The EuroBiotech Journal
RESEARCH ARTICLE
Food & Feed Biotechnology

Utilization of animal fat waste as carbon source by carotenogenic yeasts – a screening study

Ivana Marova[1], Martin Szotkowski[1], Martin Vanek[1], Marek Rapta[1], Dana Byrtusova[1], Nadzeya Mikheichyk[1], Andrea Haronikova[1], Milan Certik[2] and Volha Shapaval[3]

Abstract

Red yeast strains are ubiquitous microorganisms which accumulate substantial amounts of lipids and lipid-soluble metabolites. Red yeasts utilize many waste substrates of different origin. In this work red yeasts strains (*Rhodotorula, Sporobolomyces, Cystofilobasidium*) were used for screening of growth and metabolic activity. As a carbon source waste animal fat and its hydrolysis products were used. Hydrolysis of animal fat was tested in alkaline as well as acidic conditions. As the substrate glucose (control), glycerol, crude animal fat, acid fat hydrolyzate and hydrolysate: glucose 1:2 were used. Screening of growth and metabolic activity of red yeasts was performed by flow cytometry. Extracellular lipase production was monitored as adaptation mechanism. Carotenoids, ergosterol and ubiquinone were quantified by HPLC/PDA/MS/ESI and the biomass was evaluated gravimetrically.

All tested strains utilized fat hydrolysate and produced red coloured biomass. Cultivation in media containing non-hydrolysed fat led to strain specific induction of extracellular lipase. Amount of lipid metabolites produced by individual strains was depended on glycerol content in medium. The highest increase of lipase production was observed in *Cystofilobasidium macerans* and *Sporobolomyces shibatanus*.

Valorisation of animal fat can lead to production of unsaturated fatty acids, single cell oils, carotenoid pigments, sterols and enriched red yeast biomass.

[1]Materials Research Centre, Faculty of Chemistry BUT, Brno, Czech Republic

[2]Faculty of Biotechnical Technology, Slovak Technical University, Bratislava, Slovakia

[3]Norwegian University of Life Sciences, As, Norway

Corresponding author: I. Marova
E-mail: marova@fch.vut.cz

Published online: 27 October 2017
doi:10.24190/ISSN2564-615X/2017/04.08

Introduction

Animal fats are produced via rendering, the process in which the slaughtered animal fat by-products are converted into marketable products, including edible and inedible animal fats for food, agricultural, and industrial use. In Europe, approximately 16 million tonnes of animal fat by-products are processed annually by fat processors and renderers, where Germany, France, UK, and Spain are the main processing countries (1).

According to the ABPR (1069/2009) and the implementation regulation 142/2011, animal fat by-products are defined as either category 1 or category 2 material (1,2). The category 1 fat material consists mainly of specified risk material but includes also animal fat by-products contaminated with environmental contaminants. The category 2 material comprises condemned slaughterhouse fat by-products and dead farm stock. As a result, category 1 material is only permitted for energy generation, while category 2 materials may be used for technical applications as well. According to the above regulations, category 3 material is fat exclusively derived from approved animals, such as slaughtered animals that fit for human consumption, while it is considered as an animal by-product not intended for human consumption (1). According to the regulation, the category 3 fat has to be processed in conformance with rigorous production methods and sanitizing processing conditions. Therefore, the category 3 animal fats could be applied in the animal and fish feed chain and in the oleochemical industry. Further, due to expected growth of the meat production, an increase of the category 3 fat is expected (2).

The fatty acid composition of fats determines the nutritional and economic value. Animal and aquaculture feeds need to supply the essential omega-3 and omega-6 fatty acids via the feed. This is general limited to polyunsaturated fatty acids linoleic (C18:2, n-6), alpha-linolenic acid (ALA C18:3, n-3), arachidonic (AA C20:4, n-6) and eicosapentanoic (EPA C20:5) and in case of aquaculture feeds also docosahexanoic (DHA C22:6, n-3) (3). Animal fat is in general very low in polyunsaturated fatty acids (PUFAs) and it is high in C16:0, C18:0 and C18:1. Thus, today the minimum C18:2 (much more than C18:3) and C18:3 requirements are met by using fat rich grains in combination with plant oils (soybean, linseed oils) (4).

Certain yeasts and filamentous fungi, referred as oleaginous, are capable to accumulate up to 85% (w/w) lipid as a storage compound of the biomass (5). The complexity of fungi is accompanied by a corresponding diversity of lipid components. The oleaginous fungi accumulate lipids such as triacylglycerols (TAGs), which are generally considered as storage lipids (6). Fatty acids of TAGs are very similar to fatty acids in plant oils, where saturated and monounsaturated fatty acids dominate, and some ω6-PUFAs are present. Fatty acids derived from fungal single cell oils represent the whole spectrum from high-volume/low price to low-volume/high price. Examples for high-volume/low price fatty acids are monounsaturated fatty acids and saturated fatty acids used as surfactants, soaps, resins, stabilizers, etc. Low-volume/high price PUFAs may achieve a high market value for the pharmacy and food industry. Currently, filamentous fungi are in industrial used for the healthy PUFAs production (7).

Fungi are unique for utilizing a broad range of carbon sources including pentose sugars, variety of hydrophobic substrates and a large variety of nitrogen sources. For example, it has been shown that some species are capable of degrading lignocellulose and agricultural rest materials (rice straw, orange peels, and stillage of ethanol plants), wide range of hydrophobic rest materials (wastes of vegetable oil production, volatile fatty acids, animal rest materials) while producing a wide range of valuable metabolites (7-15).

The production of single cell oils (SCOs) by fungi has been studied for several decades. Research and development of processes has so far been focused mainly on the utilization of simple sugars as substrates, while use of hydrophobic fat-based substrates has attracted attention just recently. Therefore, limited number of reports aimed to use of different fat materials for the production of high-value fungal lipids are nowadays available (8-13).

The main goal of present study is to study possible upgrading of category 3 fat and edible animal fat into PUFA-carotenoid rich fungal biomass/oils. In order to achieve the sustainable strategy for upgrading of animal fat from SAT-TAGs to PUFA-TAGs, the screening study of the oleaginous red yeasts suitable for upgrading of animal fat will be realized. Several animal fat processing technologies were tested and different fermentation strategies for PUFA-carotenoid rich fungal biomass production were used to evaluate process and product sustainability.

Materials and Methods

Strains

Total of 16 yeast strains of the genus *Rhodotorula, Sporobolomyces, Cystofilobasidium* and *Phaffia* were enrolled into the screening study. A list of strains is introduced in **Table 1**. All strains were purchased from Culture Collection of Yeasts (CCY; Institute of Chemistry, Slovak Academy of Sciences, Bratislava, Slovak Republic) and preserved on malt agar, stored at 4 °C in darkness.

Table 1. List of red yeast strains

Nr	*Strain*	CCY
1	*Rhodotorula aurantiaca*	CCY 20-9-7
2	*Rhodotorula glutinis*	CCY 20-2-33
3	*Rhodotorula glutinis*	CCY 20-2-26
4	*Rhodotorula mucilaginosa*	CCY 20-7-28
5	*Rhodotorula mucilaginosa*	CCY 20-7-31
6	*Phaffia rhodozyma*	CCY 77-1
7	*Cystofilobasidium capitatum*	CCY 10-1-1
8	*Cystofilobasidium capitatum*	CCY 10-1-2
9	*Cystofilobasidium macerans*	CCY 17-9-3
10	*Cystofilobasidium informiniatum*	CCY 18-9-3
11	*Sporobolomyces salmonicolor*	CCY 19-4-6
12	*Sporobolomyces shibatanus*	CCY 19-20-3
13	*Sporobolomyces roseus*	CCY 19-6-4
14	*Rhodosporidium toruloides*	CCY 062-002-004
15	*Sporobolomyces pararoseus*	CCY 19-9-6
16	*Sporobolomyces metaroseus*	CCY 19-6-20

Materials

Crude animal fat was obtained from NorskProtein Company, Norway. Fat processing was done according to **Fig. 1** using mixed sample from different animal sources. The storage time was no longer than 3 months. However, the fat is usually stored by the NorskProtein for 2 weeks at maximum. Antioxidant Thermox liquid FG was added to the fat. Fatty acid composition of animal fat was verified by NorskProtein. About 80% of fatty acids are composed from the sum of palmitic acid (19.5%), stearic acid (21.8%) and oleic acid (38.8%), while content of linoleic and linolenic acids represents about 1%.

Hydrolysis of animal fat

Two types of chemical hydrolysis were tested for animal fat hydrolysis and media preparation. Acid hydrolyses were done in two repetitions in various time intervals (18-72 hours). Basic hydrolyses were done in two parallel analyses in various time ranges. After hydrolysis, glycerol was estimated as the main C source for utilization by yeasts, while limited amount of fatty acids could be used as well.

Before hydrolysis, animal fat was dissolved in chlorophorm. The stock solution of concentration c = 0.428 g/ml was used

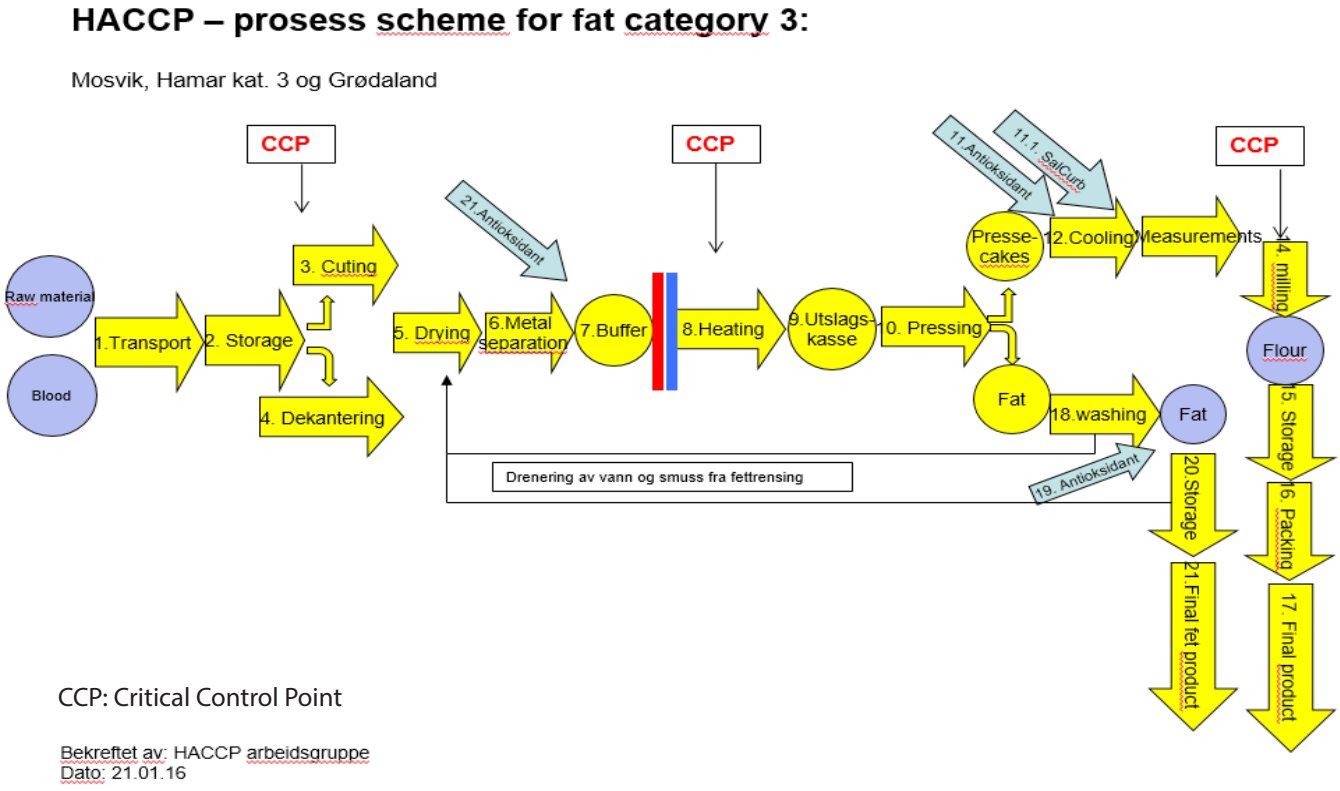

Figure 1. Animal fat processing.

with the aim to dissolve maximal amount of fat in minimal chlorophorm volume. In these experiments 150 g of fat was dissolved in 200 ml of chlorophorm (total volume cca 350 ml). Ratio of water: organic solvent mixture was 2:1, hydrolyses were done at laboratory temperature (21–22°C) and stirring at 800 rpm (revolutions per minute). Saponification with KOH was done at concentration ranges from 2.5% to 20 % for 24 and 48 hours.

Acid hydrolysis was carried out using sulfuric acid in concentration ranges of 1% to 20 % for the period of 18 to 72 hours in glass tubes shaked on shaker. Ammonium hydroxide was used as neutralizing agent. Thus, in aqueous phase $(NH_4)_2SO_4$ as nitrogen source and free glycerol as a carbon source were obtained. Fatty acids were extracted from organic phase.

Cultivation of yeasts on lipid media

Yeast strains were cultivated on glucose medium (30 g/L), fat hydrolysate (acid; 30 g/L), crude animal fat (30 g/L) and on media containing mixture of fat/hydrolysate and glucose (1:2). Two-step inoculation was performed in Erlenmeyer flasks. Optimal inoculation medium (in g/L: glucose 40, $(NH_4)_2SO_4$) 5, KH_2PO_4 5, $MgSO_4 \cdot 7H_2O$ 0.696, yeast extract 7) was used for first inoculation. The first inoculum (100 mL) was cultivated for 24 h at 28 °C under continuous lighting and shaking. Inoculum I was then transferred into 100 mL of fresh inoculum II, which was grown under the same conditions as inoculum I. After 24 h, inoculum II was transferred into Erlenmeyer flasks containing 100 mL of sterile production medium (control medium in g/L: glucose 30, $(NH_4)_2SO_4$) 5, KH_2PO_4 5, $MgSO_4 \cdot 7H_2O$ 0.696, yeast extract 7). Cultivation was carried out at 28 °C, pH 5.5, constant stirring (800 rpm) and under permanent light exposure. Fat containing media and mixed media were composed from the same compounds. The total amount of C-source in all samples was 30 g/L. Cultivations were done in triplicates. As an initial screening, samples were collected after 96 hours of cultivation for gravimetric determination of biomass and determination of metabolites.

For drop test agar (20 g/L) was added into all media. Cultivation on agar medium was done at 28°C in thermostat under permanent lighting for 48-72 hours.

Flow cytometry

For rapid screening of growth and orientational analysis of metabolic activity flow cytometry was used (Apogee, USA) according to (16). To biomass analysis a model equation $B = LALS(SSC) \cdot SALS(FSC) \cdot n$ was used. Correlation curves of three model strains of tested generi were constructed from FC and gravimetry data. For *Rhodotorula* sp. the equation f(x) = 0.6345x – 15.0276 (R^2 = 0.97), *Cystofilobasidium* sp. exhibited relationship: f(x) = 0.7704x – 18.6148 (R^2 = 0.95) and *Sporobolomyces* sp. displayed relationship f(x) = 0.7331x – 17.8355 (R^2 = 0.92). Carotenoids were determined using their autofluorescence (FC; excitation 488 nm, emission FL1 535/35 nm) in relation to total carotenoids (mg; determined by HPLC/PDA) per dry weight of biomass (determined by gravimetry). Relation of total pigments to carotenoid autofluorescence in *R.glutinis* exhibited in all 3 channels good correlation (FL1: f(x) = 4.9x – 61.26; R^2 = 0.97; FL2: f(x) = 4.18x; R^2 = 0.93; FL3: f(x) = 5.14x; R^2 = 0.92). Lipids were determined after Nile Red staining FC; excitation 488 nm, emission FL1 535/35 nm).

If not specified, the results are introduced in arbitraty units. In the preliminary study detailed quantitative analysis of flow cytometry data was not performed because of screening character of experiments, unpredictable results and supposed low production of biomass and metabolites in most of strains (see **Fig. 2**). With regard to descriptive character of data and relative high differences no statictical analysis was done.

Analysis of yeast metabolites

Biomass

The produced biomass was evaluated gravimetrically according to (15,17).

Glycerol

Glycerol concentration was analysed by RP/HPLC according to (15).

Lipid soluble metabolites

Carotenoids, ergosterol and ubiquinone were quantified by simultaneous HPLC/PDA/MS/ESI

After finishing cultivations samples were collected for extraction and analysis of yeast metabolites. Samples were then centrifuged at 5000 RPM for 10 min (Sigma 3-15, Sigma Laborzentrifugen GmbH, Germany), washed in distilled water and centrifuged again. The pellet was afterwards resuspended in acetone and mechanically disrupted by pestle and mortar. After saponificantion by ethanolic solution of KOH at 90°C extraction by diethylether was performed and the extract was dryed on rotary evaporator. Dry extract was dissolved in 1-2 mL of UV-VIS grade ethanol. Samples were then filtered using 0.45 µm PTFE filters and 10 µl of each sample was injected onto column Kinetex C18 5 µm, 150 × 4.6 mm (Phenomenex, USA) with guard column 5 µm, 30 × 4.6 mm, both equilibrated to 45°C with methanol as elution solvent (flowrate 1 mL/min), on ThermoFinnigan Surveyor HPLC system. Xcalibur software was used for chromatography data analysis. Carotenoids content was evaluated as in (15,18).

Production of lipase

Activity of lipase was determined by colorimetry using p-nitrophenylpalmitate as a substrate. Lipase hydrolyzes this substrate to form yellow-coloured p-nitrophenol, which can be determined at 410 nm. Calibration curve of p-nitrophenol was constructed in the range of 0.005 to 0.05 mmol/l. Substrate strock solution (0.0135 g/100 mL) was stored at 4°C for no longer than 24 hours. The activity unit was expressed in nmol of product per ml of medium during 1 min.

Results

Fat hydrolysis

The effectiveness of hydrolysis was monitored by determination of glycerol concentration in hydrolyzate by HPLC/PDA.

Basic hydrolysis (saponification) lead to formation of glycerol and fatty acids salts. Hydrolysis yields after 24 hour of saponification reaction by 20% KOH ranged from 70 -75% and in 48 hour acid hydrolysis approximately 80%. Saponification exhibited high yields of hydrolysis. Saponification is quick method good for extraction and separation of fatty acids, but less suitable for microorganism cultivation, due to large salt concentrations in cultivation medium. Separation of fatty acids salts and glycerol from aqeous solution is rather complicated and may be relatively expensive in industrial scale. The duration of basic hydrolysis longer than 48 hours did not exhibite substantial effect on hydrolysis yields. At the 100% effectiveness of hydrolysis, amount of glycerol in pure fat is about 10%. Thus, the concentration of glycerol in hydrolyzate should be 50 – 100 g/L, which is comparable to control cultivations.

The products of acid hydrolysis are glycerol and free fatty acids. The hydrolyzate should be neutralized before further use. The best yield of glycerol was obtained using 15% of sulfuric acid for 48 hours. The results were quite the same even with increasing concentrations of fat in samples. Acid hydrolysis is better for microorganism cultivation. Using ammonium hydroxide as neutralizing agent we obtain in aqeous phase $(NH_4)_2SO_4$ as nitrogen source and free glycerol as carbon source. Fatty acids can be easily extracted from organic phase.

For consequent cultivations acid fat hydrolyzates (30 g of fat/100 mL media) neutralized by ammonium sulphate were used in screening experiments.

Screening of growth and metabolic activity of red yeasts

For screening experiments 13 red yeast strains were used. Extreme differences in biomass production on glucose and fat were observed using flow cytometry experiments (**Fig. 2**; expressed as cell count). In **Fig. 2** are presented descriptive data of cultivation on crude animal fat and mixture of fat and glucose only. *Cystofilobasidium* genus seems to utilize fat into biomass most likely. In medium with sufficient glucose concentration yeasts can divide to higher degree. Using mixed substrate, any inhibition by some hydrolyzate/fat component can not be excluded.

Production of carotenoids (data not shown) was better in media with addition of glucose than in crude fat medium. *S. shibatanus* strain seems to be succesfull in all tested media. *C. infirmominiatum* utilized predominantly fat medium, production of pigments was quite high, similarly to biomass production.

Intracellular concentration of carotenoids (**Fig. 2**, carotenoids per cell) surprisingly exhibited no dramatic differences between acumulation of carotenoids in mixed substrate medium and control medium. Accumulation in medium with fat only was around 50% of the values in control, which is substantially better result when with biomass production.

The production of total lipids monitored by FC was quite high in *Shibatanus* sp. in control and fat media, while cultivation in mixed substrate was not successful. *Cystofilobasidium* sp. produced lipids even if in media is any glucose. Only a few of *Rhodotorula* species provides good yields of lipids in crude fat medium.

Intracellular lipid concentration (**Fig. 2**, lipids per cell) was very low in control medium with exception of one strain of *R.*

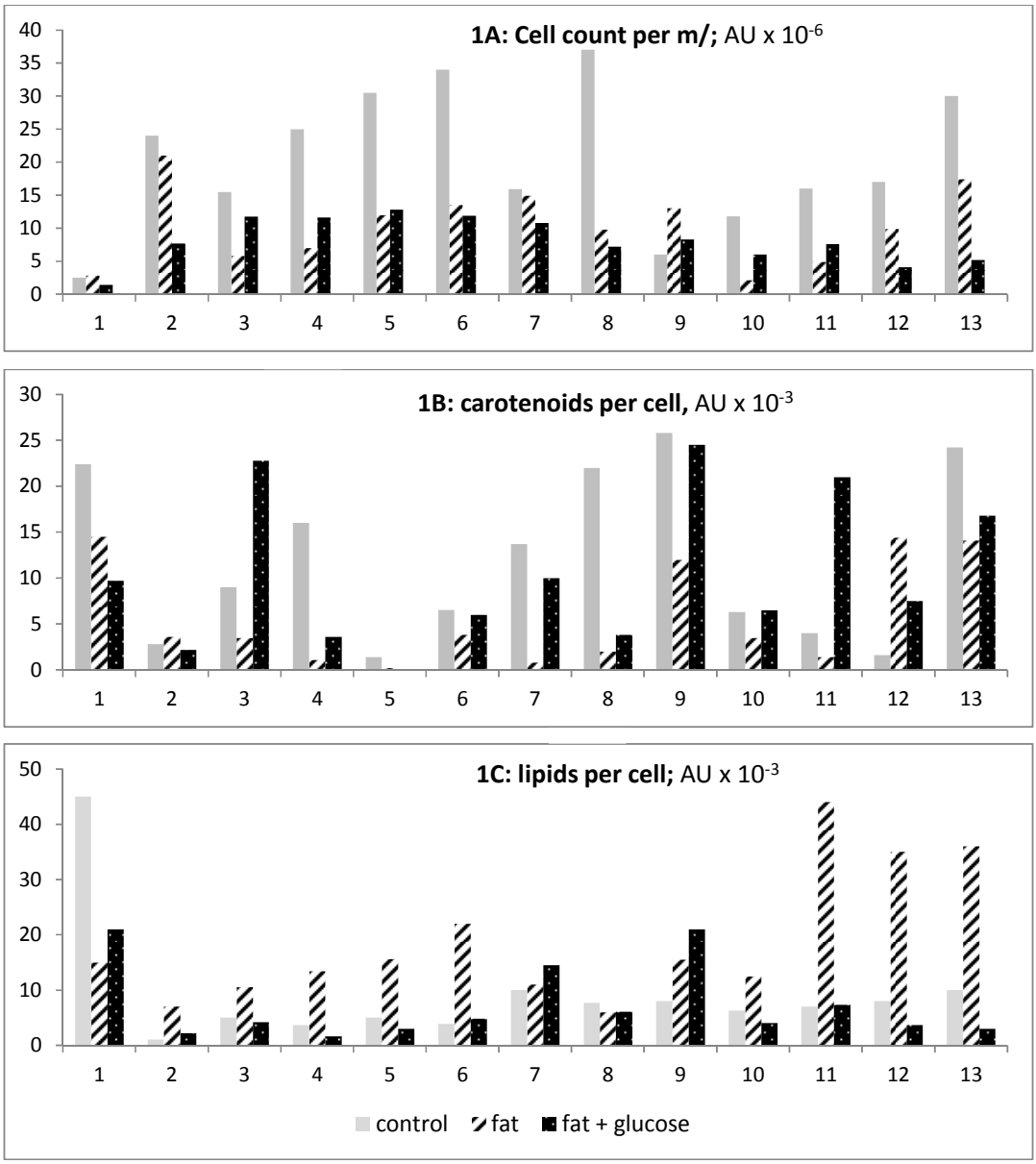

Figure 2. Flow cytometry evaluation (screening) of growth (1A) and metabolic activity of red yeasts (1B, 1C) grown in glucose medium (40 g/L), crude fat medium (5 g/L) and crude fat + glucose, 5 + 10 g/L). Data are expressed in arbitrary units. Description of strains: In all graphs the order of strains was as follows: 1… *Rhodotorula glutinis CCY 20-2-26, 2… Rhodotorula glutinis CCY 20-2-33, 3…Rhodotorula mucilaginosa CCY 20-2-31, 4… Rhodotorula mucilaginosa CCY 20-2-28, 5… Rhodotorula aurantiaca* CCY 20-9-7, 6…*Phaffia rhodozyma* CCY 77-1, 7…*Cystofilobasidium capitatum* CCY 10-1-1, 8…*Cystofilobasidium macerans* CCY 17-9-3, 9…*Cystofilobasidium infirminiatum* CCY 18-9-3, 10…*Sporobolomyces roseus* CCY 19-6-4, 11…*Sporobolomyces metaroseus* CCY 19-6-20, 12…*Sporobolomyces pararoseus* CCY 19-9-6 ,13… *Sporobolomyces shibatanus* CCY 19-20-3.

glutinis. Results of *Sporobolomyces* sp. were the best in pure fat medium, while *Cystofilobasidium* produced lipids in medium with mixed substrate. Yeasts strains of both genus *Sporobolomyces* and *Cystofilobasidium* were successful producers of lipids in control media.

Growth and production properties of selected red yeast strains

Selected strains from general screening experiments (see **Fig. 2**) were repeatly cultivated in Erlenmeyer flasks to determine growth and metabolic activity in detail. Before cultivations, orientational drop test was performed on agar plates using all tested substrates including glycerol. Results of drop-tests of selected 6 red yeast strains (*R. glutinis*, *R. mucilaginosa*, C. *capitatum*, *C. macerans*, *S.shibatanus*, *S. pararoseus)* cultivated on glucose, glycerol, crude fat, acid hydrolyzate and mixture of acid hydrolyzate + glucose are documented in **Fig. 3**. All strains were cultivated for 48 hours at permanent lighting and 28°C.

The results indicate that red yeasts are able to utilize crude animal fat and products of its hydrolysis. Different utilization of glycerol vs. glucose can be observed as a typical example

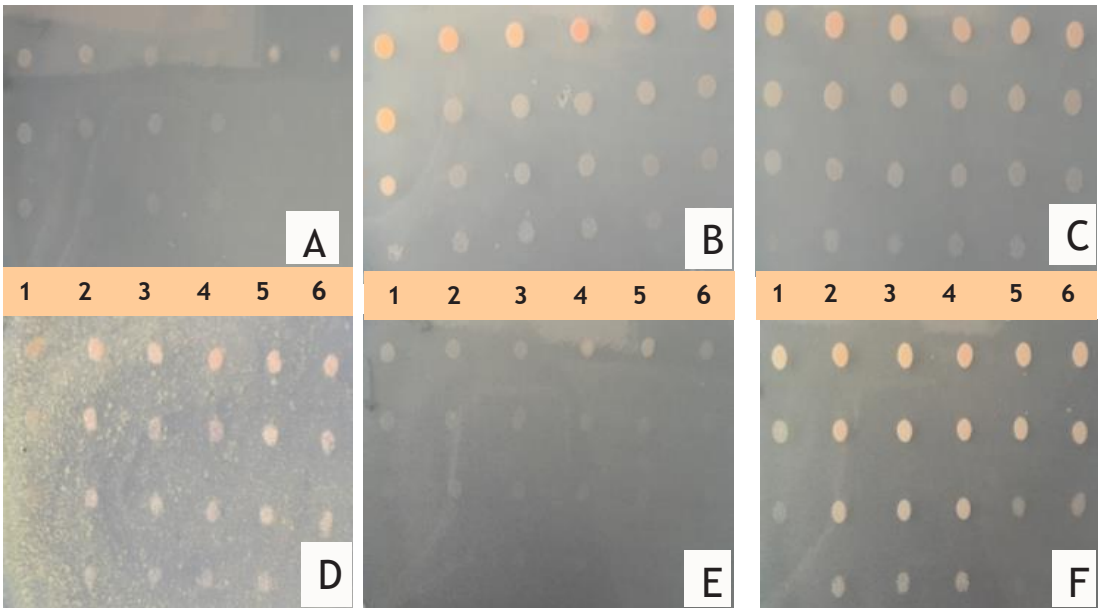

Figure 3. Drop-tests performed by tested red yeast strains growing on different fat media. A) negative control, B) positive control (glucose), C) glycerol; D)crude fat; E) acid hydrolyzate; F) acid hydrolyzate + glucose, 1:2 (all strains grown 48 hours at permanent lighting. Strains: *1-Rhodotorula glutinis, 2-Rhodotorula mucilaginosa, 3-Cystofilobasidium capitatum, 4-Cystofilobasidium macerans, 5-Sporobolomyces shibatanus, 6-Sporobolomyces pararoseus.* Lines: First line – OD = 1; 2nd line OD = 0.1, 3rd line – OD = 0,01; 4th line – OD = 0,001.

for strain specific stress response to glycerol concentration. Quite confusion results were obtained through indrop test performed with fat hydrolyzate alone; substantially lower values of biomass and produced metabolites were found also in following experiments in liguid medium.

Biomass production was further monitored in detail in 5 selected strains (see **Table 2**). The best biomass production was found in *C. macerans* (medium: fat hydrolyzate + glucose) and in *S.shibatanus* (medium: fat hydrolyzate + glucose). Relatively good results showed cultivations of all tested strains on a fat with the addition of glucose, while cultivation without glucose led to very low biomass formation.

The results introduced in **Fig. 4** show that using fat substrate production of microbial pigments is possible. Moreover, in some strains production of total carotenoids and ergosterol in medium cintaining fat hydrolyzate and glucose (1:2) was increased. It can be concluded, that red yeasts are able to utilize crude animal fat and products of its hydrolysis. Different production of lipid soluble metabolite is caused probably by different utilization of glycerol vs glucose; previously strain specific stress response was observed (15). Both *S.shibatanus* and *C.macerans* belong to oleaginous strains, because they are able to produce more than 20% of lipids per biomass (see **Table 3**).

All results obtained from 5 monitored strains undergone statistic analysis via two-way ANOVA test in IBM SPSS 23 which approved statistical significance at a probability level of $p<0.05$. Therefore, the differences in achieved production of biomass on animal fat subterates can be considered as significant in between and across selected strains.

The best producers of biomass were analyzed for production of specific red yeast metabolites. The results are documented in **Fig. 4**.

S. shibatanus is probably the best for accumulation both carotenoids and lipids in fat media. A lot of fat remain in media after the end of cultivation. More nitrogen addition to fat media should be tested to provide more potential to growth. Addition of nitrogen led to more fat consumed and higher yields. Addition of non-toxic surfactant should be beneficial, too.

Table 2. Production of biomass by selected strains of red yeasts on animal fat substrates used as C-source (g/L)

strain	control	Fat hydrolyzate	Fat hydrolyzate + glucose	Crude fat	Crude fat + glucose
C.macerans	9.80±1.15	6.45±1.22	12.70±2.54	10.75±3.24	9.35±2.28
R.mucilaginosa	3.95±0.85	1.62±0.35	3.52±0.66	3.30±0.98	2.85±0.95
C.informiniatum	3.70±0.65	1.08±0.45	1.98±0.51	1.64±0.62	2.25±0.85
S.pararoseus	4.68±0.92	2.83±0.72	3.65±0.98	2.90±1.08	4.11±1.32
S.shibatanus	10.11±0.95	5.80±1.12	9.15±2.85	5.86±2.06	9.24±3.10

Table 3. Production of pigments and lipids by selected strains of red yeasts on animal fat substrates used as C-source (g/L)

parameter	*C.macerans*		*S.shibatanus*	
	control	Fat hydrolyzate + glucose	control	Fat hydrolyzate + glucose
Biomass, g/L	9.80±1.15	12.70±2.54	10.11±0.95	9.15±2.85
Total carotenoids ug/g of dry biomass	1980±250	1650±520	570±112	680±98
Ergosterol ug/g of dry biomass	1100±192	2100±154	720±82	1615±128
Ubiquinone ug/g of dry biomass	112±20	254±28	162±34	198±12
Total lipids, %	18.2±2.5	29.8±3.6	31.1±2.0	21.3±3.1

Similarly to different adaptation to glycerol substrate, cultivation in media containing non-hydrolysed fat led to strain specific induction of lipase and utilization of crude fat. The highest increase of lipase production was observed in *C. macerans* and *S. shibatanus* (**Fig. 5**). Statistical significance of different production of pigments and lipids was approved via two-way ANOVA test at a probability level of $p<0.05$.

Yeasts were grown in glucose medium (30 g/L), fat hydrolyzate, fat hyrolyzate + glucose (1:2), crude fat medium (30 g/L) and crude fat + glucose (1:2 g/L). Lipase activity is expressed in nmol/ml.min.

Discussion

Technologies used for modifying lipids can be grouped into chemical and biological technologies. Depending on the purpose of lipid modification, different chemical methods have been developed. There are methods for separating different lipid fractions (fractionation), for producing lipid mixtures with improved properties (2) (blending) and for producing lipid products with a higher melting point (hydrogenation) (3). For changing triacylglycerol composition by rearranging of fatty acids and upgrading fat to a higher degree of unsaturation, interesterification is the most popular approach. Interesterifica-

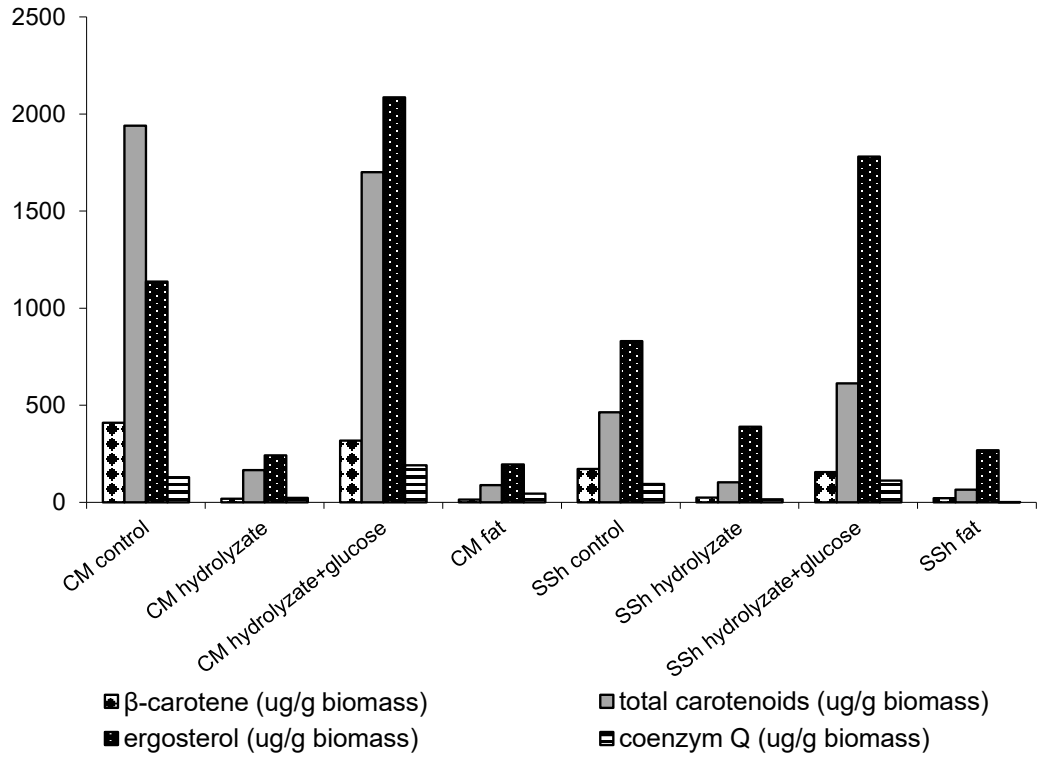

Figure 4. Production of yeast metabolites by *C. macerans* and *S. shibatanus.*

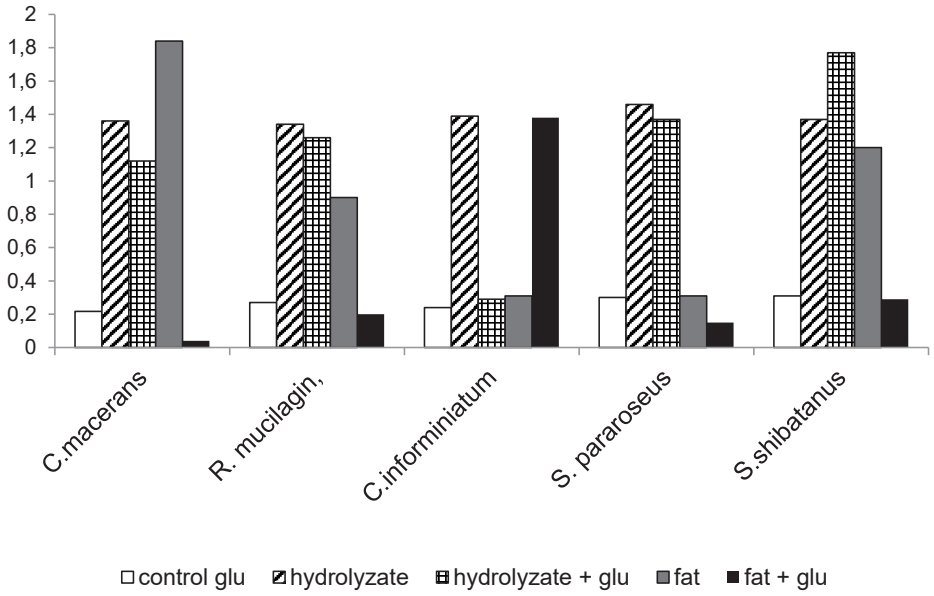

Figure 5. Production of extracellular lipase by selected red yeasts.

tion is performed by using alkoxide or enzymes (lipases) (4). The alkoxide-based interesterification process is not useful for feed or food production, since it produces randomly distributed fatty acid residues (2,5), while lipase-mediated upgradation leads to precisely structured triacylglycerols, which can be used in feed and food production processes. This process requires quite cheap equipment and produces less waste than alkoxide-based processes. Nevertheless, the commercial exploitation of lipase-mediated upgradation of the animal fats is limited, since the production of lipases is very costly (4).

A new technology for upgrading fat presented in this work is based on fermentation by oleaginous microorganisms. It is a biological technology for converting hydrophobic substrates such as vegetable oils, volatile fatty acids and animal fat into higher value single cell oils enriched in unsaturated fatty acids. Certain yeasts and fungi known as oleaginous and have the ability to accumulate from 20% up to 85% (w/w) lipids as storage compound of the biomass (6). In addition, some oleaginous microorganisms are able to perform simultaneous production of lipids and carotenoids (8,15,17,18). The oleaginous fungi accumulate lipids primarily as triacylglycerols (TAGs), which are generally considered as storage lipids. Fatty acids of TAGs are very similar to fatty acids in plant oils, where saturated and mono-unsaturated fatty acids dominate, and some ω6-PUFAs are present (5).

Fungi and yeasts can degrade a wide range of rest materials, while producing a wide range of valuable metabolites (7-18). In recent pilot study was shown that oleaginous fungi from phylum *Zygomycetes* can convert the saturated fatty acids containing triacylglycerols (SAT-TAGs) into polyunsaturated fatty acids containing triacylglycerols (PUFA-TAGs). After five days of fungal fermentation was observed that animal fat containing 46.3% of saturated fat (SAT), 40.3% of monounsaturated fat (MUFA) and 6.5% of polyunsaturated fat (PUFA) was transformed into fungal single cell oil where SAT was reduced to 33.5%, MUFA was reduced to 30.8% and PUFA was increased to 28%, where AA was 11.2%, DHA was 1% and EPA was 0.5% (7,8). Similar resuts can be expected also in red yeasts analysed in this work. Composition of fatty acid in these strains is as follows: saturated FA 20-47%, unsaturated FA 53-81% (some of them are able to produce more than 25% of ALA) (19).

In this work two strategies for upgrading of animal fat by red yeasts were tested: (i) direct upgrading, when animal fat is utilized by microorganisms in submerged fermentation and (ii) indirect upgrading, when fat is first hydrolysed and then different hydrolysis fractions are used for fermentation. The choice of the strategy depends on the type of microorganism to be used for the fermentation and fat preparation. The targeted fatty acid profile, conversion rate and biomass yield depend on the fungal strain (14). Therefore, there is a need to discover best fat upgrading strategies which are the most sustainable with respect to biomass production, lipid accumulation, and fat conversion rate.

For these purposes, a screening study was performed to select the most appropriate yeast strain(s) for upgrading of fat. Various microorganisms are capable of utilizing different hydrophobic materials (15-18). In order to use saturated triacylglycerols derived from animal fat as substrate, the types of yeasts and fungi used to upgrade them should carry out lipase-catalysed ester hydrolysis (15,16). Thus, a selection of appropriate strains, which are able to simultaneously produce lipases for ester hydrolysis and to transform and accumulate single cell oils, need to be identified. In this work induction of lipase production was confirmed in most of red yeast strains grown on crude fat substrate. This enzyme is produced extracellularly, could be better characterized and used for fat processing.

Selecting a suitable fermentation strategy and up-scaling of

fermentation is further problem. Submerged fermentation is very well developed for yeasts as well as for fungi, nevertheless, more research and development is needed in order to understand which strategy to choose in order to develop economically and environmentally sustainable fermentation processes (7,15). Regarding the cultivation conditions, yeast cells used in present work have some advantages, such as high production of biomass during relatively short time, ability to utilize waste substrates, activation of stress response mechanisms leading to overproduction of special and rare metabolites, submerged cultivation and simple manipulation.

Conclusions

The present work gives the basis to development innovative technology for upgrading of bio-based rest material, namely animal slaughter fat by-products by fungal fermentation processes. It will develop technology for conversion of animal fat materials into high-value lipids and carotenoids-rich fungal biomass for animal feed.

It can be concluded that red yeasts are able to utilize crude animal fat and products of its hydrolysis. As adaptation mechanism, strain specific extracellular lipase production was observed. Specific strain response to fat and fat hydrolysate was confirmed, which could be related to different utilization of glycerol compared with glucose and strain specific stress response. Hydrolysis of crude fat can be realized in acid and alkaline conditions. For biotechnological purposes, acid hydrolysis follwed by neutralization using ammonium sulphate and extraction of fatty acids (could be used partially as detergent) could be recommended.

Valorization of animal fat by red yeasts can lead to production of unsaturated fatty acids and single cell oils, carotenoid pigments, ubiquinone and enriched cell biomass. This biomass contains substantially better ratio of SFA/PUFA than animal fat and can be used for feeding purposes.

Acknowledgments

This work was supported by the projects „Materials Research Centre – Sustainability and Development" Nr. LO1211 of the MSMT and „Lipofungi" - Nr. 268305 of the Research Council of Norway.

Conflict of interest statement

The authors declare there is no conflict of interest.

References

1. Stephen L. Fats and Oils – Animal Based. Food Processing: Principles and Applications, Edited by Clark S, Jung S, and LamsalB. John Wiley and Sons 2014.
2. Gunstone FD. Modifying lipids for use in food. 1st Edition, 2006.
3. Doppenberg J. All About Feed. Nordic Nutrition Recommendations 2012; 23(5) 9-11.
4. Gupta R, Rathi P, Bradoo S. Lipase mediated upgradation of dietary fats and oils. Critical Reviews in Food Science and Nutrition 2003.
5. Kendrick A, Ratledge C. Lipids of selected moulds grown for production of n-3 and n-6 polyunsaturated fatty acids. Lipids 1992; 20: 15-20.
6. Kosa G, Kohler A Shapaval V, et al. Microtiter plate cultivation of oleaginous fungi and monitoring of lipogenesis by high-throughput FTIR spectroscopy. Microb Cell Fact 2017; 16: 101.
7. Shapaval V, Afseth NK, Vogt H, Kohler A. Fourier transform infrared spectroscopy for the prediction of fatty acid profiles in *Mucor* fungi grown in media with different carbon sources. Microb Cell Fact 2014; 4: 13-26.
8. Ferreira JA, et al. A Biorefinery from *Nannochloropsis* sp. microalga – Energy and CO2 emission and economic analyses. Biores Technol 2013; 135:523-32.
9. Papanikolaou S, Dimou A, Fakas S, et al. Biotechnological conversion of waste cooking olive oil into lipid-rich biomass using *Aspergillus* and *Penicillium* strains. Appl Microbiol 2013; 56: 1138-1150.
10. Aggelis G, Sourdis J Prediction of lipid accumulation – degradation in oleaginous microorganisms growing on vegetable oils. Antonie Van Leeuwenhoek 1997; 72: 159–165.
11. Papanikolaou S, Aggelis G. Lipid production by *Yarrowia lipolytica* growing on industrial glycerol in a single-stage continuous culture. Bioresour Technol 2002; 82:43–49.
12. Papanikolaou S, Aggelis G. Modeling lipid accumulation and degradation in *Yarrowia lipolytica* cultivated on industrial fats. Curr Microbiol 2003; 46: 398–402.
13. Roux-Van der Merwe MP, Badenhorst J. and Britz T.J. Fungal treatment of an edible-oil-containing industrial effluent. World J Microbiol Biotechnol 2005; 21: 947–953.
14. Papanikolaou S, Aggelis G. Lipids of oleaginous yeasts. Part II: Technology and potential applications Eur J Lipid Sci Technol 2011; 113:1052-1073.
15. Petrik S, Hároniková A, Márová I, Kostovová I, Breierová E. Production of biomass, carotenoids and other lipid metabolites by several red yeast strains cultivated on waste glycerol from biofuel production - a comparative screening study. Annals Microbiol 2013; 63: 1537-1551.
16. Lopes da Silva T, Dias C, Silva C. Effect of Medium pH on *Rhodosporidium toruloides* NCYC 921 Carotenoid and Lipid Production Evaluated by Flow Cytometry. Appl Biochem Biotechnol 2016.
17. Marova I, Carnecka M, Halienova A, Dvorakova T, Haronikova A. Use of several waste substrates for carotenoid-rich yeast biomass production. JEMA 2013; 95: S338-S342.
18. Marova I, Carnecka M, Halienova A, Koci R, Breierova E. Production of carotenoid/ergosterol-supplemented biomass by red yeast *Rhodotorula glutinis* grown under external stress. *Food Technol Biotechnol* 2010; 48(1): 56-61.
19. Kot AM, Blazejak S, Kurz A, Gientka I, Kielszek M. *Rhodotorula glutinis* - a potential source of lipids, carotenoids, and enzymes for use in industries. Appl Microbiol Biotechnol 2016; 100: 6103–6117.