Cellulase Production by Solid-state Fermentation of Marine Aspergillus ZJUBE-1 and Its Enzymological Properties

Cellulase Production by Aspergillus ZJUBE-1

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Abstract—Solid-state fermentation conditions were investigated for cellulase production by marine Aspergillus ZJUBE-1 using crop straw as the main material. When Aspergillus ZJUBE-1 was grown under the condition: ratio of rice straw to wheat bran was 1:1, the ratio of material to liquid was 1:2, the ratio of nitrogen and carbon was 5.6%, NaCl was 2.4~4.0%, temperature at 37 ℃, the maximum CMCase activity was 4705U.g⁻¹, β-Glucase (2750U.g⁻¹), and FPA (245U.g⁻¹) activities were obtained after 9-10 days of fermentation, respectively. Enzymological properties were studied, too. The optimum reaction temperature for the three kinds enzyme was 65 ℃ and the optimum reaction pH was 4-5. Keep 80 ℃ for 1 hour, CMCase still keeping 10% of the activity, 70 ℃ for 1 hour, β-glucosidase activity only loss 60%. Fe²⁺, Mn²⁺, Mg²⁺ was activator to cellulase, but Hg²⁺, Cu²⁺, Ag⁺ can inhibition strongly to cellulase activity.

Keywords—marine Aspergillus sp; solid-state fermentation; cellulase; enzymologic properties

I. INTRODUCTION

In recent years, the shortage of energy and resources has received extensively attention around the world. Cellulose is the cheapest and the most abundant renewable resources on earth. It is an effective way for low carbon and sustainable development to produce fuel, feed and chemical products by fermentation with agricultural wastes. Many studies show that the cellulose plays an important role in several areas including the use of renewable resources [1-2], development of bio-energy [3-4] and protection of environment [5-6].

There are two ways for the production of cellulose. One is solid-state fermentation, another is submerged fermentation. Both of these two methods have advantages, solid-state fermentation is much better in using of reactor and enzyme production. Therefore, industrial production of cellulose is more frequently done by the method of solid-state fermentation [7].

Three enzymes including endo-β-1,4-glucanases (EC 3.2.1.4), exo-β-1,4-glucanases (EC 3.2.1.91) and cellobiases (EC 3.2.1.21) are necessary for completely degradation of cellulose to glucose [8]. Most microorganisms are able to produce cellulose, but just fungal could produce the highest amount of cellulose. Trichoderma, Aspergillus, Rhizopus spp and myrothecium have been studied extensively [9]. As the activity of cellobiases produced by Aspergillus Niger is high, which avoiding repression effect by enzymatic hydrolysate (cellobiose). Furthermore, cellulose produced by A. niger are safe and non-toxic. Therefore, A. niger becomes one of the main strains for investigating microbial cellulose.

In this work, a new marine Aspergillus (ZJUBE-1) was screened and obtained from soil of 20-30 meters depth of East Sea. Its behavior on production of cellulase through solid-state fermentation using crop straw as substrate was investigated. The solid-state fermentation conditions were also optimized. The work would be useful for developing new marine cellulase and expanding the application of cellulose.

2 Material and Methods

2.1 Strain

The strain for cellulose production was isolated from soil of sea (depth of 20-30 meters) near Zhengjiang in our laboratory. It was identified as Aspergillus niger according to morphology of strains and sequence analysis of 18srDNA (named ZJUBE-1).

2.2 Medium

Slant culture-medium: yeast extract 0.4%, peptone 0.6%, soluble starch 1%, glucose 0.9%, potato extract solution of 7%, agar 2%, preparation with seawater, pH is adjusted to 7.0-7.4. Solid culture medium: Straw powder, bran, ammonium chloride, artificial seawater, making with a specific ratio, pH is not adjusted.

2.3 Culture condition

Preparation of spore suspension: the Aspergillus spores on the slant medium were firstly washed with a small amount of sterile artificial sea water, and then the solution was stirred with glass beads for 15 to 20 minutes. Fully disperse spores, and diluted spore suspension with concentration 5×10⁷ conidia/ml.
Solid-state Fermentation: 3 g medium in 100 ml flask, turn over mouldy substrate (koji) once after inoculating for 48 hours, add sterile water to maintain substrate moisture on the fourth or fifth day. Change fermentation conditions including composition of carbon, nitrogen, inoculum concentration and the ratio of material and water. Determine enzyme activity after inoculating for 7-8 days.

2.4 Preparation of crude enzyme solution
1g fresh mouldy substrates were soaked in 20ml distilled water, the mixture were allowed to stand for 1 h (30 °C, 100rpm) and extracts were obtained by filtering the mixtures through gauze. Then the filtrate was centrifuged at 4 °C, 5000rpm for 15min, the supernatant was crude enzyme solution.

2.5 Determination of enzyme activity and definition of enzyme activity
Content of reducing sugar: the reducing sugar (glucose) levels in enzyme solution were shown as the enzyme activity by 3, 5-dinitrosalicylic acid (DNS) assay. Drawing standard curve was according to previous report [10]. Measurement of activities of three enzymes were according to previous reports [10, 11] with slight midify as follows.
(1) Activity of CMCase: Add 0.5ml diluted enzyme solution into 2.0ml 1% (w/v) CMC-Na solution (dissolved in 0.1mol / L sodium citrate - citric acid buffer, pH 4.5), reaction at 60 °C for 30 minutes, then 2.5ml DNS was add and keep in boiling water bath for 5 minutes to stop the reaction, measure OD at 540 nm, the inactivated enzyme was used as control.
(2) Activity of β-Gluase: method is the same as the previous one. The substrate was changed with 0.5% (w/v) Salicylic acid (dissolved in acetic acid - sodium acetate buffer, pH 5.0).
(3) Activity of FPA: Add 0.5ml diluted enzyme solution and 2.0 ml, pH 4.5 sodium citrate - citric acid buffer on 50 mg filter paper (1x6 cm), reaction at 50 °C for 60 minutes, then 2.5ml DNS was add and keep in boiling water bath for 5 minutes to stop the reaction, measure OD at 540 nm.

Definition of enzyme activity: In the above reaction conditions, the amount of enzyme required to produce 1 mg glucose hydrolyzed by one gram mouldy substrate (koji) per hour, defined as an enzyme activity unit (U), namely U/g.

2.6 Enzymatic Properties
(1) Effect of pH
Measurement of enzyme activity of crude enzyme solution at pH value 3.0, 4.0, 5.0, 6.0, 7.0, 8.0 respectively, determine the optimum pH value. The crude enzyme solution was diluted with the above pH buffer respectively, kept in 45 °C water bath for 4 hours, enzyme activity was then determined.
(2) Effect of temperature
Measuring enzyme activity between 40-95°C for determination of the optimum reaction temperature. Keep enzyme solution at different temperature points between 40-95°C for 60 min, enzyme activity was then determined.
(3) Effect of metal ions
Add several different metal ions into enzyme solution with a final concentration of 4 mmol / L, and then enzyme activity was determined respectively.

Reaction conditions were pH4.5 (CMCase) or pH5.0 (β-Gluase), 50 °C for 30 min, the untreated control enzyme dilutions were used as control for calculation of relative enzyme activity.

Definition of enzyme activity: In the above reaction conditions, the amount of enzyme required to produce 1μmol glucose hydrolyzed by 1 ml enzyme solution per minute, defined as an enzyme activity unit (U), namely U/ml.

3 Results and discussion
3.1 Effect of different types and ratio of carbon source on the production of cellulase by bacterial strain
In order to investigate the effect of different carbon sources of natural fiber on the production of cellulase by Aspergillus ZJUBE-1, cheap straw, by-products like straw powder, corn stalk powder, Yellow pod powder, rice hulls or wheat bran was individually used as the only carbon source. As shown in Fig. 1, results showed that ZJUBE-1 has better ability on utilization of crop straw in solid culture. ZJUBE-1 grew faster and got the highest cellulase activity when wheat bran was used as carbon source. Wheat bran, contains not only cellulose, hemicelluloses and lignin, but also more vitamins (content of vitamin B is about 15μg g⁻¹), trace mineral (content of iron is about 146.215μg·g⁻¹) and protein (15%) when compared to other crop straw [12], which make it more suitable for the growth of bacterial strain, synthesis and secretion of enzyme. On the other hand, the activity of cellulase was low when rice hulls were used as the only carbon source. It may because of the smooth, dense and hydrophobic silica membranes and high content of lignin (21%-26%) on the surface of rice hulls [14]. These results may not only due to enough nutrition for microorganism growth was available from combined carbon sources, but also due to the function of straw powder include increasing permeability of medium and making medium more fluffy.

In order to figure out whether the effect of combined carbon source is better than single carbon source, wheat bran was then used to combine with other fibrous carbons mentioned above with ratio of 1:6 to test the effect of combined carbon source. Results showed that the activity of cellulase in both of the

![Fig. 1 Effect of carbon source species on cellulase production](image-url)
combined carbon source 1 (wheat bran + straw powder) and combined carbon source 2 (wheat bran + corn stalk powder)
were higher than that in single wheat bran. And other combined carbon sources also showed similar results.

As rice is the main food crops in southern China, the combined carbon sources (wheat bran and straw powder) were used in follow-up experiments. Firstly, effect of different ratio of wheat bran to straw powder on the production of cellulase was investigated. As shown in Fig. 2, ratio of 1:1 (wheat bran to straw powder) showed the best effect for the production of cellulase by bacterial strain. The activity of CMCase and β-Gluase increased 45% and 23% respectively, compared with that of single carbon source (wheat bran). As shown in Fig. 3, by comparing the range of enzyme activity increase between from day 5 and day 8 after cultured, β-Gluase was found to be mainly synthesized and secreted at late period of fermentation.

3.2 Effect of different types and amount of nitrogen sources on enzyme production by Aspergillus ZJUBE-1

Effect of different types of nitrogen sources on cellulase production by Aspergillus ZJUBE-1 was also investigated. Because all natural fibrous carbon sources contain a amount of organic nitrogen like protein, just inorganic nitrogen sources were changed in this experiment. As shown in Fig. 4, results showed that there was no obvious difference among different inorganic nitrogen sources on enzyme production by Aspergillus ZJUBE-1. This result indicates that the selectivity of Aspergillus ZJUBE-1 to inorganic nitrogen is not high.

The ratio of carbon to nitrogen required for production of cellulase by Aspergillus was quite different in different culture system reported by previous studies. Therefore, it is necessary to analysis the effect of different ratio of carbon to nitrogen on cellulase production by Aspergillus ZJUBE-1. Ammonium chloride was taken as nitrogen source here. Result showed that, as shown in Fig. 5, the nitrogen content is enough to meet requirements of strain growth and cellulase production when the percentage of nitrogen source increased to 5.6% of the amount of carbon source (representing 1.2% of total medium). Cellulase production was inhibited when the content of nitrogen source increased further (Fig. 5). The ratio of carbon to nitrogen in fungal cells is about 10:1, so the optimum ratio of carbon to nitrogen in fermentation medium should be larger than 10:1. The natural fiber substance is a complex nutrient, which can not only provide carbon and mineral elements, but also nitrogen in plant cells for growth of microbial. For example, content of protein in wheat bran is more than 10%. Therefore, the amount of nitrogen added into medium for production of cellulase could be reduced accordingly.

3.3 Fermentation conditions

3.3.1 Effect of temperature on cellulase production by solid-state fermentation of Aspergillus ZJUBE-1

For identifying the optimal solid-state fermentation conditions for the largest cellulase production by Aspergillus ZJUBE-1, effect of culture temperature on cellulase production by Aspergillus ZJUBE-1 was also tested. The activity of CMCase and β-Gluase were determined after inoculating for 5 day and 8 day in different culture temperatures respectively. Result showed that the activity of CMCase and β-Gluase slightly increased from 20℃-32℃; then the activity of CMCase and β-Gluase dramatically increased from 32℃-37℃ and reached the highest level at 37℃ (Fig. 6). The activity of CMCase and β-Gluase dramatically decreased thereafter (Fig. 6). The opti-
The optimal temperature for growth of most fungi is 28°C-32°C. And several previous studies reported that the optimal temperature for production of cellulase by Aspergillus was about 30°C [15-17]. However, our result here showed that the optimal temperature for growth of Aspergillus ZJUBE-1 and cellulase production was about 37°C. And Aspergillus ZJUBE-1 could still grow and be able to produce cellulase even at 50°C. These results suggest that the temperature tolerance of marine Aspergillus (ZJUBE-1) isolated by us is higher than that of terrestrial Aspergillus.

3.3.2 Effect of the ratio of material to liquid on cellulase production by solid-state fermentation of Aspergillus ZJUBE-1

One of the most important features of solid state fermentation is that there is no free water in its fermentation system. As microorganism need to growth on solid-state substance with sufficient moisture, different water content in medium should has a significant impact on growth and metabolism of microorganism. If the content of water is too low, straw material can’t fully expand, and may easy to dry due to evaporation of water, which will inhibit the growth of microorganisms in the late period of solid state fermentation. On the other hand, if the content of water is too high, it will cause agglomeration of matrix, inhibition of mycelia growth fermentation and reduce of matrix porosity which will increase resistance of oxygen transfer and be harmful to cellulase production. Therefore, it is worth investigating effect of the ratio of material to liquid on cellulase production by solid-state fermentation of Aspergillus ZJUBE-1. As shown in Fig. 7, the activity of CMCase and β-Glucase reached the highest level when the ratio of material to liquid is 1:2. The activity of CMCase and β-Glucase gradually decrease as the content of water increase (Fig. 7).

3.3.3 Effect of inoculums on cellulase production by solid-state fermentation of Aspergillus ZJUBE-1

In order to further test the effect of inoculums on cellulase production by Aspergillus ZJUBE-1, just inoculation volume of spore suspension which will be added into medium was changed. Result showed that the best ratio of spore suspension to solid materials for the production of CMCase was 1:1.5(v/w) (Fig. 8). But inoculation volume of spore suspension has little influence on the production of β-Glucase (Fig. 8).

3.3.4 Determination of incubation time on cellulase production by solid-state fermentation of Aspergillus ZJUBE-1

Effect of incubation time on cellulase production by solid-state fermentation of Aspergillus ZJUBE-1 was also analyzed in this work. The activity of CMCase and β-Glucase was measured each 24 hours from the fourth day after inoculation. Result showed that Aspergillus ZJUBE-1 exhibit the best growth rate from the third day to the fifth day after inoculation while the highest activity of CMCase and β-Glucase was reached on day 9 or day 10 after inoculation (Fig. 9).

3.4 Effect of concentration of sodium chloride (NaCl) on cellulase production by solid-state fermentation of Aspergillus ZJUBE-1

Because Aspergillus ZJUBE-1 is come from marine, salinity should have a great impact on growth of Aspergillus ZJUBE-1.
3.5 Enzymological properties of crude cellulase

3.5.1 The optimum pH value of enzyme reaction and the pH stability of the enzyme

The activity of cellulase in crude cellulase solution in different pH was measured at two time points (0 hours and 4 hours after treatment) respectively. As shown in Fig. 11, result showed that the activity of CMCase was high in pH 3.5-4.5 and the optimal pH for CMCase was 4.0; the activity of β-Gluase was high in pH 4.5-5.5 and the optimal pH for β-Gluase was 5.0. The optimal pH for FPA was confirmed to be 5.0 with the same method. These results indicate that all the cellulases produced by Aspergillus ZJUBE-1 are acidic.

It is also obvious that activity of both CMCase and β-Gluase could maintain more than 80% at pH range of 3.0-6.0 and 3.5-5.5 respectively (Fig. 11). This suggests that cellulases produced by Aspergillus ZJUBE-1 are able to maintain the stability of the enzyme activity in a wide range of pH values.

3.5.2 The optimum temperature for enzyme reaction and thermal stability of the enzyme

The activity of cellulase at different temperatures once the enzymatic reaction starts and relative activity of cellulase at different temperatures 60 minutes after reaction was measured respectively. Result showed that the optimum temperature for CMCase, β-Gluase and FPA was 65 °C (Fig. 12). It can also be seen that enzyme activity remained stable when the temperature was lower than 60 °C and CMCase showed better thermal stability than β-Gluase. CMCase can maintain 90% of its activity when the temperature was lower than 65 °C and can maintain more than 50% of its activity when the temperature reached 70 °C (Fig. 12). But heat tolerance of both CMCase and β-Gluase was significantly decreased when the temperature is higher than 65 °C (Fig. 12).

3.5.3 Effect of metal ions on enzyme activity

In this work, effect of 14 metal ions on enzyme activity was investigated. Relative enzyme activity was calculated according to enzyme activity of enzyme solution without adding any metal ions. As shown in Table 1, Fe²⁺ and Mn²⁺ dramatically activated the enzyme activity of CMCase and β-Gluase and Fe³⁺ slightly activated the enzyme activity of CMCase and β-Gluase, while Hg²⁺, Cu²⁺, Ag²⁺ strongly inhibited enzyme activity of CMCase and β-Gluase. Other tested metal ions didn’t show obvious influence on enzyme activity of CMCase and β-Gluase.
4 Conclusion

This paper reported the cellulase production from Marine aspergillus ZJUBE-1 under solid state fermentation. The results indicates that ZJUBE-1 could induced to produce cellulase using straw and bran coat of crops as carbon and nitrogen source, with the most distinctive induction by bran coat. Under mixed solid state fermentation of straw and bran coat, various kinds of nutrient composition the fungus need was balanced, and the straw powder can help to fluff out the medium and improve air permeability, so the cellulase production was enhanced largely. The bacterial strain has low selectivity and demand of Inorganic nitrogen source. The optimal condition is as follows: straw powder: bran coat 1: 1 (w/w), NaCl concentration 2.4-4.0%, spore suspension: solid medium 1: 1.5 (v/w), solid-liquid ratio 1: 2, normal PH, and after 9 or 10 d of 37℃ cultivation. The CMCase, β-Gluase and FPA activities can reach 4705 U.g⁻¹, 2750 U.g⁻¹ and 245 U.g⁻¹ respectively.

The characterization of coarse cellulase liquid shows that, the optimal tempreature of CMCase, β-Gluase and FPA is 65℃. When preserved for 1h under 65℃, the enzyme activities can still exceed 80%; after 1h preservation under 80℃, CMCase activity remained 10%; β-Gluase lost only 60% activity after 1h under 70℃ and still produce activity under 90℃. In contrast, normal cellulase has the suitable tempreature range of 45-55℃, and almost lost all the activity above 70℃. The optimal PH value of CMCase, β-Gluase and FPA is 4.0, 5.0, 5.0 respectively, and have good stability within the range 3.5-5.5. Fe²⁺, Mn²⁺ and Mg²⁺ have activation on CMCase and β-Gluase, while Hg²⁺, Cu²⁺ and Ag²⁺ have strong inhibiting effect on the enzyme activities.

Although cellulase has been commercialized at 1960s, the present cellulase still have some defects such as relatively low activity, unintegrated enzyme system, especially the lack of extreme cellulase which can tolerate particular environment like high salt, strong acid, strong alkali and low temperature. As a result, the application of cellulase is limited. For this reason, the work reported here has a positive significance for exploring industrial and market potential new cellulase as well as efficient utilization of crop staws.

References