High Yield of Ethanol Production by the Strain *Fusarium* sp. ZW-21 with Corncob Hydrolysate

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The filamentous fungus strain *Fusarium* sp. ZW-21 was used for ethanol production with corncob hydrolysate. The fermentation conditions of ethanol production from corncob hydrolysate by the strain were investigated, and the effect of temperature, pH, nitrogen source, and surfactants on the ethanol production was investigated. The two factors yeast extract and polysorbate 80 were selected for further optimization by response surface methodology. The optimal conditions for ethanol production by the strain *Fusarium* sp. ZW-21 were 50 g/L sugar of corncob hydrolysate, 10.35 g/L yeast extract, 10 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.38 g/L polysorbate 80, pH 6.0, inoculum size of 1 mL/50 mL medium, and incubation temperature of 30 °C. The fermentation period was 5 d under oxygen-limited conditions, and the ethanol yield was 24.2 g/L

Keywords: Corncob hydrolysate; Fermentation; Optimization; Ethanol; Fusarium sp.

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INTRODUCTION

Biomass-based energy has attracted the world's interest with the growing concerns of the pollution associated with fossil fuel utilization in past years. Cellulosic wastes, such as straws, stovers, and corncob, have been developed for renewable energy and chemical production at present. As a type of important renewable biofuel, ethanol has attracted attention as an alternative to petroleum-derived fuel because ethanol has merits such as it is clean and reproducible from plant wastes. Ethanol is usually produced from starch, which is edible for mankind and limited by food production. Ethanol production from the inedible cellulosic biomass is necessary for effective utilization of these raw materials. Many kinds of lignocellulosic materials, such as wood, switch grass, rice straw, wheat straw, corn stover, and sugarcane bagasse, are used for ethanol production. However, the lignin in lignocellulosic materials makes cellulose and hemicellulose resistant to hydrolysis by cellulase. Furthermore, the hydrolysate of the cellulosic materials also contains xylose, arabinose, and other substances that can inhibit yeast fermentation.

The hydrolysate of lignocellulose always contains some pentoses, such as xylose and arabinose, except for glucose (Olsson and Hahn-Hägerdal 1996), and these pentoses cannot be fermented by *Saccharomyces cerevisiae*, which is usually used for ethanol production. Thus, it is important to find other microorganisms to produce ethanol from pentoses. Some species of yeasts (*Pachysolen tannophilus*, *Pichia stipitis*), fungi (*Aspergillus*, *Rhizopus*, *Monilia*, *Neurospora*, *Fusarium*, *Trichoderma*, and *Mucor*) have been found to produce ethanol from biomass (Christakopoulos *et al.* 1989; Skory *et al.* 1997; Stevenson and Weimer 2002; Zhang *et al.* 2003; Millati *et al.* 2005; Sues *et al.* 2005;

Okamoto *et al.* 2011). It has been reported that *Fusarium* can produce anthraquinones (Baker and Tatum 1998) and convert xylitol from xylose (Liu *et al.* 2008), cutinase (Tatiana and Gabriela 2007), and polycyclic aromatic hydrocarbons removal (Jacques *et al.* 2008). As one kind of agricultural phytopathogen, *Fusarium* spp. was also found to produce ethanol (Anasontzis and Christakopoulos 2014; Wang *et al.* 2018). However, the yield of ethanol by *Fusarium* is relatively low, usually lower than 15 g/L. Yeasts are usually used for ethanol production; however, most of the yeasts cannot fully utilize the sugars xylose or arabinose in the lignocellulosic hydrolysate. In order to fully utilize the total sugars (glucose, xylose, and arabinose) in the lignocellulosic hydrolysate, novel strains should also be isolated for ethanol production. Christakopoulos *et al.* reported that the *Fusarium oxysporum* F3 could produce ethanol directly from glucose, xylose, cellobiose, and cellulose, and the ethanol concentrations could reach 14.5 g/L at 34 ℃ for 6 d (Christakopoulos *et al.* 1989). Corncob hydrolysate (mainly including glucose and xylose) was a good carbon source for ethanol production. In this work, the strain *Fusarium* sp. ZW-21 was used for ethanol production from corncob hydrolysate, and the conditions were investigated and optimized.

EXPERIMENTAL

Materials

Microorganism

Fusarium sp. ZW-21 (stored in the authors' lab in Jinan, China), was stored at 4 °C on a potato dextrose agar (PDA, containing potato extract of 20% (w/v), glucose of 20 g/L, and agar of 20 g/L) slant, and sub-cultured every three months.

Corncob hydrolysate preparation

The corncob hydrolysate was prepared from corncob with cellulase in the laboratory, and the steps can be described as follows. 1) Pretreatment: The corncob was ground, the powder was sieved, and particles sized ≤ 0.5 mm were collected. 2) HCl treatment: The pretreated materials were treated with 6% HCl at 90 °C for 1 h, then washed with water and adjusted to pH 4.8 to 5.0. 3) Cellulase hydrolysis: The powder of corncob was hydrolyzed with complex cellulase (20 Filter Paper International Unit (FPIU)/g dry mass, solid-liquid ratio was 1 : 20 at 50 °C for 48 h, and the corncob hydrolysate was obtained and concentrated by a rotary evaporator. The main sugars, such as glucose and xylose, in the hydrolysate were determined by high-performance liquid chromatography (Agilent 1100 system; Agilent Technologies, Santa Clara, CA, USA; equipped with an Aminex HPX-87P analytical column, 300 × 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA). The final concentrations of the hydrolysate used in this study were adjusted (concentrated or diluted) for the experiments in this study.

Culture

Mycelia of the strain *Fusarium* sp. ZW-21 on the PDA slant were transferred to a PDA plate and incubated at 30 ℃ for 3 d, and then the mycelia were incubated in a 250-mL Erlenmeyer flask that contained 50 mL of basal fermentation medium. The medium contained 50 g/L total sugar, 10 g/L $(NH_4)_2SO_4$, 10 g/L KH_2PO_4, and 0.5 g/L $MgSO_4 \cdot 7H_2O$, and pH 5.0).

The strain grew in 28 °C for 48 h on a reciprocal shaker at 150 rpm. Then, 1 mL seeds of the strain ZW-21 was transferred to the Erlenmeyer flasks containing 50 mL of different mediums used in the experiments below. The mouths of the flasks were sealed with plastic films for ethanol production under oxygen-limited conditions at 28 °C for 5 d.

Analytical Methods

Sugar (glucose, xylose, and arabinose) concentration was determined with a high performance liquid chromatography system (Agilent 1100, Agilent Technologies, Palo Alto, CA, USA), and the ethanol concentration was measured with a SBA-40E Biosensor (Biology Institute of Shandong Academy of Sciences, Jinan, China).

Effect of fermentation conditions on the ethanol production

The various fermentation parameters were estimated for ethanol production as follows: temperature (24, 26, 28, 30, 32, 32, 34, and 36 °C), pH (4, 5, 6, 7, 8, and 9), and the fermentation period was 5 d. The effect of different compositions in the fermentation medium on the ethanol production will be discussed in the results and discussion.

Effect of the different nitrogen sources

Yeast extract (Beijing Aoboxing Bio-tech Co., Ltd., Beijing, China), soybean meal (Shandong Jinhongchang Chemical Technology Co., Ltd., Jinan, China), corn steep (Henan Xinyang Industrial Co., Ltd., Hebi, China), $(NH_4)_2SO_4$, urea, ammonium tartrate, $NaNO_3$ and KNO_3 (these five kinds of reagents were obtained from Sinopharm Chemical Reagent Co., Ltd., Beijing, China) were used for ethanol fermentation, and the final concentration was 10 g/L.

Effect of different surfactants

Tween 20 (polysorbate 20), Tween 80 (polysorbate 80), Triton X-100 (t-octylphenoxypolyethoxyethanol), Triton X-114 ((1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol), and sodium dodecyl sulfate (SDS) were obtained from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China) and used for evaluated for ethanol production. The final concentration was 0.4 g/L.

Response surface methodology

Based on the results of the effect of nitrogen sources and surfactants conducted in the former experiments, yeast extract and polysorbate 80 were the most suitable substances for ethanol production for the strain *Fusarium* sp. ZW-21, and these two factors were further optimized by response surface methodology (RSM) for improving ethanol production.

Central composite design (CCD) was used as the experimental design for the combination of yeast extract and polysorbate 80, in addition a quadratic model was used for fitting the data obtained from the tests, and then analysis of variance (ANOVA) was performed and the parameters including R^2 value, pure error, residual error, and the lack-of-fit were calculated to evaluate the model in the experiment. The software Design Expert (version 7.0, Statease Inc., Minneapolis, MN, USA) was used for the experimental design and regression analysis.

RESULTS AND DISCUSSION

Concentration of the Sugars in the Corncob Hydrolysate

Three kinds of sugars, glucose, xylose, and arabinose, were determined, and the concentrations are shown in Table 1. The table shows that the glucose was the main sugar (approximately 60.6%), and the pentose, such as xylose and arabinose, was approximately 39.4% of the total sugars.

Table 1. Sugars in the Corncob Hydrolysate

Material	Glucose (g/L)	Xylose (g/L)	Total (g/L)
Corncob	203	132	335

Effect of the Temperature on the Ethanol Production

The influence of temperature on the ethanol production with corncob hydrolysate by the strain ZW-21 is shown in Fig. 1.

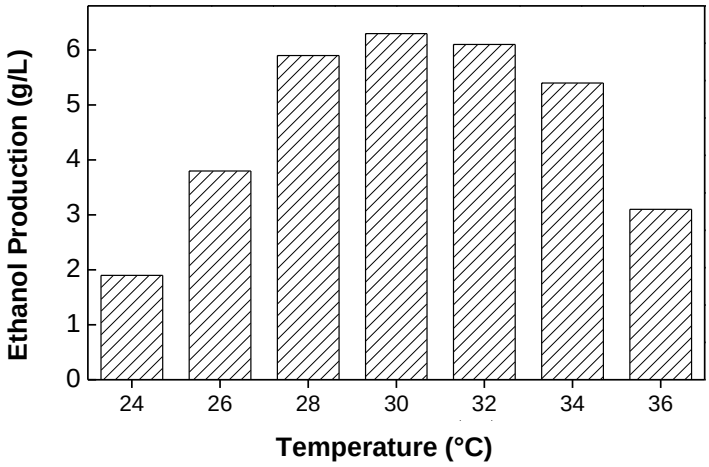

Fig. 1. Effect of the incubation temperature on the ethanol production by the strain ZW-21

It could be found that in the temperature range 24 to 36 °C, the production of ethanol increased at 24 to 30 °C and decreased rapidly at 32 to 34 °C, and the highest yield appeared at 30 °C (6.3 g/L), which was chosen for further investigation. The ethanol production might have relation to the biomass of the strain ZW-21, and the highest mycelia were observed at 30 °C, and so did the ethanol yield.

Effect of the Initial pH on the Ethanol Production

In this study, deferent pH values (4 to 9) were investigated and the results are shown in Fig. 2. It was found that pH values affected the ethanol production significantly ($P < 0.05$), and the highest ethanol yield (6.8 g/L) was reached at a pH of 6.0 when corncob hydrolysate was used as the carbon source. It was considered that under the optimal pH condition, the strain ZW-21 grew well, and more ethanol was obtained than other pH conditions.

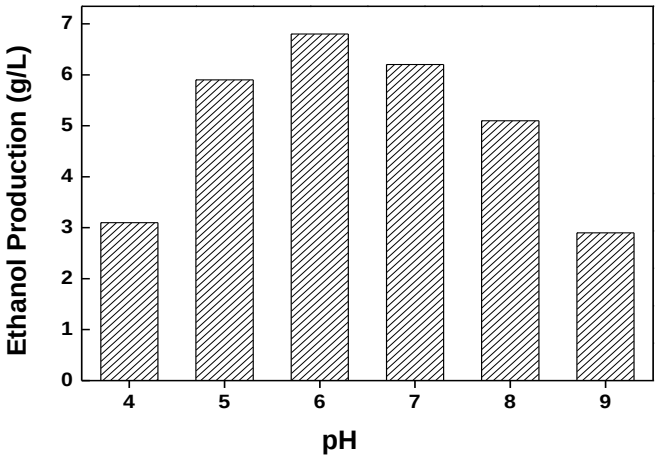

Fig. 2. Effect of pH values on the ethanol production by the strain ZW-21

Effect of the Different Nitrogen Sources on the Ethanol Production

Different nitrogen sources were used for ethanol production by the strain *Fusarium* sp. ZW-21 and the results are shown in Fig. 3.

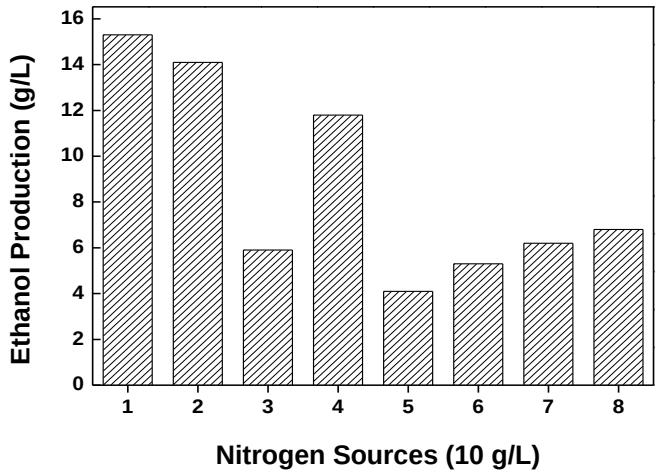

Fig. 3. Effect of the nitrogen sources on the ethanol production by the strain ZW-21: 1. Yeast extract; 2. Soybean meal; 3. $(NH_4)_2SO_4$; 4. Corn steep; 5. Urea; 6. Ammonium titrate; 7. $NaNO_3$; 8. KNO_3

It was found that the yeast extract was the optimal nitrogen source for ethanol production in this study, and the ethanol yield reached 15.3 g/L. The strain ZW-21 could produce more ethanol with the organic nitrogen sources, such as yeast extract, soybean meal, and corn steep, than the inorganic nitrogen sources such as $(NH_4)_2SO_4$, $NaNO_3$, KNO_3, or ammonium titrate. However, urea, which was considered as the organic nitrogen source, showed low ethanol production in this experiment (4.1 g/L). The organic nitrogen source such as yeast extract, soybean meal and corn steep could give more biomass of mycelia, and more ethanol production was obtained.

Effects of Different Surfactants on the Ethanol Production

The effects of different surfactants (including polysorbate 20, polysorbate 80, t-octylphenoxypolyethoxyethanol, (1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol and SDS) are shown in Fig. 4. Nonionic surfactants, such as polysorbate 20, polysorbate 80, t-octylphenoxypolyethoxyethanol, (1,1,3,3-tetramethylbutyl)phenyl-polyethylene glycol, improved the yield of ethanol during the fermentation in the experiment. However, SDS, a type of ionic surface surfactant, inhibited the growth of the strain ZW-21, and the ethanol production was also low. It was found that in the preparation of cellulosic ethanol, the addition of nonionic surfactant (Borjesson *et al.* 2007; Zhou *et al.* 2010; Seo *et al.* 2011), and the mechanism of these substances on cellulose should be elaborated from diffusion and absorption of cellulase on the lignocellulosic materials. In this study, it was also found that the nonionic surfactant could promote ethanol yield of the strain ZW-21 from hydrolysate, and the highest production reached 7.7 g/L. This was improved by these agents because of the permeability of the cell membrane, and the ethanol could be secreted easily, and nonionic surfactants used in this study was not significantly affected the mycelia production, while SDS could inhibit the growth of the strain, lower yield of mycelia were observed, and also inhibited the production of ethanol.

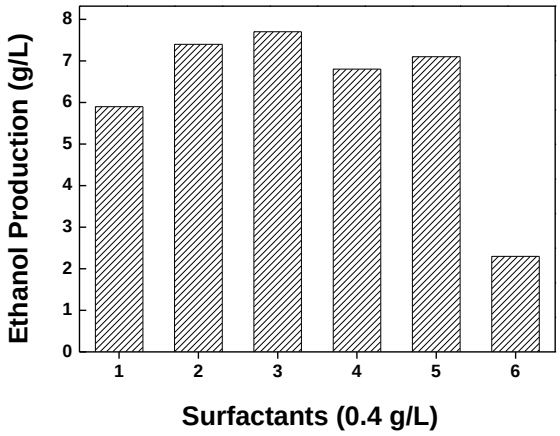

Fig. 4. Effect of different surfactants on the ethanol production by the strain ZW-21: 1. Control Check (CK); 2. Polysorbate 20; 3. Polysorbate 80; 4. (1,1,3,3-Tetramethylbutyl)phenyl-polyethylene glycol; 5. t-Octylphenoxypolyethoxyethanol; 6. SDS

Optimization of the Surfactants Addition and Yeast Extract for the Ethanol Production by RSM

In the former experiments, yeast extract and polysorbate 80 were found as the optimal nitrogen source and surfactant, respectively, for the ethanol production. In order to work out the best combination of these two factors, RSM was used for obtaining the point. The experimental design and results are listed in Table 2.

According to the results of the central composite design, the final estimated response model equation is,

$$Y = 23.58 + 0.98X_1 - 0.70X_2 - 2.46X_1^2 - 2.51X_2^2 + 1.18X_1X_2 \quad (1)$$

where Y is the response factor (ethanol production, g/L), and X_1, X_2 represent the two independent factors, such as yeast extract (g/L) and polysorbate 80 (g/L), respectively.

Table 2. Experimental Design and Results of the Central Composite Design

Run No	Yeast Extract (X_1) (g/L)	Polysorbate 80 (X_2) (g/L)	Ethanol Production (Y)(g/L)
1	8 (-1)	0.2 (-1)	18.9
2	12 (1)	0.2 (-1)	18.6
3	8 (-1)	0.6 (1)	16.2
4	12 (1)	0.6 (1)	20.6
5	10 (0)	0.4 (0)	23.5
6	10 (0)	0.4 (0)	23.8
7	10 (0)	0.4 (0)	23.3
8	10 (0)	0.2 (-1)	22.9
9	10 (0)	0.6 (1)	19.4
10	8 (-1)	0.4 (0)	20.3
11	12 (1)	0.4 (0)	22.1

The data in the brackets are the coded factor levels

The ANOVA analysis results are shown in Table 3 and Table 4. In the table, the fit of the model was checked by the coefficient of determination R^2, which was 0.9422, indicating that 94.22% of the variability in the response could be explained by this model.

Table 3. ANOVA Analysis of the Regression Model

Source	Sum of Square	Degrees of Freedom	Mean Square	F-value	P > F
Model	56.950	5	11.390	16.309	0.0041
Error	3.492	5	0.698		
Lack of Fit	3.365	3	1.122	17.712	0.0539
Pure Error	0.127	2	0.063		
Corrected Total	60.440	10			

R^2 = 0.9422; R^2_{adj} = 0.8845; coefficient of variation (CV) % = 4.00

Table 4. ANOVA Analysis of the Variables

Term	Estimate	Std Error	t Ratio	P > | t |
Intercept	23.58	0.43	55.01	＜0.0001
X_1	0.98	0.34	2.88	0.0345
X_2	-0.70	0.34	-2.05	0.0954
X_1X_2	1.18	0.41	2.81	0.0375
X_1^2	-2.46	0.53	-4.69	0.0054
X_2^2	-2.51	0.53	-4.78	0.0050

The sum of square of the model, error, lack of fit, pure error and corrected total were 56.950, 3.492, 3.365, 0.127, and 60.440, respectively; and the mean square of the model, error, lack of fit, and pure error were 11.390, 0.698, 1.122, and 0.063, respectively. The F-value and P-value of the lack of fit were 17.712 and 0.0539, respectively. The statistical significance of the model equation was evaluated by the F-test for ANOVA. The

P-value was also very low (P < 0.0041), indicating the significance of the model. The lack-of-fit was insignificant at the 5% level. The linear and the quadratic items were significant. It could be concluded that the statistical results showed a good fit between the model and the experimental data.

The response surface curves were plotted to explain the theoretical combination of yeast extract and polysorbate 80 *vs.* ethanol production (Fig. 5). The optimum concentration of yeast extract and polysorbate 80 were 10.35 g/L and 0.38 g/L, respectively, and the predicted maximum ethanol production was 23.7 g/L. In order to confirm the optimized culture conditions, three additional experiments in the shake flask were performed using the predicted medium composition, and the mean value of the ethanol production was 24.2 ± 0.3 g/L, which agreed well with the predicted yield.

Fusarium sp. could produce ethanol directly from biomass or biomass hydroysate under anaerobic or microaerobic conditions. However, The yield of ethanol by *Fusarium* was usually no more than 15 g/L. Christakopoulos *et al*. (1989) reported that the *Fusarium oxysporum* F3 could produce ethanol directly from glucose, xylose, cellobiose, and cellulose, and the maximum ethanol concentration was 14.5 g/L at 34 ℃ for 6 d. de Almeida *et al*. (2013) found that *Fusarium verticillioides* were able to co-ferment glucose and xylose, and the ethanol production from 40g/L of pre-treated sugarcane bagasse was 4.6 g/L. Genetically engineered strains usually showed higher ethanol production from corncob hydrolysate. Sun *et al.* (2018) introduced the ethanol synthesis pathway and created an ethanologenic strain *E. coli* B0013-2012PA. In shaking flask fermentation, B0013-2012PA fermented glucose to ethanol with the yield of 48.4 g/100 g sugar while xylose remained in the broth. In this paper, Corncob hydrolysate was used for ethanol fermentation by the strain *Fusarium* sp. ZW-21 and the yield was more than 24 g/L in 5 d at 30 °C, which should be highest to date when the *Fusarium* sp. strains were used.

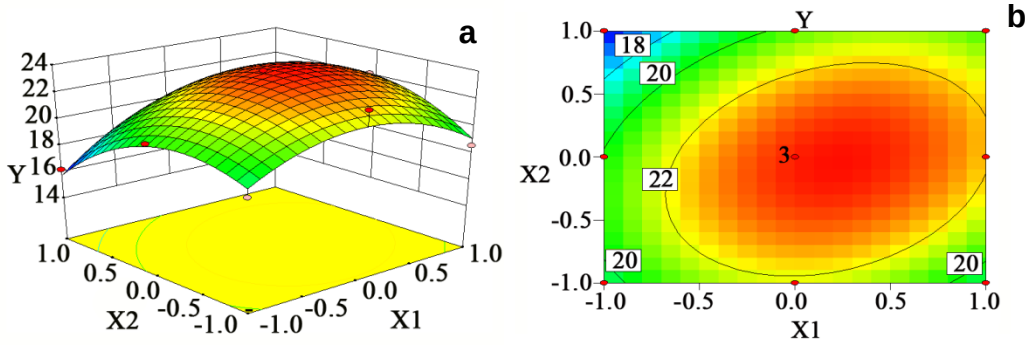

Fig. 5. Three dimensional graph and contour of the response surface plot: a. Three dimensional graph; b. Contour of the response surface

Time Course of the Ethanol Production

Fermentation time courses of the ethanol production under optimized and non-optimized conditions are given in Fig. 6. Both of the time courses showed a similar fermentation process trend. Ethanol production was low at first, and after a lag phase of approximately 24 h, ethanol production then gradually increased, and the ethanol yield reached maximum after 5 or 6 d of fermentation, and then decreased, which might have been attributed to the ethanol that was used as nutrient for the growth of the strain. Therefore, when the highest yield was reached, the fermentation should be stopped. The highest production of ethanol was 24.2 g/L in 5 d, which was much higher than the initial

medium that produced 5.9 g/L ethanol in 6 d. However, the production of ethanol by the strain ZW-21 was still low compared to that of yeasts, so mixing fermentation with yeasts and the strain ZW-21 should be investigated in future research.

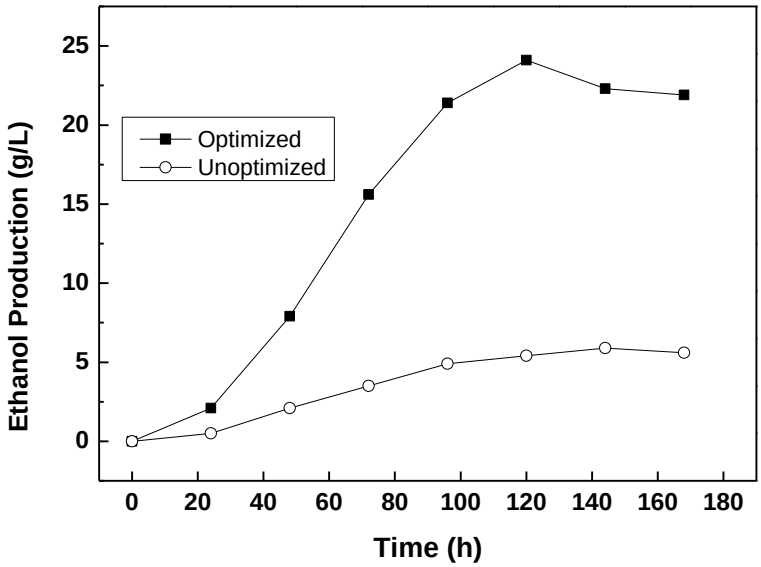

Fig. 6. Time course of the ethanol production by the strain ZW-21 using corncob hydrolysate

CONCLUSIONS

1. In this paper, the ethanol producing conditions of the strain *Fusarium* sp. ZW-21 of incubation temperature, pH, inoculum size, hydrolysate concentration, nitrogen source, and surfactant were investigated and optimized for ethanol production.

2. The optimum conditions were: 50 g/L sugar of corncob hydrolysate, 10.35 g/L yeast extract, 10 g/L KH_2PO_4 , 0.5 g/L $MgSO_4 \cdot 7H_2O$, 0.38 g/L polysorbate 80, pH 6.0, inoculum size of 1 mL/50 mL medium, and incubation temperature of 30 °C. The fermentation period was 5 d under oxygen-limited conditions, and the ethanol yield was 24.2 g/L.

3. The strain *Fusarium* sp. ZW-21 could produce ethanol from corncob hydrolysate, which was useful for biomass utilization. In the future work, a combined fermentation using both yeast and *Fusarium* sp. ZW-21 should be investigated.

ACKNOWLEDGMENTS

This work was financially supported by the National Key R&D Program of China (2018YFB1501403), Key R&D Project of Shandong Province (No. 2019LYXZ027) and Youth Science and Technology Innovation Team of Shandong Colleges and Universities (2019KJD002).

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Article submitted: March 4, 2020; Peer review completed: May 24, 2020; Revised version received and accepted: October 27, 2020; Published: November 30, 2020.
DOI: 10.15376/biores.16.1.622-632