CHAPTER 5

Bioconversion of crude glycerol into lipids and carotenoids by *Sporidiobolus pararoseus* KM281507 in an airlift bioreactor

5.1 Introduction

The third generation biodiesels feedstock specifically derived from oleaginous microorganism are considered to be a viable alternative energy resource that is devoid of the major drawbacks associated with the first and second generation biofuels (Nigam and Singh, 2011). Since the lipids from oleaginous microorganisms have many advantages over vegetable oils such as a short microbial life cycle and no need for agricultural land, they have attracted much interest as a potential non-food feedstock for biodiesel production (Bautista et al., 2012; Yen and Chang, 2015). Oleaginous microorganisms are classified as strains that can produce a high lipids content in excess of 20% by weight (Meng et al., 2009). Among various types of oleaginous microorganisms, oleaginous red yeasts have advantages over bacteria, molds and algae. This is due to their unicellular nature, relatively high growth rate and rapid lipids accumulating ability in discrete lipids bodies (Saenge et al., 2011). Not only that, but oleaginous red yeasts also can accumulate both of lipids and carotenoids in their cells, wherein β-carotene is the main component found in carotenoids (Meng et al., 2009; Zhang et al., 2011). Moreover, these yeasts have been considered as a potential oil source for the renewable biodiesel feedstock and carotenoids, natural colorant supplements (Das et al., 2007).

Biodiesel production will generate about 10% (w/w) glycerol as the main byproduct (Yang et al., 2012). As the demand for and production of biodiesel grows, the quantity of crude glycerol generated will be considerable, and its utilization will become an urgent topic (Thompson and He, 2006). Crude glycerol contains about 50–60% (w/w) of glycerol by weight in a combination with various impurities

(Gerpen, 2005; Isahak et al., 2010; Manowattana et al., 2012; Saenge et al., 2011). Recently, numerous researchers have been published on direct utilization of crude glycerol from biodiesel production as a substrate for many biotransformation processes (Chatzifragkou and Papanikolaou, 2012; Yang et al., 2012). Most biotechnological processes have been performed in a shake flask level, and ultimate commercial success will be dependent on the ability to scale-up the process (Smith, 2009). Bioreactors are, therefore, used in bioprocess development, because they provide an environment that is conducive to optimal functioning of the microorganisms (Doig et al., 2006). Moreover, choosing an appropriate bioreactor is a key step in achieving the successful cultivation of oleaginous microorganisms. An airlift bioreactor has the advantages of simple operation and low energy consumption, and thus can be used to grow oleaginous red yeasts in a relatively inexpensive manner, facilitating the commercial production of microbial oils (Yen and Liu, 2014).

In this research, we successful to investigate the scaling up of bioconversion of crude glycerol into lipids containing high oleic acid (>80%) and β-carotene, by *Sporidiobolus pararoseus* KM281507 in an 3.0 L internal-loop airlift bioreactor. Since the lipogenesis and carotenogenesis in oleaginous red yeast have been supported by oxygen content and light intensity, the effect of aeration rate and light irradiation on those products was also investigated. In this study, the new cultivation process has been developed based on the combination effect of oxygen and light to achieve the promising productions of dry cell weight (DCW), lipids, β-carotene and total carotenoids of strain KM281507.

5.2 Materials and methods

5.2.1 Microorganism and culture conditions

The oleaginous red yeast *Sporidiobolus pararoseus* KM281507 (formerly *Sporobolomyces pararoseus* TISTR5213) was obtained from the culture collection of the Thailand Institute of Scientific and Technological Research (TISTR), Pathum Thani 12120, Thailand. The inoculum preparation, production medium and cultivation condition was performed according to the method of Manowattana et al. (2012). The production medium composed of yeast extract 1.0 g, crude glycerol 55.0 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 (per liter) (Appendix A). The initial pH of the medium was adjusted to 5.63 before sterilization at 121°C for 20 min (Manowattana et al., 2012).

5.2.2 Bioconversion of crude glycerol in stirred tank and airlift bioreactors

Batch fermentation was performed in either 3.0 L stirred tanks or 3.0 L airlift bioreactors (Biostat; B. Braun, Germany) with 2.0 L working volume of the production medium. The stirred tank bioreactor, equipped with a disc turbine agitator and baffled cylindrical vessel (Biostat; B. Braun, Germany), was operated at an aeration rate of 2.0 vvm and agitation rate of 200 rpm. The internal-loop airlift bioreactor used a controlled aeration rate of 2.0 vvm. Two pH regimes during cultivation were investigated: a constant pH of 5.63 and an uncontrolled pH with initial pH at 5.63 according to the optimal condition of our previous study (Manowattana et al., 2012). The cultivation temperature was controlled at 24.0°C during the 7 days of cultivation period.

5.2.3 Factors affecting on bioconversion of crude glycerol in airlift bioreactors

Effect of aeration rate

Since carotenogenesis and lipogenesis are an aerobic process, the air flow rate and dissolved oxygen (DO) in the cultivation of oleaginous red yeast are essential factors for cell growth, lipids and carotenoids biosynthesis (Frengova and Beshkova, 2009). The effect of aeration rates of 2.0, 4.0 and the maximum capacity of bioreactor at 6.0 vvm were investigated to obtain the maximal DCW, lipids, β-carotene and total carotenoids productions by strain KM281507. The operating temperature and initial pH of the medium were described in the previous section.

Effect of light irradiation and dissolved oxygen (DO)

The effect of light irradiation on growth, lipogenesis and carotenogenesis of strain KM281507 was investigated in 3.0-L airlift bioreactors using two PL-S lamps (Philips, Netherlands). Six irradiation conditions were investigated: 1) natural light, 2) dark, 3) light 1,000 Lux, 4) light 10,000 Lux, 5) pure oxygen with natural light and 6) light 10,000 Lux plus pure oxygen. The dissolved oxygen (DO) level was monitored and controlled during the fermentation period using the DO probe (PY-D01-2S; Sartorius, Germany) by flushing the pure oxygen (O_2) into the vessel to maintain the

DO level at 60±5%. The light intensity was determined by a light meter (TM-204, Tenmars; Taiwan) at the outside surface of the bioreactor vessels, which were set to be 1,000 and the maximum capacity of 10,000 Lux. The natural light treatment used no supplemental irradiation, and the dark treatment was carried out by covering the vessel surface with three layers of aluminum foil to exclude light.

5.2.4 Analytical methods

Carotenoids extraction and determination

The intracellular carotenoids of strain KM281507 was extracted by breaking the cells (Appendix C). The process was carried out in screw cap tubes containing 10.0 mL acetone (Merck, Germany) and 1.0 g glass beads (3 mm-diam.; Superior, Germany) in the presence of 100 ppm ascorbic acid (Sigma, USA). The extraction method and quantitative analysis of carotenoids were investigated by HPLC (LC-10AT vp; Shimadzu, Japan) equipped with a C18-column (5µm, 250m × 4.6 mm; Restek, France) following the methods of Manowattana et al. (2012). The β-carotene and total carotenoids content were determined by a UV-VIS detector (SPD-10A VP; Shimadzu, Japan) at 454 nm, and expressed as volumetric production of the culture broth (g/L). The β-carotene was identified using a β-carotene standard (Sigma, USA).

Lipids extraction and determination

Extraction of lipids from the yeast cell was performed according to the modified method of Bligh and Dyer (1959). Briefly, lipids were extracted by breaking the yeast cells, in screw cap tubes using a mixture of chloroform:methanol (2:1, v/v) and glass beads. The mixture was vigorously shaken in a vortex mixer for 30 min and sonicated at 70 Hz for 30 min (Elmasonic S60H; Elma Schmidbauer GmbH, Germany). The ruptured cells and crude extracted lipids were centrifuged at 6,000 rpm (4,146 *g*) at 4°C for 10 min (Hettich MIKRO 22R; Germany), after that the clear supernatant was collected, and the organic solvent was removed by evaporation under vacuum of 300 mm bar (Rotavapor R-3; Buchi, Japan). The volumetric productivity of lipids was expressed as g/L of the culture broth. The composition of the fatty acids in the extracted lipids was investigated by derivatization of lipids to fatty acid methyl esters (FAME) following the method of Chaiyaso et al. (2012).

The FAME were analyzed using gas chromatography with a flame ionization detector (GC-FID) (GC-2010; Shimadzu, Japan) equipped with an HP-INNOWAX column (30 m × 0.25 mm, 0.25 µm film thickness; Agilent, USA) with a split ratio of 100:1. Helium was used as the carrier gas with a flow rate of 1.0 mL/min. The temperature program was 60°C (for 2 min), 10°C/min to 200°C and 5°C/min to 240°C (for 7 min). The GC-FID condition followed the EN14103:2011 method (McCurry, 2011). The fatty acid profiles were further confirmed by GC-MS analysis which was analyzed by the Science and Technology Service Center, Chiang Mai University (STSC-CMU), Thailand. GC-MS [gas chromatography with mass spectroscopy; GC 7890A: MSD 5975C (EI): Agilent, USA], was performed with a scan parameter of 50–500 amu, MS quadrupole at 150°C and MS source at 230°C (modified method from McCurry (2011)) using a DB5-MS column (30 m × 0.25 mm, 0.25 µm film thickness) with a split ratio of 100:1. Helium was used as the carrier gas with a flow rate of 1.0 mL/min. The temperature program was as indicated above.

Biomass measurement

The DCW was collected from cultivation broth. They were taken and centrifuged at 6,000 rpm (4,146 *g*) at 4°C for 10 min. The cell pellet was washed twice with *n*-hexane and once with distilled water, then centrifuged and cell pellet dried at 80°C overnight. After that, the dried cell was transferred to desiccators until a constant weight was obtained (Manowattana et al., 2012).

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.

5.3 Results and discussion

5.3.1 Bioconversion of crude glycerol in stirred tank and airlift bioreactors

In this study, two kind of bioreactors (stirred tank and airlift) were investigated for the growth and intracellular metabolites production of *Sporidiobolus pararoseus* KM281507 under two pH regimes of cultivation; uncontrolled pH (initail pH at 5.63)

and controlled pH (constant pH at 5.63). Figure 5.1 shows that, airlift bioreactor with uncontrolled pH regime enhanced the yeast DCW, lipids, β-carotene and carotenoids productions better than stirred tank bioreactor with 37, 38, 89 and 101% increasing, respectively. The maximum volumetric productions of DCW (X_{max}), lipids (L_{max}), β-carotene (β_{max}) and total carotenoids (C_{max}) of those conditions were 10.62±0.21 g/L, 3.26±0.13 g/L, 30.64±0.05 mg/L and 46.59±0.07 mg/L, respectively. Even a stirred tank bioreactor creates high turbulence to maintain transfer rates of oxygen and nutrient, but this also generates considerable shearing force that is detrimental to the yeast cells (Waites et al., 2001). In contrast, airlift bioreactors generate relative low shearing force but highly energy efficient relative to stirred bioreactors, yet the productivities of both types have been reported to be comparable (Doig et al., 2006). In this study, airlift bioreactor had a higher potential for inducing yeast DCW which led to higher lipids, β-carotene and total carotenoids productions than the stirred tank bioreactor indicating the mixing phenomenon in the airlift bioreactor occurs with no shearing force in the liquid phase (Garcia-Ochoa and Gomez, 2009). So, oleaginous red yeast strain KM281750 could grow well under without any shearing force condition. Moreover, it might be that the high level of DO in the airlift bioreactor of 50% at 4–7 days of cultivation period, greatly enhanced the specific cell growth rate (μ), compared to only 20% of DO in the stirred tank bioreactor. Similar to the result of Yen and Liu (2014) who noted that the high growth rate of *Rhodotorula glutinis* under high DO level in airlift bioreactor was observed as well as the specific lipids yield of 45% g/g was higher compared to only 25% g/g in a stirred tank bioreactor.

The airlift bioreactor was suitable to use in further developmental studies of DCW, lipids, β-carotene and total carotenoids productions under two pH regimes of cultivation. It was found that β-carotene and total carotenoids productions were increased by cultivation under the uncontrolled pH regime, while the DCW and lipids productions were increased by cultivation under controlled pH condition. The pH of the medium under the uncontrolled pH regime gradually decreased from 5.63 to 2.72 at the end of the fermentation period. Perhaps some organic acids, intermediates of the Kreb's cycle, are produced during the growth of *Sporidiobolus pararoseus* KM281507. Those acids including citric and succinic acids which have been found to be enhancers of carotenoids and lipids productions in oleaginous red yeast (Saenge et al., 2011).

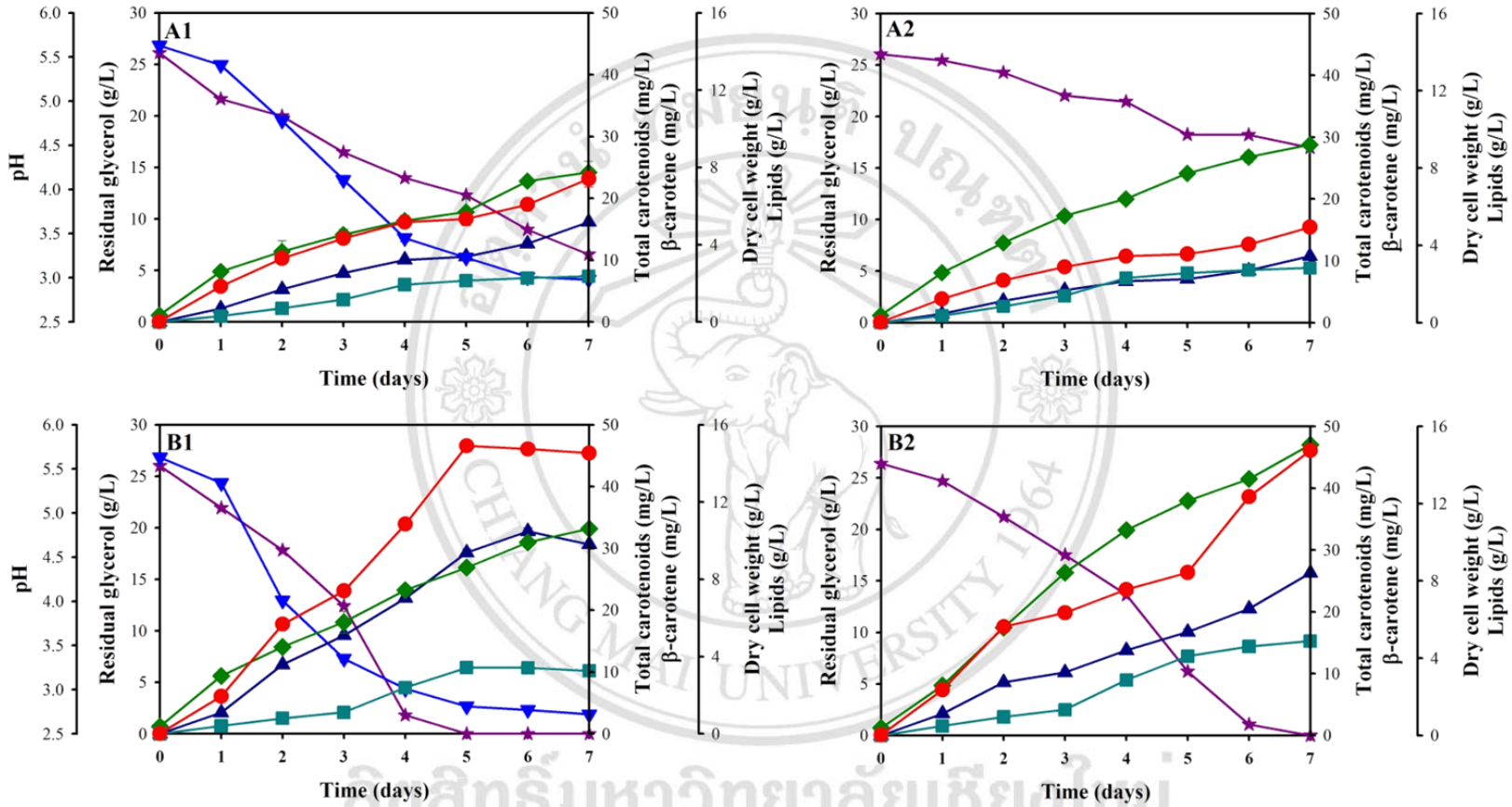

Figure 5.1 Time course of dry cell weight (◆), residual glycerol (★), pH (▼), lipids (■), β-carotene (▲) and total carotenoids (●) of *Sporidiobolus pararoseus* KM281507 in an stirred-tank bioreactor with an uncontrolled pH regime (A1), with a controlled pH regime of 5.63 (A2) and airlift bioreactor with an uncontrolled pH regime (B1) and with a controlled pH regime of 5.63 (B2)

The similar results regarding the effect of pH on carotenoids production was also reported by Hu et al. (2006), who found that the growth of *Xanthophyllomyces dendrorhous* was supported by higher pH condition whereas astaxanthin formation was occurred at lower pH. Saenge et al. (2011) reported that cultivation of *Rhodotorula glutinis* in 2 L stirred tank bioreactor under uncontrolled pH regime (initial pH at 6.0), the pH of medium declined from 6.0 to 4.3 because some organic acid were produced. Those reports supported the growth and metabolites pattern of strain KM281507 that the DCW and lipids productions might be favored by a higher pH condition while β-carotene and total carotenoids accumulation occurred at lower pH.

The maximum production of those metabolites occurred after day 5 of cultivation under uncontrolled pH regime with the lowest pH of 2.72, while 7 days of cultivation period under controlled pH was observed. Meanwhile, the crude glycerol was completely consumed after 5 days of cultivation time under the uncontrolled pH regime (Figure 5.1: B1), and for 7 days under the controlled pH condition (Figure 5.1: B2). This result indicated that cultivation strain KM281507 in an airlift bioreactor under uncontrolled pH regime had high advantage on conpletely consuming of crude glycerol while some oleaginous red yeast strains such as *Rhodotorular glutinis* (5), *Rhodosporidium paludigenum* (Yimyoo et al., 2011) could not metabolize crude glycerol completely.

Saenge et al. (2011), found that the lipids content in *Rhodotorular glutinis* were enhanced by cultivation under controlled pH condition. Similarly, this result showed that the volumetric production of lipids extracted from strain KM281507 under controlled pH was higher than uncontrolled pH condition. However, the kinetics parameters (Table 5.1), indicates that the specific lipids yield ($Y_{L/X}$) of 0.54±0.09 g/g, specific β-carotene yield ($Y_{\beta/X}$) of 4.61±0.09 mg/g and specific total carotenoids yield ($Y_{C/X}$) of 7.22±0.05 mg/g obtained by the airlift bioreactor with uncontrolled pH regime which were higher than in the controlled pH regime by 42, 250 and 260%, respectively. The results of glycerol consumtion rate (Q_S) and lipids productivity (Q_L) parameters under the uncontrolled pH were 5.21±0.05 and 0.47±0.01 g/L/d, which were significantly (p<0.05) higher than under the controlled pH regime. Therefore, production of DCW, lipids and carotenoids by strain KM281507 in airlift bioreactor

with the uncontrolled pH regime is the best choice for those metabolite productions according to the shorten cultivation time and high productivity.

Table 5.1 Kinetic parameters of batch fermentation of *Sporidiobolus pararoseus* KM281507 in a stirred tank and airlift bioreactors under uncontrolled and controlled pH regimes

Kinetic parameters	Stirred tank bioreactor		Airlift bioreactor	
	Uncontrolled pH	Controlled pH	Uncontrolled pH	Controlled pH
X_{max} (g/L)	7.74±0.57^{d*}	9.21±0.11^{c}	10.62±0.21^{b}	15.05±0.04^{a}
μ_{max} (h^{-1})	0.03±0.00^{d}	0.05±0.00^{c}	0.06±0.00^{b}	0.10±0.00^{a}
$Y_{x/s}$ (g/g)	0.33±0.01^{c}	0.71±0.01^{a}	0.26±0.00^{d}	0.52±0.00^{b}
Q_s (g/L/d)	2.79±0.01^{b}	1.30±0.01^{c}	5.21±0.05^{a}	5.21±0.04^{a}
L_{max} (g/L)	2.36±0.24^{d}	2.83±0.18^{c}	3.26±0.13^{b}	4.89±0.11^{a}
$Y_{L/S}$ (g/g)	0.20±0.01^{b}	0.31±0.02^{a}	0.14±0.01^{c}	0.20±0.01^{b}
$Y_{L/X}$ (g/g)	0.60±0.05^{a}	0.43±0.02^{bc}	0.54±0.09^{ab}	0.38±0.06^{c}
Q_L (g/L/d)	0.34±0.00^{d}	0.40±0.00^{c}	0.47±0.01^{b}	0.70±0.01^{a}
β_{max} (mg/L)	16.17±0.22^{c}	10.78±0.07^{d}	30.64±0.05^{a}	26.29±0.14^{b}
$Y_{\beta/S}$ (mg/g)	0.90±0.01^{b}	0.78±0.01^{c}	1.18±0.01^{a}	0.68±0.01^{d}
$Y_{\beta/X}$ (mg/g)	2.73±0.02^{b}	1.09±0.04^{d}	4.61±0.09^{a}	1.32±0.07^{c}
Q_β (mg/L/d)	2.31±0.02^{c}	1.54±0.01^{d}	4.38±0.11^{a}	3.76±0.12^{b}
C_{max} (mg/L)	23.14±1.30^{b}	15.43±0.21^{c}	46.59±0.07^{a}	46.08±0.42^{a}
$Y_{C/S}$ (mg/g)	1.17±0.01^{b}	1.01±0.01^{c}	1.85±0.02^{a}	1.03±0.02^{c}
$Y_{C/X}$ (mg/g)	3.54±0.06^{b}	1.41±0.01^{d}	7.22±0.05^{a}	1.99±0.02^{c}
Q_C (mg/L/d)	3.30±0.08^{b}	2.19±0.02^{c}	6.65±0.05^{a}	6.57±0.05^{a}

*Means and standard deviations of triplicate samples and different letters are significantly different by Duncan's Multiple Range Test (p<0.05)

5.3.2 Effect of aeration rate on DCW, lipids, β-carotene and carotenoids productions of strain KM281507

High aeration rate in airlift bioreactor provide high dissolved oxygen (DO) level which plays a pivotal role in carotenoids biosynthesis of all known carotenogenic microorganisms (Sanchez et al., 2013). It was observed that higher aeration rate led to significantly higher volumetric production of DCW, lipids, β-carotene and total carotenoids (Figure 5.2). Moreover, crude glycerol was consumed rapidly at 4 days of cultivation period at an aeration rate of 6.0 vvm (the maximum capacity of airlift bioreactor used in this study), while it was completely consumed at 5 and 6 days of cultivation period at aeration rates of 4.0 and 2.0 vvm, respectively. The $X_{max,}$ $L_{max,}$ β_{max} and C_{max} were increased from 11.27±0.29 g/L, 3.30±0.17 g/L, 31.48±1.18 mg/L and 47.70±0.45 mg/L, respectively, at 2.0 vvm to be 14.83±0.10 g/L, 5.70±0.40 g/L, 80.76±0.36 mg/L and 96.00±1.01 mg/L, respectively, at 6.0 vvm (32, 72, 157 and 101% increasing). These results were similar to the reports of Aksu and Eren (2007) who reported the specific growth and total carotenoids formation rates of *Rhodotorula glutinis*, changed significantly with varying the aeration rate from 0 to 2.4 vvm by 15 and 67% increasing. As well as the report of Yen and Liu (2014) who found that the high aeration rate led to a significantly higher cell growth rate of *Rhodotorula glutinis.* Meanwhile, Saenge et al. (2011) who studied the effects of aeration rate on cell growth, lipids yield, carotenoids productions and glycerol consumption by *Rhodotorula glutinis*. They found that the aeration rate significant enhanced the DCW and lipids accumulation, when it was increased from 0 to 2.0 vvm. From those mentioned result indicates that the aeration rate is an essential factor for lipids and carotenoids biosynthesis in cultivation of oleaginous red yeast (Frengova and Beshkova, 2009).

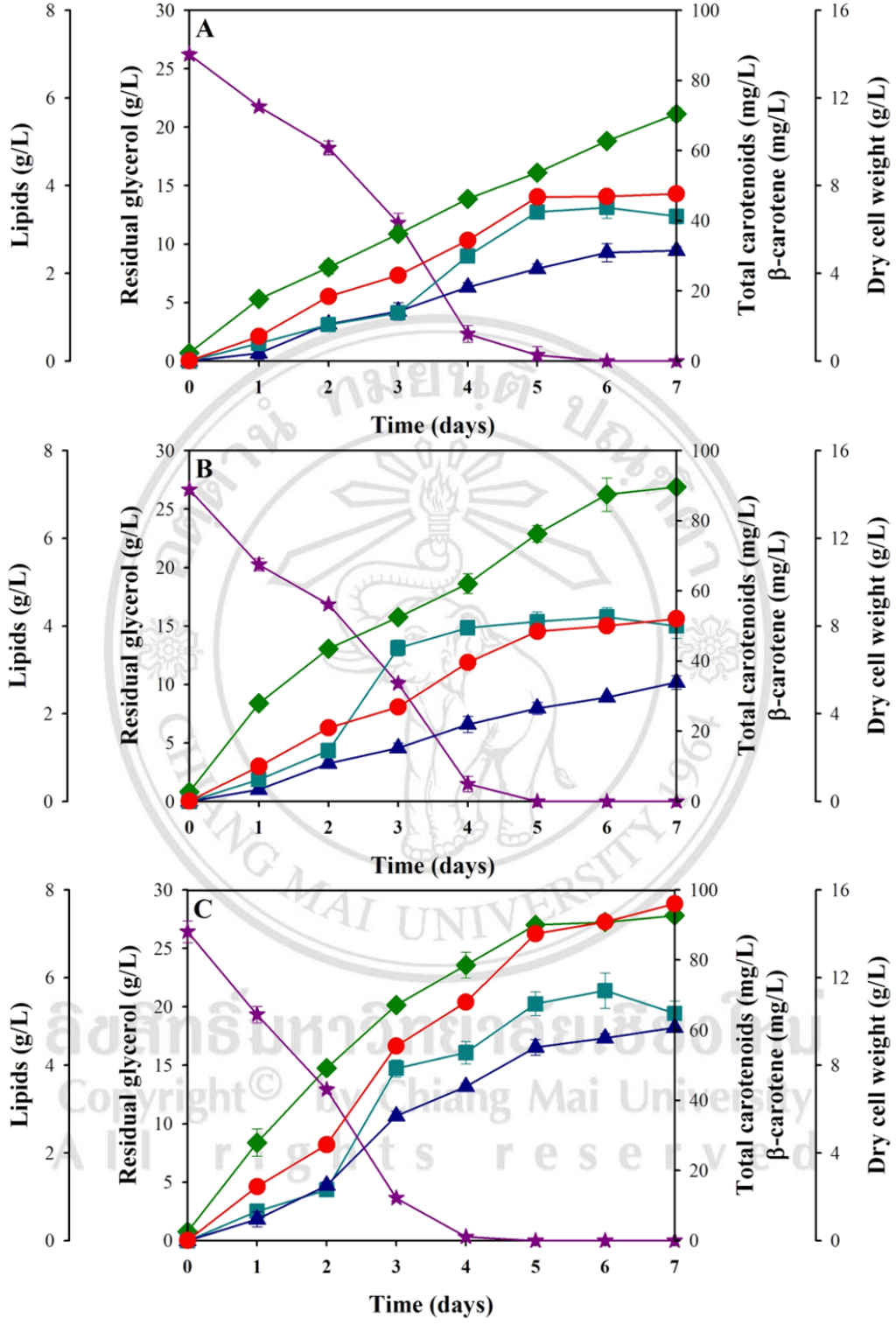

Figure 5.2 Effect of aeration rate at 2 vvm (A), 4 vvm (B) and 6 vvm (C) on the production of dry cell weight (◆), residual glycerol (★), lipids (■), β-carotene (▲) and total carotenoids (●) of *Sporidiobolus pararoseus* KM281507 in airlift bioreactor

The low level of aeration rate in the airlift bioreactor might be the reason for the low DO level, which affected cell growth and the production parameters. Oxygen is only slightly soluble in aqueous solution (Waites et al., 2001). When high biomass concentrations are used to increase productivity it also creates an enormous demand for oxygen. Consequently, the operation of such aerobic processes is generally more demanding, as it is difficult to prevent oxygen from becoming a rate-limiting factor (Waites et al., 2001). Thus, enhancement of cell growth rate and high value chemical product by increasing the aeration rate may be due to increased oxygen transfer rate, which is related to the rate of oxygen utilization in *Sporidiobolus pararoseus* KM281507. The aeration rate of 6.0 vvm enhanced growth and carotenoids production by KM281507. The maximum specific growth rate (μ_{max}), 0.10±0.00 h^{-1}, $Y_{X/S}$ of 0.51±0.01 g/g, $Y_{\beta/X}$ of 4.94±0.04 mg/g and $Y_{C/X}$ of 7.26±0.06 mg/g were supported by an aeration rate at 6.0 vvm. Even the volumetric production of lipids was enhanced by high aeration rate (6.0 vvm), the $Y_{L/X}$ was not significant with different aeration rate. From this result indicates that high DO flavored the production of DCW and carotenoids while the reverse effect was observed in specific lipids yield. Similar to the report of Yen and Zhang (2011) who found that high DO would lead to increase cell growth whereas low DO would be preferred for the lipids accumulation by *Rhodotorula glutinis* BCRC22360. Even, the DO does not support the specific lipids yield of KM281507, the oxygen supply in sufficient quantities stimulates the efficient carotenoids synthesis in many oleaginous yeast cell (Frengova and Beshkova, 2009).

5.3.3 Effect of light irradiation and dissolved oxygen on DCW, lipids, β-carotene and carotenoids productions of strain KM281507

Not only oxygen but light is also the most important factor to considerate during the production of lipids and carotenoids by oleaginous red yeast because light can improve lipogenesis and carotenogenesis (Bautista et al., 2012; Mata-Gómez et al., 2014). Oleaginous red yeasts need to prevent themselves from the light that causes cell damage, and carotenogenesis was a photoprotective mechanism (Mata-Gómez et al., 2014). Moreover, oxygen is essential for aerobic bioprocess, which is an essential factor to the substrate assimilation for improved the growth rate, lipogenesis and carotenogenesis (Gonçalves et al., 2014; Mata-Gómez et al., 2014). In this study, six

different irradiation conditions, namely natural light, dark, light 1,000 Lux, light 10,000 Lux, natural light plus pure oxygen (60±5% of DO level) and light 10,000 Lux plus pure oxygen (60±5% of DO level) were established in an airlift bioreactors under uncontrolled pH regime with 6.0 vvm of aeration rate. The result revealed that the DCW increased from 14.83±0.10 g/L of natural light to 18.08±0.50 g/L of pure oxygen plus natural light and 19.30±1.07 g/L of light 10,000 Lux plus pure oxygen with the μ_{max} of 0.16±0.00 h^{-1} (Table 5.2). Thus, oxygen and light intensity play the important role for the cell growth of strain KM281507 as described in the section above.

The limiting effects of oxygen transfer in the airlift bioreactor were studied by controlling the DO value at 60±5% by flushing pure oxygen (O_2) into the culture medium. Hence, if the rate of oxygen utilization is greater than the oxygen transfer rate, an anaerobic condition will develop, which may limit growth and productivity (Waites et al., 2001). The changing DO value during the cultivation period was monitored by a DO probe. The DO value started at 100% (day 0) and decreased to 0% at 2−3 days of cultivation time, then slightly increased after 4 days, observing the production of lipids and carotenoids rapidly increased during this period (Figure 5.3). Therefore, maintaining a high DO level by flushing with O_2 raised the amount of dissolved oxygen for developed the oxygen transfer rate and utilization by yeast cell.

Table 5.2 Kinetic parameters of batch fermentation of *Sporidiobolus pararoseus* KM281507 operated in airlift bioreactor with different light irradiation and dissolved oxygen levels

Kinetic parameters	Natural light	Dark	Light 1,000 Lux	Light 10,000 Lux	Pure oxygen (Natural light)	Light 10,000 Lux plus pure oxygen
X_{max} (g/L)	14.83±0.10^{d*}	9.32±0.420^{e}	15.21±0.35^{d}	16.75±0.28^{c}	18.08±0.50^{b}	19.30±1.07^{a}
μ_{max} (h^{-1})	0.11±0.01^{c}	0.06±0.00^{d}	0.12±0.00^{c}	0.12±0.00^{c}	0.12±0.00^{c}	0.16±0.00^{a}
$Y_{x/s}$ (g/g)	0.54±0.01^{d}	0.25±0.00^{e}	0.58±0.01^{c}	0.61±0.01^{b}	0.60±0.01^{b}	0.77±0.01^{a}
Q_s (g/L/d)	5.79±0.05^{a}	5.15±0.04^{a}	5.12±0.06^{a}	5.24±0.04^{a}	5.24±0.02^{a}	5.12±0.01^{a}
L_{max} (g/L)	5.70±0.40^{c}	3.42±0.24^{d}	5.92±0.31^{bc}	6.58±0.33^{ab}	6.39±0.58^{ab}	6.61±0.04^{a}
$Y_{L/S}$ (g/g)	0.23±0.01^{c}	0.13±0.01^{d}	0.26±0.00^{b}	0.31±0.00^{a}	0.26±0.01^{b}	0.26±0.01^{b}
$Y_{L/X}$ (g/g)	0.43±0.01^{b}	0.51±0.00^{a}	0.45±0.01^{b}	0.50±0.01^{a}	0.43±0.02^{b}	0.35±0.01^{c}
Q_L (g/L/d)	0.81±0.01^{b}	0.49±0.01^{c}	0.85±0.01^{b}	0.94±0.05^{a}	0.91±0.03^{a}	0.94±0.04^{a}
β_{max} (mg/L)	83.43±0.42^{c}	31.65±0.01^{f}	96.20±3.83^{c}	103.24±0.99^{b}	93.27±0.21^{d}	109.75±0.21^{a}
$Y_{\beta/S}$ (mg/g)	3.00±0.11^{d}	1.16±0.09^{e}	3.49±0.14^{c}	4.60±0.15^{a}	4.18±0.16^{b}	4.71±0.19^{a}
$Y_{\beta/X}$ (mg/g)	5.52±0.25^{b}	4.57±0.33^{c}	6.01±0.41^{b}	7.58±0.47^{a}	6.97±0.40^{a}	6.10±0.35^{b}
Q_β (mg/L/d)	11.91±0.32^{d}	4.52±0.01^{e}	13.74±0.41^{c}	14.75±0.50^{b}	13.32±0.28^{c}	15.68±0.40^{a}
C_{max} (mg/L)	96.69±1.11^{d}	46.21±0.26^{e}	102.51±4.17^{c}	147.49±3.41^{a}	134.63±4.38^{b}	151.00±2.71^{a}
$Y_{C/S}$ (mg/g)	3.49±0.15^{d}	1.78±0.08^{e}	3.85±0.19^{c}	6.58±0.21^{a}	5.66±0.20^{b}	6.68±0.14^{a}
$Y_{C/X}$ (mg/g)	6.42±0.45^{c}	7.05±0.43^{c}	6.62±0.50^{c}	10.83±0.59^{a}	9.44±0.38^{b}	8.65±0.42^{b}
Q_C (mg/L/d)	13.70±0.54^{d}	6.59±0.09^{e}	14.63±0.21^{c}	21.06±0.31^{a}	19.22±0.50^{b}	21.56±0.20^{a}

*Means and standard deviations of triplicate samples and different letters are significantly different by Duncan's Multiple Range Test (p<0.05)

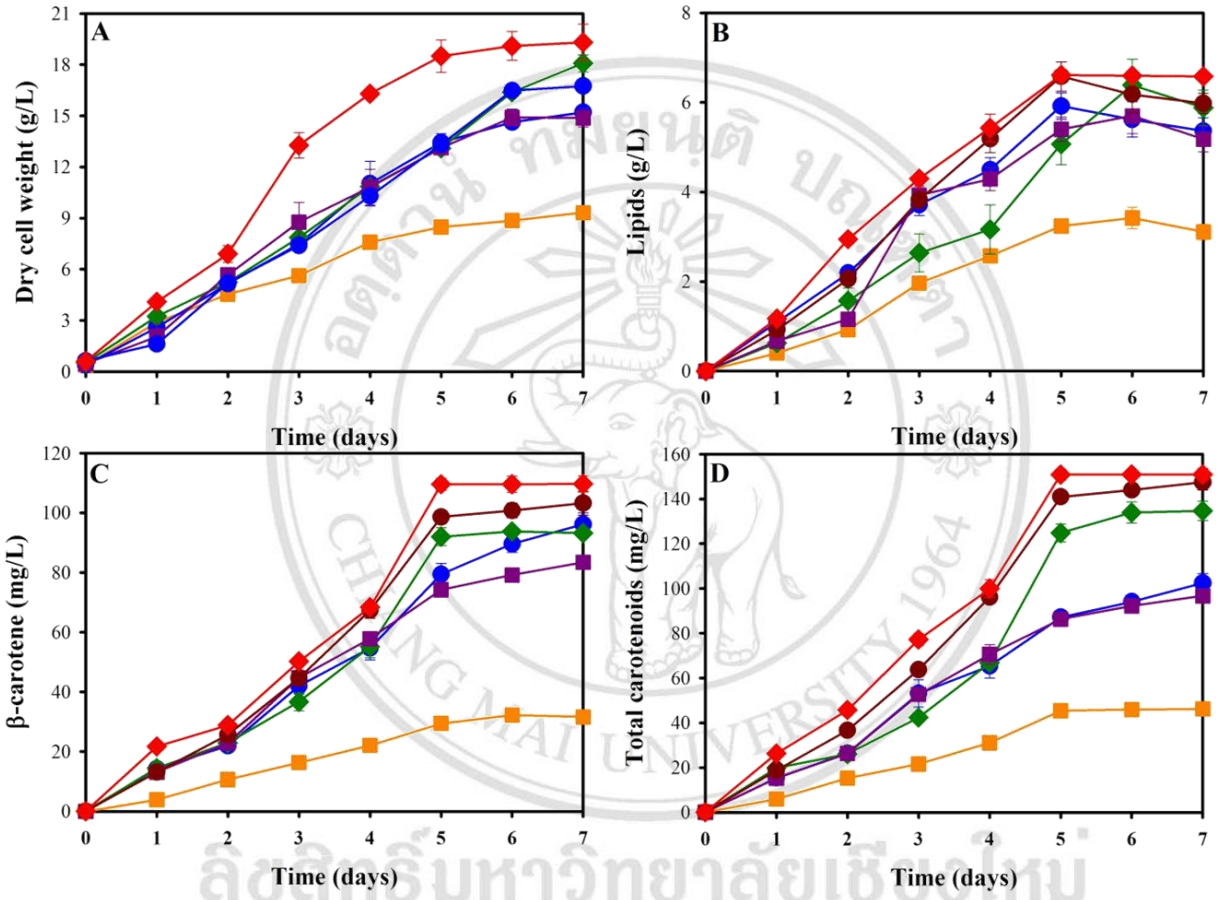

Figure 5.3 Effect of irradiation and dissolved oxygen (DO) on dry cell weight (A), lipids (B), β-carotene (C) and total carotenoids (D) on batch fermentation of *Sporidiobolus pararoseus* KM281507, when cultured under natural light (■), dark (■), light 1,000 Lux (●), light 10,000 Lux (●), pure oxygen (◆) and light 10,000 Lux plus pure oxygen (◆)

Increasing light intensity, enhancement of the volumetric productivity of DCW, lipids, β-carotene and total carotenoids were also observed. Irradiation at 1,000 Lux did not significantly enhance those parameters compared to natural light (p>0.05). It might be that natural light and low light intensity (1,000 Lux) are insufficient for carotenogenesis and lipogenesis by strain KM281507. After increasing light irradiation to the maximal capacity of 10,000 Lux, DCW, lipids, β-carotene and total carotenoids were dramatically increased. However, the best condition for those metabolites production was the light 10,000 Lux plus pure oxygen. The controlled DO condition created by adding pure oxygen with high light intensity resulted in X_{max} of 19.30±1.07 g/L, L_{max} of 6.61±0.04 g/L, β_{max} of 109.75±0.21 mg/L and C_{max} of 151.00±2.71 mg/L, respectively. These results were similar with Cheirsilp et al. (2011) who found that the high level of light intensity at 50,000 Lux supported the growth and metabolite formations of a mixed culture of oleaginous yeast *Rhodotorula glutinis* and microalgae *Chlorella vulgaris*. From our results, we found that light intensity played an important role on both carotenogenesis and lipogenesis formations. Zhang et al. (2014) noted that increasing in light intensity during the cultivation of name of microorganism, the biomass and carotenoids productions were increased whereas the lipids content was decreased. In contrast, the lipids content of strain KM281507 increased with the increasing in light intensity up to 10,000 Lux. As the evidence in Table 5.2, under dark condition, all of those parameters were low, indicating light is the most important for the levels of biosynthetic enzymes involves in carotenoid and lipids synthesis, which in turn establishes the role of white-light illumination as a stimulant (Bhosale, 2004). Cultivation strain KM281507 under the light irradiation at 10,000 Lux plus pure oxygen, the volumetric production of DCW, lipids, β-carotene and total carotenoids was promising to be the maximum level. This condition was not only supported the maximum specific growth rate (μ_{max} of 0.16±0.00 h^{-1}) but the biomass yield ($Y_{X/S}$) was promising to 0.77 g/g at day–5 days of cultivation. While, other oleaginous red yeasts required longer cultivation time (7 days) but the lowest biomass yields such as 0.24 g/g (Hu et al., 2006), 0.63 g/g in *Xanthophyllomyces dendrorhous* (Kusdiyantini et al., 1998) and 0.38 g/g in *Rhodosporidium paludigenum* (Yimyoo et al., 2011).

5.3.4 Fatty acid profile of lipids from strain KM281507

Fatty acid composition of lipids from *Sporidobolus pararoseus* KM281507 was predominated by oleic acid. The main fatty acid components were unsaturated fatty acids including oleic acid (C18:1), linoleic acid (C18:2), and palmitoleic acid (C16:1), while, saturated fatty acids including palmitic acid (C16:0), stearic acid (C18:0), and myristic acid (C14:0) were minor components (Table 5.3).

The profile of unsaturated fatty acids, especially, oleic acid was not significantly different under different conditions, except in the dark condition. Thus, the dark condition increased saturated fatty acids and decreased unsaturated fatty acids production by strain KM281507. Nevertheless, the range of oleic acid previously found in yeast was 28–66% (Meng et al., 2009). Therefore, light was an important factor for enhancing unsaturated fatty acid in this oleaginous red yeast. Moreover, the fatty acid profile and high oleic acid content (80%) of this lipids was similar to that of vegetable oil (Bautista et al., 2012) indicating the high potential as the 3rd biodiesel feedstock which can be applied in both of tropical and cold climate countries. The degree of unsaturated fatty acid in biodiesel showed the excellent fuel properties at low temperature because this biodiesel had a lower melting point than the biodiesel derived from high saturated fatty acid content (Gonçalves et al., 2014). Even the light intensity of natural light, 1,000 and 10,000 Lux did not affected on unsaturated fatty acid content, carotenoids content was dramatically affected by this factor. So, the condition that suitable for both of lipids and carotenoids productions was light 10,000 Lux plus pure oxygen (60±5% of DO level).

Table 5.3 The fatty acid profiles of crude lipids from *Sporidiobolus pararoseus* KM281507 under different batch fermentation conditions operating in airlift bioreactor

	Fatty acid	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	Others
Stirred tank	**Controlled pH**	$0.42\pm0.01^{ef*}$	8.43 ± 0.07^{h}	0.64 ± 0.03^{fg}	3.11 ± 0.04^{c}	79.58 ± 1.41^{ab}	1.19 ± 0.01^{i}	6.63 ± 2.14^{b}
bioreactor	**Uncontrolled pH**	0.41 ± 0.01^{fg}	7.58 ± 0.09^{i}	0.71 ± 0.01^{ef}	3.24 ± 0.05^{c}	77.18 ± 1.25^{b}	1.18 ± 0.00^{i}	9.70 ± 1.21^{a}
Airlift	**Controlled pH**	0.47 ± 0.02^{bc}	9.54 ± 0.14^{e}	0.68 ± 0.08^{fg}	3.47 ± 0.08^{c}	82.25 ± 1.35^{a}	1.28 ± 0.01^{g}	2.31 ± 0.12^{cd}
bioreactor	**Uncontrolled pH**	0.45 ± 0.00^{cd}	10.65 ± 0.11^{b}	0.81 ± 0.04^{de}	4.52 ± 0.08^{b}	80.28 ± 1.25^{ab}	1.24 ± 0.00^{h}	2.05 ± 0.10^{cde}
	2vvm	0.34 ± 0.00^{i}	10.41 ± 0.08^{c}	0.79 ± 0.05^{de}	3.94 ± 0.04^{bc}	80.53 ± 1.42^{ab}	1.48 ± 0.00^{f}	2.51 ± 0.14^{c}
	4vvm	0.37 ± 0.01^{h}	9.88 ± 0.05^{d}	0.82 ± 0.04^{d}	4.48 ± 0.03^{b}	81.32 ± 2.01^{ab}	1.65 ± 0.00^{e}	1.48 ± 0.24^{cde}
	6vvm	0.39 ± 0.00^{gh}	9.02 ± 0.08^{f}	0.94 ± 0.06^{bc}	4.58 ± 0.06^{b}	82.82 ± 3.12^{a}	1.94 ± 0.02^{b}	0.31 ± 0.00^{f}
	Natural light	0.37 ± 0.01^{h}	8.77 ± 0.07^{g}	0.96 ± 0.05^{bc}	3.30 ± 0.24^{c}	82.97 ± 2.14^{a}	1.95 ± 0.01^{b}	1.69 ± 0.00^{cde}
	Dark	0.60 ± 0.02^{a}	15.62 ± 0.13^{a}	0.60 ± 0.02^{g}	14.84 ± 1.10^{a}	65.45 ± 1.24^{c}	1.01 ± 0.00^{j}	1.89 ± 0.00^{cde}
	Light 1,000 Lux	0.48 ± 0.01^{b}	10.30 ± 0.11^{c}	0.88 ± 0.06^{bcd}	4.76 ± 0.58^{b}	80.23 ± 2.87^{ab}	1.82 ± 0.01^{c}	1.53 ± 0.01^{cde}
	Light 10,000 Lux	0.41 ± 0.01^{fg}	10.74 ± 0.08^{b}	1.64 ± 0.09^{a}	2.24 ± 0.14^{d}	81.68 ± 3.41^{a}	1.01 ± 0.00^{j}	2.27 ± 0.03^{cde}
	Pure oxygen (Natural light)	0.45 ± 0.02^{cd}	10.81 ± 0.09^{b}	0.86 ± 0.05^{cd}	4.45 ± 0.75^{b}	80.09 ± 2.97^{ab}	2.21 ± 0.02^{a}	1.13 ± 0.01^{ef}
	Light 10,000 Lux plus pure oxygen	0.44 ± 0.01^{de}	10.34 ± 0.14^{c}	0.97 ± 0.05^{b}	3.16 ± 0.61^{c}	81.19 ± 2.54^{abb}	1.70 ± 0.00^{d}	1.21 ± 0.01^{def}

*Means and standard deviations of triplicate samples and different letters are significantly different by Duncan's Multiple Range Test ($p<0.05$)

Many research focused on scaling up of either lipids or carotenoids productions from various strains of oleaginous red yeast. Even the lipids productivity from *Sporidiobolus pararoseus* KM281507 could not be comparable to those of various strain of *Rhodotorular glutinis* (Saenge et al., 2011; Yen and Zhang, 2011; Yen and Chang, 2015; Zhang et al., 2014), but high unsaturated fatty acid with 80% content of oleic acid of this lipids indicated the best property of the third biodiesel feed stock over other oleaginous yeast lipids. Furthermore, the carotenoids production of strain KM281507 (151.00 mg/L) was greater than those well-known carotenoids producing red yeasts; *Xanthophyllomyces dendrorhous* (27.05 mg/L) (Hu et al., 2006), *Rhodotorular glutinis* (4.20 mg/L) (Zhang et al., 2014), *Rhodotorular glutinis* (9.72 mg/L) (Yen and Chang, 2015) and *Rhodotorular glutinis* (125.75 mg/L) (Saenge et al., 2011). Therefore, the fermentation process development using internal loop airlift bioreactor under the combination effect of pH, light intensity and DO level improve the cell growth, lipids and carotenoids accumulation of high potential oleaginous red yeast strain, *Sporidiobolus pararoseus* KM281507.

5.4 Conclusions

This study indicated the enhancement of lipids and carotenoids productions of oleaginous red yeast strain KM281507 by scaling up and process developments using an internal loop airlift bioreactor. The combination effect of light irradiation 10,000 Lux plus pure oxygen with constant DO level enhanced DCW, lipids, β-carotene and total carotenoids productivities when uncontrolled pH regime with initial pH 5.63 was used. The lipids with high content of oleic acid (81%) indicated the best choice for the third biodiesel feedstock. Moreover, high volumetric carotenoids production (151.00 mg/L) with β-carotene (147.49 mg/L) or 98% of total carotenoids were the clear evidence that supported the successful of the development of fermentation process for those high value adding products from a low cost biodiesel derived-crude glycerol by the high effective oleaginous red yeast, *Sporidiobolus pararoseus* KM281507.