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Research Article

Technology Optimization of Enzymolysis of Burmuda Grass

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Abstract: Cellulose can be degraded by enzyme to glucose, which provide carbon source for ethanol fermentation. This study, taking Burmuda grass as material, analyzed effects of temperature, time, pH, PBS dosage and ratio of enzyme on reducing sugar. It came to the conclusion that the best conditions of the enzymolysis of Burmuda grass are as follows: 50°C for temperature, 32 h for time, 4.20 for pH, 2 mL for PBS dosage, 4:3 for the ratio of xylanase and cellulase, the yield of reducing sugar reached 78.63%.

Keywords: Bioethanol, cellulose, orthogonal experiment, reducing sugar, standard curve, xylanase and cellulase

INTRODUCTION

With the development of modern industry, energy has been in the limelight around the world. Nowadays, the world's energy structure is experiencing the reform from fossil energy dominated to the renewable energies' full development (Xiang-yang *et al*., 2006), renewable energy's share in energy structure will increase gradually. Biomass energy ranks the fourth energy resource among the world's primary energy consumption (Fig. 1), which is the only one to be stored and transported in all renewable energies, therefore, plays the important role in energy system (Wei *et al*., 2008; Wu *et al*., 2008).

Bioethanol is among biomass energy, the development of which can not only relief the energy shortage, but also improve and protect environment to a great degree (Yang *et al*., 2009).

Important economy entities around the world have developed bioethanol vigorously in recent 30 years, America and Brazil ranked the world top. Brail's bioethanol production focused on sugarcane, while that of America attributed to the corn (Cao and Wu, 2011). Up to now, the corn is the backbone for bioethanol production in China, which leads to competitions both in the cereal with human being and in the land with the cereal. Cellulose is a widespread biomass resource, which contributes to ethanol production.

This study, taking Bermuda grass of Poaceae as material, analyzed optimum conditions of enzymolysis (temperature, time, ratio of enzyme, pH and PBS dosage are all included), which will provide reducing sugar for the further ethanol fermentation.

MATERIALS AND METHODS

Materials: Select mature Bermuda grass in Campus of Henan University of Urban Construction, wash and dry, then shatter materials over 80 mesh screen. The temperature of electric drying oven is fixed at 70°C.

Main reagents:

- DNS reagent (Jun-Hong and Xiang-Zhen, 2010)
- **Double enzyme:** cellulase and xylanase (produced by the jade of the He Family Biological Technology Co., Ltd.)
- PBS (Phosphate Buffered Saline)

Appatus: Plant crusher, pH appratus, Paradigm 722 spectrophotometer, Steam sterilization pot with high-press, Electric drying oven with wind-drumming, thermostat water bath etc.

Analysis method: Standard curve of glucose:

- Weigh defined amount of glucose and dry it in electric drying oven with wind-drumming at 70°C till constant weight, after which weigh 0.1 g dried glucose and make into standard glucose solution of 1 mg/mL.
- According to the design in Table 1, put standard glucose solution into test tubes, then dilute to 2 mL with distilled water, thereafter, add 2 mL DNS reagent and stir completely.
- Put test tubes into boiling water bath for 6-8 min, take out and cool to room temperature.

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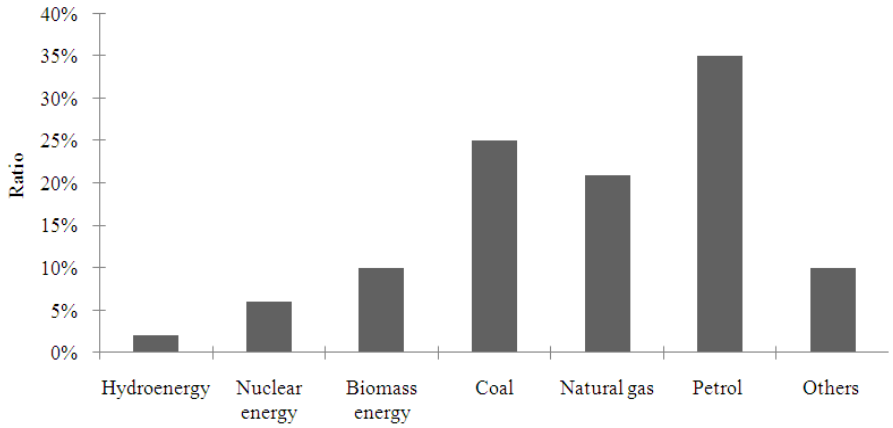

Fig. 1: Main energy demand of the world in 2005 (The International Energy Agency, 2007)

Table 1: The course of standard curve of glucose

Number of test tube	1	2	3	4	5	6	7	8
Standard glucose solution/mL	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7
Distilled water/mL	2.0	1.9	1.8	1.7	1.6	1.5	1.4	1.3
DNS reagent/mL	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0

- Measure the OD value at the wavelength of 550 nm by paradigm 722 spectrophotometer, by which the standard curve of glucose is made.

Glucose standard curve is shown in Eq. (1), in which x refers to the concentration of glucose, y refers to OD value:

$$y = 6.934x - 0.1403 \ (R^2 = 0.99) \ (x:mg/mL, y:OD \ value) \quad (1)$$

Reducing sugar yield = 0.9* the number of reducing sugar (mg/mL) *diluted ratio* the volume of hydrolysis solution/the weight of material.
Note: 0.9 is the error of calculation.

Research content: After pretreatment, cellulose degradation solution goes through enzymolysis to heighten the degradation rate. Taking reaction time, temperature, the amount of buffer solution, pH, xylanase-cellulase ratio as influencing factors and reducing sugar yield as objective, carry out single factor experiment, by which design orthogonal experiment to optimize reaction conditions, further increasing reducing sugar yield.

Weigh 1 g material, add 10 mL diluted hydrochloric acid of 1.0% and react with each other at 120°C for 60 min. Cool to room temperature, put PBS into reaction system and adjust the pH, then add double enzyme to initiate the enzymolysis.

When the reaction is completed, centrifuge the reaction solution for 7 min at the speed of 3600 r/min, take out the supernate and dilute, add 2 mL diluted solution and 2 mL DNS reagent to small test tubes, mix fully, next, put them to boiling water bath for 7 min, after cool to room temperature, measure OD value at the wavelength of 550 nm, write down the data.

By single factor experiment, design orthogonal experiment, obtain the optimum conditions of enzyme hydrolysis, providing enough carbon source for the subsequent fermentation.

RESULTS AND ANALYSIS

Effects of temperature on double enzymolysis: In the reaction system, xylanase-cellulase ratio is 4:4, the volume of PBS is 2 mL, adjust the pH to 4.6, reaction time is set to 40 h, reaction temperature ranges from 35 to 60°C, whose increasing gradient is 5°C. The results are shown in Fig. 2.

Figure 2 indicates that reducing sugar yield increases with the gradually rising temperature, which gets to the uttermost, 43.94%, when the temperature climbs to 45°C. If the temperature continues to go up, efficiency of enzymolysis declines. In enzyme-catalyzed reaction, the temperature affects both reaction rate and activity of enzymes, high temperature can results in the denaturation of enzymes.

Effects of reaction time on double enzymolysis: In the reaction system, xylanase-cellulase ratio is 4:4, the volume of PBS is 2 mL, adjust the pH to 4.6, reaction temperature is set to 45°C, reaction time ranges from 16 to 56 h, whose increasing gradient is 8 h. Effects of time on double enzymolysis are shown in Fig. 3.

As indicated in Fig. 3, at the beginning, with the increase of time, reducing sugar yield undergoes increase, accompanied by the reduced growth rate, 40 h later, reducing sugar yield amounts to 55.89%, after which the result decreases. With the reaction going on, products inhibit the activity of the enzyme, followed by the decreased reaction rate, which is in accordance with the kinetic equation of enzymatic reaction:

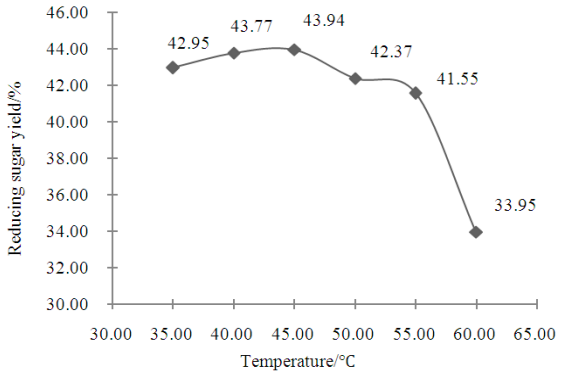

Fig. 2: Effects of temperature on enzymolysis

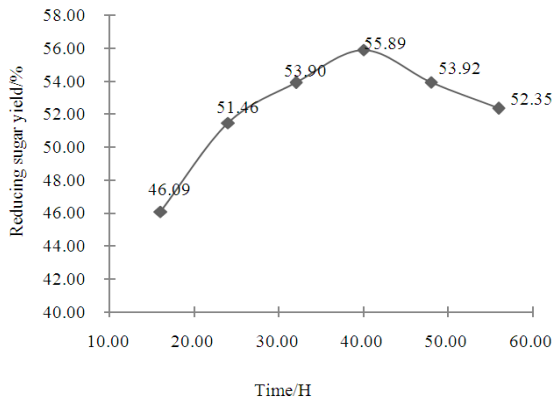

Fig. 3: Effects of reaction time on enzymolysis

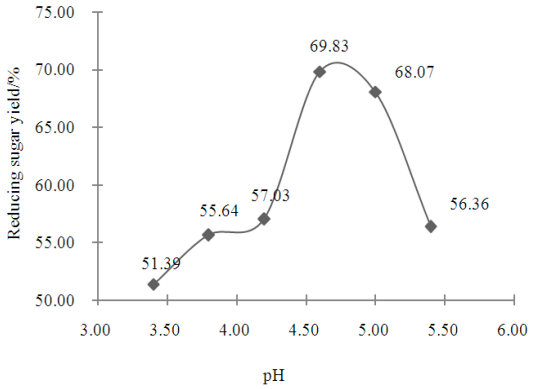

Fig. 4: Effects of pH on enzymolysis

$$S + E \overset{km}{\leftrightarrow} [SE] \overset{kn}{\leftrightarrow} S + P$$

As to the initial reaction, the concentration of composite, formed by enzyme and substrate, is very low, there is little product, therefore, feedback inhibition can be found nowhere. Reaction keeps on going, the amount of reducing sugar experiences continuous rise, which enhances feedback inhibition, leading to low reaction rate.

Effects of pH on double enzymolysis: In the reaction system, xylanase-cellulase ratio is 4:4, the volume of PBS is 2 mL, reaction temperature is set to 45°C and reaction time is limited to 40 h, adjust the pH of reaction system, make it range from 3.4 to 5.4, whose increasing gradient is 0.4. Effects of pH on double enzymolysis are shown in Fig. 4.

Data in Fig. 4 suggests that pH affects the result dramatically. Reducing sugar yield reaches 69.83% by the pH 4.60, namely, this value is most suitable to the enzyme. Strong acid or base can destroy spatial structure of the enzyme, resulting in activity loss. If the change in pH is weak, even if denaturation doesn't exist, activities of enzymes are affected to some degree. Reasons are as followed. Firstly, pH affects the dissociation state of substrates, making it impossible for the combination of enzyme and substrate, or preventing the combination from product-generation. Secondly, pH inhibits the dissociation of relevant group in active site of enzymes, followed by effects on combination of substrates and enzymes, reducing the activity of enzymes, or the dissociation of middle complex (ES) is affected, which goes against the formation of products (Wang *et al*., 2002).

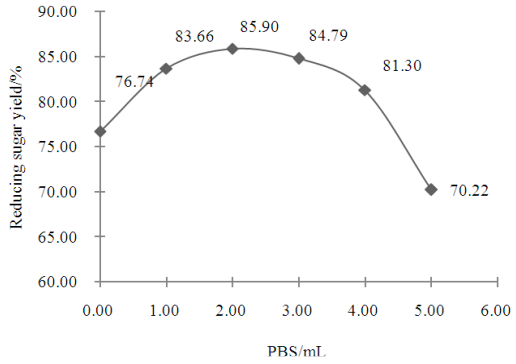

Fig. 5: Effects of PBS dosage on enzymolysis

Effects of PBS dosage on double enzymolysis: In the reaction system, xylanase-cellulase ratio is 4:4, add certain number of PBS, the volume of PBS ranges from 0 to 5 mL, whose increasing gradient is 1 mL. Reaction temperature is set to 45°C and reaction time is limited to 40 h, adjust the pH to 4.6, effects of PBS dosage on enzymolysis are shown in Fig. 5.

It is obvious in Fig. 5 that reducing sugar yield increases with the increase of PBS dosage at first, the PBS dosage of 2 mL contributes to the highest reducing sugar yield, 85.90%. When PBS dosage keeps rising, the result undergoes decline. For the increase of PBS leads to the dilutability augment of saccharification solution, which is against the reaction of enzymes and substrates.

Effects of xylanase-cellulase ratio on double enzymolysis: The xylanase-cellulase ratio is set to be 4:4, 4:2, 4:3, 4:4, 3:4 and 2:4 in turn.

In the reaction system, add 2 mL PBS, mix with xylanase and cellulase of fixed ratio, reaction temperature is set to 45°C and reaction time is limited

Table 2: Factors and level of orthogonal experiment

	Temperature/A (°C)	Time/B (h)	pH/C	PBS dosage/D (mL)	Xylanase-cellulase ratio/E
1	35	24	4.2	1	4:2
2	40	32	4.6	2	4:3
3	45	40	5.0	3	4:4
4	50	48	5.4	4	3:4

Table 3: Orthogonal experiment results and intuitive analysis table

Number/level/factor	Temperature/A	Time/ B	PH/C	PBS dosage/D	Xylanase-cellulase ratio/E	Reducing sugar yield/%
1	1	1	1	1	1	66.43
2	2	1	2	2	2	70.91
3	3	1	3	3	3	66.25
4	4	1	4	4	4	63.06
5	1	2	2	3	4	72.16
6	2	2	1	4	3	70.10
7	3	2	4	1	2	69.34
8	4	2	3	2	1	70.99
9	1	3	3	4	2	66.65
10	2	3	4	3	1	45.84
11	3	3	1	2	4	66.91
12	4	3	2	1	3	64.91
13	1	4	4	2	3	67.36
14	2	4	3	1	4	60.34
15	3	4	2	4	1	68.30
16	4	4	1	3	2	75.45
K1	272.6000	266.6500	278.8900	261.0200	251.5600	T = 1065
K2	247.1900	282.5900	276.2800	276.1700	282.3500	
K3	270.8000	244.3100	264.2300	259.7000	268.6200	
K4	274.4100	271.4500	245.5800	268.1100	262.4700	
k1	68.1500	66.6625	69.7225	65.2550	62.8900	
k2	61.7975	70.6475	69.0700	69.0425	70.5875	
k3	67.7000	61.0775	66.0575	64.9250	67.1550	
k4	68.6025	67.8625	61.3950	67.0275	65.6175	
R	6.8050	9.5700	8.3275	4.1175	7.6975	

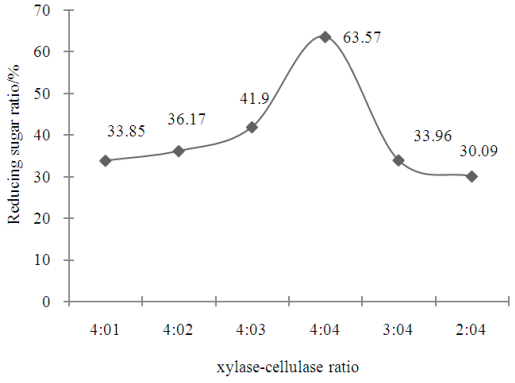

Fig. 6: Effects of xylanase-cellulase ratio on enzymolysis

to 40 h, adjust the pH to 4.6, effects of xylanase-cellulase ratio on double enzymolysis are shown in Fig. 6.

From Fig. 6, it is clear that reducing sugar yield arrives at the maximum, 63.57%, when the xylanase-cellulase ratio is 4:4, with the change in it, the result reduces, which suggests that there exists complementary function between the two enzymes. Research indicates that xylan with high concentration inhibits the activity of cellulase, while the inhibition can be relieved by the increase in the ratio of xylanase-xylosidase, thus, improving the conversion rate of cellulose (Zhao *et al*., 2011; She *et al*., 2009).

Orthogonal experiment and analysis: By the result and analysis of single factor experiment, arrange orthogonal experiment, by which optimum conditions of double enzymolysis are achieved, hence, supply carbon source for the followed fermentation processing. Orthogonal experiment design and results are shown in Table 2 and 3.

Intuitive analysis of orthogonal experiment suggests that the best combination of influencing factors is $A_4\ B_4\ C_1\ D_3\ E_2$, whose reducing sugar yield comes to 75.45%. According to the result of range analysis, the optimum condition belongs to $A_4\ B_2\ C_1\ D_2\ E_2$, which is not included in the arrangement of orthogonal experiment. With the aim of testifying the above two results, develop parallel experiment, which suggests that reducing sugar yield amounts to 78.63% by $A_4\ B_2\ C_1\ D_2\ E_2$. So the final optimum condition of double enzymolysis lays down on $A_4\ B_2\ C_1\ D_2\ E_2$.

DISCUSSION

Cellulose is difficult to be degraded, which leads to the low yield of reducing sugar, reasons are as follows:

- In the course of preparing saccharification solution from Burmuda grass, cellulose is strong in ductibility, furthermore, semi-cellulose integrates with lignin by covalent bond, forming the firm barrier, in which cellulose is imbedded, it is very difficult to destroy the barrier of lignin and semi-cellulose and break the crystal structure of cellulose (Zhao and Liu, 2008). Pretreatment and enzymolysis are incapable of breaking the barrier completely, giving rise to the low reducing sugar yield.

- There exists incomplete degradation by double enzymolysis. Research suggests that cellulose occupies the majority of the rest substance after pretreatment, which can be degraded by 3 enzymes. Endonucleases of β1, 4-glucan enzymes functions at interior of cellulose linkage, cellobiohydrolase breaks the end of polymers, βglucosidase acts on cellobiose, which converts into two molecules glucoses (Chen *et al*., 2010). In addition, ligninase and hemicellulase play important roles in degradation, which eliminate barriers for cellulose hydrolysis. In consideration of the high cost of enzymes, in this research only xylanase and cellulase are adopted to degrade cellulose.

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