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Kinetics of biological hydrogen production by the photosynthetic bacterium *Rhodobacter sphaeroides* O.U. 001

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Abstract

The kinetics and the effects of various parameters on hydrogen production by *Rhodobacter sphaeroides* O.U. 001 were investigated in a batch column photobioreactor. In particular, the effect of the inoculum age and the implementation of a light–dark cycle illumination scheme for emulating natural sunlight have been investigated in detail. The possibility of using yeast extract to replace the rather expensive vitamin mixture in the medium was also studied.

The results show that hydrogen production is decreased when the initially inoculated bacteria have a high culture age. Exposure of the bacterial culture to light–dark cycles increased the amount of hydrogen compared to continuous illumination, all other parameters remaining the same. Similarly, the use of yeast extract to replace the vitamins increased the growth and hydrogen production rates, however, with a slight reduction in the total amount of gas produced and the hydrogen fraction in the evolved gas.

Keywords: Hydrogen production; Photosynthetic bacteria; *Rhodobacter sphaeroides*; Growth kinetics; Malate consumption

1. Introduction

Hydrogen is a clean and efficient fuel, considered as a potential substitute for fossil fuels. Currently, almost all hydrogen production is linked to the consumption of fossil fuels; therefore, if hydrogen is to replace fossil fuels in the future, it has to be produced renewably and in large scale, with environmentally benign processes. Biological hydrogen production stands out as an environmentally harmless process carried out under mild operating conditions with renewable resources. Photosynthetic microorganisms like the purple bacteria, cyanobacteria and algae or fermentative microorganisms such as *E. coli* and *E. aerogenes* are commonly utilized for hydrogen production [1].

Though reports on full industrial scale hydrogen production with outdoor operation are not encountered in the literature, research on hydrogen production with laboratory-scale reactors using photosynthetic bacteria is well documented [2–8] and studies with pilot-scale reactors are increasing [9,10]. Before moving on to even larger volumes with outdoor operation, however, it is necessary to determine and evaluate the distinct effects of as many parameters as possible. For this purpose, while studies targeted at a good understanding of the related components of the bacterial metabolism [11–14] are invaluable, it is nevertheless necessary to obtain information on the overall response of the hydrogen-producing system through tightly controlled experiments with model reactors. In studies involving an approach of the latter kind with *R. sphaeroides* O.U. 001 several parameters such as substrate concentration, light intensity and initial pH have been optimized with respect to hydrogen production [2,3,6].

Either resting or growing cells have been used in previous hydrogen production studies. Resting cells are obtained by growing the bacteria up to a desired concentration, centrifuging them and placing them into the hydrogen

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Nomenclature

F-ratio	the ratio of the F-value obtained for the regression or pure error calculations to the critical F-value obtained from the upper 5% points of the F-distribution	R^2	the coefficient quantifying goodness-of-fit
		s^2	mean square about regression
		s_e^2	mean square for pure error
k_s	the substrate consumption rate constant, (h^{-1})	t	time, h
		t_d	total gas production time, h
k_c	apparent specific growth rate, (h^{-1})	V	volume of the culture, l
M	instantaneous malate concentration, mol/l	X	cell concentration (dry weight), g/l
M_0	initial molar concentration of malate (mol/l)	X_0	initial cell concentration (dry weight), g/l
P	moles of hydrogen produced, mol		
r_g	gas production rate based on culture volume, l/l/h	η	percent substrate conversion efficiency
		μ	the specific growth rate, (h^{-1})
r'_g	gas production rate based on bacterial dry cell weight, l/g/h	μ_e	the (constant) specific growth rate in the exponential phase, h^{-1}

production reactor, which is usually devoid of a nitrogen source, in order to inhibit growth [6,15]. In contrast, growing cells are obtained by inoculating the bacteria into a medium which permits both growth and hydrogen production. Little information is available on the kinetics of biological hydrogen production by growing cells [1].

The cell growth, substrate consumption kinetics and the substrate conversion efficiency of malate into hydrogen have been investigated in the present study for hydrogen production by growing cells of *R. sphaeroides* O.U. 001. Several factors affecting the performance of batch experiments have been studied for constructing a basis for scale up. Experiments in this study include the investigation of the effect of inoculum age on hydrogen production, the implementation of light–dark cycle illumination for emulation of natural sunlight and the use of yeast extract to replace the rather expensive vitamin mixture in the medium. The use of light–dark cycles is especially critical for further trials for hydrogen production involving the use of outdoor operation.

2. Materials and methods

Bacteria: R. sphaeroides O.U. 001 (DSM 5864) was used in this study. In the preparation of the inoculum, the freeze-preserved bacteria were transferred at least twice into fresh medium (activation). The amount of inoculation to the hydrogen production reactors was 10% by volume of the fresh medium.

Culture: For growth purposes the modified medium of Biebl and Pfennig [16] for purple non-sulfur bacteria was used. Malic acid (7.5 mM) was used as the carbon source and sodium glutamate (10 mM) as the nitrogen source. The liquid culture medium used for the hydrogen production was essentially the same as the growth medium except that the concentrations of malate and glutamate were 15 and 2 mM, respectively, as suggested before [3].

Photobioreactor: Rubber-tapered glass bottles with 25–50 ml of liquid volume were used for growth of the inoculum. For hydrogen production, water-jacketed glass column photobioreactors of 400 ml liquid volume were used. Details of the experimental setup and procedure were given previously [3,17].

Conditions: The photobioreactors were maintained at 32°C. The illumination was provided by 150 W tungsten lamps (100 W for routine growth and transfer procedures), adjusted to provide a uniform light intensity of 150–250 W/m^2 at the surface of the reactor. The initial atmosphere of the vessels was pure argon.

Cell growth analysis: The bacterial cell concentration was determined spectrophotometrically. It was found that an optical density of 660 nm corresponded to a cell density of 0.591 g dry weight per liter of culture.

GC analyses: The GC analyses were made to determine the composition of the evolved gas. A Hewlett-Packard Series II system with a thermal conductivity detector and a Propak Q column was used. The oven, injector and detector temperatures were 30, 40 and 50°C, respectively. Nitrogen was used as the carrier gas at a flow rate of 11 ml/min.

HPLC analyses: The HPLC analyses were made to monitor the concentration of the carbon source malate. For HPLC analysis, 1–3 ml samples taken from the reactor were centrifuged and the supernatant was analyzed by an Ion Exclusion column (Phenomenex) at 70°C with a mobile phase flow rate of 0.6 ml/min. H_2SO4 (0.005 M) (filtered and degassed) was used as the mobile phase. A differential refractometer (Knauer) was used as the detector.

Determination of pH: The pH of the culture medium was measured with a standard combination pH electrode (Mettler-Toledo) connected to an electronic transmitter (NEL Elektronik).

3. Results and discussion

Hydrogen production experiments were conducted under the conditions specified in Materials and Methods section. Table 1 summarizes the experimental conditions and the results of eight runs. Runs 2, 3 and 6 were carried out as 'control' runs, under the same conditions. Run 1 was performed under conditions very similar to these three runs, except that no activation was made. In both Runs 4 and 5, the inoculum was from a stationary phase culture instead of the usual exponential phase culture. Additionally in Run 5, the effect of light–dark cycles was studied. Yeast extract (0.2 g/l) was used to replace the standard vitamin solution in Run 7. Finally, Run 8 was conducted without liquid sampling so as not to disturb the system. In all other runs, liquid samples were collected several times during the run.

3.1. The cell growth rate

Theoretically, the cell growth rate is expressed as

$$\frac{dX}{dt} = \mu X, \tag{1}$$

where X is the cell dry weight concentration (g/l) and μ is the specific growth rate (h^{-1}), which might depend on substrate concentration and the other factors. Several models provide an expression for μ, the Monod expression being the most common. However, the growth curves obtained in this study for *R. sphaeroides* O.U. 001 could not be fitted to the Monod model or its modified versions, the Contois, Moser or Andrew's equations. Therefore, two approaches were used to characterize the growth curves in the present work. The first was based on the assumption of a constant growth rate in the exponential phase. In this case, Eq. (1) gives

$$\mu_e = \frac{\ln(X_2/X_1)}{(t_2 - t_1)}, \tag{2}$$

where μ_e is the specific growth rate in the exponential phase and X_1, X_2 are two distinct cell concentrations in the exponential phase (g/l). In this study, the values of μ_e calculated for all of the runs range between 0.035 and 0.073 (h^{-1}) and are listed in Table 2.

The second approach was the use of the logistic model, which (compared to Eq. (2)) has the additional benefit of representing the entire growth curve, including the lag

Table 1
Experimental conditions and the results of gas production

Run	Total gas (ml)	Start time (h)	Duration (h)	Cell dry weight ($g\,l^{-1}$)		pH	
				initial	final	initial	final
1	357	25	110	0.035	0.79	7.00	8.25
2	345	20	120	0.041	1.02	7.13	7.83
3	357	15	123	0.059	0.61	7.00	8.52
4	236	16	194	0.142	0.66	7.40	7.90
5	290	16	160	0.142	1.01	7.40	8.00
6	435	27	171	0.092	0.66	7.04	8.02
7	370	13	96	0.079	0.68	7.00	8.12
8	499	27	188	0.079	0.55	7.06	7.78
Average	361	20	140	0.083	0.76	7.12	8.05

n/a: not available

Table 2
Kinetic model parameters

Run number	r_g (l/l/h)	r'_g (l/g/h)	η (%)	k_s (h^{-1})	μ_e (h^{-1})	k_c (h^{-1})	X_{max} ($g\,l^{-1}$)
1	0.010	0.014	34	n/a	0.073	0.103	0.83
2	0.009	0.010	40	0.032	0.040	0.091	1.00
3	0.009	0.017	35	n/a	0.059	0.105	0.63
4	0.003	0.0047	24	0.015	0.043	0.144	0.73
5	0.005	0.0057	29	0.021	0.042	0.060	0.98
6	0.008	0.012	44	n/a	0.035	0.063	0.71
7	0.012	0.018	36	0.037	0.056	0.119	0.82
8	0.008	0.012	48	n/a	n/a	n/a	n/a
Average	0.008	0.010	36	0.026	0.044	0.098	0.81

n/a: not available

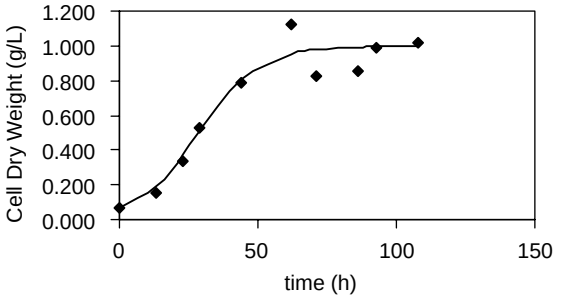

Fig. 1. The growth of cells and total gas production versus time (Run 2).

phase (if present), the exponential growth and the stationary phases. The specific growth rate for the logistic model is

$$\mu = k_c \left(1 - \frac{X}{X_{max}}\right), \quad (3)$$

where k_c is the apparent specific growth rate (h^{-1}) and X_{max} is the maximum cell dry weight concentration (g/l). Inserting Eq. (3) into (1) and integrating, the following equation for cell concentration is obtained:

$$X = \frac{X_0 \exp(k_c t)}{1 - (X_0/X_{max})(1 - \exp(k_c t))}. \quad (4)$$

The predictive power of Eq. (4) may be limited since it does not involve a substrate term [18]. However, for the purposes of batch hydrogen production experiments in this study, where the initial substrate concentrations and the inoculation volume are kept constant, the logistic model is a fair approximation of the growth curve.

Fig. 1 shows the relation between the cell dry weight and time obtained for Run 2. It includes the experimental data points as well as the fitted curve obtained based on the logistic model. The logistic model parameters (k_c and X_{max}) and μ_e values for all of the runs are listed in Table 2. The R^2 value, which quantifies the dispersion of distribution from the mean, was used as a measure of the goodness of the fit. It was found that the R^2 values for all of the logistic model fits were above 0.98, indicating excellent agreement of the model to the experimental data.

3.2. Hydrogen production

In all of the runs, hydrogen production was found to start between the mid-exponential phase and the beginning of the stationary phase after a 20 ± 7 h lag phase following the inoculation. This observation was in parallel to the findings of Arık et al. [2] and Sasikala et al. [19] who used systems including vitamins and found that hydrogen was produced in the exponential growth phase. Whereas in a previous work of Eroglu et al. [3], no vitamins were used and hydrogen production began after a lag time of 40 h, within the stationary phase.

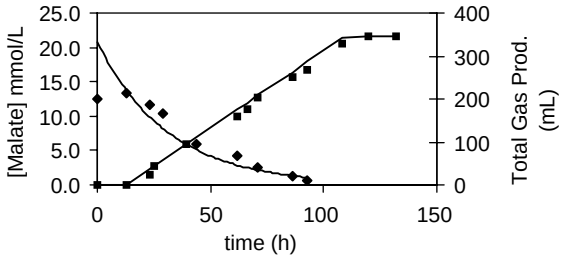

Fig. 2. Gas production and malate concentration curves (Run 2).

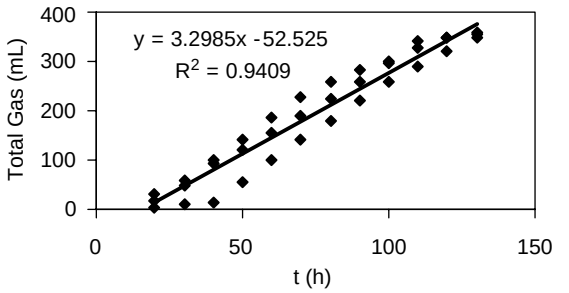

Fig. 3. Linear fit to the gas production curves of the three control runs (Runs 2,3,6).

Total gas production was 380 ± 50 ml for the control runs (Runs 2,3,6). The duration of gas production was 140 ± 30 h. The gas analyses showed 95% hydrogen and 5% carbon dioxide on an argon free basis, unless specified otherwise.

Two conventions were used for expressing the gas production rate in this study. The first is the average gas production rate per culture volume (r_g), which is calculated by dividing the total volume of gas produced by the volume of the culture and by the duration of gas production, and has the unit of l/l/h.

The second convention used is the average gas production rate per bacterial dry weight (r'_g), which is calculated by time averaging of individual rates and has the unit of l/g/h. The individual rates obtained for a certain period were calculated by dividing the volume increment of gas produced by the average cell concentration and by the duration of that period. The values obtained for hydrogen gas production rates are listed in Table 2.

Fig. 2 illustrates the gas production and malate concentration versus time data obtained for Run 2. As can be seen from Fig. 2, the total gas produced in Run 2 increases linearly with time. To investigate the general applicability of a linear model, a linear fit was applied to the gas production data of the control runs put together (Fig. 3). Statistical tests were carried out to evaluate the significance of the fit and the lack of fit, the results of which are given in Table 3. The F-ratio for the linear regression was 131 (indicating a significant fit), whereas the same ratio was calculated as 0.13 for the lack of fit (indicating an insignificant lack-of-fit).

Table 3
Regression results and significance (F) tests for the linear fit to gas production data of the three control runs (Runs 2,3,6)

Regression results			Significance of fit		Significance of lack-of-fit	
slope	y-intercept	R^2	s^2	F-ratio	s_e^2	F-ratio
3.30	−52.3	0.941	862	131	1087	0.131

To investigate whether or not liquid sampling has a significant effect on hydrogen production, Run 8 was conducted, in parallel to Run 6 (which was subjected to continuous sampling). As can be seen from Table 1, no significant changes due to absence of sampling could be observed. The slightly more hydrogen produced in the reactor in which no sampling was made could be attributed to the fact that continuous sampling may result in the reduction of the amount of the actively hydrogen-producing bacteria as a consequence of liquid volume loss due to sampling.

3.3. Substrate consumption rate and the conversion efficiency

In a previous study [3], the consumption rate of malate was found to be first order with respect to malate concentration, whereas the consumption rate of sodium glutamate was found to be second order with respect to glutamate. The expression for time dependent malate concentration obtained from the integration of the first-order consumption rate equation is

$$M = M_0 \exp(-k_s t), \tag{5}$$

where k_s is the consumption rate constant (h^{-1}) and M, M_0 are the instantaneous and initial concentrations of malate, respectively (mol/l). In Fig. 2 the fitted equation is compared with data of Run 2. The consumption rate constant for this and the other runs is between 0.015 and 0.037 h^{-1} as listed in Table 2. These are quite comparable to those obtained in the previous study [3]. All R^2 values for the fits were above 0.9.

Close examination of Fig. 2 reveals a peculiar feature of the relation of substrate consumption with hydrogen production. It can be noted that after 93 h at which malate has been practically depleted, hydrogen production continues at more or less the same rate for some time. This can be explained by the endogenous metabolism of the bacteria, which was pointed out by Gest et al. [15]. Photosynthetic bacteria are capable of accumulating large amounts of reserves such as poly-β-hydroxybutyrate (PHB) [20] that may later be degraded and used for hydrogen production. In other words, the portion of the substrate initially utilized for biosynthesis can eventually end up as a substrate for hydrogen production. Another possible reason is that the malate consumption and hydrogen production are consecutive events and a significant time delay may be seen between the two.

A particularly useful parameter for characterizing microbial hydrogen production is the substrate conversion efficiency, which is the ratio of the actual amount of hydrogen evolved to the amount expected through stoichiometric conversion of the substrate [4]. For malate, which is the primary carbon substrate used in this study, 6 moles of hydrogen are expected to be produced per mole of malate utilized, so the substrate conversion efficiency (η) is

$$\eta = \frac{100P}{6VM_0}, \tag{6}$$

where P is the moles of hydrogen produced till that time and V is the culture volume in liters.

Table 2 displays the substrate conversion efficiencies (η) for all of the runs and it is observed that the values range between 35% and 45%. A substrate conversion efficiency of 57% for the same substrate (malate) is reported in literature for *R. sphaeroides* [21].

3.4. The effect of culture age of the inoculum on hydrogen gas production

A critical parameter for hydrogen production is the age of the inoculum culture [6]. To assess the effect of this parameter, two different inoculum ages were compared in this study: an inoculum harvested from a mid-exponential phase culture (Run 3 is a typical example) and an inoculum harvested from the stationary phase (Run 4). The results were remarkably different in terms of total gas production, gas production rates and the overall substrate conversion efficiency (Tables 1 and 2). It can be suspected that the higher initial cell concentration rather than the difference in the culture age may be the reason for this difference, since high cell concentrations decrease the available light energy within the culture through absorption and scattering effects. However, since the final cell concentrations are quite close (Table 1) and the majority of the hydrogen production takes place after the cell concentration has levelled out, the difference between the two experiments are believed to be largely due to the inoculum age difference and not due to the differences in the initial cell concentration.

The reason for the poor performance of aged inocula can be attributed to the pre-culturing procedure, which is carried out in a medium promoting growth rather than hydrogen production. Large retention times within such media may be driving the bacterial metabolism to pathways other than hydrogen production, such as the production of PHB.

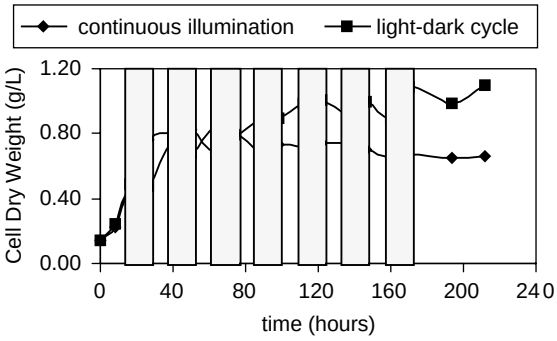

Fig. 4. Growth curves for the continuous illumination and the light–dark cycle reactors (Runs 4 and 5, respectively). Shaded regions correspond to dark periods.

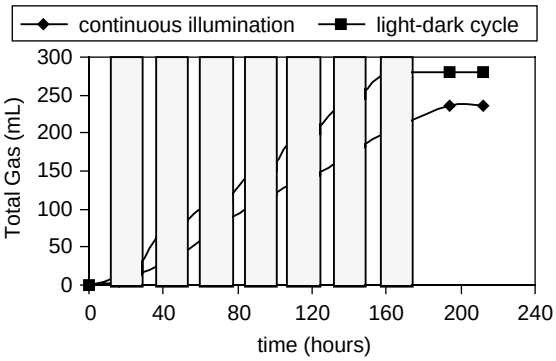

Fig. 5. Total gas production in the continuous illumination and light–dark cycle reactors (Runs 4 and 5, respectively). Shaded regions correspond to dark periods.

Relevant results in the literature seem to support or complement those obtained in this study. Fascetti et al. [22] report that continuous chemostat culturing of *R. sphaeroides* RV causes the bacteria to change their metabolism and start producing reserve products such as PHB rather than hydrogen. Jee et al. [23] reported loss of hydrogen production activity with time for batch cultures of *R. sphaeroides* S due to decline in the activity of the electron carrier ferredoxin.

3.5. The effect of light–dark cycles

Since the ultimate goal for all biological hydrogen production studies is large-scale outdoor production of hydrogen using daylight, it is desirable to operate the experimental configurations as close to outdoor conditions as possible. The natural day–night cycle results in alternating periods of light and dark. To test the performance of the hydrogen production system under such illumination conditions, an experiment was performed in which the bacteria were exposed to 14 h light–10 h dark periods (Run 5 in Tables 1 and 2). For comparison, a second reactor was operated in parallel, under continuous illumination (Run 4 in Tables 1 and 2).

Higher cell densities are achieved in the cycle reactor (Fig. 4). Fig. 5 shows that while little or no hydrogen are produced within the dark periods, the total amount of gas produced in the cycle reactor is appreciably more than the amount produced in the continuously illuminated reactor. This increase may be simply due to the higher concentration of cells reached in the cycle reactor or due to the beneficial effects of the light–dark cycles on nitrogenase, as shown by Meyer et al. [24]. Whatever the case, it can be said that not only the bacteria can survive and maintain their hydrogen production activity under natural outdoor illumination, but such an illumination is also beneficial for growth and hydrogen production.

The malate consumption curves for the light–dark cycle and continuous illumination reactors were notably different

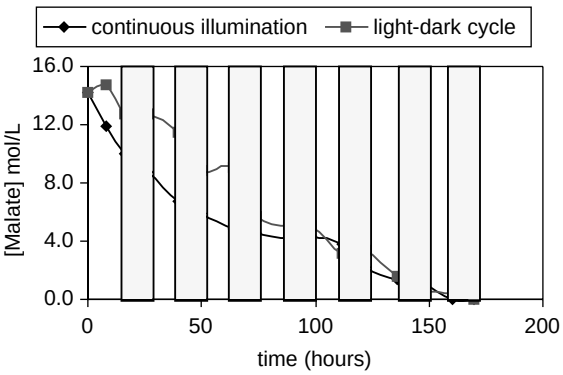

Fig. 6. Malate consumption curves for the continuous illumination and the light–dark cycle reactors (Runs 4 and 5, respectively). Shaded regions correspond to dark periods.

from each other, as shown in Fig. 6. It can be seen that the malate concentration curve for the continuous illumination reactor is distinctively smoother. It is also worth noting that considerable malate consumption occurred in the light–dark reactor in the dark periods as well.

A peculiar phenomenon encountered in this experiment was the pH change in the cycle reactor (Fig. 7). Close inspection shows a regular trend in the light–dark cycle reactor. The pH rises in the light periods and falls in the dark periods. It is probable that the decrease in the dark periods is due to the excretion of an acidic end product(s).

3.6. The use of vitamins or yeast extract

Though the use of vitamins does not enhance hydrogen production significantly, vitamins are obligatory for the long-term preservation of the bacteria. Without vitamins, the bacteria start to weaken and colonies begin to lose their characteristics. The addition of vitamins not only prevents

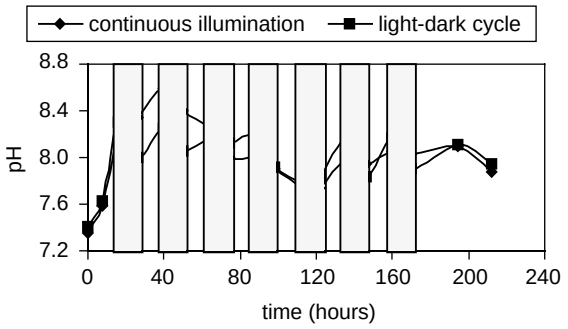

Fig. 7. pH change in the continuous illumination and the light–dark cycle reactors (Runs 4 and 5, respectively). Shaded regions correspond to dark periods.

such deterioration, but it also promotes the recovery of the strain once such a problem comes into existence.

The possibility of replacing the expensive vitamin solution (niacin, thiamine and biotin) with the relatively cheaper yeast extract is certainly desirable from an economic point of view. Yeast extract includes many of the basic nutrition materials of the bacteria, as well as vitamins that promote the selective growth of the purple non-sulphur bacteria. However, despite its nutritious potential and rich content of growth factors, it has been known for a long time that yeast extract is inhibitory for hydrogen production [25]. The reason of inhibition is the high nitrogen content of yeast.

To examine the effect of yeast extract on *R. sphaeroides* O.U. 001 the vitamin solution was replaced by 0.2 g/l yeast extract (Run 7 in Tables 1 and 2). A control reactor employing the standard vitamin solution was also operated as a reference (Run 6 in Table 1). The results are summarized in Table 1. It can be seen that both the growth and the hydrogen production rates are not affected adversely, but significantly enhanced by the presence of yeast extract. Among all the hydrogen production experiments, the highest hydrogen production rate (0.012 l/l/h) and the smallest lag (13 h) were obtained with yeast extract.

The absence of an expected inhibitory effect can be understood when the nitrogen content of yeast extract is investigated. The nitrogen content of yeast extract was determined as 8.85% by weight, corresponding to a nitrogen concentration of 1.26 mM in the medium containing 0.2 g/l yeast extract. Therefore, the total nitrogen supplied by glutamate and yeast extract still results in a favorable carbon to nitrogen ratio for hydrogen production [3].

The drawbacks of the use of yeast extract, on the other hand, are the slightly lower hydrogen fraction in the evolved gas (91% as opposed to 95%) and the lower total amount of gas evolved. However, by fine-tuning the amount of yeast extract, better results may be obtained. Fascetti and Todini [7] found that yeast extract up to concentrations of 0.5 g/l stimulated hydrogen production.

4. Concluding remarks

1. Hydrogen production starts between the mid-exponential phase and the beginning of the stationary phase.
2. The overall conversion efficiency was found to be 35–45% with malate as substrate. After the substrate is depleted, the cells probably utilize their endogenous reserves for hydrogen production.
3. It is essential to avoid inoculating an aged culture, e.g. far past the stationary phase. The inoculum to the reactor should be at the mid-exponential phase for best hydrogen production performance. Therefore, the most reasonable way to exploit the increased substrate conversion efficiency observed in later phases of hydrogen production is to use a higher inoculum percentage, rather than a denser, hence older culture.
4. More hydrogen was produced in the reactor that was exposed to light–dark cycles. Hence, natural outdoor illumination is not only feasible, but also beneficial for hydrogen production.
5. Though the use of vitamins does not enhance hydrogen production significantly, vitamins are obligatory for the long-term preservation of the bacteria. Hydrogen production using yeast extract to replace vitamins has clear advantages such as faster growth and gas production rates and reduced lag.

Acknowledgements

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