Bio-flocculation Detoxification Treatment with Acid Hydrolyzate of Corn Straw for Ethanol Production

W. Zhang,[a,*] X. Zhang,[a] L. Bao,[b] and G. Wei[c]
[a]Key Laboratory of Applied Chemistry,
Yanshan University, Qinhuangdao 066004, China
[b]Institute of Forensic Science of Ministry of Public Security P. R. C.,
Beijing 100038, China
[c]School of Materials Science and Engineering,
Beijing University of Chemical Technology, Beijing 100029, China

Original scientific paper
Received: January 6, 2012
Accepted: June 4, 2012

Lignocelluloses materials were selected for ethanol production, but certain problems occurred, such as the hydrolysis and detoxification process. A biological flocculation technology was to apply for the detoxification of acid hydrolyzate of lignocelluloses materials. Meanwhile chitosan was confirmed as the best biological flocculant which showed good detoxification effect. In order to reach the optimal detoxification effect, the Box-Behnken experimental design was carried out. The most important parameters affecting the efficiency of detoxification are dosage, temperature, reaction time, and pH of the solution. The optimal parameters were: flocculant dosage of 0.25 g L^{-1}, pH of 4, reaction time of 10 minutes, and reaction temperature of 41°C. The calculated detoxification rate was 78.2 %. Through three confirmation experiments, the practical detoxification rate was 78 %, which was close to the theoretical prediction. This model had good reliability and reproducibility.

Key words:
Lignocellulose materials, detoxification process, acid-soluble lignin, ethanol production, biological flocculation, chitosan

1. Introduction

Ethanol as a kind of clean and renewable bio-fuel to lessen the fossil fuels consumption has gained great attention in recent years.[1] Lignocelluloses (crop residues,[2] cellulose wastes,[3] municipal solid wastes[4] and so on) as the raw materials for ethanol fermentation can help avoid the food crisis and larger quantities are acquirable, permitting large-scale production.[5]

The lignocellulose material used in this experiment was waste corn straw. In China, a large amount of waste corn straw is discarded. One way for changing wastes into valuables is ethanol production from waste corn straw. However, the process was more complex than ethanol production from starch materials, and some problems such as the detoxification of hydrolysate should be solved. The process of lignocellulose materials to ethanol contains these steps: pre-treatment of lignocellulose, hydrolysis of lignocellulose, detoxification of hydrolysate, fermentation, distillation, and dehydration.

Lignocelluloses can be hydrolyzed by acids and enzymes. Acid hydrolysis is a common and fast method for producing sugars from lignocelluloses.[6–8] A number of by-products are formed during acid hydrolysis, which inhibit the fermentation process.[9,10] Pentoses can be degraded to furfural under high temperature, and the decomposition of hexoses can form 5-hydroxymethylfurfural (5-HMF).[11,12] Acetate is liberated from hemicellulose hydrolysis.[11,12] Lignin can be partially dissolved by acid to form aromatic substances.[13,14] Such acid-soluble lignin substances have the unique peaks under UV irradiation.

Furfural and other volatile substances can be removed with heat,[15] and acid substances are easy to remove by neutralization. During the hydrolyzate concentrated and thermal process, the volatile furfural was removed, but dissolved aromatic compounds (acid-soluble lignin) were difficult to remove.[16] The aromatic substances generated from lignin have a serious impact on the subsequent fermentation process.[17] At the same time, the removal of these substances is much more difficult. Preliminary experiments in our laboratory showed that these materials had a stronger inhibitory effect on fermentation than other materials.[18] Corn straw has high lignin content, so the detoxification process for the removal of acid-soluble lignin is more important.

*Corresponding author. Tel: +86 0335 8387746; E-mail address: gillianjay@126.com (Wen Zhang)

The detoxification methods of lignocelluloses hydrolysate including biological, physical, chemical and combined treatments have been reported in numerous literatures. To be specific, activated charcoal treatment,[19] alkaline treatment,[20,21] thermal filtration,[15] biological degradation[22] and ion exchange[23] were investigated. Activated charcoal treatment was proved effective for detoxification because activated charcoal could remove the lignin degradation substance from the liquid phase by adsorption.[16] Sometimes this method was combined with heat treatment for the coinstantaneous removal of furfural. However, the use of powdered activated carbon is likely to cause dust pollution and unsafe application.[24] In general, it is important to identify the main inhibitors present in the lignocelluloses hydrolyzate before choosing a suitable detoxification method or a sequence of different methods. The cost of detoxification treatment needs to be controlled. At the same time, the optimal detoxification method should be not only cheap but also suitable for the hydrolyzate.

As known, bio-flocculants are harmless to the environment and humans, indicating their potential in the replacement of existing chemical flocculants. Bio-flocculants have been used in detoxification treatment.[25] The goal of this research was to apply a biological flocculation technology for the detoxification of acid hydrolyzate, which could decrease the concentration of inhibitors caused by hydrolysis. Biological flocculant such as chitosan has strong absorption effect on chemicals like activated carbon adsorption.[26] In Saeed's (2011) work,[27] the flocculation concept was employed to recover the dissolved lignocellulosic materials of industrially produced pre-hydrolysis liquor (PHL) using two different molecular weights of chitosan. The dose of flocculant for the detoxification process is usually small, so the reducing sugar losses are less. The main purpose of detoxification in the experiment was to remove the lignin degradation products. The detoxification effect in this article was represented by the concentration variation of acid-soluble lignin.

On the other hand, Tripathi *et al.*[28] used the design of experiments (DOE) methodology to study the optimization adsorption parameters. DOE methodology can be applicable for the research of the effect of different factors, their interactions on detoxification performance and formulation characteristics. The Box-Behnken design is one of the most efficient and widely used DOE methods.[29] Box-Behnken design is a kind of second-order experimental design method based on three levels and it can evaluate the non-linear relationship between indicators and factors. The optimal value of the response can be found in the optimal region and the model of optimized area can also be obtained through the Box-Behnken design. Through the Box-Behnken design, it is easy to avoid the experiments performed under extreme conditions from which unsatisfactory results are often obtained.[30] This paper describes experiments designed to indicate interactions between four factors (dose, pH, temperature, action time) with respect to their influences on the detoxification rate by Box-Behnken methodology.

In this research, the acid hydrolysate after the optimal detoxification process treatment was used for the yeast adaptation test in order to determine whether the detoxification process was effective. Meanwhile, the most probable detoxification mechanism of CTS was also discussed.

2. Materials and methods

2.1 Acid hydrolyzates

The hydrolyzate used in the experiments was produced from waste corn straw (The corn straw was obtained form a local farm). First, the corn straw was cut into 1cm × 2 cm segment, and pulverized to obtain a cotton-like form. The raw material was then mixed with 78.5 % sulfuric acid at the solid-liquid ratio of 1:2, at temperature of 45–50 °C, and reaction time of 15 minutes. Thereupon, the mixture was diluted to form 4.7 % sulfuric acid solution. This reaction lasted 2 hours at boiling condition (100 °C). Finally, the precipitate was removed and the acid hydrolyzates were formed. The filtrate temperature was 65 °C. The acid hydrolysate was boiled for 15 minutes to remove the volatile compounds and the lost water was complemented.

2.2 The preparation of bio-flocculants

Chitosan (CTS) flocculant was prepared by adding chitosan to 10 g L^{-1} acetic acid solution to form chitosan solution (10 mg mL^{-1}). Iron-Chitosan (Fe-CTS) Flocculant was made by adding $FeCl_3$ to the chitosan solution at a Fe/CTS mass ratio of 2, and then dropping dilute NaOH solution in case of slow agitation after the solid completely dissolved, forming a solution with a certain pH value. PASP (molecular formula: $C_4H_6NO_3(C_4H_5NO_3)_mC_4H_6NO_4$; molecular mass: 1000–5000; pH (1 % aqueous solution) = 10–11) was prepared by Beijing University of Chemical Technology.[25]

2.3 Detoxification of acid hydrolyzate

A certain amount of flocculant was added into a conical flask containing the acid hydrolyzates, and treated by NaOH in order to obtain appropriate

pH, and then the flask was placed in the water bath (stirred at 100 rpm for 10–30 minutes). After the reaction, the solution was left to stand for 25 minutes. Finally, the solution was filtered through filter paper or filter cloth (pore size = 0.12 mm). The concentration of acid-soluble lignin in the solution was measured and the detoxification rate was calculated as

$$\text{DR } \% = \frac{C_1 - C_2}{C_1} \cdot 100\ \% \qquad (1)$$

where DR % is the detoxification rate, C_1 is the concentration of acid-soluble lignin in the acid hydrolyzates before detoxification, and C_2 is the concentration of acid-soluble lignin after detoxification.

During the detoxification process, a small amount of reducing sugar was absorbed. The loss rate of reducing sugar was calculated as

$$\text{LR } \% = \frac{S_1 - S_2}{S_1} \cdot 100\ \% \qquad (2)$$

where LR % is the loss rate, S_1 is the concentration of reducing sugar in the acid hydrolyzates before detoxification, and S_2 is the concentration of reducing sugar after detoxification.

2.4 Box-Behnken experimental design

Under different flocculant dosage, temperature, reaction time and pH, the value of the detoxification rate was measured. The best flocculant was chosen by single factor experiment. Then the Box-Behnken experimental design of the best flocculant was carried out.

In the present study, the Box-Behnken experimental design is applied to investigate and validate adsorption process parameters affecting the removal of acid-soluble lignin. Flocculant dose (A: 0.02–0.25 g L^{-1}), pH (B: 0–4), reaction time (C: 10–30 min), and temperature (D: 20–60 °C) are variable input parameters.

2.5 Culture media and microorganism culture

P. tannophilus 1770, which can ferment xylose and glucose, was used for ethanol fermentation.[18] The strain was stored in a tube culture at 4 °C. The Slant culture medium consisted of xylose 20 g L^{-1}, $(NH_4)_2SO_4$ 5 g L^{-1}, peptone 5 g L^{-1}, strain extract 5 g L^{-1}, KH_2PO_4 2 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 1 g L^{-1}, urea 2.5 g L^{-1}, and agar 12 g L^{-1}.

The *P. tannophilus* after activation was inoculated in the liquid culture medium in aseptic condition, and carried out under the temperature of 32 °C at 120 rpm for 48 hours to form the suspended cells liquid. The liquid medium consisted of xylose 20 g L^{-1}, $(NH_4)_2SO_4$ 5 g L^{-1}, peptone 5 g L^{-1}, strain extract 5 g L^{-1}, KH_2PO_4 2 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 2 g L^{-1} and urea 2.5 g L^{-1}.

The fermentation medium consisted of hydrolyzates, $(NH_4)_2SO_4$ 5 g L^{-1}, strain extract 2 g L^{-1}, KH_2PO_4 2 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 2 g L^{-1}.

2.6 Analytical methods

The reducing sugars were analyzed by the DNS method.[31] The reaction between 3,5-dinitrosalicylic acid and reducing sugars under heat can form reddish brown amide within a certain range, and the color depth of the reaction mixture is matched with the amount of reducing sugar. Thus, the colorimetric method could be applied to measure the sugar content of the sample. The pH was measured in a pH meter (PHS-3B, Shanghai Precision& Scientific Instrument Co. Ltd, China). The optical density (OD) of cell growth at regular intervals was determined at 600 nm using a UV-VIS Spectrophotometer (TU-1901, Purkinje General Instrument Co. Ltd, China) throughout the study. The acid-soluble lignin was measured by ultraviolet absorption.[16] The absorbance values of peaks are measured and the concentration of acid-soluble lignin can be calculated.

3 Results and discussion

3.1 The detoxification process with different flocculants

Many flocculants like aluminum salts, represented by poly-aluminum chloride (PAC), have been widely used in industrial fields.[32] Meanwhile, literature reports that these synthetic flocculants were not easily biodegraded in the natural environment.[33] In contrast, bio-flocculants are harmless to the environment and humans, indicating their potential to replace the existing synthetic flocculants. Flocculants always have a selective absorption effect on the soluble substances and the hazardous materials are removed under this absorption effect. Different flocculants were employed for various purposes according to their chemical characteristics and the types of toxins in the solutions.

In this research, four kinds of flocculants were chosen for the detoxification process including PASP, PAC, CTS and Fe-CTS. All these flocculants are bio-flocculants except PAC. The detoxification process was carried out under same conditions (pH = 2.5; temperature = 25 °C; reaction time = 10 min) for contrast. As shown in Table 1, the species and dosage of flocculants had great effect on the detoxification rate and the loss rate of sugars.

Table 1 – *Detoxification effect with different flocculants*

PASP/ g L^{-1}	DR/%	LR/%	PAC/ g L^{-1}	DR/%	LR/%	CTS/ g L^{-1}	DR/%	LR/%	Fe-CTS/ g L^{-1}	DR/%	LR/%
0.99	10	0	1.08	27.9	0	0.02	48.56	0	0.02	44.06	0
2.02	13.38	0	2.07	28.5	0	0.05	49.41	0	0.05	46.7	0
3	19.6	4.3	3.03	35.79	3.05	0.12	50.53	0.8	0.12	47	0
4.05	41.8	13.2	4.08	50.65	4.87	0.20	64.52	1.4	0.20	51.7	1.7
5.1	49.37	16.52	4.99	38.7	7.69	0.25	63.87	3.4	0.25	56.9	3.2
6.2	40.7	25.92	6.1	35.56	10.87	0.30	63.76	7.3	0.30	55.4	7.2

By increasing the flocculant concentration, the detoxification effect was strengthened because the probability of adsorption in bridge, and electrical neutralization was improved. At the same time, the adsorption of reducing sugar was increased accordingly. Among the four kinds of selective flocculants, CTS showed the best detoxification rate and the smallest adsorption of reducing sugar (the minimum dosage). In general, greater flocculant dosages lead to stronger absorption effect of reducing sugar. The common flocculant PAC did not show an ideal effect. The dosage of PASP and PAC was large (4 g L^{-1}–5 g L^{-1}) in order to attain an optimal detoxification rate, and when the dosage was small, there was no significant adsorption effect, but the small dosage of CTS and Fe-CTS (0.25 g L^{-1}) was already resulting in better adsorption. CTS and Fe-CTS had a stronger adsorption effect on the acid-soluble lignin. The possible reason was the larger number of active groups for adsorption in the CTS molecules, so the adsorption of acid-soluble lignin was better than PAC and PASP. CTS was chosen for the detoxification process and the dosage of CTS was less than 0.25 g L^{-1} to diminish the loss of reducing sugars.

3.2 Box-Behnken analysis

In order to achieve the optimum detoxification effect, the Box-Behnken experimental design was carried out in this research. According to preliminary experiments in our laboratory, the most important parameters affecting the efficiency of detoxification by CTS, were dose, temperature, reaction time, and pH of the solution. Experiments were carried out in different combinations of the parameters to analyze the combined effect of these four factors using statistically designed experiments. The range of the parameters is given in the section of Materials and Method. Twenty-nine experiments were employed in this work in order to evaluate the effects of the four main independent parameters on the acid-soluble lignin removal efficiency. A non-linear regression method was used to fit the second order polynomial to the experimental data and identify the relevant model terms. Considering all the linear terms, square terms and linear-by-linear interaction items, the quadratic response model can be described as:

$$R = \beta_0 + \sum \beta_i x_i + \sum \beta_{ii} x_{ii}^2 + \sum \beta_{ij} x_i x_j \quad (3)$$

where β_0 is the offset term, β_i is the slope or linear effect of the input factor x_i, β_{ii} is the quadratic effect of input factor x_i and β_{ij} is the linear by linear interaction effect between the input factor x_i and x_j.[33]

The results of the R (response) of detoxification rate were measured and the measured responses are listed in Table 2.

Box-Behnken experimental design model supplied a second order polynomial equation to reflect the relationship with the detoxification rate (R) and four parameters (A, B, C, and D) according to the experimental results. The final equation obtained in terms of actual factors is given below:

$$\begin{aligned} R = {} & 35.38940 + 11.82583 \cdot A - 4.81088 \cdot B - \\ & - 0.43206 \cdot C + 0.86272 \cdot D + 23.41250 \cdot A \cdot B - \\ & - 5.78250 \cdot A \cdot C - 0.95625 \cdot A \cdot D - \\ & - 0.24237 \cdot B \cdot C + 0.084688 \cdot B \cdot D + \\ & + 0.025413 \cdot C \cdot D + 417.19167 \cdot A^2 + \\ & + 0.72298 \cdot B^2 + 0.017044 \cdot C^2 - 0.014939 \cdot D^2 \end{aligned} \quad (4)$$

where R is the DR (detoxification rate %), A is the flocculant dose in the detoxification process (A: 0.02–0.25 g L^{-1}), B is the pH value of the detoxification process (B: 0–4), C is the reaction time (C: 10–30 min), and D is the temperature of the detoxification process (D: 20–60 °C).

For testing the hypothesis on the parameters of the Model, ANOVA is a statistical technique that subdivides the total variation in a set of data into component parts associated with specific sources of variation.[34] ANOVA for second-order equation in this model is presented in Table 3.

Table 2 – *Box-Behnken experimental design and results of detoxification rate*

Run	Std	A/Flocculant dose	B/pH	C/Reaction time	D/Temperature	R1/DR
26	1	0.15	2	20	40	50.5
15	2	0.15	0	30	40	61.71
25	3	0.15	2	20	40	47.5
6	4	0.15	2	30	20	35.87
28	5	0.15	2	20	40	52.94
10	6	0.25	2	20	20	48.48
2	7	0.25	0	20	40	50.8
9	8	0.05	2	20	20	42.2
27	9	0.15	2	20	40	51.95
11	10	0.05	2	20	60	52.87
14	11	0.15	4	10	40	58.31
16	12	0.15	4	30	40	49.85
12	13	0.25	2	20	60	51.5
3	14	0.05	4	20	40	54.64
4	15	0.25	4	20	40	72.22
22	16	0.15	4	20	20	33.64
17	17	0.05	2	10	40	47.48
29	18	0.15	2	20	40	46.87
13	19	0.15	0	10	40	50.78
7	20	0.15	2	10	60	46.1
23	21	0.15	0	20	60	51.32
8	22	0.15	2	30	60	54.02
18	23	0.25	2	10	40	66.32
21	24	0.15	0	20	20	48.71
5	25	0.15	2	10	20	48.28
20	26	0.25	2	30	40	50.61
1	27	0.05	0	20	40	51.95
24	28	0.15	4	20	60	49.8
19	29	0.05	2	30	40	54.9

According to the rule of ANOVA, a large value of F indicates that most of the variation in the response can be explained by the regression equation, and probability values (p values) less than 0.05 are considered to be statistically significant.[35] The result of variance analysis indicated that the second order polynomial equation adequately represented the actual relationship between the response (detoxification rate) and the parameters (dosage, pH, reaction time and temperature). At the same time, the F value for this model was large. The Model F-value of 4.39 implied the model was significant. There was only 0.45 % chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicated that the model terms were significant. In this case A, D, AC, CD, A^2, D^2 were significant model terms. Values greater than 0.1000 indicated that the model terms were non-significant. The "Lack of Fit F-value" of 3.72 implies the Lack of Fit was not significant relative to the pure error. There was a 10.84 % chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit was good. Adeq Precision measures the signal to noise

Table 3 – *ANOVA for response surface quadratic model*

Source	Sum of Squares	df	Mean Square	*F* Value	*p*-value Prob > *F*	Remark
Model	1299.32	14	92.81	4.39	0.0045	significant
A-A	107.34	1	107.34	5.07	0.0409	significant
B-B	0.85	1	0.85	0.04	0.8442	not significant
C-C	8.86	1	8.86	0.42	0.528	not significant
D-D	195.46	1	195.46	9.24	0.0088	significant
AB	87.7	1	87.7	4.15	0.0611	not significant
AC	133.75	1	133.75	6.32	0.0248	significant
AD	14.63	1	14.63	0.69	0.4196	not significant
BC	93.99	1	93.99	4.44	0.0535	not significant
BD	45.9	1	45.9	2.17	0.1629	not significant
CD	103.33	1	103.33	4.88	0.0443	significant
A2	112.9	1	112.9	5.34	0.0366	significant
B2	54.25	1	54.25	2.56	0.1316	not significant
C2	18.84	1	18.84	0.89	0.3613	not significant
D2	231.62	1	231.62	10.95	0.0052	significant
Residual	296.15	14	21.15			
Lack of Fit	267.42	10	26.74	3.72	0.1084	not significant
Pure Error	28.73	4	7.18			
Cor Total	1595.47	28				

ratio. A ratio greater than 4 was desirable. The ratio of 8.996 in this model indicated an adequate signal. This model can be used to navigate the design space.

3.3 Effect of various parameters on detoxification rate

According to the Box-Behnken experimental design and the results of detoxification rate, the variational effect of different factors on detoxification rate is plotted in Fig. 1. For example, in Fig. 1a, the points mean the DR value in four same experimental conditions (flocculant dose: 0.15 g L^{-1}; pH: 2; reaction time: 20 min; temperature: 40 °C). With the increase of bio-flocculant dose, the detoxification rate was rising also, but in order to diminish the loss of reducing sugars the dosage of CTS should be less than 0.25 g L^{-1}. The detoxification rate did not change significantly by the progressively increased reaction time and pH of the solution. The optimal temperature was 40 °C.

With the increasing temperature, the probability of inter-collision among colloidal particles, toxic impurities molecules, and CTS molecules was improved, and the flocculation was accelerated with more precipitation formation.[36] However, when the water bath temperature was too high, the stability of flocculant decreased. In this research, the acid hydrolyzate was strongly acidic and in order to avoid the large consumption of alkali, the pH was controlled from 0 to 4. The detoxification rate did not change significantly in this range. Under acidic conditions, the chitosan molecules and hydrogen ions combined to form cations, and easily formed adsorption bridging, so the impurities gathered and settled down. In our experiment, pH of 4 was preferable considering the proceeding fermentation process (fermentation pH of 4.0–4.3). In a short time, the reaction reached equilibrium, so the prolongation of reaction time did not improve the detoxification effect.

The single-factor test described the relationship between a single factor and the detoxification rate. In order to study the performance of various factors on the detoxification effect, the interaction of multiple factors on the detoxification effect was analyzed.

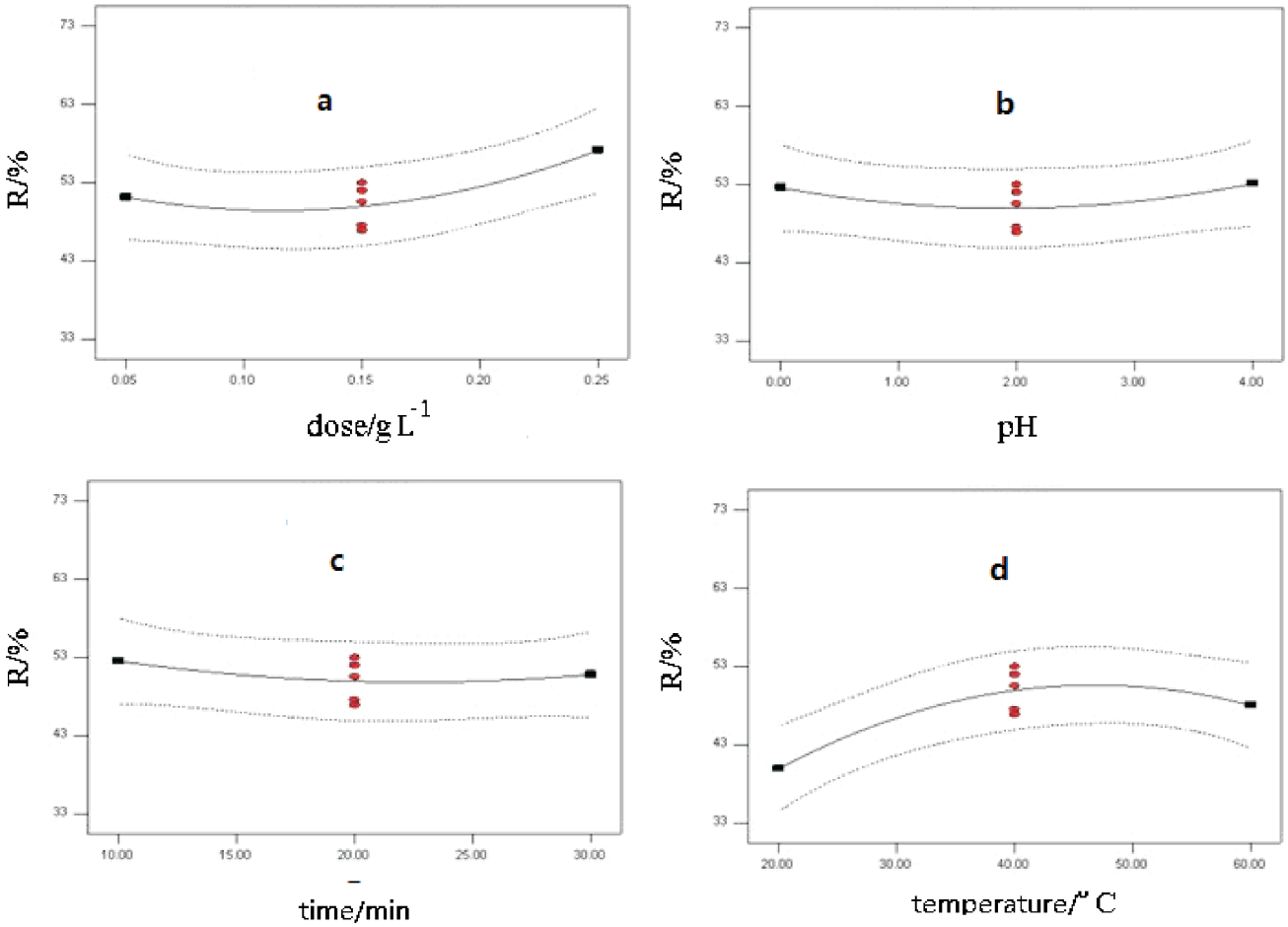

Fig. 1 – *Effect of different factors on the detoxification rate*

3.4 Effect of bio-flocculant dosage (A) and reaction time (C)

To study the interaction effect of bio-flocculant dose (A) and reaction time (C) on detoxification, experiments were carried out by varying bio-flocculant dose from 0.02 to 0.25 g L^{-1} under different reaction time from 10 to 30 minutes. The result is shown in Fig. 2 through Box-Behnken experimental design. At fixed C (for example: 10 minutes), the detoxification rate improved with an increase in bioflocculant dosage (A) for the adsorption was increased with higher concentration of flocculant, but when the C was prolonged (for example: 30 min), the detoxification rate decreased slowly to a certain limit with the increasing bioflocculant dosage (A) and then it remained almost constant; at the same time, the effect of A on the detoxification rate was not obvious. During the long contact and stirring time, the flocculating constituent was easily destabilized and this phenomenon occurred especially when the concentration of flocculant was high (the prepared flocculating particle was large).

Fig. 2 also shows that at particular A (for example: 0.05 g L^{-1}), the detoxification rate increased with an increase in C, because a certain contact time was demanded in the flocculation reaction. However, when the A was increased (for example: 0.25 g L^{-1}), the detoxification rate decreased with

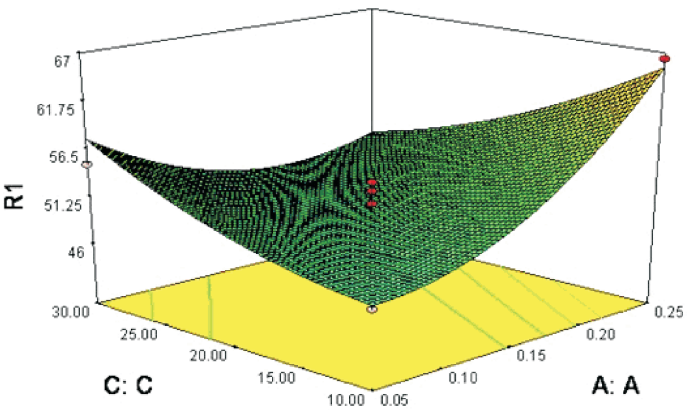

Fig. 2 – *3D response surface graph for bioflocculant dosage (A) and reaction time (C) for bio-flocculation detoxification system*

an increase in C, because the forming floccules were damaged with overlong stirring time.

3.5 Effect of pH (B) and reaction temperature (D)

To study the effect of pH and reaction temperature on toxicant removal, experiments were carried out by varying pH from 0 to 4, and *T* from 20 to 60 °C. The results are plotted in Fig. 3. This figure clearly shows that at any fixed pH, the toxicant removal increased with an increase in reaction temperature up to a certain limit, and then it remained almost constant or slightly decreased. An increase in detoxification rate with temperature can be attributed to the enhancement of intraparticle transport pore diffusion. The adsorption reaction with CTS and the toxicant is an exothermic process generally, so the temperature is not too high for this reaction.

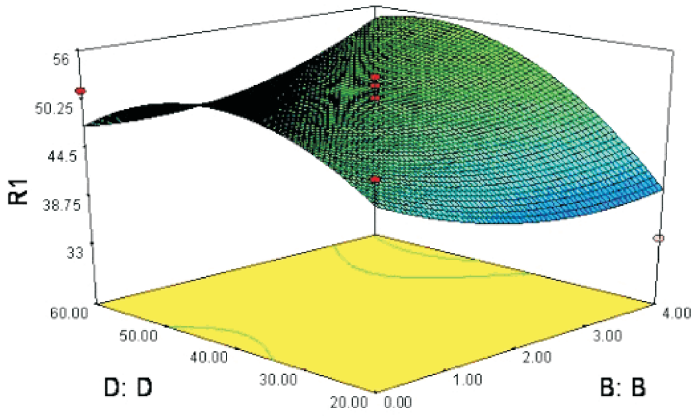

Fig. 3 – *3D response surface graph for pH (B) and reaction temperature (D) for bio-flocculation detoxification system*

Fig. 3 also shows that at particular D (reaction temperature), with an increase in B (pH), the detoxification rate did not change obviously. The detoxification process proceeded in acidic conditions all along. However, when the reaction temperature was increased, the pH of the reaction solution should have been increased, because CTS was partially dissolved in the low pH solution and the loss of bio-flocculant would cause a decrease in detoxification rate. Figure 3 could prove this conclusion. At fixed D (for example 60 °C), the detoxification rate improved with an increase in B (reaction pH).

3.6 Selection of optimal experimental conditions and optimum detoxification effect

The aim of this work is to remove a maximum amount of acid-soluble lignin with lowest possible bio-flocculant. Through the optimization design of the Design Expert 7.0, the optimal experimental conditions were obtained and the theoretical detoxification rate was calculated. The optimal parameters were: flocculant dosage of 0.25 g L^{-1}, pH of 4, reaction time of 10 minutes and reaction temperature of 41 °C. The calculated detoxification rate was 78.2 %. In three confirmation experiments, the average detoxification rate was 78 %,which was close to the theoretical prediction. This model had good reliability and reproducibility. It was also showed that the Box-Behnken model was suitable to optimize the experiments for the detoxification process.

The influence of flocculants on cell growth was considered in this experiment. The fermentation medium was synthesized from the detoxified hydrolyzate of which the reducing sugar content was 27.42 g L^{-1}. The *P. tannophilus* was inoculated in the fermentation medium. The growth curves of the yeast in different detoxified hydrolyzates are shown in Fig. 4. The cells concentration was represented by OD600 and the fermentation medium was diluted 15 times. The growth of *S. cerevisiae* was similar with *P. tannophilus*. As shown in Fig. 4, A represented the growth curve of *P. tannophilus* in the hydrolysate detoxified by CTS, and B represented the growth curve of *P. tannophilus* in the hydrolysate by the common liming detoxification treatment. The result showed that the adaptability of *P. tannophilus* in the hydrolyzate detoxified by CTS was better and after 18 hours the *P. tannophilus* went into a stable phase. The growth rate of *P. tannophilus* in the hydrolyzate detoxified by CTS was faster. In addition, the hydrolyzate detoxified

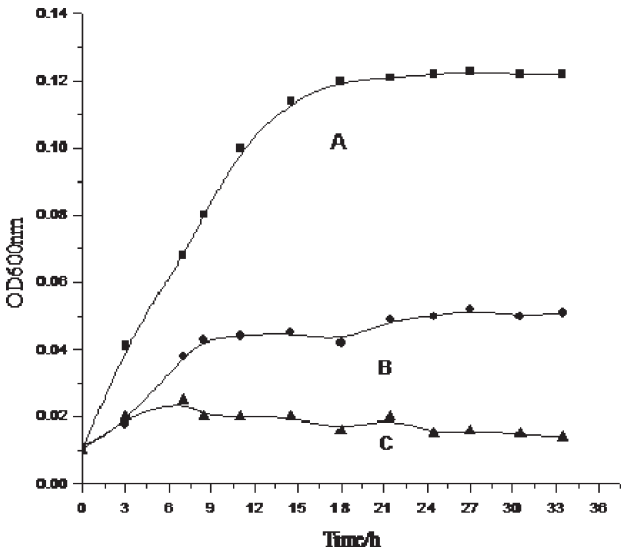

Fig. 4 – *Growth curve of P. tannophilus (A represented the growth curve of P. tannophilus in the hydrolyzate detoxified by CTS; B represented the growth curve of P. tannophilus in the hydrolyzate by the common liming detoxification treatment, and C represented the growth curve of P. tannophilus with no detoxification)*

by bio-flocculation treatment can reach the growth requirement of *P. tannophilus*. It was also showed that the bio-flocculation detoxification process was feasible.

3.7 The detoxification mechanism and ultraviolet spectrum analysis

Lignin consists of a phenol structural unit and with stable polymer structure. The acid-soluble lignin composition is very complex due to the lignin structure is very complicated. The acid-soluble lignin contained *p*-hydroxybenzoic acid, vanillic acid, *p*-hydroxybenzaldehyde, vanillin, syringaldehyde and other substances.[37] These substances are inclusive of the hydroxyphenyl structural unit (phenol). During the process of acid hydrolysis, the lignin macromolecules were decreased to small molecules and did not change its basic structure. There are many phenyl and C = O double bonds in the structure of acid-soluble lignin, and according to the UV absorption spectra, benzene and characteristic absorption were at 205 nm and 280 nm. Furfural structure also contains a large number of C = O double bonds, and the absorption peak at 280 nm is from C = O double bonds. Therefore, if we choose the 280 nm as the characteristic peak, there would be an impact on the amount of measurement results. On the basis of its characteristic absorption spectrum, we can use the UV detector to measure acid-soluble lignin content at 205 nm. The UV spectrum figures of hydrolysate before and after detoxification are shown in Fig. 5. The hydrolyzate was diluted firstly to avoid the interaction of different composition under too high concentration.

As shown in Fig. 5, A represented the UV spectrum of hydrolyzate before detoxification, and B represented the UV spectrum after detoxification. After detoxification, the absorption peaks were weakened significantly, and the detoxification process reached a good effect. This conclusion was similar with Saeed (2011).[27] In their research, CTS can remove the dissolved lignocellulosic materials and furfural.

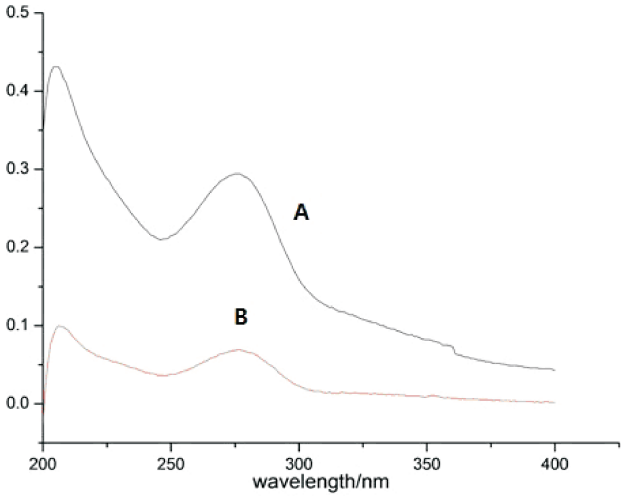

Fig. 5 – *UV spectrum of hydrolyzate before and after detoxification*

According to the results of the experiment, the most probable detoxification pathway of CTS for the removal of acid-soluble lignin was the adsorption between the chitosan molecules and the acid-soluble lignin. As we know, there are a large number of amino active groups in the chitosan molecules. In acidic conditions, the $–NH_3^+$ was formed from amido and then the hydrogen bonds effect was generated between phenolic hydroxyl (or hydroxyl) and $–NH_3^+$. Complex macromolecular structures were produced, and eventually the compound was precipitated. PASP was prepared by polycondensation of amido and carboxyl from aspartic acid, and the number of amino active groups was less than CTS in the molecules. That is why the detoxification effect of PASP was poorer than CTS. This mechanism can also explain the conclusion of part 3.1 in this manuscript. At the same time, this research also provided a new method for the removal of phenolic compounds in solution.

Another most commonly used technology for the hydrolysis of lignocellulose is enzymatic hydrolysis following diluted-acid pretreatment. The hydrolyzate after pretreatment also needs detoxification. In this case, CTS might also work to remove toxins from hydrolyzate after the diluted acid pretreatment.

4. Conclusions

In this research, a biological flocculation technology was applied for the detoxification of acid hydrolyzate, which could decrease the concentration of inhibitors caused by hydrolysis. The main purpose of detoxification in the experiment was to remove the lignin degradation products. The detoxification effect in this article was represented by the concentration variation of acid-soluble lignin.

CTS was chosen as the bio-flocculant. In order to reach the optimal detoxification effect, the Box-Behnken experimental design was carried out. The most important parameters affecting the efficiency of detoxification by CTS, are dose, temperature, reaction time and pH of the solution. The optimal parameters were: flocculant dosage of 0.25 g L^{-1}, pH of 4, reaction time of 10 minutes, and reaction temperature of 41 °C. The calculated detoxification rate was 78.2 %. In the three confirmation experiments, the average detoxification rate

was 78 %, which was close to the theoretical prediction. The adaptability of *P. tannophilus* in the detoxification hydrolysate was good and after 18 hours the *P. tannophilus* went in stationary phase.

According to the results of the experiment, the most probable detoxification pathway of CTS for the removal of acid-soluble lignin was the adsorption between the chitosan molecules and the acid-soluble lignin. In acidic conditions, the $-NH_3^+$ was formed from amido and then the hydrogen bonds effect was generated between phenolic hydroxyl (or hydroxyl) and $-NH_3^+$. Complex macromolecular structures were produced; eventually the compound was precipitated.

References

1. *Mohana, S., Acharya, B. K., Madamwar, D.,* J. Hazard. Mater. **163** (2009) 12.
2. *Lynd, L. R., Weimer, P. J., van Zyl, W. H., Pretorious, I. S.,* Microbiol. Mol. Biol. Rev. **66** (2002) 506.
3. *Sun, Y., Cheng, J.,* Bioresour. Technol. **83** (2002) 1.
4. *Sánchez, Ó. J., Cardona, C. A.,* Bioresour. Technol. **99** (2008) 5270.
5. *Lou, Y.*, Renewable Energy **5** (1994) 866.
6. *Khiyami, M. A., Pometto III, A. L., Brown, R., C.,* J. Agric. Food. Chem. **53** (2005) 2978.
7. *Kadam, K. L., Forrest, L. H., Jacobson, W. A.,* Biomass Bioenergy **18** (2000) 369.
8. *Nigam, J. N.,* J. Biotechnol. **87** (2001) 17.
9. *Hendriks, A. T. W. M., Zeeman, G.,* Bioresour. Technol. **100** (2009) 10.
10. *Purwadi, R., Taherzadeh, M. J.,* Bioresour. Technol. **99** (2008) 2226.
11. *Larsson, S., Palmqvist, E., Hahn-Hägerdal, B., Tengborg, C., Stenberg, K., Zacchi, G., Nilvebran, N.,* Microb. Technol. **24** (1999) 151.
12. *Heer, D., Sauer, U.,* Microb. Biotechnol. **1** (2008) 497.
13. *Delgenes, J. P., Moletta, R., Navarro, J. M.,* Enzyme Microb. Technol. **19** (1996) 220.
14. *Mussatto, S. I., Roberto, I. C.,* Bioresour. Technol. **93** (2004) 1.
15. *Nigam, J. N.,* J. Ind. Microbiol. Biotechnol. **26** (2001) 145.
16. *Peng, F., Zhao, X., Liu, C., Liu, D.,* Renewable Energy Resources **27** (2009) 32.
17. *Palmqvist, E., Hahn-Hägerdal, B.,* Bioresour. Technol. **74** (2000) 25.
18. *Zhang, W., Wei, G.,* Energy Source: Part A. **34** (2012) 1178.
19. *Miyafuji, H., Danner, H., Neureiter, M., Thomasser, C., Bvochora, J., Szolar, O.,* Enzyme Microb. Technol. **32** (2003) 396.
20. *Eken-Saraçoğlu, N., Arslan, Y.,* Biotechnol. Lett. **22** (2000) 855.
21. *Yang, T., Ma, M.,* China Brewing **8** (2006) 11.
22. *Roberto, I. C., Lacis, L. S., Barbosa, M. F. S.,* Process Biochem. **26** (1991) 15.
23. *Larsson, S., Nilverbrant, N. O., Jonsson, L. J., Reimann, A.,* Appl. Biochem. Biotechnol. **77/79** (1999) 91.
24. *Jin, Y., Wang, X., Gao, W.,* Journal of Beijing Polytechnic University **23** (1997) 74.
25. *Zhang, W., Xiong, R., Wei, G.,* J. Hazard. Mater. **172** (2009) 1252.
26. *Huang, Z., Sun, X., Li, Y., Ge, W., Wang, J.,* Spectroscopy and Spectral Analysis **25** (2005) 698.
27. *Saeed, A., Fatehi, P., Ni, Y.,* Carbohyd polym. **86** (2011) 1630.
28. *Tripathi, P., Srivastava, V. C., Kumar, A.,* Desalination **249** (2009) 1273.
29. *Ferreira, S. L. C., Bruns, R. E., Ferreira, H. S., Matos, G. D., David, J. M., Brandão, G. C., da Silva, E. G. P., Portugal, L. A., dos Reis, P. S., Souza, A. S., dos Santos, W. N. L.,* Anal. Chim. Acta **597** (2007) 179.
30. *Guo, C., Stine, K. J., Kauffman, J. F., Doub, W. H.,* Eur. J. Pharm. Sci. **35** (2008) 417.
31. *Miller, L. G.,* Anal. Chem. **31** (1959) 4226.
32. *Jang, J. H., Ike, M., Kim, S. M., Fujita, M.,* Biotechnol. Lett. **23** (2001) 593.
33. *Dearfield, K. L., Albernathy, C. O., Ottley, M. S., Brantner, J. H., Hayes, P. F.,* Mut. Res. **195** (1988) 45.
34. *Huiping, L. Guoqun, Z. Shanting, N. Yiguo, L.,* Comput. Mater. Sci. **38** (2007) 561.
35. *Segurola, J., Allen, N. S., Edge, M., Mahon, A. M.,* Prog. Org. Coat. **37** (1999) 23.
36. *Tian, G., Zhang, W., Wei, G., Xiong. R.,* Journal of Beijing University of Chemical Technology **35** (2008) 47.
37. *Tao, Y., Guan, Y.,* Journal of Cellulose Science and Technology **11** (2003) 42.