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Recruitment of *Cunninghamella echinulata* as an Egyptian isolate to produce unsaturated fatty acids.

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ABSTRACT

Production of microbial oils has attracted a great attention in recent years. The potential of lipid production by *Cunninghamella spp.* is the reason for using microorganisms for unsaturated fatty acids production. Microbial lipid has high similarity to the oil obtained from plants and animals in type and composition. Optimization of cultivation conditions are very important to reach higher production ratio. Therefore, Taguchi method was used to design many trials to get maximum quantity of lipids. Results showed *Cunninghamella sp.BO30* could accumulate lipids 1.43 gl^{-1} with content 34.04% at 35°C with 20 g sucrose and 5 g peptone for 9 days under static conditions. Fatty acids profile of 6-day culture of Cunninghamella sp.BO30 at optimum conditions exhibited majority of saturated fatty acids and minority of unsaturated fatty acids such as palmitic acid (%) followed by oleic and gamma-linolenic acid. On the contrary, *Cunninghamella sp.BO30* was cultured at optimum conditions for 9 days then followed by 3 days at 15ºC, fatty acids profile was completely different, where oleic acid was the dominant and percent of gamma-linolenic acid increased from 1.14 to 3.22%. Finally, *Cunninghamella sp.BO30* is Egyptian strain has ability to accumulate large quantities of unsaturated fatty acids, therefore is considered a promising strain for lipids particularly unsaturated fatty acids.

Keywords: *Cunninghamella; unsaturated fatty acids; Taguchi method.*

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INTRODUCTION

Fatty acids are the compounds that give lipids their main nutritional properties. However, there are differences regarding their physiological effect (Lehninger & Cox, 2014).Fatty acids are usually found in tissues and biological fluids, and are used in vital organism processes maintenance (Perini et al., 2010).They promote cell proper functioning and perform important functions for the organism, such as reducing the risks of cardiovascular and immunological disorders. In addition, omega-3 and omega-6 also promote blood pressure regulation, neuroprotection, mood stabilization, and inflammatory states reduction. They also help transmitting nerve impulses, among other important organism functions (Nuria et al., 2010; Perini et al., 2010).

Thus, lipids extracted from fungi may contribute to supply the demand for essential polyunsaturated fatty acids (Papanikolaou & Aggelis, 2002). The main producers of lipids are fungi, yeasts, and algae, while bacteria are bad producers (Wynn and Ratledge, 2005; Li Y. et al., 2008; Bellou et al., 2016). The lipid accumulation as a reserve storage is triggered by an excess of carbon source and one limiting nutrient, usually nitrogen. Under these conditions the carbon flux is directly channeled toward lipid synthesis and discrete oil droplets consisting of triacylglycerols are formed within the cells (Ratledge, 2004; Wynn and Ratledge, 2005).

Zygomycetes have shown to produce microbial oil from organic substances (Venkata Subhash and S. Venkata Mohan 2014, Dey et al 2011). Oleaginous Zygomycetes capable of producing lipids containing linoleic acid andγ-linolenic acid (GLA) (Ratledge 1994, Kavadia et al 2001). Genera of *Mucor*, *Mortierella*, *Absidia* and *Cunninghamella* had been extensively investigated as an alternative source for omega 6 poly unsaturated fatty acids production particularly GLA (Sancholle et al 2004).Oleaginous fungus species of *Cunninghamella* has been found to accumulate a high amount of lipid depending on the fermentation methods and culture conditions (Taha et al 2010). Of late, the focus has been to maximize PUFA production by genetic manipulation, mutagenesis, response surface methodology, as well as optimizing the media conditions of the selected strain. The aim of this work was to isolate *Cunninghamellaspp*. from different areas inside the country have ability to accumulate large quantities of unsaturated fatty acid. Subsequently, optimizing the physiological conditions for suitable growth and maximal unsaturated fatty acids production which would serve as models for bioprocess development.

MATERIAL AND METHODS

Media

Malt extract agar was used as isolation medium while production medium was composed of (in g/l): glucose 100 and yeast extract 10, with pH adjusted to pH=5.4. The 10 % (by volume) mycelial suspension of isolated culture was inoculated in 100 ml flask containing 25 ml of broth and incubated at 30^{o}C for 7 days.

Sampling and isolation Zygomycetes

Soil samples were collected from different Egyptians governorates as Giza, Gharbia, Alexandria and Al-Bohaira. One gram of each soil sample was serially diluted to 10^{-2} fold and plated on MEA plates. The plates were incubated at 28^{o}C for 3-5 days under controlled conditions. A single colony of *zygomycetes* class only was picked up and transferred repeatedly to a new MEA plate until pure cultures were obtained. These were grown on MEA slants as above and stored at 4^{o}C.

Routine and molecular identification of the fungal isolates

The isolates were identified initially by observing their morphological and microscopic characteristics **(Booth, 1971**). Then *Cunninghamellaspp.* were selected to identify it at the molecular level, the protocol used by **Abd-El-Haleem (2009)** was used. The primers used for the amplification and sequencing of 18S-rRNA-encoding genes were those described by **Suh and Nakase (1995).** The PCR products were sequenced using an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).The sequences were analyzed using the BLAST program (National Centre for Biotechnology Information) to determine the closest available database sequences.

Qualitative and quantitative techniques for lipid detection

Firstly, qualitative technique by dye (Nile-red) binding method, fungal biomass was stored in a dark with 0.5 mL PBS solution and 0.05 ml Nile-red solution (Nile red 25 µg Nile-red/ acetone 1000 ml) for 30 min **(Lim *et al*2003)**. Then, stained lipid bodies were photographed using fluorescence microscope (IX-70, Olympus, Tokyo, Japan) equipped with a CCD camera (U-CMT, Olympus, Tokyo, Japan). **Secondly**, quantitative technique by Sulfo-phospho vanillin method.**(Mishra *et al* 2014)**

Biomass determination and lipid extraction

Harvest and extraction of lipids from biomass were performed according to the procedure of **Nisha *et al*.(2009)**. Biomass production was determined by harvesting the cells by filtration followed by drying at 55–60°C overnight. The dry biomass was ground to fine powder, 1g of fungal dry powder was blended with 40 ml of chloroform/methanol (2:1), the mixtures were agitated for 20min in an orbital shaker at 20°C and then filtered with Whatman paper number 1, and 0.9% sodium chloride solution was added. The chloroform phase containing total fatty acid was obtained and then evaporated.

Methyl ester preparation and fatty acid analysis

The trans-esterification reactions were carried out using sulfuric acid as catalyst in flasks at following conditions: 30:1 molar ratio of methanol to oil, 160 rpm, 5h of reaction time, temperature at 55°C and 80% catalyst amount based on oil weight **(Liu et al 2004, Wu and Miao 2006).** The reaction mixture was cooled and undisturbed until two layers were formed in a separating funnel. The upper layer FAME was separated with petroleum ether and the final FAME product was obtained by evaporating the ether from the solution. The fatty acid methyl esters were analyzed by 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.

Taguchi method for optimizing lipid production

There are many factors affect lipid production by fungi. Temperature, pH, incubation time, carbon and nitrogen sources were tested for increasing lipid production using design of experiment (DOE) of Taguchi method in minitab17 software. Optimization process were performed with two stages as presented in tables 1 and 2, first stage includes preliminary steps to select the best carbon source (glucose, sucrose, starch and carboxy methyle cellulose) and nitrogen source (peptone, yeast extract, sodium nitrate and ammonium nitrate). In addition to, pH 4 and 7 and incubation period4,7and 10 days were tested. Second stage includes testing effect of five factors on lipid production in order to reach optimum conditions of *Cunninghamella sp. BO30.* **Table 3** shows five factors which involve temperature (15, 20, 25, 30 and 35 °C), initial pH (2, 3, 4, 5 and 6), incubation time (6, 7, 8, 9 and 10 days), different concentrations of sucrose (20, 40, 60, 80 and 100gl^{-1}) and different concentrations of peptone (1, 2, 3, 4 and 5 gl^{-1}). **Table 4** illustrates the L25 array of Taguchi design, where this design enables us to make more interactions between different levels of different factors to produce a high quantity of lipid by *cunninghamella sp.BO30*.

Table 1: Different levels of carbon and nitrogen sources

Factor	Level 1	Level 2	Level 3	Level 4
Carbon source	Glucose	Sucrose	Starch	Carboxymethyle cellulose
Nitrogen source	Peptone	Yeast extract	Sodium nitrate	Ammonium nitrate

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Table 2: L16 array of Taguchi design of carbon and nitrogen source

Experiment no.	Carbon	Nitrogen
1	1	1
2	1	2
3	1	3
4	1	4
5	2	1
6	2	2
7	2	3
8	2	4
9	3	1
10	3	2
11	3	3
12	3	4
13	4	1
14	4	2
15	4	3
16	4	4

Table 3: Taguchi design shows different factors and their levels

Factor	Level 1	Level 2	Level 3	Level 4	Level 5
Temperature °C	15	20	25	30	35
Initial pH	2	3	4	5	6
Time (days)	8	9	10	11	12
Sucrose (g/l)	20	40	60	80	100
Peptone (g/l)	1	2	3	4	5

Table 4: L25 array of Taguchi design of factors affecting lipid production

Experiment no.	Temperature level	pH level	Time level	Sucrose level	Peptone level
1	1	1	1	1	1
2	1	2	2	2	2
3	1	3	3	3	3
4	1	4	4	4	4
5	1	5	5	5	5
6	2	1	2	3	4
7	2	2	3	4	5
8	2	3	4	5	1
9	2	4	5	1	2
10	2	5	1	2	3
11	3	1	3	5	2
12	3	2	4	1	3
13	3	3	5	2	4
14	3	4	1	3	5
15	3	5	2	4	1
16	4	1	4	2	5
17	4	2	5	3	1
18	4	3	1	4	2
19	4	4	2	5	3
20	4	5	3	1	4

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21	5	1	5	4	3
22	5	2	1	5	4
23	5	3	2	1	5
24	5	4	3	2	1
25	5	5	4	3	2

Statistical Analysis

All the experiments were done in triplicate and statistical analysis was performed using Mini tab software (version 17). The values are given as means ± SD (standard deviations). Levels of significance were considered at $p \leq 0.05$ unless otherwise stated.

RESULTS AND DISCUSSION

Totally, 62 fungal isolates belong to Zygomycetes were isolated from different regions inside Egypt, 2out of 62 identified as *Cunninghamella spp.*, the first was isolated from agricultural land with beans and it was coded as BO30, whilethe second was isolated from rat dung and itwas coded as RD1. These two isolates were identified using light and scanning electron microscope as shown in figure 1 according to **Benny (2008).**

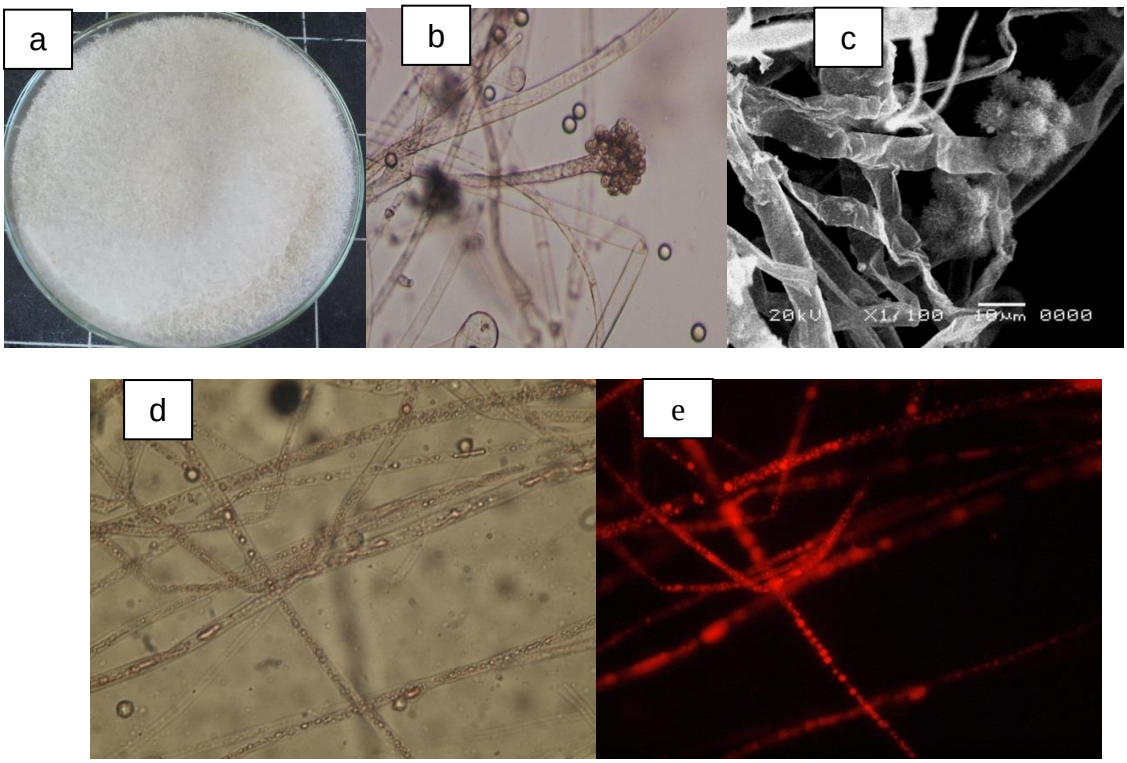

Figure 1: shows Cunninghamella sp.BO30(a) Surface growth on MEA. (b) Sporangium and sporangiophore under the light microscope. (c) Sporangium and sporangiophoreunder SEM. (d) Mycelial hyphae under the light microscope. (e) Mycelial hyphae under the fluorescence microscope.

Gema *et al*(2002)reported that Zygomycetes especially*Cunninghamella* have ability to storage large quantities of lipids as the single cell oil. These two *Cunninghamella spp.* were stained with Nile-red stain qualitatively, then followed by quantifying total lipids using sulfo-phospho vanillin (SPV) according to **Mishra *et al*.(2014). Table 5** showed *Cunninghamella sp.BO30*accumulated lipid approximately 0.53 gl^{-1} and lipid percent approximately 21.19% greater than *Cunninghamella sp.RD1* lipid quantity 0.29 gl^{-1} and lipid percent 13.88%.Consequently, *Cunninghamella sp.BO30*is considered as an oleaginous fungus where it accumulated lipids more than20% of its mycelial dry weight. *Cunninghamella sp.BO30*was identified at molecular level as *cunninghamella echinulata* and recorded in gen bank and taken accession number MG491518.

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Table 5: Dry biomass, total lipid and lipid percent of two Cunninghamella spp

Isolate	Dry biomass (g/l)	Lipid (g/l)	Lipid (%)
Cunninghamella sp.BO30	2.52 ±0.11[a]	0.53 ±0.01[a]	21.19 ±0.45[a]
Cunninghamella sp.RD1	2.09 ±0.10[b]	0.29 ±0.01[b]	13.88 ±0.06[b]

Optimization of lipid production

Taguchi method limited the number of experiments, so it is a powerful tool for investigating the effect of all parameters. Many researchers used Taguchi design for optimizing lipid production **(Enshaeieh*et al* 2014 & Chiranjeevi and Venkata Mohan, 2016)**. L16 array of Taguchi design was designed to detect the best carbon and nitrogen source for lipid production by *Cunninghamella sp.BO30*. **Table 6** showed that the trial no. 5 had the best yield in corresponding to the others, where this experiment showed that sucrose interacted with peptone give the highest quantity of lipid (0.87 gl^{-1}) with lipid content (25.16%) versus other carbon and nitrogen sources, although most previous studies showed glucose and yeast extract are the best for lipid production by many genera of Mucorales as *Mucor sp. and Cunninghamella sp*.**(Ahmed *et al*., 2006; Ling *et al.,* 2016)**. **Table 7**illustrated that lipid accumulation at pH 4 was 1.18 gl^{-1}which was more than 1.10 gl^{-1} at pH 7, but the dry biomass did not significant at different levels of pH. **Table 8**displayed that the second week is more effective than first week for lipid production, where lipid amount and lipid percent were 1.13 and 30.29% respectively at 10 days. All these preliminary optimum conditions followed L25 array design to apply interactions between different factors and their levels and detect optimum conditions to get high lipid quantity.

Table 6: Interactions between carbon and nitrogen source for lipid production using Taguchi design

Experiment no.	Dry biomass g/l	Lipid g/l	Lipid (%)
1	2.19±0.20[d]	0.49±0.030[c]	22.62±0.90[b]
2	0.75±0.02[hi]	0.13±0.002[g]	17.26±0.60[c]
3	0.91±0.02[gh]	0.13±0.001[g]	14.16±0.35[d]
4	0.91±0.08[gh]	0.09±0.004[gh]	10.02±0.59[e]
5	3.47±0.06[a]	0.87±0.021[a]	25.16±0.28[a]
6	1.84±0.11[e]	0.41±0.009[d]	22.17±0.92[b]
7	1.43±0.10[f]	0.20±0.016[f]	14.24±0.16[d]
8	0.60±0.11[i]	0.09±0.013[gh]	15.23±1.20[d]
9	2.69±0.10[b]	0.64±0.037[b]	23.70±0.61[ab]
10	2.39±0.10[cd]	0.45±0.013[cd]	18.95±0.96[c]
11	1.64±0.08[ef]	0.29±0.007[e]	17.49±0.44[c]
12	0.61±0.02[i]	0.09±0.001[gh]	14.65±0.31[d]
13	2.63±0.06[bc]	0.22±0.001[f]	8.43±0.22[ef]
14	1.44±0.04[f]	0.10±0.006[g]	7.29±0.43[fg]
15	1.53±0.05[f]	0.09±0.009[gh]	5.91±0.83[gh]
16	1.07±0.08[g]	0.05±0.002[h]	4.71±0.16[h]

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Table 7: Interactions between different pH and incubation time levels for lipid production by Cunninghamella sp.BO30

Factor	level	Dry biomass g/l	Lipid g/l	Lipid (%)
pH	**4**	**3.54±0.19^{a}**	**1.18±0.031^{a}**	**33.48±0.87^{a}**
	7	3.46±0.10^{a}	1.10±0.005^{b}	31.89±0.16^{b}
Time (Days)	4	3.12±0.11^{b}	0.54±0.035^{c}	17.32±0.84^{c}
	7	3.41±0.19^{ab}	0.76±0.061^{b}	22.38±0.58^{b}
	10	**3.73±0.12^{a}**	**1.13±0.021^{a}**	**30.29±0.72^{a}**

Table 8: Effect of main factors on lipid productivity using Taguchi design

Trial no.	Dry biomass g/l	Lipid g/l	Lipid (%)
1	0.25±0.05^{m}	0.02±0.001^{o}	7.74±0.44^{m}
2	0.53±0.06^{klm}	0.10±0.003^{n}	18.54±0.70^{ijk}
3	1.48±0.08^{fgh}	0.22±0.008^{klm}	15.14±0.58^{l}
4	1.68±0.07^{fg}	0.24±0.011^{jklm}	14.24±0.67^{l}
5	1.39±0.06^{hij}	0.26±0.013^{ijk}	18.44±1.00^{ijk}
6	1.19±0.06^{ij}	0.12±0.003^{n}	10.01±0.32^{m}
7	1.44±0.11^{ghi}	0.29±0.008^{hij}	20.18±0.59^{hi}
8	0.41±0.06^{lm}	0.07±0.002^{no}	16.86±0.42^{jkl}
9	1.49±0.08^{fgh}	0.31±0.013^{hi}	20.92±0.87^{ghi}
10	1.57±0.12^{fgh}	0.26±0.015^{ijk}	16.28±1.00^{kl}
11	1.15±0.06^{j}	0.25±0.008^{jkl}	21.86±0.75^{gh}
12	1.47±0.08^{fghi}	0.46±0.010^{ef}	31.38±0.70^{ab}
13	2.29±0.10^{d}	0.66±0.040^{d}	28.64±1.87^{bcd}
14	3.24±0.20^{b}	0.75±0.040^{c}	23.28±1.25^{fg}
15	0.65±0.06^{kl}	0.19±0.002^{m}	28.49±0.44^{cd}
16	2.68±0.08^{c}	0.75±0.042^{c}	27.82±1.57^{cde}
17	0.73±0.06^{k}	0.19±0.008^{lm}	26.53±1.10^{de}
18	1.65±0.08^{fgh}	0.42±0.007^{fg}	25.4±0.43^{ef}
19	2.03±0.08^{de}	0.39±0.003^{g}	19.4±0.17^{hij}
20	3.51±0.10^{b}	0.93±0.017^{b}	26.6±0.48^{de}
21	1.75±0.06^{ef}	0.52±0.007^{e}	29.72±0.44^{bc}
22	2.67±0.06^{c}	0.74±0.011^{c}	27.69±0.44^{cde}
23	4.20±0.16^{a}	1.43±0.031^{a}	34.04±0.74^{a}
24	1.49±0.08^{fgh}	0.33±0.018^{h}	22.14±1.24^{gh}
25	1.59±0.10^{fgh}	0.41±0.027^{fg}	25.86±1.73^{def}

L25 array design was used to apply interactions between different selected factors and their levels. **Table (8)**elucidated that experiment no. 23 is the most appropriate experiment for lipid production(1.43gl^{-1}) and dry biomass (4.2 gl^{-1}) among others. Figure 2 was designed to detect interactions and probabilities between all factors and levels to get the highest lipid production, **table (11)** illustrated that optimum conditions for *Cunninghamella sp.BO30*were; temperature 35 ˚C, pH 4, incubation time 9days, sucrose 20 g and peptone 5 g. All these results of experiment are significantly different ($p<0.05$) according to ANOVA analysis in minitab17 software.

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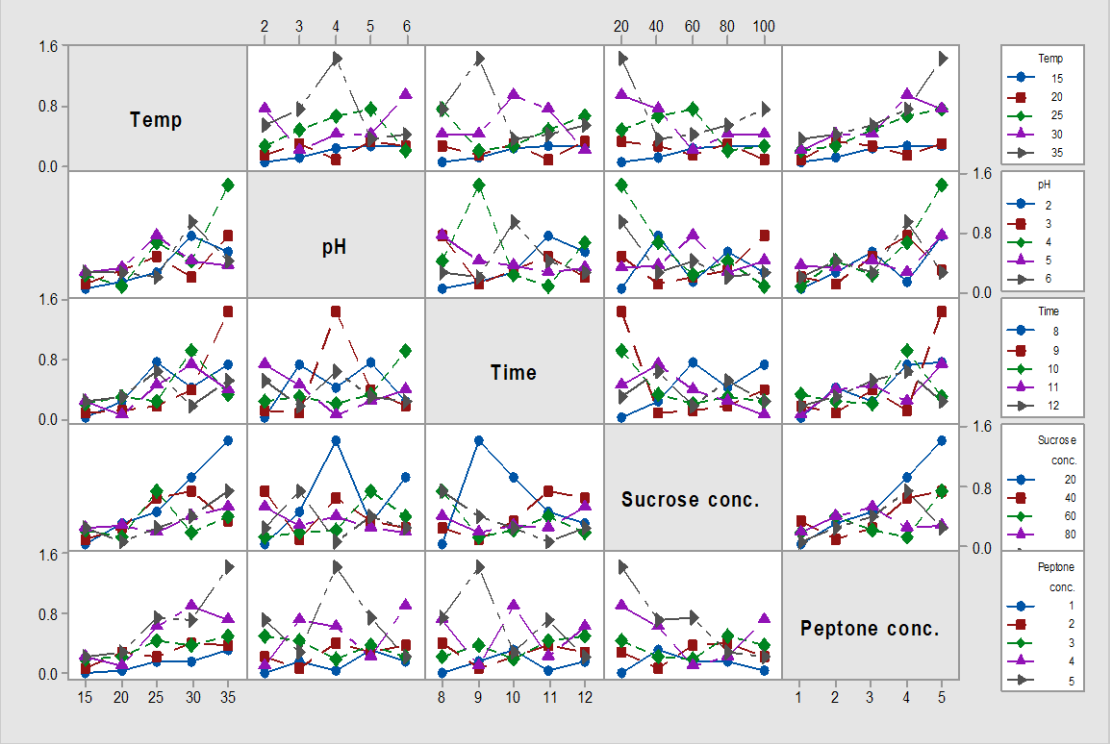

Figure 2: Interactions between different factors and their effects on lipid production by Cunninghamella sp.BO30

To evaluate effect of each factor on lipid productivity;Taguchi design was applied to detect contribution percentage for each factor in lipid productivity, **table (9)**showed different concentrations of peptone and temperature had significant effects on lipid productivity with contribution percentages 32.63 and 31.5%respectively.**Sukrutha *et al.* (2014)** reported that temperature influences growth, lipid content and fatty acid composition in Zygomycetous fungi. Whereas different concentrations of sucrose, pH and incubation time had weak effects. **Enshaeieh *et al.* (2014**) reported that temperature, glucose concentration, time of incubation and nitrogen concentration had a significant effect on lipid production, respectively. **Figure (2)** illustrated all interactions between all factors and different levels, optimum temperature after making interactions is 35, optimum pH is 4, 9 days in time, 20 g of sucrose and 5 g of peptone. Taguchi method is a modern design to increase target material production, this method was used in this study to increase lipid production from *Cunninghamella* where lipid quantity increased significantly from 0.53 to 1.43 gl^{-1} and lipid percent from 21.19 to 34.04%, therefore this method is a promising for optimization of lipid production to get the highest lipid amount.

Table 9: Main effects of factors on lipid productivity

Level	Temperature	pH	Time	Sucrose	Peptone
1	164.6	327.1	438.3	628.1	156.2
2	206.8	358.5	443.5	412.2	294.6
3	459	553.1	400	333.2	369
4	531.8	403.3	382.7	329.8	532.4
5	681.4	401.5	379.1	340.2	691.4
Delta	516.8	226	64.4	298.3	535.2
Rank	2	4	5	3	1
Percent%	31.5	13.77	3.92	18.18	32.63

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Fatty acid profile by gas chromatography mass spectroscopy

Fatty acid profile of *Cunninghamella sp.BO30*were performed at optimum conditions from Taguchi design. **Table 12 and Figure 3** showed that palmitic acid (C16:0) is the major fatty acid, followed by stearic acid (C18:0), oleic acid (C18: 1), linoleic acid (C18:2), and gamma-linolenic acids (C18:3) respectively, this result is in a harmony with **Silveira *et al.*(2010)**who reported that most of the lipids produced in the fungal biomass were mainly represented by palmitic (C16:0), oleic (C18:1) and linoleic (C18: 2) fatty acids, besides docosahexaenoic acid (DHA-C22: 6), which is a fatty acid relevant for health, as it is highly nutritional. Also, **Fakas *et al.* (2008)** detected palmitic (C16:0), stearic (C18:0), oleic (C18: 1), linoleic (C18:2), and gamma-linolenic (C18:3) fatty acids in the biomass produced by the fungus *Cunninghamella echinulata* when it was grown in Potato Dextrose Agar (PDA) medium containing glucose, whey concentrate, and hydrolyzed tomato residue, besides commercial corn gluten, corn infusion and yeast extract as nitrogen sources.

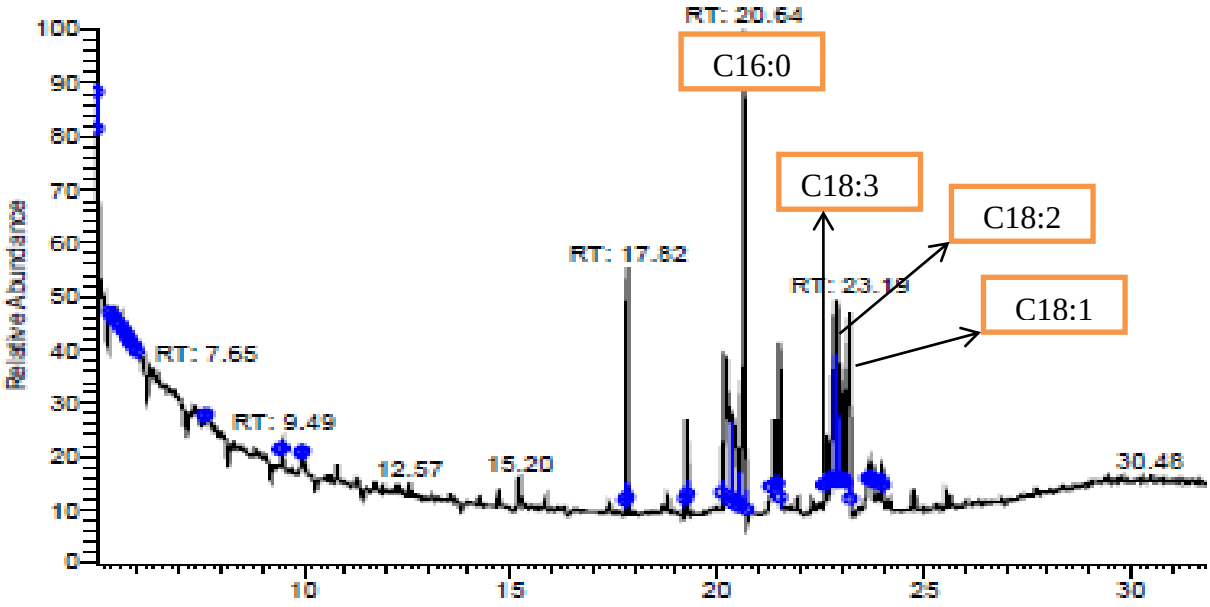

Figure 3: Chromatogram of Cunninghamella sp.BO30fatty acid profile at condition A

Degree of unsaturation in the fatty acid composition is known to influence by low temperature **(Jang *et al.*, 2005).** Therefore, *Cunninghamella sp.BO30* was cultured at optimum conditions for nine days then followed by three days at 15 ˚C. Subsequently, fatty acid profile at these conditions was significantly different. **Table 12** and **Figure 4**revealedthe presence of palmitic acid (C16:0) and stearic acid (C18:0) which sharply decreased from 54 to 32.01 and 11.75 to 2.83%, respectively. On the contrary, oleic acid (C18: 1) greatly increased from 14.15 to 43.01%. In addition to gamma-linolenic acid (C18:3) increased from 1.14 to 3.22%.Palmitoleic acid (C16:0)was appeared at 3.59%, while linoleic acid (C18:2) sharply decreased. Finally, at optimum conditions saturated fatty acid more than unsaturated fatty acid, but when these conditions followed by 3 days at 15 ˚C, saturated fatty acids sharply decreased from 76.23 to 38.51%, while unsaturated fatty acids increased from 23.73 to 50.48%.

Table 12: Fatty acids profile of Cunninghamella sp.BO30

Fatty acid composition (percentage of total fatty acid)							
Condition	**C16:0**	**C16:1**	**C18:0**	**C18:1**	**C18:2n6**	**C18:3n6**	**C20:0**
A*	**54.0**	------	**11.75**	**14.15**	**7.81**	**1.14**	--------
B**	**32.02**	**3.59**	**2.83**	**43.01**	**0.26**	**3.22**	**0.16**

A* means conditions of experiment no.23 in table 8

B** means conditions of experiment no.23 followed by 3 days at 15ºC

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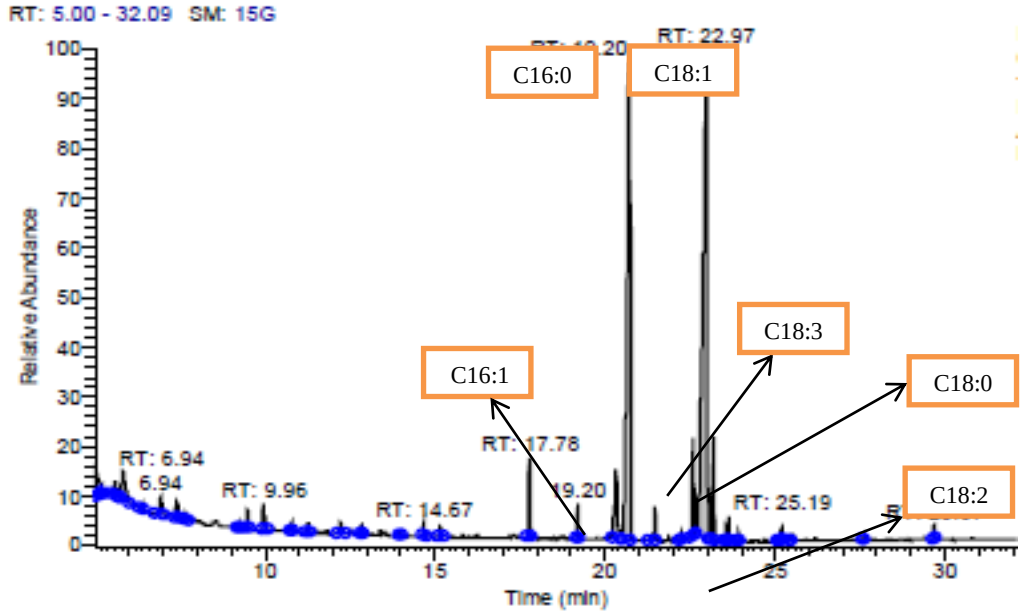

Figure 4: Chromatogram of Cunninghamella sp.BO30fatty acid profileat condition

CONCLUSION

Cunninghamella echinulata as a local strain was relatively higher producer of unsaturated fatty acids accumulated in its biomass among the tested zygomycetous fungi. Optimal conditions for maximum unsaturated fatty acids production were evaluated using Taguchi design. Lipid content of 34% was produced after nine days at 35 °C on media contains 20 grams sucrose and 5 grams peptone and initial media pH was adapted at 4. Nitrogen source and temperature were evaluated by Taguchi design as significant factors affect unsaturated fatty acids. Low temperature has influence on unsaturation rate of lipid; therefore, we conclude fatty acids profile at low temperature contains unsaturated fatty acids with high percent more than optimal temperature.

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