PREPARATION OF SECRETED PROTEINS FROM *STREPTOMYCES* SP. SIREXAA-ETO HYDROLYZE CMC AND CELLULOSE

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Abstract

One of the insect associated bacterium, *Streptomyces* sp. SirexAA-E (SirexAA-E), was described to be highly cellulolytic. SirexAA-E grew well on cellulose and other plant cell wall materials such as xylan, and secretes a suite of specialized enzymes depending on the available carbon sources in the growth medium. Non-crystalline cellulose (CMC) and chemically pretreated miscanthus were used as sole carbon sources, and secreted enzymes were compared between two culture conditions. In the presence of CMC, several prominent cellulaseswere determined by SDS-PAGE. While in the presence of miscanthus, dozens of enzymes including above cellulases were determined, thus I decided to use miscanthus as a sole carbon sources to prepare the culture supernatant of Sirex AA-E. The optimum condition of cultivation of SirexAA-E on miscanthus was determined with regard to protein yield (mg/L) and quality. It was confirmed that 5 days with 1 mL inoculum into 50 mL culture gave the highest protein yields with intact form of secreted proteins.

Keywords: SirexAA-E, CMC, SDS-PAGE, miscanthus, inoculum, secreted proteins.

Introduction

Plant biomass is the most abundant of carbon source on earth, and its deconstruction and subsequent catabolism are key components of global carbon cycling. (Schlesinger, 2000 & Klemm 2005). The main energy in plant biomass is stored in plant cell walls, primarily in the recalcitrant polysaccharides cellulose and hemicellulose (Kirk, 1987, Awungacha, 2015 & Lynd, 2002). Efficient breakdown of these insoluble polymers is difficult, and only a limited number of bacterial and fungi have this capability (Lynd, 2002). *Streptomyces*is the largest genus of Actinobacteria and over 1000 *Streptomyces* sp. from both free livings and eukaryotic symbionts are reported (Book, 2016, Lewin, 2016 & Poulsen, 2011).Because of the great potential for their capability (Lynd, 2002). *Streptomyces*is the largest genus of Actinobacteria and over 1000 *Streptomyces* sp. from both free livings and eukaryotic symbionts are reported (Book, 2016, Lewin, 2016 & Poulsen, 2011).Because of the great potential for their secondary metabolites for human health and other applications, *Streptomyces* have been capability (Lynd, 2002). *Streptomyces*is the largest genus of Actinobacteria and over 1000 *Streptomyces* sp. from both free livings and eukaryotic symbionts are reported (Book, 2016, Lewin, 2016 & Poulsen, 2011).Because of the great potential for their secondary metabolites for human health and other applications, *Streptomyces* have been the most extensively studied microorganisms in the past decades (Alvarez, 2017, Clardy, 2006, Demain, 2009 & Adams, 2011). Recently, multiple insect symbionts of *Streptomyces* were described, and a potential application for biofuels production has been identified because of their high plant cell wall degradation potential (Book, 2016).

Streptomyces is the largest genus of Actinobacteria and an ecologically important group in the soil environment. They play as essential roles in the decomposition of biomass polymers especially hemicellulose (Cantarel, 2009, Crawford, 1978, Good fellow, 1983 & McCarthy, 1992). *Streptomyces* use a wide range of carbon sources and produce antimicrobial secondary metabolites (Goodfellow, 1983 & Schlatter, 2009). Although, the cellulose-degrading ability of *Streptomyces*

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was not well described in the past, novel isolate, *Streptomyces* sp. SirexAA-E, originally isolated from the wood devastating wood wasp, was shown to possess very high cellulose-degrading potential (Fig. 1), (Bianchetti, 2013).

In this study, we examined the growth, in the presence of biomass, SirexAA-E secreted enzymes that hydrolyzed CMC and cellulose.

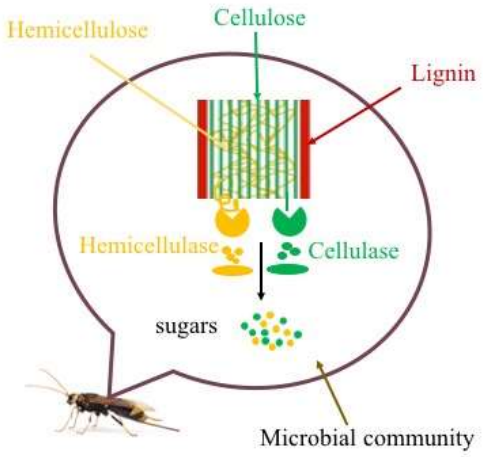

Figure 1 Schematic diagram of wood wasp symbiont microbial community that decomposes plant cell wall structures by using various cellulases and hemicellulases to produce sugars.

Materials and Methods

1 General reagents

Media for the growth and cultivation of bacterial cells were used Carboxymethyl cellulose (CMC) (Sigma-Aldrich, MO, USA), and biomass (miscanthus prepared in this study). Cellulose (Avicell) used in the cellulase activity measurements was purchased from Sigma-Aldrich.

2 Growth of organisms

Sirex AA-E was grown in YME medium Table 1. Cultures were incubated for 2 days at 30ºC. Different days and different amount of inoculum (mL of inoculum into 50 mL M63 minimum medium: are shown in (Table 2). The sole carbon source (0.5%, wt/vol) in the medium was carboxymethyl cellulose (CMC) or biomass (miscanthus). Cultures were incubated with shaking condition at 250 rpm at 30 ºC for the number of days as shown in (Table 3).

3 Preparation of secretomes

Supernatants were prepared from growing cultures by centrifuging the culture medium for 10 min at 4200×g at 4ºC to remove insoluble polysaccharides and cells. The supernatant fraction was then passed through a 0.45 µm glass fiber filter (AS ONE co., Osaka) to remove remaining cells. The concentration of secreted proteins in the supernatants was determined by Bio-Rad protein assay kit (Bio-Rad). For enzyme assays, the supernatants were concentrated to 1 mg/mL by using centrifugal ultrafiltration (VIVASPIN 20, Germany).

Table 1 Preparation of YME medium

Reagents	**Volume**
Yeast Extract Malt Extract Dextrose or D-glucose	4.0 g 10.0 g 0.4 g
Distilled H_2O to bring	1L

Table 2 Preparation of M63 minimal medium

Reagents	**Volume**
K_2HPO_4 KH_2PO_4 $(NH_4)_2SO_4$ 1 M $MgSO_4$ thiamine	53.6 g 26.2 g 10.0 g 1 mL 1 mL (1 mg/mL)
Distilled H_2O to bring	1L

Table 3 Incubation of cultures time

Inoculum (mL) in 50 mL culture	**Incubation time (days)**
1.0 mL	3 days
5.0 mL	3 days
10.0 mL	3 days
1.0 mL	5 days
5.0 mL	5 days
10.0 mL	5 days
1.0 mL	6 days
3.0 mL	6 days
5.0 mL	6 days
1.0 mL	9 days
3.0 mL	9 days
5.0 mL	9 days

4. Enzyme activity measurements

Reduced sugar assays were carried out by mixing biomass secretome preparations with polysaccharide-containing substrates including carboxymethyl cellulose CMC and cellulose. Enzyme hydrolysis reactions were carried out using 0.1 mg/mL of miscanthus secreted protein from the culture supernatant were incubated with 10 mg/mL of either CMC or cellulose in 10 mM sodium phosphate, pH 6, at 40 ºC for 20 h. The reaction will be stopped by heating for 5 min at 95 ºC. Reducing sugar content was determined by the dinitrosalicylic acid (DNS) assay. D-glucose was used to obtain a standard to quantify the amount of reducing end products. The enzyme activity in each solution (µmol reducing sugar/mg per hour) was calculated.

Results and Discussions

Growth of SirexAA-E with two different carbon sources

SirexAA-E grew well in 50 mL of M63 minimal medium containing two different carbon sources including 0.5% carboxymethyl cellulose (CMC) and 0.5% pretreated miscanthus. The

protein contents were analyzed for the culture supernatant from two different carbon sources by SDS-PAGE and also different amount of inoculum were tested, 0.5, 1.0 and 2.0 mL in 50 mL of M63 minimal medium for 7 days at 30 ºC,to test proteins amount and quality of proteins (Fig. 2). In the SDS-PAGE analysis of proteins from the CMC and biomass culture supernatants, around 47 kDa and 61 kDa proteins can be seen, respectively, which are indicative of the presence of untruncated reducing and non-reducing end cellobiohydrolases. In the following protein gel analyses, thus I utilize these two protein bands as indicators of quality of secreted proteins. From this result, among two different carbon sources in the culture medium, different amount of proteins were observed and also different inoculum showed improvement of protein amount in the culture supernatants. I concluded that the use of miscanthus as a sole carbon source in the growth medium of SirexAA-E was more relevant to prepare secreted proteins than that of CMC. Thus, I decided to optimize the secreted protein production by using the medium containing miscanthus as sole carbon source.

To optimize culture conditions, SirexAA-E was grown on 0.5% (w/v) miscanthus for 3 and 5 days at 30ºC with different amount of inoculum (1.0, 5.0 and 10.0 mL) in the 50 mL of M63 minimum medium, and analyzed by the SDS-PAGE (Fig. 3) and the concentration of proteins in the supernatant was measured (Table 4). Also the culture supernatants from 6 and 9 days at 30ºC with different amount of inoculum (1.0, 3.0 and 5.0 mL) in 50 mL of M63 minimum medium were analyzed by SDS-PAGE (Fig. 4) and the concentration of proteins in the supernatant was measured (Table 5). In the 3 days culture supernatants, the following concentrations of secreted proteins were obtained from the 1.0 mL of inoculum (0.045 µg/µL), 5.0 mL of inoculum (0.083 µg/µL), and 10.0 mL of inoculum (0.080 µg/µL), respectively. In Fig. 2.2., the SDS-PAGE analysis showed that two indicators (47 kDa and 61 kDa bands) seem to be intact. In the 5 days culture supernatants, the following concentrations of secreted proteins were obtained from the 1.0 mL of inoculum (0.358 µg/µL), 5.0 mL of inoculum (0.478 µg/µL), and 10.0 mL of inoculum (0.428 µg/µL) respectively. In Fig. 2.2., the SDS-PAGE analysis showed that two indicators (47 kDa and 61 kDa) seem to be intact.In the 6 days culture supernatants, the following concentrations of secreted proteins were obtained from the 1.0 mL of inoculum (0.154 µg/µL), 3.0 mL of inoculum (0.107 µg/µL), and 5.0 mL of inoculum (0.153 µg/µL), respectively. In Fig. 2.3., the SDS-PAGE analysis showed that two indicators (47 kDa and 61 kDa) were intact. In 9 days culture supernatants, the following concentrations of secreted proteins were obtained from the 1.0 mL of inoculum (0.144 µg/µL), followed by 3.0 mL of inoculum (0.153 µg/µL), and 5.0 mL of inoculum (0.141 µg/µL), respectively. In contrast to other samples, one of the two indicators (47 kDa and 61 kDa), 61 kDa protein seem to be proteolytically truncated. Thus, from these results, I concluded that the culture supernatant from the 5 days culture gave the highest protein production with no apparent truncation by secreted proteases.

Table 4 SDS-PAGE analysis for 3 & 5 daysof biomass secretomes

Inoculum (mL/ days)	Concentration of proteins (µg/µL)
1.0 mL-3 days	0.045
5.0 mL- 3 days	0.083
10.0 mL- 3 days	0.080
1.0 mL- 5 days	0.358
5.0 mL- 5 days	0.478
10.0 mL- 5 days	0.428

Table 5 SDS-PAGE analysis for 6 & 9 days of biomass secretomes

Inoculum (mL/ days)	Concentration of proteins (µg/µL)
1.0 mL-6 days	0.154
3.0 mL- 6 days	0.107
5.0 mL- 6 days	0.153
1.0 mL- 9 days	0.144
3.0 mL- 9 days	0.153
5.0 mL- 9 days	0.141

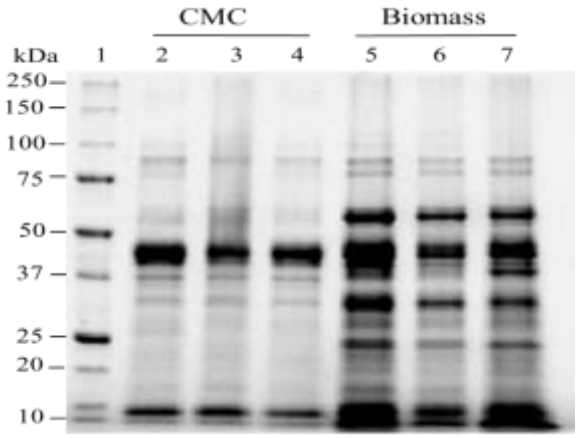

Figure 2 SDS-PAGE analysis of two difference carbon sources in 7 days inoculum.

Secretome analysis by 10% SDS-PAGE gel. SireAA-E was grown on either 0.5% CMC or biomass (miscanthus) for 7 days at 30ºC with different amount of inoculum (0.5, 1.0 and 2.0 mL) in 50 mL culture. Lane 1 is Mw marker. Lanes 2 to 4 are 0.5, 1.0 and 2.0 mL inoculum in 50 mL 0.5% CMC M63 minimum medium, respectively. Lanes 5 to 7 are 0.5, 1.0 and 2.0 mL inoculum in 50 mL 0.5% miscanthus M63 minimum medium, respectively. 10.0 µg equivalent of total secretome was used.

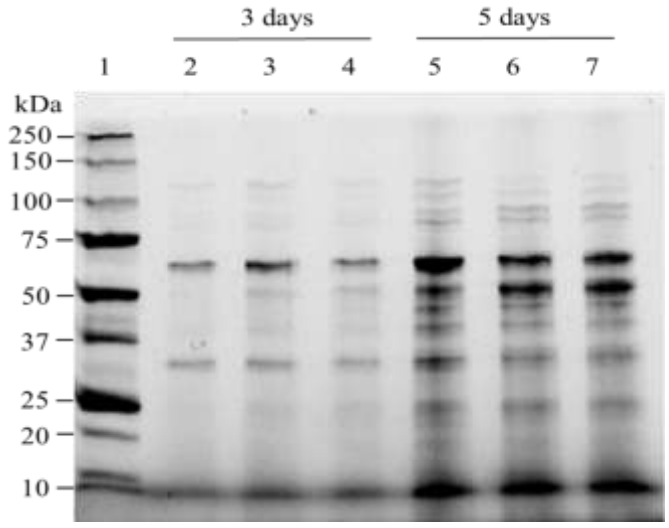

Figure 3 The SDS-PAGE analysis of biomass secretomes in 3 & 5 days inoculum Secretome analysis was done by 10% SDS-PAGE gel.

SireAA-E was grown on 0.5% miscanthus for 3 and 5 days at 30ºC with different amount of inoculum (1.0, 5.0 and 10.0 mL) in 50 mL of M63 minimum medium. Lane 1 is Mw marker. Lanes 2 to 4 are 1.0, 5.0 and 10.0 mL inoculum in 50 mL 0.5% miscanthus M63 minimum medium after 3 days respectively. Lane 5 to 7 are 1.0, 5.0 and 10.0 mL inoculum in 50 mL 0.5% miscanthus M63 minimum medium after 5 days respectively. 2.0 µg equivalent of total secretome was used.

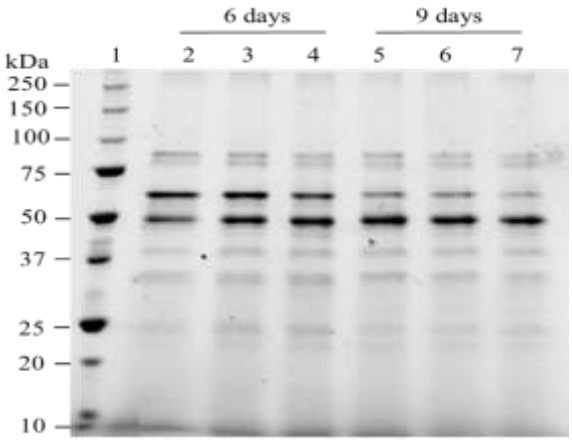

Figure 4 The SDS-PAGE analysis of biomass secretomes in 6 & 9 days inoculum.

Secretome analysis by 10% SDS-PAGE gel. SireAA-E was grown on 0.5% miscanthusfor 6 and 9 days at 30ºC with different amount of inoculum (1.0, 3.0 and 5.0 mL) in 50 mL culture. Lane 1 is Mw marker. Lanes 2 to 4 are 1.0, 3.0 and 5.0 mL inoculum in 50 mL 0.5% miscanthus M63 minimum medium after 6 days incubation, respectively. Lanes 5 to 7 are 1.0, 3.0 and 5.0 mL inoculum in 50 mL 0.5% miscanthus M63 minimum medium after 9 days incubation, respectively. 2.0 µg equivalent of total secretome was used.

Reaction of the biomass secretome

The culture supernatant containing SirexAA-E miscanthus secreted proteins from 1.0, 5.0 and 10.0 mL inoculum for 5 days at 30ºC were tested for their ability to degrade CMC and cellulose to test polysaccharide-degrading enzyme activities from three different inoculum conditions (n = 3) (Fig. 3). Secretome activity was measured after incubating with CMC or cellulose for 20h at 40ºC. The biomass secretome showed the highest polysaccharide-degrading activities for CMC substrate in all inoculum compared to the cellulose substrate. In contrast to the CMC hydrolysis reaction, the miscanthussecretome showed limited hydrolysis on the cellulose substrate due to the high crystalline nature (Fig. 5).

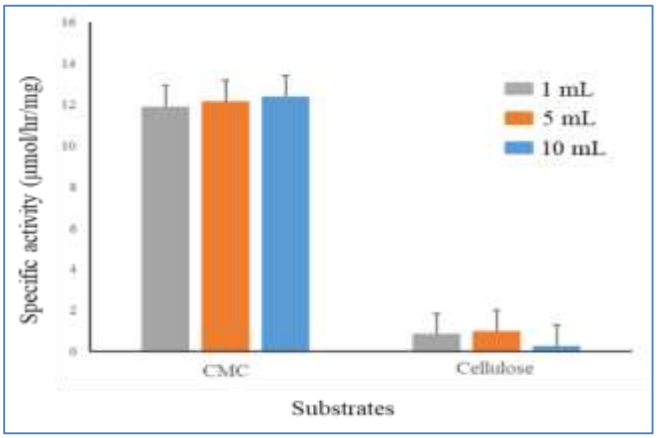

Figure 5 Determination of the specific activity (µg/hr/mg) of the culture supernatants from CMC and crystalline cellulose for polysaccharide degradation.

Different amount (1.0, 5.0 and 10.0 mL) of inoculum was tested for 5 days at 30ºC. Biomass activity from SirexAA-E culture supernatant was measured after growth on CMC and cellulose. Secretome activity was measured after incubating prepared secretomes with the substrate for 20 hrs at 40ºC. Error bars indicate the standard deviation from three independent experiments from secretome. ($P<0.05$)

Conclusion

In this study, it attempted to discover that 5 days with 1 mL inoculum into 50 mL culture gave the highest protein yields with intact form of secreted proteins. We examined the growth, in the presence of biomass, SrexAA-E secreted enzymes that hydrolyzed CMC and cellulose. The biomass secretome showed the highest polysaccharide-degrading activities for CMC substrate in 1 mL inoculum.

Acknowledgements

Special thanks go to the government of Japan through Monbukagakusho (MEXT) for awarding me a rear privilege of a fully funded scholarship to pursue my master's degree at Hokkaido University, one of the most prestigious Universities in Japan. I also give thanks to the government of the Republic of the Union of Myanmar for allowing me to pursue further studies and ensuring that my study leave was processed. I would also like to express my deepest gratitude to my supervisor Associate Professor TaichiTakasuka for his guidance, advice, and help throughout my study in Japan. Furthermore, I would like to thank Dr Ye Myint Aung, Head of Professor, Department of Chemistry, Pathein University, Myanmar for his reviewing of this research paper, support and encouragement.

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