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Cellulosic Ethanol Production Using Rice Grass (*Spartina* spp.) with Cellulase

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In this study, rice grass (*Spartina* spp.), one kind of invasive plant in the coastal areas, was investigated for cellulosic ethanol production. Firstly, the rice grass powder was used as the main carbon source for cellulase fermentation by solid-state fermentation with the strain *Trichoderma reesei* SEMCC 3.217, then optimal conditions for hydrolyzate with celluase were optimized by orthogonal experimental design and the strain *Saccharomyces cerevisiae* SEMCC 2.157 was used for ethanol fermentation, after optimized by one-factor-one-time approach, the yield of ethanol could reach 28.1 g/L in 36 h under the optimal conditions.

Key Words: Ethanol, Cellulase, Fermentation, Optimization, *Spartina* spp.

INTRODUCTION

Rice grass (*Spartina* spp.) has become a kind of serious invasive plant in coastal areas in Asia, Australia, New Zealand and North America[1]. It also spread to the Yellow River Delta these years, causing extensive damage to the natural salt marsh ecosystems in local coastal areas. So it is necessary to find a reasonable method to make full use of these plants. It has been reported that the rice grass could be used for power generation by gas-heat-electricity triple cogeneration method or for feed production[2]. However, the large-scale utilization of rice grass is limited due to the high salt content.

In these years, biomass based energy has been attracted the people's interest due to the growing concerns about the environmental impacts associated with the fossil fuels utilization[3-5]. Straws and stovers have been developed for renewable energy production at present, but only a few reports on biofuels production with rice grass. Chen *et al.*[6] reported that the rice grass could be used for biofuels production after treated by sulfuric acid. The yield of reducing sugar was 36 % (w/w, glucose and xylose) and the ethanol production was 8.9 g/100 g of dry solids (ethanol/ g dry solid) and the biolipid production was 6.4 g/100 g of dry solids (biolipid/g dry solid). In this study, rice grass was used as the main carbon resource for cellulase production and cellulosic ethanol production, which was not mentioned before as per our best of knowledge.

EXPERIMENTAL

Rice grass, colleted from Xianhe Town, Dongying City of Shandong Province, was dried at 105 ºC, crushed and sieved to an average sizes of 0.2-0.3 mm. Reagents used in this study were of analytical grade and obtained locally.

Two strains used in this study were *Trichoderma reesei* SEMCC 3.217 and *Saccharomyces cerevisiae* SEMCC 2.157, stored in the laboratory. The fungus were maintained on potato agar slants and sub-cultured fortnightly. The yeast culture was maintained on YEPD agar slants and sub-cultured weekly. In order to preparation of fungal inocula, about 5 mL of sterile distilled water containing 0.1 % of Tween 80 was introduced into the sporulated slants (incubated at 30 ºC for 7-10 d) of the fungus and the suspension was of 10^8 spores/mL was used as inoculum for cellulase production in solid-state fermentation. In the case of yeast, the culture was grown in inoculum broth for 12 h with 150 rpm (50 mL/250 mL Erlenmeyer flask) agitation on a rotary shaker and the culture was used at 10 % (v/v) as inoculum for ethanol fermentation.

Cultivation: Solid-state fermentation medium for cellulase production: rice grass powder 7.3 g, wheat bran 3.1 g, initial pH 5.0, nutrient salt solution $(NH_4)_2SO_4$ 14 g/L, KH_2PO_4 2 g/L, $CaCl_2$ 4 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L) 8 mL and the initial moisture content was adjusted to 70 % with distilled water (steriled for 0.5 h at 121 ºC).

Inoculum medium for ethanol production (g/L): Yeast extract 3, peptone 5, malt extract 3. Basal medium for ethanol production (g/L): rice grass sugar 30-100 (glucose concentration, varied with the experimental design), $(NH_4)_2SO_4$ 10, KH_2PO_4 2.6, $MgSO_4{\cdot}7H_2O$ 0.5, KH_2PO_4 0.36, $MgSO_4{\cdot}7H_2O$ 0.1.

Cellulase activity analysis: Cellulase was extracted by suspending the fermented substrate with 5-fold of citrate buffer (50 m mol/L, pH 4.8) and mixing it for 1 h at 300 rpm. And then the crude enzyme was further extracted by centrifugation (10,000 g, 20 min). The total cellulase activity (filter paper unit, FPU) was measured by the standard filter paper assay with No. 1 Waterman filter paper[7]. The filter paper enzyme activity (FPA) was expressed as FPIU/g of dry substrate (FPIU/gds). One International Unit (IU) of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of product (reducing sugar) per min at 50 ºC.

Reducing sugar, glucose and ethanol determination: The total reducing sugar was determined by the DNS assay[8]. The glucose consentration and ethanol content were measured by a SBA-40E biosensor (Biology institute of Shandong Academy of Sciences) with different enzyme membrane detectors after diluted to the optimal concentrations.

Cellulase production: Cellulase was produced by *T. reesei* SEMCC-3.217 with solid-state fermentation. The innoculum size was of 1 mL spore suspension (1×10^8 spores/mL) and the fermentation period was 7 d at 30 ºC. When the fermentation was ended, the cellulase was extracted with 50 m mol/L citrate buffer as described before. Cellulase was extracted by citrate buffer (50 m mol/L, pH 4.8), concentrated by ultrafilteration to 50 FPIU/mL for further study.

Hydrolysis of rice grass: The rice grass powder were treated with 3 % (w/v) NaOH solution (1:10, w/v) for 24 h at room temperature, adjusted to pH of 5.0-6.0 after washed by distilled water and then dried at 105 ºC before further use. The pretreated rice grass powder was treated with different cellulase dosages, substrate concentration, temperature and incubation times. The optimal conditions for hydrolysis were investigated by the orthogonal tests.

Ethanol production by rice grass: Ethanol production was studied using the enzymatic hydrolyzate of rice grass powder. The filterated hydrolysate was concentrated by evaporation to reducing sugar content of 3-10 % (w/v) and used as carbon source for ethanol production at 30 ºC, 100 rpm in the flasks. The parameters such as temperature, pH, rotation speed, medium volume and nitrogen sources were selected for further optimization.

RESULTS AND DISCUSSION

Time course of cellulase production: The results of the time course of cellulase production is shown in Fig. 1. It could be found that the cellulase could reach 32.7 FPIU/g of dry solids at 5 d fermentation and then the activity declined. So the fermentation period was 5 d.

In this study, rice grass should be a kind of good carbon source for cellulase fermentation, especially pretreated with NaOH solution, by which cellulose content in the rice grass powder increased about 32.4 % than the control group and lignin concentration decreased about 8.4 % than the control group, which were more easily for cellulase hydrolysis and fermentable sugars production[9] and could improve the cellulase production by the *Trichoderma reesei* SEMCC 3.217.

Optimization of the hydrolysis of rice grass powder: Orthogonal tests L_9 (3^4) were used for optimization of hydrolysis of rice grass powder. The experimental design is shown in Table-1 and the results and analysis are shown in Table-2.

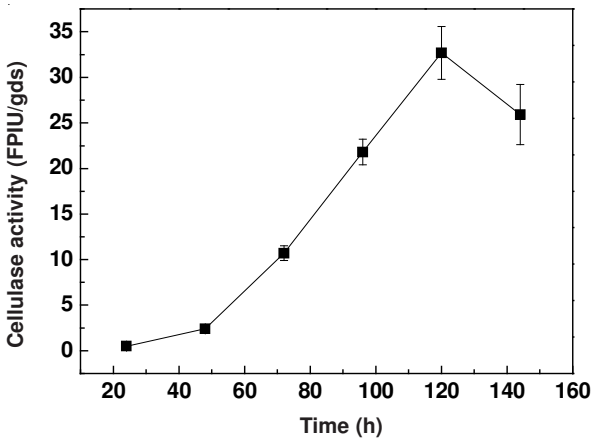

Fig. 1. Time course of the cellulase production in solid-state fermentation

TABLE-1
FACTORS AND LEVELS OF CELLULASE HYDROLYSIS IN ORTHOGONAL TESTS

Level	Cellulose dosage (FPIU/gds)	Substrate conc. (g/100 mL)	Temp. (ºC)	Reaction time (h)
1	10	1	45	12
2	15	2	50	24
3	20	3	55	36

The optimized conditions for rice grass powder hydrolysis by cellulase was: substrate concentration 2 %, cellulase activity 20 FPIU/mL, temperature 55 ºC, incubation time 36 h. Under these conditions, the hydrolysis rate could reach 51.4 %. When the cellulase dosage was over 20 FPIU/mL, the hydrolysis rate increased very slowly, indicating that at a certain concentration of substrate, the number of cellulose molecular limited reducing sugar yield and the reaction rate reached climax, so more cellulase could not produce more reducing sugar any longer[10].

Ethanol production using hydrolyzate: In order to find the optimal glucose concentration of the hydrolyzate for ethanol production, different concentrations of glucose were selected and the results are shown in Fig. 2. It was found that rice grass hydrolyzate with 60 g of glucose showed the highest ethanol conversion rate (43.7 %). So, 60 g/L glucose was used for further optimization.

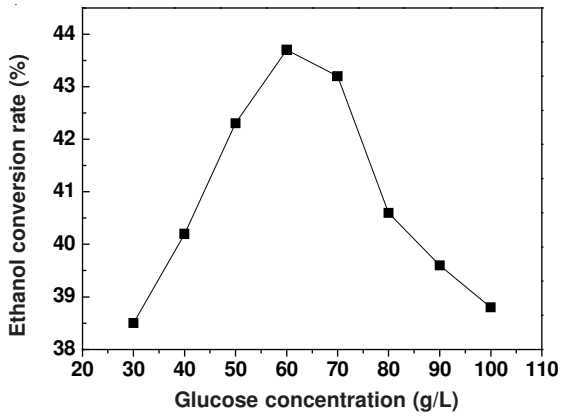

Fig. 2. Effects of glucose content on ethanol production

TABLE-2
RESULTS OF CELLULASE HYDROLYSIS IN ORTHOGONAL TESTS

Run No.	Cellulase dosage (FPIU/gds)	Substrate conc. (g/100 mL)	Temp. (ºC)	Reaction time (h)	Hydrolytic rate (%)
1	1	1	1	1	35.8
2	1	2	2	2	42.1
3	1	3	3	3	45.6
4	2	1	2	3	48.9
5	2	2	3	1	42.3
6	2	3	1	2	40.8
7	3	1	3	2	45.7
8	3	2	1	3	49.6
9	3	3	2	1	41.5
k_1	41.2	43.5	42.1	39.9	–
k_2	44.0	44.7	44.2	42.9	–
k_3	45.6	42.6	44.5	48.0	–
R	4.4	2.1	2.4	8.1	–
Optimal level	A3	B2	C3	D3	–

Glucose is very essential for ethanol production for many types of yeast. In case of rice grass hydrolyzate, the challenge lies in cellulosic ethanol fermentation is the "unusual"sugars and other chemicals. Moreover, other fermentation parameters such as temperature, pH, medium volume, rotation speed and nitrogen sources also influence the final ethanol yield, so it was important to optimize these factors. The results are shown in Figs. 3-7.

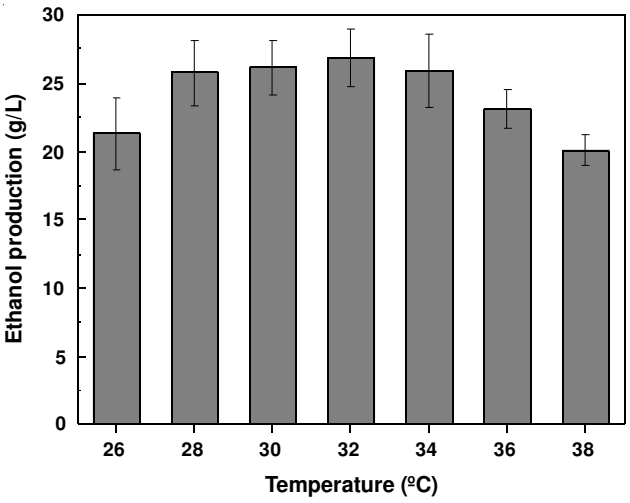

Fig. 3. Effects of temperature on ethanol production

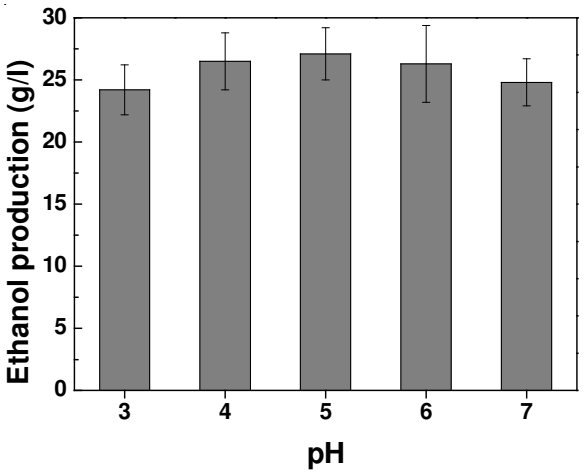

Fig. 4. Effects of pH on ethanol production

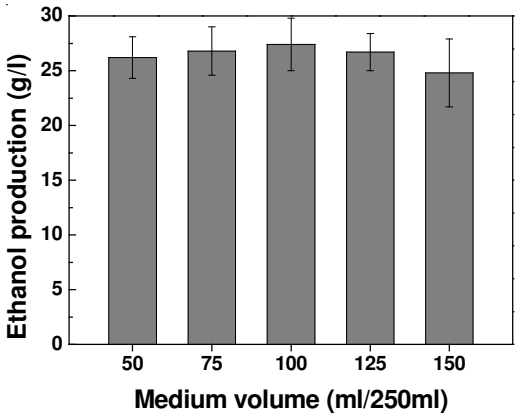

Fig. 5. Effects of medium volume on ethanol production

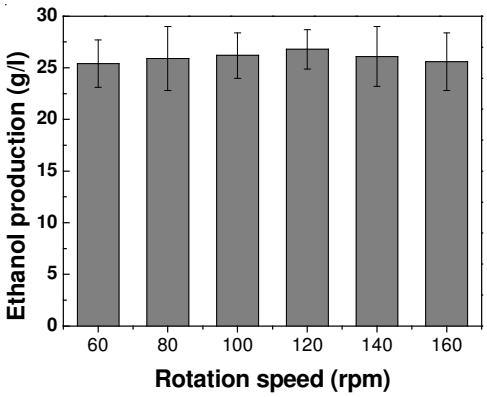

Fig. 6. Effects of swirling speed on ethanol production

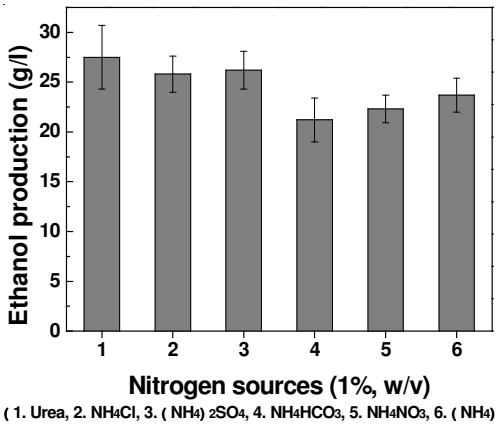

Fig. 7. Effects of nitrogen sources on ethanol production

In Fig. 3, it can be found that with the temperature raised, the ethanol production of rice grass hydrolyzate increased and reached the maximum at 32 ºC, then declined. So the optimal temperature for ethanol production by the strain *S. cerevisiae* SEMCC 2.157 should be 32 ºC. pH, medium volume and rotation speed were relative slightly influenced the ethanol production in the experiment. From the Figs. 4-6, it could be concluded that the optimal pH, medium volume and rotation speed were 5, 100 mL/250 mL flask and 120 rpm, respectively. Oxygen was not necessary during the period of ethanol biosynthesis in the fermentation, so the rotation speed was low. Nitrogen sources could affect the ethanol production significantly ($P < 0.05$) and urea was the best nitrogen source for ethanol production.

Time course of ethanol production using rice grass hydrolyzate: From Fig. 8, it could be concluded that the optimal period for ethanol fermentation by the strain *S. cerevisiae* SEMCC 2.157 was 36 h with the optimized conditions described before. And the ethanol production could reach 28.1 g/L, which was improved significantly ($p < 0.05$) than the initial basal medium. During the first 36 h of fermentation period, the ethanol production increased rapidly and the yield maintained at about 28 g/L until the fermentation was ended.

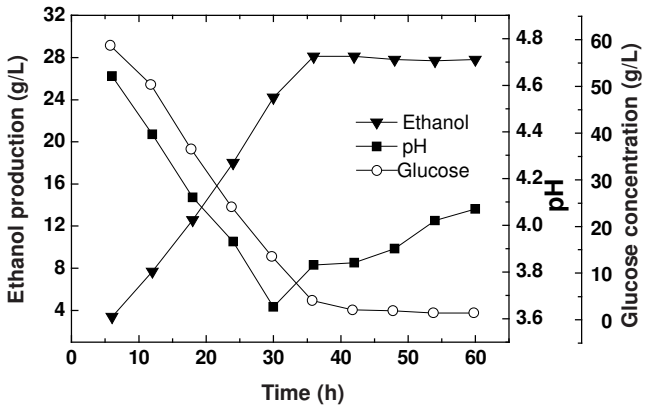

Fig. 8. Time course of ethanol production using rice grass hydrolyzate

Conclusion

In this study, the optimal techniques of the hydrolysis of rice grass was established and the conditions were: after drying the rice grass at 105 ºC, it was crushed to powders and the average particle size was 0.2-0.3 mm. The powders were pretreated with 3 % (w/v) NaOH (1:10, w/v) for 24 h at room temperature and then the pretreated rice grass powders (with concentration of 2 %, w/v) were hydrolyzed by cellulase (20 FPIU/mL, 55 ºC, 36 h) after washed by distilled water. The hydrolyzate was used for cellulosic ethanol production and the optimal conditions were: glucose concentration in the hydrolyzate was of 60 g/L, urea 10 g/L, KH_2PO_4 2.6 g/L, $MgSO_4 \cdot 7H_2O$ 0.5 g/L, inoculum size 10 % (v/v), initial pH 5, medium volume 100 mL/250 mL Erlenmeyer flask, swirling speed 120 rpm, incubation temperature 32 ºC and fermentation period 36 h. Under these conditions, the ethanol production could reach 28.1 g/L and the conversion rate of ethanol could reach 46.8 % (ethanol/glucose).

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REFERENCES

1. S.D. Hacker, D. Heimer, C.E. Hellquist, T.G. Reeder, B. Reeves, T. Riordan and M.N. Dethier, *Biol. Invas.*, **3**, 211 (2001).
2. Y.P. Dong, L. Dong, Y.Z. Jing and N. Qiang, *Trans. CSAE*, **23**, 222 (2007) (in Chinese).
3. S. Zhu, Y. Wu and Z. Yu, *Process Biochem.*, **41**, 869 (2006).
4. J.N. Nigam, *J. Biotechnol.*, **87**, 17 (2001).
5. K. Long, L. Bigot and M. Delmas, *Enzym. Microb. Technol.*, **23**, 9 (2005).
6. H.Q. Chen, W. Zhang, Z.B. Zhao, F.W. Bai and Z.B. Zhang, *Renew. Energy Resour.*, **25**, 16 (2007) in Chinese.
7. Y. Wang and T.A. McAllister, *J. Sci. Food Agric.*, **90**, 1811 (2010).
8. G.M. Miller, *Anal. Chem.*, **31**, 426 (1959).
9. S. Kim and M.T. Holtzapple, *Bioresour. Technol.*, **97**, 583 (2006).
10. Y. Fujita, S. Takahashi, M. Ueda, A. Tanaka, H. Okada, Y. Morikawa, T. Kawaguchi, M. Arai, H. Fukuda and A. Kondo, *Appl. Environ. Microbiol.,* **68**, 5136 (2002).