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Screening and Enzymatic Study of a Composite Microbial System FH3

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ABSTRACT

Five strains of fungi and 18 strains of bacteria were isolated from the soil and horse manure with cellulose as the sole carbon source. Among them, two fungal, F_9 and F_{13}, and two bacterial, B_{16} and B_{21}, showed the highest filter paper activities, which were 7.79 U g^{-1}, 9.84 U g^{-1}, 7.34 U g^{-1} and 9.68 U g^{-1}, respectively. Four microbial systems, designed as FH1 (F_{13}+B_{21}), FH2 (F_{13}+B_{16}+B_{21}), FH3 (F_9+B_{16}+B_{21}) and FH4 (F_{13}+F_9+B_{16}+B_{21}) were developed. The fermentation studies showed that the filter paper activity of the composite microbial system FH3 was higher than the others, which was 21.34 U g^{-1}. The medium with bran and filter paper as the carbon source and peptone as nitrogen source was optimal and the maximum cellulase activity was reached at 30~35ºC and pH 6.0~6.5 when FH3 was incubated for 48 h. The enzymatic reaction conditions were estimated at 45~55 ºC, pH 4.5~5.5 and the thermal stability temperature was up to 60 ºC.

Key words: Cellulose-degrading bacterium, composite microbial systems, enzyme properties

INTRODUCTION

Cellulose is the major carbohydrate polymeric compound in the plants and is the most abundant organic compound on the earth. Accordingly, its turnover in the carbon cycle is of prime importance for all the living organisms (Peter et al., 1999; Garsoux et al., 2004). Huge amounts of the cellulosic wastes, measured in billions of tons annually, are produced worldwide as the residues from the agriculture activities and industrial food processing. In China, crop straw waste exceeds 100 million tons per year (Haruta et al., 2004). The enormous potential of cellulose as a renewable source of energy was recognized only after the cellulose degrading enzymes or cellulases had been identified (Mandels, 1985). Cellulases are enzymes that hydrolyse β-1, 4-glycosidic bonds in cellulose, releasing cellobiose as the smallest product. These enzymes can be found associated with the cellulosome, an extracellular supramolecular assembly produced by some bacteria and fungi (Shoham et al., 1999; Carrard et al., 2000), but are generally secreted as independent enzymes by the cellulolytic microorganisms. Efforts have been directed to find the suitable cellulase-producing micro-organisms through the strain selection and development. *Trichoderma reesei* has been studied extensively because of its ability to produce a good cellulose

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enzyme complex that is capable of hydrolysing cellulose. However it produces little cellobiase and xylanase activities, which is a disadvantage from the point of practical saccharification. Kang *et al* (1994) found that *Aspergillus niger* was a more promising strain than *Trichoderma reesei* (Kim et al., 1997). Van Wyk et al (2003) treated various wastepaper materials with the cellulase from *viride* and converted their cellulose component into fermentation sugars. In nature, the cellulose materials are degraded with mixed group of the microorganisms. It has been reported that a mixed culture of one cellulolytic bacterium together with another noncellulolytic bacterium is ideal for degrading the cellulose. As such, the utilization of a complex microbial community could be effective in achieving the high degradation efficiency for natural cellulosic materials.

In the present study, a composite microbial system FH3 was constructed, which had a higher enzyme activity than any of the separate composting strains. The conditions for the rapid and high cellulase production by the FH3 are described. Some of the properties of the enzyme consortium are also presented.

MATERIALS AND METHODS

Experimental materials

The soil and horse faeces were collected from the cornfield and meadow of Tianjin suburb (Tianjin, China). Xinhua mensurable filter paper was cut into pieces (6cm in length and 1cm in width). Cellulose-congo red medium was composed of 0.50g K_2HPO_4, 0.25g $MgSO_4$, 1.88g cellulose powder, 0.20g congo red, 14.00g agar, 2.00g glutin, 100mL soil solution and 900mL H_2O, pH7.0. The liquid fermentation medium 1 (bacterial) was composed of 1.00g KH_2PO_4, 0.10g NaCl, 2.50g $NaNO_3$, 0.30g $MgSO_4$, 0.01g $FeCl_3$, 0.10g $CaCl_2$, 10g bran and 1000mL H_2O, pH 7.2. The liquid fermentation medium 2 (fungal) was composed of 3.00g peptone, 2.00g $(NH_4)_2SO$, 0.50g yeast extract, 4.00g KH_2PO_4, 0.30g $CaCl_2$, 0.30g $MgSO_4$, 0.20g Tween-80, 10g bran and 1000mL H_2O, pH 6.0. The peptone cellulose substrate (PCS) was composed of 1.00g yeast extract, 5.00g peptone, 2.00g $CaCO_3$, 5.00g NaCl, 20.00g cellulosic materials and 1000mL H_2O.

Screening for cellulose decomposing microbial strains

Different sample solutions were spread on the cellulose-congo red plates and incubated at 30ºC for 72h. The purified strains were obtained and inoculated on the cellulose-congo red medium and cultured at 30 ºC for six days. The strains were selected according to the value of H/C (Table 1). After that, one milliliter suspension of these strains was inoculated in the liquid medium 1 or 2 and cultured at 30 ºC for three days. By measuring the enzyme activity, some strains were chosen for the next combination experiment.

Strain combinations

Four composite microbial systems were developed according to their cellulose-degrading enzyme activities. One milliliter suspension of the four composite microbial systems was inoculated in the PCS liquid medium and incubated at 30 ºC for three days. The desired composite microbial systems were selected by its three enzyme activities and were used in the fermentation experiment.

Enzyme assays

The cellulase activities were measured by DNS (3, 5-dinitrosalicylic acid) assay (Miller, 1959), through the determination of the amount of reducing sugars from filter paper, CMC and cotton. The three reactions were performed under different experimental conditions (temperature: 50 ºC, 45 ºC and 45 ºC; time: 1h, 0.5h and 24h) and stopped by the addition of the DNS solution. The treated samples were boiled in the capped glass tubes for 10min, cooled in the water for color stabilization and the optical density was measured at A_{540}. The cellulase activities were determined from a calibration curve for glucose ($\varepsilon_{540}=3.2\times10^2$ $L.mol^{-1}.cm^{-1}$). One unit (U) of the enzyme activity was defined as the amount of enzyme that released 1µmol of reducing sugar per minute under the assay conditions. All the enzymatic assays were performed in triplicate.

RESULTS AND DISCUSSIONS

Strains screening

Twenty-three strains of cellulose-degrading

bacteria were isolated from the soil and horse feces. Eight strains were finally chosen for further study according to the diameter of the hydrolysis zones. Among them, F_9 and F_{13} were fungi, while the others were bacteria (Table 1).

Table 1-The status of H, C, and H/C of the eight strains over six days.

Strains	Time (2d)			Time (4d)			Time (6d)		
	*H/cm	C/cm	H/C	H/cm	C/cm	H/C	H/cm	C/cm	H/C
B_4	0.70±0.02	0.40±0.01	1.75±0.10	1.00±0.01	0.50±0.01	2.00±0.06	1.20±0.01	0.60±0.01	2.00±0.05
F_9	1.25±0.02	0.70±0.01	1.79±0.06	3.10±0.02	1.00±0.01	3.10±0.05	4.80±0.02	1.50±0.01	3.20±0.03
B_{12}	—	—	—	1.10±0.01	0.50±0.01	2.20±0.07	1.40±0.01	0.85±0.01	1.65±0.03
F_{13}	1.50±0.01	0.80±0.01	1.88±0.04	3.60±0.02	1.00±0.01	3.60±0.06	4.90±0.02	1.20±0.01	4.08±0.05
B_{15}	1.00±0.01	0.60±0.01	1.67±0.05	1.20±0.01	0.90±0.01	1.33±0.03	1.25±0.01	1.00±0.01	1.25±0.02
B_{16}	2.50±0.02	0.80±0.01	3.13±0.06	2.80±0.01	0.85±0.01	3.29±0.05	3.00±0.02	0.95±0.01	3.16±0.05
B_{17}	—	—	—	0.60±0.01	0.40±0.01	1.50±0.06	2.10±0.01	0.60±0.01	3.50±0.08
B_{21}	1.50±0.01	0.60±0.01	2.50±0.06	2.10±0.02	1.00±0.01	2.10±0.04	3.80±0.01	1.20±0.01	3.17±0.04

*H: the diameter of hydrolysis zone, C: the diameter of colony, H/C: the ratio of H and C.

F_9, F_{13}, B_{16} and B_{21} had a higher ability of cellulose degrading than the other strains. F_9 and F_{13} were two strains of fungi and their diameter of the hydrolysis zones developed quickly with the diversification of the diameter colony. On the sixth day, the H/C value of F_9 reached 3.2, while F_{13} reached 4.08. In the screening process, the H/C values of B_{16} and B_{21} also reached 3.0. Though the H/C value of B_{17} reached 3.5, its hydrolytic zone and colony was very low. B_{17} grew slowly and had a poor ability on cellulose decomposing.

Enzyme activity measuring

One-milliliter bacterial suspension of B_4, B_{12}, B_{15}, B_{16}, B_{17} and B_{21} was inoculated in 50mL liquid fermentation medium 1. The suspension of F_9 and F_{13} was inoculated in liquid fermentation medium 2 in the same quantity. They were incubated at 30 ºC at 160rpm for three days. The results of enzyme activities are shown in Table 2.

Table 2 - Enzyme activities of different strains.

Strain Nos	Cellulase activities		
	filter paper ($U\ g^{-1}$)	CMC ($U\ g^{-1}$)	Cotton ($U\ g^{-1}$)
B_4	3.05±0.12	10.22±0.61	0.32±0.02
F_9	7.79±0.43	23.66±0.96	0.30±0.02
B_{12}	1.17±0.08	9.68±0.48	0.18±0.01
F_{13}	9.84±0.49	27.78±1.02	0.51±0.03
B_{15}	3.41±0.17	28.86±1.05	0.56±0.03
B_{16}	7.34±0.37	39.53±1.23	0.56±0.03
B_{17}	3.81±0.19	6.09±0.30	0.38±0.02
B_{21}	9.68±0.58	60.05±1.60	0.65±0.04

Table 2 showed that all the strains of bacteria and fungi presented filter paper activity, and the largest values were obtained for F_{13} and B_{21} and the smallest value for the strain B_{12}. Strain B_{21} had the largest value for CMCase, and B_{17} had the smallest. Strain B_{21} had the largest value for cotton activity, and B_{12} had the smallest. Considering the three activities, the strains B_4, B_{12} and B_{17} presented smaller values in relation to the other strains. Therefore, strain B_{21} was the best for CMCase and cotton activities. In this study, strain's isolation was based on the filter paper enzyme activity. As cellulose material has moderate degree of polymerization and crystallization, filter paper could be used as a substitute for the natural cellulose (Li et al., 2003).

Therefore, the filter paper enzyme activity could reflect the cooperative action of three cellulases, and regarded as the basis of strain isolation. Among the eight strains of the cellulose-degrading microorganisms, F_9, F_{13}, B_{16} and B_{21} showed the higher filter paper enzyme activities, which were 7.79, 9.84, 7.34 and 9.68 U g-1, respectively. It proved that F_9, F_{13}, B_{16} and B_{21} had the highest cellulose-degrading ability. The result was consistent with H/C value.

Strain combinations

Strains F_{13} and B_{21}, which had the highest filter paper enzyme activity, were combined to form the composite microbial system FH1. Strains F_{13} with the highest filter paper enzyme activity, B_{21} with the highest CMC enzyme activity and B_{16} with the highest cotton enzyme activity were combined to form the composite microbial system FH2. Strains F_9 with a high filter paper enzyme activity, B_{21} with the highest CMC enzyme activity and B_{16} with the highest cotton enzyme activity were combined to form the composite microbial system FH3. All the four strains were combined to form the composite microbial system FH4. The four composite microbial systems were inoculated in the PCS liquid medium and incubated at 30 ºC and 160rpm for three days. The crude enzyme activities were measured and results are shown in Table 3. Table 3 indicated that the filter paper activity of the composite microbial system FH1 was 11.13 U g-1 with an increase of 13.1% and 15.0% compared to F_{13} and B_{21}, respectively. But its CMC enzyme activity and cotton enzyme activity were lower than its single strain. The filter paper activities of the composite microbial system FH2 and FH4 were lower than those of their single strains. Compared with F_{13}, B_{16} and B_{21}, the filter paper activity of FH2 decreased 45.3, 26.7 and 44.4%, the CMC enzyme activity decreased 10.3, 37.0 and 58.5%, while the cotton enzyme activity was improved by 127.5, 107.1, and 78.5%, respectively. The filter paper enzyme activity of FH4 decreased 49.2, 59.8, 46.0 and 59.1%, the CMC enzyme activity was improved by 160.2, 121.6, 55.8 and 2.5%, while the cotton activity decreased 23.3, 54.9, 58.9 and 64.6% compared with F_9, F_{13}, B_{16} and B_{21}, respectively.

Table 3 - Results of enzyme activities of composite microbial systems FH1-4.

CMS* Nos	Cellulase activities		
	filter paper (U g^{-1})	CMC (U g^{-1})	Cotton (U g^{-1})
FH1	11.13±0.56	13.80±0.69	0.21±0.01
FH2	5.38±0.27	24.92±1.25	1.16±0.06
FH3	21.34±1.07	102.89±3.14	0.75±0.04
FH4	3.96±0.20	61.57±1.08	0.23±0.02

* CMS-Composite Microbial Systems

Among the four composite microbial systems, FH3 had the highest cellulose degradation ability. All the three enzyme activities got increased compared to that of three single strains. Being related to F_9, B_{16} and B_{21}, the filter paper enzyme activity of FH3 were improved by 174.0, 190.7 and 120.5%, the CMC enzyme activity were improved by 334.9, 160.3 and 71.3%, and the cotton activity were improved by 150.0, 33.9 and 15.4%, respectively. Therefore FH3 was the best and used in further studies.

Effects of culture temperature on the enzyme production of FH3

FH3 was inoculated in the PCS liquid medium and cultured for three days at different temperatures. The relationship between the relative enzyme activity and temperature is shown in Fig. 1.

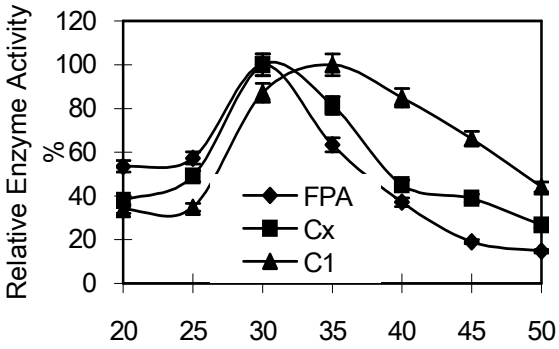

Figure 1 - Effects of culture temperature on the enzyme production of FH3 FPA-filter paper activity, Cx-CMC activity, C1-cotton activity.

Temperature is one of the most important factors affecting microorganisms' growth (Andreaus et al., 1999; Tuomela et al., 2000). Fig. 1 indicated that the filter paper activity and CMC enzyme activity were greatly affected by the temperature and reached the maximum at 30 ºC with a sharp peak. The cotton activity remained higher at 30~40 ºC and reached the maximum at 35 ºC.

Effects of initial pH of medium on the enzyme production of FH3

Another important factor significantly affecting the cellulase production was the initial pH of the medium. In this study, the FH3 was inoculated in the PCS liquid medium and incubated at 30 ºC for three days at different initial pH. The relationship between the relative enzyme activity and initial pH is shown in Fig. 2.

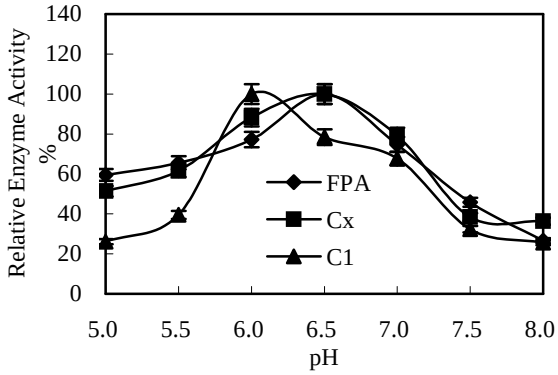

Figure 2 - Effects of initial pH value of medium on the enzyme production of FH3.

The initial pH of the medium showed a significant effect on the filter paper activity, CMC activity and cotton activity. When the initial pH value ranged from 5.5 to 7.5, the three activities had a strong variation. The filter paper activity and CMC enzyme activity reached the maximum at pH 6.5 and the cotton activity at pH 6.0.

Effects of different carbon sources on the enzyme production of FH3

Cellulase is an inducible enzyme system (Kubicek, 1993; Kubicek et al., 1993; Ryu et al., 1980). All the microorganisms produced the highest level of cellulase when grew on cellulose (Stewart et al., 1976; Wood, 1985). Bran, filter paper, glucose, sucrose, bran + sucrose, filter paper + glucose, filter paper + sucrose were used as the carbon sources in the PCS liquid medium. The nitrogen source of medium was peptone. FH3 was inoculated in the medium and incubated at 30 ºC and pH 6.0 for three days. The relationship between the relative enzyme activity and carbon sources is shown in Fig. 3.

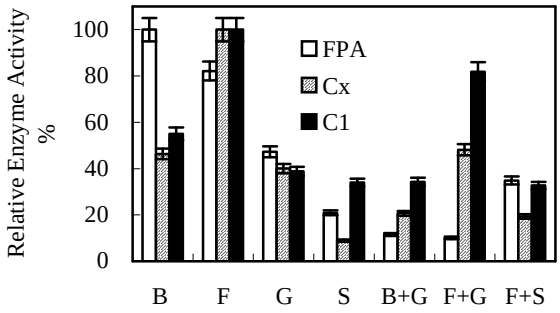

Figure 3 - Effects of carbon sources on the enzyme production of FH3 B-bran; F-filter paper; G-glucose; S- sucrose.

Evidently the filter paper activity reached the maximum value when the carbon source was bran. The three enzyme activities were higher with the filter paper as the carbon source, especially for the CMC activity and cotton activity, which reached the maximum value. Therefore, bran and filter paper were the favorable carbon sources, which could induce the FH3 to produce cellulase (Kvesitadze et al., 1999). The use of the soluble sugars can reduce certain problems during the large-scale fermentation. In this study, pure sugars gave good growth but cellulase production was very poor. This could be due to the fact that easily metabolizable substrates might prevent the enzyme synthesis (Seyis et al., 2005).

For further studies on the enzyme production by FH3, some small molecule carbon sources such as glucose and sucrose were added to the medium. Bran + sucrose, filter paper + glucose and filter paper + sucrose were used as the carbon sources in the PCS liquid medium. The results in Fig. 3 showed that the enzyme activity of FH3 decreased greatly because of glucose and sucrose. It was consistent with the inducible characteristics of the cellulase.

Effects of different nitrogen sources on the enzyme production of FH3

FH3 was inoculated in the PCS liquid medium with different nitrogen sources and incubated at 30 ºC, pH 6.0 for three days. The carbon source of medium was bran. The relationship between the relative enzyme activity and nitrogen sources is shown in Fig. 4.

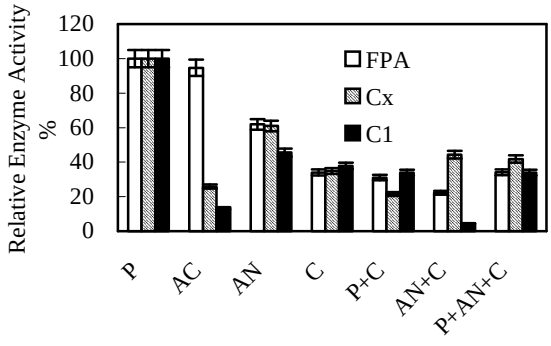

Figure 4 - Effects of nitrogen sources on the enzyme production of FH3 P-peptone; AC-ammonium citrate; AN- ammonium nitrate; C-urea.

The nitrogen source used in the production medium is one of the major factors affecting the enzyme production and level. Fig. 4 indicated that FH3 had the highest enzyme activities when peptone was used as the sole nitrogen source. The results showed that organic nitrogen source restrained FH3 to produce cellulase. Urea as a nitrogen source is widely used in industry, but it didn't induce FH3 to produce the cellulase well in this study. In order to make use of urea, peptone +

urea, ammonium nitrate + urea and peptone + ammonium nitrate + urea were used as the mixed nitrogen source for fermentation. The results showed that none of these were useful.

Relationship between culture time and enzyme production of FH3

The composite microbial system of FH3 was inoculated in the most favorable medium at 30ºC and pH 6.0 for five days. The enzyme activities of the crude enzyme were measured at 12h intervals. The variation of enzyme production with the culture time is shown in Fig. 5.

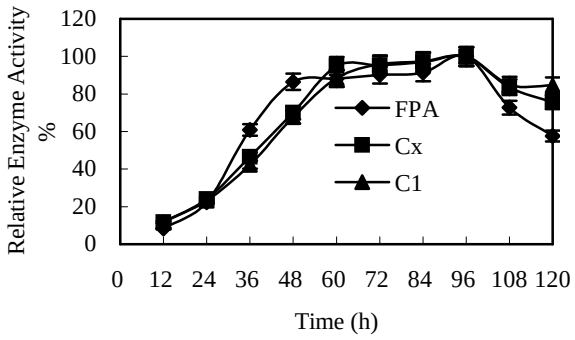

Figure 5 - Relationship between culture time and enzyme production of FH3.

The enzyme production by FH3 increased from 24h to 48h and kept constant during 48h to 96h. After 96h, the enzyme activity decreased greatly. It might be concluded that the best culture time for FH3 was from 48h to 96h.

Effects of temperature on the enzyme reaction of FH3

The variation of the enzyme activity at different temperatures is shown in Fig. 6. Filter paper activity and cotton activity were at a high level when temperature was in the range of 45~55 ºC, where they were all above 80%. The relative enzyme activity of CMC was 63.38% at 45 ºC and it exceeded 80% at 50~60ºC. The highest activities of the three enzymes were at 50 ºC. It suggested that the best enzyme reaction temperature was 50 ºC. It could prove that FH3 had a very wide range of temperature, which was a very important parameter for the industry.

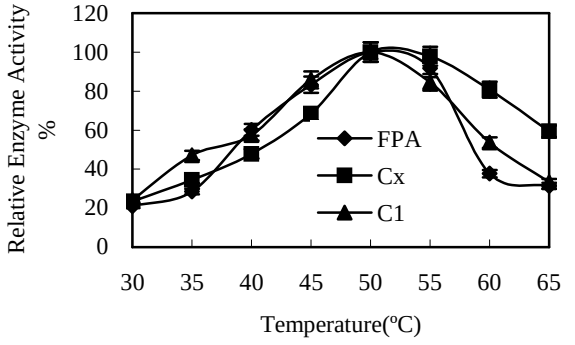

Figure 6 - Effects of temperature on the enzyme reaction of FH3.

Effects of pH on the enzyme activity of FH3

The variation of crude enzyme of FH3 at different pH (3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 and 6.5) is shown in Fig. 7.

Enzyme activity is markedly affected by pH. This is because the substrate binding and catalysis are often dependent on the charge distribution on both, substrate and, in particular enzyme molecules

(Shah et al., 2005). And the three kinds of enzyme activities of FH3 were very low under the highly acidic conditions, but they all increased slowly with the rise of pH. Filter paper and cotton activities reached the highest value at pH 4.5, and then they decreased slowly with the rise of pH. The CMC activity reached the highest at pH 5.0. Fig. 7 indicated that the filter paper and cotton activities kept a high level at pH 4.0~5.5, the range was wider than that of CMC activity which was at pH 4.5~5.5.

Thermal stability of the crude enzyme of FH3

The thermal stability is a very important aspect when considering the industrial application of the enzymes (Heck et al., 2005). The profiles of the thermal stability of cellulase at 30, 35, 40, 45, 50, 55, 60 and 65 ºC are represented in Fig. 8.

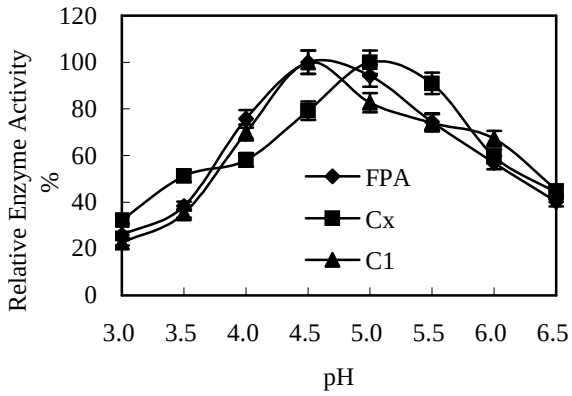

Figure 7 - Effects of pH on the enzyme activity of FH3.

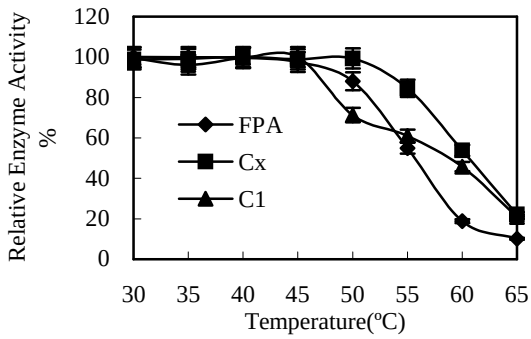

Figure 8 - The thermal stability crude enzyme of FH3.

The utilization of the enzymes in the industrial process often encounters the problem of the thermal inactivation of the enzyme. The thermal stability studies were carried out by preincubating the enzymes up to 3h in the range of 30~65ºC. The CMC activity had a better thermal stability than the other two activities. The three kinds of enzyme activities were at a higher level at 30~45ºC, and then decreased sharply above 50ºC. When the temperature reached at 65ºC, the filter paper activity, CMC activity and cotton activity decreased 68.57, 40.57 and 66.62% respectively, compared to the highest enzyme activities. From Fig. 8, it could be concluded that the thermal stability temperature was around 60ºC.

CONCLUSIONS

The FH3 was a composite microbial system with a high yield of cellulase. Its enzyme activity increased nearly two-fold compared to single

strain. The optimal enzyme production conditions of FH3 were as follows: initial pH 6.0~6.5, temperature 30~35ºC, shaker revolution 120~160 rpm and culture time 48h~96h. The optimal carbon source was filter paper and bran, and the optimal nitrogen source was peptone. The optimal conditions for enzymatic activities were: temperature 45~55ºC and pH 4.5~5.5. The thermal stability temperature of FH3 was under 60 ºC.

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