CHARACTERIZATION OF CELLULASE PREPARATION OF 
BACILLUS SP.G4 ISOLATED FROM THE TERMITES GUT

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ABSTRACT

The Bacillus sp. strain G4 isolated from termites gut can produce cellulase with high activity in rice bran medium after 72 h of incubation at 37 °C. The crude enzyme was collected by centrifugation at 6,000 rpm for 15 minutes. The precipitation of cellulase with ammonium sulfate 60 – 90 % saturation and acetone at the concentration 60 % – 90 % was studied. Results showed that ammonium sulfate 90 % saturation gave cellulase with highest purification factor 8.87 but the yield was only 59.9 %, whereas acetone at 90 % concentration gave highest yield 83.57 % with purification factor 4.38. CMCase activity of cellulase preparation obtained by acetone precipitation at 90 % was optimum at pH 7 and 60 °C. Furthermore, CMCase was stable at pH 6.0 - 7.0 and at temperature lower than 50 °C. The CMCase was activated by ion Ca2+ but inhibited by Co2+, Zn2+, Cu2+, Fe2+, and was not affected by Mg2+ and Ba2+. The CMCase was inhibited by high concentration of SDS while EDTA and Tween 80 played activated role.

Keywords: Bacillus, Cellulase, CMCase, isolation, EDTA, SDS, Tween 80, termite gut.

1. INTRODUCTION

Cellulose is the most abundant renewable biopolymer in nature. In the near future, the processes using cellulase could lead to new environmentally friendly technologies. Cellulases hydrolyze the β-1,4-glycosidic linkages of cellulose. Traditionally, they are divided into two classes referred to as endoglucanases and cellobiohydrolases. Endoglucanases (endo-1,4-β-glucanase, EGs) act on the existing or endoglucanase-generated chain ends[1, 2].

Many microorganisms such as bacteria, fungi, actinomycetes and yeasts are capable of producing extracellular cellulase enzyme [4 - 7]. Cellulase producing bacteria were isolated from various habitats like soil, hot springs, organic matters, faeces of ruminants decayed plant materials and composts [8].
Termites are one of the most important soil insects that efficiently decompose lignocelluloses with the aid of their associated microbial symbionts to simpler form of sugars monosaccharides, which later can be fermented to ethanol using yeasts. Termites are reported to dissimilate a significant proportion of cellulose (74 – 99 %) and hemicelluloses (65 – 87 %) components of lignocellulose they ingest [9].

Cellulases have a variety of application in many different industries such as food, brewery, wine, pulp and paper, textile, detergent, feed and agriculture [4, 18]. The application of produced enzymes in industry requires high thermostability along with ability to tolerate a wide pH range. Bacteria owing to their high diversity and capability to produce highly thermostable and alkali-stable enzyme, and thus may serve as highly potential sources of industrially important enzyme [4]. Among bacteria, Bacillus species can produce many types of extracellular enzymes which can hydrolyze polysaccharides. Researchers have documented the production of thermostable and alkali-stable cellulases from different Bacillus species [3, 4, 5]. For industrial use, technical enzyme preparation is preferred over purified one due to economical reason.

In this work the technical cellulase preparation from crude enzyme, produced by Bacillus sp. G4 which was isolated from Termites gut and its characteristics was investigated. The effects of temperature, pH, some metal ions, surfactant (SDS, Tween 80) and chelating agent (EDTA) on enzyme activity were studied.

2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Sample collection and isolation of cellulose degrading bacteria

Cellulose feeding termites were collected from decaying falling trees in Nghe An province.

2.1.2. Microorganism and culture

The Bacillus sp G4, isolated from gut of termites, was cultured in media containing 2 % rice bran, 1 % casein, 1 % soy flour and 1 % sodium chloride. After 72 h incubation at 37 °C with shaking at 150 rpm, the cultured broth was centrifuged at 6000 rpm for 20 min. The supernatants were used for futhercellulase purification.

2.2. Methods

2.2.1. Enzyme activity assay

Endo-β-1,4-glucanase (CMCase) activity was determined by incubation of 0.5 ml enzyme with 1ml CMC in 0.05 M sodium citrate buffer (pH 4.8) at 50 °C. After 30-min reaction, 1.5ml of dinitrosalicylic acid (DNS) was added and boiled in a water bath for 5 min to stop the reaction. The resulted samples were then cooled to room temperature and were measured at absorbance 540 nm (A_{540}). One unit of CMCase activity was defined as the amount of enzyme that could hydrolyze CMC and release 1µmol of reducing sugar (referring as glucose) within 1min reaction at 50 °C [10].
2.2.2. Determination of protein concentration

Protein concentration was determined by Lowry’s method using bovine serum albumin as standard [11].

2.2.3. Purification of cellulase

The crude cellulase was precipitated by the addition of cold ammonium sulfate of 60 – 90 % saturation or by cold 60 – 90 % acetone. The precipitate was centrifuged at 8,000 rpm for 15 min and dialyzed against acetate buffer (pH 4.8) to obtain the technical enzyme preparation.

2.2.4. Influence of temperature and pH on endoglucanase activity of technical preparation

Temperature optimum. To 1ml of 1 % CMC in 0.05 M citrate buffer (pH 4.8), 0.5 ml of enzyme were added and incubated at various temperatures (20, 30, 40, 50, 60, 70, 80, 90 °C) for 30 min [3]. The CMCase activity was then measured as describe in 2.3.

Temperature stability. 1ml of enzyme in 0.05 citrate buffer (pH 4.8) was incubated at various temperatures (30, 40, 50, 60, 70, 80, 90 °C) for 30 minute [3]. The residual activity was measured according to enzyme assay 2.3.

pH optimum. Adding 0.5 ml of enzyme to 1ml of 1 % CMC at various pH values. Various pH values were obtained by changing the buffer solution as follows: 50 mM citrate buffer for pH 3.0 - 7.0; 50 mM phosphate buffer for pH 6.0 - 9.0 and 50 mM Tris buffer for pH 8 - 11.

pH stability. One ml cellulase preparation was incubated in buffer with various pH values 3.0 - 11 at 30 °C for 30 min. The residual activity was measured.

2.2.5. Effect of metal ions, SDS, Tween 80 and EDTA on CMCase activity of technical preparation

One ml of enzyme in 20 mM citrate with each of (Co²⁺, Ba²⁺, Ca²⁺, Fe²⁺, Mg²⁺, Zn²⁺) metal ions at final concentration 0.005 M or ethylenediamine-tetraacetic acid (EDTA), Tween 80 and sodium dodecyl sulfate (SDS) at final concentrations of 1, 5, 10 mM were incubated at 30 °C for 30 min. After incubation, the residual activity was measured.

3. RESULTS AND DISCUSSION

3.1. Methods for obtaining technical cellulase preparation from B. subtilis G4

Table 1. Technical cellulase preparation by ammonium sulfate precipitation.

<table>
<thead>
<tr>
<th>Ammonium sulfate precipitation (%)</th>
<th>Total activity (UI/ml)</th>
<th>Total Protein (mg/ml)</th>
<th>Specific activity (UI/mg)</th>
<th>Purification factor</th>
<th>Recovery yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>0.53</td>
<td>1.16</td>
<td>0.15</td>
<td>5.63</td>
<td>25.60</td>
</tr>
<tr>
<td>70</td>
<td>1.04</td>
<td>1.61</td>
<td>0.65</td>
<td>8.13</td>
<td>50.24</td>
</tr>
<tr>
<td>80</td>
<td>1.19</td>
<td>1.76</td>
<td>0.67</td>
<td>8.38</td>
<td>57.48</td>
</tr>
<tr>
<td>90</td>
<td>1.24</td>
<td>1.80</td>
<td>0.71</td>
<td>8.87</td>
<td>59.90</td>
</tr>
<tr>
<td>Crude enzyme</td>
<td>2.07</td>
<td>25.05</td>
<td>0.08</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>
**Bacillus** sp G4 was cultivated at 37 °C for 72 h. After removing the cells, the obtained crude enzyme was precipitated by ammonium sulfate of 60 – 90 % saturation or 60 – 90 % acetone. The recovery and purification factor of technical cellulase preparation were presented in Table 1 and 2.

Both ammonium sulfate and acetone precipitation of crude enzyme resulted in maximum recovery yield and purification fold at 90 % final concentration. Acetone precipitation was selected over ammonium sulfate precipitation due to better recovery yield. The result was similar to previous study of Ekundayo et al. [17]. In the 90 % acetone precipitation, the recovery yield 83.57 % and purification fold 4.38 could be achieved. Meanwhile by ammonium sulfate 90 % saturation precipitation a recovery yield 59.9 % and 8.87-fold purification could be achieved. According to Ekundayo, the recovery yield of 70 % with purification fold of 3 was obtained by 80 % acetone precipitation. The specific activity of the precipitated CMCase was 0.26 UI/mg [17], which was slightly lower than 0.35 UI/mg in this study.

**Table 2. Technical cellulase preparation by acetone precipitation.**

<table>
<thead>
<tr>
<th>Acetone (%)</th>
<th>Total activity (UI/ml)</th>
<th>Total Protein (mg/ml)</th>
<th>Specific activity (UI/mg)</th>
<th>Purification factor</th>
<th>Recovery yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>60</td>
<td>0.65</td>
<td>2.35</td>
<td>0.29</td>
<td>3.63</td>
<td>31.40</td>
</tr>
<tr>
<td>70</td>
<td>1.32</td>
<td>4.16</td>
<td>0.32</td>
<td>4.0</td>
<td>63.77</td>
</tr>
<tr>
<td>80</td>
<td>1.56</td>
<td>4.60</td>
<td>0.34</td>
<td>4.25</td>
<td>75.36</td>
</tr>
<tr>
<td>90</td>
<td>1.73</td>
<td>4.90</td>
<td>0.35</td>
<td>4.38</td>
<td>83.57</td>
</tr>
<tr>
<td>Crude enzyme</td>
<td>2.07</td>
<td>25.05</td>
<td>0.08</td>
<td>1</td>
<td>100</td>
</tr>
</tbody>
</table>

3.2. Effect of temperature on CMCase and stability of technical cellulase

Technical enzyme preparation showed activity over a broad range of temperature (30 – 90 °C) with the maximum activity at 60 °C. Significant residual activity of 36 % remained even at elevated temperature of 90 °C (Fig. 1). The CMCase activity of technical preparation was stable at temperature under 50 °C (Fig. 2). The optimum temperature of CMCase was lower than some of other Bacillus strain (65 °C by CH43 and 70 °C by RH 68) [12], but higher than Mucor circinelloides (55 °C) [13]. The thermal stability of CMCase activity of technical preparation (0 – 50 °C) was similar to those from other Bacillus strain [12] but lower than that from Mucor circinelloides (0 – 70 °C) [13]. Microbial cellulase with varying thermostability (50 - 70 °C) for different time period had been documented [17].

![Figure 1. Effect of temperature on CMCase activity.](image)
For industrial application, highly thermotolerant enzymes are required, for which either the natural microflora may be screened or the enzyme may be tailored by protein so that can withstand and work at elevated temperatures during process condition.

3.3. Effect of pH on CMCase activity and stability of technical cellulase

Activity assay of CMCase was done in reaction mixture at varying pH by using appropriate buffer. It was found that enzyme had got activity in a wide range of pH (Fig. 3). Maximum
activity was expressed at pH 7 (2.14 UI/ml), however, significantly high activity remained at pH 6 (2.04 UI/ml), pH 5 (1.9 UI/ml) (Fig. 4). According to some previous studies, the optimal pH was 5.0 - 6.5 for those from *Bacillus* strain [12], 6.0 - 7.0 from *Aspergillus niger* [14] and 5.0 - 7.0 from *Lysobacter* sp [15]. The partially purified cellulase was stable at pH 6.5 - 7.5, which was higher than those from *Mucor circinelloides*, 4.0 - 7.0 [13].

3.4. Effect of divalent ions on CMCase activity

As shown in Fig. 5, Mg$^{2+}$ did not effect the enzyme activity, while Ba$^{2+}$ greatly activated the purified cellulase. Fe$^{2+}$, Cu$^{2+}$, Zn$^{2+}$, Co$^{2+}$ inhibits the purified cellulase activity but Ca$^{2+}$ activate the enzyme. The result are almost similar to the studies by Saha [13]. Mg$^{2+}$, NH$_4^+$ did not effect the cellulase from *Rhizopus oryzae* [16]. While Co$^{2+}$ and Mn$^{2+}$ activated that from *Mucor circinelloides* [13].

![Figure 5. Effect of divalent ion on CMCase activity.](image)

3.5. Effect of additives on CMCase activity

Enzyme assay was performed in presence of detergent (SDS, Tween 80) and EDTA. A comparison between the achieved residual activity using those material with final concentration of 1, 5, 10 mM. As indicated in Table 3, SDS inhibit the enzyme activity in concentration at 5 mM and 10 mM, while Tween 80 and EDTA activated its activity at al concentration.

<table>
<thead>
<tr>
<th>Additives</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Concentration (mM)</td>
</tr>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>SDS</td>
<td>118</td>
</tr>
<tr>
<td>EDTA</td>
<td>134</td>
</tr>
<tr>
<td>Tween 80</td>
<td>127.6</td>
</tr>
</tbody>
</table>
Characterization of cellulase preparation of Bacillus sp. G4 isolated from the termites gut

Similar result have been found by Nema et al., where EDTA increased cellulase activity for cellulase produced from Bacillus cereus [15]. In contradiction to EDTA the residual enzyme activity using SDS decreased considerably with increasing concentration. With SDS concentration of 10 mM the residual enzyme activity was decreased to 0.35 UI/ml. At a concentration of 1 mM it was higher than that activity of the purified cellulase. This result agrees well with Lin et al [14] and Yin et al [3].

4. CONCLUSION

The potential of Bacillus sp G4 strain isolated from termites gut for production of cellulase was demonstrated. Acetone precipitation gave better recovery yield than ammonium sulfate. The cellulase preparation obtained by acetone precipitation at 90% exhibited optimum activity at pH 7 and 60 °C. Furthermore CMCase was stable at pH 6.0 - 7.0 and at temperature lower than 50 °C. The CMCase of technical preparation was activated by Ca²⁺ but inhibited by Co²⁺, Zn²⁺, Cu²⁺, Fe²⁺, and was not affected by Mg²⁺ and Ba²⁺. The CMCase was inhibited at high concentration of SDS, while EDTA and Tween 80 played activated role.

REFERENCES


TÔM TẮT

XÁC ĐỊNH DẠC TÍNH CỦA CHẾ PHÁM CELLULASE KỸ THUẬT TƯ VỊ KHU HUAN BACILLUS SP.G4 PHẠM LẤP TƯ RUỘT MÓI

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Chúng vi khuẩn Bacillus sp G4 phân lập từ ruột mồi có khả năng sinh cellulase cao trên môi trường có độ pH từ 7.2 tới 9 độ 37°C. Enzym thế thu nhân từ môi trường tổng cộng được liệt kê ở độ 6000 vòng/phút trong 15 phút để loại bỏ tế bào. Giống cụ thể thu nhân chế phẩm cellulase kí thuật được thực hiện bằng phương pháp kết tủa enzym với ammonium sunphate bảo hòa và acetone ở các nồng độ từ 60 – 90%. Kết quả cho thấy ammonium sunphate bảo hòa 90% cho khả năng tính sản phẩm cellulase tối đa 8.87 lần nhưng hiệu suất thu hồi chỉ đạt 59,9% trong khi acetone ở nồng độ 90% cho hiệu suất thu hồi cellulase cao nhất 83,57% với độ tính sản phẩm 4,38 lần. Chế phẩm cellulase kí thuật thu nhân bằng kết tủa enzym ở nồng độ 90% cho hoạt tính CMCase cực đại ở pH 7, nhiệt độ 60°C. Enzym ổn định ở pH 6 – 7 và nhiệt độ < 50°C. Cellulase được hoạt hóa bởi ion Ca2+ nhưng bị kiềm hóa bởi các ion Co2+, Zn2+, Cu2+, Fe2+, Fe3+; enzym không bị ảnh hưởng bởi ion Mg2+ và Ba2+. Cellulase kí thuật bị kiềm hóa bởi SDS ở nồng độ cao, trong khi EDTA và Tween 80 đồng thời hoạt hóa enzym.

*Từ khóa: Bacillus, Cellulase, CMCase, phân lập, EDTA, SDS, Tween 80, ruột mồi.