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EFFECT OF FAT SUBSTRATE ON EXTRACELLULAR LIPOLYTIC ACTIVITY OF *CANDIDA BOMBICOLA ATCC 22214* *

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Key words: lipolytic activity, biosurfactants, *Candida bombicola*, lipases

The performed experiments allowed investigating a strain of *Candida bombicola ATCC 22214* yeasts from the point of view of its potential to manufacture extracellular lipase and its activity in relation to the applied fat substrate. Oleic acid, sunflower oil and a post-deodorizing condensate were used as culturing substrates. It was found that the best agent stimulating yeast enzymatic system to produce lipases was the applied post--deodorizing condensate. The highest activity of approximately 1.90 μmol/min/mL was observed on the fifth day of culturing. No lipolytic activity was recorded in culture media in which oleic acid was used as a substrate.

INTRODUCTION

Biosurfactants are surface active compounds that are synthesized by microorganisms. A common feature of these substances is their amphoteric character. The combination of a polar and non-polar particle in a single molecule endows it with several unique properties, including its potential to reduce surface tension. Thanks to this property, surfactants have been widely used in many branches of industry, such as: pharmaceutical, food, cosmetic, paper, textile, metallurgic, oil, and agriculture industries [Daniel *et al.*, 1999; Kosaric, 1993].

In addition to their synthesis of glycolipids, microorganisms, apart from biosurfactants, can also manufacture other compounds assisting them in this process, *e.g.* lipases.

Lipases are found in gastric, pancreatic and intestinal juices of vertebrates as well as in digestive juices of many invertebrates. They also occur in plants. However, their principal commercial source are microorganisms, such as: bacteria, yeasts and moulds [Trzcińska *et al.*, 2000; Maia *et al.*, 2001].

In recent years, a considerable increase of interest in this group of enzymes has been observed. They are utilized in pulp and paper industry, chemical industry and, in the field of food technology, in dairy and fat industries as triglyceride modifiers. Furthermore, they are used as detergent enzymes [Kim *et al.*, 2000]. In addition, they also take part in the synthesis of biosurfactants following an appropriate preparation of the fat raw material contained in the culturing media [Schmid & Verger, 1998; Ducret *et al.*, 1995].

Fungi provide an important source of lipolytic enzymes. Fresh attempts are being made to find new fungal strains for their synthesis, *e.g. Fusarium solani* that are capable of utilising cheap components of culture media.

The synthesis of extracellular lipases by microorganisms depends on components contained in the culture medium and the intensity of their production is influenced by various sources of carbon and nitrogen. However, production of lipases depends primarily on the fat raw material utilised in the process of culture [Maia *et al.*, 2001; Pimentel *et al.*, 1996].

Manufacture of esters of fatty acids is also possible using lipase derived from *Candida ruginosa* or employing pancreatic lipase derived from *Chromobacterium viscosum* and protease from *Bacillus subtilis* [Schmid & Verger, 1998].

It is evident from the presented properties of lipases that they can constitute an important element of biosynthesis of biosurfactants.

The objective of this research project was to assess the possibilities of manufacturing lipolytic enzymes by a strain of *Candida bombicola ATCC 22214* yeasts and to determine their activity depending on the fat substrate applied in media.

MATERIAL AND METHODS

EXPERIMENTAL MATERIAL: *Candida bombicola ATCC 22214* yeasts obtained from the *American Type Culture Collection* were used to synthesise biosurfactants.

CULTURE MEDIUM: Three experimental culture media contained the following compounds mixed in the amount of 100 g/L: oleic acid, sunflower oil, post-deodorizing condensate (after refining of rape oil, obtained in fat plant in Kruszwica), glucose and yeast extract in the amount of 5 g/L. The fourth culture media with the initial composition of glucose, post-deodorizing condensate of 100 g/L and yeast extract of 5 g/L was additionally supplemented with glucose

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from the third day on (25 g/L/day, 50% glucose solution). The initial value of the medium was corrected on the level of pH 5.8 (15% HCl). A precise composition of the applied culture media is shown in Table 1.

TABLE 1. Composition of culturing media.

Medium constituents [g/L]	Culture			
	1	2	3	*4
	with oleic acid	with sunflower oil	with post-deodorizing condensate	
Glucose	100	100	100	100
Yeast extract	5	5	5	5
Oleic acid	100	-	-	-
Sunflower oil	-	100	-	-
Post-deodorizing condensate	-	-	100	100

* In the course of culturing, the medium was supplemented with glucose in the amount of 25g/L/day.

GROWTH CONDITIONS: *Candida bombicola* yeasts were grown on culture media at the temperature of 30°C until the fourteenth day, irrespective of the applied fat substrate. Medium pH was maintained at the level of 3.5 using 5 mol/L NaOH. Optimal pH conditions for the growth of biosurfactants were selected on the basis of our own studies [Gumienna *et al.*, 2001].

ANALYTICAL METHODS: The following parameters were measured in the course of cultivation: increase of biomass [Zhou & Kosaric, 1995], content of reducing sugars – according to the method described by Miller [1959]. Throughout the process, pH value and growth of biosurfactant content were monitored. The content of biosurfactants was measured extracting them from the substrate with ethyl acetate, purifying by hexane and drying at a temperature of 30°C for 4 h and at 45°C for 3 h [Zhou & Kosaric, 1995].

Examination of lipolytic activity. In order to investigate lipolytic activity, the authors applied methodology developed by Mayordomo *et al.* [2000] as well as by Winkler and Stuckman [1978], introducing their own modifications. Lipolytic activity was determined on the basis of the hydrolytic reaction of p–nitrophenyl palmitate (p–NPP) caused by the enzyme secreted into the medium. The indicator compound that allowed determining the enzymatic activity was p–nitrophenol (p–NP). The absorbance coefficient for p–NP was measured at the wavelength of 410 nm.

The assumed unit of enzymatic activity in the experiment was the amount of p–NP μmols released in 1 minute at a temperature of 30°C by 1 mL of post-culture liquid.

Apart from examining the lipolytic activity of media, the authors also assessed optimal enzyme activity in the culture liquid in relation to pH of the reaction environment (Table 2).

The course of analysis. Post-culturing liquid was prepared by separating the biomass and centrifuging it for 20 min at 3000 rpm. The obtained biomass was dried (60°C – 4h and 105°C – 3h) and the supernatant was decanted to a distributor of 250 mL capacity, the water layer was separated, which was then filtered by a filter (until a clear filtrate was obtained).

TABLE 2. Effect of pH change of the reaction environment on lipolytic activity of post-culturing liquid after the fifth day of culturing.

pH	V_{max} [μmol/15/min]	U/mL [μmol/min/mL]	*Relative activity [%]
3.0	6.99	1.17	63.20
3.5	11.10	1.85	100.00
3.8	6.22	1.03	55.70
6.5	3.26	0.54	29.20

* The highest lipolytic activity of post-culturing liquid after the fifth day of culturing with post-deodorizing condensate.

Reaction mixture (2.0 mL) was added to test tubes. The reaction mixture was made of 100 mL phosphate or phthalic buffer (pH 3 – 3.8 – phthalic, pH 6.5 – phosphate), 0.1 g of Arabic gum, and 0.4 g of Triton X–100. Next, a solution of 225 μL of p–NPP was added and diluted in 2-propanol at the concentration of 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.5, 7.0 and 8.0 mmol/L. The obtained mixture was emulsified for 5 min in an ultrasound bath followed by a 3 min water bath at a temperature of 30°C. After initial heating of the mixture, it was supplemented with 400 μL of post-culturing liquid and then incubated for 15 min at a temperature of 30°C.

The reaction was terminated by heating samples to the temperature of 80°C for 2 min in a sealed test tube, then cooling them and adding 264 μL of 5% Trisma Base solution. Absorbance was measured at 410 nm wavelength.

Analysis of fatty acids. Analyses were performed on the post-deodorizing condensate and sunflower oil obtained from hexane fractions developed in the course of isolation and purification of biosurfactants. Gas chromatography was used to analyse the composition of fatty acids in the examined samples. The 0.1 g samples for gas chromatography examination were esterified according to methodology elaborated by Byczyńska and Krzywański [1969].

The examined sample in the amount of 0.1 g was saponified in 0.5 mol/L KOH phials heating them for 15 min at a temperature of 65–70°C. This was followed by methylation of fatty acids in the presence of concentrated H_2SO_4 at a temperature of 65–70°C by keeping in a water bath for 15 min. After cooling, 200 μL of internal standard solution containing 2 mg of methyl ester of heptadecanoic acid was added. Methyl esters were transferred to the hexane layer by means of water.

Chromatographic separation of the obtained methyl esters was performed in Hewlett–Packard 5890 gas chromatographer with a flame-ionising detector (FID) and a "split/splitless" system (1:50). An Innowax capillary column (30 m × 0.25 mm × 0.25 μm) with helium as a carrier gas was applied (1.5 mL/min.). Chromatographic separation was conducted at programmed temperature from 60°C to 200°C (at the rate of 12°C/min) and at the final temperature, which was maintained for 20 min.

Acid identification was carried out on the basis of retention times of standard compounds (Supelco 37 Comopnent Tame Mix), while quantitative interpretation was conducted on the basis of the peak area on the basis of an internal norm – methyl ester of heptadecanoic acid. The assay error did not exceed 2% for each of the quoted acids.

Statistical analysis. Single factor analysis of variance and a significance test were performed. Use was made of Statistica 5.0.

RESULTS AND DISCUSSION

The performed investigations on various culture media aimed at determining lipolytic potentials of the examined strain of *Candida bombicola* yeasts depending on the lipid substrate applied in the medium.

The obtained results indicate that the application in the experimental culture of oleic acid as fat raw material did not stimulate yeasts to develop an extracellular enzymatic apparatus characterised by lipolytic properties since no lipolytic activity was recorded in this substrate (Figure 1). On the other hand, culturing media in which sunflower oil and a post-deodorizing condensate were used as substrates and which contained esters of fatty acids promoted the production of lipolytic enzymes (Figures 2, 3 and 4). This observation was corroborated by Mayordomo *et al.* [2000], who emphasized that lipase production was initiated by substrates containing ester in which one of the radicals contained organic acid. Furthermore, they stressed that lipases can break down esters even of such complex compounds as cholesterol.

Lipolytic activity cultivation of yeasts growing on a medium with sunflower oil reached its maximum of 0.913 μmol/min/mL on the third day of cultivation (Figure 2), while in treatments containing post-deodorizing condensate, the highest activity was observed on the fifth day of culturing reaching, respectively, 1.85 μmol/min/mL and 1.97 μmol/min/mL (medium enriched with glucose) (Figures 3 and 4).

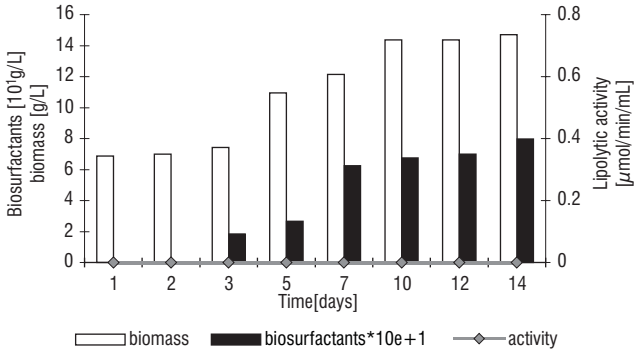

FIGURE 1. Correlations between lipolytic activity, biomass and biosurfactants production in the course of culturing on medium with oleic acid.

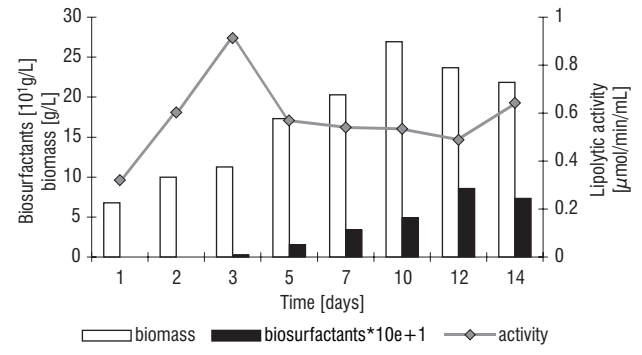

FIGURE 2. Correlations between lipolytic activity, biomass and biosurfactants production in the course of culturing on medium with sunflower oil.

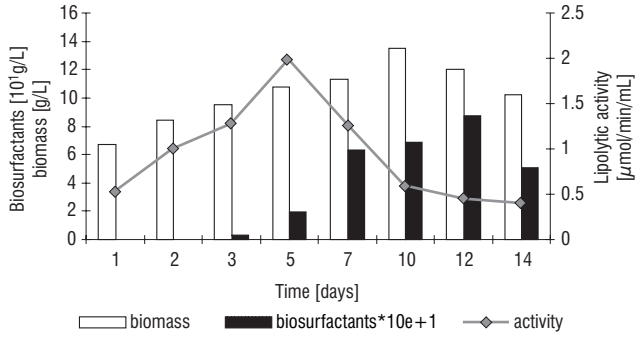

FIGURE 3. Correlations between lipolytic activity, biomass and biosurfactants production in the course of culturing on medium with post-deodorizing condensate.

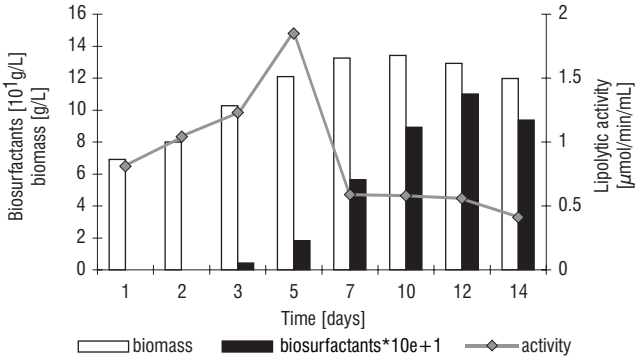

FIGURE 4. Correlations between lipolytic activity, biomass and biosurfactants production in the course of culturing on medium with post-deodorizing condensate enriched with glucose.

Two media with a post-deodorisation condensate applied in this study showed similar lipolytic activities, which did not differ from each other significantly (α=0.05), even though one of the substrates was enriched with glucose during yeasts culturing. Apparently, glucose did not have any influence on the biosynthesis of extracellular lipase production of *Candida bombicola* yeasts (Figures 3 and 4).

In their experiments carried out on lipase produced by *Aspergillus nidulans,* Mayordomo *et al.* [2000] came forward with a thesis different from the one presented above. They investigated substrates containing glucose or glucose and olive oil as the sole source of carbon. They observed that olive oil induced lipase production.

Lower lipolytic activity observed in our study in the case of yeast growing on medium with sunflower oil in comparison with cultures on post-deodorizing condensate, can probably be attributed to differences in fat substrate availability.

The observed difference in lipolytic activities of cultures containing these two substrates also involved the way of linkage of lipolytic activity with biomass growth. Increased lipolytic activity in the medium containing post-deodorizing condensate occurred in the early stages of yeast physiologic growth, *i.e.* from day 1 to day 5 of culturing (Figures 3 and

4). On the other hand, in the case of the medium with sunflower oil, similar activity was shown both by growing cells as well as by those which were in stationary phase (Figure 2). Therefore, in the case of the medium with sunflower oil, no correlation was found between biomass production and lipolytic activity, because lipolytic activity remained on the same level from the third day on (~ 0.55 μmol/min/mL). A different response of this strain of yeasts to this medium can only be explained by the effect of the type of substrate on the yeast lipolytic activity (Figure 2).

In their studies Maia *et al.* [2001] arrived at similar conclusions showing that different fat substrates affected the lipolytic activity of *Fusarium solani* moulds. They applied oils: corn, olive, sesame, palm and coconut but obtained the best lipolytic activity on the substrate containing sesame oil – 0.889 μmol/min/mL.

Additionally, the research for optimum activity of extracellular lipase was done, when the enzyme was produced by yeast in the course of growth on media with ester substrate. As it can be seen from Table 2, in the case of lipases secreted by *Candida bombicola* yeasts, their optimum activity occurred at pH ~ 3.5. This optimum coincided with conditions in which the biosynthesis of biosurfactants was carried out. However, this response is not typical for enzymes of the hydrolases class as shown by studies on the activity of lipase derived from *Aspergillus nidulans*, where optimum for its activity occurred at pH 6.5, while on environment by pH 3.5, this lipase reached only 21% of its activity [Mayordomo *et al.*, 2000].

In addition, such researchers as Winkler and Stuckmann [1978], Mosmuller *et al.* [1992], Kim *et al.* [2000] and Maia *et al.* [2001] conducted investigations on lipase activity in basic environments reporting their optimal action also in this environment.

However, in the light of the obtained results, the fact of low pH of the extracellular lipase produced by *Candida bombicola* yeasts can be attributed to high adaptation capabilities of this strain to conditions in which the process of surfactant biosynthesis took place.

Simultaneously, authors of this study made an attempt to elucidate the mechanism of action of lipases on the applied fat substrate and their link with biosynthesis surface active compounds. According to Schmid and Verger [1998], lipases may take part in surfactant synthesis by preparing the fat raw material found in the medium.

The confirmation of this thesis may be found in the first days of yeasts culturing when changes in the concentration of C_{18} unsaturated fatty acids, both on media with sunflower oil and post-deodorizing condensate, were recorded. Utilisation of these fats in the course of culturing ranged from 60 to 95% in the case of condensate and at 72% – for sunflower oil – in both cases after the 14th day of culturing (Figures 5, 6 and 7). Acids containing from 16 to 18 carbons in a molecule are best utilised by yeasts for glycolipid biosynthesis [Rau *et al.*, 1996].

Changes occurring in the fat substrate referred primarily to C_{18} fatty acids with 1 or 2 double bonds. In the sample with sunflower oil, the content of the dominating linolic acid in the medium after day 3, dropped from 184.0 mg/mL to 57.4 mg/mL, *i.e.* 68% control sample. On the other hand, on the fifth day, when the yeast lipolytic activity reached its maximum value, the content of this acid increased by 10%

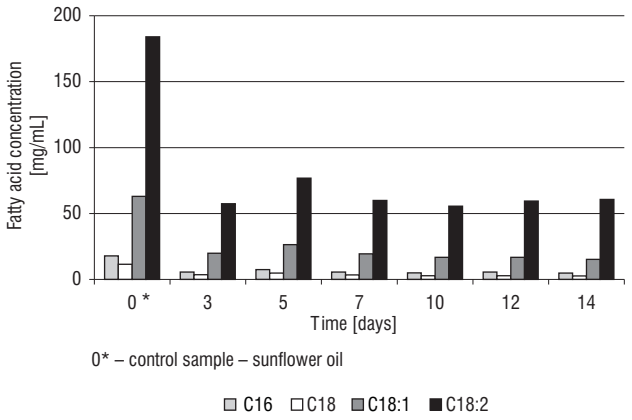

FIGURE 5. Changes in fatty acid content in successive days of culturing on medium with sunflower oil.

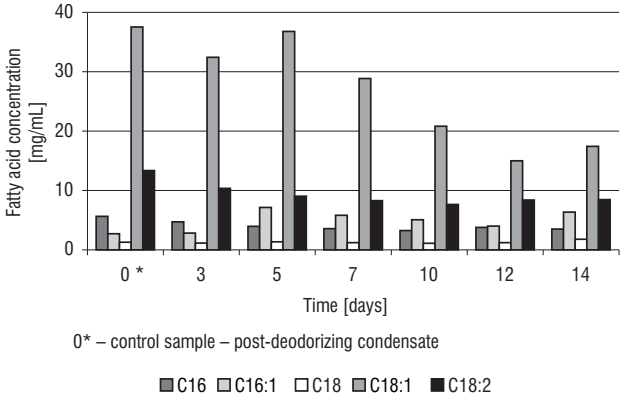

FIGURE 6. Changes in fatty acid content in successive days of culturing on medium with sunflower oil post-deodorizing condensate.

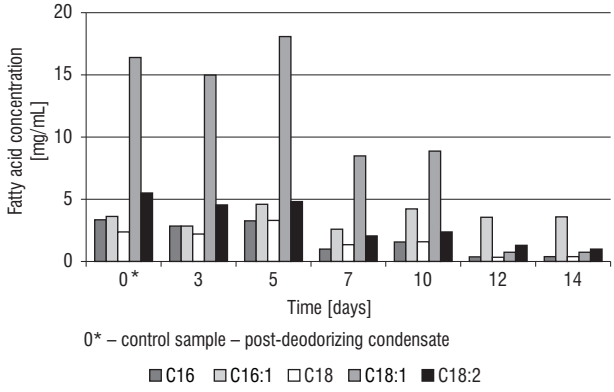

FIGURE 7. Changes in fatty acid content in successive days of culturing on medium with sunflower oil post-deodorizing condensate enriched with glucose.

in proportion to day 3 (Figure 5). Similar relationships were observed in media with post-deodorizing condensates in which the main acid undergoing quantitative changes was oleic acid. After the third day of cultivation, the concentration of this linolic acid in media decreased, respectively: from 37.5 mg/mL to 32.4 mg/mL and from 16.4 mg/mL to 14.8 mg/mL, *i.e.* from 10% to 15% control sample. On the other hand, after day 5, the concentration of this acid in both media increased by 20% in proportion to day 3 (Figures 6 and 7). It was also on this day that the highest lipolytic activity was recorded. Afterwards, both lipolytic

activity and acid concentration in experimental media decreased, while a growth of glycolipid biosynthesis was observed (Figures 2, 3, 4, 5, 6 and 7).

Summing up, the applied post-deodorizing condensate, as a substrate for biosurfactant biosynthesis, can, on the one hand, be a competitive source of hydrophobic carbon in relation to sunflower oil and oleic acid, but on the other, it can be an effective factor inducing production of extracellular lipases by the examined strain. As a waste raw material from fat industry, it can very well be utilised for the biosynthesis of surface active compounds as a cheap source of hydrophobic carbon. The efficiencies of surface active compounds achieved in the course of culturing on media with: oleic acid – 79.8 g/L, sunflower oil – 85.6 g/L and post-deodorizing condensate – 64 and 120 g/L corroborate this thesis (Figures 1, 2, 3 and 4). The highest values of lipolytic activities obtained in cultures with the post-deodorizing condensate indicate high adaptation potentials of yeast enzymatic system to medium composition, culturing conditions and glycolipid biosynthesis.

CONCLUSIONS

1. *Candida bombicola* yeasts manufacture lipolytic enzymes only in the presence of ester substrate: sunflower oil and post-deodorizing condensate.

2. The highest lipolytic activity was achieved by yeasts in cultures on media containing post-deodorizing condensate. On the fifth day of culture this activity was 1.85 μmol/min/mL.

3. Glucose added to medium in the course of culturing did not influence the lipolytic activity.

4. It was found that pH $\sim$ 3.5 was optimal for extracellular lipase production by *Candida bombicola* yeasts.

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WPŁYW SUBSTRATU TŁUSZCZOWEGO NA ZEWNĄTRZKOMÓRKOWĄ AKTYWNOŚĆ LIPOLITYCZNĄ *CANDIDA BOMBICOLA ATCC 22214*

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W pracy przebadano szczep drożdży *Candida bombicola ATCC 22214* pod względem możliwości wytwarzania zewnątrzkomórkowej lipazy i jej aktywności w zależności od zastosowanego substratu tłuszczowego. Jako substrat do podłoży hodowlanych stosowano: kwas oleinowy, olej słonecznikowy oraz kondensat podezodoryzacyjny. Zauważono, że kondensat podezodoryzacyjny najlepiej pobudza aparat enzymatyczny drożdży do produkcji lipaz zewnątrzkomórkowych. W piątym dniu hodowli uzyskano najwyższą ich aktywność wynoszącą około 1,90 μmol/min/mL (rys. 3 i 4). Natomiast podłoża hodowlane, w których substratem był kwas oleinowy nie wykazywały żadnej aktywności lipolitycznej (rys. 1).