UDC 579.2

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BIOSYNTHESIS OF LIPIDS IN TWO OIL-DEGRADING FUNGI – *CEPHALOSPORIUM HUMICOLA* AND *MUCOR CLOBOSUS*

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БИОСИНТЕЗ ЛИПИДОВ У ДВУХ НЕФТЕДЕГРАДИРУЮЩИХ ГРИБОВ – *CEPHALOSPORIUM HUMICOLA* AND *MUCOR CLOBOSUS*

Abstract. We study the effect of the carbon sources – glucose and hexadecane – on the fatty acid composition and activity of enzymes, actually or potentially involved in the biochemistry of oleaginicity of two oil degrading fungi, namely, *Cephalosporium humicola* and *Mucor globosus*. The carbon sources markedly affect the fatty acid composition of the fungi in question. The lipids extracted from hexadecane grown cells contain not only saturated fats found in fungi grown on glucose substrates but also linoleic and linolenic acids. In both fungi we observe a dramatic decrease in the stearic acid content from 16,0-29.0% to 2,8-5.9% by replacing glucose by hexadecane. The synthase activity of fatty acids is found in cells growing both on glucose and hexadecane substrates. Isocitrate lyase is absent in cells growing on glucose substrates and is found when grown on hexadecane substrates. The activity of adenosine triphosphate is as follows: citrate lyase is completely inhibited by hexadecane. Carnitine acetyltransferase is detected in cells grown on glucose substrates. The activity of this enzyme increases tenfold in the case when hexadecane is the source of carbon. The high activity of pyruvate kinase on glucose decreases by 80%–90% when grown on a hydrocarbon substrate. Analysis of the results and products of linoleic and linolenic acids shows that the hydrocarbon substrate increases the biosynthesis of 18:2 and 18:3 acids, and, hence, Δ12/Δ15-desaturase enzymes, which are responsible for the conversion of 18:1-to-18:2 and 18:2-to-18:3 acids. Although the specific activities of the enzymes vary between the fungi, the developmental profiles for all enzymes are virtually similar.

Key words: fungi, carbon sources, enzyme activity, fatty acids.

Аннотация. Изучено влияние источников углерода – глюкозы и гексадекана на жирно-кислотный (ЖК) состав и активность основных ферментов, реально или потенциально участвующих в биохимии олеофильности двух углеводороддеградирующих грибов – *Cephalosporium humicola* и *Mucor globosus.* Источники углерода оказали значительное влияние на ЖК-состав исследованных грибов. Липиды, экстрагированные из клеток, культивированных на гексадекане, содержали не только насыщенные ЖК, обнаруженные в глюкозных вариантах, но также имели линолевую и √ - линоленовую кислоты. В обоих грибах наблюдалось резкое уменьшение содержания стеариновой кислоты – от 16,0-29,0 % до 2,8-5,9 %, при замене глюкозы на гексадекан. Активность синтеза ЖК была обнаружена в клетках, растущих как на глюкозе, так и на гексадекане. Изоцитратлиаза отсутствовала в клетках, растущих на глюкозе, но была обнаружена при росте на гексадекане. Активность АТФ: цитрат лиазы была полностью подавлена на гексадекане. Карнитин ацетилтрансфераза была обнаружена в клетках, выращенных на глюкозе. Активность этого фермента возрастала десятикратно, в случае когда источником углерода был гексадекан. Высокая активность пируваткиназы на глюкозе уменьшалась на 80-90 % при росте на углеводородном субстрате. Анализ результатов по продукцию линолевой и √ - линолевой кислот показывает, что углеводородный субстрат повышает биосинтез 18:2 и 18:3 кислот, и следовательно, ферментов Δ12-десатуразы и Δ 15 десатуразы, являющихся ответственными за преобразование 18:1 к 18:2 и 18:2 к 18:3 кислот. Несмотря на то, что специфическая активность отдельных ферментов исследуемых грибов имела некоторые различия, динамика изменения активностей практически для всех ферментов была одинакова.

Ключевые слова: грибы, источники углерода, ферментативная активность, жирные кислоты.

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Filamentous fungi are metabolically versatile organisms with a very wide distribution in nature [1; 15]. The utilization of hydrocarbon substrates by filamentous fungi has received considerable attention since the observation by Miyoshi in 1895 that fungi can attack paraffins [12]. The direct incorporation of hydrocarbon substrates into the lipids of filamentous fungi has received little attention, although it has been suggested that filamentous fungi do not incorporate substrate hydrocarbons directly and without degradation to the acetate level [16]. Biosynthesis of fatty acids is achieved in the multi-enzyme complex fatty acid synthase and ends with palmitic acid (C16:0). C16:0 is then the starting material for dehydrated fatty acids and longer fatty acids which determines the central role this specific fatty acid plays in the biosynthesis of fatty acids in Eubacteria and higher organisms [7; 14]. C16:0 is present in all eukaryotes.

The catabolism of hydrophobic substrates, such as alkanes, fatty acids and triglycerides, is a quite complex metabolism which involves several metabolic pathways taking place in different subcellular compartments. An important characteristic of alkane assimilation by yeasts and fungi is the metabolic flow of carbon atoms from alkane substrates to synthesis of all cellular components via fatty acids, which is quite different to compare to such conventional substrates like carbohydrates. In the course of studies on the mineralization of various crude oils by microorganisms in oil-contaminated soils, we isolated several fungi that were capable of growth on hydrocarbons [3]. The aim of this study was to determine the relevance of inducible hydrocarbon-degrading pathways in alterations in lipid composition of two of these fungi, *Mucor globosus 11* and *Cephalosporium humicola EI*, and to build a better understanding of lipid metabolism in order to develop methods of enhancing the production of desirable compounds.

Materials and methods

Mucor globosus 11 and *Cephalosporium humicola EI* used during present investigation were isolated from oil-contaminated soils near Baku and maintained in the fungi collection of the Laboratory of Biochemistry and Physiology of Soil Microorganisms. The medium for fungi cultivation was composed of 2% (v/v) hexadecane or 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 10 days at 28°C on a rotary shaker. Flasks were periodically removed from the incubator and mycelia were collected by centrifugation at 22000 rpm for 20 min minutes and washed once with distilled water. Determinations of total biomass, lipids as well as lipid methanolysis and analysis by gas chromatography have been performed as described elsewhere [9; 13]. All enzymes were assayed using previously published procedures. Isocitrate lysase (ICL) was assayed as described by Armitt et al. [2], ATP: citrate lyase (ACL), fatty acid synthase (FAS), glucose-6-phosphate dehydrogenase (G6PD), and malic enzyme (ME) as described by Wynn et al. [17], carnitine acetyltransferase (CAT) as described by Kawamoto et al. [10], and pyruvate kinase (PK) as described in Worthington Enzymes Manual [16], Acetyl-CoA carboxylase (ACC) was assayed as described by Matthews *et al.* [11]. Diacylglycerol acyltransferase (DAGAT) activity was determined using the assay described by Kamisaka et al. [8]. All experiments were carried out in triplicate at least and data are presented as mean ± S.E.M.

Results and discussion

According to the preliminary experiments, both *C. humicolaEI* and *M. globosus 11* were able to grow on the medium with hexadecane as the sole carbon source and accumulated 67.0 and 49.5% lipid in their biomass respectively (corresponding to 8.7 and 5.9 g lipid/l). Both fungi demonstrated a similar and a typical growth and lipid production profile in fermentation (Fig.1). Higher yield biomass values and lipid production were observed for *C. humicola EI* to compare to *M. globosus 11*. The lipid content of the

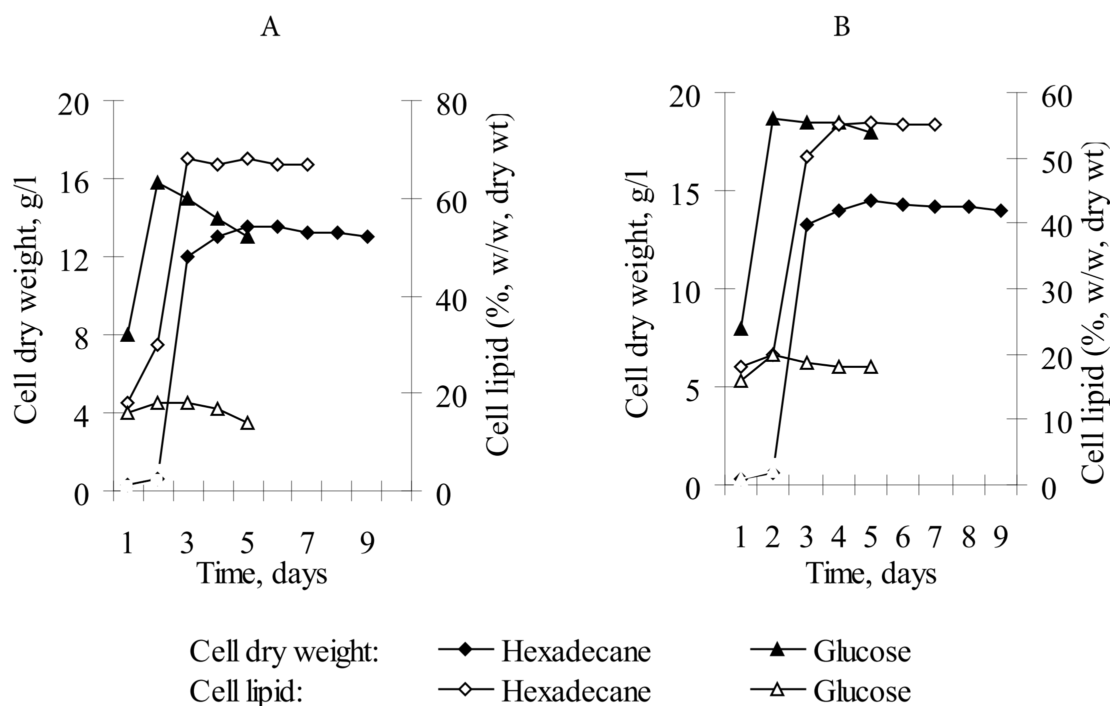

Fig 1. Growh of and lipid accumulation in *Cephalosporium humicola* (A) and *Mucor globosus* (B) grown on a medium with glucose and hexadecane

same fungi grown on glucose as the sole carbon source were 15.8 and 13.7%, respectively. From these observations it is clear that hexadecane can be employed as a carbon source for microbial lipid production.

The chromatographic analysis of microbial oil samples revealed an array of fatty acids, both saturated and unsaturated. The fatty acid profiles showing fatty acid composition expressed in the form of relative percentages of total fatty acids are presented in Table 1. Both fungi under study demonstrated great variations with respect to the level of fatty acid percentage. The results showed that the mycelium of M. globosus grown on glucose contained five kinds of fatty acid, including myristic, palmitic, palmitoleic, stearic and oleic acids. In *C. humicola* EI grown on glucose, 98.6% of total fatty acid was comprised as myristic, palmitic, stearic and oleic acids. The composition of oil from *M. globosus 11* included largely saturated fatty acids, such as myristic, palmitic, stearic acids. From *C. humicola EI* the highest percentage of saturated fatty acids found were palmitic (52.0%) and stearic (16.0%) acids. In both fungi, *M. globosus 11* and *C. humicola EI* during the growth on glucose substrate, the unsaturated fatty acids constituted 20.0 and 28.0% of the total fatty acids. In tested fungi grown on glucose no detectable amounts of C18 polyunsaturated fatty acid were found. Carbon sources analysis showed its marked influence on the fatty acids make-up of these species. The oil samples from hexadecane grown cells not only contained all the above noted saturated fatty acids, but also had linoleic and √-linolenic acids. In both fungi dramatic decrease in content of stearic acid from 16.0-29.0% to 2.8-5.9% was observed with change in carbon source from glucose to hexadecane.

Table 1

Percentage fatty acid composition of *Cephalosporium humicola EI* and *Mucor globosus 11* grown on a medium with glucose and hexadecane

Culture/ substrate	Fatty acid concentration (w/w) in total mycelial lipid							
	C14:0	C16:0	$^{\Delta}$C16:1	C18:0	$^{\Delta 9,}$C18:1	$^{\Delta 9,12}$C18:2	$^{\Delta 6,9,12}$C18:3	Other
C. humicola								
hexadecane	1.9	53.0	7.3	2.8	15.0	9.0	10.0	2.0
glucose	2.6	52.0	trace	16.0	28.0	trace	trace	1.4
M. globosus								
hexadecane	2.6	49.0	4.7	5.9	12.0	9.0	14.0	2.8
glucose	3.0	46.0	12.0	29.0	8.0	trace	12.0	2.0

A range of enzymes involved, or potentially involved, in the biochemistry of oleaginicity were studied in both fungi. These enzymes included those belonging to the lipid biosynthetic pathway itself, such as ACC, FAS and DAGAT; ACL, which is implicated in the production of cytosolic acetyl-CoA for lipid synthesis in oleaginous yeasts [4; 6]; and a range of enzymes potentially involved in the generation of NADPH for fatty acid synthesis, ME, G-6-PDH. Part of our study was aimed at gaining more knowledge in a areas with a focus on desaturation of fatty acids. The overall picture of the enzymatic activities in *C. humicola EI* and *M. globosus 11* are given in the Table 2. Although the specific activities of the enzymes varied to some degree between the fungi, the developmental profiles practically for all enzymes were similar (Fig. 2).

Cytosolic acetyl-CoA is metabolized to malonyl-CoA by ACC. ACC is the first committed step in the synthesis of fatty acids providing two of its three carbons to the fatty acid synthase. The activity of fatty acid synthase was readily detected in glucose-grown cells as well as hexadecane cells, indicating active de novo fatty acid biosynthesis. The fatty acid synthase and polyketide synthase enzymes are remarkably similar in their function with differences apparent primarily in the detailed programming. These two enzymes constitute a metabolic branch point between primary and secondary metabolism. Fatty acids enter a second branch point between membrane lipid biosynthesis (functional fats) and fat accumulation (storage fats) and this switch is controlled by DAGAT enzyme. ICL was absent in the extract from the cells grown on glucose but it was detected when the fungi were grown on hexadecane. This is a marker enzyme for the glyoxylate bypass of the citric acid cycle which is induced in cells grown on carbon sources that are metabolized via acetyl-CoA rather than pyruvate. This pathway serves to replenish the shortfall of C4 citric acid cycle intermediates, which occurs due to diversion of carbon from the citric acid cycle for biosynthesis. The appearance of isocitrate lyase confirms that growth on hexadecane induces the activity of the glyoxylate cycle.

Acetyl-CoA is synthesized in the mitochondria of fungi and must be transported to the cytoplasm for the use in fatty acid synthesis. Transport of acetyl-CoA to the cytoplasm is thought to be mediated by ATP: citrate lyase and/or carnitine acetyl transferase.

ATP: citrate lyase was detected in cells when glucose was the carbon source. This is a cytosolic enzyme that is involved in the generation of cytosolic acetyl-CoA from citrate after its export from the mitochondria. When the fungi were grown on hexadecane, however, no ATP:citrate lyase activity was detected. b-oxidation of fatty acids in the peroxisomes of fungi liberates acetyl-CoA, which is translocated to the mitochondria for energy generation. As a result, when the fungi were grown on a hexadecane-based carbon

Table 2

Enzyme activities (nmol/min/mg protein) in *Cephalosporium humicola EI* and *Mucor globosus 11* grown on glucose and hexadecane

Enzyme	*Cephalosporium humicola*		*Mucor globosus*	
	Glucose	Hexadecane	Glucose	Hexadecane
ME	35	18	22	11
ACL	30	-	26	-
G-6PGDH	1100	200	980	120
FAS	33	40	23	22
ACC	2.5	4.8	3.1	5.3
DAGAT	0.7	2.1	0.4	1.7
ICL	-	58	5	40
CAT	34	350	22	230
PK	820	160	600	67

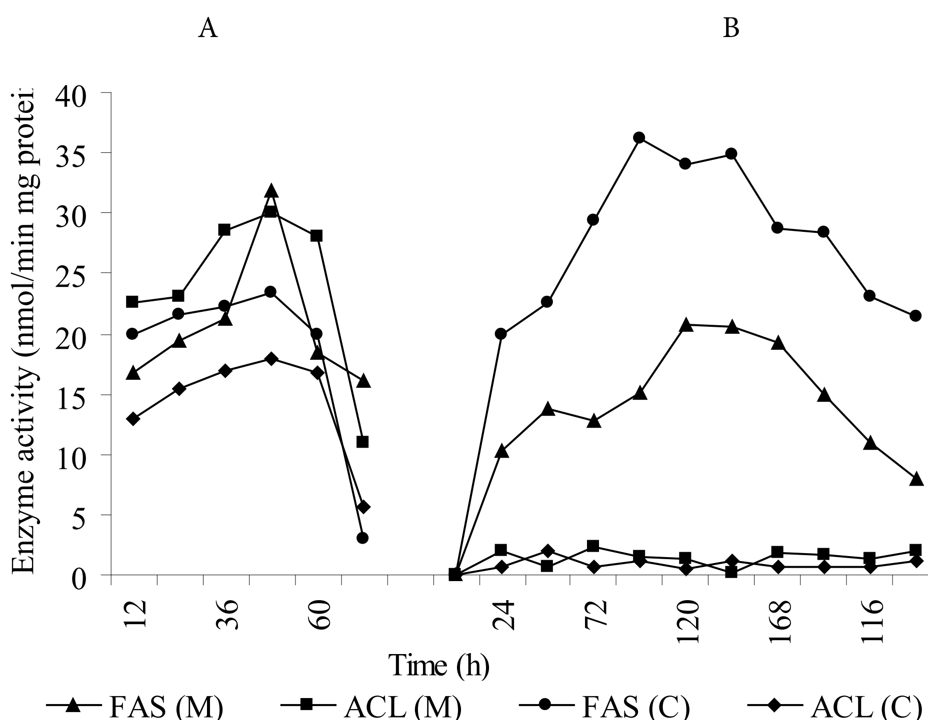

Fig 2. Activities of FAS and ACL in *Cephalosporium humicola EI* (C) and *Mucor globosus 11* (M) during growth on glucose (A) and hexadecane (B).

source, the acetyl-CoA flux was into, not out of the mitochondria; ATP: citrate lyase activity was therefore not required in such cells and was completely repressed.

Carnitine acetyltransferase is an enzyme involved in the translocation of acetyl-CoA inside the cell. This enzyme was detected in glucose-grown cells and its activity was increased tenfold when hexadecane was the carbon source. The increased activity of this enzyme was a result of the increased requirement for acetyl-CoA transportation between cellular compartments in cells grown on a hexadecane-based carbon source. The changes in the activities of this enzyme, as

well as of isocitrate lyase and ATP: citrate lyase, confirm, that when the cells were cultivated on sole hydrocarbon, the metabolism of the fungus underwent changes too.

Malic enzyme is believed to play a key role in provision of reduced nicotinamide adenine dinucleotide phosphate (NADPH) for both fatty acid biosynthesis and fatty acid desaturation. Therefore, it is an important factor controlling the extent of lipid accumulation not by reducing the supply of carbon but the supply of reducing (H+) equivalents. Proposed roles of malic enzyme in other eukaryotic microorganisms are somewhat sketchy. Although malic enzyme has been known for many years, its role may vary significantly from one organism to another. However, occurrence of malic enzyme in tested fungi is clearly implicated to be in the provision of NADPH to drive desaturation and elongation reactions leading to the formation of polyunsaturated fatty acids.

Pyruvate kinase is the last enzyme in the glycolytic pathway of sugar catabolism. It catalyzes the irreversible conversion of phosphoenolpyruvate into pyruvate by the addition of a proton and the loss of a phosphate group, which is transferred to ADP. High activity of pyruvate kinase was detected in glucose-grown cells, but was decreased by 80.0-90.0% when the fungi were grown on hexadecane. These observations surmised that the residual pyruvate kinase activity was not involved in the provision of pyruvate for biosynthesis in cells of fungi. It was hypothesized that malic enzyme activity (which increased under these conditions) was responsible for the generation of pyruvate needed for biosynthetic purposes.

Glucose-6-phosphate dehydrogenase is an enzyme of the oxidoreductase class that catalyzes the oxidation of glucose-6-phosphate to a lactone, reducing NADP+ to NADPH. The reaction is the first step in the pentose phosphate pathway of glucose metabolism. The lower activity of glucose-6-phosphate dehydrogenase in cells grown on hexadecane reflected the decreased carbon flux through the hexose monophosphate pathway.

At the inspection the results presented in Table 1 appear to indicate production or increased production of linoleic and √-linolenic acid in tested fungi with the change in carbon source from glucose to hexadecane. As well known, biosynthetic route responsible for √-linolenic acid in the fungi is n-6 route, which involves the following consecutive reactions: oxidation of hexadecanol to palmitic acid, elongation of palmitic acid to stearic acid, selective desaturation of the latter to oleic acid catalyzed by Δ9 stearoyl-CoA desaturase and then similarly, to linoleic acid and linolenic acid catalysed by Δ12 oleoyl-CoA desaturase and Δ6 linoleyl-CoA desaturase, respectively. The comparison of composition of saturated fatty acids to that of unsaturated ones from each of the oil samples indicated that out of 20.0% of total fatty acids from M. globosus, and 28.0% from C. humicola grown on qlucose were unsaturated fatty acids. These dates increased to 40 and 41.3% when substrate replaced with hexadecane. Our results suggest that biosynthesis of 18:2 and 18: 3 acids, and consequently, the enzymes Δ12-desaturase and Δ 15 desaturase, which are responsible for the conversion of 18:1 to 18:2 and 18:2 to 18:3 acids, are affected by hydrocarbon.

These studies offer an inside look into exploring the possibility of further use of such microbial lipids, as at least a supplement to other edible fats and for other non-edible industrial purposes.

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