

EFFECT OF AVOCADO OIL ON THE INDUCTION OF YEAST HYDROLASES

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Summary. Hydrolases catalyzing on the ester bonds, commonly known as lipases (EC 3.1.1) are one of the most important groups of enzymes with significant industrial importance. They are produced by plants, animals and microorganisms, but microbial lipases enjoying the greatest interest. The aim of the study was to assess the effect of avocado oil used as a hydrophobic substrate in *Yarrowia lipolytica* W29 yeast culture media on the synthesis of extracellular lipases. The study compares the metabolic activity of yeast on a medium with avocado oil and mediums with of various carbon sources (olive oil, glucose). The obtained results indicate that the lipolytic activity of yeast *Yarrowia lipolytica* W29 can be induced by the presence of avocado oil in the medium. A lower concentration of oleic acid with a higher concentration of palmitic acid in avocado oil, however, in relation to olive oil results in about 2.5 times lower yeast activity – at the level of about 0.047 ± 0.02 U/cm³.

Key words: hydrolases, lipases, avocado oil, lipolytic activity, *Yarrowia lipolytica*

INTRODUCTION

Hydrolases of glycerol esters (EC3.1.1.3) commonly referred to as lipases are defined as a group of enzymes which natural function is the hydrolysis of triacylglycerol to free fatty acids, mono-, diacylglycerol and glycerol. Hydrolases constitute over 75% of the total production of biocatalysts. Yeasts are the rich source of hydrolytic enzymes [Sharma et al. 2001, Houde et al. 2004].

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The production of enzymes by these microorganisms is of particular interest to the food, cosmetic and pharmaceutical industries, where the use of non-pathogenic, taxonomically stable, classified as GRAS (Generally Recognized As Safe), microorganisms is required. In the food industry lipases are widely used in lipid modification, in synthesis of structured fats, but also in the synthesis of aroma and flavour compounds, including low molecular weight esters formed as pure compounds in situ or synthesized from the proper chemical compounds by biotransformation [Schmid and Verger 1998, Villeneuve 2007].

The condition for efficient production of lipases by microorganisms is the creation of optimal conditions for their cultivation. The basic assumptions in the biosynthesis of these enzymes is the selection of an appropriate carbon and nitrogen source and their appropriate quantity in the culture medium, as well as optimal temperature, proper acidity and adequate concentration of dissolved oxygen [Elibol and Ozer 2000].

An important element in obtaining effective lipase production by microorganisms is the presence of a lipid carbon source in the culture medium. Depending on the species, type and even strain of the microorganisms, different lipid substances are preferred. Among the most commonly used by scientists, plant-based carbon sources there is a mention of olive oil or sunflower oil, less frequently rapeseed, soy, sesame or corn oil. Lipase inducers, used in microbial cultures, are also tripalmitin, trimyristin, tristearin and surfactants, for example Tween 20, Tween 80 or Triton X-100 [Benjamin and Pandey 1996, Sharma et al. 2001].

Due to the high level of monounsaturated fatty acids, avocado oil could be also a valuable lipase inducer. Compared to other vegetable fats, it is distinguished by a high concentration of oleic acid in particular, but also of saturated palmitic acid. Linoleic acid belonging to the group of polyunsaturated fatty acids (PUFA) is present in lower concentrations, while linolenic acid, myristic acid and eicosanoic acid are present in very little amounts. The level of these fatty acids is variable and depends on, among others, on variety, maturity, the origin region of the fruit [Araujo et al. 2018]. In addition to the desired fatty acids, avocado oil contains many other bioactive ingredients, including tocopherol or phytosterols, mainly sitosterol, but also Δ -5-avenasterol and campesterol [Akpabio et al. 2011].

In the context of this article, suitable studies were conducted on the use of avocado oil to the synthesis of extracellular lipolytic enzymes in yeasts *Yarrowia lipolytica* W29. The lipolytic activity of *Yarrowia* yeasts, grown on media with the addition of avocado oil, was compared with the activity obtained on media with olive oil or glucose. The effect of different concentrations of avocado oil (1–10%; m/v) in the culture medium was also analysed.

MATERIALS AND METHODS

Materials

The materials used for preparing the media YPG and YPGA (yeast extract, peptone, glucose, agar-agar) were obtained from BTL Łódź (Poland). Extra virgin olive oil was from Spanish, avocado oil was from Olivado (USA). Acetone, methyl and ethyl

alcohol (99.8%), heptane were purchased from POCH (Poland). *p*-Nitrophenyl laurate was synthesized in laboratory from lauryl chloride (Sigma-Aldrich) and *p*-nitrophenol (POCH), according to a method described by Furniss et al. [1989]. The experimental part of the work was carried out with the use of *Yarrowia lipolytica* W29 (ATCC20460) yeasts.

Culture conditions

In the first stage, an inoculum was prepared. The strain of yeast *Yarrowia lipolytica* W29 was transplanted from the agar slopes into a flat-bottomed flask containing 100 cm³ of YPG medium (medium composition: yeast extract 10 g · dm⁻³, peptone 20 g · dm⁻³, glucose 20 g · dm⁻³). Culturing was carried out for 24 hours at 28°C with shaking at 140 rpm. Then, 1 cm³ of the inoculum was introduced into liquid culture media having the following composition:

- peptone – 20 g · dm⁻³, yeast extract – 10 g · dm⁻³
- peptone – 20 g · dm⁻³, yeast extract – 10 g · dm⁻³, glucose – 20 g · dm⁻³
- peptone – 20 g · dm⁻³, yeast extract – 10 g · dm⁻³, extra virgin olive oil – 20 g · dm⁻³
- peptone – 20 g · dm⁻³, yeast extract – 10 g · dm⁻³, avocado oil*

*Avocado oil was used in three different concentrations: 10 g · dm⁻³, 20 g · dm⁻³ and 100 g · dm⁻³.

The experimental cultures were carried out at 28°C and 140 rpm in the IKA KS 4000 ic control shaker. The individual markings for each media were made accordingly after 24, 48, 72 and 96 hour of yeast culture.

Dry mass measurement

The 50 cm³ of medium was centrifuged at 5000 rpm for 10 min (Centrifuge MPW-223). The supernatant was discarded, and the cells in the case of the medium containing peptone, yeast extract and glucose, was rinsed with 30 cm³ of distilled water. Cells from media containing olive oil and avocado oil were pre-rinsed with 30 cm³ of mixture of acetone/ethanol (10 : 10, v/v) (in order to rinse the remaining oil fraction), followed by a second washing with distilled water. Biomass was oven-dried at 105°C (SML 32/250 Zelmet) until it reached a constant weight. The results of biomass yield were given with respect to 1 dm³ of medium (g d.w. of cells · dm⁻³).

Determination of lipase activity

In order to determine the lipolytic activity, spectrophotometric method to measure the progress of hydrolysis of *p*-nitrophenyl laurate at a wavelength of 410 nm was used. By unit of the lipase enzymatic activity (U) such amount of the enzyme was assumed that is capable of releasing 1 μmol of *p*-nitrophenol per minute under the assay conditions at 37°C. Briefly, 0.3 mM of substrate (*p*-nitrophenyl laurate) was dissolved in 2.0 cm³ of heptane and biocatalyst was added. 15.0 cm³ of post-culture liquid was used in a role of biocatalyst. Hydrolysis reaction was performed on a magnetic stirrer Heidolph MR Hei

– standard function of heating at 37°C, and after 5 and 10 min, absorbance was measured in UV/Vis spectrophotometer at 410 nm. In addition, a blank test of reaction mixture without adding the substrate was performed.

Determination of fatty acid composition by means of gas chromatography

Fatty acids were converted to volatile methyl esters in accordance with PN-EN ISO 5509: 2001. The method of gas chromatography (GC YL 6100 gas chromatograph) with a capillary column (the BPX type 30 m × 0.25 mm) and a flame ionization detector was used to determine the composition of fatty acids in the mediums after cultivation of yeast.

The program for the determination of fatty acids: the initial temperature was set to 70°C and the sample was held at this temperature for 30 seconds, followed by a temperature increase to 160°C at a rate of 15°C/min, successively the temperature was being increased to 200°C at a rate of 1.1°C/min. and the sample was kept at this temperature for 6 minutes, in the last step the temperature was being increased to 225°C at a rate of 30°C/min.

Statistical analysis

Test results were analysed using the Statgraphics Plus software and the Excel spreadsheet. The statistical analysis of the results was carried out using a one-way analyses of variance with the Tukey test at the significance level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Due to the significant influence of the composition of the culture media on the lipolytic activity of microorganisms, this study compares the ability of yeasts to produce enzymes from the hydrolase class, multiplying them on media differentiated in terms of carbon source. Cultures were carried out with the addition of glucose, olive oil, avocado oil and for the comparative purpose, culture without the carbon source (YP medium).

The highest lipolytic activity was observed in the culture of yeast grown in the medium with glucose – Figure 1. This activity changed sinusoidally over the analysed time, and the highest value of $0.84 \pm 0.06 \text{ U} \cdot \text{cm}^{-3}$ was achieved on the second day of incubation. The medium consisting of peptone and yeast extract was characterized by the lowest lipolytic activity. The maximum value of activity in this medium was $0.012 \pm 0.01 \text{ U} \cdot \text{cm}^{-3}$ (the first day of cultivation) and was over 12 times lower than the lipolytic activity of cells from the same day of culture in the medium containing glucose. Such a low activity may be due to the fact that the medium did not contain the carbon source, which is needed for the metabolic processes of these microorganisms.

Among the hydrophobic substrates, olive oil, turned out to be a better lipase inducer than avocado oil. In its presence, the highest lipolytic activity of yeast, per 24 hours of cultivation, reached the value of $0.12 \pm 0.03 \text{ U} \cdot \text{cm}^{-3}$, so approximately around 2.5 times higher than in the medium with avocado oil, where this value was $0.047 \pm 0.02 \text{ U} \cdot \text{cm}^{-3}$.

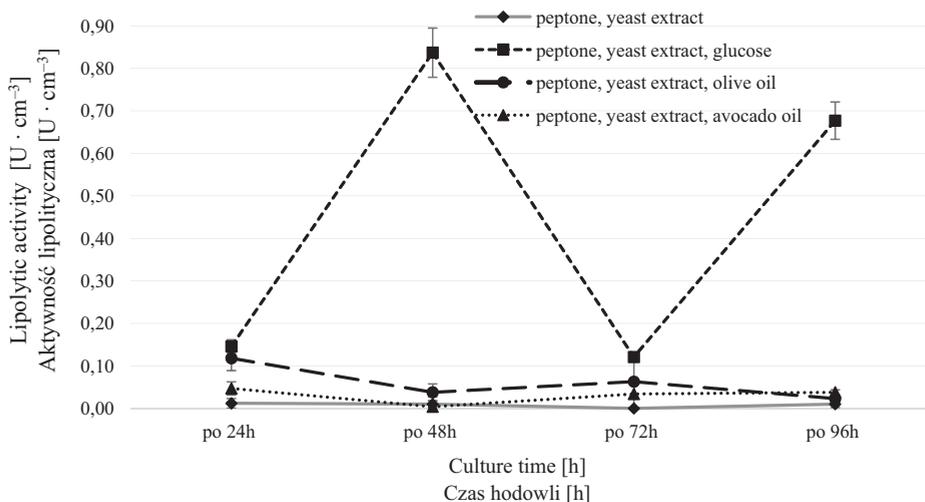


Fig. 1. The extracellular lipolytic activity of *Yarrowia lipolytica* W29 yeast in mediums with diversified of carbon source

Rys. 1. Zewnątrzkomórkowa aktywność lipolityczna drożdży *Yarrowia lipolytica* W29 w podłożach o zróżnicowanym źródle węgla

From the second day of cultivation, the lipolytic activity of yeasts in the medium with avocado oil began to show an upward tendency, and at 96 hours of cultivation it was 1.5 times higher than in the medium with olive oil ($0.038 \pm 0.01 \text{ U} \cdot \text{cm}^{-3}$ and $0.023 \pm 0.01 \text{ U} \cdot \text{cm}^{-3}$ accordingly).

Olive oil is recognized as one of the best lipase inducers in numerous scientific publications. Krzyczkowska and Kozłowska [2017] tested the lipolytic activity of *Yarrowia lipolytica* yeasts strain W29 in culture medium with various vegetable oils. The cultivation with the addition of olive oil was characterized by the highest activity, where the highest value of $3.05 \pm 0.18 \text{ U} \cdot \text{cm}^{-3}$ was obtained in 48 hours of cultivation. The lowest catalytic activity, similarly to the research in this paper, was obtained in culture containing only peptone and yeast extract.

The results presented in this paper were confirmed by the research of the team of Corzo and Revah [1999], which analysed, inter alia, the influence of various carbon sources on the lipolytic activity of the yeast *Yarrowia lipolytica* 681. Of the tested substrates, glucose turned out to be the most effective lipase inducer. In the 36 hour cultivation, the lipolytic activity was shown at the level of $27.9 \pm 3.11 \text{ U} \cdot \text{cm}^{-3}$ – in the cultivation with olive oil it was slightly lower and amounted to $25.3 \pm 2.10 \text{ U} \cdot \text{cm}^{-3}$. On the third day of cultivation, the lipolytic activity of yeast in both media was at a similar level ($30.2 \pm 3.21 \text{ U} \cdot \text{cm}^{-3}$ for the culture medium with glucose and $30.9 \pm 1.0 \text{ U} \cdot \text{cm}^{-3}$ for the medium with olive oil).

In a study conducted by Niaz et al. [2013] with the participation of fungi from *Trichophyton* sp. was shown a beneficial effect of glucose on extracellular lipolytic activity. In the culture mediums with sugar, the activity of fungal lipases was approximately $75.6 \pm 0.08 \text{ U} \cdot \text{cm}^{-3}$, and for the substrates containing a hydrophobic carbon source (olive oil) approximately $67.7 \pm 0.16 \text{ U} \cdot \text{cm}^{-3}$.

Due to a certain difference in the lipolytic activity of *Yarrowia* yeast grown on hydrophobic carbon sources, the study carried out the determination of the fatty acid profile in olive oil and avocado oil (Table 1).

The results show that in both oils, the dominant acid is oleic acid (C18:1), present in a concentration of 78.6 or 52%, for olive oil and avocado oil, respectively. Palmitic acid (C16:0) also plays a significant role, its content is approximately 10.3% in olive oil and approx. 28.1% in avocado oil. In olive oil, stearic acid (C18) (approximately 3.6%) is also present among the saturated acids. Among polyunsaturated acids (PUFA), linolenic acid (C18:3) is dominant – approximately about 4.6%. A slightly different composition of unsaturated acids is found in avocado oil. **The unsaturated acids** include: palmitoleic acid (C16:1) at a concentration of 2.5%, gadoleic acid (C20:1) 2.3% and eicosatrienoic acid (C20:3) 6.6%.

The composition of avocado oil and olive oil is confirmed by data from literature. In general, it is assumed that avocado oil, compared to many other vegetable fats, is distinguished by a high concentration of oleic acid, but also of saturated palmitic acid. Acids such as linolenic acid, myristic acid and eicosanoic acid tend to occur in very small amounts. However, it should be remembered that the level of these fatty acids is variable and depends, among others, on the variety, maturity, region and geographical area from which the fruit comes [Araújo et al. 2018]. Although the literature data show that the level of saturated fatty acids in avocado oil is similar to sunflower oil, soybean oil, peanut oil or

Table 1. Fatty acid composition and their percentage content of the olive and avocado oil

Tabela 1. Skład kwasów tłuszczowych i ich procentowa zawartość w oliwie z oliwek i oleju z awokado

Type of oil / Rodzaj oleju	% total fatty acids / % kwasu tłuszczowego					
	SFAs NKT		MUFAs JKT		PUFAs WKT	
Olive oil Oliwa	16:0	18:0	18:1	18:2 (n-6)	18:3 (n-3)	20:3 (n-3)
	10.3 ±0.23	3.6 ±0.05	78.6 ±0.17	4.6 ±0.22	20:5 (n-3)	2.4 ±0.02
Avocado oil Olej z awokado	16:0	20:0	16:1	18:1 (n-9)	20:1 (n-9)	20:3 (n-3)
	28.1 ±0.85	2.6 ±0.04	2.5 ±0.03	52.0 ±0.95	2.3 ±0.04	6.6 ±0.03

Data represent mean standard deviation of 3 independent samples, where: SFAs – saturated fatty acids, MUFAs – monounsaturated fatty acids, PUFAs – polyunsaturated fatty acids.

Podane wartości stanowią średnie odchylenia standardowe z 3 analiz; gdzie: NKT – nasycone kwasy tłuszczowe, JKT – jednonienasycone kwasy tłuszczowe, WKT – wielonienasycone kwasy tłuszczowe

olive oil, in the case of oils used in this research, the concentration of these acids was slightly different (higher by 4,2% of saturated fatty acid content in avocado oil).

The authors of numerous scientific papers emphasize that the production of microbial lipases increases with a relative increase in the concentration of C18:n acid esters [Zarevucka 2012]. Oleic acid is considered to be one of the best inducers of lipolytic activity. Assembly of Csutak et al. [2018] while investigating the effect of fatty acids on lipase production by yeast *Rhodotorula glutinis* CMGB-RG5 showed that lipase inducers include butyric and oleic acid, and the presence of palmitic acid inhibits lipase synthesis. This dependency can also be seen with high probability in the results of this study (Fig. 1). Lipolytic activity of *Yarrowia* being around 1.5–2.5 times higher in the olive oil medium as compared to the medium with avocado oil can be explained by a higher concentration of oleic acid and a lower content of palmitic acid.

Due to the fact that the extracellular lipolytic activity of *Yarrowia lipolytica* W29, determined in the post-culture fluid, may be dependent on the intensity of cell multiplication in the medium, the yield of yeast cell biomass was determined in the further part of the study. In order to show the possible influence of the hydrophobic carbon source, the determination of the biomass yield was also performed for the substrate with glucose and the so-called “reference medium” was the medium consisting of peptone and yeast extract – Figure 2.

Within the analysed cultivation time, the yield of yeast dry matter in the media including the carbon source showed an upward trend. The highest yield of cellular biomass was obtained in media with avocado oil. Within four days of culture, the biomass yield increased over 5-fold (with a value of 3.5 ± 0.08 g d.m. · dm⁻³ on the first day of culture to 18.4 ± 0.01 g d.m. · dm⁻³ on the fourth day of cell growth).

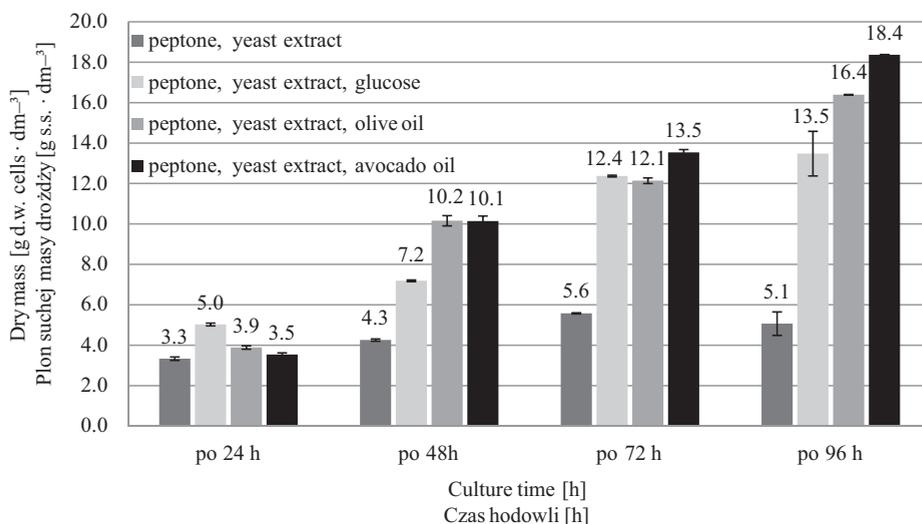


Fig. 2. Dry mass of yeast *Yarrowia lipolytica* W29 grown on media containing various carbon substrates

Rys. 2. Płon suchej masy drożdży *Yarrowia lipolytica* W29 namnażanych na podłożach zawierających zróżnicowane substraty węglowe

Olive oil also exhibited a beneficial effect on yeast dry matter gain. After of 24 hrs of cell culture, the biomass yield was similar to the results from avocado oil – that amounted to approximately $3.9 \pm 0.75 \text{ g d.m.} \cdot \text{dm}^{-3}$. The maximum value at $16.4 \pm 0.33 \text{ g d.m.} \cdot \text{dm}^{-3}$ was obtained on the last day of cell culture.

Slightly lower yield of cellular biomass could be observed in the medium with glucose. The highest cell concentration was noted for the substrate in the last analysis on the culture and it was based on the scale of approximately $13.5 \pm 1.10 \text{ g d.m.} \cdot \text{dm}^{-3}$. Standing out against all the others is a specific score of $5.0 \pm 0.07 \text{ g d.m.} \cdot \text{dm}^{-3}$ obtained after the first day of cultivation. The higher content of cells in the medium with glucose in the initial phase of yeast growth may result from easier absorption of simple sugar, compared to vegetable oils, which, in order to be used as a carbon source, must be hydrolysed to free fatty acids.

The lowest yield of dry matter was obtained in the substrate without the carbon source – consisting only of peptone and yeast extract. The highest value of the medium at $5.6 \pm 0.03 \text{ g d.m.} \cdot \text{dm}^{-3}$ was observed on the third day of cell culture. Compared to the mediums with carbon components, this value was about two times lower. At 96 hours of cultivation, this difference became even more significant – from 2.5 to 3.5 times lower in cell biomass yield, compared to carbon media.

In research conducted by Fabiszewska et al. [2014a], yield cell biomass *Yarrowia lipolytica* KKP 339 after 72 hrs of culture in medium with glucose was equal to $10.2 \text{ g d.m.} \cdot \text{dm}^{-3}$ and the substrate with olive oil $11.3 \text{ g d.m.} \cdot \text{dm}^{-3}$. In other studies, carried out by Fabiszewska et al. [2014b], in the exact same researched yeast strain, after 65 hour of cultivation in YPG medium (with glucose), the yeast biomass yield was at $9.44 \text{ g d.m.} \cdot \text{dm}^{-3}$, and the culture medium with olive oil, was in the range of $11\text{--}11,5 \text{ g d.m.} \cdot \text{dm}^{-3}$. These results are very similar to those obtained in this particular study.

Although avocado oil was a less effective inducer of lipolytic activity in relation to olive oil, an attempt was made in the further part of the study to evaluate the effect of different concentrations of this oil on the growth and metabolic activity of yeasts *Yarrowia lipolytica* W29. The relatively high concentration and variety of unsaturated fatty acids in the avocado oil suggests that proper optimization of the different parameters of the culture of microorganisms, including lipid substrate concentrations allows to achieve their high catalytic activity.

As part of this work, three media were tested, containing respectively 1, 2% (the concentration used in the first part of the study) and addition of 10% of avocado oil. The extracellular lipolytic activity was measured spectrophotometrically at 24-hour intervals. The results are shown in Figure 3.

The highest extracellular lipolytic activity was shown by yeast cells grown in medium with the highest concentration of avocado oil (10%). The maximum activity at the level of $0.789 \pm 0.01 \text{ U} \cdot \text{cm}^{-3}$ was achieved on the third day of cultivation. That value was almost 80 times higher in the analysed time in comparison to the other substrates. The lowest lipolytic activity was observed in the medium with the addition of 2% avocado oil. There was none lipase activity recorded in the supernatant between 2 and 3 day of *Yarrowia* yeast cultivation. Similar results were obtained as well for the medium with 1% oil addition (negligible lipolytic activity on the second and third day of cultivation). It is

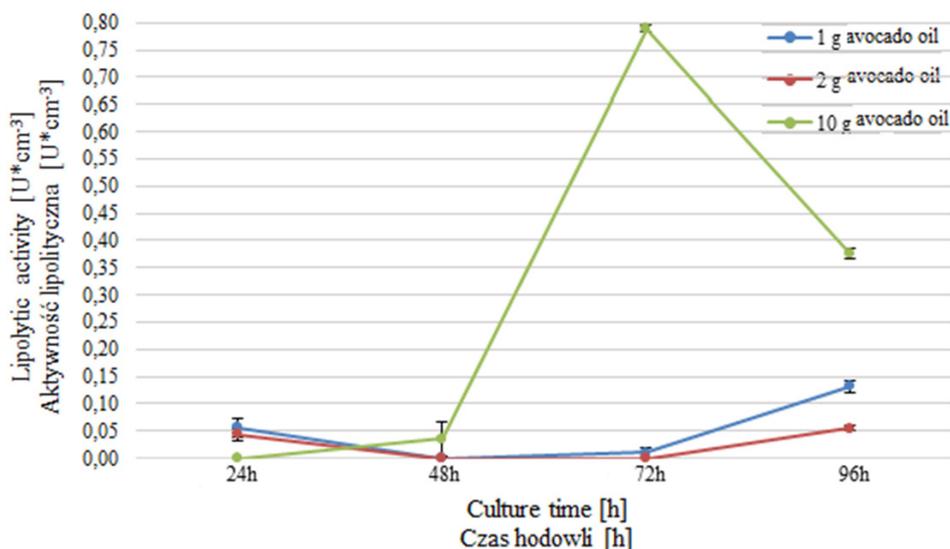


Fig. 3. The extracellular lipolytic activity of *Yarrowia lipolytica* yeast grown on media with various concentrations of avocado oil

Rys. 3. Zewnątrzkomórkowa aktywność lipolityczna drożdży *Yarrowia lipolytica* namnażanych na podłożach o różnym stężeniu oleju z awokado

presumed that the lack of yeast lipolytic activity in media with lower concentrations of avocado oil may be caused by, usage of a lipid substrate as a carbon source by microorganisms, that is necessary for growth and maintenance of vital functions. The complete consumption of avocado oil in the metabolic pathway inhibited the synthesis and secretion of lipases to the medium.

Effect of concentration of avocado oil to the induction of lipases was also studied by Martínez-Corona et al. [2019]. The strain *Kluyveromyces marxianus* was used in the cultivation. Six different concentrations of oil were used: from 1 to 4.5%. The highest lipolytic activity was observed in the medium with the addition of oil at the level of 3.5% ($4.69 \pm 0.23 \text{ U} \cdot \text{cm}^{-3}$). The organisms grown in media with low oil concentration (1–3%) were characterized by relatively low activity. Similarly to the present study, the medium with 2% addition of substrate, showed lower lipolytic activity, than in the medium with 1% oil concentration.

Effect of substrate concentration in the culture medium, on lipolytic activity was also studied by Turati et al. [2019]. They cultivated the fungi called *Penicillium* sp. and the substrate used was olive oil in concentrations ranging from 0.3 to 3.0%. Measurement of lipolytic activity was performed on the 3rd day of cultivation, and the obtained results showed the highest lipolytic activity ($1.62 \text{ U} \cdot \text{cm}^{-3}$) in the medium with the addition of 0.5% oil. The lowest lipolytic activity was obtained in cultivation with an addition of 2% of olive oil.

CONCLUSIONS

The study showed that the extracellular lipolytic activity of *Yarrowia lipolytica* yeasts can be induced by both simple sugars and hydrophobic substrates, with inclusion of avocado oil. Avocado oil, compared to olive oil, has a lower influence on lipolytic activity of yeasts, which may be due to their different fatty acid composition. In the avocado oil used in the research, there is approximately 26% less oleic acid and approximately 18% more palmitic acid compared to olive oil, which may contribute to lower lipolytic activity of yeast cells. Avocado oil used as a carbon source in microbiological media contributes to a significant increase in cellular biomass – it is proportional to the oil concentration in the media.

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WPLYW OLEJU Z AWOKADO NA INDUKCJĘ HYDROLAZ DROŹDŹOWYCH

Streszczenie. Hydrolazy katalizujące rozpad wiązania estrowego zwane powszechnie lipazami (EC 3.1.1) to jedna z ważniejszych grup enzymów, o istotnym znaczeniu przemysłowym. Wytwarzane są przez rośliny, zwierzęta i mikroorganizmy, przy czym największym zainteresowaniem cieszą się lipazy mikrobiologiczne. Celem pracy była ocena wpływu oleju z awokado stosowanego jako substrat hydrofobowy w podłożach hodowlanych drożdży *Yarrowia lipolytica* W29 na syntezę zewnątrzkomórkowych lipaz. W pracy porównano aktywność metaboliczną drożdży na podłożu z olejem z awokado, z podłożami o zróżnicowanych źródłach węgla (oliwa, glukoza). Uzyskane wyniki wskazują, iż aktywność lipolityczna drożdży *Yarrowia lipolytica* W29 może być indukowana obecnością oleju z awokado w podłożu. Niższe stężenie kwasu oleinowego przy jednoczesnym wyższym stężeniu kwasu palmitynowego w oleju z awokado, w stosunku do oliwy skutkuje jednak ok. 2,5-krotnie niższą aktywnością drożdży – na poziomie ok. $0,047 \pm 0,02 \text{ U} \cdot \text{cm}^{-3}$.

Słowa kluczowe: hydrolazy, lipazy, olej z awokado, aktywność lipolityczna, *Yarrowia lipolytica*

