Bio-H_2 from Wastewater of Food Industries Using Mixed Culture: Experiments and Modelling

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This article describes the performance of a single stage (SS) reactor for hydrogen generation using a mixed culture of dark (*Clostridium sp.*) and photo-fermentative (*Rhodopseudomonas sp.*) bacteria under dynamic conditions. Simulated wastewater, rich in food grade rice starch, was used as the initial carbon substrate. The effect of the ratio of the initial concentration of dark to photo-fermentative bacteria on the cumulative H2 production was assessed. The dynamic trends of concentrations of bacteria and volatile fatty acids as well as the system pH and cumulative hydrogen production were monitored. The experimental data were fitted in modified Gompertz model to comparatively assess the hydrogen production potentials of the individual bacteria and the mixed culture used in the SS reactor. A clear enhancement of H2 production of the order of three fold was obtained from the SS system over the individual dark fermentation process when the ratio of the initial concentration of dark to photo bacteria was maintained at 1:2. The significant increase in the cumulative hydrogen production of the SS reactor compared to the bioprocess using individual bacteria could be explained by the existence of a commensal relationship between the dark and photo-fermentative bacteria.

INTRODUCTION

Biomass has been of renewed interest as a convenient energy resource because of its various favorable attributes besides its natural abundance including its high energy potential, environmentally friendly nature and complete renewability (Herbert & Krishnan 2016). Biomass can become a dependable energy vector after its conversion to more readily utilisable forms of biofuels. Biomass can be converted to fuel energy via either thermochemical (gasification or pyrolysis) or biological/biochemical (anaerobic digestion or fermentation) routes (Biswas *et al*. 2006; Biswas *et al*. 2007; Cherubini 2010). Biomass exhibits an extremely diversified nature in the context of composition and chemical characteristics depending on its origin. The most commonly available forms of biomass used for bioenergy production include lignocellulosic and starchy residues originating from forestry, agriculture, agro-forestry, food processing, agro-food and municipal sectors (Bilba *et al*. 2007; Ujor *et al*. 2014; Zhao *et al*. 2016). Among all thebiomass-derived renewable biofuels, biohydrogen has long been identified globally as the cleanest, greenest and most efficient energy source (Padovani *et al*. 2016). If implemented properly, biohydrogen can significantly contribute to achieving the much sought after global goals - i) Reduction of an excessive dependence on fossil feedstocks, ii) Abatement of GHG emission and consequent global warming (Azwar *et al*. 2014). In the last few decades, extensive researchhas been carried out on the production of biohydrogen via dark and photofermentation routes with the successful generation of hydrogen gas from various biowastes (Azwar *et al*. 2014; Ghimire *et al*. 2015; Hay *et al*. 2013). However, these processes havesome constraints including low hydrogen yields, incomplete energy recovery from the feedstocks and high operational cost of production (Ghimire *et al*. 2015). To introduce biohydrogen into the fuel market as an efficient alternative fuel to conventional fossil fuels, the production volume of biohydrogen processes need to be increased several folds with a simultaneous minimisation of production costs. A convenient solution to this problem is the systematic integration of the dark and photofermentative hydrogen production processes (Kapdan & Kargi 2006; Uyar

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et al. 2009). This integration can be done either by using a sequential two-stage (STS) system of bioreactors or a single stage (SS) bioreactor (Chen *et al*. 2008; Liu *et al*. 2010). The utilisation of the metabolic end products (volatile fatty acids, VFAs) of the dark fermentative microbes by the photofermenting microbes forms the biochemical basis of this integration. Although STS processes have already been given much research attention, SS processes have gained interestrecently. Unlike STS systems,both dark and photofermentative microorganisms are used together as mixed cultures in a single bioreactor for the simultaneous production of hydrogen from the dark and photo routes in the SS system (Ding *et al*. 2009; Liu *et al*. 2010). The cumulative amount of hydrogen yielded by the two organisms in the SS system is greater than that obtained fromindividual operations. From a general scientific viewpoint, it isexpected that the two microorganisms will have specific interactions in the system affecting the overall hydrogen outcome (Ghosh *et al*. 2016). The major metabolic interactions prevailing betweendark and photofermentative microorganisms in the SS system can be described as commensalism where the photofermentative bacteriaobtains benefits in the form ofcarbon substrates required for their growth and survival from the dark fermentative microorganisms (Ghosh *et al*. 2016). The SS system is advantageous over the STS system due tothe lesserenergy and timerequired as it uses a single bioreactor instead oftwo separate bioreactors. The wastewaters generated from different food processing industries are rich in fermentable carbohydrates and hence can be applied as a convenient feedstock in fermentative biohydrogen production (Cappelletti *et al*. 2011; Kapdan & Kargi 2006). The utilisation of suitable waste biomass or biomass rich wastewater feedstocks in place of pure carbohydrates as the initial substrate in SS systems can significantly decrease the operating costs of the bioprocess while increasing its sustainability.

The present study aimed to investigate the performance of an SS biohydrogen system constructed through the integration of a dark (*Clostridium sp.*) and photofermentative (*Rhodopseudomonas sp.*) pair of microorganisms utilised as mixed culture. The mixed culture has been applied for the production of hydrogen using carbohydrate-rich simulated wastewater of local food processing industries as an initial feedstock in small scale batch mode experiments. The identification of the exact type of interactions between the two microorganisms in the SS system has been demonstrated scientifically. The effect of some operating parameters, namely system pH, temperature, initial substrate concentration, initial ratio of concentrations of dark to photo fermentative bacteria on microbial interactions and hydrogen productivity of the SS bioreactor system have alsonbeen studied.

MATERIALS AND METHODS

Microbial Strains and Media

The dark fermentative Clostridium sp. and photofermentative Rhodopseudomonas sp. were purchased from the microbial type culture collection centre (MTCC), India. Both microorganisms were revived and maintained primarily in their specific media in 100 mL Erlenmeyer flasks. The composition of the medium for the Clostridium sp. contained glucose (5 g/L), soluble starch (1 g/L), yeast extract (3 g/L); beef extract (10 g/L), peptone (10 g/L), NaCl (1 g/L), sodium acetate (3 g/L) and L-cysteine-HCl (0.5 g/L). The pH of the medium was adjusted to 7 and was incubated at 37oC for 48 h.

The growth medium of Rhodopseudomonas sp. consisted of acetate (3 g/L), yeast extract (0.3 g/L), sodium glutamate (1 g/L), KH2PO4 (0.5 g/L), MgCl2, 6H2O (0.4 g/L), NaCl (0.4 g/L), NH4Cl (0.4 g/L), CaCl2, 2H2O (0.05 g/L), Vitamin B12 solution (10 mg in 100 mL H2O) 0.40 mL, trace element solution 1.00 ml and L-cysteine-HCl (0.3 g/L). The trace element solution was composed of ZnSO4, 7H2O (0.10 g/L), MnCl2, 4H2O (0.03 g/L), FeSO4, 7H2O, 0.2 (g/L), H3BO3 (0.30 g/L), CoCl2, 6H2O (0.20 g/L), CuCl2, 2H2O (0.01 g/L), NiCl2, 6H2O (0.02 g/L), Na2MoO4, 2H2O (0.03 g/L). The initial pH of the medium was adjusted to 7. The starter culture flask was incubated at 32±5oCunder illuminated conditions of 4.5 Klux intensity for 72 h.

The basal medium for the cultivation of a mixed culture of Clostridium sp. and Rhodopseudomonas sp. consisted of partially acid hydrolysed crude rice starch (5 g/L), yeast extract (2 g/L), sodium glutamate (1.0 g/L), KH2PO4 (1 g/L), K2HPO4 (1.5 g/L), MgCl2, 6H2O (0.4 g/L), CaCl2, 2H2O (0.1 g/L), NaCl (0.5 g/L), L-cysteine-HCl (0.3 g/L), trace element solution 1 mL and Vitamin B12 solution 0.40 mL. The pH of the medium was adjusted to 7.0 ± 0.2 using HCl or NaOH solution.

The chemicals used were purchased from E Merck and were of analytical grade. All growth media were sterilised by autoclaving at 121oC for 15 minutes. Argon gas was used for the establishment of strict anaerobic conditions in all cases.

Simulation of Starchy Wastewater and Experimental Procedures

The starchy wastewater used as initial feedstock in the individual dark fermentation and SS processes was simulated in our laboratory. Powdered crude rice starch was acidified to pH 3 with concentrated H2SO4 and boiled at 100oC for 20 minutes to facilitate partial hydrolysis of starch. The solution was then cooled down to room temperature and the pH was adjusted to 7 with NaOHbefore beingused as the carbon substrate in the individual dark fermentation processes and SS systems. The SS fermentations were conducted in small scale reactors (250 mL Erlenmeyer flasks) with 200 mL basal medium. The medium was bubbled with argon gas for 15 minutes and after inoculation, the air headspace of the flasks was replaced by argon to establish a strict anaerobic environment. The SS reactors were inoculated with a mixed biomass of dark and photofermentative microorganisms maintaining specific ratios. The mixed culture inocula were preparedusing cultures of individual dark and photo microorganisms of known biomass concentrations. The SS reactors were incubated at 32±5°C and under a light intensity of 4.5 Klux. All batch mode experiments were performed in duplicate.

Analytical Methods

The concentrations of substrate and VFAs were analysed in thesamples. The samples were centrifuged at 7000 rpm for 10 minutes and the clear supernatant was used for the analysis. The samples were acidified and boiled for 90 minutes to facilitate complete hydrolysis of starch to glucose residues. The concentration of residual glucose was determined using the 3,5-dinitro salicylic acid (DNS) method. The starch concentration was determined in terms of glucose equivalents. For the VFA analysis, a high performance liquid chromatography (HPLC, Perkin-Elmer, USA) instrument equipped with a C18 analytical column (250 × 4.6 mm) and a refractive index detector (Shimadzu, Japan) was used with a column temperature of 30oC. 8.5 mM H2SO4 was used as the mobile phase at a flow rate of 0.6 mL/min. Pure acetic acid and butyric acid were used as standards.

The produced gas mixture was collected through a graduated water column containing a solution of 50% (w/v) NaOH to remove CO_2. For the determination of the hydrogen content produced in the biogas, samples were collected from the reactor headspace with gas-tight glass syringes and analysed by a gas chromatography (GC, Thermo Scientific) instrument equipped with a TCD detector and a 2m stainless steel 5 Å molecular sieve column. Argon was used as the carrier gas at a flow rate of 70 mL/min and pure hydrogen was used as the standard. Theoperational temperatures of the detector and injector were kept at 100°C and the column temperature at 60°C.

Biomass concentrations of dark and photofermentative microorganisms in individual systems were determined using a direct dry cell weight method. 5mL of the liquid sample was filtered through a pre-weighted 0.45µm filter paper (milipore) and dried at 105°C until the weight was constant. The weight of biomass was calculated by subtracting the weight of the filter paper. For the determination of biomass concentration in the mixed biomass samples, the gram-staining method was used along with the dry cell weight method. The samples were gram-stained and the ratio of the cell numbers of dark and photo microorganisms were determinedusing a microscope (Optika, Italy). Using the ratio of the cell numbers, the respective biomass weight of dark and photo microorganisms were calculated from a combined biomass weight. The light intensity was measured using a digital luxmeter (Lutron, USA).

RESULTS AND DISCUSSION

Individual dark fermentation was performed in a batch mode using partially hydrolysed crude rice starch as the carbon substrate for the Clostridium sp., The total duration of fermentation was 50 hours. Figure 1 depicts the concentration profiles of biomass formation, substrate consumption and the cumulative formation of hydrogen. The starch concentration declined to 0.34 g/L from an initial concentration of 5 g/L, indicating a good utilisation of starch by the dark fermentative organisms. Biomass concentration increased steadily up to 30h before entering a stationary

stage which lasted till 50h with a slight decline of biomass concentration. Hydrogen production started after a lag phase of around 5h and increased accordingly with the increase inbiomass. The cumulative hydrogen production reached a maximum value of 220mL around 35h and declined thereafter. The decline in biomass growth and H2 production was becauseof adropp in the pHof the medium to below 4.5 due to the formation of volatile fatty acids (VFAs) and their accumulation in the medium (Figure 2).

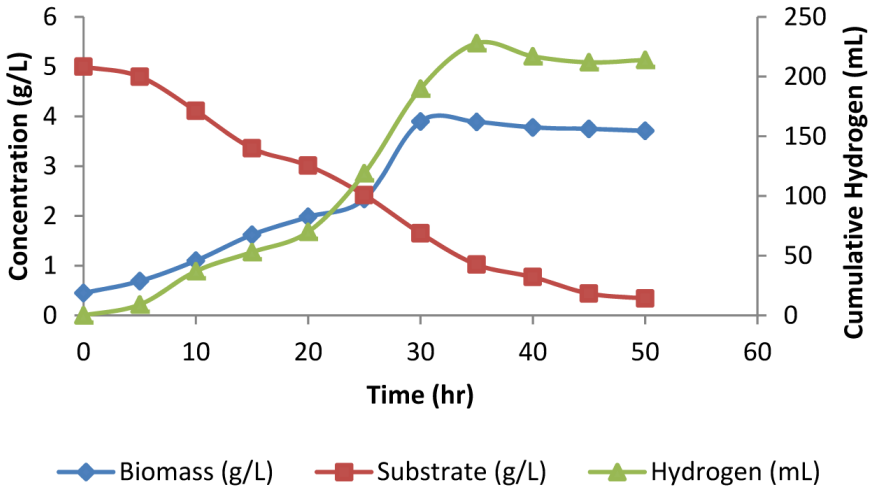

Figure 1. The biomass and hydrogen production profile along with substrate consumption.

The analysis of the liquid samples revealed that acetate was formed as the major VFA with a simultaneous formation of butyrate at lower concentrations (Figure 2). Both VFAs were observed to be growth-associatedand maximised at 30h, at which biomass growth was also at a maximum. The accumulation of VFAs in the medium resulted in a sharp decrease of pH shifting it from the functional range (5.5-7.5) and contributed to product inhibition of hydrogen formation. At this pH, the transition of the solventogenic phase of Clostridium sp. was initiated by the consumption of VFAs, which was reflected in the slight increase in pH after 35hr (Figure 2). Since acetate was the major VFA product, it could serve as the carbon substrate of the PNSB when combined in a single stage (SS) bioreactor system. The maximum amount of acetate produced in dark fermentation was 3.26 g/L (≈54mM), which was lower than the known inhibitory concentration of acetate for PNSBs (>60mM).

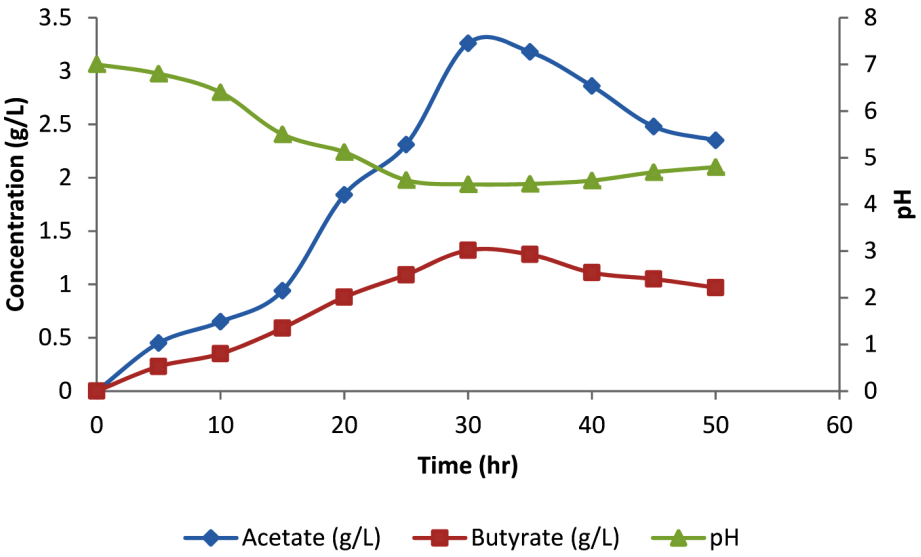

Figure 2. Production of acetate and butyrate and the change of pH of the fermentation medium.

The photofermentative production of H2 by the PNSB, Rhodopseudomonas sp., was studied using 3 g/L acetate as the carbon substrate. The PNSB consumed acetate at a slower rate but produced higher amounts of cumulative hydrogen (Figures 3 and 4). This was attributed to the fact that PNSB uses light energy to drive cellular metabolic

activities allowing a greater conversion of the carbon substrate to H2. Hydrogen formation increased exponentially up to 50h and showed no decline. According to reported research findings, the duration of hydrogen production by PNSBs could last for a few days (more than 150-170hr) (Padovani *et al.* 2016). Therefore, it was also expected that in this case, H2 production could be sustained further beyond 50h till exhaustion of the available substrates. The amount of H2 produced by Rhodopseudomonas sp. was found to be much higher (470mL) than the Clostridium sp. (Figure 4), so it was expected that their combination in a SS system could generate higher amounts of cumulative H2. The pH of the mediumof the photofermentative system tended towards a basic range due to the acetate consumption by the Rhodopseudomonas sp.

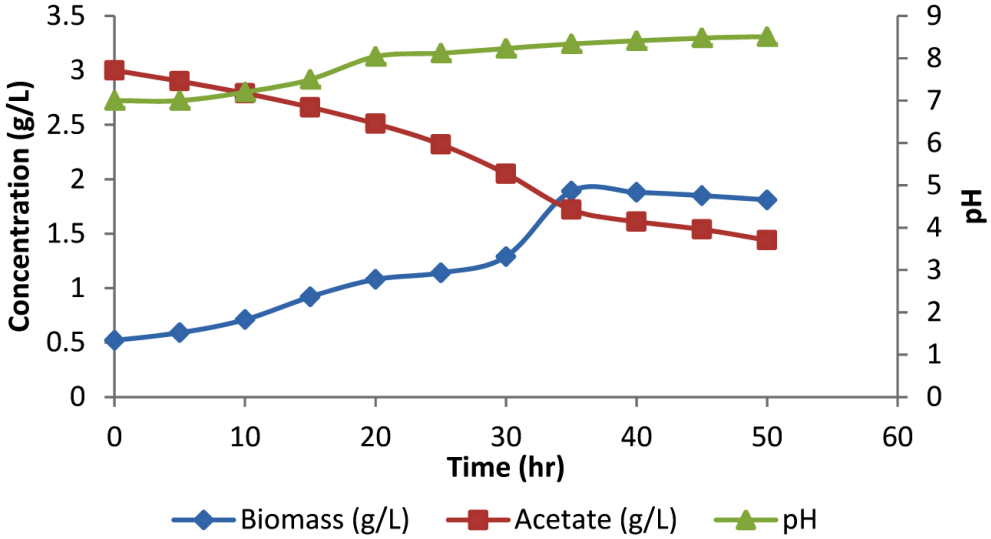

Figure 3. Biomass formation, acetate consumption and pH changes of the photofermentative system.

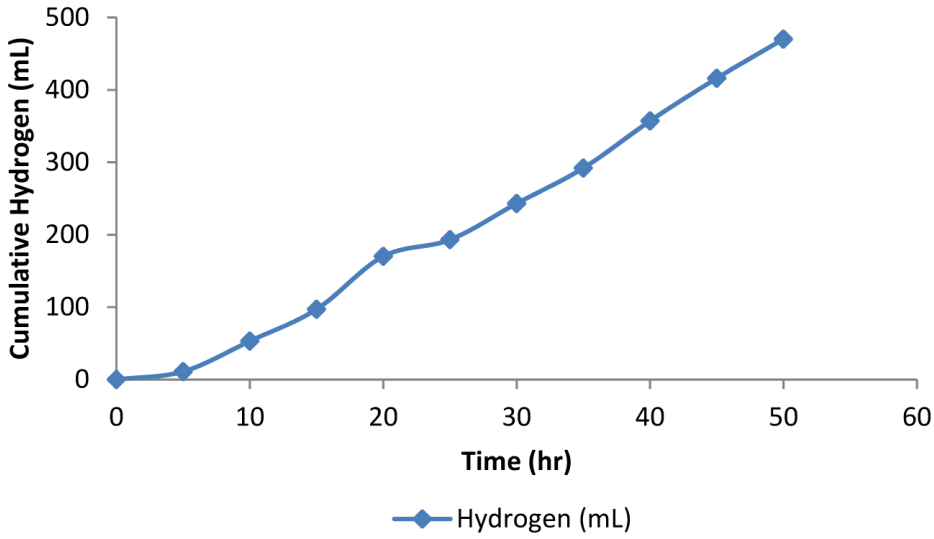

Figure 4. Cumulative hydrogen production by Rhodopseudomonas sp.

In the SS integrated system, combinations of dark and photo fermentative organisms resulted in higher amounts of cumulative H2. This was due to the co-metabolism of starch to H2 and VFAs by the Clostridium sp. and the subsequent fermentation of VFAs to H2 by the photofermentative Rhodopseudomonas sp. When the dark and photo fermentative microorganisms were mixed in a 1:1 (w/w) ratio higher amounts of H2 were obtained as compared to dark fermentation alone, as depicted in Figure 3A. In such a combined system, the dark fermentative microbes dominated the photofermentative ones, since the rate of production of acetate was higher than the rate of consumption by the PNSB. This led to the decline of pH to below 6.5 at 15hr and ultimately to 4.8 (data not shown), which severely restricted H2 production by the PNSB and thus, the lessened increment in cumulative H2.

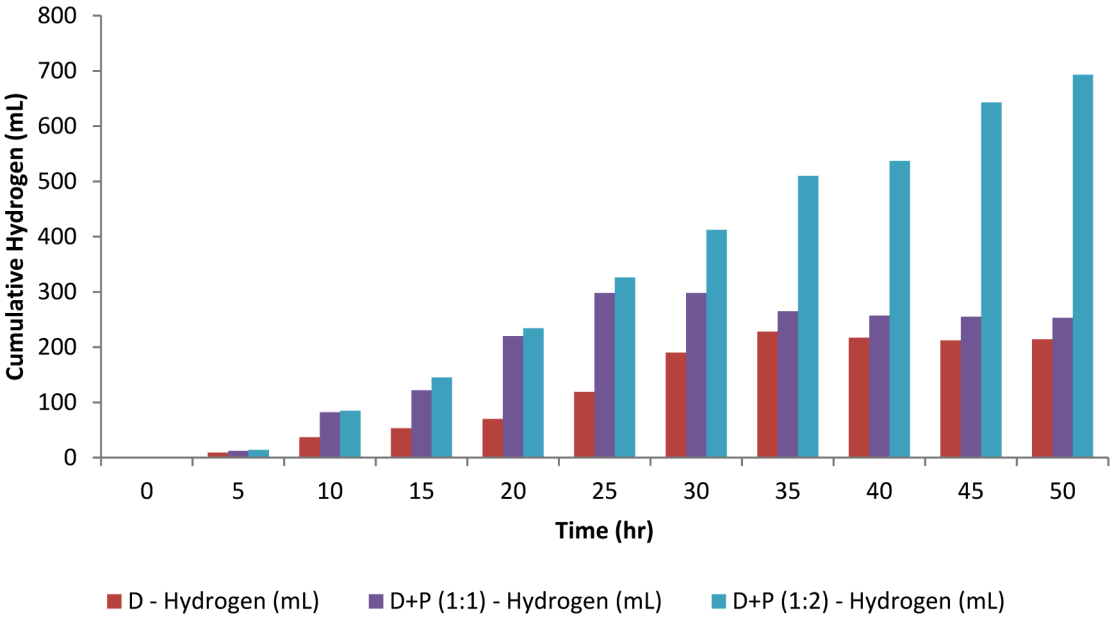

Figure 5. The comparative profile of cumulative hydrogen generation in individual dark (D) and SS systems with varying ratios (1:1, 1:2) of dark and photofermentative microorganisms (D+P (1:1), D+P (1:2)).

Increasing the concentration of photofermentative microorganisms in the mixed culture led to an increment of cumulative H2 production. When the ratio of the initial concentration of dark and photo fermentative microbes was kept at 1:2 (w/w) in the SS reactor, hydrogen production increased to higher levels (Figure 5). An almost three fold increase in the total amount of hydrogen was obtained from the SS integrated system (693mL) using a dark/photo ratio of 1:2, as compared to individual dark fermentation (228mL). In this case, the acetate consumption increased due to the higher amounts of photo fermentative microbes in the system, which in turn regulated the pH to near the optimal value of the PNSB. This particular fact facilitated the active metabolic performance of the PNSB leading to a substantial production of H2. The maximum value was also significantly higher than the one obtained from individual photofermentation (470mL), although, the initial biomass concentration of the PNSB used here was lesser. These observations clearly affirmed that the integration of dark and photo fermentative microbes in the SS system enhanced hydrogen production as compared toindividual dark or photo fermentation systems. The initial biomass ratio of the two microorganisms plays a crucial role in the performance of the SS system and hence should be optimised more stringently in order to enhance combined H2 production.

All the experimental data obtained for cumulative hydrogen production in individual dark and SS systems have been correlated with the modified Gompertz equation (Argun *et al.* 2008), expressed as follows:

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

The values of the model constants (Table 1) were determined through regression analysis where P is the maximum hydrogen production potential (mL); Rm is the maximum rate of hydrogen production (mL/h) and λ (hr) is the duration of the lag phase for hydrogen production. The tabular data shows that the highest cumulative hydrogen was produced from the SS system using a 1:2 ratio of the dark to photo microorganisms. A three-fold increase inhydrogen production in comparison to the individual dark fermentation was also clearly noticed in the same system.

Table 1. Gompertz equation constants for individual dark fermentation and SS integrated systems

System	P (mL)	R_m (mL/hr)	λ (hr)	R^2
Dark	237.4	9.58	10.3	0.9641
SS (D:P-1:1)	273.7	19.69	6.854	0.9534
SS (D:P-1:2)	852.5	18.73	7.814	0.9967

The time-wise change of the biomass of the dark and photo microorganism in the SS systems is presented in Table 2. As mentioned earlier, when a dark/photo ratio of 1:1 was used, the pH of the system ultimately fell below the functional range for the PNSB, which resulted in the stagnation of growth of the photoorganisms after 20h, hindering its H2 production. Incontrast, when the initial biomass of the PNSB was doubled in the inoculum, their growth was not inhibitedand continued to grow till 50h. In this case, the higher biomass of the photo microorganisms consumed a higher amount of acetate upon its production by the dark microbe thus maintaining the pH to a non-inhibitory range throughout the operation. These results indicated that the optimisation of the initial biomass concentrations of the dark and photo fermentative microbes could significantly enhance the H2 production performance of the SS system.

Table 2. Simultaneous growth of dark and photo microorganisms in the SS systems

Time (h)	SS Dark:Photo (1:1)		SS Dark:Photo (1:2)	
	Dark (g/L)	Photo (g/L)	Dark (g/L)	Photo (g/L)
0	0.5	0.5	0.5	1
10	0.91	0.57	0.95	1.13
20	1.87	1.08	1.75	1.95
30	3.78	1.14	3.86	2.18
40	3.58	1.18	3.66	2.39
50	3.64	1.11	3.57	2.45

CONCLUSIONS

The findings of the present study can enhance the ongoing research on SS systems in a substantial manner. Dark (Clostridium sp.) and photofermentative (Rhodopseudomonas sp.) microorganisms have been integrated in the SS system to enhance the production of hydrogen over an individual dark fermentative system. In the SS system, a synergistic growth of both microorganisms was observed indicating the prevalence of a positive interaction between them. In this case, the interaction couldbe identified as commensalism since in the SS system, the photo microbes depended on the dark microbes for their carbon source required for growth and H2 production. Moreover, the SS system generated three fold higher amounts of H2, when a balanced initial biomass of the dark/photo microbes (1:2) was used. Hence, it was clear that an interactive association of dark and photofermentative microorganisms was beneficial for H2 production in the SS system and should be studied in more detail. However, the operation of a higher scale SS system for H2 production using a mixed culture of dark/photo microbes will require a priori knowledge on the growth kinetics of both Clostridium sp. and Rhodopseudomonas sp. to facilitate efficient performance. For continuous hydrogen production using specific reactors, important operating parameters like dilution rate (based on specific growth rates) and light intensity should be optimised alongside pH, temperature, initial substrate concentration and the ratio of initial biomass of the dark to photo microbes. Proper exploitation of abundantly available prospective renewable feedstocks like carbohydrate rich food wastes and food processing wastes for H2 production in SS systems will make the process economically viable and sustainable.

ACKNOWLEDGEMENTS

Shiladitya Ghosh sincerely acknowledges the financial support extended by the World Bank in the form of a technical education quality improvement programme (TEQIP) phase II junior research fellowship.

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