Influence of different natural media on production of myco-diesel

S A Shafiq1 and R A Chechan2

1Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq
2Department of Food Science, College of Agricultural Engineering Science, University of Baghdad, Baghdad, Iraq
roquibaa.ali@coagri.uobaghdad.edu.iq
shathaali2007@yahoo.com

Abstract. The current study was an attempt to produce myco-diesel using six oleaginous fungal isolates Aspergillusfumigates, Aspergillusterreus, Aspergillusflavus, Trichodermaharizanum, Penicilliumsp. and Fusariumgraminearum during the culturing on natural culture media from various types of agro-wastes as prospective nutritional sources (almonds husks, peanut husks and sunflower husks). The results of biomass (dry weight) showed the higher biomass value was obtained using, almonds husks firstly (4.54, 4.64, 3.30, 3.04, 1.02, 1.00) g / L for six oleaginous fungal isolates A. fumigatus, A. terreus, T. harizanum, F. Graminearum. Penicilliumsp. and Fusarium graminearum respectively then followed peanut husks and sunflower husks. Likewise, the height yield of lipids using almonds husks for six oleaginous fungal isolates were recorded high lipid productivity compared with peanut husks and sunflower husks (0.77, 1.13, 0.30, 0.45, 0.05, 0.03) g/ L respectively. Furthermore, the qualitative analysis of the lipids contents by gas chromatography were identified the presence of palmitic, oleic acids, stearic acid and linoleic acid in all isolates or fatty acid methyl ester analysis showed the predominant of saturated fatty acid than unsaturated fatty acid this indicates that the fungal lipid obtained has the same properties of biodiesel and the saturated forms give more favorable properties of biodiesel and for optimized biodiesel.

1. Introduction
Oleaginous fungi are a favorable feedstock for a sustainable myco-diesel industry and a good alternative for fossil fuels [1]. They were known for the production microbial oil, which can be accumulated up to 70% of intracellular lipid of their biomass under specific condition. [2]. The application of oleaginous fungi for biodiesel production are very few although they have several advantages over conventional plant and algal resources as they can be easily grown in bioreactors, have short life cycles, display rapid growth rates, are unaffected by space, light or climatic variation [1]. Fungi are an attractive source of lipids for use in biodiesel production [3]. Many researches such as yeast strains Rhodospiridium sp., Rhodotorula sp. and Lipomyces sp.[4] and from molds some species of genus Aspergillus like Aspergillusterreus has a wide spectrum in production of biodiesel[5;6], also Trichoderm spp., Mucocircinelloides and Gliocladiumroseum [7]. Several species of fungi were able to accumulate significant amounts of intra-cellular lipids, this lipid production can be optimized by adding supplementary nutrients to culture media and/or by altering
culture conditions during growth [8]. Oleaginous fungi have the ability to manufactures and accumulate high quantities of tri acyl glycerols inside their cells. This tri acyl glycerols can be simply converted to biodiesel through a process named transesterification through the conversion of methanol and tri acyl glycerols with potassium hydroxide as a catalyst [9]. The use of microbial systems for biodiesel production although not exploited industrially until now. Biodiesel can be produced from inorganic acid such as (HCl or H2SO4)[10]. Alkal NaOH and KOH [11] and free immobilized lipase [12]. The purpose of this research to produce biodiesel by local Iraqi oleaginous fungal isolates after selective new natural media with low costs.

2. Material and Methods

2-1-Oleaginous fungal isolates

Six selected oleaginous fungal isolates Aspergillus fumigatus, Aspergillusterreus, Aspergillus flavus, Trichodermaharizanum, Penicillium sp. and Fusariumgraminearum isolated from different Iraqi environments were obtained from department of biology –college of science –Mustansiriyah university.

2-2-Preparation of Agro-Wastes Media

The agro-wastes media were prepared according to [13] 50g from each of almonds husks (Prunus dulcis), peanut husks (Arachis hypogaea) and sunflower husks (Helianthus annuus) which were obtained from Baghdad markets. The husks cut into small pieces then added one liter of distilled water and boiled for 30 minutes and cooled and put in a blender for 5 minutes then filtered using medical gauze and complements the volume supernatant of distilled water to 1000 ml and adjusted the pH to 6.0 and each 500ml medium were prepared in 1000 ml Erlenmeyer flask and sterilized in autoclave (121°C and under 15 lbs/In2 pressure for 20 minutes) cooled and added 5 discs fungal isolates each 500ml from medium and incubated for 14 days at 30°C.

2-3-Determination of Biomass for Oleaginous Fungi

To select the highest lipid producer among the six isolates of oleaginous fungi which were cultured in basal medium yeast extract sucrose (yeast extract: 15g, sucrose:150 g/l of distilled water) and different agro-wastes media almonds husks, peanut husks and sunflower husks. 500ml medium were prepared in 1000 ml erlenmeyer flask and added 5 discs in diameter 5mm for each selected fungal isolate and the final pH was adjusted to 6.0, then incubated for 14 days at 30°C, and the biomass growth of fungi was observed on 14 days. A clear biomass mat was obtained by filtered using (whatman No. 2) filter paper inside a biological safety cabinet. The collected biomass was washed twice with distilled water and dried at 60°C for 24 hours or until constant weight was achieved then the dry weight was estimated gravimetrically mg/l [14].

2-4-Extraction of Fungal Lipid

The lipids of oleaginous fungal were extracted from dried biomass using chloroform, methanol 2:1(v/v) chloroform: methanol. Both dried samples of fungal isolates were crushed with a mortar and pestle by simultaneously adding (10 ml of chloroform, 5 ml of methanol) withdraw 8 ml from this mixture and added to 1g of biomass was vortexed for 5 minutes and prepare solution saline (7.3g of NaCl, 10ml of water) withdraw 2 ml was added each tube following vortexing for 5 minutes. Then sample tubes were centrifuged at 3000 rpm for 15 minutes and the lower layer of methanol, water and NaCl was removed by pasteur pipette, residual of solvent was dried then estimated gravimetrically mg/l and to determine the ratio of extracted lipids in compare to the cell dry weight [15].

2-5- Biodiesel Production and Analysis by Gas Chromatography

Total lipids were extracted from the dried biomass from the previous item. The fatty acid compositions by oleaginous fungal isolates were determined according to the method of [16] with some modifications. The (FAMEs) were produced by transesterification reaction. 2 ml of methanol with
sulfuric acid (2.5% v/v H$_2$SO$_4$/CH$_3$OH) as a catalyst was added to the crude lipid 100 mg. The reaction was progressed for 45 min at 90°C (water bath). Then, 1 mL H$_2$O and 2 ml n-hexane were added. The (FAMEs) were dissolved into the n-hexane. The solution was centrifuged at 2000 rpm for 15 min to compact the water from the hexane phase containing (FAMEs), which was then transferred into glass vials by using Pasteur pipettes. The (FAMEs) in n-hexane were analyzed using a gas chromatograph after adding 0.1ml of solution (KOH, methanol 1% w/ v) and1ml heptane to the (FAMEs) .

A gas chromatography (GC) analyzer followed to general company for plant oils –Baghdad –Iraq , (GC-17A model by Shimadzu Inc., Kyoto, Japan) was used to analyze the weight proportions of the fatty acidsequipped with an supelco wax tm 10 capillary column (Agilent, Japan, length 30 m 0.32 mm ID, 0.25 Mm flimthikness, substance fused silica) and a flame ionized detector (FID).The operating conditions were as follows : oven temperature 180 °C , and helium air were used as carrier gas The injector temperatures 250 °C and the detector temperatures 260 °C. The Statistical analysis was performed with spssversion 11.0 statistical software package. Data were expressed as least significant difference (LSD). Comparisons between fungal isolates and type of natural medium were performed with analysis of ANOVA two ways.

3. Results and Discussion

3.1 Different natural media for production of lipid

The production of lipids has been extensively studied as a means of reducing costs by using of low-cost carbon sources , and thus becoming competitive with traditional energy crops for lipids production from the data presented in (Figure 1)indicates that there is significant differences at p<0.05 among the six fungal isolates and the type of natural medium , the results showed the higher biomass of dry weight value were recorded at almonds husks medium then peanut husks medium and sunflower husks medium at selected oleaginous fungal isolates A. fumigatus , A.terreus (4.54 , 4.64) g/l,(3.82, 2.24) g/l and (2.22 , 1.74) g/l respectively.In contrast the rest of fungal isolates T.harizanum ,F.graminearum , P.sp. and A. flavus were recorded low biomass.

![Figure 1](image_url)

**Figure 1.** Fungal biomass dry weight g / l in different natural liquid media at pH 7 and incubated for 14 days, 120 rpm at 30°C.
Figure 2. Total lipid dry weight (g/L) in different natural liquid media at pH 7 and incubated for 14 days, 120 rpm at 30°C.

Also the total lipid from the biomass was extracted and estimated as shown in (Figure 2), the high significant differences among A. fumigatus, A. terreus and the rest of fungal isolates T. harizanum, F. graminearum, Penicillium sp. and Fusarium graminearum in different natural media.

The high lipids using almonds husks for isolates A. fumigatus, A. terreus recorded (0.77, 1.13) g/l, compared with peanuts medium, while sunflower husks medium was a poor medium for lipid productivity by all fungal isolates.

The use of these natural media for production of biodiesel or mycodiesel has been studied recently through the use of wastes as substrates for the production of lipids for biofuels and the use of cheap carbon sources like husks of cereals. However, reducing production costs associated with the fermentation process which is still of essential importance to increase the economic viability. Different lignocellulosic substrates and organic wastes have been tested as carbon sources for the production of lipids such as rice hull hydrolysate, corncobs, rice straw hydrolysate, wheat straw, sawdust. [17; 18; 19]. However, the production of biofuels from cellulose biomass generally requires the previous conversion of cellulotic material into simple sugars, and subsequent conversion of these sugars into biofuels. Recently numerous studies have been focused on the finding of renewable feedstock from residual materials in order to decrease the cost of production of biodiesel. Microbial oils or single cell oils (SCOs), produced by the oleaginous microorganisms have been studied as promising alternatives to vegetable or seed oils. Different types of agro-industrial wastes have been suggested as prospective nutritional sources for microbial cultures. Since the most abundant residue from agricultural crops is ligno cellulotic biomass, by product has been given top-priority consideration as a source of biomass for biodiesel production. [10, 11, 20].

3.2 Biodiesel production and analysis by Gas Chromatography -GC
In order to compare the potential utilities of the extracted total lipid as biodiesel feedstock, fatty acid composition (FAME) of the six oleaginous fungal isolates A. fumigatus, A. terreus, A. flavus, T. harizanum, Penicillium sp. and F. graminearum in liquid medium yeast extract sucrose medium and natural media for different kinds of cereals husks which were extracted by acid methanolysis during transesterification process of fungal lipids extract to fatty acid methyl esters then determined by gas chromatography in the presence of standards of fatty acids by general company for vegetale oils - baghdad - as shown in table 1 and table 2. The fatty acids profiles by GC with Retention Time RT
showed the presence of Palmitic acid and Oleic acid mostly in all selective oleaginous fungal isolates both of YES medium and Natural media which recorded the highest concentrations among the others types of fatty acids such as stearic acid, linolenic and myristic acid.

**Table 1.** Fatty acid composition of extracted total lipids from selected oleaginous fungal isolates on YES medium by GC

| Fatty acids (FA) | A. fumigatus | A. terreus | T. harizanum | P. sp. | A. flavus | F. graminearum |
|------------------|--------------|------------|--------------|--------|-----------|----------------|
| Palmitic C16     | 65.37        | 15.26      | 36.41        | 20.41  | 17.30     | 4.80           |
| Palmitolic C16.1 | 2.93         | _          | _            | _      | _         | 5.26           |
| Stearic C18      | 27.19        | _          | _            | _      | _         | 8.49           |
| Oleic C18.1      | _            | 47.00      | 63.58        | 65.89  | 71.54     | 70.55          |
| Linolenic C18.2  | _            | 37.73      | _            | 13.695 | 12.13     | 13.13          |

**Table 2.** Fatty acid composition of extracted total lipids from selected oleaginous fungal isolates on different natural media by GC

| Fatty acids (FA) | (%) The extracted total lipids by GC of selected oleaginous fungal isolates | A. terreus (sunflower husks) | A. terreus (peanut husks) | T. harizanum (Almond husks) | RT |
|------------------|---------------------------------------------------------------------------|----------------------------|--------------------------|-----------------------------|----|
| Palmitic C16     | 22.5803                                                                   | 16.3835                   | 33.2898                  | 4.801                       |
| Myristic C14     | 12.6377                                                                   | _                         | _                        | 2.831                       |
| Stearic C18      | _                                                                         | _                         | _                        | 8.495                       |
| Oleic C18.1      | 64.7820                                                                   | 35.7831                   | 66.7102                  | 9.028                       |
| Linolenic C18.2  | _                                                                         | 47.8333                   | _                        | 10.397                      |

Palmitic acid, or hexadecanoic acid, is the most common fatty acid (saturated) found in animals, plants and microorganisms. Its chemical formula is CH₃(CH₂)₁₄COOH and it is a major component of the biodiesel [21]. Oleic acid or octadecenoic acid is an unsaturated fatty acid that is the most widely distributed and abundant fatty acid in nature. It has the formula CH₃(CH₂)₇CH=CH(CH₂)₇COOH, synthesis of oleic acid from stearic acid [22]. Stearic acid is one of the most common saturated fatty acids found in nature following palmitic acid. Linoleic acid is a polyunsaturated also octadecenoic acid while myristic acid, also called tetradecanoic acid, is a common saturated fatty acid[22]. Higher number of saturated fatty acid than unsaturated fatty acid in the (FAME) analysis indicates that the
fungal oil obtained has properties similar to those of biodiesel and the saturated forms give more favorable properties of biodiesel and for optimized biodiesel, it should contain both long chain saturated and poly unsaturated fatty acids, thus indicating the produced oil suitability for high quality biodiesel production[20 ; 23]. The results are in agreement with [24] who noticed the predominant of Palmatic acid and hexadecanoic acid in production of mycodiesel by the isolate oleaginous fungus Aspergillus terrus. Furthermore [25] revealed that Octadecanoic acid was reached up to 50% of total fatty acids and higher than hexadecanoic acid of the lipid extracted of Fusarium sp. and Aspergillus fumigates when grew on yeast extract glucose medium. Likewise [26] showed that the lipid extracted of Aspergillusawamori mainly contained higher fraction of saturated fatty acids like palmatic, stearic and myristic acid than unsaturated fatty acids oleic and linolenic acid. The biodiesel quality is dependent on the fatty acid profile of the oil used as feedstock for its production. Therefore, for the production of biodiesel, the lipid obtained should be analyzed and compared with standard specifications for renewable diesel Figure 3.

![Wet fungal Biomass, Dry fungal Biomass, Mycodiesel](image)

**Figure3.** Wet and dry fungal biomasses of A.terreus was shown with the extracted mycodiesel

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