ISOLATION AND PURIFICATION OF CELLULASE FROM ALKALINE-TOLERANT BACILLUS SUBTILIS

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ABSTRACT

Lignocellulosic biomass is one of the most abundant renewable resource in the world. It has high potential to be used as material for biorefinery process because it yields fermentable sugar that can be converted to various bioproducts via microorganism functions. To make the biorefinery process success, the efficient hydrolysis process is needed to be improved to release the maximum amounts of sugars from lignocellulosic biomass. Natural environment is an important source to find an efficient cellulase producing bacteria. Previously, an alkaline-tolerant cellulase-producing bacterium, *Bacillus subtilis* strain MSB9, was screened and isolated from Botanic garden in Mahasarakham province, Thailand. In this study, we focused on the purification and characterization of cellulase enzyme produced by *B. subtilis* strain MSB9. The crude cellulase enzyme was partially purified and concentrated by ammonium sulfate precipitation and fractionated by using size exclusion chromatography using sephacryl S-100 HR column. Two of purified cellulase have relative molecular mass of 35 and 45 kDa, as determined by SDS-PAGE combined with CMC-zymogram.

Keywords: *Cellulase, Purification, Thermophilic bacteria, Bacillus, Thailand*

1. INTRODUCTION

Lignocellulosic biomass is one of the most important renewable material for biorefinery worldwide. It is the main component of plant cell walls. Mainly, it composed of three main components, cellulose (a homologous polymer of glucose molecules connected by β-1,4 linkages), hemicelluloses (a heterologous polymer of 5- and 6-carbon sugars), and lignin (a complex aromatic polymer) (1). Based on these characters, it draws economic interest to develop processes for effective conversion and utilization of lignocellulosic biomass as inexpensive sugars or carbon sources in fermentation processes.

For biorefinery, the step to convert lignocellulosic biomass to fermentable sugars is a major bottleneck (2) for the aspects of time and cost of the process. To achieve the success for lignocellulose conversion, the development of cellulase enzyme is needed with high efficiency enzyme, low cost, stress-tolerance properties.

In this study, we aimed to isolate and purify cellulase enzyme produced by an alkaline-tolerant bacterium, *Bacillus subtilis* strain MSJ9 that was screened and isolated from Botanic garden in Mahasarakham province, Thailand.

2. MATERIALS AND METHODS

2.1. Culturing condition for cellulase production

Bacillus subtilis strain MSB9 was previously screened and isolated from soil samples in Botanic garden in Mahasarakham province, Thailand (3). This bacterium is able to grow in carboxymethylcellulose (CMC) agar plates (0.5% CMC, 0.1% $NaNO_3$, 0.1% K_2HPO_4, 0.1% KCl, 0.05% $MgSO_4$, 0.05% yeast extract, 1.5% agar) (4) at pH 10.0. Therefore, it can be categorized to be an alkaline-tolerance bacterium.

The total cellulase activity was performed by transferring 100 µl of overnight culture to 10 ml of fresh CMC broth media, and cultures were incubated at 45 °C for 48 h. Then the crude cellulase enzyme from supernatant fractions of each bacterial isolates were tested for activity to digest two different cellulose substrates, filter paper and CMC using standard method (5) and the sugar products were measured by dinitrosalicylic (DNS) assay (6). Enzyme activity was defined as the amount of enzyme that released 1 µmol of glucose per min. Protein determination was done by using Bio-Rad protein assay kit with protocol described in manual. All experiments were performed with three replicates.

2.2. Enzyme preparation and purification

After incubation in CMC broth at 45°C for 48 h, the culture was centrifuged and

supernatants were used as crude cellulase enzyme. 200 ml of the crude enzyme was brought to 80% saturation with ammonium sulfate. Pellet was collected by centrifuged at 10,000 rpm for 20 min, and dissolved in 5 ml of 50 mM sodium phosphate buffer (pH 5.0) for dialysis.

Before protein dialysis, dialysis membrane (Float-A-Lyzer, Spectrum Lab, USA) with 10 kDa MWCO was prepared as described in the manual. Dissolved pellet was dialyzed against 30 mM sodium phosphate buffer (pH 5.0) at 4 ^{o}C with three changes of buffer. This solution was applied to a Sephacryl S-100 column (16 x 600 mm; HiPrep 16/60 Sephacryl S-100 HR, GE Healthcare Life Science, USA) equilibrated with 30 mM sodium phosphate buffer (pH 5.0) at a flow rate of 0.2 ml/min. The cellulase fractions were pooled and concentrated by Vivaspin-500 column (GE Healthcare Life Science, USA) with 10 kDa MWCO. 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.

To generate the zymogram pattern, concentrated cellulase fraction was applied to 12% gel SDS-PAGE (containing 1% CMC). Then, SDS was removed by soaking the gel in wash buffer (30 mM sodium phosphate buffer containing 40% isopropanol (pH 7.2)) for 1 h. The gel was soaked in equilibrated buffer (30 mM sodium phosphate buffer (pH 7.2)) for 1 h, and transferred into renaturing buffer (30 mM sodium phosphate buffer, 5 mM β-mercaptoethanol, 1 mM EDTA (pH 7.2)) at 4 ^{o}C overnight. The renatured gel was stained with 1% congo red solution for 15 min, and washed with 1 M NaCl solution. The band of cellulase was seen as a clear zone against background.

3. RESULTS AND DISCUSSION

To monitor cellulase activities of *Bacillus subtilis* strain MSB9, crude cellulase enzyme was first prepared by culturing this bacterium in CMC broth media at 45 °C for 48 h. The total cellulase activity was determined by standard method (5) using filter paper and CMC as substrates. The result was shown that FPase and CMCase activity are 2,022 and 4,297 U/L, respectively.

Next, cellulase enzyme produced by *Bacillus subtilis* strain MSB9 was prepared and purified. Crude cellulase enzyme of MSB9 was prepared from supernatant fraction of bacterial culture as described in method. After protein precipitation by adding ammonium sulfate, sample pellet was dialyzed, and fractionated by Sephacryl S-100 column. Each collected fractions were tested for cellulase activity using CMC and

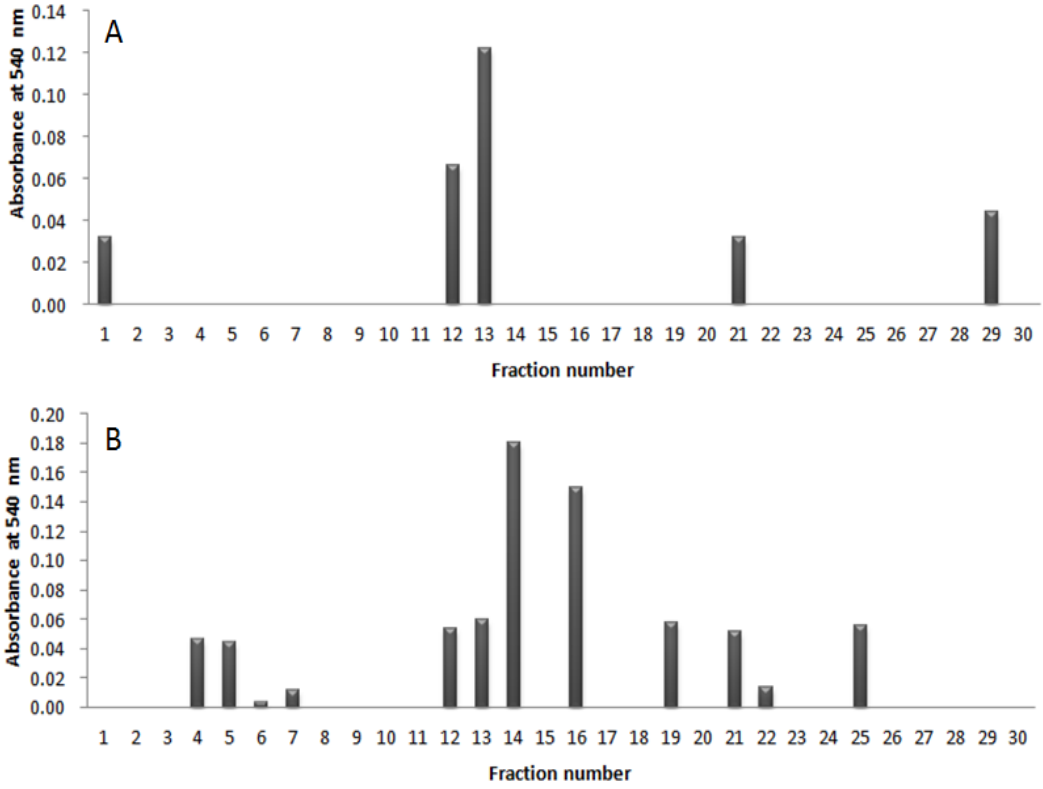

Figure 1. The enzyme activity of *B. subtilis* strain MSB9 in each fraction by using different substrate. (A) CMC and (B) Filter paper.

filter paper as substrates (Figure 1). The cellulase activities were determined based on the absorbance at 540 nm of DNS assay. The results showed that fraction number 13 and 14 have the highest CMCase and FPase activities, respectively.

To analyze with SDS-PAGE and CMC zymogram, fraction number 12 and 13 were pooled together and concentrated again, and the pooled sample then was analyzed by SDS-PAGE and CMC-zymogram as described in methods (Figure 2). SDS-PAGE analysis of pooled fraction (fraction number 12 and 13) from MSB9 protein samples revealed at least three major polypeptides of apparent size 20, 35, and 45 kDa after stained with Coomassie brilliant blue R-250. However, zymogram analysis, using CMC as substrate, revealed two polypeptides with approximately 35 and 45 kDa bands showed clear zone indicating the CMCase activity. Based on SDS-PAGE and CMC-zymogram analysis, the purified CMCase enzymes produced from MSB9 was shown to have molecular weight about 35 and 45 kDa. In this study, we did not perform zymogram using filter paper as substrate, we hypothesized that FPase enzyme should have molecular weight larger than CMCase (45 kDa) because the fraction that has the highest FPase activity is fraction number 14 and eluted out from the column later than fraction number 13.

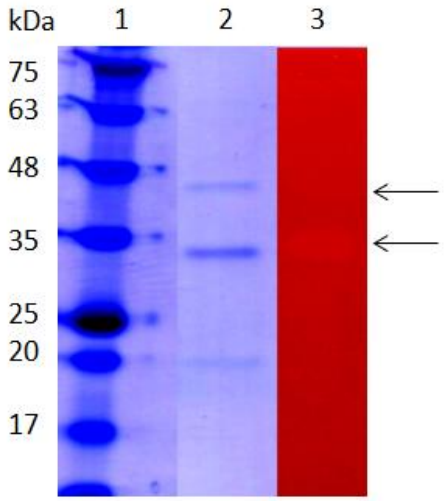

Figure 2. SDS-PAGE (lane 2) and CMC-zymogram (lane 3) analysis of pooled fraction number 12 and 13 protein samples isolated from *B. subtilis* strain MSB9.

We, next, aim to study for more details about this enzyme, including finding optimal condition for enzyme activity, and kinetic properties. The identification of polypeptide sequence should be done by using LC-MS/MS to track back to the gene encoding to this enzyme.

4. CONCLUSION

The newly bacterial isolate, *B. subtilis* strain MSB9, is a potent cellulolytic bacterium because it can tolerate at high pH condition, which makes it become a good candidate for industrial applications. Here, we identified at least two types of cellulase enzymes that have CMCase activity. We further aim for characterization of these CMCase and also FPase from fraction number 14, which helps to extend the application of these enzymes to various types of cellulose substrates.

5. ACKNOWLEDGEMENT

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