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FACTORS INFLUENCING SYNTHESIS OF EXTRACELLULAR LIPASES BY *YARROWIA LIPOLYTICA* IN MEDIUM CONTAINING VEGETABLE OILS

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ABSTRACT

The aim of this study was to evaluate lipase activity of *Yarrowia lipolytica* KKP 379 in media containing selected vegetable oils as the sole carbon source. The highest activity for extracellular *Yarrowia lipolytica* lipases was obtained in a corn oil medium, which was almost two times higher compared to lipase activity in olive oil medium, while the lowest lipolytic activity was observed in medium containing rice and peanut oils. Literature suggested that free fatty acids, especially oleic acid present in olive oil, are good inducers of microbial lipase synthesis, however the results of this study do not support the hypothesis that high oleic acid content stimulates high lipolytic activity. Neither the relationship between the percentage of individual fatty acids in triacylglycerols of vegetable oils nor their content at the *sn*-1,3 positions influenced the activity of extracellular lipases synthesized by *Yarrowia lipolytica* KKP 379. Several hypothesis are given regarding the presence of other factors which may determine differences in extracellular lipolytic activity of yeast grown on different vegetable oils, such as presence of metal ions, the content of free fatty acids and critical micellar concentration.

Keywords: Lipase, lipase activator, oleic acid, triacylglycerol structure, vegetable oil, *Yarrowia lipolytica*

INTRODUCTION

Yarrowia lipolytica is the only species within the genus *Yarrowia*, a commonly-occurring yeast in nature and used in many industries **(Sharma *et al.*, 2001; Brigida *et al.*,2014)**. It is grouped with other non-conventional yeasts which include species such as *Pichia pastoris*, *P. guilliermondii* and *Kluyveromyces lactis* **(Spencer *et al.*, 2002)**. *Y. lipolytica* is a model microorganism used to study lipid metabolism in the cells of oleaginous organisms **(Beopoulos *et al.*, 2009a)**, the genus is also a model microorganism for protein secretion, peroxisome biogenesis, the respiratory chain, dimorphism, and hydrophobic substrate utilization **(Fickers *et al.*, 2005a)**. Industrial interest in *Y. lipolytica* has increased in the last half a century from the 1960s when the yeast were used as a source of SCP (single-cell protein). *Y. lipolytica* is known to have high secretory activity, and among the most desirable metabolites are citric acid, γ-lactones and enzymes (proteases, RNase, phosphatase, esterase and lipases) **(Spencer *et al.*, 2002; Barth and Gaillardin, 1997)**.

The lipolytic activity of *Y. lipolytica* was first described by **Peters and Nelson (1948a,b)**, but the genes encoding for lipase proteins (EC 3.1.1.3, triacylglycerol hydrolase) were discovered recently. There are two fractions of *Y. lipolytica* lipases, extracellular (secreted out of the cell) and intracellular (enzymes located in the cytosol and structures associated with the cell wall). The lipases and esterases are encoded by *LIP* family genes. The *LIP2* gene, which encodes for the proenzyme of Lip2p lipase, is the primary source of extracellular lipolytic activity for *Y. lipolytica* **(Fickers *et al.*, 2005a; Pereira-Meirelles *et al.*, 2000; Fickers *et al.*, 2011)**.

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 (from 55 to 83%) in the glycerol esters of the vegetable oil, and oleic acid is considered to be an inducer of the *LIP*2 promotor **(Barth and Gaillardin, 1997; Wang *et al.*, 2008; Darvishi *et al.*, 2009)**. It should be noted, however, that extracellular lipases expressed by different yeast strains are not equally activated by the presence of olive oil and other vegetable oils are sometimes used in the production of microbial lipases **(Domínguez *et al.*, 2003)**.

The objective of our study was to investigate the effect of various vegetable oils on the synthesis of *Y. lipolytica* extracellular lipases in shaken cultures, along with attempt to identify factor(s) influencing enzyme induction in the presence of lipid carbon sources. Ten different vegetable oils were used as carbon sources. They were chosen according to several criteria. Olive oil was tested as it was the most often used hydrophobic carbon source in microbiological lipase production, as well as conventional oils available in Central and Eastern Europe. Olive oil is not a typical vegetable oil for Central and East European climate zones and is characterized by a relatively high price compared with popular sunflower or rapeseed oils. As the costs of microbial culture medium are 25 to 30% of the total expenditures for enzyme production, this issue is important for the commercial cultivation of yeast for biocatalytic purposes **(Burkert *et al.*, 2004)**. Additionally, some vegetable oils such as sesame oil and canola oil were used in the study to differentiate the range of substrates used in culture medium. The activity of lipases synthesized by *Y. lipolytica*, in addition to analysis of vegetable oil fatty acid composition, allowed to discuss the role of oleic acid in synthesis of extracellular lipolytic proteins.

MATERIAL AND METHODS

Chemicals and culture media

p-nitrophenyl laurate was synthesized in the laboratory **(Vogel *et al.*, 1996)**. Swine pancreatic lipase (100-400 U/mg) and TRIS-HCl buffer (1 M) were purchased from Sigma-Aldrich (Poznań, Poland), bile salts from Fluka (Germany) and other chemicals were purchased from POCH (Gliwice, Poland).

YPG medium (2% glucose, 1% yeast extract, 2% peptone, pH 5.0) and modified YPG medium (2% vegetable oil, 1% yeast extract, 2% peptone, pH 5.0) were used for the cultivation of yeast, and prepared with distilled water. Glucose, peptone and yeast extract were purchased from BTL (Łódź, Poland). Commercially-available vegetable oils (olive, peanut, rapeseed, grape seed, sunflower, canola with red palm, sesame, flaxseed, corn and rice oils) were used as the sole carbon sources in the medium.

Microorganism and culture conditions

Y. lipolytica KKP 379 was purchased from the Collection of Industrial Microorganisms at the prof. Wacław Dąbrowski Institute of Agricultural and Food Biotechnology in Warsaw. *Y. lipolytica* was stored in liquid nitrogen. The inoculum was generated in 100 ml YPG medium and incubated for 24 h at 28 °C in a IKA KS 4000 ic control shaker at 150 rpm. Shaken cultures were carried out in 100 ml of liquid medium with vegetable oil as the sole carbon source, where

0.1% v/v inoculum was added and incubated for 65 h at 28°C with a rotation of 150 rpm. There was chosen the optimal time of incubation for extracellular lipase activity measurement **(Fabiszewska, 2013)**. Yeast biomass was centrifuged in a MPW - 351R centrifuge at 6784 rcf for 10 minutes at 10 °C and dry cell mass measured by the thermogravimetric method at 105°C. Yeast biomass yield was determined on the basis of raw biomass weight and dry cell mass.

Determination of lipase activity

Measures of enzymatic activity were carried out using the spectrophotometric method previously described by **Krzyczkowska *et al*. (2009)** and modified according to **Kapturowska *et al.* (2012)**. The method was based on the hydrolysis of *p*-nitrophenyl laurate, where 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.

Fatty acids composition in vegetable oils

The analysis of fatty acids composition of the triacylglycerols in vegetable oils was carried out after derivatization to methyl esters by the AOCS Ce 1-62 method and evaluation by a gas chromatography system with a FID detector (Agilent Technologies 7890A GC). Separation of fatty-acid methyl esters was accomplished using a Supelcowax 10 capillary column (30 m, 0.32 mm, 0.25 µm) with helium as the carrier gas. Autosampler injection was 1 × 1 ml with a flow distribution of 50:1 and the gradient program was as follows: from 200 °C to 260 °C at a rate of 4 ºC/min, then 260 °C for 10 min. Injector temperature was 250 °C and detector temperature was 260 °C. Identification of fatty acids was based on peak retention times by comparing with the retention time of standard samples.

Structure of triacylglycerols in vegetable oils

In order to determine the composition of fatty acids in the triacylglycerols of vegetable oil, a selective enzymatic hydrolysis was performed according to the method developed by the Department of Chemistry at Warsaw University of Life Sciences **(Bryś, 2005)**. For this purpose, 0.4 g of vegetable oil was added to 8 ml of 1 M Tris-HCl, 0.5 ml 2.2 % calcium chloride solution, 0.2 ml 1 % aqueous solution of bile salts and 200 mg of pancreatic lipase (regioselective lipase specific to the sn-*1,3* bonds in the molecules of triacylglycerols) was added to start the hydrolysis (40 °C, 20 min). The reaction was stopped by addition of 15 ml of ethanol and 5 ml 6 M hydrochloric acid, hydrolysis products were extracted with diethyl ether and dried with magnesium sulfate. An extract was applied to silica gel TLC plates, the plates were washed with a solution of hexane, diethyl ether and acetic acid (50:50:1) and developed with iodine. The 2-monoacylglycerols were extracted with 20 ml of diethyl ether, and after gel filtration, the solvent was removed using a rotary evaporator (Büchi R-200/B-490) and the 2-monoacylglycerols dissolved in 2 ml hexane.
After derivatization to methyl esters according to DIN EN ISO 5509:2000 method, the fatty acid composition was determined using an YL6100 gas chromatograph coupled with a FID detector. Separation of compounds was accomplished on BPX70 column (60 m, 0.25 mm and 0.25 µm) using nitrogen as the carrier gas and a phase flow of 1.2 ml/min. The gradient program was as follows: 70 ° C for 0.5 min; from 70 to 160 °C at a rate of 15 °C/min, from 160 to 200 °C at a rate of 1.1 °C/min, 200 °C for 12 min, from 200 to 225 °C at a rate of 30 °C/min and 225 °C for 1 min. Injector temperature was 225 °C and detector temperature 250 °C. Identification of fatty acids was based on peak retention time by comparing with the retention time of standard samples.

Statistical analysis

Statistical analyses were performed of repeated measurements with one-way ANOVA followed by Tukey's multiple comparison test and analysis of correlation using STATISTICA 10.0 (Statsoft, Poland). P-values of $p \leq 0.05$ were considered to be statistically significant. The Shapiro-Wilk test was used to check if the populations were normally distributed, while Levene's test and the Brown-Forsythe test were used to assess the equality of variances for a variable calculated for groups. All experiments were repeated 5 times.

RESULTS

Y. lipolytica KKP 379 were incubated (shaken cultures) in media containing vegetable oil as the sole carbon source (peanut, rice, sesame, flaxseed, grape seed, sunflower, rapeseed, canola with red palm, olive and corn oil) and assayed after 65 h for biomass yield and extracellular lipase activity (Fig. 1 and 2). The highest average biomass yield (16.6 g d.m./dm^3) (d.m. – dry mass) was obtained in medium where peanut oil was used as the sole carbon source (Fig. 1). Significantly lower biomass yields were achieved in flaxseed and corn oil media, at 10.5 and 13.1 g d.m./dm^3, respectively. For the remaining seven oils, biomass yield did not differ significantly from that in peanut oil medium.

Greater variability was seen for extracellular lipase activity (Fig. 2). The highest average lipolytic activity was found in the supernatant from the corn oil medium (0.362 U/cm^3) and lower activity was seen in canola and red palm oil medium (0.280 U/cm^3), though this difference was not statistically significant from corn oil. The lowest activity was seen for peanut and rice oil media (0.068 and 0.089 U/cm^3 respectively). The activity was 2-fold higher when flaxseed, sesame and grape seed oil media (0.137, 0.151 and 0.151 U/cm^3, respectively) were used, though with respect to the latter two oils, the differences were not statistically significant. These five oils were classified into the same homogeneous group.

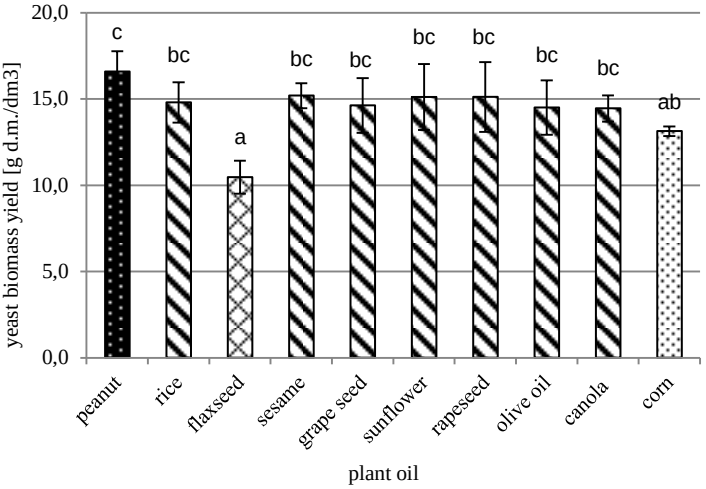

Figure 1 Influence of vegetable oil on *Y. lipolytica* KKP 379 biomass yield after 65 h of shaking culture. Homogeneous groups designated on the basis of Tukey's test were identified by letters and different posts. Means were separated into statistically different groups (a, ab, bc and c).

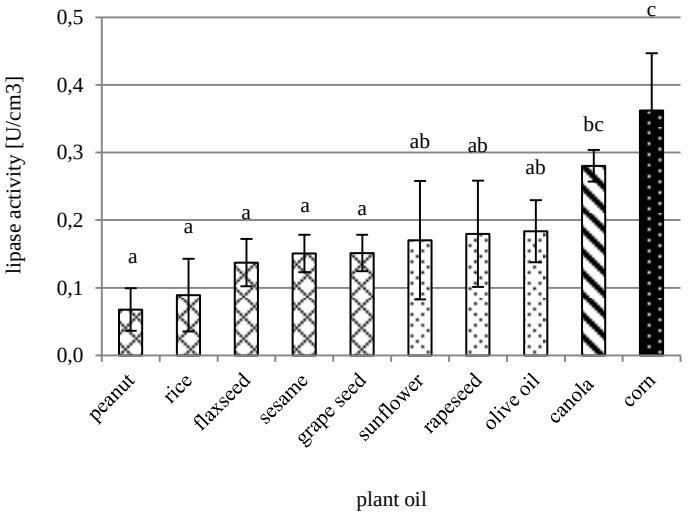

Figure 2 Influence of the vegetable oil on extracellular *Y. lipolytica* KKP 379 lipase activity after 65 h of shaking culture. Homogeneous groups designated on the basis of Tukey's test were identified by letters and different posts. Means were separated into statistically different groups (a, ab, bc and c).

Intention of the authors was to correlate lipolytic activity with fatty acid composition for each oil used in the study. All oils had a high content of unsaturated fatty acids in the triacylglycerol molecules (Codex Alimentarius), where the differences in physicochemical and sensory properties of these oils are due to fatty acid composition. Fatty acid composition was determined for each oil (Table 1), which coincided with the characteristics in the **Codex Alimentarius** and papers by **Przybylski *et al*. (2005a,b)**. The highest oleic acid content (C 18:1) was found in olive oil (over 70% of the fatty acid content) and the highest palmitic acid content was measured in canola (12.53%) and rice oils (15.23%) (Table 1). Grape seed, sunflower (extracted from sunflower seeds) and corn oils (derived from maize embryos) were characterized by a high percentage of linoleic acid (C 18:2), which accounted for greater than 50 % of all fatty acids in the oils. Flaxseed oil was characterized by a high content (52.65 %) of polyunsaturated linolenic acid (C 18:3), also found in rapeseed (8.08 %) and canola oils (6.01 %). Peanut oil was characterized by a relatively high content of behenic acid (C 22:0, 2.62 %) and lignoceric acid (C 24:0, 1.39 %), while rice oil had a high palmitoleic acid content (C 16:1, 4.06 %).

Table 1 Fatty acid composition of vegetable oils [% total fatty acid content in oil]

Fatty acids symbol	Fatty acids name	Peanut oil	Rice oil	Flaxseed oil	Sesame oil	Grape seed oil	Sunflower oil	Rapeseed oil	Olive oil	Canola and red palm oil	Corn oil
C14:0	Myristic acid	0.03	0.26	0	0.02	0.07	0.07	0.05	0	0.29	0.03
C16:0	Palmitic acid	9.47	15.23	5.87	9.32	8.06	6.62	4.05	10.27	12.53	11.06
C16:1	Palmitooleic acid	0.13	4.06	0.07	0.15	0.15	0.13	0.24	0.81	0.25	0.13
C17:0	Margaric acid	0.07	0.04	0.06	0.04	0.05	0.04	0.05	0.07	0.06	0.06
C17:1	Margarooleic acid	0.05	0.02	0	0	0.04	0.03	0.09	0.13	0.06	0.03
C18:0	Stearic acid	3.53	2.06	4.35	5.77	3.8	3.27	1.93	3.15	2.37	1.78
C18:1	Oleic acid	59.26	44.93	20.38	40.05	25.39	30.4	64.47	77.86	57.35	28.38
C18:2	Linoleic acid	19.96	30.26	15.85	43.19	60.45	58.25	18.47	6.17	18.66	56.31
C18:3	Linolenic acid	0.5	0.93	52.65	0	0.85	0.05	8.08	0.63	6.01	0.8
C20:0	Arachidic acid	1.47	0.87	0.16	0.62	0.27	0.24	0.58	0.41	0.53	0.41
C20:1	-	1.15	0.53	0.10	0.19	0.19	0.16	1.26	0.29	0.98	0.28
C22:0	Behenic acid	2.62	0.26	0.16	0.13	0.41	0.78	0.3	0.11	0.25	0.12
C22:1	Erucic acid	0.21	0.06	0	0	0.05	0.04	0.07	0	0	0
C24:0	Lignoceric acid	1.39	0.42	0.12	0.08	0.16	0.24	0.16	0.05	0.13	0.17
C24:1	Nervonic acid	0	0	0	0	0	0	0.12	0	0.12	0

Table 2 Correlation ratios for the relationship between *Y. lipolytica* KKP 379 biomass yield, extracellular lipolytic activity and individual fatty acid content of triacylglycerols in vegetable oils. Coefficients with an asterisks were statistically significant.

Correlated feature, a content of fatty acid in oil	Extracellular lipolytic activity	Biomass yield
According to all positions (*sn*-1, *sn*-2 and *sn*-3) for all tested oils		
C14:0	0.079940	0.173209
C16:0	0.115897	0.173501
C16:1	-0.341705	0.110367
C17:0	0.178193	-0.143933
C17:1	0.136691	0.324232
C18:0	-0.428441	-0.065694
C18:1	-0.091586	0.528368
C18:2	0.243609	0.090760
C18:3	-0.116265	-0.842646*
C20:0	-0.450066	0.648235*
C20:1	-0.085839	0.502861
C22:0	-0.478569	0.539497
C22:1	-0.583727	0.601967
C24:0	-0.502063	0.500337
C24:1	0.320873	0.125692
Sum of saturated fatty acids	-0.236866	0.371906
Sum of unsaturated fatty acids	0.202387	-0.340217
According to *sn*-1 and *sn*-3 positions for five selected oils (corn oil, canola and red palm oil, olive oil, rice oil and peanut oil)		
C16:0	-0.100567	-0.241602
C18:0	-0.751072	0.864908
C18:1	-0.492777	0.596541
C18:2	0.622278	-0.692906
Sum of unsaturated fatty acids	0.758170	-0.520597

Legend: *statistically significant at 95 % at confidence level

The concentrations of individual fatty acids in vegetable oils were correlated with *Y. lipolytica* KKP 379 biomass yield and extracellular lipolytic activity. Literature reports oleic acid (C18: 1) and linoleic acid (C18: 2) as stimulators of lipase microbial activity (**Fickers *et al*., 2005a; Wang *et al*., 2008; Fickers *et al*., 2005b)**, therefore, a high oleic acid concentration was expected to affect synthesis and activity of yeast lipases. Contrary to expectations, there was no significant correlation between these two dependent variables and the percentage of particular fatty acids in triacylglycerols or the percentage of saturated and unsaturated fatty acids in triacylglycerols (Table 2). The content of oleic acid did not explain the highest extracellular lipase activity in the corn oil medium (28.38%), while in the olive oil medium, lipolytic activity was significantly reduced, even though olive oil contained the highest amount of C18:1 acid among all the oils (77.86%). Extracellular lipolytic activity was lowest in the presence of rice and peanut oils, which were characterized by a two-fold higher content of oleic acid (44.93 and 59.26%, respectively) in comparison to corn oil. No correlation was seen between linoleic acid content and *Y. lipolytica* lipase

activity (Table 2). The average amount of linoleic acid in the corn oil was 56.31% of the total fatty acid content, while grape seed oil (60.45%) and sunflower oil (58.25%) were characterized by a higher concentration of this compound, and olive oil at 6.17% (C18:2 acid).

Y. lipolytica synthesizes *sn*-1,3-regioselective extracellular lipase Lip2 **(Fickers *et al*., 2011; Hadebal, 1991)**, and the main products of lipase-catalysed triglyceride hydrolysis were 2-monoacylglycerols and free fatty acids. The hypothesis was that high lipolytic activity would be associated with the content and type of fatty acids present at the *sn*-1 and *sn*-3 positions in the triacylglycerols. The existence of such a relationship was evaluated by examining the structure of the five selected vegetable oils (Table 3) which had the greatest impact on lipase activity: peanut and rice oil (characterized as weak inducers of lipolytic activity) as well as corn, olive, canola with red palm oil. A detailed analysis of the triacylglycerol structure yielded fatty acid contents at the external positions of glycerol esters (*sn*-1,3). Table 3 shows which fatty acids were dominant in the selected vegetable oils and their content (greater than 1.5%).

However according to the data in Table 3 no correlations were found between lipolytic activity and vegetable oil parameters (Table 3). Regardless, a detailed description of all oils is given (Table 4), which confirmed that while the oils differed significantly in chemical composition, there was no direct relationship between extracellular lipase activity, total content of unsaturated fatty acids in the triacylglycerol molecule or their concentration at the *sn*-1,3 or *sn*-2 positions (Table 3 and 4). Moreover, the percentage of unsaturated fatty acids at the *sn*-1,3 positions of triacylglycerols in peanut and rice oils (50.3 and 45.5 %, respectively) were lower compared to olive, canola and the corn oils (54.8, 52.5 and 55.9 %, respectively), but not sufficiently significant to play an important role in the induction of lipase expression in yeast cells.

Table 3 The structure of triacylglycerols in individual vegetable oils.

Fatty acid	Summary content of particular fatty acid in oil [%]	Content of a particular fatty acid in *sn*-1,3 or *sn*-2 position [%]		Portion of a particular fatty acid in *sn*-1,3 positions [%] ***
		sn-2*	*sn*-1,3**	
Peanut oil (lipase activity 0.068 U/cm^3)				
C 16:0	9.47	10.4	9.0	6.0
C 18:0	3.53	3.0	3.8	2.5
C 18:1	59.26	42.7	67.5	45.0
C 18:2	19.96	43.9	8.0	5.3
Rice oil (lipase activity 0.089 U/cm^3)				
C 16:0	15.23	9.0	18.3	12.2
C 18:0	2.06	1.8	2.2	1.5
C 18:1	44.93	53.5	40.6	27.1
C 18:2	30.26	35.6	27.6	18.4
Olive oil (lipase activity 0.184 U/cm^3)				
C 16:0	10.27	9.4	10.7	7.1
C 18:0	3.15	3.0	3.2	2.2
C 18:1	77.86	71.0	81.3	54.2
C 18:2	6.17	16.6	1.0	0.6
Canola and red palm oil (lipase activity 0.280 U/cm^3)				
C 16:0	12.53	9.0	14.3	9.5
C 18:0	2.37	2.2	2.5	1.6
C 18:1	57.35	57.0	57.5	38.4
C 18:2	18.66	26.6	14.7	9.8
C 18:3	6.01	5.2	6.4	4.3
Corn oil (lipase activity 0.362 U/cm^3)				
C 16:0	11.06	10.3	11.4	7.6
C 18:0	1.78	3.3	1.0	0.7
C 18:1	28.38	42.7	21.2	14.2
C 18:2	56.31	43.7	62.6	41.7

Legend: * Content of a particular fatty acid based upon the content of all fatty acids in the sn-2 position of the triacylglycerol; ** Content of a particular fatty acid based upon the content of all fatty acids in the sn-1,3 positions of the triacylglycerol; *** Portion of a particular fatty acid at the sn-1,3 positions based upon the content of individual fatty acids in oil minimized by the content of fatty acid in sn-2 position

Table 4 Characteristics of vegetable oils used in media for the cultivation of *Y. lipolytica*. Oils are ranked according to increasing lipolytic activity, and the letters denote statistical differences in lipase activity seen in Figure 2

Vegetable oil	Content of saturated fatty acids in oil [%]	Content of unsaturated fatty acids in oil [%]	Unsaturated and saturated fatty acids ratio	Content of unsaturated fatty acids in oil in *sn*-1,3 position [%]
Peanut oil [a]	18.7	81.3	4.3 : 1	50.3
Rice oil [a]	19.2	80.8	4.2 : 1	45.5
Olive oil [ab]	14.1	85.9	6.1 : 1	54.8
Canola and red palm oil [bc]	16.5	83.5	5.1 : 1	52.5
Corn oil [c]	14.1	85.9	6.1 : 1	55.9

DISCUSSION

Y. lipolytica has been widely investigated for lipase activity in media containing various carbon sources, as yeast can efficiently degrade and oxidise many hydrophobic substrates such as fats, oils, alkanes and fatty acids **(Fickers *et al*., 2005a)**. It is well known that the presence of glucose inhibits extracellular lipase activity, and supplementation with a stimulator is necessary to induce synthesis of the enzyme (**Fickers *et al*., 2003, 2005a,b)**. It should be mentioned that glucose demonstrated a repressive mechanism also on *Y. lipolytica* KKP 379 lipase production **(Fabiszewska *et al*., 2014)**, so the addition of hydrophobic carbon source to the YP medium was essential. Lipid carbon sources are convenient substrates for lipase production, and one of the best lipase inducers, olive oil, has a high oleic acid content **(Fickers *et al*., 2005a)**; some other plant oils were investigated as well **(Darvishi *et al*., 2009; Domínguez *et al*., 2003).**

A thorough review of the current state of art in the field of inducers of lipase synthesis and an attempt to suggest possible interpretations in view of our results will be presented in course of discussion. As the correlation between lipase activity and fatty acid content in vegetable oil used as carbon source failed, some new hypothesis has been formulated why *Y. lipolytica* yeast produce lipases with various activity in medium with different vegetable oils.

Fatty acids in microbial lipases synthesis

As it was described above olive oil is considered an extracellular lipase activator due to the 55-83% content of oleic acid, which is an inducer of the *LIP2* gene promotor **(Fickers *et al*., 2005a)**. The existence of two pathways regulating *LIP2* gene expression was suggested **(Fickers *et al*., 2011)**. One involves β-oxidation and is related to *POX2* gene regulation, which is sensitive to the presence of fatty acids **(Gurvitz *et al*., 2006)**, while the second is regulated *SOA*-related genes and associated with triacylglycerol metabolism **(Desfougères *et al.* 2009)**.

Fickers *et al.* (2004) observed the highest *LIP2* gene activity in a modified strain of *Y. lipolytica* (LgX64.81) in the presence of 0.5 % oleic acid, but failed to confirm a direct correlation between the level of *LIP2* gene expression and lipase synthesis. However, it was suggested that lipase synthesis could be regulated at the level of secretion and therefore, the higher the extracellular lipase activity, the more enzymatic proteins were transported from the interior of the cell into the environment **(Fickers *et al.*, 2004)**. Oleic acid present in olive oil was considered the best inducer of lipolytic activity in *R. chinensis* CCTCC M201021 **(Wang *et al*., 2008)**, and **Hsu *et al.* (2008)** reported that olive oil and oleic acid induced translation of genes encoding lipolytic enzymes in *C. rugosa*.

These observations are contradicted in the present study and also by other authors. For example **Dalmau *et al.* (2000)**, observed maximum *C. rugosa* ATCC 14830 extracellular lipase activity in a medium containing palmitic acid as the sole carbon source. The highest activity of cell-bound lipase was achieved with a triolein substrate, while oleic acid stimulated lipase synthesis, but to a much lesser extent than other lipid carbon sources **(Dalmau *et al.*, 2000)**. *C. rugosa* ATCC 14830 was also used by **Wei *et al.* (2004)** to study the stimulatory effect of selected fatty acids on lipase synthesis, and the highest lipase activity was seen in oleic acid-containing medium. Of note, no lipase synthesis was observed when myristic acid or dodecanoic acid were used. Enzyme activity was also affected by fatty acid carbon chain length (greater number of carbon atoms in the chain results in higher specific activity of yeast enzymes) and the presence of unsaturated bonds in the molecule (higher activity observed in medium containing oleic acid than with stearic acid) **(Wei *et al.*, 2004)**.
Interestingly, **Corzo and Revah (1999)** reported oleic acid (12 g/dm^3) as a lipase inhibitor for *Y. lipolytica* 681, while the presence of oleic acid exceeding 2 g/ dm^3 in the culture medium resulted in a significant reduction in the level of lipase expression for *C. rugosa* **(Gordillo *et al.*, 1995)**. Oleic acid was the final product of triacylglycerol hydrolysis and a reduction in lipolytic activity due to product inhibition may have occurred.
Some authors postulate that extensive absorption of oleic acid inside cells of *C. rugosa* coincides with intensive secretion of extracellular lipases. In turn, a small amount of lipase is essential for the hydrolysis of triacylglycerols to glycerol and free fatty acids **(Montesinos *et al.*, 1996).** This hypothesis complements the conclusions of **Pereira-Meirelles *et al.* (2000)** reporting high extracellular *Y. lipolytica* lipase activity was associated with the lipid carbon source depletion in the medium.
Although, the authors of the present study did not use oleic acid as an individual carbon source in medium, they have proved that high content of this fatty acid in vegetable oil did not correspond with high extracellular lipase activity. It is possible that for *Y. lipolytica* KKP 379 strain oleic acid (as the final product of triacylglycerol hydrolysis of olive oil) could even act as an inhibitor in lipase synthesis as it suggested **Corzo and Revah (1999)**.

Vegetable oils in microbial production of lipases

Vegetable oils have been used in the microbial production of lipases, and hydrophobic carbon sources are suitable substrates activating the synthesis of lipolytic enzymes in yeast and filamentous fungi **(Wang *et al.*, 2008; Darvishi *et al.*, 2009)**. It is noteworthy that diverse strains of *Y. lipolytica* have different preferences for the lipid substrate used in the culture medium **(Guerzoni *et al.*, 2001)** depending on different metabolic activity. Thus, for the purposes of the study a wild-type strain *Y. lipolytica* KKP 379 was used to determine the different extracellular lipase activity in medium with vegetable oils. It should be also mentioned that so far there is no single universal method for determining the activity of triacylglycerol hydrolases, so it was impossible to compare the results taking the values of lipolytic activity into account, but we were able to compare the final effect observed in each study.
According to **Domínguez *et al.* (2003)**, biomass yield of *Y. lipolytica* CECT 1240 (ATCC 18942) culture in medium supplemented with glucose, oleic acid, olive oil or sunflower oil was independent of the presence of lipid carbon sources, but the hydrophobic carbon source determined the final activity of extracellular lipases, indicating some vegetable oils can stimulate lipase activity to a greater extent than olive oil. For *Y. lipolytica* CECT 1240, higher lipase activity was achieved in culture with sunflower oil rather than olive oil **(Domínguez *et al.*, 2003)**, while olive oil and corn oil were considered the best inducers of lipase synthesis in *Y. lipolytica* 681. The use of vegetable oils as the sole carbon source in the medium resulted in satisfactory yeast biomass yields compared to medium containing glucose **(Beopoulos *et al.*, 2009a)**. **Corzo and Revah (1999)** investigated the substrate specificity of *Y. lipolytica* 681 lipases and the enzyme had the same activity when hydrolysing olive oil, tributyrin and tricaprilin substrates. Significantly lower lipase activity was achieved when triolein was used, and the lowest activity with corn oil **(Corzo and Revah, 1999)**. **Kebabci and Cihangir (2012)** evaluated the effect of vegetable oils (olive, extra-virgin olive, canola, corn, sunflower and soybean oils) as the sole carbon source on lipase production by three different strains of *Y. lipolytica* (NBRC 1658, IFO 1195 and a local wild-type strain). Maximum lipolytic activity was seen for *Y. lipolytica* NBRC 1658 in canola oil medium and *Y. lipolytica* IFO 1195 in soybean oil medium, while the carbon source had no effect on the synthesis of lipases by the local wild-type strain. No significant differences in growth were seen in media containing vegetable oils, though various yeast strains did have a preference with respect to the vegetable oil, which can stimulate lipolytic activity **(Kebabci and Cihangir, 2012)**.
Our results on the lipase-activating role of olive oil are similar to those obtained by some other authors. They do not support the thesis that olive oil is the best lipase inducer. Moreover, the few results presented by other authors claimed that corn oil could be a better lipase activator in culture medium. Unfortunately, none of authors had explained any reason of the observation. In our study we have managed to show that the high lipase activity may not be correlated with the fatty acid content in vegetable oil used as carbon source in culture medium.

Lipid metabolism in *Y. lipolytica* cells

Papanikolaou *et al.* (2001) demonstrated that during the growth of yeast cells in media containing a lipid carbon source, synthesis, modification of the lipid composition and the accumulation of intracellular lipid fractions occurs. Lipids, which are accumulated in the form of lipid bodies inside the yeast cell, may impede the absorbance of free fatty acids from the environment. When considerable amounts of triglycerides are stored in the cell, lipids inside the cell are utilized by the microorganism, which is energetically favourable compared to the transport of substrate from the external environment.
Y. lipolytica cells can accumulate fatty acids in the form of lipid bodies by *de novo* synthesis from fatty acids precursors such as acetyl-CoA and malonyl-CoA, or by an *ex novo* mechanism from fatty acids present in the culture medium **(Beopoulos *et al.*, 2009a,b)**. **Papanikolaou *et al.* (2002, 2003)** suggested that *de novo* synthesis may also occur from free fatty acids in the yeast cell. These studies suggest the activation of lipase expression may be related to the accumulation of specific fatty acids inside yeast cells, whereas *de novo* synthesis of lipid compounds is insufficient. Hence, the yeast cells increase lipase synthesis to obtain these compounds from the environment **(Beopoulos *et al.*, 2009a,b; Papanikolaou *et al.*, 2002)**.

Stereoselectivity of *sn*-1,3 lipases

A review of literature addressing the substrate specificity of *Y. lipolytica* lipases with respect to the fatty acids in outer positions (*sn*-1,3) of the triacylglycerol molecules indicated that lipases show little tendency toward stereospecific hydrolysis of ester bonds differentiating between the *sn*-1 or *sn*-3 position. According to **Aloulou *et al.* (2007)**, the Lip2p enzyme synthesized by *Y. lipolytica* showed a slight stereospecificity towards the *sn*-3 position of glycerol trioleate. Further research could also evaluate the stereospecificity of the lipases produced by *Y. lipolytica* KKP 379 and the triacylglycerol structure of vegetable oils, where these two positions are compared (treated as equal in this study). This may serve to explain the effect of vegetable oil type in the culture medium on *Y. lipolytica* lipolytic activity. Furthermore, despite a symmetrical arrangement of the fatty acids at *sn*-1 and *sn*-3 positions, some important differences in the acid content at the outer positions were shown *inter alia* for corn oil **(Brockerhoff and Yurkowski, 1966)**.
There are two possibilities for desymmetrization of the molecule, the enantiospecific hydrolysis of one enantiomer in a racemic mixture or discrimination between two ester groups in a prochiral molecule containing two identical acyl groups at the *sn*-1 and *sn*-3 positions. The latter effect is well known for different lipases, for example, *Candida antarctica* B lipase, which catalyses the desymmetrization of 3-alkylglutaric acid diesters **(Jung *et al.*, 2013)**. The highest enantiomeric excess was achieved in the case of allyl esters owing to interactions between the olefin bond and the hydrophobic amino acids residues at the enzyme active site. The olefin drives the favourable conformation change in the first tetrahedral intermediate, called the "olefin effect". The "olefin effect" between the substrate and enzyme is the critical factor increasing the enantioselectivity of *C. antarctica* B lipase-catalysed desymmetrization of prochiral 3-alkylglutaric acid diallylesters **(Jung *et al.*, 2013)**.
In the case of triacylglycerols, this problem was investigated using trioctanoin and triolein as model compounds. Hydrolysis reactions were carried out in the presence of 25 different lipases, where 17 catalysed the reaction at the *sn*-1 position for triolein, and 12 at the *sn*-1 position of trioctanoin. The enantiomeric excess varied from 100% (for bacterial *lipase* from *Pseudomonas aeruginosa* and *Pseudomonas* sp. for *sn*-1 and *Candida antarctica* A *lipase* for *sn*-3) to 3 %, and was lower with triolein than with trioctanoin **(Rogalska *et al.*, 2000)**.

Enzyme-surfactant interactions and uptake of fatty acid into the yeast cell

The first stage of fatty acid assimilation is contact between the molecule and the yeast cell surface. There are two hypotheses concerning the mechanisms, one of which involves the direct adhesion of the acid molecules to a special cell structures activated by oleic acid or other fatty acids. Several modifications in cell structure were identified, likely related to hydrophobic substrate transport, e.g., protrusions at the cell surface, decreased thickness of the cell wall and periplasmic space as well as membrane invaginations. Many findings support the second hypothesis that surfactants such as liposan, synthesized directly by the yeast *Y. lipolytica* can facilitate uptake of fatty acids due to their emulsifying properties **(Fickers *et al.*, 2005a)**. The differences between lipolytic enzyme activity for yeast cells cultivated in medium with different vegetable oils may be related to the activation of the surfactant synthesis. **Reis *et al.* (2009)** reported that lipases are activated by binding to an insoluble emulsified substrate, and the rate of triglyceride lipolysis depends strongly on the specific area of the emulsion drops. Therefore, enzyme-surfactant interactions have an important impact on the regulation of lipase catalysis and the so called "quality of interface" **(Reis *et al.*, 2009)**. Moreover, a self-regulatory system was discovered for *sn*-1,3-regioselective pancreatic lipase. Released after triacylglycerol hydrolysis, amphiphilic *sn*-2 monoacylglycerols quickly fill the interface between the hydrophilic and hydrophobic phases, displacing free fatty acids, glycerol,

triacylglycerols compounds and even lipase molecules. Therefore, the decrease in substrate concentration (triglyceride) and the increase in product concentrations cause a reduction in the number of enzyme molecules present at the interface **(Reis *et al.*, 2009)**. Due to the existence of many similarities between the properties of pancreatic lipase and the lipolytic enzymes of *Y. lipolytica* **(Najjar *et al.*, 2011)**, a similar reaction mechanism is expected for both.

Composition of vegetable oils and its correlation with *Y. lipolytica* lipase activity

Vegetable oils are multi-component mixtures, in which triacylglycerols constitute the largest part (over 90%). Oils also contain free fatty acids, mono- and diacylglycerols, phospholipids, metal ions, peroxides, waxes, sterols, chlorophylls, carotenoids, tocopherols and phenolic compounds **(Choe *et al.*, 2006)**. An important parameter characterizing vegetable oils is the content of free fatty acids, which is measured by the acidic number (number of 0.1 N KOH mg which neutralizes 1 g of oil). Perhaps, the initial concentration of free fatty acids determined the level of lipase synthesised by *Yarrowia*, which was supported by the work of **Kohlwein and Paltauf (1984)**, suggesting the existence of at least two membrane transporter systems in *Y. lipolytica* (formerly *Saccharomycopsis lipolytica*) for fatty acids with chain lengths of 12 to 14 and 16 to 18 carbon atoms. The transport of these compounds can be determined by the concentration of fatty acids in the culture medium, and a high content of free fatty acids may have a negative effect on the synthesis of lipases.

Lipolysis of triacylglycerides generates highly interfacial active molecules that can compete with the enzyme at the interface and/or modify the protein activity via molecular interactions **(Reis *et al.*, 2009)**. Amphiphilic molecules (including the fatty acids, mono- or diacylglycerols) can form three-dimensional structures called micelles. In aqueous solutions containing low concentrations of the amphiphilic componds, the molecules are single and gradually adsorbed at the interface, and after saturation of the interface, the concentration of amphiphilic products increases and micelles begin to form. The parameter, which describes the concentration of the amphiphilic compound in solution, above which it exists in an aggregated form, is the critical micelle concentration (CMC). Lipases are enzymes which catalyse a reaction at the interface, and the optimal concentration of enzyme molecules and triacylglycerols at the interface should be maintained. Furthermore, lipase access to substrate molecules is affected by micelle formation and limited availability of the triacylglycerol substrate to the enzyme active site **(Guerzoni *et al.*, 2001)**. Not to mention the fact that products of triglyceride lipolysis change the pH of the aqueous phase, which can strongly affect the protein-ionic surfactant and protein-substrate interactions **(Reis *et al.*, 2009)**. pH also had a strong influence on the active conformation of the lipase catalytic site due to the impact on amino acid protonation of the enzyme molecule.

Sterols, besides triacylglycerols, are lipid compounds stored in yeast cells in the form of lipid bodies, and are a very important cell building blocks involved in the biosynthesis and selective permeability of cell membranes as well as the energetic processes of the cell. On the other hand, free sterols and fatty acids accumulating in yeast cells can cause toxic effects under certain conditions, and maintaining a balance between the biosynthesis and degradation of sterols and triacylglycerols determines cell homeostasis. These processes depend on a proper regulation of *inter alia* hydrolytic enzymes, including triacylglycerol hydrolases **(Grillitsch and Daum, 2011)**. However, analysis of the sterol content in vegetable oils **(Codex Alimentarius, 2013)** shows that sterols cannot be inducers of lipase activity, in the light of the results presented in this paper. The total content of sterols in corn oil was as low as 22 g/ dm^3 of oil (including a major fraction of β-sitosterol), peanut oil contains only 3 g/ dm^3, while the highest content was found in rice oil (10 to 31 g/dm^3). These data suggested there was no correlation between the sterol concentration in vegetable oil and the extracellular lipase activity of *Y. lipolytica* KKP 379, keeping in mind the highest activity was achieved in a medium containing corn oil and the lowest when peanut oil and rice oil were used.

Vegetable oils may contain trace amounts of metals such as iron and copper. According to the **Codex Alimentarius (2013)**, refined oils may contain up to 1.5 mg Fe/kg and 0.1 mg Cu, and 5.0 mg Fe/kg and 0.4 mg Cu for virgin oils **(Kohlwein and Paltauf, 1984)**. *Y. lipolytica* lipase activity is positively influenced by the presence of these ions (Ca^{2+}, Mg^{2+}), while others (Zn^{2+}, Cu^{2+}, Ni^{2+} or Hg^{2+}) inhibited lipase activity **(Sharma *et al.*, 2001, Pignede *et al.*, 2000; Zhao *et al.*, 2011)**. The content of these ions in the oils used in this study may be an important factor, which determined whether an individual vegetable oil stimulated microbial lipase production (or not).

CONCLUSIONS

Vegetable oils are valuable sources of carbon and good inducers of *Y. lipolytica* lipolytic activity. Data from other authors' reports as well as our results suggested that oleic acid was not the only factor determining the high lipolytic activity in medium containing vegetable oil. No relationship was seen between the activity of extracellular lipases synthesized by *Y. lipolytica* KKP 379 and the percentage of individual fatty acids in the triacylglycerols of the vegetable oils, or their content at positions *sn*-1,3 or *sn*-2 in triglyceride molecule. Yet to be elucidated which factors were responsible for the differences in lipolytic enzyme activity of *Y. lipolytica* cultured in media with various vegetable oils.

Authors proposed several hypothesis, which were shortly described and discussed, but they need further investigations in the field of microbiology, food analysis, biotechnology and biophysics. Further work is required to verify the relationship between intracellular lipid storage and lipase synthesis, the effect of vegetable oils on the synthesis of surfactants and the impact of metal ions in oils on lipase production. The threshold levels of fatty acids, which are essential to initiating gene expression for lipolytic enzymes, and the concentration of free fatty acids, which affects the formation of micelles characterized by the critical micelle concentration (CMC), should be investigated. The possibility of the synthesis of other (than *LIP2*) lipases, which can be induced by different vegetable oils should be checked as well.

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