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Butanol Production from Halophyte Seepweed *Suaeda salsa* by Simultaneous Saccharification and Fermentation

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In this study, the halophyte seepweed *Suaeda salsa* was used for butanol production by simultaneous saccharification and fermentation. The main nutrients of *S. salsa* was estimated at first and then the influence of different factors in simultaneous saccharification and fermentation, such as substrate concentration, cellulase dosages and $CaCO_3$ were investigated and optimized by the one-factor-at-a-time approach and further optimized by response surface methodology. The optimal conditions for butanol production by simultaneous saccharification and fermentation were: seepweed 34.5 g (0.8-1.0 mm in length, pretreated by 4 % NaOH), mixed with cellulase 23.7 FPIU/g seepweed and nutrition solution (yeast extract 1.0 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, $CaCO_3$ 4.8 g/L, ammonium acetate 2.2 g/L, *p*-aminobenzoic acid 0.001 g/L, vitamin B1 0.001 g/L, biotin 0.00001 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, $MnSO_4{\cdot}7H_2O$ 0.01g/L, NaCl 0.01 g/L, $FeSO_4{\cdot}7H_2O$ 0.01g/L. Inoculum volume of 10 % (v/v) and the medium volume was 50/250 mL flask, under these conditions, the butanol yield could reach 3.5 g/L after 72 h fermentation.

Key Words: Butanol, Simultaneous saccharification, Fermentation, Optimization, *Suaeda salsa*.

INTRODUCTION

In these years, biomass based energy such as ethanol, butanol and biodesel have attracted many people's attention due to the environmental pollution of the traditional fossil fuels. Among these biofuels, butanol is a kind of superior and cleaner fuel oxygenate than ethanol. As bioenergy, it has many advantages: octane numbers of butanol is 113 and 94, while that of 111 and 94 for ethanol[1]. So butanol will be extensively used in the future. Seepweed *Suaeda salsa*, a kind of perennial secreting halophyte, which was widely distributed in saline areas of China. Young seedling of the plant could be used for vegetable and the seeds contain about 25 % of oil[2]. The *S. salsa* oil could be used to produce biodiesel[3]. However, there is no report on the butanol production from the seepweed *S. salsa*. In this study, *S. salsa* was used as substrate for butanol production using *Clostridium acetobutylicum* with simultaneous saccharification and fermentation and the medium for butanol production also optimized.

EXPERIMENTAL

Strain and culture: Clostridium acetobutylicum S-159 used for the experiments was obtained from the collection of our laboratory. The details of culture were: pretreated dried seepweed 20 g, mixed with cellulase 15 FPIU/g seepweed and nutrition solution (yeast extract 1 g/L, KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, $CaCO_3$ 2 g/L, ammonium acetate 2.2 g/L, *p*-aminobenzoic acid 0.001 g/L, vitamin B1 0.001 g/L, biotin 0.00001 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, $MnSO_4{\cdot}7H_2O$ 0.01 g/L, NaCl 0.01 g/L, $FeSO_4{\cdot}7H_2O$ 0.01 g/L inoculum volume of 10 % (v/v) and the medium volume was 50/250 mL flask, incubated at 37 ºC under stable fermentation. For the seed culture, the seepweed was replaced by 60 g/L of glucose and incubated at 37 ºC for 24 h.

Seepweed pretreatment: Seepweed was collected from Cangzhou City, Hebei Province. After harvest, the seepweed was dried at 80 ºC, chopped (average length was of 0.8-1.0 mm). Then 10 g of the seepweed powder was placed in the 250 mL Erlenmeyer flask and 4 % of NaOH solution was added, treated at 30 ºC for 24 h, then neutralized with 5 mol/L H_2SO_4 solution. After filtration, the seepweed was washed several times and dried at 80 ºC for further use.

Analysis: Crude protein, cellulose, nitrogen free extract, ash, fat and moisture content were analyzed according to the methods described in the literature[4]. The total cellulase activity (Filter Paper Unit, FPU) was measured by the standard filter paper assay[5]. The filter paper enzyme activity (FPA) was expressed as FPIU/mL. One International Unit (IU) of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of product per min at 50 ºC. The fermentation products

(ethanol, butanol and acetone) were analyzed as described by Qureshi *et al.*[6].

Design-expert (Version 7.0, STAT-EASE Inc., Minneapolis, USA) was used for experimental designs and statistical analysis of the data in this study. The analysis of variance (ANOVA) was used for the statistical parameters estimation.

RESULTS AND DISCUSSION

Main nutrient ingredients of seepweed (SW): The cellulose in seepweed was more than 20 % of the dry mass and the crude protein content was higher than corn stover or wheat straw (generally lower than 5 % of dry mass)[7]. So seepweed was a kind of excellent material for butanol fermentation. The main nutrient ingredients of the dried halophyte *S. salsa* are given in Table-1.

TABLE-1
MAIN NUTRIENT INGREDIENTS OF THE DRIED HALOPHYTE *Suaeda salsa* (%)

Moisture content	Crude protein	Crude cellulose	Crude fat	Nitrogen free extract	Crude ash
4.6	8.7	20.7	1.9	44.3	19.8

Optimization of the butanol production with pretreated seepweed

Single factor optimization: In the previous study, three main factors (seepweed, cellulase and $CaCO_3$) were found essential for butanol production and single factor optimization was conducted and the results were shown in Figs. 1-3. As shown in Fig. 1, it could be concluded that the amount of seepweed in the medium influence the butanol production significantly ($p < 0.05$). When the seepweed level was 30 g/L, the butanol yield could reach 2.7 g/L and then the production growth rate slow down. So 30 g/L of seepweed was selected for further study. Cellulase is very important for seepweed hydrolyzating. From Fig. 2, it could be found that with the cellulase activity increased, the production of butanol was also increased. Considering the costs of cellulase, 25 FPIU/g seepweed (2.9 g/L of butanol) was selected for further optimization. $CaCO_3$ is usually used as buffer in the butanol production and it could regulation the pH of broth during the fermentation period and its influence on the butanol production was shown in Fig. 3. It could be concluded that the optimal $CaCO_3$ level was 5 g/L.

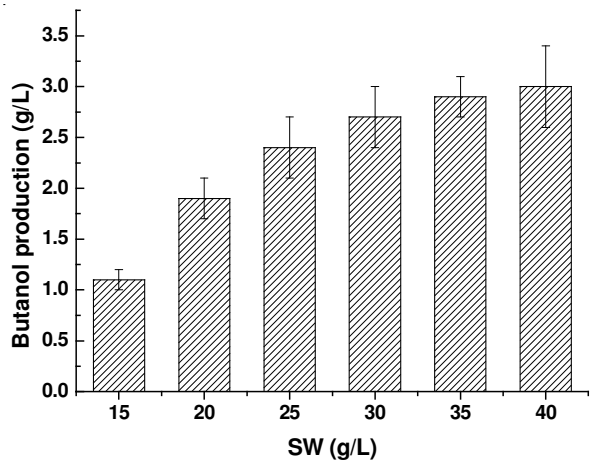

Fig. 1. Effect of seepweed on the butanol production

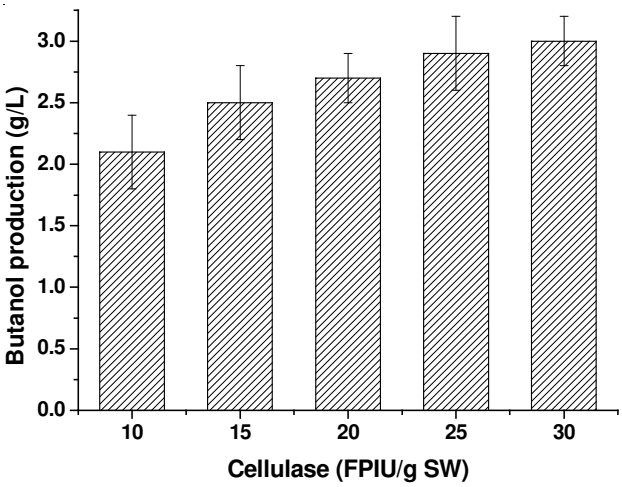

Fig. 2. Effect of cellulase dosages on the butanol production

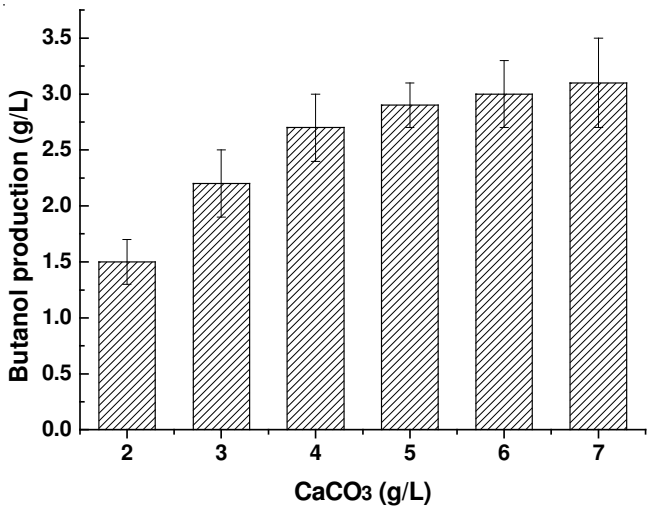

Fig. 3. Effect of $CaCO_3$ on the butanol production

Box-Behnken design and response surface methodology: The three factors (seepweed, cellulase and $CaCO_3$) were further optimized by Box-Behnken design and response surface methodology analysis. The experimental design and results were listed in Table-2.

The experimental results were analyzed by ANOVA (Table-3) and central composite design was fitted with the second-order polynomial model:

$$Y = 3.20 + 0.49A + 0.025B + 0.062C - 0.30AB - 0.18AC + 0.50BC - 0.34A^2 - 0.71B^2 - 0.49C^2$$

where Y is the response factor (butanol production, g/L) and A, B and C represent seepweed, cellulase and $CaCO_3$, respectively. The F-value of 11.63 implies the model is significant. There is only a 0.74 % chance that a "Model F-value" this large could occur due to noise. The fit of the model was estimated by the coefficient of determination R^2, which was calculated to be 0.9544, indicating that about 95 % of the variability in the experiments could be explained by the model. It was considered as very high correlation[8] when the R^2-value was higher than 0.9. The statistical significance of the model equation was evaluated by the F-test for ANOVA. The *p*-value was also very low (0.0074) indicating the significance of the model. The coefficient of variation (CV) indicates the degree of

TABLE-2
EXPERIMENTAL DESIGN AND RESULTS OF BOX-BEHNKEN DESIGN

Run No.	Variables			Observed butanol production (g/L)	Predicted butanol production (g/L)
	A (SW, g/L)	B (cellulase, FPIU/g SW)	C ($CaCO_3$, g/L)		
1	1(35)	1(30)	0(5)	2.5 ± 0.2	2.4
2	1(35)	-1(20)	0(5)	3.1 ± 0.1	2.9
3	1(35)	0(25)	1(6)	2.5 ± 0.3	2.7
4	1(35)	0(25)	-1(4)	2.9 ± 0.4	3.0
5	-1(25)	1(30)	0(5)	1.8 ± 0.1	2.0
6	-1(25)	-1(20)	0(5)	1.2 ± 0.2	1.3
7	-1(25)	0(25)	1(6)	2.2 ± 0.1	2.1
8	-1(25)	0(25)	-1(4)	1.9 ± 0.1	1.6
9	0(30)	1(30)	1(6)	2.7 ± 0.3	2.6
10	0(30)	1(30)	-1(4)	1.4 ± 0.1	1.5
11	0(30)	-1(20)	1(6)	1.6 ± 0.1	1.5
12	0(30)	-1(20)	-1(4)	2.3 ± 0.3	2.4
13	0(30)	0(25)	0(5)	3.2 ± 0.2	3.2
14	0(30)	0(25)	0(5)	3.3 ± 0.2	3.2
15	0(30)	0(25)	0(5)	3.1 ± 0.3	3.2

TABLE-3
ANALYSIS OF VARIANCE AND REGRESSION ANALYSIS FOR THE CELLULASE PRODUCTION

Source	Sum of squares	Degree of freedom	Mean square	F-value	P > F
Model	6.23	9	0.69	11.63	0.0074
Residual	0.30	5	0.059	–	–
Lack of fit	0.28	3	0.092	–	–
Pure error	0.020	2	0.01	–	0.0991
Corrected total	6.52	14	–	–	–

precision with which the treatments are compared. A lower CV means a higher reliability of the experiment. The relatively lower value of CV (10.25 %) demonstrated the performed experiments were reliable. The lack of fit *p*-value of 0.0991 implied the lack of fit is not significant relative to the pure error.

The response surface curves are plotted to explain the interaction of the variables and to determine the optimum level of each variable for maximum response. The response surface contour curves are shown in Figs. 4-6. The model predicted the optimal values (coded) of the variables were A = 0.89, B = -0.25, C = -0.22 and the values of seepweed, cellulase and $CaCO_3$ were 34.5, 23.7 and 4.8 g/L, respectively. The predicted butanol production was 3.4 g/L. In order to confirm the optimized culture conditions, three additional experiments in the Erlenmeyer flasks were performed using the predicted medium composition. The mean value of the butanol production was 3.5 g/L, which agree well with the predicted value. This result demonstrates the validity of the response model.

Conclusion

In this study, seepweed was first used as the main carbon source for the butanol production. Three effective factors such as seepweed, cellulase and $CaCO_3$ were optimized by one-factor-at-a-time approach and Box-Behnken design with response surface analysis and the composition of optimized medium was: pretreated dried seepweed 34.5 g (0.8-1.0 mm in length, pretreated by 4 % NaOH), mixed with cellulase 23.7 FPIU/g seepweed and nutrition solution (yeast extract 1.0 g/L,

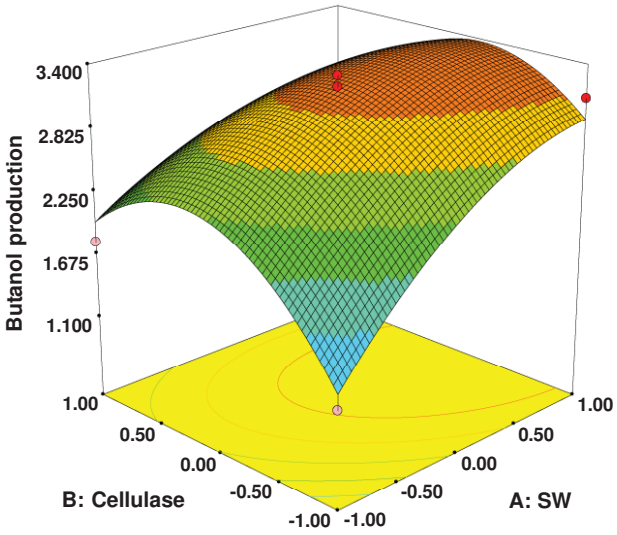

Fig. 4. Response surface curve for butanol production showing the interaction between seepweed and cellulase

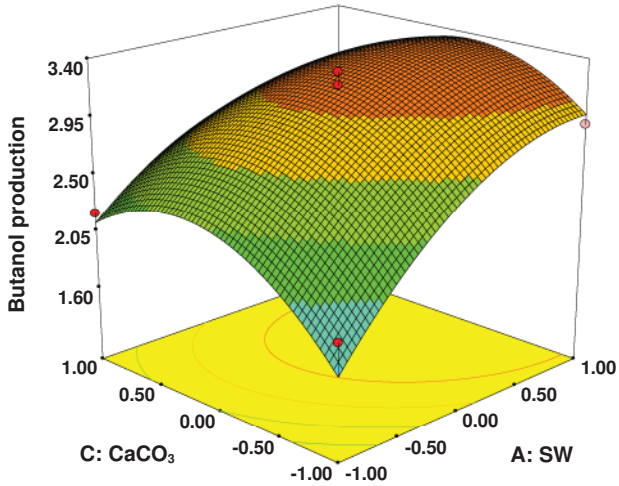

Fig. 5. Response surface curve for butanol production showing the interaction between seepweed and $CaCO_3$

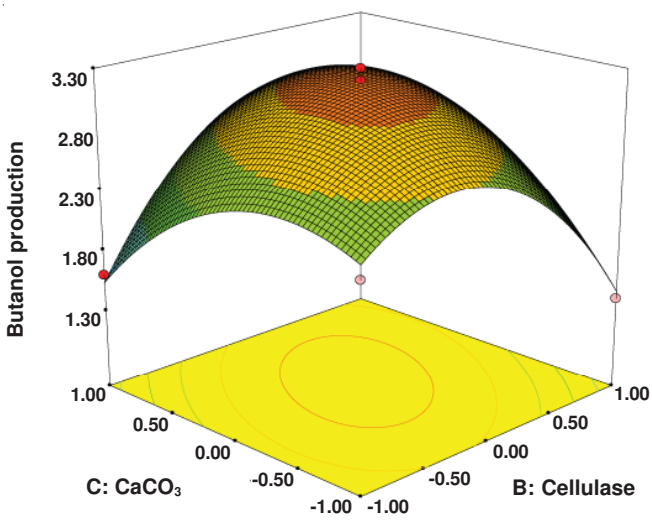

Fig. 6. Response surface curve for butanol production showing the interaction between $CaCO_3$ and cellulase

KH_2PO_4 0.5 g/L, K_2HPO_4 0.5 g/L, $CaCO_3$ 4.8 g/L, ammonium acetate 2.2 g/L, *p*-aminobenzoic acid 0.001 g/L, vitamin B1 0.001 g/L, biotin 0.00001 g/L, $MgSO_4 \cdot 7H_2O$ 0.2 g/L, $MnSO_4 \cdot 7H_2O$ 0.01g/L, NaCl 0.01 g/L, $FeSO_4 \cdot 7H_2O$ 0.01 g/L inoculum volume of 10 % (v/v) and the medium volume was 50/250 mL flask, incubated at 37 ºC under stable fermentation for 72 h. Under the optimized conditions, the butanol production could reach 3.5 g/L butanol.

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