1. INTRODUCTION

1.1. Background and motivations

The twenty first century global enhanced population and contemporary lifestyle of mankind is gearing up for a confrontation with energy requirement. Already, the Earth primary energy resources are nearly dwindling down. In 2015, the global total fossil fuel reserve levels were 892 billion tonnes of coal, 186 trillion cubic meters of natural gas, and 1,688 billion barrels of crude oil. At present stage of extraction estimations proved that the reserves of coal would be exhausted in 113 years, the last cubic meter of natural gas in 2069, and the entirety of crude oil reserves by 2067 (BP, 2015). Simultaneously, fossil fuels emit greenhouse gases which leads to increase of temperature in Earth’s atmosphere (Ugarte DG, 2003). In 2014 global carbon emissions were estimated from fossil fuel use i.e around 9.795 gigatonnes (Gt). Fossil fuel emissions were 0.6% above in 2013 and 60% above in 1990 (Zeng et al., 2015). About 44% of Carbon Dioxide (CO$_2$) emissions were accumulated in the atmosphere, 26% in the ocean, and 30% on land from 2005 to 2014 itself. The accumulation of atmospheric CO$_2$ level in 2014 was 43% above the level when the Industrial Revolution started in 1750 (Zeng et al., 2015).

Revelle and Suess express their first concern as:

"Thus human beings are now carrying out a large scale geophysical experiment of a kind that could never have happened in the past nor be reproduced in the future. Within a few centuries we are returning to the atmosphere and oceans the concentrated organic carbon stored in sedimentary rocks over hundreds of million years" (Revelle & Suess, 1957)
The greenhouse gases like CO$_2$ and other heat trapping gases have elevated the greenhouse effect resulting in rise of the Earth’s surface temperature. When compared to several other greenhouse gases CO$_2$ is one of the most important gas for global warming. Electricity production, transportation, industry, commercial and residential, agriculture activities were the primary emitting sources of CO$_2$. Wherein transportation and electricity generation are the largest contributors of recent decades (Meyer & Dafermos, 2014). The improvements on compatible mitigation of greenhouse gas by chemical and biological process are the most extensive task in this century. To preserve Earth’s atmosphere and reduce the global warming risks, humanity needs renewable energy resources without emitting CO$_2$.

1.2. Renewable energies

The search for renewable energies without damaging the bionomical balance is been a ubiquitous emphasis of research. First generation biofuels are the alternative to fossil fuels and have now achieved competent levels of production. But it needs worlds 1% of arable land food resources for providing 1% of global transport fuels, as the first generation biofuels are mainly extracted from maize, sugarcane, sugar beet, food and oil crops (FAO, 2008; FAO, 2007; IEA, 2006) by mechanical, chemical and biological methods. In case of second generation biofuels, they are the biggest anticipation for intensive demand of energy and they are usually produced from wastes (agricultural, solid, organic and animal wastes etc.,) by using micro organisms (Moore, 2008).

In the year 2014 and 2015 global status report evidenced the contribution of renewable energy for human global energy consumption (19.2%) and to generate the electricity (23.7%) respectively. This energy is coming from traditional biomass (8.9%), heat energy (4.2%) (geothermal and solar heat), hydro electricity (3.9%) and
electricity from wind, solar, geothermal, and biomass (2.2%). Global investments for renewable technologies is more than US$286 billion in 2015, mainly countries like China and the United States are the biggest investors for wind, hydro, solar and biofuels (REN21, 2016). Nearly 7.7 million jobs were estimated to be associated with the renewable energy industries, with solar photovoltaics (IRENA, 2015).

While entering the theme of renewable resources, hydrogen secures its crucial role in concern of energy security, environmental and socio-economic issues of the world. At present among environmental problems, biohydrogen is the keystone to preserve the ecosystem. Due to these environmental merits, biohydrogen will grow fast in the next decade in the automotive fuel market and other fields (Das & Veziroglu, 2008).

**Figure:1** (a) Hydrogen - 80% constituted element in Great Orion Nebula and main ingredient of the visible universe; (b) Vial of glowing ultrapure hydrogen; (c) Photobiological hydrogen producing microalga in mass culture (d) and biological hydrogen production on reactor [tiny bubbles].
1.3. **Bio(logical)hydrogen**

In Greek words “bio” meaning is life + “hydro” meaning water + genes meaning is born of or forming is known as biohydrogen. The biohydrogen are usually obtained from biodegradable organic materials of plants, animals and microorganisms such as bacteria and microalgae (Benemann, 1998; Demirbas, 2009). Environmental friendly biological hydrogen is competent and one of the most advantageous, clean and renewable biofuels for future. When compared to hydrocarbon fuels, molecular hydrogen has two times high energy yield (122 kJ/g). There are many conventional methods, followed for the production of hydrogen such as photo electrochemical, steam reforming of methane and hydrocarbons, non-catalytic partial oxidation of fossil fuels, etc. Among all, biological hydrogen production is more efficient and eco friendly (Kapdan & Kargi, 2006; Lindblad, 1999).

About 200 million years ago, living biomass fossilized and formed fossil fuels. Also biomass is attractive, plenteous, potential and suspicious natural gift with varied number of environmental benefits. The biomass already has these grade of excellence, and serves as the cost effective primary sustainable energy provider to next generation energy crisis.

1.4. **Microalgal biohydrogen production**

On organic evolution, prokaryotic and eukaryotic microbes altered lifeless Earth's surface into prospering diversity of living organisms by the process of photosynthesis (Barber, 2007). These microbes have the capacity to capture the energy and changed our planet destiny by transforming the atmosphere. Thereon microalgae are more successful in harvesting the sunlight and transmitting it into beneficial resources. Microalgae have the capability to generate hydrogen under peculiar conditions with the help of an enzyme called hydrogenase (Adams, 1990; Albracht,
According to nature of micro algae, the hydrogen reaction takes place in two different ways, one is consumption of hydrogen for their metabolic process (Weaver et al., 1980) and the other is by forming hydrogen gas by reducing the protons on aid of hydrogenase (Meyer & Gagnon, 1991; Peters et al., 1998; Voordouw & Brenner, 1985; Voordouw et al., 1989).

The micro algal photobiological hydrogen production primarily requires, indispensable pristine energy from sun (Gaffron & Rubin, 1942) and a period of dark condition with anaerobic incubation for inducing the productiveness (Ghirardi et al., 1997; Happe et al., 1994; Roessler & Lien, 1984). Hence biological hydrogen production by green algae is of great concern due to its eco friendly nature and its utilization of the earth’s inexhaustible resources like sunlight, water and more importantly CO$_2$ capturing nature from the atmosphere.

**Figure:2** Biological hydrogen production by green microalgae

Recent CO$_2$ capturing methods are relatively costly with high energy consumption for resulting marginal benefits to mitigate them (Skjanes et al., 2007). When estimating recent emission of CO$_2$ by fossil fuels only 3-6% of CO$_2$ was
captured indirectly with the help of plants (Hutchinson et al., 2007). While compared with terrestrial plants, microalga has 10 times more ability in capturing the CO$_2$ efficiently and advantageously (Usui & Ikenouchi, 1997).

During large-scale hydrogen production, photobioreactor is a major cost factor and usually bioreactors are characterized by surface area for light irradiation (Benemann, 1997). The bioreactor volume and critical optical length determine the productivity of biohydrogen. Another important factor is microalgal strain produce high yield of biohydrogen only at peculiar conditions and photosynthetic efficiencies in dense cultures were achieved at full solar intensities. Biohydrogen production is frequently carried out in two different stages and atmospheric conditions, the first stage is cell growth followed by the hydrogen evolution.

1.5. Major benefits of biohydrogen

**Economic impacts**
- Sustainability and fuel diversity
- Increased number of rural manufacturing jobs
- Increased investments in plant and equipment
- International competitiveness
- Reducing the dependency on imported petroleum

**Environmental impacts**
- Reducing of greenhouse gas emissions
- Reducing of air pollution
- Non-carbon fuel

**Energy security**
- Peaceful and domestic targets
- Reducing use of fossil fuels
- Ready availability
- Domestic distribution
- Renewability
## 1.6. Important milestones of microalgal hydrogen production

<table>
<thead>
<tr>
<th>Year</th>
<th>Findings</th>
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<tr>
<td>1939</td>
<td>German researcher Hans Gaffron, at University of Chicago, observed that the algae, <em>Chlamydomonas reinhardtii</em> (a green-algae), would sometimes switch from the production of oxygen to hydrogen (Gartner, 2002).</td>
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<td>1997</td>
<td>Professor Anastasios Melis discovered that the deprivation of sulfur will cause the algae to switch from producing oxygen to hydrogen and the enzyme, hydrogenase, was responsible for this reaction. (Energy_report, 2000)</td>
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<td>2006</td>
<td>Researchers from University of Bielefeld and University of Queensland have genetically changed the single-cell green alga <em>C. reinhardtii</em> to produce large amount of hydrogen (Dnaindia, 2006)</td>
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<tr>
<td>2007</td>
<td>When copper is added to the medium to block oxygen generation algae will switch from the production of oxygen to hydrogen (Surzycki et al., 2007)</td>
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<td>2007</td>
<td>Anastasios Melis explained the solar-to-chemical energy conversion efficiency in tlaX mutants of <em>C. reinhardtii</em>, where they have achieved 15% efficiency, proving that truncated Chl antenna (Kirst et al., 2012) size would minimize wasteful dissipation of sunlight by individual cells (Tetali et al., 2007)</td>
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<td>2008</td>
<td>Solar-to-chemical energy conversion efficiency in tlaR mutants of <em>C. reinhardtii</em> was achieved up to 25% efficiency out of a theoretical maximum of 30% was studied by Anastasios Melis (Melis, 2008)</td>
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<td>2009</td>
<td>University of Tennessee, Knoxville and Oak Ridge National Laboratory stated that the process was 10 times more efficient when the temperature increased (Iwuchukwu et al., 2010)</td>
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2011 | Bioengineered enzyme increases the rate of algal hydrogen production about 400 percentage (Yacoby et al., 2011)

2011 | Argonne's Photosynthesis Group showed that platinum nanoparticles can be linked to key proteins in algae to produce hydrogen fuel five times more efficiently (Utschig et al., 2011a; Utschig et al., 2011b)

2013 | *C. reinhardtii* Photosystem II produces in direct conversion of sunlight where 80% of the electrons end up in the hydrogen gas (Volgushева et al., 2013)

| Table:1 Important finding of microalgal biohydrogen production

### 1.7. **Hydrogenase**

The oxidation process of water in photosystem-II and light driven reaction from photosystem-I generates electrons that are subsequently transported to Fe-S protein ferredoxin with the help of a catalyst reversible hydrogenase enzyme (Fig. 3) (Benemann, 1996; Miura, 1995). These electrons are received by hydrogenase enzyme in algal chloroplast and combine them with protons (H⁺) to generate photobiological hydrogen (Fig. 4) (Adams, 1990; Melis et al., 2000). During hydrogen generation, the limited level of carbon dioxide condition proposes competition between hydrogenase pathway and carbon dioxide fixation pathway (Kessler, 1973; Kessler, 1974). Furthermore, the major source of energy for cellular reactions is generated in thylakoid membrane through electron transportation via hydrogenase pathway and photosynthetic phosphorylation (Arnon et al., 1961). Even so, hydrogenase enzyme is highly sensitive to oxygen, which permanently inactivates the enzyme’s activity (Akkerman et al., 2002; Ghirardi et al., 1997; Ghirardi et al., 2000). Therefore the micro alga was subjected to implemented in sulfur deprivation on anaerobic photofermentation to activate reversible hydrogenase.
Figure: 3 The complex structure of ferredoxin and hydrogenase in microalgae (Source: NCBI)

Figure: 4 Photosynthetic hydrogen production under anaerobic condition. Starch release the H+/e- and provide the NADPH to hydrogenase for hydrogen production. PSI: photosystem I reaction; LHCII: light harvesting antenna proteins; PQ: plastoquinone pool; Cyt b$_{6f}$: cytochrome b$_{6f}$ complex; PC: plastocyanin; Fd: ferredoxin; Hyd: hydrogenase [Source: (Hankamer et al., 2007)].
The deprivation of sulfur affects the oxygenic photosynthesis at reversible rate of decline and also respiratory oxygen consumption on micro algae but in case of mitochondrial respiration no considerable changes were observed (Melis et al., 2000; Wykoff et al., 1998). In such nutrient restricted condition the reversible inhibition of photosystem-II reduces the level of D1 polypeptide and promotes active photobiological hydrogen production (Hahn et al., 2004; Melis et al., 2000; Zhang et al., 2002).

1.8. Seaweed as a potential substrate

The rapid growth in biofuel industries requires both economically and environmentally friendly resources for fermentation process. The demands of resource stepped into search for alternative sustainable feedstock. In the year 2008, 87 gigaliters of liquid biofuels were produced by using food crops such as sugarcane and corn (Somerville et al., 2010). In this production figures got elevated year by year and directly affected the feedstock’s of world needs (Gasparatos et al., 2013). Hence it is an important issue to prevent food feedstock and focus on alternative resources with renewable and economically cheap.

Figure: 5 Seaweed deposition in seashore of Gulf of Mannar, Tuticorin coast and wastage of rich nutritional resource.
The elevation of microalgal biomass requires nutrient rich substrate. To enrich the cultural growth certain micro and macro nutrients are essential, as well grown microalgal biomass reflects the active production of photobiological hydrogen. Seaweeds are multi cellular, photosynthetic, marine macro algae with high amount of carbohydrate (Matsumura et al., 2014; Wei et al., 2013) and growth promoting phyto hormones (IAA and IBA), cytokinins, auxins, gibberellins, antibiotics, trace elements, vitamins, amino acids, fiber, micro and macro nutrients (Blunden et al., 1996; Khan et al., 2009; Thirumaran et al., 2009; Zhang, 1997). Seaweeds are differentiated by colors like red, green, brown and randomly present on beaches and shorelines. Seaweeds are fixed and free-floating nature and also utterly essential for innumerable marine creatures, both as food and as habitat. It also contains anti-inflammatory, anti-microbial and anti-cancer agents.

The medicinal effects of seaweeds have been known before thousands of years ago. The ancient Romans renowned these applications and used them to treat wounds, burns, and rashes. The ancient Egyptians also used them as a treatment for breast cancer. In manufacturing sector many seaweeds are used to produce effective binding agents (emulsifiers) in commercial goods like toothpaste and fruit jelly, and popular softeners (emollients) in organic cosmetics and skin-care products (NOAA, 2014). Seaweeds are rich in sugar content with negligible lignin and it can be degraded biologically into simple sugars. Due to this seaweed are attracted as a feedstock for bio-hydrogen production in recent years (John et al., 2011; Shi et al., 2011; Shi et al., 2013). There are several seaweed species suggested to fermentative biohydrogen production such as green seaweed (Codium fragile), red seaweed (Gelidium amansii, Porphyra tenera, Gracilaria verrucosa), and brown seaweed (Laminaria japonica, Undaria pinnatifida, Hizikia fusiforme, Ecklonia stolonifera)
(Jung et al., 2011). The intact cell membrane of seaweed usually slows down the process of autolysis, in such cases it prolongs the biohydrogen production (Carrere et al., 2010). Therefore it is necessary to accelerate the biohydrogen production and also fermentation process. The pretreatment is the process to solubilize the seaweed biomass nutrients leading to accelerate the utilization of substrate easily.

Normally heat, acid, alkaline and microwave irradiations are used to destroy the cell membrane of biomass (Cui & Shen, 2012; Liu & Cheng, 2010). In future, the usage of cheaper biomass and efficient biological hydrogen production processes competes with the conventional hydrogen production processes (Das & Veziroglu, 2008). When compared with terrestrial biofuel crop the marine-based cultivation has more advantages, lack of competition, high rate productivity and more importantly there is no need for fresh water and fertilizers for the growth (Horn et al., 2000a; Matsumura et al., 2014). With this rationale seaweed recently bought attention and consideration as an ideal biomass for biohydrogen production (Enquist-Newman et al., 2014; Takeda et al., 2011; Wargacki et al., 2012).

The advancement of hydrogen-intensive research work for the sustained production of biohydrogen has initiated by the process of genetically modified microbes for enhancing the production, improvement of the bioreactor designs, employing different solid matrices for the immobilization of whole cells, metabolic engineering, development of two-stage processes, etc. Finally the utilization of cheaper and renewable biomass as raw materials and efficient biohydrogen production process created positive results in biohydrogen generation technology.
1.9. *Valoniopsis pachynema* (G. Martens) Børgesen

The seaweed *Valoniopsis pachynema* is filamentous, one and a half inches long green macro algae (Chlorophyceae) forming stiff, spongy mats of tangled filaments, on intertidal rocks, dead corals or hard substrates (Fig. 6). Seaweed *V. pachynema* usually grows on the substrate and covers completely giving a ball like appearance. In dead reef, they spread over the entire area and forms small, green, hairy clumps, cushion like appearance. Due to its thick turf like appearance it is popularly called as Astro-turf algae. The seaweed *V. pachynema* are usually grazed upon by some crabs and sea urchins (Marinelifeindia, 2014).

![Image](image.png)

**Figure:6** Morphology of seaweed *V. pachynema*, hairy, cushion like appearance and ball like separate colonies.

The plant body parts are not differentiated into stem, leaves, roots and vascular system therefore it is called as thallus. The filamentous and branched *V. pachynema* thallus are attached with substrate like dead corals or rocks by a rhizoidal structure. The habitat of *V. pachynema* is usually in the lower littoral zones where they grow abundantly in a luxuriant manner on rocky substrates where usually high waves occur.
It grows throughout the year and widely spread in tropical seas. They are usually found in the Indian Ocean islands like Andaman Islands, Chagos Archipelago, Diego Garcia Atoll, Laccadive Islands, Maldives, Nicobar Islands, Réunion, Seychelles, Palk Bay and Gulf of Mannar regions along with some other countries (Guiry, 2016; Marinelifeindia, 2014)

1.9.1. Taxonomic position

Empire : Eukaryota

Kingdom : Plantae

Subkingdom : Viridiplantae

Infrakingdom : Chlorophyta infrakingdom

Phylum : Chlorophyta

Subphylum : Chlorophytina

Class : Ulvophyceae

Order : Cladophorales

Family : Valoniaceae

Genus : Valoniopsis

Species : pachynema

1.9.2. Geographical distribution

The seaweed *V. pachynema* are usually found in Hawaiian Archipelago, Indonesia, Formosa, Malayan Archipelago, Philippines, Northern and Eastern Australia, New Caledonia, Gulf of California, Baja California, West Indies, Mozambique, East Africa, Mauritius, Sri Lanka, Pakistan, Bermuda, India and Spain.
Environmental conditions for growth of *V. pachynema* (EOL, 2016):

- Depth range: 0 - 54.3703 (m)
- Temperature range: 24.169 - 25.100 (°C)
- Nitrate: 0.134 - 1.026 (µmol/L)
- Salinity: 35.376 - 35.563 (PPS)
- Oxygen: 4.703 - 4.869 (ml/l)
- Phosphate: 0.135 - 0.177 (µmol/l)
- Silicate: 0.846 - 1.52 (µmol/l)

Kumar et al. (2009) studied the nutritional property of some seaweed species and compared with them. The results demonstrated that *V. pachynema* (476.67 ±6.2%) has rich calcium level when compared with 9 important seaweeds. Whereas (Venkatesalu et al., 2012) team of researchers studied the seasonal variation in the fatty acid compositions of astro-turf seaweed.

In future, biohydrogen definitely becomes a competitor for conventional hydrogen production (Das & Veziroglu, 2008). Hence the present work is focused on seaweeds, a natural abundant resource present in sea shore as raw material for biological hydrogen generation by using photobioreactor. The less known and less studied, Astro-turf alga *V. pachynema* is selected for detailed investigation about the cultural growth and biological hydrogen generation by employing microalga *C. vulgaris* MSU-AGM 14.
1.10. Objectives

The main objective of the present study deals with optimization of photobiological hydrogen production from freshwater microphyte *Chlorella vulgaris* MSU-AGM 14 employing seaweed *V. pachynema* as substrate.

- Characterization of the physio-chemical properties of the seaweed *V. pachynema* for biohydrogen production
- Isolation and identification of the microalgal strain from pond water sample
- Processing of the raw material of *V. pachynema* by aqueous and sulfuric acid extraction
- Surface structure examination of the seaweed before and after treatment of acid by using Scanning Electron Microscopy (SEM)
- Estimation of the mineral contents in the hydrolysates by Scanning Electron Microscopy with Energy-dispersive X-ray spectroscopy (SEM-EDAX)
- Assessment of biohydrogen productivity of the microalga *C. vulgaris* MSU-AGM 14 by introducing it into aqueous and acid hydrolysate as substrate
- To optimize the biohydrogen productivity different variables such as substrate concentration, pH, Temperature and carbon dioxide level were optimized by using Response Surface Methodology
- Quantitative and qualitative estimation of biohydrogen production by using Gas Chromatography