CHAPTER 2

Literature Review

2.1 Food waste

Food waste has been estimated to increase by 44% between of 2005 to 2025. Because of the rapid economic development that is mostly expected in Asian nations. It is predicted that the largest increase of food waste generation in Asia could be from 278 million to 416 million tons, which would contribute to global anthropogenic emissions ranging from 8 to 10% (Thi et al., 2015). Among developing countries in South East Asia, Thailand has the highest food waste generation rate of 0.40 kg/day per head and around 9,312,788 tons/year of total food waste (Sharp and Sang-Arun, 2012).

The pollution control department (PCD) of Thailand drafted the national 3R (reduce, reuse, recycle) strategy and 3R act to promote and support the 3R implementation for management organic waste in Thailand. The 3R strategy aims to increase organic waste utilization by 50% before 2026. This strategy can greatly contribute to climate change migration by avoiding landfill gas emissions, supply organic fertilizer for cultivation, and generating alternative energy. Moreover, the government of Thailand encouraged people to separate food waste at sources in some cities to reinforce 3R strategy implementation (Sharp and Sang-Arun, 2012).

Food waste is defined as any food that is wasted, degraded or lost during production or at consumer level. Food waste consists of around 300–600 mg/g starch, 60–100 mg/g proteins and 70–300 mg/g lipids, which make it a promising source of nutrients in fermentation process after hydrolysis (Lau et al., 2014). The major components of the food waste is starch, which can be hydrolyzed to fermentable sugars (Matsakas et al., 2014). The fermentable sugars can be used as a carbon source for the growth and high value metabolite production e.g. ethanol (Hong and Yoon, 2011; Kim et al., 2011; Li et al., 2011; Ma et al., 2009; Matsakas et al., 2014), hydrogen gas

(H_2) (Elbeshbishy et al., 2011; Kim et al., 2010; Yasin et al., 2011), methane (Kastner et al., 2012; Papanikolaou et al., 2011), and microbial oil a feedstock for biodiesel by various oleaginous microorganisms (Lau et al., 2014).

Lau et al. (2014) studied the cultivation of *Chlorella vulgaris* by enzymatic-food waste hydrolysate. The results revealed that *Chlorella vulgaris* showed the biomass production of 0.9 g/g (glucose). Moreover, microalgae biomass composed of carbohydrates 400 mg/g, proteins 200 mg/g and lipids 200 mg/g, respectively.

Pisutpaisal et al. (2014) produced the hydrogen and methane from food waste in a two-stage 5–L continuous stirred tank reactors system. The first-stage hydrogen and the second-stage methane were produced under mesophilic fermentation with the initial pH 6 and 7 and hydraulic retention time of 12 and 24 h, respectively. The results revealed that the hydrogen and methane yields were 292.7 and 391.6 mL/g (total volatile solid) at the steady stage operation.

Kim et al. (2011) studied the ethanol production from food waste that consisted of carbohydrate (65% w/w). Food waste was hydrolyzed by using amylolytic enzyme which yielded glucose of 0.63 g glucose/g total solid. Enzymatic hydrolysis and ethanol fermentation by using amylolytic enzyme and *Saccharomyces cerevisiae* were conducted by separated hydrolysis and fermentation process (SHF) and the maximum of ethanol yield of 0.43 g ethanol/g total solids was obtained.

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.

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 biodiesel feedstock.

2.2 Oleaginous yeast

Oleaginous yeasts are single cell oil (SCO) (Dong et al., 2016) which can be accumulated lipids in their cell more than 20% (w/w). Oleaginous yeasts have a fast growth rate and lipids synthesis (Subramaniam et al., 2010). Lipids derived from oleaginous yeast, known as microbial lipids. These lipids have fatty acid compositions similar to vegetable oil which can use as the third-generation biodiesel feedstock. The main fatty acid composition are inducing of palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1) and linoleic acid (C18:2) (Thevenieau and Nicaud, 2013). This generation biodiesel feedstock is based on improvements in the production of microbial-derived biomass which is not food competitors, better environmental performance and particularly in terms of lower greenhouse gas emissions (Gambelli et al., 2017). The production of microbial lipids has many advantages over the vegetable oils such as shorter culture period, easy to harvest, no need of agricultural land, easy cultivability, seasonal independence and favorable fatty acid composition similar to plant oil (Kolouchová et al., 2015; Xu et al., 2012).

The diversity of oleaginous yeast considered as oleaginous as shown in Table 2.1. The typical genera and majority of studies of yeasts which considered as oleaginous are *Sporidiobolus*, *Rhodosporidium*, *Rhodotorula*, *Yarrowia*, *Trichosporon*, *Cryptococcus*, *Candida* and *Lipomyces* (Sitepu et al., 2014). There are many types of carbon sources that have been used for growth and lipogenesis of oleaginous yeasts as shown in Table 2.2. Most of oleaginous yeasts can accumulate lipids more than 20–40% of their dry cell weight. The main requirement for high lipids production is a culture medium with an excess of carbon source and other limitation of nutrients, mostly nitrogen. Moreover, some oleaginous yeasts species can accumulate both lipids and carotenoids. The morphology of colony of these strains is red, orange and pink color such as *Rhodotorula*

sp., *Rhodosporidium* sp., *Sporidiobolus* sp. and *Sporobolomyces* sp. The benefits of carotenoids are several important biological actions as provitamin A, antioxidant, anticarcinogenic activities and immunomodulation (Saenge et al., 2011b).

Table 2.1 Diversity of oleaginous yeast species

Taxonomic placement	**Genus, species (synonyms or anamorph)**
Phylum Ascomycota	*Myxozyma melibiosi*
Subphylum Saccharomycotina	*Myxozyma udenii*
Class Saccharomycetes	*Lipomyces lipofer* (syn. *Torulopsis lipofera*)
Order Saccharomycetales	*Lipomyces lipofer*
Family Lipomycetaceae	*Lipomyces tetrasporus* (syn. *Zygolipomyces lactosus*)
	Lipomyces starkeyi
	Lipomyces doorenjongii
	Lipomyces kockii
Family Metschnikowiaceae	*Kodamaea ohmeri* (syn. *Candida guilliermondii*)
	Metschnikowia pulcherrima (anamorph *Candida pulcherrima*)
	Metschnikowia gruessii (syn. *Nectaromyce reukaufii*)
Family Wickerhamomycetacaea	*Cyberlindnera jadinii* (syn. *Candida utilis, Pichia jadinii, Candida guilliermondii*)
	Cyberlindnera jadinii (syn. *Candida utilis, Pichia jadinii, Candida guilliermondii*)
	Cyberlindnera saturnus (syn. *Lindnera saturnus, Hansenula saturnus*)
	Candida freyschussii
	Wickerhamomyces ciferrii (syn. *Hansenula ciferrii*)
Family Debaryomycetaceae (*Schwanniomyces* clade)	*Schwanniomyces occidentalis*
Family Debaryomycetaceae (*Candida fragi* clade)	*Kurtzmaniella cleridarum* (syn. *Candida cleridarum*)

Table 2.1 (Continued)

Taxonomic placement	Genus, species (synonyms or anamorph)
Family Debaryomycetaceae (*Yamadazyma* clade)	*Candida diddensiae*
Family Debaryomycetacaea (Lodderomyces-Spathaspora clade)	*Candida tropicalis*
Family Dipodascaceae	*Geotrichum fermentans* (syn. *Trichosporon fermentans*) *Geotrichum histeridarum* *Magnusiomyces magnusii* (syn. *Endomyces magnusii*) *Galactomyces candidus* (anamorph *Geotrichum candidum,* syn. *Oospora lactis, Oidium*) *Galactomyces pseudocandidus* (syn. *Geotrichum vulgare*)
Family Saccharomycetaceae Order Saccharomycetales	*Torulaspora delbrueckii* *Trigonopsis variabilis*
Family Incertae sedis	*Yarrowia lipolytica* (syn. *Candida lipolytica*)
Phylum Basidiomycota Subphylum Pucciniomycotina Class Cystobasidiomycetes Order Cystobasidiales *Rhodotorula minuta* clade	*Rhodotorula minuta*
Class Microbotryomycetes Order Leucosporidiales *Leucosporidiella* clade	*Leucosporidiella creatinivora*
Order Sporodiobolales *Rhodosporidium* clade	*Rhodosporidium paludigenum* *Rhodosporidium sphaerocarpum* *Rhodotorula mucilaginosa* *Rhodotorula colostri*
Rhodotorula glutinis clade	*Rhodotorula graminis*

Table 2.1 (Continued)

Taxonomic placement	Genus, species (synonyms or anamorph)
	Rhodotorula glutinis *Rhodosporidium babjevae* *Rhodosporidium diobovatum* *Rhodosporidium toruloides* (syn. *Rhodotorula gracilis*)
Sporidiobolus ruineniae clade	*Rhodosporidium fluviale*
Rhodotorula glacialis clade	*Rhodotorula glacialis*
Order Incertae sedis	*Rhodotorula terpenoidalis*
Subphylum Agaricomycotina Class Tremellomycetes	*Cryptococcus terreus* *Cryptococcus terricola* (syn. *Cryptococcus terricolus*)
Order Filobasidiales	
Cryptococcus aerius clade	*Cryptococcus aerius* (syn. *Cryptococcus albidus* var. *aerius*)
Cryptococcus albidus clade	*Cryptococcus adeliensis* *Cryptococcus albidus*
Cryptococcus floriforme clade	*Cryptococcus wieringae* *Cryptococcus oeirensis*
Order Tremellales	
Hanaella clade	*Hannaella* aff. *zeae*
Cryptococcus aurantia lineage	*Tremella encephala*
Cryptococcus victoriae clade	*Cryptococcus victoriae* *Cryptococcus* aff. *taibaiensis*
Bulleromyces clade	*Cryptococcus laurentii* *Cryptococcus aureus* (syn. *Rhodotorula aurea*)

Table 2.1 (Continued)

Taxonomic placement	Genus, species (synonyms or anamorph)
Filobasidiella clade	*Filobasidiella neoformans* (syn. *Cryptococcus neoformans*) *Cryptococcus podzolicus*
Order Cystofilobasidiales *Guehomyces* clade	*Guehomyces pullulans* (syn. *Trichosporon pullulans, Endomycopsis vernalis, Endomyces vernalis*)
Order Trichosporonales *Trichosporon gracile* clade	*Trichosporon cacaoliposimilis* *Trichosporon loubieri*
Trichosporon cutaneum clade	*Trichosporon guehoae* *Trichosporon oleaginosus* *Trichosporon cutaneum* *Trichosporon dermatis*
Cryptococcus humicola clade	*Cryptococcus humicola* (syn. *Candida humicola*) *Cryptococcus musci* *Cryptococcus ramirezgomezianus*
Trichosporon ovoides clade	*Trichosporon coremiiforme*
Trichosporon brassicae clade	*Trichosporon domesticum* *Trichosporon montevideense* *Trichosporon brassicae*
Order Incertae sedis	*Cryptococcus curvatus* (syn. *Candida curvata, Apiotrichum curvatum*)
Class Ustilaginomycetes Order Ustilaginales *Pseudozyma* clade	*Pseudozyma aphidis*
Subphylum *Incertae sedis*; perhaps Ustilaginomycotina	*Moniliella spathulata* (syn. *Trichosporonoides spathulata*)

Source: Sitepu et al. (2014)

Table 2.2 Lipids production from various types of oleaginous yeasts

Yeast strains	Biomass (g/L)	Lipids (g/L)	Lipids content (% w/w)	Conditions	Carbon source	References
Rhodosporidium toruloides 21167	18.51	11.7	63.2	28°C, pH 4.0, 180 rpm	Hydrolysate of cassava starch	Wang et al. (2012)
Rhodosporidium toruloides	26.71	18.7	70.0	30°C, pH 6.0, 200 rpm	Glycerol	Xu et al. (2012)
Cryptococcus curvatus	17.31	5.8	33.5	28°C, pH 5.5, 200 rpm	Hydrolysate of wheat straw	Yu et al. (2011)
Lipomyces starkeyi	14.74	4.6	31.2	28°C, pH 5.5, 200 rpm	Hydrolysate of wheat straw	Yu et al. (2011)
Rhodotorula glutinis ATCC 204091	14.00	3.5	25	28°C, pH 5.5, 200 rpm	Hydrolysate of wheat straw	Yu et al. (2011)
Rhodosporidium toruloides Y4	12.18	7.1	58.3	30°C, pH 6.0, 200 rpm	Glucose + Sulphate	Wu et al. (2011)
Rhodotorula mucilaginosa	11.99	6.2	51.7	30°C, pH 6.0, 100 rpm	Molasses	Karatay and Dönmez (2010)
Rhodotorula mucilaginosa TJY15a	10.89	5.0	45.9	28°C, pH 6.0, 160 rpm	Hydrolysate of cassava starch	Li et al. (2010)
Lipomyces starkeyi DSM 70295	9.41	6.4	68.0	30°C, pH 5.0, 120 rpm	Sewage sludge	Angerbauer et al. (2008)

Table 2.2 (Continued)

Yeast strains	Biomass (g/L)	Lipids (g/L)	Lipids content (% w/w)	Conditions	Carbon source	References
Rhodosporidium toruloides Y4	19.48	12.1	62.1	30°C, pH 6.0, 200 rpm	Glucose	Wu et al. (2010)
Rhodosporidium toruloides Y4	13.38	8.7	65.0	30°C, pH 6.5, 200 rpm	Hydrolysate from lignocellulose	Hu et al. (2009)
Trichosporon fermentans	36.26	12.8	35.3	25°C, pH 6.0, 160 rpm	Molasses	Zhu et al. (2008)
Rhodotorula glutinis	6.80	2.7	39.7	30°C, pH 6.0, 150 rpm	Levoglucosan	Liang et al. (2013)
Lipomyces starkeyi AS 2.1560	9.04	5.3	58.6	30°C, pH 6.0, 200 rpm	Glucose	Lin et al. (2011)
Rhodotorula glutinis	38.22	21.1	55.2	24°C, pH 6.0, 150 rpm	Glucose	Yen et al. (2014)
Rhodotorula glutinis ATCC 204091	24.39	9.0	36.9	35°C, pH 6.0, 112 rpm	Glycerol	Easterling et al. (2009)
Yarrowia lipolytica Po1g	11.39	6.7	58.8	28°C, pH 6.5, 160 rpm	Hydrolysate from sugar cane	Tsigie et al. (2011)
Rhodotorula glutinis TISTR 5159	9.97	6.05	60.7	30°C, pH 6.0, 3.0 vvm	Crude glycerol	Saenge et al. (2011b)
Rhodotorula glutinis BCRC 22360	43.51	25.5	58.6	24°C, pH 6.0, 150 rpm	Glucose	Zhange et al. (2011)

Table 2.2 (Continued)

Yeast strains	Biomass (g/L)	Lipids (g/L)	Lipids content (% w/w)	Conditions	Carbon source	References
Rhodotorula glutinis	13.00	4.55	35.0	37°C, pH 5.5, 180 rpm	Corn starch wastewater	Xue et al. (2010)
Yarrowia lipolytica	13.38	8.7	65.0	25°C, pH 6.0, 170 rpm	Food waste and Municipal wastewater	Chi et al. (2011)
Aspergillus oryzae	36.26	12.8	35.3	30°C, pH 5.5, 160 rpm	Potato processing wastewater	Muniraj et al. (2013)
Cryptococcus terricala JCM 24523	6.80	2.7	39.7	37°C, pH 5.5, 180 rpm	Starch through consolidated bioprocessing	Tanimura et al. (2014)
Lipomyces starkeyi	4.61	1.14	24.71	30°C, pH 5.0, 150 rpm	Monosodium glutamate wastewater	Liu et al. (2012)
Rhodosporidium fluviale DMKU-SP314	14.3	7.9	55.24	28°C, pH 5.5, 150 rpm	Glucose and xylose	Poontawee et al. (2017)
Rhodotorula glutinis TR29	16.2	10.5	64.8	25°C, pH 5.0, 200 rpm	sugar beet molasses	Taskin et al. (2016)

2.2.1 Lipids biosynthesis

Oleaginous yeasts accumulate lipids via two different pathways namely *de novo* and *ex novo* lipids biosynthesis. The *de novo* synthesis is the synthesis of lipids from the acetyl-CoA and malonyl-CoA building blocks. Whereas, the *ex novo* lipids accumulation pathway involving the uptake of fatty acids, oils and triacylglycerol (TAG) from the culture (Beopoulos and Nicaud, 2012).

2.2.1.1. *De novo* lipids biosynthesis

When sugars or similarly metabolized compounds are used as the substrate, the process is called "*de novo* lipids accumulation" (Figure 2.1). The *de novo* lipids biosynthesis in oleaginous microorganisms is non-growth associated process. This process is conducted due to change of intracellular concentration of metabolites after nitrogen depletion (Probst et al., 2016). The limitation of nitrogen is the most efficient type of limitation for inducing lipids accumulation. In nitrogen limited conditions the organisms continues to assimilate the carbon source but the cell proliferation stops as nitrogen is required for the nucleic and protein synthesis. Under these conditions the carbon flux is diverted towards lipids synthesis, leading to an accumulation of TAG within discrete lipids bodies in the cell (Christophe et al., 2012).

In oleaginous microorganisms, the fatty acid biosynthesis starts with the conversion of acetyl-CoA into malonyl-CoA and malonyl-ACP. Acetyl-CoA is transported into the cytosol and derives from the breakdown of citric acid which had previously accumulated inside the mitochondria. The presence of ATP-citrate lyase (ACL) has been identified as being responsible for the formation of acetyl-CoA in oleaginous microorganisms (Martinez et al., 2015). This enzyme is absent in non-oleaginous yeasts. No organism can accumulate more than 20% of its biomass as triacylglycerol and lacks ACL activity has been found (Christophe et al., 2012). In addition, the unique characteristic associated with lipids accumulating organisms is the activity of isocitrate dehydrogenase (TCA cycle). The isocitrate dehydrogenase is dependent on the present of AMP (adenosine monophosphate) which is absent in non-oleaginous species. The step of lipids biosynthesis is descripted by Christophe et al. (2012).

1) Glucose via glycolysis leads to pyruvate that is proton-linked transported into the mitochondrion.
2) In the mitochondrion the enzyme pyruvate decarboxylative dehydrogenase converts pyruvate into acetyl-CoA.
3) Acetyl-CoA reacts with oxaloacetate to give citrate.
4) Under limitation of nitrogen and aerobic conditions, a rapid decrease in intracellular concentration of AMP.
5) The activity of AMP deaminase increases as a way of scavenging additional ammonium ions for synthesis of cell material.

$$AMP \rightarrow IMP + NH4^{+}$$

6) AMP is the alteration of isocitrate dehydrogenase in the Krebs cycle. The decreasing of AMP is results in inhibition of isocitrate dehydrogenase activity and accumulation of citrate until a critical value is reached.
7) Citrate enters the cytoplasm is exchanged via a citrate/malate translocase with intracellular malate and is cleaved by the ATP-citrate lyase into acetyl-CoA and oxaloacetate.

$$Citrate + ATP + CoA \rightarrow Acetyl\text{-}CoA + Oxaloacetate + ADP + Pi$$

8) Acetyl-CoA is converted into palmitic acid by lipogenesis pathway, which is the primer for longer chain saturated or unsaturated fatty acids.
9) Acetyl-CoA also acts as the primer for synthesis of malonyl-Acyl carrier protein and acetyl-ACP.
10) Malonyl-CoA is obtained by carboxylation of acetyl-CoA by an acetyl-CoA transacetylase or carboxylase and then transferred to an acyl carrier protein (ACP) by malonyl transacetylase, with release of CoA.
11) Malonyl-ACP allows adding carbon 2 units to the primer acetyl-ACP obtained by transfer of the acetyl group of acetoacetyl-ACP.
12) A β-ketoacyl-ACP synthase adds the C2-unit of malonyl-ACP on an acetyl ACP decarboxylating the malonyl group and causing release of an ACP to give acetoacetyl-ACP.
13) Acetoacetyl-ACP reductase leads to β-hydroxybutyryl-ACP by regeneration of $NADP^{+}$ from NADPH and H^{+}.

14) The dehydration of β-hydroxybutyryl-ACP by a β-hydroxyacyl-ACP dehydratase produces a crotonyl-ACP which is reduced by an enoyl-ACP reductase into butyryl-ACP with regeneration of a NAD^+.
15) Elongation of the fatty acid chain is continued by the cycling addition of C2-unit in the form of the acyl group from malonyl-ACP.

Acetyl-CoA + 7malonyl-CoA + 14NADPH + $14H^+$ → Palmitic acid + $7CO_2$ + 8CoA + $14NADP^+$ + $6H_2O$

16) Then specific enzymes, desaturase and/ or elongase convert plamitic acid into unsaturated or polyunsaturated fatty acid (PUFA) or into longer fatty acyl chains.
17) Desaturase catalyzes the introduction of double bond into the fatty acid chain and elongase in a sequence similar to that of C2-unit cycling addition.

2.2.1.2 *Ex novo* lipids biosynthesis

The utilize of fats or hydrophobic materials as the substrate for lipids accumulation is a growth-coupled process that formation of free lipids material occur in the presence of nitrogen in the culture medium. The fatty that material can be used as a carbon source such as free fatty acids, vegetable oils, fish oils and industrial fats. These fatty acids can be used for growth and transformation to different fatty acids. The present of exogenous *n*-alkanes and fatty acids are strongly inhibited the enzymes of fatty acid synthetase and ATP-citrate lyase. Thus, the *ex novo* biosynthesis of lipids material cannot occur at the same time de novo process (Martinez et al., 2015).

Free fatty acids are converted to acyl-CoA esters by acyl-CoA synthetase. Then, the β-oxidation process is degraded the acyl-CoA esters into smaller chain acyl-CoA ester and acetyl-CoA. These reactions are providing the energy necessary for cell growth, maintenance and production of intermediate metabolites. These processes are repeated several times until the degradation of the fatty acid is completed. Moreover, these are depended on the concentration of the substrate, the presence of acetyl-CoA and on the ratio of NAD^+/NADH. The yeast cells can degrade fatty acid or incorporate as TAG into lipids bodies. The main interest of ex novo lipids biosynthesis is associated with the modification of the hydrophobic substrate to produce high-value polyunsaturated fatty acids (Papanikolaou, 2011).

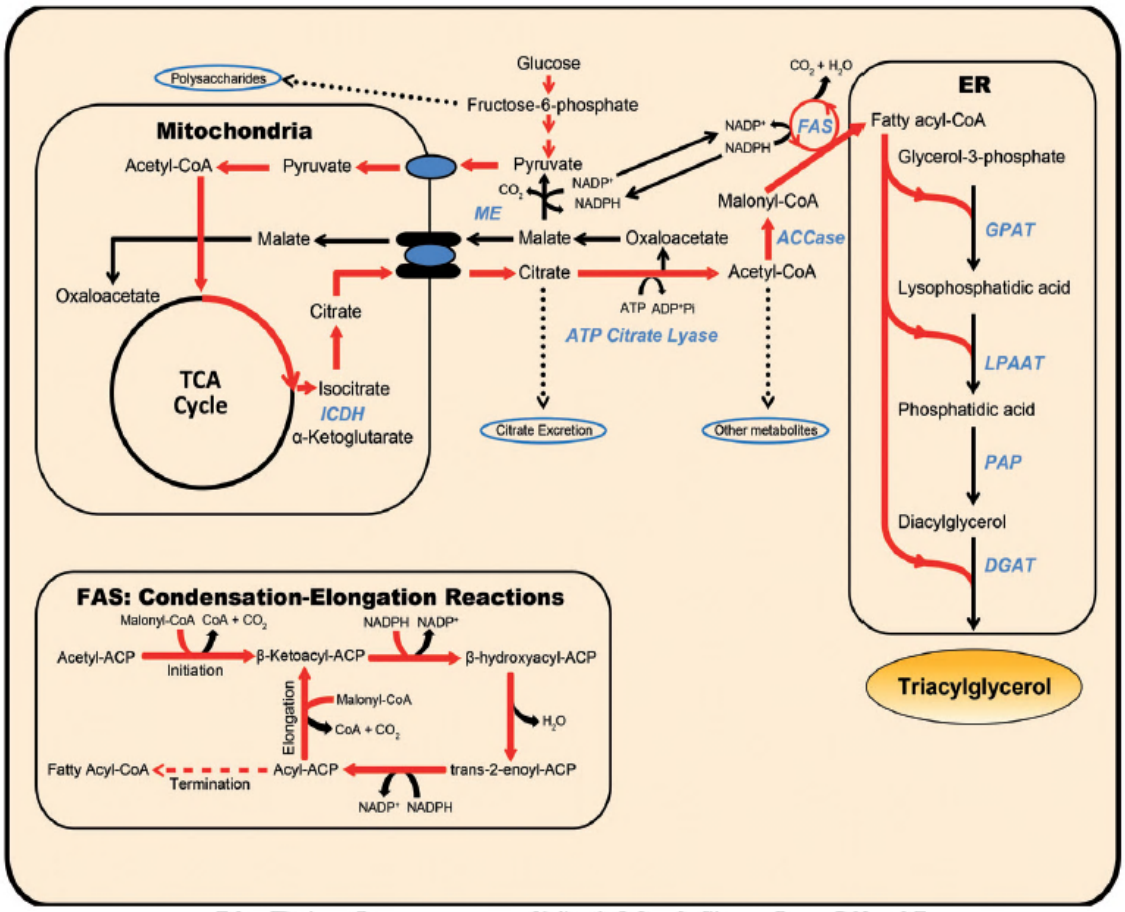

Figure 2.1 Biochemistry of lipids accumulation in oleaginous yeast. Thick red arrows indicate flux of carbon from glucose to TAG. Abbreviations: (ER) endoplasmic reticulum, (TCA) tricarboxylic acid, (ICDH) isocitrate dehydrogenase, (ME) malic enzyme, (FAS) fatty acid synthase, (ACCase) acetyl-CoA carboxylase, (GPAT) glycerol-3-phosphate acyltransferase, (LPAAT) lysophosphatidic acid acyltransferase, (PAP) phosphatidate phosphatase, (DGAT) diacylglycerol acyltransferase

Source: Probst et al. (2016)

2.2.2 Carotenoids biosynthesis

Carotenoids are nature pigments of widely disposed classes of structurally and functionally diverse color from red to yellow present in a various of bacteria, fungi, algae and plants (Kirti et al., 2014). The carotenoids pigmented yeasts or red yeast have an advantage over algae, bacterial and fungi. Because of yeast are unicellular and high growth rate with utilization of low-cost fermentation medium. The biosynthesis of carotenoids is a specific feature of the *Rhodosporidium*, *Rhodotorula* species, *Sporidiobolus* species and *Phaffia* genera. Red yeasts can be produced carotenoids around 50–350 μg/g dry cell weight (Chandi and Gill, 2011). The carotenoids are lipophilic isoprenoid molecules containing double bonds that form a light absorbing chromophore. In metabolic system, the phytoene is converts into cyclic carotenes by a series of desaturation and cyclization reaction. The chromophore will lengthen during the initiation process of conjugated double bonds. Moreover, The carotenoids structures can modified by various processes such as cyclization, double bond migration, rearrangement, isomerization, hydrogenation, dehydrogenation, introduction of oxygen function and combination of these process (Priatni, 2014). The carotenoid structure that produced by red yeast e.g. astaxanthin, β-carotene, canthaxanthin and torulene are presented in Figure 2.2 (Mata-Gómez et al., 2014).

Carotenoids are sensitive to reactions such as oxidation and isomerization and also to light, heat, acid and oxygen, due to the present of double bonds in the structure. They are hydrophobic molecules with less or no solubility in water that found in hydrophobic areas of cell (Cardoso et al., 2016).

Carotenoids are natural pigments that can applications as colorants, food supplements, medical, cosmetic and biotechnological purposes (Kirti et al., 2014). In addition, carotenoids are many beneficial functions to human life such as antioxidant, anti-inflammatory, anti-carcinogen and chemopreventive agent for some cancer diseases (Priatni, 2014).

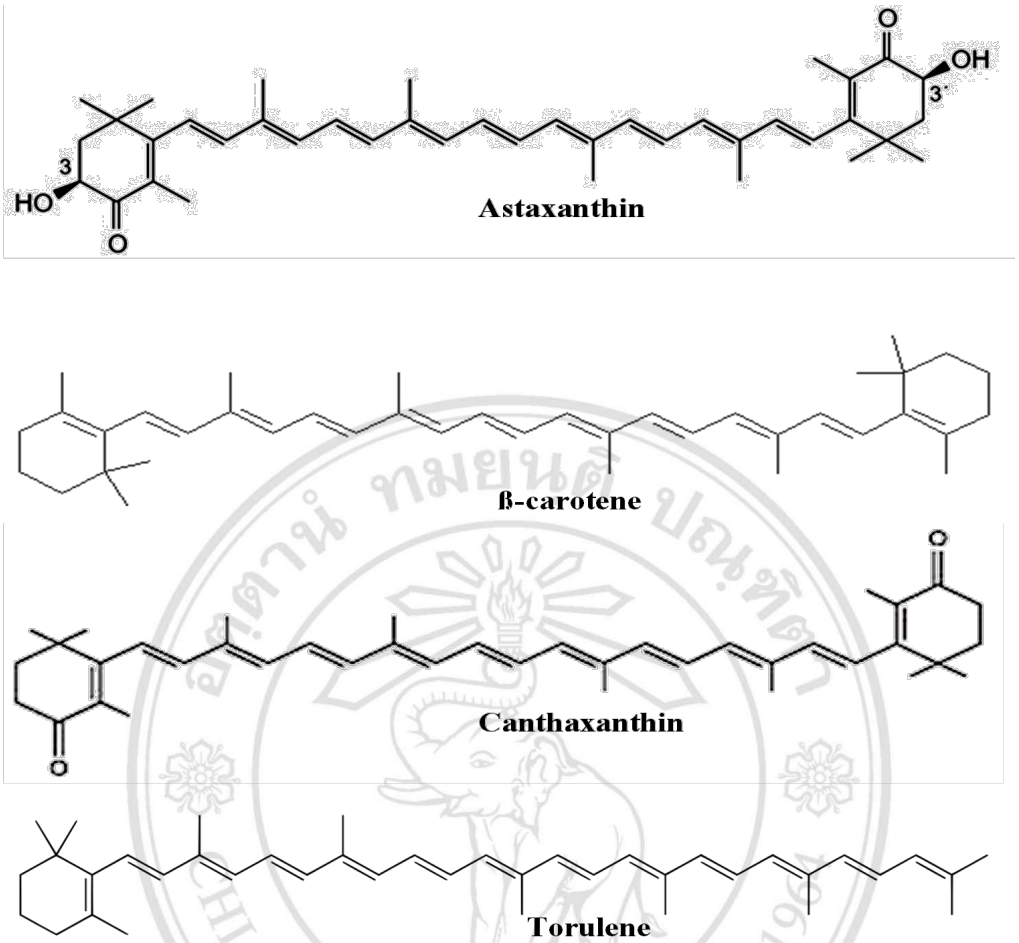

Figure 2.2 Molecular structures of carotenoids

Source: (Mata-Gómez et al., 2014).

2.2.2.1 Carotenoids biosynthesis pathway

Carotenoids are a number of isoprenoid compounds that synthesized by tail-to-tail linkage of two molecules of geranyl geranly-pyrophosphate (GGPP). The conjugated double bond in carotenoids structure is the chromophore for light-absorbing which gives these compounds an attractive color. In the metabolic system, the precursor phytoene converts into cyclic carotenes by a series of desaturation and cyclization reaction (Priatni, 2014). The pathway of carotenoids biosynthesis by yeast is divided into three general steps (Figure 2.3) (Mata-Gómez et al., 2014) as;

1) **Mevalonate pathway**: Synthesis starts with conversion of acetyl CoA to 3-hydroxy-3-methyl glutaryl-CoA (HMG-CoA), catalyzed by HMG-CoA synthase. Then, HMG-CoA is converted to mevalonic acid (MVA) that is the first precursor of isoprene biosynthesis pathway. Mevalonic acid is phosphorylated by MVA kinase and decarboxylation into isopentenyl pyrophosphate (IPP).
2) **Isoprene biosynthesis pathway**: Isopentenyl pyrophosphate (IPP) is isomerized to dimethyllayl pyrophosphate (DMAPP) with the addition of three IPP molecules to DMAPP, which catalyzed by prenyl transferase into geranyl geranly-pyrophosphate (GGPP). The condensation of GGPP of two molecules produces the phytoene (the first C_{40} carotene of the pathway) that is subsequently desaturated to form lycopene.
3) **Carotenogenic pathway**: Several cyclic carotenoids are derived from lycopene, as β-carotene, γ-carotene, torulene, torularhodin and astaxanthin when it receives many reactions.

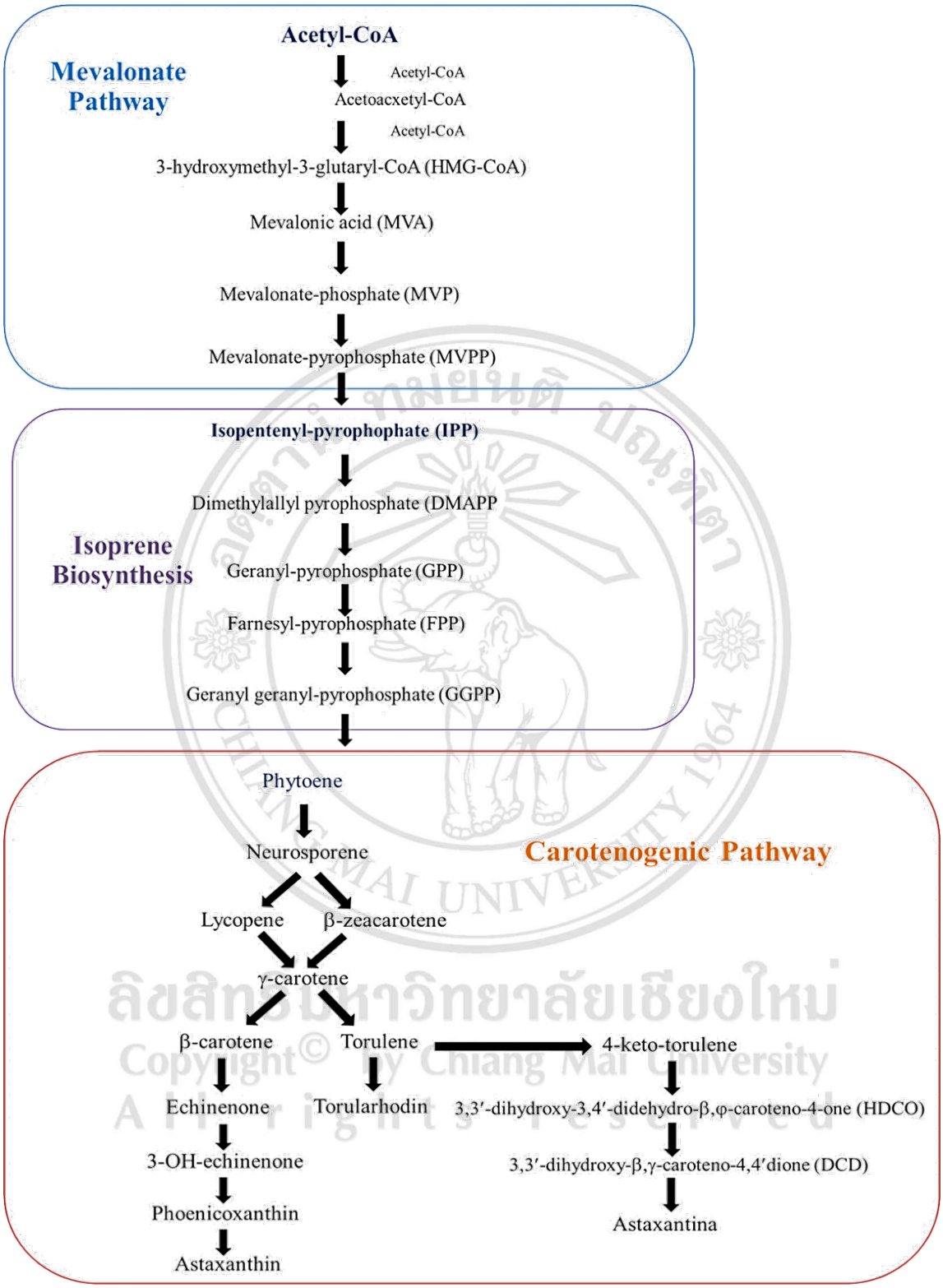

Figure 2.3 Biosynthesis of carotenoids

Source: Zoz et al. (2015)

2.2.3 Factors influencing the production of lipids and carotenoids in yeasts

1) The C:N ratio

The C:N ratio is an important factor for lipogenesis and carotenoids synthesis in oleaginous yeasts. At high C:N ratio leads to high lipids and carotenoids production (Braunwald et al., 2013). Oleaginous yeast channel carbon towards the accumulation of triacylglycerol (TAG) as a storage lipids 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). Saenge et al. (2011b) reported that both of lipids and carotenoids productions are required a medium with an excess of carbon source and limitation of other nutrient, mostly nitrogen source. The limitation of nitrogen condition, cell propagation can be inhibited. Both of biomass concentration and lipids content is relevant to lipids yield. At high content of lipids production was obtained but production yields might be low due to the decrease of biomass. Therefore, in order to obtain higher yields of the products, a high biomass should be achieved firstly. Then the optimum C:N ratio could be adjusted for enhancing the contents of lipids and carotenoids (Saenge et al., 2011b).

Kraisintu et al. (2010) studied the biomass and lipids productions of *Rhodosporidium toruloides* DMKU3-TK16 by varying C:N ratio. They found that the nitrogen source, glucose level and C:N ratio had significant effects on biomass and lipids productions. The increasing of C:N ratio decreased biomass but increased lipids content. The lipids productivity was slightly increased to 0.035 g/L/h at the highest C:N ratios of 140, with a maximum lipids content of 62.3%.

Somashekar and Joseph (2000) studied the inverse 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 (10:1) which was 15 times higher than high C:N ratio (160:1). While, this high C:N ratio increased 55% lipids compared 20% lipids at low C:N ratio.

2) Minerals and other growth factors

Minerals such as magnesium (Mg), potassium (K), sulphate (SO_4^{2-}), phosphorus (P), phosphate (PO_4^{3-}) and calcium (Ca) are the important factor on the growth and lipids production of oleaginous red yeasts at lower concentration (Sha, 2013).

Wu et al. (2010) reported that phosphate is the essential elements for cell growth and lipids accumulation. It is mostly incorporated into phospholipids, nucleic acid and coenzyme that influence on stored lipids in the cell.

Some agents such as detergent additives, oil, and surfactants can be increased lipids and carotenoids productivity. Supplementation of the culture medium with surfactants can alter the physiological properties of microorganisms, improve metabolite production, stimulate growth and respiration, and change the organization and permeability of cell membranes (Saenge et al., 2011a).

Saenge et al. (2011a) studied the effect of surfactant on lipids and carotenoids productions of oleaginous red yeast *Rhodotorula glutinis* TISTR5159 using palm oil mill effluent as carbon source. In this experiment, a number of surfactants including Tween 20, Tween 80, and gum arabic at 1.0% concentration were investigated. The results showed that there were significant increases in the biomass, lipids and carotenoids productions, compared to the control to which no surfactant was added. From three surfactants, only Tween 20 effectively increased both lipids content and carotenoids productions. The highest of biomass, lipids content, and carotenoids productions of 7.07 g/L, 38.15% and 125.94 mg/L, respectively. While, this strain produced the biomass, lipids content and carotenoids of 6.29 g/L, 29.15% and 115.76 mg/L, respectively, in the presence of surfactant.

3) Temperature

The optimal temperature for growth, lipids accumulation and carotenoids production is depended on species of oleaginous red yeasts. Generally, the optimal temperature of oleaginous red yeast is 25°C. High or low temperature is effects on cell growth and lipids production. During yeasts cell accumulation, decreasing temperature from optimal growth is resulted in increasing of the lipids content and lipids production (Lamers et al., 2016). The melting point of unsaturated fatty acid is lower than saturated fatty acid and short chain fatty acids lower than long chain fatty acid. Hence, the

decreasing temperature is results in increased level of unsaturated and short chain fatty acid (Sha, 2013).

However, the several enzymes involve in carotenoids biosynthesis in eukaryote and prokaryote microorganisms, such as β-carotene hydroxylase, lycopene cyclase and phytoene desaturase and the enzyme activity are stimulated at higher temperature of 30°C (Malisorn and Suntornsuk, 2008). Zhang et al. (2014) studied the effect of temperature for the growth and carotenoids production of *Rhodotorula glutinis* under two different temperatures of 24°C and 30°C. The results showed that the maximum biomass and carotenoids were 17.9 g/L and 1.21 mg/L at 30°C, respectively. At 24°C, this strain produced biomass and carotenoids were only 15.8 g/L and 0.91 mg/L, respectively.

Hadi and Ivan (2006) studied the effect of temperature on the growth and carotenoids production of *Sporobolomyces ruberrimus* H110 at four temperature values of 19, 23, 27 and 31°C. The results showed that the maximum concentration of total carotenoids was 3.84 mg/g including torularhodin (3.70 mg/g) and β-carotene (0.14 mg/g) at 19°C. However, the maximum specific growth rate was obtained (μ_{max} = 0.094 1/h) at 27°C.

4) pH value

The optimum pH value for the growth of oleaginous red yeast is different and varies from pH 3.0–7.0. However, the optimal pH for lipids accumulation is lower than for optimal growth (Sha, 2013). *Lipomyces starkeyi* could produce highest lipids yield of 7.5 g/L at pH 5.0 while the highest growth was 15.12 g/L at pH 6.5 (Angerbauer et al., 2008). Karatay and Dönmez, (2010) studied the effect of medium pH value on lipids accumulation of *Candida lipolytica* in molasses medium. The experiments were performed at pH 4.0, 5.0, 6.0 and 7.0. The results revealed that this strain produced lipid content of 31.5, 37.1 and 34.4% (w/w) at pH 4.0, 6.0 and 7.0, respectively while the maximum lipids content was 59.9% (w/w) at pH 5.0.

Hadi and Ivan (2006) studied the effect of different pH on the growth and carotenoids production by *Sporobolomyces ruberrimus* H110 using glycerol as carbon source at pH 3.5, 4.0, 4.4, 5.0, 5.5, 6.0, 6.6, 7.0, 7.6 and 8.2, respectively. The results revealed that pH had an obvious effect on the biomass and carotenoids productions.

This strain produced the maximum biomass and carotenoids of 11.3 g/L and 38.75 mg/L at pH 6.0. While, the biomass and carotenoids productions were 6.81 g/L and 13.75 mg/L at pH 3.5.

5) Aeration rate

Aeration rate is one important factor for oleaginous red yeast on cell growth and lipids levels (Saenge et al., 2011b). The aeration rate indicates the dissolved oxygen (DO) level in the culture medium (Kumar et al., 1999). DO level highly influence on fatty acid profile in lipids of oleaginous red yeast. Moreover, the oxygen supply strongly influences the respiration and enzyme activities in β-carotene biosynthesis pathway (Malisorn and Suntornsuk, 2008).

Saenge et al. (2011b) studied the effect of aeration rate on cell growth, lipids yield and carotenoids production of *Rhodotorula glutinis* using crude glycerol as carbon source. They found that aeration rate was significant affected to cell mass and lipids accumulation. When increasing aeration rate from 0 to 2 vvm was resulted in high biomass and lipids yield of 8.17 and 4.32 g/L, respectively.

Malisorn and Suntornsuk (2008) studied the optimal DO on biomass and β-carotene productions of *Rhodotorula glutinis* by varying the DO of 40, 60 and 80%, respectively. The results revealed that increasing DO enhanced biomass and β-carotene productions. At DO level of 40 and 60%, this strain produced the biomass of 2.09 and 2.41 g/L, with the β-carotene of 162 and 183 μg/L, respectively. Whereas, the maximum biomass and β-carotene were 2.7 g/L and 201 μg/L at 80% DO level.

6) Light intensity

Light is an important factor for the production of microbial carotenoids. Oleaginous red yeast need to prevent them from the light because of it causes cell damage. The carotenogenesis is a protoprotective mechanism (Mata-Gómez et al., 2014).

Yen at al. (2011) reported that light irradiation increased the carotenoids production of *Rhodotorula glutinis* under different light irradiation conditions of 2 LED (light emitting diode) lamps, 3 LED lamps and no light irradiation. The results revealed that light irradiation with 2 LED lamps increased β-carotene content of 24.60±0.25 μg/g

while only 14.69±1.42 μg/g was obtained under without irradiation. However, at higher light intensity (3 LED lamps) led to lower β-carotene content of 17.38±0.57 μg/g. It might be the effect from light damage caused by high light intensity.

Zhang et al. (2014) studied the carotenoids production of *Rhodotorula glutinis* under different irradiation conditions (without light, 2 LEDs and 3 LEDs). The results showed that the maximum carotenoids concentration was 2.6 mg/L in the batch cultivation with 3 LED lamps. While, the carotenoids concentration only 1.2 mg/L was obtained in the batch without light. The oleaginous red yeast produces the carotenoids pigment for the protection against photo-oxidative damage of cell (Zhang et al., 2014).

2.3 Biodiesel

Biodiesel is the renewable energy resources, which is produced from renewable biomass by transesterification of triacylglycerol (TAG), yielding monoalkyl esters of long-chain fatty acids with short-chain alcohols (biodiesel) and glycerol (by-product) (Lin et al., 2013). The transesterification is a process to convert TAG to biodiesel that involves multiple steps of reaction between triglycerides or fatty acid and alcohol. In this process can be used various alcohols such as ethanol, butanol, propanol, methanol and amyl alcohol. However, methanol and ethanol are often used for the commercial because of its low cost and its physical and chemical advantages. The reaction can be carried out in the presence of an inorganic catalyst (acids and alkalies) or lipase enzyme (Behera et al., 2014). Biodiesel contributes no net carbon dioxide or sulfur to the atmosphere and emits less gaseous pollutants than normal diesel (Meng et al., 2009).

2.3.1 Types of transesterification based on catalyst

2.3.1.1 Homogeneous catalyst

The reaction of transesterification is a reversible reaction and proceeds essentially by mixing the reactants in which the catalysts is a liquid acid or a liquid base (Figure 2.4) (Naik et al., 2010).

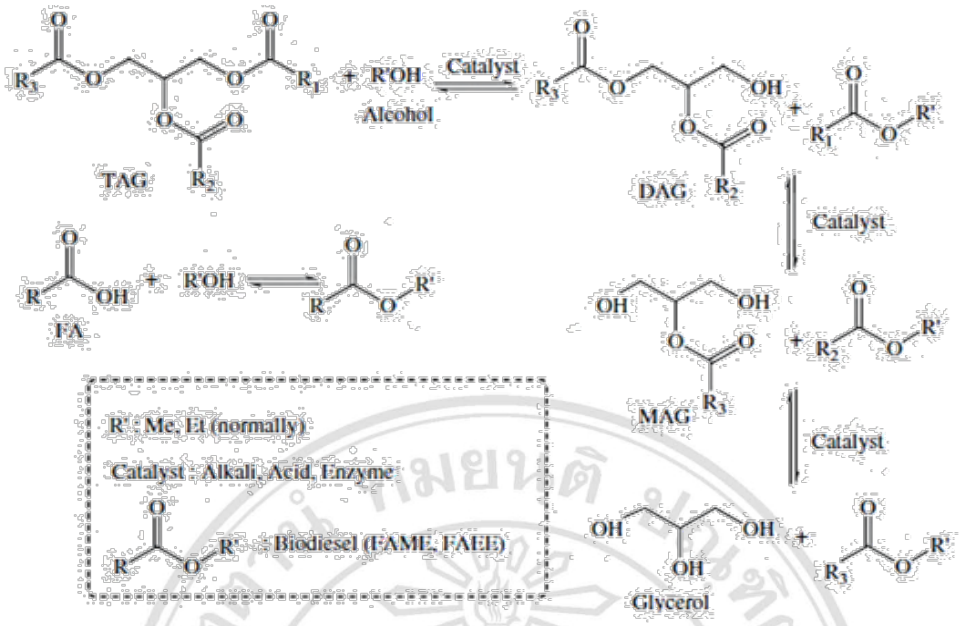

Figure 2.4 Biodiesel production by transesterification. Abbreviations: triacylglycerol (TAG), diacylglycerol (DAG), monoacylglycerol (MAG), fatty acid (FA)

Source: Xu et al. (2013)

2.3.1.2 Heterogeneous catalyst

Saponification reaction is the cause to decreases the conversion of oil to methyl ester due to high free fatty acid (FFA) content oil which is not possible to perform a basic transesterification process. The solid catalyst is suggested for high free fatty acid containing oil. For example, solid acid catalysts that can simultaneously catalyze the transesterification of triglycerides and esterification of free fatty acid (FFA) present in oil to methyl esters. Solid acid catalysts have the strong potential to replace homogeneous catalysts, eliminating separation, corrosion and environmental problems (Naik et al., 2010).

The solid acid catalyzed biodiesel production by simultaneous esterification and transesterification of low quality oil containing high FFA. The reaction mechanism of simultaneous esterification and transesterification using Lewis acid is as shown in Figure 2.5. The esterification occurs between free fatty acids and methanol whereas transesterification occurs between triglyceride and methanol adsorbed on acidic site (L^+) of catalyst surface. The interaction of the monoacylglycerol

(MAG) with acidic site of the catalyst forms carbocation. The nucleophilic attack of alcohol to the carbocation produces a tetrahedral intermediate. During esterification the tetrahedral intermediate removes water molecule to form one mole of ester. The transesterification mechanism can be extended to TAG and di-acylglycerol (DAG). In the reaction sequence the TAG is converted stepwise to DAG and MAG and finally glycerol. The tetrahedral intermediate formed during reaction eliminates DAG, MAG and glycerol when TAG, DAG and MAG come in contact with the acidic sites, respectively, to give one mole of ester in each step. In cases, esterification and transesterification produce methyl ester, the same final product. Also, as shown in Figure 2.5, the catalyst is regenerated after the simultaneous esterification and transesterification reactions. The utilize of excess alcohol favors forward reaction and thus maximizes the ester yield (Naik et al., 2010).

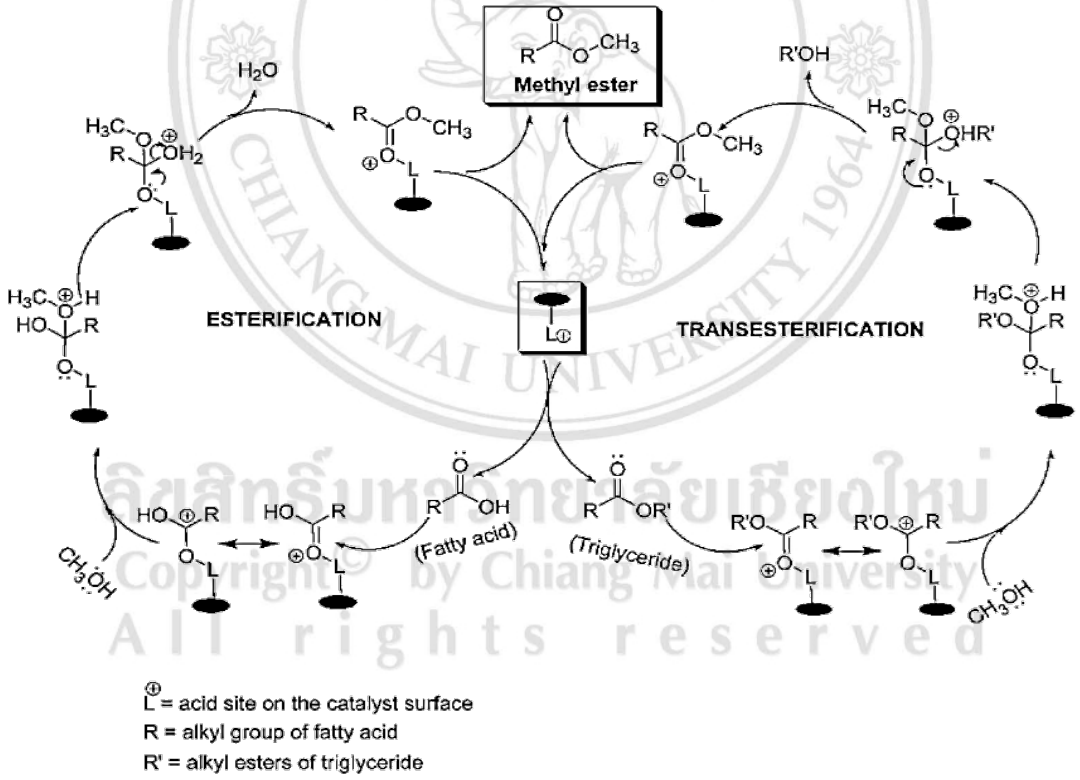

Figure 2.5 Solid acid catalyzed simultaneous esterification and transesterification

Source: Naik et al. (2010)

2.3.2 Generation of biodiesel

2.3.2.1 The first-generation of biodiesel

The first-generation of biodiesel is a substitute of diesel which is produced though transesterification of vegetable oil, residual oils and animal fats and based on mature and well-established technologies. This generation can offer some CO_2 benefits and can help to improve domestic energy security (Naik et al., 2010). As the production of first-generation biodiesel is mainly from agricultural food crops, this has induced competition for both agricultural land, water resources and the high energy requirement for the oil extraction process (Gambelli et al., 2017). The main disadvantage of first generation biofuels is the food-versus-fuel debate, one of the reasons for rising food prices is due to the increase in the production of these fuels (Alam et al., 2012).

2.3.2.2 The second-generation of biodiesel

The second-generation of biodiesel is produced from nonedible plant oils such as Jatropha, jojoba and waste oils such as cooking grease and animal fats. These generations have encouraged development due to the rising cost of edible plant oils and public debate of the "food versus fuel" issue of first generation (Sitepu et al., 2014).

These second-generation biofuels could significantly reduce CO_2 production, do not compete with food crops and some types can offer better engine performance. When commercialized, the cost of second generation biofuels has the potential to be more comparable with standard diesel, and would be most cost effective route to renewable, low carbon energy for road transport (Alam et al., 2012). However, these oils may not be sufficiently abundant to meet global needs, and animal fats perform poorly in cold weather as they do not fit the specifications (Sitepu et al., 2014).

2.3.2.3 The third-generation of biodiesel

The third-generation of biodiesel is under development using the microbial oil such as microalgae, bacteria, yeasts and mold. The single cell oils (SCO) or microbial oil systems that produce and store oil in their cell, have attracted significant research attention recently, for the rising price of petroleum (Sitepu et al., 2014).

Oleaginous microorganism can convert substrates such as carbon dioxide, sugars, organic acids, starch based substrate and lignocellulose-based substrate to oil or

lipids. While some species produce intracellular neutral lipids continuously, most cell types require stressors of limitation of nutrient to stimulate lipids synthesis. Once the cells produce the lipids, they are harvested and lysed by solvent, mechanical and enzymatic for the releasing the lipids. The lipids are separated from the cell fraction and the neutral lipids undergoes chemical refining to produce a biodiesel or other target molecule by releasing the glycerol from the individual fatty acids (Soccol et al., 2017). The current barrier in microbial lipids production is developing a robust system that is cost competitive with petroleum-based fuels. This can be performed by the development of strains able to convert low cost substrates, grow quickly to high density, produce larger quantities of neutral lipids, and development of improved harvesting and dewatering technologies (Sitepu et al., 2014).