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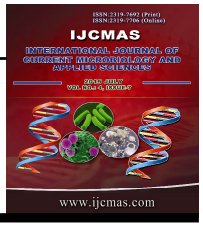

Original Research Article

Single Cell Oil of Oleaginous Fungi from Lebanese Habitats as a Potential Feed Stock for Biodiesel

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Keywords

Lebanese environment, Oleaginous fungi, Fatty acids; agro-industrial Waste products, Biodiesel, Single cell oil

A B S T R A C T

Single cell oils (SCOs) accumulated by oleaginous fungi have emerged as a potential alternative feedstock for biodiesel production. Five fungal strains were isolated from the Lebanese environment namely *Fusarium oxysporum*, *Mucor hiemalis*, *Penicillium citrinum*, *Aspergillus tamari*, and *Aspergillus niger* that have been selected among 39 oleaginous strains for their potential ability to accumulate lipids (lipid content was more than 40% on dry weight basis). A wide variations were recorded in the environmental factors that lead to maximum lipid production by fungi under test and were cultivated under submerged fermentation on medium containing glucose as carbon source. The maximum lipid production was attained within 6-8 days, at pH range 6-7, 24 to 48 hours age of seed culture, 4 to 6.10^7 spores/ml inoculum level and 100 ml culture volume. Eleven culture conditions were examined for their significance on lipid production using Plackett-Burman factorial design. Reducing sugars and nitrogen source were the most significant factors affecting lipid production process. Maximum lipid yield was noticed with 15.62, 14.48, 12.75, 13.68 and 20.41g/l for *Fusarium oxysporum*, *Mucor hiemalis*, *Penicillium citrinum*, *Aspergillus tamari*, and *Aspergillus niger* respectively. A verification experiment was carried out to examine model validation and revealed more than 94% validity. The profile of extracted lipids from each fungal isolate was studied using thin layer chromatography (TLC) indicating the presence of monoacylglycerols, diaacylglycerols, free fatty acids, triacylglycerols and sterol esters. The fatty acids profiles were also determined by gas-chromatography coupled with flame ionization detector (GC-FID). Data revealed the presence of significant amount of oleic acid (29-36%), palmitic acid (18-24%), linoleic acid (26.8-35%), and low amount of other fatty acids in the extracted fungal oils which indicate that the fatty acid profiles were quite similar to that of conventional vegetable oil. The cost of lipid production could be further reduced with acid-pretreated lignocellulotic corncob waste, whey and date molasses to be utilized as the raw material for the oleaginous fungi. The results showed that the microbial lipid from the studied fungi was a potential alternative resource for biodiesel production.

Introduction

Biomass-based biofuel production represents a pivotal approach to face high energy prices and potential depletion of crude oils reservoirs, to reduce greenhouse gas emissions, and to enhance a sustainable economy (Zinoviev *et al.*, 2010). Biodiesel

is an attractive alternative to diesel fuel due to its positive environmental characteristics. It is non-toxic, biodegradable, has a favorable emissions profile, and is produced from a variety of renewable resources including: soybean, palm, sunflower, rapeseed, jatropha, and waste oils (Al-Widyan and Al-Shyoukh, 2002; Bouaid *et al.,* 2007). However, growing concerns over the use of food crops for fuel production, as well as the rising global energy demand has put pressure on the biodiesel industry to find alternative sources of oil (Ruan *et al.,* 2012).

Attention has shifted to non-edible oil sources such as those produced from oleaginous microorganisms (those organisms with lipid content in excess of 20%). Microbial lipids are viewed as a possible alternative for industrial production because their fatty acid composition is similar to that of vegetable oils, as well as the fact that they are rich in polyunsaturated fatty acids (Huang *et al.,* 2009). The major fatty acids present in the lipids produced by oleaginous microorganisms are myristic acid (C14:0), palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2), and linolenic acid (C18:3); all of which can be converted to biodiesel through a transesterification reaction (Ruan *et al.,* 2012).

In general, lipids are accumulated in oleaginous organisms due to the presence of the ATP-dependent citrate lyase. Under nutrient limited conditions, especially nitrogen limitation with an abundant carbon source (high C/N ratio), AMP deaminase is up-regulated eventually leading to the presence of citrate in the cell cytosol. Citrate is cleaved by the ATP-dependent citrate lyase producing acetyl-CoA, which is used in fatty acid biosynthesis. It is by this pathway that oleaginous microorganisms shift their carbon sources away from growth and toward lipid production (Ratledge, 2004).

Glucose is the carbon source most commonly employed for growth of oleaginous fungi and lipid production (Saxena *et al.,* 2008; Zhao *et al.,* 2008). To reduce the cost of microbial oils, exploring other carbon sources instead of glucose is very important especially for such oils applied to biodiesel production. It was reported that xylose, glycerol, corn straw, molasses, whey and other agricultural and industrial wastes could be used as the carbon sources for microbial oils accumulation (Liang and Jiang, 2013).

The biochemical route of lipid production from lignocellulosic biomass typically involves biomass pretreatment, enzymatic hydrolysis of pretreated biomass to generate simple sugars, and fermentation of sugars to lipids. Pretreatment, a key step, involves the application of physical, chemical, thermal, or biological forces to disrupt the lignin-carbohydrate complex (LCC) in the cell walls of plants in lignocellulosic biomass and to increase the sugar yield of enzymatic hydrolysis (Yang and Wyman, 2008; Balan, 2014). Some of the leading pretreatment technologies that have been successfully scaled up to industrially relevant conditions include Ammonia Fiber Expansion (AFEX), Dilute Acid (DA), Steam Explosion, and Organosolve. Enzymatic hydrolysis applies enzyme cocktails comprising cellulases and hemicellulases to hydrolyze the major polysaccharides (cellulose and hemi-cellulose) into simple sugars (Van Dyk and Pletschke, 2012).

Since the sugars derived from lignocellulosic biomass are mostly glucose and xylose, oleaginous strains that consume both glucose and xylose are preferred, and the ability to consume minor sugars such as

arabinose, mannose, or galactose is also desirable (Huang *et al.,* 2013). Submerged culture is the dominant culture method used in the literature for lipid production from lignocellulosic biomass, but solid-state fermentation is also a viable option (Zhang and Hu, 2012). However, solid-state fermentation has drawbacks in areas such as heat and mass transfer, scale-up and cell and lipid harvest.

The application of a complete factorial design would require 2^n experiments if n factor have to be investigated. In the present case, eleven variables would lead to 1024 trials, which is a very large number. Using a fraction of the factorial design without losing information about the main effects of variables (Ooikaas *et al.,* 1998) can reduce the number of experiments. The Plackett-Burman experimental design, a fractional design, (Plackett and Burman, 1946 and Yu *et al.,* 1997) was used in this research to reflect the relative importance of various nutritional factors on lipid production by the tested fungal strains.

In the present study some environmental and physiological factors that affect the single cell oil production by five fungal isolates namely *Fusarium oxysporum*, *Mucor hiemalis*, *Penicillium citrinum*, *Aspergillus tamari*, and *Aspergillus niger* when grown on glucose as a carbon source have been studied, the nutritional requirements of these fungi were optimized using Plackett-Burman statistical design, the lipid and fatty acids profile were determined, also the ability of these fungi to accumulate lipids when grown on whey, date molasses and acid pretreated corncob lignocellulose wastes were tested.

Materials and Methods

Microorganisms

Five fungal strains were isolated from the Lebanese environment and were identified on the basis of molecular study as: *Fusarium oxysporum*, *Mucor hiemalis*, *Penicillium citrinum*, *Aspergillus tamari*, and *Aspergillus niger* and have been selected among 39 oleaginous strains for their potential ability to accumulate lipids (El-haj *et al.,* 2015).

Reagents and chemicals

All reference substances and chemicals were all purchased from Fluka. All other chemicals used were of analytical grade and were obtained from recognized chemical suppliers.

Maintenance of the stock culture

Fungal isolates used throughout the present work was maintained on Czapek Dox agar slants and stored at 4°C with regular transfer at monthly intervals. For long preservation 25% glycerol was used or in the form of spore suspension with 25% glycerol.

Seed culture preparations and fermentation technique

The fungal spore suspensions were prepared from 96 hours old Czapek Dox agar slants cultures using sterile distilled water and the spores were counted using haemocytometer then were used as standard inocula ($1x10^6$-10^8 spores /ml medium) unless otherwise stated.

The fermentation medium used during the optimization of incubation time, initial pH, inoculum level, age of the seed culture and the culture volume, was carbon rich and nitrogen limited to induce lipid accumulation and contained (g/L) : (glucose, 60; yeast extract, 1; NH4Cl, 1; KH_2PO_4, 2; $MgSO_4.7H_2O$, 0.75; $CaCl_2.2H_2O$, 0.05; $ZnSO_4.7H_2O$, 0.01; $FeCl_3.6H_2O$, 0.01; Na_2HPO_4, 1; pH 6.).

Aliquots of 50 ml medium was dispensed in 250 ml Erlenmeyer flasks, inoculated with the single cell oil (SCO) (4×10^7 spores /ml, 48 hours age of seed culture) producers unless otherwise stated, and then incubated at 28°C, under shaken conditions (160 rpm), where triplicate samples were set up to determine dry weight and lipid content.

Physiological and environmental factors affecting the lipid production

Effect of incubation period

Aliquots of 50 ml fermentation medium were dispensed in 250ml Erlenmeyer flasks, sterilized, inoculated with 4×10^7 (spores/ml) standard inocula of each of the selected fungi one at a time and incubated at 28°C for different time intervals (2, 4, 6, 8, 10 and 12 days) under shaken conditions (using a reciprocal shaker with 160 rpm).

pH relations

The effect of different starting pH values on the lipid production by the selected fungi one at a time investigated using initially adjusted fermentation medium either with 1N HCl or NaOH to pH values ranging from 3 to 9. All the pH adjustments were carried out by means of PYE UNICAM pH meter.

Age of seed culture

Aliquots (50 ml) of the seed medium (Sabouraud dextrose broth) were dispended in 250 ml Erlenmeyer flasks, sterilized and mixed with standard inocula(4×10^7 spores/ml each of the selected fungi one at a time.) of the selected fungal strain one at time. The culture flasks were then shaken at 28˚C for different time intervals (24, 48, 72 and 96 hrs). At the end of the incubation, 4% (v/v) were transferred as standard inocula to the fermentation media.

Effect of inoculum size

In order to test the effect of the inoculum level on lipid production and fungal biomass, the sterilized seed medium was dispended in 250 ml Erlenmeyer flasks (50ml each), received different inoculum levels (2×10^7, 4×10^7, 6×10^7, 8×10^7, and 10×10^7 spores/ml), incubated for the optimum seed culture age, and then inoculated to fermentation medium and incubated for the optimum incubation period.

Effect of culture volume

In order to test the effect of culture volume on lipid production by the fungal strain under test, 250 ml Erlenmeyer flasks were allowed to receive different volumes (25, 50, 75 and 100 ml) of the fermentation medium. After sterilization, each flask was received the appropriate inoculum level of each of the selected fungi one at a time and incubated at 28˚C under shaken conditions.

Optimization of the nutritional factors affecting lipid production using multifactorial statistical design: **Plackett-Burman design**

Eleven independent variables (glucose, yeast extract, NH_4Cl, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, $ZnSO_4.7H_2O$, $FeCl_3.6H_2O$, Na_2HPO_4, xylose and glycerol) were screened in twelve combinations organized according to the Plackett-Burman design matrix described in the results section. For each variable, a high (+) and low (-) level was tested. All trials were performed in duplicates and the averages of obtained lipid concentration were treated as the response for each trial. The main effect of each variable was determined with the following equation:

$E_{xi} = (\sum M_{i+} - \sum M_{i-}) / N$

Where; E_{xi} is the variable main effect, M_{i+} and M_{i-} are lipid concentration in trials where the independent variable (x_i) was present in high and low levels, respectively, and N is the number of trials divided by two. A main effect figure with positive sign indicates that the high level of this variable is nearer to optimum and a negative sign indicates that the low level of this variable is nearer to optimum. Using Microsoft Excel, statistical t-values for equal unpaired samples were calculated for determination of variable significance.

Single cell oil production using agro-industrial waste products

Date molasses, whey and dilute acid pretreated corncob lignocellulosic waste were used instead of glucose and xylose as a carbon source in the optimized fermentation medium, to test the ability of oleaginous fungi to accumulate lipids when grown on agro-industrial wastes.

Date molasses used throughout the present work was obtained from Al-Shifaa factory at Damascus, Syria. Samples were collected in sterile flasks and kept at 4ºC for further study. Reducing sugars content was estimated using the dinitrosalicylic acid method according to Miller (1959). Date molasses (400g) was dissolved in 1L distilled water, filtered by the glass wool to remove the none dissolved particles and centrifuged at 3000 rpm for 10 minutes to obtain a clear solution and then diluted to obtain a 80 g/l reducing sugars content, unless otherwise indicated (El-haj *et al.*, 2012).

The whey used was obtained from Alchakour farms, Alchouf-Mount Lebanon. The whey was first rotary evaporated to reduce the water content and then the reducing sugars was determined and their content readjusted to obtain 80 g/l reducing sugars concentration unless otherwise stated (pH adjusted to 6).

The corncob wastes were air dried, ground into powder using a blender (Brown, Germany) and subjected to dilute acid pretreatment(DA) (1% w/w H_2SO_4, 121˚C, 2 h, 10% corncob dry solid). After pretreatment, the sample was filtered with four layers of cheese cloth and the hydrolysate liquid was neutralized using $CaCO_3$ to a pH of 6 and the reducing sugars content was adjusted to 80 g/l unless otherwise indicated (Ruan *et al.*, 2012).

Determination of Biomass yield (dry weight) of oleaginous microorganisms

At the end of the incubation period the cells were harvested by filtration, washed twice with distilled water and dried at 60˚C till constant weight whereby the weight of the dried biomass was calculated.

Extraction of lipid compounds

Lipids were extracted following the method of Bligh and Dyer (1959).

The obtained biomass (dry weight) was transferred to 50 ml centrifugal tube and 15 ml of HCl (4M) were added and the mixture was kept at room temperature for 30 min before it was kept at -80°C for 2 hours and subsequently in boiling water for 20 min. This freezing/thawing process was repeated 3 times in order to break up the cells. Thirty milliliters chloroform/methanol (1:1) were added into the tube, shacked vigorously with a vortex and then centrifuged at 5000 rpm for 10 min. The lipid containing chloroform layer (the lower layer) was dried using rotary evaporator before it was weighed for lipid content estimation.

Residual reducing sugars determination

Reducing sugars were determined using dinitrosalicylic acid method according to Miller (1959).

Analysis of extracted lipid by thin layer chromatography (TLC)

Commercially available TLC plates (5 ×20 cm) pre-coated with 0.25 mm thick layer of silica gel G (Adsorbosil Plus 1, Altech, Deerfield, IL, USA) were used. The solvent was n-hexane-diethyl ether-acetic acid (90:10:2). The bands were observed after staining the TLC plate by Iodine vapor and the obtained Rf values were compared to those obtained with lipid standards (Khan *et al.,* 2014). Rf values were calculated using the formula, Rf= distance travelled by the analyte/distance travelled by the mobile phase.

Determination of fatty acids profile by GC-FID

A known amount (0.2g) of each extracted lipid was added into a glass tube and reacted with 0.6 ml of KOH (0.5N) in methanol at 80°C for 10 min, then 1 ml of Boron trifluoride methanol complex was added to each tube and incubated at 80°C for 10 min. Two milliliters of distilled water was added to stop the reaction, the suspension was vortexed vigorously. Equal volume of hexane was added to each tube and mixed thoroughly. After 30 min the upper phase containing the fatty acids methyl esters (FAMEs) were taken out and then transferred into a new clean tube for gas chromatography (GC) analysis. The gas chromatography (7890A, Agilent Technologies, USA) was equipped with a flame ionization detector (FID) and a capillary column DB225 (30 m × 0.25 m × 0.25 μm film thickness). The injector temperature was 220°C; the initial column temperature was 160°C and rose to the final temperature of 190°C at a rate of 3°C per min; and the detector temperature was 270°C. The carrier gas was helium at a flow rate of 0.9 mL/min. FAME composition of lipids was determined by comparing the retention time and peak area of samples with the internal standard and FAME mix (Sigma, Ireland).

Determination of biodiesel properties of SCOs (Khot *et al.,* 2012)

Different physicochemical fuel properties namely density, kinematic viscosity, SN, IV, HHV, CN, TAN and FFA were determined by using predictive models and mathematical equations for the transesterified SCOs of the five oleaginous fungal isolates.

Density values were estimated by predictive model using equation (1) based on Kay's mixing rule (Pratas *et al.,* 2011). The density database of individual pure fatty acid methyl ester compounds (Lapuerta *et al.,* 2010) was used for calculations.

$$\rho = \sum(c_i \rho_i) \quad (1)$$

Where, c_i is the concentration (mass fraction) and ρ_i is the density of component (individual fatty acid methyl ester) present in biodiesel.

Kinematic viscosity values (40°C; $mm^2\ s^{-1}$) were calculated using equation (2), a modified Grunberg-Nissan equation for biodiesel (Knothe and Steidley, 2011):

$$\nu_{mix} = \sum A_c \times \nu_c \quad (2)$$

Where, ν_{mix} = the kinematic viscosity of the biodiesel sample (mixture of fatty acid alkyl esters), A_c = the relative amount (%/100) of the individual neat ester in the mixture (as determined by, GC-FID) and ν_c = obtained

from the kinematic viscosity database of individual FAME present in biodiesel (Knothe and Steidley, 2011).

SN and IV were calculated empirically from FAME composition, with the help of Eq. (3) and (4), respectively:

$SN = \sum (560 \times A_i)/MW_i$ (3)
$IV = \sum (254 \times D \times A_i)/MW_i$ (4)

where, A_i is the percentage, D is the number of double bonds and MW_i is the molecular mass of each fatty acid methyl ester (Azam *et al.,* 2005; Gunstone *et al.,* 2007).

The higher heating value (HHV) also known as gross heat of combustion or heat content of an oil or biodiesel depends on the SN and IV of that fuel and therefore, for calculation of HHVs (expressed as MJ kg^{-1}), Eq. (5) has been used (Demirbas, 1998).

$HHV = 49.43 - [0.041 (SN) + 0.015 (IV)]$ (5)

Cetane Number (CN) was calculated using the multiple linear regression equation Eq. (6) (Tong *et al.,* 2011):

$CN = 1.068 \sum (CN_i W_i) - 6.747$ (6)

Where, CN_i represent reported CN of pure fatty acid methyl ester available in database (Tong *et al.,* 2011) and W_i is the mass fraction of individual fatty ester component detected and quantified by GC-FID.

Results and Discussion

Physiological and environmental factors affecting the lipid production

Effect of incubation period

It was noticed that the optimum incubation period for lipid production by *Mucor hiemalis, Aspergillus tamarii* and *Aspergillus niger* was 6 days that leads to 7.68±0.24 (Fig. 2), 8.80±1.01 (Fig. 4) and 14.29±0.46 g/l (Fig. 5) lipid yield respectively. In contrast, the optimum incubation period was 8 days for *Fusarium oxysporum* and *Penicillium citrinum* to accumulate lipids with a yield of 8.57±0.55 (Fig. 1) and 10.68±0.65 (Fig. 3) respectively. Concerning the biomass produced, it was noticed an increase in biomass production during the time intervals to reach their maximum in day 6 for *Mucor hiemalis, Aspergillus tamarii* and *Aspergillus niger* (19.82±0.33, 13.73±0.68 and 22.97±0.50g/l respectively) and in day 8 for *Fusarium oxysporum* and *Penicillium citrinum* (19.63±1.03 and 16.63±0.54 respectively). After reaching their optimum, the results (Figs 1, 2, 3, 4, 5) showed a decrease in biomass and lipid yield. The residual reducing sugars concentration decreases during increasing time intervals indicating that these sugars were consumed by the oleaginous fungi to grow and accumulate lipids. As results indicate the optimum incubation period for each fungal strain was used for further study.

pH relations

Results indicate that the best pH that leads to maximum biomass and lipid production was 6 for *Mucor hiemalis,* and 7 for *Fusarium oxysporum, Penicillium citrinum, Aspergillus tamarii* and *Aspergillus niger* leading to a lipid yield of 9.08±0.10 (Figure 7), 10.01±0.14 (Figure 6), 12.22±0.55 (Figure 8), 11.09±0.59 (Figure 9) and 18.92±1.03 (Figure 10) respectively. Data in figures 6, 7, 8, 9 and 10 showed that there is a net decrease in biomass and lipid production for initial pH values above and below the pH range 6-7 indicating that the tested oleaginous fungi prefer a slight acidic or neutral pH to grow and accumulate lipids which have been demonstrated in many studies (Subramaniam *et al.,* 2010; Ageitos *et al.,* 2011).

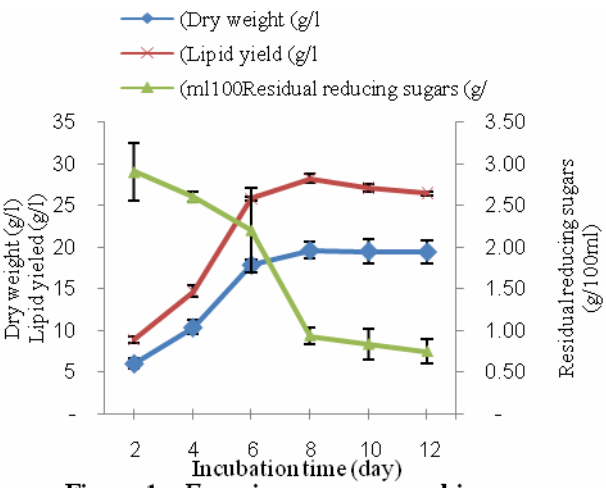

Figure 1 : *Fusarium oxysporum* : biomass and lipid production as affected by incubation time

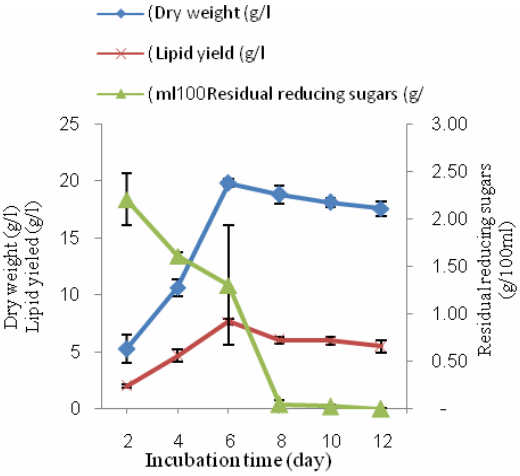

Figure 2 : *Mucor hiemalis*: biomass and lipid production as affected by incubation time

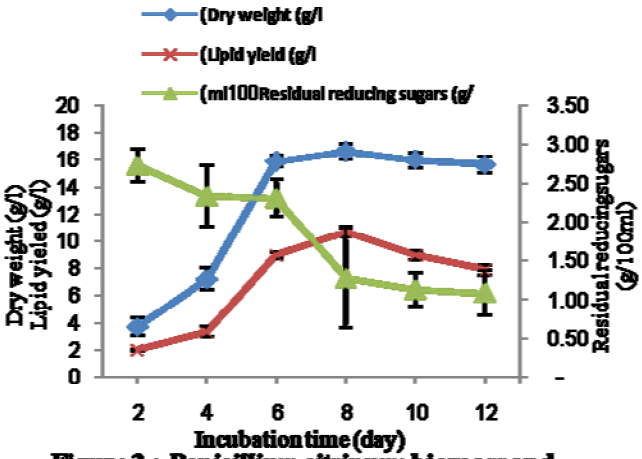

Figure 3 : *Penicillium citrinum*: biomass and lipid production as affected by incubation time

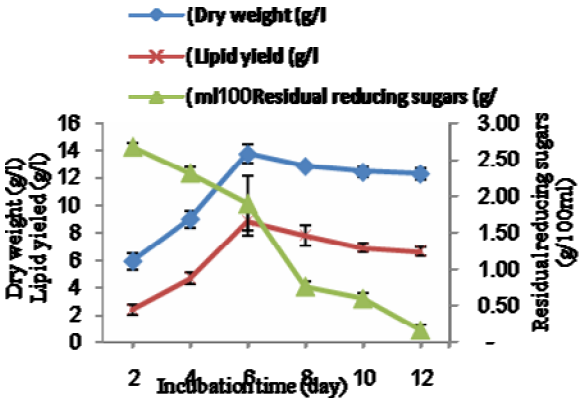

Figure 4: *Aspergillus tamarii*: biomass and lipid production as affected by incubation time

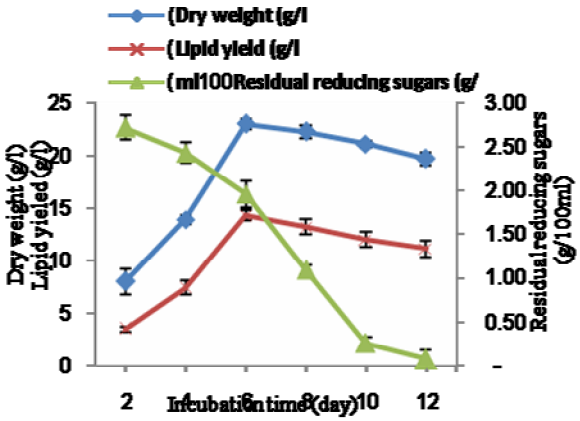

Figure 5 : *Aspergillus niger*: biomass and lipid production as affected by incubation time

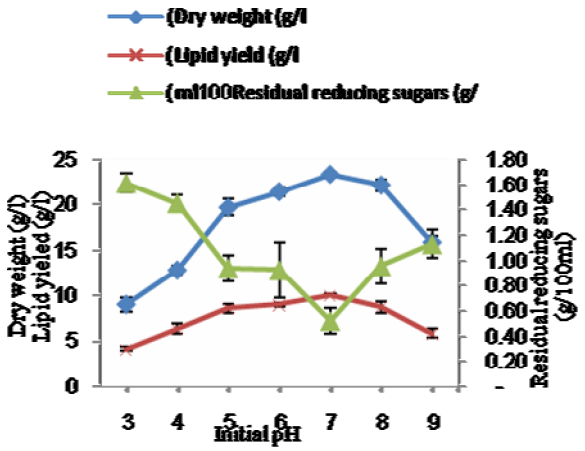

Figure 6:*Fusarium oxysporum*: lipid and biomass production of as affected by the medium initial pH

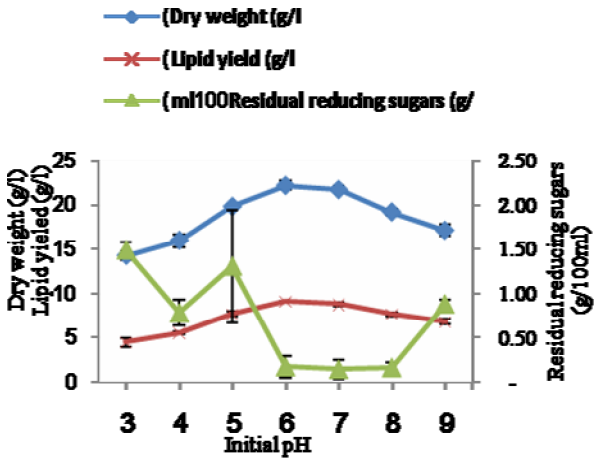

Figure 7:*Mucor hiemalis* : lipid and biomass production of as affected by the medium initial pH

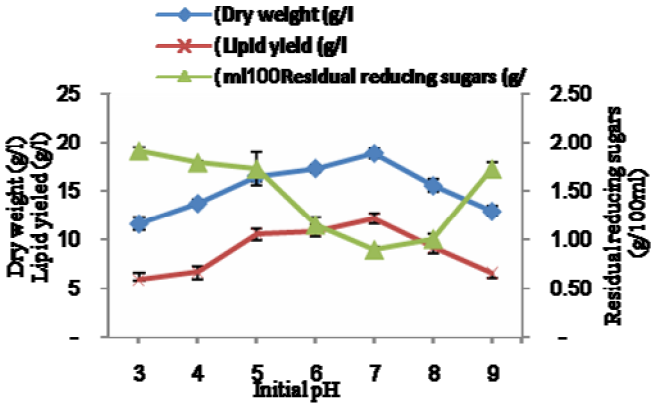

Figure 8: *Penicillium citrinum*: lipid and biomass production of as affected by the medium initial pH

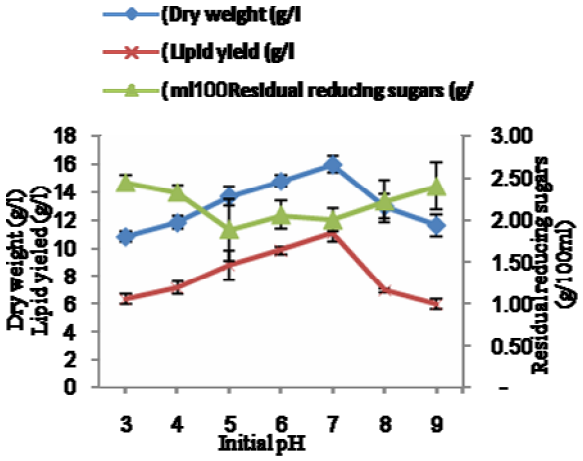

Figure 9: *Aspergillus tamarii* : lipid and biomass production of as affected by the medium initial pH

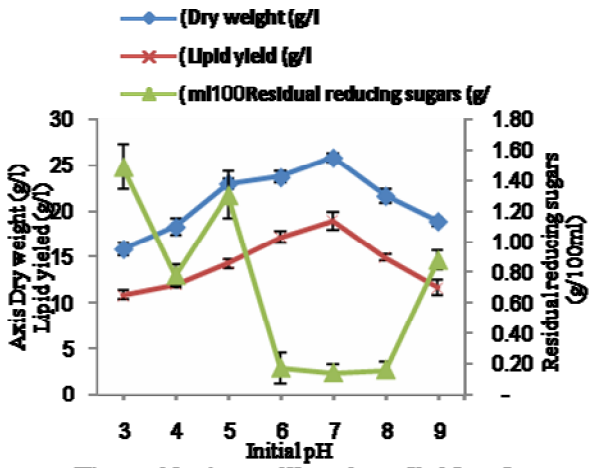

Figure 10: *Aspergillus niger* : lipid and biomass production of as affected by the medium initial pH

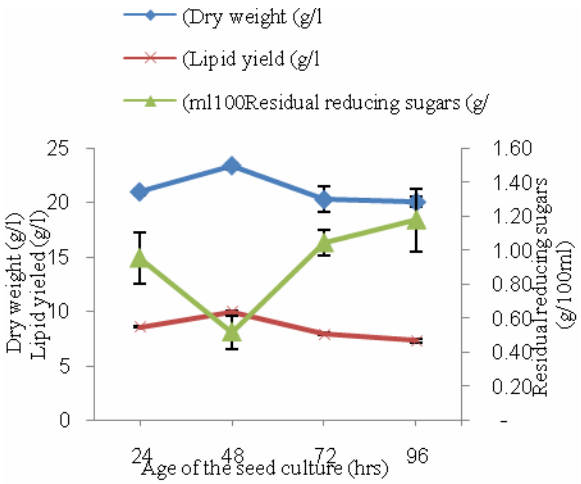

Figure 11: ***Fusarium oxysporum*****: lipid and biomass production as affected by the age of seed culture**

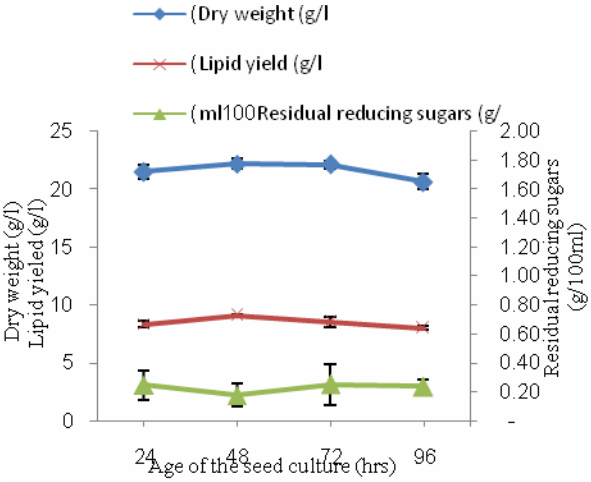

Figure 12:*Mucor hiemalis* : lipid and biomass production as affected by the age of seed culture

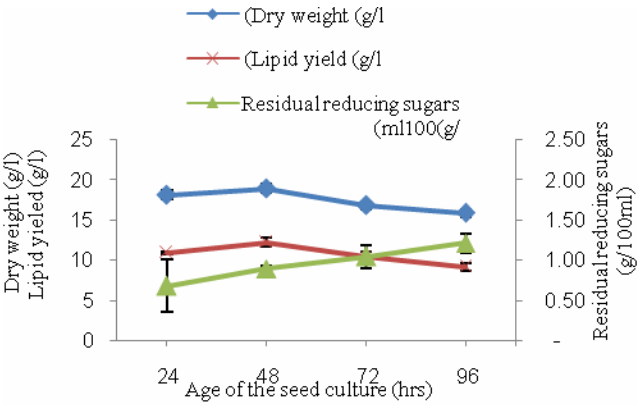

Figure13: ***Penicillium citrinum*****: lipid and biomass production as affected by the age of seed culture**

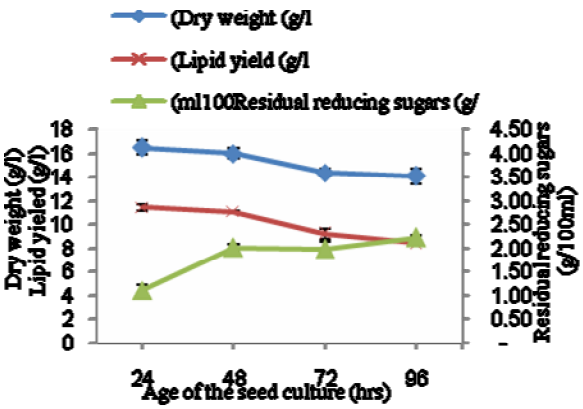

Figure 14: ***Aspergillus tamarii*****: lipid and biomass production as affected by the age of seed culture**

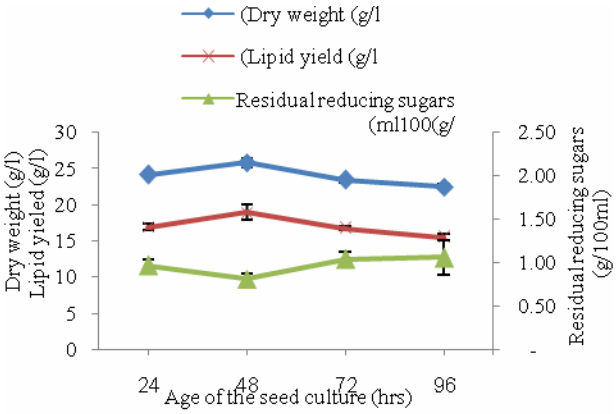

Figure 15: ***Aspergillus niger:*** **lipid and biomass production as affected by the age of seed culture**

The effects of pH on fungal metabolism include availability of metal ions, cell permeability, and enzymatic activity. Lower pH increases iron availability, while higher pH increases enzymatic activity (Webster and Weber, 2007). As a result the best initial pH for each fungal strain was used for further study.

Age of seed culture

Data in figures 11, 12, 13 and 15 revealed that the best fungal strain seed culture age was 48 hrs for *Fusarium oxysporum, Mucor hiemalis, Penicillium citrinum,* and *Aspergillus niger* that leads to maximum lipid production (10.01±0.13, 9.08±0.10, 12.22±0.55 and 18.92±1.03 g/l respectively), dry weight (23.41±0.29, 22.18±0.46, 18.95±0.52, 25.82±0.48 g/l respectively), while the data in figure 14 revealed that the best *Aspergillus tamarii* seed age was 24 hrs leading to 11.42±0.38 g/l lipid yield and 16.48±0.34 dry weight. As the inoculum age increase; there is a clear decrease in the amount of lipids produced. These findings were concurrent with previous studies using 24 hrs and 48 hrs seed culture (Huang *et al.*, 2009; Ruan *et al.*, 2012). For further study, the optimum age of seed culture was used for each fungal strain.

Effect of inoculum size

The results revealed that maximum lipid concentration, biomass were obtained with inoculum size of 4×10^7 spores/ml for *Mucor hiemalis, Penicillium citrinum* (Figs. 17 and 18) and 6×10^7 spores/ml for *Fusarium oxysporum, Aspergillus tamarii* and *Aspergillus niger* (Figs. 16, 19 and 20) that lead to lipid production of about 9.08±0.10 (Fig. 17), 12.22±0.55 (Fig. 18), 10.69±0.23 (Fig. 16), 11.70±0.07 (Fig. 19) and 19.12±0.85 g/l (Fig. 20) respectively.

The difference in lipid and biomass production between those obtained with optimum inoculum level and those obtained with others are slightly different. Xia *et al.* (2011) and Khot *et al.* (2012) utilized inoculum levels within the range 1 $x10^6$-10^8spores/ml.

Effect of culture volume

The results shown in figures 21, 22, 23, 24 and 25 indicate that the culture volume did not affect significantly biomass and lipid production. The finite volume of a culture medium means that it can only contain limited nutrients for the microorganism. An increased culture volume has effect on the aeration and oxygen availability, but this problem can be solved by high increased shaking rate. Most fungi are obligate aerobes, requiring molecular oxygen for their growth; insufficient oxygen supply increases the nutritional demand and thereby decreases fungal growth (Webster and Weber, 2007). The shaking rate used throughout the study was 160 rpm and 100ml culture volume was chosen for further experiments.

Optimization of the nutritional factors affecting lipid production using multifactorial statistical design: Plackett-Burmen design

In screening and optimizing the factors affecting lipid production, it is very important to test as much factors as possible and to identify the significance of each of them. Plackett-Burman design offers good and fast screening procedure and mathematically computes the significance of large number of factors in one experiment, which is time saving and maintain convincing information on each component (Srinivas *et al.*, 1994).

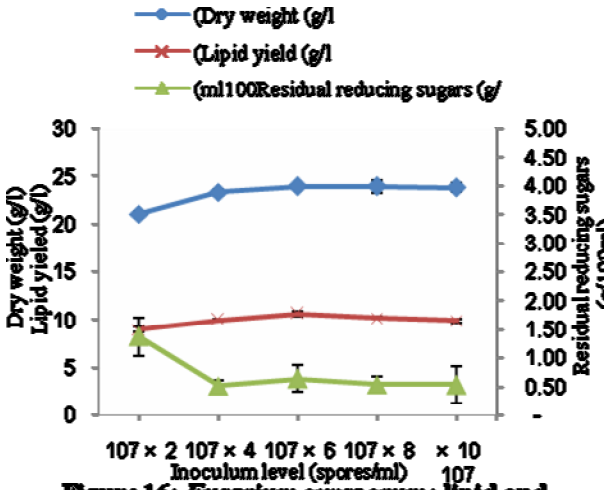

Figure 16: *Fusarium oxysporum* : lipid and biomass production as affected by inoculum level

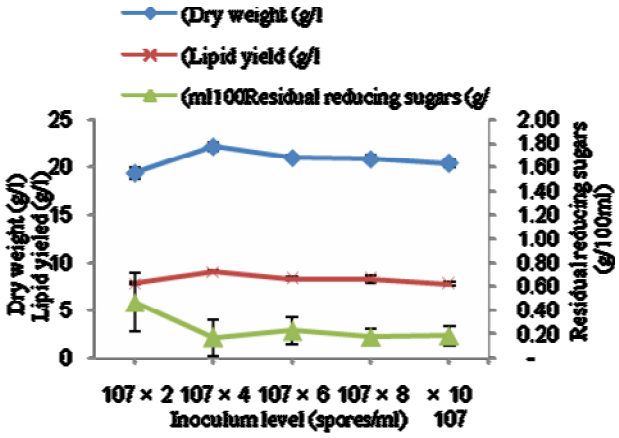

Figure 17: *Mucor hiemalis* : lipid and biomass production as affected by inoculum level

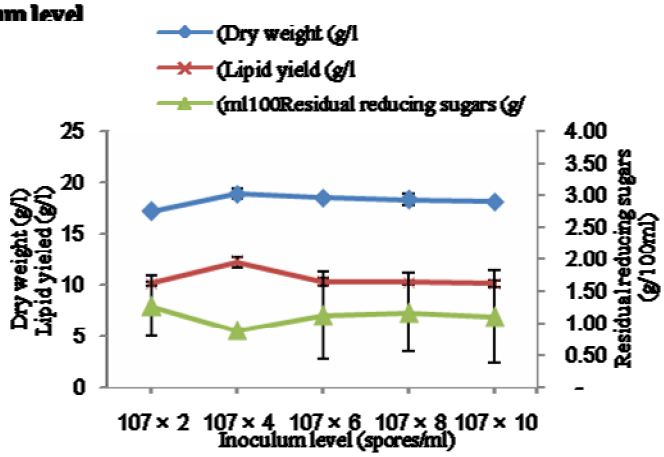

Figure 18: *Penicillium citrinum*: lipid and biomass production as affected by inoculum level

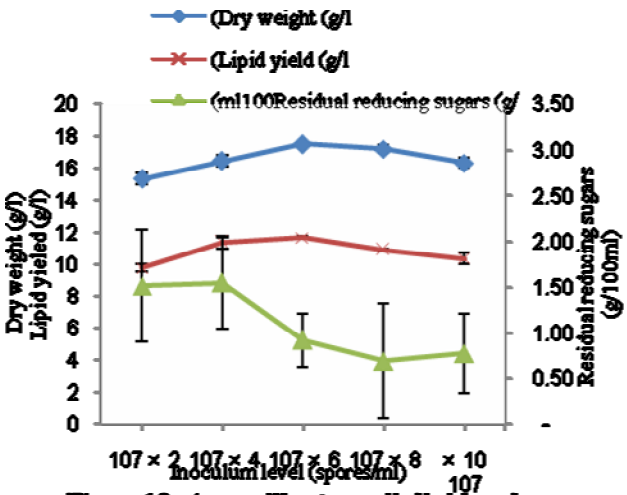

Figure19: *Aspergillus tamarii* : lipid and biomass production as affected by inoculum level

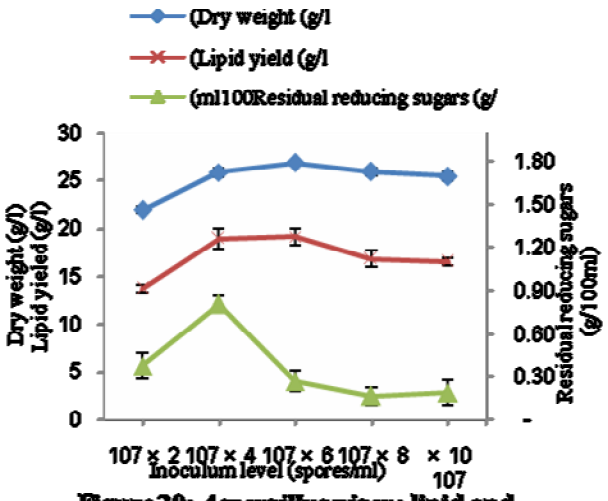

Figure20: *Aspergillus niger* : lipid and biomass production as affected by inoculum level

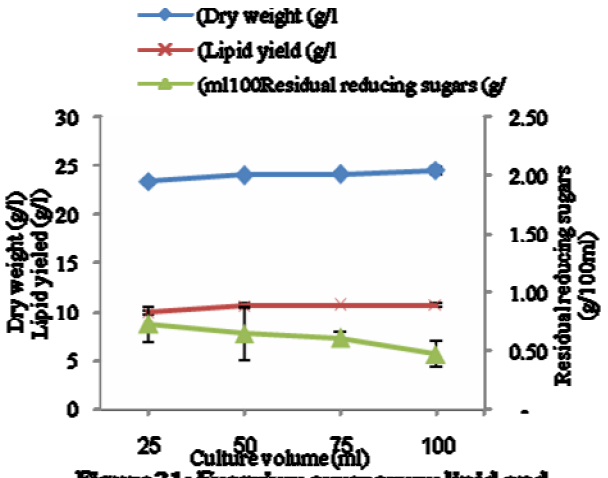

Figure 21: *Fusarium oxysporum*: lipid and biomass production as affected by culture v

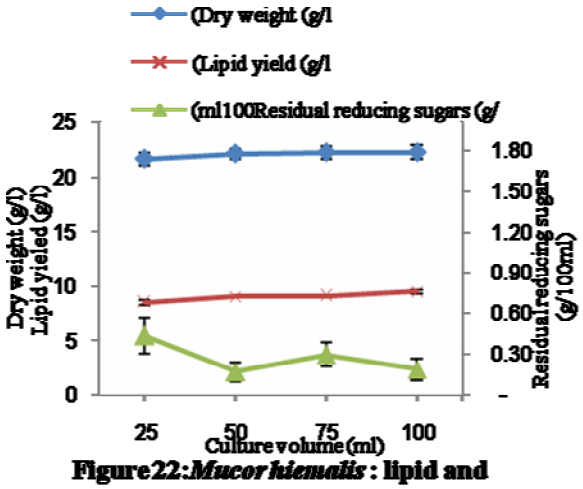

Figure 22: *Mucor hiemalis*: lipid and biomass production as affected by lume

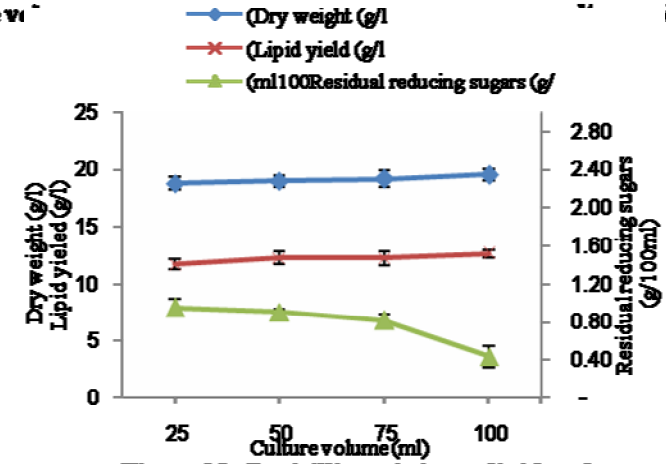

Figure 23: *Penicillium citrinum*: lipid and biomass production as affected culture volume

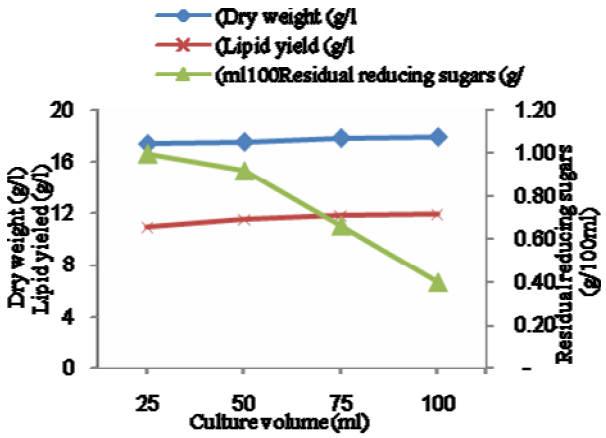

Figure 24: *Aspergillus tamarii*: lipid and biomass production as affected by culture volume

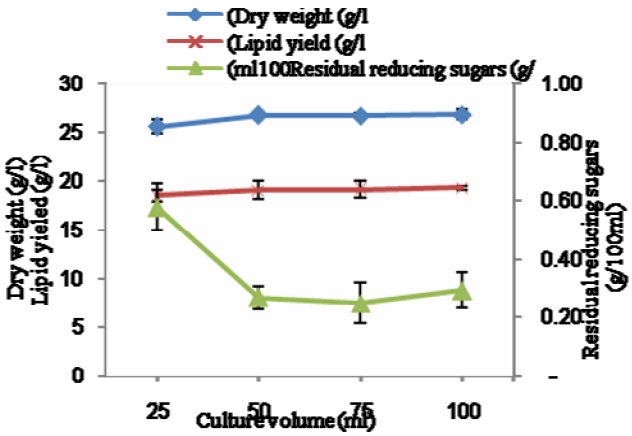

Figure 25: *Aspergillus niger*: lipid and biomass production as affected by culture volume

The design is recommended when more than five factors are under investigation (Abdel-Fattah *et al.*, 2002). The influence of eleven factors including carbon, nitrogen and metal ions on lipid production was tested (Table 1).

The variation in lipid production for *Fusarium oxysporum, Mucor hiemalis, Penicillium citrinum, Aspergillus tamarii* and *Aspergillus niger* was ranging from 7.45±0.57 to 16.42±0.84, from 9.43±0.54 to 15.48±0.65, from 6.55±0.41 to 12.75±0.71, from 6.12±0.35 to 14.02±0.31 and from 7.61±0.19 to 21.71±0.42g/l respectively (Table 1). It was revealed that thes factors under test showed a strong influence on lipid production. Statistical analysis showed that initial glucose and yeast extract concentrations (Figure 26), initial glucose and glycerol concentrations (Figure 27), initial yeast extract and ferric chloride concentrations (Figure 28), initial yeast extract and ammonium chloride concentrations (Figure 29), and initial ammonium chloride and zinc sulfate concentrations (Figure 30) had a significant effect on lipid production by *Fusarium oxysporum, Mucor hiemalis, Penicillium citrinum, Aspergillus tamarii* and *Aspergillus niger respectively*, whereas the other factors affected slightly the lipid production process.

At the end of the optimization experiment, a condition nearer to the optimum one was achieved. Near optimum nutritional conditions for lipid production by *Fusarium oxysporum* are (g/l): (Glucose, 80; yeast extract, 1.5; NH_4Cl, 1.5; KH_2PO_4, 3; $MgSO_4.7H_2O$, 1; $CaCl_2.2H_2O$,0.1; $FeCl_3.6H_2O$, 0.02; Na_2HPO_4, 1.5; Xylose, 5 ; Glycerol, 2; C/N=52.50), by *Mucor hiemalis* (g/l): (Glucose, 80; yeast extract, 1.5; NH_4Cl, 1.5; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; $FeCl_3.6H_2O$, 0.02; Na_2HPO_4, 1.5; Xylose, 10 ; Glycerol, 2; C/N=55.50), by *Penicillium citrinum* (g/l): (Glucose, 40; yeast extract, 1.5; NH_4Cl, 1.5; KH_2PO_4, 3; $MgSO_4.7H_2O$, 1; $CaCl_2.2H_2O$,0.1; $ZnSO_4.7H_2O$, 0.02; $FeCl_3.6H_2O$, 0.02; Na_2HPO_4, 1.5; Xylose, 10 ; Glycerol, 1; C/N=31.00), by *Aspergillus tamarii* (g/l): (Glucose, 80; yeast extract, 1.5; NH_4Cl, 1.5; KH_2PO_4, 3; $MgSO_4.7H_2O$, 1; $CaCl_2.2H_2O$,0.1; $ZnSO_4.7H_2O$, 0.02; $FeCl_3.6H_2O$, 0.02; Na_2HPO_4, 0.5; Xylose, 5 ; Glycerol, 1; C/N=52.00),and by *Aspergillus niger* are (g/l): (Glucose, 80; yeast extract, 1.5; NH_4Cl, 1.5; KH_2PO_4, 3; $MgSO_4.7H_2O$, 0.5; $ZnSO_4.7H_2O$, 0.02; $FeCl_3.6H_2O$, 0.02; Na_2HPO_4, 1.5; Xylose, 5 ; Glycerol, 1; C/N=52.00).

The tested oleaginous fungi can accumulate lipids using glucose, xylose and glycerol as carbon source. An optimally high carbon-to-nitrogen (C:N) ratio is key to allowing cells to reach their maximal lipid storage capacity and lipid yield. Lipid accumulation per liter of culture is usually optimal at molar C:N ratio exceeding 50 and near 100 (Ageitos *et al.*, 2011). The limitation of many elements can induce lipid accumulation but usually is nitrogen limitation which was used for this purpose because it was the most efficient type of limitation for inducing lipid accumulation (Wynn *et al.*, 1999; Nigam 2000; Beopoulos *et al.*, 2009). In nitrogen limited conditions the organisms continues to assimilate the carbon source but the cell proliferation stops as nitrogen is required for the protein and nucleic synthesis (Christophe *et al.*, 2012).

Table.1 Randomized Plackett-Burman experimental design for evaluating factors influencing lipid production by *Fusarium oxysporum*, *Mucor hiemalis*, *Penicillium citrinum*, *Aspergillus tamarii* and *Aspergillus niger*

Variables / Trials	glucose	yeast extract	NH_4Cl	KH_2PO_4	$MgSO_4.7H_2O$	$CaCl_2.2H_2O$	$ZnSO_4.7H_2O$	$FeCl_3.6H_2O$	Na_2HPO_4	xylose	glycerol	Lipid production (g/l)				
												F. oxysporum	*M. hiemalis*	*P. citrinum*	*A. tamarii*	*A. niger*
1	+	-	+	-	-	-	+	+	+	-	+	11.00±0.61	15.48±0.65	7.20±0.54	7.69±0.42	21.71±0.42
2	+	+	-	+	-	-	-	+	+	+	-	14.25±0.54	13.44±0.47	12.95±0.71	9.65±0.60	11.67±0.32
3	-	+	+	-	+	-	-	-	+	+	+	11.38±0.42	13.07±0.81	8.25±0.62	8.59±0.48	12.09±0.48
4	+	-	+	+	-	+	-	-	-	+	+	11.76±0.38	13.19±0.78	8.27±0.52	8.67±0.52	10.39±0.24
5	+	+	-	+	+	-	+	-	-	-	+	11.79±0.47	12.46±0.36	6.93±0.47	9.87±0.64	13.18±0.43
6	+	+	+	-	+	+	-	+	-	-	-	16.42±0.84	11.26±0.72	10.89±0.57	14.02±0.31	15.13±0.88
7	-	+	+	+	-	+	+	-	+	-	-	11.54±0.65	10.12±0.63	12.39±0.64	11.60±0.46	19.30±1.04
8	-	-	+	+	+	-	+	+	-	+	-	8.70±0.47	9.56±0.25	10.76±0.69	9.62±0.52	19.63±0.87
9	-	-	-	+	+	+	-	+	+	-	+	12.71±0.68	13.16±0.48	7.62±0.93	8.03±0.38	9.59±0.60
10	+	-	-	-	+	+	+	-	+	+	-	10.25±0.49	10.52±0.61	8.44±0.50	7.32±0.68	10.80±0.59
11	-	+	-	-	-	+	+	+	-	+	+	14.03±0.91	12.89±0.74	10.43±0.46	10.89±0.71	15.02±0.56
12	-	-	-	-	-	-	-	-	-	-	-	7.45±0.57	9.43±0.54	6.55±0.41	6.12±0.35	7.61±0.19
13	0	0	0	0	0	0	0	0	0	0	0	10.74±0.16	9.56±0.15	12.59±0.36	11.93±0.18	19.34±0.23

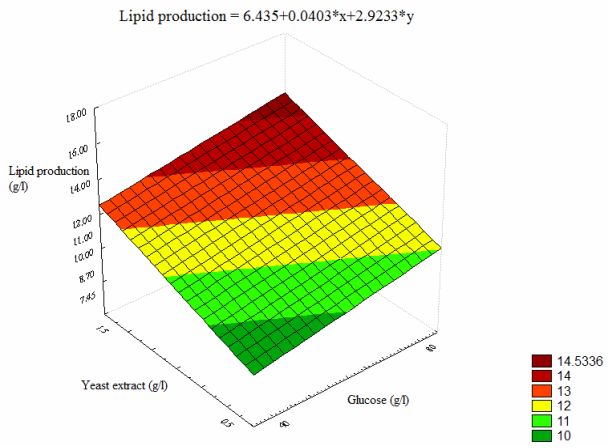

Figure 26: Effect of initial glucose and yeast extract concentrations on lipid production by *F. oxysporum*

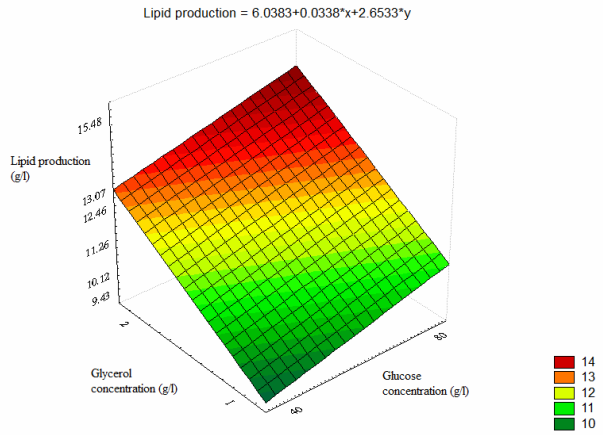

Figure 27: Effect of initial glucose and glycerol concentrations on lipid production by *Mucor hiemalis*

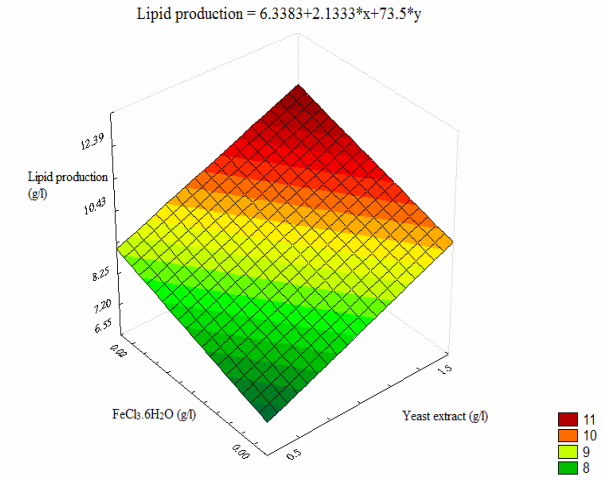

Figure 28: Effect of initial yeast extract and ferric chloride concentrations on lipid production by *Penicillium citrinum*

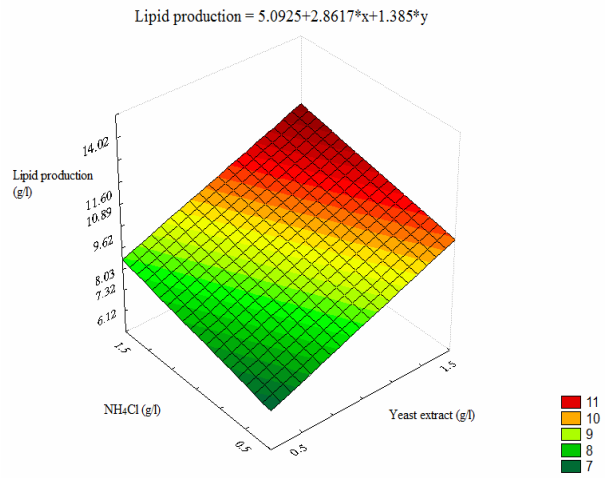

Figure 29: Effect of initial yeast extract and ammonium chloride concentrations on lipid production by *A. tamarii*

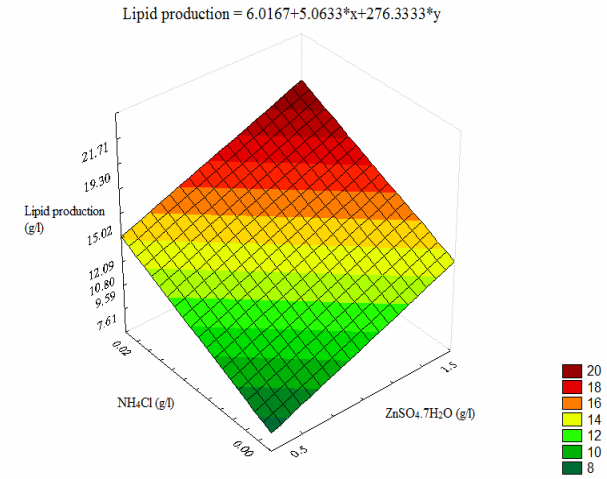

Figure 30: Effect of initial ammonium chloride and zinc sulfate concentrations on lipid production by *A. niger*

Glucose is one of the carbon sources for the growth of all oleaginous microbes, but other monosaccharides, such as fructose, mannose, and galactose, can support growth in certain species, also glycerol and xylose are used as carbon source for lipid accumulation (Jin *et al.,* 2014).

Verification experiments were applied to examine model validation and to evaluate the basal fermentation medium versus the optimized conditions. Verification result revealed 95.13%, 93.54%, 98.46%, 97.57% and 94.01% validity and showed 15.62, 14.48, 12.75, 13.68 and 20.41 g/l lipid production for *Fusarium oxysporum, Mucor hiemalis, Penicillium citrinum, Aspergillus tamarii* and *Aspergillus niger* respectively, which represents about 1.45, 1.51, 1.02, 1.15 and 1.06 fold increase in lipid production respectively, when compared to the control basal medium. The importance of the experiment was not only the optimization of nutritional factors but it proved the ability of tested fungi to grow and accumulate lipids even in the presence of new medium constituents such as xylose and glycerol which leads to an increase in lipid production by *Fusarium oxysporum* and *Mucor hiemalis* whereas for *Penicillium citrinum, Aspergillus tamarii* and *Aspergillus niger,* there is a minor change in lipid production.

Analysis of extracted lipid by thin layer chromatography (TLC)

TLC is a simple and efficient technique for separation of different classes of lipids with good resolution. The Rf values of detected lipids were as follows: monoacylglycerols (0.054-0.091), diacylglycerols (0.107-0.147), free fatty acids (0.200-0.272), triacylglycerols (0.636-0.643), and sterol esters (0.836-0.887). A similar lipid profile for tested oleaginous fungi (Figure 31) was obtained indicating the presence of monoacylglycerols, diacylglycerols, free fatty acids, triacylglycerols, and sterol esters.

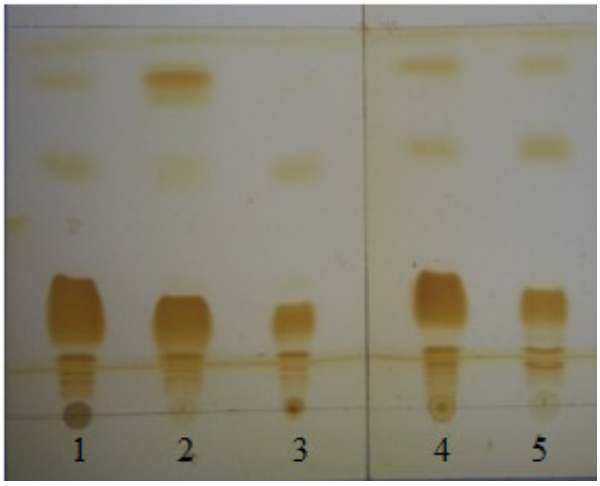

Figure 31: TLC plates: lipid profiles of (1) *Fusarium oxysporum* (2) *Mucor hiemalis*, (3) *Penicillium citrinum*, (4) *Aspergillus tamarii*, (5) *Aspergillus niger*

Determination of fatty acids profile by GC-FID

The fatty acid composition of SCOs was analyzed for all five oleaginous fungi in order to ascertain and compare their potential as biodiesel feedstock.

The lipid profiles are qualitatively similar but differ quantitatively among all five tested fungi (Table 2). All the fungal SCOs in the present study were found to contain a high fraction of saturated, and monounsaturated, mainly of C16 and C18 series, which is considered a potential feature to indicate the fuel quality of fungal based diesel. This composition is quite similar to the commonly used vegetable oil feedstock for biodiesel such as rapeseed, soybean, sunflower and palm (Christophe *et al.,* 2012). In contrast, the fatty acid profiles of oleaginous algae and cyanobacteria show a dominance of C14 and C16 fatty acids with *Chlorella* sp. being rich in C18 (Hu *et*

al., 2008). Among saturated fatty acids, palmitic acid (C16:0) was found to be present with values varying between 18.09% (*A.niger*) and 24.92% (*A. tamarii*) for all the fungi (Table 2).

Low amounts of stearic acid (C18:0) were also observed in the lipid profile of all fungi with the highest content of 11.09% for *A. tamarii* followed by 11.04% for *A. niger*. Venkata and Venkata (2011) reported high contents of stearic acid for *Aspergillus* sp. (48-57%), which is contradictory with our results for the *Aspergillus* sp. Also,Vicente *et al.* (2010) reported low contents of stearic acid in *Mucor circinelloides* (7%) and M*ucor. isabellina* (1%) (Liu and Zhao, 2007). In contrast, high quantities of palmitic acid were detected for all tested fungi ranging from18.09 (*A. niger*) to 22.75 (*M. hiemalis*). Venkata and Venkata (2011) reported low quantities of palmitic acids for *Aspergillus* sp., while it was dominant in *M. circinelloides* (20.7%) (Vicente *et al.,* 2010) and *M. isabellina* (28%) (Liu and Zhao, 2007).

Among monounsaturated fatty acids, oleic acid (C18:1) was shown to be dominant in SCOs of all the five fungal strains with highest content for *A.niger* (35.87 %) followed by the other four strains with a content of 30 - 34% (Table 2). Oleic acid contents have been found to be 0.1-1.6, 28 and 55.5% in lipids of *Aspergillus* sp., *M. circinelloides* and *M. isabellina*, respectively (Venkata and Venkata, 2011; Vicente *et al.,* 2010; Liu and Zhao, 2007).

Fungal SCOs usually differ from most vegetable oils in being rich in PUFAs (poly-unsaturated fatty acids) and hence are mainly exploited for PUFA production. In the present study, the major PUFA member was determined to be linoleic acid (C18:2) with contents ranging from 26.88 (*A. tamari)* to 34.84% (*F.oxysporum)* in the SCOs of all tested fungal strains (Table 2).

The content of linolenic acid (C18:3) was found to be very low (0.04-1.25%) and PUFAs with four double bonds(Arachidic acid) were between 0.25 and 1.02% (Table 2). This is in contrast with the biodiesel obtained from *M. circinelloides* and *A. terreus* MTCC 6324 wherein higher PUFA contents of C18:3 (22.5%) and C15:4, C17:4, C19:4, C32:3, C33:4 (9%), respectively were obtained (Kumar *et al.,* 2010, Vicente *et al.,* 2010).

Biodiesel properties of transesterified fungal SCOs (FAMEs)

The direct measurement of fuel properties of biodiesel is quite complex with high cost, high error in reproducibility and requiring a considerable amount of fuel sample (Liu and Zhao, 2007). Therefore, prediction models and mathematical equations have been developed to predict biodiesel properties from FAME composition (Tong *et al.,* 2011; Demirbas, 1998). In the present study, the different physicochemical biodiesel properties were determined for all five SCOs using these models and/or equations based on FAME profiles and the results are summarized in table 3. Among physical properties of biodiesel, the density values as determined from prediction models were found to be in the range for the five tested fungi as per the standard norms, and therefore, these isolates are considered suitable for biodiesel production based on the fuel density values. The calculated kinematic viscosity values (3.55 – 3.82) at 40°C for all fungal samples (Table 3) were in the range of the biodiesel standard specifications. Iodine value (IV), saponification number (SN) and higher heating value (HHV) are three important chemical properties of biodiesel attributed to the fatty acid profile.

Table.2 FAME composition of fungal oils as analyzed by GC-FID and compared to vegetable oils (Christophe *et al.*, 2012)

Species.	Myristic acid (C14:0)	Palmitic acid (C16:0)	Palmitoleic acid (C16:1)	Stearic acid (C18:0)	Oleic acid (C18:1)	Linoleic acid (C18:2)	Linolenic acid (C18:3)	Arachidic acid (C20 :4)
F.oxysporum	0.01	22.31	0.98	5.98	34.32	34.84	0.04	0.25
M. hiemalis	0.01	22.75	1.03	5.92	34.22	33.28	0.35	0.27
P. citrinum	0.36	20.07	1.73	10.80	29.61	27.58	1.25	0.35
A. tamarii	0.01	24.92	0.96	11.09	29.99	26.88	0.47	0.67
A.niger	0.01	18.09	0.44	11.04	35.87	29.54	0.60	1.02
Oil palm	ND	32-59	ND	1-8	27-52	5.14	traces	ND
Sunflower	ND	3-10	ND	1-10	14-65	20-75	traces	ND
Soya	ND	7-14	ND	1-6	19-30	44-62	4-11	ND
Peanut	ND	6-12.5	ND	2.5-6	37-61	13-41	1	ND
Cottonseed oil	ND	27-28	ND	0.92	13-18	51-57	traces	ND
Soybean oil	ND	11	ND	4	22	53	8	ND
Rapeseed oil	ND	3-14	ND	0.9-2	56-64	22-26	8-10	ND
Corn oil	ND	12	ND	0.9	25	61	0.48	ND

Table.3 Biodiesel properties of the trans-esterified SCOs of oleaginous fungi

property	Fungal strains					US biodiesel standard ASTM D6751*	EU biodiesel standard EN 14214*
	F.oxysporum	*M. hiemalis*	*P. citrinum*	*A. tamarii*	*A. niger*		
Density ($g.cm^{-3}$)	0.86	0.84	0.80	0.85	0.87	NS	0.86-0.90
Kinematic viscosity (40°C; mm^2s^{-1})	3.82	3.75	3.55	3.65	3.77	1.9-6.0	3.5-5.0
SN	196.71	195.03	187.83	194.75	196.41	NS	NS
IV	119.95	117.56	112.00	113.95	118.07	NS	120 max
HHV ($MJ\ kg^{-1}$)	39.57	39.67	40.05	39.74	39.61	NS	NS
CN	52.44	53.90	54.11	52.00	51.76	47-65	51 min
Concentration of linolenic acid (C18:3)	0.04	0.35	1.25	0.47	0.60	NS	12 max
FAME having ≥4 double bonds (%)	0.25	0.27	0.35	0.67	1.02	NS	1 max

*(Leung *et al.*, 2010).

SN: saponification number, IV: iodine value, HHV: higher heating value, CN: cetane number, FAME: fatty acid methyl ester. NS: not specified, max: maximum, min: minimum.

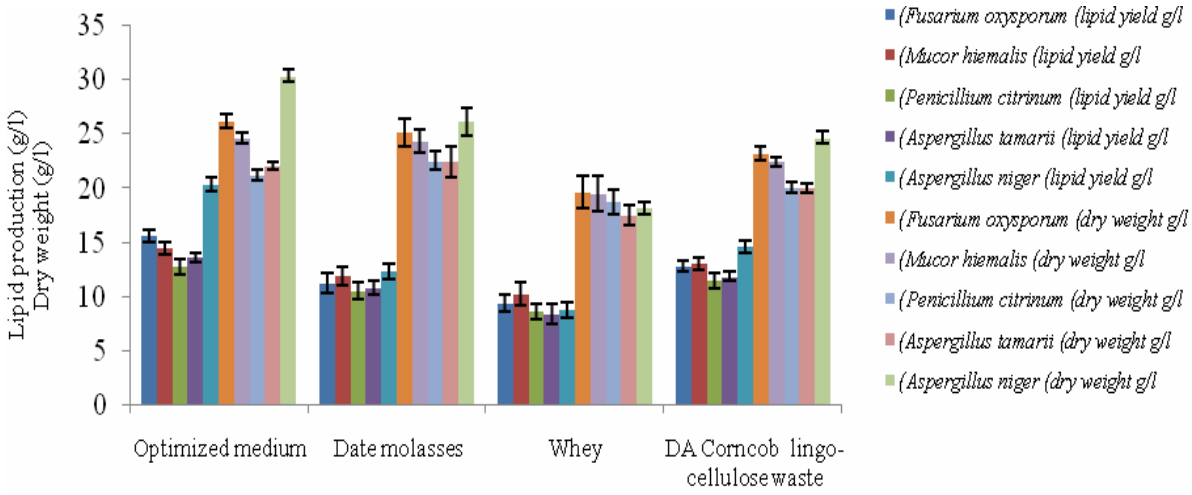

Figure 32:single cell oils and biomass production of oleaginous fungi using optimized medium and agro-industrial waste products

The IV is a crude measure of degree of unsaturation of the biodiesel and is often used in connection with its oxidative stability. The SN indicates the amount of TAG present in total lipid and HHV depends upon both IV and SN. Therefore, in the present study the SN and IV were calculated empirically from fatty ester composition of transesterified total lipids. The predicted IVs were below the EN14214 specification (120 maximum) and suggest good oxidative stability of the transesterified oils from the tested fungal isolates. The calculated SNs were found to be comparable for all the isolates while HHVs of approximately 40 MJ kg^{-1} were similar to methyl esters of vegetable oils (Leung *et al.*, 2010).

For biodiesel, CN has been found to increase with an increasing weight percentage of saturated and long chain fatty ester. In fact, methyl esters of stearic acid (C18:0), which is of relevance to biodiesel, have been found to possess the highest CN (> 80) (Knothe, 2005; Knothe *et al.*, 2005). In the present study, the calculated CNs for all fungal isolates were found to be between 52-54 (Table 3), and comparable with the predicted values reported for biodiesel obtained from other oleaginous fungi (Liu and Zhao, 2007).These values are in the acceptable range of international biodiesel standard norms indicating their suitability as a fuel.

Single cell oil production using agro-industrial waste products

Some agroindustrial waste products as cheap carbon sources were tested for their ability to produce biodiesel, data in figure 32 revealed that the highest lipid and biomass production were obtained by using dilute acid pretreated corncob lignocellulosic wastes when compared to optimized medium leading to 12.84±0.54, 13.08±0.54, 11.55±0.71, 11.88±0.41 and 14.61±0.62 g/l lipid production by *Fusarium oxysporum*, *Mucor hiemalis*, *Penicillium citrinum*, *Aspergillus tamarii* and *Aspergillus niger* respectively**,** whereas lipid production obtained with the oleaginous fungi when grown on whey and date molasses was ranged from 8.42 to 10.24 g/l, and from 10.52 to 12.34 g/l respectively. Makri *et al.* (2010), André *et al.* (2010) Fakas *et al.*

(2009) reported 22.3, 39, 53% lipid content of *Rhodotorula glacialis, Aspergillus niger and Cunninghamella echinulata* respectively, when grown on glycerol as a substrate, also Zhu *et al.* (2008) have reported the production of lipids by *Trichosporon fermentans* with lipid content of 63, 58, and 37% when cultivated on sucrose, xylose and molasses respectively.

Also, data revealed that the lipid content was ranged from 44.75 to 55.32% for *F. oxysporum,* from 48.81 to 58.21% for *M. hiemalis,* from 46.69 to 57.41% for *P. citrinum,* from 48.38 to 59.16 for *A. tamarii* and from 47.21 to 59.25% for *A.niger* when grown on date molasses and diluted acid pretreated corncob lignocellulosic material which indicate that the present study constitute a strong basis for large-scale industrial production of single cell oils using the tested oleaginous fungi grown on agro-industrial waste products.

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