Chapter 1

Introduction

1.1 Energy

Energy is an important currency for human society. The inevitable role of energy in economic development of a country can hardly be avoided that dictates the technological developments, social progresses and improvement in the quality of life. The world population growth and rapid economic progresses are expected to result in considerable increase in the demand for energy. The International Energy Agency (IEA) has reported in the reference scenario that the world's primary energy need is projected to grow by 55% between 2005 and 2030, at an average annual rate of 1.8%. Majority of the world energy needs are supplied through fossil fuels such as coal, natural gases and petrochemical sources. Fossil fuels will remain the dominant source of primary energy, accounting for 84% of the overall increase in demand between 2005 and 2030 (IEA, 2007).

The world is facing a formidable fuel crisis due to the declining non-renewable energy resources popularly known as 'Peak Oil', while the energy demand is exploding (Campbell, 2008). The World Energy Forum has predicted that fossil-based oil, coal and gas reserves are going to be exhausted in less than another 100 years (Sharma and Singh, 2009). Driven by such increasing demand and dwindling fuel production, the cost of petroleum fuel has gone up sky high in recent times, which has ominously jeopardized the economic progresses of a nation. Despite the fuel crisis, increasing concentration of CO_2 and other heat trapping greenhouse gases (GHGs) in the atmosphere, primarily due to the combustion of fossil fuels is clearly the prime responsible for rapid warming of the

planet (Shay, 1993). Given the projected uprising trend in world population growth and energy demands, the most challenging issue facing the international community today is how to attain a sustainable economic development *vis-à-vis* environmental security. Such growing concerns of significant global climate change and national energy security resurgent our quest for renewable sources of energy.

1.2 Renewable energy

Renewable energy is a form of energy that is produced from natural sources like sunlight, wind, hydropower, geothermal and biomass, which are naturally replenished. Renewable energy sources have the potential to provide energy with negligible emissions of air pollutants and greenhouse gases, and can put our civilization on a more sustainable footing. Development of renewable sources of alternative energy has become crucial in the national and international efforts towards maximum self-reliance - the cornerstone of our energy security strategy.

Currently, renewable energy supplies ~18% of the world's energy consumption, counting traditional biomass, large hydropower, and 'new' renewables like wind, solar, geothermal and biofuels. Traditional biomass, primarily used for cooking and heating, represents about 13% of global energy consumption (Kumar *et al*., 2010). Hydropower is another source of renewable energy that converts the potential energy or kinetic energy of water into mechanical energy and electrical energy. It represents 3% of global energy demand and is growing modestly, primarily in developing countries (IEA, 2008). New renewables such as wind, solar, geothermal and biofuels represent 2.4% and are growing very rapidly in developed countries and also in some developing countries (REN, 2007).

At present, most of these renewable energy sources (hydropower, wind, solar and geothermal) target the electricity market, while the majority of world energy consumption (about two thirds) is derived from liquid fuels (Campbell, 2008; Hankamer *et al*., 2007). This has stimulated recent interest to explore alternative sources for petroleum-based fuels and much of the attention has been focused on biomass-derived liquid fuels or biofuels (Haag, 2007; Schneider, 2006). However, biofuels still represent a minor

proportion of total fuel consumption in the world, accounting for 0.3% of world's energy consumption (Kumar *et al*., 2010). Liquid biofuels are available in two forms, bioalcohols and biodiesel. The first one is commonly used in combination with gasoline and the second corresponds to an alternative fuel option for diesel.

1.3 Petroleum fuel scenario of India

India ranks 7th as the world's energy producer accounting for about 2.5% of the world's total annual energy production and world's 5th largest energy consumer with about 3.5% of the global primary energy demand (IEA, 2007; Planning Commission, Govt. of India, 2007). Despite being amongst the largest energy producer, India is a net importer of energy largely due to huge imbalance between energy consumption and production. About 30% of India's total primary energy needs are being met by petroleum oil, of which 76% are imported. Rapid economic expansion will continue to drive up India's energy demand. India needs to sustain an 8 - 10% economic growth for the next 25 years to eradicate poverty and meet its human development goals (Planning Commission, Govt. of India, 2007). In the reference scenario, primary energy demand in India will be more than doubles by 2030, growing on an average by 3.6% per year (IEA, 2007). The projected crude oil demand of India will be in between 350 - 486 million tones (Mt) by 2030 of which only about 35 Mt could be met by domestic production (Planning Commission, Govt. of India, 2007). India is facing formidable challenges in meeting its energy demands.

India's transportation fuel requirements are unique in the world. India consumes almost five times more diesel fuel than gasoline, whereas all other countries in the world use more gasoline than diesel fuel (Khan *et al*., 2009). Thus, search for alternatives to diesel fuel is of special importance in India. Bioalcohols are unsuitable substitutes for diesel engines, because of their low cetane numbers along with poor energy content per unit biomass (Bhattacharyya and Reddy, 1994; Rao and Gopalkrishnan, 1991). Therefore, biodiesel is the only option to fulfill the requirements in future.

1.4 Biodiesel

Biodiesel is chemically monoalkyl esters of long chain fatty acids derived from renewable feedstocks, such as vegetable oils or animal fats. The history of using vegetable oil as an alternative fuel dates back to 1900, when Rudolph Diesel used peanut oil as fuel in the World Exhibition in Paris. It was found that vegetable oils, in general, have acceptable cetane number and calorific values as compared to the conventional diesel. However, the major problem with the direct use of vegetable oils as fuel of compression ignition (CI) engine is their high viscosity and low volatility. High viscosity of raw vegetable oils interfere with the fuel injection and atomization contributing to incomplete combustion, nozzle clogging, injector coking, severe engine deposits, ring sticking and gum formation leading to engine failure (Konthe, 2005; Meher *et al*., 2006; Singh and Rastogi, 2009). Because of these problems, vegetable oils need to be modified to bring their combustion related properties closer to those of diesel fuel. One possible method to overcome the problem of high viscosity of vegetable oils is their chemical modification to esters, what is nowadays called as 'biodiesel'.

Biodiesel has emerged as the most able alternative to petroleum diesel fuel owing to its eco-friendly characteristics and renewability (Krawczyk, 1996). It burns in conventional diesel engines with or without any modifications while reducing pollutions (100% less sulfur dioxide, 37% less unburned hydrocarbons, 46% less carbon monoxide, and 84% less particulate matter) in comparison to the conventional diesel fuel (McMillen *et al.*, 2005). The basic feedstocks for the production of first generation biodiesel were mainly edible vegetable oils like soybean, rapeseed, sunflower and safflower. The use of first generation biodiesel has generated a lot of controversy, mainly due to their impact on global food markets and on food security for diverting food away from the human food chain. The second generation biodiesel produced by using non-edible oil sources like used frying oil, grease, tallow, lard, karanja, jatropha and mahua oils (Alcantara *et al.*, 2000; Becker and Francis, 2002; Canakci and Gerpen, 2001; Dorado *et al.*, 2002; Ghadge and Raheman, 2006; Mittelbach *et al.*, 1992). Nevertheless, the cost of biodiesel production is still a major obstacle for large scale commercial exploitation; mainly due to

the high feed cost of vegetable oils (Lang *et al.*, 2001). Moreover, the first as well as the second generation biodiesel based on terrestrial plants initiate land clearing and potentially compete with net food production (Chisti, 2008; Marsh, 2009). The focus of researchers has now been shifted to the next generation biodiesel. The third generation biodiesel is both promising and different; it is based on simple microscopic organisms that live in water and grow hydroponically, i.e. microalgae.

The possibilities of biodiesel production from edible oil resources in India is almost impossible, as primary need is to first meet the demand of edible oil that is being imported. India accounts for 9.3% of world's total oil seed production and contributes as the fourth largest edible oil producing country. Even then, about 46% of edible oil is imported for catering the domestic needs (Jain and Sharma, 2010). So the non-edible oil resources like *Jatropha*, pongamia, mahua etc., seem to be the only possibility for biodiesel production in the country. The Government of India has duly realized the importance of biodiesel and introduced a nationwide programme under the National Biodiesel Mission (NBM) in 2003 with the aim of achieving a target of meeting 13.4 Mt of biodiesel (@ 20% blending) from *Jatropha curcas* by 2012 and to achieve the target about 27 billions of planting materials are required to be planted over 11.2 million hectares of land (Planning Commission, Govt. of India, 2003). At the current rate of consumption, if all petroleum-derived transport fuel is to be replaced with biodiesel from *Jatropha* oil, *Jatropha* would need to be grown over an area of 384 million hectares, which is more than 100% of the geographic area of India (Khan *et al*., 2009). Therefore, India must find additional, reliable, cost-effective and sustainable feedstock for biodiesel production. In this context, biodiesel from microalgae seems to be a suitable substitute for diesel fuel in the long run.

1.5 Microalgae: viable feedstocks for biodiesel

Microalgae are a diverse group of photosynthetic organisms whose systematic is based on the kinds and combinations of photosynthetic pigments present in different species. They can grow in various environmental conditions and are able to produce a wide range of chemical products with applications in feed, food, nutritional, cosmetic and

pharmaceutical industries. These are primitive organisms with a simple cellular structure and a large surface to volume body ratio, which gives them the ability to uptake large amount of nutrients. While the mechanism of photosynthesis in microalgae is similar to that of higher plants, they have the ability to capture solar energy with an efficiency of 10 - 50 times higher than that of terrestrial plants (Li *et al*., 2008). Moreover, because the cells grow in aqueous suspension, they have more efficient access to water, CO_2 and other nutrients. For these reasons, microalgae are capable of producing more the amount of oil per unit area of land in comparison to that of all other known oil-producing crops (Chisti, 2007; Haag, 2007). The per hectare yield of microalgal oil has been projected to be 58,700 - 136,900 litre $year^{-1}$ depending upon the oil content of algae, which is about 10 - 20 times higher than the best oil producing crop, i.e. palm (5950 litre ha^{-1} $year^{-1}$, Chisti, 2007). The most acclaimed energy crop, i.e. *Jatropha* has been estimated to produce 1892 litre ha^{-1} $year^{-1}$ only. More importantly, due to aquatic in nature, algae do not compete for arable land for their cultivation; they can be grown in freshwater or saline, and salt concentrations up to twice that of seawater can be used effectively for few species (Aresta *et al.*, 2005; Brown and Zeiler, 1993). The utilization of wastewater that is rich in nitrogen and phosphorus may bring about remarkable advantages by providing N and P nutrients for growing microalgae which in turn will remove N and P from the wastewaters (Mallick, 2002). This implies algae need not compete with other users for freshwater (Campbell, 2008). On top of these advantages, microalgae grow even better when fed with extra carbon dioxide, the main greenhouse gas. If so, these tiny organisms can fix CO_2 from power stations and other industrial plants, thereby cleaning up the greenhouse problem. Each ton of algae produced consumes about 1.8 ton of CO_2 (Chisti, 2007). Thus, the integrated efforts to cleanup industrial flue gas with microalgal culture by combining it with wastewater treatment will significantly enhance the environmental and economical benefits of the technology for biodiesel production by minimizing the additional cost of nutrients and savings the precious freshwater resources.

1.6 Selection of potent strains

Realizing the oil yielding potentialities with much faster growth rate and efficient CO_2 fixation, microalgae appear to be the best option as a renewable source of biodiesel that has the potentiality to completely replace the petroleum diesel fuel. However, the lipid content in the microalga required to be high, otherwise the economic performance would be hard to achieve.

Each species of microalga produces different ratios of lipids, carbohydrates and proteins. Nevertheless, these tiny organisms have the ability to manipulate their metabolism through simple manipulations of the chemical composition of the culture medium (Behrens and Kyle, 1996), thus high lipid productivity can be achieved. Physiological stresses such as nutrient limitation/deficiency, salt stress and high light intensity have been employed for directing metabolic fluxes to lipid biosynthesis of microalgae. Few reports are available, where attempts have been made to raise the lipid pool of microalgal species. Table 1.1 summarizes those studies.

Table 1.1: Review on increased lipid content of some microalgae under various specific conditions

Microalga	**Growth condition**	**Lipid content (% dcw)**	**Reference**
Botryococcus braunii	Brown resting state	86 17*	Brown *et al.* (1969)
Chlorella vulgaris	Nitrogen limitation	53 12*	Piorreck *et al.* (1984)
Chlorella emersonii	Nitrogen limitation	63 29*	Illman *et al.* (2000)
C. minutissima		57 31*	
C. vulgaris		40 18*	
C. pyrenoidosa		23 11*	
Nannochloris sp. UTEX LB1999	Nitrogen limitation	51 31*	Takagi *et al.* (2000)

Table 1.1-continued

Microalga	Growth condition	Lipid content (% dcw)	Reference
Chlorella protothecoides	Heterotrophy with 0.1% glucose under nitrogen limitation	55 15*	Miao and Wu (2004)
	Heterotrophy with corn powder hydrolysate under nitrogen limitation	55 15*	Xu *et al.* (2006)
Dunaliella sp.	1M NaCl	71 64*	Takagi *et al.* (2006)
Chlorella sp.	Iron limitation	57 8*	Liu *et al.* (2008)
Chlorella sp.	Heterotrophy with 1% sucrose	33 15*	Rattanapoltee *et al.* (2008)
Neochloris oleoabundans	Nitrogen deficiency	56 29*	Gouveia and Oliveira (2009)
Nannochloropsis oculata NCTU-3	2% CO_2	50 31*	Chiu *et al.* (2009)
Nannochloropsis sp. F&M-M24	Nitrogen deficiency	60 31*	Rodolfi *et al.* (2009)
	Phosphorus deficiency	50 31*	
Nannochloropsis oculata	Nitrogen limitation	15 8*	Converti *et al.* (2009)
Chlorella vulgaris		16 6*	
Choricystis minor	Nitrogen and phosphorus deficiencies	60 27*	Sobczuk and Chisti (2010)
Haematococcus pluvialis	High light intensity	35 15*	Damiani *et al.* (2010)
	High light intensity under nitrogen deficiency	33 15*	
Chlorella protothecoides	Heterotrophy with sweet sorghum hydrolysate under nitrogen limitation	50 15*	Gao *et al*. (2010)

* Lipid content of control culture

Exceptionally, an oil content of 86% (dcw) was reported in the brown resting state colonies of *Botryococcus braunii*, while the green active state colonies were found to account for 17% lipid of the dry weight (Brown *et al*., 1969). However, the major obstacle in focusing *B. braunii* as an industrial organism for biodiesel production is its poor growth rate (Dayananda *et al.*, 2007).

Nitrogen limitation/deficiency has been found to lead to lipid accumulation in a number of microalgal species. For instance, Piorreck *et al*. (1984) reported an increased lipid content from 12 to 53% of dry cell weight (dcw) in *Chlorella vulgaris* under nitrogen-limited condition. Unlike the green algae, the blue-green algae viz. *Anacystis nidulans* and *Oscillatoria rubescens* contained the same quantities of lipid at different nitrogen concentrations. It was observed by Illman *et al*. (2000) that four species of *Chlorella* (*C. emersonii*, *C. minutissima*, *C. vulgaris* and *C. pyrenoidosa*) could accumulate lipid up to 63, 57, 40 and 23% respectively, on dry weight basis in low N medium. These values in control vessels were, respectively 29, 31, 18 and 11% in the above order. Takagi *et al*. (2000) observed an increase in intracellular lipid pool up to 51% (dcw) against 31% under control condition in *Nannochloris* sp. UTEX LB1999 grown in continuous nitrate (0.9 mM)-fed medium with 3% CO_2 purging. *Chlorella protothecoihedes* also shown a rise in lipid content from 15 to 55% (dcw), when grown heterotropically with glucose (1%) under reduced nitrogen concentration (Miao and Wu, 2004). Similarly, *C. protothecoihedes* depicted a lipid pool of 55% (dcw) when grown heterotrophically with corn powder hydrolysate under nitrogen limitation (Xu *et al*., 2006). Gao *et al.* (2010) used sweet sorghum hydrolysate instead of corn powder in *C. protothecoihedes* culture and lipid yield of 50% (dcw) was recorded. Nitrogen limitation/ starvation also enhanced the lipid content in *Neochloris oleoabundans*, *Nannochloropsis oculata* and *Chlorella vulgaris* (Converti *et al*., 2009; Gouveia and Oliveira, 2009).

Limitation of phosphate was also found to enhance lipid accumulation in *Ankistrodesmus falcutus* and *Monodus subterraneus* (Khilam *et al.*, 1997; Khozin-Goldberg and Cohen, 2006). Rodolfi *et al*. (2009) have screened 30 microalgal strains for lipid production, among which the marine genus *Nannochloropsis* sp. F&M-M24 emerged as the best candidate for algal oil production (50% under phosphorus deficiency against 31% control). Sobczuk and Chisti (2010) observed an increase in intracellular lipid content up to 60% (dcw) against 27% under control in *Choricystis minor* under simultaneous nitrate and phosphate deficiencies. Furthermore, iron limitation has been reported to stimulate lipid accumulation in the microalga *C. vulgaris* (57% of dcw against 8% under control condition, Liu *et al*., 2008).

In addition to nutrient limitation/deficiency, other stress conditions may also enhance lipid accumulation in microalgae. Takagi *et al*. (2006) studied the effect of NaCl on accumulation of lipids and triacylglycerides in the marine microalga *Dunaliella* sp. Increase in initial NaCl concentration from 0.5 M (sea water) to 1.0 M resulted into a higher intracellular lipid yield (71% dcw). Lipid yield was also increased in sucrose-supplemented culture (Rattanapoltee *et al*., 2008). Damiani *et al*. (2010) studied the effects of continuous high light intensity (300 µmol photons m^{-2} s^{-1}) on lipid accumulation in *Haematococcus pluvialis* grown under nitrogen-sufficient and nitrogen-deprived conditions. A lipid yield of 33 – 35% was recorded under the high light intensity as compared to 15% yield in control culture. Nitrogen deprivation was however, not found to raise the lipid content of *H. pluvialis* culture.

Nutrient limitations/deficiencies or physiological stresses required for accumulation of lipid in cells is associated with reduced cell division (Ratledge, 2002). The overall lipid productivity is therefore, compromised due to the low biomass productivity. For instance, Scragg *et al.* (2002) studied the energy recovery of *Chlorella vulgaris* and *C. emersonii* grown in Watanabe's medium and a low nitrogen medium. The results showed that the low nitrogen medium, although induced higher lipid accumulation in both the test algae with high calorific values, the overall energy recovery was lower with the low nitrogen medium than that with the Watanabe's medium. A commonly suggested countermeasure is to use a two-stage cultivation strategy, dedicating the first stage for cell growth/division in nutrient sufficient medium and the second stage for lipid accumulation under nutrient starvation or other physiological stresses. To get maximal biomass and lipid yield CO_2 can also be utilized. Chiu *et al*. (2009) reported an increased accumulation of lipid (from 31 to 50% dcw) in the stationary phase cultures of *Nannochloropsis oculata* NCTU-3 grown under 2% CO_2 aeration.

1.7 Lipid biosynthetic pathway

Lipid metabolism, particularly the biosynthetic pathways of fatty acids and triacyl glycerols (TAGs), has been poorly studied in algae. Based upon the sequence homology and some shared biochemical characteristics of a number of genes and/or enzymes

isolated from algae and higher plants those are involved in lipid metabolism, it is generally believed that the basic pathways of fatty acids and TAGs biosynthesis in algae are directly analogous to those demonstrated in higher plants (Hu *et al.*, 2008). The ability of algae to adapt to various environmental conditions is reflected in an exceptional variety of lipid patterns as well as with their ability to modify lipid metabolism to synthesize a number of unusual compounds (Guschina and Harwood, 2006; Thompson, 1996; Wada and Murata, 1998). It should be noted here that algal lipid research is still fragmentary.

In algae, *de novo* synthesis of fatty acids occurs primarily in chloroplast (Roessler *et al*., 1994). A generalized scheme for fatty acid biosynthesis is shown in Fig. 1.1. Overall, during fatty acid biosynthesis, 16- or 18-carbon fatty acids are synthesized which are used as the precursors for synthesis of chloroplast and other cellular membranes as well as the synthesis of neutral storage lipids.

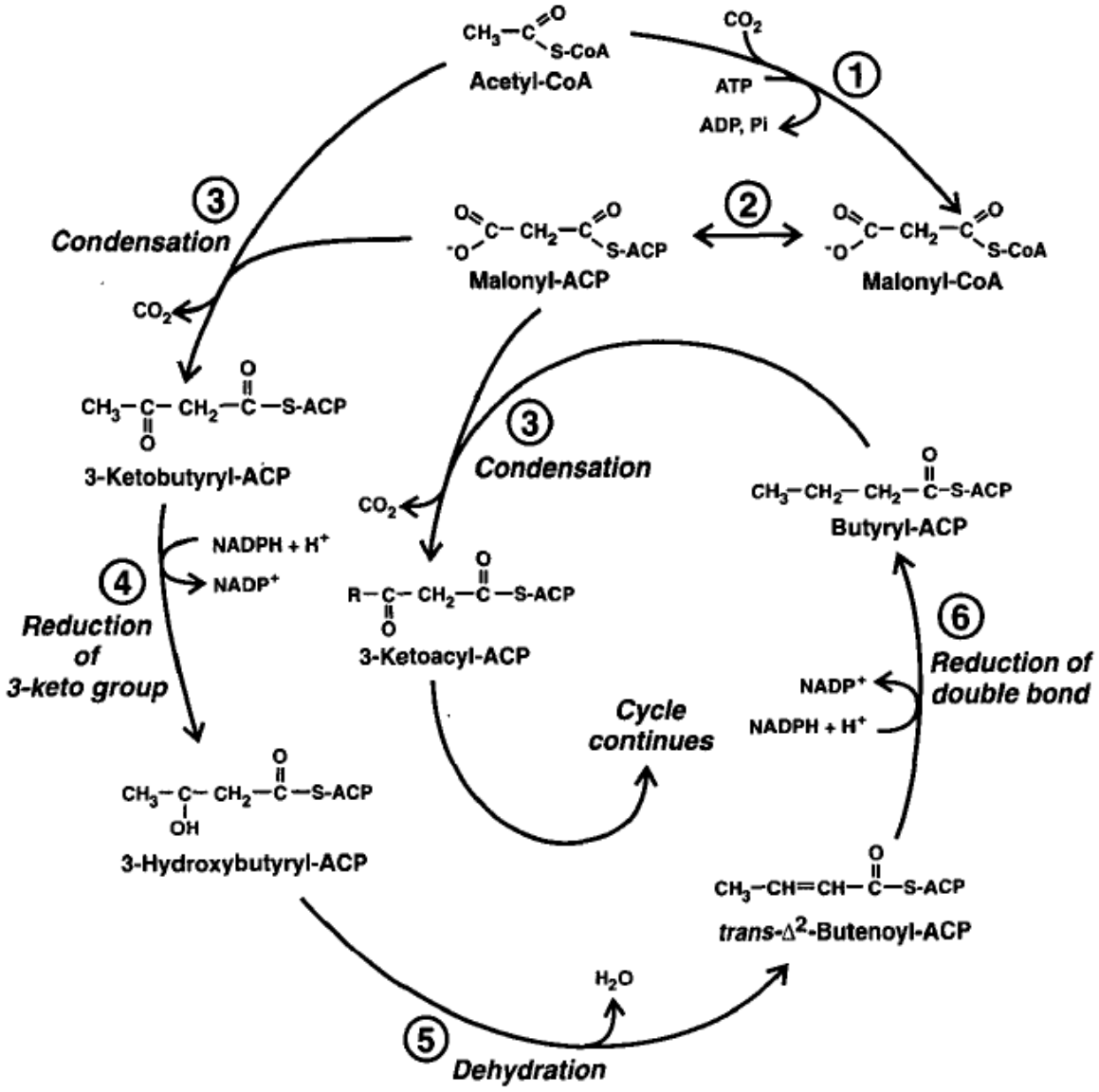


Fig. 1.1: The pathway of fatty acid biosynthesis.

Enzymes: (1) Acetyl-CoA carboxylase (2) Malonyl-CoA-ACP transacylase, (3) 3-ketoacyl-ACP synthases, (4) 3-ketoacyl-ACP reductase, (5) 3- hydroxyacyl-ACP dehydrase, and (6) Enoyl-ACP reductase (Ohlrogge and Browse, 1995).

The committed step in fatty acid synthesis is the conversion of acetyl-CoA to malonyl-CoA, catalysed by acetyl-CoA carboxylase (ACCase). This reaction actually takes place in two steps. In the first step, which is ATP dependent, CO_2 (from HCO_3^-) is transferred by the biotin carboxylase portion of ACCase to nitrogen of a biotin prosthetic group attached to the ε-amino group of a lysine residue. In the second reaction, catalyzed by the carboxyltransferase, the activated CO_2 is transferred from biotin to acetyl-CoA to form malonyl-CoA (Ohlrogge and Browse, 1995). However, before entering the fatty acid synthesis pathway, the malonyl group is transferred to a protein cofactor, acyl carrier protein (ACP). ACP is a small (9 kD) acidic protein that contains a phosphopantethein prosthetic group to which the growing acyl chain is attached as a thioester. After transfer to ACP, the malonyl-thioester enters into a series of condensation reactions with acyl-ACP (or acetyl-CoA) acceptors. These reactions result in the formation of a carbon-carbon bond and release of CO_2 that was added by the ACCase reaction. Removal of this CO_2 helps to drive this reaction forward, making it essentially irreversible.

At least three separate condensing enzymes are required to produce an 18- carbon fatty acid. The first condensation of acetyl-CoA and malonyl-ACP to form a four-carbon product is catalyzed by 3-ketoacyl-acyl carrier protein synthase III (KAS III, Jaworski *et al.*, 1989). A second condensing enzyme KAS I is believed responsible for producing chain lengths from 6 to 16 carbons. Finally, elongation of the 16 carbon palmitoyl-ACP to stearoyl-ACP requires a separate condensing enzyme, KAS II. The initial product of each condensation reaction is a 3-ketoacyl-ACP. Three additional reactions occur after each condensation to form a saturated fatty acid. The 3-ketoacyl-ACP is reduced at the carbonyl group by the enzyme 3-ketoacyl-ACP reductase, which uses NADPH as the electron donor. The next reaction is dehydration by hydroxyacyl-ACP dehydratase. Each round of fatty acid synthesis is then completed by the enzyme enoyl-ACP reductase, which uses NADH or NADPH to reduce the trans-2 double bond to form a saturated fatty acid. The combined action of these four reactions leads to the lengthening of the precursor fatty acid by two carbons until the saturated chain of a palmitic (16:0) or a stearic acid (18:0) is formed (Subrahmanyam and Cronan, 1998). At last, ACP-thioesterase cleaves the acyl chain and liberates the fatty acid. To obtain longer or unsaturated chains, elongases and desaturases are required, which act on palmitate or

stearate. These enzymes are located in endoplasmic reticulum membrane and mitochondrial matrix.

Like other higher plants and animals, microalgae are able to biosynthesize triglycerides to store energy. Triacylglycerol (TAG) biosynthesis in algae has been proposed to occur via the direct glycerol biosynthetic pathway (Fig. 1.2).

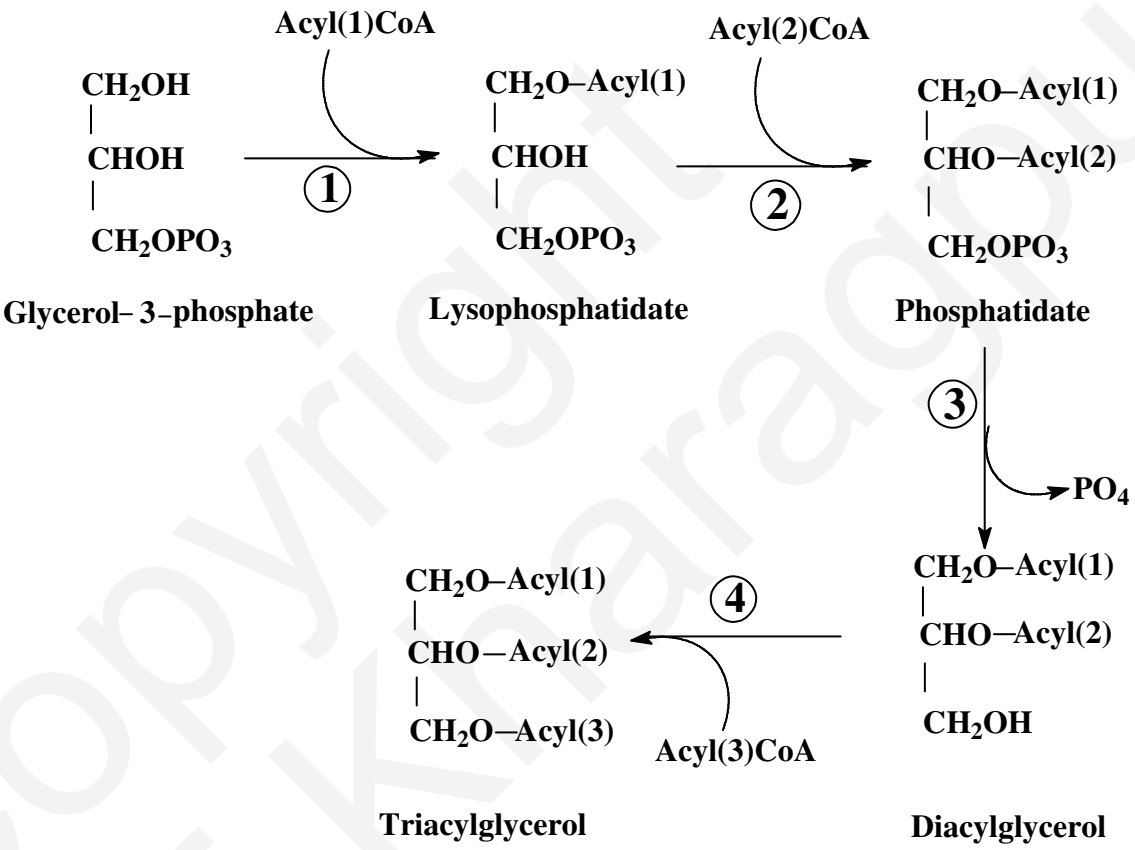


Fig. 1.2: Biosynthetic pathway of triacylglycerol.
Enzymes: (1) Glycerol-3-phosphate acyl-transferase, (2) Lysophosphatidate acyl-transferase, (3) Phosphatidic acid phosphatase, and (4) Diacylglycerol acyl-transferase (Hu *et al*., 2008; Roessler *et al*., 1994).

The first step of TAG synthesis is the condensation (acylation) of glycerol-3-phosphate with an acyl-CoA to form lysophosphatidate, which is catalyzed by glycerol-3-

phosphate acyl-transferase (GPAT). This enzyme exhibits the lowest specific activity of TAG synthesis pathway, and is suggested to be potentially the rate limiting step (Cao *et al.*, 2006; Coleman and Lee, 2004). The lysophosphatidate is then further condensed, catalyzed by lysophosphatidate acid acyl-transferase (LPAT), with another acyl-CoA to produce phosphatidate (Athenstaedt and Daum, 1999). Afterwards, phosphatidate can be dephosphorylated by phosphatidic acid phosphatase (PAP) to produce diacylglycerol. At last, synthesis of TAG is catalyzed by diacylglycerol acyl-transferase (DGAT), which incorporates the third acyl-CoA into the diacylglycerol molecule. This enzyme is also known as an important regulator of TAG synthesis pathway (Oelkers *et al.*, 2002; Sandager *et al.*, 2002). TAGs can then be stored in oil bodies in cytoplasm (Murphy, 2001).

1.8 Genetic engineering approaches

High oil yielding transgenic microalgae could be a promising source for biodiesel production. However, the biotechnological processes based on transgenic microalgae are still in their infancy. In manipulation of genetically modified algae for high oil content, acetyl-CoA carboxylase (ACCase) was first isolated from the diatom *Cyclotella cryptica* by Roessler (1990) and then successfully transformed into the diatoms *C. cryptica* and *Navicula saprophila* (Dunahay *et al.*, 1995, 1996; Sheehan *et al.*, 1998). A plasmid was constructed that contained *acc*1 gene driven by the cauliflower mosaic virus 35S ribosomal gene promoter (CaMV35S) and the selectable marker *npt*II from *E. coli*. Introduction of plasmids into the diatoms was mediated by microprojectile bombardment. The *acc*1 was overexpressed with the enzyme activity enhanced by 3-fold. These experiments demonstrated that ACCase could be transformed efficiently into microalgae, although no significant increase in lipid accumulation was observed in the transgenic diatoms (Dunahay *et al.*, 1995, 1996). Thus, there is no success story with respect to lipid overproduction in microalgae using the genetic engineering approach till date.

Extensive studies have also been carried out on enhancement of lipid production using genetic engineering approaches in different bacterial and plant species, which may

provide valuable background for future studies with microalgae. Some of these studies are summarized in Table 1.2.

Table 1.2: Trials to enhance lipid biosynthesis in transgenic organisms (modified from Courchesne *et al.*, 2009)

Gene (enzyme)	Source species	Receiver species	Result	Reference
fabH (KAS III)	*Escherichia coli*	*Brassica napus*	Change in the fatty acid composition, cell growth arrested	Verwoert *et al.* (1995)
acc1 (ACCase)	*Cyclotella cryptica*	*Cyclotella cryptica*	3-fold rise in ACCase activity, no change in lipid content	Dunahay *et al.* (1995, 1996)
		Navicula saprophila	3-fold rise in ACCase activity, no change in lipid content	
acc1 (ACCase)	*Arabidopsis* sp.	*Brassica napus*	2-fold rise in plastid ACCase, 6% rise in fatty acid content	Roesler *et al.* (1997)
LPAT	*Saccharomyces cerevisiae*	*Brassica napus*	6-fold rise in oil content	Zou *et al.* (1997)
fabF (KAS II)	*E. coli*	*E. coli*	Toxic to cell	Subrahmanyam and Cronan (1998)
accA, accB, accC, accD (ACCase)	*E. coli*	*E. coli*	6-fold rise in fatty acid synthesis	Davis *et al.* (2000)
are1 and *are2* (DGAT)	*Arabidopsis thaliana*	*Saccharomyces cerevisiae*	9-fold rise in TAG content	Bouvier-Nave *et al.* (2000)
KAS III	*Spinacia oleracea*	*Nicotiana tabacum*	Rise in palmitic acid, lipid content reduced	Dehesh *et al.* (2001)
		Arabidopsis sp.		
		Brassica napus		
DGAT	*Arabidopsis* sp.	*Arabidopsis* sp.	70% rise in lipid content	Jako *et al.* (2001)

Table 1.2-continued

Gene (enzyme)	Source species	Receiver species	Result	Reference
acc1 (ACCase)	*Arabidopsis* sp.	*Solanum tuberosum*	5-fold rise in TAG content	Klaus *et al.* (2004)
acs (acetyl-CoA synthase, ACS)	*E. coil*	*E. coli*	9-fold rise in ACS activity	Lin *et al.* (2006)
malEMt and *malEMc* (malic enzyme, ME)	*Mortierella alpina* and *Mucor circinelloides*	*M. circinelloides*	2.5-fold rise in lipid accumulation	Zhang *et al.* (2007)
fadD, ACCase, thioesterase (TE)	*E. coil*	*E. coli*	20-fold rise in fatty acid synthesis	Lu *et al.* (2008)
wri1	*Brassica napus*	*Arabidopsis thaliana*	40% rise in oil content	Liu *et al.* (2010)

The cytosolic ACCase from *Arabidopsis* sp. was overexpressed in *Brassica napus* (rapeseed) plastid. The fatty acid content of the recombinant was 6% higher than the control (Roesler *et al.*, 1997). In prokaryotes like *Escherichia coli*, overexpression of four ACCase subunits resulted into 6-fold rise in the rate of fatty acid synthesis (Davis *et al,* 2000), confirming that the ACCase catalyzed committing step was indeed the rate-limiting step for fatty acid biosynthesis in this strain. However, as mentioned in Table 1.2, no significant enhancement in lipid production in diatoms *C. cryptica* and *N. saprophila* was observed after transformed with the *acc1* gene. This indicates that overexpression of ACCase enzyme alone might not be sufficient to enhance the whole lipid biosynthetic pathway (Sheehan *et al.*, 1998). Nevertheless, Klaus *et al*. (2004) achieved an increase in fatty acid synthesis and a more than 5-fold rise in the amount of TAG in *Solanum tuberosum* (potato) by overexpressing the ACCase from *Arabidopsis* in the amyloplasts of potato tubers.

Trials in overexpressing the KAS II subunit of fatty acid synthase (FAS) in *E. coli* were carried out to facilitate the C2 concatenation. However, this manipulation was found highly toxic for the *E. coli* cells (Subrahmanyamand and Cronan, 1998). In another trial, an *E. coli* KAS III was overexpressed in rapeseed (Verwoert *et al.*, 1995), which caused a major change in the fatty acid composition and also affected the growth of the plant cells significantly. Similarly, overexpression of KAS III from *Spinacia oleracea* in *Nicotiana*

tabacum, *Arabidopsis* sp. and *Brassica napus* resulted into a reduction in lipid synthesis and increase in the accumulation of palmitic acid (Dehesh *et al*., 2001). Transformation of rapeseed with a putative *sn*-2-acyl-transferase gene from *Saccharomyces cerevisiae* was carried out by Zou *et al.* (1997), leading to overexpression of seed LPAT activity. This enzyme is involved in TAG formation and its overexpression led to increase in oil content from 8% to 48% on seed dry weight basis. However, it was cautioned that the steady-state level of diacylglycerol could be perturbed by an increase of LPAT activity in the developing seeds. Transformations of *S. cerevisiae* with the *Arabidopsis* DGAT were performed by Bouvier-Nave *et al.* (2000). About 600-fold rise in DGAT activity in the transformed *S. cerevisiae* was observed, which led to a 9-fold increase of TAGs accumulation. DGAT gene has also been overexpressed in the plant *Arabidopsis* and it was shown that the oil content was enhanced in correlation with the DGAT activity (Jako *et al.*, 2001). All these results suggest that the reaction catalyzed by ACCase, FAS, LPAT and DGAT are important rate-limiting steps in lipid biosynthesis. However, in microalgae reports regarding the overexpression of these enzymes except ACCase are not available.

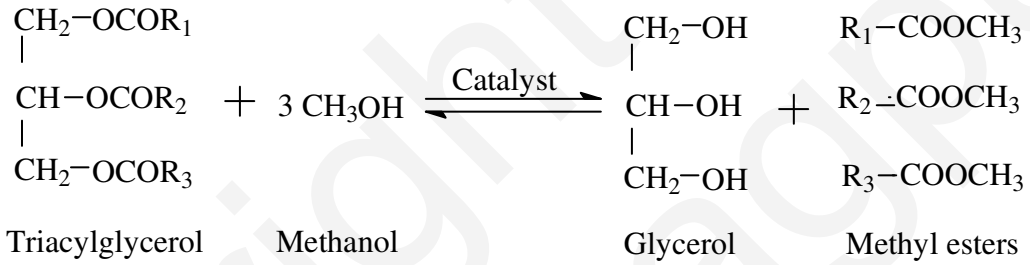
A few enzymes that are not directly involved in lipid metabolism have also been demonstrated to influence the rate of lipid accumulation. For instance, it was observed by Lin *et al.* (2006) that by overexpressing the *acs* gene in *E. coli*, the ACS activity was increased by 9-fold, leading to a significant increase in the assimilation of acetate from the medium, which can contribute to lipid biosynthesis. The genes coding for malic enzyme (ME) from *Mucor circinelloides* (malEMt) and from *Mortierella alpina* (malEMc), respectively, were overexpressed in *M. circinelloides* which led to a 2.5-fold increase of lipid accumulation (Zhang *et al.*, 2007). Lu *et al*. (2008) reported a 20-fold enhancement of fatty acid productivity of *E. coli* by combining four targeted genotypic changes: deletion of the *fadD* gene encoding the first enzyme in fatty acid degradation, overexpression of the genes encoding the endogenous ACCase, and overexpression of both an endogenous thioesterase (TE) as well as a heterologous plant TE. Most recently, overexpression of *wri1* gene from *Brassica napus* in transgenic *Arabidopsis thaliana* resulted into 40% increased seed oil content (Liu *et al.*, 2010).

1.9 Lipid extraction

Lipids are a diverse group of biological substances made up primarily of polar (free fatty acids, phospholipids and sphingolipids) and non-polar compounds (triglycerides, diglycerides, monoglycerides and sterols). They bind covalently to carbohydrates and proteins to form glycolipids and lipoproteins, respectively. The possibility of lipids to bind to other molecules and the ability of different solvent mixtures to solubilize lipid classes has led to the concept of total lipid extraction. Several methods are developed for total lipid extraction depending on the combination of solvents (Bligh and Dyer, 1959; Booij and Van den Berg, 1994; Christie, 1976; de Boer, 1988; Folch *et al.*, 1957; Gardner *et al.*, 1985; Smedes and Askland, 1999). Most of these methods were carried out on animal tissue. Because of large variations in algal cell shape, size, cell wall structure and characteristics of algal lipids, various lipid extraction methods may work differently on different algal species (Shen *et al.*, 2009). Therefore, in addition to lipid content of algal cells, lipid extraction method can also significantly affect the lipid yield. However, the extraction procedures and efficiencies for plant material, especially for algae, are not well established. Converti *et al*. (2009) demonstrated that the most effective lipid extraction method in *Nannochloropsis oculata*, among those taken into consideration, was the combination of ultrasound with the Folch method (1957). Most recently, Lee *et al*. (2010) tested various methods, including autoclaving, bead-beating, microwave, sonication, and a 10% NaCl solution, to identify the most effective cell disruption method in microalgae *Botryococcus* sp., *Chlorella vulgaris*, and *Scenedesmus* sp. The total lipids from these microalgae were extracted by following Bligh and Dyer method (1959). This method is widely used for extraction of lipid, which involves solvent extraction and gravimetric determination. The choice of Bligh and Dyer method was justified on the basis that chloroform and methanol quantitatively extracted all lipid classes, produced the highest lipid yields and was a relatively precise method (Randall *et al.*, 1991; Roose and Smedes, 1996).

1.10 Transesterification

Biodiesel is produced by transesterification process, where triglycerides are transformed into fatty acid methyl ester (FAME) in presence of a monohydroxy alcohol, such as methanol and a catalyst, an alkali or acid, with glycerol as a byproduct (Hoydonckx *et al*., 2004). The emergence of transesterification can be dated back as early as 1846 when Rochleder described glycerol preparation through ethanolysis of castor oil (Formo, 1954). This process has now been widely used to reduce the high viscosity of triglycerides. The transesterification reaction is represented by the general equation as given below, where R_1, R_2 and R_3 are long hydrocarbon chains.



Transesterification is a reversible reaction and proceeds essentially by mixing of the reactants. The process of transesterification is affected by various factors like molar ratio of alcohol to oil, type of alcohol, type and amount of catalyst, reaction time and temperature, and purity of reactants.

One of the most important variables affecting the yield of ester is the type of alcohol and its molar ratio to triglyceride. Stoichiometrically, three moles of alcohols are required for each mole of triglyceride to yield three moles of fatty acid alkyl esters and one mole of glycerol. However, transesterification is an equilibrium reaction in which a large excess of alcohol is required to drive the transesterification reaction to completion. Freedman and Pryde (1982) investigated the tranesterification of soyabean oil with methanol using 1% concentrated sulfuric acid as catalyst. The highest conversion with acid catalyst was obtained at a molar ratio of 30:1 (alcohol:oil, Table 1.3). Ramadhas *et al*. (2005) reported 6:1 molar ratio during acid-catalyzed transesterification, and 9:1 molar ratio during alkaline-catalyzed transesterification to be the optimum amount for

biodiesel production from high free fatty acids (FFA) rubber seed oil. Karmee and Chadha (2005) reported 92% conversion with karanja oil using 10:1 molar ratio (methanol:oil) at 60 ^{0}C in presence of 1% KOH as catalyst. A pseudo-first-order of acid-catalyzed reaction of waste frying oil in the presence of a large excess of methanol, which drove the reaction to completion by 4 h using an oil:methanol:acid ratio of 1:245:3.8 at 70 ^{0}C (Zheng *et al*., 2006). Presence of sufficient amount of methanol during the transesterification reaction is essential to break the glycerine-fatty acid linkages (Al-Widyan and Al-Shyoukh, 2002). But excess of methanol should be avoided because it makes the ester recovery process complicated and raised the biodiesel cost. Among the alcohols, methanol and ethanol are used most frequently, especially methanol because of its low cost, and its physical and chemical advantages (Ma *et al*., 1999). Being polar and shortest chain alcohol, methanol can quickly react with triglycerides.

Catalyst is usually used to improve the reaction rate and yield. The transesterification reaction can be catalyzed by alkalis, acids, or enzymes. The alkalis used for transesterification are NaOH, KOH and corresponding sodium and potassium alkoxides such as sodium methoxide, sodium ethoxide etc. Several researchers have reported the kinetics for both acid- and alkali-catalyzed transesterification reactions. Raheman and Phadatare (2004) reported a 75% conversion with karanja oil using 6:1 molar ratio (methanol:oil) at 60 ^{0}C with 1.3% KOH for 30 min. 1% KOH was reported as the optimal amount for alkaline transesterication reaction of karanja oil by Meher *et al*. (2006). Leung and Guo (2006) tried three different alkali catalysts, i.e. NaOH, KOH, and CH_3ONa; CH_3ONa proved to be the best one because CH_3ONa dissociates into CH_3O^- and Na^+ and does not form any water as side product. Yang *et al*. (2009) reported a yield of 99% of methyl esters in *Idesia polycarpa* oil using 6:1 molar ratio of methanol to oil, 1% KOH and reaction time of 40 min at 30 ^{0}C. Recently, Anwar *et al.* (2010) transesterified okra seed oil using CH_3ONa as a catalyst. KOH and NaOH are commonly used base catalyst for biodiesel preparation. However, during the separation of the final products from glycerol, KOH has been found to be more convenient. Potassium soap being softer than sodium soap does not block of the bottom of the separating funnel and can be removed easily. Hence, KOH as catalyst is preferred over NaOH (Leung and Guo, 2006; Sharma and Singh, 2007).

Alkali catalyst can catalyze faster than an acid catalyst even at room temperature (Freedman *et al.*, 1986; Guan *et al.*, 2009). However, one limitation to the alkali-catalyzed process is its sensitivity to the purity of reactants; the alkali-catalyzed system is very sensitive to both water and FFA. The presence of water may cause saponification under alkaline condition (Basu and Norris, 1996; Liu, 1994). Also, FFA can react with an alkali catalyst to produce soaps and water. Saponification not only consumes the alkali catalyst, but also the resulting soaps can cause the formation of emulsions, which creates difficulties in downstream recovery and purification of the biodiesel. Thus, dehydrated vegetable oil with less than 2% free fatty acids, an anhydrous alkali catalyst and anhydrous alcohol are necessary for alkali-catalyzed systems (Freedman *et al.*, 1984; Jeromin *et al.*, 1987). Ma *et al*. (1998) studied the transesterification of beef tallow catalyzed by NaOH in presence of FFA and water. Without adding FFA and water, the apparent yield of beef tallow methyl esters was highest (42%). When 0.6% of FFA was added, the yield was reduced to less than 5% under any level of water addition. When 0.9% of water was added, without addition of FFA, the apparent yield was about 17%.

The starting materials used for base-catalyzed transesterification should meet certain specifications. The triglycerides should have lower free fatty acid and all material should be substantially anhydrous. These limitations can be eliminated by using acid catalysts. Acids used for transesterification include sulfuric, phosphoric, hydrochloric and organic sulfonic acids. Canakci and Garpen (1999) studied the effects of process variables on acid-catalyzed transesterification of soyabean oil. It was reported that ester conversion reached 98% at a molar ratio of 30:1 (methanol:oil) with 3% sulphuric acid as catalyst at 60 ^{0}C. Mahamad and Ali (2002) also conducted experiments on transesterification of waste palm oil into biodiesel under acid-catalyzed condition. The best process combination was 2.25 M H_2SO_4 with 100% excess ethanol, which reduced the specific gravity from an initial value of 0.92 to final value of 0.87 in about three hour of reaction. Wang *et al*. (2008) optimized the conditions for biodiesel production from trap grease by response surface methodology (RSM). They reported that a 35:1 molar ratio of methanol to oil with 11.3% sulfuric acid gave maximum ester conversion of 90% after 4.6 h at 90 ^{0}C.

As acid-catalyzed transesterification is a relatively slow process, many researchers have combined both acidic and alkaline catalysts in a two-step reaction in which the acid treatment converts the FFA into esters while the alkaline catalyst is performing the transesterification. This process has been developed by Canacki and Garpen (2001) using yellow and brown grease having FFA content of more than 10%. Using acid catalyzed pretreatment followed by an alkali-catalyzed final reaction, the transesterification reaction was completed in much less time than would be possible with acid-catalyzed transesterification alone. Veljkovic *et al*. (2006) produced biodiesel from high FFA tobacco seed oil by using two step transeterification. These workers reduced the FFA content of the oil to less than 2% through acid catalyst before transesterifying with an alkali catalyst to complete the reaction. Ghadge and Raheman (2006) used a central composite rotatable design to study the effect of methanol quantity, acid concentration and reaction time on the reduction of free fatty acids content of mahua oil during its pretreatment for making biodiesel. The optimum combinations for reducing the acid level of mahua oil to less than 1% after pretreatment was 9:1 methanol-to-oil ratio, 1.24% v/v H_2SO_4 catalyst and 1.26 h reaction time at 60 ^{0}C. Sahoo *et al.* (2007) used zero-catalyzed reaction (to remove organic matters and impurities from oil using toluene) and acid esterification (using H_2SO_4) prior to alkaline esterification to reduce the acid value from 22 to 2%. Sharma and Singh (2007) also favored acid esterification prior to alkaline transesterification with karanja oil as feedstock having FFA of 2.5% using H_2SO_4. In the same manner, the acid value of jatropha which corresponds to 14% FFA was reduced to less than 1% by using H_2SO_4 (Tiwari *et al.*, 2007). Wang *et al.* (2007) tried a new catalyst, ferric sulphate, as an alternate to sulphuric acid and have reported much better conversion (97%) of biodiesel from waste cooking oil of high FFA by a two step catalytic process. Recently, Sharma and Singh (2010) also produced biodiesel from kusum (*Schleichera triguga*) seed oil by two step transesterification. An economic analysis however, has shown that the acid-catalyzed procedure, being a one-step process, is more economical than the alkali-catalyzed process, which requires an extra step to convert free fatty acids to methyl esters to avoid soap formation (Zheng *et al.*, 2006).

The homogeneous catalysts require neutralization and separation from the reaction mixture. To accomplish this, water, solvents and energy are needed. To

overcome these, heterogeneous catalysts were tested by researchers, where separation was possible without using solvent and also showed easy regeneration. The heterogeneous catalysts being used by researchers include alkaline-earth oxides, zeolites, hydrotalcites, MgO and CaO (Corma *et al.*, 1998; DiSerio *et al*., 2004; Dossin *et al.*, 2006; Grylewicz, 2000). Furuta *et al*. (2006) tested amorphous zirconia solid catalysts, TiO_2/ZrO_2 (11 wt% Ti) and Al_2O_3/ZrO_2 (2.6 wt% of Al) and reported more than 95% conversion of soyabean oil. The zinc stearate immobilized on silica gel (ZS/Si) was the most effective catalyst in simultaneously catalyzing the transesterification of triglycerides and esterification of free fatty acid present in waste cooking oil to methyl esters (Jacobson *et al*., 2008). Most recently, Gao *et al.* (2010) tried a solid base catalyst KF/Ca - Al hydrotalcite for biodiesel production from palm oil. These workers reported that a 12:1 molar ratio of methanol to oil with 5% catalyst gave maximum ester conversion of 98% after 5 h at 65 ^{0}C.

Although chemical transesterification using an alkaline catalysis process gives high conversion levels of triglycerides to their corresponding methyl esters in short reaction times, the reaction has several drawbacks: it is energy intensive, recovery of glycerol is difficult, the catalyst has to be removed from the product, alkaline waste water requires treatment, and FFA and water interfere the reaction. Enzymatic catalysts like lipases are able to effectively catalyze the transesterification of triglycerides in either aqueous or non-aqueous systems, which can overcome the problems mentioned above (Fuduka *et al*., 2001). In particular, the by-products, glycerol can be easily removed without any complex process, and also that FFA contained in waste oils and fats can be completely converted to alkyl esters. Transesterification of triglycerides using lipase have been studied by many researchers (Abigor *et al.*, 2000; Breivik *et al.* 1997; Chang *et al.*, 2009; De *et al.*, 1999; Dizge and Keskinler, 2008; Li *et al.*, 2009; Linko *et al.*, 1998; Mittelbach, 1990; Nelson *et al.*, 1996; Nie *et al.*, 2006; Royon *et al.*, 2007; Selmi and Thomas, 1998; Shah and Gupta, 2007; Shieh *et al.*, 2003; Watanabe *et al.*, 2002; Wu *et al.* 1999). However, the major drawback in commercialization of lipase-catalyzed biodiesel production is its high production cost and slow reaction rate.

Various oils have been in use in different countries as raw materials for biodiesel production owing to their availability. The different processes, reagents and catalyst used for biodiesel production from various feedstocks are summarized in Table 1.3. It can be observed from the table that methanol is the most frequently used alcohol in the process of making biodiesel from vegetable oils. A higher molar ratio of alcohol:oil is required for acid-catalyzed transesterification than alkali-catalyzed one. Among the acid catalysts H_2SO_4 and HCl are preferred. KOH and NaOH are mostly used base catalysts, especially KOH for transesterification. The catalyst concentration generally varied between 1 - 2%. The higher reaction temperature has been preferred for faster reaction rates, however it is limited by the boiling point of the alcohol used. The reactions are also affected by molar ratio of triglyceride to alcohol and reaction time. Thus, all these parameters needed to be studied and optimized for increasing the biodiesel yield.

Table 1.3: Biodiesel production from various feedstocks using different transesterification processes

Feedstock	Transester-ification step	Alchol		Catalyst		Reaction temperature (^{0}C)	Duration	Biodiesel conversion (%)	Reference
		Type	Molar ratio (Alchol : Oil)	Type	Concentration				
Soyabean oil (*Glycine max*)	Single step	Methanol	30:1	H_2SO_4	1%	60	44 h	>95	Freedman and Pryde (1982)
Soyabean oil (*Glycine max*)	Single step	Methanol	30:1	H_2SO_4	3%	60	48 h	98	Canakci and Garpen (1999)
Brown grease	Two step	Methanol Methanol	10:1 6:1	H_2SO_4 KOH	15% 1%	60	30 min 8 h	75	Canakci and Garpen (2001)
Soyabean oil (*Glycine max*)	Single step	Methanol	3.4:1	Lipase (*Rhizomucor miehei*)		37	6.3 h	92	Shieh *et al.* (2003)
Karanja oil (*Pogomia pinnata)*	Single step	Methanol	6:1	KOH	1.3%	60	30 min	75	Raheman and Phadatare (2004)
Rubber seed oil (*Ficus elistica*)	Two step	Methanol Methanol	6:1 9:1	H_2SO_4 KOH	0.5% 0.5%	45	30 min 30 min	>95	Ramadhas *et al.* (2005)
Karanja oil (*Pogomia pinnata)*	Single step	Methanol	10:1	KOH	1.0%	60	1.5 h	92	Karmee and Chadha (2005)
Waste frying oil	Single step	Methanol	245:1	H_2SO_4	3.8 M	70	4 h	99	Zheng *et al.* (2006)
Waste frying oil	Single step	Methanol	7.5:1	NaOH KOH CH_3ONa	1% 1% 1%	70	30 min 30 min 30 min	85 86 89	Leung and Guo (2006)
Karanja oil (*Pogomia pinnata)*	Single step	Methanol	6:1	KOH	1%	65	2h	98	Meher *et al.* (2006)
Tobaco (*Nicotiana tabacum*)	Two step	Methanol Methanol	18:1 6:1	H_2SO_4 KOH	1% 1%	60	25 min 30 min	91	Veljkovic *et al.* (2006)

Table 1.3-continued

Feedstock	Transester-ification step	Alchol		Catalyst		Reaction temperature (^{0}C)	Duration	Biodiesel conversion (%)	References
		Type	Molar ratio (Alchol : Oil)	Type	Concentration				
Mahua oil (*Madhuca indica*)	Two step	Methanol Methanol	9:1 7:1	H_2SO_4 KOH	1.2% 0.7%	60	1.26 h 30 min	98	Ghadge and Raheman (2006)
Soyabean oil (*Glycine max*)	Single step	Methanol	40:1	TiO_2/ZrO_2 Al_2O_3/ZrO_2 K_2O/ZrO_2	(11 wt% Ti) (2.6 wt% Al) (3.3 wt% K)	250	2 h 2 h 2 h	>95 >95 >95	Furuta *et al.* (2006)
Waste cooking oil	Two step	Methanol Methanol	10:1 6:1	Fe_2SO_4 KOH	2% 1%	95 65	4 h 1 h	97	Wang *et al.* (2007)
Polanga oil (*Calophyllum inophyllum*)	Triple step (First step by tolune)	Methanol Methanol	6:1 9:1	H_2SO_4 KOH	0.7 % 1.5 %	65	2 h 4 h	>99	Sahoo *et al.* (2007)
Jatropha oil (*Jatropha curcas*)	Two step	Methanol Methanol	7.5:1 4:1	H_2SO_4 KOH	1.4% 1.5 %	60	88 min	>99	Tiwari *et al.* (2007)
Trap grease	Single step	Methanol	35:1	H_2SO_4	11.3%	90	4.6 h	90	Wang *et al.* (2008)
Waste cooking oil	Single step	Methanol	18:1	ZS/Si catalyst	3%	200	10 h	98	Jacobson *et al.* (2008)
Iigiri oil (*Idesia polycarpa*)	Single step	Methanol	6:1	KOH	1%	30	40 min	99	Yang *et al.* (2009)
Kusum oil (*Schleichera triguga*)	Two step	Methanol Methanol	10:1 8:1	H_2SO_4 KOH	1% 0.7 %	50	1 h 1 h	97	Sharma and Singh (2010)
Palm oil	Single step	Methanol	12:1	KF/Ca – Al hydrotalcite	5%	65	5 h	98	Gao *et al.* (2010)
Okra oil (*Hibiscus esculentus*)	Single step	Methanol	7:1	CH_3ONa	1%	65	2 h	97	Anwar *et al.* (2010)

1.11 Microalgal biodiesel production

Microalgal biodiesel production is relatively new and not very well explored. Few reports are available, where attempts have been made to produce biodiesel from algae (Table 1.4). Miao and Wu (2006) reported that lipid extracted from the heterotrophically-grown microalga, *Chlorella protothecoides*, transformed into biodiesel with a yield of 63% under 1:1 weight ratio of H_2SO_4 to oil, and 56:1 molar ratio of methanol to oil at 30 ^{0}C for a reaction time of 4 h . Xu *et al.* (2006) characterized the biodiesel obtained from the *C. protothecoides* oil by acid-catalyzed transesterification. The most abundant fatty acid methyl ester (FAME) in *C. prothecoides* biodiesel was methyl oleate (61% of total FAME) followed by methyl linoleate (17%) and methyl palmitate (13%). Subsequently, Li *et al.* (2007) showed that it was feasible to grow *C. protothecoides* in a commercial-scale bioreactor. Using 75% immobilized lipase, these researchers claimed ~98% conversion could be obtained in 12 h when the reaction condition with respect to solvent type, water content and pH were optimized. Hossain and Sallesh (2008) studied biodiesel production from *Oedogonium* and *Spirogyra* species using NaOH as catalyst. Algal oil and biodiesel production was higher in *Oedogonium* sp. than *Spirogyra* sp. Umdu *et al*. (2009) studied the effects of Al_2O_3 supported CaO and MgO catalysts in the transesterification of lipid of *Nannochloropsis oculata*. These researchers found that pure CaO and MgO were not active, and CaO/Al_2O_3 catalyst showed the highest activity. Biodiesel yield was increased up to 98 from 23% under CaO/Al_2O_3 catalyzed reaction.

Lipid extracted from *Neochloris oleabundans* was found to have an adequate fatty acid profile and iodine value according to the biodiesel specifications of European standard (Gouveia *et al.*, 2009). Converti *et al.* (2009) analyzed the fatty acid methyl esters in biodiesel produced from *N. oculata* and *C. vulgaris.* The most abundant composition was methyl palmitate, which was 62 and 66%, respectively in *N. oculata* and *C. vulgaris* biodiesel. However, the concentration of linolenic acid (18%) in *N. oculata* could not meet the requirement of European legislation for biodiesel. Johnson and Wen (2009) prepared biodiesel from the microalga *Schizochytrium limacinum* by direct transesterification of algal biomass. Parameters such as free glycerol, total glycerol, acid number, soap content, corrosiveness to copper, flash point and viscosity met the ASTM and European standards,

while the water and sediment content, as well as the sulfur content did not pass the standard. Damiani *et al*. (2010) studied biodiesel production from *Haematococcus pluvialis* using potassium hydroxide as the catalyst. The major constituent of *H. pluvialis* biodiesel was palmitic acid followed by linoleic, oleic and linolenic acid methyl ester. The iodine value was within the limit established by European standard. Chinnasamy *et al.* (2010) produced biodiesel by a two steps transesterification process (acid-catalyzed followed by base-catalyzed) from a consortium of 15 native algae cultivated in carpet industry wastewater. Algal methyl esters were predominated by linolenic, linoleic, palmitic and oleic acid. The biodiesel was found to contain 0.0155 and 0.0001% bound and free glycerin, respectively, and met the ASTM and European standard specifications.

Table 1.4: Attempts on biodiesel production from microalgae

Name of the alga	Transesterification process with % conversion	Properties of biodiesel		Reference
		Major ester	Physical property	
Chlorella protothecoides	H_2SO_4-catalyzed (63%)	NC	Density: 0.86 kg l^{-1}, Viscosity: 5.2 cSt, Flash point: 115 ^{0}C, Acid value: 0.37 mg KOH g^{-1}, Heating value: 41 MJ kg^{-1}	Miao and Wu (2006)
	H_2SO_4-catalyzed (63%)	methyl oleate: 61%, methyl linoleate: 17%, methyl palimate: 13%	Density: 0.86 kg l^{-1}, Viscosity: 5.2 cSt, Flash point: 115 ^{0}C, Solidifying point: 12^{0}C Acid value: 0.37 mg KOH g^{-1}, Heating value: 41 MJ kg^{-1}	Xu *et al.* (2006)
	Lipase-catalyzed (98%)	methyl oleate: 65%, methyl linoleate: 18%, methyl palimate: 10%	NC	Li *et al.* (2007)
Oedogonium sp.	NaOH-catalyzed (95%)	NC	NC	Hossain and Sallesh (2008)
Spirogyra sp.	NaOH-catalyzed (93%)			
Nannochloropsis oculata	Heterogeneous catalyst (Al_2O_3 supported CaO & MgO) (98%)	NC	NC	Umdu *et al.* (2009)

Table 1.4-continued

Neochloris oleabundans	BF_3-catalyzed (NR)	methyl oleate: 38% methyl palimate: 17% methyl sterate: 14% methyl linolenate: 8%	Iodine value: 72	Gouveia *et al*. (2009)
Nannochloropsis oculata	Acid-catalyzed (NR)	methyl palmitate: 62%, methyl linolenate: 18%, methyl linoleate: 12%, methyl oleate: 6%	NC	Converti *et al*. (2009)
Chlorella vulgaris		methyl palmitate:66%, methyl linolenate:12%, methyl linoleate:11%, methyl oleate:7%		
Schizochytrium limacinum	H_2SO_4-catalyzed (66%)	methyl palmitate :57%, methyl ester of C22:6 : 30%	Viscosity: 3.87 cSt Flash point: 204^0C, Moisture content: 0.11%, Acid value: 0.11 mg KOH g^{-1}, Total glycerin: 0.097%, Free glycerin: 0.003%,	Johnson and Wen (2009)
Haematococcus pluvialis	KOH-catalyzed (NR)	methyl palmitate: 23%, methyl linoleate: 20%, methyl oleate: 19%, methyl linolenate: 16%	Iodine value: 111	Damiani *et al*. (2010)
A consortium of 15 native microalgae	Acid-catalyzed followed by base-catalyzed (64%)	methyl linolenate: 28% methyl linoleate: 20, methyl palmitate: 16%, methyl oleate:12%	Bound glycerin: 0.0155%, Free glycerin: 0.0001%	Chinnasamy *et al*. (2010)

NR: not reported, NC: not characterized.

1.12 Fatty acid methyl esters and fuel properties

As stated before, biodiesel is the best substitute for diesel due to its physical properties, which are close to those of diesel. Diesel is a petroleum-derived fuel, which is a mixture of hydrocarbons obtained from crude oil in the distillation range of 250-350 ^{0}C. The length of hydrocarbon chain in petroleum oils varies in between 12 to 50 carbon atoms. Diesel is the mixture of C_{15} to C_{18} hydrocarbons. Diesel contains only carbon and hydrogen atoms, which are arranged in straight or branched chain structures, as well as aromatic configurations. Diesel may contain both saturated and unsaturated hydrocarbons.

Biodiesel has different chemical structure than the conventional diesel fuel. Biodiesel is monoalkyl esters of long chain fatty acids derived from vegetable oils. Several types of vegetable oils, with a varied composition in fatty acids, can be used for the preparation of biodiesel. The oils from different sources contain 10 common types of fatty acids which have between 12 to 24 carbons, with over 90% of them being between 16 to 18 carbons. The common fatty acids present in vegetable oils are listed in Table 1.5.

Table 1.5: Common fatty acids present in vegetative oils and their chemical structure

Common name	**Systemic name**	**Chemical Structure**	**Common acryonym**
Lauric acid	Dodecanoic acid	$CH_3(CH_2)_{10}COOH$	C 12:0
Myristic acid	Tetradecanoic acid	$CH_3(CH_2)_{12}COOH$	C 14:0
Palmitic acid	Hexadecanoic acid	$CH_3(CH_2)_{14}COOH$	C 16:0
Stearic acid	Octadecanoic acid	$CH_3(CH_2)_{16}COOH$	C 18:0
Oleic acid	Octadecenoic acid	$CH_3(CH_2)_7CH=CH(CH_2)_7COOH$	C 18:1
Linoleic acid	Octadecadienoic acid	$CH_3(CH_2)_4CH=CHCH_2CH=CH(CH_2)_7COOH$	C 18:2
Linolenic acid	Octadecatrienoic acid	$CH_3CH_2CH=CHCH_2CH=CHCH_2CH=CH(CH_2)_7COOH$	C 18:3
Archidic acid	Eicosanoic acid	$CH_3(CH_2)_{18}COOH$	C 20:0
Behenic acid	Decosanoic acid	$CH_3(CH_2)_{20}COOH$	C 22:0
Erucic acid	Docosenoic acid	$CH_3(CH_2)_7CH=CH(CH_2)_{11}COOH$	C 22:1
Lignoceric acid	Tetracosanoic acid	$CH_3(CH_2)_{22}COOH$	C 24:0

Fuel properties of biodiesel which are influenced by the fatty acid profile and, in turn, by the structural features of various fatty acid esters are cetane number and ultimately exhaust emission, heat of combustion, cold flow, oxidative stability, viscosity, and lubricity. Structural feature of a fatty acid ester molecule that influence the physical and fuel properties are chain length and degree of unsaturation (Knothe, 2005). Since biodiesel is produced in quite differently scaled plants from vegetable oils of varying origin and quality, it is necessary to install a standardization of fuel quality to guarantee engine performance without any difficulties.

Cetane number (CN) is widely used as diesel fuel quality parameter related to the ignition delay time and combustion quality. Higher the cetane number better is the ignition properties (Meher *et al.*, 2006). High cetane numbers ensure good cold start properties and minimize the formation of white smoke. The longer the fatty acid carbon chains and the more saturated the molecules are, higher are the cetane numbers (Bajpai and Tyagi, 2006). According to Knothe *et al.* (2003), high cetane numbers are observed for esters of saturated fatty acids such as palmitic and stearic acids.

The oxidation stability decreased with increase in the contents of polyunsaturated methyl esters (Ramos *et al.*, 2009). The limitation of unsaturated fatty acids is also necessary due to the fact that heating of higher unsaturated fatty acids results in polymerization of glycerides. This can lead to the formation of deposits in the machines (Mittelbach, 1996).

One of the major problems associated with the use of biodiesel is its poor cold flow property, indicated by relatively high cloud point and pour point. Saturated fatty acids have significantly higher melting points than unsaturated fatty acids and in a mixture the former crystallizes even at room temperature. Thus biodiesel fuels derived from fats or oils with significant amounts of saturated fatty compounds will display higher cloud points and pour points. Viscosity also increases with the increasing degree of saturation and chain length (Knothe, 2005). Unsaturated fatty acid exhibited better lubricity than saturated species (Kenesey *et al.*, 2003). Heat of combustion increases with chain length and decreases with unsaturation (Goering *et al*. 1982). The increase in heat content results from a gross increase in number of carbon and hydrogen as well as increase in the ratio of these elements relative to oxygen. Thus biodiesel containing saturated and mono-unsaturated fatty acids exhibits improved fuel properties (Liu and Zhao, 2007).

1.13 Waste utilization for algal cultivation

The disposal of waste is increasingly becoming a problem and new paradigm is to regard waste as resources for sustainable development. In agricultural countries like India, waste discharges from agriculture, agro-based industries and city sewages are the main sources of water pollution. Conventional wastewater treatment systems do not seem to be

the definitive solution to pollution and eutrophication problems. They suffer mainly from two drawbacks: cost and lack of nutrient recycling (Eisenberg *et al.,* 1981). Secondary sewage treatment plants are specifically designed to control the quantity of organic compounds in wastewaters. Other possible pollutants including nitrogen and phosphorus are only slightly affected by this type of treatment (Gates and Borchardt, 1964). Owing to the ability to use nitrogen and phosphorus for growth, algae can successfully be cultivated in such type of wastewaters (Mallick, 2002). This has been evolved from the early work of Oswald (Oswald, 1953) using microalgae in tertiary treatment of municipal wastewater. The widely used microalgae cultures for nutrient removal are *Chlorella* (Lee and Lee, 2001; Gonzales *et al.*, 1997), *Scenedesmus* (Martinez *et al.*, 1999, 2000) and *Spirulina* (Olguín *et al.*, 2003). Nutrient removal efficiency of *Nannochloris* sp. (Jimenez-Perez *et al.*, 2004), *Botryococcus brauinii* (An *et al.*, 2003), and *Phormidium* sp. (Dumas *et al.*, 1998; Laliberte *et al.*, 1997) has also been investigated. One of the well-known algae containing bioprocesses for wastewater treatment is high-rate algal ponds (HRAP) (Cromar *et al.*, 1996; Deviller *et al.*, 2004). Recently, corrugated raceways (Craggs *et al.*, 1997; Olguín *et al.*, 2003), triangular photobioreactors (Dumas *et al.*, 1998), and tubular photobioreactors (Molina *et al.*, 2000) have been developed for nutrient removal.

Among agro-industries, aquaculture contributes significantly to the generation of wastewaters. The main source of potentially polluting waste in fish culture is feed-derived. Intensified aquaculture follows extensive use of feed inputs which in turn increases organic matter as unconsumed and undigested feed and fish excreta. The direct discharge of aquaculture waste is internationally disapproved because the organic and nutrient loads cause eutrophication of the receiving waters. Qian *et al.* (1996) reported the collapse of a prawn industry in China for reasons attributed to outbreak of pathogenic bacteria caused by increased nutrient load. Environmental concerns and limitation in water availability are some of the factors that make recirculation systems an important option for the aquaculture industry. Reused water systems reduce water replacement rates and waste volume. Various studies have shown the efficiency of algae biofilters in removing nitrogen from fish effluents (Cohen and Neori, 1991; Jimenez del Rio *et al.*, 1996; Schuenhoff *et al.*, 2003). Most works on the use of algae to treat effluent water from aquaculture has used integrated systems with seaweeds of the genera *Ulva* and *Gracilaria.* Vandermeulen and Gordin (1990) reported the

mariculture experiments with *Ulva lactuca* in an integrated system of finfish, *Sparus aurata.* These workers found that *U. lactuca* efficiently removed up to 85% of total ammonia nitrogen (TAN) from fishpond effluent. Jimenez del Rio *et al.* (1996) obtained dissolved inorganic nitrogen removal by the seaweed biofilter *U. rigida.* Species of *Gracilaria* have been identified as an effective alternative for nutrient bioremediation (Neori *et al.*, 2004). For example, Jones *et al.* (2001) showed that *G. edulis* removed around 95% of ammonium originating in shrimp cultivation. Buschmann *et al.* (1996) reported that *G. chilensis* was capable of removing 95% of ammonium and 32% of orthophosphate from an integrated system (seaweed/salmon). Neori *et al.* (1998) found that *G. conferta* removed 34% of ammonium and around 25% of orthophosphate from a polyculture system (mollusks/fish/seaweed). The biofiltration capacity of *G. birdiae* and *G. caudata* was confirmed by the significantly reduced concentration of three nutrients analyzed (PO_3^{-3}, NH_4^- and NO_3^-) over a study period of 28 days in shrimp pond effluents (Marinho-Soriano *et al.*, 2008, 2009). However, the uses of microalgae to treat effluent water from aquaculture are very scanty in the scientific literature. Dumas *et al.* (1998) investigated the use of a cyanobacterium, *Phormidium bohneri*, to remove dissolve inorganic nutrients from rainbow trout effluent. The average efficiencies of ammonium and orthophosphate removal were 82 and 85% respectively, over a one month period. This method generates a potentially valuable algal biomass in parallel with waste treatment via uptake of inorganic nutrients by the algae.

Poultry waste is another area to be addressed. An immense quantity of waste is generated with poultry production. Effluents from poultry farming contain high concentrations of nitrogen, phosphorus, and organic matter in both soluble and particulate forms, the composition mainly depending on animal nutrition and farming practices (Edwards and Daniel, 1992; Kelleher, 2002). Agricultural land disposal methods have traditionally been used to solve poultry waste management, however, the recent intensive farming context has overflowed the natural capacity of the farm surrounding lands to cope with poultry wastes. Bitzer and Sims (1988) reported that excessive application of poultry litter in cropping systems can result in nitrate (NO_3) contamination of groundwater. On the other hand, anaerobic digestion, although particularly suitable for the range of organic matter concentrations commonly found in these wastes, are not efficient in nutrients

removal. In this context, the development of cost-effective technologies, which support a simultaneous carbon oxidation and nutrient recovery, is crucial in the establishment of sustainable farming. Microalgae-based systems can significantly reduce both organic matter and nutrients present in poultry litter and generate a potentially valuable algal biomass for biodiesel production. To the best of our knowledge, cultivation of microalge in urban wastewater, swine wastes, dairy manure and cattle residues has been reported by several authors (De la Noüe and Basseres, 1989; De la Noüe and Proulx, 1988; Fallowfield, 1999; Lincoln and Hill, 1980; Mulbry and Wilkie, 2001; Mulbry *et al.*, 2008; Pizarro *et al*., 2006; Wilkie and Mulbry, 2002), but not a single report dealing with direct use of poultry litter by microalgae has been appeared in the scientific literature, except the work of Mahadevaswamy and Venkataraman (1986), where bioconversion of poultry litter for biogas production and utilization of the effluent for production of the blue-green alga *Spirulina platensis* was studied. Therefore, the use poultry litter with microalgal culture by combining it with wastewater treatment and biodiesel production is a very promising eco-friendly strategy in the present scenario.

In view of the above discussion, the present investigation was undertaken with the following specific objectives:

1. To screen lipid accumulation potential of microalgae from laboratory-grown cultures as well as from local isolates.
2. To optimize the condition(s) for maximum lipid accumulation in the selected microalgal species.
3. To check the feasibility of waste utilization for algal cultivation and lipid accumulation.
4. To establish the transesterification process for algal oil.
5. To determine the fuel properties of algal biodiesel.