Purification and identification of bacterial cellulase activity of *Bacillus subtilis* W48 for biofuel production

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Abstract

Lignocellulosic biomass is a renewable, inexpensive, and abundant resource with high potential for biofuel production to implement the sustainable energy worldwide. An important key of biofuel production is the hydrolysis of lignocellulosic biomass to fermentable sugars. Screening for a novel cellulase, as a biocatalyst, is challenge for development of biofuel production to be the economic and environmental friendly process. Natural environment is an important source to find an efficient cellulase producing bacteria. Previously, Bacillus subtilis strain W48 was screened and isolated from rainforest park at Assumption University, Bangkok, Thailand. In this study, we focused on the purification and characterization of cellulase enzyme produced by B. subtilis strain W48. The crude cellulase enzyme was collected by culture grown at 45°C in carboxy-methyl-cellulose (CMC) media. Then, it is concentrated by ammonium sulfate precipitation and fractionated by using size exclusion chromatography using sephacryl S-100 HR column. In this study, the fractions that has highest CMCase activity (0.017 mg/ml), as endoglucanase, and FPase activity (0.013 mg/ml), exoglucanase, are fraction no. 13 and 11, respectively.

Keywords: *Bacillus subtilis* W48, Cellulase, Purification

Introduction

Nowadays, the increasing of industrial growth worldwide leads to the rising of energy demand. Generally, the main source of energy to supply the need is obtained from natural petroleum. Therefore, availability of natural petroleum is decreased and the price increases, which make people try to find a new renewable energy resource.

Cellulose is one of the most abundant organic material. It is the main structural component of the primary cell wall of green plants, algae, and the oomycetes (Verma *et al.*, 2012). Cellulose is an interesting material for biofuel production, because it is available in agricultural waste. Using cellulose for the process can contribute to increase the value of waste, and help to reduce environmental problem.

However, to produce biofuels from cellulose, it is needed to be hydrolyzed to small molecule of sugar by the activity of cellulase enzyme. Cellulase is the enzyme that microbes produce to break down cellulose to smaller compounds, including sugars that can be utilized by microbes (Tomme P. *et al*., 1995). The biological function of cellulose has been studied for many years, especially cellulase produced by fungi and bacteria (Tomme P. *et al*., 1995). Additionally, many studies demonstrated the novel cellulase enzymes that have high efficiency to degrade cellulose. However, to apply cellulase for biofuel production, the special properties and characters are required, for example, high temperature tolerance.

In our previous study, about 300 different bacterial isolates were screened from various soil samples obtained from different locations in Thailand (Yasurin *et al.*, 2013). 10 bacterial isolated that grew on CMC agar and showed cellulase activity higher than *Cellulomonas*, a common cellulase producing bacteria, were selected for secondary screening in different carbon sources. Among 10 isolates, *B. subtilis* strain W48 has high cellulase specific activity compared to other isolates up to 4.9 U/mg protein.

Therefore, in this research, we focused on the purification and characterization of cellulase enzyme produced by *B. subtilis* strain W48. The enzyme activity was monitored and its molecular property was described here.

Materials and Methods

A. Production of extracellular cellulase enzyme

The *B. subtilis* strain W48 (Yasurin *et al.*, 2013) was inoculated in CMC broth and incubated in shaking incubator at 45 °C for 48 h, 150 rpm. Crude extracellular cellulase was collected by centrifuging at 5,000 rpm for 15 min to obtain the cell-free supernatant as the crude cellulase.

B. Purification of cellulase enzyme

The crude cellulase was precipitated by ammonium sulfate and kept in 4^{o}C for 12 h. The cellulase precipitates were collected by centrifugation at 5,500 rpm for 15 min at 4^{o}C then dissolved in 5 ml sodium phosphate buffer (pH 7.0) followed by dialysis for 24 h. The concentrated protein was collected by using spin column with 10 kDa MWCO (Balanchandrababu *et al*., 2012).

C. Size exclusion chromatrography

The 3 ml concentrated protein was separated by using sephacryl S-100 HR column. The column was equilibrated by 50 mM sodium phosphate buffer (pH 7.0). The entire purification was carried out at 4°C. The fraction was collected at 1 ml/min flow rate. Concentrated cellulase fraction was analyzed by 12% gel SDS-PAGE to determine the molecular weight of purified enzyme sample by comparing with standard protein marker (BLUeye Protein Ladder, RBC Bioscience, China). Protein samples were stained with Coomassie Brilliant Blue R-250.

D. Cellulase activity assay

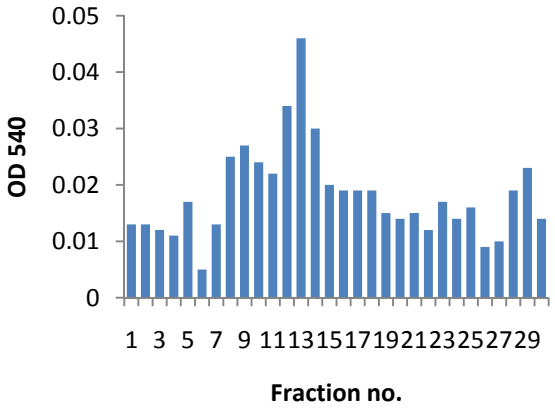
Dinitrosalicylic acid (DNS) assay were performed with the standard method to determine amounts of reducing sugars in the samples (Miller, 1959). The reducing sugar amounts of hydrolysates were measured by the spectrophotometer at 540 nm. Endoglucanase (CMCase) and exoglucanase or cellobiohydrolase (FPase) activity were determined by using CMC and filter paper as substrate, respectively. 0.2% w/v of substrates were mixed in 50 mM phosphate buffer (pH 7.0) containing 500 µl of each enzyme fraction. Each samples were incubated in shaking incubater at 45 °C for 2 h, 150 rpm. Then the hydrolysis reaction was stopped by heating the sample to 95 °C for 10 min. The amounts of reducing sugars in the samples were calculated based on the relationship of absorbance value and glucose amount in the glucose standard curve.

Results and Discussion

Cellulase purification using Size exclusion chromatrography

The extracellular fraction of *B. subtilis* strain W48 culture grown in CMC broth media was collected as crude enzyme. Then enzyme was concentrated by using ammonium precipitation method. The dialyzed enzymes was loaded into sephacryl S-100 HR column. Each enzyme fractions were collected separately and aliquoted to test their activity with CMC and filter paper substrate. Figure 1 shows the CMCase and FPase activity of each fraction. The highest CMCase (0.017 mg/ml) and FPase (0.013 mg/ml) activities were detected in fraction no. 13 and 11, respectively. The endoglucanase (CMCase) showed higher activity than exoglucanase (FPase) when compare within the same fraction.

(a)



(b)

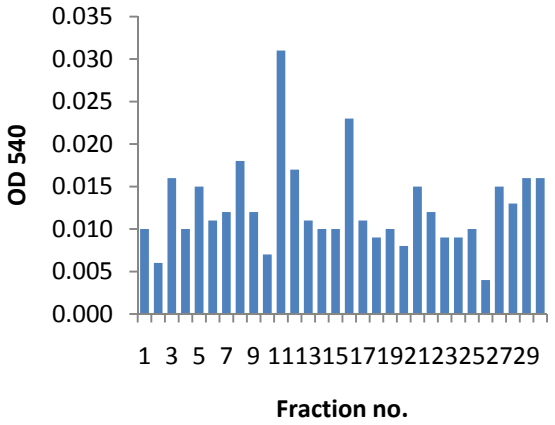


Figure 1. The enzyme activity of *B. subtilis* strain W48 in each fraction by using different substrate (a) CMC and (b) Filter paper.

To determine the size of enzyme molecule, the active fractions were pooled together and re-concentrated by using spin column with 10 kDa MWCO again. Then, the pooled active fractions was analyzed by SDS-PAGE as shown in Fig 2. The results showed one major polypeptide of approximate size 40 kDa after stained with Coomassie brilliant blue R-250.

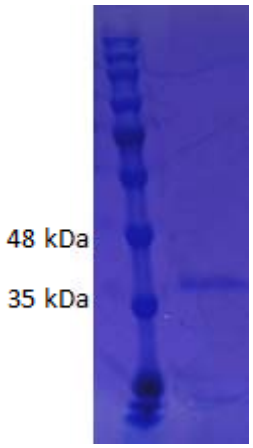


Fig. 2. SDS-PAGE analysis of cellulase-containing fraction of *B. subtilis* strain W48

The endoglucanase (CMCase) specifically cleaves the internal bonds of the cellulose polymer chain, while exoglucanase (FPase) specifically cleaves the end of cellulose polymers. To maximize the release of fermentable sugar for biofuel production, the combined functions of these enzymes are required. The *B. subtilis* strain W48 showed high endoglucanase (CMCase) activity, so this result indicates that the *B. subtilis* strain W48 has potential to apply for biofuel production using lignocellulosic biomass as raw material. However, the addition of exoglucanase from other sources is suggested.

The results from this work was related to the previous study. The total cellulase activity on different carbon source as inducer were observed, and the result showed that the highest total cellulase activity was showed when growing *B. subtilis* strain W48 in CMC media (Yasurin *et al.*, 2013). This indicated that different carbon sources have an effect on the expression of endoglucanase (CMCase) activity.

Conclusion

It was found that *B. subtilis* strain W48 produced cellulase showed the higher the endoglucanase (CMCase), 0.017 mg/ml than the exoglucanase or cellobiohydrolase (FPase) activity, 0.013 mg/ml. The further works are to identify the type of 40 kDa-sized cellulase from the active fraction by using LC-MS/MS. The kinetic of purified cellulase; endoglucanase

(CMCase) and exoglucanase or cellobiohydrolase (FPase), will be also determined to provide the fundamental knowledge and information for application in biofuel production process in the future.

Acknowledgement

The authors would like to thank King Mongkut's University of Technology, North Bangkok (Research University Grant No. KMUTNB-GOV-57-41) and The Institute for the Promotion of Teaching Science and Technology, Thailand (IPST) (Research Grant No. 022/2555) for financial support of this work.

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