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Preparation of Levulinic Acid Using Cellulase and Solid Acid Synergistic Hydrolysis for Rice Straw

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Levulinic acid was prepared from cellulase and SO_4^{2-}-TiO_2/clay solid acid synergistic hydrolysis for rice straw. Orthogonal experiment and response surface methodology were applied to carry out optimum analysis on main parameters. The aim of this study is to obtain the optimal reaction conditions needed to degrade rice straw by cellulase and to synthesize levulinic acid with solid acid. The orthogonal experiment result shows that when the reaction temperature is 50 °C, the volume of the enzymolysis solution is 30 U g^{-1} of dried straw powder, the vibration reaction time is 8 h, the raw material granularity is 80 grit and the average content of reducing sugar is 15.67 mg mL^{-1}. Response surface analysis indicates that under a reaction temperature of 215.94 °C, a reaction time of 34 min and a solid acid dosage of 7.10 %, the levulinic acid yield can reach 23.52 %. The synergistic hydrolysis of cellulase and SO_4^{2-}-TiO_2/clay solid acid on rice straw had the benefit of increasing the levulinic acid yield. The existing reaction conditions are mainly the same as the experimental reaction conditions. As compared to the technology without the use of cellulase hydrolysis, the method in this study produced levulinic acid with 4.79 % increased yield.

Key Words: Levulinic acid yield, Rice straw, Cellulase, Solid acid, Synergistic hydrolysis.

INTRODUCTION

Levulinic acid (LA) is a new type of high-performance platform chemical compound[1-3]. Its molecule contains a carbonyl group and a carboxyl group. It has good reactivity and can be used for esterification, oxidation reduction, substitution and polymerization reactions. Therefore, it is widely used in products such as biopharmaceuticals, chiral reagents, lubricants, coatings and printing inks and electronics, among others.

At present, two major methods are used to produce levulinic acid (i) the catalyzed hydrolysis of furfuryl alcohol and (ii) the hydrolysis of biomass. In the former method, furfuryl alcohol has to be prepared first and then hydrolysis is performed

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in an acid medium. In the latter method, biomass containing cellulose and starch is used as a raw material. A reaction is then carried out at a high temperature and the material is subjected to heat treatment with catalysis reaction using an inorganic strong acid to obtain the monosaccharide. 5-HMF was formed after dehydration, levulinic acid was generated after hydrolysis in acid medium. Both methods of levulinic acid production have disadvantages, such as low yield, long reaction time, many by-products and in particular, the liquid inorganic acid catalysts not only corrode equipment but also separate from the product with difficulty.

In accordance with Green Chemistry needs, biodegradation and solid acid hydrolysis technologies have developed rapidly[4]. Solid acid is a new type of catalyst which is reusable and easy to separate from liquids. Furthermore, enzymic hydrolysis requires only mild conditions with low energy consumption and no pollution. A report is available in literature[5] on the preparation of levulinic acid using inorganic acid hydrolysis of rice straw, but none on the production of levulinic acid using cellulase and solid acid synergistic hydrolysis for rice straw. This study uses rice straw as raw material and cellulase and solid acid concerted catalysis technology to degrade rice straw to prepare levulinic acid. This is in an effort to address the disadvantages of the conventional preparation method, such as high cost, low yield, environmental pollution, *etc*.

EXPERIMENTAL

Rice straw (South China); cellulose (2×10^4 U mL-1) (Worthington Biochemical Corp.); SO_4^{2-}-TiO_2/clay solid acid catalyst (South Company).

The experiment and test equipment mainly include high-performance liquid chromatograph (HPLC) (LC-10AVP Plus, Japan); digital water-bath with a constant temperature vibrator (SHZ-82, China); and a stainless steel high-pressure reactor with a diameter of Φ 40 mm, a volume of 300mL and a designed pressure of 15 MPa (MD-300, China, Weihai).

Enzymolysis of rice straw: Pulverize the dried rice straw using 20, 40, 60, 80 and 100 grit sieves. Then use dilute sulfuric acid with a concentration of 5 % and a liquid-solid ratio of 10:1 to soak it for 1 h. Wash the filter residue with distilled water and leave it to parch dry at 80 °C as well as to keep the weight stable. Add a certain amount of cellulase into the rice straw powder which has gone through the acid treatment. Then use distilled water to adjust the solid-liquid ratio and carry out vibration hydrolysis under a certain temperature condition. Finally, measure the content of reducing sugar in the reaction solution when the reaction is over.

Synthesis of levulinic acid: Heat the reaction in the high-pressure reactor after putting the above-mentioned zymohydrolysis solution and rice straw residue inside. Include a certain amount of solid acid and rigidly control every reaction factor. Then measure the levulinic acid yield after the reaction is over. Meanwhile, carry out comparative trials after directly hydrolyzing the rice straw which has not gone through the zymohydrolysis process with cellulase.

Measuring and computing: 3,5-Dinitrosalicylic acid (DNS) reagent method was used to measure the total reducing sugar in the straw zymohydrolysis solution. Analyze samples through the HPLC to detect the levulinic acid yield[6]. The method for computing is:

$$\text{Content [Reducing Sugar]} = \frac{\text{Weight of reducing sugar in reaction solution}}{\text{Volume of the reaction solution}} \times 100\% \quad (1)$$

$$\text{Yield [LA]} = \frac{\text{Weight of levu lim ic acid in reaction solution}}{\text{Initial weight of rice straw}} \times 100\% \quad (2)$$

RESULTS AND DISCUSSION

Determination of rice straw enzymolysis conditions: Based on the explorative experiment, a single-factor experiment is carried out. Add distilled water and cellulase liquid into the 4 g straw powder which has gone through the acid treatment in order to let the total amount of enzyme reach 120 U (*i.e.*, 30 U g^{-1} of straw powder). Place it in a constant temperature water-bath vibrator to induce reactions under different temperatures and time. The results are shown in Figs. 1-4.

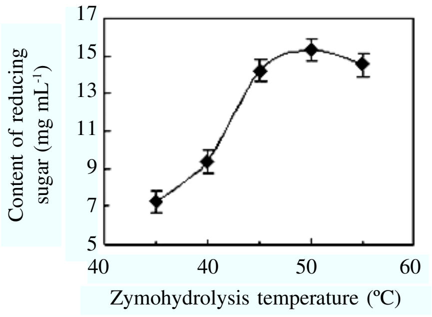

Fig. 1. Effects of zymohydrolysis temperature on the content of reducing sugar. The zymohydrolysis solution is composed of 30 Ug^{-1} of dried straw powder. The raw material granularity is 80 grit. The vibration reaction duration is 8 h

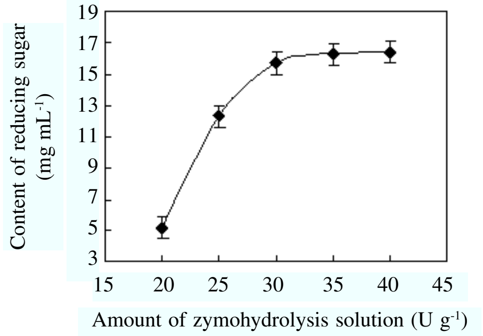

Fig. 2. Effects of the amount of zymohydrolysis solution on the content of reducing sugar. The zymohydrolysis temperature is 50 °C, the raw material granularity is 80 grit and the vibration reaction duration is 8 h

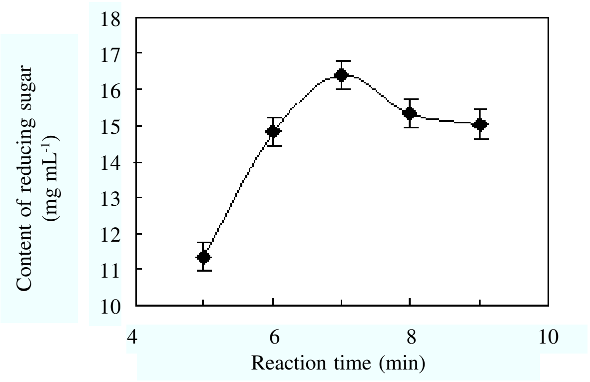

Fig. 3. Effects of reaction time on the content of reducing sugar. The zymohydrolysis temperature is 50 ºC, the zymohydrolysis solution is 30 U g-1 of dried straw powder and the raw material granularity is 87 grit

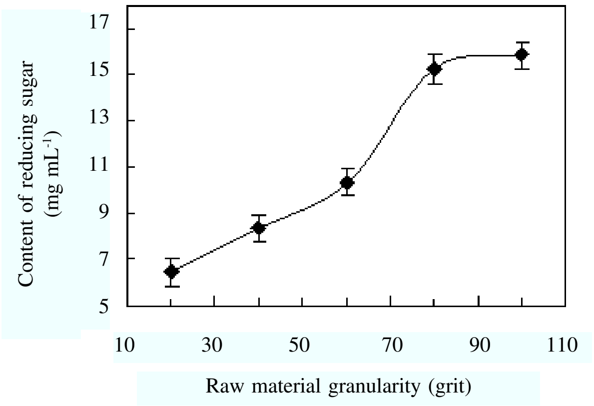

Fig. 4. Effects of raw material granularity on the content of reducing sugar. The zymohydrolysis temperature is 50 ºC, the zymohydrolysis solution is 30 U g-1 of dried straw powder and the reaction time is 8 h

The reaction temperature plays an important role in the zymohydrolysis process. When the temperature is 35-50 °C, with the increase in temperature, the reducing sugar content in the reaction system soars (Fig. 1). Around 50 °C, the yield is 15.29 %. When the temperature is higher than 50 °C, the yield has the tendency to decrease, which can indicate that the increase in temperature can increase the activation energy in the reaction system in a move to boost the reaction speed, the high temperature can also cause cellulase to lose activity. As such, very high temperatures reduce the effective concentration of cellulase in the reaction system and lead to the decrease in hydrolysis capacity of the system.

The dosage of zymohydrolysis solution directly affects the hydrolysis speed and the reducing sugar content. When the zymohydrolysis solution dosage is lower than 30 U g^{-1} dried straw powder, as the zymohydrolysis solution dosage increases, the release of reducing sugar increases remarkably. However, when it exceeds 30 U g^{-1}, the increase in reducing sugar yield slowed down (Fig. 2). This is because the increase in the zymohydrolysis solution dosage actually means the increase in the contact area between the zymohydrolysis solution and the cellulase in rice straw powder. However, when the contact area reaches its peak, the excess cellulase cannot participate in the reaction. Consequently, the increase in reducing sugar yield is slowed down.

The content of reducing sugar in the reaction system also depends on the zymohydrolysis time. As the reaction time is prolonged, the content of reducing sugar grow gradually and when the reaction time is over 8 h, the rate of increase is remarkably reduced (Fig. 3). This is because as the reaction time is prolonged, the amount of by-products increase in the reaction system, which leads to the self-decomposition of reducing sugar and the condensation reaction with by-products. This indicates that under the current conditions, it tends to be stable only after 8 h of zymohydrolysis reaction.

Raw material granularity affects the content of the reducing sugar and the hydrolysis speed. As the raw material granularity increases from 20 grit to 80 grit, the reducing sugar yield increases from 6.47 to 15.45 mg mL^{-1} (Fig. 4). However, the increasing degree of yield increase is relatively small at a granularity higher than 80 grit. Taking into account the pulverizing cost, it is recommended to apply the 80 grit. The effects of raw material granularity on the content of reducing sugar may be linked to the fact that machine pulverization can destroy the bonding layer of lignin, hemicellulose and cellulose, reduce the degree of crystallinity of these three compounds and change the crystalline structure of cellulose[7]. This is conducive to glycosidic bond fission which can increase the reducing sugar yield and the hydrolysis speed. At the same time, the smaller the granules, the greater the contact area with the zymohydrolysis solution and thus the higher the sugar yield.

After testing the above-mentioned parameters, the optimum reaction conditions are obtained through 4-factor and 3-level orthogonal analysis. As soon as the reaction temperature reaches 50 °C, the zymohydrolysis solution dosage becomes 30 U g^{-1} dried straw powder. The vibration reaction time is 8 h. The raw material granularity should be maintained at 80 grit. After three repeated trials under the same conditions, the average content of reducing sugar in the reaction system was found to be 15.67 mg mL^{-1}.

Levulinic acid synthesis of solid acid: The zymohydrolysis solution prepared under the above-mentioned optimum and the residues were placed into a high-pressure reactor to synthesize levulinic acid with solid acid as catalyst. Based on the explorative experiment, implementation of single-factor analysis of the reaction temperature, reaction time, solid acid dosage and solid-liquid ratio was done to study the effects of the above-said factors on the levulinic acid yield.

The activity of the reaction system depends on the temperature. Adding solid acid with a constant mass fraction of 7% into the reaction solution after zymohydrolysis and maintaining a 0.5 h reaction time, it is possible to carry out contrast experiments under different reaction temperatures. The levulinic acid yield is shown in Table-1. When the temperature is below 220 °C, the yield of levulinic acid increases with temperature and beyond 220 °C, the yield decreases.

TABLE-1
EFFECT OF DIFFERENT REACTION CONDITIONS ON THE LEVULINIC ACID (LA) YIELD

Item	Reaction condition	LA yield (%)	Item	Reaction condition	LA yield (%)
Reaction temperature (°C)	180	16.57	Solid acid dosage (%)	5	17.47
	200	18.33		6	21.32
	220	22.16		7	22.93
	240	20.53		8	20.81
Reaction time (min)	15	18.53	Liquid-solid ratio	6:1	18.68
	20	21.67		8:1	20.09
	30	22.49		10:1	22.87
	40	21.52		12:1	19.65

When the reaction temperature is 220 °C, the solid acid dosage is 7 % and the liquid-solid ratio is 10:1, then under different reaction times, the levulinic acid yield increases as the time expands. When the reaction time exceeds 0.5 h, the yield of levulinic acid demonstrate a gradual reduction tendency (Table-1).

Catalysts increase the reaction speed. When the reaction temperature is 220 °C, the liquid-solid ratio is 10:1 and the reaction time is 0.5 h. The levulinic acid yield with different solid acid dosages is shown in Table-1. When the solid acid dosage is 7 %, the levulinic acid yield reaches its peak value. However, the continuous increase in solid acid does not lead to a remarkable increase in the levulinic acid yield.

When the solid acid dosage is constant, the change in the liquid-solid ratio is actually the change in H^+ concentration in the reaction system. Therefore, the levulinic acid yield is related to the liquid-solid ratio. The results of the levulinic acid yield with different liquid-solid ratios at 220 °C, the solid acid dosage is 7 % and the reaction time is 0.5 h are presented in Table-1. The levulinic acid yield reaches its peak when the liquid-solid ratio is 10:1.

Response surface analysis (RSA): Based on the central composite design principle, the selection of the four factors *i.e.*, reaction temperature (A), reaction time (B), solid acid dosage (C), liquid-solid ratio (D) are important. The levulinic acid yield is set as the responsive value and the 4-factor and 3-level response-surface experiment is designed, as shown in Table-2.

The results of combining the single-factor experiment data, the application of the Design-expert software (version 7) to implement quadratic multivariate regression fitting and the use of quadratic model for analysis are shown in Table-3.

TABLE-2
LEVEL AND CODE IN THE RESPONSE-SURFACE EXPERIMENT

Code	Name	-1 Level	1 Level
A	Temperature (°C)	200	240
B	Time (min)	20	40
C	Amount of solid acid (%)	6	8
D	Liquid: solid rate	8	12

TABLE-3
MODELS AND VARIANCE

Source	Sum of squares	Df	Mean square	F Value	P Value Prob > F	
Model	242.14	8	30.267	9.36	< 0.0001	Significant
Error	58.17	18	3.232			

R-squared = 0.806

In the quadratic multivariate regression analysis, all parameters *i.e.*, A (temperature), B (time), C (amount of solid acid) and D (liquid: solid rate), A^2, B2, C^2 and D^2 are statistically significant at 95 % confidence level. 80.63 % of response variability can be explained by those significant predictors. The equation obtained from analysis is:

$$\text{Levulinic acid yield} = -707.33 + 4.968A + 2.064B + 28.873C + 9.247D - 0.011A^2 - 0.030B^2 - 1.996C^2 - 0.430D^2 \quad (3)$$

In order to simplify the explaination of relationship between levulinic acid yield and those predictors, the response surface 3D plots are used, which are shown in Figs. 5-7. In Fig. 5, as the reaction time is extended and the solid acid dosage increases, the levulinic acid yield increases continuously. The yield reaches its highest value when the solid acid dosage is around 7 %. A continuous increase in solid acid

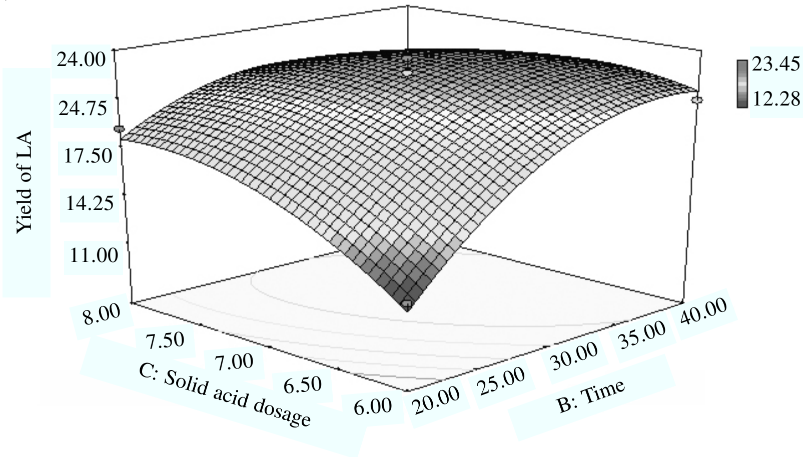

Fig. 5. Effects of reaction time and solid acid dosage on the levulinic acid yield

dosage lead to the decrease in the levulinic acid yield. The β-1, 4-glycosidic bonds between large molecules are destroyed by H^+ action[8], so when the H^+ concentration is on the low side, the catalytic activity in the system is correspondingly low. As the solid acid dosage increases, the H^+ concentration becomes relatively big in the reaction system, which is beneficial for reaction acceleration. Therefore, the levulinic acid yield is increased. However, as time expands, levulinic acid's self-decomposition and condensation reaction with its by-products increases accordingly. Hence, when the solid acid dosage reaches a certain value, the expansion of reaction time lead to a reduction in the levulinic acid yield.

As the reaction temperature and time increase, the levulinic acid yield increase accordingly, but when the temperature is around 220 °C, it begins to slow down (Fig. 6). This is because when the reaction temperature is lower than 220 °C, the increase in temperature and the extension of the reaction time increase the activity of the reaction system, as well as curb some subsidiary reactions. However, when the temperature is relatively high, the levulinic acid in the reaction system begins to decompose[9]. On the other hand, the increase in reaction time accelerate the reactions between levulinic acid and its by-products. As a result, after examining these experimental conditions, the suitable hydrolysis temperature range is found to be from 210-230 °C.

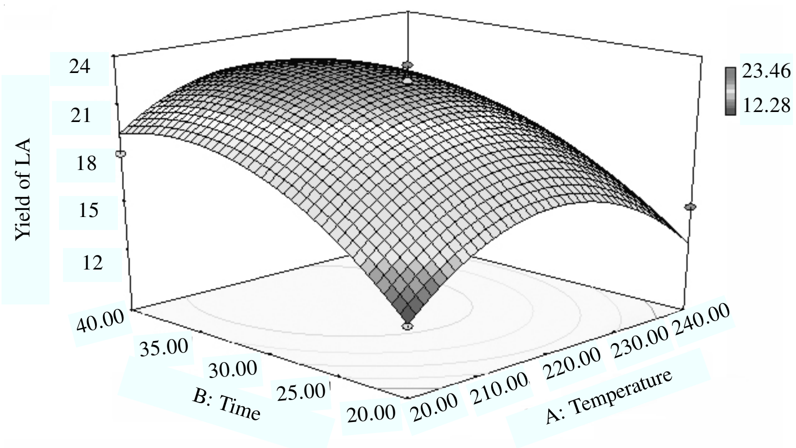

Fig. 6. Effects of reaction time and temperature on the levulinic acid yield

The solid acid dosage and reciprocal effects of temperature on the levulinic acid yield is shown in Fig. 7. Under some temperature conditions, the increase in solid acid dosage increase the levulinic acid yield. When the solid acid dosage is fixed, the extremely high temperatures cause the levulinic acid yield to decrease. In other words, under lower temperatures, the catalytic activity of solid acid is relatively low, such that increasing temperature increases the activity of the reaction system. However, under high temperatures beyond the limit, levulinic acid accelerate the

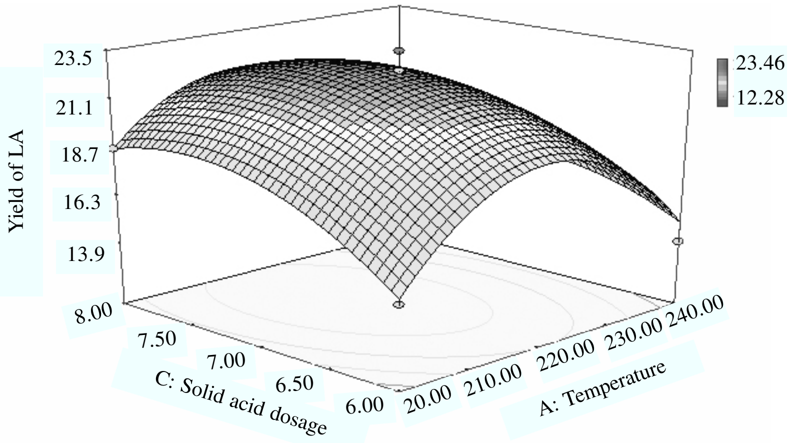

Fig. 7. Effects of reaction temperature and solid acid dosage on the levulinic acid yield

decomposition. Thus, higher temperatures lead to a decrease in the levulinic acid yield. At the same time, when the H^+ concentration is relatively high enough that the hydrolysis can be curbed and the levulinic acid yield can decrease.

Optimum reaction conditions are obtained through the analysis and evaluation of the effects of a two-factor reciprocal reaction. When the reaction temperature reaches 215.94 °C, the reaction takes 34 min to produce a levulinic acid yield of 23.52 % given that the solid acid dosage is maintained at 7.10 % mass fraction and the liquid-solid ratio is at 10.7:1. Data were verified in accordance with the requirements of a technological condition. The average result of the three experiments is 23.16 %. The predicated value and the true value are close to each other, indicating that the technology is practical. In comparison, the direct use of solid acid to implement the hydrolysis of rice straw without going through zymohydrolysis much like the present used reaction conditions gave a levulinic acid yield of only 18.73 %. The old method yields a remarkable 4.79 % drop in comparison with the method formulated in this study. This is because cellulase can rupture the β-1, 4-glycosidic bond, then hydrolyze to form reducing sugar[10,11]. At the same time, cellulase can rupture the crystalline structure of cellulose, accelerating the hydrolysis of solid acid on rice straw and allowing the hydrolysis to be more complete in a step forward reaction for increased levulinic acid yield.

Conclusion

(1) Cellulase is used to hydrolyze rice straw. When the zymohydrolysis temperature is set at 50 °C, the zymohydrolysis solution reaches 30 U g^{-1} dried straw powder and the vibration process is 8 h, the average content of reducing sugar in the reaction system is 15.67 mg mL^{-1}. (2) The hydrolysis reaction of cellulase on rice straw is conducive to the increase in the levulinic acid yield. Cellulase synergizes solid acid catalysts to hydrolyze rice straw and form levulinic acid. The optimized reaction conditions are (a) reaction temperature of 215.94 °C, (b) reaction time of

34 min and (c) solid acid dosage of 7.10 %. The levulinic acid yield under these conditions is 23.52 %. As compared to the one without the use of zymohydrolysis under the same reactions conditions, a 4.79 % yield increase is observed with the new method formulated in this study.

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