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SIGNIFICANCE OF NITROGEN LIMITED MEDIUM (NLM) COMPONENTS ON LIPID PRODUCTION *Lipomyces starkeyi* Y853

SIGNIFIKANSI KOMPONEN *Nitrogen Limited Medium* (NLM) TERHADAP PRODUKSI LIPID *Lipomyces starkeyi* Y853

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Abstract

Oleaginous yeasts, such as *Lipomyces starkeyi*, can convert carbon and nitrogen sources into lipids up to 50% of the cell's dry weight. A high lipid percentage was achieved (53.5%) using Nitrogen Limited Medium (NLM), which indicates that the components of NLM play a role in the production of biomass and lipids. Nevertheless, the statistical analysis of the role of NLM components on lipid production has not yet been conducted. Thus, this research was designed and carried out to determine the role of NLM components in lipid production using Plackett-Burman Design. The results show that the variables had an insignificant impact on lipid production based on the ANOVA test (P >0.05). Additionally, main effect plots were generated to identify the negative and positive effects of the components. The graph indicates that peptone and yeast extract (YE) are essential components in high concentrations to increase lipid production. This result was due to the insufficient concentration used in this experiment (YE= 0.5 g/L; peptone= 0.3 g/L) compared to the optimal conditions (YE= 8 g/L; peptone= 3 g/L). Therefore, further research should be conducted with the addition of external factors (pH, temperature, shaker speed) to acquire more significant results on biomass lipid production.

Keywords: Lipid production; *Lipomyces starkeyi*; Nitrogen Limited Medium (NLM); Plackett-Burman

Abstrak

Khamir oleaginous, seperti Lipomyces starkeyi, mampu mengubah sumber karbon dan nitrogen menjadi lipid hingga 50% dari berat kering sel. Persentase lipid yang dihasilkan oleh L. starkeyi mencapai 53.5% menggunakan Nitrogen Limited Medium (NLM) sebagai media kulturnya karena dapat meningkatkan akumulasi lipid. Hal tersebut menunjukkan komponen NLM memiliki peran dalam produksi biomassa dan lipid. Namun, pengaruh signifikan dari masing-masing komponen belum diketahui secara kuantitatif. Oleh karena itu, Desain Plackett-Burman (PBD) digunakan untuk analisis enam variabel pada dua level guna menentukan kontribusi masing-masing terhadap produksi biomassa dan lipid. Hasil penelitian menunjukkan bahwa tidak ada variabel yang signifikan terhadap produksi lipid berdasarkan P-value uji ANOVA ≥0.05 (95%). Selain itu, main effect plot digunakan untuk mengamati efek negatif dan positif komponen terhadap produksi biomassa dan lipid. Keduanya menunjukan bahwa pepton dan yeast extract merupakan komponen yang diperlukan dalam konsentrasi tinggi untuk meningkatkan kedua produksi. Hal tersebut terjadi karena konsentrasi yang digunakan dalam eksperimen ini terlalu rendah (yeast extract= 0.5 g/L; pepton= 0.3 g/L) dibandingkan dengan kondisi optimal (yeast extract= 8 g/L; pepton= 3 g/L). Untuk penelitian selanjutnya, penambahan faktor eksternal (pH, suhu, kecepatan shaker) diperlukan untuk memperoleh hasil yang lebih signifikan terhadap produksi lipid.

***Kata Kunci**: Lipomyces starkeyi; Nitrogen Limited Medium (NLM); Plackett-Burman; Produksi lipid*

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INTRODUCTION

Microorganisms can obtain a specific compound from resources made available within their environment. For example, they can convert carbohydrate compounds into lipids, which is seen in oleaginous yeast. Oleaginous yeast is a type of single-cell fungus that is capable of accumulating lipid up to more than 20% of its biomass. Two genera of oleaginous yeast are *Lipomyces* and *Rhodotorula*, which are capable of accumulating lipids up to 70% of the weight of the biomass produced (Ratledge & Wynn, 2002). *Lipomyces starkeyi* is one of the oleaginous yeasts that has the potential to be used on an industrial scale for lipid production due to its triacylglycerol (TAG) composition. The TAG composition produced by *L. starkeyi* primarily consists of palmitic and oleic acids, which are also present in vegetable oils. Therefore, TAG accumulated by *L. starkeyi* can serve as a substitute for vegetable oil as a feedstock for biodiesel production (Yamazaki et al., 2019). One benefit of utilizing microbes as lipid producers is their relatively short life cycle which requires less labor and is more cost-effective when scaled up to industrial standards (Li et al., 2008). Additionally, *L. starkeyi* specifically strain Y853 has been demonstrated to exhibit the highest lipid accumulation among the other six strains of *Lipomyces,* with lipid content up to 50% (Mar'atussholihah, 2023). Therefore, the objective of this experiment is to analyze the impact of nitrogen-limited medium (NLM) components on lipid production using *L. starkeyi* Y853.

L. starkeyi has been demonstrated to utilize a diverse range of carbon sources for lipid production, including glucose (Oguri et al., 2012), xylose (Zhao et al., 2008), glycerol (Liu et al., 2017), starch (Liu et al., 2013), and industrial waste (Angerbauer et al., 2008). Glucose is a typical carbon source utilized in the cultivation of *L. starkeyi* for lipid production because *Lipomyces* is capable of converting glucose first into lipids compared to other carbon sources. Moreover, *L. starkeyi* has been demonstrated to produce the highest lipid yields when utilizing a carbon source in the form of D-glucose (Oguri et al., 2012). Mar'atussholihah (2023) indicates that *L. starkeyi* Y853 has a lipid yield of 4.36 g/L at the 48th hour with a biomass of 8.14 g/L when using NLM due to the medium utilizing glucose as its primary carbon source. Hence, glucose was selected as the carbon source to achieve the highest lipid production by *L. starkeyi*.

The medium selected for this experiment is a Nitrogen-Limited Medium (NLM), which contains glucose, peptone, yeast extract, KH_2PO_4, $Na_2HPO_4.7H_2O$, and $MgSO_4$ (Jiru et al., 2016). This medium was selected based on the observation that *L. starkeyi* is capable of producing high lipids in low nitrogen concentrations (Li et al., 2008). Additionally, the concentration of metal compounds (Mg^{2+}) has been shown to influence the metabolic rate of yeast when present in low concentrations (Dzurendova et al., 2020). However, a deeper investigation of the specific role of each NLM component on biomass and lipid accumulation by *L. starkeyi* Y853, with statistical analysis, had not been conducted.

The Plackett-Burman Design is a quantitative experimental methodology that identifies critical factors that impact the production of a given product. This statistical test uses a multitude of factors, with observations from a total of 'n' factors and observations of 'n+1' experiments (Vanaja & Rani, 2007). The objective of this study was to determine the significance of the components in NLM on the lipid production of *L. starkeyi* Y853, utilizing a Plackett-Burman Design (PBD) with six variables.

MATERIALS AND METHODS

Materials

Lipomyces starkeyi InaCC Y853 was obtained from the Indonesian Culture Collection (InaCC). Mediums and chemicals which were used were Potato Dextrose Agar (PDA) (Merk Millipore); Glucose Peptones Yeast (GPY) media consisting of glucose (Merck Millipore), peptone (Oxoid), and yeast extract (Merck Millipore); Nitrogen Limited Medium (NLM) with composition glucose (Merck Millipore), peptone (Oxoid), yeast extract (Merck Millipore), KH_2PO_4 (Merck Millipore), $Na_2HPO_4.7H_2O$ (Supelco), and $MgSO_4$ (Supelco); chloroform (Merck Millipore); and methanol (Merck Millipore).

Cell Proliferation with Liquid Glucose Peptones Yeast (GPY) Medium

Strain *L. starkeyi* Y853 was cultivated into liquid GPY medium composition consisting of glucose 100 g/L; peptone 3 g/L; and yeast extract 8 g/L. Inoculum was taken as much as 10% (v/v) into 45 mL liquid GPY medium, incubated at 28 °C for 24 h in the shaking water bath (B-One) at 180 rpm (Jiru et al., 2016).

Cultivation of *Lipomyces starkeyi* in Liquid Nitrogen Limited Medium (NLM)

The results of subculturing in the GPY medium were measured in the optical density (OD) until it reached a value of 0.8 using a UV-VIS spectrophotometer (Thermo Scientific). Subsequently, the effect of the NLM composition in lipid production was observed with six variables at two-factor levels (Table 1). Then, *L. starkeyi* was cultivated in liquid NLM by the experimental design (Table 2), which references the Plackett-Burman Design (PBD). The components and levels were determined by the PBD method. The medium was prepared in 50 mL and then incubated in the shaking water bath (B-One) for 72 h at 28 °C with agitation set at 180 rpm (Jiru et al., 2016).

Table 1. Determination of components and levels using the Plackett-Burman Design (PBD)

Component	Variable code	Low level (g/L)	High level (g/L)
Glucose	A	10	23
Peptone	B	0	0.3
Yeast extract	C	0	0.5
KH_2PO4	D	0	7
$Na_2HPO_4.7H_2O$	E	0	2
$MgSO_4$	F	0	1.5

Table 2. Experiment design using Plackett-Burman Design

Run order	A*	B*	C*	D*	E*	F*
1	23	0.3	0	7	0	0
2	10	0	0	7	2	1.5
3	10	0.3	0	0	0	1.5
4	10	0.3	0.5	7	0	1.5
5	23	0	0	0	2	1.5
6	10	0	0	0	0	0
7	10	0.3	0.5	0	2	0
8	23	0	0.5	0	0	0
9	10	0	0.5	7	2	0
10	23	0.3	0	7	2	0
11	23	0	0.5	7	0	1.5
12	23	0.3	0.5	0	2	1.5

Note: Variable with * symbol is in g/L

Determination of Dry Biomass Weight of *Lipomyces starkeyi* Y853

A 15 mL of the sample was taken from cultivation of NLM, then centrifuged at 5,000 rpm for 10 minutes. The sample was washed with distilled water (15 mL) twice. Subsequently, the washed sample was moved to a conical bottle and dried in the oven (Despatch) at 60 °C until the weight reached constant (Amir et al., 2015).

Lipid Extraction and Determination of Lipid Weight of *Lipomyces starkeyi* Y853

Lipid extraction was conducted using the modified Bligh and Dyer (1959) method. A 35 mL of the sample was taken from cultivation of NLM, then centrifuged at 5,000 rpm for 10 minutes. The sample was washed with distilled water (15 mL) twice. Subsequently, 7 mL of 4 M HCl is added to the sample and heated in a mini water bath for 2 h at 60 °C. After that, 14 mL of chloroform: methanol (2:1) solution was added to the sample and placed at room temperature for 3 h. Then, the sample was centrifuged at 5,000 rpm for 10 minutes to separate the liquid phase (upper phase) and organic phase (lower phase), which contain the lipid. The lipid content was extracted with a pipette and evaporated in the mini water bath (B-One) at 80 °C in the fume hood until the lipid remains in the bottom of the bottle. Lipid evaporation result was weighed and recorded (Amir et al., 2015). The lipid content was

determined using the following formula; lipid content (%) $= \frac{\text{lipid weight (g)}}{\text{dry biomass weight (g)}} \times 100\%$ (Amir et al., 2015).

Determination of Sugar Consumption in Liquid Nitrogen Limited Medium (NLM)

A 35 mL sample was taken from cultivation of NLM, then centrifuged at 5,000 rpm for 10 minutes. The supernatant taken using the pipette is transferred into a conical tube. Then, 1 mL of the supernatant sample is transferred into a test tube, followed by adding the 2 mL distilled water and 1 mL of DNS reagent consisting of 2,3-dinitro salicylic acid powder 10 g/L; Ka-Na tarter 18.2 g/L; and NaOH 10 g/L. The sample was heated with hot water for 2 minutes. Then, the sample was cooled with ice water at 4 °C for 1 minute, removed, and transferred to a cuvette. The absorbance value was measured at a wavelength of 540 nm using a UV-VIS spectrophotometer. The obtained absorbance value was plotted on a standard curve to determine the concentration of glucose in the sample (Julaeha et al., 2016).

Data Analysis

Plackett-Burman Design (PBD) was utilized to optimize the experimental conditions. The PBD employs a two-level design (Table 1) as a factor for observing the screening of significant effects on lipid and biomass production by *L. starkeyi* Y853 with six variables. The data will be tabulated using Microsoft Office Excel 2020 and then analyzed using Minitab ver. 19. The output will be in the form of ANOVA calculation tables and main plot effects. The calculation of the PBD can be obtained using the formulas shown in Eq. 1–4. $\text{Diff A} = \Sigma A\,(H) + \Sigma A(L)$ (Eq.1); $\text{Eff A} = x_{A(H)} - x_{A(L)}$ (Eq.2); $\text{Mean sq. A} = \frac{(\Sigma A(H) - (\ ))}{\text{total trial}}$ (Eq.3); $\text{F value} = \frac{\text{Mean sq.factor}}{\text{Mean sq.error}}$ (Eq.4) (Stanbury et al., 1995).

RESULTS

Biomass Dry Weight (g/L), Lipid Weight (g/L), Lipid Percentage (%), and Percentage Sugar Reduction (%)

The lowest lipid weight was in experiment 8 and the highest in experiment 11 with a yield of 0.0286 g/L and 1.6 g/L respectively (Table 3). The lowest biomass yield was in Experiment 10 and the highest in Experiment 12 were 8.533 g/L and 15.767 g/L, respectively. The highest percentage of lipids was obtained in experiment 1 at 12.37%, while the lowest value was observed in experiment 8 at 0.24%.

Table 3. Experiment design with the result of lipid weight (g/L), biomass dry weight (g/L), lipid content (%), and sugar reduction (%)

Experiment	A*	B*	C*	D*	E*	F*	Biomass dry weight (g/L) ± std	Lipid weight (g/L) ± std	Lipid content (%) ± std	Sugar reduction (%) ± std
1	23	0.3	0	7	0	0	10.622	1.3143	12.37	0,93
2	10	0	0	7	2	1.5	9.978	0.3429	3.44	0.69
3	10	0.3	0	0	0	1.5	11.444	0.6857	5.99	2.07
4	10	0.3	0.5	7	0	1.5	13.289	0.4000	3.01	0.72
5	23	0	0	0	2	1.5	11.644	0.2286	1.96	2.51
6	10	0	0	0	0	0	9.356	0.2286	2.44	2.10
7	10	0.3	0.5	0	2	0	14.000	0.2000	1.43	1.01
8	23	0	0.5	0	0	0	11.822	0.0286	0.24	1,58
9	10	0	0.5	7	2	0	9.356	0.4000	4.28	0,48
10	23	0.3	0	7	2	0	8.533	0.4286	5.02	0,09
11	23	0	0.5	7	0	1.5	14.022	1.6000	11.41	0,96
12	23	0.3	0.5	0	2	1.5	15.767±0,33	1.4286 ± 0.12	9.06 ± 0,96	2,78 ± 0.29

Note: Variable with * symbol is in g/L; A= glucose; B= peptone; C= yeast extract; D= KH_2PO4; E= $Na_2HPO_4.7H_2O$; F= $MgSO_4$

The result of the percentage of sugar reduction ranges from 0.09% to 2.78%, with the lowest value obtained in experiment 10, while the highest was observed in experiment 12. The highest values for biomass dry weight, lipid weight, lipid content, and percentage sugar reduction were observed when a high level of glucose (variable A) was utilized, while the lowest values were obtained when using

a low level of MgSO4 (variable F). The results are then submitted for further analysis using MiniTab ver. 19, which generates an Analysis of Variance (ANOVA) table and main effect plot.

Effects of Nitrogen Limited Medium (NLM) Components on Biomass Production by *Lipomyces starkeyi* Y853

Biomass harvesting was conducted through the drying of the biomass using an oven. The objective was to determine the dry weight of the biomass produced by *L. starkeyi* Y853. The result of dry-weight biomass will be used as a basis for calculating the lipid content, which is essential to determine which components of NLM are responsible for biomass production. As illustrated in Table 4, the P-value of the model is statically significant ($P \leq 0.05$). The highest value was observed for the $Na_2HPO_4.7H_2O$ variable (0.756), meanwhile, the lowest value was observed for yeast extract (0.008). The order of the P-values from highest to lowest, is as follows $Na_2HPO_4.7H_2O$ > glucose > peptones > KH_2PO_4 > $MgSO_4$ > yeast extract.

Table 4. Analysis of variance (ANOVA) calculation result for biomass weight (g/L)

Variable code	Factor	Df	Adj SS	Adj MS	F-value	P-value
	Model	6	48.6254	8.1042	6.41	0.030
	Linear	6	48.6254	8.1042	6.41	0.030
A	Glucose	1	2.0725	2.0725	1.64	0.257
B	Peptone	1	4.6588	4.6588	3.68	0.113
C	*Yeast Extract*	1	23.1824	23.1824	18.33	0.008
D	KH_2PO_4	1	5.6485	5.6485	4.47	0.088
E	$Na_2HPO_4.7H_2O$	1	0.1359	0.1359	0.11	0.756
F	$MgSO_4$	1	12.9273	12.9273	10.22	0.024
	Error	5	6.3244	1.2649		
	Total	11	54.9498			

Note: Df= degrees of freedom; SS= sum of squares; MS= mean squares

Figure 1 depicts the mean variation of each variable utilized in the NLM between low and high levels of biomass production. The result indicates that three variables (peptone, yeast extract, and $MgSO_4$) have positive effect, while the other three variables (glucose, KH_2PO_4, and $Na_2HPO_4.7H_2O$) has minimal effect.

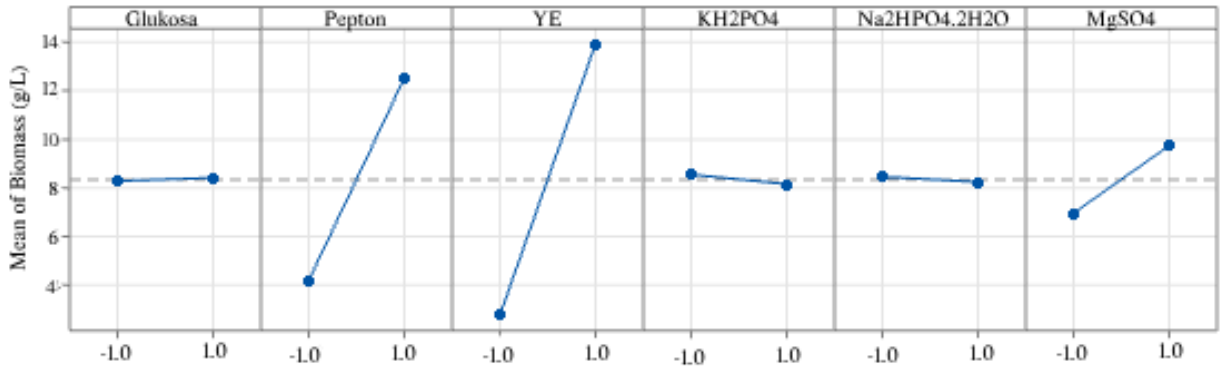

Figure 1. Main effect plot between nitrogen-limited medium **(**NLM) component with biomass weight (g/L)

Effects of Nitrogen Limited Medium (NLM) Components on Lipid Production by *Lipomyces starkeyi* Y853

ANOVA was conducted to determine which variables that influence on lipid production by *L. starkeyi* Y853 in NLM. Based on

Table **5**, the P-value associated with the model is not statistically significant ($P > 0.05$). The highest value was observed for the yeast extract variable (0.680), while the lowest value was observed for the glucose variable (0.204). Therefore, it can be concluded that none of the variables exhibited a statistically significant impact on lipid production. Thus, the effect of each NLM component on lipid production by *L. starkeyi* Y853 will be observed using the main effects plot (Figure 2).

Table 5. Analysis of variance (ANOVA) calculation result for lipid weight (g/L)

Variable code	Factor	Df	Adj SS	Adj MS	F-value	P-value
	Model	6	1.64338	0.27390	0.91	0.550
	Linear	6	1.64338	0.27390	0.91	0.550
A	Glucose	1	0.6401	0.6401	2.14	0.204
B	Peptone	1	0.221	0.221	0.74	0.430
C	*Yeast Extract*	1	0.0572	0.0572	0.19	0.680
D	KH_2PO_4	1	0.2368	0.2368	0.79	0.415
E	$Na_2HPO_4.7H_2O$	1	0.12577	0.12577	0.42	0.546
F	$MgSO_4$	1	0.36251	0.36251	1.21	0.321
	Error	5	1.49800	0.29960		
	Total	11	3.14138			

Note: Df= degrees of freedom; SS= sum of squares; MS= mean squares

Figure 2 illustrates the mean variation of each variable used in the NLM process between low and high levels of lipid production. The graph indicated that three variables (peptone, yeast extract, and $MgSO_4$) have a positive effect, two variables (glucose and KH_2PO_4) have minimum impact and one variable ($Na_2HPO_4.7H_2O$) has a negative effect.

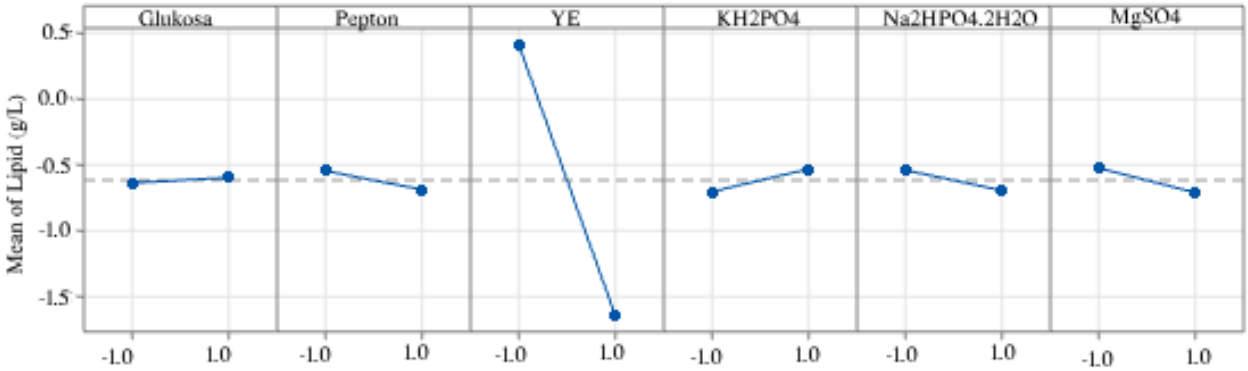

Figure 2. Main effect plot between nitrogen-limited medium **(**NLM) component with lipid weight (g/L)

Effects of Nitrogen Limited Medium (NLM) Components on Percentage of Sugar Reduction by *Lipomyces starkeyi* Y853

As illustrated in Table 6, the P-value of the model indicates a statistically significant difference in the percentage of sugar reduction ($P < 0.05$). The highest value was observed for the peptone variable (0.676) while the lowest value (0.004) was observed for the KH_2PO_4. This value indicates that KH_2PO_4 plays a significant role in increasing the percentage of sugar reduction.

Table 6. Analysis of variance (ANOVA) calculation result for percentage of sugar reduction (%)

Variable code	Factor	Df	Adj SS	Adj MS	F-value	P-value
	Model	6	0.000704	0.000117	5.35	0.043
	Linear	6	0.000704	0.000117	5.35	0.043
A	Glucose	1	0.000026	0.000026	1.20	0.323
B	Peptone	1	0.000004	0.000004	0.20	0.676
C	*Yeast Extract*	1	0.000006	0.000006	0.28	0.619
D	KH_2PO_4	1	0.000558	0.000558	25.43	0.004
E	$Na_2HPO_4.7H_2O$	1	0.000005	0.000005	0.24	0.643
F	$MgSO_4$	1	0.000104	0.000104	4.76	0.081
	Error	5	0.000110	0.000022		
	Total	11	0.000814			

Note: Df= degrees of freedom; SS= sum of squares; MS= mean squares

Figure 3 represents a main effect plot that illustrates the mean variation of each variable used in the NLM between low and high levels on the percentage of sugar reduction by *L. starkeyi* Y853.

The results indicate that four variables (peptones, yeast extract, KH_2PO_4, and $Na_2HPO_4.7H_2O$) have a negative effect, while two variables (glucose and $MgSO_4$) have a positive effect.

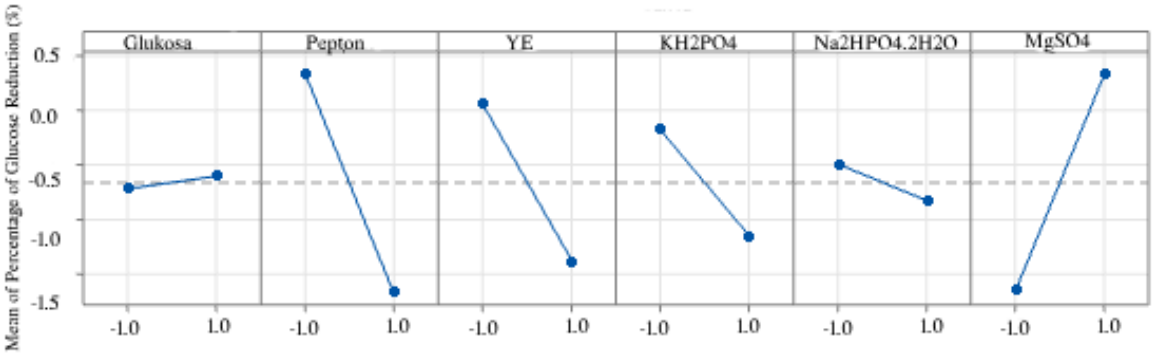

Figure 3. Main effect plot between nitrogen-limited medium **(**NLM) component with the percentage of glucose reduction (%)

DISCUSSION

The four variables demonstrated the most optimal outcomes when a high level of the glucose component (variable A) was utilized because the results are influenced by glucose, which serves as the primary carbon and energy source. Glucose is also a sugar that has been demonstrated to produce high biomass and lipids in comparison to other types of sugar (fructose, sucrose, and lactose) (Vijaya Kumar et al., 2010). Moreover, research by Zhao et al. (2008), indicates that glucose is the first carbon source utilized in comparison to other types of sugar. This is evidenced by a reduction quantity of glucose during the initial 72 hours.

The use of low levels of $MgSO_4$ (variable F) resulted in low results. Consequently, a low concentration of $MgSO_4$ will require low results as well. This finding is not aligned with the observations reported in other studies. The presence of low concentrations of magnesium and SO_4^{2-} can stimulate lipid production, with excess carbon sources redirected into the lipid synthesis process (Morales-Palomo et al., 2023). Additionally, the results may also be influenced by interactions between other components. To illustrate, experiment 12 (Table 3.) displays all variables at a high level except KH_2PO_4, because each component plays a specific in the production of biomass and lipids. Glucose serves as the primary source of energy, nitrogen is involved in metabolic processes, and inorganic components act as cofactors or coenzymes during the lipid production by *L. starkeyi* (Bevilacqua et al., 2008; Liu et al., 2013; Saenge et al., 2011). Therefore, each component plays a specific role and interacts with the other to produce higher biomass and lipids.

The main effect plot (Figure 1, Figure 2, & Figure 3) shows the impact of each factor on the overall performance of the system of which parameters exert the greatest influence on performance. The horizontal line indicates that the average characteristic is consistent across all parameter variants. If the plot is non-horizontal, it can be concluded that there is a significant effect (Kaur et al., 2023). The effect was observed based on the direction of the sign of the effect to determine whether the average response value increased or decreased. If the effect of the parameter is positive, the resulting value indicates that the response is high when the levels are high. Conversely, when the effect is negative, it indicates that the average response at the low level resulted in high production outcomes (Antony, 2023).

The two main effect plots (Figure 1 & Figure 2) indicate that yeast extract and peptones had a positive effect on lipid and biomass production. In contrast, the negative effects observed were the result obtained in the presence of $Na_2HPO_4.7H_2O$, which led to a reduction in lipid production, and $MgSO_4$, which had a similar negative effect on biomass production. These findings suggest that the variables displaying negative effects were present at relatively low concentrations and could potentially enhance lipid and biomass production. Figure 3 shows that $MgSO_4$ and glucose have a positive effect on sugar consumption and the other variables have a negative effect. Similarly, when variables with positive effects have high levels, lipid and biomass production alongside the percentage of sugar reduction is correspondingly elevated.

All these results are influenced by several factors, one of them is the use of glucose as the main carbon source. Glucose is known to enhance the production of biomass and lipids by *L. starkeyi*. The utilization of glucose by yeast can result in higher biomass and lipid production when compared with other sugars (fructose, sucrose, and lactose) (Liu et al., 2013; Vijaya Kumar et al., 2010). Moreover, Zhao et al. (2008) indicated that glucose is the primary carbon source utilized, in comparison to other types of sugar. This is supported by an observed reduction of glucose levels during the initial 72-hour period. Nevertheless, an excessively high glucose concentration can result in a reduction in biomass and lipids because elevated glucose concentrations result in diminished biomass and lipid accumulation (Liu et al., 2013).

In addition to glucose, nitrogen is a nutrient that plays a crucial role in cellular growth and metabolism, including reproduction and lipid accumulation. The nitrogen sources used were yeast extract and peptone, which were found to have a positive impact on biomass and lipid production by *L. starkeyi* Y853. The impact of both yeast extract and peptone compositions did not yield statistically significant results for lipid production (

Table **5**), while the yeast extract does have a significant effect on biomass production (Table 4). Nitrogen will also enhance the stress tolerance of yeast, which will increase the ATPase activity. A high concentration of nitrogen will increase the ATPase activity on the plasma membrane (Zhongfeng, 2010). However, an excess of nitrogen will have an inhibitory effect on yeast survival, resulting in cell death and the inhibition of the fermentation process (Tesnière et al., 2013). Therefore, peptone and yeast extract resulted in a negative outcome (Figure 3) and significantly impacted glucose consumption (

Table 6). YE is one of the nitrogen sources in NLM and plays a crucial role in biomass production where the compounds contain micronutrients that contribute to the growth of yeast cells. Gao et al. (2013) stated that YE is a valuable nitrogen source that stimulate cell growth, leading to high biomass production. This is evidenced by the biomass production at 5.6 g/L with lipid of 4.3 g/L by *Mortierella isabelline* when using YE as the sole nitrogen source.

Nevertheless, the findings of Liu et al. (2013) indicated that yeast extract and peptone, which are include as organic nitrogen, show a minimal impact on biomass and lipid by *L. starkeyi.* This is due to the fact that other organic nitrogen, such as glutamine, urea, and arginine, demonstrate a more significant impact on biomass and lipid production. Those nitrogen sources can also significantly increased lipid accumulation, specifically in *Rhodosporodium toruloides.* Evans and Ratledge (1984) demonstrated that *Rs. toruloides* CBS 14 yield lipids up to 50% (w/w) when organic nitrogen is used as their source, due to the role of these compounds in regulating the flow of carbon to the precursors of lipid biosynthesis.

Inorganic sources also play a role in biomass and lipid accumulation, specifically magnesium, sulfur, potassium, sodium, and phosphorus. Those inorganic, which are from $MgSO_4$, KH_2PO_4, and $Na_2HPO_4.7H_2O$, have been demonstrated to influence lipid production by *L. starkeyi* Y853 (Saenge et al., 2011). When compared to other inorganic compounds, $MgSO_4$ has been observed to utilize a positive impact on the main effect graph of NLM components on biomass, lipid production, and percentage of sugar reduction (Figure 1,Figure 2, & Figure 3). Magnesium from $MgSO_4$ plays vital role in cell growth, glucose consumption, and ethanol fermentation (Li et al., 2020). The consumption of glucose is increased when the concentration of magnesium is maintained at a high level, because magnesium plays a role in the metabolic process, specifically functioning as a cofactor for several enzymes that are involved in the fermentation process and also protect yeast under conditions of stress. This conclusion is supported by the findings of Dombek and Ingram (1986), which demonstrate that a magnesium concentration at 0.5 nM can extend the exponential growth phase, thereby increasing yeast biomass. The cell density of yeast indicates that the energy demand is greater, resulting in increased glucose consumption by yeast, since glucose serves as the primary energy source for *L. starkeyi* in nitrogen-limited conditions. Additionally, the presence of SO_4^{2-} ions at low concentrations was proven to stimulate lipid production, with excess carbon sources redirected toward the synthesis of lipids. This was demonstrated in a study by Morales-Palomo et al. (2023), where low levels of SO_4^{2-} also yielded low levels of lipid at 0.3 g/g by *Yarrowia lipolytica*. This is because low

concentrations of SO_4^{2-} is crucial and function as a coenzyme that facilitates lipid production in oleaginous yeast. The process involves the rerouting of excessive carbon to the lipid synthesis process.

As illustrated in Figure 1, KH_2PO_4 exerts a minimal impact on lipid production by *L. starkeyi* Y853. The presence of phosphate (PO_4^{3-}) has been demonstrated to affect biomass and lipid production by oleaginous microbes, as evidenced by studies on *Mucor circinelloides* isolates, which produce low levels of lipids in high phosphate concentrations (Dzurendova et al., 2020). Furthermore, the production of lipids is enhanced when KH_2PO_4 has a low concentration, where *Rhodosprodium toruloides* Y4 has an output of 11.4 g/L in environments with low KH_2PO_4 concentrations (Li et al., 2006). The production of lipids in conditions of low phosphate concentration will be regulated by the diacylglycerol (DAG) mechanism, the supply of NADPH, and the activity of isocitrate dehydrogenase, increasing lipid production (Morales-Palomo et al., 2023).

The utilization of $Na_2HPO_4 \cdot 7H_2O$ in NLM by *L. starkeyi* Y853 serves as a stimulus for biomass and lipid production. The P-value for $Na_2HPO_4.7H_2O$ is high (

Table **5**), indicating that this component has a minimal impact on biomass production. This result would be inaccurate since $Na_2HPO_4.7H_2O$ can promote biomass and lipid production. This has been demonstrated to induce lipid production by *Rhodosporidium toruloides*, as it enhances the absorption of carbon sources for essential metabolic processes and lipid biosynthesis (Osorio-González et al., 2023). Further study by Chang (1986) demonstrated that *Candida tropicalis* is capable of producing high biomass when utilizing Na_2HPO_4. Therefore, $Na_2HPO_4 \cdot 7H_2O$ can be employed as a stimulus for growth and high lipid and biomass production. While the presence of salt as a nutrient can be affected negatively on the consumption of glucose by *L. starkeyi*. The sodium from $Na_2HPO_4 \cdot 7H_2O$ has been observed to inhibit glucose consumption, since salt stress-inducing affects on yeast cells resulting in the need for additional energy as a form of self-defense. The requirement for supplementary energy can lead to increased glucose consumption when the salt concentration is low (Casey et al., 2013). However, high salt concentration can cause yeast cell death, consequently reducing the glucose consumption process (Morphis et al., 2017).

The factors from the external environment have the potential to influence the lipid production of *L. starkeyi*. These include pH, temperature, and shaker speed. Oleaginous yeast can produce high levels of lipids with low pH values because it helps suppress bacterial growth, preventing contamination (Ageitos et al., 2011; Ratledge & Cohen, 2008). The study by Angerbauer et al. (2008) revealed that the highest lipid production was observed at a pH of 5.0, while the highest lipid yield per liter was recorded at a pH of 6.5. Subsequently, the temperature can influence the growth of yeast cells and the conversion of glucose. That is the reason why the lipid production rate was at its highest during the growth phase at a temperature of approximately 28 °C (Suutari et al., 1993). Additionally, other studies have indicated that the optimal temperature is 30 °C (Ganatsios et al., 2017; Liu et al., 2012). The final variable that may potentially influence the results is the speed of the shaker. An increase in agitation rates will increase cell production, as well as an increase in glucose consumption by the cell. Calvey et al. (2016) demonstrated that a speed of 200 rpm resulted in a high lipid content of up to 55%. However, an excessive agitation rate (300 rpm) will reduce lipid production. Another oleaginous yeast, *Rs. toruloides* produces the highest lipid content during an agitation rate of 150 rpm at 28 °C using NLM as the medium (Kraisintu et al., 2010). Therefore, environmental variables can significantly impact lipid production with various treatments.

CONCLUSION

The analysis using the PBD yielded inconclusive results regarding the impact of the selected factor on lipid production by *Lipomyces starkeyi* Y853, due to insignificant values ($P > 0.05$). The component that exhibited the most positive effect on lipid production was peptones, while yeast extract demonstrated the greatest positive impact on biomass production. Meanwhile, the components that exhibited a negative impact on lipid production are yeast extract and magnesium sulfate, where magnesium sulfate hurts biomass production. It is necessary to incorporate additional variables

beyond the medium components (pH, temperature, and shaker speed) to achieve more significant outcomes for lipid accumulation by *L. starkeyi* Y853.

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