Identification of newly-isolated microorganisms containing valuable polyunsaturated fatty acids

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The fatty acid compositions of 21 cultures of microorganisms isolated from soil or freshwater sites in Kazakhstan were studied. *Pleurosigma sp*. contained significant amounts of arachidonic acid while several cultures had appreciable eicosapentaenoic acid, in addition to various 18C polyunsaturated acids. Cultures containing the highest amount of polyunsaturated fatty acids were: *Pleurosigma sp., Petromyces alliaceus, Rhodotorula mucilaginose, Yarrowia lipolytica, Auerobasidium commune* and they were selected for further study. They may prove useful as new sources for the production of nutritionally-valuable polyunsaturated fatty acids.

Keywords: Arachidonic acid; Fungi; Microalgae; Polyunsaturated fatty acids; Eicosapentaenoic acid.

Abbreviations: FAs - fatty acids; PUFAs - polyunsaturated fatty acids; FAME - fatty acid methyl ester; HPLC – high performance liquid chromatography; FID - flame ionizing detector; AA - arachidonic acid; EPA - eicosapentaenoic acid.

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Introduction

Polyunsaturated fatty acids (PUFAs) are currently attracting increasing attention because of their important nutritional, industrial or pharmaceutical uses. They are the structural components of cell membrane lipids and lipoprotein complexes in brain and spinal cord and in other tissues, for example heart, liver, and eyes. In living organisms, they affect transport processes and stimulate cellular responses, including lipid metabolism, immune responses and adaptation to the various environmental factors [1-2]. PUFAs are essential for the growth and development of the body and play an important role in the prevention and treatment of ischemic heart disease, hypertension, diabetes, rheumatoid arthritis and other inflammatory and immune diseases and cancer [3-4].

PUFAs are converted via cyclooxygenase, lipoxygenase or epoxygenase reactions into biologically-active metabolites such as the eicosanoids, resolvins and protectins. These metabolites have widespread biochemical and

physiological activities [5]. For a discussion of the relative importance of n-3 and n-6 PUFAs in the diet see also [6-8].

According the location of the first double bond PUFAs belong to two classes or series (n-6 or n-3 acids). The most significant representatives of the n-6 PUFAs are linoleic (18:2), γ-linolenic (18:3), dihomo-γ-linolenic (20:3), and arachidonic (20:4, AA) acids; the n-3 PUFAs include α-linolenic (18:3), stearidonic (18:4), eicosapentaenoic (20:5, EPA), and docosahexaenoic (22:6) acids [9].

Traditional sources of PUFAs are vegetable oils such as sunflower, linseed, soybean, rapeseed as well as marine sources such as fish oils. However, in recent decades, there has been interest in using microorganisms as promising producers of PUFAs. With the increase in demand for PUFAs as well as the decline in fish stocks, there has increased attention paid to microorganisms which, after all, are the primary sources of the 20 or 22C PUFAs in fish oil. Moreover, microorganisms show high growth rates [10] and can be cultivated under conditions which do not compete with land for other food production.

It is believed that the PUFA yields from microorganism are in the order microalgae > fungi > bacteria [11]. Moreover, the content of individual PUFAs can exceed 60% of the total fatty acids (FAs) under optimal culture conditions or after genetic modification. Even in the different species within one genus, PUFA contents may vary significantly. For example, it has been shown that the *Mortierella* fungus can accumulate arachidonic acid from 4.2% (strain *M. gracilis VKM-F-1493*) to 55.2% (strain *M. alpina NRRL A-10995*) of the total FA [12] under similar growth conditions.

Due to PUFA importance, the PUFA-containing products have wide applications. Plant oils containing PUFAs are used in food and medicine. PUFAs isolated from microorganisms are used in infant formulas, adult diets, as food additives and pharmaceutical precursors, as well as in aquaculture [13].

In this study, we have collected, isolated, and cultivated a variety of microorganisms from different soil and freshwater habitats in Kazakhstan. The overall aim was to select species containing significant quantities of PUFAs that might be useful for further commercial exploitation.

Materials and Methods

Microorganisms and culture conditions

15 cultures of microscopic fungi and yeast and 6 microalgae strains were initially isolated from the soil or freshwater sources in Kazakhstan, purified, identified and kept at the local collection of al-Farabi Kazakh National University. Microorganisms were isolated by dilution plating method in different selective medium [14]. The purity of cultures was monitored by seeding onto solid nutrient medium and by microscopy [14].

For lipid analysis, microalgae were grown in 250 ml flasks on a 16:8 h (L:D) cycle at room temperature in Fitzgerald' medium as previously described [15]. Fungi were grown in Petri dishes prepared with Sabouraud medium, containing 20 g dextrose, 1.5 g peptone, 1.5 g casein hydrolysate per L of water (pH 5.7) at 29°C for 10 days. The yeast were grown in 500 ml flasks with 200 ml of Czapek medium (30 g sucrose, 1.2 g sodium nitrate, 1.1 g dipotassium phosphate, 0.5 g magnesium sulphate, 0.5 g potassium chloride, 1.0 g ferrous sulphate per L of water; pH 7.3), under conditions previously described [16]. The initial optical density was 0.1, and all organisms were collected in the stationary phase. Absorbance was measured at 590 nm. Microalgae and yeast cells were harvested by centrifugation (1000 g); fungal biomass was collected from Petri dishes using a pair of tweezers.

Identification of microorganisms

Identification of microorganisms based on morphological, physiological and biochemical features was carried out according to the methods described previously [17-21]. To clarify the species of microorganisms, some genetic identification was also performed. DNA extraction was done using CTAB method [22], followed by amplification of two 18S rRNA microalgae fragments using two pairs of primers: 5'-TACCTGGTTGATCCTGCCAGTA-3' as forward and 5'-ATTACCGCGGCTGCTGGCACC-3' as reverse, and 5'-TAGAGGTGAAATTCT-3' as forward and 5'-GGGCATCACAGACCTG-3' as reverse [23, 24]. For fungi and yeast, an amplification of the internal transcribed spacer (ITS) has been done using primers ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3' as forward) and ITS4 (5'-TCCTCCGCTTATTGATATGC-3' as reverse) [25]. The PCR mix contained 20 ng of DNA for microalgae and 40 ng of DNA for fungi and yeast, 1 unit of Taq DNA Polymerase, 0.2 mM of each dNTP, 1X PCR buffer (Fermentas, Thermo Scientific, Waltham, Massachusetts, USA), 2.5 mM $MgCl_2$, and 10 pM of each primer. The total volume was 30 µl. PCR amplification program included a long denaturation at 95°C for 7 min; 30 cycles at 95°C - 30 s, 55°C - 40 s, 72°C - 1 min; the final elongation was at 72°C for 7 min. The PCR was performed using a GeneAmp PCR System 9700 Thermal Cycler (Applied Biosystems, Grand Island, New York, USA). Purification of PCR products from unbound primers was performed by an enzymatic method using Exonuclease I and Shrimp Alkaline Phosphatase (Fermentas, Thermo Scientific, Waltham, Massachusetts, USA)[26]. Sequencing reactions were performed using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Grand Island, New York, USA) according to the manufacturer's instructions, followed by fragments separation on an automatic genetic 3730xl DNA analyzer (Applied Biosystems, Grand Island, New York, USA). The nucleotide sequences of cultures were analyzed using SeqMan (DNA Star) software. Sequences were identified by BLAST algorithm in GenBank. A subsequent nucleotide sequence was aligned with the sequences deposited at the international GenBank database. In cases of dispute, phylogenetic trees were built using the Neighbor-Joining method [27].

Lipid extraction

Lipid extraction was carried out initially according to Kates [28] followed by the method of Garbus et al. [29]. Total lipids were pre-extracted from 10-100 mg of biomass using 2 ml of isopropanol heated at 70°C during 30 min to inactivate endogenous lipases (twice). The isopropanol extracts were combined, dried under a stream of nitrogen and then redissolved in 3 ml of chloroform/methanol (2:1, by volume). Total lipids were further separated using the method of Garbus et al. [29]. For this, 2 ml of Garbus solution (2 M KCl in 0.5 M phosphate buffer, pH 7.4) was added, mixed and centrifuged at 200 g for 5 min to separate two layers. The lower chloroform fractions were collected, and the solvents were evaporated under a stream of nitrogen. Total lipid extracts were stored in chloroform at -20°C under nitrogen until further analysis.

Fatty acid analysis

Aliquots of the total lipid extracts were used for fatty acid methyl ester (FAME) preparation. FAMEs were prepared by transmethylation with 2.5% H_2SO_4 (v/v) in dry methanol / toluene (2:1, by volume) at 70°C for 2 h. A known amount of C24:1 was added as an internal standard, so that subsequent quantification of peaks (and, consequently, lipids) could be performed. FAMEs were extracted with HPLC grade hexane after addition of 5% (w/v) NaCl. A Clarus 500 gas chromatograph with a flame ionizing detector (FID) (Perkin-Elmer 8500, Norwalk, CT, USA) and fitted with a 30 m x 0.25 mm i.d. capillary column (Elite 225, Perkin Elmer) was used for separation and analysis of FAs. The oven temperature was programmed as follows: 170°C for 3 min, increased to 220°C at 4°C/min), and then held at 220°C for 15 min. FAMEs were identified routinely by comparing retention times of peaks with those of G411 FA standards (Nu-Chek Prep. Inc., Elysian, MN, USA). Perkin-

Elmer Total Chrom Navigator software was used for data acquisition. When double bond positions were not confirmed by mass spectrometry [30], then no specific identity is given.

Results and discussion

Comparison of nucleotide sequences deposited at the GenBank database showed the following results. The cultures of microalgae 1 - 6v shared the highest similarities, respectively, with *Chlorococcum sp. LU9* (Accession No. JQ360519.1), *Chlorococcum infusionum* (Accession No. KF861549.1), *Scenedesmus obliquus strain LU16* (Accession No. JQ360526.1), *Sphaerocystis sp. BUM11019* (Accession No. KC218490.1), *Oocystis lacustris* and *Oocystis sp. MCCS 033* (Accession No. DQ887507.1 and GQ414516.1), *Pleurosigma sp. GGM-2004* (Accession No. AY485515.1).

The fungi and yeast cultures shared the highest similarities: 1g with *Penicillium bilaiae* (Accession No. HG326278.1), 2g with *Penicillium raistrickii strain FRR 1044* (Accession No. AY373927.1), 3g with *Emericellopsis pallida strain XJURML-3* (Accession No. EU045572.1), 4g with *Petromyces alliaceus* (Accession No. EF447422.1), 5g with *Penicillium restrictum strain FRR 332* (Accession No. AY373928.1), 6g with *Penicillium citrinum* (Accession No. KJ154959.1), 7g with *Penicillium aculeatum* (Accession No. EU818695.1), 8g with *Aspergillus terreus strain ATCC 12238* (Accession No. JQ070071.1) and *Aspergillus terreus isolate wb464* (Accession No. AF455426.1), 9g with *Aspergillus sp. DHE9-25* (Accession No. JQ693975.1), 10g with *Mucor circinelloides strain S2-3* (Accession No. JX537952.1), 11g with *Chalastospora obclavata isolate CBS 124120* (Accession No. FJ839616.1) and *Chalastospora cetera* (Accession No. JN383482.1) and *Chalastospora ellipsoidea isolate CBS 121331* (Accession No. FJ839608.1); 12d with *Yarrowia lipolytica strain M7* (Accession No. HM011048.1), 13d with *Rhodotorula mucilaginosa isolate 4502* (Accession No. AF515474.1), 14d with *Yarrowia lipolytica isolate JKL507* (Accession No. HQ718589.1), 15d with *Aureobasidium pullulans strain LDT-1* (Accession No. JN207852.1). Building of phylogenetic trees allowed to specify the taxonomy of cultures.

The total fatty acid composition of selected microorganisms is presented in Table 1 and 2. Our results showed that in most microalgae polyunsaturated FAs were a dominant fraction in comparison to saturated and monoenoic FAs with the exception of the diatom alga *Pleurosigma sp*.: in this alga monoenoic FAs were the major components. The sum of total PUFAs in green microalgae varied from 55.9 to 64.5% of total FAs, the diatom alga contained 33.2% of FAs. Our data showed that the principal FAs in green microalgae were palmitic (C16: 0), palmitoleic (C16: 1n-7), oleic (C18: 1n-9), linoleic (C18: 2n-6) and α-linolenic (C18: 3n-3) or γ-linolenic (C18: 3n-6) acids. The relative amounts of linoleic acid and α-linolenic acid varied between 12-26% and 22-32%, respectively. In *Pleurosigma sp*. the major FAs were palmitic and palmitoleic. Also this alga accumulated significant amounts of AA (12.2%) and EPA (8.5%) (Table 1). Other algal species studied in this work, contained only trace amounts of long-chain PUFA, but they accumulated α-linolenic acid in relatively large amounts, from 22% in *Chlorococcum influsionum* to 24.5 in *Oocystis rhomboideus* (Table 1). It is interesting that in a previous study on FA composition of *Chlorococcum sp*., much lower amounts of C18:3 (as the sum of both isomers) were detected (only about 10% of the total FAs) [31]. This may have been due to species or growth condition effects.

In the present study, the dominant FAs in all selected species of fungi were palmitic, oleic and linoleic acids. Stearic acid (C18: 0) was detected in all fungi at the relative amounts not exceeding 7% of total FAs. Linoleic acid was found in these fungal species at the relatively large amounts (about 50% of total FAs), except

Table 1. Fatty acid contents of some isolated microalgae, % of total FA.

Culture	1v	2v	4v	5v	6v
Species	*Chlorococcum sp.*	*Chlorococcum infusionum*	*Dictyochlorella globosa*	*Oocystis rhomboideus*	*Pleurosigma sp.*
C16:0	14.4	13.4	12.3	14.4	15.3
C16:1*	18.0	9.6	8.5	12.0	49.6
C16:2n-6	3.5	2.3	1.3	6.8	3.0
C16:3n-3	3.5	6.0	6.9	2.3	4.7
C16:4n-3	9.1	5.7	5.6	2.2	0.4
C18:0	1.7	n.d.**	n.d.	n.d.	0.5
C18:1*	10.0	13.9	16.5	9.1	1.4
C18:2n-6	12.7	17.7	11.7	25.6	1.4
C18:3n-6	1.2	2.7	n.d.	0.3	0.7
C18:3n-3	22.8	22.0	32.2	24.5	2.3
C18:4n-3	2.1	5.2	3.1	0.8	n.d.
C20:3n-3	1.0	1.5	1.9	2.0	n.d.
C20:4n-6	n.d.	n.d.	n.d.	n.d.	12.2
C20:5n-3	n.d.	n.d.	n.d.	n.d.	8.5
Total PUFAs	55.9	63.1	62.7	64.5	33.2

*C16:1 and C18:1– the sum of n-9 and n-7 isomers; **n.d. = not detected.

Mucor circinelloides where the level of this FA accounted for 24% of total FA (Table 2). It should be noted that in a previous study on the same species, the level of linoleic acid has been found to be about 4 times lower than in our study [32], pointing to variability probably due to growth conditions. In addition, *M. circinelloides* accumulated quite appreciable amount (14.3% of total FA) of γ-linolenic acid (Table 2). In general, in all fungi studied in the present work, PUFAs were more abundant than monoenoic or saturated FAs. The sum of the total PUFAs ranged from 38.7% in *M. circinelloides* to 70% in *Penicillium bilaiae* (Table 2). In most of the fungal species studied EPA was present at 3-8% of total FA with the maximum observed in *Petromyces alliaceus* (Table 2). AA was detected in small amounts (about 1% of total FAs) only in one species, *Aspergillus sp.* (Table 2).

Our study showed that in the yeast species studied unsaturated FAs were predominant over saturated FAs. Monoenoic FAs were the major class among unsaturated FAs but the PUFA, linoleic acid was abundant in two of the three isolates. Total PUFAs ranged from 21.6% to 43.6% of total FAs. In *Auerobasidium commune* palmitic, palmitoleic, stearic, oleic and α-linolenic acids were the dominant FAs; the percentage of the latter reached 16% of total FAs. *Yarrowia lipolytica* mainly contained palmitic, palmitoleic, oleic and linoleic acids. The relative amount of linoleic acid was about 36% of total FA; this value was about 3 times larger than the value reported by Wang et al.

Table 2. Fatty acid contents of some isolated fungi and yeast, % of total FA.

Culture	6g	5g	1g	7g	4g	9g	10g	13d	12d	15d
Species	*Penicillium citrinum*	*Penicillium restrictum*	*Penicillium bilaiae*	*Penicillium aculeatum*	*Petromyces alliaceus*	*Aspergillus sp.*	*Mucor circinelloides*	*Rhodotorula mucilaginose*	*Yarrowia lipolytica*	*Auerobasidium commune*
C16:0	16.6	19.1	16.3	14.5	14.8	15.5	16.9	7.6	7.6	19.0
C16:1*	1.2	2.1	1.1	0.9	1.2	0.8	5.9	1.5	11.6	13.7
C16:2n-6	n.d.**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.6	2.5
C17:0	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.0	5.9
C18:0	2.5	4.2	2.6	2.2	5.5	6.8	4.2	0.7	n.d.	8.7
C18:1*	25.9	18.7	8.7	20.6	18.5	15.9	32.1	46.6	39.4	20.5
C18:2n-6	47.2	46.4	64.6	56.7	46.9	55.0	23.8	40.4	36.0	3.1
C18:3n-6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	14.3	n.d.	n.d.	n.d.
C18:3n-3	n.d.	n.d.	0.5	n.d.	2.4	3.3	0.6	3.2	n.d.	16.0
C18:4n-3	n.d.	n.d.	n.d.	1.1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C20:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.5	5.6
C20:2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	2.3	n.d.
C20:4n-6	n.d.	n.d.	n.d.	n.d.	n.d.	1.1	n.d.	n.d.	n.d.	n.d.
C20:5n-3	5.4	5.1	4.9	3.1	8.2	n.d.	n.d.	n.d.	n.d.	n.d.
C22:1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	5.0
C22:2	n.d.	1.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
C24:0	1.2	3.2	1.3	0.9	2.5	1.6	2.2	n.d.	n.d.	n.d.
Total PUFAs	52.6	52.7	70	60.9	57.5	59.4	38.7	43.6	38.9	21.6

*C16:1 and C18:1– the sum of n-9 and n-7 isomers; **n.d. = not detected.

[33] in the previous study on *Y. lipolytica*. Palmitic, oleic and linoleic acids were dominant in *Rhodotorula mucilaginose.* The level of the latter (40.4% of total FAs) was much higher than previously reported for *Rhod. mucilaginose AMCQ10C* [34] (Table 2). None of the yeasts contain appreciable amounts of the nutritionally-important long-chain PUFAs such as AA or EPA.

Several of the algae or fungi isolated contained significant amounts of fatty acids which would be important as nutritional supplements. These included AA and EPA in *Pleurosigma sp.*, EPA in *Petromyces alliaceus*, linoleic acid in *Rhodotorula mucilaginose* and *Yarrowia lipolytica* and α-linolenic acid in *Auerobasidium commune*. In fact, most of the fungi studied here were capable of accumulating appreciable amounts of EPA. In our study, the PUFA yields from microalgae and fungi were at the same level, but the PUFA yield from the yeast was lower. Thus, we have been able to isolate a number of microorganisms from natural sources which could be very useful for further commercial exploitation as sources of various PUFAs.

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