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Optimization of Hydrogen Gas Production Conditions from *Egyptian Chlamydomonas Sp*

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Abstract

Hydrogen gas could provide attractive options as ideal fuel for a world, in which environmental friendly and economically sustainable manner. Microalgae have the ability to bio-synthesis hydrogen gas. Algal H_2 does do not generate any toxic or polluting bi-products and could potentially offer value-added products derived from algal biomass. In this work, the feasibility of coupling sulfur deprivation and light on hydrogen production by *Chlamydomonas sp* grown in photobioreactor was investigated. The cells growth, hydrogen production, total carbohydrate and chlorophyll content were determined. The results showed that, under optimum condition, algae cells were required 168 hr (7days) to reach the late logarithmic phase (the algal dry weight 4.11 g/L). Whereas the algae cells were needed about 18~22 days to reach this value (3.55 g/ L) when grow in optimum medium. The concentration of Chlorophyll (5.65%) and carbohydrate (39.46%) were accumulated in algae cells grow in S-deprives medium coupled with dark condition over that did in algae cells cultured in optimum medium. After about a 24 h of cultivation, photo-production of H_2 was observed for C. *sp* either in absence or presence of sulfate. But under sulfur deprivation coupled with dark condition, higher H_2 gas was obtained after 16 hr (7 several days) of incubation period. In new design photobioreactors (PhBRs), after 18 days of cultivation, the volume of H_2 gas in was found to be 450 ml in cells grow in sulfur-deprived culture). This value was 360 ml in cells grow under optimal condition.

Keywords: Biohydrogen gas; Microalgae; Hydrogenase enzyme and *chlamydomonas sp*.

1. Introduction

Nowadays, the world's depend mainly on limited supplies of oil, coal, and natural gas as energy sources. The main important problems that humanity faces during the last 25 years are the decrease of fossil fuels and the increase of the environmental pollution. The photosynthetic biomass, as the largest renewable resource, represents an imported idea for the solution of these problems. However, hydrogen is consider as one of the alternative fuels to meet the global energy requirements and its consumption as a fuel is completely deprived of carbon dioxide emissions as compared to conventional fuels. Thus, efficient use of hydrogen as a fuel resource is of utmost importance as a clean and high-energy gas [1]. The hydrogen metabolism pathway of green microalgae and cyanobacteria can produce H_2, in the following reactions: $2H_2O$+ light energy $\rightarrow O_2^+ + 4H^+ + 4e \rightarrow O_2^+ + 2H_2$. However, there are three pathways that could be describe in green algae: two light driven H_2-photoproduction pathways, and a third, light in dependent, fermentative H_2 pathway coupled to starch degradation [2].

Hydrogen gas is thought to be the ideal fuel for a world in which air pollution has been elevated, global warming has been arrested, and the environment has been protected in an economically sustainable manner. However, hydrogen gas can be produced by some chemical process but producing hydrogen from biomass has been declared as innovative and promising biotechnology methods. Microalgae produce hydrogen by adopting a two-stage process, in stage (1) CO_2 fixed in the presence of sunlight through photosynthesis and allowing O_2 production. In stage (2) hydrogen is produced by the degradation of stored organic compounds via bioreactor under sulfur deprivation. However, there are three methods by which hydrogen can be produced from algae, namely (1) biochemical process (microalgae can split water into hydrogen and oxygen under controlled conditions), (2) gasification (During gasification, biomass is converted into a gaseous mixture comprising primarily mixture of H_2,COand methane (CH_4), by applying heat under pressure can be separation of H from synthesis -gas and (3) steam reforming (The fermentation of algal biomass lead to produces methane gas, then by traditional steam reformation(SMR) techniques can be used to derive hydrogen from Methane).Thus, microalgae can be preferred feedstock for high energy density, feasible liquid transportation fuels. Microalgal biofuel production have much interest of scientific researchers and entrepreneurs around the world that: Asia-Pasific Economic Cooperation APEC [3], algal productivity can offer high biomass yields per acre of cultivation, [4-6]. Algae cultivation strategies can minimize or avoid competition with arable land and nutrients used for conventional agriculture [7, 8] Algae can utilize low water quality (waste water, produced water, and saline water), thereby reducing competition for limited freshwater supplies [9]. Algae can recycle carbon from CO_2 rich flue emissions from stationary sources, including power plants and other industrial emitters, and [10] algal biomass is compatible with the integrated biorefinery vision of producing a variety of fuels and valuable adding by-products.

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Microalgae are the eventual approaches that make hydrogen production cost-effective and sustainable. The marine algae are considered an important biomass source; however, their utilization as an energy source is still quite low. In more recent, the microalgae biotechnology is grown basis on biology and genetics interaction results for production now industrial products attractive to commercial enterprises for agriculture, human health, and the production of fine chemicals. Therefore, the importance of being able to harness biotechnology approaches to generate algae with desirable properties for the production of biofuels and co-products. However, the basic biology of algal growth and metabolite accumulation using modern analytical approaches will provide a wealth of hypotheses for potential algae species improvements to produce hydrogen, the methods of cultivation, harvesting (developing low-energy methods to harvest microalgae cells) and extraction (low cost-effective bioenergy carrier extraction techniques) of bio-products process of algae in commercial settings is important to as novel biotechnology. This is means that in order to meet the energy demand of Egypt an unfeasibly large area of land must be set aside for biofuel and this not would certainly compromise of food production. Is it possible, therefore, in photobiological hydrogen production, the bioreactor design, hybrid, and integrated systems, metabolic engineering, and associated genetic manipulations that would be needed to make bio-hydrogen a commercially viable fuel for the global economy. Finally, in order to optimize the bio-hydrogen production process microalgae such *as C. reinhardtii*, different strategies have been suggested, like optimization of the light intensity , pH regime, and nutrient medium composition (nitrogen or sulphate).

2. Materials and Methods

2.1. Sample Collection and Isolation of Microalgae

Freshwater samples (150 mL) were collected from three location of Nile river. Microalgae samples (50 mL) were enriched with 50 mL of basal standard medium (BSM). The pH was adjusted to 7.5 with 1 M NaOH before autoclaving and the addition of microalgae. The microalgal cells were maintained in basal standard medium (BSM) for three weeks in a controlled culture room at 25 ± 1 ◦C with 12:12-h light-dark cycles using 100 µE·m−2 ·s −1 intensity of cool-white fluorescent light and continuous agitation at 110 rpm.

After initial cultivation of the mixed cultures, unicellular microalgae were subjected to isolation by the cell washing. Briefly, a 20-µL aliquot of the mixed microalgae culture was placed on a sterile glass slide under an inverted microscope to pick up a single microalgae cell with a Pasteur pipette containing a cotton-wool filter and connected to a flexible plastic hose. Each microalgae cell was then sequentially washed in six 20-µL drops of basal standard medium (BSM). Subsequently, the drop with a single microalgae cell was transferred to a test tube with 1 mL of basal standard medium (BSM), followed by inoculation onto Petri plates containing basal standard medium (BSM) supplemented with 1.5% (w/v) of agar. Repeated streaking on the nutrient agar plate and routine microscopic examination ensured the purity of the cultures. Single individual colonies appearing on plates (~7 days) were inoculated into BSM liquid medium and grown for 14 days at the described conditions.

Morphological Identification of Microalgae Isolated strains were preliminarily identified using standard morphological features [11].

2.2. Microalgae Strains and Growth Cultivation Under Aerobic Conditions

The cultures of *C. sp* grown photoautotrophically on a basal standard medium (BSM) in Algae Unit, NRC. The composition of growth medium is shown in Table 1.The cells were grow in two 2000 mL flasks both containing 1500 mL of BSM at room temperature (28 ±2), and illuminated from two sides with cool-white fluorescence lamps, which provided an average incident light intensity of about (~20 Em^{-2}PAR). CO_2 (bubbled with ~3% CO in air) was used as a carbon source.

2.3. Cultivation of *C. sp* Cells Under Anaerobic Conditions for Hydrogen Gas Production

The concentrated the *C. sp* cultures by centrifugation and used sterile technique to transfer highly concentrated pellets into one of two bioreactors were tightly sealed , then flushed for 20 min with O_2-free N_2 gas (99.99%) and connected to custom-built gas collection cylinders, placed on magnetic stirrer and illuminated from opposite sides of the stirrer with white fluorescent light. 500 mL photoreactors each containing approximately 300mL of either regular medium or sulfur-deprived medium [12] (Table 1 and Figure 1). The pH of both culture mediums was adjusted to 7.2by the automated addition of CO_2 gas. The media was maintained by adding the fresh cultures twice a week with their respective culture medium.

After another day, to ensure sufficient concentration of cells in the two media, a haemocytometer was used to count cells in either Basel regular medium or sulfur-deprived medium as shown in Fig. (1). Cultures were grown at a temperature of 28^{o}C and at a light intensity of 70 mmol photons m^2s^{-1}, supplied on both sides. The culture was provided by bubbling a mixture of air enriched with 3% CO_2 volume fraction.

2.4. Determination of the Hydrogen Gas Produced

The gas produced was collected from the top of the reactor by displacement under water and measured daily.

2.5. Algal Growth Measurements

Algae cell density was measured by cell counting using the hemacytometer and a BH-2 light microscope (Olympus, Tokyo) operated at a magnification of 200 x.

2.6. Growth Measurements and Harvesting

The growth of *Chlamydomonas sp* was monitoring every day through cultivation period by determining the dry weight (d. w) and optical density at 680 nm methods. The cells were harvested at the stationary phase, by centrifugation at 6,000 *g* (4°C) for 15 min and the cells masses was stored at -20°C until analysis.

2.7. Quantification of Chlorophyll and Carbohydrate

Algae cells were taken directly from the culture (about 5 mL of algal suspension for each tests) and centrifuged for 5 min at 1500 ×g. The pellets were stored at -20ºC until all samples were ready for processing.

2.7.1. Chlorophyll Measurements

Total chlorophyll content was determined spectrophotometrically (Visible and Ultraviolet Absorption Spectroscopy) in 95% ethanol (v/v) by the method of Lichtenthaler and Wellburn [13].

2.7.2. Determination of Total Carbohydrates

Total carbohydrate contents was recorded on 5 ml culture samples aseptically taken from the both cultures media at both the beginning and end of the hydrogen production phase. The total carbohydrate content was determined using the phenol/sulfuric acid method, using D+ glucose as a standard [14].

2.7.3. Determination of Dissolved Oxygen

Culture dissolved oxygen was measured by the dissolved oxygen meter electrode

2.7.4. Determination of pH

Culture pH values was measured by the pH meter electrode

2.8. Gas Collection H_2 and Concentration Measurement

Gas produced by algae cells was accumulated in the inverted graduated pipe by replacing an equal volume of water (Fig. 2). The volume of H_2 was calculated, at intervals time during 169 hr.

B-New design photobioreactor for hydrogen production from microalgae as follow in: Fig. (3 and 4): Schematic diagram of microalgal photobioreactor design for hydrogen gas production as follow:

1. Magnetic sterile
2. Perspex tube (65 Cm X 10 Cm)
3. Dissolved oxygen meter electrode
4. pH meter electrode
5. Gas outlet
6. Algal sample
7. Hydrogen gas measurement
8. Water
9. Hydrogen Gas

3. Results and Discussion

The optimal cell cultivation and hydrogen production, and main nutrient element (nitrogen, phosphate and sulfate) and other inorganic trace minerals are imperative supplements for carbohydrate based feedstock's. It is known well that phosphate and nitrogen are required for optimal hydrogen production. The elements like Fe, Ni, Mg and Zn are crucial supplements and have an importance role in the enzymatic activity of hydrogen production (NiFe-hydrogenases, (FeFe-hydrogenases, and (Fe-hydrogenase) [8]. On the other hand, the traditional industrial methods for H_2 production are quite costly. It is imaginable to design bioreactors on small scale using microalgae as bioreactors are categorically a pre-requisite for large-scale hydrogen production by microorganisms [15]. So we design a photo-bioreactors depends on the use processes linked with micro-algae or cyanobacteria, which the productivity of a photo-bioreactor is light-dependent. The deferring photochemical efficiency, absorption coefficient and size, the light regime including light and dark cycles is hypothetically is much more determining than biological factors [16]. Thus, in design a photo-bioreactors, the ratio of surface to volume is a pre-requisite, is a way to access an economical, rapid multiplication and high density of the microalgae culture [17].

3.1. Cultivation of *Chlamydomonas* in a Small Bioreactor for Hydrogen Production

3.1.1. Effect of Sulfur-Deprived and Light on *Chlamydomonas* Growth

The effect of light and sulfur-deprivedon the algae cells growth in term of total count (x 10^6) was presented in Table 2 and fig. 5 and 6. Algae cells quant's at the beginning of experiment to the late growth phase were recorded. Algae cells cultured in dark or light only neededabout48 hr to reach the late logarithmic phase at the density of about 20× 10^6 cells/mL. Then, the algae cells cultured in light were tented to increase over than that in cells grown in the dark. For example, at 144 h incubation time, the cell density in light (55 × 10^6 cells/mL) was about 1.5 times that did in the dark (35 × 10^6 cells/mL). At the late growth phase (168 h incubation), the count of cells were found to be 65x 10^6 compared with that of 40x10^6in culture grow in dark condition. However, the counts of algae grown under

coupled combination of Sulfur-deprived either with + light (SDL) or dark condition SDD) were quite different. This action indicated that growth of algae cells was induced during light illumi

3.1.2. Effect of Sulfur-Deprived and Light on Hydrogen Production by *Chlamydomonas*

As shown in Table 3, *C. sp* cultures were able to produce H_2 under all growth conditions. Hydrogengas production in the bioreactors, measured by the displacement of and fig. 7 and 8 water in inverted graduated cylinders (mL), appeared around 24 h after the establishment of anaerobic cultures, around ~ 4-10 h in all culture (data not shown). [18] reported that algae grown under photoautotrophic had high ability to produceH_2 soon after the establishment of anaerobic condition. However, H_2(the solubility of H_2 is 754 nmol ml at 28°C in H_2O) is takes time to saturate the culture liquid and build enough pressure to displace water in the collecting system. Therefore, the about 7 h delay in the start of visible H_2in photoautotrophic. The initial rates of visible H_2 production were about ~5 ml (10 h)in photoautotrophic cultures. The high output of H_2wasobserved in algae cultures grown in S-depressive condition coupled with dark (195± 5 ml) then in S-depressive cultures coupled with light (100± 3 mL) illumination (Table 3). Photoautotrophic cells produced about 2 times under dark conditions. In general, production of hydrogen gas was direct proportion to Sulfur-deprived. Algae cells grown in percent sulfur produce low hydrogen during light illumination that the sealed culture maintained aerobic condition and hydrogenase gene could not be induced (Fig. 7 and 8), which might result from the liquid phase shifted into anaerobic condition a head of the gas phase [18]. Kosourov, *et al.* [19], reported that the hydrogen production of green algae is dependent on hydrogenase gene expression and electron transportation from ferredoxin to hydrogenase. In *C. sp* about 80% of electrons are needed by sulfur-deprived, which comes from PSII-catalyzed H_2O oxidation, while the remaining electrons were most probably generated from endogenous substrate degradation. Thus, the culture growth medium played an essential role in the algae hydrogen production. Moreover, it could be noted that hydrogenase enzyme activity and electron transport chain in mitochondria clearly and significantly playing an important role in hydrogen production [18].

3.2. Carbohydrate and Chlorophyll Contents

As shown in Table 4, more Carbohydrate (CAR) was accumulated in algae cells grow under dark condition in either SD or SSD medium when compared to algae cultures grow under continuous light illumination. The highest content of CAR content in algae cells were 39.14 ± 6.54%, and 36.54% in SSD and RSD cultures, respectively. While, under light illumination, these values were 27.32 ± 7.42 and 30.12 ± 5.16%in cells grow in SSL and SSL, respectively. Under illumination condition, *Chlamydomonas* grow in normal (3.76%) or S-deprived medium (3.45%) had approximately the similar the total chlorophyll contents. While, under dark condition, the cells grow in the S-deprived medium (5.65%) had a high Chlorophyll content (Table 4) than that did in normal culture (4.66 %).

The carbohydrate and other bio-molecule catabolism was need for sulfur-deprived *C. sp* H_2 production. The change in the total cellular carbohydrate and chlorophyll contents in cultures of S-deprived microalgae was reported. The good correlation between H_2 production and start of anaerobic condition was observed, than that in H_2 production and CAR accumulation under degree of light illumination [20]. According to Tsygankov, *et al.* [20], the accumulation of carbohydrate (as starch) varies during growth of algae under light–dark cycles, the cells harvested after 4 h of light had the highH_2 production due to a high starch accumulation.

3.3. Cultivation of *Chlamydomonas sp* in a New Design Photobioreactor for Cultivation of Microalgae under Anaerobic for Hydrogen Gas Production

In the second experiment, total counts and photoproduction of H_2 gas has been examined in either normal or sulfur-deprived *Chalmydomonas* cultures under illumination conditions, placed in new design photobioreactors (Fig 3 and 4, PhBRs) for 18 days. The results demonstrate that algae cells counts ml^{-1} (10^{-6}) increased gradually as a function of incubation time (Table 5). Under illumination condition, the total counts of cells grow in normal medium was high as that did in Sulfur-deprived Medium, at intervals incubation time. After 18 days, these values were $65x10^{-6}$ and $50x10^{-6}$, respectively. However a good statistical coloration was found between algae cells counts ml^{-1} (10^{-6}) and interval incubation time, with R^2 ranged from 0.956 to 0.979 (Fig. 9).

In new design photobioreactors (Fig 3 and 4, PhBRs), the production H_2 gas in algae cultures for 18 days, has been examined in either normal or sulfur-deprived *Chalmydomonas* cultures under illumination conditions. Remarkably, at all interval incubation time (18 days), H_2 gas per ml of the algae sulfur-deprived culture is the much high yield ranges 15 – 450 ml(table 6 and fig. 10) over that reported for a normal culture (10 – 360 ml). Thus, the production H_2 gas by algae cells cultures is high affected by growth in PhBR. These experiments in combination with studies of the direct production in normal flasks clearly show that H_2 production in algae mainly depend significantly, on the cultivation methods.

The total CAR and chlorophyll contents in *Chalmydomonas* cultures under illumination conditions coupled with either in normal S or sulfur-deprived (SD), in new design photobioreactors (Table 7) for 18 days was determined. The total carbohydrate (CAR) content was significantly increased in algae cells grow in illumination condition coupled with SD medium (39.54%± 6.54) when compared with that in cells grow under continuous light illumination in optimal medium (30.54% ± 6.54). Also, the higher chlorophyll content was obtained in algae cells grow in illumination condition coupled with SD medium (4.65%) than that in cell grow in optimal medium (3.94%).

4. Conclusion

We use Egyptian green microalgae *Chlamydomonas* sp for production huge amount of hydrogen gas.

Under sulfur deprivation coupled dark condition, showed a highest hydrogen gas after 16 hr (7 several days) incubation time.

In new design photobioreactors, the production hydrogen gas in algae cultured for 18 days, showed a remarkably increased in sulfur-deprived culture.

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Fig-1. *Chlamydomonas sp* cells were grown in regular medium and sulfur-deprived medium

Fig-2. Dark anaerobic cultivation conditions for hydrogen gas production from *Chlamydomonas* sp

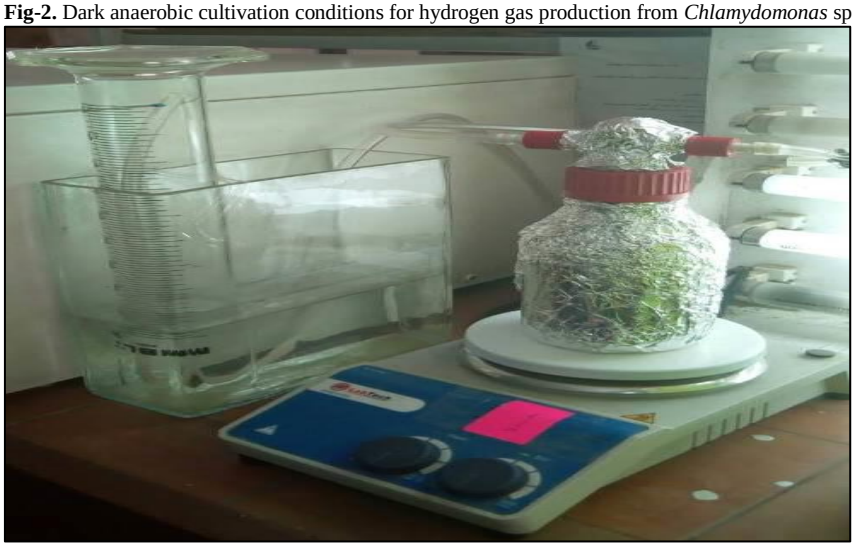
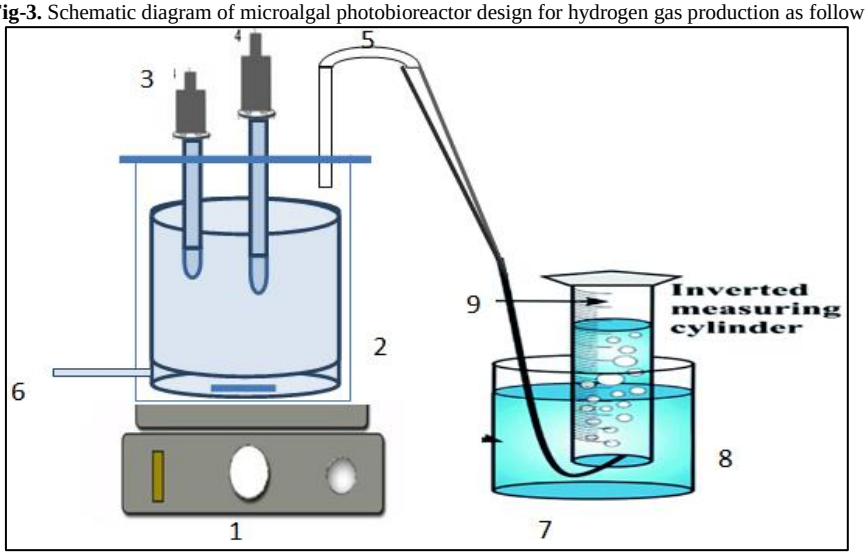


Fig-3. Schematic diagram of microalgal photobioreactor design for hydrogen gas production as follow



1. Magnetic sterile
2. Perspex tube (65 Cm X 10 Cm)
3. Dissolved oxygen meter electrode
4. pH meter electrode
5. Gas outlet
6. Algal sample
7. Hydrogen gas measurement
8. Water
9. Hydrogen Gas

Fig-4. New design photobioreactorfor cultivation of microalgae under anaerobic for hydrogen gas production

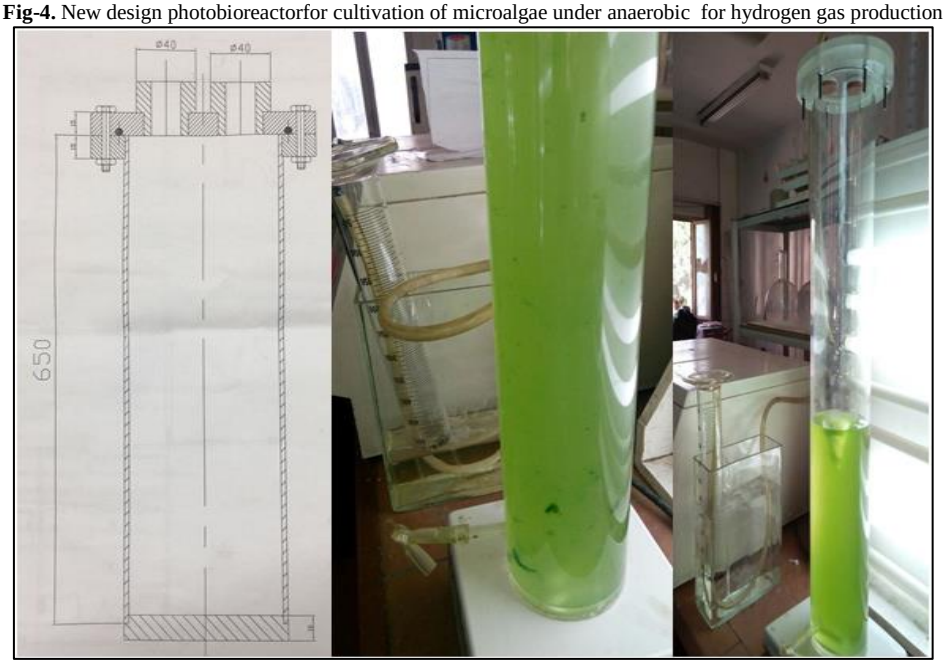


Fig-5. Changes in cells count of *Chlamydomonas* cultivated in regular medium and sulfur-deprived exposed to light and dark

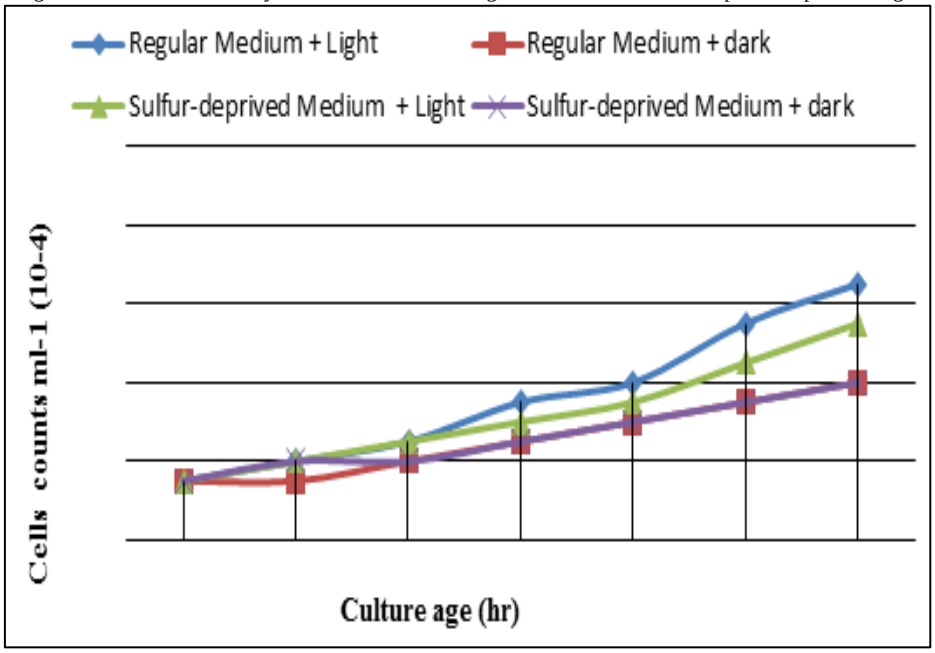


Fig-6. Effect of sulfur-deprived and light on *Chlamydomonas* growth

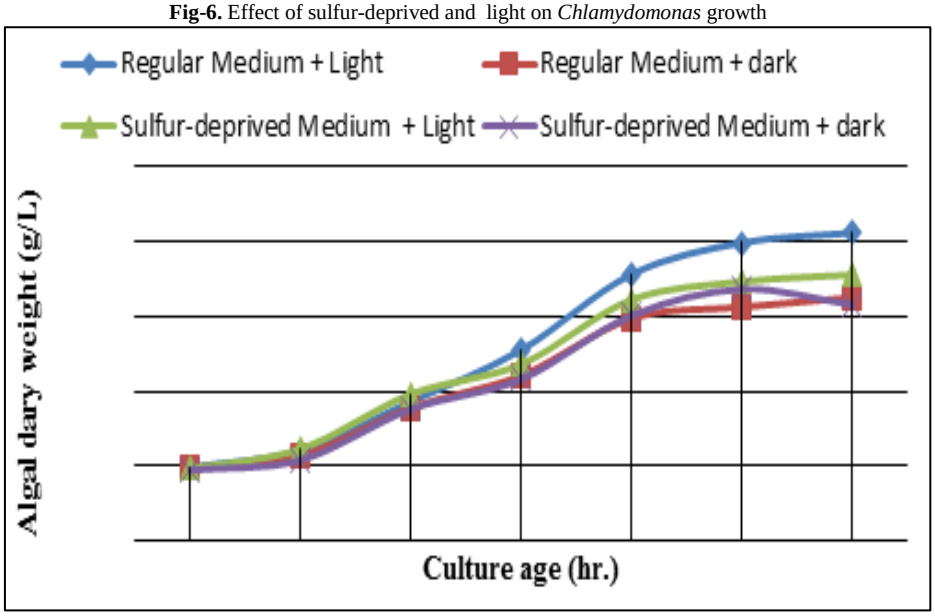


Fig-7. Mean volume of gas produced by Chlamydomonas displacement in graduated cylinder in different media exposed to light and dark

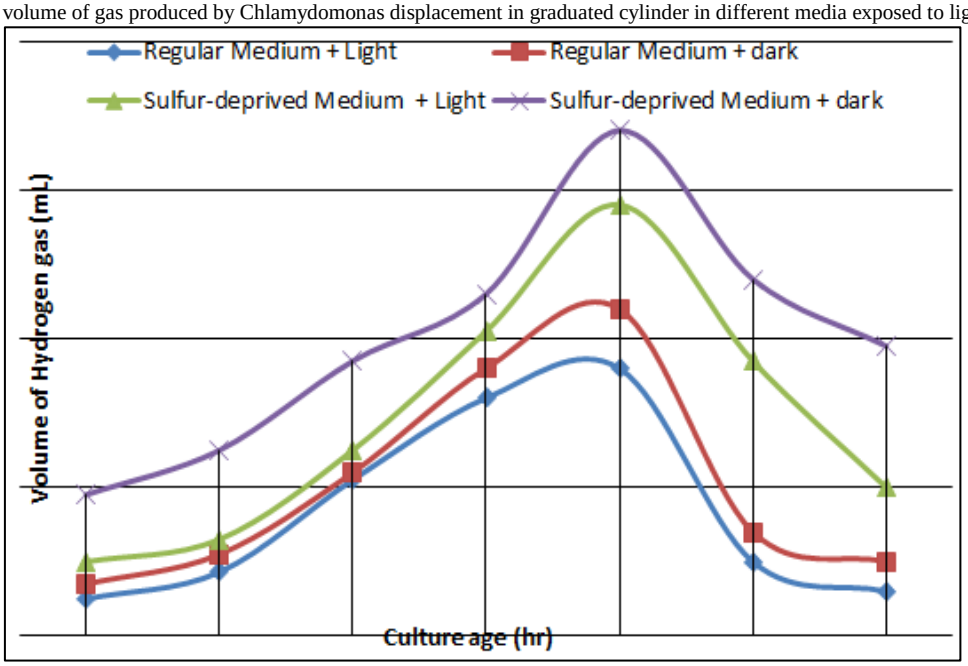


Fig-8. Mean volume of gas produced by Chlamydomonasri displacement in graduated cylinder in different media exposed to light and dark at 120 hr

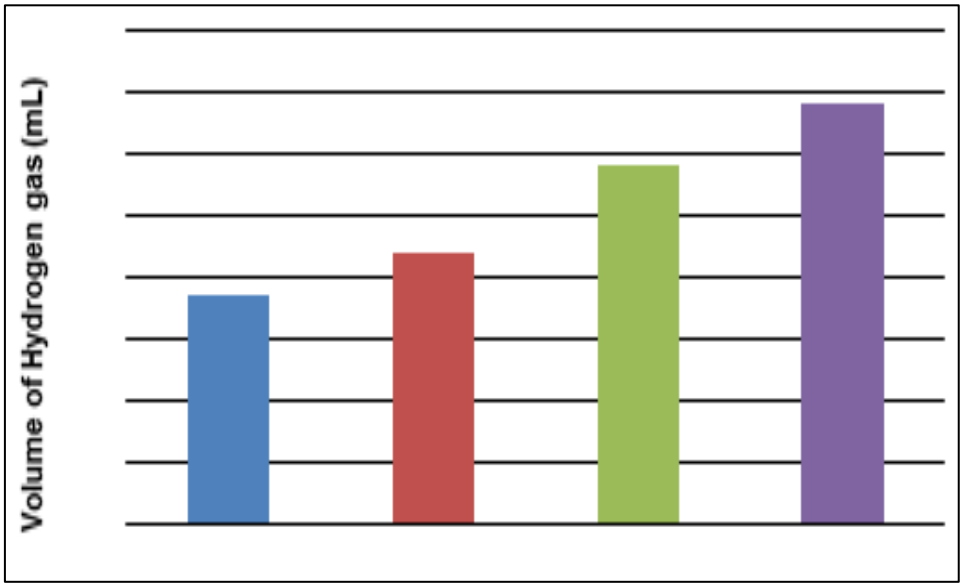


Fig-9. Changes in cells counts of Chlamydomonas cultivated new design photobioreactor for cultivation of microalgae under anaerobic and Sulfur- deprived for hydrogen gas production

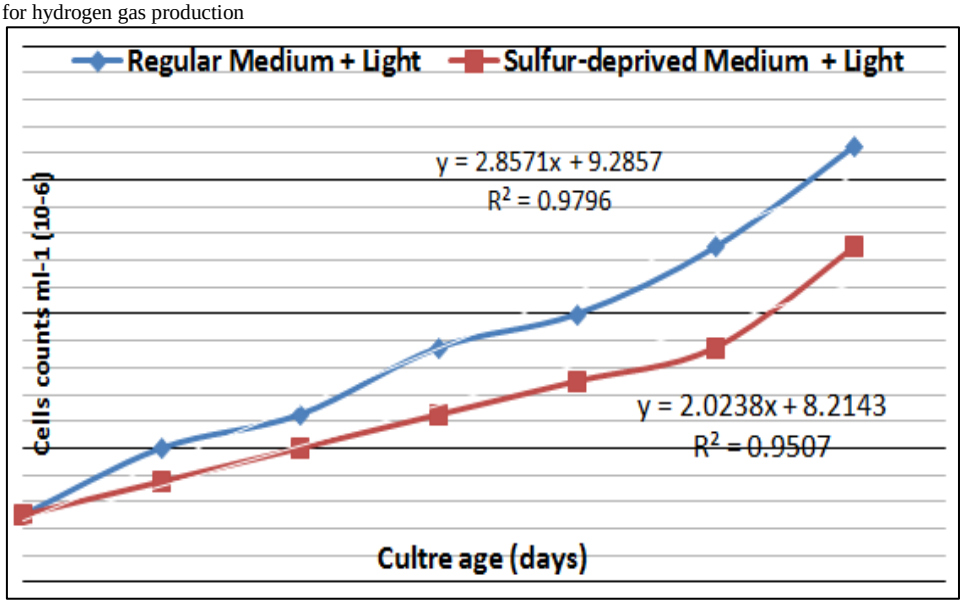


Fig-10. Mean volume of gas produced by Chlamydomonas cultivated new design photobioreactor under and aerobic and Sulfur-deprived displacement in graduated cylinder

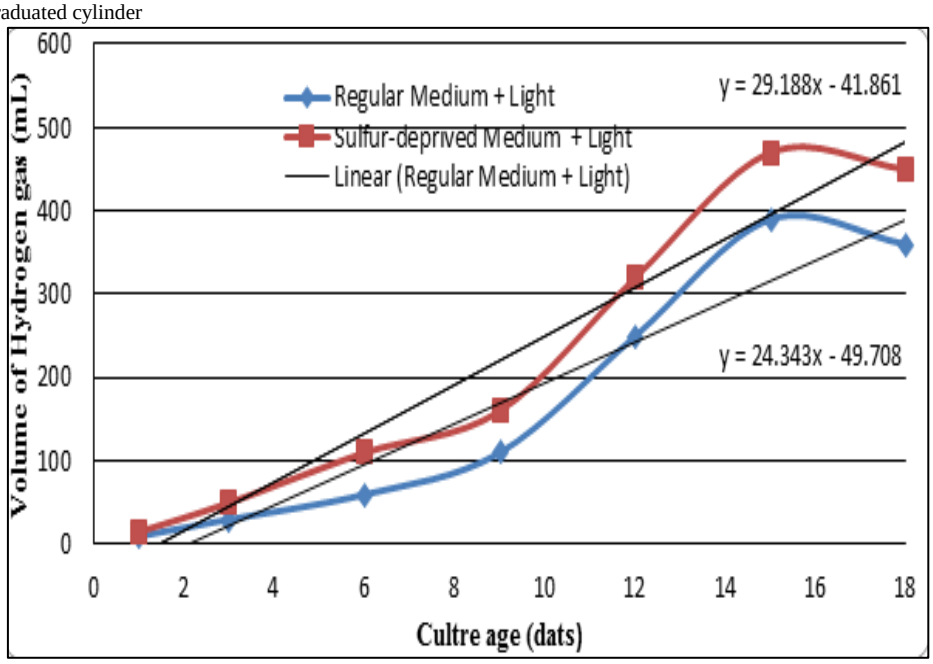


Table-1. Medium recipe for regular medium and sulfur-deprived medium

Regular Medium		**Sulfur-deprived Medium**	
Stock Solutions	**Concentrations (mg/L)**	**Stock Solutions**	**Concentrations**
KH_2PO_4-$7H_2O$	100	KH_2PO_4-$7H_2O$	100
K_2HPO4	130	K_2HPO_4	130
$FeCl_3$	12.5	$FeCl_3$	12.5
$MgSO_4$-$7H_2O$	300	$MgCl_2$-$6H_2O$	247
$CaCl_2$	47.5	$CaCl_2$	47.5
H_3BO_3	4.0	H_3BO_3	4.00
$ZnSO_4$-$7H_2O$	4.0	$ZnCl_2$	0.474
$MnSO_4$-4H2O	1.6	$MnCl_2$-$4H_2O$	0.266
COCl2-6H2O	0.8	$COCl_2$-$6H_2O$	0.800
$CuSO_4$	0.16	$CuCl_2$-$2H_2O$	0.0427
NH_4Moltbdate	0.8	NH_4Moltbdate	0.80
Na_3citrate-2H2O	100	Na_3citrate-$2H_2O$	100
NH_4NO3	300	NH_4NO_3	300

Table-2. Changes in cells counts of *Chlamydomonas sp*

	in regular medium and sulfur-deprived exposed to light and dark			
Culture age (hr.)	**Cells counts ml^{-1} (10^{-6})**			
	Regular Medium+ Light	Regular Medium + dark	Sulfur-deprived Medium + Light	Sulfur-deprived Medium + dark
24	15	15	15	15
48	20	15	20	20
72	25	20	25	20
96	35	25	30	25
120	40	30	35	30
144	55	35	45	35
168	65	40	55	40

Table-3. Mean volume of gas produced by *Chlamydomonas* displacement in graduated cylinder in different media exposed to light and dark

Culture age (hr.)	Regular Medium + Light	Regular Medium + dark	Sulfur-deprived Medium + Light	Sulfur-deprived Medium + dark
	Volume of Hydrogen gas (mL)	Volume of Hydrogen gas (mL)	Volume of Hydrogen gas (mL)	Volume of Hydrogen gas (mL)
24	25	35	50	95
48	43	55	65	125
72	105	110	125	185
96	160	180	205	230
120	200	220	290	340
144	50	70	185	240
168	30	50	100	195

n=3 for each treatment

Table-4. Total Chlorophyll and total carbohydrates content of *Chlamydomonas* sp cultivated in regular medium and sulfur-deprived exposed to light and dark

Cultivation comedians	Total Chlorophyll (%)	Total carbohydrates (%)
Regular Medium + Light	3.45	27.32
Regular Medium + dark	4.66	36.54
Sulfur-deprived Medium + Light	3.76	30.12
Sulfur-deprived Medium + dark	5.65	39.46

Table-5. Changes in cells counts of Chlamydomonas cultivated new design photobioreactor for cultivation of microalgae under anaerobic and Sulfur- deprived for hydrogen gas production

Culture age	Cells counts ml^{-1} (10^{-6})	
(days)	Regular Medium + Light	Sulfur-deprived Medium + Light
0	10	10
3	20	15
6	25	20
9	35	25
12	40	30
15	50	35
18	65	50

Table-6. Mean volume of gas produced by Chlamydomonas cultivated new design photobioreactor under and aerobic and Sulfur-deprived displacement in graduated cylinder

Culture age	Volume of Hydrogen gas (mL)	
(days)	Regular Medium + Light	Sulfur-deprived Medium + Light
1	10	15
3	30	50
6	60	110
9	110	160
12	250	320
15	390	470
18	360	450

n=3 for each treatment

Table-7. Total Chlorophyll and total carbohydrates contents of Chlamydomonas cultivated new design photobioreactor under and aerobic and Sulfur-deprived displacement in graduated cylinder

Culture age (hr.)	**Regular Medium + Light**	**Regular Medium + dark**	**Sulfur-deprived Medium + Light**	**Sulfur-deprived Medium + dark**
	Volume of Hydrogen gas (mL)	**Volume of Hydrogen gas (mL)**	**Volume of Hydrogen gas (mL)**	**Volume of Hydrogen gas (mL)**
24	25	35	50	95
48	43	55	65	125
72	105	110	125	185
96	160	180	205	230
120	200	220	290	340
144	50	70	185	240
168	30	50	100	195
n=3 for each treatment				