

## Isolation, Purification and Characterization of Cellulases from *Streptomyces* Strains in the Gut of the Termite *Odontotermes formosanus*

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**Abstract:** An exoglucanase ( $C_1$ ), two endoglucanases ( $C_x$ ) and a  $\beta$ -glucosidase ( $\beta G$ ) were isolated from the fermentation broth of two *Actinomycete* strains in the gut of the termite *Odontotermes formosanus*. These enzymes were purified sequentially by ammonium sulfate precipitation, and Sephadex G100 and DEAE-Sephadex A50 column chromatographies. Both *Actinomycete* strains were identified as belonging to *Streptomyces* spp. based on their morphological, growth, physiological and biochemical characteristics. The molecular masses of  $C_1$ , two  $C_x$  enzymes and  $\beta G$  were determined by SDS-PAGE to be 76.9, 22.3, 66.2 kD and 31.9 kD, respectively. The optimal pH was 5.6 for  $C_1$  and 5.0 for  $C_x$  and  $\beta G$ , respectively; the optimal reaction temperature was 50 °C for  $C_x$  and  $C_1$ , and 40 °C for  $\beta G$ , respectively. Moreover, these enzymes retained more than 50% activity even at a temperature as high as 70 °C. These enzymes could be activated by  $Fe^{2+}$  and  $Ca^{2+}$ , but inhibited by  $Mn^{2+}$ ,  $Cu^{2+}$ ,  $Zn^{2+}$  and  $Co^{2+}$ . These results indicate that these cellulases originated from *Streptomyces* strains in *O. formosanus* gut are acidic, heat-tolerant enzymes and that their prosthetic groups may be  $Fe^{2+}$  and  $Ca^{2+}$ . Therefore, these enzymes has potential in industrial applications for the decomposition of insoluble cellulose.

**Key words:** exoglucanase; endoglucanase;  $\beta$ -glucosidase; *Streptomyces*; *Odontotermes formosanus*

## 白蚁肠道二株链霉菌株所产纤维素酶的分离、纯化及其特性

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**摘 要:** 通过硫酸铵沉析、依次过葡聚糖 G100 和阴离子交换树脂 DEAE 葡聚糖 A50 柱, 从白蚁(*Odontotermes formosanus*)肠道 2 株放线菌株发酵液中获得 1 种葡聚糖外切酶( $C_1$ )、2 种葡聚糖内切酶( $C_x$ )和 1 种  $\beta$ -葡萄糖苷酶( $\beta$ -glucosidase,  $\beta G$ )。该 2 株放线菌株在形态、生长、生理生化特性上显示属于链霉菌属种类。SDS-PAGE 电泳分析表明上述 4 种酶的分子质量分别为 76.9、22.3、66.2、31.9kD。外切酶在 pH 5.6, 内切酶和  $\beta$ -葡萄糖苷酶在 pH 5.0; 以及内切酶和外切酶在 50 °C,  $\beta$ -葡萄糖苷酶在 40 °C 时, 有最大酶活性, 甚至温度高达 70 °C 时, 这些酶仍有 50% 以上的酶活性。二价阳离子如  $Fe^{2+}$ 、 $Ca^{2+}$  在质量浓度 100mg/L 条件下对酶有激活作用, 而  $Mn^{2+}$ 、 $Cu^{2+}$ 、 $Zn^{2+}$ 、 $Co^{2+}$  对酶活起抑制作用。结果表明来自白蚁肠道 2 株链霉菌株所产的纤维素酶可能以  $Fe^{2+}$  和  $Ca^{2+}$  作为辅基, 属于酸性、耐热性酶。这类酶具备用于工业上分解不溶性的纤维素的能力。

**关键词:** 葡聚糖外切酶; 葡聚糖内切酶;  $\beta$ -葡萄糖苷酶; 链霉菌; 白蚁

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Cellulose is the most abundant carbohydrate in cell walls but is not assimilated by most organisms. The degra-

dation of cellulose by cellulases has been utilized in the bio-conversions of agricultural and forest wastes, bioethanol

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production, manufacture of recycled paper and pulp<sup>[1]</sup>, biopolishing of textiles<sup>[2]</sup>, processing of fruit juices and beverages. Such enzymatic degradation requires synergistic action of several cellulases comprising, a) endo-(1,4)- $\beta$ -*D*-glucanase [EC3.2.1.4], sometimes referred to as endoglucanase, carboxymethyl cellulase (CMCase),  $C_x$ -cellulase or avicelase which initiates random attack on crystalline cellulose producing cellodextrins, cellobiose and glucose; b) exo-(1,4)- $\beta$ -*D*-glucanase, or  $C_1$ -cellulase, there are at least two types: 1) 1,4- $\beta$ -*D*-glucan cellobiohydrolase [EC3.2.1.91] which removes cellobiose units one by one from the non-reducing ends of cellulose chains; 2) 1,4- $\beta$ -*D*-glucan glucohydrolase [EC3.2.1.74] which removes glucose molecules one by one from non-reducing ends of the chain; 3)  $\beta$ -1,4)-*D*-glucosidase [EC3.2.1.21] or cellobiose which hydrolyses cellobiose to glucose. Some organisms have evolved a mechanism to utilize cellulose by symbiosis with other organisms. Cellulases are of various types, currently classified into approximately 100 families on the basis of the primary structure. The cellulose degrading function of these enzymes is thought to be due to their enzymatic structure, and is regulated by condition such as pH, temperature, cation concentration.

Termites are a fascinating group of insects that have attracted the interest of many researchers due to their ability to feed on complex biopolymers such as wood. This is due to the symbiotic interaction with microorganisms capable of cellulose degradation. Inoue et al.<sup>[3]</sup> reported endo- $\beta$ -1,4-glucanase,  $\beta$ -glucosidase, endo- $\beta$ -1,4-xylanase and  $\beta$ -xylosidase from the lower termite *Reticulitermes speratus*. Kouame et al.<sup>[4]</sup> reported two  $\beta$ -glycosidase from the termite *Macrotermes subhyalinus*. Rouland et al.<sup>[5]</sup> also reported two  $\beta$ -glycosidases from the termite *Macrotermes muelleri* and from its symbiotic fungus *Termitomyces* sp.. Matsuura et al.<sup>[6]</sup> also reported  $\beta$ -glucosidase from cuckoo fungus which mimics termite eggs. In general, flagellates and bacteria occur in the gut of lower termites, while higher termites possess only bacteria. Actinomycetes are a little isolated from termites. Hungate<sup>[7]</sup> isolated for the first time a cellulose-decomposing actinomycete *Micromonospora propionici*, from the alimentary canal of the worker termite of the wood-eating species *Amitermes minimus*. Pasti et al.<sup>[8]</sup> reported cellulolytic activity of actinomycetes from termites *Macrotermes amitermes*.

In the present paper, we report the cellulases from two *Streptomyces* strains (actinomycetes) in termite *Odontotermes*

*formosanus* gut and their isolation, purification and characterization.

## 1 Materials and Methods

### 1.1 Microbial strains and identification

Termite *O. formosanus* species was collected from the plantation of Jiangxi Agricultural University, China. The aggregated termites were transferred into the laboratory, surface sterilized using 1 g/100 mL  $HgCl_2$  solution for 3 min submergence, washed 3 times with 75% ethanol solution, successively rinsed with distilled water and blotted dry on sterilized filter papers. The intestinal tracts were then removed aseptically with fine-tipped forceps onto sterilized slides and the contents of the gut were transferred into sterile tubes with water to prepare a suspension. Subsequently 0.2 mL of microbial solution at a dilution of  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$  from the contents of the gut were spreaded on Gause' s No.1 medium plate in Petri dishes respectively. After incubation at 30 °C, a single actinomycete colony was transferred onto a Petri dish with congo red dye<sup>[9]</sup>. Cellulose degradation was indicated by a clear zone around the colonies. Target actinomycetes with rapid growth and bigger hydrolysis zone were selected.

In order to identify selected actinomycetes, purified colonies were spreaded onto Gause' s No.1 medium, beef-peptone-agar medium, salt-starch-agar medium, and yeast-agar medium to observe mycelium growth characteristics. Characteristics for their mycelia were observed under light microscope on slides taken from Gause' s No.1 medium. Samples were then tested for gluten liquefaction, milk solidification, milk peptonizing, starch hydrolysis, nitrate reduction, growth on cellulose substrate, production of melanin for physiological and biochemical features.

### 1.2 Enzymatic activity assays

#### 1.2.1 Cellulase

Cellulase activity was measured from the release of a reducing sugar on quantitative filter paper strip (Whatman) by the combined actions of all the activities present in the cellulase complex. The combined enzyme sample (0.5 mL) was treated with substrate (paper strip 50 mg in 1.5 mL, 0.05 mol/L, pH 4.5 citric acid buffer, 50 °C, 1 h). 3,5-Dinitrosalicylic acid reagent (DNS, 1.5 mL) was added and the sample boiled for 5min, then diluted with distilled water to 20mL and  $A_{540nm}$  determined. One unit of enzymatic activity (FPase) was defined as the amount of enzyme that releases 1  $\mu$ g of reducing sugar per minute under the assay conditions<sup>[10]</sup>.

### 1.2.2 Exoglucanase

This activity was measured from the release of a reducing sugar of degrease cotton. The enzyme sample (0.5 mL) was treated with substrate (50 mg in 1.5 mL, 0.05 mol/L, pH 5.0 citric acid buffer, 45 °C, 24 h). Staining and determining were performed as described at 2.2.1 section. One unit of enzymatic activity ( $C_i$ ) was defined as the amount of enzyme that releases 1  $\mu$ g of reducing sugar per hour under the assay conditions<sup>[10]</sup>.

### 1.2.3 Endoglucanase

This activity was measured from the release of a reducing sugar of carboxymethylcellulose (CMC). The enzyme sample (0.5 mL) was treated with substrate (0.51 g/100 mL in 1.5 mL, 0.05 mol/L, pH 5.0 citric acid buffer, 50 °C, 30 min). Staining and determining were performed as described at 2.2.1 section. One unit of enzymatic activity ( $C_x$ ) was defined as the amount of enzyme that releases 1  $\mu$ g of reducing sugar per minute under the assay condition<sup>[10]</sup>.

### 1.2.4 $\beta$ -Glucosidase

This activity was measured from the release of glucose from salicin (2-(hydroxymethyl) phenyl- $\beta$ -D-glucopyranoside). The enzyme sample (0.5 mL) was treated with substrate (0.5 g/100 mL in 1.5 mL, 0.05 mol/L, pH 5.0 citric acid buffer, 50 °C, 30 min). Staining and determining were performed as described at 2.2.1 section. One unit of enzymatic activity ( $\beta$ G) was defined as the amount of enzyme that releases 1  $\mu$ g of reducing sugar per minute under the assay condition<sup>[10]</sup>.

### 1.3 Preparation, isolation and purification of cellulases

Three actinomycetes selected were inoculated into liquid medium for fermentation as single or multi-strains. The detail of this method is similar to that outlined below and in Table 1.

Two actinomycetes strains ( $A_2:A_3 = 1:2$  inoculation proportion, 8% total inoculation quantity) with high cellulose degradation activity were inoculated into fermentation medium (1% bran, 0.5% beef extract, initial pH 5.0, at 30 °C, 80 mL medium quantity per 250 mL, 150 r/min shaker speed for 120 h). The fermentation mixture (100 mL) was centrifuged ( $10000 \times g$ , 30 min, 4 °C). A cell-free supernatant with cellulases was collected.

The supernatant containing cellulases was precipitated using 30% — 60% ammonium sulfate. The precipitate collected was filled into dialysis tubing, dialyzed in citric acid buffer under 4 °C for 12 — 18 h, replacing buffer every 3 — 4

h to remove ammonium sulfate. Cellulase activity was determined as above and protein relative content was expressed as  $A_{280nm}$ .

Enzyme collection above (5 mL) was loaded onto an Sephadex G100 column (2.6 cm  $\times$  20 cm) equilibrated with Tris-HCl buffer (0.05 mol/L, pH 8.5). The enzymes were eluted in the same buffer at the rate of 18 mL/h. The elution of the proteins was monitored at 280 nm. The same active fractions were pooled, concentrated against PEG8000, 3 h). The fraction with single enzyme and protein peak was subjected to SDS-PAGE electrophoresis. The fraction with multiple enzyme and protein peaks was further purified following DEAE-Sephadex A50 column equilibrated with the same buffer. Bound enzymes were eluted from the column by a stepwise increase in NaCl (0 — 0.5 mol/L) in the same buffer and rate. The fractions with the major peak of enzyme activity and protein were concentrated as above for SDS-PAGE.

### 1.4 Zymogram analysis

The analysis was performed by SDS-PAGE discontinuous gel electrophoresis as described by Guo Yaojun<sup>[11]</sup> using a 12% separating gel, concentrated gel (5%) and  $\beta$ -galactosidase (116 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD), lactate dehydrogenase (35 kD), REase Bsp981 (25 kD),  $\beta$ -lactoglobulin (18.4 kD), and lysozyme from egg white (14.4 kD) as a standard protein. Enzyme bands were stained with Coomassie blue R250.

### 1.5 Effect of pH and temperature on activity of cellulases

The enzymes of FPase,  $C_i$ ,  $C_x$ ,  $\beta$ G were added to relevant substrate mediums at different pH ranges (pH 4.0, 4.6, 5.0, 5.6, 6.0) respectively, and incubated for 10 min at 50 °C. The optimal temperature for activity of each enzyme was determined by incubating the enzymes in different temperatures (30, 40, 50, 60, 70, 80 °C) at pH 5.0. The residual activities of enzymes were measured as described above.

### 1.6 Effect of cation on cellulases

The enzymes of FPase,  $C_i$ ,  $C_x$ ,  $\beta$ G were incubated (15 min, 50 °C, pH 5.0) with 100 mg/L metal chloride including Fe, Ca, Mn, Cu, Zn, Co. Each assay was initiated by the addition of respective substrate and enzyme activities determined as before.

## 2 Results and Analysis

### 2.1 Acquisition of cellulolytic actinomycetic strains and their classification

Three actinomycete strains were obtained by diluting

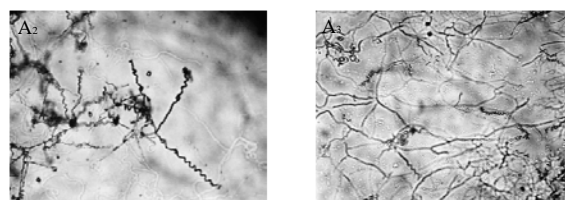
content of *O. formosanus* gut to  $10^{-5} - 10^{-7}$ , screening using Gause' s No.1 medium and cellulose-congo red medium with increased diameter of dissolving ring at the rate of 0.5 cm/d and growth diameter of colony at the rate of 0.25 cm/d. After inoculating the three strains in liquid fermentation medium (30 °C, 150 r/min, for 120 h), all three strains generate cellulolytic enzymes respectively (Table 1).  $A_1$  and  $A_3$  strains have greatest ability for production of enzymes. Except for  $C_1$  enzyme activity, FPase,  $C_x$  and  $\beta G$  from  $A_2$  are notably lower in  $A_1$  and  $A_3$ . Total cellulolytic activity of  $A_2$  was 88.6% of  $A_1$  and 90.15% of  $A_3$ . Its enzymatic activity of  $\beta G$  was 90.38% of  $A_1$  and 91.53% of  $A_3$ , while its enzymatic activity of  $C_x$  was only 59.9% of  $A_1$  and 64.51% of  $A_3$ . However, the results of enzymatic activity from mixed strain fermentation is out of imagination. Mixing fermentation of two strains ( $A_2 + A_3$ ) with respective lower enzymatic activity produced stronger activity than the other combinations. The combined activity of FPase,  $C_1$ ,  $C_x$  and  $\beta G$  was 114.56%, 115.00%, 193.28%, 111.81% of  $A_2$ , 103.28%, 115.00%, 124.69%, 102.33% of  $A_3$ , and 101.50%, 121.05%, 115.78%, 101.05% of  $A_1$  respectively. Therefore, we choose the  $A_2$  and  $A_3$  combination to investigate further.

**Table 1** Enzyme activity (EA) unit of 120-h fermentation broth of individual and combined inoculum strains for degradation cellulose

Name of strains	EA of FPase/U	EA of $C_1$ /U	EA of $C_x$ /U	EA of $\beta G$ /U
$A_1$	465	20	1217	478
$A_2$	412	20	729	432
$A_3$	457	19	1130	472
$A_1+A_2$	412	19	930	424
$A_1+A_3$	424	18	1193	437
$A_2+A_3$	472	23	1409	483
$A_1+A_2+A_3$	443	19	1187	454

Morphological characteristics of the different strains under the microscope show that mycelia of  $A_2$  is a helical aerial hypha, while  $A_3$  is a loose, curly and partial spiry aerial hypha (Fig.1). Both strains grow on beef extract peptone agar (NA), inorganic salt starch agar (ISA), yeast agar (TA), Gause' s No.1, and potato dextrose agar (PDA), but the color of their aerial mycelia (AM) and substrate mycelia (SM) are somewhat different. For instance, SM of  $A_3$  are grey on ISA whereas  $A_2$  are yellow; SM of  $A_2$  is fulvous on YA, whereas  $A_3$  are yellow; such is the case for AM of  $A_3$  which is dust colored on ISA, whereas  $A_2$  is off-white; and AM of  $A_2$  is grey on YA, whereas  $A_3$  is beige. Their colors are similar on NA, Gause' s No.1 and PDA. Physiological and biochemical

characteristics of  $A_2$  and  $A_3$  strains also show some difference.  $A_3$  can produce melanin, while  $A_2$  can not.  $A_2$  can peptonize milk, while  $A_3$  can not. Both can reduce nitrate and grow onto cellulose substrate, but they can not solidify milk. Due the these characteristics we make the preliminary conclusion that  $A_2$  and  $A_3$  are two different strains, and both belong to *Streptomyces* spp. according to Bergey' s Manual of Determinative Bacteriology.

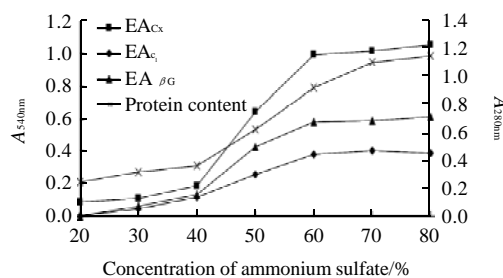


**Fig.1** Morphological characteristics of  $A_2$  and  $A_3$  strains under microscope ( $\times 400$ )

2.2 Isolation and purification of cellulolytic enzymes from fermentation broth of mixing *Streptomyces* strains  $A_2 + A_3$

2.2.1 Isolation of cellulolytic enzymes with partition salt-out of ammonium sulfate

Fig.2 shows that activity of  $C_x$ ,  $C_1$  and  $\beta G$  increased slowly with increased content of protein precipitation when concentration of ammonium sulfate ranges from 20% to 40%. Enzymatic activity barely increases with increased content of protein sediment at concentration of ammonium sulfate from 60% to 80%. This indicates that the precipitate fraction at this range does not contain cellulolytic enzymes. Between 40% and 60% ammonium sulfate, enzymatic activity increase rapidly with increased amount of precipitate. The precipitate fraction obtained at concentration of ammonium sulfate between 40% — 60% is the target fraction comprising all three cellulolytic enzymes. Therefore, we collected this fraction to dialyze for removal salt and other small molecules.



**Fig.2** Effect of salting out with different concentrations of ammonium sulfate on the recoveries of  $C_1$ ,  $C_x$  and  $\beta G$  activities and protein content

### 2.2.2 Purification of cellulolytic enzyme with gel chromatography of Sephadex G100

Four fractions containing protein peaks A, B, C and D respectively (Fig.3) were pooled. Of these, three peaks were associated with cellulase: the B protein peak correlated with  $C_1$ ; the C protein peak correlated with  $C_{x-1}$ ; and the D peak correlated with  $C_{x-2}$  and  $\beta G$ . The C fraction was subsequently concentrated for purity testing by electrophoresis. The D fraction was further separated using anion exchange resin of DEAE-Sephadex A50. The A fraction proteins were discarded they were without cellulolytic activity.

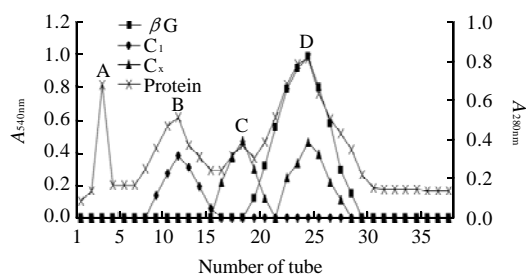


Fig.3 Elution profile of salting out precipitation products on Sephadex G100 column

The D-1 peak showed activity of  $\beta G$ , while the D-2 peak showed activity of  $C_{x-2}$  when absorption values at 280 nm (protein) are compared that at 540 nm (for enzymatic activity) (Fig.4). The D-1 and D-2 peaks were concentrated by polyethyleneglycol 8000 prior to electrophoresis analysis. As described above, the combination of  $A_2$  and  $A_3$  strains yield a  $C_1$ , two  $C_x$  and a  $\beta G$  enzyme. Comparing enzymes from the  $A_2 + A_3$  mixture with enzymes from  $A_2$  or  $A_3$ , it appears that *Streptomyces*  $A_2$  and  $A_3$  produce the same enzyme in the case of  $C_1$  and  $\beta G$ , while it is possible that the two  $C_x$  proteins originate from the  $A_2$  and  $A_3$  strain respectively. Alternatively a  $C_x$  origin  $A_2$  or  $A_3$ , and another  $C_x$  origin both of  $A_2$  and  $A_3$ . The origin of the variable forms of the  $C_x$  protein may be a question for further investigation.

### 2.2.3 Cellulolytic enzyme purification with anion exchange chromatography of DEAE-sephadex $A_{50}$

The D-1 peak showed activity of  $\beta G$ , while the and D-2 peak showed activity of  $C_{x-2}$  when absorption values at 280 nm (protein) are compared that at 540 nm (for enzymatic activity) (Fig.4). The D-1 and D-2 peaks were concentrated by polyethyleneglycol 8000 prior to electrophoresis analysis. As described above, the combination of  $A_2$  and  $A_3$  strains yield a  $C_1$ , two  $C_x$  and a  $\beta G$  enzyme. From comparing enzymes from the  $A_2 + A_3$  mixture with enzymes from  $A_2$  or

$A_3$ , it appears that *Streptomyces*  $A_2$  and  $A_3$  produce the same enzyme in the case of  $C_1$  and  $\beta G$ , while it is possible that the two  $C_x$  proteins originate from the  $A_2$  and  $A_3$  strain respectively. Alternatively a  $C_x$  origin  $A_2$  or  $A_3$ , and another  $C_x$  origin both of  $A_2$  and  $A_3$ . The origin of the variable forms of the  $C_x$  protein may be a question for further investigation.

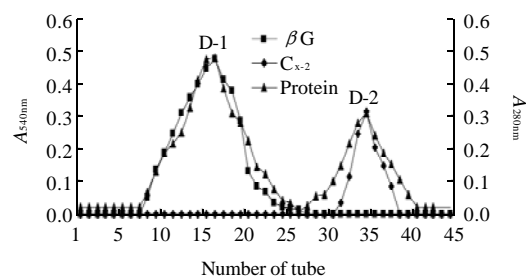
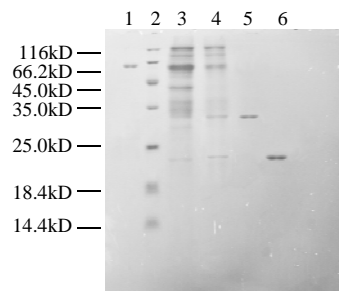


Fig.4 Elution profile of fraction D on DEAE-Sephadex A50 column

### 2.3 Purity identification and determination of molecular weight

The peaks of C, D-1 and D-2 corresponded to  $C_{x-1}$ ,  $\beta G$  and  $C_{x-2}$ , which each give single band (Fig.5). This demonstrates three pure enzymes were obtained. In the case of  $\beta$ -galactosidase (116 kD), bovine serum albumin (66.2 kD), ovalbumin (45 kD), lactate dehydrogenase (35 kD), REase Bsp981 (25 kD),  $\beta$ -lactoglobulin (18.4 kD), lysozyme from egg white (14.4 kD) as standard protein, the equation  $\lg y = -0.9357x + 5.0546$  was reduced on the relationship between relative mobility of standard protein ( $x$ ) and common logarithm of relative molecular mass of standard protein ( $\lg y$ ). Molecular mass of  $\beta G$  was calculated as 31.9 kD, while the molecular mass of both  $C_x$  proteins were calculated as 22.3 kD ( $C_{x-2}$ ) and 64.5 kD ( $C_{x-1}$ ) respectively. The fraction of  $C_1$  was not used for electrophoresis, but we can infer that the second band downward belongs to  $C_1$  because the mixture generates five bands. The top band firstly exhibited agreed with the protein of A peak as Sephadex G100. The molecular mass of  $C_1$  was calculated as 76.9 kD on its relative mobility.



1.  $C_{x-1}$ ; 2. Marker; 3. Crude enzymes; 4. Enzymes salted-out; 5.  $\beta G$ ; 6.  $C_{x-2}$ .  
Fig.5 SDS-PAGE of fermentation supernatant, salting out precipitation products, and purified  $\beta G$  and  $C_x$

## 2.4 Characteristics of cellulolytic enzymes

Optimum pH value of  $\beta G$  and  $C_{x-1}$  were pH 5.0, while  $C_1$  was optimally active at pH 5.6 (Fig.6), owing to their strongest activity being observed at these pH values. The three cellulases are therefore all acidic enzymes.

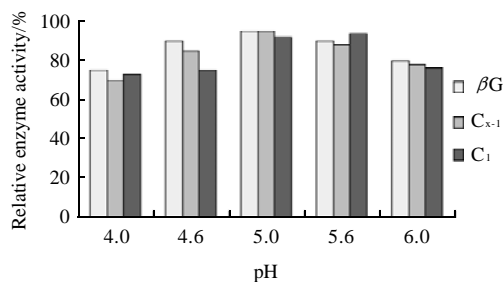


Fig.6 Effect of pH on relative activities of three enzymes

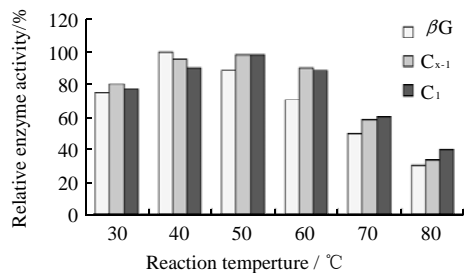


Fig.7 Effect of temperature on relative activities of three enzymes

The optimum temperature of  $\beta G$  was 40 °C, while  $C_{x-1}$  and  $C_1$  were 50 °C based on highest enzyme activity tested over a range of temperatures (Fig.7). Even when temperatures as high as 70 °C were used, these three enzymes retained more than 50% of their enzymatic activity at pH 5.0.

Metal cations notably influenced total cellulolytic enzymatic activity (FPase) for  $\beta G$ ,  $C_{x-1}$  and  $C_1$  compared to control samples (Fig.8).  $Fe^{2+}$  and  $Ca^{2+}$  enhanced enzyme activity, whereas  $Cu^{2+}$  and  $Co^{2+}$  weakened their activity,  $Mn^{2+}$  and  $Zn^{2+}$  had little effect on their activity. It is probable that  $Fe^{2+}$  and  $Ca^{2+}$  are a prosthetic group of these enzymes.  $Cu^{2+}$  and  $Co^{2+}$  might have given birth to effect by the way of antagonism to  $Fe^{2+}$  and  $Ca^{2+}$ .

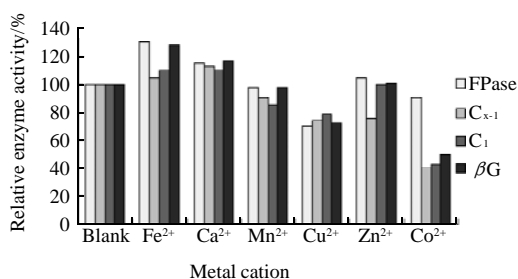


Fig.8 Effect of metal ions on relative activities of three enzymes

## 3 Discussion

Independent molecular studies have revealed that the majority of microbial gut symbionts have not yet been cultured, including actinobacterial clusters associated with termite guts<sup>[12]</sup>. The reason is thought to be that background bacteria impede the growth of actinomycetes on isolation plates. Removal of common background bacteria is one of the important steps towards culturing the previously little cultured microbes. We use specific actinomycete medium, Gause' s No.1, to remove common background bacteria and obtain two *Streptomyces* strains with cellulolytic enzyme production. The plates without specific actinomycete medium such as PDA and YA contained bacteria forming smears resulting in the cancellation of actinomycete taxa. This result agrees with the report of Kurtboke et al.<sup>[13]</sup> about termite *Coptotermes lacteus* gut microflora. This also implies that culture independent techniques can be designed to obtain target microbes from diverse floral profiles of the termite gut.

Since actinomycete taxa are prone to be inhibited by other gut bacteria, how can the taxa occur in the termite gut. Earlier research have shown that actinobacterial clusters do not accidentally occur in the intestinal lumen, but are intimately associated with the absorptive epithelia of the intestine<sup>[14]</sup>. The epithelium forms distinct sites for the attachment of symbiotic microorganisms.

The fore- and midgut of all termites are aerobic, but paunch, colon and hindgut are anaerobic. A constant diffusion of oxygen through the paunch epithelium readily support its use for aerobic and facultative anaerobic microorganisms and the gut contents remain anaerobic<sup>[15]</sup>. The termite gut is a well-aerated anaerobic gradient system where aerobic microbes are also present<sup>[16]</sup>. Therefore, two aerobic *Streptomyces* isolated can exist in the this environment. The two strains differ from Hungate' s isolated Actinomycete<sup>[8]</sup>, *Micromonospora propionici* which belongs to anaerobic microbe.

The cellulases isolated directly from free-cell fermentation broth of two *Streptomyces* strains indicates these enzymes are extracellular production. Both of the isolates are of secreting three cellulolytic enzyme ability, of which there are two enzymes with the same molecular mass:  $\beta G$  and  $C_1$ , while there may be different molecular masses for  $C_x$ . Zhu Huiyuan et al.<sup>[17]</sup> showed the  $C_x$  molecular mass range to be between 23–146 kD. Wu Xianrong et al.<sup>[18]</sup> reviewed  $C_1$  molecular mass range and showed it to be between 38–118 kD. Molecular

masses for  $C_{x-1}$  and  $C_1$  isolated are in accordance with the expected size range, but the molecular mass of  $C_{x-2}$  (22.3 kD) isolated is small for its expected size. In addition, the molecular mass of  $\beta$  G is lower than the other  $\beta$ -glycosidases reported<sup>[4,6,19]</sup>, only 31.9 kD.

In general, bacterial cellulases are active in neutral pH while enzymes of actinomycetes origin are active in acidic range. Our research show similar results:  $C_x$  and  $\beta$  G have strongest activities at pH 5.0; while another  $C_x$  is highest at pH 5.6. On the other hand, these three enzymes are of strongest activity at higher temperature:  $\beta$  G is 40 °C, while the two  $C_x$  are best at 50 °C. Even at temperatures up to 70 °C, three enzymes exhibit more than 50% residual activity. Industrial application of cellulases is plagued by such problems as catalytic constant towards insoluble substrates, low thermostability, high degree of product inhibition, and low adsorption coefficient towards the substrate. Paul et al.<sup>[20]</sup> observed that glucose, the end product, enhanced endoglucanase synthesis and activity. The endoglucanase originated from *Bacillus* isolated in mound soil of the termite *Odontotermes obesus*. This indicates synthesis and activity of endoglucanase from this isolate were not subject to end product regulation. Therefore the three cellulolytic enzymes identified here (which are heat-tolerance and acidic), and the two *Streptomyces* strains with secreting extracellular enzymes may have industrial applications for the decomposition of insoluble cellulose.

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