REVIEW OF LITERATURE

The four major issues regarding energy resources like increased energy prices, exhaustion of fossil fuel reserves and tremendous rise in atmospheric carbon dioxide levels have led to the search of biomass for energy generation and the strategies to produce them in bulk in a cost-effective manner (Ho et al., 2014). Biomass has a promising role as renewable resources in order to form various types of biofuels (John et al., 2011). Presently, bioethanol production primarily uses substrates generated from sucrose, starch crops and lignocellulosic wastes (Nigam and Singh, 2011). However, the use of feed and fodder for bioethanol scale-up production give rise to a number of issues, due to the exploitation of arable lands and water resources. Cost goes up when lignocellulosic substrates are converted into ethanol which cannot be compromised. Their lignin content leads to tedious saccharification method which affects the upstream and downstream processing (Sun and Cheng, 2002).

Microalgae is currently the most sought-after alternative substrate for third generation bioethanol production (Chisti, 2007). The microalgae with abundant starchy and cellulosic content prove to be exceptional substrates. The starchy microalgal biomass can be harnessed for bioethanol generation as they can be cultivated easily and they have the efficiency to fix greenhouse emissions. Starch and cellulose are much easily converted into fermentable sugar unlike the lignocellulose for microbial synthesis of bioethanol (Domozych, 2012). Hence, before fermentative production of bioethanol, the polysaccharide content of microalgae must be hydrolyzed to sugars which can be fermented.

2.1 Need of bioethanol

Liquid fuel demand is increasing with the growing population over the world. Biodiesel and bioethanol are the emerging non-conventional liquid fuels for transportation. Bioethanol is the trending biofuel all around the world which can be synthesized in large amount by utilizing the sugar obtained from starch or cellulose-containing biomass. Brazil is the leading one in this field (Chiaramonti, 2007). Atmospheric carbon dioxide gets regulated and the growth of biomass for bioethanol generation lowers other greenhouse gases as well. Bioethanol is very much eco-friendly and contributes less to pollution in the environment which is another concern for human health and well being. Talking about the famous Kyoto Protocol, the Canadian Government has made its mark in the reduction of greenhouse gas emissions by 6% (Champagne, 2007). One can easily locate the petrol pumps selling the ethanol blended with gasoline in the present scenario. The applications of such a non-toxic fuel range from power generation to thermo-chemical reactions for industrial use (Petrou and Pappis, 2009). Ethanol produced from biological sources could replace octane enhancers also (Champagne, 2007).

Feedstock crops possess a lot of banes if used as substrates for biofuel production because of the effect on food supply and security. Keeping these issues in view, the focus has shifted on microalgae, which is a sustainable alternative to give large-scale biofuel to the world. Scientists and researchers have discussed that without competing with the feedstocks, algae can produce the bulk amount of biofuels (Subhadra and Edwards, 2010). The expansion of microalgal biofuel has witnessed the significance of the choice of suitable substrate like algae with high starch content to produce ethanol. Alternative substrates mark the future of biofuels

as it is a reliable and promising bioprocessing strategy. Huge investments are being made by the popular aviation as well as petroleum companies.

2.2 Challenges in bioethanol production

Based on abundance and availability, the feedstock is selected. Bioethanol through conventional processing methodologies is included in as first-generation biofuels category while that obtained from lignocellulosic belongs to the second generation category (Chiaramonti, 2007). Bioprocessing of ethanol using fermentable sugar from popular sources like beet, cane, molasses, corn is well acquainted. It is quite convenient to directly ferment the sugar present in the feedstock and make bioethanol but the cost of preparation in such cases is high and indeed leads to food scarcity.

Large-scale cultivation of the energy crops is also harmful as it involves pollution of agricultural land with chemical fertilizers and biocontrol ecosystem service losses (Subhadra and Edwards, 2010). Lignocellulosic materials and starch-containing wastes like crop residues, sawdust, can be subsequently hydrolyzed and utilized for biofuel scale up.

2.3 Algae as a potential source for bioethanol

Third generation biofuels wherein algae are used as a major feedstock overcomes the drawbacks and the issues related to the first and second generation biofuels as discussed in the previous section. This particular point stimulates the interest in algae-derived biofuels. The history of algae as biofuel feedstock started in the 1950s (Chen et al., 2010) when it was in its infant stage with a very little awareness in the field of science and technology. As the issue of fuel crisis was outspread in the world, extensive research on algal biofuels and the use of algae for carbon dioxide sequestration started all around the world to mitigate

environmental issues and to combat fuel prices to balance the economy (Borowitzka, 2008). From 1978 to 1996, $25 million was spent on research related to the algal biofuels at the National Renewable Energy Lab (NREL). This investment gave way to the current scenario of algal biofuel (Waltz, 2009). Algae are photosynthetic species (Nigam and Singh, 2011) which can be autotrophic or heterotrophic and are used to take sunlight, carbon dioxide to assimilate and form carbohydrate which is further harnessed for fuel bioprocessing (Vunjak-Novakovic et al., 2005; Ratledge and Cohen, 2008; De Morais and Costa, 2007).

The ethanol production is done after hydrolyzing the accumulated carbohydrates in algae. Microalgae are the unicellular, photosynthetic microorganisms. Macroalgae are multicellular and are similar to higher plant varieties (Chen et al., 2010). Algae generate huge quantities of carbohydrate biomass and are used as inexpensive raw materials (Subhadra and Edwards, 2010; Packer, 2009). Algenol Biofuels Inc. is a pioneer company which has contributed to the development of a strategy for ethanol production using appropriate bioreactors in its biorefinery. Several by-products and residual feed are generated during the fuel generation process, which is useful.

Microalgae are unicellular eukaryotes capable of performing photosynthesis and assimilate environmental CO_2 in the form of carbohydrates and lipids, which are further utilized for the production of biofuels. The assimilated carbon can be stored either in the form of lipid or carbohydrate. Microalgae can also grow efficiently in wastewater and reduce the organic content of wastewater and therefore help in wastewater treatment. Microalgae have been chosen as the promising alternative substrate for the production of biofuels because of a number of reasons. First, they have very higher biomass productivity. Second, they do not

directly compete with the food crops as they do not arable land and fresh water for their growth. Third, they have high carbohydrate/ lipid content for the production of biofuels.

Microalgae can mitigate the problem of high degree of carbon dioxide in the atmosphere as they can exploit the green house gases released from power station(s) and various industrial sources in an efficient manner (Singh et al., 2011). These attributes of microalgae are now constantly being investigated and therefore, bulk cultivation of microalgae is done to generate Bioethanol feedstock for fermentation (Huntley and Redalje, 2007; Rosenberg et al., 2008; Subhadra and Edwards, 2010). Starch and glycogen in the algal species such as *Chlorella, and also Scenedesmus,* is more than 50% of the total dry cell weight (Ueda et al., 1996). The assimilation of cellulose by the microalgal biomass contributes in bioethanol production procedure (Chen et al., 2010). Table 2.1 sums up the microalgal substrates which are fermentable (Packer, 2009; Lam and Lee, 2015; Velazquez-Lucio et al., 2018).

Optimal nutrients can be easily provided to the microalgal species. Aquaculture suits microalgal growth. The lack of roots and stems favours the microalgal cultivation in aquaculture. The self-contained microalgal biomass is very productive and is mostly unicellular in nature. Almost no energy is spent by the microalgae for movement of starch in the tissues. If optimal conditions are the present, very rapid growth of the microalgae occurs and it has been observed that the doubling period of *Chlamydomona*s is very short i.e. 6 hours (Chen et al., 2010). Taking advantage of bulk production of microalgae and its bioconversion to fuel generation, the technology can work at an industrial level where carbon dioxide fixation and biofuel generation can be done (Sheehan, 2009).

2.4 Cultivation of microalgae

The process of cultivating microalgae has been in practice since the 1950s. The photobioreactor designs and configurations available in literature have been only used for investigational purposes, and have not been successful for large-scale cultivation operations (Olaizola, 2000). Knowledge of gas holdup, hydrodynamics, and transport properties inside a real microalgae culture, as well as the operating parameters of the photobioreactor, are essential for successful scale-up and optimization for mass cultivation of microalgae, and a lack of the same has made commercial-scale microalgae culturing a costly affair (Olaizola, 2000). Of the available photobioreactor configurations, bubble columns and airlift photobioreactors are very promising for culturing algae on a large scale (Ugwu et al., 2008). They are also compact and easy to construct and operate. Since gas injection is used for mixing as well as introduction of nutrient gases into the system, they provide low shear stress to algae along with proper mixing and mass transfer (Ugwu et al., 2008; Merchuk et al., 2000; Miron et al., 1999; Zittelli et al., 2003).

Airlift photobioreactors supply a controlled concentration of CO_2 (with air and nitrogen), typically by sparging the gas into the algal culturing media, where the bubbles help in distributing the gas and agitating the culture as they move. In bubble columns even though there are light and dark regions present inside the reactors (thus allowing for the flashing light effect), research has shown that properly ordered mixing strategies must be introduced to facilitate movement of cells between these zones (Greenwell et al., 2009).

Table 2.1: The carbohydrate content of some microalgae species (REF)

Microalgae species	Carbohydrate content (% dry weight)	References
Chlamydomonas rheinhardii	22.6	(Milano et al., 2016)
Chlorella pyrenoidosa	26	(Becker, 1994)
Chlorella vulgaris	20.99	(Wang et al., 2013)
Chloroccum sp.	32.5	(Harun et al., 2011)
Chlorella sorokiniana	35.67	(Cheng et al., 2014)
Dunaliella salina	32	(Becker, 1994)
Euglena gracilis	14-18	(Becker, 1994)
Mychonastes afer	28.4	(Guo et al., 2013)
Nannochloropsis oculata	8	(Biller and Ross, 2011)
Porphyridium cruentum	40	(Biller and Ross, 2011)
Porphyridium cruentum	40-57	(Milano et al., 2016)
Scenedesmus abundans	41	(Guo et al., 2013)
Scenedesmus dimorphus	21-52	(Becker, 1994)
Spirogyra sp.	33-64	(Becker, 1994)
Scenesdesmus obliquus	15-51.8	(Ho et al., 2012)
Synechoccus sp.	15	(Becker, 1994)
Tetraselmis maculate	15	(Becker, 1994)
Tetraselmis sp.	24	(Schwenzfeier et al., 2011)
Tetraselmis suecica	15-50	(Bondioli et al., 2012)

Thus the draft tube and split airlift reactor are a better choice for microalgae cultivation. Both of these airlift reactors provide efficient circulation leading to ordered mixing and movement of cells between the light and dark phases. When gas flows through the microalgae culture inside the reactor, the gas holdup and bubble dynamics determine the transfer of the gasses from the gas phase to the liquid phase, and also the transfer of oxygen (produced during photosynthesis) from the liquid to the gas phase.

Gas-liquid interaction is affected by the local gas holdup, bubble frequency, chord length and velocity, interfacial area, and mass transfer. These parameters are critical not only to ensure that the nutrients are supplied to the cells at an effective rate but also to avoid oxygen build-up in the medium. Some studies on estimating the gas holdup, bubble dynamics, and mass transfer in airlift reactors are available in the literature. There are also many correlations for calculating the flow dynamic properties of the system, but the empirical or semi-empirical nature of these studies limits their applicability.

As the culture grows and increases in density, the rheological properties of the system such as density, viscosity, and surface tension also change. These dynamic changes in the physical properties of the medium, in turn, alter the structure, size, and frequency of the gas bubbles, and hence the bubble dynamics of the system. Hence, it is essential to study the mass transfer inside the medium. Very few studies in the literature have addressed this essential issue.

Most of these studies do not deal with a biological system, and hence fail to take into account the rheological changes in the system. Also, to truly understand the overall mass transfer coefficient, it is important to separately analyze the local mass transfer coefficient and the local interfacial area. Thus, a study of the overall parameters is insufficient, making it

essential to investigate the changes in the local gas hold-up, bubble dynamics, and mass transfer coefficient as the physical properties such as optical density, viscosity, and surface tension of the system change (Contreras et al., 1998). Also, during the process of photosynthesis, microalgae generate oxygen, which is transferred from the liquid to the gas phase. As the culture photosynthesizes and grows in density, more and more oxygen is produced, which tends to accumulate inside the medium. Accumulation of oxygen hinders the process of photosynthesis and is detrimental to the growth of algae (Greenwell et al., 2009). This further emphasizes to fully understand the mass transfer process in real microalgae culturing systems. Another crucial step in the process of optimizing the growth and biomass productivity of microalgae cultures is the integration of the dynamic and kinetic growth studies.

An efficient photo-bioreactor design should have sufficient light penetration as well as its distribution inside the bioreactor. Mass transfer, efficient mixing and desired temperature and pH can contribute to the significant enhancement of microalgal growth. Also, for cost-effective development of photo-bioreactor requires the inclusion of capital and operational costs.

2.5 Enhancement of microalgal starch/ carbohydrate content

The microalgae can produce and accumulate either lipid or starch as their carbon and energy reserves depending upon its species. The starch or carbohydrate producing microalgae generally stores a small quantity of lipid under normal conditions, but under specific growth conditions, some of the microalgae can overproduce lipids. The starch produced in the microalgae can be used as a source of energy and carbon source for the process like nuclear division, DNA replication, and cytokinesis (Brányiková et al., 2011). The cellular

composition of microalgal starch is mainly governed by two factors. The first is the rate of synthesis of starch and second is its consumption into the process like cell division. Therefore for increasing the starch content, it is necessary to inhibit the processes which utilize the starch and the conditions favoring the starch production should be optimized (Brányiková et al., 2011).

2.5.1 Protein synthesis inhibition by cycloheximide

Cycloheximide is a protein synthesis inhibitor in eukaryotes which is synthesized by *Streptomyces griseus*. The antibiotic cycloheximide binds to the 60S subunit of the ribosome where it inhibits the translocation step of the protein synthesis. The presence of cycloheximide prevents the cells from undergoing nuclear division which leads to inhibition of cell division. Therefore, the cells stop its energy consuming processes and the carbon flows to the starch synthesis which leads to the accumulation of starch in the cells. Cycloheximide interferes with protein synthesis and redirects the carbon flux into an energy-rich compound like carbohydrate which inhibits the cell growth (Douskova et al., 2008). Brányiková *et al.* reported that *Chlorella* sp. synthesized starch efficiently up to 60% of its dry weight with Cycloheximide treatment (Brányiková et al., 2011; Zachleder and Vtov, 2011).

2.5.2 Inhibition of lipid synthesis pathways by cerulenin

Cerulenin is an antibiotic which prevents steroid and fatty acid biosynthesis process. Cerulenin interacts in equal ratio with β-keto-acyl-ACP synthase. This enzyme is a part of the seven subunits of fatty acid synthases (FAS). It interferes with the interaction of malonyl-CoA and FAS. Starch and lipid get accumulated in the microalgae as major energy reserves. However, the starch and lipid content of microalgae depends on the type of species

(LOEBLICH III, 1984). The fatty acid synthesis inhibition diverts the carbon flow towards the starch synthesis and leads to accumulation of carbohydrates inside the cells. Kwok *et al.* observed that inhibition of lipid synthesis by cerulenin upregulated the cellulose synthesis and increased the cellulose content of *Crypthecodinium cohnii* Biecheler strain 1649 microalgae (Kwok and Wong, 2005). Figure 2.1 shows the lipid and starch synthesis pathway in which inhibition of fatty acid synthase enzyme was shown.

2.5.3 Nutrient limitation strategy

The use of inhibitors for overproducing the starch in microalgae is useful in the basic research but it could not be used in industrial scale due to its economic and environmental constraints. Macro-elements (N, P and S) limitation conditions suppressed the cell cycle events by preventing the energy and carbon flow from the starch to cell processes in autotrophic microalgae growth. The increase in starch content was reported by many scientists when the microalgae were grown under nutrient limitation conditions (Klein, 1987; Ball et al., 1990). The exact mechanisms of nutrient-limited induced starch synthesis are still unknown and nonspecific (Ballin et al., 1988). However, its application in growing microalgae for large scale is both advantageous in economic and environmental point of view.

Nitrogen is required for the biosynthesis of nucleic acid, protein and photosynthetic pigments therefore affect the biomass yield (Berdalet et al., 1994). In many microalgae, under nitrogen limitation, the biosynthesis of carbohydrates or lipids is enhanced, instead of proteins (Markou et al., 2012). When microalgae are grown under nitrogen deprived condition, cell division is inhibited and the carbon flow switches towards the starch synthesis. In a study performed by Klein *et al.*, they reported that in *Chlamydomonas reinhardtii,* protein and

chlorophyll content was decreased while carbohydrate content was increased about six-fold when the nitrogen source was exhausted (Klein, 1987). Ballin *et al.* cultivated the microalgae *Scenedesmus quadricauda* and reported that the synthesis of macromolecules like RNA, DNA and proteins was arrested under nitrogen lacking condition. This inhibition leads to the diversion of the carbon flow towards the starch synthesis (Ballin et al., 1988). Behrens *et al.* studied the cultivation of *Chlorella vulgaris* under nitrogen sufficient and nitrogen limitation conditions. They found that microalgae accumulated the starch approximately 20% of its dry weight under nitrogen sufficient conditions, whereas the starch content increased up to 55% of its cellular weight under nitrogen limitation growth condition (Behrens et al., 1989).

Nitrogen limitation was found as an efficient technique to enhance the carbohydrate concentration in microalgae. It was observed that *C. vulgaris* stored carbohydrates in nitrogen deprived situations up to 51.3% post four days of continuous starvation. Maximum productivity of the biomass and carbohydrates were 1.437 as well as 0.631 g L^{-1} d^{-1}. Microalga was found to be an excellent substrate for bioethanol fermentation with high glucose (93%) content (Ho et al., 2013b).

The starch and lipid synthesis in microalgal cells i.e. in *Chlorella zofingiensis*, was analyzed under various nitrogen limiting conditions. Cell growth and chlorophyll depletion occurred under nitrogen starved condition which was contrary to the nitrogen-replenished cells. In the case of nitrogen starvation, starch synthesis preceded lipid storage because post second day of starvation, starch degraded to balance lipid biosynthesis. Lipid was synthesized for long-term energy accumulation while starch was a quick response to the stress conditions(Zhu et al., 2014a).

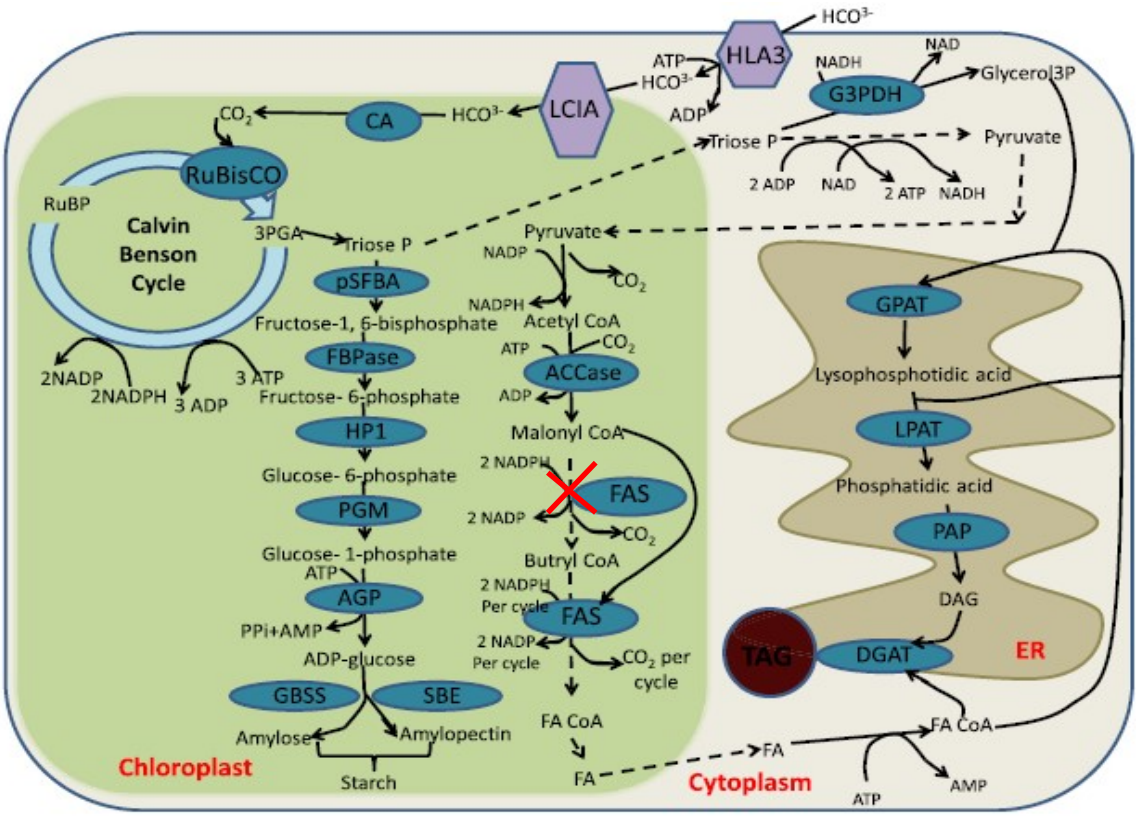

Figure 2.1: Blockage of lipid synthesis pathway by cerulenin in microalgae and accumulation of starch (Subramanian et al., 2013).

Microalgal growth in nitrogen-starved conditions was found to be 0.48 day^{-1} which accounted to be less than nitrogen replenished conditions (1.02 day^{-1}). Interestingly, nitrogen deprived conditions generated stress which actually led to the accumulation of starch. Carbohydrate content and starch accumulation improved 37 % and 4.7 folds, respectively. 66.7 % of starch increment with 66.9 % of dry weight (DW) was observed a (Zhu et al., 2014b). The two-stage processing strategy for starch enhancement was done using nitrogen depletion strategy. But, the specific growth rate was less than that seen for nitrogen-assisted growth. The starchy material of the freshwater strain, *C. vulgaris* strain P12, increased 8 fold, up to 41.0% of dry cell biomass (Dragone et al., 2011).

Zu *et al.* 2014 revealed that the cell growth in nitrogen deficient condition was quite less than that under nitrogen sufficient condition. Also, nitrogen limitation stimulated the storage of starch. After just 1 day of nitrogen deficiency, starch and carbohydrate concentration was enhanced by 4.7-fold and 37 %, respectively. The maximum carbohydrate concentration increased up to 66.9 % of dcw of which 66.7 % was starch (Zhu et al., 2014b). Dragone *et al.* proposed a two-stage cultivation strategy for carbohydrate enhancement in which the accumulation of starch was found under nitrogen-deficient conditions. However, the cell growth was less than that found under nitrogen-replenished growth conditions. The starch concentration of *C. vulgaris* increased which was found to be 8-fold greater than the control (Dragone et al., 2011).

A semi-batch cultivation method for microalgae was used to promote the carbon-dioxide fixation efficiency, cell growth and carbohydrate productivity. Under the optimal operation condition (semi-batch system of 50% replacement), the highest CO_2 fixation rate, carbohydrate productivity, and bioethanol yield reached 1546.7 ± 57.5 mg L^{-1} d^{-1}, 467.6 ± 29.2 mg L^{-1} d^{-1}, and 0.202 ± 0.007 g/g biomass, respectively (Ho et al., 2012). Cheng *et al*. evaluated a two-stage cultivation system for enhancing carbohydrate and starch storage in *Chlorella* sp. AE10. The maximum carbohydrate concentration was observed as 77.6% while the maximum starch content was found as 60.3%. The starch productivity was measured 0.311 g L^{-1} day^{-1} and the carbohydrate productivity was calculated as 0.421 g L^{-1} day^{-1} from 0^{th} day to 6^{th} day (Cheng et al., 2017).

The phosphorus is also one of the most important macro-elements for the cells and the main element of energy-carrying molecules and other macromolecules like DNA and RNA. Phosphorus is necessary for nucleic acid biosynthesis and various cellular metabolites; hence

phosphorus limitation ceases the various cellular metabolic processes (Wijffels and Barbosa, 2010). The phosphorus limitation mainly affects RNA synthesis and protein synthesis. The inhibition of RNA and protein synthesis did not affect the photosynthesis and therefore accumulation of starch was obtained (Ballin et al., 1988). Phosphorus limitation in *Chlorella vulgaris* blocked the cell division and reduced the biomass synthesis and completely stopped after 12 h in the absence of phosphorus in the medium (Brányiková et al., 2011). In a similar study, Brányiková *et al.* demonstrated that under sulphur limited condition *Chlorella vulgaris* accumulated and maintained a high starch content of 60% (w/w) (Brányiková et al., 2011).

Sulphur is also an essential macro-elements required for metabolisms of lipid, protein and molecules associated with electron transport chains (Yildiz et al., 1994). It also affects the synthesis of starch content in microalgae. The microalga, *Tetraselmis subcordiformis*, accumulates starch autotrophically or mixotrophically. The maximum starch content was obtained as 35 % (w/w) under the normal growth conditions. The starch content was increased in *T. subcordiformis* using the sulfur starvation condition which led to direct starch overproduction and starch content of 62.1 % (w/w/) was obtained (Yao et al., 2012).

2.6 Pretreatment of microalgae for carbohydrate extraction

Pretreatment of the microalgal biomass is a very important step for the production of ethanol from microalgae because it provides the accessibility of carbohydrate to microorganisms and resulted in improvement of the fermentation process (Harun et al., 2014; Demuez et al., 2015). Pretreatment causes the breakdown of the cell walls of microalgal cells and modifies the intracellular carbohydrate structures. The carbohydrates of microalgae are found in its cell wall in the form of cellulose and stored in the plastids as starch. The cell wall of microalgae is mainly composed of pectin, cellulose and sulphated polysaccharides (Scholz et

al., 2014). In the microalgae cells, starch is present in the form of semi-crystalline granulose particles mainly composed of amylose and amylopectin (Huang et al., 2017). The granulous form starch is more stable due to its crystalline structure and small water content. Hence, pretreatment is necessary to change their structure through different pretreatment methods. There are different types of pretreatment methods i.e. acidic, alkali, enzymatic and some other mechanical methods like, ultrasonication, microwave, bead beater, and homogeniser through which microalgal biomass can be broken down and used as a suitable substrate for ethanol fermentation.

2.6.1 Acidic pretreatment

The acidic pretreatment of algal biomass can be used with concentrated or diluted acids. The concentrated acids need low temperature and less time for the biomass pretreatment. However, the diluted acid requires longer time, high temperature and pressure value to achieve the favourable hydrolysis efficiency (Gírio et al., 2010). The use of acids at high temperature involves the formation of undesired products like furfural and hydroxyl methyl furfural which inhibit the microorganisms' growth and fermentation process (Ruiz et al., 2015). Acid-based methods are commonly used with sulphuric acid and hydrochloric acid with concentration (1-10%), temperature (60°C to 180°C) and time (10-120 min).

Harun *et al* 2011 investigated the influence of acid pretreatment on microbial biomass for bioethanol production. They optimized acid concentration, temperature, microalgae loading and pretreatment time and obtained the highest bioethanol concentration 7.20 g/L when the pretreatment step was performed with 15 g/L of microalgae at 140°C using 1% (v/v) of sulphuric acid for 30 min. It was found in the study that temperature is the most critical factor amongst the parameters investigated during acid pretreatment of microalgae for bioethanol

production (Harun and Danquah, 2011). In another study, Nguyen *et al*. reported the dilute acid hydrothermal pretreatment of *Chlamydomonas reinhardtii* UTEX 90 which was used as a feedstock for production of bioethanol. The 5% (w/v) of algal biomass was pretreated with varying concentration of sulphuric acid (1-5%) at 100 to 120°C for 15 to 120 min. After the pretreatment, the maximum glucose release was found as 58% (w/w) at optimum conditions of pretreatment (3% sulphuric acid at 110°C for 30 min). The hydrolyzed sugar present in the pretreated slurry was fermented by *Saccharomyces cerevisiae* S288C and an ethanol yield was obtained as 29.2% (w/w) from algal biomass (Nguyen et al., 2009). Miranda *et al.* 2012 studied the acid pretreatment optimization of *Scenedesmus obliquus* using sulphuric acid (0.05 N–10 N) for production of ethanol. They compared the physical and physicochemical methods for hydrolyzing the algal biomass and found that highest sugar extraction efficiency was obtained with H_2SO_4 at 120°C for 30 min (Miranda et al., 2012).

2.6.2 Alkali pretreatment

In the alkaline-based method, sodium hydroxide is mainly used as the alkaline agent. The cell wall of microalgae is ruptured by salvation and saponification reactions resulted in the formation of pores in the cell wall. This method allows the intracellular compounds to come out of the cells and decreases the size and crystallinity of starch and cellulose (Harun et al., 2011).

Harun *et al.* 2011 investigated the alkaline pretreatment of *Chlorococcum infusionum* biomass using NaOH for bioethanol production. The alkaline pretreatment forced the microalgae cells to release the entrapped polysaccharide and breakdown the polysaccharides into fermentable sugars. The effects of three parameters; the concentration of NaOH, temperature and the pretreatment time were examined on the glucose yield and bioethanol

production. The highest glucose yield was determined to be 350 mg/g with 0.75% (w/v) of NaOH at 120°C for 30 min and the maximum bioethanol yield obtained was 0.26 g ethanol/g algae (Harun et al., 2011). In another study, Sui *et al.* performed the polysaccharide extraction from *Chlorella sp.* to recover the total water-soluble cell wall polysaccharides and they found that significant amount of carbohydrates could be extracted with a mildly acidic solution of sodium chlorite. The yield was recovered as polysaccharide (19-22%), water soluble carbohydrate (13-19%) and 2% NaOH soluble carbohydrate (3-6%). They also reported that alkaline hydrogen peroxide pretreatment of cells provided 19% yield of polysaccharide (Sui et al., 2012).

2.6.3 Free hydroxyl mediated thermal pretreatment

The hydroxyl radical-aided thermal pretreatment uses hydroxyl radicals produced during the Fenton's reaction. The hydroxyl radicals react with hydroxyl groups of the carbohydrate present in the cell wall. Therefore, the crystalline structure of the cell wall and hydrogen-bonds were disrupted (Lodha and Chaudhari, 2007).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \cdot OH \text{ (Fenton's reaction)}$$

The hydroxyl radical aided thermal pretreatment does not affect the high-value substances extraction and carbohydrate recovery from microalgae. Therefore it is one of the suitable methods for algal biomass digestion. This method uses a very low concentration of hydroxyl radical and short reaction time for biomass digestion and provides good saccharification results (Gao et al., 2015b). For the lignocellulosic biomass, a high concentration of hydroxyl radical with longer pretreatment time is used for pretreatment (Gao et al., 2015b). The wood decaying microorganisms use the

hydroxyl radicals to digest the lignocellulosic materials and break down the biopolymer into their monomeric forms (Ke et al., 2011).

Gao *et al.* investigated the effects of different variables like hydroxyl radical concentration, pretreatment temperature and pretreatment time, on the glucose release of different algal biomass. They used different macroalgae (*Ulva prolifera*, *Macrocystis pyrifera*, *Gelidium amansii*, and *Porphyra umbilicalis*) and four microalgae (*Scenedesmus quadricanda, Chlorella sorokiniana*, *Chlamydomonas hedleyi* and *Haematococcus pluvialis*) for the pretreatment study. The hydroxyl radical-aided thermal pretreatment provided the 100% pretreatment efficiency in terms of carbohydrate concentration (Gao et al., 2015b). In another study, Gao *et al*. used the hydroxyl radicals and hot water pretreatment (IHRHW) method for recovery of sugars from the brown macroalgae *Macrocystis pyrif*era. The optimum conditions used for the pretreatment were 100°C, 30 min, 11.9 mM $FeSO_4$. They reported that IHRHW method provided the 88.1% digestibility of macroalgae under the optimum experimental condition (Gao et al., 2015a).

2.6.4 Ultrasonication pretreatment

The ultrasound process uses the sound waves and creates the cavitation phenomenon including the formation of bubbles in the liquid medium. The bubbles are collapsed due to condensation of gases creating the shock waves and very high temperature and pressure regions are generated. The elevated temperature and pressure create the microjets which allow the solvents to penetrate into the cell wall and causes to rupturing of the cells (Luo et al., 2014). Jeon *et al*. reported that an ultrasonication pretreatment of *Scenedesmus obliquus* biomass between 10-60 min facilitates the accessibility of bacteria to ferment the sugars present in the biomass. They found that the best pretreatment was obtained with a time period

of 15 min and this method could be an alternative for cell disruption where acidic and alkali pretreatment could be used (Jeon et al., 2013).

Ferreira *et al.* extracted the compounds like carbohydrates, lipids, proteins, and pigments from *Chlorella. vulgaris, Nannochloropsis oculata*, and *Scenedesmus obliquus* using the low-frequency ultrasound and different solvents as biorefinery concept (Ferreira et al., 2016). The effect of ultrasound pretreatment was demonstrated through the deformation of the crystalline structure of starch and enhancement in water uptake capacity of the starch granules. This pretreatment enhances the accessibility of enzymes and overall increases the hydrolysis efficiency (Zheng et al., 2013).

2.6.5 Enzymatic pretreatment

The enzymatic pretreatment has been used extensively in the starch processing industry for liquefaction and saccharification (Hasan et al., 2006). Enzymatic hydrolysis has shown promising results when compared with other pretreatment methods (Demuez et al., 2015). The advantages of the enzymatic hydrolysis include high specificity of enzymes, no harsh conditions of reactions, and ease to perform at industrial scale. The main drawback of this pretreatment is that enzymes are very expensive (Günerken et al., 2015). Different enzymes can be used for performing the enzymatic hydrolysis among which cellulases, amylases, and amyloglucosidases are employed to hydrolyze the microalgae cell wall polysaccharides. It is commonly observed that proteases are used in the hydrolysis of glycoproteins in microalgal cell walls (Günerken et al., 2015; Pirwitz et al., 2016), leading to better extraction of the valued metabolites. Liang *et al.* preferred a combination of alkaline and neutral proteases for cell disruption. Endo-β-(1, 4)-D-glucanase breaks the cellulose linkages while exo- β-(1, 4)-D-glucanase break down cellulose into oligosaccharides. β-glucosidase gives glucose and

maltose by degrading glycosidic bond. Zheng *et al.* enhanced the hydrolytic effect of the enzymes with better shelf life using a mixture of cellulases with different kinds of polymers. The enzymes can hydrolyse the intracellular carbohydrates depending on the permeability of the microalgal species (Zheng et al., 2016). Table 2.2 depicts the enzymes used for microalgae biomass hydrolysis and their saccharification rates (Velazquez-Lucio et al., 2018).

Saïdane-Bchir *et al.* studied the effects of five treatment methods grinding, thermal shock, enzymatic, acidic and basic treatments on microalgae to disrupt the cells. They found that enzymatic hydrolysis provided the maximum sugar release with a yield of 82.2% (w/w). The maximum ethanol production was obtained with basic treatment with a yield of 43% (Saïdane-Bchir et al., 2016). Cell wall disruption and hydrolysis are two essential aspects of biomass preparation due to the fact that the majority of carbohydrates are entrapped within the cell wall or intra-cellularly as the energy-storage molecules in the form of starch (Hernández et al., 2015).

2.7 Fermentation processes

The sugars derived from microalgae can be used as a substrate for bioethanol production. The commonly used fermentation processes are separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF).

2.7.1 Separate hydrolysis and fermentation (SHF)

In SHF process, both hydrolysis and fermentation processes are performed separately in different reactors/vessels (Xiros et al., 2013). Using the different reactors allow the reactions

to happen at their respective optimum conditions such as temperature, pH, agitation and time (Harun and Danquah, 2011).

Table 2.2: Enzymes used for hydrolyzing some algal species in different studies

Microalgae species	Enzymes	Conditions	Saccharification rate	References
Gracilaria verrucosa	cellulase and β-glucosidase	cellulase (20 FPU/g of dry biomass) β-glucosidase (60 U/g of dry biomass)	87%	(Kumar et al., 2013)
Chlorella vulgaris Chlamydomonas reinhardtii	Glucanase Protease	Glucanase (0.3 ml/g biomass); Protease (0.2 ml/g biomass); 50°C, pH 4.5 for 5 hours	86-96%	(Mahdy et al., 2016)
Dunaliella tertiolecta	Amylo-glucosidase	0.4ml enzyme/g biomass, 55°C, pH 5.5	80.9%	(Lee et al., 2013)
Chlorella sorokiana	Cellulases Amylases	Celluclast-1.5L (20Ul/g biomass) Novozyme188 (10uL/g biomass) 55°C, pH 4.5 for 72 hours	100%	(Hernández et al., 2015)
Chlorella vulgaris	Cellulase Pectinase Xylanase β-glucosidase Amylase Chitinase Lysozyme Sulfatase	Cellulase (0.122FPU/mg); Pectinase (240 IU/mg protein) Amylase (16 FAU/ml); β- glucosidase(10U/ml); Xylanase (100U/ml); Chitinase (0.2 U/ml); Lysozyme (4000U/ml); Sulfatase (50U/ml), 50°C, pH 4.8 for 72 hours	79%	(Kim et al., 2014)

Chlamydomonas reinhardtii	α-amylase Amylogluco sidase	120KNU/g (300AGU/ml); 50-65°C and pH 4.5-5.5for 10-60 minutes	56%	(Choi et al., 2010)

Thus more products could be formed during the reactions. However, this process has some limitation like in case of acidic and alkali hydrolysis, extra neutralizing agents are required. In the case of enzymatic hydrolysis, the accumulation of reducing sugars formed after the hydrolysis leads to inhibition of enzymes.

The SHF process was used for the production of ethanol by some recent studies. Ho et al. studied the dilute acid hydrolysis of *Scenedesmus obliquus* biomass for the production of ethanol. The glucose concentration as 16-16.5 g/L was found after the hydrolysis which was further used by *Zymomonas mobilis* for production of ethanol and 0.213 g of ethanol/ g of biomass was produced under the fermentation conditions at 30°C and pH of 6.0 (Ho et al., 2013c). Harun *et al.* carried out a similar study where they used an SHF process for the production of ethanol from *C. infusion*. However, they used alkali (concentration of 0.75% of NaOH at 120°C for 30 min) instead of acids for hydrolyzing the algal biomass. The highest ethanol yield was obtained as 26.1% (g of ethanol / g of microalgae) by *S. cerevisiae* at 30°C and 200 rpm for 72 h. Table 2.3 shows the bioethanol production in some studies from using different microalgae through SHF and SSF (Harun et al., 2014).

2.7.2 Simultaneous saccharification and fermentation (SSF)

The process of biofuels production on a large scale becomes tedious and economically challenging. To ease the process, SSF wherein simultaneously both hydrolysis and fermentation can be done within the reactor (Hahn-Hägerdal et al., 2007). Thus, substrate inhibition could be mitigated as direct conversion of bioethanol using yeast culture is done

(Xiros et al., 2013). The process has a low enzyme requirement and improved bioethanol productivity (Lin and Tanaka, 2006). The boons of this particular method range from single fermenter usage reduced production time with lesser contamination issues (Lin and Tanaka, 2006). On the contrary, the difficulty lies in achieving similar physical, chemical or physicochemical conditions for saccharification process and as well as fermentative production methods (Gupta et al., 2012). The commercialization of the process is challenged by the recycling process of both enzymes and yeast culture which hampers the scale-up process (Olofsson et al., 2008).

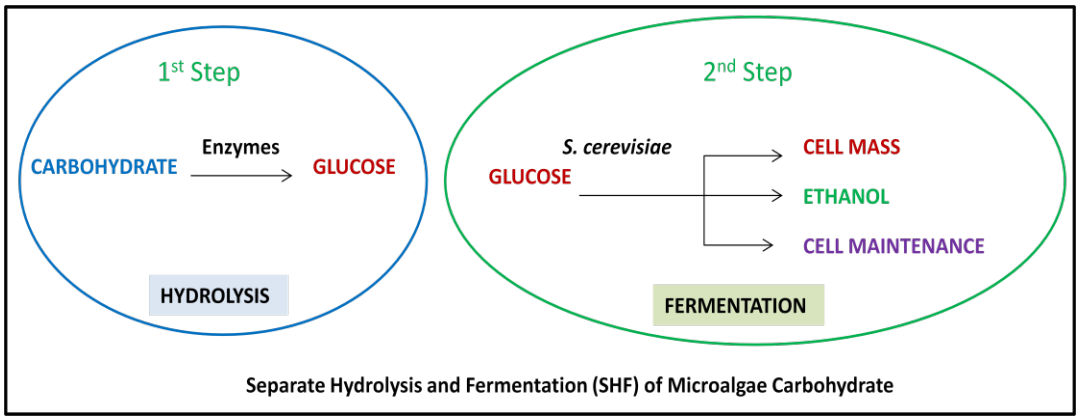

Figure 2.2:- Separate hydrolysis and fermentation of algae carbohydrate

Literature from previous research work on SSF technique have revealed the benefits of the process in case of *C. vulgaris* as feedstock. Endoglucanase with β-glucosidase and amylases helped in saccharification and the culture of *Zymomonas mobilis* was considered for the fermentation. The maximum bioethanol production was found to be 4.27 g/L using 20 g/L algae at 30°C, pH 6.0, in 60 hours (Ho et al., 2013a). The bioethanol production achieved through SSF was 12.2% more than that achieved in SHF methodology using enzymes. Research reports have stated this fact with evidence that improved bioethanol generation could be obtained through the SSF process (Harun et al., 2014). It is often observed that

contamination in the anaerobic system and faulty operating procedures lead to the failure of SSF process and thus the required amount of bioethanol is not formed by the saccharification process followed by the fermentative production (Ho et al., 2013a; Harun et al., 2014). SSF was selected as an efficient process to enhance the bioethanol yield through repeated-batches using immobilized yeast cells. Combined sonication and enzymatic hydrolysis of *Chlamydomonas mexicana* generated 10.5 and 8.48 g/L of ethanol in SSF and SHF, respectively (El-Dalatony et al., 2016).

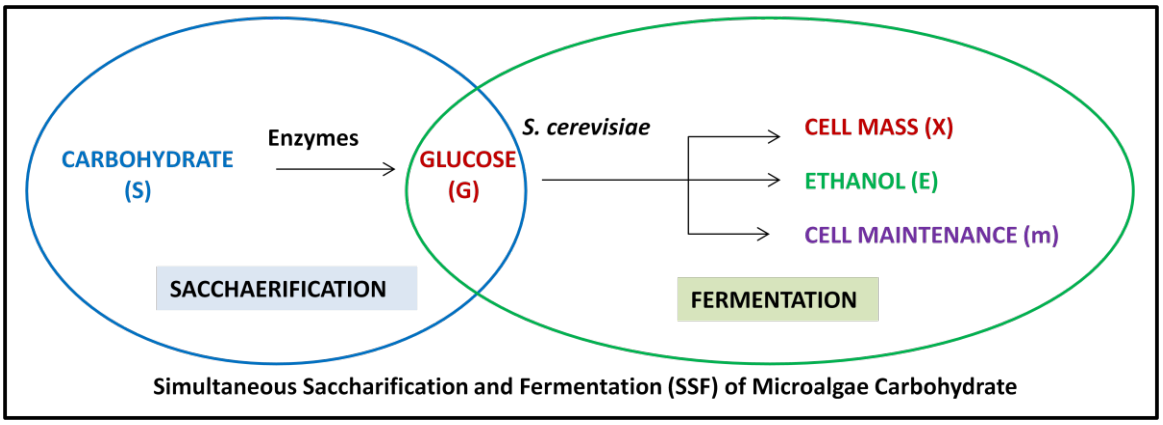

Figure 2.3 Simultaneous saccharification and fermentation of algae carbohydrate

The bioethanol industry has been energized by the cultivation of microalgae. Both biodiesel using lipid content and bioethanol using carbohydrate content in microalgae have high potentiality. Enzymatic as well as chemical hydrolysis is used to produce simple reducing sugars before going for the typical yeast fermentation method. Technical, as well as economic considerations, are to be taken care of while focusing on the feasibility of bioethanol production by utilizing microalgal biomass.

Table 2.3: Bioethanol production in some studies from using different microalgae through SHF and SSF

Fermentation Type	Algae Biomass	Pretreatment and Hydrolysis	Fermentation	Bioethanol Yield	Referen-ces
		Source (Treatment conditions)	Source (Process conditions)		
SHF	*Chlorella vulgaris*	Endoglucanase, 0.61 U mL^{-1}; β-glucosidase, 0.30 U mL^{-1}; amylase, 0.75 U mL^{-1} (200 rpm and 45°C)	*Zymomonas mobilis* (30°C)	0.178 g ethanol/g of algae	(Ho et al., 2013a)
SHF	*C. vulgaris*	1 % H_2SO_4 (121°C for 20 min)	*Zymomonas mobilis* (30°C)	0.233 g ethanol/g of dry algae	(Ho et al., 2013a)
SHF	*Scenedesmus abundans*	Dilute H_2SO_4 + Cellulase	*Saccharomyces cerevisiae*	0.103 g of ethanol/ g of algae	(Guo et al., 2013)
SHF	*Dunaliella tertiolecta*	HCl/ H_2SO_4 Cellulose +amyloglucosidase (pH 5.5 and 55 °C)	*Saccharomyces cerevisiae*	0.14 g ethanol/g residual biomass	(Lee et al., 2013)
SSF	*Saccharina japonica*	*Bacillus licheniformis* (30°C for 7.5 days)	*Pichia angophorae* (30°C for 13 h)	0.077g of ethanol / g of biomass	(Jang et al., 2012)
SSF	*Chlamydomonas fasciata*	Glutase-AN (Ultrasonicated for 30 min)	*Saccharomyces cerevisiae* (40°C for 30 h)	0.194 g-ethanol/g-dry microalgae	(Asada et al., 2012)
SSF	*Chlorella vulgaris*	Endoglucanase, 0.61 U mL^{-1}; β-glucosidase, 0.30 U mL^{-1}; amylase, 0.75 U mL^{-1}	*Zymomonas mobilis* (30°C)	0.214 g ethanol/g of microalgae	(Ho et al., 2013a)

SSF	*Chlamydomonas mexicana*	Cellulase (30°C)	*Saccahromyces cerevisiae* (30°C pH 5.0)	0.5 g of ethanol/ g of extracted sugar	(El-Dalatony et al., 2016)

2.8 Mathematical modelling of bioethanol production

The experimental behaviour of a bioprocess can be predicted and estimated through mathematical modelling (Jang et al., 2012). The interrelation of various functional variables involved in the process can be therefore computed. The biological processes involve different process parameters which are analyzed in this way and help in process design (Jang et al., 2012). Mathematical modelling of the SSF process has been stated in several kinds of literature. A cybernetic model (unstructured) for SSF process was developed in case of starch derived ethanol production using *S. cerevisiae* (recombinant) through Metropolis Monte Carlo methodology along with sensitivity study. The main emphasis was given on product inhibition based on fixed process parameters which were used to analyze process behaviour in varied conditions (Ochoa et al., 2007).

A kinetic model was developed for SSF through starch hydrolysis using amyloglucosidase produced by *Zymomonas mobilis* (Lee et al., 1992). The model parameters were predicted using an unstructured model with no initial process conditions (Jang and Chou, 2013). Similarly, the SSF based mathematical model wherein food waste was converted into ethanol was done. The predicted parameters were matched with experimental process parameters thorough Matlab Simulink. Some instances of SSF modelling stated the use of modified Michaelis-Menten and Monod-type equations as well which were beneficial in bioprocess design (Davis, 2008).

OBJECTIVES

Based on the review of literature following are the objectives of the present study:

2.9 Objectives of the research work

1. To isolate and identify native microalgae for having a substantial content of carbohydrates (mainly starch) for bioethanol production

2. To grow microalgae in different culture conditions (media, pH, temperature, inoculum, and agitation rate) and in different photobioreactors to achieve a high yield of algal biomass

3. To enhance the carbohydrate/starch content in microalgae by blocking the protein and lipid synthesis and also using nutrient limitation strategies

4. To select best pretreatment methods for extraction of carbohydrate from isolated microalgae

5. To produce bioethanol from algal starch through Separate hydrolysis and fermentation (SHF) and Simultaneous Saccharification and fermentation (SSF) processes