The main purpose of the study was to enhance lipid production through formulation of lipid production medium and optimization of natural microalgae isolates by biochemical and genetic engineering approaches for increased Acetyl CoA Carboxylase (ACCase) activity. Over exploitation of fossil fuel for automobiles and industries leads to petroleum demand (Barbara, 2007). Therefore in future energy crisis would be the main problem.

The first generation biodiesel feed stock raised a food crisis (Mitchell, 2008) and was not able to compete with petroleum products. The second generation biodiesel feed stock based on non-food resource were require a pretreatment (Sanderson, 2011) which in turn increased the cost of fuel production. Microalgae that belong to third generation biodiesel feed stock is aimed to replace petro diesel (Hu et al., 2008) but still wide researches is required to make them a suitable candidate for biodiesel production.

Various species of microalgae differ in their growth requirement. Therefore, the sampling location and spot is an important factor for the selection of microalgae strains (De Morais and coasta., 2007). For the present study fresh and marine water samples were collected from each site with visible algal population located in Chennai, Cuddalore, Trichy, Salem, Rameshwaram of Tamil Nadu and from Pudhucherry union territory.

Phytoplankton population in water system is related to many environmental factors such as temperature, pH, light, soluble gases and the level of dissolved
inorganic nutrients. These characters are determining the conditions required for the growth of microalgae isolates (Chynoweth et al., 2001). Hence, it is necessary to know abiotic properties of water samples at the time of collection. The pH of fresh water filtrates was between 7.5 and 8.3 and of marine water was between 9.8 and 11.5. This helped in standardising microalgae growth in laboratory conditions.

Among microalgae, green algae (Chlorophyta) comprise more lipid storing microalgae (Bruton et al., 2009). Hence the filtrates were categorized based on abundance green algae members into four types viz., A (very good sample), B (good sample), C (average sample) and D (Unsuitable sample). This was necessary to reduce the time duration for the unimicroalgal isolation by water filtrate categorising. Among water filtrates, AY filtrate showed the presence of Neochloris sp. and an unknown microalgal strain and high number of Scenedesmus obliquus was observed in MS filtrate. The overall possible strains in the water filtrates observed includes., Scenedesmus obliquus, Chlorella sp., Scenedesmus quadricauda, Dunaliella tertiolecta, Chlorella minutissima, Chlorella marina and Dunaliella salina. Compared to marine filtrates CS.1, CU.S, PU and RS, the fresh water filtrates MS and AY were observed with more viable green algae members. Still equal chances were given to both fresh and marine filtrates and isolation procedure was performed.

General methods employed for microalgae isolation are serial dilution and plating method, random sampling and inoculation method, centrifugation and plating technique. Among the above said methods centrifugation and plating method is found to be effective for unialgal isolation (Parvin et al., 2007). Therefore in the present study isolation procedure was performed by centrifugation and plating.
technique. Among the seven microalgae isolates, two were of marine origin, namely CS.1 and CU.S and five were fresh water isolates namely MS1, MS2, MS3, AY1 and AY2. Most of the natural isolates resembled *Chlorella* sp. and *Scenedesmus* sp. based on the traditional microscopic identification of isolates. For the reference purpose, two strains namely *Chlorella vulgaris* designated as Ch.Ac Ref I and *Scenedesmus obliquus* designated as Sc.Ac Ref II strains were obtained from Centre for Advanced Science (CAS) in Botany at University of Madras and included in the present study.

Axenization is a process that is necessary to obtain a pure viable single species devoid of other contaminants. Only axenic microalgae cultures have scope in industrial sectors for the production of highly valuable products. Usual methods for axenization include with density gradient centrifugation, UV irradiation, filtration and antibiotic treatment (Kim *et al.* 1999). In the present study, combination of antibiotics were used for axenization of natural isolates which includes Meropenum (2 µg/mL) and Nystatin (5 µg/mL). This combined antibiotic treatment was quite effective for the axenization of all the natural isolates. The axenic strains were confirmed with inoculation in BBM agar and incubated at 37°C for three days which would be helpful in selecting completely axenated unialgal strains.

Accumulation of lipids in the microalgae cells mainly depended on diverse factors. These include growth temperature, pH, nutritional imbalances (carbon, nitrogen, phosphorous, and silicate), the growth regime (autotrophic, mixotrophic, or heterotrophic), the age of the culture, and specific microalgal strain (Ratledge and Cohen, 2008). The above mentioned factors are the deciding factors of the biochemical composition of microalgae. For the selection of specific algae
medium, cultivation of fresh water microalgal isolates were carried out with three synthetic medium that are different in its nutrient components *viz.*, BBM a suitable green algae medium, Bristol medium a simplified or older form of BBM and TAP medium a quite complex medium. Marine isolates were cultivated in BBM-NaCl, Bristol-NaCl and TAP-NaCl (each medium was prepared with 2% NaCl incorporated in the corresponding medium). Depending upon their nutrient requirement, microalgae isolates would grow well in particular medium. Further high biomass and lipid producing isolates and their specific medium could be selected.

After screening in synthetic media the marine isolates CS.1 showed minimum protein and carbohydrate content (0.3212 mg/L and 0.117 mg/L respectively) and low lipid content in BBM-NaCl medium and CU.S showed only high protein content (1.932 mg/L) in TAP- NaCl medium. Both the isolates produced less than 1 mg of total biomass in dry weight and their lipid content was less than 0.01mg/L. This result implied that they were unable to adapt in laboratory conditions. Hence, their lipid content was low. Based on these reasons they were excluded for further screening procedures.

Both the reference strains were producing low lipid (<0.003 mg/L) in BBM. This infers that instead of lipid content they store energy in the form of cellular protein. Ch.Ac Ref I and Sc.Ac Ref II had a protein content of 33.8 and 23.3 mg/L respectively. Among the fresh water isolates only AY2 produced a maximum of 97 mg/L biomass (in dry weight) and lipid of 0.026 mg/L in BBM. MS3 isolate produced a maximum of 112mg/L biomass (in dry weight) and lipid of 0.3mg/L in Bristol medium. Therefore after screening in synthetic medium, AY2 and MS3 and
their specific synthetic medium BBM and Bristol respectively were selected for medium optimization studies to attain enhanced lipid production.

Instead of lipid induction TAP medium supported beta carotene production in MS3 (0.11mg/L) and in Sc.Ac Ref II (0.230mg/L). Though the chlorophyll a content reached the maximum, the biomass was not high and lipid production was negligible in TAP medium. Therefore TAP medium was not found suitable for lipid optimization studies.

Before scaling up from lab scale to large scale, the efficiency of microalgal strain to grow in large scale should be confirmed (Brennan and Owende., 2010). With this concept, large scale modes namely static, submerged and aerated type cultivation were applied for microalgae cultivation. Static method of cultivation lack mixing process and was time consuming. Submerged cultivation type was found to be a suitable method to increase the culture volume only up to 700 mL (Chisti et al., 2007). So it was not suitable for large scale cultivation due to limitation in medium supplementation. Only aerated type provided with aerator and stirrer was found effective for cultivation of both Neochloris sp. and Asterarcys sp. in large scale.

In this study identification of selected microalgal isolates was carried out using molecular techniques. Microalgae identification based on single gene was not sufficient to characterize an isolate into particular species (Coleman., 2006). Hence, the present study was targeted to amplify microalgae ribosomal cluster region including SSU partial, ITS 1, 5.8s, ITS2 and LSU partial sequence for molecular characterization of an algae. The obtained ribosomal cluster sequences were analysed through BLAST. This sequence of MS3 strain was observed with
99% similarity with the existing ribosomal region of *Asterarcys quadricellulare*. Based on phylogenetic tree it was confirmed that the microalgae (MS3) isolated in the present study was *Asterarcys* sp (NCBI accession no KM893430). For AY2 ribosomal cluster sequence BLAST analysis revealed 99 % similarity with the existing ribosomal region of *Neochloris Vigensis*. Based on the phylogenetic tree it was confirmed that the microalgae (AY2) isolated in the present study was *Neochloris* sp (NCBI accession no KM893429).

One of the major limitations in algae biodiesel production is the abundant nutrient and water requirement. Therefore in this study, Efforts were taken to overcome the above mentioned issues and effluent system was applied for microalgae cultivation and simultaneous lipid induction. Since industrial effluents consist of abundant nutrient in the form of nitrate, phosphate, chloride, ammonia, sulphate and heavy metals, they could be utilized by algae for their growth. As per Li et al., (2008) statement, the heavy metals such as cadmium, iron, copper and zinc increase the lipid content in many microalgae. For instance cadmium treated cells of *Euglena gracilis* under illumination showed increased lipid production (Einicker-Lamas et al., 2002). The exact mechanism is still unclear (Chisti et al., 2007).

The overall ability of the fresh water isolates and reference strains adaptation in industrial effluent was tested. The four industrial effluents at various stages were collected from sago (SIE), sugar distillery (SUI, SU II), textile dyeing (TDE I, TDE II) and silk processing industries (CW, RW). Only isolates that are highly tolerant to heavy metals could grow in these effluent medium.
Based on evans blue staining observation, only Asterarcys sp. and Neochloris sp. were able to adapt in both effluents of textile dyeing industry (TDE I and TDE II) and silk processing industry (CW and RW). Hence, these four effluents and microalgal isolates were selected for further studies. In the present work, effluent based lipid production was applied with autotrophic condition to support photosynthetic activity of microalgae under stress condition.

It was observed that Asterarcys sp. growth rate in TDE I (0.143) was less than growth rate in TDE II (0.586). In CRWS the growth rate of Asterarcys sp. in CW (0.245) was less than the growth rate in RW (0.496). Similarly Neochloris sp. growth rate in TDE I (0.117) was less than growth rate in TDE II (0.632). In CRWS the growth rate of Neochloris sp. in CW (0.174) was less than the growth rate in RW (0.560). Compared to TDE II and RW, the other two effluents TDE I and CW were highly concentrated. Difference in growth rate among effluents denoted that isolates were under stress condition due to toxicity.

Previously many research works were conducted on reducing the toxicity of the effluent for biodiesel production. Bhatnagar et al. (2010) investigated on untreated carpet industry effluent as a growth medium for Chlamydomonas, Chlorella and Scendesmus sp. They also conducted research on diluted (10-15%) municipal sewage water which contained adequate quantities of nutrients rather than nutrient load in raw effluent to support algal growth.

In the present study instead of diluting with additional water different approach was made. This is the first study to combine effluents from various stages of same industry for better algal growth. It would reduce the excess water requirement for algal cultivation. Also the nutrient load in the diluted form in
combined effluent would support the algal growth. Hence, TDE I and TDE II from textile dyeing industry were combined to produce TDES medium, CW and RW from silk processing industry were combined to produce CRWS medium.

From Table 17 it is observed that pH, Total Dissolved Solids, Total suspended Solids, Total Hardness, Biological Oxygen Demand and Chemical Oxygen Demand were considerably reduced in TDES and CRWS combined effluent medium after the growth of Asterarcys sp. and Neochloris sp. In TDES medium, both Asterarcys sp. and Neochloris sp. produced lipid of 0.07 mg/L and 0.057 mg/L respectively. These isolates reduced heavy metal concentrations in this medium and the reduction as follow, Asterarcys sp. and Neochloris sp. reduced 0.001 mg/L of lead and zinc whereas Neochloris sp. reduced 0.001 mg/L of chromium in TDES medium.

In CRWS medium, both Asterarcys sp. and Neochloris sp. produced lipid of 0.089 mg/L and 0.104 mg/L respectively. The heavy metal concentrations after cultivation in this medium were as follows, Asterarcys sp. reduced 0.002 mg/L of copper and 0.001 mg/L of nickel whereas Neochloris sp. reduced 0.002 mg/L of copper. The relevance of heavy metal reduction and lipid enhancement was unknown but it is clear that this combined effluent medium can be used for the formulation of lipid production medium.

Still the concentrations of all the dissolved nutrients and heavy metals were high in combined effluent medium. In further steps, attempts were made to reduce the effluent medium toxicity by diluting with synthetic media previously tested for the isolates (In this study). Previously Lim et al. (2010) diluted the textile wastewater with BBM and used for microalgae cultivation. They pointed out that among ten microalgae screened; Chlorella vulgaris was identified as the best strain.

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The alga was adapted 100% in textile water but still more diluted proportions gave higher biomass.

Also the amount of lipid produced after cultivation in combined effluent by Asterarcys sp. and Neochloris sp. were not sufficient. Therefore formulated medium was required for high lipid production. Four different medium were formulated as SET I, II, III and IV in three proportions (25:75, 50:50 and 75:25). SET I and II (prepared with Bristol) were used for the cultivation of Asterarcys sp. and SET III and IV (prepared with BBM) were used for Neochloris sp.

Lipid profiling is the most useful indicator of the feed stock capacity in producing cost effective liquid biodiesel (Brennan and Owende, 2010). Total biomass productivity and lipid accumulation should be necessarily correlated to determine the actual neutral lipid content in total biomass. From Table 18, it is observed that the amount of lipid produced by Asterarcys sp. cultivation in SET I (75:25) medium was 8.33 % higher than Bristol medium. The same isolate produced 6.45 % high lipid in SET II (50:50) medium than Bristol medium. The amount of lipid produced by Neochloris sp. cultivation in SET III (25:75) medium was 12.12 % higher than BBM. The same isolate produced 14.07 % high lipid in SET IV (50:50) medium than BBM. Based on total lipid percentage the above mentioned proportions in formulated medium sets were selected.

Further it is necessary to know the neutral lipid content in microalgae isolates. Nile Red based fluorescent imaging of in vivo lipid (Chen et al., 2011) was an effective method in visualization and estimation of neutral lipid accumulation within cells. This Nile red imaging and quantification methods were applied in the present study. According to Nile red fluorescence imaging, SET II (50:50) medium
was suitable for Asterarcys sp. and SET IV (50:50) medium was suitable for Neochloris sp.

In addition to lipid percentage and neutral lipid imaging, lipid characterisation also important to select better proportion in each formulated medium set. Hence, final selection of suitable formulated medium was performed after FT-IR analysis of lipid samples from isolates cultivated in formulated medium. FT-IR was used to detect lipid concentrations from trace level to 250µg/ml (Dean et al., 2010). Infrared based analysis of biological samples would give accurate biochemical information about the samples. Always lipid particles are detected in the lower IR absorbance due to C-H₂ group in the 3000-2800 cm⁻¹ region and as well as by the presence of spectral peak at 2926 cm⁻¹ which denote the presence of lipid compound (Mourant et al., 2003). Final selection of formulated media was based on the peak at 2926 cm⁻¹ and near band ranges in FT-IR spectra. For Neochloris sp. SET III (25:75) medium was found suitable for lipid production and SET II (50:50) medium was found suitable for lipid production in Asterarcys sp.

The optimized formulated medium was selected for the optimization of ACCase. The light source selection is necessary for maintaining ACCase activity. In this study five light sources viz., white, red, blue, green and yellow were provided for the isolates cultivated in formulated medium. As an outcome of light source application to isolates, yellow fluorescent light for Neochloris sp. and white fluorescent light for Asterarcys sp. were selected for further ACCase optimization studies.

For biochemical optimization, citrate an allosteric activator of ACCase and biotin an analog of ACCase were tested individually for the isolates. Among the
different concentration of citrate; the ACCase activity was maximum for Neochloris sp. and Asterarcys sp. at a concentration of 0.05% and 0.01% respectively. It was confirmed by ACCase assay and FT-IR analysis. From the FT-IR spectra, it was observed that biotin had no effect on ACCase activity of both the isolates. Biochemical optimization revealed citrate induced the ACCase activity in Neochloris sp. and Asterarcys sp. Further this result was supported by SEM analysis, citrate supplemented microalgal culture are showing distinct morphology rather than biotin supplemented culture.

HPLC analysis can act as a useful alternative to GC and many available liquid chromatographic methods for quantification of FAMEs (Di Nicola et al., 2008). Quantification of FAMEs will be accurate by HPLC method. Hence, after optimization, the total lipid extracts were converted into fatty acid methyl esters (FAMEs) and analysed through HPLC. FAME mixture was quantified according to the HPLC chromatogram. The chromatogram of Neochloris sp. after optimization with 0.05% citrate produced 88.84% methyl stearate and 8.49% of methyl palmitate. Asterarcys sp. optimized with 0.01% citrate produced 73.33% methyl stearate and 10.07 % of methyl palmitate.

Microalgae with short chain fatty acids are preferred rather than a larger fatty acid spectrum, containing a molecular structure more than 18 carbons (Belarbi et al., 2000). For example Chlorella pyrenoidosa contained short, medium and long chain fatty acids in the range of C16 to C24. Lee et al. (2010) stated that the most commonly synthesized fatty acids from microalgae had chain lengths from C16 to C18, similar to those of higher plants, and the derivatives palmitic, stearic,
oleic and linolenic acid were recognized as the most common fatty acids derivatives in biodiesel.

Gas Chromatography (GC) analysis reveals about structural fragments and Mass Spectroscopy (MS) could provide molecular mass and baseline drift informations (Knothe et al., 2003). In the present study FAME compounds were identified using GC-MS analysis. The optimised *Asterarcys* sp. with 0.01% citrate consisted of fatty acids from C16 to C18 which includes C(16:0), C(17:2), C(18:1) and C(18:3). Major fatty acid fragment in this mixture was identified as 9, 12, 15 octadecatrienoic acid (alpha linolenic acid) through mass spectrum fragmentation pattern and respective molecular formula is C_{18}H_{30}O_{2}. This mixture could be a more appropriate biodiesel mixture and either it could be used as a proportion or major biodiesel source for engine systems.

The optimised *Neochloris* sp. with 0.05% citrate consisted of fatty acids ranged from C14 to C16 which includes C(14:2), C(15:1), C(16:0) and (C16:1). Major fatty acid fragment in this mixture was identified as palmitic acid through mass spectrum fragmentation pattern and respective molecular formula is C_{16}H_{32}O_{2}. Due to low cetane value this mixture cannot perform alone as biodiesel. Hence, it can be used as a biodiesel blend.

The biochemical optimization of ACCase enzyme was quite helpful in producing enhanced and desirable lipid feed stock and thus it could support biodiesel production. In order to avoid impurities and free fatty acid content, still attempts are made in this study for molecular characterisation of accD gene to produce recombinant strains for increased lipid production.
The increased accD gene expression in *Chlorella sorokiniana* might account for the increased lipid content in stationary phase (Wan *et al.*, 2011). Within *Chlorella* species the accD gene expression vary according to the environmental conditions and the circumstances induced metabolic pathway of microalgae (Chen *et al.*, 2011).

In this study, regarding *Asterarcys* sp. biochemical approach was successful and lipid accumulation increased up to 8%. The increased lipid (8%) of *Asterarcys* sp. may be due to autophagy like mechanism with response to heavy metal toxicity in effluents (Jin *et al.*, 2012). After so many attempts, the amplification of accD gene was not successful from *Asterarcys* sp. The cellular stress due to effluent treatment might result in pigment and chloroplast reduction but it doesn’t mean that there is a reduction in the accD gene copy number (Ahmad *et al.*, 1990). Sometime there are chances of accD gene deletion or replacement with cytochrome C (cyt C) gene. Hence, accD gene was categorized into miscellaneous gene family. Therefore considering the above reasons, further molecular work was carried out with only *Neochloris* sp.

Microalgae transformation efficiency is strongly species dependent, so that the transformation method should be carefully selected and optimized for each microalga. A wide variety of transformation methods had been used to transfer DNA into microalgal cells including glass bead or silicon carbide whisker used agitation method (Dunahay *et al.*,1995), electroporation (Chen *et al.*,2011), biolistic microparticle bombardment and *Agrobacterium tumefaciens* mediated gene transfer (Cheney *et al.*,2001).
Glass bead based transformation was selected for the present study due to their successful rate in microalgae transformation and also this is easy and cost effective process. The present study was performed with glass bead based transformation for pET-32a-accD vector transformation inside microalgae cells. Huan et al. (2000) stated that according to microalgae strain antibiotic sensitivity varies. For an example, Phaeodactylum tricornatum was sensitive to ampicillin. Therefore NPTII marker (neomycin phosphatase transferase II) or ampicillin resistant (Amp') gene were suggested as selective antibiotic marker. In the present study, Neochloris sp. was identified as ampicillin sensitive. Hence, Neochloris sp. clones were selected on BBM agar using Amp' gene as selective marker. Further relative quantification method was performed to know the expression level of accD gene transcript in the transformed cells.

The expression of accD gene was quantified using real time PCR. Two unknown sets of clones, one negative and positive control were tested. Based on Cp value expression level of clone 1 and clone 2 of Neochloris sp. gene was higher than accD calibrator which served as a positive control. In addition target accD gene transcript was measured with known GAPDH reference as T: R (Target: Reference), known as normalized ratio (Pfaffl, 2004). According to normalized ratio value, clone-1 Neochloris sp. did not show a positive accD expression pattern. Only clone-2 of Neochloris sp. was confirmed to show three fold over expression of accD gene construct. From the present work it is concluded that the optimized Neochloris sp. (clone-2) can serve as suitable candidate for better biodiesel feed stock and simultaneous utilisation of industrial effluent system. Also the formulated medium prepared in this study can be useful as lipid production medium for microalgae
strains in future. This *Neochloris* sp (clone 2) will provide benefit to the society by providing biodiesel feed stock, reducing pollutants in industrial effluents and ultimately reducing the biodiesel cost.