PRINT ISSN 1119-8362
Electronic ISSN 2659-1502

Full-text Available Online at
https://www.ajol.info/index.php/jasem
http://www.bioline.org.br/ja

J. Appl. Sci. Environ. Manage.
Vol. 26 (12) 2113-2124 December 2022

Investigation and Scaling of Hydrogen Production by *Klebsiella* sp. ABZ11 for Optimal Yield and the Kinetics of Batch Fermentation Process

*[1]MOHAMMED, A; [2,3]MOHD FIRDAUS, A; [2]ZAHARAH, I; [1]MOHAMMED, IL; [1]MOHAMMED, JN; [1]BALOGU, TV

*[1]Department of Microbiology, IBB University, Lapai Niger State, Nigeria.
[2]Department of Biosciences, Faculty of Science, Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor, Malaysia.
[3]Taiwan-Malaysia Innovation Centre for Clean Water and Sustainable Energy (WISE Centre), Universiti Teknologi Malaysia, 81310 Johor Bahru, Johor, Malaysia.

*Corresponding Author Email: ibrahimnusaiba2@gmail.com, abdmohammed@ibbu.edu.ng
Co-Authors Email: firdausw@utm.my, zaharah@utm.my, ndejiko@gmail.com; misahlegbo@gmail.com, tovin2009@yahoo.com

ABSTRACT: The slow metabolism of bacteria at low temperatures affects the catalytic efficiency of enzymes and productivity. This article investigates the use of a psychrotolerant bacteria (*Klebsiella* sp. ABZ11) for biohydrogen production, yield and scaling at optimal temperature, pH and glucose in a batch fermentation process within a 2-liter bioreactor using Response Surface Methodology (RSM) to report the process performance. The results show 33.5°C, pH 6.75 and 9.15 g glucose as the optimal conditions. Scale-up yielded 137.56 mol/L biohydrogen, 22.13% more than production under optimized conditions. Biomass grew at 0.081/h and doubled in 17 h with 0.71 g cells to reach maximum production. Compared to 0.062/h, 22 h with 0.87 g cells in optimal condition to achieve maximum biohydrogen production. This result shows the potential of biohydrogen production using Antarctic psychrotolerant bacteria at mesophilic temperature.

DOI: https://dx.doi.org/10.4314/jasem.v26i12.28

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Cite this paper as: MOHAMMED, A; MOHD FIRDAUS, A; ZAHARAH, I; MOHAMMED, I. L; MOHAMMED, J. N; BALOGU, T. V. (2022). Investigation and Scaling of Hydrogen Production by *Klebsiella* sp. ABZ11 for Optimal Yield and the Kinetics of Batch Fermentation Process. *J. Appl. Sci. Environ. Manage.* 26 (12) 2113-2124

Dates: Received: 21 November 2022; Revised: 19 December 2022; Accepted: 27 December 2022; Published: 31st December 2022

Keywords: Biohydrogen production; Optimization; Scale-up; Kinetics

Current global energy demand is increasing rapidly with continued dependence on fossil fuels (Holechek et al., 2022). Fossil fuel consumption has led to the emission of greenhouse gases worldwide, leading to pollution and climate change (Bchtold, 2018; Andrews, 2017). The situation urgently requires an alternative renewable energy source that is more environmentally friendly. Hydrogen is considered an alternative energy carrier due to its renewability and high energy yield per unit mass (122 kJ/g), which is 2.75 times higher than conventional fuels (Patel et al., 2015). Furthermore, hydrogen combustion produces only water as a by-product, making it an attractive energy of the future (Christopher and Dimitrios, 2012). Hydrogen is mainly produced from fossil fuels, an energy-intensive and costly inefficient process (Kumar et al. 2019). However, due to the widespread acceptance of hydrogen as the energy of the future, biological routes have been seen as a cleaner and better alternative for production. Biohydrogen is produced through a fermentation process using waste and microorganisms as catalysts (Prakash et al., 2018). The enormous waste in our environment as a substrate for production makes hydrogen a cheaper energy source (Prabakar et al., 2018). Due to increasing access to cold regions and energy saving opportunities in their microbial potential. Research has recently focused on using these strains as inoculum for biohydrogen production (Alvarez-Guzmn et al., 2016; de la Cueva et al., 2018). Psychrophilic bacteria grow best at 15°C – 20°C < 20°C – 40°C for rapid growth and metabolism by psychrotolerant strains. Therefore, general abundance of psychrotolerant strains in cold environments and rapid growth at ambient temperatures are advantages that makes them a better tool for biotechnological applications (Rodrigues and

Silva, 2016; Rizzo and Lo, 2018). Furthermore, due to their versatile nature, psychrotolerant bacteria show better nutritional and environmental diversity than psychrophilic strains (Shen et al., 2017). Most biohydrogen production studies using psychrophilic bacteria as catalysts display low hydrogen yield as problem due to the slow metabolic rate and prolonged lag time (Coultate, 2009; Lu et al., 2012; Witarsa and Lansing, 2015).

This persistent problem in cold adapted hydrogen bacteria need to be addressed for practical applications to improve the hydrogen yield of the process. Therefore, the objective of this study is to investigate the biohydrogen production, yield and scaling by psychrotolerant bacteria at optimal temperature, pH and glucose in a 2-liter bioreactor.

MATERIALS AND METHODS

Media and Bacterial Strain: In this study, *Klebsiella* sp. ABZ11 (GenBank accession no. KX266892) previously obtained from Antarctic seawater was used (Mohammed et al., 2018). Bacterial cells were grown on Marine 2216 agar plates and maintained at 4°C.

Experimental Design: The Central Composite Design (CCD) component of RSM with 3 factorial levels yielded batch fermentation values to study the effect of temperature, pH and glucose concentration using Design-Expert version 2.4 (Table 1). The independent values for the variables entered into the RSM software were obtained from previous preliminary experiments (data not shown). Table 2 shows the variables and the corresponding data areas.

Table 1. Variables and corresponding levels for optimization of biohydrogen production

Variables	Factors	Unit	Low levels	High levels
X_1	Glucose	g/L	10	12.5
X_2	Temperature	ºC	30	37
X_3	pH	–	6	7.5

Table 2. RSM experimental design

	Factor 1	Factor 2	Factor 3	Response 1	Response 2
Run	A: Glucose Concentration (g/L)	B: Temperature (°C)	C: pH	Hydrogen production (mol/L)	Hydrogen yield (mol H_2/mol glucose)
1	10.00	37.00	7.50	58.36	1.0
2	10.00	37.00	6.00	60.24	1.0
3	12.50	30.00	6.00	57.17	0.8
4	09.15	33.50	6.75	110.15	2.1
5	11.25	33.50	8.01	77.62	1.2
6	11.25	33.50	6.75	50.55	0.8
7	12.50	37.00	7.50	61.39	0.8
8	11.25	39.39	6.75	0.00	0.0
9	11.25	33.50	6.75	51.55	0.8
10	12.5	37.00	6.00	36.99	0.5
11	10.00	30.00	6.00	88.08	1.5
12	11.25	33.50	6.75	49.55	0.7
13	11.25	33.50	6.75	47.55	0.7
14	11.25	33.50	5.49	67.16	1.0
15	11.25	33.50	6.75	53.55	0.8
16	11.25	33.50	6.75	49.55	0.7
17	10.00	30.00	7.50	76.12	1.3
18	12.50	30.00	7.50	71.48	1.0
19	13.35	33.50	6.75	75.49	1.0
20	11.25	27.61	6.75	45.09	0.7

Equation was obtained in both models.

Optimization of Biohydrogen Production: The batch fermentation was performed in 150 mL serum bottles containing 130 mL of production medium and the pH was adjusted accordingly with 0.1 M potassium phosphate buffer. The medium composition contained a filter sterilized solution of glucose and beef extract (Table 3). The beef extract was added in a mass ratio (30/1) to the total glucose (Chen et al. 2012). One (1) mL trace element solution containing 100 mg $ZnCl_2.7H_2O$, 30 mg $MnCl_2.4H_2O$, 300 mg H_3BO_3, 200 mg $CoCl_2.6H_2O$, 10 mg $CuCl_2.2H_2O$, 20 mg $NiCl_2.6H_2O$, 30 mg Na_2MoO_4 per liter (Kruse et al., 2018) was added to each medium for effective growth. The media were inoculated with 10% (v/v) of the initial biomass (1.0 OD600) and fermented at appropriate temperature conditions. Experimental bottles sealed with rubber stoppers and aluminium caps and a hydrogen-producing fermentation run at 150 rpm. Hydrogen production was monitored periodically at 2 hours from start up to 6 hours, then periodically at 6 hours to the end of the fermentation period (Kumar et al., 2012). One (1) mL of biogas was withdrawn with an airtight syringe (Agilent Technology) and analyzed in a gas chromatography thermal conductivity detector (GC-TCD) (Agilent 7890B) equipped with five columns (two Hayesep Q80/100 SS, one Hayesep Q 80/200 and two Molsieve 5A 60/80 SS) (Mohammed et al., 2018). The injection temperature and the detector temperature were 250 and 250°C, respectively. Helium gas was used as the

carrier gas. Biomass growth was measured by intermittently collecting and centrifuging 1 ml of production medium and filtering through a 0.22 mm membrane. The supernatant was used to determine sugar content using a 3, 5-dinitrocylisalic acid method (Cheng et al., 2017).

Table 3. Medium composition for inoculum propagation in optimization and scale-up experiments

Experiments	Runs	Glucose (g/L)	Nitrogen mass ratio (1/30) (g/L)	volume (ml)	Head space (ml)
Optimization	1	10.00	0.33	130	20
	2	10.00	0.33		
	3	12.50	0.42		
	4	09.15	0.31		
	5	11.25	0.38		
	6	11.25	0.38		
	7	12.50	0.42		
	8	11.25	0.38		
	9	11.25	0.38		
	10	12.50	0.42		
	11	10.00	0.33		
	12	11.25	0.38		
	13	11.25	0.38		
	14	11.25	0.38		
	15	11.25	0.38		
	16	11.25	0.38		
	17	10.00	0.33		
	18	12.50	0.42		
	19	13.35	0.45		
	20	11.25	0.38		
Scale-up	1	18.3	0.61	1800	200

Biohydrogen Scale-up Experiment: The scale-up experiment was performed in a fabricated 2L fermenter (Fig. 1). The optimal conditions for various parameters obtained in the previous experiment were used. The bioreactor produced was a 2L Schott bottle fitted with two fittings: one connected to a medium length hose connected to an airbag for collecting evolved biogas, one connected to another hose and closed with a clip. The second port was the pathway used to assess pH, cell growth and glucose levels at fermentation intervals. This port also served as a pH control channel. The bioreactor was seeded with 1800 ml medium in 1.0 M phosphate buffer solution (pH 6.75). The medium prepared comprised 18.3 g/l glucose, 0.61 g/l bovine extract in equal parts by mass (Table 2) medium and was filter sterilized through 0.2 µm pores PTFE membrane filter (Kruse et al., 2018). Two (2) ml in drops of trace element solution was supplemented into the fermentation medium to provide minerals for effective cell growth (Kruse et al., 2018). The bioreactor was inoculated with 180 ml (10% v/v) fresh bacterial solution (1.0 OD600). Then sealed with a rubber stopper, aluminum cap and completely wrapped in aluminum foil. The aluminum foil wrapper provides an effective medium for dark fermentation and prevent light interference in the fermentation process. The fermentation was carried out at 33.5°C with agitation at 150 rpm using a previously used magnetic stir bar before capping the scotch bottle (Matsumura et al., 2014). This is intended to ensure greater contact between the substrate and bacteria and a higher mass transfer in the fermentation medium (Kumar et al., 2015). Biogas, pH and sugar content were sampled periodically for 2 hours during the initial 6-hour fermentation period. Then 6 h intervals until the end of the fermentation time (48 h). Sugar and pH were measured by withdrawing 2 ml of medium using a syringe and needle and filtering through a 0.22 mm membrane at each interval. The collected cells were used to determine cell growth at each interval and the supernatants were used to determine sugar content using a 3, 5-dinitrocylisalic acid method (Xiao et al., 2013). The pH was readjusted by injecting 5 M NaOH solution into the bioreactor at intervals. The biogas produced was directed into an airbag using a hollow rubber hose connected to the sampling port and the total gas measured at intervals using a calibrated syringe. Then 1 ml of the produced biogas was analyzed by GC-TCD (Agilent 7890B) as previously explained to determine the produced hydrogen gas content.

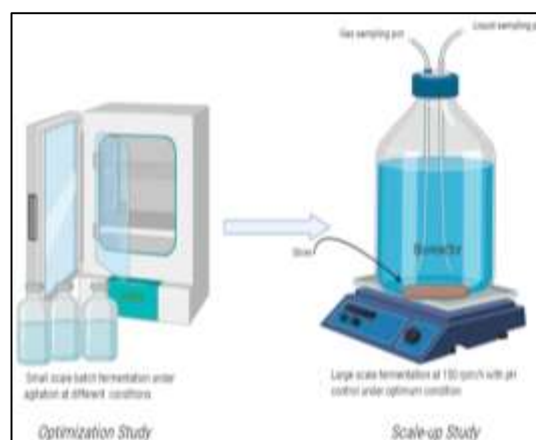

Fig 1. Experimental design for optimization and scale-up studies (Created with BioRender.com)

Analytical Methods: Cell growth was assessed by measuring dry cell weight (DCW). The results were analyzed using multivariate ANOVA in SPSS version

24. The biohydrogen production was calculated in mol/l according to the following equation.

$$\text{Production (mol/L)} = \frac{\text{HP in mol/L}}{\text{HS in mol/L}} \text{x}116.17 \qquad 1$$

Where HP = hydrogen production; HS = Hydrogen in standard gas; 116.17 represent the standard hydrogen moles in the mass balance and was calculated using Ideal gas law

$$n = \frac{PV}{RT} = \frac{150 \times 0.9869\, atm \times 48\text{ L}}{0.0821\text{ atm/L} \times 298\text{ K}} = \frac{7105.68\text{ atm/L}}{24.4658\text{ atm/L}} = 290.433 \text{ moles} \qquad 2$$

Where; P = Size of the gas tank × molar mass of H_2 (150 × 0.9869) = 148.035 atm, R = 0.0821atm/mol K (constant), T = 25°C+273 = 298 K and V = 48 L. But the Standard gas contain 40 % Hydrogen. Therefore, Hydrogen moles = 0.4 × 290.433 moles = 116.17 moles

The biohydrogen yield was calculated according to the following equation

$$\text{Yield in mol/mol} = \frac{\text{Hydrogen Produced mol/L}}{\text{Glucose Consumed mol/L}} \qquad 3$$

Kinetic Parameters: Kinetic parameters (specific growth rate, maximum growth rate, max; yield of biomass to substrate, Yx/s; maximum biomass, Xmax; yield of biohydrogen to substrate, Yp/s; doubling time, t_d; maximum productivity and total productivity) were described previously calculated (Stanbury et al., 2013).

RESULTS AND DISCUSSION

Experimental Distributions: The normal and random distribution of the experiments were examined using the residual plots shown in Fig. 2. This was done to minimize errors in the experiments and to ensure that the results obtained are accurate and reliable. From the plots; Normal plot of Residual, Residual vs Predicted, Residual vs Run and Predicted vs Actual, it was clear that all experiments were normally distributed without human intervention. In addition, 98.82% and 96.65% of the RSM models also agree with the normal and random distribution of the experiments in this study.

Model Fitness for Biohydrogen Production: Regression analyses from the RSM model designed for optimization studies showed R_2 values of 0.9882 and 0.9784 with 98.82% and 96.65% (Tables 4 and 5) for hydrogen production and hydrogen recovery, respectively. The ANOVA results indicate good correlation and high consistency of the models. Furthermore, the closeness of adjR_2 to preR_2 in both models also indicates a high accuracy of the response. The coefficients of variation (CV) describe the high accuracy of the experimental models and indicate the possibility of reproducibility.

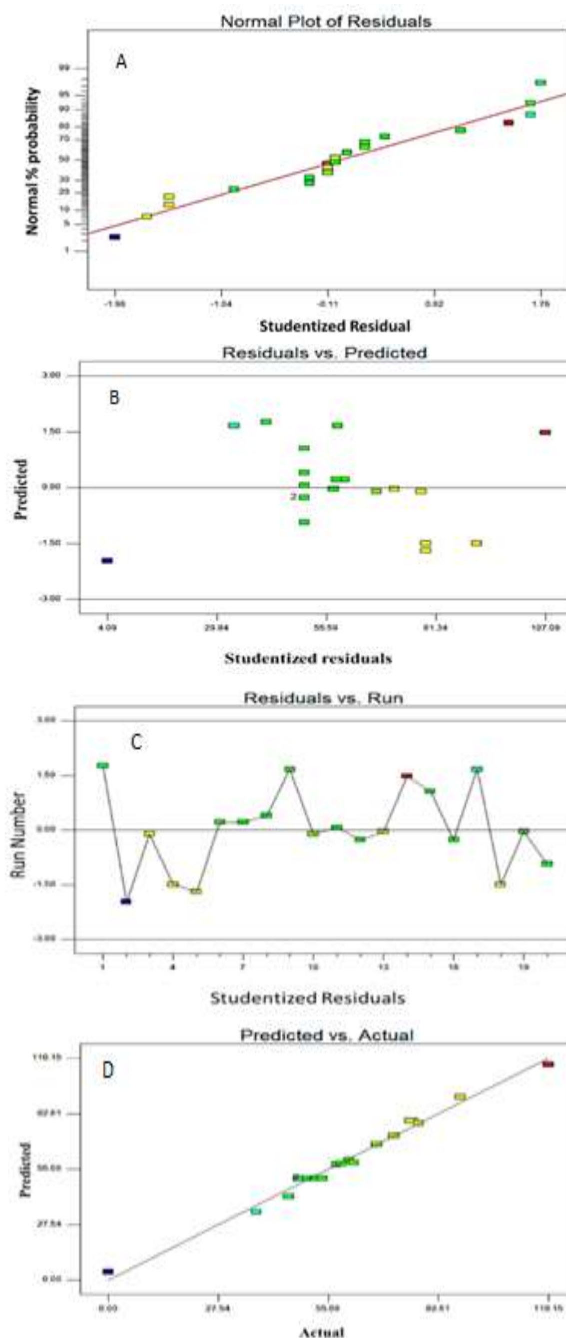

Fig 2. Diagrams for probability distribution of parameters in biohydrogen production

In addition, a second-order quadratic equation was obtained in both models. Quadratic equations for biohydrogen production/yield:

$$HP = 50.17 - 8.35A + 11.11B + 3.11C + 15.09A^2 - 9.76B^2 + 7.87C^2 + 1.92AB + 6.57AC + 2.52BC$$

$$HY = 0.75 - 0.26A - 0.18B + 0.047C + 0.28A^2 - 0.15B^2 + 0.12C^2 + 0.038AB + 0.08AC + 0.038BC$$

Where HP and HY are biohydrogen production and yield; A, B and C represent glucose, temperature and pH, respectively.

Table 4. Model fitness for biohydrogen production

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	9120.23	9	1013.36	92.86	0.0001	Significant
A	952.63	1	952.63	87.29	0.0001	
B	1685.13	1	1685.13	154.41	0.0001	
C	132.02	1	132.02	12.1	0.0059	
A^2	3281.04	1	3281.04	300.65	0.0001	
B^2	1371.99	1	1371.99	125.72	0.0001	
C^2	891.62	1	891.62	81.7	0.0001	
A*B	29.38	1	29.38	2.69	0.1319	
A*C	345.19	1	345.19	31.63	0.0002	
B*C	50.85	1	50.85	4.66	0.0562	
Residual	109.13	10	10.91			
Lack of Fit	88.3	5	17.66	4.240.0695		Not significant
Pure Error	20.83	5	4.17			
Cor Total	9229.36	19				

$\mathbf{R^2}$ = 0.9882, **CV** = 5.56 %, $_{adj}\mathbf{R^2}$ = 0.977, $_{pre}\mathbf{R^2}$ = 0.927

NB: A = glucose, B= temperature and C= pH

Table 5. Model fitness for biohydrogen yield

Source	Some of Squares	DF	Mean Square	F Value	Prob > F	
Model	3.20	9	0.36	50.34	0.0001	significant
A	0.92	1	0.92	130.61	0.0001	
B	0.45	1	0.45	63.6	0.0001	
C	0.03	1	0.03	4.2	0.0677	
A^2	1.11	1	1.11	156.51	0.0001	
B^2	0.31	1	0.31	44.23	0.0001	
C^2	0.2	1	0.2	28.36	0.0003	
A*B	0.011	1	0.011	1.59	0.2356	
A*C	0.061	1	0.061	8.67	0.0147	
B*C	0.011	1	0.011	1.59	0.2356	
Residual	0.071	10	7.07×10^{-3}			
Lack of Fit	0.056	5	0.011	3.71	0.0882	Not significant
Pure Error	0.015	5	3.00×10^{-3}			
Cor Total	3.27	19				

$\mathbf{R^2}$ = 0.9784, **CV** = 5.48%, $_{adj}\mathbf{R^2}$ = 0.9591, $_{pre}\mathbf{R^2}$ = 0.8641

NB: A = glucose, B= temperature and C= pH

Effects of Temperature, pH and Glucose on Biohydrogen Production: The impact of various factors on biohydrogen production and yield in fermentations was examined in the optimization experiments. This bacterium has shown ability to produce hydrogen in fermentation process using different carbon sources as reported by different scientists (Niu et al., 2010; Pugazhendhi and Thamaraiselvi, 2017). A minimum hydrogen production of 36.99 mol/L was observed at 37°C, pH 6.5 and 12.5 g/L glucose. While maximum hydrogen production of 110.15 mol/l was seen at 33.5°C, pH 6.75 and 9.15 g/l glucose. This indicated lower glucose and temperature condition optimal for biohydrogen production due to more impact, despite isolation of the bacterium from a psychrophilic environment. However, no hydrogen production was seen at 39°C with an initial pH of 6.75 and 11.25 g/L glucose, suggesting that increasing the temperature to 39.39°C with 11.25 g/L glucose at pH 6.75 completely stops hydrogen production of the strain. It was attributed to the denaturing of enzymes due to inactivation of the bacterium above its growth temperature condition (Mohammed et al., 2018). The 3D response surface plot at fixed pH and different temperature and glucose levels is shown in Fig. 3. Biohydrogen production decreases with increasing temperature and glucose concentration. However, at fixed temperature and variable pH and glucose concentration, biohydrogen production increases with a decrease in glucose and an increase in pH to 6.75. When glucose concentration was fixed, pH and temperature varied. Biohydrogen production increased significantly with a drop in temperature to 33.5°C.

Higher temperatures impair the performance of most cold-active bacteria due to their impact on metabolic activity with negative consequences for biogas production in the fermentation process (Franzmann et al., 1997; Sivagurunathan et al., 2017). In this study, it was found that 33.5 °C is optimal for biohydrogen production, which is consistent with the results of previous researchers who used mesophilic bacteria as a biocatalyst in the fermentation process. For example, Ferreira et al., (2018) reported an optimal production of 194.9 ml h-1 l-1 at 30ºC. While Mishra et al. (2017) produced a maximum of 123 ml/h of hydrogen from *Bacillus anthracis* PUNAJAN 1 at 35 °C. This

demonstrates robust hydrogen production compared to other psychrophilic strains whose productivity are inhibited by lower temperature conditions. However, they observed a decrease in production with further increases in temperature above 35 °C, similar to the findings of this study

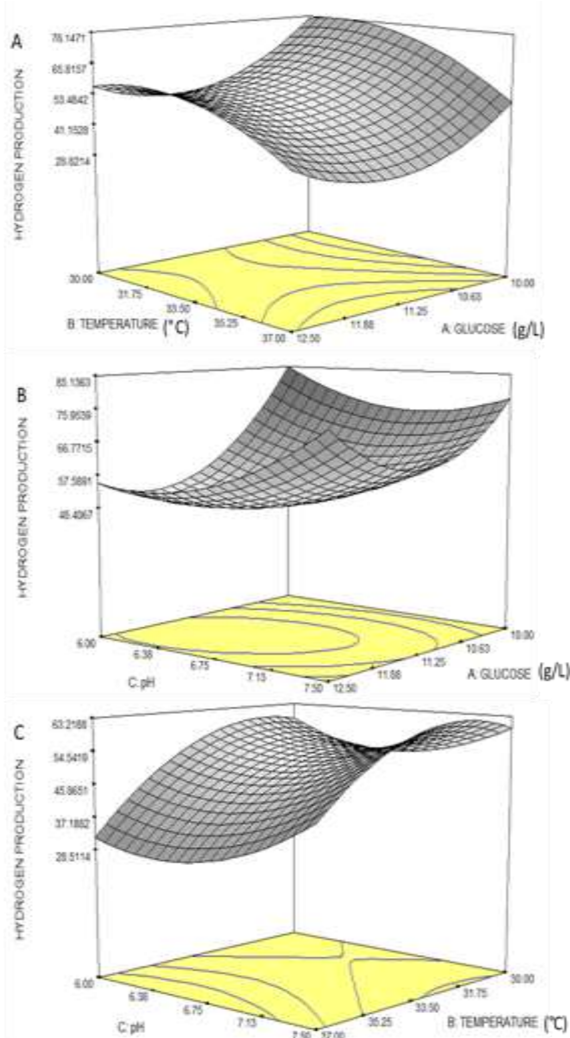

Fig 3. Response surface plot for biohydrogen production. A; pH level was fixed while temperature and glucose levels varied. B; Temperature was fixed with varying pH and glucose. C; Glucose fixed, pH and temperature varied.

Effects of Temperature, pH and Glucose on Biohydrogen Yield: The maximum biohydrogen yield in this study was 2.1 mol H_2/mol glucose at 33.5°C, initial pH 6.75 and 9.15 glucose in g/L (Fig. 4). The results showed that at fixed pH and variable temperature and glucose concentration, reducing the glucose concentration to 11.25 g when the temperature decreased from 37 to 33.5°C increased the biohydrogen yield. However, further reducing the glucose concentration to 10 g increased the biohydrogen yield. The increased temperature increased the biohydrogen yield to an optimum at 33.5°C and a further increase to 35°C showed a slight decrease in yield.

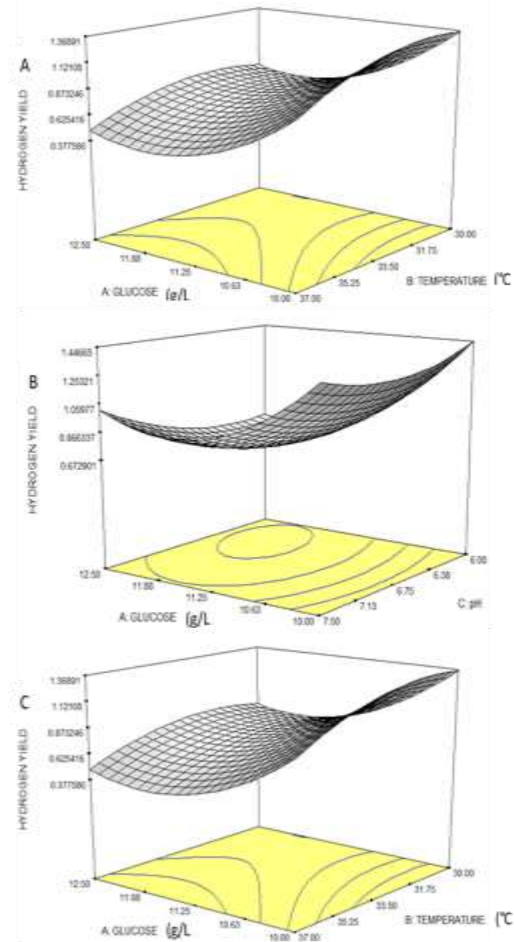

Fig 4. Response surface for biohydrogen yield. A; pH level fixed while glucose and temperature varied. B; Temperature was fixed while pH and glucose were varied. C; glucose fixed, pH and temperature varied

In contrast, the 3D surface plot for biohydrogen yield shows that decreasing glucose concentration increases biohydrogen yield. A similar effect was found by Wu et al., (2011) who reported a decrease in yield when the glycerol concentration was slowly increased from 10 to 70 g/L. Furthermore, their results agreed with those of Chookaew et al. (2014), who also found increased hydrogen production at low substrate concentrations with *Klebsiella* sp. TR17. This result indicates higher metabolism and hydrogen production at ambient temperature conditions due to increased conversion of acetyl-CoA to biohydrogen (Chookaew et al., 2012). However, under psychrophilic conditions, lower hydrogen production may have been observed due to increased production of acidic metabolites that inhibit biomass metabolic activities. In this study, the highest biohydrogen yield of 2.1 mol

H_2/mol glucose (at 33.5 °C, pH 6.75 and 9.15 g/L glucose concentration) is higher than that reported for the psychrophilic G088 strain (1. 93 mol H_2/mol glucose at 37°C, pH). 8.0) (Alvarez-Guzmn et al., 2017) and for psychrophilic N92 (1.7 mol H_2/mol glucose at 29 °C, initial pH 6.86, glucose concentration 28.4 g/L) (de la Cueva et al., 2018). Rapid metabolism of simple and complex substrates such as glucose, sewage sludge and glycerol by *Klebsiella* strains have been reported (Niu et al., 2010; Li et al., 2018). Low substrate increases the growth flexibility and energy efficiency of hydrogenases and directs metabolism to pathways less by-products (Wang et al., 2008).

Production Scale-up in 2 L Fermenter: A scale-up study was performed to evaluate biohydrogen production under optimized conditions obtained from the RSM. The total amount of glucose (9.15 g/L) used was doubled to compensate for the sugar required for the scale-up study in a 2L bioreactor. While other parameters such as pH and temperature have been adjusted as they were obtained in the optimal condition. The entire biogas and biohydrogen production in the scale-up experiment with *Klebsiella* sp. ABZ11 are shown in Figure 5.

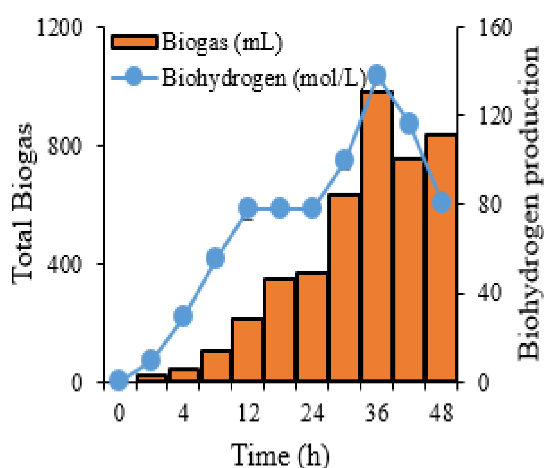

Fig 5. Time course of total biogas and biohydrogen production in scale-up experiment

The accumulation of biogas and biohydrogen started in 2 hours and gradually increased to a peak in 12 hours. The observed immediate hydrogen production indicates a short lag phase, similar to the report by Niu et al. (2010). Then the biogas and biohydrogen production stabilized between 12 and 24 h before reaching a peak value (979 ml biogas and 137,562.30 mol/l biohydrogen) at 36 h, after which a decrease was observed. As a result, a decrease in cell mass in the fermentation vessels is observed after 36 hours. This could be due to feedback inhibition from the accumulated hydrogen pressure on the bacteria reducing substrate metabolism. The hydrogen pressure on the bacteria may have shifted the process mechanism from ferredoxin (Fd) reduction to Fd or NADH oxidation, resulting in the oxidation of hydrogen to protons with a subsequent decrease in hydrogen production, consistent with the results of Chong et al., (2009). Dong et al. (2009) and van et al. (2003) also confirmed the decrease in hydrogen production when the built-up partial pressure prevents the synthesis of fatty acids into hydrogen or causes a shift in the metabolic pathway. The metabolites produced here would not have explained the decline in biohydrogen production, since the pH is readjusted at intervals, which neutralizes the metabolites formed before fermentation.

Cell growth, mean glucose concentration and pH change during fermentation time are shown in Fig. 6. The increase in pH from 6.75 to 5.10 in the medium may have been the cause of the high glucose consumption in 2 h with very little biohydrogen production in the start. Oxidative phosphorylation is an energy-intensive process that coincides with log phase, which demand the expression of metabolic enzymes for substrate degradation (Yin & Wang, 2019). The pH was adjusted to 6.75 by injecting NaOH solution into the medium before fermentation proceeded. This slightly stabilizes glucose levels and pH between 4 and 6 hours. However, these dominant factors had no effect on bacterial growth due to the steady increase in cell count observed after a lag time of 2 hours to a peak (0.73 g/L) in 36 hours before falling. Meanwhile, after 4 hours of fermentation, the pH and glucose uptake stabilize until the end of the fermentation. The biohydrogen scale-up experiment showed a maximum biohydrogen production of 137.56±2.30 mol/L at 36 h. Stirring potentially improved the availability of glucose on a larger scale due to a more homogeneous distribution, which may have influenced the process leading to maximum biohydrogen production. Reniati et al. (Reiniati et al., 2017) reported a 2-fold yield of biohydrogen when the stirring speed was increased from 100 to 700 rpm. These increased NAD+/NADH production, which consequently increased hydrogenase enzyme and biohydrogen production in the exponential growth phase (Xia et al., 2015). In addition, the high metabolic activity, the increased substrate uptake and the short lag phase show a rapid adaptation to the fermentation conditions. This confirmed the possibility of large-scale biohydrogen production using this bacterium at mesophilic temperature conditions with pH control and agitation. In the scale-up experiment, biohydrogen production of 137.56 ml was observed within 36 h fermentation at 0.73 g/l cell dry biomass and 18.3 g/l glucose uptake. While the scale up experiment yielded

110.15 mol/L biohydrogen with 0.82 g/L cell weight and 9.5 g/L glucose uptake.

Kinetics of Biohydrogen Production: An increase in production during fermentation is possible by understanding the kinetic parameters involved (Chen et al., 2001). The specific growth rate, maximum specific growth rate and cell doubling time in our RSM optimization study were 0.031 h -1, 0.062 h -1 and 22.3 h, respectively (Table 5). The maximum production, maximum productivity and total productivity are 110.15 mol/l, 3.70 mol/l/h and 1.90 mol/l/h, respectively. Activation of the formate lyase pathway, triggered by anaerobic conditions in the medium in the presence of the facultative anaerobic biocatalyst, could have resulted in high biohydrogen production through rapid glucose synthesis in the process (Leonhartsberger et al., 2002; Chen et al., 2009). This could account for the maximum production, maximum productivity, and overall productivity achieved.

Table 6. Kinetics of biohydrogen production and optimization studies

Serial number	Parameters	Optimized condition (130 mL)	Scale-up condition (1800 mL)
1	Specific growth rate (μ)	0.031 h^{-1}	0.040 h^{-1}
2	Maximum growth rate (μ_{max})	0.062 h^{-1}	0.081 h^{-1}
3	Yield of biomass towards substrate (*Yx/s*)	0.85 g/L	0.71 g/L
4	Yield of biohydrogen towards substrate (*Yp/s*)	129.58 mol/L	191.85 mol/L
5	Maximum biomass (*Xmax*)	0.87 g/L	0.73 g/L
6	Doubling time (t_d)	22.3 h	17.3 h
7	Maximum production (*Pmax*)	110.15 mol/L	137.56 mol/L
8	Maximum productivity	3.70 mol/h	3.82 mol/h

The yield coefficient of biomass formation was 0.85 g/g, which means that 0.85 g of biomass was obtained from each gram of glucose. The biohydrogen recovery coefficient was 122 mol/L/g, showing that 122 mol/L of biohydrogen was recovered from each gram of glucose. From the yield of biomass and the formation of biohydrogen it can be concluded that the fermentation has favoured the formation of biohydrogen more than the formation of biomass.

This means that increasing the temperature from 30°C to 33.5°C accelerated the fermentation process with an increase in productivity to 110.15 mol/L with an increase in biomass from 0.74±1.02 to 0.87±0.04 biomass under optimized conditions.

The highest biohydrogen production shown in the exponential growth phase of the cells (Figure 6) indicates the production of biohydrogen as the primary metabolite as previously observed (Mu et al., 2005). The performance of the system in this study was compared to understand the variance in productivity, cell growth and glucose uptake.

This is because similar bioprocess parameters were used but with different pH conditions due to the control of pH in the scale-up experiment at each 2-hour interval. The scale-up experiment produced a biohydrogen yield of 137.56 mol/L, 22.13% higher than that of the optimized small-scale system, as also previously observed (Sekoai et al., 2019).

A scale-up experiment under controlled pH has been shown to produce more biohydrogen than an uncontrolled pH study (Faloye et al., 2014).

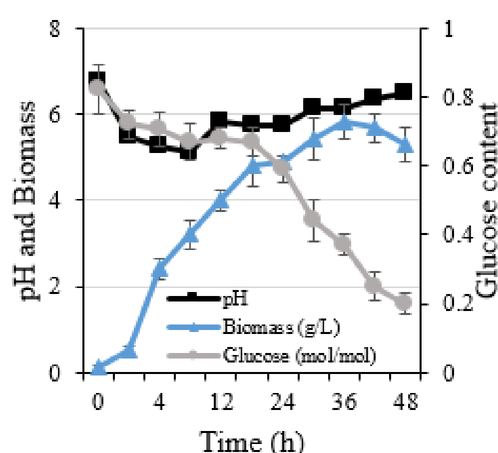

Fig 6. *Time course of cell growth, glucose content and pH in scale-up experiment*

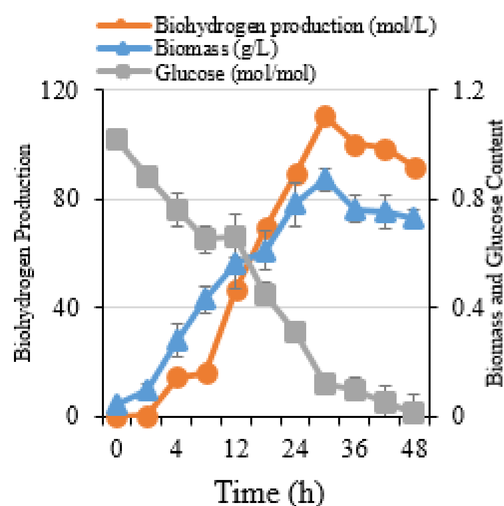

Fig 7. Relationship between biohydrogen production, glucose content and biomass formation in optimization study

Comparison of Biohydrogen Production to Studies: Performance in batch and pH-controlled experiments was compared to previous studies as shown in Tables 7 and 8. The maximum biohydrogen production, given in ml/l, was converted to mol/l by the formula: produced H_2 × volume H_2 density (0.00523) / molar mass H_2 (2.015 g/mol). Production under varying temperature conditions was observed with a maximum biohydrogen ranging from 110.15 mol/l to 0.37 mol/l. The moles of hydrogen produced by this study were higher than previous studies from a relatively smaller carbon source. The fermentation with *T. thermosaccharolyticum* IIT BT-ST148 was carried out at thermophilic temperature, which gives our process an energetic advantage due to the possibility to work at room temperature and saves production costs. Also, at ambient temperature, they showed a good ability to produce hydrogen as psychrophiles, even when both bacteria come from a very low temperature environment. Although equal or higher hydrogen yields than other mesophiles were observed in large-scale production, the differences in hydrogen yields with *B. thuringiensis* RH1 can be attributed to different substrates used as the carbon source in the production process. Therefore, further studies are needed to evaluate biohydrogen production by *Klebsiella* sp. ABZ11 using various wastes available in the environment to further reduce production costs.

Table 7. Comparison of biohydrogen production to batch fermentations

Bacterial	(T) °C	Glucose (g/L)	Maximum production (mol/L)	Reference
Klebsiella pneumoniae ECU-15	37	35.62	14.16	Niu et al., 2010
Clostridium sp. YM1	37	20	9.07	Abdeshahian et al., (2014)
T. thermosaccharolyticum IIT BT-ST1	60	12	10.37	Roy et al., (2014)
Clostridium beijerinckii YA001	40	10	8.07	An et al., (2014)
Sejongia marina M02	20	23	0.37	Alvarado-Cuevas et (al., 2015)
Klebsiella sp ABZ11	30	9.15	110.15	This study

Maximum biohydrogen production for all strains are converted from mL to mol/L except for this study.

Table 8. Comparison of biohydrogen production to previous scale-up studies

Bacterial	(T) °C	Carbon type	Carbon source (g/L)	Bioreactor size (L)	Medium volume (L)	Yield (mol/mol glucose)	Reference
Clostridium species	35	Glucose	10	10	7	2.0	Patel et al., (2015)
Klebsiella sp. WL1316	37	Glucose	40	5	3.5	1.4	Li et al., (2018)
B. thuringiensis RH1	37	RM wastewater	100%	3.5	2.5	1.6	Ramu et al., (2020)
Klebsiella sp ABZ11	33.5	Glucose	18.3	2	1.8	2.1	This study

RM; Rice Mill

Conclusion: The influence of temperature, pH and glucose maximized the hydrogen production by *Klebsiella* sp. ABZ11 to 110.15 mol/l and 137.56 mol/l for optimization and scaling in batch fermentation with a yield of 2.1 mol H_2/mol glucose.

Kinetics show increased hydrogen production and decreased biomass with agitation and pH control. The study demonstrates increased hydrogen production under mesophilic conditions using cold-active bacteria.

Acknowledgements: This work was supported by the UTM Flagship Research Grant (Grant Numbers 01G27, 00G98, 02E13) awarded to Z.I. and the UTM Tier 1 Research University Grant (Grant Numbers 07H31 and 19H14) awarded to M.F.A-W. A. Mohammed would like to thank Tertiary Education Trust Fund (TETFund) Nigeria and IBB University, Lapai, Niger state, Nigeria for the provided PhD scholarship.

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