ISSN 1330-9862
(FTB-1662)

preliminary communication

Cellulase Production by *Trichoderma koningii* AS3.4262 in Solid-State Fermentation Using Lignocellulosic Waste from the Vinegar Industry

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Received: March 23, 2006
Accepted: July 5, 2006

Summary

Cellulase production was carried out in solid-state fermentation using the waste from the vinegar industry as the substrate for *Trichoderma koningii* AS3.4262. This waste is porous and easy to degrade by cellulolytic fungi. The effects of water content, initial pH value in solid substrate and culture temperature on cellulase synthesis were observed for optimal production in flask fermentors. An orthogonal layout was employed in the statistical process and better cellulase activity was obtained in the fermentation batch. The optimal filter paper cellulase (FPAse) activity of 6.90 IU/g of substrate dry matter (SDM), and carboxylmethyl cellulase (CMCase) activity of 23.76 IU/g SDM were obtained after 84 h of incubation with media containing vinegar waste, with optimal moisture content of 50 %, pH=5.0, incubation temperature of 30 °C, and additional nutrients of inorganic salts in a certain amount. To produce cellulase on a larger scale, a deep trough fermentor with forced aeration was used, so that FPAse activity of 5.87 IU/g SDM and CMCase activity of 12.98 IU/g SDM were reached after 84 hours of solid-state fermentation. Results indicate the excellent scope of utilizing vinegar waste as solid substrate for commercial production of cellulase employing fungi.

Key words: cellulase, vinegar waste, solid-state fermentation, *Trichoderma koningii*, filter paper activity, CMCase activity, orthogonal test

Introduction

Cellulase production is the most important step in the economical production of ethanol, single cell protein and other chemicals from renewable cellulosic materials. To date, the production of cellulase has been widely studied in submerged culture processes, but the relatively high cost of enzyme production has hindered the industrial application of cellulose bioconversion (*1*). It has been reported that solid-state fermentation is an attractive process to produce cellulase economically due to its lower capital investment and lower operating expenses (*2*). Another approach to reduce the cost of cellulase production is the use of lignocellulosic materials as substrates rather than expensive pure cellulose (*3*). In prior publications, abundant agricultural residues such as corn stover, wheat straw, rice straw, bagasse, *etc*. were used in cellulase production (*4*). Although these raw materials are cheaper, pretreatment is generally required to improve the utilization ratio of lignocellulosic materials and the cost is still considerable. In China, vinegar is an impure diluted solution of acetic acid obtained from foodstuff and wheat bran by fermentation beyond the alcohol stage and used as a traditional condiment and preservative. Through centuries, vinegar has been produced from many materials, including molasses, sorghum, fruits, berries, melons, coconut, honey, beer, maple syrup, potatoes, beets, malt, grains and whey. Lots

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of porous solid supports are needed in production to enforce air transfer and make aerobic *Acetobacter* alive. Usually, some agricultural materials such as foodstuff, wheat shell and rice bran rich in starch and lignocellulose are employed in vinegar production. As a result, a large amount of waste residue is generated in the vinegar industry, which is usually not used, and often causes environmental pollution. It is an important issue to deal with the residue both for the comprehensive utilization of lignocellulosic resources and for the prevention of environmental pollution. Since most of starch and soluble ingredients in the vinegar raw material have been degraded by bacteria in the production process, the vinegar residue has a very high void fraction. Therefore, it is well ventilated and the cultured cellulolytic fungi can get enough oxygen easily. In this work, the residue without further pretreatment was used as a substrate in solid--state fermentation to produce cellulase.

Materials and Methods

Microorganism

Trichoderma koningii AS3.4262 was cultivated on potato dextrose agar containing 1.5 % agar and incubated at 30 °C for 7 days until complete sporulation. The spores from slants were suspended in sterile water. The suspension was used as inoculum (10^7 spores/mL).

Lignocellulosic substrate

The residue of (3±1) mm size was obtained from a local vinegar manufacturer, Yangxin Hengqingtang Ya Pear Fermenting Co., Ltd, China.

Medium

The seed medium was that of Mandels *et al.* (*5*). The basic substrates used were the wheat bran (150 g) and vinegar waste (100 g). The medium was adjusted to certain moisture and pH by the addition of 0.4 M HCl. The sterilization was made at 121 °C for 15 min and the water content of the substrate was 50 % in most experiments with the exceptions pointed out in the text.

Cellulase production

Small scale experiments were carried out in conical flasks (500 mL). Each flask was filled with 100 g of wet substrate. After inoculation with 10 % spore suspension, the flasks were put in an incubator and kept at constant temperature, 27, 30 and 33 °C. Pilot scale production of cellulase was performed in a stainless steel fermentation chamber, which was 280 mm in height and 240 mm in diameter. The thickness of the solid substrate layer was 20 cm and the surrounding temperature was controlled at 28–30 °C by water bathing. Air with over 90 % humidity blew through the bottom of the cultivation chamber by the forced aeration. The air flux was 10 L/min. Long gauze bags (3×25 cm) filled with substrate were embedded in the packed bed for sampling (Fig. 1). Samples were collected from the bed at 2, 6, 10, 14 and 18 cm height. The new holes were filled with backup substrate in time.

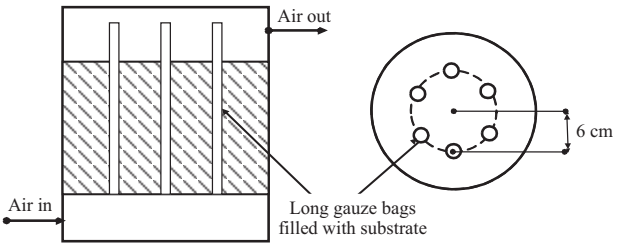

Fig. 1. A layout of the stainless steel fermentation chamber. Long gauze bags filled with substrate were embedded in the packed bed for sampling

Analysis methods

Samples were collected every 12 h during fermentation for the determination of moisture, cellulase activity and total reducing sugars. The fermented mass produced was mixed with 25 volumes of buffer at pH=4.8 to extract cellulase, stirred at 200 rpm at room temperature for 1 h and filtered through cotton cloth of 200 mesh. Filter paper activity (FPAse) and carboxylmethyl cellulase (CMCase) were measured according to the method recommended by Ghose (*6*) and expressed as international units (IU). A piece of filter paper (6×1 cm) or 5 mL of carboxylmethyl cellulase solution (1 %) was mixed with diluted enzyme solution, and then kept at 50 °C for 30 min. The amount of reducing sugar was determined by the dinitrosalicylic acid (DNS) method (*6*). One international unit of cellulase activity is the amount of enzyme which releases one μmol of glucose per min during the hydrolysis reaction. Enzyme activities were calculated for 1 g of substrate dry matter (SDM).

Results and Discussion

Effect of water content in the substrate

The appropriate moisture of substrate is one of the critical factors influencing the solid-state fermentation (SSF), and is governed by the requirements of the microorganism. Experiments with different substrate moisture were carried out in flasks. The culture temperature was kept at (30±1) °C. The enzyme production by *Trichoderma koningii* is shown in Fig. 2. The optimal water fractions in the solid substrate appear to be 40 to 60 % (by mass). Under these culture conditions, cellulase activity of 4.3 IU FPAse/g SDM and 10.5 IU CMCase/g SDM were obtained. The results indicated a positive relationship between cellulase production and moisture when the water content was lower than 40 %. It was observed that the moisture enabled better utilization of the substrate by microorganisms and the efficiency of mass transfer in the solid phase particles depended on the substrate characteristics and the appropriate moisture. But further increase in moisture influenced the enzyme production negatively. It reduced surface area of the particles, and made the water film thicker, which affected the accessibility of the air to the particles. The free water of the substrate determined the void space which is occupied by air. Since the transfer of oxygen affected the growth and metabolism, the substrate should contain suitable amount of water to enhance mass transfer.

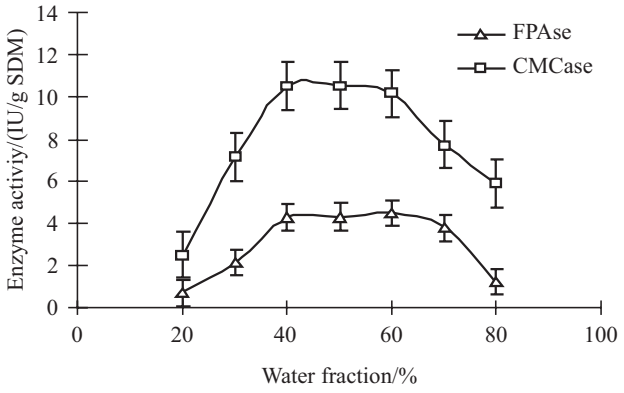

Fig. 2. The moisture of the substrate affected cellulase production. The temperature and cultivation time were 30 °C and 72 h. The initial pH was not adjusted

Therefore, the water content of solid substrates is one of the key factors in cellulase production. In this study, the optimal moisture fraction was taken as 50 %.

Effect of initial pH value on cellulase production

There exists a strong influence of initial pH of the medium on enzyme production. To evaluate the effects of initial pH value in solid substrate on cellulase synthesis, the initial pH values were adjusted by the addition of HCl or NaOH to 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0. The results of six batches of solid state fermentation in flasks with different initial substrate pH values are shown in Fig. 3. The cultivation period for each batch was 3 days. It was observed that the original residue of pH=3 was unsuitable for cellulase production since the low pH value resulted in poor growth. In contrast, if the initial pH value of the substrate ranged from 5.0 to 6.0, no significant difference was observed on cellulase yield. Optimal cellulase production was obtained at an initial pH of 5.0, and it may change slightly during the fermentation process.

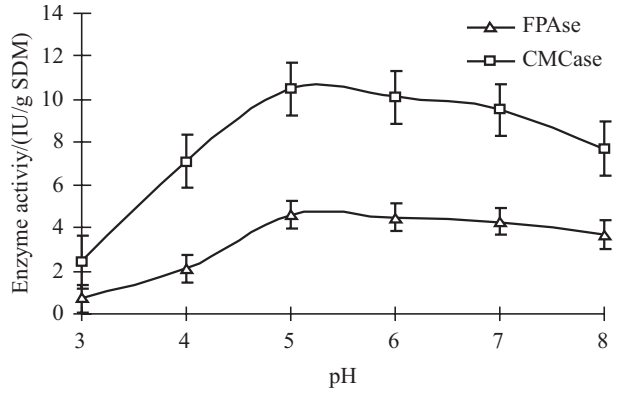

Fig. 3. The influence of initial pH value of the substrate on cellulase production. Temperature and cultivation time were 30 °C and 72 h, respectively

Effect of temperature and time

The incubation temperature is a factor regulating the enzyme synthesis. Sun *et al.* (7) found that maximal growth and cellulase production by *Trichoderma* sp. were at 25–35 °C. The temperature maintained in the SSF system by *Trichoderma koningii*, in general, is in the range of 27–33 °C, and it depends on the growth kinetics of the microorganism rather than on the enzyme produced. When the temperature was changed from 27 to 30 °C, the ultimate yield of FPAse activity was raised to a certain extent. Further increase in the temperature did not result in a corresponding increase in the yield.

The time of fermentation had a great effect on enzyme production, as the maximum filter paper activity of 4.64 IU/g SDM (Fig. 4a), CMCase activity of 10.42 IU/g SDM (Fig. 4b) were obtained after 84 h of fermentation, but further culture resulted in reduced enzyme yield. Similar trend was also reported in cellulase production using *Trichoderma* sp. Proper cultivation time was significant for growth and production. The decrease in activity after 84 h may be due to denaturation of the enzyme, resulting from variation in pH and cellular metabolism during fermentation.

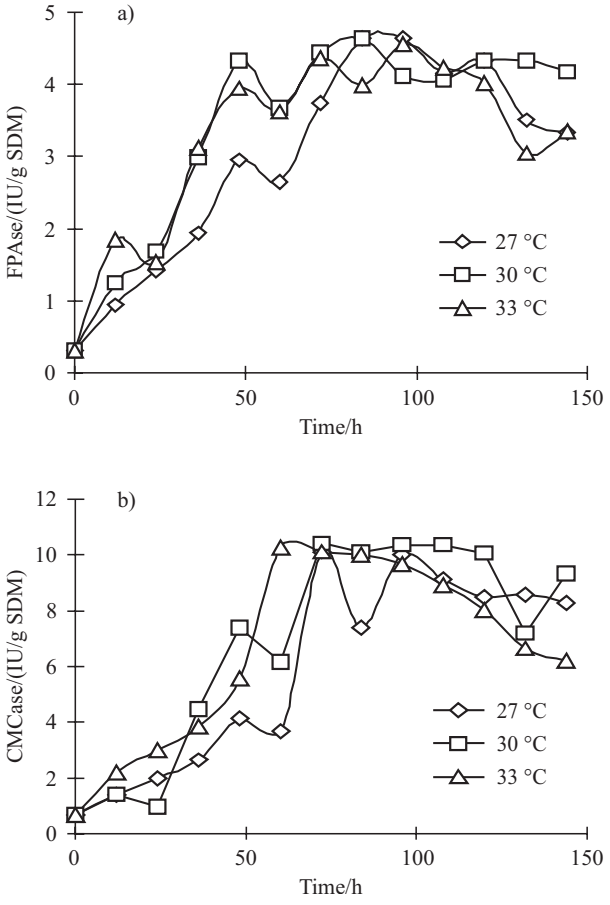

Fig. 4. Effect of temperature on cellulase production. Fig. 4a shows the effect of temperature on FPAse production, and Fig. 4b shows the effect of temperature on CMCase production. Initial pH=5.0

Statistical design of fermentation medium

The cultural media and conditions were considered to meet the nutritional demands of the producer organism. According to the varieties of media, the optimal medium was obtained by designing an orthogonal lay-

out L_9 (3^4) in the cultures. The levels of factors of culture medium are shown in Table 1. Different quantities of vinegar waste were tested for cellulase production in solid--state fermentation with 50 % water content of substrate at 30 °C. The sampling was at the 84th hour. The amounts of vinegar waste, $(NH_4)_2SO_4$, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$ were regarded as correlated factors of the culture medium.

The results of the optimization of culture medium are presented in Table 2, which summarizes the influence of the four factors (the quantities of vinegar waste, $(NH_4)_2SO_4$, KH_2PO_4 and $MgSO_4{\cdot}7H_2O$) on mycelial cellulase production. Scores mean the sum of two kinds of enzyme activity, including FPAse and CMCase, which was taken as a comprehensive evaluation. The R values showed that the quantity of vinegar waste was a more important factor than other culture conditions in the orthogonal layout L_9 (3^4), because R value of vinegar waste (17.88) was higher than that of KH_2PO_4 (11.64), $(NH_4)_2SO_4$ (10.19) and $MgSO_4{\cdot}7H_2O$ (9.41). The sequence of influences on cellulase from more to less is vinegar waste, KH_2PO_4, $(NH_4)_2SO_4$ and $MgSO_4{\cdot}7H_2O$. In the fourth experiment, the yields of mycelial FPAse and CMCase reached 6.90 IU/g SDM and 23.76 IU/g SDM, respectively.

The optimum quantity of vinegar waste was 40 %. A higher quantity of vinegar waste will result in poor fungal growth and low cellulase activity for its limited nutrition. On the other hand, smaller quantity may cause blocked interspace. Therefore, bad ventilation decreased cellulase production. It is now generally accepted that a low oxygen flux through hydrolysis restrains cellulase accumulation, because this fungus is a kind of strict aerobe.

It was reported that good cellulase yield can be obtained with ammonium compound as the nitrogen source (*7*). Though the addition of organic nitrogen sources such as beef extract and peptone resulted in increased growth and enzyme production, as was reported before (*7*), they were not an effective replacement for inorganic nitrogen sources because of their higher cost. More literature data indicate that the source of nitrogen should be inorganic for better results (*8,9*). Results suggest that inorganic salts can enhance cellulase synthesis to a significant level (FPAse 6.90 IU/g SDM and CMCase 23.76 IU/g SDM). An increase in cellulase activity was observed when enriching medium with 1 % ammonium sulphate, but further increase in the concentration did not improve cellulase production. Additional supply of nitrogen sources influenced the CMCase activity to a certain extent,

Table 1. L_9 (3^4) orthogonal design for optimization of culture medium

Factors	*w*(vinegar waste) / %	*w*($(NH_4)_2SO_4$) / %	*w*(KH_2PO_4) / %	*w*($MgSO_4{\cdot}7H_2O$) / %
	A	B	C	D
Level 1	30	0.5	0.1	0.02
Level 2	40	1.0	0.2	0.05
Level 3	50	1.5	0.3	0.10

% is the percentage of the dry mass

Table 2. Orthogonal test according to the orthogonal design L_9 (3^4)

Experimental group	A	B	C	D	Enzyme activity		Scores
					FPAse IU/g (SDM)	CMCase IU/g (SDM)	
1	1 (30)	1 (0.5)	1 (0.1)	1 (0.02)	5.21	15.18	20.39
2	1	2 (1.0)	2 (0.2)	2 (0.05)	6.79	18.17	24.96
3	1	3 (1.5)	3 (0.3)	3 (0.10)	6.47	15.96	22.43
4	2 (40)	1	2	3	6.90	23.76	30.66
5	2	2	3	1	6.64	20.93	27.57
6	2	3	1	2	6.51	20.92	27.43
7	3 (50)	1	3	2	6.65	11.98	18.63
8	3	2	1	3	6.78	20.56	27.34
9	3	3	2	1	5.53	19.12	24.65
I_j	67.78	69.68	75.16	72.61			
II_j	85.66	79.87	80.27	71.02			
III_j	70.62	74.51	68.63	80.43			
R	17.88	10.19	11.64	9.41			
Optimization	A_2	B_2	C_2	D_3			

I_j is the total scores of level 1; II_j, those of level 2; III_j, those of level 3; R means the maximum of I_j, II_j and III_j minus the minimum of I_j, II_j and III_j

whereas the influence on FPAse activity seemed comparatively lower. The effect of other additional salts on enzyme yield was tested using the basal medium with the addition of phosphorus, potassium and magnesium, so KH_2PO_4 and $MgSO_4{\cdot}7H_2O$ were also added to the growth substrate. However, the addition of KH_2PO_4 at concentrations above 0.3 % led to a significant reduction in enzyme synthesis. It was inferred that supplementation with KH_2PO_4 at a certain concentration was sufficient for enhancing enzyme production. Total cellulase enzyme titers with inorganic salt-enriched samples were filter paper activity of 6.90 IU/g SDM and CMCase activity of 23.76 IU/g SDM, compared with 4.64 IU/g SDM and 10.42 IU/g SDM of the blank (Fig. 4). These results have shown that although vinegar waste itself can act as a source of carbon, nitrogen or minerals as well as growth factor, some adjustments in ingredients are also necessary.

Solid-state fermentation in packed bed fermentor

Bioreactor design and operation for cellulase production by SSF requires much more attention. For solid-state fermentation at laboratory scale, cellulase is commonly produced in flasks. Flasks are suitable for investigation in the laboratory because they are easy to handle. If lots of separate flasks are used, then all flasks should be representative and individual flasks can be removed daily without disrupting other samples. But if it comes to an industrial scale, a bioreactor often used for large-scale cellulase production similar to the flask is tray. The tray technique is simple, but requires a large area and is difficult to automate and is therefore labour-intensive, and it also allows relatively little control over the cultural conditions. The packed bed has a potential for cellulase production. This bioreactor deserves further attention because it operates without damaging fungal mycelia. The time course of cellulase synthesis in a deep trough fermentor by *Trichoderma koningii* is shown in Fig. 5. It was found that the mass transfer in the deep trough fermentor with forced aeration may be effectively inhibited. The cellulase activity on top and at the bottom of the bioreactor was promoted, because the air there was rich in oxygen, and the heat dissipated easily. Cellulase activity on top and at the bottom of the bioreactor was higher, whereas that in the middle was lower. After 84 h of solid-state fermentation, the maximum FPAse activity of 5.87 IU/g SDM was reached, while the maximum CMCase activity was 12.98 IU/g SDM. Although the deep trough fermentor gave a little lower productivity and less uniform quality, it can produce cellulase on a large scale. There was only 100 g of wet substrate in each flask, whereas about 3000 g of wet substrate was in the fermentation chamber. Therefore, the total yield of the bioreactor was at least 20 times more than that of a flask.

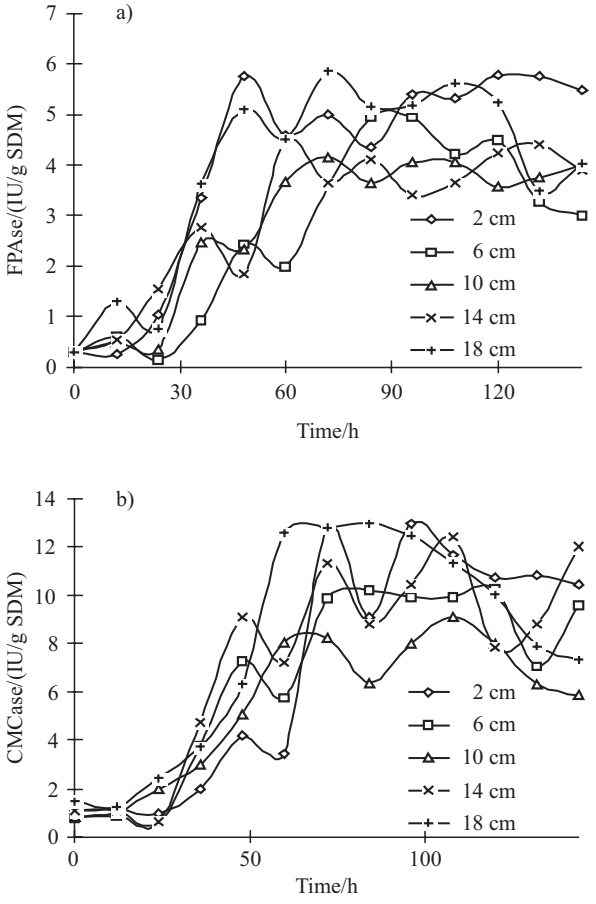

Fig. 5. Effects of bed height and culture time on cellulase activity in the solid-state fermentation reactor. Five spots were monitored. Figs. 5a and 5b represent FPAse and CMCase, respectively. The temperature and initial pH were 30 °C and 5.0

Conclusions

In accordance with the results, taking all the influencing factors and the results into consideration, the optimal cultural process was considered as follows: media and cultural conditions including vinegar waste 40 %, $(NH_4)_2SO_4$ 1 %, K_2HPO_4 0.2 %, $MgSO_4{\cdot}7H_2O$ 0.1 %, pH=5.0, culture temperature 30 °C and culture time 84 hours. The results indicate the suitability of using cheap and abundantly available vinegar waste as solid substrate for large-scale production of cellulase in an SSF system in order to reduce the high costs. Maximum utilization of this waste can also contribute to efficient solid-waste management, where continuous accumulation of industrial wastes poses a serious environmental problem. This process therefore has a high potential for comprehensive utilization of renewable lignocellulosic resources. However, it is necessary to further optimize the fermentation conditions in a bigger fermentor to achieve the demands of large-scale production.

Acknowledgements

The authors thank Prof. George T. Tsao for his contribution to this research program.

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