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EFFECT OF GROWTH CONDITIONS ON BIOSYNTHESIS OF OIL BY *MORTIERELLA RAMANNIANA*.

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The effects of culture conditions (pH 4.0–5.0, 25–35°C, 48–96 h) on cell growth, lipid accumulation and lipid composition (fatty acids, mono-, di-, and triacylglycerols) are reported for *Mortierella ramanniana* var. *angulispora* IFO 8187 cultivated in shake flask in glucose medium. The highest yield of biomass (22.4 g/L), lipids (3.9 g/L) and γ-C18:3 (178.1 mg/L or 8.8 mg/g dry cell weight) was observed at pH 4.5 and 30°C after 96 h of cultivation. The percentage of fatty acids in the oil changed upon the pH, cultivation time, and to a minor extent upon the growth temperature. The highest variations were observed for the dominating fatty acids: C18:1 (35.0%–45.3%), C16:0 (26.5%–32.4%), C18:2 (8.0%–13.7%) and for γ-C18:3 (4.1%–8.2%). Among the lipid classes, triacylglycerols (63.5%–72.7%) dominated followed by free fatty acids (14.5%–15.3%). The percentage of monoacylglycerols increased about 2-fold (9.1%–20.0%) and that of diacylglycerols decreased about 3-fold (3.6%–1.2%) during the last 24 h of cultivation. C18:1 and C16:0 dominated in all the lipid classes. The characteristic fatty acids in triacylglycerols were γ-C18:3, C20:0, C20:1 and C22:0.

INTRODUCTION

Microbial synthesis of fatty acids having medical and nutritional values has been considered as an attractive alternative for their traditional sources [Ratledge, 1991; Leman, 1993, 1993a, 1994]. The latter include evening primrose oil, fish oils, and porcine liver and adrenal glands, used for the production of γ-linolenic acid (γ-C18:3, GLA), icosapentaenoic- (IPA) and docosahexaenoic (DHA) acids, and

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arachidonic acid (ARA), respectively. Microbial production of these acids requires, however, a definite compromise to be found since a large number of variables affects the fatty acid biosynthesis, with the medium composition, pH, oxygen supply and growth rate being of the greatest importance [Hansson & Dostálek, 1988; Sumner *et al.*, 1969; Kendrick & Ratledge, 1992; Neidleman, 1987; Šajbidor *et al.*, 1988]. Yet, the cell concentration, the oil content of the cell and the fatty acid content of the oil are three variables that determine the characteristic pattern to the production of certain fatty acids by microorganisms [Kennedy *et al.*, 1993].

The up-to-date studies on screening microorganisms for polyunsaturated fatty acid (PUFA) synthesis have resulted in the selection of the best performing organisms [Ratledge, 1991, 1993; Suzuki & Yokochi, 1984; Kendrick & Ratledge, 1992a; Nakahara *et al.*, 1993]. Among them, filamentous fungi of the *Mucorales* and *Mortierella* groups are the potential PUFA producers. The commercial processes for production of GLA have been developed in the U.K and Japan which use *Mucor javanicus* or *M. circinelloides* and *Mortierella isabellina*, respectively [Ratledge, 1991, 1993]. The Japanese studies on a range of *Mortierella* species under diffe-rent conditions resulted in the selection of mutant strain of *M. ramanniana* with enhanced GLA productivity when grown in a continuous culture system [Nakahara *et al.*, 1993; Kamisaka *et al.*, 1990].

In Poland, oleaginous microorganisms were considered so far in terms of potential applications to the fat industry or animal feeding [Makarewicz & Gogolewski, 1983; Leman, 1991]. This research is first domestic approach that presents a preliminary studies in regard to the possibility of producing microbial oil rich in pharmacologically active fatty acids, GLA particularly [Leman, 1993, 1993a, 1994]. In the study, *M. ramanniana* var. *angulispora* IFO 8187 recommended by Japanese authors [Nakahara *et al.*, 1992] for production of GLA-rich oil was used.

In this work we report on the effects of pH, temperature and cultivation time on the growth, lipid production and oil composition in *M. ramanniana* var. *angulispora* IFO 8187, to verify its fatty acid producing abilities, with an emphasis on GLA synthesis, using shake flask cultures and glucose medium.

MATERIAL AND METHODS

MICROORGANISM AND MEDIUM: *Mortierella ramanniana* var. *angulispora* IFO 8187 was purchased from the culture collection of the Institute of Fermentation, Osaka, Japan. The microorganism was maintained on malt extract agar slants at 8°C and was transferred every third month. The basal medium used for fermentation and preparation of inoculum was generally the same as in the studies by Japanese authors [[Nakahara *et al.*, 1992] and contained in 1 L distilled water: glucose, 50 g; $CuSO_4$ x $5H_2O$, 0.2 mg; $MnSO_4$ x $4H_2O$, 1.0 mg; $CaCl_2$ x $2H_2O$, 0.1 g;

$FeSO_4$ x $7H_2O$, 10.0 mg; $MgSO_4$ x $7H_2O$, 0.3 g; KH_2PO_4, 3.0 g; NH_4NO_3, 3.0 g. The pH of the medium was adjusted to 4.0, 4.5 or 5.0 with 1 mol/L HCl before the medium was autoclaved at 121°C for 15 min.

SHAKE FLASK EXPERIMENTS: The inoculum was grown in 200 mL of the medium with pH 5.0 at 30°C for 48 h. The shake flask of 500 mL capacity containing 200 mL of the medium was inoculated with 20 mL of the inoculum and incubated at 25°C, 30°C or 35°C on a New Brunswick Scientific G25 rotary shaker at 250 rev/min for 48 h, 72 h and 96 h. After incubation, the cells were separated from the medium by vacuum filtration through a Shott funnel with a sintered glass disc 17G1 and washed with deionized water. The wet biomass was weighed and kept frozen at –10°C before being analysed for dry cell weight, lipid content and lipid composition. At least three independent cultivations were performed for each experiment.

ANALYTICAL METHODS: The dry cell weight was measured by drying a known wet weight of the biomass in an oven at 105°C to a constant weight, and then calculated as grams of dry biomass per 1 L of culture medium. Lipids were extracted from the thawed biomass with 2:1 v/v chloroform-methanol at a 5:1 v/v solvent-biomass ratio followed by vacuum evaporation of the solvent at 50°C. The total lipid content was measured by weighing the extract dried to a constant weight in a vacuum oven over P_2O_5 at 55°C and calculated as grams of dry oil per 1 L of culture medium.

The oil was fractionated into monoacylglycerols (MAG), diacylglycerols (DAG), triacylglycerols (TAG) and free fatty acids (FFA) by ascending thin layer chromatography on 1 mm Silica 60 gel (Merck, Germany) plates (20 x 40 mm) with hexane-ethyl ether-methanol-acetic acid, 90:20:3:2 by volume. The chromatograms were developed in iodine vapours and the particular lipid fractions were scraped off and extracted with ethyl ether. After the sorbent was separated by filtration through a Shott funnel and the solvent was vacuum evaporated at 50°C, the fractions were weighed and their percentage in the oil was calculated.

The fatty acid composition of the oil and its fractions was determined by gas chromatography on the fatty acid methyl esters (FAMEs) prepared by the Peisker method in the modification by Żegarska *et al.* [1991], using chloroform-methanol--H_2SO_4, 100:100:1 by volume. The FAMEs were analysed in a Pye-Unicam gas chromatograph with a flame-ionization detector and PP88 Pye-Unicam integrator, using 2.7 m x 4 mm glass column packed with 10% BDS on Gas-Chrom Q (100/120 mesh) support. Argon was used as a carrier gas at the rate of 60 mL/min. The column, vaporizer and detector were operated at 195°C, 225°C and 250°C, respectively. Peaks of the sample FAMEs were identified by using standard FAMEs (Applied Science Laboratory, U.S.A.) and the weight percentages of sample FAMEs were calculated from peak areas given by the integrator.

RESULTS

THE YIELD OF BIOMASS AND OIL

The yields of biomass and oil of *M. ramanniana* var. *angulispora* IFO 8187 were influenced by the growth conditions (Figure 1). During the cultivation, the yield of biomass increased from about 10% to about 65% after 72 h and from

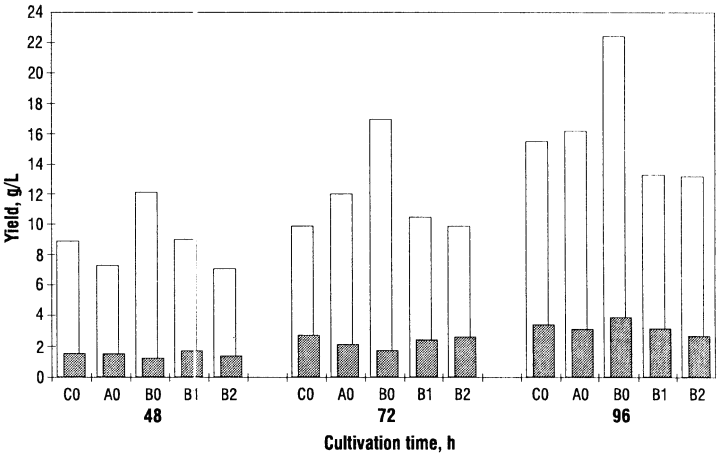

Figure 1. The yields of biomass (□) and oil (□) during cultivation of *M. ramanniana* var. *angulispora* IFO 8187 in glucose medium at pH 4.0 (A), 4.5 (B), 5.0(C) and 30°C (0), 25°C (1), and 35°C (2). Results are means from three independent experiments.

about 49% to about 125% after 96 h of cultivation depending on pH and temperature. The respective values for the yield of oil were about 40% to about 86% and about 88% to about 225%. Independent of the cultivation time, the highest yield of biomass was found when the cultivation was performed at pH 4.5 and 30°C. In these pH and temperature values, the yield of oil was the lowest up to the 72nd hour of cultivation, after which time it increased about 3-fold compared to that after 48 h cultivation and reached the maximum by the end of cultivation. Depending on pH and temperature, the yield of oil was lower by about 4–10-fold than of biomass. Only at the optimum pH and temperature conditions, oil accumulation distinctly increased, being about 5.7-fold lower than accumulation of biomass after 96 h of cultivation. Under non-optimum growth conditions the accumulation of oil during cultivation increased by about 2-fold being from 4.1-fold to 5.2-fold lower than accumulation of biomass after 96 h of cultivation.

FATTY ACID COMPOSITION OF OIL

The fatty acid profile did not differ depending on the growth conditions (Table 1). Oleic acid (C18:1), palmitic acid (C16:0), linoleic acid (C18:2) and γ-C18:3 were the major fatty acids in the oil. However, the growth conditions affected the fatty acid percentages in the oil. The highest variations were observed for the fatty acids dominating in the oil, *i.e.* C18:1 (3–8%), C18:2 (4–5%), C16:0 and γ-C18:3 (2–5%), and the lowest for stearic acid (C18:0, 1–3%) and minor fatty acids (0–2%). The changes in the fatty acid percentages were affected differently by the time, pH and temperature of cultivation, with the latter having the least effect.

Compared to the fatty acid percentages in the oil produced in the optimum conditions (pH 4.5, 30°C, 96 h), lower pH or temperature of cultivation increased the percentage of polyunsaturated fatty acids: C18:2 by about 46% and about 22%, respectively, and γ-C18:3 by about 81% and about 37%, respectively. The percentages of other fatty acids, except C14:0, decreased at pH 4.0 in the range from about 46% (C18:0) to about 5% (C16:0). At lower temperature of cultivation, the percentage of C14:0, C16:1 and C18:1 decreased by about 39%–60%, and that of C16:0 increased by about 10%. No effect of lower temperature of cultivation was observed on the percentage of C18:1 and minor fatty acids. Higher pH or temperature of cultivation increased the percentage of C16:0 by about 13%–16%. At higher pH, also the percentage of C14:0, C16:1, C18:2 and minor fatty acids increased by about 64%, 29%, 49% and 89%, respectively,

Table 1. Fatty acid composition (%) of oil produced by *M. ramanniana* var. *angulispora* IFO 8187 under different growth conditions.

Fatty acid	Cultivation time, h	pH*			Temperature**, °C	
		4.5	4.0	5.0	25	35
C14:0	48	2.2	1.6	1.4	1.0	1.8
	72	4.6	2.2	3.0	1.0	1.7
	96	2.5	3.3	4.1	1.0	1.6
C16:0	48	29.5	28.8	26.5	31.7	33.2
	72	28.0	27.3	29.0	33.1	32.7
	96	28.6	27.2	32.4	31.4	33.1
C16:1	48	4.8	4.0	5.5	2.3	2.9
	72	6.3	5.5	5.1	2.2	2.0
	96	5.6	5.1	7.2	2.3	2.3
C18:0	48	3.2	3.0	2.5	3.4	4.1
	72	3.6	2.6	3.1	3.5	5.5
	96	5.7	3.1	3.2	3.5	4.9
C18:1	48	44.7	43.7	41.0	43.8	48.4
	72	44.0	45.3	37.6	45.6	48.8
	96	44.2	40.8	35.0	44.9	47.5
C18:2	48	8.7	9.7	13.7	9.3	5.6
	72	8.0	9.7	13.7	8.9	5.4
	96	8.3	12.1	12.4	10.1	6.1
γ-C18:3	48	6.2	8.0	8.2	6.0	3.3
	72	4.8	6.5	5.5	4.9	3.0
	96	4.3	7.8	4.1	5.9	3.7
Minor	48	0.7	1.2	1.3	2.5	0.8
	72	0.6	0.7	3.0	0.8	1.7
	96	0.9	0.7	1.7	0.9	0.8

* 30°C, ** pH 4.5. Results are means from three independent experiments

while those of C18:0, C18:1 and γ-C18:3 decreased by about 44%, 21% and 5%, respectively. Lower cultivation temperature decreased the percentage of fatty acids, except C16:0 and C18:1, in the range from about 50% (C16:1) to about 11% (minor fatty acids).

The time of cultivation at the optimum pH and temperature did not affect much the fatty acid percentages, except of those for C18:0 that increased by about 80%, and γ-C18:3 that decreased by about 30%. Compared to this, the reverse relationship for these fatty acids was observed in the oil produced at lower pH or temperature of cultivation, *i.e.* the percentage of C18:0 decreased and that of γ-C18:3 increased. At higher pH or temperature of cultivation, the percentage of C18:0 fluctuated, and that of γ-C18:3 decreased at lower pH but it increased at both lower and higher temperature of cultivation. Irregular changes with different tendencies were observed in the percentage of the other fatty acids during the cultivation.

COMPOSITION OF OIL PRODUCED IN OPTIMUM GROWTH CONDITIONS

The oil produced in the optimum growth conditions was composed of MAG, DAG, TAG and FFA. Two last-mentioned lipid classes made together 78%, with an evident domination of TAG in the oil after 72 h of cultivation (Table 2). The DAG percentage of oil was the lowest. The percentage of particular lipid classes, except FFA, changed during the cultivation. The highest variation was observed for MAG, which increased about 2-fold, and for DAG, which decreased about 3-fold. The percentage of TAG decreased by about 9%.

Table 2. Percent composition of monoacylglycerols (MAG), diacylglycerols (DAG), triacylglycerols (TAG) and free fatty acid (FFA) in *M. ramanniana* var. *angulispora* IFO 8187 oil produced in optimum growth conditions (pH 4.5, 30°C).

Lipid class	Cultivation time, h	Percentage in oil
MAG	72	9.1
	96	20.0
DAG	72	3.6
	96	1.2
TAG	72	72.7
	96	63.5
FFA	72	14.6
	96	15.3

Results are means from three independent experiments

The major fatty acids in all the lipid classes were C18:1 and C16:0 which made 74%, 81%, 69% and 81% in MAG, DAG, TAG and FFA, respectively (Table 3). The characteristic fatty acids in TAG were γ-C18:3, MAG- and DAG-missed C20:0, and DAG-missed C20:1 and C22:0. The percentage of C14:0 and C16:1 was on a similar level in all the lipid classes. The highest percentage of C16:0 was in FFA, of C18:0 in TAG, of C18:1 in MAG and DAG, and of C18:2 and γ-C18:3 in MAG.

Table 3. Percent composition of fatty acids in monoacylglycerols (MAG), diacylglycerols (DAG), triacylglycerols (TAG) and free fatty acids (FFA) of *M. ramanniana* var. *angulispora* IFO 8187 oil produced in optimum growth conditions (pH 4.5, 30°C) after 96 h of cultivation.

Fatty acid	Percentage of fatty acids			
	MAG	DAG	TAG	FFA
C14:0	1.5	1.6	2.5	1.6
C16:0	22.3	30.5	27.7	35.9
C16:1	4.0	3.5	3.7	3.9
C18:0	4.9	5.8	7.8	4.9
C18:1	51.9	50.8	41.5	44.9
C18:2	10.9	7.0	7.5	6.1
γ-C18:3	4.6	0.9	2.5	1.9
α-C18:3	n.d.	n.d.	0.7	n.d.
C20:0	n.d.	n.d.	0.4	0.2
C20:1	0.3	n.d.	0.6	0.3
C22:0	1.7	n.d.	5.0	0.2

n.d. — not detected. Results are means from three independent experiments

DISCUSSION

The yield and composition of oil produced by microorganisms and the cell growth are interrelated, yet, no simple relationship exists between those variables [Ratledge, 1991]. This is so because a high yield of biomass with the ability for fattening that ensures high oil content has to be agreed with possibly the highest content of a desired metabolite within the oil. However, the yield of oil and the content of a desired metabolite are usually negatively correlated [Ratledge, 1991, 1993]. A compromise is then necessary that would satisfy the demands for a reasonably high content of both oil and metabolite. In searching the solution for this problem, the approach reported by Kennedy *et al.* [1993] sounds appealing.

The ability of *M. ramanniana* var. *angulispora* IFO 8187 for lipid production was studied herein in the physiologically permitted range of temperature (20–35°C) [Sumner *et al.*, 1969] and pH (3.0–6.0) [Nakahara *et al.*, 1992] in glucose medium optimized by Nakahara *et al.* [1992]. Opposite to the report of the Japanese authors [Nakahara *et al.*, 1992] we found that the optimum conditions for cell growth were the same as for oil accumulation, with the yield of biomass being about 5.7-fold higher than of oil. The yield of γ-C18:3 was also the highest under the optimum growth conditions (Figure 2), although, the oil percentage of γ-C18:3 was relatively low (Table 1). The yield of *M. ramanniana*-produced γ-C18:3 reported in the literature ranges from 8.1 mg/g to 39.4 mg/g dry cell weight [Yamada, 1990; Hansson & Dostálek, 1988; Stredanska & Šajbidor, 1992; Roux *et al.*, 1994]. The higher values in this range refer either to the conditions optimum for γ-C18:3 production or to strains with enhanced productivity of this fatty acid. In our study, the highest and most stable accumulation of γ-C18:3 in the oil was found after the cultivation at pH 4.0 and 30°C, when the biomass/oil yield ratio ranged from 4.8 to 5.2. Such ratio

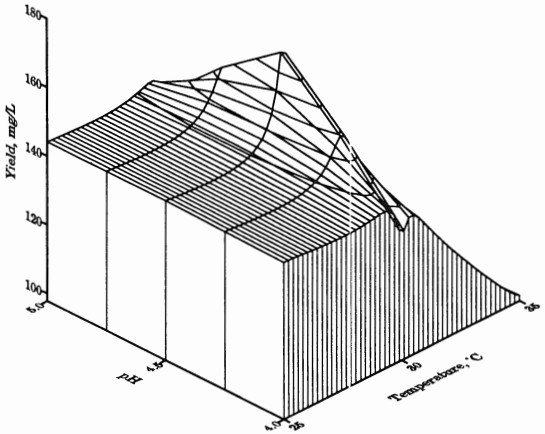

Figure 2. The effect of pH and temperature on the yield of γ-C18:3 after 96 h cultivation of *M. ramanniana* var. *angulispora* IFO 8187 in glucose medium. Results are means from three independent experiments.

values agree with those reported by Nakahara *et al.* [1992] for flask culturing of the fungus and should be attempted in the process of biosynthesis when γ-C18:3 is a desired metabolite.

The oil produced in optimum growth conditions was mainly in the form of TAG in which C18:1, C16:0 and C18:2 dominated, with the γ-C18:3 percentage being about 2-fold lower than in MAG, yet, about 3-fold higher than in DAG. The latter agrees with the findings by Kamisaka *et al.* [1990] who reported on the higher incorporation of γ-C18:3 into TAG than DAG.

Compared to the optimum conditions, lower pH and temperature of cultivation caused that the percentage of polyunsaturated fatty acids (C18:2, γ-C18:3) in the oil increased and that of C18:0 and minor fatty acids decreased. This agrees again with the opinion that more unsaturated oil is produced at lower growth temperature and lipid content of cell [Kates & Baxter, 1962; Sumner & Morgan, 1969; Hansson & Dostálek, 1988]. In the oil produced at the pH higher than the optimum pH, an about 2-fold increase in the percentage of minor fatty acids and their content variations during cultivation, were characteristic. This would suggest that in the conditions closed to the physiologically extreme values, the cell metabolism changes and the fatty acid synthesis and degradation processes become activated [Kates & Baxter, 1962].

Despite some differences, our results drive us to the same conclusions as those already reported in the literature regarding both general principles for oil biosynthesis and *M. ramanniana* characteristics.

CONCLUSIONS

The direct proportional relationship between the yields of biomass and oil, and the reverse proportional relationship between the yield of oil and its percentage of γ-C18:3, were observed under the optimum growth conditions (pH 4.5, 30°C) of *M. ramanniana* var. *angulispora* IFO 8187 cultured for 96 h in shake flasks in glucose medium. The values of pH and temperature lower than optimum growth conditions favoured the synthesis of γ-C18:3 by the fungus; thus they should be con-

sidered when the fermentation is to be optimized for the synthesis of this metabolite. The high yield of oil compensated for the low percentage of γ-C18:3 in the oil, for the yield of this fatty acid was the highest after the cultivation at the optimum growth conditions, which again needs a consideration when optimizing the process for production of the γ-C18:3-rich oil.

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WPŁYW WARUNKÓW HODOWLI NA WYDAJNOŚĆ I SKŁAD TŁUSZCZU GRZYBA STRZĘPKOWEGO *MORTIERELLA RAMANNIANA*.

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W celu sprawdzenia uzdolnień do syntezy kwasów tłuszczowych, w tym zwłaszcza kwasu γ-linolenowego (γ-C18:3), badano wpływ pH (4,0–5,0), temperatury (25°C–35°C) i czasu hodowli (48–96 h) grzyba strzępkowego *Mortierella ramanniana* var. *angulispora* IFO 8187 na wzrost i produkcję tłuszczu podczas hodowli wstrząsanych w pożywce z glukozą. Największą wydajność biomasy (22,4 g/L), tłuszczu (3,3 g/L) i kwasu γ-C18:3 (178,1 mg/L tj. 8,8 mg/g suchej biomasy) stwierdzono po 96 h hodowli w pH 4,5 i temp. 30°C (rys. 1, 2). Zmiany procentowego udziału kwasów tłuszczowych w tłuszczu były zależne od pH i czasu hodowli, a w mniejszym stopniu od temperatury (tab. 1). Największym wahaniom ulegała zawartość kwasów tłuszczowych dominujących w tłuszczu tj. C18:1 (35.0–45,3%), C16:0 (26,5–32,4%), C18:2 (8,0–13,7%) i γ-C18:3 (4,1–8,2%) (tab. 1). Frakcyjny skład tłuszczu wykazywał dominację triacylogliceroli (63,5-72,7%) i wolnych kwasów tłuszczowych (14,5–15,3%) (tab. 2). Udział monoacylogliceroli zwiększył się około dwukrotnie (9,1–20,0%), a diacylogliceroli zmniejszył się trzykrotnie (3,6-1,2%) w czasie ostatnich 24 h hodowli. Dominującymi kwasami tłuszczowymi wszystkich frakcji były C18:1 i C16:0 (tab. 3). Frakcja triacylogliceroli charakteryzowała się udziałem kwasu γ-C18:3, C20:0, C20:1 i C22:0.