THE PRODUCTION OF THE HIGHLY UNSATURATED FATTY ACID EICOSAPENTAENOIC ACID BY FUNGAL SOLID STATE FERMENTATION

by
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DECLARATION

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ABSTRACT

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. Fish oil as the main dietary source of EPA holds several disadvantages and alternative sources and production processes such as microbial fermentation are increasingly being investigated.

Therefore the aim of the first part of this study was to evaluate brewers' spent grain (BSG) as substrate for the production of 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 studied. Consequently, fungal inoculated BSG was incubated at 22°C for three days to obtain optimal fungal growth, before the incubation temperature was lowered to 16°C for the following eight days. Cultures were then harvested and dried, followed by lipid extraction and analyses using gas chromatography.

All the strains were found to produce EPA on BSG, while addition of the LSO improved the EPA yield of most strains. 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.

During the second part of the study eight *Mortierella* strains were used to study EPA production via solid state fermentation of sunflower press cake (SPC). Similar culture conditions and analytical methods were used as in the first part of the study. The effect of supplementing the SPC substrate with 10% (w/w) LSO was studied with regard to the supplement's impact on EPA production and on the highly unsaturated fatty acid (HUFA) profile of the fermented substrate.

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Addition of LSO improved EPA yield of most strains on SPC, leading to a reduction in the average arachidonic acid (ARA):EPA ratio from 50.68 to 3.66. The ratio of HUFA to saturated and monoenoic fatty acids, was increased significantly ($t=5.75$, $p=0.05$) by the addition of LSO, with higher desaturation levels among the 20-carbon fatty acids. Addition of LSO also had a positive effect ($r = 0.9291$, $p = 0.001$) on the relative amount of long chain fatty acids ($C \geq 20$) produced. The strains which produced the highest levels of EPA on SPC supplemented with LSO 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.

Fungi belonging to the genus *Mortierella* successfully converted LSO supplemented agro-processing wastes, such as BSG and SPC, to materials containing EPA, thereby adding value to these substrates. These EPA-enriched waste substrates could eventually find applications as animal or fish feed or as a source of EPA and other HUFA for the growing omega-3 market in the neutraceutical and therapeutics industry.

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OPSOMMING

Langketting omega-3 vetsure soos eikosapentaenoë suur (EPS) is noodsaaklik vir die regulasie van kritiese biologiese funksies in mense en ander soogdiere. Visolie, die mees belangrike EPS-bron in die dieet, hou verskeie nadele in en alternatiewe bronne sowel as produksie-prosesse, soos mikrobiologiese fermentasie, word dus toenemend ondersoek.

Die doel van die eerste gedeelte van hierdie studie was dus om gebruikte brouersgraan (GBG) te ëvalueer as ‘n substraat vir die produksie van EPS deur soliede staat fermentasie met 29 fungus isolate wat verskillende *Mortierella* spesies verteenwoordig. Die uitwerking van byvoeging van 10% (m/m) lynsaadolie (LSO) op EPS-produksie is ook bepaal. Gevolglik is fungus-geïnokuleerde GBG vir drie dae by 22°C geïnkubeer om optimale fungusgroei te verkry, waarna die inkubasie temperatuur verlaag is na 16°C vir die volgende agt dae. Kulture is hierna ge-oes en gedroog, gevolg deur lipied ekstraksie en analise met behulp van gaschromatografie.

Al die isolate het EPS geproduseer op die GBG substraat, terwyl byvoeging van LSO die EPS-opbrengs van die meeste isolate verbeter het. Die isolate wat die hoogste vlakke van EPS op GBG wat met LSO verryk is, geproduseer het, was *Mortierella antarctica* Mo 67 en *Mortierella epicladia* Mo 101, wat onderskeidelik 2.8 mg en 2.5 mg EPS per g GBG geproduseer het.

Tydens die tweede gedeelte van die studie is agt *Mortierella* isolate gebruik om die produksie van EPS deur soliede staat fermentasie van sonneblom perskoek (SPK) te ondersoek. Kultuurtoestande en analitiese metodes soortgelyk aan die eerste gedeelte van die studie is gebruik. Die uitwerking van byvoeging van 10% LSO tot die SPK substraat is ondersoek met betrekking tot die impak van die byvoeging op EPS

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produksie asook op die profiel van hoogs onversadigde vetsure (HOVS) van die gefermenteerde substraat.

Die byvoeging van LSO tot SPK het die EPS opbrengs van meeste isolate verbeter en het tot ‘n verlaging in die gemiddelde arachidoonsuur (ARS):EPS verhouding vanaf 50.69 tot 3.66 gelei. Die verhouding van HOVS tot versadigde en mono-onversadigde vetsure, is betekenisvol ($t=5.75$, $p=0.05$) verhoog deur die byvoeging van LSO, met hoër vlakke van onversadigheid onder die 20-koolstof vetsure. Byvoeging van LSO het ook ‘n positiewe uitwerking ($r = 0.9291$, $p = 0.001$) op die relatiewe aantal langketting vetsure ($C \geq 20$) gehad. Die isolate wat die hoogste vlakke van EPS geproduseer het op LSO-verrykte SPK, was *Mortierella alpina* Mo 46 en *Mortierella basiparvispora* Mo 88, wat onderskeidelik 6.4 mg en 5.8 mg EPS per g SPK geproduseer het.

Fungi wat aan die genus *Mortierella* behoort, het LSO-verrykte agro-prosesserings afvalprodukte, soos GBG en SPK, suksesvol omgeskakel na materiale wat EPS bevat, en sodoende waarde toegevoeg aan hierdie substrate. Die EPS-verrykte afvalsubstrate kan uiteindelik toepassings vind as diere- of visvoer of as bron van EPS of ander HOVS vir die groeiende omega-3 mark in die neutraseutiese en terapeutiese industrie.

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ABBREVIATIONS

AACC	American Association of Cereal Chemists
ALA	alpha-linolenic acid (C18:3 n-3)
ARA	arachidonic acid (C20:4 n-6)
ATCC	American Type Culture Collection
BSG	brewers' spent grain
C/N	carbon to nitrogen
DGLA	dihomo-gammalinolenic acid (C20:3 n-6)
DHA	docosahexaenoic acid (C22:6 n-3)
DNA	deoxyribonucleic acid
DPA	docosapentaenoic acid (C22:5 n-6)
EL	elongase
EPA	eicosapentaenoic acid (C20:5 n-3)
g	gram
ETA	eicosatrienoic acid (C20:3 n-3)
GLA	gamma-linolenic acid (C18:3 n-6)
GRAS	generally regarded as safe
HUFA	highly unsaturated fatty acid
LA	linoleic acid (C18:2 n-6)
LSO	linseed oil
MA	mead acid (C20:3 n-9)
mg	milligram
ml	millilitre
Mo	Mortierella
m/m	mass per mass
NADP	nicotinamide adenine dinucleotide phosphate
OA	oleic acid (C18:1 n-9)
PCB	polychlorobiphenyl
SD	standard deviation
SE	standard error
SCO	single cell oil
SFF	solid state fermentation
SmF	submerged fermentation
SPC	sunflower press cake
USA	United States of America
w/w	weight per weight
β	beta
Δ	delta
$	dollar
ω	omega

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Introduction

The production of the highly unsaturated fatty acid eicosapentaenoic acid by fungal solid state fermentation

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Motivation

Over the past decades highly unsaturated fatty acids (HUFA) as food additives have attracted increasing attention among researchers, consumers and biotechnology entrepreneurs alike (Higashiyama *et al.*, 2002; Naylor *et al.*, 2009). Based on the position of the first double bond in relation to the methyl terminus of the hydrocarbon chain, these fatty acids may be grouped into two main classes, ω-3 (n-3) and ω-6 (n-6) fatty acids (Sijtsma and De Swaaf, 2004). Both classes contain fatty acids that are essential for the regulation of critical biological functions in mammals, including man (Certik and Shimizu, 1999).

Omega-3 HUFA such as eicosapentaenoic acid (EPA, 20:5 n-3) and docosahexaenoic acid (DHA, 22:6 n-3) are essential to the regulation of the cardiovascular, digestive, immune and neurological systems in mammals (Qiu, 2003; Sijtsma and De Swaaf, 2004). Humans cannot synthesise adequate levels of these HUFA from precursor fatty acids and long chain ω-3 HUFA have to be included in the diet (Dyal and Narine, 2005). Several food products and supplements enriched with ω-3 HUFA are now commercially available.

Marine fish also require ω-3 HUFA for optimal growth and health, as they typically do not possess the elongation and desaturation enzymes required to produce ω-3 HUFA from shorter chain precursors (Craig and Helfrich, 2002). The main dietary source of ω-3 HUFA for both mammals and farmed marine fish is fish oil (Ward and Singh, 2005; Naylor *et al.*, 2009) and therefore the ω-3 HUFA market and aquaculture industry are interrelated. However, there is growing concern about the sustainability of marine fish oil as a source of ω-3 HUFA, mainly because of the increasing demand for fish oil by the aquaculture and pharmaceutical industries (Hasan *et al.*, 2007). The demand for omega-3 oil is predicted to grow from $13bn in 2008 to more than $17.6bn in 2013 according to Euromonitor (Starling, 2009). HUFA-enriched infant formula is gaining popularity with a predicted demand of 4000 tons of ARA

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and DHA per year (Kyle, 2005). Alternative sources of ω-3 HUFA oil are therefore increasingly being explored.

Recent research has focused on microbial production of ω-3 HUFA as a sustainable and safe alternative source to fish oil, as well as on improving the economical competitiveness of microbial lipids with fish and plant oils (Certik and Shimizu, 1999). Due to the high production cost in comparison with conventional oils from natural sources, very few microbial oils have been commercialised successfully. According to Peng and Chen (2008), the high production cost of microbial oils is the major barrier to its commercialisation. The only HUFA currently commercially available from an algal source is DHA (Harwood and Guschina, 2009). Algal EPA production has low productivity and high production cost due to relatively complex technology (Lewis *et al.*, 1999).

The major commercial source of EPA therefore remains marine fish oil (Cheng *et al.*, 1999), which presents problems in terms of oxidative stability, sensory quality, long term sustainability and safety, due to toxins and pollutants entering the marine food chain (Sijtsma and De Swaaf, 2004; Ratledge, 2005). Microbial EPA-containing oils are usually produced using submerged fermentation (SmF) by algal or fungal strains in a liquid medium. For these production processes to compete more favourably with EPA extraction from fish oil, several biotechnological strategies have been explored. These include using inexpensive feedstocks, selection and optimisation of high-yield strains and reducing the production and processing steps for oil recovery from cells (Certik and Shimizu, 1999).

Submerged fermentation often requires high capital investment e.g. the total capital investment for fungal lipase production was 78% higher by SmF than by solid state production (Couto and Sanroman, 2006). Also, SmF utilises relatively expensive feedstocks, whereas inexpensive raw materials or agro-processing waste products could be utilised as solid substrates in solid state fermentation (SSF), thereby enhancing economic competitiveness

(Stredansky *et al.,* 2000). The latter fermentation process is also ideal for the production of low volume – high cost products (Pandey *et al.*, 2000), such as EPA.

It was found that the rate of lipid and HUFA production by fungi may be higher during solid state fermentation than during submerged fermentation (Stredansky *et al.*, 2000), while the specific yield of EPA was also found to be higher during SSF (Jang *et al.*, 2000). In addition, filamentous fungi belonging to the genus *Mortierella* were identified as good candidates for SSF. These fungi also have higher reported EPA productivity than algae in SmF: 1350 mg/L with *Mortierella alpina* (Shimizu *et al.*, 1989) as opposed to 695 mg/L with the algal diatom *Nitzschia laevis* (Ward and Singh, 2005).

An objective of the current study was therefore to evaluate two agro-processing waste products, brewers' spent grain (BSG – Chapter 2) and sunflower press cake (SPC – Chapter 3), as substrates for HUFA and EPA production by SSF. In both cases, SSF was performed using different representatives of the oleaginous fungal genus *Mortierella.* A secondary objective was to establish a cost-effective alternative production process for EPA by manipulating culture conditions to enhance EPA yield. Linseed oil, an oil rich in a ω-3 precursor, was added to the substrate before fermentation and the incubation temperature was manipulated to improve conversion of the added oil to EPA. Enhanced EPA yields thus obtained would increase the economic competitiveness of the SSF process of fungal EPA production.

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Chapter 1

Literature review:
The production of the highly unsaturated fatty acid eicosapentaenoic acid by fungal solid state fermentation

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1. Highly unsaturated fatty acids (HUFA) as food, feed and supplements

1.1 Introduction

Over the past 30 years the production of highly unsaturated fatty acids (HUFA) has attracted increasing attention among researchers. The transition of HUFA from “academic curiosities” to important neutraceuticals was brought about by overwhelming evidence of the dietary significance of HUFA, as well as of the lack of safe, sustainable sources of HUFA in the plant and animal kingdoms. Micro-organisms are increasingly being viewed as the only realistic source of highly unsaturated oils (Ratledge, 2005). Within the microbial world, filamentous fungi and more specifically those belonging to the genus *Mortierella,* were identified as potential sources of HUFA, especially since it is known that some of the best producers of highly unsaturated fatty acids resides within this genus (Dyal and Narine, 2005).

The term “highly unsaturated fatty acids” normally refers to a group of fatty acids with long unbranched hydrocarbon chains containing more than 18 carbon atoms and more than four *cis* double bonds (Craig and Helfrich, 2002; Gawrich and Soubias, 2008). However, throughout this dissertation, the term “HUFA” will refer to unbranched long chain fatty acids with more than 18 carbon atoms and more than two double bonds. Based on the position of the double bonds in the carbon chain, HUFA may be classified into two main groups; ω-3 (or n-3) fatty acids and ω-6 (or n-6) fatty acids (Zhu, 2002). The former is characterised by the first double bond (as counted from the methyl group at ω-end of the carbon chain) being situated on the third carbon atom (Figure 1), while the latter is characterised by this double bond being on the sixth carbon atom counting from the ω-end of the carbon chain (Bell and Tocher, 2008).

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cis-5,8,11,14,17 – eicosapentaenoic acid

Figure 1. Molecular structure of eicosapentaenoic acid (EPA), a representative of the ω-3 fatty acids (www.cyberlipid.org/fa/acid0003.htm)

In nature, HUFA occur widely in algae, bacteria, ferns, fish, fungi, mammals (within renal, neural and reproductive tissues), mosses and plants. To date, the most highly unsaturated fatty acid known to occur in a living structure is C34:9 n-6, discovered in a freshwater crustacean in Europe (Rezanka and Dembitsky, 2004). This ω-6 HUFA contains 34 carbon atoms in its fatty acyl chain (hence the prefix “C34” before the colon), as well as 9 double bonds (indicated by the number after the colon) of which the first one is situated at the sixth carbon atom counted from the ω-end of the carbon chain (hence the suffix “n-6”).

1.2 The role of HUFA and EPA in mammal health

Highly unsaturated fatty acids are essential for regulation of critical biological functions in humans and mammals (Certik *et al.*, 1999). These molecules are required in almost every organ of the body, contributing to the function, fluidity and structure of cell membranes (Gawrich and Soubias, 2008), including regulating permeability and transport of nutrients. These fatty acids also control the expression of certain genes, affecting processes such as fatty acid biosynthesis and cholesterol transport in the body, thereby preventing diseases like coronary heart disease, arrhythmia (by reducing blood pressure), rheumatoid arthritis, diabetes mellitus and psoriasis (Cheng *et al.*, 1999). Highly unsaturated fatty acids serve as precursors to a wide variety of biologically active molecules such as prostaglandins, leukotrienes, thromboxanes and hydroxyl-fatty acids, which regulate critical immunological

and hormonal functions (Bajpai *et al.*, 1991; Dyal and Narine, 2005). Deficiencies in HUFA may lead to abnormalities of the skin, to defects in the cardiovascular, endocrine, renal, respiratory, nervous and reproductive systems, as well as to immune and inflammatory disorders (Certik and Shimizu, 1999; Yaqoob, 2003).

In eukaryotic cells two essential fatty acids, linoleic acid (LA, C18:2 n-6) and α-linolenic acid (ALA, 18:3, n-3), play an important role in the anabolism of HUFA (Certik and Shimizu,1999). These fatty acids serve as precursors for the ω-6 and ω-3 series of HUFA respectively, and are thus further elongated and desaturated (Figure 2). However, the anabolic pathways for the two series of HUFA are in competition with each other, since both uses the same set of enzymes. It was found that ∆6-desaturases show higher substrate specificity for LA than for ALA, leading to the preferential synthesis of ω-6 HUFA (Certik *et al.*, 1998).

Linoleic acid (ω-6) and alpha-linolenic acid (ω-3) are converted to long chain ω-3 HUFA, such as eicosapentaenoic acid (EPA, C20:5 n-3) and docosahexaenoic acid (DHA C22:6 n-3) by the consecutive action of desaturase and elongase enzymes (Figure 2), either starting with a Δ-6 desaturation or with a Δ-9 elongation via eicosatrienoic acid (Meyer *et al.*, 2004). The Δ-3-desaturases (Figure 2, double arrow), which link the ω-6 and ω-3 pathways in many eukaryotes, are not present in mammals (Meyer *et al.*, 2004). Within mammal liver and brain cells, DHA can be synthesized from the ω-3 precursors via the Sprecher pathway (Pereira *et al.*, 2004). By this pathway, the Δ-4 double bond in DHA is inserted as the result of a complicated series of reactions. These involve the elongation to a C24 fatty acid, a second Δ-6 desaturation and the final chain shortening by β-oxidation which takes place in cellular peroxisomes. However, there is accumulating evidence that the rate of EPA and DHA synthesis in mammals cannot always satisfy demand, due to insufficient levels of the relevant desaturase and elongase enzymes (Streekstra, 1997; Zhu, 2002). This necessitates a dietary source of EPA which, in turn, is a direct precursor for the series 3 prostaglandins. These prostaglandins are biologically important compounds

with hormone-like action on the constriction of smooth muscles. Eicosapentaenoic acid also prevents blood platelet aggregation, reduces the risk of arteriosclerosis (Dyal and Narine, 2005) and has strong anti-inflammatory properties (Qiu, 2003).

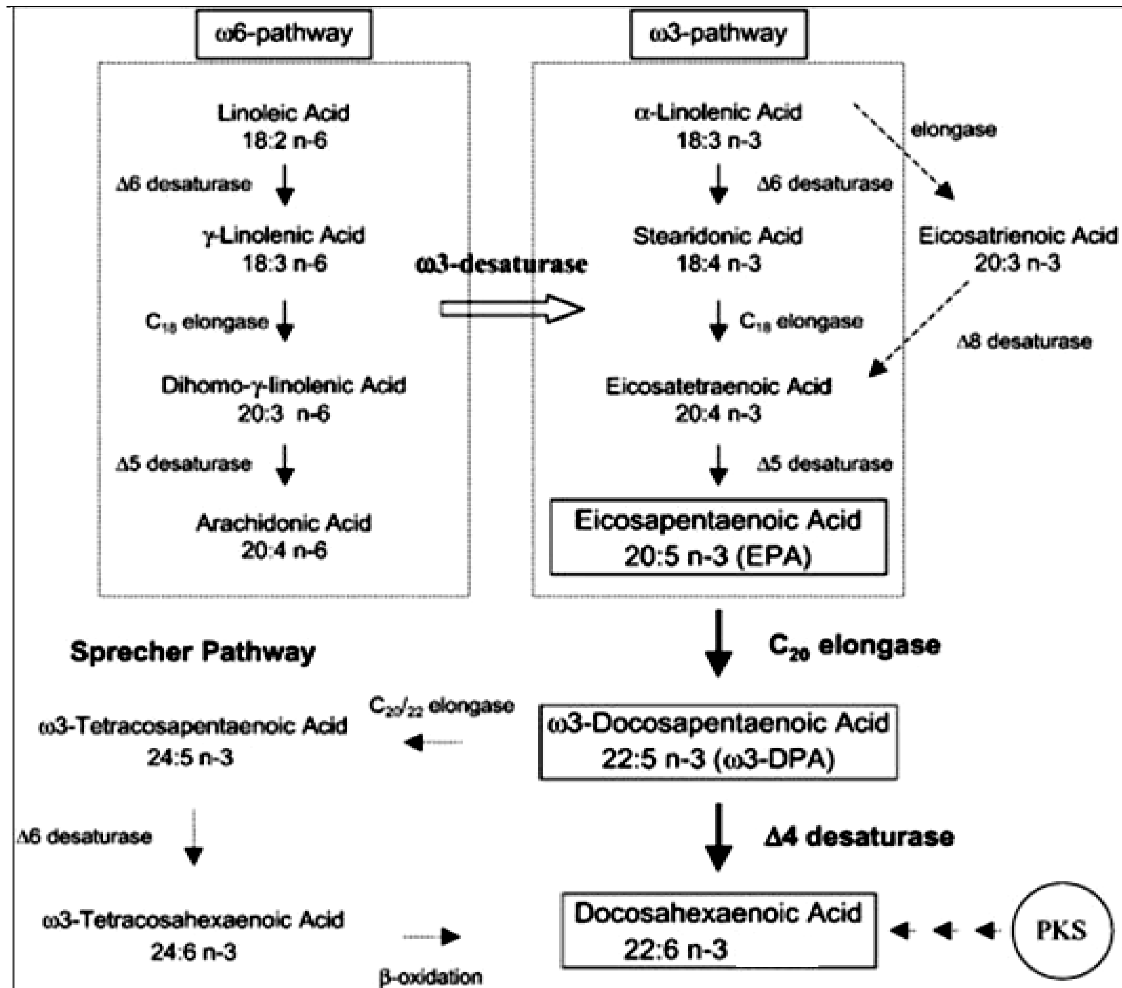

Figure 2. Diagrammatic representation of the biosynthetic pathways of ω-6 and ω-3 HUFA production in eukaryotic (mammal and microbial) cells (Adapted from Pereira *et al.*, 2004)
(PKS: polyketide synthase system)

As mammals cannot synthesize adequate amounts of long chain ω-3 HUFA, these HUFA have to be added to the diet from external sources (Certik and Shimizu, 1999; Dyal and Narine, 2005). Due to the recognised nutritional

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value of ω-3 HUFA, several food products enriched with these fatty acids are now commercially available e.g.:

- A Costa Rican dairy, Dos Pinos, produces ω-3 fortified milk using micro-encapsulated Norwegian fish oil (Starling, 2009).
- Scientists from New Zealand have developed a process of encapsulating emulsified fish oil containing ω-3 fatty acids in milk proteins used for cheese production. Emulsification reduced the levels of fatty acid oxidation significantly, thereby also reducing “fishy” flavours in the cheese (Daniells, 2009).
- Coca-Cola produces a “superjuice” fortified with docosahexaenoic acid (DHA, C22:6 n-3) advertised as a “memory-booster” (Starling, 2009).
- Fish fillets coated with a mixture of chitosan and fish oil (containing ω-3 fatty acids) was found to be not only nutritionally superior, but had a longer shelf-life than uncoated fillets (Halliday, 2009).

The main dietary source of ω-3 HUFA, from which various products such as those mentioned above are produced, is marine fish oil. However, there is growing concern over the sustainability of fish sources, since the demand for crude fish oil in the aquaculture industry is rapidly increasing (Hemre, 2002; Jang and Yang, 2008).

1.3 The role of HUFA and EPA in aquaculture

The HUFA market and aquaculture industry are interrelated. As wild fish populations are becoming depleted due to over-exploitation, aquaculture continues to grow. Globally, the aquaculture sector had an annual production value of US$ 56.47 billion in 2000 (Tacon, 2003). The fastest growing sector of the aquaculture industry is in the farming of carnivorous fish as these are the most popular for human consumption. Marine fish require more ω-3 HUFA for optimal growth and health than freshwater fish, as marine species typically

do not possess the elongation and desaturation enzymes required to produce ω-3 HUFA from shorter chain precursors (Craig and Helfrich, 2002).

Carnivorous marine fish require high levels of ω-3 HUFA in their diets. In nature this is primarily supplied by smaller pelagic oily fish and plankton rather than by biosynthesis (Barclay *et al.*, 2005). Mediterranean fish species require a diet containing between 0.9 and 1.3% total ω-3 HUFA (Alexis, *ca.*1996). The optimal dietary ratio of EPA:DHA varies between fish species (Sargent *et al.*, 1999). In aquaculture feeds for carnivorous fish, of which the natural diet consists of smaller fish and krill, ω-3 HUFA are usually supplied via fish oil and fish meal.

While freshwater fish can convert ALA to EPA and DHA, marine fish are characterised by insufficient activity of their Δ5-desaturase enzymes and therefore require a diet containing the essential fatty acids, EPA and DHA (Lall, 2000). Farming of carnivorous marine fish therefore uses vast quantities of harvested fish for the industrial manufacture of fish meal and ω-3 rich fish oil. While fish meal could be successfully substituted with plant derived proteins, the substitution of fish oil is a limiting factor in marine aquaculture (Pike and Barlow, 2002).

According to Naylor *et al.* (2000) 2,5 – 5,0 kg of sea fish biomass is needed for every 1 kg of farmed carnivorous fish produced, leading to global over-exploitation of natural fish stocks. Substituting fish oil with plant-derived lipids is not a perfect solution as vegetable oils are generally high in ω-6 fatty acids such as LA. According to Pike and Barlow (2002), the presence of high levels of ω-6 fatty acids results in the production of predominantly ω-6 HUFA such as docosapentaenoic acid (DPA, C22:5 n-6) due to the activity of Δ4-desaturase. The substrate preference of this enzyme for the more abundant ω-6 fatty acids effectively blocks elongation of ω-3 ALA to EPA and DHA in fish. These factors have intensified the search for cost-efficient sustainable sources of HUFA as an alternative to fish oil.

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2. Sources of HUFA

Natural sources of HUFA include plants, fish, marine animals and micro-organisms.

2.1 Plants

Plant seed oils like soybean oil, canola oil, flaxseed oil, walnut, perilla seed oil and purslane oil are important sources of the two essential eighteen carbon fatty acid precursors of HUFA mentioned previously (Figure 2). Perilla seed is known to be the best plant source of ALA, since this fatty acid may represent 54% to 65% of the total fatty acid content of the seeds (Kurowska *et al.*, 2002). On the other hand the precursor for the ω-6 series of HUFA, i.e. LA, is the most common polyunsaturated fatty acid in seeds of walnut, peanut, sunflower, grape, maize sesame and soybeans. Another eighteen carbon fatty acid, gamma-linolenic acid (GLA, 18:3 n-6), occurs in some plants, with evening primrose and borage seeds being some of the most commonly known sources (www.cyberlipid.org/fa/acid0003.htm Accessed 25/10/09)

Highly unsaturated fatty acids, with more that eighteen carbons in the acyl chain, cannot be synthesised by higher plants (Certik *et al.*, 1998). Genetic manipulation of crop plants to produce longer chain HUFA would seem to be the most feasible and sustainable means of economical production in the long term. However, the acceptance and inclusion of genetically modified oils, particularly in infant formulation, currently the main commercial application of HUFA, is unlikely in the medium term. Nevertheless, several major industrial companies have engaged in genetic manipulation of plants for improved HUFA production (Ratledge, 2005).

Genes encoding the various fatty acid desaturases and elongases were transferred from micro-organisms and inserted into plant DNA. Several genes encoding elongation and desaturation enzymes were expressed seed-specifically in *Arabidopsis thaliana* (Robert *et al.*, 2005). It was however,

calculated that successful HUFA production in plants will come at considerable metabolic cost to the plant as 18 mol of NADPH is needed to produce one mol of fatty acid. The production of HUFA throughout the entire plant – in the leaves, stem and roots – would leave the plant without sufficient energy and unable to grow properly. This metabolic cost will increase with the amount of double bonds and chain length of the target HUFA (Ratledge, 2005). It is therefore obvious that numerous problems must still be overcome before plants could be successfully engineered to produce recombinant HUFA. If HUFA is to be produced in the seed alone, enormous scientific challenges still exist in finding appropriate genes and in inserting it in the plant genome so that it could be expressed during seed formation. The large scale production of HUFA in agricultural crops is therefore not a commercially viable option in the near future.

2.2 Fish and marine animals

The major source of ω-3 HUFA in the human diet is fish and fish oil capsules (Ward and Singh, 2005; Bell and Tocher, 2008). These contain twenty- and twenty-two carbon fatty acids, notably EPA, docosapentaenoic acid (DPA, C22:5 n-3) and DHA. The oils in these capsules are derived mainly from “oily” fish; principally herring, mackerel, sardine and salmon. Marine fish acquire HUFA primarily from their diet of plankton, smaller pelagic fish or added fish oil (in the case of aquaculture) rather than by biosynthesis (Barclay *et al.*, 2005).

The demand for HUFA in fish feeds, pharmaceutical applications and human nutrition is growing rapidly. However, the inclusion of HUFA from fish oil in foods or infant formulas has some disadvantages. Fish oil is often unsuitable because of contamination of the fish by environmental pollution (PCB’s, dioxins and mercury) and organoleptic problems associated with the typical fishy smell and unpleasant taste (Certik and Shimizu, 1999; Kyle, 2005). Attempts to improve the organoleptic characteristics of fish oil remain largely unsuccessful (Barclay *et al.*, 2005). The HUFA from fish oil sources are also

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not very stable and oxidative degeneration leads to the formation of toxic short chain oxidation products. A specific problem regarding infant formulas is that fish oils generally contain EPA, an undesirable component in these formulas, because it leads to reduced arachidonic acid (ARA, C20:4 n-6) levels in infants (Lapillonne *et al.*, 2000). This has been correlated with reduced rates of weight gain in preterm infants (Carlson *et al.*, 1992).

Fish oil production was around 1.2 million tons per annum (2002), 70% of which went to feed farmed fish (Sijtsma and De Swaaf, 2004). Most of this fish oil is produced in only one region of the world i.e. Chile and Peru, which makes the oil market highly susceptible to climatic fluctuations e.g. the El Nino phenomenon (Hemre, 2002). The unreliable supply as well as the previously mentioned organoleptic and quality issues related to fish oil, has lead to an intensified search for safe, sustainable sources of HUFA as an alternative to fish oil.

2.3 Micro-organisms

Micro-organisms capable of producing HUFA with 5 or more double bonds and 20 or more carbon atoms include lower fungi, bacteria and marine micro-algae (Table 1; Sijtsma and De Swaaf, 2004). Marine organisms such as marine bacteria, algae and mosses are thought to be the primary producers of HUFA in the marine food chain. Although marine fish (and mammals) have some capacity for the *de novo* biosynthesis of ω-3 HUFA, most seem to originate in their diet from plankton and micro-algae (De Swaaf, 2003).

Although a variety of micro-organisms have the ability to synthesise and/or accumulate HUFA (De Swaaf, 2003) only some, the so-called oleaginous or ‘oil-bearing’ micro-organisms, can accumulate lipids in their cells up to the equivalent of 50% of dry biomass (Higashiyama *et al.*, 2002). These lipids function as a reserve storage material in the form of triacylglycerols (triglycerides).

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Table 1. HUFA produced by micro-organisms (adapted from Certik and Shimizu, 1999)

HUFA	Potential microbial source
GLA (n-6) (gamma linolenic acid)	**Fungi** (*Mucor circinelloides*, *Mucor mucedo, Mortierella isabellina*, *Mortierella ramanniana*, *Cunninghamella echinulata*, *Cunninghamella elegans*, *Cunninghamella japonica*, *Rhizopus arrhizus*, *Thamnidium elegans*) **Algae** (*Spirulina platensis*, *Chlorella vulgaris*)
DGLA (n-6) (dihomo-GLA)	**Fungi** (*Mortierella* spp., especially *M.alpina, Conidiobolus nanodes, Saprolegnia ferax*)
ARA (n-6) (arachidonic acid)	**Fungi** (*Mortierella* spp., especially *M.alpina, Conidiobolus nanodes, Entomophthora exitalis, Blastocladiella emersonit*) **Algae** (*Porphyridium cruentum*, *Sargassum salicifolium, Euglena gracilis*)
MA (n-9) (mead acid)	**Fungi** (*Mortierella alpina*)
EPA (n-3) (eicosapentaenoic acid)	**Fungi** (*Mortierella alpina, Mortierella elongata, Pythium irregulare, Pythium ultimum*) **Algae** (*Chlorella minutissima, Monodus subterraneus, Polysiphnia latifolium, Porphyridium cruentum, Phaeodactylum tricornutum, Nannochloropsis oculata, Amphidinium carteri, Thalassiosira pseudonana, Pavlova lutheri, Glossomastix chrysoplastos*) **Bacteria** (*Shewanella putrifaciens*)
ETA (eicosatetraenoic acid)	**Fungi** (*Mortierella alpina*)
DPA (docosapentaenoic acid)	**Fungi** (*Mortierella alpina*)
DHA (docosahexaenoic acid)	**Fungi** (*Thraustochytrium aureum, Thraustochytrium roseum, Schizochytrium SR21, Schizochytrium aggregatum*) **Algae** (Microalgae MK8805, *Crypthecodinium cohnii, Gyrodinium nelsoni, Amphidinium carteri, Gonyaulax polyedra, Pavlova lutheri*) **Bacteria** (*Vibrio* spp., *Rhodopseudomonas* spp.)

It is well known that HUFA can be obtained from oleaginous micro-organisms as an alternative to agricultural, animal or fish oil products (Certik and Shimizu, 1999). However, at present the contribution of microbial HUFA to the oil industry is small, but there are reasons to increase its application in future. High growth rates of micro-organisms on a variety of substrates (ideally residues from food production), ease of manipulation and readily induced mutation, suggest that micro-organisms are ideal for HUFA production. Highly unsaturated fatty acid-containing oils produced by single cell micro-organisms are known as single cell oils (SCO) and have several advantages over oils obtained from plants, fish and animals. These advantages include:

- Oils produced by single cell organisms often contain only a single fatty acid as main, if not sole, HUFA. By contrast, oils obtained from plants, fish and animals always contain a range of saturated and unsaturated fatty acids. A dietary requirement for a single pure HUFA may therefore be met with a specific SCO (Wynn *et al.*, 2005).
- Some valuable or unusual HUFA, which have a more favourable cost to selling price ratio than other freely available HUFA, are produced exclusively by micro-organisms and are not available from other sources (Sijtsma and De Swaaf, 2004).
- Microbial sources can supply oils with a concentrated HUFA content and controlled quality (Certik and Shimizu, 1999).
- The oxidative stability of microbial ω-3 lipids is generally higher than that of fish oils (Sijtsma and De Swaaf, 2004).
- Microbial HUFA can be produced from sustainable raw materials (Sijtsma and De Swaaf, 2004).
- Knowledge of genetics and biochemical pathways within micro-organisms may provide tools for the development of novel production systems and targeted products (Sijtsma and De Swaaf, 2004).

While different fungal species belonging to the order Mucorales produce oil with species-specific fatty acid profiles (Dyal *et al.*, 2005), oleaginous fungi of the genus *Mortierella* are reported to accumulate large amounts of lipids in their cells (Streekstra, 1997). In order to achieve lipid accumulation, an oleaginous organism requires a growth medium with an excess of carbon

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substrate and a limiting amount of nitrogen (Ratledge, 2005). As the organism grows, it exhausts the nitrogen, but continues to assimilate the carbon source, which is channelled into lipid synthesis with the resulting accumulation of triacylglycerols within the cell as discrete lipid droplets. When placed in the same nitrogen limiting growth medium, non-oleaginous organisms either tend to cease further cell proliferation or continue to assimilate the available carbohydrate, diverting it into glycogen, mannans and other storage polysaccharides. Thus, although nitrogen supplementation usually stimulates microbial growth, lipid-accumulation is triggered by conditions of nitrogen depletion (De Swaaf *et al.*, 2003). The C/N ratio can therefore be manipulated to achieve either growth or HUFA-production (Dyal and Narine, 2005). Excess carbon stimulates the formation of higher concentrations of long chain HUFA.

2.3.1 **Bacteria**

Oleaginous bacteria are restricted to marine species; often halotolerant, psychrophilic and piezophilic (Russell and Nichols, 1999). The role of these prokaryotes in the marine food chain is not fully understood, but there is increasing speculation that intestinal HUFA-producing bacteria may contribute to the HUFA requirements of marine animals and fish. On the other hand, some bacteria may contain unusual fatty acids not found in other systems and generally do not produce triacylglycerols, but instead produce poly-β-hydroxybutyrates and alkanaotes as storage polymers, rendering them less suitable as HUFA producers.

The levels of HUFA in bacteria are not as high as in some species of fungi and algae (Sijtsma and De Swaaf, 2004). Nevertheless, many bacteria have the ability to assimilate exogenous HUFA present in the culture (Russell and Nichols, 1999). Apart from bacteria that have the ability to incorporate exogenous HUFA, there are a few such as *Flexibacter polymorphus, Shewanella gelidimarina, Shewanella hanedai* and *Colwellia psychrerythraea* which possess metabolic pathways of *de novo* HUFA synthesis, such as the

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PKS pathway (Figure 2). These bacteria produce ARA, DHA and/or EPA fatty acids (Russell and Nichols, 1999).

Nichols *et al.*, (1993) stated that bacteria possess distinct advantages over algae or fish oils as sources of HUFA. Culturing of algal species usually require highly regulated, expensive growth conditions in terms of nutrients, light quantity and quality, as well as oxygenation and carbon dioxide levels. In contrast, most bacteria are not fastidious, and can often be grown on the waste products of other agricultural or industrial processes. Secondly, unlike fish oils, which represent a finite and often unreliable resource, bacteria, because of their ability to be cultured and maintained almost indefinitely, can be considered as an easily renewable and reliable resource for the production of HUFA.

2.3.2 **Algae**

Microalgae (diatoms and dinoflagellates) are the major primary producers of a wide range of HUFA in marine environments (Wynn *et al.*, 2005). These HUFA which include EPA and DHA are essential dietary components of higher marine organisms. The majority of dinoflagellate algal species are photosynthetic.

Currently, one of the only two HUFA in commercial production is DHA marketed by Martek Biosciences Corp. (Kyle, 2005). The process utilises a marine heterotrophic dinoflagellate algae *Crypthecodinium cohnii* (Dufernez *et al.*, 2008). The commercial production of DHA from this algal species has been conducted since the early 1990’s and several patents have been filed by various researchers (Sijtsma and De Swaaf, 2004). As a source of HUFA, *C. cohnii* is considered the most reliable and potentially useful DHA producing micro-organism. The strain *C. cohnii* ATCC 30772 was found to accumulate lipid to over 20% of its dry weight biomass, while the DHA content of the total lipids may range from 30 to 50% (De Swaaf *et al.*, 2003; Sijtsma and De Swaaf, 2004). DHA constitutes around 40% of algal biomass produced

commercially in 200 m^3 stirred tank fermentors and 240 tons of DHA was produced in 2003. The SCO produced is used exclusively for infant formulations (Kyle, 2005) which are currently marketed in more than 60 countries (Harwood and Guschina, 2009).

Apart from *C. cohnii*, other algae which are potentially good sources of HUFA include *Pavlova lutheri* known for the ability to produce EPA and DHA (Tonon *et al.*, 2003). Algae in the class Pinguiophyceae (e.g. *Glossomastix chrysoplastos*) characteristically produce large amounts of EPA (Hsiao and Blanch, 2006). Species representing the genus *Spirulina*, i.e. *S. platensis*, was found to produce oil with concentrations of GLA up to 23.6% of the total lipids, while *Porphyridium cruentum* synthesised EPA in amounts ranging between 26.5 and 40.4% of fatty acids (Cohen *et al.*, 1992).

2.3.3 **Fungi**

Several fungal genera contain highly oleaginous species which have the ability to produce and accumulate high amounts of HUFA (Streekstra, 1997). Similar to oleaginous algae, these fungi are increasingly being investigated as potential sources of HUFA. *Pythium irregulare, Pythium ultimum, Mortierella alpina* and *Mortierella elongata*, were identified as good sources of ARA and EPA (Cheng *et al.*, 1999; Ward and Singh, 2005). *Pythium irregulare* was considered to be one of the most promising fungal species for the commercial production of EPA (Zhu, 2002). However, *M. alpina* was also regarded as an excellent source of HUFA, including ARA and EPA (Certik and Shimizu, 1999; Dyal and Narine, 2005). Consequently, this *Mortierella* species is used for the production of ARASCO, a single cell oil containing mainly ARA, produced for inclusion in infant formula. ARASCO constituted 60% of the total single cell oils produced commercially in 2003 (Kyle, 2005).

The Thraustochytrids (e.g. *Schizochytrium*) are a group of osmeoheterotrophic chromistan fungus-like protists, ubiquitous in marine environments and sandy sediments. These primitive fungi are taxonomically aligned with heterotrophic algae and can synthesise and accumulate high

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amounts of DHA and EPA (Lewis *et al.*, 1999). Fungi like *Thraustochytrium aureum, Thraustochytrium roseum* and *Schizochytrium aggregatum* are reported to be good sources of DHA (Certik and Shimizu, 1999). Oil containing DHA is also produced commercially by *Schizochytrium* spp. (Kyle, 2005). Production of HUFA by these fungi has been so successful that their HUFA have been used in a wide variety of products on the market today. According to Jain and Kumar (2004), the yields of DHA and EPA can be enhanced beyond their natural levels by modifying the viscosity of the production medium.

3. Microbial HUFA production processes

Microbial HUFA and EPA have been produced by fermentation processes which can be classified as either submerged (liquid) fermentation (SmF) or solid state fermentation (SSF).

3.1 **Submerged fermentation**

In submerged fermentation (SmF), micro-organisms are cultivated in a liquid medium in which all nutrients necessary for growth are dissolved. Usually, most of the sources of nutrients are water-soluble and in instances where one or more of the required nutrients are oily, a suitable emulsifier is also added to the medium. In classical SmF systems, mixing is performed by means of a shaker or stirrer to allow gas transport, mass transfer and to disperse nutrients (Hilton, 1999).

Historically the first commercial production of SCO containing HUFA was performed by SmF in 1985. J. and E. Sturge Ltd (UK) produced about two tons of GLA-containing oil per batch in 220 m^3 stirred tank fermentors (Ratledge, 2005). This oil was produced as a supplement for the food-additive market, but could not compete with cheaper plant-based sources of GLA such as evening primrose oil, borage oil and blackcurrant seed oil. The company

was sold in 1990, but it laid the technological foundation for future SCO processes.

Submerged fermentation can be performed in closed systems (fermentation vessels or bioreactors) or open pond systems (Wen and Chen, 2005). The oldest and simplest systems for cultivation of phototrophic algae were in open ponds. This was used for cultivation of phototrophic species such as *Pavlova lutheri* and *Spirulina acutus*, which produced oil with high quantities of eighteen carbon unsaturated fatty acids.

Open cultivation systems are dependent on the weather and climate; hence product quality and quantity may vary (Anderson *et al.*, 2002). Due to susceptibility for contamination with bacteria and predation by protozoa, phototrophic cultivation in open ponds is only feasible when suitable selective conditions, such as high salinity, are used (Sijtsma and De Swaaf, 2004). Covering ponds with transparent material alleviates some of the disadvantages, but it was suggested that enclosed photobioreactors would enhance commercial algal production (Anderson *et al.*, 2002).

During SmF all the required nutrients and culture inoculum may be added simultaneously and such a process is known as “batch fermentation”. If at least one of the ingredients, such as acetic acid as a carbon source, is added at intervals, the process is known as “fed-batch fermentation” (De Swaaf *et al.*, 2003). The choice of whether to use the former or the latter method depends on the micro-organism used, composition of the growth medium and the target HUFA.

Submerged fermentation processes are used for the production of the only two HUFA currently successfully commercialised, namely DHA (produced by algae) and ARA (produced by fungi). Most micro-algal species are obligate photoautotrophs, but a limited number are capable of growing in the dark with an organic substrate as the sole carbon source (Wen and Chen, 2005). These heterotrophic algal species are now used commercially for large-scale DHA production in closed SmF systems. For heterotrophic algae like

Crypthocodinium cohnii and *Amphidinium spp.* the fermentation process can be controlled and oil production can continue throughout the year, as there is no seasonal or climatic dependence. It was found that when using ethanol as the carbon source, fed-batch cultivation of *C. cohnii* results in high DHA productivity, while batch cultivation was not suitable for this alga grown on ethanol as carbon source (De Swaaf *et al.*, 2003).

The fungus *Mortierella alpina* is used in the commercial production of ARA by continuous fed-batch SmF. At present, this is the only example of a commercially successful fungal lipid production process (Streekstra, 2005). This process was patented in 2005 by Suntory Limited and the invention covers several species e.g. *Mortierella elongata, Mortierella exigua, Mortierella hygrophila* and *Mortierella schmuckeri* (Akimoto *et al.*, 2005). This patent was however preceded by several patents filed by Barclay for Martek Corporation on ARA production by *Mortierella* species e.g. *M. schmuckeri* (Barclay, 2004). The same company patented the use of *Pythium insidiosum* for the SmF production of ARA which is "substantially free" of EPA (Kyle, 1997).

3.1.1 **Eicosapentaenoic acid production by SmF**

Currently the most promising process for the commercial production of EPA is by submerged fermentation (SmF) with heterotrophic algae (Wen and Chen, 2005). The heterotrophic diatom *Nitzschia laevis* was found to yield 695 mg EPA per litre in fed-batch culture (Wen and Chen, 2005).

In SmF with *Mortierella spp.* the ARA concentration in the fungal oil is usually higher than that of EPA (Certik and Shimizu, 1999). Fermentation with *Mortierella spp.* such as *M. alpina* or *Mortierella elongata* usually produces ARA, as well as a range of other HUFA such as EPA, GLA and dihomo-gamma-linolenic acid (DGLA, C20:3 n-6) (Sakuradani *et al.*, 2005) (Figure 2). Since production of infant formula requires pure ARA and DHA and the presence of EPA is considered to be undesirable (Ratledge, 2005), cultivation

conditions are usually manipulated to control the ARA:EPA ratio (Cheng *et al.*, 1999).

In the HUFA biosynthetic pathway, low temperatures encourage desaturation and also increase the ω-3:ω-6 HUFA ratio (Harwood and Guschina, 2009). Therefore, a technique known as 'temperature shifting' is used for the manipulation of the growth conditions to enhance EPA production. Incubation of fungal cultures at 20°C for the first 5 days of growth encourages biomass formation and ARA production. Cultures are subsequently incubated at temperatures as low as 12°C, which activates the temperature dependent desaturase enzymes to transform ARA to EPA (Jang *et al.*, 2000). In *M. alpina* the conversion of ARA (C20:4) to EPA (C20:5) at low incubation temperatures appears to be catalysed by ∆17 desaturase enzymes and can also be enhanced by exogenous addition of α-linolenic acid (ALA, 18:3 n-3) (Wen and Chen, 2005). *Mortierella alpina* therefore produces more EPA when grown at a low temperature and when the culture is supplemented with ALA (Ratledge, 2005).

Interestingly, a fungus unrelated to *Mortierella*, i.e. *Pythium irregulare* ATCC10951, was recommended for EPA production as a result of its ability to produce high quantities of EPA by submerged fermentation at room temperature (Zhu, 2002). This SmF process employed combinations of food processing wastes such as sweet whey permeate and soymeal waste, combined with crude soybean oil or linseed oil.

The yield for EPA production achieved by SmF, as recorded in literature, vary notably as can be seen from the data listed below:

- *Mortierella alpina,* 28.8-32.0 mg EPA/g dry fungal biomass (Bajpai *et al.*, 1991)
- *Mortierella alpina,* 600 mg EPA/L (Bajpai *et al.*, 1992)
- *Mortierella alpina* IS-4, 67 mg EPA/g dry fungal biomass; 1880 mg/L (Shimizu *et al.*, 1989c)

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- *Mortierella alpina* 20-17, 41.5 mg EPA/g dry biomass; 1350 mg/L (Shimizu *et al.*, 1989)
- *Pythium irregulare,* 42 mg EPA/g fungal biomass; 1400 mg/L (Cheng *et al.*, 1999). *P. irregulare* was grown on laboratory scale in shake-flask in nutrient broth containing 1% soymeal waste stream. EPA yields were enhanced by addition of 4% crude soybean oil to stimulate bioconversion of LA and ALA to EPA, coupled with a temperature shift from 25°C to 12°C during incubation.

3.2 **Solid state fermentation**

During solid state fermentation (SSF) micro-organisms are grown on a moist solid substrate which acts as an inert support and/or a nutrient source in the absence of free-flowing water (Pandey *et al.*, 1999), simulating low moisture fermentation reactions occurring in nature (Emelyanova, 1996). Solid state fermentation had its origin in food production and is widely used in the production of traditional Oriental foods and alcoholic beverages such as koji, sake and tempeh (Sato and Sudo, 1999). The process has since been successfully adapted for the commercial production of extracellular enzymes such as proteases and lipases (Mahanta *et al.*, 2008), and microbial metabolites for example alkaloids, biopesticides, antibiotics and organic acids (Pandey *et al.,* 2000).

During SSF microbial growth occurs mainly on the surface, penetrating inwards from the outer layers of the substrate. The culture environment is therefore heterogeneous in terms of cell growth, aeration, temperature, moisture and nutrient content (Sato and Sudo, 1999). Consequently, the factors contributing to high production are not fully understood and this also makes the process difficult to control. Nevertheless, SSF has gained increasing attention from industry as it may give similar or higher yields than corresponding submerged processes, delivering stable products with lower energy requirements and smaller volumes of polluting effluents (Pérez-Guerra

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et al., 2003), leading to a lower environmental impact. In addition, exhausted biomass, rich in assimilable proteins, can be used as feed for animals or fish (Conti *et al.*, 2001).

When micro-organisms capable of rapid growth on the substrate are used in SSF, conditions of absolute sterility may not be essential. In other cases, the micro-organisms may be unable to compete favourably with contaminants and the use of a bioreactor and aseptic cultivation would be essential. This could make the process less economical than the corresponding SmF process (Pandey *et al.,* 2000). However, when the choice of micro-organism and culture conditions allow for SSF it may be notably less costly to operate than SmF; for example, the production of ligno-cellulosic enzymes by SSF is generally cheaper than by SmF. Tengerdy (1998) compared the production costs of cellulase by SSF and SmF and found that SSF was a hundred times less expensive than SmF ($0.2/kg vs. $20/kg respectively).

The ability of micro-organisms to grow on a solid substrate is a function of their requirements of water activity, their capacity of adherence and penetration into the substrate and their ability to assimilate complex compounds in the substrate. Due to their physiological, enzymological and biochemical properties, the filamentous fungi are the micro-organisms best adapted to SSF (Pérez-Guerra *et al.*, 2003). In nature fungi typically grow on solid substrates with lower moisture levels such as decaying wood, seeds, roots and bones (Bhargav *et al.*, 2008). Filamentous fungi such as species belonging to the genus *Mortierella* can penetrate into solid substrates by a hyphal mode of growth.

Solid substrates, utilised as solid inert supports and/or sources of nutrients for fungal growth during SSF, are typically low cost raw materials or by-products of the agro-food industry, thus rendering the process economically viable (Stredansky *et al.*, 2000). Several food industry by-products were identified as potential substrates and carbon sources for SSF. These include rice bran, wheat bran, spent malt grains, peanut meal residue, sweet potato residue or mixtures of sweet potato residue and rice bran as substrate (Jang *et al.*,

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2000). It has also been found that fungi grown on agro-industrial residues can simultaneously decrease the levels of anti-nutrient compounds and partially hydrolyse substrate biopolymers, thereby increasing the nutritional value of the fermented bioproduct (Slavikova and Certik, 2005). Fermented substrates with improved protein and fungal oil content could find applications in food, feed, pharmaceutical and veterinary fields (Jang and Yang, 2008).

3.2.1 **Highly unsaturated fatty acid and EPA production by SSF**

There is general consensus that, by using *Mortierella spp.,* SSF yields higher fatty acid productivity than SmF (Dyal and Narine, 2005). Lai *et al.* (1998) found that SSF yielded nine times the amount of dihomo-gamma-linolenic acid (DGLA) than SmF. This comparison was however not based on large scale studies and according to Lindberg and Molin (1993), scaling up is technically more problematic in SSF than in SmF. Scaling up of SSF presents several challenges such as medium sterilisation, agitation to provide homogeneity, heat and gas transfer, moisture content and contamination control (Pérez-Guerra *et al.*, 2003). However, Stredansky *et al.* (2000) assessed the scalability of SSF production of HUFA and found that HUFA production by smaller and larger scale SSF were similar. The production of HUFA by SSF has also been investigated by several other researchers.

Slugen *et al.* (1994) filed a Czech. patent for the use of mixtures of prefermented solid substrates with enhanced HUFA content as inexpensive food and feed supplements (Certik and Shimizu, 1999). The main claim of the patent was the enrichment of cereal materials with nutritionally important lipids (such as ARA and GLA) by SSF with filamentous fungi belonging to the Mucoraceae. In an example fermented barley biomass was produced which contained 18 mg GLA/g bioproduct. According to Bhargav *et al.* (2008) the production of GLA via SSF is the most extensively studied of all HUFA using this fermentation process, and GLA was produced commercially in Japan with representatives of the genera *Mortierella* and *Mucor* (Conti *et al.*, 2001). It was also demonstrated that another mucoralean fungus, *Cunninghamella japonica,* could be employed to produce GLA via SSF of several cereal grains

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(Emelyanova*,* 1996). It was found that rice and millet gave the highest yields, which ranged from 7.03 to 7.87% (w/w) GLA. Using SSF in combination with yet another mucoralean fungus, i.e. *Thamnidium elegans,* Certik *et al.* (2006) enriched cereals with GLA. Later, the same research group managed to enrich several cereals with GLA, DGLA, ARA and EPA using SSF with *T. elegans* and *M. alpina* (Certik *et al.*, 2008).

Using SSF Jang *et al.* (2000) evaluated HUFA production on rice bran, wheat bran, peanut meal residue and sweet potato residue, all supplemented with linseed oil or soybean oil before fermentation. Trials revealed that rice bran was the most effective substrate with the highest yields of LA, ARA, EPA and total HUFA, yielding 47.8 mg, 54.5 mg, 12.8 mg and 122.2 mg per g of substrate carbon, respectively. The second most effective was peanut meal residue, followed by wheat bran and sweet potatoes residues. These results were achieved by fermentation with *M. alpina*.

Totani *et al.* (1987) and Stredanska *et al.* (1993) studied ARA production during SSF with *M. alpina* and achieved yields of 13 mg and 36 mg per g potato/dextrose paste and dehulled millet, respectively. More recently Jang and Yang (2008) optimised the production of HUFA (including ARA and EPA) in a solid-state column reactor packed with rice bran. They found that lowering of the incubation temperature on the fifth day (temperature shifting) increased EPA yield relative to ARA yield. Cultures of rice bran fermented by SSF had a higher ω-3 content in the total fatty acids than the corresponding SmF cultures. They also found that the upper layers of the fermented substrate had significantly higher final levels of HUFA and particularly of EPA than the lower levels. A yield of 12 mg EPA per gram of substrate carbon was achieved and the authors concluded that the HUFA-enriched rice bran could be used as an inexpensive food and feed supplement. The fermented product could be dried and used directly as feed, which would reduce down-stream processing and extraction costs.

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4. Brewers' spent grain and sunflower press cake as potential substrates for SSF aimed at producing HUFA (including EPA)

4.1 Brewers' spent grain

According to Mussatto *et al.* (2006) brewers' spent grain (BSG), a by-product from beer production, is an excellent inert support for fungal SSF. Although BSG does not contain high levels of carbohydrates compared to other cereals (Table 2), its physical properties such as particle size, specific density, high moisture content and water holding capacity favour fungal growth. Furthermore, its porous nature prevents agglomeration and allows better aeration than other moistened cereals. On average, about 20 kg (dry mass) of BSG is produced per 100 litres of beer (Mussatto *et al*., 2006). The annual production of BSG in Europe is estimated to amount to 3.5 million tons (dry mass) per year (Erasmus, 2009), while the South African beer brewing industry alone creates ~520 000 tons of BSG per year (calculated from production figures available at http://www.sablimited.co.za).

Table 2. Comparison between the general composition of brewers' spent grains and wheat

Component	BSG (% of dry mass) (Erasmus, 2009)	Wheat (% of dry mass) (Wang *et al*., 1968)
Protein	21.7	17.4
Ash	3.3	1.7
Fat	9.0	1.9[a]
Crude fibre	16.8	2.6
Carbohydrates	49.2[b]	76.5

a: ether extract

b: calculated by difference

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Thus, the abundance of this relatively protein-rich by-product attracts the attention of biotechnologists studying SSF. Not surprisingly, BSG and mixtures of BSG with other agro-processing by-products were also evaluated as substrates for bacterial SSF, aimed at producing xantham gum and succinoglycan polymers (Stredansky *et al.*, 1999). It was found that the polymer yield with SSF was two to four times higher than the yield obtained with the corresponding SmF process.

4.1.1 *The use of BSG for the production of HUFA and EPA by fungal SSF*

BSG [containing no detectable levels of ARA, EPA, DHA or GLA (Table 3)], was evaluated, in combination with other cereal by-products, for the production of GLA by *M. isabellina* M-14 (Slugen *et al.*, 1994). The same workers also described the production of EPA (17.8% of fatty acids) by *M. alpina* grown on a mixture of barley, thresh and linseed oil. Certik and Shimizu (1999) later also mentioned that SSF of cereals could be used for the production of HUFA and EPA.

Conti *et al.* (2001) screened fungal strains belonging to the order Mucorales for GLA production on mixtures of dry cereals that were moistened with a nutrient solution. These substrates consisted of pearl barley, spent malt grains, millet and rice. It was found that a strain representing *Cunninghamella elegans* produced 14.2 mg GLA per gram of a mixture of barley, spent malt grains and peanut oil. According to the authors, the BSG was incorporated into the substrate mixture, because these absorbing, porous particles that are practically devoid of nutrient substances, serve as an inert support, preventing cereal grain agglomeration.

Table 3. **The fatty acid profile of brewers' spent grain (BSG)** (Adapted from Erasmus, 2009)

Fatty acid	Fatty acid generic name	Level[a] in BSG
C14:0	Myristic	0.2
C16:0	Palmitic	22.6
C16:1	Palmitoleic	0.1
C18:0	Stearic	2.0
C18:1 n-9	Oleic (OA)	12.3
C18:2 n-6	Linoleic (LA)	51.4
C18:3 n-3	Alpha-linolenic (ALA)	5.1
C18:3 n-6	Gamma-linolenic (GLA)	0.0
C20:0	Arachidic	0.4
C20:1 n-9	Gadoleic	1.0
C20:2	Eicosadienoic	0.2
C20:3 n-6	*cis*-8,11,14-Eicosatrienoic (ETA)	0.1
C20:3 n-3	*cis*-11,14,17-Eicosatrienoic	0.2
C20:4 n-6	Arachidonic (ARA)	0.0
C20:5 n-3	Eicosapentaenoic (EPA)	0.0
C22:0	Behenic	1.4
C22:1 n-9	Erucic	1.0
C24:0	Lignoceric	0.4
C24:1 n-9	Nervonic	0.2

[a]Values refer to % fatty acid of total fat.

The disadvantages of agglomeration of substrate particles during SSF are also described by Couto and Sanroman, (2006), who concludes that the optimal particle size would be a compromise. Smaller particles are more easily colonised as it provides a larger surface area for microbial growth, but also agglomerate more easily, whereas larger particles provide less surface area for growth but better aeration. BSG thus appear to be the appropriate

compromise in terms of particle size for the SSF production of GLA with *C. elegans* (Conti *et al.*, 2001).

BSG was also incorporated into growth substrates by Stredansky *et al.* (2000). Cereal grains (finger millet, rice or pearled barley) or apple pomace were mixed with spent malt grains, the latter added to obtain adequate porosity of the solid growth substrate. These substrates were also supplemented with vegetable oils (coconut, peanut, sunflower or linseed oil) and a nutrient solution containing inorganic nitrogen. On a combination of 28.5% barley, 5.75% spent malt grains, 5.75% linseed oil and 60% nutrient solution, *Pythium ultimum* achieved a yield of 3.5 mg EPA per g wet substrate by SSF.

The choice of substrate, organism and the type of fermentation process directly affect the yield of the target HUFA. This was confirmed by the work done by Stredansky *et al.* (2000), which showed that *P. ultimum* produced HUFA faster when cultivated by solid state than by liquid fermentation. The total lipids, ARA and EPA content after 12 days in the latter were 2.9 mg/ml, 0.31 mg/ml and 0.21 mg/ml culture broth respectively. Conversely the values were as high as 35.0 mg/g, 2.10 mg/g and 1.14 mg/g wet substrate, respectively, after only 7 days of cultivation on the barley based solid substrate.

Slavikova and Certik (2005) reported on the use of BSG in SSF with *Thamnidium elegans* and *M. alpina*. A variety of agricultural residues were combined and carbon sources and oils were added. The aim of the study was to produce a HUFA-enriched fermented bioproduct as an inexpensive feed and food supplement. *M. alpina* converted a mixture of wheat bran and spent malt grains (3:1 w/w) to a bioproduct with 42.3 mg ARA/g fermented substrate. The process of SSF of a combination of peeled barley, linseed oil and spent malt grains (0.5:1:3 w/w) with *M. alpina* yielded a bioproduct containing 23.4 mg EPA and 36.6 mg ARA per g fermented substrate. These results were also presented by Certik and Slavikova (2004). Later, Certik and co-workers (2006) used a mixture of wheat flakes and BSG (3:1 w/w) and

produced 7.2 mg GLA/g bioproduct fermented with *T. elegans.* BSG added as an internal support increased the GLA yield significantly.

4.2 **Sunflower press cake**

Bautista *et al.* (1990) studied the lignocellulosic fraction of sunflower meal and concluded that it is a suitable fermentation substrate for fungal SSF. Also, it has been stated that a by-product of the sunflower oil industry, sunflower oil cake has potential as substrate for industrial fermentations (Ramachandran *et al.*, 2007). This is mainly due to the relatively high protein content of 27 to 34%, as well as the high energy and fibre content of sunflower press cake (SPC) (Table 4; Alexis, *ca.* 1996).

The annual global production of sunflower meal was 9.6 million tons in 2005 (Gill *et al.*, 2006) and production is steadily increasing. In the year ending March 2009, approximately 364 754 tons of SPC was available in South Africa, most of which was locally produced (Animal Feed Manufacturers Association, 2009). This supply is expected to rise rapidly with the production of biodiesel from sunflower oil.

Currently, SPC is mainly utilised as ruminant animal feed. The average price of SPC is also much lower than that of fish meal ($94 vs. $641 per ton in 2005), although fish meal has double the protein content (Gill *et al.*, 2006). The use of SPC as fish meal replacement in animal/fish diets is therefore receiving urgent consideration, but there are some hurdles in the application of SPC as fish or poultry feed. The protein in SPC is low in lysine, methionine and cystine.

Commercial SPC does not contain many antinutritional factors except for high fibre, lignocellulose, phytic acid (4.3% according to Miller *et al.*, 1986) and chlorogenic acid, which may reduce the bioavailability of some amino acids like lysine (Gill *et al.*, 2006). High temperature extrusion may reduce the phytic acid content of SPC, but the phenolic compounds, fibre and

lignocellulose remain problematic in the use of SPC as a fish meal replacement.

Solid state fermentation of sunflower by-products has been evaluated in several biotechnological processes. These include production of protein-enriched feeds by SSF with *Aspergillus tamarii* (Hongyan, 1999), biogas by anaerobic fermentation (Jagadesh *et al.*, 1996) and the antibiotics cephamycin C (Kota and Sridhar, 1999) and clavulanic acid (Sircar *et al.*, 1998) by bacterial SSF with *Streptomyces clavuligerus.*

Table 4. Composition of sunflower press cake (SPC) (Mérida *et al.*, 2010)

Nutrient	Level in SPC (% of dry mass)
Moisture	10,7
Protein	35.0
Ash	7.0
Fat	1.2
Crude fibre	23.6
N-free extract	33.3

4.2.1 *The use of SPC for the production of HUFA and EPA by fungal SSF*

Sunflower press cake (SPC) contains higher levels of LA than ALA and is therefore more suitable for the production of ω-6 HUFA than ω-3 HUFA in eukaryotes (Figure 2). Consequently, sunflower may act as one of the major sources of the ω-6 precursor, LA in the human diet (www.cyberlipid.org). However, due to its fatty acid profile and the presence of anti-nutrients such as chlorogenic acid, SPC and sunflower oil are seldom used as substrates for fermentation with the aim of producing fish feeds. The high level of crude fibre in SPC may also interfere with the digestion of other nutrients in fish (Alexis, *ca.* 1996). This led Davies (1985) to erroneously report negative digestibility

coefficients for sunflower meal with sea bream. According to Pike and Barlow (2002), the high levels of ω-6-fatty acids in vegetable oils hinder the synthesis of EPA and DHA. Hemre (2002) reported on the low levels of EPA in the liver and fillet of Atlantic salmon fed on a diet substituting fish oil with sunflower oil and reiterates the urgent need for alternatives to fish oil. When Maina *et al.* (2003) fed tilapia on a diet containing SPC, the lipid profile of both the diet and the fish contained LA as the major fatty acid. Tilapia was however capable of converting some of the eighteen carbon fatty acids in the diet to longer chain HUFA.

For the above-mentioned reasons, no reference could be found on the use of SPC for the production of HUFA by SSF. However, Slavikova and Certik (2005) and Weber (2003) reported on the effect of sunflower oil on the growth of *Mortierella spp.* and subsequent production of HUFA by SSF. Weber described the spontaneous fermentation with *Mortierella spp.* when sunflower oil was disposed of onto rotting compost during winter in Germany. The formation and movement of large storage lipid droplets in the cytoplasm of the fungal hyphae was studied by fluorescence microscopy. Lipid formation was increased at low temperature. In another study, it was found that addition of 50% (w/w) sunflower oil to SSF cultures of *Thamnidium elegans* and *M. alpina,* grown on a mixture of wheat bran and spent malt grains, increased the yield of GLA (Slavikova and Certik, 2005).

5. Conclusions

According to market analysts Frost and Sullivan, the wider European market for omega-3 ingredients obtained from marine, algal and flaxseed sources, is predicted to reach a staggering $1.6bn by 2014 (Frost and Sullivan, 2009). These predictions are based on an annual growth of 24% in consumer demand (Daniells, 2009). However, the major global demand for omega-3 oil will continue to come from the aquaculture sector. Between 1997 and 2020 fish consumption in developing countries is expected to rise by 57% to 98.6 million metric tons (International Food Policy Research Institute, 2004). The

average compounded annual growth rate for aquaculture was 9.2% since 1970 (Erasmus, 2009) with a total global aquaculture production of 30 Mton in 1997 (Hardy and Tacon, 2002).

The ever-increasing demand for omega-3 HUFA is currently met with fish oil, but this is not a sustainable long term option (Naylor *et al.*, 2000). At present, 1.2 million tons of fish oil is produced annually of which 70% is used to feed farmed fish (Hemre, 2002; Sijtsma and De Swaaf, 2004).

Fish oil also poses risks in terms of toxins such as dioxins, polychlorobiphenyls, organo-heavy metals and mercury entering the marine food chain (Ratledge, 2005) and has an objectionable taste, which makes it an undesirable option for the neutraceutical/pharmaceutical industry (Sijtsma and De Swaaf, 2004).

Fish oil contains high levels of EPA and DHA, the two major ω-3 HUFA required in the aquaculture industry (Sargent *et al.*, 1999). The production of EPA and DHA by alternative methods such as fermentation is the subject of current research. The cost of submerged fermentation processes prohibits the successful commercialisation of some HUFA, but could be reduced by using inexpensive processing by-products as fermentation feedstocks (Du Preez *et al.*, 1997; Peng and Chen, 2008). The production of EPA by fermentation of solid by-product substrates with *Mortierella* fungi could provide a safe alternative to fish oil and alleviate the environmental pressure on wild fish stocks.

From the above it is clear that the global demand for HUFA, especially EPA, is on the increase and that alternative sources to fish oil are urgently needed, thus the objectives of this study were firstly to evaluate two agro-processing waste products, brewers' spent grain (BSG) and sunflower press cake (SPC), as substrates for EPA production by SSF. In both cases, SSF was performed using different representatives of the oleaginous fungal genus *Mortierella.* A secondary objective was to establish a cost-effective alternative production process for EPA by manipulating culture conditions to enhance EPA yield. An

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oil rich in the ω-3 precursor ALA, i.e. linseed oil, was added to the substrate before fermentation and the incubation temperature was manipulated to improve conversion of the added oil to EPA.

To achieve the above mentioned goals BSG was evaluated as substrate for the production of EPA by SSF with different representatives of the genus *Mortierella*. Also, the effect of linseed oil supplementation on EPA production by the cultures was determined (See Chapter 2). Subsequently, SPC was evaluated as substrate for the production of HUFA by SSF with a number of representatives of *Mortierella.* During this study the effect of supplementing the SPC with linseed oil was also studied with regard to the supplement's impact on EPA production and the fatty acid profile of the fermented substrate (See Chapter 3).

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Quote:

“Growth is always accompanied by stretching.” - Anonymous

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Chapter 2

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

Parts of this chapter have been accepted for publication in the Proceedings of the ICC International Grains Symposium held at the University of Pretoria, Sanlam Conference Centre, Pretoria, South Africa from 3 to 5 Feb 2010 (Appendix 1). Also, parts of this chapter have been published in Jacobs *et al.* (2009) Biologia 64: 871—876.

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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 and 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 and Shimizu, 1999; Zhu, 2002). The main dietary source of ω-3 HUFA is marine fish oil (Dyal and Narine, 2005; Ward and 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 and Singh, 2005).

Several terrestrial micro-organisms can produce lipids and HUFA, accumulating up to 80% of their biomass as lipids. Micro-organisms that are able to accumulate over 25% lipid on a dry cell biomass basis, are called oleaginous organisms (Higashiyama *et al.*, 2002; Dyal and Narine, 2005). This study focused on HUFA production by saprophytic filamentous soil fungi belonging to the orders Mucorales and Mortierellales (Cavalier-Smith, 1998; Dyal and Narine, 2005). The oil produced by these fungi has GRAS status (Cohen and 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).

SSF processes involve growth of micro-organisms 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 (Pérez-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 solid state fermentation (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 520 000 tons of brewers' spent grain (BSG) per year (http://www.sablimited.co.za). The unfermented substrate contains ~21% crude protein and has a total oil content of ~9% of which more than half is linoleic acid (C18:2 n-6), a precursor for the ω-6 HUFA (Erasmus, 2009). To enhance ω-3 HUFA production by oleaginous micro-organisms 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 and 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 and 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 were depleted (Bajpai *et al.*, 1991), 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

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of SSF cultivation. This temperature shift activates the desaturase enzymes catalysing the conversion of arachidonic acid to EPA in oleaginous fungi (Zhu, 2002; Cohen and Ratledge, 2005).

The production of EPA by *Mortierella* strains during SSF with BSG as sole substrate has not been evaluated. Solid state fermentation studies by Jang *et al.* (2000), Stredansky *et al.* (2000), and Slavikova and 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 brewers' spent grain 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 linseed oil supplement on EPA production was determined while these fungi were used in SSF of the spent grain.

Materials and Methods

Micro-organisms 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 (gram per litre): 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

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400 Stomacher for 2 minutes) 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 to provide optimal moist conditions for SSF in the absence of free-flowing water. Dried spent grain was then treated by one of the following two methods before inoculation: Ten percent (w/w) LSO was added to half of the substrate treatments whereas the rest of the treatments were not supplemented with LSO. Flasks were covered with cotton wool and cloth lids 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 the treatments (with and without linseed oil supplementation). 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. The effects of humidity, air-flow and light intensity were not evaluated and these parameters therefore remained at ambient levels.

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 minutes) 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 x 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 [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 (Pérez-Guerra *et al.*, 2003). Apart from the addition of LSO as the EPA precursor, several other factors such as incubation temperature (Dyal and Narine, 2005), carbon to 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).

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An important factor impacting on growth of *Mortierella* is the level of aeration of the cultures. Lindberg and 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 and 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.

Table 1. Lipid content and eicosapentaenoic acid produced by representatives of the genus *Mortierella* while growing on brewers' spent grain (BSG) with or without linseed oil (LSO) supplementation.

Fungal Strain Number	Species	Total lipids[a]			Eicosapentaenoic acid [b]		
		[c]BSG - LSO % (w/w)	[c]BSG +LSO % (w/w)	% Increase lipids	[c]BSG - LSO (mg/g)	[c]BSG +LSO (mg/g)	% Increase EPA
Mo 018	*Mortierella 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	-25.0
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

Table 1 (continued …).

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Fungal Strain Number	Species	Total lipids[a]			Eicosapentaenoic acid [b]		
		[c]BSG - LSO % (w/w)	[c]BSG +LSO % (w/w)	% Increase lipids	[c]BSG - LSO (mg/g)	[c]BSG +LSO (mg/g)	% Increase EPA
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%)

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) (Table1). 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.

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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 and Shimizu, 1999), but vary in the amount of lipid produced between and within species. The total lipid content is important (Dyal and 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 and Shimizu, 1999; Jang *et al.*, 2000).

When LSO enriched BSG was used as substrate 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

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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 (Stredansky *et al.*, 2000; Jang *et al.*, 2000; Slavikova and 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. alpina.* The solid substrates were also supplemented with nitrogen sources such as yeast extract and nitrates. Rice bran fermentation produced *ca.* 6 mg EPA/g substrate and production was increased to ca. 11 mg EPA/g by the addition of 1% LSO. Better results were also obtained by Slavikova and 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 and Singh, 2005), but as lipid and HUFA production are triggered by nitrogen limitation (Koike *et al.*, 2001), the

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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 interspesific 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 and 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 (Figure 1A).

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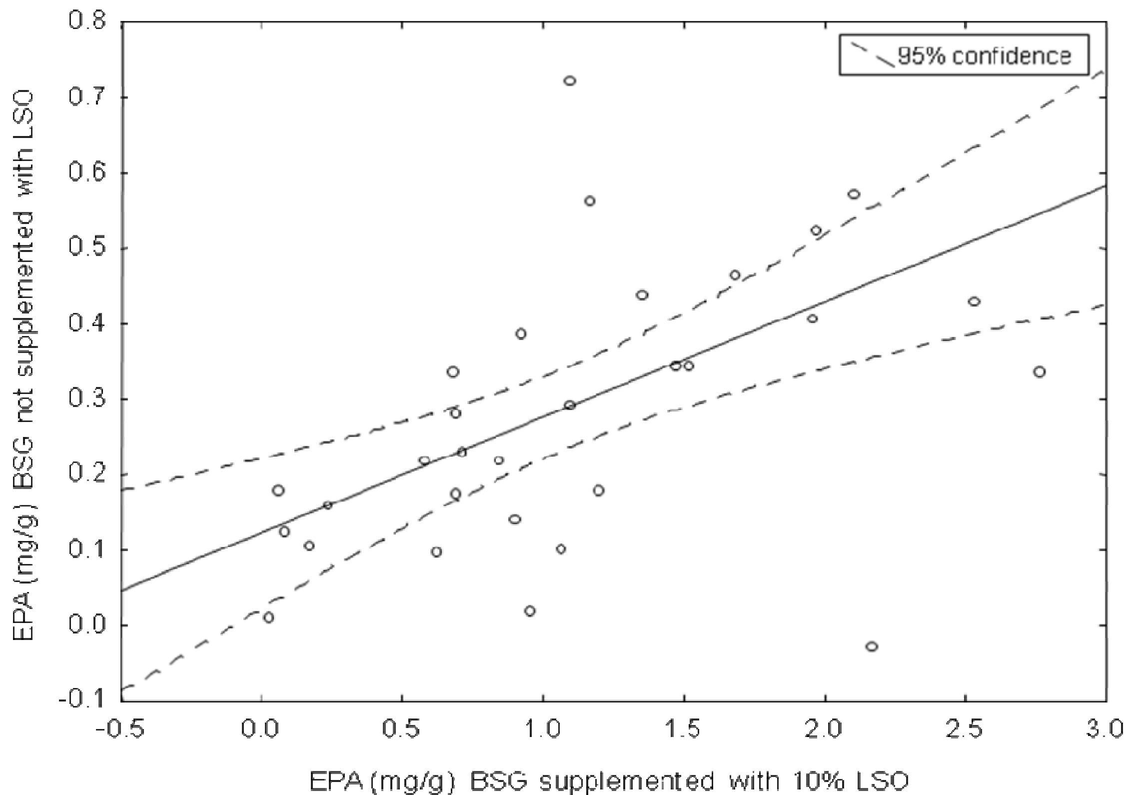

Figure 1A. Scatterplot of EPA content of the fermented BSG supplemented with 10% (w/w) LSO plotted against the EPA content of the fermented BSG not supplemented with LSO.
(BSG: brewers' spent grain; EPA: eicosapentaenoic acid; LSO: linseed oil)

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.

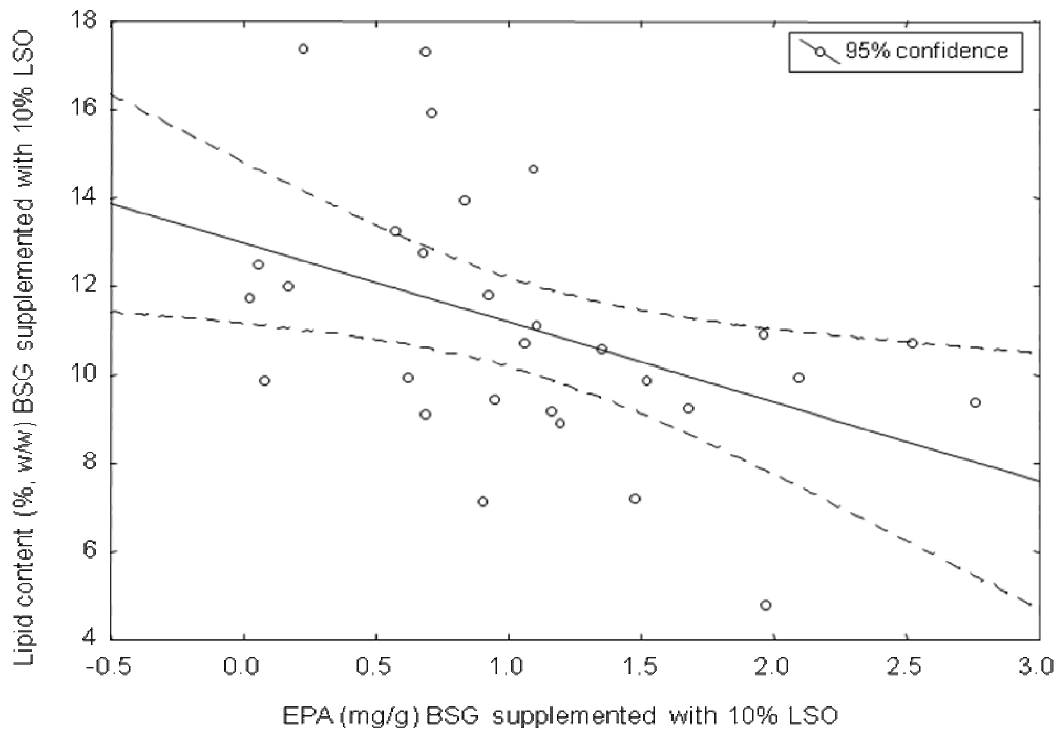

Figure 1B. Scatterplot of EPA content of the fermented BSG supplemented with 10% (w/w) LSO plotted against the total lipid content of the fermented BSG supplemented with 10% LSO.

(BSG: brewers' spent grain; EPA: eicosapentaenoic acid; LSO: linseed oil)

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 (Figures 1 B and C respectively).

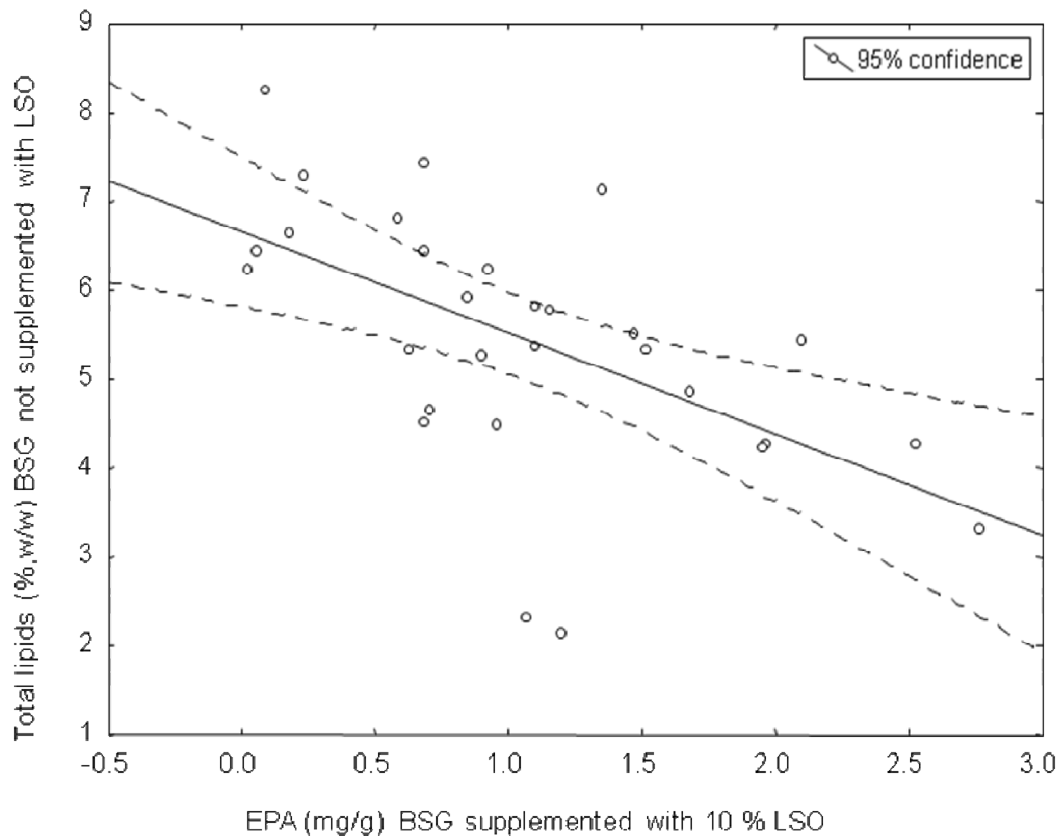

Figure 1C. Scatterplot of EPA content of the fermented BSG supplemented with 10% (w/w) LSO plotted against the total lipid content of the fermented BSG not supplemented with LSO.

(BSG: brewers' spent grain; EPA: eicosapentaenoic acid; LSO: linseed oil)

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 (Figures 1 B and 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.

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Conclusions

This study proved that the production of eicosapentaenoic acid by solid state fermentation 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.

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Chapter 3

Sunflower press cake as a substrate for eicosapentaenoic acid production by representatives of the genus *Mortierella*

Parts of this chapter have been accepted for publication in the Proceedings of the ICC International Grains Symposium held at the University of Pretoria, Sanlam Conference Centre, Pretoria, South Africa from 3 to 5 Feb 2010 (Appendix 1).

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Introduction

Eicosapentaenoic acid (EPA, C20:5 n-3) is a metabolically important omega-3 highly unsaturated fatty acid (ω-3 HUFA), 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 HUFA by elongation and desaturation of shorter chain fatty acids, these HUFA have to be included in the diet (Certik and Shimizu, 1999; Dyal and Narine, 2005). Currently the main dietary source of ω-3 HUFA is marine fish oil (Sijtsma and De Swaaf, 2004). However, there is growing concern about the sustainability of this source of ω-3 HUFA oil, mainly because of increasing demand for fish oil by the aquaculture and pharmaceutical industries (Certik and Shimizu, 1999; Hasan *et al.*, 2007). Alternative sources of ω-3 HUFA oil are therefore explored.

Recent research focused on microbial and especially algal production of ω-3 HUFA 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 HUFA. These fungi commonly occur in soil (Streekstra, 1997) and are known to produce a range of microbial oils and HUFA, including EPA, γ-linolenic acid (GLA, C18:3 n-6), dihomo-γ-linolenic acid (DGLA, C20:3 n-6) and the ω-6 arachidonic acid (ARA, C20:4 n-6), 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 (Figure 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.

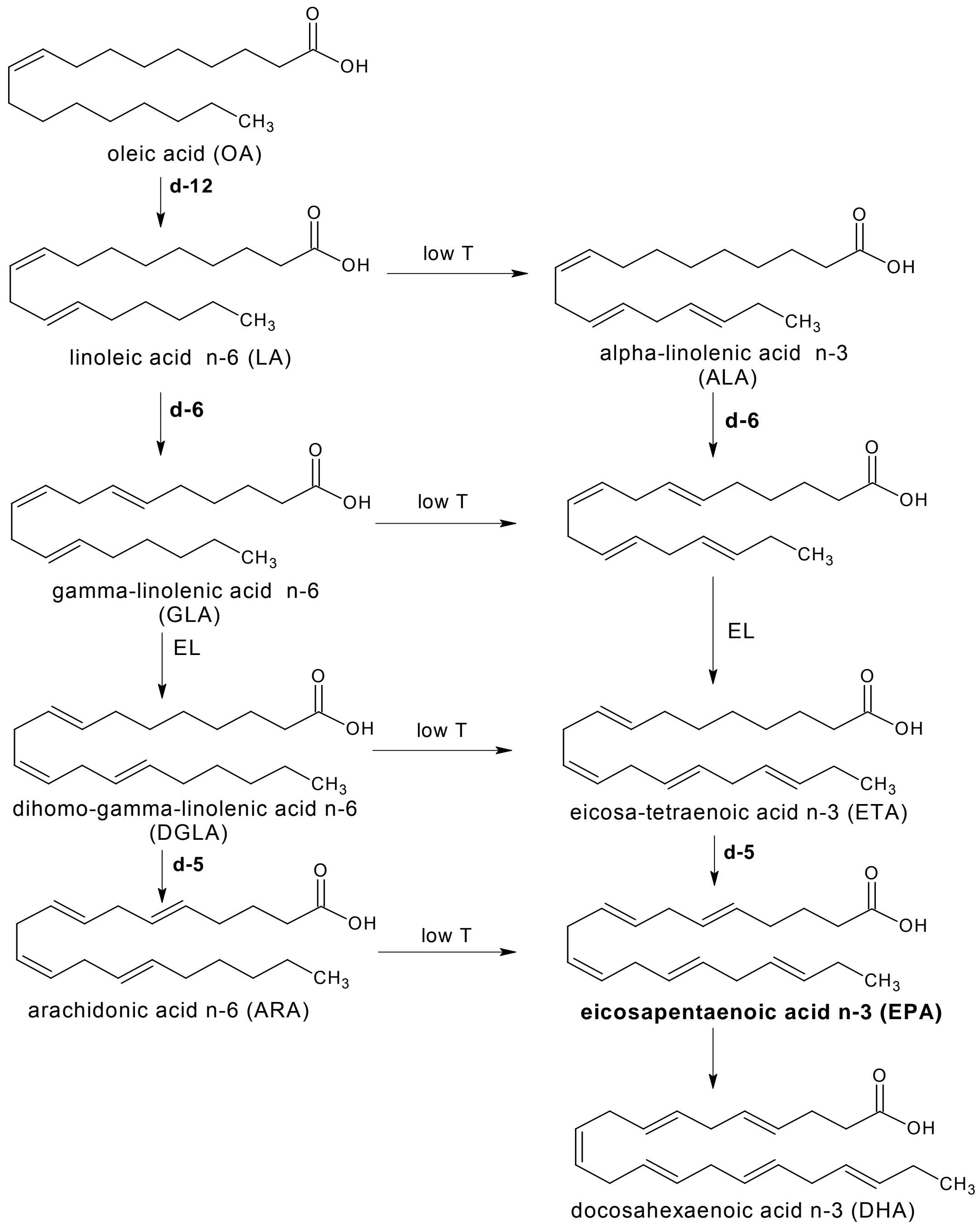

Figure 1. Biosynthetic pathways of the production of poly-unsaturated fatty acids including EPA by eukaryotes (adapted from Certik & Shimizu, 1999 and Pereira *et al.*, 2004)
(d = desaturase, EL = elongase, T = temperature, ω = omega)

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Microbial HUFA-containing oils are usually produced by submerged fermentation within a liquid medium. However, it was demonstrated that the rate of HUFA 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 HUFA 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 or 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 by-products presents an attractive alternative substrate to be used during SSF. The global agro-processing industry generates millions of tons of cereal derived by-products 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 by-products are expected to increase substantially in the near future as a result of the growing biofuel industry which generates by-products 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 (Chapter 2). 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 ~57% of the ω-3 precursor α-linolenic acid (ALA, C18:3 n-3), improved the EPA yield of most strains.

Bautista *et al.* (1990) studied the lignocellulosic fraction of sunflower meal and concluded that it is a suitable fermentation substrate for fungal SSF. However, the production of EPA by *Mortierella* strains grown on SPC using SSF has not

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been evaluated previously. 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.*

Materials and Methods

Fungal isolates and inoculum preparation

A liquid inoculum was prepared from each of 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 (gram per litre): 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 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 20 g 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 2 ml of the homogenised inoculum for both LSO treatments. The inoculated cultures

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were incubated at 22°C for three days to obtain optimal fungal growth. To enhance HUFA production the temperature was subsequently lowered to 16°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 after 5 days growth at 25°C. Fermented substrate was milled and oven dried (50°C; 48 h) and the moisture content was determined by AACC Method 44-20 (http://www.aaccnet.org/approvedmethods/).

Total lipids and EPA contents were determined on the complete dried fermented biomass. Lipids were extracted from the fermented biomass using solvent extraction on the Soxhlet apparatus. 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. Separation was performed on a polyethylene glycol-based capillary column, (30 m x 0.32 mm ID Omegawax 320 with 0.25 µm film thickness, Supelco, Catalogue Number 24152) using the following conditions:

Carrier gas	:	Helium at 76 kPa
Detector temperature	:	300 °C
Injection temperature	:	250 °C
Gases	:	Air at 420mL/min
		H_2 at 40mL/min
Auxiliary gas	:	N_2 at 10mL/min
Split vent flow	:	79cm^3/min
Oven temperature programme:		140°C (hold 5 min) to 240°C

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@ 4°C/min (hold 10 min)

Run time : 41 minutes

Injection volume : 1µL, split ratio 50:1

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 24 cultures (containing eight triplicates), as well as three un-inoculated 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, version 7.1; Statsoft, Inc., Tulsa, Oklahoma, 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 Table 1.

Compared to previous results, obtained when similar experiments were conducted with BSG as substrate for SSF (Chapter 2), SPC as substrate generally led to the production of higher levels of EPA. However, SPC lacks in the ω-3 precursor for EPA production, i.e. ALA (Figure 1).

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

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increased the amount of EPA produced by the isolates (Table 1). This supports previous findings using BSG supplemented with LSO as substrate for EPA production (Chapter 2), 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).

Table 1. Final lipid content, arachidonic acid and eicosapentaenoic acid produced by representatives of the genus *Mortierella* grown on sunflower press cake (SPC) with or without linseed oil (LSO) supplementation

Fungal Strain Number	Species	Total lipids[a]			Arachidonic acid			Eicosapentaenoic acid		
		SPC - LSO % (w/w)	SPC +LSO % (w/w)	% Increase lipids	SPC - LSO (mg/g)	SPC +LSO (mg/g)	% Increase ARA	SPC - LSO (mg/g)	SPC +LSO (mg/g)	% Increase EPA
Mo 46	*Mortierella alpina*	7.50 (0.20)	13.37 (0.12)	5.78	12.48 (0.47)	24.24 (1.12)	94.26	0.08 (0.00)	6.44 (0.43)	7972.74
Mo 47	*M. selenospora*	7.35 (0.51)	12.77 (0.06)	5.42	5.47 (0.21)	7.07 (0.88)	29.09	0.38 (0.04)	2.36 (0.26)	523.96
Mo 50	*M. alpina*	8.93 (0.12)	13.77 (0.83)	4.84	5.48 (0.85)	17.09 (3.55)	211.98	0.11 (0.01)	4.82 (0.94)	4381.68
Mo 88	*M. basiparvispora*	7.70 (0.04)	13.50 (0.31)	5.80	13.48 (1.28)	21.73 (1.44)	61.21	0.12 (0.03)	5.81 (0.58)	4760.24
Mo 101	*M. epicladia*	8.23 (0.26)	13.56 (0.51)	5.33	7.90 (0.30)	14.40 (2.33)	82.32	0.22 (0.02)	4.21 (0.64)	1780.60
Mo 102	*Mortierella spp*	10.37 (0.21)	16.32 (0.85)	5.95	0.86 (0.16)	2.13 (0.43)	147.14	0.11 (0.00)	0.50 (0.16)	352.72
Mo 114	*Mortierella spp*	10.14 (0.19)	16.30 (0.30)	6.16	1.09 (0.15)	2.31 (0.35)	112.67	0.11 (0.00)	0.58 (0.10)	435.61
Mo 130	*Mortierella spp*	9.86 (0.17)	15.60 (0.30)	5.74	3.74 (0.23)	6.25 0.21)	67.13	0.21 (0.01)	1.74 (0.06)	718.74
SPC control	Un-inoculated	14.78 (0.21)	22.64 (0.82)	7.86	0.16 (0.00)	0.24 (0.01)	52.85	0.16 (0.00)	0.24 (0.01)	52.85

Values represent the mean of 3 repetitions
The standard deviation (SD) of each mean is indicated in brackets
SPC: sunflower press cake; LSO: linseed oil; ARA: arachidonic acid; EPA: eicosapentaenoic acid

In the non-fermented SPC substrate 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, C18:2 n-6) than the ω-3 precursor (ALA) (Maina *et al.*, 2003)**,** therefore cultures prepared with the *Mortierella* strains growing on SPC were characterized 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 (Figure 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).

Under conditions of carbon limitation in the growth substrate, oleaginous fungi can utilise lipids as an energy source for growth (Weber and Tribe, 2003; Dyal and Narine, 2005). *Mortierella* isolates could therefore catabolise the accumulated 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).

To further investigate the mechanism whereby additional LSO was able 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 lower total levels of fatty acids with chain lengths of less than 20 carbon atoms, indicating that relatively more long chain fatty acids were produced by these isolates (Figure 2). LSO addition had a positive effect ($r = 0.9291$, $p = 0.001$) on the relative amount of long chain fatty acids produced.

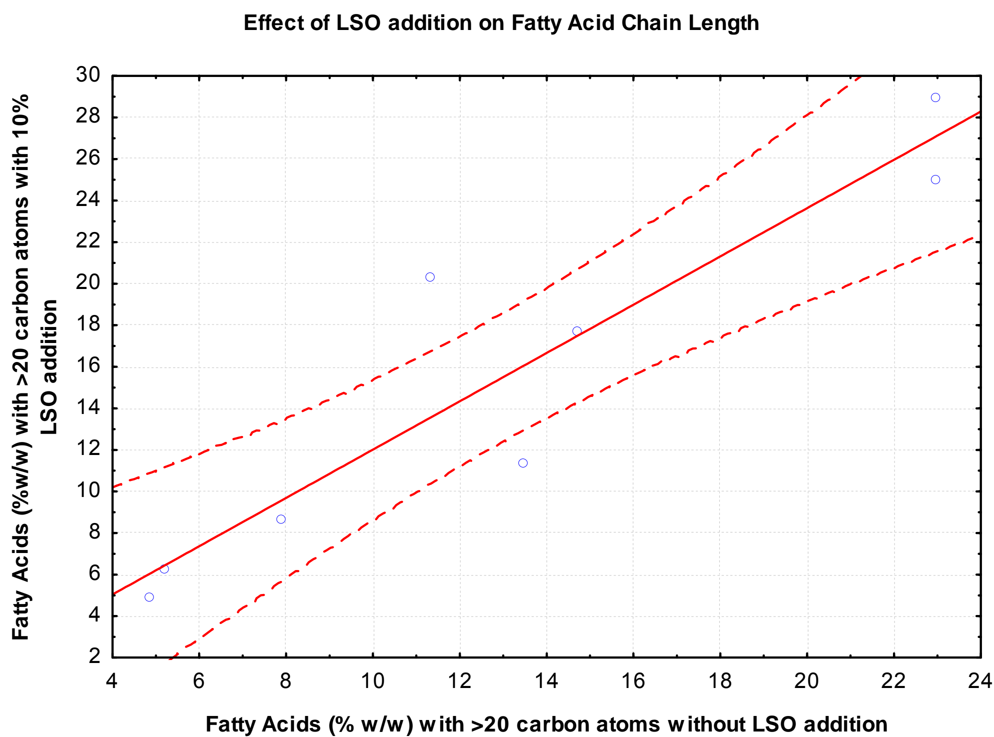

Figure 2. Correlation of the percentage of long chain (C = 20 or more) fatty acids, relative to total fatty acids, produced by eight fungal isolates grown on sunflower press cake with or without addition of linseed oil (LSO)

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 HUFA, containing two or more double bonds; to saturated and monoenoic fatty

acids, was increased significantly (t=5.75, p=0.05) by addition of LSO to the substrate (Figure 3).

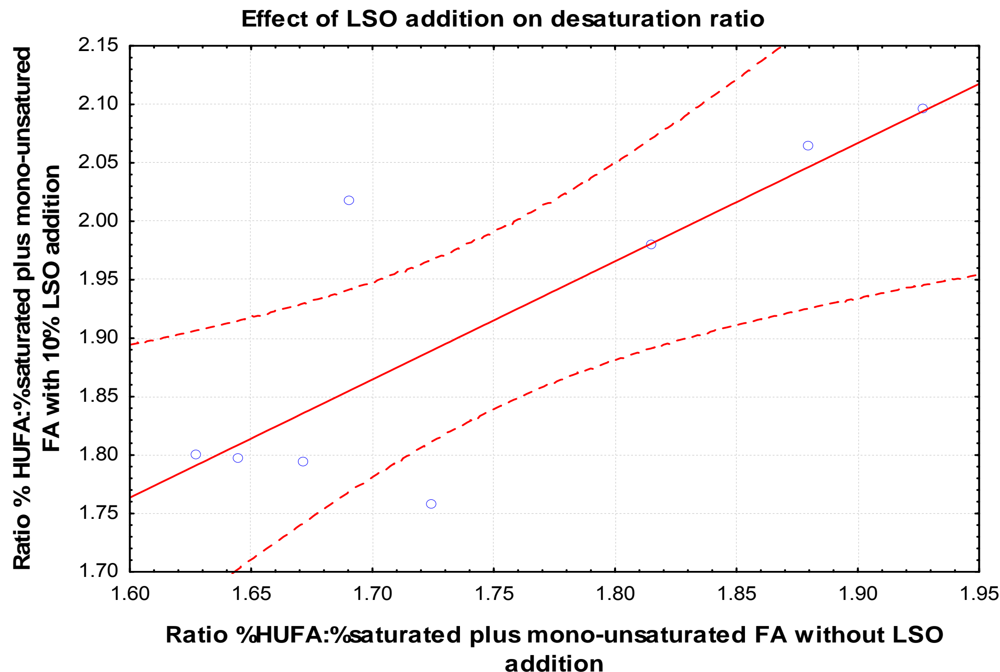

Figure 3. Correlation of the desaturation ratios - % HUFA (fatty acids with two or more double bonds): % saturated and monoenoic fatty acids, among the 14 to 24 carbon fatty acids of eight fungal isolates grown on SPC with or without addition of LSO

(FA: fatty acids; HUFA; highly unsaturated fatty acids; LSO: linseed oil; SPC: sunflower press cake)

Thus, the activity of fatty acid desaturase enzymes seems to have been enhanced by LSO addition, indicating that in addition to increasing the levels of EPA, the levels of other HUFA such as ARA, GLA and DGLA were also increased by supplementing the SPC with LSO.

When desaturation ratios among only the 18-carbon fatty acids were similarly determined for the cultures with and without additional LSO, it was found that the level of saturation of these fatty acids was not significantly increased by the

addition of LSO to the substrate (results not shown; r = 0.3069, p = 0.460). This indicated that the high levels of ALA introduced to the cultures by addition of LSO, were effectively metabolized by the cultures. Some of these metabolic products would have been 20-carbon HUFA, since cultures to which LSO was added were characterized by higher desaturation levels among their 20-carbon HUFA than cultures which received no LSO. This was evident from the positive correlation in desaturation ratios of these fatty acids observed between cultures with no additional LSO and those that were supplemented with this oil (Figure 4, r = 0.9140, p = 0.002).

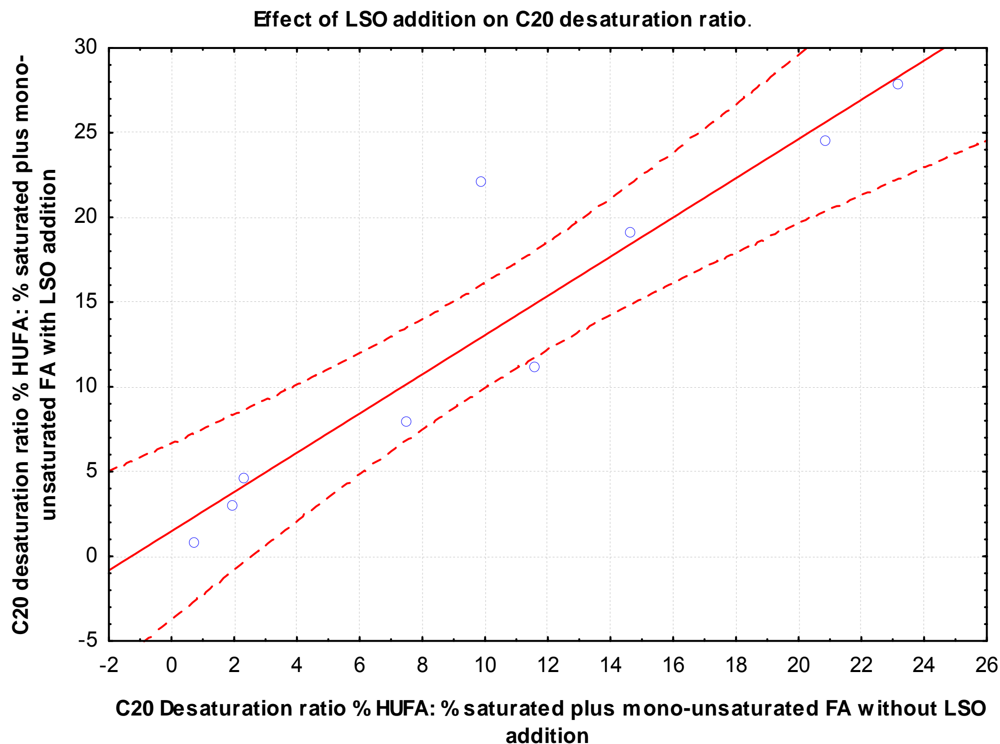

Figure 4. Correlation of the desaturation ratios - %HUFA: % saturated and monoenoic fatty acids, among the 20 carbon fatty acids of eight fungal isolates grown on SPC with or without addition of LSO

(FA: fatty acids; HUFA; highly unsaturated fatty acids; LSO: linseed oil; SPC: sunflower press cake)

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It is therefore evident that the fungal ∆6 and ∆5 desaturase and elongase enzymes (Figure 1) successfully incorporated and converted the ALA in the added LSO to C20 HUFA such as EPA and ARA.

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

During our study fermentation with *Mortierella* fungi produced ARA as well as a range of other HUFA, including EPA, GLA and DGLA (Figure 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 (Chapter 2).

The challenge in fungal HUFA production is to modify the lipid composition of the product to contain higher levels of the desired HUFA (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°C. These measures increased the enzyme activity along the ω-3 biosynthetic pathway and lead to the formation of higher levels of EPA.

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Conclusions

Fungi from the genus *Mortierella* can successfully convert LSO supplemented lignocellulose-rich wastes, such as SPC, to materials containing HUFA, thereby adding value to these substrates. Fungal strains showing the most potential were *M. alpina* Mo 46 and *M. basiparvispora* Mo 88, producing the highest 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 ω-3 HUFA-enriched agroprocessing by-products. The SPC enriched with HUFA which is 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 industry.

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Chapter 4

Conclusions and recommendations

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Conclusions and Recommendations:

During this study we investigated fungal production of eicosapentaenoic acid (EPA) as a sustainable alternative to the use of fish oil to meet the ever-increasing global demand for omega-3 oil.

Results of this study, as well as of others (Slugen *et al.*, 1994; Jang *et al.*, 2000; Slavikova and Certik, 2005; Certik *et al.*, 2008; Jang and Yang, 2008), proved that the production of EPA by fungal fermentation of solid agro-processing by-products with *Mortierella spp.* could provide an alternative source of ω-3 oil and alleviate the environmental pressure on wild fish stocks. This study achieved its main objective by proving that EPA could be successfully produced by solid state fermentation of supplemented brewers' spent grain (BSG) or sunflower press cake (SPC) using *Mortierella* fungi. All the *Mortierella* strains produced lipids containing the target HUFA on these low-cost substrates. The average EPA content was significantly ($p=0.00$) enhanced from 0.29 (SD = 0.18) mg to 1.08 (SD=0.71) mg per g fermented BSG by the addition of linseed oil to the substrate before fermentation (Chapter 2). Two strains, *Mortierella antarctica* Mo 67 and *Mortierella epicladia* Mo 101, respectively produced 2.8 mg and 2.5 mg EPA per g of linseed oil supplemented BSG. These strains most efficiently converted the added EPA precursor in the linseed oil by enzymatic desaturation and elongation to produce higher levels of EPA.

Solid state fermentation of SPC with *Mortierella* strains generally led to accumulation of higher levels of EPA than of the BSG substrate (Chapter 3). The substrate was therefore more suitable for EPA production by *Mortierella* spp., possibly due to its nutritional composition and/or physical properties such as porosity, moisture retention and particle size. Linseed oil supplementation again improved the EPA yield in the majority of *Mortierella* strains.

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The strains showing the most potential on SPC were *M. alpina* Mo 46 and *M. basiparvispora* Mo 88, producing 6.4 and 5.8 mg EPA per g of linseed oil supplemented SPC respectively. These levels are in agreement with the findings recorded in literature such as Jang *et al.* (2000), who recorded yields of *ca.* 6 mg EPA/g and *ca.* 11 mg EPA/g fermented rice bran without and with linseed oil addition respectively. The rice bran was also supplemented with nitrogen sources such as yeast extract.

The major application of ω-3 HUFA is as feeds for the aquaculture industry. It is therefore suggested that the fermented SPC could be evaluated as marine fish feed. The effect of SSF with *Mortierella* strains (Mo 46 and Mo 88) on the levels of fibre, antinutrients (such as phytic acid and chlorogenic acid), as well as on the levels of nutrients (such as proteins and lipids) should be assessed by analyses. The fermentation process altered the fatty acid profile of the SPC substrate (Chapter 3) and is also expected to modify the amino acid profile. Ultimately, the suitability of fermented EPA-enriched SPC as feed for marine aquaculture should be evaluated in carefully controlled feeding trials.

As the secondary objective was to improve the economic competitiveness of microbial EPA production, the feedstock costs were kept at a minimum for the purposes of this study. The cost and advantages of the basic solid substrate should be considered in a thorough economic feasibility study. Brewers' spent grain might for instance be a lower cost substrate than rice bran or SPC, but the higher substrate costs could be offset by higher EPA yields. Addition of supplements such as nitrogen or linseed oil should also be carefully evaluated to determine the economic benefits.

It should also be noted that the strains producing the highest yields of EPA on BSG differed from the strains with the most efficient conversion of SPC. This demonstrates that the relative amounts of HUFA produced can vary between *Mortierella* species (Dyal and Narine, 2005) and also that each strain has its own individual optimal combination of nutrients and substrate characteristics for growth and EPA production. Screening and fermentation trials (Chapters 2

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and 3) were performed under conditions selected by performing preliminary trials. The preliminary trials (results not shown) were performed to select the combination of conditions most favourable for fungal growth and EPA production by the majority of the screened isolates. These trials investigated the performance of a diverse selection of *Mortierella* isolates with various inoculation techniques and concentrations, incubation temperatures and linseed oil supplementation concentrations.

The culture temperature used during fermentation trials reported on in Chapters 2 and 3 were adapted during incubation to provide optimal fungal growth conditions (at 22°C) for the first three days of cultivation, followed by eight days at 16°C. It is known that lower incubation temperatures enhance EPA production with concurrent reduction in the ARA:EPA ratio (Jang *et al.*, 2000). Whereas maintaining the lower incubation temperature would constitute an added expense under local conditions, it could actually lead to a reduction in production cost in the European climate. This factor should also be built into the techno-economic evaluation model.

The *Mortierella* strains which converted the LSO most efficiently to EPA should be further investigated to optimise growth conditions during scaling-up of solid state fermentation. Results of scaled-up trials should also be subjected to economic evaluation and compared with corresponding larger scale submerged fermentation. According to Lindberg and Molin (1993) scaling up is technically less problematic in submerged fermentation. However, Stredansky *et al.* (2000) assessed the scalability of solid state production of HUFA and found good correlation between small- and larger-scale cultivation results. Humphrey (1998) remarked that scale-up is “still an art and not an exact science”, but the design of large scale bioreactors for solid state fermentation has made significant advances in recent years (Couto and Sanroman, 2006).

Scaling-up should be investigated with the specific end use in mind. For some applications scale-up might be premature considering the current EPA yield.

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However, for other applications such as EPA-enriched fish feed, the level of 0.6% EPA (w/w) produced by *Mortierella alpina* Mo 46 on SPC falls within the range of 0.5 to 2.0% ω-3 HUFA reportedly required in the diet of marine fish (Craig and Helfrich, 2002).

It is recommended that a techno-economic evaluation model should be developed based on the information accumulated in the course of this study and also in larger scale trials. The model should incorporate all the process parameters such as capital investment in equipment/bioreactors, cost of feedstocks/substrates, supplements, temperature, aeration as well as downstream processing for oil recovery and purification for specific applications. This model should be used to compare fungal solid state fermentation with submerged fermentation as well as extraction from fish oil as a means of producing EPA. The cost benefits of each of the process variables should be determined, thereby designing the most cost-effective EPA production process.

There are certain benefits of fungal production of EPA that would be difficult to quantify, such as the environmental benefit of a sustainable source of ω-3 HUFA. The effect of depleting wild fish stocks as a source of fish oil and EPA for aquaculture will only be appreciated by future generations. The reliability, oxidative stability and consistent quality of fungal HUFA is superior to that of fish oil-derived HUFA. Wild fish landings are affected by seasonal and climatic changes such as the El Nino phenomenon, especially in Chile and Peru, the two countries which dominate the fish oil export market (Hemre, 2002). Another factor which should favour the economical competitiveness of fungal EPA is the safety of the product. The process of production of ARA for infant formula by fermentation with *Mortierella alpina* has GRAS status (Zeller, 2005), whereas there is speculation about the extent of the risk of pollutants such as dioxin, PCB’s and mercury which could accumulate in fish oil (Kyle, 2005).

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Whereas the economic evaluation model might prove that the production of EPA and other HUFA by micro-organisms such as *Mortierella spp.* is currently not financially viable, the above-mentioned factors should be taken into account. Legislatory measures have been introduced to minimize the use of fish oil in aquaculture feeds (Naylor *et al.*, 2009). With the global emphasis on climate change and sustainability, policy-makers might consider incentives to encourage the development of environment "friendlier" production processes such as fungal solid state fermentation for EPA production.

In this study, fungi belonging to the genus *Mortierella* successfully converted linseed oil supplemented agro-processing wastes, such as BSG and SPC, to materials containing EPA, thereby adding value to these substrates. The EPA-enriched bioproducts produced in this manner could eventually find applications as animal or fish feed or as a source of EPA for the growing ω-3 market in the neutraceutical and therapeutics industries.

In conclusion it was demonstrated in this study that the process of fungal conversion of low-cost processing by-products to EPA-enriched products by solid state fermentation is feasible. The process could be 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.

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Appendix 1

Adding value to the by-products of cereal processing by fungal production of highly unsaturated fatty acids

This paper has been accepted for publication in the Proceedings of the ICC International Grains Symposium held at the University of Pretoria, Sanlam Conference Centre, Pretoria, South Africa from 3 to 5 Feb 2010. It contains excerpts of Chapters 2 and 3.

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Adding value to the by-products of cereal processing by fungal production of highly unsaturated fatty acids

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Introduction

The processing of cereals as agricultural crops or biofuels generates millions of tons of by-products annually. The South African beer brewing industry alone produces approximately 520 000 tons of brewers' spent grain per year. These residues not only lead to economic losses for the industry, but could constitute an environmental hazard.

Currently cereal by-products are mainly utilised as animal and fish feeds. This application is limited by the high fibre and low protein levels generally present in these by-products. The presence of antinutrients (phytic acid, chlorogenic acid and other phenolic compounds) and the low levels of highly unsaturated fatty acids (HUFA) in most of these by-products also limit its use, especially as feeds for monogastric animals and fish.

HUFA such as eicosapentaenoic acid (EPA) are essential to the regulation of the cardiovascular, immune, digestive and neurological systems in mammals and fish. Arachidonic acid (ARA) is of nutritional importance in fish egg and larvae development (Ogata *et al*., 2004). These long chain fatty acids have to be included in the diets of mammals and fish (Dyal and Narine, 2005). Currently the main dietary source of HUFA is marine fish oil. As demand for crude fish oil for the aquaculture industry increases and the market for dietary

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omega-3 supplements expands by 24% annually, there are concerns over the sustainability of marine and fish sources of HUFA (Jang *et al.,* 2000).

Recent research has focused on HUFA production by micro-organisms as a sustainable and safe alternative to fish oil (Ward and Singh, 2005). Fungi of the genus *Mortierella* are used for industrial production of some of these valuable HUFA and could be grown directly on the cereal by-products.

The aim of this study was to enhance the quality of the by-products and to provide an alternative application for cereal by-products as a source of HUFA. Cereal by-products enriched with HUFA could find applications as food, feed, or pharmaceutical or veterinary products.

Materials and methods

Substrate preparation

Dried solid brewers' spent grains (BSG) and sunflower press cake (SPC) were treated by one of two methods before inoculation. In both treatments 20 g aliquots of cereal substrate were distributed in conical flasks and a 70% moisture level obtained by adding water. Ten percent (m/m) linseed oil (LSO) was then added to half of the substrate treatments. The rest of the treatments were not supplemented with LSO. All treatments were sterilised by autoclaving (121°C, 20 min).

Fungal isolates and inoculum preparation

A liquid inoculum was prepared from each of 8 fungal strains representing the genus *Mortierella* (Table 1). These indigenous fungal strains were originally isolated from soil and maintained in the culture collection of the University of Stellenbosch, South Africa.

Cultivation conditions

Cultures representing each isolate were inoculated with 2 ml of the homogenised inoculum for both LSO treatments. The inoculated cultures were incubated at 22°C for 3 days to obtain optimal fungal growth. The

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cultivation temperature was subsequently lowered to 16°C to enhance HUFA production and the cultures were incubated for a further 8 days (Figure 1).

Figure 1. ***Mortierella spp.*** **growing on agar medium and brewers' spent grain.**

Analyses

The cultures were harvested, dried and analyses were performed on the total dried fermented bioproduct containing the fungal biomass (Jacobs *et al.*, 2009). The total lipids, EPA and ARA levels of the dried fermented biomass were determined by gas chromatography with flame ionisation detection.

Results and discussion

It is generally accepted that the target concentration of omega-3 HUFA required in marine fish diets is ~1% m/m, depending on the fish species. In fresh water fish species, less omega-3 but relatively higher levels of dietary omega-6 are required (Ogata *et al.*, 2004).

Neither of the cereal substrates tested had significant initial levels of EPA or ARA. Solid-state fermentation with *Mortierella spp.* improved the composition of the total lipids by increasing the levels of omega-3 EPA and omega-6 ARA in both the fermented BSG and SPC. The highest EPA yield of 0.6% (m/m) was achieved with *Mortierella alpina* Mo 46 in the sunflower substrate supplemented with LSO (Table 1).

Table 1. Lipid and EPA production during fermentation of LSO supplemented cereal by-products with *Mortierella spp.*

Fungal strain number	**Species**	**Total lipids[a]**		**Eicosapentaenoic acid[b]**	
		BSG[+]	**SPC[+]**	**BSG[+]**	**SPC[+]**
	Substrate control	14.4	22.6	0.2	0.2
Mo 46	*Mortierella alpina*	9.2	13.4	1.7	6.4
Mo 47	*M. selenospora*	11.0	12.8	1.1	2.4
Mo 50	*M. alpina*	9.2	13.8	1.2	4.8
Mo 88	*M. basiparvispora*	7.2	13.5	1.5	5.8
Mo 101	*M. epicladia*	10.7	13.6	2.5	4.2
Mo 102	*Mortierella spp.*	10.9	16.3	2.0	0.5
Mo 114	*Mortierella spp.*	9.9	16.3	1.5	0.6
Mo 130	*Mortierella spp.*	12.0	15.6	0.2	1.7
Mean		10.0	14.4	1.5	3.3
Std Dev		1.5	1.4	0.7	2.3

a: gram lipid per 100g oven dried bioproduct

b: mg EPA per g oven dried bioproduct

+: 10% (m/m) LSO was added to substrate before fermentation

EPA: eicosapentaenoic acid; LSO: linseed oil; BSG: brewers' spent grain (values are averages of results obtained with duplicate experiments) SPC: sunflower press cake (values are means of results obtained with triplicate experiments).

The omega-6 ARA production by the *Mortierella* isolates was improved by the addition of linseed oil to the substrate (Figure 2). In the sunflower by-product the highest ARA yield of 2.4% (m/m) was achieved with *Mortierella alpina* Mo 46.

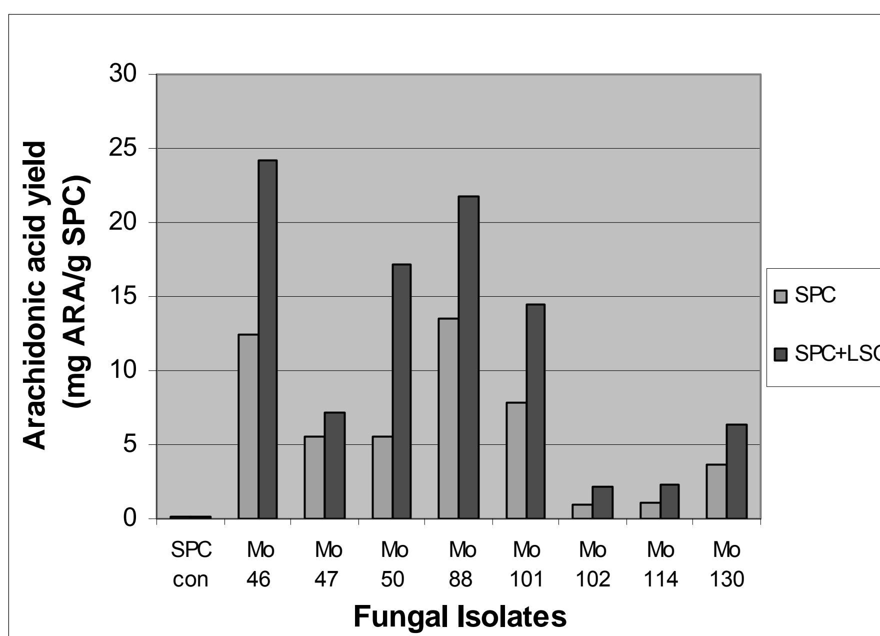

Figure 2. Effect of the addition of LSO on the levels of arachidonic acid produced during fermentation of SPC with fungi of the genus *Mortierella.* (ARA: arachidonic acid; LSO: linseed oil; SPC: sunflower press cake)

Conclusions

Fermentation with oleaginous fungi of the genus *Mortierella* produced HUFA-enriched cereal bioproducts. *M. alpina* Mo 46 produced the highest levels of EPA and ARA and could be investigated for larger scale fermentation and value-addition to cereal by-products. HUFA-enriched bioproducts have potential applications as animal or fish feed or could be a source of EPA and ARA for the growing HUFA market in the infant formula, neutraceutical and therapeutics industry.

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Adding Value to the By-products of Cereal Processing by Fungal Production of Highly Unsaturated Fatty Acids

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Figure 1: Fungi of the genus *Mortierella* producing highly unsaturated fatty acids while growing on agar medium and brewers' spent grain.

Analyses

The cultures were harvested, dried and analyses were performed on the total dried fermented bioproduct containing the fungal biomass[2]. The total lipids, EPA and ARA levels of the dried fermented biomass were determined by gas chromatography with flame ionisation detection.

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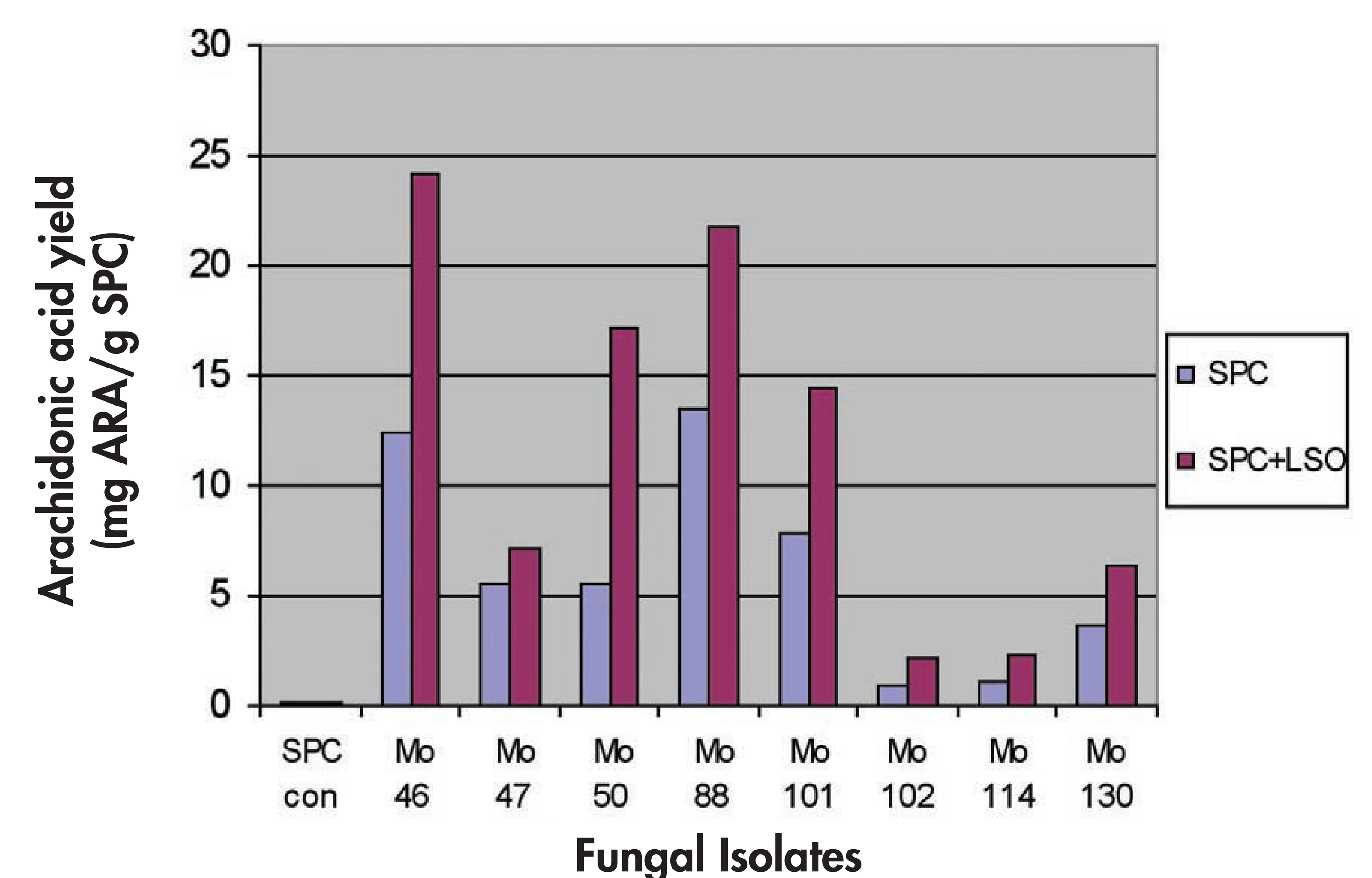

Figure 2: The effect of the addition of LSO on the levels of arachidonic acid produced during fermentation of SPC with fungi of the genus *Mortierella*. ARA: arachidonic acid; LSO: linseed oil; SPC: sunflower press cake.

CONCLUSIONS

Fermentation with oleaginous fungi of the genus *Mortierella* produced HUFA-enriched cereal bioproducts. *M. alpina* Mo 46 produced the highest levels of EPA and ARA and could be investigated for larger scale fermentation and value-addition to cereal by-products. HUFA-enriched bioproducts have potential applications as animal or fish feed or could be a source of EPA and ARA for the growing HUFA market in the infant formula, neutraceutical and therapeutics industry.

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