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Bio-Hydrogen Production using a Two-Stage Fermentation Process

[1]W.M. Alalayah, [1]M.S. Kalil, [1]A.A.H. Kadhum, [1]J.M. Jahim, [1]S.Z.S. Jaapar and [2]N.M. Alauj
[1]Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Malaysia
[2]Yemen Gas Company, P.O. Box 13465, Sana'a, Republic of Yemen

Abstract: A two-stage fermentation process consisting of dark and photo-fermentation periods was carried out in a batch reactor. In the first stage, glucose was fermented in the dark stage using *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564; CSN1-4) to produce acetate, CO_2 and H_2. The acetate produced in the first stage is fermented to H_2 and CO_2 by *Rhodobacter sphaeroides* NCIMB 8253 for further hydrogen production in the second, illuminated stage. The yield of hydrogen in the first stage was about 3.10 mol H_2 (mol glucose)$^{-1}$ at a glucose concentration of 10 g L^{-1}, pH 6±0.2 and 37°C and the second stage yield was about 1.10-1.25 mol H_2 (mol acetic acid)$^{-1}$ at pH 6.8±0.2 and 32°C, without removal of the *Clostridium* CSN1-4. The overall yield of hydrogen in the two-stage process, with glucose as the main substrate was higher than single-stage fermentation.

Key words: Dark fermentation, photo-fermentation, hydrogen production, two-stage process

INTRODUCTION

Among various processes of biological hydrogen production, anaerobic fermentation, commonly termed dark fermentation, has several advantages over other methods. Apart from its simple technology, it can utilize a wide range of substrates, both pure as well as waste products and does not rely on light availability. However, the major bottleneck of the dark fermentation process stems from a lower achievable yield of hydrogen per mole of substrate (Das and Iu, 2001). The pathways and experimental evidence cited in the literature reveal that at most four moles of hydrogen are produced from each mol of glucose consumed during acetate fermentation, as shown in Eq. 1 (Hawkes *et al*., 2002; Nath and Das, 2004). The theoretical maximum hydrogen yield from dark fermentation of glucose is 4 moles H_2 (mol glucose)$^{-1}$ if acetic acid was only Volatile Fatty Acid (VFA) formed (Yokoi *et al*., 2001; Liu *et al*., 2003; Liu and Shen, 2004; Lay, 2001).

A combination of dark and light fermentations, as shown in Eq. 1 and 2, could be expected to reach as close to the theoretical maximum production of 12 moles of H_2 (mol glucose)$^{-1}$ equivalent as possible, according to the following reactions:

- **Stage 1:** Dark fermentation by CSN1-4 (anaerobes)

$$C_6H_{12}O_6+2H_2O \rightarrow CH_3COOH+2CO_2+4H_2 \quad (1)$$

- **Stage II:** Photo-fermentation by RS8253 (photosynthetic bacteria)

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

Some studies have combined dark fermentation with photofermentation to enhance hydrogen productivity from food processing waste water and sewage sludge. One way to overcome the economic restriction of biological hydrogen production is to associate this process with waste treatment, as practical applications of hydrogen production cannot utilize expensive synthetic culture media (Yokoi *et al*., 2002; Han and Shin, 2004).

Renewable resources, such as biomass and agricultural and domestic wastes, are attractive raw materials for bio-hydrogen production on a large scale. Sequential dark and light anaerobic fermentations are used for bio-hydrogen production from carbohydrate-rich biomasses or waste materials. The first step is the enzymatic hydrolysis of the biomass to a highly concentrated sugar solution, followed by dark fermentation by acetogenic-anaerobic organisms to produce (VFA), hydrogen and CO_2, the organic acids produced by dark fermentation can be further fermented by the photo-heterotrophic bacteria such as *Rhodobacter* spp. to produce CO_2 and H_2 (Kapdan and Karig, 2006; Manish and Banerjee, 2008; Kotay and Das, 2008; Ni *et al*., 2006).

Corresponding Author: M.S. Kalil, Department of Chemical and Process Engineering, Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Malaysia Tel: +60389216419 Fax: +60389216148

In present study, *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564; CSN1-4) and *Rhodobacter sphaeroides* NCIMB 8253 (RS8253) were used in a two-stage fermentation process for maximization of hydrogen yield via combined dark and photo-fermentation processes, respectively. Glucose was fermented by CSN1-4 in the first stage to produce acetate and then consumed by RS8253 to produce hydrogen.

MATERIALS AND METHODS

Microorganisms and culture conditions: A stock culture of CSN1-4 was obtained from the culture collection maintained at the Biotechnology Laboratory, Chemical and process Engineering Department, UKM (Cirilo *et al*., 2008). The stock culture was grown on TYA agar medium, that containing 40 g glucose, 2 g yeast extract, 6 g Bacto-Tryptone, 3 g ammonium acetate, 10 mg $FeSO_4.7H_2O$, 0.5 g KH_2PO_4, 0.3 g $MgSO_4 \bullet 7H_2O$ and added 15 g agar Bacteriological (No.1) per litre of distilled water. Single colonies (three to five) of CSN1-4 from TYA agar culture were transferred anaerobically into 10 mL 15% potato-glucose (PG) medium (PG containing; 150 g fresh mashed potato, 0.5 g $(NH_4)_2SO_4$, 10 g glucose and 3 g $CaCO_3$) and these were incubated anaerobically at 37°C for 30 h. A 1 mL aliquot of this culture was transferred into 9 mL of fresh PG medium, which was incubated in the same conditions at 37°C for 18 h and used as inoculum (Kalil *et al*., 2003).

For photo-fermentation, RS8253 culture stock was obtained from the culture collection maintained at the Biotechnology Laboratory, Chemical and process Engineering Department, UKM. The organism was cultured in modified Biebl and Pfennig growth medium for the hydrogen production experiment. The amount of inoculum used in the hydrogen production medium was 10% by volume and the bacterium was anaerobically grown in liquid medium at 32°C under a light intensity of 5.2±0.5 klux with a tungsten lamp (100 W) at a distance of 25 cm as the light source from one face. The initial pH of the growth medium was maintained to 6.8±0.2 after was dropped from first stage. Argon gas was used to create anaerobic conditions.

Experimental procedure: Dark fermentation was carried out at 37°C in a 500 mL Duran bottle. At the top of the bottle, there was an inlet for the medium and outlets for gases. CSN1-4 was grown in PG for 30h and used as the inoculum. The inoculum (10% v/v) was transferred into fermentation medium (TYA) containing 40 g glucose, 2 g yeast extract, 6 g Bacto-Tryptone, 3 g ammonium acetate, 10 mg $FeSO_4.7H_2O$, 0.5 g KH_2PO_4 and 0.3 g $MgSO_4.7H_2O$ per litre of distilled water. The culture pH was not controlled during fermentation. The initial pH of the TYA medium was adjusted to 6.0±0.2 with 5 M NaOH and sterilized at 121°C for 15 min (Alalayah *et al*., 2008).

The fermentation temperature was maintained at 37°C using a water bath. The evolved gas mixture from the reactor, containing both hydrogen and carbon dioxide (Kaushik *et al*., 2006), was passed through a 5 M KOH solution to absorb most of the carbon dioxide (Wooshin *at al*., 2006) and then analyzed by Gas Chromatography (GC).

Photo-fermentation was performed in another vessel of the same dimensions with a light source as the photo-bioreactor. The fermentation temperature was maintained at 32°C. Illumination was provided by 100 W tungsten lamps, with a distance of 25 cm from one face. The initial pH of the growth medium was maintained to 6.8±0.2. For the hydrogen production study, the spent medium from the first stage was centrifuged in some run and not centrifuged in others, before inoculation with 10% (v/v) RS8253 to study the effect of bacteria removed on fermentation processes. The evolved gas from the headspace of each of the reactors was channeled through a 5 M KOH solution, which stripped most of CO_2 from the gas stream.

Analytical method: The composition of the gas liberated from fermentation processes was analysed using gas chromatography (SRI 8610C, USA) with a helium ionization detector. The temperatures of the oven, injector and detector were 50, 100 and 150°C, respectively. The cell biomass concentration was estimated as the Dry Cell Weight (DCW) by measuring the optical density at 660 nm and then related the optical density to the DCW. The cells were centrifuged at 10,000 (rpm) for a period of 15 min in a refrigerated centrifuge (Kubota-5220, Japan).

The reducing-sugar (glucose) content of the medium was estimated using the Miller method (Miller, 1959). The glucose concentration in the medium was measured using the 3, 5-dinitrosalicylic acid (DNS) assay for total reducing sugars. A 1 mL aliquot of the sample and 2 mL aliquot of the DNS reagent mixture were combined in a test tube. The mixtures were placed in a boiling water bath for 5 min and then diluted with 10 mL of distilled water. The absorbance at 550 nm was recorded for all samples and the glucose concentration was calculated from a standard calibration curve.

RESULTS

Dark fermentation using CSN1-4: Results showed the highest yield of hydrogen was observed when the initial glucose concentration was 10 g L^{-1} and then decreased

Table 1: Effect of initial glucose concentration on hydrogen production by CS N1-4

Initial glucose (g L^{-1})	Duration of gas production (h)	Volume of hydrogen production (mL)	Residual glucose concentration (g L^{-1})	Glucose consumption (%)
1	24	232	0.28	72.00
5	34	225	1.50	63.00
10	48	388	1.82	81.80
20	48	203	8.20	59.00
40	49	113	26.50	33.75

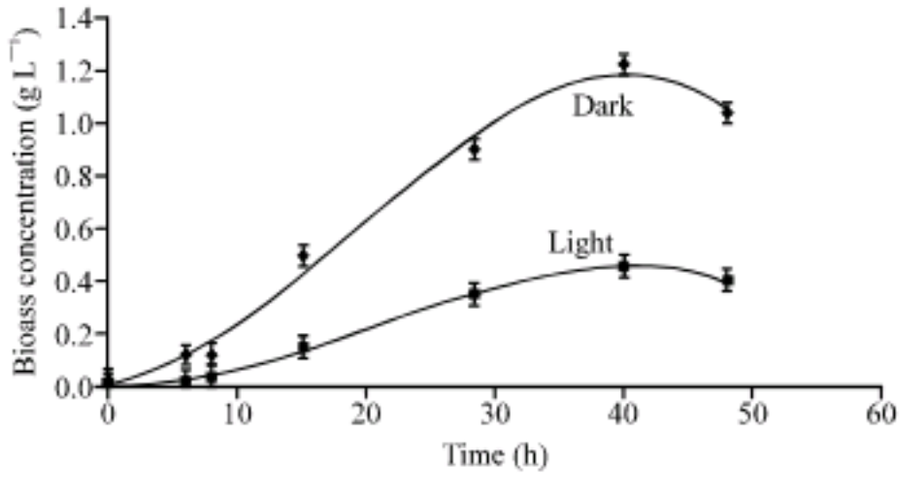

Fig. 1: The effects of light on growth of CSN1-4 in fermentation

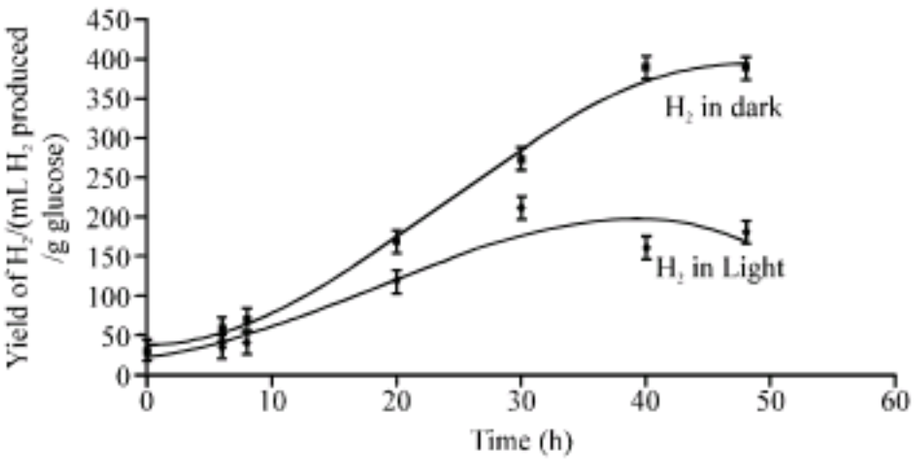

Fig. 2: The effects of light on H_2 production by CSN1-4

with increasing glucose concentration as indicated in (Table 1). The value of hydrogen yield was based on a glucose utilization of 81.8%.

Effect of light on H_2 production in the first stage with CSN1-4: The results show the maximum yield of hydrogen production by CSN1-4 was at the initial pH of 6±0.2, 10 g L^{-1} initial glucose and 37°C in the dark stage. The yield of hydrogen was 3.10 mol H_2 (mol glucose)$^{-1}$ in the dark fermentation Fig. 1 and 2, while at the same conditions of temperature, initial pH and initial glucose concentration, but in the presence of light, the maximum yield of hydrogen was 1.40 mol H_2 (mol glucose)$^{-1}$, the higher specific growth rate and doubling time, as determined by the biomass concentration measured by OD660 nm. This indicates that the light reduced the growth and therefore the hydrogen production. This reduction in growth is likely due to the increased temperature, as previously reported (Alalayah *et al*., 2009).

Table 2: Effects of initial nitrogen sparging on biomass concentration and hydrogen production using CSN1-4 in batch reactor

Process	Biomass concentration (g L^{-1})	Hydrogen production rate (mL h^{-1})
With sparging	1.130	8.11
Without sparging	0.801	6.45
Percentage increasing (%)	41.080	25.74

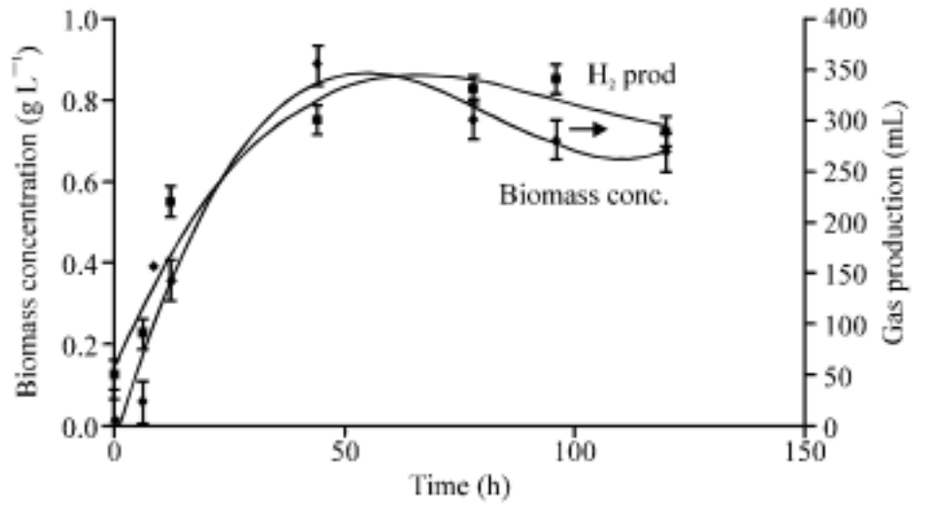

Fig. 3: Profile of biomass and H_2 production by removed CSN1-4 and no autoclave

Effect of nitrogen sparging on H_2 production in the first stage: The effects of sparging the fermentation system with nitrogen were studied for dark and light fermentations to ensure a total removal of oxygen. It was observed that the biomass concentration and hydrogen production at an initial pH of 6±0.2, initial glucose concentration of 10 g L^{-1} and 37°C were increased by sparging with nitrogen (Table 2).

Combination of dark and photo-fermentation: Spent medium from the glucose-fed hydrogen fermentation by CSN1-4 exhibited a high total organic acid concentration, composed predominantly of acetic acid, together with a small amount of butyric acid and ethanol. The spent medium was used for photo-production of hydrogen in the second stage by RS8253 using different route, irrespective of its composition and with a separation of the accompanying metabolites. A volume of inoculum 10% (v/v) of light-grown RS8253 cells were transferred into the spent medium of the dark fermentation. The culture was incubated anaerobically at 32°C with light at 5.8 klux intensity.

Effect of removing CSN1-4: The yield of hydrogen in the second stage was 0.4 mol H_2 (mol acetic acid)$^{-1}$, as shown in Fig. 3. The spent medium used for photo-fermentation in a subsequent second stage was adjusted to 32°C and pH 6.8±0.2. Acetic acid was presumably the sole substrate for hydrogen production in the second stage.

Effect of co-existing CSN1-4 and RS8253: From the results in this experiment, the yield of hydrogen in the second stage was 0.6 mol H_2 (mol acetic acid)$^{-1}$ under the first stage conditions of 37°C, pH 6.0±0.2 (Fig. 4). Without

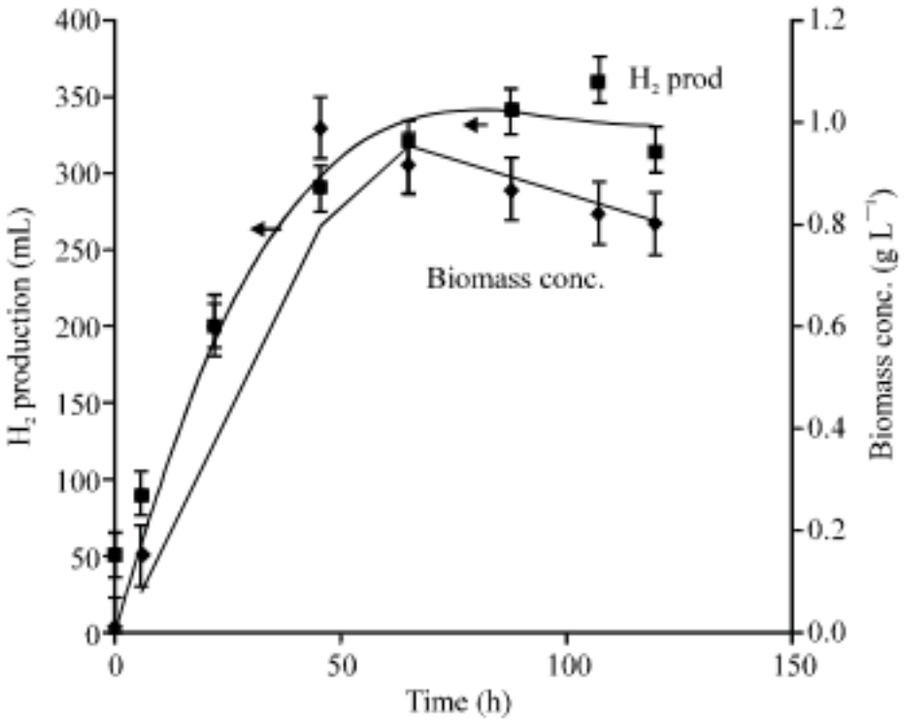

Fig. 4: Profile of biomass and H_2 production with CSN1-4 and RS 8253 were co-fermentation

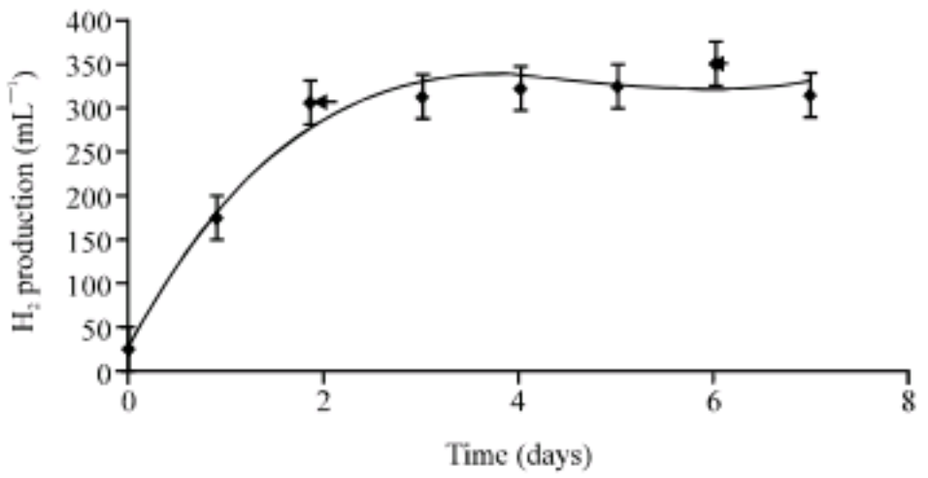

Fig. 5: Profile of H_2 production without condition adjustments

adjusting the conditions, light was added after 48 h to allow RS 8253 to grow and produce hydrogen. The overall yield of hydrogen in the two-stage process, based on glucose as the initial substrate, was higher than the single-stage process by 0.6 mol H_2/spent media.

Effect of adding RS 8253 to the CSN1-4 culture: In this experiment, the spent medium from the first stage was used for photo-fermentation in the second stage by adding RS8253 to the existing CSN1-4 fermentation without any adjustment to the conditions. The overall yield increased by 0.5 mol. H_2 (mol acetic acid)$^{-1}$ as presented in Fig. 5.

Effect of autoclaving the CSN1-4 culture prior to photo-fermentation: Here, the CSN1-4 dark fermentation effluent was autoclaved, followed by addition of inoculum of RS 8253 culture. The yield of hydrogen in the second stage was 1.10-1.25 mol H_2 (mol acetic acid)$^{-1}$ (Fig. 6). The medium used for photo-fermentation in the second stage was first adjusted to pH 6.8±0.2 and 32°C. The overall

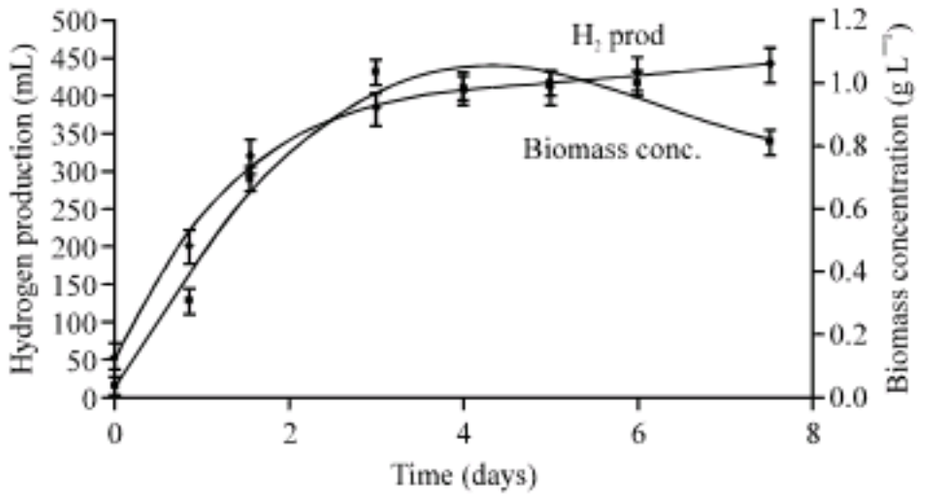

Fig. 6: Profile of biomass and H_2 production by autoclaving CSN1-4 before the addition of RS 8253

yield of $H_2$from glucose by the two-step process of dark and photo-fermentation increased two folds compared to the dark process alone.

DISCUSSION

Dark fermentation in first stage was studied using TYA medium containing glucose as the principal substrate. In dark fermentation, the initial medium pH of 6.0±0.2 and a temperature of 37°C were optimal for producing hydrogen by CSN1-4; these parameters were maintained during second stage fermentation. Most of the light fermentation studies were performed with pure volatile fatty acids (VFAs), such as acetic or butyric acids, using different pure *Rhodobacter* species (Kotay *et al*., 2008; Shi *et al*., 2004). However, there have been a limited number of light fermentation studies on dark fermentation effluents for bio-hydrogen production (Shi and Yu, 2004; Oh *et al*., 2004; Asada *et al*., 2006).

Since present study carried out photo-fermentation on spent broth of CSN1-4. Most of the reported studies on light fermentations employed single pure *Rhodobacter* culture and no systematic investigations have been reported on the use of different *Rhodobacter* species or their mixtures for bio-hydrogen production from dark fermentation effluents. It's well known that glucose is the easiest monosaccharide to be used as energy source. It has already been reported that substrate inhibition gets predominant at higher glucose concentration because this modifies the metabolic pathways (Fabiano and Perego, 2002; Oh *et al*., 2003).

The lower H_2 production indicates that the carbon flux at high glucose concentrations is more directed to the production of reduced by-products such as ethanol and/or organic acids (Oh *et al*., 2003). Since alcohol production involves the consumption of hydrogen in the form of reducing equivalents such as NADH, it is inevitable that fermentation conditions that favour the

metabolism of sugar to alcohols reduce hydrogen production. Niel *et al*. (2003), also studied substrate and product inhibition of hydrogen production during sucrose fermentation by the thermophilic bacteria *Caldicellulosiruptor saccharolyticus*. They found hydrogen was a strong inhibitor, when allowed to accumulate in the culture. However, the extent of inhibition by hydrogen was dependent on the density of the culture. Without the initial nitrogen sparging, lower hydrogen production and biomass concentration were observed. Since, CSN1-4 is an anaerobic bacterium, precautions were taken to control the presence of oxygen in the culture after medium sterilization and during inoculation (Kaushik *et al*., 2006).

Other investigators have used various strategies to enhance the anaerobic fermentation environment, including using an anaerobic chamber for inoculation and maintaining a constant stream of nitrogen gas over the culture headspace during fermentation, or until the microorganism is capable of producing enough metabolic gases to fill the culture headspace. It was described from the results that the spent medium was used for photoproduction of hydrogen in the second stage light grown cells of RS8253, were inoculated in the spent medium of dark fermentation (Nath *et al*., 2005).

Acetic acid was presumably the sole substrate for hydrogen production in the second stage. However, estimation of total yield of hydrogen in the two stage process was done on the basis of initial glucose content, since glucose (present in TYA media of dark fermentation) was converted to acetic acid and no additional substrate was supplied therein.

The overall yield of hydrogen in the two-stage process, based on glucose as the initial substrate, was higher compared to the single-stage process, as also reported in a previous study with both dark and photo-fermentation using another strain of *Rhodobacter* (Oh *et al*., 2004). The two-step process of dark and photo-fermentation can increase the overall yield from glucose to H_2 by two fold compared to the dark process alone. The estimation of the total hydrogen yield in the two-stage process was done on the basis of the initial glucose content, since glucose (present in TYA media of dark fermentation) was converted to acetic acid and no additional substrate was supplied therein.

CONCLUSIONS

The overall hydrogen yield from RS8253 in a second, photo-fermentation stage was increased by using spent medium from a dark fermentation by CSN1-4 in a two-stage batch fermentation process. The yield of hydrogen in the first stage was about 3.10 mol H_2 (mol glucose)$^{-1}$ at an initial glucose concentration of 10 g L^{-1}, an initial pH of 6±0.2 and a temperature of 37°C and the trace of light and nitrogen sparging were cleared on hydrogen yield and reported in this work. The highest yield of hydrogen in the second stage was about 1.10-1.25 mol H_2 (mol acetic acid)$^{-1}$ when the conditions were adjusted to pH 6.8±0.2 and 32°C, with the spent CSN1-4 culture autoclaved prior to photo-fermentation. The overall yield of hydrogen in two-stage process, based on glucose as the substrate, was higher than the single-stage process.

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