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Single cell oil (SCO) production by *Fusarium* species using cheese whey as a substrate

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Abstract

In this study single cell oil (SCO) production by *Fusarium* species using deproteinized whey as carbon source was investigated. The total lipid accumulated in the biomass of the fungi examined ranged from 0.1467 to 0.8661 mg lipid per mg wet weight. Gas chromatography analysis of the synthesized microbial lipids from the *Fusarium* species revealed that the major constituents were palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2). Therefore, the whey medium was found to be effective for promotion of the accumulation of substantial amounts of lipids by *Fusarium* species.

Key words: single cell oil, whey, *Fusarium* species

Introduction

Whey, a valuable by-product of the cheese industry, is the abundant liquid fraction that remains after the milk has been curdled and drained during the cheese-making process (Akpinar-Bayizit et al., 2009; Koutinas et al., 2009). Although the chemical composition of whey differs according to the types of cheese produced and particularly according to the type of milk used for the cheese production, whey typically constitutes about 85-90 % of the milk volume used for transformation into ripened cheese, and it retains about 55 % of the milk nutrients. The principal whey components are lactose (44-52 gL^{-1}), proteins (6-8 gL^{-1}) and minerals (4.3-9.5 gL^{-1}) (Jelen, 1992). The biological properties of individual whey constituents have become a focus of scientific interest as potential ingredients of so-called functional or health-promoting foods in relation to excellent nutritional value (Kosseva et al., 2009; Golfinopoulos et al., 2011). However, in most cases, the whey is wasted rather than used, causing a significant loss of resources and major environmental pollution, because of its high biochemical (BOD) and chemical oxygen (COD) demands and making treatment prohibitive due to the cost (Athanasiadis et al., 2004; Koutinas et al., 2009; Guimarães et al., 2010; Vamvakaki et al., 2010; Tavares et al., 2011; 2012).

In the frame of extensive efforts on the bioremediation of this high polluting liquid waste, a strategy of remarkable importance, has been devised for the biotechnological valorization of whey, involving the simultaneous reduction of the pollution load and use as fermentation substrate for conversion into higher-value end products, such as microbial biomass (single-cell protein - SCP) and microbial lipids (single-cell oil - SCO) (Fakas et al., 2008; 2009a,b; Vamvakaki et al., 2010). SCOs, microbial lipophilic compounds, may be defined as sources of high-valued oils, including cocoa butter equivalent (CBE) fats and polyunsaturated fatty acids of nutritional and pharmaceutical importance. The microorganisms, such as bacteria, yeasts, molds and microalgae that accumulate more than 25 % of their biomass as microbial oil are generally termed as oleaginous due to their similarity of their oils in fatty acid composition to vegetable oil. Compared with the production of vegetable oils, the culture of oleaginous microorganisms is not affected by the

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seasons or climate; furthermore, oleaginous microorganisms can accumulate lipids within a short period of time. In addition, the use of many oleaginous microorganisms to produce lipids provides some advantage for such various inexpensive agro-industrial sources as whey with regard to the enormous variability of the fatty acid composition, depending on the carbon source used for the cultivation of the cells (Akpinar, 1997; Papanikolaou et al., 2004; Dyal et al., 2005; Papanikolaou et al., 2007; Fakas et al., 2009a). The biomass production and lipid yield by various molds utilizing whey are summarized in Table 1.

There is current interest in some molds as suitable alternatives for sources of SCOs, as these fungi have the capacity to convert a number of raw materials into a series of value-added products, such as lipids-rich-in polyunsaturated-fatty-acids- (PUFAs) having more than 18 carbon atoms. A number of fatty acids in this range has been associated with particularly health promoting effects, including γ-linolenic acid (GLNA, C18:3ω-6 all *cis* 6,9,12-octadecatrienoic acid), dihomo-γ-linolenic acid (DHGLNA, C20:3ω-6 all *cis* 8,11,14-octadecatrienoic acid), arachidonic acid (ARA, C20:4ω-6 all *cis* 5,8,11,14-eicosatetraenoic acid), eicosapentaenoic acid (EPA, C20:5ω-3 all *cis* 5,8,11,14,17) and docosahexaenoic acid (DHA, C22:6ω-3 all cis 4,7,10,13,16,19). Similar to vitamins, these essential fatty acids are nutrients that must be provided in the diet because they cannot be synthesized within the body and are, thus, of commercial importance (Aggelis et al., 1988; Lechevalier and Lechevalier, 1988; Gill, 1997; Larsson et al., 2006).

Fusarium spp. are a widespread group of fungi and commonly associated with plants. Most species are saprophites, however, some species could produce mycotoxins that affect human and animal health if they enter the food chain (Chand and Srinivasan, 1984a; 1984b). To date, no relevant research is available concerning the potential of *Fusarium* spp. that could be utilized in microbial lipid production. Bhatia and Arneja (1978) observed that during initial stages of growth of *F. oxysporum* (up to 1 h of incubation) the phospholipid fraction exhibited relatively higher specific activity values indicating its rapid rate of synthesis. Naim et al. (1985) studied the effect of yeast extract, beef extract, corn steep liquor and molasses addition to cultivation medium on the growth of *F. oxysporum*. Yeast or beef extracts and corn steep liquor stimulated fungal growth, however treated molasses were found unsuitable. On the contrary the total lipids were remarkably high at molasses concentrations of 8 % and 9 %. Naqvi et al. (1997) investigated the fourty seven species of soil fungi for the presence of lipids/fats via surface culture method using two different media, namely Pruess media (Pruess et al., 1934) and modified Pruess media. The capabilities of fungal species to produce lipids were: *F. acuminatum* 4.36 %, *F. oxysporum* 44.96 %, *F. semitectum* 1.30 % and *F. solani* 3.58 %. It is concluded that modified Preuss medium, containing ammonium nitrate as nitrogen source and glucose as carbon, was rather suitable for biomass production and lipid synthesis than Pruess media.

Azeem et al. (1999) stated that the fatty acid profile of microbial lipids obtained from *Aspergillus sydowii*, *Fusarium oxysporum* and *F. equisetti* had a high percentage of unsaturated fatty acids, particularly oleic acid (62.9, 52.4 and 46 % respectively). These microbial oils had a similarity to the edible oils, such as groundnut and palm oil, as containing C16 and C18 fatty acids esterified in the form of triacylglycerols.

Table 1. Biomass and lipid yield on whey media by various mold species

Molds	References	Biomass (mg)	Lipid yield (gL^{-1})
M. isabellina	Fakas et al., (2009b)	7800	2.0
	Papanikolaou et al., (2004)	8400-10400	3.7-2.0
Mucor spp. RRL001	Ahmed et al., (2006)	28000	5.0
Aspergillus spp.	Akpinar-Bayizit et al., (2010)	3700-13350	1.10-7.72
C. *echinulata*	Fakas et al., (2008)	6200	3.3
	Papanikolaou et al., (2004)	4100-13500	3.8-0.4

Ellis et al. (2002) determined that the most prominent fatty acids found in the mycelium of *F. oxysporum* B1 were 18:1, 16:0 and 18:0, which amounted 47 % of total fatty acids.

Since the potential of *Fusarium* spp. for value-added lipids is rather unnoticeable, we aimed to analyze the biomass, lipid accumulation and fatty acid profiles of five *Fusarium* species, namely, *Fusarium culmorum*, *Fusarium solani*, *Fusarium verticillioides*, *Fusarium graminearum* and *Fusarium semitectum* in a liquid media containing deproteinized cheese whey, an inexpensive and plentiful carbon substrate, as well as glucose, a growth promoter.

Materials and Methods

Fungal cultivation and preparation of inoculum

The examined fungal cultures of *Fusarium culmorum*, *Fusarium solani*, *Fusarium verticillioides* (formerly named as *Fusarium moniliforme*), *Fusarium graminearum* (also called as *Gibberella zeae*) and *Fusarium semitectum* were provided from the culture collections of Food Engineering and Plant Protection Departments of Uludag University Faculty of Agriculture. All fungal cultures were maintained on potato dextrose agar (PDA, Difco) slants at 4±1 °C and subcultured every month till further use. Approximately 1 cm^2 of mycelia, grown for 4 days on PDA slants, was aseptically removed and used as the inoculum for a 150 mL shake flask with 50 mL of basic medium.

Growth media and fungal growth

The molds were pre-cultivated in 150 mL Erlenmeyer flasks containing 50 mL of basic medium (Table 2, 3) for 48 h. After pre-cultivation cells were transferred into 2 L Erlenmeyer flasks containing 1000 mL basic medium, which were orbitally shaken at 160 rpm in a shaker incubator. The batch cultivation was carried out at 25±1 °C for 2 days until the glucose was exhausted. The initial pH of medium was adjusted to 6.0-6.5 and sterilized at 121 °C for 15 minutes. The denaturated protein was vacuum filtered; the pH of supernatant (peptonized whey) was adjusted to 6.0 with H_2SO_4 and re-sterilized at 121 °C for 15 minutes (Akpinar, 1997).

Harvesting and lipid extraction

Fungal cells were harvested by vacuum filtration through two thickness of Whatman GF/A filter paper (7 cm diameter), and washed with distilled water (2 x 500 mL). The mycelia were transferred into a pre-weighed shake flask and the washed wet cells were dried at 80 °C for 24 h, basically when

Table 2. Composition of optimized basic medium

Compound		gL^{-1}
Nitrogen source	KH_2PO_4	7
	Na_2HPO_4	2
Carbon source	Yeast extract	1.5
	Ammonium chloride	2
	Glucose	10
	Whey powder	20
The trace element solution	$CaCl_2 \cdot 2H_2O$	0.5
	$FeSO_4 \cdot 7H_2O$	0.1
	$ZnSO_4 \cdot 7H_2O$	0.1
	$MnSO_4 \cdot 2H_2O$	0.001
	$CuSO_4 \cdot 5H_2O$	0.005

Table 3. Properties of whey powder

Lactose (%)	75.5
pH	6.3-6.8
Titritable acidity (% LA)	0.18
Fat (%)	1.5
Protein (%)	12
Total ash (%)	8
Moisture (%)	3

a constant weight was achieved (Kendrick and Ratledge, 1990). All of the experiments were performed in duplicate. The total lipid contents of the fungal mycelia were determined using the Folch et al. (1957) method. Approximately 5 g wet cells were extracted into 150 mL chloroform/methanol (2:1, v/v) at 5±1 °C overnight. The cellular debris was removed from the organic phase by filtration and washed with 75 mL chloroform/methanol (2:1, v/v). The two organic extracts were transferred to a separating funnel and washed twice with 100 mL of distilled water. After each washing the organic phase was kept, residual water was removed with anhydrous $MgSO_4$ and the solvent was evaporated under vacuum until absolute dryness. Lipid material was dissolved in a minimal volume of diethyl ether, quantitatively transferred to a preweighed vial and the ether was removed under a stream of nitrogen. The sample was dried in a vacuum desiccator over P_2O_5 for 24 h, reweighed and dissolved in 3 mL petroleum ether for further fatty acid analysis.

Preparation of fatty acid methyl esters

Cold esterification was carried out to obtain fatty-acid methyl esters (FAMES) of the algal lipids according to the IUPAC (International Union of Applied and Pure Chemistry) method (Commission Regulation (EC) No 796/2002 of 6 May 2002). The analysis of fatty acid was performed with Perkin-Elmer-Thermoquest Trace Gas Chromatograph (GC) coupled with Perkin-Elmer-Thermoquest Trace mass spectroscopy (MS) detector. A fused silica capillary column, SP-2330 (30 m×0.25 mm inside diameter, 0.25 μm film thickness) was used for GC system. The temperature program was set up from 50 °C to 220 °C with a ramp of 5 °C min-1, of 8 min isotherm at 120 °C and 220 °C. Both the injector and detector temperatures were 250 °C. Helium was used as the carrier gas (1 mL min^{-1}). The injection volume was 0.5 μL with a split rate of 150:1. For the MS detection interface temperature was 230 °C, an ionization energy (EI) of 70 eV, full scan acquisition mode, a source temperature of 150 °C, scan range of 50-300 amu and scan rate of 1s^{-1} were used.

The individual constituents showed by GC were identified by referencing GC/MS spectra of authentic standards and identified compounds in the TUBITAKMRC, WILEY and NIST/EPA/NIH Mass Spectral Libraries. The relative concentrations of each constituent of fatty acid was expressed as (%) total fatty acids.

Results and discussion

In most oleaginous molds, the lipids are present both in the cell membranes and in the cytosol. The accumulation of lipid in oleaginous molds is known to occur when there is a depletion of growth nutrients, other than carbon, preventing cell proliferation and allowing the accumulation of lipids in the cells (Kendrick and Ratledge, 1990). As data on *Fusarium* species for SCO production is very limited, this is the first detailed report focusing on *Fusarium* with respect to lipid and fatty acid production using whey as carbon source, which is particularly known as a good carbon source for utilization by yeasts for microbial lipid synthesis.

The maximum biomass-specific productivities of the molds cited in Fig. 1a were approximately equivalent for *F. verticillioides*, *F. culmorum* and *F. solani*, as 937.50, 780 and 778.75 mg. The biomass of *F. verticillioides* was eleven-fold higher than *F. semitectum*, nonetheless the ratio of lipid yield within these species was two-fold. It was determined that *F. culmorum* had five-fold higher biomass than *F. graminearum*, however, on the contrary the lipid yield of *F. culmorum* was half-fold higher. Lipid contents in wet biomass of examined mold species were between 67.5 and 221.25 mg (Fig. 1b). *F. graminearum* had the highest content of lipid in wet biomass with 86.61 %, followed by *F. semitectum* (80.60 %). From these observations, it is clear that whey could be employed for lipid production by all of the *Fusarium* spp. examined as a C-source.

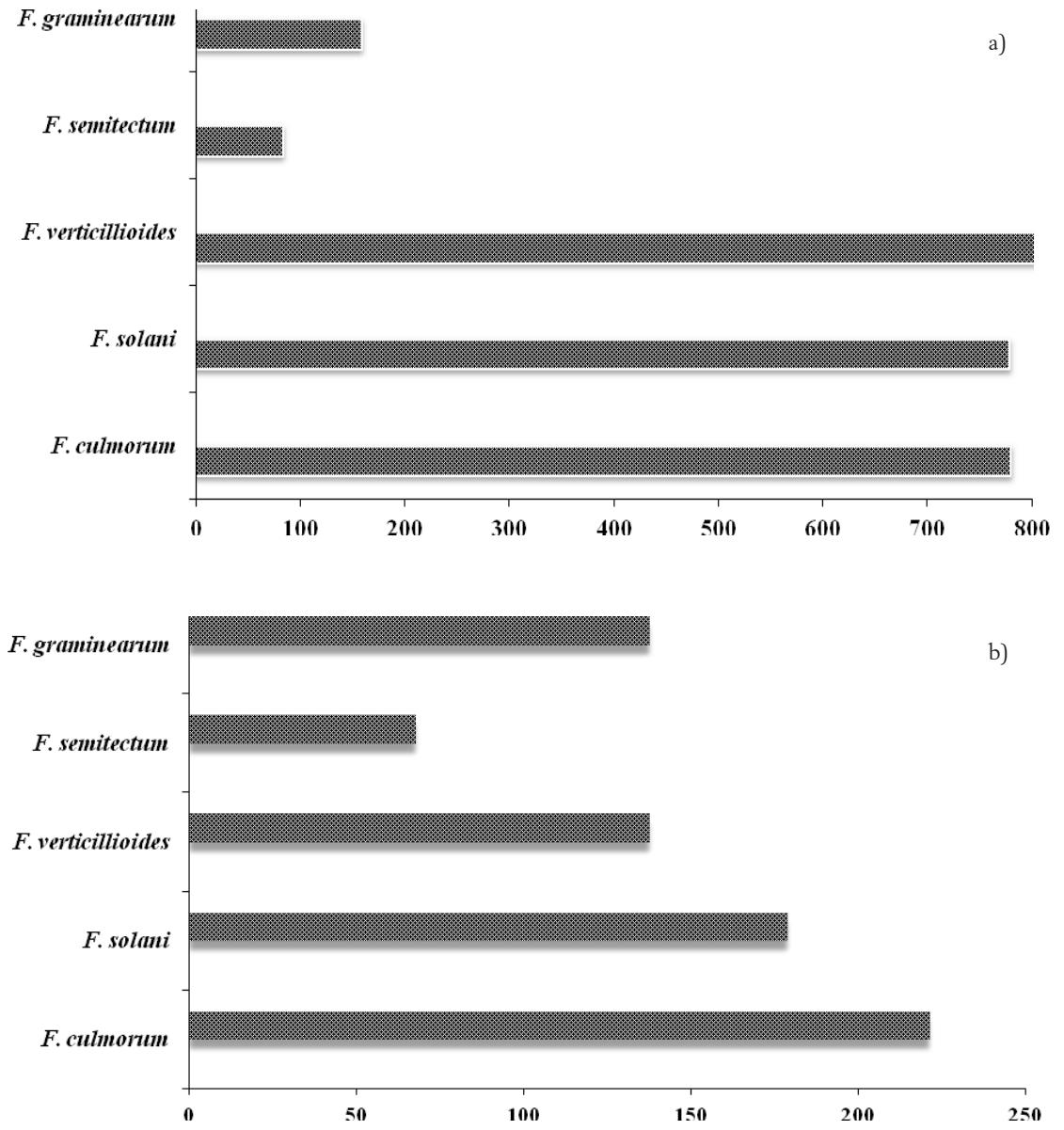

Figure 1. a) Biomass (mg wet weight) and b) lipid yield (mg lipid per mg wet weight) by *Fusarium* species

The fatty acid composition of total lipids determined by capillary gas chromatography, as well as total lipid contents of examined *Fusarium* spp. are presented in Table 4. The chromatographic analysis revealed an array of both saturated and unsaturated fatty acids. The data indicated the presence of identified 17 fatty acid compounds with different chain lengths, ranging from 10 to 20 carbons. The saturated and unsaturated portions represented 22.17 to 40.13 % and 59.89 to 78.16 % of total fatty acids, respectively. Although not being most dominant polyunsaturated fatty acids ranged from 32.06 % in *Fusarium semitectum* to 57.20 % in *Fusarium solani*.

In the analysed *Fusarium* species, palmitic acid and stearic acid were the most abundant saturated fatty acids. The content of C16:0 was highest in *Fusarium solani* with 20.88 % of total FAMEs and lowest levels were in *F. graminearum* with 11.86 % of total FAMEs. Stearic acid content varied from 5.76 % to 17.78 %. Furthermore, lauric acid (C12:0), myristic acid (C14:0), pentadecanoic acid (C15:0) and

Table 4. Fatty acid composition of microbial oil samples in *Fusarium* species

	F. culmorum	*F. solani*	*F. verticillioides*	*F. semitectum*	*F. graminearum*
Fatty Acids (%)					
Saturated					
>C16:0	2.81	2.19	2.43	3.15	3.60
C16:0	13.07	20.88	17.02	18.31	11.86
C18:0	6.39	5.76	6.17	17.78	6.41
C20:0	0.32	0.27	0.43	0.89	0.30
Unsaturated					
Monounsaturated					
C16:1, *cis*-9	0.93	0.70	0.81	2.33	0.84
C18:1, *cis*-9	33.11	12.79	28.92	24.86	31.18
C18:1, *trans*-9	nd	0.25	nd	0.64	0.31
Polyunsaturated					
C18:2, *cis*-9,12	35.89	51.57	35.08	21.52	36.13
C18:3, *cis*-9,12,15	4.21	1.79	1.83	1.95	4.51
C18:3, *cis*-6,9,12	0.11	0.10	0.12	0.16	0.16
C20:3, *cis*-5,8,11	0.02	0.17	0.19	0.28	0.23
C20:4, *cis*-5,8,11,14	0.80	nd	nd	nd	0.05
C20:5, *cis*-5,8,11,14,17	0.04	0.05	0.07	0.12	0.03
<C20:5	3.05	3.52	6.93	8.03	4.43
Saturated (%)	22.59	29.10	26.05	40.13	22.17
Unsaturated (%)	78.16	70.94	73.95	59.89	77.87
Monounsaturated	34.04	13.74	29.73	27.83	32.33
Polyunsaturated	44.12	57.20	44.22	32.06	45.54

nd - not determined

arachidic acid (C20:0) were detected in the mold species tested either in trace levels or not detected.

Among the monounsaturated fatty acids (MUFAs) oleic acid (c18:1, cis-9) was the most abundant fatty acid in all *Fusarium* species examined ranging from 12.79 to 33.11 %. Total MUFAs accounted for 13.74 to 34.04 %. Despite a relatively low biomass, *F. graminearum* was found to be second highest producer of monounsaturated fatty acids with the percentage of 32.33 %. With a six-fold high biomass production *F. culmorum* was also a good source for MUFAs (34.04%). Ievleva and Bragintseva (1984) reported that palmitic acid was the dominant fatty acid in *Fusarium sambucinum* grown in a fermenter utilising molasses and ethanol as carbon sources.

LA, ALNA, GLNA, eicosatrienoic acid (C20:3 all *cis*-5,8,11; ω-9), ARA and EPA represented the predominant proportions of PUFAs. The amount of LA averaged from 21.52 % of total FAMEs of *F. semitectum* to 51.57 % of total FAMEs in *F. solani*. Fatty acid studies on molds are mainly based on GLNA, EPA, DHA and ARA production due to their health promoting functions (Aggelis et al., 1990; Higashiyama et al., 2002; Ahmed, 2006; Dyal et al., 2005; Fakas et al., 2008).

Total polyunsaturated fatty acids of C18 and C20 accounted for 32.06 to 57.20 %, the lowest being in *F. semitectum* and the highest being in *F. solani*. There were large variations in PUFAs within all species examined. ARA was detected only in *F. culmorum* and *F. graminearum* in very small

quantities. EPA was detected in all species ranging from 0.03 to 0.12 % with the highest amount detected in *F. semitectum*. The oil produced by these organisms generally approximated the oil produced by plants in that they contained C16 and C18 fatty acids esterified in the form of triacylglycerols. It is interesting to note that very long-chain polyunsaturated fatty acids (longer than 18 carbons), which are not found in whey, have been detected in the SCOs of *Fusarium* species. In general the composition of accumulated microbial lipid has been stated to reflect the composition of the utilized carbon source. Detection of 20 carbon fatty acids in SCOs of *Fusarium* species indicated the possibility of desaturase/elongase activity through the enzymatic steps of fatty acid synthesis.

Conclusion

Microbial lipids are in the forefront of biotechnological products for many years. However, SCO to be considered as commercially viable products, have to compete with the abundance of oils available from agricultural sources. *Fusarium* species are microorganisms of interest since they have potential to utilize numerous agro-industrial residues, such as whey, molasses, olive mill waste, with the added bonus of reducing the pollution due to by-products. In the present study the total lipid content and fatty acid distribution of five *Fusarium* species in batch cultivation on whey media was investigated. *Fusarium* species displayed a significant growth and a notable production of essential fatty acids such as LA and ALNA. Since certain *Fusarium* fungi, i.e. *F. verticillioides*, *F. proliferatum* and *F. graminearum*, are reported to produce mycotoxins under certain conditions in maize and grains, in this study mycotoxin formation was not evaluated as the main aim was to investigate whey as carbon source for microbial lipid production. Therefore, further research should focus on prevention of mycotoxin producing conditions aside with selection of high lipid-accumulating *Fusarium* species.

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Ulje Fusarium spp. iz sirutke kao supstrata

Sažetak

Cilj ovog rada bio je istražiti mogućnost sineteze ulja *Fusarium* spp koristeći deproteiniziranu sirutku kao izvor ugljika. Ukupni lipidi akumulirani u biomasi gljiva utvrđeni su u rasponu od 0,1467-0,8661 mg lipida po mg biomase. Plinskom kromatografijom utvrđeno je da su glavni sastojci sintetiziranih mikrobnih lipida *Fusarium* spp: palmitinska kiselina (C16:0), palmitoleinska kiselina (C16:1), stearinska kiselina (C18:0), oleinska kiselina (C18:1) i linolna kiselina (C18:2), te se može zaključiti da je sirutka učinkovit medij za sintezu lipida *Fusarium* spp.

Ključne riječi: ulje mikrobnog porijekla, sirutka, *Fusarium* spp.

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