Pertanika J. Trop. Agri. Sci. 47 (3): 905 - 921 (2024)

PERTANIKA JOURNALS

TROPICAL AGRICULTURAL SCIENCE

Journal homepage: http://www.pertanika.upm.edu.my/

Optimization of Medium for Lipid Production from *Lipomyces maratuensis* InaCC Y720 Using Statistical Experiment Design

Liya Audinah[1], Atit Kanti[2] and Miftahul Ilmi[1]*

[1]Faculty of Biology, Universitas Gadjah Mada, Jl. Teknika Selatan, Sekip Utara, Yogyakarta 55281, Indonesia
[2]Research Center for Biosystematics and Evolution, National Research and Innovation Agency, Republic of Indonesia, Jl. Raya Jakarta-Bogor Km.46, Cibinong 16911, Indonesia

ABSTRACT

Lipomyces maratuensis InaCC Y720 is a potential novel oleaginous yeast. Media-based production optimization has never been carried out using this strain. This study aims to define an optimized medium from 12 medium component factors, where the Taguchi method is used for screening significant factors of medium and the response surface methodology (RSM) is used to optimize the concentration of significant factors. According to Taguchi, glucose, yeast extract, and magnesium sulfate ($MgSO_4$) have a significant influence on lipid accumulation, with their concentrations maintained at optimal levels through RSM optimization. Conversely, potassium dihydrogen phosphate, sodium hydrogen phosphate, and calcium chloride inhibit lipid accumulation, and copper(II) sulfate has the least influence, categorizing them as eliminated factors. The RSM-optimized medium increased lipid content by 3.6-fold compared to the initial medium. Glucose and yeast extract showed a positive correlation with lipid accumulation, suggesting potential for further optimization, while the optimum concentration for $MgSO_4$ was 0.15 g/L. This study is intended to serve as a reference for increasing lipid accumulation by *L. maratuensis* InaCC Y720.

Keywords: Initial media, nutrients, oleaginous yeast, optimized media, response surface methodology, Taguchi method

ARTICLE INFO

Article history:
Received: 06 November 2023
Accepted: 20 February 2024
Published: 20 August 2024

DOI: https://doi.org/10.47836/pjtas.47.3.19

E-mail addresses:
liya.audinah@mail.ugm.ac.id (Liya Audinah)
atityeast@gmail.com (Atit Kanti)
m.ilmi@ugm.ac.id (Miftahul Ilmi)
*Corresponding author

INTRODUCTION

The expansion of microorganisms as renewable energy continues to be developed. One of them is the development of lipid accumulation from microbes. Microbial lipids, also known as single-cell oil (SCO), show the potential to substitute palm oil in biodiesel production due to their rapid

ISSN: 1511-3701
e-ISSN: 2231-8542

production, minimal labor needs, resilience to seasonal changes, and ease of industrial-scale processing (El Kantar et al., 2021; Mhlongo et al., 2021). Microbes that can accumulate lipids of more than 20% from their dry biomass are referred to as oleaginous microorganisms (Anandan et al., 2016).

In the lipid accumulation by microbes, the composition of the media (macronutrients, micronutrients) and physical factors (temperature, pH, production time, and agitation) are crucial factors that can influence lipid accumulation (Duman-Özdamar et al., 2022; Sarkar et al., 2023). Lipid accumulation in oleaginous microorganisms occurs when nitrogen nutrients for cells are limited, and carbon is abundant (Qin et al., 2017). Regarding media composition, various macronutrients (C, N, Mg, P, Ca, Na, K) and micronutrients (Fe, Zn, Co, Cu, Mn) are combined to determine the effects of enhancement or inhibition on lipid production. Therefore, there is potential for higher lipid production under optimal cultivation media conditions.

In several studies optimizing cultivation media for lipid production by oleaginous microorganisms, statistical approaches such as the Taguchi method and response surface methodology (RSM) are utilized. The Taguchi method enhances the quality and performance of products and processes by systematically identifying and optimizing factors influencing variation. Meanwhile, RSM aims to study the relationship between input variables (factors) between input variables (factors) and the output response in complex systems. Based on the Taguchi method, it is known that glycine as an N source gives higher productivity (1.5 times), followed by urea and cellulose as a carbon source, significantly increasing biomass (2.4 times) and lipid (2.3 times) productivity in *Desmodesmus subspicatus* (Sarkar et al., 2023). Applying RSM, Duman-Özdamar et al. (2022) determined a carbon-to-nitrogen (C/N) ratio of 175 g/g for maximal oil production by *Cutaneotrichosporon oleaginosus* and a C/N ratio of 140 g/g for *Yarrowiaa lipolytica*.

Lipomyces maratuensis InaCC Y720 is a novel oleaginous yeast. It can produce up to 3.7 g/L of fatty acids when grown in a medium containing glucose and malt extract (Yamazaki et al., 2017). Based on its ability to accumulate high lipids, *L. maratuensis* InaCC Y720 has a potential for improvement through optimization of its growth medium, which has not been explored. For that reason, this study aims to define an optimized medium and determine the influence of component media on lipid production by *L. maratuensis* InaCC Y720 using the Taguchi method and RSM approach.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

Lipomyces maratuensis InaCC Y720 is an Indonesian culture collection (InaCC) isolated from Maratua Island, East Kalimantan, Indonesia (Yamazaki et al., 2017). The strain was grown on potato dextrose agar (PDA, Oxoid, United

Kingdom) and kept in a refrigerator. Seed culture was prepared by subculturing the yeast in potato dextrose broth (PDB, Oxoid, United Kingdom) at 28ºC with 200 rpm agitation for 48 hours.

Screening of Significant Medium Component using Taguchi

Medium components are screened using the Taguchi method with the "bigger is better" for the signal-to-noise ratio (SNR) parameter. The design of the experiment Taguchi consists of three levels and 11 factors (Table 1) that result in 27 medium variations. Level 2 medium compositions were based on the initial medium, according to Holdsworth and Ratledge (1988). All chemicals used in the medium were manufactured by Merck (Germany).

The experiment was carried out by inoculating 1 ml seed culture (OD_{600} = 0.8) into 50 ml varied medium in a 250 ml Erlenmeyer flask and then incubating at 28ºC with 200 rpm agitation for 72 hr. According to Bligh and Dyer (1959), yeast biomass and lipids were determined after 72 hr of incubation. All experiments were done in triplicates.

Optimization of Significant Factors Using RSM Method

Based on the Taguchi results, three significant factors (a, b, and c) were selected as independent variables and lipid production (mg/L) as dependent variables (y). The three significant factors are (a) glucose, (b) yeast extract, and (c) $MgSO_4$. Their concentrations were optimized using Box-Behnken design (BBD) and RSM. Each variable was studied in three levels (−1, 0, +1), and the experimental design included 15 runs with three replicates (Table 1). The mathematical relationship between the response variable (lipid production) and variables (a, b, and c) was projected according to the polynomial equation:

$$y = x + xa + xb + xc + xa^2 + xb^2 + xc^2 + xab + xac + xbc \quad \text{[Eq. 1]}$$

where, y = Dependent variables; x = Dependent variables; a = Glucose; b = Yeast extract; c = $MgSO_4$.

The precision of the above polynomial model was evaluated by the coefficient of determination (parity plot) and normal probability, and the *F*-test determined the statistical significance. The experiments

Table 1
Coded values of response surface methodology using three levels Box-Behnken design

Independent variable (g/L)	Symbol	Level		
		Low (-1)	Middle (0)	High (+1)
Glucose	a	30	50	70
Yeast extract	b	1.5	3.0	4.5
Magnesium sulfate	c	1.5	3.0	4.5

were carried out by adding 1 ml seed culture (OD_{600} = 0.8) to 50 ml medium in a 250 ml Erlenmeyer flask and then incubating at 28ºC with 200 rpm agitation for 72 hr. Biomass and lipids were collected after 72 hr and determined according to Bligh and Dyer (1959).

Growth Profile

The growth profile was observed by growing the yeast in 50 ml optimized medium (1 ml inoculum with OD_{600} = 0.8) at 28ºC and 200 rpm agitation for 72 hr. Biomass, lipid, glucose, and total nitrogen were measured every 12 hr. All experiments were done in triplicate.

Lipid Extraction and Analysis

Biomass was harvested by centrifugation (Hermle-Z326K, Germany) at 1,746 x *g* for 10 min, washed twice with 35 ml of distilled water, and dried in the oven at 70°C. Biomass is measured gravimetrically by comparing wet weight to dry weight. Lipid extraction was carried out according to Bligh and Dyer (1959). The cells were disrupted by adding 10 ml of 4 M hydrogen chloride (HCl, Merck, Germany) for 2 hr, then a mixture of chloroform (Merck, Germany) and methanol (Merck, Germany) (2:1 v/v ratio) was added before incubated for another 2 hr. The mixture was then centrifuged at 1,746 x *g* for 10 min, and the lower layer was transferred to a new glass vial and dried in the oven at 70°C. Lipid measured gravimetrically. The content of lipids was determined by comparing lipid weight to dry biomass weight, and lipid yield was determined by a gram of lipid per gram consumed glucose.

Analysis of Glucose Content

Reducing sugar is determined by the 3,5-dinitrosalicylic acid (DNS) method. DNS solution was carried out by dissolving 1 g DNS powder (Merck, Germany), 20 ml 2 M sodium hydroxide (NaOH, Merck, Germany), and 30 g Rochelle salt (Merck, Germany), then adding distilled water until the volume reached 100 ml. Reducing sugar concentration was measured by adding 1 ml sample, 1 ml DNS reagent (Merck, Germany), and 2 ml distilled water in a test tube heated for 1 min at 100°C in a water bath and then moving it into cold water. The absorbance of the sample was measured by a spectrophotometer at 540 nm (Wood et al., 2012).

Analysis of Total Nitrogen

The total nitrogen (%) in the sample was determined using the Kjeldahl method. Add 1 ml sample, 0.7 g catalyst (i.e., a combination of 250 g sodium sulfate [Na_2SO_4, Merck, Germany] + 5 g copper(II) sulfate [$CuSO_4$, Merck, Germany] + 0.7 g selenium [Merck, Germany]), and 4 ml concentrated sulfuric acid (H_2SO_4, Merck, Germany) into Kjeldahl flask, then destructed at 100°C in a fume hood until the color became light green. The sample cooled down, and 10 ml of distilled water was then distilled by adding 20 ml of sodium hydroxide-titanium (II) oxide (NaOH-TiO,

i.e., sodium hydroxide [NaOH 40%, Merck, Germany] + sodium thiosulfate [NaS_2O_3 5%, Merck, Germany]). The distillation used boric acid (H_3BO_3) 4%, which was given methyl red-bromo cresol green indicator (Mr-BCG, Smart Lab, Indonesia). This process ran until 60 ml distillate was obtained. 0.02 N hydrochloric acid (HCl, Merck, Germany) titrated the distillate. The residual nitrogen was measured as follows:

$$\text{Total nitrogen (\%)} = \frac{(\text{Titration volume} \times 0.02 \times 14)}{\text{Sample weight}} \times 100\% \quad \text{[Eq. 2]}$$

Data Analysis

The data was analyzed using Taguchi, RSM, and analysis of variance (ANOVA) on Minitab software (ver. 18) (Hamzaçebi, 2021; Roy, 1990).

RESULTS AND DISCUSSION

Screening of Significant Medium Component for Lipid Production by *L. maratuensis* InaCC Y720

The Taguchi method was utilized to screen significant factors and their concentration in the growth medium that affect lipid accumulation by *L. maratuensis* InaCC Y720. Subsequently, RSM was applied to optimize the concentrations of the identified significant factors as determined by the Taguchi method. *L. maratuensis* InaCC Y720 was cultured under 12 factors with three levels (Table 2) at 28℃ and 200 rpm for 72 hr. Biomass and lipids were collected for evaluation. Screening by Taguchi is presented in the SNR analysis of variance (Table 3) and the main effect plot for SNR (Figure 1).

SNR analysis of variance (Table 3) showed the significance and contribution of each factor to lipid accumulation. Three significant factors and their contributions were shown: yeast extract (16.88%), $MgSO_4$ (16.83%), and glucose (14.20%). The contribution of other factors is as follows: calcium chloride ($CaCl_2$, 10.69%), manganese sulfate ($MnSO_4$, 9.6%), zinc sulfate ($ZnSO_4$, 8.35%), sodium hydrogen phosphate (Na_2HPO_4, 7.93%), cobalt(II) nitrate ($Co(NO_3)_2$, 4.64%), potassium dihydrogen phosphate (KH_2PO_4, 3.61%), ammonium chloride (NH_4Cl, 3.36%), iron(III) chloride ($FeCl_3$, 1.98%), and copper(II) sulfate ($CuSO_4$, 1.36%).

Certain metal ions serve as micronutrients, supporting fungal growth and cellular metabolic activity. Zinc (Zn) deficiency impacts spore germination and fungal cell proliferation. Magnesium (Mg) functions as an intracellular divalent cation crucial for DNA and adenosine triphosphate (ATP) synthesis, as well as stimulating fatty acid synthesis. Mg deficiency in yeast can lead to distorted cell division, abnormal cell shape, and reduced viability, resulting in delays or alterations in the cell cycle. Mangan (Mn) serves various roles, such as acting as an intracellular regulator for enzymes, stimulating protein synthesis, and participating in thiamine biosynthesis. Iron (Fe) and copper (Cu) are recognized as cofactors. Phosphorus is a vital element in cells, commonly found in nucleic acids, phospholipids, and coenzymes, and can be stored as polymetaphosphate in cells.

Table 2
Factors and levels of experimental design Taguchi

No.	Factors	Level 1	Level 2	Level 3
1	D-glucose (g/L)	10	30	50
2	NH_4Cl (g/L)	0	0.5	1.0
3	Yeast extract (g/L)	0	1.5	3.0
4	KH_2PO_4 (g/L)	0	7.0	14.0
5	Na_2HPO_4 (g/L)	0	2.0	4.0
6	$MgSO_4.7H_2O$ (g/L)	0	1.5	3.0
7	$CaCl_2.2H_2O$ (g/L)	0	0.1	0.2
8	$FeCl_3.6H_2O$ (g/L)	0	0.008	0.016
9	$ZnSO_4.7H_2O$ (g/L)	0	0.001	0.002
10	$CuSO_4.5H_2O$ (g/L)	0	0.0001	0.0002
11	$Co(NO_3)_2.6H_2O$ (g/L)	0	0.0001	0.0002
12	$MnSO_4.5H_2O$ (g/L)	0	0.0001	0.0002

Note. NH_4Cl = Ammonium chloride; KH_2PO_4 = Potassium dihydrogen phosphate; Na_2HPO_4 = Disodium phosphate; $MgSO_4 \cdot 7H_2O$ = Magnesium sulfate heptahydrate; $CaCl_2 \cdot 2H_2O$ = Calcium chloride dihydrate; $FeCl_3 \cdot 6H_2O$ = Ferric chloride hexahydrate; $ZnSO_4 \cdot 7H_2O$ = Zinc sulfate heptahydrate; $CuSO_4 \cdot 5H_2O$ = Copper(II) sulfate pentahydrate; $Co(NO_3)_2 \cdot 6H_2O$ = Cobalt(II) nitrate hexahydrate; $MnSO_4 \cdot 5H_2O$ = Manganese(II) sulfate pentahydrate

Table 3
Analysis of variance of the mean plot for signal-to-noise ratio

Factors	df	SS	Contribution	MS	F	P
Glucose	2	194.025	14.200	97.012	24.630	0.039*
NH_4Cl	2	45.915	3.360	22.958	5.830	0.146
Yeast extract	2	230.573	16.880	115.287	29.270	0.033*
KH_2PO_4	2	49.313	3.610	24.657	6.260	0.138
Na_2HPO_4	2	108.388	7.930	54.194	13.760	0.068
$MgSO_4$	2	229.999	16.830	114.999	29.200	0.033*
$CaCl_2$	2	146.086	10.690	73.043	18.540	0.051
$FeCl_3$	2	27.118	1.980	57.056	3.440	0.225
$ZnSO_4$	2	114.113	8.350	9.315	14.490	0.065
$CuSO_4$	2	18.629	1.360	31.687	2.360	0.297
$Co(NO_3)_2$	2	63.374	4.640	65.465	8.050	0.111
$MnSO_4$	2	130.929	9.600	3.939		
Error	2	7.877	0.580			
Total	26	1,366.34	100			

Note. Df = Degrees of freedom; SS = Sum of squares; MS = Mean squares; * = Significant at P-value <0.05; NH_4Cl = Ammonium chloride; KH_2PO_4 = Potassium dihydrogen phosphate; Na_2HPO_4 = Disodium phosphate; $MgSO_4$ = Magnesium sulfate; $CaCl_2$ = Calcium chloride; $FeCl_3$ = Ferric chloride; $ZnSO_4$ = Zinc sulfate; $CuSO_4$ = Copper(II) sulfate; $Co(NO_3)_2$ = Cobalt(II) nitrate; $MnSO_4$ = Manganese(II) sulfate

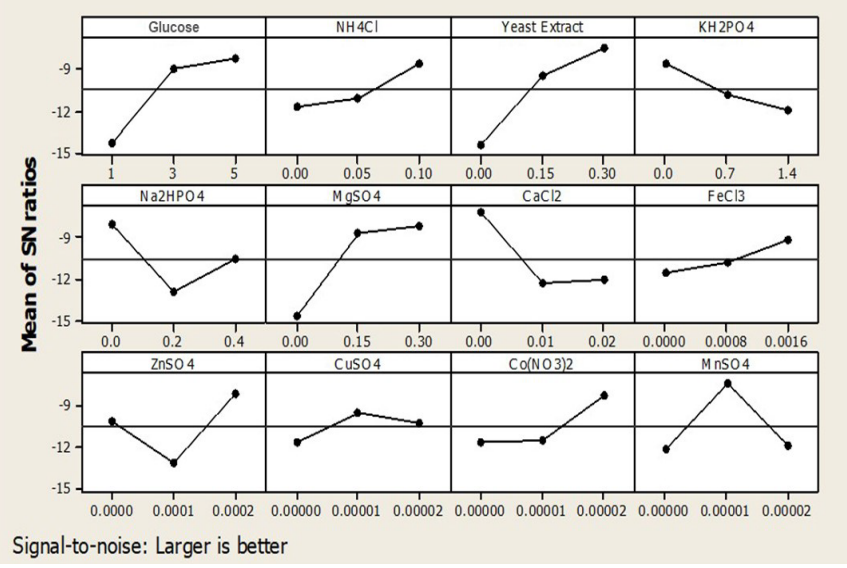

Figure 1. Main effect plot for signal-to-noise ratios (SNR) of lipid production using Taguchi method

Note. x-axis = Concentration of the factors (g/L) ; y-axis = The ratio of the mean (signal) to the standard deviation (noise) ; NH_4Cl = Ammonium chloride; KH_2PO_4 = Potassium dihydrogen phosphate; Na_2HPO_4 = Disodium phosphate; $MgSO_4$ = Magnesium sulfate; $CaCl_2$ = Calcium chloride; $FeCl_3$ = Ferric chloride; $ZnSO_4$ = Zinc sulfate; $CuSO_4$ = Copper(II) sulfate; $Co(NO_3)_2$ = Cobalt(II) nitrate; $MnSO_4$ = Manganese(II) sulfate

However, an excess concentration of metal ions can function as an inhibitor. Calcium (Ca) plays a role in metabolic responses, cell membrane stabilization, budding, and protein synthesis in cell walls. Two hypotheses explain the lipid-triggering effect of calcium deficiency. The first involves antilipolytic pathways mediated by the calcium-sensing receptor (CaSR), and the other centers on calcium ions' role in the basal sensitivity of the sterol-sensing mechanism in the sterol regulatory element binding protein (SREBP) pathway. Reduced calcium in the endoplasmic reticulum alters sterol distribution, enhancing SREBP activation and initiating neutral lipid synthesis (Arigony et al., 2013; Dzurendova et al., 2021; Ouedraogo et al., 2017; Wang et al., 2017). Studies regarding the effect of manganese on lipid accumulation in yeast are still few. Some studies suggest a positive impact on lipogenesis in *Mucor plumbeus* (Yoo et al., 1982), *Mortierella* sp. (Šajbidor et al., 1992), and *M. circinelloides* (under phosphorus-limiting conditions) (Dzurendova et al., 2020). However, in *Cunninghamella bainieri* 2A1, Mn appears to have no significant effect (Manikan et al., 2014).

Several studies highlight the influence of media components on lipid accumulation. A study by Shuib et al. (2014) showed that media containing ammonium tartrate, glucose, and metal ions (Mg^{2+}, Mn^{2+}, Fe^{3+}, Cu^{2+}, Ca^{2+}, Co^{2+}, and Zn^{2+}) increased lipid content by *C. bainieri*

2A1. In addition, it was observed that the cessation of lipid accumulation was caused by reduced enzyme activity as well as depletion of metal ion concentrations in the medium. A study by Zhao et al. (2016) showed that cobalt significantly inhibited the growth of *Y. lipolytica* but led to a slight increase in lipid content. Consistent findings from past to present studies suggest that phosphate limitation consistently increases lipid content. It was reported in *Rhodotorula glutinis* (Granger et al., 1993), *Rhodosporidium toruloides* Y4 (Wu et al., 2010), and a recent study by Morales-Palomo et al. (2023) revealed that *Y. lipolytica* ACA DC 50109 under phosphate limitation (0 g/L), the lipid content and lipid yield increased to 44.4% w/w lipid per dry weight. Phosphate ions (PO_4^{3-}) are vital for cofactors, phosphorylated proteins, and RNA/DNA synthesis. Limitation in PO_4^{3-} significantly impact cellular physiology and metabolism, hindering biomass production while promoting lipid accumulation (Wang et al., 2017). Dzurendova et al. (2020) studied the effects of metal in phosphorus-limiting media on lipid accumulation by *M. circinelloides*. Key points include Zn^{2+} enhancing biomass, Mg^{2+} optimizing both biomass and lipid production, Fe^{3+} deficiency causing inhibited growth, and reported lipid increase with Ca and Cu deficiency.

This study applied the "larger is better" approach in Taguchi for the main effect plot, where higher points on the horizontal axis signify increased lipid accumulation. The main effects observe each level concentration and offer valuable insights for factor and level assessments (Minitab, n.d.a). This plot draws trends and shows how each level influences *L. maratuensis* InaCC Y720 lipid accumulation. It makes this a consideration for this research in evaluating and determining the factors and their levels that will be optimized in RSM.

This study divides the factors into three categories: (1) optimized materials, (2) maintained materials, and (3) eliminated factors. Based on Figure 1, the higher the lipids accumulated, the more significant factors showed higher concentrations. Therefore, they are optimized with RSM and increased by one level (concentration) to find the optimum level. $MnSO_4$, $ZnSO_4$, $Co(NO_3)_2$, NH_4Cl, and $FeCl_3$ were categorized as maintained factors. They showed a contribution and a trend of increasing levels directly proportional to the increase in lipid accumulation. Therefore, Level 3 of them was applied in RSM even for NH_4Cl as the main N source, except $MnSO_4$ at Level 2. For the factors eliminated are KH_2PO_4, Na_2HPO_4, $CaCl_2$, and $CuSO_4$. Adding KH_2PO_4, Na_2HPO_4, and $CaCl_2$ in the media showed the highest lipid accumulation at the lowest level. It means that a higher concentration can inhibit lipid accumulation. However, at certain concentrations, it may increase lipid accumulation. While $CuSO_4$ showed the lowest percentage of contribution to increasing lipid accumulation.

Optimization of Conditions to Increase Lipid Production in *L. maratuensis* InaCC Y720

Based on our consideration and explanation above, the new formula medium for optimization in RSM contained (g/L) 0.1 NH_4Cl, 0.0016 $FeCl_3$, 0.00002 $ZnSO_4$, 0.00002 $Co(NO_3)_2$, and 0.00001 $MnSO_4$. This study applied the BBD in RSM, resulting in 15 variations. The BBD involves fitting a second-order polynomial equation to experimental data, enabling us to identify optimal conditions for the desired outcome. The experimental responses are presented in Table 4.

In the BBD experimental design, the ninth design was predicted to yield the highest lipids (1.68 g/L), but both the second and ninth designs in the experiments produced 1.66 g/L. With small residual values and no significant deviation from the prediction model, the second design was selected as the optimized media composition, utilizing less glucose (50 g/L compared to 70 g/L in the ninth design). Papanikolaou et al. (2002) explained that each microorganism has a different tolerance level to glucose concentrations in the environment. High initial glucose concentrations allow inactivating enzymes involved in lipid synthesis in some microorganisms. Studies by Mondala et al. (2012), 40 and 60 g/L glucose showed no difference in lipid production. In Braunwald et al. (2013), after 48 hr of incubation, no significant differences were noted among C/N ratios (glucose and NH_4^+) of 20, 70, and 120 by *R. glutinis*. However, higher lipid accumulation was apparent after 120 hr of incubation.

Table 4
Optimizing Lipomyces maratuensis InaCC Y720 *lipid content and comparing data with model predictions*

Run	Glucose (g/L)	Yeast extract (g/L)	$MgSO_4$ (g/L)	Lipid content (g/L)		
				Experiment	Predicted	Residues
1	50	4.5	4.5	0.40	0.49	-0.09
2	50	4.5	**1.5**	**1.66**	**1.61**	**0.05**
3	70	3.0	4.5	0.56	0.45	0.11
4	30	3.0	4.5	0.76	0.73	0.03
5	70	1.5	3.0	0.48	0.54	-0.06
6	50	3.0	3.0	1.03	0.87	0.16
7	50	1.5	1.5	0.45	0.36	0.09
8	30	4.5	3.0	0.99	0.92	0.07
9	**70**	**3.0**	**1.5**	**1.66**	**1.68**	**-0.02**
10	30	3.0	1.5	0.59	0.70	-0.11
11	50	3.0	3.0	0.99	0.87	0.12
12	70	4.5	3.0	1.31	1.33	-0.02
13	30	1.5	3.0	0.27	0.25	0.02
14	50	1.5	4.5	0.24	0.28	-0.04
15	50	3.0	3.0	0.59	0.87	-0.28

Note. $MgSO_4$ = Magnesium sulfate; The highlighted figures = Highest results in the experiments

The RSM can estimate the equation model of the medium concentration to obtain the optimum response of lipid accumulation. The experiment of the BBD was fitted with polynomial regression (Table 5) as follows:

$$y = -2.6187 + 0.2537a + 9.6074b + 7.5336c + 0.0122a^2 - 6.9568b^2 - 1.2901c^2 + 0.952ab - 1.0560ac - 11.5873bc \quad \text{[Eq. 3]}$$

where, y = lipid yield (g/L), a = glucose concentration (g/L), b = yeast extract concentration (g/L), c = $MgSO_4$ concentration (g/L).

Based on the ANOVA of the polynomial equation model (Table 5), yeast extract and the interaction of glucose*$MgSO_4$ and yeast extract*$MgSO_4$ exhibit p-values of less than 0.05, signifying their significant impact on lipid accumulation. The lack-of-fit value is crucial for assessing the reliability of the regression model derived from the experimental design (Minitab, n.d.c). In this case, the model's lack-of-fit *p*-value is greater than 0.05, indicating no significant difference. It implies that the response data aligns well with the model and that no statistically significant difference exists in responses.

In the contour plot (Figure 2), the correlation between glucose and yeast extract

Table 5
Analysis of variance for the polynomial model for optimization of lipid production (P<0.05)

Factors	df	Seq SS	Adj SS	Adj MS	*F*	*P*
Regression	9	2.811	2.811	0.312	9.000	0.013*
Linear	3	2.029	0.404	0.135	3.880	0.089
Glucose	1	0.024	0.029	0.029	0.830	0.403
Yeast extract	1	1.066	0.296	0.296	8.520	0.033*
$MgSO_4$	1	0.717	0.182	0.182	5.240	0.071
Square	3	0.106	0.106	0.035	1.020	0.459
Glucose*Glucose	1	0.015	0.009	0.008	0.250	0.636
Yeast extract*Yeast extract	1	0.088	0.090	0.090	2.610	0.167
$MgSO_4$*$MgSO_4$	1	0.003	0.003	0.003	0.090	0.777
Interaction	3	0.676	0.676	0.225	6.500	0.035*
Glucose*Yeast extract	1	0.003	0.003	0.003	0.090	0.771
Glucose*$MgSO_4$	1	0.401	0.401	0.401	11.570	0.019*
Yeast extract*$MgSO_4$	1	0.272	0.272	0.272	7.830	0.038*
Residual error	5	0.173	0.173	0.034		
Lack-of-fit	3	0.055	0.055	0.018	0.310	0.821
Pure error	2	0.118	0.118	0.059		
Total	14	2.985				

Note. R^2 = 0.9419; df = Degrees of freedom; Seq SS = Sequential sum of squares; Adj SS = Adjusted sum of squares; Adj MS = Adjusted mean square; * = Significant at *P*-value <0.05; $MgSO_4$ = Magnesium sulfate

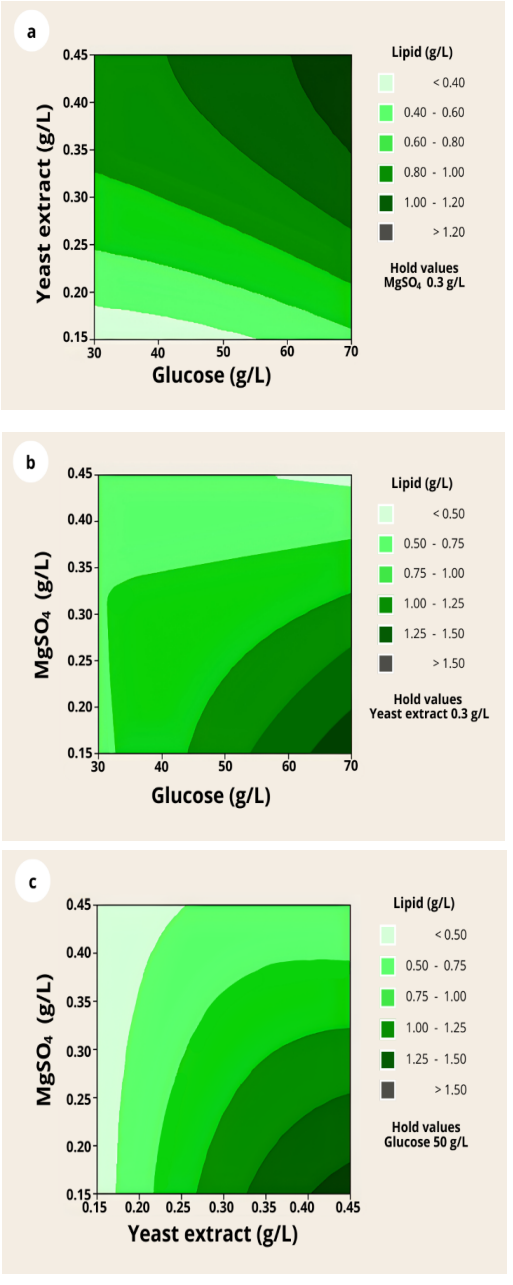

Figure 2. Contour plot of the glucose, yeast extract, and magnesium sulfate ($MgSO_4$) interaction in lipid accumulation. (a) Glucose*Yeast extract, (b) Glucose*$MgSO_4$, and (c) Yeast extract*$MgSO_4$

indicated that elevating the concentration increases lipid accumulation. While $MgSO_4$ showed the opposite interaction. Based on Taguchi and RSM, 0.15 g/L is the optimum concentration for $MgSO_4$. Glucose and yeast extract have not reached their peak point, meaning they can be increased to determine the optimal concentration. However, as explained above, longer incubation may be necessary at higher glucose concentrations. Glucose is a carbon source for forming lipid chains, and a high C/N ratio has been proven to stimulate increased lipid production (Lopes et al., 2020). Yeast extract contains amino acids and trace elements essential for biomass, growth, and lipid metabolism. These include glycine, proline, histidine, alanine, tyrosine, cysteine, arginine, asparagine, glutathione, dextran, mannan, trehalose, vitamin B, Fe^{3+}, Mg^{2+}, Mn^{4+}, K^{+}, Se^{2-}, Na^{+}, Zn^{2+}, Ca^{2+}, and Cu^{2+} (Ardiyanti & Guntoro, 2019; Tomé, 2021). In lipid metabolism, B vitamins play a role. Pyridoxine (B6) functions as a coenzyme, and inositol (B8) is a precursor of phosphatidylinositol, the main constituent of phospholipid membranes (Perli et al., 2020).

The model's statistical analysis included an assessment of accuracy and the normality of the residuals. The R^2 value indicates the polynomial equation's suitability to the experimental data. The parity plot (Figure 3) shows an R^2 value of 94.19%, meaning that the polynomial model cannot explain only around 5.81% of the total variation.

In addition, the residual data were analyzed using the Kolmogorov-Smirnov. It evaluates whether the residuals' distribution

significantly deviates from a normal distribution (Minitab, n.d.b). Figure 4, the probability plot of lipids showed a *p*-value of 0.085 (> 0.05). It suggests that the prediction model is not statistically significant for the experiment, indicating that the distribution of the residuals does not significantly differ from a normal distribution.

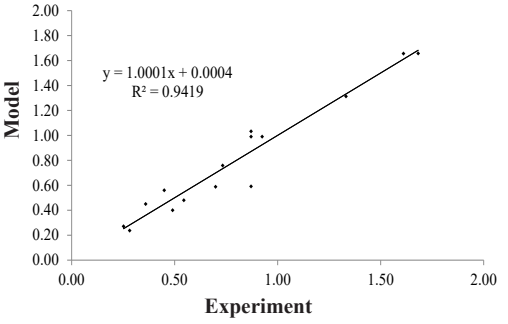

Figure 3. Parity plot of the model to the experiment (R^2 = 0.9419)

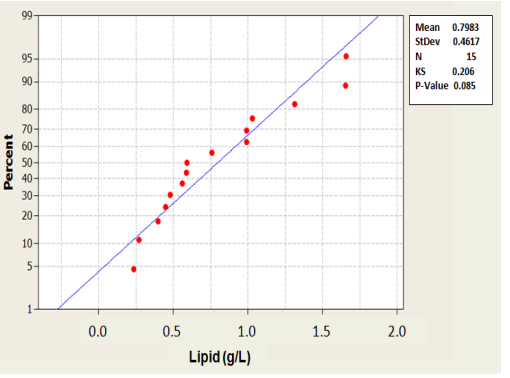

Figure 4. Normal probability plot for lipid production

Note. StDev = Standard deviation; N = Sample sizes or number of observations; KS = Kolmogorov-Smirnov

Profile of Lipid, Biomass, and Consumption of Carbon and Nitrogen

This study compares the profile of lipid, biomass, consumption of carbon and nitrogen of *L. maratuensis* InaCC Y720 in both media. The optimized medium by RSM, determined as the highest lipid production, comprises the following concentrations in g/L: 5.0 glucose, 0.45 yeast extract, 0.15 $MgSO_4$, 0.1 NH_4Cl, 0.0016 $FeCl_3$, 0.00002 $ZnSO_4$, 0.00002 $Co(NO_3)_2$, and 0.00001 $MnSO_4$. The results of this comparison are presented in Figure 5.

The optimized medium obtained 5.26 g/L biomass, 1.57 g/L (31.7%) lipid, with a yield of 0.0157, while the initial medium obtained 4.96 g/L biomass, 0.43 g/L (8.7%) lipids, with a yield of 0.0043. The optimized medium exhibited a significant 3.6-fold increase in lipid content. This improvement is attributed not only to the optimization of glucose, yeast extract, and $MgSO_4$ concentrations by RSM but also to the exclusion of KH_2PO_4, Na_2HPO_4, $CaCl_2$, and $CuSO_4$ based on Taguchi selection.

In this study, a decrease in lipid content percentage was observed (despite an increase in biomass) in the media after 60 hr, both in the initial and optimized media (Figure 5). It might be caused by the exhaustion of carbon in media that was detected to be depleted before 48 hr (Figure 6). Similar findings were reported by X. Zhang et al. (2017); there was a decrease in lipid content after 48 hr on glucose and 60 hr on glycerol. At the same time, the carbon source was detected as exhausting after 36 hr for the glucose medium and 48 hr for the glycerol medium, respectively.

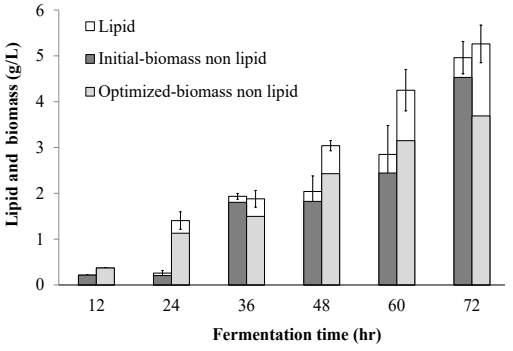

Figure 5. Lipid and biomass on initial and optimized medium

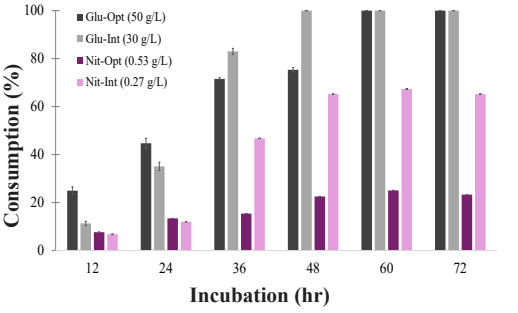

Figure 6. Glucose and nitrogen consumption (%) in initial and optimized media

Note. Glu = Glucose; Nit = Nitrogen; Opt = Optimized media; Int = Initial media

This study measured nitrogen consumption rates to identify when nitrogen limitation triggers accumulation (Figure 6). After 72 hr of incubation, nitrogen consumption reached 65.21% in the initial medium and 23.30% in the optimized medium. It assumed that the Kjeldahl Method is not suitable for this. It only measures nitrogen bound to organic components (proteins, amino acids, nucleic acids) and ammonium in samples (Muñoz-Huerta et al., 2013). In media, NH_4Cl is the main source of inorganic nitrogen, and yeast extract is the source of organic nitrogen and vitamins. It assumed that NH_4Cl was depleted in the media and that nitrogen was detected from extracellular enzymes and yeast extract. It is supported by the optimized media, which has a higher yeast extract concentration, thus explaining why lipid accumulation occurred when nitrogen was still present in this study.

L. Zhang et al. (2022) explain some effective strategies for enhancing lipid yields, such as optimizing parameters, employing two-stage systems, metabolic engineering, selective mutagenesis, and co-culture systems. Parameter optimization including physical parameters (agitation speed, temperature, inoculum age and size, optical density (OD), operation modes, feeding strategies, and fermentation period) and chemical parameters (concentration and source of carbon and nitrogen, C/N ratio, pH, micronutrients, and inhibitor concentration). In our study, some chemical parameters are optimized.

Yamazaki et al. (2017) reported that *L. maratuensis* InaCC Y720 accumulated 3.70 g/L total fatty acids after ten days in the 5G5M medium (50 g/L glucose, 50 g/L malt extract). This study only achieved 43% of that amount (1.66 g/L) within three days. It has shorter incubation and uses fewer carbon sources. Nonetheless, this research successfully optimized the Bligh Dyer medium for lipid accumulation by *L. maratuensis* InaCC Y720. It has optimized the concentration of media components and eliminated components considered inhibitors of lipid accumulation by *L. maratuensis* InaCC Y720.

Lipomyces, especially *L. starkeyi*, is a frequently utilized species in lipid production research using oleaginous yeast. *L. starkeyi* InaCC Y604 (isolated from Indonesia) grown in nitrogen-limited mineral medium (-NMM) ($MgSO_4$ 1.5 g/L, KH_2PO_4 7 g/L, Na_2HPO_4 5 g/L, $FeSO_4$ 0.08 g/L, $ZnSO_4$ 0.01 g/L, $CaCl_2$ 0.1 g/L, $MnSO_4$ 0.1 g/L, $CuSO_4$ 0.002 g/L, and $CoCl_2$ 0.002 g/L) containing glucose (50 g/L) and xylose (50 g/L) achieved lipid content and biomass 17% (w/w) and 25.02 g/L (60 hr), respectively (Agustriana et al., 2020). In a subsequent study, Juanssilfero et al. (2021) introduced variations in other carbon sources in the design (fructose, galactose, mannose, and cellobiose). It showed mixed glucose and xylose still the highest even compared with a single carbon source (lipid content and biomass of 64.19% (w/w) and 34.49±0.38 g/L [60 hr]).

In contrast, in this study, the attained lipid content and biomass were only 31% (w/w) and 5.25 g/L (60 hr). Apart from genetic differences, variations also exist in the C/N ratio, carbon concentration, sources, and extraction methods. Nevertheless, the potential and lipid production capabilities of *L. maratuensis* InaCC Y720 remain unexplored and open to optimization.

CONCLUSION

This study enhanced lipid production by *L. maratuensis* InaCC Y720 by applying Taguchi and RSM designs. The optimized medium resulted in a 3.6-fold increase in lipid production compared to the initial medium. Glucose, yeast extract, and $MgSO_4$ emerged as significant factors influencing lipid accumulation. $MnSO_4$, $ZnSO_4$, $Co(NO_3)_2$, NH_4Cl, and $FeCl_3$ showed a positive influence. KH_2PO_4, Na_2HPO_4, and $CaCl_2$ inhibit lipid accumulation, and $CuSO_4$ has the lowest contribution. According to RSM results, the optimized medium comprises the following concentrations in g/L: 5.0 glucose, 0.45 yeast extract, 0.15 $MgSO_4$, 0.1 NH_4Cl, 0.0016 $FeCl_3$, 0.00002 $ZnSO_4$, 0.00002 $Co(NO_3)_2$, and 0.00001 $MnSO_4$.

ACKNOWLEDGMENTS

This study was part of the project funded by Universitas Gadjah Mada, Indonesia through Rekognisi Tugas Akhir (RTA) Grant (no. 3143/UN1.P.III/DIT-LIT/PT/2021) and the government of the Republic of Indonesia through Prioritas Riset Nasional (PRN) Grant (no. 006/E4.1/AK.04.PRN/2021).

REFERENCES

Agustriana, E., Juanssilfero, A. B., Andriani, A., Fahrurrozi., Pangestu, R., & Yopi. (2020). Single cell oil production by wild type strain *Lipomyces starkeyi* Y604. In *IOP Conference Series: Earth and Environmental Science* (Vol. 439, No. 1, p. 012002). IOP Publishing. https://doi.org/10.1088/1755-1315/439/1/012002

Anandan, R., Dharumadurai, D., & Manogaran, G. P. (2016). An introduction to Actinobacteria. In D. Dhanasekaran & Y. Jiang (Eds.), *Actinobacteria - Basics and biotechnological applications*. IntechOpen. https://doi.org/10.5772/62329

Ardiyanti, C. A. P., & Guntoro. (2019). Yeast extract production from spent brewer's yeast. *BIOEDUKASI: Jurnal Pendidikan Biologi*, *12*(1), 52–60. https://doi.org/10.20961/bioedukasi-uns.v12i1.27404

Arigony, A. L. V., de Oliveira, I. M., Machado, M., Bordin, D. L., Bergter, L., Prá, D., & Henriques, J. A. P. (2013). The influence of micronutrients in cell culture: A reflection on viability and genomic stability. *BioMed Research International*, *2013*, 597828. https://doi.org/10.1155/2013/597282

Bligh, E. G., & Dyer, W. J. (1959). A rapid method of total lipid extraction and purification. *Canadian Journal of Biochemistry and Physiology*, *37*(8), 911–927. https://doi.org/10.1139/o59-099

Braunwald, T., Schwemmlein, L., Graeff-Hönninger, S., French, W. T., Hernandez, R., Holmes, W. E., & Claupein, W. (2013). Effect of different C/N ratios on carotenoid and lipid production by *Rhodotorula glutinis*. *Applied Microbiology and Biotechnology*, *97*, 6581–6588. https://doi.org/10.1007/s00253-013-5005-8

Duman-Özdamar, Z. E., dos Santos, V. A. P. M., Hugenholtz, J., & Suarez-Diez, M. (2022). Tailoring and optimizing fatty acid production by oleaginous yeasts through the systematic exploration of their physiological fitness. *Microbial Cell Factories*, *21*, 228. https://doi.org/10.1186/s12934-022-01956-5

Dzurendova, S., Zimmermann, B., Kohler, A., Reitzel, K., Nielsen, U. G., Dupuy-Galet, B. X., Leivers, S., Horn, S. J., & Shapaval, V. (2021). Calcium affects polyphosphate and lipid accumulation in Mucoromycota fungi. *Journal of Fungi*, *7*(4), 300. https://doi.org/10.3390/jof7040300

Dzurendova, S., Zimmermann, B., Tafintseva, V., Kohler, A., Horn, S. J., & Shapaval, V. (2020). Metal and phosphate ions show remarkable influence on the biomass production and lipid accumulation in oleaginous *Mucor circinelloides*. *Journal of Fungi*, *6*(4), 260. https://doi.org/10.3390/jof6040260

El Kantar, S., Khelfa, A., Vorobiev, E., & Koubaa, M. (2021). Strategies for increasing lipid accumulation and recovery from *Y. lipolytica*: A review. *Oilseeds and fats, Crops and Lipids*, *28*, 51. https://doi.org/10.1051/ocl/2021038

Granger, L.-M., Perlot, P., Goma, G., & Pareilleux, A. (1993). Effect of various nutrient limitations on fatty acid production by *Rhodotorula glutinis*. *Applied Microbiology and Biotechnology*, *38*, 784–789. https://doi.org/10.1007/BF00167145

Hamzaçebi, C. (2021). Taguchi method as a robust design tool. In P. Li, P. A. R. Pereira, & H. Navas (Eds.), *Quality control - Intelligent manufacturing, robust design and charts*. IntechOpen. https://doi.org/10.5772/intechopen.94908

Holdsworth, J. E., & Ratledge, C. (1988). Lipid turnover in oleaginous yeasts. *Microbiology*, *134*(2), 339–346. https://doi.org/10.1099/00221287-134-2-339

Juanssilfero, A. B., Salsabila, P., Agustriana, E., Andriani, A., Fahrurrozi., Perwitasari, U., & Sutrisno, A. (2021). Microbial lipid production by the yeast *Lipomyces starkeyi* InaCC Y604 grown on various carbon sources. In *IOP Conference Series: Earth and Environmental Science* (Vol. 762, No. 1, p. 012073). IOP Publishing. https://doi.org/10.1088/1755-1315/762/1/012073

Lopes, H. J. S., Bonturi, N., Kerkhoven, E. J., Miranda, E. A., & Lahtvee, P.-J. (2020). C/N ratio and carbon source-dependent lipid production profiling in *Rhodotorula toruloides*. *Applied Microbiology and Biotechnology*, *104*, 2639–2649. https://doi.org/10.1007/s00253-020-10386-5

Manikan, V., Kalil, M. S., Omar, O., Omar, O., Abdul Kader, A. J., & Abdul Hamid, A. (2014). Effects of Mg^{2+}, Fe^{3+}, Mn^{2+} and Cu^{2+} ions on lipid accumulation by *Cunninghamella bainieri* 2A1. *Sains Malaysiana*, *43*(3), 443-449.

Mhlongo, S. I., Ezeokoli, O. T., Roopnarain, A., Ndaba, B., Sekoai, P. T., Habimana, O., & Pohl, C. H. (2021). The potential of single-cell oils derived from filamentous fungi as alternative feedstock sources for biodiesel production. *Frontiers in Microbiology*, *12*, 637381. https://doi.org/10.3389/fmicb.2021.637381

Minitab. (n.d.a). *Interpret the key results for analyze Taguchi design*. https://support.minitab.com/en-us/minitab/help-and-how-to/statistical-

modeling/doe/how-to/taguchi/analyze-taguchi-design/interpret-the-results/key-results/

Minitab. (n.d.b). *Interpret the key results for Normality test*. https://support.minitab.com/en-us/minitab/21/help-and-how-to/statistics/basic-statistics/how-to/normality-test/interpret-the-results/key-results/

Minitab. (n.d.c). *Lack-of-fit and lack-of-fit tests*. https://support.minitab.com/en-us/minitab/21/help-and-how-to/statistical-modeling/regression/supporting-topics/regression-models/lack-of-fit-and-lack-of-fit-tests/

Mondala, A. H., Hernandez, R., French, T., McFarland, L., Santo Domingo, J. W., Meckes, M., Ryu, H., & Iker, B. (2012). Enhanced lipid and biodiesel production from glucose-fed activated sludge: Kinetics and microbial community analysis. *AIChE Journal*, *58*(4), 1279–1290. https://doi.org/10.1002/aic.12655

Morales-Palomo, S., Tomás-Pejó, E., & González-Fernández, C. (2023). Phosphate limitation as crucial factor to enhance yeast lipid production from short-chain fatty acids. *Microbial Biotechnology*, *16*(2), 372–380. https://doi.org/10.1111/1751-7915.14197

Muñoz-Huerta, R. F., Guevara-Gonzalez, R. G., Contreras-Medina, L. M., Torres-Pacheco, I., Prado-Olivarez, J., & Ocampo-Velazquez, R. V. (2013). A review of methods for sensing the nitrogen status in plants: Advantages, disadvantages and recent advances. *Sensors*, *13*(8), 10823–10843. https://doi.org/10.3390/s130810823

Ouedraogo, N., Savadogo, A., Somda, M. K., Tapsoba, F., Zongo, C., & Traore, A. S. (2017). Effect of mineral salts and nitrogen source on yeast (*Candida utilis* NOY1) biomass production using tubers wastes. *African Journal of Biotechnology*, *16*(8), 359–365. https://doi.org/10.5897/AJB2016.15801

Papanikolaou, S., Muniglia, L., Chevalot, I., Aggelis, G., & Marc, I. (2002). *Yarrowia lipolytica* as a potential producer of citric acid from raw glycerol. *Journal of Applied Microbiology*, *92*(4), 737–744. https://doi.org/10.1046/j.1365-2672.2002.01577.x

Perli, T., Wronska, A. K., Ortiz-Merino, R. A., Pronk, J. T., & Daran, J.-M. (2020). Vitamin requirements and biosynthesis in *Saccharomyces cerevisiae*. *Yeast*, *37*(4), 283–304. https://doi.org/10.1002/yea.3461

Qin, L., Liu, L., Zeng, A.-P., & Wei, D. (2017). From low-cost substrates to Single Cell Oils synthesized by oleaginous yeasts. *Bioresource Technology*, *245*(Part B), 1507–1519. https://doi.org/10.1016/j.biortech.2017.05.163

Roy, R. K. (1990). *A primer on the Taguchi method*. Society of Manufacturing Engineers.

Šajbidor, J., Koželouhov'a, D., & Ĉert'ik, M. (1992). Influence of some metal ions on the lipid content and arachidonic acid production by *Mortierella* sp. *Folia Microbiologica*, *37*, 404–406. https://doi.org/10.1007/BF02899897

Sarkar, S., Bhowmick, T. K., & Gayen, K. (2023). Enhancement for the synthesis of bio-energy molecules (carbohydrates and lipids) in *Desmodesmus subspicatus*: Experiments and optimization techniques. *Preparative Biochemistry and Biotechnology*, *54*(3), 343-357. https://doi.org/10.1080/10826068.2023.2241898

Shuib, S., Wan Nawi, W. N. N., Taha, E. M., Omar, O., Abdul Kader, A. J., Kalil, M. S., & Abdul Hamid, A. (2014). Strategic feeding of ammonium and metal ions for enhanced GLA-rich lipid accumulation in *Cunninghamella bainieri* 2A1. *The Scientific World Journal*, *2014*, 173574. https://doi.org/10.1155/2014/173574

Tomé, D. (2021). Yeast extracts: Nutritional and flavoring food ingredients. *ACS Food Science and Technology*, *1*(4), 487–494. https://doi.org/10.1021/acsfoodscitech.0c00131

Wang, W.-A., Liu, W.-X., Durnaoglu, S., Lee, S.-K., Lian, J., Lehner, R., Ahnn, J., Agellon, L. B., & Michalak, M. (2017). Loss of calreticulin uncovers a critical role for calcium in regulating cellular lipid homeostasis. *Scientific Reports*, *7*, 5941. https://doi.org/10.1038/s41598-017-05734-x

Wood, I. P., Elliston, A., Ryden, P., Bancroft, I., Roberts, I. N., & Waldron, K. W. (2012). Rapid quantification of reducing sugars in biomass hydrolysates: Improving the speed and precision of the dinitrosalicylic acid assay. *Biomass and Bioenergy*, *44*, 117–121. https://doi.org/10.1016/j.biombioe.2012.05.003

Wu, S., Hu, C., Jin, G., Zhao, X., & Zhao, Z. K. (2010). Phosphate-limitation mediated lipid production by *Rhodosporidium toruloides*. *Bioresource Technology*, *101*(15), 6124–6129. https://doi.org/10.1016/j.biortech.2010.02.111

Yamazaki, A., Kanti, A., & Kawasaki, H. (2017). Three novel lipomycetaceous yeasts, *Lipomyces maratuensis* sp. nov., *Lipomyces tropicalis* sp. nov., and *Lipomyces kalimantanensis* f.a., sp. nov. isolated from soil from the Maratua and Kalimantan Islands, Indonesia. *Mycoscience*, *58*(6), 413–423. https://doi.org/10.1016/j.myc.2017.06.002

Yoo, J.-Y., Lee, H.-C., Shin, D.-H., & Min, B.-Y. (1982). Production of fungal lipids effects of vitamins metabolic intermediates and mineral salts on the growth and lipid accumulation of *Mucor plumbeus*. *Korean Journal of Food Science and Technology*, *14*, 151–155.

Zhang, L., Lee, J. T. E., Ok, Y. S., Dai, Y., & Tong, Y. W. (2022). Enhancing microbial lipids yield for biodiesel production by oleaginous yeast *Lipomyces starkeyi* fermentation: A review. *Bioresource Technology*, *344*(Part B), 126294. https://doi.org/10.1016/j.biortech.2021.126294

Zhang, X., Chen, J., Yan, S., Tyagi, R. D., Surampalli, R. Y., & Li, J. (2017). Lipid production for biodiesel from sludge and crude glycerol. *Water Environment Research*, *89*(5), 424–439. https://doi.org/10.2175/106143017x14839994523424

Zhao, L., Li, B., Xiong, D., Zhang, H., Tang, X., Zhang, H., Song, Y., & Yang, S. (2016). Cocoa-butter-equivalent production from *Yarrowia lipolytica* by optimization of fermentation technology. *American Journal of Biochemistry and Biotechnology*, *12*(4), 196–205. https://doi.org/10.3844/ajbbsp.2016.196.205