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# Effects of Olive Oil and Tween 80 on Production of Lipase by *Yarrowia* Yeast Strains

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**Abstract:** Lipase is one of the most commonly used biocatalysts in the food, pharmaceutical and cosmetic industries, and can be produced by *Yarrowia lipolytica* yeast. Despite the intensive studies of lipase from *Yarrowia*, there are still many open questions regarding the enzyme secretion process, especially by new isolates of this genus as well as the effect of substrates or surfactants, or both on the production of lipase. This research focused on the effect of olive oil and surfactant Tween 80 including the optimisation of the concentration of these compounds on the production of lipase by some novel *Yarrowia* isolates. Moreover, the optimal environmental parameters (pH, temperature) of crude enzyme synthetised by *Yarrowia* strains were determined. All investigated strains were able to produce lipase in both intracellular and extracellular fractions. The extracellular lipase activities were higher than the intracellular ones (*Y. divulgata* Y.02062 and *Yarrowia lipolytica* 854/4 147 U/L, 80 U/L and 474 U/L, 122 U/L, respectively). In the case of extracellular lipase, supplementing olive oil and Tween 80 enhanced significantly the synthesis and secretion of the enzyme. The lipase activity can even be enhanced by 20 times higher from 25 U/L to 474 U/L in the case of *Yarrowia lipolytica* 854/4 strain. In the case of intracellular, supplementation of Tween 80 generally reduces lipase activity except for the *Y. lipolytica* 1/4 strain, which was affected by two times the increase. The optimised concentration of olive oil and Tween 80 were determined for *Y. divulgata* Y.02062, *Y. divulgata* 5257, *Y. lipolytica* 1/4, and *Yarrowia lipolytica* 854/4 strains as 1.6% olive oil and 0.09% Tween 80, 1.6% olive oil and 0.06% Tween 80, 1.4% olive oil and 0.09% Tween 80 as well as 1.6% olive oil and 0.065% Tween 80, respectively. The optimum pH and temperature of crude lipases (intra and extracellular) synthetised by the tested *Yarrowia lipolytica* and *Y. divulgata* yeast strains were found to be pH 7.2 and 37 °C, respectively. Our results confirmed that the new isolate *Y. divulgata* is a very promising species for further development for industrial use as *Y. lipolytica*.

**Keywords:** *Yarrowia lipolytica*; *Yarrowia divulgata*; olive oil; surfactant; RSM



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## 1. Introduction

The lipase (triacylglycerol hydrolase; EC 3.1.1.3) belongs to the group of serine hydrolase enzymes that catalyse the hydrolysis of various fats and oils. In contrast to esterases, lipases break the ester bonds in water-insoluble fatty acid esters at the interface between the aqueous and oily phases [1,2]. The application of lipase enzymes has expanded dynamically in recent years, because of their favourable properties in industrial processes [3]. Lipase enzymes are widely used in the food industry including meat, dairy, and bakery products, as well as in the fat, oil and beverage industries. In addition, these enzymes have great potential for the pharmaceutical and healthcare sectors [4–6]. Furthermore, they also

play a significant role in biosensor technology [7] such as for the determination of pesticide methyl-parathion, olive oil, triglycerides, triacetin, tributyrin, triolein, etc.

Among the members of the *Yarrowia* clade, *Y. lipolytica* is most studied due to its metabolic products [8], such as lipase, protease [9], erythritol, mannitol [10], and citric acid [11]. Strains of the species *Y. lipolytica* also known as "oily yeast" are commonly isolated from substrates containing lipids or proteins such as cheese or sausage, and they are able to accumulate large amounts of lipids. Numerous experiments have shown that *Y. lipolytica* is particularly cold-tolerant and can therefore survive in refrigerated temperatures and cause food spoilage [12,13]. The other species of the genus *Yarrowia*, such as *Y. divulgata*, *Y. porcina*, and *Yarrowia bubula* also seem to have high application potential in the food industry. Some strains of these species appeared to be much better sweetener producers when grown on glycerol than *Y. lipolytica* [8,14]. Wild-type and genetically modified strains of lipase-producing *Yarrowia* are also suitable for alkane and fatty acid bioconversion [15]. *Yarrowia* yeasts have been reported to produce extracellular, intracellular and cell wall-bound lipase enzymes [16] in lipid- or oil-containing substrates. However, the lipase produced by these yeasts remains generally inside of the cell (bound to the cell wall) and is only secreted into the culture medium when the carbon source in the medium becomes scarce, i.e., in the transition to diauxie (when more than one substrate is used) or to stationary phase. Hydrophobic substrates are considered to be the most efficient carbon sources, which stimulate the production of lipolytic enzymes by microorganisms. Among them, great attention has been paid to vegetable oils (olive oil, sunflower, corn, palm, rapeseed, soybean oil) and pure fatty acids (palmitic acid, oleic acid) [17,18]. Research has proven that among the natural vegetable oils, olive oil proved to be an effective inducer in terms of lipase production because of the high content of oleic and linoleic acids [19,20]. In addition to vegetable oils, oil industry residues can be promising natural substrates for growth, and lipase production by *Y. lipolytica* [18,21]. The quality of substrate and inducer as well as the presence of surfactants play an important role in the enzyme synthesis. This phenomenon can vary from genus to genus, species to species and even from strain to strain.

So far, because of the industrial importance, most studies focused only on the *Y. lipolytica* species, and less attention was paid to other ones, especially new isolates. In the last decade, a number of new strains were isolated and identified by colleagues of the Institute of Food Science and Technology, Hungarian University of Agriculture and Life Science (Hungary). No doubt that studies of these new isolates have significant importance and results will contribute new evidence to the community to understand this yeast genus as well as to develop industrial applications. In our study, the effects of olive oil and Tween 80 on the production of *Yarrowia* isolates were investigated.

## 2. Materials and Methods

### *2.1. Materials*

The artificial substrate p-nitrophenyl laurate was purchased from Sigma-Aldrich Chemical Co. (Budapest, Hungary). All other chemicals, and reagents are analytical grades and either from Sigma-Aldrich Co., VWR International LLC. (Debrecen, Hungary) or Reanal Ltd. (Budapest, Hungary).

Strains, media and growth conditions: Three *Yarrowia lipolytica* strains, two *Y. divulgata* strains, one *Y. porcina* and one *Y. bubula* strain were used in this study (Table 1) and were kindly provided by the National Collection of Agricultural and Industrial Microorganisms (NCAIM, Budapest, Hungary). These strains were isolated from various sources such as meats, and cheeses.

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 for 24 h, then stored at 4 °C until use.

**Table 1.** *Yarrowia* strains used in this study.

| Strain | Source of Isolation |
|---|---|
| *Yarrowia divulgata* Y.02062 | Chicken breast |
| *Yarrowia divulgata* 5257 | Minced pork leg |
| *Yarrowia porcina* Y.02102 | Minced pork leg |
| *Yarrowia bubula* 441/4 | Minced pork leg |
| *Yarrowia lipolytica* 854/4 | Minced pork leg |
| *Yarrowia lipolytica* 1/4c | Edam cheese |
| *Yarrowia lipolytica* 6/3 | Cottage cheese |

### *2.2. Methods*

#### 2.2.1. Enzyme Fermentation

Yeast cells from agar slants were inoculated in 250 mL flasks containing 150 mL basal medium containing 20 g/L glucose, 20 g/L peptone, and 10 g/L yeast extract. The modified media were prepared by supplementing the basal medium with different concentrations of olive oil and/or Tween 80. Enzyme fermentations were initiated with 5 v/v% 24 h inoculum cultures, using a shaker at 130 rpm in a volume of 125–200 mL at 28 °C for 148 h. The fermentation medium was prepared by dissolving 20 g glucose, 6.4 g peptone and 10 g yeast extract in 1 L tap water and then sterilised at 121 °C for 30 min. Depending on the experimental design, fermentation broth was supplemented with olive oil and/or Tween 80 in different concentrations.

Meanwhile, the extracellular enzyme activities were monitored daily during the fermentation process, whereas the intracellular activity was determined from samples taken at 72 h after 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.

#### 2.2.2. Optimisation of Medium Composition for Maximising Lipase Production

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. Meanwhile, two medium components namely olive oil ($X_1$, g/L) and Tween 80 ($X_2$, g/L) were selected as independent variables based on preliminary experiments, whereas the lipase enzyme activities (Y) were used as dependent variables.

The second-order polynomial model (Equation (1)) was used to obtain the response surfaces and thus estimate the optimum points that provide maximal lipase production.

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3{X_1}^2 + b_4{X_2}^2 + b_5X_1X_2 \quad (1)$$

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.

#### 2.2.3. 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 [17]. Briefly, 100 µL of 25 mM p-nitrophenyl laurate (Sigma-Aldrich Chemical Co.) dissolved in 96% ethanol and 0.725 µL Sorensen's phosphate buffer (pH 7.2) were preheated at 37 °C for 15 min. 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.

#### 2.2.4. Determination of Optimum pH

The pH optimum of both intracellular and extracellular lipase enzyme activities was checked using 72 h ferment broth containing 0.05% Tween 80 and 1% olive oil. Sorensen's phosphate buffers were prepared in the pH range of pH 5–8 and the lipase activity was assayed at 37 °C. The results were presented in terms of relative activity.

#### 2.2.5. Determination of Temperature Optimum

The temperature optimum of the enzyme activity was determined in the range from 30 °C to 45 °C with 5 °C stepwise using Sorensen's phosphate buffer pH 7.2. Enzyme samples were produced by fermentation in a medium containing 0.05% Tween 80 and 1% olive oil for 72 h. The results were presented in terms of relative activity.

### *2.3. Statistical Analysis*

The experimental data were collected and preprocessed in Microsoft Excel spreadsheet in the Office365 Software package. Results are generally expressed as the mean ± SE of different independent replicates. Two-way analysis of variables (two-way ANOVA) was conducted using STATISTICA software (version 9.0) to test the differences between the variances. Data were considered significant at $p < 0.05$.

## 3. Results and Discussion

### *3.1. Strain Selection for Lipase Production*

The extracellular lipase production of seven different *Yarrowia* strains was screened in the basal medium (Figure 1). The fermentation was performed for 5 days, and lipase activity was monitored at specified time intervals.

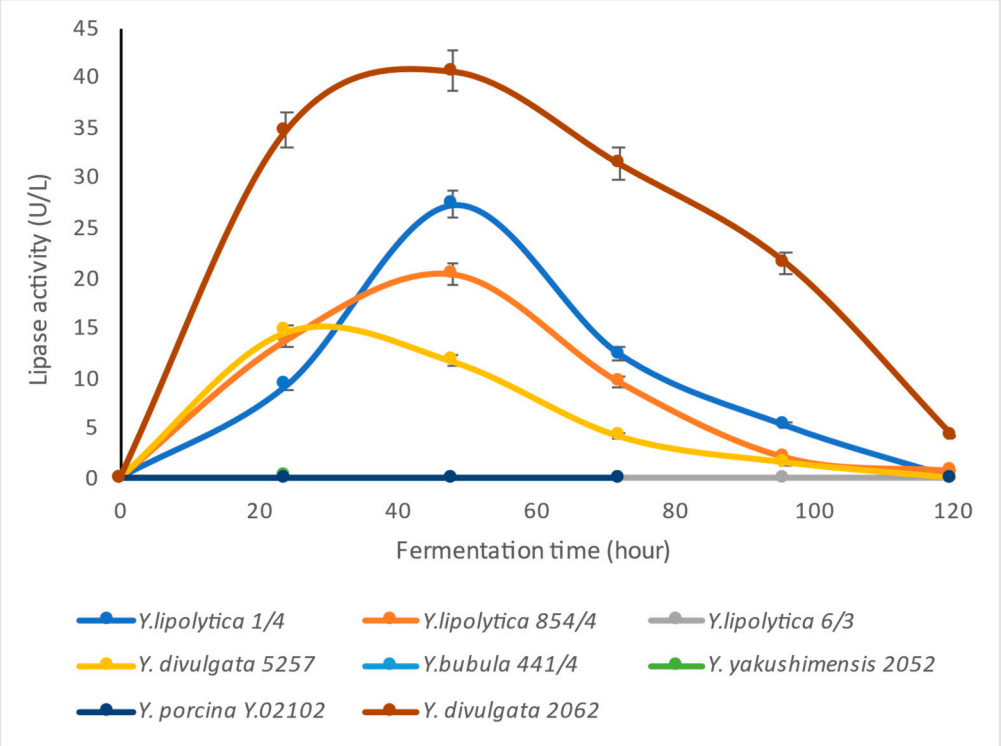


**Figure 1.** Extracellular lipase activity of new *Yarrowia* isolates.

In the case of *Yarrowia porcina* Y.02102, *Yarrowia bubula* 441/4, and *Yarrowia lipolytica* 6/3 strains, no lipase activity was detected during fermentation for 5 days in media without any supplementation. *Yarrowia divulgata* 5257 strain reached the maximal activity on the first day of fermentation (14.6 U/L), while the highest enzyme activity can be observed on day 2 in the cases of *Yarrowia lipolytica* 1/4 (27.3 U/L), *Yarrowia lipolytica* 854/4 (20.4 U/L) and *Y. divulgata* Y.02062 (40.56 U/L) strains. The lipase activity dropped sharply at 72 h in all cases of *Y. lipolytica* strains. The best value was given by *Y. divulgata* Y.02062 at 48 h, with a maximum of 40.56 U/L. In contrast to the other strains, the lipase activity was also detected at hour 72 of fermentation by this strain. Two strains of *Y. divulgata*, as well as *Y. lipolytica* 1/4 strain and *Y. lipolytica* 854/4 strain achieved higher activities in the screening process, therefore these were selected for further studies. Lipase production of *Y. lipolytica* was investigated by several researchers on raw glycerol [13], hydrophobic substrate [15], olive oil [22,23] and sunflower oil [24], and this species is generally claimed to be an excellent lipase producer (20–1000 U/mL), even on an industrial level.

### *3.2. Effects of Olive Oil and Tween 80 on Lipase Production*

It is well known that olive oil is an excellent carbon source and has a high inducer effect on the secretion of lipase by *Yarrowia lipolytica* yeast [25]. This effect is associated with oleic acid, which is considered an inducer of the LIP2 promoter [17]. In our study, 1% olive oil was added into the basal medium as an inducer and a substrate for both *Y. lipolytica* and new isolate *Y. divulgata* as well. The fermentation process was carried out for 144 h (6 days) and extracellular lipase activity was monitored daily. The enzyme activity of *Y. lipolytica* 854/4 strain was 13 U/L at 24 h, increased to 25 U/L at 48 h and then dropped to around 20 U/L at the end of the fermentation process (Figure 2). In the case of the *Y. lipolytica* 1/4 strain, the extracellular lipase activity peaked (131 U/L) at 48 h, then dropped to 44 U/L at 72 h and then to 24 U/L at the end of fermentation. The lipase activity of the *Y. divulgata* Y.02062 strain exhibited maximum value (81 U/L) at 72 h and the kept unchanged until 144 h of fermentation. Darvishi and co-workers [19] as well as Paludo and co-workers [25] also found the maximum extracellular lipase (34.6 U/mL and 2.23 U/mL) from *Y. lipolytica* by adding 10 g/L olive oil (1%) into the fermentation medium. However, Pignède and co-workers [26] found that the addition of 50 g/L exhibited a higher effect (2000 U/mL) on the enzyme secretion than 10 g/L olive oil (1500 U/mL). Iqbal and Rehman [27] reported an increase in growth and lipase production of *Bacillus subtilis* by supplementing 0.5–1% olive oil. They also reported the highest enzyme titers (1800 U/mL) in the case of the addition of 1% olive oil. The optimum inducer concentration was found to be 0.5% (*v*/*v*) sunflower oil in the study of lipase activity produced by *R. delemar* [28]. In our study, similar trends were observed in all investigated strains. The addition of 1% olive oil was found to be an effective inducer for the production of lipases by *Y. lipolytica* 1/4, *Y. lipolytica* 854/4, *Y. divulgata* 5257 and *Y. divulgata* Y.02062 strains. The stimulating effect of olive oil on lipase production has also been confirmed by other studies [13,14,16]. Galvagno et al. [13] reported a significant increase in lipase production when an organic nitrogen source and 10 g/L of olive oil were added. The lipase production by the *Y. lipolytica* 681 strain was significantly increased ($p < 0.05$) by supplementation of olive or corn oil as a carbon source and inductor [13]. Nunes et al. [16] also found that olive oil had a significant stimulating effect on the production of intracellular lipase by *Y. lipolytica.* At the same time, Domínguez et al. [24] observed that sunflower oil was the best lipolytic inducer, even better than olive oil. The maximum activity was detected on day 8 of fermentation.

The effect of Tween 80 on the lipase production by *Yarrowia* yeast was investigated using a basal medium with the addition of 1% olive oil and 0.05% Tween 80 based on preliminary studies as well as a report from Domiguez et al. [24]. Both extracellular and intracellular activities were monitored daily during the fermentation process for 6 days (Figure 2, Table 2). It was found that, in the presence of Tween 80, all investigated *Yarrowia* strains produced higher lipase activities in both intracellular and extracellular fractions. In the case of the extracellular lipase, the *Y. lipolytica* 854/4, *Y. lipolytica* 1/4 and *Y. divulgata*

Y.02062 strains secreted the highest activities with values of 474 U/L, 221 U/L and 147 U/L, respectively, whereas in the case of intracellular lipase, the highest lipase activity (273 U/L) was produced by *Y. lipolytica* 1/4 strain.

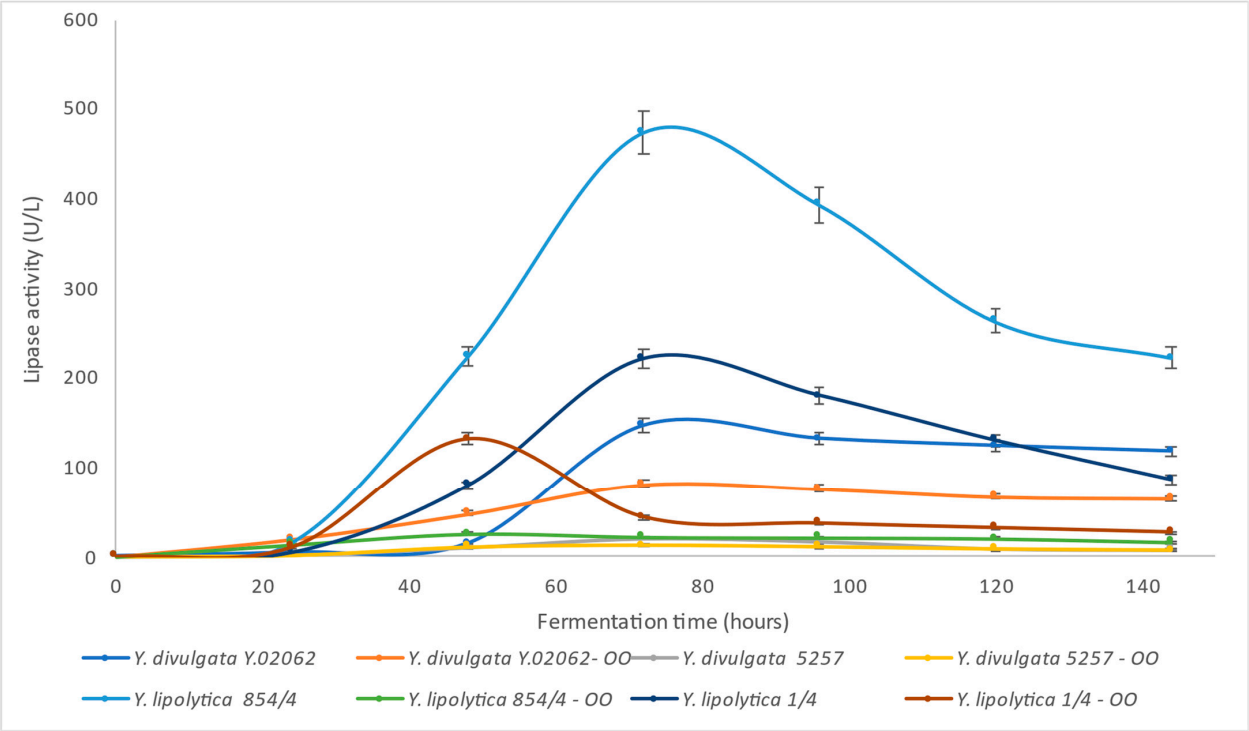


**Figure 2.** Extracellular lipase activities produced by *Yarrowia* strains in different media. OO: olive oil.

**Table 2.** Intracellular lipase activity of different *Yarrowia* yeast strains at 72 h.

| Strain | Lipase Activity (U/L) | |
|---|---|---|
| | Olive Oil (1%) | Olive Oil (1%) and Tween 80 (0.05%) |
| *Y. divulgata* 5257 | 22 | 8 |
| *Y. divulgata* Y.02062 | 117 | 80 |
| *Y. lipolytica* 1/4 | 126 | 273 |
| *Y. lipolytica* 854/4 | 467 | 122 |

The maximum of extracellular lipase activity produced by the *Y. lipolytica* 854/4 strain (Figure 2) in a medium containing only olive oil (without Tween 80) was 25 U/L at 48 h of fermentation. This value is about 20 times lower than one (474 U/L at 72 h) with Tween 80. The reverse trend was observed in the case of intracellular lipase activity. Meanwhile, the maximum intracellular activity in the medium without Tween 80 was 467 U/L, whereas in the medium with Tween 80, this value was only 122 U/L (Table 2). It means that the addition of Tween 80 caused an increase in extracellular activity and a decrease in intracellular activity.

In the case of the *Y. lipolytica* 1/4 strain, the maximum extracellular lipase activity measured in the medium with Tween 80 was 221 U/L at 72 h, which was significantly lower than the one produced by the *Y. lipolytica* 854/4 strain. In the case of intracellular lipase, the supplement of Tween 80 had a positive effect meaning an increase in activity from 126 U/L to 273 U/L with Tween 80. Interestingly, the intracellular activity of this strain was significantly higher with Tween 80 than without it.

In the case of the *Y. divulgata* Y.02062 strain, the maximum extracellular lipase activity was determined in both media at 72 h, and the lipase activity in the medium containing

Tween 80 (147 U/L) was about two times higher than the one (81 U/L) without Tween 80. The intracellular lipase activity decreased significantly from 117 U/L to 80 U/L with the supplementation of Tween 80 in the fermentation medium (Table 2).

In the case of the *Y. divulgata* 5257 strain, the maximum extracellular lipase activity (Figure 2) was detected after 72 h of fermentation. It was noted that the maximum extracellular activities (20 U/L and 13 U/L) detected at 72 h were very low even in media with or without Tween 80. In the case of the intracellular activity (Table 2), the highest enzyme activities (22 U/L and 8 U/L) were observed in the media with and without Tween 80 supplementation, respectively.

Tween 80 is well known as a surfactant and non-ionic polyoxyethylene detergent. The hydrophobic part usually consists of an alkyl chain and the hydrophilic part is made of uncharged ethylene oxide units [29]. Generally, Tween 80 stimulates lipase biosynthesis and secretion as it increases cell permeability and facilitates lipase excretion across the cell membrane [30]. This phenomenon has been attributed to altered cell permeability or surfactant effects on cell-bound lipase. Nevertheless, surfactants do not only increase lipase production, and their effect appears to depend on both the surfactant and strain studied [24]. Corzo and Revah [22] also confirmed that the extracellular lipase activity can be increased by the addition of Tween 80 in the concentration range of 0.5 to 2 g/L without any change in the biomass concentration. In our study, Tween 80 was found to be effective in enhancing the extracellular lipase activity for all strains, whereas it was effective for the intracellular activity in the case of *Y. lipolytica* 1/4 strain only.

### *3.3. Optimisation of Extracellular Lipase Production*

Optimisation of the concentration of olive oil and Tween 80 for maximising extracellular lipase production by selected strains was performed using central composite design (CCD) a part of the response surface method (RSM) [31]. A set of experimental runs with two independent variables (olive oil and Tween 80) was designed (Table 3) and enzyme activities were assayed at different fermentation times. The results at 72 h fermentation and the CCD matrix are presented in Table 3.

**Table 3.** CCD experimental design and lipase enzyme activities of different *Yarrowia* strains.

| Run | Transformed Level | | Real Concentration | | Lipase Activity at 72 h (U/L) | | | |
|---|---|---|---|---|---|---|---|---|
| | Olive Oil | Tween 80 | Olive Oil (%) | Tween 80 (%) | *Y. lipolytica* 854/4 | *Y. lipolytica* 1/4 | *Y. divulgata* Y.02062 | *Y. divulgata* 5257 |
| 1 | −1.41 | 0 | 0.295 | 0.05 | 305 | 74 | 99 | 120 |
| 2 | −1 | 1 | 0.5 | 0.07 | 301 | 181 | 134 | 107 |
| 3 | 0 | 1.41 | 1 | 0.0782 | 612 | 354 | 258 | 222 |
| 4 | 1 | 1 | 1.5 | 0.07 | 570 | 221 | 217 | 192 |
| 5 | 1.41 | 0 | 1.705 | 0.05 | 599 | 202 | 238 | 282 |
| 6 | 1 | −1 | 1.5 | 0.03 | 240 | 137 | 191 | 182 |
| 7 | 0 | −1.41 | 1 | 0.0218 | 233 | 96 | 78 | 151 |
| 8 | −1 | −1 | 0.5 | 0.03 | 289 | 114 | 147 | 96 |
| 9 | 0 | 0 | 1 | 0.05 | 504 | 166 | 158 | 182 |
| 10 | 0 | 0 | 1 | 0.05 | 530 | 173 | 161 | 187 |

Analysis of variances (ANOVA) showed that in the case of the *Y. lipolytica* 854/4 strain, the variances of linear and quadratic variables as well as the interaction between them were significantly similar at 95% and 90% levels, respectively (Table 4). Additionally, in the cases of strains *Y. lipolytica* 854/4, 1/4, *Y. divulgata* Y.02062 and 5257, the lack of fit (LoF) values of the models were significant at 85%, 93%, 98% and 92% levels, respectively. In the case of

the *Y. lipolytica* 1/4 strain, similarities of variances of two linear variables were significant at the 96% level, while the ones of two quadratic variables were at the 93% level.

**Table 4.** Analysis of variances of two factors olive oil and Tween 80 in the optimisation experiments.

| Factor | *Y. lipolytica* 854/4 | | *Y. lipolytica* 1/4 | | *Y. divulgata* Y.02062 | | *Y. divulgata* 5257 | |
|---|---|---|---|---|---|---|---|---|
| | F-Value | *p*-Value | F-Value | *p*-Value | F-Value | *p*-Value | F-Value | *p*-Value |
| Olive oil (%) (L) | 151.59 | 0.05 | 315.51 | 0.04 | 5563.74 | 0.01 | 1491.02 | 0.02 |
| Olive oil (%) (Q) | 41.28 | 0.10 | 84.76 | 0.07 | 61.16 | 0.08 | | |
| Tween 80 (%) (L) | 290.21 | 0.04 | 1425.55 | 0.02 | 3789.22 | 0.01 | 133.10 | 0.06 |
| Tween 80 (%) (Q) | 66.31 | 0.08 | 104.31 | 0.06 | 52.19 | 0.09 | 40.67 | 0.10 |
| Olive oil × Tween 80 | 75.76 | 0.07 | 3.11 | 0.33 | 162.65 | 0.05 | | |
| Lack of fit (LoF) | 24.69 | 0.15 | 117.26 | 0.07 | 1137.95 | 0.02 | 92.20 | 0.08 |

L: linear, Q: quadratic.

Meanwhile, in the case of the *Y. divulgata* Y.02062 strain, the variances of different variables of the full second-order polynomic model were significantly similar by at least 91%, whereas in the case of the *Y. divulgata* 5257 strain, the partial model was used and the similarities of variances of independent variables were significant at 90% or higher levels (Table 4). The regression analysis was completed based on the results of ANOVA and the models Equations (2)–(5) were built to describe the changes in extracellular lipase activities at 72 h fermentation vs. the concentration of Tween 80 and olive oil.

$$Y_1 = 518 + 159\,X_1 - 110\,{X_1}^2 + 220\,X_2 - 139\,{X_2}^2 + 159\,X_1\,X_2 \quad (2)$$

$$Y_2 = 170 + 61\,X_1 - 41\,{X_1}^2 + 129\,X_2 + 46\,{X_2}^2 + 8\,X_1\,X_2 \quad (3)$$

$$Y_3 = 159 + 81\,X_1 + 11\,{X_1}^2 + 67\,X_2 + 10\,{X_2}^2 + 20\,X_1\,X_2 \quad (4)$$

$$Y_4 = 180 + 100\,X_1 + 30\,X_2 - 20\,{X_2}^2 \quad (5)$$

where

$Y_1$ is the lipase activity of the *Y. lipolytica* 854/4 strain at 72 h of fermentation.
$Y_2$ is the lipase activity of the *Y. lipolytica* 1/4 strain at 72 h of fermentation.
$Y_3$ is the lipase activity of the *Y. divulgata* Y.02062 strain at 72 h of fermentation.
$Y_4$ is the lipase activity of the *Y. divulgata* 5257 strain at 72 h of fermentation.
$X_1$ is the olive oil concentration (%).
$X_2$ is the Tween 80 concentration (%).

The significant levels of regressed coefficients were investigated by t-probe and almost all values (except the interaction between two variables in the case of *Y. lipolytica* 1/4 strain) are fit at more than 90% level. Surface plots of lipase activities vs. the olive oil and Tween 80 concentration were demonstrated in Figure 3. Based on the results, the optimal concentration of independent variables (olive oil and Tween 80) was 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* Y.02062 strain: 1.6% olive oil, 0.09% Tween 80.
- *Y. divulgata* 5257 strain: 1.6% olive oil, 0.06% Tween 80.

These values were validated and confirmed experimentally by fermentation of the relevant strain using media supplemented with the above-mentioned concentration.

The lipase production of the *Y. lipolytica* Wt-11 mutant yeast was also increased by Tween 80 at a concentration of 0.1%, but a higher amount had an inhibitory effect [32]. In our study, *Y. divulgata* Y.02062 and *Y. divulgata* 5257 resulted in the highest lipase activities in the media containing 1.6% olive oil, and 0.09% Tween 80 or 0.06% Tween 80, respectively.

Boekema et al. [33] found that a concentration of 0.1% Tween 80 dramatically increased the extracellular lipase activity in the bacteria *Burkholderia glumae*.

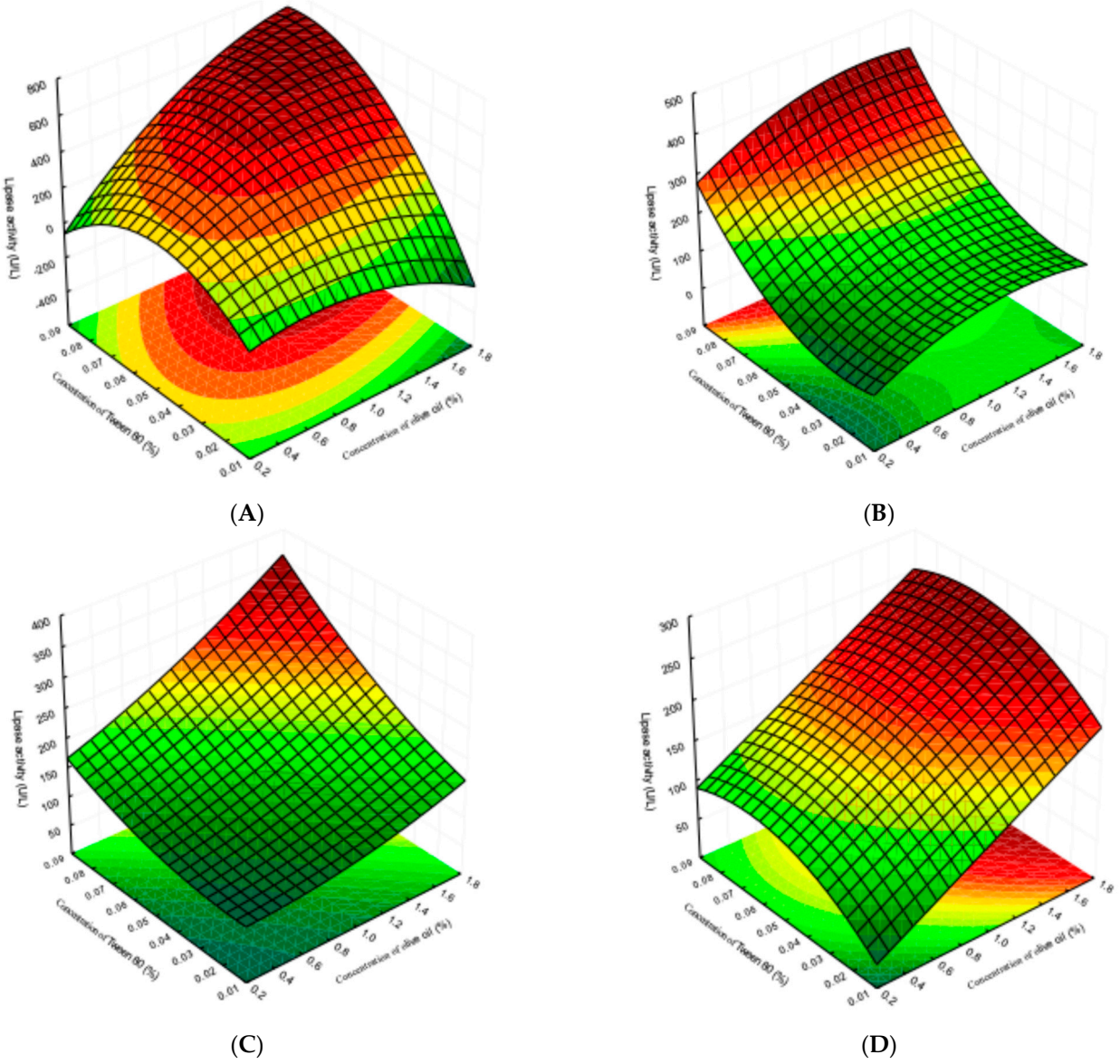


**Figure 3.** Surface plots of the lipase activity from different strains vs. concentration of olive oil and Tween 80. (**A**) *Y. lipolytica* 854/4 strain, (**B**) *Y. lipolytica* 1/4 strain, (**C**) *Y. divulgata* Y.02062 strain, (**D**) *Y. divulgata* 5257 strain.

The most commonly used olive oil concentration is 1%. In contrast, the most promising Tween 80 concentration is between 0.05 and 0.09%. Papagora et al. [31] isolated lipase-producing yeasts from dry-salted olives (Thassos variety), which were later identified as *Debaryomyces hansenii* yeasts. During screening, 0.1% Tween 80 was added to the medium, which promoted lipase production. Tween 80 was added to the production medium, which contains 1% olive oil, as preliminary experiments have shown that this is the optimal ratio, as higher concentrations of Tween 80 have been shown to inhibit lipase activity [34].

Olive oil is considered an activator for extracellular lipase due to the 55–83% content of oleic acid, which is an inducer of the LIP2 gene promotor. Nevertheless, not all types of extracellular lipases are activated by olive oil, and it is thought that cell-bound lipases are not stabilised by oleic acid [35]. The production of extracellular lipase by *Y. lipolytica*

KKP 379 was induced by the lipids present in the medium containing olive oil. In YPG, no stimulation effect was achieved because of the lack of hydrophobic substrates. However, a similar pattern of lipase production in this medium was also observed for *Y. lipolytica*, which can be used to interpret the mechanisms of lipolytic enzyme synthesis in yeast cells [35].

### *3.4. Partial Characterisation of Crude Lipases from Yarrowia Yeasts*

The optimum pH and temperature of both extracellular and intracellular crude enzyme preparations produced by all the tested *Yarrowia* strains were determined (Figure 4).

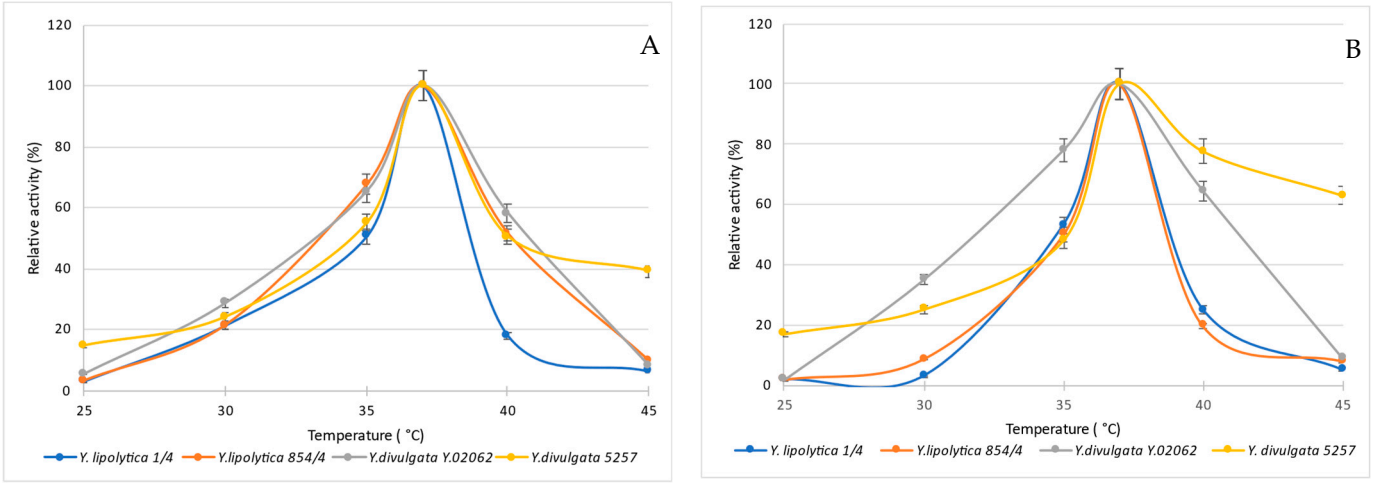


**Figure 4.** Optimum temperature of the extracellular (**A**) and intracellular (**B**) lipase enzymes of the four *Yarrowia* strains.

In the cases of extracellular fractions, the lipase activities exhibited the same trends from 25 °C to 37 °C and peaked at 37 °C (100%). Then the activities dropped drastically. The enzyme activity from the *Y. lipolytica* 1/4 strain dropped to around 20% of the highest activity at 40 °C and to 10% at 45 °C. In the cases of the intracellular fractions, similar trends were observed. An increase in incubation temperature from 25 °C to 37 °C resulted in an increase in lipase activity even in different profiles. The maximum activities were assayed at 37 °C for all crude enzyme preparations. The optimum temperature of extracellular ones was also 37 °C. Corzo and Revah [22] observed that the maximal lipase activity of strain *Y. lipolytica* 681 was at 37 °C. The optimal temperature for lipase activity of the *Y. lipolytica* CBS 6303 strain and *Y. lipolytica* LgX64.81 mutant strain was also 37 °C [15,23]. Carvalho et al. [8] determined the optimum range of 35–40 °C for the lipase enzyme produced by the *Y. lipolytica* IMUFRJ 50682 strain.

In the case of the *Y. divulgata* 5257 strain, both intra- and extracellular fractions exhibited more stability at 45 °C than lipase from other investigated strains. In incubation at this temperature, 63% and 39% of the maximum activities were assayed in the intracellular and extracellular fractions, respectively. The *Yarrowia lipolytica* Lip2 is unstable at high temperatures and rapidly loses its activity at temperatures higher than 40 °C [35]. The thermostability of Lip2 can be improved by engineering combined disulphide bonds between different regions (interregional disulphide bonds). The optimum temperature of lipase from the *Y. lipolytica* 4S mutant strain increased from 35 °C to 40 °C, whereas the ones from the mutants 5S strain and 6S strain to 55 °C [36], respectively. The disulphide bounds generally stabilise the folded form of proteins, thus increasing their thermostability. In our case, a speculation can be made, that the lipase protein from the *Y. divulgata* 5257 strain is richer in disulphide bounds.

In the case of the effect of pH on lipase enzyme activity, all investigated fractions exhibited optimum pH at pH 7.2 using the Sorensen buffer series (Figure 5). In addition,

significantly lower enzyme activity was observed at the two extreme pH values, pH 5 and pH 8. In the case of pH 7.5, lipase activities of *Y. lipolytica* 1/4 and *Y. divulgata* 5257 strains were significantly higher than other cases of pHs. However, while *Y. lipolytica* 1/4, *Y. divulgata* 5257, *Y. lipolytica* 854/4, and *Y. divulgata* NCAIM Y.02062 showed almost the same intracellular activities, whereas the extracellular activities of all preparations were sufficiently different. The pH can affect the state of protonation of amino acids functional groups, causing conformational change in the active site and inhibiting the effective binding of the substrate. Carvalho et al. [5] reported that the pH optimum of the lipase enzyme produced by strain *Y. lipolytica* IMUFRJ 50682 was in the pH 6–7 range. pH 7 was also considered optimal for the lipase activity of *Y. lipolytica* by others [15,23]. Interestingly, while some authors [12,35] tested lipase activity at pH 7.2, no one found that pH 7.2 is the optimal pH for the lipase enzyme from *Yarrowia*. The disulphide bonds could also affect the pH stability of the enzyme [35]. Li et al. [35] engineered lipase from *Yarrowia* by insertion of multiple disulphide bounds and they found that the stable pH range of the 5S strain and 6S strain extended from pH 4.0 to 9.0 (wild-type strain) to pH 3.0 to 11.0 (mutant strains). More than 75% of its original activity was retained after incubation for 24 h [35].

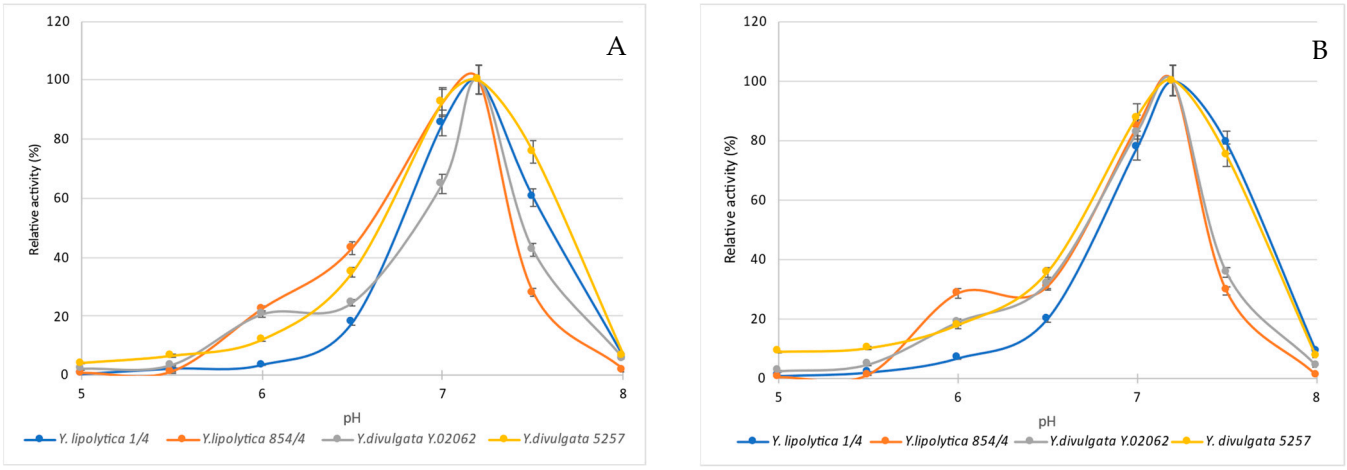


**Figure 5.** Optimum pH of the extracellular (**A**) and intracellular (**B**) lipase enzymes of *Yarrowia* strains.

## 4. Conclusions

Both intracellular and extracellular lipase enzymes can be produced by some *Yarrowia* isolates (*Y. lipolytica* 854/4, *Y. lipolytica* 1/4, *Y. divulgata* Y.02062, *Y. divulgata* 5257) using olive oil as substrate. Both olive oil and Tween 80 have a stimulating and enhancing effect on lipase production, but the optimal concentration of inducers may vary from strain to strain. Additionally, the presence of olive oil induced the secretion of extracellular lipase enzymes. In all cases of tested strains, the lipase activity of the extracellular fraction was significantly higher than the intracellular one. All crude enzyme preparations have optimum temperature and pH at 37 °C and pH 7.2, respectively. Our results showed that the lipase production of the *Y. divulgata* Y.02062 strain (from the NCAIM) was as good as *Y. lipolytica*, and it is very promising for further development for industrial applications.

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**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available due to the data handling regulation of the Hungarian University of Agriculture and Life Science (www.uni-mate.hu).

**Conflicts of Interest:** The authors declare no conflict of interest.

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