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Original Research

Waste Frying Oil as Substrate for Lipase Production by *Geotrichum candidum* Strains

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Abstract

The study was carried out on 9 yeast strains of *Geotrichum candidum.* The purpose of the study was to determine the ability of the yeast strains to produce extra- and intracellular lipases in shake cultures in a medium containing 2% and 3% of rapeseed oil used as a carbon source, after deep frying of Frenchfries. Biosynthesis of these enzymes in an AK-210 bioreactor at a working volume of 5 L was analyzed. Lipase activity was determined using olive oil (JL_O) and glycerol tributyrate (JL_T) as substrates.

The results obtained in shake cultures showed that the best carbon source was the medium containing 2% of olive oil, while the best producer of extracellular lipases (198 JL_{ZO} mL^{-1}) was the strain of *G. candidum* MSK_3-11.

Lipase synthesis in the bioreactor was begun at the exponential phase of growth. The activity of extracellular lipases amounted to 88 JL_{ZO} mL^{-1} at biomass yield of 14 g L^{-1} at a stationary phase.

Irrespective of the strain and the medium, both extra- and intracellular activity was higher with the use of olive oil than with tributyrin.

Keywords: *Geotrichum candidum*, lipases, waste frying oil, rapeseed oil

Introduction

Hydrolases of glycerol esters [E.C.3.1.1.3], also referred to as lipases, are common in nature. The main sources they originate from are microorganisms, e.g. bacteria, fungi and yeast, animal organs and plants [1, 2]. The great interest of biotechnological industries in microbiological lipases is due to their specific properties, such as stability within a wide pH range in high temperatures [3] and organic solvents, and also due to the fact that they do not need cofactors [4, 5], but exhibit substrate specificity and enantiospecificity of catalyzed reactions [6]. Due to these capabilities, the most commercially important field of application for hydrolytic lipases is their addition to detergents [5]. In the food industry, for example, lipases are extensively used in dairy products for the hydrolysis of milk fat. Current applications include the flavour enhancement of cheese, the acceleration of cheese ripening, the manufacturing of cheese-like products [7]. In the paper industry, lipases are used to remove triglycerides and waxes from the pulp [4]. Many pharmaceutical companies use lipase preparations for the production of optically active semi-products in industrial quantities [8]. Besides this, they are used in textile and cosmetic industries [9]. Lipases are also used for biodiesel production [10].

Lipolytic enzymes have already found many applications, but it is worth noting that a steadily increasing interest in them has been observed in recent years in the oil industry, where they can be used for utilization of by-products containing high amounts of fats, oils and slurry, which are an

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enormous threat to the environment [11]. Bioremediation for waste disposal is a new avenue in lipase biotechnology. Oil spills during rigging and refining, oil-wet night soils, and lipid-tinged wastes in lipid processing factories and restaurants can all be treated by the use of lipases of different origins. Lipases have been used extensively (ex situ or in situ) for the degradation of wastewater contaminants such as olive oil from oil mills [12]. The treatment process generally consists of the cultivation of lipase-producing microbial strains in the effluents. Wakelin and Forster [13] investigated the microbial treatment of waste from fast-food restaurants for the removal of fats, oil and greases. They cultivated pure and mixed microbial strains and *Acinetobacter* was the most effective of the pure cultures, typically removing 60–65% of the fatty material whose initial concentration had been 8 g/l. The effectiveness of the mixed culture, MC1, was variable, with the removal efficiency ranging from 29% for rapeseed oil to 73% for restaurant grease.

As more and more fatty waste is continuously generated from industry, it is very important to find new biotechnological ways to valorize this substrate. In the present study, we also used a waste product, which was rape oil after deep frying of French fries and yeast *Geotrichum candidum* as a lipases-producer strain.

The purpose of the present study was to determine

1) the ability of nine yeast strains of *Geotrichum candidum* to produce extra- and intracellular lipases in a medium containing this substrate, and
2) biosynthesis of these enzymes, using a selected strain 3-11 in an AK-210 bioreactor.

Materials and Methods

Microorganisms

Nine strains of *Geotrichum candidum* were used in this study (Table 1). These strains were grown on YM-agar slants at 30°C for 48 h and then stored in a refrigerator at +4°C by yearly transfers.

Media

A mineral medium used for the study contained: 5% peptone, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.1% $NaNO_3$ at pH 6.0 [14]. The medium for the process in a bioreactor consisted of 1% peptone, 0.1% $MgSO_4 \cdot 7H_2O$ and 0.1% $NaNO_3$ at pH 6.0.

The carbon source was rapeseed oil (*kujawski* oil) previously used several times for deep frying of French fries at 190°C, in the amounts of 2% and 3% (shake cultures) and 2% in the bioreactor. The medium was sterilized at 121°C for 20 minutes.

Shake-Flask Cultivations

The shake cultures were conducted in 250 mL Erlenmayer's flasks containing 50 mL of the medium on an Elpan rotary shaker at 170 rpm, at 30ºC for 7 days.

Yeast suspension was prepared in 0.1% Tween 80 and were diluted to a final concentration of 10^7 cells mL^{-1} (estimated by microscopy). The media were inoculated with 1 ml of cell suspension of *G. candidum*.

At first, the pH was measured and the samples (5 mL) collected after 3, 5 and 7 days. Following this, the entire culture was centrifuged (1000 x g for 10 min), using a K24D centrifuge and cooled at the same time. The biomass obtained after double washing with 1 mL of 0.05 M Tris-HCl buffer at pH 8.0 was suspended in 2 mL of the buffer and then frozen. After thawing, it was disintegrated using an HD 2070 ultrasonic disintegrator at 90% power for 10 min. The suspension was centrifuged as described above. Lipolytic (intracellular) activity and protein content were determined in the supernatant.

The inoculated cultures for the bioreactor were performed as described above, in medium containing bactopepton 5%, for 48 h.

Fermenter Cultivations

The process was carried out in a 10 L stirred tank bioreactor AK-210 with a working volume of 5 L at 30°C.

Table 1. Applied microorganisms.

Strain	Symbols used in figures	Source
Geotrichum candidum 1	O1	Laboratory collection, WUELS
Geotrichum candidum Sc 12	Sc 12	Cammembert cheese
Geotrichum candidum KB 6	X6	Brie cheese
Geotrichum candidum KB 5	X5	Brie cheese
Geotrichum candidum PH 1	PH	Chicken feathers
Geotrichum candidum SS 47 D_2	D2	Malt
Geotrichum candidum SS 32 B_1	B1	Malt
Geotrichum candidum $MSK_3$11	3-11	Malt
Geotrichum candidum CCM 8265	CCM	Czech Collection of Microorganisms

Aeration rate was fixed at 1 volume of air per volume of medium and per minute (vvm), agitation speed was adjusted to 700 min^{-1}. The inoculum accounted for 5%. The process was performed with no adjustment of pH. Samples (15 mL) were collected at frequencies shown in the Figures below. The pH and the biomass [X], oil residues [O] were measured and extra- and intracellular lipolytic activity were determined as described above.

Analytical Methods

Extracellular (JL_Z) and intracellular (JL_W) lipolytic activities were determined using tributyrin (JL_{ZT}, JL_{WT}) and olive oil (JL_{ZO}, JL_{WO}) as described by Rywińska et al. [15].

Lipolytic activity was expressed in the amounts of μM of 0.05 M of NaOH necessary for neutralization of fatty acids released by lipases present in 1 mL of the culture liquid within 1h.

Protein content was determined using Lowry's method [16].

Microbial biomass concentration [X] was measured by dry weight estimation. 10mL of cell suspension from the culture was extracted twice, using petroleum ether (2 x 2.5mL) in order to dissolve the fatty fraction. After centrifuging (at 4000 rpm for 5 min) and the isolation of the ether fractions, the biomass was separated using a 0.45 μm pore-size membrane filter (Millipore) and dried at 105°C until constant weight was reached, cooled in desiccator and weighed.

The residue oil [O] was also determined. The ether fractions obtained with biomass determinations were evaporated at 50°C in a weighing bottle and dried again at 105°C until constant weight was reached, cooled in a desiccator and weighed.

Results and Discussion

The results obtained in this study show that, under the conditions of the investigation, all the yeast strains exhibited the capability of extra- and intracellular lipase biosynthesis. However, the values of lipolytic activity varied, depending on the oil concentration in the medium and length of the process and the substrate used for the enzymatic reaction.

Markedly higher values were obtained with olive oil. The maximum extracellular activity of 198 μM mL^{-1} h^{-1} was obtained with a 3-11 strain, on day 3 of the culture, both in the medium containing 2% and 3% of the olive oil (Figs. 1A and 1C). The same value on day 3 was observed with strain B1 in the medium containing more carbon source (Fig. 1C). Strain 3-11 also exhibited the highest

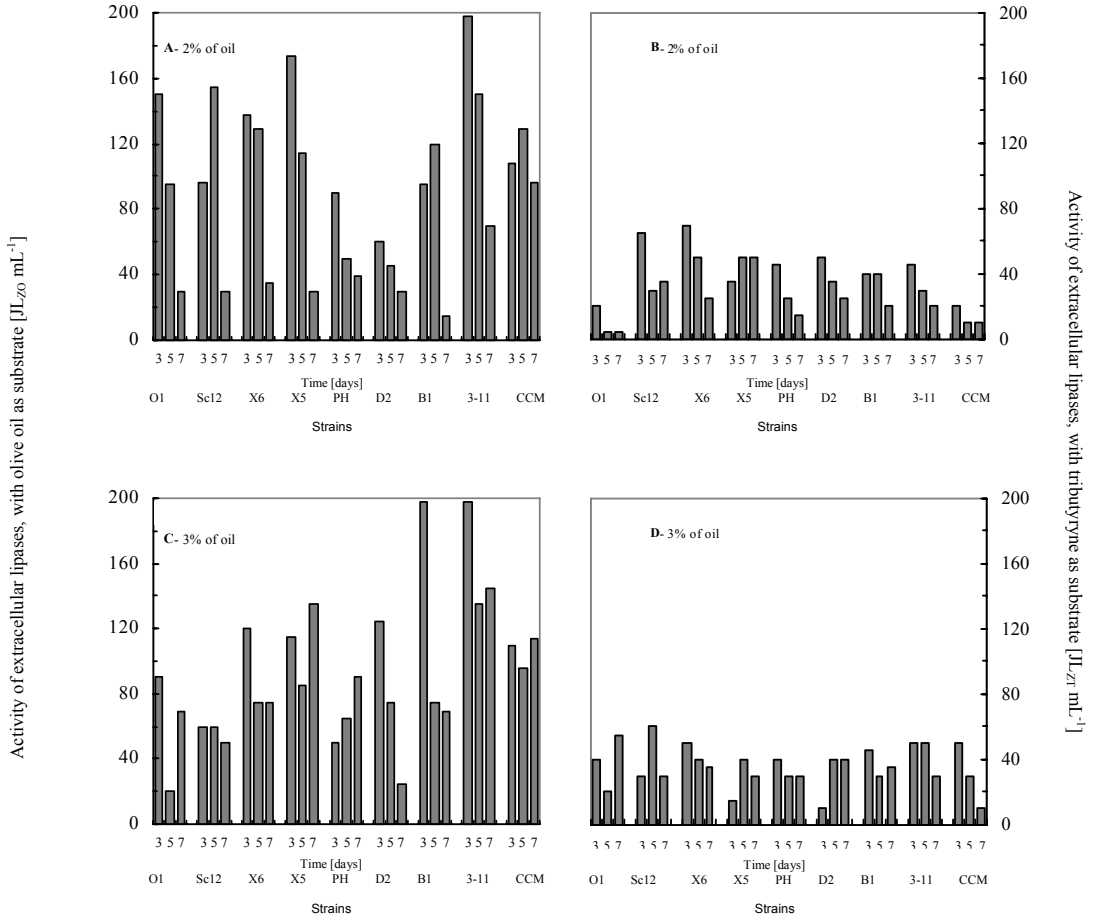

Fig. 1. Effect of oil concentration 2% - (A, B), 3% - (C, D) on extracellular lipases, with olive oil ZO – (A, C) and tributyrin ZT – (B, D) as substrate, induction in strains *Geotrichum candidum*.

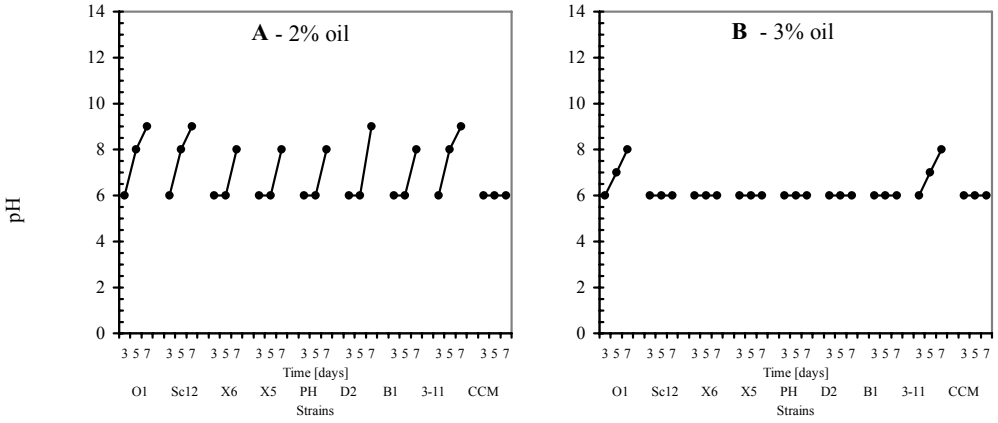

Fig. 2. Change of pH during shaking cultures of *Geotrichum candidum* strains in medium with 2% (A) and 3% (B) of oil.

extracellular lipase activity in the medium containing another by-product of the oil industry, i.e. 2% sludge, but the activity was 23% lower [17]. The authors of the present study used rapeseed oil as a carbon source for lipase biosynthesis by *G. candidum* in earlier investigations [15].

It is worth noting that they used 1% crude oil and the values of lipase activity were slightly higher, especially with the strains X5 (260 μM mL^{-1} h^{-1}) and B1 (225 μM mL^{-1} h^{-1}). In the present study, the majority of the yeast strains under investigation enhanced extracellular lipase activity, both with the use of olive oil and tributyrin, when the medium containing 2% of oil was used (Figs. 1A and 1B). In this medium, the pH was increased to 9.0 (Fig. 2A), while the pH of the cultures containing 3% of oil remained unchanged (Fig. 2B), except those containing strains O1 and 3-11.

No changes in the pH and lower values of lipase activity were likely due to the fat sticking to the yeast cells, consequently preventing them from access to other ingredients of the medium as well as dissolved oxygen. Such a negative impact of fat on the cells of microorganisms was observed earlier by other authors [17, 18].

When tributyrin was used as a substrate and the medium contained 2% of oil, the highest extracellular activity was observed with the strains X6 (70 μM mL^{-1} h^{-1}) and Sc12 (65 μM mL^{-1} h^{-1}) (Fig. 1B). When the oil concentration increased, a greater impact of the strains Sc12 (60 μM mL^{-1} h^{-1}) and O1 (55 μM mL^{-1} h^{-1}) was observed (Fig. 1D). These values confirmed a greater preference of the lipases produced by yeast strains under investigation to hydrolyze ester bonds made up by long-chain fatty acids rather than short-chained butyric acid present in tributyrin. Similar results obtained with the same strains, yet growing under other conditions, were reported in earlier studies [15, 17]. Similar findings were also reported with the use of the strain *G. candidum* NRRL Y-553 [14].

The maximum lipolytic activity of most yeast strains under investigation was observed on day 3 (Fig. 1), and later, especially in the cultures containing lower amounts of oil, the activity was found to be markedly reduced (Fig. 1A). The drastic decreases in lipolytic activity were likely due to both increasing pH and gradually decreasing amounts of the substrate.

Similar conclusions were reported by Corzo and Revah [19], while Zarevúcka et al. [20] attributed the reductions in lipase production to enhanced activity of proteolytic enzymes produced at the same time.

Conversion of the activity into milligram of protein allowed us to compare the production of intracellular and extracellular proteins. The results obtained in our studies show that irrespective of the carbon source and the substrate used in enzyme tests, the activity of intracellular lipases in shake cultures was higher than extracellular activity (Figs. 3 and 4).

Taking into account the results obtained in the studies, the strain *G. candidum* 3-11 was selected for the continuous culture performed in an AK-210 bioreactor, with no pH adjustment, using 2% of kujawski rapeseed oil. On a large scale, the cultures of the yeast of *G. candidum* cause a number of problems [21]. The process in the present investigation was performed using 1% of peptone. Under these modified conditions, the process lasted 92 h, but the medium still contained trace amounts of the substrate. After 30 h, the yeast reached a stationary phase of growth at the biomass yield of 14 g L^{-1} (Fig. 5).

As can be seen in Fig. 5, the production of extracellular lipases began in the exponential phase and was more intense (with lipases determined with the use of olive oil) than that observed in the stationary phase. The type and quantity of carbon substrates are important factors affecting the initiation of lipase synthesis in the cells of microorganisms. When the medium contains one carbon source, the enzyme production coincides with the growth of cells [22]. When two carbon substrates are used (most frequently carbohydrates and fats), lipase production usually starts after depletion of the available carbon source, e.g. glucose [2], which usually occurs at the end of the exponential phase of the growth of the microorganism. In the present study, about 20 h after the culture was finished, extracellular lipase activities determined with olive oil as a substrate, were maintained at almost the same level (88 JL_{ZO} mL^{-1}). This activity was almost twice as low as that observed with this strain in shake cultures with 2% (198 μM mL^{-1} h^{-1}) of *kujawski* oil. The difference was likely due to peptone content of the bioreactor reduced to 1%. A markedly higher activity (20 μM mL^{-1} min^{-1}) was observed by Kamimura et

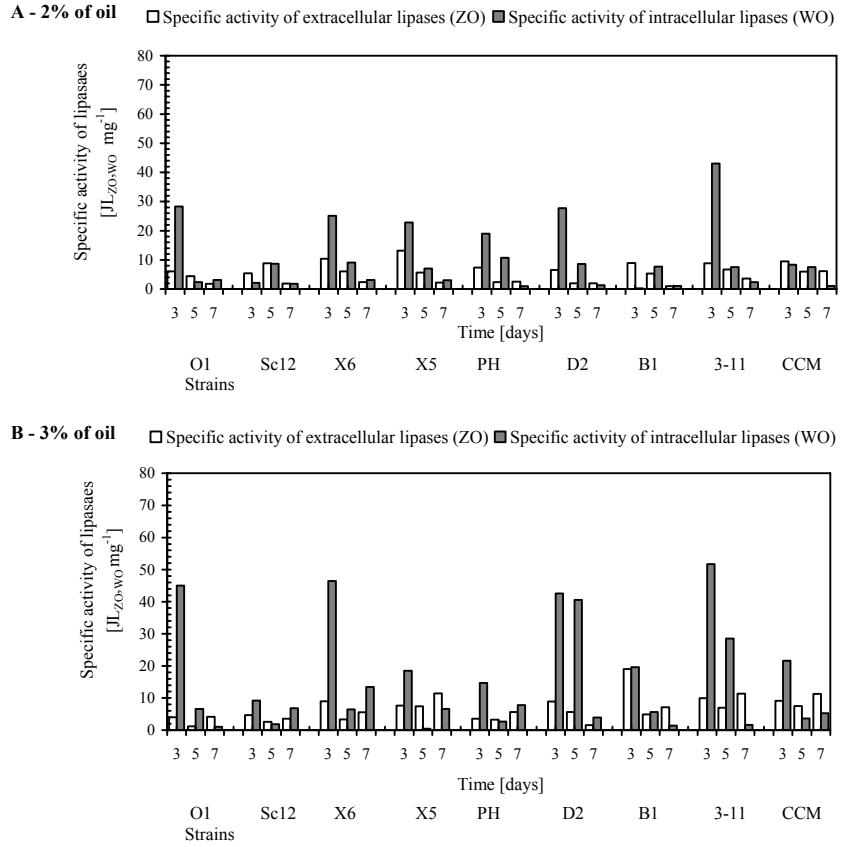

Fig. 3. Effect of oil concentrations on extracellular (ZO) and intracellular (WO) lipases (with olive oil as substrate) induction in *Geotrichum candidum* strains.

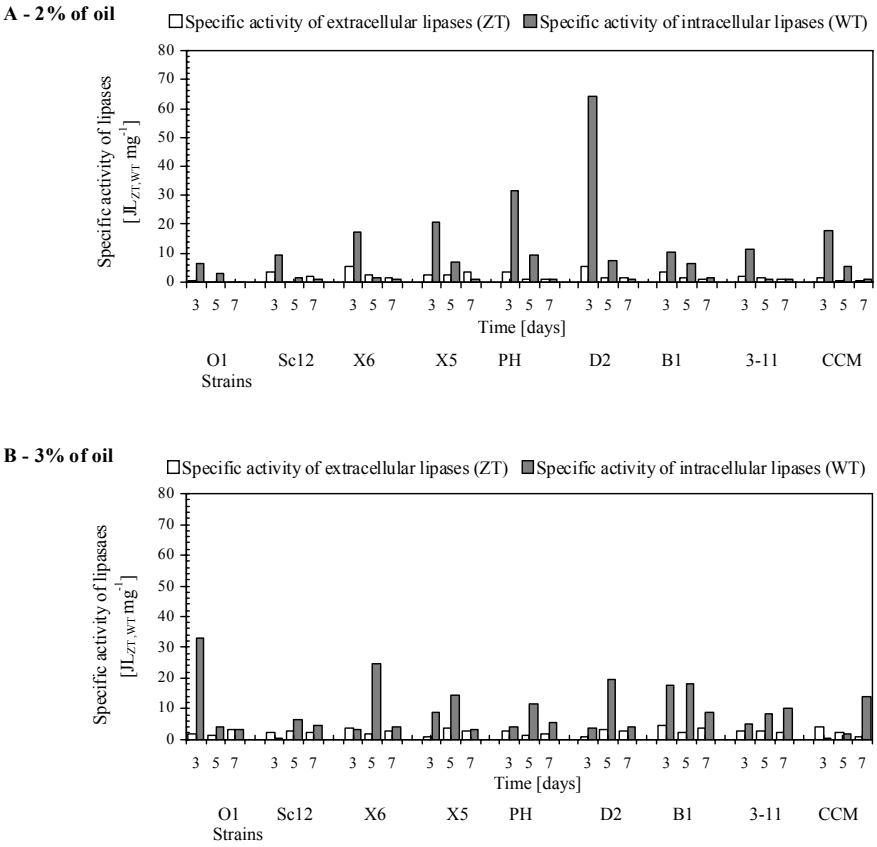

Fig. 4. Effect of oil concentrations on extracellular (ZT) and intracellular (WT) lipases (with tributyryne as substrate) induction in *Geotrichum candidum* strains.

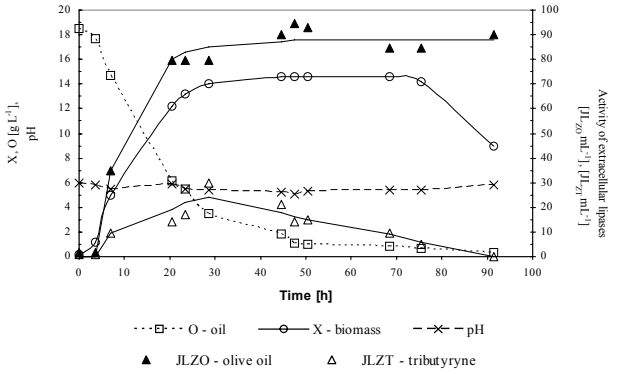

Fig. 5. Biosynthesis of extracellular lipases, in a batch culture of *Geotrichum candidum* 3-11 strain in bioreactor AK-210, in medium of 2% oil.

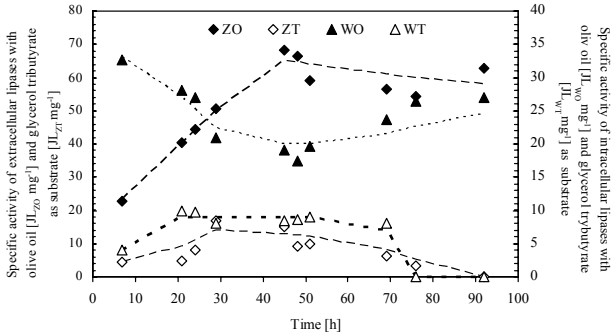

Fig. 6. Biosynthesis of extra- and intracellular lipases, in a batch culture of *Geotrichum candidum* 3-11 strain in bioreactor AK-210, in medium of 2% oil.

al. [23], who studied the yeast of Geotrichum sp. in the culture containing 1% of olive oil. Higher activities were also observed by Corzo and Revah [19] in the cultures of Yarrowia lipolytica in the medium containing 1% of olive oil (32 μM mL^{-1} min^{-1}).

When tributyrin was used as a substrate, maximum activity was observed after 28 h (28.0 JL_{ZT} mL^{-1}), and the values were drastically decreasing (Fig. 5).

After 45 h, the activity of extracellular lipases determined with olive oil (68.2 JL_{ZO}/mg) reached its maximum value, and afterwards it was slightly decreasing (Fig. 6).

Specific extracellular lipase activity determined in the presence of tributyrin was three-fold lower than that observed when the enzymes were determined with the use of oil.

In turn, intracellular lipase activity determined with olive oil as substrate reached the highest value (32.72 JL_{WO} mg^{-1}) in a shorter time, which was likely due to trace amounts of proteins present in the cell, especially at the beginning of the culture. In contrast to the shake cultures, specific intracellular activity of the lipases produced in the bioreactor was lower than specific extracellular activity (Fig. 6).

The highest value of specific intracellular activity of lipases was determined with glycerol tributyrate and amounted to 14.04 JL_{WT} mg^{-1} (Fig. 6).

It is worth noting that the pH of the culture was changing both in our studies (Fig. 5) and other investigations [19, 23]. In general, pH was decreasing until a stationary phase and a maximum lipolytic activity was reached, where afterwards it started to increase. In our investigations on the cultures in the bioreactor, no drastic changes in pH were observed – it was maintained at 6.0-5.1. Similarly, in the bioreactor culture with strain of *Geotrichum* sp., in synthetic medium containing 1% of olive oil [23], the pH of the culture decreased from 5.6 to 4.5 until the stationary phase was reached, and afterwards it started to increase to 5.6. When Corzo and Revah [19] used the yeast of *Yarrowia lipolytica*, the differences in the pH were significant; at first it was decreasing from 6.0 to 3.5, and next increasing to 8.0. According to Corzo and Revah [19], the decreasing pH resulted from organic acid production, while the increases were due to degradation of these compounds by proteases.

Summing up, it can be concluded that lipase production with the use of oil industry waste products may encounter many problems, especially those connected with finding appropriate conditions for biosynthesis of these enzymes and change scale of biosynthesis from shake cultures to the bioreactor.

Conclusions

1. All the yeast strains of *G. candidum* under investigation were able to produce extra- and intracellular lipases.
2. The intensity of lipase biosynthesis depended on the concentration of the carbon source – extracellular lipase production was most efficient with the use of 2% of *kujawski* rapeseed oil, while the most efficient intracellular lipase production was observed with the medium containing 3% of oil.
3. Most of the strains reached the highest lipase activity on day 3 of the shake culture.
4. Higher extra- and intracellular lipase activities were observed when olive oil was used as substrate.
5. The highest extracellular lipase activity (198 JL_{ZO} mL^{-1} on day 3) in shake cultures in the medium containing both 2% and 3% of oil, was observed with the strain *G. candidum* 3-11.
6. The strain *G. candidum* 3-11 in the bioreactor with 2% of *kujawski* oil produced smaller amounts of extracellular lipases than in shake cultures (88 JL_{ZO} mL^{-1}).

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