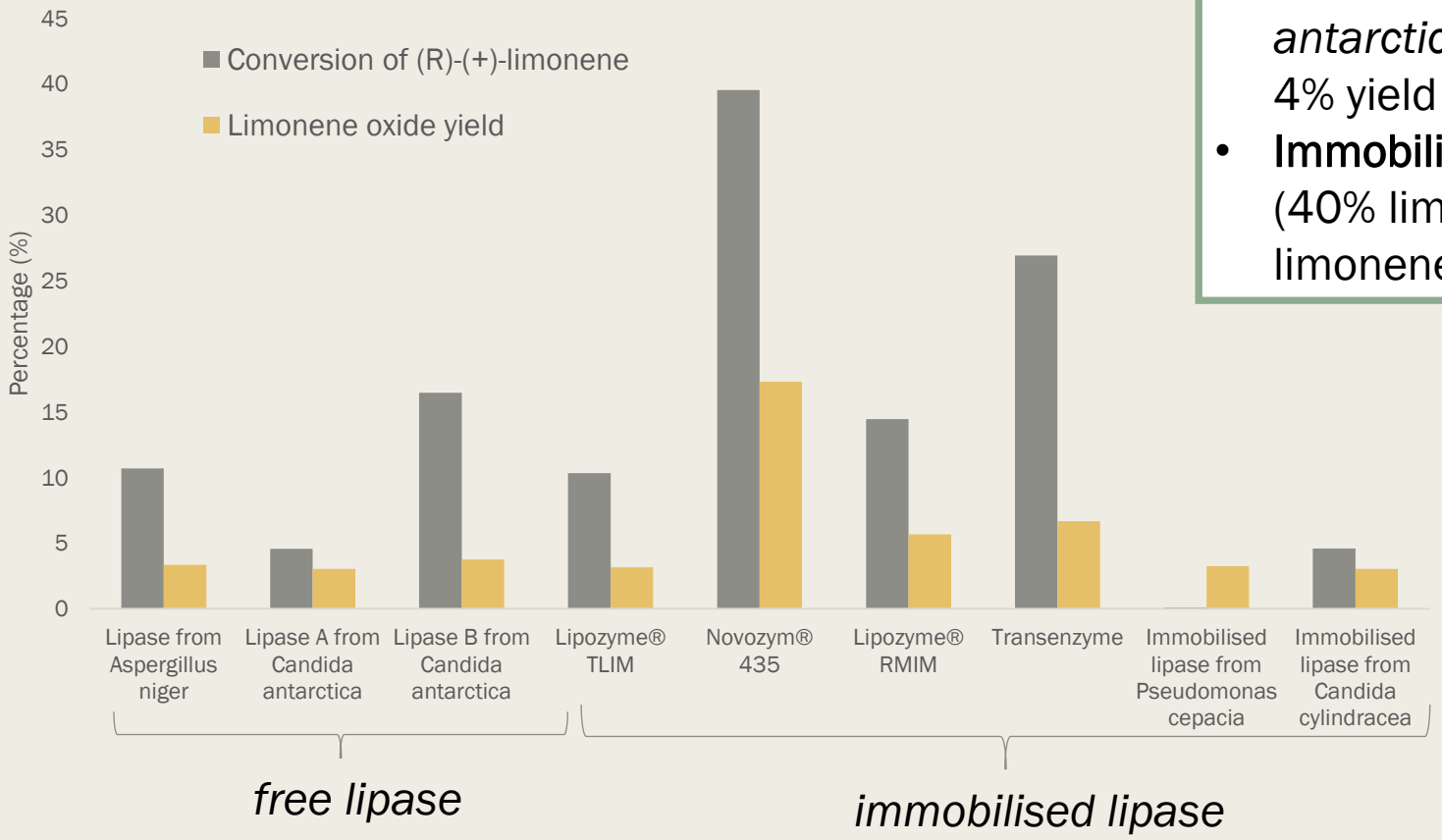
  → 30 min mixing at room temperature;

3. **Analysis:** GC-MS, GC-FID

Biocatalysts

1. Lyophilized lipases (43.89 µg/mL) from:
   - *Aspergillus niger*
   - lipase A from *Candida antarctica*
   - lipase B from *Candida antarctica*
2. Immobilized lipases (11 mg):
   - Lipozyme® TL IM –*Thermomyces lanuginosus* in silica gel
   - Novozym® 435 – lipase B from *Candida antarctica* in PMMA
   - Transenzyme – *Geobacillus stearothermophilus* in acrylic resign
   - Lipozyme® RM IM –*Rhizmucor miehei* in anionic exchange resin
   - lipase from *Candida cylindracea* in sol-gel
   - lipase from *Pseudomonas cepacian* in sol-gel

Free enzyme vs Immobilized enzyme



Best performance:

- **Free enzyme:** lipase B from *Candida antarctica* (16% limonene conversion, 4% yield in limonene oxide)
- **Immobilized enzyme:** Novozym® 435 (40% limonene conversion, 17% yield in limonene oxide)

What we choose:

1. Lipase from Aspergillus niger (used in previous bienzymatic system)
2. Novozym® 435 (best overall results)
3. Lipozyme® RM IM (good conversion and yield)

✓ Immobilized enzymes provide higher enzymatic activity

Effect of reaction temperature on the enzymatic process

Monoenzymatic samples:
- 1.6 M octanoic acid
- 1.6 M substrate
- 0.1 M PBS pH 8
- Lipase:
  - 43.89 µg/mL solution lipase *Aspergillus niger*
  - 11 mg Novozym® 435, Lipozyme® RM IM
- 0.44 mM H_2O_2

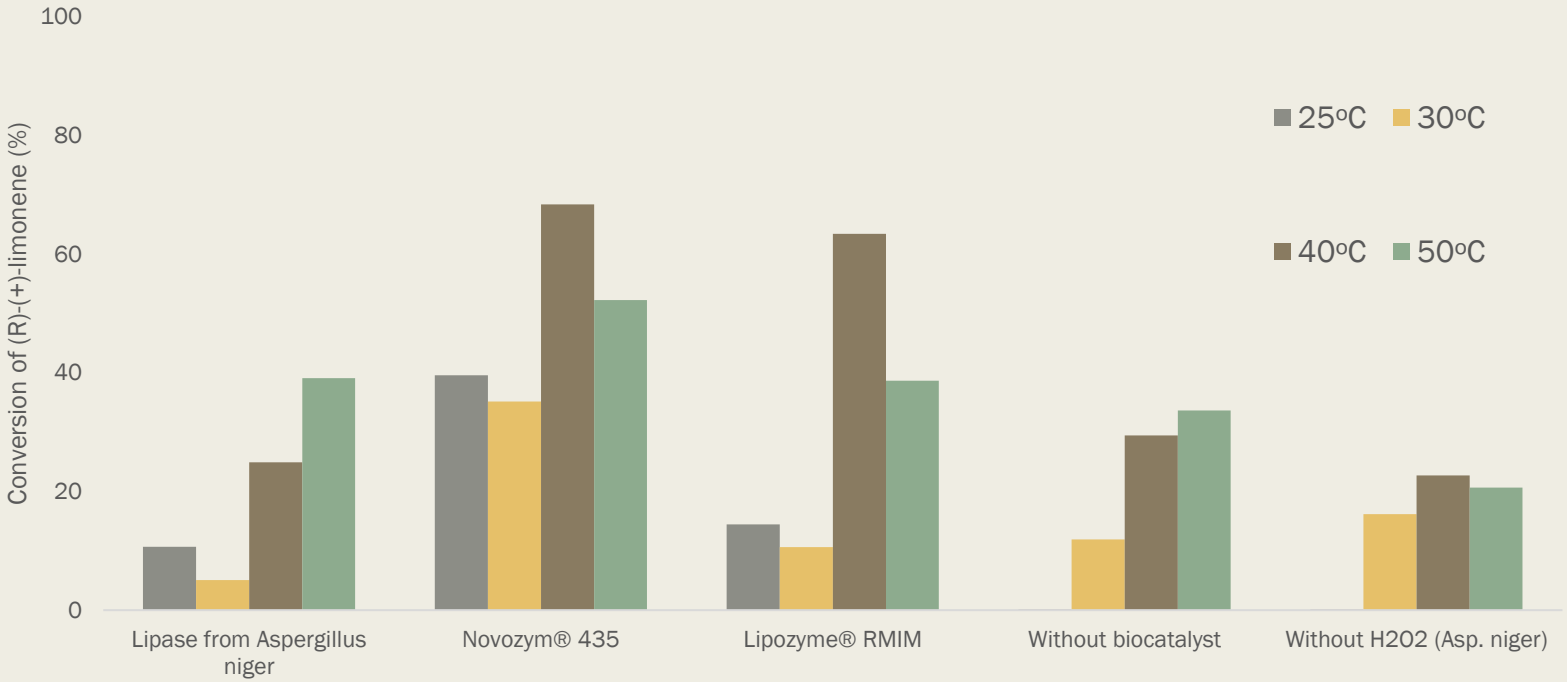
Samples without biocatalyst:
- 1.6 M octanoic acid
- 1.6 M substrate
- 0.1 M PBS pH 8
- 0.44 mM H_2O_2

Samples without hydrogen peroxide:
- 1.6 M octanoic acid
- 1.6 M substrate
- 0.1 M PBS pH 8
- 43.89 µg/mL solution lipase *Aspergillus niger*

1. **Reaction:** 24 h, 25 – 50 °C, 1000 rpm (thermoshaker)
2. **Pretreatment after reaction:**
   - extraction - 1:1 = sample : ethyl acetate → 30 min mixing at room temperature;
3. **Analysis:** GC-MS, GC-FID

We tested the system behavior at the following temperatures:
- ✓ 25 °C
- ✓ 30 °C
- ✓ 40 °C
- ✓ 50 °C

Effect of reaction temperature on the enzymatic process



Limonene oxide yield (%)	25ºC	30ºC	40ºC	50ºC
Lipase from Aspergillus niger	3	3	3	3
Novozym® 435	17	22	9	9
Lipozyme® RMIM	6	5	3	4
Without biocatalyst	3	3	3	3
Without H_2O_2 (Asp. niger)	0	0	0	3

Best:

- Temperature: 40 °C (Novozym® 435, Lipozyme® RM IM)
- Performance overall: Novozym® 435

- Yield decreases at 40 °C (higher energy input → epoxide is transformed)

What we choose:

- Reaction temperature 40 °C (more than half of limonene is converted)

Possible bienzymatic systems

A sample contains:
- 1.6 M octanoic acid
- 1.6 M substrate
- 0.1 M PBS pH 8
- Lipase:
  - 43.89 µg/mL solution lipase Aspergillus niger
  - 11 mg Novozym® 435, Lipozyme® RM IM
- 2.81% v/v hydrolase (Re-LEH, CH55-LEH, Tomsk-LEH*)
- 0.44 mM H_2O_2

*thermophilic

Procedure:

1. **Reaction:** 24 h, 25 – 50 °C, 1000 rpm (thermoshaker)
2. **Pretreatment after reaction:**
   - extraction - 1:1 = sample : ethyl acetate → 30 min mixing at room temperature;
   - derivatization - 100 µL extract, 150 µL pyridine, 66 µL acetic anhydride → 24 h, 25 °C, 1000 rpm (thermoshaker)
3. **Analysis:** GC-MS, GC-FID, GC*-FID

Combinations:
- Novozym® 435: 40 °C
  - Re-LEH
  - CH55-LEH
  - Tomsk-LEH
- Lipozyme® RM IM: 40 °C
  - Re-LEH
  - CH55-LEH
  - Tomsk-LEH
- *Aspergillus niger*: 25 °C (previous system)
  - Re-LEH
  - CH55-LEH

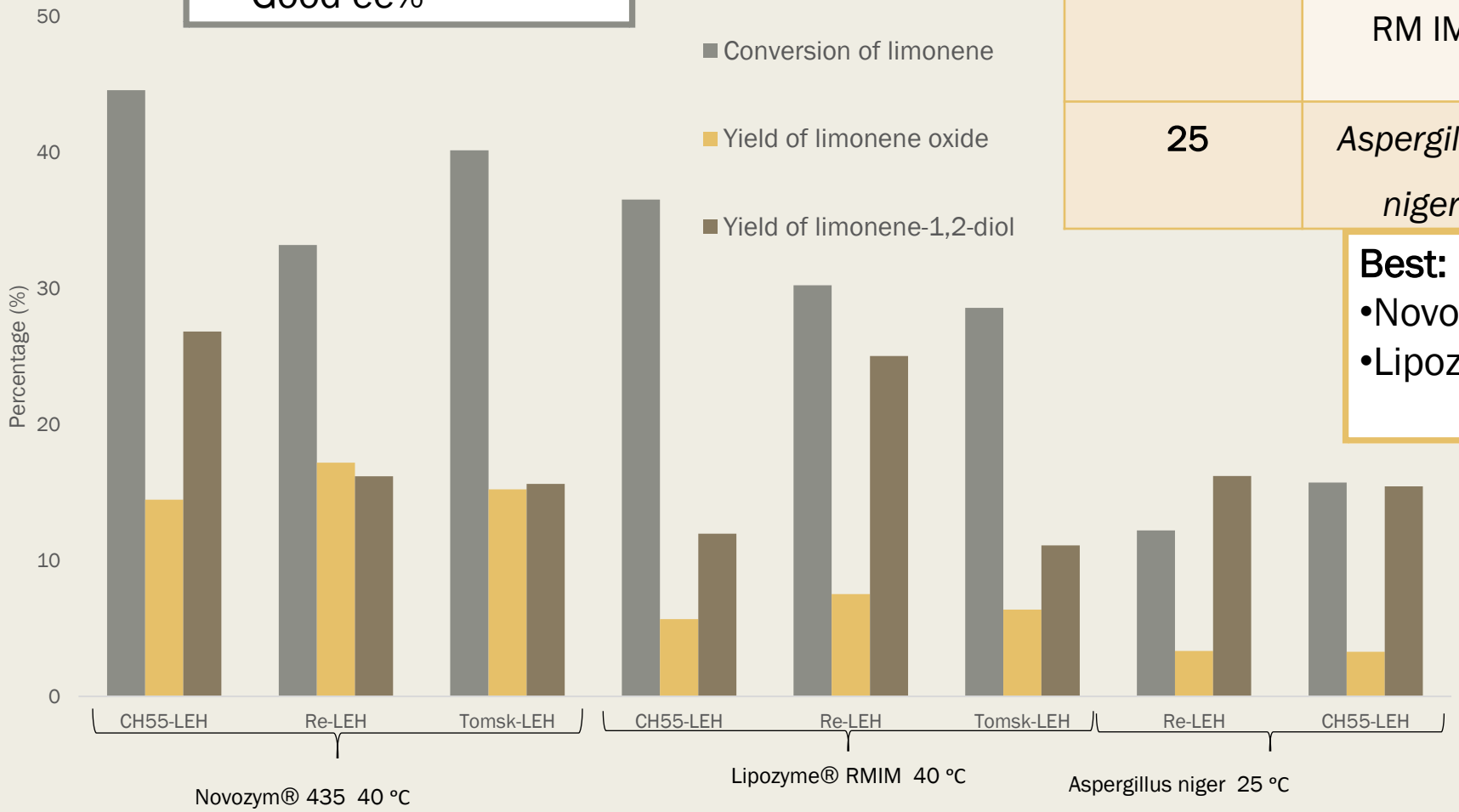
Goals:
1. High substrate conversion
2. High diol yield
3. Enantiopure diol

Possible bienzymatic systems

***Aspergillus niger*: 25 °C**
- Low conversion
- Good diol yield
- Good ee%

React. t °C	Lipase	Hydrolase	ee%
40	Novozym® 435	CH55-LEH	96
		Tomsk-LEH	89
		Re-LEH	89
	Lipozyme® RM IM	CH55-LEH	88
		Tomsk-LEH	89
		Re-LEH	97
25	*Aspergillus niger*	CH55-LEH	89
		Re-LEH	91

Best:
- Novozym® 435 – CH55-LEH (40 °C)
- Lipozyme® RM IM – Re-LEH (40 °C)



Conclusions

1. The behavior of free compared to immobilized lipase enzyme
   - the immobilized enzymes provide higher enzymatic activity, converting larger amounts of substrate compared to the free (dissolved) enzyme;
   - Novozym® 435 and Lipozyme® RM IM exhibited good ability to transform limonene into limonene oxide.
2. The influence of reaction temperature on the system efficiency
   - the immobilized lipases managed to convert more than half of the substrate present in the sample at a temperature of 40 ^{o}C;
3. The possible combinations of lipases and hydrolases for a bienzymatic cascade process
   - promising results were obtained for the couple

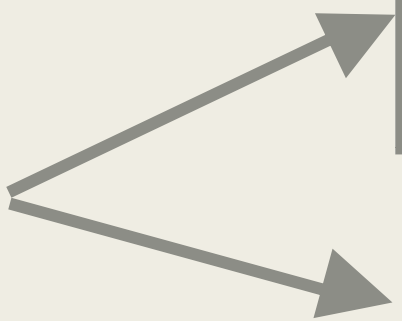
   Lipozyme® RM IM and Re-LEH

   Novozym® 435 and CH55-LEH

Future perspectives

- The attachment of the second enzyme (hydrolase) to the immobilized lipase composite.

Promising candidates:
- Novozym® 435 – CH55-LEH
- Lipozyme® RM IM – Re-LEH



Bio-composite characterization
- Enzyme loading
- Recovery of the enzyme activity
- Structural characterization (ex. FTIR)

Bio-composite tests
- Evaluation of the bio-composite for the limonene biotransformation

Thank you for your attention!