Pakistan Journal of Biological Sciences 13 (14): 674-682, 2010
ISSN 1028-8880

Applications of the Box-Wilson Design Model for Bio-hydrogen Production using *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564)

[1]W.M. Alalayah, [1]M.S. Kalil, [1]A.A.H. Kadhum, [1]J. Jahim, [2]A. Zaharim, [4]N.M. Alauj and [1,2,3]A. El-Shafie
[1]Department of Chemical and Process Engineering,
[2]Unit of Fundamental Engineering Studies,
[3]Department of Civil and Structural Engineering,
Faculty of Engineering and Built Environment,
Universiti Kebangsaan Malaysia, 43600 UKM Bangi, Malaysia
[4]Aden Refinery Company, P.O. Box (3003) Little 110 Aden, Yemen

Abstract: Box-Wilson Design (BWD) model was applied to determine the optimum values of influencing parameters in anaerobic fermentation to produce hydrogen using *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564). The main focus of the study was to find the optimal relationship between the hydrogen yield and three variables including initial substrate concentration, initial medium pH and reaction temperature. Microbial growth kinetic parameters for hydrogen production under anaerobic conditions were determined using the Monod model with incorporation of a substrate inhibition term. The values of μ_{max} (maximum specific growth rate) and K_s (saturation constant) were 0.398 h^{-1} and 5.509 g L^{-1}, respectively, using glucose as the substrate. The experimental substrate and biomass-concentration profiles were in good agreement with those obtained by the kinetic-model predictions. By varying the conditions of the initial substrate concentration (1-40 g L^{-1}), reaction temperature (25-40°C) and initial medium pH (4-8), the model predicted a maximum hydrogen yield of 3.24 mol H_2 (mol glucose)$^{-1}$. The experimental data collected utilising this design was successfully fitted to a second-order polynomial model. An optimum operating condition of 10 g L^{-1} initial substrate concentration, 37°C reaction temperature and 6.0±0.2 initial medium pH gave 80% of the predicted maximum yield of hydrogen where as the experimental yield obtained in this study was 77.75% exhibiting a close accuracy between estimated and experimental values. This is the first report to predict bio-hydrogen yield by applying Box-Wilson Design in anaerobic fermentation while optimizing the effects of environmental factors prevailing there by investigating the effects of environmental factors.

Key words: Bio-hydrogen production, renewable energy, anaerobic fermentation, Box-Wilson design model

INTRODUCTION

There has been a renewed research interest on biological hydrogen production because of the growing global environmental concerns regarding depletion of fossil fuel and expected drastic environmental condition in coming future. Hydrogen is considered as promising, alternative to fossil fuel and clean energy carrier without any emission of carbon dioxide or hazardous material on burning in contrast to other conventional fuels. Producing H_2 from renewable feedstock could potentially alleviate many environmental, social and political problems associated with using fossil fuels (Barreto *et al*., 2003). Several processes may be applied to produce hydrogen including electrolysis of water, thermo catalytic reformation (steam reforming) of hydrogen-rich organic compounds and biological processes (Rosen and Scott, 1998). However, a critical analysis of the steam-reformation route illustrates that emissions from this process are a major contributor to global greenhouse gases (Dunn, 2002).

The steam reformation and electrochemical routes to hydrogen are energy intensive and rely on the use of fossil fuels (Dunn, 2002; Midillia *et al*., 2005). Therefore, the steam-reforming and electrochemical-decomposition routes cannot be considered sustainable or environmentally friendly. Biological routes mediated by different microorganisms can produce hydrogen from reduced carbon compounds or from water. The two main routes under consideration include bio photolysis and

Corresponding Author: W.M. Alalayah, Department of Chemical and Process Engineering,
Faculty of Engineering and Built Environment, Universiti Kebangsaan Malaysia,
43600 UKM Bangi, Malaysia

anaerobic fermentation. In the direct and indirect bio photolysis routes, hydrogen is produced from water in the presence of sunlight (Nath and Das, 2004). The direct route involves the splitting of water in a single step, while in the indirect route several steps are involved and the end products are hydrogen and oxygen (Kotay and Das, 2008). The degradation of complex organic molecules by anaerobic microorganisms to produce hydrogen is another biological route, termed dark fermentation (Das and Veziroglu, 2001).

Fermentative production of hydrogen is an exciting area of technological development that offers a potential means to produce hydrogen from a variety of renewable resources. Through fermentation processes, hydrogen gas can be produced directly from high concentrations of renewable substrates such as sugars or even wastewater. The theoretical yield of hydrogen from glucose fermentation can be estimated by a known metabolic pathway, giving a maximum yield of four moles of hydrogen per mole of glucose when acetic acid is produced as the terminal metabolite. Many studies have reported that hydrogen can be produced from wastewater or solid waste by mixed/pure cultures in batch or chemostat reactors (Fang and Liu, 2002; Lin and Lay, 2004; Noike and Mizuno, 2000; Ueno *et al*., 1995), but with a wide fluctuation in hydrogen-production performance. The relatively unstable and unpredictable biological hydrogen-production processes are primarily dependent on fermentation conditions such as pH (Fang and Liu, 2002; Zhu and Yang, 2004; Khanal *et al*., 2004) and hydraulic or solid retention time. Recent reports pointed out that *Clostridium* species were the dominant microorganisms in anaerobic hydrogen-fermentation processes (Iyer *et al*., 2004; Andreesen *et al*., 1989; Wang and Wan, 2009; Cebeci and Sonmez, 2006), but their contributions in hydrogen production have not yet been identified quantitatively.

Clostridia are known as classical acid producers and usually ferment glucose to butyrate, acetate, carbon dioxide and molecular hydrogen (Alalayah *et al*., 2009b). Several statistical-design approaches used to optimise the hydrogen yield in fermentation processes have been reviewed (Wang and Wan, 2009). Among the different approaches, fractional factorial designs are common choices. A full factorial design is often considered impractical due to the requirement for a large number of experiments to accurately predict the response. In comparison, a fractional factorial-design approach suffers from its ability to accurately predict all positions of the factor space equidistant from the centre (rotatability).

Another approach to investigate the impact of the experimental variables on hydrogen production is to use a response-surface design. Central Composite Design (CCD) and Box-Wilson Design (BWD) are response surface designs which are commonly chosen for the purpose of response optimisation (Cebeci and Sonmez, 2006).

In the present study, the Monod model was applied to the microbial growth kinetic parameters for *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564) (hereafter referred to as CSN1-4) using glucose as a substrate and the hydrogen yield was optimised using the Box-Wilson Design (BWD) to develop a predictive model for the hydrogen yield. This is the first report to investigate the effect of environmental parameters on optimum hydrogen production by applying Box-Wilson Design model and may help the researchers at industrial or laboratory scale to investigate the influence of factors and estimate the near about accurate hydrogen yield in anaerobic/aerobic fermentation.

MATERIALS AND METHODS

Microbial strain and preculture development: The CSN1-4 culture stock was obtained from a culture collection maintained at the Chemical Engineering Department, UKM and reported previously by (Alalayah *et al*., 2009a; Kalil *et al*., 2003).

Culture media: A solution of 15% PG medium per litre of distilled water was used as a growth medium for the inoculum. This medium was incubated in boiling water for one hour and then filtered through cotton cloth. The filtrate was sterilised in an autoclave at 121°C for 15 min. TYA medium was used for the preculture as well as main culture and the composition of this medium per litre of distilled water was 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 (Alalayah *et al*., 2008).

Experimental procedure: The experimental methods reported in this work were adapted from earlier studies published in the literature (Alalayah *et al*., 2009a, b; Wooshin *et al*., 2006).

Statistical analysis: The variation between the experimental data points and those predicted by the Monod models with the substrate was estimated by testing of the hypothesis by population variance using least-squares regression and the statistical significance of the parameters obtained by nonlinear curve fitting; the hydrogen yield was optimised using a Box-Wilson Design (BWD) to develop a predictive model using the (Statistica 7.0) software program.

KINETIC MODELLING DEVELOPMENT

Biomass-growth kinetics by the monod model: The Monod equation empirically fits a wide range of data satisfactorily and is the most commonly applied model of microbial growth. The values of specific growth rate (μ_{max}) and saturation constant (K_s) were estimated following the Monod model by regression analysis. The temperature was held at 37°C during experiments.

Model of substrate-and biomass-concentration profiles: To determine the simulated values of substrate concentration as a function of time the following expression was used (Shuler and Kargi, 2002):

$$\mu_{max} = \left(X_0 + Y_{\frac{x}{s}} S_0 \right) t = [X_0 + Y_s (S_0 + K_s)] \mathrm{Ln} \left(\frac{X_0 + Y_{\frac{x}{s}} (S_0 + S)}{X_0} \right) - K_s Y_{\frac{x}{s}} \mathrm{Ln} \left(\frac{S}{S_0} \right) \tag{1}$$

From the above expression, the simulated substrate profile with time was determined using the Wegstein convergence method of successive substitutions in each iteration (Wu *et al*., 2006). The simulated values of cell-mass concentration, X, were calculated by the following relation:

$$X = X_0 + Y_{\frac{x}{s}} (S_0 - S) \tag{2}$$

Substrate-inhibition model: At high substrate concentrations, bacterial growth is inhibited by the substrate. The degree of substrate inhibition can be described by Andrews (1968):

$$\mu = \frac{\mu_{max} S}{K_s + S + (\frac{S^2}{K_i})} \tag{3}$$

where, μ_{max} is the maximum specific growth rate, K_s is the saturation constant for glucose and S is the inhibition constant for glucose and *S* is the glucose concentration. The values of μ_{max}, K_s and K_i can be obtained (by Lineweaver-Burk plot) and the relationship between specific growth rate and substrate concentrations thus determined.

Simulation of biomass-and substrate-concentration profiles: The growth kinetics of CSN1-4 during batch fermentation can be described for total biomass formation by this model:

$$R_x = \frac{dx}{dt} = \mu X = \frac{\mu_{max} S}{K_s + S + (\frac{S^2}{K_i})} X \tag{4}$$

where, R_x is the rate of change of cell concentration and related to the cell concentration X by the specific growth rate. The carbon-source consumption rate (Rs) can be expressed as:

$$R_x = \frac{dx}{dt} = -\frac{\mu}{Y_{\frac{x}{s}}} X - mX \tag{5}$$

where (S) is the concentration of substrate utilised for total biomass formation and (m) is the maintenance energy.

Hydrogen-production model using a Box-Wilson design method: A second-order polynomial mathematical model was employed to represent the yield of hydrogen (y) as a function of reaction temperature, initial medium pH and initial glucose concentration. The general form of this model for these three variables is represented by the following regression formulation:

$$y = a_0 + a_1 T + a_2\ pH + a_3 S + a_4 T{*}pH + a_5 T{*}S + a_6 S{*}pH + a_7 T^2 + a_8 pH^2 + a_9 S^2 \tag{6}$$

The model was evaluated based on the experimental results, with optimum values sought for the three independent variables. The total number of experiments N was computed according to the following equation:

$$N = 2^P + 2P + 1 \text{ then, } N = 2^P + 2P + 1 = 15$$

Here (P) is the number of variables and an experimental design based on the Box-Wilson method was used to organise the experiments (Cebeci and Sonmez, 2006; Badiea and Mohana, 2008). In order to design the experiments, model 6 was evaluated with respect to the experimental response. Terms (a_0-a_9) in this model are coefficients of the multiple regression analysis. The operating range of the variables is given in Table 1.

Table 1: Variables and levels that selected from the experimental study

	Levels		
Variables	-1	0	1
Reaction temperature (°C)	28	32	37
Initial medium pH	5	6	8
Initial glucose concentration (g L^{-1})	10	20	40

RESULTS AND DISCUSSION

Kinetics of cell growth by the Monod model: The Monod equation was used to develop a model of biomass growth for hydrogen production using CSN1-4. The values of the specific growth rate (μ_{max}) and substrate constant (K_s) estimated by Lineweaver-Burk linearisation were 0.40 h^{-1} and 5.5 g LG^{1}, respectively, using TYA medium and glucose as the growth substrate. The maximum specific growth rate depends on temperature and initial pH medium. It should be noted that the temperature was kept constant during growth experiments while pH was not controlled (Alalayah *et al*., 2008). Both of these values were found to be lower than some reported previously (Nath *et al*., 2008) but were within the reported range (Kumar *et al*., 2000; Horiuchi *et al*., 2002). Experimental data and those predicted produced by the Monod kinetic model for substrate and biomass concentrations over the course of the fermentation are shown in Fig. 1 and 2.

The experimental conditions were 10 g LG^{1} initial glucose concentration, 37°C reaction temperature and 6.0±0.2 initial medium pH. Figure 1 and 2 show few relatively insignificant fits between the experimental data and predictions, perhaps due to either product or substrate inhibition as reported previously (Kumar *et al*., 2000). The presence of a gas phase in the reactor at high partial pressures of hydrogen resulted in a lowering of the hydrogen production as evaluated by Kumar *et al*. (2000), Horiuchi *et al*. (2002). Apparently, as in the present process the product is a gas, the trace effect of product inhibition can be neglected. Testing of variance methods was applied to investigate and evaluate the statistical significance of the proposed model output with the experimental data points. It revealed that there was slight or no significant evidence against the null hypothesis, indicating that all residuals had a random normal distribution of less than 5% random error.

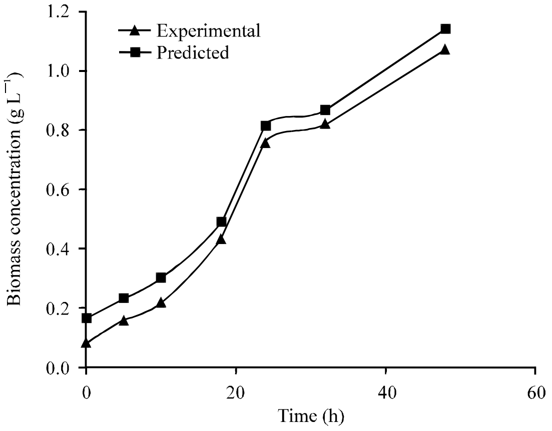

Fig. 1: Experimental data and kinetic model prediction for biomass concentration as a function of reaction time using Monod model

Substrate-inhibition model: The influence of glucose concentration on the specific growth rate was obtained in batch reactors inoculated at different initial glucose concentrations, as Shown in Fig. 3. Model 3 (Andrews' model) was used to describe the relationship between substrate concentrations and the specific growth rate. Substrate inhibition was observed at glucose concentrations greater than 10 g LG^{1}. The values of maximum specific growth rate, $\mu_{max,}$ substrate constant for glucose, K_S and the inhibition constant for glucose, K_i, were estimated by Lineweaver-Burk plotting that reported by Najafpour (2007) and Shuler and Kargi (2002). The effect of substrate inhibition based on Andrews' model can be used to predict the growth rate (Ghose and Tyagi, 1979).

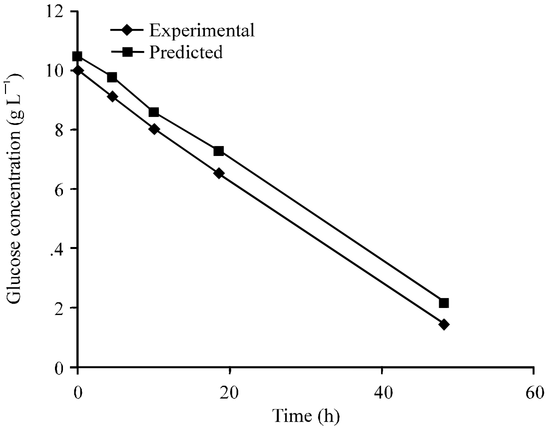

Fig. 2: Experimental data and kinetic model prediction for substrate concentration as a function of reaction time using Monod model

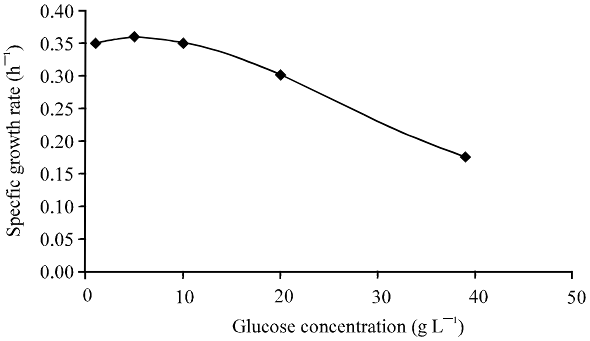

Fig. 3: Profile of set of glucose concentrations with specific growth rate using CSN1-4 in batch reactor

Analysis of Box-Wilson design experimental results: Prediction of the Hydrogen Yield (HY) under any experimental approach is considered to be a highly stochastic process and requires a nonlinear mathematical procedure. The BWD is a statistical technique to investigate the impact of the experimental variables on the response output that use Central Composite Design (CCD) to use a response surface design, which are commonly chosen for the purpose of response optimization. BWD always based on Newton statistical method and also depend on the numbers of the variables (Montgomery, 1976; Box and Wilson, 1951). The hydrogen produced from glucose during fermentation was considered as a response variable and the combinations of computed values at different factor-level were treated statistically to develop the response surface model.

A nonlinear least-squares regression program based on the Gauss-Newton Method (GNM) was used to fit the experimental data of hydrogen yield to construct the model 6 and it was used by Badiea and Mohana (2008) who reported that the BWD model was used to relate the response and three variables inputs. This fitting provided the predicted hydrogen yield (y), the residual error and the coefficients (a_n) of the equation. The fitted response, y, for coded variables in the form of a matrix is shown in Table 2 and presented as model 7:

$$y = 71.80+7.85T_c+8.64pH_c+7.30S_c-0.408(T_c*pH_c)-2.23(T_c*S_c)-1.22(pH_c*S_c)-4.680T_c^2-6.044pH_c^2-4.23S_c^2 \quad (7)$$

The above model represents the best form of the mathematical model that relates the hydrogen yield (y) to the three variables in terms of coded levels with a high coefficient of determination (R^2 = 0.92). An equivalent equation, in terms of the actual levels, will be more useful in estimating the response for any desired conditions in the range of the independent variables.

Table 2: Box-Wilson design statistical calculations of hydrogen yields

Exp. run No	Coded factors T_c	 pH_c	 S_c	Real factors T	 pH	 S	H_2 yield (%)
1	-1	-1	-1	28	5	10	28.00
2	1	-1	-1	37	5	10	54.00
3	-1	1	-1	28	8	10	45.21
4	1	1	-1	37	8	10	66.56
5	-1	-1	1	28	5	40	49.80
6	1	-1	1	37	5	40	67.08
7	-1	1	1	28	8	40	65.61
8	1	1	1	37	6	10	77.75
9	-1.73	0	0	25	8	10	41.06
10	1.73	0	0	40	4	20	66.60
11	0	-1.73	0	32	4	20	46.02
12	0	1.73	0	32	6	20	71.07
13	0	0	-1.73	32	6	5	45.09
14	0	0	1.73	32	6	20	71.07
15	0	0	0	32	6	20	71.07

T_c: Temperature coded; pH_c: pH coded; S_c: Substrate concentration coded

Development of the response model

Calibration of the response model and effect of the variable factors on response: A least-squares regression program based on the Gauss-Newton Method (GNM) was used to verify model 6 by using the set of 10 experimental runs and fitted the results well (R^2 = 0.91). A multiple regression analysis was performed on the experimental data to estimate the regression coefficient for model 8. Table 3 shows the values of these coefficients and statistically insignificant terms for the model which represented the suitable form of the mathematical model relating the hydrogen yield, y, to the three variables in terms of levels.

$$y = -649.6-26.47T_c+58.66pH_c+4.99S_c-0.32T_c^2-3.25pH_c^2-0.042S_c^2 \quad (8)$$

The residuals between the experimental and predicted hydrogen yields are important indicators for demonstrating the effectiveness of the proposed model for mapping the experimental data and hence for predictions. The maximum response of hydrogen yield in this model, recorded near the optimal factor setting, was 80%, which is comparable to that obtained with the optimum factors in the experimental of 77.75%. The effect of the reaction-temperature factor on the hydrogen yield in model 8 predicted increased hydrogen yields with increasing temperature, while the observed response of hydrogen yield decreased as the temperature increased above 37°C. Kaushik *et al*. (2006) reported agreement with this observation and other studies reported values in the same range (Alalayah *et al*., 2008; Nath *et al*., 2008). Figure 4 shows that the lowest hydrogen yield was at 25°C and the highest hydrogen yield was at 37°C based on an initial medium pH of 6.0±0.2 and an initial glucose concentration of 10 g LG^{1}.

An increase in hydrogen yield was also associated with increasing initial medium pH from 4.0 to 6±0.2 and the hydrogen yield decreased when the initial medium pH was greater than 6.0±0.2. The optimal pH of 6.0±0.2 showed a

Table 3: Regression coefficients of the response surface model for hydrogen yield

Term	Coefficients	Regression coefficients	P
constant	a_0	-649.677	S
T	a_1	26.479	S
pH	a_2	58.663	S
S	a_3	4.990	S
T*pH	a_4	-0.065	NS
T*S	a_5	-0.350	NS
S*pH	a_6	-0.062	NS
T^^{2}	a_7	-0.320	S
pH^^{2}	a_8	-3.252	S
S^^{2}	a_9	-0.041	S

Where statistically insignificant (p>0.05); NS: Insignificant; S: Significant

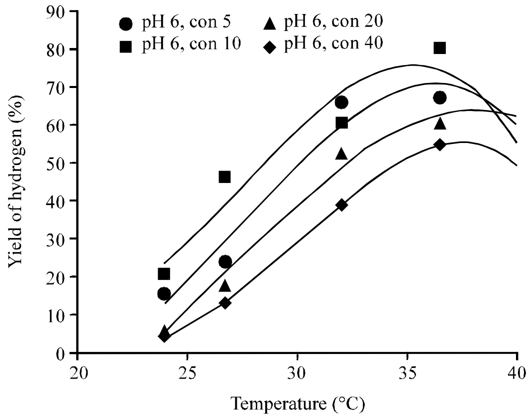

Fig. 4: Relations between hydrogen yield with reaction temperature at different initial glucose concentrations and fixed initial medium pH

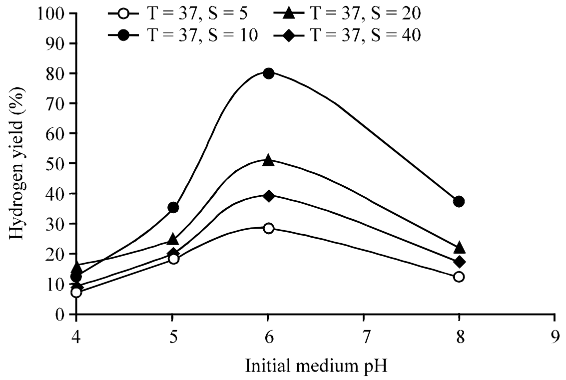

Fig. 5: Relations between hydrogen yield with initial medium pH at different initial glucose concentrations and fixed reaction temperature, T: Temperature, pH: Initial medium and S: Initial glucose concentrations g LG^1

significant improvement in the hydrogen yield compared to pH values at 7.0±0.2 and 8±0.2, as reported previously by several researches (Wooshin *et al*., 2006; Fang and Liu, 2002) and statistically shown in Fig. 5.

The model predicted that the lowest hydrogen yields were at the initial medium pH of 4.0 ±0.2 and the highest hydrogen yield were at the initial medium pH of 6.0 ±0.2 based on a reaction temperature of 37EC and an initial glucose concentration of 10 g LG^1.

The effects of glucose content in the culture media on fermentation were evaluated at initial concentrations from 1-40 g LG^1. As shown in Fig. 6, the highest yield of hydrogen was observed when the initial glucose concentration was 10 g LG^1 and it decreased with increasing glucose concentration based on a reaction temperature of 37°C and initial medium pH of 6.0 ±0.2.

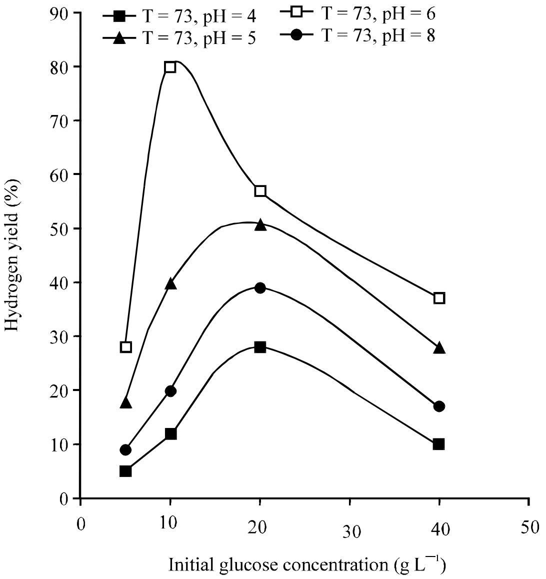

Fig. 6: Relations between hydrogen yield with initial glucose concentrations at different initial medium pH and fixed reaction temperature

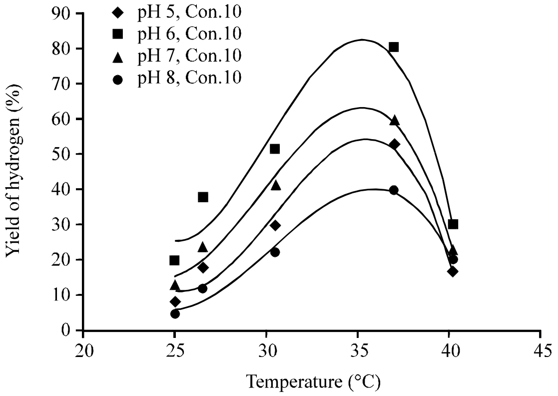

Fig. 7: Relations between hydrogen yield with reaction temperature at different initial medium pH and fixed initial glucose concentrations

Validation of the response model and effect of the variable factors on response: Using the same design method and the least-squares regression based on the Gauss-Newton method used to validate model 6, the five remaining experimental runs were evaluated and fitted (R^2 = 0.89). A validation study was performed for each of the three factors under evaluation, in which the model prediction was compared against values reported in the literature. The hydrogen yield was computed for reaction temperatures in the range of 25-40°C. Figure 7 shows the lowest hydrogen yields were at the initial medium pH of 8±0.2 and the highest hydrogen yield was at an initial medium pH of 6.0±0.2, both at an initial glucose

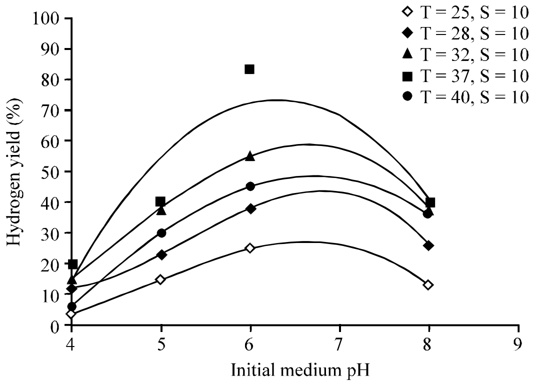

Fig. 8: Relations between hydrogen yield with initial medium pH at different reaction temperature initial and fixed glucose concentrations

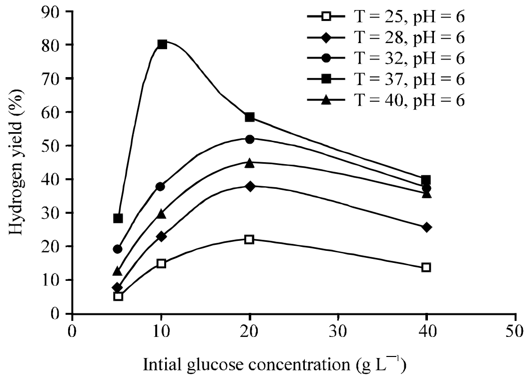

Fig. 9: Relations between hydrogen yield with initial glucose concentrations at different reaction temperature and fixed initial medium pHs, T: Temperature, pH: Initial medium and S: Initial glucose concentrations g LG^{1}

concentration of 10 g LG^{1} and different reaction temperatures. This observation was compatible with the previously published experimental data (Alalayah *et al.*, 2008).

Increased hydrogen yield at an initial pH of 6 ±0.2 in batch pure/mixed cultures has been observed by several researchers (Mu *et al.*, 2008). In addition, Liu and Fang 2002 reported an initial pH value of 5.5 as the optimum for maximum hydrogen yield. Under similar conditions, (Fang *et al.*, 2006) observed maximum yields at pH 4.5 and 5.0, respectively. It should be noted that pH ranges from 6.0 to 8.0 are preferred by the former microorganisms (Wooshin *et al.*, 2006; Fang and Liu, 2002; Nath *et al.*, 2008; Fang *et al.*, 2002a, b). Figure 8 shows the low hydrogen yields at 25°C, an initial glucose concentration of 10 g LG^{1} and different initial medium pH values. Figure 9 shows the low hydrogen yields at 25°C, an initial medium pH of 6.0±0.2 and different initial glucose concentrations.

CONCLUSIONS

The results of this investigatory studies on hydrogen production using CSN1-4 has successfully paved the path to estimate the target goals by applying mathematical modeling for the hydrogen production in fermentation process. Extract of our research work is to present the computational estimation and experimental verification of expected hydrogen yield as a clean fuel from glucose using CSN1-4 by optimizing the values of all influencing environmental parameters on the basis of Box Wilson Design model. The Monod model, with incorporation of a substrate-inhibition term was also used to determine the growth kinetic parameters for hydrogen yield. The three experimental factors under consideration were including the initial glucose concentration, initial pH and reaction temperature presenting significant interactions among each other. The predicted hydrogen using computational estimation (80%) was in close proximity to the experimental hydrogen yield (77.77%) under the optimized operating conditions as described before. This study may assist the researchers at industrial scale and laboratory scale to find computational estimation of maximum hydrogen yield under different influencing parameters.

ACKNOWLEDGMENTS

The authors thanks to Prof. Dr. Yoshino Sadazo, Kyushu University, Japan, who provided us with CSN1-4 and Dr. Ehsan Ali, Universiti Kebangsaan Malaysia for the valuable discussions during my studies. This research was supported by the UKM- GUP-KPB-08-32/128 grant.

NOMENCLATURE

μ_{max} = Maximum specific growth rate
K_s = Saturation constant
K_i = Inhabitation constant for glucose
a_i = Coefficients of estimated model
P = Number of variables
Si = Significant
NS = Not significant
X_0 = Initial biomass concentration g LG^{1}
X = Biomass concentrations g LG^{1}
S_0 = Initial glucose concentration g LG^{1}
S = Glucose concentrations g LG^{1}
$Y_{x/s}$ = Yield of biomass

REFERENCES

Alalayah, W.M., M.S. Kalil, A.H. Kadhum, J.M. Jahim and N.M. Alaug, 2008. Hydrogen production using *Clostridium saccharoperbutylacetonicum* N1-4 (ATCC 13564). Int. J. Hydrogen Energy, 33: 7392-7396.

Alalayah, W.M., M.S. Kalil, A.H. Kadhum, J.M. Jahim and N.M. Alaug, 2009a. Effect of environmental parameters on hydrogen production using *C. saccharoperbutylacetonicum* N1-4(ATCC 1356 4). Am. J. Environ. Sci., 5: 80-86.

Alalayah, W.M., M.S. Kalil, A.A.H. Kadhum, J.M. Jahim, S.Z.S. Jaapar and N.M. Alauj, 2009b. Bio-hydrogen production using a two-stage fermentation process. Pak. J. Biol. Sci., 12: 1462-1467.

Andreesen, J.R., H. Bahl and G. Gottschalk, 1989. Introduction to the Physiology and Biochemistry of the Genus *Clostridium*. In: Biotechnology Handbooks: *Clostridia*, Minton, N.P. and D.J. Clarke (Eds.). Plenum Press, New York.

Andrews, J.F., 1968. A mathematical model for the continuous culture of microorganism utilizing inhibitory substrates. Biotechnol. Bioengin., 10: 707-723.

Badiea, M.A. and N.K. Mohana, 2008. Effect of fluid velocity and temperature on the corrosion mechanism of low carbon steel in industrial water in the absence and presence of 2-hydrazino benzothiazole. Korean J. Chem. Eng., 25: 1292-1299.

Barreto, L., A. Makihira and K. Riahi, 2003. The hydrogen economy in the 21st century: a sustainable development scenario. Int. J. Hydrogen Energy, 28: 267-284.

Box, G.E.P. and K.B. Wilson, 1951. On the experimental attainment of optimum conditions. J. R. Statist. Soc., 13: 1-45.

Cebeci, Y. and I. Sonmez, 2006. Application of the Box-Wilson experimental design method for the spherical oil agglomeration of coal. Fuel, 85: 289-297.

Das, D. and T.N. Veziroglu, 2001. Hydrogen production by biological processes: A survey of literature. Int. J. Hydrogen Energy, 26: 13-28.

Dunn, S., 2002. Hydrogen futures: Toward a sustainable energy system. Int. J. Hydrogen Energy, 27: 235-264.

Fang, H. and H. Liu, 2002. Effect of pH on hydrogen production from glucose by a mixed culture. Bioresour. Technol., 82: 87-93.

Fang, H.H.P., H. Liu and T. Zhang, 2002a. Characterization of a hydrogen-producing granular sludge. Biotechnol. Bioeng., 78: 44-52.

Fang, H.H.P., T. Zhang and H. Liu, 2002b. Microbial diversity of a mesophilic H2- producing sludge. Applied Microbiol. Biotechnol., 58: 112-118.

Fang, H.H.P., C. Li and T. Zhang, 2006. Acidophilic biohydrogen production from rice slurry. Int. J. Hyd. Energy, 31: 683-692.

Ghose, T.K. and R.D. Tyagi, 1979. Rapid ethanol fermentation of cellulose hydrolysate.II. Product and substrate inhibition and optimization of fermenter design. Biobutanol. Bioeng., 21: 1401-1420.

Horiuchi, J.I., T. Shimizu, K. Tada, T. Kanno and M. Kobayashi, 2002. Selective production of organic acids in anaerobic acid reactor by pH control. Bioresour. Technol., 82: 209-213.

Iyer, P., M.A. Bruns, H. Zhang, S.V. Ginkel and B.E. Logan, 2004. H_2 producing bacterial communities from a heat-treated soil inoculum. Applied Microbiol. Biotechnol., 66: 166-173.

Kalil, M.S., K.W. Pang, Y. Wan Mohtar Wan, S. Yoshino and R.A. Rakmi, 2003. Direct fermentation of palm oil mill effluent to acetone-butanol-ethanol by solvent producing clostridia. Pak. J. Biol. Sci., 6: 1273-1275.

Kaushik, N., K. Anish and D. Debabrata, 2006. Effect of some environmental parameters on fermentive hydrogen production. Can. J. Microbiol., 52: 525-535.

Khanal, S.K., W.H. Chen and S. Sung, 2004. Biological hydrogen production: Effects of pH and intermediate products. Int. J. Hydrogen Energy, 29: 1123-1131.

Kotay, S.M. and D. Das, 2008. Biohydrogen as a renewable energy resource–prospects and potentials. Int. J. Hydrogen Energy, 33: 258-263.

Kumar, N., P.S. Monga, A.K. Biswas and D. Das, 2000. Modeling and simulation of clean fuel production by *Enterobacter cloacae* IIT-BT 08. Int. J. Hydrogen Energy, 25: 945-952.

Lin, C.Y. and C.H. Lay, 2004. Carbon/nitrogen-ratio effect on fermentative hydrogen production by mixed microflora. Int. J. Hydrogen Energy, 29: 41-45.

Midillia, A., M. Aya, I. Dincer and M.A. Rosen, 2005. On hydrogen and hydrogen energy strategies: I: current status and needs. Renewable Sustainable Energy Rev., 9: 255-271.

Montgomery, D.G., 1976. Design and Analysis of Industrial Experiments. 3rd Edn., John Weley and Sons, Inc., New Delhi.

Mu, Y., G. Wang and H.Q. Yu, 2008. Response surface methodological analysis on biohydrogen production by enriched anaerobic cultures. Enzyme Microbiol. Technol., 38: 905-913.

Najafpour, G.D., 2007. Biochemical Engineering and Biotechnology. 1st Edn., Elsevier, New York, pp: 51-56.

Nath, K. and D. Das, 2004. Improvement of fermentative hydrogen production: Various approaches. Applied Microbiol. Biotechnol., 65: 520-529.

Nath, K., M. Muthukumar, A. Kumar and D. Das, 2008. Kinetics of two-stage fermentation process for the production of hydrogen. Int. J. Hydrogen Energy, 33: 1195-1203.

Noike, T. and O. Mizuno, 2000. H_2 fermentation of organic municipal wastes. Water Scin. Technol., 42: 155-162.

Rosen, M.A. and D.S. Scott, 1998. Comparative efficiency assessments for a range of hydrogen production processes. Int. J. Hydrogen Energy, 23: 653-659.

Shuler, M.L. and F. Kargi, 2002. Bioprocess Engineering: Basic Concepts. 2nd Edn., Prentice-Hall, Englewood Cliffs, New Jersey, ISBN-13: 9780130819086, pp: 171.

Ueno, Y., T. Kawai, S. Sato, S. Otsuka and M. Morimoto, 1995. Biological production of hydrogen from cellulose by natural anaerobic microflora. J. Ferment. Bioeng., 79: 395-397.

Wang, J. and W. Wan, 2009. Experimental design methods for fermentative hydrogen production: A review. Int. J. Hydrogen Energy, 34: 235-244.

Wooshin, P., H.H. Seung, E.O. Sang, E.L. Bruce and K. Ins, 2006. Removal of headspace CO_2 increases biological hydrogen production. Environ. Sci. Tech. Am. Chem. Soc., 39: 4416-4420.

Wu, S.Y., C.H. Hung, C.N. Lin, H.W. Chen HW, A.S. Lee and J.S. Chang, 2006. Fermentative hydrogen production and bacterial community structure in high-rate anaerobic bioreactors containing silicone-immobilized and self-flocculated sludge. Biotechnol. Bioeng., 93: 934-946.

Zhu, Y. and S.T. Yang, 2004. Effect of pH on metabolic pathway shift in fermentation of xylose by *C. tyrobutyricum*. J. Biotech., 110: 143-157.