Al-Saeed University Journal of Applied Sciences
journal@alsaeeduni.edu.ye
Vol (7), No(2), Dec., 2024
ISSN: 2616 – 6305 (Print) ISSN: 2790-7554 (Online)

Optimization of Cultural Conditions for Enhanced Lipid Accumulation by a Local Isolate of *Cunninghamella sp*. using Response Surface Methodology

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Received:11 /11/2024 **Accepted: 2/10/2024**
Journal Website: **https://journal.alsaeeduni.edu.ye**

تحسين الظروف المزرعية لتحسين انتاج الدهون بواسطة عزلة محلية من فطر الكانينجهاميلا باستخدام منهجية الاستجابة السطحية

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الملخص

يعد إنتاج الدهون الميكروبية بواسطة الفطريات الدهنية مصدرًا واعدًا لإنتاج الأحماض الدهنية المتعددة غير المشبعة، والتي تعتبر مواد ذات قيمة للاستخدامات الغذائية والصيدلانية، ويعد تحسين ظروف البيئية والزراعية أمرًا ضروريًا، لتعزيز إنتاج الدهون الميكروبية. يهدف هذا البحث إلى تحسين إنتاج الدهون باستخدام عزلة محلية من فطر الكانينجهاميلا، حيث تم دراسة تأثير عدة عوامل مثل مصدر الكربون، ومصدر النيتروجين، ودرجة الحموضة، ووقت التحضين على تراكم وإنتاج الدهون بواسطة فطر الكانينجهاميلا بطريقة تقليدية تعتمد على تغيير عامل واحد في كل مرة، أظهرت النتائج أن أفضل مصدر للكربون كان الجلوكوز، وأفضل مصدر للنتروجين كان نترات الصوديوم. كما وجد أن القيمة المثلى للأس الهيدروجيني (درجة الحموضةpH) هي 6.0 وأن أفضل وقت للتحضين هو 5 أيام، بالإضافة الى ذلك، تم تحسين تركيز الجلوكوز، ونترات الصوديوم، ودرجة الحموضة، لتحسين إنتاج الدهون باستخدام منهجية الاستجابة السطحية (RSM)، وتم تطبيق تصميم مركب مركزي(CCD) ، واستخدام نموذج الانحدار متعدد الحدود مع الحد التربيعي لتحليل البيانات التجريبية باستخدام تحليل التباين (ANOVA)، وأظهرت نتائج التحليل أن التركيز الأمثل للجلوكوزكان 38.28 جم/لتر، ونترات الصوديوم 0.48 جم/لتر، وذلك عند قيمة pH بلغت 5.79، مما أدى إلى تراكم للدهون بنسبة 25.4% (وزن/وزن)، وكشف النموذج التربيعي أن الأس الهيدروجيني (درجة الحموضة) كان العامل الأكثر تأثيرًا في تحسين انتاج الدهون بواسطة فطر الكانينجاميلا، مما يدل على قدرة هذه العزلة المحلية في تحقيق تراكم فعال لهذه الدهون.

الكلمات المفتاحية: فطر كانينجهاميلا، الأحماض الدهنية العديدة غير المشبعة، الدهون الميكروبية، التحسين، منهجية الاستجابة السطحية.

Optimization of Cultural Conditions for Enhanced Lipid Accumulation by a Local Isolate of *Cunninghamella sp*. using Response Surface Methodology

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Abstract

Microbial lipid production by oleaginous fungi offers a potential promising source for producing polyunsaturated fatty acids (PUFAs), valuable compounds for nutraceutical and pharmaceutical applications. Optimization of culture conditions is essential for enhancement of microbial lipid production. The aim of this study is to improve the lipid synthesis using a local oleaginous mold of *Cunninghamella sp*. The effects of several factors including carbon source, nitrogen source, pH and incubation time on lipid accumulation by *Cunninghamella sp* were investigated conventionally (one-variable-at-a-time). The results showed that the most effective carbon source was glucose and sodium nitrate was the best nitrogen source for lipid synthesis. The optimum pH and incubation time were found to be 6.0 and 5 days, respectively. Furthermore, glucose concentration, sodium nitrate and pH were further optimized to maximize lipid production using response surface methodology (RSM). A central composite design (CCD) was applied, and a polynomial regression model with a quadratic term was used to estimate the experimental data using analysis of variance (ANOVA). Results of RSM-CCD optimization indicated that the optimal concentrations of glucose and sodium nitrate were 38.28 g/L glucose, 0.48 g/L, respectively, at a pH value of 5.79, resulting in a lipid accumulation of 25.4% (w/w). The quadratic model revealed that pH was the most influential factor in lipid synthesis by *Cunninghamella sp*., a local isolate demonstrating efficient lipid accumulation potential.

Keywords: *Cunninghamella sp*; polyunsaturated fatty acids; microbial lipids; optimization; response surface methodology.

Introduction:

Lipids, a major macronutrient category encompassing oils and fats, play diverse roles in metabolism. They serve as an energy reserve in both animal and plant bodies, form the primary component of cell membranes, and act as precursors for biosynthesis of various hormones (Akpinar-Bayizit, 2014).

Lipids or oils derived from microorganisms are known alternatively as single-cell oils (SCOs) (Mhlongo, *et al.,* 2021). SCOs are hydrophobic compounds primarily composed of triacylglycerols which are derived from oleaginous microorganisms such as molds, yeasts, algae and bacteria (Rossi et al., 2011). Oleaginous microorganisms are lipid-producing organisms that can accumulate oils to over 20% of the total dry cell mass within their cells or mycelia (Chebbi *et al.,* 2019; Ratledge and Wynn, 2002). These microorganisms are emerging as promising cell factories for the production of SCOs (Leong *et al.,* 2018). SCOs possess significant industrial and economic value as potential to replace costly plant- or animal-based lipids. They have many applications and can be used as alternative to costly lipid sources such as fish oil, borage oil, cocoa butter, and evening primrose oil, as feedstock for second-generation biofuel production and as precursors for the synthesis of several oleochemical compounds (Kalampounias, *et al.,* 2024; Robles-Iglesias, *et al.,* 2023 ; Lewis *et al.,* 2000). The chemical composition of lipids produced by oleaginous fungi primarily consists of unsaturated or polyunsaturated fatty acids (PUFAs). Among these PUFAs, the fatty acid gamma-linolenic acid (GLA—$^{\Delta 6,9,12}$C18:3) is found in varying concentrations within the cellular lipids of these microorganisms (Bellou *et al.,* 2016; Papanikolaou and Aggelis, 2019). It is noteworthy that the gamma (γ) isomer of linolenic acid (GLA), rather than the more common alpha (α) isomer, is a unique characteristic of Zygomycetes (Ratledge, and Wynn, 2002; González-Fernández *et al.,* 2020).

Lipids containing GLA are highly valuable commodities for both the nutraceutical and pharmaceutical industries due to their properties such as anti-thrombotic, anti-irritant, and particularly anti-cancer. They have also demonstrated effective in treating various diseases, including inflammatory disorders, atopic eczema, and rheumatoid arthritis (Ratledge and Wynn, 2002; Ratledge, 1994). Despite the fact that vegetable oils containing GLA are generally expensive, the potential for developing a fermentation bioprocess

to produce significant amounts of fungal oils rich in GLA from low-cost substrates offers significant promise (Bellou *et al.*, 2016; Fazili *et al.*, 2022; Athenaki *et al.*, 2018).

The global demand for lipids is expanding at a faster rate than traditional production methods can meet due to the limited arable land and increase in population growth. Production of lipids by oleaginous microorganisms, with their distinctive properties and applications in the food, pharmaceutical, chemical, and energy industries, provides a promising and sustainable alternative oil source (Huang *et al.*, 2017).

Microbial SCO production offers several benefits over traditional animal and vegetable oils. Microbial sources have shorter life cycles, exhibit rapid growth rates, are easier to cultivate in bioreactors, are readily scalable, and are unaffected by climate changes (Li *et al.*, 2008) which make them ideal candidates for industrial-scale production (Dong, 2016).

Cunninghamella species are oleaginous fungi, belong to Zygomycetes, capable of synthesizing and accumulating polyunsaturated fatty acids (PUFAs). Among PUFAs, the bioactive GLA, which has potential anti-tumor activities, and docosahexaenoic acid (DHA), which is essential for human health, especially for brain and eye development, are particularly noteworthy (Kalampounias *et al.*, 2024; Manikan *et al.*, 2015).

In order to improve the productivity and yield of microbial lipids through fermentation processes, culture conditions and nutritional factors must be optimized. Lipid accumulation by oleaginous microorganisms and fatty acid content differ depending on the environmental conditions such as pH, incubation time, incubation temperature and the kind of employed microorganism as well as the type and availability of key nutrients (Ageitos *et al.*, 2011)

Fermentation optimization can be achieved using a "one variable at a time" method to determine the optimal conditions for maximizing growth and lipid synthesis. However, optimization using response surface methodology (RSM) has been used much more frequently in recent years to optimize medium content and culture conditions to improve the efficacy of fermentation process (Awad *et al.*, 2011). When compared to traditional optimization methods, RSM is a highly helpful technique that minimizes the number of tests, saving time and chemicals utilized in the experiments. RSM

also yields accurate response predictions, which makes it a favorable choice for experimental design (Singh *et al.*, 2013). The main designs in RSM include the Box–Behnken design (BBD), central composite design (CCD), user-defined design, D-Optimal design, historical data design and one-factor design. The BBD and CCD are the two most popular RSM statistical techniques and BBD only offers three levels for a single numerical variable, compared to five for CCD (Al-Shorgani *et al.*, 2015 and Bezerra *et al.*, 2008). Additionally, RSM offers an experimental model that forecasts the connections and interplay between a collection of experimental variables and observed results enabling the optimization of experimental setting (Zheng *et al.*, 2008).

The objective of the present study was to optimize lipid synthesis by RSM with CCD and analyzing the impact of glucose concentration, sodium nitrate concentration and pH as well as their interactions on lipid synthesis using a local fungal isolate of *Cunninghamella sp*

MATERIALS & METHODS:

Microorganism Strain:

Cunninghamella sp is a newly local fungal isolate which was recovered from a local soil from Taiz City. *Cunninghamella sp* was presumptively identified according to its morphology by light microscope. This fungal isolate was stored in potato dextrose agar (PDA) slant agar at 4℃.

Inoculum Preparation and Culture Conditions:

Fresh inoculum of *Cunninghamella sp* was prepared by transferring mycelium from PDA into a fresh potato dextrose broth (PDB) and incubated at 28°C for 48 hours. This fresh inoculum was then used to inoculate the production medium.

Lipid production was conducted in batch fermentation using flask scale (250 mL conical flasks) with a working volume of 100 mL. The production medium used for lipid production was basal medium. *Cunninghamella sp* was grown on basal medium that contains 50 g/L glucose, 0.5 g/L yeast extract, 0.4 g/L $MgSO_4.7H_2O$, 2 g/L KH_2PO_4 , 0.5 g/L $CaCl_2$, 0.05 g/L $CuSO_4$ $.5H_2O$, with an initial pH of 6.0. A 10% (v/v) of fresh mycelium from PDB was added as inoculum to 100 mL of basal media in 250 mL conical flasks and the mixture was then incubated under constant conditions for five days at 28°C. After the incubation period, the fungal culture was extracted using filtration

and the biomass was collected and repeatedly washed using purified water to eliminate any remaining medium (Youssef et al., 2020).

Verification of the Quadratic Model:

The predicted optimum conditions by the empirical model were validated using a set of batch culture fermentation under optimum conditions of glucose, sodium nitrate and initial pH.

Dry Cell Weight Determination:

Fungal biomass were harvested by centrifugation at 3000 rpm for 20 minutes followed by two rinsing the biomass pellet twice with 50 mL sterile distilled water. Samples were dried in hot air oven at 95 °C until reach constant weights. Biomass was expressed as dried weight in gram per liter of growth medium.

Lipid Extraction:

Extraction of lipid was conducted according to Folch *et al.*, (1957). After drying the mycelium biomass of Cunninghamella spp., the dried biomass was then crushed into a powder using a mortar and pestle, and was added to a mixture of 20 mL chloroform and 10 mL methanol and the mixture was stirred for 30 minutes. Filtration by filter paper (Wattmann No.1) was used to seperate the mixtures and sodium chloride solution 0.9% was added. Lipids-containing solvent was isolated using a separating funnel. The layer on the bottom of NaCl, methanol, and water were eliminated, and the residual of solvent was dried and then weighted. The lipid content was estimated by using Eqution (1) according to Nisha *et al.*, (2009) as follow:

$$\text{Lipid content (\%)} = \frac{\text{Weight of lipid (g)}}{\text{Weight of dried biomass (g)}} \times 100 \quad \text{(Equation 1)}$$

Experimental Design of Culture Conditions for Lipid Synthesis Optimization by *Cunninghamella sp*:

Optimization of culture conditions influencing the lipid biosynthesis by *Cunninghamella sp* were estimated in two steps, preliminary optimization using covenantal method of one-variable-at-a-time (OVAT) and final optimization (secondary) using RSM with CCD.

Preliminary optimization for lipid production by a local isolate of *Cunninghamella sp* was conducted to evaluate the impact of various parameters including various carbon sources, nitrogen sources and initial pH and incubation time using (OVAT) method. The tested carbon sources include glucose, fructose, lactose, maltose, sucrose and starch. To choose the

best nitrogen source that give the highest lipid production, yeast extract (the original nitrogen source in basal medium) was replaced with three other nitrogen sources, one at a time, on equal nitrogen bases (0.5 g/L); sodium nitrate, peptone, and ammonium acetate. The influence of initial pH value of the fermentation medium on fungal biomass growth and lipid accumulation was studied which ranged from pH 4 to 9. Incubation time of the fermentation culture was varied from 4 days to 7 days in order to estimate the suitable period for maximal lipid production by local strain of *Cunninghamella sp*.

Secondary optimization for lipid production was carried out based on the results obtained from the preliminary optimization. Three variable parameters including glucose concentration, sodium nitrate concentration and initial pH were chosen at two levels utilizing the design of experiment (DOE) by RSM based on CCD. The chosen ranges of the three variables are as follow: glucose concentration 20 – 60 g/L, sodium nitrate concentration 0 – 1 g/L and initial pH of the medium 4 – 9. Table (1) shows the ranges of the tested three variables.

Table (1): The level of variable parameters affecting lipid production by *Cunninghamella sp*

Parameter	Unite	Level (-1)	Level (+1)
Glucose (X_1)	**g/L**	**20**	**60**
Sodium nitrate (X_2)	**g/L**	**0**	**1**
pH (X_3)	**-**	**4**	**9**

Experimental design by RSM-CCD with six-center point replications gave 20 experimental runs which were conducted with various combinations of three independent variables (Table (2)). These experiments took place in batch fermentation cultures of *Cunninghamella sp* within 250 mL conical flasks, each containing a working volume of 100 mL. The inoculum size was 10% for each culture and the incubation temperature was set at 28°C for 5 days incubation time. Statistical analysis, performed by an Analysis of Variance (ANOVA) with DOE software was employed to determine the best culture conditions for the maximum lipid production by the strain of *Cunninghamella sp*.

Table (2): Experimental design matrix using RSM-CCD for three variables for lipid optimization by *Cunninghamella sp*

Run No.	X_1: Glucose (g/L)	X_2: Sodium nitrate (g/L)	X_2: pH
1	60.00	0.05	9.0
2	60.00	1.00	4.0
3	40.00	0.53	2.2
4	40.00	0.53	6.5
5	20.00	1.00	9.0
6	40.00	0.53	6.5
7	40.00	0.53	6.5
8	60.00	0.05	4.0
9	20.00	1.00	4.0
10	40.00	0.53	6.5
11	73.64	0.53	6.5
12	40.00	0.53	6.5
13	40.00	0.00	6.5
14	6.36	0.53	6.5
15	20.00	0.05	4.0
16	40.00	0.53	10.7
17	60.00	1.00	9.0
18	40.00	0.53	6.5
19	20.00	0.50	9.0
20	40.00	1.32	6.5

Results and Discussion

Previous studies have identified that incubation temperature, incubation period, initial pH value and the sources of carbon and nitrogen play as crucial factors effecting lipid synthesis by oleaginous microorganisms (Enshaeieh *et al.,* 2014). In this investigation, the initial optimization step utilized a "one variable at a time" experimental method to determine the major parameters necessary for maximizing lipid synthesis by a local isolate of *Cunninghamella sp.* The preliminary optimization of lipid production utilizing OVAT used to

optimize carbon source, nitrogen source, initial medium pH and incubation time.

Impact of Different Carbon Sources on Lipid Synthesis by *Cunninghamella sp:*

The type of carbon source in a fermentation medium significantly affects the amount and the composition of lipids produced by various oleaginous fungal species, making it as an essential medium component for fungal growth. This is due to their unique metabolic processes.

Glucose is the most common sugar often utilized by oleaginous fungi for biomass growth and lipid accumulation under nitrogen-limited conditions (Carvalho *et al.,* 2018). Furthermore, the concentration of glucose in the culture medium was another key factor that boosted lipid production by oleaginous molds. The optimal glucose concentration for achieving maximal lipid accumulation in oleaginous molds is varied within the range of 20 to 80 g/L (Lei *et al.,* 2024). Al-Hawash *et al.,* (2018) found that presence of low glucose concentrations in culture medium resulted in lower lipid yield, whereas rising the amounts of glucose boosts lipid content, peaking at approximately 50g/L with a lipid synthesis content of 39.02% of biomass.

The impact of various carbon sources on lipid production by *Cunninghamella sp* was investigated in this study. The tested carbon sources included monosaccharides, disaccharides, and polysaccharides such as glucose, fructose, lactose, maltose, sucrose, and starch. The experiments were conducted using a basal medium, inoculum amount of 10% (v/v), incubation temperature of 28°C, incubation time of 7 days and under static condition.

Results in this study demonstrated that the lipid biosynthesis and fungal biomass growth by *Cunninghamella sp* was significantly influenced by the carbon type supplied in the culture medium. It was observed that all tested carbon sources were consumed by the isolate *Cunninghamella sp* while the biomass growth and lipid content were varied based on the carbon source used. Among the tested carbon sources, glucose yielded the highest lipid accumulation, followed by sucrose, as illustrated in Figure (1). Starch, on the other hand, resulted in the lowest fungal growth and lipid production. This can be attributed to the fact that glucose is a simple carbon source which is more easily metabolized by the fungus *Cunninghamella sp*. As a result, it

leads to greater lipids and biomass production compared to more complex carbon sources such as starch (Nasr *et al.*, 2017; Nisha and Venkateswaran, 2011).

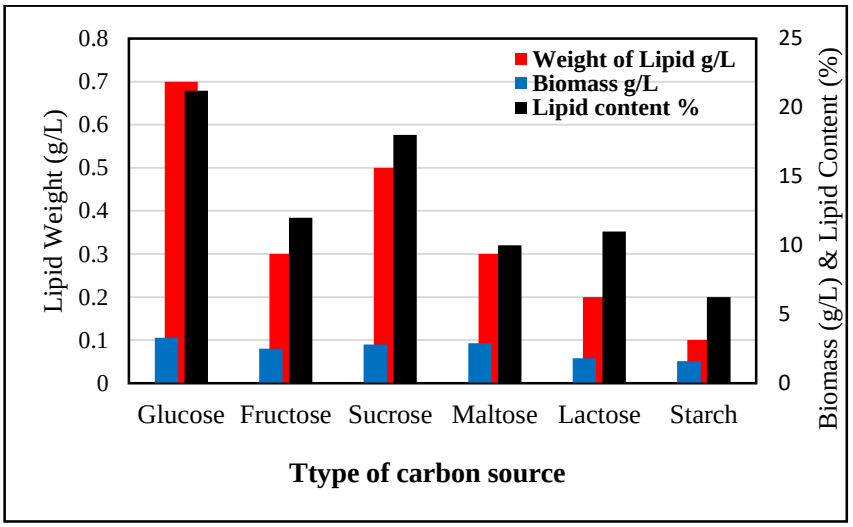


Figure (1): Impact of various carbon sources on lipid synthesis by *Cunninghamella sp*

Several studies have reported that glucose yielded the highest lipid content among various carbon sources using oleaginous mold *Cunninghamella sp* (Ahmed *et al.*, 2006; Papanikolaou *et al.*, 2007; Shrivastava *et al.*, 2008; Sukrutha *et al.*, 2014). A study by Varma *et al.*, (2018) to investigate the effect of carbon sources on the synthesis of γ-linolenic acid (GLA) using *Cunninghamella elegans* CFR C07 demonstrated that glucose was the most effective carbon source for synthesis of GLA lipid. Our findings are consistent with these studies, indicating that the best source of carbon for synthesis of lipid using a local isolate of *Cunninghamella sp* is glucose. Glucose as a common carbon source is known to improve both growth and lipid synthesis in oleaginous molds (Ochsenreither *et al.*, 2016).

Impact of Different Nitrogen Sources on Lipid Synthesis by *Cunninghamella sp:*

Depletion of nitrogen source is concerned as the most influencing parameter for lipogenesis in majority oleaginous fungal species. Initially, when there is an adequate amount of nitrogen source, oleaginous fungi utilize the available source of carbon in the medium for growth and cell proliferation, producing lipid-free biomass that contains functional lipids. Once the

nitrogen in the culture medium is exhausted in the presence of accessible carbon, oleaginous fungi then initiate the accumulation and storage of lipids (Economou *et al.*, 2011).

In this study, the impact of different nitrogen sources on lipid biosynthesis by *Cunninghamella sp* was investigated. This was conducted using four different nitrogen sources in a basal medium contains glucose (50 g/L) as the sole carbon source under optimal conditions of incubation temperature at 28°C, initial pH of 6.0 and under static condition. The results of this study indicated that sodium nitrate was the optimal inorganic nitrogen source for maximum lipid content (22.7%) followed by yeast extract (Figure (2)). In terms of biomass growth and lipid production, yeast extract showed the highest fungal growth as 3.3 g/L, and the produced lipid weight was 0.7 g/L, whereas the lipid content was 21.2% as illustrated in Figure (2). In contrast, the lowest lipid yield, in terms of lipid/dry biomass ratio, was observed with peptone and ammonium acetate.

The results in this study are in agreement with that previously reported by Sheekh *et al.*, (2019) who reported that addition of sodium nitrate to the growth medium of microalga *Desmodesmus intermedius* enhanced lipid production by 60% compared to the control. Similarly, Abdella *et al.*, (2020) found that sodium nitrate produced the highest lipid production by *Aspergillus spp* with 53.3% lipid/dry biomass, followed by ammonium sulfate (45.3%) and ammonium oxalate (39.6%) while the lowest lipid yield was obtained with ammonium chloride as 34.3% lipid/dry biomass. In addition, Moubasher *et al.*, (2016) demonstrated that supplementing the growth medium of *Fusarium oxysporum* with sodium nitrate as a nitrogen source led to maximize lipid accumulation (23.65%) and biomass (2.10 g/L). Ali et al., (2017) reported that utilization of sodium nitrate as a nitrogen source for lipid production by *Penicillium brevicompactum* resulted in the highest lipid accumulation. In contrast, Ramírez-Castrillón *et al.*, (2017) and Xing *et al.*, (2012), reported different findings, showing that the most appropriate nitrogen source for lipid production was ammonium sulfate using *Mortierella isabellina* and *Meyerozyma guilliermondii*, respectively.

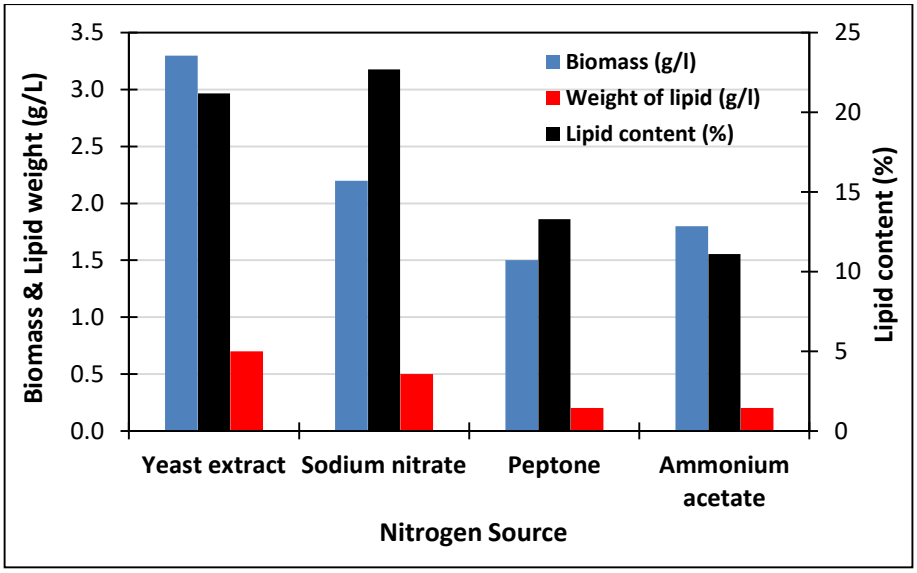


Figure (2): Impact of nitrogen source on lipid synthesis by *Cunninghamella sp.*

Impact of Initial pH on Lipid Synthesis by *Cunninghamella sp*:

The influence of initial medium pH on lipid synthesis by oleaginous *Cunninghamella sp* was studied over a range between pH 4.0 to pH 9.0 by adjusting the medium's pH before autoclaving by dilute HCl/NaOH. The results in this study found that the lipid production was maximized when *Cunninghamella sp* was cultured at pH 6.0, resulting in the highest biomass growth and lipid content. The results also indicated that the total lipid content extremely minimize at pH higher than 8.0 (Figure(3)). This observation is supported by Sukratha *et al.,* (2014) who reported that the highest lipid accumulation by *Cunninghamella blakesleeana* was reached at pH 6.0. Moreover, Shoaib *et al.,* (2014) indicated that pH 6.0 was the optimal for the growth and lipid production by *Aspergillus wentii*. Additionally, this outcome is close to the optimal pH of 5.5 for lipid production by *Aspergillus awamori* as reported by Venkata and Venkata (2014).

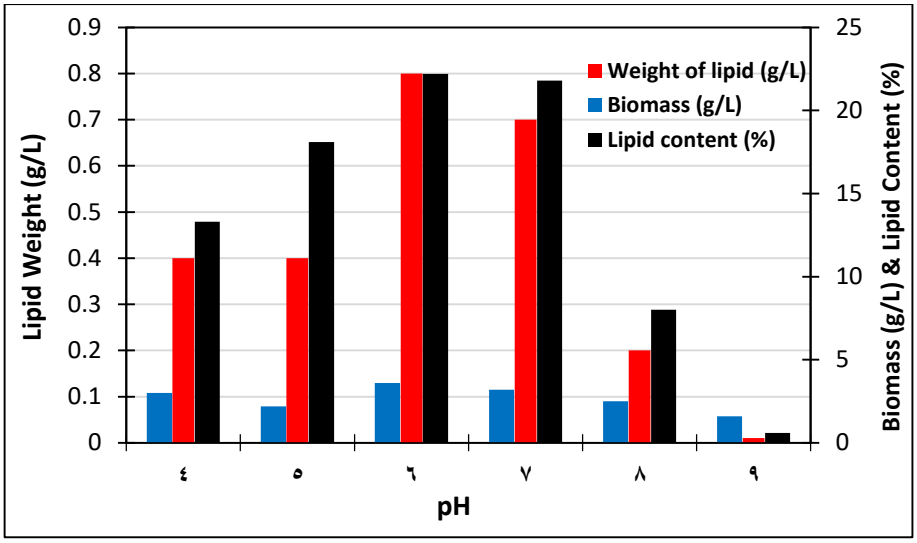


Figure (3): Impact pH on lipid synthesis by *Cunninghamella sp*.

Studies have shown that a pH range of 5.0 to 6.0 is the optimal pH for the highest lipid accumulation and growth of the majority of oleaginous molds (Ali and El-Ghonemy 2014; Ruan *et al.*, 2014; Jiru *et al.*, 2017; Ali *et al.*, 2017). Previous studies have indicated that pH is a critical environmental factor that can induce stress responses in fungal cells, subsequently influencing their growth, lipid accumulation, and the composition of the lipids produced (Xia *et al.*, 2011; Mironov *et al.*, 2018).

Impact of Incubation Period on Lipid Synthesis by *Cunninghamella sp:*

The incubation time significantly influences lipid accumulation in oleaginous fungal species and the optimal incubation durations varying depends on the fungal species, fermentation medium and growth conditions (Nisha and Venkateswaran, 2011).

The current study found that on the fifth day of growth, there was the greatest biomass growth and lipid synthesis (Figure (4)). The incubation time influenced fungal biomass formation and lipid synthesis, as a longer incubation period allowed for more effective use of the obtainable source of carbon in the growth culture, leading to greater lipid content. Shorter incubation periods resulted in less substrate utilization by *Cunninghamella sp*, resulting in reduced biomass formation and lipid accumulation. These results are consistent with findings by Kumar and Banerjee (2013) who

reported that most lipid synthesis by *Aspergillus spp* occurred later five days of incubation.

In addition, Ali and El-Ghonemy (2014) noted that both *Trichoderma viride* and *Aspergillus spp* reached most lipid synthesis after five days of cultivation. Abdelhamid *et al.*, (2019) also indicated that *Penicillium commune* reached the highest lipid synthesis using a basal liquid medium after 5 days of incubation.

The maximum lipid synthesis by *Cunninghamella sp* was found to occur after 5 days of cultivation under constant conditions at 28°C and pH of 6.0, based on the findings from the first step of sequential optimization. Moreover, the best sources of carbon and nitrogen were found to be glucose and sodium nitrate, respectively.

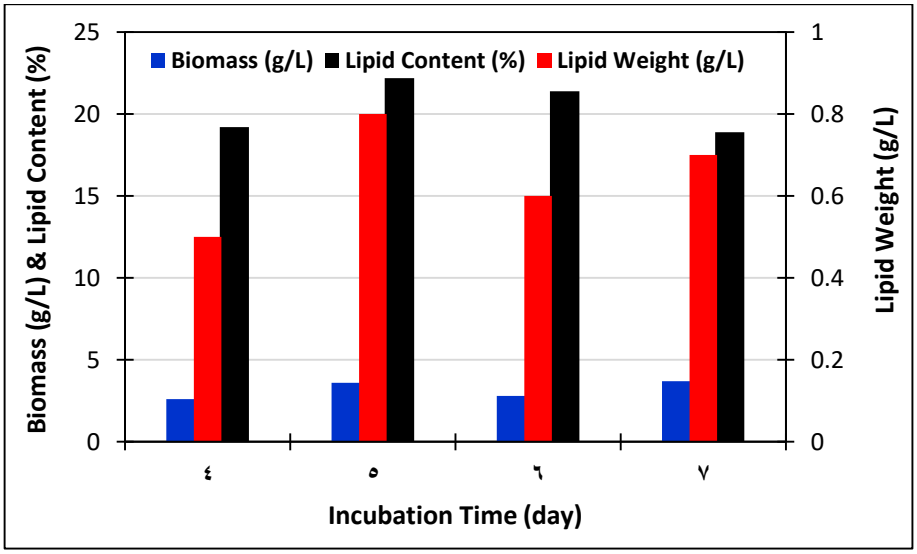


Figure (4): Impact of incubation period on lipid synthesis by *Cunninghamella sp.*

Optimization of Glucose, Sodium nitrate and pH using RSM:

In order to optimize the quantities of glucose and sodium nitrate in the fermentation medium, and the initial medium' pH were further optimized using RSM-CCD, to enhance lipid production by this local isolate of *Cunninghamella sp.*

RSM has been effectively employed for optimization of several bioproducts fermentation (Lin *et al.*, 2011; Ranjan *et al.*, 2013b). In this

regards, the impact and the interaction between three experimental variable factors (glucose, sodium nitrate, and pH) were determined to enhance and maximize lipid production by *Cunninghamella sp.* using RSM-CCD. Experimental design by RSM-CCD was employed, resulting in 20 experimental tests. After conducting these experiments, the lipid production content was ranged from 0.6% to 25%, as shown in Table (3).

Table (3): Experimental design for three experimental factors and results of lipid accumulation by *Cunninghamella sp.*

Run No.	Variable parameters			Response
	X_1: Glucose (g/L)	X_2: Sodium nitrate (g/L)	X_3: pH	Lipid content (%)
1	60.00	0.05	9.0	0.9
2	60.00	1.00	4.0	10
3	40.00	0.53	2.2	0.6
4	40.00	0.53	6.5	22
5	20.00	1.00	9.0	2.5
6	40.00	0.53	6.5	21
7	40.00	0.53	6.5	24.2
8	60.00	0.05	4.0	11.5
9	20.00	1.00	4.0	3.4
10	40.00	0.53	6.5	22.5
11	73.64	0.53	6.5	4.8
12	40.00	0.53	6.5	25
13	40.00	0.00	6.5	15
14	6.36	0.53	6.5	0.8
15	20.00	0.05	4.0	1.5
16	40.00	0.53	10.7	0.0
17	60.00	1.00	9.0	0.6
18	40.00	0.53	6.5	23.8
19	20.00	0.50	9.0	1.0
20	40.00	1.32	6.5	10

The results were analyzed using one-away ANOVA (analysis of variance) suitable for the experimental design, as shown in Table(4). The ANOVA analysis of the quadratic regression model indicates that the model is significant ($p< 0.0001$).

The Model F-value of 48.8 suggests a high level of significance, as it is the ratio of the mean square regression to the mean square residual. Additionally, the Model P-value (Prob > F) is very low (0.0001), further confirming the model's significance.

Table (4): Analysis of variance (ANOVA) for the quadratic polynomial model of lipid accumulation by *Cunninghamella sp*.

Source	Sum of Squares	DF	Mean^2	F value	p-value (Prob > F)
Model	**1749.961**	**9**	**194.4401**	**48.81932**	**< 0.0001**
Glucose (X_1)	**33.30549**	**1**	**33.30549**	**8.362222**	**0.0161**
Sodium nitrate (X_2)	**1.556602**	**1**	**1.556602**	**0.390826**	**0.5459**
pH (X_3)	**36.7703**	**1**	**36.7703**	**9.232155**	**0.0125**
X_1 X_2	**3.38**	**1**	**3.38**	**0.848638**	**0.3786**
X_1 X_3	**43.245**	**1**	**43.245**	**10.8578**	**0.0081**
X_2 X_3	**0.08**	**1**	**0.08**	**0.020086**	**0.8901**
X_1^2	**693.0196**	**1**	**693.0196**	**174.0008**	**< 0.0001**
X_2^2	**205.0166**	**1**	**205.0166**	**51.47483**	**< 0.0001**
X_3^2	**881.8126**	**1**	**881.8126**	**221.4023**	**< 0.0001**
Residual	**39.82852**	**10**	**3.982852**		
Lack of Fit	**28.54018**	**5**	**5.708037**	**2.52829**	**0.1658**
Pure Error	**11.28833**	**5**	**2.257667**		
Cor Total	**1789.79**	**19**			
Std. Dev.= 2.0	**Mean = 10.06**				
R^2 = 0.97	**Adj R^2 = 0.95**		**Adequate Precision = 17.5**		

To determine the significance of each variable factor and consequently, the pattern of the reciprocal interactions among the test variables, the p-values were employed as a tool. Table (4) provides the F-value, accompanying p-values, and the coefficient estimate. The model terms are considered significant when the value of P is less than 0.0001. The study's test variables, X_1, X_2, X_3, X_1 X_2, X_1 X_3, X_2 X_3, X_1^2, X_2^2, and X_3^2 (where $X_1$is glucose, $X_2$is

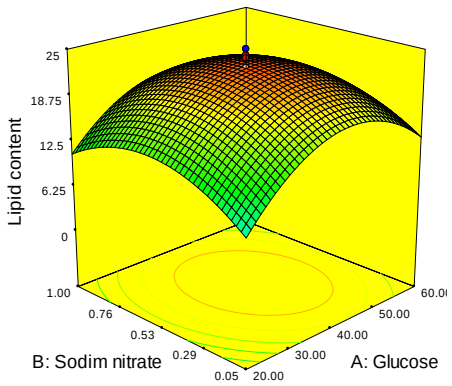
sodium nitrate and $X_3$is pH), appear to be significant model terms based on the coefficient estimates and corresponding p-value. X_1, X_3, X_1 X_3, X_1^2, X_2^2, and X_3^2 ($P < 0.05$) have significant effect on lipid production. However, it was observed that $X_2$and the interactions between X_1 X_2 and X_2X_3 were not significant ($P > 0.05$).

ANOVA was used in a quadratic regression study to assess the model coefficients' significance. The strength of the interaction between each independent variable is also reflected in the impact of every variable, as shown through the p-values. The model in terms of real variables and lipid synthesis as the expected response is presented in Equation 2, as follow:

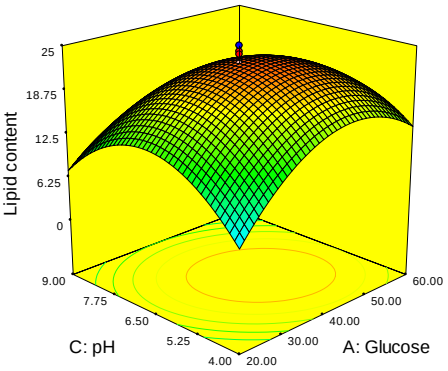
$$\begin{aligned}\text{Lipid content } (\%\text{w/w}) &= -75.58363 + 1.79714 \times \text{Glucose} + 24.65162 \\ &\times \text{Sodim nitrate} + 17.35919 \times \text{pH} - 0.068421 \times \text{Glucose} \\ &\times \text{Sodim nitrate} - 0.046500 \times \text{Glucose} \times \text{pH} + 0.084211 \\ &\times \text{Sodim nitrate} \times \text{pH} - 0.017261 \times \text{Glucose}^2 - 20.64178 \\ &\times \text{Sodim nitrate}^2 - 1.24613 \\ &\times \text{pH}^2 \qquad \text{(Equation 2)}\end{aligned}$$

The goodness of fit of the model was assessed using the coefficient of determination (R^2). The R^2 expresses the extent to which the independent variables and their interactions account for the variability observed in the experimental responses. According to Alharbi *et al.*, (2022) the R^2 value denotes the optimal situation in which the model fully explains the variation in actual data. The study's R^2 value was 0.977, meaning that the model could account for 97.77% of the response variability. Furthermore, all regression models' adjusted R^2 value were high and nearly matched the R^2 value that were anticipated, demonstrating a remarkable agreement between the actual and expected findings. A ratio larger than four indicates adequate precision, which measures the signal-to-noise ratio (Fawzy *et al.*, 2022). In this study, the predicted R^2 of 0.8597 was reasonably close to the adjusted R^2 of 0.9577, and the adequate precision ratio of 17.566 indicated a sufficient signal.

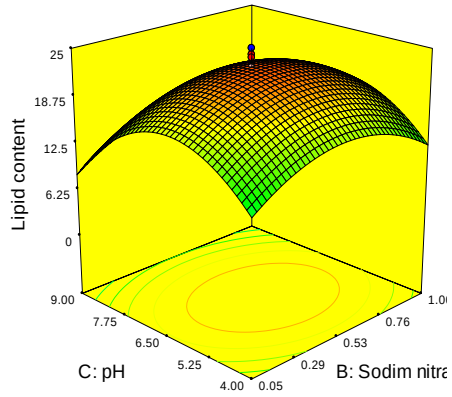
The primary and interaction impacts of the covariates on the response value were better understood with the use of three-dimensional response surface plots, which visually displayed the regression equation. The response surfaces of glucose and pH, glucose and sodium nitrate, and sodium nitrate and pH on lipid synthesis are shown in Figures (5a–c), respectively.



(a)



(b)



(c)

Figure (5): Response surface plot of lipid content using *Cunninghamella sp* observing the interaction between(a)impact of glucose and sodium nitrate contents on lipid synthesis, (b) impact of glucose and pH on lipid synthesis, (c)impact of sodium nitrate and pH on lipid synthesis.

Figure (5a) illustrates the influence of varying glucose and sodium nitrate concentrations on lipid synthesis. The highest lipid synthesis was achieved with concentration of glucose 50 g/L and a sodium nitrate concentration of 0.63 g/L. Increasing the glucose concentration resulted in a continuous rise in lipid synthesis.

Figure (5b) demonstrates the combined impacts of glucose and pH on lipid synthesis. The surface plot indicates that, with a constant sodium nitrate concentration of 0.53 g/L, the highest lipid synthesis occurred at a concentration of glucose 50 g/L and a pH of 6.5.

Figure (5c) shows the influence of sodium nitrate and pH on lipid synthesis. The 3D plot reveals no significant interaction between sodium nitrate and pH, as detailed in Table (4).

Model Validation:

Based on Table (3), the highest lipid content produced by *Cunninghamella sp* was 25% (w/w) was achieved with glucose, sodium nitrate, and pH levels set at 40.0 g/L, 0.53 g/L, and 6.5, respectively.

The quadratic model proposed optimal conditions for maximal lipid accumulation to be 38.28 g/L of glucose, 0.48 g/L of sodium nitrate, and a pH of 5.79, with a predicted lipid production of 23.55%. The model's validity was confirmed through validation experiments conducted in triplicates under the estimated optimal conditions. These experiments resulted in a lipid production of 25.4%, closely matching the predicted value and confirming the model's adequacy as show in Table (5).

Table (5): Model validation of optimized factors on lipid production under optimized conditions

Optimized Conditions (g/L)			Lipid Content (%)	
Glucose	**Sodium nitrate**	**pH**	**Experimental**	**Predicted**
38.28	**0.48**	**5.79**	**25.4%**	**23.55%**

Numerous studies have optimized lipid synthesis via RSM. Shrivastava *et al.* (2008) optimized fermentation conditions for the oleaginous fungus *Cunninghamella echinulata var. elegans* using a CCD, resulting in a lipid synthesis of 19.8 ± 0.35 mg/g of dry cell weight. Bardhan *et al.,* (2019) obtained the maximum lipid synthesis of 1.73 g/L by *Penicillium citrinum* using CCD-based RSM.

Additionally, Shoaib *et al.,* (2018) achieved a maximum lipid synthesis of 40% from *Aspergillus wentii* biomass when cultivated in a growth culture containing 50 g/L glucose, 1 g/L nitrate, and 1.5 g/L phosphate using RSM. Optimization of lipid production by oleaginous microorganisms using RSM technique exhibited that RSM–based optimization is an efficient technique for maximal lipid generation.

Conclusion:

Cunninghamella sp., a local fungal isolate, was used successfully for lipid production. Enhanced lipid synthesis was optimized through the utilization of *Cunninghamella sp* using RSM-CCD. The optimized conditions for the maximum lipid-enriched biomass were optimized. A maximum lipid content of 25.4 % was obtained using RSM-based CCD after 5 days of incubation at pH 5.79 and glucose, sodium nitrate concentrations of 38.28 g/L and 0.48g/L, respectively. Thus, it can be concluded that the lipid-enriched biomass was successfully optimized using a local isolate of *Cunninghamella sp.*

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