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Biosynthesis of highly unsaturated fatty acids by hydrocarbon degrading microorganisms

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ABSTRACT

Disruption of polyunsaturated fatty acids (PUFA) metabolism leads to many diseases. In this study, producers of γ-linolenic acid (GLA), arachidonic acid (ARA) and eicosapentaenoic acid (EPA) were selected: *Cephalosporium humicola IE* (on glucose, dry biomass – 14 g/l, total lipids – 18-20%, GLA in lipids – 12.0%), *Mucor globosus 11* (respectively – 15 g/l, 18% and 5%) and *Pythium irregulare LX* (on glucose, dry biomass – 14.5 g/l, total lipids – 18-20%, 9.2 and 7.8% of ARA and EPA, respectively). On crude oil as the only source of carbon, the amount of biomass of the specified fungi decreases by 3-4 times, whereas the quantity of lipids and highly unsaturated fatty acids increases in four and 1.2 - 3.4 times, respectively. The maximum γ-linolenic acid in *M. globosus* and *C. humicola* was detected at neutral pH. Optimum volume of inoculate was 2.0-4.0%, nitrogen source NH_4NO_3, a carbon-nitrogen ratio 34:1. For biosynthesis of ARA and EPA by *P. irregulare*, the optimum nitrogen source was NH_4Cl, pH 7.0- 8.0 and C/N - 50:1 at 28°C. The process of adaptation to stressful situation under crude oil motivated the increase of the rate of membrane phospholipids with high quantity of unsaturated fatty acids.

Key words: *Cephalosporium humicola*, *Mucor globosus*, *Pythium irregulare*, single cell oils, polyunsaturated fatty acids, omega, optimization

Abbreviations

C/N = Carbon/ Nitrogen
PUFA = Polyuunsaturated fatty acids
EPA = Eicosapentaenoic acid
GLA = Gamma-linolenic acid
ARA = Arachidonic acid
SCO = Single-cell oil
HPLC = High-Performance Liquid Chromatography
UV = Ultra violet
AMEA = Azerbaijan National Academy of Sciences
ME = Malic enzyme
ACL = ATP citrate lyase
G-6PGDH = glucose-6-phosphate dehydrogenase
FAS = Fatty acid synthase
ACC = Acetyl-CoA carboxylase
DAGAT = Diacylglycerol acyltransferase
ICL = Isocitrate lysase
CAT = Carnitine acetyltransferase,
PK = Pyruvate kinase

Introduction

Polyunsaturated fatty acids (PUFA), which have 18 and up carbon and two or more double bounds in their structure, participate in whole normal activities of all organisms, from prokaryotes to multicellular eukaryotes. These compounds play a vital role including energy source and structural components in the mammalian cells. Not surprisingly, disruption of lipids and fatty acids metabolism leads to many diseases such as skin, nervous system, immune system, inflammation, cardiovascular, atherosclerosis, endocrine system, kidneys, respiratory and reproductive systems and obesity (Certik & Shimizu, 1999).

Currently, the elderly as well as children are in high demand for unsaturated fatty acids, as nutritional supplements. Unsaturated fatty acids, especially the long-chain and high unsaturated fatty acids are advised through diet in order to improve coronary heart problems, retinal eye and brain functions. Eicosapentaenoic acid (EPA; 20:5n-3;

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omega three or ω-3), gamma-linolenic acid (GLA; 18:3n-6; omega six or ω-6) and arachidonic acid (ARA; 20:4n-6; omega 6 or ω-6) are not synthesized in the human body and they must be added to foods. Synthesis of these PUFAs goes by carbon chain elongation and desaturation. PUFAs metabolic phases are carried out by Δ-6 desaturase, Δ-5 desaturase and elongase enzymes. Mammals are not able to synthesis long-chain PUFA because of the lack of compatible enzymes. Therefore, they are obliged to adopt them with food (Jin et al., 2008).

Natural sources of PUFA are plants, animals and microorganisms. But, throughout history, people have only utilized the nutrition oils of plants and animals such as butter and lard. Microbial oil or "single-cell oils"(SCO) is relatively new concept, and for the first time has been proposed in 20th century (Ratledge, 2004). However, the cost of SCO is rather high than plant and animal oils, so that obtaining oil by microbiological pathways are less likely in industrial scales, even in the near future (Higashiyama et al., 2002; Armenta & Mercia, 2013). In this regard, recent attempts have been directed only onto biosynthesis of the high value polyunsaturated fatty acids as food supplements, which cannot be produced by the plants (Sijtsma & Swaaf, 2004; Laoteng et al., 2011; Uemura, 2012).

Microorganisms that synthesize the PUFA are fungi, bacteria and seaweeds (Ratledge et al., 2001; Kyle, 1996, 1997. However, bacteria are not suitable because they do not contain high amounts of triacylglycerol and also they have unusual fatty acids and lipids, that the other systems do not deal with them. Oleaginous micromycetes are economically feasible sources of PUFA. Fungi are an alternative to fish oil due to their high content of PUFA. In addition, there were no other unsaturated fatty acids in the lipid composition of micromycetes, and they also have unique PUFA (Kennedy et al., 1993). At the present time, we can say that microorganisms are not utilized to production of PUFA. However, there are a number of serious reasons for continuing research. Firstly, microorganisms, which produce high amount of PUFA, should be selected, secondly, the microbial stability of lipids oxidation is higher than fish oil (Ratledge, 2012), third, PUFA can be produced from cheaper raw materials. In addition, knowledge gained from genetics and biochemistry of biosynthesis of PUFA is useful for new production systems or products. Thus, the study of biosynthesis, metabolism and regulation of lipids in fungi, not only has theoretical interest, but it is also very important for modern medicine, veterinary and agriculture.

The purposes of this work were the selection of the fungi species for biosynthesis of PUFA, determination of the conditions for obtaining the required high amounts of the product as well as to study the effect of stress conditions on the synthesis of fatty acids and lipogenesis.

Materials and Methods

Screening and isolation of oleaginous fungi from oil-contaminated soil

The screening of fungi was carried out using the Microorganism Culture Collection of the Institute of Microbiology at Azerbaijan National Academy of Sciences. The fungi already have been isolated from oil-contaminated soils near Baku, Azerbaijan. The species of class Zygomycetes, in order Mucorales, *Mucor mucedo*, *M. circinelloides*, *M. globosus 11*, and order Mortierellales, *M. alpina*, *M. elongate*, *M. spinosa*, *M. humilis*, *M. isabellina*, and *Cephalosporium humicola IE*, which have the ability to synthesize the C-18 fatty acids, were cultured by deep cultivation method in rotary incubator shaker 220 rpm (Shimizu et al., 1988; Bajpai et al., 1991; Saxena et al., 1998; Papanikolaou & Aggelis, 2003; Papanikolaou et al., 2004; Ratledge, 2004; De & Kumar, 2005; Szczesha-Antczak et al., 2006; Tao & Zhang, 2007; Fakas et al., 2007, Fakas et al., 2008a, 2008b). The composition of broth medium was 3% glucose, 0.2% sodium nitrate, 0.1% potassium dihydrogen phosphate, 0.05% potassium chloride, 0.05% magnesium sulphate heptahydrate, and 0.001% iron (II) sulphate heptahydrate (pH 5.5). Culture flasks (500 ml) containing 200 ml of the medium were inoculated with vegetative mycelium and cultivated for 5-10 days at 28°C. Firstly, flasks were periodically removed from the incubator and mycelia were collected by centrifugation at 22000 rpm for 20 minutes and washed once with distilled water. Then lipids were extracted and analyzed by HPLC to determine fatty acids compositions (Bajpai & Bajpai, 1992; Andreishcheva et al., 1999).

Moreover, micromycetes in contaminated soils are considered to be the object of the experiment. For this purpose, gray-brown oil-contaminated topsoil samples, from Absheron, Azerbaijan, were collected by sterile trowel after dismissal (1.5-2 cm) and thrown into jars. General profile of the soil was characterized as follows: the sample was not taken from the area of flora, poor soil structure, moisture 26.4%, the amount of humus 1-2%, weakly alkaline, the oil amount was 2.8% in dry weight soil. Agar-agar 2% and

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Capek-Dock medium used for primary cultivation of fungi. The composition of Capek-Dock medium was (g/l): sucrose - 30, $NaNO_3$ - 2, K_2HPO_4 - 1, $MgSO_4 \times 7H_2O$ - 0.5, KCl - 0.5; $FeSO_4 \times 7H_2O$ - 0.01, agar - 20, pH – 5.0- 5.5, streptomycin - 20 µg. The antibiotic was sterilized through membrane filters and added to medium before culturing, in order to prevent of bacterial contamination. In all cases, mediums pH reduced to 5.0- 6.0 by HCl 10% or $NaHCO_3$ (Das et al., 2001).

In isolation stage, first 60-100 g soil samples were cleaned from small stones and screened in 2.5 mm perforated sieve, homogenized, wetted until the formation of pasta shape and cultured on the solid medium by loop (4 point in each Petri dish) and then incubated aerobically, as well as it was done in incubator shaker with 220 rpm in the broth medium at 5-10°C for 10-30 days. The fungi were identified under the microscope through morphology characteristics (Waterhouse, 1968; Van der Plaats-Niterink, 1981). The cultures were maintained in two ways, suspensions of spores and slant tube methods in the Capek-Dock medium (Maheshwari, 2005; Borkovich & Ebbole, 2010).

The initial distinction of oleaginous fungi was done by Black Sudan B staining protocol, consequently the fat droplets was observed in the blue or grayish color of the cells under microscope. As a result, 24 strains were collected. The biomass was separated from 100 ml cultures by centrifugation with 6000 rpm for 15 minutes, and then washed twice by distilled water, next filtered by Whatman No. 1 filter paper; biomass weight was stable at 65°C. The samples dried up to determine of oil-polluted rate of soil; the oil was extracted by Soxhlet extractor by using petroleum or hexane (Thakur et al., 1989). Finally, according to Eroshin et al. (1996), in order to screen of the most strongest producer, the selected micromycetes were cultured in the solid Capek-Dock medium containing different concentrations of aspirin (0.25, 0.5, 0.75 and 1.0 g/l) at 25°C and at last diameter of the grown colonies were measured every 12 hours (Eroshin et al., 1996).

Rate and qualitative analysis of lipids

The extraction of lipids was used according to Folch (1957) and Bligh & Dyer (1978) methods. The lyophilized biomass were agitated with 4.0 ml distilled water + 5.0 ml chloroform + 10 ml methanol in a tube for 5 minutes, and then were kept shaking 3-4 hours at room temperature. Extraction was continued for 30 minutes, after adding 5.0 ml chloroform + 5.0 ml distilled water, filtered and poured into separator funnel. Filters were washed by 12.5 ml chloroform, and united at last. After stratification methyl alcohol + water layer were divided in separator funnel completely, the chloroform layer was brought to a constant weight, poured in a pear-shaped vacuum flask and evaporated in vacuum. The total lipid found in biomass as follows:

$$TL(\%) = \frac{\mathrm{m2} - \mathrm{m1}}{a} 100$$

where TL – Total lipids (%), m1 – the empty weight of flask (g), m2 – the weight of flask with biomass (g), a – the weight of biomass (g).

The lipids were separated to neutral and polar fractions by precipitation through cold acetone. For dissolving during the separation of fractions, a portion of the lipid solution (100 mg) was carried into 15 ml centrifuge test-tubes. The solution was evaporated up to 0.2-0.3 ml under nitrogen stream at 30°C, then 5 ml acetone and 0.1 ml of $MgCl_2 \times 6H_2O$ in methanol solution 10% was added, mixed and cooled in ice water bath for an hour. The suspension was precipitated in centrifuge at 2500 rpm for 3-5 minutes. Supernatant was cleared out by Pasteur-pipette. Sediment was washed and made suspension in 1 ml of cold acetone; the suspension was cooled in ice water bath and precipitated. The process of washing was repeated twice, and precipitated phospholipids were dried in nitrogen stream on KOH in the vacuum desiccators. The dried sediment was weighted and dissolved in determined volume of chloroform. The combined acetone supernatant was evaporated; the neutral lipids are weighted and dissolved in determined volume of chloroform. The dry weight is determined by a portion of the chloroform solution. Both non-acetone-soluble and acetone-soluble fractions were analyzed by silica acid impregnated paper chromatography. The polar lipids fractionations were carried out in chloroform/methanol/water (65:25:4, by vol) and the neutral lipids fractionations were performed in hexane/diethyl ether/acetic acid (85:25:4, by vol) systems by thin-layer chromatography. The lipids were transferred on silufol boards by drops of petroleum ether or benzene (neutral lipid), chloroform, or chloroform/methanol (9:1, by vol, polar lipid) 5-10% solution. Separated compounds were identified by the standard lipid preparation, as well as these systems have been assessed by Rf. The amount of lipid components was determined by densitometry. The fatty acids composition of lipids was determined by HPLC. For this purpose, the mixture of methylated fatty acids was divided by methanolic acid into UV detector (λ=250 nm) KOBO marked (Czech

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Republic) the liquid chromatography. The structure of fatty acids was also determined by mass-spectrum analysis (Folch, 1957; Bligh & Dyer, 1978).

The number of double bounds was calculated by "the number of iodine" protocol: first ethanol was added over lipid sample in 50 ml flasks, and then 12.5 ml of 0.1 N ethanol solution of iodine was added and mixed. A control flask only had 25 ml ethanol and the same amount of ethanol solution of iodine. 100 ml distilled water was added to both of flasks, then strapped, shaken, and after 5 minutes titration with the thiosulfate solution 0.1 N was continued until appearance of the yellow color. Then, 1 ml of starch solution was added, titration lasted until disappear of the blue color. Finally the iodine number was calculated as follows:

$$IN = (V1–V2) \times 0.0127 / a$$

where IN – measured with the amount of iodine combined with 100 g lipid (g) , V1 – the volume of thiosulfate solution 0.1 N used in titration in control variant (ml), V2 – the volume of thiosulfate solution 0.1 N used in titration in tested variant (ml), 0.0127 – the titration of thiosulfate according to iodine, a – lipid sample weight (g) (Yasuda, 1931).

Selection of physicochemical factors of the medium

The effects of changes in the initial pH (4–8) in the medium, as well as temperature (15, 21 and 28°C) were studied on the amount of biomass, lipid and PUFA compositions. In ''temperature shifting technique'' first fungi were cultured for 3, 4 or 5 days at 28°C, after incubation temperature was reduced to 15°C and the biomass was collected in 7th day. The effect of different concentrations of inoculums (0.5, 1, 2, 3, 4 and 5%, 1.5×10^6 spore per ml) were studied on biomass, lipid and PUFA synthesis after 6 days at 28°C.

As a source of nitrogen, CO, $(NH_2)_2$, $NaNCO_3$, NH_4Cl, NH_4NO_3 and yeast extract were used in Capek-Dock medium. The amount of nitrogen compounds in accordance with medium (0.33 g of nitrogen per liter of medium) was 0.7, 1.95, 1.23, 0.92 and 0.33 g, respectively. Initial pH was 6.0-6.2 (if necessary, the pH was increased by 40% KOH). Yeast extract and glucose as a source of nitrogen and carbon with a ratio of 20:1, 34:1, 50:1, 75:1, 100:1, respectively, were used to study the effect of C/N. Cultures were grown in medium containing glucose, yeast extract and 0.1% KH_2PO_4 at 28°C for 5 days aerobically. In the next experiments 1.0% palmitic, stearic, and miristic acids, as well as flax and cotton oils were added to the basic substrate crude oil (1.0%), and finally were analyzed by chromatography. The effects of exogenous phosphorus composition (K_2HPO_4) were studied on cell lipids and their fatty acids structure at different concentrations (0.5, 1, 2 and 5 g/l) at 28°C- 30°C for 7 days, aerobically. The amount of phosphorus in the broth medium with crude oil was 89, 178, 356 and 890 mg per liter.

Stress situation

Crude oil with 1, 2, 3, 5 and 10% concentrations were added to broth medium containing glucose. Cultivation was 5 days.

Results

The results of screening of fungi are presented in Table 1. The data in the table determined that the two micromycetes species – *Mucor globosus* 11 and *Cephalosporium humicola IE* are suitable in accordance with the requirements. In the next experiment, the isolation of lipogenous fungi from soil showed sensitivity of four isolated fungi to aspirin among 24 isolated strains after 5 days. The diameters of measurements have presented in Figure 1. The growth and lipid accumulation in *C. humicola IE*, *M. globosus 11* and *P. irregulare LX* as the producer of PUFA in the substrates glucose and crude oil, which were used in medium, have determined in Figure 2. The comparative analysis of lipid fractions composition indicated that all three fungi contained neutral lipid, triacylglycerol, diacylglycerol, monoacylglycerols, free fatty acids, polar lipids, free sterols, sterols esters, cardiolipin, glycolipid, phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, lysophosphatidylcholine, phosphatidic acid and some fractions were not identified. The activity of multienzyme complex of fatty acid synthesis system had a certain position in this work (Table 2). The initial pH in the medium with glucose to product of maximum amounts of biomass was 6.0, 0.6 and 0.7 in *C. humicola*, *M. globosus* and *P. irregulare*, but in medium with crude oil substrates was lower, 5.0, 5.0 and 6.0, respectively. But, the optimal pH to biosynthesis of PUFA was 0.7, 0.7 and 0.8, respectively. The growth curve of *C. humicola IE*, *M. globosus 11*, and *P. irregulare LX* in medium with glucose were formed within 4, 5 and 5 days at 28°C, 6, 6 and 7 days at 21°C and 9, 7 and 11 days at 15°C, respectively. Consequently to optimize of lipid production the cultures were grown for 3, 4 or/and 5 days at 28°C, and followed by the incubation temperature was reduced to 15°C and biomass accumulated of 7 days. The data in Table 3 showed that effect of different nitrogen sources on biomass,

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lipids and PUFA. The effect of carbon-nitrogen ratio on the biomass, lipid and PUFA has been shown in Table 4. The sixteen different mediums combination were used to study the effect of C/N on productivity of ARA and EPA in biomass (Table 5). The results of using exogenous fat and fatty acids as essential or additional source of carbon in medium have been presented in Table 6. The graphics of vegetable oil and fatty acids showed a positive impact on the growth and lipid production (Figure 3). The results showed that the intensity of lipogenesis depended on the quantity of additive phosphate to the medium (Table 7). The results presented in Table 8 showed that more effect of phosphate concentration on palmitic, linoleic and linolenic acids of the lipid composition was in *M. globosus* 11. The effect of phosphate concentration was different in the other two fungi. Adaptation of microorganisms to the different stress situation was important due to the changes of lipid and fatty acid composition. The results of growth, lipogenesis, polar lipids, neutral lipids and unsaturated fatty acids especially increasing the proportion of phospholipids of fungi, showed the fact of increase in degree of unsaturation; in different concentrations of crude oil were added to medium confirmed this fact, despite the delay in growth of them.

Table 1. *The composition of fatty acids in AMEA collection cultures of micromycetes*

Micromycetes	**Lipid %**	**The main polyunsaturated fatty acids in %**					
		18:3	20:3	20:4	18:3	20:3	20:4
		Glucose			Crude oil		
Mucor globosus 11	18 ±0.5	12 ±0.5	1.0 ±0.01	1 ±0.05	15.6±1	trace	trace
M. mucedo	20±0.6	5 ±0.2	0.4 ±0.02	2 ±0.04	8.5 ±0.5	trace	trace
M. circinelloides	19 ±0.7	2 ±0.2	0.2 ±0.01	1 ±0.05	5.2 ±1	trace	trace
Mortierella alpina	21 ±0.1	5 ±0.7	0.2 ±0.03	1 ±0.04	5.4 ±1	trace	trace
M. spinosa	20 ±1	2 ±0.1	1.1 ±0.01	2 ±0.03	4 ±1	trace	trace
M. humilis	17 ±0.9	4 ±0.8	1.5 ±0.01	1 ±0.02	7 ±1	trace	trace
M. isabellina	18 ±0.7	2 ±0.1	1.3 ±0.02	2 ±0.01	5 ±0.9	trace	trace
M. elongata	21±0.7	6 ±0.5	1.0 ±0.01	1 ±0.01	7 ±0.8	trace	trace
Cephalosporium humicola IE	18 ±1	12±0.9	1.9 ±0.2	1 ±0.03	17±1.3	trace	trace

Table 2. *Enzyme activities (nmol/min/mg protein) in fungi grown on carbon*

Enzyme	***C. humicola***		***M. globosus***		***P. irregulare***	
	glucose	**crude oil**	**glucose**	**crude oil**	**glucose**	**crude oil**
ME	35±1.2	18±1.3	22±2.1	11±1.2	24±2.4	13±1.1
ACL	30±0.9	-	26±2.6	-	28±1.2	-
G-6PGDH	1100±2.3	200±1.2	980±2.1	120±1.5	1060±1.5	17±0.8
FAS	33±0.8	40±1.3	23±1.8	22±1.3	27±1.6	33 ±0.7
ACC	2.5±0.4	4.8±2.3	3.1±0.9	5.3±1.9	6.4±0.1	5.9±1.4
DAGAT	0.7±0.1	2.10.3	0.4±0.1	1.7±0.4	2.6±0.5	1.9±0.2
ICL	-	58±1.6	5±1.1	40±1.6	7±0.5	46±1.4
CAT	34±1.2	350±2.3	22±1.1	230±2.1	29±0.7	310±1.8
PK	820±2.6	160±2.1	600±1.7	67±2.6	703±0.9	89±2.1

ME: malic enzyme, ACL: AT P citrate lyase, G-6PGDH: glucose-6-phosphate dehydrogenase, FAS: fatty acid synthase, ACC: Acetyl-CoA carboxylase, DAGAT: Diacylglycerol acyltransferase, ICL: Isocitrate lysase, CAT: carnitine acetyltransferase, PK: pyruvate kinase.

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Table 3. *The amount of biomass, lipid compositions and PUFA in different nitrogen sources*

	$CO(NH_2)_2$	$NaNO_3$	NH_4Cl	NH_4NO_3	Peptone	Yeast extract
			glucose			
M. globosus 11						
Biomass, g/l	13.8±0.6	14.0±0.7	11.3±0.5	10.0±0.5	13.2±0.6	16.1±0.8
Lipid, %	20.0±1.0	18.5±0.9	18.5±0.9	18.0±0.9	22.0±0.1	20.0±1.0
PUFA	12.3±0.6	12.1±0.6	13.5±0.6	14.0±0.7	12.0±0.6	12.0±0.6
C. humicola						
Biomass, g/l	12.3±0.6	13.5±0.6	12.1±0.6	11.2±0.6	15.0±0.7	15.4±0.7
Lipid, %	21.0±1.0	20.5±1.0	19.0±0.9	18.0±0.9	25.1±1.2	22.0±1.1
PUFA	5.0±0.2	5.0±0.2	5.8±0.2	6.0±0.3	5.0±0.2	5.0±0.2
P. irregulare						
Biomass, g/l	13.0±0.6	12.0±0.6	12.0±0.6	12.0±0.6	13.5±0.7	14.0±0.7
Lipid, %	20.0±1.0	19.5±0.9	19.0±0.9	19.0±0.9	22.0±1.0	20.5±1.0
PUFA	30.1±1.5	32.5±1.6	35.8±1.7	35.2±1.7	30.4±1.0	30.1±1.5
			crude oil			
M. globosus 11						
Biomass, q/l	9.0±0.4	9.8±0.4	8.0±0.4	7.6±0.3	11.0±0.5	11.2±0.5
Lipid, %	39.0±1.8	37.6±1.7	36.4±1.7	36.0±1.7	40.4±2.0	39.0±1.7
PUFA	16.0±0.8	16.0±0.8	18.0±0.9	18.5±0.9	16.0±0.8	16.0±0.8
C. humicola						
Biomass, g/l	6.3±0.3	6.5±0.3	6.0±0.3	6.1±0.3	8.0±0.4	8.3±0.4
Lipid, %	47.0±2.3	45.0±2.2	43.0±2.1	43.0±2.1	50.2±2.5	48.3±2.3
PUFA	24.0±1.2	23.0±1.1	26.8±1.3	26.9±1.3	25.0±1.2	25.2±1.2
P. irregulare						
Biomass, q/l	7.5±0.3	7.8±0.3	7.2±0.3	7.0±0.3	9.2±0.4	9.8±0.4
Lipid, %	39.0±1.3	38.0±1.3	35.0±1.6	35.0±1.7	46.1±2.3	45.0±2.2
PUFA	42.5±2.1	42.0±2.1	45.7±2.2	44.8±2.2	42.0±2.1	42.0±2.1

Discussion

It is known that the lipid composition of microorganisms have important role in adoption of hydrocarbon substrates, because the hydrophobic parts of the cell increase as a result of lipid accumulation, and this improves the conditions for absorption of lipophilic substrates (Eroshin et al., 1996).

In accordance with the analysis, the lipid compositions of *M. globosus 11* and *C. humicola IE* do not contain eicosane acids, but a sufficient amount of C18:3 were synthesized in crude oil substrates and this topic was the reason to select them as next object of the experiments. The study of lipid composition of *C. humicola IE* had special interest, which has the ability to synthesize antibiotics cephalosporin because there is no information about synthesis of PUFA in literatures, even about lipogenesis. A high oleaginous and aspirin sensitivity strain of the fungus selected as the research object was identified as *Pythium irregulare* and called *Pythium irregulare LX*.

Replacement of the substrate glucose with crude oil had a great impact on the lipid fractions. The polar lipids were increased from 22% up to 27% in *C. humicola IE* and also from 13% to 39.8% in *P. irregulare LX*, but not a big change in *M. globosus 11*. The maximum biomass was accumulated in substrates crude oil in proportion to glucose were equals 28.6% in *C. humicola IE*, 27.2% in *M. globosus 11* and 43.6% in *P. irregulare LX*, respectively. The amount of lipid in dry biomass (%) was 58.4% in *C. humicola IE*, 56.7 % in *M. globosus 11*, and 64.1% *P. irregulare LX*, respectively, whereas the amount of lipids was as low as 3- 3.5 times in glucose. The analysis of HPLC demonstrated that the rise the level of lipid in biomass was due to increase the share unsaturated fatty acids (EPA, ARA and GLA) along with

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reduction of the saturated fatty acids. In *M. globosus 11* and *C. humicola IE*, the PUFA were more in the diacylglycerol, triacylglycerol and polar lipids composition, and almost there were no free fatty acids. EPA and ARA were collected at the polar lipids, free fatty acids, and diacylglycerol compositions in *P. irregulare LX*. In the triacylglycerol compositions, the level of C20:4, C20:5 acids were too low during growth. Large amounts of C20:4 and C20:5 acids in polar lipids compositions showed the important role of them in plastic function of cell membrane.

To some extent, there was a difference between the enzymatic activities of fungi, but their activity dynamics in accordance with the change of the substrates were almost the same.

The initial pH to produce maximum amount of GLA was 7.0 in *M. globosus 11*. In this rate the amount of accumulated biomass was slightly less than pH 6.0, while the amount of lipogenesis and GLA was more (0.52 g/l), In addition to 0.21 g/l ARA was also synthesized. Initial pH in *C. humicola IE* (production of GLA) was 6.0 (0.48 g/l PUFA). In addition to 0.20 g/l arachidonic acid was also synthesized. Initial pH in *P. irregulare LX* (production of GLA and EPA) was 7.0 (1.4 g/l PUFA). Generally, at various pH, the amount of saturated and unsaturated fatty acids decrease with increasing pH (Xian et al., 2001; Dyal et al., 2005; Xia et al., 2011). However, some studies have shown that changing in pH did not affect the composition of lipid and fatty acids (Ratledge & Botham, 1977; Davies, 1988).

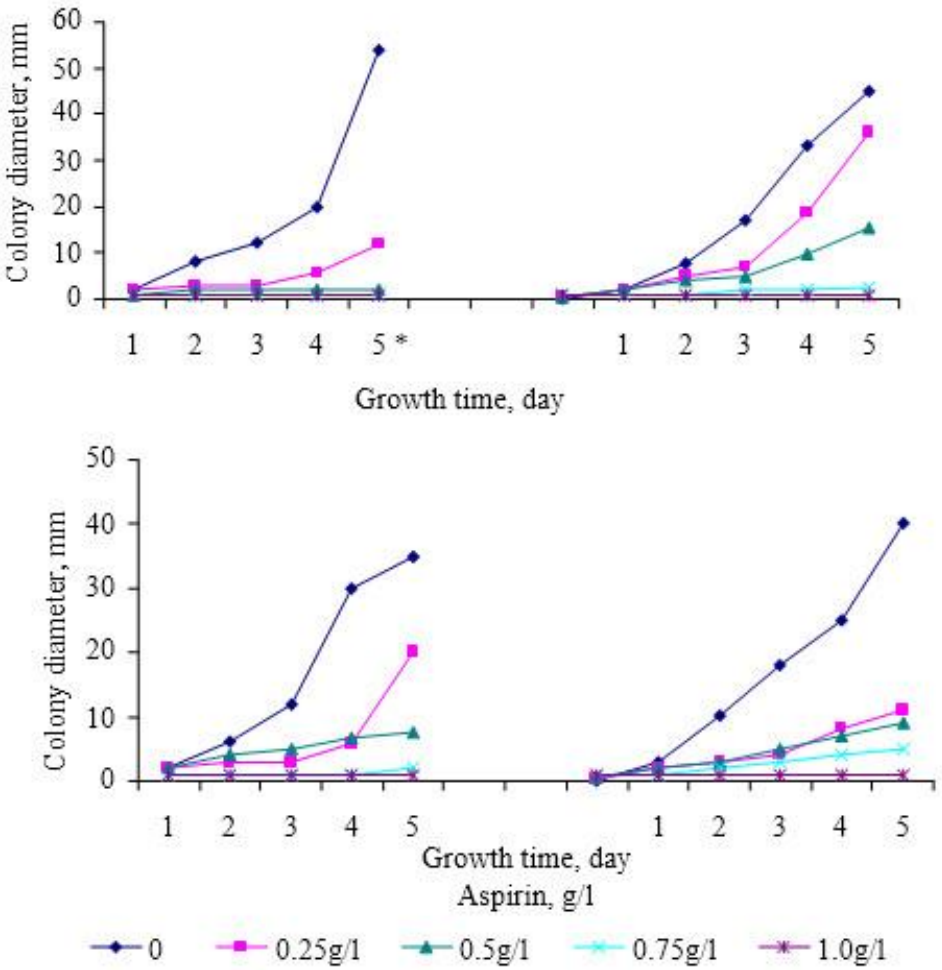

Figure 1. *The change of dimensions of colonies diameter in the high sensitivity aspirin.*

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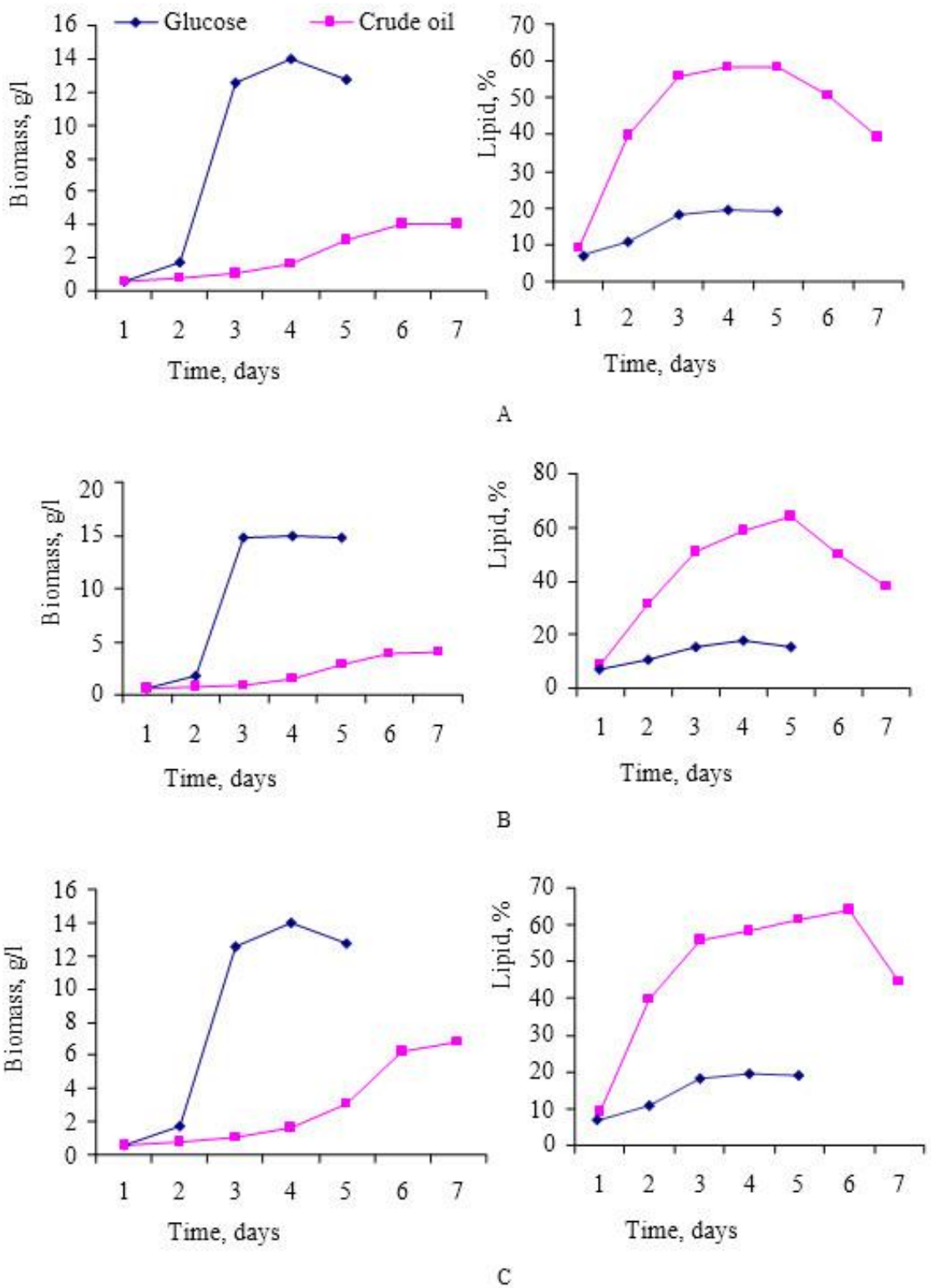

Figure 2. *Growth and lipid accumulation in C. humicola IE (A), M. globosus 11 (B) and P. irregulare LX (C) grown on a medium with glucose and crude oil substrates.*

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Table 4. *The effect of C/N ratio on the biomass, lipid and PUFA*

Fungi	carbon-nitrogen ratio				
	20:1	34:1	50:1	75:1	100:1
M.globosus 11					
Biomass, g/l	18.3	16.1	16.0	14.2	10.5
Lipid, %	18.2	20.0	23.1	20.0	20.0
PUFA	11.0	12.0	11.0	10.0	10.0
C.humicola					
Biomass, g/l	17.1	15.4	15.4	15.4	15.4
Lipid, %	21.8	22.0	23.0	24.0	22.0
PUFA	3.0	5.0	4.0	4.0	3.0
P. irregulare					
Biomass, g/l	16.5	14.0	14.0	14.0	14.0
Lipid, %	20.3	20.5	22.6	20.4	20.1
PUFA	28.1	30.1	29.1	28.2	26.1

* All indicators were $P \leq 0.05$.

Table 5. *The C/N ratio due to a change in the amount of glucose-yeast extract medium*

№	glucose, % (w/v)	Yeast extract, % (w/v)	C/N	№	glucose, % (w/v)	Yeast extract, % (w/v)	C/N
1	1	0.25	16	9	3	.25	48
2	1	0.50	8	10	3	0.50	24
3	1	0.75	5.3	11	3	0.75	16
4	1	1.0	4	12	3	1.0	12
5	2	0.25	32	13	4	0.25	64
6	2	0.50	16	14	4	0.50	32
7	2	0.75	10.6	15	4	0.75	21.4
8	2	1.0	8	16	4	1.0	16

Table 6. *The effect of exogenous fat and fatty acids on the amount of PUFA*

Fungi	Amount of PUFA, %					
	Palmitic acid	Stearic acid	Miristic acid	Flax oil	Cotton oil	control
M. globosus 11	27.5±0.9	19.0±0.8	16.0±0.6	26.0±1.2	28.0±1.3	16.0±0.8
C.humicola IE	29.0±1.2	25.5±1.1	25.0±1.0	31.0±1.5	31.0±1.5	25.0±1.1
P. irregulare LX	47.0±2.1	42.0±2.0	42.0±1.9	49.0±2.4	50.0±2.4	42.0±2.0

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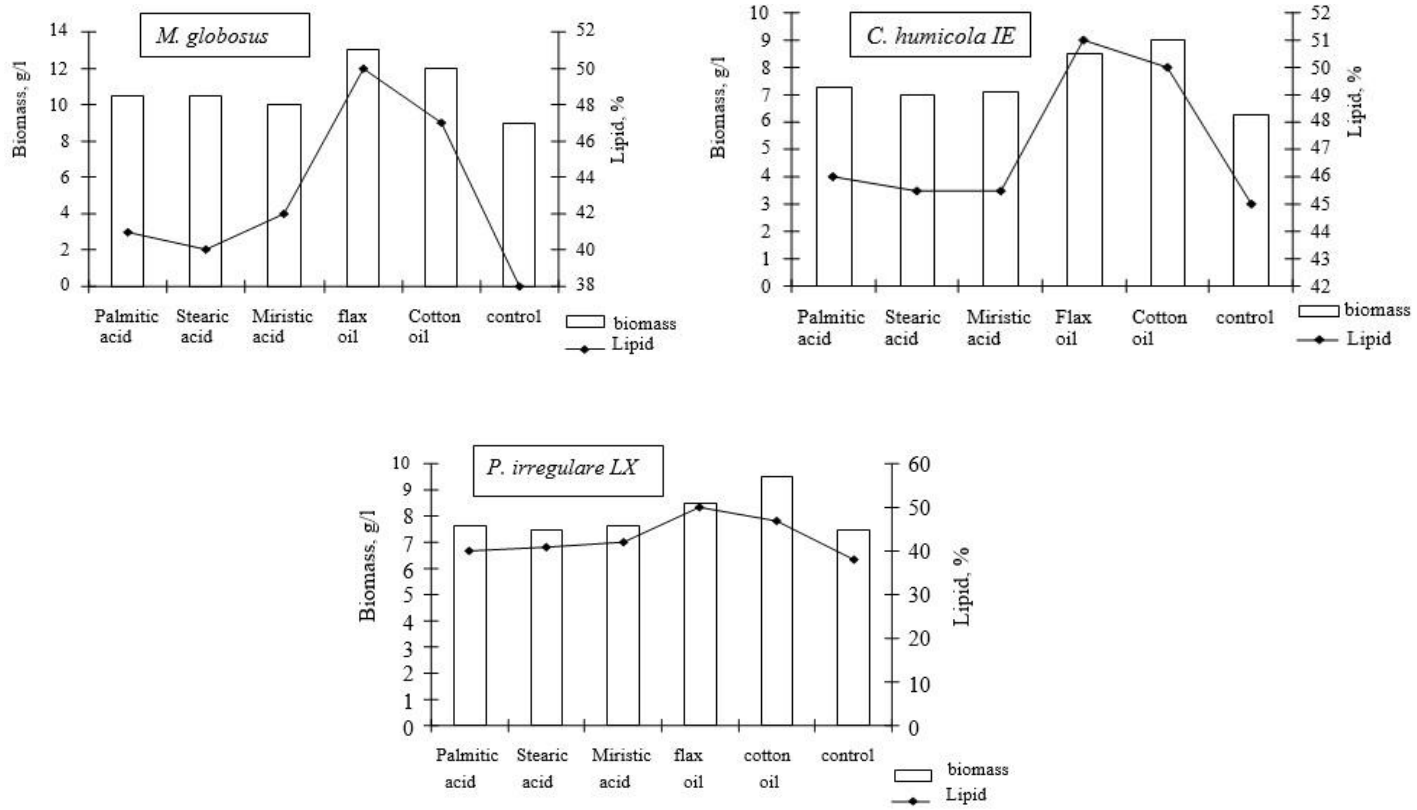

Figure 3. *The effect of fatty acids and vegetable oils on growth and lipid production of fungi.*

Table 7. *The effect of different phosphate concentration on biomass and lipid*

phosphate concentration	*M. globosus 11*		*C. humicola IE*		*P. irregulare*	
	Biomass, g/l	**Lipid, %**	**Biomass, g/l**	**Lipid, %**	**Biomass, g/l**	**Lipid, %**
0 (control)	1.2	14.0	1.0	16.0	0.8	16.0
0.5 g/l	7.5	20.0	5.0	12.0	5.5	22.0
1.0 g/l	9.0	38.0	6.3	45.0	7.5	38.0
2.0 g/l	8.5	32.0	5.2	40.0	4.5	34.0
5.0 g/l	5.0	25.0	5.0	35.0	4.0	31.0

Stationary growth phase was very short in all three fungi. The peak of biomass in growth curves was fallen down dramatically, in a very short period of time. However, the reason was due to the lack of oxygen or lysis of the biomass. It should be noted that we received the results did not coincide with other published data, several authors try to prove that the increase of growth and lipids occur in below the optimum temperature (O'Brien et al., 1993; Cheng et al., 1999; Stredansky et al., 2000). However, in our studies, the amount of lipid was significantly higher at 28°C. Decreasing the cultivation temperature was caused increasing of the rate of C16:1 and C18:1 fatty acids of lipid composition in C. *humicola IE* and *M. globosus 11*. The optimal biomass production was occurred at 28°C stable temperature, whereas the results of "temperature shifting technique" showed that the greatest production of GLA, ARA and EPA were occurred at 28°C for 5 days followed by at 15°C for 2 days.

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Table 8. *The effect of phosphate concentration on the amount of fatty acids*

Fatty acids	Total amount, %											
	M. globosus 11				*C. humicola IE*				*P. irregulare LX*			
	the amount of phosphate, %											
	0(control)	0.05%	0.1%	0.5%	0	0.05%	0.1%	0.5%	0	0.05%	0.1%	0.5%
Lipid,%	24.0	30.0	37.0	25.5	30.0	34.0	35.0	35.0	20.0	29.5	32.0	42.5
C <14	2.7	2.6	2.6	2.0	2.8	1.9	1.9	2.0	trace	trace	trace	trace
C16:0	51.0	48.0	40.0	35.0	45.0	35.0	26.0	22.0	40.0	34.5	20.3	31.7
C16:1	9.5	9.0	8.5	8.5	8.2	7.5	7.3	7.2	5.2	5.5	5.5	5.1
C18:0	9.0	6.4	5.9	5.9	8.9	4.8	3.5	3.5	7.5	4.2	2.6	2.0
C18:1	11.0	14.0	10.0	10.0	15.6	10.5	15.0	15.0	5.0	5.0	5.0	5.4
C18:2	6.0	7.0	12.0	15.2	2.0	8.0	9.0	9.9	8.1	10.0	13.4	15.3
C18:3	8.0	9.0	13.0	15.0	5.2	13.1	16.9	18.0	8.0	10.8	12.2	14.7
C20:1	3.0	3.0	2.0	2.0	4.5	0	0	0	1.2	1.2	1.2	2.0
C20:3	0	1.0	2.0	2.0	0	3.1	3.8	3.8	5.5	5.2	10.0	11.0
C20:4	0	0	2.0	2.4	0	5.0	7.0	9.0	9.5	11.5	15.3	18.5
C20:5	0	0	2.0	2.0	0	0	0	0	10.0	12.5	14.5	17.7

All indicators were $P \leq 0.05$.

The biomass was raised with the increase in the amount of inoculums would have increased. However the problem was caused by lack of O_2 because of an increase in viscosity of culture in broth medium. Stearic acid and unsaturated fatty acids were synthesized increasingly in high and low inoculums through changes in the activity of desaturase enzymes, which converse saturated fatty acids to unsaturated fatty acids aerobically. Therefore, their activities are stopped after oxygen consuming (Ratledge, 2004; Jenke-Kodama & Dittmann, 2009).

Optimal inorganic source of nitrogen was $NaNO_3$ (0.58 g/l PUFA), and organic nitrogen source was peptone (0.7 g/l PUFA) in *M. globosus 11*. Sources of inorganic nitrogen were $NaNO_3$ (0.70 g/l PUFA) and CO (NH_2) (0.71 g/l PUFA), organic nitrogen source was equally optimal; PUFA yield was about 1.0 g/l in *C. humicola IE* (GLA synthesis). Affordable inorganic nitrogen source were $NaNO_3$ and CO (NH2) (1.1 g/l PUFA), as well as source of organic nitrogen was peptone (1.85 g/l PUFA) in *P. irregulare LX* (GLA, EPA and ARA synthesis). The ratio of carbon and nitrogen has an important role in the fermentation process (Ykema et al., 1988). The highest products of EPA and ARA were in C/N = 32 (2% glucose and 0.25% yeast extract- 20 mg /g and 15.3 mg /g. The results were similar according to the results of Cheng (1999).

Chromatography analysis of the fatty acid compositions showed 0.8% miristic, 22.3% palmitic, 0.4% palmitoleic, 2.0% stearic, 16.7% oleic, 57.6% linoleic and 0.3% linolenic acids in used flax oil, in cotton oil 5.1% palmitic, 0.5% palmitoleic, 2.5% stearic, 18.2% oleic, 0.5% eicosane, 15.5% linoleic and 57.4% linolenic acids (25% saturated, 17% monounsaturated, 58,0% PUFA. The presented indicators proved that suitable substrate for the synthesis of the fungal PUFA flax oil. It should be noted that, the increase in the quantity of oil was recorded until just rises to 2.0%. There were not an increase in higher concentrations; it is associated with activation of acetyl CoA carboxylase, a key enzyme in lipid synthesis (Šajbidor et al., 1992; Kyle, 1996; Totani et al., 2000).

The maximum amount of lipids biosynthesis was in 2.0 g/l of phosphate. Phosphorus deficiency in the early days of growth was resulted in increases in the synthesis of the amount of palmitic acid, decrease in linoleic and linolenic acids and decline in the degree of saturation of lipid in *M. globosus 11*. However, after some time degree of saturation began to increase gradually. The effect of phosphate concentration was different in the other two fungi. Linolenic acid was decreased due to the increase in palmitic acid in *C. humicola IE*. The phosphoric shortage in lipid of *P. irregulare LX* was resulted in eicosane acids. Phosphorus

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deficiency also was caused decrease eicosane acids in the lipid of *P. irregulare*. In all cases, an increase in saturation degree of SCO was accompanied by a decrease in the polar lipid fraction, especially phospholipids. It should be noted that some researchers insist on the presence of certain phospholipids are obligatory to activity of desaturase system (Russell, 1989; Kates et al., 1984; Jackson et al., 1998).

It was found that the growth of fungi was delayed significantly by increasing the concentration of crude oil. In addition, the amount of lipids and unsaturation degree of fatty acids was increased in biomass. An increase in portion of polar lipids was caused increase in the unsaturation degree; to clarify of this fact, the fatty acids composition of neutral and polar lipids was analyzed. The amount of oleic and linoleic acids had changed in neutral fraction due to effects of crude oil. Increasing of extra oils to medium up to 3.0% concentration were increased the rate of oleic acid gradually, but decreased at 5.0% and 10%. One of the most interesting changes was the increase in the amount of less than 14C fatty acids. The mentioned above growth condition, the fatty acids composition and unsaturation of polar lipids degree were changed more than neutral lipid. The amount of saturated and monounsaturated fatty acids of polar fraction were raised increasing in crude oil concentration in medium. The amount of short-chain fatty acids was increased up to 5-10%. Thus, fatty acids, particularly the mechanisms of elongation and desaturation, had played important role in the regulation of permeability of membrane lipids. The results showed increase in the proportion of polar lipids to free sterols due to the effect of crude oil in composition of membrane lipids, which was associated with an increase in proportion of polar lipids in the total lipid fraction. It can be concluded that the process of adaptation of micromycetes to a stressful situation caused by crude oil, induced an increment in the rate of membrane phospholipids with high quantity of unsaturated fatty acids.

In this study, the certain physicochemical optimal conditions were found for fungal growth of *Cephalosporium humicola IE, Mucor globosus 11* and *Pythium irregulare LX*- to produce maximum fatty acids, including γ-linolenic (GLA), arachidonic (ARA) and eicosapentaenoic (EPA) acids by microbiological methods. It was shown also that crude oil and exogenous waste fats can be cost-effective as an alternative substrate. Moreover, these fungi can prevent environmental pollutions through these wastes. Oil in addition to being as a substrate, it can also be stressful, therefore the unsaturation level of fatty acids in membrane phospholipids were increased in these fungi and thereby the maximum highly unsaturated fatty acids were achieved.

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