CHAPTER 2

Literature review

This study emphasizes on the usage of crude glycerol from the biodiesel production plant for biomass and high added value production by the selected oleaginous red yeast, *Sporidiobolus pararoseus* KM281507. The lipids and carotenoids are the major products of this oleaginous red yeast, which have been explained in this section.

2.1 Biodiesel production

Biodiesel is an alternative diesel fuel (Chatzifragkou and Papanikolaou, 2012; Gerpen, 2005; Thompson and He, 2006). It has the potential to reduce the reliance on petroleum fuel and reduce air pollutant emissions from diesel engines (Chatzifragkou and Papanikolaou, 2012; Hayyan et al., 2010). Biodiesel can be produced from a great variety of feedstocks, which are vegetable oils e.g. soybean, cottonseed, palm, peanut, rapeseed, sunflower, coconut and animal fats (usually tallow) as well as not edible oil and waste cooking oils. In the recent years, the third generation biodiesel feedstock of microbial lipids/oils have been evaluated as high potential feedstock for biodiesel production since they have an appropriate fatty acid profile, do not compete with food supply and do not require agricultural land. Moreover, the oleaginous microorganisms can grow well on waste or low-grade substrate as carbon source in a well-controlled large scale bioreactors with high efficiency by increasing of biomass yield and reduction of production cost of biomass and oil (Bautista et al., 2012).

Biodiesel is mostly produced by transesterification of triacylglycerol or TAG (fats and oils) with a short chain alcohol such as ethanol and methanol (Chatzifragkou and Papanikolaou, 2012; Gerpen, 2005). This reaction requires a catalyst, usually a strong base e.g. NaOH and KOH and new chemical compounds called fatty acid methyl or ethyl esters (biodiesel) have been produced (Gerpen, 2005). In the transesterification reaction, one molecule of TAG reacts with three molecules of methanol in the presence

of either KOH or NaOH and generate three molecules of fatty acid methyl esters (FAME) and one molecule of glycerol as shown in Figure 2.1 (Chatzifragkou and Papanikolaou, 2012; Gerpen, 2005; Ibeto et al., 2009).

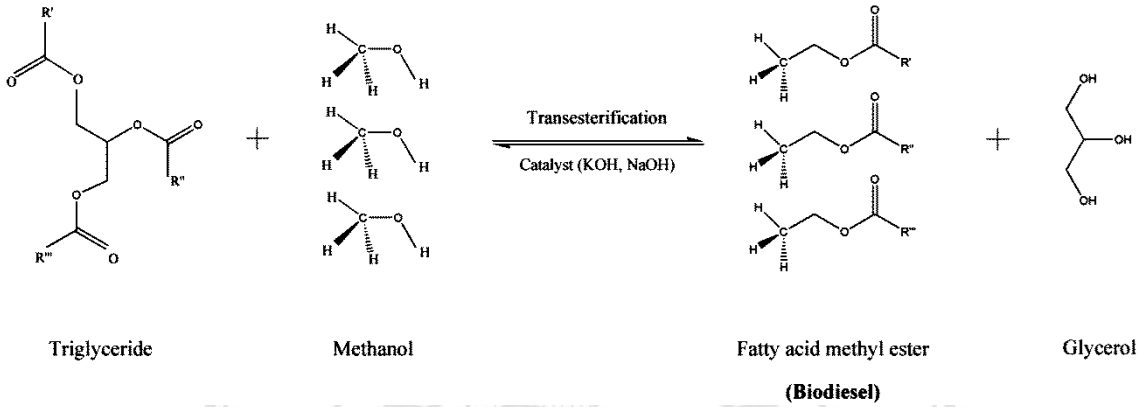


Figure 2.1 Transesterification reaction for production of biodiesel
Source: Ibeto et al. (2009)

There are many advantages of biodiesel over the fossil diesel (Ignacimuthu, 2012).

1) Biodiesel is biodegradable, harmless and decreases smoke emission considerably.
2) Biodiesel can be almost exclusively produced from renewable materials.
3) Fatty acid methyl ester contain little sulfur content (about 0.001%) and this undesired compound is completely absent in fatty acid ethyl esters.
4) Biodiesel emits about the same CO_2 that is absorbed during cultivation of oil crop.
5) There are numerous social and economic advantages for its use, particularly in developing countries.
6) Biodiesel represents a suitable outlet for the vegetable oil industry, serving as an important tool for market regulation.
7) Biodiesel can be used as blends or as neat fuels.
8) Biodiesel increases engine lifetime due to superior lubrication.
9) Biodiesel can be produced with a straight forward technology particularly in the case of methyl esters (methanolysis).

2.2 Crude glycerol from biodiesel production

Crude glycerol is the principal byproduct of biodiesel production. It occurs in fats and oils at a level of approximately 10% by weight. As the 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). The excess glycerol is generated may become an environmental problem, since it cannot be disposed of in the environment (da Silva et al., 2009). Methanol and alkali catalyst contents require crude glycerol to be treated as hazardous waste. Moreover, the purification of crude glycerol for further usage is industrially almost infeasible because of the high processing cost (Chatzifragkou et al., 2011a; Gerpen, 2005; Isahak et al., 2010). However, the usage of this low–grade glycerol is a big challenge as it cannot use in food and cosmetic industries (Pachauri and He, 2006).

Crude glycerol obtained from the biodiesel production plant via the transesterification reaction (methanolysis) is very low value byproduct because of its high impurities (Pachauri and He, 2006; Thompson and He, 2006). The chemical composition of crude glycerol obtained from biodiesel production are varied with the type of catalyst used, transesterification efficiency, recovery efficiency of the biodiesel, other impurities in the feedstock and type of alcohol used (Yang et al., 2012). Generally, the pH of crude glycerol derived from alkali-catalyzed transesterification is relative high around pH 10–12 (Nicol et al., 2012). Therefore, crude glycerol contains about 50–60% of glycerol and others impurities of 40–50% by weight (Gerpen, 2005; Isahak et al., 2010; Manowattana et al., 2012; Saenge et al., 2011).

In the recent year, crude glycerol has been used in the production of many high value added products via a microbial conversion (Table 2.1). The impurities in crude glycerol, physico-chemical parameters of medium and cultivation conditions are the important considerations in that intention of the bioconversion of crude glycerol to higher value products (Bautista et al., 2012; Nicol et al., 2012).

Table 2.1 Bioconversion of crude glycerol to high value added products

Products	Commercial utility	Microorganisms	References
1, 3-propanediol	Monomer for bioplastics	*Klebsiella pneumoniae*	Oh et al. (2008)
		Clostridium butyricum	Chatzifragkou et al. (2011b)
Biosurfactant	Amphiphilic, petroleum-based products, emulsifiers and dispersants.	*Pseudomonas aeruginosa*	Nitschke et al. (2005)
		Candida bombicola	Ashby et al. (2005)
Butanol	Fuel	*Clostridium pasteurianum*	Taconi et al. (2009)
Carotenoids	Anticancer agent, Provitamin A source, food colorants, photo protectant, feed additive mainly in aquaculture and cosmetic preparations	*Xanthophyllomyces dendrorhous*	Certik et al. (2005)
		Rhodotorula glutinis	Saenge et al. (2011)
		Rhodosporidium paludigenum	Yimyoo et al. (2011)
		Sporidiobolus pararoseus	Valduga et al. (2014)
		Sporobolomyces roseus	Davoli et al. (2004)
Citric acid	Tart/fruity flavor to foods and beverages, additive in detergents, pharmaceuticals, cosmetics and toiletries	*Yarrowia lipolytica*	Papanikolaou et al. (2002)
Docosahexaenoic acid (DHA)	Omega-3 fatty acid, cosmetic industry and serves as a versatile building block	*Schizochytrium limacinum*	Ethier et al. (2011)

Table 2.1 (Continued)

Products	Commercial utility	Microorganisms	Referents
Eicosapentaenoic acid (EPA)	Omega-3 fatty acid, precursor for prostaglandin-3	*Pythium irregulare*	Athalye et al. (2009)
Erythritol	Sugar alcohol for use as a food additive	*Yarrowia lipolytica*	Rymowicz et al. (2009)
Ethanol	Fuel	*Kluyvera cryocrescens*	Choi et al. (2011)
		Klebsiella pneumoniae	Oh et al. (2008)
Hydrogen	Fuel	*Rhodopseudomonas palustris*	Sabourin-Provost and Hallenbeck (2009)
		Enterobacter aerogenes	Ito et al. (2005)
Lipase	Enzyme that catalyzes the hydrolysis of fats	*Staphylococcus caseolyticus*	Volpato et al. (2008)
Lipids	Biodiesel feedstock, dietary supplements and health foods	*Schizochytrium limacinum*	Liang et al. (2010a)
		Cryptococcus curvatus	Liang et al. (2010b)
		Rhodotorula glutinis	O'Grady and Morgan (2011)
		Chlorella protothecoides	Chen and Walker (2011)
Polyhydroxyalkanoates (PHAs)	Biopolymer	*Pseudomonas* sp.	Ashby et al. (2004)

2.3 Bioconversion of crude glycerol into high value products by oleaginous red yeast

Oleaginous yeasts are considering as the abundant sources of oils and fats because their cell membrane structures always contain lipids. The oleaginous yeasts produce high lipids content more than 20% by weight (Meng et al., 2009). Moreover, many type of oleaginous yeast can accumulate both of lipids and carotenoids in their cell, which is designed as a group of "oleaginous red yeast". This research emphasizes on bioconversion of crude glycerol into biomass, carotenoids and lipids production by the effective oleaginous red yeast. This section focuses on the literature review on the bioconversion of biodiesel-derived crude glycerol to the high value chemical produced by various types of oleaginous red yeasts. The scope of this study could be summarized in the Figure 2.2.

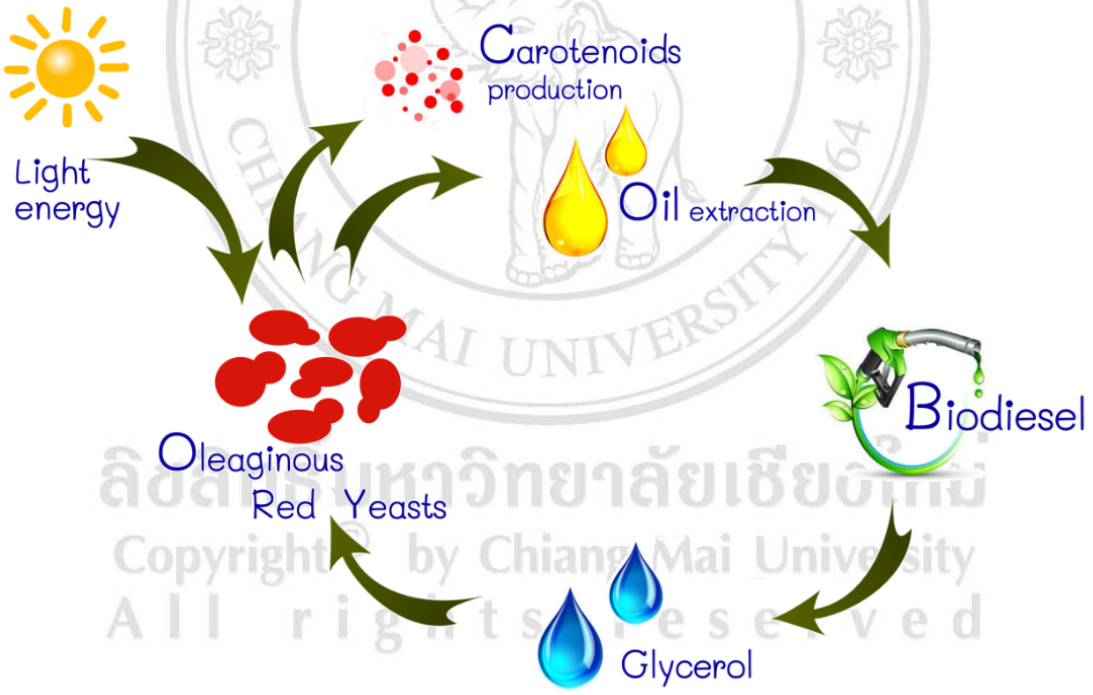


Figure 2.2 Schematic flow chart for the productions and utilization of lipids and carotenoids from crude glycerol by the effective oleaginous red yeast

2.4 Lipids production

One of an alternative use of crude glycerol is as a source of carbon and energy for the cultivation of oleaginous yeast/red yeasts, which show the good ability to grow and accumulate intracellular lipids when cultivate in crude glycerol (Easterling et al., 2009). In addition, production of microbial lipids, the third generation biodiesel feedstock, by various oleaginous yeasts represents an alternative way to valorize this industrial waste as a starting material for microbial lipids production, may decrease the production cost of the whole process, in a biodiesel plant unit (Chatzifragkou and Papanikolaou, 2012).

Among heterotrophic microorganisms, oleaginous yeasts have increasingly been reported as good triacylglycerols (TAG) producers. These simple lipids have similar composition and energy value to plant oils and animal fats, but their productions do not compete for food resources, in particular if they are based on inexpensive carbon sources e.g. agriculture and industrial wastes/by-products including lignocellulose, whey and crude glycerol. Furthermore, oleaginous yeasts have a short process cycle, and their productions are not subjected to seasonal and cyclical weather variations (Papanikolaou and Aggelis, 2011).

2.4.1 Lipids accumulation and fatty acid synthesis in oleaginous red yeast

Lipids accumulation in oleaginous yeast begins when it exhausts a nutrient from the medium (it is usually nitrogen), but an excess of carbon (in the form of glycerol) is still assimilated by the cells and is converted into TAG, while lipids is synthesized during the balance phase of growth at nearly the same rate (Meng et al., 2009). The major site for fatty acid synthesis is the cytosol (Sul and Smith, 2008). However, acetyl-CoA that constitutes the precursor of intracellular biosynthesis of fatty acid derived from breakdown of citric acid that under some conditions have been previously accumulated inside the mitochondria and then are transported into the cytoplasm (Papanikolaou and Aggelis, 2011).

The glycolysis product is pyruvic acid, which passes through the mitochondria membrane to the mitochondria matrix. Pyruvate-dehydrogenase catalyzes the formation of acetyl-CoA from pyruvic acid and acetyl-CoA either enters inside the Krebs cycle or

is transported again into the cytoplasm in order to enhance biosynthesis of cellular fatty acids (Papanikolaou and Aggelis, 2011). Moreover, citrate therefore accumulates in the mitochondria. An efficient citrate efflux system exists in the mitochondria membrane for the export of citrate (in exchange for malate). Moreover, citrate enters the cytosol and is cleaved by ATP: citrate lyase (ACL) to give acetyl-CoA and oxaloacetate. Then, acetyl-CoA is used for fatty acid biosynthesis. Thus oxaloacetate is converted via malate dehydrogenase to malate, which is then used as the counter ion in the citrate efflux system (Papanikolaou and Aggelis, 2011; Ratledge, 2004). The metabolic pathway of lipids biosynthesis is presented in Figure 2.3.

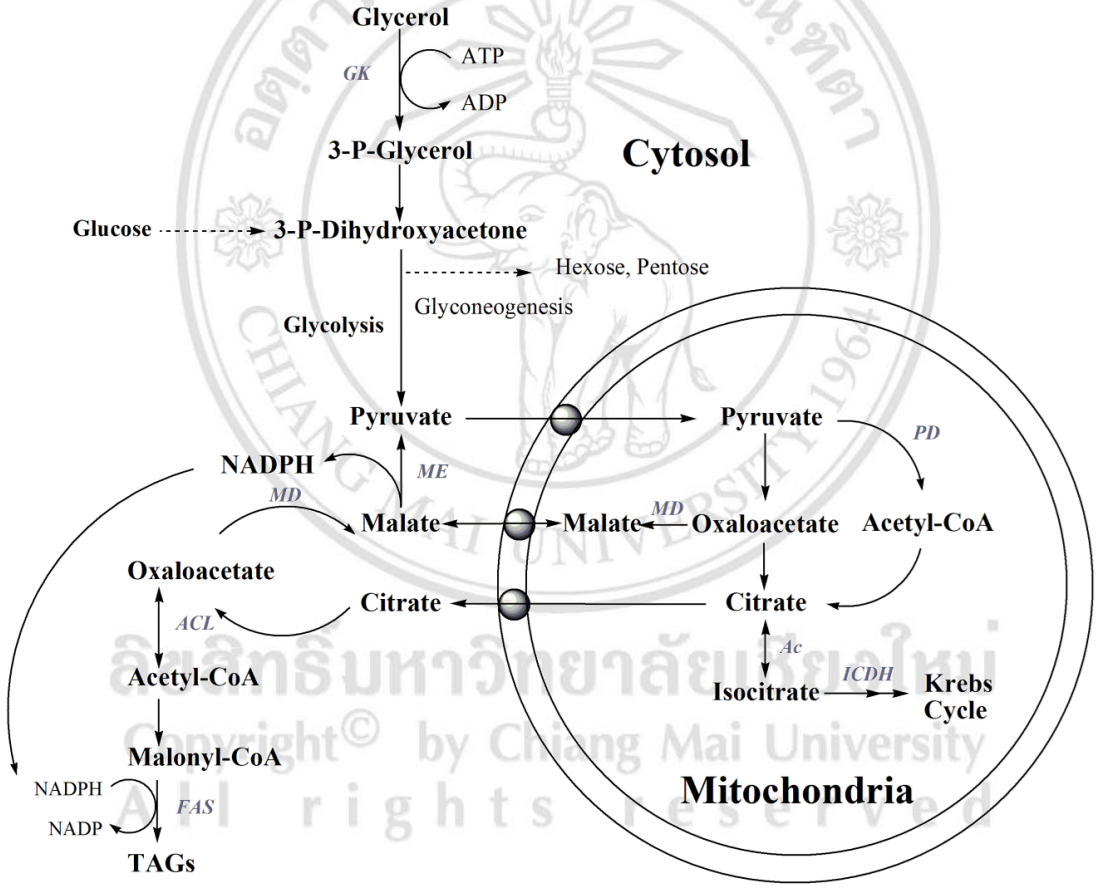


Figure 2.3 Pathway of triacylglycerol synthesis in the oleaginous yeasts. Enzyme: GK, glycerol kinase; PD, pyruvate dehydrogenase; Ac, acotinase; ICDH, iso-citrate dehydrogenase; MD, malate dehydrogenase; ME, malic enzyme; ACL, ATP-citrate lyase; FAS, fatty acid syntheses

Source : Papanikolaou and Aggelis (2011)

2.4.2 Lipids compositions in oleaginous red yeast

The microbial lipids compose more lipids than TAG and free fatty acids (FFA). In fact, not all lipids obtained from microbial biomass are suitable for producing of biodiesel. Only lipids incorporated with fatty acid ester linkages (also refer to as saponifiable lipids) and FFA can be transesterified to be fatty acid alkyl ester (FAAE) or biodiesel. The list of number of carbons, double bonds and structural formula of fatty acid usually found in microbial lipids are shown in Table 2.2. These microbial lipids are categorized as energy storage and structural lipids. The energy storage lipids are neutral lipids (mono-, di- and tri-acylglycerols or MAG, DAG and TAG) and free fatty acid or FFA. The structural lipids are the polar lipids, including phospholipids, sphingolipids, glycolipids and sterol esters (Bautista et al., 2012). As conventional vegetable oils, microbial saponifiable lipids and free fatty acids can be converted into biodiesel through a transesterification and esterification reaction with methanol in the presence of a suitable catalysts e.g. strong base and lipase. The lipids extracted from oleaginous yeasts/red yeasts also contain a fraction of non-saponifiable lipids, which consists of carotenoids, sterols and tocopherols. These lipids do not have fatty acid ester linkages, so they cannot be transformed to biodiesel (Bautista et al., 2012).

Many carbon sources have been reported to be the high potential substrate for lipids production by oleaginous yeasts/red yeast. The most oleaginous microorganisms prefer simple sugar, like glucose, to grow, but the use of glucose or other pure sugars to obtain microbial biomass for the production of biodiesel is not economically (Bautista et al., 2012). Therefore, nowadays the production of microbial oils is predominantly addressed to transformation of raw materials, by-products and lignocellulose (Rossi et al., 2011). Raw materials that are basically rich in sucrose, sugar cane, either in the form of cane juice or cane molasses and beet molasses, have been extensively used for microbial growth. Although this type of carbon source is probably the best among the low cost raw materials, the presence of heavy metals in high concentrations may cause a critical problem during fermentation because they can inhibit the growth of oleaginous microorganisms, influence the pH of the substrate and are involved in the inactivation of the enzymes associated with biosynthesis of products (Bautista et al., 2012).

According to the economic point of view, many researchers focus on the utilization of by-products obtained from industrial section to be the carbon source for growth and lipids accumulation by oleaginous yeast/red yeast. Among various kinds of by-products, crude glycerol shows good potential to be high efficiency low cost carbon source (Easterling et al., 2009). Nowadays, lipids production by oleaginous yeasts/red yeast is focused on selection and development of yeasts as converters of crude glycerol into lipids for biodiesel production, since it is the major by-product of the biodiesel production process. The biotransformation of crude glycerol into lipids is therefore regarded as a promising way to decrease the cost of biodiesel process through simultaneous reutilization of its major byproduct (Rossi et al., 2011; Chatzifragkou and Papanikolaou, 2012). As the mentioned information, crude glycerol is the good substrate for lipids production by various types of oleaginous yeast/red yeast which might be a sustainable biodiesel feedstock e.g. *Pichia kudriavzevii* (Sankh et al., 2013), *Rhodotorula glutinis* (Saenge et al., 2011), *Rhodotorula graminis* (Galafassi et al., 2012), *Rhodosporidium toruloides* (Xu et al., 2012b), *Trichosporonoides spathulata* (Kitcha and Cheirsilp, 2012) and *Yarrowia lipolytica* (Rakicka et al., 2015). The name of oleaginous yeast/red yeasts that capable to grow and accumulate intracellular lipids as well as theirs fatty acid profiles are listed in Table 2.3.

Table 2.2 Number of carbons, double bonds and structural formula of fatty acids

Fatty acids	**Number of carbons and double bonds**	**Chemical structure**
Caproic acid	C6:0	$C_5H_{11}COOH$
Caprylic acid	C8:0	$CH_3(CH_2)_6COOH$
Capric acid	C10:0	$CH_3(CH_2)_8COOH$
Lauric acid	C12:0	$CH_3(CH_2)_{10}COOH$
Myristic acid	C14:0	$CH_3(CH_2)_{12}COOH$
Pentadecanoic acid	C15:0	$CH_3(CH_2)_{13}COOH$
Palmitic acid	C16:0	$CH_3(CH_2)_{14}COOH$
Palmitoleic acid	C16:1	$CH_3(CH_2)_5CH=CH(CH_2)_7COOH$
Stearic acid	C18:0	$CH_3(CH_2)_{16}COOH$
Oleic acid	C18:1	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$
Linoleic acid	C18:2	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$
Linolenic acid	C18:3	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$
Arachidic acid	C20:0	$CH_3(CH_2)_{18}COOH$
Eicosenoic acid	C20:1	$CH_3(CH_2)_7CH=CH(CH_2)_9COOH$
Behenic acid	C22:0	$CH_3(CH_2)_{20}COOH$
Eurcic acid	C22:1	$CH_3(CH_2)_7CH=CH(CH_2)_{11}COOH$

Source: Tyson et al. (2004)

Table 2.3 Fatty acid composition of various types of microbial lipids

Oleaginous yeasts	Crude glycerol (g/L)	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2	References
Pichia kudriavzevii	23.00	NA*	29.30	1.00	8.89	41.90	9.22	Sankh et al. (2013)
Rhodotorula glutinis	60.70	0.87	16.80	0.81	3.68	45.75	17.92	Saenge et al. (2011)
Rhodotorula graminis	54.00	NA	20.49	NA	7.36	43.62	19.44	Galafassi et al. (2012)
Rhodosporidium toruloides	70.00	1.60	29.10	1.00	17.80	38.10	9.70	Xu et al. (2012b)
Trichosporonoides spathulata	56.40	1.10	35.10	2.10	7.50	41.10	11.20	Kitcha and Cheirsilp (2012)
Yarrowia lipolytica	40.00	NA	20.00	9.30	NA	51.00	10.40	Rakicka et al. (2015)

*NA: Not Available

2.5 Carotenoids production

Carotenoids are yellow to orange-red pigments that are ubiquitous in nature (Waites et al., 2001). They are a group of 600 molecules which can be found in plants and microorganisms, including bacteria, algae, molds and yeasts (Frengova and Beshkova, 2009), but are not synthesized in animals (Rock, 1997). Carotenoids are soluble in nonpolar solvents, including edible fats and oils but they are not soluble in water. The color of carotenoids is the result of the presence of a conjugated double bond system in the molecules. The electron excitation spectra of systems are of interest for elucidation of their structure and for qualitative and quantitative analysis (Belitz and Grosch, 1986).

Carotenoids are sensitive to heat, light and oxygen. Therefore, the extraction of carotenoids must be performed away from direct sunlight and where possible, solutions should be protected from oxidation by maintaining a nitrogen atmosphere or by adding antioxidants e.g. vitamin E, vitamin C and derivatives (King, 1978). Membranes of microorganisms, plants and animals contain carotenoids as pigments as direct constituents of their phospholipids bilayer (Figure 2.4). The rod-like structure of carotenoids molecules are often terminated with polar groups. The molecular dimensions of typical carotenoids matching the thickness of the hydrophobic membrane core, are directly responsible for the localization and orientation of pigment molecules within the membrane and for effects on the membrane properties (Gruszecki, 1999). Model studies have revealed several effects of carotenoids on the structure and dynamics of lipids membranes. Restrictions to the motional freedom of lipids due to the hydrophobic interactions with rigid rod-like molecules of carotenoids are the main cause of the effects on the membrane properties such as the increase in the membrane rigidity and thermal stability or the increase in the penetration barrier to molecular oxygen and other small molecules (Fuji Health Science, 2012; Gruszecki, 1999).

Carotenoids represent one of the broadest groups of natural antioxidants with significant biological effect and numerous industrial applications (Certik et al., 2009). Industrially, carotenoids are used in pharmaceuticals, neutraceuticals and animal feed additives, as well as colorants in cosmetics and foods. Scientific interest in dietary

carotenoids has increased in the recently years because of their beneficial effects on human health such as lowering the risk of cancer and enhancement of immune system function, which are attributed to their antioxidant potential (Das et al., 2007).

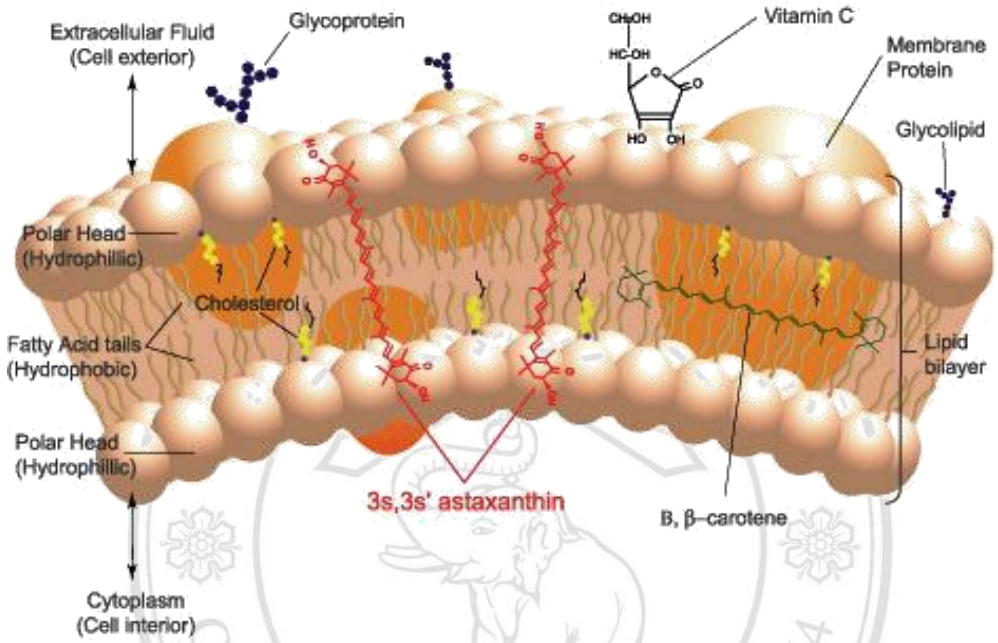


Figure 2.4 The orientation of astaxanthin and β-carotene in phospholipids bilayer
Source: Fuji Health Science (2012)

Despite the availability of a variety of natural and synthetic carotenoids, there is currently renewed interest in microbial sources. Many type of microorganisms could accumulate several types of carotenoids as a part of their response to various environmental stresses (Bhosale, 2004; Certik et al., 2009). Microbial production of carotenoids, when compared with extraction from vegetables or chemical synthesis seems to be of paramount interest mainly because of the problems of seasonal and geographic variability in the production and marketing of several of the colorants of plant origin (Certik et al., 2009). Moreover, the increasing interest in microbial sources of carotenoids is related to consumer preference for natural additives and the potential cost effectiveness of creating carotenoids via microbial biotechnology. Moreover microbial production of carotenoids are an environmental friendly method compared to chemical production and able to meet the increasing demand of natural carotenoids (Das et al., 2007).

The most interesting carotenoids producing microorganism is a group of red yeast and oleaginous red yeasts e.g. *Rhodotorula, Rhodosporidium, Sporidiobolus, Sporobolomyces, Cystofilobasidium, Kockovaella* and *Xanthophyllomyces* (formerly *Phaffia*). The major pigments found in oleaginous red yeast are β-carotene, γ-carotene, torulene, torularhodin and astaxanthin (Dufosse, 2006). Among those red yeasts, genera *Rhodotorula* is one of main carotenoids producer with predominant synthesis of β-carotene, torulene and torularhodin (Davoli et al., 2004; Libkind and van Broock, 2006; Maldonade et al., 2008). Red yeasts in the genus *Cystofilobasidium* and *Dioszegia* have also found to synthesize these three pigments (Certik et al., 2005). Some of carotenoids from red yeasts are modified with oxygen-containing functional groups e.g. astaxanthin which is almost exclusively formed by *Xanthophyllomyces dendrorhous* (Certik et al., 2005).

The efficiency of the carbon source conversion into high growth rate and carotenoids accumulation in red yeast and the optimization of the growth medium with respect to reduce of production cost, have been the subject of intensive studies (Certik et al., 2009). Numerous carbon sources, including pentose and hexose sugars, various disaccharides, glycerol, ethanol, methanol, oils/fats, alkanes or a wide variety of wastes derived from agricultural production e.g. molasses, grape must, sugar cane molasses, whey lactose, coconut milk, radish brine and rice bran, have been used for biotechnological production of carotenoids and derivatives (Aksu and Eren, 2005; Buzzini, 2000; Dominguez-Bocanegra and Torres-Munoz, 2004; Frengova et al., 2004; Malisorn and Suntornsuk, 2008; Roadjanakamolson and Suntornsuk, 2010).

In general, high grade and high concentration of carbon source support the growth of red yeast and total carotenoids production (Aksu and Eren, 2005), however the low cost carbon sources have to be considered. Therefore, many researchers focus on using crude glycerol as a carbon source and combine with optimization process to increase of yeasts growth and carotenoids production, because its price is very low (Fan et al., 2010; Nicol et al., 2012). For example, Yimyoo et al. (2011) who reported that *Rhodosporidium paludigenum* DMKU3-LPK4, the red yeast isolated from soil samples in the northern part of Thailand. It exhibited the capacity to produce carotenoids with the application of glycerol as the sole carbon source. The carotenoids concentration was 3.42 mg/L under the optimal condition. While, Saenge et al. (2011) reported that crude

glycerol could be used as the sole carbon source for carotenoids production by *Rhodotorula glutinis* TISTR5159, the relatively high carotenoids production of 135.25 mg/L was obtained. Moreover, crude glycerol could enhance the total carotenoids contents over than pure glycerol by 19.0% when by *Sporobolomyces pararoseus* TISTR5213 was cultivated with this glycerol (Manowattana et al., 2012).

2.5.1 Carotenoids biosynthesis in oleaginous red yeast

All carotenoids are synthesized via two pathways that lead to the formation of isopentenyl diphosphate (IPP) and/or dimethylallyl diphosphate (DMAPP), the pathway building units. Eukaryotes (except Euglenophyta) and archeabacteria typically follow the mevalonate pathway (MVA) producing both IPP and DMAPP precursors (Sanchez et al., 2013). The starting materials of mevalonate (MVA) pathway such as acetyl-CoA, pyruvate, and 3-P-Glycerol (G3P) are the metabolites of glycolysis pathway (Figure 2.5). Carotenoids production has been greatly enhanced by the balanced and increased formation of the metabolites. Pyruvate is required as a precursor in many metabolic pathways and, presumably, more available than G3P for isoprenoid biosynthesis (Das et al., 2007).

In the MVA pathway, acetyl-CoA is condensed to form acetoacetyl-CoA by acetyl-CoA acetyltransferase. Then, the conversion of acetyl-CoA to 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) is catalyzed by HMG-CoA synthase. HMG is then reduced by HMG-CoA reductase finally to obtain mevalonate. Next, mevalonate is sequentially phosphorylated, first by mevalonate kinase and then by phosphomevalonate kinase (Das et al., 2007). Then, it undergoes a decarboxylation that results in the formation of IPP. This IPP is transformed to its isomer DMAPP by IPP isomerase (Kuzuyama, 2002). Condensation of IPP with DMAPP produces geranyl diphosphate (GPP) through the action of GPP synthase. The addition of IPP to GPP produces farnesyl diphosphate (FPP) and the addition of one more IPP unit yields the C_{20} compound geranylgeranyl diphosphate (GGPP) (Sanchez, 2013). Condensation of two molecules of GGPP leading to phytoene, the first C_{40} carotene of the pathway, which undergoes desaturation to form lycopene (Goodwin, 1993).

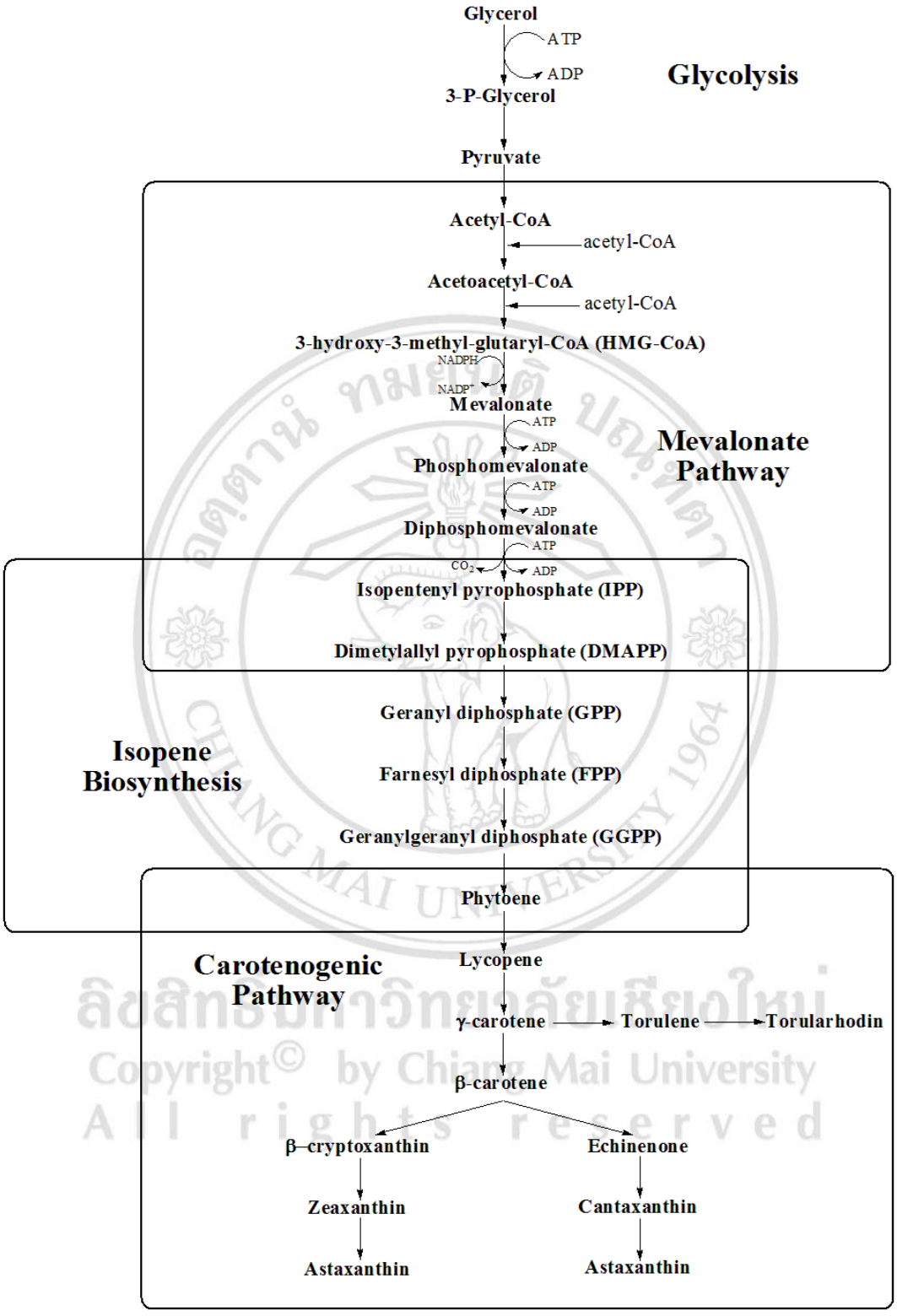


Figure 2.5 Biosynthesis of carotenoids from glycolysis pathway to carotenogenic pathway by oleaginous red yeast

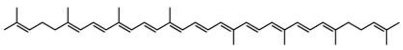
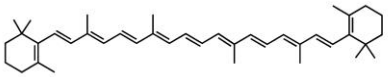
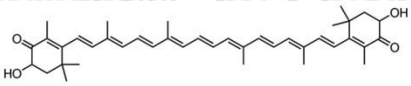
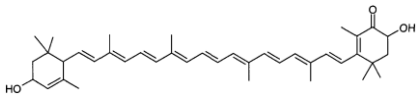
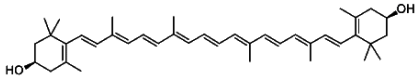
Source : Das et al. (2007)

Lycopene acts as precursor of cyclic carotenoids and undergoes a number of metabolic reactions (e.g. cyclization) to form β-carotene, γ-carotene, torulene, torularhodin or astaxanthin. The γ-carotene is the major branch point and acts as the precursor for β-carotene and torulene. Hydroxylation and oxidation of torulene by mixed function oxidase lead to the formation of torularhodin (Frengova and Beshkova, 2009).

2.5.2 Carotenoids compositions in oleaginous red yeast

The major composition of carotenoids of red yeasts are β-carotene, γ-carotene, torulene, torularhodin and astaxanthin (Dufosse, 2006). The structure and major commercial utility of carotenoids in red yeasts is shown in Table 2.4.

Table 2.4 Structure and major commercial utility of carotenoids

Carotenoids	**Structures**	**Major commercial utility**
Lycopene		Against cardiovascular diseases, prostate cancer and cosmetic preparations
β-carotene		Anticancer agent, provitamin A source, food colorants, photo protectant and cosmetic preparations
Astaxanthin		Feed additive mainly in aquaculture and as food colorants
Lutein		Prevention of age-related macular degeneration (AMD), cosmetic preparations
Zeaxanthin		Prevention of age-related macular degeneration (AMD)

Source: Bhosale (2004)

1) β-carotene is produced on a large scale by chemical synthesis, and also plant sources and fermentation by red yeasts. The majority of natural sources contain the all-*trans* isomer as the dominant geometrical isomer as is the case for synthetic β-carotene. The various preparations differ in the composition of geometrical isomers and in the presence of α-carotene and other carotenoids, particularly biosynthetic intermediates (Dufosse, 2006).

2) Astaxanthin is present in all salmon fishes, various marine animals as well as in some flowers. Astaxanthin is responsible for the pink color customers require from a healthy salmon (Liaaen-Jensen, 2004). There are also reports of beneficial actions of astaxanthin for human health, so its use in supplements is of interest. The biotechnological production of astaxanthin from microalgae, yeasts and bacteria is the subject of intensive investigation, though synthetic astaxanthin remains the market leader (Dufosse, 2006). Some red yeasts carotenoids are modified with oxygen-containing functional groups e.g. astaxanthin which is almost exclusively formed by *Xanthophyllomyces dendrorhous* (Certik et al., 2005).

3) Torulene and torularhodin, red yeasts of the genus *Rhodotorula* synthesize carotenoids, mainly torularhodin and torulene accompanied by very small amounts of β-carotene. Most of the research has focused on the species *Rhodotorula glutinis*, though other species such as *Rhodotorula gracilis*, *Rhodotorula rubra*, and *Rhodotorula graminis* have been studied. These yeasts have potential as feed products rather than as health supplements. Moreover, optimization studies have mainly resulted in an increased yield of torulene and torularhodin, which are of minor interest, though some did succeed in increasing the β-carotene content up to about 70 mg/L (Dufosse, 2006).

2.6 Optimization of carotenoids and lipids productions by oleaginous red yeast

In order for current biotechnology research to continue revolutionizing industries, new processes must be developed to transform current research into viable market products. Specifically, attention must be directed toward the industrial processes of cultivation of cells, tissues, and microorganisms (Harada et al., 1997).

The performance of improved strains selected from a screening process using small-scale fermentation vessels must be validated in large-scale bioreactors. Once validated, the improved strains will be used for commercial production. The process of transferring a laboratory fermentation process to an industrial operation is referred to as scale-up. The goal of scale-up is to recreate in large production bioreactors the optimal conditions in which the improved strains are cultivated in the laboratory. This is accomplished by controlling environmental conditions, including power input, mixing ability, oxygen transfer, shear stress, heat transfer, media sterilization and seed culture preparation etc. (Vanot and Sergent, 2005).

There are several major topics to consider in scaling up laboratory processes to the industrial level. In general, scale-up is accomplished for a discrete system through laboratory and pilot scale operations. The steps involved can be broken down into seven topics that require some elaboration (Vanot and Sergent, 2005):

1. Strain improvements
2. Optimization of medium composition and cultural conditions
3. Oxygen supply required by cells to achieve the proper metabolic activities
4. Selection of an operative mode for culture process
5. Measurement of rheological properties of cultural broth
6. Modeling and formulation of process control strategies
7. Manufacturing sensors, bioreactors and other peripheral equipment

Steps 1 and 2 should be determined in the laboratory using shake flasks or small size bioreactors. Steps 3–7 are usually determined in the pilot plant. The importance of the pilot plant is, however, not limited to steps 3–7. The pilot plant also provides the cultured broths needed for downstream processing and can generate information to determine the optimal cost structure in manufacturing and energy consumption as well as the testing of various raw materials in the medium (Vanot and Sergent, 2005).

2.6.1 Optimization of medium composition and cultural conditions in shaking flask level

Cellular organisms require specific internal conditions for optimal growth and function. Understanding yeast requirement is important for successful cultivation of yeast in the laboratory but also for optimization of industrial fermentation process. Elemental composition of yeast cell gives a broad indication as to the nutritional requirements of the yeast cell. Similar to yeasts, oleaginous red yeasts require essential elements from their growth environment from simple food sources which need to be available at the macronutrient level in the case of C, H, O, N, P, K, Mg and S or at the micronutrient level in the case of trace elements. Oleaginous red yeasts are chemoorganotrophs as they use organic compounds as a source of carbon and energy. Yeasts can use a wide variety of substances as nutrient sources (Marova et al., 2011).

In order to improve the production yield of lipids and carotenoids pigments, so that subsequently decrease the cost of this biotechnological process, diverse studies have been performed by optimizing the culture conditions, including nutritional and physical factors. Factors such as nature and concentration of carbon and nitrogen sources, minerals, vitamins, pH, aeration, temperature, light irradiation, and stress have a major influence on cell growth, yield of lipids and carotenoids by oleaginous red yeasts (Certik et al., 2005; Marova et al., 2011).

1) Carbon source: lipids and carotenoids accumulation by oleaginous red yeasts depends mostly on nutrient limitation conditions when excess carbon is presented in the medium (high C/N ratio). Nutrient limitation prevents cells from being generated, while the carbon excess is converted into lipids and carotenoids. Not only C/N ratio, but many published studies also report that phosphorus, magnesium, zinc, or iron limitation lead to lipids and carotenoids accumulation in model oleaginous red yeasts (Rossi et al., 2011). Numerous sources, including pentose and hexose sugars, various disaccharides, glycerol, ethanol, methanol, oils, n-alkanes or a wide variety of wastes derived from agricultural production (e.g. molasses, grape must, sugar cane molasses, whey lactose, coconut milk, radish brine, rice bran), have been considered as potential carbon sources for biotechnological production of carotenoids (Aksu and Eren, 2005; Buzzini, 2000;

Dominguez-Bocanegra and Torres-Munoz, 2004; Frengova et al., 2004; Malisorn and Suntornsuk, 2008; Roadjanakamolson and Suntornsuk, 2010). In general, the increase in sugar concentration increased the growth of yeast and total carotenoids production (Aksu and Eren, 2005), however the low cost carbon sources have to be considered according to low production cost aspect.

2) Nitrogen limitation: the chemical composition and concentration of nitrogen source in the medium might also be a means of physiological control and regulation of metabolism in microorganisms (Certik et al., 2009). Nitrogen limitation is the most efficient form of nutrient limitation for lipogenesis and carotenogenesis induction, leading to the highest values of substrate/lipids or substrate/carotenoids conversion yield, and lipids and carotenoids content within biomass. Therefore, nitrogen limitation is commonly used to induce lipogenesis and carotenogenesis in oleaginous red yeast and the utilization of cultural media with appropriate C/N ratio is crucial to maximize lipids and carotenoids production. Moreover, lipids and carotenoids accumulation typically occurs during the latter stages of cultivation in many microorganisms, that is, after growth has ceased. Thus, nutrient depletion appears to be associated with lipogenesis and carotenogenesis. A high C/N ratio in the culture medium has been used extensively to promote lipids and carotenoids accumulation (Rossi et al., 2011; Sanchez et al., 2013).

3) pH value: pH is one of most important environment parameters affecting cell growth, lipids and carotenoids production (Saenge et al., 2011). The optimal growth pH value is different and response to a wide range of pH varies with oleaginous red yeast strain (Rattray et al., 1975). Moreover, it has been found that the pH optimal for lipids and carotenoids accumulation lower than that for optimal growth. For example, highest cell growth of *Xanthopyllomyces dendrorhous* was observed at around pH 6.0, while a highest astaxanthin was presented around pH 4.0 (Hu et al., 2006). Moreover, *Rhodotorula glutinis* could accumulate lipids and carotenoids at the optimum pH ranging between 5 and 6 (Schneider et al., 2013).

4) Temperature: temperature is considered to be the main physical factor directly controlling growth rate and plays an important role in the biosynthesis pathway of lipids and carotenoids in oleaginous red yeast (Bhosale, 2004). The optimal temperature for high growth rate and higher production for each oleaginous red yeast strains are different but generally around 25°C but do not above 30°C. Very high or very low temperature affects the cell growth, lipids and carotenoids accumulation, which higher temperature will cause the denaturation of specific lipogenesis/ carotenogenesis enzymes involve mesophilic oleaginous red yeasts (Aksu and Eren, 2007; Sha, 2013). In case of lipids production, during yeast cells lipids accumulation, dropping temperature from optimal growth generally results in increasing of the lipids content and influence lipids composition. Usually, melting point of unsaturated fatty acids is lower than saturated fatty acids and for short chain fatty acids (SCFA) lower than long chain fatty acids (LCFA). Thus, temperature decreasing results in increasing level of unsaturated and short chain fatty acids, for example increasing ratio of linoleic acid to oleic acid (Sha, 2013). Similar with carotenoids production, cultivation temperature has a strong influence on intracellular carotenoids content, carotenoids profile and cell growth rate of red yeast (Sanchez et al., 2013).

5) Light irradiation: carotenoids plays important roles in light harvesting and photoprotection of photosynthetic organisms (Sanchez et al., 2013). Carotenogenesis in many microorganisms is regulated by light. However, the intensity and protocol of illumination vary with the microorganism. Irrespective of whether increases or decreases in illumination time and/or intensity lead to improvements in carotenoids yield. There are two aspects to the theory of photo-induction. The first is that improvements of the volumetric production of carotenoids (mg/L) are generally associated directly with improved growth of the microorganism. Thus, the effect of light on the growth of the microorganism plays an important role in establishing the authentic role of white-light illumination as a stimulant of carotenoids production. The second aspect to be considered is that increases in the cellular accumulation (mg/g) of carotenoids are associated with increased activity of enzymes involved in carotenoids biosynthesis (Frengova and Beshkova, 2009). For example, an increased carotenoids accumulation of *Rhodotorula glutinis* was observed in the light irradiation treatment.

The maximum carotenoids concentration reached 2.6 mg/L in the three LED lamps, which increased nearly by 1.4 mg/L compared with the control group (1.2 mg/L) (Zhang et al., 2014).

6) Aeration rate and dissolved oxygen (DO): Oxygen plays important roles in lipids and carotenoids biosynthesis (Sanchez et al., 2013). Lipogenesis and carotenogenesis are aerobic process, and the air flow rate in the oleaginous red yeast culture is an essential factor to assimilate the substrate as well as for growth rate, biomass, lipids and carotenoids synthesis. The effect of aeration is dependent on the species of the oleaginous red yeasts strain (Frengova and Beshkova, 2009). For example, the cultivation of *Rhodotorula glutinis* at the low DO was suggested for the purpose of lipids production. Under high DO control, resulted in rapid cell growth to the maximum biomass of 56.6 g/L, as compared to 35.8 g/L in the low DO. However, the lipids content obtained in the high DO experiment was 47.3±7.2%, which was obviously lower than the value of 63.4±5.6% that obtained in the low DO condition (Yen and Zhang, 2011). In contrary with the result of Yen and Liu (2014) who found that the relatively low level of DO in the airlift bioreactor led to the low level of cell growth of *Rhodotorula glutinis*. In order to raise the DO in the airlift bioreactor, the aeration rate was varied at 1.0, 1.5, 2.0 and 2.5 vvm. The results found that a higher aeration rate led to a significantly higher cells growth rate. The maximum biomass concentration of 25.40 g/L was obtained at 2.0 vvm of aeration rate.

Once a fermentation medium is developed, the next phase is to optimize the media composition to achieve the best possible benefit. The traditional method of media optimization is to alter one ingredient at a time until its optimum concentration is identified while the remaining ingredients are held constant. Recently, new techniques such as statistical experimental design have become available to assist fermentation scientists. The performance of improved strains selected from a screening process using small-scale fermentation vessels must be validated in pilot-scale bioreactor. Once validated, the improved strains will be used for commercial production. The process of transferring a laboratory fermentation process to an industrial operation is referred to as scale-up. The goal of scale-up is to recreate in large production bioreactor the optimal

conditions in which the improved strains are cultivated in the laboratory (Vanot and Sergent, 2005).

2.6.2 Scale up of carotenoids production in bioreactor

1) Stirred tank bioreactor, in the conventional stirred tank bioreactor, mixing and bubble dispersion are achieved by mechanical agitation; this requires a relatively high input of energy per unit volume. Baffles are used in stirred reactors to reduce overtaxing. A wide variety of impeller sizes and shapes is available to produce different flow patterns inside the vessel; in tall bioreactors, installation of multiple impellers improves mixing. Stirred bioreactors are used for free- and immobilized enzyme reactions, and for culture of suspended and immobilized cells. Care is required with particulate catalysts which may be damaged or destroyed by the impeller at high speeds (Doran, 1995).

2) Airlift bioreactor, the airlift bioreactors are accomplished without mechanical agitation. Airlift reactors are often chosen for culture of plant and animal cells and immobilized catalysts because shear levels are significantly lower than in stirred vessels. The airlift configuration confers a degree of stability to liquid flow compared with bubble columns; therefore, higher gas flow rates can be used without incurring operating problems such as slug flow or spray formation. Several empirical correlations have been developed for liquid velocity, circulation time and mixing time in airlift reactors (Doran, 1995). Airlift bioreactor systems provide some advantages as the list below (Willium, 2002).

Simple design with no moving parts or agitator shaft seals, for less maintenance, less risk of defects and easier sterilization.

1) Lower shear rate, for greater flexibility.
2) The system can be used for growing both plant and animal cells.
3) Efficient gas-phase disengagement.
4) Large, specific interfacial contact-area with low energy input.
5) Well-controlled flow and efficient mixing and well-defined residence time for all phases.

6) Increased mass-transfer due to enhanced oxygen solubility achieved in large tanks with greater pressures.
7) Large-volume tanks possible, increasing the output.
8) Greater heat-removal compares to conventional stirred tanks.

While, the main disadvantages are listed as below

1) Higher initial capital investments due to large scale processes. Greater air through put and higher pressures needed, particularly for large-scale operation.
2) Low friction with an optimal hydraulic diameter for the riser and down comer.

In general, conventional agitation tanks are often used for the cultivation of oleaginous red yeast, due to the relatively high oxygen transfer coefficient that can be achieved in such devices. Nevertheless, airlift bioreactors are attractive alternatives to agitations tanks, as they are both relatively simple and cheap to operate (Yen and Liu, 2014). For example, Yen and Liu (2014) reported that the comparison of *Rhodotorula glutinis* growth in an agitation and airlift bioreactors. The growth of *Rhodotorula glutinis* in the agitation tank was better than in the airlift bioreactor, based on a 1.0 vvm of aeration rate. The greatest biomass in the stirred tank and airlift bioreactors were about 20.8 and 16.6 g/L, respectively. Nevertheless, the rapid cell growth in the agitation tank led to a lower lipids content compared to the airlift bioreactor, the average lipids content in the agitation tank was only about 25.0±4.0%, far less than the 45.0±3.0% achieved in the airlift bioreactor (Yen and Liu, 2014).