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Arachidonic-acid production by species of *Mortierella*†

V.K. Eroshin, E.G. Dedyukhina, T.I. Chistyakova, V.P. Zhelifonova, C.P. Kurtzman and R.J. Bothast*

A growth-inhibiting, aspirin-containing medium was developed to select arachidonic-acid-(ARA)-producing *Mortierella* species and to determine the fatty-acid content of 87 *Mortierella* strains. ARA was detected in 66 strains from 33 species and its production may prove useful for systematic studies on *Mortierella* spp. The ARA content of the 66 producing strains tested ranged from 4% to 55% of total lipids. Most of the ARA-producing strains of *Mortierella* (59 strains) grown on potato/glucose/agar synthesized < 20% ARA. Four strains produced between 20% and 25% ARA and three produced > 40%. An inverse relationship was observed between ARA and oleic-acid contents.

Key words: Arachidonic acid, fatty acids, fungi, lipids, *Mortierella*.

Arachidonic acid (ARA), 5,8,11,14-all*cis*-eicosatetraenoic acid (ω-6 type), possesses a number of unique biological activities and is a precursor of prostaglandins, leucotrienes and a large group of C_{20} compounds which have various regulatory effects in humans. ARA is also an elicitor of phytoalexins in plants and may be used for preventing plant disease (Bostock *et al.* 1981). In recent years, ARA has been widely used in a number of fields, including pharmaceuticals, cosmetics and agriculture.

Currently, the main sources of ARA are bovine and porcine liver, adrenal glands and sardines. However, the ARA content per unit dry weight of these sources is only 0.2% (Bajpai *et al.* 1991). Consequently, microorganisms are an attractive potential source of ARA.

The ability of microorganisms to produce polyunsaturated fatty acids of the ω-6 group can have taxonomic value (Shaw 1966; Losel 1988). The most active ARA-producing fungi belong to the class *Zygomycetes*, especially the genus *Mortierella* (Totani & Oba 1987; Yamada *et al.* 1987, 1989; Shimizu *et al.* 1989; Shinmen *et al.* 1989, 1993; Bajpai *et al.* 1991). However, selection of ARA-producing strains has been restricted by the absence of convenient screening methods. It is known that aspirin (*O*-acetylsalicylic acid) inhibits oxygenation reactions in prostaglandin synthesis by acetylating the terminal amino group in prostaglandin synthase (Vane 1971). Aspirin at 1 mM inhibits synthesis of ARA metabolites in yeasts and Botha *et al.* (1992) and Botha & Kock (1993) proposed using growth in the presence of 1 mM aspirin as a taxonomic criterion for yeasts.

The aim of the present study was to determine the effect of aspirin on growth of ARA-producing and non-producing strains, and to investigate ARA-production in species of *Mortierella*.

Materials and Methods

Microorganisms and Growth

Mortierella strains were obtained from the All-Russian Culture Collection of Non-pathogenic Microorganisms (VKM), Puschchino, Russia, the ARS Culture Collection (NRRL), Peoria, Il, and the Centraalbureau voor Schimmelcultures (CBS), Baarn, The Netherlands. They were all grown in Petri dishes containing potato/glucose/agar medium with and without aspirin for 15 days at

V.K. Eroshin, E.G. Dedyukhina, T.I. Chistyakova and V.P. Zhelifonova are with the Institute of Biochemistry and Physiology of Microorganisms, 142292 Pushchino, Moscow Region, Russia. C.P. Kurtzman is with the Microbial Properties Research Unit, and R.J. Bothast is with the Fermentation Biochemistry Research Unit, both of the National Center for Agricultural Utilization Research, USDA, Agricultural Research Service, 1815 N. University Street, Peoria, IL 61604, USA; fax: 309 681 6686.
*Corresponding author.

†Names used in this article are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by USDA implies no approval of the product to the exclusion of others that may also be suitable.

Table 1. Lipid and fatty-acid composition of *Mortierella* strains grown on potato/glucose/agar without aspirin.

Species	Strain	Lipid [% (w/w) of dry biomass]	Fatty acids [% (w/w) of total lipids]											Growth with aspirin at 0.84 g/l
			14:0	15:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:3	20:4 (ARA)	
Section *Isabellina*														
M. isabellina	NRRL 1757	37	0.7	0.2	23.5	1.5	1.8	57.4	12.3	2.6	–	–	–	+
	VKM-F-510	17	1.4	0.6	22.2	–	0.6	48.9	16.8	9.3	0.2	–	–	+
	VKM-F-525	24	0.9	0.6	26.1	tr	2.1	46.2	18.2	5.2	0.6	–	–	+
	VKM-F-526	31	1.0	0.7	26.2	–	tr	46.3	19.1	6.7	–	–	–	+
	VKM-F-528	21	1.0	0.5	26.0	–	3.0	44.8	12.2	11.9	0.5	–	–	+
	VKM-F-1628	30	0.8	0.7	22.9	–	1.2	45.6	23.3	5.0	0.4	–	–	+
M. isabellina var. *atrogrisea*	VKM-F-527	24	1.1	0.7	26.5	–	3.6	44.1	11.1	12.2	0.7	–	–	+
M. vinacea	VKM-F-724	32	0.9	0.6	23.4	–	3.2	39.7	19.9	12.1	0.2	–	–	+
M. longicollis	VKM-F-532	23	0.7	1.1	28.5	–	4.9	38.5	14.5	11.8	–	–	–	+
	VKM-F-537	16	0.9	1.1	22.8	tr	2.3	43.3	18.9	10.7	–	–	–	+
	VKM-F-654	26	0.6	1.0	21.5	–	3.2	45.1	16.4	10.8	1.4	–	–	+
	VKM-F-665	27	0.7	0.7	25.6	–	4.5	44.2	12.2	11.1	0.6	–	–	+
Section *Stylospora*														
M. sepedonioides	NRRL 6425	10	3.0	0.9	32.6	–	6.2	38.6	6.6	1.6	4.4	–	6.2	+
M. stylospora	VKM-F-1207	24	0.1	0.9	23.0	–	15.8	32.4	27.0	0.8	–	–	–	+
M. lignicola	VKM-F-1438	29	1.6	1.7	24.2	–	7.0	39.3	9.9	3.2	4.3	1.1	7.8	–
M. humilis	VKM-F-1611	8	2.2	1.3	32.0	1.8	2.7	35.2	7.2	3.3	2.4	2.2	9.6	–
	VKM-F-1636	7	3.1	1.8	34.4	2.5	4.0	27.4	9.2	2.9	–	1.2	13.4	–
	VKM-F-1650	5	3.9	1.6	23.6	–	10.8	24.0	6.6	6.8	1.1	3.6	15.0	–
	VKM-F-1652	10	1.9	0.8	32.0	2.0	4.6	28.8	7.7	3.4	2.6	2.4	13.9	–
Section *Alpina*														
M. globalpina	VKM-F-1527, CBS 360.70	15	0.8	1.3	24.4	–	11.6	16.9	15.6	5.5	0.6	3.0	20.2	–
M. alpina	NRRL A-10995	7	0.4	0.8	13.7	–	5.4	3.7	3.5	3.3	0.7	2.9	55.2	–
	VKM-F-1609	5	tr	tr	18.7	–	9.9	11.5	8.7	3.5	–	5.4	42.4	–
	VKM-F-1630	9	2.7	0.7	16.6	–	1.9	47.4	17.7	11.4	1.8	–	–	+
M. angusta	VKM-F-1401, CBS 293.61	4	3.2	2.2	25.6	2.4	1.2	30.4	16.0	19.1	–	–	–	+
M. elasson	VKM-F-1417, CBS 220.29	1	5.2	6.2	22.3	–	4.5	4.8	41.9	15.2	–	–	–	+
M. strangulata	VKM-F-1387	9	1.8	1.4	14.2	0.6	12.0	32.1	7.1	5.4	1.5	4.0	16.0	–
M. alliacea	VKM-F-1526, CBS 894.68	7	2.5	1.3	13.4	2.7	4.7	53.3	5.0	3.0	3.2	2.6	8.2	–
M. pusilla	VKM-F-1436	10	2.6	0.9	10.1	2.4	8.2	39.7	8.0	4.3	4.8	2.3	16.2	–
M. simplex	VKM-F-1535, CBS 751.68	1	0.9	3.6	20.8	1.2	2.9	41.3	29.4	–	–	–	–	+
M. globulifera	VKM-F-1408, CBS 108.68	14	2.0	1.7	16.6	–	7.0	46.8	12.4	6.0	0.9	0.6	6.0	+
	VKM-F-1448, CBS 746.68	12	2.7	2.3	22.4	–	4.5	45.7	11.5	5.8	–	–	5.0	+
	VKM-F-1495, CBS 417.64	7	2.3	2.0	29.8	–	8.2	39.9	9.7	4.4	–	–	–	+

Section *Jenkinia*													
M. bainieri	NRRL 6716	25	3.4	0.2	20.5	–	12.4	44.7	5.1	2.8	0.7	2.4	7.7	–
M. jenkinii	VKM-F-949	10	3.6	1.2	26.5	0.9	11.9	45.7	1.1	2.6	–	0.2	6.3	–
	VKM-F-1442	11	3.9	1.7	31.1	2.3	6.3	42.5	3.2	2.2	–	0.9	5.9	–
M. dichotoma	VKM-F-1407,CBS 221.35	13	7.5	2.3	32.6	1.1	7.9	32.0	6.2	3.2	–	0.4	6.8	–
M. sclerotiella	VKM-F-1099	2	3.6	1.4	20.6	2.4	4.8	39.6	7.5	5.4	–	3.0	11.8	–
Section *Hygrophila*														
M. nana	VKM-F-1400, CBS 309.52	19	0.6	0.9	20.2	–	3.9	42.5	19.2	12.0	0.6	–	–	+
	VKM-F-1421	19	1.0	0.7	21.8	–	2.4	49.1	15.1	9.9	–	–	–	+
M. hygrophila	VKM-F-919	6	3.3	tr	16.8	–	4.6	52.1	8.4	6.5	–	tr	8.3	–
	VKM-F-1629	8	4.7	0.7	17.4	–	1.0	40.3	11.0	5.8	–	1.2	14.4	–
M. marburgensis	VKM-F-529	8	6.1	1.7	19.4	1.3	3.7	35.4	10.4	6.4	–	0.4	15.2	–
	VKM-F-976	10	2.7	1.8	14.8	–	12.4	37.3	8.6	6.7	1.3	1.7	12.6	–
	VKM-F-977	6	1.2	1.9	27.9	–	10.8	29.8	8.7	4.7	–	2.1	12.8	–
	VKM-F-1386, CBS 220.58	6	0.9	1.4	13.1	–	16.8	26.3	7.4	5.9	0.9	2.5	17.4	–
	VKM-F-1612	6	1.6	1.0	32.3	–	10.7	31.0	5.9	4.6	0.3	1.5	10.3	–
	VKM-F-1613	12	0.9	0.6	19.2	1.8	6.2	40.6	10.8	4.5	1.9	1.7	10.6	+
M. minutissima	VKM-F-1639	8	2.4	1.9	18.7	–	7.0	26.8	7.0	5.7	0.6	2.5	23.2	–
	VKM-F-1771	12	1.8	0.8	19.5	–	8.8	36.4	4.6	4.7	1.1	2.5	17.5	–
	VKM-F-1884	7	1.1	2.1	25.3	–	5.7	29.6	10.3	4.2	–	3.4	18.4	–
M. beljakovae	VKM-F-1608	17	0.7	0.7	24.0	–	1.4	47.6	10.2	5.3	0.9	0.8	8.3	–
M. sclerotiella	VKM-F-1099	2	3.6	1.4	20.6	2.4	4.8	39.6	7.5	5.4	–	3.0	11.8	–
M. sarnyensis	VKM-F-1638	15	0.3	0.1	36.8	–	0.4	32.1	15.4	1.5	–	–	13.4	–
M. nigrescens	VKM-F-1439	7	5.8	1.1	31.3	2.6	5.1	36.3	6.1	3.5	–	1.9	6.2	–
M. zychae	VKM-F-861	10	2.5	2.0	25.2	1.0	8.5	34.1	11.8	3.7	1.4	0.7	9.2	–
	VKM-F-1621	7	2.1	1.5	17.7	0.6	6.4	29.1	7.5	4.1	6.8	4.2	19.8	–
	VKM-F-1622	5	1.9	1.7	17.1	–	12.1	29.0	14.8	5.9	4.8	1.0	11.9	–
	VKM-F-1623	9	2.5	1.4	19.7	1.8	2.6	42.2	6.5	3.0	8.4	1.8	9.2	–
	VKM-F-1624	4	3.0	1.3	18.5	3.5	4.3	30.9	7.2	3.8	10.1	4.1	13.4	–
M. gemmifera	VKM-F-1631	10	2.0	0.5	20.2	–	11.6	40.3	8.9	3.4	0.6	1.6	10.9	–
	VKM-F-1252	11	–	–	–	–	–	–	–	–	–	–	10.3	–
M. elongata	VKM-F-524	3	3.3	3.3	21.0	1.6	7.0	42.9	9.7	4.1	1.0	–	6.2	–
	VKM-F-1614	9	2.6	0.8	20.9	1.6	10.7	31.4	7.4	5.5	0.5	3.2	14.5	–
	NRRL 5246	3	1.1	1.6	18.6	1.1	1.2	30.1	45.7	0.6	–	–	–	+

Table 1. Lipid and fatty-acid composition of *Mortierella* strains grown on potato/glucose/agar without aspirin—continued.

Species	Strain	Lipid [% (w/w) of dry biomass]	Fatty acids [% (w/w) of total lipids]											Growth with aspirin at 0.84 g/l
			14:0	15:0	16:0	16:1	18:0	18:1	18:2	18:3	20:1	20:3	20:4 (ARA)	
Section *Polycephala*														
M. reticulata	VKM-F-1405, CBS 241.33	10	1.9	1.0	14.4	1.1	8.6	42.5	4.8	4.0	1.8	1.0	18.6	–
M. parvispora	VKM-F-523	14	1.5	0.6	19.5	1.9	5.0	39.0	9.8	4.6	1.1	2.2	13.9	–
	VKM-F-536	11	1.0	1.2	14.7	1.0	4.5	48.5	7.7	6.3	1.4	2.1	11.5	–
	VKM-F-950	16	2.7	1.0	26.2	–	3.9	48.5	6.2	2.5	–	2.0	7.2	–
	VKM-F-1547	13	3.0	0.8	33.8	0.8	4.5	45.5	4.4	1.6	0.5	1.7	6.4	–
	VKM-F-1610	16	2.1	1.1	27.3	1.1	7.7	42.8	7.5	2.4	0.4	1.4	6.2	–
M. pulchella	VKM-F-1531, CBS 441.68	12	0.5	0.6	9.9	0.5	4.7	23.8	5.7	7.2	2.4	5.2	25.0	–
M. gamsii	VKM-F-1640	4	2.2	1.6	20.1	–	6.5	38.9	7.1	3.5	0.8	3.6	15.9	–
	VKM-F-1647	11	1.5	1.1	16.0	–	5.2	27.1	9.8	5.7	–	8.7	25.0	–
	VKM-F-1402, CBS 749.68	20	3.2	1.0	20.3	–	11.8	41.1	6.5	3.9	1.4	2.4	8.5	–
	VKM-F-1529	12	4.2	1.1	22.7	1.9	6.1	37.4	5.5	4.3	2.0	3.8	11.1	–
M. nantahalensis	NRRL 5216, CBS 610.70	6	0.9	0.5	13.6	–	25.7	31.8	11.7	1.5	0.7	1.5	6.5	–
M. polycephala	NRRL A-9455	12	1.2	0.8	18.8	2.1	4.5	35.3	7.6	5.0	0.9	4.7	15.8	–
	VKM-F-953	6	3.2	0.7	14.7	0.7	2.1	23.8	3.3	6.0	–	2.8	41.8	–
M. spinosa var. *sterilis*	VKM-F-1534, CBS 665.68	14	0.6	0.6	30.3	1.5	8.9	34.9	7.6	1.7	0.9	1.2	10.8	–
M. spinosa	VKM-F-1055	18	4.0	1.4	20.2	0.9	7.9	30.1	6.7	5.8	1.2	3.4	17.3	+
	VKM-F-1491,CBS 870.68	11	5.6	0.4	22.7	0.4	10.5	29.9	6.8	4.7	0.4	2.6	15.9	–
	VKM-F-1641	22	2.0	0.7	17.8	–	7.4	41.2	12.1	5.3	0.7	2.9	9.9	–
	VKM-F-1642	8	0.4	1.8	17.9	–	3.5	28.0	13.2	5.4	–	3.8	25.3	–
	VKM-F-1643	8	3.3	1.3	18.0	0.9	5.8	33.1	9.1	5.2	1.2	3.3	18.8	–
	VKM-F-1646	17	3.5	0.8	23.1	0.8	7.3	42.9	9.1	3.3	0.6	1.5	7.2	–
Not assigned														
M. indolhii	NRRL 5248, CBS 460.75	10	6.0	0.6	43.3	1.1	6.5	27.0	2.9	3.3	–	1.4	7.9	–
M. gracilis	VKM-F-1493, CBS 445.68	17	6.9	0.4	24.3	2.9	3.5	49.7	4.9	1.9	–	1.2	4.2	–

– —Not detected; tr—trace.

25°C. Aspirin was added at 0.42, 0.84 or 1.66 g/l being dissolved in ethanol and then added to the medium before autoclaving at 108°C for 30 min.

Lipid Analyses

Mycelia were harvested from slants and dried at 70°C under vacuum. The potato/glucose medium was analysed for fatty acids to check that the medium contained no lipids. Fatty acids were determined in dried cells according to the method of Sultanovich *et al.* (1982). Fatty-acid methyl esters were prepared by adding acetyl/chloride (0.3 ml) and methanol (1.5 ml) to a flask with dry mycelium (20 to 50 mg). The flask was held at 80°C to complete solvent evaporation. n-Hexane (0.2 ml) was added to extract the fatty-acid methyl esters which were analysed by GC using a flame-ionization detector and a glass column (200 × 0.3 cm) containing 15% Reoplex-400 (polar coating) on Chromaton N-AW (0.16 to 0.20 mm). The column was operated at 200°C and argon was used as the carrier gas. The methyl esters were identified using standard esters. Identification of ARA was confirmed by GC-MS. Individual fatty acids were determined as percentages of total fatty acids. Total lipids were calculated as the sum of fatty acids, using heptadecanoic acid (17:0) or *n*-docosane (22:0) as internal standards.

Results and Discussion

Screening for ARA-producing Fungi

In the first series of experiments, the effect of aspirin concentration on growth of fungi was determined. Aspirin at 1.66 g/l inhibited growth of both ARA-producing and non-producing strains whereas 0.42 g/l allowed growth of all strains. However, 0.84 g/l had a selective effect upon the growth of ARA-producing and non-producing strains. Consequently, this concentration was used to study the growth of *Mortierella* strains varying in their ability to synthesize ARA. The results are presented in Table 1.

All strains that did not produce ARA grew on a medium containing 0.84 g aspirin/l but most ARA-producing *Mortierella* species (61 strains) could not grow on this medium. Although five ARA-producing strains were able to grow with aspirin at this concentration, the ARA content of these strains did not exceed 11% of total lipids. These results suggest that a medium with 0.84 g aspirin/l may be used for preliminary selection of ARA-producing fungal strains.

Distribution of the ARA-producing Capability in Mortierella *Species*

ARA was detected in 66 strains from 33 species. The data on lipid content and ARA content of the lipids for these 66 *Mortierella* strains are summarized in Table 1. ARA content ranged from 4% to 55% of total lipids. Four strains produced up to 25% ARA and three strains produced 42% to 55% ARA. Consequently, although ARA-producing ability is widely distributed in species of *Mortierella*, high ARA production is rather rare. According to the data available in the literature, the ARA content of fungal lipids usually ranges from 2% to 15% (Shimizu *et al.* 1989), although there are some reports of *Mortierella* strains that produce > 30% of total lipids as ARA (Totani & Oba 1987; Totani *et al.* 1987; Bajpai *et al.* 1991; Shinmen *et al.* 1993). The highest ARA content reported (> 40% of total fatty acids) was found by Totani et al. (1987) in *Mortierella* grown on potato/glucose/agar.

According to Milko (1974), *Mortierella* species are subdivided into seven sections mainly on the basis of morphological criteria. Section *Isabellina* includes species forming coloured colonies, section *Stylospora* includes species forming stylospores and sections *Alpina, Jenkina, Dichotoma, Hygrophila,* and *Polycephala* are distinguished from each other chiefly by sporangiophore morphology. Some modifications to Milko's classification of *Mortierella* have been given by Gams (1977). It should be mentioned that no ARA-producing strains were found among all three known *Mortierella* species in the section *Isabellina*: *M. isabellina, M. vinacea* and *M. longicollis*. All the species tested belonging to section *Jenkinia* produced ARA. In other sections, both ARA-producing and non-producing strains were found. According to another classification (Shinmen *et al.* 1989), the genus *Mortierella* can be subdivided into two subgenera, *Mortierella* and *Micromucor*. It has been suggested that the subgenus *Mortierella* comprises species that produce ARA whereas in the subgenus *Micromucor* (*M. ramanniana, M. isabellina, M. vinacea, M. humicola* and *M. nana*) do not produce ARA. The present data are in agreement with that observation; no ARA-producing strains being found in the species tested that belong to the genus *Micromucor*: *M. isabellina* and *M. vinacea* (section *Isabellina*), *M. nana* (section *Hygrophila*) or *M. ramanniana*. The results indicate that ARA synthesis is a characteristic feature of certain *Mortierella* species and may be useful in systematic studies of the genus *Mortierella*.

Fatty-acid analyses showed that ARA-producing strains contained mainly fatty acids from C_{14} to C_{20}. Unsaturated fatty acids were predominant, total unsaturated fatty acids ranging from 57% to 78% of total lipids. Unidentified fatty acids $\geq C_{20}$ were observed in some strains. An inverse relationship between ARA and oleic-acid contents in the lipids was determined, though no biochemical explanation can be offered for this at the moment.

The different fatty-acid compositions of the ARA-producing strains indicate that strain selection should be based on the application involved. For example, strains producing moderate amounts (10% to 20%) of ARA and large amounts of other essential fatty acids (γ-linolenic, 18:3 ω-6; dihomo-γ-linolenic, 20:3 ω-6) could have nutritional applications whereas those with high proportions of ARA (20:4 ω-6) and eicosapentaenoic acid (20:5 ω-3) could have plant-health applications. However, strains with high ARA, 20:3 (ω-6) and 20:5 (ω-3) contents would be undesirable in a case where ARA purity is desirable because of the difficulties in separation of acids with the

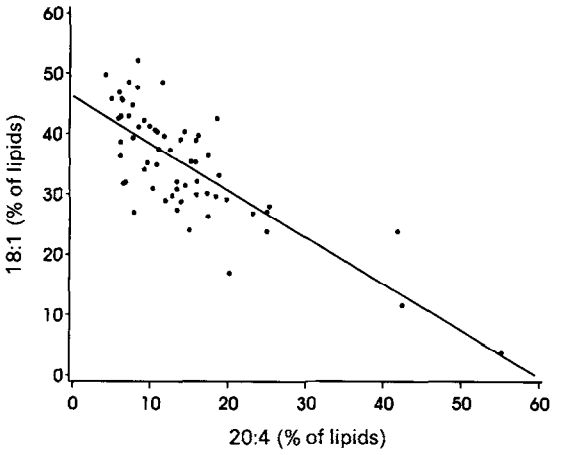

Figure 1. Correlation ($r = -0.77$) between arachidonic-(20:4)- and oleic-(18:1)-acid contents of total lipids.

same chain length but slightly different unsaturation levels.

In conclusion, an aspirin-containing medium can be used as a selective inhibitor of growth of ARA-producing strains for preliminary selection of ARA producers. The present results indicate that ARA synthesis is a characteristic feature of some *Mortierella* species and that this could be used in systematic studies of the genus *Mortierella* as well as for fermentative production of ARA.

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