Fungal oil with EPA in *Mucor circinelloides* cultivated on thin stillage from corn-to-ethanol production

by

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ABSTRACT

This study demonstrates the potential of the oleaginous fungus *Mucor circinelloides* for synthesizing oil rich in polyunsaturated fatty acids when cultivated in thin stillage (TS) and centrifuged thin stillage (CTS) from corn-to-ethanol production. Biomass yield, oil yield and its EPA content were studied at different cultivation periods and carbon to nitrogen ratios. The fungal biomass yield (g/L) and its oil content (g/L) were found to be twice as high in TS as in CTS when harvested after two to six days. EPA content doubled in TS and increased by 2.6 times in CTS when the cultivation period was extended from three to six days. Glycerol supplementation at 20g/L of C led to an 18% increase and 63.6% decrease in fungal oil yield in TS and CTS respectively. Glycerol addition also increased EPA content by 33% in TS and 60% in CTS. Adding 5g/L N using ammonium sulfate as a nitrogen source enhanced the fungal oil yield by 34% in TS and 26% in CTS and EPA content by 32% in TS and 12% in CTS. Urea at 0.5 g/L of N increased the EPA content by 22% in TS and 33% in CTS and oil content by 12% in TS and 13% in CTS. Potassium nitrate at 5g/L of N increased EPA content by 28% in TS and 33% in CTS at 0.5 g/L of N dosage and increase in oil yield by 18% in TS and decreased oil yield in CTS by 37%.

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CHAPTER I

INTRODUCTION AND OVERVIEW OF THE RESEARCH

1. Introduction

Corn ethanol production in the United States has been growing with 13 billion gallons produced in 2012. For every gallon of ethanol produced, 5–6 gallons of stillage is generated (Rasmussen et al., 2014) which is composed of corn fiber, oil, protein, other unfermented components of the grain and yeast cells (Kim et al., 2008). These compounds are ideal nutrients for microorganisms (Dowd et al., 1993; Kim et al., 2008).

This whole stillage is then centrifuged to produce a liquid called thin stillage and a solid fraction called wet distillers grains. Less than 50% of the thin stillage (TS) is recycled back as backset for liquefaction of ground corn (Sankaran et al., 2010). The rest goes through multiple effect evaporators requiring substantial amounts of energy to make a condensed syrup, that later ends up in dried distillers grains and solubles (DDGS) (Kim et al., 2008) and is sold at low margins as animal feed (Moreau et al., 2011).

During the whole downstream process, evaporation of TS is an energy-intensive process and a major cost to ethanol plant. Therefore developing an efficient way to use thin stillage and increase the product value will enhance the economy of the dry milling ethanol production chain.

Thin stillage has been used as a carbon source for growing the fungus. For example *Ganoderma lucidum* was grown to produce polysaccharides (Hsieh et al., 2005). Van Leeuwen et al. (2010) performed a thorough study of using thin stillage for growing a variety of fungi which can be used as animal feeds (Van Sambeek et al., 2014a and 2014b), human

food (tests in progress) and sources of nutraceuticals (tests in progress). The group recently reported using thin stillage for growing oleaginous fungus *Mucor circinelloides* in an air-lift bioreactor which led to a high biomass concentration and high oil content (Mitra et al., 2012). Ahn et al. (2011) also reported using thin stillage to grow bacterium *Clostridium pasteurianum* for butanol production. Using anaerobic digestion for treating thin stillage for improving water quality and energy efficiency in dry milling ethanol plant was also investigated (Alkan-Ozkaynak and Karthikeyan, 2011). Liang et al., (2012) proposed using thin stillage for growing the fungus *Pythium irregulare* to produce eicosapentaenoic acid (EPA).

1.1. **Thesis Organization**

This thesis is organized into four chapters. The first chapter is a common introduction on the ethanol industry, the biggest challenge faced and the current trends in negating the problem.

The second chapter focusses on the literature review of an essential fatty acid called eicosapentaenoic acid, its importance and the necessity for current project

The third chapter is a journal paper on "Fungal oil with EPA in *Mucor circinelloides* cultivated on thin stillage from corn-to-ethanol production" submitted to Bioresource Technology. The chapter focusses on producing fungal oil rich in polyunsaturated fatty acids (PUFA) and the various conditions tested to obtain higher PUFA content in the fungal oil.

The fourth chapter addresses the general problems faced during this research and recommendations for future research.

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CHAPTER 2

LITERATURE REVIEW

2. Eicosapentaenoic acid

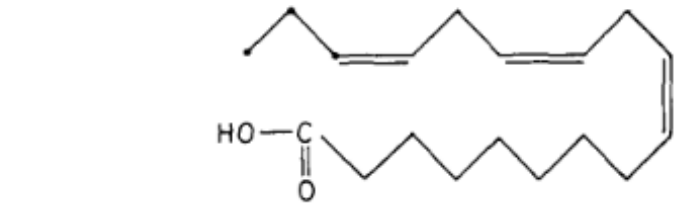
2.1. Introduction

Eicosapentaenoic acid (EPA) is a polyunsaturated fatty acid (PUFA) that is commonly found in marine animals and phytoplanktons. It contains 20 carbon atoms with five double bonds (20:5) (Fig.1). The double bonds are arranged with the last one located three carbon atoms from methyl end of the chain, and is, therefore, referred to as an omega-3 or (n-3) fatty acid. Other members of the omega-3 family are docosahexaenoic acid (DHA; 22:6), and a-linolenic acid (18:3). Although linolenic acid can be obtained from vegetable sources, e.g. linseed oil contains up to 58% α -linolenic acid, EPA and DHA are obtained from marine fish oils only, although the original source is the plankton at the base of the marine food chain.

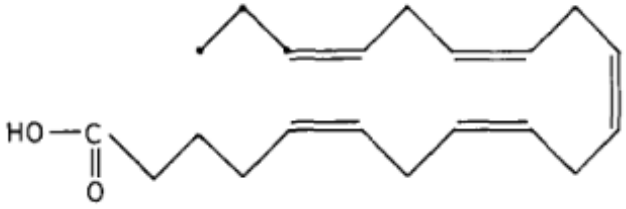
2.2. Health benefits

The omega-3 fatty acids have been shown to be of major importance in the prevention or treatment of a range of human diseases or disorders. Physiological effects of the omega-3 fatty acids have been observed in three main areas (Table 1). In the heart and circulatory system, these lipids have been shown to have beneficial effects in the prevention or treatment of atherosclerosis (Dyerberg, 1986; Dratz and Deese, 1986), thrombosis (Urakaze et al., 1986), hypertriglyceridaemia (Phillipson et al., 1985) and high blood pressure (Mortenson et al., 1983). Beneficial physiological effects have also been observed in the inflammatory area, related to treatment of asthma, arthritis, migraine, psoriasis and nephritis (Lands, 1986;

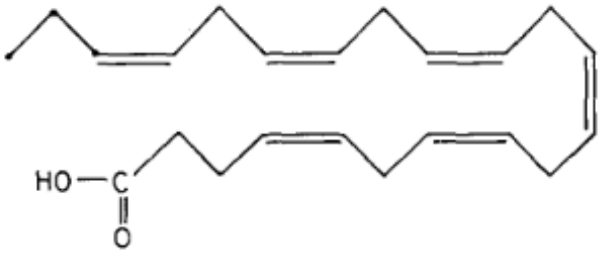
Kermer et al., 1987; Ziboh et al., 1986; Robinson et al., 1986; Braden and Caroll, 1986). Potential applications have also been proposed in treatment of breast, prostate and colon cancers (Reddy and Maruyama, 1986; Weber et al., 1986).



a. ∝ – linolenic acid (all - cis - 9, 12, 15 - octadecatrienoic acid)



b. EPA (all - cis - 5, 8, 11, 14, 17 - eicosapentaenoic acid)



c. DHA (all - cis - 4, 7, 10, 13, 16, 19 - docosahexaenoic acid)

Fig.1 Chemical structures of omega-3 fatty acids. Adapted from Bajpai and Pramod (1992)

Possible targets of omega-3 activity

Heart and circulatory	Disease class	
	Inflammatory	Cancers
Atherosclerosis	Asthma	Breast
Thrombosis	Arthritis	Prostrate
Hypertriglyceridaemia	Migraine headaches	Colon
High blood pressure	Psoriasis	
	Nephritis	
	Diabetes	
	Graft rejection	

Fig. 2 Possible targets of omega-3 activity. (Adapted from Bajpai and Pramod, 1992).

Industrial production of EPA has gained more attention recently due to the proven clinical importance of EPA in reducing the risk of cardiovascular diseases, lowering of plasma cholesterol and decreasing the incidence of breast, colon and pancreatic cancers, in addition it plays an important role in controlling various biological processes as it is a precursor for a number of vital eicosanoid signaling compounds (Frank et al., 1991) (De Caterina, 2011).

2.3. **Importance of current research**

EPA is an essential fatty acid as humans lack the ability to produce it in the body but they would require to ingest it for maintaining good health. The main EPA source is through dietary supplements (Gill et al., 1997). Although fish oil is the main commercial source of EPA as a dietary supplement, there are many limitations on its wider usage such as high purification cost, complex composition, potential heavy metal contamination and unacceptable odor. Recently, fish oil was found to interfere with chemotherapy causing cancer cells to become less sensitive to such treatments (Roodhart et al., 2011), due to the

presence of 12-oxo-5, 8, 10-heptadecatrienoic acid and hexadeca-4, 7, 10, 13-tetraenoic acid which, even in minute quantities, induces resistance to a wide spectrum of chemotherapeutic agents. As a result, microbial EPA may be a promising alternative. Using thin stillage to produce eicosapentaenoic acid (EPA, C20:5, n-3) by fungal fermentation provides another outlet for this currently underutilized material.

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CHAPTER 3

Fungal oil with EPA in *Mucor circinelloides* cultivated on thin stillage from corn-to-ethanol production

A paper to be submitted to Journal of Bioresource Technology

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3.1. **Abstract**

This study demonstrates the potential of the oleaginous fungus *Mucor circinelloides* for synthesizing oil rich in polyunsaturated fatty acids when cultivated in thin stillage (TS) and centrifuged thin stillage (CTS) from corn ethanol production. Oil yield and its EPA content were studied at different cultivation period and carbon to nitrogen ratios. EPA content doubled in TS and increased by 2.6 times in CTS when cultivation period was

extended from three to six days. Glycerol addition at 20g/L of C increased EPA content by 33% in TS and 60% in CTS. Supplementing ammonium sulfate at 5g/L of N enhanced EPA content by 32% in TS and 12% in CTS. Urea at 0.5 g/L of N increased the EPA content by 22% in TS and 33% in CTS. Potassium nitrate at 5g/L of N increased EPA content by 28% in TS and 33% in CTS at 0.5 g/L of N dosage.

Abbreviations

TS, thin stillage; CTS, centrifuged thin stillage; SCOD, soluble chemical oxygen demand; EPA, eicosapentaenoic acid; DWB, dry weight basis; GC, gas chromatography; TFA, total fatty acids; PUFA, poly unsaturated fatty acids;

Keywords

Mucor circinelloides, PUFA, Thin stillage, Oleaginous, Fungal lipids

1. Introduction

Thin stillage (TS) is an underused low-value coproduct obtained in the corn-ethanol industry and a very good source of carbon and nitrogen for single-cell protein production. One promising application of TS is for fungal cultivation, not only for protein, but also for oil production. The fungal oil contains valuable polyunsaturated fatty acids (PUFA) with valuable nutrient content such as omega-3 fatty acids, particularly when the mold *Mucor circinelloides* was used (Xia et al., 2011). The aim of this project was to determine which nutrient factors enhance the fungal oil yield and its PUFA content, particularly, the longer chain, 20:5 n-3 eicosapentaenoic acid (EPA), which is a scarce and essential fatty acid.

Studies have suggested that carbon to nitrogen ratio in the growth media is an important parameter that can affect the biomass yield and the fungal lipid content. Hansson and Dostalek (1988) and Yokochi and Suzuki (1986) reported that high C: N ratio in the media increased the total fungal lipid content in *Mortierella ramanniana* which in turn affected the PUFA yields. Xia et al. (2011) reported that nitrogen depletion plays a key role in stimulating lipid accumulation of *M. circinelloides* and that fungus produces lipid within 24 hours of nitrogen depletion. Thus a higher C: N ratio of the growth media stimulates more oil accumulation in the fungal cell. Thin stillage usually contains about 5 g/L total nitrogen against a chemical oxygen demand of about 100 g/L (Rasmussen et al., 2014), which is equivalent to roughly 40 g/L carbon, the C: N ratio being approximately 8:1. Not all the nitrogen is in available form and much of the carbon is not used by the fungi. It is not clear, therefore, whether the C: N ratio in thin stillage imposes limits on fungal growth.

It is likely that additional carbon introduced to thin stillage, will increase the fungal lipid content and not the biomass yield. Mitra et al. (2012a) reported an increase of 32% in cellular oil content due to addition of crude glycerol as an additional carbon source to the TS but no pronounced effect on the biomass yield. Thus, supplementing TS with glycerol and varying the carbon dosage, thereby increasing the C: N ratio, is expected to increase the fungal oil content (Mitra et al., 2012a) and EPA content oil as well (Shimizu et al., 1988). Another carbon source that has been found to be the best for EPA production is linseed oil with 58% alpha linolenic acid. This fatty acid is a precursor for EPA synthesis and enhances EPA production (Shimizu et al., 1989); (Bajpai et al., 1991).

Excess nitrogen addition to thin stillage is expected to benefit the biomass production and be counterproductive to oil production. However, the study by Bajpai and Pramod (1992)

on the production of EPA from several microorganisms reported that both the concentration and source of the nitrogen play an important role in increasing the PUFA yields of the fungal oil. This factor could make *Mucor circinelloides* produce more PUFA in the oil at lower C: N ratio in TS and CTS as opposed to higher C: N ratio as proven by Xia et al. (2011). Thus the fatty acid profiles of the fungal oil for different C: N ratios should be analyzed.

Yongamanitchai and Ward (1989) and Erwin (1973) in their study, concluded that the nitrogen content in the medium alters the proportion of saturated to unsaturated fatty acids in fungi. Hansson and Dostalek (1988) reported that the media containing potassium nitrate as nitrogen source resulted in higher production of total lipid content in *M. ramanniana* as compared to ammonium salts. However, *Mortierella isabellina* produced highest lipid content when ammonium sulfate was used (Yokochi and Suzuki 1986). Though *M.circinelloides* has been observed to produce more oil under nitrogen deficient conditions (Xia et al., 2011), varying the additional dosage of nitrogen using organic and inorganic sources to the TS and CTS might increase the PUFA yield in the fungal oil. Therefore *M. circinelloides* might also prefer some specific added nitrogen source in the TS and CTS to produce more oil.

The duration of cultivation is an important parameter for the rate of nitrogen depletion in TS during the growth phase. It is expected that longer the duration of growth, more stresses would be experienced by the fungi and more oil would be synthesized (Bajpai and Pramod 1992).

Based on the above results, we hypothesized that the fungus *M. circinelloides* is capable of synthesizing oil containing EPA by changing C: N ratio of the media and the duration of growth.

2. Material and methods

2.1. Thin stillage (TS)

Thin stillage was obtained from Lincolnway Energy (Nevada, IA, USA), a local dry-grind corn ethanol plant and stored at 4 °C prior to use. One batch of TS was used for the entire study and its nitrogen content was determined to assess the C: N ratio. The thin stillage was also filtered to remove the solid particles and centrifuged at 5000*g for 15 minutes. Both TS and centrifuged TS (CTS) were used as fungal growth media following heat sterilization at 121 °C for 15 min. To differentiate between the oil in fungal biomass and oil in solid particles, the experiments were also carried out on CTS to obtain the fatty acid composition in fungal oil alone, without corn particle interference (Mitra et al., 2012b).

2.2. Inoculum preparation

The fungal inoculum for the shake flasks was prepared by adding 1 vial of 2ml spore suspension into 1 L heat sterilized (121 °C for 15 min) YM broth in a 2L Erlenmeyer flask with foil covered mouth. These inoculum flasks were then incubated at 150rpm, 30°C for 24h. The mycelial pellets were then filtered using Whatman No.1 filter paper, rinsed with sterile saline solution to remove media components, homogenized and 10 ml of that sample, roughly 0.019g ± 0.002 (dwb) was added to heat sterilized TS and CTS for uniformity.

2.3. Fungal cultivation

The flasks containing 100 ml of TS and CTS with inoculum were incubated on a shaker at 150 rpm, 30°C for 4 days. At the end of cultivation period, the biomass was harvested.

2.4. Fungal cultivation conditions tested

2.4.1. Duration of cultivation

M. circinelloides was grown on TS and CTS at 30°C, 150 rpm and harvested on different days (day 2 through day 6 except day 5) in order to study their nitrogen utilization, biomass yield, oil yield and fatty acid profile of fungal oil. Six days of cultivation was chosen to understand how the prolonged duration of growth affects the biomass and total oil. All the experiments were done in triplicate.

2.4.2. Effect of higher C: N ratio by glycerol addition

Studies were conducted for growing *M. circinelloides* in TS and CTS by supplementing crude glycerol stoichiometrically such that the C: N ratio differed by a significant amount. The fungi were also grown in TS and CTS without additional glycerol as a control. The period of growth chosen was 4 days at 30°C and 150rpm, followed by harvesting and drying. Day 4 was chosen for cultivation so that the fungi could experience stressful conditions due to limited nitrogen (Mitra et al., 2012a) and produce more PUFAs in the oil. Temperature was maintained at 30°C throughout the experiment and the pH was not changed. The experiments were done in triplicate.

2.4.3. Effect of lower C: N ratio and additional nitrogen source

Studies were conducted for growing the fungi on TS and CTS after adding different sources of nitrogen, such as urea, potassium nitrate and ammonium sulphate with an assumed high and low dosage 5 g of N/L and 0.5 g of N/L addition respectively. The fungi were also grown under nitrogen limiting condition (TS and CTS only) as a control. The fungus was allowed to grow for 4 days at 30°C and 150rpm, then harvested and dried. Day 4 was chosen

for cultivation assuming the fungi would experience stressful conditions and produce more PUFAs in the oil. The experiments were done in triplicate.

2.5. Analytical methods

2.5.1. Nitrogen estimation in TS and CTS

Samples from TS and CTS, before and after fungal cultivation were analyzed for total nitrogen content using the Elementar CN Vario-Max that uses the Dumas method. The initial nitrogen content in the TS was estimated by filtering the sample using filter paper and separating the corn solids, to find the nitrogen content present in soluble form and in corn solids (Sankaran et al., 2010). The decrease in nitrogen content was correlated with corresponding oil yield and PUFA content in the fungal oil (Rasmussen et al., 2014).

2.5.2. Fungal biomass yield

The fungal biomass with attached corn solids in TS and without corn solids in CTS were filtered out from the media using a stainless steel screen of pore size 1mm. The biomass was oven dried at 80 °C for 12 h to reduce the initial moisture content and then in a vacuum oven for 10 h at 40 °C to avoid the potential of heat–drying effects at high temperature. The dried biomass were subjected to moisture test to ensure complete drying. The dried solids were measured gravimetrically and biomass yield was reported in g dried biomass per litre of stillage.

2.5.3. Fungal oil extraction using organic solvents

The oven-dried fungal cells were subjected to the Folch, Lees and Stanley method for fungal oil extraction (Christi and Han, 2010).

2.5.4. Fatty acid analysis of lipids

The dried fungal biomass were subjected to fatty acid profiling using gas chromomatography. The internal standard was prepared by adding 200 mg of methyl heptadecanoate (C 17:0) to 100ml of methanol. The fungal biomass including 3 ml of internal standard were treated with 6% sulphuric acid in methanol for 2 days at 60 °C. The FAMEs were extracted by hexane and washed with water and then analysed with a Hewlett–Packard 5890 series II GC equipped with a flame ionization detector and a SPB-2340 fused silica column (60 m × 0.25 mm id and 0.20 μm film thickness) (Supelco, Bellefonte, PA, USA). The initial oven temperature was 100 °C, the oven temperature program was ramped up from 100 to 240 °C at a rate of 4 °C/min and the injector and detector temperatures were 250 °C. The sample injection volume was 1 μL. The carrier gas (helium) flow rate was 1 mL/min. All analyses were done in duplicate.

2.6. Statistical analysis

Linear models were performed using the Generalized Linear Mixed Model (GLIMMIX) procedure in SAS to determine the significant differences among various treatments. Differences of Least Square Means were calculated and significant difference was declared at $p = 0.05$ by Student's t test. All treatments were carried out in triplicates (except fatty acid analysis) and the results are shown as the means of three replicates ± standard deviation (SD)

3. Results and discussion

3.1. Thin stillage characterization

Thin stillage contained 6.3% (w/v) total solids with a pH of 3.5 to 4.2. Freeze dried TS solids were found to contain 8.0 ± 2.0% oil (g of oil in 100g stillage solids) (Mitra et al.,

2012a). Hence TS with 6.3% total solids contained 5.0 ± 1.2 g oil/ L. 2g/L(dry) of corn solids were retained when unsettled TS was passed through a screen with an opening size of 1mm. The total solids in CTS were found to be approximately 0.5% and the total oil content was calculated to be 0.4 ± 0.1 g oil/ L. The initial nitrogen in TS and CTS were 2.85 g/L and 1.8 g/L respectively. Taking the COD content in initial TS and CTS to be 100g/L (Rasmussen et al., 2014) and 45g/L respectively (Mitra et al., 2012a), the C: N ratio for TS and CTS were calculated to be 14:1 and 9:1 respectively.

3.2. Cultivation period

The fungal biomass was found to have a lag phase of less than 5 hours in TS and 1 day in CTS. The lag phase was followed by log phase until day 2 when the fungi grew steadily in both TS and CTS and then slowly reached the stationary phase from days 2 to 6. (Fig. 1). The nitrogen content in the media decreased steadily during the 2 d growth phase indicating that nitrogen content in the media is responsible for the growth of fungal biomass. However, during the stationary phase, nitrogen utilization by the fungal biomass is very limited and the fungi might have started producing more PUFA in the oil due to stresses attributed by longer duration of growth (Fig. 1).

The results (Table 1) agreed with the hypothesis. The composition of major fatty acids (%) in thin stillage such as palmitic acid, stearic acid, oleic acid and linoleic acid (Liang et al., 2012) (Mitra et al., 2012a) (Table 1) closely matched with the fungal oil, indicating that the fungi might have adsorbed/assimilated the oil from thin stillage (Srinivasan and Viraraghavan, 2010). However, the fungal oil additionally contained linolenic acid and eicosapentaenoic acid indicating that when *M. circinelloides* was cultivated longer, it could be stressed due to lesser availability of nutrients in stillage and

starts producing PUFA, particularly linolenic acid and eicosapentaenoic acid. Also, the nutrients in the growth media such as corn oil could have been utilized as a carbon source for intracellular lipid production thereby aiding the fungi to synthesize PUFA in fungal oil, as suggested by (Mitra et al. 2012a). The fungal oil contained about 55-60 % PUFA in TS and 53-58% in CTS. The PUFA content in the oil was found to increase with longer duration of growth in TS and CTS.

Visual observations showed that the fungi grew as branched mycelia in TS, entrapping the corn solids and leaving behind a clearer effluent (Mitra et al., 2012a) and as compact pellets on the CTS. This was also evident from the current results (Fig 1), which show that the dry fungal biomass yield and oil content were nearly double in TS than in CTS from days 2 through 6. Several factors contributed to the lower yields of CTS among which lower nitrogen and insoluble sugar contents were the most influential (Mitra et al. 2012a). Both the biomass and fungal oil content were found to increase over the period of growth in TS and remain constant in CTS, indicating a direct relationship between fungal biomass content and its oil yield in both the media. There was a significant difference ($p < 0.05$) between the fungal biomass and its oil content when grown in TS and CTS from days 2 to 6. Since the TS originally contained an oil concentration of 5.0 ± 1.2 g oil/ L, the fungus was capable of increasing the oil content in the biomass by 19% on day 2 to 60% on day 6 in TS. In a similar experiment conducted by Mitra et al. (2012b), the total yield of lipids in *M. circinelloides* was almost twice as high as in mixotrophically productive algae.

To our knowledge, this is the first study to report that *M. circinelloides* is capable of synthesizing EPA in both TS and CTS. The EPA content was found to double when the cultivation period was extended from 3 days to 6 days in TS and increased by 2.3 times

during the same period of growth in CTS. The highest composition of EPA was obtained on day 6 (Table 1).

3.3. Variation in C: N ratio with addition of glycerol

The results (Fig. 2) agreed with the hypothesis that higher C: N ratio does not significantly change the biomass yield ($p > 0.05$) but increases the fungal lipid content in TS. The biomass yield was found to almost remain constant despite the addition of 20g/L of glycerol but an insignificant increase ($p > 0.05$) of 15% and 18% in fungal oil yield was observed in TS for 10g/L and 20g/L of glycerol dosage respectively. The fungi when grown at a higher C: N ratio to the initial TS, synthesized valuable PUFA, evident from increase in linolenic acid and EPA composition in the fatty acid profile of fungal oil. An increase of 50% and 56% in linolenic acid (mg/g DW) and 29% and 33% (mg/g DW) in EPA for 10g/L and 20g/L addition of glycerol respectively were observed in TS (Table 2). The PUFA content of fungal oil varied between 54-56 % control cells and glycerol supplemented TS media. The PUFA content in TS when 10g/L and 20g/L glycerol were added increased by 11% and 18% respectively.

Contrary to the observations made on TS, the glycerol addition turned out to be undesirable to the fungal oil production in CTS, decreasing the yield by 71% and 66% for 10g/L and 20g/L dosage respectively. The possible explanation could be that the glycerol addition might have exceeded the maximum threshold of C: N ratio in the CTS media, beyond which the fungi might have not been able to synthesize oil. Since the initial nitrogen content was already low in CTS (1.8g/L), the glycerol addition could have been detrimental for the fungi to synthesize oil due to limited availability of nutrients in CTS media. The majority of the nitrogen in the media was probably unavailable for the biomass to utilize,

which is indicated by decrease in biomass yield by 8% and 15% for 10g/L and 20g/L dosage respectively. Had the nitrogen been utilized by the fungi, the biomass yields would have been more than the control cells. Though the overall fungal oil yield reduced drastically, the fatty acid composition (Table 2) reveals that the carbon addition in CTS resulted in 37.5% and 59.4% increase in EPA composition for 10g/L and 20g/L glycerol dosage respectively. EPA content was found to be the highest at 20g/L glycerol dosage in CTS. From the overall fungal oil yield standpoint even though the glycerol addition proved to be undesirable in the CTS media, it is evident that EPA was more favorably synthesized by the fungi. Further studies would be required to establish an optimum glycerol dosage, which would increase the fungal oil yield with a higher fraction linolenic acid and EPA. Dosage of glycerol beyond 20g/L was not chosen in order to use TS as the primary carbon source.

The fungi, having shown the capability of synthesizing EPA with glycerol as carbon source, might probably prefer carbon sources having high linolenic acid content such as linseed oil in order to synthesize fungal oil containing much higher EPA content. Studies need to be done using linseed oil, soybean oil, glucose and perilla oil as carbon sources, keeping in mind the cost involved and their feasibility in large-scale plants as all these sources are known precursors suitable for EPA production (Bajpai and Pramod 1992). The combined effect of most suitable carbon source and higher duration of growth should also be researched if it leads to higher PUFA yields in fungal oil.

3.4. Variation in C: N ratio with an additional source of nitrogen

The nitrogen sources were selected based on organic and inorganic sources. Urea was chosen as the organic source and both potassium nitrate and ammonium sulfate were chosen as inorganic sources. The compounds were added at a specified high (5g/L) and low dosage

(0.5g/L) of N, and their C: N ratios, effects on biomass yield, fungal oil content and EPA content were determined (Fig. 3). The fatty acid composition of the fungal oil for each source and dosage were tabulated in Tables 3 and 4.

Studies of higher dosage of N (5g/L) with C: N ratio of 5:1 in TS and 2.5: 1 in CTS revealed that addition of inorganic source increased the fungal oil content when grown on TS when compared to the control cells. Potassium nitrate and ammonium sulfate at 5g/L dosage increased the fungal oil yield by 19% and 34% with the PUFA contents in the oil increasing by 13% and 29.1% respectively. Urea decreased the fungal oil yield by 3% and PUFA content in the oil by 5.6% in TS.

There was no biomass growth on CTS media when urea (5g/L) was dosed. Probably organic sources of nitrogen are not favorable for growth of *M. circinelloides* at a lower C: N ratio. More experiments with varying dosages of urea should be tested to confirm this hypothesis. Visual observations revealed that even at low dosage, urea removed the characteristic yellow color of the biomass grown in TS, whereas addition of other nitrogen sources did not have any effect on the fungal biomass color. High potassium nitrate dosage did not have any effect on the fungal oil yield in CTS. High ammonium sulfate dosage was found to be the most favorable nitrogen source in CTS as well, increasing the oil yield by 26% in CTS. Considering both TS and CTS media, ammonium sulfate at 5g/L was found to be the best nitrogen source from a fungal oil yield and PUFA content standpoint. The fatty acid analysis of the fungal oil (Table 3) showed ammonium sulfate dosage increased the EPA content by 31.5% in TS and 12% in CTS.

Studies of lower dosages of N (0.5g/L) with C: N ratio 11: 1 in TS and 7:1 in CTS showed that the organic source of nitrogen (urea) was more favorable for oil production,

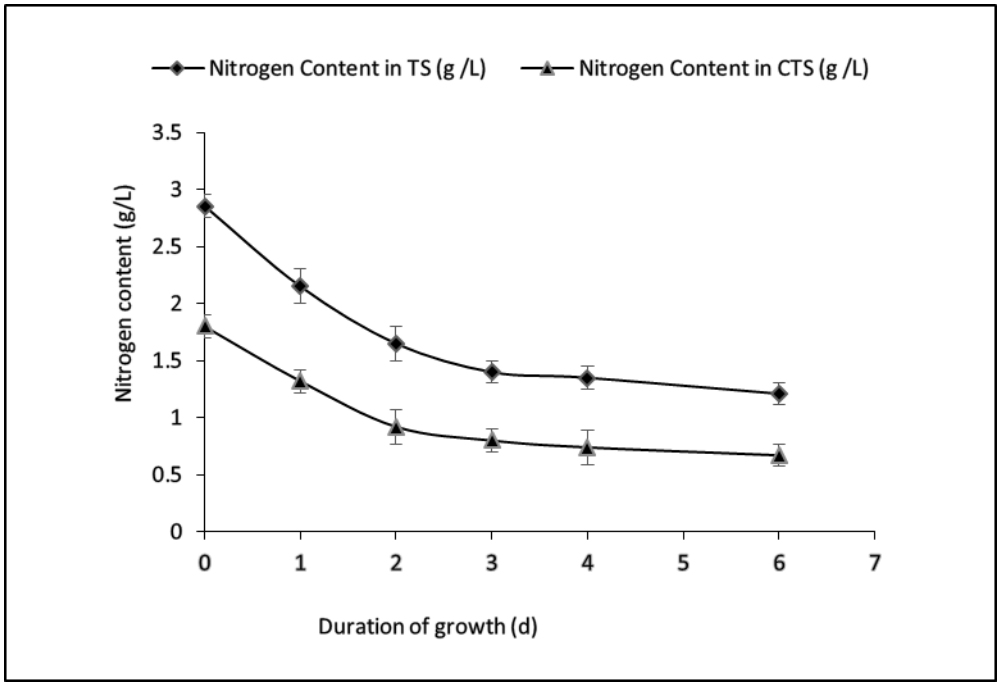
increasing the fungal oil yield by 12% and 13% and also the EPA content by 22% and 33% in TS and CTS respectively. Thus, the fungus is capable of synthesizing an oil with the highest PUFA content at 5g/L dosage of ammonium sulfate or 0.5 g/l dosage of urea. The fact that urea contains 47% N and ammonium sulfate 21% could have contributed substantially to the efficacy of urea at lower dosages. The carbon content of urea could also have contributed to the useful organic substrate for oil synthesis.

One more interesting conclusion from this study was that an optimum C: N ratio for producing high PUFA content in fungal oil couldn't be arrived at because the fatty acid composition of the oil depended on the source of carbon or nitrogen added to the medium rather than the C: N ratio. For a given C: N ratio, the fatty acid profile was found to vary for different sources of nitrogen added in same amount of N, evident from Tables 3 and 4. Fig.4 contains *Mucor circinelloides* filaments depicting intracellular oil bodies (Mitra et al., 2012)

4. Conclusions

This work provided five main findings: (1) *M. circinelloides* cultivated in TS and CTS synthesizes linolenic acid and EPA; (2) EPA content in fungal oil increased > 50% by extending cultivation from 3 to 6 days; (3) Increasing C: N ratio by glycerol addition increased oil production and PUFA content on TS, but was counterproductive on CTS; (4) Glycerol supplementation increased EPA production in TS and CTS respectively; (5) Ammonium sulfate and urea increased fungal oil production and EPA content but nitrates enhanced only EPA content and decreased oil yield.

5. Figures



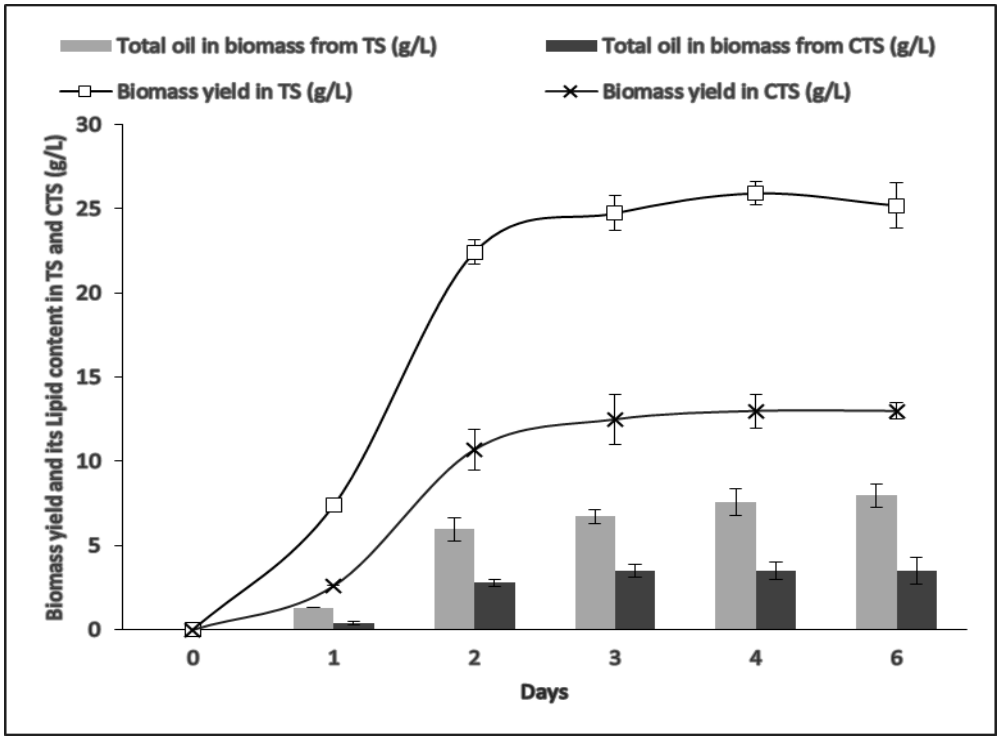


Fig. 1. Change in nitrogen content in TS and CTS during the growth of *M. circinelloides* for 6 days (top); fungal biomass and oil yield in TS and CTS during 6 days of cultivation (bottom)

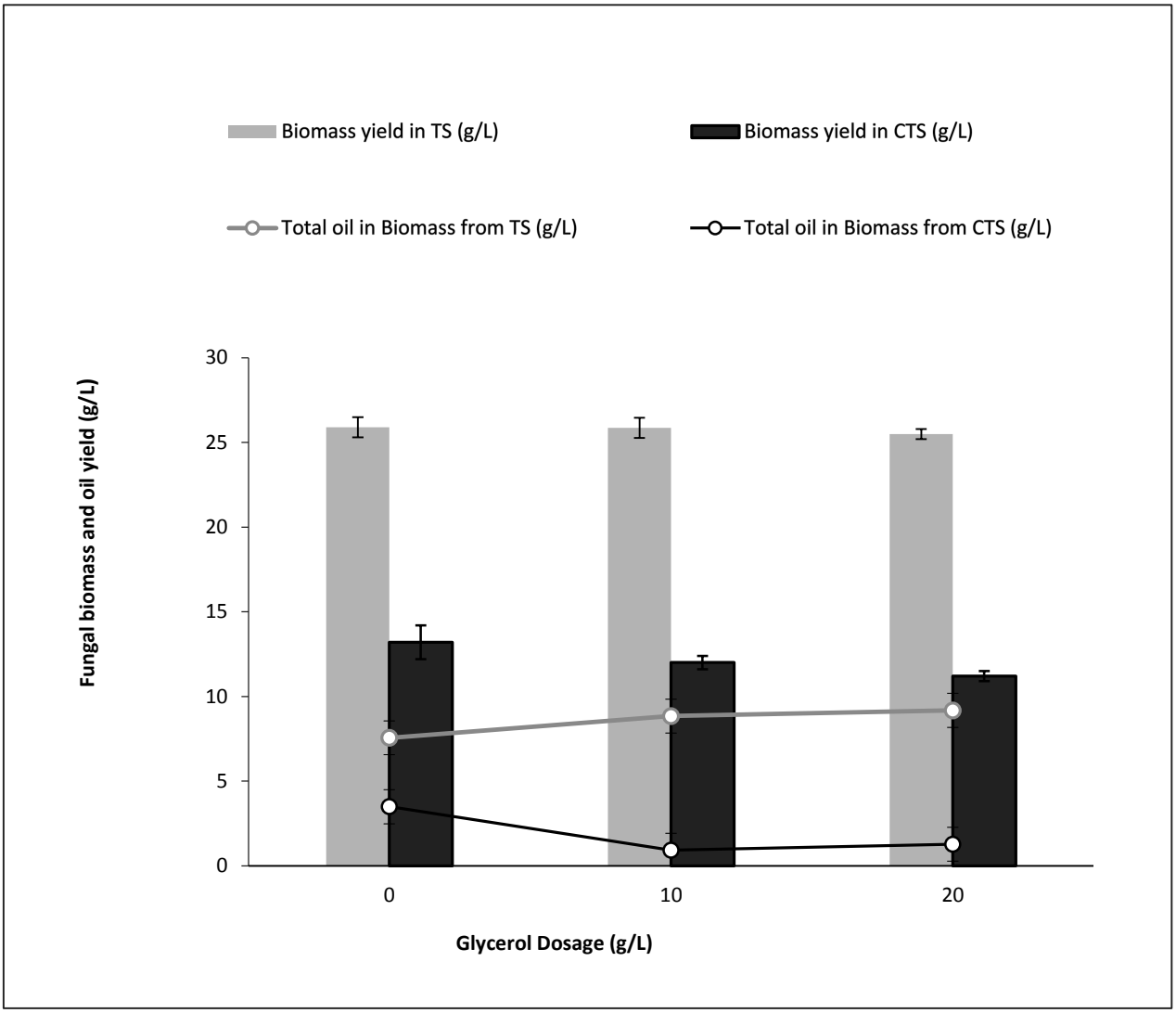


Fig. 2. Biomass and oil yield of *M. circinelloides* in TS and CTS for different dosage of Carbon using glycerol

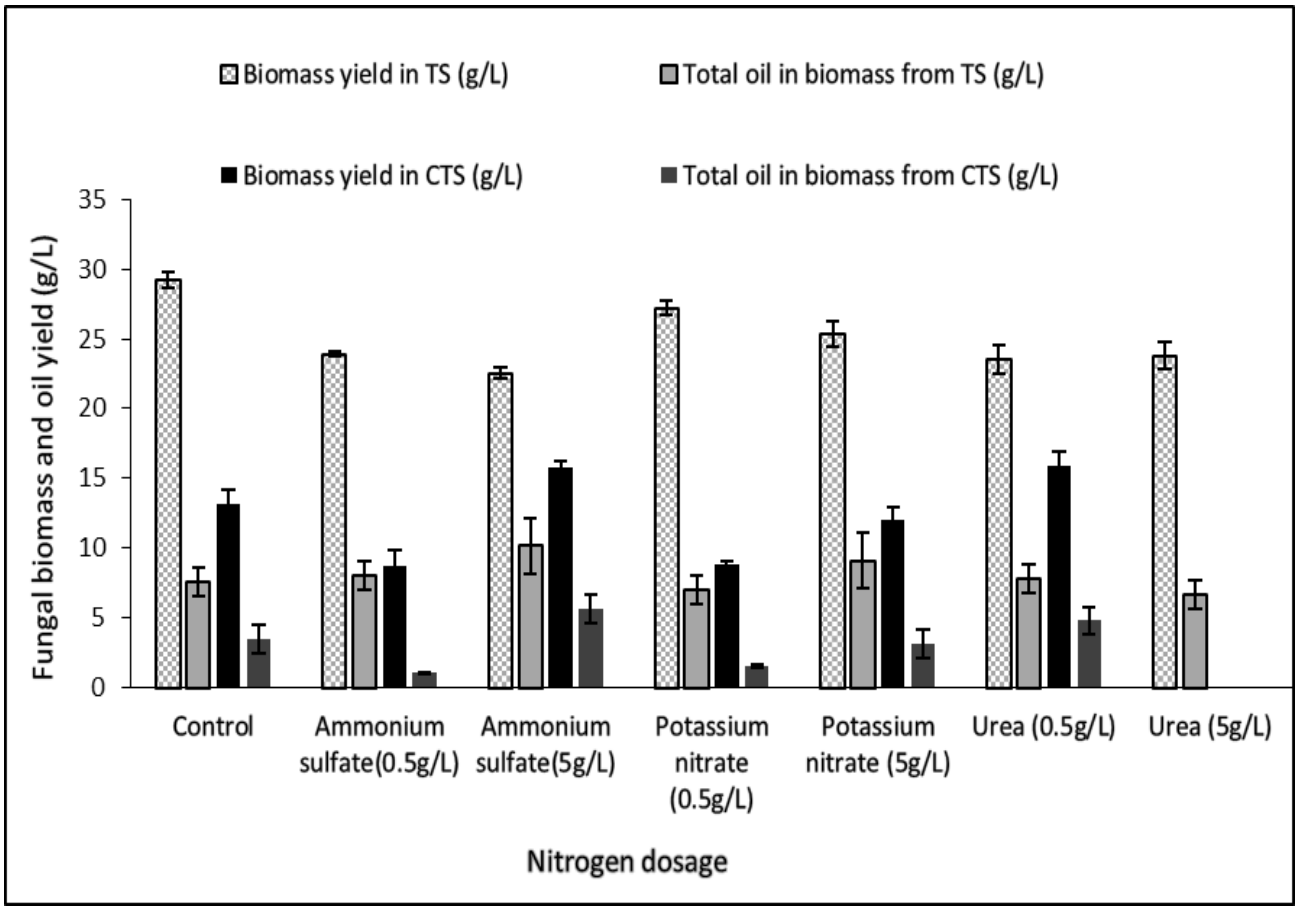


Fig. 3. Biomass and oil yield of *M. circinelloides* in TS and CTS for different dosage of nitrogen using urea, potassium nitrate and ammonium sulfate as nitrogen source

Fig.4 Micrographs (100x 10magnification) of *Mucor circinelloides* filaments depicting intracellular oil bodies. Adapted from (Mitra et al., 2012)

6. Tables

Table 1. Fatty acid profile of fungal biomass grown in thin stillage and centrifuged thin stillage for different growth periods. Compositional analysis was done by GC with prior transesterification to FAMEs. Data are means ± SD, n = 2

Fatty acid	Unit	TS [a]	Day 2 [b]		Day 3		Day 4		Day 6	
			TS	CTS	TS	CTS	TS	CTS	TS	CTS
16.0±0.3	%TFA	16.5±1.8		10.4±0.3	12.6±0.7	12.5±0.7	12.6±0.4	12.3±0.3	13.6±0.6	12.4±1.2
C 18:0	%TFA	2.2±0.1	3.4±0.4	1.0±0.1	2.5±0.4	2.7±1.0	2.5±0.2	3.4±1.6	3.4±0.5	4.3±0.4
C 18:1	%TFA	27.8±1.6	23.6±2.0	35.4±2.1	25.4±0.3	30.6±1.0	28.6±3.0	27.1±1.4	28.0±2.0	25.2±1.8
C 18:2	%TFA	53.0±1.8	55.0±3.0	50.0±0.2	56.3±4.1	50±4.0	52.4±2.7	51.4±2.2	50.0±3.2	52.2±1.6
C 18:3	%TFA	-	2.1±0.5	3.0±0.3	2.8±0.3	3.7±1.0	3.3±0.4	4.8±1.2	3.9±0.4	5.1±0.6
C 20:0	%TFA	0.3±0.0	-	-	0.4±0.3	-	0.4±0.3	-	1.0±0.1	-
C 20:5	%TFA	-	-	-	0.4±0.1	0.39±0.2	0.4±0.1	0.6±0.3	0.6±0.2	0.9±1.0
EPA content	mg/g of DW	-	-	-	1.0±0.1	1.1±0.2	1.3±0.1	1.5±0.3	2.0±0.2	2.6±1
TFA content	mg/g of DW	-	266.0±10.0	261.0±9.0	272.0±7.0	280.0±12.0	292.0±11.0	265.0±12.0	316.0±12.0	271.0±20.0
PUFA content	% TFA	53.0±1.8	57.1±3.5	53.0±0.5	59.5±4.5	54.1±5.2	56.2±3.2	56.8±3.7	54.5±3.8	58.2±3.2
PUFA content	mg/g of DW	-	151.6±9.3	138.3±1.3	161.8±12.3	151.5±14.5	164.1±9.3	150.5±9.8	172.2±8.2	157.7±8.6

[a] The fatty acid profile of freeze dried TS oil taking the mean values reported by Liang et al., (2012) and Mitra et al., (2012a)
[b] The fungal biomass grown in TS and harvested on day 2

Table 2. Fatty acid profile of fungal biomass grown in thin stillage and centrifuged thin stillage for different dosages of glycerol. Compositional analysis was done by GC with prior transesterification to FAMEs. Data are means ± SD, n = 2

Fatty acid	Unit	Glycerol dosage in TS			Glycerol dosage in CTS		
		Control [a]	10g/L	20g/L	Control [b]	10g/L	20g/L
	C:N	13:1	16.5:1	20:1	9:1	15:1	21:1
C 16:0	%TFA	12.6±0.4	12.8±0.8	13.1±0.6	12.3±0.3	11.1±0.6	11.8±0.4
C 18:0	%TFA	2.5±0.2	2.6±0.3	2.2±0.7	3.4±1.6	4.2±0.4	3.9±1.1
C 18:1	%TFA	28.6±3.0	30.8±1.3	29.1±1.2	27.1±1.4	30.2±0.4	31±0.9
C 18:2	%TFA	52.4±2.7	47.8±1.2	48.9±1.5	51.4±2.2	38.2±0.9	42.3±1.1
C 18:3	%TFA	3.3±0.4	5.6±0.2	6.1±0.2	4.8±1.2	15.2±0.4	10.1±0.8
C 20:0	%TFA	0.4±0.3	-	-	-	-	-
C 20:5	%TFA	0.4±0.1	0.6±0.2	0.6±0.3	0.6±0.3	0.9±0.5	1.4±0.1
EPA content	mg/g of DW	1.3±0.1	1.7±0.2	1.8±0.3	1.5±0.3	2.4±0.5	3.7±0.1
TFA content	mg/g of DW	292.0±11.0	343.0±10.0	361.0±20.0	265.0±12.0	77 ±10.0	91±10.0
PUFA content	% TFA	56.2±3.2	54.0±1.6	55.6±2.0	55.8±3.7	54.3±1.8	53.8±2.0
PUFA content	mg/g of DW	164.1±9.3	185.2±5.4	200.7±7.2	150.5±9.8	41.8±1.4	48.9±1.8

[a] Biomass grown on thin stillage without glycerol addition and harvested at day 4
[b] Biomass grown in centrifuged thin stillage without glycerol addition and harvested at day 4.

Table 3. Fatty acid profile of fungal biomass grown in thin stillage and centrifuged thin stillage for 5g/L dosage of different nitrogen sources. Compositional analysis was done by GC with prior transesterification to FAMEs. Data are means ± SD, n = 2
[a] The biomass grown on thin stillage and centrifuged thin stillage without nitrogen addition and harvested at day 4

Fatty acid	Unit	Control[a]		Potassium nitrate (5g/L)		Urea (5g/L)		Ammonium sulfate (5g/L)	
		TS	CTS	TS	CTS	TS	CTS	TS	CTS
	C:N	13:1	9:1	5:1	2.5:1	5:1	2.5:1	5:1	2.5:1
C 16:0	%TFA	12.6±0.4	12.3±0.3	14.8±0.5	12.1±0.5	13.7±0.4	-	14.7±0.2	13.2±1.2
C 18:0	%TFA	2.5±0.2	3.4±1.6	2.2±0.8	2.9±0.3	3.2±0.3	-	2.1±0.4	1.7±0.7
C 18:1	%TFA	28.6±3.0	27.1±1.4	30.2±1.1	33.1±1.2	28.3±0.6	-	30.3±1.1	27.6±0.6
C 18:2	%TFA	52.4±2.7	51.4±2.2	50.1±1.9	44.2±1.4	51.4±1.9	-	49.4±1.6	53.4±2.2
C 18:3	%TFA	3.3±0.4	4.8±1.2	1.9±0.7	5.3±0.8	2.9±0.9	-	2.3±0.9	2.2±0.3
C 20:0	%TFA	0.4±0.3	-	0.4±0.3	0.6±0.4	-	-	0.9±0.2	0.7±0.6
C 20:5	%TFA	0.4±0.1	0.6±0.2	0.5±0.2	0.6±0.2	0.4±0.1	-	0.4±0.1	0.4±0.1
EPA content	mg/g DW	1.3±0.3	1.5±0.1	1.8±0.1	1.5±0.4	1.3±0.3	-	1.9±0.1	1.7±0.7
TFA content	mg/g DW	292.0±11.0	265.0±12.0	359.0±20.0	261.0±10.0	283.0±10.0	-	445.0±20.0	359.0±20.0
PUFAcontent	% TFA	56.2±3.2	55.8±3.7	52.5±2.8	50.1±2.4	54.7±2.9	-	52.1±2.6	56±2.6
PUFA content	mg/g DW	164.1±9.3	150.5±9.8	188.5±10.0	130.5±6.2	154.8±8.2		231.8±11.5	201±9.3

[a] The biomass grown on thin stillage and centrifuged thin stillage without nitrogen addition and harvested at day 4

Table 4. Fatty acid profile of fungal biomass grown in thin stillage and centrifuged thin stillage for 0.5g/L dosage of different nitrogen sources. Compositional analysis was done by GC with prior transesterification to FAMEs. Data are means ± SD, n = 2

		Control[a]		Potassium nitrate (0.5g/L)		Urea (0.5g/L)		Ammonium sulfate (0.5g/L)	
Fatty acid	**Unit**	**TS**	**CTS**	**TS**	**CTS**	**TS**	**CTS**	**TS**	**CTS**
	C:N	13:1	9:1	11:1	7:1	11:1	7:1	11:1	7:1
C 16:0	%TFA	12.6±0.4	12.3±0.3	12.4±0.3	12.1±0.4	11.6±0.8	11.4±0.2	14.1±0.2	13.7±0.2
C 18:0	%TFA	2.5±0.2	3.4±1.6	2.4±0.3	4±0.4	2.3±0.2	2.6±0.4	2.7±0.1	3.8±0.3
C 18:1	%TFA	28.6±3.0	27.1±1.4	29.7±1.1	27.2±0.7	26.8±1.2	24.5±1.1	29.6±1.2	24.3±1.1
C 18:2	%TFA	52.4±2.7	51.4±2.2	52.8±2.4	44.4±1.3	51.5±1.9	53.1±1.7	50.3±1.9	44.0±2.5
C 18:3	%TFA	3.3±0.4	4.8±1.2	1.6±0.5	7.7±0.3	2.2±0.5	1.5±0.4	2.2±0.2	12.1±0.3
C 20:0	%TFA	0.4±0.3	-	0.4±0.2	1.0±0.2	0.4±0.1	0.4±0.1	0.8±0.1	-
C 20:5	%TFA	0.4±0.1	0.6±0.3	0.4±0.1	1.2±0.3	0.5±0.1	0.7±0.1	0.4±0.1	1.6±0.1
EPA content	mg/g DW	1.3±0.3	1.54 ±0.1	1.1±0.3	2.3±0.4	1.7±0.2	2.2±0.3	1.4	2.0±0.3
TFA content	mg/g DW	292.0±11.0	265.0±12.0	259.0±10.0	178.0±10.0	332.0±10.0	306.0±10.0	338.0±10.0	121.0±10.0
PUFA content	% TFA	56.2±3.2	55.8±3.7	54.8±3.0	53.3±2.0	54.2±2.5	55.3±2.2	52.9±2.2	57.7±2.9
PUFA content	mg/g DW	164.1±9.3	150.5±9.8	141.9±7.8	94.8±3.6	179.9±8.3	169.2±6.7	178.8±7.4	69.8±3.5

[a] The biomass grown on thin stillage and centrifuged thin stillage without nitrogen addition and harvested at day 4

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CHAPTER 4

GENERAL SUMMARY AND CONCLUSION

General discussion and research problems

The fungus *Mucor circinelloides* was found to be capable of synthesizing EPA when grown in both TS and CTS. Although the EPA content per g dry biomass was lower compared to *Pythium irregular* (Liang et al., 2012) when grown on TS, more conditions need to be tested before arriving at a concrete conclusion about the capability *Mucor circinelloides* to synthesize EPA.

The problem with the fungal growth and oil production is the inconsistency in fatty acid profiles obtained when repeating experiments with different fungal strains of the same fungi. Different fungal spore suspensions could yield different fatty acid profile when experiments are performed in different batches.

In the experiments carried out by (Mitra et al., 2012) with one particular *Mucor* strain, the fatty acid profile of the fungi in different growth media is shown in Fig.1. The author reported that the fungus was capable of synthesizing gamma linolenic acid when cultivated in TS but no EPA was found. In the results from our experiment using a different fungal strain, the fungus was found to synthesize EPA when grown in TS for 3 days without altering conditions much and EPA content increasing during longer growth periods

The experiments in this project were done using shake flasks. Using a 2-L bio reactor and repeating the conditions could lead to higher level of EPA content because of aeration (Erwin, 1973).

Lipid class	Total fatty acid composition (wt %)		
	Mucor-YM_{oil}	Mucor-TS_{oil}	TS_{oil}
C 13:0	12.5 ± 1.4	NA	NA
C 14:0	2.3±0.1	NA	NA
C15:0	0.6±0.0	NA	NA
C16:0	15.8±0.4	15.7±1.0	15.0±0.2
C16:1	6.3±0.0	NA	NA
C18:0	4.0±0.2	2.3±0.1	2.2±0.0
C18:1	24.4±0.7	29.6±0.5	28.7±0.2
C18:2	15.7±0.2	50.0±1.6	52.5±0.2
C18:3	17.5±0.1	1.4±0.1	NA
C20:0	0.6±0.0	1.2±0.1	1.5±0.1
C22:0	0.4±0.0	NA	0.1±0.1

Fig. 1 The fatty acid composition in oil obtained from *Mucor circinelloides* when grown in TS at 37 °C for 2 days in an air lift bioreactor. (Mitra et al., 2012)

Recommendation for future research

There are several other experimental parameters that can be modified in the fungal growth process which can have an effect on the EPA content in the oil.

Effect of temperature

Liang et al. (2012) determined that temperature plays a vital role in EPA production. Jiang and Chen (2000) and Wen and Chen (2001) found that generally, high temperature leads to faster cell growth and higher cell density while low temperature stresses

increase unsaturated fatty acid content. The reason attributed was that ‘cells need to maintain membrane fluidity at low temperature, by increasing the degree of unsaturation in fatty acids’. Liang et al. (2012) also found that EPA was produced by the fungus *Pythium irregulare* in TS at high temperature, owing to low DO content due to solid particles in TS getting attached to fungal biomass and blocking oxygen transfer and limiting the cell growth. Xia et al. (2011) and Mysyakina and Funtikova (2008) reported that *M. circinelloides* has best growth temperatures ranging between 24°C to 30°C. However, Mitra et al. (2012) reported the highest biomass yield at 37°C when grown in TS for 2 days. Hence, changing the incubation temperature to below 24°C or above 40°C during the exponential growth phase should provide stressful conditions for the fungal biomass and may lead to more production of PUFA by *M. circinelloides*.

Temperature shift strategy studied by Liang et al. (2012) on *P. irregulare* grown in TS reportedly produced more EPA in a shorter time as compared to harvesting the fungi with same temperature throughout the growth experiment. Also Converiti et al. (2009) reported lipid content in biomass being 2.5 times higher on *C. vulgaris* when incubation temperature was changed from 30°C to 25°C during the growth experiment. Thus implementing this strategy on *M. circinelloides*, during the stationary phase, which is between day 2 and 3, Mitra et al. (2012) found that this results in higher PUFA content in oil

This strategy can also be extended to a novel method of inducing a sudden temperature shock during these fungi’s exponential growth phase, by subjecting the growth media to a low temperature of about 15°C by refrigerating or a high temperature from 40°C which might enhance the PUFA content in fungi.

Effect of ageing

Microorganisms store their energy in the form of lipids rich in both saturated and unsaturated fatty acids. Shizmu et al. (1989) reported that the oleaginous fungi *M. alpina* when harvested on the 6th day and then further allowed to stand for 7 days at 28°C produced 1.6 times more EPA than that determined at the initiation of ageing. This effect of ageing has not been studied on *M. circinelloides*. Therefore after harvesting the fungi, they can be incubated at normal temperatures such as 28°C and stressful temperatures discussed earlier, for one week and their lipid fatty acid profile can be compared.

Effect of pH

An initial pH of 6.0 – 7.6 was found to be optimal for EPA production in fungi and algae (Yongmanitchai and Ward, 1991; Bajpai et al., 1992). The pH in thin stillage is about 4.5 and so changes in pH could be effective for the fungi to produce more EPA. The best pH for lipid production varies with fungal species, but lipid content is almost unchanged between pH 5.9 to 7.5 (Weete, 1980).

Based on the above results, we can hypothesize that the fungus *M. circinelloides* will be capable of synthesizing more eicosapentanoic acid (EPA 20:5 n-3) when two or more conditions are favorable for the fungi to grow in the TS and CTS media.

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