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Optimization of lipid production by locally isolated *Rhodotorula toruloides* using response surface methodology

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ABSTRACT

Biodiesel has received increasing interest as green and sustainable fuel replacing the depleting fossil-based diesel. Oleaginous yeast has been known able to produce lipids as biodiesel feedstock during nutrient limitation. Recently, an oleaginous yeast identified as *Rhodotorula toruloides* was successfully isolated from a highly acidic runoff water sample. This study was conducted to evaluate and optimize the lipid production of the *Rhodotorula toruloides* using response surface methodology. Sudan test was conducted to evaluate the lipid production by the yeast qualitatively. A central composite experimental design was adopted to optimize pH, temperature and C/N ratio for lipid production. The interaction of the process variables was studied. The Sudan test demonstrated colour changes that occurred on media containing yeast, indicating its ability to produce lipids at all tested parameters. Statistical data analysis showed a high coefficient of determination (R^2) determined at 0.9183 with a predicted optimum lipid yield of 5.6% produced at pH 5.6, 27.7°C and a C/N ratio of 107.29. The optimized conditions for the yeast were further tested and the resulting observed lipid yield was 7.7%. The finding of this study provides insightful knowledge for the large-scale production of microbial oil using oleaginous yeast.

Keywords: *Rhodotorula toruloides*, lipid production, optimization, response surface methodology

INTRODUCTION

With the increasing demand for global energy requirements, the search for clean and renewable energy is critical. Renewable bioenergy plays a vital role in replacing fossil-based fuels as alternative energy with low emissions and environmental impact. Biodiesel has been recognized as one of the most promising owing to its similar physicochemical properties to petro-diesel (Zahan & Kano, 2018). Biodiesel is a mixture of animal fats, vegetable oils, or waste cooking oils synthesized via the transesterification of triacylglycerols (TAGs) with short-chain alcohols (Bozbas, 2008). Biodiesel that has determined properties such as viscosity, density and melting point can be mixed with common diesel fuel. Furthermore, biodiesel can be applied as a substitute for low-carbon heating oil.

Concerns over food security and large land usage of oil-rich crops, and biodiesel from non-edible renewable sources have become a biotechnological interest. The issues associated with costly fertilizer, water demand and season-dependent crops cause oil increases making microbial oil a feasible alternative (Somacal et al., 2020). Oleaginous microorganisms including yeasts, algae, bacteria, and moulds are capable of accumulating lipids in their biomass (Musa et al., 2018). In comparison with plant oils, microbial oils offer the benefits of a rapid life cycle with the ability to utilize cheap substrates and feasibility for large-scale cultivation. Wild strains of microorganisms also can be metabolically engineered to advance lipid production (Liang & Jiang, 2013).

Among oleaginous microorganisms, yeast has been known promising produce TAGs similar to vegetable oils and also endotoxins-free which are pronounced in bacterial species (Patel et al., 2016). Yeast is unicellular and has a relatively high growth rate and rapid lipids accumulating ability in discrete lipids bodies compare to bacteria, moulds and algae (Athenaki et al., 2017). Yeasts can store lipids up to 40% of their biomass but under nutrient limitation conditions, they may accumulate lipids to levels exceeding 70% of their biomass. According to Beopoulos and Nicaud (2012), oleaginous yeasts that are recognized as among the best oil producers are commonly from *Rhodotorula*, *Cryptococcus*, *Candida*, *Trichosporon*, *Rhizopus*, *Yarrowia* and *Lipomyces* genera. *Rhodosporidium toruloides* previously known as *Rhodotorula glutinis* or *Rhodotorula gracilis* is a non-pathogenic basidiomycetous fungus that is pink in colour and can store lipids exceeding 70% of the biomass dry weight (Zhu et al., 2012).

Recently, an oleaginous yeast strain was isolated from a runoff water sample collected from a river located in Ranau, Sabah. The strain exhibited pink colonies on nutrient agar known as an oleaginous yeast capable of lipid production and identified as *Rhodotorula toruloides* by Geoffrey et al. (2018). Zhao et al. (2012) have demonstrated that crude lipids from *R. toruloides* could yield up to 67.2% biodiesel by enzymatic transesterification indicating that this yeast species could be a potential feedstock for biodiesel production. The sampling site has been reported to possess high sulphate and elevated concentrations of dissolved heavy metals like Al, Mn, Fe, Cu and Zn (Low

et al., 2020). The seepage of water was analyzed with a low pH value of around 2.90 – 3.75. Microorganisms originating from an extreme environment like mining sites may exhibit diverse tolerability towards pH, temperature and nutrients.

This study aimed to optimize the production of lipids by the *Rhodotorula toruloides* using response surface methodology (RSM). RSM is a statistical and mathematical technique based on the fit of a polynomial equation to the experimental data useful in designing the experiments and evaluating the affecting variables (Bazerra et al., 2008). The optimization study was conducted by varying the operating conditions (pH, temperature and carbon-to-nitrogen ratio) by adopting the central composite design (CCD) of RSM. The resulting lipid production was further analysed qualitatively and quantitatively. The findings of this study will lead to the discovery of important parameters for the optimal production of biofuel.

MATERIALS AND METHODS

Preparation of *Rhodotorula toruloides* Culture

The *Rhodotorula toruloides* yeast was isolated from runoff water at Ranau River, Sabah (Low et al., 2020). The yeast cultured was streaked on Yeast Peptone Dextrose (YPD) (Sigma) agar plate and incubated at 30°C for 72 h. For inoculum preparation, a single colony of 72-h grown yeast was inoculated into 150 ml sterilized YPD broth (Sigma) and was incubated for 20 h at 30°C with an agitation of 180 rpm (Saran et al., 2017).

Production of Lipid by *Rhodotorula toruloides*

Approximately 10% of yeast inoculum (OD_{600} = 1.8 – 2.2) was inoculated into 150 ml culture media. The pH and C/N ratio of the media was adjusted accordingly as designed by the response surface methodology (RSM). The culture was grown at the designed temperature with 200 rpm agitation. The cultivation was carried out until it reached the mid-exponential phase about 20 h cultivation. Samples were collected for the determination of lipid yield (Saran et al., 2017).

Experimental Design and Statistical Analysis

The statistical analysis of lipid production by *Rhodotorula toruloides* yeast was performed using Design Expert (V 6.0.8) software. The central composite design (CCD) of response surface methodology (RSM) was used to study the interaction of process variables (temperature, pH, C/N ratio) and to predict the optimum process condition for lipid production (Yaakob et al., 2011). The process variables and levels in the CCD are tabulated in Table 1. The lipid yield (%) was taken as a response to the design experiment. The total number of experiments was 20 and were conducted as designed by RSM with triplicate samples to evaluate the error.

Table 1 Process variables and levels in central composite design

	Name	Units	–1 Level	+1 Level
A	pH	–	4	9
B	Temperature	Celcius	24	30
C	C: N ratio	molar	50	120

Qualitative Assay of Lipid Production

Test tubes containing 5 mL of 18-h grown yeast were added with a few drops of Sudan IV stain (Sigma) as a qualitative indicator for lipid production (Patel et al., 2016). The formation of reddish-orange stains on the media indicates a positive assay. Sudan IV solution was prepared by mixing 75 mL of 96% ethyl alcohol in the organic solvent with 0.5 g of Sudan IV powder.

Quantification of Lipid

Lipid extraction was conducted following the method by Bligh and Dyer (1959) with some modifications. Samples from the cultivation media were centrifuged to obtain the biomass pellet and dried at 60°C until reaching a constant weight. Approximately 5 mg of dried samples were grounded into powder and dissolved in 1 ml distilled water:chloroform:methanol (Sigma) mixtures at a 1:1:1 ratio. The mixture was vortexed and left on the bench for 2 h. The samples then were centrifuged for 5 mins at 5,500 rpm resulting in two layers of solutions. The top layer containing methanol and water was discarded while the bottom layer containing lipid and chloroform was transferred into a pre-weighed glass tube. The glass tube was placed inside the speed-vac to dry the solvent for about one and a half hours. After drying, the glass tube containing the crude lipid was weighed. The lipid mass and lipid yield of extracted lipid were determined using Eq. 1 and Eq 2.

$$\text{Lipid mass (g)} = (\text{Weight of glass tube} + \text{Extracted oil}) - (\text{Weight of glass tube}) \quad \ldots (1)$$

$$\text{Lipid yield (\%)} = \frac{(\text{lipid mass (g)}}{\text{sample weight (g)}} \times 100\% \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

RESULTS AND DISCUSSION

Qualitative Assay of Lipid Production

The complete design matrix of central composite design (CCD) and the interaction effect of variables on the response variable (lipid yield) are given in Table 2. The data shows that different level of variables contributes a different response. The qualitative assay for lipid production is shown in Figure 1. The presence of lipids in the culture was visually observed based on the colour change in comparison with the control media (absence of yeast culture). The Sudan IV test is a biochemical technique that qualitatively visualizes the presence of fatty acid compounds. The control media formed two layers of solution of media and Sudan IV solvents indicating the absence of lipid formation (Mane & Raut, 2016). As can be seen, all 20 samples exhibited a homogenous, reddish-coloured solution implying the presence of a lipid compound. The organic solvent in the Sudan IV reagent was responsible to separate lipid from biomass culture and further stained it.

Table 2 Matrix of central composite design and the experimental value for response variables of lipid production.

Run	Variables			Responses
	pH	Temperature (°C)	C/N ratio	Lipid yield (%)
1	4	30	50	4.15
2	6.5	27	85	6.58
3	6.5	32	85	3.40
4	9	24	120	3.56
5	4	24	120	3.99
6	6.5	27	85	5.41
7	6.5	27	143.86	3.79
8	2.3	27	85	5.61
9	4	30	120	4.62
10	9	30	120	3.39
11	9	30	50	3.37
12	6.5	27	26.14	3.39
13	4	24	50	2.64
14	6.5	27	85	4.72
15	6.5	27	85	5.41
16	10.7	27	85	2.05
17	6.5	27	85	4.62
18	6.5	22	85	1.94
19	6.5	27	85	5.85
20	9	24	50	1.59

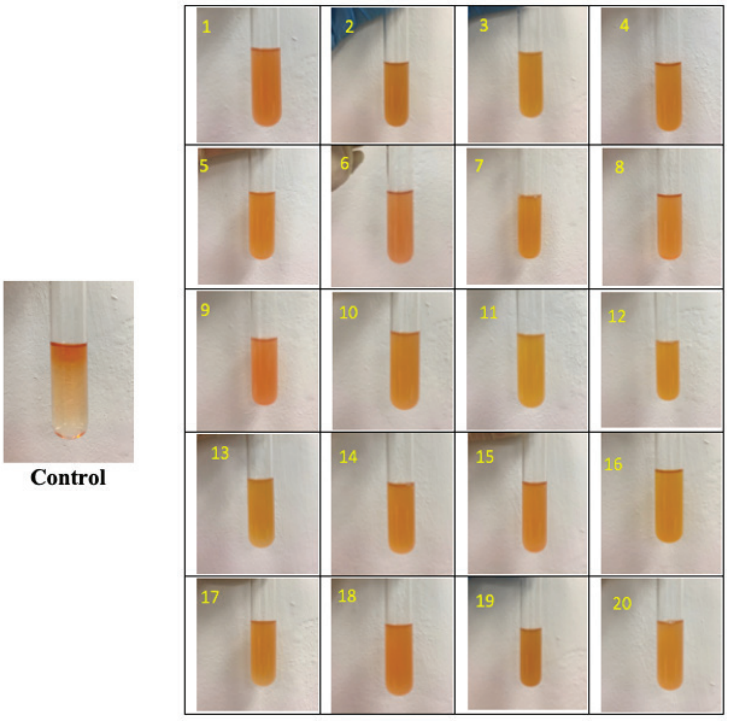

Figure 1 Colour change of media containing 18-h grown *Rhodotorul toruloides* yeast in YPD after staining with Sudan IV reagent in comparison with the control sample (non-cultured). The label numbers correspond to the designated run of experiments in Table 2

Central Composite Design and Data Analysis

Figure 2 presents the coefficient of determination (R^2) which is an indicator of how well the model fits the data. The predicted and experimental yield of lipids was found to be relatively close. The R^2 value for lipid yield determined at 0.9183 indicates an adequate model for all response variables. The empirical model should be at least 0.75 to adequately explain most of the variability in the assay reading (Mane & Raut, 2016). Furthermore, the ANOVA data in Table 3 signifies the model for lipid yield was significant with a *p*-value was <0.050 and F-values (12.35) were higher than the tabulated F-value (3.02). A smaller *p*-value indicates a significant corresponding coefficient (Yi et al., 2010). As can be seen, both temperature (B^2) and C:N ratio) (C^2) demonstrated a significant effect on lipid production ($p<0.05$). The findings showed that the ability to produce lipids was critically affected by the C/N ratio of culture. According to Galafassi et al. (2012), the yeast would produce lipids under a stress condition when the medium has excess carbon and nitrogen limitation. In the condition, where the cells run out of nitrogen, cells would not multiply but the excess carbon substrate would be assimilated continuously to produce lipids (Li et al., 2006). Temperature played an important role too in lipid production as temperature variations can lead to changes in the fatty acid composition and affect the lipid yield (Zlatanov et al., 2010).

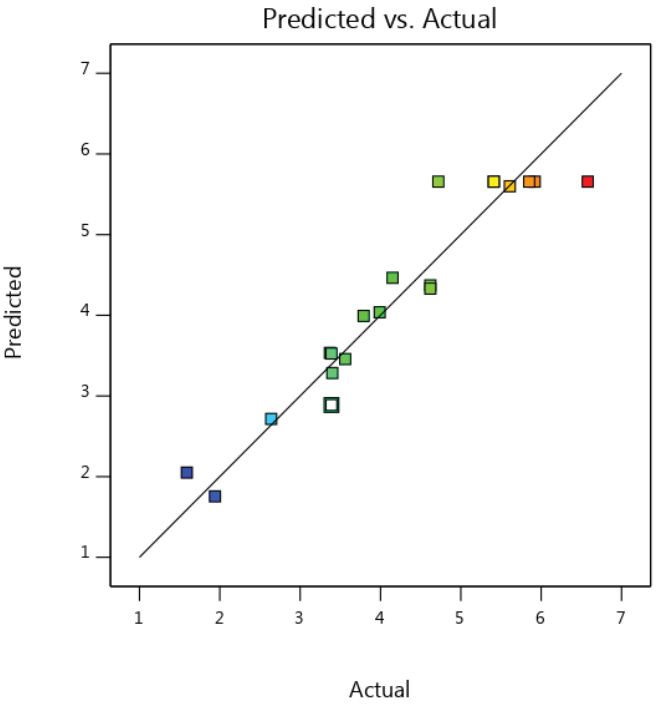

Figure 2 Predicted-actual values plots for lipid yield by *Rhodotorula toruloides*

Table 3 Analysis of variance (ANOVA) of response variables for lipid yield

Source	Sum of square	df	Mean Square	*F*-value	*p*-value	
Model	31.68	9	3.52	12.49	0.0002	significant
A-pH	1.95	1	1.95	6.90	0.0253	
B-Temperature	2.82	1	2.82	10.00	0.0101	
C-C:N ratio	1.47	1	1.47	5.22	0.0454	
AB	0.0351	1	0.0351	0.1245	0.7315	
AC	0.0036	1	0.0036	0.0128	0.9121	
BC	1.00	1	1.00	3.55	0.0889	
A^2	0.8618	1	0.8618	3.06	0.1110	
B^2	17.72	1	17.72	62.86	< 0.0001	
C^2	8.85	1	8.85	31.39	0.0002	

Process Variables' Interaction with Lipid Production

A 3-dimensional surface plot was used to investigate the interaction of tested processing parameters. The model is plotted to represent the lipid yield as a function of 2-factor as shown in Figure 3 to Figure 5. The contour plot in Figure 3 illustrates the interaction of reaction temperature and time on lipid yield. The increment of lipid yield with the

increase of reaction temperature and pH up to a critical point from 24°C to 27°C and 4 to 6.5, respectively. Madani et al. (2017) reported an optimum temperature of two groups of Oleaginous yeasts in the range of 25 – 30°C and 35 – 45°C, respectively. However, above the optimum processing condition, the yield of lipids decreased which could be due to the inhibition of yeast growth at a higher temperature and pH conditions.

The interactions of C/N ratio and pH on lipid yield are exhibited in Fig. 4. As can be seen, the pattern of interaction was observed similar to the trend of temperature-pH interaction, as evidenced by the 3D response surface plots (Figures 2a, 3a) and contour plots (Figures 2b, 3b). The effect of interaction could be more obvious if the range of the tested parameters were bigger. Meanwhile, Figure 5 presents the interaction between the C/N ratio and temperature on lipid yield. The yield increased as the C/N ratio increased from 50 to approximately 85 and the pH of 24 and approximately 28°C. The elliptical nature of the contour plot in lipid yield indicates the interaction of the C/N ratio and temperature is considerable. Carbon is needed to produce carbon dioxide and ethyl alcohol while nitrogen can boost cell growth as well as lipid accumulation in a yeast cell. Lipid accumulation is obtained when the carbon-nitrogen imbalance, normally occurred during nitrogen limitation (Ratledge & Wynn, 2002). During nitrogen stress conditions, the yeast would stop growing while the available carbon will be assimilated into the cell and will be stored as single-cell oil (Fei et al., 2011). Ageitos et al. (2011) and Qadeer et al. (2017) have reported the optimal C/N ratio for the production of lipids by oleaginous yeast was ranging from 65 to 100.

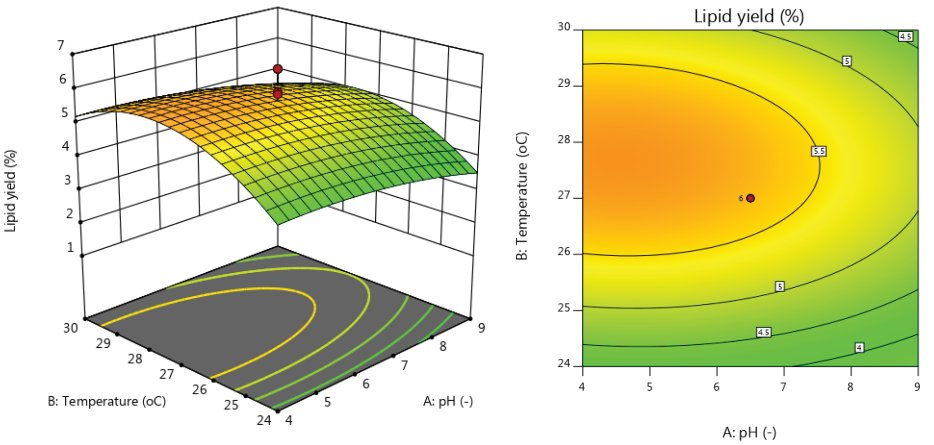

Figure 3 Interaction between temperature and pH to lipid yield (a) 3D response surface plots (b) contour plot

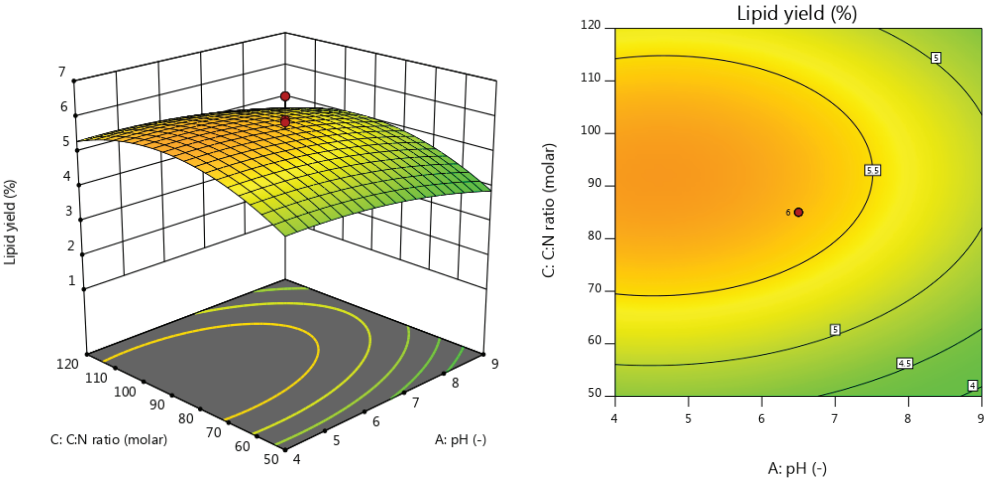

Figure 4 Interaction between C/N ratio and pH to lipid yield (a) 3D response surface plots (b) contour plot

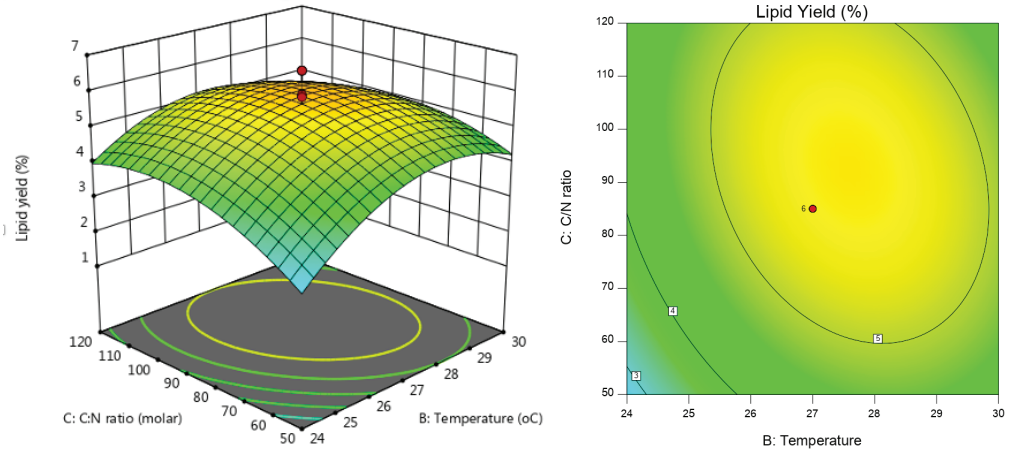

Figure 5 Interaction between C/N ratio and temperature to lipid yield (a) 3D response surface plots (b) contour plot

Optimization of Lipid Production

Based on the results obtained, the suggested optimum levels for all variables from the quadratic model in this study were the pH level of 5.62, the temperature of 27.69°C and the C/N ratio of 107.29, yielding lipids of about 5.6%. The numerical optimization conditions of each response were demonstrated in Figure 6. The numerical optimization condition for the C/N ratio was 107.293. The C/N ratio plays a vital role especially in lipid production as the value of nitrogen will create a stressful environment for the yeast to produce lipids. Furthermore, nutrient imbalance in the culture medium has to be high (Beopoulos et al., 2009). The model suggested that the optimum condition for pH was

5.62. The finding was in agreement with Jiru et al. (2017) that reported optimal pH of 5.5 for maximum lipid production. Validation was carried out to evaluate the conditions predicted by the RSM. The culture was done with a pH level of 5.62, the temperature of 27.69°C and C/N ratio of 107.29. The lipid yield achieved was 7.7% which was close to the prediction by the model whereas the biomass productivity was 1.43 g/L/day. The yield of lipids was found much lower than in the reported literature. Alok et al. (2017) reported oleaginous yeast like *Rhodotorula toruloides*, *Rhodotorula toruloides*, *Yarrowia lipolytica* and *Cryptococcus curvatus* are species that are able to accumulate more than 60% lipids in their cellular component. Some other important factors have to be explored further to increase lipid production. Coradetti et al. (2018) reported *R. toruloides* is relatively resistant to a variety of stresses, such as osmotic stress and high salinity. In addition, the newly isolated acid-tolerant *Rhodotorula toruloides* yeast probably requires molecular characterization studies to understand its behaviour for lipid production.

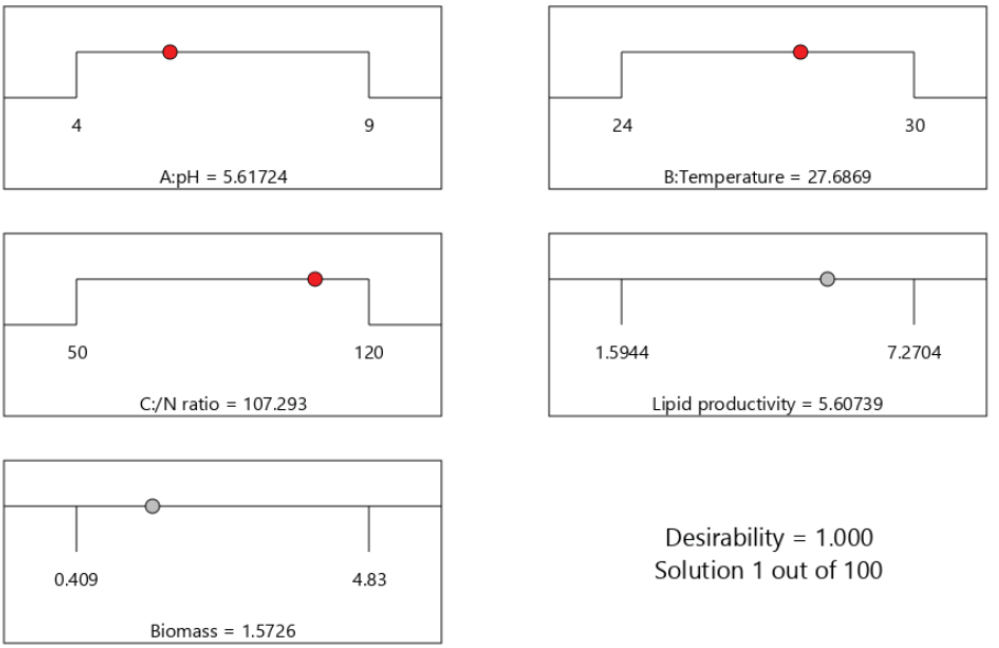

Figure 6 Numerical initial optimization conditions of each response

CONCLUSION

Sudan qualitative assay demonstrated *Rhodotorula toruloides* can produce lipids. Based on the findings, it can be concluded that *Rhodotorula toruloides* could produce a high yield of lipids at optimum processing conditions. Based on the findings, approximately 5.6% lipid was produced at optimal conditions determined at pH 5.6, the temperature of 27.68°C and the C/N ratio of 107.29. The RSM technique was found successful in optimizing the conditions for lipid production. For future studies, the cultivation of the lipid producer can be applied in low-cost media like agricultural wastes on a bench scale and upscale studies to enhance the production of lipids. The findings of this study will open the door for economical and industrially feasible production of biofuel in future.

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