CHAPTER 3

Screening of oleaginous red yeast producing lipids and carotenoids and its optimization production by using crude glycerol as carbon source

3.1 Introduction

Crude glycerol is a main byproduct of biodiesel production process (Xu et al., 2012a). As the biodiesel production grows, the quantity of crude glycerol generated will be considerable, and it utilization will become an urgent topic (Thompson and He, 2006; Xu et al., 2012a). Moreover, crude glycerol has little value and disposal may be difficult, which purification of crude glycerol is industrially almost infeasible because of the high processing cost. In addition, crude glycerol is very low value because of the high impurities (Pachauri and He, 2006; Thompson and He, 2006). The impurities composition of crude glycerol are varied with the type of catalyst used, the transesterification efficiency, recovery efficiency of the biodiesel, other impurities in the feedstock, and type of alcohol used as well as the recovery of alcohol and catalyst (Yang et al., 2012). Moreover, crude glycerol obtained from transesterification contains about 50–60% by weight of glycerol and other impurities are 40–50% by weight (Gerpen, 2005; Isahak et al., 2010; Manowattana et al., 2012; Saenge et al., 2011). The methanol and alkali catalyst contents require crude glycerol to be treated as hazardous waste (Chatzifragkou et al., 2011a; Gerpen, 2005; Isahak et al., 2010). So, the impurities and other physico-chemical parameters can be important considerations in the intention of the bioconversion of crude glycerol to higher value products (Nicol et al., 2012).

Crude glycerol derived from biodiesel production has been interesting for biotransformation into high value chemical products through microbial fermentation. In terms of bioconversion methods for crude glycerol utilization, researcher has also

investigated the utility of oleaginous yeasts. So, oleaginous yeasts are considered as abundant sources of oil and fats because their cell membranes structures always contain lipids. These yeasts that can produce high lipids content or more than 200 mg/g of their biomass (20% by weight) are identified as "oleaginous" (Meng et al., 2009). Under favorable conditions, some oleaginous yeast can accumulate lipids up to 700 mg/g of their dry weight by consuming carbohydrates and hydrocarbons (Zhang et al., 2011). Moreover, many type of yeast can accumulate lipids and carotenoids in their cell, which is a group of "oleaginous red yeast". Thus carotenoids are yellow to orange-red pigments that are ubiquitous in nature (Waites et al., 2001). The most of these added carotenoids are chemically synthesized there is an increasing interest for carotenoids of biological origin because of the public concern over the safety of artificial food colorant (Iturriaga et al., 2005). The increasing interest in microbial sources of carotenoids is related to consumer preferences for natural additives and the potential cost effectiveness of creating carotenoids via microbial biotechnology. Moreover microbial production of carotenoids is an environmental friendly method compared to chemical production and able to meet the increasing demand of natural carotenoids (Das et al., 2007). The past of several years, many researchers found that crude glycerol could be used as the sole carbon source for carotenoids production by oleaginous red yeast (Saenge et al., 2011). Saenge et al. (2011) reported that the relatively high carotenoids production of 135.25 mg/L and highest lipids production of 6.05 g/L were obtained from *Rhodotorula glutinis* TISTR5159 by using crude glycerol as a carbon source. Moreover, crude glycerol from biodiesel production process could increase total carotenoids contents over than pure glycerol about 18.70% when cultured by *Sporobolomyces pararoseus* TISTR5213 (Manowattana et al., 2012).

Nowadays, crude glycerol is interesting as the sole carbon source for oleaginous red yeasts (Yang et al., 2012) because its price is very low (Fan et al., 2010; Nicol et al., 2012). Oleaginous red yeasts are the most efficiency microorganism that can use crude glycerol for theirs high growth rate and production of high value chemical metabolites. There are many reports involve in the usage of crude glycerol as the good feedstock for carotenoids and lipids productions. For examples, Kusdiyantini et al. (1998) revealed that pure glycerol is a potential carbon source for astaxanthin production from *Phaffia*

rhodoxyma. This red yeast produced astaxanthin up to 33.7 mg/L when the initial glycerol concentration of 37.8 g/L was used. While, Yimyoo et al. (2011) who found that the newly isolate *Rhodosporidium paludigenum* carotenoids production. Under optimal conditions, carotenoids production of strain DMKU3-LPK4 was 3.42 mg/L. Recently crude glycerol has been reported as a good carbon source for carotenoids production. Manowattana et al. (2012) reported that crude glycerol, an industrial waste obtained from biodiesel plant, was a good carbon source for carotenoids production from various types of red yeasts including *Rhodotorula rubra* TISTR5134, *Sporobolomyces* sp. TISTR5899 and *Xanthophyllomyces dendrorhous* TISTR5730. Additionally, Cutzu et al. (2013) reported that the mutant *Rhodotorula glutinis* 400A15 was a good biocatalyst of choice for the bioconversion of crude glycerol. The highest carotenoids production yield of 14.07 mg/L was obtained when the crude glycerol concentration hot higher than 7.5%. Similar to the report of Saenge et al. (2011) who found that the medium containing 9.5% of crude glycerol could enhance total carotenoids to 135.25 mg/L. Moreover, crude glycerol and glycerol were reported as a supplementary carbon source for β-carotene production by *Blakeslea trispora* (Mantzouridou et al., 2008) as well as astaxanthin and β-carotene productions by *Phaffia rhodozyma* and *Sporobolomyces roseus* (Ananda and Vadlani, 2010).

Optimization of medium composition using a traditional technique of "one-variable-at-a-time" is the time consuming and it does not include interactive effects among the variables. Additionally, using response surface methodology (RSM) can provide a statistical model which helps us to understand the interaction between variable factors at varying levels and to calculate the optimal level of each variable factor for a given target (Geiger, 1997; Saenge et al., 2011). Based on the optimization aspects mentioned above, in this study, the crude glycerol obtained from biodiesel production process was used as the sole carbon source for enhancing the production of lipids, β-carotene and total carotenoids by *Sporobolomyces pararoseus* TISTR5213 using Plackett-Burman screening methodology and RSM optimization applied in a central composite design (CCD).

3.2 Materials and methods

3.2.1 Microorganisms

Nine strains of red yeasts were kindly given by the culture collection section of the Thailand Institute of Scientific and Technological Research (TISTR). The strains were *Sporobolomyces* sp. TISTR5899, *Sporobolomyces pararoseus* TISTR5213, *Sporobolomyces shibatanus* TISTR5563, *Sporobolomyces nylandii* TISTR5581, *Rhodosporidium toruloides* TISTR5123, *Rhodotorula rubra* TISTR5134 and TISTR5158, *Dioszegia* sp. TISTR5792 and *Xanthophyllomyces dendrorhous* TISTR5730. All yeasts were maintained in glycerol stock at -20°C (Kusdiyantini et al. 1998).

3.2.2 Inoculum preparation

The yeast malt-extract medium for inoculum preparation contains the following composition (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 (Appendix A). The glycerol stocks (1.0 mL) of red yeast 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 (Maldonade et al., 2008). The starter culture with the OD_{600} of 1.0 was then 10.0% (v/v) inoculated to the basal medium by batch fermentation.

3.2.3 Raw materials

Crude glycerol obtained from the biodiesel production process was kindly given by the Energy Research and Development Institute (ERDI), Chiang Mai University, Thailand. It was transferred into a separating funnel and allowed to settle overnight. The upper layer was discarded while the lower layer was used as the crude glycerol. Methanol content in crude glycerol was removed to obtain demethanolized crude glycerol heating at 85°C for 1 h (Thompson and He 2006). The concentration of glycerol and the proximate analysis were explained in Appendix B. Crude glycerol composed of glycerol (56.30±0.62%) and other impurities of methanol (15.09±0.63%),

lipids content (10.85±0.14%), ash content (6.12±0.05%), moisture content (6.07±0.05%) and other component (by difference) (5.57±1.49%), respectively.

3.2.4 Screening of carotenoids and lipids producing oleaginous red yeasts

The carotenoids and lipids production of oleaginous red yeasts were screened in 2 conditions. The first condition was; cultivation in basal medium supplemented 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 0.25 g (Appendix A). The second condition was cultivated in basal medium by replacement pure glycerol with crude glycerol (BMC). The medium compositions were similarly to BMP medium, but pure glycerol was replaced by 20.0 g of crude glycerol. Batch cultures were performed in 250 mL Erlenmeyer flasks containing 50 mL of medium. The initial pH of each medium was adjusted to 6.0 by H_3PO_4 before sterilization (121°C for 15 min) and the carotenoids and lipids production were carried out by incubating in the incubator shaker (Kühner, Switzerland) at 25°C, with a shaking speed of 200 rpm for 5 days (Maldonade et al., 2008).

3.2.5 Effect of impurity in crude glycerol

Crude glycerol from biodiesel production process has high impurity (Chatzifragkou and Papanikolaou, 2012). Methanol is largely used for transesterification reaction. So, the unreacted methanol remains in the aqueous phase. Depending on separation process, methanol concentration in the aqueous phase is varied (Xu et al., 2012b). The effect of methanol concentration on the growth, carotenoids and lipids productions by *Sporobolomyces pararoseus* TISTR5213 was investigated by adding methanol into the basal medium supplemented with 34.0 g/L demethanolized crude glycerol to obtain the final concentration of methanol (0–10%) and cultivated under the optimum condition according to the previous study of Manowattana et al. (2012). The basal medium supplemented with 34.0 g/L of crude glycerol (without demethanolization) was used as the control experiment.

3.2.6 Screen of factors affecting carotenoids and lipids productions

The Plackett-Burman design was employed to screen for components of the basal medium that support the growth of *Sporobolomyces pararoseus* TISTR5213, β-carotene, total carotenoids and lipids productions yield. The eight components, codes and the levels of each component were evaluated to determine whether the key ingredients were significantly affected including; yeast extract (X_1, 0.2–0.5 g/L), demethanolized crude glycerol (X_2, 10–50 g/L), KH_2PO_4 (X_3, 2–10 g/L), $(NH_4)_2SO_4$ (X_4, 2–10 g/L), K_2HPO_4 (X_5, 1.5–7.5 g/L), $MgSO_4.7H_2O$ (X_6, 0.1–2.0 g/L), $MnSO_4.H_2O$ (X_7, 0.1–1.0 g/L) and NaCl (X_8, 0.1–2.0 g/L) as follow in Table 3.1. The Plackett-Burman design of each factor was examined at two levels: –1 as the lower level and +1 as the highest level and based on the first-order polynomial model as follows:

$$Y = \beta_0 \Sigma \beta i Xi \quad (1)$$

Where Y was the response of production yield, β_0 was the model intercept, βi was the linear coefficient, and Xi was the level of the independent variables.

This model did not describe interaction among those factors. It was used for screening and evaluating the important factors that influenced the response. The magnitude of the coefficient of positive or negative indicated the corresponding impact on the titer. The coefficient value approaches to zero, which implied small or no effect. The p-value was the probability that the coefficient results from a random process. A low p-value indicates a significant effect. The significance of each variable was determined by applying the F-value (Plackett and Burman, 1946).

Table 3.1 Experiment variables at various levels used in the lipids and carotenoids productions 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
Demethanolized crude 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. The dry cell weight (DCW) of *Sporobolomyces pararoseus* TISTR5213, β-carotene, total carotenoids and lipids productions yield were carried out in triplicate. The average of observed and predicted values by the equation models were shown as response *Y* (Table 3.4, 3.6, 3.8 and 3.10). Based on a regression analysis of the variables, a confidence level of 95% ($p<0.05$) for each factor was considered to have a significant effect on the DCW, β-carotene, total carotenoids and lipids productions. 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.

3.2.7 Optimization of significant variables using response surface methodology (RSM)

An experimental design using CCD was used to estimate the coefficients in a mathematical model, predict the response, and check the applicability of the model. The

pH and temperature factors were investigated with the one variable of demethanolized crude glycerol, which obtained from the Plackett-Burman design for the DCW, β-carotene, total carotenoids and lipids productions. The pH and temperature are most important environment parameters affecting cell growth, lipids, β-carotene and carotenoids productions. However, it brings the changes in many biosynthetic pathways (Bhosale, 2004; Saenge et al., 2011). 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 ±α, 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 contained also depends on certain properties required for the design. The CCD always contained twice as many star points as factors in the design. The star points represented 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 (Haaland, 1989). In this experimental design, the statistical software package Design Expert 6.0.10 was used in the design of the experiments, the analysis of the experimental data, and ingeneration of 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*-ratio:

$$Y = \beta_0 + \Sigma\beta iXi + \Sigma\beta iiXi^2 + \Sigma\beta ijXiXj \qquad (2)$$

Where *Y* represented the response variable (DCW, lipids, β-carotene or total carotenoids production yield), β_0 was the interception coefficient, βi the coefficient for the linear effect, βii the ij^{th} coefficient of the interaction effect, and *XiXj* were input variables that influence the response variable *Y*. The response variable in each trial was the average of the three replicates.

pH and temperature were the most important conditions on the DCW, lipids, β-carotene and total carotenoids production yield. The pH and temperature are considered to be the main physical factor directly controlling growth rate thus plays an

important role in the biosynthesis of lipids and carotenoids in microorganism (Bhosale, 2004; Frengova and Beshkova, 2009). In general, the effects of pH and temperature on the cell growth, lipids, β-carotene and carotenoids accumulations vary with the different microorganisms, medium composition and operation conditions (Hu et al., 2006). A range of pH level in this study between 5–7 with a boundary of 4–8 for ±α and temperature between 20–30°C with the boundary of 15–35 for ±α were selected in the experimental design. The CCD consisted of 3 center point and 14 non-center points. The experiments consisted of 17 runs with no blocking and the design matrix is provided in Table 3.2.

Table 3.2 Experimental codes, ranges and levels of independent variables in the response surface methodology experiment

Variables	**Units**	**Symbol codes**	**Levels**				
			-α	**Low**	**Center**	**High**	**+α**
Demethanolized crude glycerol	g/L	X_2	19.77	30.0	45.0	60.0	70.23
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

3.2.8 Analytical methods

Carotenoids analysis

Ten milliliters of 5 days-olds culture broth was taken from each flask and centrifuged at 6,000 rpm (4,146 g) at 4°C for 10 min (Hettich MIKRO 22R; Germany). The cell pellet was washed twice with *n*-hexane (LabScan, Thailand) and once with distilled water. The β-carotene content of cell pellet was extracted by a method which ruptured the yeast cell, carried out in screw cap tube (25×150 mm), containing 10.0 mL acetone (Merck, Germany) and glass beads (4.0 g, 3 mm-diam.; Superior, Germany). The mixture was vigorously shaken in a vortex mixer for 15 min in the presence of 100 ppm ascorbic acid (Sigma, USA). The ruptured cell was centrifuged at 6,000 rpm (4,146 *g*) at 4°C for 10 min and the clear supernatant was collected and dried by

flushing it with N_2, then re-dissolved in 1.0 mL *n*-hexane (Manowattana et al., 2012). The extract was filtered through a nylon membrane filter (0.2 μm, FilTrex, USA) and subjected to a high performance liquid chromatography (HPLC) analysis. A modified method of Wang et al. (2007), HPLC analysis was performed on the analytical HPLC (Shimadzu, Japan) equipped with C18 column (4.6 mm × 250 mm; 5μm, Restek, USA). 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 β-carotene and total carotenoids content were detected by UV-VIS detector (SPD-10A VP; Shimadzu, Japan), which operated at 454 nm; in a linear gradient for 45 min, maintaining this proportion until the end of the run (Wang et al., 2007). The retention time of β-carotene extracted from *Sporobolomyces pararoseus* TISTR5213 was 37.86 min and quantified against peak area calibrations calculated from the standard curves of the β-carotene standard (Sigma, USA), as mg of equivalent to β-carotene/L of culture broth (Appendix C).

Lipids extraction

Extraction of lipids from biomass was performed according to the modified method of Bligh and Dyer (1959). Lipids were extracted with a mixture of chloroform and methanol (2:1, v/v) for 1 h. The ruptured cell and extracted lipids were centrifuged at 6,000 rpm (4,146 *g*) at 4°C for 10 min, and the clear supernatant was collected and removed by evaporation under vacuum using a rotary evaporator (Rotavapor R-3; Buchi, Japan) (Appendix D). Lipids content was expressed in the volumetric of the extracted lipids in culture broth (g/L), and in the percentage of the extracted lipids in relation to the dried biomass (% g/g).

Biomass measurement

Dry cell weight (DCW) of each flask was collected from 5 day olds cultivation broth, then centrifuged at 6,000 rpm (4,146 *g*) at 4°C for 10 min and washed twice as described above before drying at 80°C overnight and then transferred to desiccators until a constant weight was obtained (Manowattana et al., 2012).

Statistical analysis

All experiments were carried out as triplicate samples. The data were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range Test (p<0.05). The statistical software package SPSS v.17 was used in the analysis of the experimental data.

3.3 Results and discussion

3.3.1 Screening of carotenoids and lipids producing oleaginous red yeasts

The results revealed that nine strains of red yeasts could grow and produce the high value chemicals (β-carotene, total carotenoids and lipids) in basal medium supplemented with pure glycerol (BMP) and basal medium supplemented with crude glycerol (BMC). The evidence from Table 3.3 shows that the red yeast *Sporobolomyces pararoseus* TISTR5213 showed the highest β-carotene, total carotenoids and lipids production yield of 0.62±0.05 mg/L, 1.72±0.16 mg/L and 1.59±0.01 g/L in BMP and 1.17±0.01 mg/L, 1.77±0.01 mg/L and 2.05±0.02 g/L in BMC, respectively. In comparison, BMC increased the β-carotene, total carotenoids and lipids content over BMP by approximately 47.00, 2.82 and 22.44%, respectively. Crude glycerol could enhance lipids, β-carotene and carotenoids synthesis because it contained trace elements e.g. Ca, K, Mg, Na, P, and S in term of ash content (Thompson and He, 2006). The percentage of ash content in crude glycerol used in this study was 6.12±0.05%. Trace elements have been demonstrated to act as stimulants for growth of oleaginous red yeasts, which had a stimulatory effect on lipids and carotenoids synthesis. The observed effect of trace elements on the biosynthesis of specific lipids and carotenoids in oleaginous red yeasts may be explained by an activation or inhibition mechanism by selected metal ions on specific lipogenesis and carotenogenic enzymes, in particular, on specificity of the desaturases which are involved in β-carotene, other carotenoids and lipids biosynthesis (Frengova and Beshkova, 2009).

Table 3.3 DCW, β-carotene, total carotenoids and lipids productions yield of nine red yeasts cultivated in pure glycerol (BMP) and crude glycerol (BMC)

Yeast strain	Pure glycerol				Crude glycerol			
	DCW (g/L)	β-carotene (mg/L)	Total carotenoids (mg/L)	Lipids (g/L)	DCW (g/L)	β-carotene (mg/L)	Total carotenoids (mg/L)	Lipids (g/L)
TISTR5123	6.17 ± 0.10^{c*}	0.18 ± 0.02^{b}	0.48±0.04^{d}	1.36±0.01^{b}	3.59 ± 0.10^{a}	0.41 ± 0.05^{c}	0.59±0.06^{c}	1.68±0.02^{d}
TISTR5134	7.60 ± 0.44^{b}	0.17 ± 0.01^{b}	0.37±0.01^{d}	1.22±0.02^{c}	3.84 ± 0.46^{a}	0.25 ± 0.02^{e}	0.35±0.02^{f}	1.78±0.02^{bc}
TISTR5158	8.24 ± 0.40^{a}	0.41± 0.06^{ab}	0.48±0.05^{d}	0.49±0.00^{f}	3.47 ± 0.21^{ab}	0.35 ± 0.03^{cd}	0.56±0.04^{cd}	1.80±0.02^{b}
TISTR5213	5.73 ± 0.18^{c}	0.62 ± 0.05^{a}	1.72±0.16^{a}	1.59±0.01^{a}	3.49 ± 0.39^{ab}	1.17 ± 0.01^{a}	1.77±0.01^{a}	2.05±0.02^{a}
TISTR5563	2.98 ± 0.08^{d}	0.67 ± 0.04^{a}	1.04±0.02^{b}	0.21±0.00^{g}	2.89 ± 0.30^{bc}	0.32 ± 0.04^{d}	0.49±0.01^{e}	1.82±0.02^{b}
TISTR5581	7.50 ± 0.36^{b}	0.71 ± 0.05^{a}	1.09±0.05^{b}	0.60±0.00^{ef}	2.92 ± 0.46^{bc}	0.94 ± 0.04^{b}	1.36±0.05^{b}	1.74±0.04^{c}
TISTR5730	6.98 ± 0.40^{b}	0.37 ± 0.05^{ab}	0.69±0.04^{c}	0.96±0.01^{d}	2.73 ± 0.20^{c}	0.12 ± 0.02^{f}	0.32±0.01^{f}	1.25±0.01^{f}
TISTR5792	8.40 ± 0.67^{a}	0.04 ± 0.01^{b}	0.16±0.01^{e}	0.71±0.02^{e}	2.48 ± 0.41^{c}	0.11 ± 0.00^{f}	0.20±0.01^{g}	1.43±0.03^{e}
TISTR5899	5.92 ± 0.27^{c}	0.19 ± 0.01^{b}	0.38±0.02^{d}	1.18±0.20^{c}	3.42 ± 0.39^{ab}	0.33± 0.01^{d}	0.52±0.02^{de}	1.01±0.04^{d}

*Means and standard deviations of triplicate samples

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

3.3.2 Effect of methanol in crude glycerol on lipids and carotenoids productions from *Spolobomyces pararoseus* TISTR5213

Crude glycerol from biodiesel production plant usually consists of glycerol and many chemical substances including water, salts, methanol, and traces of tri-, di- and monoacylglycerols (Hájek and Skopal, 2009). Methanol is largely used as the alcohol for producing biodiesel by transesterification reaction because of not expensive alcohol and good reactivity in the alkaline synthesis reaction (Chatzifragkou and Papanikolaou, 2012). After transesterification, methanol is present in glycerol phase at around 15–40% (w/w) (Hájek and Skopal, 2009; Manowattana et al., 2012; Thompson and He, 2006; Xu et al., 2012b). The crude glycerol used in this study composed of glycerol (56.30±0.62% w/w), methanol (15.09±0.63% w/w), lipids content (10.85±0.14% w/w), ash content (6.12±0.05% w/w), moisture content (6.07±0.05% w/w) and other components (5.57±1.49% w/w).

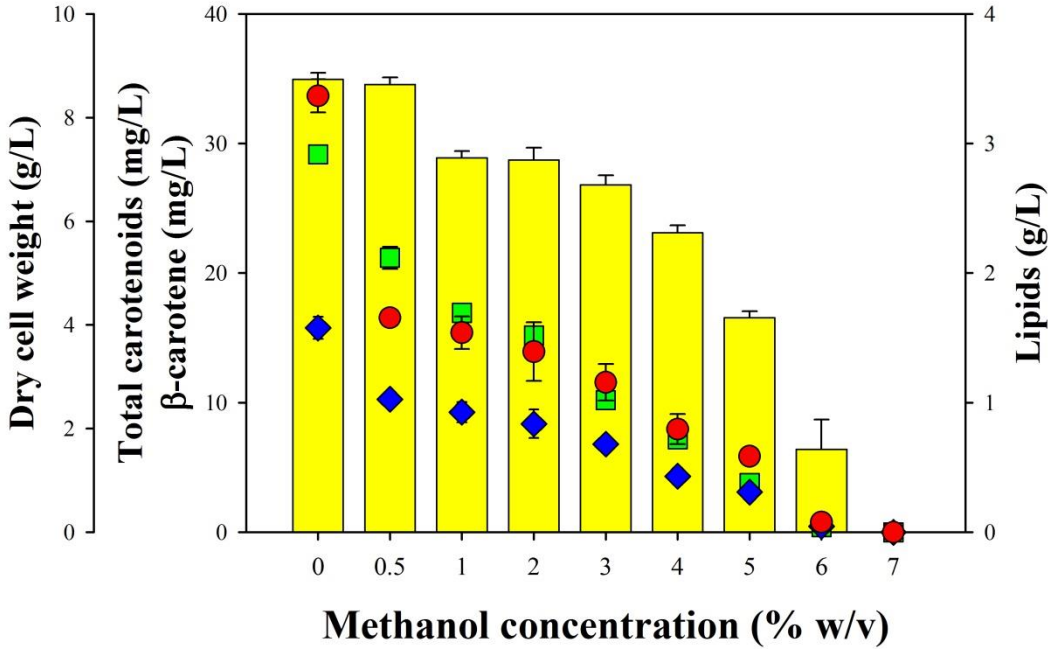

Figure 3.1 The effect of methanol on dry cell weight (bar), β-carotene (■), total carotenoids (●) and lipids (◆) productions of *Sporobolomyces pararoseus* TISTR5213

The effect of methanol on DCW, lipids, β-carotene and total carotenoids productions of strain KM281507 was studied by adding the pure methanol (0–1.0% w/v) into the basal medium supplemented with 34.0 g/L of either crude glycerol or demethanolized crude glycerol (Manowattana et al., 2012). The lipids, β-carotene and total carotenoids productions in the control experiment (basal medium) supplemented with crude glycerol (0.5% w/w of methanol) were 1.69±0.09 g/L, 10.26±0.32 mg/L and 16.55±0.16 mg/L, respectively. However, after the removal of methanol from the crude glycerol (demethanolized crude glycerol), the yeast biomass was 8.64±0.13 g/L obtained from crude glycerol did not significant different from the demethanolized crude glycerol (8.74±0.12 g/L). While, lipids, β-carotene and total carotenoids were significantly (p<0.05) enhanced up to 2.92±0.03 g/L, 25.76±0.85 mg/L and 33.67±1.28 mg/L, respectively. As the results from Figure 3.1, it was observed that the maximum production of those metabolites was achieved when demethanolized crude glycerol was used as a sole carbon (C) source. Even oleaginous yeast strain KM281507 could grow in the presence of methanol, it was observed that this strain could not grow well under the presence of methanol exceed 1.0% (w/v) and completely stopped the growth at 6.0% (w/v) methanol.

The capability of tolerating methanol concentrations dependent on the different microorganism (Chatzifragkou and Papanikolaou, 2012). Effect of methanol on growth and metabolite productions of various oleaginous red yeasts were studied by adding methanol into the media. For example, methanol concentration of 0.8% (w/v) did not showed significantly effect on the biomass and lipids formation by *Rhodosporidium toruloides* Y4. The biomass and lipids were slightly decreased only 5.4 and 6.9%, respectively when compared to the absence of methanol condition (Yang et al., 2014). Moreover, Xu et al. (2012b) reported that the biomass and lipids yield of Rhodosporidium toruloides decreased by 5.0 and 23.0%, respectively, under the presence of methanol (0.8% w/w). Methanol inhibited the cell growth, lipids and carotenoids productions of strain KM281507. It probably that the alteration of the fluidity of cell membranes by the methanol (Yang et al., 2014). Thus, demethanolized crude glycerol was the best choice of C source for the cell growth, lipogenesis and carotenogenesis by *Sporidiobolus pararoseus* KM281507.

3.3.3 Screening of significant variables using the Plackett-Burman design

The results of Plackett-Burman experimental design of DCW are presented in Table 3.4. Different medium components at various concentrations were investigated. An evaluation of the fit of the model using, the proximate correlation coefficient (R^2) to 1.0 indicated better fitting of the predicted values from the equation to the experimental values. From Table 3.4, it shows the values of DCW generated using the predicted model. The standard order of condition number 6 gave the highest yield of DCW, which was 11.78 g/L. The following first-order polynomial equation for biomass production in term of DCW of *Sporidiobolus pararoseus* TISTR5213:

$$Y\ (\text{DCW}) = -\ 0.07 + 0.37X_1 + 0.13X_2 + 0.12X_3 - 0.16X_4 + 0.27X_5 + 0.24X_6 - 1.19X_7 - 0.06X_8 \quad (3)$$

Table 3.4 Twelve-trial Plackett-Burman design matrixes for eight variables and the predicted DCW

STD order	X_1*	X_2	X_3	X_4	X_5	X_6	X_7	X_8	DCW (g/L)	
									Actual	Predicted
1	5.0	10.0	10.0	2.0	1.5	0.1	1.0	2.0	2.67	3.03
2	5.0	50.0	2.0	10.0	1.5	0.1	0.1	2.0	6.50	6.99
3	0.2	50.0	10.0	2.0	7.5	0.1	0.1	0.1	8.76	9.22
4	5.0	10.0	10.0	10.0	1.5	2.0	0.1	0.1	3.77	3.38
5	5.0	50.0	2.0	10.0	7.5	0.1	1.0	0.1	8.13	7.64
6	5.0	50.0	10.0	2.0	7.5	2.0	0.1	2.0	11.78	11.32
7	0.2	50.0	10.0	10.0	1.5	2.0	1.0	0.1	5.29	5.68
8	0.2	10.0	10.0	10.0	7.5	0.1	1.0	2.0	1.95	1.59
9	0.2	10.0	2.0	10.0	7.5	2.0	0.1	2.0	1.81	2.17
10	5.0	10.0	2.0	2.0	7.5	2.0	1.0	0.1	3.78	4.27
11	0.2	50.0	2.0	2.0	1.5	2.0	1.0	2.0	6.31	5.92
12	0.2	10.0	2.0	2.0	1.5	0.1	0.1	0.1	1.96	1.50

*X_1 Yeast extract (g/L); X_2 Demethanolized crude glycerol (g/L); X_3 KH_2PO_4 (g/L); X_4 $(NH_4)_2SO_4$ (g/L); X_5 K_2HPO_4 (g/L); X_6 $MgSO_4.7H_2O$ (g/L); X_7 $MnSO_4.H_2O$ (g/L); X_8 NaCl (g/L)

The R-square (R^2) value of DCW was 98.02% 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 DCW of *Sporobolomyces pararoseus* TISTR5213. The greater magnitude of the coefficient indicated that, a large effect on the response. Variables at confidence levels greater than 95% ($p<0.05$) were considered significant. Table 3.5 shows that the factors with *p*-values less than 0.05 were considered to have significant effects on DCW. The yeast extract, demethanolized crude glycerol and K_2HPO_4 were significantly with the confidence level of 95% on DCW.

Table 3.5 Estimated effects, linear regression coefficients and corresponding *F*-ratio and *p*-values for the DCW for eight variables using the Plackett-Burman experiment design

Source	**DCW (g/L)**			
	Effect	**Coefficient**	***F*-values**	***p*-values**
Main Effects		5.226	18.550	0.018[a]
X_1*	1.758	0.879	12.710	0.038[a]
X_2	5.138	2.569	108.550	0.002[a]
X_3	0.955	0.478	3.750	0.148[b]
X_4	-1.302	-0.651	6.970	0.078[b]
X_5	1.618	0.809	10.770	0.046[a]
X_6	0.462	0.231	0.880	0.418[b]
X_7	-1.075	-0.538	4.750	0.117[b]
X_8	-0.112	-0.056	0.050	0.835[b]
R^2	98.02%			

*X_1 Yeast extract (g/L); X_2 Demethanolized crude glycerol (g/L); X_3 KH_2PO_4 (g/L); X_4 $(NH_4)_2SO_4$ (g/L); X_5 K_2HPO_4 (g/L); X_6 $MgSO_4.7H_2O$ (g/L); X_7 $MnSO_4.H_2O$ (g/L); X_8 NaCl (g/L)

[a]significant at $p<0.05$, [b]not significant at $p<0.05$

The results of Plackett-Burman experimental design of lipids are presented in Table 3.6. Different medium components at various concentrations were investigated. An evaluation of the fit of the model using, the proximate correlation coefficient (R^2) to 1.0 indicated better fitting of the predicted values from the equation to the experimental values. From Table 3.6 shows that the information of values of lipids generated using the predicted model. The standard order of condition number 6 gave the highest yield of lipids, which was 1.96 g/L. The following first-order polynomial equation for lipids of *Sporidiobolus pararoseus* TISTR5213:

$$Y \text{ (lipids)} = -0.01 + 0.01X_1 + 0.02X_2 + 0.01X_3 - 0.02X_4 + 0.01X_5 + 0.14X_6 + 0.02X_7 + 0.25X_8 \quad (4)$$

Table 3.6 Twelve-trial Plackett-Burman design matrixes for eight variables and the predicted lipids production yield

STD order	X_1*	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Lipids (g/L)	
									Actual	Predicted
1	5.0	10.0	10.0	2.0	1.5	0.1	1.0	2.0	0.59	0.81
2	5.0	50.0	2.0	10.0	1.5	0.1	0.1	2.0	1.69	1.36
3	0.2	50.0	10.0	2.0	7.5	0.1	0.1	0.1	1.10	1.13
4	5.0	10.0	10.0	10.0	1.5	2.0	0.1	0.1	0.27	0.41
5	5.0	50.0	2.0	10.0	7.5	0.1	1.0	0.1	0.64	0.97
6	5.0	50.0	10.0	2.0	7.5	2.0	0.1	2.0	1.96	1.93
7	0.2	50.0	10.0	10.0	1.5	2.0	1.0	0.1	1.31	1.17
8	0.2	10.0	10.0	10.0	7.5	0.1	1.0	2.0	0.86	0.64
9	0.2	10.0	2.0	10.0	7.5	2.0	0.1	2.0	0.61	0.83
10	5.0	10.0	2.0	2.0	7.5	2.0	1.0	0.1	0.96	0.63
11	0.2	50.0	2.0	2.0	1.5	2.0	1.0	2.0	1.62	1.76
12	0.2	10.0	2.0	2.0	1.5	0.1	0.1	0.1	0.25	0.22

*X_1 Yeast extract (g/L); X_2 Demethanolized crude glycerol (g/L); X_3 KH_2PO_4 (g/L); X_4 $(NH_4)_2SO_4$ (g/L); X_5 K_2HPO_4 (g/L); X_6 $MgSO_4.7H_2O$ (g/L); X_7 $MnSO_4.H_2O$ (g/L); X_8 NaCl (g/L)

The R^2 value of lipids was 84.13% 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 lipids of *Sporobolomyces pararoseus* TISTR5213. The greater magnitude of the coefficient indicated that, a large effect on the response. Variables at confidence levels greater than 95% ($p<0.05$) were considered significant. Table 3.7 shows that the factors with *p*-values less than 0.05 were considered to have significant effects on lipids. Demethanolized crude glycerol was determined to be the only one significant factor ($p<0.05$), with the confidence level of 95%. Although, the *F*-value showed that demethanolized crude glycerol showed positive effects on lipids.

Table 3.7 Estimated effects, linear regression coefficients and corresponding *F*-ratio and *p*-values for the lipids production yield for eight variables using the Plackett-Burman experiment design

Source	**Lipids (g/L)**			
	Effect	**Coefficient**	***F*-values**	***p*-values**
Main Effects		0.988	1.990	0.309[b]
X_1*	0.060	0.030	0.060	0.824[b]
X_2	0.797	0.398	10.420	0.048[a]
X_3	0.053	0.027	0.050	0.843[b]
X_4	-0.183	-0.092	0.550	0.511[b]
X_5	0.067	0.033	0.070	0.805[b]
X_6	0.267	0.133	1.170	0.359[b]
X_7	0.017	0.008	0.000	0.950[b]
X_8	0.467	0.233	3.580	0.155[b]
R^2	84.13%			

*X_1 Yeast extract (g/L); X_2 Demethanolized crude glycerol (g/L); X_3 KH_2PO_4 (g/L); X_4 $(NH_4)_2SO_4$ (g/L); X_5 K_2HPO_4 (g/L); X_6 $MgSO_4.7H_2O$ (g/L); X_7 $MnSO_4.H_2O$ (g/L); X_8 NaCl (g/L)

[a]significant at $p<0.05$, [b]not significant at $p<0.05$

The results of Plackett-Burman experimental design of β-carotene are presented in Table 3.8. Different medium components at various concentrations were investigated. An evaluation of the fit of the model using, the proximate correlation coefficient (R^2) to 1.0 indicated better fitting of the predicted values from the equation to the experimental values. From Table 3.8 shows that the information of values of β-carotene generated using the predicted model. The standard order of condition number 6 gave the highest yield of β-carotene, which was 5.98 mg/L. The following first-order polynomial equation for β-carotene of *Sporidiobolus pararoseus* TISTR5213:

$$Y\ (\beta\text{-carotene}) = -0.69 + 0.29X_1 + 0.04X_2 + 0.17X_3 - 0.10X_4 + 0.07X_5 + 0.33X_6 - 0.37X_7 - 0.07X_8 \quad (5)$$

Table 3.8 Twelve-trial Plackett-Burman design matrixes for eight variables and the predicted β-carotene production yields

STD order	X_1*	X_2	X_3	X_4	X_5	X_6	X_7	X_8	β-carotene (mg/L)	
									Actual	Predicted
1	5.0	10.0	10.0	2.0	1.5	0.1	1.0	2.0	2.21	2.29
2	5.0	50.0	2.0	10.0	1.5	0.1	0.1	2.0	0.98	2.14
3	0.2	50.0	10.0	2.0	7.5	0.1	0.1	0.1	2.94	3.53
4	5.0	10.0	10.0	10.0	1.5	2.0	0.1	0.1	3.21	2.55
5	5.0	50.0	2.0	10.0	7.5	0.1	1.0	0.1	3.54	2.38
6	5.0	50.0	10.0	2.0	7.5	2.0	0.1	2.0	5.98	5.40
7	0.2	50.0	10.0	10.0	1.5	2.0	1.0	0.1	1.90	2.56
8	0.2	10.0	10.0	10.0	7.5	0.1	1.0	2.0	0.61	0.53
9	0.2	10.0	2.0	10.0	7.5	2.0	0.1	2.0	0.05	0.13
10	5.0	10.0	2.0	2.0	7.5	2.0	1.0	0.1	0.97	2.13
11	0.2	50.0	2.0	2.0	1.5	2.0	1.0	2.0	2.56	1.90
12	0.2	10.0	2.0	2.0	1.5	0.1	0.1	0.1	0.61	0.02

*X_1 Yeast extract (g/L); X_2 Demethanolized crude glycerol (g/L); X_3 KH_2PO_4 (g/L); X_4 $(NH_4)_2SO_4$ (g/L); X_5 K_2HPO_4 (g/L); X_6 $MgSO_4.7H_2O$ (g/L); X_7 $MnSO_4.H_2O$ (g/L); X_8 NaCl (g/L)

The R^2 value of β-carotene was 79.03% 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 β-carotene of *Sporobolomyces pararoseus* TISTR5213. The greater magnitude of the coefficient indicated that, a large effect on the response. Variables at confidence levels greater than 85% (p<0.15) were considered significant. Table 3.9 shows that the factors with *p*-values less than 0.15 were considered to have significant effects on β-carotene. Demethanolized crude glycerol was determined to be the only one significant factor (p<0.15), with the confidence level of 85%. Although, the *F*-value showed that demethanolized crude glycerol showed positive effects on β-carotene.

Table 3.9 Estimated effects, linear regression coefficients and corresponding *F*-ratio and *p*-values for the β-carotene production yield for eight variables using the Plackett-Burman experiment design

Source	**β-carotene (mg/L)**			
	Effect	**Coefficient**	***F*-values**	***p*-values**
Main Effects		2.130	1.410	0.426[b]
X_1*	1.370	0.685	2.640	0.203[b]
X_2	1.707	0.853	4.100	0.136[a]
X_3	1.357	0.678	2.590	0.206[b]
X_4	-0.830	-0.415	0.970	0.397[b]
X_5	0.437	0.218	0.270	0.640[b]
X_6	0.630	0.315	0.560	0.509[b]
X_7	-0.330	-0.165	0.150	0.722[b]
X_8	-0.130	-0.065	0.020	0.887[b]
R^2	79.03%			

*X_1 Yeast extract (g/L); X_2 Demethanolized crude glycerol (g/L); X_3 KH_2PO_4 (g/L); X_4 $(NH_4)_2SO_4$ (g/L); X_5 K_2HPO_4 (g/L); X_6 $MgSO_4.7H_2O$ (g/L); X_7 $MnSO_4.H_2O$ (g/L); X_8 NaCl (g/L)

[a]significant at p<0.15, [b]not significant at p<0.15

The results of Plackett-Burman experimental design of total carotenoids are presented in Table 3.10. Different medium components at various concentrations were investigated. An evaluation of the fit of the model using, the proximate correlation coefficient (R^2) to 1.0 indicated better fitting of the predicted values from the equation to the experimental values. From Table 3.10 shows that the information of values of total carotenoids generated using the predicted model. The standard order of condition number 6 gave the highest yield of total carotenoids, which was 15.68 mg/L. The following first-order polynomial equation for total carotenoids of *Sporidiobolus pararoseus* TISTR5213:

$$Y \text{ (total carotenoids)} = 2.51 - 0.16X_1 + 0.19X_2 + 0.17X_3 - 0.22X_4 - 0.46X_5 + 2.31X_6 + 1.45X_7 - 0.92X_8 \quad (6)$$

Table 3.10 Twelve-trial Plackett-Burman design matrixes for eight variables and the predicted total carotenoids production yields

STD order	X_1*	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Total carotenoids (mg/L)	
									Actual	Predicted
1	5.0	10.0	10.0	2.0	1.5	0.1	1.0	2.0	2.45	4.04
2	5.0	50.0	2.0	10.0	1.5	0.1	0.1	2.0	9.37	7.18
3	0.2	50.0	10.0	2.0	7.5	0.1	0.1	0.1	11.85	10.11
4	5.0	10.0	10.0	10.0	1.5	2.0	0.1	0.1	8.27	7.14
5	5.0	50.0	2.0	10.0	7.5	0.1	1.0	0.1	5.29	7.48
6	5.0	50.0	10.0	2.0	7.5	2.0	0.1	2.0	10.22	11.96
7	0.2	50.0	10.0	10.0	1.5	2.0	1.0	0.1	15.68	16.81
8	0.2	10.0	10.0	10.0	7.5	0.1	1.0	2.0	1.92	0.33
9	0.2	10.0	2.0	10.0	7.5	2.0	0.1	2.0	0.44	2.03
10	5.0	10.0	2.0	2.0	7.5	2.0	1.0	0.1	8.22	6.03
11	0.2	50.0	2.0	2.0	1.5	2.0	1.0	2.0	16.54	15.41
12	0.2	10.0	2.0	2.0	1.5	0.1	0.1	0.1	2.14	3.88

*X_1 Yeast extract (g/L); X_2 Demethanolized crude glycerol (g/L); X_3 KH_2PO_4 (g/L); X_4 $(NH_4)_2SO_4$ (g/L); X_5 K_2HPO_4 (g/L); X_6 $MgSO_4.7H_2O$ (g/L); X_7 $MnSO_4.H_2O$ (g/L); X_8 NaCl (g/L)

The R^2 value of total carotenoids was 89.07% 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 total carotenoids of *Sporobolomyces pararoseus* TISTR5213. The greater magnitude of the coefficient indicated that, a large effect on the response. Variables at confidence levels greater than 95% ($p<0.05$) were considered significant. Table 3.11 shows that the factors with *p*-values less than 0.05 were considered to have significant effects on total carotenoids. Demethanolized crude glycerol was determined to be the only one significant factor ($p<0.05$), with the confidence level of 95%. Although, the *F*-value showed that demethanolized crude glycerol showed positive effects on total carotenoids.

Table 3.11 Estimated effects, linear regression coefficients and corresponding *F*-ratio and *p*-values for the total carotenoids production yield for eight variables using the Plackett-Burman experiment design

Source	**Total carotenoids (mg/L)**			
	Effect	**Coefficient**	***F*-values**	***p*-values**
Main Effects		7.699	3.060	0.194[b]
X_1*	-0.792	-0.396	0.160	0.715[b]
X_2	7.585	3.793	14.850	0.031[a]
X_3	1.398	0.699	0.500	0.529[b]
X_4	-1.742	-0.871	0.780	0.441[b]
X_5	-2.752	-1.376	1.950	0.257[b]
X_6	4.392	2.196	4.980	0.112[b]
X_7	1.302	0.651	0.440	0.556[b]
X_8	-1.752	-0.876	0.790	0.439[b]
R^2	89.07%			

*X_1 Yeast extract (g/L); X_2 Demethanolized crude glycerol (g/L); X_3 KH_2PO_4 (g/L); X_4 $(NH_4)_2SO_4$ (g/L); X_5 K_2HPO_4 (g/L); X_6 $MgSO_4.7H_2O$ (g/L); X_7 $MnSO_4.H_2O$ (g/L); X_8 NaCl (g/L)

Demethanolized crude glycerol was only one factor significant for lipids, β-carotene and total carotenoids productions. Moreover, the response of DCW found that demethanolized crude glycerol was only one factor significant and related with all of product. Therefore, to maximize DCW, lipids, β-carotene and total carotenoids productions, the concentration of demethanolized crude glycerol should be shifted the levels for higher RSM via the CCD.

3.3.4 Optimization of significant variables using response surface methodology (RSM)

Response surface methodology (RSM) designs are usually used to obtain precise information about factor effects including magnitude and direction. From the previous section (3.3.3) found that demethanolized crude glycerol could serve as only one factor affected on lipids, β-carotene and total carotenoids productions by *Sporobolomyces pararoseus* TISTR5213. The reason might be it contained some trace elements as already mentioned in the previous section. For RSM designs, three main factors, namely demethanolized crude glycerol concentration, pH and temperature, were selected for the optimization of DCW, lipids, β-carotene and total carotenoids productions. The CCD experiment led to a total 17 sets of experiments with no blocking. The low, center and high levels of each variable and the experimental design and respective experimental results are shown in Table 3.12, 3.14, 3.16 and 3.18 for DCW, lipids, β-carotene and total carotenoids productions, respectively.

Table 3.12 The CCD matrixes for the experiment design and predicted responses of DCW

STD order	Variables codes			DCW (g/L)	
	X_2*	X_9	X_{10}	Actual	Predicted
1	30.00	5.00	20.00	6.30	6.40
2	60.00	5.00	20.00	7.67	7.97
3	30.00	7.00	20.00	14.28	12.62
4	60.00	7.00	20.00	11.30	11.60
5	30.00	5.00	30.00	4.92	3.71
6	60.00	5.00	30.00	7.46	8.21
7	30.00	7.00	30.00	13.61	12.40
8	60.00	7.00	30.00	15.31	14.30
9	19.77	6.00	25.00	4.99	6.92
10	70.23	6.00	25.00	10.49	9.85
11	45.00	4.32	25.00	8.31	7.91
12	45.00	7.68	25.00	16.58	18.27
13	45.00	6.00	16.59	7.13	7.26
14	45.00	6.00	33.41	6.12	7.27
15	45.00	6.00	25.00	8.02	8.40
16	45.00	6.00	25.00	8.86	8.40
17	45.00	6.00	25.00	8.54	8.40

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

Three main factors, namely demethanolized crude glycerol concentration, pH and temperature, were selected for the optimization of DCW. The CCD experiment led to a total 17 runs of experiments with no blocking. The low, center and high levels of each variable and the experimental design and respective experimental results (actual and predicted values) are shown in Table 3.12. The strain TISTR5213 was cultivated in 17 different conditions to obtain the maximum DCW production yield in batch cultivation using 250 mL Erlenmeyer flasks containing 50 mL of medium with 5 days cultivation period. The seed culture was 10% (v/v) inoculated to the batches fermentation. The results were obtained by the CCD were analyzed by ANOVA (Table 3.13). The CCD generated a quadratic equation for DCW production (Y) as a function of demethanolized crude glycerol concentration (X_2), pH (X_9) and temperature (X_{10}) are given as follows:

$$Y\,(\mathrm{DCW}) = -8.40 + 0.87X_2 + 3.08X_9 + 0.00X_{10} - 0.01X_2^2 + 1.66X_9^2 - 0.40X_{10}^2 - 0.65X_2X_9 + 0.73X_2X_{10} + 0.62\,X_9X_{10} \quad (7)$$

As shown in Table 3.12, the run orders of 3, 8 and 12 enhanced DCW to the levels of 14.28, 15.31 and 16.58 g/L, respectively. The quadratic mathematic model in equation 7 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_2, and X_2^2 were significant as shown in Table 3.13.

In Table 3.13, the probability *p*-values of the model was relatively low (0.0040), indicating a significant model. The coefficient of variation for the model (R^2 = 0.9218) were represented.

From Figure 3.2, it is obvious that DCW was strongly affected by variable of pH value. the DCW was increased from 8.31 g/L at lowest point to 8.86 g/L at the center point to 16.58 g/L at high point of pH value, respectively.

Table 3.13 Analysis of variance (ANOVA) of the quadratic model for response variables. The probability values (*p*-values) of parameter and egression of estimated coefficients of the second order polynomial for response variables are shown for DCW

Probability	**DCW (g/L)**			
	SS	**MS**	**F-value**	***p*-value**
model	192.41	21.38	9.17	0.0040[a]
Linear effect				
X_2*	10.33	10.33	4.43	0.0733[b]
X_9	129.53	129.53	55.54	0.0001[a]
X_{10}	0.00	0.00	0.00	0.9930[b]
Quadratic effect				
X_2^2	0.00	0.00	0.00	0.9901[b]
X_9^2	30.98	30.98	13.28	0.0082[a]
X_{10}^2	1.80	1.80	0.77	0.4082[b]
Interaction effect				
X_2X_9	3.37	3.37	1.44	0.2686[b]
X_2X_{10}	4.28	4.28	1.83	0.2177[b]
X_9X_{10}	3.04	3.04	1.30	0.2913[b]
Residual	16.32	2.33		
Lack of Fit	15.97	3.19	17.77	0.0541[b]
Pure Error	0.36	0.18		
Cor Total	208.74			
Coefficient of determination				
R^2				0.9218
Adj R^2				0.8212

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

[a]significant at $p<0.05$, [b]not significant at $p<0.05$

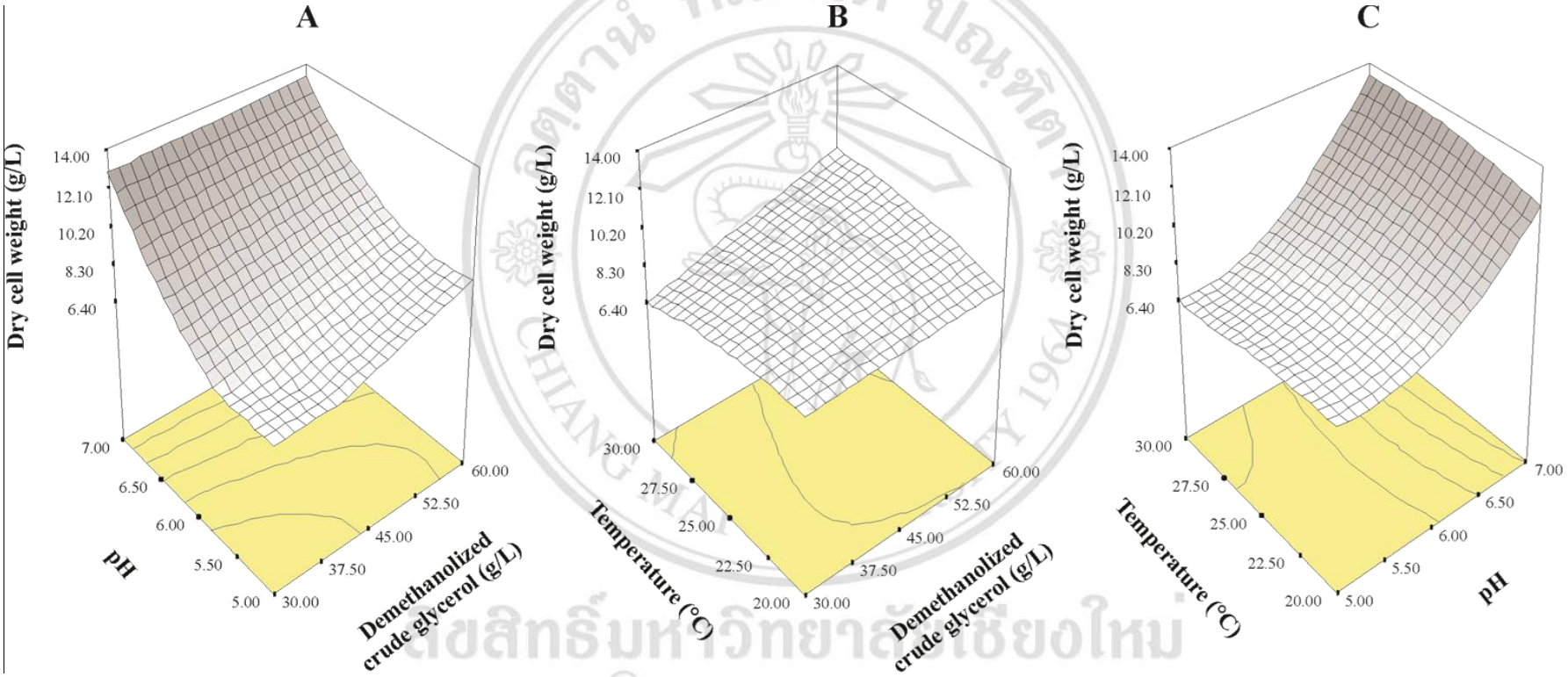

Figure 3.2 Dry cell weight in three-dimension for quadratic response surface optimization. The comparison was made between demethanolized crude glycerol and pH (A), temperature and demethanolized crude glycerol (B), temperature and pH (C)

Table 3.14 The CCD matrixes for the experiment design and predicted responses of lipids production yield

STD order	Variables codes			Lipids (g/L)	
	X_2*	X_9	X_{10}	Actual	Predicted
1	30.00	5.00	20.00	1.37	1.48
2	60.00	5.00	20.00	1.60	1.48
3	30.00	7.00	20.00	4.17	3.73
4	60.00	7.00	20.00	3.49	3.77
5	30.00	5.00	30.00	0.58	0.03
6	60.00	5.00	30.00	1.49	1.66
7	30.00	7.00	30.00	4.04	3.89
8	60.00	7.00	30.00	5.93	5.55
9	19.77	6.00	25.00	0.64	1.12
10	70.23	6.00	25.00	2.62	2.52
11	45.00	4.32	25.00	1.95	2.05
12	45.00	7.68	25.00	6.93	7.21
13	45.00	6.00	16.59	1.40	1.37
14	45.00	6.00	33.41	1.25	1.66
15	45.00	6.00	25.00	1.91	1.96
16	45.00	6.00	25.00	2.18	1.96
17	45.00	6.00	25.00	1.87	1.96

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

Three main factors, namely demethanolized crude glycerol concentration, pH and temperature, were selected for the optimization of lipids production. The CCD experiment led to a total 17 runs of experiments with no blocking. The low, center and high levels of each variable and the experimental design and respective experimental results (actual and predicted values) are shown in Table 3.14. The strain TISTR5213 was cultivated in 17 different conditions to obtain the maximum lipids production yield in batch cultivation using 250 mL Erlenmeyer flasks containing 50 mL of medium with 5 days cultivation period. The seed culture was 10% (v/v) inoculated to the batches fermentation. The results were obtained by the CCD were analyzed by ANOVA (Table 3.15). The CCD generated a quadratic equation for lipids production (Y) as a function of demethanolized crude glycerol concentration (X_2), pH (X_9) and temperature (X_{10}) are given as follows:

$$Y \text{ (lipids)} = 1.96 + 0.42X_2 + 1.54X_9 + 0.08X_{10} - 0.05X_2^2 + 0.94X_9^2 - 0.16X_{10}^2 + 0.01X_2X_9 + 0.41X_9X_{10} + 0.40X_9X_{10} \quad (8)$$

As shown in Table 3.14, the run orders of 3, 8 and 12 enhanced lipids production yield to the levels of 4.17, 5.93 and 6.93 g/L, respectively. The quadratic mathematic model in equation 8 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_2, X_9, X_9^2, X_2X_{10} and X_9X_{10} were significant as shown in Table 3.15.

In Table 3.15, the probability p-values of the model was relatively low (<0.0001), indicating a significant model. The coefficient of variation for the model (R^2 = 0.9739) were represented. The interaction term between the temperature and demethanolized crude glycerol concentration levels (X_2X_{10}) and the pH and cultivation temperature level (X_9X_{10}) had a relatively low p-value less than 0.05 at 0.0346 and 0.0362, respectively. From the p-values, it was deduced that the temperature and demethanolized crude glycerol concentration levels interacted as well as the cultivation temperature and pH.

Table 3.15 Analysis of variance (ANOVA) of the quadratic model for response variables. The probability values (*p*-values) of parameter and egression of estimated coefficients of the second order polynomial for response variables are shown for lipids production

Probability	**Lipids (g/L)**			
	SS	**MS**	**F-value**	***p*-value**
model	50.32	5.59	28.99	< 0.0001[a]
Linear effect				
X_2*	2.36	2.36	12.25	0.0100[a]
X_9	32.18	32.18	166.90	< 0.0001[a]
X_{10}	0.10	0.10	0.51	0.4987[b]
Quadratic effect				
X_2^2	0.03	0.03	0.15	0.7099[b]
X_9^2	10.02	10.02	51.96	0.0002[a]
X_{10}^2	0.28	0.28	1.47	0.2648[b]
Interaction effect				
X_2X_9	0.00	0.00	0.00	0.9566[b]
X_2X_{10}	1.32	1.32	6.85	0.0346[a]
X_9X_{10}	1.29	1.29	6.68	0.0362[a]
Residual	1.35	0.19		
Lack of Fit	1.29	0.26	9.09	0.1020[b]
Pure Error	0.06	0.03		
Cor Total	51.67			
Coefficient of determination				
R^2				0.9739
Adj R^2				0.9403

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

[a]significant at $p<0.05$, [b]not significant at $p<0.05$

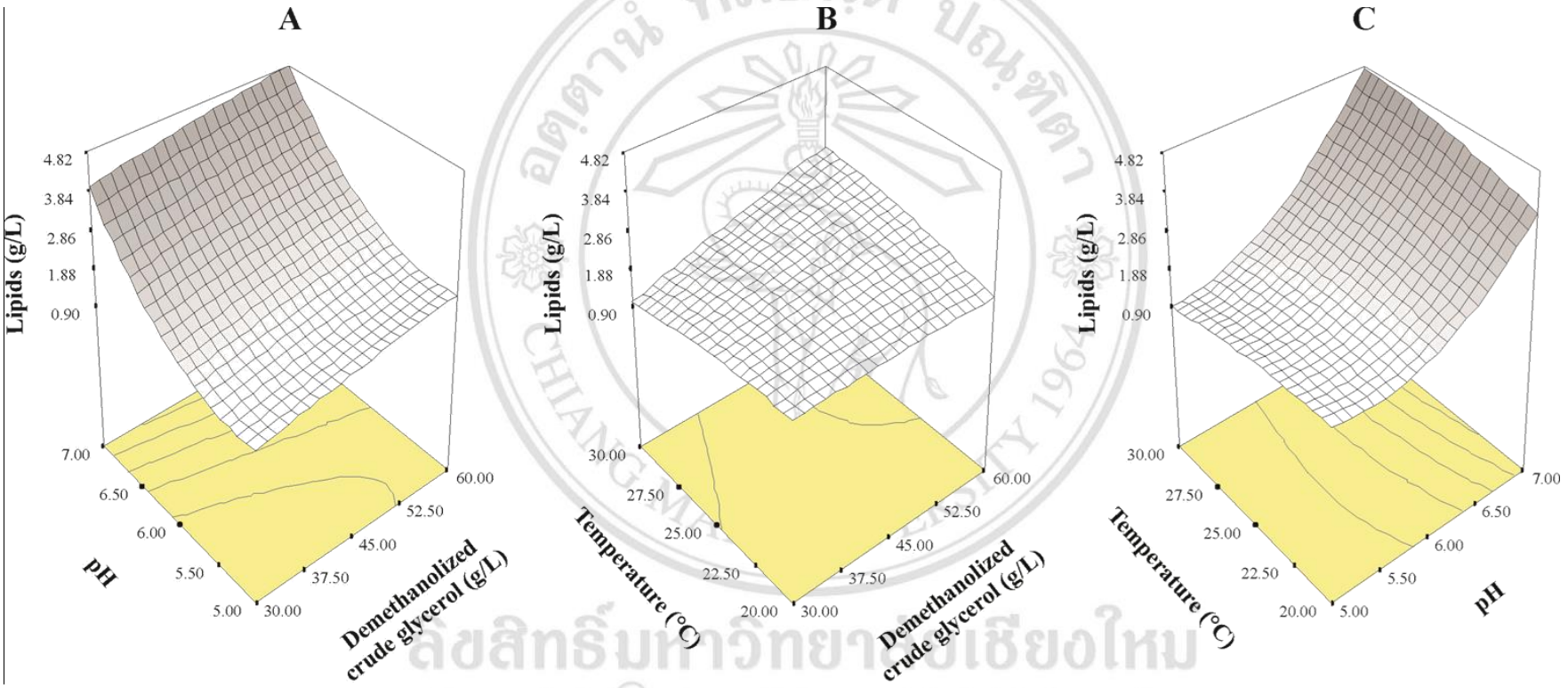

Figure 3.3 Lipids in three-dimension for quadratic response surface optimization. The comparison was made between demethanolized crude glycerol and pH (A), temperature and demethanolized crude glycerol (B), temperature and pH (C)

From Figure 3.3, it is obvious that lipids production yield was strongly affected by demethanolized crude glycerol concentration. The lipids production yield increased when the demethanolized crude glycerol concentration was elevated from 0.64 g/L at 19.77 g/L demethanolized crude glycerol (low point $-\alpha$) to 1.87 g/L at 45.0 g/L demethanolized crude glycerol (center point) and reached 2.62 g/L at 55.04 g/L demethanolized crude glycerol. These results indicate that demethanolized crude glycerol could use as an effective carbon source on growth and lipids production yield of *Sporobolomyces pararoseus* TISTR5213 as well as *Rhodosporidium toruloides* (Xu et al., 2012b) and *Rhodotorula glutinis* (Saenge et al., 2011). However, at a demethanolized crude glycerol concentration higher than 60.0 g/L, lipids production yield was decreased.

Environmental conditions affect microbial growth and product formation. Two main fermentation factors; pH (X_9) and cultivation temperature (X_{10}) were selected for the optimization study of lipids production. From Table 3.14 and Figure 3.3, low and high temperatures (16.59°C and 33.41°C) decreased lipids and high pH values (pH 7.68) significantly enhanced lipids production yield. The maximum lipids production yield of 6.93 g/L was obtained at the highest point of pH values. In addition, their interactions can affect the lipids production yield.

Table 3.16 The CCD matrixes for the experiment design and predicted responses of β-carotene production yield

STD order	Variables codes			β-carotene (mg/L)	
	X_2*	X_9	X_{10}	Actual	Predicted
1	30.00	5.00	20.00	12.94	12.35
2	60.00	5.00	20.00	20.41	20.95
3	30.00	7.00	20.00	11.59	11.89
4	60.00	7.00	20.00	15.04	15.55
5	30.00	5.00	30.00	9.99	9.72
6	60.00	5.00	30.00	18.11	18.06
7	30.00	7.00	30.00	5.50	5.20
8	60.00	7.00	30.00	7.77	8.60
9	19.77	6.00	25.00	11.81	12.44
10	70.23	6.00	25.00	23.50	22.53
11	45.00	4.32	25.00	15.92	16.26
12	45.00	7.68	25.00	8.59	7.92
13	45.00	6.00	16.59	15.55	15.23
14	45.00	6.00	33.41	7.18	7.17
15	45.00	6.00	25.00	26.57	26.84
16	45.00	6.00	25.00	26.66	26.84
17	45.00	6.00	25.00	27.22	26.84

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

Three main factors, namely demethanolized crude glycerol concentration, pH and temperature, were selected for the optimization of β-carotene production. The CCD experiment led to a total 17 runs of experiments with no blocking. The low, center and high levels of each variable and the experimental design and respective experimental results (actual and predicted values) are shown in Table 3.16. The strain TISTR5213 was cultivated in 17 different conditions to obtain the maximum β-carotene production yield in batch cultivation using 250 mL Erlenmeyer flasks containing 50 mL of medium with 5 days cultivation period. The seed culture was 10% (v/v) inoculated to the batches fermentation. The results were obtained by the CCD were analyzed by ANOVA (Table 3.17). The CCD generated a quadratic equation for β-carotene production (Y) as a function of demethanolized crude glycerol concentration (X_2), pH (X_9) and temperature (X_{10}) are given as follows:

$$Y\ (\beta\text{-carotene}) = 26.84 + 3.00X_2 - 2.48X_9 - 2.39X_{10} - 3.31X_2^2 - 5.21X_9^2 - 5.53X_{10}^2 - 1.23X_2X_9 - 0.07X_2X_{10} - 1.02X_9X_{10} \quad (9)$$

As shown in Table 3.16, the run orders of 15, 16 and 17 enhanced β-carotene production yield to the levels of 26.57, 26.66 and 27.22 mg/L, respectively. The quadratic mathematic model in equation 9 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_2, X_9, X_{10}, X_2^2, X_9^2, X_{10}^2, X_2X_9 and X_9X_{10} were significant as shown in Table 3.17.

In Table 3.17, the probability *p*-values of the model was relatively low (<0.0001), indicating a significant model. The coefficient of variation for the model (R^2 = 0.9950) were represented. The interaction term between the pH and demethanolized crude glycerol concentration levels (X_2X_9) and the pH and cultivation temperature level (X_9X_{10}) had a relatively low *p*-value less than 0.05 at 0.0026 and 0.0070, respectively. From the *p*-values, it was deduced that the pH and demethanolized crude glycerol concentration levels interacted as well as the cultivation temperature and pH.

Table 3.17 Analysis of variance (ANOVA) of the quadratic model for response variables. The probability values (*p*-values) of parameter and egression of estimated coefficients of the second order polynomial for response variables are shown for β-carotene production

Probability	**SS**	**MS**	**F-value**	***p*-values >F**
model	818.30	90.92	156.19	< 0.0001[a]
Linear effect				
X_2[*]	122.95	122.95	211.21	< 0.0001[a]
X_9	84.02	84.02	144.34	< 0.0001[a]
X_{10}	78.26	78.26	134.44	< 0.0001[a]
Quadratic effect				
X_2^2	123.26	123.26	211.74	< 0.0001[a]
X_9^2	306.52	306.52	526.56	< 0.0001[a]
X_{10}^2	344.63	344.63	592.03	< 0.0001[a]
Interaction effect				
X_2X_9	12.19	12.19	20.94	0.0026[a]
X_2X_{10}	0.04	0.04	0.06	0.8141[b]
X_9X_{10}	8.26	8.26	14.19	0.0070[a]
Residual	4.07	0.58		
Lack of Fit	3.83	0.77	6.15	0.1457[b]
Pure Error	0.25	0.12		
Cor Total	822.37			
Coefficient of determination				
R^2				0.9950
Adj R^2				0.9887

[*]X_2 Demethanolized crude glycerol (g/L); X_9 pH; X_{10} Temperature (°C)

[a]significant at $p<0.05$, [b]not significant at $p<0.05$

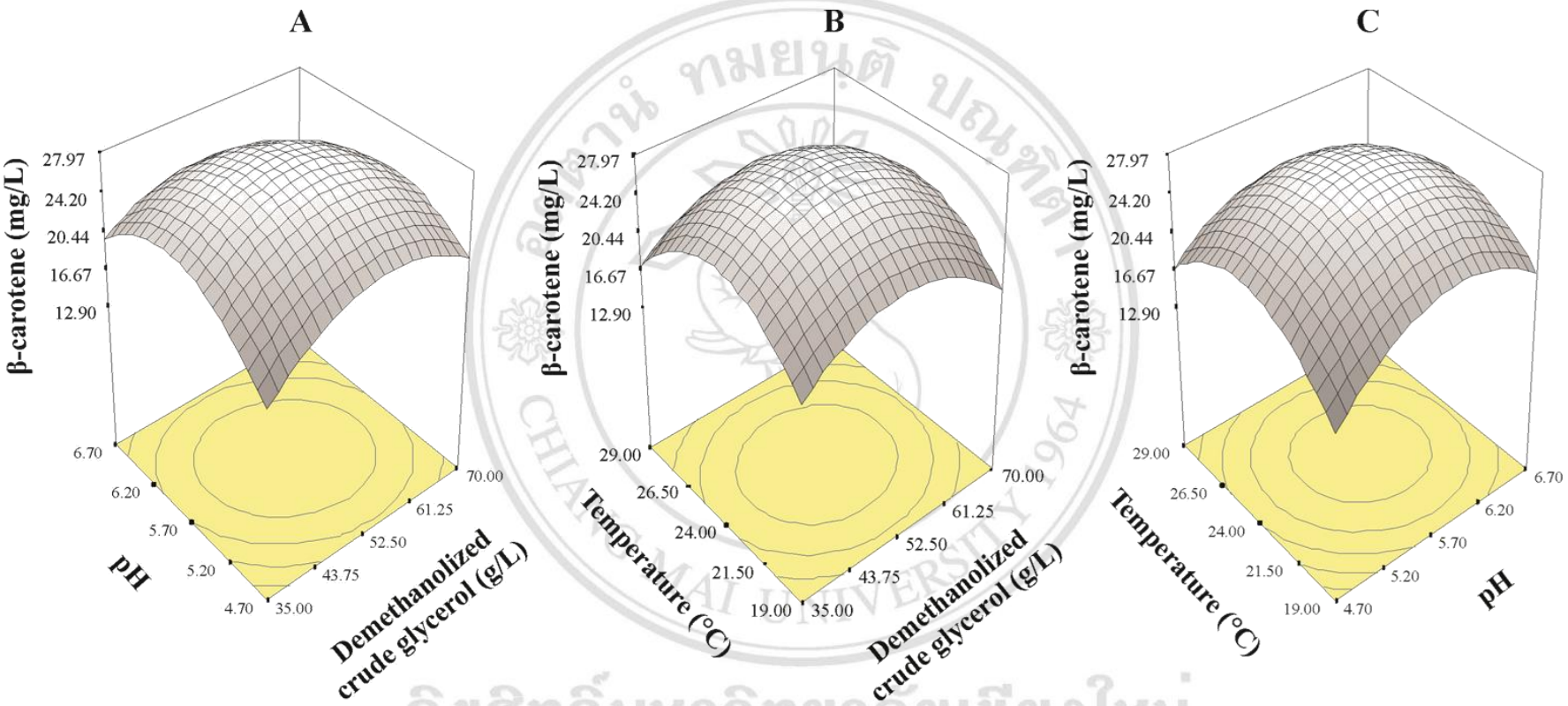

Figure 3.4 β-carotene in three-dimension for quadratic response surface optimization. The comparison was made between demethanolized crude glycerol and pH (A), temperature and demethanolized crude glycerol (B), temperature and pH (C)

From Figure 3.4, it is obvious that β-carotene production yield was strongly affected by demethanolized crude glycerol concentration. The β-carotene production yield increased when the demethanolized crude glycerol concentration was elevated from 11.81 mg/L at 19.77 g/L demethanolized crude glycerol (low point –α) to 26.66 mg/L at 45.0 g/L demethanolized crude glycerol (center point). These results indicate that demethanolized crude glycerol could use as an effective carbon source on growth and β-carotene production yield of *Sporobolomyces pararoseus* TISTR5213 as well as *Phaffia rhodozyma* (Kusdiyantini, 1998) and *Sporobolomyces ruberrimus* (Razavi et al., 2007). However, at a demethanolized crude glycerol concentration higher than 60.0 g/L, β-carotene production yield was decreased. A similar result of Saenge et al. (2011) was observed in *Rhodotorula glutinis*, at higher crude glycerol concentration than 95.0 g/L, cell growth and carotenoids production decreased. The possible reason for this effect is that a high concentration of glycerol results in a high osmotic pressure which inhibits the metabolic activity of yeast cell (Zhu et al., 2008).

Environmental conditions affect microbial growth and product formation. Two main fermentation factors; pH (X_9) and cultivation temperature (X_{10}) were selected for the optimization study of β-carotene production. From Table 3.16 and Figure 3.4, low and high temperatures (16.59°C and 33.41°C) and pH values (4.32 and 7.68) significantly decreased β-carotene production yield. The maximum β-carotene production yield of 27.22 mg/L was obtained at the center point (pH 6.0 and 25°C). In addition, their interactions can affect the β-carotene production yield.

Table 3.18 The CCD matrixes for the experiment design and predicted responses of total carotenoids production yield

STD order	Variables codes			Total carotenoids (mg/L)	
	X_2^*	X_9	X_{10}	Actual	Predicted
1	30.00	5.00	20.00	19.54	19.73
2	60.00	5.00	20.00	30.83	30.30
3	30.00	7.00	20.00	27.03	26.29
4	60.00	7.00	20.00	25.88	27.16
5	30.00	5.00	30.00	18.73	16.45
6	60.00	5.00	30.00	27.42	27.16
7	30.00	7.00	30.00	14.28	13.80
8	60.00	7.00	30.00	16.01	14.82
9	19.77	6.00	25.00	22.16	23.64
10	70.23	6.00	25.00	33.45	33.38
11	45.00	4.32	25.00	20.23	21.46
12	45.00	7.68	25.00	16.41	16.60
13	45.00	6.00	16.59	29.86	29.26
14	45.00	6.00	33.41	14.11	16.12
15	45.00	6.00	25.00	47.25	47.28
16	45.00	6.00	25.00	48.02	47.28
17	45.00	6.00	25.00	46.81	47.28

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

Three main factors, namely demethanolized crude glycerol concentration, pH and temperature, were selected for the optimization of total carotenoids production. The central composite design (CCD) experiment led to a total 17 runs of experiments with no blocking. The low, center and high levels of each variable and the experimental design and respective experimental results (actual and predicted values) are shown in Table 3.18. The strain TISTR5213 was cultivated in 17 different conditions to obtain the maximum β-carotene production yield in batch cultivation using 250 mL Erlenmeyer flasks containing 50 mL of medium with 5 days cultivation period. The seed culture was 10% (v/v) inoculated to the batches fermentation. The results were obtained by the CCD were analyzed by ANOVA (Table 3.17). The CCD generated a quadratic equation for total carotenoids production (Y) as a function of demethanolized crude glycerol concentration (X_2), pH (X_9) and temperature (X_{10}) are given as follows:

$$Y \text{ (total carotenoids)} = 47.28 + 2.90X_2 + 1.45X_9 - 3.91X_{10} - 6.63X_2^2 - 9.99X_9^2 - 8.69X_{10}^2 - 2.42X_2X_9 + 0.04X_2X_{10} - 2.30X_9X_{10} \quad (10)$$

As shown in Table 3.18, the run orders of 15, 16 and 17 enhanced β-carotene production yield to the levels of 47.25, 48.02 and 46.81 mg/L, respectively. The quadratic mathematic model in equation 10 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_2, X_9, X_{10}, X_2^2, X_9^2, X_{10}^2, X_2X_9 and X_9X_{10} were significant as shown in Table 3.17.

In Table 3.19, the probability *p*-values of the model was relatively low (<0.0001), indicating a significant model. The coefficient of variation for the model (R^2 = 0.9911) were represented. The interaction term between the pH and demethanolized crude glycerol concentration levels (X_2X_9) and the pH and cultivation temperature level (X_9X_{10}) had a relatively low *p*-value less than 0.05 at 0.0039 and 0.0051, respectively. From the *p*-values, it was deduced that the pH and demethanolized crude glycerol concentration levels interacted as well as the cultivation temperature and pH.

Table 3.19 Analysis of variance (ANOVA) of the quadratic model for response variables. The probability values (*p*-values) of parameter and egression of estimated coefficients of the second order polynomial for response variables are shown for total carotenoids production

Probability	**Total carotenoids (mg/L)**			
	SS	**MS**	**F-value**	***p*-value**
model	2045.94	227.33	86.58	< 0.0001[a]
Linear effect				
X_2*	114.50	114.50	43.61	0.0003[a]
X_9	28.55	28.55	10.87	0.0132[a]
X_{10}	208.39	208.39	79.36	< 0.0001[a]
Quadratic effect				
X_2^2	496.16	496.16	188.96	< 0.0001[a]
X_9^2	1124.51	1124.51	428.27	< 0.0001[a]
X_{10}^2	851.79	851.79	324.40	< 0.0001[a]
Interaction effect				
X_2X_9	47.01	47.01	17.90	0.0039[a]
X_2X_{10}	0.01	0.01	0.00	0.9515[b]
X_9X_{10}	42.30	42.30	16.11	0.0051[a]
Residual	18.38	2.63		
Lack of Fit	17.64	3.53	9.50	0.0980[b]
Pure Error	0.74	0.37		
Cor Total	2064.32			
Coefficient of determination				
R^2				0.9911
Adj R^2				0.9796

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

[a]significant at $p<0.05$, [b]not significant at $p<0.05$

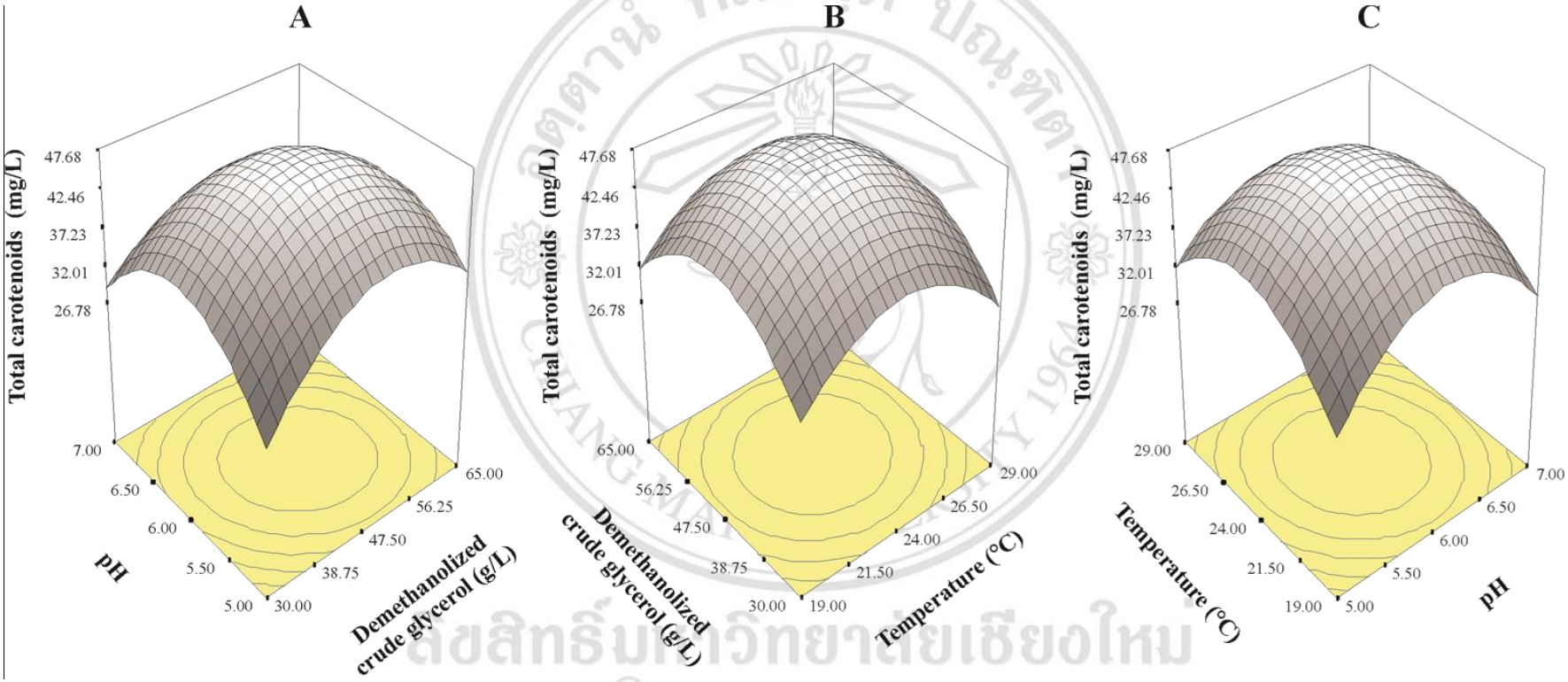

Figure 3.5 Total carotenoids in three-dimension for quadratic response surface optimization. The comparison was made between demethanolized crude glycerol and pH (A), temperature and demethanolized crude glycerol (B), temperature and pH (C)

From Figure 3.5, it is obvious that total carotenoids production yield was strongly affected by demethanolized crude glycerol concentration. The total carotenoids production yield increased when the demethanolized crude glycerol concentration was elevated from 22.16 mg/L at 19.77 g/L demethanolized crude glycerol (low point $-\alpha$) to 48.02 mg/L at 45.0 g/L demethanolized crude glycerol (center point). These results indicate that demethanolized crude glycerol could use as an effective carbon source on growth and total carotenoids production yield of *Sporobolomyces pararoseus* TISTR5213 as well as *Phaffia rhodozyma* (Kusdiyantini, 1998) and *Sporobolomyces ruberrimus* (Razavi et al., 2007). However, at a demethanolized crude glycerol concentration higher than 60.0 g/L, total carotenoids production yield was decreased. A similar result of Saenge et al. (2011) was observed in *Rhodotorula glutinis*, at higher crude glycerol concentration than 95.0 g/L, cell growth and carotenoids production decreased. The possible reason for this effect is that a high concentration of glycerol results in a high osmotic pressure which inhibits the metabolic activity of yeast cell (Zhu et al., 2008).

Environmental conditions affect microbial growth and product formation. Two main fermentation factors; pH (X_9) and cultivation temperature (X_{10}) were selected for the optimization study of total carotenoids production. From Table 3.18 and Figure 3.5, low and high temperatures (16.59°C and 33.41°C) and pH values (4.32 and 7.68) significantly decreased total carotenoids production yield. The maximum total carotenoids production yield of 48.02 mg/L was obtained at the center point (pH 6.0 and 25°C). In addition, their interactions can affect the total carotenoids production yield.

The results obtained from the CCD were then analyzed by ANOVA. Moreover, the response, X_2, X_9 and X_{10} were coded in terms of variable, i.e. demethanolized crude glycerol, pH and temperature, respectively. Values of "*P*-value>*F*" less than 0.05 indicate model terms were significant. The quadratic mathematic model in equation 2 was further simplified, corresponding to the *p*-value in the model terms. In this case, *p*-value less than 0.05 indicated significant model terms and values, whereas greater than 0.05 indicated non significant model terms. The terms of interaction effect found that X_2X_9 and $X_9\ X_{10}$ were significant for β-carotene and total carotenoids productions, while X_2X_{10} and $X_9\ X_{10}$ were significant for lipids production. The DCW was only one response, which had not interaction effect. Moreover, the probability *p*-values of the model was relatively low, indicating a significant model. The coefficient of determination for the DCW (0.9218), β-carotene (0.9950), total carotenoids (0.9911) and lipids (0.9739) were represented.

From Figure 3.2–3.5, DCW, lipids, β-carotene and total carotenoids production yields were strongly affected by demethanolized crude glycerol concentration. The β-carotene production and total carotenoids yields increased when the demethanolized crude glycerol concentration were elevated from 11.81 mg/L and 22.16 mg/L at 19.77 g/L demethanolized crude glycerol (low point –α) to 26.66 mg/L and 48.02 mg/L at 45.0 g/L demethanolized crude glycerol (center point) and reached 23.50 mg/L and 33.45 mg/L at 70.23 g/L demethanolized crude glycerol (high point +α).While, the DCW and lipids were increased from 4.99 g/L and 0.64 g/L at lowest point to 8.86 g/L and 2.18 g/L at the center point to 10.49 g/L and 2.62 g/L at high point, respectively. These results indicated that demethanolized crude glycerol could use as an effective carbon source on DCW, β-carotene, total carotenoids and lipids productions of *Sporobolomyces pararoseus* TISTR5213. However, at a concentration of demethanolized crude glycerol higher than 60.0 g/L, β-carotene and total carotenoids production yield were decreased. A similar result of Saenge et al. (2011) was observed in *Rhodotorula glutinis*, at higher crude glycerol concentration than 95.0 g/L, cell growth and carotenoids production decreased. The possible reason for this effect is that a high concentration of glycerol results in a high osmotic pressure which inhibited the metabolic activity of yeast cell (Zhu et al., 2008). Two main fermentation factors; pH

(X_9) and cultivation temperature (X_{10}) of environmental factor were selected for the optimization study of DCW, β-carotene, total carotenoids and lipids productions. Their interactions could affect the DCW, β-carotene, total carotenoids and lipids production yield. Thus, low and high temperatures (16.59°C and 33.41°C) found that at low temperature gave the highest yields of DCW, β-carotene, total carotenoids and lipids productions, while at low pH values (4.32) was good condition for β-carotene and total carotenoids but DCW and lipids production increased at high pH value (7.68). Similar result has been reported by Malisorn and Suntornsuk (2008) in β-carotene production yield of *Rhodotorula glutinis* DM28, which was increased with elevated pH and moderate temperature (pH 6.0 and 30°C) and with further increases in both factors an adverse effect was observed. Therefore, the pH and temperature are most important environment parameters affecting cell growth, carotenoids and lipids productions. Moreover, They bring the changes in many biosynthetic pathways, including carotenoids and lipids biosynthesis (Bhosale, 2004; Saenge et al., 2011).

3.3.5 Validation of CCD optimization model

To confirm the applicability of the model, DCW, β-carotene, total carotenoids and lipids production by *Sporobolomyces pararoseus* TISTR5213 were carried out by cultivation this strain under the optimal conditions suggested of 55.04 g/L demethanolized crude glycerol, pH 5.63 at 24.01°C. From the experimental results, a higher yield of DCW, lipids, β-carotene and total carotenoids productions of 8.83±0.05 g/L, 4.00±0.06 g/L, 27.41 mg/L and 53.70±0.48 mg/L, respectively, was obtained with a higher than predicted value from 7.90 g/L, 3.62 g/L, 26.84 mg/L and 46.90 mg/L, respectively (Figure 3.6). This result indicated that the model could be used to predict the maximum of DCW, lipids, β-carotene and total carotenoids production yield.

Figure 3.6 shows the DCW, β-carotene, total carotenoids and lipids production behaviors of *Sporobolomyces pararoseus* TISTR5213 under the optimal conditions. This strain needed cultivation time up to 5 days for the highest β-carotene, total carotenoids and lipids accumulation in its cell. As the evidence from Figure 3.6, β-carotene, total carotenoids and lipids productions were growth-associated and increased during the log phase of growth. Similar to the results of Bhosale and Gadre

(2001) reported that the carotenoids production of *Rhodotorula glutinis* was a growth-associated product. Moreover, Aksu and Eren (2005) reported that the carotenoids production of *Rhodotorula mucilaginosa* was a growth-associated product and the highest β-carotene content was obtained at the end of log phase (5 days).

As evidence of Figure 3.6, the pH changing profile related to the increasing of DCW, β-carotene, total carotenoids and lipids production yields. The pH was rapidly decreased from pH 5.63 to pH 2.60 at the 5 days of cultivation period, which the DCW, β-carotene, total carotenoids and lipids content were also rapidly increased and reached to 8.83±0.05 g/L, 27.41±0.20 mg/L, 53.69±0.48 mg/L and 4.00±0.06 g/L, respectively. Moreover, the decreasing of pH was obviously related to the demethanolized crude glycerol consumption in term of residual glycerol. When, the glycerol content in the media was almost completely consumed by *Sporobolomyces pararoseus* TISTR5213, the pH was also decreased to the lowest of pH 2.60 at 5 days of cultivation period. Similar to the result of Hu et al. (2006) who reported that under the uncontrolled pH condition, pH was decreased until the end of culvationtime and related with the glucose consumption. The decreasing of pH during β-carotene, total carotenoids and lipids productions from 5.63 to 2.60 which may indicate the production of acids. To date, there have been no reports about the acid production and decreasing of pH during carotenoids production production. Similar result was observed in the studies of Saenge et al. (2011), the pH of the medium declined from 6.0 to 4.3 when crude glycerol was used as the substrate for carotenoids production from *Rhodotorula glutinis* TISTR5159.

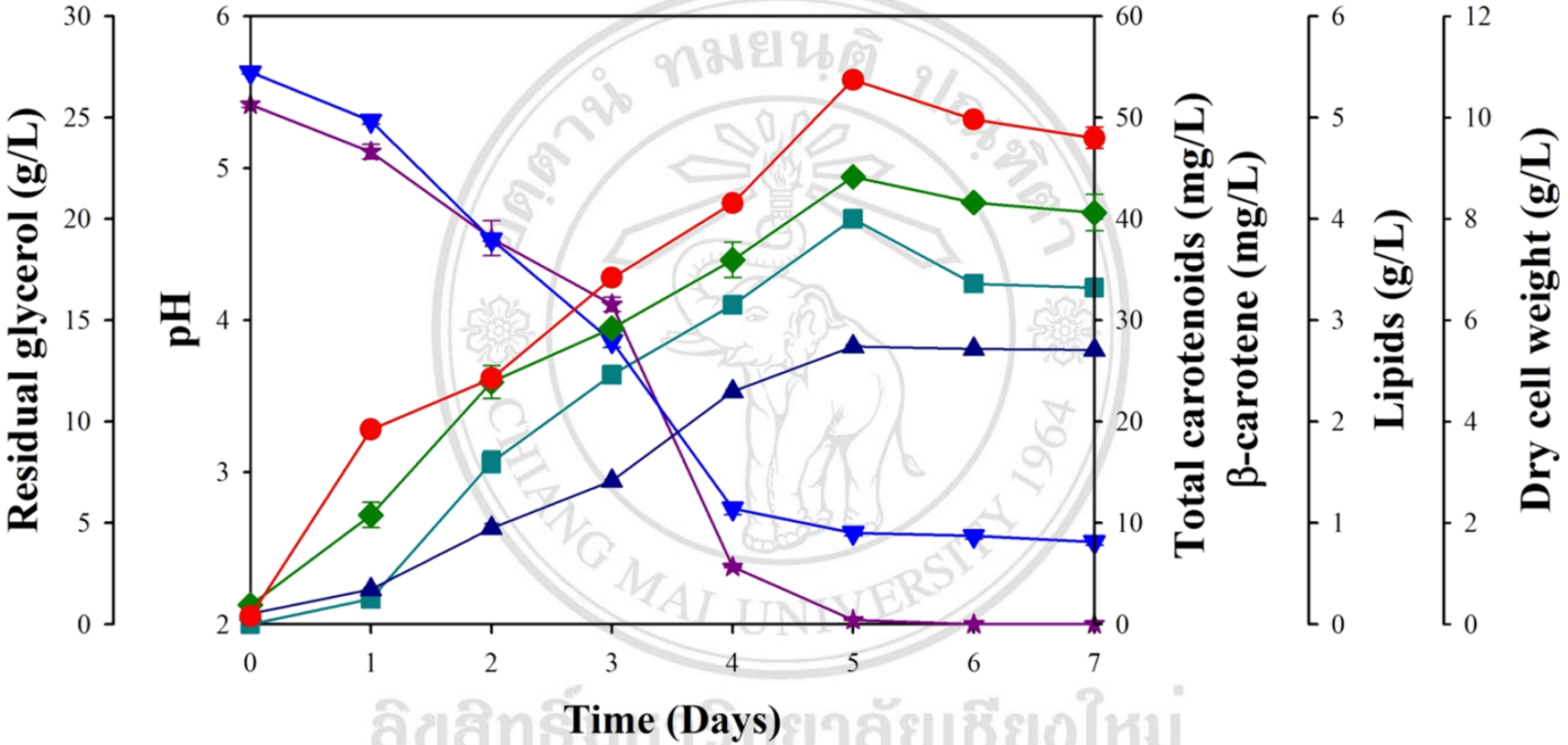

Figure 3.6 Time course of dry cell weight (◆), residual glycerol (★), pH (▼), lipids (■), β-carotene (▲) and total carotenoids (●) by *Sporobolomyces pararoseus* TISTR5213 under optimal conditions

3.4 Conclusions

Crude glycerol was the good carbon source and considered to be a cost effective raw material for lipids β-carotene and total carotenoids production from oleaginous red yeasts. *Sporobolomyces pararoseus* TISTR5213 had a great potential for bioconversion of crude glycerol into lipids and carotenoids. This oleaginous red yeast produced maximum DCW, total carotenoids and lipids production yield of 3.49±0.39 g/L, 1.77±0.01 mg/L and 2.05±0.02 g/L, respectively, when cultivated in the basal medium supplemented with crude glycerol (20.0 g/L) as a carbon source. Methanol, an impurity presented in crude glycerol showed strongly effect on the growth, lipids, β-carotene and total carotenoids production of *Sporobolomyces pararoseus* TISTR5213. The highest DCW, lipids, β-carotene and total carotenoids of 9.40±0.12 g/L, 2.92±0.03 g/L, 25.76±0.85 mg/L and 33.67±1.28 mg/L, respectively, were obtained, with the basal medium supplemented with 34.0 g/L of demethanolized crude glycerol (absent of methanol). The Plackett-Burman design was employed to select factor affecting to enhance lipids, β-carotene and total carotenoids production yield by batch cultivation of *Sporobolomyces pararoseus* TISTR5213. The linear model was established using the Plackett-Burman design to select demethanolized crude glycerol as only one factor that exerted the highest influence on lipids, β-carotene and total carotenoids productions. After that, three main factors, namely demethanolized crude glycerol concentration, pH and temperature, were then selected for the optimization of β-carotene production by RSM. The second-order quadratic model generated by the CCD to simulate the optimal conditions for the highest β-carotene production when the demethanolized crude glycerol was increased from 20.0 g/L (2.0%) to 55.04 g/L (5.5%), initial pH at 5.63 and 24.01°C for 5 days. Using this experimental design, the DCW, lipids, β-carotene and total carotenoids production were increased 8.83±0.05 g/L, 4.00±0.06 g/L, 27.41 mg/L and 53.70±0.48 mg/L, respectively. Furthermore, our study has shown that *Sporobolomyces pararoseus* TISTR5213 has great potential for bioconversion of demethanolized crude glycerol to valuable lipids, β-carotene and total carotenoids in the view of efficient renewable resource utilization.