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The production of eicosapentaenoic acid by representatives of the genus *Mortierella* grown on brewers' spent grain

Annali Jacobs[1], Alf Botha[2] & Willem H. Van Zyl[2]

[1] *CSIR Biosciences, P O Box 395, Pretoria 0001, South Africa; e-mail: ajacobs@csir.co.za*
[2] *University of Stellenbosch, Private Bag x1, Stellenbosch 7602, South Africa*

Abstract: Brewers' spent grain (BSG) was evaluated as substrate for the production of eicosapentaenoic acid (EPA) by solid-state fermentation with 29 fungal strains representing different *Mortierella* species. The effect of a 10% (w/w) linseed oil (LSO) supplement on EPA production was also determined. All the strains produced EPA on the substrate, while addition of the LSO improved the EPA yield of most strains. The strains producing the most EPA in the absence of additional LSO generally also produced the most EPA when LSO was added to the BSG. The strains, which produced the highest levels of EPA on BSG supplemented with LSO were *Mortierella antarctica* Mo 67 and *Mortierella epicladia* Mo 101, which respectively produced 2.8 mg and 2.5 mg EPA per g of BSG.

Key words: eicosapentaenoic acid; *Mortierella*; spent grain; HUFA; linseed oil.

Abbreviations: BSG, brewers' spent grain; EPA, eicosapentaenoic acid (C20:5 n-3); HUFA, highly unsaturated fatty acids; LSO, linseed oil; SSF, solid-state fermentation.

Introduction

Highly unsaturated fatty acids (HUFA) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid are essential to the regulation of the immune, cardiovascular, digestive and neurological systems in mammals (Qiu 2003; Sijtsma & de Swaaf 2004). As humans cannot synthesise adequate levels of HUFA from precursor fatty acids, long chain ω-3 HUFA have to be included in the diet (Certik & Shimizu 1999; Zhu 2002; Dyal & Narine 2005;). The main dietary source of ω-3 HUFA is marine fish oil (Dyal & Narine 2005; Ward & Singh 2005), but recent research has focused on microbial production of ω-3 HUFA mainly by marine organisms as an alternative sustainable and safe source (Ward & Singh 2005).

Several terrestrial microorganisms can produce lipids and HUFA, accumulating up to 80% of their biomass as lipids. Microorganisms that are able to accumulate over 25% lipid on a dry cell biomass basis, are called oleaginous organisms (Higashiyama et al. 2002; Dyal & Narine 2005). This study focused on HUFA production by saprophytic filamentous soil fungi belonging to the orders Mucorales and Mortierellales (Cavalier-Smith 1998; Dyal & Narine 2005). The oil produced by these fungi has the GRAS (generally recognised as safe) status (Cohen & Ratledge 2005) and *Mortierella* species have found industrial applications in the production of food enzymes, oils, β-carotene and other food-related products (Streekstra 1997).

Solid-state fermentation (SSF) processes involve growth of microorganisms on moist solid substrates to produce valuable microbial metabolites in the absence of free-flowing water. These processes may have yields similar to or higher than the corresponding submerged fermentation processes (Pèrez-Guerra et al. 2003). Microbial HUFA-containing oils are usually produced by liquid fermentation. Stredansky et al. (2000) showed that the rate of lipid and HUFA production by *Pythium ultimum* was higher during SSF than during submerged fermentation, while Jang et al. (2000) found that the specific yield of HUFA was higher during SSF. Despite this, the use of SSF for the production of HUFA has received less attention than submerged fermentation, due to technical problems, such as contamination control, scaling-up and lipid extraction (Zhu 2002). Traditionally agricultural products like cereals, soybeans or rice have been used as solid substrates, but the use of inexpensive agricultural, industrial or food-processing wastes is attracting increasing attention among researchers (Cheng et al. 1999; Stredansky et al. 2000).

The South African beer brewing industry produces approximately 290 000 tons of brewers' spent grain (BSG) per year (http://www.sablimited.co.za/). The unfermented substrate contains ~19% crude protein and has a total oil content of ~9% of which half is linoleic acid (C18:2 n-6), a precursor for the ω-6 HUFA (A. Jacobs, unpublished results). To enhance ω-3 HUFA production by oleaginous microorganisms on substrates such as BSG, an oil rich in the ω-3 precursor could to be added to the substrate before fermentation (Shimizu et al. 1989; Jang et al. 2000; Dyal & Narine 2005). Linseed

oil (LSO), which contains ∼57% of the ω-3 precursor α-linolenic acid (C18:3 n-3), could be considered as a supplement.

An important factor in the biotechnological production of fungal lipids is the incubation temperature. This affects the growth rate and other metabolic activities, such as substrate consumption and pH changes, as well as the production of HUFA. Lindberg & Molin (1993) found that at lower incubation temperatures*Mortierella alpina* produced lipids with a higher degree of unsaturation, as well as a higher EPA content. Zhu (2002) utilised the "temperature shifting" technique for production of EPA by *Pythium*. Higher yields of EPA (ω-3), at the expense of arachidonic acid (ω-6) yields, were obtained with fungal cultures that were first incubated at 25 °C until nutrients such as the nitrogen source (Bajpaj et al. 1991) were depleted, and then incubated at 12 °C, compared to cultures that were only incubated at the higher temperature. Similarly, Jang et al. (2000) increased *Mortierella alpina* production of total HUFA and EPA by 12.0% and 84.4%, respectively, by shifting the culture temperature from 20 °C to 12 °C on the fifth day of SSF cultivation. This temperature shift activates the desaturase enzymes catalysing the conversion of arachidonic acid to EPA in oleaginous fungi (Zhu 2002; Cohen & Ratledge 2005).

The production of EPA by *Mortierella* strains during SSF with BSG as sole substrate has not been evaluated. SSF studies by Jang et al. (2000), Stredansky et al. (2000), and Slavikova & Certik (2005) made use of other fungal species and substrates or combinations of substrates.

With the above as background, the aim of this study was to employ the "temperature shifting" technique in the evaluation of BSG as the sole substrate for the production of ω-3 HUFA, i.e. EPA, using fungal strains representing different species of the oleaginous genus *Mortierella.* In addition, the effect of a LSO supplement on EPA production was determined while these fungi were used in SSF of the spent grain.

Material and methods

Microorganisms and inoculum preparation

A liquid inoculum was prepared from each of 29 strains representing the genus *Mortierella* listed in Table 1. Twenty-eight of these strains were obtained from the culture collection of Stellenbosch University, South Africa, while the other one was a reference HUFA-producing strain, *Mortierella alpina* ATCC 32223, obtained from the American Type Culture Collection (http://www.atcc.org/).

The inoculum medium contained (g/L): dextrose, 10.0; yeast extract, 5.0; NH_4Cl, 1.0; $MgSO_4.7H_2O$, 0.25; KH_2PO_4, 0.5; $CaCl_2$, 0.05. Mycelium of each of the 29 fungal isolates 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 2 min) and the viability thereof was confirmed by determining the amount of viable colony forming units per volume.

Substrate preparation

Aliquots of 20 g dried BSG were distributed in conical flasks and 80 mL water were added. Dried spent grain was then treated by one of the following two methods before inoculation: 10% (w/w) LSO was added to half of the substrate treatments whereas the rest of the treatments were not supplemented with LSO before autoclave sterilisation.

Cultivation conditions

Duplicate samples (containing 20 g BSG) of each isolate were inoculated with 2 mL of the homogenised inoculum for both LSO treatments. Fermenting BSG was incubated at 22 °C for three days to obtain optimal fungal growth and the temperature was then lowered to 16 °C for the following eight days to enhance HUFA production.

Analyses

All analyses and calculations were performed in duplicate on the total dried fermented BSG substrate containing the fungal biomass. The cultures were harvested, homogenised (Colworth 400 Stomacher for 2 min) and the viability of the fungal biomass was confirmed by performing fungal counts with the pour plate method, using Potato Dextrose Agar (Oxoid CM 129) as enumeration medium. Fermented substrate was milled and oven dried (50 °C, 48 h). The moisture content was determined by AACC Method 44–20.

The total lipids and EPA content of the dried fermented BSG biomass were determined according to the method of Folch et al. (1957). Lipids were extracted from the fermented biomass using a 2:1 (v/v) mixture of chloroform:methanol and weighed. Lipids were then derivatised to methyl esters with methanolic NaOH solution, followed by addition of BF_3/methanol complex. The fatty acid methyl esters were analysed by gas chromatography with flame ionisation detection. The instrument used was a Varian 5890 Series 11 Gas Chromatograph and separation was performed on a polyethylene glycol-based capillary column, (30 m × 0.32 mm ID Omegawax 320 with 0.25 μm film thickness, Supelco, Catalogue Number 24152). Peaks were identified by using a reference 37 component fatty acid methyl ester mixture (Supelco, Catalogue Number 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 58 cultures (plus 2 un-inoculated cultures) containing 29 duplicates (and one duplicate representing un-inoculated BSG). To determine the significance of the differences between treatment means (Schefler et al. 1979), a Student's *t*-test on pairs of data sets was used (STATISTICA, version 7.1; Statsoft, Inc., Tulsa, Oklahoma, USA; http://www.statsoft.com/). Using the same software correlation matrices were constructed to determine the relationship between the lipid content, the EPA content, as well as the % increase in EPA and lipid content after addition of LSO.

Results and discussion

Due to their physiological, enzymological and biochemical properties, filamentous fungi are well adapted to SSF of cereal by-products (Perez-Guerra et al. 2003). It is known that apart from the addition of LSO as the EPA precursor, several other factors such as incubation temperature (Dyal & Narine 2005), carbon to

Table 1. Lipid content and EPA produced by representatives of the genus *Mortierella* while growing on BSG with or without LSO supplementation.

Fungal strain No.	Species	Total lipids[a]			Eicosapentaenoic acid[b]		
		BSG – LSO % (w/w)[c]	BSG + LSO % (w/w)[c]	% Increase lipids	BSG – LSO (mg/gb)[c]	BSG +LSO (mg/g)[c]	% Increase EPA
Mo 018	*M. kuhlmanii*	6.2	11.7	5.5	0.38	0.92	142.1
Mo 031	*M. sclerotiella*	4.6	15.9	11.3	0.23	0.71	208.7
Mo 035	*M. vinacea*	4.5	9.4	4.9	0.02	0.96	4700.0
Mo 038	*M. alpina*	7.3	17.4	10.1	0.16	0.24	50.0
Mo 046	*M. alpina*	4.9	9.2	4.3	0.46	1.68	265.2
Mo 047	*M. selenospora*	5.4	11.0	5.6	0.72	1.11	54.2
Mo 050	*M. alpina*	5.8	9.2	3.4	0.56	1.16	107.1
Mo 059	*M. antarctica*	5.9	13.9	8.0	0.22	0.85	286.4
Mo 063	*M. horticola*	4.5	9.1	4.6	0.17	0.69	305.9
Mo 064	*M. sarnyensis*	8.2	9.8	1.6	0.12	0.09	−245.0
Mo 066	*M. basiparvispora*	6.6	11.9	5.3	0.10	0.18	80.0
Mo 067	*M. antarctica*	3.3	9.4	6.1	0.33	2.77	739.4
Mo 070	*M. basiparvispora*	7.4	17.3	9.9	0.28	0.69	146.4
Mo 073	*M. alpina*	2.1	8.9	6.8	0.18	1.20	566.7
Mo 074	*Mortierella* spp.	5.4	9.9	4.5	0.57	2.11	270.2
Mo 077	*M. alpina*	5.3	9.9	4.6	0.10	0.63	530.0
Mo 080	*M. dichotoma*	5.8	14.6	8.8	0.29	1.10	279.3
Mo 081	*M. parvispora*	6.8	13.2	6.4	0.22	0.59	168.2
Mo 083	*M. sarnyensis*	2.3	10.7	8.4	0.10	1.07	970.0
Mo 084	*M. epicladia*	5.3	7.1	1.8	0.14	0.90	542.9
Mo 088	*M. basiparvispora*	5.5	7.2	1.7	0.34	1.48	335.3
Mo 089	*Mortierella* spp.	6.2	11.7	5.5	0.01	0.03	200.0
Mo 092	*M. antarctica*	4.2	4.8	0.6	0.52	1.97	278.8
Mo 094	*Mortierella* spp.	6.4	12.7	6.3	0.33	0.69	109.1
Mo 099	*M. angusta*	6.4	12.5	6.1	0.18	0.06	−66.7
Mo 101	*M. epicladia*	4.3	10.7	6.4	0.43	2.53	488.4
Mo 102	*Mortierella* spp.	4.2	10.9	6.7	0.41	1.96	378.0
Mo 114	*Mortierella* spp.	5.3	9.9	4.6	0.34	1.52	347.1
ATCC 32223	*M. alpina*	7.1	10.6	3.5	0.44	1.36	209.1
BSG control	Un-inoculated	6.7	14.4	7.7	ND	0.24	-

[a] Gram lipid per 100g oven dried fermented BSG biomass. [b] Milligram EPA per g oven dried fermented BSG biomass. [c] Values are the average of duplicate values. ND, not detected at levels above detection limit (0.1%).

nitrogen ratio (Jang et al. 2000) and cultivation process (Stredansky et al. 2000) influence the production of EPA, which may vary between species. The fact that all 29 *Mortierella* strains investigated were able to grow and produce EPA indicates that a suitable combination of fermentation parameters was selected for this study (Table 1).

An important factor impacting on growth of *Mortierella* is the level of aeration of the cultures. Lindberg & Molin (1993) found that doubling the air-flow rate of *Mortierella alpina* also doubled the biomass and lipid production rates in submerged fermentation. During our own SSF experiments, growth first appeared on the surface of the BSG, indicating that aeration could improve growth for most of the fungal isolates. According to Conti et al. (2001) spent malt grains are not rich in nutrient substances, but are porous and allow better aeration than other moistened cereals. It provides an excellent inert support for fermentation.

Except for aeration, successful cultivation by SSF also depends on factors such as moisture content, layer thickness, incubation temperature and availability of nutrients in the substrate (Emelyanova 1996; Stredansky et al. 2000; Conti et al. 2001). Optimum substrate nutrient composition, as well as optimum growth conditions, could however vary dramatically between representatives of the genus *Mortierella* (Dyal & Narine 2005). This could have led to the wide range in lipid content of the BSG fermented by the different strains (Table 1).

Lipids are used as energy source during the exponential growth phase of oleaginous fungi. When nutrients such as nitrogen become depleted in the growth medium, a metabolic shift occurs and fungal cells switch from growth to lipid accumulation (Koike et al. 2001; Zhu 2002; Dyal et al. 2005). Higashiyama et al. (1999) found that in submerged fermentation with *M. alpina* intracellular lipid accumulation commences after the initial 2-day period of rapid fungal growth. However, the rate at which this occurs and the balance between utilisation and accumulation of lipids vary between fungal strains. Thus, depending on the fungal strain used and its growth phase, the lipid content either decreases as lipids are utilised as energy source for cell growth, or increases as lipids accumulate in the biomass. These characteristics of oleaginous fungi may also contribute to the wide range in lipid content of the BSG fermented by the different strains, observed in Table 1.

For BSG without additional LSO as substrate, the highest lipid content was obtained with *M. sarnyensis* Mo 064 (8.2%, w/w), while the lowest lipid content was found in the BSG fermented with *M. alpina* Mo 073

(2.1%, w/w) (Table 1). Addition of 10% LSO (w/w) to the BSG before sterilization only increased the lipid content of the autoclaved substrate by 7.7% (Table 1). This apparent anomaly could be explained by the partial hot extraction of lipids during the sterilization process. It must also be noted that since the fungal biomass could not be separated from the fermented substrate, it was impossible to determine how much of the LSO was assimilated by the fungal biomass and how much was absorbed to the BSG during the subsequent fermentation process.

In our study, cultures of *M. sclerotiella* Mo 031 accumulated the most lipids during fermentation of LSO supplemented BSG (Table1). The lipid content of these cultures was found to be 11.3% more than cultures of this strain growing on BSG without the LSO supplement. The cultures prepared with BSG supplemented with LSO that had the lowest lipid content, were those of *M. antarctica* Mo 092. The lipid content of these cultures was lower than the control supplemented with LSO and only 0.6% more than cultures of this strain growing on BSG without the LSO supplement. As lipid in the non-fermented BSG increased by 7.7% when LSO was added (Table 1), cultures prepared with LSO supplemented BSG displaying a lower increase after incubation, utilised more LSO for growth than for lipid accumulation. The only cultures that showed a net increase in lipids during incubation were those of *M. sclerotiella* Mo 31, *M. alpina* Mo 38, *M. antarctica* Mo 59, *M. basiparvispora* Mo 70, *M. dichotoma* Mo 80 and *M. sarnyensis* Mo 83. These results are in agreement with the findings recorded in literature. It is known that *Mortierella* isolates can efficiently utilise, incorporate and modify exogenously added oils, e.g. LSO in their cells (Certik et al. 1999), but vary in the amount of lipid produced between and within species. The total lipid content is important (Dyal & Narine 2005), but the major challenge is to change the final composition of the lipid fraction to contain a higher percentage of longer chain fatty acids with a higher degree of unsaturation (Certik et al. 1999; Jang et al. 2000).

When LSO enriched BSG was used as substrate to during our study, only three strains (*M. sarnyensis* Mo 064, *Mortierella* spp Mo 089, and *M. angusta* Mo 099) produced EPA at quantities close to the lowest analytical detection limit (0.1% EPA). The results obtained for these three strains should therefore be considered as insignificant. However, taking into account all the results on the EPA content of cultures listed in Table 1, it was found that EPA content was significantly ($p < 0.05$) more when the BSG substrate was supplemented with LSO (1.08 mg/g BSG; SD = 0.71), than in the absence of the supplement (0.29 mg/g; SD = 0.18). Thus, compared to cultures grown in the absence of additional LSO (Table 1), addition of LSO to the BSG resulted in a mean increase of 436.8% in the average EPA content of the fermented biomass (SD = 850.8; SE = 160.6; $t = 2.470$; $P = 0.02$). This confirms previous work by Shimizu et al. (1989) who found that strains of the genus *Mortierella* produced higher levels of EPA when 1% LSO was added to the liquid fermentation medium. Jang et al. (2000) found a 92% increase in EPA production when 1% LSO was added to solid rice bran substrate.

Cultures of *M. antarctica* Mo 067 had the highest EPA content (2.77 mg/g BSG), which represented an increase of 739.4% with the addition of the LSO supplement (Table 1). However, this EPA content is still lower than obtained by other workers with similar experiments (Jang et al. 2000; Stredansky et al. 2000; Slavikova & Certik 2005). Studies by Stredansky et al. (2000) using *Pythium ultimum* to ferment pearled barley supplemented with 5.75% LSO yielded 3.5 mg EPA/g wet substrate. Spent malt grain was added to the substrate to improve porosity of the solid substrate. Similarly, Jang et al. (2000) fermented several solid substrates with *M. alpine*. The solid substrates were also supplemented with nitrogen sources such as yeast extract and nitrates. Rice bran fermentation produced ∼6 mg EPA/g substrate and production was increased to ∼11 mg EPA/g by the addition of 1% LSO. Better results were also obtained by Slavikova & Certik (2005) who fermented mixed solid substrates containing peeled barley, LSO and spent malt grain (0.5:1:3 w/w) with *M. alpina* and produced 23.4 mg EPA/g substrate.

The low yield in EPA obtained during our study with BSG as substrate indicates that this substrate is not rich in nutrients (Conti et al. 2001) or that the nutrients are not in a form readily available to the majority of the fungi tested. Addition of nitrogen prior to fermentation could improve the growth and subsequent EPA production of the fungi on the substrate (Jang et al. 2000). HUFA production correlates positively with fungal growth (Ward & Singh 2005), but as lipid and HUFA production are triggered by nitrogen limitation (Koike et al. 2001), the nitrogen added should preferably be fully utilised during the exponential growth phase.

In general, no correlation was observed between the EPA content and the lipid content of cultures without LSO addition listed in Table 1. This may perhaps be partly ascribed to the interspecific diversity in the activity of the Δ6–desaturase enzymes catalysing the desaturation of the C18 fatty acids. These enzymes have a substrate preference for the ω-6 precursor, C18:2 n-6, above the ω-3 precursor, α-linolenic acid (C18:3 n-3) (Cohen & Ratledge 2005). The fungi therefore synthesise ω-6 arachidonic acid (C20:4 n-6) more readily than the corresponding ω-3 EPA (C20:5 n-3). Since the ω-6 precursor (C18:2 n-6) is the major fatty acid in the lipids of non-supplemented BSG, it is not surprising that the isolates did not produce excessive amounts of the ω-3 HUFA, EPA, on this substrate.

The increase in EPA content of the fermented substrate that was observed when BSG was supplemented with LSO, did not correlate with lipid or EPA levels on non-supplemented BSG, indicating that the isolates differ in their utilisation, incorporation and conversion of the exogenously added ω-3 rich oil. This variation could be due to different levels and/or rates of production of

fatty acid desaturase and elongase enzymes required for the production of EPA from LSO. However, the EPA content of cultures grown on BSG supplemented with LSO showed a positive correlation with the EPA content of cultures grown on BSG without the LSO supplement ($r = 0.617$; $p = 0.000$), indicating that LSO supplementation proportionally increased the EPA content of cultures (Fig. 1A). Addition of LSO to the BSG created a substrate containing relatively more of the ω-3 than the ω-6 precursor. This may have resulted in partial redirection of the enzymatic fatty acid desaturation and elongation reactions along the ω-3 synthesis pathway towards the formation of EPA. Although the arachidonic acid content was not monitored, it could be assumed that the arachidonic acid:EPA ratio in the fermented biomass was lowered by the addition of LSO.

A negative correlation existed between the EPA content of cultures grown on BSG supplemented with LSO, and the lipid content of cultures grown on BSG with ($r = -0.446$; $p = 0.015$) and without ($r = -0.5654$; $p = 0.001$) the LSO supplement (Fig. 1B and Fig. 1C, respectively). Thus, taking into account that depletion of nutrients in the medium results in a metabolic shift towards lipid accumulation and EPA production (Cheng et al. 1999; Koike et al. 2001; Zhu 2002; Dyal et al. 2005), these correlations indicate that cultures with low EPA levels lacked sufficient activity of the conversion enzymes or were not sufficiently stressed by nutrient and lipid exhaustion to switch to HUFA and EPA production. Also, the negative correlation between lipid and EPA content (Figs 1B,C) indicates that optimization for EPA production using SSF with *Mortierella*, growing on LSO enriched BSG, will most probably result in a trade off between lipid and EPA content of the culture.

Conclusions

This study proved that the production of EPA by SSF of supplemented BSG using *Mortierella* fungi is feasible. All the *Mortierella* strains produced lipids containing the target HUFA and in the majority of strains EPA accumulation was enhanced by the addition of LSO to the substrate. The strains producing the most EPA in absence of additional LSO generally also produced the most EPA when the LSO was added to the BSG.

Generally, the addition of LSO to cultures of those strains with a relatively low lipid content resulted in the highest EPA content. Two such strains were *Mortierella antarctica* Mo 67 and *Mortierella epicladia* Mo 101, which respectively produced 2.8 mg and 2.5 mg EPA per g of LSO supplemented BSG. These strains enzymatically converted the LSO more efficiently to EPA and should be further investigated to optimise growth conditions during SSF. Parameters to be investigated include oxygen availability, other waste substrates, C:N ratio, pH, incubation temperature, the addition of nutrients and minerals, layer thickness and moisture content. Successful optimisation and scale-up of this process could add value to BSG or other agroprocessing by-products. The process is relatively inexpensive when compared to submerged fermentation and may provide a pure, safe and sustainable source of the ω-3 long chain fatty acid, EPA, normally obtained from fish oil.

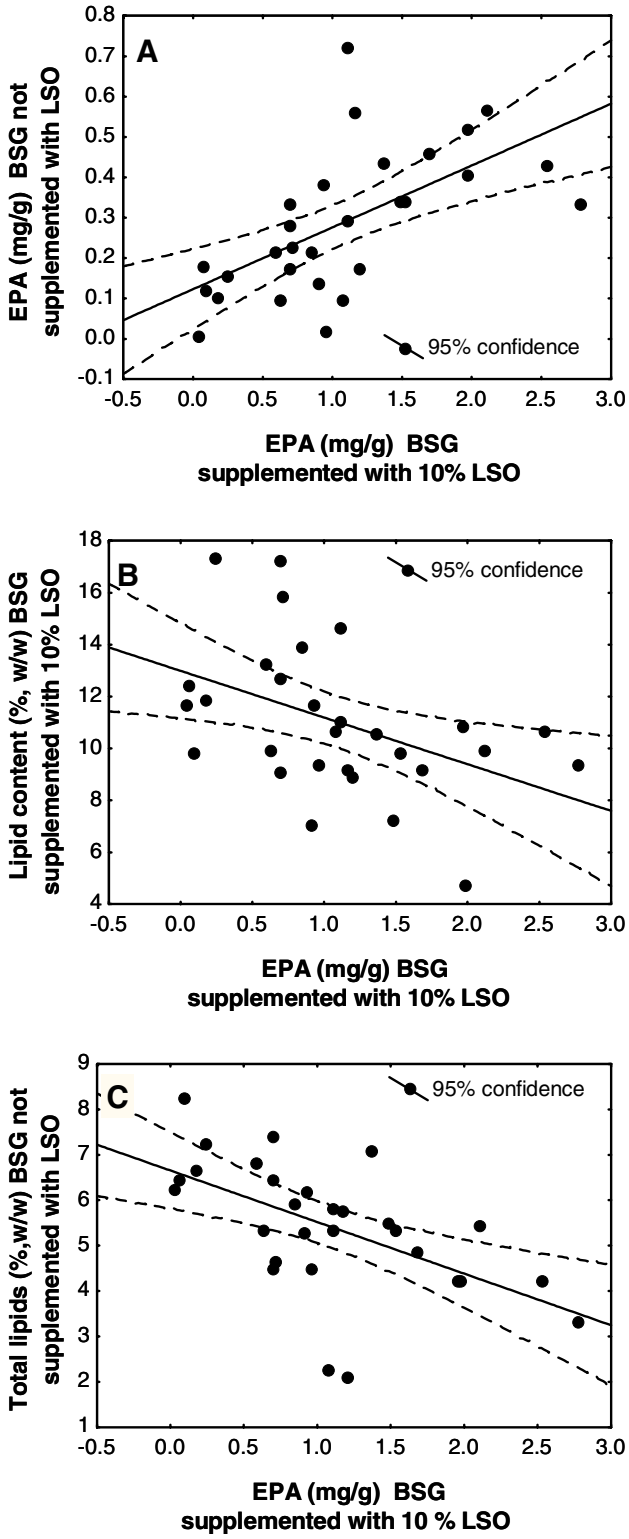

Fig. 1. Scatter plots of the EPA content of the fermented BSG supplemented with 10 % (w/w) LSO plotted against (A) the EPA content of the fermented BSG not supplemented with LSO, (B) total lipid content of the fermented BSG supplemented with 10 % LSO, and (C) total lipid content of the fermented BSG not supplemented with LSO.

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