

Simultaneous Saccharification and Fermentation of Corn Straw to Lactic Acid

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The utilization of corn straw to produce lactic acid could largely save the resource as well as decrease the amount of agricultural waste. In this study, orthogonal experimental design was adopted to investigate the optimum conditions for cellulase production from corn straw, and then the cellulase was further used to produce lactic acid through simultaneous saccharification and fermentation (SSF) process. $(\text{NH}_4)_2\text{SO}_4$ and Tween 80 were also used to test their effects on lactic acid production. The optimum conditions were determined as follows: solid-to-liquid ratio was $\zeta = 1 : 3$, pH of the substrate mixture was 4.5, inoculum size of *Trichoderma koningii* was $G = 12\%$ (v/m), fermentation time was $t = 84$ h. Corresponding maximum cellulase of $H = 2630.3 \text{ U g}^{-1}$ straw was achieved. With the produced cellulase added, the maximum lactic acid concentration of $\gamma = 20.2 \text{ g L}^{-1}$ could be achieved inoculated with lactic acid bacteria T50 at $\vartheta = 45^\circ\text{C}$. As compared with control (non-addition), addition of $w = 2\%$ $(\text{NH}_4)_2\text{SO}_4$ could increase the lactic acid by 3.6%, while addition of Tween 80 could shorten the fermentation time from $t = 108$ h to $t = 72$ h. The result indicated that utilization of corn straw as substrate to produce cellulase and lactic acid was applicable and could reduce pollution, thus this was worth investigating further.

Key words:

Cellulose, simultaneous saccharification and fermentation (SSF), lactic acid, corn straw, *Trichoderma koningii*

1. Introduction

Large quantities of agricultural and agro-industrial residues are generated from diverse agricultural and industrial practices. In China, corn straw residue is the most voluminous. It is reported that six million tons would be produced in China. Such a substrate has gained great attention due to its potential to produce valuable products such as lactic acid and ethanol.^{1–4} The conventional techniques for achieving these products include acid or enzyme hydrolysis of cellulose followed by fermentation of the resulting soluble sugars. Acid hydrolysis has high requirement for the reactor and would cause pollution, while the by-products generated during the process need appropriate treatment.^{5–8} Enzymatic hydrolysis of cellulose has the advantage of few by-products, but also the drawback of slow rate due to strong inhibition by glucose and cellobiose, and the large quantity of enzyme usage would enhance the cost.^{9–11} Simultaneous saccharification and fermentation (SSF) is a possible method to substantially decrease the glucose inhibition since the glucose could be utilized promptly during this process. Furthermore, separate reactors are replaced by a single reactor, which could

save the capital investment. In SSF, the cellulose first has to be hydrolyzed to glucose, then the various products can be obtained by subsequent fermentation. Therefore, if the microorganisms used for fermentation are compatible with the cellulase system, the SSF process could be more efficient.^{12–13} In this study, the saccharification of corn straw by the *Trichoderma koningii* cellulase enzyme system was carried out with fermentation of glucose formed by a thermophilic facultative anaerobe.

Lactic acid was chosen as the present product since it is one of the most important organic acids with a wide range of food-related and industrial applications, especially in the production of biodegradable plastics.^{14–16} The greatest obstacle in producing lactic acid at the industrial scale is in the high costs for substances and purification. Utilization of cellulosic substrates to lactic acid has been studied by several researchers,^{17–20} but most of them adopted the industrial cellulase which would increase the total cost. This study aimed to develop a fermentation process to produce cellulase and lactic acid at a lower cost by utilizing cellulosic substrates. The optimum conditions for enzyme production as well as the affecting factors for lactic acid production by SSF were determined in this study.

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2. Materials and methods

2.1 Materials

2.1.1 Substrates

Corn straw was obtained from the Department of Botany, Jia Mu Si University, Jia Mu Si City, China. The raw materials were milled to a particle diameter of $d_p = 0.5\text{--}1$ mm, then dipped in an ammonia liquor (8 %) for 24 h at normal temperature. The solid was recovered by filtration, and then washed repeatedly with tap water until neutral. Subsequently, they were dried at $\vartheta = 105$ °C to attain constant weight. The pretreated straw was used for enzyme fermentation and subsequent hydrolysis. The composition of pretreated straw was $w = 40.96$ % cellulose, $w = 28.57$ % hemicellulose and $w = 18.83$ % lignin.

2.1.2 Microorganism

Trichoderma koningii used in this study was obtained from the College of Plant Science, Jilin University, China. It was maintained at $\vartheta = 4$ °C on potato dextrose agar slants and sub-cultured every month. The microorganisms for inoculation were prepared at $\vartheta = 28$ °C for 4 d to produce spores. Then the spores were scraped and dissolved in a sterilized water solution to the desired concentration 10^6 mL⁻¹. The solution was utilized for fermentation with the mentioned inoculum size. Lactic acid bacteria T50 was selected in our laboratory, it was thermophilic facultative anaerobe. The cultures were maintained at $\vartheta = 4$ °C on slants MRS culture.

2.2 Experimental design

2.2.1 Cellulase production from corn straw using *Trichoderma koningii*

The cellulase production experiment was conducted in a $V = 500$ mL conical flask by using *Trichoderma koningii*. The conical flask was loaded with $m = 50$ g dry pretreated corn straw and citric acid-sodium citrate buffer. Every $V = 100$ mL citric acid-sodium citrate buffer contained $m = 2$ g (NH₄)₂SO₄, $m = 0.1$ g KH₂PO₄, $m = 0.05$ g MgSO₄ · 7H₂O. All the flasks were sterilized at $\vartheta = 121$ °C for $t = 15$ min. Then the *Trichoderma koningii* suspended solution made as mentioned under 2.1.2 was inoculated into the system. The orthogonal experiment was designed to investigate the influence of main parameters, namely the fermentation time, pH, inoculum size, solid-to-liquid ratio. Corresponding factors and levels are shown in Table 1.

Table 1 – Factors and level in the orthogonal experiment

Factor	Time, t/h	pH	Inoculum size, G/%	ξ_{SL}
(I)	72	4.0	4	1 : 2
(II)	84	4.5	8	1 : 3
(III)	96	5.0	12	1 : 4

2.2.2 Lactic production from corn straw by SSF

The SSF was carried out at different temperatures for $t = 6$ d in incubation chambers. The batch experiments were conducted in $V = 500$ mL flasks. Each conical flask was loaded with $m = 20$ g dry pretreated corn straw and $V = 200$ mL tap water, after sterilized at $\vartheta = 121$ °C for $t = 20$ min, the media was inoculated with lactic acid bacteria and wet cellulase yeast, and the inoculum size was $\varphi = 2.5$ % and 300 FPA g⁻¹ respectively. The pH was controlled by adding $m = 3$ g CaCO₃ in every flask.

2.3 Analytical methods

The sample was centrifuged at 3500 rpm for $t = 20$ min. The supernatant was used to measure the concentration of lactic acid, cellulase and reducing sugar. Reducing sugar was determined by the DNS method.²¹ The cellulase was determined according to the methods by Ghose (1987), one unit (U) of enzyme activity was defined as the amount of enzyme that releases $m = 1$ µg product per min. Sugar composition was determined by high performance liquid chromatography, with a NH₂ column (4.6 mm · 250 mm) and refractive index detector. Ethyl nitrile (ethyl nitrile: water $\psi_{E+CN/H_2O} = 70 : 30$) was used as a mobile phase with a flow rate of $Q = 1$ mL min⁻¹. Lactic acid was determined by using C₁₈ column (4.6 mm · 250 mm) and ultraviolet absorption detector, sulfuric acid of $c = 0.01$ mol L⁻¹ at $Q = 0.7$ mL min⁻¹ was used as the mobile phase and the detection was made at $\lambda = 210$ nm. The injection volume was $V = 10$ µL. The academic degradation rate (I) of cellulose in corn straw was calculated according to the following formula:¹⁹

$$I = \frac{m_L + m_R}{m_M} \cdot 0.9 \cdot 100$$

Where I : academic degradation rate (%), m_L : lactic acid (g), m_R : reducing sugar (g), m_M : cellulose in the substrate (g).

It was called academic degradation since the sugar cannot transfer to lactic acid completely.

3. Results and discussion

3.1 Optimization of cellulase production conditions by Orthogonal experiment

The orthogonal experimental design and the corresponding results are shown in Table 2. The $L_9(3^4)$ orthogonal experiments could determine the optimum conditions as well as the importance of the parameters. The range analysis was applied to clarify the importance of the sequence of parameters in the orthogonal experiments. Table 3 shows that range (F) of factor D was $F = 744.3$, which ranked the first. Factor A was $F = 342.3$, which ranked the second. Factor C was $F = 311.9$, which ranked the third. Factor B was $F = 217.9$, which ranked the last. The greater F value of a factor represents greater effect on the enzyme production. According to the range, the order of the factor sequence was: solid-to-liquid ratio > fermentation time > inoculum size > pH. The optimal conditions for improving enzyme was determined as $A_2B_2C_3D_2$, as follows: fermentation time of $t = 84$ h, pH of 4.5, inoculum size of $G = 12$ %, solid-to-liquid ratio of $\zeta_{S/L} = 1 : 3$. Under these conditions, the enzyme production was carried out based on the procedure mentioned in 2.2.1, the corresponding result was $H = 2630.3$ U g^{-1} straw.

The solid-to-liquid ratio could also be regarded as the effect of moisture on enzyme production. High moistures resulted in decrease of substrate porosity, which in turn prevented oxygen penetration; this might cause bacterial contamination. While on the other hand, low moisture content might lead to poor accessibility of nutrients resulting in poor microbial growth. In this study, the mass ratio of $\zeta = 1 : 3$ demonstrated that the initial moisture of $s = 75$ % was beneficial to the enzyme production. When it came to fermentation time, $t = 84$ h was determined as the best. Prolonged time resulted in the decrease of the cellulase activity. That might be because the microorganism itself would compete with the limited substrates, which caused a decrease in activity. From the industrial point of view, the fermentation time should be as short as possible, given the same enzyme result. As for the inoculum size, $G = 12$ % was superior to 4 % and 8 %, the abundant quantity of microorganism inside the system could utilize the substrate sufficiently and produce the desired product. While the least important factor in this study was determined to be pH. That did not mean that the pH was trivial to the enzyme production, only that the levels in this design did not show the importance of this factor. In fact, pH could affect physiological phenomena, such as fungal growth and enzyme stability, the result in this study demonstrated the acidic condition was suitable for the enzyme production.²³

Table 2 – Orthogonal experimental design and results

No.	A	B	C	D	cellulase activity $H/U\ g^{-1}$
	time, t/h	pH	inoculum size, $G/\%$	$\zeta_{S/L}$	
1	72	4.0	4	1 : 2	1204.7
2	72	4.5	8	1 : 3	2288.4
3	72	5.0	12	1 : 4	2003.1
4	84	4.0	8	1 : 4	2346.2
5	84	4.5	12	1 : 2	1981.3
6	84	5.0	4	1 : 3	2195.8
7	96	4.0	12	1 : 3	2587.8
8	96	4.5	4	1 : 4	2235.9
9	96	5.0	8	1 : 2	1652.9

Table 3 – Range analysis of the orthogonal experimental design

	A	B	C	D
	time, t/h	pH	inoculum size, $G/\%$	$\zeta_{S/L}$
□	5496.2	6138.7	5636.4	4838.9
□	6523.3	6505.6	6287.5	7072
□	6476.6	5851.8	6572.2	6585.2
□/3	1832.1	2046.2	1878.8	1613
□/3	2174.4	2168.5	2095.8	2357.3
□/3	2158.9	1950.6	2190.7	2195.0
R	342.3	217.9	311.9	744.3
S	2	4	3	1
O	84	4.5	12 %	1 : 3

Where R is the range analysis, S is factor importance sequence, O is the corresponding optimal conditions.

3.2. Analysis of enzymatic hydrolysate

In order to test the hydrolysis effect of the enzyme produced, pretreated corn straw was utilized to be hydrolyzed by the enzyme. The hydrolysis experiment was performed according to the conditions determined in the earlier study.²⁴ Sugar composition of enzymatic hydrolysate assayed by high performance liquid chromatograph (HPLC) is shown in Fig. 1. Since corn straw contained cellulose and hemicellulose, the main sugar released after enzymatic hydrolysis of pretreated corn straw was glucose, cellobiose and xylose at a mass ratio of $\zeta = 3.9 : 1 : 1.7$. During the SSF process, the glu-

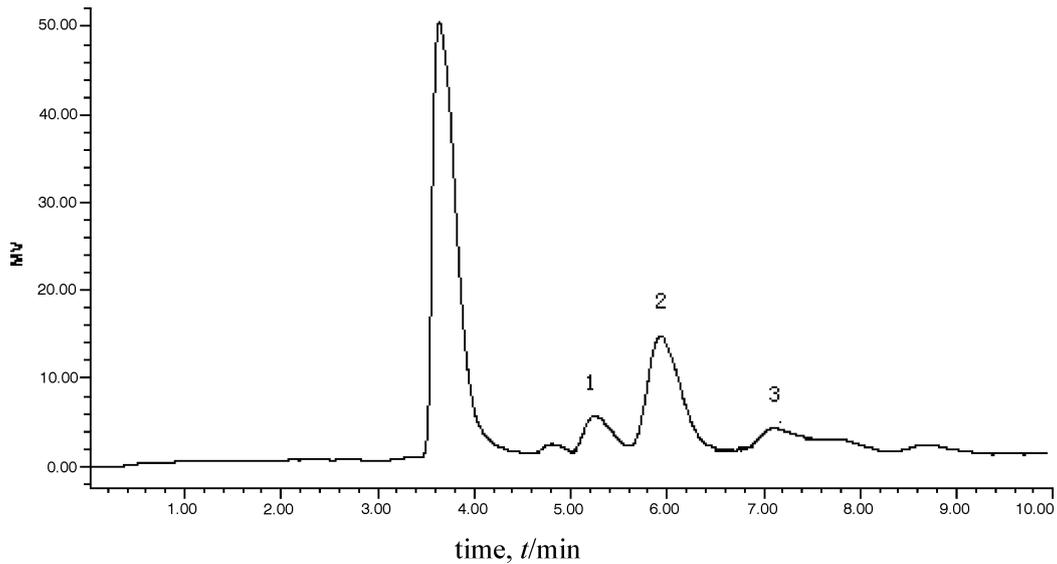


Fig. 1 – Composition analysis of hydrolysis liquid for corn straw using the produced cellulose: 1–xylose, 2–glucose, 3–cellobiose

cose was utilized by the lactic acid bacteria and resolved the inhibitor caused by the accumulation of glucose, then the cellulase and bacteria was further utilized as substrate to produce the desired product.

3.3 Production of lactic acid by SSF with strain T50 and produced cellulase

3.3.1 Effect of temperature on lactic acid production

T50 performed well at $\vartheta = 48\text{ }^{\circ}\text{C}$ during culture: the proper temperature for lactic acid production from corn straw during the SSF process was still unknown. Thus, the fermentation experiments of T50 were carried out at $\vartheta = 40\text{ }^{\circ}\text{C}$, $45\text{ }^{\circ}\text{C}$ and $50\text{ }^{\circ}\text{C}$. The results shown in Fig. 2 indicate that the concentration of lactic acid at $\vartheta = 45\text{ }^{\circ}\text{C}$ was higher than that at $40\text{ }^{\circ}\text{C}$ and $50\text{ }^{\circ}\text{C}$. Fig. 3 demonstrates that the concentrations of reducing sugar at $\vartheta = 45\text{ }^{\circ}\text{C}$ and $50\text{ }^{\circ}\text{C}$ were equal on the whole, both higher than that at $\vartheta = 40\text{ }^{\circ}\text{C}$. It could be concluded that $\vartheta = 45\text{ }^{\circ}\text{C}$ was the optimum SSF temperature that could satisfy the hydrolysis of corn straw and fermentation of lactic acid.

During the prophase of SSF, the concentration of reducing sugar increased rapidly, but as the process continued, it decreased gradually and reached a steady level. This might be because the lactic acid bacteria did not acclimatize to the environment in the prophase of SSF and the metabolizability was very slow, thus the reducing sugar was accumulated. However, with time, the enhancement of metabolizability of lactic acid bacteria resulted in the utilization of the reducing sugar. Meanwhile, the cellulase activity decreased and the hydrolysis speed was very slow. Thus, the concentration of the reducing sugar decreased gradually.

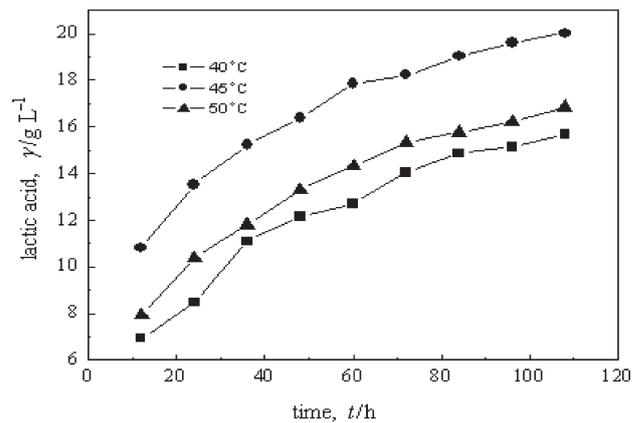


Fig. 2 – Effect of temperature on lactic acid with T50
■ 40 °C ● 45 °C ▲ 50 °C

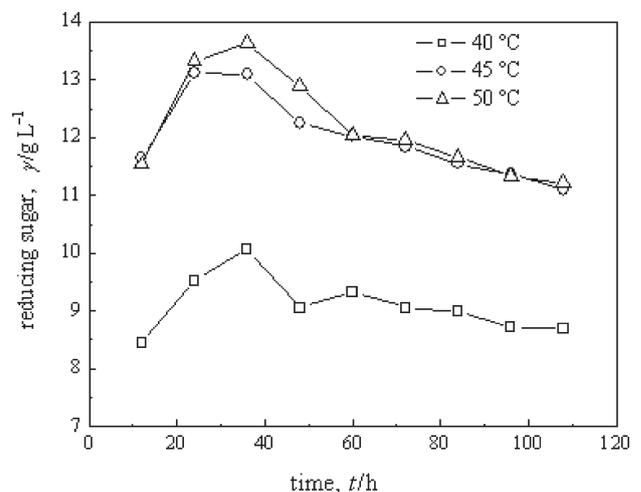


Fig. 3 – Effect of temperature on reducing sugar with T50
■ 40 °C ○ 45 °C △ 50 °C

As shown in Fig. 4, at the starting stage of SSF, the academic degradation rate of cellulose in corn straw increased rapidly. With time, the cellulase activity decreased, that should be restrained by the production of lactic acid and cellobiose. Thus, the academic degradation rate increased slowly and finally reached a steady level. The effect of temperature on the academic degradation rate showed a similar trend with the lactic acid production. The maximum academic degradation rate of $I = 68.4\%$ was obtained at $\vartheta = 45\text{ }^{\circ}\text{C}$.

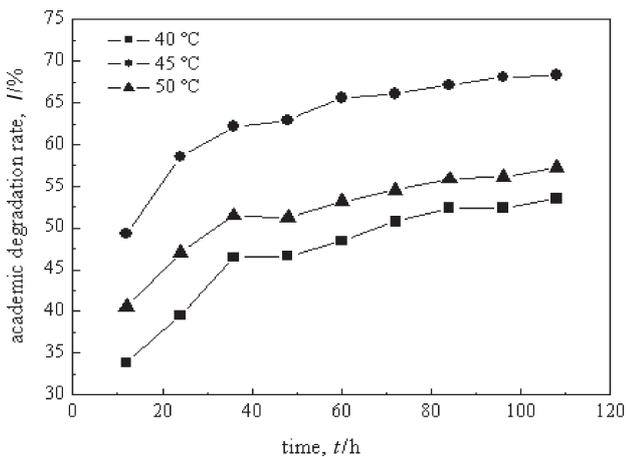


Fig. 4 – Effect of temperature on academic degradation rate
 ■ 40 °C ● 45 °C ▲ 50 °C

3.3.2 Effect of $(\text{NH}_4)_2\text{SO}_4$ and Tween 80 on lactic acid production

Nitrogen is the necessary nutrient for micro-organism growth.²⁵ In this experiment, different concentrations of $(\text{NH}_4)_2\text{SO}_4$ were added to test its effect on lactic acid production; the experiment was performed according to the optimal conditions mentioned in 2.2.2. The fermentation result shown in Fig. 5 demonstrates that $w = 2\%$ of $(\text{NH}_4)_2\text{SO}_4$

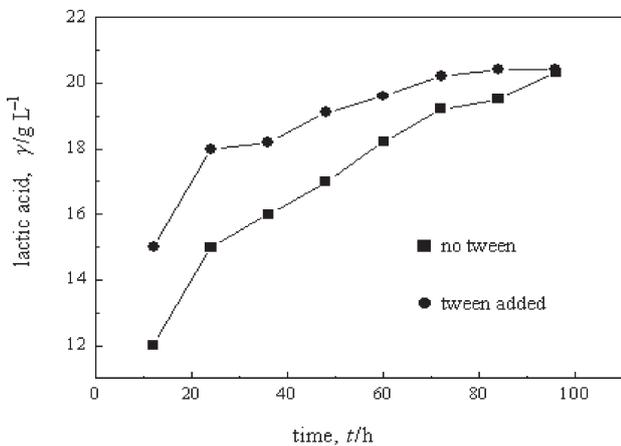


Fig. 5 – Effect of Tween 80 on lactic acid
 ■ no tween ● tween added

could achieve the maximum lactic acid production, 3.6 % the second, and no addition the last. When the $(\text{NH}_4)_2\text{SO}_4$ was further increased, the yield of lactic acid decreased rapidly. This might be because the osmotic pressure of fermentation liquor was higher than that of the cytoplasm, which could have prevented the bacteria from absorbing the nutrient.

Besides the proper nitride, different mechanisms were proposed for the positive effect of surfactant added to enzymatic hydrolysis of cellulose. The surfactant could change the nature of the substrate by increasing the available cellulose surface or by removing inhibitory lignin. Based on kinetic analysis, Kaar and Holtzaple have found that surfactants could promote the availability of reaction sites, which would increase the hydrolysis rate.²⁶ The surfactant could also increase the stability of the enzymes and thus reduce enzyme denaturation during hydrolysis. In order to test the effect of surfactant, 1 mL Tween-80 was added into a conical to determine its influence on lactic acid. At the prophase of SSF, the concentration of lactic acid with added Tween-80 was far higher than that of the normal substrate. However, at the anaphase of SSF, the former was only a little higher than the latter. Although the addition of Tween-80 did not increase the lactic acid production, the added one could attain a maximum concentration of $\gamma = 20.4\text{ g L}^{-1}$ in 72 h, while the one without the addition could reach this level in $t = 108\text{ h}$. Since $m = 20\text{ g}$ pre-treated corn straw was dissolved into the tap water, that meant $\gamma = 20.4\text{ g L}^{-1}$ lactic acid could be produced from $\gamma = 100\text{ g L}^{-1}$ pre-treated corn straw. Thus, it was obvious that the surfactant would not contribute to lactic acid fermentation, but significantly shorten the fermentation time. The result was comparable with a similar study carried out by Shigenobu, M., in that study, $\gamma = 24\text{ g L}^{-1}$ of L-lactic acid was produced from $\gamma = 100\text{ g L}^{-1}$ of untreated raw corncob.²⁷

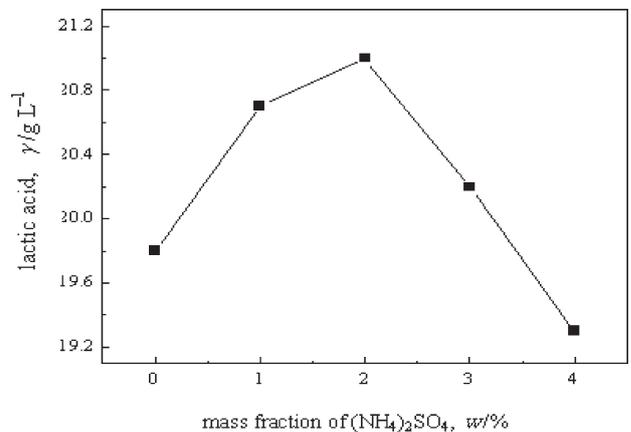


Fig. 6 – Effect of mass fraction of $(\text{NH}_4)_2\text{SO}_4$ on lactic acid

4. Conclusion

Corn straw was utilized for cellulase production and then the product was further used for lactic acid fermentation. The optimum conditions for cellulase as well as for lactic acid were determined, the influential factors such as Tween 80 and $(\text{NH}_4)_2\text{SO}_4$ were determined. The result of orthogonal experiment design for cellulase production showed that the optimal conditions were fermentation time of $t = 84$ h, pH of 4.5, inoculum size of $G = 12$ %, ratio of corn straw to liquid of $\zeta = 1 : 3$. The importance sequence for the factors were: solid-to-liquid ratio > fermentation time > inoculum size > pH. The produced cellulase was proved capable of successfully transferring the corn straw to glucose, cellobiose and xylose. The ideal temperature of lactic acid production from corn straw by the SSF process was determined as $\vartheta = 45$ °C. The addition of $w = 2$ % $(\text{NH}_4)_2\text{SO}_4$ could increase the lactic acid by 3.6 %. The utilization of Tween 80 inside the substrate could shorten the fermentation time from $t = 108$ h to $t = 72$ h. Lactic acid production by corn straw through the SSF process could largely save the material costs and recycle the resource, which makes this method environmentally as well as economically significant.

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List of symbols

A, B, C, D	– factors in the orthogonal experimental design
R	– range analysis
G	– inoculum size, % (v/m)
t	– time
H	– enzyme activity, U g^{-1}
γ	– mass concentration, g L^{-1}
ϑ	– temperature, °C
w	– mass fraction, %
l	– length, m
V	– volume, mL
m	– mass, g
c	– concentration, mol L^{-1}
Q	– flow rate, mL min^{-1}
λ	– wave length, nm

I	– academic degradation rate, %
ζ	– mass ratio
$\zeta_{\text{S/L}}$	– ratio of solid to liquid
s	– moisture, %
ψ	– volume ratio

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