Microbial Biohydrogen Production by *Rhodobacter sphaeroides* O.U.001 in Photobioreactor

Nitai Basak[1, 2*] and Debabrata Das[2]

***Abstract*-Pure strains of Purple Non-Sulfur (PNS) bacteria: *Rhodobacter sphaeroides* strain O.U 001 was studied for its efficiency to produce biohydrogen using the photofermentative pathways. An annular photobioreactor was being designed aiming the uniform light distribution. In prototype annular photobioreactor, at an initial pH of 6.8±0.2, temperature 32 ± 2 ◦C, inoculum volume 10% (v/v), inoculum age of 48 h, light intensity of 15±1.1 W m^{-2} and stirring at 250 rpm; the average H_2 production rate was about 6.55±0.05 ml H_2 h^{-1} l^{-1} media for *Rhodobacter sphaeroides* strain O.U 001 (initial Malic acid concentration of 2.01 g l^{-1}). The biohydrogen production was found to be microbial mixed growth associated function.**

***Keywords*-Biohydrogen, photobioreactor, photofermentation, purple non-sulfur (PNS) bacteria.**

I. INTRODUCTION

Hydrogen gas is recognized as a potential energy carrier since it does not cause the "greenhouse effect" and liberates large amounts of energy per unit weight in combustion (143 GJ $tonne^{-1}$). Hydrogen serves as a medium in which primary energy sources (nuclear and /or solar energy) can be stored, transmitted and utilized [1]. Fermentative biohydrogen production processes are operated in ambient temperatures and atmospheric pressure, and hence less energy consuming as compared to thermo chemical and electrochemical processes [2]. In photofermentative hydrogen production, purple non-sulfur (PNS) bacteria can utilize small-chain organic acids as electron donors using light energy from sunlight alone or in combination with one or more artificial illumination [3]. Energy from light enables PNS bacteria to overcome the thermodynamic barrier in the conversion of malic acid into hydrogen. PNS bacteria have the ability to use light energy in a wide range of absorption spectra (522-860) nm and lack in oxygen-evolving activity, which otherwise might cause oxygen-inactivation problems [4]. The mechanism of photofermentative hydrogen generation in PNS bacteria is a membrane bound electron transfer process controlled by nitrogenase enzymes [5].

Optimal light utilization and optimal penetration of light in the photobioreactor is essential for higher yield of H_2 production by PNS bacteria.

Manuscript received on 14th July 2007. The work is supported by Department of Biotechnology, Govt. of India. NB author thanks the authority Dr. B. R. Ambedkar N.I.T. Jalandhar, India for granting leave and providing all possible helps for attending the WCECS 2007 conference under TEQIP.
[1]Department of Biotechnology and Leather Technology, Dr B R Ambedkar National Institute of Technology Jalandhar, India-144 011
[2] Fermentation Technology Laboratories, Department of Biotechnology, Indian Institute of Technology Kharagpur, India-721302
[*] Author for correspondence: Fax: (091) 181-2690320,
E-mail: basakn812@yahoo.com

Both Tungsten and Luminescence light can be useful with a uniform light intensity of 7.3±1.5 W m^{-2} for cell growth and 11.7±2.9 W m^{-2} for hydrogen generation in an anoxic environment. For both biomass growth and photo production of hydrogen by PNS bacteria, light intensity higher than optimum did not cause inhibition [6]. Closed tubular photo bioreactors using cyanobacteria and algae for laboratory use, pilot plant and large-scale technical production had been reported in the literature [7-9]. In the present study we report the construction of a prototype annular triple jacketed photobioreactor where the light was applied axially giving uniform light distribution through out the reactor.

II. MATERIALS AND METHODS

A. Microorganisms strain and growth conditions

Rhodobacter sphaeroides gave highest hydrogen-producing rate (260 ml mg^{-1} h^{-1}), with a photo-energy conversion efficiency of 7% [ratio of energy yielded (by combustion of produced hydrogen) to incident solar energy], determined using a solar simulator [10]. In the present study, we used *Rhodobacter sphaeroides* strain O.U.001, which was received as a gift from Dr. K. Sasikala (Environmental Microbial Laboratory, JNTU, Hyderabad, India). The cells are ovoid, 0.5–0.7 µm wide and 2–2.5 µm long, gram-negative and motile. The organism was grown photo-heterotrophically with DL Malic acid (1.0 g l^{-1}) and L Glutamic Acid (1.48g l^{-1}) and for H_2 production the carbon and nitrogen source were DL Malic acid (2.01 g l^{-1}) and L Glutamic Acid (0.3 g l^{-1}) in modified Biebl & Pfennig media [11]. Apart from carbon and nitrogen sources, 1 l of modified Biebl & Pfennig medium contained 0.2 g $MgSO_4.7H_2O$, 0.4 g NaCl, 0.4 g Yeast Extract, 0.5 g KH_2PO_4, 0.05 g $CaCl_2.2H_2O$, 5 ml Ferric citrate solution (0.1%wt./vol) and 1 ml trace element solution (SL-7). The trace element solution (SL-7) contained (in mg/l) $ZnCl_2$, 70; H_3BO_3, 60; $MnCl_2.4H_2O$, 100; $CoCl_2.6H_2O$, 200; $CuCl_2.2H_2O$, 20; $NiCl_2.6H_2O$, 20; $(NH_4)_2MoO_4.2H_2O$, 40; HCl (25% v/v), 1ml/l.

In all cases of media preparation, KH_2PO_4 and $CaCl_2.2H_2O$ were taken in 10% of the total volume of the media and sterilized separately, later on added into the main media in the laminar hood. The media and all glassware were autoclaved at 121 ◦C and 1.05 kg cm^{-2} steam pressure for 15 min. The initial pH of the medium was maintained at pH 6.8±0.2 [12]. The medium pH value was determined by a pH meter (EUTECH Instruments, Singapore). Cells were grown in 250 ml Erlenmeyer flasks containing 100 ml medium at 32 ± 2 ◦C and about 7.3±1.5 W m^{-2} light intensity (measured by digital Power meter, Lutron, Taiwan), illuminated by tungsten

filament lamp. The gas phase part of the flask was flushed with argon (99.99% pure) for 10 min to ensure anaerobic conditions. The strain was routinely maintained anaerobically on nutrient agar as stabbed culture.

B. Hydrogen production in batch fermentor, experimental set up

The designing of the annular prototype photobioreactor was made considering the principal demands [13] such as: (a) accommodating reactor shapes with high ratios of irradiated surface area to volume; (b) uniform flux on the irradiated surfaces; (c) high efficiency for collecting and delivering light radiation; (d) being based on existing and affordable technologies; and (e) compactness. Experiments were performed in a prototype triple jacketed annular photobioreactor (made of glass) of working volume 1 l placed on a magnetic stirring platform using the free suspended cells of the bacterial strains (Fig. 1). The water jacket was at outermost layer, attached with a circulating water bath for maintaining constant temperature (32±2°C). In the annular cylindrical space the microbial suspension culture (48 h inoculum ages) was taken. The Luminine tubular light source was in the central axis of triple jacketed cylindrical bioreactor giving 15±1.1 W m^{-2} light intensity, uniformly distributed throughout the microbial suspension. The gas phase part of the photobioreactor was flushed with argon (99.99% pure) for 10 min to ensure anaerobic conditions. For shake flask study, the experiment was performed in 1l Erlenmeyer flasks. Illumination was provided externally with a light intensity of 15±1.1 W m^{-2} at a distance of 30 cm and stirring at 250 rpm. The produced gas mixture was allowed to pass through 30% KOH solution for specific absorption of carbon dioxide.

The filtered gas was collected in a graduated water displacement system containing saline solution at ambient temperature and pressure. To check the reproducibility of the data, the three different batch processes were repeated under same experimental condition and the results were expressed as the average of the data obtained in triplicate.

C. Analytical methods

The liquid sample was collected from the sealed photobioreactor through the sampling point with respect to time to measure the cell dry mass concentration, pH (data not shown) and residual substrate (malic acid) concentration. Cell dry weight concentration was determined by centrifuging cell suspension at 10000 rpm for 20 min (Model name 4K15 and 2K15, SIGMA, Germany and for 1.5 ml eppendorf tube, SPINWIN) and then washing the pellet thrice with distilled water. Thereafter, the samples were dried at 100±5 °C until constant weight, cooled in desiccators and weighed.

The supernatant separated after the centrifugation was subjected to the analysis of residual malic acid concentration. Malic acid (as substrate) in the exhaust fermented broth was analyzed by Reverse Phase High Performance Liquid Chromatography (RPHPLC) (Shimadzu, Japan). 2–3 ml sample was filtered by 0.45 µm disposable unit, 20 µl of the sample was injected into the C-18 column (SGE make, Australia, organo-silane ligands, pore size 5µm). Dual λ absorbance detector (λ = 210 nm, Waters 2487) was used at 25°C. 50% Methanol in water (filtered, mixed and degassed) was used as the mobile phase at a flow rate of 1.0 ml min^{-1}. The usual retention time for the pure malic acid solution (concentration 1.0 g l^{-1}) was around (2.9-3.0) minute.

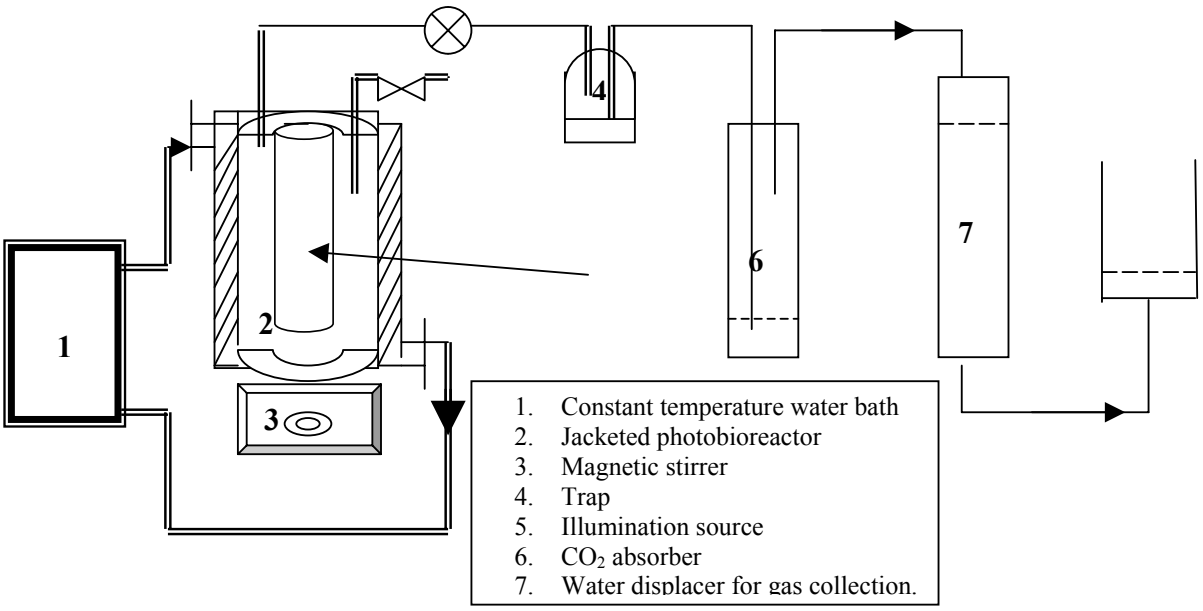

Fig.1: Experimental set up using triple jacketed prototype nnular photobioreactor used for Photofermentative hydrogen production

The same retention time for malic acid was obtained when filtered modified Biebl & Pfennig media (both before and after fermentation) were passed through same RPHPLC. The standard curve was prepared for known concentration of malic acid vs. its peak height, obtained in HPLC chromatogram.

Gas samples were taken from sampling point by gas syringe at desired time interval to measure the gas composition. The gas composition was analyzed by gas chromatograph (GC; Perkin–Elmer, USA) with thermal conductivity detector (TCD), using 80/100 Porapak-Q (3.2 mm diameter x 2 m length) column. The oven, injector and detector temperatures were 80, 150 and 200°C, respectively. N_2 was used as the carrier gas at 20 ml min^{-1} flow rate and 550 kPa pressures. The chromatogram was developed and analyzed using software, Turbochrome Navigator (version 4.1) Perkin–Elmer Corporation (Fig. 2).

The results were expressed as the average of the data obtained from experiments done in triplicate. The data was found to be reproducible within experimental limits of ±5% and the values reported in the figures were the average of the measurements. All the data obtained during the study were analyzed by Origin 6.0 software.

B. Studies of photofermentative hydrogen production in batch and suspension culture

Theoretically 6 mol of H_2 can be obtained per mole of malic acid as par following equation:

$$C_4H_6O_5 + 3H_2O \rightarrow 4CO_2 + 6H_2 \quad \ldots (1)$$

Aliquots of light-grown cells (inoculum age 48 h) , containing about 0.56–0.6 g l^{-1} cells in case of *Rhodobacter sphaeroides* strain O.U.001 was inoculated into the Hydrogen production media. Photofermentative H_2 production started after about 10 h and the total duration of H_2 production in individual batch process was 115±5 hour. The graph showing H_2 production rate with respect to time gave the measure of highest rate of H_2 production at the time t_{max} (Fig.4). It was found that: (i) the hydrogen production rate in the near approaching stationary phase was higher than that of logarithmic phase and (ii) H_2 production rate was higher in the Prototype annular photobioreactor than in the shake flask condition.

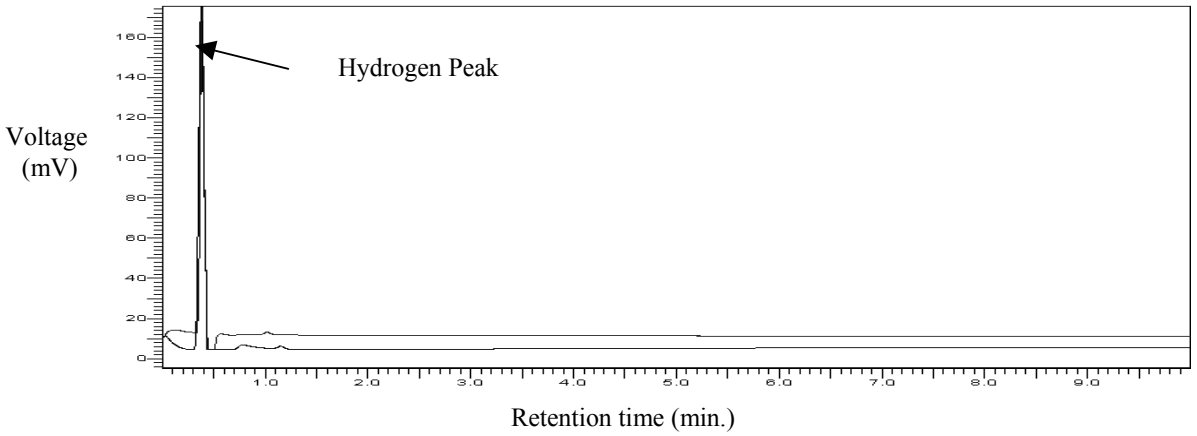

Fig.2: Gas chromatogram data by *Rhodobacter Sphaeroides* O.U. 001 in Modified Biebl & Pfennig production medium

III. RESULTS AND DISCUSSION

A. Cell growth characteristics of PNS bacterial strain

The cells were cultivated in the shake flask in anoxygenic condition with optimum condition related to carbon and nitrogen source in growth media [11], light intensity (7.3±1.5 W m^{-2}), pH (6.8±0.2) and shaking at 250 rpm. The cell growth characteristic (Fig.3) showed the starting of exponential growth phases in 48±4 h. The stationary phases were attained at 80±4 h for *Rhodobacter sphaeroides* strain O.U.001 with a cell dry mass concentration of 1.2±0.02 g l^{-1}.

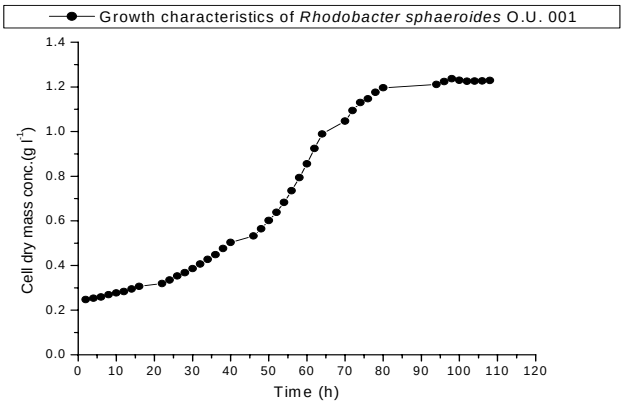

Fig.3: Growth curve of *Rhodobacter sphaeroides* O.U.001

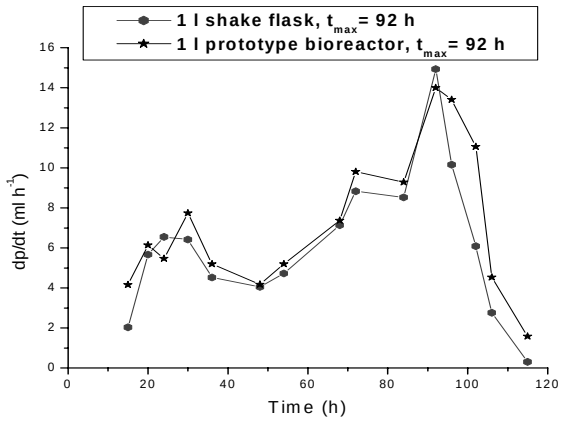

Fig.4: Hydrogen production rate (dp /dt) vs. time (t) of *Rhodobacter sphaeroides* strain O.U.001, t_{max} = 92 h, Both in shake flask and prototype annular photobioreactor

Table 1 summarized the comparative cumulative hydrogen production data by this PNS bacterium. There were substantial increases in cumulative H_2 production, average H_2 production rate, volumetric H_2 production rate, specific H_2 production rate, yield, substrate (malic acid) conversion efficiency, maximum cell dry mass concentration and cell dry mass concentration at t_{max} in the prototype photobioreactor. These attribute the fact that uniform light distribution in the annular photobioreactor made it possible to achieve the higher photochemical efficiency and productivity in photofermentative H_2 generation process using PNS bacteria.

Table 1: Comparative H_2 production value in Shake flask and annular Photobioreactor for PNS bacteria *Rhodobacter sphaeroides* O.U.001

Parameter	Shake flask	Annular Photobioreactor
Cumulative H_2 production (ml)	649.5	753.9
Maximum cell dry mass conc. (gl^{-1})	1.12	1.21
Cell dry mass conc. (g l^{-1}) at t_{max}	1.008	1.101
Average volumetric H_2 production rate (ml H_2 h^{-1} l^{-1} media)	5.65	6.55
Specific H_2 production rate (ml H_2 h^{-1} g^{-1} cell)	5.043	5.418
Yield (mol of H_2 mol^{-1} of substrate)	4.3	4.5
Conversion of Malic acid (%)	44.96	49.98

Working Volume : 1 lit
Total Production time : 115 h
t_{max} (h) : 92 h
Running time = 120 h, temperature = 32±2 ˚C, Shaking = 250 rpm, Illumination intensity = (15±1.1) W m^{-2}

C. Luedeking-Piret model for the photofermentative hydrogen production

Luedeking-Piret model (as per following equation) was employed to relate photofermentative hydrogen production rate to the cell growth [14]:

$$1/x\, dP/dt = \alpha\, 1/x\, dx/dt + \beta \quad \ldots (2)$$

Where, α = Growth associated constant
β = Non-growth associated constant (h^{-1})
P = Cumulative hydrogen production (ml)
x = Cell dry mass concentration (g l^{-1})
Specific H_2 production rate (ν) = 1/x dP/dt and Specific growth rate (μ) = 1/x dx/dt

These ν values were then plotted against specific growth rate values (μ) in the mixed growth model. The plot of specific H_2 production rate (ν) vs. specific growth rate (μ) for the combined results of the above batches (Fig.5) showed that H_2 production was mixed growth associated product for the PNS bacteria *Rhodobacter sphaeroides* strain O.U. 001 in photofermentative condition. In the present work, the α and β values for *Rhodobacter sphaeroides* strain O.U. 001 were 175.29 and 6.394 h^{-1} respectively with the R^2 value 0.9699 (Fig. 5). The model explained the possible reason of the decrease of the hydrogen production with a decrease in the cell mass at late exponential phase and its dependency on the cell growth.

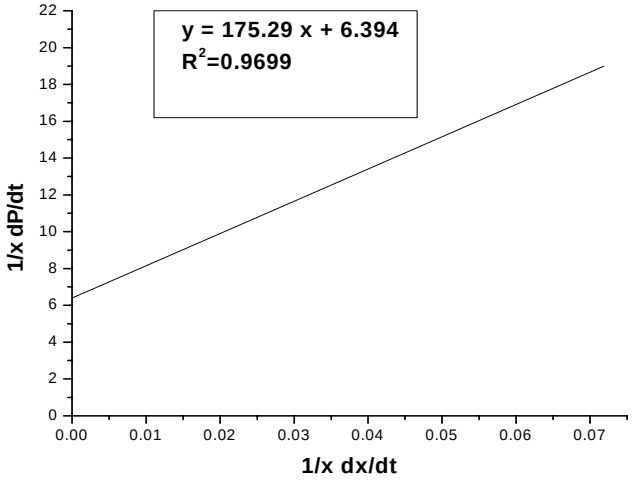

Fig. 5: Luedeking- Piret kinetic model for the hydrogen Formation kinetics in 1 l Prototype annular Photobioreactor *Rhodobacter sphaeroides* strain O.U. 001

IV. CONCLUSIONS

Rate of Hydrogen production varies even in same bioreactor in similar environment but with different working volume, because of difference in surface area of illumination.

In the present study, the prototype model of photobioreactor increased the photochemical efficiency in terms of higher percentage of substrate conversion, yield and cumulative hydrogen production rate. The obtained results had also been quantitatively confirmed by fitting of the Luedeking Piret model to the experimental data. The yield could probably have been more and the cost may be lowered if the same annular prototype photobioreactor was employed in continuous immobilized system using some suitable transparent but inert polymeric beads, instead of a free suspended batch culture. The further improvement of photofermentative H_2 production using this annular photobioreactor may be achieved by (i) maintaining low partial pressure during H_2 collection to make the reaction thermodynamically more favourable; (ii) identifying best possible transparent but inert polymeric beads for its effective utilization in the immobilized cell growth process for continuous H_2 production. Further research is now necessary to develop this laboratory study into the scale up study and thereby into practical reality for microbial photofermentative biohydrogen production.

Nomenclature

P	cumulative hydrogen production (ml)
t	fermentation time (h)
t_{max}	fermentation time at which highest rate of H_2 production was obtained (h)
x	cell dry mass conc. (g l^{-1})
x_{max}	maximum cell dry mass conc. (g l^{-1})
dx/dt	rate of change of cell dry mass conc. (g l^{-1} h^{-1})
dP/dt	hydrogen production rate (ml h^{-1})

Greek letters

α	growth associated constant for product formation
β	non growth associated constant for product formation (h^{-1})
μ	specific growth rate (h^{-1})
μ_{max}	maximum specific growth rate (h^{-1})

REFERENCES

[1] D. Das and T.N. Veziroglu, "Hydrogen production by biological processes: a survey of Literature", *Int. J. Hydrogen Energy*, vol. 26, 2001, pp. 13-28.

[2] J.R. Benemann, "Feasibility analysis of photobiological hydrogen production", *Int. J.Hydrogen Energy*, vol. 22, 1997, pp. 979-987.

[3] K. Nath and D. Das, "Improvement of fermentative hydrogen production: various Approaches", *Appl. Microbiol. Biotechnol.,* vol. 65, 2004, pp. 520-529.

[4] I. Akkerman, M. Janssen, J. Rocha and R.H. Wijffels, "Photobiological hydrogen production: photochemical efficiency and bioreactor design", *Int. J. Hydrogen Energy*, vol. 27, 2002, pp. 1195–1208.

[5] N. Basak and D. Das, "The prospect of purple non-sulfur (PNS) photosynthetic bacteria for hydrogen production: the present state of the art", *World J. Microbiol. Biotechnol.,* vol. 23, 2007, pp. 31-42.

[6] K. Sasikala, Ch. V. Ramana and P.R. Rao, "Environmental regulation for optimal biomass yields and photo production of hydrogen by *Rhodobacter sphaeroides* O.U.001", *Int. J. Hydrogen Energy*, vol.16, 1991. pp. 597-601.

[7] Y.K. Lee and C.S. Low, "Effect of photobioreactor inclination on the biomass of an outdoor algal culture", *Biotechnol. Bioeng.* , vol.38, 1991. pp. 995-1000.

[8] O. Pulz and P. Kretschmer, "Perspectives of phototrophic microorganisms in environment protection and ecology", *Acta Biotechnol.* , vol. 6, 1993, pp. 517-520.

[9] O. Pulz and K. Scheibenbogen, "Photobioreactors: design and performance with respect to light energy input", *Adv. Biochem. Eng. Biotechnol.* , vol. 59, 1998, pp. 123-152.

[10] J. Miyake and S. Kawamura, "Efficiency of light energy conversion to hydrogen by photosynthetic bacteria *Rhodobacter sphaeroides*", *Int. J. Hydrogen Energy,* vol. 12, 1987, pp. 147-149.

[11] H. Koku, I. Eroglu, U. Gunduz, M.Yucel and L. Turker, "Kinetics of biological hydrogen production by the photosynthetic bacterium *Rhodobacter sphaeroides* O.U.001", *Int.J. Hydrogen Energy,* vol. 28, 2003, pp. 381-388.

[12] K. Sasikala, Ch. V. Ramana and P.R. Rao, "Regulation of simultaneous hydrogen photoproduction during growth by pH and glutamate in *Rhodobacter sphaeroides* O.U.001", *Int. J. Hydrogen Energy*, vol. 20, 1995, pp. 123-126.

[13] J.M.Gordon, "Tailoring optical systems to optimized photobioreactors", *Int. J. Hydrogen Energy*, vol. 27, 2002, pp. 1175–1184.

[14] R. Luedeking and E.L. Piret, "A kinetic study of the lactic acid fermentation: batch process at controlled pH", *J. Biochem. Microbiol. Technol. Eng.* , vol.4, 1959, pp. 231-241.