Environmental and Experimental Biology (2014) 12: 15–27 *Original Paper*

Production of fatty acids by *Mortierella* and *Umbelopsis* species isolated from temperate climate soils

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Abstract

Soil fungi from the genera *Mortierella* and *Umbelopsis* are known as effective producers of polyunsaturated fatty acids, among which industrially the most important is arachidonic acid. Primary screening for the best arachidonic acid producers was conducted on malt extract with gelatin and potato dextrose agar with or without aspirin. Only five isolates were able to grow on potato dextrose agar with aspirin. They did not contain arachidonic acid but they produced relatively high amounts of other unsaturated fatty acids such as oleic and linoleic acids. All isolates producing arachidonic acid produced also lignoceric acid. From all tested liquid media the highest yield of arachidonic acid and total yield of fatty acids was obtained using medium containing soybean powder. Some of the isolates produced higher amounts of total fatty acids and arachidonic acid in media containing fewer amounts of carbon sources and at low incubation temperature.

Key words: arachidonic acid; fatty acid; lignoceric acid; *Mortierella*; *Umbelopsis*.
Abbreviations: ARA, arachidonic acid; GC-MS, gas chromatography-mass spectrometry; HPLC, high-performance liquid chromatography; MEG, malt extract gelatin; MSCL, Microbial Strain Collection of Latvia; PDA, potato dextrose agar; PUFAs, polyunsaturated fatty acids; *r*, Pearson correlation coefficient; R^2, determination coefficient; SD, standard deviation.

Introduction

For several decades it is known that soil fungi from the genera *Mortierella* and *Umbelopsis* are effective producers of polyunsaturated fatty acids (PUFAs; Jareonkitmongkol et al. 1992; Eroshin et al. 1996; Botha et al. 1999). Industrially the most important fatty acid is arachidonic acid (ARA), which is used in medicine, pharmacology, cosmetics, the food industry (Dyal, Narine 2005) and agriculture (Eroshin, Dedyukhina 2002).

The genus *Mortierella* belongs to the order Mucorales within the subphylum Mucoromycotina (Hibbett et al. 2007). Members from the former *Mortierella isabellina*-group are now classified as *Umbelopsis* (*Umbelopsis isabellina, Umbelopsis ramanniana*; Meyer, Gams 2003) and are classified along with the genus *Mortierella* in the family Mortierellaceae (Hoffmann et al. 2013).

The most studied species from *Mortierella* genus is *Mortierella alpina* but not all isolates of this species produce ARA. For example, in the study by Eroshin et al. (1996) it was found that isolate VKM-F-1630 did not produce this fatty acid but two isolates produced it at a level up to 55.2% of total lipids, which was the highest among all tested *Mortierella* species. *Mortierella gamsii, Mortierella minutissima, Mortierella elongata* and *Mortierella humilis* were observed to produce ARA up to 25, 23.2, 6.2 and 15% of total lipid content, respectively (Eroshin et al. 1996). Strains of *Mortierella globulifera* were able to grow on agar media containing aspirin and they were weak producers of ARA. In the investigation of Botha et al. (1999) one isolate of *Mortierella clonocystis* was observed to produce ARA 29.6% of total lipid content. There is lack of information about the species *Mortierella macrocystis* and its ability to produce ARA. This species is common in forest soils of the Northern temperate region (Grantina et al. 2012; Grantina-Ievina et al. 2013a). *U. ramanniana* does not produce ARA, but isolates of this species can produce other fatty acids in high concentrations, for example, γ-linolenic acid up to 18.3% of lipid content (Hiruta et al. 1996). *U. isabellina* can produce lipids up to 37% of total biomass (Eroshin et al. 1996).

Primary screening for the ARA production can be conducted using growth media containing aspirin (acetylsalicylic acid). Aspirin inhibits oxygenation reactions in prostaglandin synthesis by acetylating the terminal amino group in prostaglandin synthase, and it inhibits synthesis of ARA metabolites (Vane 1971; Botha et al. 1992). *Mortierella* strains that do not produce ARA grow on medium containing 0.84 g L^{-1} aspirin, but most ARA-producing strains cannot grow on such medium (Eroshin et al. 1996). The precursors of ARA are palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3) and dihomo-γ-linolenic acid (20:3) (Lounds et al. 2007). Different techniques exist

for mycelial aging after incubation, which can enhance the lipid and ARA content (Jin et al. 2009). In contrast to other organisms, *Mortierella* spp. are able to produce odd chain saturated and unsaturated fatty acids (Shimizu et al. 1991; Eroshin et al. 1996).

The ecological reason why these fungi accumulate lipids is storage of lipids as carbon reserves, mainly in sporangium spores and/or zygospores (Ansell, Young 1983; Weber, Tribe 2003).

Isolates used in other investigations have been isolated from countries with warm climate, for example, from Taiwan (Chen et al. 1997; Jang et al. 2005) and South Africa (Botha et al. 1999). According to the CBS-KNAW Fungal Biodiversity Centre strain database, a part of the isolates used in specific investigations (Eroshin et al. 1996) originated from temperate climate soils, for example, from Russia, Sweden, Netherlands, and Germany. Additionally, several cultures previously used in investigations originate from warmer climatic regions. For example, *M. alpina* CBS 528.72 (*M. alpina* ATCC 32222) was isolated in North Carolina (Singh, Ward 1997; Nisha, Venkateswaran 2011), and *M. alpina* CBS 754.68 in India (Samadlouie et al. 2012). Since it is known that one of the adaptation mechanisms of fungi to cold environments is increase of unsaturated fatty acids in constituent and membrane lipids (Robinson 2001), it was hypothesized that isolates from temperate climate soils would be good producers of fatty acids and ARA in particular.

The aim of the investigation was to test 19 isolates from temperate climate soils: *M. alpina* (eight isolates), *M. clonocystis, M. gamsii, M. globulifera, M. humilis, M. macrocystis, M. minutissima, M. elongata*, *Mortierella* spp. (two isolates), *U. isabellina* and *U. ramanniana* for fatty acid production using growth media and conditions suggested as the most suitable in previous investigations.

Materials and methods

Fungal strains

All tested strains were obtained from the Microbial Strain Collection of Latvia (MSCL). They were all isolated in previous investigations from various soils of Latvia. *Mortierella alpina* MSCL 1098, *Mortierella alpina* MSCL 1254, *Mortierella alpina* MSCL 1271, *Mortierella clonocystis* MSCL 1200, *Mortierella globulifera* MSCL 1244, *Mortierella* sp. MSCL 1052 and *Mortierella* sp. MSCL 1053 were isolated from aspen stands on former agricultural soil (Grantina-Ievina et al. 2012). *Mortierella alpina* MSCL 959, *Mortierella gamsii* MSCL 948, *Mortierella humilis* MSCL 967, *Umbelopsis ramanniana* MSCL 941, *Umbelopsis isabellina* MSCL 951 and *Mortierella macrocystis* MSCL 1136 were isolated from Norway spruce stands on sod-podzolic soil (Grantina et al. 2012; Grantina-Ievina et al. 2013b). *Mortierella alpina* MSCL 959, *Mortierella gamsii* MSCL 948 and *Umbelopsis ramanniana* MSCL 941 were isolated from the soil sampled in February when the average air temperature was below 0 °C. *Mortierella minutissima* MSCL 1377, *Mortierella alpina* MSCL 1378, *Mortierella alpina* MSCL 1379, *Mortierella elongata* MSCL 1380 and *Mortierella alpina* MSCL 1381 were isolated from an organic potato field (Grantina-Ievina, unpublished data), and *Mortierella alpina* MSCL 1314 was isolated from vermicompost (Grantina-Ievina et al. 2013a).

Chemicals

The following chemicals were used: malt extract produced by Biolife Italiana S.r.l. (Milan, Italy); gelatin 200 BLOOM from Latplanta (Sigulda, Latvia); potato dextrose agar from Laboratorios Conda S.A. (Madrid, Spain); aspirin ASA-Grindex from Grindex (Riga, Latvia); D-glucose from Penta (Prague, Czech Republic); soybean meal from GPRIC (Riga, Latvia); soluble starch, urea, n-hexane, acetone from Sigma-Aldrich (St. Louis, USA); yeast extract from Bio-Rad (Marnes-La-Coquette, France); KH_2PO_4, $MgSO_4$ $7H_2O$ and N,O-bis(trimethylsilyl)trifluoroacetamide from Merck (Darmstadt, Germany); B-group vitamins Multi-B strong from Vitabalans Oy (Hämeenlinna, Finland); rapeseed oil “Ideal” from Iecavnieks Ltd. (Iecava, Latvia); n-heptadecane from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Cultivation conditions

The primary screening of *Mortierella* and *Umbelopsis* strains for the production of ARA was conducted on 2% malt extract with 30% gelatin (MEG) at incubation temperature 20 ± 2 °C for two weeks (Botha et al. 1999) and on potato dextrose agar (PDA) with or without aspirin (0.84 g L^{-1}) at incubation temperature 25 ± 2 °C for two weeks (Eroshin et al. 1996). After incubation plates with PDA were kept for one week at 4 °C for mycelium aging.

Cultivation of seven isolates with the highest content of ARA was repeated on both screening media to test fatty acid production stability. They were then cultivated in liquid cultures in 250 mL Erlenmeyer flasks in 50 mL media volume at 25 ± 2 °C for six days, on a rotary shaker with agitator spin 150 rpm. Four media were used. Medium S was composed (in g L^{-1}) of D-glucose 50.35 and soybean meal 18.30 (Samadlouie et al. 2012). Medium C was composed (in g L^{-1}) of soluble starch 100.00, yeast extract 5.50, urea 1.00, KH_2PO_4 3.75, and $MgSO_4$ 7 H_2O 1.00 (Chen et al. 1997). Medium V was composed (in g L^{-1}) of D-glucose 80.00, yeast extract 11.00, KH_2PO_4 3.80, $NaNO_3$ 3.40, $MgSO_4$ 7 H_2O 0.50, and B-group vitamins 1.12 (Zeng et al. 2012). Medium ME was composed of malt extract 20 g L^{-1}.

Additionally, two isolates *M. alpina* MSCL 959 and *M. alpina* MSCL 1381 were grown at 25 ± 2 °C for 12 days in 300 mL of medium S and ME, respectively, in 500 mL Erlenmeyer flasks, on a rotary shaker with agitator spin 125 rpm. The seed culture was cultivated in 250 mL Erlenmeyer flasks in 50 mL media volume at 25 ± 2 °C for five days,

and a 300 mL volume was inoculated with 4% (v/v) of seed culture. For chemical analysis two average samples from every 12 flasks were taken.

In order to test the ability of isolates to grow at low incubation temperature, all isolates that were able to grow on MEG were grown for four weeks at 12 ± 2 °C. Additionally to this, at low incubation temperature 1% rapeseed oil was added to the media for four isolates, as suggested in the investigation of Singh and Ward (1997).

Since it has been reported that older mycelia can form lipid droplets outside the mycelium (Wang et al. 2011) as was observed in the case of *M. alpina* MSCL 1098 on malt extract agar used for maintaining of seed cultures, the large droplets were collected by pipette tip and subjected to GC-MS.

Chemical analysis

After incubation mycelia were harvested from agar or liquid media and lyophilized. The spectrum and quantity of fatty acids was determined using gas chromatography-mass spectrometry (GC-MS; GCMS-QP2010, Shimadzu, Kyoto, Japan). For the extraction approximately 100 mg of lyophilized fungal biomass was ground in a mortar, precisely weighed and a defined amount of internal standard n-heptadecane solution was added. In the next step 3 g of n-hexane and acetone solution was added, and extractions were incubated for 15 min in the ultrasonic bath "Sonorex" (Bandelin Electronics, Berlin, Germany) and for 12 h at 20 ± 2 °C. After that extracts were filtered through a 0.45 μm MS PTFE Syringe Filter (Membrane Solutions, USA) and to 0.5 g of the extract 20 μL of the N,O-bis(trimethylsilyl) trifluoroacetamide were added followed by 0.5 h incubation at 50 °C. One μL of obtained solution was analyzed by GC-MS or flame ionization detector YL 6100 GC (Kyounggi-do, Republic of Korea). Samples from liquid media were subjected to two extractions with three analytical replicates of each extraction. Individual fatty acids were determined as percentage of total fatty acid content. Total lipids were calculated as the sum of fatty acid content using an internal standard. Lyophilized biomass of two isolates was subjected to supercritical CO_2 extraction by the company Pharmidea Ltd. (Olaine, Latvia). The conditions for the extraction were as follows: separator operated at 80 °C and 320 atm pressure 3 h, 1.4 mg CO_2 per hour. The obtained nonpolar fraction was analyzed as described previously with GC-MS.

The collected extramycelial liquid droplets were subjected to HPLC on Waters Alliance 2690 and MS Waters Micromass Quattro Micro API with column Waters Atlantis HILIC Silica, 2.1 × 150 mm, 3 μm.

Statistical analysis

F-test, *t*-test and correlation analysis were performed with Excel (Microsoft, USA). Significance was evaluated at $p < 0.05$ level. Both Pearson correlation (r) and determination coefficients (R^2) were determined. Cluster analysis with average distance was conducetd using the R package (R Core Team 2013).

Results

Results of primary screening

Only five isolates were able to grow on PDA with aspirin: *Mortierella* sp. MSCL 1052, *Mortierella* sp. MSCL 1053, *M. globulifera* MSCL 1244, *U. ramanniana* MSCL 941 and *U. isabellina* MSCL 951. On PDA without aspirin they did not produce ARA but they produced relatively high amounts of other unsaturated fatty acids such as oleic acid (18:1) and linoleic acid (18:2) at levels of 40 and 19 % of total fatty acid content, respectively (Tables 1 and 2).

Four isolates were not able to grow on MEG: *Mortierella* sp. MSCL 1052, *M. macrocystis* MSCL 1136, *U. ramanniana* MSCL 941 and *U. isabellina* MSCL 951. The results of the other tested isolates on MEG are given in Tables 3 and 4. The highest percentage of ARA was observed for two *M. alpina* isolates, 54 and 53%.

The highest total amount of fatty acids on PDA without aspirin was obtained from isolates *Mortierella* sp. MSCL 1053, *M. gamsii* MSCL 948 and *M. humilis* MSCL 967 (Table 2). The highest total amount of fatty acids on MEG was obtained from isolates *M. globulifera* MSCL 1244, *Mortierella* sp. MSCL 1053 and *M. elongata* MSCL 1380 (Table 4). The correlation coefficient (r) between the content of ARA and precursors of ARA (palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2), linolenic acid (18:3) and dihomo-γ-linolenic acid (20:3)) estimated as mg g^{-1} dry mass on PDA without aspirin was on average 0.73 ± 0.15 and on MEG 0.74 ± 0.06. The highest correlation was between linoleic acid and ARA on MEG ($r = 0.82$) and between dihomo-γ-linolenic acid and ARA on PDA ($r = 0.92$).

Fig. 1 illustrates the results of the cluster analysis using average distance according to the amounts of the fatty acids on MEG and PDA without aspirin (data from the Tables 2 and 4).

For further experiments, seven isolates were chosen according to the ARA yield per one Petri dish on MEG or PDA without aspirin, taking into account that the total amount of ARA per Petri dish differed on both used media (Fig. 2). The chosen isolates were *M. alpina* MSCL 959 (0.80 mg on MEG), *M. alpina* MSCL 1381 (0.45 mg on MEG), *M. elongata* MSCL 1380 (0.42 mg on MEG), M. alpina MSCL 1379 (0.42 mg on MEG), *M. alpina* MSCL 1098 (0.25 mg on MEG), *M. gamsii* 948 (0.96 mg on PDA) and *M. humilis* MSCL 967 (0.85 mg on PDA).

Content of extramycelial liquid droplets

According to the results of GC-MS, the extramycelial liquid droplets from the isolate *M. alpina* MSCL 1098 had 1.56% fatty acid content (mainly palmitic acid). Analysis of extramycelial liquid with HPLC method showed that it

Table 1. Percentage of individual fatty acids from all fatty acids on Potato Dextrose Agar without aspirin arranged by the percentage of arachidonic acid (20:4). –, not detected)

Isolate, No. in MSCL	14:0	15:0	16:1	16:0	17:0	18:3	18:2	18:1	18:0	20:4	20:3	20:1	20:0	22:0	24:1	24:0
M. alpina 1271	0.3	1.2	–	11.2	–	0.7	0.9	6.2	2.3	69.0	–	–	0.7	1.7	–	5.8
M. alpina 1378	0.7	1.0	0.3	15.0	–	1.1	0.5	6.4	5.2	42.0	1.3	0.7	1.1	7.6	6.2	12.0
M. clonocystis 1200	1.1	1.5	1.1	18.0	–	2.7	12.0	23.1	1.7	34.0	2.5	0.3	–	0.9	0.7	0.8
M. alpina 1098	1.2	1.7	–	24.0	0.5	0.6	0.8	7.4	9.8	32.0	–	–	1.5	6.1	4.3	10.0
M. alpina 959	0.6	0.8	–	26.0	–	0.4	1.0	8.0	7.6	29.0	–	0.5	1.6	5.5	0.9	18.0
M. elongata 1380	0.4	0.8	0.8	19.0	0.8	2.2	8.7	25.8	6.8	24.0	2.6	1.3	0.3	1.3	0.8	1.0
M. alpina 1381	1.3	1.4	0.7	18.0	0.4	2.6	8.6	36.5	4.8	23.0	1.6	1.0	–	0.4	0.3	0.5
M. gamsii 948	0.6	0.5	0.2	18.0	–	2.8	7.1	29.6	9.2	21.0	3.1	0.9	0.5	1.5	0.8	2.4
M. alpina 1379	1.3	1.1	–	16.0	–	0.7	0.5	8.5	10.0	21.0	–	0.7	2.4	11.0	6.3	20.0
M. alpina 1314	1.0	0.5	0.3	30.0	0.3	3.8	7.0	18.0	12.0	17.6	2.1	0.6	0.9	1.9	0.1	3.6
M. alpina 1254	1.4	2.3	–	25.0	–	0.2	0.5	3.9	8.3	11.0	–	1.1	2.8	11.0	1.1	32.0
M. minutissima 1377	1.7	2.4	–	31.0	0.6	–	0.5	8.0	12.0	10.0	–	0.6	2.5	11.0	4.6	15.0
M. humilis 967	0.9	0.7	1.3	30.0	0.2	1.6	4.5	23.4	5.9	7.3	0.9	2.4	0.9	4.8	3.8	10.0
M. macrocystis 1136	1.7	0.6	–	27.0	0.7	1.4	4.6	51.9	9.1	2.5	0.6	0.3	–	–	–	–
Mortierella sp. 1053	0.5	0.2	1.6	14.0	–	8.7	30.0	41.5	2.2	–	–	0.2	0.1	–	–	0.2
M. globulifera 1244	0.8	0.6	1.8	20.0	–	8.7	21.0	41.2	3.2	–	–	0.4	–	0.6	–	1.4
Mortierella sp. 1052	0.6	0.6	1.1	16.0	–	11.0	15.0	38.1	3.3	–	–	–	–	–	–	–
U. ramanniana 941	1.3	0.7	1.2	27.0	–	10.0	13.0	42.0	5.8	–	–	–	–	–	–	–
U. isabellina 951	0.8	0.6	2.6	21.1	0.32	3.7	14.1	51.0	3.3	–	–	0.5	0.6	0.5	–	1.0

Table 2. Amount of fatty acids (mg g-1 dry mass) on Potato Dextrose Agar arranged by arachidonic acid (20:4) content. –, not detected

Isolate, No. in MSCL	14:0	15:0	16:1	16:0	17:0	18:3	18:2	18:1	18:0	20:4	20:3	20:1	20:0	22:0	24:1	24:0	Total
M. gamsii 948	0.30	0.30	0.14	11.00	–	1.60	4.20	17.40	5.40	12.00	1.80	0.50	0.30	0.90	0.50	1.40	57.74
M. clonocystis 1200	0.30	0.40	0.30	5.00	–	0.80	3.30	6.60	0.50	9.30	0.70	0.10	–	0.30	0.20	0.20	28.00
M. alpina 1271	0.03	0.10	–	1.10	–	0.10	0.10	0.60	0.20	7.00	–	–	0.10	0.20	–	0.60	10.13
M. elongata 1380	0.08	0.15	0.15	3.50	0.15	0.40	1.60	4.60	1.20	5.10	0.50	0.20	0.10	0.20	0.10	0.20	18.23
M. alpina 1378	0.07	0.10	0.02	1.30	–	0.10	0.04	0.53	0.50	3.80	–	0.07	0.10	0.70	0.60	1.10	9.03
M. humilis 967	0.40	0.30	0.60	140	0.10	0.80	2.10	11.10	2.80	3.40	0.40	1.10	0.40	2.30	1.80	4.70	46.30
M. alpina 1381	0.10	0.10	0.06	1.50	0.03	0.20	0.70	2.90	0.40	1.90	0.10	0.10	–	0.03	0.02	0.04	8.18
M. alpina 1379	0.10	0.08	–	1.20	–	0.05	0.04	0.64	0.80	1.50	–	0.05	0.20	0.80	0.50	1.50	7.46
M. alpina 959	0.03	0.04	–	1.20	–	0.02	0.05	0.40	0.40	1.40	–	0.02	0.10	0.30	0.04	0.90	4.00
M. alpina 1098	0.04	0.06	–	0.90	0.02	0.02	0.03	0.24	0.40	1.10	–	–	0.10	0.20	0.20	0.40	3.71
M. alpina 1314	0.03	0.02	0.01	1.03	0.01	0.13	0.24	0.62	0.41	0.60	0.07	0.02	0.03	0.06	0.01	0.12	3.43
M. alpina 1254	0.04	0.07	–	0.80	–	0.01	0.02	0.13	0.30	0.35	–	0.04	0.10	0.40	0.04	1.00	3.30
M. minutissima 1377	0.04	0.06	–	–	0.02	–	0.01	0.22	0.30	0.30	–	0.02	0.10	0.30	0.10	0.40	1.87
M. macrocystis 1136	0.15	0.05	–	2.50	0.06	0.13	0.40	4.80	0.80	0.20	0.06	0.03	–	–	–	–	9.18
Mortierella sp. 1053	0.30	0.10	1.10	9.30	–	5.80	20.00	27.00	1.50	–	–	0.20	0.10	–	–	0.10	65.50
M. globulifera 1244	0.20	0.10	0.40	4.20	–	1.80	4.40	8.80	0.70	–	–	–	–	0.10	–	0.30	21.00
Mortierella sp. 1052	0.10	0.10	0.20	3.40	–	2.20	3.20	8.00	0.70	–	–	–	–	–	–	–	17.90
U. ramanniana 941	0.06	0.03	0.05	1.20	–	0.40	0.60	1.90	0.30	–	–	–	–	–	–	–	4.54
U. isabellina 951	0.01	0.01	0.05	0.37	–	0.07	0.25	0.89	0.06	–	–	0.01	0.01	0.01	–	0.02	1.75

Table 3. Percentage of individual fatty acids from all fatty acids on malt extract gelatin arranged by the percentage of arachidonic acid (20:4). –, not detected

Isolate, No. in MSCL	7:0	8:0	9:0	14:0	15:0	16:1	16:0	17:0	18:3	18:2	18:1	18:0	20:4	20:3	20:1	20:0	22:0	24:1	24:0
M. alpina 1271	–	–	0.4	0.7	1.7	–	21.0	–	0.5	2.0	10.2	5.1	54.0	–	–	–	–	–	4.5
M. alpina 959	–	–	–	0.5	0.9	–	16.2	–	1.6	2.1	9.9	3.3	53.0	0.9	1.1	–	2.9	–	8.2
M. alpina 1379	–	–	–	0.4	0.3	–	15.6	–	1.1	1.0	25.1	7.7	42.0	1.6	–	–	1.9	–	3.9
M. alpina 1381	–	0.7	0.8	0.6	0.3	–	16.3	–	1.2	1.7	18.5	9.1	42.0	1.2	–	–	2.3	–	5.7
M. alpina 1254	–	–	–	1	2.2	–	24.6	–	–	2.6	13.4	4.8	40.3	–	–	–	2.3	–	8.7
M. alpina 1314	–	–	–	0.6	0.7	0.8	14.9	0.2	2.5	18.7	18.4	1.0	38.5	1.9	0.2	0.13	0.5	–	1.3
M. clonocystis 1200	–	–	–	1.3	2	–	31.5	–	0.7	3.4	25.5	3.4	28.0	–	–	–	–	–	4.4
M. alpina 1378	–	–	–	0.4	0.5	–	23.3	–	0.8	0.8	28.2	11.1	27.1	1.3	–	–	2.5	–	4.3
M. gamsii 948	2.5	6.1	6.7	1.6	1.8	–	18.2	–	2.8	4.2	22.4	7.6	23.2	–	–	–	0.8	–	2.3
M. alpina 1098	–	–	2.3	1.6	1.2	–	30.0	–	–	2.9	14.4	19.0	20.0	–	–	–	–	–	7.7
M. elongata 1380	–	0.1	0.1	1.2	0.4	–	16.2	–	2.1	11.0	16.1	20.7	14.2	4.6	1.4	1.9	4.1	1.0	4.1
M. humilis 967	0.3	0.7	0.7	0.6	0.2	–	21.0	–	3.9	3.4	26.9	9.6	9.2	2.3	4.6	1.0	2.9	2.1	8.8
M. minutissima 1377	–	–	–	1.5	1.6	1.0	31.0	0.6	–	1.1	44.2	4.5	2.6	–	–	1.0	4.0	0.3	6.4
Mortierella sp. 1053	–	–	–	0.5	–	–	14.0	–	8.3	31.0	41.2	2.3	–	–	0.2	0.1	–	–	–
M. globulifera 1244	–	–	–	0.3	–	–	9.6	–	12.6	24.3	49.5	2.6	–	–	0.4	–	–	–	–

Table 4. Amount of fatty acids (mg g^{-1} dry mass) on malt extract gelatin arranged by arachidonic acid (20:4) content. –, not detected

Isolate, No. in MSCL	7:0	8:0	9:0	14:0	15:0	16:1	16:0	17:0	18:3	18:2	18:1	18:0	20:4	20:3	20:1	20:0	22:0	24:1	24:0	Total
M. elongata 1380	–	0.05	0.05	0.60	0.20	–	8.10	–	1.00	5.40	8.10	10.00	6.00	2.30	–	0.70	2.00	0.50	2.00	47.00
M. alpina 959	–	–	–	–	–	–	1.20	–	0.10	0.20	0.70	0.20	4.00	0.10	0.10	–	0.20	–	0.60	7.40
M. alpina 1314	–	–	–	0.05	0.06	0.07	1.33	0.01	0.22	1.67	1.65	0.09	3.45	0.17	0.02	0.01	0.04	–	0.12	8.95
M. alpina 1381	–	0.05	0.05	0.40	0.02	–	1.10	–	0.10	0.10	1.30	0.60	2.70	0.10	–	–	0.20	–	0.40	7.12
M. alpina 1271	–	–	0.10	0.03	0.06	–	0.80	–	0.02	0.10	0.40	0.20	2.00	–	–	–	–	–	0.20	3.91
M. alpina 1254	–	–	–	0.06	0.10	–	1.20	–	–	0.10	0.60	0.30	2.00	–	–	–	–	–	0.30	4.66
M. alpina 1379	–	–	–	0.02	0.02	–	0.80	–	0.05	0.05	1.20	0.40	2.00	0.10	–	–	0.10	–	0.20	4.94
M. gamsii 948	0.25	0.60	0.60	0.10	0.20	–	1.70	–	0.30	0.40	2.00	0.70	1.90	–	–	–	0.10	–	0.20	8.80
M. alpina 1378	–	–	–	0.03	0.03	–	1.30	–	0.04	0.04	1.50	0.60	1.50	0.10	–	–	0.14	–	0.20	5.48
M. alpina 1098	–	–	0.20	0.10	0.10	–	2.10	–	–	0.20	1.00	1.30	1.40	–	–	–	–	–	0.50	6.90
M. humilis 967	0.01	0.10	0.10	0.10	0.02	–	2.50	–	0.50	0.40	3.10	1.10	1.10	0.30	0.50	0.10	0.30	0.30	1.10	11.62
M. clonocystis 1200	–	–	–	0.04	0.10	–	1.00	–	0.02	0.10	0.80	0.10	0.90	–	–	–	–	–	0.10	3.16
M. minutissima 1377	–	–	–	0.04	0.04	0.03	0.85	0.02	–	0.03	1.22	0.12	0.07	–	–	0.03	0.11	0.01	0.18	2.17
Mortierella sp. 1053	–	–	–	0.30	–		8.90		5.30	19.00	2.74	1.40	–	–	0.10	0.10	–	–	–	60.84
M. globulifera 1244	–	–	–	0.22	–		6.40		8.50	16.30	33.10	1.80	–	–	0.20	–	–	–	–	66.52

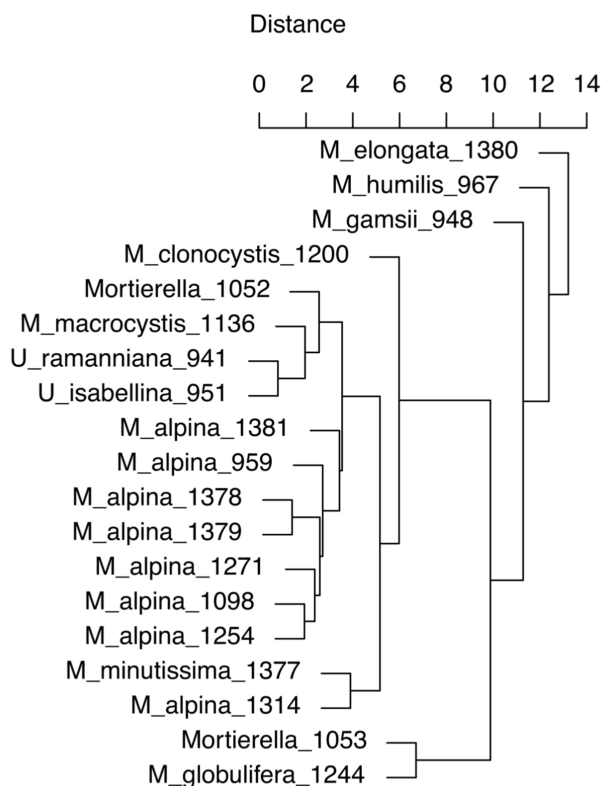

Fig. 1. Results of the cluster analysis with average distance of tested isolates according to the amount of all detected fatty acids separately, mg g^{-1} dry mass, on malt extract medium with gelatin and potato dextrose agar without aspirin.

contained also several aminoacids (phenylalanine, leucine, hydroxyproline, proline and glutamine) and choline (data not shown).

Fatty acid production in liquid media

Not all of the seven isolates were able to grow in particular liquid media (S, C, V and ME). *M. gamsii* MSCL 948 was able to grow only in medium V, and *M. elongata* MSCL 1380 and *M. humilis* MSCL 967 only in media S and C.

After six days of incubation in 50 mL of medium S, the total yield of fatty acids varied from 2.95 to 17.07 mg g^{-1} dry mass, corresponding to 0.3 to 1.7% fatty acid content in biomass. The yield of ARA varied from 0.10 to 0.69 mg g^{-1} dry mass (up to 17.04 ± 1.46 mg L^{-1}) and the percentage of ARA of total fatty acid content varied from 2.5 to 8.6% (Table 5) or from 0.1 to 0.7 % from dry biomass. The correlation coefficient (*r*) between the content of ARA and precursors of ARA was on average 0.75. The correlation coefficient between the content of ARA and linoleic acid was 0.55, and with dihomo-γ-linolenic acid it was 0.82. Dihomo-γ-linolenic acid was produced only by two isolates, whitch had the highest production of ARA.

After 12 days of incubation of isolate *M. alpina* MSCL 959 in 300 mL of medium S, the average total yield of fatty acids was 17.42 mg g^{-1} dry mass (241.97 ± 177.79 mg L^{-1}, $n = 6$), which was 2.5 times higher than in a volume of 50 mL.

The ARA amount in 300 mL volume of S medium, in comparison with 50 mL volume, decreased from 0.60 to 0.23 mg g^{-1} dry mass (from 17.04 to 3.25 mg L^{-1}) but the

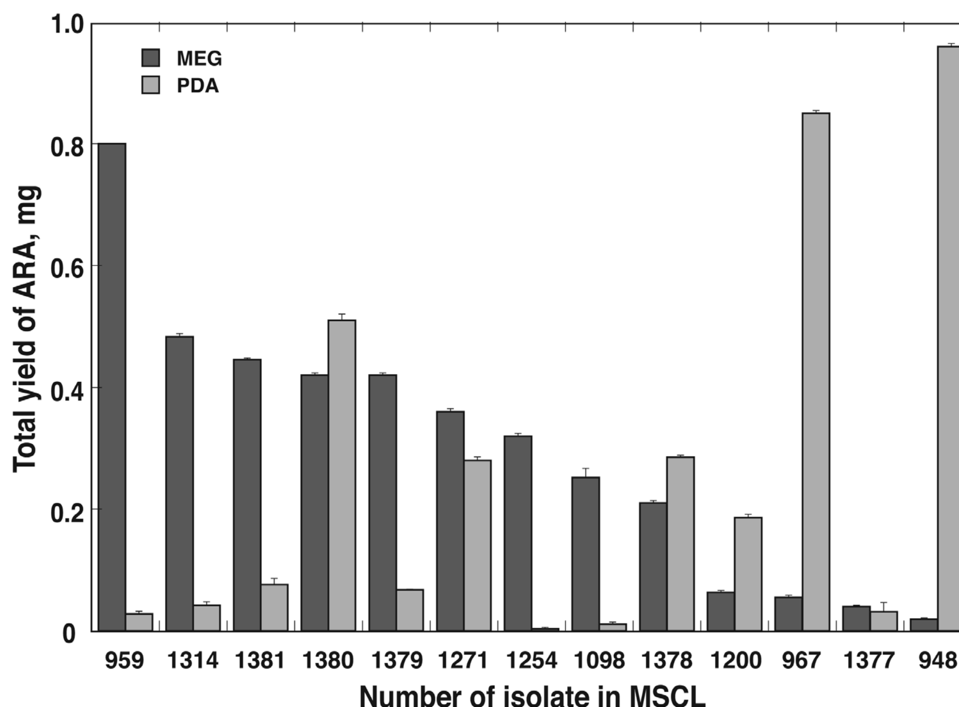

Fig. 2. The total yield of arachidonic acid (ARA) obtained from one Petri dish on malt extract medium with gelatin (MEG) and potato dextrose agar (PDA) without aspirin. Tested isolates are indicated with their numbers in Microbial Strain Collection of Latvia (MSCL). Error bars indicate standard deviations (±SD), $n = 2$.

Table 5. The percentage of all detected fatty acids, total yield of fatty acids and yield of arachidonic acid (mg g^{-1} dry mass) produced on medium S. Data are means from two replicates ± SD. [a], incubation time 12 days in 300 mL of medium; other isolates have been cultivated for five days in 50 mL; –, not detected

Fatty acid	*M. alpina* MSCL 959	*M. alpina* MSCL 959[a]	*M. alpina* MSCL 1098	*M. alpina* MSCL 1379	*M. elongata* MSCL 1380	*M. alpina* MSCL 1381	*M. humilis* MSCL 967
13:0	0.64 ± 0.58	–	0.48 ± 0.40	2.47 ± 1.42	0.73 ± 0.72	–	–
14:0	1.82 ± 0.11	–	1.05 ± 0.24	0.97 ± 0.39	1.73 ± 0.19	2.24 ± 0.57	1.73 ± 0.08
15:0	0.30 ± 0.01	15.59 ± 4.17	0.36 ± 0.05	0.17 ± 0.07	0.85 ± 0.09	0.19 ± 0.12	0.22 ± 0.05
16:0	41.69 ± 0.82	30.80 ± 2.36	21.61 ± 1.76	20.04 ± 1.18	19.12 ± 0.46	22.89 ± 0.85	28.81 ± 0.61
17:0	0.30 ± 0.04	4.54 ± 0.51	0.77 ± 0.13	0.35 ± 0.08	2.77 ± 0.33	0.10 ± 0.15	0.29 ± 0.02
18:3	1.43 ± 0.06	0.52 ± 0.56	1.06 ± 0.14	0.85 ± 0.14	2.82 ± 0.16	1.37 ± 0.12	1.96 ± 0.08
18:2	7.62 ± 0.86	21.19 ± 2.17	5.69 ± 0.22	6.52 ± 0.85	14.28 ± 1.42	5.01 ± 0.53	23.38 ± 0.63
18:1	21.73 ± 0.26	17.22 ± 1.86	30.91 ± 2.03	30.05 ± 1.56	27.16 ± 0.71	43.51 ± 2.24	22.28 ± 0.64
18:0	9.67 ± 0.13	7.36 ± 1.52	16.65 ± 1.48	20.72 ± 1.04	16.24 ± 0.62	12.62 ± 0.69	12.59 ± 0.28
20:4	8.65 ± 0.42	1.94 ± 1.48	6.79 ± 0.88	4.52 ± 0.65	4.57 ± 0.47	5.62 ± 0.35	2.52 ± 0.11
20:3	0.42 ± 0.09	–	–	–	1.13 ± 0.04	–	–
20:1	0.49 ± 0.06	–	0.96 ± 0.27	1.03 ± 0.26	0.63 ± 0.02	0.52 ± 0.08	0.16 ± 0.08
20:0	0.58 ± 0.04	–	0.90 ± 0.18	1.05 ± 0.16	1.12 ± 0.03	0.48 ± 0.19	0.65 ± 0.01
22:0	1.04 ± 0.12	–	1.98 ± 0.23	2.29 ± 0.38	2.58 ± 0.19	1.02 ± 0.32	1.32 ± 0.11
24:1	0.35 ± 0.06	–	–	–	0.48 ± 0.22	0.43 ± 0.23	0.99 ± 0.10
24:0	3.27 ± 0.34	0.85 ± 0.56	10.20 ± 1.69	8.97 ± 2.38	3.98 ± 0.67	4.02 ± 1.98	3.10 ± 0.36
Yield of fatty acids	6.95 ± 2.64	17.42 ± 10.42	2.95 ± 0.40	2.53 ± 0.31	17.07 ± 8.91	3.82 ± 0.28	7.56 ± 0.15
Yield of ARA	0.60 ± 0.23	0.23 ± 0.15	0.17 ± 0.03	0.10 ± 0.01	0.69 ± 0.10	0.18 ± 0.02	0.19 ± 0.01

Table 6. The percentage of all detected fatty acids, total yield of fatty acids and yield of arachidonic acid (mg g^{-1} dry mass) produced on medium S. Data are means from two replicates ± SD. [a], incubation time 12 days in 300 mL of medium; other isolates have been cultivated for five days in 50 mL; –, not detected

Fatty acid	*M. alpina* MSCL 959	*M. alpina* MSCL 959	*M. alpina* MSCL 1379	*M. alpina* MSCL 1381	*M. alpina* MSCL 1381[a]
14:0	–	–	–	–	2.59 ± 0.22
15:0	–	–	–	–	0.86 ± 0.05
16:1	–	–	–	–	0.76 ± 0.10
16:0	53.56 ± 0.99	24.00 ± 1.81	26.96 ± 0.75	29.47 ± 0.54	21.31 ± 0.72
17:0	2.83 ± 0.10	4.81 ± 0.54	3.90 ± 0.16	2.47 ± 0.19	0.71 ± 0.06
18:3	1.30 ± 0.10	1.87 ± 0.07	0.94 ± 0.05	6.31 ± 0.32	2.36 ± 0.10
18:2	2.14 ± 0.21	3.06 ± 0.50	2.05 ± 0.23	8.39 ± 0.47	2.84 ± 0.11
18:1	19.15 ± 0.58	25.00 ± 0.48	19.29 ± 0.41	24.33 ± 0.31	35.36 ± 0.76
18:0	11.82 ± 0.32	22.67 ± 0.44	29.71 ± 0.33	15.99 ± 0.50	12.97 ± 0.23
20:4	2.69 ± 0.41	8.58 ± 0.56	3.31 ± 0.19	6.35 ± 0.63	7.93 ± 0.31
20:1	–	0.80 ± 0.28	0.79 ± 0.22	0.45 ± 0.10	0.70 ± 0.05
20:0	0.58 ± 0.07	1.40 ± 0.29	2.24 ± 0.18	1.09 ± 0.44	0.73 ± 0.06
22:0	1.43 ± 0.56	2.60 ± 0.49	4.23 ± 0.35	0.96 ± 0.55	1.73 ± 0.13
24:1	–	–	–	–	1.06 ± 0.15
24:0	2.89 ± 0.73	4.18 ± 0.46	4.63 ± 0.32	3.95 ± 0.40	7.15 ± 0.70
26:1	–	–	–	–	0.18 ± 0.00
26:0	–	–	–	–	1.02 ± 0.20
Yield of fatty acids	7.42 ± 0.43	7.95 ± 0.58	8.90 ± 0.27	12.45 ± 2.61	8.56 ± 0.56
Yield of ARA	0.20 ± 0.02	0.67 ± 0.10	0.30 ± 0.01	0.80 ± 0.21	0.68 ± 0.05

amount of palmitic acid (16:0) was on average the same 2.92 and 5.32 mg g^{-1} dry mass (82.24 ± 3.78 and 73.85 ± 54.05 mg L^{-1}). Several isolates in 50 mL of medium S produced odd chain fatty acid tridecylic acid (13:0), which was not observed on solid media.

The production by six isolates in medium C was lower than in medium S. The highest amount of ARA was obtained in the case of *M. alpina* MSCL 1379, 3.28 ± 1.54 mg L^{-1}, which corresponded to 3.73 ± 0.86 % of total fatty acid content (data not shown). The correlation coefficient (r) between the content of ARA and precursors of ARA was on average 0.86. The correlation coefficient between the content of ARA and linoleic acid was 0.98, but dihomo-γ-linolenic acid was not produced in this medium.

In the medium V the highest ARA yield was obtained by *M. alpina* MSCL 1098, 2.54 ± 0.44 mg L^{-1} (data not shown). The correlation coefficient (r) between the content of ARA and precursors of ARA was on average 0.84. The correlation coefficient between the content of ARA and linoleic acid was 0.97, but dihomo-γ-linolenic acid was not produced in this medium.

In medium ME, the highest amount of fatty acids and the highest yield of ARA (3.68 mg L^{-1}) was obtained by *M. alpina* MSCL 1381 (Table 6). The correlation coefficient (r) between the content of ARA and precursors of ARA was on average 0.49. The correlation coefficient between the content of ARA and linoleic acid was 0.77, but dihomo-γ-linolenic acid was not produced in this medium.

M. alpina MSCL 1381 was cultivated also in 300 mL volume for 10 days. The prolonged cultivation in a larger volume of medium reduced the total content of fatty acids and yield of ARA, and changed the spectrum of fatty acids (Table 6). Little amounts of such fatty acids as ximinic (26:1) and cerotic acids (26:0) were observed. Trace amounts of cerotic acid were observed also in the mycelium of *M. alpina* MSCL 959 and *M. alpina* MSCL 1098 grown on PDA without aspirin (data not shown).

In liquid media the highest ARA yield was obtained for *M. alpina* MSCL 959 (17.04 mg L^{-1}) in 50 mL volume of medium S. The highest total yield of fatty acids was by *M. elongata* MSCL 1380 in medium S, 338 mg L^{-1}. In other media the best yields of fatty acids was significantly lower, 118 mg L^{-1} by *M. alpina* MSCL 1381 in medium ME (300 mL volume), 58 mg L^{-1} by *M. alpina* MSCL 1098 in medium V and 24 mg L^{-1} by *M. alpina* MSCL 959 in medium C. In this investigation, the best liquid media were those containing the lowest concentration of carbon source: 1.7% of maltose in medium ME and 5% of glucose in medium S. Lower production was observed using higher concentrations of carbon source, as 8% glucose in medium V and 10% soluble starch in medium C (Fig. 3). The coefficient R^2 between the concentration of carbon source and total amount of fatty acids was 0.97.

The two fatty acids with the highest production rates were palmitic acid (16:0) and oleic acid (18:1). The highest amounts of these fatty acids were produced on medium S. The highest amount of palmitic acid was 82.24 mg L^{-1} (*M. alpina* MSCL 959) and 64.43 mg L^{-1} (*M. elongata* MSCL 1380) in 50 mL volume and 73.85 mg L^{-1} (*M. alpina* MSCL 959) in 300 mL volume. The highest amount of oleic acid was 85.86 mg L^{-1} (*M. elongata* MSCL 1380) and 50.87 mg L^{-1} (*M. alpina* MSCL 1381) in 50 mL volume and 36.22 mg L^{-1} (*M. alpina* MSCL 959) in 300 mL volume.

From the obtained data it can be concluded that dihomo-γ-linolenic acid is one of the most important ARA precursors because the highest ARA yields in liquid media were obtained by isolates that produced dihomo-γ-linolenic acid.

Content of the oil after supercritical CO_2 extraction

Total yield of fatty acids from isolate *M. alpina* MSCL 959 grown in 300 mL of the medium S for 12 days and extracted with solution of n-hexane/acetone was 17.42 ± 10.42 mg g^{-1} dry mass. In the supercritical CO_2 extraction, similar amounts of fatty acids was obtained, 19.05 ± 1.00 mg g^{-1} dry mass. The proportion of individual fatty acids was also similar (Fig. 4).

Production of fatty acids at low incubation temperature and with added rapeseed oil

The poduction of total fatty acids at low incubation temperature (12 ± 2 °C) was higher by three isolates of the 13 that were able to grow on MEG, in comparison with that on the same growth media but at incubation temperature 20 ± 2 °C: *M. alpina* 1271 [11.23 mg g^{-1} dry mass in comparison with 3.19 mg g^{-1} dry mass (Table 4)], *M. alpina*a MSCL 1381 (8.83 mg g^{-1} dry mass in comparison with 7.12 mg g^{-1} dry mass) and *M. alpina* MSCL 1314 (11.86 mg g^{-1} dry mass in comparison with 8.95 mg g^{-1} dry mass). Two isolates produced similar amounts of fatty acids at both

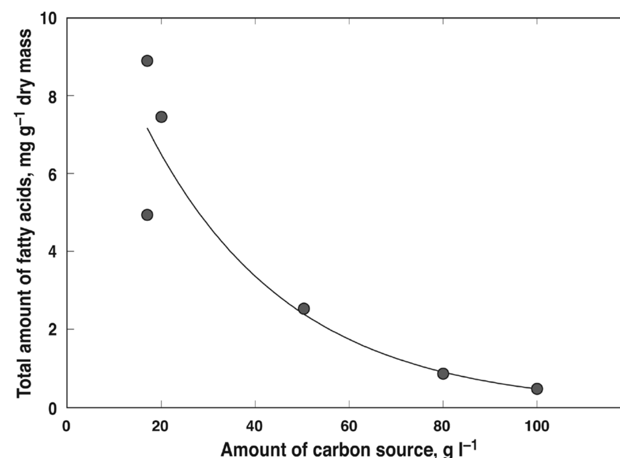

Fig. 3. The correlation between the amount of the carbon source in the growth media and total amount of fatty acids in the case of *M. alpina* MSCL 1379. 1.7% of maltose in medium ME, 5% of glucose in medium S, 8% of glucose in medium V and 10 % of soluble starch in the medium C.

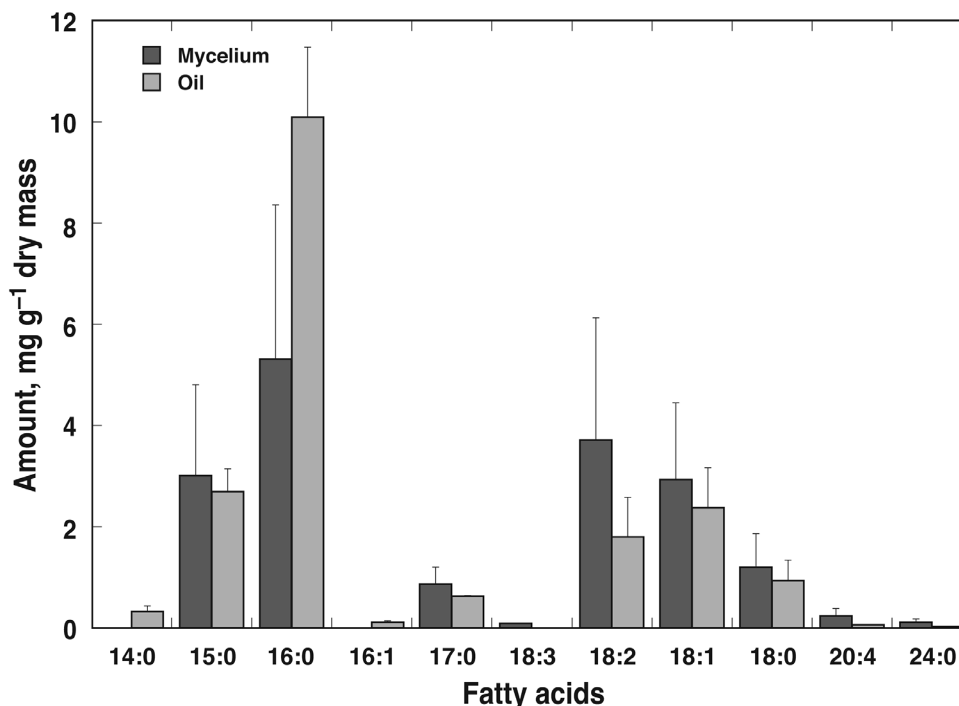

Fig. 4. The fatty acid content of the mycelium of *M. alpina* MSCL 959 grown in 300 mL of medium S for 12 days and in the oil after the critical CO_2 extraction estimated as mg g^{-1} dry mass of the mycelium. Error bars indicate standard deviations (±SD), $n = 2$.

incubation temperatures: *M. clonocystis* MSCL 1200 (3.14 mg g^{-1} dry mass) and *M. elongata* MSCL 1380 (46.67 mg g^{-1} dry mass). The other isolates produced significanlty lower total amounts of fatty acids at low incubation temperature, although the incubation time was longer. The total yield of ARA per Petri dish at low incubation temperature was increased only in the case of two isolates: *M. alpina* MSCL 1314 and *M. elongata* MSCL 1380 (Fig. 5, for comparison see Fig. 2). The total yield of ARA per Petri dish at low incubation temperature and with 1% of rapeseed oil was higher in the case of one isolate, *M. alpina* MSCL 1254 (Fig. 5). The low incubation temperature caused the production of eicosatetraenoic acid (20:4). The highest amount of this fatty acid was obtained in the case of the isolate *M. elongata* MSCL 1380, 4.08 mg g^{-1} dry mass (data not shown). Various odd chain saturated and unsaturated fatty acids were also present (15:0, 17:0 and 17:1, data not shown).

Discussion

Results of the primary screening

Several features of the fatty acid spectrum of tested isolates on PDA without aspirin corresponded to the results obtained by Eroshin et al. (1996) but some differed. For example, the percentage of oleic and linoleic acids produced by *M. globulifera* MSCL 1244 corresponded to the level of *M. globulifera* isolates tested by Eroshin et al. (1996) or were higher as in the case of linoleic acid (Table 1). Five isolates of *M. alpina* did not produce palmitoleic acid (16:1) in contrast with the previous study (Table 1 and 2). Considering other species, palmitoleic acid was produced by *M. globulifera* MSCL 1244 and *M. gamsii* MSCL 948, which was not observed by Eroshin et al. (1996). All isolates on PDA without aspirin produced the odd chain fatty acid pentadecylic acid (15:0), and eight isolates produced another odd chain fatty acid margaric acid (17:0), which was not observed by Eroshin et al. (1996). Furthermore, several isolates produced also arachidic acid (20:0), behenic acid (22:0), nervonic acid (24:1) and lignoceric acid (24:0), which were not found by Eroshin et al. (1996) (however, the authors stated that unidentified fatty acids ≥ C_{20} were observed in some strains), but were observed in the investigation by Wang et al. (2005) with the exception of nervonic acid produced by a *M. alpina* isolate. Lignoceric acid was found to be produced by *M. alpina* isolate 1S-4 at all growth temperatures employed (Jareonkitmongkol et al. 1992). Three *M. alpina* isolates produced dihomo-γ-linolenic acid (20:3), which was produced by two *M. alpina* isolates in the investigation of Eroshin et al. (1996) and by Δ5 desaturase-defective mutants of *M. alpina* (Sakuradani, Shimizu 2009).

The highest percentage (54 and 53%) of ARA on MEG was observed for two *M. alpina* isolates (Table 3), which corresponded to the production by the best isolates of this species in the investigation of Botha et al. (1999). Similarly, the ARA content of *M. clonocystis* MSCL 1200 corresponded to this investigation. The ARA content of *M. gamsii* MSCL 948 was 23.2%, which was higher than that by the *M. gamsii* isolate used in the mentioned investigation. The authors reported that other fatty acids produced on MEG were

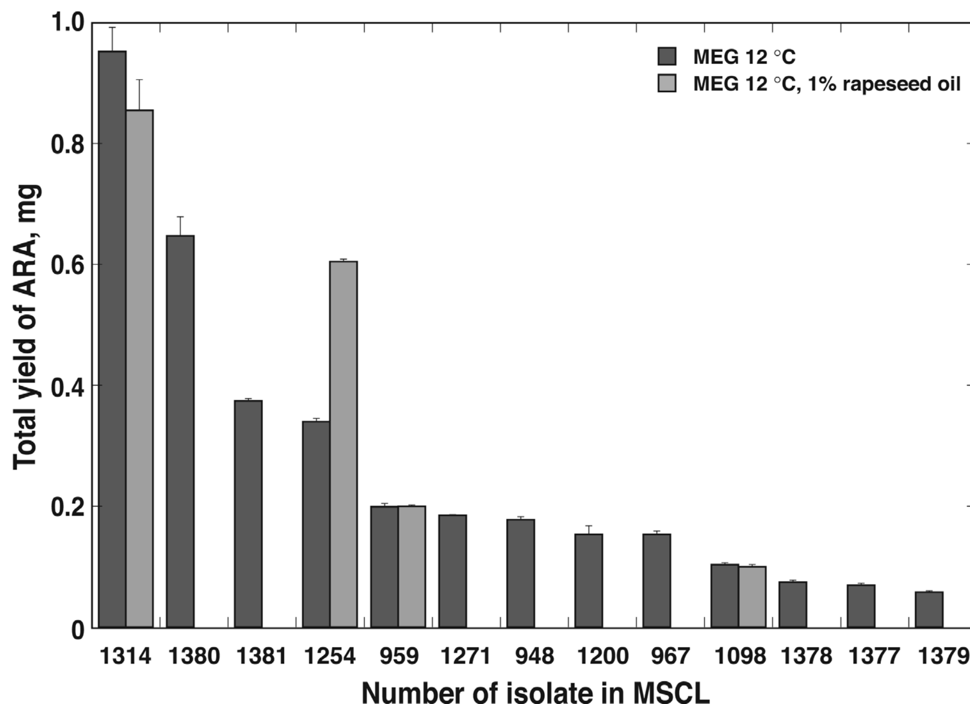

Fig. 5. The total yield of arachidonic acid (ARA) per Petri dish on malt extract medium with gelatin (MEG) at low incubation temperature (12 ± 2 °C) with and without addition of rapeseed oil (1%). Tested isolates are indicated with their numbers in Microbial Strain Collection of Latvia (MSCL). Error bars indicate standard deviations (±SD), $n = 2$.

palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and γ-linoleic acid (18:3). The tested isolates produced also myristic acid (14:0), a part of the isolates produced pentadecylic acid (15:0), dihomolinolenic acid (20:3), gadoleic acid (20:1), arachidic acid (20:0), behenic acid (22:0), nervonic acid (24:1) and lignoceric acid (24:0). Five isolates produced enanthic acid (7:0), caprylic acid (8:0) and pelargonic acid (9:0), which in the case of *M. gamsii* MSCL 948 constituted together 15.3% of all fatty acids (Table 3). Caprylic acid was observed in oil produced by *M. isabellina* M2 isolate (Xing et al. 2012).

The second cultivation of seven isolates on both media (PDA and MEG) showed that all *M. alpina* isolates were stable fatty acid producers and that the total amount of fatty acids did not differ significantly between both cultivation times. In the case of other isolates on PDA, the percentage of individual fatty acids did not significantly differ, but the total yield of fatty acids was significantly lower, which might be explained by sensitivity of these species to the degeneration in media rich in soluble sugars, as observed in the case of particular *Mortierella* species (Weber, Tribe 2003).

It is known that fatty acid composition profiles can be used to differentiate species from *Mortierella* and *Micromucor* genera (Amano et al. 1992) but have not been applied as potential chemotaxonomic markers to many strains from one taxon (Frisvad et al. 2008). Fig. 1 illustrates the results of cluster analysis with average distance according to the amounts of fatty acids on MEG and PDA without aspirin (data from the Tables 2 and 4). All *M. alpina* isolates were grouped together with the exception of *M. alpina* MSCL 1314, which was located separately together with *M. minutissima* MSCL 1377. According to the work of Wagner et al. (2013), the species *M. minutissima* needs revision or nomenclatural synonymization. Close to this group were isolates *Mortierella* sp. MSCL 1052, *M. macrocystis* MSCL 1136, *U. ramanniana* MSCL 941 and *U. isabellina* MSCL 951. According to the molecular phylogeny analysis of the Mortierellales, the genus *Umbelopsis* is an outgroup (Wagner et al. 2013) but is seems that the profiles of fatty acids are not so distinct. The other isolates were more distinct from *M. alpina* and *Umbelopsis* sp. groups. *M. globulifera* formed a completely separate group also in the molecular phylogeny analysis of Wagner et al. (2013).

According to the results of GC-MS the extramycelial liquid droplets from the isolate *M. alpina* MSCL 1098 contained only 1.56% of fatty acids. Analysis of extramycelial liquid with HPLC method gave information that this liquid contains also several aminoacids and choline. Wherewith, it can be concluded that extramycelial droplets are not lipid droplets as it was stated by Wang et al. (2011).

Production of fatty acids in liquid media

The production of ARA and total yield of fatty acids in the medium S was significantly lower than observed for

M. alpina by Samadlouie et al. (2012), 27% of ARA from oil and 25% of oil from dry biomass after six days of incubation at 21 °C and agitator spin 185 rpm on a rotary shaker. In the case of *M. elongata* MSCL 1380, the yield of ARA (15.98 ± 1.90 mg L^{-1}), linoleic acid (50.09 ± 5.84 mg L^{-1}) and α-linolenic acid (9.74 ± 0.74 mg L^{-1}) was several times higher than the results reported for this species by Jang et al. (2005) in the medium containing 2% soluble starch. Additionally to these PUFAs, this isolate produced also fatty acid 20:3 in the amount of 3.87 ± 0.18 mg L^{-1}, and the total yield of PUFAs was 79.68 mg L^{-1}.

After 12 days of incubation in the case of isolate *M. alpina* MSCL 959 in 300 mL of the medium S the average total yield of fatty acids was 17.42 mg g^{-1} dry mass (241.97 ± 177.79 mg L^{-1}), which was 2.5 times higher than in a volume of 50 mL, but still more than ten times lower than that reported by Samadlouie et al. (2012), 32% of oil from dry biomass after ten days of incubation at 25 °C and agitator spin 185 rpm on a rotary shaker. Comparing these results with the investigation of Jang et al. (2005), the total amount of PUFAs in our study was 80.88 ± 35.99 mg L^{-1}, which was within the range of production by *M. alpina* isolates from Taiwan. Isolates from a temperate climate show similar results to common isolates from other soils but they produce lower levels of fatty acids, compared to the best producers such as *M. alpina* ATCC 32222 (Jang et al. 2005).

The production by six isolates in medium C was lower than in medium S. In the study by Chen et al. (1997) the highest ARA yield in optimized incubation conditions after five days of incubation at 20 °C was 3885 mg L^{-1}. In the present investigation the highest amount of ARA was obtained in the case of *M. alpina* MSCL 1379, 3.28 ± 1.54 mg L^{-1}, which corresponded to 3.73 ± 0.86 % of total fatty acid content.

Using medium V Zeng et al. (2012) obtained ARA 10 g L^{-1} after seven day incubation at 23 °C and 130 rpm, while in the present study the highest ARA yield obtained, in the case of *M. alpina* MSCL 1098, was 2.54 ± 0.44 mg L^{-1}, which was significantly lower. Additionally to probably different gene expression levels of he tested isolates, these differences might be caused by slightly different contents of B vitamin complex used. Additionally to thiamin (B_1), nicotinamide (B_3), D-pantothenate (B_5) and pyridoxine (B_6) were used in the investigation by Zeng et al. (2012), while the vitamin complex used in the present investigation contained also B_2, B_{12} and biotin, and the concentrations of D-pantothenate and pyridoxine were lower than the optimal concentrations for the vitamins.

The production in medium ME was higher than in media C and V. *M. alpina* MSCL 1381 was cultivated also in 300 mL volume of medium ME for 10 days. The prolonged cultivation in a larger volume of the medium reduced the total content of fatty acids and yield of ARA, and changed the spectrum of fatty acids (Table 6). Little amounts of such fatty acids as ximinic (26:1) and cerotic acids (26:0) were observed. Trace amounts of cerotic acid were observed in the mycelium of *M. alpina* MSCL 959 and *M. alpina* MSCL 1098 grown on PDA without aspirin. These fatty acids are rarely produced by fungi from the *Mortierella* genus. Low amounts of these two fatty acids have been observed in yeast transformants overexpressing *M. alpina* fatty acid Δ9-desaturase genes or the *S. cerevisiae OLE1* gene (MacKenzie et al. 2002). The two fatty acids with the highest production rates were palmitic acid (16:0) and oleic acid (18:1), as observed also in other screening experiments (Eroshin et al. 1996).

In the supercritical CO_2 extraction, similar amounts of fatty acids were obtained as in extraction with n-hexane/acetone. The proportions of individual fatty acids were also similar (Fig. 4). The supercritical CO_2 extraction method has been described as an effective method for the extraction of fatty acids from fungal mycelia in some previous studies (Certik, Horenitzky 1999; Walker et al. 1999).

In this investigation the best liquid media were those containing the lowest concentration of carbon source: 1.7% of maltose in medium ME and 5% of glucose in medium S. Higher concentrations of carbon source gave lower production: 8% of glucose in medium V and 10% of soluble starch in medium C (Fig. 3). This observation was in contrast to other studies where high yields of fatty acids were obtained in media containing soluble starch and glucose in high concentrations (Chen et al. 1997; Zeng et al. 2012). In the investigation of Jang et al. (2005), in the case of *M. alpina* ATCC 32222, the lowest yield was obtained in medium containing 2% maltose and the best yields of fatty acids were obtained at 6% glucose and 10% soluble starch. However, a 12% concentration of soluble starch gave a lower yield of fatty acids (Jang et al. 2005).

From the obtained results it can be concluded that dihomo-γ-linolenic acid is one of the most important ARA precursors, as the highest ARA yields in liquid media were obtained by isolates that produced dihomo-γ-linolenic acid, although the concentrations of dihomo-γ-linolenic acid were low as detected also in other investigations (Jareonkitmongkol et al. 1992).

Production of fatty acids at low incubation temperature and with added rapeseed oil

The production of total fatty acids at low incubation temperature (12 ± 2 °C) was increased only in the case of three isolates of 13 that were able to grow on MEG. Two isolates produced similar amounts of fatty acids at the two incubation temperatures. The other isolates produced lower total amounts of fatty acids at low incubation temperature, although the incubation time was longer. The total yield of ARA per Petri dish at low incubation temperature was increased only in the case of two isolates. The total yield of ARA per Petri dish at low incubation temperature and with 1% of rapeseed oil was increased in

the case of one isolate, *M. alpina* MSCL 1254 (Fig. 5). As shown in other investigations, low incubation temperature causes the production of eicosatetraenoic acid (20:4) (Jareonkitmongkol et al. 1993). The highest amount of this fatty acid was obtained by isolate *M. elongata* MSCL 1380, 4.08 mg g^{-1} dry mass.

Summarizing the results of all experiments it can be concluded that every tested isolate produced PUFAs and 14 isolates produced ARA, but in terms of the quantity they were not effective producers. This can be explained by their isolation conditions at 20 °C. It might be possible to isolate good ARA producers if the isolation temperature is 5 °C (Botha et al. 1999). However, three strains isolated from soil sampled in winter were not better producers than the strains isolated during the active vegetation period or from vermicompost. The production efficiency more depended on the growth medium. However, the tested isolates produced a broad spectrum of fatty acids, ranging from short chain (C_7–C_9) to very long chain fatty acids (C_{26}). Higher fatty acid yields were obtained on solid growth media, at lower carbon source concentrations and, particularly, at low incubation temperature.

Acknowledgements

This investigation was supported by the European Regional Development Fund funded project "Development of prophylactic skin regenerating polysaccharide and glycoprotein containing products of plant and fungal origin, use of them to elaborate compositions for hygiene and cosmetic products" No 2010/0292/2DP/2.1.1.1.0/10/APIA/VIAA/134.

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Received 27 December 2013; received in revised form 20 January 2014; accepted 22 February 2014