CHAPTER 4

Results and Discussion

4.1 The composition of rice residue from food waste

Rice residue from food waste was daily collected from the canteen of the Faculty of Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand during September to October of the first semester, academic year 2015. After removal of contaminated solid waste residues e.g. bone, meat, egg and vegetable residues, it was dried, ground to be the fine particle and kept at 4°C. The proximate analysis of dried rice residue was determined according to the AOAC method (Helrich, 1990). The result showed that it composed of moisture content (7.18±0.34%), crude fat (0.75±0.01%), ash content (0.38±0.03%), crude protein (1.29±0.03%) and carbohydrate content (90.00±0.01%) as shown in Table 4.1

Table 4.1 Compositions of rice residue from food waste

Compositions	**Content (% of dry weight)**
Moisture content	7.18±0.34*
Crude fat	0.75±0.01
Ash content	0.38±0.03
Crude protein	1.29±0.03
Carbohydrate content	90.00±0.01

*Means and standard deviations of triplicate samples

Carbohydrate (90% of dry weight) was the major composition of rice residue from food waste. This component can be hydrolyzed by either acid or enzymes to produce fermentable sugars, which have been used as carbon sources for lipids and carotenoids productions by various types of oleaginous yeasts. For example, Uçkun Kiran et al. (2014) reported that food waste could be used as carbon source for lipids production of

oleaginous microorganisms which could be converted to biodiesel via transesterification. While, Pleissner et al. (2013) have reported that food waste hydrolysate could be used as carbon and nutrient sources for microalgae *Schizochytrium mangrovei* and *Chlorella pyrenoidosa* for theirs growth and metabolite production. In this study, food waste was hydrolyzed by amylolytic fungal *Aspergillus awamori* and *Aspergillus oryzae*. The hydrolysate composed of glucose, free amino nitrogen (FAN), and phosphate of 31.9, 0.28 and 0.38 g/g, respectively. The results showed that both microalgae biomass reached in carbohydrate, lipids, ω-3 fatty acids and proteins up to 300–400, 300, 150 and 100 mg/g, respectively. While, cultivation in glucose, the relative low content of the carbohydrate, lipids, ω-3 fatty acids and proteins were 200–300, 150, 100 and 50 mg/g, respectively. Moreover, the fatty acids presented in crude lipids of both strains were suitable for biodiesel production (Pleissner et al., 2013).

Zeng et al. (2017) studied the utilization of acid-hydrolysate from food waste for microbial lipids and protein productions by *Rhodosporidium toruloides* Y2. The results revealed that this strain produced the biomass, lipids and protein of 32.1, 7.3 and 7.0 g/L, respectively. The lipids from this strain had oleic acid (C18:1) content about 50% of the total fatty acid which was similar to the plant oil, indicating this lipids could be used as a biodiesel feedstock.

4.2 Production of rice residue hydrolysate

The enzymatic-rice residue hydrolysate contained reducing sugars of 168.02±0.02 g/L and production yield ($Y_{p/s}$) of 0.960 g/g, while acid rice-hydrolysate was 128.55±0.04 g/L and production yield of 0.734 g/g. These results indicated that enzyme hydrolysis using α-amylase (EC 3.2.1.1) and amyloglucosidase or AMG (EC 3.2.1.3) yielding high content of glucose because of the specificity of these amylolytic enzymes (Sundarram and Murthy, 2014), while acid hydrolysis showed lower reducing sugar. It might be that acid has limitation for hydrolysis under high temperature and pressure conditions. Furthermore, the formation of undesired products e.g. furans, carboxylic acid and phenolic compounds from acid hydrolysis is the main drawback of this method. Those products have been reported as the microbial growth inhibitors (Karimi et al., 2006).

4.3 Screening and isolation of oleaginous red yeast for lipids and carotenoids productions

The screening of oleaginous red yeast from flowers and leaves samples obtained from Doi Inthanon National park, Chiang Mai 50160, Thailand, the culture collection of Thailand Institute of Scientific and Technological Research (TISTR) and the Division of Biotechnology, Faculty of Agro-Industry, Chiang Mai University, Chiang Mai 50100, Thailand were studied. Sixty-seven of red yeast isolates were obtained. After cultivation in the basal medium supplemented with glucose as a carbon source, found that nine isolates could accumulate lipids in their cell more than 10% (w/w) as shown in Figure 4.1. However, only two strains of red yeast isolate C7 and isolate C10 could accumulate lipids in theirs cell more than 20% (w/w) and produce maximum lipids content of 22.44±1.08 and 21.58±0.05% (w/w), respectively (Figure 4.1A).

Figure 4.1B shows that red yeast isolate C7 produced the volumetric productions of total carotenoids and β-carotene of 0.94±0.08 and 0.43±0.01 mg/L, with the yield of total carotenoids and β-carotene of 188±0.12 and 85±0.01 μg/g, respectively. While, isolate C10 produced the volumetric production of total carotenoids and β-carotene of 0.86±0.01 and 0.26±0.00 mg/L, with the yield of total carotenoids and β-carotene of 161.0±0.11 and 50±0.18 μg/g, respectively. Moreover, red yeast isolate C20 produced the highest of the volumetric production of total carotenoids and total carotenoids yield of 2.49±0.08 mg/L and 545±0.09 μg/g, while yeast isolate C14 gave the highest β-carotene and β-carotene yield of 1.74±0.12 mg/L and 355±0.11 μg/g, respectively.

4.4 Screening of oleaginous red yeast for lipids and carotenoids productions using rice residue hydrolysate from food waste as a carbon source

Nine isolates (selected from section 4.3) were cultivated in basal medium supplemented either enzymatic or acid rice residue hydrolysate. Figure 4.2A shows that, red yeasts isolate C7, isolate C10, *Diozegia* sp. TISTR5792 and isolate TC32 could produce the maximal lipids content of 24.26±0.56, 23.69±0.91, 22.43±1.09 and 23.07±0.80% (w/w), respectively, when cultivated in the basal medium supplemented with enzymatic-rice residue hydrolysate. While, cultivation in basal medium supplemented with acid-rice residue hydrolysate showed the lipids content of

21.84±0.56, 22.41±0.23, 19.32±0.80 and 19.00±0.50% (w/w) by red yeast isolate C7, isolate C10, *Diozegia* sp. TISTR5792 and isolate TC32, respectively (Figure 4.3A).

The carotenoids production revealed that red yeasts isolate C7, isolate C10, *Diozegia* sp. TISTR5792 and isolate TC32 produced total carotenoids of 0.82±0.05, 1.57±0.05, 1.64±0.35 and 2.68±0.09 mg/L, with the total carotenoids yield of 150±0.10, 280±0.22, 400±0.13 and 595±0.90 μg/g, respectively. The β-carotene production of those isolates were 0.44±0.00, 0.94±0.01, 1.41±0.90 and 2.17±0.19 mg/L, with the β-carotene yield of 80±0.04, 170±0.55, 340±0.04 and 480±0.05 μg/g, respectively, when cultivated in the basal medium supplemented with enzymatic-rice residue hydrolysate (Figure 4.2B).

While, cultivation in the basal medium supplemented with acid-rice residue hydrolysate demonstrated that the total carotenoids of 0.74±0.13, 1.24±0.01, 1.03±0.02 and 1.88±0.10 mg/L, with the total carotenoids yield of 150±0.04, 260±0.6, 290±0.94 and 470±0.85 μg/g, respectively. For the β-carotene of their isolates were 0.21±0.00, 0.65±0.44, 0.79±0.07 and 1.28±0.06 mg/L, with the β-carotene yield of 40±0.51, 140±0.63, 220±0.23 and 320±0.10 μg/g, respectively (Figure 4.3B).

These results indicated that enzymatic-rice residue hydrolysate supported the lipids and carotenoids productions better than glucose and acid hydrolysis. It might be that rice residue from food waste contained not only carbohydrate, but other component also found in this starchy material (Table 4.1). Crude lipids, crude fat and some trace elements in term of ash content may enhance the growth and lipids production of oleaginous red yeast (Subramaniam et al., 2010). Sha (2013) reported that trace element such as magnesium (Mg), potassium (K) and calcium (Ca) are normally essential for growth and lipids accumulation in oleaginous microorganisms.

Moreover, cultivation in basal medium supplemented with enzymatic-rice residue hydrolysate showed higher lipids and carotenoids contents than acid-rice residue hydrolysate. It might be that acid rice hydrolysate may contain furfural or hydroxymethylfurfural (HMF) which usually occurred during heat-process combined with acid hydrolysis. These compounds have been reported as the inhibitor of microbial growth by reducing enzymatic and biological activities, leading to low productivity (Zhang et al., 2013). The lipids accumulations of these four strains were confirmed by

staining with Sudan black B (Table 4.2). The high intensity of black color indicated high content of lipids which accumulated in yeast cell (Patnayak and Sree, 2005).

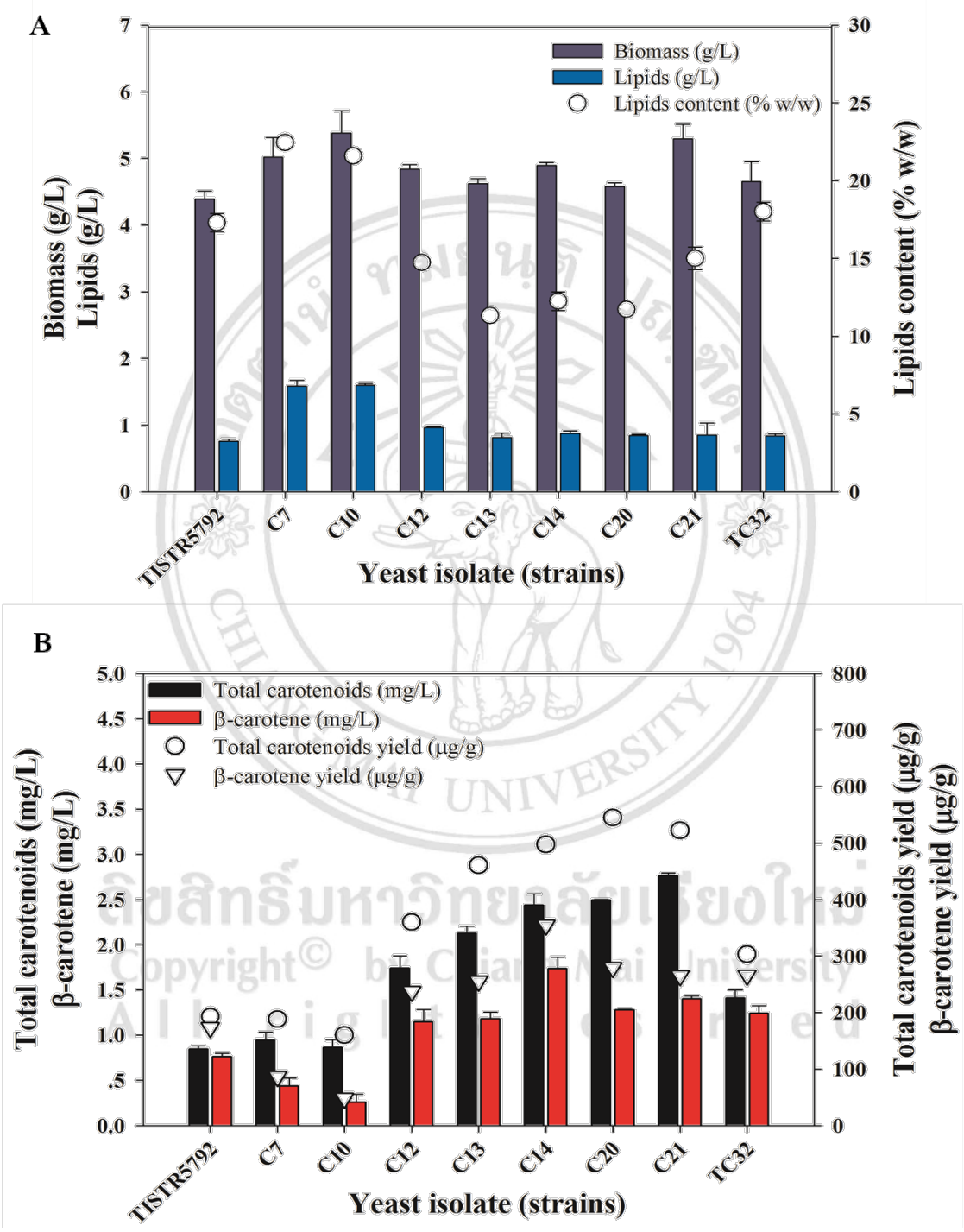


Figure 4.1 Productions of biomass and lipids (A) and carotenoids (B) by nine red yeast strains cultivated in the basal medium supplemented with glucose as carbon source

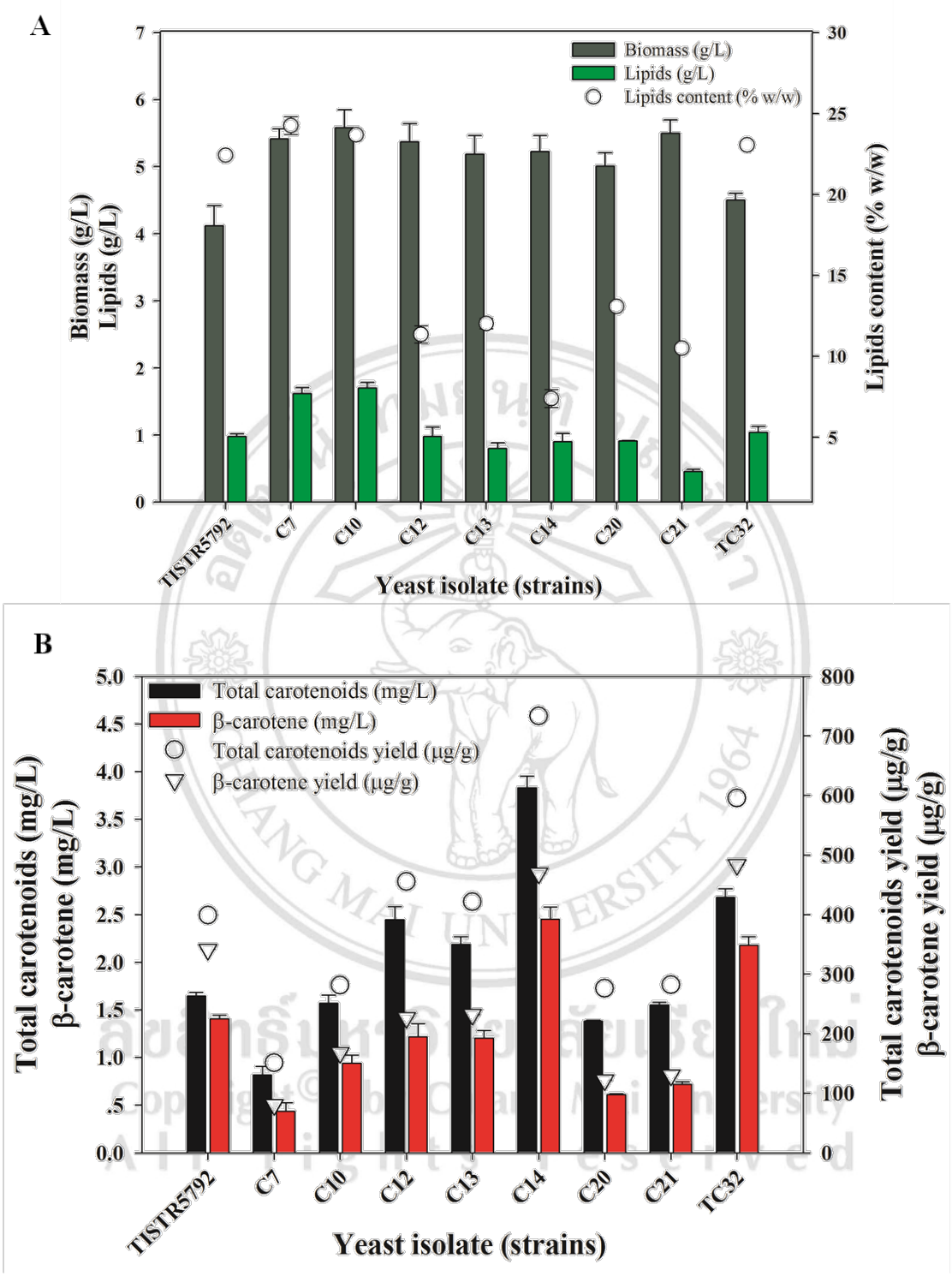


Figure 4.2 Productions of biomass and lipids (A) and carotenoids (B) by nine red yeast strains cultivated in the basal medium supplemented with enzymatic-rice residue hydrolysate as carbon source

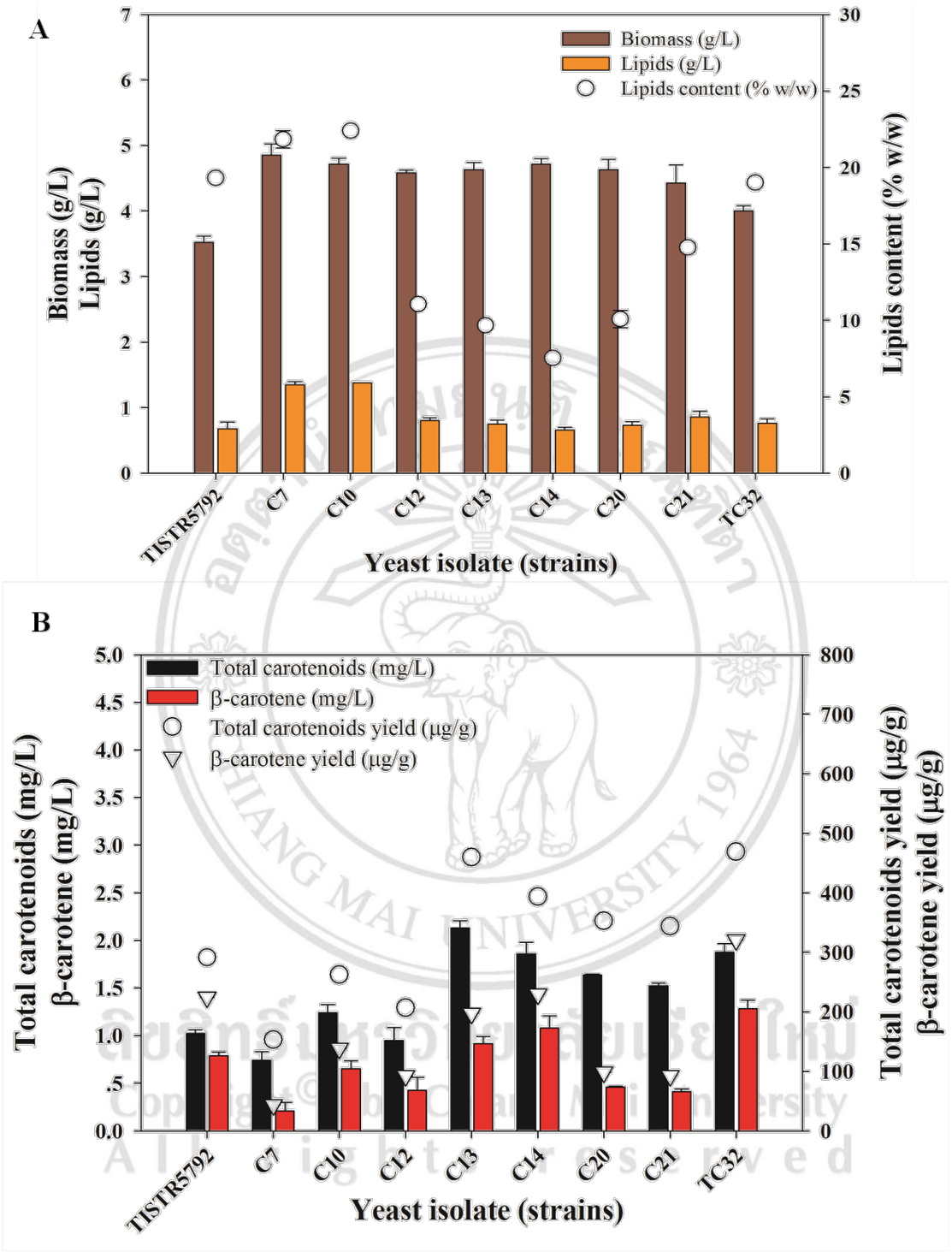
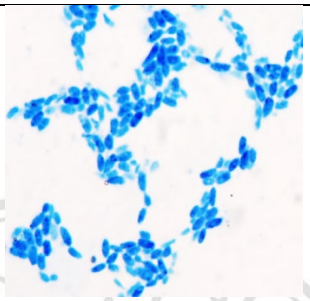
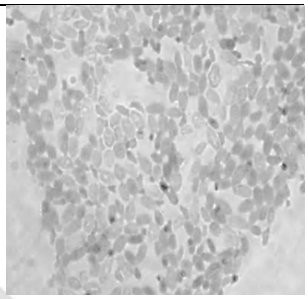
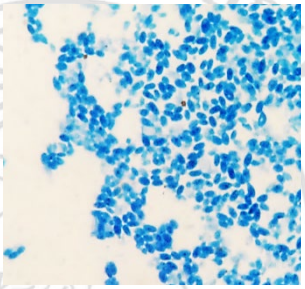
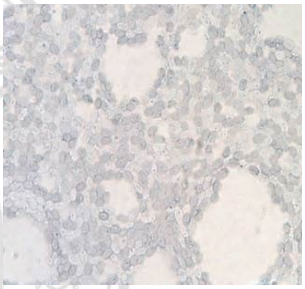
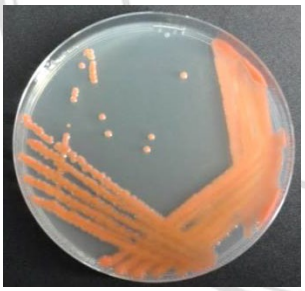
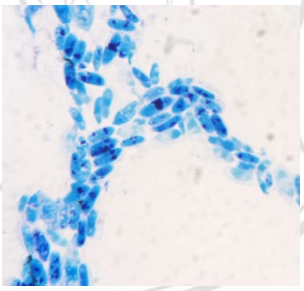
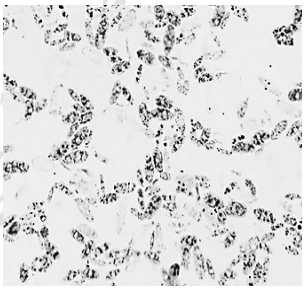
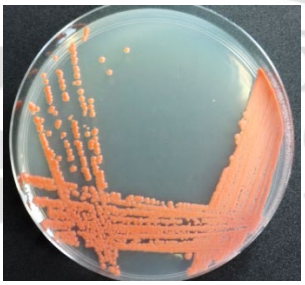
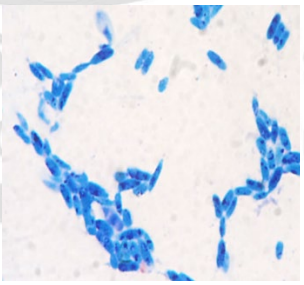
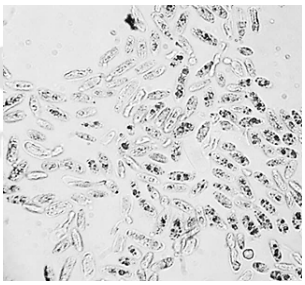


Figure 4.3 Productions of biomass and lipids (A) and carotenoids (B) by nine red yeast strains cultivated in the basal medium supplemented with acid-rice residue hydrolysate as carbon source

Table 4.2 The morphology of colony and cell and intracellular lipids of oleaginous yeast (100X)

Yeast isolate	Morphology of colony	Morphology of cell*	Intracellular lipids**
Isolate C7			
Isolate C10			
Isolate TC32			
Diozegia sp. TISTR5792			

* Methylene blue staining technique

**Sudan black B staining technique

4.5 Screening of oleaginous red yeast for lipids and carotenoids productions using rice residue from food waste as a carbon source

The ability of direct bioconversion of rice residue to biomass, lipids and carotenoids of nine isolates (selected from section 4.3) were also investigated. The results revealed that only two isolates of *Diozegia* sp. TISTR5792 and isolate TC32 could directly use rice residue for theirs growth and accumulated the maximum lipids content of 18.00±0.83 and 21.67±0.02% (w/w), respectively. Similar with the result of Wild et al. (2010), who revealed that the lipids production from *Lipomyces starkeyi* in starch medium was 40% (w/w), which was higher than glucose medium (30% w/w). It was found that the newly isolate TC32 could produce high volumetric production of lipids in basal medium supplemented with rice residue of 1.26±0.01 g/L. In contrast, *Diozegia* sp. TISTR5792 could produce total carotenoids and β-carotene of 0.85±0.10 and 0.51±0.04 mg/L which were higher than newly isolate TC32 with the total carotenoids and β-carotene of 0.64±0.00 and 0.33±0.01 mg/L, respectively. The ability of direct bioconvert of starch and rice residue was confirmed by the α-amylase and AMG activities as presented in Table 4.3. The results found that *Diozegia* sp. TISTR5792 and isolate TC32 could produce extracellular amylolytic enzymes with α-amylase activity of 0.25±0.18 and 0.54±0.09 U/mL and AMG activity of 0.020±0.00 and 0.023±0.00 U/mL, respectively. It was also demonstrated that the newly isolate TC32 could produce lipids content and amylolytic enzymes activity higher than *Diozegia* sp. TISTR5792. Hence, the newly isolate TC32 has been selected for this study.

Table 4.3 Characteristics of oleaginous red yeasts TC32 and TISTR5792 when cultivation in basal medium supplemented with either soluble starch or rice residue from food waste as a carbon source

Characteristics	Isolate TC32		TISTR5792	
	Soluble starch	Rice residue	Soluble starch	Rice residue
Biomass (g/L)	2.49±0.22^{c*}	5.82±0.30^{a}	3.37±0.38^{b}	5.29±0.30^{a}
Lipids (g/L)	0.63±0.07^{c}	1.26±0.01^{a}	0.70±0.21^{c}	0.97±0.01^{b}
Lipids content (% w/w)	24.00±1.7^{a}	21.67±0.02^{a}	21.65±3.9^{a}	18.41±0.83^{b}
Total carotenoids (mg/L)	0.45±0.01^{c}	0.64±0.00^{b}	0.54±0.03^{bc}	0.85±0.10^{a}
Total carotenoids yield (μg/g)	180.7±0.09^{a}	110.0±0.00^{c}	160.2±0.10^{b}	160.7±1.40^{b}
β-carotene (mg/L)	0.24±0.00^{b}	0.33±0.01^{b}	0.41±0.01^{a}	0.51±0.04^{a}
β-carotene yield (μg/g)	96.4±0.22^{b}	56.7±0.09^{c}	121.7±0.14^{a}	96.4±0.07^{b}
α-Amylase activity (U/mL)	0.14±0.04^{c}	0.54±0.09^{a}	0.21±0.10^{bc}	0.25±0.18^{b}
AMG activity (U/mL)	0.036±0.00^{a}	0.023±0.00^{b}	0.031±0.00^{a}	0.020±0.00^{b}

*Means and standard deviations of triplicate

[a]Different letters in same row indicate significantly treatments difference (p<0.05) according to the statistical analysis Duncan's multiple range test

4.6 Identification of oleaginous red yeast

The identification of oleaginous red yeast isolate C7, C10 and amylolytic oleaginous yeast TC32 were investigated. The 26S rDNA sequences of their isolates were partially determined and subjected to BLAST searching of the National Center Biotechnology Information (NCBI) databases. Alignment results of rDNA sequences of their isolates revealed that the sequence isolate C7, C10 and newly amylolytic oleaginous red yeast TC32 were found to have 99, 97 and 100% similarity with the D1/D2 26S rDNA sequences of strain *Rhodotorula glutinis* CBS2203, *Rhodosporidium* sp. APSS849 and *Sporidiobolus pararoseus* CBS7716, respectively. The position of each strain in phylogeny and a number of sequences were selected from GenBank database for the construction of a phylogenetic tree as shown in Figure 4.4–4.6. The sequences of isolate C7, C10 and TC32 have been deposited to the GenBank under the accession number of KM281508, KX281510 and KX709872, respectively.

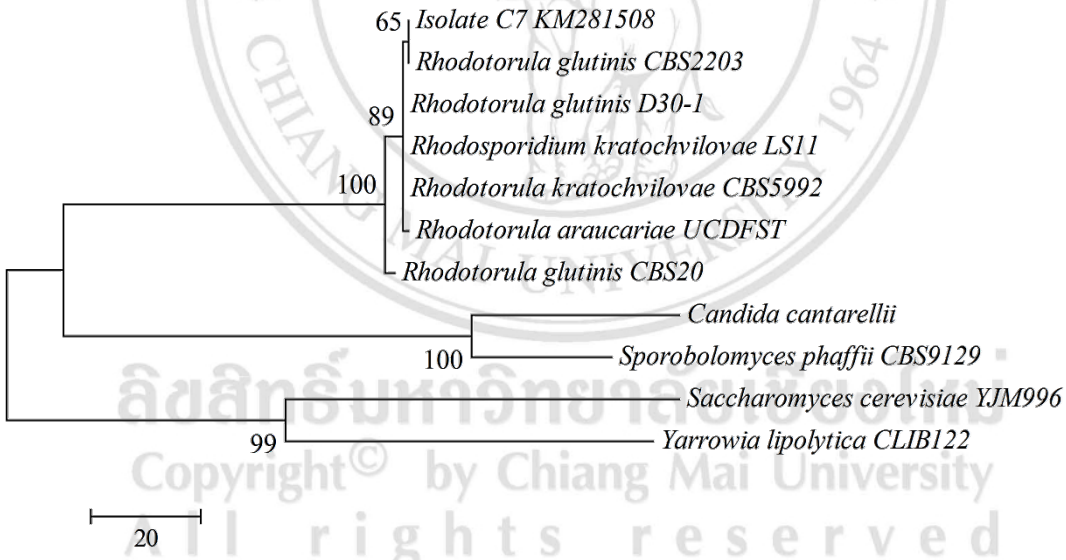


Figure 4.4 Phylogenetic relationships between *Rhodotorula glutinis* KM281508 and other 26S rDNA sequence of published strains

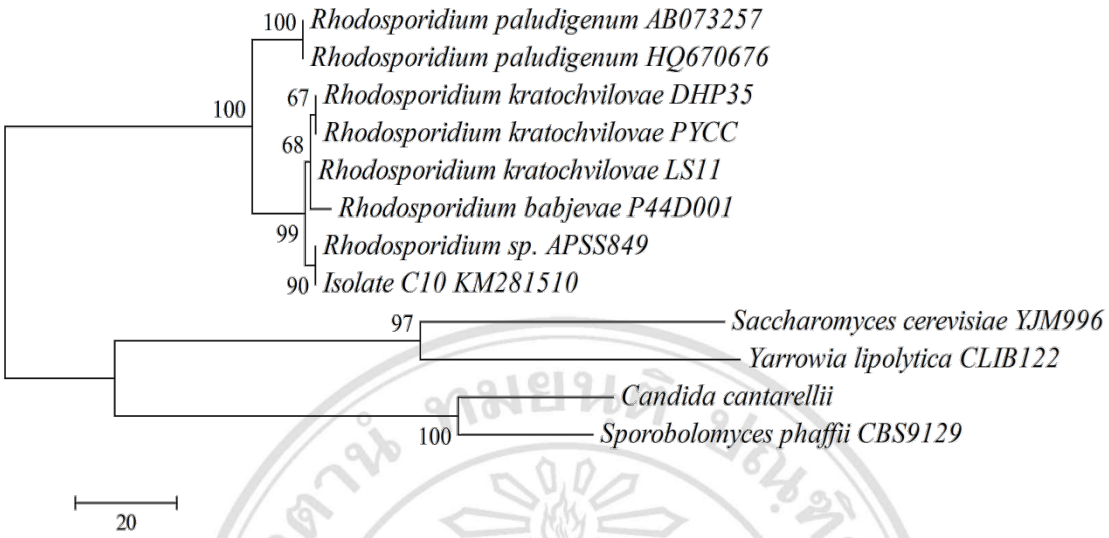


Figure 4.5 Phylogenetic relationships between *Rhodosporidium* sp. KX281510 and other 26S rDNA sequence of published strains

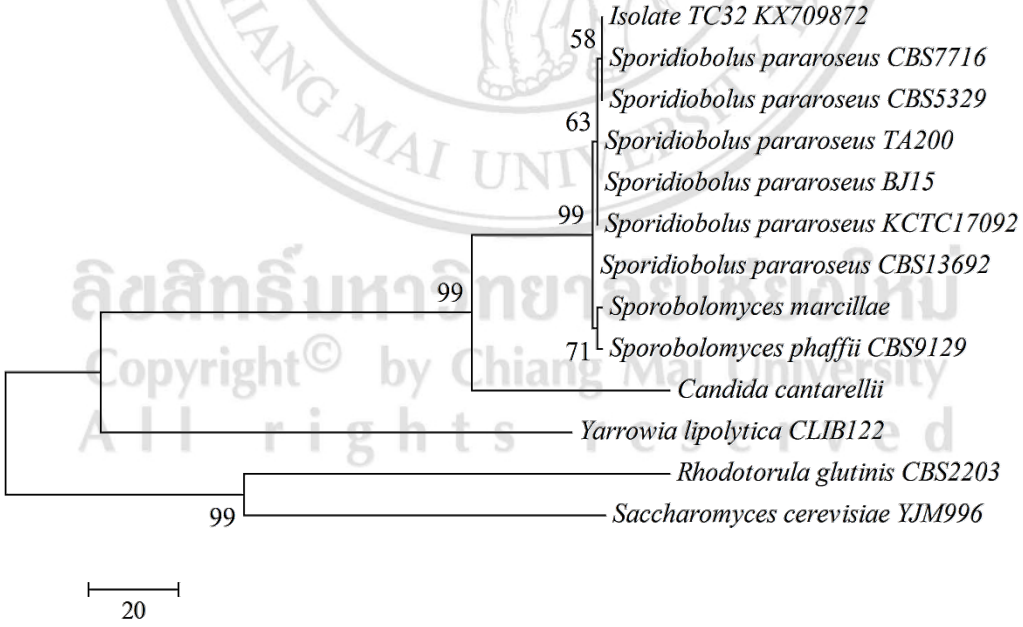


Figure 4.6 Phylogenetic relationship between *Sporidiobolus pararoseus* KX709872 and other 26S rDNA sequence of published strains

4.7 Effect of medium compositions on biomass, lipids and carotenoids productions by selected oleaginous red yeast *Sporidiobolus pararoseus* KX709872

Various medium components at different concentrations were investigated using the Plackett-Burman design. The designed matrix for the screening of significant variables for productions of biomass, lipids, lipids content, total carotenoids, total carotenoids yield, β-carotene and β-carotene yield and corresponding responses are shown in the Table 4.4. The variables at confidence level above 90% (p<0.1) was considered significant factor. The results revealed that, only one factor of C:N ratio which expressed in term of rice residue concentration, was significant and influenced on all response including biomass, lipids, lipids content, total carotenoids, total carotenoids yield, β-carotene and β-carotene yield with the p-values of 0.011, 0.004, 0.093, 0.000, 0.001, 0.008 and 0.007, respectively (Table 4.5). The C:N ratio is the important factor for lipogenesis and carotenoids synthesis in oleaginous red yeasts. At high C:N ratio leads to high lipids and carotenoids productions (Braunwald et al., 2013). Oleaginous yeasts store lipids mainly in the form of triacylglycerols (TAG) in intracellular lipids bodies during limitation of nitrogen source period (Saenge et al., 2011a). However, the growth of yeast, lipids content and carotenoids are significantly influenced by the C:N ratio of the cultivation medium which depends on the studied yeast species and the cultivation conditions (Kolouchová et al., (2015). The corresponding responses of the biomass (Y_1), lipids (Y_2), lipids content (Y_3), total carotenoids (Y_4), total carotenoids yield (Y_5), β-carotene (Y_6) and β-carotene yield (Y_7) were expressed in terms of the following regression equation 4.1–4.7

Biomass (g/L) = $-0.612 +0.181X_1 +0.073X_2 +0.150X_3 +0.794X_4 -0.851X_5 -6.463X6 - 0.126X_7 +0.574X_8$ (4.1)

Lipids (g/L) = $-1.279 +0.074X_1 +0.021X_2 +0.094X_3 +0.625X_4 -0.427X_5 +2.994X_6 - 0.112X_7 +0.094X_8$ (4.2)

Lipids content (% w/w) = $6.019 +0.368X_1 +0.215X_2 +0.618X_3 +4.235X_4 -1.745X_5 +36.685X_6 -0.721X_7 -0.175X_8$ (4.3)

Total carotenoids (mg/L) = $-2.820 +0.115X_1 +0.096X_2 +0.127X_3 +0.196X_4 -0.646X_5 - 5.039X_6 -0.135X_7 +0.392X_8$ (4.4)

Total carotenoids yield (μg/g) = $-20.621 + 0.854X_1 + 1.180X_2 + 1.303X_3 + 2.658X_4 - 3.618X_5 - 20.599X_6 - 1.072X_7 + 2.895X_8$ (4.5)

β-carotene (mg/L) = $-1.504 + 0.058X_1 + 0.090X_2 + 0.043X_3 + 0.150X_4 - 0.267X_5 - 4.306X_6 - 0.064X_7 + 0.246X_8$ (4.6)

β-carotene yield (μg/g) = $-10.260 + 0.395X_1 + 1.024X_2 + 0.456X_3 + 2.261X_4 - 0.921X_5 - 22.695X_6 - 0.419X_7 + 1.831X_8$ (4.7)

As shown in Table 4.4, run order 4 with different combination of medium component levels enhanced maximum volumetric production of lipids and lipids content of 4.62 g/L and 43.07% (w/w), respectively. Thus, in order to enhance the lipids production, the medium component of run order 4 was selected to be as the suitable cultivation medium for the next experiment.

Table 4.4 Twelve-trial Plackett-Burman design matrixes for eight variables and actual value of seven responses

Run	Experimental values								Responses						
	X_1**(C:N)	X_2	X_3	X_4	X_5	X_6	X_7	X_8	Biomass (g/L)	Lipids (g/L)	Lipids content (% w/w)	Total carotenoids (mg/L)	Total carotenoids yield (µg/g)	β-carotene (mg/L)	β-carotene yield (µg/g)
1	6:1(50.0)*	0.5	10.0	0.1	0.2	0.01	10.0	5.0	12.17	2.49	20.48	4.90	402.3	2.55	209.7
2	15:1 (50.0)	7.0	1.0	1.0	0.2	0.01	1.0	5.0	12.36	3.43	27.31	5.62	454.9	3.46	280.1
3	18:1 (25.0)	7.0	10.0	0.1	2.0	0.01	1.0	0.5	5.02	0.95	20.50	0.80	160.1	0.49	97.6
4	23:1 (50.0)	0.5	10.0	1.0	0.2	0.1	1.0	0.5	10.03	4.62	43.07	3.67	365.9	1.27	126.6
5	7:1 (50.0)	7.0	1.0	1.0	2.0	0.01	10.0	0.5	7.19	1.51	22.28	1.36	188.7	0.99	137.0
6	15:1 (50.0)	7.0	10.0	0.1	2.0	0.1	1.0	5.0	10.30	3.23	30.03	4.84	469.6	2.71	263.0
7	4:1 (25.0)	7.0	10.0	1.0	0.2	0.1	10.0	0.5	5.01	0.92	17.57	0.60	119.8	0.50	100.7
8	3:1 (25.0)	0.5	10.0	1.0	2.0	0.01	10.0	5.0	5.35	0.88	17.14	0.64	118.8	0.44	81.9
9	9:1 (25.0)	0.5	1.0	1.0	2.0	0.1	1.0	5.0	6.03	0.88	16.17	0.65	108.3	0.55	91.1
10	7:1 (50.0)	0.5	1.0	0.1	2.0	0.1	10.0	0.5	5.34	0.80	16.56	0.23	43.8	0.19	36.1
11	3:1 (25.0)	7.0	1.0	0.1	0.2	0.1	10.0	5.0	5.37	0.92	18.61	0.65	121.7	0.42	77.7
12	18:1 (25.0)	0.5	1.0	0.1	0.2	0.01	1.0	0.5	3.48	0.48	14.49	0.05	15.5	0.04	11.3

*Values in parentheses are the concentration of rice residue from food waste (g/L)

**X_1: C:N ratio (Rice residue (g/L)), X_2: K_2HPO_4 (g/L), X_3: KH_2PO_4 (g/L), X_4: NaCl (g/L), X_5: $MgSO_4.7H_2O$ (g/L), X_6: $MnSO_4.H_2O$ (g/L), X_7: $(NH_4)_2SO_4$ (g/L) and X_8: Yeast extract (g/L)

Note: The observed values of lipids (g/L), biomass (g/L), lipids content (% w/w), total carotenoids (mg/L), total carotenoids yield (µg/g), β-carotene (mg/L) and β-carotene yield (µg/g), were the mean values of triplicate

Table 4.5 Corresponding *p*-values for productions of biomass, lipids, lipids content, total carotenoids, total carotenoids yield, β-carotene and β-carotene yield for eight variables by the Plackett-Burman design experiment

Variables	Symbol codes	*p*-value						
		Biomass (g/L)	**Lipids (g/L)**	**Lipids content (% w/w)**	**Total carotenoids (mg/L)**	**Total carotenoids yield (μg/g)**	**β-carotene (mg/L)**	**β-carotene yield (μg/g)**
C:N ratio (Rice residue)	X_1	0.004*	0.011*	0.093*	0.000*	0.001*	0.008*	0.007*
K_2HPO_4	X_2	0.459	0.710	0.736	0.036*	0.011*	0.086*	0.022*
KH_2PO_4	X_3	0.095*	0.082*	0.238	0.007*	0.003*	0.197	0.078*
NaCl	X_4	0.292	0.185	0.388	0.378	0.160	0.604	0.279
$MgSO_4.7H_2O$	X_5	0.072*	0.101	0.467	0.007*	0.017*	0.132	0.352
$MnSO_4.H_2O$	X_6	0.376	0.472	0.447	0.077*	0.266	0.196	0.279
$(NH_4)_2SO_4$	X_7	0.137	0.055*	0.185	0.006*	0.005*	0.092*	0.094*
Yeast extract	X_8	0.019*	0.288	0.848	0.002*	0.002	0.018*	0.013*
Confidence levels		90%	90%	90%	90%	90%	90%	90%
R^2		0.9727	0.9511	0.8200	0.9946	0.9944	0.9654	0.9730

*Significant at $p \leq 0.10$

4.8 Optimization of cultivation condition using response surface methodology (RSM) via a central composite design (CCD)

4.8.1 Optimization of condition for biomass and lipids productions

The influence of C:N ratio (X_1), initial pH of production medium (X_9), cultivation temperature (X_{10}) and agitation rate (X_{11}) on biomass, lipids and lipids content of strain KX709872 were investigated using a central composite design (CCD). The CCD matrix for 27 sets of experimental and actual responses for biomass, lipids and lipids content are presented in Table 4.6, while the analysis of variance (ANOVA) for a response surface quadratic model for all responses is shown in Table 4.7.

The CCD generated a quadratic equation for volumetric production of biomass, volumetric production of lipids and lipids content as a function of C:N ratio (X_1), pH (X_9), temperature (X_{10}) and agitation rate (X_{11}) as equation 4.8–4.10

Biomass (g/L) = $14.11 + 4.75X_1 - 0.32X_9 - 0.23X_{10} + 1.04X_{11} - 0.45X_1^2 - 0.56X_9^2 - 1.20X_{10}^2 - 1.20X_{11}^2 + 4.37\times10^{-3}X_1X_9 - 0.11X_1X_{10} + 0.97X_1X_{11} - 0.038X_9X_{10} + 0.22X_9X_{11} + 0.44X_{10}X_{11}$ (4.8)

Lipids (g/L) = $6.31 + 1.63X_1 - 0.14X_9 - 0.57X_{10} + +0.58X_{11} - 0.57X_1^2 - 0.44X_9^2 - 0.70X_{10}^2 - 0.92X_{11}^2 - 0.076X_1X_9 - 0.27X_1X_{10} + 0.69X_1X_{11} + 0.10X_9X_{10} + 0.14X_9X_{11} - 0.15X_{10}X_{11}$ (4.9)

Lipids content (% w/w) = $44.78 + 0.99X_1 + 0.30X_9 - 4.37X_{10} + 1.48X_{11} - 4.41X_1^2 - 1.23X_9^2 - 1.54X_{10}^2 - 4.06X_{11}^2 - 1.76X_1X_9 - 1.10X_1X_{10} + 3.46X_1X_{11} + 1.07X_9X_{10} + 0.042X_9X_{11} - 2.69X_{10}X_{11}$ (4.10)

In this experiment, a *p*-value less than 0.05 indicated significant model terms and values, whereas greater than 0.10 indicated nonsignificant model terms. The terms of The quality of the models was expressed in terms of the R^2, C.V., *F*-values and *p*-values (Table 4.7). The R^2 values of the models for biomass, lipids and lipids content were relatively high at 0.9669, 0.9753 and 0.8061, respectively. Generally, the closer R^2 is to 1, the stronger the model and the better it predicts the response (Chaiyaso et al., 2011). These data demonstrated that up to 81–97% of the variations in volumetric productions of biomass and lipids, and lipids content could be described by these equations (4.8–4.10). The coefficient of variation (C.V.) indicates the degree of precision with which the treatments are compared and is a good index of reliability of the experiment (Zhang et al., 2012). The C.V. value of the models for biomass, lipids and lipids content

were relatively low at 0.1217, 0.1223 and 0.1593, respectively. The lower value of C.V. indicated a better precision and reliability of the experiment.

Table 4.6 The CCD matrix for the experimental design and actual responses for biomass, lipids and lipids content

Std Order	Coded levels				Y_1**	Y_2	Y_3
	X_1*	X_9	X_{10}	X_{11}			
1	18:1 (25.0)	5.00	20.00	100.00	6.98	2.28	32.57
2	25:1 (75.0)	5.00	20.00	100.00	14.44	4.94	34.19
3	18:1 (25.0)	7.00	20.00	100.00	6.23	2.61	41.93
4	25:1 (75.0)	7.00	20.00	100.00	14.03	4.29	30.55
5	18:1 (25.0)	5.00	30.00	100.00	6.5	2.32	35.64
6	25:1 (75.0)	5.00	30.00	100.00	12.9	3.82	29.59
7	18:1 (25.0)	7.00	30.00	100.00	6.27	2.16	41.66
8	25:1 (75.0)	7.00	30.00	100.00	10.49	2.73	26.04
9	18:1 (25.0)	5.00	20.00	200.00	7.12	2.87	39.56
10	25:1 (75.0)	5.00	20.00	200.00	15.93	7.61	47.79
11	18:1 (25.0)	7.00	20.00	200.00	6.02	2.26	37.49
12	25:1 (75.0)	7.00	20.00	200.00	16.47	7.26	44.12
13	18:1 (25.0)	5.00	30.00	200.00	6.21	1.43	22.97
14	25:1 (75.0)	5.00	30.00	200.00	17.17	5.07	29.54
15	18:1 (25.0)	7.00	30.00	200.00	6.23	2.01	32.29
16	25:1 (75.0)	7.00	30.00	200.00	17.46	6.08	34.83
17	0:1 (0.0)	6.00	25.00	150.00	0.74	0.12	16.33
18	27:1 (100.0)	6.00	25.00	150.00	24.12	7.71	31.97
19	23:1 (50.0)	4.00	25.00	150.00	12.93	5.08	39.3
20	23:1 (50.0)	8.00	25.00	150.00	11.08	3.81	34.4
21	23:1 (50.0)	6.00	15.00	150.00	9.8	4.70	47.93
22	23:1 (50.0)	6.00	35.00	150.00	9.03	2.11	23.3
23	23:1 (50.0)	6.00	25.00	50.00	6.89	1.43	20.77
24	23:1 (50.0)	6.00	25.00	250.00	11.98	3.63	30.31
25	23:1 (50.0)	6.00	25.00	150.00	13.71	6.66	48.6
26	23:1 (50.0)	6.00	25.00	150.00	14.77	6.17	41.75
27	23:1 (50.0)	6.00	25.00	150.00	13.86	6.09	43.99

*X_1: C:N ratio (Rice residue (g/L)), X_9: pH, X_{10}: Temperature and X_{11}: Agitation rate

**Y_1: Biomass (g/L), Y_2: Lipids (g/L) and Y_3: Lipids content (% w/w)

Table 4.7 The *p*-value, *F*-value, coefficient of variation (C.V.) and coefficient of determination (R^2) of the predicted second order polynomial models for biomass, lipids and lipids content

Coefficient	Biomass (g/L)		Lipids (g/L)		Lipids content (% w/w)	
	p-value	*F*-value	*p*-value	*F*-value	*p*-value	*F*-value
Model	<0.0001**	25.05	<0.0001**	33.85	0.0167**	3.56
X_1*	<0.0001**	298.07	<0.0001**	269.11	0.3974	0.77
X_9	0.2636	1.38	0.1694	2.14	0.7937	0.072
X_{10}	0.4190	0.70	<0.0001**	33.04	0.0023**	14.93
X_{11}	0.0026**	14.25	<0.0001**	33.82	0.2155	1.71
X_1^2	0.1503	2.36	0.0002**	29.50	0.0032**	13.49
X_9^2	0.0816	3.61	0.0013**	17.40	0.3251	1.05
X_{10}^2	0.0014**	16.96	<0.0001**	44.14	0.2235	1.65
X_{11}^2	0.0015**	16.82	<0.0001**	76.10	0.0054**	11.45
X_1X_9	0.9899	$1.68x10^{-4}$	0.5419	0.39	0.2274	1.62
X_1X_{10}	0.7567	0.10	0.0470**	4.90	0.4411	0.63
X_1X_{11}	0.0137**	8.33	0.0001**	32.28	0.0279**	6.25
X_9X_{10}	0.9119	0.013	0.4207	0.70	0.4553	0.60
X_9X_{11}	0.5230	0.43	0.2797	1.28	0.9760	$9.41x10^{-4}$
$X_{10}X_{11}$	0.2158	1.71	0.2556	1.43	0.0761	3.77
Lack of fit	0.1419	6.44	0.2940	2.77	0.2901	2.82
C.V.	0.1217		0.1223		0.1593	
R^2 of model	0.9669		0.9753		0.8061	

*X_1: C:N ratio (Rice residue (g/L)), X_9: pH, X_{10}: Temperature and X_{11}: Agitation rate, **Significant at $p \leq 0.05$

According to Table 4.7, the results indicated that C:N ratio (X_1) and agitation rate (X_{11}) had significant effect on lipids and biomass productions, while temperature (X_{10}) had a significant effect on lipids production and lipids content. The interaction term between C:N ratio and agitation rate (X_1X_{11}) was significant for all responses of lipids production, biomass and lipids content (p<0.05). Furthermore, the interaction term between the C:N ratio and temperature (X_1X_{10}) were also significant for lipids production.

As shown in Figure 4.7, it was found that the interaction term of C:N ratio and agitation rate level (Figure 4.7 (B3)) was strongly influenced on volumetric production of biomass, as a clear evidence from the positive coefficient in equation 4.8. The result revealed that the biomass was increased from 0.74 g/L at C:N ratio of 0:1 (low point –α) to 14.77 g/L at C:N ratio of 23:1 (center point) with agitation rate 150 rpm (Table 4.6). The C:N ratio and agitation rate enhanced biomass, lipids and lipids content because of the level of lipids and biomass biosynthesis in oleaginous yeast depends mostly on high C:N ratio and oxygenation (Saenge et al., 2011a). At high level of aeration and carbon source were increased of ATP synthesis which was required for the biomass formation. Kavšček et al. (2015) found that lower of ATP affects to the efficiency of enzyme and other energy consuming cellular activities. While, the interaction term between C:N ratio and pH (X_1X_9) (Figure 4.7 (B1)), C:N ratio and temperature (X_1X_{10}) (Figure 4.7 (B2)), pH and temperature (X_9X_{10}) (Figure 4.7 (B4)), pH and agitation rate (X_9X_{11}) (Figure 4.7 (B5)) and between temperature and agitation rate ($X_{10}X_{11}$) (Figure 4.7 (B6)) were nonsignificant model term for volumetric productions of biomass.

As shown in Figure 4.8, it was found that the interaction term between C:N ratio and temperature (X_1X_{10}) (Figure 4.8 (L2)) and between C:N ratio and agitation rate (X_1X_{11}) (Figure 4.8 (L3)) were significant for volumetric productions of lipids (p<0.05). Moreover, the interaction term of between C:N ratio and temperature (X_1X_{10}) was also found to influence volumetric production of lipids (Figure 4.8 (L2)). As the evidence from the negative coefficient of the equation 4.9, decreasing of lipids production under high temperature of 35°C (high point +α) was observed. The results showed that the volumetric productions of lipids decreased from 6.66 to 2.11 g/L when the temperature increased from 25°C (center point) to 35°C (high point +α) (Table 4.6). It might be that the enzyme activities, regulation and transport systems are in generally affected

enormously by high cultivation temperature (Anastassiadis and Rehm, 2006). While, the interaction term of between C:N ratio and agitation rate (X_1X_{11}) was found to enhance the volumetric production of lipids (Figure 4.8 (L3)). The volumetric production of lipids increased from 0.21 g/L at C:N ratio of 0:1 (low point –α) to 6.66 g/L at C:N ratio of 23:1 (center point) with agitation rate 150 rpm (Table 4.6). When oleaginous yeasts enter the limiting phase of nitrogen sources, they generate biomass at the expense of the accumulated lipids. The difference between oleaginous yeasts and non-oleaginous yeasts is the key enzyme that found in oleaginous yeast namely; ATP citrate lyase, which involves in converting citrate to be Acetyl-CoA and oxaloacetate. Acetyl-CoA is the initial building block for lipids biosynthesis (Sha, 2013). Braunwald et al. (2013) studied the effect of C:N ratio on carotenoids and lipids production by oleaginous red yeast *Rhodotorula glutinis*. The results revealed that both of lipids and carotenoids productions were increased at high C:N ratio. While, Saenge et al. (2011a) found that the C:N ratio contributed a significant effect on biomass, lipids content and carotenoids productions by *Rhodotorula glutinis* TISTR5159 which cultivated in palm oil mill effluent (POME).

Whereas, the interaction term between C:N ratio and pH (X_1X_9) (Figure 4.8 (L1)), pH and temperature (X_9X_{10}) (Figure 4.8 (L4)), pH and agitation rate (X_9X_{11}) (Figure 4.8 (L5)) and between temperature and agitation rate ($X_{10}X_{11}$) (Figure 4.8 (L6)) were nonsignificant model term for volumetric productions of lipids.

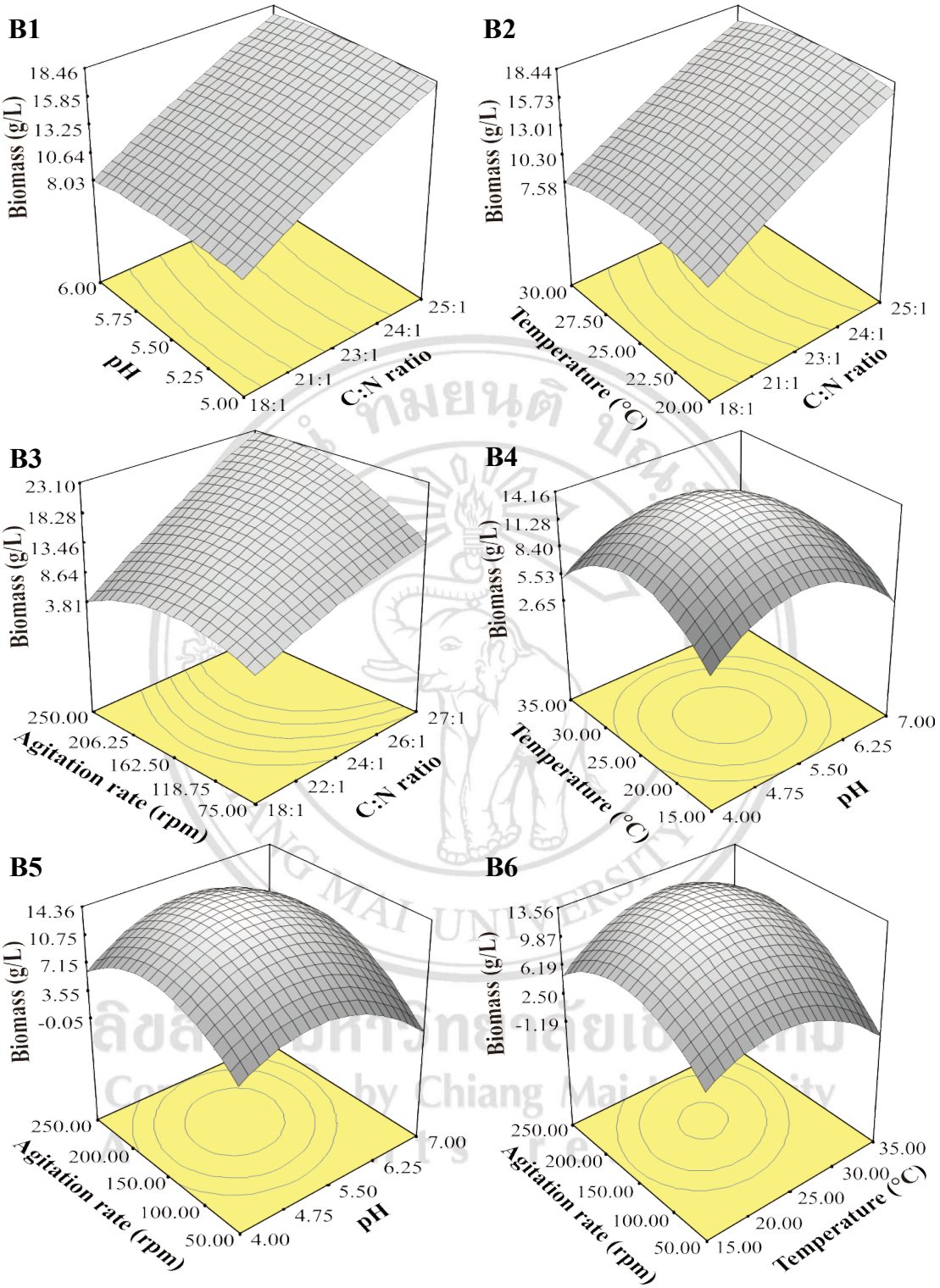


Figure 4.7 Volumetric production of biomass in 3D graphic for quadratic response surface optimization

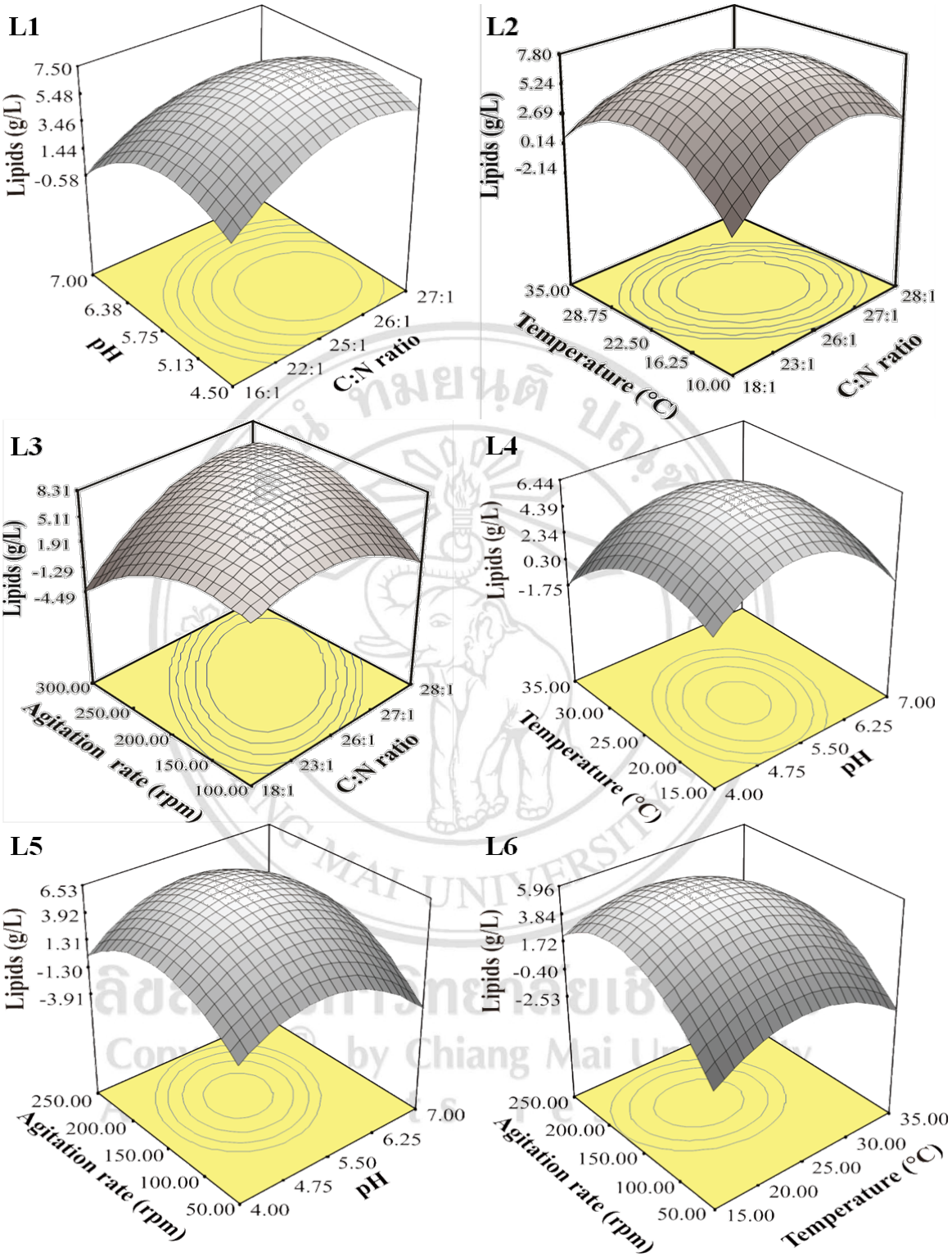


Figure 4.8 Volumetric production of lipids in 3D graphic for quadratic response surface optimization

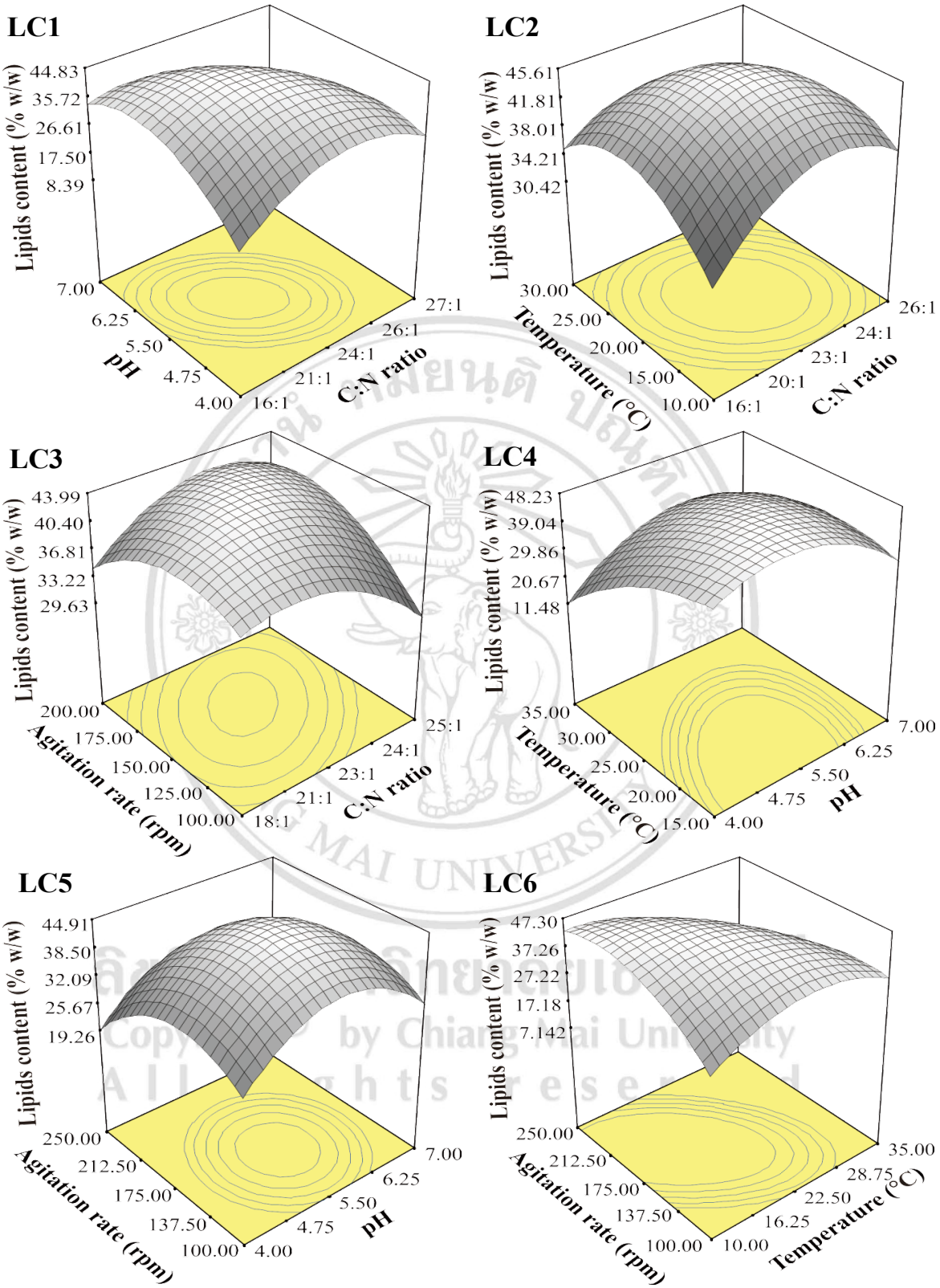


Figure 4.9 Lipids content in 3D graphic for quadratic response surface optimization

From Figure 4.9 (LC3) shows that the significant interaction term of between C:N ratio and agitation rate (X_1X_{11}) was found to enhance the lipids content, as a clear evidence from the positive coefficient in equation 4.10. The result revealed that the lipids content was increased from 16.33% (w/w) at C:N ratio of 0:1 (low point –α) to 48.6% (w/w) at C:N ratio of 23:1 (center point) with agitation rate 150 rpm (Table 4.6). The limitation of nitrogen source and low oxygenation increased lipids accumulation and reduced cell growth resulting in the high lipids content. As shown in Table 4.6 and Figure 4.9 (LC3), the lipids content was decreased from 48.60 to 30.31% (w/w) when the agitation rate increased from 150 rpm (center point) to 250 rpm (high point +α). According to Kavšček et al. (2015) who found that accumulation of lipids can not only be induced by limitation of nitrogen, but also by reduction of oxygen supply. However, the lipids content was decreased from 30.31 to 20.77% (w/w) when the agitation rate decreased from 150 rpm (center point) to 50 rpm (low point –α) as shown in Table 4.6 and Figure 4.9 (LC3). The low oxygenation or completely anaerobic conditions had strongly effected on lipids accumulation and cell growth that resulted in decreasing of lipids content and reduced cell growth of yeast cell (Kavšček et al., 2015).

Whereas, the interaction term between C:N ratio and pH (X_1X_9) (Figure 4.9 (LC1)), C:N ratio and temperature (X_1X_{10}) (Figure 4.9 (LC2)), pH and temperature (X_9X_{10}) (Figure 4.9 (LC4)), pH and agitation rate (X_9X_{11}) (Figure 4.9 (LC5)) and between temperature and agitation rate ($X_{10}X_{11}$) (Figure 4.9 (LC6)) were nonsignificant model term for lipids content.

4.8.2 Optimization of cultivation condition for carotenoids production

The influence of C:N ratio (X_1), initial pH of production medium (X_9), cultivation temperature (X_{10}) and agitation rate (X_{11}) on total carotenoids, total carotenoids yield, β-carotene and β-carotene yield of strain KX709872 were investigated using a central composite design (CCD). The CCD matrix for 27 sets of experimental and actual responses for total carotenoids, total carotenoids yield, β-carotene and β-carotene yield are presented in Table 4.8. While, the analysis of variance (ANOVA) for a response surface quadratic model for all responses is shown in Table 4.9.

The CCD generated a quadratic equation for volumetric production of total carotenoids and β-carotene and the yield of total carotenoids and β-carotene as a

function of C:N ratio (X_1), pH (X_9), temperature (X_{10}) and agitation rate (X_{11}) as equation 4.11–4.14

Total carotenoids (mg/L) = $-30.40040 + 0.12X_1 + 16.26X_9 - 0.85X_{10} - 0.04X_{11} - 7.25\times10^{-4}X_1^2 - 1.82X_9^2 + 4.42\times10^{-3}X_{10}^2 - 5.92\times10^{-5}X_{11}^2 - 6.95\times10^{-3}X_1X_9 - 2.91\times10^{-3}X_1X_{10} + 5.23\times10^{-4}X_1X_{11} + 0.11X_9X_{10} + 8.93\times10^{-3}X_9X_{11} + 4.35\times10^{-7}X_{10}X_{11}$ (4.11)

Total carotenoids yield (µg/g) = $-168.90 + 17.67X_1 + 819.24X_9 - 155.32X_{10} - 3.69X_{11} - 0.055X_1^2 - 90.94X_9^2 + 1.76X_{10}^2 + 1.97\times10^{-3}X_{11}^2 - 2.35X_1X_9 - 0.22X_1X_{10} + 0.03X_1X_{11} + 10.80X_9X_{10} + 0.33X_9X_{11} + 0.03X_{10}X_{11}$ (4.12)

β-carotene (mg/L) = $-4.97 + 0.08X_1 + 4.04X_9 - 0.36X_{10} - 0.02X_1 - 2.46\times10^{-4}X_1^2 - 0.36X_9^2 + 5.36\times10^{-4}X_{10}^2 - 6.07\times10^{-6}X_{11}^2 - 7.81\times10^{-3}X_1X_9 - 9.13\times10^{-4}X_1X_{10} + 1.02X_1X_{11} - 1.44\times10^{-3} X_9X_{10} + 8.79\times10^{-4}X_9X_{11} + 4.75\times10^{-4}X_{10}X_{11}$ (4.13)

β-carotene yield (µg/g) = $713.54 + 7.06X_1 + 109.08X_9 - 67.22X_{10} - 1.91X_{11} - 0.01X_1^2 - 4.25X_9^2 + 1.06X_{10}^2 + 2.68X_{11}^2 - 1.18X_1X_9 - 0.05X_1X_{10} + 7.00\times10^{-3}X_1X_{11} + 0.09X_9X_{10} - 0.09X_9X11 + 0.06X_{10}X_{11}$ (4.14)

In this experiment, a *p*-value less than 0.05 indicated significant model terms and values, whereas greater than 0.10 indicated nonsignificant model terms. The quality of the models was expressed in terms of the R^2, C.V., *p*-values and *F*-value (Table 4.9). Three-dimensional (3D) plots were used to represent the predicted model equation and thus the interaction between the different factors and the response (Derrien et al., 2017). The response surface plots of influencing factors of the total carotenoids, total carotenoids yield, β-carotene and β-carotene yield are shown in Figure 4.10–4.13.

The interaction term between C:N ratio and agitation rate (X_1X_{11}), C:N ratio and pH (X_1X_9), C:N ratio and temperature (X_1X_{10}), pH and temperature (X_9X_{10}), pH and agitation rate (X_9X_{11}) and between temperature and agitation rate ($X_{10}X_{11}$) were nonsignificant model term for all responses of total carotenoids (Figure 4.10 (T1–T6)), total carotenoids yield (Figure 4.11 (TY1–TY6)), β-carotene (Figure 4.12 (C1–C6)) and β-carotene yield (Figure 4.13 (CY1–CY6)) at $p<0.05$.

The probability (*p*-values) of models for total carotenoids, total carotenoids yield, β-carotene and β-carotene yield were more than 0.05, indicating a nonsignificant model.

It might be that culture medium component obtained from Plackett-Burman design which used in this experiment might be support on the biomass and lipids productions. According to the previously study that selected the medium component of run order 4 which was high C:N ratio and low concentration of nitrogen for high lipids content (Table 4.4). Since, the C:N ratio and concentration of nitrogen were influence on carotenoids production. From Table 4.4, at high C:N ratio of 23:1 (run order 4) gave the highest of lipids and lipids content of 4.62 g/L and 43.07% (w/w) and resulted in lower the total carotenoids and β-carotene of 3.67 and 1.27 mg/L, respectively. At low C:N ratio of 15:1 (run order 2), the highest of total carotenoids and β-carotene of 5.62 and 3.46 mg/L were obtained, while the lipids and lipids content were 3.43 g/L and 27.31% (w/w). Braunwald et al. (2013) reported that the carotenoids production was decreased at high C:N ratio (low nitrogen source). In contrast, lipids accumulation was increased at high C:N ratio. Somashekar and Joseph (2000) reported the reverse relationship between carotenoids and lipids formation of *Rhodotorula gracilis* according to the C:N ratio. They found that *Rhodotorula gracilis* produced the highest total carotenoids at low C:N ratio in the medium (10:1) which was 15 times higher than high C:N ratio (160:1). Whereas, this strain could produce lipids up to 55% (w/w) at high C:N ratio which was higher than low C:N ratio of 20% (w/w).

The R^2 values of the models for total carotenoids, total carotenoids yield, β-carotene and β-carotene yield by stain KX709872 were 0.7120, 0.5853, 0.5869 and 0.5407, respectively. The coefficient of variation (C.V.) indicates the degree of precision with which the treatments are compared and is a good index of reliability of the experiment (Zhang et al., 2012). The C.V. value of total carotenoids, total carotenoids yield, β-carotene and β-carotene yield were relatively high at 0.5910, 0.7161, 0.6300 and 0.7480, respectively. A high value of C.V. showed a lesser precision and reliability of the experiments conducted. The results revealed that those models were not accurately fit the data. It might be that culture medium component obtained from Plackett-Burman design which used in this experiment supported the biomass production and lipogenesis more than carotenogenesis.

Table 4.8 The CCD matrix for the experimental design and actual responses for total carotenoids, total carotenoids yield, β-carotene and β-carotene yield

Std Order	Coded levels				Y_4**	Y_5	Y_6	Y_7
	X_1*	X_9	X_{10}	X_{11}				
1	18:1 (25.0)	5.00	20.00	100.00	2.35	337.15	1.39	199.2
2	25:1 (75.0)	5.00	20.00	100.00	1.95	134.76	1.22	84.3
3	18:1 (25.0)	7.00	20.00	100.00	2.03	325.14	1.29	207.2
4	25:1 (75.0)	7.00	20.00	100.00	1.64	116.74	1.35	96.4
5	18:1 (25.0)	5.00	30.00	100.00	0.80	122.44	0.48	74.4
6	25:1 (75.0)	5.00	30.00	100.00	1.31	101.42	1.06	81.8
7	18:1 (25.0)	7.00	30.00	100.00	2.84	453.34	1.28	203.4
8	25:1 (75.0)	7.00	30.00	100.00	0.12	11.53	0.08	7.9
9	18:1 (25.0)	5.00	20.00	200.00	1.37	192.83	0.71	99.1
10	25:1 (75.0)	5.00	20.00	200.00	4.52	283.87	1.53	95.9
11	18:1 (25.0)	7.00	20.00	200.00	1.76	292.93	0.88	146.2
12	25:1 (75.0)	7.00	20.00	200.00	4.54	275.80	1.38	83.6
13	18:1 (25.0)	5.00	30.00	200.00	1.65	265.51	1.04	166.9
14	25:1 (75.0)	5.00	30.00	200.00	1.31	76.44	0.89	51.9
15	18:1 (25.0)	7.00	30.00	200.00	2.24	360.15	0.98	157.5
16	25:1 (75.0)	7.00	30.00	200.00	4.10	234.96	1.13	64.6
17	0:1 (0.0)	6.00	25.00	150.00	0.02	24.13	0.01	18.8
18	27:1 (100.0)	6.00	25.00	150.00	3.06	126.75	1.06	43.9
19	23:1 (50.0)	4.00	25.00	150.00	2.53	195.77	1.49	115.1
20	23:1 (50.0)	8.00	25.00	150.00	0.52	47.05	0.09	8.1
21	23:1 (50.0)	6.00	15.00	150.00	7.29	743.74	3.32	338.8
22	23:1 (50.0)	6.00	35.00	150.00	0.29	32.56	0.05	6.0
23	23:1 (50.0)	6.00	25.00	50.00	0.06	8.98	0.06	8.0
24	23:1 (50.0)	6.00	25.00	250.00	5.45	455.18	2.13	177.4
25	23:1 (50.0)	6.00	25.00	150.00	3.21	233.83	1.12	81.9
26	23:1 (50.0)	6.00	25.00	150.00	3.50	237.22	1.39	94.3
27	23:1 (50.0)	6.00	25.00	150.00	2.85	205.58	0.97	69.9

*X_1: C:N ratio (Rice residue (g/L)), X_9: pH, X_{10}: Temperature and X_{11}: Agitation rate

**Y_4: Total carotenoids (mg/L), Y_5: Total carotenoids yield (μg/g), Y_6: β-carotene (mg/L) and Y_7: β-carotene yield (μg/g)

Table 4.9 The *p*-value, *F*-value, coefficient of variation (C.V.) and coefficient of determination (R^2) of the predicted second order polynomial models for total carotenoids, total carotenoids yield, β-carotene and β-carotene yield

Coefficient	Total carotenoids (mg/L)		Total carotenoids yield (μg/g)		β-carotene (mg/L)		β-carotene yield (μg/g)	
	p-value	*F*-value	*p*-value	*F*-value	*p*-value	*F*-value	*p*-value	*F*-value
Model	0.0997	2.12	0.3752	1.21	0.3701	1.22	0.4995	1.01
X_1*	0.1471	2.40	0.2519	1.45	0.4250	0.68	0.1173	2.85
X_9	0.9999	$1.30x10^{-8}$	0.7385	0.12	0.4154	0.71	0.7945	0.07
X_{10}	0.0130**	8.48	0.0411**	5.24	0.0138**	8.30	0.0401**	5.30
X_{11}	0.0150**	8.04	0.1232	2.75	0.1888	1.94	0.5207	0.44
X_1^2	0.1571	2.28	0.3372	1.00	0.3050	1.15	0.6142	0.27
X_9^2	0.1548	2.31	0.5166	0.45	0.5402	0.40	0.9503	$4.06x10^{-3}$
X_{10}^2	0.7191	0.14	0.2174	1.69	0.3679	0.88	0.1366	2.55
X_{11}^2	0.6309	0.24	0.8852	0.02	0.9174	0.01	0.6949	0.16
X_1X_9	0.8064	0.06	0.4617	0.58	0.5663	0.35	0.4587	0.59
X_1X_{10}	0.3146	1.10	0.4936	0.50	0.5036	0.48	0.7410	0.11
X_1X_{11}	0.0838	3.56	0.3346	1.01	0.4534	0.60	0.6579	0.21
X_9X_{10}	0.4360	0.65	0.5075	0.47	0.9829	$4.77x10^{-4}$	0.9911	$1.30x10^{-4}$
X_9X_{11}	0.5315	0.42	0.8395	0.04	0.8966	0.02	0.9066	0.01
$X_{10}X_{11}$	0.9998	$9.87x10^{-8}$	0.8552	0.03	0.4867	0.52	0.4593	0.58
Lack of fit	0.0457**	21.28	0.0102**	97.19	0.0851	11.15	0.0209**	47.33
C.V.	0.5910		0.7161		0.6300		0.7480	
R^2 of model	0.7120		0.5853		0.5869		0.5407	

*X_1: C:N ratio (Rice residue (g/L)), X_9: pH, X_{10}: Temperature and X_{11}: Agitation rate, **Significant at $p \leq 0.05$

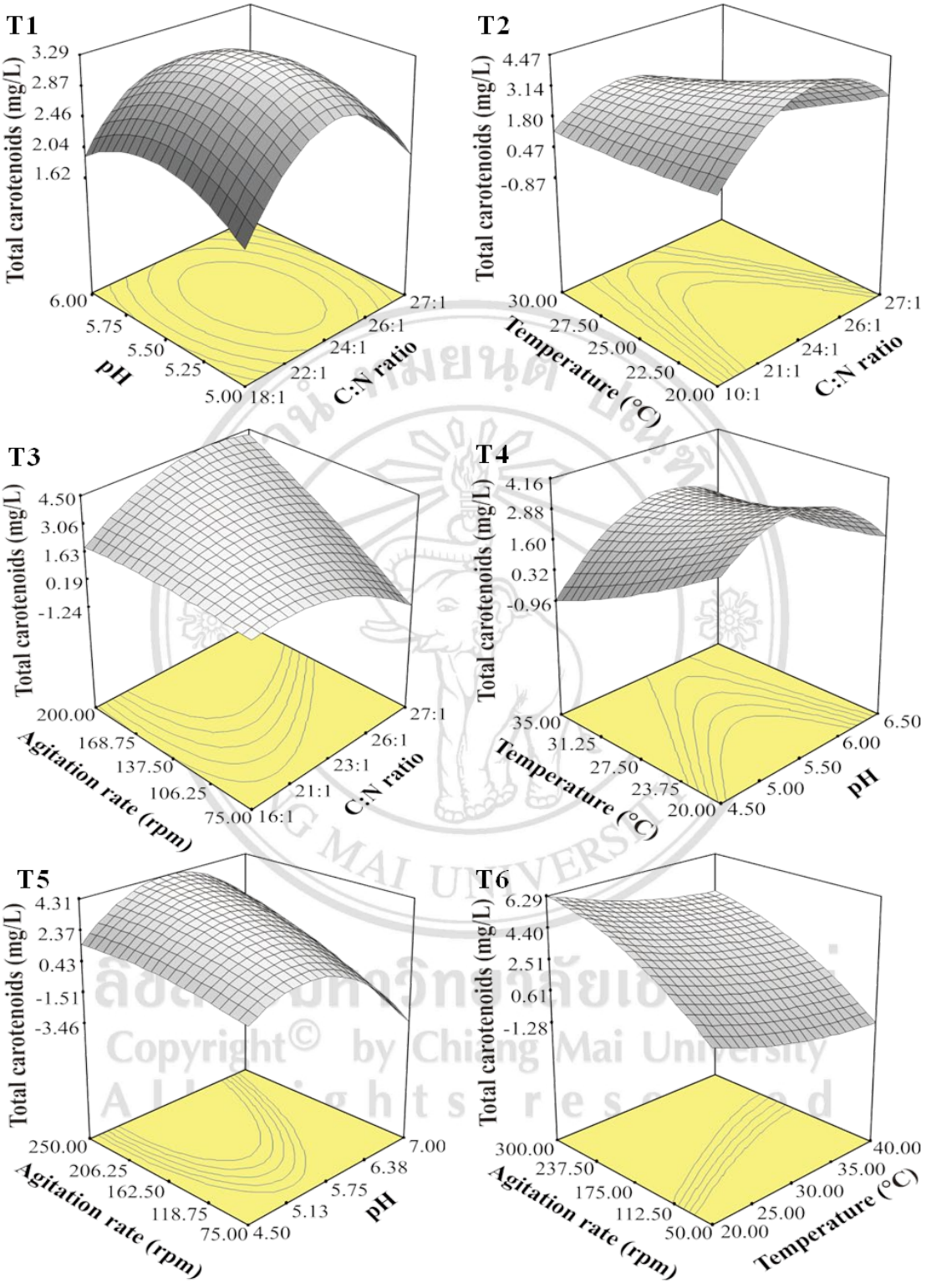


Figure 4.10 Volumetric production of total carotenoids in 3D graphic for quadratic response surface optimization

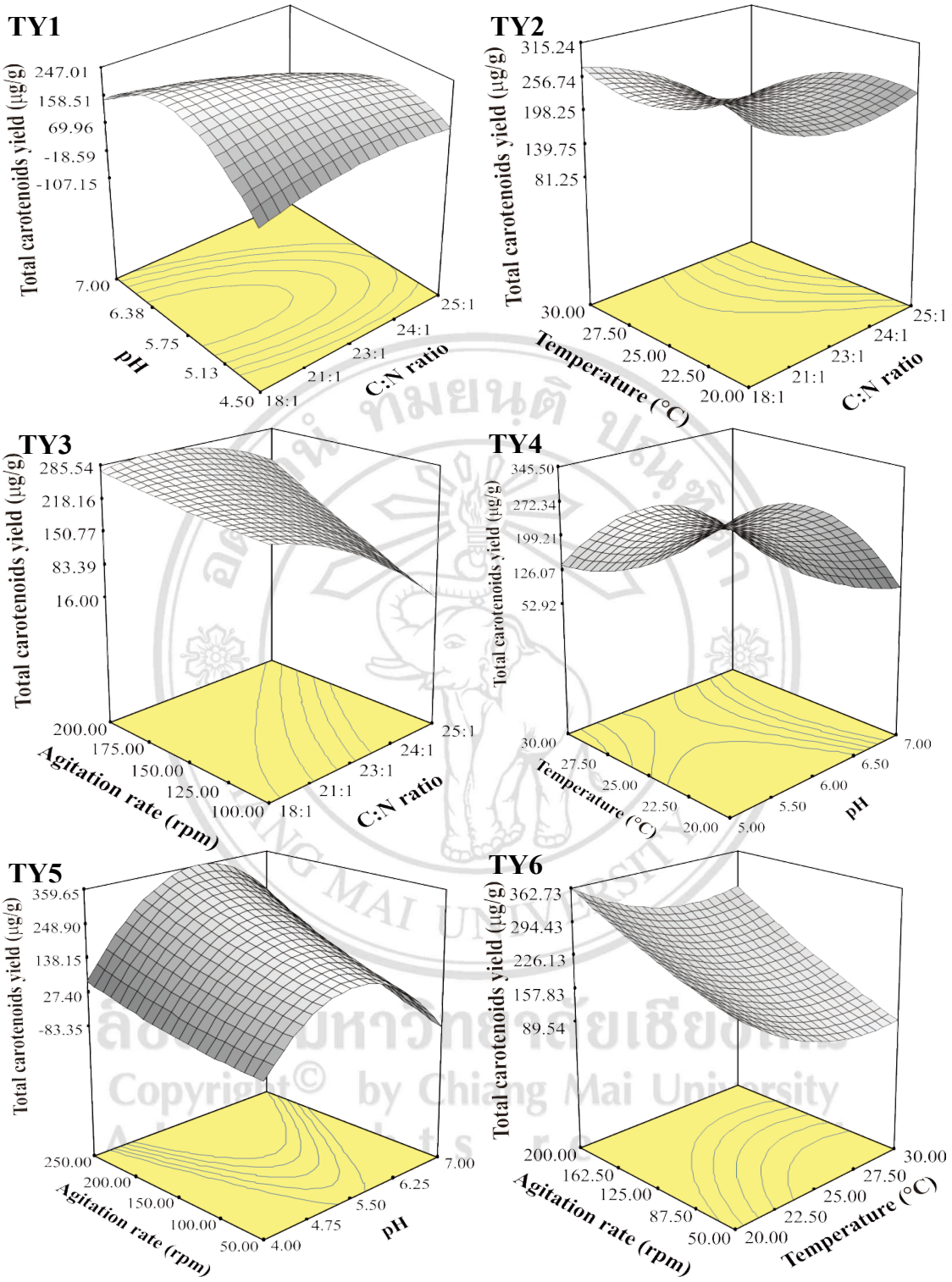


Figure 4.11 The yield of total carotenoids in 3D graphic for quadratic response surface optimization

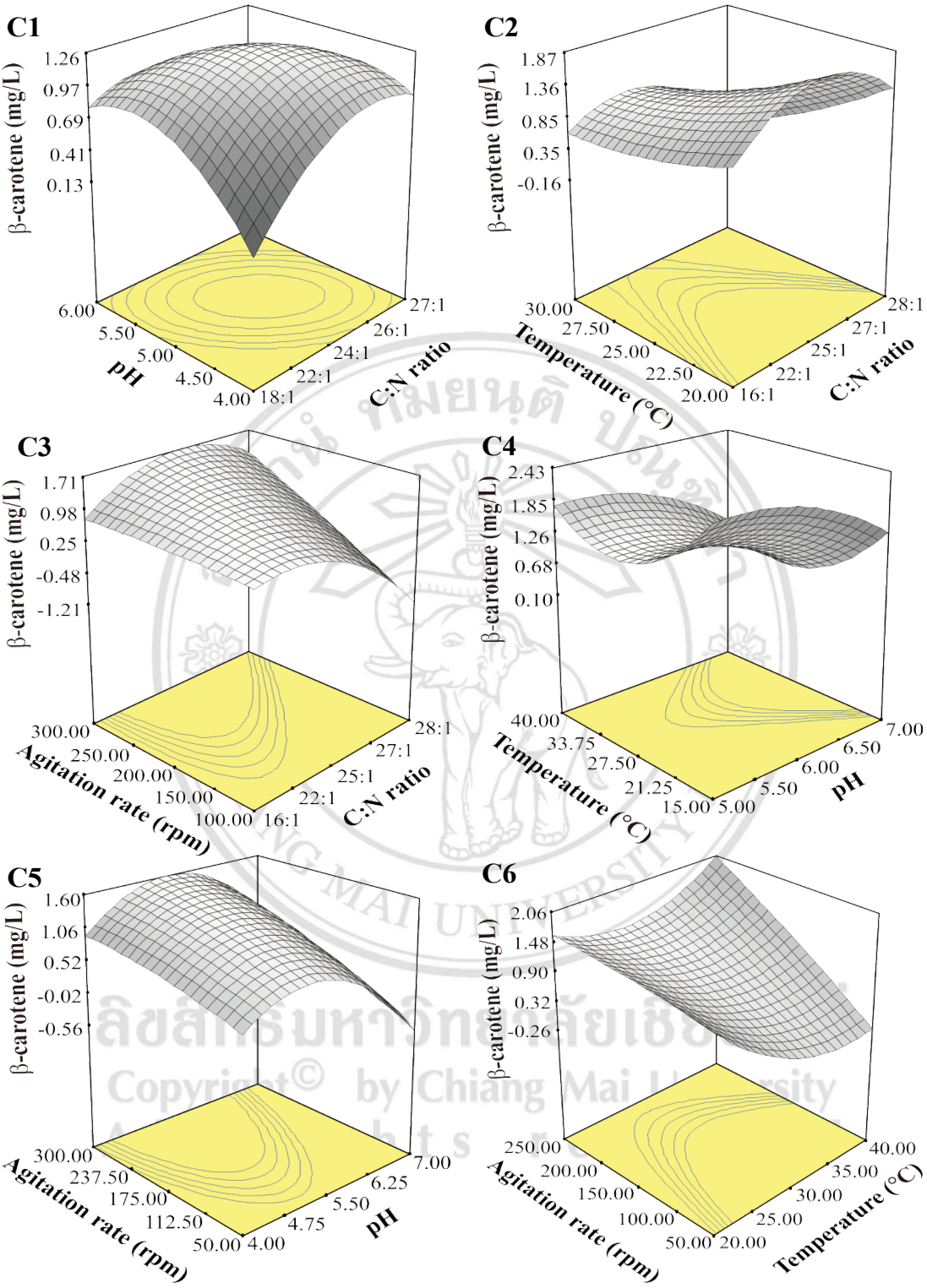


Figure 4.12 Volumetric production of β-carotene in 3D graphic for quadratic response surface optimization

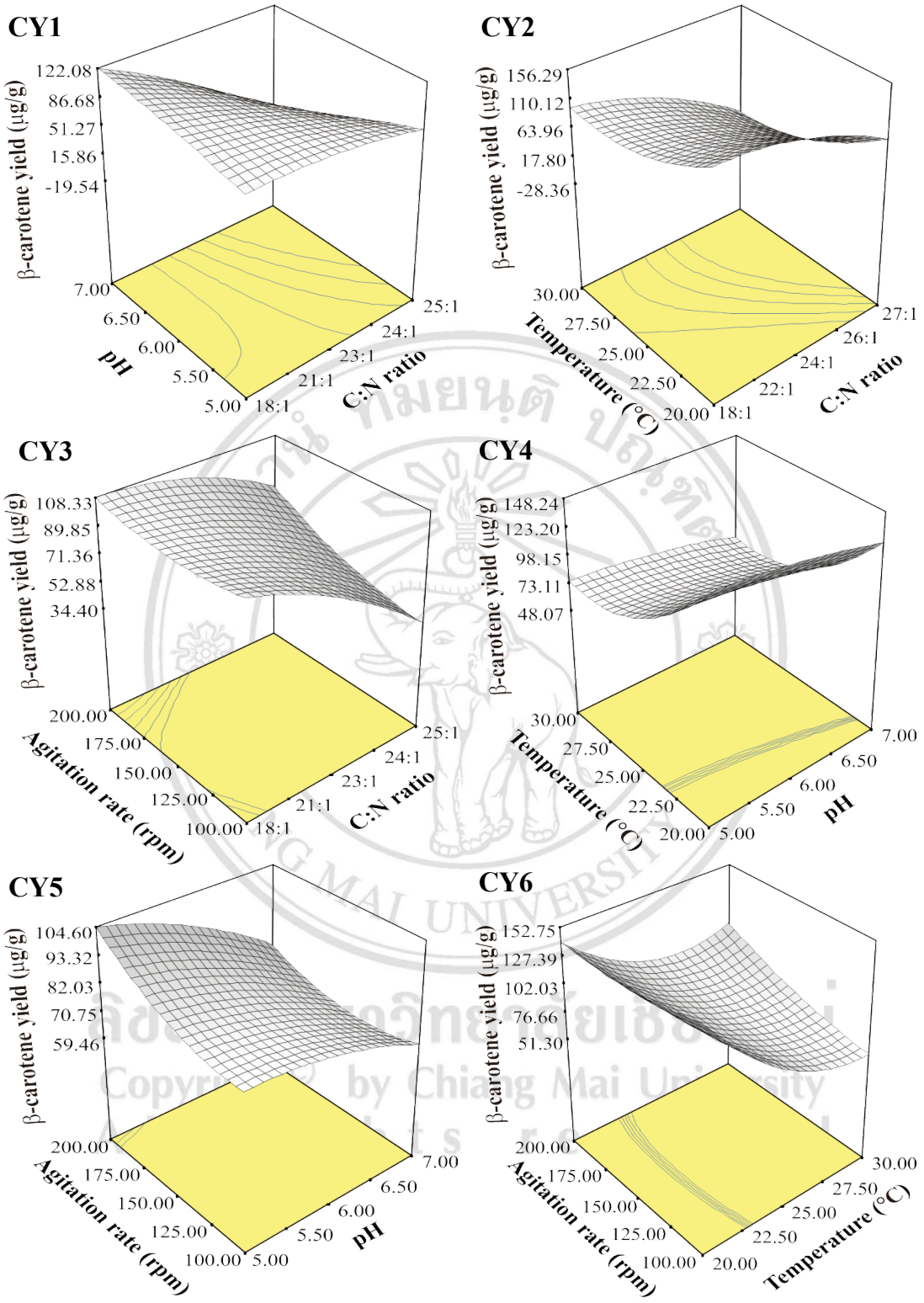


Figure 4.13 The yield of β-carotene in 3D graphic for quadratic response surface optimization

4.9 Validation of the model for biomass, lipids and carotenoids productions

To confirm the applicability of the CCD optimization model, the biomass, lipids and carotenoids productions by strain KX709872 was cultivated under the optimal conditions for the maximal the productions of biomass, lipids and carotenoids. From the experimental results as shown in Table 4.10, the three validating model for maximum biomass, lipids and carotenoids were obtained.

The optimal conditions for the maximum biomass (C:N ratio of 25:1, pH of 5.44, temperature of 25.34°C and agitation rate of 192.73 rpm) gave the highest biomass, lipids and carotenoids productions. From this condition, the volumetric productions of biomass and lipids and lipids content of strain KX709872 were 17.96±0.07 g/L, 8.99±0.33 g/L and 49.22±0.12% (w/w), respectively. The volumetric productions of total carotenoids and β-carotene and the yield of total carotenoids and β-carotene yield were 6.81±0.06 mg/L, 2.71±0.08 mg/L, 387±14.11 µg/g and 154±8.53 µg/g, respectively.

The optimal conditions for the maximum volumetric production of lipids (C:N ratio of 25:1, pH of 5.45, temperature of 22.36°C and agitation rate of 199.40 rpm) gave high biomass, lipids and lipids content of 17.69±0.44 g/L, 8.35±0.19 g/L and 49.48±0.41% (w/w). Whereas, the volumetric productions of total carotenoids and β-carotene obtained from this conditions less than the optimum conditions for the maximum biomass.

The optimal conditions for the maximum lipids content (C:N ratio of 24:1, pH of 5.36, temperature of 20.05°C and agitation rate of 170.96 rpm) showed that the biomass, lipids and carotenoids productions decreased with decreasing C:N ratio of 24:1 and agitation rate of 170.96.

Hence, the optimal conditions of biomass which showed highest biomass, lipids and carotenoids productions was selected to be as the suitable cultivation conditions for the next experiment. The lipids production, biomass and lipids content of strain KX709872 under the optimal conditions, were higher than the report of Xue et al. (2010). In their study, lipids content of 30.00% (w/w) was produced by *Rhodotorula glutinis* using corn starch wastewater supplemented with waste syrup as carbon source. While, in this study, higher lipids contents of 49.22 and 56.61% (w/w) was obtained

when cultivated in 50 mL of Erlenmeyer flask and 5.0–L stirred tank bioreactor, respectively. Schneider et al. (2012) revealed that *Rhodotorula glutinis* could produce carotenoids only 0.13 mg/L using the culture medium supplemented potato processing wastewater. While, in this study higher carotenoids of 6.81±0.06 mg/L was obtained when cultivated in 50 mL of Erlenmeyer flask.

Table 4.10 The predicted and actual value of biomass, lipids and lipids content validating the fitness of model

Model	Biomass*		Lipids**		Lipids content***	
	Predicted	Actual	Predicted	Actual	Predicted	Actual
Biomass (g/L)	19.27	17.96±0.07[a]	18.56	17.69±0.44[b]	15.84	15.95±0.49[c]
Lipids (g/L)	7.71	8.99±0.33[a]	7.97	8.35±0.19[b]	7.34	7.13±0.06[c]
Lipids content (% w/w)	42.22	49.22±0.12[a]	46.53	49.48±0.41[a]	49.63	47.15±0.15[b]
Total carotenoids (mg/L)	3.38	6.81±0.06[a]	3.53	2.71±0.23[b]	3.57	2.16±0.02[c]
Total carotenoids yield (μg/g)	220	387±14.11[a]	218	152±17.80[b]	218	133±5.41[c]
β-carotene (mg/L)	1.45	2.71±0.08[a]	1.54	0.83±0.09[b]	1.70	0.60±0.08[c]
β-carotene yield (μg/g)	105	154±8.53[a]	103	46±6.30[b]	103	40±1.67[b]

[a]Different letters in same row indicate significantly treatments difference (p<0.05) according to the statistical analysis Duncan's multiple range test

*C:N ratio of 25:1, pH of 5.44, temperature of 25.34°C and agitation rate of 192.73 rpm

**C:N ratio of 25:1, pH of 5.45, temperature of 22.36°C and agitation rate of 199.40 rpm

***C:N ratio of 24:1, pH of 5.36, temperature of 20.05°C and agitation rate of 170.96 rpm

4.10 Scale-up production in stirred tank bioreactor

The growth and production of lipids and carotenoids behavior of *Sporidiobolus pararoseus* KX709872 under the optimal conditions in 5.0–L stirred tank bioreactor with 2.5–L optimized medium were investigated. Figure 4.14 shows that the growth, lipids accumulation and activities of α-amylase and AMG were simultaneously increase in days–4 of cultivation time. In the early phase of cultivation, rice residue from food waste concentration rapidly decreased from 75.0 g/L to 3.0 g/L after days–4 of cultivation period. Meanwhile, the biomass increased rapidly from 0.05 to 16.33 g/L within days–4 of cultivation period. In this period, the biomass increased with maximum specific growth rate (μ_{max}) of 0.011/h. The volumetric production of biomass and lipids, and lipids content reached 16.33±0.49 g/L, 8.75±0.13 g/L and 56.61±0.04% (w/w), respectively, at days–4 of cultivation period. The highest lipids productivity of 2.188 g/L/d was obtained. After this period, the growth and metabolites production of this oleaginous red yeast was slightly decreased indicating that either limitation of carbon source or decreasing of pH to 3.08±0.02.

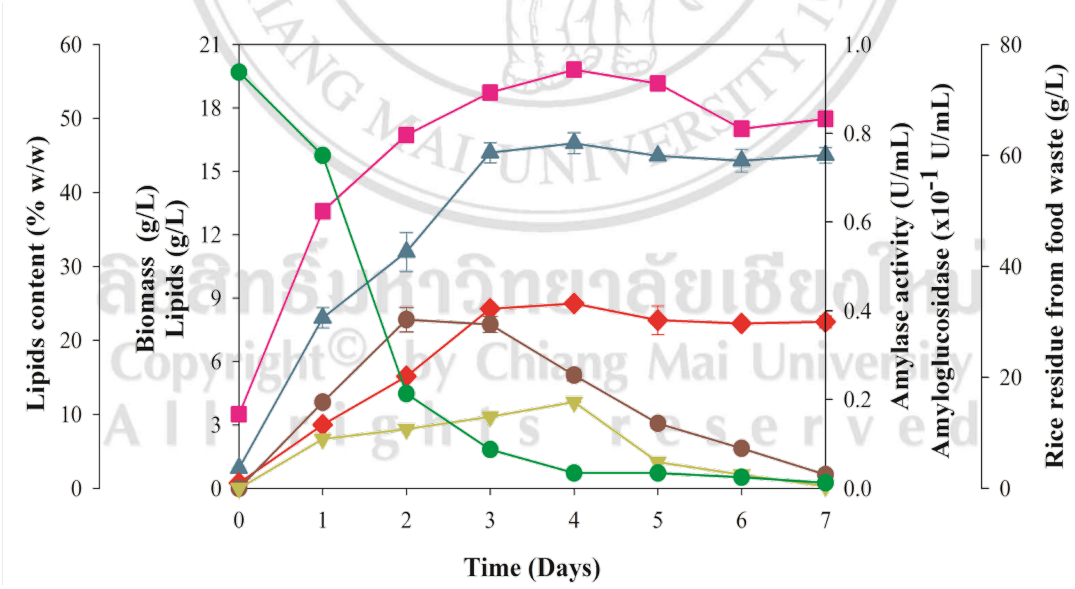


Figure 4.14 Time course of biomass (▲), lipids (◆), lipids content (■), α-amylase activity (●), AMG (▼) and rice residue from food waste (●)

Moreover, the carotenoids production of *Sporidiobolus pararoseus* KX709872 was highest at days–6 as shown in Figure 4.15. The maximum of total carotenoids and β-carotene were 7.10±0.09 mg/L and 2.99±0.06 mg/L, with the yield of total carotenoids and β-carotene of 600±0.06 and 252±0.01 µg/g, respectively. The highest total carotenoids and β-carotene productivity of 1.18 and 0.50 mg/L/d were obtained. It was observed that the carotenoids synthesis was to be contrasted with lipids production. The total carotenoids and β-carotene were increased, while lipids production was slightly decreased. Since, the limitation of carbon source was result to low C:N ratio. Somashekar and Joseph (2000) found that *Rhodotorula gracilis* could produce the highest total carotenoids at low C:N ratio in the medium which was 15 times higher than high C:N ratio. Under the condition of high C:N ratio, available carbon will be used in favor to accumulation of lipids rather than carotenoids synthesis. These results were supported by the changing behavior of color intensity of yeast cell of strain KX709872 under the optimal condition is shown in Figure 4.16. The changing of color intensity of yeast cell was corresponding to the increasing of carotenoids production of *Sporidiobolus pararoseus* KX709872. From Figure 4.14, it observed that the lipids production of this oleaginous red yeast was slightly decreased after days–4 of cultivation period. While, the carotenoids production was increased resulting in high intensity of red color of yeast cell (Figure 4.15).

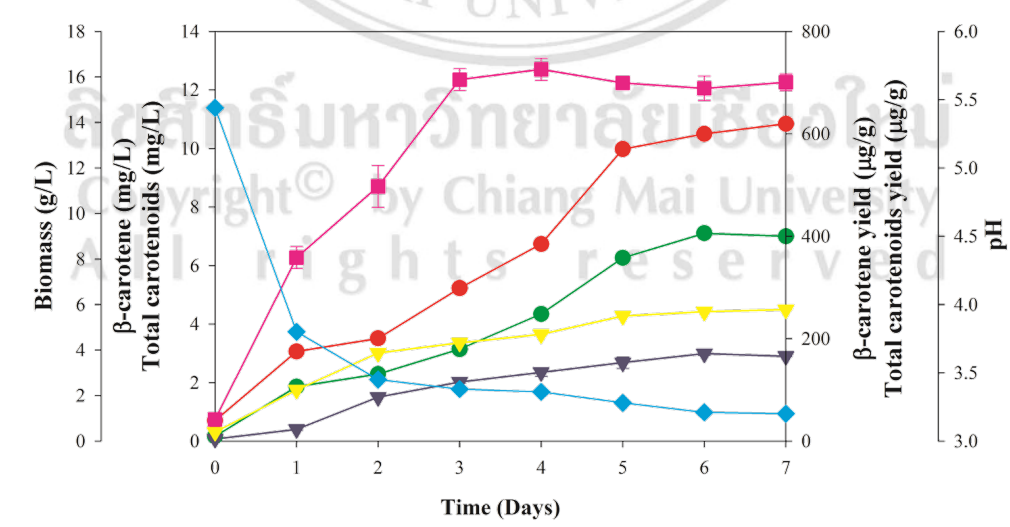


Figure 4.15 Time course of biomass (■), total carotenoids (●), total carotenoids yield (●), β-carotene (▼), β-carotene yield (▼) and pH (◆)

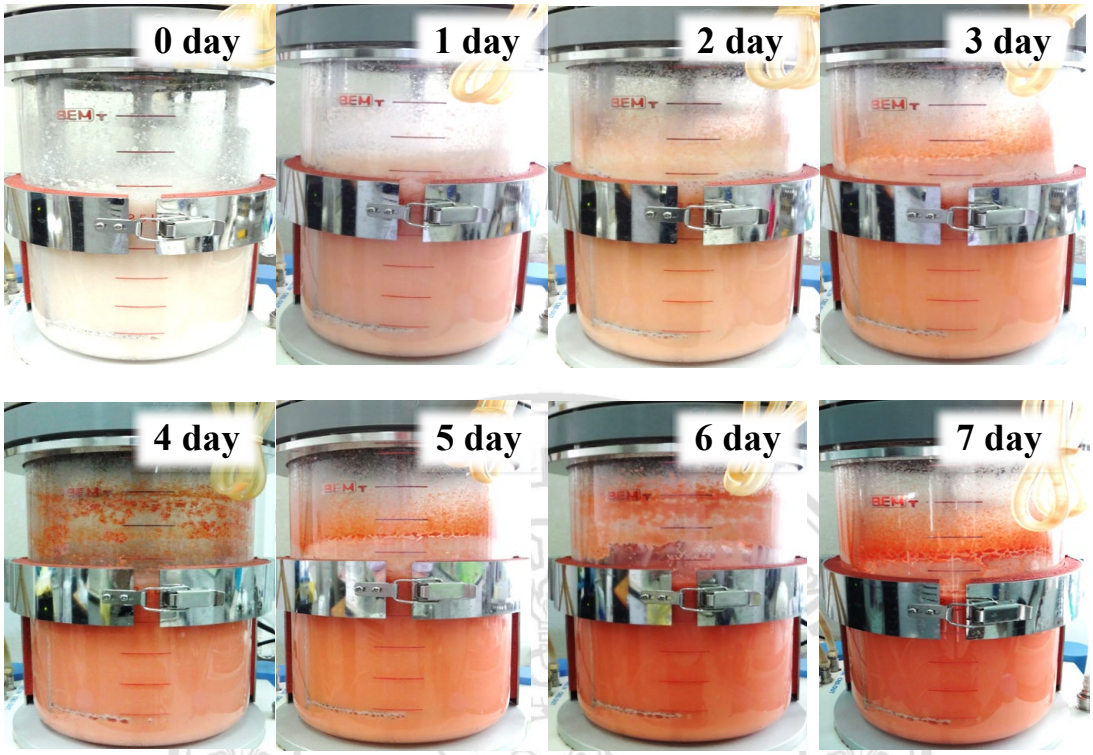


Figure 4.16 The color changing behavior in 5.0–L stirred tank bioreactor by *Sporidiobolus pararoseus* KX709872

The comparison of the volumetric productions of biomass and lipids of *Sporidiobolus pararoseus* KX709872 with other oleaginous microorganism using starchy material as carbon source is shown in Table 4.11. Muniraj et al. (2013) demonstrated that the amylolytic oleaginous filamentous fungus *Aspergillus oryzae* could produce the biomass, lipids and lipids content of 8.75 g/L, 3.5 g/L and 40% (w/w), respectively, when cultivated in potato processing wastewater. Xue et al. (2010) also reported that *Rhodotorula glutinis* produced 4.55 g/L of lipids and 35% (w/w) of lipids content using corn starch wastewater as carbon source. While, Chi et al. (2011) revealed that the lipids production of yeast strain *Yarrowia lipolytica* were only 0.03 g/L or 11.5% (w/w) which cultivated in food waste and municipal wastewater. Schneider et al. (2012) have studied the microbial production of oils for biodiesel production and high-quality pigments of oleaginous red yeast *Rhodotorula glutinis* using potato processing wastewater as a carbon source. The results revealed that *Rhodotorula glutinis* could produce biomass, lipids, lipids content and carotenoids only 0.91 g/L, 0.10 g/L 14.28% (w/w) and 0.13 mg/L at days–2 of cultivation time in

medium supplemented potato processing wastewater. After this period, the biomass, lipids and carotenoids productions were slightly decreased.

Among those amylolytic oleaginous yeasts, *Cryptococcus terricala* exhibited high lipids content of 61.96% (w/w) at days−10 of cultivation using starch through consolidated bioprocessing as carbon source. However, the slow rate of lipids production with a lipids productivity of 0.302 g/L/d was obtained (Tanimura et al., 2014). This study revealed that *Sporidiobolus pararoseus* KX709872 produced lipids up to 8.75 g/L with a lipids content of 56.61% (w/w) in days−4 of cultivation period, resulting in the highest lipids productivity of 2.188 g/L/d. So, *Sporidiobolus pararoseus* KX709872 showed high ability of direct bioconversion of rice residue from food waste for lipids and biomass productions.

Table 4.11 The biomass and lipids productions cultivated with starchy materials by various oleaginous yeast strains

Oleaginous microorganisms	Carbon source	Biomass (g/L)	Lipids (g/L)	Lipids content (% w/w)	Lipids productivity (g/L/d)	Saturated/ Unsaturated fatty acid (%)	Oleic acid (%)	Total carotenoids (mg/L)	References
Rhodotorula glutinis	Corn starch wastewater	13.00	4.55	35.0	0.910	NA*/NA	NA	NA	Xue et al. (2010)
Yarrowia lipolytica	Food waste and Municipal wastewater	0.25	0.03	11.5	0.006	NA/NA	NA	NA	Chi et al. (2011)
Aspergillus oryzae	Potato processing wastewater	8.75	3.5	40.0	0.700	39.2/60.8	30.3	NA	Muniraj et al. (2013)
Cryptococcus terricala JCM 24523	Starch through consolidated bioprocessing	4.88	3.02	61.96	0.302	9.37/90.63	73.17	NA	Tanimura et al. (2014)
Rhodotorula glutinis	Potato processing wastewater	0.91	0.10	14.28	0.100	41.0/59.0	35.0	0.13	Schneider et al. (2012)
Sporidiobolus pararoseus KX709872	Rice residue from food waste	17.96	8.99	49.22	1.670	34.63/65.37	60.94	6.81	This study**
Sporidiobolus pararoseus KX709872	Rice residue from food waste	16.33	8.75	56.61	2.188	28.85/71.15	62.13	7.10	This study***

*Not Available, **Cultivated in 250–mL Erlenmeyer flask, ***Cultivated in 5.0–L stirred tank bioreactor

4.11 Fatty acid composition of lipids from *Sporidiobolus pararoseus* KX709872

The fatty acid compositions of the lipids from *Sporidiobolus pararoseus* KX709872, cultivated in 5.0–L stirred tank bioreactor. It composed of oleic acid (C18:1) as the main fatty acid of 62.13±0.04% followed by palmitic acid (C16:0), stearic acid (C18:0), linoleic acid (C18:2) and myristic acid (C14:0) of 20.55±0.25, 5.58±0.16, 8.35±0.33 and 0.73±0.03%, respectively. This fatty acid composition was similar to the lipids from reported *Sporidiobolus pararoseus* when cultivated on glucose as carbon source was oleic acid (66.8%), palmitic acid (21.7%), stearic acid (3.72%), linoleic acid (2.9%) and myristic acid (1.11%) (Han et al., 2016). This result indicated that the main fatty acid composition of lipids from this strain were long-chain fatty acid with 16 and 18 carbon atoms (total 82.68%), especially C18:1 or oleic acid. These lipids are the good feedstock for biodiesel production because its lipids composition was similar to the vegetable oil. The vegetable oil with fatty acid chain length of C16 and C18 has been reported as the good feedstock for biodiesel production (Gen et al., 2014).

Figure 4.17 shows the type of lipids obtained from *Sporidiobolus pararoseus* KX709872 during cultivation in 5.0–L stirred tank bioreactor. It was observed that the saponified and unsaponified lipids were simultaneously increased in day–1 of cultivation time. At days–4 of cultivation period, lipids obtained from KX709872 was predominated by saponified lipids of 8.41 g (96.11%) while unsaponified lipids were only 0.34 g (3.89%). Normally, lipids from oleaginous yeast can be classified as neutral and polar, based on the polarity of the molecular head group. Neutral lipids are comprised of neutral saponified lipids (TAG and free fatty acids) and unsaponified lipids (hydrocarbons, sterols, waxes and pigments). Whereas, polar lipids can be classified as phospholipids and glycolipids, which are saponified lipids because they contain fatty acids (Viegas et al., 2015). After days–4 of cultivation period, the saponified lipids were slightly decreased. It might be that the carbon source was not enough for growth and lipids production. As evidence from Figure 4.14, rice residue from food waste concentration was rapidly decreased from 75.0 g/L to 3.0 g/L after days–4 of cultivation period. When strain KM709872 enters the limiting phase of carbon source, this strain might use the accumulated lipids as another source of carbon, resulting in the decreasing of saponified lipids. According to Yang et al. (2015) who

reported that the oleaginous yeasts can use hydrophobic substrates such as fatty acid, fats and alkanes, as carbon source for growth and lipids production. Moreover, Papanikolaou et al. (2011) reported that the fatty acids from yeast are dissimilated for growth needs or become a substrate for intracellular biotransformation. The fatty acids would be degraded into smaller chain of acyl-CoA and acetyl-CoA, thus, firstly the necessary energy for cell growth and secondly the formation of organic substance that become the precursors for the synthesis of cellular materials (Papanikolaou et al., 2011).

The property of biodiesel affected by the quality of biodiesel feedstock, highly percentage of saturated fatty acids let the biodiesel with poor cold flow properties, while feed stocks with highly unsaturated fatty acid structures (such as oleic acid) result in biodiesel having better performance. Degree of unsaturation has a significantly effect on cold flow properties, as more unsaturation results in greatly improve low temperature performance which can use in cold climate countries (Dwivedi and Sharma, 2015; Verma et al., 2016). Hence, the quality of lipids from *Sporidiobolus pararoseus* KX709872 was also confirmed by using thin layer chromatography (TLC). The bands were observed after staining the TLC plate by iodine vapor which react only the double bond found in fatty acid. According to the Figure 4.18, bands of biodiesel or fatty acid methyl ester (FAME) produced from crude lipids of strain KX709872 (lane 4–6) were similar to the band of FAME obtained from olive oil (lane 2) indicates that crude lipids from this strain has high potential as the third-generation of biodiesel feedstock because of its high oleic acid content.

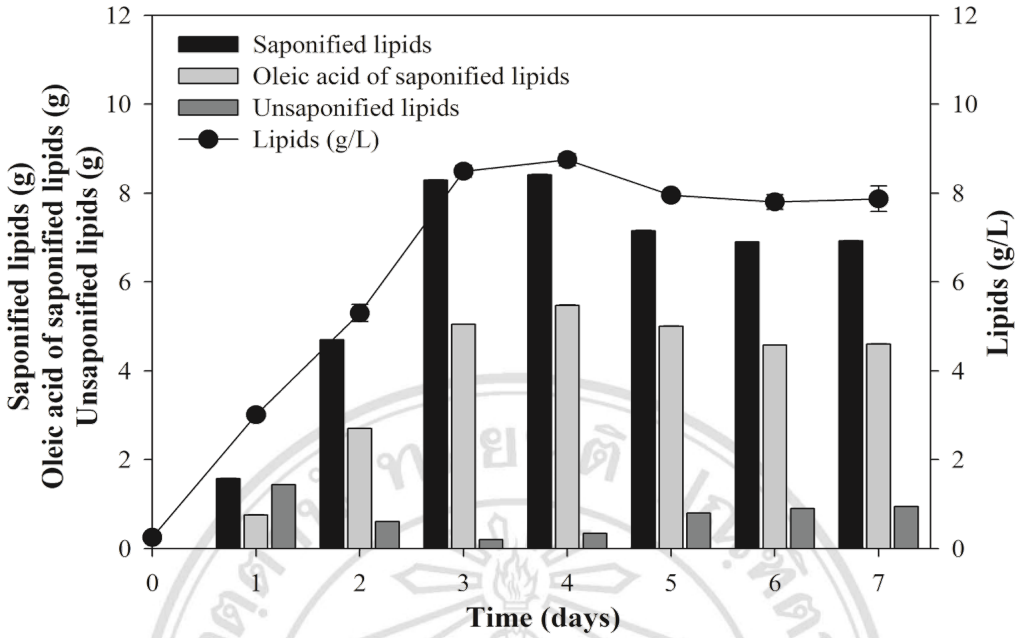


Figure 4.17 Type of lipids obtained from strain KX709872 during cultivation in 5.0–L stirred tank bioreactor

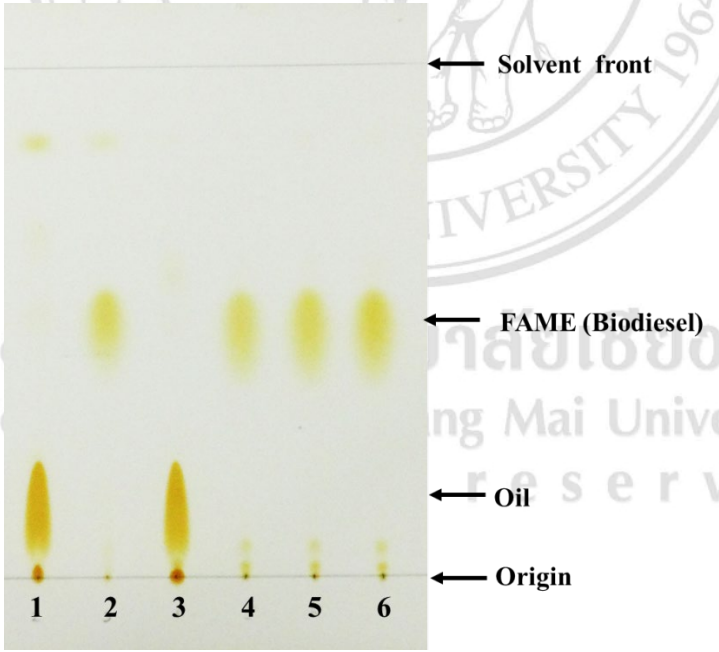


Figure 4.18 TLC of fatty acid methyl ester (FAME) or biodiesel. Lane 1: olive oil. Lane 2: FAME from olive oil. Lane 3: lipids from *Sporidiobolus pararoseus* KX709872. Lane 4–6: FAME from lipids-obtained from *Sporidiobolus pararoseus* KX709872