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Pretreatment Condition Optimization of Raw Materials for Biogas Fermentation and its Effect Research

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The present study is to inhibit the acidification and enhance biogas production in the Biogas fermentation process. Pig manure as fermentation substrate was pre-fermented by *Clostridium butyricum* to enhance the butyric acid content and reduce the propionic acid content in the biogas fermentation acidification phase. During pre-fermentation, Plackett-Burman(P-B) design were adopted to screen significant factors, and Central Composite Design(CCD) were used to optimizedthe pre-fermentation condition. It demonstrated that among the five factors, namely, the moisture content, inoculation time, inoculation size, temperature, initial pH, only the moisture content and temperature were selected as critical factors. The optimization experiment results for the moisture content, inoculation time and temperature by CCD were determined to be 62.04%,27.22h, and 37.13°C, respectively. Under the optimum condition, the result of verification experiment showed that 21.13 g/kg was the maximum butyric acid yield. The pre-fermentation experiment and maximum butyric acid yield of 21.55 g/kg was obtained with a corresponding mathematical model established. This proved that statistical method was a powerful tool for the optimization of butyric acid fermentation. To monitor the dynamic changes of *clostridium butyricum* in the fermentation system by the method of Real-Time PCR (RT-PCR), it is indicated after analysis that dynamic changes of the amount of *clostridium butyricum*are in conformity with the trend of butyric acid yield.The pre-fermented manure was used for biogas production and biogas production increased by 19.02% in comparison with the control (without its pre-fermentation).

Key words: Butyric acid type pre-fermentation, Biogas fermentation, Response surface method, Real-time PCR.

At present, the large scale organic acidaccumulation leading to acid inhibitionoften occurred in the process of biogas fermentation, which was caused by the raw material of hydrolysis acidification speed rather than gas production. This is because acid-producing anaerobes increase quickly and Methane-producing bacteria reproduce slowly. It often appeared “acidosis”phenomenon severe case, biogas generator thus cannot be run normally[1]. If the concentration of butyric acid increased in acid producing stage, it could not only reduce the acidic terminal of fermentation products; also, compared with the acetic acid producing pathway, the butyric acid producing pathway could reduce the production of NADH+$H^+$and hadpromotion impact to accelerateGlucose metabolism process and then improved the biogas production[2-5]. In order to promote butyric acid type pre-fermentation, the optimization of fermentation condition is very necessary. In the current study, pig manure was adopted as substrate material. Plackett-Burman (P-B) design were adopted to screen significant factors, and Central Composite Design(CCD) were

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used to optimize the pre-fermentation condition.STATISTICA software is adopted to analyze it, in order to conclude the key influence factors and the optimal fermentation conditions. The method of Real-Time PCR (RT-PCR) was drawn on, with the specific primers which were designed to monitor the trend of dynamic changes of *clostridium butyricum* in the fermentation system.

MATERIALS AND METHODS

Strain and inoculation preparation

C.butyrium B1 was acquired from Beijing Agro-Biotechnology Research Center which was screened and stored at 4°C.Spores of *C.butyricum* were heat shocked at 80°C for 10 min followed by transferring to glucose-based fresh medium.

Basal medium composition(g/L):Tryptone5 g/L, Beef extract 5 g/L, Glucose5 g/L, Yeast extract powder10 g/L, Resazurin0.5 ml/L(0.1%), Salt solution40mL(salt solution preparation: $CaCl_2$•$2H_2O$ 0.25 g/L, KH_2PO_4 1 g/L, K_2HPO_4 1 g/L, $MgSO_4$•$7H_2O$ 0.5 g/L, $NaHCO_3$ 10 g/L, NaCl 2 g/L, stored at minus 4 degrees Celsius).0.5g/L cysteinewas added after the first boiling, then through the nitrogen boiling for 15 minutes, and repackaging into anaerobic bottles or anaerobic tube, after sterilizing in115°C for 15 min. *Clostridium butyricum* suspension at 37 °C for 12 ~ 16h by static culture after culture as inoculum.

The pretreatment of the raw material fermentation

The test used manure from Fangshan, Beijing, China, naturally dried and crushed to the complex, over 40 mesh sieve stored for testing.

Detection Method of VFA

Fermentation liquid was centrifugated by 8 000 r/min for 10 min, filtering the supernatant by pin type water phase filter(13 mm × 0.22 μm), Using Shimadzu HPLC LC-20AT and the UV-VIS detector: SPD -20A(UV spectrometry detection); Chromatographic column: ZorbaxSB-Aq(5μm 4.6 mm×150mm); 20 mmol·L^{-1} of NaH_2PO_4 as the mobile phase(pH adjusted with phosphoric acid 2.0, over 0.22 μm membrane, plus one percent by volume of acetonitrile). The flow rate was 1 mL·min^{-1},column temperature was 35°C and injection volume 5 μL; diode array test wavelength of 210 nm. The mobile phase with 0.45 μm microporous membrane filter and ultrasonic degassing. Before using column chromatography, cleaning system 0.5~1 h by pure methanol, and then eluted the column with a mobile phase until baseline was flat.

Determination method of RT-PCR

Utilizethe method of Real-Time PCR (RT-PCR), with the specific primers which were designed to monitor the trend of dynamic changes of *clostridium butyricum* in the fermentation system. The total DNA of fermentation samples was extracted by FastDNA SPIN Kit, DNA purified by DNA purification kit as a template for Real-Time PCR amplification. Amplification system:SYBR GREEN PCR MASTER MIX 10 μL, primer 2 ul, DNA template 1 μL, ddH2O 5 ul. The amplification conditions were 95°C for 2 min; 35 cycles of 94°C for 1 min,60°C for 2 min,and 72°C for 2 min; and a terminal extension step at 72°C for 5 min. The PCR procedure is one cycle denaturing at 94°C for 2min, 40 cycles which involves denaturing at 94°C for 20s,annealingat 58°Cand extending for 45s. upstream primer:5'-AGCGTTGTCCGGATTTACTG-3', downstream primer:5'-TTCGCCACT GGTATTCTTCC-3[6].

Experiment design and statistical analysis

(I)Plackett-Burman design. The Plackett-Burman(P-B) design,an effective technique for fermentation conditions optimization, was used to pick factors that significantly influenced butyric acid production. This experimental design was a two-level factorial design, which could indentified the critical physic-chemical parameters required, in order to elevated butyric acid yield by screening *n* variables in *n*+1 experiments[7-9]. The technique is based on the first-order polynomial regression equation:

$$Y = \beta_0 + \sum \beta_i X_i \quad ...(1)$$

Where *Y* is the response(butyric acid yield), β_0 is the model intercept, β_i is the linear coefficient and X_i is the level of the independent variable. 8 runs of P-B design matrix generated by the STATISTICA10.0 (StatSoft Inc., USA) was multiplied(Table 1 run 8), and then used to investigate the effects of environment factors on the butyric acid type fermentation by *C.butyricum*. The five factors were examined to investigate the key factors significantly affecting the production of butyric acid. The experimental design for the screening of the variables is showed in Table 1. All

the variables were denoted as numerical factors and investigated at two widely spaced intervals designated as -1(low level) and +1(high level). The effects of individual parameters on butyric acid production were calculated by the following equation:

$$E_x = \frac{\sum Y(+1) - \sum Y(-1)}{n/2} \quad ...(2)$$

Where *E* is the effect of parameter under the experiment conditions, and $\sum Y(+1), \sum Y(-1)$ are the sum of responses (butyric acid production) of trial at which the parameter was at its higher and lower levels, respectively, and *n* is the total number of trials.

(II) Central composite design. The response surface methodology was used to optimize the screened variables for enhanced butyric acid production based on Central Composite Design(CCD), which is helpful to investigate linear, quadratic, and cross-product effects of the three reaction condition variables on the butyric acid production[10-13].The experimental data were analyzed by RSM using the following second-order polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i X_i + \sum_{i=1}^{n} \beta_{ii} X_i + \sum \sum_{i<j=1}^{n} \beta_{ij} X_i Y_j \quad ...(3)$$

where Y is the response(butyric acid production); Xi and Xj are the coded independent variables and β_0, β_i, β_{ii}, β_{ij} are intercept, linear, quadratic, and interaction constant coefficients, respectively. Subsequently, The maximum response variable and the corresponding variables were estimated from eq.(3).

STATISTICA 10.0 was applied for analysis of variance (ANOVA) of experimental data and regression analysis. Response surfaces and contour plots were exploited for regression analysis to obtain the fitting quadratic polynomial equations, corresponding to the change of the stagnation point and the other two variables.

RESULTS AND DISCUSSIONS

Effect of environmental conditions in butyric acid production

Objective to investigate the effect of the condition factors of butyric acid type fermentation, the relative importance of five factors, including the moisture content,inoculation time,inoculation size,temperature, and initial pH were investigated by P-B design, As shown in Table 2, the main effect of each variable in butyric acid yield was estimated to the average difference between the two measurement of the high level(+1) and the low level(-1) of the factors. After the estimated regression coefficients, the important factors of interest identified by the variable (response) after the analysis of variance (ANOVA) (Table 2)According to the SS, P and E, the importance of factors can be listed as moisture content> temperature> inoculation time> initial pH>inoculation size.

When the confidence levels greater than 95% can be considered as influencing butyric acid yield, significantly, moisture content was significantly higher than 99.9% confidence levels for butyric acid yield. From the *P*-value analysis,

Table 1. Plackett-Burman experimental design for the screening of significant process variables affecting butyric acid production

Code	Temperature (°C)	Inoculation time /h	Inoculation doze /%	Moisture content /%	Initial pH	Butyric acid production[a]/ g·kg^{-1}
1	20	10	10	95	5	1.15
2	20	30	10	65	5	6.98
3	20	30	2	65	9	8.30
4	20	10	2	95	9	4.06
5	40	10	10	65	9	14.02
6	40	30	10	95	9	3.56
7	40	30	2	95	5	4.40
8	40	10	2	65	5	13.00

a) Butyric acid yield was produced by per kg pig manure

moisture content and temperature had a substantial positive impact on butyric acid yield.

When E> 0, that goal expectation is positive, then showed positive significant effect, anyway, is shown as a negative significant effect. As can be seen from the E values, inoculum time, inoculum size, moisture content of these three factors on the butyric acid fermentation is negative

Table 2 Effect of factors in P-B design on butyric acid production

Factors	*Df*	*SS*	*F*	*P*	E[b)]
Temperature	1	26.280	35.37	0.027	3.62
Inoculation time	1	10.112	13.61	0.066	-2.25
Inoculation doze	1	2.055	2.77	0.238	-0.93
Moisture content	1	106.017	142.68	0.007	-7.28
Initial pH	1	2.431	3.27	0.212	1.10
Error	2	1.486			
Total SS	7	148.381			

b)Effect value of factor.

Table 3. Experimental design matrix and results

Code	A: Moisture content(%)	B: Inoculation time(h)	C: Temperature(°C)	Butyric acid ($g \cdot kg^{-1}$)
1	75	35	30	13.35
2	65	8.18	35	10.28
3	48.18	25	35	18.85
4	75	15	40	13.94
5	65	25	43.41	19.20
6	65	25	26.59	9.21
7	75	15	30	10.25
8	65	41.82	35	18.28
9	55	35	40	17.92
10	55	15	30	14.34
11	81.82	25	35	13.60
12	55	35	30	16.70
13	65	25	35	21.14
14	55	15	40	16.63
15	65	25	35	21.81
16	75	35	40	17.83

Table 4. ANOVA for response surface quadratic model for butyric acid production

Source	*SS*	*Df*	Mean square	*F*-value	*P*-value
A(L)	26.5804	1	26.58041	11.99487	0.013412
A(Q)	27.1878	1	27.18784	12.26898	0.012784
B(L)	42.4801	1	42.48011	19.16988	0.004677
B(Q)	53.4008	1	53.40079	24.09802	0.002686
C(L)	59.4108	1	59.41076	26.81012	0.002059
C(Q)	54.5615	1	54.56151	24.62181	0.002547
AB	1.3987	1	1.39866	0.63117	0.457191
AC	2.7159	1	2.71592	1.22561	0.310661
BC	0.0098	1	0.00979	0.00442	0.949160
Error	13.2959	6	2.21598		
Total SS	222.8588	15			

Table 5. The main VFA content in the pre-fermentation product

	Acetic acid	Propionic acid	Butyric acid
Experimental group	33.78	8.82	21.13
Control group	41.76	10.89	10.11

for acid fermentation, and the temperature and the initial pH of the acid fermentation shown a positive significant. Since this experiment was butyric acid pre-fermentation biogas, taking into account the practical application of the fermentation process to achieve better efficiency, it will select the training time for central composite experimental.

The optimal level of the key factors (moisture content, temperatureandinoculation time)and their interaction on yield of butyric acid further to explored the influence of the Centre Composite Design (CCD), and it is used to develop condition variables, the correlation betweenthe butyric acid yields. Complete design matrix and butyric acid yield in different condition variables are listed in Table 3.

The butyric acid yield was in the range from 9.21 to 21.81 g/L. By applying multiple regression analysis on the experimental data, and the following second-order polynomial equation was established to explain the butyric acid yield:

$$Y = -162.131 + 1.575A - 0.017A^2 + 1.13B - 0.024B^2 + 6.472C - 0.097C^2 + 0.004AB + 0.012AC - 0.001BC \quad ...(4)$$

Where Y is the predicted butyric acid yield rate; A, B and C are the coded values of moisture content, temperature andinoculation time, respectively.

The analysis of variance (ANOVA) was conducted to test the significance of the fit of the second-order polynomial equation for the experimental data as shown in Table 4.

ANOVA of the fitting model equations indicated that it was highly significant($P \leq 0.01$), and the lack of fit was not significant($P > 0.05$). The coefficient of determination (R^2) was 0.9403, which could explain 94.03% variation of the response variable; only 5.97% cannot explained by the model. It represented a good agreement between experimental and predicted values and implied that eq.(4) could describe the influence of the moisture content, inoculation time, temperature on the study of butyric acid production is very good.

ANOVA of the fitting model also showed that the linear and quadratic effect of inoculation time and temperature on butyric acid production rate were extremely significant($P \leq 0.01$), indicating that these terms had great impact on the butyric acid production rate. However, the intersection of all factors squared is not significant($P \leq 0.05$).

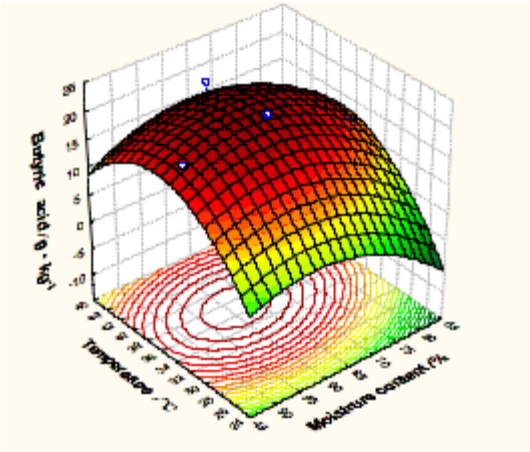

Fig. 1. The effect of moisture content and temperature on the butyric acid fermentation production surface figure and contour plots

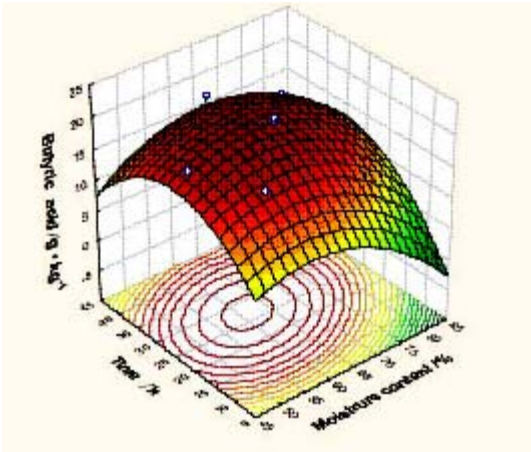

Fig. 2. The effect of moisture content and inoculation time on the butyric acid fermentation production surface figure and contour plots

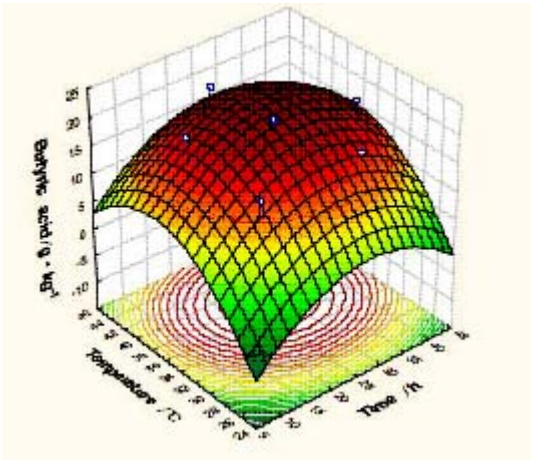

Fig. 3. The effect of inoculation time and temperature on the butyric acid fermentation production surface figure and contour plots

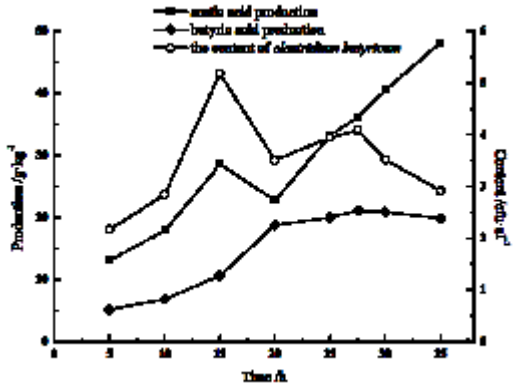

Fig. 4. The dynamic change of *Clostridium butyricum* in the fermentation and main volatile acid changes

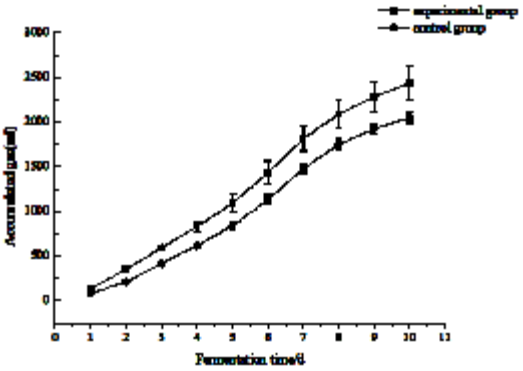

Fig. 5. Pretreatment of *C.butyricum* influence accumulative biogas production

Subsequently, the maximum butyric acid production was estimated from eq.(4) at the moisture content of 62.04%, the inoculation time of 27.22 h and the temperature of 37.13°C.

The response surface and contour plots were shown in Figures 1-3, which described the interactions between two variables by keeping the other variables at zero level for butyric acid yield. The figure shows that, in the design of boundary, each response surface plot had a obvious peak and corresponding contour plot had clear highest point, which denoted that the maximum butyric acid yield could be reached the design boundary. The butyric acid yield rate increased with increasing the moisture content, inoculation time and temperature to the optimal levels, and decreased with a further increase. Lower and higher levels of the intersection of all factors did not lead to higher butyric acid yield. The shape of the response surface curves showed a moderate interaction between the tested variables.

Validation of the models

Validation was executed under conditions of prediction the model as follow: moisture content62.04%, Inoculation time27.22h and temperature37.13°C. Under the above optimized condition, the maximum yield of butyric acid based on pre-fermentation was estimated as 22.55 g·kg^{-1}. The results were further verified by triplicate experiments, and the maximum butyric acid yield was 21.81 g·kg^{-1} (Figure 4). It indicates that the experimental value obtained was in consistent with the value of the calculation model.

The RSM designs in many recent biotechnological researches have been successfully applied in the present investigation. .However, no single report was obtained on butyric acid type fermentation in biogas fermentation.

In this article, we adopted pig manure as substratematerial;significantly enhance butyric acid production in the system by butyric acid type pre-fermentation so as to promote butyric acid type fermentation in biogas fermentation, providing important data for subsequent methane fermentation tests.

The change of clostridium butyricum in the pre-fermentation process

To analysis the dynamic changes of clostridium butyricum in the fermentation system by the method of Real-Time PCR (RT-PCR), As

shown in Figure 4, the strain in adapt to the environment after 10-15 h exponential phase in its proliferation after injected *clostridium butyricum*, when the number of bacteria reaching the highest 15h, the first peak appears, and butyric acid does not reach the maximum output, when the proliferation of Clostridium butyricum, acetogenic pathway than the production of butyric acid to provide more energy are rapidly increasing production of acetic acid, eq.(5-6) as follow:

$C_6H_{12}O_6+4H_2O+2NAD^++4ADP+4Pi\rightarrow 2CH_3COO^-+6H^++2H_2+2HCO_3^-+2NADH+4ATP$ ....(5)

$C_6H_{12}O_6+2H_2O+3ADP+3Pi\rightarrow CH_3CH_2\ CH_2COO^-+3H^++2HCO_3^-+3ATP$ ...(6)

When *Clostridium butyricum* into the stationary phase at 20h, when production reached a peak of butyric acid, acetic acid production is also a corresponding increase, in the acid production of the highest 27.5h, as well Clostridium butyricum reached the second peak, after 27.5h, with the system in the acidity increases, the number of *Clostridium butyricum* presented a trend of attenuation.

The effect of butyric acid Pre-fermentation on biogas production

In the preprocessing stage to adding *Clostridium butyricum* for pre-fermentation, measured main volatile acid content in the fermented product as shown in table 5, from table 5 shows, after access clostridium butyricum for fermentation, the butyric acid/acetic acid ratio in the product increased by 158.4% than the control group, propionic acid content was reduced by 19%, the total acidity reduced 4% than the control group, from acetic acid and propionic acid by the end of the acid ratio 6.5:1 to 2.33:1, so as to solve the often due to low pH value of the negative feedback. Pre-fermentation product then access biogas slurry fermentation, gas production test results shown in Figure 5, the experiments showed that preprocessing phase access *Clostridium butyricum*B1 than the average total gas production in the control group increased by 19.02%. Test gas is biogas generated by the content of each component: CH_4 accounted for 57.56%, CO_2 accounted for 40.62%, N2 and O2 accounted for 1.82%, the content of CH4 using standard has reached ignition.

CONCLUSIONS

P-B design and Central Composite Design were adopted to screen the key factors and determine optimal culture conditions which strengthened butyric acid yield by*Clostridium butyricum* B1. It turned out that this statistical method provides an effective and feasible approach for the condition optimization for butyric acid production.The following conclusions could be drawn.

1. Moisture content, the incubation time and temperature and so on various factors of indicators too high or too low are obvious influence on the yield of butyric acid, butyric acid type fermentation effectively as well.The optimal conditions of butyric acid pre-fermentation Moisture were as followed:62.04% moisture content,27.22h fermentation period at of 37.13°C. The predicted and experimental butyric acid yields were found to be 22.55 $g\cdot kg^{-1}$ and 21.81 $g\cdot kg^{-1}$, respectively.
2. The dynamic change of Clostridium butyricum were analyzed by the method of Real-timePCR (RT-PCR) in the fermentation system, and number of *Clostridium butyricum* in 15 h and 27.5 h appeared two peaks, The first peak is related to bacteria own breeding, and the second peak coincided with the time of extremum butyric acid production. Dynamic changes of the amount of clostridium butyricum in conformity with the trend of butyric acid yield and acetic acid yield.
3. Gas experiments show that after butyric acid pre-fermentation for biogas fermentation, compared to control in the process of the fermentation, the methane gas production increased by 19.02%.This may be due to: first, after pre-fermentation, butyric acid/ acetic acid increased significantly reduced the acid terminal,portion slows down the phenomenon of acidosis; Second,it is to a certain extent to reduce the content of propionic acid, and then reduced the biogas fermentation inhibition of mixed bacteria in the system, especially reducing the inhibitory activity of the methane bacteria, thus improving the production of biogas.

This experiment can be further explored to develop into biogas pretreatment agent experimental basis.

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