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Bio-hydrogen production from acid hydrolyzed wheat starch by photo-fermentation using different *Rhodobacter sp*

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ABSTRACT

Hydrogen gas production from sugar solution derived from acid hydrolysis of ground wheat starch by photo-fermentation was investigated. Three different pure strains of *Rhodobacter sphaeroides* (RV, NRLL and DSZM) were used in batch experiments to select the most suitable strain. The ground wheat was hydrolyzed in acid solution at pH = 3 and 90 °C in an autoclave for 15 min. The resulting sugar solution was used for hydrogen production by photo-fermentation after neutralization and nutrient addition. *R. sphaeroides* RV resulted in the highest cumulative hydrogen gas formation (178 ml), hydrogen yield (1.23 mol H_2 mol $^{-1}$ glucose) and specific hydrogen production rate (46 ml H_2 g^{-1} biomass h^{-1}) at 5 g l^{-1} initial total sugar concentration among the other pure cultures. Effects of initial sugar concentration on photo-fermentation performance were investigated by varying sugar concentration between 2.2 and 13 g l^{-1} using the pure culture of *R. sphaeroides* RV. Cumulative hydrogen volume increased from 30 to 232 ml when total sugar concentration was increased from 2.2 to 8.5 g l^{-1}. Further increases in initial sugar concentration resulted in decreases in cumulative hydrogen formation. The highest hydrogen formation rate (3.69 ml h^{-1}) and yield (1.23 mol H_2 mol^{-1} glucose) were obtained at a sugar concentration of 5 g l^{-1}.

1. Introduction

Hydrogen gas is an energy carrier with high energy content (122 kJ g^{-1}) and it is considered as a potential fuel source of the future. Therefore, some of the recent studies on hydrogen production are devoted primarily to the development of new technologies for economical and efficient hydrogen gas production.

The well known hydrogen production technologies such as steam reforming or partial oxidation of hydrocarbons and electrolysis of water are efficient, but energy intensive processes requiring high temperatures (>850 °C) and energy input [1]. Some recent studies are concentrated on solar thermochemical processes for reforming [2] and photo-electrochemical processes for water splitting to reduce the cost of energy requirement [3]. Some promising results were obtained with these approaches in terms of high hydrogen yields and low energy requirements [2].

Biological processes offer unique advantages for hydrogen gas production as compared to chemical processes due to operation under mild conditions and low energy requirements. Major bio-hydrogen production methods are bio-photolysis of water and dark/photo fermentation of carbohydrate rich raw materials such as waste biomass [4,5]. Dark and photo-fermentations are the most common approaches for biological hydrogen production. Hydrogen production by dark fermentation is relatively well known technology and carried out under anaerobic conditions by

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certain bacterial species such as *Clostridium* and *Enterobacter* [6,7]. The dominant culture of *Clostridia* can be easily obtained by heat treatment of biological sludge [8,9]. A wide range of substrates from sugar to complex carbohydrates or biomass to waste materials such as domestic or agricultural residues, and wastewater can be used in dark fermentation [8–11].

Photo-fermentation is performed by anaerobic, photo-heterothrophic bacteria like *Rhodobacter*, *Rhodopseudomonas* in the presence of light by using organic acids such as acetic and butyric acids as substrate for hydrogen production [12–16]. Photoheterothrophic bacteria are also capable of hydrogen production from simple sugars as glucose, fructose and sucrose [17–19]. Efficient hydrogen gas production from complex carbohydrates containing wastewaters such as tofu and olive mill wastewater by photo-fermentation has also been achieved [20–22]. The end products of photo-fermentation of some carbohydrates were reported as organic acids [19,21].

Utilization of dark fermentation effluent containing VFAs as substrate for the photo-fermentation in sequential production is a known practice [23,24]. Alternatively, dark and photo-fermentations can be realized in a single stage by combined fermentation [25,26]. The reactions of hydrogen production from carbohydrates by dark and photo-fermentations and theoretical yields of hydrogen formation can be summarized as follows.

Two moles of acetic acid and 4 moles of H_2 are produced from 1 mol glucose when acetic acid is the only VFA produced in dark fermentation.

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

When glucose is used as the substrate for photo-fermentation, 12 moles of H_2 should be produced per mole of glucose on the basis of the following reaction

$$C_6H_{12}O_6 + 6H_2O + \text{“light energy”} \rightarrow 12H_2 + 6CO_2 \quad (2)$$

For sequential or combined dark and photo-fermentations, acetic acid produced from the dark fermentation is used as the substrate for the photo-fermentation as described below.

$$\text{Dark fermentation}: \quad C_6H_{12}O_6 + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2CH_3COOH \quad (3)$$

$$\text{Photo-fermentation}: \quad 2CH_3COOH + 4H_2O + \text{“light energy”} \rightarrow 8H_2 + 4CO_2 \quad (4)$$

Although, the maximum theoretical hydrogen yield from glucose is 12 mol H_2 mol^{-1} glucose by combined dark and photo-fermentations, hydrogen yields are in reality lower than theoretical estimations since part of the substrate is used for microbial growth and maintenance [4,5]. It was estimated that hydrogen yield of 8 mol H_2 mol^{-1} glucose will be sufficient for economical bio-hydrogen production [27]. The reported yields so far are around 2.5 mol H_2 mol^{-1} glucose for dark fermentation [9] and 7 mol H_2 mol^{-1} glucose for combined [25] or sequential fermentations [26]. Extensive investigations are needed to improve the yield and rate of bio-hydrogen production. Development and selection of most suitable cultures with high hydrogen production capabilities, utilization of inexpensive and carbohydrate rich raw materials (i.e., waste biomass), optimization of environmental conditions and media compositions and overcoming substrate and product inhibitions are the major approaches followed to improve bio-hydrogen production economy.

In the light of the aforementioned studies, the most suitable approach to improve hydrogen yield is either sequential fermentation or combined dark and photo-fermentations. Production of hydrogen gas from different carbohydrates by dark fermentation has been extensively studied [6–11]. However, fermentation of carbohydrates to hydrogen gas by photo-fermentation using *Rhodobacter sp* was not studied extensively. Therefore, this study was designed to investigate the hydrogen gas production from glucose solution obtained by acid hydrolysis of wheat starch in photo-fermentation. The hydrogen production potentials of three different *Rhodobacter* strains were compared in terms of cumulative hydrogen formations, hydrogen yields and specific hydrogen production rates. Effects of initial total sugar concentrations on hydrogen formation by the selected strain were also investigated and the most suitable sugar concentration was determined.

2. Materials and methods

2.1. Organisms and growth media

Three different *Rhodobacter sphaeroides* strains were used in the experiments. *R. sphaeroides* (NRRL B-1727) was obtained from USDA National Center for Agricultural Utilization Research, Peoria, IL, USA. *R. sphaeroides* (DSMZ-158) was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) in lyophilized form. *R sphaeroides* RV was obtained from Dr. Miyake of Japan [28].

Rhodobacter cultures were first grown on the growth medium which were than transferred to the hydrogen production medium. The growth medium had the following composition: acetic acid (3 g l^{-1}), butyric acid (3 g l^{-1}), NH_4Cl (0.25 g l^{-1}), yeast extract (0.5 g l^{-1}), K_2HPO_4 (2.8 g l^{-1}), KH_2PO_4 (3.9 g l^{-1}), $Na_2MoO_4 \cdot 2H_2O$ (0.75 mg l^{-1}), $FeSO_4 \cdot 7H_2O$ (10 mg l^{-1} from $FeSO_4 \cdot 7H_2O$ EDTA complex), $MgSO_4 \cdot 7H_2O$ (0.2 g l^{-1}) at $pH_i = 7.0$. The organisms were grown for three days at 30 °C under 3000 lux illumination using an incubator shaker at 100 rpm. The cultures were transferred to hydrogen production medium which had the same composition as the growth medium with the only exception that 10 mmol Na-glutamate was used instead of 0.25 g l^{-1} NH_4Cl in the production medium.

2.2. Hydrolysis of ground wheat

The powdered wheat of −200 mesh was hydrolyzed at pH = 3 (adjusted by H_2SO_4) at 90 °C for 15 min in an autoclave. The conversion yield of starch to total sugar was 85%. The solid phase of the hydrolysate was separated by centrifugation at 8000 rpm. The sugar solution was neutralized to pH = 7 by addition of 10 M NaOH.

2.3. Experimental setup and procedure

Batch photo-fermentation experiments were carried out in 0.3 L serum bottles (Isolab-Germany Boro 3.3) with 0.25 L

reaction volume. Silicone rubber stoppers and screw caps were used to avoid gas leakage from the bottles. The cells cultivated in hydrogen production media were separated by centrifugation and washed with 5 g l^{-1} NaCl solution before inoculating the experimental bottles. The initial biomass concentration was kept constant at $X_o = 0.3 \pm 0.02$ g l^{-1} and initial total sugar concentration was varied between 2 and 13 g l^{-1}. No external N and P sources but only $Na_2MoO_4 \cdot 2H_2O$ (0.75 mg l^{-1}) and $FeSO_4 \cdot 7H_2O$ (10 mg l^{-1} from $FeSO_4 \cdot 7H_2O$ EDTA complex) were added to the sugar solution. The NH_4–N concentration in the hydrolyzed wheat starch was 0.4 mg g^{-1} wheat powder corresponding to 0.44 mg NH_4–N g^{-1} total sugar. Therefore, NH_4–N concentration in the experiments was not at inhibitory level for the photo-fermentation. The oxidation reduction potential (ORP) was adjusted to nearly −200 mV by passing argon gas through the head space and the initial pH was adjusted to 7. The bottles were placed in an incubator shaker at 30 °C, 3000 lux illumination and 100 rpm.

2.4. *Analytical methods*

Samples removed from the liquid phase everyday were centrifuged at 8000 g and the clear supernatants were used for analysis of total sugar (TS) and total volatile fatty acids (TVFA) Total sugar content was determined by the acid–phenol spectrometric method [29]. TVFA analysis was carried out by using analytical kits (Spectroquant, 1.01763. 0001, Merck, Darmstadt, Germany) and a PC spectrometer (WTW Photolab S12). Hydrogen gas was sampled from the head space of the bottles by using gas-tight glass syringes. Hydrogen gas concentration in the gas phase was determined by using a gas chromatograph (HP Agilent 6890). The column was Alltech, Hayesep D 80/100 6″ × 1/8″ × 085″. Nitrogen gas was used as carrier with a flow rate of 30 ml min^{-1} and the head pressure was 22 psi. Temperatures of the oven, injector, detector, and filament were 35 °C, 120 °C, 120 °C, and 140 °C, respectively. The amount of total gas produced was determined by water displacement method everyday using sulfuric acid (2%) and NaCl (10%) containing solution. Hydrogen gas volume was determined by multiplying total gas volume by the hydrogen concentration.

Biomass concentration in the inoculum was determined by filtering 20 ml sample through a 0.45 μm millipore filter and drying at 105 °C until the constant dry weight [30]. pH and ORP of the fermentation medium were monitored by using a pH and ORP meters with relevant probes (WTW Scientific, Germany). pH of the medium was controlled at pH = 7 by addition of 10 M NaOH when necessary. ORP values varied between −100 and −300 mV during the course of photo-fermentation, in general.

3. Results and discussion

3.1. *Comparison of different* Rhodobacter *species for hydrogen production*

Fig. 1 depicts time course of variation of cumulative hydrogen by different *Rhodobacter* species when the initial total sugar concentration was 5 ± 0.2 g l^{-1}. Hydrogen gas production was completed within 90 h with *R. sphaeroides* RV. Hydrogen production rate was slower with *R. sphaeroides* NRLL and *R. sphaeroides* DSMZ. The highest cumulative hydrogen gas production (178 ml) was obtained with the *R. sphaeroides* RV. Cumulative hydrogen volumes obtained with RS-NRLL and RS-DSMZ were 113 ml and 135 ml, respectively at the end of 200 h.

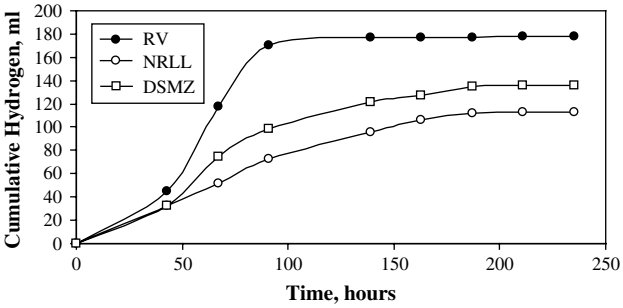

Fig. 1 – Time course of variation of cumulative hydrogen formation for different *Rhodobacter* strains at 5 ± 0.2 g l^{-1} initial total sugar concentration.

Cumulative hydrogen formation data depicted in Fig. 1 were correlated with the Gompertz equation and the constants were determined by regression analysis. The Gompertz equation has the following form:

$$H(t) = P \exp\left\{ -\exp\left[\frac{R_m e}{P}(\lambda - t)\right] + 1 \right\} \quad (5)$$

where "*H*" is the hydrogen formation at any time (ml), "*P*" is the maximum potential hydrogen formation (ml); "R_m" is the maximum rate of hydrogen formation (ml h^{-1}), "λ" is the duration of the lag phase, "*e*" is 2.718 and "t" is the time (h). Table 1 summarizes the Gompertz equation coefficients for different *Rhodobacter* species. The highest cumulative hydrogen (178.3 ml) and formation rate (3.69 ml h^{-1}) were obtained with the RS-RV culture. However, the lag phase was the lowest (11.2 h) with the RS-NRLL culture.

Specific hydrogen production rate (SHPR, ml H_2 g^{-1} biomass h^{-1}) and hydrogen yield are also important parameters used in comparison of different *Rhodobacter* cultures. The SHPR was determined by dividing the R_m (ml h^{-1}) obtained from the Gompertz equation to initial amount of cell concentration ($SHPR = R_m/(V_o X_o)$). SHPRs obtained with different cultures at $TS_0 = 5 \pm 0.2$ g l^{-1} initial total sugar concentration are depicted in Fig. 2. The highest specific rate of hydrogen formation (SHPR = 46 ml H_2 g^{-1} biomass h^{-1}) was obtained with

Table 1 – Gompertz equation constants for hydrogen production by different *Rhodobacter* species at 5 g l^{-1} initial total sugar concentration.

Type of culture	P (ml)	R_m (ml H_2 h^{-1})	λ (h)	R^2
RV	178.3	3.7	31.5	0.999
NRLL	115.3	0.9	11.2	0.999
DSMZ	135.1	1.5	19.9	0.998

R. sphaeroides RV. The other pure cultures resulted in relatively lower specific rates. The SHPRs obtained with the RS-DSMZ and RS-NRRL cultures were 20 ml H_2 g^{-1} biomass h^{-1} and 11 ml H_2 g^{-1} biomass h^{-1}, respectively. Fig. 3 depicts hydrogen yield coefficients for different *R. sphaeroides* cultures at an initial sugar concentration of 5 ± 0.2 g l^{-1}. The highest yield was obtained with *R. sphaeroides* RV (1.23 mol H_2 mol^{-1} glucose). The yields obtained by RS-DSMZ and RS-NRLL were 0.97 mol H_2 - mol^{-1} glucose and 0.81 mol H_2 mol^{-1} glucose, respectively.

3.2. *Effects of sugar concentration on hydrogen formation by* R. sphaeroides RV

R. sphaeroides RV was used to investigate the effects of initial sugar concentration on hydrogen formation by photo-fermentation of acid hydrolyzed ground wheat since this strain performed better than the other *Rhodobacter* species in previous experiments. Hydrogen production potential of RV from different substrate was studied by Miyake et al. and it was shown that culture is capable of converting different organic acids to hydrogen by high conversion efficiency [13–15,24]. However, hydrogen production capability of this culture from sugar solution was not reported. The initial total sugar concentration obtained from hydrolysis of the wheat starch was varied between 2 ± 0.1 and 13 ± 0.1 g l^{-1} and the initial biomass concentration was kept constant at 0.30 ± 0.02 g l^{-1}.

Fig. 4 depicts time course of variation of cumulative hydrogen formation (CHF) by *R. sphaeroides* RV at different initial sugar concentrations. CHF with 2 ± 0.1 g l^{-1} initial sugar content was only 30 ml at the end of 230 h incubation period. An increase in total sugar concentration to 3 g l^{-1} increased CHF to 115 ml. The highest CHF (232 ml) was obtained with 8.5 g l^{-1} total sugar concentration. Further increases in the initial sugar concentration resulted in decrease in CHF due to formation of VFAs in concentrations above 2.5 g l^{-1} which are known to be inhibitory to *Rhodobacter* in photo-fermentation [15,23]. CHF was only 21 ml at 9.5 g l^{-1} total sugar concentration and almost no hydrogen formation was observed at a sugar content of 13 g l^{-1}. Low hydrogen gas formation at high initial sugar concentrations could be due to substrate or product inhibition. The end products formed after photo-fermentation of carbohydrates were reported as acetic and butyric acids [19,21]. In addition, high concentration of organic acids could have inhibition effect on hydrogen production during photo-fermentation [13,15] probably high concentrations of volatile fatty acids (VFAs) formed at sugar contents above 8.5 g l^{-1} inhibited the nitrogenase enzyme in *R. sphaeroides* RV reducing hydrogen gas formation.

Variation of sugar utilization by *R. sphaeroides* RV with time at different initial total sugar concentrations is depicted in Fig. 5. Total sugar consumption was 70% for $TS_0 = 2.2$ g l^{-1} and slightly increased to around 80% for $TS_0 = 3.0$–8.5 g l^{-1}. Final sugar concentrations for $TS_0 = 9.5$ and 13 g l^{-1} were obtained as 2.5 g l^{-1} and 1.5 g l^{-1}, respectively. The results indicate that *R. sphaeroides* RV can use hydrolyzed wheat starch sugar as substrate for hydrogen production. These results are in agreement with the reported results in the literature. Carbohydrate utilization like glucose, fructose, xylose, maltose and sucrose as substrate by *Rhodobacter* for hydrogen production were investigated and it was shown that *Rhodobacter* can completely consume sugar and can produce hydrogen [17–21]. However, hydrogen production yields are lower than the theoretical yields probably because of partial utilization of carbon source for growth and maintenance.

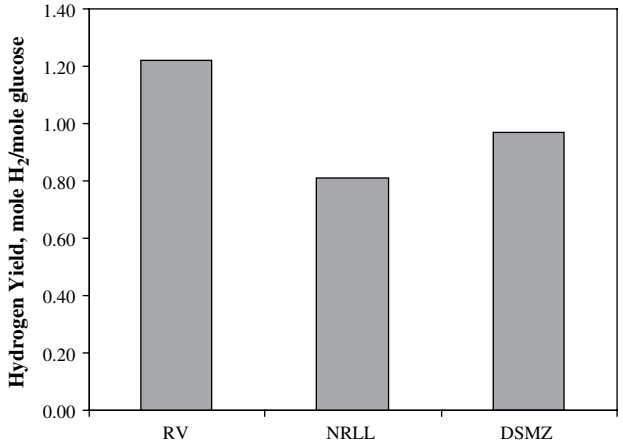

Fig. 3 – Hydrogen yield coefficient for different *R. sphaeroides* cultures at 5 ± 0.2 g l^{-1} initial total sugar concentration.

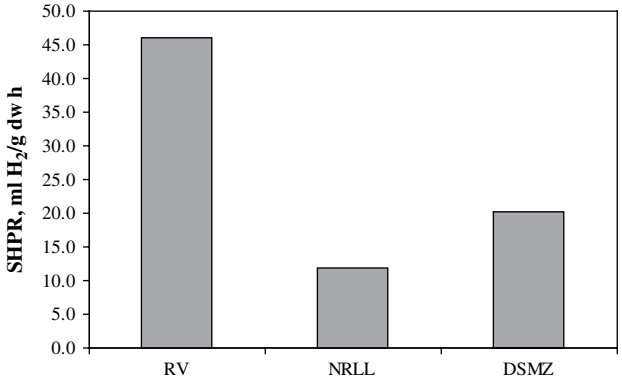

Fig. 2 – SHPR for different *Rhodobacter sphaeroides* cultures at 5 ± 0.2 g l^{-1} initial total sugar concentration.

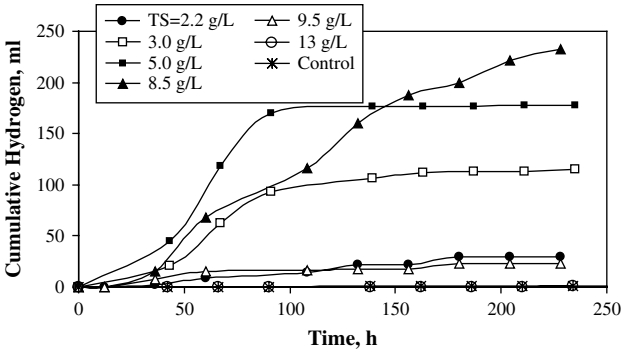

Fig. 4 – Time course of variation of cumulative hydrogen formation by *R. sphaeroides* RV at different initial total sugar concentrations.

The rate and the extent of hydrogen formation by *R. sphaeroides* RV strain were determined by fitting the experimental data in Fig. 6 to the Gompertz equation for different sugar concentrations and the results are presented in Table 2. An increase in initial sugar concentration from 2.2 g l^{-1} to 8.5 g l^{-1} increased the maximum potential hydrogen volume from 34 ml to 253 ml. Further increases in sugar content above 8.5 g l^{-1} adversely affected hydrogen formation. Despite a long lag phase, the highest hydrogen production rate (3.69 ml h^{-1}) was obtained with a sugar content of 5 g l^{-1}. Hydrogen production rates for sugar contents above and below 5 g l^{-1} were considerably lower than that obtained at 5 g l^{-1} sugar content. Low hydrogen formation rates at low initial sugar contents (<5 g l^{-1}) was due to substrate limitation. However, at high sugar contents above 5 g l^{-1}, inhibition caused by high concentrations of VFA formed from light fermentation of sugar was probably the major reason for low hydrogen formation rates [13,15]. This product inhibition effect is clearly shown in Fig. 5 depicting variation of SHPR with the initial sugar content. SHPR increased from 3.15 ml g^{-1} h^{-1} to 46 ml g^{-1} h^{-1} with the increase in total sugar concentration from 2.2 g l^{-1} to 5 g l^{-1} indicating substrate limitations at sugar contents below 5 g l^{-1}. A substantial decrease in the SHPR was observed for sugar contents above 5 g l^{-1} due to inhibition effects of high VFA concentrations produced at high sugar contents (product inhibition).

Hydrogen yield coefficient also varied with the initial total sugar concentration. The yield coefficient was relatively low (0.60 mol H_2 mol^{-1} glucose) at a total sugar concentration of 2.2 g l^{-1}. Increase in initial sugar concentration resulted in substantial increase in hydrogen yield up to sugar content of 5 g l^{-1} as shown in Fig. 7. The yield of hydrogen formation was nearly 1.23 mol H_2 mol^{-1} glucose for the initial total sugar contents between 3 and 5 g l^{-1}. Again, the major reason for low yields at low initial sugar contents is substrate limitation. Further increases in sugar content above 5 g l^{-1} resulted in substantial decrease in hydrogen yields due to inhibitory effects of high VFA concentrations formed. Hydrogen yield decreased to 0.11 mol H_2 mol^{-1} glucose and further to 0.05 mol H_2 mol^{-1} glucose for the initial total sugar concentrations 9.5 g l^{-1} and 13 g l^{-1}, respectively. These results indicated that the optimal initial sugar concentration for hydrogen production by *R. sphaeroides* RV was around 5 g l^{-1}. High sugar concentrations resulted in formation of VFAs in high concentrations causing inhibition on nitrogenase enzyme of the *R. sphaeroides* RV reducing hydrogen yields.

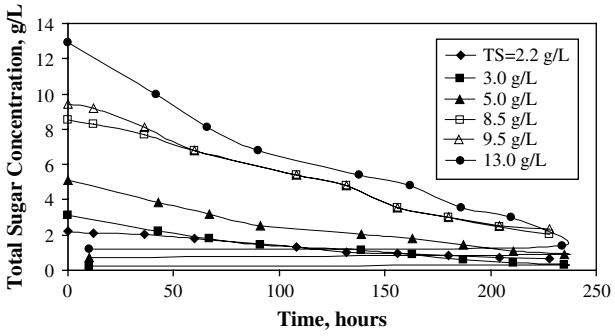

Fig. 5 – Variation of sugar concentration with time in light fermentation by RS-RV for different initial sugar concentrations.

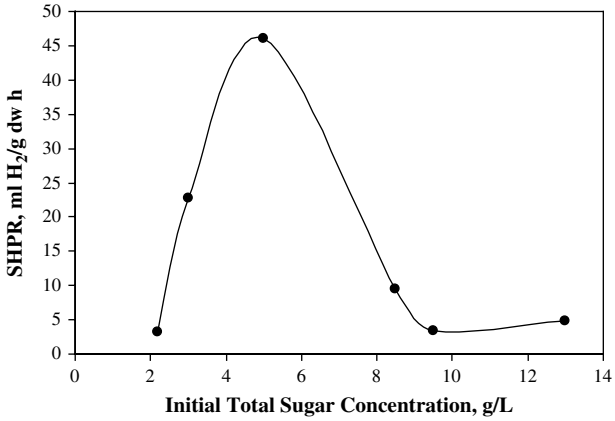

Fig. 6 – Variation of SHPR with the initial total sugar concentration for *R. sphaeroides* RV.

There are limited studies on hydrogen formation from carbohydrates by photo-fermentation. Jeong et al. [17] indicated that *R. sphaeroides* is capable of hydrogen production from glucose in the presence and absence of light. It was observed that the culture can completely consume up to 20 g l^{-1} glucose concentration producing nearly 200 ml cumulative hydrogen with a 2 ml H_2 g^{-1} VSS h^{-1} specific production rate under light. Hydrogen production potential and rates by *R. sphaeroides* were lower under dark fermentation [17]. Similarly, hydrogen production by photosynthetic bacteria under illumination from different sugars such as glucose, fructose, xylose, maltose and sucrose was also reported although hydrogen production was relatively low as compared to the production from organic acids [18]. Fang et al. observed 100 ml cumulative hydrogen production from around 5.4 g l^{-1} initial glucose concentration when glucose was completely utilized by *R. sphaeroides*. It was also shown that *R. sphaeroides* can convert glucose to organic acids with conversion yield of 0.18 mol acetate mol^{-1} glucose 0.42 mol-butyrate mol^{-1} glucose [19]. Hydrogen production by *R. sphaeroides* from tofu wastewater with high carbohydrate content was also studied. The culture effectively reduced the total organic carbon (TOC) content of the wastewater and produced hydrogen gas [20]. Further studies with the same wastewater and culture showed that the end products of the hydrogen production were acetic and butyric acids [21].

Table 2 – Gompertz equation constants for hydrogen production by *Rhodobacter sphaeroides* RV at different initial total sugar concentrations.

Total sugar (g l^{-1})	P (ml)	R_m (ml H_2 h^{-1})	λ (h)	R^2
2.2	34	0.21	29.18	0.927
3.0	113	1.83	32.35	0.999
5.0	178	3.69	31.47	0.999
8.5	253	1.57	27.78	0.997
9.5	21	0.26	7.25	0.959
13.0	0.22	0.39	0.39	0.955

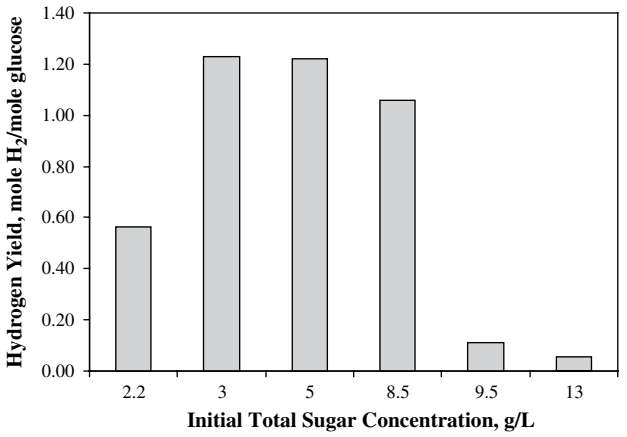

Fig. 7 – Variation of hydrogen yield coefficient with the initial total sugar concentration for *R.sphaeroides* RV.

4. Conclusions

Hydrogen production from photo-fermentation of sugar solution obtained from acid hydrolysis of wheat powder (WP) by different *Rhodobacter* strains was investigated. The results indicated that *R. sphaeroides* can use simple sugars or hydrolyzed wheat starch for hydrogen gas production with different rates and yields. The highest specific hydrogen production rate ($46\,ml\,H_2\,g^{-1}$ biomass h^{-1}) and the yield ($1.23\,mol\,H_2$ - mol^{-1} glucose) were obtained with the *R. sphaeroides* RV culture. The other pure cultures provided substantially lower rates and hydrogen yields.

The initial sugar concentration significantly affected the rate and extent of hydrogen production and also hydrogen yield in photo-fermentation of hydrolyzed sugar by the *R. sphaeroides* RV. Hydrogen production potential increased up to sugar concentration of $8.5\,g\,l^{-1}$ and decreased for higher sugar concentrations. The highest rate and yield of hydrogen formation were obtained with an initial sugar content of $5\,g\,l^{-1}$. Low sugar contents caused substrate limitations and high sugar contents resulted in product inhibition due to high VFA concentrations formed at high sugar contents. Hydrogen yield was $1.23\,mol\ H_2\,mol^{-1}$ glucose for sugar contents between 3 and $5\,g\,l^{-1}$.

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