SUNFLOWER PRESS CAKE AS A SUBSTRATE FOR EICOSAPENTAENOIC ACID PRODUCTION BY REPRESENTATIVES OF THE GENUS *MORTIERELLA*

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Long chain omega-3 fatty acids such as eicosapentaenoic acid (EPA) are essential for the regulation of critical biological functions in humans and other mammals. EPA production via solid state fermentation of sunflower press cake was investigated using eight fungal strains representing the genus *Mortierella*. During this study the effect of supplementing the sunflower press cake substrate with 10% (w/w) linseed oil was studied with regard to the supplement's impact on EPA production and the polyunsaturated fatty acid profile of the fermented substrate. The addition of the linseed oil improved the EPA yield of most strains, leading to a reduction in the average arachidonic acid:EPA ratio from 50.68 to 3.66. The ratio of polyunsaturated to saturated fatty acids was increased significantly (t=5.75, p=0.05) by the addition of linseed oil, with higher desaturation levels among the 20-carbon fatty acids. The strains that produced the highest levels of EPA on sunflower press cake supplemented with linseed oil were *Mortierella alpina* Mo 46 and *Mortierella basiparvispora* Mo 88, which produced 6.4 mg and 5.8 mg EPA per g of sunflower press cake, respectively.

Keywords: Eicosapentaenoic acid; Linseed oil; Mortierella; PUFA; Sunflower press cake

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INTRODUCTION

Eicosapentaenoic acid (EPA, C20:5n3) is a metabolically important omega-3 polyunsaturated fatty acid (ω-3 PUFA), which plays a pivotal role in the regulation of mammalian cardiovascular, digestive, immune, and neurological systems. Since mammals cannot synthesize adequate amounts of ω-3 PUFA by elongation and desaturation of shorter chain fatty acids, these PUFA have to be included in the diet (Dyal and Narine 2005). Currently the main dietary source of ω-3 PUFA is marine fish oil (Sijtsma and De Swaaf 2004). However, there is growing concern about the sustainability of this source of ω-3 PUFA oil, mainly because of increasing demand for fish oil by the aquaculture and pharmaceutical industries (Hasan et al. 2007; Certik and Shimizu 1999). Alternative sources of ω-3 PUFA oil are therefore being explored.

Recent research has focused on microbial and especially algal production of ω-3 PUFA as a sustainable and safe alternative to fish oil (Ward and Singh 2005). Representatives of the genus *Mortierella*, which belong to the group of oleaginous or "oil-bearing" fungi, have been identified as an alternative source of dietary PUFA. These fungi commonly occur in soil (Streekstra 1997) and are known to produce a range of microbial oils and PUFA, including EPA, gamma-linolenic acid (GLA, C18:3n6),

dihomo-gamma-linolenic acid (DGLA, C20:3n6), and the ω-6 arachidonic acid (ARA, C20:4n6), which has GRAS status (Zeller 2005). Interestingly, it was found that the precursor substrates of ω-3 EPA and ω-6 ARA are in competition for the same desaturase and elongase enzymes (Fig. 1). Also, cultivation conditions can be manipulated to control the ARA:EPA ratio e.g. more EPA is produced by addition of an ω-3 precursor or by lowering the incubation temperature.

Microbial PUFA-containing oils are usually produced by submerged fermentation within a liquid medium. However, it was demonstrated that the rate of PUFA production by *Pythium ultimum* was higher during solid state fermentation (SSF) than during submerged fermentation (Stredansky et al. 2000). Similarly, Jang et al. (2000) found that the specific yield of PUFA was higher during SSF, and it was stated by Pérez-Guerra et al. (2003) that these fermentation processes may have yields similar to or higher than the corresponding submerged fermentation processes.

Substrates used for SSF include agricultural products such as cereals, soybeans, and rice, but lignocellulosic residues and industrial or food-processing wastes are increasingly studied as potential substrates for this type of process (Cheng et al. 1999). The abundance of these relatively inexpensive byproducts presents an attractive alternative substrate to be used during SSF. The global agro-processing industry generates millions of tons of cereal derived byproducts annually. The South African beer brewing industry alone creates 520,000 tons of brewers' spent grain BSG) per year (http://www.sablimited.co.za). Volumes of lignocellulosic byproducts are expected to increase substantially in the near future as a result of the growing biofuel industry, which generates byproducts such as sunflower press cake (SPC).

Recently, BSG was evaluated as substrate for the production of EPA by solid state fermentation with a number of fungal strains representing different *Mortierella* species (Jacobs et al. 2009). It was found that all the strains were able to produce EPA on BSG and that the addition of a 10% (w/w) linseed oil (LSO), containing ~43% of the ω-3 precursor α-linolenic acid (ALA, C18:3n3), improved the EPA yield of most strains. However, the production of EPA by *Mortierella* strains grown on SPC using SSF has not been evaluated previously. Bautista et al. 1990 studied the lignocellulosic fraction of sunflower meal and concluded that it is a suitable fermentation substrate for fungal SSF. Consequently, the aim of this study was to evaluate SPC, supplemented with 10% (w/w) linseed oil (LSO), as substrate for the production of EPA by SSF, using fungal strains representing different species of the genus *Mortierella*.

EXPERIMENTAL

Fungal Isolates and Inoculum Preparation

A liquid inoculum was prepared from each of the eight strains representing the genus *Mortierella* listed in Table 1. These strains, originally isolated from soil, were obtained from the culture collection of the University of Stellenbosch, South Africa.

The inoculum medium contained (grams per litre): dextrose, 10.0; yeast extract, 5.0; NH_4Cl, 1.0; $MgSO_4.7H_2O$, 0.25; KH_2PO_4, 0.5; and $CaCl_2$, 0.05. Mycelium of each of the eight fungal strains was inoculated into baffled conical 250 mL flasks containing 50

mL inoculum medium and cultivated at 22°C for seven days with reciprocal shaking. Each inoculum was then aseptically homogenised (Colworth 400 Stomacher for two minutes) and the viability confirmed by determining the amount of viable colony forming units per volume of inoculum.

Substrate Preparation

Dried solid SPC was treated by one of two methods before inoculation. In both treatments 20g aliquots of substrate were distributed in conical flasks and water was added to a 70% moisture level. Ten percent (w/w) LSO was then added to half of the substrate treatments. The rest of the treatments were not supplemented with LSO before autoclave sterilisation.

Cultivation Conditions

Triplicate cultures, representing each isolate, were each inoculated with two milliliters of the homogenised inoculum for both LSO treatments. The inoculated cultures were incubated at 22^{o}C for three days to obtain optimal fungal growth. To enhance PUFA production the temperature was subsequently lowered to 16^{o}C and the cultures were incubated for a further eight days.

Analyses

All analyses and calculations were performed in triplicate on the total dried fermented substrate containing the fungal biomass. The cultures were harvested, homogenised (Colworth 400 Stomacher for two minutes), and the viability of the fungi in the biomass was confirmed by performing fungal counts by the pour plate method, using Potato Dextrose Agar (Oxoid CM 129) as enumeration medium. Fermented substrate was milled and oven dried (50^{o}C; 48 h), and the moisture content was determined by AACC Method 44-20 (http://www.aaccnet.org/approvedmethods/).

Total lipids, EPA, and ARA contents were determined on the complete dried fermented biomass. Lipids were first extracted from the fermented biomass samples by the Soxhlet method (using the Buchi B-811 Extraction system) with petroleum ether as the extraction solvent. Fatty acids were then derivatised to their fatty acid methyl esters (FAMES) using methanolic sodium hydroxide and a 14% boron trifluoride in methanol mixture. The FAMES were analysed by gas chromatography with flame ionisation detection. The instrument used was the GC 6890N from Agilent Technologies and separation was performed on a polyethylene glycol-based capillary column (Omegawax 320, 30 m x 0.32 mm ID with 0.25 µm film thickness, Supelco, Cat. No. 24152). Identification of FAMES was achieved by comparing their retention times with that of a reference 37-component FAME reference mixture (Supelco, Cat. No. 47885-U).

Experimental Design and Statistical Analyses

The experimental layout consisted of two treatments (either not supplemented or supplemented with LSO); each treatment comprised of a group of 24 cultures (containing eight triplicates), as well as three uninoculated substrate controls. To determine the significance of the differences between treatment means (Schefler 1979), a Student's t-

test on pairs of data sets was used (STATISTICA, ver. 7.1; Statsoft, Inc., Tulsa, OK, USA [www.statsoft.com]).

RESULTS AND DISCUSSION

The moistened SPC substrate is a highly suitable medium for fungal growth in terms of physical and nutritional composition. The lignocellulosic fibrous structure is porous and allows sufficient aeration, thereby providing an excellent inert support for fermentation. The results obtained when SPC was used as substrate for EPA production by different *Mortierella* strains using SSF are listed in Tables 1 and 2.

Table 1. Final Lipid Content as Produced by Representatives of the Genus *Mortierella* Grown on Sunflower Press Cake (SPC) with or without Linseed Oil (LSO) Supplementation

		Total lipids[a]			
Fungal Strain Number	Species	[a]SPC - LSO % (w/w)	% Decrease in lipids relative to SPC control[b]	[a]SPC + LSO % (w/w)	% Decrease in lipids relative to SPC control[b]
Mo 46	*Mortierella alpina*	7.50 (0.20)	7.28	13.37 (0.12)	9.27
Mo 47	*M. selenospora*	7.35 (0.51)	7.43	12.77 (0.06)	9.87
Mo 50	*M. alpina*	8.93 (0.12)	5.85	13.77 (0.83)	8.87
Mo 88	*M. basiparvispora*	7.70 (0.04)	7.08	13.50 (0.31)	9.14
Mo 101	*M. epicladia*	8.23 (0.26)	6.55	13.56 (0.51)	9.08
Mo 102	*Mortierella spp.*	10.37 (0.21)	4.41	16.32 (0.85)	6.32
Mo 114	*Mortierella spp.*	10.14 (0.19)	4.64	16.30 (0.30)	6.34
Mo 130	*Mortierella spp.*	9.86 (0.17)	4.92	15.60 (0.30)	7.04
SPC control	Un-inoculated	14.78 (0.21)	-	22.64 (0.82)	-

Values represent the mean of 3 repetitions
The standard deviation (SD) of each mean is indicated in parentheses
a: gram total lipid per 100g SPC with or without LSO supplementation
b: difference between "a" in unfermented SPC control and "a" in SPC sample fermented by fungal strain
LSO: linseed oil; SPC: sunflower press cake

Under conditions of carbon limitation in the growth substrate, oleaginous fungi can utilise lipids as an energy source for growth (Dyal and Narine 2005; Weber and Tribe 2003). When carbon in the SPC became depleted due to fungal growth, *Mortierella* isolates could therefore catabolise the SPC lipids, and this would explain the lower final levels of lipids in fermented SPC (mean 8.76% w/w, SD 1.23 $p<0.05$) compared to the non-fermented substrate (14.78% w/w) (Table 1). However, *Mortierella* isolates can also incorporate exogenously added oils e.g. LSO into their cells. In cultures where LSO was added to the substrate, the fermented SPC also had lower final lipid levels (mean 14.40% w/w, SD 1.43, $p<0.05$) than non-fermented SPC (22.64% w/w), indicating that the added LSO and accumulated lipids were catabolised as an energy source for cell growth (Weber and Tribe 2003).

Table 2. Final Arachidonic Acid and Eicosapentaenoic Acid Content as Produced by Representatives of the Genus *Mortierella* Grown on Sunflower Press Cake (SPC) with or without Linseed Oil (LSO) Supplementation

Fungal Strain Number	Species	Arachidonic acid			Eicosapentaenoic acid		
		[a]SPC -LSO (mg/g)	[a]SPC +LSO (mg/g)	% Increase ARA[b]	[a]SPC -LSO (mg/g)	[a]SPC +LSO (mg/g)	% Increase EPA[b]
Mo 46	*Mortierella alpina*	12.48 (0.47)	24.24 (1.12)	94.2	N/D -	6.44 (0.43)	-
Mo 47	*M. selenospora*	5.47 (0.21)	7.07 (0.88)	29.3	0.38 (0.04)	2.36 (0.26)	521.1
Mo 50	*M. alpina*	5.48 (0.85)	17.09 (3.55)	211.9	0.11 (0.01)	4.82 (0.94)	4281.8
Mo 88	*M. basiparvispora*	13.48 (1.28)	21.73 (1.44)	61.2	0.12 (0.03)	5.81 (0.58)	4741.7
Mo 101	*M. epicladia*	7.90 (0.30)	14.40 (2.33)	82.3	0.22 (0.02)	4.21 (0.64)	1813.6
Mo 102	*Mortierella spp.*	0.86 (0.16)	2.13 (0.43)	147.7	N/D -	0.50 (0.16)	-
Mo 114	*Mortierella spp.*	1.09 (0.15)	2.31 (0.35)	111.9	N/D -	0.58 (0.10)	-
Mo 130	*Mortierella spp.*	3.74 (0.23)	6.25 (0.21)	67.1	0.21 (0.01)	1.74 (0.06)	728.6
SPC control	Un-inoculated	N/D -	N/D -	-	N/D -	N/D -	-

Values represent the mean of 3 repetitions
The standard deviation (SD) of each mean is indicated in parentheses
a: mg fatty acid per g fermented SPC with or without LSO supplementation
b: % increase in mg fatty acid per g fermented SPC, due to LSO supplementation prior to fermentation
ARA: arachidonic acid; EPA: eicosapentaenoic acid; LSO: linseed oil; N/D: not detected; SPC: sunflower press cake
Limit of detection = 0.10%

Compared to previous results, obtained when similar experiments were conducted with brewers' spent grain (BSG) as substrate for SSF (Jacobs et al. 2009), SPC as substrate generally led to the production of higher levels of EPA. However, SPC lacks is lacking in the essential ω-3 precursor for fungal EPA production, i.e. ALA (Fig. 1).

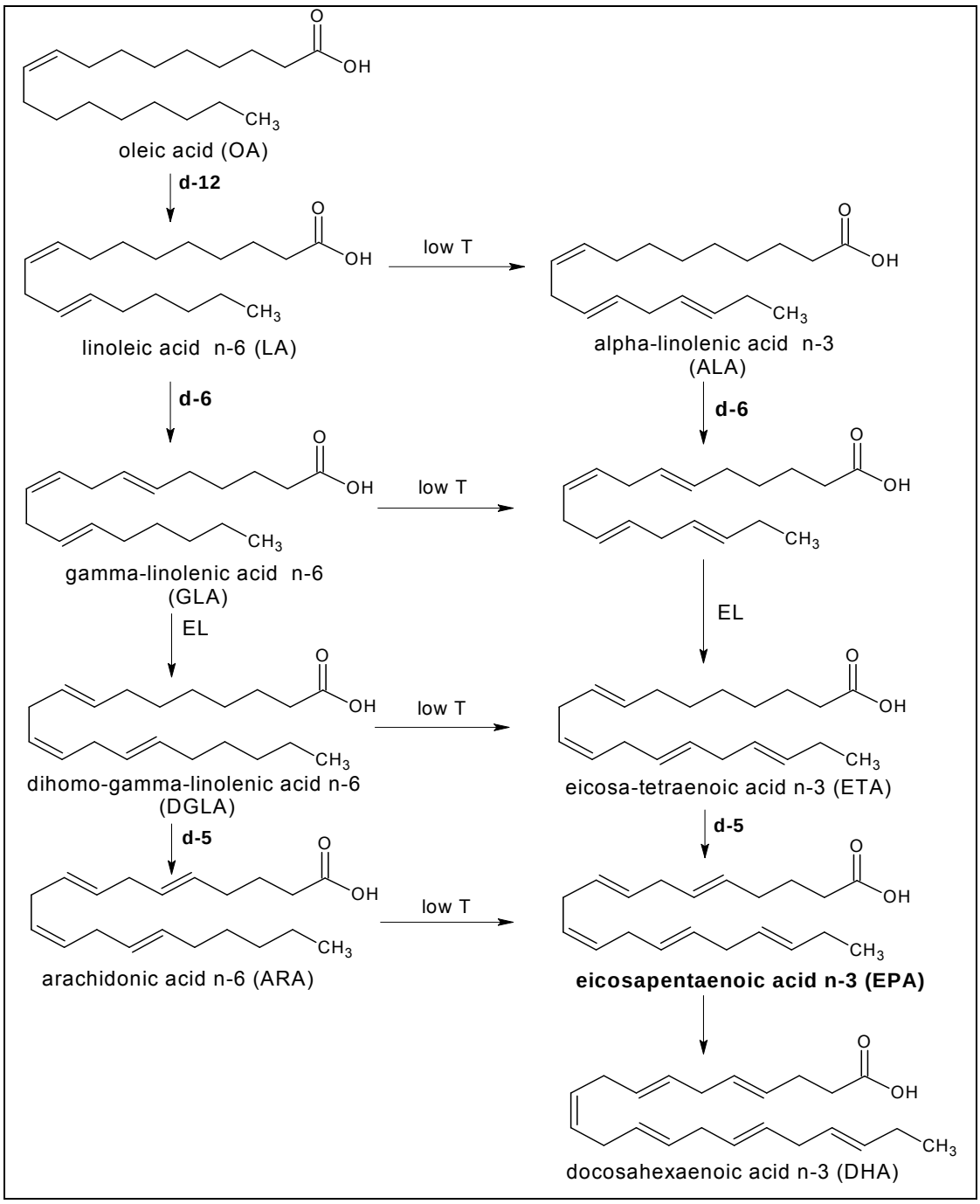

Fig. 1. Biosynthetic pathways of the production of poly-unsaturated fatty acids and EPA by eukaryotes (adapted from Certik and Shimizu 1999, and Pereira *et al*. 2004).
(T = temperature, d = desaturase, EL = elongase, ω = omega)

Representatives of the genus *Mortierella* can utilise, incorporate, and modify exogenously added oils in their cells (Certik et al. 1998), and therefore LSO containing the ALA precursor was added to SPC used as substrate in the present study. It was found that addition of 10% (w/w) LSO significantly ($p<0.05$) increased the amount of EPA

produced by the isolates (Table 2). This supports previous findings using BSG supplemented with LSO as substrate for EPA production (Jacobs et al. 2009), as well as the results of Jang et al. (2000), who found a 92% increase in EPA production when 1% LSO was added to solid rice bran substrate. When LSO was added to the substrate, SPC cultures of *M. alpina* Mo 46 and *M. basiparvispora* Mo 88 had the highest EPA content (6.44 and 5.81 mg/g SPC respectively).

In the non-fermented SPC substrate as well as in the added LSO, the levels of both ARA and EPA were below the analytical detection limit (<0.1% of total fatty acids) so that the ARA:EPA ratio could not be determined. SPC contains higher levels of the ω-6 precursor, i.e. linoleic acid (LA, C18:2n6) than the ω-3 precursor (ALA) (Maina et al. 2003), therefore cultures prepared with the *Mortierella* strains growing on SPC were characterised by a relatively high ARA:EPA ratio. Also, it is known that the Δ6 desaturase responsible for the formation of the next double bond in the desaturation of a C18:2 fatty acid (Fig. 1), shows substrate preference for the ω-6 precursor, leading to the formation of higher levels of ARA than EPA (Certik et al. 1998). The addition of LSO raises the level of the ω-3 precursor in the substrate and subsequently increases the level of EPA produced (Jang et al. 2000). In our study, this was reflected in the significant ($p<0.05$) reduction in the average ARA:EPA ratio from 50.68 (SD=55.05) to 3.66 (SD=0.39).

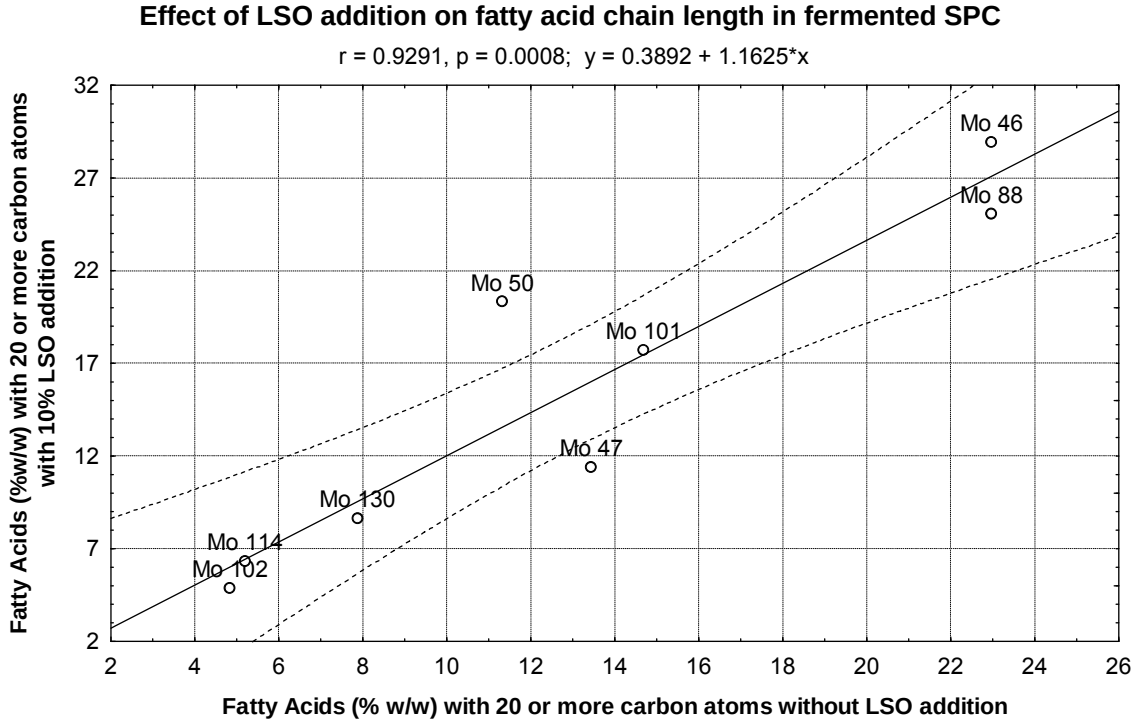

Fig. 2. Correlation of the percentage of long chain (C = 20 or more) fatty acids, relative to total fatty acids, produced by eight fungal isolates grown on sunflower press cake (SPC) with or without addition of linseed oil (LSO)
The long chain fatty acids with 20 or more carbon atoms include: C20:0, C20:1n9, C20:3n6, C20:3n3, C20:4n6, C20:5n3, C22:0, C22:1n9, C24:0 and C24:1n9
Confidence interval represented by the dotted lines = 95%

To further investigate the mechanism whereby added LSO was utilised to enhance EPA production in the *Mortierella* cultures, the effect of LSO addition on net elongation and desaturation levels of cellular long-chain fatty acids was determined.

It was found that the ratio of shorter to longer chain fatty acids was lower for all isolates which produced relatively higher levels of EPA and ARA, i.e. Mo 46, Mo 47, Mo 50, Mo 88 and Mo 101. The production of substantial amounts of EPA and ARA corresponded with higher total levels of fatty acids with chain lengths of more than 20 carbon atoms, indicating that relatively more long chain fatty acids were produced by these isolates (Fig. 2). LSO addition had a positive effect ($r = 0.9291$, $p < 0.05$) on the relative amount of long chain fatty acids produced.

The effect of LSO addition on saturation of cellular long-chain fatty acids within the SPC fermented with *Mortierella* cultures was also determined. The ratio of PUFA, containing two or more double bonds, to saturated and monoenoic fatty acids was firstly determined among the 18-carbon fatty acids only. Desaturation levels of cultures with and without additional LSO did not differ significantly in this group (results not shown; $r = 0.3069$, $p = 0.460$), although high levels of 18-carbon PUFA (ALA, C18:3n3) had been introduced to the cultures by addition of LSO. This indicated that the 18-carbon ALA was effectively metabolised by the cultures. Cultures in which the level of ALA was reduced relative to the unfermented SPC substrate showed a corresponding increase in EPA levels and were therefore capable of converting ALA to EPA effectively (Fig. 3).

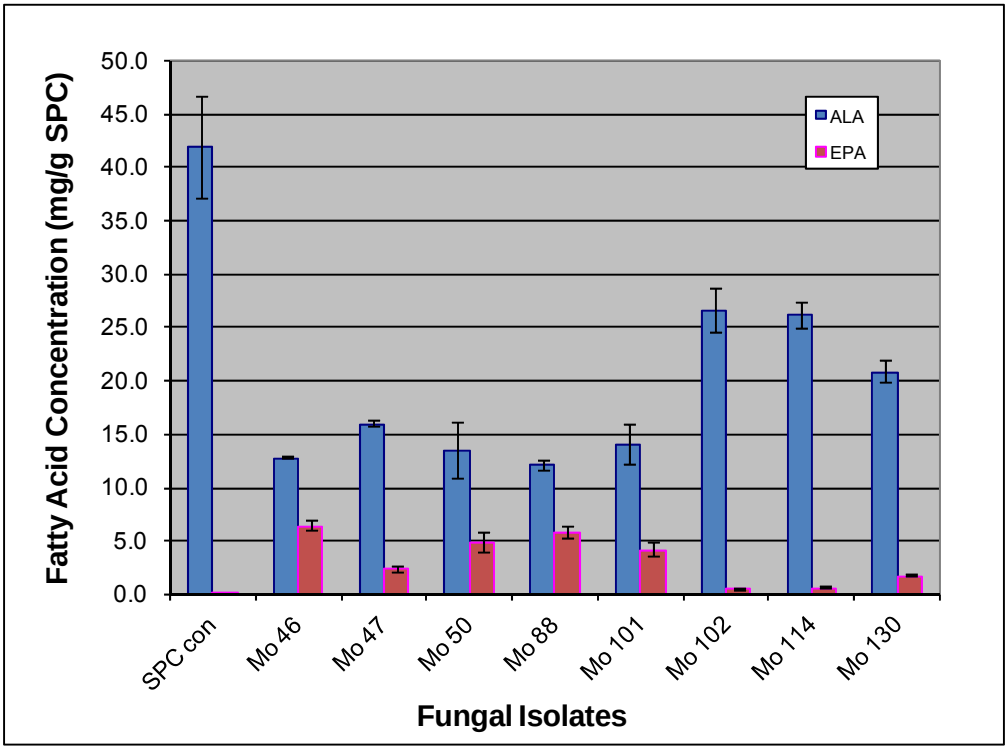

Fig. 3. The effect of fungal fermentation on the levels of the ω-3 fatty acids, ALA and EPA, in SPC supplemented with LSO before fermentation
(ALA: α-linolenic acid; EPA: eicosapentaenoic acid; LSO: linseed oil; SPC: sunflower press cake)

The desaturation ratios were then also determined among the 20-carbon fatty acids. Some of the products of fungal metabolism of ALA would have been EPA and other 20-carbon PUFA, since cultures to which LSO was added were characterised by higher desaturation levels among their 20-carbon PUFA than cultures that received no LSO (results not shown; r = 0.914, p = 0.002). It is therefore evident that the fungal Δ6 and Δ5 desaturase and elongase enzymes (Fig. 1) successfully incorporated and converted the ALA in the added LSO to C20 PUFA such as EPA and ARA. To illustrate the difference in final fatty acid profiles, an example of the profiles of SPC fermented by Mo 46, with and without LSO supplement, is presented as the overlay of chromatograms in Fig. 4. The increase in ALA, ARA, EPA and other long chain fatty acid levels due to the addition of LSO is evident in this chromatogram.

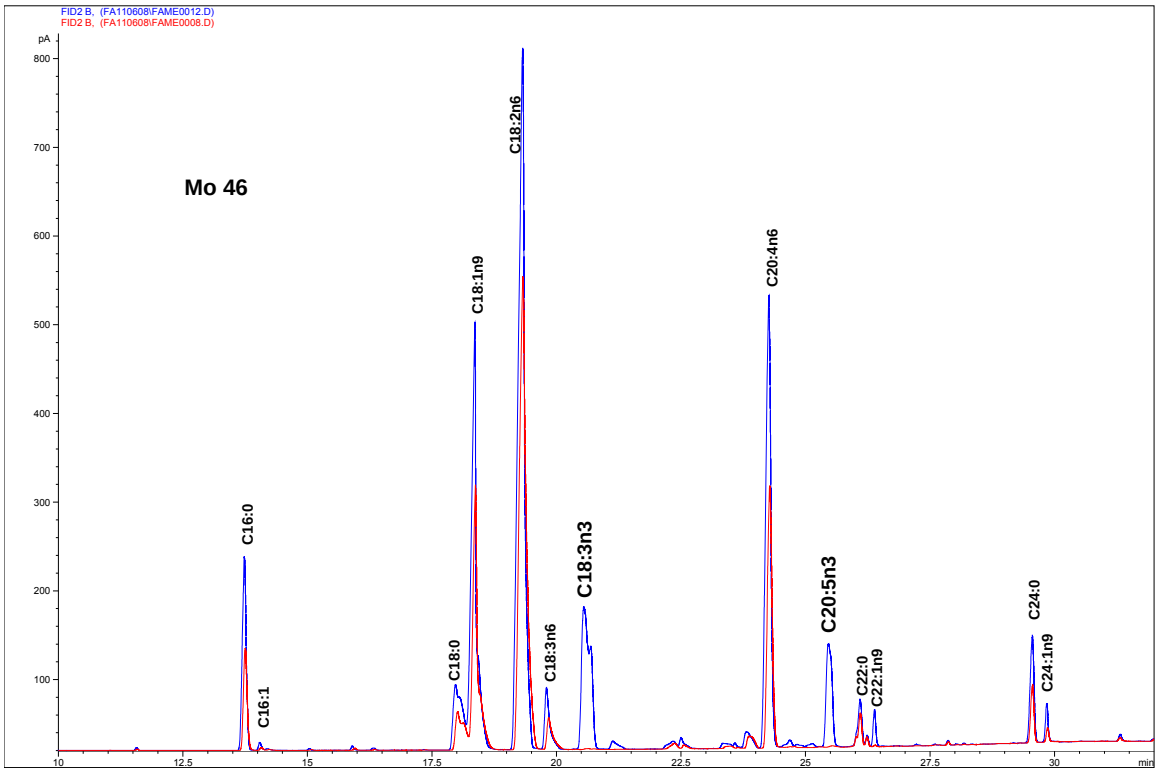

Fig. 4. Combined chromatogram representing the FAMES in SPC on which *Mortierella alpina* Mo 46 was grown, without (red) and with (blue) LSO supplementation.
(C18:3n3: α-linolenic acid; C20:4n6: arachidonic acid; C20:5n3: eicosapentaenoic acid; FAMES: fatty acid methyl esters; LSO: linseed oil; SPC: sunflower press cake)

The findings mentioned above confirmed the reports that most *Mortierella spp.* are able to utilise exogenously added lipids to produce PUFA by desaturation and elongation of the fatty acids of the added oils (Certik et al. 1998).

During our study fermentation with *Mortierella* fungi produced ARA as well as a range of other PUFA, including EPA, GLA, and DGLA (Fig. 1). This corresponded with reports of previous studies (Sakuradani et al. 2005) and also confirmed findings obtained by SSF of brewers' spent grain as an alternative substrate (Jacobs et al. 2009).

The challenge in fungal PUFA production is to modify the lipid composition of the product to contain higher levels of the desired PUFA (Certik and Shimizu 1999). EPA is regarded as a valuable neutraceutical and has several clinical applications e.g. as a potential anti-inflammatory agent (Wen and Chen 2005). On the other hand, the production of infant formula requires pure ARA (and docosahexaenoic acid) and for this purpose the presence of EPA is considered undesirable (Ratledge 2005). The precursor substrates of EPA and ARA are in competition for the same desaturase and elongase enzymes, but cultivation conditions can be manipulated to control the ratio of ARA:EPA produced during fermentation (Cheng et al. 1999).

In this study the amount of the ω-3 EPA was successfully increased relative to the amount of ω-6 ARA produced. This was achieved by the addition of LSO, containing the ω-3 precursor, as well as by cultivation at the lower temperature of 16^{o}C. These measures increased the enzyme activity along the ω-3 biosynthetic pathway and led to the formation of higher levels of EPA.

CONCLUSIONS

1. Fungi from the genus *Mortierella* can successfully convert linseed oil supplemented lignocellulose-rich wastes, such as sunflower press cake (SPC), to materials containing polyunsaturated fatty acids, thereby adding value to these substrates.
2. Fungal strains showing the most potential were *Mortierella alpina* Mo 46 and *M. basiparvispora* Mo 88, producing the highest eicosapentaenoic acid (EPA) content of 6.44 and 5.81 mg EPA/g SPC respectively. These isolates are currently being investigated in larger scale trials for the production of omega-3-enriched agroprocessing byproducts.
3. The SPC enriched with polyunsaturated fatty acids, which are produced in this manner, could eventually find applications as animal or fish feed or as a source of EPA and ARA for the growing ω-3 market in the neutraceutical and therapeutics industries.

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