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Conversion of Cost-Effective agricultural wastes into valued oil using the fungus *Curvularia* Sp*:* Isolation, Optimization and Statistical analysis

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The fungus *Curvularia* was isolated from a soil sample and identified at the molecular level using 18S rDNA. Subsequently, various growth parameters were optimized using one variable at a time (OVAT), Plackett-Burman Statistical Design (PBD) and Response Surface Methodology (RSM). Results of OVAT indicated that the best carbon source, nitrogen source, agitation conditions, and incubation temperature were sucrose, yeast extract, static conditions, and 30°C, respectively. On the other hand, the best carbon/nitrogen ratio was 30/1. Moreover, the results of PBD indicated that yeast extract, incubation days, and KCl concentration were found to be critical factors affecting lipid accumulation. Therefore, Central Composite Design (CCD) was utilized to determine the optimum levels of these significant factors. Afterward, the optimum parameter values were as follows: yeast extract 2.2 g/L, KCL 0.00, and incubation time of 201 h. Under the optimized conditions, *Curvularia* sp. produced a total biomass of 13.5 g/L with 38.5% lipid content. Also, results proved that orange peel and molasses are potentially valuable sources for the production of single-cell oil with oil yield of 3.7 g/L and 4.2 g/L, respectively. Fatty acid profiles from cultivation on sucrose indicated that they contain (54.13%) SFAs, (24.51%) MUFAs, and (21.35%) PUFAs. On the other hand, fatty acid profile from cultivation on molasses resulted in the presence of (51.28 %) SFAs, (23.95%) MUFAs, and (24.75 %) PUFAs. Whereas, when cultivated on orange peel give (51.13%) SFAs, (28.93%) MUFAs, and (19.93%) PUFAs. This study ensure the ability of Curvularia to convert agricultural waste into valued oils.

Keywords: Agricultural Wastes, fungi, Statistical Analysis, oil.

INTRODUCTION

The main sources of oil and fat in the world are derived from agricultural products (vegetable oil) and the remainder amount is coming from animal and marine sources (fish oils) (Akpinar-Bayizit, 2014). Concerning the nutritional problems accompanying the rapid growth of world's population, it is essential to find new sources for oil supplement. Therefore, present biotechnological research has focused on the commercial exploitation of microorganisms for the production of oils.

As a matter of fact, not all microorganisms can be considered as abundant sources of lipids , though, like all living cells, lipids occurs naturally in the microbial system as a component of the cell membrane, playing essential structural and functional roles in microbial biology (Dowhan and Bogdanov, 2013).

A small percentage of microorganisms have the ability to accumulate intracellular lipids in the stationary growth phase under nutrient limitation

conditions, those organisms are defined as oleaginous microorganisms (Bharathiraja et al., 2017) and the oil produced is termed single cell oils (SCOs) (Ratledge, 2004).

Oleaginous organisms such as fungi, yeasts, algae and bacteria can accumulate oil beyond 20% of their biomass under appropriate cultivation conditions (Karamerou *et al*., 2016). Depending on the type of microorganism, fatty acid profiles of SCOs may vary, making them highly suitable for diverse industrial applications (Subramaniam *et al*., 2010). The production of microbial oil has many advantages compared to vegetable oils for the following reasons: the cultivation of microorganisms does not require huge space, oil can be produced in a much shorter time, and less affected by environmental factors (Li et al., 2008). Recently, the discipline of single cell oil production from oleaginous filamentous fungi has attracted great attention by the virtue of fast growth rate, accumulation of some added-value fatty acids, ease of scaling up in bioreactors, and the ability to use inexpensive renewable and agro-industrial wastes as substrates for fungal growth (Yehia et al., 2017).

Interestingly, the composition of fungal lipids has attracted much attention in many sectors of industry, as it has appeared to have high similarity to plant oils, so that, it is considered as an alternative feedstock and also a source for biodiesel production (Papanikolaou and Aggelis, 2011). Additionally, it can be utilized as substitutes for expensive lipids, which are rarely found in plant or animal kingdoms, and finally SCO could be used as substitutes for several important exotic fats like cocoa butter and shea butter (Wei et al., 2017).

The amount and quality of the lipids produced can vary not only from one organism to another, but also with age, developmental stage, nutritional and environmental conditions (Donot et al., 2014).

Consequently, optimization of the production conditions is an essential step for a higher lipid production under economical cost and for further industrialization (Jiru et al., 2017).

The traditional approach for optimization design is to use one factor at a time (OVAT), while keeping the other factors constant. This approach has many disadvantages such as: time consuming, boring, and does not study the interaction between the tested factors (Song *et al*., 2007). To overcome these disadvantages, statistical methods were used, which provide efficient ways for screening a large number of variables simultaneously, predicting the significant parameters affecting the yield and determining the interactions among the selected variables. Among these approaches, Plackett–Burman (PB) Design, this is a good tool to determine the significance among large number of factors. In this method, detailed information about each factor is obtained in a minimum number of experiments, which subsequently can minimize the cost of production (Srinivas et al., 1994). Another statistical method is Response Surface Methodology (RSM), which predicts the optimum levels of the key factors in the cultivation process. Moreover, it explains the individual and interactive effects of the studied independent variables on the response levels (Liu et al., 2003; Francis et al., 2003).

In order to achieve sustainable and economical production of lipids by oleaginous microorganisms, cheap and abundantly available growth substrates that have zero or negative value are required (Pan et al., 2009). Examples of these substrates are sugarcane or beet molasses and raw glycerol (Christophe et al., 2012).

Based on the above-mentioned facts, this study aims to isolate and identify fungal isolate capable of storing high amount of lipids. The lipids content of the selected isolate was subsequently optimized using different statistical designs of experiment such as PB and CCD. Moreover, different wastes were examined as alternative substrates for fungal growth and lipid production.

MATERIALS AND METHODS

Soil Sampling

Three soil samples from different sites of Bani mazar (El-Minya governorate) were collected from a depth of 5-15 cm below the soil surface, sealed in sterile sampling polyethylene bags, and stored at 4°C until further manipulation.

Isolation of Fungi

One gram of each soil sample was individually suspended in 9 mL of sterile distilled water, serially diluted 10-fold and plated on potato dextrose agar medium (pH 6), and chloramphenicol was added to the medium at a concentration 100 mg/L to prevent bacterial growth. The plates were incubated at 30 °C for 5 days. Pure colonies were obtained by repetitive streaking on PDA agar plates and stored at 4°C.

Selection of Oleaginous Fungi by Nile Red Staining

All fungal strains were screened based on their capability of lipid accumulation by rapid

estimation with Nile Red staining (Kimura et al., 2004). Fungal biomass were incubated in the 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 drying in the air. Fluorescence microscopy studies were performed using Olympus BX 40 microscope.

Molecular Identification of the Selected Isolate.

Molecular identification of the selected isolate with the highest lipid content was done based on PCR amplification of the 18S rDNA gene. Genomic DNA was extracted using AMSHAG-DNA Extraction Kit (Elrashdy and Abd-El-Haleem, 2005). 0.1 µg genomic DNA was used as a template for the PCR reaction and two primers were used for the amplification of 18S-rDNA encoding genes (Suh and Nakase, 1995). The PCR was conducted on Thermo PCR machine (Thermo Fisher, USA). 1% agarose gel containing ethidium bromide stain was used to separate the amplified PCR fragments using gel electrophoresis technique. The electrophoresis was performed at 100 V in 1X TBE buffer then visualized using Multi Image gel documentation system. PCR products were purified using gel purification kit (Elrashdy and Abd-El-Haleem, 2005) and sequenced using 373 API DNA sequencer. The sequences were analyzed using BLAST online tool of the National Centre for Biotechnology Information (NCBI) to find out the homology percentage between the obtained sequences and the sequences of the existing species in the database. Confirmed sequence was submitted to the GenBank database.

Culture Media and Optimization of Culture Conditions for Biomass Production and Lipid Accumulation

Cultivation of the fungus was first performed on Czapek Dox's basic 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) 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 a Time (OVAT)

The influence of different: temperatures (10, 20, 30 and 40 ˚C); carbon sources (30 g/L of; sucrose, glucose, lactose, glycerol, starch and carboxy methyl cellulose (CMC); nitrogen sources (2 g/L; sodium nitrate, ammonium chloride, urea, yeast extract, peptone and glutamic acid) ; C/N ratios (30:1, 30:2, 30:4, 30:6, 15:2, 60:2, 60:4); and agitation conditions (static and shaking at 150 rpm) were studied. Aliquots of 50 mL medium in 250 mL Erlenmeyer flasks were prepared in triplicates, for each experiment. One disk from the margin of 5 days aged cells on agar PDA was used as inoculum. Dry biomass, lipid yield, and lipid content were determined.

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, number of 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 a low (-1) levels, 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 and lipid content was considered as the response in each trial.

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

$$Y = \beta O + \Sigma \beta\, iXi \qquad (1)$$

Where, Y is the response or dependent variable (lipid content) and it will always be the variable we aim to predict, βo is the model intercept βi is the linear coefficient, and Xi is the level of the independent variable. The statistical analysis output will be used to calculate the significance of the variables depending on their nature; and their positive or negative effects on the lipids production process.

Central Composite Design (CCD)

RSM was employed to optimize the concentrations of the effective parameters selected by Packett-Burman Design experiment. Three parameters were studied independently including, yeast extract, number of incubation days, and KCl concentration. Each variable was studied at five different levels (-1.681, -1, 0, +1, +1.681). A set of 20 experiments were employed as shown in (Table 3) and both the minimum and the maximum ranges of each variable 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 the statistical calculation, the relationship between the coded and the actual values is described by Eq.2:

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

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:

$$Y = \beta_0 + \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_{11}X_{11} + \beta_{22}X_{22} + \beta_{33}X_{33} + \beta_{12}X_1X_2 + \beta_{13}X_1X_3 + \beta_{23}X_2X_3 \ldots\ldots\ldots\ldots (3)$$

Where: *Y* is the predicted response; X_1, X_2, X_3 are input variables which influence the response variable Y*;* β_0, intercept; β_1, β_2 and β_3 linear coefficients; β_{11}, β_{22} and β_{33}, squared or quadratic coefficients β_{12}, β_{13}, and β_{23} interaction coefficients

Statistical Analysis

All the experiments were conducted in triplicates. 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, USA). The data of lipid yield was subjected to Analysis of Variance (ANOVA).

Determination of Dry Biomass, Lipid Yield, and Lipid Content

At the end of the incubation period of each experiment the mycelia mats (in triplicate) was harvested by simple filtration method using Whatman No.1 filter paper and washed 3 times with distilled water followed by drying at 60^0C 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.

Lipid yield and content were assessed using phosphovanillin method described by (Helal et al., 2006). The standard calibration curve was performed using canola oil as a standard.

Lipid Production using Low Cost Substrates

The carbon source in the optimized media was replaced by molasses and/or orange peel. Orange peels were sun dried, grounded into fine powder, and preserved in desiccator until further utilization. The fungi were inoculated in the production medium and were kept in a shaker incubator at 150 rpm.

Lipid Extraction

Extraction of lipids was performed according to the method reported by Bligh and Dyer (1959). The dry biomass was grounded in a mixture of chloroform: methanol (2:1) and agitated for 20 min at 200 rpm at room temperature, followed by centrifugation at 6000 rpm for 10 min to recover the solvent phase. The same process was repeated twice. Finally, the solvent was evaporated, all the samples were dried under vacuum, and the amount of oil was determined using gravimetric method.

Transesterification of the Extracted Lipids

The obtained fungal lipids were mixed vigorously with 20 ml of methanol and 2 ml of concentrated sulphuric acid for 2 hours at 70ºC. The mixture was allowed to cool at room temperature, thereafter, was transferred to the separating funnel to obtain two layers, an upper methyl ester layer and a lower glycerol layer. The methyl ester layer was collected and analyzed using GC-MS technique (Madonna et al., 2016). Agilent 6890N Gas Chromatograph machine connected to Agilent 5973 Mass Spectrometer were employed using the following settings; 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, 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 fungal isolates were isolated from 3 different soil samples from Bani mazar. These isolates were tested for lipid accumulation using Nile red stain. After Nile red staining, 6 fungal isolates, designated as F1, F2, F3, F4, F5 and F6, exhibited strong fluorescence under fluorescent microscope. Among the 6 isolates, isolate F4 showed the strongest fluorescence in comparison to the other isolates. Therefore, isolate F4 was selected for further studies. Nile red stain seems preferable for intracellular lipid determination, as it

reacts only with hydrophobic compounds and emits strong red fluorescence signal, which can be detected by fluorescence spectroscopy (Spiekermann et al., 1999). The fluorescence intensity of the stained cells under UV light depends on lipid concentration. Microorganisms that do not accumulate lipids emit light fluorescence, because they contain almost no lipid granules (Dawes, 1990).

Identification of the Selected Isolate

Molecular identification was done by sequencing the 18S rRNA encoding gene. The sequence was analyzed using Blast program and showed 98% similarity with *Curvularia sp.* and subsequently coded as *Curvularia sp.* strain GH2 with accession number MG590070.

Optimization of Biomass and Lipid Yield using OVAT:

Effect of agitation rate on Growth and Lipid Production of *Curvularia*

Generally, the dissolved oxygen concentration is critical for microbial growth. The concentration of dissolved oxygen in the medium was controlled by the rotation speed of the shaker, which subsequently influenced on biomass and lipid production. The results indicated that both static and shaking conditions resulted in the same biomass. On the other hand, there was slightly increase in lipid yield and percentage under static condition (Figure 1). In consistence with our results, Kirrolia et al., 2012, Ali and El-ghonemy 2014 and Ali et al., 2017 found that static incubation produced higher quantities of lipid than the amount produced under shaking condition.

Effect of Temperature

Figure 2 represents the effect of different temperatures on biomass and lipid yield of *Curvularia sp.,* indicating that there was no detected growth at 10°C and at 40°C, consequently, there was no lipids accumulation observed under these conditions. On the other hand, maximum biomass (15.36 ± 0.02 g/L), lipid yield (3.39 ±0.03 g/L) and lipid content (22%) were observed at a temperature of 30°C. Accordingly, it was selected for further studies. In agreement with this result, Carlile et al., 2001 reported that 30°C is the optimum temperature for maximum biomass and lipid content, as the activity of all fungal enzymes was high at this temperature range. Also, Farooq et al., 2005 found that 30°C was the optimum temperature for *F. oxysporium* growth and was drastically reduced below 15°C and above 35°C (Kendrick and Ratledge, 1992; Mamatha, 2009).

Effect of Different Carbon Sources

Carbon is one of the most important essential elements required by the living organisms. Utilization of various carbon sources is mainly dependent upon the enzymatic system of the fungus and its ability to utilize certain forms or its capability to convert complex carbon compounds into simpler forms, which may be easily utilized by the fungus (Ramjegathesh and Ebenezar, 2012). Herein, all carbon sources tested were found to support both the growth and lipid accumulation of fungus at different levels of the tested carbon source. As shown in Figure (3), the highest biomass (14.06 ± 0.02 g/L), lipid yield (3.15 ± 0.04 g/L) and lipid content 22.4% were obtained. On the other hand, CMC had the lowest biomass (5.34 ± 0.009 g/L) and lipid yield (0.06 ± 0.015 g/L). Obviously, sucrose was the best carbon source for lipid production by *Curvularia sp*. This is in agreement with Papanikolaou et al., (2010) who reported that *Thamnidium elegans* gave the maximum concentration of total lipid content (9.3 g/L) upon utilization of sucrose as the sole carbon source, whereas glucose and fructose resulted in the accumulation of 9.2 and 8.8 g/L lipids, respectively.

Effect of Different Nitrogen Sources

Different levels of cell biomass and lipids were obtained using different organic and inorganic nitrogen sources (Figure 4). The results showed that urea was a poor nitrogen source that gave the lowest cell biomass and lipid yield. Yeast extract was found to be the most suitable nitrogen source with biomass (14.81± 0.02 g/L), lipid yield (3.45± 0.03 g/L), and lipid content 23.3%. Similarly, Gao et al., 2013 found that *M. isabellina* gave the maximum lipid yield (64.2%) using yeast extract-rich media as a nitrogen source. Yeast extract contains proteins, short chain peptides, carbohydrates, free amino acids nucleic acids, essential heavy metals, and vitamins.

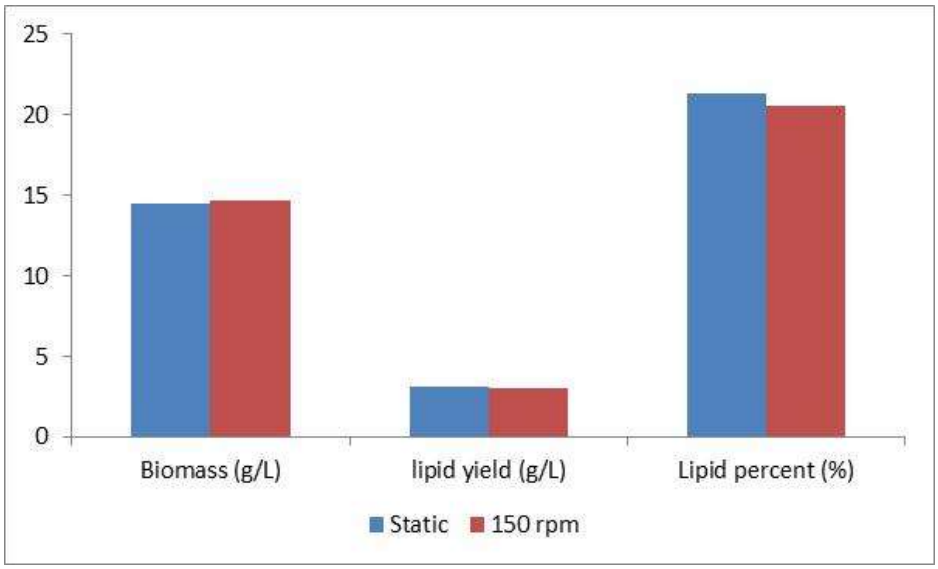

Figure1; Effect of aeration conditions (shaking and static) on biomass and lipid production by *Curvularia sp.*

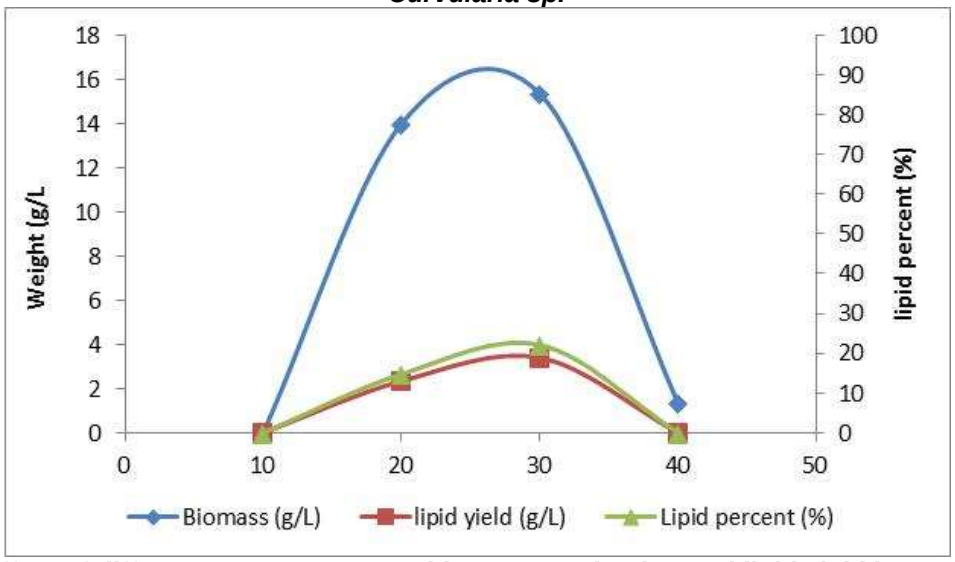

Figure 2; Effect of different temperatures on biomass production and lipid yield by *Curvularia sp.*

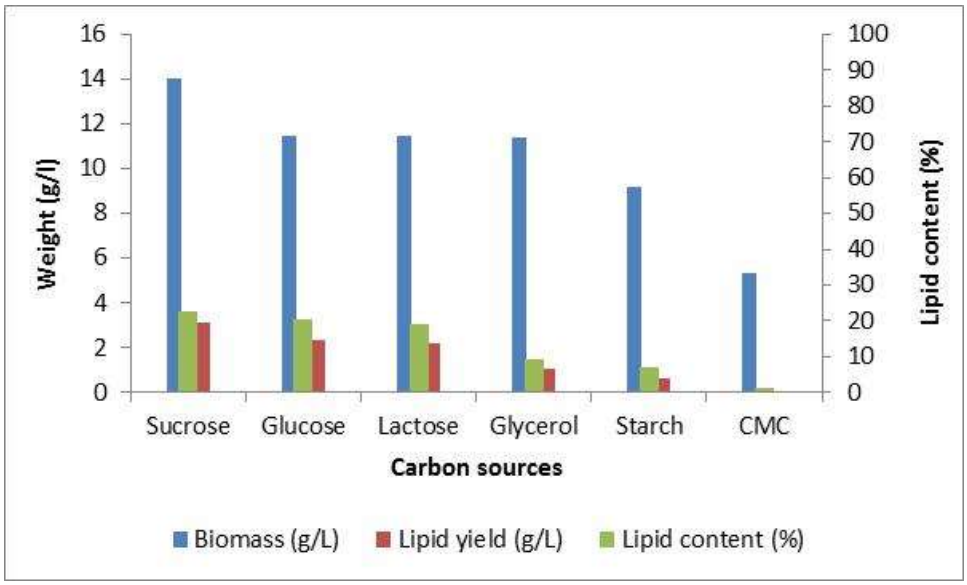

Figure 3; Effect of different carbon sources on biomass production and lipid yield by *Curvularia sp*.

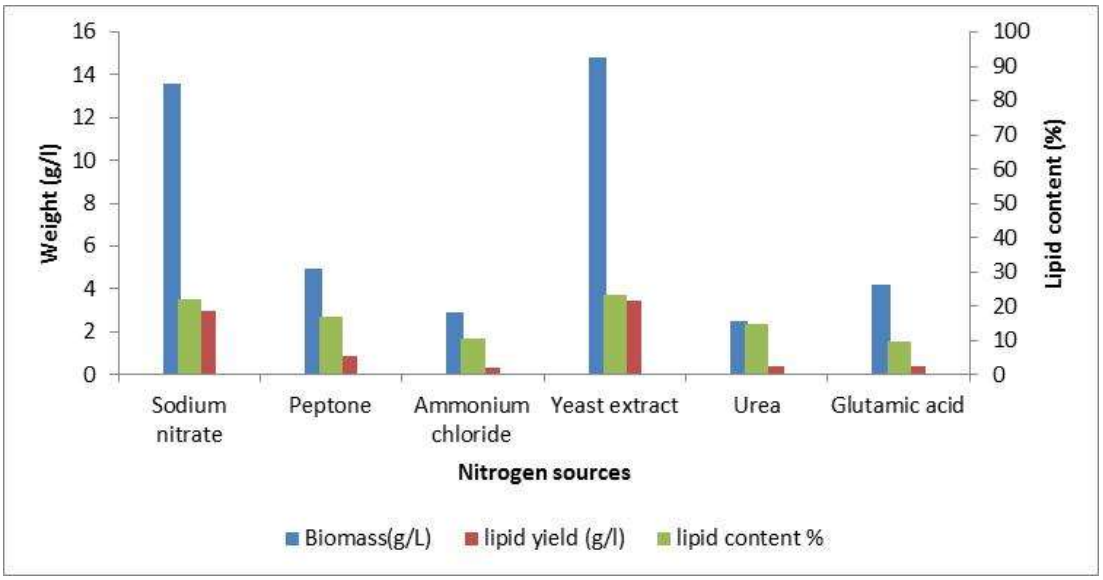

Figure 4; Effect of different nitrogen sources on biomass, lipid yield, and lipid content by *Curvularia sp*

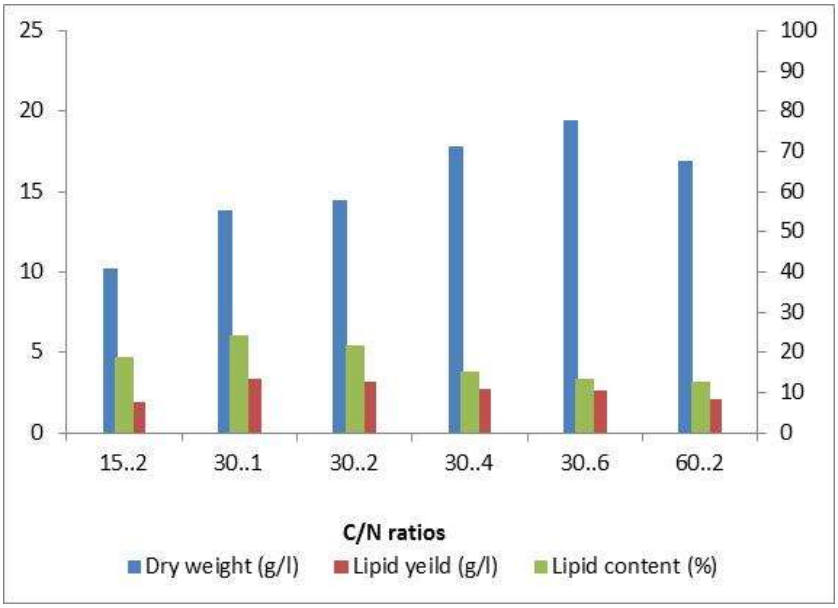

Figure 5; Effect of different carbon to nitrogen ratio on biomass, lipid yield and lipid content by *Curvularia sp.*

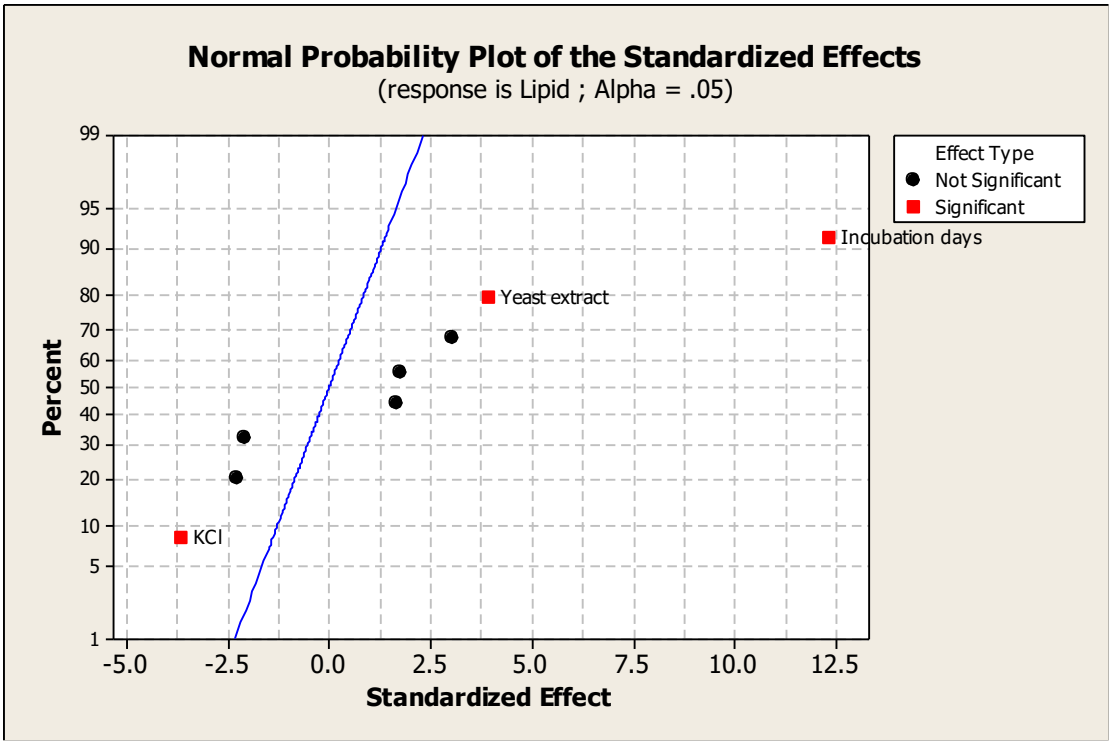

Figure 6; Normal Probability Plot of variables effects on lipid accumulation

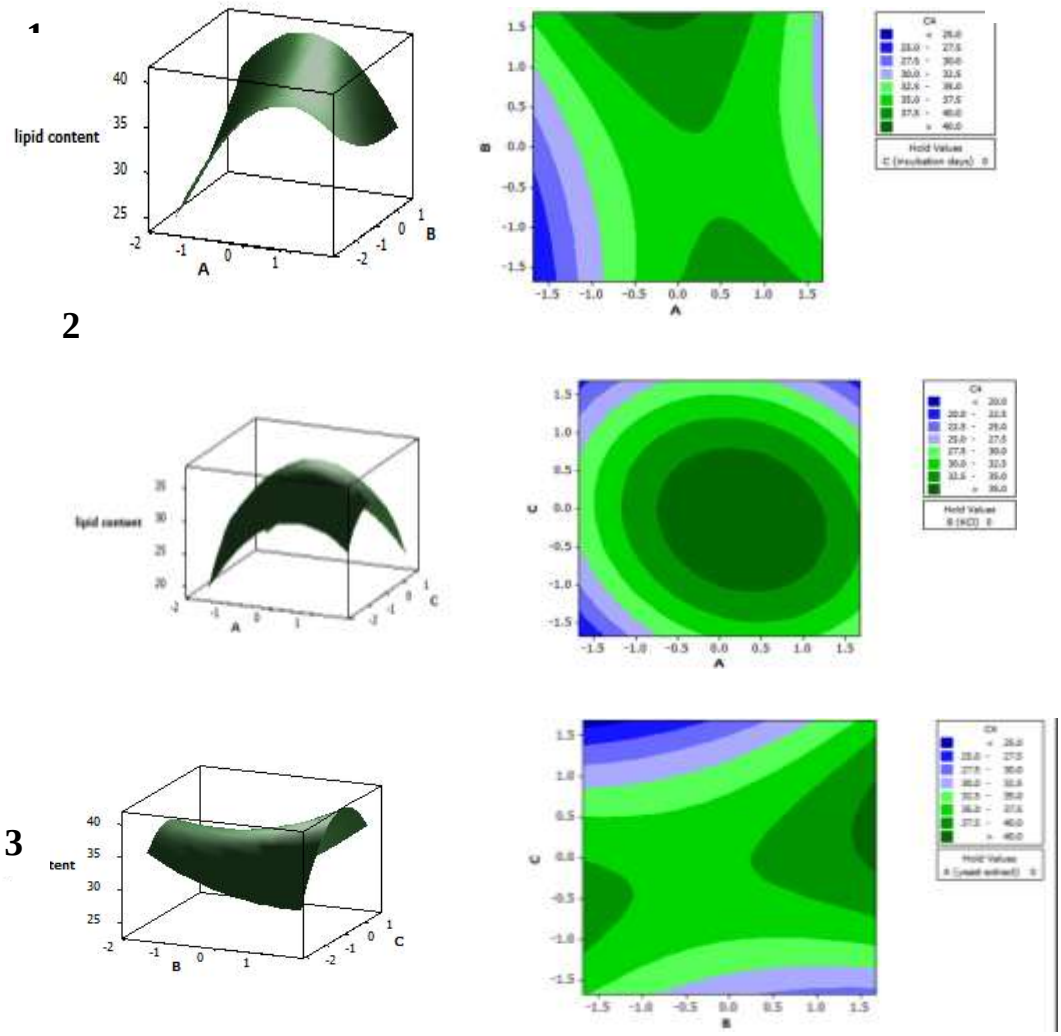

Figure 7(A);Surface plot & (B) Contour plot showing the interactive effects of (1) yeast extract and KCL (2) Yeast extract and incubation time (3) KCL and incubation time

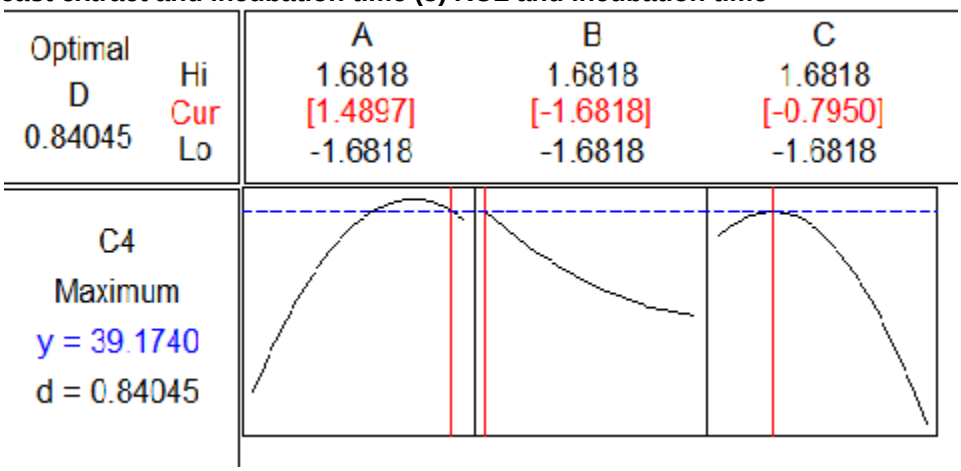

Figure 8; Response Optimizer for optimum concentration of variables

Therefore, it is supposed to enhance growth of fastidious microorganisms by acting as both carbon and nitrogen sources simultaneously. In addition, it is easily decompose and utilized by microorganisms (Seesuriyachan et al., 2011). Moreover, Ratledge and Wynn, (2002) mentioned that yeast extract is rich in glutamate, which lead to increase lipid production in *R. toruloides*.

3.3.5. 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 5. As noticed, low C: N ratio (15:2) caused reduction in biomass and lipid yield, which could be attributed to the exhaustion of sucrose that was in low concentration at the preliminary stage of growth. (Subhash and Mohan, 2014). Whereas, maximum lipid yield (3.35 ± 0.03g/L) was obtained at ratio 30:1. As explained by Mandal and Mallick, 2009, the rapid depletion of low nitrogen concentration slow the fungal growth, while, the high concentration of sucrose existed in the medium was directed towards lipid formation.

An increase in biomass and a decrease in lipid yield were observed by increasing yeast extract concentration while keeping a constant sucrose concentration (30:2 and 30:4), as high concentration of nitrogen source in the presence of carbon source stimulate the cell towards the production of biomass (Subhash and Mohan, 2014). At a ratio of 30:6, the maximum biomass (19.48 g/L ± 0.02) and the lower lipid yield was obtained, suggesting that the utilization of both high concentration of carbon and nitrogen for biomass production over several days of incubation (Subhash and Mohan, 2014). A decrease in biomass production and lipid accumulation was observed at high C: N ratio (60:2), this can be explained by the previous findings, which illustrated that high substrate concentrations had inhibitory effects on both fungal growth and lipids accumulation (Gao et al., 2013).

Virtually, the nutrient imbalance in the culture medium, particularly high C/N ratio (greater than 20), plays a pivotal role for both fungal growth and lipids accumulation (Beopoulos et al., 2009; Papanikolaou and Aggelis, 2011).

Screening of significant variables using PBS

Basically, PBD represents an uncomplicated and quick method for the screening of a large number of variables in one experiment comparing to other statistical design strategies (Eltarahony et al., 2016). Two level of eight tested variables in the design were examined along with 12 experimental run conducted in triplicate, indicating variation of lipid content of *Curvularia* sp. ranging from 6.21 to 27.31 %, which represent 0.33 and 3.18 g/l, respectively (Table 1.)

This variation reveals the strong influence of medium components for attaining higher lipid accumulation, which illustrates the importance of using optimization process. The correlation between the independent variables and lipids content was determined through multiple-regression mathematical model. The regression analysis of PBD model indicates the coefficient values for each parameter (Table 5), where the significance of each coefficient is checked by its *p*-values and t-value. Variables with high t-value and small *p*-values (less than 0.05) were considered to have a significant effect on the response (Liu et al., 2003). Accordingly, yeast extract (*p*-value 0.029), incubation days (*p*-value 0.001), and KCl (0.035) played significant roles in the model response (lipid content).

Goodness of the model was checked from the correlation coefficient (R^2) values and the adjusted R^2. The value of R^2 is (0.9856) indicates that the total variation of 98% for in the fungus lipid content is attributed to the independent variables and only about 2% of the total variation cannot be explained by the model. The closer the value of R^2 to 1 is, the stronger the model and a better correlation between the experimental and the predicted values occurs (Wang and Lu, 2005; Vasconcelos et al., 2000).

Normal Probability Plot **(**Fig. 6**)** shows the effect of different variables on lipid yield, where yeast extract and incubation days had positive effects on the model responses, since they lie on the right hand side of the line. Meanwhile, KCl had a negative effect on the lipids content by being on the left hand side of the line.

By applying ANOVA, the first order equation that represents the optimum lipids content as a function of the studied independent factors was obtained as follows:

Lipid % = 15.2 + 1.49 Sucrose + 1.93 Yeast extract + 0.813 KH_2PO_4 - 1.12 $MgSO_4$ - 1.79 KCl + 0.852 $FeSO_4.7H_2O$ - 1.03 pH + 6.04 Incubation days

Ultimately, the nitrogen concentration was the key factor affecting lipids accumulation (Subhash and Mohan, 2011). During the growth phase, the synthesis of proteins and nucleic acids is usually dependent on the presence of sufficient nitrogen source in the growth medium, so that, when

nitrogen concentration decreases, the growth rate also decreases and the synthesis of both proteins and nucleic acids tends to cease. At this stage, the lipid accumulation occurs and increases with more depletion of nitrogen from the medium (Ratledge, 2002).Wynn et al., (1999) explained the importance of nitrogen concentration for ARA production, as it has an obvious 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*.

However, sufficient incubation time resulted in efficient utilization of the nutrients in the growth medium, which resulted in a higher accumulation of lipid granules. Basically, the production of PUFA decreased gradually in prolonged cultivation time due to auto-cell lysis (Bajpai and Bajpai, 1992).

For further optimization using CCD, all variables with a positive effect on lipids accumulation were fixed at high levels and those variables that affected negatively were maintained at low levels.

Optimization of Significant Variables Using CCD

CCD was employed to determine the optimum values of the three significant factors, which produce the maximum yield of lipid and their interactions with each other. In this study, 20 experiments with different levels and combinations of yeast extract concentrations (A), KCl concentrations (B), and incubation time (C) were Performed. The design matrix and the results of CCD experiments were demonstrated in Table (4). As noticed, the maximum lipids content was observed at the axial point (run 7) with 41%.

The adequacy of the model was checked by ANOVA, which demonstrated 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 6.

The significance of each coefficient in the experimental model, as well as the significance of linear, quadratic and interaction effects of the variables, was elucidated in (Table 7). The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value of 95.0% indicates to the accuracy of the model (Table 7). This ensures a satisfactory fitting of the quadratic model to the experimental data. The adjusted $R^2$of 90.4% indicates a good agreement between the experimental and predicted values and implies that the model is reliable for maximum lipids production from *Curvularia sp.*

Furthermore, the adjusted R^2 corrects the R^2 values for the sample size and for the number of terms in the model. In this case, the adjusted R^2 value is very close to the R^2value. All these considerations indicate a good adequacy of the second-order polynomial model proposed for explaining lipids accumulation by *Curvularia sp.*

To evaluate the relationship between the lipids content and the independent variables, and also to assign the optimal concentration of each component involved in the maximum lipids content, the equation of second-order polynomial was proposed to define the predicted lipid content (Y) in terms of the independent variables:

Eq. (1) $Y=37.2 + 1.18\ A + 0.928B - 0.649\ C - 2.45\ A^2 + 0.60\ B^2 - 2.80\ C^2 -1.44\ AB - 0.75AC +1.64\ BC$

Where Y is the response variable (Lipid content) and A, B and C is yeast extract, KCl and incubation days respectively.

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).

Generally, the surface plots were convex shape, suggesting that there are well-defined optimal variables. Further, as the variable ranges were appropriate, the optimum lies in the design space. Figure (7 -1) indicates an antagonistic interaction effect between yeast extract and KCl. Keeping the number of incubation days at center level, a higher value of lipids content was attained with increasing concentration of KCl and decreasing concentrations of yeast extract. In addition, a significant interaction was exhibited as indicated by contour plot (Figure 7 -1).

On the other hand, more than 35% of the lipids could be obtained by maintaining yeast extract concentration and incubation days at their intermediate levels as deduced from Figure (7-2).

Table 1: Plackette-Burman Design matrix for screening of critical factors influencing lipid production by *Curvularia sp.*

Variables / Trials	Sucrose	Yeast extract	KH_2PO_4	Mg_2SO_4	KCl	$FeSO_4.7H_2O$	pH	Incubation days	Lipid content%	
									Experimental	Predicted
1	1	1	-1	1	1	-1	1	-1	6.21	6.96
2	-1	-1	-1	1	1	1	-1	1	15.97	15.98
3	-1	1	-1	-1	-1	1	1	1	22.21	23.59
4	-1	-1	-1	-1	-1	-1	-1	-1	8.65	8.00
5	1	1	1	-1	1	1	-1	1	27.31	26.66
6	-1	1	1	-1	1	-1	-1	-1	9.26	9.90
7	1	-1	-1	-1	1	1	1	-1	7.79	7.04
8	-1	1	1	1	-1	1	1	-1	12.28	10.90
9	1	-1	1	1	-1	1	-1	-1	10.68	12.07
10	1	-1	1	-1	-1	-1	1	1	22.63	22.63
11	-1	-1	1	1	1	-1	1	1	13.85	13.85
12	1	1	-1	1	-1	-1	-1	1	25.4	24.68

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	15.000	30.00	45.00
yeast extract	g/L	0.500	1.00	1.50
KH_2PO_4	g/L	0.500	1.00	2.00
$MgSO_4.7H_2O$	g/L	0.250	0.50	1.00
KCl	g/L	0.250	0.50	1.00
$FeSO_4.7H_2O$	g/L	0.005	0.01	0.02
pH	-	4.5	5.5	6.5
Incubation days	-	4	7	10

Table 3: Experimental levels of the effective variables used in CCD

Variables	Coded levels Experimental values				
	-1.681	-1	0	1	1.681
Yeast extract (g/L)	0.75	1.00	1.50	2.00	2.50
Kcl (g/L)	0.000	0.125	0.250	0.500	0.750
Incubation days	6	8	10	12	14

Table 4: Central Composite Design of the significant factors for lipids production by *Curvularia sp.*

Run order	Yeast extract (A)	Kcl (B)	Incubation days (C)	Lipid content%	
				Experimental	Predicted
1	0	0	0	37.63	37.23
2	0	0	-1.68179	29.32	30.40
3	0	0	0	37.22	37.23
4	1.68179	0	0	31.02	32.29
5	1	1	-1	33.99	33.01
6	0	-1.68179	0	35.86	37.39
7	0	1.68179	0	41.50	40.52
8	-1	-1	-1	31.77	30.57
9	-1	-1	1	26.89	27.49
10	1	-1	1	32.59	31.23
11	0	0	0	36.94	37.23
12	-1	1	1	34.98	35.52
13	-1.68179	0	0	29.03	28.30
14	1	1	1	32.68	33.50
15	0	0	0	37.25	37.23
16	0	0	0	37.60	37.23
17	1	-1	-1	38.26	37.33
18	-1	1	-1	31.05	32.02
19	0	0	0	36.84	37.23
20	0	0	1.68179	28.75	28.21

Table 5: Statistical analysis of Plackett-Burman and predict illustrating regression coefficients of the tested variables on lipids content of *Curvularia* sp.

Term	Effect	Coef.	SE Coef.	T	P
Constant		15.193	0.4902	30.99	0.000
Sucrose	2.970	1.485	0.4902	3.03	0.056
Yeast extract	3.852	1.926	0.4902	3.93	0.029
KH_2PO_4	1.627	0.813	0.4902	1.66	0.196
$MgSO_4$	-2.236	-1.118	0.4902	-2.28	0.107
KCl	-3.579	-1.789	0.4902	-3.65	0.035
$Fe_2SO_4.7H_2O$	1.704	0.852	0.4902	1.74	0.181
pH	-2.054	-1.027	0.4902	-2.09	0.127
Incubation days	12.087	6.044	0.4902	12.33	0.001
S = 1.69817 R^2= 98.56% R^2(adj) = 94.72%					

Table 6: ANOVA for lipid content according to response surface quadratic model.

Source	DF	Seq SS	Adj SS	Adj MS	F value	*p*. value
Regression	9	278.714	278.714	30.9683	20.93	0
Linear	3	36.681	36.681	12.2271	8.26	0.005
Square	3	199.154	199.154	66.3845	44.87	0
Interaction	3	42.88	42.88	14.2932	9.66	0.003

Table 7: Estimated regression coefficients for second order polynomial model

Term	Coef	SE Coef	T	*p*. value
Constant	37.2336	0.4961	75.057	0
A	1.184	0.3291	3.597	0.005
B	0.9283	0.3291	2.82	0.018
C	-0.6499	0.3291	-1.974	0.077
A*A	-2.4515	0.3204	-7.651	0
B*B	0.6098	0.3204	1.903	0.086
C*C	-2.8021	0.3204	-8.746	0
A*B	-1.4421	0.43	-3.353	0.007
A*C	-0.7541	0.43	-1.754	0.11
B*C	1.6467	0.43	3.829	0.003
R^2= 95.0%		R^2 (adj) = 90.4%		

Table 8: Growth of *Curvularia sp.* on low cost substrates

Carbon source	Biomass g/L	Lipid content g/L
Sucrose	13.47	4.8
Molasses	16.36	4.2
Orange peel	21.14	3.7

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

Fatty acids		Percentage of each fatty acid		
		Sucrose	Molasses	Orange peel
Caproic acid	(C6)	0.072	0.96	0.57
Caprylic acid	(C8)	0.15	0.15	0.78
Capric acid	(C10)	0.018	0.017	0.09
Undecanoic acid	(C11)	0.016	0.014	0.077
Lauric acid	(C12)	0.039	0.033	0.14
Tridecanoic acid	(C13)	0.018	0.018	0.096
Myristoleic acid	(C14)	0.046	0.047	0.24
Myristic acid	(C14)	0.52	0.53	0.49
Cis-10-Pentadecenoic acid	(C15)	0.076	0.072	0.28
Pentadecenoic acid	(C15)	0.13	0.22	0.36
Palmitoleic acid	(C16)	2.07	1.74	2.09
Palmitic	(C16)	40.21	38.31	36.24
Cis-10-Heptadecenoic	(C17)	0.13	0.22	0.43
Heptadecenoic	(C17)	0.19	0.31	0.48
Gama-linolenic	(C18)	0.13	0.11	1.72
Linolenic	(C18)	20.33	23.84	13.36
Oleic	(C18)	21.22	20.45	22.93
Elaidic	(C18)	0.59	0.99	1.57
Stearic	(C18)	11.17	9.13	7.89
Arachidonic	(C20)	0.11	0.11	0.58
Cis-5,8,11,14,17 -Eicosapentaenoic	(C20)	0.19	0.12	0.51
Cis-8,11,14-Eicosatrienoic	(C20)	0.11	0.11	0.58
Cis-11,14-Eicosadienoic	(C20)	0.13	0.15	0.52
Cis-11-Eicosenoic	(C20)	0.15	0.16	0.42
Cis-11,14,17-Eicosatrienoic	(C20)	0.11	0.10	0.55
Arachidic	(C20)	0.20	0.18	0.38
Heneicosanoic	(C21)	0.10	0.11	0.42
Cis-4,7,10,13,16,19-Docosahexaenoic (C22)		0.13	0.11	2.12
Cis-13,16-Docosadienoic	(C22)	0.11	0.11	0
Erucic	(C22)	0.09	0.13	0.35
Behenoic	(C22)	0.40	0.45	1.01
Tricosanoic	(C23)	0.33	0.24	0.67
Nervonic	(C24)	0.13	0.14	0.61
Lignoceric	(C24)	0.57	0.61	1.44

With respect to prospective contour plot, it reveals that interaction effect between both factors was negligible on lipids content, as referred by a circular shape.

The interaction effect of KCl concentrations and the number of incubation days on lipid content was illustrated in 3D surface and 2D contour plots (Figure 7-3). Minimax or saddle shape surface plot displayed a mutual interaction. As noticed, the lipids content reached the maximum value by increasing the concentration of KCl, while keeping the number of incubation days at the intermediate level (8-12 days). Clearly, saddle-shape contour plot revealed a significant interaction between KCl concentration and the number of incubation days that affected lipids content (Figure 7-3).

Finally, Response Optimizer Tool was used to identify the exact optimum values of each tested variable that leads to achieving the response goals. The results of the response optimizer at optimum conditions for maximum lipids production are shown in Figure (8). It was observed that the desirability (d) value was 0.979, which is closed to one, indicating the setting seems to achieve favorable results for maximizing the lipids content value. The optimum parameter values were as follows: yeast extract 2.2 g/L, KCl 0.00 g/L, and incubation time of 201 h.

Verification of Model

To assess the accuracy and to verify the generated model, the experiments were carried out in triplicates under the optimized conditions, and the data were compared to the predicted data. The control basal-medium was run in parallel experiment. The average amount of lipids was obtained in these experiments of optimized medium was 5.2 g/L of oil, which was extracted from 13.5 g of fungal biomass and corresponds to a lipids content of 38.5%. This value of lipid content matches very well the value predicted from the fitted model (39%). This confirms the accuracy and validity of the model. Furthermore, the optimized model enhanced the lipids content by 1.66-fold in comparison to the control basal-medium, which gave lipids yield of 3.2 g/L, corresponding to 23.1% lipids content.

3.7. Lipid Production Using Low Cost Substrates as a Carbon Source

Several 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, such as complex and simple sugars, nitrogen substances, and inorganic salts. All these compounds 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 *Curvularia sp.* on various low-cost substances were studied. It was found that these substrates support both the growth and lipids production to a considerable extent. Molasses, an industrial by-product that is used in many biotechnological applications (Arshad et al., 2014; Xia et al., 2014) was tested for the biomass and lipids production. The results showed that molasses was the most suitable carbon source for growth and lipids production with biomass 16.3 g/L and lipids content 4.2 g/L (Table 8). It is known that molasses-based cultivation media are indefinite media, which contains different saccharides, nitrogen compounds, and many other substances that affect growth at different rates depending on molasses batches (Olbrich, 2006).

The ability of *Curvularia sp.* to grow 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). In agreement with these studies, Bagy et al., 2014 proved the ability of 6 oleaginous fungi namely: *Alternaria alternata*, *Cladosporium cladosporioides*, *Epicoccum nigrum*, *Fusarium oxysporum*, *Aspergillus parasiticus,* and *Emericella nidulans* for bio-diesel production using sugarcane molasses as substrate.

After molasses, orange peels waste is a highly economical substrate for oil production by *Curvularia* sp. with lipid yield of 3.7 g/L, this was attributed to the high value of C/N ratio of orange peel, which is suitable for lipid production (Chen and Chang 1996). Also, the composition of orange peel made it a suitable substrate for fungal growth, as it contains protein, soluble sugar, starch, cellulose, hemicellulose, lignin, ash, pectin, fat, and other compounds, which include organic acids (citric acid, oxalic acid, malonic acid, and malic acid), and vitamins such as vitamin C (Rivas et al., 2008). Gema et al., 2011 showed that cultivation of the fungi *Cunninghamella echinulata* on orange peel resulted in lipid yield of 15–20 mg of oil per gram of dry mass.

Fatty Acid Composition

It is well known that fatty acid composition of SCOs vary greatly depending upon the species, cultivation conditions, and nutritional factors, especially, (carbon, nitrogen, temperature, pH, incubation time, C/N ratio, and DO) (Subramaniam et al., 2010). Thus, in our study, fatty acid composition of the obtained lipids was performed to estimate the compositional changes with respect to substrate variation (sucrose, molasses, and orange peel).

Fatty acids profile of the fungal lipids, obtained from *Curvularia sp.* cultivated on sucrose, contain 54.13% SFAs, 24.51% MUFAs, and 21.35% PUFAs with the highest percent (40.21%) of palmitic acid C16:0, followed by 21.22% oleic acid C18:1 n-9, and finally linoleic acid C18:2 n-6 with 20.33%, as shown in (Table 9).

On the other hand, fatty acids profile of the obtained lipids, when the fungus was cultivated on molasses, demonstrated the presence of 51.28 %SFAs, 23.95% of MUFAs, and 24.75 % of PUFAs. The most dominant fatty acid was palmitic acid (38.31%), followed by linoleic acid (23.84 %), and oleic acid (20.45%). Whereas, when the fungus was cultivated on orange peel, the lipids obtained were found to contain 51.13% SFAs, 28.93% MUFAs, and 19.93% PUFAs (Table 9). Palmitic acid (36.24%) was considered as the most prevalent fatty acid, followed by oleic acid (22.93%) then linolenic acid (13.36 %).

Interestingly, by comparing fatty acid profiles of the tested carbon sources, significant differences were observed as follow: The highest percentage of omega-6 was PUFAs (24.42%), especially linolenic acid was obtained by cultivating *Curvularia sp.* with molasses. Meanwhile, The highest percent of omega-3 PUFAs (3.18%), especially Cis-4, 7, 10, 13, 16, and 19-docosahexaenoic acid, was obtained by cultivating the fungus on orange peel waste. Finally, the highest amount of MUFAs was obtained by utilizing orange peel (28.93%) mainly as omega-9 oleic acid.

CONCLUSION

The present study explored the capacity of isolated oleaginous fungus; *Curvularia sp.* for lipid accumulation under different growth conditions. Process optimization of the culture medium and culture conditions efficiently improved the lipids content of the fungus up to 39 %. In addition, the feasibility of using inexpensive and renewable waste substrate as carbon source for oil production. The two waste materials orange peel and molasses, was also demonstrated. Fatty acid profiles of the produced lipids showed the presence of saturated, monounsaturated, and polyunsaturated fatty acids. Thus, the study provides a clear evidence that *Curvularia sp.* is a promising candidate for economic production of single cell oil that contain significant amounts of valuable unsaturated fatty acids.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

GA and HE designed and performed the experiments and also wrote the manuscript. ME designed statistical experiments and do data analysis. DA reviewed the manuscript. All authors read and approved the final version.

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