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Biohydrogen Production from Hydrolysates of Selected Tropical Biomass Wastes with *Clostridium Butyricum*

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Biohydrogen production has received widespread attention from researchers in industry and academic fields. Response surface methodology (RSM) was applied to evaluate the effects of several key variables in anaerobic fermentation of glucose with *Clostridium butyrium*, and achieved the highest production rate and yield of hydrogen. Highest H₂ yield of 2.02 mol H₂/mol-glucose was achieved from 24 h bottle fermentation of glucose at 35 °C, while the composition of medium was (g/L): 15.66 glucose, 6.04 yeast extract, 4 tryptone, 3 K₂HPO₄, 3 KH₂PO₄, 0.05 L-cysteine, 0.05 MgSO₄·7H₂O, 0.1 MnSO₄·H₂O and 0.3 FeSO₄·7H₂O, which was very different from that for cell growth. Sugarcane bagasse and *Jatropha* hulls were selected as typical tropical biomass wastes to produce sugars via a two-step acid hydrolysis for hydrogen production. Under the optimized fermentation conditions, H₂ yield (mol H₂/mol-total reducing sugar) was 2.15 for glucose, 2.06 for bagasse hydrolysate and 1.95 for *Jatropha* hull hydrolysate in a 3L fermenter for 24 h at 35 °C, with H₂ purity of 49.7–64.34%. The results provide useful information and basic data for practical use of tropical plant wastes to produce hydrogen.

Hydrogen is a clean fuel that only produces water as its environmentally benign product in combustion, and therefore attracts attentions from researchers in the world¹. The specific energy of hydrogen (122 kJ/g) is 2.75 times that of regular hydrocarbon fuels². Annual global production of lignocellulosic biomass is about 2.20 × 10¹² Kg (dry weight) from agriculture and forestry residuals, energy crops, aquatic plants and algae³. These rich and sustainable lignocellulosic resources potentially produced H₂ with low cost from biomass via hydrolysis and fermentation⁴.

South China is located in subtropical and tropical regions that were suitable to grow energy or economic crops, such as sugarcane and *Jatropha curcas* L⁵. In China, sugarcane production increased from 21.12 in 1978 to 128.2 million tons in 2013, which produced large amount of bagasse as waste in sugar industry⁶. Large amount of bagasse was produced as industrial waste, which could be used to produce biofuels such as bioethanol and biohydrogen^{7,8}. On the other hand, *Jatropha* is a promising energy crop for biodiesel industry. More than 2,000 biodiesel production plants had been built up in China by 2007 with many residual hulls produced^{9,10}. *Jatropha* hulls were previously studied to produce 2,3-butanediol and biogas^{11,12}.

Lignocellulosic biomass has complex structure composing of cellulose, hemicellulose and lignin, which should be disrupted before hydrolyzing cellulose and hemicellulose to soluble sugars¹³. Various types of agricultural residues (such as rice straw and corn stover) were hydrolyzed with dilute-acid for the production of hydrogen^{14,15}. Compared with hydrochloric acid, nitric acid and phosphoric acid, sulfuric acid was more effective in producing fermentable sugars¹⁶. Most hemicellulose was hydrolyzed in the first step. The second step was then performed

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Factor (g/L)	Variables	Levels ^a	
		−1	1
Glucose	X ₁	15	25
Yeast extract	X ₂	3.5	6.5
Tryptone	X ₃	3	5
K ₂ HPO ₄	X ₄	2	4
KH ₂ PO ₄	X ₅	2	4
L-Cysteine	X ₆	0.05	0.15
MgSO ₄ ·7H ₂ O	X ₇	0.0	0.1
FeSO ₄ ·7H ₂ O	X ₈	0.2	0.4

Table 1. Variables and their levels employed in Plackett-Burman design. ^ax₁ = (X₁ − 20)/5; x₂ = (X₂ − 5)/1.5; x₃ = (X₃ − 4)/1; x₄ = (X₄ − 3)/1; x₅ = (X₅ − 3)/1; x₆ = (X₆ − 0.1)/0.05; x₇ = (X₇ − 0.05)/0.05; x₈ = (X₈ − 0.3)/0.1.

Run	Variable level								H ₂ yield (mol/mol) ^a
	X ₁	X ₂	X ₃	X ₄	X ₅	X ₆	X ₇	X ₈	
1	−1	−1	−1	1	−1	−1	1	−1	1.39 ± 0.08
2	−1	1	−1	−1	1	−1	1	1	1.50 ± 0.02
3	−1	−1	1	−1	−1	1	1	1	1.41 ± 0.07
4	−1	−1	−1	1	−1	−1	1	−1	1.36 ± 0.05
5	−1	1	1	1	−1	−1	−1	1	1.44 ± 0.03
6	1	1	−1	−1	−1	1	−1	−1	1.30 ± 0.09
7	−1	1	−1	1	1	1	−1	−1	1.41 ± 0.05
8	1	−1	1	1	1	−1	−1	−1	1.28 ± 0.05
9	1	−1	−1	−1	1	−1	−1	1	1.32 ± 0.05
10	1	1	1	1	1	1	1	1	1.35 ± 0.01
11	1	−1	−1	1	−1	1	1	1	1.23 ± 0.04
12	1	1	1	−1	−1	−1	1	−1	1.34 ± 0.05

Table 2. Plackett-Burman design variables (in code levels) with H₂ yield as response for 24 h bottle fermentation at 35 °C with 130 rpm shaking. ^aValues were given by mean ± standard deviation (n = 3).

Variable	Coefficient	Std. Error	t-Value	p-Value
Intercept	1.3644	0.0054	251.54	<0.0001 ^b
X ₁	−0.0611	0.0054	−11.27	0.0015 ^b
X ₂	0.0256	0.0054	4.71	0.0181 ^b
X ₃	0.0078	0.0053	1.45	0.2439
X ₄	−0.0161	0.0053	−3.00	0.0579
X ₅	0.0167	0.0056	2.96	0.0593
X ₆	−0.0122	0.0053	−2.27	0.1077
X ₇	0.0067	0.0055	1.22	0.3112
X ₈	0.0083	0.0055	1.52	0.2260

Table 3. Effects and statistical analysis of variables^a. ^aR² = 0.9858, R² (Adj) = 0.9478. ^bStatistical signification at 95% of confidence level (p < 0.05).

at severer conditions to open up the cellulose structure for improving the accessibility of enzymes for hydrolysis, or to hydrolyze cellulose with acid¹⁷. Therefore, two-step dilute acid hydrolysis process was one of the effective pretreatment methods.

Clostridium butyricum as a model bacterium with clear metabolic pathways, was widely used in hydrogen production^{18–20}. Preliminary results suggested that the optimized fermentation medium by response surface methodology (RSM) improved the growth of *C. butyricum* effectively²¹. However, the most optimal culture medium for the strain growth was not optimal for H₂ yield. To the best of our knowledge, the hydrolysate of *Jatropha* hulls for biohydrogen production has not yet been reported in any previous work. This study aims to (i) optimize the fermentation media for biohydrogen production using glucose as carbon source by RSM, and (ii) the results was further used to efficiently produce biohydrogen from the hydrolysates of bagasse and *Jatropha* hulls by two-step acid hydrolysis.

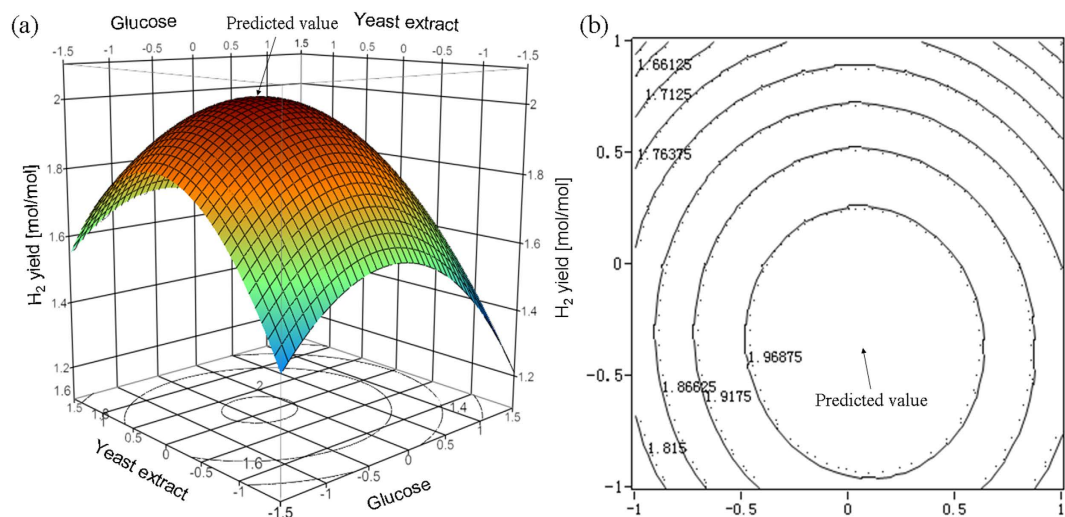


Figure 1. (a) Response surface plot and (b) corresponding contour of the mutual effects of glucose and yeast extract on H_2 yield (24 h bottle fermentation at 35 °C with 130 rpm shaking).

Test	Glucose (g/L)	Yeast extract (g/L)	H_2 yield (mol/mol) ^a
1	20	5.0	1.62 ± 0.12
2	18	5.5	1.63 ± 0.03
3	16	6.0	1.78 ± 0.02
4	14	6.5	1.51 ± 0.03
5	12	7.0	1.33 ± 0.02
6	10	7.5	1.31 ± 0.28
7	8	8.0	1.20 ± 0.01

Table 4. Experimental results along the path of the steepest ascent for 24 h bottle fermentation at 35 °C with 130 rpm shaking. ^aValues were given by mean ± standard deviation (n = 3).

Run	Coded variable level		Real variable level		H_2 yield (mol/mol-TRS) ^a	
	X ₁	X ₂	X ₁	X ₂	Observed	Predicted
1	1	-1	17	5.5	1.62 ± 0.03	1.61
2	0	0	16	6.0	2.02 ± 0.05	2.00
3	-1.41	0	14.59	6.0	1.84 ± 0.01	1.86
4	-1	-1	15	5.5	1.79 ± 0.08	1.78
5	0	0	16	6.0	1.99 ± 0.02	2.00
6	0	0	16	6.0	1.98 ± 0.01	2.00
7	0	0	16	6.0	2.01 ± 0.02	2.00
8	0	-1.41	16	5.29	1.64 ± 0.03	1.66
9	0	1.41	16	6.71	1.71 ± 0.07	1.72
10	-1	1	15	6.5	1.85 ± 0.09	1.83
11	0	0	16	6.0	2.01 ± 0.02	2.00
12	1.41	0	17.41	6.0	1.61 ± 0.02	1.61
13	1	1	16	6.5	1.63 ± 0.04	1.63

Table 5. Levels of the factors, experimental design and the results of the central composite design. ^aValues were given by mean ± standard deviation (n = 3).

Results and Discussion

Glucose and hydrolysates were fermented with *C. butyrium* in both bottles (batch) and a fermenter. Single-factor bottle experiments were conducted to find best culture medium for hydrogen production from glucose with results given in supplementary materials (Figure S1). Linear relation between dry cell weight (DCW) and optical density at 650 nm (OD_{650}) was plotted in supplementary materials (Figure S2). Both H_2 yield and DCW versus

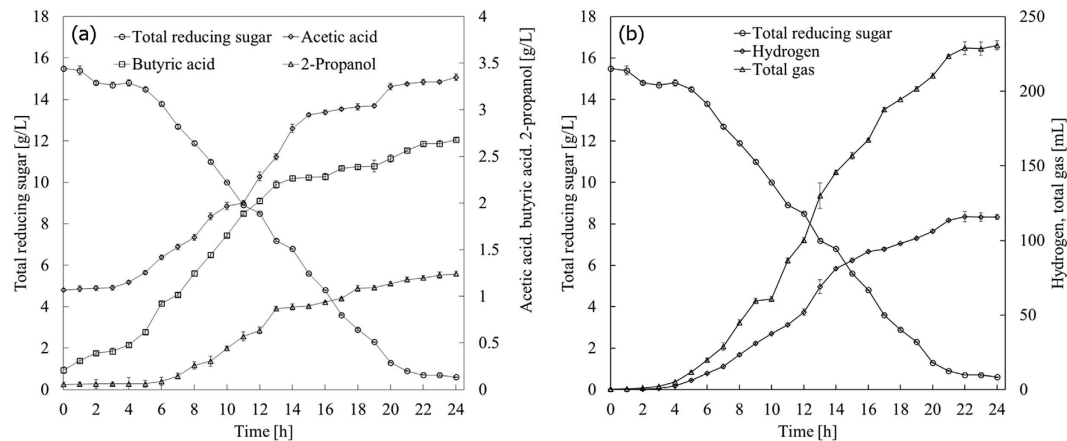


Figure 2. H₂ and by-products production versus time in bottle fermentation of bagasse hydrolysates under the optimized medium at 35 °C with 130 rpm shaking (a) liquid products (b) gas products.

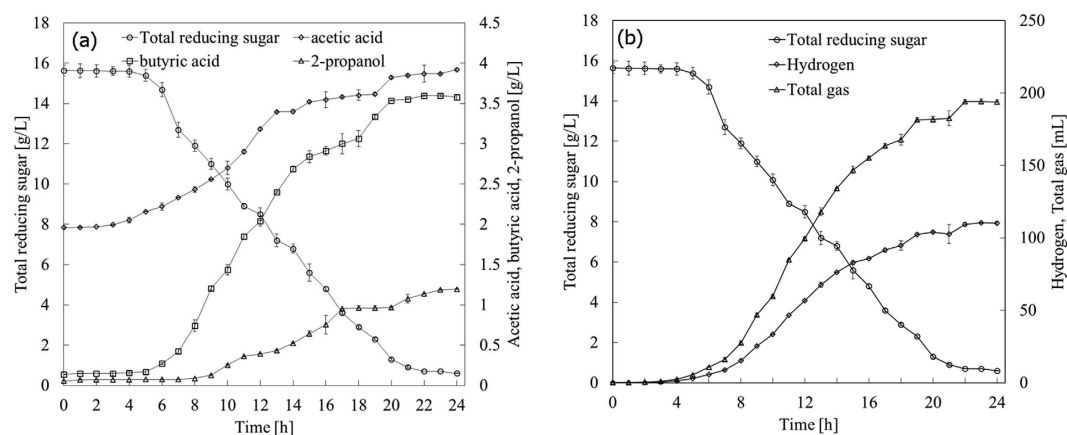


Figure 3. H₂ and by-products production versus time in bottle fermentation of *Jatropha* hulls hydrolysates under the optimized medium at 35 °C with 130 rpm shaking (a) liquid products (b) gas products.

Variable	Coefficient	Std. Error	<i>t</i> -Value	<i>p</i> -Value
Intercept	2.002	0.0088	227.04	<0.0001 ^b
X ₁	-0.0894	0.0070	-12.83	<0.0001 ^b
X ₂	0.0211	0.0070	3.03	0.0191 ^b
X ₁ X ₂	-0.0125	0.0098	-1.27	0.2454
X ₁ X ₁	-0.1329	0.0075	-17.77	<0.0001 ^b
X ₂ X ₂	-0.1579	0.0075	-21.12	<0.0001 ^b

Table 6. Significance test of regression coefficient^a. ^aR² = 0.9918, R² (Adj) = 0.986. ^bStatistical signification at 95% of confidence level (*p* < 0.05).

different variables were illustrated in supplementary materials (Figure S3). Plackett–Burman (PB) design, experimental results and analysis with the help of software JMP package (version 10, SAS Institute Inc., Raleigh, NC) were demonstrated in Tables 1–3, respectively. Study on the path of steepest ascent could be observed in Table 4. Central composite design (CCD), experimental results and analysis were summarized in Tables 5 and 6. Response surface plot and corresponding contour, canonical analysis for RSM were given in Fig. 1 and Table 7, respectively. Products versus time in bottle fermentation of hydrolysates of bagasse and *Jatropha* hulls were showed in Figs 2 and 3. Composition analysis of bagasse and *Jatropha* hulls was given in Table 8. Products produced from glucose and hydrolysates in both bottles and fermenter were presented in Table 9. Table 10 Compares hydrogen yield in this study with reported data. All the experiments were repeated more than two times and the standard deviation

Source	Degree of freedom	Sum of square	Mean square	F-value	Probability > F
Model	5	0.330755	0.066151	170.1574	<0.0001
Error	7	0.002722	0.000389		
C. total	12	0.333477			
Lack of fit	3	0.001641	0.000547	2.0264	0.2528
Pure error	4	0.001088	0.000270		
Canonical analysis					
	X ₁	X ₂	Predicted H ₂ yield: 2.02 (mol/mol)		
Eigen value	-0.3402	0.0804			
Actual value	15.6598	6.0402			

Table 7. Analysis of variance and canonical analysis for the parameters of RSM.

Biomass	Glucan (%)	Xylan (%)	Galactan (%)	Arabinan (%)	Mannan (%)	Lignin (%)	Ash (%)	Extractives (%)
Bagasse	42.05 ± 0.62	19.79 ± 2.81	2.47 ± 0.29	1.79 ± 0.02	1.12 ± 0.05	20.82 ± 0.12	1.98 ± 0.25	9.29 ± 0.01
<i>Jatropha</i> hulls	36.95 ± 1.35	9.78 ± 1.35	0.82 ± 0.03	0.30 ± 0.00	2.47 ± 0.53	27.9 ± 0.1	1.19 ± 0.19	18.61 ± 0.03

Table 8. Structural carbohydrates and lignin in bagasse and *Jatropha* hulls^a. ^aAll data were given by mean ± standard deviation (n = 3).

(σ) was within the range of 0.002 to 0.11 (mol/mol) for H₂ production, and 0.008 to 0.09 (g/L) for microbial growth. Detailed results were presented and discussed below.

Single-factor experiments. Single-factor experiments were performed to verify factors in fermentation medium that affected H₂ yield, and to provide basic data for following experiments. Batch (bottle) fermentation was performed at 35 °C for 24 h with the culture medium conditions given in Method section and shaking rate of 130 rpm. It can be concluded from Figure S1 that fermentation medium producing the highest H₂ yield of 1.67 mol H₂/mol-glucose contained (g/L): 15 glucose, 5 yeast extract, 3 K₂HPO₄, 3 KH₂PO₄, 0.05 L-cysteine, 0.05 MgSO₄·7H₂O, 0.1 MnSO₄·H₂O and 0.3 FeSO₄·7H₂O, as compared to the yield of 1.28 mol H₂/mol-glucose (increased by 30.47%) with the initial medium. Microbial growth was also studied under different variable concentrations in culture medium. Regression curve presents well-linear relationship between DCW and OD₆₅₀ (Figure S2). It was found that the optimal concentrations of variables for microbial growth and H₂ yield were different (Figure S3). Obviously, the optimal concentrations of glucose, L-cysteine, MgSO₄·7H₂O and FeSO₄·7H₂O were 30, 0.1, 0.15 and 0.1 g/L for DCW, respectively, while they were 15, 0.05, 0.05 and 0.3 g/L for H₂ production (Figure S3). The most optimal culture medium for the strain growth was not the maximum for hydrogen production.

PB design. PB design was used to identify variables that had significant effects on H₂ production. The medium compositions were: glucose, yeast extract, tryptone, K₂HPO₄, KH₂PO₄, L-cysteine, MgSO₄·7H₂O and FeSO₄·7H₂O (Table 1). Both high and low levels (1 and -1) of each variable were chosen based on the above preliminary single-factor experiments. Table 2 gave the PB experiment results in 24 h batch fermentation at 35 °C with 130 rpm, while the statistical analysis of PB experiment data was summarized in Table 3. The significance of each variable was determined by their corresponding *p*-values. Factors evidencing *p*-values of less than 0.05 were considered to be significant effects on the response²², and were subsequently studied in further optimization studies. In this case, X₁ (glucose; *p* = 0.0015) and X₂ (yeast extract; *p* = 0.0181) were statistically significant in the production of H₂ (*p* < 0.05). X₁ had the greatest negative impact on H₂ yield. On the other side, X₂ had positive effect on H₂ yield. X₃ (tryptone), X₅ (KH₂PO₄), X₇ (MgSO₄·7H₂O) and X₈ (FeSO₄·7H₂O) were set at their high levels according to the positive effects although they were insignificant to H₂ yield. Factors such as X₄ (K₂HPO₄) and X₆ (L-cysteine) with negative effects were selected on their low levels. To approach the optimum response, a fitted first-order model equation for H₂ yield (Y) was obtained from the PB design experiments:

$$Y = 1.3644 - 0.0611X_1 + 0.0256X_2 + 0.0078X_3 - 0.0161X_4 + 0.0167X_5 - 0.0122X_6 + 0.0067X_7 + 0.0083X_8 \quad (1)$$

The coefficient of each variable in Eq. (1) represents the effect weight of the variable on H₂ yield. The quality of the fitted polynomial model equation was expressed by the coefficient of determination (R²). The obtained R² was 0.9858, indicating that 98.58% variability in the response could be explained by the model, but only 1.42% variability was not explained. Meanwhile, the value of adjusted determination coefficient (Adj R² = 0.9478) was very high, which advocates a high significance of the model²³. These results show that the response equation provides a suitable model for the PB design experiments.

Path of the steepest ascent. Experiments were started from zero level in PB design. If *t*-value was positive, climbing direction would increase and vice versa²⁴. Overall, step size was determined by the above *t*-values

Substrate	Reactor	H ₂ yield (mol/mol)	Acetic acid (AC) (g/L)	Butyric acid (BC) (g/L)	2-propanol (g/L)	AC/BC Ratio	Gas volume (mL)	Composition (%): H ₂	CO ₂
Glucose	Fermenter	2.15 ± 0.03	3.87 ± 0.12	3.35 ± 0.09	0.84 ± 0.01	1.15	8120 ± 37.98	64.34 ± 0.47	35.66 ± 0.40
	Bottle	2.03 ± 0.02	3.14 ± 0.06	2.87 ± 0.04	1.27 ± 0.02	1.09	233 ± 3.79	52.69 ± 0.14	47.31 ± 0.81
Bagasse hydrolysate	Fermenter	2.06 ± 0.02	3.46 ± 0.09	3.34 ± 0.07	1.36 ± 0.03	1.03	8532 ± 33.51	53.92 ± 0.61	46.08 ± 0.09
	Bottle	1.99 ± 0.03	2.28 ± 0.03	2.47 ± 0.02	1.24 ± 0.03	0.9	230 ± 5.69	50.16 ± 0.09	49.84 ± 0.04
<i>Jatropha</i> hulls hydrolysate	Fermenter	1.95 ± 0.06	2.37 ± 0.01	3.54 ± 0.03	1.15 ± 0.01	0.67	8767 ± 88.77	49.70 ± 0.49	50.29 ± 0.20
	Bottle	1.89 ± 0.02	1.97 ± 0.01	3.43 ± 0.03	1.20 ± 0.01	0.57	195 ± 1.49	56.62 ± 1.30	43.38 ± 0.24

Table 9. Comparisons of product yields from glucose, hydrolysates of bagasse and *Jatropha* hulls for H₂ production for 24 h fermentation at 35 °C with 130 rpm stirring/shaking in both reactors^a. ^aAll data were given by mean ± standard deviation (n = 3).

Raw biomass	Carbon resource	Microorganism	Reducing Sugar (g/L)	H ₂ yield (mol/mol)
Bagasse	Glucose Xylose	<i>C. butyricum</i>	20	1.73 ¹⁸
Bagasse	Glucose Xylose	<i>Clostridium</i> sp.	10	0.8 ¹⁹
Starch	Hexose	<i>C. butyricum</i>	5	2.0 ²⁰
Glucose	Glucose	<i>C. butyricum</i> ATCC19398	3	1.8 ³⁷
Glucose	Glucose	<i>C. butyricum</i>	2.5	1.4–2.3 ³⁸
Glucose	Glucose	<i>Clostridium</i> sp.	20	0.85 ³⁶
Glucose	Glucose	<i>C. butyricum</i>	15.64	2.15 ^a
Bagasse	Glucose Xylose	<i>C. butyricum</i>	15.64	2.06 ^a
<i>Jatropha</i> hulls	Glucose Xylose	<i>C. butyricum</i>	15.64	1.95 ^a

Table 10. Comparison of biohydrogen yield in this study with reported data. ^aData in this study in 3L fermenter.

(Table 3) and single-factor experiments. According to *t*-values (Table 3) and single-factor results, step size of 2 and 0.5 g/L was set for glucose and yeast extract, respectively. Glucose shows decreasing trend but an increasing trend for yeast extract. The concentrations of other factors were steadfast in all trials at their optimal levels. It was observed that H₂ yield increased along the path from test 1 to 3, reached the peak of 1.78, and decreased from test 4 to 7. This means that the optimal level was close to that in test 3 (16.0 g/L glucose and 6.0 g/L yeast extract). In Table 4, the highest H₂ yield was 1.78 mol H₂/mol-glucose for test 3. In these experiments, H₂ yield was remarkably improved. This suggests that the steepest ascent method was an effective technique to determine an optimal level. However, the optimal values for these two variables need to be determined *via* the following CCD.

RSM and CCD. A series of experiments were carried out *via* RSM to obtain an optimal combination of glucose and yeast extract. Table 5 gave five different coding levels, full experimental design and the results of CCD, with the center point set based on the results in steepest ascent test (Table 4).

Table 6 summarizes the significance test of regression coefficients. Using multivariate regression analysis of the observed data (Table 6), the obtained model illustrates the relationship between glucose (*X*₁) and yeast extract (*X*₂) corresponding to H₂ yield (*Y*) as below:

$$Y = 2.002 - 0.00894X_1 + 0.0211X_2 - 0.0125X_1X_2 - 0.1329X_1^2 - 0.1579X_2^2 \quad (2)$$

where *Y* was the predicted H₂ yield, *X*₁ and *X*₂ were the coded values of glucose and yeast extract. The regression model (Eq. 2) can be used to predict the range of H₂ production for various levels of the selected variables.

Determination and adjusted determination coefficients (*R*² and Adj *R*²) were calculated as 91.72% and 85.8% for the regression, demonstrating the agreement between the experimental and predicted H₂ yields that provides a good estimation of the response within the range of process conditions.

Analysis of variance (ANOVA) is essential to test the significance and adequacy of model. High *t*-value reveals an adequate explanation of the variation of data regarding to their mean value. The *t*-test and *p*-values served to examine the significance of each coefficient, which also illustrated the interaction strength among independent variables²⁵. The *t*-test of each significant variable level was given in Table 6. In this model term, the linear and quadratic of *X*₁, *X*₁*X*₁ and *X*₂*X*₂ (*p* < 0.05) were recognized as the significant factors²⁶, suggesting that H₂ yield was directly related to these two main factors (glucose and yeast extract). However, the interaction between glucose and yeast extract (*X*₁*X*₂, *p* > 0.05) seemed to be less significant on H₂ production. *F*-values of the model and lack of fit were 170.16 and 2.03, respectively (Table 7), while model *p*-values and *p*-values of lack of fit were <0.0001 and 0.2528, suggesting that the model was good fit to the experimental data.

Three dimensional (3D) response surface and corresponding contour of glucose and yeast extract were presented in Fig. 1. The response surface (Fig. 1a) displays an obvious convex, revealing the optimum conditions

were well-defined. The contour plot was almost circular, suggesting that the interaction of glucose and yeast extract had a less significant effect on H₂ production. In the surface plot, H₂ yield achieved the highest value within the studied region. Canonical correlation analysis (CCA) is a multivariate technique focused on determining the relationship among groups of variables in a datum set²⁷. CCA was further conducted (Table 7), at X₁ = -0.3402 (glucose, 15.66 g/L) and X₂ = 0.0804 (yeast extract, 6.04 g/L), the predicted maximum H₂ yield corresponding to these values was 2.02 mol H₂/mol-glucose.

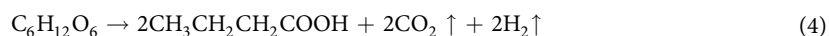
Experimental validation of the optimized medium. In order to verify the model (Eq. 2) adequacy in predicting the maximum yield of H₂, experiments were performed under optimum medium composition with 3 repetitions. H₂ yield was 2.03 ± 0.02 mol H₂/mol-glucose after 24 h bottle fermentation that agrees excellently with the predicted value (2.02 mol H₂/mol-glucose).

In summary, RSM is an efficient tool to optimize the medium composition for hydrogen production by *C. butyrium*. The optimized medium was (g/L): 15.66 glucose, 6.04 yeast extract, 4 tryptone, 3 K₂HPO₄, 3 KH₂PO₄, 0.05 L-cysteine, 0.05 MgSO₄·7H₂O, 0.1 MnSO₄·H₂O and 0.3 FeSO₄·7H₂O with 2.03 mol H₂/mol-glucose after 24 h. On the other hand, previous study gives the optimum fermentation medium for the growth of *C. butyricum* was composed of (w/v): 2% glucose, 0.5% pectin, 0.2% casein, 3.98% soyabean cake extract, 0.1% (NH₄)₂SO₄, 0.124% NaHCO₃, 0.37% corn steep flour, 0.02% MnSO₄·H₂O, 0.02% MgSO₄·7H₂O and 0.002% CaCl₂ at pH 7.5²¹. The optimized media for growth and H₂ production were different. All the above experiments were conducted in sealed 100 mL bottles. The obtained optimized results were further used for the production of H₂ from actual biomass wastes below.

Hydrogen from hydrolysates of bagasse and *Jatropha* hulls. Bagasse and *Jatropha* hulls mainly consist of cellulose and hemicellulose and lignin^{11,18}. The chemical compositions of bagasse and *Jatropha* hulls were analyzed according to the technical report from US National Renewable Energy Laboratory (NREL)^{28,29} (Table 8). Bagasse consisted of 25.17 wt% hemicellulose and 42.05 wt% cellulose, but 13.37 wt% hemicellulose and 36.95 wt% cellulose for *Jatropha* hulls. Bagasse and *Jatropha* hulls were hydrolyzed by two-step dilute acid hydrolysis in an autoclave. After reaction, the hydrolysates were neutralized, concentrated, detoxified, and fermented under the optimized medium obtained from glucose. Figures 2 and 3 illustrated liquid and gas products vs. time in the bottle fermentation of hydrolysates from bagasse and *Jatropha* hulls, respectively. For the fermentation of bagasse hydrolysate (Fig. 2), as time increased, yields of H₂, acetic acid and butyric acid increased with the decreasing TRS. These liquid products rose very slowly before 4 h as the strain was in the lag phase for a short adaptation to the new environment³⁰. In Fig. 2a, after 5 h, acetic acid and butyric acid grew rapidly to 3.25 and 2.48 g/L at 20 h (vs. 1.26 and 0.62 g/L at 5 h), and slightly rose further to 3.35 and 2.68 g/L at 24 h, respectively. Acetate and butyrate rose slightly after 15 h and stopped increasing after 20 h with the consumption of TRS because the accumulation of acetate and butyrate caused a sharp drop in culture pH and inhibited the growth of bacteria. 2-Propanol also presented similar rising trend. Similarly, H₂ production (Fig. 2b) rose sharply from 3.05 to 115.87 mL at time from 4 to 22 h, and changed little until 24 h. At the same time, total gas rose much higher from 5.29 at 4 h to 228.9 mL at 22 h because CO₂ was produced. For the fermentation of hydrolysate of *Jatropha* hulls (Fig. 3a), acetic acid and butyric acid increased rapidly to 3.87 and 3.59 g/L at 22 h from 2.16 and 0.17 g/L at 5 h. However, more butyric acid (3.52 g/L) produced at 24 h from the hydrolysate of *Jatropha* hulls than that of bagasse (2.68 g/L). The initial acetic acid concentration from the hydrolysate of *Jatropha* hulls (1.96 g/L) was higher than that of bagasse (1.06 g/L) that may inhibit the further production of acetic acid. More acetic acid was produced from the hydrolysate of bagasse (2.28 g/L) than *Jatropha* hulls (1.97 g/L) at 24 h (Table 9). Contrary, the production of butyric acid from the hydrolysate *Jatropha* hulls (3.43 g/L) was more than that of bagasse (2.47 g/L). The growth of strains was likely to be inhibited by the acetate produced when grown on media containing glucose³¹. So, less H₂ was produced from hydrolysate of *Jatropha* hulls (110.4 vs. 115.87 mL for bagasse hydrolysate at 24 h) via acetic route discussed below.

Sugars were also fermented in a 3L fermenter for 24 h at 35 °C with 130 rpm stirring (Table 9). H₂ yield from glucose is 2.15 (mol H₂/mol-glucose), which is slightly higher than the experiment in bottle fermentation (2.02 mol H₂/mol-glucose) because hydrogen pressure in bottle fermentation increased as gas accumulated in the fixed 100-mL bottle that may prevent hydrogen production. But, no pressure rose for the fermenter since it was connected on-line to an empty gas bag. Previous study shown that partial pressure of H₂ (pH₂) was an extremely important factor for continuous H₂ synthesis³². The hydrogen evolution rate and yield were improved by 10% and 15%, respectively, by reducing the H₂ partial pressure through pumping out produced gas in a fermentor with silicone rubber³³. So, high pressure prevented hydrogen production in bottle fermentation. The hydrogen production also affected by its concentration. As hydrogen concentration increased, H₂ synthesis decreased and metabolic pathways shifted towards the production of more reduced substrates, such as lactate, ethanol, acetone, butanol or alanine³². On the other hand, acids produced in the bottles without neutralization may also hamper the production of H₂. Acetate accumulation caused a sharp drop of culture pH and subsequent inhibition of bacterial hydrogen production, that were reported previously³⁴. For fermenter experiments, H₂ yield from hydrolysates of bagasse and *Jatropha* hulls is 2.06 and 1.95 (mol H₂/mol-TRS), which reached 95.8% and 90.7% of that from pure glucose, respectively.

Generally, glucose or other reducing sugars (e.g., xylose) were the preferred carbon source for fermentation with overall reactions as below³⁵:



According to Eq. (3), 4 mol H₂/mol-glucose was obtained with acetic acid and CO₂ as by-products. However, if butyric acid was formed as by-product, only 2 mol H₂/mol-glucose was produced (Eq. 4). The ratio of acetic/butyric acids obtained could be related to the production of H₂. Previous studies showed that the increase of the ratio was accompanied by increased production of H₂³⁵. In Table 9, it was well-confirmed in fermenter that acetic/butyric acid ratio was 0.67, 1.03 and 1.15 (g/g) for *Jatropha* hulls, bagasse and glucose, corresponding to their H₂ yield (mol/mol-TRS) of 1.95, 2.06 and 2.15, respectively. As comparison, bottle experiments with lower acetic/butyric acid ratio (g/g) of 0.57, 0.9 and 1.09 for hydrolysates of *Jatropha* hulls, bagasse and glucose had lower H₂ yield (mol/mol-TRS) of 1.89, 1.99 and 2.03 (Table 9). Gas is composed of 49.7–64.34% H₂ and 35.66–50.29% CO₂ from anaerobic fermentation in bottles and fermenter. H₂ from glucose (64.34%) had much higher concentration than those from hydrolysates of bagasse (53.92%) and *Jatropha* hulls (49.7%) in fermenter. The concentrations of H₂ produced from glucose and bagasse in fermenters were higher than those in bottles (64.34% and 53.92% vs. 52.69% and 50.16%). However, H₂ produced from *Jatropha* hulls (49.7%) in fermenter was less pure than that in bottles (56.62%).

Table 10 compares biohydrogen production in this work with other previous studies from various types of biomass with different microorganisms. In those previous studies, the yield of H₂ fermented by bagasse hydrolysate was 0.8–1.73 mol H₂/mol-TRS^{18,19}, and 0.85–2.3 mol H₂/mol-glucose^{36–38}. This study produced comparable biohydrogen yields to the reported studies (1.95–2.15 vs. 0.8–2.3 mol H₂/mol-TRS).

Conclusions

Fermentation medium for H₂ production from glucose with *Clostridium butyricum* was optimized by response surface methodology with the highest H₂ yield of 2.02 mol H₂/mol-glucose for batch fermentation at 35 °C for 24 h. The optimized medium was composed of (g/L): 15.66 glucose, 6.04 yeast extract, 4 tryptone, 3 K₂HPO₄, 3 KH₂PO₄, 0.05 L-cysteine, 0.05 MgSO₄·7H₂O, 0.3 FeSO₄·7H₂O and 0.1 MnSO₄·H₂O. Higher H₂ yield of 2.15 mol H₂/mol-glucose was achieved for 24 h in a well-controlled fermenter. The hydrolysates of bagasse and *Jatropha* hulls from two-step dilute acid hydrolysis were further successfully fermented to hydrogen with yields of 2.06 and 1.95 (mol H₂/mol-total reducing sugars) in fermenter, respectively. Higher H₂ yield from glucose and bagasse hydrolysate was due to their high acetic/butyric acid ratio *via* acetic route for hydrogen production. It is also found that optimized condition for H₂ production was not the best condition for microbial growth. Tropical biomass wastes as inexpensive raw materials can be effectively produce biohydrogen.

Methods

Materials. Substrates [glucose (99.8%), xylose (99.9%), fructose (99.5%), galactose (99.8%) and mannose (99.7%)], yeast extract, L-cysteine and tryptone were bought from Bomei Biotech Co., Ltd. (Heifei, Anhui). Calcium carbonate (99.8%), K₂HPO₄ (99%), KH₂PO₄ (99.8%), Na₂HPO₄ (99%), NaCl (99.5%), MnSO₄·H₂O (99%), MgSO₄·7H₂O (99%), FeSO₄·7H₂O (99%), glycerol (99%), ethanol (99.7%) and sulfuric acid (98%) were bought from Xilong Chemical Factory Co., Ltd. (Shantou, Guangdong). Standard sugars of glucose, xylose, arabinose, mannose and galactose (purity > 99%) were bought from Sigma-Aldrich (Shanghai). Acetic acid (99.5%), butyric acid (99.7%) and 2-propanol (99.9%) were from Aladdin Industrial Corporation (Shanghai). Activated carbon (powder, 97%) was purchased from Fengchuan Chemical Reagent Co., Ltd (Tianjin).

Jatropha hulls were purchased from Yunnan Shenyu New Energy Co., Ltd. (Chuxiong, Yunnan). Bagasse was bought from Dehong (Yunnan). Biomass samples were dried at 45 °C until constant weight (WFO-710, EYELA, Tokyo Rikakikai Co., Ltd.), ground in a pulverizer (9FC-15, Xudong Machinery Manufacturing Co., Ltd., Leshan, Sichuan) and sieved through 80 mesh for analysis and hydrolysis.

Microorganism and batch (bottle) fermentation. *C. butyricum* (CICC 20763) used for producing H₂ was purchased from China Center of Industrial Culture Collection (CICC, Beijing). The culture was maintained on corn meal agar stab at 4 °C. The seed medium composed of (g/L): 20 glucose, 5 yeast extract, 5 tryptone and 3 NaCl with pH 6.5. To make the seed cultivation, a loop of *C. butyricum* from a fresh slant tube was inoculated into a serum bottle (250 mL) containing 50 mL seed medium and then incubated in a rotary shaker (ZWY-2102C, Zhicheng Analytical Instrument Manufacturing Co., Ltd., Shanghai) at 35 °C with 130 rpm for 12 h.

The initial medium for anaerobic fermentation was composed of (g/L): 25 glucose, 10 tryptone, 5 Na₂HPO₄, 3 NaCl and 0.05 FeSO₄·7H₂O. A 4% (v/v) of the seed culture was inoculated into the medium for fermentation. After sterilization at 121 °C for 20 min in an autoclave (HVE-50, Hirayama Manufacturing Corp., Tokyo), substrates with *C. butyricum* (about 20 mL, initial pH 6.5) were loaded into 100-mL bottles in an anaerobic incubator (YQX-II, CIMO Medical Instrument Manufacturing Co., Ltd., Shanghai) to maintain anaerobic environment by nitrogen purge (99.999% N₂, Meisel Gas Products Co., Ltd., Kunming, Yunnan) and sealed by rubber-aluminium cap. The bottles with samples were put in an incubator shaker (ZWY-2102C) at 130 rpm and 35 °C for batch fermentation. After fermentation (up to 24 h), gas was collected in a bag (500 mL) by a 50 mL syringe (Agilent Inc., Palo Alto, CA) and its volume (e.g., up to 233 mL at 24 h) was measured by the same syringe. Gas was analyzed by gas chromatograph (GC) and liquid sample by high performance liquid chromatograph (HPLC), ultraviolet (UV)-visible spectrophotometer and biosensor introduced in detail below.

PB design. PB design was applied to select factors that significantly influenced H₂ yield, based on the first-order (linear) model as below²²:

$$Y = \beta_0 + \sum \beta_i X_i, \quad i = 1, 2, \dots, k \quad (5)$$

where Y was H₂ yield as response; X_i was coded independent factor and β_i was linear coefficient; β₀ was intercept value. Each variable had two-levels, high and low, coded by (+1) and (−1), respectively.

Path of steepest ascent method. The method of steepest ascent given by Box and Wilson is a procedure for moving sequentially along the direction of the maximum increase in response³⁹. The direction in which H₂ yield increased most rapidly was that of steepest ascent, started from the zero level of variables (significant factors) in PB design, while the step size was decided by the estimated coefficient ratio from Eq. 5, together with practical experience. This test was disused until response no longer increased. As a result, the steepest ascent method allowed factors approaching the optimal level and gave a more limited region for RSM optimization⁴⁰.

CCD and RSM. CCD was employed to optimize the two most significant factors (glucose and yeast extract) for the maximum H₂ yield, screened by PB design. These two independent factors were studied at five different levels (−1.41, −1, 0, +1 and +1.41), and a set of 13 experiments were carried out (Table 5). The behavior of the experiment was explained by the following second-order polynomial equation²³:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j \quad i = 1, 2, \dots, k \quad (6)$$

where Y was the predict response, β_0 was intercept, X_i and X_j were input variables which influenced the response Y, β_i was linear coefficient, β_{ii} was quadratic coefficient, and β_{ij} was interaction coefficient. The second-order polynomial coefficients were also calculated.

Composition analysis of bagasse and *Jatropha* hulls. Composition analysis of bagasse and *Jatropha* hulls was conducted according to the standard US NREL protocols^{28,29}. All the raw materials were dried at 45 °C until achieving constant weight. Biomass sample (2–10 g, oven dry weight, ODW) was extracted in a Soxhlet extractor with deionized water and ethanol at boiling points for 12–24 h, respectively. The extracted solutions were dried in a flask to remove water and ethanol at 45 to 60 °C by a rotary evaporator. The extracted solid biomass (0.3 ± 0.01 g, dried) was hydrolyzed in a 25-mL tube containing 4.98 ± 0.01 g concentrated sulfuric acid (72 wt%) stirred by a magnetic bar at 30 °C water bath for 1 h, and then diluted to 4 wt% sulfuric acid solution by adding 84 ± 0.01 mL deionized water. The diluted solution with biomass sample was put in a 100 mL bottle sealed by a rubber-aluminum cap and autoclaved at 121 °C for 60 ± 1 min (HVE-50) for second-step hydrolysis, at the same time, a reference standard sugar mixture (glucose, xylose, galactose, arabinose and mannose) with similar concentrations to the sugars in biomass sample was also put in the autoclave to calibrate the decomposition of each sugar. The filtrated supernatant was collected for acid-soluble lignin determination by using UV-Visible spectrophotometer at 240 nm wavelength (UV-1800, Shimadzu, Kyoto). The supernatant was neutralized to pH 5.0–6.0 with calcium carbonate for sugar analysis described below. The solid residual separated from the hydrolysate by filtration (0.22 μm pore size) was oven-dried for acid-insoluble lignin determination. Crucibles containing residues were placed in a muffle furnace (4–10, Ever light medical equipment Co., Ltd, Beijing) at 575 ± 25 °C for 24 h to determine ash weight.

Hydrolysis of *Jatropha* hulls and bagasse. Two-step dilute acid hydrolysis was conducted in a 500-mL high-pressure autoclave (FCFD05-30, Yantai Jianbang Chemical Mechanical Co. Ltd., Shandong). In the first-step hydrolysis, bagasse or *Jatropha* hulls (20 g, dry weight) mixed with 2 wt% sulphuric acid solution with solid-liquid ratio (SLR) of 1:10 (g/g) was hydrolyzed at 130 °C for 1 h⁴¹. After reaction, solid residue was filtrated (0.22 μm pore size), washed and dried for second-step hydrolysis in 4 wt% sulphuric acid solution with 15 g solid residue, SLR of 1:10 (g/g) at 150 °C for 1 h⁴¹. Hydrolysates from both steps were mixed together, and neutralized to pH 6.0 with calcium hydroxide. After filtration (0.22 μm pore size), the solution was concentrated to 15.66 g/L TRS by a rotary evaporator at 60–70 °C. The concentrated solutions (100 mL) were further detoxicated with 2 wt% activated carbon at 50 °C for 2 h, and used for the following fermentations. The hydrolysates were fermented under the optimized medium obtained from glucose in bottles because they mainly contained hexose and pentose sugars with minor of acids, furfural, hybrid protein and other toxic components that were neutralized and detoxified.

Fermentation in a fermenter. Fermentation of glucose and hydrolysates (about 1 L) with *C. butyricum* was also carried out in a 3L fermenter (LiFlus GX, Hanil Science Industrial Co. Ltd., Incheon, South Korea) at 35 °C for 24 h with 130 rpm stirring. Its pH was controlled at 6.5 by adding 1 M sodium hydroxide or 1 M hydrochloric acid. Gas generated (up to 8.8 L) during the fermentation process was collected into a gas bag (10 L) on-line and its volume was measured after fermentation using a wet gas meter (LMF-1, Shanghai A.K. Instruments Co., Ltd.), where water was saturated by NaHCO₃ to avoid absorption of CO₂. Gas and liquid products were analyzed described below.

Analytical methods. Gas was analyzed by GC (7820A, Agilent, Palo Alto, CA) equipped with four columns [three packed columns: one Porapak Q (9 ft × 1/8 in.) and two Porapak N (3 ft × 1/8 in.), and one Molecular Sieve 5A (6 ft × 1/8 in.)] with two thermal conductivity detectors (TCD). In this work, no CH₄, CO, C₂H₆ and C₂H₂ gases were detected. Produced gases (H₂ and CO₂) were only separated by Porapak Q (CH₄ together with CO were also monitored even though their peaks were overlapped). Helium (99.999% purity, Guangruida Co. Ltd., Kunming, Yunnan) was used as carrier gas. Gas sample was calibrated with a standard gas mixture (35% H₂, 2% CH₄, 25% CO₂; v/v) (Huatae Gas Co. Ltd., Foshan, Guangdong), and diluted 1–5 times into five gradients with nitrogen (99.9999%, Guangruida Co. Ltd.). All the standard calibration curves obtained had R² > 0.999. H₂ yield was defined as below:

$$\text{Hydrogen yield (mol/mol)} = \frac{PV_{H_2}/RT}{N} \quad (7)$$

where P (0.8×10^5 Pa in Kunming at an altitude of 1,896 m, calculated by Barometric formula⁴²) is atmospheric pressure; V_{H_2} is gas volume (m^3); R is perfect gas constant ($8.3144, m^3 \cdot Pa \cdot mol^{-1} \cdot K^{-1}$); T is gas temperature (298 K), and N is mole number of substrate (e.g., glucose, TRS, glycerol) before fermentation (mol).

Liquid samples (glucose, xylose, acetic acid and butyric acid) after fermentation were measured by HPLC (LC-20A, Shimadzu) equipped with a refractive index detector (RID, Shimadzu) setting at 55 °C and Aminex HPX-87H column (Bio-Rad, Hercules, CA). Running temperature was 60 °C and 0.005 M H_2SO_4 was applied as mobile phase with a flow rate of 0.6 mL/min. Each product was calibrated by its standard solutions with five different concentrations (e.g. 1, 2, 3, 4, 5 g/L). Similarly, sugars from the composition analysis of biomass were analyzed by the HPLC and Hi-Plex Pb column. The mobile phase was de-ionized water at 0.5 mL min^{-1} . Temperatures of the detector and the column oven were 55 °C and 70 °C, respectively. Concentrations of the monomeric sugar standards were 0.05, 0.1, 0.5, 1, 1.5 and 2 g L^{-1} . All the standard calibration curves for HPLC analysis obtained with $R^2 > 0.998$ were used for quantitative calculation.

Microbial mass (DCW, g/L) was determined through measuring the absorbance of broth at 650 nm (OD_{650}) by the UV-visible spectrophotometer. One unit of optical density (OD_{650}) was estimated to be equal to 0.36 g/L DCW ($y = 0.2835x + 0.0846, R^2 = 0.9974$, Figure S2). Besides cross-checked by HPLC, glucose in liquid samples after fermentation was mainly quickly determined using a biosensor (SBA-40, Institute of Biology, Shandong Academy of Sciences, Jinan) calibrated with glucose standard solution (100 mg/dL). TRS in the hydrolysates after two-step hydrolysis or fermentation was determined by the UV spectrophotometer using dinitrosalicylic acid (DNS) method⁴³ calibrated with five different concentrations (0, 0.2, 0.4, 0.6, 0.8 and 1.0 g/L) with $R^2 > 0.9999$.

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Author Contributions

D.J. (PhD student) designed the study, carried out the experimental work, and drafted the manuscript. Z.F. (supervisor) supervised the study and revised the manuscript. S.-x.C. helped to design CCD experiment and revised the manuscript. X.-f.T. revised the manuscript. T.-c.S. helped to perform the experiment on growth curve. All authors read and approved the final manuscript.

Additional Information

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