Original Research Article   https://doi.org/10.20546/ijcmas.2017.612.082

Screening and Characterization of Oleaginous Fungi from Irish Soil for Growth under Low Carbon Substrates

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A B S T R A C T

In this study oleaginous microorganisms were screened from Irish soils and are characterized under low cost carbon substrates for economic lipid production. Total of 247 fungi cultures were isolated from soil samples collected from various parts Ireland. Oleaginous 50 isolates were screened on glucose and starch at 3% concentrations respectively. The maximum lipid content in biomass was observed in the isolate I16-3, up to 44.3%. The lipid yield with respect to glucose consumed, $Y_{L/C}$ (g lipids/g glucose consumed), $Y_{L/C}$ values were in the range of 4.7 -14.0 g lipids/100 g of glucose consumed. The lipid content in biomass ranged from 19% to 39%; the maximum lipid content (39%) was observed in the same isolate I16-3. Interestingly, the isolate had a slightly higher lipid yield (4.3 g/L) when grown on 3% starch medium than 3% glucose medium (4.1 g/L). In addition to that 19 isolates were positive (+ve) for amylase secretion with a production of 34U/mL. *Trichoderma* sp., *Penicillium* sp., *Aspergillus* sp., *Mortierella* sp., *Zygomycetes* sp., *Acremonium* sp. and *Umbiliopsis* sp are the seven genera in which the oleaginous fungi were grouped.

Introduction

In recent days microbial lipids are considered as one of the potential alternative to Biodiesel production. In nature several microorganisms when grown under nutrient stress conditions (High C: N) ratio including bacteria, fungi, yeast and algae capable of producing microbial oils (1-6). Utilization of low cost materials for production of lipids is major concern for commercialization. The number of oleaginous microorganisms currently in use for energy production is limited; for example, of the 600 yeasts only 25% are known to produce lipids and among 60,000 fungal species less than 50% are known to accumulate lipids (7). Although the numbers seem large, the number of oleaginous microorganisms in use is limited, since the microbial lipid production research and application until now is focused on production of special lipids rich in poly unsaturated fatty acids (such as gamma linolenic acid and arachidonic acid) (1, 8-12). Oleaginous fungi used for these special lipids production are mainly cultures belonging to

the Mucorales order (13). Such mucaraceous oleaginous fungi for biodiesel production has a limited scope as their microbial oils would contain a high linolenic acid content, which does not satisfy the European standards (EN14214 and 14213) for biodiesel production (14). Hence search for newer organisms with potential oil yielding capacity becomes essential. While searching for hyper oleaginous microbes, their environment in which these organisms grow and produce oils, their adaptability are to be considered.

Feedstocks for the second generation biodiesel include biomass, agricultural and forestry residues, and biowastes (15). In order to utilize these residues for lipid production, oleaginous microorganisms should have respective enzymes to breakdown complex organic molecules, like cellulose, starch, etc. Otherwise external enzymes need to be added, thus increasing the lipid production cost. It is reported that when oleaginous fungi *Mortierellais abelliana ATHUM* 2935 and *Cunninghamella echinulata* ATHUM 4411were grown on renewable carbon sources (starch, pectin and lactose) their lipid yields were greatly influenced by secreted enzymes (amylase and polygalucturonase) (16).

Hence, it is of particular interest to isolate more and more oleaginous microorganisms with the capability to secrete enzymes(5). Oleaginous yeasts with xylose assimilating capability for utilizing ligno-cellulosic wastes for the second generation biodiesel production is attempted (17). Furthermore, the oleaginous microorganisms must be safe to human beings, local ecology and local environment. Diversity of oleaginous microorganism form Irish soils is explored and their lipid production capability was studied, in addition lipid production on complex carbon sources and their respective fatty acid content is also described. Finally biodiesel properties of lipids produced by oleaginous

microorganisms were evaluated and their feasibility was reported in this chapter.

Materials and Methods

Soil sample collection

Fifty undisturbed soil samples were collected from various locations in western Ireland and used for isolation of oleaginous fungi. Upper layer (5-15 cm) undisturbed soils were collected with sterile polyethylene bags, brought to the laboratory and used immediately for isolation studies.

Isolation of oleaginous fungi

Fungi were isolated from soil samples by means of the serial dilution and plating method. Ten gram of each soil sample was suspended in 90 mL of sterile distilled water added in 250 mL conical flasks; the flasks were shaken vigorously for five minutes. The suspensions were serially diluted up to one thousand folds. One mL of the one thousand dilution aliquot was transferred aseptically in a Class II biosafety cabinet (Fisher Scientific, Ireland) onto sterile Petri dishes and then 10-15 mL of molten PDA medium was poured into the Petri dishes. Filter sterilized Kanamycin (35ppm) was added into the sterilized PDA medium before plating. The plates were incubated at 30°C for 5 days. Single fungal colonies were isolated and transferred repeatedly to new plates until pure cultures were obtained, which were maintained in PDA slants and stored at 4°C.

Screening for lipid production

Cultures with a good mycelial morphology were selected for screening. Mycelium was taken from disks using a cork borer from the actively growing area and was pre-cultured in the Yeast Extract Malt Extract (YEME) broth for 2 days. The composition of the medium

was (g/L): glucose, 10; peptone, 5; yeast extract, 3; and malt extract, 3. After two days, the mycelium was harvested, and then mycelium with a wet weight less than 10 g was transferred to 10 mL sterile plastic tubes (Fisher scientific, Ireland). 5-8 sterile glass beads (5 mm diameter) were added and the mycelium was homogenised using vortex for two min. 0.8 mL of the homogenised mycelium was then transferred into 250 mL conical flasks containing 50 mL of the lipid producing medium, which consisted of (g/L): glucose, 30; yeast extract, 1.5; KH_2PO_4, 7; Na_2HPO_4, 5; $MgSO_4$, 1.5; $FeCl_3$, 0.08; $ZnSO_4$, 0.01; $CaCl_2$, 0.1; $MnSO_4$, 0.1; $CuSO_4$, 0.1; and $CO(NH_3)_2$, 0.1. The medium had a pH value of 5.5. Glucose in this medium was replaced with 30 g/L of starch while studying the capability of isolated oleaginous fungi in lipid production with starch as the carbon source.

Then, the cultures were incubated in an orbital shaker incubator (Fisher Scientific, Ireland) at a shaking speed of 160 rpm and an incubation temperature of 30 ± 1^oC. Flasks were periodically removed from the shaker incubator and the samples were filtered using 0.09 mm stainless steel sieve (Starsdet, Germany). Filtrates were centrifuged again at 5000 rpm for 5 min and used to analyze glucose, starch and amylase. The biomass was used for extraction of lipids.

Analytical methods

Residual glucose analysis

Remaining glucose in the culturing supernatant was analyzed using the DNS method (Miller, 1959). 1 mL of the supernatant samples was added into 15mL glass tubes and the liquid volume in the tubes was equalized to 3mL by adding distilled water. The tubes were then added with 3 mL of DNS reagent and were placed in a water bath at 90^oC for 5 min. 1 mL of 40% Rochelle salt solution (potassium sodium tartrate) was added to the tubes when the contents were still warm. After the tubes were cooled to the ambient temperature, the intensity of a dark red color was read at the wavelength of 510 nm using a spectrophotometer (Hach Lange, Ireland). 1 mL of distilled water was added in a 15mLglasstube and used as the blank sample; the procedure mentioned above was repeated. A series of glucose solutions with known glucose mass (0–500µg) were run in parallel for the purpose of calibration.

Residual starch analysis

Residual starch in the culturing supernatant was analyzed according to the phenol sulphuric acid method (Dubois *et al.,* 1956). 0.1 mL of the supernatant sample was added in 15 mL glass tubes and the liquid volume was made up to 1 mL with distilled water. 1 mL of phenol and 5 mL of 96% sulphuric acid solution were added into the tubes. The contents were well mixed by inverting the tubes several times and left undisturbed for 10 min. Then, the tubes were shaken and placed in a water bath at 25-30 ^{o}C for 20 min. The contents were cooled at ambient temperature and the colour intensity was read at the wavelength of 490 nm using the spectrometer. The blank sample was 1 mL of distilled water with the procedure mentioned above repeated. The same procedure was followed to standard glucose solutions containing 0 - 500 µg glucose for the purpose of calibration.

Screening for amylase secretion

Preliminary screening of fungi with amylase secretion was conducted on 2% starch agar medium in plates. A disk of actively growing mycelium was placed in the centre of the plates, incubated at 30°C and examined after 24 - 48 h. α-amylase producing cultures were selected by the formation of a clearance zone

when the plates were flooded with iodine solution (1% iodine in 2% potassium iodide, w/v). Amylase secretion of positive cultures was quantified after incubating these species with the lipid producing medium containing 3% starch for five days. The amylase activity was measured by mixing 1 mL of starch solution with 1 mL of properly diluted culturing supernatant in a tube, followed by incubation at 27 $^{\circ}$C for 15 min. Then 2 mL of DNS solution was added and the mixture was heated in a boiling water bath for 5 min; when the tubes were still warm, 1 mL of potassium sodium tartrate solution was added and the tubes were cooled to ambient temperature. The liquid volume was then made up to 10 mL with distilled water and the colour intensity was read at the wavelength of 560 nm using the spectrophotometer. One unit of amylase activity (IU/mL) was defined as the number of mg of maltose liberated by 1 mL of 1% enzyme solution per 5 min (Bernfeld, 1955).

Biomass analysis

Fungal biomass was analyzed by filtering the contents in conical flasks using 0.09 mm stainless steel sieve (Starsdet, Germany). Filtered biomass was washed twice with distilled water and then dried at 100 $^{\circ}$C until a constant weight.

Lipid extraction

Lipid extraction from dried biomass was performed according to the Bligh and Dyer method. 100 mg of dry biomass was weighed accurately and added into a *reacti* vial (Fisher Scientific, Ireland). The lipids were extracted from the biomass using 3 mL of chloroform and methanol mixture (2:1; v/v) overnight by shaking the tubes gently on a mini shaker (Starsdet, Ireland). Then lipids were separated by adding 1 mL of water followed by centrifugation (Sigma Aldrich, Ireland) at

3000 rpm for10 minutes. The lower liquid phase containing lipids was then transferred using Pasteur pipette; the solvents in the extracts were evaporated using dry nitrogen gas and the lipids were measured gravimetrically (Bligh and Dyer, 1959).

Equations

The lipid content in biomass, $Y_{L/X}$, was calculated using Equation 3.1:

$$Y_{L/X} = \frac{L_{max}}{X} \text{[Eq. 3.1]}$$

Where,

L_{max}, maximum lipid yield, g/L; and

X, biomass yield corresponding to the maximum lipid yield, g/L.

The amount of biomass yield with respect to the substrate consumption, $Y_{X/C}$, is calculated using Equation. 3.2:

$$Y_{X/C} = \frac{X}{(C_i - C_f)} \text{[Eq.3.2]}$$

$Y_{X/C}$, biomass yield with respect to carbon substrate consumed, g biomass/g substrate;

X, fungal biomass yield at time t, g/L;

C_i, initial carbon substrate concentration, g/L; and

C_f, carbon substrate concentration at time t, g/L.

The lipid yield with respect to carbon substrate consumption, $Y_{L/C}$, was calculated using Equation 3.3:

$$Y_{L/C} = \frac{L_{max}}{(C_i - C_f)} \text{[Eq. 3.3]}$$

Where,

C_f, carbon substrate concentration at time t corresponding to the maximum lipid yield, g/L.

DNA extraction

Three loops of oleaginous fungal cultures were scrapped from PDA plates and inoculated in YEME broth (Section 3.2.3) and incubated for 24 – 48 hrs in a shaker incubator (Fisher Scientific, Ireland) at a shaking speed of 160 rpm at 30 ± 1°C.Genomic DNA of the cultures was extracted using Cambio Microbial DNA Isolation Kit (CAMBIO, UK) according to the manufacturer's protocol.

PCR amplification

Polymerase chain reaction (PCR) amplification of 18s rDNA gene was carried out using a master cycler gradient thermo cycler (Eppendorf, Germany). PCR reactions were performed in 25-µl volumes containing 1µl of DNA, 12 µl of PCR master Mix (Applied Biosystems, UK), 2 µl each forward and reverse primers and 8.0 µl of PCR water (Sigma, Ireland). The forward primer was EF4 (5'-GGAAGGGRTGTATTTATTAG-3') and the reverse primer was EF3 (5'-TCCTCTAAATGACCAAGTTTG-3'). The thermo cycle patterns were as follows: 94 ^{o}C for 3 min (one cycle); 94 ^{o}C for 1 min, 50 ^{o}C for 1 min, 72 ^{o}C for 2 min (40 cycles); and 72 ^{o}C for 10 min (one cycle). PCR products with a correct size were confirmed by 1% agarose gel electrophoresis.

Nucleotide sequencing

The sequencing of purified PCR products containing 1.5-1.6 kb of 18s rDNA was carried out by means of automated sequencing (Euro Fins Biotech Pvt. Ltd.,

Germany). The sequence data generated were subjected to homology search through Basic Local Alignment Search Tool (BLAST) of National Centre for Biotechnological Information (NCBI) (www.ncbi.nlm.nih.gov/Blast). New gene sequences of isolates were submitted to the NCBI.

Results and Discussion

Growth and microbial lipid production of isolates in the glucose containing medium

Among the fifty fungal isolates screened for lipid production, 30 isolates were able to convert glucose into lipids with the lipid contents in biomass ranging from 20% to 44% within 7 days of cultivation.Therefore, these 30 isolates were designated as oleaginous. Glucose supported biomass growth (4.4 to 11.8g biomass yield/L medium) and was consumed by all the 30 cultures (11.0 to 29.3 g glucose consumed/L medium) (Fig. 1). The maximum lipid content in biomass was observed in the isolate I16-3, up to 44.3%. The lipid yield with respect to glucose consumed, $Y_{L/C}$ (g lipids/g glucose consumed), $Y_{L/C}$ values were in the range of 4.7 -14.0 g lipids/100 g of glucose consumed. The higher $Y_{L/C}$ values demonstrate the higher the substrate conversion efficiency of the isolated oleaginous fungi. The isolate I16-3 had the maximum $Y_{L/C}$ of 14.0g of lipids yield per 100 g of glucose consumed. Eight isolates (I1A1, I1A3, I1A5, I5-8, I8-1, I14-5, I16-3 and I18-1) had $Y_{L/C}$ higher than 10g of lipids yield per 100 g of glucose consumed. In the study, glucose was not completely utilized by any of the selected fungi; hence occurrence of lipid turnover was not observed. With the glucose concentration of 30 g/L the maximum $Y_{L/C}$ values obtained for *Mortierella isabelliana* and *Cunninghiamella echinulata* were 10.6 and 11.3 g lipids/100 g of glucose consumed, respectively(16). Our present study identified oleaginous fungi with higher

$Y_{L/C}$ values. The literature also reports much lower $Y_{L/C}$ values. For example, *Z. moelleri* MUCLL1430 had $Y_{L/C}$ of 4.23 g lipids/100 g of glucose consumed, *M. ramannia* ATHUM2922 had 5.0 g lipids/100 g of glucose consumed, *R. stolonifer* LGAM (9)1 had 4.5 g lipids/100 g of glucose consumed, *R. stolonifer* BPIL 1676 had 2.8 g lipids/100 g of glucose consumed, and *Mucor rouxianus* CBS120-08 had 1.2 g lipids/100 g of glucose consumed when cultivated on glucose(18).

Growth and microbial lipid production of isolates in the starch containing medium

All the selected fifty isolates were grown on the medium containing 3% starch to test their capability in lipid accumulation with starch as the carbon substrate. After 7days of incubation 19 cultures were able to accumulate lipids; interestingly all the 19 cultures were among the 30 cultures which were able to accumulate lipids in the medium containing 3% glucose (19). Other 11 cultures did not accumulate lipids in the starch containing medium; this might be because

glucose is a simple carbon source. A noticeable amount of biomass was produced (4.8 - 15.0 g/L) and the maximum biomass value obtained (among the 19 cultures) in the starch containing medium was higher than in the glucose containing medium (15.0 against 11.8g/L), confirming that starch is a good substrate carbon for oleaginous fungi (16, 19). All the 19 cultures utilized starch above 15 g/L (Table 1). The lipid content in biomass ranged from 19% to 39%; the maximum lipid content (39%) was observed in the same isolate I16-3. Interestingly, the isolate had a slightly higher lipid yield (4.3 g/L) when grown on 3% starch medium than 3% glucose medium (4.1 g/L). Moreover, the maximum lipid yield obtained by Isolate I16-3 (4.3 g/L) is comparable with other researchers' data. For instance, 3.7 and 3.8 g/L of lipids were produced by *Mortierella isabelliana* and *Cunninghiamella echinulata* when grown on 3% starch (16). 3.4 g/L microbial lipids were produced when *Aspergillus oryzae* cultured on 4% starch. Our isolates possessed a higher lipid yield than reported species.

Fig.1 Characterization of isolated oleaginous fungi cultured in the glucose containing medium
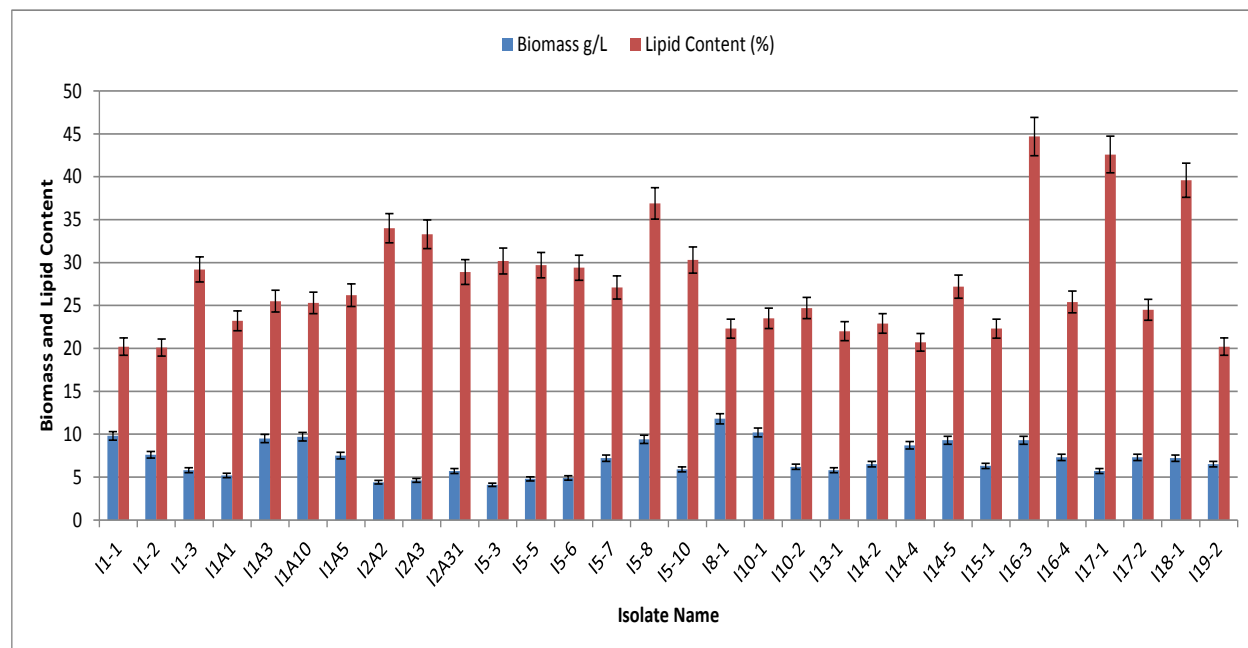
Fig.2 Characterization of isolated oleaginous fungi cultured in the starch containing medium
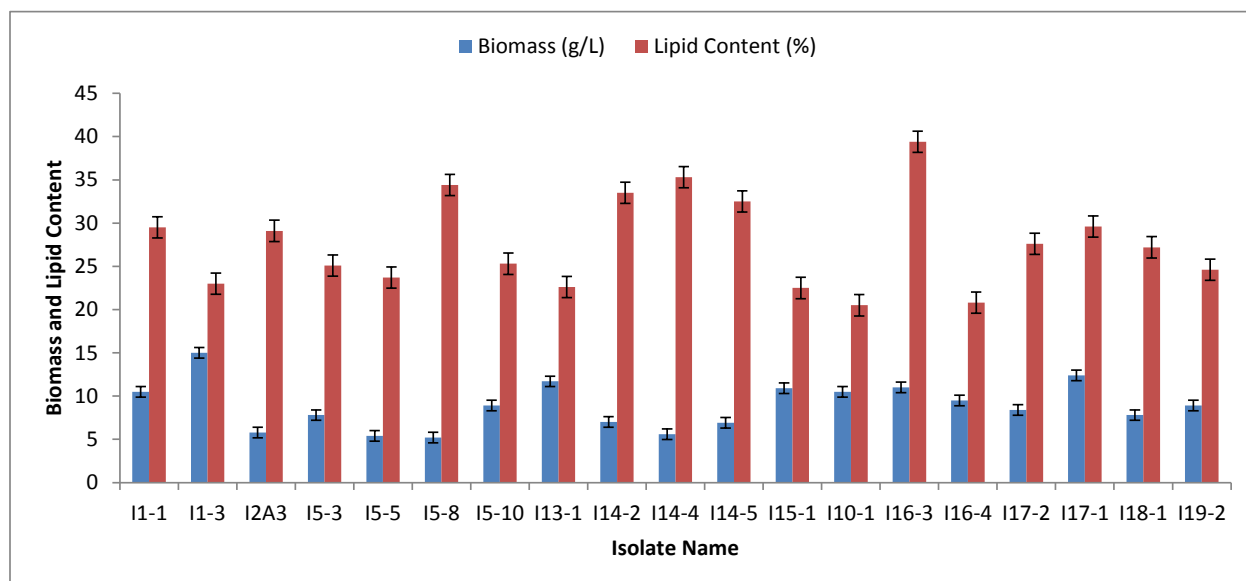
Table.1 Screening of oleaginous fungi with amylase secretion

Isolate name	Width of the clearing zone(mm)	Width of the biomass(mm)	Hydrolytic capacity
I1-1	10±2	10±3	1.00
I1-3	5±2	8±1	0.63
I2A3	3±1	10±3	0.30
I5-3	3±1	5±4	0.60
I5-5	2±2	5±5	0.40
I5-8	9±1	5±2	1.80
I5-10	5±2	5±1	1.00
I13-1	7±1	4±3	1.75
I14-2	4±3	7±2	0.57
I14-4	5±2	10±1	0.50
I14-5	2±3	7±3	0.29
I15-1	12±1	10±2	1.20
I15-4	3±2	2±1	1.50
I16-3	20±2	10±3	2.00
I16-4	3±3	10±3	0.30
I16-5	6±2	4±4	1.50
I17-1	9±3	1±5	9.00
I18-1	7±2	8±2	0.88
I19-2	3±1	3±1	1.00

In this study, seven isolates (I1-1, I1-3, I13-1, I15-1, I16-3, I17-1 and I19-2) had $Y_{L/C}$ values above 10 g lipids/100 g of glucose consumed and a maximum value of 19.3 g lipids/100 g of glucose consumed was observed in the isolate I16-3 (Table 1). $Y_{L/C}$ values of 14.9 and 15.2 g lipids/100 g of glucose consumed were obtained when *Mortierella isabelliana*

and *Cunninghiamella echinulata* were cultured on the medium containing 3% starch. Much lower $Y_{L/C}$ of 3.5 g lipids/100 g of starch consumed was obtained when *Aspergillus oryzae* was grown on 4% starch. These results suggest that Isolate I16-3 is a potential starch utilizing culture; this is probably because of its high amylase activity.

Amylase is the primary enzyme responsible for starch hydrolysis. It is reported that, when complex carbon sources are used for microbial lipid production, the capability of oleaginous fungi in secretion of amylase is critical to obtain high lipid yields (16). Hence, in this chapter, all 50 isolates were screened for amylase secretion on plates and the positive ones were tested with the lipid producing medium containing 3% starch. The results showed that, among the 50 isolates screened, 19 isolates were positive (+ve) for amylase secretion with the width of the clearing zones ranging from 2 to 20 mm and the width of the biomass ranging from 3 to 40 mm (Table 1). The result shows that the isolates which were positive were all oleaginous. The isolate I16-3 achieving the highest lipid yield possessed the highest amylase activity with a 20 mm clearing zone in plates and 34U/mL of amylase was secreted in the liquid medium (Table 1).

In order to use low-cost starchy substrates as raw materials for microbial lipid production, it is important to use oleaginous fungi with amylase secretion capability so as to reduce the lipid production cost. However, in the literature very few oleaginous microbes with amylase secretion capability for utilization of starchy wastes have been reported; they were *Aspergillus oryzae*, *M. isabelliana ATHUM 2935* (0.12 IU/mL) and *C. echinulata* ATHUM 4411 (0.19IU/mL)*(16)*. Other studies on use of starch based wastes for microbial lipid production either utilize commercial amylase or followed an initial step of cultivating amylase positive cultures

on starchy waste to hydrolyse starch and then employed oleaginous microorganisms on hydrolysate for lipid production. For instance, alpha amylase and gluco amylase were added to hydrolyze cassava starch for lipid production by micro algae *Chlorella protothecoide* (20-22). Li *et al.,* (2010) used *Streptomyces fibuligera* amylase secreting culture to produce crude amylase, which was then added to cassava starch hydrolysate for lipid production by *Rhodotorula mucilaginosa* TJY15a. In the present study, 19 oleaginous fungi were observed to have amylase secretion, which can be useful for low-cost lipid production by using starchy wastes.

Molecular identification of oleaginous fungi

Sequencing of 18s rDNA of the total 30 from fifty oleaginous isolates shows that they belonged to seven different genus: *Trichoderma* sp., *Penicillium* sp., *Aspergillus* sp., *Mortierella* sp., *Zygomycetes* sp., *Acremonium* sp. and *Umbiliopsis* sp. (Fig. 2). Among the seven genera, 13 isolates showed 99% similarity with already reported sequences of reference strains identified by BLAST analysis and the sequences were submitted to the NCBI gene bank (Acc. No: JF895924, JF895925, JF895926, JF895927, JF895928, JF895929, JF895930, JF912414, JF912415, JF912416, JF912417, JF912418 and JF912419). Out of the 30 isolates, seven, ten, seven and three belongs to *Trichoderma* sp., *Penicillium* sp., *Aspergillus* sp. and *Zygomycetes* sp., respectively; one each of *Mortierella* sp., *Acremonium* sp. and *Umbliopsis* sp.was identified (Table 1). Most of the studies in the literature related to oleaginous fungi used the Mucorales order; very few, to our best knowledge, only one genus *Aspergillus* which is non mucaraceous fungi have been used for lipid production. In addition, oleaginous fungi belonging to the Mucorales order are always used for

production of lipids with special long- chain poly unsaturated fatty Acids (PUFA), such as gamma linolenic acid (GLA) and arachidonic acid (AA). These fatty acids have nutritional importance. The present study explored fungi which were capable of producing lipids and did not belong to the Mucorales order. Non mucarales fungi are attractive alternative fungi for microbial lipid production; *Aspergillus* a known oleaginous fungus belongs to the Deutromycetes order, *Tricoderma* belongs to the Hypocreales order, and *Penicillum* belongs to the Eurotiales order.

Furthermore, their amylase secretion could be an additional advantage when using starch wastes for low-cost lipid production. *Acremonium* sp. is well known for hydrolysis of lignocellulosic materials (23), while in this study, it is found that *Aceremonium* sp. (I18-1) possessed starch utilizing capability and oleaginous nature. This is reported in the first time to the best of our knowledge. Therefore, this culture might be used for simultaneous saccharification and fermentation (SSF) of lignocellulosic materials for lipid production.

This could be an attractive option for producing the second generation biofuels. When the microbial oil is used for biodiesel applications, European standards (EN14214 and 14213) regulate that the PUFA content in the microbial oil should not be more than 12%. Our study has explored non mucaraceous oleaginous fungi whose PUFA contents presumably would not exceed the limit.

This paper dealt with fungi in lipid production with the media containing glucose and starch was studied. Many non mucaraceous oleaginous fungi with amylase secretion capability were reported, which could be used for low cost lipid production using starchy waste materials as carbon substrates.

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