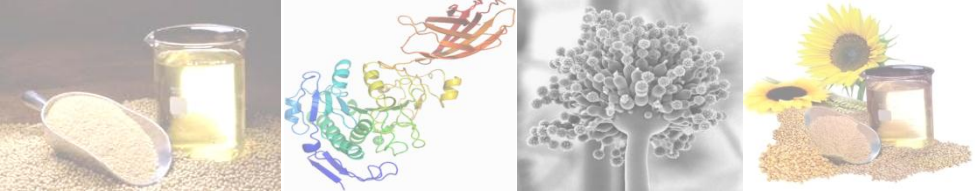
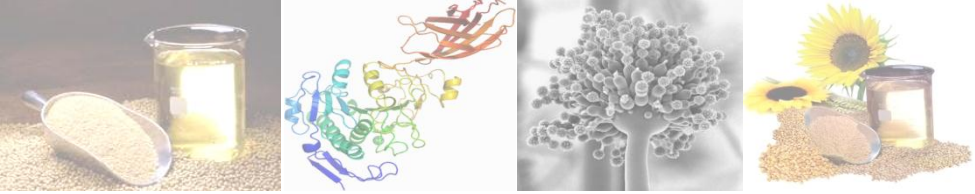


PROJECT:

Ethylic biodiesel production using intra and extracellular lipases from thermophilic fungi



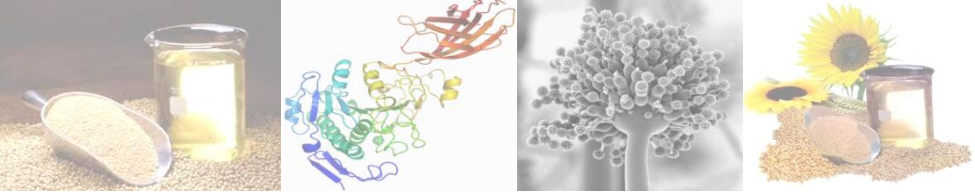
Teaching Staff:

Responsible:

Roberto da Silva (Departamento de Química e Ciências Ambientais)

Collaborators:

- Eleni Gomes (Departamento de Biologia)
- Gustavo O. B. Rodriguez (Depto de Química e Ciências Ambientais)
- João C. Thoméo (Depto de Engenharia e Tecnologia de Alimentos)
- Maurício Boscolo (Depto de Química e Ciências Ambientais)



Students:

Post-doctorate (1)

• Ana Lúcia Ferrarezi Duarte (IBILCE/UNESP)

PhD student (1)

• Janaína Pires Borges (Dr. Biotecnologia – Unesp Araraquara)

MSc students (4)

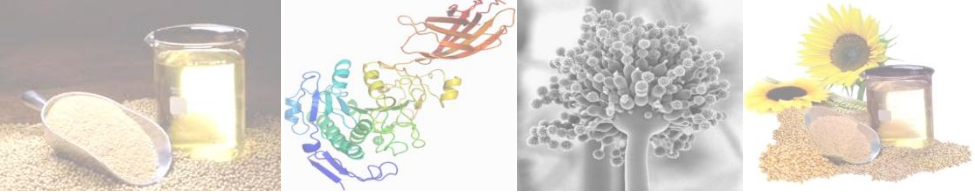
•José Carlos Quiles Junior (Química - IBILCE/UNESP)

• Rafaela Rodrigues* (Microbiologia – IBILCE/UNESP)

• Thiago H. K Ohe** (Química - IBILCE/UNESP)

• Barbara Martineli Bonine** (Microbiologia - IBILCE/UNESP)

* continues in PhD ** discontinued

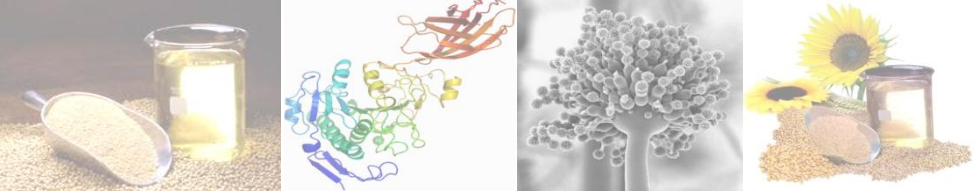


Students:

Scientific initiation students (5)

- Barbara Garcia São José** (Bacharel em Ciências Biológicas)
- Pedro Henrique Vendramini (Química Ambiental – IBILCE/UNESP)
- Marcos Rechi Siqueira (Química Ambiental – IBILCE/UNESP)
- Jéssica F. Alves dos Santos (Química Ambiental – IBILCE/UNESP)
- Felipe Tomasello Correa (Química Ambiental – IBILCE/UNESP)

** discontinued

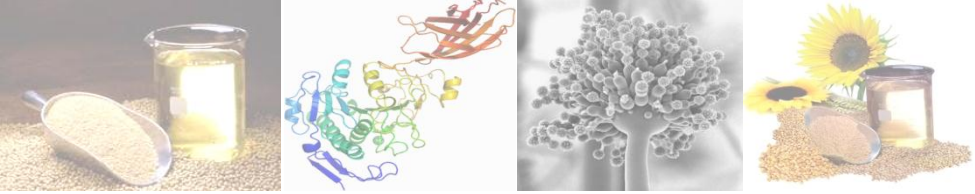


Summary

Biodiesel is an alternative to the conventional diesel, and can be produced from the transesterification reaction of triglycerides found in vegetable oils or animal fats and alcohols such as methanol or ethanol.

A promising route of this reaction is the enzymatic catalysis using lipases. This study aimed at selecting fungal strains capable of producing lipolytic enzymes with transesterification property to be used for the production of biodiesel via ethylic transesterification.

The ability of transesterification of enzymes was evaluated in both free, immobilized on supports and also, immobilized in its own hyphae. The results obtained so far are presented below.



Objective

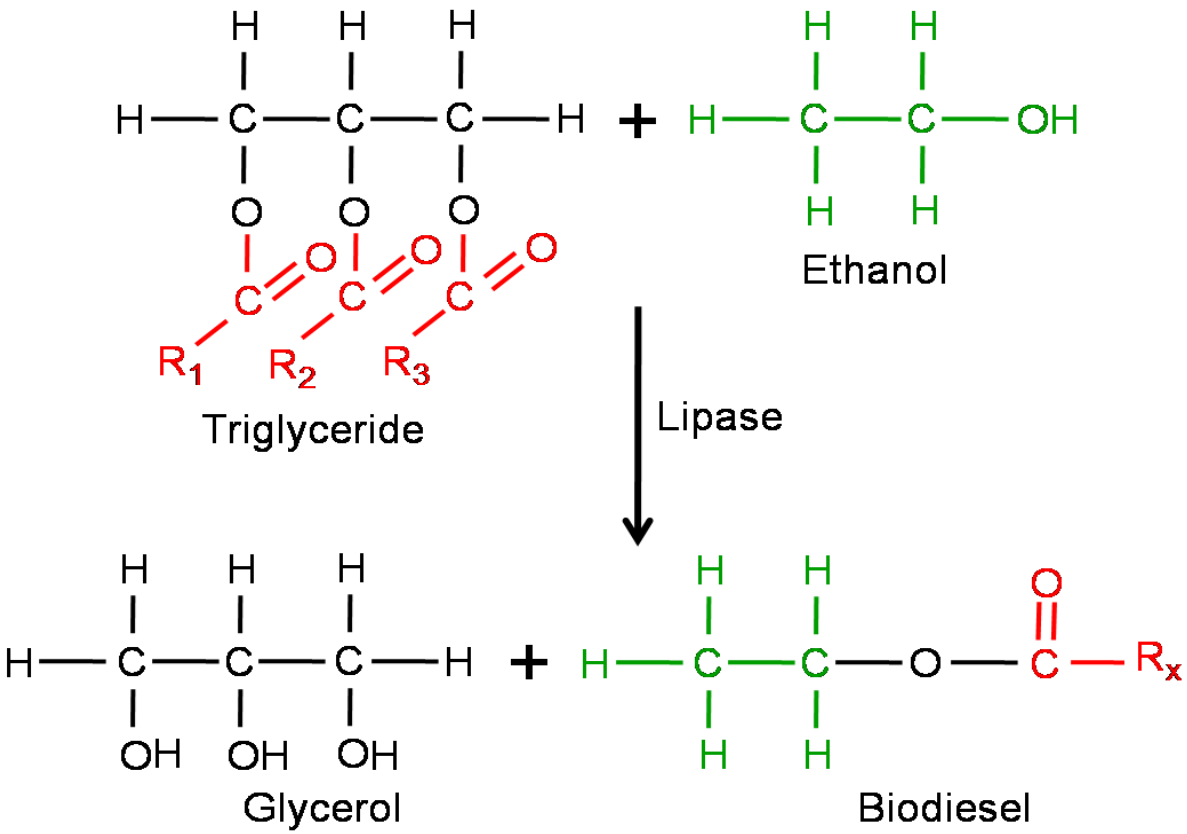
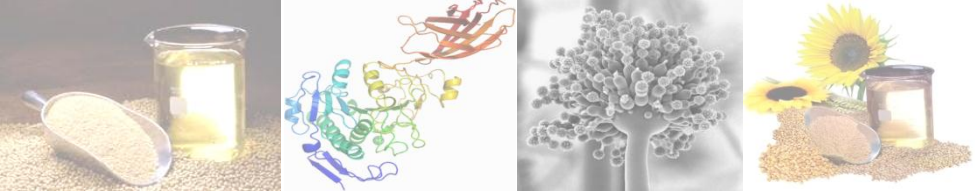


Figure 1. Chemistry of the reaction for biodiesel production. R1, R2, and R3 are alkyl groups with different lengths attached to glycerol of the triglyceride molecule. Lipase promotes the catalysis of the triglyceride hydrolysis to produce free fatty acid and transfers the free fatty acid to the ethanol molecule to form the mono-alkyl ester (or diesel) and crude glycerol.



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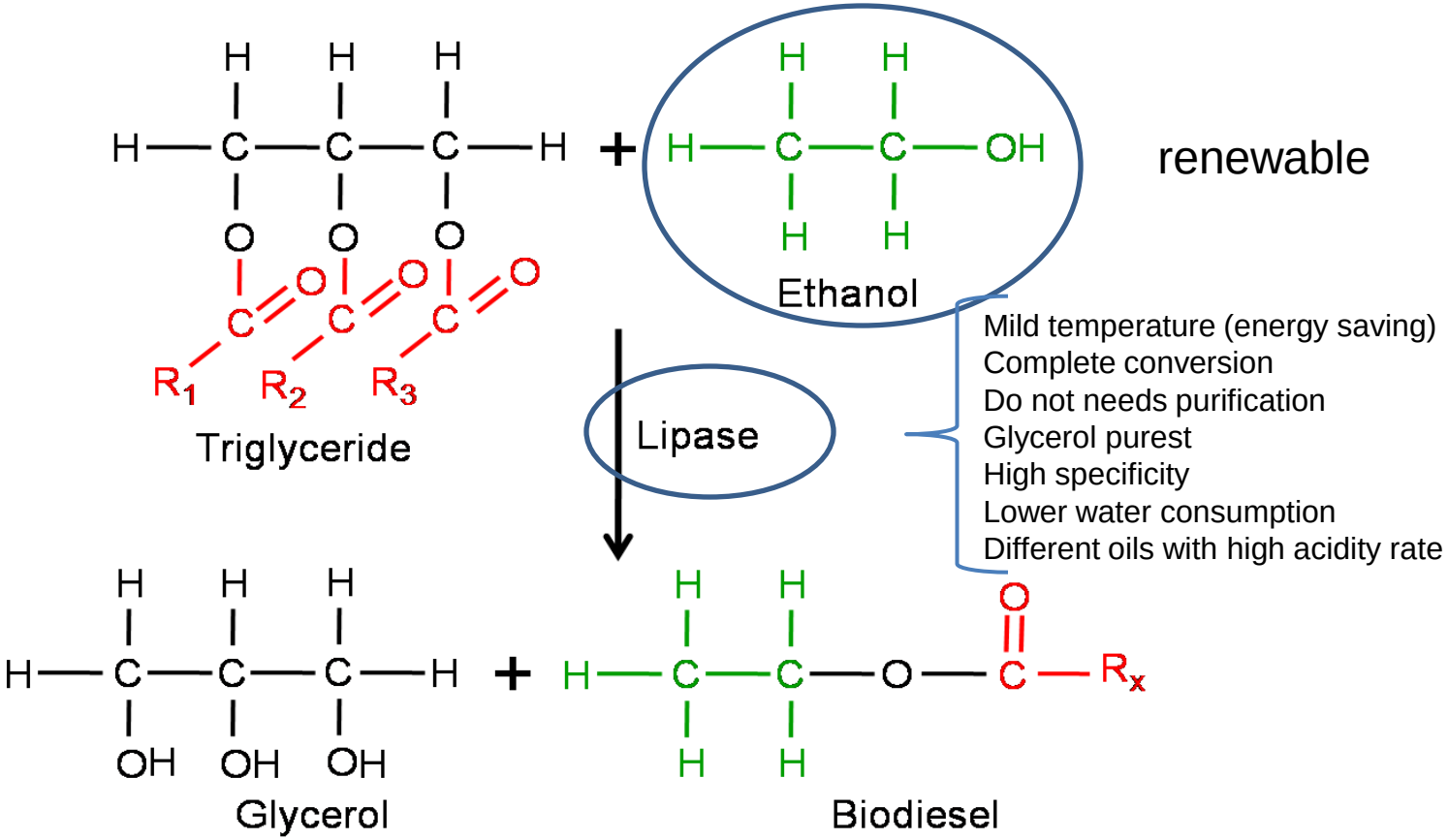

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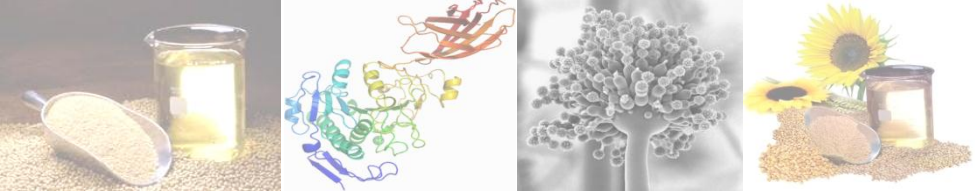
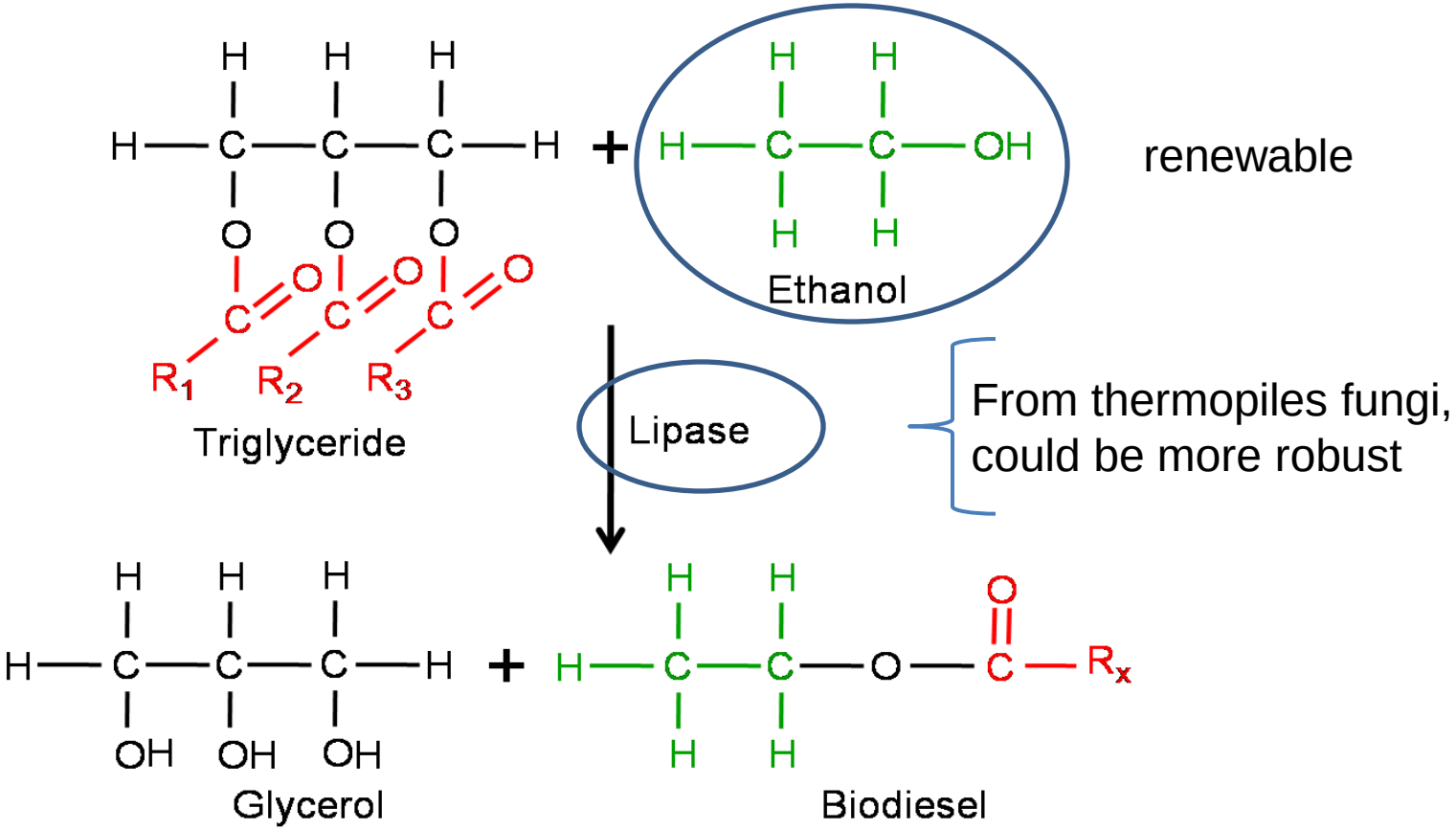


Objective



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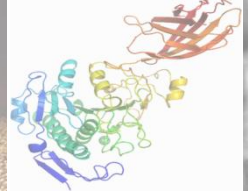
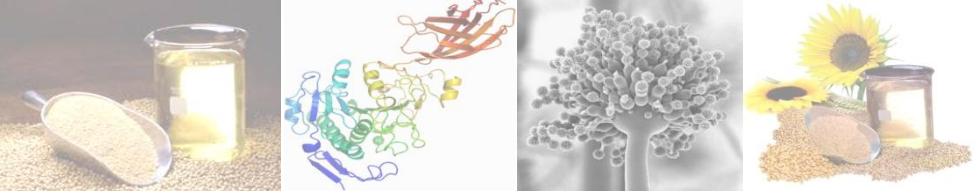


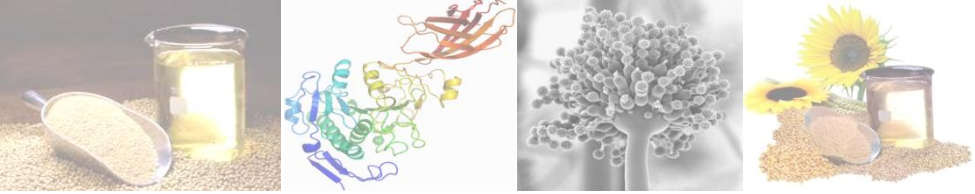
Advantages of enzymatic biodiesel

Advantages of Enzymes in the Production of Biodiesel
Mild temperature (energy saving)
Complete conversion
No purification
Glycerol purest
High specificity
Lower water consumption
Different oils, no requirement of refined oils with low acidity
Enzyme can be genetically transformed



Advantages of ethilic biodiesel

Advantages of Ethanol in the Production of Biodiesel
Ethanol is renewable product, not derived from petroleum.

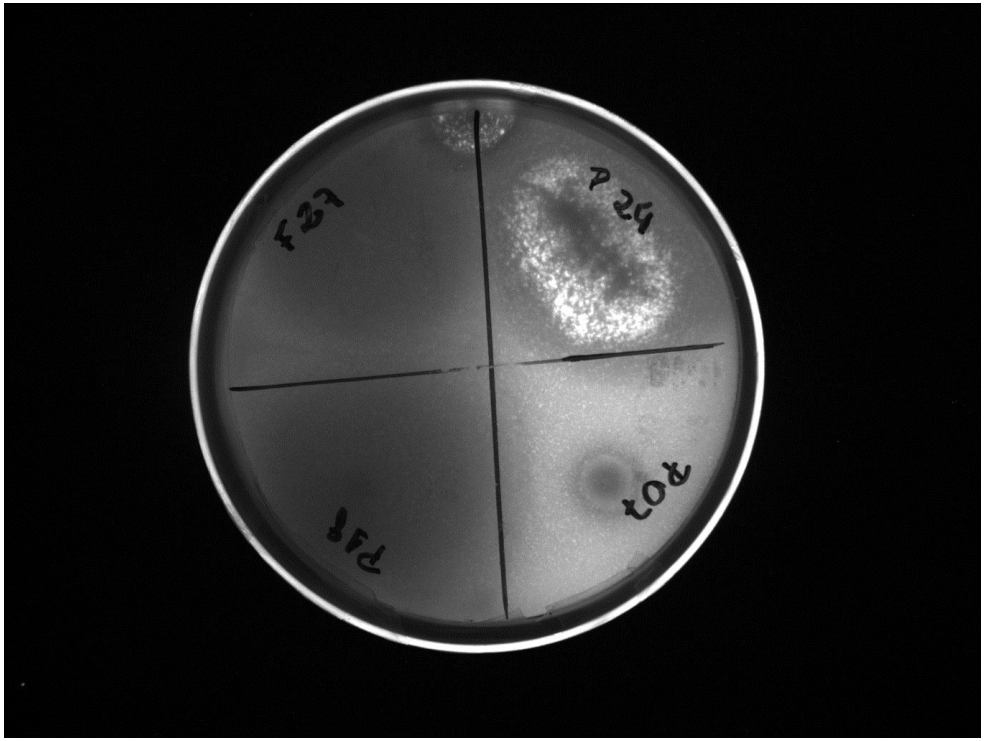


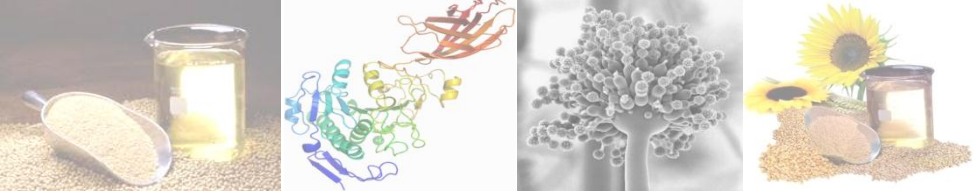
- ***Test lipolytic activity (zymogram)***

54 thermophilic

32 mesophilic

Fungi



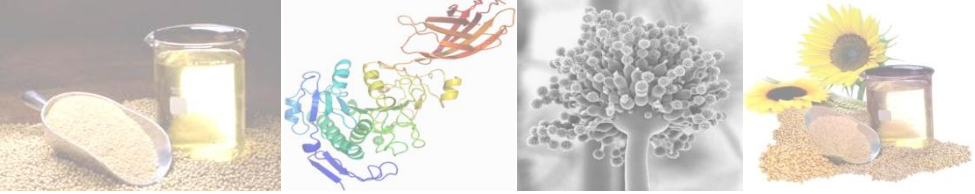


Main results

A. Isolation of Microorganisms

54 Fungi tested

Thermophilic fungi	**Hydrolysis (9)**	**Esterification (6)**	**Transesterification (4)**
Thermomucor indicae-seudadticae N31	x	x	x
Rhizomucor pusillus.	x	x	x
Thermomyces lanuginosus TO-03	x	-	-
Thermomyces lanuginosus TO-05	x	x	x
Thermomyces lanuginosus ROB	x	-	-
Myceliophtora heterotalica (F2.1.4)	x	-	-
Myceliophtora sp (F2.1.1)	x	x	-
Myceliophtora sp (F2.1.3)	x	x	-
Myceliophtora thermophila (M7.7)	x	x	x

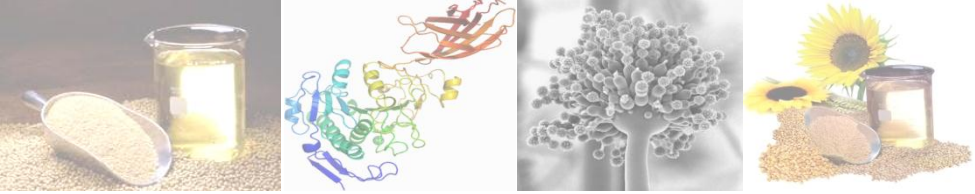


Main results

A. Isolation of Microorganisms

32 Fungi tested

Mesophilic fungi	Hydrolysis (10)	Esterification (7)	Transesterification (3)
Fusarium sp1 F01	x	x	-
Fusarium sp2 B26	x	x	-
Fusarium sp4 G02	x	x	x
Trichoderma sp1 G03	x	-	-
Trichoderma sp2 G17	x	x	-
Trichoderma sp3 G22	x	-	-
Aspergillus nigri sect G08	x	-	-
Aspergillus flavus sect B20	x	x	-
Fusarium sp P24	x	x	x
Acremonium sp	x	x	x



MAIN RESULTS

A. Isolation of Microorganisms

At least 7 different strains of fungi with transesterifying activity and positive forbiodiesel synthesis, were isolated and identified. Some until genera some until species, including:

1) *Thermomucor indicae-seudaticae**
2) *Myceliophtora thermophila**
3) *Rhizomucor pusillus*
4) *Thermomyces lanuginosus*

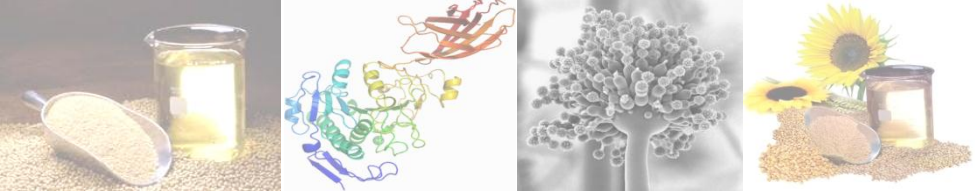
Thermophilic fungi

5) *Acremonium sp (Mesophilic)*
6) *Fusarium sp (Mesophilic)*
7) *Aspergillus sp (Mesophilic)*

Mesophilic fungi

* novelty

Other potential are in the process of identifying

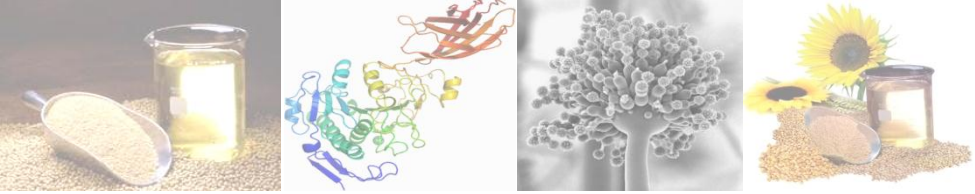


B. Imobilizations

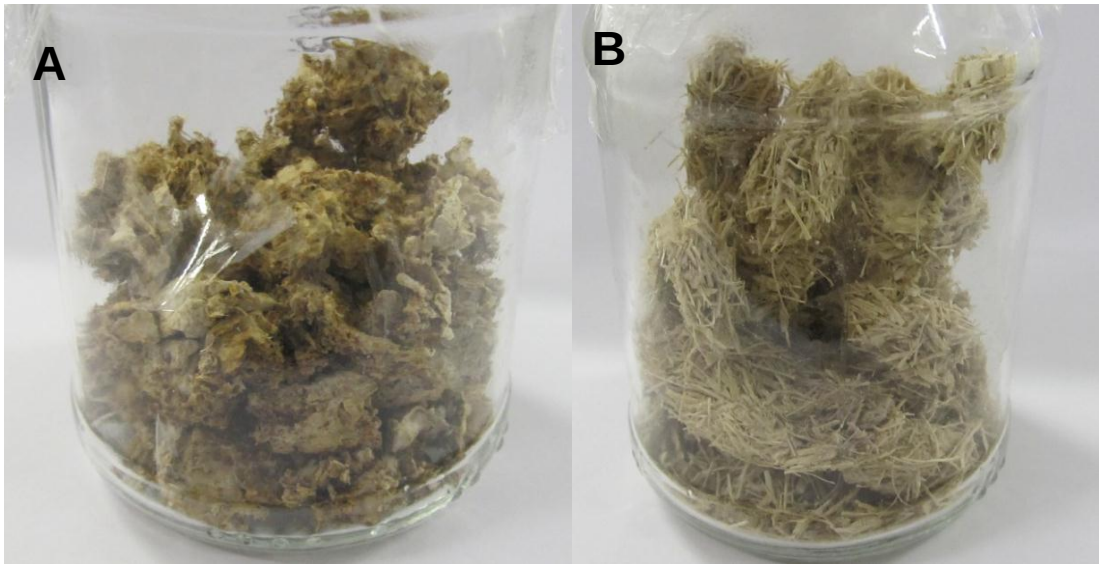
1) The lipase *Myceliophtora* spp. was immobilized in calcium alginate and chitosan being possible to reuse for 6 and 12 times consecutively, respectively.

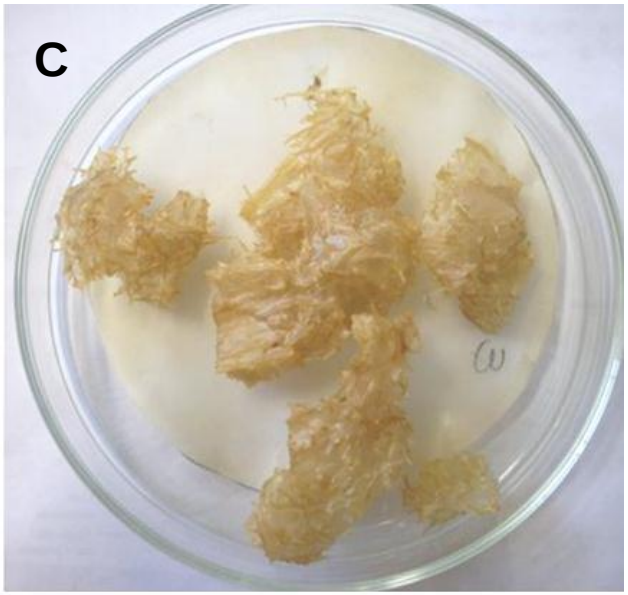
2) In the culture medium itself (hyphae) *R. pusillus*
T. lanuginosus and *T. indicae-seudaticae*

3) Vegetables bushings: *Myceliophtora sp* and *R. pusillus*. Transesterification were improved compared to free enzyme, with yields of 70 and 80% respectively in the conversion of substrates into esters.



B. Imobilizations





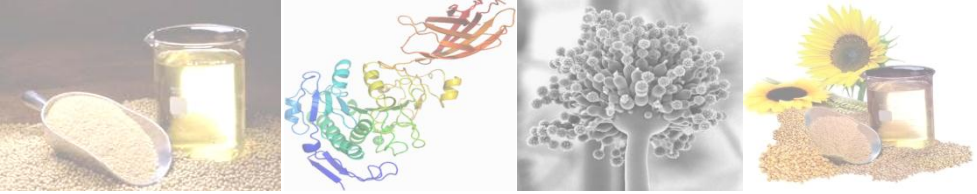
Whole cell imobilization

Myceliophthora thermophila

(A) = Wheat bran
(B) = Sugarcane bagasse

Thermomucor indicae-seudaticae

(C) Vegetal sponge (Loofah sponges)



C. Quantification and identification of esters by gas chromatography

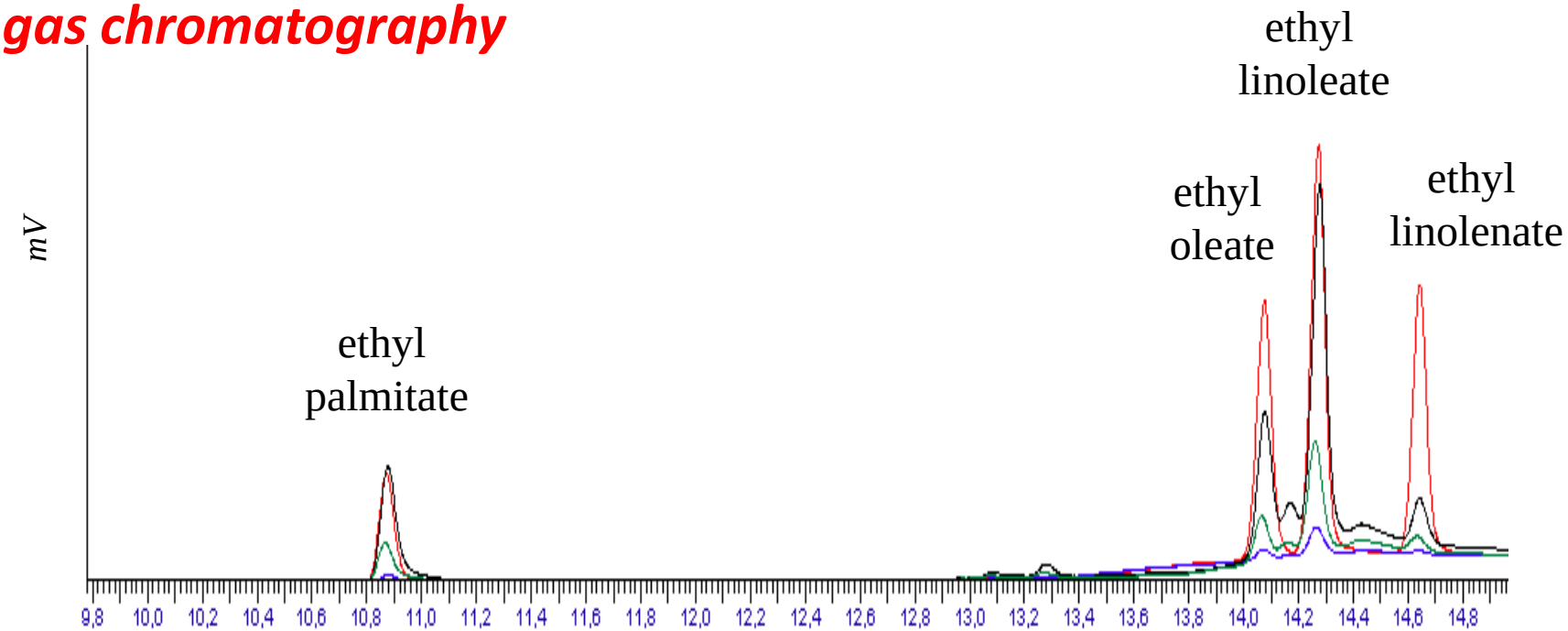
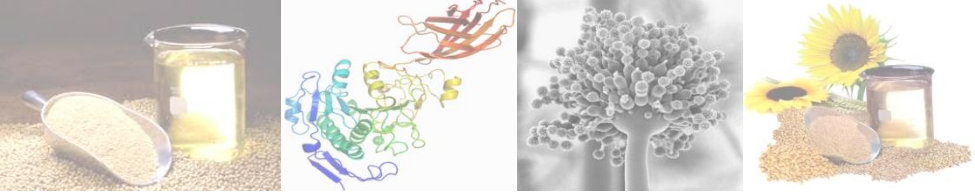


Figure 8: Chromatograms of ethyl esters standards: palmitate, oleate, linoleate and linolenate (red) and the syntheses performed with immobilized hyphae in wheat bran (black), soybean meal (blue) and sugar cane bagasse (green).

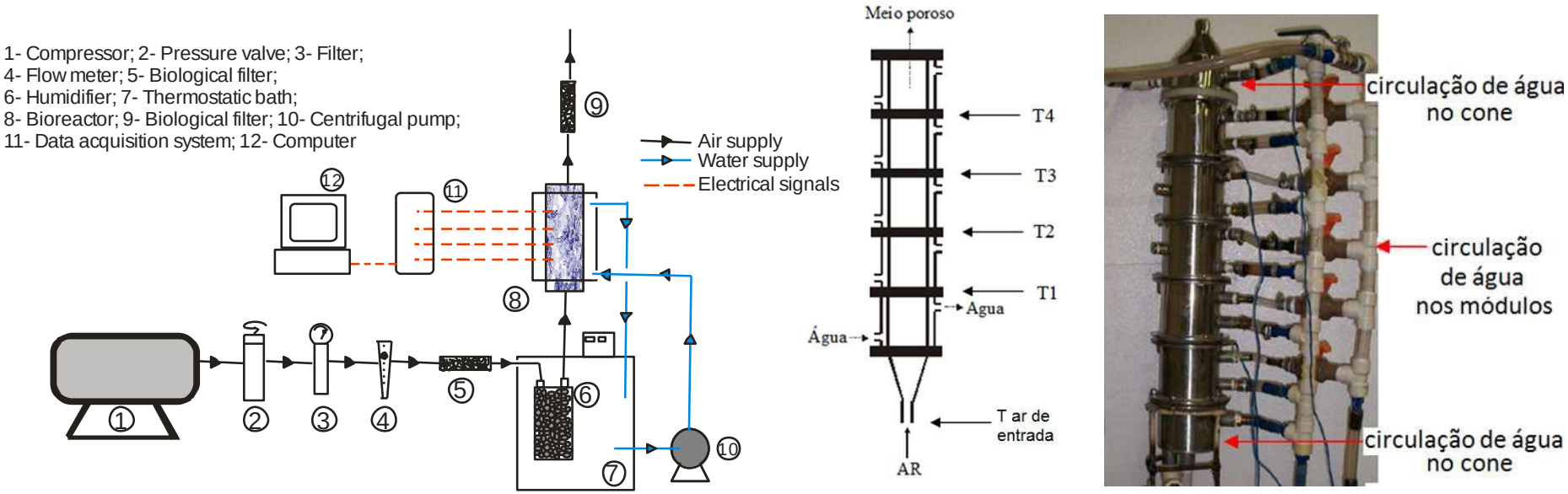
Fungy = *Fusarium* sp (lactis), substrate = Soybean oil



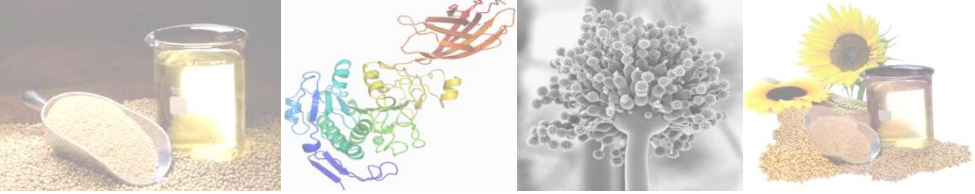
D. Construction of bioreactors

1) A bench top Solid State fermentation (SSF) bioreactor has already been built and operated successfully for the production of fungal lipases with *Myceliophtora* sp.

2) A second larger bioreactor is being developed for production of lipases.



Bioreactor Fixed Bed For SSF (a - Schematic, b - Side View), (T air input and T1 to T4 = thermocouple)



E. Use of Ultrasound

The use of ultrasound irradiation in the transesterification reaction for producing biodiesel from soybean oil and ethanol led to increased conversion of ethyl esters from 92 to 99% showing a very significant effect when used with commercial enzyme.

E. Use of Ultrasound

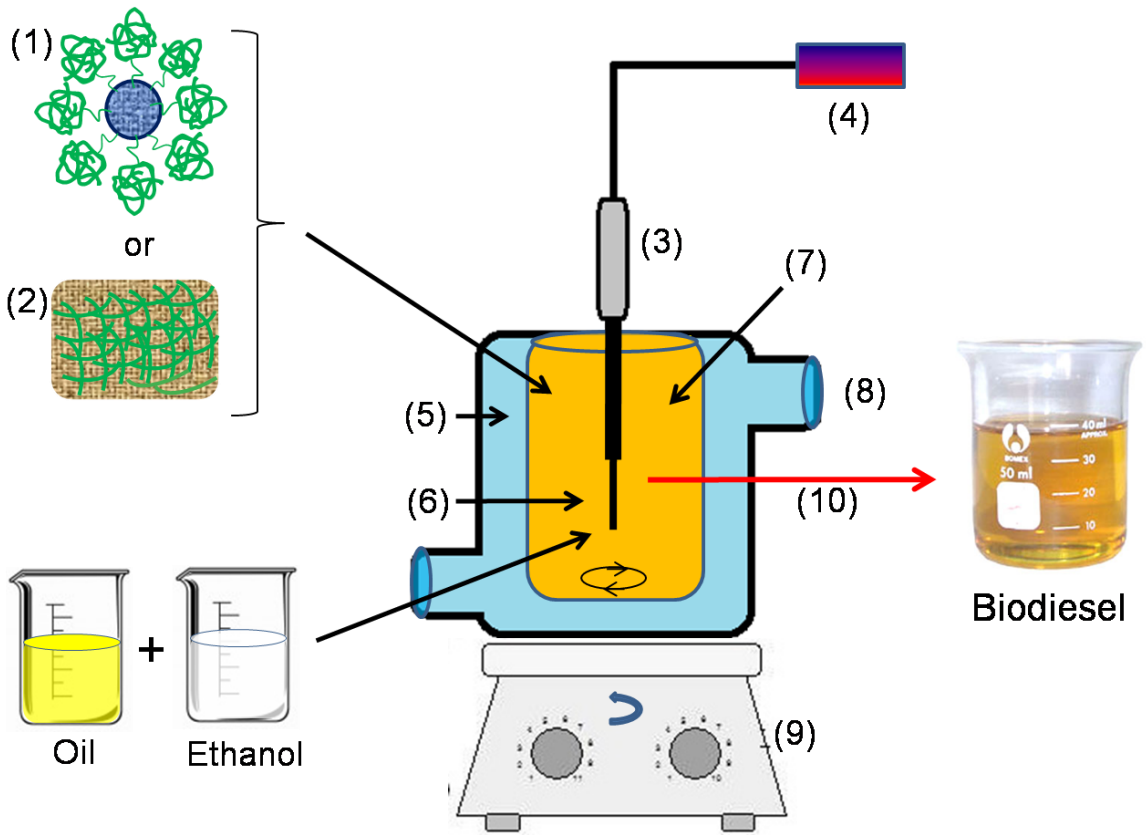
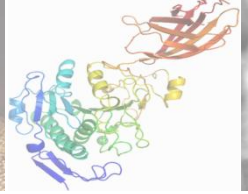


Figure 2. Experimental set-up for enzymatic biodiesel production with ultrasound: (1) immobilized enzyme, (2) immobilizes mycelium, (3) ultrasound probe, (4) power supply, (5) thermostated vessel, (6) reaction vessel, (7) solvent, (8) water inlet/outlet, (9) stirring equipment, (10) Product of reaction after reaction time and removal of glycerol phase.

Thesis and dissertations in this project

Thesis concluded in the project (1)

-Ana Lucia Ferrarezi. Produção de lipases por fungos termofílicos imobilizados e a sua utilização para produção de Biodiesel por transesterificação. 2011. Tese (Ciências Biológicas (Microbiologia Aplicada) - Universidade Estadual Paulista Júlio de Mesquita Filho. Bolsa Fapesp (Orientadora Eleni Gomes)

Dissertations concluded in the project (4)

-Thiago Hideyuki Kobe Ohe. Produção de biodiesel etílico com uso de lipases extracelulares de fungos termofílicos. 2011. Dissertação (Química) - Universidade Estadual Paulista Júlio de Mesquita Filho. Bolsa CNPq (Orientador Roberto da Silva)

-Janaina Pires Borges. Produção de lipases por fungos termofílicos e mesofílicos e uso na produção de biodiesel etílico. 2012. Dissertação (Química) - IBILCE – UNESP. Bolsa FAPESP 2010/03555-5 (Orientador Roberto da Silva).

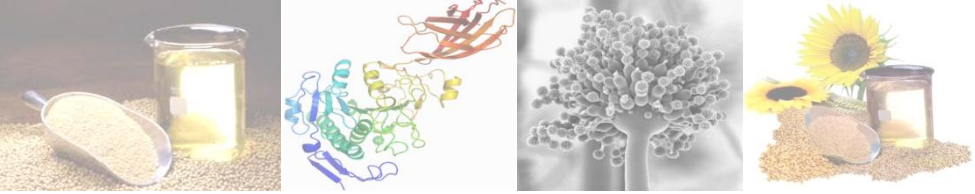
-Bárbara Martineli Bonine. Produção de lipase pelo fungo *myceliophthora sp. f 2.1.4, caracterização e imobilização da solução enzimática bruta. 2012. Dissertação (Microbiologia) - Instituto de Biociências, Letras e Ciências Exatas-Unesp-SJRP. (Orientadora Eleni Gomes).*

-Rafaela Rodrigues de Brito. *Isolamento de fungos produtores de lipases catalisadoras de reações de transesterificação para produção de biodiesel. 2012. Dissertação (Microbiologia) - Instituto de Biociências, Letras e Ciências Exatas-Unesp-SJRP. (Orientadora Eleni Gomes).*

Completion of course work (monographi) (2)

-Daniele Heiras Pivetta. Purificação parcial e caracterização bioquímica das lipases produzidas pelo fungo termofílico Rhizomucor pusillus.. 2011. Trabalho de Conclusão de Curso. (Graduação em Bacharelado em Física Biológica) - Instituto de Biociências Letras e Ciências Exatas de São José do Rio Preto. Orientador: Gustavo Orlando Bonilla Rodriguez.

- Bárbara Garcia São José. Produção de lipases por fungos termofílicos e mesofílicos e uso na produção de biodiesel etílico. 2011. Trabalho de Conclusão de Curso. (Graduação em Bacharelado em Biologia) - Instituto de Biociências Letras e Ciências Exatas de São José do Rio Preto. Orientador: Roberto da Silva.



Scientific papers in this project

Articles resulting from this project

1. de Vasconcellos, Adriano ; Paula, Alex S. ; Luizon Filho, Roberto A. ; Farias, Lucas A. ; Gomes, Eleni ; Aranda, Donato A.G. ; Nery, José G. Synergistic effect in the catalytic activity of lipase Rhizomucor miehei immobilized on zeolites for the production of biodiesel. Microporous and Mesoporous Materials (Print), v. 163, p. 343-355, 2012.

2. Casciatori FP, Laurentino CL, Lopes KCM, Souza AG, Thoméo JC. Stagnant effective thermal conductivity of agro-industrial residues for solid state fermentation. *International Journal of Food Properties (in press). 2012.*

3. Screening and fermentation studies of thermophilic fungus *Thermomucor indicae-seudaticae* N31 for extracellular lipase production (*editing*).

4. Hyphae immobilization of newly thermophilic fungus *Thermomucor indicae-seudaticae N31 for biodiesel application and lipase characterization* (*editing*).

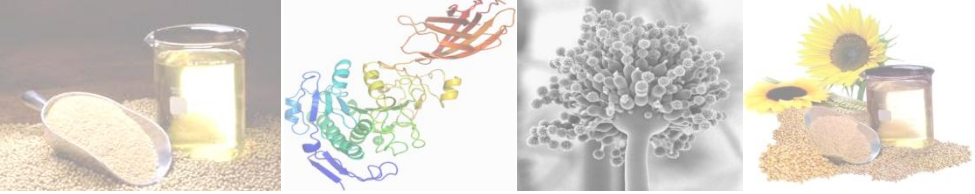
5. Immobilization of fungus's hyphae thermophilic *Rhizomucor pusillus* for biodiesel production *(editing).*

6. Optimization of ethylic biodiesel production by ultrasonic irradiation using soybean oil and lipase of *Thermomyces lanuginousus (editing).*

7. Production and application of lipases of *Fusarium* sp. in the production of ethyl biodiesel *(editing).*

8. Production of lipase from the fungus Myceliophthora thermophila by SSF: characterization, and application for ethyl biodiesel production *(editing).*

9. Isolation and screening of fungi producers of lipases with transesterification actions *(editing).*



Overview

Remarks:

We can point to two main difficulties in the development of this project:

- Development of bioreactors for production of the enzyme in the solid state fermentation (SSF).

Fortunately, we have received a visiting researcher, Prof. Dr Jochen Mellmann, the Leibniz Institute of Agricultural Engineering Potsdam-Bornim (Germany), who is a respected bioreactors researcher, who is now collaborating with this subject.

- The second difficulty is in the process of immobilizing enzymes.

The use of free enzymes in transesterification reactions is not efficient because, in aqueous reaction, it is not favored. So, there is the need for immobilization.

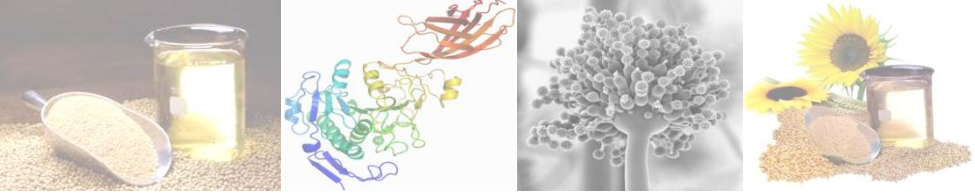
Most of the immobilized lipases were inefficient in converting the biodiesel. Two causes may have contributed to this:

a) enzymes or b) the supports employed. In both cases, they can be sensitive even to the ethanol and solvent used as reactants in the reaction medium. In the case of the enzyme, it could be denatured, and in the case of the support it could be disintegrated.

We are in collaboration with Dr. Benevides Pessela – Research Institute for Food Sciences (CIAL), Autonomous University of Madrid (Spain)

Highlights

As shown before, our best results for transesterifications were obtained with the immobilization of fungal mycelia, which were confirmed by GC analysis. The results of this project generated one Doctoral thesis, four Masters degree and two monographs. Two papers have been accepted, one is under review and six are being finalized for submission to peer reviewed journals.



Others publications

Articles published that took advantage of infrastructure acquired with the financial resources of the project (indirect production)

Merheb-Dini, Carolina ; Garcia, Graziele Aparecida Chiuchi ; Penna, Ana Lúcia Barretto ; Gomes, Eleni ; da Silva, Roberto . Use of a new milk-clotting protease from Thermomucor indicae-seudaticae N31 as coagulant and changes during ripening of Prato cheese. Food Chemistry , v. 130, p. 859-865, 2012.

2. Ellen C. Giese, Robert F. H. Dekker, Aneli M. Barbosa, Maria de Lourdes Corradi da Silva and Roberto da Silva. Production of β-(1,3)-glucanases by Trichoderma harzianum Rifai: Optimization and Application to Produce Gluco-oligosaccharides from Paramylon and Pustulan. *Fermentation tchnology.* Volume 1, 2012.

3. GUEZ, Marcelo A U, Diaz, A.B., Ory, I, Blandino, A., GOMES, E., Caro, I. Xylanase production by *Aspergillus awamori* under solid state fermentation conditions on tomato pomace. Brazilian Journal of Microbiology 42: 1585-1597, 2011.

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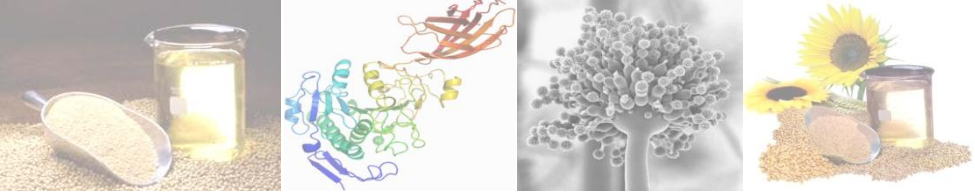

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Indirect production

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