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RESEARCH PAPER

Efficient Concomitant Production of Lipids and Carotenoids by Oleaginous Red Yeast *Rhodotorula glutinis* Cultured in Palm Oil Mill Effluent and Application of Lipids for Biodiesel Production

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Abstract *Rhodotorula glutinis* TISTR 5159 is oleaginous red yeast that accumulates both lipids and carotenoids. It was cultured in palm oil mill effluent (POME) with only the addition of ammonium sulfate and Tween 20 as a suitable nitrogen source and surfactant, respectively. Response surface methodology (RSM) was applied to optimize initial chemical oxygen demand (COD) in POME, C/N ratio, and Tween 20 concentration for concomitant production of lipids and carotenoids. Among three investigated factors, C/N ratio contributed a significant effect upon lipid and carotenoids production. Analysis of response surface plots revealed that the optimum C/N ratio for the biomass was 140, while that for lipid content and carotenoids were higher at 180 and 170, respectively. The high level of the nitrogen source (with a low C/N ratio) enhanced the biomass, making the accumulation of lipids and carotenoids less preferable. Hence, the two-stage process was attempted as an optimal way for cell growth in the first stage and product accumulation in the second stage. The lipid yield and carotenoid production obtained in the two-stage process were higher than those in the one-stage process. In the semi-continuous fermentation, *R. glutinis* TISTR 5159 accumulated high lipid content and produced a considerably high concentration of carotenoids during long-term cultivation. Additionally, efficient COD removal by *R. glutinis* TISTR 5159 was observed. The biodiesel produced from yeast lipids was composed mainly of oleic and palmitic acids, similar to those from plant oil.

Keywords: carotenoids, lipid, palm oil mill effluent, response surface methodology, *Rhodotorula glutinis*

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1. Introduction

Microbial oils are produced in lipids formed by oleaginous microorganisms, including bacteria, yeasts, moulds, and algae. They are now attracting interest as a promising potential feedstock for biodiesel production due to their fatty acid compositions being similar to that of vegetable oils [1]. Compared with vegetable oil production, the oleaginous microorganism culture is more advantageous as it is affected neither by seasons nor climate. *Rhodotorula glutinis* is an oleaginous red yeast that can accumulate both lipids and carotenoids [2]. It has an advantage over algae, fungi, and bacteria due to its relatively high unicellular growth rate and capacity to rapidly accumulate lipids when utilizing low-cost fermentation media such as nutritional residues from agriculture and industry. In addition to lipids, carotenoids are gaining importance as natural food colorants because of the possible safety hazards of chemical colorants. Hence, there is a great potential for using *Rhodotorula* carotenoids in foods and feeds.

Palm oil processes can be categorized into dry and wet milling standard processes. The wet process of milling palm oil is the most common way of extracting palm oil, especially in Thailand. It is estimated that for each ton of

crude palm oil that is produced, 5 ~ 7.5 tons of water are required, and more than 50% of this water ends up as palm oil mill effluent (POME) [3]. The high chemical oxygen demand (COD) in POME makes its disposal a pollution problem. Therefore, the use of POME as an inexpensive medium for fermentation processes has long been of industrial interest. The possibility of reusing POME as fermentation media is largely due to the fact that POME contains high concentrations of carbohydrates, proteins, and nitrogenous compounds. In addition, POME is also rich in minerals and contains vitamins, which may provide valuable nutrients to stimulate cell growth and product formation [4-6].

Statistical experimental design techniques, especially the response surface methodology (RSM), are very useful tools for optimizing process parameters. They can provide statistical models that help elucidate the interactions among parameters at varying levels and calculate the optimal level of each parameter for a given target [7,8]. Application of RSM in medium optimization can not only improve growth and production, but also reduce process variability, development time, and overall costs. To date, optimization of lipid production using glucose and glycerol has been studied by several researchers [1,9,10]. However, the use of POME for concomitant production of lipids and carotenoids has not been studied.

It is well established that the lipid yield coefficient of oleaginous yeasts is high when these organisms are grown in a medium with a high C/N ratio. For instance, with a low nitrogen medium, they channel carbon towards the accumulation of triacylglycerol as a storage lipid [2]. Some agents, such as detergent additives, oil, and surfactants have been known to increase lipid and carotenoid productivity [11]. Supplementation of the fermentation medium with surfactants can alter the physiological properties of microorganisms, improve metabolite production, stimulate growth and respiration, and change the organization and permeability of cell membranes [12]. In this study, concomitant production of lipids and carotenoids by cultivating *R. glutinis* TISTR 5159 in POME was attempted. Firstly, a suitable nitrogen source and surfactant were screened. The effects of the initial COD in POME, C/N ratio, and surfactant concentration on the production of lipids and carotenoids were simultaneously investigated using RSM. The batch fermentation was then carried out in a 2.0 L bioreactor equipped with pH control and aeration systems. The long-term cultivation of *R. glutinis* TISTR 5159 in POME was performed in semi-continuous fermentation mode. In addition, the lipids obtained from yeast were converted to biodiesel and its fatty acid composition analyzed.

2. Materials and Methods

2.1. Microorganism and media

Rhodotorula glutinis TISTR 5159 obtained from the Thailand Institute of Scientific and Technological Research was used for the production of lipids and carotenoids. A plate culture was incubated at 30°C for 24 h. The cells were transferred to 250 mL Erlenmeyer flasks containing 50 mL of culture medium (g/L: glucose 10, peptone 5, yeast extract 3, and malt extract 3). The flasks were incubated at 30°C and 200 rpm for 24 h for seed culture. Palm oil mill effluent (POME) was obtained from Srijareon Palm oil mill (Krabi, City, Thailand). The original characteristics of the POME after filtration by filter paper were listed as: pH 4.5; COD 48 g/L; total nitrogen 262 mg/L; reducing sugar 12 g/L; oil and grease 280 mg/L.

2.2. Screening of nitrogen sources and surfactants

Flasks containing 90 mL diluted POME (COD = 10 g/L) were sterilized at 121°C for 15 min and inoculated with seed culture (10% v/v). To study the effects of the nitrogen source, various organic and inorganic nitrogen sources, including yeast extract, peptone, urea, ammonium sulfate, ammonium chloride, and ammonium nitrate, were each added to the diluted POME to obtain a C/N ratio of 160. Three surfactants (Tween 20, Tween 80, gum Arabic) were screened for enhancing lipid and carotenoid production. All cultures were grown at 30°C and 200 rpm for 72 h at least in duplicate. Samples were taken to measure biomass, lipid content, carotenoid production, and COD removal.

2.3. Medium optimization through RSM

A Box-Behnken design with three variables at three levels was followed to determine the response pattern and synergy of the variables. According to this design, 15 runs were conducted containing three replications at the central point for estimating the purely experimental uncertainty variance. The relationship of the variables was determined by fitting a second-order polynomial equation to the data obtained from the 15 runs. A uniform design and analysis of data were executed. The response surface analysis was based on the multiple linear regressions, taking into account the main, quadratic, and interaction effects, in accord with the following equation:

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j \quad (1)$$

where Y is the predicted response, x_i and x_j the variables or parameters, β_0 the offset term, β_i the linear effect, β_{ij} the first order interaction effect, and β_{ii} the squared effect. The goodness of fit of the model was evaluated by the coefficient of determination (R^2) and the analysis of variance (ANOVA). Response surface plots were developed to indicate optimum

conditions by using the fitted quadratic polynomial equations obtained by holding one of the independent variables at a constant value and changing the levels of the other two variables.

2.4. Fermentation in bioreactor

The batch fermentation was carried out in a 2.0 L bioreactor. The bioreactor contained 1.0 L of optimized medium from RSM. The culture conditions were 10% (v/v) seed culture and a temperature of 30°C. Agitation was provided by two turbine impellers located 5 and 10 cm above the bottom of the vessel. The impeller speed was 100 rpm. The aeration rate was 2 vvm. The pH was monitored and maintained at 6.0 with 2.5 N NaOH. In the semi-continuous fermentation, the start-up of the process was the same as for the batch operation. Following a growth period of 48 h, half of the content of the medium (500 mL) was removed and 500 mL of fresh medium added. During the 336 h of total cultivation, this process was repeated every 48 h. The time courses of the biomass, lipid content, production of carotenoids, and COD removal were determined.

2.5. Analytical methods

Biomass was harvested by centrifugation and dried to constant weight in vials [13]. Extraction of lipids and carotenoids from the biomass was performed according to the modified procedure of Bligh and Dyer [14]. The total carotenoid content was determined spectrophotometrically and β-carotene (Sigma, City, State, Country) was used as the standard [15]. The method for converting extracted lipids to fatty acid methyl esters (FAME) involved hydrolysis of the lipids followed by esterification. The fatty acid composition in the FAME was analyzed using a HP6850 Gas Chromatography equipped with a cross-linked capillary FFAP column (length 30 m, 0.32 mm I.D, 0.25 μm film thickness) and a flame ionization detector. The operating conditions were as follows: inlet temperature 290°C; oven temperature initial 210°C hold 12 min ramp to 250°C at 20°C/min hold 8 min; detector temperature 300°C. The fatty acids were identified by comparison of their retention times with those of standards, quantification being based on their respective peak areas, and normalized.

COD, total nitrogen (TN), oil, and grease were determined according to the procedures used in the standard methods [16]. The C/N ratio was calculated from COD/TN. The nitrogen content in the organic nitrogen source was calculated by assuming that the yeast extract, urea, and peptone contained 11 (w/w), 46.65 (w/w), and 36.6% (w/w) of nitrogen, respectively. Total reducing sugar concentration was determined using the 3,5-dinitrosalicylic acid (DNS) method [17]. The statistical significance of the results was evaluated by one-way ANOVA (analysis of variance) and Duncan's multiple range tests ($P < 0.05$) using the SPSS version 10 software.

3. Results and Discussion

3.1. Effect of nitrogen source

The low nitrogen level in the POME (262 mg/L) and the resultant high C/N ratio prompted the need for supplementation with an external source of nitrogen. Although a nitrogen source has long been known to promote growth, its high concentration also suppresses the biosynthesis of lipids and other secondary metabolites. A number of researchers have tested the effects of a nitrogen source on biomass and the lipid content of various microorganisms [1,18,19]. In this study, the POME was supplemented with several organic and inorganic nitrogen sources. The effects of the nitrogen source on biomass, lipid content, and production of carotenoids of *R. glutinis* TISTR 5159 are shown in Table 1. Addition of either organic or inorganic nitrogen sources was found to increase the biomass, lipid content, and carotenoid production. The highest values of biomass, lipid content, and carotenoid concentration were obtained with a yeast extract (6.33 g/L, 32.63%, and 129.94 mg/L, respectively), followed by ammonium sulfate (6.29 g/L, 29.15%, and 115.76 mg/L, respectively),

Table 1. Effect of nitrogen source on biomass, lipid content, carotenoids production, and COD removal of *R. glutinis* TISTR 5159 cultured in POME

Nitrogen source	Biomass (g/L)	Lipid content (%)	Carotenoids production (mg/L)	COD removal (%)
Control	4.15 ± 0.09^{e}	20.97 ± 1.65^{c}	85.26 ± 0.01^{f}	40.50 ± 1.41^{f}
Yeast extract	6.33 ± 0.01^{a}	32.63 ± 0.08^{a}	129.94 ± 1.68^{a}	65.13 ± 1.20^{a}
Urea	5.44 ± 0.01^{b}	29.11 ± 1.41^{b}	109.11 ± 1.55^{c}	53.92 ± 1.40^{c}
Peptone	4.46 ± 0.03^{d}	26.89 ± 2.04^{b}	99.55 ± 1.09^{d}	43.48 ± 1.27^{e}
Ammonium sulfate	6.29 ± 0.16^{a}	29.15 ± 1.41^{b}	115.76 ± 2.49^{b}	59.29 ± 1.40^{b}
Ammonium nitrate	4.80 ± 0.11^{c}	27.77 ± 0.01^{b}	105.85 ± 1.57^{c}	46.99 ± 1.59^{d}
Ammonium chloride	4.19 ± 0.04^{e}	26.82 ± 1.27^{b}	92.74 ± 1.24^{e}	43.00 ± 1.41^{e}

Values are means ± SD. Control: the culture without addition of nitrogen source. Different letters in the same column indicate significant treatments difference ($P < 0.05$).

Table 2. Effect of surfactant on biomass, lipid content, carotenoid production, and COD removal of *R. glutinis* TISTR 5159 cultured in POME added with ammonium sulfate as nitrogen source

Surfactant	Biomass (g/L)	Lipid content (%)	Carotenoids production (mg/L)	COD removal (%)
Control	6.29 ± 0.16^{b}	29.15 ± 1.41^{c}	115.76 ± 2.49^{b}	59.29 ± 1.40^{b}
Tween 20	7.07 ± 0.23^{a}	38.15 ± 1.46^{a}	125.94 ± 1.95^{a}	66.85 ± 1.57^{a}
Tween 80	6.64 ± 0.28^{ab}	34.15 ± 1.37^{b}	118.92 ± 1.33^{b}	65.12 ± 1.54^{a}
Gum arabic	6.62 ± 0.42^{ab}	33.45 ± 1.41^{b}	115.32 ± 1.09^{b}	64.43 ± 1.20^{a}

Values are means ± SD. Control: the culture without addition of surfactant. Different letters in the same column indicate significant treatments difference ($P < 0.05$).

while peptone, ammonium nitrate, and ammonium chloride gave poor growth and lower lipid content and carotenoid production. Addition of yeast extract to increase carotenoid production was also previously reported for *Rhodotorula* sp. cultured in sugarcane molasses [20].

The biomass and lipid content obtained with ammonium sulfate were comparable to those with yeast extract. This suggested that certain essential amino acids could be synthesized from inorganic nitrogen sources by *R. glutinis* TISTR 5159. The COD removal when using ammonium sulfate as a nitrogen source was also found comparable with yeast extract (Table 1). The possible conversion of organic compounds in POME to lipids and carotenoids by *R. glutinis* is of importance for waste treatment and valorization. The POME corresponds to carbohydrates in nature, indicating the possible presence of pentose. The presence of pentose in POME has been reported previously and its most likely source is the palm cell wall [4]. Water-soluble carbohydrates, in terms of glucose, reducing sugars, and pectin, are also present in the soluble fractions of POME. Although the yeast extract gave the highest biomass, lipid content, and carotenoid production, its high cost was considered to have a negative impact on its economic use in industrial-scale processes. Therefore, in order to improve the economic parameters of lipids and carotenoids, a cheaper ammonium sulfate was selected as a suitable nitrogen source for further study.

3.2. Effect of surfactant

Some surfactants have been known to increase lipid and carotenoid productivity [11]. The mechanism of this stimulation is not completely clear, but the agents appear to cause different alterations in membrane fluidity [21]. Enhancement of cell permeability by surfactants depends on the chemical composition of the cell, such as the membrane sterol content [22]. In this study a number of surfactants, including Tween 20, Tween 80, and gum Arabic at a 1% concentration, were tested for enhancement of lipid and carotenoid production by *R. glutinis* TISTR 5159. The yeast was cultivated in POME with the addition of ammonium sulfate as the nitrogen source. The results are shown in Table 2. There were significant increases in the amounts of biomass and lipid content when the surfactant was added, compared to the control to which no surfactant was added. Of these three surfactants, only Tween 20 effectively increased both lipid content and carotenoid production. The COD removal was also improved by addition of the surfactant. This could be due to the emulsifying action of the surfactant on hydrocarbon-based compounds, which degraded them into more metabolizable substances that the microbes digest more efficiently [23].

The highest amounts of biomass, lipid content, and production of carotenoids (7.07 g/L, 38.15%, and 125.94 mg/L, respectively), as well as the highest COD removal (66.85%), were obtained when Tween 20 was added to the POME. This result was in accord with reports for performance of other microorganisms. It was reported that Tween 20, when added into a medium, stimulated the growth, improved the growth rate, and hence enabled excretion of the product out of the cells [24]. Previous research has found that use of Tween 20 as a carbon source increased lipid accumulation, but was not suitable for cell growth [25]. In contrast, addition of Tween 20 in POME enhanced both cell growth and production of lipids and carotenoids of *R. glutinis* TISTR 5159.

3.3. Medium optimization through RSM

Since most industrial experiments usually involve many variables, a full factorial design entails a large number of experiments. To reduce the number of experiments to a practical level, only a small set from all the possibilities is normally selected. The significance of the variables can be explained as follows. The basic physiology of lipid accumulation in microorganisms has been well studied. A nutrient imbalance in the culture medium is known to trigger lipid accumulation in oleaginous microorganisms. Lipid production requires a medium with an excess of sugar or similar components and other limited nutrients, usually nitrogen. Thus, the oleaginous potential is critically affected by the carbon-to-nitrogen (C/N) ratio of the culture. At a high C/N ratio, when cells run out of nitrogen, they cannot multiply and excess carbon substrate is assimilated continuously to produce storage lipids. In this study, POME was thought to be suitable for lipid production

Table 3. Experimental range and levels of the three independent variables used in RSM in terms of actual and coded factors and experimental data for the three-factor with three-level response surface analysis

Trial	Independent variables			Dependent variables		
	COD (g/L)	C/N ratio (–)	Tween 20 (g/L)	Biomass (g/L)	Lipid content (%)	Carotenoids production (mg/L)
	x_1	x_2	x_3	Y_1	Y_2	Y_3
1	1(40)	1(180)	0(1.0)	7.54	37.50	124.26
2	1(40)	−1(140)	0(1.0)	8.05	35.13	116.56
3	−1(10)	1(180)	0(1.0)	7.33	38.48	118.58
4	−1(10)	−1(140)	0(1.0)	7.65	33.35	122.15
5	1(40)	0(160)	1(1.5)	7.85	36.92	139.56
6	1(40)	0(160)	−1(0.5)	7.82	35.55	138.28
7	−1(10)	0(160)	1(1.5)	7.60	35.88	135.82
8	−1(10)	0(160)	−1(0.5)	7.30	35.73	138.15
9	0(25)	1(180)	1(1.5)	7.50	42.56	155.23
10	0(25)	1(180)	−1(0.5)	7.55	37.40	144.29
11	0(25)	−1(140)	1(1.5)	7.50	34.03	132.58
12	0(25)	−1(140)	−1(0.5)	7.00	33.24	125.47
13	0(25)	0(160)	0(1.0)	7.50	37.16	132.57
14	0(25)	0(160)	0(1.0)	7.60	37.86	132.32
15	0(25)	0(160)	0(1.0)	7.55	37.44	133.39

Note: values in parentheses are the actual independent variables x_1: COD; x_2: C/N ratio; x_3: Tween 20 concentration; Y_1: biomass; Y_2: lipid content; Y_3: carotenoids production.

since it contains high COD [3]. In addition to the C/N ratio, the levels of initial COD and Tween 20 may also affect the growth and the lipid and carotenoid production of *R. glutinis* TISTR 5159.

Consequently, three main fermentation factors, COD (x_1), C/N ratio (x_2), and Tween 20 (x_3), were selected for optimization of biomass, lipid content, and carotenoid production of *R. glutinis* TISTR 5159 using RSM. The Box-Behnken design leading to a total 15 sets of experiments was carried out (Table 3). The results obtained by the Box-Behnken design were analyzed by ANOVA (Table 4). The second-order regression equations for biomass (Y_1), lipid content (Y_2), and carotenoid production (Y_3) as a function of COD (x_1), C/N ratio (x_2), and Tween 20 (x_3) are given as follows:

$$\text{Biomass } (Y_1) = 6.8465 - 0.0614\,x_1 - 0.00979\,x_2 + 4.1166\,x_3 + 0.000861\,x_1^{\,2} + 0.0000469\,x_2^{\,2-} - 0.7250\,x_3^{\,2} + 0.000292\,x_1x_2 - 0.01167\,x_1x_3 - 0.01375\,x_2x_3 \quad (2)$$

$$\text{Lipid content } (Y_2) = -54.6700 + 0.8510\,x_1 + 1.0230\,x_2 - 20.5300\,x_3 - 0.00869\,x_1^{\,2} - 0.00292\,x_2^{\,2} + 1.9520\,x_3^{\,2-} - 0.00313\,x_1x_2 + 0.04067\,x_1x_3 + 0.1090\,x_2x_3 \quad (3)$$

$$\text{Carotenoid production } (Y_3) = -182.6500 - 0.05528\,x_1 + 4.3270\,x_2 - 110.8700\,x_3 - 0.03069\,x_1^{\,2} - 0.01367\,x_2^{\,2} + 48.3900\,x_3^{\,2} + 0.00939\,x_1x_2 + 0.1200\,x_1x_3 + 0.09575\,x_2x_3 \quad (4)$$

Table 4. Regression of coefficients and analysis of variance of the second order polynomial for response variables

Coefficient	Biomass (g/L)	Lipid content (%)	Carotenoids (mg/L)
	Y_1	Y_2	Y_3
β_0	6.8465*	−54.6700*	−182.6500*
Linear			
x_1	−0.0614*	0.8510	−0.05528
x_2	−0.00979	1.0230*	4.3270*
x_3	4.1166	−20.5300	−110.8700
Interaction			
x_1x_2	0.000292	−0.00313*	0.00939*
x_1x_3	−0.01167	0.04067	0.1200
x_2x_3	−0.01375	0.1090*	0.09575*
Quadratic			
$x_1^{\,2}$	0.000861*	−0.0869	−0.03069
$x_2^{\,2}$	0.0000469	−0.00292*	−0.01367*
$x_3^{\,2}$	−0.7250	1.9520	48.3900
Variability			
R^2 of model	0.93	0.97	0.93
F value of model	49.00	40.53	269.4
$P > F$	0.020	0.024	0.003
CV of model	3.61	4.89	5.75

x_1, x_2, and x_3 are COD, C/N ratio, and Tween 20 concentration, respectively.
*means significant at 5% level.

The models fitted satisfactorily to the experimental data as indicated by their goodness of fit expressed by R^2 and P

values. The respective R^2 values of the models for Y_1, Y_2, and Y_3 were 0.93, 0.97, and 0.93. This indicated that up to 93 ~ 97% of the variations in biomass, lipid content, and carotenoid production can be explained by these equations. The coefficients of variance (CV) indicate the degree of precision with which the experiments are compared. The lower reliability of the experiment is usually indicated by a high CV (> 15). In the present case, acceptable CV values were observed for the model of biomass, lipid content, and carotenoid production (3.61, 4.89, and 5.75, respectively). This denotes that the experiments performed were reliable. The P values of the models for biomass,

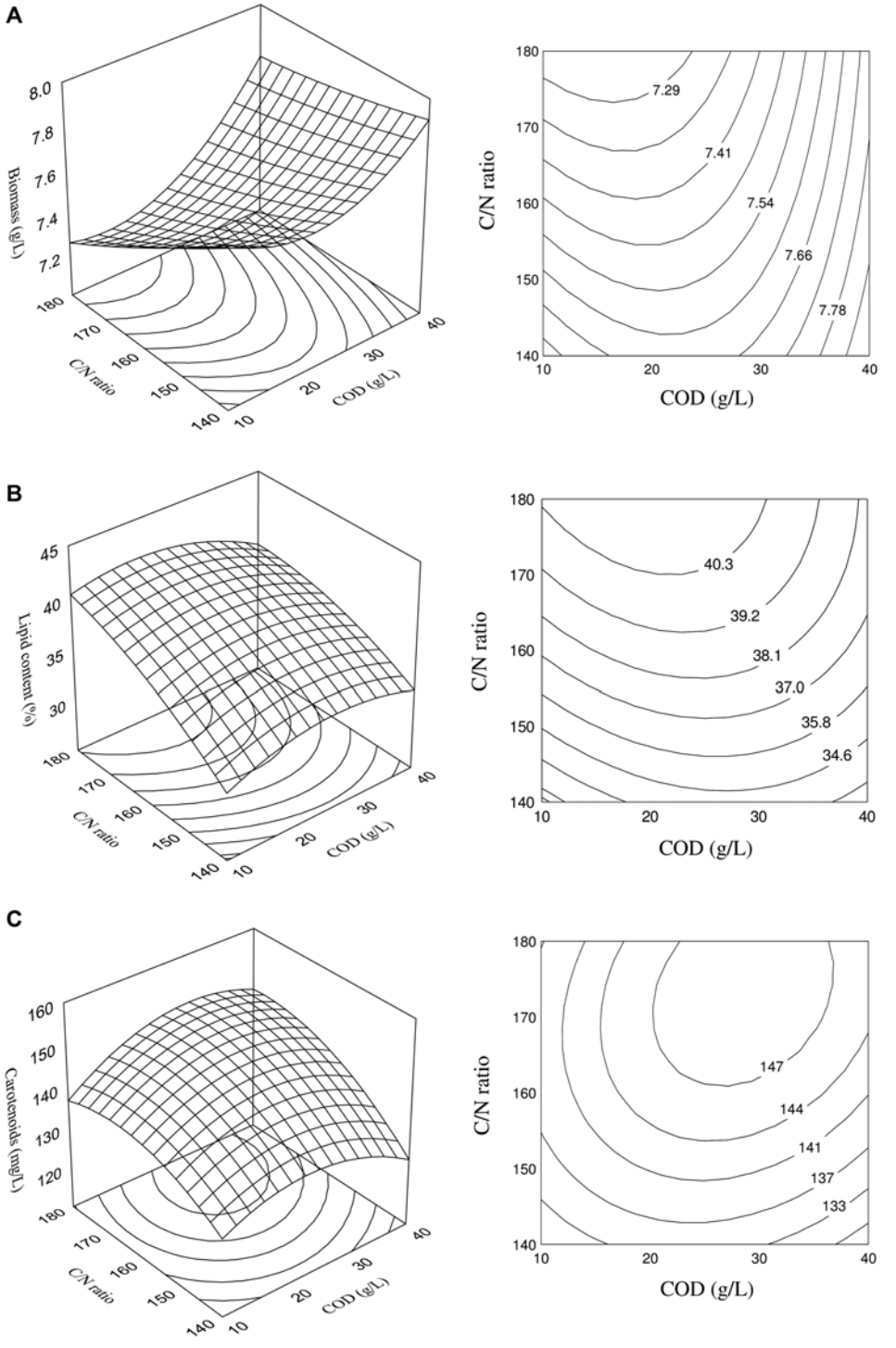

Fig. 1. Response surface plots and contour plots for the effects of COD (x_1) and C/N ratio (x_2) on biomass (A), lipid content (B), and carotenoid production (C) when the Tween 20 concentration was fixed at 1.5 g/L.

Table 5. Comparison of predicted responses from each optimum condition with different criteria

Criteria	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)	Carotenoids (mg/L)
Maximum biomass[a]	7.91	32.78	2.59	126.26
Maximum lipid content[b]	7.32	41.08	3.00	147.81
Maximum carotenoids[c]	7.47	39.74	2.96	148.77

[a]COD of 40 g/L and C/N ratio of 140; [b]COD of 25 g/L and C/N ratio of 180; [c]COD of 30 g/L and C/N ratio of 170.

lipid content, and production of carotenoids were 0.020, 0.024, and 0.003, respectively. The P value 0.05 indicates the significance of the coefficients. The statistical significance of the model equation was also confirmed by F values of the model, which were 49.00, 40.53, and 269.4 for biomass, lipid content, and carotenoid production, respectively.

Further statistical analysis showed that the C/N ratio (x_2) had a significant effect on lipid and carotenoid production, while COD (x_1) had a significant effect on biomass ($P < 0.05$) (Table 4). The terms of x_1x_2 and x_2x_3, indicating the interactions of C/N ratio with COD and Tween 20, respectively, were also significant for lipid and carotenoid production. However, the effect of Tween 20 concentration (x_3) was insignificant on all responses in the examined range. Thus, the mathematical model was simplified by using Tween 20 concentration (x_2) terms at optimum value (1.5 g/L). Then, in further regression analysis, the main, quadratic, and interaction effects of x_1 and x_2 were maintained. The second order regression equation for biomass (Y_1), lipid content (Y_2), and carotenoid production (Y_3) as a function of COD (x_1) and C/N ratio (x_2) generated a regression relationship as given in Eq. (5-7):

$$Y_1 = 11.39028 - 0.0788\ x_1 - 0.0304\ x_2 + 0.000861\ x_1^2 + 0.0000468\ x_2^2 + 0.000292\ x_1x_2 \quad (5)$$

$$Y_2 = -81.073 + 0.912\ x_1 + 1.186\ x_2 - 0.00869\ x_1^2 - 0.00292\ x_2^{2-} - 0.00313\ x_1x_2 \quad (6)$$

$$Y_3 = -204.077 + 0.1247\ x_1 + 4.470\ x_2 - 0.03069\ x_1^2 - 0.01367\ x_2^2 + 0.00939\ x_1x_2 \quad (7)$$

Regression models were employed to develop response surface plots as shown in Fig. 1. The response surface and contour plots of biomass, lipid content, and carotenoid production illustrated the effects of the COD and C/N ratio. Based on response surface plots, the interaction between the two variables and their optimum levels can be easily understood and located. Fig. 1A shows that the biomass increased with increasing COD, up to 40 g/L and decreasing C/N ratio, to 140. Conversely, the maximum lipid content was obtained at a moderate COD of 25 g/L and increased C/N ratio, up to a high level of 180 (Fig. 1B). The maximum production of carotenoids was obtained at a COD of 30 g/L and relatively high C/N ratio of 170 (Fig. 1C). When the C/N ratio remained constant, the production of lipids and carotenoids decreased with an increase in the COD above optimum levels. Since the optimum condition for the biomass was different from that for the lipid content, the lipid yield per liter (biomass × lipid content) of each optimal condition was compared (Table 5). The optimum conditions for the lipid content (COD of 25 g/L and C/N ratio 180) gave the highest lipid yield of 3.00 g/L. Although the optimum conditions for the biomass (COD of 40 g/L and C/N ratio 140) gave the highest biomass of 7.91 g/L, only 32.78% of the lipid content was accumulated and resulted in a lower lipid yield of 2.59 g/L. In addition, since the optimum conditions for carotenoid production (COD of 30 g/L and C/N ratio of 170) was similar to that for the lipid content, a comparable lipid yield of 2.96 g/L was obtained. The lipid content of *R. glutinis* TISTR 5159, using POME in this study, was higher than that reported by Xue *et al.* [26]. In their study, only 20% of the lipid was accumulated by *R. glutinis* when using monosodium glutamate wastewater as the culture medium. This might be due to the higher carbon content in the POME compared with that in monosodium glutamate wastewater.

The level of lipid synthesis in oleaginous microorganisms depends mostly on a high C/N ratio [27,28]. This is attributed to the induction of nitrogen-scavenging reactions, the effect of which is lowered levels of adenosine monophosphate (AMP). This consequently disrupts the citric acid cycle due to dependence of the isocitrate dehydrogenase reaction on AMP. The influence of the C/N ratio on carotenoid production has, however, remained unevaluated until now. A study by Somashekar and Joseph [2] found that a medium with a high C/N ratio tended to produce lipids rather than carotenoids. As the culture growth progresses, a change in the C/N ratio is expected with lower levels of nitrogen. The conditions for lipid and carotenoid production might become available at the later stage of culture growth.

The increased growth, lipid content, and carotenoid production of *R. glutinis* TISTR 5159 cultured in the POME were obtained when sufficient Tween 20, as an activator, was supplied with a suitable COD and C/N ratio. This suggests that Tween 20 might influence the enzyme activities in the lipid and carotenoid biosynthetic pathway of *R. glutinis* TISTR 5159. It was reported that several enzymes involved in lipid and carotenoid biosynthesis in prokaryotic and eukaryotic cells are stimulated by Tween

Table 6. Experimental results of biomass, lipid content, lipid yield, and carotenoids production by *R. glutinis* TISTR 5159 in one and two-stage processes

Strategy/Criteria	Biomass (g/L)	Lipid content (%)	Lipid yield (g/L)	Carotenoids (mg/L)
One-stage process[a]				
Maximum lipid content	7.59 ± 0.37	40.85 ± 1.83	3.10 ± 0.14	153.73 ± 12.01
Two-stage process[b]				
First stage	8.58 ± 0.25	34.99 ± 0.19	3.00 ± 0.05	126.51 ± 2.13
Second stage	8.82 ± 0.18	51.85 ± 0.64	4.58 ± 0.11	176.45 ± 3.19

[a]The cells were grown under condition for maximum lipid content for 72 h; [b]the cell was grown under condition for maximum biomass for 48 h in the first stage and then transferred to be cultured under condition for maximum lipid content for 24 h in the second stage.

20 [29]. These include, for example, phytoene desaturase, β-carotene hydroxylase, and lycopene cyclase.

Bioprocesses for lipid production may be designed around the ability of *R. glutinis* to grow and accumulate large amounts of lipids. In this study, the predicted condition for maximizing lipid content was experimentally tested in a one-stage process. A two-stage process was also attempted and compared to the one-stage process, in which cell growth was promoted in the first stage and lipid production was then enhanced in the second stage. The experimental results are shown in Table 6. The first stage corresponded to the maximum biomass production obtained with a high COD of 40 g/L and low C/N ratio of 140. At this stage, the highest biomass of 8.58 g/L was obtained while the lipid content and carotenoid production were 34.99% and 126.51 mg/L, respectively. The second stage corresponded to the establishment of lipid accumulation with COD of 25 g/L and high C/N ratio of 180. At this stage, the lipid content and carotenoids were enhanced up to 51.85% and 176.45 mg/L, respectively. Hence, the overall lipid yield and carotenoids increased from 3.10 g/L and 153.73 mg/L in the one-stage process, up to 4.58 g/L and 176.45 mg/L in the two-stage process. The two-stage system was also found suitable for the production of carotenoids by *Xanthophyllomyces dendrorhous* [30].

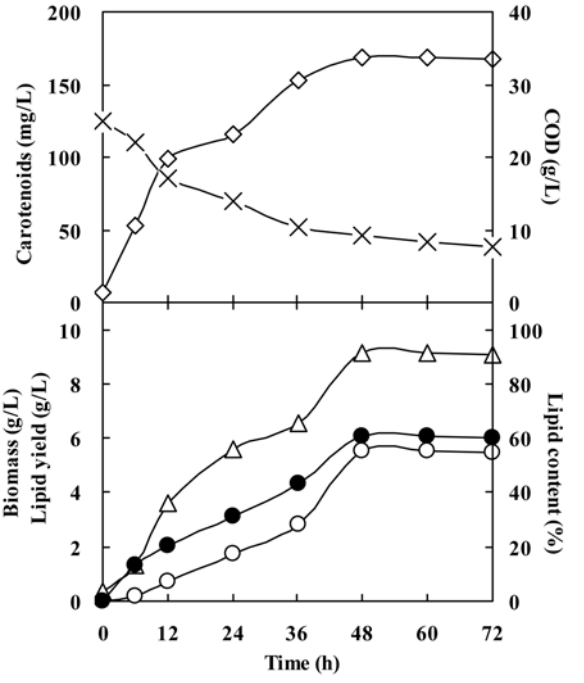

Fig. 2. Batch fermentation of *R. glutinis* TISTR 5159 with optimized medium in a stirred bioreactor. The pH was controlled at 6.0 and the aeration rate was 2.0 vvm. Biomass (open triangle), lipid yield per liter (open circle), lipid content (filled circle), carotenoids (open diamond), and COD (cross).

3.4. Batch and semi-continuous fermentation

To further increase the growth and production of lipids and carotenoids, batch fermentation was carried out in a 2.0 L bioreactor equipped with pH control and aeration systems (Fig. 2). The conditions for maximizing lipid content in a one-stage process were applied. As *R. glutinis* is an aerobic microorganism, fermentation in a bioreactor with aeration had a profound effect on lipid and carotenoid production. The biomass and lipid content reached 9.15 g/L and 60.62%, respectively, after 48 h of fermentation. This thus led to a lipid yield of 5.55 g/L. The carotenoid concentration was also increased up to 188.31 mg/L. The COD removal in the batch fermentation was also evaluated to reveal that 69.60% of the COD was reduced.

In semi-continuous fermentation, a portion of the culture is withdrawn at intervals and fresh medium is added to the system. This method has the advantages of the continuous and batch operations. There is no need for a separate inoculum vessel, except at the initial startup. Furthermore, time is not wasted through non-productive activities such as cleaning and resterilization. Another advantage of this operation is that little control is required [31]. In the semi-continuous fermentation of *R. glutinis* TISTR 5159 cultured in POME, the first fill-and-withdraw operation was introduced at 48 h and repeated five times during the 336 h of fermentation. Time courses of biomass, COD, carotenoids, lipid yield, and lipid content are shown in Fig. 3. The specific rates include the specific growth rate,

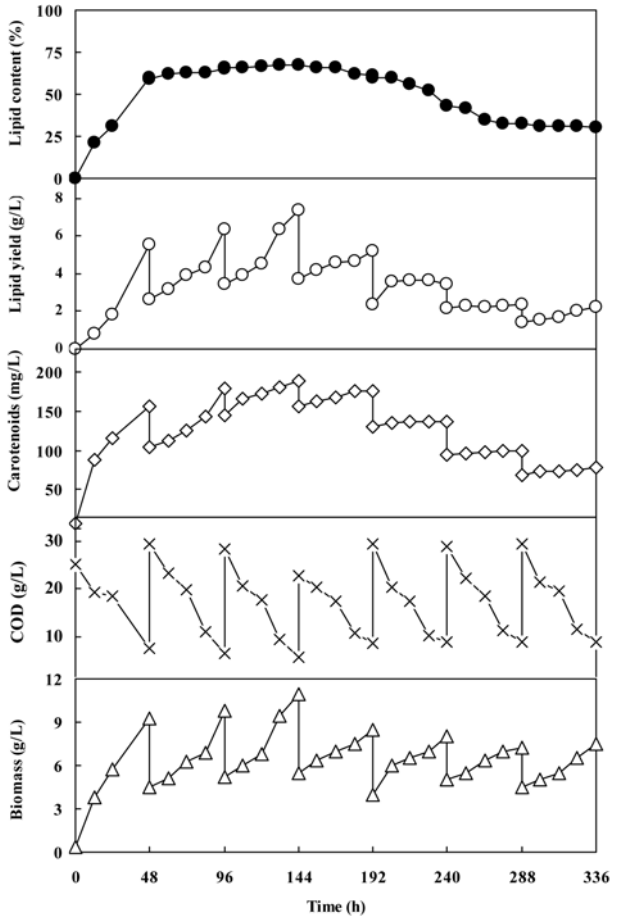

Fig. 3. Semi-continuous fermentation of *R. glutinis* TISTR 5159 with optimized medium in a stirred bioreactor. The pH was controlled at 6.0 and the aeration rate was 2.0 vvm. The fill-and-draw operation started at 48 h and the interval of reset was 48 h. Biomass (open triangle), lipid yield per liter (open circle), lipid content (filled circle), carotenoids (open diamond), and COD (cross).

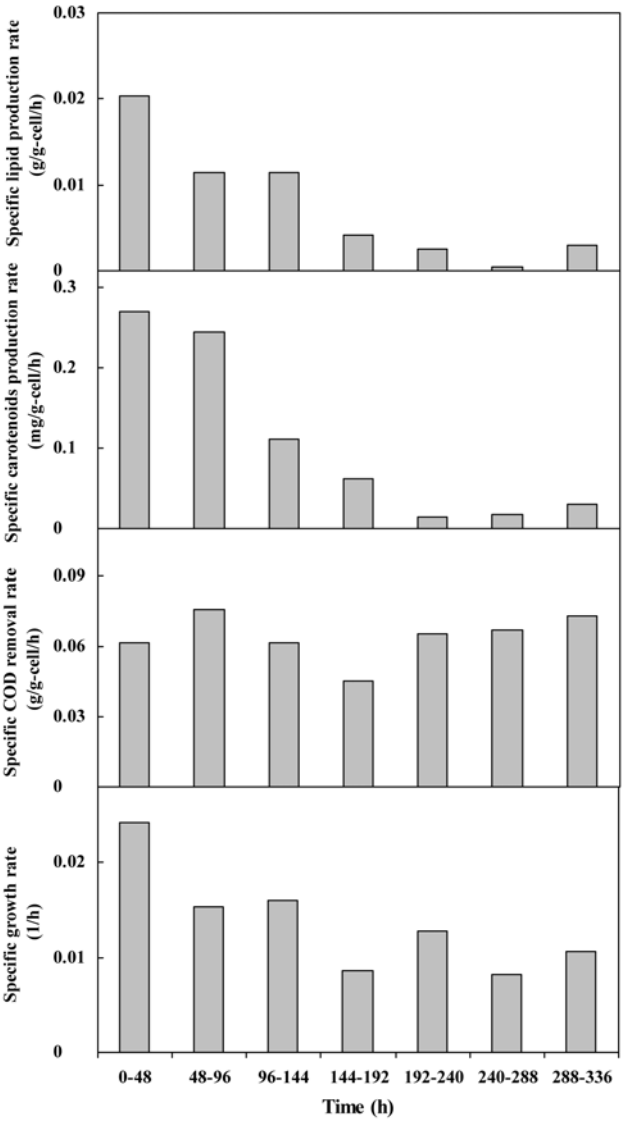

Fig. 4. Time courses on specific growth rate, specific COD removal rate, specific carotenoid production rate, and specific lipid production rate of *R. glutinis* TISTR 5159 in semi-continuous fermentation.

specific COD removal rate, specific production rates of carotenoids and lipids, and are shown in Fig. 4. It was obvious that cells grew fast during the initial stage. The biomass increased rapidly from 0.37 to 9.3 g/L within 48 h. In this period, cell growth was linear and the biomass increased with the highest specific growth rate of 0.024 /h. In the periods of 48 ~ 96 h and 96 ~ 144 h, the specific growth rate decreased to 0.016 /h, and after 144 h, it further decreased and fluctuated at levels of 0.009 ~ 0.013 /h. Therefore, the increase in biomass after 144 h was smaller than that before 144 h.

The maximum lipid yield of 7.4 g/L and maximum carotenoid concentration of 188.55 mg/L were achieved at 144 h. This was due to the maximum biomass of 10.9 g/L and the high lipid content of 67.27% obtained at 144 h. The lipid content was maintained above 60% until 204 h and gradually decreased and reached 30% at the end of fermentation. Final biomass, lipid yield, and carotenoid production were 7.5, 2.25, and 77.81 mg/L, respectively. The effective COD removal was continued throughout the 336 h of fermentation with the overall specific COD removal rate of 0.06 g/g-cell/h. The specific lipid production rate was highest at 0.021 g/g-cell/h in the initial period of 0 ~ 48 h and reduced to 0.011 g/g-cell/h after 48 h and to 0.004 g/g-cell/h after 144 h. The specific carotenoid production rate was highest at 0.269 mg/g-cell/h in the initial period of 0 ~ 48 h and reduced to 0.110 mg/g-cell/h after 96 h and to 0.062 mg/g-cell/h after 144 h. Nonetheless, effective COD removal and moderate cell growth were observed during 144 ~ 336 h, the lipid and carotenoid producing ability drastically decreased. This phenomenon

Table 7. Fatty acids composition of biodiesel derived from lipid of *R. glutinis* TISTR 5159 cultured in POME

Distribution of fatty acids	%
Lauric acid C12:0	0.18
Myristic acid C14:0	1.04
Palmitic acid C16:0	20.37
Palmitoleic acid C16:1	0.83
Heptadecanoic acid C17:0	1.38
Stearic acid C18:0	10.33
Oleic acid C18:1	47.88
Linoleic acid C18:2	7.31
Linolenic acid C18:3	0.85
Behenic acid C22:0	1.50
Lignoceric acid C24:0	1.03
Not identified	7.30

could be explained by the cells potentially using most of the consumed substrate to maintain their activities in long-term cultivation rather than accumulate as lipids and carotenoids.

3.5. Biodiesel production from yeast lipids

For the production of biodiesel, the preparation of fatty acid methyl esters (FAME) was carried out in two steps. After the hydrolysis reaction, esterification with methanol was carried out using acid catalysis. Table 7 shows the fatty acid composition of biodiesel derived from yeast lipids and their contents. The data show that the main fatty acids were long-chain fatty acids with 16 and 18 carbon atoms, including oleic acid (47.88%), palmitic acid (20.37%), stearic acid (10.33%), and linoleic acid (7.31%). The lipids from other oleaginous yeasts also contain mainly long-chain fatty acids with 16 and 18 carbon atoms. *Rhodosporidium toruloides* Y4 contained four main fatty acids, including oleic acid (46.9%), palmitic acid (20%), stearic acid (14.6%), and linoleic acid (13.1%), when grown on glucose [1]. While *R. toruloides* Y4 was grown on lignocellulose biomass hydrolyzate, it contained mainly oleic acid (50%) and palmitic acid (33%) [32]. *Rhodotorula mucilaginosa* TJY15a also contains mainly oleic acid (63.5%) and palmitic acid (22.3%) when using cassava starch hydrolyzate as a substrate [33]. The similar fatty acid compositional profile to that of plant oil indicates that lipids from yeast possess great potential as biodiesel feedstock.

The high content of unsaturated fatty acids (oleic acid) would evidence lower oxidative stability, but excellent fuel properties at low temperatures, which is an advantage in operation in winter. However, compared with the commonly used soybean oil and rapeseed oil as feedstock for biodiesel production in the US and EU, the FAME derived from *R. glutinis* TISTR 5159 cultured in POME were more saturated. Soybean oil contains mostly linoleic acid and oleic acid at 53.7 and 23.3%, respectively, while rapeseed oil also contains similar fatty acids at 23.3 and 64.4%, respectively [34]. Therefore, the lipids from *R. glutinis* TISTR 5159 tend to give favorable properties to the biodiesel derived from soybean oil and rapeseed oil. These include an increased cetane number (CN), decreased NOx emissions, shorter ignition delay time, and oxidative stability. It is known that fatty acid distribution impacts the CN values. Considering the percentage distribution of fatty acids shown in Table 7 and the empirical equation [35], the yeast lipids could produce biodiesel with CN values higher than 54. The minimal requirement for CN values have been set at 47, 49, and 51 by biodiesel standards ASTMD 6751 (USA), DIN 51606 (Germany), and EN 14214 (European Organization), respectively. Since the biodiesel produced from lipids of *R. glutinis* TISTR 5159 meet these standards, it has a great potential for biodiesel production.

4. Conclusion

Improved biomass, lipid content, and carotenoid production of *R. glutinis* TISTR 5159 cultured in POME have been achieved by medium optimization using RSM. The proposed model equation illustrated the quantitative effect of variables and also the interactions among the variables on biomass, lipid content, and carotenoid production. The optimized conditions showed that cell growth required a relatively high COD and low C/N ratio, while the lipid and carotenoid production required lower COD but higher C/N ratio. The two-stage process for cell growth in the first stage and lipid accumulation in the second stage significantly improved lipids and carotenoid production. In semi-continuous fermentation, *R. glutinis* TISTR 5159 accumulated a significant amount of lipid content and produced considerably high concentration of carotenoids during long-term cultivation. Moreover, efficient COD removal by *R. glutinis* TISTR 5159 was also observed. This study has showed the potential use of *R. glutinis* TISTR 5159 for lipid and carotenoid production, as well as COD removal from POME.

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