2. Review of Literature

2.1. Historical background

At present global energy demand depends on petroleum products, upcoming scarcity and depletion of fossil fuel reserves raised a need for alternative renewable fuel resources (Rittmann. 2008). Biodiesel is an alternative diesel fuel mainly from renewable biological sources such as vegetable oils and animal fats. It is environmental friendly due to its properties viz., biodegradable, nontoxic and has low emission profiles. The usage of biodiesel as fuel has the potential to reduce the pollutant level (Krawczyk, 1996).

The Biodiesel production is not something new because the concept of using vegetable oil as fuel dates back to 1895. Rudolf Diesel developed the first diesel engine which was run with groundnut oil as fuel in 1900 (Bijalwan et al., 2006). The use of vegetable oils for engine fuels may seem insignificant at that time but such oils gained importance like petroleum and the coal tar products at present (Babu and Devaradjane, 2003).

After many decades, the awareness about environment and energy security among the people to search for an alternative fuel that could burn with less pollution at affordable cost. With the available petroleum reserves in 1930s, vegetable oil was used only in emergency situation. Recently, the increased crude oil prices, limitation in fossil oil reserves and environmental concern has renewed the focus on biodiesel feed stock again. Another problem is the global warming mainly due to continuous and increased usage of petroleum will intensify local air pollution and magnify the green house gas emission and its adverse effect on environment.
2.2. Biodiesel

Biodiesel is defined as a fuel comprised of mono-alkyl esters of long chain fatty acids derived from vegetable oils or animal fats (Vicente et al., 2007). It has similar physico-chemical properties of conventional fossil fuel and can consequently, entirely or partially substitute fossil diesel fuel in compression ignition engines (Pasqualinoa et al., 2006).

Considerable research has been done on vegetable oils as diesel fuel. Those research included palm oil, soybean oil, sunflower oil, coconut oil, rapeseed oil and tung oil. Animal fats have not studied well as vegetable oils because of the natural property differences among them. Microbial oil sources from algae, bacteria and fungi also have been investigated (Shay. 1993). Terpenes and latexes also were studied as diesel fuels. Among all, microalgae have been believed as a sustainable source of fatty acid methyl ester to serve as diesel fuel (Nagel and Lemke, 1990).

2.3. Developments in biodiesel feedstock

Biodiesel has come across three generation; the usage of first generation biofuel from food based crops viz., corn, sunflower, vegetable oil, rape seed oil instead of fossil fuel has been estimated to reduce green house gas (GHG) emission up to 20%. The GHG emission can be avoided through carbon capture by crop when they grow (Searchinger et al., 2008). According to the World Bank, Food crop usage for biofuel was the biggest factor in driving up the food prices (Mitchell, 2008). For an example, In USA at the end of 2010 corn price increased from $3.49 to $6.10 due to their usage in bio-ethanol production.

Second generation biodiesel were targeted from cellulose, hemicelluloses or lignin biomass. The biomass originated from non food sources such as corn stalks
and kernels, waste from forest, fast growing plants and weeds, grass like switch grass (*Panicum virgatum*) and so on. The biomass was considered as easily available and low cost material but it required a pre-treatment with chemicals or enzymes before fuel conversion (Sanderson, 2011).

Third generation biodiesel, Scientists are searching for an apt biomass for biodiesel production. Biodiesel is ought to compete and replace petro diesel due to its renewable nature. They are considered to replace liquid based transportation fuel demand in future. Algae was cultivated for food supplement in many centuries, but the concept of using algae for fuel was first proposed by Meier in 1955 (Hu et al., 2008). Later they were acknowledged as a good lipid source for biodiesel production. In 1978 the USA started the National Aquatic Species Program (NASP) for algae fuel research. During this program, around 3000 algae isolates and their valuable information on growth and photosynthesis were gathered. After 18 years of research, in 1996 the program came to an end with the conclusion that biofuel could not compete with the dropping oil prices at that time (Sheehan, 1998). Now the concept of microalgae biodiesel has been renewed by many countries because of fuel hikes, energy and environmental security.

Ramos et al. (2009) stated that as a third generation feed stock, microalgae offer novel aquatic biomass systems with higher lipid yield and lower water demand for cultivation. Direct transesterification process would allow desired fatty acid methyl ester (FAMEs) production. At present most of the research activities are targeted to improve lipid production through algae strain optimization, novel reactor design and process integration with auxiliary by products.
2.4. Microalgae as biodiesel feed stock

Jain and Sharma (2011) explained about microalgal lipids which are divided into three types namely; crude lipids, neutral lipids and total lipids. Crude lipids include neutral lipids and pigments. Neutral lipids are comprised of triglycerides, free fatty acids, hydrocarbons, sterols, wax, sterol esters and free alcohols. Total lipids are comprised of pigments, phospholipids, glycolipids, and neutral lipids. More number of microalgal species were identified as suitable candidate for biodiesel feed stock preparation due to their high growth, lipid content, and ability to grow in non-arable land. The extraction method from lab scale to large scale would decide the actual triglyceride recovery from microalgal strain (Chisti, 2008).

According to ASTM (American Society for Testing and Materials) standard the microalgal lipid is desirable for the conversion into biodiesel (Sheehan et al., 1998). The quantity and quality of lipid types do vary according to microalgal species. The lipid type, especially their amount of saturation and carbon chain length decide the biodiesel quality (Xu et al., 2006). The steps involved in generating microalgal biodiesel are as follows, optimization of nutrients required for algal growth, biomass recovery and lipid extraction which altogether increased the biodiesel production cost. In order to reduce the biodiesel production cost industrial effluent systems were applied for microalgae cultivation (Grima et al., 2003).

Microalgae were desirable for biodiesel production as compared to crop based biodiesel feedstock because of the following reasons: (1) fast growth rate could lead to high biomass yield, (2) do not interfere with food security concerns like plant derived vegetable oil, (3) microalgae diesel has less greenhouse gas emissions and contaminants when compared to petroleum based fuels,
(4) cultivation in non-arable land, (5) utilize industrial flue gas and effluents as nutrient source, (6) biomass could be harvested daily (Chisti, 2007).

Due to these favourable features microalgae are widely targeted and exploited for biodiesel production. The following microalgae are known to be the biodiesel producer’s *viz.*, *Botryococcus braunii*, *Neochloris oleoabundans*, *Chlorella* sp., *Dunaliella primolecta* etc. Still there is a need to find the maximum lipid producing strains for biodiesel production (Wen *et al.*, 2006). Therefore the present study was initiated with isolation procedures.

### 2.5. Limitations of microalgae feed stock

Many microalgae are able to grow rapidly and produce lipid in the form of triacylglycerol (TAG) or free fatty acid, thus referred as oleaginous algae (Sheehan *et al.*, 1998). The quantification of chlorophyll-a pigment is necessary; it directly denoted the microalgae photosynthetic rate (Lee *et al.*, 1998). Algal growth is mainly decided by the availability of nutrients, temperature, light and pH (Yang *et al.*, 2011).

From environmental point of view microalgae are attractive biofuel resource because they could consume carbon dioxide and could grow on marginal land by utilising waste or salt water. In contrast to other biofuel such as corn based ethanol, soy or palm based biodiesel, microalgae feed stocks derived biofuel would not directly compete with the resources necessary for agricultural food production (Dismukes *et al.*, 2008). Development of waste water based microalgae cultivation system would be helpful in recycling of organic and inorganic compounds.
The major disadvantages of microalgae cultivation for biodiesel production are the low biomass and oil concentration due to the light penetration limit of microalgae cells. The small size of algal cells and water content made the harvest process difficult and need energy consuming process for drying the harvested biomass. It increases the algal feed stock cost. When compared to conventional agricultural farming microalgae farming is found to be more complicated and costly. However these problems were expected to be minimized by strain optimization procedures.

2.6 Unialgal isolation and cultivation

Hu et al. (2008) proposed a rough estimation about members of microalgae lineage and only a small fraction was identified from the total existing species. Identifying new microalgae strains and their optimization and genetic engineering methods could advance the microalgae utilization in biofuel sector.

Unialgal isolation is difficult and time consuming process, in many studies mixed cultures were used for the removal of nutrient from wastewater and simultaneous biodiesel production (Chisti, 2008). In genetic engineering approach of microalgae for enhanced lipid production, mixotrophic cultures should be avoided. Only axenated unialgal strains are required to maintain the specificity of molecular cloning and gene expression studies.

Traditional microalgae identification was based on vegetative cell shape, chloroplast position and pyrenoids, motility pattern and developmental stages. Some species were not easily recognizable by this method and need more specific identification method. In order to avoid misinterpretation in species identification, specific molecular characterization is required. DNA barcoding method is an
important molecular method would allow even nonspecialist to identify green algae members (Kelling et al., 2010).

The internal transcribed spacer (ITS) region of the nuclear ribosomal operon had been proposed as a DNA barcode for green algae and land plants (Moniz and Kaczmarska, 2010). ITS locus was found in between the large and small nuclear ribosomal subunit genes and includes the 5.8S rDNA gene. (Fama’et al., 2002).

2.7. Optimization of microalgae for increased lipid production

Enhancing microalgae lipid production is necessary to reduce the biodiesel feedstock cost and its sustainability. Under nutrient stress green algae strains could accumulate large quantities of lipids. The oil content of microalgae ranged from 16 to 68% dry weight and the oil yield from microalgae can reach up to 136,900 L/ha compared to other plant crops, which ranged from 172 to 5950 L/ha (Chisti, 2007). Further optimization of culture conditions could enhance the lipid content of microalgae.

Chisti (2007) stated microalgae to be the most favourable source for biodiesel. The fatty acids attached to the triglycerides within the algal cells can be both short and long chain hydrocarbons. The short chain length acids are ideal for obtaining biodiesel. Genetic modification of microalgae has received little attention. Molecular level engineering can be used to potentially increase photosynthetic efficiency which in turn enhances the biomass and growth rate.

Strain optimization is needed for the production of food products, pharmaceuticals and bio-fuel. Fortman et al. (2008) stated that decades of work
produced a considerable knowledge base for making microbial engineering an ideal strategy for producing bio-fuel. Although ethanol was currently dominating the biofuel market because of some of its inherent physical properties it is less sufficient to meet global fuel demand.

The study on regulatory mechanism of oil accumulation in microalgae and approaches for making microalgae diesel equally competitive to petro diesel is really effortful work. The improvement in lipid quality could help in fuel cost reduction and thus it could compete with conventional fuels. Meng et al. (2009) supported the developments in microalgae engineered strains through biochemical and genetic engineering for biodiesel production would be a potential and promising source for future energy security. The manipulation and regulation of microalgae lipid biosynthesis would be helpful in extend the application to industry level.

Miao and Wu (2006) studied the lipid content of *Chlorella protothecoides* that was grown by heterotrophic cultivation and reported oil reserves as high as 55%, which is four times higher than autotrophically grown cells. So it is believed that heterotrophic cultivation could result in higher production of biomass and accumulation of high lipid in cells.

At first, heterotrophic condition was ought to favour microalgae lipid production, Jin et al. (2012) proposed that due to autophagy like mechanism played at this condition is known to be an important reason for lipid production. Previously Ahmad et al. (1990) pointed that the chloroplast reduction do not reduce the accD gene copy number, under heterotrophic condition, instead chloroplast are changed to another kind of plastids to perform necessary action during stress condition.
2.8. Large scale cultivation of microalgae

For the first time successful cultivation of microalgae without microbial contamination was achieved by Mahadevaswamy et al. (1981) and after that so many researchers tried to attain axenic microalgae cultivation. Kaur et al. (2010) has carried out preliminary work on algal diversity and selection of suitable strains for renewable biodiesel feedstock production. The selected strains, namely Ankistrodesmus sp., Scenedesmus sp., Euglena sp., Chlorella sp., Chlorococcum sp. and Navicula sp. were known to accumulate high intracellular lipid content as their storage product. These strains could be successfully exploited for algal oil and its biochemical conversion into biodiesel.

Open raceway pond is of low cost and easy to build; usually 10 to 20 cm deep is recommended and the deeper ponds would lead to shading effect to algae culture system (Huntley and Redalje, 2007). The other drawbacks are due to the shallow depth and large area is needed to produce large volume of biomass and highly susceptible for evaporation and simultaneous contamination (Pulz, 2001).

Especially enclosed photobioreactors are effective in overcoming the contamination and evaporation problem faced in open type cultivation. Also the biomass productivity could average 13 times more than that of a traditional raceway pond. The recovery of biomass from the system is considered as another expensive step, using photobioreactor the cost for algal recovery could be reduced up to 30 times (Chisti, 2007). Rengasamy. (2008) have successfully cultivated Botryococcus braunii in open raceway pond without any contamination and carried out subsequent biogas production. He denoted that the development of sophisticated
culture and screening techniques, microalgae biotechnology and genetic improvement could meet the challenging demands of the food, pharmaceutical and fuel resources.

2.9. Environmental impact of industrial efﬂuents

Efﬂuent derived from municipal, agricultural and industrial activities could be a source of nutrients for microalgae cultivation that signiﬁcantly reduced the operational costs of algal production systems. Efﬂuent constituent was varied depending upon the source of production. Mostly efﬂuent contains three types of contaminants viz., pathogens (such as bacteria, viruses and parasitic worms) and organic particles (such as Urea, proteins, sugars, humus, human and animal waste and lipids) and inorganic constituents (like ammonia, nitrates, phosphates, sulphates and chlorides). These excess nutrients cause water pollution if not treated properly. At first in1970s the concept of algal cultivation in efﬂuent for tertiary treatment process was initiated (Lardon et al., 2009).

For the past 30 years, limnologists were investigating about the physico-chemical parameters of water bodies due to efﬂuent contamination. Recently many research works are undergoing in lab scale regarding heavy metal scavenging by biological process (Shaik et al., 2009). According to the United States Environmental Action Group (USEAG), heavy metal pollution had threatened the health of more than 10 million people worldwide. Around 53 elements were classiﬁed as heavy metals and if their densities exceed 5 g cm$^{-3}$, they were known as universal pollutants (Sharma, 2011).

Conventional physical and chemical technologies such as membrane ﬁltration, coagulation, precipitation, ﬂotation, adsorption, ion exchange, chemical reduction, ultrasonic mineralization, electrolysis and advanced chemical
oxidation methods are used in effluent treatment. Some of these methods are termed to be effective and also they possess underlying drawbacks such as high cost, intensive energy requirements, and generation of hazardous by products and sludge which cause a secondary pollution. At present most of the industries are following conventional method because of their less time consumption (Robinson et al., 2001).

2.10. Industrial effluents as lipid production medium

Most of the industrial sectors faced an economic crisis because of effluent management through conventional physical and chemical processes. Instead our natural system is having its own candidates such as bacteria, fungi, and algae to remove these anthropogenic factors by metal binding capacity. Algae are capable to uptake abundant nutrients from domestic wastewater efficiently than any other biological process. This gives more economic value for microalgae and up gradation to secondary level treatment to tertiary level (Oswald et al., 1992).

Due to their large surface volume, high affinity, metal binding groups on cell surface and efficient metal uptake and storage, they could easily absorb and metabolize heavy metals. They could accommodate trace metal up to 10% in their total biomass (Rajamani et al., 2007). In addition to trace metals that are essential for metabolic activity, they could also sequester heavy metals. Metal elements could not chemically degrade; undesirably they are accumulated in food chain. Hence, it is necessary to remove them from contaminated site and also from effluent before discharging into water bodies.

Often there is competition between trace and heavy metal in effluent for microalgae cellular binding and uptake. Therefore the aquatic organisms like
zooplankton, oyster and fish which are consuming those microalgae with heavy metal, biologically transmit heavy metal in food chain. Human consumption of these aquatic organisms could result in gradual cellular, organ damage and cancer. Due to anthropogenic activities, previously unavailable heavy metals are now biologically available either in the form of food or water.

Einicker-Lamas et al. (2002) reported that heavy metals like cadmium, iron, copper and zinc were found to increase the lipid content in some microalgae. In *Euglena gracilis* high cadmium level increased the lipid content in all three culture system including autotrophic, heterotrophic and mixotrophic condition. Membrane lipid was increased in the form of phosphoglyceride and sterol content was lower. *E. gracilis* was highly sensitive to copper and zinc. Similarly the effect of iron on *Chlorella vulgaris* growth and lipid accumulation was investigated by Liu et al. (2009). They reported that the culture in late exponential growth phase with Fe$^{3+}$ at different concentrations, showed an increased total lipid content up to 56.6% in total biomass.

2.11. Fatty acid biosynthesis of microalgae

Several microalgae species could be induced to accumulate substantial quantities of lipid, often greater than 60% of their dry biomass (Richmond, 2004). In stationary phase, neutral lipids are dominating than other lipid types especially in the form of triglycerides (Miao et al., 2004).

De novo fatty acid synthesis, a fundamental pathway required for the biogenesis of membrane and storage lipids. Using Acetyl-coenzyme A (Acetyl-CoA) as the initial primer, this synthesis occur through the sequential condensation of two carbon units, which are derived from malonyl-CoA. The generation of malonyl-CoA from acetyl-CoA is
the first committed step in fatty acid biosynthesis catalysed by \textit{Acetyl CoA Carboxylase} (\textit{ACCase}). Citrate is an allosteric activator of \textit{ACCase} (Fig. 1).

![Diagram of fatty acid biosynthesis](image)

**Fig. 1: Triacylglycerol (TAG) biosynthetic pathway** (Courchesne \textit{et al.}, 2009)

Since \textit{ACCase} was the key regulator of fatty acid biosynthesis, the genetics of this enzyme should be thoroughly studied (Lu \textit{et al.}, 2008). Later on
Wan et al. (2011) reported that lipid content increased in stationary phase of *Chlorella sorokiniana* in response to increased accD gene expression level. Overall lipid production was not increased because maintainence of rapid growth and lipid accumulation at the same time is difficult.

ACCase is classified into two types namely homomeric and heteromeric ACCase. Only heteromeric ACCase catalysed the *de novo* fatty acid biosynthesis. ACCase is composed of four subunits which are as follows; one sub unit (accD) was encoded by the plastid genome and the other three sub units (accA, accB and accC) were encoded by the nuclear genome (Sasaki et al., 2004). Chloroplast follows a light mediated pathway through redox cascade which in turn regulate enzymes that are involved in metabolic pathways.

The plastidic ACCase (heteromeric ACCase) activity is modulated by light, this light dependent cascade of fatty acid biosynthesis is partly activated by ACCase (Lombard and Moreira, 2011). Plastidic ACCase is a multi enzyme complex composed of biotin carboxyl carrier protein (BCCP), biotin carboxylase, and a carboxyltransferase complex made up of α and β subunits.

### 2.12. Microalgae fattyacid: a suitable biodiesel feedstock

Microalgae synthesized fatty acids have chain length ranging from C16 to C18, quite similar to higher plants such as palmitic, stearic, oleic and linolenic acids. These are renowned form of biodiesel feedstock. Fatty acids play an important role in cell membrane, in energy storage and involved in metabolic synthesis (Orhan et al., 2003).
Meng et al. (2009) suggested that a high content of C18:0 and C18:1 in microorganisms is a prerequisite for biodiesel production due to similar properties of high value oil, oxidative stability and larger potential adaptability in industrial production. C16:0 has also been proposed as a suitable fatty acid in renewable diesel production. It is also a common fatty acid in microalgae as well as in oil from higher plants. Both the oxidative stability and combustion of diesel fuel at different temperatures are important features in deciding biodiesel quality.

2.13. Lipid profiling

Stehfest et al. (2005) reported that compared to chromatographic method Nile red based methods are efficient and quick for neutral lipid estimations. There might be changes in Nile red efficiency depending on algal species. Recently FT-IR based methods are involved in identifying the cellular components including lipids in response to nutrient limitations and stress conditions.

Fourier transform infrared (FT-IR) spectroscopy analysis for specific molecular groups is an alternative approach. The compounds are identified by their absorption bands in relation to a range of molecular vibrational modes (Murdock and Wetzel, 2009). Macromolecules like proteins, lipids, carbohydrates and nucleic acids could be quantified approximately with this technique.

At first Cooksey et al. (1987) proposed that Nile Red (NR) staining and fluorescence method is a promising high throughput sensing technique for lipid studies. Nile red staining is the best method to determine the neutral lipid content of many algal strains (Lee et al., 1998). NR diffuses through algal cell walls and binds to the lipid droplets (Elsey et al., 2007).
The FT-IR spectroscopy is known to be reliable, quick and easily accessible tool for identifying and characterising microalgae strains (Dean et al., 2010). FT-IR spectroscopy method is a rapid method requires minimum volume of sample for analysis. Recently FT-IR method becomes an indispensable tool for quantitative analysis in industrial sector (Liang et al., 2013). The overall biochemical analysis of the extracted lipid is better done by FT-IR spectroscopy analysis. It gives specific molecular information about the macromolecules present in biomass such as protein, carbohydrate and lipids that were identified based on their absorption bands.

Considering the merits and demerits from the literature review, the present study was designed. In this study Acetyl CoA Carboxylase enzyme was the prime target in combined biochemical and genetic engineering approach for enhanced lipid production. After primary screening of natural microalgae isolates, lipid production medium and biochemical optimization of selected isolates was performed for ACCase activity. Followed by accD gene cloning approaches were performed for biochemically optimized microalgae strain. This would optimize a natural isolate with enhanced ACCase activity and lipid production for the purpose of better biodiesel feed stock.