Thesis of the PhD Dissertation

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Investigation of biotechnologically significant metabolites produced by *Yarrowia* yeasts

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1. INTRODUCTION

Yarrowia lipolytica is one of the best-known and most extensively studied species within the *Yarrowia* genus, primarily due to its ability to produce and secrete large quantities of important metabolic products, which justifies its industrial application and intensive research interest. This non-pathogenic yeast is listed as GRAS (Generally Recognized As Safe), making it safe for use in food applications. *Y. lipolityca* exhibits strong lipolytic and proteolytic activities, as well as the ability to produce pigments. In addition to the food industry, *Y. lipolytica* is also utilized in the detergent industry, healthcare, and the pharmaceutical sector. It shows promising potential for biofuel production and plays an important role in environmental protection.

Lipase is an enzyme that belongs to the group of serine hydrolases and catalyzes the hydrolysis of triglycerides into glycerol and free fatty acids at the oil-water interface. Lipases are ubiquitous in nature; many plants, animals, and microorganisms are capable of producing them. Lipase is known not only for its biotechnological applications but also for its biological significance. In plants, lipase enzymes play an important role particularly in seed germination and in the ripening process of the fruit itself. In humans and animals, lipases are produced in the digestive system, mainly in the saliva, stomach, and pancreas, where they play a key role in digestion. In industry, microbial lipases are most commonly used because the microorganisms that produce them can be easily cultivated and utilized. While lipases produced by bacteria and filamentous fungi were traditionally used, scientific research has also focused on yeasts. In fungi, lipases are often found extracellulary, although they can also occur in a cell wall-bound or intracellularly. The role of the extracellular enzyme is to break down lipid substrates into smaller molecules that the cells can absorb from the environment and utilize. On the other hand, intracellular lipases hydrolyze lipids stored inside the cells as reserve nutrients, in contrast to extracellular lipases. Cell wall-bound lipases are essential in cell structure formation and in the synthesis of specific lipids, including membrane-forming lipid compounds.

Hydrophobic substrates are among the most effective carbon sources that stimulate the production of lipolytic enzymes by microorganisms. Among them, vegetable oils — such as olive oil, sunflower oil, corn oil, palm oil, rapeseed oil, and soybean oil — and pure fatty acids like palmitic acid and oleic acid receive particular attention. olive oil is considered an effective inducer of lipase production among natural vegetable oils due to its high oleic acid and linoleic acid content. In addition to vegetable oils, by-products from the oil industry can also be used as natural substrates to promote the growth of *Yarrowia lipolytica* and for lipase production. The quality of the substrate and inducing agent, as well as the presence of surfactants, play an important role in enzyme synthesis. This phenomenon can vary not only between genera, but also between species and even strains.

In addition to lipase production, the *Yarrowia* genus can also be utilized for the production of pigments, which are used as colorants in a wide range of industries. Biological pigments are found in all living matter; they provide attractive colors and play a fundamental role in the development of organisms. The use of natural pigments is becoming more widespread, as synthetic dyes have been associated with adverse effects including hyperactivity

in children, allergic reactions, and even carcinogenicity. As a result, many artificial colorants have already been banned. Beyond these health concerns, the precursors used in the production of synthetic pigments may be harmful to industrial workers, and the environment though waste generated during the process. Considering these factors, manufacturers are strongly encouraged to produce natural pigments. Using microorganisms offers several advantages: they can be easily cultivated, the pigments are simple to extract, seasonal availability is not an issue, and they are capable of producing a wide range of pigment colours. Moreover, they are non-allergenic, non-toxic, non-carcinogenic, and biodegradable. In addition, they can have positive health effects, as some possess antioxidant properties or function as provitamins.

The presence of amino acids and certain trace elements has a beneficial effect on the production of melanin-like brown pigment. Optimising the quality and quantity of these substances in the fermentation medium has a major impact on the efficiency of the process

Based on all the information, the main goal of this study was to explore the biotechnological applicability of *Yarrowia* species - especially with regard to their lipase and pigment production by fermentation - that have been less studied. I am convinced that, in addition to *Y. lipolytica*, other species can also be useful for various industrial uses.

2. OBJECTIVES

In industry, lipase enzyme production primarily relies on bacteria and filamentous fungi, with yeasts being utilized to a lesser extent. However, recent research increasingly suggests that yeast certain strains, including members of the *Yarrowia* genus, are also effective producers of both lipases and pigments. Under optimized environmental conditions and with appropriate substrate, they may even outperform traditional organisms. While *Yarrowia lipolytica* is the most extensively studied species in the genus, other *Yarrowia* species also hold industrial potential. Therefore, the aim of my research was to optimize the fermentation processes for both lipase enzyme and brown pigment production in less-characterized *Yarrowia* isolates. In addition to optimizing fermentation conditions, the investigation of using industrial by-products as natural substrates for lipase production was conducted in line with the growing emphasis on the circular economy. Regarding the brown pigment, the goal was to evaluate its stability, which is a key consideration for potential future industrial applications. The experimental work included the following tasks:

- Investigation of growth and lipase activity in selected *Yarrowia* strains
- Analysis of lipase production in extracellular and intracellular fractions
- Experiments to enhance lipase production include the following:
    - Application of different vegetable oils (e.g., olive oil) and agro-industrial pellets as natural substrates
    - Evaluation of the effect of surfactants (Tween 80 and Triton X-100)
    - Optimization of fermentation parameters (temperature, pH, inoculum volume, and aeration rate)
- Screening of *Yarrowia* strains for pigment production
- Experiments to enhance pigment production include the following
    - Optimization of environmental parameters affect fermentation (temperature, pH, inoculum size and age, aeration rate)
    - Investigation of key components and their concentrations affecting pigment yield in the most promising pigment-producing strain
- Pigment stability analysis under various temperature and light conditions, including boiling, room temperature, refrigerated storage, and exposure to light vs. darkness.

3. MATERIAL AND METHODS

3.1. *Yarrowia* strains used in the experiments

During my research work, 12 strains and isolates belonging to the *Yarrowia* genus were used: *Y. bubula* 441/4, *Y. divulgata* NCAIM Y.02062, *Y. divulgata* 5257, *Y. divulgata* 445/4, *Y. lipolytica* 854/4, *Y. lipolytica* 1/4, *Y. lipolytica* 6/3, *Y. porcina* NCAIM Y.02102, *Y.porcina* 859/4, *Y. yakushimensis* NCAIM Y.02049, *Y. yakushimensis* NCAIM Y.02050, *Y. yakushimensis* NCAIM Y.02052.

Strains were kindly provided by the National Collection of Agricultural and Industrial Microorganisms (NCAIM, Budapest, Hungary).

3.2. Applied media

In all cases, the culture media were sterilized in autoclave at 121 °C for 15 minutes.

Agar slants

The strains were grown on YEPD agar slants, containing 5 g/L yeast extract, 20 g/L glucose, 10 g/L peptone, and 25 g/L agar.

Medium Used for Pigment Screening

For pigment production screening, a tyrosine-containing agar medium was used and poured into Petri dishes. The composition of the medium was 5 g/L yeast extract 5 g/L peptone 4.504 g/L lactic acid 1.819 g/L tyrosine 0.106 g/L $MnSO_4 \cdot H_2O$, 25 g/L agar.

Inoculum medium

Inoculum medium contained 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract.

Media used for lipase fermentation

Basal fermentation medium

For the lipase fermentation YEPD medium was used with the following composition: 20 g/L glucose, 6.4 g/L peptone, 10 g/L yeast extract

YEPD broth containing natural substrate and Tween 80

During the optimization experiments, YEPD media supplemented with 1% of various natural substrates (vegetable oils and oilseed cakes) and 0.5 g/L Tween 80 were used. The vegetable oils applied in the study — including olive oil (used as a control), walnut oil, grape seed oil, sunflower oil, rapeseed oil, sesame oil, corn oil, coconut oil, and waste frying oil — were all commercially available. The oilseed cakes (from pumpkin seed, golden flaxseed, sunflower seed, peanut, and hemp seed) were kindly provided by the Department of Cereal and Industrial Crop Processing Technology.

Media used for pigment fermentation

Basal fermentation medium

For the pigment fermentation experiments, a basal medium was used, consisting of 4 g/L KH_2PO_4, 2.5 g/L $MgSO_4 \cdot 7H_2O$ 0.106 g/L, $MnSO_4 \cdot 5H_2O$.

Supplemented Fermentation Media

To enhance pigment production, three variants of the basal medium were prepared: In the first case, the basal medium was supplemented with 0.27 g/L tyrosine. In the second case, in addition to tyrosine, the medium was further supplemented with 1 g/L each of glycine, L-glutamine, and L-asparagine. In the third variant, the medium included tyrosine, glycine, L-glutamine, and L-asparagine, as well as 5.46 g/L lactic acid to promote further pigment synthesis.

3.3. Methods

Determination of pH

The pH values of the samples were determined using a Mettler Toledo SevenMulti™ pH meter. The calibration of the pH meter was performed with pH 4 and pH 9 calibration solutions.

Cell count determination

Yeast cell counts were determined using the Bürker- chamber method with an Olympos microscope. A tenfold diluted sample was prepared, and 20 µL of the diluted suspension was pipetted into the Bürker- chamber. Using the microscope, cells were counted in 10 squares of equal size. The results were then averaged, and the cell concentration was calculated and expressed in cells/mL, taking the dilution factor into account.

Lipase fermentation

Yeast cells from agar slants were inoculated in 250 mL flasks containing 150 mL basal medium and incubated for 24 hours at 130 rpm and 28°C. During the screening experiments and when determining the optimal environmental parameters enzyme fermentations were initiated with 5 v/v% 24 h inoculum cultures. In the optimization of the inoculum size, cell concentrations of 10^6 cells/mL, $5*10^6$ cells/mL, and 10^7 cells/mL were used. The fermentation was carried out in a shaker at 130 rpm in a volume of 125–200 mL at 28 °C for 5 to 13 days. Meanwhile, the extracellular enzyme activities were monitored daily during the fermentation process, whereas the intracellular activity was determined after cell disruption from samples taken at 72 h.

Lipase enzyme activity assay

The lipase activity was assayed by determining the amount of p-nitrophenol liberated from the artificial substrate p-nitrophenyl laurate by the enzyme solution. Briefly, 100 µL of 25 mM p-nitrophenyl laurate dissolved in 96% ethanol and 0.725 µL Sorensen's phosphate buffer

(pH 7.2) were preheated at 37 °C. Then 25 µL of the appropriately diluted sample was pipetted into the test tubes to initiate the enzyme reaction. After 10 min of incubation at 37 °C, the reaction was stopped by adding 250 µL of 0.1 N sodium carbonate solution. The absorbance of the samples was read at 405 nm. The activity assays were performed in duplicate.

Cell disruption

For cell disruption, 25 mL of ferment broth was centrifuged at 10,000 rpm at 4 °C for 10 min. The supernatant was collected separately and used as extracellular lipase fraction. The wet cells were collected and washed twice with 5 mL McIlvaine buffer (pH 6.5). Finally, the cells were resuspended in 2 mL McIlvaine buffer (pH 6.5) and disrupted by French Press high-pressure homogeniser at 800 psi. Generally, at least two three cycles were performed to ensure the disruption process, after which the intracellular enzyme activity was determined, using the same procedure as the extracellular lipase activity assay.

Central composite design (CCD)

Central composite design — one commonly used technique in the response surface methodology (RSM) — was used to optimise the concentration of medium compositions for enhancement of lipase production. In the first case, olive oil (x_1) and Tween 80 (x_2), while in the second case, Tween 80 (x_1) and Triton X-100 (x_2) were selected as independent variables based on preliminary experiments, whereas the lipase enzyme activities (Y) were used as dependent variables. For both factors, the center point was 0.05%, and the step size was 0.025%. The second-order polynomial model was used to obtain the response surfaces and thus estimate the optimum points that provide maximal lipase production.

$$Y = b_0 + b_1 * x_1 + b_2 * x_1^2 + b_3 * x_2 + b_4 * x_2^2 + b_5 * x_1 x_2$$

Where Y is the dependent variable, X_1, X_2 are independent variables for olive oil and Tween 80, b_1–b_5 are the coefficients for the independent variables, b_1, b_2 are linear effects, b_3, b_4 are squared effects and b_5 are interaction term, b_0 are the intercept.

Pigment fermentation

As the first step of the pigment fermentation, yeast cells were transferred from slant agar into the inoculum medium under sterile conditions. The yeast cultures were then incubated for 24 hours at 28°C and 130 rpm. Following this, 5% (v/v) of the inoculum culture was transferred into the fermentation medium (with the exception of the inoculum optimization experiments, where cell concentrations of 10^6, 5×10^6, and 10^7 cells/mL were used). The fermentation was carried out at 28°C and 130 rpm, except during experiments investigating the effect of aeration, where 100 rpm and 160 rpm were also tested. Fermentation lasted for at least 6 days, and

pigment production was monitored by taking periodic samples throughout the cultivation process.

Determination of pigment production

Pigment production was quantified using a Unicam Helios Alpha spectrophotometer. Samples were centrifuged at 10,000 rpm for 10 minutes in Eppendorf tubes to remove cells and debris. The absorbance of the supernatant was read at a wavelength of 400 nm.

4. RESULTS AND DISCUSSION

4.1. Investigation of lipase production by *Yarrowia* strains

Over the past few decades, the industrial application of lipase enzymes has significantly increased. Lipases are now widely used in the food, pharmaceutical, paper, cosmetic, and energy industries. Among the various sources, microbial lipases are the most commonly employed in industrial processes. While lipases produced by bacteria and filamentous fungi have long been used, recent scientific advancements have brought growing attention to lipases derived from yeast. For these reasons, the first part of my PhD research focused on evaluating the lipase activity of various *Yarrowia* yeast strains.

4.1.1. Selection of lipase-producing strains

As the first step of my research, screening of *Yarrowia* strains was performed for lipase production in YEPD broth. No lipase activity was observed during the 5-day fermentation in the case of *Y. porcina* NCAIM Y.02102, *Y. bubula* 441/4, and *Y. lipolytica* 6/3 strains. The *Y. divulgata* 5257 reached its maximum activity on the first day of fermentation. However, the highest enzyme activities were recorded on the 2nd day for the strains *Y. lipolytica* 1/4, *Y. lipolytica* 854/4, and *Y. divulgata* NCAIM Y.02062. In all *Y. lipolytica* strains tested, lipase activity showed a sharp decline between 48 and 72 hours. The highest lipase activity - 40.56 mU at 48 hours - was obtained from *Y. divulgata* NCAIM Y.02062, which unlike the other strains, still exhibited detectable lipase activity at 72 hours. Based on their superior enzyme production, the strains *Y. divulgata* NCAIM Y.02062, *Y. divulgata* 5257, *Y. lipolytica* 1/4, and *Y. lipolytica* 854/4 were selected for further investigation.

4.1.2. The effect of olive oil and Tween 80 on lipase production by *Y. lipolytica* and *Y. divulgata* strains

Following the selection of the most promising strains, optimization experiments were conducted to enhance lipase enzyme activity. The basal medium was supplemented with 1% olive oil as an inducer, and the fermentation process was carried out for 148 hours, with daily monitoring of extracellular lipase activity. The effect of Tween 80 on lipase production by *Yarrowia* yeasts was also investigated in a medium containing 1% olive oil. Tween 80 was applied at a concentration of 0.05%, and both extracellular and intracellular lipase activities were measured daily for 6 days. In general, the presence of Tween 80 resulted in increased lipase activity in both extracellular and intracellular fractions across all tested *Yarrowia* strains. For the *Y. lipolytica* 854/4 strain, the maximum extracellular lipase activity in the olive oil + Tween 80 medium was observed at 72 hours, reaching 474 U/L, while in the absence of Tween 80, a peak of only 25 U/L was observed at 48 hours. In contrast, the maximum intracellular activity without Tween 80 was 467 U/L, while with Tween 80 it was only 122 U/L. For the *Y. lipolytica* 1/4 strain, the extracellular lipase activity peaked at 131 U/L at 48 hours in the olive oil-only medium, whereas in the presence of Tween 80, the maximum activity reached 221 U/L at 72 hours. This value was significantly lower than the one observed for *Y. lipolytica* 854/4. As

for intracellular activity, Tween 80 had a positive effect, increasing the value from 126 U/L to 273 U/L. In the case of *Y. divulgata* NCAIM Y.02062, maximum extracellular activity was observed at 72 hours in both media, but the activity in the Tween 80-containing medium (147 U/L) was approximately double that of the medium without Tween 80 (81 U/L). The intracellular lipase activity was 117 U/L in the olive oil-only medium, and 80 U/L in the medium containing both olive oil and Tween 80. For *Y. divulgata* 5257, maximum extracellular lipase activity was also detected after 72 hours of fermentation, but the activity levels were very low in both media: 20 U/L with Tween 80 and 13 U/L without. As for intracellular activity, the highest values were 22 U/L and 8 U/L, in the presence and absence of Tween 80, respectively.

4.1.3. Investigation of Lipase production by *Yarrowia yakushimensis* strains

The extracellular lipase producing capacity of lesser known strains — *Y. yakushimensis* NCAIM Y.02049, NCAIM Y.02050, and NCAIM Y.02052 — was examined in YEPD medium supplemented with 1% olive oil over a two-week fermentation period. Based on the measured lipase activity, it was evident that the *Y. yakushimensis* NCAIM Y.02050 strain produced very low levels of lipase, with enzyme activity not even reaching 1 U/L throughout the fermentation. From day 3, clear differences began to emerge between this strain and the other two strains (Y.02049 and Y.02052), whose enzyme activity started to rise significantly after day 8. The highest lipase activity was observed on day 13, with *Y. yakushimensis* NCAIM Y.02052 reaching 98 U/L, and Y.02049 reaching 85 U/L. When compared to other high-performing strains — *Y. lipolytica* 1/4, *Y. lipolytica* 854/4, *Y. divulgata* 5257, and *Y. divulgata* NCAIM Y.02062 — these values fall within a similar range under identical fermentation conditions with 1% olive oil. Following confirmation of the inducing effect of olive oil in *Y. yakushimensis* strains, the impact of Tween 80 was also investigated in a 7-day fermentation. The YEPD medium was supplemented with 1% olive oil and 0.05% Tween 80, and both extracellular and intracellular lipase activities were monitored for strains NCAIM Y.02049 and NCAIM Y.02052. The results demonstrate that, similar to *Y. lipolytica* 1/4, *Y. lipolytica* 854/4, *Y. divulgata* 5257, and *Y. divulgata* NCAIM Y.02062, the addition of Tween 80 in combination with olive oil enhanced extracellular lipase activity in *Y. yakushimensis* strains as well. Similar to the other *Yarrowia* strains, intracellular lipase activity of these strains was lower than the extracellular activity, highlighting a similar secretion pattern.

4.1.4. Effect of olive oil and Tween 80 concentration on extracellular lipase production

The optimization of olive oil and Tween 80 concentrations for maximizing extracellular lipase production by the selected *Yarrowia* strains was carried out using response surface methodology, specifically employing a central composite statistical design. Mathematical model equations were established, allowing the determination of the optimal concentrations of olive oil and Tween 80 required for maximum enzyme production in each strain. Among the tested strains, the highest enzyme activity values were recorded for *Y. lipolytica* 854/4. Additionally, significant differences were observed in the enzyme activity values obtained under the various tested conditions for this strain. In contrast, for the other three strains (*Y. lipolytica* 1/4, *Y. divulgata* 5257, and *Y. divulgata* NCAIM Y.02062), no substantial differences

were detected — neither between activity values across different settings nor in their respective maximum values. Overall, it can be concluded that within the tested range, increasing the concentration of both olive oil and Tween 80 led to enhanced enzyme activity. Based on the results, the optimal concentrations of the independent variables (olive oil and Tween 80) within the tested range were determined as follows:

- *Y. lipolytica* 854/4 strain: 1.6% olive oil, 0.065% Tween 80
- *Y. lipolytica* 1/4 strain: 1.4% olive oil, 0.09% Tween 80
- *Y. divulgata* NCAIM Y.02062 strain: 1.6% olive oil, 0.09% Tween 80
- *Y. divulgata* 5257 strain: 1.6% olive oil, 0.06% Tween 80.

4.1.5. Effect of aeration on lipase production

Due to the limited literature is available on the effect of aeration on lipase secretion in *Yarrowia* yeasts, this was also investigated. It was found that the most effective agitation speed for *Y. lipolytica* 1/4, *Y. lipolytica* 854/4, *Y. divulgata* 5257, and *Y. divulgata* NCAIM Y.02062 was 130 rpm. Under this condition, lipase production was significantly more efficient, as these four yeasts produced 2-3 times more enzyme than at 100 or 160 rpm. Therefore, this aeration rate was applied in the further experiments.

4.1.6. Optimization the amount of inoculum

Optimizing the amount of inoculum is also a critical factor in fermentation processes. The effect of initial cell concentration on lipase production in *Y. lipolytica* 854/4, *Y. divulgata* 5257, *Y. divulgata* NCAIM Y.02062, and three *Y. yakushimensis* strains at cell concentrations of 10^6, $5*10^6$, and 10^7 CFU/mL were examined. The lowest concentration (10^6 CFU/ml) was not the most effective for any strain. However, no significant differences in enzyme production were observed between $5*10^6$ and 10^7 CFU/mL, so a 24-hour inoculum at the optimal concentration for each strain was applied in the following fermentations.

4.1.7. Enhancing extracellular lipase production using Tween 80 and Triton X-100

To enhance enzyme production, the effect of Triton X-100 and Tween 80 on lipase activity was investigated using a central composite design (CCD) with five *Yarrowia* strains (*Y. lipolytica* 854/4, *Y. yakushimensis* NCAIM Y.02049, *Y. yakushimensis* NCAIM Y.02052, *Y. divulgata* NCAIM Y.02062, *Y. divulgata* 5257) in YEPD broth with olive oil. Statistical analysis confirmed that both Tween 80 and Triton X-100 had significant effects on enzyme production. While higher concentrations (0.07-0.09%) of Tween 80 were more effective, Triton X-100 was beneficial at lower concentrations (0.01-0.02%). Based on the results, the optimal concentration of the independent variables (Tween 80 and Triton X-100) can be determined as follows:

- *Y. yakushimensis* NCAIM Y.02049, NCAIM Y.02052, and *Y. divulgata* NCAIM Y.02062*:* 0.09% Tween 80, 0.01% Triton X-100
- *Y. lipolytica* 854/4: 0.09% Tween 80, 0.03% Triton X-100
- *Y. divulgata* 5257: 0.05% Tween 80, 0.05% Triton X-100

Following the optimization, all five *Yarrowia* strains exhibited enzyme activity levels that were orders of magnitude higher than the initial activity. The most substantial increase, a 101-fold rise in lipase activity was detected in the *Y. lipolytica* 854/4 strain compared to its initial value. In the *Y. divulgata* 5257, a 58-fold increase was observed, followed by a 55-fold increase in the *Y. divulgata* NCAIM Y.02062, a 28-fold increase in the *Y. yakushimensis* NCAIM Y.02049, and a 25-fold increase in the *Y. yakushimensis* NCAIM Y.02052. In the case of the two *Y. divulgata* strains, the highest lipase activity was measured when all three components — olive oil, Tween 80, and Triton X-100 — were present. For the remaining three strains, maximum enzyme activity was achieved in media supplemented only with olive oil and Tween 80.

4.1.8. Partial characterization of crude lipases derived from *Yarrowia* yeasts

The extracellular and intracellular crude lipases produced by *Y. lipolytica* 1/4, *Y. lipolytica* 854/4, *Y. divulgata* 5257, *Y. divulgata* NCAIM Y.02062, *Y. yakushimensis* NCAIM Y.02049, and *Y. yakushimensis* NCAIM Y.02052 were partially characterized in terms of their temperature and pH optima. The temperature optimization was conducted in the range of 30°C to 45°C, and the highest enzyme activity was observed at 37°C for both extracellular and intracellular fractions. For pH optimization, Sörensen phosphate buffers of varying pH values (from pH 5 to pH 8) were used at the optimal temperature of 37°C. The results indicated that pH 7.2 was the most favourable condition for lipase activity for both extracellular and intracellular crude enzyme fractions.

4.1.9. Applicability of vegetable oils and oilseed cakes for lipase enzyme production

Vegetable oils

Various vegetable oils (olive oil as a control, coconut oil, walnut oil, grapeseed oil, sesame oil, sunflower oil, corn oil, wate frying oil, and rapeseed oil) were compared as natural substrates for lipase enzyme production of *Y. lipolytica* 854/4 and *Y. yakushimensis* NCAIM Y.02052 strain at a 1% concentration. The results showed that olive oil and sunflower oil were the best inducers for both strains. Specifically, *Y. yakushimensis* NCAIM Y.02052 exhibited the highest lipase activity when olive oil was used as substrate, while *Y. lipolytica* 854/4 performed best with sunflower oil. In addition, waste frying oil, as a potentially recyclable waste for enzyme fermentation, demonstrated promising efficiency compared to several other tested oils.

Oilseed Cakes

Due to the increasing importance of circular economy principles, an experiment was designed to investigate the feasibility of using industrial by-products (oilseed cakes) for lipase enzyme fermentation. Five different oilseed pellets — pumpkin seed, golden flaxseed, sunflower seed, peanut, and hemp seed were tested. Among them, golden flaxseed pellet proved to be the best inducer for both *Y. lipolytica* 854/4 and *Y. yakushimensis* NCAIM Y.02052.

However, while *Y. lipolytica* 854/4 reached maximum lipase activity at 24 hours, *Y. yakushimensis* NCAIM Y.02052 did not achieved this until 72 hours.

4.1.10. Optimization of natural substrate concentrations for extracellular lipase production

The effects of different concentrations (0.5%, 1%, and 2%) of the most promising plant oils (olive and sunflower), pellet (golden flaxseed), and waste frying oil on the lipase production for both strains were investigated. The results generally showed that the higher the substrate concentrations, the higher the enzymatic activity. Olive oil and sunflower oil were the most effective inducers for both yeast strains, at both 1% and 2%, and 2% concentrations, respectively. The highest lipase activity was observed with a 2% olive oil substrate. Although the waste frying oil was not the most efficient inducer, increasing its concentration in the fermentation medium significantly enhanced the lipase activity. In contrast, the golden flaxseed pellet was the least effective substrate, yielding the lowest lipase activity at all tested concentrations.

4.2. Investigation of pigment production by *Yarrowia* strains

Another major focus of my research was to study brown pigment production in *Yarrowia* yeast strains. Pigments are widely used as colouring agents in several industries, including the food, pharmaceutical, dye, cosmetic, and textile industries. It is well known that synthetic dyes may have adverse health effects, and their production can be harmful to the environment. As a result, the market for synthetic pigments is declining, while the demand for naturally derived microbial pigments is growing. For these reasons, the brown, melanin-like pigment was chosen for further research.

4.2.1. Screening of *Yarrowia* strains for pigment production

Seven different *Yarrowia* strains (*Y. porcina* 859/4, *Y. divulgata* NCAIM Y.02062, *Y. lipolytica* 1/4, *Y. divulgata* 445/4, *Y. lipolytica* 6/3, *Y. lipolytica* 854/4, and *Y. divulgata* 5257) were screened on agar containing tyrosine. All strains produced pigments, but at different levels. For further optimization experiments *Y. lipolytica* 6/3, *Y. lipolytica* 854/4, *Y. divulgata* NCAIM Y.02062, and *Y. divulgata* 5257 strains were selected and tested in submerged fermentation. The fermentation medium was first optimised by supplementing the basal medium (containing tyrosine) with different amino acids - glycine, L-glutamine, and L-asparagine - and, in some cases, lactic acid. Among the tested strains, *Y. lipolytica* 6/3 exhibited the highest pigment production in the medium supplemented with amino acids. Between the two *Y. divulgata* strains, *Y. divulgata* 5257 proved to be the better pigment producer. Based on these results, for further research, *Y. lipolytica* 6/3and 854/4, were selected and the less well-studied strains of *Y. divulgata* NCAIM Y.02062 and *Y. divulgata* 5257.

4.2.2. Effect of medium composition

Medium optimization was conducted experiments using four selected strains in submerged fermentations. To enhance pigment production, the basic fermentation medium was

supplemented with tyrosine, glycine, L-glutamine, L-asparagine, and lactic acid components, and inoculated it with 5% inoculum culture. One of the media contained only tyrosine as a supplement, another contained all components, while a third one contained all of the listed components except for lactic acid. During sampling, I monitored the pH changes and absorbance values at 400 nm to track pigment production. The incubation period lasted at least one week. Among the strains used, *Y. lipolytica* 6/3 was the most successful in terms of pigment production when grown in a medium supplemented with amino acids. Comparing the two *Y. divulgata* strains, *Y. divulgata* 5257 was the better pigment producer. There is some debated in the literature about the effect of lactic acid as an inducer, but my experiments showed that its inclusion led to increased pigment synthesis in most strains compared to the basal medium. Overall, the most effective medium for all strains was the one supplemented with amino acids only. The results also suggest that a neutral pH range is favourable for pigment synthesis.

4.2.3. Effect of the buffer type of the fermentation medium on pigment production

Given that pH affects pigment production, the required pH was achieved using different buffer systems (McIlvaine and Sørensen) and also adjusted the pH with NaOH in media supplemented with amino acid. The results were then, compared to a control medium prepared by water. When lactic acid was tested in combination with buffers and the pH was adjusted to neutral, pigment synthesis was generally enhanced. The most effective was the medium contained NaOH for *Y. lipolytica* 6/3, *Y. divulgata* NCAIM Y.02062, and *Y. divulgata* 5257, whereas for *Y. lipolytica* 854/4, the McIlvaine buffer without lactic acid supplementation was the best. However, no significant differences were observed between the different initial media. Based on economic aspects, the control medium prepared with water was applied for further studies.

4.2.4. Effect of agitation speeds on lipase production

The effect of different agitation speeds (100 rpm, 130 rpm, and 160 rpm) on pigment synthesis was investigated, since *Y. lipolytica* is an obligate aerobic yeast and aeration can enhance metabolic processes and therefore increase pigment production. The results showed that the optimum speed varied between strains, but overall, aeration had no significant effect on pigment synthesis. Consequently, 130 rpm was used for further experiments.

4.2.5. Effect of inoculum age on pigment production

The effect of inoculum age on pigment production was also investigated. 16-, 24-, and 48-hours inoculum were used for initiation of fermentation. The findings, supported by literature data, showed that while inoculum age influences fermentation efficiency, a 24-hour inoculum was the most practical choice based on economic and technological aspects.

4.2.6. Effect of initial inoculum cell density on pigment production

The effect of initial inoculum cell density on pigment production was studied using concentrations of 10^6, $5*10^6$, and 10^7 cells/mL. The results showed that cell concentration had a significant effect on pigment synthesis. The optimum concentrations were $5*10^6$ CFU/mL for *Y. lipolytica* 6/3, 10^6 CFU/mL for *Y. lipolytica* 854/4 and *Y. divulgata* 5257, and 10^7 CFU/mL for *Y. divulgata* NCAIM Y.02062. These values were used in further fermentations.

4.2.7. Effect of Mn^{2+} concentration on pigment production

Trace elements also affect pigment synthesis, so the effect of Mn^{2+} at different concentrations (0, 0.1, 0.2, and 0.5 g/L) was investigated. The results clearly showed that Mn^{2+} had a positive effect on the pigment synthesis of *Yarrowia*, and the higher the Mn^{2+} concentrations, the higher the pigment yields. A concentration of 0.5 g/L Mn^{2+} resulted in the highest absorbance. During the optimisation process, *Y. lipolytica* 6/3 was the most efficient pigment producer among the tested strains, achieving a sevenfold increase in absorbance compared to the initial value.

After the optimization process, higher absorbance values were obtained for all four *Yarrowia* yeast strains, compared to the initial values, indicating an increase in pigment production. The most significant increase, a 7.1-fold rise, was observed in the *Y. lipolytica* 6/3 strain. A 2.6-fold increase was achieved in the *Y. lipolytica* 854/4 and *Y. divulgata* 5257 strains, while the smallest increase (1.63 fold) in absorbance was recorded for the *Y. divulgata* NCAIM Y.02062.

4.2.8. Investigation of pigment stability

For potential applications in the food industry, the stability of the pigments produced by *Yarrowia* strains was investigated including boiling, freezing, and storage under different light and temperature conditions. Boiling tests showed that the pigment absorbance remained unchanged after 30 minutes indicating high thermal stability. Storage tests under different temperature and light conditions (room temperature vs. refrigeration and freezing at -18°C, and exposure to light vs. darkness) demonstrated that pigment stability remained high over time under all conditions. The results confirmed that *Yarrowia* pigments do not degrade over long storage periods under various environmental conditions.

5. CONCLUSIONS AND RECOMMENDATIONS

5.1. Lipase enzyme production

During the selection of *Yarrowia* strains in YEPD broth, significant differences was observed in lipase enzyme activity among the various strains. In addition to the widely used *Yarrowia lipolytica*, lipase activity was also detected in the tested strains of *Y. divulgata* and *Y. yakushimensis*, while the available *Y. porcina* and *Y. bubula* strains did not produce lipase enzymes during the 5-day fermentation without the presence of an inducing compound. Both extracellular and intracellular lipase activity was detected; however, in all strains, significantly higher activity values were found in the extracellular fraction.

Optimization studies aimed at enhancing enzyme production (effect of olive oil and Tween 80; agitation speed; inoculum size; different concentrations of Triton X-100 and Tween 80) resulted in several-fold increases in activity. Compared to the initial lipase activity, the following increases were measured: 101-fold for *Y. lipolytica* 854/4, 58-fold for *Y. divulgata 5257*, 55-fold for *Y. divulgata* NCAIM Y.02062, 28-fold for *Y. yakushimensis* NCAIM Y.02049, and 25-fold for *Y. yakushimensis* NCAIM Y.02052.

The pH and temperature optima of extracellular and intracellular crude lipase enzymes from six selected *Yarrowia* strains (*Y. lipolytica* 1/4, *Y. lipolytica* 854/4, *Y. divulgata* NCAIM Y.02062, *Y. divulgata* 5257, *Y. yakushimensis* NCAIM Y.02049, and *Y. yakushimensis* NCAIM Y.02052) were determined. For both fractions, the optimal pH was 7.2, while significantly lower enzyme activity was detected at the extreme pH values (pH 5 and pH 8). Regarding temperature, lipase activity showed a consistent increasing trend between 25°C and 37°C, reaching a maximum at 37°C, after which it decreased sharply.

Plant oils, including olive oil (as control), coconut oil, walnut oil, grapeseed oil, sesame oil, sunflower oil, corn oil, waste frying oil, and rapeseed oil were applied at 1% concentration in YEPD medium supplemented with 0.05% Tween 80. They all proved to be suitable substrates for extracellular lipase production by *Y. lipolytica* 854/4 and *Y. yakushimensis* NCAIM Y.02052. Based on the results, olive oil, followed by sunflower oil, proved to be the most effective in both *Yarrowia* strains. Waste frying oil also showed promise. It outperformed several plant oils (grapeseed, corn, rapeseed) in both yeast strains.

In addition to the plant oils, five different oilseed press cakes (pumpkin seed, golden flaxseed, sunflower seed, peanut, and hemp seed) were investigated as natural substrates. The highest lipase activity was achieved using golden flaxseed pellet, followed by pumpkin seed pellet.

When testing natural substrates (olive oil, sunflower oil, waste frying oil, golden flaxseed press cake) at concentrations of 0.5%, 1%, and 2%, the 2% concentration proved to be the most effective for both yeasts. For both *Y. lipolytica 854/4* and *Y. yakushimensis* NCAIM Y.02052, olive oil was determined as the best inducer. Golden flaxseed pellet was the least effective substrate at all concentrations, likely due to its lower fatty acid content.

For future research, it would be worthwhile to explore the use of waste frying oil as a substrate, especially by increasing its concentration. Although the maximum enzyme activity detected in media containing waste frying oil did not reach the levels observed with the best-

performing plant oils (olive and sunflower). Its potential large-scale application could be economically viable due to its low cost of purchase, and it contributes to waste valorization. Similarly, increasing the concentration or applying solid-state fermentation for golden flaxseed pellet could be considered. Its integration into lipase fermentation would support both circular economy aims and environmental sustainability.

5.2. Brown pigment production

The *Yarrowia* strains were screened for pigment production using an agar diffusion method on nutrient agar containing tyrosine. There were noticeable differences among the strains regarding the amount of pigment produced. The most efficient pigment-producing strain was *Y. lipolytica* 6/3. In addition, *Y. lipolytica* 854/4, as well as the lesser-studied *Y. divulgata* NCAIM Y.02062 and *Y. divulgata* 5257 strains were selected for further experiments.

To enhance pigment production, optimization of the fermentation conditions was performed, including medium composition, effect of pH, agitation speed, inoculum size and age, and Mn^{2+} ion concentration. As a result, the following increases were achieved in absorbance at 400 nm compared to the initial values: a 7.1-fold increase for *Y. lipolytica* 6/3, 2.6-fold for *Y. lipolytica* 854/4, 2.62-fold for *Y. divulgata* 5257, and 1.63-fold for *Y. divulgata* NCAIM Y.02062. Among the optimization parameters, the increase in manganese concentration had the most significant effect on pigment production.

After optimizing pigment fermentation, the stability of the produced colorant under various conditions was examined. It was found that the absorbance did not change during 30 minutes of boiling. The pigment's colour remained stable for all strains throughout the 5-month study period, regardless of whether it was stored at room temperature in the dark, in light, at 4 °C, or frozen at –18 °C

Before any potential industrial application, it would be highly advisable to investigate the stability of the produced melanin-like brown pigment under acidic conditions, as many food products have a pH lower than neutral (often for preservation purposes).

It is also recommended to identify the pigment type using high-performance liquid chromatography (HPLC) techniques.

6. NEW SCIENTIFIC RESULTS

1. By examining the effects of different inducers, it was found that olive oil proved to be the most effective inducer for both intracellular and extracellular lipase production in various *Yarrowia* strains. Additionally, the application of surfactants such as Tween 80 and Triton X-100 enhanced extracellular lipase activity. The highest lipase activity in YEPD broth was achieved during the optimization of olive oil and Tween 80 concentrations, under the following conditions: *Y. lipolytica* 854/4: 1.6% olive oil and 0.065% Tween 80, *Y. lipolytica* 1/4: 1.4% olive oil and 0.09% Tween 80, *Y. divulgata* NCAIM Y.02062: 1.6% olive oil and 0.09% Tween 80, *Y. divulgata* 5257: 1.6% olive oil and 0.06% Tween 80.
   It was confirmed that combining Tween 80 and Triton X-100 could reduce the optimized olive oil concentration required for extracellular lipase production. The following combinations significantly increased lipase activity in YEPD medium containing 1% olive oil: *Y. lipolytica* 854/4: 0.09% Tween 80 and 0.03% Triton X-100, *Y. yakushimensis* NCAIM Y.02049, NCAIM Y.02052, and *Y. divulgata* NCAIM Y.02062: 0.09% Tween 80 and 0.01% Triton X-100, *Y. divulgata* 5257: 0.05% Tween 80 and 0.05% Triton X-100.

2. The optimal environmental conditions for crude extracellular and intracellular lipase enzymes produced by the following *Yarrowia* strains were determined: *Y. lipolytica* 1/4, *Y. lipolytica* 854/4, *Y. divulgata* 5257, *Y. divulgata* NCAIM Y.02062, *Y. yakushimensis* NCAIM Y.02049, and *Y. yakushimensis* NCAIM Y.02052. The optimal pH was 7.2 (using Sørensen buffer), and the optimal temperature was 37°C.

3. Waste frying oil as an inexpensive and environmentally polluting waste product can be used for lipase production by *Yarrowia* yeast strains. Similar lipase titer was achieved in a YEPD medium containing 0.5% waste frying oil and 0.05% Tween 80, then in 0.5% olive oil substrate, after 24 hours of fermentation with *Y. lipolytica* 854/4 and *Y. yakushimensis* NCAIM Y.02052 strains.

4. The composition of medium was optimized to enhance the production of brown, melanin-like pigment. The best results with *Y. lipolytica* 6/3 were achieved using a medium containing 0.27 g/L tyrosine, 0.5 g/L Mn^{2+} ions, and 1 g/L of additional amino acids (glycine, L-glutamine, and L-asparagine). The fermentation was carried out with a 5×10^6 CFU/mL inoculum and shaking at 130 rpm for 24 hours.

5. I The stability of the brown, melanin-like pigment produced by *Yarrowia* yeast was examined. It was found that the pigment derived from the *Y. lipolytica* 6/3, *Y. lipolytica* 854/4, *Y. divulgata* NCAIM Y.02062, and *Y. divulgata* 5257 strains was highly stable under various conditions. The colour did not change after boiling for 30 minutes and it remained stable during storage at room temperature (both in light and dark) for 5 months, as well as at 4°C and –18°C.

7. PUBLICATIONS

7.1. Papers published in scientific journals

Sipiczki, G., Micevic, S. S., Kohari-Farkas, C., Nagy, E. S., Nguyen, Q. D., Gere, A., & Bujna, E. (2024). Effects of Olive Oil and Tween 80 on Production of Lipase by *Yarrowia Yeast* Strains. *Processes*, *12*(6), 1206. https://doi.org/10.3390/pr12061206

Végh, R., **Sipiczki, G**., Csóka, M., Mednyánszky, Z., Bujna, E., & Takács, K. (2024). Chemical Characterization, In Vitro Analysis of Functional Properties, and Volatile Profiling of Sixteen Nutraceuticals Marketed as "Superfood". *Applied Sciences*, *14*(23), 11069. https://doi.org/10.3390/app142311069

Végh, R., **Sipiczki, G**., & Csóka, M. (2023). Investigating the antioxidant and color properties of bee pollens of various plant sources. *Chemistry & Biodiversity*, *20*(7), e202300126. https://doi.org/10.1002/cbdv.202300126

Sun, W., Nguyen, Q. D., **Sipiczki, G**., Ziane, S. R., Hristovski, K., Friedrich, L., Visy, A., Hitka. G,. Gere, A., & Bujna, E. (2022). Microencapsulation of *Lactobacillus plantarum* 299v strain with whey proteins by lyophilization and its application in production of probiotic apple juices. *Applied Sciences*, *13*(1), 318. https://doi.org/10.3390/app13010318

Vu, V., Farkas, C., Riyad, O., Bujna, E., Kilin, A., **Sipiczki, G**., Sharma, M., Usmani, Z, Gupta, K. V., & Nguyen, Q. D. (2022). Enhancement of the enzymatic hydrolysis efficiency of wheat bran using the *Bacillus* strains and their consortium. *Bioresource Technology*, *343*, 126092. https://doi.org/10.1016/j.biortech.2021.126092

Sipiczki, G., Eszterbauer, E., Nagy, E. Sz.; Kiss, Zs., Bujna, E., (2022). *Yarrowia* törzsek pigment termelésének vizsgálata. Élelmiszer Tudomány Technológia, p.68

7.2. Conference posters

Nagy, E. Sz., Bujna, E., **Sipiczki, G**., Farkas, Cs., Belo, I., Lopes, M., Braga, A., Mesquita, D. P., Ferreira, P., Nguyen, D. Q. (2018) Optimization of lipase production by *Yarrowia divulgata,* 3rd International Conference on Food Science and Technology, pp. 136-137., http://www.foodconf.hu/files/BOA2018.pdf

Nagy, E. Sz., Bujna, E., **Sipiczki, G**., Farkas, Cs., Belo, I., Lopes, M., Braga, A., Mesquita, D. P., Ferreira, P., Nguyen, Q. D.. (2019) Enhancement of lipase production by *Yarrowia divulgata,* XX EuroFoodChem Conference, pp. 284., Book of Abstracts of the XX EuroFoodChem Congress

Sipiczki G., Eszterbauer, E., Eszterbauer E, Szinger, D.; Bujna E., (2020) Production of Pigment by *Yarrowia*, Magyar Mikrobiológiai Társaság (MMT) pp. 34., A Magyar Mikrobiológiai Társaság 2020. évi Nagygyűlése és a XIV. Fermentációs Kollokvium: Absztraktfüzet

Sipiczki, G., Bujna E. (2020) A pH és a levegőztetés hatása a *Yarrowia* élesztők pigment termelésére, ITT -Ifjú Tehetségek Találkozója 2020, Konferenciakiadvány p. 335.

Sipiczki, G., Kovács, O. L., Bujna E. (2021) Production and stability of pigment by *Yarrowia*, 4th International Conference on Biosystems and Food Engineering, Book of abstract Paper E447.

Sipiczki, G, Bujna, E. (2021) *Yarrowia* törzsek pigment termelésének optimálása, ITT -Ifjú Tehetségek Találkozója

Sipiczki, G, Bujna, E. (2022) Effect of aeration and manganese concentration on pigment production by *Yarrowia* yeast, 4th International Conference on Food Science and Technology, pp. 75., http://www.foodconf.hu/files/BOA2022.pdf

Sipiczki, G., Bujna E. (2023) Effect of agitation speeds on lipase production by *Yarrowia* yeasts, 5th International Conference on Biosystems and Food Engineering, ISBN 978-615-01-8151-6