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Detection of Lipids Production Potential of Soil Bacteria and Fungi in Ekiti State University

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A B S T R A C T

This study was conducted to determine the lipid producing potential of microbial isolates from different soil samples from Ekiti State University. The media used were sterilized in an autoclave at 121^{0}C for 15minutes, while serial dilution; isolation of colonies; identification and characterization of isolates were carried out from five different soil samples namely: kitchen effluent soil, cultivated, uncultivated soils, soil from dump site and palm oil spill site. The results showed that different fungi which include; *Fusarium* sp, *Scopulariopsis* sp, *Verticullium* sp, *Aspergillus* sp, *Geotrichum* sp, *Trychophyton* sp were detected from different soil samples. Bacterial species *Enterobacter* sp., *Bacillus cereus* were from cultivated soil while *Bacillus* sp., *Escherichia coli, Staphylococcus aureus* were detected from kitchen effluent soils respectively. Bacterial species *Serratia marcescens*, *Bacillus cereus* and *Enterobacter* sp. were isolated from uncultivated soil. Soil from dump site *Escherichia coli* and *Bacillus cereus* were detected and from palm oil spill site *Bacillus sp* and *Staphylococcus aureus* were detected. Fungal isolates screened for lipid content indicated only four fungal produced lipids with the highest production from isolate SDS 10 (7.98 g/L) and least in CS1(1.13 g/L). Gas Chromatography-MS showed that caproic acid had the highest concentration in the fungal isolates. The selected fungal isolates were identified using molecular genotypic identification via the 18SrRNA gene sequencing as *Purpureocillium lilacinum*, *Fusarium oxysporum*, *Aspergillus flavus* and *Rhizopus oryzae*. The best carbon source for lipid production for the fungal isolates was glucose (8.32 g/L) and least in starch (3.33 g/L). The highest (8.45 g/L) lipid production was recorded in fermentation medium supplemented with sodium nitrate from *Fusarium oxysporum* with the least (2.46 g/L) in the medium supplemented with peptone from *Rhizopus oryzae*. In conclusion, microorganisms identified from this study could be used to produce large amount of lipids for biofuel production.

Introduction

In microorganisms, lipid content and composition vary with the organisms particular metabolic machinery but can be manipulated by culture condition like temperature, growth stage at harvest available nutrient and pH. The rate of lipid accumulation in oleaginous microorganisms typically increases during nitrogen-limitation when cells division is inhibited funnelling carbon into triglycerides [TAGS] (Meng *et al.,* 2009). The biological production

of lipids using oleaginous microorganisms like micro algae, yeast, fungi and bacteria have been widely studied (Cheh *et al.,* 2015). Lipids accumulate in cells biomass majorly in the form of triglycerides, free fatty acids (FFA), polar lipids, sterols, hydrocarbon and pigments. Lipids accumulate in different locations in the cells and plays important role due to their specific cellular functions. Lipids bodies consisting primarily of TAG and sterol esters surrounded by a phospholipid monolayer rich in characteristics lipid body proteins, are present in the cytoplasm as a form of energy storage (Ryckebosch *et al.,* 2013).

The rising price of petroleum products and environmental concern associated with the emissions from combustion of these fuels, has led to an interest in the search for alternatives of petroleum products and one among them is biodiesel. Biodiesel, which is a mixture of fatty acid alkyl esters, can be used as a good substitute for petroleum (Ryckebosch *et al.,* 2013). One of the conventional methods to produce biodiesel was to trans esterify plant oils with methanol (Krawczy, 1996). It can be produced by transesterification of vegetable oils or animal fats, which yields fatty acids methyl esters.

Oleaginous microorganisms are historically defined as organisms in which the lipid contents exceeds 20%, and are thus promising candidates for producing fatty acids as sustainable biofuel precursors. Harvested biomass needs to be broken down in a downstream process, also referred to as a bio-refinery in which biofuels, value-added co products, and energy can be obtained (Clark *et al.,* 2009). The bio-refinery concept is a mass-based industry especially for microbial cell biomass which can be converted to multiple products (Hughes *et al.,* 2013). Including fuels and value added petrochemical replacement products.

In a bio-refinery process, the extraction of intracellular lipids has been identified as a crucial element linking the upstream bio-synthesis and downstream up grading steps According to a recent micro algal biofuel design report in a bio-refinery process lipid extraction efficiency has the largest impact on the cost of micro algal biofuel (Davis *et al.,* 2014). Microbial oil can be produced by the cultivation of algae, bacteria or fungi. Microalgae such as *Cholera* sp, *Nannochloropsis* sp and *Scenedesmus* sp are promising candidates for biofuel production as a result of their especially high lipid productivity, and rapid growth compared to other energy crops (Chisti *et al.,* 2010). Nowadays, limited stock of petroleum-derived fuel resources combined with perpetually increasing demands for energy due to rapid industrialization and population growth has troubled many governments and organizations across the world (Shafiee *et al.,* 2009).

Moreover, the combustion of fossil-derived fuels has led to increasing emission of greenhouse gases such as carbondioxide (CO_2), leading to global climate change and posing threats to the biosphere (Chisti, 2007). In order to achieve sustainable development, the critical issues noted above and the gradually rising fossil-derived fuel prices have called for the needs to search alternative sustainable and renewable energy sources (Demirbas *et al.,* 2011). Biofuel, produced from biomass, are promising alternatives to fossil-derived due to several distinct advantages including carbon neutrality, reduced emissions of gaseous pollutants e.g Carbon monoxide, Carbon dioxide, and Sulfur oxides, continuous availability of biomass feedstocks, and their safety for production by farming (Li *et al.,* 2011)**.**

Lipids isolated from oleaginous microorganisms have been used as components in coatings, paints, personal care products, production of fine chemicals and bio diesel thereby decreasing the dependency on vegetable and mineral oil. Fatty Acid Methyl Ester originating from the lipids of oleaginous microorganism e.g. algae, fungi show identical fuelling properties compared to conventional diesel and could be used in modern cars without major adaptation (Yuan *et al.,* 2005). Oleaginous bacteria, such as *Arthrobacter* sp, (Mengs *et al.,* 2009), *Rhodococcus opaque* (Alvarez *et al.,* 2002) and *Acinetobacter calcoaceticus* (Alvarez, *et al.,* 2002) can accumulate oil content up to 87% of their dry biomass and have high growth rates generating large amounts of biomass during short cultivation times.

Materials and Methods

The materials used for this study were plastic wares and glass wares; beakers, test tubes, petri dishes, conical flask, test tubes, measuring cyclinder, Bijou bottles, MacCartney bottles, syring and sterile universal bottles. Other materials include inoculating loop, nose mask, aluminium foil paper, spatula, examination gloves, non-absorbent, cotton wool, labelling tape, water proof permanent markers, Bunsen burner, cork borrer, distilled water and hand trowels. The media used were; Potato Dextrose agar, Nutrient agar, Christensen's medium (Oxoid), peptone water (Oxioid), Triphenyltetrazolium chloride (TTC) motility agar (Hardy Diagnostics) and

Simmons citrate agar (HiMedia), Triple sugar agar, MR-VP broth (Hi Media). Reagents and chemicals included; 70% ethanol, Gram's stain reagent, 0.5ml of Kovac's reagent, 3% hydrogen peroxide, Sudan black solution (0.3g of the powder stain in 100ml of 70% ethanol), and Ampicillin.

Collection of soil samples

The sample was collected from palm oil mill soil from Ekiti State University Teaching and Research Farm, cultivated soil sample in Ekiti State University Teaching and Research Farm, uncultivated soil sample, soil from a particular dump site at Ekiti State University Campus hostel (Achiever's lodge) and soil from kitchen effluent.

Sterile universal bottle was used to store the samples collected. The samples were collected using a disinfected hand trowel and spatula and labelled appropriately

Sterilization method

Cleaning of materials and disinfection of working areas

Glass wares and plastic petri wares were washed with detergent, rinsed thoroughly with distilled water and air dried before and after each use.

Preparation and sterilization of media

Media were prepared according to manufacturer's specification and sterilized in an autoclave at 121^{o}C and for 15 minutes.

Isolation of Oleaginous Microorganisms

Serial Dilution

A 250ml conical flask containing 50ml of sterile distilled water and ten series of test tubes each containing 9ml of sterile distil water was set up in a test tube rack and appropriately labelled.

Isolation of colonies for pure culture and Stock culture preparation

The several fungi grown on the potato dextrose agar plates were aseptically subcultured on a fresh medium,

Identification and characterization of isolates

The isolated organisms were characterized and identified based on their cultural, morphological and biochemical tests Jorgensen *et al.,* (2015).

The colonies were sub-cultured, Gram stain and subjected to biochemical tests such as oxidase, catalase, coagulase, indole, Motility test, urease test, Utilization test, oxidase test, Voges-Proskauer Test, Methyl Red Test.

Fungal isolates were identified on the basis of morphological and biochemical characteristics. The morphological features of are the appearance and colony colour on the agar medium.

Screening for oleaginous fungi

The oleaginous fungi colonies were initially streaked onto YEPD (yeast, peptone and dextrin) slant and grown for 3-5 days.

Determination of lipid content

To determine the lipid content in the fungi cells, lipids were extracted dried and weighed. This is a fast procedure allowing complete lipid extraction.

Briefly, a 50-mL sample was centrifuged at 5000*g* for 5 min, after which the fungi was washed twice with 50 mL of distilled water, then added into 10 mL of 4 M HCl, and incubated at 60 °C for 1 to 2 h.

Molecular Identification of Isolates

The four fungal isolates with the highest lipid production capacity were taken further for molecular identification using 18SrDNA.

DNA Extraction

DNA extraction was carried out on the isolates using the Zymo Fungal DNA extraction kit according to manufacturer's instructions. The purity and concentration of the extracted DNA was evaluated using a NANODROP (ND 1000) Spectrophotometer (Thermo Scientific, USA). All the samples showed a DNA yield between 5ng - 25ng, and the extracted DNA was optimally pure showing A260/A280 between 1.60-1.80.

PCR Amplification of the ITS Gene

Polymerase chain reaction was carried out to amplify the ITS gene of the fungi using the primer pair ITS-1 (5'-TCCGTAGGTGAACCTGCGG) and ITS-4 (5'-TCCTCCGCTTATTGATATGC) The PCR reaction was carried out using the Solis Biodyne 5X HOT FIREPol Blend Master mix

DNA Sequencing and phylogenetic analysis

Sequencing of the purified PCR products was done using a commercial service provider (Macrogene, South Korea). Sequences of the isolates were manually edited in chromas and checked for presence of artifacts or chimeric structures using the Mallard software.

Results and Discussion

Biochemical and morphology characteristics of microbial isolates from soil samples

The biochemical and morphological characteristics of bacterial isolates from soil samples is shown Tables 1 and 2. The five bacteria genera isolated include *Enterobacter* sp., *Bacillus cereus*, *Escherichia coli*, *Staphylococcus aureus* and *Serratia marcescens*. Similarly, the cultural characteristics of the fungal isolates from soil samples are shown Table 3. The eleven fungal isolates belonged to six (6) different genera were identified as *Fusarium* sp., *Aspergillus* sp., *Scopulariopsis* sp., *Verticullium* sp., *Geotrichum* sp. and *Trichophyton* sp.

Frequency of occurrence of bacteria isolated from the soil samples

The frequency of occurrence of bacteria isolated from the different soil samples is represented in Table 4. A total of fourteen bacterial isolates belonging to five genera were isolated from soil viz; *Enterobacter* sp., (14.29 %), *Escherichia coli* (21.43%), *Staphylococcus aureus* (14.29 %). However, *Bacillus cereus* had the highest frequency of occurrence (42.86%) while *Serratia marcescens* had the lowest frequency of occurrence (7.14%).

Distribution of Bacterial and Fungal Isolates from Different soil samples

Table 5 shows the bacterial and fungal isolates encountered in different soil samples. The results showed that bacterial species such as *Enterobacter* sp., *Bacillus cereus* and fungal species such as *Fusarium* sp., *Scopulariopsis* sp., *Verticullium s*p., *Aspergillus* sp were all isolated from cultivated soil. In kitchen effluent soil, only bacterial species (*Bacillus* sp., *Escherichia coli, Staphylococcus aureus*) were encountered. Also, bacterial species (*Serratia marcescens*, *Bacillus cereus, Enterobacter* sp.) and fungal species (*Aspergillus niger*) were isolated from uncultivated soil. As shown in Table 5 *Bacillus* species was isolated from the different sources of soil samples.

Lipid screening of fungal from soil

The lipid accumulation capacity of the isolated colonies revealed by Sudan Black B staining showed that out of a total 14 bacterial isolates. Eight bacterial were positive for Sudan black staining reaction (Table 6). Meanwhile eight fungal isolates were positive for Sudan black staining out of the eleven fungal isolates (Table 7).

Quantitative Screening of the fungal production of Biomass and lipid yield

The quantitative screening of fungal isolates from soil for lipid production is shown in Table 8. The fungal isolates displayed lipid production ranged from 1.13 to 7.98g/L while the biomass ranged from 4.23 to 13.32 g/L (Table 8).

PCR amplification

The quantified DNA of the four isolates with highest lipid production was subjected to polymerase chain reaction (PCR) to produce several copies of DNA and run through gel electrophoresis to determine the quality and separate it unto different bands, viewed under ultra violet transilluminator, sequenced and the sequence was analyzed using Basic Local Alignment Search Tool (BLAST) to determine the strain of the organism (Plate 1).

Identification of fungi strains

The ITS 1 and 4 gene sequences of the four fungal isolates were compared with the Gene Bank on National Centre for Biotechnology (NCBI) and the phylogenetic tree showed that strains are member different genes. The percentage similarities of the organisms with the sequence revealed *Purpureocillium lilacinum* (99 %),

Fusarium oxysporum strain FAZI25-3-2 (99%), *Aspergillus flavus* enrichment culture (96%), *Rhizopus oryzae* strain: PCNB1276 (99%) (Table 9).

Effect of carbon source on lipid production

Figure 1 shows the effect of carbon source on lipid production from fungal isolates. The carbon sources exhibited varied lipid production. Highest lipid production 8.32 g/L from *Aspergillus flavus* was recorded from a medium supplemented with glucose, followed by lactose (5.05 g/L), sucrose (4.23 g/L), starch (3.33 g/L) while the medium supplemented with maltose had least lipid production 2.43 g/L. Similarly, *Fusarium oxysporum* produced the second highest lipid production when supplemented with glucose (6.12 g/L) while maltose supplementation recorded the lowest lipid production (1.45 g/L). *Purpureocillium lilacinum* grown on supplemented with maltose medium had second highest lipid production 2.43 g/L.

Effect of nitrogen source on lipid production

Figure 2 shows the effect of nitrogen source on lipid production by fungal isolates. The fungal cells grew more quickly on fermentation medium supplemented with sodium nitrate which reached the highest lipid yield production (8.45 g/L) followed by *Fusarium oxysporum.* While the medium supplemented with peptone recorded the least lipid production from *Rhizopus oryzae* 2.46 g/ L.

Effect of incubation temperatures on lipid production

The effect of incubation temperatures (15^{o}C – 50^{o}C) for the biosynthesis of lipid from fungal isolates is represented (Figure 4). The lipid production increased with increase in incubation temperature until maximum activity was attained at 30^{o}C with 8.67 g/L by *Aspergillus flavus* before it declined.

The basic physiology of lipid accumulation in microorganisms has been well studied (Lu-Xia *et al.,* 2008). It is known that when the culture medium lacks the nitrogen source, the isocitric dehydrogenase (ICDH) was suppressed, therefore the tricarboxylic acid circulation (TCA) was blocked. Extra carbon source was transformed to triglyceride (TAG) by a series of enzymes like the citric acid lytic enzyme, the malic acid enzyme, the fatty acid synzyme, thus completed the fat accumulation. In this study, high lipid producing microorganisms were screen by the principle of high fat contents and fast growth on carbon limited culture medium as alternative sources for biodiesel production.

Four oleaginous fungal strains (*Purpureocillium lilacinum, Fusarium oxysporum, Aspergillus flavus* and *Rhizopus oryzae*) were isolated from soil samples around Ekiti State University Ado Ekiti. The reason for soil was the fact that it contains high amount of carbon arising from old wood and plants while nitrogen content is low.

However, very few studies had been done directly on isolating microorganisms from natural environment, which transform substrates to biodiesel. Pan *et al.,* (2009) isolated 20 strains which belong to 13 different species of fat fungi from oil-rich soil and nut samples. This indicates there is abundant lipid producing microorganisms in natural environment (Pan *et al.,* 2009). Therefore, screening fat producing microorganisms from natural environment, on one hand, it will expand the oil microorganism species resources, on the other hand, it also possibly obtains oil microorganism that have potential industrial application values.

However, oleaginous fungi have been considered as potential oil sources for biodiesel production because they accumulate large amounts of lipids which may accumulate up to 86 and 57% of lipids in dry biomass, respectively (Fakas *et al.,* 2007).

This is in line with the results obtained from this study in which fungal isolated produced a considerable amount of Lipid; *Purpureocillium lilacinum* (57.83%), *Fusarium oxysporum* (50.94%), *Aspergillus flavus* (59.91%) and *Rhizopus oryzae* (51.77%).

Ratledge (1984) showed that many species of microorganisms can accumulate lipids efficiently, such as *Schizochytriu*m sp., *Arthrobacter* sp., *Bacillus* sp., *Candida* sp., *Cryptococcus* sp., *Aspergillus* sp.. The results of this study is similar to work down by Ykema *et al.,* (1986) who reported that *Apiotrichum curvatum* simultaneously accumulated about 20% carbohydrate in the cells along with the 50% lipid. Yamauchi *et al.,* (1983) used ethanol as substrate with *Lipomyces starkeyi* and achieved a biomass density of 150 g LP1 with a lipid content of 54%.

Table.1 Frequency and Percentage Distribution of Bacterial Recovered from the Soil

Isolated organisms	Occurrence	Percentage (%)
***Enterobacter* sp.**	2	14.29
Bacillus cereus	6	42.86
Escherichia coli	3	21.43
Staphylococcus aureus	2	14.29
Serratia marcescens	1	7.14
Total	14	100.00

Table.2 Distribution of Microbial Isolates from Different Soil Samples

Soil samples	Bacterial isolates	Suspected Fungal isolates
Cultivated soil	*Enterobacter* sp., *Bacillus cereus.*	*Fusarium* sp., *Scopulariopsis* sp., *Verticullium s*p., *Aspergillus* sp.,
Kitchen effluent soil	*Bacillus* sp., *Escherichia coli, Staphylococcus aureus*	
Uncultivated soil	*Serratia marcescens*, *Bacillus cereus, Enterobacter* sp.	*Aspergillus niger*.
Soil from Dumpsite soil	*Escherichia coli, Bacillus cereus.*	*Geotrichum* sp., *Fusarium* sp.,
Palm oil spill soil	*Bacillus* sp., *Staphylococcus aureus.*	*Fusarium* sp., *Trichophyton* sp.

Table.3 Quantitative screening of the fungal isolates for Biomass and lipid yield

Isolates code	Biomass (g/L)	Lipid yield (g/L) Lipid (%)
CS 1	6.02	1.13 18.77
UCS 7	8.23	4.76 57.83
CS 5	4.78	1.19 24.89
UCS 6	12.19	6.21 50.94
CS 2	11.32	4.02 35.51
SDS 9	4.23	2.19 51.77
SDS 10	13.32	7.98 59.91
POS 11	6.82	2.12 31.29

Plate.1 Polymerase chain reaction of isolated fungal amplification on agarose gel

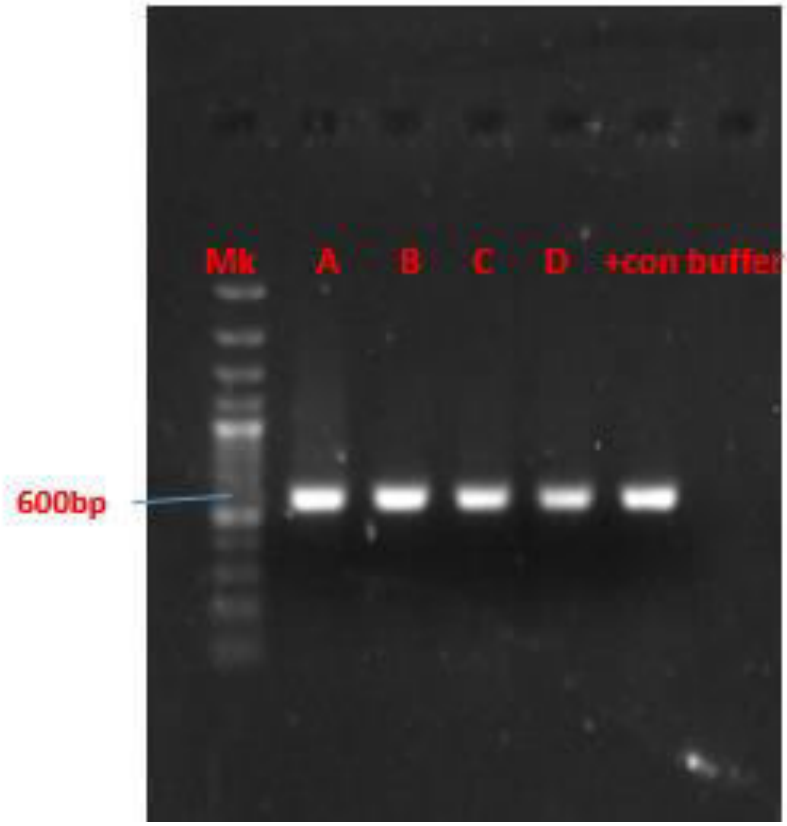


Figure.1 Phylogenetic tree of the isolated Fungai

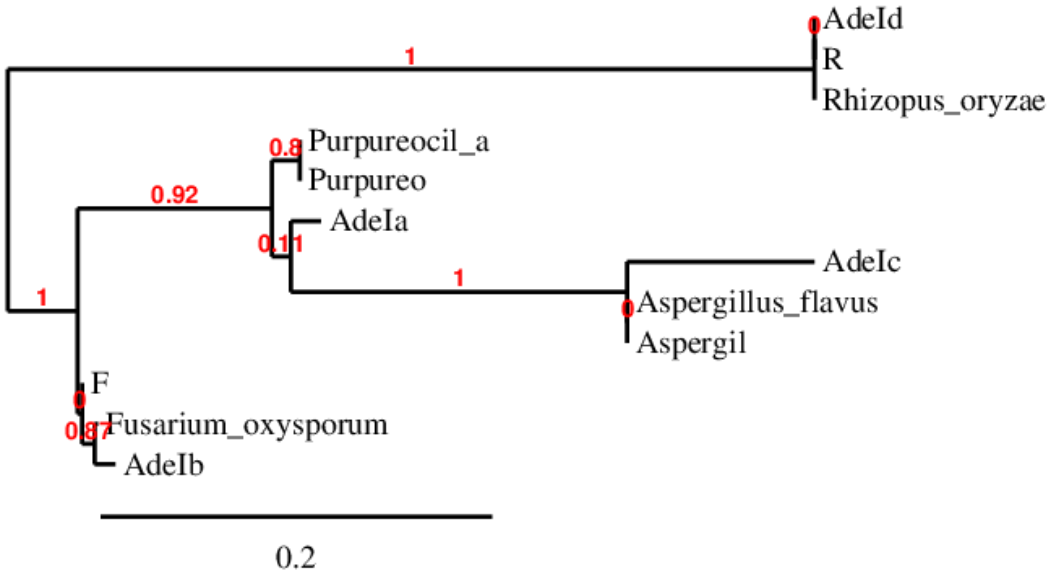


Figure.2 Effect of carbon sources on lipid production by fungal isolates

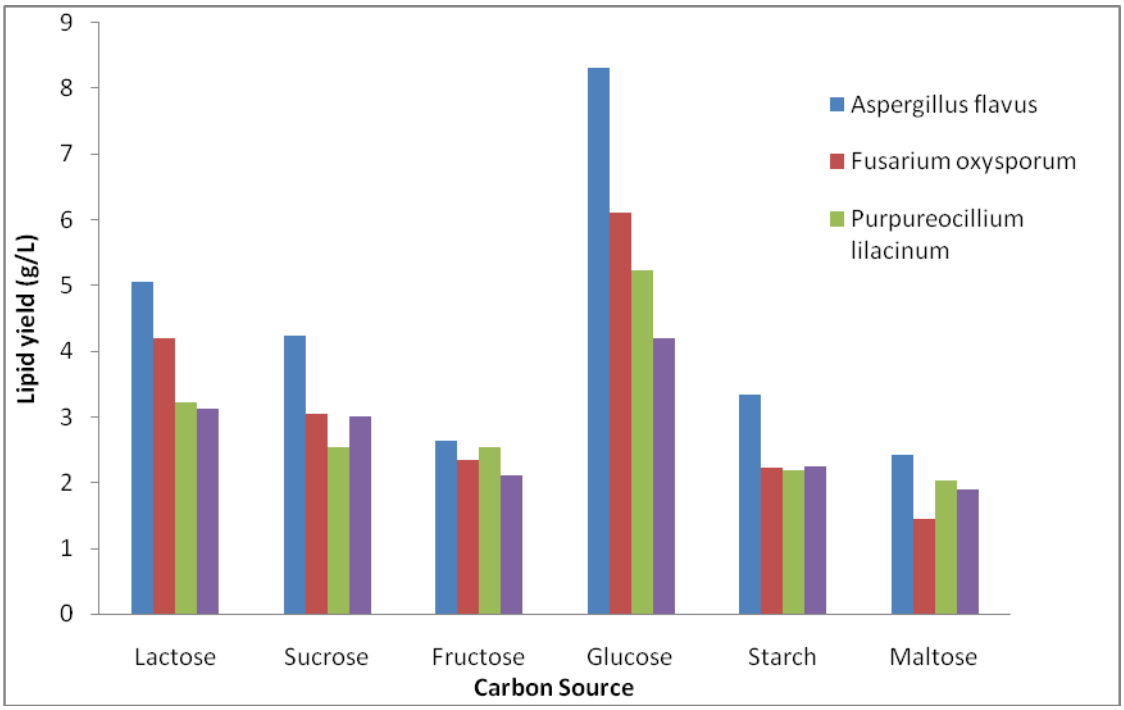


Figure.3 Effect of nitrogen sources on lipid production by fungal isolates

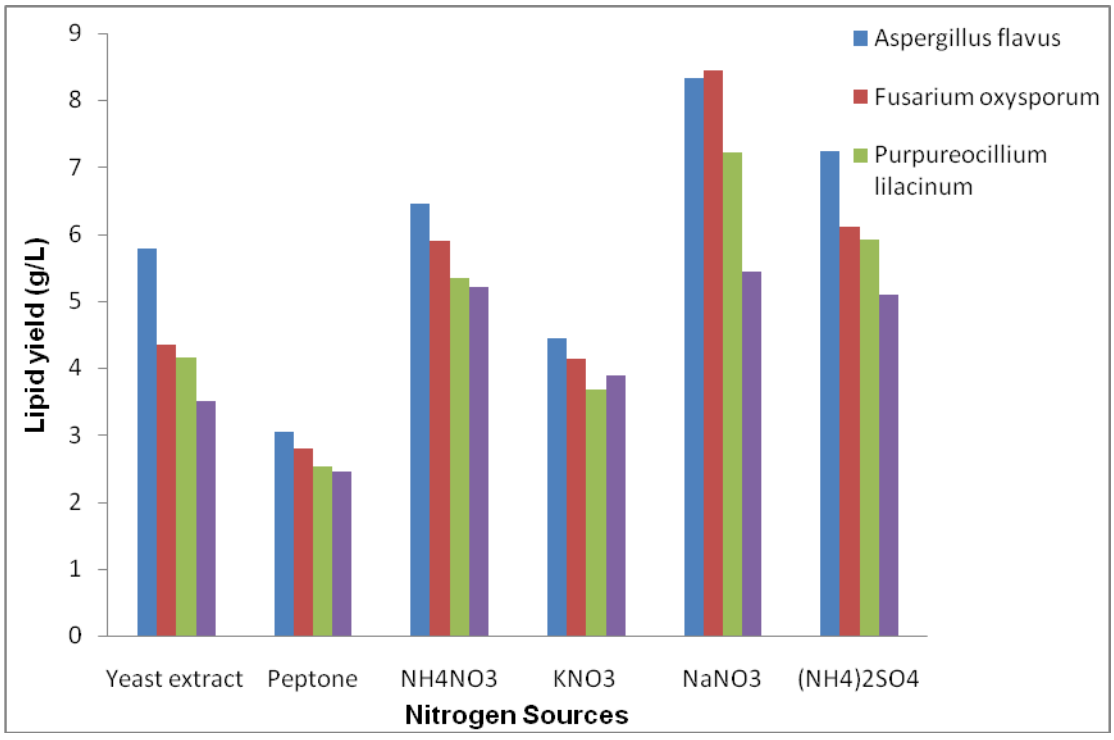


Figure.4 Effect of pH on lipid production by fungal isolates

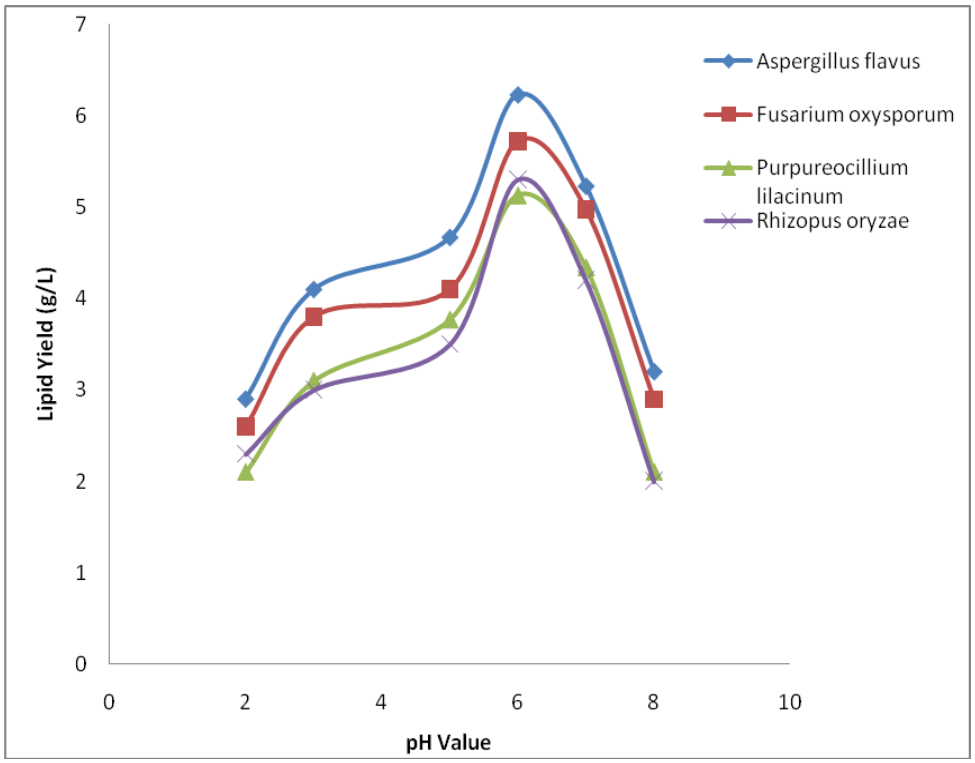
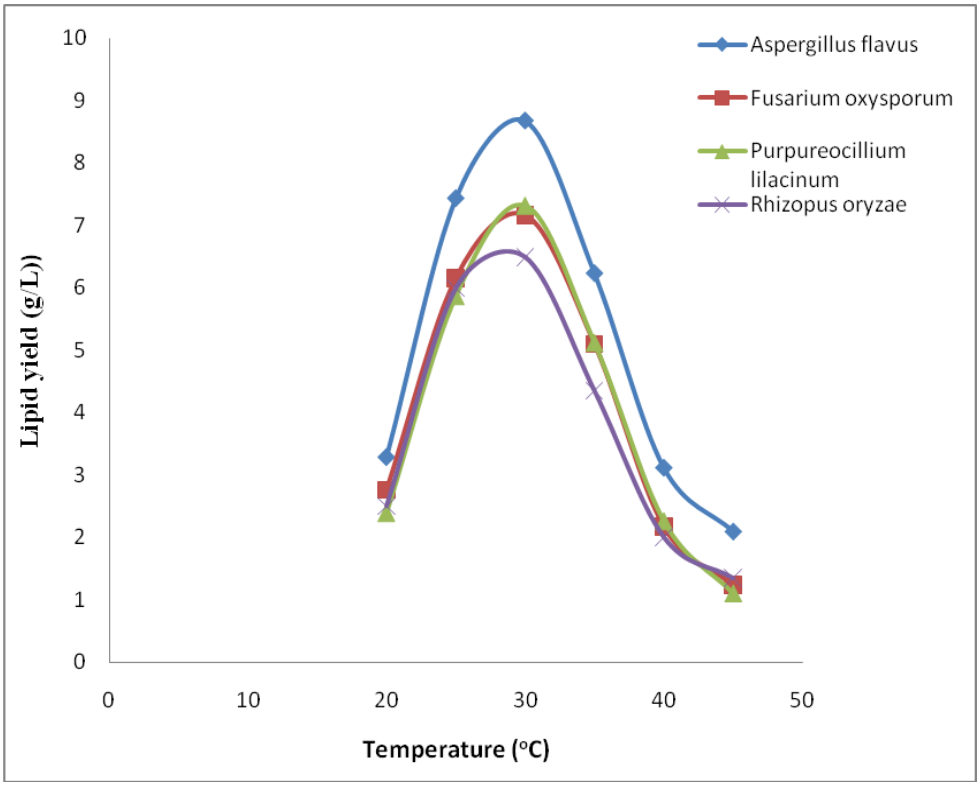


Figure.5 Effect of temperature on lipid production by fungi isolates



In terms of nitrogen source, this study showed that most of oleaginous yeast could utilize peptone for lipid accumulation. All the fungal isolates utilized sodium nitrate and $(NH4)_2SO_4$ for lipid accumulation, although the lipid content of *Aspergillus flavus* with $NaNO_3$ was lower than *Fusarium oxysporum* $NaNO_3$. Peptone did not promote lipid accumulation in the fungal isolates. Several studies reported that organic nitrogen sources such as peptone and yeast extract were more suitable for lipid accumulation than inorganic nitrogen sources (Wu *et al.,* 2010).

However, some oleaginous fungi such as *Candida lipolytica* and *Rhodotorula mucilaginosa* could utilize $(NH_4)_2SO_4$ for lipid accumulation (Karatay and Deonmez, 2011). This was similar to the result of the present study which revealed that *Aspergillus flavus*, *Fusarium oxysporum* could utilize $(NH_4)_2SO_4$ for lipid production.

In this study, a simple method was used in screening oleaginous fungi from the soil. Eleven fungal strains were isolated and it was shown that only four (4) fungal strains accumulated microbial lipids with a higher yield when fed with carbohydrates. This is an efficient way of producing a large amount of microbial lipid as alternative sources for bio-fuels production in future. Therefore, obtained results constitute a solid foundation for using the promising strains in producing microbial lipid. Further studies are required to investigate its physiology characters of oleaginous fungi isolated from this study.

Recommendations

From the findings of this study oligeanous micrrorganisms can serve as a source of biofuel in the face of increasing demand for cleaner energy. Therefore, efforts for the production of biofuels from oligeanous micrrorganisms should be intensified as these can serve as an alternative source of energy. Also, effective methods for titer and productivity improvement should be developed, possibly through the use of fed batch processing or immobilized cell cultures. More laboratory research has to be directed into the use of genetic engineering methods of selected organisms in future research to optimize the production of biodesel by oligeanous microorganisms.

Author Contributions

B. O. Adebayo: Investigation, formal analysis, writing—original draft.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical Approval Not applicable.

Consent to Participate Not applicable.

Consent to Publish Not applicable.

Conflict of Interest The authors declare no competing interests.

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