Bioresource Technology 107 (2012) 368–375

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Value-added oil and animal feed production from corn-ethanol stillage using the oleaginous fungus *Mucor circinelloides*

Debjani Mitra [a,b,c], Mary L. Rasmussen [b,c], Priyanka Chand [a,d], Venkat Reddy Chintareddy [e], Linxing Yao [b], David Grewell [a,d], John G. Verkade [e], Tong Wang [b], J. (Hans) van Leeuwen [a,b,c,d,*]

[a] Biorenewable Resources and Technology Program, Iowa State University, Ames, IA 50011, USA
[b] Department of Food Science and Human Nutrition, Iowa State University, Ames, IA 50011, USA
[c] Department of Civil, Construction and Environmental Engineering, Iowa State University, Ames, IA 50011, USA
[d] Department of Agricultural and Biosystems Engineering, Iowa State University, Ames, IA 50011, USA
[e] Department of Chemistry, Iowa State University, Ames, IA 50011, USA

ARTICLE INFO

Article history:
Received 8 October 2011
Received in revised form 5 December 2011
Accepted 6 December 2011
Available online 14 December 2011

Keywords:
Thin stillage
Mucor circinelloides
Oleaginous
Value-addition
Fungal lipids

ABSTRACT

This study highlights the potential of oleaginous fungus, *Mucor circinelloides* in adsorbing/assimilating oil and nutrients in thin stillage (TS), and producing lipid and protein-rich fungal biomass. Fungal cultivation on TS for 2 days in a 6-L airlift bioreactor, resulted in a 92% increase in oil yield from TS, and 20 g/L of fungal biomass (dry) with a lipid content of 46% (g of oil per 100 g dry biomass). Reduction in suspended solids and soluble chemical oxygen demand (SCOD) in TS were 95% and 89%, respectively. The polyunsaturated fatty acids in fungal oil were 52% of total lipids. Fungal cells grown on Yeast Malt (YM) broth had a higher concentration of γ-linolenic acid (17 wt.%) than those grown on TS (1.4 wt.%). Supplementing TS with crude glycerol (10%, v/v) during the stationary growth phase led to a further 32% increase (from 46% to 61%) in cellular oil content.

1. Introduction

Corn ethanol production in the United States has doubled from 6500 in 2007 to 13,230 MGY in 2010 (Renewable Fuels Association, 2011). For every gallon of ethanol produced, 5–6 gallons of stillage is generated (Rasmussen et al., in press) which is composed of corn fiber, oil, protein, other unfermented components of the grain and yeast cells (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). In a study by Moreau et al. (2011), it was found that TS contained the highest amount of corn oil on a dry basis, among the various pre- and post-fermentation corn fractions in the dry-grind corn ethanol process. In recent years, recovery of corn oil from post-fermentation corn fractions has drawn considerable interest for use as a biodiesel feedstock (Noureddini et al., 2009).

The presence of oil in a microbial growth medium increases the lipid accumulation in the microbial cells, and the composition of the accumulated intracellular lipid reflects the length of the carbon chain and structure of the oil source (Szczesna-Antczak et al., 2006). Certain mucoralean fungal strains like *Mucor circinelloides* are known to be efficient producers of intracellular lipases, which display very high hydrolytic and synthetic activities (Szczesna-Antczak et al., 2006). Fungal biomass has also been documented as excellent biosorbent material (Ozsoy et al., 2008) capable of removing oil from oil–water emulsions (Srinivasan and Viraraghavan, 2010). Based on the above facts, we hypothesized that growing *M. circinelloides* on TS would help in both oil recovery and production since the fungal cells would not only adsorb and remove the oil from TS but would also be able to metabolize the corn oil and use the end products (fatty acids) for further lipogenesis. Since, triglycerides are the main constituent of both corn and fungal lipids (Vicente et al., 2009), they could serve as suitable feedstock for biodiesel production, which could then be used

Abbreviations: TS, thin stillage; CTS, centrifuged thin stillage; SLPM, standard liters per minute; CDS, condensed distillers' solubles; DDGS, dried distillers grains with solubles; TAG, triacylglycerol; FFA, free fatty acids; SCOD, soluble chemical oxygen demand.

* Corresponding author at: Department of Civil, Construction and Environmental Engineering, Iowa State University, Ames, IA 50011, USA. Tel./fax: +1 515 294 5251.
E-mail address: leeuwen@iastate.edu (J. (Hans) van Leeuwen).

0960-8524/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.biortech.2011.12.031

in-house by the corn-ethanol plants or sold. Our hypothesis is further supported by the fact that *M. circinelloides* is oleaginous in nature and accumulates high levels of lipids in its mycelia (Vicente et al., 2009). More significantly, its oil has been found to be rich in γ-linolenic acid, which has a growing market demand due to its high value as a nutraceutical (Ratledge, 2004). Xia et al. (2011) tested the effect of culture conditions such as pH and temperature on growth and lipid production by *M. circinelloides* in a synthetic medium. In this study, fatty acid characteristics of Mucor oil, especially the presence of high-value PUFAs were examined by growing the fungi in both a synthetic (YM), and a complex organic medium (TS).

Despite the numerous favorable impacts of biodiesel production, its economic aspect suffers from the high cost of raw material (Miao and Wu, 2006). Plant-based oil feedstocks account for almost 85% of total biodiesel production cost and also lead to food vs. fuel controversy (Miao and Wu, 2006). Microbial lipids in contrast, do not require fertile land and also have much higher yields (Meng et al., 2009). Especially mucoralean fungi are known to grow rapidly and contain almost 40–50% oil (Sergeeva et al., 2008; Vicente et al., 2009). These are also excellent enzyme producers and can be grown on various substrates. For example, *Rhizopus microsporus* (var. *oligosporus*) has been successfully grown on TS from corn ethanol plants (Rasmussen et al., in press) for use as animal feed. To our knowledge, this is the first study of TS as a growth medium for the production of *M. circinelloides* biomass and fungal oil. Our study also reports the change in fatty acid composition of lipids produced by *M. circinelloides* when grown on an oil-free synthetic medium, YM broth (Difco Laboratories, Sparks, MD, USA) as compared to the oil-containing TS medium. The sustainability and profitability of biodiesel plants also requires value-addition to its co-products especially crude glycerol, which is being generated in surplus amounts (Nitayavardhana and Khanal, 2011). Due to its easy availability, inexpensive nature and existing evidence of its consumption by fungal strains (Nitayavardhana and Khanal, 2011), crude glycerol was investigated in this study as a supplementary carbon source.

Microbial cell disruption, in order to release intracellular lipids, is a fairly new technique and has been employed in a few studies. Ultrasonication assisted oil extraction was used by Cravotto et al. (2008) to extract intracellular oil from a marine microalga and higher yields were obtained with ultrasonication compared with microwaves or Soxhlet extraction. In this study, we have applied ultrasonication to disrupt the cell wall of *M. circinelloides* prior to solvent extraction. The Folch, Lees and Stanley method (Christi and Han, 2010) is one of the most popular oil extraction methods and involves the use of chloroform, which is very toxic and hazardous to human health with increasing restrictions on its use. In this study, we have investigated the efficacy of the toluene/methanol solvent pair (1:1) for oil extraction instead of chloroform/methanol, and compared the yields from both methods.

Some of the research results from this study resulted in an R&D 100 Award from R&D Magazine in 2009 (http://www.rdmag.com/Community/Blogs/RDBlog/Helping-The-Third-World/).

2. 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 in sterile 10-L carboys at 4 °C prior to use. Two batches of stillage were used for the entire study. Both batches were chemically characterized and were noted to have similar compositions. The solids in the TS were seen to settle upon storage for 3–4 days, leaving a clearer liquid on top. The top layer was decanted and centrifuged at 5000×*g* for 15 min. Both regular thin stillage (TS) without settling (% total solids = 6.1) and centrifuged thin stillage (CTS, % total solids = 3.8) were used as fungal growth media following heat sterilization at 121 °C for 15 min.

2.2. Fungal strain, media and inoculum preparation

Lyophilized culture of *M. circinelloides* f. *lusitanicus* CBS 277.49 was purchased from CBS-KNAW Fungal Biodiversity Centre, The Netherlands. The frozen culture was soaked in sterile deionized water and revived by spread plating onto Bacto PDA (Potato Dextrose Agar, Difco Laboratories, Sparks, MD, USA) plates and incubating for 5–7 days at 37 °C until white sporulation was observed. Fungal spores and mycelia were lightly scraped off from the plates aseptically using a sterile solution of 0.1% (w/v) Bacto peptone and 0.2% (v/v) Tween 80 (Fisher Scientific, Fair Lawn, NJ, USA). The spore and mycelial suspension was then passed through a 20 mL syringe containing glass wool which removed the mycelia. The filtrate or spore suspension was mixed with Bacto potato dextrose broth (PDB) in a 1:1 ratio and glycerin was added to a final concentration of 20% (v/v). The glycerin-spore stock was distributed into sterile 2 mL cryo-vials and preserved in a −75 °C freezer. The spore count of the stock was determined by a haemocytometer and found to be $\sim 8 \times 10^4$ spores/mL.

The fungal inoculum for the shake flask studies was prepared by adding 0.2% (v/v) of spore suspension to 50 mL of heat sterilized (121 °C, 15 min) YM broth (Difco Laboratories, Sparks, MD, USA) in 200 mL Erlenmeyer flasks with foiled covered mouth. The inoculum for the 6-L bioreactor was prepared by adding 0.2% (v/v) of spore suspension to 500 mL YM broth contained in a 2-L flask with cotton plugged mouth. Centrifuged thin stillage (CTS) was also tested for inoculum preparation in an attempt to acclimate the fungal cells to stillage nutrients, for enabling higher biomass yields from the main cultivation process in TS. Inoculum flasks were incubated on a shaker at 150 rpm, 37 °C for 24 h. The fungal cells that grew as small compact mycelial pellets were then filtered using Whatman No. 1 filter paper, rinsed with sterile saline to remove media components and used as the inoculum. Quality control tests were performed to confirm uniformity in inoculum cell weight added to each cultivation batch. In a separate batch experiment, the fungal mycelial inoculum was shredded using a homogenizer to test the ability of smaller mycelial fragments in producing higher fungal growth as compared to compact pellets.

2.3. Fungal cultivation

Growth conditions of *M. circinelloides* were optimized in a series of shake flask experiments in 2 L Erlenmeyer flasks containing 500 mL of TS. The TS was heat sterilized (121 °C, 15 min) unless otherwise mentioned. A 10% (v/v) mycelial inoculum was added and the flasks were incubated on a shaker at 150 rpm agitation speed and 37 °C for 2 days or longer when required. The optimized culture conditions were scaled up in the 6-L airlift draft-tube bioreactor with a 5 L working volume. An aeration rate of 7 SLPM (standard liters per minute; 1.4 vvm) was used. The draft tube facilitated proper air circulation and mixing of the reactor contents.

2.3.1. Optimization of growth conditions in flasks

The growth of the fungus was tested in whole thin stillage (TS, total solids = 6.1%) and centrifuged thin stillage (CTS, total solids = 3.8%). Since significantly higher ($p < 0.05$) fungal biomass yield (g/L) was obtained from TS as compared to CTS (as discussed in Section 3), TS was chosen to be used in the rest of the optimization studies. The effect of sterilization on fungal growth was checked by autoclaving TS in flasks at 121 °C for 15 min. Since

sterile TS gave consistently higher fungal cell yields than non-sterile TS under the same culture conditions (as discussed in Section 3), further growth optimizations were done using autoclaved TS. The effect of pH on fungal growth was tested by adjusting the initial pH of TS to 4, 5, 6 and 7, before sterilization using 1 N HCl/1 N NaOH. The flasks with TS adjusted to different pH levels were then inoculated with mycelia and incubated under similar conditions. A range of incubation temperatures viz. 25, 30, 37 and 45 °C were examined and optimized conditions favoring highest growth were selected. In another optimization study, the length of the incubation period required for the highest biomass and oil yield was determined. Flasks containing TS inoculated with fungal mycelia were incubated for 1, 2, 4, 6, 8 and 10 days. At each time period, two flasks (duplicates) were randomly removed from the shaker and screened to determine the fungal biomass and oil yield. The effect of the physiological state of the fungal inoculum (i.e. spores vs. mycelia on the fungal growth rate and final biomass yield) was also evaluated. One set of flasks with TS was inoculated with 0.2% (v/v) spore stock, while the other set was inoculated with 10% (v/v) mycelia and the biomass yields were compared.

2.3.2. Optimization of growth and oil production conditions in 6-L airlift reactor

The optimized growth conditions determined by the shake flask studies were scaled up to the draft-tube 6 L airlift bioreactor. The fungal culture was grown for 72 h on sterile TS with a working volume of 5-L adjusted to pH 6, at 37 °C and 7 SLPM aeration rate. Samples were periodically removed from the broth during the cultivation process, filtered aseptically using a screen (pore-size of 1 mm × 1 mm) and the fungal cells were returned to the reactor. The effluent/filtrate was analyzed for total solids, COD, soluble sugars, acids, glycerol and nitrogen content. At the end of each run, the entire culture broth was filtered and the biomass yield and oil content were measured.

In another batch of fungal cultivation on TS, a supplemental carbon source in the form of crude glycerol (10%, v/v) was fed to the fungal culture and its effect on fungal oil production was tested. The crude glycerol was obtained as a by-product from a local soy-biodiesel plant. The glycerol was added to the fungal culture broth at the stage where the glycerol and lactic/acetic acid concentration in TS had just started falling. This phase indicated the exhaustion of available sugars and the shift of the fungal metabolism towards consumption of alternative carbon sources in the stillage. After glycerol addition, the fungi were allowed to grow for another 2 days. As a control, the fungal culture was grown in unsupplemented TS for the same time period. Fungal biomass and lipid yields from both cultivation batches were then measured and compared.

2.4. Analytical methods

2.4.1. Fungal biomass yield

The fungal biomass with attached stillage solids was filtered out from the culture broth using a stainless steel screen of pore size of 1 mm. The biomass was then oven dried at 80 °C for 24 h. The dried solids were measured gravimetrically and the biomass yield was reported in terms of 'grams of dried biomass per liter of stillage'.

2.4.2. Fungal biomass characterization

Proximate analysis of dried fungal cells was done to investigate its suitability as an animal feed. Total crude protein was measured by the AOAC official combustion method (AOAC 990.03) and an Elementar Vario Max Carbon Nitrogen analyzer (Elementar Analysensysteme GmbH, Hanau, Germany). Total carbohydrate content in the biomass was calculated by adding the starch, fiber and soluble sugar. Starch was measured by the amyloglucosidase/α-amylase method (Knudsen, 1997); while the amylase neutral detergent fiber method (Van Soest et al., 1991) and phenol–sulfuric acid colorimetric assay (Dubois et al., 1956) were used to measure the fiber and soluble carbohydrate contents, respectively. Total fat was measured using the SoxCap 2047 with Soxtec extraction systems (Foss Analytical AB Soxtec System Application Note AN3906 (2007)), followed by the ether extraction method for crude fat estimation (AOAC Official Method 920.39). The ash content was determined as the residue after ignition of the biomass at 600 °C for 3 h. The percent moisture content was measured by heating the samples to 105 °C and calculating the loss in moisture gravimetrically as described in the Standard Methods for the Examination of Water and Wastewater (2005). The total carbohydrate and fat measurements were performed by Dairyland Laboratories Inc., WI.

2.4.3. Chemical analyses of culture broth

The effluent TS after removal of fungal cells was analyzed for nitrogen, soluble sugars, acids, glycerol and soluble chemical oxygen demand (SCOD). The soluble sugars, lactic and acetic acids, and glycerol were measured using a Waters Model 401 HPLC system with an RI detector, equipped with a column heater, auto-sampler, and computer controller. A Bio-Rad Aminex HPX-87H column (300 × 7.8 mm; Bio-Rad Chemical Division, Richmond, CA, USA) was used with 0.012 N sulfuric acid as the mobile phase with a flow rate of 0.8 ml/min, injection volume of 20 µl, and a column temperature of 65 °C. Total crude protein was measured as described earlier. SCOD was calculated using the Hach COD kit (COD Vials 200–15,000 mg/L, High Range Plus, Hach company, Loveland, CO, USA). Total and suspended solids in TS were measured as described in the Standard Methods for the Examination of Water and Wastewater (2005).

2.4.4. Intracellular fungal-oil extraction using high-power ultrasonication and organic solvents

The oven-dried fungal cells were subjected to ultrasonication in the presence of organic solvents in order to mechanically disrupt the cell wall and enable extraction of the intracellular oil by the solvents. A Branson 450 Series bench-scale ultrasonics unit (Branson Ultrasonics, Danbury, CT) with a maximum power output of 400 W and frequency of 20 kHz was used. The ultrasonic cell-disruptor horn used was a tapered microtip titanium horn with a flat 1/8 in. (3 mm) diameter face. The solvent system comprised of methanol and toluene (1:1). Toluene was tested as a substitute for chloroform, which is the standard solvent used in the standard Folch, Lees and Stanley method (Christi and Han, 2010). As a control, the oil content (wt.%) in the fungal biomass was also measured by the standard method (Christi and Han, 2010) and compared. Other solvent systems that were investigated were – toluene, methanol, hexane, and methanol:toluene (1:5), methanol:hexane (1:1). One gram of dried fungal biomass was mixed with 20 mL of the solvent system and sonicated at 70% amplitude (494 μm_{pp}) for 3 min. The optimum time of sonication was found to be 3 min after testing 0.5, 1, 2, 3, 4 and 5 min (data not shown). The glass container with the fungal cell samples was placed in an ice bath during the sonication process to prevent temperature rise. After sonication, anhydrous magnesium sulfate was added to remove any water present and the mixture was filtered through Whatman No. 1 filter paper. The filter paper was rinsed with toluene to wash away any residual oil sticking to the paper. The solvents were removed from the filtrate using a rotary evaporator at 65 °C (Buchi rotavapor R124) and the weight of the oil was determined gravimetrically. The lipid concentration was calculated as percent oil yield (g of extracted oil per 100 g of dry biomass). The lipid productivity ($g\,L^{-1}\,day^{-1}$) obtained from each culture medium was determined as the product of biomass productivity ($g\,L^{-1}\,day^{-1}$) and lipid content (g of oil per g of biomass) as

follows: $P_L = C_L \times \Delta CDW/t$; where P_L is the lipid productivity, ΔCDW is the accumulated dry cell weight from inoculation to harvest time, t and C_L is the cellular lipid content. The efficiency of the sonication method of cell disruption was compared to other mechanical shearing techniques such as magnetic stirring (with glass beads) and rotary mixing, using the same solvent system and the same batch of fungal biomass. The oil content in TS itself was determined by freeze-drying one liter of TS and extracting the corn oil from the dried stillage solids using the sonication–solvent method. All the lipid samples were analyzed for their fatty acid composition by thin layer chromatography (TLC) and gas chromatography (GC).

2.4.5. Fatty acid analysis of lipids

The oil samples extracted from fungal biomass grown on TS and YM as well as the stillage oil samples were subjected to fatty acid profiling using GC. Methyl heptadecanoate (C17:0) was added as an internal standard to 30 mg of oil sample. The total lipids were esterified to fatty acid methyl esters (FAMEs) by treatment with 3% (v/v) sulfuric acid in methanol for 2 days at 60 °C. The FAMEs were extracted by hexane and washed with water and then analyzed with a Hewlett–Packard 5890 series II GC equipped with a flame ionization detector and a SPB-2330 fused silica column (15 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 200 °C at a rate of 5 °C/min and the injector and detector temperatures were 230 °C. The sample injection volume was 1 μL. The carrier gas (helium) flow rate was 3.5 mL/min.

The lipid classes in the oil samples were characterized by thin layer chromatography (TLC). About 50–60 mg of oil sample was dissolved in hexane (1 mL) and acetone (2 drops) and applied to a preparative TLC plate (20 × 20 cm, 500 μm thickness). The plate was developed using hexane/diethyl ether/acetic acid (70:30:1, v/v/v), then sprayed with 2,7-dichlorofluorescein and viewed under UV light. The TAG and FFA bands were scraped off and extracted twice with 10 mL of diethyl ether. The scraped DAG and polar lipids bands were extracted twice with 10 mL of chloroform/methanol (2:1, v/v). The solvents were removed by using a flow of nitrogen. The fatty acids were transesterified into FAMEs following the procedure given above. All analyses were done as duplicates.

2.5. Statistical analysis

The Analysis of Variance (ANOVA) was performed using JMP 8.0.2 (Cary, NC, USA), to determine significant differences among the various treatments. Least Significant Means Differences (LSD) were calculated 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. Optimization of M. circinelloides growth in flasks

3.1.1. Effect of corn solids on fungal morphology and biomass yields

The dry fungal biomass yield from CTS averaged about 8 g/L while unsettled (original) TS gave an average biomass yield of 20 g/L when cultivated in a 6-L airlift bioreactor for 2 days. When unsettled TS (without fungal cells) was passed through the same screen (pore size, 1 mm × 1 mm), 2 g/L (dry) of corn solids were retained. Several factors contributed to the lower yields obtained from CTS, among which lower nitrogen and insoluble sugar contents, due to the removal of suspended solids, were most influential. The suspended solids consisted of both corn solids left-over after ethanol separation from the corn mash, as well as some residual yeast cells and some possible bacterial contaminants too. Due to the higher biomass yields, TS was used in all further experiments instead of CTS. *M. circinelloides* is known to produce cellulolytic and other extracellular enzymes (Botha et al., 1997) which could have aided in degrading and utilizing the insoluble di- and polysaccharides present in TS. The total dissolved solids were quite similar in CTS (40 g/L) and TS (43 g/L), while the total nitrogen content was much lower in CTS (1.5 g/L) compared to TS (5.5 g/L).

Filamentous fungi are known to grow as loose hyphal aggregates called "mycelial clumps", or as denser, often spherical aggregates called "pellets" (Paul and Thomas, 1998). Visual observations showed distinct morphological differences in the growth of *M. circinelloides* in TS as compared to CTS. In TS it grew as branched mycelia, while in CTS it grew as compact pellets. Similar observations were made by Rasmussen et al. (in press) in the case of *Rhizopus oligosporus*, where they found a distinct change in fungal morphology from compact pellets to filaments when the fungus was grown on settled thin stillage supernatant and TS respectively. The presence of corn solids in TS might serve as supports for attachment by the fungal mycelia. We found that the fungal mycelial biomass adsorbed and separated most of the corn solid matter from the TS leaving behind a relatively clearer effluent. Since the mycelial form is known to contain higher chitosan than the yeast-like form (discussed later), the mycelial biomass of *M. circinelloides* facilitated the agglomeration of corn solids within and around the fungal filaments resulting in the efficient removal of corn solids from the thin stillage. Solids content in stillage between 3% and 6% supported good fungal growth, but 8% or more was found unfavorable for fungal cultivation and dilution was required. Concentrated thin stillage also known as "syrup", or "condensed distillers" solubles (CDS) has a high solid content (~25% total solids). When it was tested as a growth medium for *M. circinelloides* cultivation, no screenable (pore size, 1 mm) fungal growth could be seen unless the syrup was diluted.

Dimorphic growth is a typical feature of representatives from the genus *Mucor*. Sporangiospores are specialized fungal cells which germinate under favorable conditions to produce mycelia, while the yeast-like budding cells are the pre-dominant forms present under stressed conditions such as low pH, high glucose, low nitrogen or anaerobic atmosphere (Funtikova and Mysyakina, 2003). Extensive research has been done on the correlation between fungal morphology and intracellular lipid composition. The lipids in these yeast-like cells differed from mycelial cells and were found to contain higher concentrations of polar lipids and lower concentrations of unsaturated fatty acids and glycolipids, which makes it unsuitable as a biodiesel feedstock (Mysyakina and Funtikova, 2008). Therefore, careful study of the fungal morphological adaptations in response to the characteristics of the TS (e.g. solid content, nitrogen and glucose levels) is vital for lipid production. Microscopic observations of *M. circinelloides* when grown on CTS and CDS indicated more yeast-like cells than mycelia. The possible explanation could be the low nitrogen content in CTS and high solid content in CDS creating unfavorable culture conditions for the fungus and promoting its growth in yeast-like form. As a consequence, the fungal growth in CTS and CDS could not be harvested via screening (pore size, 1 mm) and required high-speed centrifugation. On the other hand, mycelia were easier to harvest and gave higher yields than the yeast-like form. Thus, our findings indicate that *M. circinelloides* should be grown in its mycelial form for ease of operation and for better quality biomass.

3.1.2. Effect of TS sterilization on fungal biomass production

Autoclaved TS was found to produce consistently high fungal biomass yields (20 ± 2 g/L, dry). However, unsterilized TS used after prolonged storage (>15 days) in carboys at 4 °C, showed lower

fungal yields (5 ± 1 g/L, dry). On plating a 20-day old TS sample on nutrient agar (Difco Laboratories, Sparks, MD, USA), bacterial growth was observed confirming contamination of TS during storage probably by psychrophilic bacteria. Bacterial growth lowered the nutrient content of TS especially sugars and prevented fungal growth. Therefore, heat sterilization was recommended as a pretreatment step for stillage before *M. circinelloides* cultivation, especially when it is stored for long periods. However, on a commercial scale, a continuous system enabling the transfer of TS directly from the corn-ethanol plant into the fungal reactor could omit the storage period and heat-sterilization steps. Unsterilized and fresh TS from the ethanol plant when used directly without storage gave a biomass yield of 18 ± 2 g/L (dry).

3.1.3. Effect of pH, temperature, inoculum preparation and incubation period on biomass production

Fungal members of the Mucorales order are reported to grow best in the pH range of 5–7 (Funtikova and Mysyakina, 2003; Mysyakina and Funtikova, 2008; Xia et al., 2011). Similar results were found in this study with a pH of 5.5 ± 1.5 giving the highest biomass yield. A temperature range of 25–30 °C is the commonly reported incubation temperature for *M. circinelloides* (Mysyakina and Funtikova, 2008; Xia et al., 2011). Our findings however indicate a higher growth rate of 15 g/L/day in TS at 37 °C as compared to 10 g/L/day at 25 °C. The highest biomass yields were 18.5 ± 0.5 g/L after 8 days at 25 °C, 19.2 ± 0.4 g/L after 5 days at 30 °C and 20.0 ± 0.3 g/L after 2 days at 37 °C. Thus, on day 2 a significantly higher ($p < 0.05$) biomass yield was observed at 37 °C compared to 25 and 30 °C. However, a temperature of 45 °C was found to be too high and resulted in negligible fungal growth. *M. circinelloides* strains are known to be isolated from human or animal sources (http://www.cbs.knaw.nl/) and could explain the preference for a higher incubation temperature observed in this study. However, no specific information could be found from the culture collection center on the specific strain used in this study.

When spore stock was used as the inoculum in the main fungal cultivation process, a long lag phase of 2 days was followed by the log phase from day 2 to day 4 (Fig. 1). A mycelial inoculum prepared in both YM and CTS produced a shorter lag phase (18–24 h) with the growth rate being highest from day 1 to day 2 of incubation. The final biomass yields on day 4 produced by the different inoculum preparations were however not significantly different ($p > 0.05$). Homogenization of the fungal inoculum did not lead to any significant increase in yields ($p > 0.05$).

3.2. Growth and oil production by M. circinelloides in an airlift bioreactor

3.2.1. Change in TS characteristics during fungal growth

During the fungal cultivation, periodic sampling of the fungal culture broth was done and the TS 'effluent' after removal of fungal biomass was analyzed for compositional changes. Sugar analysis by HPLC showed a sharp fall in the concentration of the monosaccharides present in TS (glucose and fructose) in the first 18 h of growth. Samples collected after 18 h showed a gradual fall in the concentration of the more complex sugars, maltose and DP4 + sugars (sugars with four or more monomeric units) as shown in Fig. 2a. *M. circinelloides* is known to produce α-D-glucosidase enzyme needed for breaking down and assimilating maltose sugar. It also produces other essential enzymes for sugar assimilation like β-glucosidase and amylase (Botha et al., 1997) required for breakdown of cellobiose and starch respectively. Although our HPLC was not standardized to measure the polysaccharides in TS, other research suggests that *M. circinelloides* is capable of metabolizing xylose, arabinose, cellulose, and starch, known to be present in TS (Kim et al., 2008).

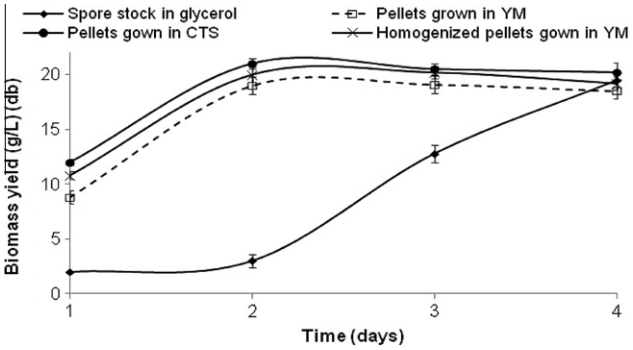

Fig. 1. Effect of inoculum preparation on the growth characteristics of *M. circinelloides* in thin stillage contained in 2-L Erlenmeyer flasks and incubated for 4 days at pH 6, 37 °C and 150 rpm agitation. Data are means ± SD, $n = 3$.

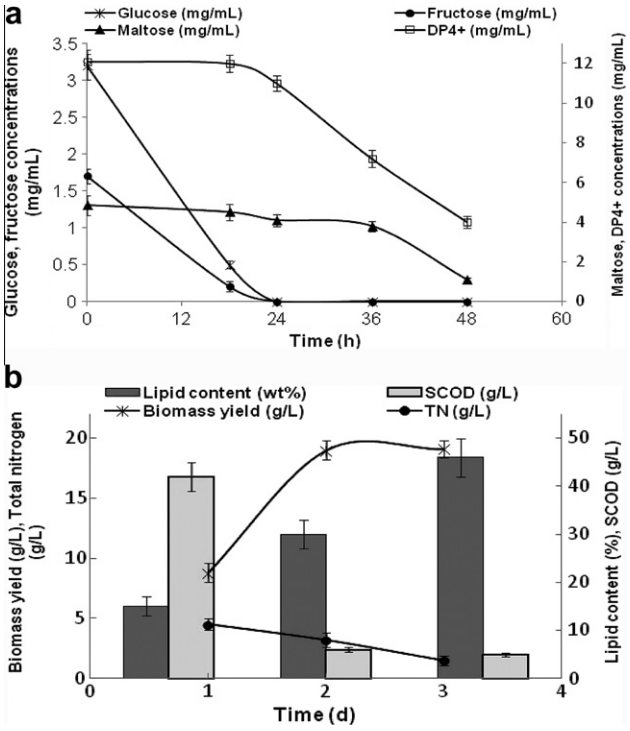

Fig. 2. (a) Change in glucose, fructose, maltose and DP4 + (sugars containing 4 or more monomers) concentrations in thin stillage during the growth of *M. circinelloides* for 48 h at pH 6, 37 °C and 7 SLPM aeration rate in a 6-L airlift bioreactor. Data are means ± SD, $n = 3$. (b) Change in fungal biomass and fungal lipid concentrations, and change in soluble chemical oxygen demand (SCOD) and total nitrogen (TN) in TS, during the growth of *M. circinelloides* for 72 h at pH 6, 37 °C and 7 SLPM aeration rate in a 6-L airlift bioreactor. Data are means ± SD, $n = 3$.

Original TS contained glycerol and organic acids (lactic and acetic) carried over from the yeast fermentation. An initial rise (between 0 and 24 h) was recorded in their concentrations in the TS effluent during *M. circinelloides* cultivation. However, culture broth samples collected after 24 h showed a gradual decrease in these concentrations, as illustrated in Fig 2b. A probable explanation could be the exhaustion of simple sugars during fungal growth on TS, triggering a shift in fungal metabolism, where the formation of glycerol and acids was replaced by their utilization as carbon sources. A similar observation was also made by Rasmussen et al. (in press) for *R. oligosporus* grown on TS. After 48 h of *M. circinelloides* cultivation in the airlift bioreactor, yellow-colored fungal mycelial clumps were observed in the bioreactor leaving the rest of the culture broth with substantially lower amounts of corn

solids. The SCOD in TS was reduced from 45 ± 4 to 5 ± 0.5 g/L; while the total solid content was decreased from 66 ± 5 to 6.6 ± 0.3 g/L and pH increased from 4.2 ± 0.3 to 5.4 ± 0.3; after 48 h of fungal growth. Initial total nitrogen load of 5.5 g/L in TS was reduced to ~0.5 g/L, a part of which could be from the soluble fraction of dead fungal cells. The filamentous nature of the fungal growth is known to increase the viscosity of the cultivation broth unlike pelleted fungal suspensions (Metz et al., 1979). In our study with *M. circinelloides* cultivation in TS, we made similar observations. The high viscosity caused by the fungal filaments led to some mixing problems with a few stagnant and oxygen-limited zones in the airlift bioreactor. A fungal biomass yield of 20.0 ± 1.2 g/L was obtained from the fungal cultivation in the bioreactor after 48 h of cultivation.

Proximate analyses of *M. circinelloides* cells grown on TS indicated higher fat and crude fiber content as compared to DDGS (Belyea et al., 2004). The protein contents of the *M. circinelloides* biomass and DDGS were quite similar (Table 1). Thus, high fiber and protein content in the fungal biomass indicated its potential application as an animal feed. It should be noted that *M. circinelloides* also has GRAS (Generally Regarded As Safe) status from the Food and Drug Administration.

3.2.2. Optimization of oil extraction

Among the different solvent systems tested, toluene:methanol (1:1) solvent system was quite comparable ($p > 0.05$) to the methanol:hexane (1:1) and chloroform:methanol (2:1) solvent pairs. The oil yields using the three solvent systems were −46.2 ± 3.1; 45.5 ± 3.5; and 43.6 ± 3.3 g oil/100 g biomass, respectively. Due to toluene being a relatively safer solvent than either hexane or chloroform, the toluene:methanol solvent pair was selected for rest of the oil extractions in this study. A significant difference ($p < 0.05$) in oil yields was observed when methanol (16.8 ± 2.3 g oil/100 g biomass), hexane (23.5 ± 1.5 g oil/100 g biomass) and toluene (21.0 ± 1.8 g oil/100 g biomass) were used as single solvents and when they were used as pairs. Among the three cell-disruption methods, both magnetic and rotary mixing took almost 12 h to produce the same oil yields (9.2 g of oil/L stillage) as achieved by the sonication method in 3 min. Sonication was also faster than the standard Folch, Lees and Stanley method (Christi and Han, 2010) which required a homogenization step before solvent addition and ~4 h of mixing/contact times. The oil yield using this standard method involving chloroform:methanol (2:1) was found to be 43% (g of oil/100 g dry fungal cells), which was comparable to the yields from the ultrasonication–solvent method.

3.2.3. Fungal oil production

Freeze dried TS solids were found to contain 8.0 ± 2.0% oil (g of oil/100 g stillage solids). Hence, TS with 6% total solids contained 4.8 ± 1.2 g of oil/L. After fungal cultivation on TS, the oil concentration in the effluent was negligible. The corn oil was almost completely removed from TS during the growth of *M. circinelloides*. The oil could have been utilized as a carbon source for intracellular lipid production by employing the fatty acids from the corn oil breakdown as precursors or as backbone to synthesize the fungal storage lipids. Or they could have been degraded into basic skeletons (such as acetyl CoA) serving biomass synthesis (Ratledge, 1989). The corn oil could also have been removed from TS via adsorption (Srinivasan and Viraraghavan, 2010).

The oil content in the dried fungal biomass grown on TS was found to be in the range of 46.0 ± 2.0% (wt of oil per 100 g of dry biomass). When grown on YM broth under the same culture conditions with only glucose as the carbon source, the biomass yield obtained was 5.0 ± 0.5 g/L (dry) and the oil content was 20.0 ± 1.5% or ~1 g/L (Table 2). Thus, the *M. circinelloides* cells were found to have much higher oil content when grown on TS, as compared to synthetic media such as YM broth or the one used by Xia et al. (2011) which resulted in cells with 30% of their dry weight as oil at the highest carbon/nitrogen ratio. The lipid content in the fungal cells was found to be the highest at the stationary phase of growth (between day 2 and day 3) under nitrogen limited conditions (Fig. 2b). An average fungal biomass yield of 20 g/L from TS with an oil content of 46% (wt of oil per 100 g of dry biomass) led to an oil yield of 9.2 g/L of TS after 3 days of incubation. Since TS originally has an oil concentration of ~4.8 g/L, fungal processing led to a 92% increase in oil production.

Crude glycerol was supplemented to TS on the fourth day of fungal cultivation when the fungal cells were seen to reach the stationary phase, possibly due to nitrogen exhaustion. After supplementation, cells were allowed to grow till day 6 and their oil content showed a 32% increase (46–61 g of oil/100 g dry biomass) as compared to the control cells. However, glycerol supplementation did not have a pronounced effect on biomass yield due to the nitrogen limited conditions and led to an increase of only 8 ± 2%.This indicated a future avenue of recycling crude glycerol generated during biodiesel production and simultaneously boosting fungal oil production.

3.2.4. Oil characterization

The nomenclature used in this study for the oil samples extracted from fungal biomass grown on YM and TS, and from TS itself, was Mucor-YM_{oil}, Mucor-TS_{oil} and TS_{oil} respectively. Table 4 shows the major lipid class distribution in Mucor-YM_{oil}, Mucor-TS_{oil} and TS_{oil} oil samples. Triacylglycerol (TAG) was found to be the most dominant class (64.5 wt.%) in TS_{oil} sample followed by free fatty acids (FFA) (6.4 wt.%). Oil from fungal cells grown on YM with glucose as the only carbon source (Mucor-YM_{oil}) also contained TAGs as the dominant lipid class (34.2 wt.%). However, oil from fungal cells grown on TS which contained a mixture of carbohydrates and oil (Mucor-TS_{oil}) was composed of FFA as the major lipid class (38.4 wt.%). This also indicated the lipase activity of *M.*

Table 1
Proximate analysis showing the chemical characteristics of *M. circinelloides* biomass grown on thin stillage (TS) for 48 h in a 6-L airlift bioreactor, harvested by screening and oven-dried at 80 °C for 24 h, as compared to the properties of DDGS. Data are means ± SD, $n = 3$.

Chemical component (%) (dry basis)	*M. circinelloides* biomass	DDGS[a]
Moisture	2.1 ± 0.3	NA
Ash	4.0 ± 0.5	4.6
Total carbohydrates	23.1 ± 1.3	44
Starch	0.8 ± 0.1	5.1
Crude fiber	19.6 ± 1.5	10.2
Soluble sugars	2.2 ± 0.2	NA
Total fat	39.4 ± 2.1	11.9
Total protein	30.4 ± 2.5	31.3

[a] Belyea et al. (2004); NA: Data not provided in referred article (Belyea et al., 2004).

Table 2
Biomass and oil yields after growing *M. circinelloides* in thin stillage (TS) and YM broth for 48 h, pH 6, 37 °C, 7 SLPM aeration rate in a 6-L airlift bioreactor. Data are means ± SD, $n = 3$.

Parameters	Growth media	
	YM	TS
Biomass yield (g/L)	5.0 ± 0.5	20.0 ± 0.5
Biomass productivity (g/L/day)	2.5 ± 0.25	10.0 ± 0.25
Oil content in fungal cells (%)	20.0 ± 1.5	46.0 ± 2.0
Oil yield (g/L)	1.0 ± 0.2	9.2 ± 1.0
Oil productivity (g/L/day)	0.5 ± 0.1	4.6 ± 0.5
PUFA content in fungal oil (% total lipids)	33.2 ± 1.2	51.4 ± 2.6

Table 3
Lipid class composition of oil extracted from fungal cells grown on YM broth (Mucor-YM_{oil}) and thin stillage (Mucor-TS_{oil}), and from thin stillage alone (TS_{oil}). The different lipid classes were separated by TLC and their quantities were measured by GC. Data are means ± SD, $n = 2$.

Lipid composition (wt.%)	Mucor-YM_{oil}	Mucor-TS_{oil}	TS_{oil}
TAG[a]	34.2 ± 0.7	14.7 ± 1.8	64.5 ± 4.3
FFA[b]	4.2 ± 0.5	38.4 ± 6.1	6.4 ± 0.8
DAG[c]	1.5 ± 0.1	8.4 ± 1.1	1.1 ± 0.3
Polar	4.1 ± 0.3	3.2 ± 0.4	0.4 ± 0.1
Total quantified lipids by TLC in lipid extract (wt.%)	44.0 ± 0.4	64.7 ± 2.3	72.4 ± 1.4
FAME[d] converted from total lipids in extract (wt.%)	69.7 ± 5.3	70.0 ± 4.3	87.5 ± 13.8

[a] TAG: Triacylglycerol.
[b] FFA: Free fatty acids.
[c] DAG: Diacylglycerol.
[d] FAME: Fatty acid methyl esters.

Table 4
Total lipid fatty acid composition of oil extracted from fungal cells grown on YM broth (Mucor-YM_{oil}) and thin stillage (Mucor-TS_{oil}), as well as oil from thin stillage alone (TS_{oil}). Compositional analysis was done by GC with prior transesterification to FAME's. Data are means ± SD, $n = 2$.

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

circinelloides, which may have hydrolyzed the TAGs in corn oil leading to the high FFA content in the Mucor-TS_{oil} samples. The fatty acid methyl ester (FAME) directly converted from the total lipid extracts did not add up to a 100% due to the unsaponifiable lipids and the presence of glycolipids and phospholipids, which have non-acyl groups that cannot be converted to FAMEs, but these non-acyl portions did contribute to the total oil weight. The FAME content (in wt.%) of Mucor-YM_{oil}, and Mucor-TS_{oil} samples was almost the same but the lipid class distribution was quite different. Also, the total estimated lipids (in wt.%) in the fungal oil samples were much lower than 100%. This result suggests that Mucor-YM_{oil} and Mucor-TS_{oil} contain large amount of unsaponifiables. Many unidentifiable TLC bands were also observed, which were probably carotenoids, hydrocarbons, cholesterol or other sterols; and some other polar complex lipids. These unknown TLC bands, which were not included in the transesterification process, represented a non-negligible quantity according to the band width shown on the TLC plate. Since these unsaponifiable lipid materials and unknowns were not accounted for, they contributed to low total lipid percentages as in Table 3. Other factors for the low total lipid values may be the presence of residual solvent in the lipid extracts, and the observed non-lipid fine particles that may be difficult to remove. The much lower total quantified lipids in Mucor-YM_{oil} than in Mucor-TS_{oil} may suggest that when the oil is fully synthesized *de novo* by the microbe, the oil will contain more non-acyl lipids.

Table 4 illustrates the fatty acid composition of the three oil samples, Mucor-YM_{oil}, Mucor-TS_{oil} and TS_{oil}. The fatty acid composition of Mucor-TS_{oil} matched closely with that of TS_{oil} and further proved that the nature of the oil present in the growth medium greatly influences the fatty acid composition of the intracellular microbial oil. The fatty acid with the highest concentration in Mucor-YM_{oil} sample was C18:1 (oleic acid) at 24.4 ± 0.7 wt.%; while Mucor-TS_{oil} and TS_{oil} samples contained C18:2 (linoleic acid) as the primary fatty acid at 50.0 ± 1.6 wt.% and 52.5 ± 0.2 wt.% respectively. This is similar to the corn oil FAME composition with C18:2 at ~57% and C18:1 at ~28% (Majoni and Wang, 2010). Mucor-YM_{oil} sample contained certain unique saturated fatty acids, such as C13:0 (tridecylic acid), C14:0 (myristic acid) and C15:0 (pentadecylic acid) and a monounsaturated fatty acid C16:1 (palmitoleic acid), which were absent in the other two oil samples. Another interesting finding was the presence of γ-linolenic acid (C18:3) in Mucor-YM_{oil} (17.5 ± 0.1 wt.%) which was much higher than that in Mucor-TS_{oil} (1.4 ± 0.1 wt.%) and not detected in TS_{oil}. This again proved that this microbe is able to synthesize lipids from media that do not contain acyl compounds, and the *de novo* synthesis and assimilation from the environment produce lipids with different composition. The presence of linolenic acid (C18:3) in fungal-based lipids is well documented and fungal oil is known to be a rich source of gamma linolenic acid (GLA), an omega 6, a PUFA of nutritional and dietary importance (Ratledge, 2004). Overall, fungal oil grown on TS was not found to be suitable for biodiesel production using the common alkaline transesterification process due to its high FFA content. However, acid esterification could be a viable alternative. On the other hand, the low percentage of both saturated and polyunsaturated fatty acids and high percentage of monounsaturated fatty acids (oleic) were favorable properties for biodiesel production (Durrett et al., 2008). TS-derived fungal oil could also have applications in the production of high-value fatty acids like linoleic and oleic acids. Considering the encouraging fungal biomass yields from TS, other oleaginous strains could be tested as part of a future study and targeted specifically towards production of high-value fatty acids such as GLA.

4. Conclusion

This work provided four main findings: (1) efficient method of converting TS into oil and animal feed with the effluent containing much lower organic load facilitating water reclamation; (2) fungal growth and morphology were affected by the solids content in TS and highest biomass and lipid yields from TS (total solids = 5–7%) were 20.0 and 9.2 g/L, respectively; (3) fatty acid composition of fungal lipids was influenced by the nature of growth medium (YM/TS); and (4) YM and TS-derived fungal oil contained high-value PUFAs. Overall, oleaginous fungal cultivation on TS has the potential to boost the profitability of the corn-ethanol industry.

Acknowledgements

The authors would like to thank the Iowa Energy Center for partial funding of this research and Lincolnway Energy, Nevada, IA for providing thin stillage for this study. We appreciate the lab facilities provided by the Department of Food Science and Human Nutrition and Iowa State University's Center for Crops Utilization Research (CCUR). We are grateful to National Science Foundation for grant support of part of this work. We would also like to acknowledge the help given by Bill Colonna, Carol Ziel, and Supriyo Das at the various stages of this research.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.biortech.2011.12.031.

References

AOAC Official Method 920.39. Fat (Crude) or Ether Extract in Animal Feeds, 16th ed. p. 25 (Chapter 4).

AOAC Official Methods, 2000. Official Methods of Analysis of AOAC International, 17th ed. AOAC International, Gaithersburg, MD.

Belyea, R.L., Rausch, K.D., Tumbleson, M.E., 2004. Composition of corn and distillers dried grains with solubles from dry grind ethanol processing. Bioresour. Technol. 94, 293–298.

Botha, A., Kock, J.L.F., Coetzee, D.J., Botes, P.J., 1997. Physiological properties and fatty acid composition in *Mucor circinelloides* f. *Circinelloides*. Antonie van Leeuwenhoek 71, 201–206.

CBS-KNAW Fungal Biodiversity Centre, 2011. <http://www.cbs.knaw.nl/> (last accessed 29.11.2011).

Christi, W., Han, X., 2010. Lipid Analysis – Isolation, Separation, Identification and Lipidomic Analysis, vol. 24, fourth ed. The Oily Press Lipid Library.

Cravotto, G., Boffa, L., Mantegna, S., Perego, P., Avogadro, M., Cintas, P., 2008. Improved extraction of vegetable oils under high-intensity ultrasound and/or microwaves. Ultrason. Sonochem. 15, 898–902.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. 28 (3), 350–356.

Durrett, T.P., Benning, C., Ohlrogge, J., 2008. Plant triacylglycerols as feedstocks for the production of biofuels. Plant J. 54, 593–607.

Folch, J., Lees, M., Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. 226, 497–509.

Funtikova, N.S., Mysyakina, I.S., 2003. Sporangiospores of the fungus *Mucor lusitanicus* 12M: correlation between lipid composition, viability, and morphology of growth upon germination. Microbiology 72 (6), 686–689.

Kim, Y., Mosier, N.S., Hendrickson, R., Ezeji, T., Blaschek, H., Dien, B., Cotta, M., Dale, B., Ladisch, M.R., 2008. Composition of corn dry-grind ethanol by-products: DDGS, wet cake, and thin stillage. Bioresour. Technol. 99, 5165–5176.

Knudsen, K.E.B., 1997. Carbohydrate and lignin contents of plant materials used in animal feeding. Anim. Feed Sci. Technol. 67, 319–338.

Majoni, S., Wang, T., 2010. Characterization of oil precipitates and oil extracted from condensed corn distillers solubles. J. Am. Oil Chem. Soc. 87, 205–213.

Meng, M., Jianming, Y., Xu, X., Zhan, L., Nie, Q., Xian, M., 2009. Biodiesel production from oleaginous microorganisms. Renew. Energy 34, 1–5.

Metz, B., Kossen, N.W.F., van Suijdam, J.C., 1979. The rheology of mold suspensions. Adv. Biochem. Eng. 11, 103–156.

Miao, X., Wu, Q., 2006. Biodiesel production from heterotrophic microalgal oil. Bioresour. Technol. 97, 841–846.

Moreau, R.A., Liu, K., Winkler-Moser, J.K., Singh, V., 2011. Changes in lipid composition during dry grind ethanol processing of corn. J. Am. Oil Chem. Soc. 88, 435–442.

Mysyakina, I.S., Funtikova, N.S., 2008. Metabolic characteristics and lipid composition of yeastlike cells and mycelium of *Mucor circinelloides* var. *lusitanicus* INMI grown at a high glucose content in the medium. Microbiology 77 (4), 407–411.

Nitayavardhana, S., Khanal, S.K., 2011. Biodiesel-derived crude glycerol bioconversion to animal feed: a sustainable option for a biodiesel refinery. Bioresour. Technol. 102, 5808–5814.

Noureddini, H., Bandlamudi, S.R.P., Guthrie, E.A., 2009. A novel method for the production of biodiesel from the whole stillage-extracted corn oil. J. Am. Oil Chem. Soc. 86, 83–91.

Ozsoy, H.D., Kumbur, H., Saha, B., van Leeuwen (Hans), J., 2008. Use of *Rhizopus oligosporus* produced from food processing wastewater as a biosorbent for Cu(II) ions removal from the aqueous solutions. Bioresour. Technol. 99 (11), 4943–4948.

Paul, G.C., Thomas, C.R., 1998. Characterisation of mycelial morphology using image analysis. Adv. Biochem. Eng.: Biotechnol. 60, 1–59.

Rasmussen, M., Khanal, S.K., Pometto III, A.L., van Leeuwen (Hans), J., in press. Water reclamation and value-added animal feed from corn ethanol stillage by fungal processing. J. Biomass Bioenergy.

Ratledge, C., 1989. Microbial Lipids, vol. 2. Academic Press, London, ISBN 0125823053, pp. 567–668.

Ratledge, C., 2004. Fatty acid biosynthesis in microorganisms being used for single cell oil production. Biochimie 86, 807–815.

Renewable Fuels Association (RFA), 2011. Ethanol industry statistics. <http://www.ethanolrfa.org/pages/statistics/> (last accessed 28.06.2011).

Sankaran, S., Khanal, S.K., Jasti, N., Jin, B., Pometto III, A.L., van Leeuwen, J., 2010. Use of filamentous fungi for wastewater treatment and production of high value fungal by-products: a review. Crit. Rev. Environ. Sci. Technol. 40 (5), 1–49.

Sergeeva, Y.E., Galanina, L.A., Andrianova, D.A., Feofilova, E.P., 2008. Lipids of filamentous fungi as a material for producing biodiesel fuel. Appl. Biochem. Biotechnol. 44 (5), 523–527.

Srinivasan, A., Viraraghavan, T., 2010. Oil removal from water using biomaterials. Bioresour. Technol. 101, 6594–6600.

Standard Methods for the Examination of Water and Wastewater, 2005. American Public Health Association/American Water Works Association/Water Environment Federation (APHA/AWWA/WEF), 21st ed. Washington, DC, USA.

Szczesna-Antczak, M., Antczak, T., Piotrowicz-Wasiak, M., Rzyska, M., Binkowska, N., Bielecki, S., 2006. Relationships between lipases and lipids in mycelia of two *Mucor* strains. Enzyme Microb. Technol. 39, 1214–1222.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber non-starch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.

Vicente, G., Bautista, L.F., Rodriguez, R., Gutierrez, F.J., Sadaba, I., Ruiz-Vazquez, R.M., Torres-Martinez, S., Garre, V., 2009. Biodiesel production from biomass of an oleaginous fungus. Biochem. Eng. J. 48, 22–27.

Xia, C., Xhang, J., Zhang, W., Hu, B., 2011. A new cultivation method for microbial oil production: cell pelletization and lipid accumulation by *Mucor circinelloides*. Biotechnol. Biofuels 4, 15.