Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org
Original Research

Effect of pH on optimization of photofermentative hydrogen production by co-culture of Rhodobacter sphaeroides-NMBL-02 and Bacillus firmus-NMBL-03

A. Pandey[1*], S. Dolly[1], D. Semwal[1], A. Pandey[2]

[1] Department of Biotechnology, Motilal Nehru National Institute of Technology (MNNIT), Allahabad, U. P., India
[2] Department of Chemistry, Motilal Nehru National Institute of Technology (MNNIT), Allahabad, U. P., India

Correspondence to: anjanap@mnnit.ac.in, apandey70@rediffmail.com
Received May 20, 2016; **Accepted** May 15, 2017; **Published** July 31, 2017

Doi: http://dx.doi.org/10.14715/cmb/2017.63.6.14

Abstract: *Rhodobacter sphaeroides* NMBL-02, photosynthetic purple non sulfur (PNS) bacteria and associated *Bacillus firmus* NMBL-03 were isolated from water sample collected from 15-20 inches beneath the surface of ponds from Northern region of India in modified Sistrom's media (120 ml) containing 3 g/L malate and 1.2 g/L ammonium sulfate. The isolation was done in air tight serum bottles (120 ml) under tungsten bulb (1.8 kLux light intensity) at 30 ºC ± 2 ºC. The PNS and heterotrophic bacteria associated with the culture was purified by clonal selection method and characterized by 16S rDNA sequencing. The PNS isolate was identified as *Rhodobacter sphaeroides* NMBL-02 (ID: 1467407, Accession BANKIT: JN256030) and associated heterotroph as *Bacillus firmus* NMBL-03 (Gene Bank Accession no.: JN 256029). The effect of initial medium pH on optimization of hydrogen production was investigated in batch process. The maximum hydrogen potential and hydrogen production rate was 2310 ± 55 ml/L and 4.75 ml/L culture/h respectively using glutamate (1.7 mmol/L) as nitrogen source and malate (22.38 mmol/L) as carbon source with 76.39% malate conversion efficiency at initial medium pH 5.0. This co-culture has the ability to produce significant amount of hydrogen in the pH range of 5.0 to 10.0 with 76.39% to 35.71% malate conversion respectively.

Key words: Purple non sulfur bacteria; H_2 production; Co-culture; pH effect.

Introduction

Hydrogen is a clean and an efficient carrier of fuel. It is widely being accepted as a potential substitute for fossil fuels. Therefore, if hydrogen is to replace fossil fuels in the future, it has to be produced renewably and in large scale, through environmentally benign process. Biological hydrogen production has been studied in detail in two groups of bacteria; anaerobic photosynthetic bacteria and anaerobic fermentative bacteria (1-3). Anaerobic photosynthetic bacteria are potent hydrogen producers using organic acids as carbon source. Therefore, these bacteria are suitable candidates for large scale-production due to their high substrate conversion efficiencies and their capability of using a wide variety of substrates either for growth or hydrogen production. Biological hydrogen production is affected by many factors such as pH, carbon source, C/N ratio, phosphate levels as well as on the nature of microbial flora. In photosynthetic hydrogen production, H_2 production is driven by nitrogenase activity, which simultaneously converts molecular nitrogen (N_2) to ammonia (NH_3), thus, H_2-producing activity of photofermentative bacteria is strongly inhibited by an excessive amount of nitrogen and unfavourable pH (4,5). Both the activity and gene expression of nitrogenase are inhibited by NH_4^+ (6-8). Therefore, the hydrogen potential is greatly affected by nitrogen sources and their concentration. The optimized production of hydrogen lies in the key understanding of the responses to culture environmental changes that influence hydrogen metabolism.

Hydrogen production from conventional dark fermentation only can produce 2-4 moles hydrogen from 1 mol hexose with the production of acetate and butyrate (2,5,9,10) leading to lowering of pH which is not suitable for further fermentation. Therefore, to solve this problem the concept of co-culture (dark fermentation and photofermentation) has been tested for further hydrogen production (11-14). The major advantage of this approach is that the substrates for photofermentation are generated *in situ* by the fermenting organism and if immediately used for photofermentation would not build up to inhibitory levels. In addition to that the acidification due to the dark fermentation could be balanced by the alkalinization brought about by photofermentation (15-16). A number of studies have been examined to test this approach with co-cultures in liquid phase or co-immobilized (17). The overall yield of 7 moles of H_2 per mole of hexose was obtained (15).

The present investigation was conducted to observe the effect of initial pH on the rate and potential of hydrogen production to determine the optimum operational pH range for hydrogen production in batch operation using co-culture of *Rhodobacter sphaeroides*-NMBL-02 and *Bacillus firmus*-NMBL-03.

Materials and Methods

Microorganisms and their isolation

Rhodobacter sphaeroides NMBL-02, photosynthetic purple non sulfur (PNS) bacteria was isolated from water sample collected from 15-20 inches beneath the

surface of ponds from Northern region of India in modified Sistrom's media (120 ml) containing 3 g/L malate and 1.2 g/L ammonium sulfate. The isolation was done in air tight serum bottles (120 ml). Samples were kept under tungsten bulb (1.8 kLux) at 30 °C ± 2 °C. The isolated PNS bacteria was tightly associated with heterotrophic bacteria and purified by clonal selection method (serial dilution) followed by plating procedure and characterized by 16S rDNA sequencing using universal primers 27 F and 1492 R (Eurofins, Bangalore, India). The PNS isolate was identified as *Rhodobacter sphaeroides* NMBL-02 (ID: 1467407, Accession BANKIT: JN256030) and associated heterotroph as *Bacillus firmus* NMBL-03 (Gene Bank Accession no.: JN 256029) and co-culture of these two microbes was used for optimization studies of molecular hydrogen production at different initial pHs (5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0 and 10.0).

Media composition

One liter of modified Sistrom's media contains Macro solution, Trace elements and Vitamin solutions (19). The macro solution contained optimized concentration of 1.7 mmol/L glutamate and 22.38 mmol/L DL-malic acid as nitrogen and carbon source respectively. The pH of the media was adjusted to 7.0. Vitamin solution was added after autoclaving the media at 103.5 kPa (15 psi) pressure and 121 °C ± 2 °C temperature for 15 min.

Experimental conditions

The batch experiments were performed in air-tight stoppered 120 ml serum bottles containing 100 ml of inoculated medium under still condition with intermittent shaking. After capping the bottle with a gas-tight rubber stopper and an aluminium cover, solution was carefully deaerated with argon for 2-3 min prior to illumination. The experimental set up was maintained at 32 °C ± 2 °C and the illumination provided with 200W tungsten lamp adjusted to provide a uniform light intensity of 1.8 kLux at the surface of batch reactors. The hydrogen produced was collected in the 20 ml gas tight disposable syringes. Initial pH of media was adjusted ranging from 5.0 to 11.0 in step of 0.5/1 unit using 0.5M NaOH to study the effect of pH on hydrogen production kinetics. Initial cell concentration and pH were set to 0.40 g dcw/L (48 h grown cells) and 7.0 respectively. The experiment was monitored till 34 days. The colony forming unit per ml (cfu/ml) was calculated in 10^{-8} dilution to know the population dynamics of PNS bacteria *Rhodobacter sphaeroides* NMBL-02 and associated heterotroph *Bacillus firmus* NMBL-03 on day 2, day 10, day 15, day 25 and day 34.

Analytical method

The bacterial cell concentration was measured by UV-Vis spectrophotometer (Labomed, USA) using the standard curve where one unit of optical density at 660 nm corresponded to 0.50 g dcw/L-medium. Hydrogen gas in the collected gas was analyzed with a gas chromatograph (Agilent 7890) equipped with a thermoconductivity detector and a capillary column (HP-PLOT/Q). Nitrogen gas served as carrier and pure hydrogen gas served as standard. The oven temperature was 90 °C, and the temperature of detector and injector was 100 °C and 70 °C respectively. The analysis of organic acids was done using HPLC (Agilent 1200) fitted with C-18 column using 0.05 M KH_2PO_4 buffer at pH 2.5 using phosphoric acid with a flow rate of 0.15 ml/min for 10 min. A modified Gompertz equation [Eq. (1)] was used to fit the cumulative hydrogen production curves for each batch reactor to obtain the hydrogen production potential P, the hydrogen production rate R_m and lag phase λ (20).

$$H = P \exp \{-\exp [R_m.e (\lambda - t)/P] + 1\} \quad (1)$$

Where, H is the cumulative hydrogen production (ml), λ the lag-phase time (h), P the hydrogen production potential (ml), R_m the maximum hydrogen production rate (ml/Lh); t the incubation time (h), e the exp(1) = 2.718. The analysis was done using SYSTAT software using Newton algorithm.

The pH of the culture medium was measured with Eutech (Merck) pH meter. The final pH of the media was taken at the end of experiment i.e. 34th day and end metabolites analyzed by HPLC. The data plotted are the average of four independent experiments.

Results and discussion

Effect of pH on hydrogen production potential and rate

pH is one of the most important factors which controls anaerobic photofermentation and hydrogen production. To assess the effect of this parameter, hydrogen production experiments were conducted under the conditions specified in materials and methods section. The optimal concentration of malate (22.38 mmol/L) and glutamate (1.7 mmol/L) was used in the present study. The cumulative H_2 production and final biomass at different initial pHs (5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0 and 10.0) is shown in Fig. 1. Maximum rate of hydrogen production (4.75 ml/Lh) took place at pH 5.0 and thereafter it started declining (Fig. 2 & 3). The corresponding average H_2 production rate and lag phase obtained at different pH is shown in Fig. 3. The plot showed that the initial pH has

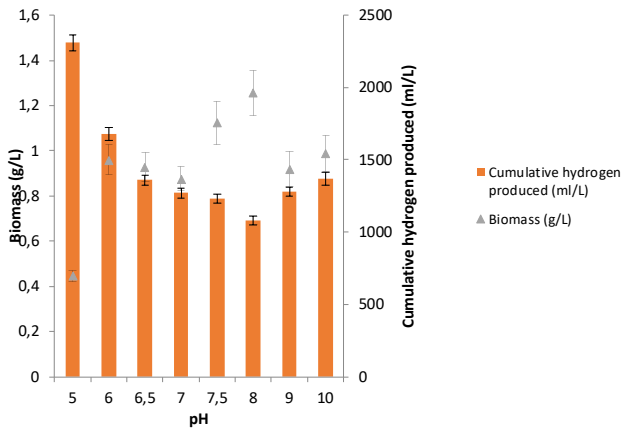

Figure 1. Effect of pH on cell concentration (biomass in g/L) and cumulative hydrogen produced (ml/L) by co-culture of *Rhodobacter sphaeroides*-NMBL-02 and *Bacillus firmus*-NMBL-03 using malate (22.38 mmol/L) as carbon source and glutamate (1.7 mmol/L) as nitrogen source at temperature 32 °C ± 2 °C under illumination of 1.8 kLux in a temperature controlled growth chamber.

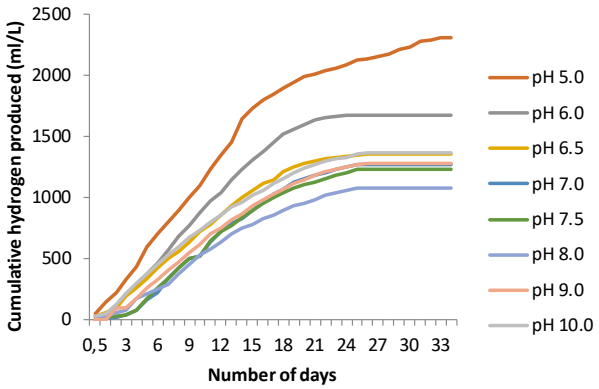

Figure 2. Effect of different pHs (5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, 10.0) on kinetics of hydrogen production by co-culture of *Rhodobacter sphaeroides*-NMBL-02 and *Bacillus firmus*-NMBL-03 using malate (22.38 mmol/L) as carbon source and glutamate (1.7 mmol/L) as nitrogen source at temperature 32 °C ± 2 °C under illumination of 1.8 kLux in a temperature controlled growth chamber.

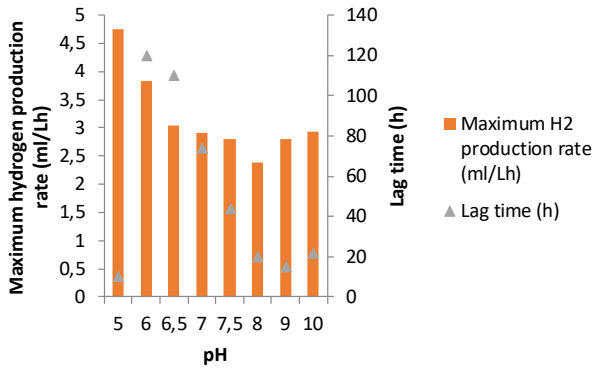

Figure 3. Effect of different pHs (5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, 10.0) on lag time (h) of hydrogen production and maximum rate of hydrogen production (ml/L culture/h) by co-culture of *Rhodobacter sphaeroides*-NMBL-02 and *Bacillus firmus*-NMBL-03 using malate (22.38 mmol/L) as carbon source and glutamate (1.7 mmol/L) as nitrogen source at temperature 32 °C ± 2 °C under illumination of 1.8 kLux in a temperature controlled growth chamber.

pronounced effect on both hydrogen production potential and hydrogen production rate. During the experiment the light intensity was kept at 1.8 kLux and the temperature was maintained at 32 ± 2 °C. The experiments were conducted at uncontrolled pH conditions. The results shown in Table 1 indicate that cumulative hydrogen production reached its maximum i.e. 2310 ± 55 ml/L at initial pH of 5.0. The results were remarkably different with respect to total gas production, gas production rates and the overall substrate conversion efficiency (Table 1) in comparison to experiments done at other pHs.

The results revealed that the initial pH did have a pronounced effect on both hydrogen production potential and hydrogen production rate. The maximum biomass yield (1.256 g/L) was at pH 8.0 with least hydrogen potential (1080 ± 28 ml/L) and % malate conversion (35.71%) and further it decreased as the pH increased from 9.0 to 10.0. At pH 5.0 maximum hydrogen potential (2310 ± 55 ml/L) and % malate conversion (76.39%) with least biomass (0.446 g/L) were obtained. The high cell concentrations decrease the available light energy within the culture through absorption and scattering effects. Maximum biomass yield was obtained at pH 7.0 in case of *Rhodobacter sphaeroides* O.U. 001 (5). The results are similar with the cell growth of *R. sphaeroides* KD 131 (21) where the biomass yield was better at the initial pH ranges of 6.8-7.2 in comparison to other pH ranges of 5.0-6.8 and 7.2-8.0 in the Sistrom's media containg DL- malic acid and glutamate. The initial pH of the medium increased from 6.5 to 8.25 whereas the initial pH of 7.0 ended up to 8.66 (5). The increase in pH during the biomass growth might be attributed due to the accumulation of polyhydroxybutyrate (PHB) in cells (18, 22). The pH changes during photosynthetic incubation of these strains exhibited similar trend, in which the pH slightly increased to 7.5-7.6 in 60-70 h of incubation and then decreased to 7.2-7.3 when hydrogen production stopped (22). Eroglu et al. (23) reported slight decrease in pH during the hydrogen production by *R.*

Table 1. Effect of initial medium pH on final pH, cumulative hydrogen produced (ml/L), duration of hydrogen production (in days), maximum rate of hydrogen production (ml/L culture/h), biomass (g/L), substrate conversion efficiency and end metabolites (fatty acids) formed by co-culture of *R. sphaeroides*-NMBL-02 and *Bacillus firmus*-NMBL-03 in batch system.

S. No.	Initial pH of the media	Final pH of the spent media	Cumulative hydrogen produced (ml/L)	Total duration of hydrogen production (days)	Maximum rate (ml/L culture/h))	Biomass (g/L)	Malate conversion efficiency (%)	Organic acids formed at the end
1.	5.0	7.0	2310 ± 55	34	4.75	0.446	76.39	butyrate, pyruvate
2.	6.0	7.58	1680 ± 44	30	3.83	0.959	55.56	butyrate, pyruvate
3.	6.5	7.62	1360 ± 34	26	3.04	0.929	44.97	butyrate, pyruvate
4.	7.0	8.16	1270 ± 32	26	2.92	0.876	42	butyrate, pyruvate
5.	7.5	8.44	1230 ± 31	26	2.79	1.123	40.67	butyrate, pyruvate
6.	8.0	8.22	1080 ± 28	26	2.38	1.256	35.71	butyrate, pyruvate
7.	9.0	8.56	1280 ± 29	25	2.81	0.918	42.33	butyrate, pyruvate
8.	10.0	8.88	1370 ± 43	25	2.93	0.987	45.3	butyrate, pyruvate

sphaeroides O.U. 001 when the culture medium contained 30 mmol/L of L-malate and 2 mmol/L of sodium glutamate with the initial pH of 7.5.

To better understand pH effect on hydrogen production, the hydrogen production rate and lag phase (λ) calculated from the plot of kinetics of hydrogen production at different initial pH (Fig 2) were plotted against the corresponding initial pH values as shown in Fig. 3. At initial pH 5.0, the hydrogen rate was maximum i.e. 4.75 ml/L culture/h, followed with declining trend upon further increase in pH. The minimum rate at 2.38 ml/L culture/h was obtained at pH 8.0. However, the hydrogen production potential and rate again increased slightly at pH 9.0 and 10.0. The lag time of hydrogen production also varied significantly by varying the initial pH of the medium. The shortest lag phase was observed at initial pH of 5.0 and 9.0 i.e. 10 h and 15 h respectively. While the lag phase obtained at pH 6.0, 6.5, 7.0, 7.5, 8.0 and 10.0 was 120 h, 110 h, 74 h, 44 h and 22 h respectively.

The results described in Table 1 indicated that hydrogen production potential reached its maximum at initial pH of 5.0. The biomass yield was minimum at that pH, while it was high at pH range of 6 to 10. It was observed during the experiment that during the growth, pH increased considerably. Final biomass concentration and pH of the spent media at the end of batch operation are also shown in Table 1. The initial pH of the medium increased for pH 5.0 to 8.0 whereas the initial pH of 9.0 and 10.0 ended up to 8.56 and 8.88 respectively. The initial medium pH of 7.5 increased to 10 in nitrogen deprived cells due to the accumulation of PHB in cells (22). The pH changes during photosynthetic incubation of *R. sphaeroides* O.U. 001 exhibited the similar trend when malic acid and glutamic acid were used as the carbon and nitrogen sources, respectively, in which the pH slightly increased to 7.5-7.6 for 60-70h of incubation and then decreased to 7.2-7.3 when hydrogen production stopped (5). Eroglu et al. (23) reported different observation in pH changes in the culture medium where a slight decline in pH occurred during the biomass growth and the pH increased during the hydrogen production by *R. sphaeroides* O.U. 001. The acid produced during the course of fermentation provides buffering effect, resulting in attainment of optimum pH level for the media having high initial pH. This observation suggests that the fermentative medium could not adapt to the rapid change in environment and thus might have been inhibited. On the other hand, at a lower initial pH level, the initial environment might not be favourable for hydrogen producers. However, with their adaptation to the environmental condition like pH, they started to produce hydrogen gradually at a moderate rate. The optimum pH for maximizing the rate of hydrogen production is dependent on both the type of microorganism and the substrates used.

Effect of pH on organic acids by HPLC analysis

The metabolite analysis of the spent media by HPLC revealed the fluctuating concentrations of fatty acids as shown in Fig. 4. The HPLC analysis of the spent media at the end of experiment indicated significant amount of butyrate and pyruvate (Fig 4). The butyrate concentration was maximum (1.34 g/L) at pH 10.0 and minimum (0.539 g/L) at pH 5.0 reflecting that maximum utilization of butyrate, the byproduct of heterotrophic partner i.e. dark fermenter (*B. firmus*) of the co-culture took place at this pH 5.0 supporting the performance and sustainability (34 days) of the batch operation for hydrogen production under this condition in comparison to the performance obtained at other pHs. At the same time at acidic pH, biomass concentration was also low with highest % malate conversion (76.39%), corroborating the optimized nitrogenase mediated hydrogen production potential i.e. 2310 ml/L. Whereas with further increase in pH or at alkaline pH butyrate concentration showed increasing trend and was maximum at pH 10.0 with correspondingly high biomass and lower hydrogen production potential and % malate conversion (Table 1). The possible explanation for enhanced hydrogen production by this co-culture is that *Rhodobacter sphaeroides* NMBL-02 utilizes DL-malic acid for its growth and hydrogen production and at the same time fixed carbon compound by it was consumed by *Bacillus firmus* NMBL-03 producing butyrate. The butyrate produced by *Bacillus firmus* NMBL-03 was detected by HPLC to show that it was further consumed by *Rhodobacter sphaeroides* NMBL-02 at appropriate pH for growth and hydrogen production. The least butyrate (0.539 g/L) at pH 5.0 substantiates the maximum hydrogen production (2310 ± 55 ml/L) and % malate conversion (76.39%). The cfu/ml of *Rhodobacter sphaeroides* NMBL-02 and *Bacillus firmus* NMBL-03 has showed the proportion 3:1 at the end of experiment. However, during the experiment till 10 days the proportion of both the organisms was 1:1. These results clearly indicate that for optimum conversion of malic acid, acidic pH is suitable using the co-culture used in the present study. However, this co-culture is also suitable for wide pH range of substrate's utilization.

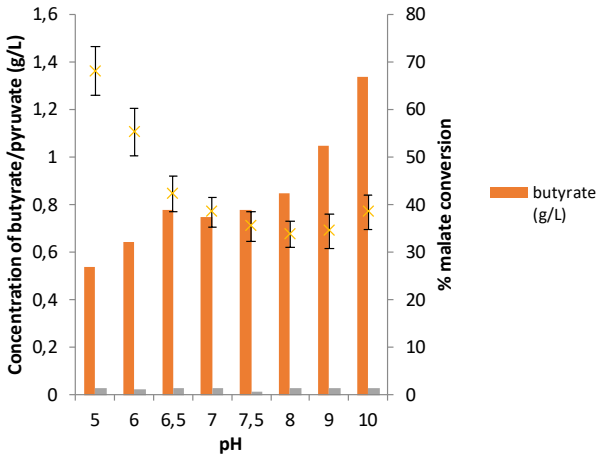

Figure 4. Effect of different pHs (5.0, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, 10.0) on concentration of butyrate/pyruvate (g/L) in spent media at the end of photofermentation and % malate conversion by co-culture of *Rhodobacter sphaeroides*-NMBL-02 and *Bacillus firmus*-NMBL-03 using malate (22.38 mmol/L) as carbon source and glutamate (1.7 mmol/L) as nitrogen source at temperature 32 °C ± 2 °C under illumination of 1.8 kLux in a temperature controlled growth chamber.

Conclusion

Hydrogen production by co-culture is significantly affected by initial pH of the optimum medium. Initial medium pH of 5.0 at a temperature of 32 °C ± 2 °C and

the illumination of 1.8 kLux was found to be the most favourable for maximizing the rate (4.75 ml/Lh) and potential of hydrogen production (2310 ± 55 ml/L). The present investigation led to determine the actual pH values that yield maximum hydrogen. This pH data could be employed in developing continuous flow reactor system for maximizing hydrogen yield by controlling the operational parameters like HRT/organic loading rate, solid retention time for specific waste treatment.

Acknowledgements
The author Dr. Anjana Pandey thankfully acknowledges the funding support received from Department of Biotechnology (DBT) and Ministry of New and Renewable Energy Sources (MNRE), Government of India, New Delhi for providing financial assistance.

References

1. Hallenbeck PC, Benemann JR. Biological hydrogen production; fundamentals and limiting processes. Int J of Hyd Energy 2002;27(11-12):1185-93.
2. Pandey A, Sinha P, Kotay SM, Das D. Isolation and evaluation of a high hydrogen producing lab isolate from cow dung. Int J of Hyd Energy 2009;34(17):7483-88.
3. Levin DB, Chahine R. Challenges for renewable hydrogen production from biomass. Int J of Hyd Energy 2010;35(10):4962-9.
4. Wang X, Jin B, Mulcahy D. Impact of carbon and nitrogen sources on hydrogen production by a newly isolated *Clostridium butyricum* W5. Int J of Hyd Energy 2008;33(19):4998-5005.
5. Nath K, Das D. Effect of light intensity and initial pH during hydrogen production by an integrated dark and photofermentation process. Int J of Hyd Energy 2009;34(17):7497-501.
6. Akkose S, Gunduz U, Yucel M, Eroglu I. Effects of ammonium ion, acetate and aerobic conditions on hydrogen production and expression levels of nitrogenase genes in *Rhodobacter sphaeroides* O.U. 001. Int J of Hyd Energy 2009;34(21):8818-27.
7. Koku H, Eroglu I, GÜndÜz U, YÜcel M, TÜrker L. Aspects of the metabolism of hydrogen production by *Rhodobacter sphaeroides*. Int J of Hyd Energy 2002;27(11-12):1315-29.
9. Hallenbeck PC. Fermentative hydrogen production: principles, progress, and prognosis. Int J of Hyd Energy 2009;34(17):7379-89.
10. Li Z, Wang H, Tang Z, Wang X, Bai J. Effects of pH value and substrate concentration on hydrogen production from the anaerobic fermentation of glucose. Int J of Hyd Energy 2008; 33(24):7413-7418.
11. Hashesh MA, Gosh D, Tourigny A, Taous A, Hallenbeck PC. Single stage photofermentative hydrogen production from glucose: An attractive alternative to two stage photofermentation or co-culture approaches. Int J of Hyd Energy 2011; 36(21):13889-13895.
12. Chen CY, Yang MH, Yeh KL, Liu CH, Chang JS. Biohydrogen production using sequential two-stage dark and photo fermentation processes. Int J of Hyd Energy 2008;33(18):4755-62.
13. Su H, Cheng J, Zhou J, Song W, Cen K. Combination of dark and photo fermentation to enhance hydrogen production and energy conversion efficiency. Int J of Hyd Energy 2009;34(21):8846-53.
14. Zhu H, Wakayama T, Asada Y, Miyake J. Hydrogen production by four cultures with participation by anoxygenic phototrophic bacterium and anaerobic bacterium in the presence of NH_4^+. Int J of Hyd Energy 2001;26(11):1149-54.
15. Miyake J, Mao XY, Kawamura S. Hydrogen photoproduction from glucose by a co-culture of a photosynthetic bacteria and *Clostridium butyricum*. J of Ferment Technol 1984;62:531-5.
16. Fang HHP, Zhu HG, Zhang T. Phototrophic hydrogen production from glucose by pure and co-cultures of *Clostridium butyricum* and *Rhodobacter sphaeroides*. Int J of Hyd Energy 2006;31(15):2223-30.
16. Asada Y, Tokumoto M, Aihara Y, Oku M, Ishimi K, Wakayama T et al. Hydrogen production by co-cultures of *Lactobacillus* and a photosynthetic bacterium, *Rhodobacter sphaeroides* RV. Int J of Hyd Energy 2006;31(11):1509-13.
17. Nath K, Muthukumar M, Kumar A, Das D. Kinetics of two-stage fermentation process for the production of hydrogen. Int J of Hyd Energy 2008;33(4):1195-203.
18. Hustede E, Steinbuchel A, Schlegel HG. Relationship between the photoproduction of hydrogen and the accumulation of PHB in non-sulphur purple bacteria. Appl Microbiol and Biotechnol 1993;39(1):87-93.
19. Pandey A, Srivastava N, Sinha P. Optimization of hydrogen production by *Rhodobacter sphaeroides* NMBL-01. Biomass and Bioenergy 2012;37:251-256.
20. Dolly S, Pandey A, Pandey BK, Gopal R. Process parameter optimization and enhancement of Photo-biohydrogen production by mixed culture of *Rhodobacter sphaeroides* NMBL-02 and *Escherichia coli* NMBL-04 using Fe nanoparticle. Int J of Hyd Energy 2015;40(46):16010-20.
21. Kim MS, Ahn JH, Yoon YS. Photobiological hydrogen production by the uptake hydrogenase and PHB synthase deficient mutant of *Rhodobacter sphaeroides*. In: Miyake J, Igarashi Y, Rogner M, editors. Biohydrogen III. Elsevier; 2004. P. 45-53.
22. Khatipov E, Miyake M, Miyake J, Asada Y. Accumulation of poly-β-hydroxybutyrate by *Rhodobacter sphaeroides* on various carbon and nitrogen substrates. FEMS Microbiol Lett 1998;162(1):39-45.
23. Eroglu K, Aslan U, GÜndÜz M, YÜcel TÜrker L. Substrate consumption rates for hydrogen production by *Rhodobacter sphaeroides* in a column photobioreactor. J of Biotechnol 1999;70(1-3):103-13.