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**Research Article**

# SINGLE CELL OIL PRODUCTION BYALTERNARIA SP. USING MOLASSES AS ASUBSTRATE

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**ABSTRACT**

This study investigate the effect of various parameters on biomass and oil production of the isolated fungus Alternaria sp., also study the feasibility of using molasses as an alternative carbon source for oil production. Thus, eight independent variables (sucrose, yeast extract, KH2PO4, MgSO4.7H2O, KCl, FeSO4.7H2O, PH, Incubation days) were studied using the Plackett-Burman statistical design technique. Data revealed that sucrose, yeast extract and incubation days were found to be critical factors affecting lipid production. Therefore, they were optimized using a response surface methodology. The optimum conditions for this fungus were in g/l: (sucrose, 35, yeast extract, 2.33, KH2PO4 2, MgSO4.7H2O 0.25, KCL 0.25, FeSO4.7H2O 0.02) pH 6.5, 30 °C, static conditions and incubation period of 160 hours. Under optimized conditions, the fungus gave the maximum biomass of 11.8 g/l, lipid yield of 5.94 g/l and lipid content of 50.3 %, respectively. Fatty acid characterization of the produced oil by gas chromatography revealed that it contains 40.31, 26.11 and 33.58 % of saturated, monounsaturated and polyunsaturated fatty acids, respectively, with linoleic acid as the most dominant fatty acid. Molasses was utilized as a carbon source and it was supports both growth and lipid production with biomass of 19.3 g/l and lipid yield of 5.6 g/l. Its oil profile was containing 41.53, 33.14, 25.33 % of saturated, monounsaturated and poly unsaturated fatty acids. Consequently, this study provide new insights for the economical single cell oil (SCO) production with valuable profile using agro industrial waste as a substrate for growth for Alternaria sp.



## INTRODUCTION

It is known that lipids are occurring naturally in the microbial system as a component of the cell membrane in the range of 6–8% of the cell dry biomass (Schulze *et al.*, 2014). A small percentage of microorganisms have the ability to synthesize and accumulate 20- 87% of their total biomass as intracellular lipids, those organisms are defined as oleaginous microorganisms (Calvey *et al*., 2016; Bharathiraja *et al*., 2017). The production of microbial oil has many advantages compared with vegetable oils as: the cultivation of microorganisms does not require huge space and can be produced in a much shorter time and less affected by environmental factors (Li *et al*., 2008, Amaretti *et al*., 2010, Thiru *et al*., 2011). Moreover, depending on the fatty acid composition, the oil produced can be exploited for human consumption and in certain valuable industrial applications (paints and coatings, detergents, cleaning products and cosmetics) and as a potential candidate for biodiesel production because its fatty acid profile has a great similarity to vegetable oils (Xu and Liu, 2017).

Recently, the tremendous attention for SCO production is directed to oleaginous filamentous fungi, which can compete at commercial scale with other lipid producers. They exhibit fast growth rate, accumulate some value added fatty which show potential for commercialization, their ability to grow well in the bioreactors, easier to scale up and their ability to consume inexpensive renewable and agro-industrial wastes as a substrates for growth (Yehia *et al*., 2017). Actually, the amount and type of lipid produced is highly affected with fungal species, nutritional requirements and culture conditions (Akpinar-Bayizit, 2014), so optimization of these conditions is

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an essential step as it result in higher lipid production under economical cost for further industrialization (Jiru *et al*., 2017). The traditional approach for optimization deal with one factor at time while keeping the others constant has many disadvantages such as a time consuming process, boring and does not study the interaction amongst the studied factors (Song *et al*., 2007). To overcome these disadvantages, statistical methods was used to provide an efficient way of screening a large number of variables at the same time. The models developed also indicate the interactions among the selected variables, one of these is Plackett–Burman design (PBD), which is a good tool to determine the significance of large number of factors and give certain information about each factor in one experiment. So this minimizes the costs of production of many processes (Srinivas *et al.,* 1994). Another statistical method is response surface methodology (RSM), which optimizes the levels of the key factors in the cultivation process (Liu *et al*., 2003; Francis *et al.,* 2003).

Although lipid production by oleaginous fungi provides a significant alternative method, the obstacle for the broader commercialization is the high production cost. The carbon sources used accounted 75% of the total costs (Andrea *et al*., 2010), so exploring other alternative carbon sources such as agricultural and industrial wastes is very important to reduce the costs of the oil produced (Liang and Jiang, 2013). One of these wastes is molasses, which is a by-product of sugar industry, consisting of up to 50% (w/w) total sugars, minerals, vitamins and other components (Najafpour and Shan, 2003). It has been developed as a cheap carbon source for production of numerous industrially important chemicals, such as lactic acid (Kotzamanidis, 2002), citric acid (wang *et al*., 2000), polysaccharide (Roukas, 1998), microalgal oils (Yan *et al*., 2011), and astaxanthin (Liu *et al*., 2012)

Therefore, based on the above-mentioned findings, the objectives of the present study were; isolation and identification of a fungal isolate capable of yielding high amount of valuable storage lipids, optimization of cultivation conditions for maximum lipid accumulation and test its ability to utilize molasses as a cheap carbon source.

## MATERIALS AND METHODS

### *Soil Sampling*

Two soil samples from a depth of 5-15 cm below the surface were collected from different sites of Quesna(El-Menoufiagovernorate). Directly, samples were sealed in sterile sampling polyethylene bags, and stored at 4°C until use.

### *Isolation and purification of fungi*

One gram of each soil sample was individually suspended in 1 mL of sterile distilled water, serially diluted 10-fold and plated on potato dextrose agar (PDA) medium, then the plates were incubated at 30°C for 5 days until pure cultures were obtained. Pure cultures were grown on PDA slants and stored at 4°C.

### *Screening for oleaginous fungi*

All fungal strains were screened for lipid accumulation by Nile red staining assay (Kimura *et al*., 2004). Directly, fungal biomass were incubated in dark with 0.5 mL of 0.1 mM phosphate buffer saline (PBS) pH 7.4 and 0.05 mL Nile-red solution (25 µg/mL in acetone). After 30 min; a thin film was prepared on a clean glass slide and retained for air-drying. Examinations performed using the fluorescence microscopy (Olympus BX 40 microscope).

### *Selection of lipid-producing fungi*

Fungal isolates with the strongest fluorescence signals were grown on aliquot of 50 mL Czapek- Dox's medium in 250 mL Erlenmeyer flasks in triplicates. The pH was adjusted to 6 using 1.0 M (HCl or NaOH) before autoclaving at 121°C for 20 min. After autoclaving and cooling each flask was inoculated with a disk aseptically removed from the actively growing outer edge of the 5 days aged mycelium on agar PDA plates. Flasks were incubated at 30ºC for 7 days under static conditions. After incubation period dry biomass, lipid yield and lipid content were determined. The isolate with the highest lipid content were selected for further studies.

### *Determination of dry biomass*

To determine biomass, at the end of the incubation period of each experiment the mycelia mats (in triplicate) of culture broth was harvested by simple filtration method using Whatman No.1 filter paper and washed 3 times with distilled water followed by drying at $60^0$C till constant weight. Dry biomass weight was determined gravimetrically, expressed in g/l according to Devi *et al*., (2009) and was crushed into fine powder and preserved in desiccators until use.

### *Determination of lipid yield and Lipid content*

Lipid yield was measured using phosphovanillin method described by (Helal *et al*., 2006). The standard calibration curve was performed using canola oil as a standard. Total lipid yield (the amount of lipid extracted from the biomass per liter of fermentation medium) and lipid content (the percentage of lipid to dry biomass) were determined.

### *Identification of the selected isolate*

Both morphological (Luttrell, 1977) and molecular identification of the selected fungal isolate was performed. Molecular identification was done based on PCR amplification of the 18S rDNA gene. Fungus was allowed to grow for 3 days and genomic DNA was prepared using AMSHAG-DNA Extraction Kit (Elrashdy and Abd-El-Haleem, 2005). Then 0.1 µg genomic DNA was used as a template for PCR reaction and the primers used for the amplification of 18S-rDNA encoding genes were those described by (Suh and Nakase, 1995). The PCR was run on Thermo PCR machine. Thereafter, 1% agarose gel containing Ethidium bromide was used to separate the PCR fragment by electrophoresis. Gel was run at 100 V in 1X TBE buffer and then visualized using the Multi Image light cabinet gel documentation. PCR product was purified using kit (Elrashdy and Abd-El-Haleem, 2005) and sequenced using 373 API DNA sequencer. The sequences were analyzed using the BLAST program (National Centre for Biotechnology Information) to find out the homology with the existing species. Confirmed sequence was submitted to the GenBank.

### *Culture media and optimization of culture conditions for biomass production and lipid accumulation*

Cultivation of the fungus was first performed on the basic Czapek-Dox's medium (in g/L: Sucrose 30, $NaNO_3$ 2, $KH_2PO_4$ 1, $MgSO_4.7H_2O$ 0.5, KCL 0.5, $FeSO_4.7H_2O$ 0.01) the pH was

adjusted to 6 using 1.0 M (HCl or NaOH )before autoclaving at 121°C for 20 min.

***Optimization of one variable at time (OVAT)***

The influence of temperature (10, 20, 30, 40 and 50°C), carbon source (sucrose, glucose, lactose, glycerol, starch and carboxy methyl cellulose (CMC)) at 30 g/l in the basal medium, nitrogen source (sodium nitrate, ammonium chloride, urea, yeast extract, peptone and glutamic acid) were incorporated at 2 g/l separately in the basal medium, different concentrations of C/N ratio (30:1, 30:2, 30:4, 30:6, 15:2, 60:2, 60:4), static and shaking conditions (150 rpm) were studied. Aliquot of 50 mL medium in 250 mL Erlenmeyer flasks was prepared in triplicate for each experiment. After autoclaving and cooling each flask was inoculated with a disk from the margin of 5 days aged cells on agar PDA solidified medium in petri dishes and incubated at 30ºC for 7 days under static conditions except for the experiment of shaking at 150 rpm. Dry biomass, lipid yield and lipid content were determined as described above

***Statistical experimental design***

***Plackett-Burman design (PBD)***

Eight independent variables (sucrose, yeast extract, $KH_2PO_4$, $MgSO_4.7H_2O$, KCl, $FeSO_4.7H_2O$, PH, incubation days) were screened in twelve combinations organized according to the Plackett-Burman design matrix (Table1). Each independent variable was set at two levels: a high (+1) and low (-1) level, the low and high values of each variable are presented in (Table 2). Along with each experiment biomass, lipid yield and lipid content were determined. The lipid content was considered as the response for each trial.

Plackett–Burman experimental design is based on the first order model (Eq.1):

$$Y = \beta_O + \Sigma\beta i X i \quad (1)$$

Where, Y is the response or dependent variable (lipid content); it will always be the variable we aim to predict, $\beta_o$ is the model intercept and βi is the linear coefficient, and Xi is the level of the independent variable. From the statistical analysis, the main effect was used to elucidate the significance of variables depending on their nature; positive or negative effect on the production process.

**Table 2** The coded and actual values of experimental variables at different levels

| Variables | Unite | Coded levels Experimental values | | |
|---|---|---|---|---|
| | | -1 | 0 | +1 |
| Sucrose | g/L | 15 | 30 | 45 |
| yeast extract | g/L | 0.5 | 1 | 1.5 |
| $KH_2PO_4$ | g/L | 0.5 | 1 | 2 |
| $MgSO_4.7H_2O$ | g/L | 0.25 | 0.5 | 1 |
| KCl | g/L | 0.25 | 0.5 | 1 |
| $FeSO_4.7H_2O$ | g/L | 0.005 | 0.01 | 0.02 |
| PH | - | 4.5 | 5.5 | 6.5 |
| Incubation days | - | 4 | 7 | 10 |

**Central composite design (CCD)**

RSM was employed to optimize the concentrations of the most effective parameters selected by Packett-Burman design using a (CCD). Three parameters were studied independently yeast extract, sucrose and incubation days. Each variable was studied at five different levels (-1.681, -1, 0, +1, +1.681) and the other variables in the study were maintained at a constant level which gave maximal yield in the PB experiments. A set of 20 experiments were employed as indicated in (Table 3) and the minimum and maximum ranges of variables were investigated (Table 4). Along with each experiment biomass, lipid yield and lipid content were determined and lipid content was considered as the response for each trial.

For statistical calculation, the relationship between the coded and actual values is described by Eq.2:

$$Xi=Ui-Ui_0/\Delta Ui \quad (2)$$

Where *Xi* is the coded value of the *i*th variable, *Ui*is the actual value of the *i*th variable, $Ui_0$ is the actual value of the *i*th variable at the center point and ΔUi is the step change of variable. The response variable (lipid content) suitable to a quadratic equation for the variables was as Eq.3:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 \quad (3)$$

Where: *Y* is the predicted response; $X_1$, $X_2$, $X_3$ are input variables which influence the response variable Y*;* $\beta_0$, intercept; $\beta_1$, $\beta_2$ and $\beta_3$ linear coefficients; $\beta_{11}$, $\beta_{22}$ and $\beta_{33}$, squared or quadratic coefficients $\beta_{12}$, $\beta_{13}$, and $\beta_{23}$ interaction coefficients**.**

**Table 1** Plackette-Burman design matrix for screening of critical factors influencing lipid production by *Alternaria sp.*

| Variables / Trials | Sucrose | Yeast extract | $KH_2PO_4$ | $Mg_2SO_4$ | KCl | $FeSO_4.7H_2O$ | pH | Incubation days | Experimental Lipid content% | Predicted Lipid content% |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | 7.62 | 7.52 |
| 2 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 8.82 | 10.31 |
| 3 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | 23.80 | 23.30 |
| 4 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | -1 | 6.55 | 5.47 |
| 5 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 1 | 14.86 | 13.78 |
| 6 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 9.54 | 10.62 |
| 7 | 1 | -1 | -1 | -1 | 1 | 1 | 1 | -1 | 1.87 | 1.96 |
| 8 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | -1 | 17.46 | 17.97 |
| 9 | 1 | -1 | 1 | 1 | -1 | 1 | -1 | -1 | 3.81 | 3.30 |
| 10 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 1 | 6.32 | 7.81 |
| 11 | -1 | -1 | 1 | 1 | 1 | -1 | 1 | 1 | 10.99 | 9.50 |
| 12 | 1 | 1 | -1 | 1 | -1 | -1 | -1 | 1 | 14.09 | 14.19 |

**Table 3** Experimental range and levels of the most effective variables used in CCD

| Variable | Coded levels Experimental values | | | | |
|---|---|---|---|---|---|
| | -1.681 | -1 | 0 | 1 | 1.681 |
| Yeast Extract g/L | 0.75 | 1 | 1.5 | 2 | 2.5 |
| Sucrose g/L | 5 | 10 | 15 | 25 | 35 |
| Incubation days | 6 | 8 | 10 | 12 | 14 |

**Table 4** Central composite design of the significant factors for lipid production by *Alternaria sp*

| Run order | Sucrose (A) | Yeast extract (B) | Incubation days (C) | Lipid content % | |
|---|---|---|---|---|---|
| | | | | Experimental | Predicted |
| 1 | 1 | 1 | -1 | 51.66 | 49.72 |
| 2 | 0 | 0 | 0 | 44.78 | 43.01 |
| 3 | 0 | -1.68179 | 0 | 29.81 | 31.23 |
| 4 | 0 | 0 | -1.68179 | 33.96 | 37.10 |
| 5 | -1 | 1 | -1 | 26.66 | 25.94 |
| 6 | -1 | -1 | 1 | 28.3 | 29.02 |
| 7 | 0 | 0 | 0 | 42.57 | 43.01 |
| 8 | -1 | 1 | 1 | 30.44 | 31.94 |
| 9 | 0 | 0 | 0 | 42.78 | 43.01 |
| 10 | -1.68179 | 0 | 0 | 23.7 | 22.74 |
| 11 | 1.68179 | 0 | 0 | 41.34 | 44.04 |
| 12 | 0 | 0 | 0 | 41.73 | 43.01 |
| 13 | 0 | 1.68179 | 0 | 41.84 | 42.15 |
| 14 | 0 | 0 | 1.68179 | 35.92 | 34.51 |
| 15 | 1 | -1 | -1 | 42.38 | 39.65 |
| 16 | 0 | 0 | 0 | 43.92 | 43.01 |
| 17 | 0 | 0 | 0 | 42.57 | 43.01 |
| 18 | 1 | -1 | 1 | 31.07 | 30.57 |
| 19 | -1 | -1 | -1 | 27.95 | 27.08 |
| 20 | 1 | 1 | 1 | 45.05 | 44.70 |

***Statistical analysis***

All experiments were conducted in triplicate. The experimental results obtained were expressed as means of standard deviation. Experimental matrix and statistical analysis of data of PBD and CCD, subsequent regression analysis, ANOVA, 3D surface plots - 2D contour plots and optimizer were performed using Minitab 15 (Minitab Inc., Pennsylvania, qand USA). The data obtained on lipid yield was subjected for Analysis of Variance (ANOVA) appropriate to the design of the experiments.

***Lipid production using low cost substrates***

At the same concentration, the carbon source in the optimized media was replaced by molasses. Then the media was autoclaved, the fungus was inoculated in the production medium and was kept in shaker at 150 rpm.

***Lipid Extraction***

Extraction of lipids was performed according to the method of Bligh and Dyer, (1959). The dry biomass was grounded with mixture of chloroform: methanol (2:1) and agitated for 20 min at 200 rpm at room temperature. Centrifuge at 6000 rpm for 10 min to recover solvent phase. The same process was repeated two times. The solvent was evaporated and samples were dried under vacuum, and then the amount of oil is recorded using gravimetric method.

***Tranesterification of extracted lipid***

Fungal lipid obtained was mixed vigorously with 20 ml of methanol and 2ml of concentrated sulphuric acid for 2 hours at 70ºC. After the completion of reaction the mixture was allowed to cool at room temperature and then transferred to the separating funnel for obtaining two layers containing upper methyl ester layer and lower glycerol layer. The methyl ester was collected and analyzed using GC-MS. It was performed with Agilent 6890N Gas Chromatograph connected to Agilent 5973 Mass Spectrometer at 70 eV (m/z 50–550; source at 230 °C and quadruple at 150 °C) in the EI mode with an HP-5ms capillary column (30 m ´ 0.25 mm i.d., 0.25 mm film thickness; J & W Scientific, USA). The carrier gas, helium, was maintained at a flow rate of 1.0 mL/min. The inlet temperature was maintained at 300 °C and the oven was programmed for 2 min at 150 °C, then increased to 300 °C at 4 °C/min, and maintained for 20 min at 300 °C. The injection volume was 1 mL, with a split ratio of 50:1. Structural assignments were based on interpretation of mass spectrometric fragmentation and confirmed by comparison of retention times as well as fragmentation pattern of authentic compounds and the spectral data obtained from the Wiley and NIST libraries.

## RESULTS AND DISCUSSION

***Isolation and Screening of oleaginous filamentous fungi***

Twenty six fungal isolates were obtained from the two collected soil samples. These isolates were tested for lipid accumulation using Nile red staining assay. After Nile red staining, four fungal isolates designated as F1, F2, F3 and F4 exhibited strong fluorescence under fluorescent microscope, so these isolates were selected for further studies. It was cleared that Nile red seems preferable for intracellular lipid determination; it was able to recognize lipid-producing and non-producing organisms, as it reacts only with hydrophobic compounds like lipids and emits strongly positive red fluorescence signals which can be detected by fluorescence spectroscopy. The fluorescence intensity of stained cells under UV light depends on the lipid concentration. Microorganisms that do not accumulate lipids are only lightly fluorescent because they contain almost no lipid granules (Dawes, 1990).

***Selection of the isolate with the highest lipid content***

To select the best isolates, lipid contents of the selected four isolates were determined (Table 5). Obviously, it is cleared that isolate F2 was exhibited the highest lipid content (22.5%). Consequently, this isolate was selected for further studies.

**Table 5** Biomass, lipid yield and lipid content of the isolates with the strong red fluorescence signals cultivated on Czapek- Dox's medium for 7 days of incubation.

| No. | Isolate name | Biomass (g/l) | Lipid yield (g/l) | Lipid content %) |
|---|---|---|---|---|
| 1 | F 1 | 9.4 | 1.8 | 19.1 |
| 2 | F 2 | 10.6 | 2.39 | 22.5 |
| 3 | F 3 | 9 | 1.8 | 20 |
| 4 | F 4 | 10.3 | 1.9 | 18.4 |

***Identification of selected isolate***

***Morphological identification***

The colony growth of the fungus on PDA was observed as white-greyish airy mycelium with clear light to dark green inner zones emanating from a common center at the initial stage of the fungal growth. The color of the mycelium changes to dark brown, green at the later part, surrounded by light grey mycelium at the margin in the Petri dish. Microscopic analysis

of the conidia and conidiophore were carried out at 40x resolution, they were typically ovoid in shape. The conidia were slightly constricted with transverse and longitudinal septa (Figure 1). Based on these characters, the oil producing isolate F2 was identified as *Alternaria sp.*

**Figure1** Microscopic features of isolate F2

### *Molecular identification*

Based on the sequence analysis of 18S rDNA gene of isolate F2 in compare to relative sequences present in the GenBank, isolate F2 was closely related to *Alternariasp.* and this confirmed the microscopic features identification. Therefore, 18S rDNA sequence of isolate F2 was submitted to the GenBank and gain accession number MG582185. Isolate F2 named and desined as *Alternariasp.* strain GH1.

### *Effect of various parameters on biomass production and lipid yield*

### *Choosing between shaking and static incubations*

A comparison between shaking and static culture incubations showed that biomass, lipid yield and lipid content were higher under static condition than shaking condition (Figure 2). Similar results were reported by Kirrolia *et al*., (2012); Ali and El-ghonemy, (2014) and Ali *et al*., (2017). They found that static condition gave lipid accumulation higher than the shaking one. This result might be due to the aeration level, which has a great effect on the fungal growth and its ability to accumulate lipids.

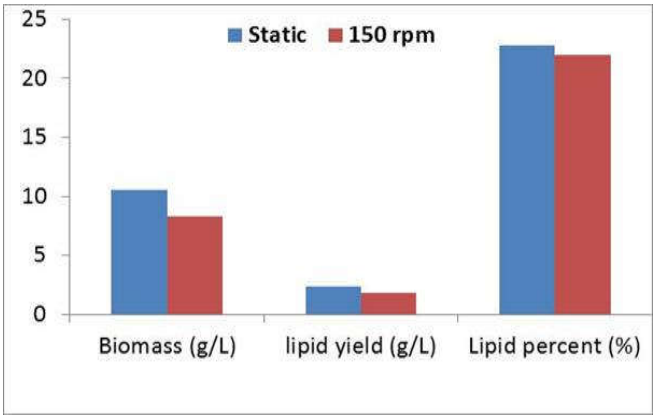


**Figure 2** Effect of shaking and static conditions on biomass, lipid yield and lipid content of *Alternariasp*GH1, respectively.

### *Effect of temperature*

Figure (3) represents the effect of different temperatures on biomass and lipid yield of *Alternariasp.,* indicating that there is no growth at 10°C and 40°C. On the other hand, at a temperature of 30°C, it produces the maximum biomass (12.02 ± 0.04 g/l), lipid yield (2.73 ± 0.08 g/l) and lipid content (22.7%). So, this temperature was selected as the optimum temperature for further studies. In agreement with this result Carlile *et al.,* (2001)reported that 30°C is optimum for maximum biomass and lipid content as the activity of all fungal enzymes was high at this temperature range. In addition, Farooq *et al*., (2005) studied the impact of temperature on *F. oxysporium* and he found that 30°C was the optimum temperature and fungal growth drastically reduced below 15°C and above 35°C. Referring to that, temperature is an important environmental parameter affects all living organisms by controlling the growth rate and was reported as an important factor in regulating fatty acid composition in fungi (Kendrick and Ratledge, 1992; Weinstein *et al*., 2000;Mamatha, 2009).

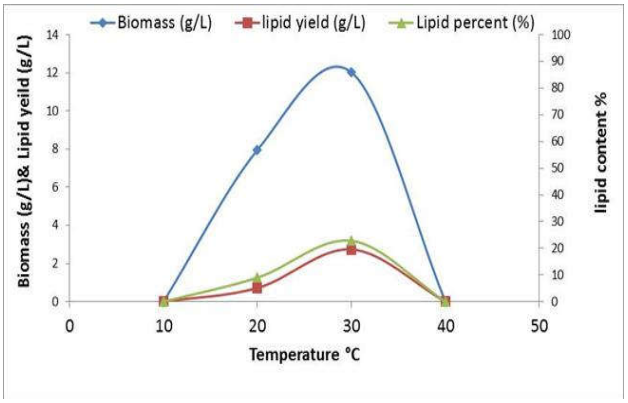


**Figure 3** Effect of different temperatures on biomass, lipid yield, and lipid content of *Alternaria sp.*

### *Effect of different carbon sources*

All carbon sources tested were found to support both growth and lipid accumulation of fungus with different levels. As shown in Figure (4), the highest biomass (12.09 ± 0.05 g/l), lipid yield (2.80 ± 0.02 g/l) and lipid content 23.15 % were recorded when *Alternaria* was grow in the presence of sucrose, followed by glucose and lactose with lipid yield 2.32 ± 0.06 g/l and 2.09 ± 0.04 g/l, respectively. On the other hand, CMC had the lowest biomass (6.41±0.01 g/l) and lipid yield (0.54 ± 0.007 g/l). According to these results sucrose was the most better carbon source for lipid production by *Alternaria sp.*. This is in agreement with the previous report by Papanikolaou *et al*., (2010)who reported that *Thamnidiumelegans* gave the maximum concentration of total lipid content 9.3 g/l when sucrose was used as a carbon source compared with glucose and fructose with lipid content 9.2 and 8.8 g/l, respectively.

Carbon is considered one of the most important essential elements required by the living organisms, the utilization of various carbon sources is mainly dependent upon enzyme system of the fungus and its ability to utilize certain simpler forms or its power to convert the complex carbon compounds into simpler one, which may be easily utilized (Ramjegathesh and Ebenezar, 2012).

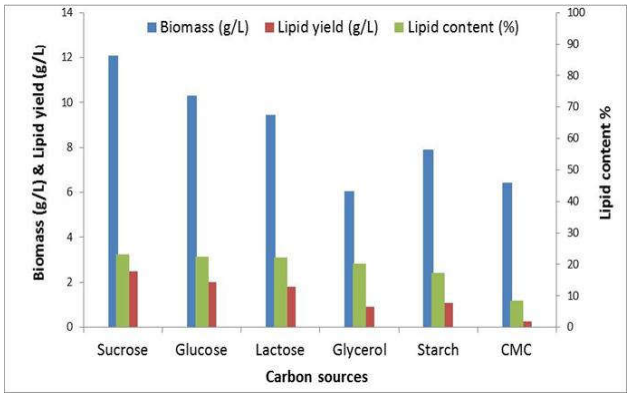


**Figure 4** Effect of different carbon sources on biomass, lipid yield and lipid content of *Alternariasp.*

### *Effect of different nitrogen sources*

Different levels of cell biomass and lipids were obtained by using different organic and inorganic nitrogen sources as represented in Figure (5). Results showed that urea was a poor nitrogen source with lowest cell biomass and lipid yield. Yeast extract was found to be the most suitable nitrogen source with biomass (12.12± 0.02 g/l), lipid yield (2.91± 0.02 g/l) and lipid content 24%. Recently, Gao *et al*., 2013 found that *M. isabellina* gave the maximum lipid yield 64.2% using media reach in yeast extractas a nitrogen source and xylose as a carbon source. Yeast extract is preferable nitrogen source for lipid accumulation by various microorganisms because it contains all metal ions and micronutrients required (Dyal *et al*., 2005). In addition, Ratledge and Wynn, (2002) mentioned that yeast extract is rich with glutamate and this lead to increase lipid production in *R. toruloides*.

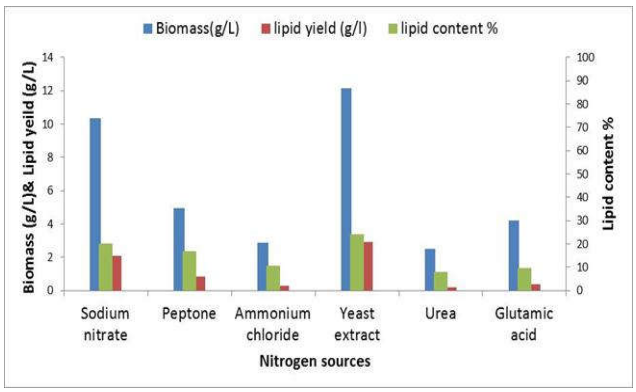


**Figure 5** Effect of different nitrogen sources on biomass, lipid yield and lipid content of *Alternariasp.*

### *Effect of C/N ratio*

Among the tested factors, C/N ratio was found to have obvious effect on growth and lipid accumulation as indicated in Figure (6). It is cleared that at low C: N ratio (15:2) a decrease in biomass and lipid yield was observed. As the low concentration of sucrose was got exhausted at preliminary stage of growth, then sucrose concentration in the growth medium become insufficient which lead to a decrease in growth rate compared with control and disability of the organism to accumulate lipid granules (Subhash and Mohan, 2014).Where, at (30:1) an increase in biomass was determined and lipid yield reached its maximum level (2.29 ± 0.02 g/l). This may be attributed to depletion of the low concentration of nitrogen (1g) from the medium for fungal growth, while sucrose is still exists at this time excess carbon was directed towards lipid formation due to nitrogen deficiency (Mandal and Mallick, 2009; Welter *et al*., 2013).

Increase in biomass and decrease in lipid yield was observed by increasing yeast extract concentration while keeping sucrose constant (30:2), as high concentration of nitrogen source in the presence of carbon source stimulate the cell to produce biomass and this pattern was also observed at (30:4 & 30:6) (Subhash and Mohan, 2014). At 60:2 maximum biomass was determined (19.45 g/l ± 0.02),while lipid yield was decreased this may be as a result of utilizing this high concentration of carbon and nitrogen for biomass production over days of incubation and the organism may need longer time for lipid production (Subhash and Mohan, 2014).

A decrease in biomass and lipid accumulation was observed at high C:N ratio ( 80:2 & 100:2), this can be explained by the previous findings which mentioned that high substrate concentrations have an inhibitory effects on the growth and lipid accumulation of oleaginous microorganisms (Gao *et al*., 2013).

The C/N ratio in the growth medium plays a pivotal role for both fungal growth and lipid accumulation. For lipid production by oleaginous microorganisms, there must be nutrient imbalance in the culture medium and specifically the C/N ratio has to be high (greater than 20) (Beopoulos *et al*., 2009; Papanikolaou and Aggelis, 2011).

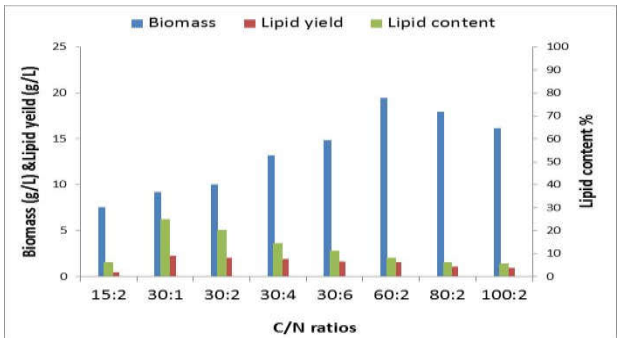


**Figure 6** Effect of different carbon and nitrogen ratios on biomass, lipid yield and lipid content of *Alternariasp.*

### *Screening of significant variables using Plackett-Burman design*

The results represented in Table 1 of the PBD for the eight tested variables revealed that there is a variation in lipid content for *Alternaria* ranging from 1.87 to 23.80 %, which represent 0.13 and 2.8 g/l, respectively. This variation indicates that these factors under test showed a strong influence on lipid accumulation. The regression analysis for the PBD indicates the coefficient values for each parameter (Table 6), where, the significance of each coefficient is checked from P values (probability of error value). The P-values are used as a tool to check the significance of each coefficient, which in turn may indicate the pattern of the interaction between the coefficients (Liu *et al*., 2003). The smaller the value of P (less than 0.05), the more significant is the corresponding coefficient (Manimekalai and Swaminathan, 1999; Prakash and Srivastava; 2005). Accordingly, sucrose, yeast extract and

incubation days played significant role on the model response (lipid content). Goodness of model can be checked from values of coefficient R-Sq and adjusted R-Sq. The value of R-Sq is (0.9740) proposed that the total variation of 97% for lipid content is attributed to the independent variables and only about 3% of the total variation cannot be explained by the model. The closer of value of R-Sq to 1 the better of correlation between the experimental and predicted values (Wang and Lu, 2005; Vasconcelosn *et al*., 2000).

**Table 6** The effect of variables in Plackett-Burman experiment

| Term | Effect | Coef | SE Coef | T | P |
|---|---|---|---|---|---|
| Constant | | 10.48 | 0.55 | 18.95 | 0 |
| Sucrose | -4.77 | -2.38 | 0.55 | -4.31 | **0.023** |
| Yeast extract | 8.17 | 4.09 | 0.55 | 7.39 | **0.005** |
| $KH_2PO_4$ | 0.04 | 0.02 | 0.55 | 0.04 | 0.974 |
| $MgSO_4$ | -0.03 | -0.01 | 0.55 | -0.02 | 0.983 |
| KCl | -3.06 | -1.53 | 0.55 | -2.77 | 0.07 |
| $FeSO_4.7H_2O$ | 2.59 | 1.29 | 0.55 | 2.34 | 0.101 |
| pH | 1.73 | 0.87 | 0.55 | 1.57 | 0.215 |
| Incubation days | 5.33 | 2.67 | 0.55 | 4.83 | **0.017** |
| S = 1.91487 | R-Sq = 97.40% | | R-Sq(adj) = 90.45% | | |

Normal probability Plot (Figure 7) show the effect of different variables on lipid yield. Where, yeast extract and incubation days have a positive effect on the model response since they lie on right hand side of the line, while sucrose has a negative effect on lipid content, as it is lie on left hand side of the line. For further optimization by the CCD, all variables with a positive effect on lipid accumulation were fixed at a high level and those variables which exerted a negative effect were maintained at a low level.

On application of ANOVA, it was found that the first order model for lipid content was fitted to the results obtained from the 20 experiments as the following equation (Eq.4).

Lipid content % = 10.5 - 2.38 Sucrose + 4.09 Yeast extract + 0.020 $KH_2PO_4$ - 0.013 $MgSO_4$ - 1.53 KCl + 1.29 $FeSO_4.7H_2O$ + 0.866 pH + 2.67 Incubation days (4)

Among different variables tested, yeast extract was significant and showed the strongest effect on lipid production. This result can be deduced based on the literature as it was demonstrated that the nitrogen concentration was the key factor affecting lipid accumulation (Subhash and Mohan, 2011). During the growth phase the synthesis of proteins and nucleic acids is dependent on the presence of nitrogen in the growth medium, so when nitrogen concentration decrease in the medium the growth rate decrease, the synthesis of proteins and nucleic acids tends to cease and lipid accumulation occurred and increased with nitrogen depletion from the medium (Ratledge, 2002). Similarly, Wynn *et al*., (1999)explained the importance of nitrogen concentration for ARA production as it effect on the maintenance of the high activity of malic enzyme which plays an important role in the provision of NADPH for lipid biosynthesis and thus regulates the extent of lipid accumulation in *M. alpina*.

Carbon concentration is also an important nutrition in the medium affecting lipid accumulation as it was suggested that lipid accumulation is directly proportional to the availability of carbon source up to certain limit (Peng *et al*., 2013; Chatzifragkou *et al*., 2010; Chatzifragkou *et al*., 2011). Carbon source in the medium is utilized for cell growth and maintenance so balanced growth occurs without any accumulation of storage lipids, in case of carbon excess and nitrogen deficiency, the excess carbon is channeled to the production of storage lipids which increase up to a maximum level until the carbon is become limited or get exhausted from the medium (park *et al*., 1990; Granger *et al*., 1993).

Regarding to the incubation time, it is also an important factor for inducing oleagenicity of an organism; enough incubation time can lead to efficient utilization of available carbon source in the growth medium leading to a higher accumulation of lipid granules. On the other hand shorter incubation time influenced the substrate utilization by the organism leading to less biomass and lipid particles formation (Subhash and Mohan, 2014). However, the production of PUFA decreased gradually in prolonged cultivation due to cell lysis (Yamada *et al*., 1987; Bajpai and Bajpai, 1992). The time needed for maximum yields of PUFA also depends on the kind of fatty acid since the ɤ-linolenic acid and linoleic acid were synthesized prior to arachidonic acid and eicosapentaenoic acid. (Li *et al*., 2008).

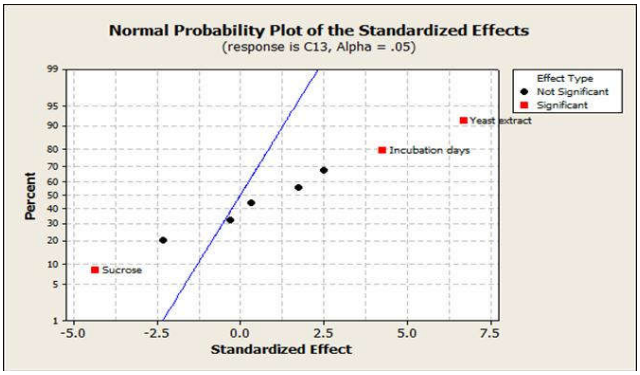


**Figure 7** Normal probability Plot of variables effects on lipid accumulation

***Optimization of significant variables using CCD***

CCD was used to determine the optimum values of the three significant factors and also to study the interactions among them. The design matrix and results of RSM experiment of the three significant factors are shown in Table (4). By applying multiple regression analysis on the experimental data, the following second-order polynomial equation **Eq. (5)** was obtained to predict lipid content in terms of coded factors.

$Y= 43 + 6.33\ A + 3.25\ B - 0.77C - 3.40\ A^2 - 2.23\ B^2 - 2.55\ C^2 + 2.80\ AB - 2.76\ AC + 1.02\ BC$ (5)

Where Y is the response variable (Lipid content) and A, B and C is sucrose, yeast extract and incubation days, respectively. The adequacy of the model was checked by the analysis of variance which demonstrates that the model is highly significant, and fitted the second-order polynomial model to explain the observed yields as this is evident from the F-value (Fisher's test) and the probability P-value (Table 7).

**Table 7** ANOVA for lipid content according to response surface quadratic model

| Source | DF | Seq SS | Adj SS | Adj MS | F | P |
|---|---|---|---|---|---|---|
| Regression | 9 | 1111.47 | 1111.472 | 123.497 | 28.17 | 0 |
| Linear | 3 | 699.62 | 699.617 | 233.206 | 53.19 | 0 |
| Square | 3 | 280.04 | 280.042 | 93.347 | 21.29 | 0 |
| Interaction | 3 | 131.81 | 131.813 | 43.938 | 10.02 | 0.002 |

The significance of each coefficient in the experimental model as well as the significance of linear, quadratic and interaction effects of the variables was determined using the T-value and the P-value (Table 8). A high T-test value and a low probability suggested a high significance (**Saban *et al.*, 2005**). It is appear that first order main effect of sucrose and yeast extract and second order effects of all variables were significant on lipid yield. This result indicates that for optimum lipid production to occur in the cell, all the three variables are required.

The R-Sq value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R2 value of 96.2% point to the accuracy of the model. This ensured a satisfactory fitting of the quadratic model to the experimental data. The adjusted R-Sq of 92.8% indicated a good covenant between the experimental and predicted values and indicated that there is only a 3.8% could occur due to noise (Table 8).The adjusted R-Sq corrects the R-Sq values for the sample size and for the number of terms in the model. In this case, the adjusted R-Sq value is very close to the R-Sq value. All these considerations indicate a good adequacy of the second-order polynomial model proposed for explaining lipid accumulation by *Alternaria sp.*

**Table 8** Estimated regression coefficients for second order polynomial model

| Term | Coef | SE Coef | T | P |
|---|---|---|---|---|
| **Constant** | **43.0088** | **0.854** | **50.362** | **0** |
| A | 6.3321 | 0.5666 | 11.175 | 0 |
| B | 3.2469 | 0.5666 | 5.73 | 0 |
| C | -0.7684 | 0.5666 | -1.356 | 0.205 |
| A*A | -3.4022 | 0.5516 | -6.168 | 0 |
| B*B | -2.2337 | 0.5516 | -4.05 | 0.002 |
| C*C | -2.5466 | 0.5516 | -4.617 | 0.001 |
| A*B | 2.8012 | 0.7403 | 3.784 | 0.004 |
| A*C | -2.7563 | 0.7403 | -3.723 | 0.004 |
| B*C | 1.0163 | 0.7403 | 1.373 | 0.2 |
| R-Sq = 96.2% | | | R-Sq(adj) = 92.8% | |

Optimization of lipid productivity requires a complete picture of all variables not just how various variables affect individually but also of the interactions between these variables (Subhash and Mohan, 2014). In order to understand the interactions between these significant factors, response surface curves were plotted (2D contour plots 3D response surface plots). These curves were the graphical representations of the regression model and act as a function of two factors at a time keeping the other factors fixed (El-Hadi *et al*., 2017).It was observed from Surface plot and contour plot of mutual interaction effect of sucrose and yeast extract that higher level of lipid content was attained with increasing both concentrations of sucrose and yeast extract, while keeping the other variables at its center levels as indicated in Figure (8 - 1).As illustrated in Figure (8- 2), antagonistic interaction was exhibited between sucrose and incubation time, where more than 40 % of lipid content was obtained by 35 g of sucrose and up to 12 days incubation.

Figure (8-3) represents the interaction between yeast extract concentration and incubation time. It showed that the maximum lipid yield appeared at high levels of yeast extract and by increasing incubation time up to around 12 day. With respect to its prospective contour plot, it reveals that interaction between yeast extract and incubation time was negligible as the contour plot was circular.

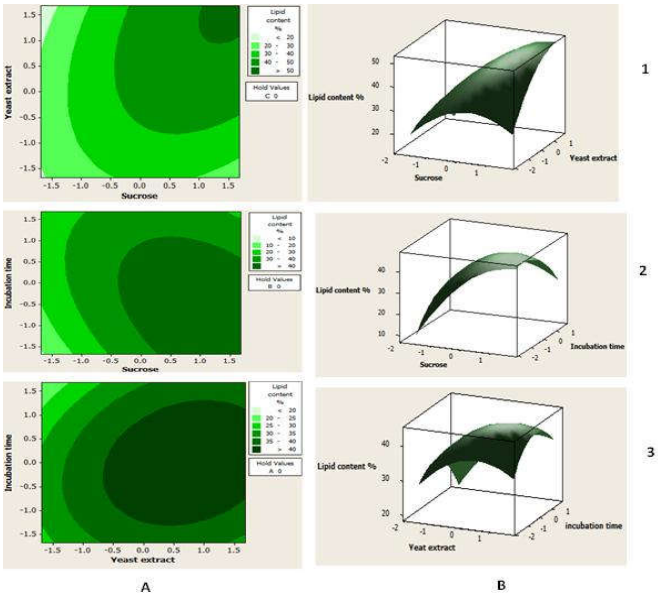


**Figure 8** (A) Contour plot & (B) Surface plot showing the interactive effects of (1) sucrose and yeast extract. (2) Sucrose and incubation time (3) Yeast extract and incubation time

Finally, responseoptimizer tool was used to identify the exact optimum values of each tested variable that leads to achieving response goals. The result of the response optimizer at optimum conditions for maximum goals shown in Figure (9).It was observed that desirability value recorded 0.979 which was closed to indicating the setting seem to achieve favorable results for maximizing lipid content value.The optimum parameter values were as follows: sucrose 35g/l, yeast extract 2.33g/l and incubation time of 160 h, respectively.

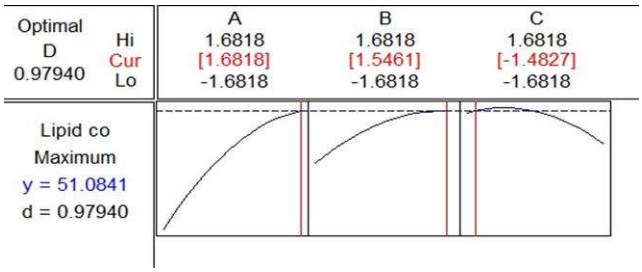


**Figure 9** Response optimizer for optimum concentration of variables

### *Verification of model*

At the end of the optimization experiments, optimum conditions for lipid production by *Alternaria sp.* are (g/l): (sucrose, 35, yeast extract, 2.33, $KH_2PO_4$ 2, $MgSO_4.7H_2O$ 0.25, KCL 0.25, $FeSO_4.7H_2O$ 0.02) pH 6.5 and incubation for 160 hours. This combination was predicted to yield 51% lipid. To test model validation and to evaluate the original medium versus the optimized medium, verification experiment were performed by cultivation *Alternaria sp.* under the above-mentioned optimal conditions and the control was the basal medium. The average amount of the lipid obtained by these experiment was 5.9 g/L of oil extracted from 11.8 g of fungal biomass amounted to a lipid content of 50 % and represent 2.2 fold increases when compared to the control basal medium that gave lipid yield of 22.5%. This validation result was in close agreement with the model-predicted response of 51%.

Therefore, the validation experiments confirmed the predicted values and the accuracy of the model equation.

***Lipid production using molasses as a carbon source***

Many by-products were produced from different industries, which are regarded as waste materials with little value; these materials still contain substances, which are economically valuable, like complex and simple sugars, nitrogen substances, and inorganic salts. All of these componentants are important for growth of different microorganisms, and therefore, there is a strong potential for using these by-products as substrates in biotechnological production (Gajdoš *et al*., 2015).

The growth and lipid production of oleaginous fungi *Alternaria sp.* on molasses were studied. It was found that molasses support both the growth and lipid production with biomass 19.3g/l and lipid yield 5.6g/l (Figure 10) compared with sucrose, with lipid yield 5.7 g/l and biomass 11.45g/l. In respect to molasses, it is an industrial by-product, which used in many biotechnological applications (Arshad *et al*., 2014; Xia *et al*., 2014; He *et al*., 2014; Ortiz *et al*., 2012). Molasses-based cultivation media are indefinite media, which containing different saccharides, nitrogen compounds, and many other substances affecting the growth at different rates depending on molasses batches (Olbrich, 2006).

The ability of *Alternaris sp.* for growth on molasses returns to the presence of sucrose cleaving enzyme invertase which cleaves sucrose that act as the main carbon source in molasses into glucose and fructose (Lazar *et al*., 2013). Similiarly, Bagy*et al.,* (2014) proved the ability of 6 oleaginous fungi namely: *Alternariaalternata*, *Cladosporiumcladosporioides*, *Epicoccumnigrum*, *Fusariumoxysporum*, *Aspergillusparasiticus* and *Emericellanidulans* for biodesel production using sugarcane molasses as substrate and *Alternariaalternata* was the highest lipid containing fungal isolate (40.7% lipids per dry mass). Also several studies on lipid accumulation by oleaginous yeasts and filamentous fungi using molasses have also been studied (Subramaniam *et al*., 2010; Zhu *et al*., 2008; Makri *et al*., 2010; André *et al*., 2010; Fakas *et al*., 2009; Chatzifragkou *et al*., 2010).

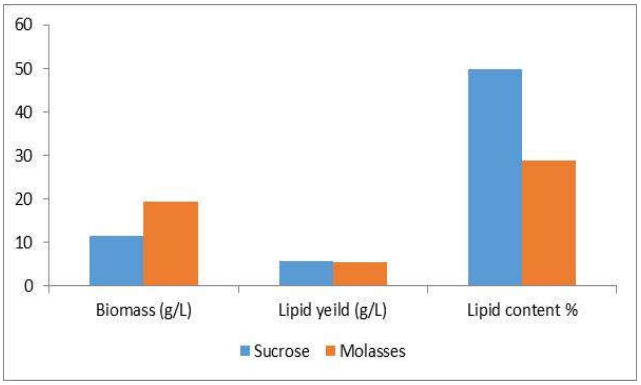


**Figure 10** Biomass, Lipid yield and Lipid content of *Alternaria sp*. grown on sucrose and molasses

***Fatty acid composition***

According to **Jacob, (1993)** the type of species and growth conditions (temperature, pH, type of substrate, variation in C/N ratio and oxygen not only influence the efficiency of lipid accumulation, but also the fatty acid profiles of the cellular lipids).Thus, the influence of different two carbon sources (sucrose and molasses) on fatty acid composition was investigated. The fatty acid profiles of lipids from the two different cultures are compared in Table (9).When the fungus was grown on sucrose the fungal oil was found to contain saturated (40.31%), monounsaturated (26.11%) and poly unsaturated (33.57%) fatty acids with linoleic acid (C18:2) (32.62%) followed by palmitic (C16:0) (32.19%) then oleic acid (C18:1) (19.33%), respectively. On the other hand, when the fungus was cultivated on molasses the lipids obtained was found to containe saturated (41.53%), monounsaturated (33.14%) and poly unsaturated (25.33%) fatty acids with palmitic acid (C16:0) (32.28%) as the most dominant. Recently, other researchers reported similar results on major fatty acid profiles (Ahmad *et al*., 2015).

Interestingly, significant differences were observed in the fatty acid composition for the two carbon sources. Culture on sucrose has a high content of omega-6 PUFA while, culture on molasses has a high content of omega-3 PUFA. Likewise, the presence of gamma linolenic acid (GLA C18:3) (1.911%) which represent one of the most important omega-6 PUFA. It was absent from the profile of lipids obtained by sucrose a carbon source.

Several commercial sugars including glucose, fructose, saccharose, and molasses have been evaluated for glinolenic acid (GLA) production by *M. isabellina* (Chatzifragkou *et al*., 2010; Kavadia *et al*., 2001; Papanikolaou *et al*., 2004).

Finally, an increase in the percent of Cis-4,7,10,13,16,19-Docosahexaenoic (DHA) (Omega-3 C22:6) from 0.12% when sucrose was used as carbon source to 1.4 % when molasses used as a carbon source. A previous study by (Gong *et al*., 2015) reported that the addition of waste molasses as a supplementary carbon source greatly increased the biomass and DHA yield. Our fatty acids profile' results found to be differ from the fatty acid composition of vegetable oils in being rich in (PUFAs), hence it may be exploited for PUFA production (Chisti, 2007).

All significant PUFAs are valuable fatty acids and are beneficial to human health, suggesting that the PUFA-rich oil produced by *Alternaria sp.* Would have high potential in food and therapeutically applications.

**Table 9** Fatty acid composition and concentrations of extracted total lipids from *Alternariasp* using sucrose and molasses as a carbon source by GC/MS

| Fatty acids | Percentage of each fatty acid | |
|---|---|---|
| | Sucrose | Molasses |
| Caproic acid (C6) | 1.22 | 0.027 |
| Caprylic acid (C8) | 0.16 | 0.43 |
| Capric acid (C10) | 0.02 | 0.05 |
| Undecanoic acid (C11) | 0.02 | 0.05 |
| Lauric acid (C12) | 0.03 | 0.05 |
| Tridecanoic acid (C13) | 0.019 | 0.05 |
| Myristoleic acid (C14) | 0.05 | 0.13 |
| Myristic acid (C14) | 0.41 | 0.39 |
| Cis-10-Pentadecenoic acid (C15) | 0.056 | 0.17 |

| | | |
|---|---|---|
| Pentadecenoic acid (C15) | 0.07 | 0.13 |
| Palmitoleic acid (C16) | 4.43 | 4.49 |
| Palmitic (C16) | 32.19 | 32.28 |
| Cis-10-Heptadecenoic (C17) | 0.13 | 0.28 |
| Heptadecenoic (C17) | 0.11 | 0.23 |
| Gama-linolenic (C18) | 0 | 1.91 |
| Linolenic (C18) | 32.62 | 20.40 |
| Oleic (C18) | 19.33 | 25.33 |
| Elaidic (C18) | 1.69 | 1.822 |
| Stearic (C18) | 4.87 | 5.76 |
| Arachidonic (C20) | 0.11 | 0.32 |
| Cis-5,8,11,14,17-Eicosapentaenoic (C20) | 0.21 | 0.38 |
| Cis-8,11,14-Eicosatrienoic (C20) | 0.11 | 0.31 |
| Cis-11,14-Eicosadienoic (C20) | 0.15 | 0.33 |
| Cis-11-Eicosenoic (C20) | 0.16 | 0.30 |
| Cis-11,14,17-Eicosatrienoic (C20) | 0.13 | 0.30 |
| Arachidic (C20) | 0.12 | 0.26 |
| Heneicosanoic (C21) | 0.08 | 0.22 |
| Cis-4,7,10,13,16,19-Docosahexaenoic ( C22) | 0.12 | 1.39 |
| Cis-13,16-Docosadienoic (C22) | 0.11 | 0 |
| Erucic (C22) | 0.13 | 0.25 |
| Behenoic (C22) | 0.26 | 0.54 |
| Tricosanoic (C23) | 0.18 | 0.32 |
| Nervonic (C24) | 0.14 | 0.36 |
| Lignoceric (C24) | 0.55 | 0.73 |

## CONCLUSION

There is a great of interest in the use of oleaginous filamentous fungi for lipid production, as they can accumulate lipids up to 70% of their biomass. The present report describes the use of a strain of the oleaginous fungi *Alternaria* for lipid production. Process optimization of the culture medium and culture conditions efficiently improved the lipid content of the fungus up to 51%. Sugar cane molasses was proved as a suitable carbon source for both fungal growth and lipid accumulation. The oil produced by this fungus contains saturated, monounsaturated and polyunsaturated fatty acids. Thus, *Alternaria sp.* represents a promising candidate for production of single cell oil.

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