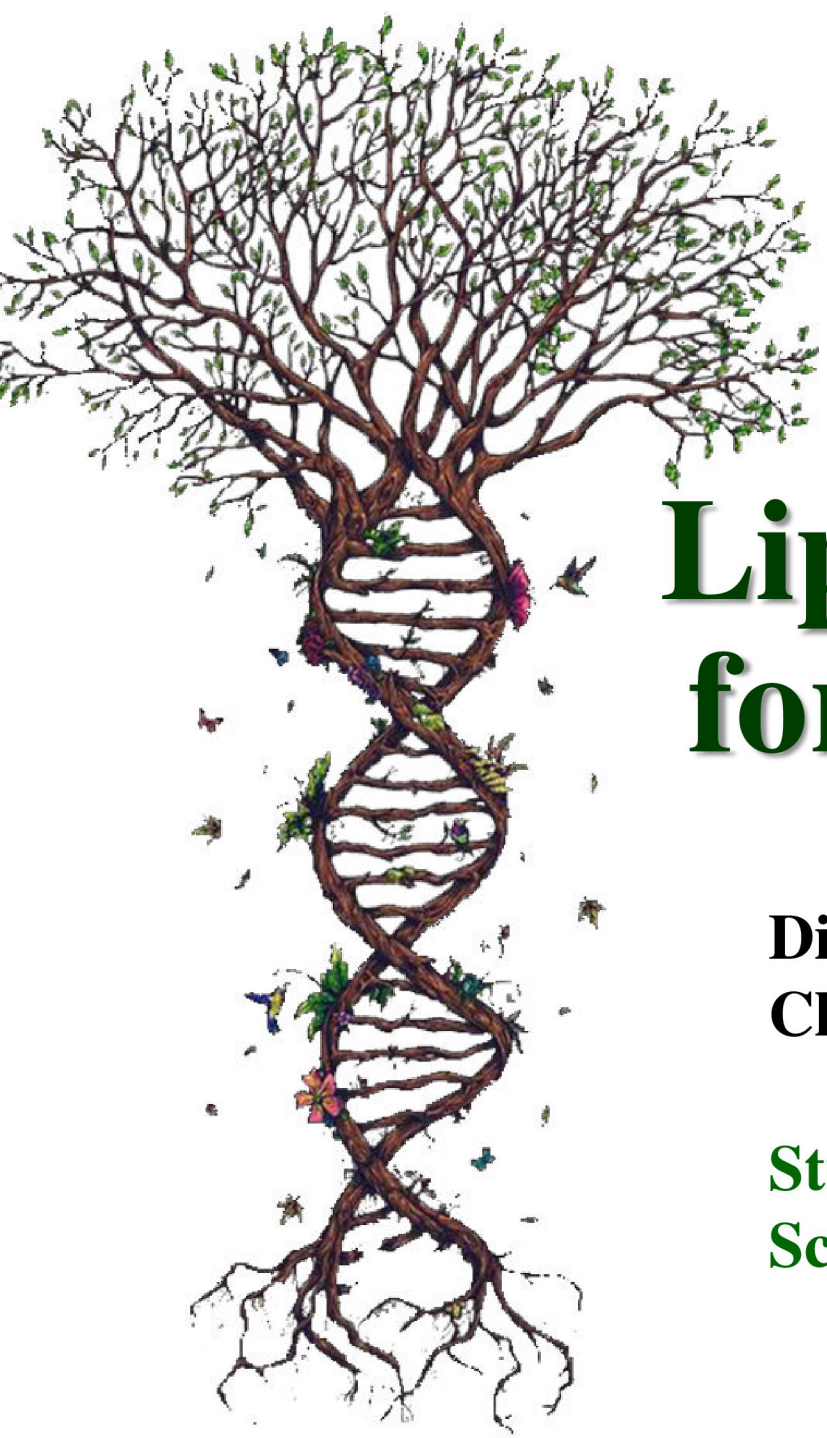

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UNIVERSITY OF BUCHAREST

NTNU
Norwegian University of Science and Technology

FACULTY OF CHEMISTRY
DEPARTMENT OF ORGANIC CHEMISTRY, BIOCHEMISTRY AND CATALYSIS

Lipase-designed biocatalysts for silymarin derivatization

Dissertation Thesis: Experimental report
Chemistry of Advanced Materials

Student: Giulia-Roxana Gheorghiță
Scientific Coordinator: Assoc. Prof. Dr. Mădălina Tudorache

February 2021, Bucharest

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

Biocatalyst preparation and characterization

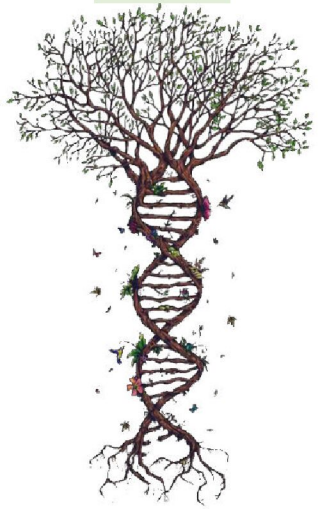
Immobilization approaches and performance

Relative enzyme activity

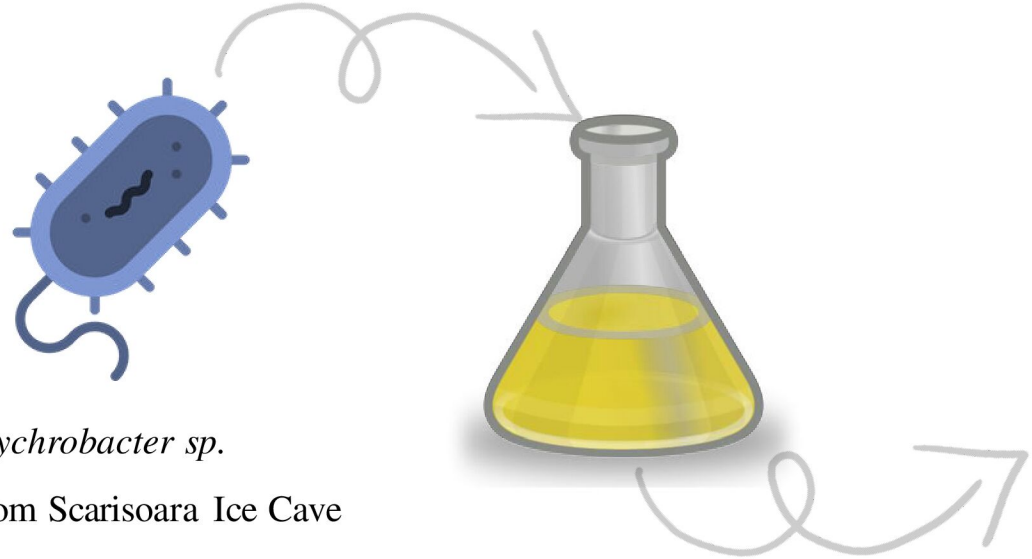
Design of the biocatalytic system

Testing the biocatalytic system

Conclusions



2. BIOCATALYST PREPARATION AND CHARACTERIZATION



Psychrobacter sp.

From Scarisoara Ice Cave

Psychrophilic microorganism

Producing cold-active extracellular lipases

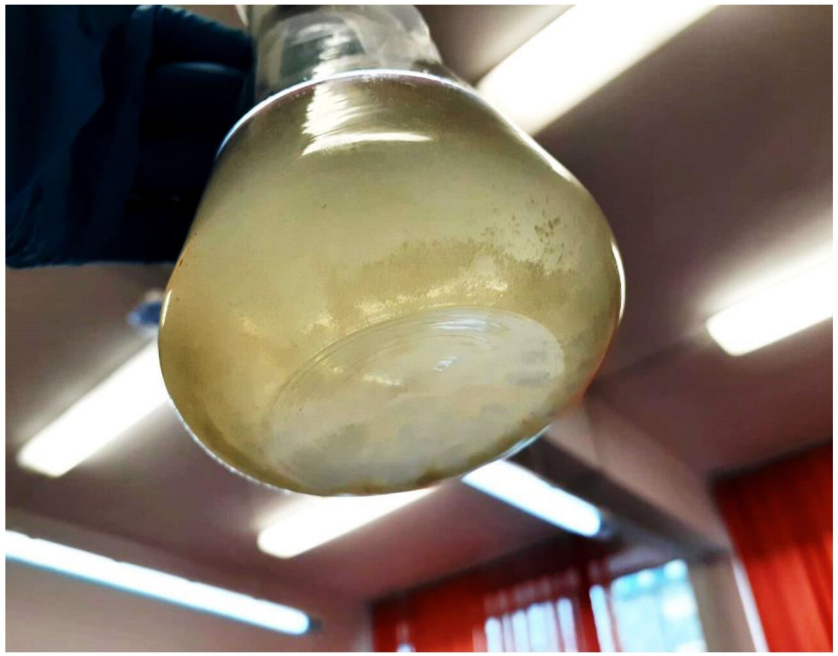
Reasoner's or Tryptic soy media

Proper cultivation media for *Psychrobacter*

15°C for 3 or 4 days under stirring

Supplimented with olive oil*

*carbon source for lipase production

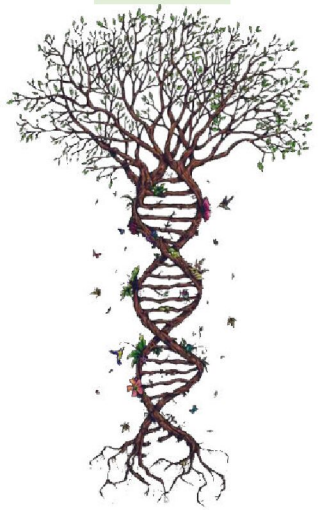


After bacterial growth: cell deposition and supernatant collection.

Supernatant contains extracellular lipases.

Extraction with organic solvent: 80% cold acetone added drop by drop onto the supernatant.

Why is precipitation needed?



3.

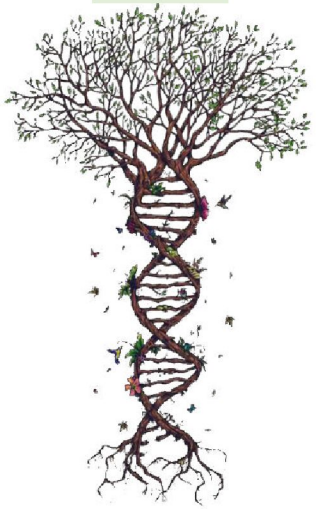
BIOCATALYST PREPARATION AND CHARACTERIZATION

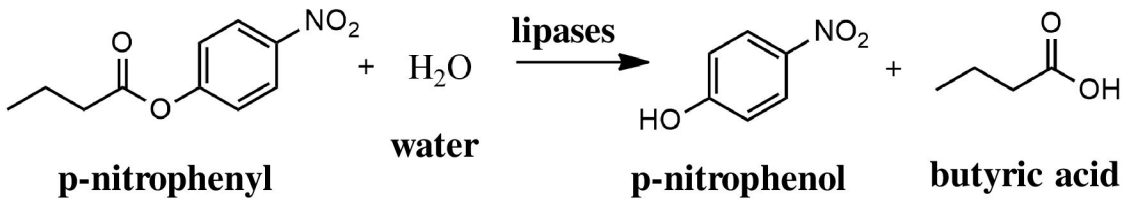
Protein concentration

After protein powder finally obtained,
resuspension in 100mM TRIS-HCl pH 8.
Sample diluted 1 to 10 for UV concentration analysis.
Concentration of the protein content
4.96 mg/ml - 6.6 mg/ml.

Enzyme kinetics

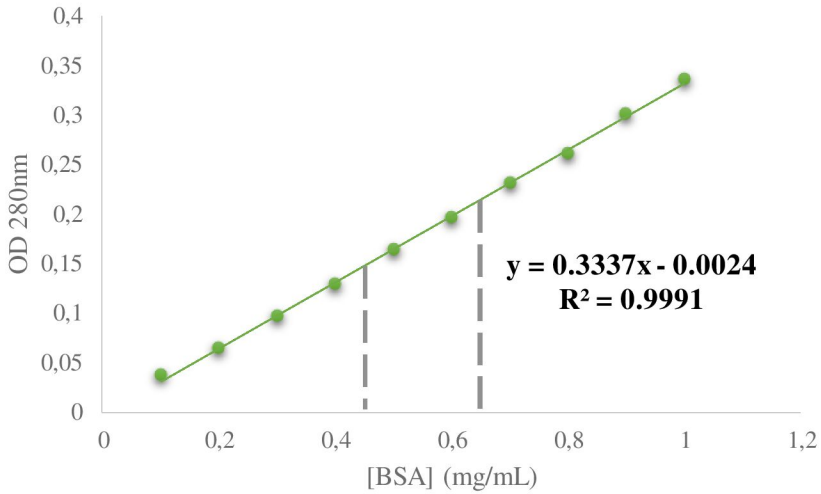
Substrate mix sonicated for emulsification:
4 750 µl 100 mM TRIS-HCl pH 7.55,
90 µl Triton100, 250 µl p-NPB.
100 µl protein extract + 900 µl substrate mix.
Measuring continuously for 30 minutes at 410nm.
Lambert Beer's Law is applied.



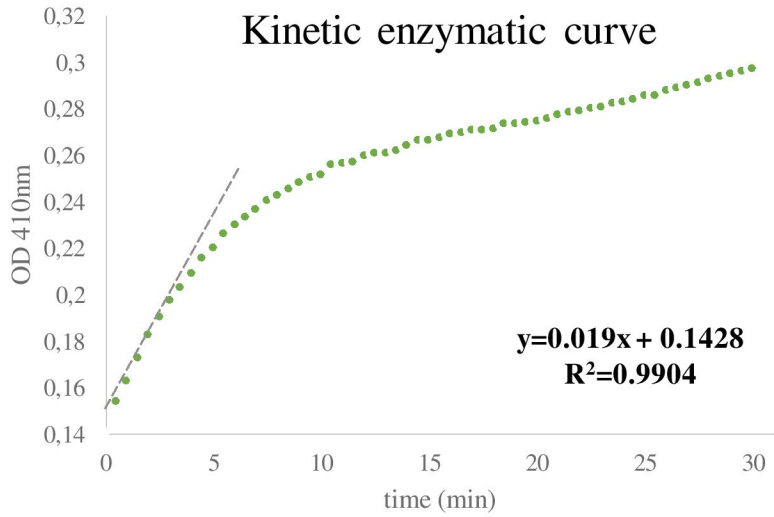


p-nitrophenyl butyrate + water → **p-nitrophenol** + **butyric acid**

Calibration curve of BSA

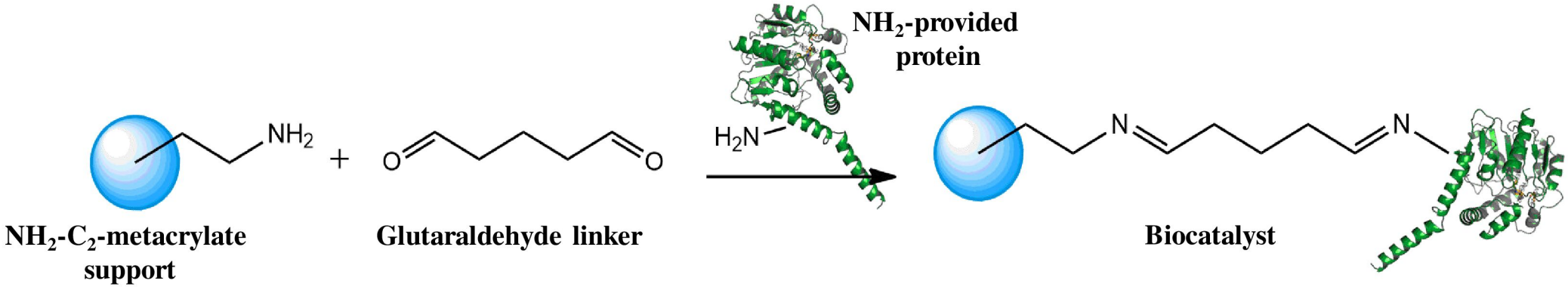


Kinetic enzymatic curve



Enzyme activity
4.25µM/min/mg protein

4. IMMOBILIZATION OF THE BIOCATALYST



NH_2-provided protein

NH_2-C_2-metacrylate support

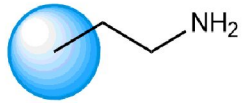
Glutaraldehyde linker

Biocatalyst

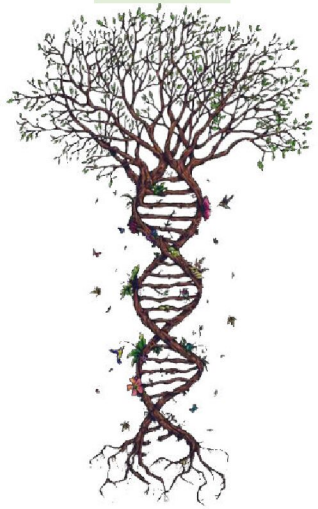
Procedure

0.1g support
0.1% glutaraldehyde
100µL protein extract
in MES (pH 4.7) or PBS (pH 7.4) buffers

Legend:



spherical beads (150–300µm) of methacrylate cross-linkers polymer functionalized with -NH_2 groups.

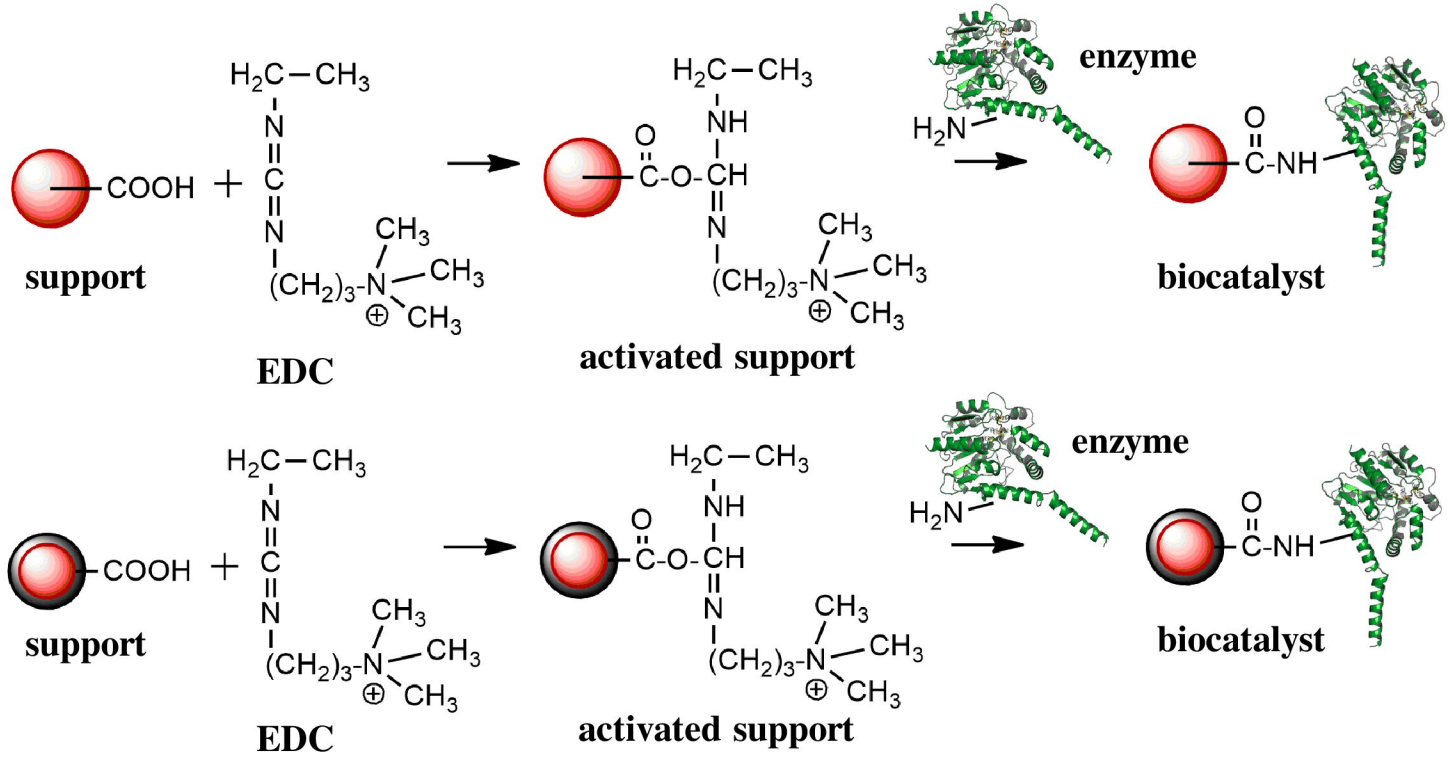


Immobilization performance

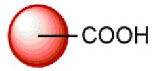
Starting concentration	Immobilized	Protein loading	Protein recovery
4.96 mg/mL	4.382 mg/mL	41.73 mg protein/g support	88.35%
	4.47 mg/mL	42.33 mg protein/g support	90.12%

5.

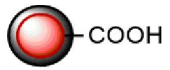
IMMOBILIZATION OF THE BIOCATALYST



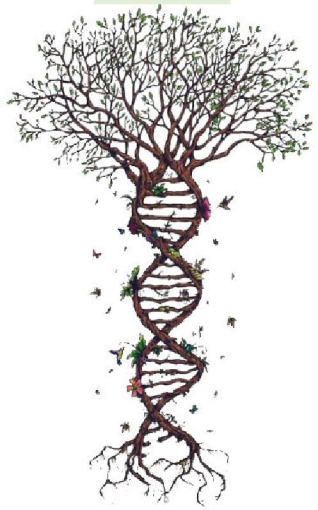
Legend:



0.5 µm to 1 µm magnetic cores coated with non-porous silica provided with fuctional groups.



50 nm, 100 nm and 200 nm beads of magnetic iron oxides covered with hydrophilic polymers with different functional groups.



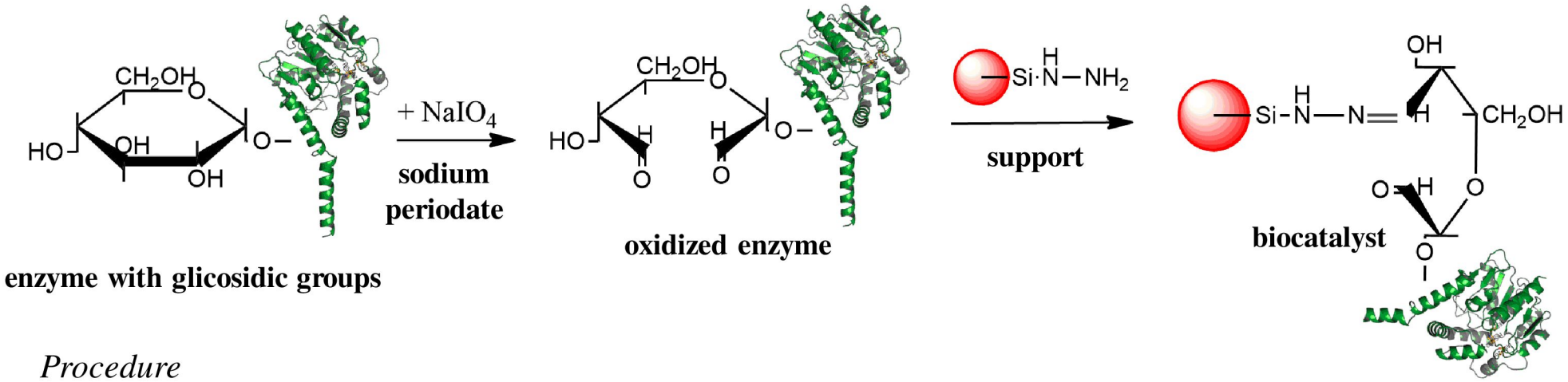
Carbodiimide method
1mL support
0.25M EDC in MES buffer
200µL protein extract

Immobilization performance

Starting concentration	Immobilized	Protein recovery	Biocatalyst
6.6 mg/mL	6.545 mg/mL	99.16%	Si-MAG-Carboxyl x Protein
	6.33 mg/mL	95.9%	Fluid-MAG-ARA x Protein

6.

IMMOBILIZATION OF THE BIOCATALYST



enzyme with glicosidic groups

oxidized enzyme

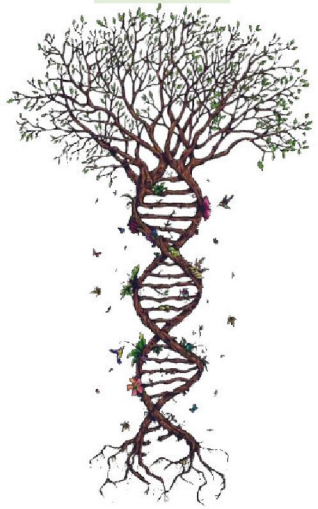
Procedure

500µL protein extract

5mg $NaIO_4$

1mL support

250µL protein extract (oxidized)



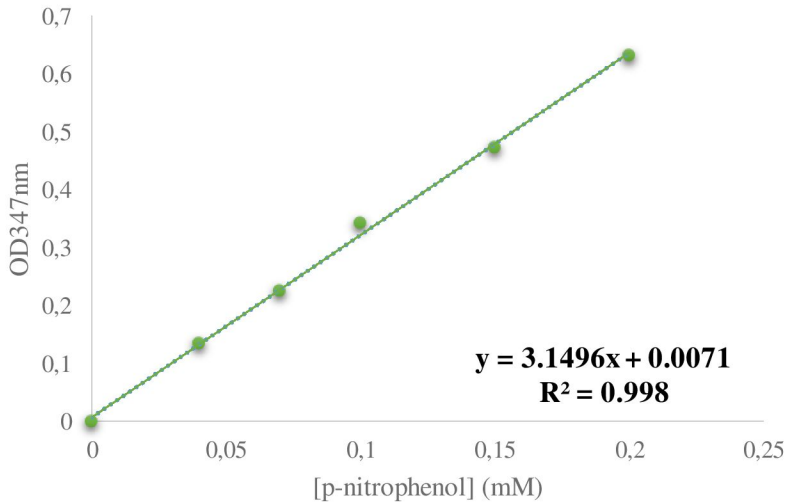
Immobilization performance

Starting concentration	Immobilized	Protein recovery	Biocatalyst
6.6 mg/mL	6.53 mg/mL	98.94%	Si-MAG-Hydrazine x Protein

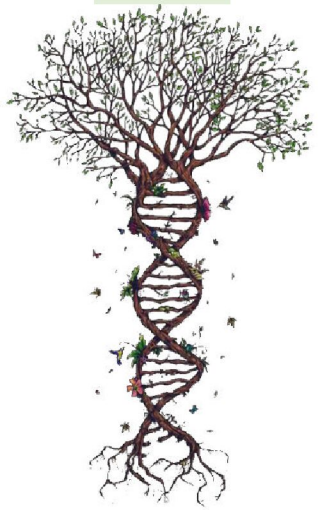
7.

RELATIVE ENZYME ACTIVITY

Enzyme activity of both free and immobilized protein
Reaction mix:
0.25 mL protein extract, 0.65 mL 0.1M TRIS-HCL pH 7,
0.1 ml 0.025M substrate dissolved in acetonotril
30 minute at 37°C
Blocking solution: 0.1M Na_2CO_3

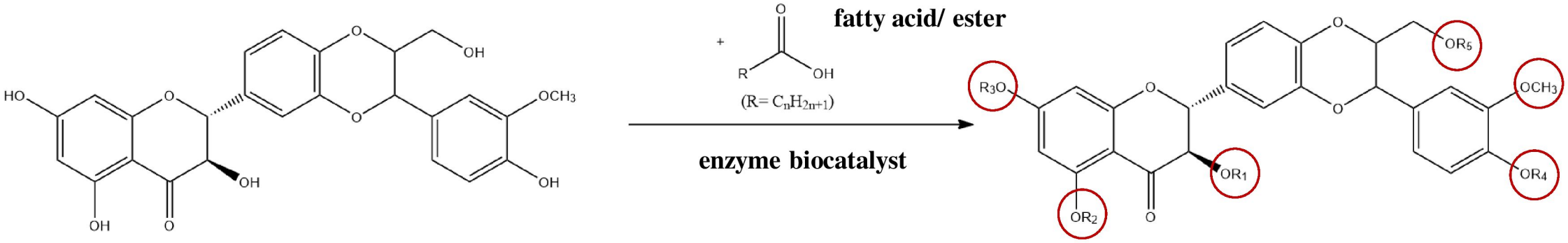


Relative enzyme activity	Biocatalysts	µM/min/mg protein
	Si-MAG-carboxyl x Protein	3.6
	Si-MAG-hydrazine x Protein	3.6
	Fluid-MAG-ARA x Protein	5.9
	NH_2-C_2-metacrylate in MES x Protein	4.8
	NH_2-C_2-metacrylate in PBS x Protein	4.4
	Free protein extract	4.6



8.

DESIGN OF THE BIOCATALYTIC SYSTEM



silybin

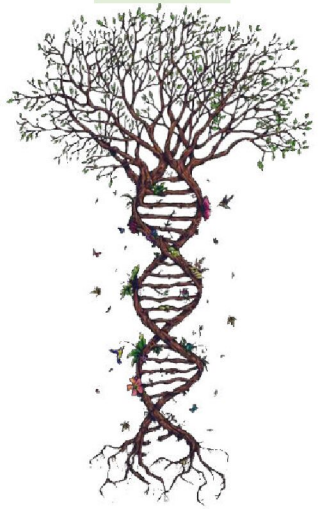
silybin esters

Reaction mixture
1mg of silybin in 1mL THF
10µL of fatty acid/ester
100µL of protein specimen
25°C for 24h at 1000rpm

centrifuged for 15 minutes at 1500rpm
0.22µm milipore filtration
Solvent evaporation at 70°C
Redissolving into mobile phase

HPLC analysis
Poroshell 120 EC-C18 column
acetonitrile and water (41/59, v/v) as mobile phase
1mL/min flow rate and 25µL injection volume
30 minutes reading
DAD detection

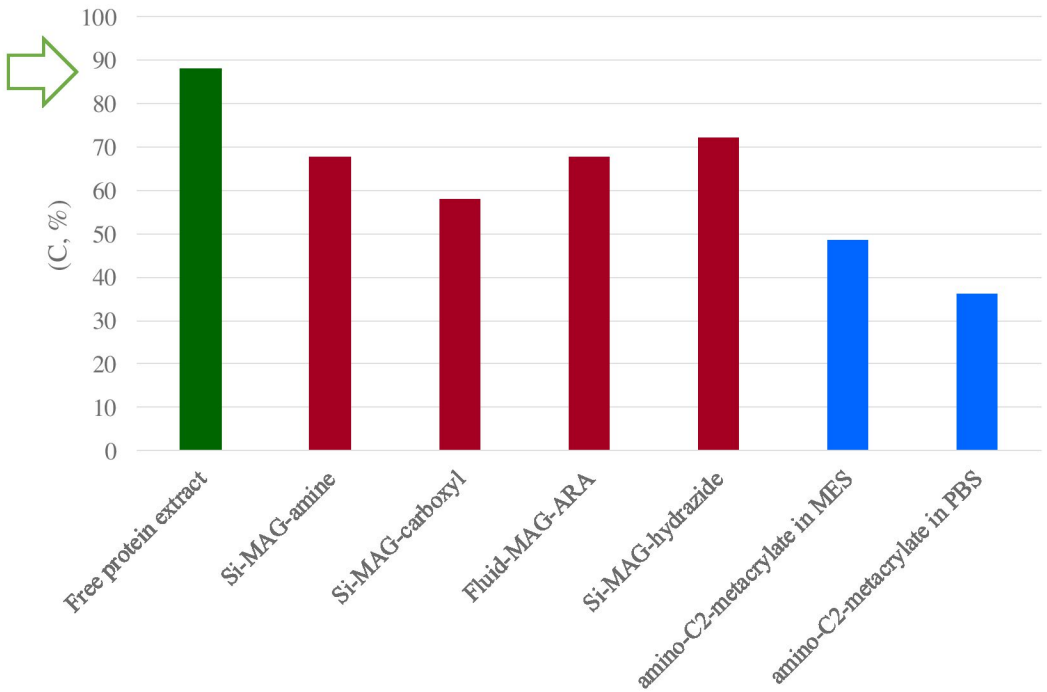
C(%) = (mass of converted susbtrate) / (initial mass of substrate) x 100



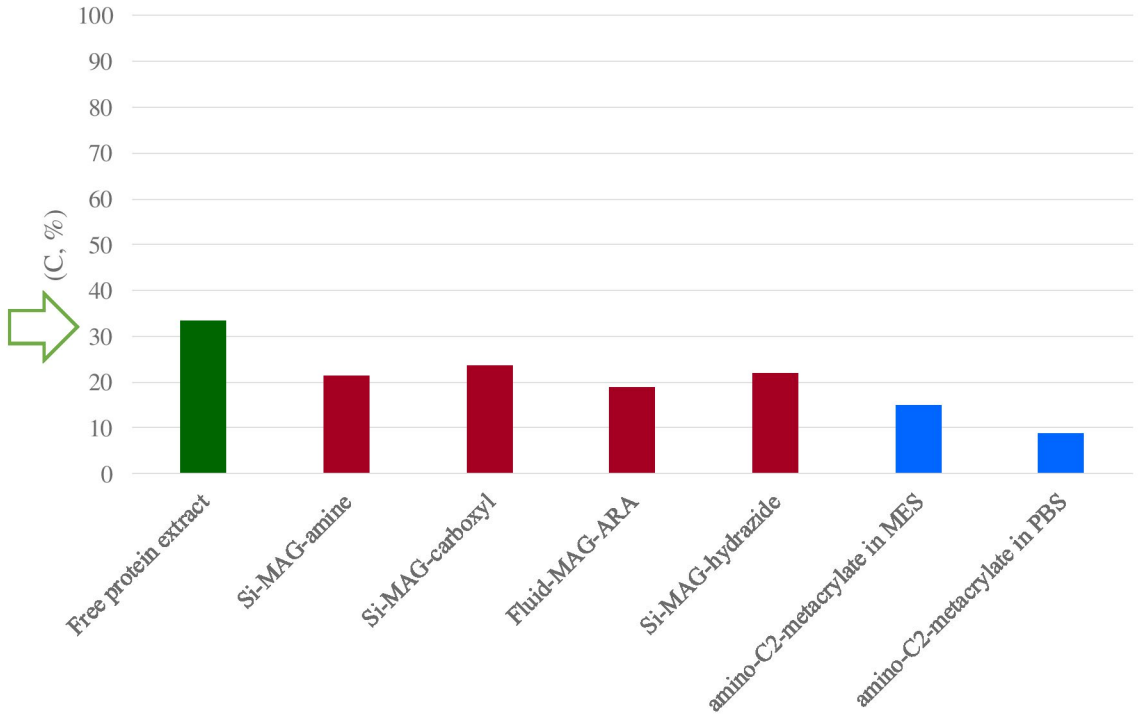
9.

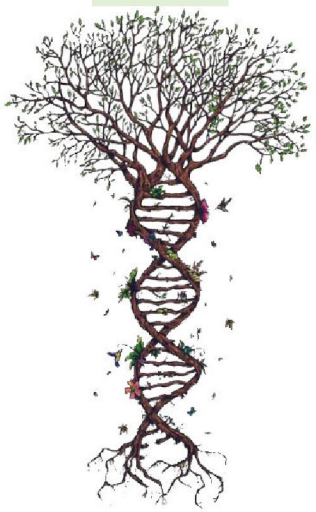
TESTING THE BIOCATALYTIC SYSTEM

Esterification of silybin with octanoic acid



Esterification of silybin with oleic acid

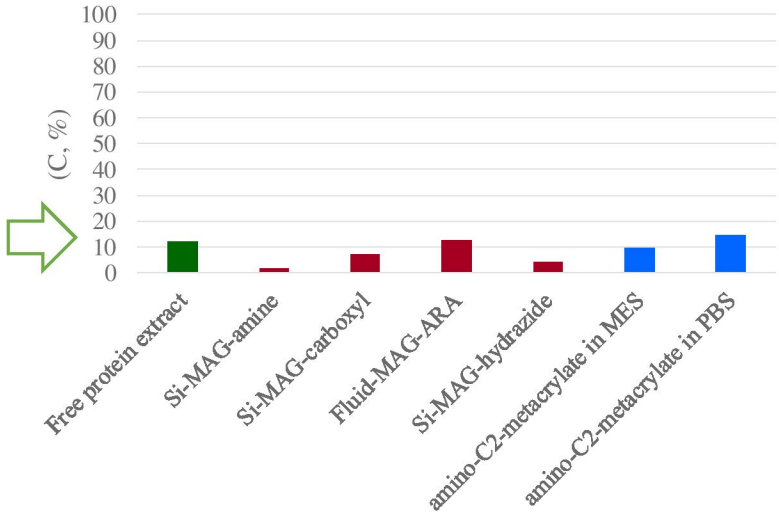


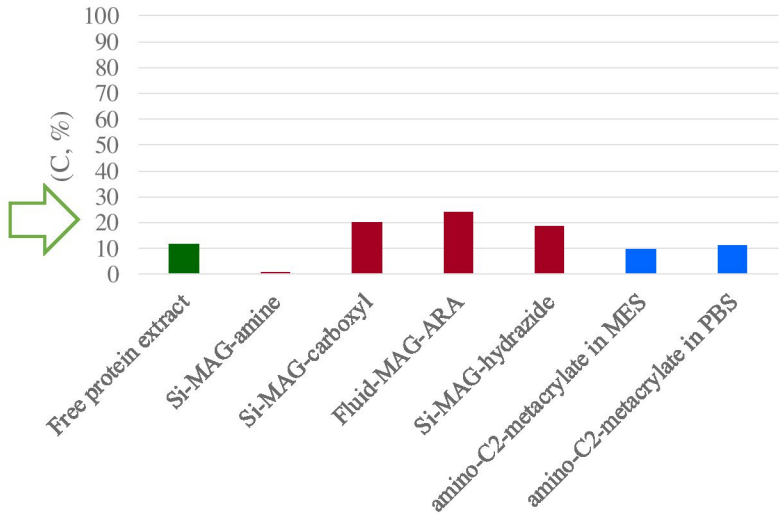


10.

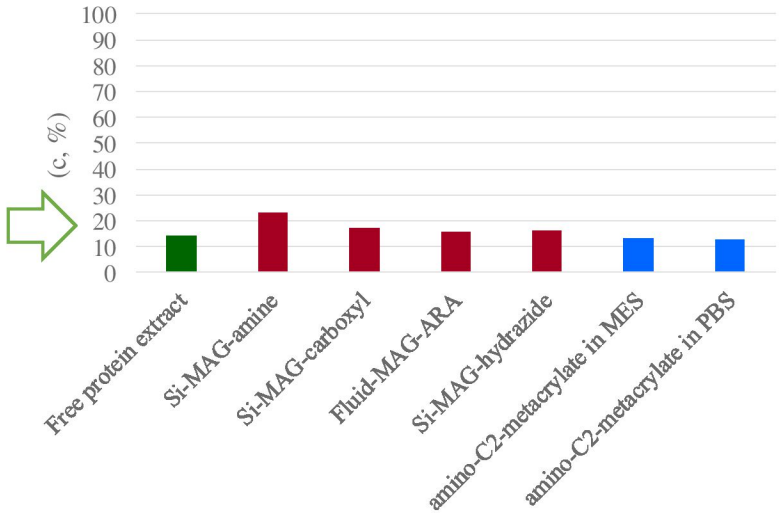
TESTING THE BIOCATALYTIC SYSTEM

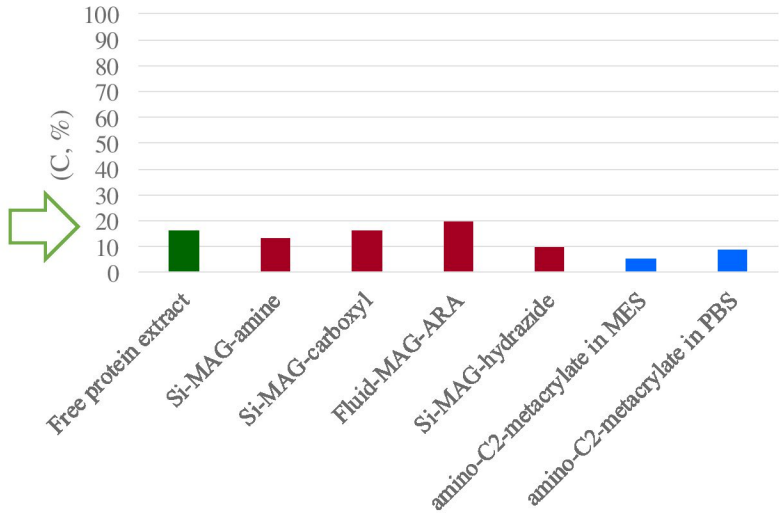
Transesterification of silybin with methyl decanoate vs. methyl laurate

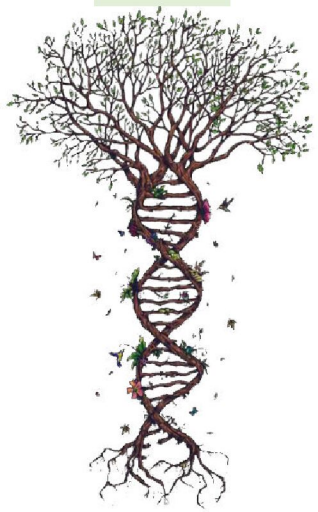




Transesterification of silybin with methyl myristate vs. methyl palmitate



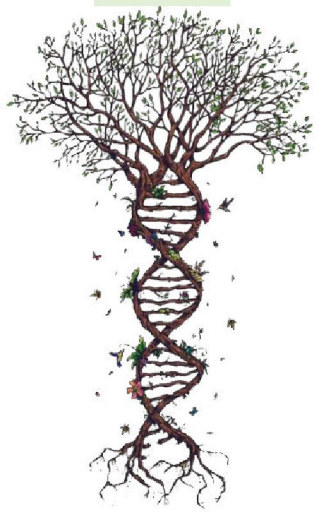




11.

CONCLUSIONS

- Following the extracellular production of proteins, thus suspended in the culture medium of the microorganism, a purification step is absolutely necessary, in order to eliminate the interferences imposed by the composition of the environment or by the production of other biomolecules by the microorganism.
- Immobilization of the protein was achieved as a compromise between stability and activity, particularly imposed by the covalent bonding.
- The protein material obtained showed a good activity in catalyzing the esterification reaction between silybin and octanoic acid.

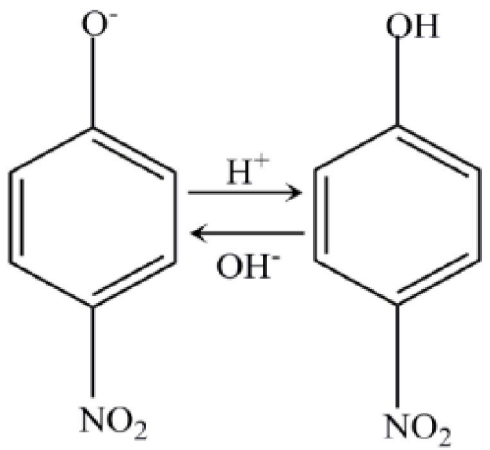


Perspectives

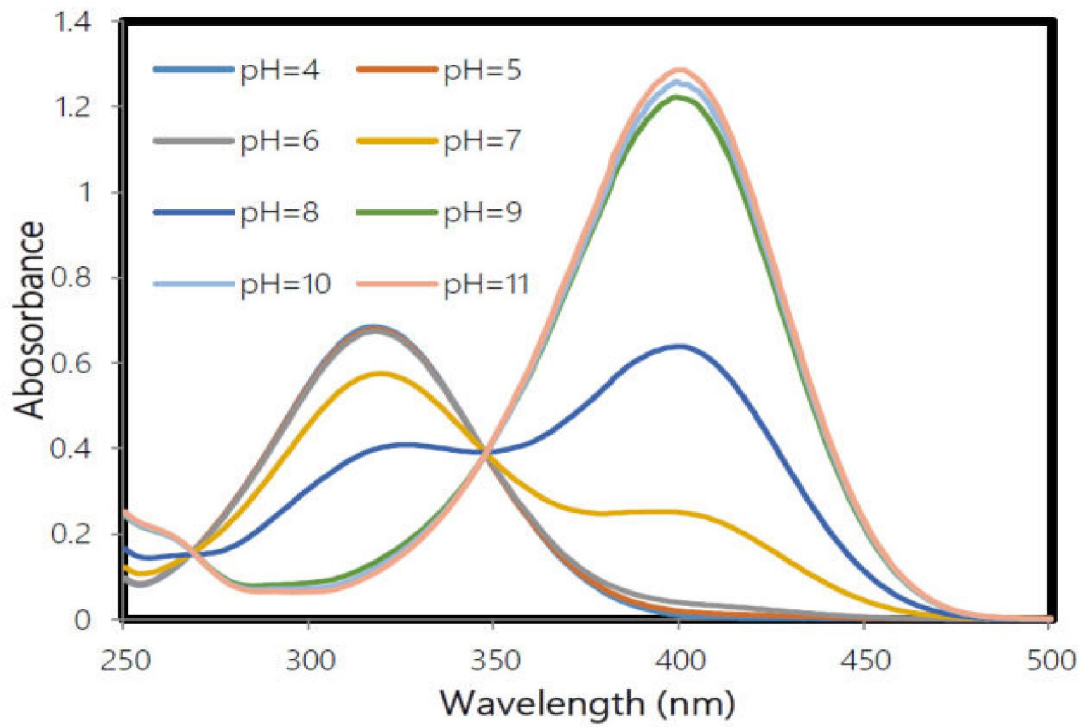
- Characterization of the free and immobilized bioctalyst through FTIR, SEM, CD.
- Optimizing the biocatalytic system in terms of good substrate conversion.
- Establishing if the cold-active character of the biocatalyst remains unaltered.

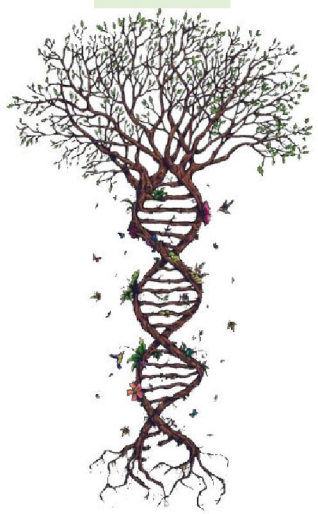
Thank you for your attention!

ANNEX



Ionized and non-ionized forms of p-nitrophenol



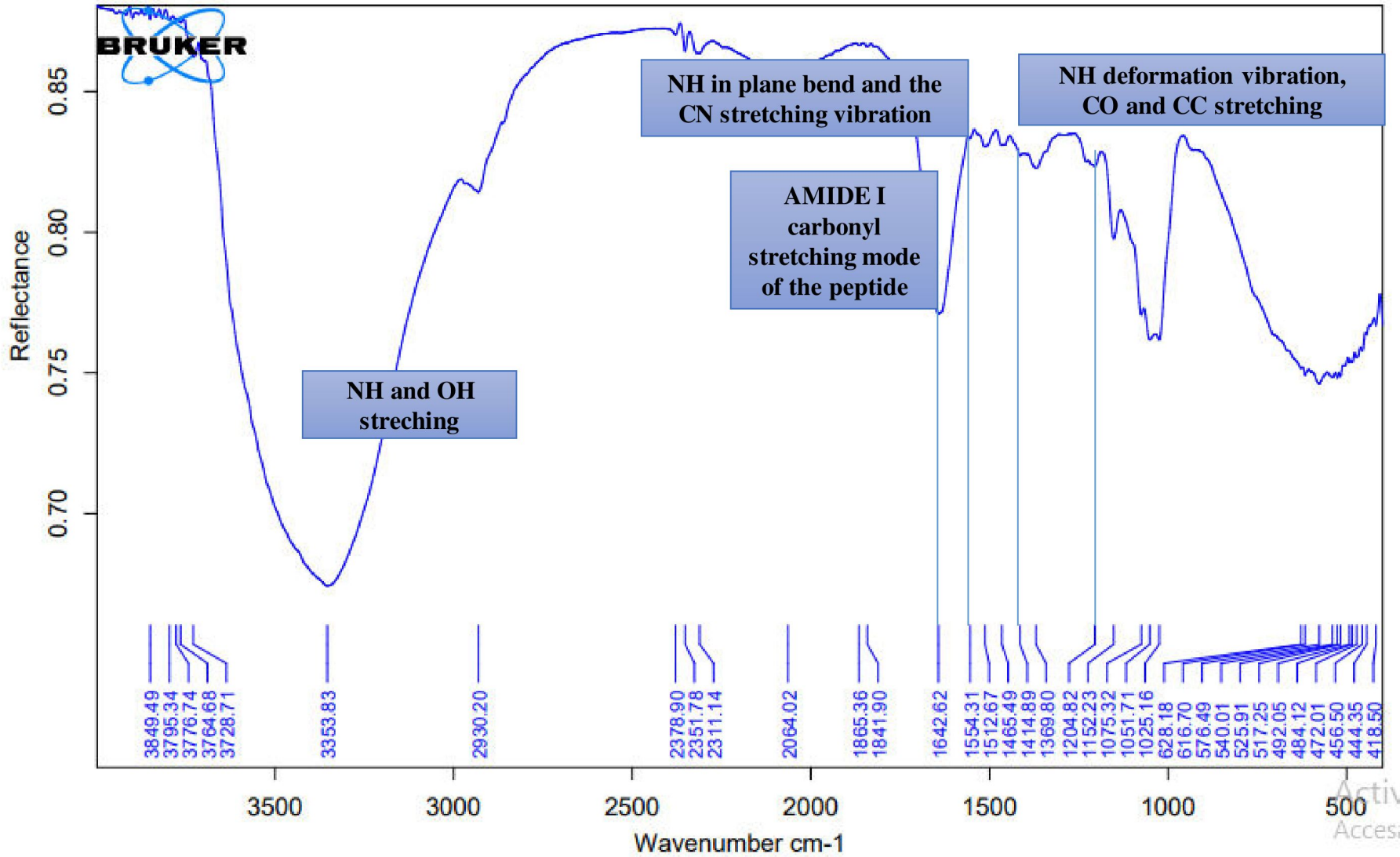


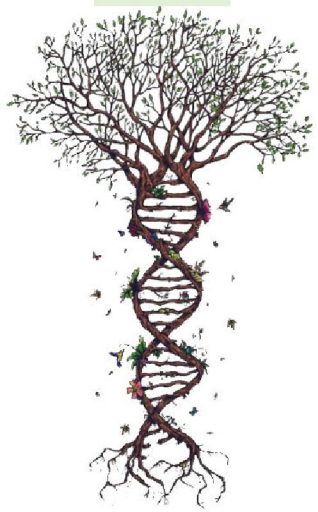
8.

IMMOBILIZATION PERFORMANCE

FT-IR analysis in the context of biological materials

- *Free protein extract*





IMMOBILIZATION PERFORMANCE

FT-IR analysis in the context of biological materials

- *Immobilization via magnetic particles support*

Legend:
Immobilized protein
free protein extract
support