PRODUCTION OF CELLULASE BY *Trichoderma viride* UNDER DIFFERENT FERMENTATION CONDITIONS

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ABSTRACT

Trichoderma viride has been widely to produce cellulase. The strain studied has the ability to fix nitrogen and produce ethylene that could reach 1.3×10^{-6} mol·L^{-1}·d^{-1}. In order to utilize this strain better, the production of different cellulolytic enzymes under different fermentation conditions was studied. The results showed that the optimal ratio of rice straw to wheat bran was 3 to 2 or 1 to 1; the optimal nitrogen source was NH_4Cl; the optimal growth temperature was 27-30 °C and the highest production of cellulose enzyme was obtained on the fourth day using solid culture with rice straw and wheat bran. The production of β-glucosidase was very low using liquid medium (Mendel's), however, the production could be enhanced when 0.2% glucose was added to the culture.

Keywords: cellulase, fixing nitrogen, ferment conditions, β-glucosidase

1. INTRODUCTION

Every year about 580 billion tons crop straw is produced, most of which is burned. This is a waste of natural resources, and also pollutes the environment. So this subject became a focus of study. *Trichoderma spp.* are cellulolytic fungi. During past few decades usefulness of microbial polysaccharides for food, pharmaceutical, petroleum, and other industry applications has been established. However, the degradation of cellulose, nitrogen shortage is the limiting factor (as the higher ratio of carbon to nitrogen). By selection, we obtained a strain which can fix nitrogen, so it could better degrade cellulolytic resources. In order to utilize the strain better, the fermentation conditions and primary cellulases production were studied.

2. MATERIALS AND METHODS

2.1 Microorganisms

The laboratory strain T_4 of *Trichoderma viride*, isolated from soil, was used in the present investigation .

2.2 Methods

2.2.1 Measurement of nitrogenase activity

This was performed according to the acetylene reduction method

2.2.2 Optimum of fermentation conditions

2.2.2.1 The effect of different fermentation times

Three grams of straw powder, 2 g of wheat bran and 10 ml of 1.0 % $(NH_4)_2SO_4$ were put into each of four 250 ml flasks, and the contents were mixed and then sterilized. Three mL (6.8×10^6 cfu/mL) of T_4 spore suspension were inoculated in the solid cultures which were incubated for 3 days, 4 days, 5 days, 6 days, respectively at 30 °C. Then crude enzyme was extracted by filter and dialyzed by distilled water. After that, cellulase activity was measured as described below.

Enzyme extraction and assays: Cellulases were extracted by suspending the fermented straw in 50 mL distilled water and at 37 °C in water bath for 1 h, shaking every 15 min. The mixture was filtered, and then supernatant dialyzed by distilled water. The dialyzed solution was used as the source of the crude enzyme. Enzyme activity on filter paper (FPA), cotton (C_1) and carboxymethylcellulose (CMC-Cx) was measured respectively to determine the ability to degrade cellulose. The measurement of cellulolytic enzymes was according to Abdulah (1985) by using DNS (3, 5 - Dinitrosalicylic acid) method. The protein content was determined by Folin-phenol method.

2.2.2.2 The effect of different ratios of straw to bran

Total content of straw and bran was 5 g in each 250 mL flask. The ratio of straw to bran was 1 to 4 , 1.5 to 1.5, 2 to 3, 2.5 to 2.5, 3 to 2, 3.5 to 1.5 and 4 to 1, respectively , Ten ml of 1.0% $(NH_4)_2SO_4$ were added to each flask after mixing and sterilization. The flasks were inoculated and incubated for 4 days at 30 °C, as described above.

2.2.2.3 The effect of different nitrogen source

The basic fermented culture: 2.5g straw , 2.5g bran , 2.0% nitrogen , 10ml distilled water .

Supplying nitrogen sources: $NaNO_3$, $(NH_4)_2SO_4$, $(NH_4)_2SO_4$, bean cake (1:1), urine, bean cake, NH_4Cl, NH_4NO_3, yeast extract

Inoculation and fermentation conditions were as described above.

2.2.2.4 The effect of oxygen

Three grams of straw powder, 2 g of wheat bran and 10 mL of 1.0 % $(NH_4)_2SO_4$ were put into 50 mL, 100mL, 150 mL and 250 mL flasks, respectively, and the contents were mixed and then sterilized. Three mL (6.8×10^6cfu/mL) of T_4 spore suspension were inoculated in the solid cultures which were incubated for 4 days at 30 °C. Then crude enzyme was extracted. Enzyme

activity and protein content were measured as described above.

2.2.2.5 The effect of cultural temperature

Three grams of straw powder, 2 g of wheat bran and 10mL of 1.0% $(NH_4)_2SO_4$ were put into each of four 250 mL flasks, and the contents were mixed and then sterilized. Three mL (6.8×10^6cfu/mL) of T_4 spore suspension were inoculated in the solid cultures which were incubated for 4 days at 20 °C, 25 °C, 27 °C, 30 °C, 35 °C, 37 °C, respectively. Then crude enzyme was extracted and enzyme activity was measured as described above.

2.2.2.6 The effect of different cultural methods

Solid culture: Three grams of straw powder, 2 g of wheat bran and 10 mL of 1.0 % $(NH_4)_2SO_4$ were put into each of four 250 mL flasks, and the contents were mixed and sterilized. Three mL (6.8×10^6 cfu/mL) of T_4 spore suspension were inoculated in the solid cultures which were incubated for 4 days. Then crude enzyme was extracted and enzyme activity was measured as described above.

Liquid state culture: Fifty mL of Mendel's medium were put into each of four 200 mL flasks. Two mL (6.8×10^6cfu/mL) of T_4 spore suspension were inoculated in the liquid cultures which were incubated at 30 °C for 3 days in an orbital shaker 160 rpm. Then crude enzyme was extracted by filter and dialyzed by distilled water. Enzyme activity was measured as described above.

Revised formula for liquid state culture: Except for the addition of 0.2% glucose to Mendel's culture, other ingredients and culture conditions was as described for liquid state culture.

Enzyme activity indication: every 30 min, 60 min or 24 h produced 1μg of glucose as 1 unit [4].

3. RESULTS AND DISCUSSION

3.1 Identification of strain

By primary identification, this strain was *Trichoderma viride*, the spore color was black-green. The growth on PDA medium was poor weakness. Conidia size was 2.6-4.0 × 2.1-3.2 μm.

3.2 The measurement of nitrogenase activity

Result showed that this strain has the ability of fixing nitrogen, and as the cultivation time increased, the content to fix nitrogen changed little. The highest nitrogenase activity was detected at four days, the production of ethylene was 1.3×10^{-6} mol·L^{-1}·d^{-1} (**Figure 1**). Because of this characteristic, this strain was special, so the fermentation conditions of it was studied .

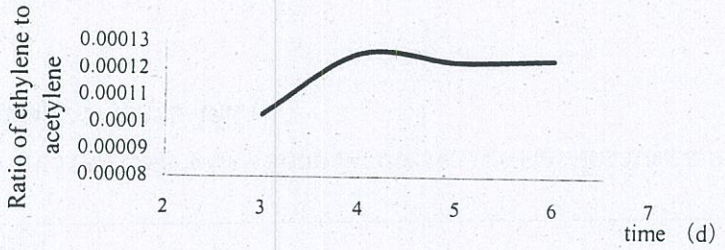

Figure 1. The ratio of ethylene to acelylene at diffenent inoculation times

3.3 The effects of different fermentation time

The results showed that the optimal time of producing enzymes was 4 days, for the filter paper enzyme fermentation for cotton enzyme. CMC-Na enzyme and the enzyme activities were 1472 u, 4168 u, 3618 u, respectively, but the prolongation of fermentation time had little effect on enzyme protein (**Figure 2**). This result also showed the cellulase activity was different. In this strain, cotton enzyme activity, CMC-Na enzyme activity were higher than filter paper enzyme activity. Although the time prolongated, the enzyme activity and enzyme protein were also changed little, sometimes enzyme activity maybe decreased, so the optimal fermentation time was important to obtain the highest enzyme production.

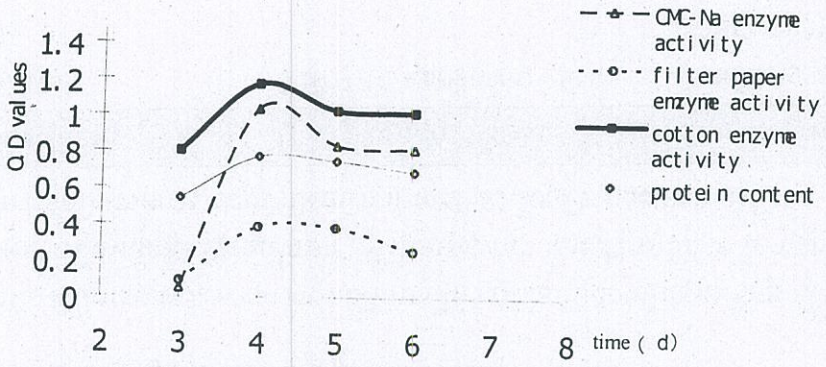

Figure 2. The effect of different fermented time

3.4 The effect of different ratios of straw to bran on enzyme production

The optimal ratios were 3:2 and 2.5:2.5 (Table 1) (either increase Carbon source ratio or nitrogen source ratio, the enzyme activity was all decreased). During the fermentation period, the optimal ratio of different culture medium was important in order to obtain the highest production of enzyme.

Table 1. The effect of different ratio of straw to bran (Optical density; O.D.)

Ratio (straw/bran)	4:1	3.5:1.5	3:2	2.5:2.5	2:3	1.5:3.5	1:4
CMC-Na	0.112	0.254	0.454	0.548	0.501	0.171	0.086
Filter paper	0.062	0.100	0.493	0.396	0.329	0.341	0.02
Cotton	0.992	1.109	1.283	1.268	0.802	1.017	0.643

3.5 The effect of different sources of nitrogen

Enzyme production was affected significantly under different nitrogen sources. The result showed that the different nitrogen source had different effect on enzyme activity. Although $(NH_4)_2SO_4$ was also used as nitrogen source in most fermentation conditions, among the different nitrogen sources the best was NH_4Cl. The cellulase activity can be seen from Table 2 .

Table 2. The effect of different nitrogen sources (Optical density; O.D)

Nitrogen source	$NaNO_3$	$(NH_4)_2SO_4$	$(NH_4)_2SO_4$: bean cake (1:1)	urine	bean cake	NH_4Cl	NH_4NO_3	yeast extract
CMC-Na	0.325	0.166	0.338	0.345	0.294	0.582	0.368	0.426
Filter paper	0.192	0.26	0.273	0.141	0.295	0.428	0.244	0.086
Cotton	0.614	0.811	0.953	0.237	0.712	1.408	1.009	0.523

3.6 The effect of oxygen

Results showed that the difference of varied flasks with the same content of solid culture was not obvious. So this strain was not strict to O_2 under air condition. The activities on filter paper (FPA), cotton (C_1) and carboxymethylcellulose (CMC-Cx) was from 1488 u , 4259 u, 3816 u, respectively to 1496 u, 4268 u, 3812 u.

3.7 The effect of temperature

Below 25 °C the growth of this strain was slow, so the ability of producing enzyme was also lower. The optimum temperature was 27 °C to 30 °C. The growth of this strain and the ability of producing enzyme were both higher at this temperature. The activities on filter paper (FPA), cotton (C_1) and carboxymethylcellulose (CMC-Cx) were 1520 u, 4282 u, 3926 u, respectively.

3.8 the effect of different cultivated condition

When this strain cultivated at different medium, the result was obviously different, especially the producing of β-glucosidase. When this strain was incubated in liquid medium, the

the producing of β-glucosidase. When this strain was incubated in liquid medium, the production of β-glucosidase was nearly zero. By analysing the difference of these two medium and adding glucose to Mendel's medium, the production of β-glucosidase was increased. So the production of β-glucosidase maybe related to glucose, this enzyme may need the induction of glucose.

4. CONCLUSIONS

4.1 The strain studied had the ability of fixing Nitrogen, this was the special character and was benefit to degrade straw.

4.2 The optimal solid culture fermentation conditions with straw and bran was: 3 to 2 or 1 to 1 ratio, with NH_4Cl as nitrogen source, incubated at 27 °C to 30 °C.

4.3 The production of β-glucosidase may need the induction of glucose.

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