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Optimization of Carotenoids Production by Red Yeast *Sporobolomyces pararoseus* TISTR5213 Using Waste Glycerol as the Sole Carbon Source

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Abstract

The usage of waste glycerol obtained from biodiesel production is a big challenge as this glycerol is not suitable for foods and cosmetics applications. Therefore, nine strains of red yeasts from the culture collection of Thailand Institute Scientific and Technological Research (TISTR) were screened for carotenoids production in yeast malt-extract medium (YM), basal medium supplemented with either pure glycerol (BMP) or waste glycerol (BMW) as the sole carbon source. The results showed that *Sporobolomyces pararoseus* TISTR5213 produced the maximum of total carotenoids at 4.87±0.08, 1.58±0.44 and 1.77±0.02 mg/L, in YM, BMP and BMW, respectively. Variable medium components of BMW were selected in accordance with the Plackett-Burman experimental design with only one factor of waste glycerol being significant. The optimization conditions for physical factors (pH and temperature levels) were then combined in further studies through the response surface methodology (RSM) approach. A quadratic model was constructed by central composite design (CCD). Using this experimental design, the total carotenoids production yield increased from 1.77 to 16.55 mg/L or about 835% higher than un-optimized BMW. The optimum conditions to achieve maximum yield of total carotenoids were; waste glycerol 34.00 g/L, initial pH at 5.64 and 23.90°C for 5 days. In comparison, the cost of carotenoids production in YM, BMP and optimized BMW were; 12.08±2.68, 15.69±4.41 and 0.90±0.02 Baht/mg carotenoids/L medium, respectively.

Keywords: Plackett-Burman design, central composite design (CCD), carotenoids, waste glycerol, *Sporobolomyces pararoseus* TISTR5213

1. Introduction

Carotenoids are yellow to orange-red pigments that are ubiquitous in nature (1). They are a group of 600 molecules which can be found in plants and microorganisms, including bacteria, algae, molds and yeasts (2) but are not synthesized in animals (3). They are used in pharmaceuticals, neutraceuticals, chemicals and animal feed additives (2), as well as colorants in cosmetics and foods (4). Carotenoids are usually C_{40} tetraterpenoids built from eight C_5 isoprenoid units joined, so that the sequence is reversed at the center (5). Carotenoids consist of two classes of molecules, the carotene which are strictly hydrocarbons e.g. lycopene, α-carotene, and β-carotene and the xanthophylls which are oxygenated derivatives e.g. lutein, α-cryptoxanthin, β-cryptoxanthin, zeaxanthin, canthaxanthin and astaxanthin (4, 6). Various genera of red yeasts, such as *Cryptococcus*, *Rhodosporidium*, *Rhodotorula*, and *Sporobolomyces* and *Xanthophyllomyces* can produce and accumulate carotenoids in their cells (2, 7). Commercial production of carotenoids using red yeasts is highly efficient because they are not only easy to manipulate in the processing schemes (8), they also have a high growth rate and are convenient for large-scale fermentation (2).

Many kinds of low cost carbon sources, such as cane sugar, glycerol, vegetable oils, *n*-alkanes, or a variety of wastes derived from agricultural production e.g. sugar cane molasses, raw coconut milk, radish brine, whey, hydrolyzed mung bean waste flour, have been considered as a potential carbon sources for biotechnological carotenoids production (8-12). Waste or crude glycerol is a by-product from biodiesel production industry. Currently, biodiesel production is increasing exponentially and waste glycerol is generated by the transesterification of vegetable oils has also been generated in large quantity. Glycerol is present in the form of its esters (triacylglyerols and derivatives) in all fats and oils (13). For every 9 kg of biodiesel produced, about 1 kg of waste glycerol by-product is obtained (14).

The usage of waste glycerol is a big challenge as this glycerol is not suitable for foods and cosmetics applications (13). In this study, we focus on using waste glycerol as the sole carbon source to increase carotenoids production by *S. pararoseus* TISTR5213, using the experimental design with the Plackett-Burman screening methodology and optimization using response surface methodology (RSM) applied in a central composite design (CCD).

2. Materials and Methods

2.1 Microorganisms

Nine strains of red yeasts were kindly given by the culture collection section of Thailand Institute of Scientific and Technological Research (TISTR). The strains were *Sporobolomyces* sp. TISTR5899, *S. pararoseus* TISTR5213, *S. shibatanus* TISTR5563, *S. nylandii* TISTR5581, *Rhodosporidium toruloides* TISTR5123, *Rhodotorula rubra* TISTR5134 and TISTR5158, *Dioszegia* sp. TISTR5792 and *Xanthophyllomyces dendrorhous* (formerly *Phaffia rhodozyma*) TISTR5730. All red yeasts were maintained in glycerol stock at -20°C (15).

2.2 Raw materials

Waste glycerol was kindly given by the Energy Research and Development Institute (ERDI), Chiang Mai University, Thailand. The composition of waste glycerol from biodiesel production process is shown in Table 1. The concentration of glycerol in waste glycerol was determined by HPLC equipped with an Aminex HPX-87H column (300 x 7.8 mm; Bio-Rad, USA). The mobile phase used 5.0 mM H_2SO_4 as an eluent with a flow

rate 0.75 mL/min and the column thermostat was set at 40°C. Glycerol was detected by an RI detector (refractive index detector RID-10A; Shimudzu, Japan); in a linear gradient in 20 min, maintaining this proportion until the end of the run (16). Proximate analysis of ash content, water content and lipid content of waste glycerol were analyzed according to the AOAC international official methods of analysis (17).

2.3 Inoculum preparation

The yeast malt-extract medium (YM) for inoculum preparation had the following compositions (per liter): yeast extract 4.0 g, malt extract 10.0 g, glucose 4.0 g and the initial pH was adjusted to pH 6.0. Glycerol stocks of red yeasts were transferred into 250 mL Erlenmeyer flasks containing 50 mL of fresh YM on an incubator shaker (Kühner, Switzerland) at 25°C, with a shaking speed of 200 rpm for 3 days for inoculum preparation (18). The starter culture was 10.0% (v/v) inoculated by batch fermentation.

2.4 Screening of carotenoids producing red yeasts

The carotenoids production of red yeasts was screened in 3 conditions. The first condition was; cultivation in yeast malt-extract medium (YM) containing (per liter); yeast extract 4.0 g, malt extract 10.0 g and glucose 4.0 g. The second condition was; cultivation in basal medium supplement with pure glycerol (BMP) containing (per liter); yeast extract 1.0 g, pure glycerol 20.0 g, KH_2PO_4 5.5 g, $(NH_4)_2SO_4$ 5.3 g, K_2HPO_4 3.7 g, $MgSO_4.7H_2O$ 0.5 g, $MnSO_4.H_2O$ 0.2 g and NaCl 0.5 g. The third condition was; cultivation in basal medium supplement with waste glycerol (BMW). The medium compositions of BMW were similarly to BMP but pure glycerol was replaced by 20.0 g of waste glycerol. The initial pH of each media was adjusted to 6.0 and the carotenoids production was carried out by incubating in the incubator shaker at 25°C, with shaking speed of 200 rpm for 5 days (18).

Table 1. The compositions of waste glycerol.

Parameters	Compositions (%)
Glycerol content	56.30±0.62
Methanol	15.09±0.63
Lipid content	10.85±0.14
Ash content	6.12±0.05
Water content	6.07±0.05
Other components (by difference)	5.57±1.49

2.5 Screening of factors affecting on carotenoids production

The Plackett-Burman design was employed to screen for components of the BMW to support growth and carotenoids production of *S. pararoseus* TISTR5213. Eight components were evaluated to determine Plackett-Burman design each factor was examined at two levels: -1 as the low level and +1 as the high level. The Plackett-Burman design with the eight factors under investigation as well as the levels of the each factor used in the experimental design is shown in Table 2, based on the first-order polynomial model as follows:

$$Y = \beta_0 \sum \beta_i X_i \quad (1)$$

Where Y is the response of total carotenoids, β_0 is the model intercept, β_i is the linear coefficient, and X_i is the level of the independent variables.

This model does not describe interaction among the factors. It is used for screening and evaluating the important factors that influence the response. The magnitude of the coefficient of positive or negative indicates the corresponding impact on the titer. The coefficient value approaches to zero, which implies small or no effect. The *p*-value is the probability that the coefficient results from a random process. A low *p*-value indicates a significant effect. The significance of each variable is determined by applying the *F*-value (19).

Table 2. Experiment variables at various levels used in the carotenoids production by *Sporobolomyces pararoseus* TISTR5213 using the Plackett-Burman design.

Variables	Units	Symbol codes	Experimental values	
			Low (-1)	High (+1)
Yeast extract	g/L	X_1	0.2	5.0
Waste glycerol	g/L	X_2	10.0	50.0
KH_2PO_4	g/L	X_3	2.0	10.0
$(NH_4)_2SO_4$	g/L	X_4	2.0	10.0
K_2HPO_4	g/L	X_5	1.5	7.5
$MgSO_4.7H_2O$	g/L	X_6	0.1	2.0
$MnSO_4.H_2O$	g/L	X_7	0.1	1.0
NaCl	g/L	X_8	0.1	2.0

In this study, eight assigned variables were screened during 12 experimental runs. Carotenoids production yield was carried out in triplicate and the average values of observed values and predicted values by the equation model were shown as response Y (Table 5). Based on a regression analysis of the variables, a confidential level of 95% ($p \leq 0.05$) for each factor was considered to have a significant effect on the carotenoids production. In this experimental design, the statistical software package Design Expert 6.0.10 (Stat-Ease, Minneapolis, MN) was used in the design of the experiments and the analysis of the experimental data.

2.5 Optimization of significant variables using response surface methodology

An experimental design using CCD is used to estimate the coefficients in a mathematical model, predict the response, and check the applicability of the model (20). The pH and temperature were investigated with the one variable of waste glycerol which obtained from the Plackett-Burman design for carotenoids production (Table 3). The CCD contained an imbedded factorial or fractional factorial matrix with center points and star points around the center point that allowed estimation of the curvature. The distance from the center of the design space to a factorial points was ±1 unit for each factor, and the distance from the center of the design space to a star point was $\pm\alpha$, where $|\alpha|>1$. The precise value of α depended on certain properties needed for the design and on the number of factors used (in this case $\alpha=1.68$). Similarly, the number of center point runs that the design

must contain also depends on certain properties required for the design. The CCD always contains twice as many star points as factors in the design. The star points represent new extreme values (low and high) for each factor in the design. To maintain rotability, the value of the α depended on the number of experimental runs in the factorial portion of the CCD. In this experimental design, the statistical software package Design Expert 6.0.10 (Stat-Ease, Minneapolis, MN) was used in the design of the experiments, the analysis of the experimental data, and the generation the response surface graphs. The significant values of the model equation and the model terms were evaluated by Fisher's test as expressed in term of the *F*-values:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ii} X_i^2 + \Sigma\beta_{ij} X_i X_j \quad (2)$$

Where Y represents the response variable, β_0 is the interception coefficient, β_i the coefficient for the linear effect, β_{ii} the ij^{th} coefficient of the interaction effect, and $X_i X_j$ are input variables that influence the response variable Y. The response variable in each trial is the average of three replicates (20).

pH and temperature were the most important conditions on carotenoids production. A range of pH level between 5-7 with a boundary of 4-8 for $\pm\alpha$ and temperature between 20-30°C with the boundary of 15-35 for $\pm\alpha$ were selected in the experimental design. It included a total of 17 experiments with three trials of center points (Table 7).

Table 3. Experimental codes, ranges and levels of independent variables in the response-surface methodology experiment.

Variables	Units	Symbol codes	Levels				
			$-\alpha$	Low (-1)	Center (0)	High (+1)	$+\alpha$
Waste glycerol	g/L	X_2	-3.60	10.0	30.0	50.0	63.64
pH	-	X_9	4.32	5.0	6.0	7.0	7.68
Temperature	°C	X_{10}	16.59	20.0	25.0	30.0	33.41

2.6 Carotenoids analysis

Ten milliliters of 5 days-olds culture broth was taken from each flask and centrifuged at 6,000 rpm at 4°C for 10 min. The cell pellet was washed twice with *n*-hexane and once with distilled water. The carotenoids content of cell pellet was extracted by a method which broke the yeast cell, carried out in screw cap tube (25 x 150 mm), containing 10.0 mL acetone and glass beads (size 3 mm, 4.0 g). The mixture was vigorously shaken in a vortex mixer for 15 min in the presence of 100 ppm ascorbic acid. The broken cell was centrifuged and the clear supernatant was collected and dried by flushing it with N_2, then re-dissolved in 1.0 mL *n*-hexane. The extract was filtered through a nylon membrane filter (0.2 μm; FilTrex, USA) and subjected to HPLC analysis. A modified method of HPLC analysis was performed on the analytical HPLC (Shimadzu, Japan) equipped with a C18 column (4.6 mm x 250 mm, 5µm; Restek, France). The mobile phase was composed of acetonitrile : dichloromethane : methanol (80:10:10, v/v/v) with a flow rate of 1.0 mL/min. The column thermostat was set at 30°C. The detector was operated at 454 nm; in a linear gradient for 45 min, maintaining this proportion until the end of the run (21).

2.7 Biomass measurement

Dried cell weight (DCW) of each flask was collected from 5 days-olds cultivation broth, then centrifuged and washed twice as described above before drying at 80°C overnight and then transferred to desiccators until a constant weight was obtained.

3. Results and Discussion

3.1 Screening of carotenoids producing yeasts

The results revealed that nine strains of red yeasts could grow and produce carotenoids in YM, BMP and BMW (Table 4). The red yeast, *S. pararoseus* TISTR5213 had the highest total carotenoids production yield of 4.87±1.08, 1.58±0.44 and 1.77±0.02 mg/L in YM, BMP and BMW, respectively. In comparison, BMW increased the total carotenoids content over BMP about 18.70%. Waste glycerol could enhance carotenoids synthesis because it had trace elements e.g. Ca, K, Mg, Na, P and S in term of ash content (22). The percentage of ash content in waste glycerol was 6.12±0.05 (Table 1). The trace elements have been demonstrated to act as stimulants for growth of red yeasts, which have a stimulatory effect on β-carotene and γ-carotene synthesis. The observed effect of trace elements on the biosynthesis of specific carotenoids in red yeasts may be explained by hypothesizing a possible activation or inhibition mechanism by selected metal ions on specific carotenogenic enzymes, in particular, on specific desaturases involved in carotenoids biosynthesis (2).

Table 4. Total carotenoids production by red yeasts cultivated in YM, BMP and BMW medium.

Red yeasts strain	YM		BMP		BMW	
	X_m [g/L]	P_m [mg/L]	X_m [g/L]	P_m [mg/L]	X_m [g/L]	P_m [mg/L]
TISTR5123	6.68 ± 0.06^{b}	0.89 ± 0.10^{c}	6.17 ± 0.10^{c}	0.48 ± 0.06^{cd}	3.59 ± 0.10^{a}	0.59 ± 0.09^{c}
TISTR5134	6.70 ± 0.26^{b}	0.66 ± 0.03^{c}	7.60 ± 0.44^{b}	0.37 ± 0.02^{cd}	3.84 ± 0.46^{a}	0.32 ± 0.03^{e}
TISTR5158	6.59 ± 0.33^{b}	1.00 ± 0.06^{bc}	8.24 ± 0.40^{a}	0.46 ± 0.07^{cd}	3.47 ± 0.21^{ab}	0.56 ± 0.06^{c}
TISTR5213	5.06 ± 0.42^{d}	4.87 ± 1.08^{a}	5.73 ± 0.18^{c}	1.58 ± 0.44^{a}	3.49 ± 0.39^{ab}	1.77 ± 0.02^{a}
TISTR5563	4.38 ± 0.20^{e}	2.68 ± 0.43^{b}	2.98 ± 0.08^{d}	1.04 ± 0.03^{b}	2.89 ± 0.30^{bc}	0.49 ± 0.01^{cd}
TISTR5581	6.53 ± 0.15^{b}	2.69 ± 1.82^{b}	7.50 ± 0.36^{b}	1.09 ± 0.08^{b}	2.92 ± 0.46^{bc}	1.36 ± 0.07^{b}
TISTR5730	8.31 ± 0.58^{a}	0.81 ± 0.01^{c}	6.98 ± 0.40^{b}	0.69 ± 0.05^{c}	2.73 ± 0.20^{c}	0.23 ± 0.01^{ef}
TISTR5792	5.84 ± 0.19^{c}	0.26 ± 0.00^{c}	8.40 ± 0.67^{a}	0.16 ± 0.01^{d}	2.48 ± 0.41^{c}	0.20 ± 0.02^{f}
TISTR5899	5.67 ± 0.68^{cd}	0.57 ± 0.01^{c}	5.92 ± 0.27^{c}	0.38 ± 0.03^{cd}	3.42 ± 0.39^{ab}	0.43 ± 0.02^{d}

*Means and standard deviations of triplicate samples

Value with different significance according to the statistical analysis Duncan's multiple range test ($p \leq 0.05$)

X_m: Biomass (dried cell weight); P_m: Product (total carotenoids)

3.2 Screening of significant variables using the Plackett-Burman design

The results of Plackett-Burman experimental design are presented in Table 5. Various medium components at various concentrations were investigated. The average of total carotenoids production of the observed values and the predicted values by the equation were taken as response *Y*. To examine the fitting quality of the model, the proximate correlation coefficient (R^2) to 1 indicated better fitting of the predicted values from equation to the experimental values. The value of R^2 was 0.9437 of the variability in the response. The magnitude and direction of the factor coefficient in the equation explained the influence of the eight medium components on the carotenoids production of *S. pararoseus* TISTR5213. The greater magnitude of the coefficient indicated a large effect on the response. Variables at confidence levels greater than 95% ($p \leq 0.05$) were considered significant. The corresponding response of the carotenoids production was expressed in terms of the following regression equation:

$$Y \text{ (total carotenoids)} = 1.702 - 0.165X_1 + 0.215X_2 + 0.051X_3 - 0.218X_4 - 0.293X_5 + 1.788X_6 + 2.551X_7 - 0.922X_8 \quad (3)$$

Table 5. Twelve-trial Plackett-Burman design matrixes for eight variables and predicted total carotenoids.

Run order	Experimental values								Total carotenoids (mg/L)	
	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Actual	Predicted
1	0.2	10.0	2.0	10.0	7.5	2.0	0.1	2.0	0.44	1.53
2	5.0	10.0	10.0	2.0	1.5	0.1	1.0	2.0	2.45	3.54
3	5.0	50.0	2.0	10.0	1.5	0.1	0.1	2.0	9.37	7.68
4	5.0	50.0	10.0	2.0	7.5	2.0	0.1	2.0	10.22	11.46
5	5.0	10.0	2.0	2.0	7.5	2.0	1.0	0.1	8.22	6.53
6	0.2	50.0	2.0	2.0	1.5	2.0	1.0	2.0	16.54	15.91
7	0.2	50.0	10.0	10.0	1.5	2.0	1.0	0.1	15.68	16.32
8	0.2	10.0	10.0	10.0	7.5	0.1	1.0	2.0	1.92	0.83
9	0.2	50.0	10.0	2.0	7.5	0.1	0.1	0.1	11.85	10.61
10	5.0	50.0	2.0	10.0	7.5	0.1	1.0	0.1	8.27	9.97
11	0.2	10.0	2.0	2.0	1.5	0.1	0.1	0.1	2.14	3.38
12	5.0	10.0	10.0	10.0	1.5	2.0	0.1	0.1	5.29	4.65

*The observed values of total carotenoids were the mean values of three experiments

$\boldsymbol{X_1}$ Yeast extract (g/L); $\boldsymbol{X_2}$ Waste glycerol (g/L); $\boldsymbol{X_3}$ KH_2PO_4 (g/L); $\boldsymbol{X_4}$ $(NH_4)_2SO_4$ (g/L); $\boldsymbol{X_5}$ K_2HPO_4 (g/L); $\boldsymbol{X_6}$ $MgSO_4.7H_2O$ (g/L); $\boldsymbol{X_7}$ $MnSO_4.H_2O$ (g/L); $\boldsymbol{X_8}$ NaCl (g/L)

The factors with *p*-values less than 0.05 were considered to have significant effects on the response and were selected for further study of optimization using CCD (Table 6). Waste glycerol was determined to be the first one significant factor, with *p*-value corresponding to 0.009 ($F = 36.86$). Waste glycerol had a positive effect on the produced total carotenoids of *S. pararoseus* TISTR5213 with in design range of 10-50 g/L, as the evidence of the minus symbol in the coefficient of the linear regression equation.

Table 6. Estimated effects, linear regression coefficients and corresponding *F*-values and *p*-values for total carotenoids for eight variables by the Plackett-Burman design experiment.

Variables	Symbol codes	Effect	Coefficient	Standard error	*F*-values	*p*-values
Yeast extract	X_1	-0.79	-0.17	1.41	0.31	0.614[b]
Waste glycerol	X_2	8.58	0.22	1.41	36.86	0.009[a]
KH_2PO_4	X_3	0.40	0.05	1.41	0.08	0.794[b]
$(NH_4)_2SO_4$	X_4	-1.74	-0.22	1.41	1.52	0.305[b]
K_2HPO_4	X_5	-1.76	-0.29	1.41	1.55	0.302[b]
$MgSO_4.7H_2O$	X_6	3.40	1.79	1.41	5.78	0.096[b]
$MnSO_4.H_2O$	X_7	2.30	2.55	1.41	2.64	0.203[b]
NaCl	X_8	-1.75	-0.92	1.41	1.54	0.303[b]

[a]Significant at $p \leq 0.05$

[b]Not significant

3.3 Optimization of significant variables using response surface methodology

Response surface designs are used to obtain precise information about factor effects including magnitude and direction (20). It was found that the waste glycerol could serve as a sole carbon source for carotenoids production of *S. pararoseus* TISTR5213. The reason could be waste glycerol contained trace elements as already mentioned above. In this study, three main factors namely waste glycerol, pH and temperature, were selected for the optimization of carotenoids production. The CCD generated a quadratic equation for carotenoids production (Y) as a function of waste glycerol (X_2), pH (X_9) and temperature (X_{10}) as follow:

$$Y(\text{total carotenoids}) = -203.443 + 1.002X_2 + 31.377X_9 + 9.543X_{10} - 0.009X_{10}^2 - 2.169X_9^2 - 0.163X_{10}^2 - 0.028X_2X_9 + 0.010X_2X_{10} + 0.250X_9X_{10} \quad (4)$$

S. pararoseus TISTR5213 was cultivated 17 experiments to obtain total carotenoids production in batch cultivation using Erlenmeyer flasks for 5 days cultivation period. As shown in Table 7, the run orders of 1, 2 and 17 with different combinations of waste glycerol, pH and temperature levels enhanced total carotenoids to production levels of 16.92, 15.28 and 14.59 mg/L, respectively (Figure 1-3). Moreover, the quadratic mathematic model in equation 3 was further simplified, corresponding to the *p*-value in the model terms. In this case, a *p*-value less than 0.05 indicated significant model terms and values, whereas greater than 0.05 indicated insignificant model terms. The terms of X_{10}, X_2^2 and X_{10}^2 were significant model terms, as shown in Table 8.

Table 7. CCD matrixes for the experiment design and predicted responses for total carotenoids production.

Run order	Coded levels			Total carotenoids (mg/L)	
	X_2	X_9	X_{10}	Actual	Predicted
1	30.0	6.0	25.0	16.92	15.48
2	30.0	6.0	25.0	15.28	15.48
3	30.0	7.7	25.0	7.29	6.45
4	10.0	7.0	20.0	8.88	5.79
5	30.0	4.3	25.0	9.34	12.23
6	50.0	5.0	20.0	13.43	10.75
7	30.0	6.0	33.4	0.92	0.51
8	63.6	6.0	25.0	8.43	7.10
9	50.0	5.0	30.0	5.50	7.14
10	10.0	7.0	30.0	0.00	1.23
11	30.0	6.0	16.6	4.93	7.39
12	50.0	7.0	20.0	6.70	8.71
13	10.0	5.0	20.0	7.18	5.63
14	-3.6	6.0	25.0	0.37	3.76
15	50.0	7.0	30.0	0.00	0.10
16	10.0	5.0	30.0	9.52	6.06
17	30.0	6.0	25.0	14.59	15.48

X_2 Waste glycerol (g/L); X_9 pH; X_{10} Temperature (°C)

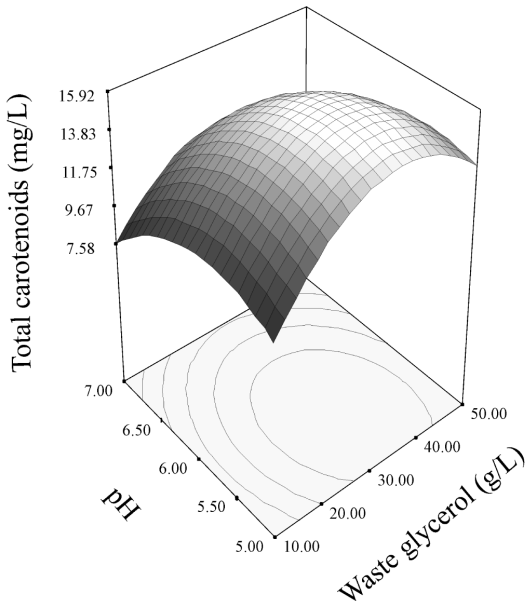

Figure 1. Total carotenoids production in three-dimension for quadratic response surface optimization. The comparison was made between pH and waste glycerol.

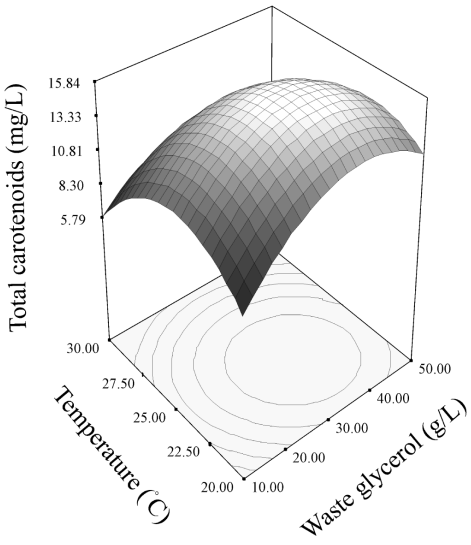

Figure 2. Total carotenoids production in three-dimension for quadratic response surface optimization. The comparison was made between temperature and waste glycerol.

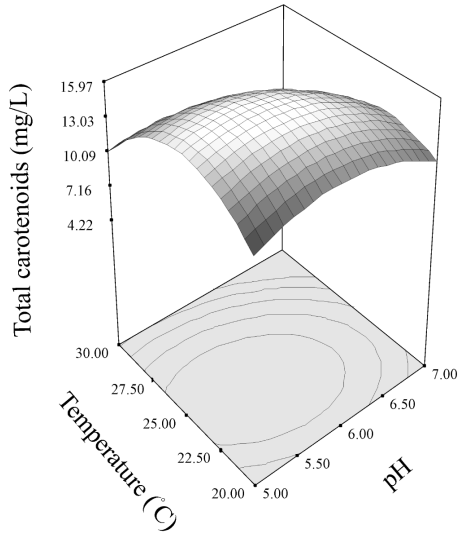

Figure 3. Total carotenoids production in three-dimension for quadratic response surface optimization. The comparison was made between temperature and pH.

In Table 8, the probability *p*-values of the model was relatively low (0.0336), indicating significant model. The coefficient of variation for the model (R^2=0.8470) was represented, and they implied a high correlation between the experimentally observed and the predicted values, similarly to the model.

Table 8. Analysis of variance (ANOVA) for the parameters of RSM fitted to the quadratic equation.

Source	SS	DF	MS	*F*-value	*p*-value>*F*	
Model	391.92	9	**43.55**	**4.31**	**0.0336**	***Significant***
X_2	13.63	1	13.63	1.35	0.2837	
X_9	40.40	1	40.40	4.00	0.0857	
X_{10}	57.08	1	57.08	5.65	0.0492	
X_2^2	142.27	1	142.27	14.07	0.0072	
X_9^2	53.03	1	53.03	5.25	0.0558	
X_{10}^2	187.23	1	187.23	18.52	0.0036	
X_2X_9	2.43	1	2.43	0.24	0.6387	
X_2X_{10}	8.18	1	8.18	0.81	0.3982	
X_9X_{10}	12.48	1	12.48	1.23	0.3033	
Residual	70.77	7	10.11			
Lack of fit	67.90	5	13.58	9.45	0.0984	***Not significant***
Pure error	2.87	2	1.44			
Total	462.69	16				
R^2	**0.8470**					

X_2 Waste glycerol (g/L); X_9 pH; X_{10} Temperature (°C)

3.4 Validation of the CCD optimization model

To confirm the applicability of the CCD optimization model, the carotenoids production by *S. pararoseus* TISTR5213 was carried out by cultivation under the optimal conditions suggested of 34.00 g/L waste glycerol, pH 5.64 at 23.90°C with 16.11 mg/L for a maximum total carotenoids production yield. From the experimental results (Figure 4), a yield of total carotenoids production of 16.55 mg/L was obtained with a higher than predicted value by 2.73%. This result indicated that the model could be used to predict the maximum yield of total carotenoids production.

The growth and carotenoids production behavior of *S. pararoseus* TISTR5213 under the optimal conditions obtained from the CCD optimization models were shown in Figure 4. This strain needed cultivation time up to 5 days for the highest carotenoids accumulation in its cell. As the evidence from Figure 4, carotenoids production seemed to be a growth-associated product and it increased during the log phase of *S. pararoseus* TISTR5213 growth. Similar to the results of Bhosale and Gadre, 2001 (23), who reported that carotenoids production from *R. glutinis* was a growth-associated product. Moreover, Aksu and Eren, 2005 (24) reported that the carotenoids production of *R. mucilaginosa* was also a growth-associated product and the highest carotenoids content was obtained at the end of log phase (5 days).

The maximum carotenoids production yield from *S. pararoseus* TISTR5213 of 16.55±0.16 mg/L and DCW of 8.64±0.13 g/L were obtained at 5 days of cultivation period and further increasing of cultivation time, decreasing of the carotenoids production yield and DCW was observed. As the results obtained from this study, it could be concluded that the carotenoids production in *S. pararoseus* TISTR5213 was formed almost parallel with the cell growth.

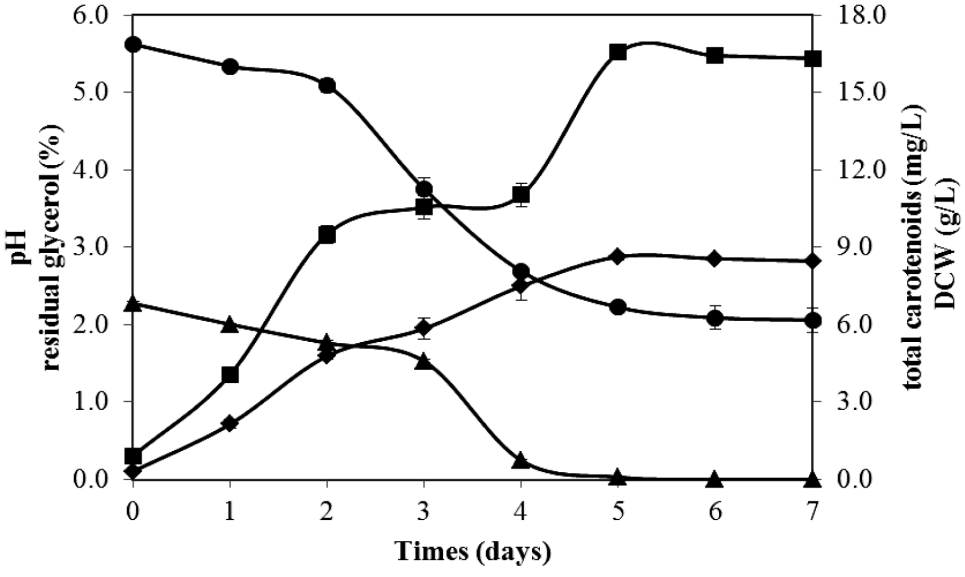

Figure 4. Time course of DCW (◆), residual glycerol (▲), pH (●) and total carotenoids (■) by *Sporobolomyces pararoseus* TISTR5213 under the optimal conditions.

3.5 Comparison of carotenoids production by *S. pararoseus* TISTR5213 with other red yeasts

A comparison of carotenoids production yield between *S. pararoseus* TISTR5213 and other red yeasts is shown in Table 9. The strain TISTR5213 used waste glycerol as a source of energy and exhibited relatively high carotenoids production. From the optimization experimental design data found that waste glycerol was important factor for carotenoids production in strain TISTR5213 which could produced total carotenoids of 16.55±0.16 mg/L at pH 5.64 and 23.90°C after 5 days. Compared to other red yeasts, strain TISTR5213 showed higher carotenoids production yield than *R. rubra* (10), *R. mucilaginosa* (25), *R. glutinis* DM28 (12) and *Sporidiobolus salmonicolor* (26). Recently, Yimyoo et al., 2011 (27) reported that *Rhodosporidium paludigenum* DMKU3-LPK4 could use pure glycerol as a sole carbon source. However, this strain showed a relatively low production yield of 3.42 mg/L when cultivated at pH 5.5 and 32°C.

3.6 The carotenoids production cost

From Table 10, the estimating cost of carotenoids production by *S. pararoseus* TISTR5213 in YM, BMP, BMW and optimized BMW were calculated. The BMW showed lower production cost than YM and BMP, respectively. In addition, the optimized BMW could enhance the production yield from 1.77 to 16.55 mg/L so the production cost was dramatically decreased from 8.56 to 1.09 Baht/mg/L or 685% reduction.

Table 9. Comparison of carotenoids production yield between *Sporobolomyces pararoseus* TISTR5213 and other red yeasts.

Microorganisms	Carbon sources	Conditions	Carotenoids production (mg/L)	References
Rhodotorula glutinis DM28	Fermented radish bine	pH 6.0, 30°C	0.20	(12)
R. glutinis	Glucose + peanut oil	pH 6.7, 28°C	13.43	(21)
R. glutinis	Hydrolyzed mung bean waste flour	pH 5.9, 30°C	3.48	(8)
R. glutinis	Sugar cane molass	pH 5.5, 20°C	3.46	(11)
R. rubra	Whey ultrafiltrate	pH 6.0, 30°C	10.20	(10)
R. mucilaginosa	Glucose	pH 5.0, 25°C	2.32	(25)
Rhodosporidium paludigenum DMKU3-LPK4	Glycerol	pH 5.5, 32°C	3.42	(27)
Sporidiobolus salmonicolor (CBS2636)	Glucose	pH 4.0, 25°C	1.02	(26)
S. pararoseus TISTR5213	Waste glycerol	pH 6.0, 23.9°C	16.55	This study

Table 10. Carotenoids production cost in various media.

Media	Cost of media (Baht/L)	Carotenoids production (mg/L)	Carotenoids production cost (Baht/mg/L)
YM	57.42	4.87 ± 0.08	12.08 ± 2.68
BMP	23.86	1.58 ± 0.44	15.69 ± 4.41
BMW	15.16	1.77 ± 0.02	8.56 ± 0.09
Optimized BMW	15.23	16.55 ± 0.16	1.09 ± 0.03

*Means and standard deviations of triplicate samples

4. Conclusion

The Plackett-Burman design and RSM were employed to enhance the carotenoids production by batch cultivation of *S. pararoseus* TISTR5213. Waste glycerol was good carbon source, and was considered to be a cost effective waste raw material for Thailand. Moreover, our study could reduce the amount of waste glycerol from biodiesel production process. The linear model was established using the Plackett-Burman design to select one medium component that exerted the highest influence on carotenoids production. The quadratic model generated by the CCD was also used to simulate the optimal conditions for high total carotenoids

production were; waste glycerol 34.00 g/L, initial pH at 5.64 and 23.90°C for 5 days. Total carotenoids production obtained from the optimized BMW was 16.55 mg/L, or 835% higher than total carotenoids obtained from un-optimized BMW. By application of an experimental design approach, the cost of total carotenoids production could be dramatically decreased and led to a maximum level of carotenoids production yield.

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