Chapter 14
Gaseous Fuels Production from Algal Biomass

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14.1 Introduction

The most abundant renewable energy available to us is solar energy. It provides 178,000 TW energy to the Earth per year (Rupprecht et al. 2006). Entrapment of such consistent source of energy has been performed by photosynthetic organisms. The interest for microalgae is growing worldwide for their ability to harness solar energy and consequently providing biomass that can be used as feedstock for renewable energy generation. The rate of CO_2 fixation was up to 6.24 kg m^{-3} day^{-1} (Cheng et al., 2006). The productivity of algae could be 10 times higher (50 ton dry weight (DW) per hectare per year) when compared with conventional agricultural crops (Murphy and Power, 2009; Wijffels, 2008).

Hydrogen is considered as a clean and renewable source of energy for the future. It may be used as a potential alternative energy source in place of fossil fuels. It has the long-term potential to reduce the dependence on hydrocarbon-based fuels. Molecular H_2 has the highest energy content per unit weight among the known gaseous fuels (143 GJ ton^{-1}) and is the only carbon-free fuel which ultimately oxidizes to water as a combustion product. Therefore, burning hydrogen not only has the potential to meet a wide variety of end user applications but also does not contribute to greenhouse emission, acid rain or ozone depletion. Although hydrogen is the most abundant element in the universe, it must be produced from other hydrogen-containing compounds such as biomass, organic wastewater or water (Kotay and Das, 2008).

In a process called biophotolysis, green algae uses light energy to generate the energy carrier i.e. H_2 from water (Skjånes et al., 2007). Since water is considered as widely available resource, in recent times, many studies are focused on the

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photobiological hydrogen production. The history of biological hydrogen production dates way back to three billion years ago when it developed photosynthesis process to convert CO_2, water and sunlight into hydrogen and oxygen. Although a comprehensive knowledge is available on biological H_2 production, a detailed emphasis is needed on basic and applied research for its application on commercial scale. A paramount requirement for understanding hydrogen production is in-depth knowledge of biochemistry and enzymology. Through understanding of these photobiological mechanisms, chemical mimicking of biophotolysis can be developed. This will, in addition, necessitate much more work on the isolation, characterization and stabilization of functional biological components.

For a stable and cost-effective hydrogen production, light conversion efficiency should be improved by at least 10 % so that it can compete with photoelectrical systems (Kruse et al., 2005; Prince and Kheshgi 2005). Metabolic engineering could prove a promising tool to increase conversion efficiencies and improve productivity. Fortunately, several advances in direct photolysis, indirect photolysis, and photo-fermentation have shown importance (Dickson et al., 2009). Although, hydrogen production from photolysis of water projects a promising future, the cumulative hydrogen production is quite low (Table 14.1). In case of *Chlamydomonas reinhardtii*, maximum cumulative hydrogen production of 3.1 ± 0.3 mL L^{-1} was observed which was quite low as compared to dark fermentative hydrogen production (Tamburic et al., 2011). Such low rate of production is the major bottleneck for large scale hydrogen production from photolysis of water.

For sustainable green energy production, biomass is considered as one of the most promising alternative. A model of decentralized power generation from hydrogen produced from biomass can be envisioned with the advent of cheap commercialization of fuel cells. There are many reports available on lab scale hydrogen production but commercial hydrogen production plants from biomass are not known in the world today. Algal biomass could prove to be a potential feedstock for biohydrogen production. This biomass is biodegradable, requires less harsher pretreat-

Table 14.1 Photobiological hydrogen production using algae

Microorganism	Photobioreactor	Rate of hydrogen production (mL L^{-1} h^{-1})
Chlamydomonas reinharditi strain 137 (mt^+)	Flat bottle	2
Chlamydomonas reinharditi	vials	2.1
Chlamydomonas reinharditi strain 137C+	vials	4.3
Chlorella sorokiniana Ce	-	1.35
Anabena cylindrica	-	0.74 µmol mg^{-1} Ch.l *a*
Synechocystis PCC6803	Flask	0.66 µmol mg^{-1} Chl *a*
Gloeobacter PCC 7421	Flask	1.35 µmol mg^{-1} Chl *a*
Nostoc muscorum	Flask	7.4 µmol mg^{-1} Chl *a*
Nostoc sp. PCC7422	Flask	100 µmol mg^{-1} Chl *a*

ment for saccharification and is produced in relatively less amount of time. Algal biomass mostly contains starch and cellulose and lacks lignin in their ultra structure. Suitable dark fermentative mixed consortia could be used to utilize such complex carbohydrates and produce hydrogen. During dark fermentation of organic material, energy carriers such as hydrogen and methane may be produced (Schink, 1997).

14.2 Photobiological Route for Hydrogen Production Using Algae

14.2.1 Hydrogen Production by Microalgae

Hydrogen production ability of green algae is coupled with photosynthesis. Water acts as direct source of electron. As the water splits to oxygen with the help of light energy and Photosystem II (PSII), the electron generated in this process is transferred to cytochrome b6f complex using plastoquinone (PQ) (Finazzi et al., 2002). Plastoquinone (PQ) then reduces plastocyanin (PC). Plastocyanin is a copper-containing electron transfer protein which then reduces Photosystem I (PSI). PSI can then reduce a ferredoxin (Eq. 14.1) or flavodoxin, which in turn reduces ($NADP^+$) and produces NADPH. The trans membrane potential of protons was used by membrane bound an ATPase to generate ATP and H^+ ions which enters into the lumen from stroma thylakoid. These protons are reduced by ferredoxin (reduced) to form molecular hydrogen via Fe-Fe hydrogenase enzymes (Eqn. 14.2).

$$Fd_{(ox)} + e^-_{(\text{from PSI})} \xrightarrow{\text{ferredoxin oxidoreductase}} Fd_{(Red)} \tag{14.1}$$

$$2H^+ + 2e^- \xrightarrow[\text{Ferredoxin}]{\text{Fe}-\text{FeH}_2 \text{ genase}} H_2 \tag{14.2}$$

The Fe-Fe-hydrogenase of green algae differs from most [FeFe]-hydrogenase as it lacks accessory Fe–S clusters at the N-terminus. It uses the electrons from ferredoxin (reduced) and combines it with the protons (H^+) to form molecular hydrogen.

14.2.1.1 Hydrogen Production by Cyanobacteria

Cyanobacteria, also called blue-green algae, belongs to the phylum of bacteria inhabiting watery environment. They obtain energy through oxygenic photosynthesis. This ability of cyanobacteria has converted early earth's reducing atmosphere in to an oxidizing one. These ancient life forms are harnessed in producing the eco-friendly energy of the future—hydrogen.

14.2.1.2 Non-heterocystous Cyanobacterial H_2 Production

Similar to microalgae, cyanobacteria also splits water and the electron thus generated is transferred to ferredoxin. Ferredoxin then channelizes these electrons to reduce $NADP^+$ to NADPH via FNR enzyme complex. NADPH then donates electron to bidirectional hydrogenase that converts proton to molecular hydrogen. In this process, bidirectional hydrogenase is Ni-Fe hydrogenase and thus plays a crucial role in conversion of solar energy and water into molecular hydrogen. These pigments system present in cyanobacteria are similar to microalgae. Cyanobacteria have large light-harvesting antenna complexes which give them an upper hand for survival in low-light conditions. Due to self-shading effect, the efficiency of large antenna systems decreases. Moreover, most of the energy from the incident light of the absorbed photons are dissipated as fluorescence and heat (Polle, 2002).

14.2.1.3 Heterocystous Cyanobacterial H_2 Production

Another category of cyanobacteria have filamentous morphology. These filamentous cyanobacteria have nitrogen fixing ability and have two types of cellular polymorphism. It has vegetative cells and heterocyst cells. Heterocystous cells are thick walled devoid of photosystem II (PS II). Thus photolysis of water doesn't take place inside the heterocysts thus anaerobic condition prevails inside the heterocyst. This anaerobic condition is very much required for nitrogenase and bidirectional hydrogenase activity. Nitrogenase is exclusively found in heterocysts. It fixes molecular nitrogen to ammonia and hydrogen is produced as a byproduct. Hydrogen is produced to maintain the redox potential inside the heterocyst. The turnover number of nitrogenase is significantly lower as compared to bi-directional [Ni-Fe] hydrogenase (Tamagnini et al., 2007). For substantial hydrogen production, larger amount of nitrogenase enzyme is required. Moreover, maintenance of nitrogenase enzyme is an energy demanding process as it consumes 16 moles of ATP to fix 1 mole of ammonia and to produce 1 mole of molecular hydrogen (Eq. 14.3).

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow H_2 + 2NH_3 + 16\left(ADP + Pi\right) \tag{14.3}$$

During nitrogen fixation, the electron allocation coefficient is defined as the ratio of electron used for nitrogen fixation to total electron consumed in the nitrogenase mediated reaction. The value of this coefficient remains in the range of 0.7 to 1. Therefore, in presence of nitrogen a large number of electrons are needed to be channelized to form hydrogen. This decreases the value of electron allocation coefficient. But scenario changes when there is a nitrogen deprived condition. In this case hydrogen is formed by expenditure of just 4 moles of ATP (Eq. 14.4).

$$2e^- + 2H^+ + 4ATP \rightarrow H_2 + 4\left(ADP + P_i\right) \tag{14.4}$$

14.2.2 Molecular Approach for Improvement of H_2 Production in Green Algae

Photosynthetic efficiency of the antenna pigments are just 10 % which implies that the rate of electron transfer from PSII to PSI is about 10 times lower than the rate of photon capture by the antenna pigments. One approach to improve photosynthetic efficiency was performed in *Chlamydomonas reinhardtii*, by truncating the chlorophyll (Chl) antenna size of PSII. Most recent studies showed the application of RNA interference or RNAi to down regulate the genes encoding for light harvesting complexes (LHC). Silencing of LCH genes lead to occurrence of less tightly stacked grana with improved photosynthetic quantum yield (81 %) and it also reduced the sensitivity towards photo-inhibition (Rosenberg et al., 2008).

The [FeFe]-hydrogenases of green algae are oxygen sensitive. This hampers hydrogen production. Therefore, protein engineering strategies are envisioned to increase the oxygen tolerance of these enzymes. Use of random and site directed mutagenesis has led to strain improvement of *Chlamydomonas* sp. which showed 10-fold improvement of oxygen tolerance (Chen et al., 2003).

14.2.3 Biohydrogen Production Using Algal Biomass as Feedstock

Microorganisms generate hydrogen for two principle reasons, first to dispose of excess reducing equivalents and second as a byproduct in nitrogen fixation. Microbial H_2 production is an attractive process for supplying a significant share of the H_2 required for the near future (Fig. 14.1).

Obligate anaerobes, facultative anaerobes, methylotrophs, and photosynthetic bacteria are well known microorganisms that have H_2 production ability. Based on the pathway that these microbes follow to evolve H_2, they are broadly categorized as either dark fermentative or photo fermentative microorganisms. Operation of dark fermentative H_2 production has low energy demand as compared to other conventional production processes. Moreover, it requires moderate process conditions which results in low cost for operation and minimal pollution generation (Angenent et al., 2004; Valdezvazquez et al., 2005). Therefore, dark fermentation has attracted greater attention of the researchers. Among a large number of microbial species, strict anaerobes and facultative anaerobic chemoheterotrophs, such as *Clostridia* and *Enteric* bacteria are efficient H_2 producers while degrading various types of carbohydrates.

Anaerobic *Clostridia* are well known group of microorganisms having greater potential for H_2 production. Immobilized *C. butyricum* was reported to produce 2 mol H_2 mol^{-1} glucose at 50 % glucose conversion efficiency. The highest theoretical yield of 4 mole H_2 from 1 mole of glucose can be achieved on following acetic acid fermentation (Taguchi et al., 1996). Formation of other metabolites like etha-

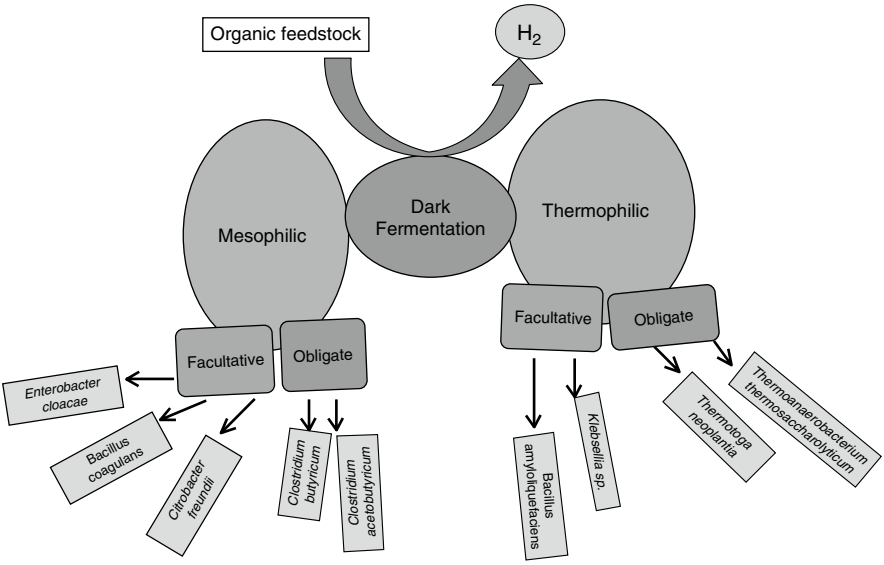

Fig. 14.1 Different domains of dark fermentative hydrogen production.

nol, propanol, butyrate, etc. leads to lower H_2 yields as formation of these metabolites compete for reducing equivalents inside the cell. For instance the conversion of one mole of glucose into butyrate is accompanied by the production of only 2 mol of H_2. *Clostridium* sp. such as *C. butyricum*, *C. welchii*, *C. pasteurianum and C. beijerinckii* are some newly isolated species that are potential H_2 producing microorganisms.

These organisms have plethora of hydrolytic enzymes which helps in degradation of organic matter to carbon dioxide and hydrogen. Such report on hydrogen production was available over 100 years ago by the biochemist Hoppe-Seyler (Stephenson and Stickland, 1932). The reduction of protons to H_2 serves to dissipate the excess electrons within the cell and generally permits additional energy generating steps in metabolism.

Plethora of microbial species is reported to produce hydrogen through dark fermentation viz. *Enterobacter, Citrobacter, Bacillus* and *Clostridium* sp. In recent times, apart from pure cultures, enriched mixed consortia are now gaining importance. By virtue of abundance of different array of hydrolytic enzymes, the mixed consortia or synthetic mixed consortia are ideal biocatalyst to utilize complex carbohydrates present in biomass for gaseous energy recovery. Nevertheless, the pursuit of ideal microbe(s) for H_2 production has attracted several researchers to screen exotic sources like hot spring, coal mine leachate, acid mine leachate, etc.

Anaerobic heterotrophic microorganisms can form hydrogen during the oxidation of organic substrates. A great advantage of fermentation is fast degradation of solids and other complex organics found in wastes and agricultural products. On the other hand, fermentation today converts only about 15 % of the energy to hydrogen.

On the other hand, biohydrogen production at thermophilic temperatures (60 °C) has many attractive advantages as compared to mesophilic temperatures (37 °C). Higher temperature condition leads to pathogenic destruction, lowers the risk of contamination by methanogenic archaea, higher rate of hydrolysis as well as H_2 yield.

The hyper thermophilic archaebacterium *Pyrococcus furiosus* produces H_2, organic acids and CO_2 from carbohydrates (Godfroy et al., 2000). Some cellulose degrading thermophiles that produce H_2 are *Anaerocellum, Caldi-cellulosiruptor, Clostridium, Dictyoglomus, Fervidobacterium, Spirocheta, Thermotoga* and *Thermoanaerobacter*. Hyperthermophilic *Thermotoga maritime* produces H_2 at 80 °C with yield of 4 mol mol^{-1} glucose which is equal to the maximal theoretical value (Schröder et al. 1994). Similar stoichiometries as of *T. maritima* were obtained for two more moderate thermophiles, *Acetothermus paucivorans* and *Acetomicrobium flavidum*, grown at 60 °C. These results showed that higher H_2 yields on hexose can be reached by extreme- and hyper-thermophiles as compared to mesophilic facultative and strict anaerobes.

Different pretreatments method could be employed to improve hydrogen production using algae as feedstock. Different physico-chemical pretreatments were employed to increase the accessibility of different complex sugars entrapped in algal biomass into simpler form. Carbohydrates in algal biomass are found as intracellular complex polymeric form bounded with rigid algal cell walls. Therefore, it is necessary to break the algal cell wall along with complex carbohydrate to facilitate the release of simple sugar. Cost of pretreatment of biomass adds significantly to overall hydrogen production process. Several methods of pretreatments such as physical (sonication, milling, grinding and pyrolysis), chemical (acid, alkali, thermal, H_2O_2) and biological methods (enzymatic, microbial) have been reported to break algal cell wall, hydrolyze the complex carbohydrates and release fermentable sugars. Each of the method has its own merits and demerits. Preference of chemical method such as acid treatment over others is because of higher conversion efficiency of polymeric carbohydrates into simpler sugars in lesser time. Physico-chemical methods are based on simpler technology but they are energy intensive processes limiting their use for commercial application. They also lead to formation of furfurals which inhibits the growth of the fermentative organisms. Biological based methods are developed to overcome these problems. It includes co-culture development where one organism will produce hydrolytic enzymes that would facilitate saccharification process.

Dark fermentative organisms can utilise simple sugars (which are released during saccharification) to produce hydrogen. Even crude enzymatic techniques were also studied for pretreatment of algal carbohydrates. But these processes are costly and time consuming with low reducing sugar yield. Very few reports were available on usage of algal biomass as feedstock for hydrogen production. In a study, algal biomass was used to produce H_2 using *Clostridium butyricum* and subsequent use of produced organic acids for H_2 production by photo fermentation using *R. sphaeroides* KD 131 (Kim et al., 2006). Tam et al. (2012) showed thermophilic hydrogen production using *Chalmydomonas reinharditti* (Nguyen et al., 2010). Nayak et al.

(2014), used amylase treated *Anabena variabilis* biomass to produce hydrogen via thermophilic dark fermentation. Similar study with *Chlorella sorokiniana* was also reported where acid-heat treated biomass was used by *Enterobacter cloacae* IITBT08 to produce hydrogen (Kumar et al., 2013). Thermophilic dark fermentation with acid-heat treated *Chlorella sorokiniana* biomass gave higher yield as compared to mesophilic process (Roy et al., 2014).

14.3 Biomethane Production Using Algae Biomass as Feedstock

Anaerobic digestion process (ADP) has been used since centuries to produce biogas. However, the first documented digestion plant was constructed in Bombay, India in 1859 (Lettinga, 2001). Utilization of biogas from a digester plant was first introduced in 1895 in Exeter, England where biogas was used for street lighting. Currently almost 15 million digesters, including small farm-based digesters, are operated in China and roughly 12 million digesters are established in India (Pathak et al., 2009).

An increasing awareness on greenhouse gas emissions and global warming has led to the global demand for alternate renewable fuels and has attracted researchers to promote ADP for industrial applications. Apart from conventional methods of treatment of sewage biosolids, livestock manure, and concentrated wastes from food industry, ADP can also serve as a significant source of renewable fuel. For instance, the anaerobic digestion (AD) of algal biomass to biogas possesses several advantages as compared to other biofuel sources and conversion techniques, such as:

- Uncritical water quality. Wastewater, brackish water and even seawater can be used for algae culturing in addition to fresh water.
- Reduced energy consuming steps.
- Maximal algal biomass utilization possible.
- Partial recycling of nutrients with AD effluent. AD releases nutrients in a potentially usable and recyclable form. The supernatant liquid rich in nitrogen and phosphorus content can be used as a fertilizer for algae culturing while the solid phase can be used as a fertilizer in agriculture or as a livestock nutrient.
- Possible integration with other technologies. ADP can be used as a co-technology for algal residues utilization after biodiesel, green diesel, bioethanol, and hydrogen production. Also, a variety of organic wastes and by-products can be co-digested with algae to produce biogas.

Nevertheless, methane production from algae suffers from certain limitations that need to be addressed prior to its industrial application, some of which are (Gujer and Zehnder 1983):

- High capital cost of algae production and AD units.

- Low algae productivity. Algae growth rate is relatively limited by low efficiency of photosynthesis, photoinhibition and carbon assimilation.
- Incomplete digestibility of algal cells. The algal biomass partially contains recalcitrant organic matter that cannot be hydrolyzed by the conventional ADP.
- Slow conversion rate. Biomass residence time in the ADP varies between 10 and 30 days.
- Unbalanced C:N ratio. A low C:N ratio can lead to the accumulation of NH_4^+ in an anaerobic digester to inhibitory levels while lack of nitrogen can limit anaerobic conversion and methane production.
- High sensitivity of the ADP. Methanogenic organisms are sensitive to fluctuations of environmental and operational parameters.

The main structural elements of macroalgae are composed of polysaccharides and their cell wall lack lignin. Based on the cell envelope components, they are classified into three major phyla: red, green and brown. Green algae contain ulvan and xylan in their cell envelopes whereas red algae contain carrageen, agar and xylose. Alginate and fucoidan are found in brown algae. In many algal classes cellulose is main structural element of cell wall. The main storage polysaccharides also vary among the three phyla of algae. Floridean starch is found in red algae; chlorophycean in green macroalgae while laminarin and mannitol in brown macroalgae. Remarkable variations are observed in the biochemical composition of microalgae, cyanobacteria and macroalgae which usually depends on several environmental factors such as temperature, salinity, light intensity and nutrient availability. Proteins and lipids account for the bulk of microalgal dry weight while carbohydrates contribute to minor component of cell dry weight. This discrepancy in the biochemical composition among different phylum or genera and also among similar species challenges the ADP of algae.

14.3.1 Principles of the Anaerobic Digestion Process

AD is a complex biological process in which organic matter is degraded into methane by naturally occurring microbial consortium comprising anaerobic bacteria and archaea. This section specifically deals with the detailed description of ADP biochemistry and microbiology, the influence of environmental and physicochemical parameters on process performance, and the importance of biogas composition and its application.

14.3.1.1 Biochemistry and Microbiology of Anaerobic Digestion

Algal biomass is a blend of organic and inorganic matter in which the organic part comprises complex polymeric macromolecules like proteins, polysaccharides, lipids, and nucleic acids that appear in particulate or colloidal form. During ADP, this organic matter is converted to final products (methane and carbon dioxide), new

biomass, and/or inorganic residues by the action of diverse groups of microorganisms. The overall process comprises multiple stages with numerous intermediate products. Generally, ADP constitutes four key steps: (1) hydrolysis, (2) fermentation or acidogenesis, (3) acetogenesis and (4) methanogenesis. The overall transformation can be described by six distinct biological processes as mentioned in Fig. 14.2.

1. Hydrolysis of colloid and particulate biopolymers to monomers.
2. Fermentation or acidogenesis of amino-acids and sugars to intermediary products (propionate, butyrate, lactate, ethanol, etc.), acetate, hydrogen, and formate.

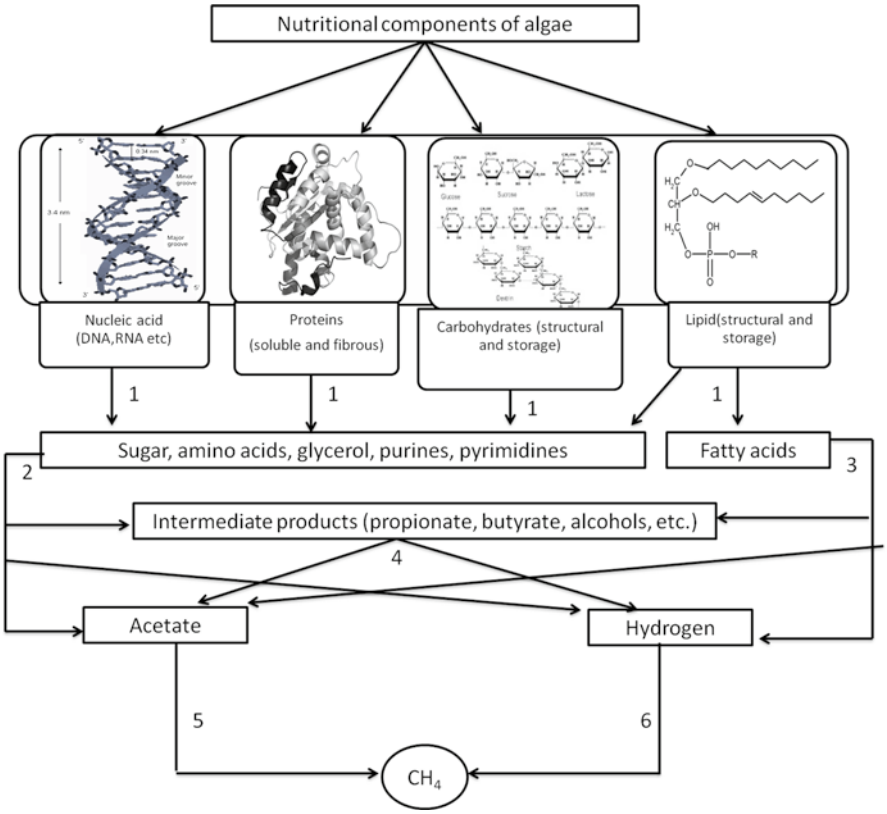

Fig. 14.2 Fate of organic components in anaerobic digestion.

3. β-oxidation of long-chain fatty acids and alcohol fermentation to volatile fatty acids (VFA) and hydrogen.
4. Anaerobic oxidation or acetogenesis of intermediary products, such as VFAs to acetate, carbon dioxide and hydrogen by obligate and facultative hydrogen producing species.
5. Transformation of acetate into methane by acetoclastic methanogens.
6. Transformation of molecular hydrogen and carbon dioxide into methane by hydrogenophilic methanogens.

Similar fermentative bacteria perform the first three steps which are sometimes referred to as acidogenesis or the acid-phase (Chen et al., 2008). Other important biological processes in AD are:

- Conversion of a variety of monocarbon compounds (e.g., formate, methanol) into acetic acid by homoacetogenic bacteria.
- Reduction of sulphur compounds to hydrogen sulphide by sulphur reducing bacteria.

14.3.2 Operational Parameters, Physicochemical Factors, and Inhibition of the Anaerobic Process

Among the microorganisms involved in AD, archaea possess the slowest growth rate and attain lowest energy during methanogenesis. Thus, for effective methane production, proper maintenance of environmental and operational parameters for archaea is the key factor. The main environmental factors for ADP are temperature, pH, alkalinity and redox potential. Operational parameters include C:N:P ratio, presence of essential micronutrients, organic loading rate (OLR), hydraulic (HRT) and solids retention time (SRT), and incoming salts and toxicants concentration. The accumulation of certain intermediates or byproducts, such as VFAs, ammonia and hydrogen sulphide, can lead to inhibition of methane production.

14.3.2.1 Temperature

The most important environmental factor for methanogenesis is temperature. The optimal temperature for growth of psychrophilic organisms is between 10 and 15 °C, mesophilic between 35 and 40 °C, and thermophilic between 58 °C and 68 °C. Compared to mesophiles and thermophiles, the rate of methane production is slowest with psychrophiles and, therefore, psychrophilic regime is rarely used for

large-scale methane production. Methanogens are highly sensitive to temperature variation. Even a minute temperature change of 2–3 °C can cause accumulation of VFAs thereby decreasing methane generation rate especially at thermophilic conditions (Speece, 1983). The activity of all anaerobic microorganisms is drastically affected by considerable temperature drop and ceases methane production. However, these microorganisms are able to recover with appropriate temperature stabilization.

14.3.2.2 pH and Alkalinity

The pH is another important environmental factor for ADP as the optimum pH varies for different groups of methanogens. The acidogens exhibit maximum activity at pH 5.5–6.5 while methanogens exhibit at pH 7.8–8.2. Thus anaerobic digesters are usually maintained in the range of 7–8 to enhance methanogenesis. At pH higher than 8, dissociation of NH_4^+ to neutral NH_3 occurs that inhibits growth of methanogens (Hansen et al., 1998). An important marker of pH persistence in anaerobic digesters is alkalinity. Usually the pH fluctuations caused by the generation of VFAs and carbon dioxide is controlled by bicarbonate alkalinity buffers at pH close to neutral. A stable ADP is characterized by the bicarbonate alkalinity in the range of 1,000 to 5,000 mg L^{-1} as $CaCO_3$. The ratio between VFAs to alkalinity should be in the range of 0.1–0.25. Further increase of VFAs to alkalinity ratio indicates possible process deterioration and requires the OLR to decrease in order to lower the VFA formation rate.

14.3.2.3 Nutrients

Stable growth of anaerobic microorganisms is dependent on availability of appropriate micro and macro nutrients. The approximate ratio of carbon to nitrogen and phosphate should be in the range of 75:5:1 to 125:5:1. Chynoweth reported the nitrogen limiting conditions when C/N ratio exceeded 15 during digestion of *Macrocystis*. The most important element for growth is nitrogen as it serves dual role: it helps in synthesis of protein and nucleic acids, and when reduced to ammonia, serves as a base to maintain neutral pH. Certain macronutrients such as iron, nickel, cobalt, molybdenum, zinc, calcium, copper and boron, are necessary for stable AD in the ppm level (Kida et al., 2001). Most of these metals serve as the principal component of enzymes' active site. For example, copper and cobalt are constituents of B_{12}-enzyme which catalyze the methanogenesis; nickel is part of factor F 430 found only in methanogenic bacteria, and molybdenum and selenium are subcomponents of formate dehydrogenase, which is part of the active site of hydrogenase and acetyl-CoA synthase.

14.3.2.4 Oxidation–Reduction Potential

A substance's affinity to either gain or lose electrons is measured by its oxidation-reduction potential (ORP). In AD, it is a measure of available oxidants (oxygen or nitrate ions) or reductants (hydrogen). A high ORP (>50 mV) indicates presence of free oxygen in the anaerobic environment, ORP between 50 and −50 mV depicts an anoxic environment with nitrates and nitrites, and an ORP lower than −50 mV implies reducing environment. At an ORP range of −50 to −100 mV, sulphate reducing microorganisms surpass methanogens for hydrogen and acetate since sulphate is a more thermodynamically favourable electron acceptor. Acid production during fermentation occurs in the ORP range of −100 to −300 mV while methanogenesis require ORP ≤ 300 mV when carbon dioxide is used as an electron acceptor and methane is formed (Gerardi, 2003).

14.3.2.5 Organic Loading Rate, Hydraulic and Solids Retention Times

The organic loading rate (OLR), hydraulic and solids retention times (HRT and SRT) also play a significant role in ADP. With rapid increase in OLR, accelerated acid formation takes place that leads to alkalinity depletion and a subsequent pH drop. The volume and capital cost of AD system depend upon HRT while SRT influences volatile solids (VS) reduction and, thus the methane yield from biomass. Any deviation in OLR, HRT and SRT can disturb ADP and inhibit methane production.

14.3.2.6 Toxicants

Methanogens are strict anaerobes and even the lowest oxygen concentrations (as low as 0.1 mg L^{-1}) can inhibit methane production. Methanogenesis using marine algae is sensitive to high salts concentration (e.g. NaCl). Nevertheless, comparable methane yield was reported from green macroalgae diluted with seawater and fresh water. In another report, methanogens were observed to be adapted to 65 g L^{-1} NaCl concentration. Moreover, desalination of macroalgae by heat and pressure resulted in less methane yield possibly due to loss of easily digestible organic matter as compared to untreated algae (Braun et al., 1981). Some algae have a tendency to accumulate heavy metals which can be detrimental to methanogenic bacteria. However, their negative effect can be decreased by precipitation with sulphide compounds. Nevertheless, by-products, including ammonia and hydrogen sulphide, at high concentrations can be toxic for bacteria. Although the nitrogen and sulphur source in AD is proteins, certain seaweeds have a high amount of sulfated carbohydrates. The neutral forms of ammonia and hydrogen sulphide are more toxic to bacteria as they can easily penetrate the cell membrane.

This toxicity of ammonia and sulphide is dependent upon the presence of metals, temperature, and pH in digesters. However, some authors have reported increasing

sulphide toxicity with increasing pH possibly due to different mechanisms of sulfide toxicity on different species which is usually associated with several factors: competition between methanogens for hydrogen and acetate; denaturation of native proteins through the formation of sulphide and disulphide cross linkage between polypeptide chains; interference with the assimilatory metabolism of sulphur; and the ability to remove essential metals (nickel, iron, cobalt) from the solution. Disruption of intracellular pH, potassium deficiency, and inhibition of a specific enzymatic reaction are associated with ammonia toxicity. The reported lethal concentration for ammonia toxicity for methanogenic microorganisms is 1.5–1.7 g L^{-1} at pH 7.4 and above (Sterling et al., 2001). However, methanogens can tolerate ammonia concentration up to 3–4 g L^{-1} at lower pH. Moreover, ADP levels of microorganisms are stable at high nitrogen concentrations (5–7 g L^{-1}). AD is inhibited by accumulation of non-ionic form of organic acids which is usually caused by decline in hydrogen utilization by acetoclastic methanogens and, therefore, leads to drop in pH. The mechanism by which organic acids inhibits ADP is probably through denaturation of cell proteins.

14.4 Anaerobic Digestion of Algae

Research on production of biogas from seaweeds and microalgae by AD started in mid-sixties. The first detailed large-scale study of seaweed cultivation and AD was performed in the Institute of Gas Technology, USA (Chynoweth et al., 2001). The composition of biogas obtained from ADP depends on the type of substrate and operational parameters. The theoretical yield of biogas can be estimated by the Bushwell equation (Eq. 14.5) (Buswell and Mueller, 1952).

$$C_cH_hO_oN_n + yH_2O \rightarrow xCH_4 + nNH_3 + (c - x)CO_2 \tag{14.5}$$

where

$$x = (4c - h - 2o - 3n - 2s)/8$$
$$y = (4c - h - 2o + 3n + 3s)/4$$

Methane yield depends on the average carbon oxidation state of the substrate. The theoretical methane yield from different algae is presented in Table 14.2. As observed from the table, lipids have lowest oxidation state and largest theoretical methane yield as compared to proteins, glycerol, and carbohydrates. Thus, macroalgae with high carbohydrate content and cyanobacteria with high protein content are theoretically poorer feedstock for methane production while microalgae with high lipid content have higher potential methane yield.

Table 14.2 Potential of different algae for methane production

Feedstock	CH_4 yield (L $g^{-1}VS$)	Purity of CH_4 (%)
Macrocystis pyrifera	0.64	59.5
Chlorella sp.	0.59	58.9
Chlorella pyrenoidosa	0.97	66.2
Anabaenopsis sp.	0.49	52.8
Chlamydomonas sp.	0.46	51.9
Laminaria sp.	0.45	51.9
Gracillaria verrucosa	0.44	49.3
Spirulina maxima	0.43	49.9
Ulva sp.	0.42	60.6
Fucus vesiculosus	0.41	50.1
Gracillaria tikvahiae	0.42	52.7

14.4.1 Anaerobic Digestion of Macroalgae

The biochemical composition varies considerably in different macroalgae species. However, cellulose is the main primary component among all algal groups. Extensive research has been conducted to study biological degradation of cellulose in recent years and the degradation mechanisms differ in anaerobic and aerobic bacteria. Anaerobic bacteria comprises multi enzyme complex—cellulosome in their cell envelope and consists of up to 11 different catalytic enzymes carried by scaffold-proteins. The enzymatic hydrolysis of algal cellulose is relatively slow and is inhibited by close association with other structural materials like polyphenols, fucoidan, protein and alginate. Thus, other species-specific sulphonated, methylated or carboxylated polysaccharides, mannitol, proteins and lipids usually determine the readily biodegradable fraction of algal biomass (Rees and Welsh, 1977).

14.4.1.1 Rhodophyta (Red Algae)

The main biochemical components of red algae consist of two sulphated polysaccharides-agars and carrageenans which are responsible for main part of cell dry weight. Both of them have an agarose backbone composed from α (1→3) linked galactose disaccharide units, connected by β (1→4) linkages. Galactose units on agars can be methylated (up to 20 %) and have few sulphate ester groups, while carrageenans can have from one to three sulphate ester groups, one for every disaccharide unit. Polysaccharides have non-uniform structure that depends on algal source, life stage, and season. Gamma Proteobacteria class, Bacteroidetes and

Planctomycetes phyla are usually able to degrade the red algal cell wall by secreting specific glycoside hydrolases, diverse agarases and carrageenases. Biodegradation of agar by a consortium of microorganisms from an anaerobic digester was studied on the biodegradation of *Gracilaria tikvahiae* and the results suggested that more than 50 % of the agar could be fermented over the course of three weeks. The rest of the agar, however, degraded slowly and complete fermentation (more than 90 %) required more than nine weeks. Overall agar was degraded faster than the rest of the organic matter in algae. This conclusion was supported by results of another experiment on *G. tikvahiae* batch digestion, where less than 50 % of the agar was fermented during 11 weeks. AD of carrageenan was studied on the biodegradation of red alga—*Eucheuma cottonii*, which has about 61.1–72.9 % of carrageenan content from dry weight. About 40 % of the biomass remained after 10 weeks of digestion but carrageenan accounted for 49.8–59.6 % of the fraction remaining (King et al., 1985). Therefore, it was concluded to be less biodegradable than rest of the algal organic matter.

Biogas production was observed to be highest during first four weeks when methane concentration ranged from 10 to 25 %. During later stages, however, methane production ceased probably due to accumulation of VFAs, out performance of the methanogens by sulphate reducing bacteria, or inhibition by sulphides. According to chemical composition, the theoretical methane yield for *G. tikvahiae* is 0.42 L g^{-1} VS and for *Gracilaria verrucosa* is 0.44 L/g VS. Biomethane potential (BMP) assays provided an experimental methane yield from *G. tikvahiae* of 0.35–0.4 L g^{-1} VS added, which corresponds to about 70–95 % of the theoretical methane yield. The methane yield from *G. verrucosa* was in the range 0.28–0.35 L $(g\ VS)^{-1}$, which corresponds to 58–77 % of the theoretical yield. A 60-d residence time was required for *Gracilaria* conversion to biogas.

14.4.1.2 Green Algae

Most frequently used feed stocks for AD consist of Ulvales, particularly *Ulva* (sea lettuce), *Enteromorpha* and *Cladophora* which are widespread all over the world specially in eutrophic ecological systems. However, *Ulva* sp. is considered as a potential feedstock for biomethane production since it has a low fraction of poorly biodegradable polyphenol materials (varies from 1 to 1.9 %). The major components of *Ulva* sp. are carbohydrates (about 40–60 % of cell dry weight), proteins (10–17 %), and lipids (1.8–3.5 %). Carbohydrates accumulate mostly as the cell envelope and consist of ulvan, cellulose, xyloglucan and glucuronan which are responsible for up to 38–54 % of the algal dry matter. In contrast to cellulose, ulvan is a water-soluble sulphated polysaccharide. The average composition of ulvan is rhamnose (16.8–45.0 % dw), xylose (2.1–12.0 %), glucose (0.5–6.4 %), uronic acid (6.5–19.0 %) and sulphate (16.0–23.2 %) but the main repeating disaccharide is ulvanobiouronic acid that is composed of aldobiouronic acid and 4-*O*-b-d-glucuronosyl-l-rhamnose. Despite the high ratio of sugars, only 8.9 % of ulvan and 16.6 % of *Ulva* organic matter is fermented by biota from the human. In another

study degradation of 32, 25.9 and 50.9 % of sugars from *Ulva* was reported after 24 h of fermentation. It was concluded that the complex chemical structure of ulvan cell polysaccahrides makes them poorly accessible for enzymatic attack. The recommended OLR and HRT for *Ulva* AD fall in the range of 0.8–1.2 g VS $L^{-1}d^{-1}$ and 15–20 d, respectively. Differences in *Ulva* composition among strains and even seasonal changes for one strain account for this observed variability (Lee, 2013).

14.4.1.3 Phaeophyceae (Brown Algae)

Alginic acid, laminarin, mannitol and fucoidan form the major organic components of brown algae. Among these, mannitol lacks polymeric structure, is soluble, and can be easily transferred into the cell. Anaerobic microorganisms can utilize mannitol effectively with the formation of acetate and hydrogen as the major products, and minor production of ethanol, formate, lactate and succinate. AD of laminarin was reported using microbial consortium isolated from the human gut where almost complete (>90 %) laminarin was used up within 24 h along with the formation of butyrate and other VFA. Alginate has a more complex molecular structure and usually forms a gel in algae. The alginate lyases are enzymes found to be responsible for alginate depolymerization. Biological degradation of soluble Na-alginate gel is 6–8 times faster as compared to Ca-alginate gel due to calcium cross bridging in the polysaccharides. Alginate depolymerization leads to formation of mixture of oligosaccharides with different length, which are further degraded to 4-deoxy-l-erythro-5-hexoseulose uronic acid. The final products of alginate degradation are glyceraldehyde-3-phosphate and pyruvate AD of fucoidan is still not reported. However, several fucoidan-degrading marine bacteria were isolated and characterized and the possibility of AD of fucoidan containing waste sludge from alginate extraction has been explored. The molecular structure of particular strains makes the AD of fucans difficult. The AD of algal proteins and polyphenols and their impact on overall digestibility still needs to be addressed. It is assumed that polyphenols associate with proteins and polysaccharides in the cell envelope that decreases their availability for biological degradation.

Brown algae are one of the most prominent algal feedstock for AD and several species have been examined including *Macrocystis pyrifera*, *Ascophyllum nodosum*, *Durvillea antarctica*, *Sargassum* spp. and *Laminaria* spp. According to the chemical composition, the theoretical methane yield of 0.52 L g^{-1} VS and 0.49 L g^{-1} VS were predicted for *M. pyrifera* and *Laminaria* sp. respectively. Researchers have reported an experimental methane yield for *M. pyrifera* of 0.43 L g^{-1} VS (82 % VS reduction) but only 0.24–0.3 L g^{-1} VS (50–60 % VS reduction) for *Laminaria saccharina*. Variability in chemical composition between these genera signifies the difference in yield. *Laminaria* has a higher content of fucoidan, laminarin and alginate but lower content of mannitol. The ratio between experimental and theoretical methane yield by *M. pyrifera* species is highly correlated with the mannitol content, in contrast to polysaccharides. Mannitol can be easily and completely degraded by anaerobic microorganisms (*Oceanography and Marine Biology, An Annual Review*, 2003).

14.4.2 Anaerobic Digestion of Cyanobacteria and Microalgae

14.4.2.1 Cyanobacteria

In contrast to algae, cyanobacteria consists of proteins as their biochemical component and lack of hard polysaccharide-based cell wall which makes them suitable for ADP. Two genera, *Arthrospira* (*Spirulina*) and *Anabaena* have been used extensively as potential feedstock for ADP. The BMP assay for a cyanobacterium mixture collected from Lake Dian resulted in a higher methane yield of 0.37 L g^{-1} VS (HRT 35 days) with a methane content of 60–65 % in the biogas. The methane yield during batch digestion of *Arthrospira platensis* and *Arthrospira maxima* species varied from 0.29 to 0.33 L g^{-1} VS corresponding to 68–77 % of the theoretical methane yield (Mussgnug et al., 2010). When municipal anaerobic sewage sludge was used as inoculum with cyanobacterium as feedstock, methane yield of 0.26 L g^{-1}VS was observed at an OLR of 0.97 g VS $L^{-1}d^{-1}$, HRT of 33 d, and temperature at 30 °C. Despite high ammonia and fatty acids concentrations (2.5 and 2 g L^{-1}, respectively) methane production was stable possibly due to high alkalinity (8 g L^{-1}) and pH of 7.55. AD stability and methane yield was highly influenced by VS concentration, OLR and HRT (Samson and LeDuyt, 1986). The methane yield and methane volumetric production rate were 0.04–0.36 L g^{-1} VS and 0.17–0.8 L $L^{-1}d^{-1}$, respectively.

14.4.2.2 Microalgae

Microalgae comprise diverse group of organisms and their predominant organic constituent vary from carbohydrates to proteins or lipids. Due to the widespread nature, fast growth rate and robustness, green microalgae serve as potential substrates for ADP. The BMP determined for *Chlamydomonas reinhardtii*, *Chlorella kessleri* and *Scenedesmus obliquus* was 0.387, 0.218 and 0.178 L g^{-1} VS, respectively (Samson and LeDuyt, 1986). The amount of biogas production correlated well with the extent of algal degradation. *C. reinhardtii* exhibited a higher cell disintegration rate in comparison to *C. kessleri* and *S. obliquus*. Number of the *Chlorella* and *Scenedesmus* species as well as several other algae (e.g. *Nannochloropsis*) have resistant trilaminar membrane-like structure containing an hydrolysable sporopollenin-like biopolymer—algaenan. The overall cell wall structure consists of four distinct layers: rigid internal, micro fibrillar, medial trilaminar and external columnar (for green algae *Coelastrum*). The major algaenan functions are protection from parasites and desiccation. *Chlorella* and *Scenedesmus* have internal rigid cell walls, either glucose-mannose type or glucosamine-type. In contrast, *C. reinhardtii* has a cell wall composed of proteins and glycoproteins. Resistant cell wall retained *S. obliquus* cells undamaged after six months of digestion (Takeda, 1988).

14.5 Current and Prospective Methods for Algae to Methane Process Enhancement

The biological production of methane from algae or cyanobacteria is generally a two-step process. First, biomass is produced where sunlight energy is captured and converted to new algal cells. Second, the energy stored in biomass is transformed into a more applicable form, such as methane gas, through the ADP. Thereafter, methane can be easily stored, transported, and used later for the production of heat or electricity. Methane can also be used as a motor fuel. The overall efficiency of methane production depends on performance of these coupled steps.

14.5.1 Anaerobic Digestion Improvement

The main objective for improving ADP is increasing the conversion efficiency while simultaneously decreasing capital and operational costs.

14.5.1.1 Inoculum Source for Anaerobic Digestion of Algae

As discussed earlier, the biochemical composition of algal biomass varies with the type of species which consists of unique compounds, such as algin, laminarin and fucoidan. Moreover, marine algal biomass has a high salt concentration that can affect anaerobic microorganisms. Thus isolation and application of microorganisms adapted for digestion of specific algae is required which is labour-intensive but, if successful, has the potential to improve algal ADP. Typically anaerobic sludge from a domestic sewage plant or marine anaerobic sediment has been used in literature for startup of the algal ADP. Several authors have reported that anaerobic organisms adapt readily to algal biomass as a sole substrate, and the inoculum source has a minor or no effect on the final methane yield and VS reduction. Application of an inoculum adapted to high ammonia concentration is a possible solution to overcome the problem of ammonia inhibition.

14.5.1.2 Process Parameters and Reactor Design

VS reduction, OLR and HRT are the most important operating parameters that affect the methane yield in ADP. Generally, the ratios of actual to theoretically calculated methane yield and VS reduction are low (typically from 0.4 to 0.6) as the degradation ability is dependent on the ability of anaerobic bacteria to hydrolyze complex organic compounds as well as by the slow rate of acetogenesis and methanogenesis stages of AD. To increase the AD efficiency, appropriate adjustments are made with process parameters and reactor design. Feedstock pretreatment and

conditioning and choice and source of specific anaerobic microorganisms can contribute in improving ADP. The general principles of digestion with non-algal feedstock can be applied to the AD of algae. Also environmental parameters have a significant impact on AD performance. With optimum process design methane yield is enhanced with increasing OLR and decreasing HRT.

The main goal of optimal reactor design is to reduce reactor volume and capital costs without compromising the maximum methane yield at high OLR and low HRT. Over the past few decades several high-rate digester configurations and their characteristics, advantages and disadvantages are described elsewhere. Decoupling HRT from SRT by anaerobic sludge immobilization is one of the strategies employed along with granulation and floc formation, biomass recycling, or membrane retention. The methane yield and methane production in a non-mixed vertical flow reactor (NMVFR) are larger and more stable at higher OLR as compared to CSTR. Another promising approach for biosolids digestion is separation of the hydrolysis and acetogenesis steps from methanogenesis, a process called a two-stage system. A two-stage anaerobic reactor system achieved stable methane production from *M. pyrifera* and *D. antarctica* with an HRT of one day for each stage (Vergarafernandez et al., 2008).

A special case of the two-stage system is preliminary treatment of macroalgae in percolation reactors with natural hydrolysis and acidogenesis processes. In percolation reactors, algae are stored in a tank yielding a drained liquid product containing VFAs and ethanol as substrates for methanogenesis. The AD of hydrolysis juices is more economically efficient as compared to digestion of whole macroalgae due to lower reactor size, energy for substrate heating, grinding and pumping. For example, the volume of digester with fixed bacteria for digestion of hydrolysis juices is 25 times smaller as compared to a CSTR digester required for whole algae (Nizami et al., 2009).

The relevant C/N ratios for microalgae are in the range of 4–6 and it was observed that the addition of carbon-rich cellulosic materials can balance the high nitrogen content. Addition of 25 and 50 % of waste paper to a mixture of *Scenedesmus* sp. and *Chlorella* sp. resulted in a 1.59- and 2.05-fold increase in the methane yield (Yen and Brune, 2007). The optimal ratio between algal biomass (*Scenedesmus* sp. and *Chlorella* sp.) and waste paper was found to be 40 % algae and 60 % paper with corresponding C:N ratio equal to 22.6. The authors also reported that paper addition stimulated cellulase activity in the anaerobic digester from 1.26 ± 0.14 mg L^{-1} min^{-1} to 3.02 ± 0.09 mg L^{-1} min^{-1} (50 % paper, C:N is equal to 18). Addition of nutrient-rich algal and cyanobacterial biomass to nutrient limited waste products that cannot be digested as sole substrate but can help to increase the process efficiency.

14.6 Conclusion

Biological production of hydrogen through dark fermentation process appears to be more suitable as compared to all other biological routes of hydrogen production. It has higher rate of hydrogen production and less energy input is required when compared with other biological routes. Renewable feedstock like algal biomass could make dark fermentative hydrogen production truly renewable. But there are certain bottlenecks regarding dark fermentative hydrogen production viz. low substrate conversion, influence of process parameters, accumulation of metabolites, etc. The spent media after dark fermentation is rich in volatile fatty acids which could be further used for biomethanation. This leads to enhanced gaseous energy recovery. Moreover, cyanobacteria and green algae could also be a promising feedstock for biogas production through anaerobic digestion process. The anaerobic digestion of algae can be applied for biofuel production.

References

Angenent, L.T., Karim, K., Al-Dahhan, M.H., Wrenn, B.A. and Domíguez-Espinosa, R. (2004). Production of bioenergy and biochemicals from industrial and agricultural wastewater. *Trends Biotechnol*., 22, 477–485.

Braun, R., Huber, P. and Meyrath, J. (1981). Ammonia toxicity in liquid piggery manure digestion. *Biotechnol. Lett*., 3, 159–164.

Buswell, A.M. and Mueller, H.F. (1952). Mechanism of Methane Fermentation. *Ind. Eng. Chem.*, 44, 550–552.

Chen, H.-C., Yokthongwattana, K., Newton, A.J. and Melis, A. (2003). *SulP*, a nuclear gene encoding a putative chloroplast-targeted sulfate permease in *Chlamydomonas reinhardtii. Planta*, 218, 98–106.

Chen, Y., Cheng, J.J. and Creamer, K.S. (2008). Inhibition of anaerobic digestion process: A review. *Bioresour. Technol*., 99, 4044–4064.

Cheng, L., Zhang, L., Chen, H. and Gao, C. (2006). Carbon dioxide removal from air by microalgae cultured in a membrane-photobioreactor. *Sep. Purif. Technol*., 50, 324–329.

Chynoweth, D.P., Owens, J.M. and Legrand, R. (2001). Renewable methane from anaerobic digestion of biomass. *Renew. Energy*, 22, 1–8.

Dickson, D., Page, C. and Ely, R. (2009). Photobiological hydrogen production from *Synechocystis* sp. PCC 6803 encapsulated in silica sol–gel. *Int. J. Hydrogen Energy*, 34, 204–215.

Finazzi, G., Rappaport, F., Furia, A., Fleischmann, M., Rochaix, J.-D., Zito, F. and Forti, G. (2002). Involvement of state transitions in the switch between linear and cyclic electron flow in *Chlamydomonas reinhardtii. EMBO Rep*., 3, 280–285.

Gerardi, M.H. (2003). The Microbiology of Anaerobic Digesters (Google eBook). John Wiley & Sons, USA, ISBN 0-471-20693-8.

Gibson, R.N. and Atkinson, R.J.A. (eds) (2003). Oceanography and Marine Biology, An Annual Review, Volume 41. CRC Press, USA.

Godfroy, A., Raven, N.D.H. and Sharp, R.J. (2000). Physiology and continuous culture of the hyperthermophilic deep-sea vent archaeon *Pyrococcus abyssi* ST549. *FEMS Microbiol*. Lett., 186, 127–132.

Gujer, W. and Zehnder, A.J.B. (1983). Conversion Processes in Anaerobic Digestion. *Water Sci. Technol*., 15(8–9), 1276–1277.

Hansen, K.H., Angelidaki, I. and Ahring, B.K. (1998). Anaerobic digestion of swine manure: Inhibition by ammonia. *Water Res*., 32, 5–12.

Kida, K., Shigematsu, T., Kijima, J., Numaguchi, M., Mochinaga, Y., Abe, N. and Morimura, S. (2001). Influence of Ni^{2+} and Co^{2+} on methanogenic activity and the amounts of coenzymes involved in methanogenesis. *J. Biosci. Bioeng*., 91, 590–595.

Kim, M., Baek, J., Yun, Y., Junsim, S., Park, S. and Kim, S. (2006). Hydrogen production from *Chlamydomonas reinhardtii* biomass using a two-step conversion process: Anaerobic conversion and photosynthetic fermentation. *Int. J. Hydrogen Energy*, 31, 812–816.

King, G.M., Guist, G.G. and Lauterbach, G.E. (1985). Anaerobic Degradation of Carrageenan from the Red Macroalga *Eucheuma cottonii. Appl. Envir. Microbiol*., 49, 588–592.

Kruse, O., Rupprecht, J., Bader, K.-P., Thomas-Hall, S., Schenk, P.M., Finazzi, G. and Hankamer, B. (2005). Improved photobiological H_2 production in engineered green algal cells. *J. Biol. Chem.*, 280, 34170–34177.

Kumar, K., Roy, S. and Das, D. (2013). Continuous mode of carbon dioxide sequestration by *C. sorokiniana* and subsequent use of its biomass for hydrogen production by *E. cloacae* IIT-BT 08. *Bioresour. Technol*., 145, 116–122.

Lee, J.W. (ed.) (2013). Advanced Biofuels and Bioproducts. Springer New York, New York, NY.

Lettinga, G. (2001). Challenge of psychrophilic anaerobic wastewater treatment. *Trends Biotechnol*., 19, 363–370.

Meher Kotay, S. and Das, D. (2008). Biohydrogen as a renewable energy resource—Prospects and potentials. *Int. J. Hydrogen Energy*, 33, 258–263.

Murphy, J.D. and Power, N. (2009). Technical and economic analysis of biogas production in Ireland utilising three different crop rotations. *Appl. Energy*, 86, 25–36.

Mussgnug, J.H., Klassen, V., Schlüter, A. and Kruse, O. (2010). Microalgae as substrates for fermentative biogas production in a combined biorefinery concept. *J. Biotechnol*., 150, 51–56.

Nayak, B.K., Roy, S. and Das, D. (2014). Biohydrogen production from algal biomass (*Anabaena* sp. PCC 7120) cultivated in airlift photobioreactor. *Int. J. Hydrogen Energy*, 39, 7553–7560.

Nguyen, T.-A.D., Kim, K.-R., Nguyen, M.-T., Kim, M.S., Kim, D. and Sim, S.J. (2010). Enhancement of fermentative hydrogen production from green algal biomass of *Thermotoga neapolitana* by various pretreatment methods. *Int. J. Hydrogen Energy*, 35, 13035–13040.

Nizami, A.S., Korres, E.N. and Murphy, J.D. (2009). Review of the Integrated Process for the Production of Grass Biomethane. *Environ. Sci. Technol.*. 43, 8496–8508.

Pathak, H., Jain, N., Bhatia, A., Mohanty, S. and Gupta, N. (2009). Global warming mitigation potential of biogas plants in India. *Environ. Monit. Assess*., 157, 407–418.

Polle, J. (2002). Truncated chlorophyll antenna size of the photosystems? A practical method to improve microalgal productivity and hydrogen production in mass culture. *Int. J. Hydrogen Energy*, 27, 1257–1264.

Prince, R.C. and Kheshgi, H.S. (2005). The Photobiological Production of Hydrogen: Potential Efficiency and Effectiveness as a Renewable Fuel. *Crit. Rev. Microbiol*., 31(1), 19–31.

Rees, D.A. and Welsh, E.J. (1977). Secondary and Tertiary Structure of Polysaccharides in Solutions and Gels. Angew. *Chemie Int. Ed. English*, 16, 214–224.

Rosenberg, J.N., Oyler, G.A., Wilkinson, L. and Betenbaugh, M.J. (2008). A green light for engineered algae: Redirecting metabolism to fuel a biotechnology revolution. *Curr. Opin. Biotechnol*., 19, 430–436.

Roy, S., Kumar, K., Ghosh, S. and Das, D. (2014). Thermophilic biohydrogen production using pre-treated algal biomass as substrate. *Biomass and Bioenergy*, 61, 157–166.

Rupprecht, J., Hankamer, B., Mussgnug, J.H., Ananyev, G., Dismukes, C. and Kruse, O. (2006). Perspectives and advances of biological H_2 production in microorganisms. *Appl. Microbiol. Biotechnol*., 72, 442–449.

Samson, R. and LeDuyt, A. (1986). Detailed study of anaerobic digestion of *Spirulina maxima* algal biomass. *Biotechnol. Bioeng*., 28, 1014–1023.

Schink, B. (1997). Energetics of syntrophic cooperation in methanogenic degradation. *Microbiol. Mol. Biol. Rev*., 61, 262–280.

Schröder, C., Selig, M. and Schünheit, P. (1994). Glucose fermentation to acetate, CO_2 and H_2 in the anaerobic hyperthermophilic eubacterium *Thermotoga maritima*: Involvement of the Embden-Meyerhof pathway. *Arch. Microbiol*., 161, 460–470.

Skjånes, K., Lindblad, P. and Muller, J. (2007). BioCO_2—A multidisciplinary, biological approach using solar energy to capture CO_2 while producing H_2 and high value products. *Biomol. Eng.*, 24, 405–413.

Speece, R.E. (1983). Anaerobic biotechnology for industrial wastewater treatment. *Environ. Sci. Technol.*, 17, 416A–427A.

Stephenson, M. and Stickland, L.H. (1932). Bacterial enzyme liberating molecular hydrogen. *Biochem. J.*, 26, 712–724.

Sterling, M., Lacey, R., Engler, C. and Ricke, S. (2001). Effects of ammonia nitrogen on H_2 and CH_4 production during anaerobic digestion of dairy cattle manure. *Bioresour. Technol.*, 77, 9–18.

Taguchi, F., Yamada, K., Hasegawa, K., Taki-Saito, T. and Hara, K. (1996). Continuous hydrogen production by *Clostridium* sp. strain no. 2 from cellulose hydrolysate in an aqueous two-phase system. *J. Ferment. Bioeng*., 82, 80–83.

Takeda, H. (1988). Classification of *Chlorella* strains by cell wall sugar composition. *Phytochemistry*, 27, 3823–3826.

Tamagnini, P., Leitão, E., Oliveira, P., Ferreira, D., Pinto, F., Harris, D.J., Heidorn, T. and Lindblad, P. (2007). Cyanobacterial hydrogenases: Diversity, regulation and applications. *FEMS Microbiol. Rev*., 31, 692–720.

Tamburic, B., Zemichael, F.W., Maitland, G.C. and Hellgardt, K. (2011). Parameters affecting the growth and hydrogen production of the green alga *Chlamydomonas reinhardtii*. *Int. J. Hydrogen Energy*, 36, 7872–7876.

Valdezvazquez, I., Riosleal, E., Esparzagarcia, F., Cecchi, F. and Poggivaraldo, H. (2005). Semi-continuous solid substrate anaerobic reactors for H_2 production from organic waste: Mesophilic versus thermophilic regime. *Int. J. Hydrogen Energy*, 30, 1383–1391.

Vergarafernandez, A., Vargas, G., Alarcon, N. and Velasco, A. (2008). Evaluation of marine algae as a source of biogas in a two-stage anaerobic reactor system. *Biomass and Bioenergy*, 32, 338–344.

Wijffels, R.H. (2008). Potential of sponges and microalgae for marine biotechnology. *Trends Biotechnol*., 26, 26–31.

Yen, H.-W. and Brune, D.E. (2007). Anaerobic co-digestion of algal sludge and waste paper to produce methane. *Bioresour. Technol*., 98, 130–134.