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Kinetics of two-stage fermentation process for the production of hydrogen

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ABSTRACT

Two-stage process described in the present work is a combination of dark and photofermentation in a sequential batch mode. In the first stage glucose is fermented to acetate, CO_2 and H_2 in an anaerobic dark fermentation by *Enterobacter cloacae* DM11. This is followed by a successive second stage where acetate is converted to H_2 and CO_2 in a photobioreactor by photosynthetic bacteria, *Rhodobacter sphaeroides* O.U. 001. The yield of hydrogen in the first stage was about 3.31 mol H_2 (mol glucose)$^{-1}$ (approximately 82% of theoretical) and that in the second stage was about 1.5–1.72 mol H_2 (mol acetic acid)$^{-1}$ (approximately 37–43% of theoretical). The overall yield of hydrogen in two-stage process considering glucose as preliminary substrate was found to be higher compared to a single stage process. Monod model, with incorporation of substrate inhibition term, has been used to determine the growth kinetic parameters for the first stage. The values of maximum specific growth rate (μ_{max}) and K_s (saturation constant) were 0.398 h^{-1} and 5.509 g l^{-1}, respectively, using glucose as substrate. The experimental substrate and biomass concentration profiles have good resemblance with those obtained by kinetic model predictions. A model based on logistic equation has been developed to describe the growth of *R. sphaeroides* O.U 001 in the second stage. Modified Gompertz equation was applied to estimate the hydrogen production potential, rate and lag phase time in a batch process for various initial concentration of glucose, based on the cumulative hydrogen production curves. Both the curve fitting and statistical analysis showed that the equation was suitable to describe the progress of cumulative hydrogen production.

1. Introduction

Among various processes of biological hydrogen production, anaerobic fermentation, commonly termed as dark fermentation has several advantages to its credit over the others. Apart from its simple technology, it can utilize a wide gamut of substrates, both pure as well as waste products, and does not rely on light availability [1]. However, the major bottleneck of dark fermentation process stems from lower achievable yield of hydrogen per mol of substrate and thus the process is not economically viable in its present form. The pathways and experimental evidences cited in the literature reveal that at most 4 mol of hydrogen could be obtained from each mol of glucose as substrate [2,3] during acetate fermentation. It is reported that twice this yield would be sufficient for economic hydrogen fermentation [4]. Therefore, amelioration of hydrogen productivity by increasing its yield has become a challenging R&D task in hydrogen biotechnology. Two-stage

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Nomenclature

μ_{max}	maximum specific growth rate, h^{-1}
k_c	apparent specific growth rate, h^{-1}
K_I	inhibition constant, $g\,l^{-1}$
K_S	saturation constant, $g\,l^{-1}$
X	dry biomass concentration, $g\,l^{-1}$
X_{max}	maximum dry cell mass concentration, $g\,l^{-1}$
X_0	initial biomass concentration, $g\,l^{-1}$.
$H(t)$	cumulative volume of hydrogen production, ml
P	gas production potential, ml
R_m	the maximum production rate, $ml\,h^{-1}$
λ	lag time, h
t	incubation time, h
S	substrate (glucose) concentration, $g\,l^{-1}$
S_0	initial substrate concentration, $g\,l^{-1}$

fermentation process, described in the present work, is an approach to that end. The synergy of the process lies in the maximum utilization of the substrate which otherwise fails to achieve complete conversion due to thermodynamic limitation. An interesting combination of photoheterotrophic and hyperthermophilic hydrogen fermentation was tested in an European Union 5th Framework project, coordinated by the Wageningen University, The Netherlands. The project was aimed at the development of an integrated process in which biomass is utilized for the biohydrogen production in two steps [5]. In the first stage, the biomass is fermented to acetate, carbon dioxide and hydrogen in a thermophilic dark fermentation. In a separate photobioreactor acetate is converted to hydrogen and carbon dioxide. The combination could be expected to reach as close to the theoretical maximum production of 12 mol of H_2 $(mol\,glucose)^{-1}$ equivalent as possible, according to the following reactions:

(i) Stage I—dark fermentation (facultative anaerobes)

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2.$$

(ii) Stage II—photofermentation (photosynthetic bacteria)

$$2CH_3COOH + 4H_2O \rightarrow 8H_2 + 4CO_2.$$

Lee et al. [6] have studied the combination of purple nonsulfur photosynthetic (PNS) bacteria and anaerobic bacteria for efficient conversion of wastewater into hydrogen. In their study, effluents from different carbohydrate fed reactors have been used for hydrogen production. In another study Kim et al. (2001) [7] have combined dark fermentation with photofermentation to improve hydrogen productivity from food processing wastewater and sewage sludge. Broth from the dark fermentation by *Rhodopseudomonas palustris* P4 has been used for photosynthetic hydrogen production by the same strain [8]. In the present study two-stage fermentation has been carried out using glucose as substrate in the first stage and spent medium obtained in the first stage has been used for photofermentation in the second stage. One of the objectives of the present study was to investigate the suitability of spent medium from dark fermentation for production of hydrogen in a subsequent photofermentation. The other objective was the prediction of kinetic behavior of two-stage fermentation process. In general, the fermentation model can be subdivided into a growth model, a substrate model and a product model [9]. Monod model has been applied for prediction of growth kinetic parameters of *Enterobacter cloacae* DM11 using glucose as substrate in the first stage. A model based on Logistic equation has been used to describe the growth of *Rhodobacter sphaeroides* O.U. 001 in the second stage. Modified Gompertz equation was applied to estimate the hydrogen production potential, rate and lag phase time in a batch process for various initial concentration of glucose, based on the cumulative hydrogen production curves.

2. Materials and methods

2.1. Microorganisms and culture conditions

E. cloacae DM11 was used as the microorganism for the first stage. This is a mutant of *E. cloacae* IIT-BT-08 which has already been reported for biohydrogen production [10]. Nutrient broth (Himedia Laboratories), consisting of pectic digests of animal tissue 5.0 g, sodium chloride 5.0 $g\,l^{-1}$, beef extract 1.5 $g\,l^{-1}$, yeast extracts 1.5 $g\,l^{-1}$ was used as growth medium for the microorganism. Prior to inoculation the pH of the medium was adjusted to 6.0 ± 0.2 and sterilized at 121 °C and 15 Psi pressure for 15 min. The organism was grown at 37 °C in an incubator–shaker at 200 rpm, kept overnight. The strain was routinely maintained aerobically on nutrient agar by weekly transfer under aseptic condition to the slants. For photofermentation, *R. sphaeroides* O.U. 001, kindly provided by Dr. Ch Sasikala, Environmental Microbial Laboratory, JNTU, Hyderabad, India, has been used. The organism was grown photoheterotrophically with malate (30 mM) and sodium glutamate (10 mM) as carbon and nitrogen sources, respectively (using Biebl and Pfenning's basal media) at 34 ± 1 °C and about 2000–2500 lx light intensity under anaerobic environment. Initial pH of the growth medium was maintained at 6.8 ± 0.2 [11].

2.2. Experimental procedure

Dark fermentation was carried out in a custom designed vertical tubular bioreactor (glass make) of working volume of 600 ml (height: 36 cm and O.D: 7.5 cm.) with a water jacket. At the top of the bioreactor, there was an inlet for the medium and outlets for the argon and hydrogen gases. The reaction temperature was maintained at 37 °C by circulation of water from a constant temperature circulating water bath. The production media (MYG) contained 1% (w/v) malt extract, 0.4% (w/v) yeast extract and 1% (w/v) of glucose. Overnight seed culture of *E. cloacae* DM11 (10%, v/v) grown in nutrient

broth was used as inoculum. After inoculation the reactor was flushed with pure argon gas (99.99%) for a period of 5 min to ensure anaerobic condition. The medium was agitated at a speed of 100 rev $(min)^{-1}$ with the help of a magnetic stirrer. Unless stated otherwise the duration of batch fermentation was 72 h. Photofermentation was performed in a second glass column photobioreactor of same dimensions with water jacket. Temperature was maintained at 34 °C. Illumination was provided by 100-W lamps, of approximately 5500 ± 500 lx intensity [assuming 1 lx = 0.015 W yellow light of wavelength ($\lambda = 555$ nm)] from a distance of 30 cm. For hydrogen production study, the spent medium from dark fermentation was centrifuged for separation of biomass and the supernatant was diluted with sterile deionized water to the required concentration and inoculated with 10% (v/v) overnight culture of *R. sphaerodies* O.U.001 in the photobioreactor. Light grown cells containing about $0.15-0.17\ g\,l^{-1}$ cells of *R. sphaeroides* O.U. 001, were inoculated in the spent media of dark fermentation. The evolved gas mixture from the headspace of each of the reactor consisting of both carbon dioxide and hydrogen was channeled through an absorber containing 30% (w/v) KOH solution, which stripped CO_2 from the gas stream. Hydrogen gas was collected in a gas collector by the displacement of 10% saline water at room temperature of 25 °C and pressure of 760 mmHg.

2.3. Analytical methods

The composition of the gas was analyzed by a gas chromatograph (Perkin-Elmer) with Thermal conductivity detector (TCD) using 80/100 Porapak-Q (3.2 mm dia × 2 m length) column. The oven, injector and detector temperature were 80, 150 and 200 °C, respectively. Nitrogen was used as carrier gas at a flow rate of 20 ml $(min)^{-1}$ and a pressure of 80 Psi. Biomass concentration was measured with a spectrophotometer (Model: Novaspec) at a wavelength of 660 nm. For cell centrifugation rotor speed was maintained at 6000*g* for a period of 15 min at 4 °C in a refrigerated centrifuge (Sigma 4K 15). Reducing sugar (glucose) content of the medium was also estimated spectrophotometrically (Model: Novaspec, wavelength = 540 nm) using dinitrosalicylic acid (DNS) reagent [12]. Total volatile fatty acid (VFA) content of the fermentation broth (in terms of acetic acid $mg\,l^{-1}$) was estimated titrimetrically after titrating the distillate (obtained by simple distillation of broth) against standard NaOH solution [13]. The same was also confirmed with GC by flame ionization detector (FID) using DB-FFAP capillary column (0.25 mm dia, 30 mm long, nitroterephthalic acid modified polyethylene glycol bonded). During FID analysis the temperature of the oven, injector and detector were 170, 250 and 250 °C, respectively. Intensity of light was measured by 'Lutron' digital luxmeter (Taiwan make) having range of 0–50,000 lx. The photobioreactor is externally illuminated and therefore the incident light intensities are considered as the light intensities on the outer surface of the illumination side.

2.4. Modeling and data fitting

Modeling and data fitting for the biomass growth, substrate utilization and hydrogen production by anaerobic fermentation during stage 1 was carried out by writing a program in C (compiled in GCC) following Monod model with subsequent incorporation of substrate inhibition factor. Kinetic parameters were determined by the method of least squares. Parameters of logistic and Gompertz equations were determined by nonlinear curve fitting using a software program Curve expert 1.3.

2.5. Statistical analysis

The variation between the experimental data points and those predicted by the Monod models with substrate inhibition was estimated by testing of hypothesis by population variance using Chi-square statistic. Kolmorgorov–Smirnov goodness of fit test was applied to assess the statistical significance of the parameters obtained by nonlinear curve fitting of cumulative hydrogen production data by modified Gompertz equation [14].

3. Kinetic model development

3.1. Biomass growth kinetics in stage 1 by Monod model

Monod equation empirically fits a wide range of data satisfactorily and is the most commonly applied unstructured, nonsegregated model of microbial growth [15]. The values of specific growth rate (μ_{max}) and saturation constant (K_S) were estimated following Monod model by regression analysis of Lineweaver–Burk linearized equation.

3.2. Modeling of substrate and biomass concentration profiles

To find out the simulated values of substrate concentration as a function of t the following expression was used:

$$\mu_{max}(X_0 + Y_{X/S}S_0)t = [X_0 + Y_{X/S}(S_0 + K_S)]\ln\left[\frac{X_0 + Y_{X/S}(S_0 - S)}{X_0}\right] - K_S Y_{X/S}\ln\frac{S}{S_0}. \quad (1)$$

From the above expression, simulated substrate profile with t was determined by using Wegstein convergence method of successive substitutions on each iteration [16]. Also simulated values of cell mass concentration, X were calculated by using the following relation:

$$X = X_0 + Y_{X/S}(S_0 - S). \quad (2)$$

3.3. Substrate inhibition model

At high substrate concentrations, microbial growth rate is inhibited by the substrate. A substrate inhibition model of the following form is proposed:

$$\mu = \frac{\mu_{max}S}{K_S + S + S^2/K_I}. \quad (3)$$

This is known as Andrew's model and suggests a nonlinear relationship between the specific growth rate μ, and substrate

concentration S [17]. For calculating kinetic parameter values from above equation method of least square was used.

3.4. Simulation of biomass and substrate concentration profiles

$$\frac{dX}{dt} = \mu X = \frac{\mu_{max} S}{K_S + S + \frac{S^2}{K_I}} \cdot X, \tag{4}$$

$$\frac{dS}{dt} = \frac{-1}{Y_{X/S}} \frac{\mu_{max} K_S}{K_S + S + \frac{S^2}{K_I}} \cdot X. \tag{5}$$

It can be integrated to give an expression for simulated values of S as a function of t:

$$\begin{aligned}\mu_{max}(X_0 + Y_{X/S}S_0)t &= [X_0 + Y_{X/S}(S_0 + K_S)] \times \ln\frac{X_0 + Y_{X/S}(S_0 - S)}{X_0} + K_S Y_{X/S} \ln\frac{S}{S_0} \\ &+ K_I(X_0 + Y_{X/S}S_0)\left[S - S + \frac{(X_0 + Y_{X/S}S_0)}{Y_{X/S}}\right] \times \ln\left(\frac{X_0 + Y_{X/S}(S_0 - S)}{X_0}\right).\end{aligned} \tag{6}$$

3.5. Biomass growth kinetics in stage 2 by logistic model

To study the growth kinetics of *R. sphaeroides* O.U. 001 in the second stage of the two-stage fermentation process logistic equation has been used. Recently the logistic model, as a sigmoidal shaped model, has been a most popular one due to its 'goodness of fit' and has been widely used in describing the growth of microorganism. The use of logistic model has a benefit of representing the entire growth curve including the lag phase (if present), the exponential growth and the stationery phases [18]. In this paper, we develop an equation based on the logistic model with growth-associated production of hydrogen in photofermentation. The logistic equation for cell concentration for a batch reactor can be represented as

$$X = \frac{X_0 e^{k_c t}}{1 - \frac{X_0}{X_{max}}(1 - e^{k_c t})}. \tag{7}$$

This equation shows the relationship of biomass and the fermentation time, which is used to fit the experimental data of biomass concentration. Logistics constants were determined by using a software program, Curve expert 1.3, for fitting curves.

3.6. Cumulative hydrogen production by modified Gompertz equation

The cumulative hydrogen production in the anaerobic reactor can be modeled by modified Gompertz equation [19], which can be written as

$$H(t) = P \exp\left\{-\exp\left[\frac{R_m \cdot e}{P}(\lambda - t) + 1\right]\right\}. \tag{8}$$

This was found to be a suitable equation for describing the progress of cumulative gas production obtained from the experiment, where $H(t)$ represents cumulative volume of hydrogen production (ml), P the gas production potential (ml), R_m (ml h^{-1}) the maximum production rate, λ (h) the lag time, t incubation time (h) and e, the exp(1) = 2.718. The typical cumulative hydrogen production curve was nonlinearly modeled by the above equation. The values of P, R_m and (for each set of experiment) were determined by best fitting the cumulative hydrogen production data for the above equation using a software program, Curve expert 1.3.

4. Results and discussions

4.1. Dark fermentation in stage 1

Dark fermentation in stage 1 was studied using MYG medium having glucose as the principal substrate. As it has already been reported [19] that during dark fermentation initial medium pH of 6.5 ± 0.2 and temperature of 37 °C are most suitable in terms of maximum productivity of hydrogen by *E. cloacae* DM11, these parameters were maintained in the present study. The cumulative volume of hydrogen production was found to increase with increase in initial glucose concentration in the medium. However, beyond 1% (w/v) of initial glucose concentration noticeable decrease in hydrogen production took place (Table 1). At 1% (w/v) glucose concentration, the molar yield of hydrogen produced was 3.31 mol H_2 (glucose)$^{-1}$ assuming NTP conditions. This was based on glucose utilization of 74% (data not shown) and taking purity of hydrogen as 88%. Wu and Lin [20], observed a similar trend between the substrate concentration and the cumulative hydrogen production in which the maximum cumulative production was found at 4% (w/v) of molasses concentration using a mixed culture. Cumulative volume of hydrogen production also reached its maximum with 1% (w/v) of initial glucose concentration compared to the higher concentrations. It has already been reported that substrate inhibition gets predominant at higher glucose concentration because this modifies the metabolic pathways [21,22]. 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 [22]. Since alcohol production involves the consumption of hydrogen in the form of reducing equivalents such as NADH, it is inevitable that fermentation conditions that favor the metabolism of sugar to alcohols reduce hydrogen production. van Niel et al. [23], 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.

4.2. Kinetics in stage 1 by Monod model

Monod equation was used to model biomass growth kinetics for the hydrogen production using *E. cloacae* DM11 in stage 1. The values of specific growth rate (μ_{max}) and saturation constant (K_S) were estimated by Lineweaver–Burk linearized equation. The values of maximum specific growth rate (μ_{max})

Table 1 – Kinetic parameters of cumulative hydrogen production for different initial glucose concentration calculated from nonlinear regression of modified Gompertz equation for stage 1

Glucose concentration (% w/v)	P (ml H_2)	R_m (ml h^{-1})	λ (h)	Correlation coefficient	Residual sum of squares	D^a
0.2	442	9.78	6.06	0.996	14.45	0.1538
0.4	455	8.47	12.63	0.996	15.99	0.1538
0.6	570.54	10.02	11.58	0.996	18.47	0.1474
0.8	752.38	2.32	3.34	0.996	15.99	0.4196
1	1019.38	13.92	4.04	0.996	29.047	0.2154
1.2	927.64	17.65	2.05	0.990	45.08	0.1538
1.4	796.73	14.14	1.00	0.992	34.82	0.2308

[a] Kolmogorov–Smirnov statistical indicator.

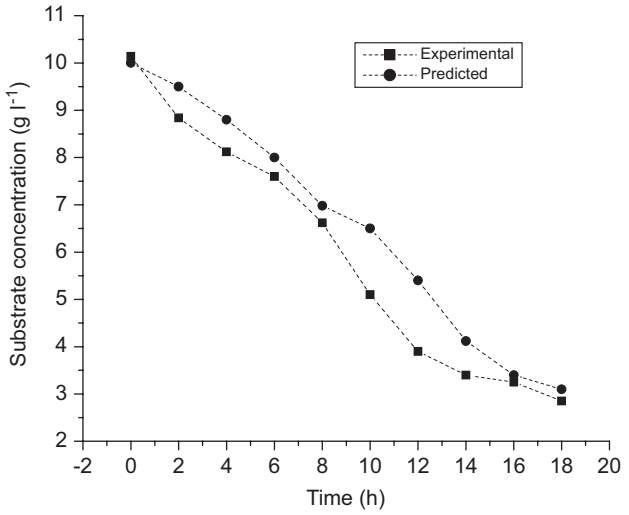

Fig. 1 – Experimental data and kinetic model prediction for substrate concentration as a function of reaction propagation time using Monod model. Experimental conditions: batch operation, volume of medium: 250 ml, temperature 37 °C and initial medium pH: 6.0 ± 0.2.

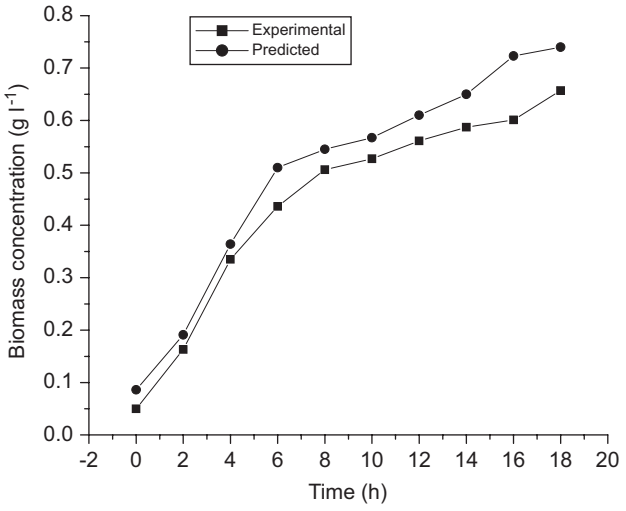

Fig. 2 – Experimental data and kinetic model prediction for biomass concentration as a function of reaction propagation time using Monod model. Experimental conditions: batch operation, volume of medium: 250 ml, temperature 37 °C and initial medium pH: 6.0 ± 0.2.

and K_S (saturation constant) were 0.40 h^{-1} and 5.51 g l^{-1}, respectively, using glucose as substrate. Both the values of μ_{max} and K_s were found lower than those reported for *E. cloacae* IIT-BT-08 ($\mu_{max} = 1.12\,h^{-1}$ and $K_S = 8.9\,g\,l^{-1}$) [24]. The maximum specific growth rate depends on temperature and pH of the medium [25]. Although temperature was kept constant during growth experiment, pH was not controlled. This might have resulted in the reduced value of μ_{max}. Moreover, the saturation constant (K_s) and maximum specific growth rate hold some physical significance. The kinetic constants obtained from this work can be used as design parameters for hydrogen producing bioprocesses with a variety of reactor configuration. For instance, the μ_{max} value 0.40 h^{-1} suggests that the continuous culture should not be performed at dilution rates close to or above 0.40 h^{-1}. As the K_S value (5.51 g l^{-1}) represents the substrate concentration required to achieve 50% of the maximum growth rate, it could become a guideline for adjusting the most efficient substrate concentration in the feed [26].

Experimental data and those predicted by the Monod kinetic model for substrate and biomass concentration in course of reaction propagation time are presented in Figs. 1 and 2. The little deviation between the experimental and predicted values might be due to either product or substrate inhibitions. Since in the present process the product is a gas, effect of product inhibition can be ignored. However, Van Groenestijn [27] observed that presence of gas phase in the reactor at higher partial pressure of hydrogen resulted in low overall production of hydrogen. This fact clearly exemplifies the inhibition due to product hydrogen gas. Product inhibition could be mitigated by keeping the partial pressure of hydrogen at some lower levels. The effect of substrate inhibition was taken into consideration using a model based on Andrew's equation in this study. This is however different

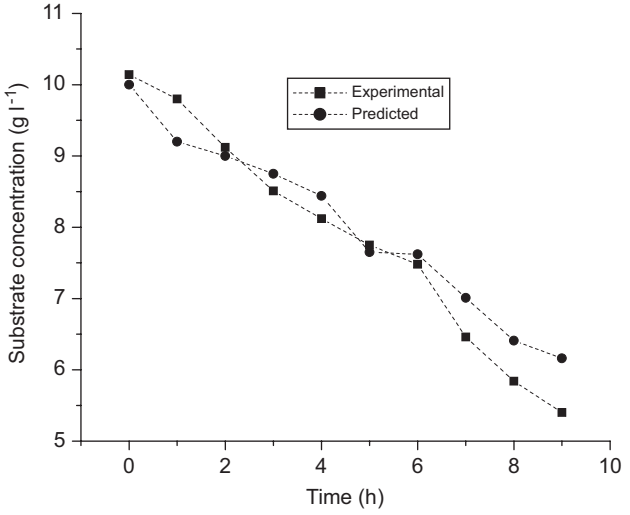

Fig. 3 – Experimental and simulated profile of substrate concentration as a function of reaction propagation time using substrate inhibition model. Experimental conditions: batch operation, volume of medium: 250 ml, temperature 37 °C and initial medium pH: 6.0 ± 0.2.

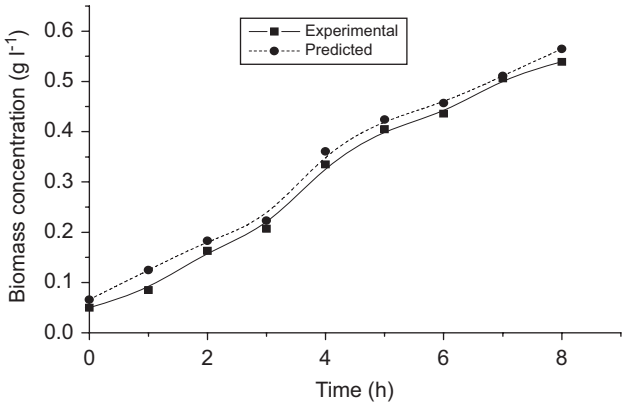

Fig. 4 – Experimental and simulated profile of biomass concentration as a function of reaction propagation time using substrate inhibition model. Experimental conditions: batch operation, volume of medium: 250 ml, temperature 37 °C and initial medium pH: 6.0 ± 0.2.

from the previously reported [28] inhibition kinetics of the strain *E. cloacae* IIT-BT-08, where modified Andrew's model fitted well to predict the substrate inhibition. The models developed according to Eqs. (4) and (5) were used to study the effects of substrate inhibition on the substrate utilization and biomass concentration profiles. Figs. 3 and 4 depict experimental and simulated values of substrate and biomass concentration as a function of reaction propagation time using substrate inhibition model. These values have good resemblance as compared to the experimental results. The little deviation between the experimental data and those predicted by the model might be due to the assumption that $Y_{x/s}$ remained constant over the system irrespective of time of fermentation. This was assumed by ignoring the effect of maintenance energy on cell growth and metabolism. Testing of variance methods was applied to find out the statistical significance of the experimental data points and those predicted by the model. It revealed that there were little or no real evidences against the null hypothesis indicating that all residuals had a random normal distribution of less than 5% random error.

4.3. *Photofermentation of spent medium in stage 2*

Spent medium from glucose-fed hydrogen fermentation exhibited a high total organic acid concentration (about 2000–2500 mg l^{-1}) and was comprised predominantly of acetic acid, together with a small amount of butyric acid and ethanol. However, the spent medium was used for photoproduction of hydrogen in the second stage irrespective of its composition and without the separation of accompanying metabolites. Light grown cells of *R. sphaeroides* O.U. 001 (containing about 0.15–0.17 g l^{-1} cells), were inoculated in the spent medium of dark fermentation [19]. Since, with 1% (w/v) of glucose concentration in the MYG medium maximum cumulative volume of hydrogen was obtained in the first stage, the spent medium from the same was used for photofermentation in a subsequent second stage. The yield of hydrogen in the second stage was 1.5–1.72 mol H_2 (mol acetic acid)$^{-1}$. 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 MYG media of dark fermentation) was converted to acetic acid and no additional substrate was supplied therein. The overall yield of hydrogen in two-stage process considering glucose as initial substrate was found higher compared to a single stage process. Oh et al. [8] have reported both dark and photofermentation using the same strain of *R. palustris* P4. The conversion yield of acetate to H_2 was estimated to be 2.4–2.8 mol H_2 (mol acetate)$^{-1}$ indicating that the overall yield from glucose to H_2 by a two-step process of the dark and photofermentation can be increased by twofold compared to that by the dark-only process [8]. However, volumetric H_2 production rate in photofermentation was too low for the two-step process to be economically practical. But according to the authors the fact that a single strain can carry out both dark fermentation and photofermentation has an important implication to the process development for economic H_2 production.

4.4. *Kinetics of stage 2 by logistic model*

Effect of initial acid concentration, expressed in terms of total acetic acid, in the spent media from dark fermentation have profound effect on hydrogen production as well as on biomass concentration in photofermentation in the second stage. To study the effect of initial acetic acid concentration in photoproduction of hydrogen, spent media from four different batches having four different concentration of acetic acid were selected. A light grown culture was used as inoculum at an initial concentration of 0.17 g cells (l media)$^{-1}$. The profile of biomass concentration with time (Fig. 5) indicates that biomass concentration increases appreciably with a high initial acetic acid concentration of 2.5 g l^{-1} for set 4, whereas

for the remaining sets there is only marginal increase in biomass concentration with increase in acetic acid concentration. Oh et al. [8] also reported that *R. palustris* P4 could utilize acetate as a carbon and an energy source for biomass growth and hydrogen production under light conditions.

Although Eq. (7) is implicit in its dependence on substrate concentration, however for the batch hydrogen production experiments in the second stage fermentation of this study where the initial substrate concentration and volume of inoculum are kept constant, the logistic model is a fair approximation of the growth kinetics [18]. The logistic model parameters (k_c and X_{max}) for the growth of *R. sphaeroides* O.U. 001 using various initial concentration of acetic acid in the spent medium from stage 1 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. Both X_0 and X_{max} values that are determined by the logistic model are quite similar to that of the experimental values. For initial acetic acid concentration of 2.5 g l^{-1} in the spent medium the maximum specific growth rate was found to be the highest.

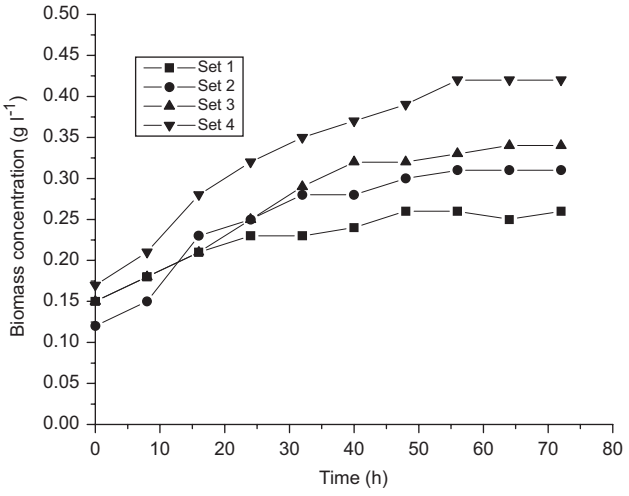

Fig. 5 – Biomass concentration profile for various initial acetic acid concentration. Concentration of acetic acid (g l^{-1}) in set 1: 0.35; set 2: 0.55; set 3: 0.95; set 4: 2.5. Temperature: 34 °C, light intensity 5000 ± 500 lx and initial pH of the medium was adjusted to 6.8.

4.5. *Cumulative hydrogen production by modified Gompertz equation*

Cumulative hydrogen production curves were fitted by the modified Gompertz equation with various initial concentration of glucose as the substrate. The values of *P*, R_m and λ were determined by best fitting the cumulative hydrogen production data for the above equation using a software program, Curve expert 1.3 (Table 1). The overall magnitude of regression obtained in the range 0.990–0.996 indicated a strong correlation between the experimental data and the fit. The calculated values of *D* of Kolmogorov–Smirnov test, for determining the random normal distribution of residuals (i.e. the difference between the estimation and experiment) ranged from 0.14 to 0.31, which was less than the table value of 0.36. This indicates that all residuals had a random normal distribution of less than 5% random error. The values of different coefficients are given in Table 1. It reveals that maximum hydrogen production potential (*P*) was obtained with 1% (w/v) of glucose concentration however the rate of production was maximal at 1.2% glucose concentration. The value of R_m, the maximum production rate, was 13.917 ml h^{-1} which is much higher than those reported by Liu et al. [29] and Lee et al. [30] but the value of λ, the lag phase time, is in good agreement with those obtained in the literature [30–32]. However, it is somewhat difficult to compare the values of these parameters even under comparable conditions as microorganisms and the substrates are different.

The cumulative hydrogen production curve from the photofermentation of the spent medium was also fitted by the modified Gompertz equation. The spent medium which was used at this stage was obtained in a batch of dark fermentation of the MYG medium originally containing 1% (w/v) of glucose. The values of *P*, R_m and λ were determined by best fitting the cumulative hydrogen production data for the above equation using the same software. Both the overall magnitude of regression (0.996), and the value of *D* of Kolmogorov–Smirnov test agreed well between the experimental data and the fit. The values of different coefficients are given in Table 3.

Table 2 – Kinetic model parameters of the logistic equation for various initial acetic acid concentration in the spent media from dark fermentation

Run no.	Initial acetic acid concentration (g l^{-1})	X_{max} (g l^{-1})	X_0 (g l^{-1})	k_c (h^{-1})	R^2
1	2.5	0.44	0.06	0.09	0.993
2	0.95	0.39	0.045	0.05	0.988
3	0.55	0.31	0.06	0.04	0.992
4	0.35	0.25	0.04	0.05	0.991

R^2: Residual sum of squares.

Table 3 – Kinetic parameters of cumulative hydrogen production calculated from nonlinear regression of Gompertz equation in stage 2

Kinetic parameters	Values
P (ml H_2)	615.65
R_m (ml h^{-1})	16.15
λ (h)	14.32
Correlation coefficient	0.996
Residual sum of squares	18.47
D (Kolmorgorov–Smirnov test)	0.161

5. Conclusion

Spent medium from dark fermentation by *Enterobacter cloacae* DM11 is found to have the ability to photoproduce hydrogen by *Rhodobacter sphaeroides* O.U. 001 in a two-stage batch fermentation process. The yield of hydrogen in the first stage was about 3.31 mol H_2 (mol glucose)$^{-1}$ and that of in the second stage was about 1.5−1.72 mol H_2 (mol acetic acid)$^{-1}$. The overall yield of hydrogen in two-stage process considering glucose as preliminary substrate was found to be higher compared to a single stage process. Substrate inhibition model following Andrew's equation was found suitable for hydrogen production using glucose as substrate in the first stage of fermentation compared to the classical Monod model. For initial acetic acid concentration of 2.5 g l^{-1} in the spent medium the maximum specific growth rate was found to be 0.099 h^{-1} using logistic model in the second stage. Both the curve fitting and statistical analysis showed that the modified Gompertz equation was suitable to describe the progress of cumulative hydrogen production. For the first stage, the value of R_m, the maximum production rate, was 13.917 ml h^{-1} and λ, lag phase time was 4.04 h using 1% (w/v) of glucose concentration, whereas for the second stage the value of R_m was 16.15 ml h^{-1} and λ, 14.32 h. However, it is worthwhile to note that the overall yield of hydrogen obtained in the present study is much lower than the theoretical maximum value. Hence, this constitutes an important topic for a further study. Introducing continuous immobilized reactor instead of batch, maintaining higher concentration of cells in the inoculum and optimizing the light intensity might be some of the options that could be explored for the amelioration of hydrogen productivity in two-stage process.

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