

FAMES Profile of Oil Produced by Oleaginous Fungi Isolated from Fermented Beverage Wastewaters and Soil

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Abstract

Fungal strains isolated from fermented maize (*ogi*) (PW) and sorghum-based brewery wastewaters (BW) and two soil isolates were evaluated for oleaginicities. The fungal isolates from the wastewater that had lipid content of at least 20% of their biomass were identified by both culture methods and internal transcribed spacer (ITS) 1-5.8S-ITS2 ribosomal DNA sequencing. The isolates were identified to be *Aspergillus fumigatus* (PW8), *Aspergillus flavus* (PW10), *Candida tropicalis* (PW16) and *Aspergillus tubingensis* (PW3), *Trichosporon luoberi* (BW7), *Aspergillus* sp. (BW4) and *Candida tropicalis* (BW1; BW3). FAMES composition was determined for the four strains with the highest lipid content by acid-catalyzed transesterification and analyzed by Gas Chromatography-Flame Ionization Detector (GC-FID). Palmitoleic acid was the dominant fatty acid in *M. circinelloides* and *T. reesei*, and the best producers of capric and lauric acids were *Aspergillus fumigatus* and *Aspergillus* sp. (BW4), respectively. These fatty acids are beneficial in making cosmetics and pharmaceuticals (antimicrobials and dietary supplements). The analysis of the FAMES profile in the species indicated low amounts or absence of some key long chain fatty acid (LCFA) constituents of biodiesels. Based on the FAMES profile of *M. circinelloides* investigated, this strain could hold promise for use as feedstock for biodiesel with genetic engineering and a tailored lipid production favouring enrichment of LCFA.

Keywords: Fungal lipids, wastewater, fatty acid methyl ester, GC-FID

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Introduction

Filamentous fungi have broad application as production hosts in the industry mainly for their capacity to secrete metabolites (Peberdy, 1994, Punt et al., 2002; Wosten, 2019). Microbial oils are secondary metabolites produced by bacteria, yeast, fungi and microalgae and are accumulated within specific organelles like lipid bodies within the cell (Kosa and Ragauskas, 2011). A microorganism is said to be oleaginous when it can accumulate more than 20% of biomass as lipids (Ratledge and Wynn,

2002; Thevenieau and Nicaud, 2013). Microbial systems that can produce and store oil have attracted significant research attention in recent years (Bharathiraja et al., 2017), especially triacylglycerols (TAGs) produced from oleaginous microorganisms as the supplementary sources of conventional oil for biodiesel production (Thevenieau and Nicaud, 2013). Oleaginous fungi include *Mucor circinelloides*, *Candida tropicalis*, *Pythium ultimum*, *Morietella isabellina*, *Aspergillus terreus*, *Claviceps purpurea*, *Pellicularia praticola* (Thevenieau and

Nicaud, 2013). These fungi can also grow by utilizing sugarcane molasses, soluble starch and wheat straw (Lin et al., 2010). Nitrogen limitations in the substrate are required for the accumulation of lipids (Ratledge 2002; Calvey et al., 2016).

Microbial oil extraction processes are simpler to handle and require less labor (Li and Wang, 1997; Li et al., 2008) compared to plant oil extraction procedures. Plant oil extraction requires the processing of the specific plant tissue (Atabani et al., 2013) as opposed to microbial oil extraction in the aqueous phase or ultrasonication (Zhang et al., 2014). The procedure for oil extraction is too expensive to apply for production on a large scale, therefore the viability of production of microbial oil is dependent on the high conversion of substrate to intracellular lipids combined with high extraction efficiency at low energy consumption (Bharathiraja et al., 2017).

Biodiesels are defined as the fatty acid alkyl monoesters (FAMES) derived from renewable sources, such as vegetable oils and animal fats. They are produced by transesterification of the lipid with an alcohol especially methanol (Yap et al., 2011). The environmental benefits of using biodiesel over petroleum diesel include biodegradability, lower sulfur and aromatic hydrocarbon content and the reduction in the emissions of carbon monoxide, carbon dioxide and particulate matter (Meher et al., 2006; Sawangkeaw and Ngamprasertsith, 2013). The main disadvantage of this process is the unavailability of feedstocks and substrates, which accounts for 70% of the final cost of the biodiesel (Hanna, 1991; Thevenieau and Nicaud, 2013).

There is an increase in the demand for renewable fuel due to the limited energy resources and the environmental hazards that fossil fuels pose to the environment. Biodiesel is being increasingly investigated as an alternative to fossil fuels in modified combustion engines for transport as well as in engines for power generation (Gavrilescu and Chisti, 2005; Bharathiraja et al., 2014).

The composition and the yield of microbial oils are affected by cultural conditions such as substrates, incubation period, nitrogen source, pH and aeration of the culture medium (Liu et al., 2010). The yield and type of lipid are dependent on several factors like the type of organism, culture conditions and the substrate chosen (Ledesma-Amaro et al., 2016). The use of pure fungal isolates from environmental

sources as feedstock for the production of fatty acid methyl esters relevant in biodiesel, and pharmaceuticals and cosmetics was investigated.

Materials and Methods

Source of fungal isolates

Mucor circinelloides (IYN 13) and *Trichoderma reesei* (IYN 15) are laboratory stock strains (unpublished data) that were isolated from the soil. These strains were selected because of other reports on their oleaginicity. All the other fungi from this study were obtained during the screening of untreated wastewater from two sources- fermented cereal "ogi" (PW) and a brewery (BW) in Ogun State, Nigeria. The brewery uses sorghum as the substrate for its fermentation process and corn was the substrate for *ogi*. The wastewater samples were collected in sterile 1 litre plastic bottles and were processed within 24 hours of sample collection.

Enrichment and isolation of pure isolates from the wastewater samples

Ten (10) ml of the wastewater sample was added into a 250 ml flask containing 50 ml sterilized enrichment medium. Enrichment medium was composed of (g/l) D- xylose 100 g, yeast extract 1 g, KH_2PO_4 2.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.75 g, Na_2HPO_4 1 g, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.2 g, FeCl_3 0.01 g, ZnCl_2 0.1 g, Rose Bengal 50 mg/L, 3.3 ml of chloramphenicol solution (10000U/mL) and set at pH 7.0 (Abu-Elreesh and Abd-El-Haleem, 2014). The mixture of the wastewater and the enrichment medium was then cultured at 28°C, with agitation using orbital shaker at 180 rpm for 48 hours. Serial dilution was carried out on an aliquot of the incubated medium, and cultured on PDA (Himedia, India) supplemented with chloramphenicol and Rose Bengal Chloramphenicol agar (Oxoid, UK). Incubation was done at 35°C for 5 days. Pure cultures were then obtained from mixed culture plates. Morphological appearances of the inoculated plates (at room temperature) were observed and distinct colonies were subcultured to obtain pure isolates which were then maintained on Potato Dextrose Agar slants and stored at -20°C.

Culturing of the laboratory stock isolates

Isolates *M. circinelloides* (IYN 13) and *T. reesei* (IYN 15) were cultured on PDA +Cam (PDAC) and Rose Bengal Chloramphenicol (RBC) (Oxoid, UK).

Classical and Molecular identification of the fungal isolates

Cultural identification of the fungal isolates

The isolates were identified based on their growth patterns on PDAC and RBC and by microscopy. Pure cultures were obtained for all the strains and were stored on PDA slants

Molecular identification of the fungal isolates

The laboratory strains (IYN 13 and IYN 15) had been previously authenticated by morphological and molecular methods (unpublished). DNA extracted from all the other fungal strains was done using ZR Fungal/Bacterial DNA MiniPrep™ kit (Zymo, USA), according to the manufacturer's instructions. The DNA extractions and sequencing analyses were performed at the University of Lagos. Polymerase Chain Reaction (PCR) of the extracted genomic DNAs from the 7 isolates was done in a GeneAmp PCR system 9700 PCR thermal cycler. Each 25 µl master mix consisted of 2.5 µl of 10x PCR buffer, 1 µl of 25mM MgCl₂, 1 µl each of forward (ITS5F: GGAAGTAAAAGTCGTAACAAGG) and reverse (ITS4R: TCCTCCGCTTATTGATATG) primers (Inqaba, South Africa), 1 µl of DMSO, 2 µl of 2.5mM dNTPs, 0.1 µl of 5µg/µl Taq DNA polymerase, 3 µl of 10ng/µl DNA and 13.4 µl Nuclease free water. The PCR conditions were as follows: Initial denaturation at 94°C for 5 mins, followed by 36 cycles of denaturation at 94°C for 30 s, annealing at 54°C for 30 s, elongation at 72°C for 45 s, a final elongation step at 72°C for 7 mins and hold temperature at 10 °C. The amplicons were visualized on Safe view-stained 1.5% agarose electrophoresis gels. The expected size of the amplicons was about 650 bp and the DNA ladder used was Hyperladder™ 1kb (Bioline, TN, USA).

The PCR amplicons were sequenced at the DNA Sequencing Facility of the Bioscience Center, International Institute of Tropical Agriculture, Ibadan, Oyo using 3130XL genetic analyzer (Applied Biosystems, CA, USA). The sequences were checked for quality and assembled using BioEdit (version 7.2.5) Sequence Alignment Editor (Hall, 1999). The consensus sequence obtained for each isolate was compared to the GenBank nucleotide data library using the Basic Local Alignment Search Tool, BLAST software (Altschul et al., 1990) at the National Centre for Biotechnology Information (NCBI (<http://www.ncbi.nlm.nih.gov>) website (Nsa et al., 2020). The sequences were submitted to GenBank and accession numbers have been assigned to the isolates.

Microscopic screening for Lipid production

The microscopic screening of the pure isolates for intracellular lipid accumulation was performed by a

modified Sudan Black B method (Thancharoen et al., 2017). Yeast Extract Malt Extract Agar (YEMEA) of basal medium composition (g/l): glucose, 10; peptone, 5; yeast extract, 5; and malt extract, 3 and agar-agar 15 was used for culturing the organisms in glass tubes for 48 hours (Liu *et al.*, 2010). The two-day-old isolates were smeared, heat-fixed, flooded with Sudan Black B stain and kept for 15 minutes until the stain turned yellowish-green. The stain was rinsed and counterstained with safranin for 30 seconds. It was thereafter air-dried, blotted and observed under a light microscope (Bresser LCD 40 x1400 Germany) at a100 x magnification. Intracellular lipid accumulation in fungal cells was determined based on the density of globules /retention of Sudan Black B in cells.

Quantitative examination of lipid production in the isolates/Liquid production medium

Lipid production medium was prepared as described by Abu-Elreesh and Abd-El-Haleem, (2014) containing (in g/L): yeast extract 0.5, MgSO₄.7H₂O 0.4, KH₂PO₄ 2.0, CaCl₂ 0.5, CuSO₄.5H₂O 0.05 and sodium molybdate 0.005; and 5% glucose (w/v), pH 6 and dispensed into 250 ml Erlenmeyer flasks. Each flask was inoculated with a fungus (positive for intracellular lipid accumulation) and incubated at 28° ± 2°C with a shaking speed of 180rpm for 5 days.

Effects of carbon sources on accumulation and FAMES composition of the isolates

Lipid production medium was prepared as described above and sucrose was used as an alternative carbon source to glucose. Thirty (30) ml of the medium was dispensed into 100 ml Erlenmeyer flask in triplicates and inoculated with 5-day old fungal cultures and incubated with shaking at 180 rpm, 28°C ± 2°C for 5 days. The fungal biomass was harvested from each culture .

Lipid Yield (dry weight of lipid, lipid extraction process and lipid content) Estimation

Dry weight of the biomass: Samples were filtered using a pre-weighed sterile Whatman filter paper inserted in a funnel placed in conical flasks and allowed to drain completely. The biomass was washed twice with distilled water and then dried in a hot air oven at 70°C to a constant weight. The dry weights of the biomass were recorded and used for lipid extraction.

The lipid extraction process was carried out according to a modified Bligh and Dyer method (Muniraj et al., 2017). The dried fungal masses

were crushed in a mortar and pestle and centrifuged at 10,000 rpm for 20 mins. A mixture of chloroform, methanol and distilled water (2:1:1) was added to the centrifuged biomass and spun again for 20 mins. The lower liquid phase (chloroform layer containing lipid) was extracted and dispensed into pre-weighed Bijoux bottles and evaporated using nitrogen gas. The remaining lipids were weighed and recorded.

The lipid content and the biomass yield of each fungal isolate were estimated using the equations described by Muniraj et al., 2017.

The lipid content in biomass = $Y_{L/X}$,

$$Y_{L/X} = \frac{L}{X}$$

Where, L, maximum lipid yield, g/L and X, biomass yield that corresponds to the volume of the medium.

Determination of Fatty-Acid Methyl Esters (FAMES) profile by Gas Chromatography-Flame Ionization Detector (GC-FID)

The lipid extract from the fungal isolates was transesterified by the addition of methanol, concentrated hydrochloric acid and water in the ratio 10:1:1 as described by Patel et al., (2016); the mixture in glass tubes was centrifuged and the upper phase removed using hexane. The hexane phase was passed through a gas chromatographic

column. Gas chromatography was carried out on the transesterified extract. To determine FAMES, 5-point serial dilution calibration standards (0.25, 0.50, 1.00, 2.00, 4.00 ppm) were prepared from the stock and used to calibrate the GC-FID. Determination of the levels of FAMES was done using Agilent 7820A gas chromatography coupled to a flame ionization detector fitted with a DB-1 capillary column coated with 5% Phenyl Methyl Siloxane (30m length x 0.32mm diameter x 0.25µm film thickness) (Agilent Technologies). After calibration, the samples were analyzed and chromatogram and its fatty acid concentrations obtained.

Results

Isolation and enumeration of pure fungal isolates from the wastewater samples.

A total of twenty-five fungal isolates were obtained from 10⁻⁵ PDA dilution plates of the brewery wastewater and fermented cereal *ogi* wastewater. The isolates from the brewery and *ogi* wastewaters were labelled BW and PW, respectively. From the mixed culture plates (**Fig.1**), nine isolates were obtained from the brewery wastewater and sixteen isolates from the *ogi* wastewater. The laboratory strains (previously isolated from the soil) *M. circinelloides* and *T. reesei* were maintained on PDA.



Figure 1. Some mixed fungal culture plates from **A.** Brewery Wastewater (dominated by yeasts) **B.** *Ogi* Wastewater (dominated by yeasts) and **C.** *Ogi* Wastewater (moulds).

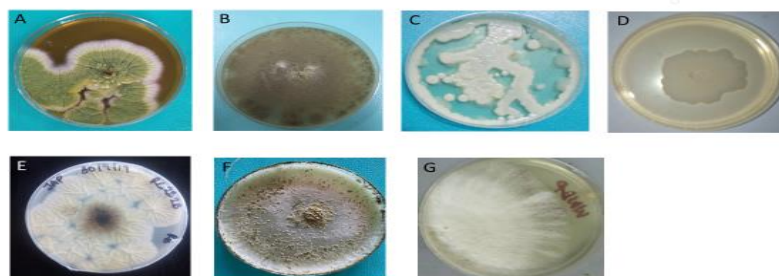


Figure 2. Pure cultures of fungal strains. A. *Aspergillus flavus*, B. *Aspergillus fumigatus*, C. *Candida tropicalis* D. *Candida tropicalis*, E. *Trichoderma reesei*, F. *Mucor circinelloides*, G. – *Aspergillus* sp.

Figure 2. Pure cultures of fungal species . **A.** *Aspergillus flavus*, **B.** *Aspergillus fumigatus*, **C.** *Candida tropicalis* **D.** *Candida tropicalis*, **E.** *Trichoderma reesei*, **F.** *Mucor circinelloides*, **G.** *Aspergillus* sp. *Microscopic Screening of isolates for lipid accumulation.*

Ten out of the 27 pure culture fungal isolates (**Fig. 2**) that were screened on YEMEA displayed significant oil accumulation including *M. circinelloides*

and *Trichoderma reesei*. The isolates BW1, BW3, BW4 and BW7, PW3, PW8, PW10 and PW16 were positive for oil accumulation based on the amount of Sudan black dye retained in their cells (**Fig. 3**)

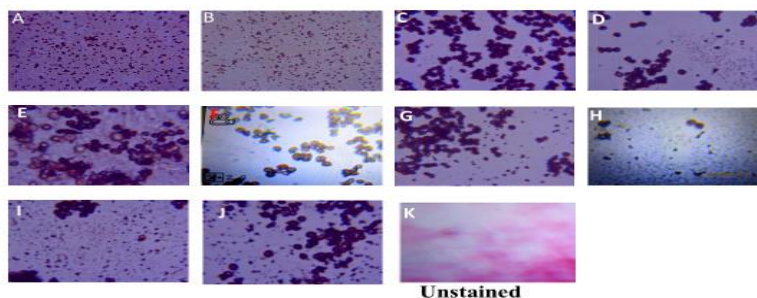


Figure 3. Micrographs of oil accumulation surrounding the cells of screened fungal isolates **A** – BW1, **B** – BW3, **C** – BW4, **D** – BW7, **E** – PW3, **F** – PW8, **G** – PW10, **H** – PW16, **I** – *T. reesei*, **J** – *M. circinelloides*, **K** – Unstained

Figure 3. Micrographs of oil accumulation surrounding the cells of screened fungal isolates **A** – BW1, **B** – BW3, **C** – BW4, **D** – BW7, **E** – PW3, **F** – PW8, **G** – PW10, **H** – PW16, **I** – *T. reesei*, **J** – *M. circinelloides*, **K** – Unstained

Quantitative Determination of lipid content of the screened isolates

The amount of oil produced by the strains that were positive for oil accumulation was measured as a fraction of cell weight/volume of the medium. The lipid yield of the strains, BW 1, BW 3, BW 4, BW 7,

PW 3, PW 8, PW 10, PW 16, *M. circinelloides* and *T. reesei* were determined (**Table 1**). Out of these 10 strains, the highest oil producers were *M. circinelloides* 8.05 g/L; (41.3%), *T. reesei* 4.2 g/L (28.7%), BW4 3.18 g/L; (35.3%), PW10 5.27 g/L; (34.6%) PW16 2.26 g/L; (29.9%).

Table 1: Lipid Yield Estimation for screened isolates.

Isolate	Dry cell weight(g/L)	Lipid yield (w/v)	Lipid content (%)
BW1	5.13	1.47	28.6
BW3	7.43 ± 0.00	1.90 ± 0.01	25.6
BW4	9.00	3.18	35.3
BW7	7.87 ± 0.001	2.23 ± 0.00	28.3
PW3	7.20	2.43	33.7
PW8	2.27 ± 0.00	0.423 ± 0.01	18.6
PW10	15.20	5.27	34.6
PW16	7.56	2.264	29.9
<i>Mucor circinelloides</i>	19.65	8.05	41.3

Table 2: Molecular identification of the screened isolates.

Isolate	Source	Isolate Identification	Accession Number
BW1/IYN75	Brewery Wastewater	<i>Candida tropicalis</i>	MT3777705
BW3/IYN76	Brewery Wastewater	<i>Aspergillus tubingensis</i>	MT3777706
BW4/IYN74	Brewery Wastewater	<i>Aspergillus</i> sp.	MT3777704
BW7/1YN71	Brewery Wastewater	<i>Trichosporon loubieri</i>	MT3777701
PW3/1YN77	Ogi Wastewater	<i>Candida tropicalis</i>	MT3777707
PW8/1YN72	Ogi Wastewater	<i>Aspergillus flavus</i>	MT3777702
PW10/IYN73	Ogi Wastewater	<i>Aspergillus fumigatus</i>	MT3777703
PW 16/IYN78	Ogi Wastewater	<i>Candida tropicalis</i>	MT3777708
IYN13	Soil	<i>Mucor circinelloides</i>	MT421897
IYN15	Soil	<i>Trichoderma reesei</i>	MT421898

Molecular identification of the fungal isolates

With the BLASTn tool, the DNA sequences of the ITS-1, 5.8S rRNA and ITS2 regions for each isolate were compared to the ITS1 sequences deposited in NCBI-GenBank and a species match was called by an identity threshold of > 99%. The sequences of the fungal isolates obtained from this study have been deposited in GenBank and their Accession numbers issued have been indicated (**Table 2**). The BW1, BW3, BW4 and BW7, PW3, PW8, PW10 and PW16 were identified as *Candida tropicalis*, *Aspergillus tubingensis*, *Aspergillus* sp., *Trichosporon loubieri*, *Candida tropicalis*, *Aspergillus flavus*, *Aspergillus fumigatus* and *Candida tropicalis*, respectively.

FAMES analysis of the oil extracts obtained from the isolates

Based on the lipid production yield, *M. circinelloides*, *T. reesei*, PW 10 (*Aspergillus fumigatus*) and BW 4 (*Aspergillus* sp.) were selected for FAMES analysis. FAMES profiles for the isolates *M. circinelloides* and *T. reesei* were determined with glucose and sucrose while only glucose was used for PW 10 and PW 4.

T. reesei and *M. circinelloides* did not contain any short-chain FAMES, caproic acid (C6:0) and caprylic (C8:0) when either sucrose or glucose was the

substrate. The principal fatty acid methyl ester of lipid extracts from *T. reesei* (85.94%, 87.31%) and *M. circinelloides* (50.41%, 87.33%) was the long-chain fatty acid- palmitoleic acid (C16:1). Whereas, its yield in *Aspergillus fumigatus* and the other *Aspergillus* sp. (BW4) was 1.70% and 1.32% respectively. Palmitic acid (C16:0) was the second most abundant long-chain fatty acid from *T. reesei* (7.11%) and *M. circinelloides* (6.37%). The other long-chain FAMES were present at concentrations of 3-4% in *T. reesei* and 3-11% in *M. circinelloides* (**Table 3**). *A. fumigatus* was enriched in the medium-chain, capric acid (C10:0) (95.37%) and had a low concentration of long-chain FAMES (~3%). The other *Aspergillus* sp. (BW4) had lauric acid as the principal FAME (69.99%), 16.27% of medium-chain FAME, capric acid and 13.36% long-chain FAMES. The Oleic acid and elaidic acid concentrations were highest in *Aspergillus* sp. (BW4). Among the four species, *Aspergillus* sp. (BW4) had the greatest number of FAMES undetected (caproic acid, caprylic acid, linoleic acid, linoleic acid, linoleic acid, arachidic acid, linolenic acid heptadecanoic acid) whereas *A. fumigatus* had 17 FAMES. Tridecanoic acid was only present in *M. circinelloides*. The extracts from *T. reesei* and *M. circinelloides* were high in MUFA while

the *Aspergillus* species were high in saturated fatty acids.

Table 3: FAMES profile of acid-catalyzed transesterified oil extracts of the fungal strains

FAMES (Mg/L)	<i>T. reesei</i>		<i>M. circinelloides</i>		<i>A. fumigatus</i>	<i>Aspergillus</i> sp.
	Sucrose	Glucose	Sucrose	Glucose	Glucose	Glucose
Caproic Acid (C6:0)	-	-	-	-	0.71	-
Caprylic Acid (C8:0)	-	-	-	-	0.05	-
Capric Acid (C10:0)	3.37	1.14	15.11	0.91	95.37	16.26
Undecanoic Acid (C11:0)	-	0.16	1.80	-	0.04	0.38
Tridecanoic Acid (13:1)	ND	ND	-	0.38	ND	-
Lauric Acid (C12:0)	2.75	1.05	10.43	1.08	0.18	69.99
Myristic Acid (C14:0)	0.059	1.70	0.83	0.23	0.10	0.18
Myristoleic Acid (14:1)	1.31	0.47	6.42	0.50	0.03	-
Pentadecanoic Acid (C15:0)	0.39	0.08	1.44	0.07	0.14	0.21
Palmitic Acid (C16:0)	0.38	7.11	2.47	6.37	0.13	0.72
Palmitoleic Acid (C16:1)	85.94	87.31	50.41	87.33	1.70	1.32
Heptadecanoic Acid (C17:1)	1.76	1.25	5.98	1.58	0.03	-
Stearic Acid(C18:1)	0.51	0.58	-	0.67	0.01	0.80
Linoleic Acid (C18:2)	ND	-	1.55	0.17	-	-
Elaidic Acid (C18:1)	2.43	0.21	0.64	0.21	0.07	6.81
Linolelaidic Acid (C18:2)	0.23	0.35	1.74	0.39	0.33	-
Arachidic Acid (20:0)	0.30	-	1.19	-	0.01	-
Linolenic Acid (C18:3)	0.41	-	-	-	0.10	-
Oleic Acid(C18:1)	0.13	0.08	-	0.11	0.01	0.34
Saturated %	39.2	11.57	9.53	11.30	96.45	88.81
Monounstaurated %	59.2	88.43	90.06	88.53	1.98	9.20
Polyunsaturated %	1.5	0.0	0.41	0.16	0.33	2.00

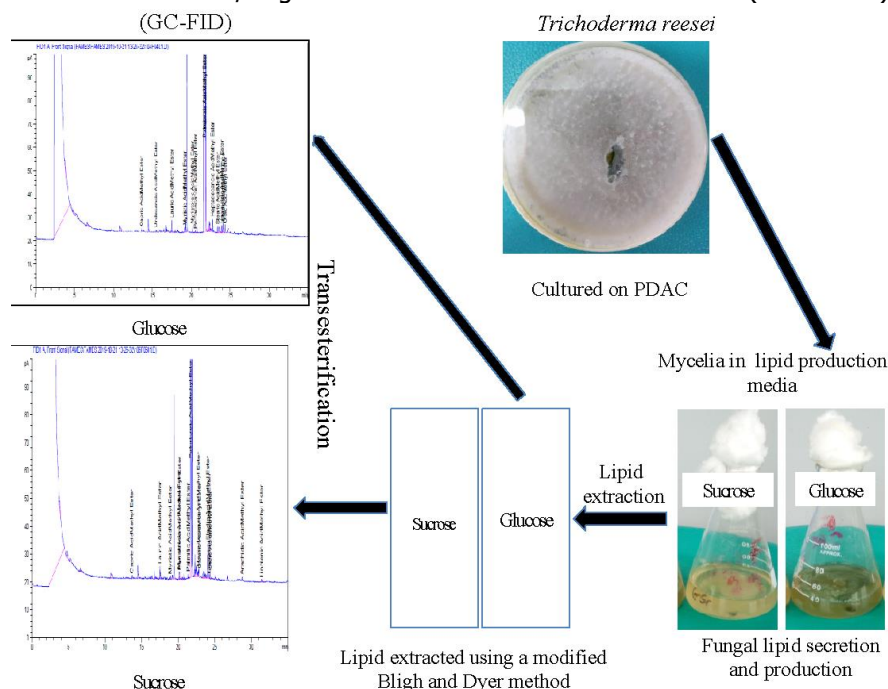


Figure 4. Flowchart of oil production for *Trichoderma reesei* from its cultivation on potato dextrose agar to oil extraction and GC-FID chromatogram of the FAMES.

Discussion

The prospects of isolating microorganisms from wastewater and soil for the production of microbial oils have been extensively studied (Muniraj *et al.*, 2015; Muniraj *et al.*, 2017; Sheerin *et al.*, 2017). A total of 25 fungal strains were isolated from fermented maize (*ogi*) wastewater (16) and sorghum-based brewery wastewater (9). The new fungal isolates and two existing laboratory fungal strains, *M. circinelloides* and *T. reesei* isolated from the soil were screened for oleaginity based on the accumulation of lipid in their globules and also the quantification of oil produced by Modified Sudan Black B Method (Muniraj *et al.*, 2017; Thanchaeron *et al.* 2017) (Table 1) Eight selected strains that had at least 20% oil accumulation capability were identified by culture-based methods and ITS-rDNA sequencing to be *Aspergillus fumigatus* (PW8), *Aspergillus flavus* (PW10), *Candida tropicalis* (PW16) and *Aspergillus tubingensis* (PW3), *Trichosporon luoberi* (BW7), *Aspergillus sp.* (BW4) and *Candida tropicalis* (BW1 and BW3). It was not surprising that *Aspergillus* spp. and *C. tropicalis* were the most commonly encountered oleaginous microbes from the fermented beverage wastewater as they have been reported as part of the fungal community involved in the fermentation of maize (*ogi*) (Anumudu *et al.*, 2018).

C. tropicalis isolated from a cassava processing wastewater source had a similar lipid yield of 29.4% (Ashika *et al.*, 2017) to the *C. tropicalis* isolates from our study (BW1-28.6%, PW3-33.7%, PW 16 - 29.9%). The other oleaginous yeast identified from brewery wastewater was *Trichosporon loubieri*; other species *T. cutaneum*, *T. fermentans*, *T. capitatum* and *T. oleaginosus* are known to accumulate lipids (Zhu *et al.*, 2008; Wu *et al.*, 2011; Chen *et al.*, 2013; Kourist *et al.*, 2015). Oleaginous isolates of *Aspergillus* genera including *A. flavus*, *A. fumigatus* and *A. tubingensis* have been reported from environmental samples (Abu-Elreesh and Abd-El-Haleem, 2014; Kadhim and Alrubayae, 2019 Shafiq, 2017; Khahim and Alrubayae, 2019; Abu-Elreesh and Abd- El-Halleem, 2014). This study reports the isolation of *A. tubingensis* from brewery wastewater accumulating 25.6% of its biomass as lipids. Intasit *et al.*, 2019 reported a strain of *A. tubingensis* (TSIP9) accumulating 25.57% lipid when cultivated on a non-sterile palm empty fruit branch biomass. It was not surprising that *A. flavus* had the lowest amount of lipids as it had the least dry cell weight. It was observed that the soil isolate, *M. circinelloides* had the highest lipid content (41.3%), and biomass (19.65g/L). *T. reesei* had a yield of 28.7% with a dry cell weight (DCW) of 14.60 g/L comparable to a report where *T. reesei* isolated from wood could produce 30 g/L and 32.4% of DCW

(Bharathiraja et al., 2017). The potential of strains of *M. circinelloides* and *T. reesei* as oleaginous fungi and feedstock for biodiesel production has been widely reported (Bhanja et al., 2014; Qiao et al., 2018; Zhao et al., 2018; Kumar et al., 2014; Bharathiraja et al., 2017).

FAMES profiles were determined for the four fungal isolates *M. circinelloides*, (glucose, sucrose) *T. reesei* (glucose, sucrose) *Aspergillus fumigatus* PW10 (glucose) *Aspergillus* sp. (BW 4) (glucose). Transesterified oil extract from *M. circinelloides* and *T. reesei* in this study showed higher amounts of unsaturated fatty acid while the two *Aspergillus* species examined were high in saturated fatty acids. Palmitoleic acid was the most abundant fatty acid methyl ester of *M. circinelloides* and *T. reesei* regardless of the cultivation medium being glucose or sucrose. However, the use of glucose as substrate enhanced the recovery of palmitic acid methyl ester from 0.38% to 7.11% and 2.47% to 6.37% in *M. circinelloides* and *T. reesei* respectively. Overall, for *T. reesei*, the FAMES profile generated from sucrose and glucose were not overly different from each other, but in *M. circinelloides*, lauric acid and capric acid were enriched when sucrose was the substrate.

FAMES profile was dependent on the type of carbon source - glucose or sucrose (**Table 3; Fig. 4**). The effect of carbon sources on fatty acid composition and lipid accumulation in oil-producing moulds have been reported (Liu et al., 2010; Thanana and Dina, 2014). The major fatty acid produced in the *M. circinelloides* is palmitoleic acid which accounted for 50.41% and 87.3% in sucrose and glucose respectively. In *T. reesei*, palmitoleic acid methyl ester was the major fatty acid, having 85.94% and 87.31% with sucrose and glucose respectively. The palmitoleic acid composition of *M. circinelloides* and *T. reesei* has been reported to be indicative of its use as an excellent feedstock for the production of single-cell oil (Bhanja et al., 2014).

The finding here that medium-chain saturated capric acid was the dominant FAME in *A. fumigatus* and the second most abundant in *Aspergillus* sp. (BW4) contrasts the report from Ascii et al. (2020) who showed that the FAMES profile of the six *A. fumigatus* strains studied were dominated by long-chain fatty acids and suitable for use as biodiesel.

In this study, Palmitoleic acid was produced at much higher concentrations by *M. circinelloides* and *T. reesei* at concentrations higher than observed from plant oils like Macadamia (28%) (Matthan et al.,

2009). Palmitoleic acid, the most abundant FAME in *T. reesei* and *M. circinelloides* is known to improve biodiesel properties (Knothe, 2009).

M. circinelloides was the most promising fungus for use as biodiesel but its profile ratios need to be improved for such use. In general, hexadecanoic (palmitic, C16:0), octadecanoic acid (stearic, C18:0) octadecenoic (oleic, C18:1) octadecatrienoic acid (linolenic, C18:3) and octadecenoic (linoleic, C18:2) fatty acids are present among other FAMES in different quantities in biodiesel (Li et al., 2019). In comparison with the profiles from *M. circinelloides* strains used as feedstock for biodiesel (Qiao et al., 2018; Reis et al., 2011; Carvalho et al., 2017) one or more of these FAMES were absent or present at very low levels in the oleaginous fungi for such consideration. Its relevant long-chain FAMES profile with sucrose, was palmitic (2.47%), linoleic (1.55%), differed from that of the FAMES profile of palmitic (6.37%), stearic (0.67%), oleic (0.11%), linoleic (0.17%), and linolenic (0.17%) from glucose.

The FAMES profiles of the oleaginous strains investigated had low levels or total absence of these necessary FAMES suggesting that the oils extracted from these isolates under the culture conditions are not fit for use as biodiesel. Further optimization of cultivation and lipid extraction conditions and genetic engineering may improve fatty acid methyl ester profiles of the most promising isolate *M. circinelloides* for its use as feedstock for biodiesel production.

The FAMES profile of the fungal isolates from this work shows that they would be a good feedstock for the production of pharmaceuticals and cosmetics. Lauric acid and capric acid are used in the cosmetic industry as emollients, dispersing agents for other chemicals, solvents and antioxidants. They can inhibit the growth of *Propionibacterium acne*, act as intestinal anti-inflammatory and alleviate oxidative stress (Huang et al., 2014; Lee and Kang, 2017). Palmitoleic acid is used to improve cold flow and also acts as a stabilizer for biodiesel (Knothe, 2010). These fatty acids have also been reported to have effects on the inhibition of *Candida albicans* (Clément et al., 2007; Murzyn et al., 2010).

Conclusion

In conclusion, among all the oleaginous species assayed, the FAMES profile of *M. circinelloides* contained a better composition of biodiesel relevant fatty acids although at low proportions. Optimization

of culture conditions, transesterification, extraction procedures and genetic manipulation for this isolate might be recommended for future research for obtaining better biodiesel production.

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