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Extracellular Lipases of *Yarrowia lipolytica* Yeast in Media Containing Plant Oils—Studies Supported by the Design of Experiment Methodology

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Abstract: Lipases are enzymes of great application importance in the food industry, in the cosmetic and detergent industries, in pharmacy and medicine, and in organic chemistry. Among lipases of various origins, those from microorganisms are currently the most commonly used. An excellent producer of lipases seems to be the nonconventional *Yarrowia lipolytica* yeast, but the biosynthesis of valuable metabolites depends on many factors. This study aimed to investigate the biodiversity of extracellular enzymes produced by four strains of *Y. lipolytica*, and to determine the optimal conditions of catalysis for the enzymes, according to temperature and pH, in a model hydrolysis reaction. Based on the obtained results, the biodiversity and strain dependence in lipase biosynthesis were observed. Using a Central Composite Design, it was found that temperature is the main factor in determining lipase activity. The enzymes produced by four different strains exhibited other substrate specificity, which was investigated using Latin square design methodology. Only two examined yeast strains, KKP 379 and W29, produced extracellular lipases at a high activity level towards medium- and long-chain fatty acid esters. Moreover, extracellular lipase from wild-type strain KKP 379 was further characterized, followed by exploring the activity of whole-cell biocatalyst and lyophilized enzyme solutions, and it was acknowledged that it was a "true" lipase with the highest affinity to *p*-nitrophenyl oleate.

Keywords: design of experiment; lipase; *Yarrowia lipolytica*; specific activity; esterase activity

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1. Introduction

Nearly 30% of commercially produced enzymes are used in the food industry [1]. Lipases belong to an essential group of biocatalysts with high application potential in the food industry, and at the same time have significant physiological importance. Microorganisms are the main source of hundreds of commercially available lipolytic enzymes. Despite significant advances in the genetic engineering of industrial strains, finding the optimal synthesis conditions of enzymes is still necessary [2,3].

Lipases (EC 3.1.1.3) catalyze the hydrolysis or formation of triacylglycerols, playing an essential role in the digestion, transport, and processing of dietary lipids. These enzymes catalyze both the hydrolysis of esters formed from glycerol and long-chain fatty acids in high-water-content environments, and the synthesis of triacylglycerol esters in organic solvents at low water activity, with a certain substrate specificity and activity under a

specific temperature and pH. Moreover, lipases are enzymes characterized by high regio- and stereospecificity [4].

Microbial lipases have many applications, including organic synthesis, in the food and detergent, paper and oleochemical, cosmetic, medicine, and waste treatment industries [5]. Whole-cell lipases and esterases have become increasingly important in recent years, because the organic solvent-tolerant cells allow the production of optically active alcohols, acids, esters, and lactones. The majority of commercially significant lipases are produced by *Candida rugosa*, *C. antarctica*, *Aspergillus niger*, *Thermomyces lanuginosus*, *Rhizomucor miehei*, and *Rhizopus arrhizus* [6].

Yarrowia lipolytica is a commonly occurring yeast species in nature, considered to be non-pathogenic with GRAS status. The high secretion activity of *Y. lipolytica* is well known, and the most desirable yeast metabolites are citric acid, 2-phenylethanol, γ-decalactone, erythritol, and enzymes: RNase, phosphatases, esterases, and lipases. The genes of the *LIP* family encoding for *Y. lipolytica* lipase proteins were discovered in recent decades [7,8]. The main extracellular lipase of *Y. lipolytica* is the Lip2p protein encoded by the *LIP2* gene. It is a *sn*-1,3-regioselective enzyme, and the products of the catalyzed reaction are mainly 2-monoglycerides. Other *Y. lipolytica* lipases have been cloned and characterized, although knowledge about them is still small [9,10].

It is widely known that the microbial production of lipases is strongly influenced by culture conditions such as carbon and nitrogen sources, the presence of lipid sources, minerals, pH, temperature, inoculum age, the presence of activating and inhibitory compounds, and other factors. There is still intrigue as to whether strain differences exist in lipase enzyme profiles. This study aimed to investigate the biodiversity of extracellular enzyme properties produced by four strains of *Y. lipolytica*, and to determine the optimal conditions of catalysis for the enzymes, according to temperature and pH, in a model hydrolysis reaction. Moreover, extracellular lipase from wild-type strain KKP 379 was characterized, followed by exploring the activity of whole-cell biocatalyst and lyophilized enzyme solutions.

2. Materials and Methods

2.1. Yeast Strains and Culture Conditions

In this study, the following yeast strains were used: *Y. lipolytica* KKP 379, purchased from the Collection of Industrial Microorganisms at the Prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology—State Research Institute (Warsaw, Poland); *Y. lipolytica* W29 (ATCC® 20460™); *Y. lipolytica* ATCC® 18942™; and *Y. lipolytica* JM23 (ATCC® 90812™). The latter three strains were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). *Y. lipolytica* KKP 379 has been widely examined in the Department of Chemistry WULS. All strains had acknowledged hydrolytic activity, and were stored in glycerol stocks at −20 °C.

Inoculum batch-agitated cultures of yeast strains were provided in a YPO medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L olive oil) for 24 h at 28 °C. Experimental shaking cultures were carried out for 48 and 65 h in a rich liquid medium (YP; 10 g/L yeast extract, 20 g/L peptone, pH = 5.0) and minimal medium (YNB—Yeast Nitrogen Base with Amino Acids; Sigma-Aldrich, Poznań, Poland). Both media were supplemented with 20 g/L plant oil as the carbon source. The oils used were commercially available first-cold-pressed plant oils: extra virgin olive oil (Olitalia, Forli, Italy), first-cold-pressed peanut oil (Olitalia, Forli, Italy), rice oil (Alfa One, Bangkok, Thailand), and corn oil (Olitalia, Forli, Italy), purchased from the local supermarket in Warsaw (Poland). An inoculum of 0.1% (*v*/*v*) was added, and the yeasts were incubated at 28 °C with a rotation of 150 rpm. The culture time was chosen according to previous studies [11].

2.2. Design of Experiments in the Study

The scope of the work included several stages, described in detail in Figure 1. Each of the 5 stages involved a specific objective. In the subsequent stages, different culture

media were used, and various statistical methods were applied to support the design of the experiments and data analysis.

	Experimental step	Aim of the step	Culture medium	Statistical methodology
1	Shaken cultures of four *Y. lipolytica* strains ATCC 90812, W29, ATCC 18942, KKP 379	• Comparison of extracellular lipolytic activity of the yeast strains • Investigation on the impact of temperature and pH	YPO rich medium	Central Composite Design (CCD)
2	Shaken cultures of three *Y. lipolytica* strains ATCC 90812, W29, KKP 379	• Evaluation of impact of fatty acid composition and acid value of plant oils used in culture media on extracellular lipase activity	YNB minimal medium or YP rich medium with 4 plant oils peanut)	Latin square design
3	Shaken cultures of three *Y. lipolytica* strains ATCC 90812, W29, KKP 379	• Determination of the substrate specificity of hydrolytic extracellular enzymes in the supernatant (cell-free extract)	YPO rich medium	ANOVA Tukey's post hoc test
4	Shaken cultures of one *Y. lipolytica* strain KKP 379	• Purification of extracellular lipase • Investigation specificity of the enzyme	YPO rich medium	ANOVA Tukey's post hoc test
5	Immobilization of biocatalysts from *Y. lipolytica* strain KKP 379	• Exploration of the potential of yeast lipases with varied purification degrees (free or immobilized form)	YPO rich medium	ANOVA Tukey's post hoc test

Figure 1. A diagram with the objectives of the steps of the study and a general methodology applied.

2.3. Lipase Purification

The lipases from *Y. lipolytica* KKP 379 were purified according to the method described by Jasińska et al. [12]. Briefly, after the freeze-drying process of the supernatant of the *Y. lipolytica* cultures, the obtained preparations were purified by ion-exchange chromatography with a linear gradient elution, with 0.7 M NaCl and 15 mM Tris-HCl (pH = 6.8), as well as by molecular sieves with a 50 mM phosphate buffer (pH = 7.0), and the active fractions were then concentrated by centrifugation. Samples of the same volume were collected manually.

2.4. Hydrolytic Activity Assay

Measures of the enzymatic activity were carried out using the spectrophotometric method, based on the hydrolysis of *p*-nitrophenyl laurate [11]. One unit of enzyme activity was defined as the enzyme quantity that liberated 1 µmol of *p*-nitrophenol per minute under the assay conditions at 37 °C. For the reaction, *p*-nitrophenyl laurate was used, and the lipase had a specificity towards insoluble or at least poorly soluble long-chain fatty acids, in contrast to esterase, whose activity was found to be highest towards more water soluble substrates.

To determine the lipase specificity of biocatalysts, a modified protocol was used for the hydrolytic activity assay. The following fatty acid esters of *p*-nitrophenol were used in the same concentration (0.3 mmoles in 2 mL of heptane): butyric (C4:0), caprylic (C8:0), lauric (C12:0), palmitic (C16:0), stearic (C18:0), and oleic (C18:1). *p*-Nitrophenyl esters were synthesized in the reactions of equimolar amounts of a specific acyl chloride, *p*-nitrophenol, and triethylamine [13]. To investigate the impact of pH and temperature on lipase activity, the substrate solution was mixed with a phosphate buffer solution, and a model reaction was provided in variable temperatures. Detailed descriptions can be found in the Section 2.10.1.

2.5. Determination of Protein Content

The amount of protein released from the yeast cells was measured by a modified spectrophotometric Lowry's method at 750 nm [12]. Bovine serum albumin was used as the protein standard (Sigma Aldrich, Poznań, Poland).

2.6. Acid Values of Plant Oils

The acid values of the plant oils were determined by titrimetric methods, according to PN-EN ISO 660:2021-03 [14]. The acid value was expressed as the number of milligrams of KOH needed to neutralize the free fatty acids contained in 1 g of fat. The principle of the method is to titrate the fat solution in the solvent with a standard KOH solution. The free fatty acids contained in the fat are then neutralized.

2.7. Synthesis of Fatty Acid Methyl Esters (FAME) and Determination of Fatty Acid Composition of the Studied Oils

Fatty acid derivatization was provided using methanol solutions of KOH [15]. Methyl heptadecanoate was used as an internal standard. A 4 mg lipid sample in a screw-capped 10 mL glass tube was hydrolyzed and methylated with 4 mL of 0.5 M KOH in methanol at 60 °C for 30 min. After cooling to room temperature, 2 mL of water was added to the solution, and then the FAMEs were extracted with 4 mL of n-hexane. The hexane phase was analyzed with GC-MS, namely with an Agilent Technologies 68790 N GC gas chromatograph coupled with a mass spectrometer (Agilent Technologies, Santa Clara, CA, USA), and the separation of compounds was accomplished on an HP 5-MS column. Helium was used as the carrier gas, and the gas flow was 1.2 mL/min. Individual fatty acids were identified based on retention times, comparing them with reference acids.

2.8. Determination of Triacylglycerol Structure of Plant Oils

To determine the composition of fatty acids in the *sn*-2 position of the triacylglycerols of the vegetable oils, selective enzymatic hydrolysis was performed. The method was modified by using a lipolytic preparation from Sigma-Aldrich—immobilized 1,3-*sn*-regiospecific lipase from *Thermomyces lanuginosus* (≥3000 U/g). The hydrolysis products were extracted and an extract was applied to silica gel TLC plates to isolate 2-monoacylglycerols. After derivatization to methyl esters, the fatty acid composition of the plant oils was determined with GC-MS, according to the method described in the previous subsection.

2.9. Lipase Immobilization

Before immobilization, the yeast biomass obtained by the centrifugation of cells from the supernatant was subjected to the process of lysis. The biomass (4 g) was suspended in 36 mL of distilled water, and then 10 g of glass beads with a diameter of 5 mm were added. The suspension with the beads was shaken for 30 min, and then the biomass was separated from the liquid by centrifugation, and the obtained fractions (disintegrated biomass and cell-free extract) were subjected to the determination of hydrolytic activity, as stated in the Section 2.4.

According to the protocol of Jasińska et al. [12], a cell-bound lipase preparation from *Y. lipolytica* KKP 379 was immobilized on Lewatit VP OC 1065 (a macroporous resin of poly (methyl methacrylate)). Briefly, after activating the beads in ethanol and drying them under a vacuum, 1 g of beads and 15 mL of lipase solution were shaken at 4 °C for 14 h. Then, the immobilized preparation was filtered, washed with distilled water, and dried for 24 h.

2.10. Design of Experiment Methodology and Statistical Analysis

Statistica 13.1 software (TIBCO Software Inc., Palo Alto, CA, USA) was used to perform the DoE experiments. The significance level was determined at 0.05. ANOVA analyses, accompanied by Tukey's post hoc test, were performed.

2.10.1. Central Composite Design

Due to the need to perform multiple combinations of two selected variables (temperature and pH), a response surface methodology of DoE (design of experiment) was applied as a statistical tool for investigating the impact of culture conditions on the crude extracellular extract of *Y. lipolytica* yeast strains. Two separate experimental schemes and two parallel cultures for each design were prepared, based on simplex designs for ternary mixtures. The schemes varied in assumptions as to the maximum amount of carbon sources. Within a particular experimental scheme, it was possible to evaluate three different mixture designs: a simplex lattice design (medium 1–6, Table 1), a simplex centroid design (medium 1–7, Table 1), and a simplex extended design (medium 1–10, Table 1).

Table 1. Experiment design—Central Composite Design.

No.	Temperature (°C)	pH
1	25.0	6.3
2	25.0	7.7
3	45.0	6.3
4	45.0	7.7
5	20.9	7.0
6	49.1	7.0
7	35.0	6.0
8	35.0	8.0
9	35.0	7.0
10	35.0	7.0

2.10.2. Latin Square Design

A design of the experiment was applied as a tool in investigating the effect of plant oils on *Y. lipolytica* lipase activity. A 4 × 4 Latin square design method was used for the experiments provided in flasks (Table 2).

Table 2. Experiment design—Latin square design 4 × 4, column factor—type of culture medium (YP—rich medium or YNB—minimal medium), row factor—yeast strain, tested factor—plant oil.

Yeast Strain	Medium			
	YP	YNB	YP	YNB
Y. lipolytica KKP 379	olive oil	corn oil	peanut oil	rice oil
Y. lipolytica W29	rice oil	peanut oil	corn oil	olive oil
Y. lipolytica ATCC 90812	peanut oil	rice oil	olive oil	corn oil
Y. lipolytica ATCC 18942	corn oil	olive oil	rice oil	peanut oil

Latin square designs are used when the number of input values is greater than two, and the effect of the factor under study is strongly masked by the influence of dominant confounders that are known and can be controlled. The Latin square itself is a square matrix in which no row or column contains two of the same words. Each tested factor must occur in the same number, for example, in a 4 × 4 Latin square (Table 2). There are 4 column factors, 4 row factors, and 4 test factors. The main purpose of this method is to separate the influences of interfering factors. The tested factor was the type of plant oil used in the culture medium as the sole carbon source. The interfering factors were the *Y. lipolytica* strain and the culture medium (YNB minimal medium or YP rich medium), due to the likely masking effect of these factors on the effect of the examined factor. The main analytical tools at this stage were the multi-factor analysis of the variance of the main effects, and the main effects diagrams.

3. Results

3.1. Strain-Dependent Extracellular Lipase Activity

In the first stage of the current work, the influence of pH and temperature on extracellular lipase activity in a model reaction of *p*-nitrophenyl laurate hydrolysis was assessed. Within this part of the experiment, a Central Composite Design, with the two above-mentioned tested variables, was applied. The results, in the form of Pareto charts and contour plots with the response surfaces, are presented in Figures 2 and 3, respectively.

The results showed that the extracellular lipases produced by three out of four examined strains, namely ATCC 90812, W29, and KKP 379, showed similar levels of activity at different conditions of the hydrolysis reaction. Only the ATCC 18942 strain stood out from the others, producing enzymes at a very low level.

The optimal conditions for achieving the highest reaction yield were determined. As can be seen in Figure 2, temperature (linear dependence) was the significant factor in evaluating lipase activity for all the examined strains. The optimal temperature conditions ranged from c.a. 20 °C to 40 °C. Interestingly, *Y. lipolytica* W29 showed the opposite effect to the other strains, because in this case, the lower the temperature, the higher the activity. The strains also differed according to the optimal pH of the buffer (Figure 3).

3.2. Substrate Specificity of Y. lipolytica Extracellular Lipases in Crude and Purified Lipase Solutions

The subsequent part of the current work was devoted to the examination of the substrate specificity of *Y. lipolytica* extracellular lipases. In this case, firstly, four plant oils (olive, rice, corn, and peanut) were analyzed for their total fatty acid composition in oils and fatty acid composition in triacylglycerols at the *sn*-2 position (Table 3).

Table 3. Fatty acid composition in plant oils (%, all positions in triacylglycerols *sn*-1,2,3), fatty acid composition in *sn*-2 position of triacylglycerols in plant oils (abbreviations: C16:1—palmitoleic acid, C17:1—heptadecenoic acid, C18:1—oleic acid, C18:2—linoleic acid, C18:3—linolenic acid, C20:1—eicosenoic acid, C22:1—erucic acid, C24:1—nervonic acid) and acid values of the studied plant oils.

Plant Oil	Position of Fatty Acid in Triacylglycerols	Fatty Acid Content (%)									Acid Value (mg KOH/g)
		C16:1	C17:1	C18:1	C18:2	C18:3	C20:1	C22:1	C24:1	Sum of Saturated Fatty Acids	
Olive oil	*sn*-1,2,3	0.45	0.1	72.9	9.4	0.6	1.4	-	0.1	14.4	0.66
	sn-2	2.41	-	72.37	11.50	4.96	-	-	-		
Rice oil	*sn*-1,2,3	0.15	-	41.7	35.9	1.7	0.6	0.1	0.65	15.1	0.39
	sn-2	1.42	-	52.78	26.23	6.23	-	-	-		
Corn oil	*sn*-1,2,3	-	0.1	24.4	58.6	0.55	0.8	0.15	-	15.0	0.25
	sn-2	2.05	-	29.18	64.46	0.75	-	-	-		
Peanut oil	*sn*-1,2,3	0.15	0.1	59.3	19.5	0.1	1.2	-	0.1	8.9	0.55
	sn-2	1.15	-	63.89	23.76	0.80	-	-	-		

All the oils had a high content of unsaturated fatty acids in the triacylglycerol molecules. The highest oleic acid content (C18:1) was found in olive oil (72.9%), a lower content in peanut oil (59.3%) and rice oil (41.9%), and the lowest content in corn oil (24.4%) (Table 3).

The highest content of linoleic acid (C18:2) was also determined; therein, the highest amounts of this compound were detected in corn oil (58.5%) and rice oil (35.9%). It is worth emphasizing that the sum of the oleic and linoleic acid contents in all four plant oils varied, from 78% for rice oil and 83% for corn oil. Other fatty acids were present in small amounts of less than 2%.

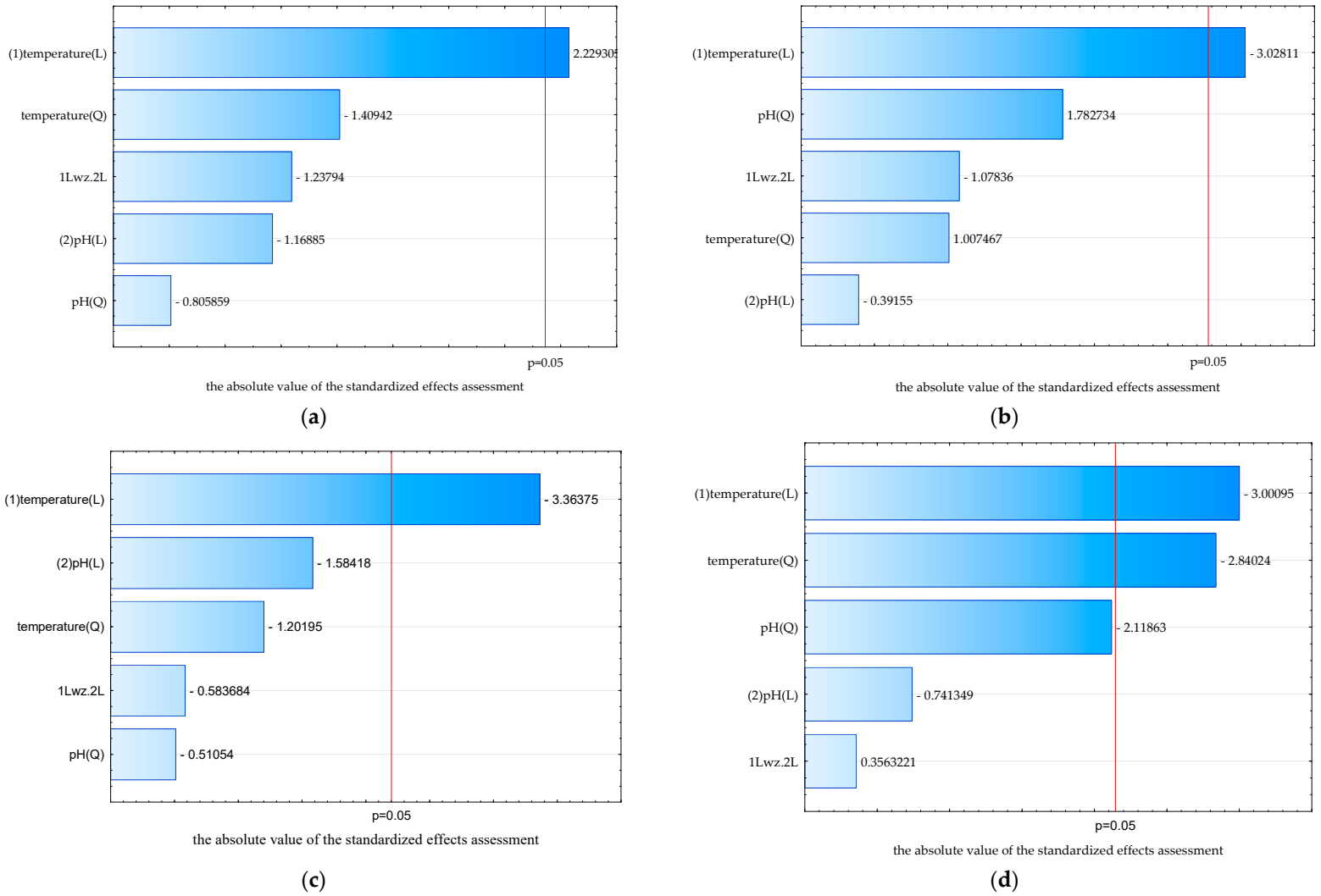

Figure 2. Pareto charts indicating significantly important factors influencing the extracellular hydrolytic activity of (**a**) *Y. lipolytica* ATCC 90812; (**b**) *Y. lipolytica* W29; (**c**) *Y. lipolytica* ATCC 18942; and (**d**) *Y. lipolytica* KKP 379. Abbreviations: L—linear effect, Q—quadratic effect, 1L wz 2L—the interaction of the effects.

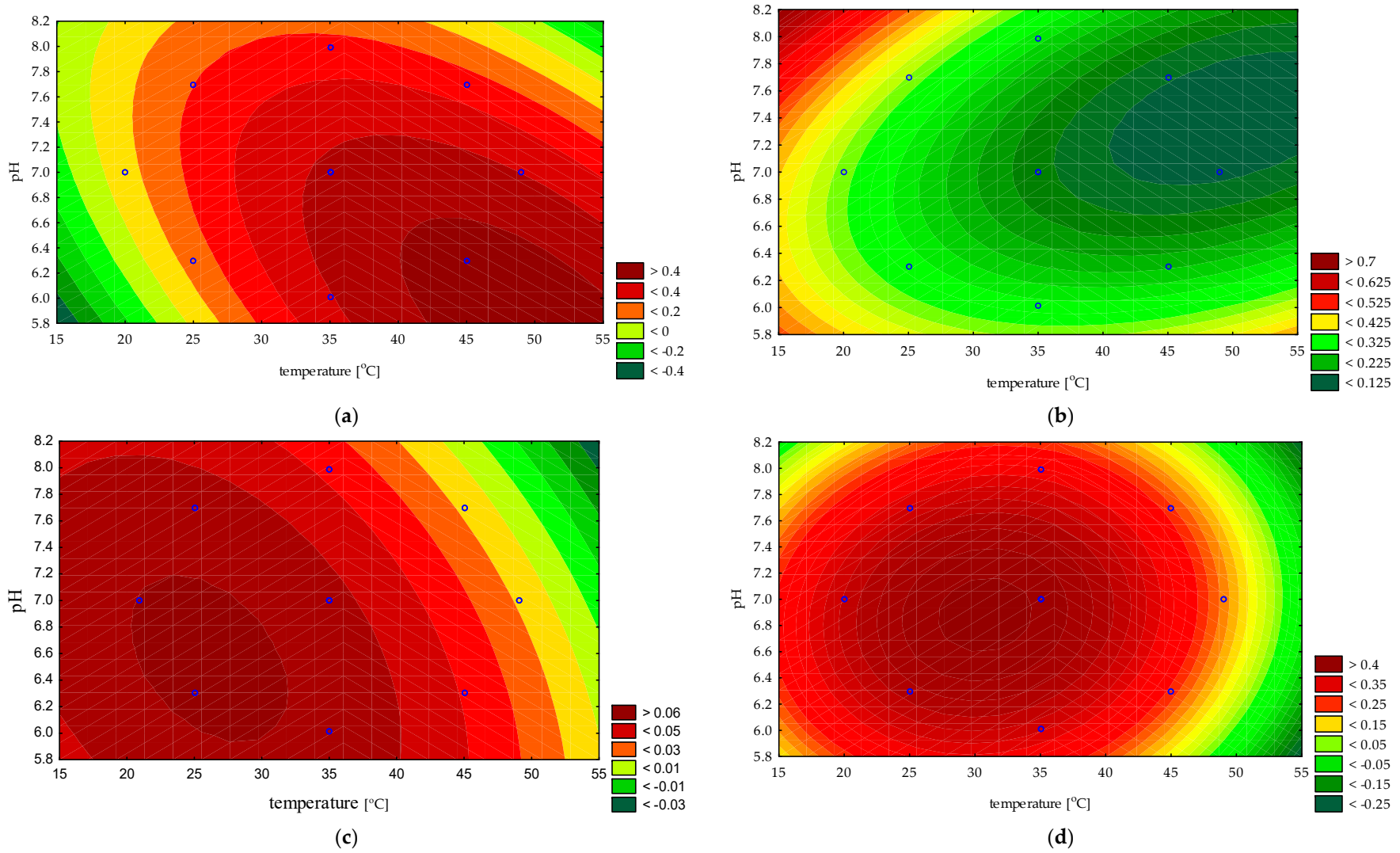

Figure 3. Response surfaces with second-degree polynomial models for the extracellular hydrolytic activity of (**a**) *Y. lipolytica* ATCC 90812; (**b**) *Y. lipolytica* W29; (**c**) *Y. lipolytica* ATCC 18942; and (**d**) *Y. lipolytica* KKP 379, cultured in YPO medium. Bullet points represent experimental points included in the design.

Considering the regioselectivity of the enzyme, oils were analyzed in this study, and were characterized as similarly rich in oleic and linoleic acids at the *sn*-2 triacylglycerol position as in the *sn*-1,3 position (Table 3). The highest oleic acid content was observed in olive oil, and the highest linoleic acid content in corn oil. The fatty acid residues were distributed proportionally in the molecules of triacylglycerols, so this factor had no significant effect on the level of synthesis of the lipases.

It was assessed whether there may be a correlation between the production of extracellular lipases by the lipolytic yeast species and the level of free fatty acids present in the plant oil used as the sole carbon source in the medium. The acid values were then determined, and the results of these measurements are presented in Table 3. The acid values of the oils (free fatty acid content) were higher for olive oil (0.66) and peanut oil (0.55) than for rice oil (0.39) and corn oil (0.25 mg KOH/g).

The effect of the yeast strain used, the type of plant oil in the culture medium, and the type of the medium were investigated in relation to the extracellular activity of lipases synthesized by *Y. lipolytica* yeast cells using a Latin square design. The results of the experiment are presented in the form of main effects plots in Figure 4. The analysis of variance of the experimental data showed no significant effect of the two factors, i.e., plant oil and yeast strain, on the extracellular hydrolytic activity and specific activity. Only the type of medium used was statistically significant.

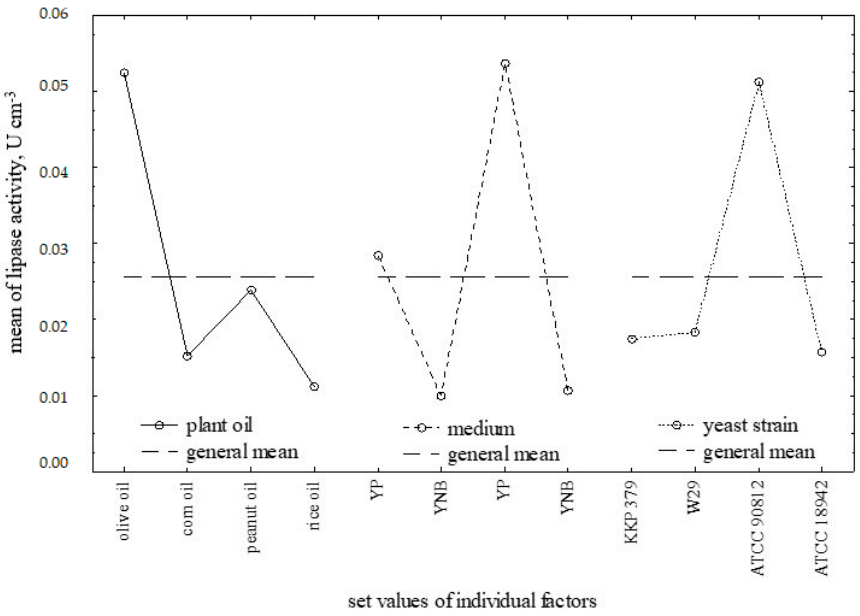

Figure 4. Main effects plot for *Y. lipolytica* extracellular lipase activity after 65 h shaken cultures. The first third of the figure shows lipase activity obtained when the medium was supplemented with plant oils; the second third of the figure shows lipase activity in two media—YP rich medium or YNB minimal medium; and the third part of the figure shows lipase activity according to four different yeast strains.

The next stage of the current work was the determination of the substrate specificity of the enzymes in the supernatant (cell-free extract) from the cultures of three *Y. lipolytica* strains. It is worth mentioning that the ATCC 18942 strain, due to its very low production of lipolytic enzymes (Figure 3), was excluded from further research. After 48 h of yeast cultivation in the YPO medium, the activities of supernatants towards different *p*-nitrophenyl esters were measured. At this stage, proteins with hydrolytic activity were not purified and thus their activity was determined in a mixture of various enzymes, including hydrolytic ones, secreted by yeast cells into the medium. The esters used for this part of the research were derivatives of fatty acids: butyric acid (C4:0), lauric acid (C12:0), and oleic acid (C18:1). Derivatives of these three fatty acids were selected because they enabled the determination

of the affinity of enzyme proteins for short-, medium-, and long-chain carbon sources. Analyzing the data presented in Figure 5, it can be observed that the highest hydrolytic activities were obtained for the two yeast strains of *Y. lipolytica*, i.e., KKP 379 and W29. Both of these strains showed a high affinity for the substrates used, but the highest was noted for esters of medium-chain fatty acids. The hydrolytic activity for the C12 ester was 0.199 U/mL and 0.143 U/mL for the KKP 379 and W29 strains, respectively. *Y. lipolytica* KKP 379 also showed an activity of 0.107 and 0.100 U/mL for the butyric and oleic acid esters. In the case of the W29 strains, these activities were slightly lower, at 0.071 and 0.088 U/mL, respectively.

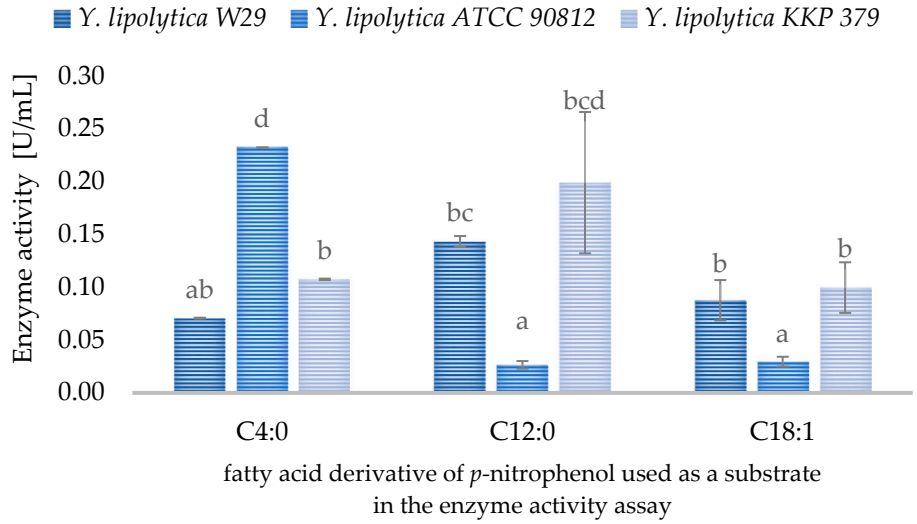

Figure 5. The hydrolytic activity of lipases in crude enzyme extract (supernatant) from three *Y. lipolytica* yeast strains towards different fatty acids esters of *p*-nitrophenol. Means with the same letter (a–d) did not differ significantly ($\alpha = 0.05$).

In the case of the ATCC 90812 strain, the hydrolytic enzymes present in this supernatant had the highest activity towards *p*-nitrophenyl butyrate (0.139 U/mL), but in the other two cases (for laurate and oleate esters), the activity was significantly lower compared to other strains. It can therefore be concluded that the cell-free extract of this strain was dominated by esterases (EC 3.1.1.1) and/or the amount of lipolytic proteins was small, while for the strains KKP 379 and W29, lipases (EC 3.1.1.3) were in the majority and/or the activity of these enzymes was relatively high. Due to the observed high activity about the oleic acid ester, comparable to the activity of the W29 strain, and the higher activity about the lauric acid derivative, the KKP 379 strain was selected for the next step, the purpose of which was to purify lipolytic enzymes.

The purification of enzymes by ion-exchange chromatography was carried out on the supernatant from a shaken culture of the yeast strain *Y. lipolytica* KKP 379. The strain was chosen because of its relatively high extracellular "true" lipase activity (Figure 5). A total of 80 protein-containing fractions were obtained, of which the hydrolytic activity was measured in 40 fractions in a *p*-nitrophenyl laurate hydrolysis reaction (Figure 6).

Analyzing Figure 6, two peaks, characterized by hydrolytic activity, can be observed. The first was for fractions 15–17, for which the hydrolytic activity was approx. 0.250 U/mL, and the second was for fractions no. 59–63, where an activity of about 0.450 U/mL was noted. It can be concluded that the purified supernatant contained enzymes with different charges, and the obtained electropherogram showed bands corresponding to sizes of approx. 30 and 40 kDa (Supplementary Materials, Figure S1). The molecular weight of Lip2p lipase is 38 kDa [16], which may confirm the presence of this lipase in the supernatant of the KKP 379 strain and a second lower-molecular-weight enzyme, e.g., a protease, which is also secreted by this yeast, and has a molecular weight of 30 kDa [17].

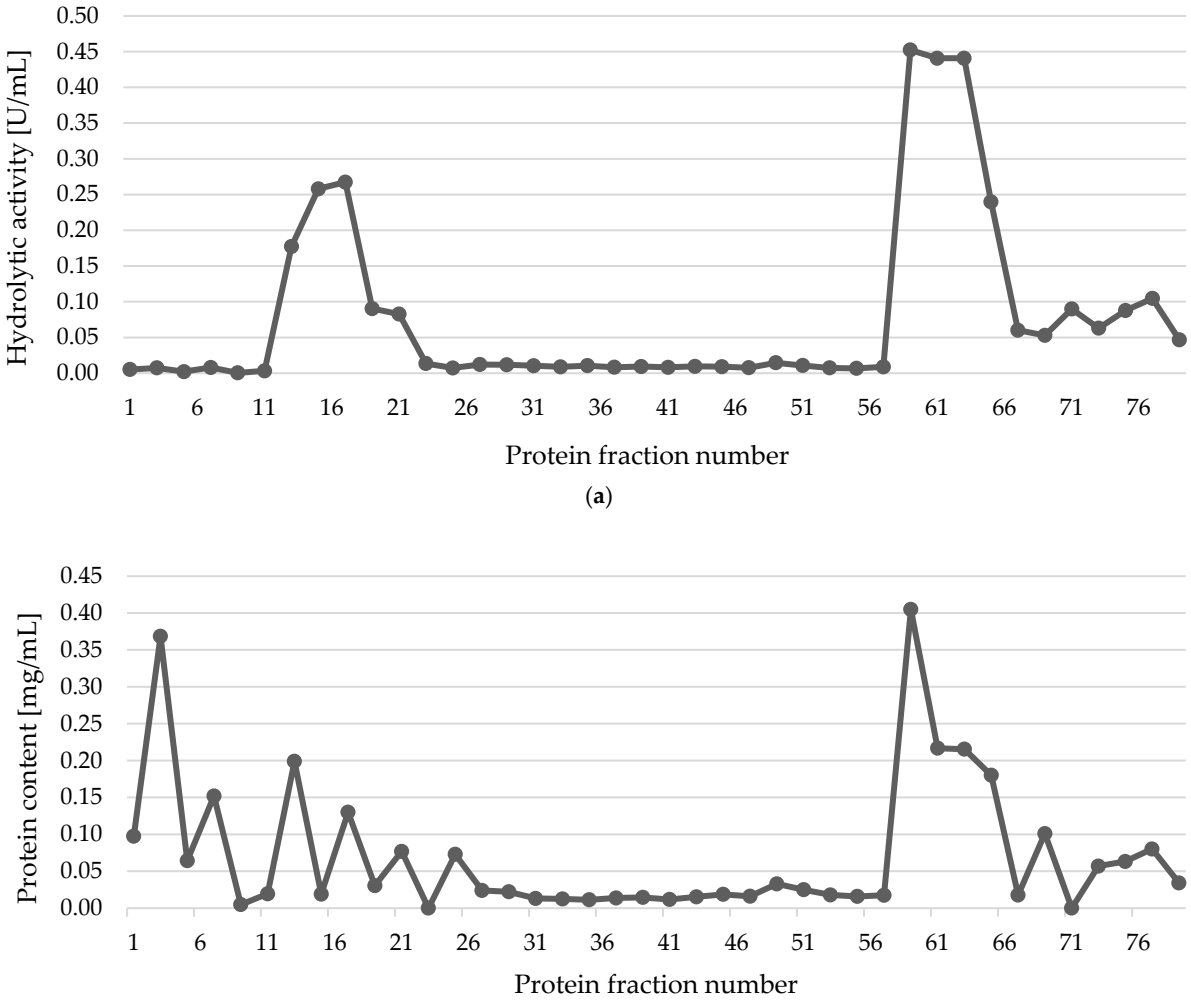

Figure 6. Hydrolytic activity (**a**) and protein concentration (**b**) of *Y. lipolytica* KKP 379 protein fractions purified by ion-exchange chromatography.

In an effort to prove that it is a "true" lipase, we decided to conduct an additional experiment in which the specific activities of the extracellular lipase from *Y. lipolytica* KKP 379 (fraction 60) towards different fatty acids esters of *p*-nitrophenol, i.e., butyric, caprylic, lauric, palmitic, stearic, and oleic acids esters, were determined (Figure 7).

A purified fraction of the lipase exhibited high hydrolytic activity, and the highest specific activity was observed for butyric acid ester (0.93 U/mg). The elongation of the fatty acid side chain resulted in a decrease in activity; for the caprylic acid ester, the specific activity was 0.62 U/mg, then 0.29 U/mg for the laurate ester, and in the case of the palmitate and stearate esters, the obtained values were comparable, at 0.12 and 0.10 U/mg, respectively. Interestingly, the specific activity measured towards *p*-nitrophenyl oleate started increasing again, and the value was on the level of the C8 ester, at 0.56 U/mg.

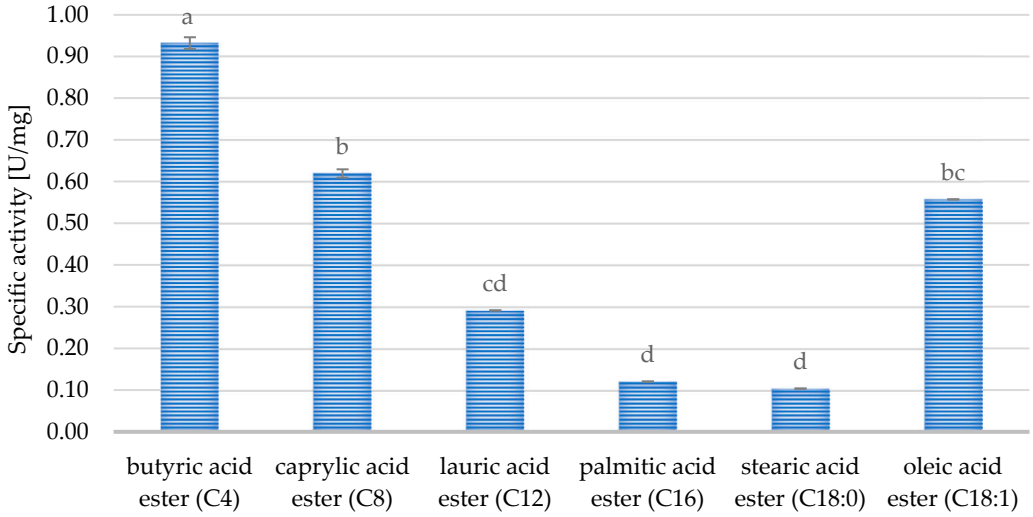

Figure 7. The specific activity of extracellular lipase from *Y. lipolytica* KKP 379 (fraction 60) towards different fatty acids esters of *p*-nitrophenol. Means with the same letter (a–d) did not differ significantly ($\alpha = 0.05$).

3.3. Crude Extracellular Lipase Preparations as Potential Biocatalysts

Various studies have confirmed that the enzymes produced by *Y. lipolytica* yeast can be used as biocatalysts in enzymatic reactions. Both purified and crude extracellular lipases, as well as whole yeast cells, have been applied in, e.g., biodiesel synthesis, ring-opening polymerization, kinetic resolution, and flavor ester synthesis [6,10]. To compare the activity of enzymes obtained from shaken cultures of *Y. lipolytica* KKP 379, the hydrolytic activity of proteins in the model reaction of *p*-nitrophenyl laurate hydrolysis was evaluated. The following fractions of lipases were applied: extracellular lipases present in the crude solution (unpurified supernatant), and intracellular lipases from the yeast biomass both before and after cell disintegration, as well as the extract of intracellular enzymes obtained after cell disintegration. The results of the hydrolytic activity measurements are presented in Table 4.

Table 4. The hydrolytic activity of crude enzyme solution and yeast biomass of *Y. lipolytica.* Means with the same capital letter (A,B) or lowercase letter (a–c) did not differ significantly ($\alpha = 0.05$).

Source of Lipases	Hydrolytic Activity [U/mL]	Hydrolytic Activity [U/g DM]
Crude enzyme solution (supernatant)	0.07 ± 0.09^{B}	-
Yeast biomass before disintegration	-	$146.70^{b} \pm 1.50$
Yeast biomass after disintegration	-	$54.02^{a} \pm 6.32$
Enzyme solution after yeast cell disintegration	0.37 ± 0.08^{A}	-
Immobilized cell-bound lipase preparation	-	$232.00^{c} \pm 5.20$

Intracellular enzymes, namely cell-bound ones and those from the cytosol, showed a very high enzymatic activity of 146.70 U/g (Table 4). As a result of cell disintegration using the glass beads, the cell structure was damaged, and the enzymes were released into the solution, which is reflected in the reduced activity of the yeast biomass after disintegration (54.02 U/g) compared to the initial state. This is also confirmed by the activity of the supernatant obtained after the disintegration process, which also showed a hydrolytic activity of 0.37 U/mL. The preparation obtained by physical adsorption of the supernatant after the disintegration of cells on Lewatit VP OC 1065 resin also exhibited satisfactory

hydrolytic activity, and 232.00 U/g was achieved. Enzymatic kinetics was also evaluated for crude extracellular lipase immobilized on the support (Figure 8). It was revealed that the enzymatic preparation retained 50% of its activity after 60 min of the hydrolysis reaction, in comparison to the activity measured after 15 min (80% after 30 min and 63% after 40 min).

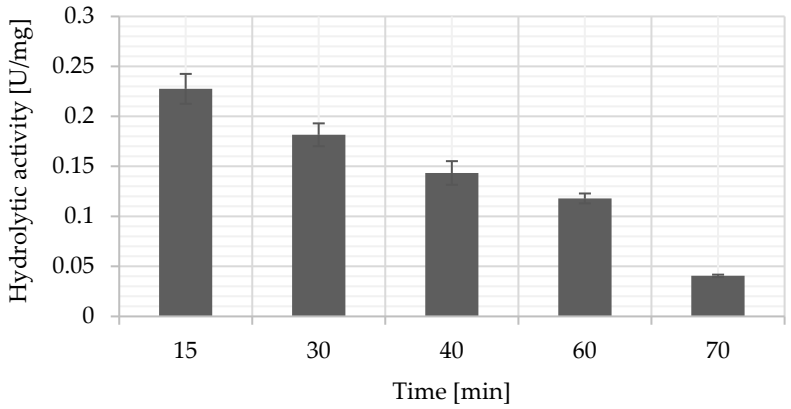

Figure 8. Hydrolytic activity for crude extracellular lipases of *Y. lipolytica* KKP 379 immobilized on Lewatit support, determined by the hydrolysis of *p*-nitrophenyl laureate under optimal reaction conditions, at different times.

4. Discussion

Based on the obtained results, it cannot be denied that the investigated factor, which was plant oil type, did not affect the hydrolytic activity and specific activity of the four different *Y. lipolytica* strains, although there was no significant effect of the carbon source used in the culture media. However, some dependencies and directions for further work on stimulating lipase synthesis in plant oil media were indicated. Within strains of *Y. lipolytica* yeasts, a large variability in physiological abilities is observed, and much research is devoted to the search for special features among strains of this species. Furthermore, metabolite biosynthesis is often dependent not only on specific yeast strains, but also on culture conditions, mainly carbon and nitrogen sources, oxygenation, temperature, or pH [18–21]. This strain-dependent behavior was described by Gottardi et al. [9], who conducted a study in which 20 strains of *Y. lipolytica* of various origins (Po river, dairy products, or refrigerated food) were compared in terms of their lipolytic and proteolytic profiles, as well as their possible solutions for the valorization of untreated cheese whey. Very often, as in the current study, statistical methods are used to assess or select many factors, optimize the biosynthesis of a selected metabolite, or screen strains; e.g., in the optimization of lipase production, using fractional factorial design to evaluate inoculum concentration, temperature, and agitation [20]; or in the optimization of pH, culture time, and glucose concentration using Response Surface Methodology for high-yield citric acid production [21]. In the case of the biosynthesis of extracellular lipases, a lot of research in the scientific literature has been devoted to the use of appropriate carbon sources in the form of lipids, especially those with a high content of oleic acid.

In the present study, the strain *Y. lipolytica* KKP 379 produced a "true" lipase, showing high activity towards oleic acid ester in the model reaction of the hydrolysis of *p*-nitrophenyl esters. The activity of extracellular lipases was higher than for laurate esters, but lower than for butyric acid ester. In this study, hypotheses which assumed that there exists a minimum threshold concentration of free fatty acids present in plant oil (used in culture medium as a sole carbon source), which is essential for initiating the expression of extracellular lipases, were acknowledged. On the other hand, no connection between the production of lipases, the total composition of fatty acids in triacylglycerols, and their concentration in the *sn*-2 positions of triacylglycerols in plant oils was found. Plant oils are certainly good lipase secretion stimulators, but their connection with enzyme activity is not straightforward, and is influenced by other factors.

The assumption that oleic acid serves as an inducer of *LIP2* gene expression has been explored by Fickers et al. [22]. Most authors consider olive oil as an excellent carbon source for stimulating the synthesis of lipases in oleaginous yeast, including *Y. lipolytica*. The stimulatory action of olive oil on microbial lipase synthesis is attributed to the high content of oleic acid in glycerol esters, which is an inducer of the *LIP2* promotor [11]. However, it should be noted that extracellular lipases expressed by different *Y. lipolytica* strains are not equally activated by the presence of olive oil and other plant oils used in the production of microbial lipases [23]. Some plant oils have been shown to stimulate lipase activity of the yeast to a greater extent than olive oil [11,24].

Fatty acids may be largely free and unbound in the triacylglycerols of plant oils. Therefore, yeast cells could absorb free fatty acids without the need for a high production of lipases, as confirmed by Domínguez et al. [23], who observed a lower hydrolytic activity of *Y. lipolytica* CECT 1240 (ATCC 18942) in a medium with oleic acid than in a medium containing olive oil. It can be deduced that there exists a level of free fatty acids that activates lipase synthesis, but exceeding the concentration gives a signal to inhibit enzyme production.

Olive oil and corn oil were recognized as the best inducers of lipase synthesis in *Y. lipolytica* 681 cultures [25]. Likewise, Kebabci and Cihangir [24] evaluated the effect of plant oils (olive, extra virgin olive, canola, corn, sunflower, and soybean oils) on lipase production by three different strains of *Y. lipolytica*. The maximum hydrolytic activity was detected for *Y. lipolytica* NBRC 1658 in the canola oil medium, and *Y. lipolytica* IFO 1195 in the soybean oil medium, while the carbon source did not affect the synthesis of lipases by the local wild-type strain. In turn, for *Y. lipolytica* CECT 1240, a higher lipase activity was achieved in a culture with sunflower oil rather than olive oil [23]. Akpinar and Ucar [25] were not able to find simple relationships between hydrolytic activity and the composition of the lipid substrate present in the medium (plant oil or fish oil) when growing 22 different *Y. lipolytica* yeast strains.

Kamzolova et al. [26] do not confirm the hypothesis that oleic acid is the only or the main stimulator of the synthesis of lipolytic enzymes by the yeast *Y. lipolytica*. The authors prepared cultures in which olive oil, rapeseed oil, and sunflower oil were used as lipid substrates. In the medium containing rapeseed oil, the activity concerning the olive oil was 5% higher, and the highest hydrolytic activity was achieved in the medium containing sunflower oil. The results by Fabiszewska and Białecka-Florjańczyk support the hypothesis that a high oleic acid content in triacylglycerol molecules is not the only or main factor that stimulates the high hydrolytic activity of *Y. lipolytica* yeast, and that there are other factors determining enzyme synthesis, which the authors of the present study did not investigate.

Comparing the results of different authors may prove difficult or even impossible, due to the lack of a standardized method for determining lipase activity, their wide variety, different culture conditions, and the different strains used in other authors' studies. Nevertheless, the results obtained so far show that the extracellular hydrolytic activity of the *Y. lipolytica* species is a result of at least several factors, and it is most likely a mistake to correlate it only with the oleic acid content of the plant oil used as a carbon source in the culture medium.

In addition to the factors mentioned above that make it difficult to compare results between different research centers, another important piece of this puzzle is the appropriate selection of the substrate for the determination of hydrolytic activity, which was also undertaken in the current work. Similarly, Faiz et al. [27] also used two esters of *p*-nitrophenyl, namely laurate and butyrate esters, for the comparison of the activity of esterases synthesized by *Anoxybacillus gonensis* A4. The hydrolytic activity of the esterases for these two substrates was 48 U/L and 347 U/L, respectively. Based on these results, it can be seen that the esterases hydrolyze both of these substrates; however, the activity against *p*-nitrophenyl butyrate was about seven times higher compared to that against *p*-nitrophenyl laurate. The determination of the substrate specificity of esterases towards short-chain fatty acids allowed for the development of a method for the determination

of these enzymes, using *p*-nitrophenyl butyrate and fatty acid esters with a carbon chain length of more than 10 carbon atoms that have been reserved for lipases [27].

Ester bonds are certainly hydrolyzed by both esterases and lipases, but these enzymes differ in the reactions they perform. "True" esterases (EC 3.1.1.1) hydrolyze primarily short-chain esters, and, unlike lipases, the interfacial activation phenomenon is not observed in them. Furthermore, only lipases and cutinases are active in low water/high organic solvent environments [28,29].

Finally, *Y. lipolytica* KKP 379, the strain that showed the most interesting properties in terms of the production of both extra- and intracellular lipases as well as their specificity, and the strain to which the most space was devoted in the current study, can be used as a biocatalyst in esterification reactions, confirming the findings of Zieniuk et al. [30]. In their study, freeze-dried biomass of this yeast species was used in the synthesis of butyl esters of 3-phenylpropanoic acid and its derivatives. Reactions proceeded with 75–98% conversion, but most interestingly, these values were higher compared to the commercial preparation, which was lipase B from *C. antaractica* (CALB). Both enzymes exhibited different substrate specificities, which were also observed for the esterification of phenylacetic acids, and the *Y. lipolytica* biomass, unlike CALB, was not able to proceed with these reactions [30]. In the future, further research should investigate, e.g., the stability of the enzyme, its reusability, or the impact of solvents on immobilized biocatalysts. Mangiagalli et al. investigated CALB-immobilized catalyst aggregation when using short-chain alcohols as solvents. The conformations were only partially prevented by immobilization, and alcohols modified the texture of the solid support, promoting the enzyme release [31].

Notably, enzymatic productivity measures were provided to investigate substrate disappearance over time, at a prescribed temperature under specified reaction conditions. According to Siddiqui et al. [32] and Gomes et al. [33], this is the only measure that reliably summarizes the durability and reaction yield (a measure of substrate conversion) of an enzymatic process. Kinetic productivity analysis was employed to assess the catalytic capacity of the immobilization carrier for affecting the productivity of the crude extracellular enzyme preparation. Our results also revealed that for commercial use, the stability of enzymatic activity should be improved.

5. Conclusions

Issues concerning the activation of microbial lipase synthesis are complex, and the experiments in this study indicated many factors influencing this process. Lipase biosynthesis is also dependent on the strain used. It can be hypothesized that there are other factors that mask the effect of plant oil on extracellular yeast lipase activity. This scientific problem could be solved by experiments involving a simpler system of substrates, e.g., a mixture of fatty acids and triacylglycerols, consisting of three identical acid residues. It is important to emphasize that statistical methods of design of experiment may support the research. Still, their application should be accompanied by a good understanding of the biological process and the factors influencing it. Moreover, yeast strains are also sources of highly active intracellular lipases, and fractions of lipases may be used as biocatalysts in both their immobilized form and as commercial preparations, increasing the range of the applications of this enzyme.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app142311449/s1, Figure S1: SDS-PAGE gel for proteins purified from the culture broth of the yeast *Y. lipolytica* KKP 379. Fractions: 1, 2, 3—10-fold concentrated fractions after purification with an ion-exchanger; 4—fraction after the stage of precipitating the proteins with acetone in the raw supernatant; 5—raw supernatant.

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