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A Study on Biohydrogen Production based on Biophotolysis from Cyanobacteria

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ABSTRACT

Hydrogen (H_2) has long been promoted as an ideal fuel, as it permits a completely clean combustion and has great potential to provide clean power needed for transport and electricity generation. The unicellular, nitrogen-fixing cyanobacterium *Cyanothece sp.* ATCC 51142 is a promising strain with a remarkable capability of producing large quantities of H_2. Under anaerobic condition, the cyanobacterium carries out the biological fixation of atmospheric nitrogen (N_2) into ammonia (NH_3), concurrently producing H_2 as by-product. The aim of this thesis was to improve our understanding of the growth and H_2 production of *Cyanothece sp.* ATCC 51142 in order to develop a continuous and practical cyanobacterial H_2 production process. In order to effectively handle incompatible requirements between the cyanobacterial growth and its sequential H_2 production, a novel two-stage chemostat photobioreactor (PBR) system was designed and developed, with an aim to improve H_2 production yield as well as extend its production duration. The system has been operated non-stop for consecutive 30 days without any losses in its performance and subsequently demonstrated a remarkable improvement in H_2 production, with more than 6.4 times higher yield than that of a single-stage batch system. With the continuous mode of operation, a continuous collection of produced biomass from the PBR is also permitted (more than 7.3 times improvement in biomass yield than that of a single-stage batch system). At an industrial scale, this biomass could undergo further downstream processing to generate a multistreamline of high valued by-products such as e.g. vitamins, pharmaceuticals and human nutrition, which can subsequently contribute to a significant improvement in an economic viability of biohydrogen process.

Keywords: Hydrogen Generation, Biophotolysis, Cyanothece, Photobioreactor, Bio-Hydrogen, Nitrogenase, Hydrogenase.

INTRODUCTION

Biochemical production of hydrogen as a by-product of the metabolism of microorganisms is a relatively new area of technological development, and a promising renewable energy source for the future. Since the possibility of such reactions, occurring in living organisms was demonstrated more than half a century ago, the process of biophotolysis conducted by cyanobacteria has been actively studied over the past 35 years. During this time, the basic molecular mechanisms of hydrogen take up by living systems were studied. Systems of water biophotolysis, which have two common elements, such as the electron transport chain of photosynthesis, including a water decomposition system and hydrogen formation catalysts, are conceptually considered as direct and indirect biophotolysis. As it is known, the process of direct biophotolysis involves the use of light energy of absorbed by the photosynthetic apparatus for the water cleavage with the formation of oxygen and for the generation of low-potential reducing agents followed by the reduction of protons and the production of hydrogen.

Biological H_2 production processes can be classified into two groups, depending on whether the

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process is light-dependent or not. Dark fermentation is a light-independent process. The light-dependent processes can be further classified into either a photosynthetic or a fermentation process.

Dark Fermentation

Dark fermentation is by far considered as the most practical biological H_2 production process, due to its high volumetric H_2 productivity, an ease of process operation and inexpensive feedstock, usually organic acids collected from various sources of wastewater. This process is carried out most efficiently by strict anaerobes such as *Clostridium* and facultative anaerobic bacteria such as *Escherichia coli*. During the operation, these bacteria are cultivated using carbohydrate-rich organic substrates such as glucose, sucrose under dark environment. These substrates are degraded by an oxidative reaction to provide building blocks and metabolic energy for bacterial growth. The oxidation also generates electrons, which, in an oxic environment, would reduce O_2 to water. In anaerobic conditions, other compounds such as protons need to act as electron acceptor and are subsequently reduced to form H_2.

Dark Fermentation Redox Pathways:

$Pyruvate + CoA + 2Fd_{oxidised} \rightarrow Acetyl\text{-}CoA + 2Fd_{reduced} + CO_2$

$2H^+ + 2Fd_{reduced} \rightarrow H_2 + 2Fd_{oxidised}$

Photofermentation

Photofermentation is a light-dependent photoheterotrophic process carried out by purple non-sulphur photosynthetic bacteria, using carbon substrates, typically organic acids and alcohols, together with the solar energy captured by their photosystem to produce H_2. Similar to dark fermentation, organic acids are initially oxidise to produce ATP and electrons, at which the latter are used to reduce Fd. But in this case, reduced Fd is reoxidised by the nitrogenase enzymes, which catalyse the N_2-fixation reaction. During nitrogen-fixing reaction, electrons reduce dinitrogen (N_2) into ammonia (NH_3), at the same time producing H_2 as by-product. With a pure N_2 atmosphere, the ratio of an electron allocation for the N_2 reduction and H_2 formation would be at 3:1. However, in the absence of N_2, bacteria are capable of diverting all of the fermentative electrons towards H_2 production, enhancing the stoichiometric H_2 yield by a factor of 4.

Photofermentation Reaction:

$2CH_3COOH\ (acetic\ acid) + 4H_2O \rightarrow 4CO_2 + 8H_2$

Biophotolysis

Biophotolysis is a completely sustainable H_2 production process, as H_2 is produced from the world's two most plentiful resources – sunlight and water. Once it has been used up as a fuel, H_2 also returns to water. Biophotolytic H_2 production process can be classified into two types – direct and indirect.

For direct biophotolysis, the photosystem II (PSII) protein complex within the chlorophyll (Chl) of green algae and cyanobacteria captures sunlight and utilises this energy to dissociate water into its constituents – protons (H+), electrons (e-) and oxygen (O_2). The photosynthetic electrons are transported by Fd and other intermediates to the hydrogenase enzyme, which catalyses the recombination reaction of proton-electron to eventually produce H_2. However, as hydrogenase is rapidly inactivated by photosynthetically evolved O_2, the direct biophotolytic H_2 production is

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only possible when the cultures are incubated under some special conditions such as sulphur deprivation of green alga *Chlamydomonas reinhardtii* (*C. reinhardtii*) culture. Fundamentally, sulphur is a key component of the *C. reinhardtii*'s photosynthetic mechanism, since it is required to repair the D1 protein, a major component of the PSII reaction centre. As a result, sulphur-limitation leads to the significant reduction in the rate of photosynthesis and its concurrent O_2 evolution, while having minimal effect on the cellular respiration rate. Due to the greater rate of respiration than photosynthesis of the cells, after about 24 to 30 hr of sulphur deprivation, the *C. reinhardtii* culture would become anaerobic in the light and subsequently start to produce H_2.

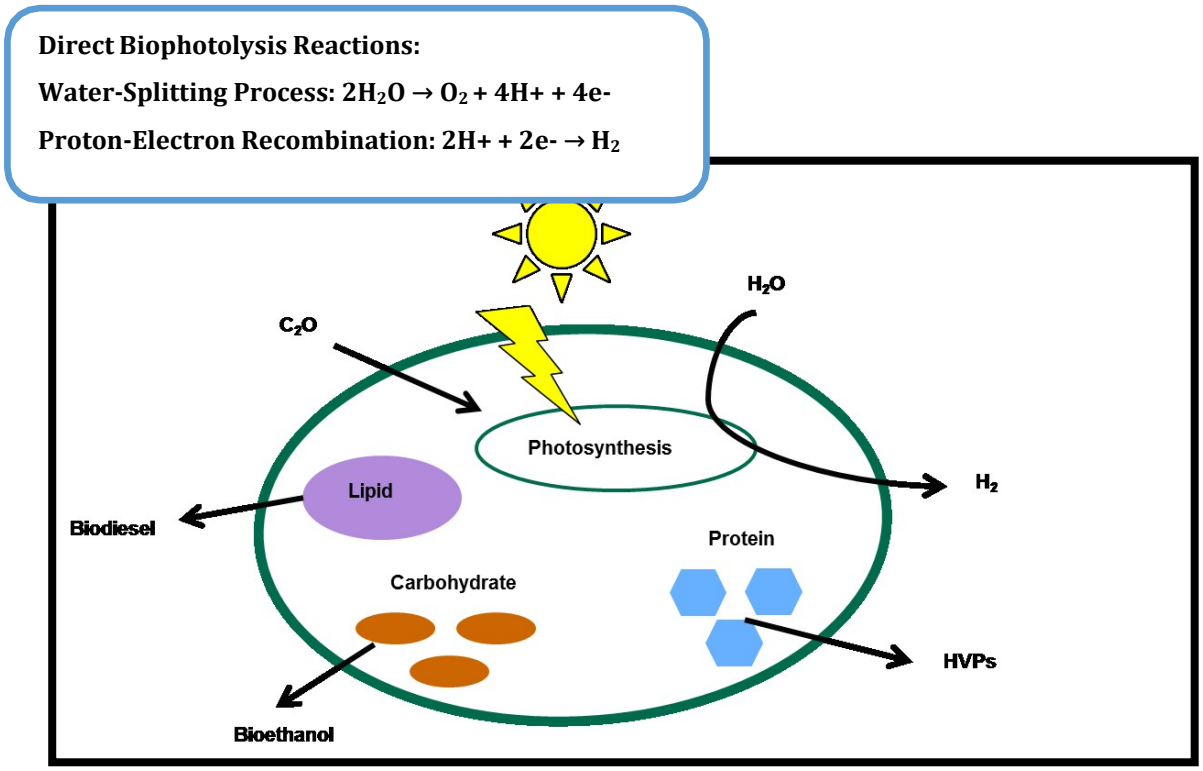

Figure 1: Schematic Diagram of Microalgal Biorefinery

In the case of indirect biophotolysis, electrons from water are used in the carbon fixation of the Calvin cycle and subsequently stored in some form of endogenous reserve carbohydrates, which are later used to produce H_2. The process, which re-converts carbohydrates into electrons, is known as cellular respiration. The indirect bio photolytic H_2 production is usually found in many nitrogen-fixing cyanobacteria (blue-green algae), in which photosynthetic O_2 evolution and its incompatible H_2 production reactions are temporarily or physically separated from each other.

Indirect Biophotolysis Reactions:

Oxygenic Photosynthesis: $6CO_2 + 6H_2O \rightarrow C_6H_{12}O_6 + 6O_2$

Oxygenic Photosynthesis

Photosynthesis is a radical energy storage system naturally possessed by oxygenic photoautotrophs plants, green algae and cyanobacteria (Blankenship 2008). The process consists of two subprocesses, taking place in sequence:

(1) Light-dependent reaction and

(2) Light-independent reaction (dark reaction).

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The photosynthetic light reaction begins with the absorption of light by two essential light-harvesting antenna systems - chlorophyll (Chl) a and phycobilins. Pigmented Chl a biomolecules are grouped together to form light-harvesting complex (LHC) proteins. LHC proteins arrange themselves into light-harvesting antennae, which are bound into two multi-membrane protein complexes: photosystem I (PSI) and PSII. In contrast, phycobilins are only present in cyanobacteria, at which they are organised together in large multi-protein complexes, called phycobilisomes (PBSs). The light-harvesting photosystems and most of the photosynthetic electron transport chain involved in the photosynthesis process are located within the thylakoid membrane: an internal membrane system that separates the cytoplasm of cyanobacterial cells from their lumen.

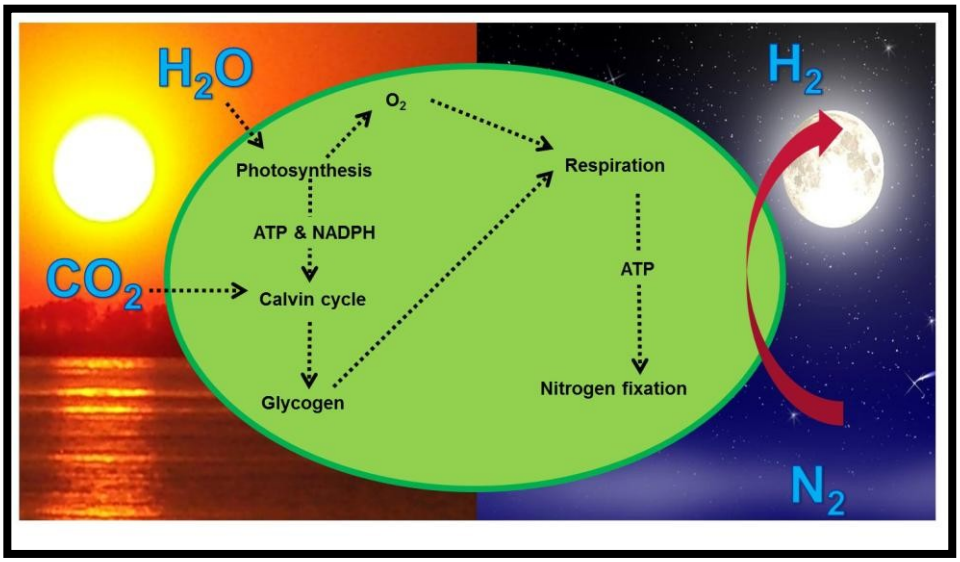

Figure 2: Schematic Diagram of Biophotolytic H_2 Production of *Cyanothece* 51142. Adapted From (Welsh, Liberton et al. 2010)

Table 1: Different Biological H_2 Production Processes and Their Advantages and Disadvantages

Process	Dark Fermentation	Photofermentation	Biophotolysis
Organisms	Anaerobes and Facultative anaerobes	Purple, Non-sulphur photosynthetic bacteria	Green algae and Cyanobacteria
Substrate	Organic acids	Organic acids	Water
Light Dependence	No	Yes	Yes
H_2 Productivity (mL/L/hr)	1000-1500	12-83	2.5-13
Purity (%)	40-60	90-96	Almost 100

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Advantages	• Simple operation • High Productivity • Capable of utilising various wasted substrates	• High Purity • Nearly complete conversion • Capable of using wasted substrates	Very abundant inexpensive substrate
Disadvantages	• Many side products • Low productivity	• Low light conversion efficiencies • Low H_2 production rate	• Incompatibility between photosynthetically evolved O_2 and H_2 evolving catalyst • Extremely low production rate

Selection of Studied Species: Introduction to *Cyanothece* sp. ATCC 51142

Cyanothece sp. ATCC 51142 (*Cyanothece* 51142) is a marine, unicellular, non-heterocystous, nitrogen-fixing cyanobacterium (blue-green alga), which was isolated from the intertidal sands of the Texas Gulf coast (temperature varies within a range of 20 – 35 ℃). Individual cells of *Cyanothece* 51142 are spherical and have approximately 4 to 5 μm in diameter. Its cell division proceeds by binary fission. An oxygenic photosynthesis of *Cyanothece* 51142 is virtually identical to higher plants and green algae, except for the difference in accessory pigments. The cyanobacterium captures the solar energy using two essential light-harvesting units - chlorophyll a and phycobilins. Chlorophyll a is housed inside the thylakoid membranes, whereas phycobilins, usually organised into hemispherical phycobilisomes, which are attached to the outside of the thylakoid membranes. As the laboratory wild type (WT) *Cyanothece* 51142 strain has an ability to aerobically produce H_2, albeit the highest rate was observed under anaerobic conditions it is used as a model organism for bioenergy research and studies on how a unicellular organism balances multiple, often incompatible, photosynthetic and N2-fixation processes within the same cell Like many oxygenic cyanobacteria, *Cyanothece* 51142 possesses both hydrogenase and nitrogenase enzymes, which are directly involved with its biological H_2 formation reaction. In addition to standard photoautotrophic conditions, *Cyanothece* 51142 has advantageous growth flexibility and is able to grow under various conditions - photo/chemoheterotrophic and mixotrophic conditions.

MATERIALS AND METHOD

❖ Stock Culture Preparation

Cyanothece 51142 cells were scraped off an agar plate and re-suspended in a sterilised 50 ml Conical flask filled with autoclaved ASP2+N medium. This procedure was performed inside a laminar flow hood, a device that filters ambient air to minimise particulate concentrations. Before making cell transfer, the working surface of the sterile hood and the nitrile lab gloves were washed with 70% ethanol in order to ensure a sterile environment during the early stages of cyanobacterial growth. Reasonably, this is the period when the culture is most vulnerable to contamination. The 50 ml flask was kept under the optimal growth conditions recommended in the literature: at 30 ℃, with starting pH of 7.4 and under continuous cool-white fluorescent illumination of 46 μmol /m^2 /s. The mixing was provided by a mechanical shaker. After approximately 5 days of cultivation, the culture was scaled up to a volume of 200 ml. Once again,

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a sterile hood was used to transfer the contents of the 50 ml flask into a 200 ml flask, which was then topped up with freshly made and sterile ASP2+N medium. As the cell density of *Cyanothece* 51142 increased, the culture became greener and less transparent. The entire growth process lasted less than 2 weeks. At that point, fully grown stock cultures were ready to be used for experiments (200 ml for ICL flat-plate PBR and 400 ml for Sartorius PBR).

❖ Analytical Techniques

➢ Spectrophotometry

Spectrophotometry was used to determine the OD of *Cyanothece* 51142 culture by light absorption. ODs, measured in both absorbance units (AU) and signal (mV), have a linear relationship with the Chl content and the dry biomass concentration of that culture. A Lambda 40 UV / Vis Spectrometer from Perkin-Elmer Instruments running the UV WinLab software was used to measure OD of the culture at three distinct wavelengths of 620, 678 and 750 nm. Cyanobacterial samples were analysed in a 1.5 ml cuvette and the instrument was autozeroed with the light absorbed measurement from a cuvette filled with ASP2 medium

❖ Growth of *Cyanothece* sp. ATCC 51142

The major reason behind my decision of choosing a *Cyanothece* 51142 strain as the studied microorganism came from the fact that this cyanobacterium is a wild-type strain, which has an exceptional ability to produce H_2 at the highest rate among all of previously investigated strains. The quantity of produced biohydrogen is closely related to the quantity of functioning *Cyanothece* 51142 cells, which in turn directly determines the number of light-harvesting complexes, oxygen evolving centres, nitrogenase enzymes and other reaction centres that are critical to the H_2 production process. In addition to enhancing the gas production, the production of *Cyanothece* 51142 biomass also facilitates the accumulation of essential bioactive products such as proteins, vitamins and amino acids

❖ Choice of Photobioreactor

All of growth experiments were carried out in the Sartorius tubular PBR, due to its major advantage of an automated parameter controlling system. The temperature of the aqueous culture is measured by a Pt100 Type 25-3 thermocouple and is controlled by means of a heated water jacket that encompasses the central vessel of the PBR. pH and dissolved oxygen concentration (pO_2) are measured by probes from Hamilton – Easyferm : Plus K8 160 pH electrode and Oxyferm FDA 160 oxygen tension probe, respectively. The growth of the cyanobacterium is real-time monitored by the Sartorius Fundalux II OD probe, containing an LED that operates in the 870-910 nm wavelength range. The OD calibration was performed by directly extracting a 6 ml cyanobacterial sample from the tubular reactor at regular time intervals, and analysing its light-scattering properties at 620, 678 and 750 nm using a spectrophotometer. This OD measurement, initially in absorbance units, was converted to units of chlorophyll and then dry biomass concentration. In addition, the PBR also provides a high level of operational flexibility, as it can be operated as an open system, allowing a continuous supply of gaseous CO_2 for autotrophic growth condition.

The cyanobacterial growth is monitored using a photodiode operating with an absorption bandwidth of 650 – 700 nm. In addition, a membrane-inlet mass spectrometry (MIMS) system was also incorporated into the reactor for *in situ* measurement of dissolved H2 gas in the aqueous phase.

❖ Batch Hydrogen Production of *Cyanothece* 51142

In order to induce anaerobiosis inside the air-incubated *Cyanothece* 51142 culture, without the use

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of inert gas sparging, organic glycerol was used as sole carbon source for the batch cultivation of this microbe. Glycerol was added into the medium to the final concentration of 50 mM, as high rates of H_2 production were previously reported under this particular concentration. Sterile pure air was sparged throughout the liquid culture for 5 minutes before the PBR was completely sealed in order to provide saturated O_2 and N_2 for respiration and N_2-fixation respectively. Under photoheterotrophic growth conditions, the culture progressed through all five common growth phases (similar to other microorganisms) – from the initial lag phase up to an eventual death phase. As generally expected, during the lag phase of approximately 10 hours, a minimal change in dry biomass concentration of the culture is observed, as cells are adapting to the new environment.

❖ RESULTS AND DISCUSSION

➢ Growth Cycle of *Cyanothece* 51142

Commonly, the growth cycle of microorganism can be characterised into five distinct phases:

- ✓ Lag(adaptation)
- ✓ Exponential (light and nutrient-sufficient)
- ✓ Linear (light-limited)
- ✓ Stationary(nutrient-limited) and
- ✓ Cell death

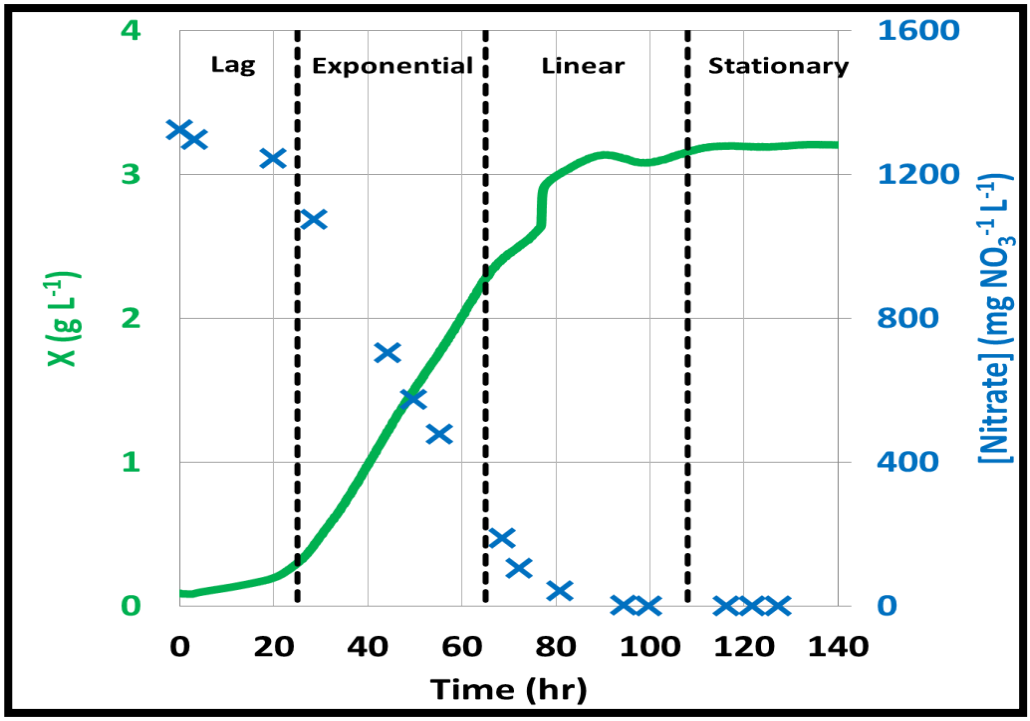

Figure 3: Four Common Growth Phases of The Cyanobacterial Culture. An Increase in Biomass Concentration (Green Line) was Observed in Parallel with The Decrease In NO_3 / Substrate

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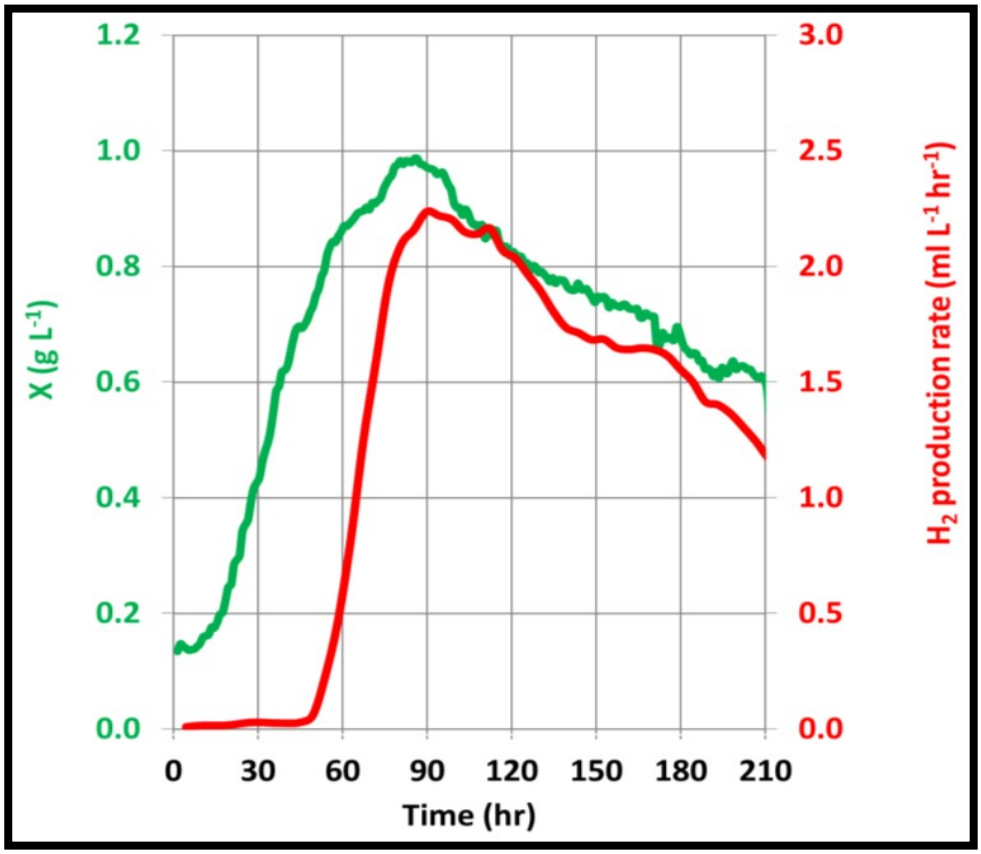

Figure 4: A Whole H_2 Production Consists of Two Interconnected Sub-Stages – (1) The Growth Stage, For First 70 hr, and (2) The H_2 Production Stage, Beginning From Time = 55 hr. Abbreviations: (1) Initial Lag (2) Exponential (3) Linear (4) Stationary and (5) Death Phases.

Biohydrogen generation by the batch cyanobacterial culture, cultivated within a novel ICL flat-plate PBR, was facilitated under very well-controlled condition. The biological response of H_2 production process to individual changes in temperature and light intensity was subsequently monitored. Glycerol was demonstrated as an effective anaerobic inducer onto cyanobacterial cultures for all of experiments. Under 35 ℃ and 92 μmol m-2 s-1, the obtained H_2 production rate of 2.13 ml H_2 /L/ hr (X = 1 g L-1) and its corresponding solar-to-H_2 energy conversion efficiency of 0.716 % are comparable to previously conducted studies. Uptake kinetics of key substrates – glycerol and nitrate – as well as cellular respiration were successfully computed using logistic models.

CONCLUSION

Due to low power density of traditional energy crops, there are significant requirements for arable land and agricultural resources (such as water and fertiliser), which has led to the food versus fuel and food versus forest issues. Photosynthetic microalgae - green algae and cyanobacteria – possess a number of superior features over terrestrial plants, and could replace crops for biofuel production. A hydrogen economy would require development of more-efficient PEM fuel cell and H_2 storage technologies as well as sustainable and economical H_2 production. The direct biohydrogen production can be carried out in three biological processes: dark fermentation, photofermentation and biophotolysis.

FUTURE PROSPECTS

The future of biological hydrogen production depends not only on research advances, *i.e.* improvement in efficiency through genetically engineering microorganisms and/or the development of bioreactors, but also on economic considerations (the cost of fossil fuels), social acceptance, and the development of hydrogen energy systems.

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I pay tribute to My Parents for lifting me up till this phase of life. I thank them for their love, trust, patience, support and bearing all kind of stress to make me what I am.

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