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EvoSCO

Adaptive laboratory evolution strategies to increase the lipid accumulation process in oleaginous microorganisms and microbial oil production using agroindustrial residues as substrates

Brochure for the PNRR project no. 760238/28.12.2023, code PNRR/2023/C9/MCID/I8 CF 162/31.07.2023

evosco.unibuc.ro

About the project

Yarrowia lipolytica is a model oleaginous yeast and a source of neutral intracellular lipids. Currently, on an industrial scale, only polyunsaturated fatty acids of microbial origin are obtained using genetically modified strains of *Y. lipolytica*, and their production cost remains high due to low accumulation rates. The aim of this project is to develop Adaptive Laboratory Evolution (ALE) strategies for the generation of hyper-productive oleaginous strains. The methodology was developed and initially applied on *Y. lipolytica* strains and then extended to other oleaginous microorganisms of industrial interest, with significant contributions to the elucidation of the evolutionary molecular mechanisms associated with lipogenesis.

Microbial oils

Oleaginous eukaryotic microorganisms, as well as certain bacterial species, are at the forefront of biotechnological research due to their ability to accumulate lipids (i.e., triacylglycerols – TAGs) in the form of microbial oils (single cell oils – SCOs), with major industrial applications, particularly feedstock in biodiesel production. Currently, despite the use of agro-industrial residues as low-cost substrates, the limited productivity of oleaginous microorganisms leads to high production costs.

Main objectives

Yarrowia lipolytica, *Rhodosporidium toruloides* and *Cunninghamella echinulata* are subjected to adaptive laboratory evolution under specific conditions, in order to obtain naturally occurring clones with improved oleaginous character. Clones with increased lipogenic potential are selected on solid carbon-free medium and characterized biochemically, genotypically and transcriptomically, with annotation of differentially expressed genes by Gene Ontology. Optimized strains are evaluated at bioreactor scale using agro-industrial residues as carbon sources.

About Us

Project director

Professor **George Aggelis** is one of the pioneers in the field of microbial lipids, who started working in the field 35 years ago in France, performing his PhD thesis.

He has published so far two books, four book chapters, 15 review papers, 131 research papers and one patent and has participated in many national and international conferences and meetings receiving significant international recognition.

He has participated in 32 research projects and he is an active member of the Editorial Board of four journals.

Since December 2005, Prof. Aggelis has been working at the **University of Patras**, **Department of Biology**, remaining in the field of microbial lipids.

Ana-Maria Lecu, Ana-Maria Tănase, George Aggelis, Irina Lascu, Viorica Corbu, Ana-Maria Georgescu

EvoSCO Team

The project team is highly interdisciplinary and diverse, comprising both experienced researchers who have successfully collaborated in previous research projects and young promising researchers, namely PhD students, from:

- the Department of Genetics, Faculty of Biology, University of Bucharest, Romania – *Csutak Ortansa, Tănase Ana Maria, Corbu Viorica, Ionela Avram, Ionescu Robertina, Gulea Chiciudean Iulia, Lascu Irina, Lecu Ana-Maria, Georgescu Ana-Maria, Vasile Camelia;*
- the Department of Botany and Microbiology, Faculty of Biology, University of Bucharest, Romania – *Holban Alina, Barbu Ilda, Gheorghe Barbu Irina*;
- the Research Institute of the University of Bucharest (ICUB), Romania – *George Aggelis, Grumezescu Alexandru, Vasile Otilia, Niculescu Adelina, Florea Eusebiu Cristian*;
- the Department of Biology, University of Patras, Greece – *George Aggelis*.

The Research Institute of the University of Bucharest (ICUB) has the required infrastructure existing in the Research Platform in Biology and System Ecology, suitable for realizing the ALE experiments and characterizations. The project also benefits from updated wet-lab equipment granted by the project's funds, in order to fulfill the ALE-related activities within the proposed timeframe.

Adaptive Laboratory Evolution (ALE) and oleaginous microorganisms

What is ALE?

ALE is an experimental approach that exploits natural selection under controlled laboratory conditions to obtain microorganisms with improved or desired phenotypes.

By applying defined selective pressures over multiple generations, ALE drives the accumulation of beneficial mutations, resulting in strains with enhanced fitness without requiring prior genetic modification.

How do we apply ALE?

ALE is applied by exposing a parental strain to alternating nitrogen- and carbon-limiting conditions that promote lipid accumulation and the proliferation of high lipid content cells. Through serial batch cultivation, adapted variants with improved oleaginous phenotypes outcompete the parental strain. Evolved populations and isolated clones are screened and characterized, enabling the selection of new strains (clones) with up to a twofold increase in lipid production.

Yarrowia lipolytica

Oleaginous model yeast, capable of accumulating lipids up to 40-50 % of CDW;

Capable of metabolizing diverse carbohydrates and agro-industrial residues;

Tolerates moderate osmotic stress and varying pH, making it robust for industrial processes;

Genetically tractable: well-established tools for metabolic engineering.

Rhodosporidium toruloides

Oleaginous red yeast, accumulating lipids up to 70 % of CDW;

Produces a variety of compounds (e.g., carotenoids);

Capable of metabolizing diverse carbohydrates and agro-industrial residues;

Highly adaptable to varying growth conditions and tolerant to inhibitory or toxic compounds, (e.g. furfural).

Cunninghamella echinulata

Oleaginous filamentous fungus, accumulating lipids up to 30-40 % of CDW;

Produces SCO rich in polyunsaturated fatty acids (e.g. gamma-linolenic acid – GLA);

Capable of metabolizing diverse carbohydrates and agro-industrial residues;

Forms mycelial pellets, facilitating downstream processing in bioreactors.

Organization

Work packages

This project is divided into **5 major work packages**, organized in accordance with the logical progression of the project, starting with the generation of improved oleaginous phenotypic clones through ALE on glucose, followed by their cultivation in bioreactor systems with agro-industrial residues serving as the sole carbon source, for comprehensive multi-omic characterizations and complete assessment of their biotechnological potential at an industrial scale, concluding with the dissemination of these results.

WORK PACKAGES

WP1 — ALE for *Yarrowia lipolytica*

- ALE experiment
- Clone characterization
- Genomic analysis
- Data correlations for key gene identification

WP2 — ALE for *Rhodosporidium toruloides*

- ALE experiment
- Clone characterization
- Genomic analysis
- Data correlations for key gene identification

WP3 — ALE for *Cunninghamella echinulata*

- ALE experiment
- Clone characterization
- Genomic analysis
- Data correlations for key gene identification

WP4 — Agro-industrial residue cultivations

- Batch flask cultivations
- Bioreactor cultivations
- Detailed characterization
- Productivity analysis

WP5 — Management and dissemination

- Project management
- Dissemination & outreach
- Networking and industry contact

Website

A web page was established, containing general information about the project, status updates, events, photographies and more (https://evosco.unibuc.ro/).

Meetings

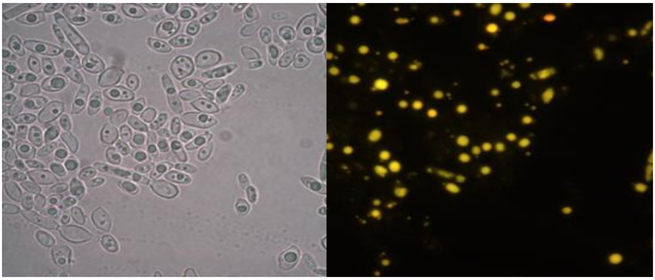
The EvoSCO team holds regular meetings with the project director, both in-person and online, for efficient organization, as well as for reviewing and discussing the results obtained along the way.

Meeting in Bucharest, February 2025

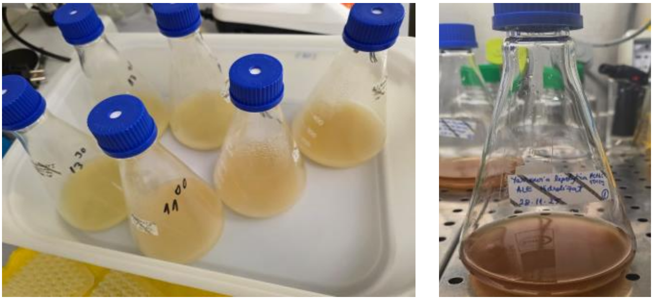
Meeting in Bucharest, April 2024

Results

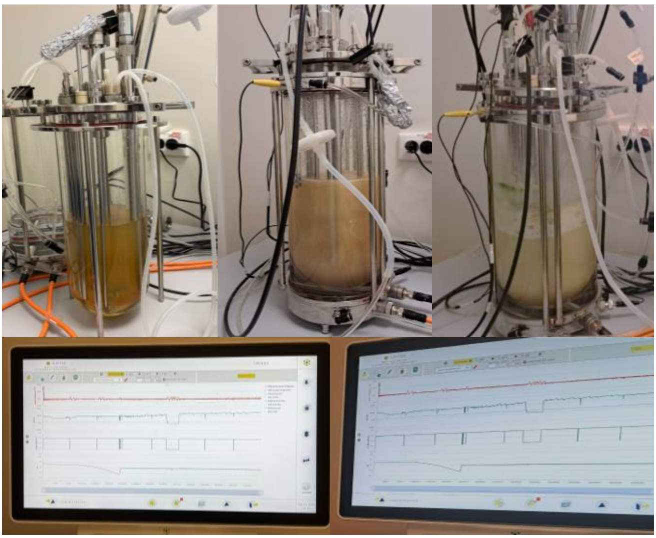
Yarrowia lipolytica



Fluorescence microscopy images showing lipid accumulation (lipid droplets) visualized by Nile Red staining



500 mL flask cultivations on glucose (left) and agro-industrial residue (i.e., cotton lignocellulosic hydrolysate) (right)



4L bioreactor batch cultivations of *Yarrowia lipolytica*

Larger lipid droplets

During ALE, Nile Red staining was performed on fresh, viable cells after each round of evolution. With successive rounds, an increase in the size of lipid droplets within the yeast cells was observed, which was later confirmed quantitatively.

Reduced cultivation time on agro-industrial residues

After several ALE cultivations with glucose, two high lipid-yielding clones were selected for further experiments using agro-industrial residues. On glucose, the clones produced twice the lipid yield of the parental strain (~37 % vs ~15 %). When grown on agro-industrial residues, the clones required shorter cultivation times, and lipid production remained doubled compared to the parental strain (~31 % vs ~15 %), making the process more cost-effective.

Bioreactor batch cultures

Yarrowia lipolytica was cultivated in a 4L bioreactor for 5-7 days. Initial cultivations were performed on glucose, after which a high lipid-yielding clone was selected for subsequent cultivation on agro-industrial residues. While lipid production remained comparable between the two substrates, differences in biomass accumulation were observed.

Results

Larger lipid droplets

During ALE, Nile Red staining was performed on fresh, viable cells after each round of evolution. With successive rounds, an increase in the size of lipid droplets within the yeast cells was observed, which was later confirmed quantitatively.

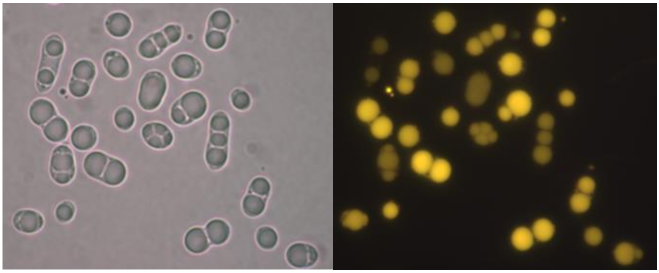
Significantly increased lipid yield in *R. toruloides* clones

After several ALE cultivations on glucose, high lipid-yielding clones were obtained. These evolved clones exhibited an approximately 1.6-fold increase in lipid yield compared to the parental strain (~55 % vs ~35 %), indicating a clear improvement in lipid accumulation as a result of the applied evolutionary pressure.

Maintained lipid productivity on agro-industrial residue

When tested on agro-industrial residues, specifically a cotton-derived lignocellulosic hydrolysate from textile waste, the selected clones retained their enhanced lipid-producing capacity. Despite the presence of inhibitory compounds (e.g., furfural), lipid yields remained nearly 1.6-fold higher than those of the parental strain (~40 % vs ~25 %), with unchanged cultivation times, resulting in increased productivity within the same timeframe and improved cost-efficiency.

Rhodosporidium toruloides



Fluorescence microscopy images showing lipid accumulation (lipid droplets) visualized by Nile Red staining

500 mL flask cultivation of a selected clone (left) and subsequent lipid extraction and processing steps, including Folch extraction with reflux (middle) and lipid sample concentration by rotary evaporation (right)

Pieces of cotton fabric treated with acid during hydrolysis (left), the resulting lignocellulosic hydrolysate (middle) and 500mL flasks cultivations of parental and evolved strains incubated under usual conditions (left)

Results

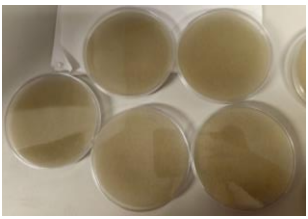
Cunninghamella echinulata

Lipid extract obtained from the culture (left) and the lipid extraction process using Folch method with reflux (right)

250mL baffled flask cultivations on liquid media using different carbon sources

C. echinulata cultivated on different solid media

Microscopy images showing spores and mycelium



ALE protocol on solid high C/N media

Fluorescence microscopy images showing lipid accumulation (lipid droplets) in spores, visualized using Nile Red staining

High lipid yield across multiple carbon sources

C. echinulata exhibited differential responses when cultivated on sucrose, xylose, molasses, and glucose. Sucrose yielded the highest lipid accumulation, reaching 40.72%, while xylose and glucose supported production at 30.11% and 33.26%. Molasses, however, resulted in the lowest lipid content (8.16%).

Modified ALE protocol for *C. echinulata* evolution

To adapt the ALE strategy for *C. echinulata*'s filamentous growth and pellet formation in liquid media, a solid culture medium was employed to promote lipid accumulation, targeting the selection of robust spores. This modified approach relied on the assumption that fungal mycelia storing high amounts of oil will produce oil-rich spores, which, when transferred to carbon-free media, allow only the most resilient strains to thrive and progressively increase lipid production through successive rounds of selection.

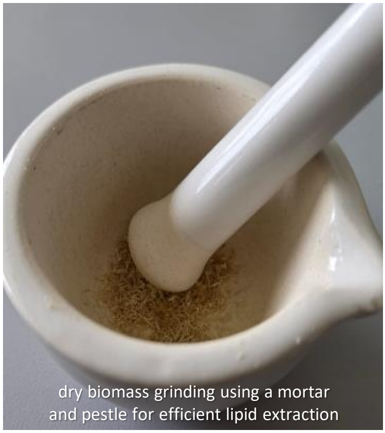
Localization of lipid droplets in spores

Nile Red staining of spores retrieved from ALE culture media revealed that lipid droplets are indeed present within the spores, highlighting the key role of spores in growth sustainability under carbon-limited conditions.

Photo collage

Yarrowia lipolytica culture on lignocellulosic hydrolysate

Y. lipolytica biomass scraping after drying at 80 °C



dry biomass grinding using a mortar and pestle for efficient lipid extraction

Folch extraction under reflux

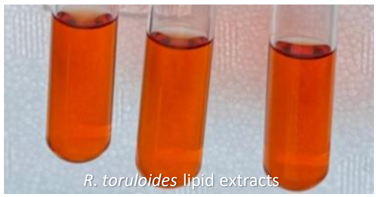
organic-aqueous phase separation

solvent removal by rotary evaporation

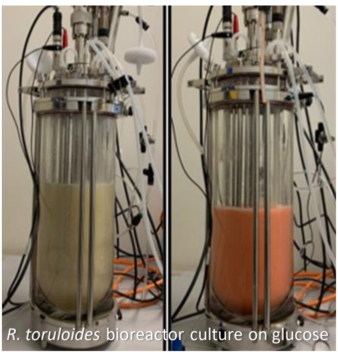
Y. lipolytica lipid extract

R. toruloides biomass before lipid extraction

R. toruloides biomass after lipid extraction



R. toruloides lipid extracts



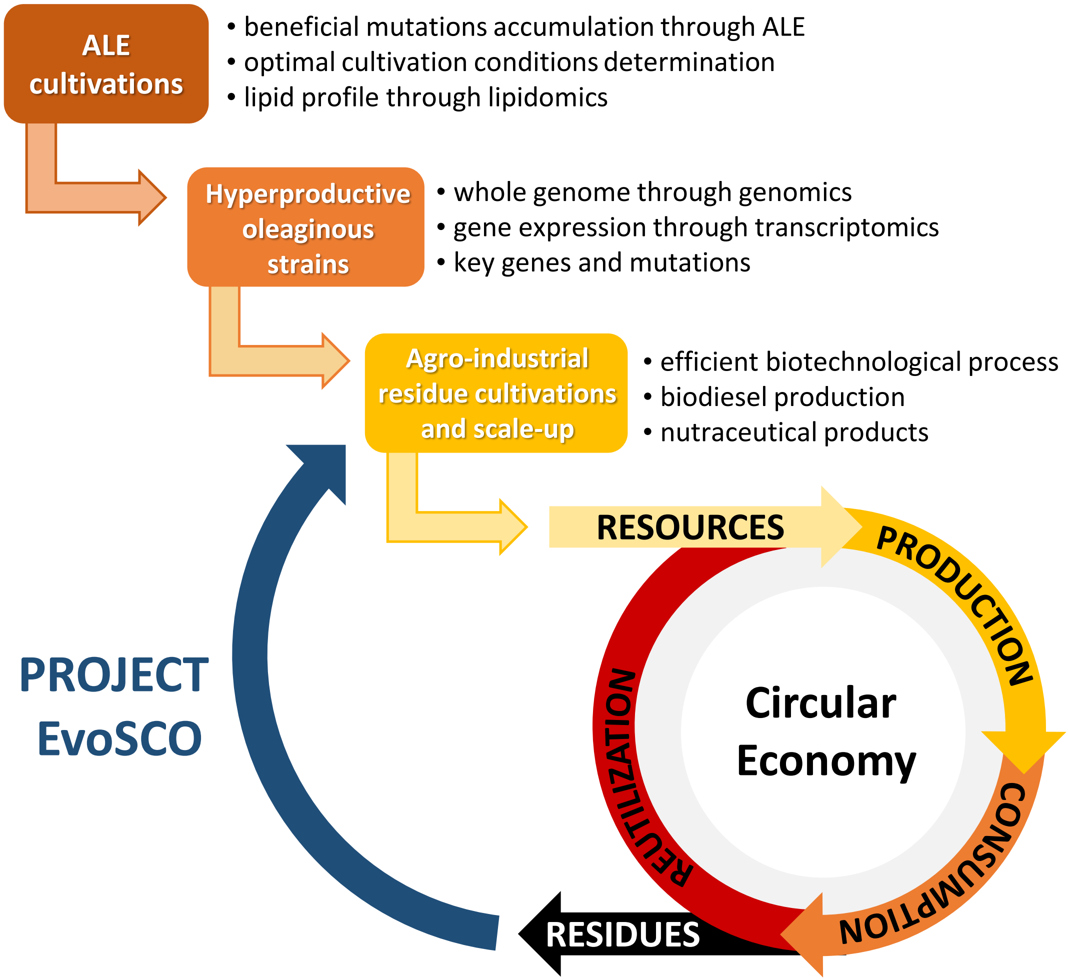
R. toruloides bioreactor culture on glucose

R. toruloides bioreactor culture on molasses

Cunninghamella echinulata mycelial pellets grown on liquid YPG media

Points to adress

Benefits for industry, society and economy



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