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Research article

Isolation and screening of promising oleaginous *Rhizopus sp*. and designing of Taguchi method for increasing lipid production

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Key words: *Rhizopus sp.*, Taguchi method, SFAs, USFAs, PUFAs, γ-lenolenic acid.

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Abstract

Oleaginous Zygomycetes have the ability to accumulate large amounts of lipids rich in polyunsaturated fatty acids. A total of 62 isolate from oleaginous Zygomycetes were isolated. 27 out of 62 identified as *Rhizopusspp* and screened for production of high quantity of lipid. 9 out of 27 have the capability of lipid production in high amount. *Rhizopus sp. Strain GB2* was selected for further optimization where it produces lipids equal to 1.01gl^{-1} with lipid content34.21%. Taguchi method was used to increase lipid production where many factors at different levels were selected for optimization process. Sucrose, peptone and temperature have a direct effect on lipid production with contribution percentage (25.8, 25.33 and 25.26%) respectively, in contrast pH and time have weak effect on lipid production. GC profile showed that saturated fatty acid (SFAs) more than unsaturated fatty acid (USFAs), where palmitic acid and oleic acid were dominant 43.68 and 18.36, respectively. On the other hand, when *Rhizopus sp.GB2* incubated at 15°C for three days after full incubation period, GC profile shifted to USFAs more than SFAs, where palmitic acid decreased to 40.55% and oleic acid increased to 44.43%, also γ-lenolenic acid (2.79%)and linoleic acid (0.10%) were appeared as polyunsaturated fatty acid (PUFAs). Therefore, *Rhizopus sp.GB2* is considered as promising oleaginous filametous fungi that can be used in industrial application for PUFAs production.

Introduction

Oils and fats are both classed as lipids; lipids are substances of vegetable and animal origin that widely found in nature and form the third major group of macronutrients after proteins and carbohydrates [1]. Lipids have many metabolic roles, for example, they act as storage materials in animals, plant and microbial cells and are also responsible for the structure of cell membranes, and protect the body against cold and other environmental influences, etc. One of their important physiological roles is that they are precursors of hormone-like compounds, which is performed mainly by polyunsaturated fatty acids [2-3]. Human body can produce saturated and mono-unsaturated fatty acids from food components, but it cannot synthesize polyunsaturated fatty acids. In other words, these have to be supplied externally from specific foodstuffs such as leafy vegetables and fish. This explains why polyunsaturated fatty acids are called "essential fatty acids" [4]. Polyunsaturated fatty acids (PUFAs) are valuable additives for food and pharmaceutical products due to their beneficial influence on human health [5]. Common examples include γ-linolenic acid (GLA) [6]. GLA is akey precursor for the biosynthesis of several prostaglandins that additionally presents anti-inflammatory and anti-cancer [7-8]. Therefore, the interest in PUFAs has attracted the attention for many researchers recently. The major source of PUFAs is marine fish. Their global stocks are gradually limited, resulting in inadequate production in the near future. Besides, the despicable tastes and odors of fish oil, shows that some fish especially salmon, sardine, tuna and hake, are often contaminated with heavy metals (cadmium, lead and mercury) and organic pollutants (dioxins, dioxin-like compounds and furans) that are toxic to human [9]. Related to health risks derived from the environment contaminant found in fish, a remarkable promotion of fish consumption as a source of omega-3 PUFAs has been reviewed scientifically for adverse and beneficial effects [10]. Thus, several sources containing various types of PUFAs are proposed alternatively, like, microalgae, bacteria, yeast, fungi and transgenic plants. Lipids Production from fungi have been obviously targeted to Zygomycetes especially of the genera Cunninghamella, Mucor, Mortierella, Rhizopus and Zygorhynchus [7, 11]. However, lipid accumulation was observed when the organisms were under stress conditions, for example, a high carbon to nitrogen ratio in the growth medium [12]. The aim of this work was to isolate and screen lipid-

producing *Rhizopus spp.,* and to optimize various process parameters for lipid production using Taguchi method to get a promising oleaginous *Rhizopus sp.*

Experimental

Materials and Methods

Chemical and reagents

Most of solvents and chemicals used were purchased from Sigma–Aldrich.

Sampling and isolation of oleaginous Zygomycetes

Soil samples were collected from various localities inside Egypt. 1 g of each soil sample was individually suspended in 1 ml of sterile distilled water, serially diluted to 10^{-2} fold and plated on malt extract agar (MEA) purchased from oxoid. The plates were incubated at 30°C for 3 days under controlled conditions. Single colony of fungus was isolated and transferred repeatedly to a new MEA plate until pure cultures were obtained. Pure cultures were grown on MEA slants and stored at 4°C until use.

Media used and cultivation conditions

MEA was used as screening medium while production medium was composed of (in g/l): glucose 100 and yeast extract 10, with pH adjusted to 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°C for 7 days.

Standard curve of lipid

The standard lipid stocks were prepared using commercial canola oil at 20 mg in 10 ml chloroform. Different volumes of standard oil solution equal to concentration from to was added to empty tubes. The tubes were kept at 60 °C for 10 min to evaporate the solvent then 100 µl of water was added. Afterthat sample was prepared by following sulfo-phospho vanillin reaction methods. Teflon-covered glass vials were used throughout all experiments [13].

Screening of oleaginous zygomycetes

Two techniques were followed for screening oleaginous *zygomycetes* for lipid production,. Firstly, qualitative analysis by dye (Nile-red) binding method, fungal biomass was stored in 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 [14]. 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 analysis by Sulfo-phospho vanillin, phosphovanillin reagent was prepared and lipid quantified according to [13].

Classical and molecular identification of the fungal isolates

Observing the morphological characteristics (color, texture appearance, and diameter of the colonies) and microscopic characteristics were performed to identify fungal isolates cultivated on potato dextrose agar medium**,** using light and electron microscope. To identify fungal isolates at the molecular level, the protocol used by [15] was used. The primers used for the amplification and sequencing of 18S-rRNA-encoding genes were those described by [16]. 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.

Cell dry mass determination and lipid extraction

Biomass production was determined by harvesting the cells by filtration followed by drying at 55–60 °C overnight or until constant weight then determined. 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 20 min in an orbital shaker at 20°C and then filtered with Whatman paper number 1, then sodium chloride solution 0.9% was added. The solvent containing lipids was separated then evaporated then lipids were determined [17].

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 [18-19]. 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.

Evaluation effects of different parameters on lipid production by taguchi method

Design of experiment (DOE) was done by taguchi method for evaluation effects of different factors on lipid production by fungi viz. carbon source, nitrogen source, incubation period, pH and temperature. Figure 1 shows many steps were carried out during optimization process where include selection the best carbon and nitrogen source, selection optimum pH, and the best incubation time. This stage followed by designing five factors at different five levels by Taguchi method. These factors include temperature, initial pH, incubation period, different concentrations of carbon source, and different concentrations of nitrogen source to enhance lipid production by *Rhizopus sp.GB2.*

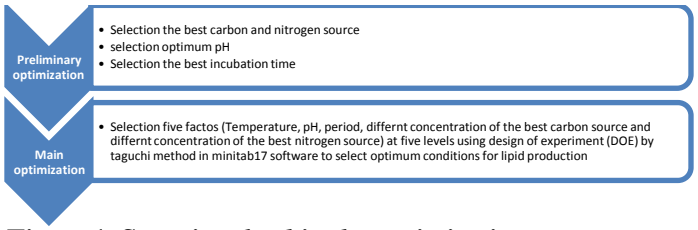

Figure 1. Steps involved in the optimization process

i) Preliminary optimization

Preliminary optimization aimed at selection optimum conditions for lipid production by *Rhizopus sp.GB2*, this stage include many steps as selection the best carbon and nitrogen source, optimum pH, and optimum incubation time in Table 1 and Table 2. This stage followed by main optimization.

ii) Main optimization

Taguchi method was used in this process where five factors and their five levels were designed. Table 3 shows five factors which involve temperature (15, 20, 25, 30 and 35^{o}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 100 gl^{-1}) and different concentrations of peptone (1, 2, 3, 4 and 5 gl^{-1}). Table 4 illustrates L25 array of Taguchi design, where this design enable us to make more interactions between different levels of different factors to produce high quantity of lipid by *Rhizopus sp.GB2.*

Table 1. Preliminary optimization shows different factors and their levels

Factor	Level 1	Level 2	Level 3	Level 4
Carbon source	Glucose	Sucrose	Starch	CMC
Nitrogen source	Peptone	Yeast extract	Sodium nitrate	Ammonium nitrate
pH	4	7	--	--
Incubation time (days)	4	7	10	--

Table 2. L16 array of Taguchi design

Trial 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. Main optimization by Taguchi design shows different factors and their levels

Factor	Level 1	Level 2	Level 3	Level 4	Level 5
Temperature (^{O}C)	15	20	25	30	35
Initial pH	2	3	4	5	6
Time (days)	5	6	7	8	9
Sucrose (g/l)	20	40	60	80	100
Peptone (g/l)	1	2	3	4	5

Table 4. L25 array of different factors and their levels

Trial 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
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

Results and Discussion

Screening of filamentous fungi for lipid production

Sixty-two fungal isolates belongs to Zygomycetes, 27 out of 62 identified as Rhizopusspp using light and scanning electron microscope in figure 2 according to [20]. Zygomycetes are potential sources either of polyunsaturated fatty acids or lipid suitable for biodiesel production [21-23]. Productions of fungal oils have been obviously targeted to Zygomycetes especially of the genera Cunningghamella, Mucor, Mortierella, Rhizopus and Zygorhynchus [7, 24]. *Rhizopus spp.* were screened for lipid formation qualitatively then quantitatively. Firstly, preliminary screening of *Rhizopus spp.* for high lipid formation using Nile-red staining technique qualitatively, 9 out of 27 can form high quantity of lipid as oil droplets, these nine isolates followed by secondary screening. Secondary screenings for target isolates were carried out quantitatively by using SPV method to estimate quantity of lipid produced by these isolates. Phospho-vanillin reagent was prepared by initially dissolving 0.6 g vanillin in 10 ml absolute ethanol; 90 ml deionized water and stirred continuously. Subsequently 400 ml of concentrated phosphoric acid was added to the mixture, and the resulting reagent was stored in the dark until use. To ensure high activity, fresh phospho-vanillin reagent was prepared shortly before every experiment run. For SPV reaction of the algal culture for lipid quantification, a known amount of biomass in 100 µl water, which are either suspended in a known volume of liquid culture or harvested via centrifugation at 4000 RPM for 5 min, was used. 2 mL of concentrated (98%) sulfuric acid was added to the sample and was heated for 10 min at 100 ºC, and was cooled for 5 min in ice bath. 5 mL of freshly prepared phospho-vanillin reagent was then added, and the sample was incubated for 15 min at 37 ºC incubator shaker at 200 rpm. Absorbance reading at 530 nm was taken in order to quantify the lipid within the sample [13], in addition to select the most potent oleaginous *Rhizopus spp.* using canola oil as standard for calculation quantity of lipid produced from selected fungal isolates.

All nine *Rhizopus spp.* which screened for lipid production considered oleaginous fungi except two *Rhizopus spp.* MICE 1 and RAT 6, where these two species can produce lipid less than 20 % of mycelial dry weight, therefor these two species considered non oleaginous fungi. In Table 1 ANOVA analysis in minitab17 statistical software was used to select the most potent *Rhizopus spp.* for lipid production. Table 5 shows *Rhizopus spp.GB2* is the highest for total lipid production and lipid percentage among other *Rhizopus spp.*, where this isolate produced 1.01 $gl^{-1}$and 34.21% as lipid percentage. Using the alignment search tools (http://blast.ncbi.nlm.nih.gov), the18S rDNA homology value of strain GB2 showed similarity (99%) with *Rhizopusoryzae*. The GenBank accession number for strain GB2 nucleotide sequence is MG518370. *Rhizopusoryzae-JSK3, Rhizopus sp.JSK6, Rhizopus sp. JSK8, Rhizopus sp. JSKp* were isolated by [25] which

produces 13, 15, 10 and 12% of mycelial dry weight (1.59, 6.73, 1.25 and 2.56 gl^{-1}, respectively).

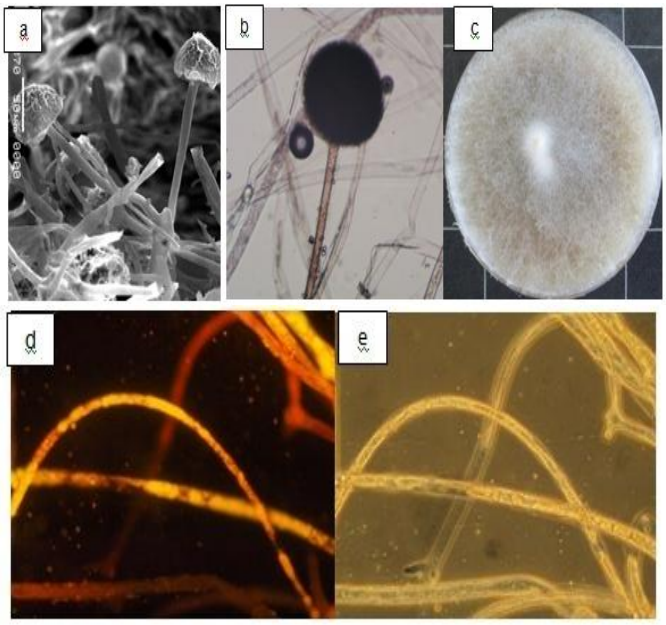

Figure 2. shows *Rhizopus sp. GB2.*pictures (a) surface growth on MEA. (b) sporangium and sporangiophore under light microscope. (c) sporangium and sporangiophore of under SEM. (d) mycelial hyphae under light microscope. (e) mycelial hyphae under fluorescence microscope using Nile-red stain.

Table 5. Screening of *Rhizopus spp.* for lipid production using SPV method

Rhizopussp	Dry biomss (g/l)	Total lipid (g/l)	Lipid (%)
CA1-2	1.89 ± 0.12^{cd}	0.41 ± 0.02^{d}	21.71 ± 0.74^{e}
CB4	1.26 ± 0.11^{e}	0.38 ± 0.01^{d}	29.49 ± 0.54^{b}
FB2	3.6 ± 0.08^{a}	0.93 ± 0.04^{b}	25.24 ± 1.75^{cd}
FC	3.45 ± 0.18^{a}	0.94 ± 0.02^{ab}	26.93 ± 0.50^{c}
GA11	2.57 ± 0.16^{b}	0.65 ± 0.04^{c}	23.73 ± 0.78^{de}
GB2	**2.88 ± 0.08^{b}**	**1.01 ± 0.01^{a}**	**34.21 ± 0.40^{a}**
MICE1	1.68 ± 0.08^{d}	0.27 ± 0.01^{e}	15.87 ± 1.07^{g}
RAT6	1.93 ± 0.11^{cd}	0.37 ± 0.02^{d}	18.69 ± 0.53^{f}
SF	2.02 ± 0.04^{c}	0.60 ± 0.01^{c}	29.57 ± 0.73^{b}

Enhancement of lipid production using taguchi method

i) Preliminary optimization process

Different factors were used in this process as carbon, nitrogen source, temperature, pH, and incubation time. Taguchi design was used for optimizing lipid production [26-27]. L16 array of Taguchi design was used to detect the best carbon and nitrogen source for lipid production by most potent fungus, table 6 shows trial no. 5 is the best trial among other trials, this trial shows sucrose with peptone give highest quantity of lipid (3.11gl^{-1}) with lipid content (35.31%) among other carbon and nitrogen sources as shown in table 6 but most previous studies showed glucose and yeast extract are the best for lipid production by many genera of mucorales as *Mortierella sp., Mucor sp. and cunninghamella sp.* [28-30]. Two levels of pH were used, table 7 shows pH 4 is better than pH 7 for lipid production. Table 8 illustrates the period between 7 and 10 days is optimum for lipid production. All these optimum conditions followed by main optimization stage to apply interactions between different factors and their levels to get high lipid quantity. All these results are significantly different ($p<0.05$) according to ANOVA analysis in minitab17 software.

ii) Main optimization process

This process was carried out to apply interactions between different selected factors and their levels. Taguchi method was used to design this experiment where L25 array design was used in table 4. Table 9 shows that trial no. 23 is the best trial for lipid production(1.69gl^{-1}) among others, where conditions of this trial were temperature 35^{o}C, pH 4, incubation time 6 days, sucrose 20 g and peptone 5 g which showed in table 11.

Table 6. Effect of carbon and nitrogen source on lipid production by *Rhizopus sp.GB2* using Taguchi design

Trial no.	Dry biomass (g/l)	Total lipid (g/l)	Lipid (%)
1	**2.76 ± 0.21^{b}**	**0.92 ± 0.03^{c}**	**33.20±1.22^{ab}**
2	0.91± 0.10^{de}	0.15 ± 0.00^{f}	16.60± 0.48^{efg}
3	0.39± 0.06^{ij}	0.07 ± 0.00^{hi}	17.36±0.20^{ef}
4	0.65± 0.06^{efgh}	0.15 ± 0.00^{f}	23.25± 0.28^{d}
5	**3.11 ± 0.10^{a}**	**1.10 ± 0.01^{a}**	**35.31± 0.44^{a}**
6	0.72± 0.04^{defg}	0.22 ± 0.01^{e}	31.03± 1.84^{bc}
7	0.49± 0.06^{ghi}	0.12 ± 0.00^{fg}	24.19± 0.41^{d}
8	0.53± 0.08^{fghi}	0.10 ± 0.00^{gh}	18.20± 0.42^{ef}
9	2.91± 0.08^{ab}	0.99 ± 0.02^{b}	34.15± 0.86^{a}
10	1.40± 0.12^{c}	0.40 ± 0.04^{d}	28.92± 2.86^{c}
11	0.33± 0.06^{ij}	0.06 ± 0.00^{hi}	19.07± 0.68^{e}
12	0.40± 0.04^{hij}	0.05 ± 0.00^{hi}	13.65± 0.49^{g}
13	0.21± 0.02^{j}	0.07 ± 0.00^{hi}	32.35± 0.54^{ab}
14	0.65± 0.05^{efgh}	0.12 ± 0.00^{fg}	18.01± 0.62^{ef}
15	0.92± 0.08^{d}	0.15 ± 0.01^{f}	15.93± 0.63^{fg}
16	0.77± 0.06^{def}	0.05 ± 0.00^{i}	6.25± 0.42

Table 7. Effect of different pH levels on lipid production by *Rhizopus sp.GB2*

pH	Dry biomass g/l	Total lipid g/l	Lipid (%)
4	**4.06 ± 0.08^{a}**	**1.30 ± 0.07^{a}**	**32.16 ± 1.87^{a}**
7	3.61 ± 0.06^{b}	1.07± 0.02^{b}	29.58 ± 0.6^{a}

Table 8. Effect of different incubation periods on lipid production by *Rhizopus sp.GB2*

Period	Dry biomass g/l	Total lipid g/l	Lipid (%)
4	3.67 ±0.08^{b}	0.98 ± 0.05^{b}	26.83 ± 1.40^{b}
7	**3.87 ±0.06^{a}**	**1.37 ± 0.004^{a}**	**35.47 ± 0.11^{a}**
10	3.95 ±0.06^{a}	1.27 ± 0.08^{a}	32.17 ± 2.23^{a}

Table 9. Effect of main parameters on lipid production by *Rhizopus sp.GB2* using Taguchi design

Trial no.	Dry biomass g/l	Total lipid g/l	Lipid (%)
1	0.32 ± 0.04^{k}	0.10 ±0.001^{l}	32.16 ±0.44^{efg}
2	0.75 ± 0.06^{j}	0.27 ±0.008^{jk}	35.66 ±1.20^{bcdef}
3	1.16 ± 0.08^{hi}	0.42 ±0.018^{hi}	35.85 ±1.62^{bcde}
4	0.89 ± 0.06^{ig}	0.29 ±0.003^{jk}	32.73 ±0.42^{defg}
5	1.63 ± 0.06^{f}	0.27 ±0.010^{jk}	16.78 ±0.63^{k}
6	1.57 ± 0.06^{fg}	0.48 ±0.006^{gh}	30.45 ±0.42^{gh}
7	0.81 ± 0.06^{j}	0.29 ±0.004^{jk}	35.85 ±0.58^{bcde}
8	0.67 ± 0.06^{j}	0.13 ±0.002^{l}	20.20 ±0.42^{jk}
9	1.57 ± 0.05^{fg}	0.49 ±0.013^{gh}	31.29 ±0.88^{fgh}
10	1.47 ± 0.06^{fg}	0.30 ±0.008^{jk}	20.60 ±0.56^{jk}
11	0.71 ± 0.06^{j}	0.27 ±0.021^{jk}	38.42 ±3.01^{abc}
12	1.99 ± 0.02^{de}	0.83 ±0.058^{b}	41.74 ±2.96^{a}
13	2.08 ± 0.17^{de}	0.63 ±0.032^{de}	30.45 ±1.54^{gh}
14	2.41 ± 0.06^{bc}	0.73 ±0.023^{c}	30.26 ±0.98^{gh}
15	0.87 ± 0.02^{j}	0.23 ±0.010^{k}	26.93 ±1.21^{hi}
16	2.23 ± 0.06^{cd}	0.82 ±0.017^{b}	36.61 ±0.75^{bcd}
17	1.31 ± 0.06^{gh}	0.40 ±0.021^{i}	30.26 ±1.64^{gh}
18	1.91 ± 0.10^{e}	0.52 ±0.005^{fg}	27.21 ±0.26^{hi}
19	1.17 ± 0.08^{h}	0.25 ±0.011^{jk}	21.34 ±0.91^{j}
20	2.55 ± 0.06^{b}	0.82 ±0.073^{b}	32.06 ±2.88^{efg}
21	1.47 ± 0.08^{fg}	0.40 ±0.025^{i}	26.94 ±1.74^{hi}
22	2.00 ± 0.17^{de}	0.69 ±0.020^{cd}	35.34 ±1.01^{cdefg}
23	**4.29 ± 0.10^{a}**	**1.69 ±0.023^{a}**	**39.45 ±0.54^{ab}**
24	1.47 ± 0.06^{fg}	0.57 ±0.015^{ef}	38.89 ±1.00^{ab}
25	1.35 ± 0.16^{gh}	0.32 ± 0.017^{j}	$^{23.45}$ 1.30^{ij}

All these results of trials are significantly different ($p<0.05$) according to ANOVA analysis in minitab17 software. To evaluate effect of each factor on lipid productivity, Taguchi design was used to detect contribution percentage for each factor in lipid productivity, table 10 and figure 3 show peptone, sucrose and temperature had significant effects on lipid productivity, respectively, whereas pH and incubation time had weak effects. Temperature, glucose concentration, time of incubation and nitrogen concentration had significant effect on lipid production, respectively [26].

Fatty acid profile by gas chromatography

FAME composition showed higher ratios of saturated fatty acid than unsaturated fatty acid at optimum conditions which optimized by Taguchi design, this result completely agreed with [31]. Table 12 shows SFAs include palmitic acid and stearic acid as dominant fractions 43.68, 17.72%, respectively, and other SFAs had low percentage as pentadecanoic acid, myristic acid, margaric acid and arachidic acid. While as, oleic acid is considered the major fraction among UFAs, this full agreement with [31].

On the contrary, FAME percentages were completely changed and new PUFAs were appeared when incubation time was increased for extra 3 days at 15^{o}C, SFAs were decreased to 47.82%, in contrast, USFAs were sharply increased to 51.45%. Broughton [32] proved that incubation at 15°C produced the highest unsaturation index value therefore suggest temperature has an effect on fungal lipid composition, and that lower temperatures may increase lipid unsaturation levels. Also, low temperature leads to appearance two omega six PUFAs γ-lenolenic acid (2.79%) and linoleic acid (0.10%). *Rhizopus sp.* has been found to produce C18:3 n6, which produced 4.37 mg/g of dry substrate [33].

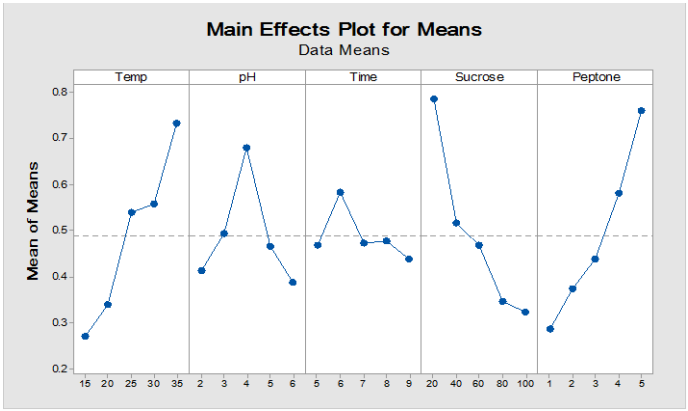

Figure 3. Main effect of each factor and its influence on lipid production by *Rhizopus sp.GB2.*

Table 10. Main effects of factors on lipid production by *Rhizopus sp. GB2*

Level	Temp.	pH	Time	Sucrose	Peptone
1	0.2701	0.4128	0.4685	0.7870	0.2874
2	0.3402	0.4939	0.5846	0.5177	0.3730
3	0.5396	0.6793	0.4732	0.4674	0.4388
4	0.5593	0.4672	0.4775	0.3463	0.5817
5	0.7325	0.3884	0.4379	0.3233	0.7608
Delta	0.4623	0.2909	0.1467	0.4637	0.4735
Rank	3	4	5	2	1
Percent	25.26%	15.90%	7.71%	25.33&	25.8%

Table 11. Optimum conditions for lipid production by *Rhizopus sp. GB2*

Factor	Level description	Level
Temperature	35^{o}C	5
pH	4	3
Incubation time	6 days	2
Sucrose	20 g	1
Peptone	5 g	5

Table 12. GC profile of fatty acid obtain from validation trials (trial no. 23)

Fatty acid name	Type	Fatty acid (%) at Condition A*	Fatty acid (%) at Condition B**
Myristic acid (C14:0)	SFAs	8.13	1.20
Pentadecanoic acid(C15:0)	SFAs	9.25	1.28
Palmitoleic acid (C16:1n9c)	MUFAs	0.3	4.13
Palmitic acid (C16:0)	SFAs	43.68	40.55
Margaric acid (C17:0)	SFAs	1.24	0.10
Gamma-linolenic acid (C18:3n6)	PUFAs	----	2.79
Oleic acid (C18:1n9c)	MUFAs	18.36	44.43
Stearic acid(C18:0)	SFAs	17.72	4.53
Linoleic acid (C18:2n6c)	PUFAs	----	0.10
Arachidicacid (C20:0)	SFAs	0.35	0.16

Conclusion

The results of this study showed that the isolate strain of *Rhizopusoryzae* is a promising fungus for lipid production particularly unsaturated fatty acids. This study revealed that Taguchi method is considered excellent in optimization processes particularly lipid production where this method showed that nitrogen, carbon source and temperature have significant effects on lipid production. Also, optimum conditions were detected by this method, where lipid yields was 1.69gl^{-1} with lipid content 39.45% by *Rhizopusoryzaeat 35°C* and pH 4 for 6 days. This ability shows the potential of this local strain in industrial lipid production.

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